Uma visão estrutural sobre componentes do processo de adaptação metabólica de tumores.

Tese apresentada ao Departamento de Física e
Ciência Interdisciplinar do Instituto de Física de São
Carlos, Universidade de São Paulo, de acordo com as
normas do concurso público de títulos e provas para
obtenção do título de Livre-Docente (EDITAL
ATAc/IFSC-19/2017).

Andre Luis Berteli Ambrosio

Agosto de 2017

Em branco.

Agradecimentos

O ponto em comum ao longo de toda a minha trajetória, já há 16 anos, é a Sandra. Sem ela – sem sombra de dúvidas – eu não chegaria nem perto de onde estou hoje. Sua seriedade, determinação, perseverança, e principalmente, sua contagiante paixão pela descoberta científica, ainda hoje formam a bases de minhas decisões. Mesmo que pudesse realizar o controle negativo na minha vida, duvido que estaria melhor, e mais feliz, do que se eu não a tivesse encontrado.

Agradeço a todos com quem interagi e que, de uma maneira ou de outra, contribuíram para minha formação e trajetória. Agradecimento especial aos meus orientadores, co-orientadores e supervisores, colaboradores, colegas e amigos de formação. Me espelho no que cada um tem de melhor.

Agradeço o LNBio e o CNPEM, na pessoa do Dr. Kleber Franchini, pela oportunidade e apoio incondicional, em todos os níveis; pelo suporte institucional, pelas inúmeras oportunidades de colaborações com meus colegas pesquisadores e pela liberdade e confiança nas decisões que dizem respeito ao andamento de nossa linha de pesquisa. Com certeza o LNBio é um lugar único no país para o desenvolvimento da ciência. Faço um agradecimento especial aos excelentes membros permanentes, principalmente à Alessandra Girasole. Muito obrigado aos alunos e aos pós-doutorandos que fazem ou fizeram parte do PMT desde 2009; todos sempre abraçaram nossas ideias como causa própria e são a força motriz da nossa linha de pesquisa.

Agradeço a FAPESP por todo o financiamento provido ao longo desses anos, na forma de Auxílios de Pesquisa e Bolsas.

Expresso minha enorme felicidade e gratidão à minha mãe, Sueli, ao meu pai, José Renato, e à minha irmã, Cíntia. Graças aos seus valores, sacrifícios e apoio, pude estudar e seguir o meu caminho.

Não menos importante, dedico este documento ao meu filho Bruno, o resultado mais importante da minha colaboração com Sandra. Bruno foi concebido em laboratório (literalmente), graças ao pioneirismo de Patrick Steptoe e Robert Edwards e da habilidade da equipe da Dra. Paula Navarro (FMRP, USP). Que Bruno cresça com o pensamento livre e seja justo e feliz.

“To a man with only a hammer, everything looks like a nail.” – Mark Twain

Em branco.

SUMÁRIO

1.	SOBRE A ORGANIZAÇÃO DESTE DOCUMENTO	7
2.	CONTEXTUALIZAÇÃO DO PROBLEMA BIOLÓGICO: O METABOLISMO TUMORAL	8
3.	PROTEÍNAS DE INTERESSE.....	11
3.1.	Glutaminases (2009 - presente).....	11
3.1.1.	Contribuições do candidato na área.	17
3.2.	Fatores de transcrição induzíveis por hipóxia, HIF (2011 - presente)	21
3.2.1.	Contribuições do candidato na área.	28
3.3.	Carreador mitocondrial de piruvato, MPC (2013 – presente).....	30
3.3.1.	Contribuições do candidato na área.	33
3.4.	Prolil-hidroxilases, PHD (2012 – presente).....	36
3.4.1.	Contribuições pretendidas.....	38
3.5.	Canal ânion-seletivo dependente de voltagem 2, VDAC-2 (2016 – presente).....	40
3.5.1.	Contribuições pretendidas.....	42
3.6.	Fator de transcrição MondoA (2013 – presente)	43
3.6.1.	Contribuições pretendidas.....	44
4.	CONSIDERAÇÕES FINAIS	46
5.	REFERÊNCIAS	47

Em branco.

1. SOBRE A ORGANIZAÇÃO DESTE DOCUMENTO

Quando da confecção desta tese, são praticamente 8 anos trabalhando como pesquisador independente no LNBio. Durante todo esse tempo, dividi, e ainda divido, a coordenação do laboratório de pesquisas multidisciplinares no assunto do processo de adaptação metabólica de tumores, com a Dra. Sandra Dias. O objetivo do nosso laboratório é contribuir para o entendimento das bases moleculares que permitem aos tumores suprir suas elevadas demandas por energia e blocos biossintéticos; estas demandas estão diretamente associadas às altas taxas de proliferação celular. Neste sentido, combinamos nossa experiência nas áreas de biologia estrutural, molecular e celular, visando sempre uma validação complementar das hipóteses levantadas. Obviamente, o objetivo máximo é o de contribuir com o desenvolvimento de novas e mais eficientes oportunidades terapêuticas, todavia, passando antes pela ciência básica.

Como já descrito em meu memorial circunstanciado, o tema da pesquisa e a aproximação multidisciplinar ao problema foram concebidos e herdados do trabalho desenvolvido durante meu pós-doutoramento nos Estados Unidos. Naquela ocasião, Sandra e eu presenciamos o renascimento do interesse da comunidade científica no metabolismo de tumores e participamos ativamente da descoberta da importância das enzimas glutaminase como potenciais alvos para o tratamento neste contexto.

Ao longo dos últimos anos, como pesquisador independente no LNBio, dentre vários outros trabalhos, contribuí para a demonstração inédita de que a isoenzima GAC é a mais relevante glutaminase em câncer e também o principal alvo para a descoberta de novos fármacos. Também, nosso grupo foi o pioneiro em determinar as estruturas cristalográficas de todas as isoenzimas das glutaminase de mamíferos (GAC de camundongo e KGA e GLS2 humanas). Descrevemos um mecanismo molecular inédito de ativação e inibição alostérica destas dessas proteínas, que envolve a formação de filamentos. Para tanto, fizemos uso combinado de microscopia eletrônica de transmissão para a proposição de uma estrutura molecular.

Através da cristalografia, fomos os primeiros em identificar um ligante intracelular não-covalente para a família dos fatores de transcrição HIF. HIF, que regula a expressão de mais de 5 % do genoma humano, permite que tumores sobrevivam em condições de baixa disponibilidade de oxigênio e nutrientes. Por último, realizamos a inédita purificação do carreador mitocondrial de piruvato humano, juntamente com a confirmação de sua atividade autônoma em vesículas artificiais. Até o momento, este é o único trabalho que descreve produção recombinante e funcional desta classe de proteínas de membrana e abriu as portas para estudos estruturais.

Em vista do acima exposto, espero, através deste documento, demonstrar minha capacidade de produzir uma linha de pesquisa própria, coerente e continuada. Intrinsecamente associado a isso, está a captação de recursos perante a FAPESP e o exercício da docência, principalmente na área de pós-graduação, orientando dissertações e teses (6 concluídas e 2 em andamento), e supervisionando pós-doutores (1 concluída e 3 em andamento).

Compilo, portanto, cinco artigos dos quais sou autor de correspondências, já publicados em revistas internacionais indexadas, além de um outro manuscrito, em fase de revisão (Anexos I, II e III). Também, descrevo outros trabalhos em andamento, tão relevantes quanto, obviamente ainda dentro do tema do metabolismo tumoral. Estes são desenvolvidos juntamente com pesquisadores do Brasil, Estados Unidos e Reino Unido. A intenção é garantir a internacionalização de nossa pesquisa e também estar alinhado com as fronteiras tecnológicas voltadas para a biologia estrutural. Tenho plena convicção que estes trabalhos colaborativos renderão excelentes frutos em um futuro próximo.

2. CONTEXTUALIZAÇÃO DO PROBLEMA BIOLÓGICO: O METABOLISMO TUMORAL

Tumor é um termo genérico, associado a um grupo de mais de 200 doenças que podem se desenvolver em praticamente qualquer parte do corpo humano. O ponto em comum dessas doenças é que as células que as constituem, independentemente de sua origem, e em função de mudanças genéticas acumuladas, crescem e multiplicam de maneira anormal e descontrolada. À essa massa atípica de tecido é dado o nome de neoplasia.

Neoplasias (ou tumores) classificados como “benignos” são normalmente localizados e restritos ao órgão de origem, e respondem bem a tratamentos clínicos. Em casos mais graves, porém, células neoplásicas se desprendem da massa primária e podem ser levadas pela corrente sanguínea ou pelo sistema linfático a locais distantes de sua origem, onde formam novas colônias; este processo é chamado de metástase. Os tumores metastáticos são considerados “malignos” e comumente referidos como câncer.

Apesar das definições um tanto quanto simples, a variedade e a complexidade inerentes a essas doenças, do ponto de vista molecular, tornam extremamente desafiadores tanto o seu entendimento e caracterização, quanto a associação de uma terapia adequada. Neste sentido, em paralelo ao desenvolvimento de novas alternativas de diagnóstico e tratamento, grande parte dos esforços da comunidade científica estão voltados à determinação e organização dos mecanismos moleculares básicos de origem e propagação do câncer.

O câncer se desenvolve ao longo do tempo, à medida que alterações genéticas, epigenéticas e outras aberrações cromossômicas se acumulam nas células. Além do mais, estas alterações devem ser positivamente selecionadas, do ponto de vista evolutivo e clonal. Tais características, que lenta e coletivamente transformam uma célula normal em pré-cancerosa e, em última instância, em câncer, são chamadas de “pilares da transformação celular” (1). São oito no total e encontram-se resumidos a seguir. Embora cada uma dessas marca de seleção contribua para o desenvolvimento da doença, não é até que uma célula exiba o conjunto completo (ou quase) de características, pode-se dizer que o câncer desenvolveu completamente.

- i. **Divisão celular autossuficiente.** Células são organizadas em tecidos e os tecidos, por sua vez, em órgãos com funções específicas, como os pulmões, fígado, coração, cérebro e pele. As células de cada órgão devem se comunicar e funcionar coletivamente para o correto desenvolvimento e funcionamento do respectivo órgão. Quando o crescimento é necessário, as células coletivamente enviam sinais para outras células para serem divididas. Células de câncer, por outro lado, não se comportam como membros dessa “equipe”. Eles controlam sua própria proliferação, produzindo os próprios sinais de crescimento, superativando receptores de sinal ou ativando oncogenes;
- ii. **Insensibilidade aos sinais de interrupção da divisão celular.** Assim como existem sinais que estimulam a proliferação celular, existem sinais que a interrompem. As células cancerosas são capazes de interromper ou ignorar essas mensagens inibitórias. Normalmente, isso é resultado de mutações ou alterações em genes supressores de tumores, que normalmente controlam a resposta de uma célula a estímulos internos e externos de saída do ciclo de divisão celular;
- iii. **Resistência à morte celular programada.** Quando as células ficam velhas ou são danificadas, estas são programadas para se autodestruir através de um processo controlado, chamado de apoptose. Esta é a forma do corpo de limitar o crescimento e descartar células com material genético comprometido, a fim de evitar a propagação de erros. Células cancerosas evitam o ciclo normal da morte celular e continuam a se acumular no corpo. Os sinais de apoptose podem ser interrompidos quando genes supressores de tumores sofrem mutações ou deixam de ser expressos;

- iv. **Potencial replicativo ilimitado.** O acúmulo de bilhões de células necessárias para a formação de um tumor requer divisão celular descontrolada, evitando apoptose e senescência replicativa. Na divisão celular normal, uma pequena porção do final de cada cromossomo, em uma região chamada telômero, é removida sempre que o DNA é copiado. Eventualmente, a perda de telômeros atinge um ponto crítico e a célula não pode mais dividir e replicar. Desta forma, células saudáveis autolimitam sua replicação, mas a ativação de uma enzima chamada telomerase pode manter telômeros e permitir que a célula continue a replicar indefinidamente. Mais de 90 % das células de câncer "imortalizadas" ativaram a telomerase, enquanto a maioria das células normais não o fazem (2).
- v. **Criação do próprio suprimento de sangue.** O crescimento tumoral requer um maior suprimento de sangue para fornecer oxigênio e nutrientes à massa celular acumulada. Um tumor é capaz de estimular a formação de novos vasos sanguíneos (angiogênese) e glóbulos vermelhos (eritropoiese), a partir da regulação de expressão gênica;
- vi. **Capacidade de invadir outros órgãos.** Como mencionado acima, células cancerosas, ao contrário das células normais, podem romper as barreiras do tecido de origem e se espalhar de um órgão para o outro (metástase). Muitas vezes, as células fazem isso penetrando nos vasos sanguíneos recém-formados pelo próprio tumor;
- vii. **Evasão do sistema imunológico.** Ao funcionar adequadamente, o sistema imunológico do corpo detecta e destrói células estranhas e anormais. Embora o processo não seja totalmente compreendido, há evidências de que as células cancerosas são capazes de evadir a destruição das defesas imunitárias do corpo até certo ponto, o que lhes permite proliferar e invadir outros tecidos.
- viii. **Reprogramação do metabolismo energético e biossintético.** Mesmo com um aumento do suprimento de sangue, as células no interior de um tumor podem ser privadas de oxigênio e nutrientes. Isso seria prejudicial às células normais, que usam oxigênio para converter a glicose em energia através do processo de metabolismo aeróbico. As células cancerosas têm a capacidade de mudar o metabolismo de glicose aeróbico para anaeróbico (livre de oxigênio) (glicólise) para permitir que as células privadas de oxigênio continuem a produzir energia e a sobreviver. Todo um rearranjo das vias tradicionais de anabolismo e catabolismo se seguem.

Dentre todas as características brevemente descritas acima, o processo de adaptação metabólica de tumores constitui o tema principal de minha linha de pesquisa e, portanto, será abordado em mais detalhes a seguir.

Para realizar a divisão, uma célula qualquer deve duplicar seu genoma, proteínas e lipídios, ou seja, toda a sua biomassa, e montar esses elementos em células filhas. O aumento das taxas de divisão celular no câncer exige que as vias metabólicas sejam quase que completamente redesenhadas e/ou adaptadas, quando comparadas com células normais proliferativas (3). As primeiras observações sobre as alterações metabólicas características de tumores, principalmente a elevada captação de glicose, foram feitas há quase um século pelo fisiologista alemão Otto Warburg (4), e lhe renderam o Prêmio Nobel em Fisiologia de 1931. Porém, o campo do metabolismo do câncer tornou-se um tema de renovação interesse somente na última década.

Hoje em dia, é evidente que as alterações metabólicas associadas à tumorigênese abrangem todos os níveis conhecidos de interação entre a célula e os metabólitos (5). Primeiro, a célula transformada apresenta uma capacidade aumentada de captar os nutrientes necessários. Em segundo lugar, células remodelam a forma como tais nutrientes, uma vez internalizados, são preferencialmente atribuídos a vias metabólicas. Por último, o metabolismo diferencial desses

nutrientes exerce efeitos de longo alcance sobre o destino celular, entre os quais estão as alterações na diferenciação das próprias células cancerosas, bem como dos componentes do microambiente tumoral.

Dois nutrientes principais, absolutamente necessários pelas células em crescimento, são o carboidrato glicose e o aminoácido glutamina. Ambos fornecem um conjunto de intermediários de carbono para síntese de macromoléculas e garantem balanço dos níveis de agentes redutores utilizados na geração de ATP ou manutenção do equilíbrio redox (5).

A descoberta de que as células cancerosas utilizam excessivamente a glicose, mesmo na presença de oxigênio, é chamado de "efeito Warburg" (4). À este efeito, também conhecido como "glicólise aeróbica", está associada uma presença elevada de proteínas de transporte de glicose (GLUT). Este é talvez um dos efeitos mais encontrados em câncer de maneira geral (6), e frequentemente associado à maus prognósticos (7–9). Múltiplos mecanismos e vias de sinalização promovem a absorção de aumentada glicose nas células cancerosas. O fator de transcrição induzível por hipóxia, HIF, aumenta a expressão de GLUT e hexokinase, e a sinalização aberrante de PI3K/Akt aumenta a expressão especificamente de GLUT1 e a translocação dessa proteína para a membrana celular, assim como os oncogenes KRAS e BRAF (5, 6, 10).

A alta demanda de células tumorais proliferativas por glutamina foi descrita pela primeira vez na década de 1950 pelo fisiologista americano Harry Eagle. Eagle demonstrou que o crescimento ótimo de células HeLa cultivadas requer um excesso molar de glutamina em meio de cultura de 10 a 100 vezes em relação a outros aminoácidos (11). No contexto metabólico, glutamina é talvez o nutriente mais versátil, podendo doar carbonos e nitrogênio para vias anabólicas e produção de energia (12). Possui também a capacidade de reabastecer os intermediários do ciclo TCA (anaplerose) e promover a síntese de nucleotídeos, proteínas e lipídio; além disso, está envolvida na sinalização celular e no controle de expressão gênica [146].

Basicamente, o envolvimento da célula tumoral no processo de adaptação metabólica é multifatorial. Vai desde a otimização da captação de nutrientes através das membranas, passando por rearranjos em seus metabolismos, em diferentes compartimentos, e terminando, muitas vezes, no núcleo, com efeitos diretos ou indiretos na expressão de genes, que outrora seriam fortemente regulados em células normais. Nesse contexto, os assuntos de interesse de meu grupo navegam por esses diferentes aspectos, associados a diferentes tipos de proteínas. Não necessariamente em ordem de incorporação do projeto, ou mesmo relevância, mas conduzimos pesquisa em: i) enzimas (glutaminases e prolil hidroxilases), ii) fatores de transcrição (HIF e MondoA) e iii) proteínas de membrana (Carreador mitocondrial de piruvato e porina VDAC2).

A seguir, apresento as bases contextuais e as nossas contribuições para o entendimento das estruturas e funções das proteínas acima. Os temas foram sendo incorporados de maneira lógica e coerente, e de acordo com oportunidades; todavia, sempre associados com uma percepção de originalidade e potencial de novidade das descobertas. Em paralelo, sempre vislumbrei também maneiras de aprender e incorporar em nosso grupo um conhecimento técnico-experimental que seja compatível com a fronteira em biologia estrutural.

3. PROTEÍNAS DE INTERESSE

3.1. Glutaminases (2009 - presente)

Glutaminases são enzimas que catalisam a hidrólise do aminoácido glutamina (Gln), produzindo glutamato (Glu), concomitante com a geração de um íon amônio. De maneira geral, a existência de múltiplas isoenzimas em mamíferos foi primeiramente demonstrada por Hans Krebs, com base na detecção de parâmetros cinéticos tecido-específicos e suscetibilidade a inibição pelo produto de catálise, enquanto testava a conversão de glutamina em glutamato em extratos teciduais de ratos (13). Até o momento, quatro isoformas de glutaminases já foram descritas em humanos, as quais são codificadas por dois genes distintos (Figura 1). As isoformas conhecidas coletivamente como GLS1, a *Kidney-type* glutaminase (KGA) e a Glutaminase C (GAC), são geradas por *splicing* alternativo do gene *GLS* (2q32.2). Em contraste, as isoformas GLS2, *Liver-type* glutaminase (LGA) e Glutaminase B (GAB), originam do gene *GLS2* (12q13.3) através do uso alternativo de pontos de iniciação de transcrição e promotores (14).

Em células normais, a expressão de KGA é ubíqua, sendo encontrada em todos os tecidos com atividade de glutaminase com exceção do fígado (15, 16), apesar de ter sido reportada também em células endoteliais hepáticas (17). A variante GAC é predominantemente expressa em músculo cardíaco humano e pâncreas, ocorrendo também na placenta, rim e pulmão, porém, ausente no cérebro e fígado (18). Por sua vez, os transcritos derivados do gene *GLS2* foram identificados também em tecidos extra hepáticos, como cérebro, pâncreas e células de câncer de mama (19), contrariando as descobertas anteriores de que sua expressão estaria restrita ao fígado adulto (15, 20).

Os dois genes parálogos que codificam para as glutaminases humanas supostamente se originaram a partir de um gene ancestral comum por duplicação seguida de evolução divergente (21, 22). O gene *GLS* humano tem 82 kb (quilobases) e se divide em 19 éxons (21) (Figura 1). O transcrito de KGA é composto por 18 éxons e formado pela junção dos éxons 1-14 e 16-19; o transcrito de GAC compreende apenas os 15 primeiros éxons (18, 21). O gene *GLS2* humano tem comprimento de aproximadamente 18 kb e se divide em 18 éxons (22) (Figura 1). O transcrito canônico de GAB é formado pela junção de todos os 18 éxons do gene, enquanto que o éxon 1 está ausente no transcrito de LGA (14, 20).

Embora localizados em cromossomos diferentes, os genes *GLS* e *GLS2* humanos apresentam grau considerável de similaridade sequencial. Desconsiderando o éxon adicional presente em *GLS*, as principais diferenças entre as sequências codantes de ambos os genes se encontram nos éxons localizados nas extremidades dos genes. Por exemplo, os éxons 1 compartilham 62,5% de similaridade entre si, porém codificam para 129 aminoácidos em KGA e apenas 61 aminoácidos em GAB. Estas sequências codificadas pelo éxon inicial contém os sinais envolvidos no direcionamento mitocondrial e processos de translocação (Shapiro et al. 1991; Gómez-Fabre et al. 2000). Do mesmo modo, o éxon 19 de *GLS* e o éxon 18 de *GLS2*, que codificam para a região C-terminal de ambas, apresenta a menor similaridade sequencial (29,4%). Foi demonstrado que esta região da proteína GAB está envolvida no reconhecimento de módulos de interação PDZ (Olalla et al. 2001), enquanto que em KGA, a porção equivalente sinaliza para ubiquitinação, via reconhecimento do motivo KEN (25). Nota-se que as diferenças mais significativas entre as regiões codantes de *GLS* e *GLS2* compreendem motivos ou domínios envolvidos em direcionamento a organelas e interações proteína-proteína, o que pode explicar, pelo menos em parte, as diferenças de função e regulação. Por sua vez, os éxons 3-17 dos transcritos de

mRNA de *GLS* e *GLS2* tem o mesmo comprimento e apresentam alta similaridade sequencial (78%).

Figura 1. Transcritos de mRNA dos genes humanos de glutaminases. **(A)** Gene *GLS* humano e transcritos alternativos KGA e GAC. **(B)** Gene *GLS2* humano e transcritos alternativos GAB e LGA. Cada gene é representado com seus íntrons em linhas sólidas e éxons em caixas enumeradas. As regiões promotoras também são indicadas na extremidade 5' de cada gene, incluindo o promotor alternativo do gene *GLS2* no íntron 1. Linhas rosas tracejadas indicam os éxons que formam os transcritos de KGA e GAB, e as linhas verdes escuras pontilhadas compreendem os éxons envolvidos na geração dos transcritos GAC e LGA. O sítio de início da transcrição é marcado por uma seta e enumerado como +1. Imagem adaptada de (26).

De maneira geral, desde bactérias até eucariotos, o domínio glutaminase canônico e autônomo consiste de uma estrutura globular compacta, tipicamente compreendida por aproximadamente 300 aminoácidos e dividida em dois subdomínios (27). Um dos subdomínios é inteiramente formado por α -hélices, enquanto que o outro é misto, contendo tanto α -hélices quanto folhas- β . Na junção entre os dois subdomínios é formado o bolsão catalítico, que abriga o resíduo de serina característico do sítio ativo (Figura 2).

Figura 2. A estrutura do domínio glutaminase apresenta dois subdomínios e o sítio ativo está localizado na interface de ambos. **(A)** A estrutura cristalográfica de GAC de camundongo em complexo com glutamato (Glu) obtida por nosso grupo (PDB 3SS5) está representada em *cartoon*. O domínio I (Ile226-Pro286 e Cys429-Leu538, em verde) é composto por uma folha- β de cinco cadeias antiparalelas ($\beta 2 \downarrow \beta 1 \uparrow \beta 5 \downarrow \beta 4 \uparrow \beta 3 \downarrow$) cercada por seis α -hélices e diversos *loops*. O domínio II (Phe287-Thr428, em magenta) consiste basicamente de sete α -hélices. **(B)** L-Glutamato faz ligações de hidrogênio com os resíduos do sítio ativo; o grupamento amino da cadeia principal se liga a Glu386, Asn340, Tyr419 e Asn393; no ramo de cadeia lateral, a porção carboxila faz interações polares com Ser291, Tyr471 e o nitrogênio da cadeia principal de Val489.

Todavia, sabe-se atualmente que as glutaminases humanas são enzimas mais complexas em termos de arquitetura, possuindo estruturas com domínios adicionais flanqueando o domínio glutaminase, conforme representado na Figura 3.

Figura 3. Representação dos multidomínios das glutaminases KGA, GAC, GAB e LGA, codificadas pelos genes *GLS* (2q32.2) e *GLS2* (12q13.3). Os números indicam as posições na sequência primária de aminoácidos de cada proteína. A linha tracejada indica a região de processamento das glutaminases imediatamente após sua entrada na mitocôndria. MS: *mitochondrial signaling sequence*. NRB: *nuclear receptor box* (motivo LXXLL, L= leucina e X = aminoácido qualquer). ANK: domínio formado por repetições de anquirina. PDZ-br: PDZ (PSD95/Dlg/ZO1) *binding region*.

O comportamento cinético distinto das isoenzimas GLS e GLS2 é comumente utilizado para distinguir entre as isoformas de glutaminases humanas. As eficiências catalíticas das isoformas GLS são consideravelmente aumentadas na presença de fosfato inorgânico (P_i), enquanto que o acúmulo do produto da catálise, Glu, causa inibição (Curthoys et al. 1976; Curthoys & Watford 1995; Elgadi et al. 1999). Em constraste, ambas as isoformas GLS2 são insensíveis ao P_i (29) e não apresentam inibição por Glu (14, 30).

As principais diferenças cinéticas foram observadas em relação à dependência do ativador P_i , que é baixa para GLS2 e alta para GLS1; à relativa afinidade pelo substrato Gln, que é maior em GLS1 do que nas isoformas GLS2; e ao efeito inibitório de Glu, uma característica única reportada apenas para as isoenzimas GLS1 (15, 31). O mecanismo através do qual o P_i ativa as enzimas GLS1 foi demonstrado por nosso grupo a partir de dados estruturais de cristalografia por difração de raios X da proteína GAC (29). O P_i se liga no interior do bolsão catalítico, resultando na estabilização alostérica dos tetrâmeros e facilitando a entrada do substrato por competir com o produto, Glu, garantindo a ciclagem da enzima. Esta competição de P_i *versus* Glu pela ligação ao sítio ativo explica a diminuição do efeito inibitório de Glu na presença de concentrações crescentes de P_i (32).

Na presença de P_i , tanto KGA quanto GAC sofrem polimerização extensa e específica (33–35). Além disso, o equilíbrio tetrâmero-dímero é dependente de concentração, conforme demonstrado para a isoforma GAC, a qual sofre polimerização mesmo na ausência de P_i , apenas elevando-se a concentração da enzima (35). Há uma correlação direta entre a tendência de oligomerização e os níveis de atividade das isoenzimas GLS1, sendo GAC a enzima mais ativa e com maior capacidade de se polimerizar. Deste modo, o inibidor GLS1-específico, BPTES [*bis*-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide], atua justamente de modo a prevenir a formação dos superoligômeros induzidos por P_i (29, 36). O mecanismo de inibição foi elucidado a partir de dados estruturais das isoformas GLS1 cristalizadas. A inibição por BPTES é alcançada através de um mecanismo não competitivo que interfere na ativação alostérica induzida por P_i : o domínio glutaminase de KGA humana forma um tetrâmero altamente simétrico contendo duas moléculas de BPTES que estão posicionadas nas interfaces dímero-dímero; a ligação do BPTES desencadeia uma mudança conformacional dos resíduos do sítio ativo e congela a enzima em um tetrâmero inativo estável (35, 37, 38).

A estrutura multidomínios das glutaminases de mamíferos (Figura 3) é uma evidência da provável multifuncionalidade destas proteínas (26). Além do domínio catalítico, funcional e estruturalmente bem caracterizado, as glutaminases apresentam regiões N- e C-terminais contendo motivos e sequências que podem estar envolvidos em funções diversas. Os 15 primeiros aminoácidos localizados no N-terminal das isoformas de glutaminase, com exceção da variante LGA, correspondem à sequência de sinalização mitocondrial. O sítio de iniciação da transcrição e o promotor alternativo de LGA estão contidos no íntron 1 do gene *GLS2*, portanto, esta isoforma não contém o éxon 1, onde se localiza o peptídeo sinal. De fato, análises de bioinformática da proteína LGA humana não encontraram uma sequência de sinalização mitocondrial no N-terminal, entretanto, um peptídeo sinal secretório foi predito para os 16 primeiros aminoácidos da proteína LGA transcrita a partir do segundo ATG (14).

Ainda na região N-terminal, todas as quatro isoformas de glutaminase apresentam um motivo de interação com receptores nucleares (*NR box*, LXXLL): a sequência LEDLL nas isoformas GLS1 (resíduos 139 – 143) e LGDLL para GLS2 (resíduos 72 – 76 de GAB e 35 – 39 de LGA). A existência de resíduos carregados flanqueando motivos *NR box* parece contribuir para a afinidade da interação de coativadores com receptores nucleares (39), condição que também é atendida pelas glutaminases. Em GLS, existem lisinas localizadas a N-terminal (posições 130 e 132 de GLS humana) e ácidos glutâmicos a C-terminal (posições 149 e 152 de GLS humana) do motivo *NR box*; em GLS2, há uma arginina a N-terminal (posição 71 de GAB humana) e dois ácidos glutâmicos a C-terminal (posições 82 e 85 de GAB humana) do motivo.

A partir do resíduo 76 de GLS humana, o N-terminal se assemelha estruturalmente ao domínio *EF-hand*, formado por motivos do tipo hélice-*loop*-hélice, no qual o *loop*, usualmente formado por 12 resíduos, apresenta sítios de ligação a íons Ca^{2+} . Domínios *EF-hand* estão presentes em proteínas como calmodulina (CaM, *calcium-modulated protein*), envolvida em sinalização celular (40), e troponina-C, importante componente da contração muscular esquelética e cardíaca (41). Proteínas contendo este domínio estão sujeitas a mudanças

conformacionais cálcio-dependentes que expõem sítios de ligação a peptídeos alvos (42). Entretanto, apesar de sua conformação estrutural *EF-hand-like*, as glutaminases de mamíferos não apresentam os resíduos necessários para a coordenação dos íons Ca^{2+} (29). Portanto, até o momento, nenhuma função foi descrita para esta região.

Na porção central das glutaminases, encontra-se o domínio catalítico, que é altamente conservado desde bactérias até seres humanos (27), como já mencionado acima. Por sua vez, o C-terminal de glutaminases de mamíferos, com exceção de GAC, apresenta motivos conhecidos de interação proteína-proteína denominados repetições ANK (revisado no tópico 1.2 adiante). O C-terminal único de GAC, por sua vez, é formado por 47 resíduos e corresponde a uma região conformacionalmente heterogênea, que não pôde ser modelada nas análises estruturais de raios X (29). Até o momento, nenhuma função foi atribuída para a região C-terminal de GAC.

A região C-terminal de glutaminases mamíferas com exceção de GAC também apresenta motivos denominados *KEN box*, cujas letras representam a sequência de aminoácidos (lisina – ácido glutâmico – asparagina) que o compõe. Os substratos do ciclossomo/complexo promotor de anáfase E3 ubiquitina ligase ligado à sua proteína ativadora Cdh1 (APC/C-Cdh1) são direcionados para degradação através de motivos de reconhecimento específicos, entre os quais, o *KEN box* (43). Foi demonstrado que GLS1 é substrato de APC/C-Cdh1 em linfócitos T proliferativos (25).

Conforme mencionado anteriormente, os últimos 4 aminoácidos da extremidade C-terminal de GAB humana, ESMV, correspondem a uma sequência consenso necessária para a interação com proteínas contendo domínio PDZ, um dos módulos mais comuns envolvidos em interações *scaffolding*, uma vez que se ligam às proteínas alvo e desencadeiam a formação de complexos funcionais específicos na membrana (44). Até o momento, foram identificadas duas proteínas contendo domínio PDZ e que são parceiras de interação de GAB: α -1-sintrofina (SNT) e *glutaminase-interacting protein* (GIP) (24). Segundo Márquez et al. (2009), a hipótese de GLS2 cerebral sendo regulada por proteínas PDZ é atraente porque pode fornecer um mecanismo de controle tanto da síntese de glutamato quanto do direcionamento de glutaminase cerebral a compartimentos celulares extra-mitochondriais, como o núcleo, onde GLS2 já foi localizada (24).

Glutamina é o aminoácido mais abundante no plasma humano, perfazendo mais de 20% do *pool* de aminoácidos livres no sangue e 40% no músculo (46). A manutenção de altos níveis de Gln no sangue fornece uma fonte pronta de carbono e hidrogênio para biossíntese, energética e homeostase celulares que pode ser explorada por células tumorais para impulsionar o crescimento tumoral (47, 48). Após entrar nas células, Gln deve seguir entre dois caminhos: (a) contribuir para a biossíntese de nucleotídeos e de uridina difosfato N-acetilglicosamina (UDP-GlcNAc); (b) ser convertida a Glu pelas glutaminases (GLS ou GLS2) (Figura 4). Glu, por sua vez, pode contribuir para a síntese de glutatona e tem papel fundamental como precursor ou substrato para a síntese de vários aminoácidos, ácidos nucleicos, nucleotídeos e metabólitos (recentemente revisado em (49) (Figura 6). Portanto, o metabolismo de Gln é importante para o reabastecimento do reservatório de intermediários do ciclo do TCA (anaplerose), os quais são removidos da mitocôndria para participar das reações de biossíntese (cataplerose) (50).

Figura 4. Principais destinos metabólicos e biossintéticos de Gln. Ao entrar na célula através de transportadores como SLC1A5, Gln pode ser utilizada na síntese de nucleotídeos e uridina difosfato N-acetilglicosamina (UDP-GlcNAc) ou ser convertida em Glu pelas glutaminases. Por sua vez, Glu contribui para a síntese de glutathione ou é convertido em α -cetoglutarato (α -KG) através de duas enzimas diferentes: glutamato desidrogenase (GLUD) ou aminotransferases, cujos subprodutos de catálise são, respectivamente, NH_4^+ e aminoácidos. α -KG entra no ciclo do ácido tricarboxílico (TCA), o qual fornece elétrons para a cadeia transportadora da mitocôndria na geração de ATP através da geração de NADH e FADH_2 . Oxaloacetato (OAA) pode ser convertido em aspartato para a síntese de nucleotídeos. Alternativamente, α -KG pode ser usado na produção de acetil-CoA e lipídeos através do processo chamado de carboxilação redutiva (RC). Imagem retirada de (51).

A importância da glutaminólise para a manutenção do fenótipo maligno coloca as glutaminases na posição de alvos terapêuticos potenciais para o tratamento do câncer. A expressão de GAC, a isoforma GLS1 mais ativa, é aumentada em diversos tipos de câncer, sugerindo que o *splicing* alternativo do gene *GLS* contribua para um fluxo glutaminolítico mais elevado em câncer (29, 52–55). Por sua vez, GLS2 parece ter uma função mais complexa em câncer. GLS2 é induzida pelo supressor tumoral p53 e tem características supressoras de tumor contra gliomas, glioblastomas, câncer de pulmão e hepatocarcinomas, reduzindo proliferação, migração e formação de colônia *in vitro* (29, 52–59). GLS2 também exibe habilidades supressoras de tumor através da regulação negativa da sinalização por PI3K/AKT, sendo que a expressão de GLS2 foi associada com maior taxa de sobrevivência de pacientes HCC (hepatocarcinoma celular) (60). Um estudo recente mostrou que GLS2 sequestra a *small* GTPase Rac1 de modo a suprimir metástase (61). Além disso, foi demonstrado que GLS2 tem a função de inibir migração e invasão em células HCC via Dicer-miR-34a-Snail, a partir de sua interação direta com a proteína Dicer (62). A consequente estabilização de Dicer facilita a maturação de miR-34a que, por sua vez, reprime a expressão da proteína Snail, fator de transcrição envolvido em processos de transição epitélio-mesênquima (EMT) (62, 63). Entretanto, GLS2 parece apoiar crescimento e promover resistência a radiação ionizante em alguns tipos de câncer (64).

Adicionalmente, GLS2 foi apontada como alvo crucial da oncoproteína N-Myc em neuroblastoma (65, 66).

Muitos oncogenes e supressores tumorais, tais como Myc (67), Ras (68), Rho GTPases (69), TGF β (70), PTEN (71) e p53 (56), foram descritos como envolvidos no metabolismo de Gln glutaminase-dependente. Notavelmente, o fator de transcrição oncogênico Myc estimula a captação e degradação de Gln por GLS1 para sustentar a viabilidade celular e a anaplerose do ciclo de Krebs (67). Conhecido por regular miRNAs (72) e estimular proliferação celular (Eilers & Eisenman 2008), Myc reprime a transcrição de miR-23a e miR-23b, resultando em expressão aumentada de seu alvo, *GLS*, e consequentemente, na regulação positiva do catabolismo de Gln (74). miRNAs desempenham papel central na regulação pós transcricional da expressão gênica ao anelar nas regiões 3' não traduzidas dos mRNAs alvos para promover a degradação do transcrito ou a repressão de sua tradução (75). Similarmente, em células leucêmicas humanas cultivadas em Gln, o fator nuclear kappa B (NF- κ B) p65 intensificou o metabolismo de Gln ao diminuir a expressão de miR-23a através de sua ligação direta ao promotor gênico (76).

Transformação dirigida pelo oncogene K-ras também mostrou induzir dependência a Gln para promover crescimento e sobrevivência (68, 77). Inibição do metabolismo de Gln pelo *knockdown* de *GLS* foi suficiente para comprometer crescimento de câncer dirigido por K-ras, indicando uma vulnerabilidade metabólica (78). Por sua vez, o fator de transcrição oncogênico c-Jun foi identificado como regulador primário da expressão de *GLS* em células de câncer de mama, sendo capaz de se ligar diretamente à região promotora do gene *GLS* e elevar a sua expressão (79).

3.1.1. Contribuições do candidato na área.

Meu primeiro contato com as glutaminases foi durante o pós-doutoramento no laboratório do Dr. Richard Cerione (Rick), na *Cornell University*. Naquela ocasião, em paralelo à descoberta realizada em seu grupo, a de que essa proteína era um inédito e potencial alvo em câncer, me foi dada a responsabilidade de produzir e purificar glutaminase em grandes quantidades – a partir de fonte recombinante – visando posteriores ensaios de ativação e inibição *in vitro*. Estes ensaios enzimáticos foram planejados para complementar a publicação original da descoberta (69).

Já se sabia da existência das múltiplas isoenzimas humanas, que seriam produzidas a partir de dois genes distintos, porém o número de publicações eram baixo. Todavia, começando então a estudar o assunto, percebi que havia uma grande e errônea simplificação das proteínas, a começar pela sua nomenclatura em artigos. KGA, ou *Kiney-type Glutaminase*, foi inicialmente isolada no início da década de 1970, em grande parte, pelos laboratórios dos doutores Norman Curthoys e Elling Kvamme. A outra proteína produzida pelo gene *GLS*, chamada de Glutaminase C, ou GAC, foi descrita pela primeira vez em 1999. Porém, ambas ainda eram coletiva e frequentemente ou chamadas de GLS1 em publicações, mesmo quando somente uma das duas proteínas era estudada. Inclusive, em um dos manuscritos seminais em metabolismo tumoral, publicado em 2008 pelo laboratório do Dr. Chi van Dang, a simplificação errônea foi mantida.

O trabalho desenvolvido no laboratório do Rick havia isolado GAC como alvo do inibidor 968. Todavia, eles possuíam somente o clone da KGA de rato para expressão recombinante em bactérias. Esse clone tinha sido gentilmente fornecido Curthoys. Realizei então a subclonagem de GAC de camundongo em vetor de expressão bacteriano, a partir de biblioteca de cDNA disponível no nosso laboratório.

Com sucesso, purifiquei tanto KGA, como GAC; os ensaios de atividade e inibição que se seguiram foram incorporados ao manuscrito (69). Como ainda não estava claro para nenhum de

nós como fazer a correta distinção entre KGA e GAC, ao longo desse manuscrito ambas foram indiscriminadamente referidas como “isoformas de KGA”.

Independentemente, após a conclusão do trabalho, todos estávamos automaticamente interessados em conhecer a estrutura cristalográfica do complexo GAC:968. Em 2008-2009, somente estava disponível no banco de dados PDB a estrutura do domínio glutaminase de GLS1, comum a KGA e GAC (PDB ID 3czd); lembrando que, como detalhado acima, estas são complexas proteínas multidomínios. Por meses, trabalhei incessantemente na cristalização de GAC e KGA completas e do domínio glutaminase comum à duas, visando obter a estrutura do complexo proteína:inibidor. Inicialmente, obtive inúmeros cristais do domínio glutaminase na presença de 968. Conjuntos de dados foram coletados nos CHESS (*Cornell High-Energy Synchrotron Source*). Todavia, nunca observamos a presença de 968 ligado à proteína, por meio da detecção de distribuição de densidade eletrônica característica.

Naquela época, consegui também cristalizar GAC na presença de 968 e coletar bons dados de difração no APS (*Advanced Photon Source*). Também, nunca observei a presença do inibidor ligado à proteína completa. Ainda, obtive cristais para KGA, porém, não foi possível coletar dados de difração além de 10 Å de resolução máxima.

Há décadas, se sabia que as glutaminases GLS1 (KGA e GAC) eram dependentes da presença de íons fosfato para completa ativação de suas atividades catalíticas sobre glutamina. Uma observação que fizemos quando dos ensaios de atividades para o manuscrito original, foi a de que GAC era muito mais ativa e eficiente que KGA sobre o substrato, mesmo com ambas dividindo sítios ativos idênticos e em condições análogas de fosfato. Porém, naquela ocasião, optamos por não incluir estas observações, por não estarem completamente caracterizadas. Foi nesse momento que encerrei meu pós-doutorado, para assumir a vaga de pesquisador independente no LNBio.

Já no Brasil, dedicando esforços para estabelecer as bases de meu laboratório, junto com a Sandra, foi natural a continuidade deste trabalho sobre as atividades catalíticas das glutaminase. Mais importante, estava claro para nós que GAC e KGA eram proteínas bem distintas, do ponto de vista enzimático, e mereciam ser caracterizadas como tais. Assim, nosso primeiro trabalho na área, como pesquisadores independentes, foi publicado na PNAS, no início de 2012. Neste trabalho, reportamos a clara e inédita distinção cinética entre GAC e KGA, também comparadas a LGA (uma das glutaminase expressas pelo gene *GLS2*), insensível a fosfato. Adicionalmente, demonstramos que GAC, especificamente, era a glutaminase mais adequada para suprir as elevadas demandas metabólicas em modelos agressivos de câncer de mama, e descrevemos a primeira estrutura cristalográfica de uma glutaminase contendo múltiplos domínios (29).

Ainda em 2011-2012, o mecanismo aceito para a ativação das glutaminases dependentes de fosfato, previamente estabelecido pelo Dr. Curthoys, envolvia uma transição de dímeros inativos para tetrâmeros ativos, quando na presença desse íon. Esse modelo de ativação foi tido como verdadeiro em nosso manuscrito original. Porém, ao estudarmos a fundo a transição oligomérica destas proteínas quando ativadas, percebemos que espécies bem maiores que tetrâmeros e heterogêneas em população, em termos de parâmetros hidrodinâmicos, estavam sendo formadas.

Foi então que conduzimos uma série de experimentos de microscopia eletrônica de transmissão (TEM) para avaliar visualmente a distribuição destas espécies oligoméricas. Estes estudos foram feitos em colaboração com o Dr. Rodrigo Portugal, do Laboratório Nacional de Nanotecnologia (LNNano), também no campus do CNPEM. Publicamos, em agosto de 2013, um inédito mecanismo de ativação para as glutaminases, baseado na formação de filamentos supra-tetraméricos (35). Mais ainda, demonstramos que a extensão do filamento estava diretamente relacionada aos níveis de atividade das diferentes isoenzimas. GAC, a glutaminase mais ativa,

formava os filamentos mais longos. KGA e LGA, ambas possuindo domínios ANK C-terminal, formavam filamentos curtos, com pouca atividade (KGA), ou não formavam filamentos, também não respondendo a fosfato (LGA).

Em um momento anterior, havíamos nos encontrado com o Dr. Chi van Dang em um congresso internacional da área. Na ocasião, ele nos ofereceu amostras de um outro novo inibidor de glutaminase, chamado de BPTES, também desenvolvido pelo Dr. Curthoys. Chi nos propôs que fizéssemos a determinação do mecanismo de inibição, em nível molecular. Ainda neste manuscrito de 2013, na JBC, descrevemos que BPTES era um inibidor alostérico, que causava o rompimento dos filamentos, outrora ativos.

Em meados de 2012, como consequência da nossa publicação na PNAS, fomos procurados pelo médico geneticista George Calin, do MD Anderson Cancer Center, em Houston, TX. Dr. Calin vinha se dedicando a investigar mecanismos moleculares envolvidos no desenvolvimento de câncer colorretal e havia descoberto que nesse tipo de tumor era comum a superexpressão de um longo RNA não codificador (lncRNA) conhecido como CCAT2. Seu grupo havia observado, mais especificamente, que uma variante alélica de CCAT2, com uma base guanina (G) no lugar de timina (T), estava relacionada com risco aumentado de câncer colorretal. Ele notou ainda que a troca desse nucleotídeo gerava alterações metabólicas na célula e levantou a hipótese de que lncRNA seria um regulador de glutaminase.

Nosso encontro resultou em uma colaboração que envolveu pesquisadores de sete países: Brasil, Estados Unidos, Espanha, Holanda, Croácia, Itália e Romênia. Demonstramos, por meio de diversas técnicas, que a variante alélica G de CCAT2 interage mais fortemente com um complexo proteico conhecido como CFIm, induzindo um aumento na expressão de GAC em detrimento da isoforma KGA; essa preferência por GAC favoreceria um maior consumo de glutamina pelas células tumorais, levando ao aumento da proliferação e metástase (55). De minha parte, contribuí, junto com membros de meu grupo, para a produção das CFIm recombinante e estudos bioquímicos e biofísico de sua interação com CCTA2. Nossa colaboração com o Dr. Calin ainda continua ativa, agora com a submissão de novas propostas de pesquisa, todavia ainda dentro do assunto de lncRNA e câncer.

Um aspecto das glutaminase humanas que sempre nos intrigou, é o fato de que, como mencionado na sessão anterior, estas existem na forma de várias isoenzimas em humanos e todas são proteínas multidomínios. Uma linha de pesquisa derivada em nosso laboratório investiga a estrutura e função destes, e um manuscrito foi recentemente publicado em JBC (80). Basicamente, a filogenia dessas enzimas medicamente relevantes permanece desconhecida, levando-nos a investigar sua origem e evolução. Usando sequências de glutaminas procarióticas e eucarióticas, construímos uma árvore filogenética cuja topologia sugeriu que a arquitetura multidomínios foi herdada de antepassados bacterianos, provavelmente simultaneamente com a hospedagem do endossimbionte da proto-mitocôndria. Propusemos um modelo evolutivo em que o aparecimento da isoforma enzimaticamente mais ativa, GAC, que é expressa em diversos tipos de câncer, foi um evento de retrotransposição tardia que ocorreu em peixes da classe Chondrichthyes. As repetições de ankyrina (ANK) nas glutaminases foram adquiridas no início da sua evolução. Para obter informações sobre o enovelamento ANK, resolvemos duas estruturas cristalográficas de alta resolução das repetições de ANK de ambas as isoformas, de glutaminase GLS1 (KGA) e GLS2 (GAB e LGA). Descobrimos que ANK de glutaminase formam contatos intramoleculares únicos através de dois motivos altamente conservados; Curiosamente, esse arranjo oclui uma região geralmente envolvida em interações proteína-proteína mediadas por ANK. Também resolvemos a estrutura cristalográfica de KGA completa. Coletivamente, as estruturas explicam a capacidade comprometida dessas proteínas de se montar em filamentos supra-tetraméricos cataliticamente ativos, como mostrado anteriormente para GAC.

Entre os meses de agosto de 2012 e janeiro de 2013, um aluno de mestrado sob minha orientação, trabalhando na caracterização cinética e determinação estrutural das isoenzimas GLS2, teve a oportunidade de realizar um estágio BEPE FAPESP no *Structural Genomics Consortium* (SGC), em Oxford, UK. Na ocasião, sob a orientação do Dr. Wyatt Yue, o aluno determinou a estrutura cristalográfica inédita do domínio glutaminase de GLS2, que está sendo descrita agora em um manuscrito em fase final de preparação em nosso grupo e que, de maneira inédita, estabelece esta proteína como um novo fator pró-tumoral em câncer de mama. Adicionalmente, esta interação de sucesso com o SGC abriu portas para novas colaborações e publicações com o laboratório do Dr. Yue, como será descrito em sessões posteriores.

De minha parte, essas pesquisas foram financiadas por três Auxílios Regulares FAPESP, sob minha responsabilidade: 2010/05003-0, 2012/14298-9 e 2014/20673-2. Uma dissertação de mestrado e duas teses de doutorado, sob minha orientação, foram concluídas. A lista das publicações que resultaram desta linha de pesquisa, até o momento, encontram-se a seguir. As versões completas dos manuscritos estão disponíveis nos Anexos I.1 a I.4.

- **Anexo I.1:** Cassago, A., Ferreira, A. P. S., Ferreira, I. M., Fornezari, C., Gomes, E. R. M., Greene, K. S., Pereira, H. M., Garratt, R. C., Dias, S. M. G., and **Ambrosio, A. L. B.** (2012) Mitochondrial localization and structure-based phosphate activation mechanism of Glutaminase C with implications for cancer metabolism. *Proc. Natl. Acad. Sci.* **109**, 1092–1097; **Co-autor de correspondência.**
- **Anexo I.2:** Ferreira, A. P. S., Cassago, A., Goncalves, K. d. A., Dias, M. M., Adamoski, D., Ascencao, C. F. R., Honorato, R. V., de Oliveira, J. F., Ferreira, I. M., Fornezari, C., Bettini, J., Oliveira, P. S. L., Paes Leme, A. F., Portugal, R. V., **Ambrosio, A. L. B.**, and Dias, S. M. G. (2013) Active Glutaminase C Self-assembles into a Supratetrameric Oligomer That Can Be Disrupted by an Allosteric Inhibitor. *J. Biol. Chem.* **288**, 28009–28020; **Co-autor de correspondência.**
- **Anexo I.3:** Redis, R. S., Vela, L. E., Lu, W., Ferreira de Oliveira, J., Ivan, C., Rodriguez-Aguayo, C., Adamoski, D., Pasculli, B., Taguchi, A., Chen, Y., Fernandez, A. F., Valledor, L., Van Roosbroeck, K., Chang, S., Shah, M., Kinnebrew, G., Han, L., Atlasi, Y., Cheung, L. H., Huang, G. Y., Monroig, P., Ramirez, M. S., Catela Ivkovic, T., Van, L., Ling, H., Gafà, R., Kapitanovic, S., Lanza, G., Bankson, J. A., Huang, P., Lai, S. Y., Bast, R. C., Rosenblum, M. G., Radovich, M., Ivan, M., Bartholomeusz, G., Liang, H., Fraga, M. F., Widger, W. R., Hanash, S., Berindan-Neagoe, I., Lopez-Berestein, G., **Ambrosio, A. L. B.**, Gomes Dias, S. M., and Calin, G. A. (2016) Allele-Specific Reprogramming of Cancer Metabolism by the Long Non-coding RNA CCAT2. *Mol. Cell.* **61**, 520–534;
- **Anexo I.4:** Pasquali, C. C., Islam, Z., Adamoski, D., Ferreira, I. M., Righeto, R. D., Bettini, J., Portugal, R. V., Yue, W. W., Gonzalez, A., Dias, S. M. G., and **Ambrosio, A. L. B.** (2017) The origin and evolution of human glutaminases and their atypical C-terminal ankyrin repeats. *J. Biol. Chem.* **292**, 11572–11585; **Co-autor de correspondência.**

3.2. Fatores de transcrição induzíveis por hipóxia, HIF (2011 - presente)

Células normais quiescentes captam glicose para produção de energia, precursores biossintéticos e poder redutor através de vias oxidativas, como a fosforilação oxidativa, o ciclo do ácido cítrico (também conhecido como ciclo de Krebs), e a via das pentoses fosfato, além da reciclagem de doadores de elétrons como NADH e NADPH. Em neoplasmas, no entanto, a proliferação acelerada tem sido associada a um fenômeno descrito como glicólise aeróbica. Neste caso, a maioria do carbono derivado do consumo da glicose é secretada na forma de lactato mesmo em situações de alta disponibilidade de oxigênio no microambiente celular. Este fenômeno foi observado pela primeira vez em meados da década de 20 pelo fisiologista alemão Otto Warburg, que atribuiu essa alta produção de lactato a um defeito incomum das mitocôndrias (4, 81, 82). Posteriormente suas observações foram chamadas de O “efeito Warburg”, e já foi demonstrado em diversos tipos de câncer com taxas de glicólise 200 vezes maior do que em células normais, devido ao aumento concomitante na captação e metabolismo de glicose. Essa característica é explorada em exame clínico, que utiliza glicose marcada e permite a detecção de alguns tumores, a técnica é conhecida como do FDG-PET (83). O desenvolvimento do câncer está intrinsecamente associado a reprogramação do metabolismo energético através da regulação de genes responsáveis pelo transporte de glicose, bem como da via glicolítica, de tal maneira que esse fenômeno é hoje aceito como um dos pilares de transformação celular associada ao câncer (HANAHAHAN E WEINBERG, 2011).

O efeito da glicólise aeróbica é somente 5% eficiente em produção de ATP quando comparada com a fosforilação oxidativa, no entanto, deve-se considerar que a super-ativação desta via em células cancerosas em alto estado de proliferação e elevada captação de glicose em uma taxa glicolítica alta o suficiente, faz com que a porcentagem final de ATP produzida pela glicólise possa exceder à produzida pela via oxidativa e em contraste com a respiração mitocondrial, a geração de energia pela glicólise é independente da presença de oxigênio no ambiente celular, conferindo assim aos tumores a capacidade de sobrevivência e a possibilidade de migração para áreas hipóxicas.

Em relação a difusão do oxigênio dos vasos sanguíneos as células, isto ocorre em um limite entre 100-200 μm de distância para a adequada oxigenação celular. Em tumores, as células rapidamente se proliferam e alcançam uma alta densidade celular, as quais facilmente esgotam o suprimento de nutrientes e oxigênio a partir da vascularização normal, superando assim o alcance e a capacidade de irrigação sanguínea. Esse cenário é um evento muito comum no processo canceroso, e devido à alta densidade celular, rapidamente levam à condições de moderada à severa hipóxia. Diversos mecanismos adaptativos contra hipóxia foram desenvolvidos pelas células e organismos. Um dos exemplos que podemos citar, é o aumento da transcrição do gene da eritropoietina que regula a produção de glóbulos vermelhos e assim elevar a oxigenação sanguínea (84, 85).

A homeostase do oxigênio é um dos processos mais importantes a serem regulados, pois tanto o excesso como sua escassez podem levar à toxicidade através de estresse oxidativo ou da morte celular programada. Em resposta à manutenção da homeostase do oxigênio, ocorre o acúmulo nuclear do fator de transcrição HIF-1 (*Hypoxia-induced factor 1*), que medeia as respostas adaptativas às mudanças nos níveis de oxigênio em tecidos e exerce um papel chave nesse processo. HIF-1 foi inicialmente identificada a aproximadamente 25 anos, através de sua atividade de ligação à DNA em extratos nucleares que foram sujeitos a hipóxia (86), e sua síntese é regulada pela ativação da fosfatidilinositol 3 quinase (PI3K) e pela via da MAPK (*Mitogen-activated protein kinase*) (87).

Atualmente são conhecidos mais de 100 genes cuja expressão é controlada pela HIF-1. Isso corresponde a aproximadamente 5% do genoma e esses genes podem ser agrupados em grandes grupos funcionais, como por exemplo, HIF-1 é responsável pela transcrição dos genes

relacionados a proliferação celular, sobrevivência celular, motilidade, metabolismo energético, formação de metástases, eritropoiese e angiogênese, remodelamento de matriz extracelular, regulação do pH, apoptose entre outros (87, 88). A hipóxia é um forte sinal para angiogênese em tumores e um dos principais alvos diretos da HIF-1 é a transcrição do VEGF (*Vascular Endothelial growth factor*) que rapidamente pode expandir regiões pouco vascularizadas em tumores sólidos através do recrutamento de células endoteliais e estimular a sobrevivência e proliferação celular.

Uma via metabólica muito importante em processo canceroso é a via do metabolismo energético, da qual HIF regula muitos dos genes envolvidos nessa via coordenando então diversas etapas, das quais podemos citar o aumento dos transportadores de glicose 1 e 3 (GLUT), hexokinases 1 e 2 (HK), fosfofrutokinase (PFK), lactato-desidrogenase (LDH), fosfoglicerato-kinase 1 (PGK), glicose fosfato isomerase (PGI), piruvato kinase M, dentre muitos outros (89–91). O aumento da expressão desses genes, leva diretamente ao aumento da captação e metabolismo de glicose, suprimindo assim a elevada demanda energética de células em alto estado proliferativo.

Outros processos além da hipóxia podem levar a estabilização de HIF-1, uma delas são mutações em genes supressores de tumor como o *von Hippel-Lindau* impede que HIF seja degradada em condições de normoxia, o que leva a formação de tumores de células renais (RCC), cerebelo, retina, feocromocitoma, tumores pancreáticos de ilhotas e saco endolinfático (ELSTs). Outras mutações também levam a estabilidade de HIF, como os genes de p53, Rb, Bcl2, Myc, ARF e Ras. HIF pode ainda ser estabilizada e ativada por outros processos celular, por exemplo, através dos hormônios e citocinas inflamatórias, pela via da PI3K/Akt, pela atividade das quinases dependentes de ciclina (CDKs) levando a aumento da expressão ou degradação lisossomal de HIF e através da regulação de micro-RNAs levando ao aumento ou redução da sua expressão, como miR-20b que suprime a HIF-1 α em células de osteossarcoma e portanto, baixos níveis de miR-20b levam ao estímulo e atividade de HIF (84, 92).

De modo geral, independente se HIF é regulada em condições de hipóxia ou através de outros processos celulares, é irrefutável que o controle da expressão de tantos genes por HIF-1 é considerado o exemplo mais extenso de controle metabólico coordenado em nível transcricional já descrito em qualquer organismo (93). Desta forma, a presença de HIF-1 é sempre associada com mau prognóstico e altos níveis de mortalidade de pacientes com câncer, à resistência a radio e quimioterapias, ao aumento do potencial metastático e recentemente foi tida como um dos cinco marcadores para análise de imuno-histoquímica para câncer de mama (84, 85).

HIF-1 é expressa em todos os tipos celulares e funcionalmente é um heterodímero composto das subunidades HIF-1 α (*Homo sapiens*, 826 aminoácidos) e HIF-1 β também conhecida como ARNT (*Aryl Hydrocarbon Receptor Nuclear Translocator*) (*Homo sapiens*, 789 aminoácidos), pertencentes à família de fatores de transcrição bHLH-PAS (*basic Helix-Loop-Helix PAS*) que se ligam à elementos responsivos, HRE, em genes alvo, cuja sequência consenso é 5' – RCGTG – 3' (onde R pode ser A ou G).

A subunidade HIF-1 α tem uma meia-vida de aproximadamente 5 minutos e é altamente regulada pela tensão intracelular de oxigênio (94). A regulação direta da estabilidade e a ativação de HIF-1 se dá principalmente por modificações pós-traducionais, onde a hidroxilação das prolinas 402 e 564 pelas prolin hidroxilases (PHDs) leva à rápida degradação de HIF através da degradação proteossomal. Além disso a hidroxilação do resíduo de asparagina 803 pelo fator FIH (*inhibitor HIF1*) resulta na inabilidade de HIF ligar-se a proteínas transativadoras. O resumo do mecanismo de regulação da HIF-1 α em condições de normoxia e sua atividade em condições hipóxicas é ilustrado na figura 5 abaixo.

Figura 5. Mecanismo de regulação da HIF-1α e sua atividade. Em condições de normoxia (parte superior), HIF sofre regulação pós traducional através da hidroxilação de dois resíduos de prolina, seguido de reconhecimento do complexo VHL destinando-a a degradação pelo 26s proteossomo. Na parte inferior da figura observamos o mecanismo de atividade da HIF em condições de hipóxia, onde a prolil-hidroxilação é inibida pela ausência de oxigênio e a subunidade α das HIFs irá se heterodimerizar com HIF-1β, o complexo no núcleo reconhece os genes que contêm os elementos responsivos a hipóxia (HRE), o que levará a transcrição de mais de 100 genes no genoma. Imagem adaptada de <https://research.medicine.umich.edu/office-research/biomedical-research-core-facilities/dna-sequencing> e <http://www.expertreviews.org/>.

Ao todo, são conhecidos três genes distintos que codificam para subunidades α: HIF-1α, contendo 826 aa, HIF-2α, contendo 870 aa e HIF-3α com 669 aa, sendo as HIF-1α e -2α as mais estudadas. Todas possuem semelhanças na organização estrutural quanto aos seus domínios funcionais, detalhados a seguir, além do domínio de interação com DNA (bHLH). As HIF apresentam dois domínios do tipo PAS (família Per-ARNT-Sim), chamados de PASa (mais próxima da região N-terminal) e PASb; estes domínios são amplamente encontrados em proteínas envolvidas na transmissão de sinal, funcionando como módulo sensorial da tensão de oxigênio, potencial redox ou intensidade de luz, já identificado em mais de 2000 proteínas entre os mais diversos organismos. Demonstrou-se ainda esse domínio como sendo um elemento de interação proteína-proteína (95).

Uma peculiaridade das subunidades α é a presença do domínio de degradação dependente de oxigênio (ODDD), onde estão os resíduos de prolina que em condições de normoxia serão hidroxilados por uma família de proteínas conhecida como Prolil-hidroxilases (PHDs), a prolil-hidroxilação levará então a ubiquitinação de HIF-α pela proteína supressora de tumor pVHL (*von Hippel-Lindal*) e posteriormente ocorre a interação com o complexo da E3 ubiquitina ligase destinando então as subunidades α para degradação através do complexo proteossomal 26s(96).

As subunidades 1 e 2 apresentam ainda dois domínios de transativação (estímulo da transcrição), designados NTAD (na porção n-terminal) e CTAD (na porção c-terminal), além de sequências consenso de sinalização nuclear (NLS). Apenas NTAD é encontrado na subunidade 3. Apesar da alta conservação de domínios funcionais, HIF-1 α compartilha apenas 43% e 36% de identidade sequencial com HIF-2 α e HIF-3 α , respectivamente enquanto HIF-2 α e HIF-3 α são apenas 37% idênticas. A subunidade HIF-1 β (789 aa, em média 30% idêntica à subunidades α) é compreendida pelos domínios bHLH, PASa, PASb e possui somente um domínio de transativação C-terminal, CTAD. Todas as características estruturais das três subunidades α , como descritas acima, estão ilustradas na Figura 6.

Figura 6. Representação esquemática da estrutura dos domínios que constituem as isoformas α da HIF em humano. Os domínios de importância funcional estão representados da seguinte forma: bHLH - domínio hélice-loop-hélice básico de ligação à sequências consenso HRE, PAS - domínio de homologia Per-ARNT-Sim com repetições internas a e b, ODD - domínio de degradação dependente de oxigênio, TAD - domínios de transativação N- e C- terminais respectivamente. Sequências de sinalização nuclear, NLS, são encontradas nas subunidades 1 α e 2 α . Adaptado de: Costa, M. e Ke, Q. *Molecular Pharmacology*, 2006.

O mecanismo de ativação da HIF-1 α , além de requerer a heterodimerização com a subunidade β , requer a interação com proteínas co-ativadoras diversas, como CBP/p300, SRC-1 (*Steroid Receptor Coactivator-1*), Smad3 (*Mothers against decapentaplegic homolog 3*), HNF4 (*Hepatocyte Nuclear Factor 4*), AP-1 (*Activator protein-1*), Tie-2 (*V-ets erythroblastosis virus E26 oncogene homolog 1*) (97). Esta interação ocorre através do domínio C-TAD, que em particular, foi demonstrada como essencial para ativar a transcrição de genes específicos regulados por HIF-1 (98).

A subunidade HIF-2 α ativa a transcrição de um grande número de genes, dentre os quais vários são igualmente regulados por HIF-1 α . No entanto, alguns deles são expressos somente pela HIF-2 α , como o gene da EPO (eritropoietina), *TGFA* (fator de crescimento) e *ANGPT2* (relacionado ao remodelamento de vasos sanguíneos) (99). Keith et al., 2012, observaram em modelos animais que, quando silenciado o gene da HIF-2 α , ocorreu o crescimento e progressão tumoral. Isso indica que HIF-1 α e HIF-2 α , desempenham papéis diferentes no desenvolvimento e progressão do câncer (99, 100).

Diferentemente de HIF-1 α e HIF-2 α , a subunidade HIF-3 α é um pouco restrita quanto a expressão, comumente presente em cérebro, cerebelo, coração, pulmão, rim e músculo esquelético (101, 102). HIF-3 α foi a última subunidade a ser identificada, em 1998, e é capaz de heterodimerização com a HIF- β em humanos e o complexo formado também se liga aos HREs. Recentemente em células de hepatoma, HIF-3 α foi caracterizada por retardar o aumento celular, reduzir a vascularização e por romper o complexo formado entre HIF-1 α -ARNT-DNA (101, 103). Não temos conhecimento de estudos que correlacionam as subunidades -3 α e -2 α em termos de antagonização ou amplificação de função transcricional.

A subunidade β da HIF-1 é normalmente encontrada em todos os tecidos, tanto em situações de normoxia quanto em hipóxia. Em condições normais de oxigênio (normoxia), ela está envolvida com a regulação do metabolismo de agentes xenobióticos, neste caso formando um complexo com a proteína AhR (*Aryl hydrocarbon Receptor*) (104–106). Já em condições de hipóxia, se complexa com as subunidades α da HIF e atuam na transcrição dos genes já mencionados acima.

Das três subunidades α da HIF, 3α foi a última delas a ser identificada e é distinta das demais em diversos sentidos. Por exemplo, o gene que codifica para HIF- 3α é regulado por HIF- 1α e não por HIF- 2α , onde apresenta seu ápice de expressão entre 8 à 16 horas em hipóxia, enquanto HIF- 1α tem seu ápice de expressão entre 4 à 8 horas (102, 107, 108). Em sua sequência de aminoácidos, HIF- 3α apresenta somente uma prolina (P492) que é hidroxilada pelas PHD's, e posteriormente levam a ligação ao pVHL ao contrário das demais HIFs que são hidroxiladas em dois resíduos de prolina. HIF- 3α é encontrada no citoplasma e no núcleo sobre condições de normoxia, o que indica que somente a hidroxilação de uma prolina não seja suficiente para levar a sua degradação via proteossomo (108, 109). HIF- 3α é a menor das subunidades devido à ausência da região do CTAD, o que a impede a atividade de transativação com proteínas como p300, que é essencial para a atividade transcricional. A organização do gene da *HIF3 α* consiste de 19 éxons e três posições alternativas para início da transcrição são localizados nos exons 1a, 1b e 1c e, desta forma é exclusivamente suscetível a regulação pós traducional (109). São preditas 10 isoformas de *splicing* alternativo das quais seis delas foram confirmadas em nível proteico, sendo a IPAS (*inhibitory PAS domain protein*) a primeira isoforma identificada em camundongo, e seu homólogo em humano é chamado de HIF- $3\alpha 4$. Além de IPAS, outras cinco isoformas variantes de *splicing* foram identificadas para HIF- 3α , sendo referidas como HIF- $3\alpha 1$, HIF- $3\alpha 3$, HIF- $3\alpha 5$, HIF- $3\alpha 7$ e HIF- $3\alpha 9$ e esta última sendo a mais longa de todas com 669 aa diferindo da isoforma $3\alpha 1$ por alguns aminoácidos presente no N-terminal (108). A única região comum entre todos *splicings* é o domínio PASb e, de forma geral as isoformas - 3α diferem grandemente no N- e no C-terminal, onde ocorre a perda parcial ou total no domínio bHLH (109, 110). Enquanto outras sofrem perda total ou parcial do C-Terminal incluindo o ODD e NTAD (- $3\alpha 4$). A Figura 7 abaixo ilustra os domínios presentes em cada isoforma, indicando a posição onde ocorrem variações devido aos *splicings*, em amarelo.

Figura 7. Representação esquemática dos domínios que constituem HIF- 3α e suas variantes por *splicing* ou uso de promotores alternativos (os locais de ambos eventos são marcados em amarelo). Adaptado de (107). Os *splicings* contêm os domínios bHLH - domínio hélice-loop-hélice básico, PAS - domínio de homologia Per-ARNT-Sim com repetições internas 1 e 2, PAC - região associada aos Pas2 (PASb = PAS2 + PAC), ODD - domínio de degradação dependente de oxigênio, NTAD - domínios de transativação N-terminal. A isoformas 1 é tida como a isoformas canônica, em termos de numeração e composição. Todas as variações em regiões especificam geradas por *splicing* alternativo, que determinam as outras isoformas, são circundadas em amarelo.

Quanto à sua expressão, varia de acordo com célula e tecido específico, em humanos seus transcritos estão presentes em diversos tecidos como coração, cérebro, placenta, fígado, rim, pulmão, pâncreas, músculo esquelético e cartilagem em diferentes padrões (108). Em câncer, sua expressão foi reportada em linhagens celulares de Ad4 e Caki-1 (rim), SKOV-3 (ovário), HeLa (útero), DU45 (próstata), MSTO e MTT (mesotelioma) e A549 (pulmão) (108–110).

A isoforma 3 α 4 sofre um *splicing* na metade do domínio PASb acarretando uma mudança de 50% da sequência de aminoácidos quando comparada com as outras isoformas. Ela é também a melhor caracterizada das isoformas de *splicing*, e sua atividade é sempre reportada como sendo um regulador negativo das demais subunidades *alpha* das HIFs. Em camundongos é mais expressa em células purkinje no cerebelo e no epitélio da córnea. Sua expressão na córnea correlata com o fato dela interagir diretamente com HIF-1 α e HIF- β , em condições de hipóxia, como durante o sono, onde a córnea é recoberta pela pálpebra, formando assim heterodímeros inativos impedindo então o complexo HIF-1 α :HIF- β de transcrever genes como o VEGF, mantendo então o fenótipo avascular da córnea (101, 110). Já em câncer renal (RCC células), a ligação de HIF-3 α 4 interage com HIF-2 α levando também a formação de complexos inativos, reduzindo a expressão de genes envolvidos em hipóxia e progressão do câncer, sendo sugerido assim como uma opção terapêutica para diversos tipos de câncer onde HIF-1 α e -2 α são expressas (111). Estruturalmente a isoforma 3 α 4 contém o domínio de ligação ao DNA (bHLH), dois domínios tipo PAS (PASa e PASb), faltando o domínio ODD e N-TAD e, portanto, é insensível a redução dos níveis intracelulares de oxigênio, ao contrário da isoforma -3 α 9 que contém todos domínios funcionais, bHLH, PASa e PASb, ODD e NTAD. De todas as isoformas HIF-3 α , a IPAS é a mais estudada do ponto de vista celular, e em relação às outras isoformas, em muitos trabalhos é reportada apenas como HIF-3 α sem fazer especificidade sobre qual das isoformas se trata, dificultando assim a comparação de atividade para cada um dos *splicing* gerados.

Desde sua descoberta, HIF-3 α sempre tem sido reportada como sendo regulador negativo para as demais isoformas e sua atividade transcricional permaneceu desconhecida. Esse fato é em parte devido à ausência do domínio de transativação CTAD o que reduziria sua atividade transcricional e impediria a ligação a proteínas transativadoras. No entanto, esse estereotipo foi recentemente quebrado e a atividade transcricional da isoforma HIF-3 α 9 foi confirmada em *zebrafish*, no qual regulou a expressão de 58 genes exclusivos como o *sqrdl*, *mclb*, *zp3v2*, *igfbp-1b*, *redd1*, *mlp3c*, genes envolvidos na via de sinalização *Jak-Stat* e nas vias de sinalização do receptor NOD-like. Além disso, houve a expressão de outros 97 genes também regulados pela HIF-1 α (103, 108, 112, 113). Além deste genes, foi demonstrada a atividade de transcrição a genes não relacionados diretamente a hipóxia o que levou ao remodelamento do epitélio pulmonar através da ligação ao elemento responsivo bem como a indução da expressão no gene SOX-2, que desempenha um papel importante na manutenção das propriedades de células de câncer pluripotentes (114, 115).

Recentemente foi reportada a atividade não-transcricional de HIF-3 α 1 em células de tumor colorretal onde está superexpressa tanto em camundongo quanto em tecidos de pacientes, e sua localização celular é reportada somente no citosol. Em decorrência disso, autores observaram que houve o crescimento celular em tamanho e aumento na quantidade de colônias formadas através da ativação da via de sinalização JAK-STAT3, que é conhecida por promover proliferação e sobrevivência celular. No entanto o mecanismo pela qual HIF-3 α 1 ativa essa via ainda não foi elucidada (116).

A existência de tantas isoformas tornou o estudo da HIF-3 α ainda mais desafiador no sentido caracterizar as suas respectivas expressões e funções celulares, qual forma delas é a mais ativa ou se as condições de hipóxia levam a diferentes papéis cada uma. De acordo com as

características estruturais das isoformas confirmadas em nível proteico, o domínio PAS é um fator comum entre elas, exceto para a isoforma -3α4 que possui uma sequência diferente neste domínio. De modo a obter informações estruturais da HIF-3α que possam auxiliar na compreensão de sua atividade relacionada a transcrição de genes e também nas novas funções que têm sido associadas a ela, nosso interesse maior foi o de desenvolver um estudo estrutural que envolvesse o domínio PASb da HIF-3α que gerassem resultados que pudessem ser entendidos e aplicados a todas as isoformas.

A atividade biológica e a regulação dos processos celulares ocorrem através da interação entre proteínas ou mesmo de proteínas com íons, pequenas moléculas orgânicas ou inorgânicas e com macromoléculas, como o DNA e o RNA. Um dos mediadores destas interações são os domínios do tipo PAS (Per-ARNT-Sim). Essa nomenclatura foi estabelecida após a verificação de uma região conservada entre a proteína do ciclo circadiano (*PERIOD*) de drosófila, a proteína de vertebrados *Aryl Hydrocarbon Nuclear Translocator* (ARNT) e a proteína reguladora do desenvolvimento *single-minded* de drosófilas (*SIM*) originando a nomenclatura PAS (Henry & Crosson, 2011; Möglich, Ayers, & Moffat, 2009).

Estes domínios são encontrados frequentemente nos três reinos da vida (bactérias, archaea e eucariotos) com uma cópia no genoma ou em cópias múltiplas (em *tandem*) dentro de uma única proteína (117). Ao longo da evolução, os domínios PAS foram selecionados pela grande habilidade de se ligar a diferentes cofatores e ligantes, tornando-se sensores capazes de perceber parâmetros ambientais, como a incidência de luz, pressão parcial de um gás e potencial redox. Além disto, existem muitos domínios PAS que não necessitam de íons ou cofatores para exercer sua função celular (117, 118).

A identidade sequencial nestas proteínas é muito baixa (em torno de 20%) quando comparadas às estruturas primárias, entre as sequências de humano, bactéria (*E. coli*), alga do tipo clamidomonas e plantas (*Arabidopsis*), que apresentam 12, 22 e 18% de identidade, respectivamente. No entanto as características estruturais são bastante conservadas e, sua estrutura terciária é caracterizada pelo arranjo antiparalelo de cinco folhas β enoveladas partindo no N-terminal para o C-Terminal, composta aproximadamente por 100 a 150 aminoácidos (118). Um exemplo típico de sua topologia pode ser visualizado na figura 8 onde observamos o arranjo de topologia e suas estruturas terciárias.

Figura 8. Estrutura de topologia e cristalográfica dos domínios tipo PAS, ilustrando a comparação entre os organismos Humano, *E.coli*, *Chlamydomonas reinhardtii* e *Arabidopsis thaliana*.

As interações proteína:proteína nos domínio tipo PAS ocorrem normalmente através da superfície formada pelas folhas β , enquanto que os diferentes tipos de ligantes (pequenas moléculas) que interagem com estes domínios ficam abrigados em bolsões hidrofóbicos formados entre as α -hélices e as folhas beta (117, 120). No caso das HIF's, são encontrados dois domínios PAS em tandem, denominados PASa e PASb, e até o presente momento, não foi identificada a necessidade de cofatores ou ligação com íons para sua atividade funcional, ocorrendo simplesmente a heterodimerização através dos domínios PAS com a HIF-1 β ou ARNT (*aryl hydrocarbon receptor nuclear translocator*).

3.2.1. Contribuições do candidato na área.

Em meados de 2010, com o bom andamento das pesquisas em glutaminases, senti a necessidade de começar a expandir os assuntos do laboratório, com foco em estudos estruturais. Como mencionei em meu memorial, parte do meu projeto de doutorado previa a cristalização do receptor nuclear PPAR γ humano (um fator de transcrição) em complexo com fragmentos de seu elemento responsivo. Apesar de, durante o doutoramento, não ter conseguido alcançar tal objetivo, ainda estava motivado a tentar cristalizar um complexo proteína:DNA.

Ao estudar as revisões que eram frequentemente publicadas, ficou claro para mim que HIF seria um alvo interessantíssimo, com bastante potencial de descobertas inéditas e coerente com o tema do laboratório. Naquela ocasião, existiam somente estruturas cristalográficas do domínio PASb das subunidades 1 α ou 2 α em complexo com o domínio equivalente em 1 β . Durante 2011 e 2012, como tema de uma dissertação de mestrado de uma aluna, inúmeros esforços foram dedicados para o objetivo de cristalizar o heterodímero de HIF com seu elemento responsivo. Conseguimos purificar diversas construções das duas proteínas, combinando diferentes domínios. Adquirimos também vários oligonucleotídeos da sequência alvo de HIF. Todavia, o sucesso na cristalização não foi possível.

Com a aproximação do final do projeto de mestrado, no início de 2013, decidimos que uma alternativa mais segura para a conclusão da dissertação seria a cristalização do domínio PASb de HIF-3 α humana, que ainda continuava desconhecida. A construção da proteína, para expressão em sistema bacteriano, e suas respectivas purificação e cristalização foram rapidamente alcançadas com sucesso. Dados de difração a altíssima resolução para esses cristais foram coletados em setembro de 2013, no síncrotron PETRA III, na Alemanha. Para nossa completa surpresa, após a conclusão da construção do modelo atômica, observamos uma cavidade bastante volumosa ($\sim 500 \text{ \AA}^3$), dentro da qual se encontrava uma molécula de lipídeo bacteriano.

Os próximos meses, já durante o doutoramento da aluna, foram dedicados para a caracterização do(s) lipídeo(s) que estavam interagindo com a proteína recombinante. Ensaios *in vitro* mostraram que a proteína é capaz de se ligar a diferentes tipos de lipídeos, os quais foram submetidos à análise por espectrometria de massas através da extração da fase orgânica da amostra; foram identificados dois liso-fosfolipídeos. De modo a explorar a cavidade hidrofóbica presente na estrutura e identificar outros lipídeos que possam também se ligar a proteína, realizamos ensaio de delipidação e titulação de ácidos graxos livres com diferentes comprimentos da cadeia carbônica e níveis de insaturação, que pudessem estar presentes no organismo humano. Como resultado observamos uma alta afinidade com ácidos graxos oleico e linoleico, ambos com alta afinidade, na faixa de nanomolar.

O trabalho foi publicado em agosto de 2015 (121). Esta é a primeira, e até hoje, a única, descrição de um ligante intracelular não-covalente de HIF. Pode, possivelmente, ditar a expressão do gene dependente em resposta à metabolismo biossintético e/ou energético, tal como a acumulação ou depleção de lipídeos, em exposição à hipóxia. Mais ainda, estes

resultados evidenciam, de maneira inédita, que um domínio PAS eucariótico pode servir como um sensor para os níveis intracelulares de lipídeos.

De minha parte, essas pesquisas foram financiadas por dois Auxílios Regulares FAPESP, sob minha responsabilidade: 2010/05003-0 e 2012/14298-9. Uma dissertação de mestrado e uma tese de doutorado, sob minha orientação, foram concluídas. Atualmente está em andamento uma outra dissertação de mestrado, cuja proposta de pesquisa é dedicada ao estabelecimento dos modelos celulares para o estudo de quais lipídeos interagem com a proteína e descoberta dos potenciais efeitos dessa interação na regulação da expressão genica mediada por HIF, como colocado acima. Já estamos começando a realizar experimentos de espectrometria de massas para detectar/identificar potenciais lipídeos na proteína imunoprecipitada. Sandra é co-orientadora neste projeto. A publicação que resultou desta linha de pesquisa, até o momento, encontra-se listada a seguir. A versão completa do manuscrito está disponível no Anexos II.1.

- **Anexo II.1:** Fala, A. M., Oliveira, J. F., Adamoski, D., Aricetti, J. A., Dias, M. M., Dias, M. V. B., Sforça, M. L., Lopes-de-Oliveira, P. S., Rocco, S. A., Caldana, C., Dias, S. M. G., and **Ambrosio, A. L. B.** (2015) Unsaturated fatty acids as high-affinity ligands of the C-terminal Per-ARNT-Sim domain from the Hypoxia-inducible factor 3 α . *Scientific Reports* **5**, 12698.
Co-autor de correspondência.

3.3.Carreador mitocondrial de piruvato, MPC (2013 – presente)

The processing of pyruvate is a critical branch of carbon metabolism within the cells, both from the standpoint of energy and biosynthesis. Therefore, pyruvate gaining access to the mitochondria is an essential step that provides the cell with its energy currency, ATP, besides providing for the carbon skeleton for the synthesis of diverse molecules. Although the existence of a carrier for pyruvate has been anticipated for more than 40 years (122), its molecular identity remained unknown until recently.

For the cytosolic pyruvate to have access to the mitochondrial matrix, three layers must be crossed: the outer mitochondrial membrane (OMM), the intermembrane space and the inner mitochondrial membrane (IMM). The transport through the outer mitochondrial membrane is easily accomplished by major non-selective channels, since this membrane is completely permeable to small molecules and ions. The movement across the inner mitochondrial membrane is, on the other hand, much more restrictive and typically involves the presence of specific transporters, which lead to the question of how pyruvate can go through the inner mitochondrial membrane (figure 9).

Figure 9: Cellular metabolism of pyruvate. Carrier of the mitochondrial pyruvate (MPC), highlighted in red circle, is located at the IMM and responsible for the import of pyruvate from the intermembrane space to the mitochondrial matrix. Adapted from (123).

In June 2012, two papers published simultaneously in *Science* by independent research groups, identified two internal mitochondrial membrane (IMM) proteins, MPC1 and MPC2, as essential for the transport of pyruvate in yeast (*S. cerevisiae*), *Drosophila* and humans (124, 125). In yeast, a third member, MPC3, has also been identified. MPC2 and MPC3 share 79% sequence identity to each other and there is evidence that they are products of a recent gene duplication event. These proteins have three predicted transmembrane helices (124) (figure 10). Although each of the subunits have about 15 kDa of molecular weight (or about 130 amino acids), the complex is suggested to possess 150 kDa, indicating a decamer, but its exact stoichiometry is

not yet known. MPC1 cannot be a part of this complex without MPC2, whereas MPC2 can form the complex without MPC1 (125). This indicates that MPC2 is the major structural unit of the multimeric complex.

Mutant yeast strains, *mpc1Δ* and *mpc2Δ*, deleted individually for each subunit, showed growth defects in medium lacking leucine, in glucose medium and in glycerol medium [Bricker DK, et al. 2012]. Also, *mpc1Δ* and *mpc2Δ mpc3Δ* cells grew more slowly in amino acid-free medium (124), the growth defect being rescued by leucine or valine or both. *mpc2Δ* had somewhat reduced growth, while *mpc3Δ* showed no phenotype in amino acid free medium. *mpc1Δ* phenotype was rescued by yeast, *Drosophila* and human MPC1 (124, 125). *mpc2Δ mpc3Δ* phenotype was rescued in yeast by either MPC2 or MPC3 (124). Mutant *Drosophila* strains lacking MPC1 were defective in carbohydrate metabolism, and were rescued with wild-type MPC1 (125). These mutant strains showed elevated levels of pyruvate and amino acids that can interconvert with glycolytic intermediates, and depleted levels of acetyl CoA and amino acids that can interconvert with TCA cycle intermediates. Similarly, *mpc1Δ* and *mpc2Δ* yeast strains had elevated levels of pyruvate and CoA and depleted levels of malate and acetyl CoA. This indicates that the mutants are unable to convert cytosolic pyruvate to mitochondrial acetyl CoA to drive the TCA cycle.

A Mpc1

Mpc2

B

Figure 10: (A) Sequence alignment of MPC1 and MPC2 between several organisms, highlighting conserved transmembrane regions, delimited by the red boxes. (B) Representation of the transmembrane regions (TM1, TM2 and TM3) and predicted orientation in the membrane for MPC1 and MPC2, as predicted by MEMSAT3 [5]. Figures adapted from [Bricker DK, et al. 2012].

Herzig *et al.* have found that less than 2% of the amount of valine and leucine metabolized by the WT is metabolized in the mutant yeast strains (125). They found this to be the result of impairment on the activity of the lipoyl-dependent complexes such as pyruvate dehydrogenase and α -ketoglutarate dehydrogenase in *mpc1 Δ* , *mpc2 Δ* and *mpc2 Δ 3 Δ* cells grown in amino acid free medium. They determined the defective step in the lipoic acid synthesis pathway in *mpc1 Δ* cells to be upstream to acetyl CoA metabolism. The phenotype was similar to pyruvate dehydrogenase deficient cells. Since pyruvate dehydrogenase activity was normal in *mpc1 Δ* cells in rich medium (124, 125), MPC proteins seemed to act upstream of pyruvate dehydrogenase, proving its involvement with the transport of pyruvate from the cytosol to the mitochondria.

Isolated mitochondria from *mpc1 Δ* , *mpc3 Δ* and *mpc2 Δ mpc3 Δ* cells grown in lactate medium showed decreased pyruvate uptake compared to WT despite a normal mitochondrial membrane potential (124). Isolated mitochondria from *mpc1 Δ* cells were also shown to be defective in pyruvate uptake by Bricker *et al.* (125). Pyruvate transport, however, was normal in *mpc2 Δ* mitochondria (124). The difference in sensitivity of *mpc2 Δ* and *mpc3 Δ* strains in amino acid-free medium and lactate medium was explained by the discovery that in amino acid free medium yeast expressed mainly MPC2 whereas in lactate medium they mainly expressed MPC3. Thus, it seems likely that MPC2 and MPC3 have different non-redundant functions under different growth conditions.

Heterologous MPC1 and MPC2 from mouse functionally complement each other in *mpc* deleted yeast strains. Depletion of either MPC1 or MPC2 in mouse fibroblasts resulted in slight decrease in pyruvate-driven oxygen consumption and a stronger decrease in the presence of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), which stimulates maximal respiration (125). This suggests that murine MPC1 and MPC2 function in pyruvate transport similar to yeast and *Drosophila*. When MPC1 and MPC2 from mouse were coexpressed in the bacterium *Lactococcus lactis*, four-fold pyruvate uptake was detected compared to the empty vector control or expression with either MPC alone (124). This pyruvate uptake depended on a proton electrochemical gradient, and was sensitive to the mitochondrial pyruvate carrier inhibitor UK5099.

Families with defective mitochondrial pyruvate transport have mutations in their DNA sequences, which result in the Leu79His and Arg97Trp substitutions (125). Leu 79 is conserved amongst the MPC1 orthologs, while Arg 97 is conserved amongst the orthologs of both MPC1 and MPC2. Cells from the affected individuals had impaired pyruvate-driven oxygen consumption. Expression of WT MPC1 in these cells completely or partially rescued the pyruvate oxidation defect. Leu79His and Arg97Trp alleles of MPC1 were much less effective in rescuing yeast *mpc1 Δ* growth defect compared to human WT MPC1, suggesting that MPC1 function is evolutionarily conserved from yeast to man.

Concerning to the molecular aspects of the MPC, as previously mentioned, it has been proposed that the complex has a molecular weight of 150 KDa, much bigger than the molecular weight of the MPC1 and MPC2 monomers, based on its migration pattern on non-denaturing electrophoresis (125). However, this conclusion is still controversial, since such a technique is influenced mainly by the shape and net charge of the molecule rather than its molecular size. A proper experimental approach would be required for a reliable determination of the molecular size of the complex, such as analytical ultra-centrifugation and/or size-exclusion couple to multi-angle static light scattering, which could only be achieved if either endogenous or recombinant MPC were purified from membrane fractions. Another open question is the relative stoichiometry of the two subunits.

In this context, we proposed this research in our lab which aims to contribute towards the understanding of the underlying mechanisms of pyruvate shuttling across the mitochondrial membrane; this is a basic and crucial mechanism for cell energy and biosynthetic pathways. The first question we would like to approach is related to the molecule assembly of the MPC, in regards to its size and subunit composition. Our hope is that once we can reconstitute the functional MPC complex *in vitro*, structural studies can also be performed, such as cryo-electron microscopy and x-ray crystallography, with the intent of providing the structural basis for pyruvate shuttling across the complex. Divakaruni and coworkers (126) reported that clinically relevant concentrations of thiazolidinediones (TZDs) are capable of strongly and specifically inhibit the activity of mitochondrial pyruvate carrier in a variety of cell types. The TZDs, also known as glitazones, were introduced in the late 1990s and are a class of medications used in the treatment of diabetes mellitus type 2, and have long been believed to act specifically on the peroxisome proliferator-activated receptors (PPAR). We also set as a goal for this project to contribute to the understanding of the TZD-dependent inhibition mechanism of the MPC complex. The complex formation between MPC and TZDs may also help stabilize the overall complex and perhaps will facilitate complex purification and extraction. Usually, ligands can increase the overall hydrophilicity of complexes, helping improving its stability. This especially holds good for membrane proteins as they have short loops connecting the transmembrane helices. Second, binding of a ligand may stabilize one specific conformation of membrane protein thereby increasing the structural homogeneity of the protein sample (127–129), thus facilitating crystallization.

3.3.1. Contribuições do candidato na área.

Apesar dos temas acima tratarem de enzimas (glutaminases) e fatores de transcrição (HIF), esse, na verdade, é o segundo trabalho de nosso grupo visando estudos estruturais proteínas de membrana; porém, é de fato o primeiro no tema de metabolismo tumoral. Este projeto foi derivado de uma experiência nossa anterior, bem-sucedida, com uma proteína associada à membrana mitocondrial do fungo causador da doença Vassoura-de-Bruca, em cacaueiros; mais precisamente, durante o desenvolvimento de nosso projeto colaborativo com o Dr. Gonçalo Amarante Pereira, da Unicamp, dentro da chamada SMOLBnet 2.0, da FAPESP (janeiro de 2011 a dezembro de 2012). Na ocasião, tentamos produzir a Oxidase Alternativa (AOX), uma proteína também associada à membrana mitocondrial, de *M. perniciosus*, e de grande interesse para o desenvolvimento de pequenos inibidores químicos. Obtivemos sucesso no aprendizado e implementação de protocolos de preparação e extração das proteínas de extratos de membranas, seleção por detergentes e purificação.

Mais de quatro décadas atrás, no início de 1970, Andrew Halestrap (Universidade de Bristol) demonstrou pela primeira vez a natureza ativa, isto é, dependente de proteína, do transporte de piruvato do citosol para a mitocôndria. Em fevereiro de 2012, participei de um simpósio da série Keystone, em Banff, no Canadá, cujo tema era Câncer e Metabolismo. Na ocasião, Dr. Jared Rutter (*University of Utah*), palestrante, apresentou a identidade das proteínas MPC, encarregadas desse processo previsto por Halestrap. Alguns meses mais tarde, seu trabalho seria publicado na *Science*, em paralelo com o trabalho do laboratório do Dr. Jean-Claude Martinou (*Université de Genève*).

Os laboratórios dos doutores Rutter e Martinou isolaram e identificaram, de forma independente, MPC1 e MPC2, e propuseram sua associação funcional em uma estrutura oligomérica de 150 kDa; este oligômero seria formado pela associação entre as duas subunidades. Essas importantes publicações abriram as portas para estudos subsequentes que caracterizaram ainda mais o papel e a regulação do transporte de piruvato dependente de MPC,

no entanto, exclusivamente no contexto mitocondrial. Independentemente, criticismos à essas publicações, partindo principalmente do próprio Andrew Halestrap, foram constantemente levantadas; e persistem até a data, sobre a natureza funcionalmente independente do MPC1: MPC2. Rapidamente, se tornou consenso que o isolamento e a purificação até a homogeneidade de MPC1 e MPC2, e subsequente reconstituição em vesículas artificiais seria o experimento obrigatório para medir o transporte de piruvato dependente de MPC.

Quando assisti a palestra do Dr. Rutter, em 2012, estávamos trabalhando com AOX. Tínhamos adquiridos reagentes e equipamentos para o projeto; uma pós-doutoranda de meu grupo, que conduzia as pesquisas com AOX, tinha também participado de um curso sobre purificação de proteínas de membrana. Devido ao fato de MPC ser totalmente inédito, e termos uma certa capacidade técnica, decidi que seria interessante nos aventarmos no tema de MPC.

Ainda em 2012, tive a oportunidade de conversar com dois dos maiores especialistas mundiais em estudos estruturais de proteínas de membrana sobre nossos planos para MPC. Um desses especialistas é a Dra. Isabel de Moraes, diretora do *Membrane Protein Laboratory* (MPL), localizado no síncrotron *Diamond Light Source*, em Didcot, no Reino Unido. Com o plano de pesquisa traçado, durante o ano de 2013, tive uma aluna de graduação estagiando em meu laboratório, que realizou a montagem do sistema de expressão a ser usado, que seria leveduras, e também a adaptação de um vetor e subclonagem de dez pares homólogos de MPC, desde humanos, passando por plantas, mosca, mosquito, até nematódeos.

A pesquisa em nosso laboratório foi então projetada com o objetivo de ajudar a responder à questão relevante, e de longa data, que diz respeito à verdadeira natureza dos transportadores de piruvato glicolítico através da membrana mitocondrial interna. Então, em meados de 2014, recrutei um pós-doutorando, que assumiu o projeto. Logo em seguida, um aluno de doutorado se juntou. Desde então, considero que temos tido enorme sucesso nessa empreitada. Nosso primeiro manuscrito, descrevendo justamente a purificação e reconstituição funcional em vesículas artificiais está em fase de revisão. Na verdade, aliado a nossos esforços, o sucesso desse trabalho se deve enormemente à colaboração contínua da Dra. Isabel de Moraes, que, na verdade, nos ajuda até hoje, inclusive hospedando membros de meu grupo no MPL. Também, tivemos a enorme felicidade de conhecer o laboratório do Dr. Pietro Ciancaglini, da USP de Ribeirão Preto, que foi fundamental para o estabelecimento dos ensaios de transporte *in vitro*. Foi por meio da Dra. Eneida de Paula, da Unicamp, que chegamos até o Dr. Ciancaglini.

Neste manuscrito, que ajuda a resolver a questão da real funcionalidade de MPC, como proposto em 2012, relatamos a primeira produção heteróloga MPC na literatura, já com o homólogo humano. Foram planejadas estratégias de coexpressão e purificação para isolar o complexo funcional esperado de 150 kDa, que, no entanto, permitiu apenas a purificação dos componentes individuais e em pesos moleculares aparentes mais baixos. Mais especificamente, MPC1 foi purificado com sucesso, mas co-eluído com uma chaperona do hospedeiro, e apenas com muitos traços de MPC2; não foi observada atividade de transporte *in vitro* para esta proteína.

Por outro lado, mostramos que MPC2 humano purifica até a homogeneidade na ausência total de MPC1, novamente, apesar de uma co-expressão recombinante e induzível. Fornecemos então a primeira demonstração de que o MPC2 pode funcionar como um transportador autônomo, de forma oligômeros. Mais importante ainda, a coleção de parâmetros funcionais descritos no nosso artigo está de acordo com as publicações anteriores e recentes, incluindo dos doutores Halestrap, Rutter e Martinou, que sugerem que o transporte de piruvato mitocondrial pela MPC é um processo rápido e específico, depende da co-importação de prótons e do equilíbrio redox. Nosso artigo culmina com a confirmação de que MPC2 humano pode funcionar como um transportador autônomo em um modelo celular levedura.

Além dos pontos acima mencionados, também propomos um simples ensaio de atividade de transporte *in vitro* que não requer o uso de substratos radioativos, o que muitas vezes é uma limitação séria para muitos laboratórios, incluindo o nosso.

Essas pesquisas foram, e ainda são, financiadas por Auxílios Regulares FAPESP, sob minha responsabilidade: 2012/14298-9, 2014/20673-2 e 2017/117665. Uma tese de doutorado está em andamento, assim como um projeto de pós-doutoramento. Uma cópia do manuscrito em revisão (título e lista de autores abaixo) encontra-se no anexo III.1.

- **Anexo III.1:** Nagampalli, R. S. K., Quesñay, J. E. N., Adamoski, D. Islam, Z., Birch, J., Sebinelli, H. G., Ascensão, C. F. R., Fala, A. M., Pauletti, B. A., Consonni, S. R., Oliveira, J. F., Silva, A. C. T., Franchini, K. G. Paes Leme, A. F., Ciancaglini, P., Moraes, I., Dias, S. M. G., and **Ambrosio, A. L. B.** (2017) Human mitochondrial pyruvate carrier 2 as an autonomous and selective transporter. *Under review*.

3.4. Prolil-hidroxilases, PHD (2012 – presente)

As PHDs pertencem à família das enzimas dioxigenase, que fazem a hidroxilação de resíduos de prolina, requerendo oxigênio, ferro e 2-oxoglutarato (2-OG) para a sua atividade catalítica. A sua baixa afinidade com o oxigênio – de cerca de 2 a 10 vezes superiores às concentrações fisiológicas de oxigênio – permite com que as enzimas atuem como sensores de oxigênio, sendo classificadas como tal (130). Três isoformas das PHDs (PHD1, PHD2 e PHD3) foram identificadas e os seus substratos são conhecidos como sendo bastante diversos e específicos de cada isoforma. Uma quarta isoformas também é descrita, porém, bem menos estudada.

PHD1 controla o nível de expressão do *fator-kappa* das células B ativadas (NF-kB) por inativação mediada por hidroxilação do inibidor de NF-kB quinase (131). Além disso, PHD1 está envolvida na proliferação celular através da degradação de um regulador do ciclo celular da ciclina D1 em uma maneira dependente da atividade de hidroxilase (132). A subunidade maior da RNA polimerase II Rbp1 tem sido mostrado como o outro substrato para PHD1, e a prolil hidroxilação é necessária para a sua degradação dependente de VHL.

PHD2 é considerada crítica na regulação da via de HIF, embora a atividade de hidroxilase seja necessária para a regulação de outras vias de sinalização incluindo fosforilação cofilina e a degradação da proteína NDRG3 (133, 134). Especificamente, a angiogênese aumentada e o aumento dos níveis do fator de crescimento endotelial vascular (VEGFA) e eritropoietina (EPO) foram observados em nocaute condicional de PHD2 (135, 136). Tais observações, juntamente com que já havia sido descrito para HIF, reforçam a presença EPO circulante e concomitante aumento da eritropoiese (87), o que implica que a ativação de HIF por moduladores das PHDs poderia ser benéfico para pacientes com doenças relacionadas a anemia e com a isquemia. Assim, abordagens farmacológicas como inibidores da atividade hidroxilase das PHDs afim de manipular a via de HIF têm sido estudadas para o tratamento de doenças sistêmicas e locais relacionadas com a hipóxia.

Por outro lado, PHD3 desempenha papéis importantes no desenvolvimento neural, na função do sistema imunológico, na migração celular e na apoptose (137). O fator de ativação de transcrição 4 e o homólogo humano da proteína de *C. elegans* clock protein CLK-2 foram identificadas como substratos não-HIF para PHD3 (138, 139). Actina não-muscular também é hidroxilada em resíduos de prolina por PHD3 e a inibição da atividade PHD3 leva ao aumento de filamentos de F-actina (140). Distintamente da sua atividade de hidroxilase, PHD3 pode atuar como uma proteína que está presente na regulação de diversas vias de sinalização (141, 142).

Uma quarta prolil hidroxilase foi descoberta, esse gene está presente em mamíferos e também foi identificado em *zebrafish*. Ela difere das demais PHDs devido a sua localização celular, pois sua sequência contém um domínio transmembrana e ela está associada ao retículo endoplasmático com seu domínio catalítico voltado para o lúmen do retículo. PHD4 em ensaios *in vitro* foi capaz de hidroxilar o peptídeo referente as prolinas de HIF no domínio de degradação dependente de oxigênio, no entanto especula-se que essa proteína tenha outros substratos devido ao alto grau de redundância por outros substratos (143).

Em termos de sequência primária, os genes humanos das PHDs são bem conservados, especialmente no domínio do sítio ativo. PHD1 e PHD2 contém um sinal de localização nuclear no N-terminal e PHD2 contém ainda uma sequência de exportação nuclear e motivo ZF-Mynd, o que pode permitir a interação com outras proteínas. Ambas as enzimas são relatadas como citoplasmáticas, no entanto, ambas foram recentemente encontradas no núcleo, consistente com os peptídeos de sinalização acima, sugerindo que a sua atividade sobre HIF também ocorre no núcleo. A Figura 11 mostra a arquitetura dos domínios das PHDs.

Figura 11. Representação esquemática da arquitetura das PHDs 1-4. As quatro isoformas possuem domínio catalítico, requerido para função enzimática. Adicionalmente PHD1 possui um motivo de localização nuclear (NLS), PHD2 apresenta os motivos de exportação para o núcleo (NES), de interação com outras moléculas (zf-Mynd) e o sinal de localização nuclear (NLS), PHD4 possui um domínio transmembrana (DTM) além do domínio catalítico.

PHDs catalisam a hidroxilação dependente de Fe (II) e oxigênio de dois resíduos específicos de prolina. Sob condições de normóxia a hidroxilação de ODD (Pro-402 e Pro-564) nas HIF-1 α e -2 α , favorece a ligação do supressor de tumor VHL levando-as a poli-ubiquitinação e sinalizando para degradação proteossômica (144). Em condições de hipóxia, a prolil-4-hidroxilação é reduzida, e HIF-1 α e HIF-2 α tornam-se estabilizadas e translocam-se para o núcleo onde heterodimerizam-se com a subunidade constitutivamente expressa HIF-1 β e regulam a expressão dos diversos genes-alvo.

Apesar das PHDs possuírem domínios catalíticos parecidos, elas exibem tendência quanto a isoforma a ser hidroxilada, HIF-1 α ou 2 α , e quanto ao sítio de hidroxilação (CDD ou NODD), por exemplo, PHD3 preferencialmente hidroxila a HIF-2 α em diversas linhagens celulares e PHD1 e 2 preferencialmente hidroxilam HIF-1 α (145). Além destas atividade específica das PHDs, a PHD3 é a única isoforma que está sob controle transcricional de HIF-1 α , e sua expressão é elevada em tecidos de câncer de pescoço e cabeça e, apesar desta enzima não possuir sinal de localização nuclear, ela está presente tanto no citoplasma quanto no núcleo, indicando assim que possa ter sido carregada através da interação com outras proteínas. Recentemente um ensaio utilizando siRNA contra PHD3 levou a regulação do p27, proteína relacionada ao bloqueio do ciclo celular na fase G1 (146), sugerindo desta forma que as prolil hidroxilases estão envolvidas também em outras vias celulares, direta ou indiretamente, sendo este mais um motivo importante no estudo desta família de proteínas. Até o presente momento somente a estrutura do domínio catalítico da isoforma PHD2 foi elucidado por cristalografia de raios X (*pdb*: 3HQR)(147).

Apesar do rápido acumulo de informações na literatura no que diz respeito as funções in vitro e celular das PHDs, pouca informação foi reportada quanto à sua estrutura molecular. Somente a PHD2, que é a hidroxilase mais estudada desta classe do ponto de vista estrutural, e até o presente momento, apenas a estrutura do seu domínio catalítico, foi elucidada por cristalografia de raios-X. Tal informação experimental foi um ponto importante para a identificação e teste de inibidores desta prolil hidroxilase (148). Muitos esforços para a determinação estrutural da PHD1 de humanos e mamíferos e outros organismos foram apresentadas ao longo dos anos, porém com pouco sucesso.

Recentemente, outros organismos foram selecionados e utilizados como modelos alternativos para estudar doenças humanas, como cardiopatologias e câncer. Um dos muitos exemplos é o rato-toupeira-pelado (*Naked mole rate-NMR*, *Spalax sp*) e a toupeira (*Blind mole*

rate-BMR, Heterocephalus glaber). Estes roedores têm um estilo de vida muito peculiar: sua vida subterrânea é formada por grandes colônias e são constantemente submetidos a intensas fontes de estresse, como a escassez de alimentos, condições de baixo oxigênio, escuridão, inundação, exposição a um grande número de patógenos e predação. Além de todas estas atribuições que os tornam vulneráveis, eles possuem uma vida longa em torno de 30 anos, muito acima do esperado para os roedores (149).

Câncer representa ~23% de mortalidade humana (150). Em roedores, tais como camundongos e ratos a mortalidade por câncer é muito elevada, alcançando 90% em algumas estirpes (151). Ao longo de um período de 40 anos, milhares de indivíduos de NMR e de BMR foram alojados e estudados em diferentes regiões do mundo, lugares com características mais quentes como deserto e mais frias como os polos, e apesar destes pequenos roedores (~100-200 gr.) apresentarem uma vida extremamente longa (> 20 anos), nenhum deles jamais desenvolveu tumores espontâneos e nem demonstram nenhuma alteração fenotípicas relacionadas com o envelhecimento.

Eles têm desenvolvido um complexo adaptativo exclusivo para viver no subsolo, incluindo uma capacidade única para lidar com extrema hipóxia e hipercapnia (152). Além disso podem realizar trabalho aeróbico intensivo sob pressões baixas de O₂, devido ao aumento da massa muscular, alta densidade de vasos sanguíneos e mitocôndrias, resultando em redução da distância de difusão de oxigênio e fornecimento de oxigênio eficiente mesmo em baixa capilar PO₂ (152, 153). A espécie *Spalax sp* possui adaptações em genes relacionadas com a hipóxia na estrutura e função, como a elevada expressão de VEGF e p53 que abriga duas mutações no sítio de ligação do DNA idênticas às mutações do gene p53 em tumores mais comuns (153–155).

Outro modelo alternativo para o estudo fisiológico, em especial os sistemas digestivo e cardiovascular, são as cobras. Especialmente répteis do gênero *Python sp*. *Pythons* não utilizam a tática de caçar suas presas, como as demais cobras. Sua abordagem tática é a de esperar a presa se aproximar e, como resultado disto, eles têm uma resposta fisiológica rápida a alimentação e jejum. Durante o curto período de digestão, menos de 24 horas, seus órgãos praticamente duplicam de tamanho e o seu aumento de atividade é em torno de 10 e 40 vezes, o que leva a um aumento na absorção de nutrientes tais como glicose e transportadores e enzimas desta via. O processo inverso também ocorre rapidamente, o seu coração, fígado, pâncreas e rins reduzem a massa e retornam ao repouso rapidamente e sua atividade volta a ser reduzida.

É bem conhecido que a maioria das respostas fisiológicas acima, em especial para a adaptação às condições de hipóxia, angiogênese, eritropoiese, e a adaptação dos processos metabólicos, são controlados por HIFs. Portanto, o envolvimento direto de PHDs nestes processos é uma hipótese muito plausível. Neste contexto e do ponto de vista molecular, nosso interesse é estudar as PHDs destes organismos incomuns e, sobretudo, aprender como eles se comparam com os homólogos humanos. A compreensão de uma possível regulação e supressão do tumor relacionada com as PHDs nestes organismos podem fornecer pistas sobre os mecanismos e as diferenças em comparação com os seres humanos e, portanto, poderia ajudar a encontrar novos alvos para a terapia anticâncer por ativação das mesmas condições para as células humanas ou revelar mecanismos moleculares importantes que são ignorados até agora.

3.4.1. Contribuições pretendidas.

Como descrito acima, PHDs são os principais reguladores da presença de HIF nas células. O projeto relacionado às prolil-hidroxilases foi planejado em colaboração com o Dr. Christopher Schofield, professor no Departamento de Química da Universidade de Oxford. Esta colaboração é fruto do trabalho realizado no projeto BEPE FAPESP desenvolvido pela aluna Angela Maria Fala durante o período de mestrado em que esteve no *Structural Genomics Consortium* (SGC) na

Universidade de Oxford. Angela foi a aluna responsável pelas pesquisas em HIF e lipídeos, bem como PHDs, em nosso laboratório. Ambos os trabalhos foram tema de sua tese de doutorado pela Unicamp, a qual defendeu em julho de 2017. Os resultados obtidos pela aluna durante o BEPE foram de suma importância para despertar o interesse do professor Schofield; estendemos a colaboração além do SGC, desde 2013, continuamos com projeto aqui no LNBio. Visamos expressar e cristalizar homólogos não-convencionais de PHD, pois o trabalho de outros laboratórios mais estabelecidos na área, como o do próprio Dr. Schofield, tenta, há anos cristalizar homólogos e de mamíferos, como camundongo. Até o momento, temos identificamos condições de cristalização para PHD1 e PHD3. O laboratório do Dr. Schofield realiza os ensaios de atividade, que não são triviais, para confirmar a funcionalidade das enzimas recombinantes que produzimos. Com a conclusão do doutorado da aluna responsável, o laboratório do Dr. Schofield está levando também adiante a otimização das condições de cristalização.

3.5. Canal ânion-seletivo dependente de voltagem 2, VDAC-2 (2016 – presente)

As primeiras observações por microscopia eletrônica de transmissão da mitocôndria definiram sua morfologia básica: uma matriz envolvida por uma membrana interna, uma membrana externa e um espaço intermembranas (156). Recentemente, as técnicas de crio-microscopia eletrônica de partículas isoladas e crio-tomografia eletrônica têm fornecido dados 2D e 3D descrevendo interações entre complexos do sistema de fosforilação oxidativa da mitocôndria e gerando novas percepções de como estes supercomplexos estão estruturados dentro da mitocôndria (157).

A matriz mitocondrial contém uma mistura concentrada de centenas de enzimas, incluindo o complexo da piruvato desidrogenase e as enzimas do ciclo do ácido cítrico, a via de beta-oxidação de ácidos graxos e as vias de oxidação de aminoácidos. Contém ainda várias cópias do DNA genômico mitocondrial, dos ribossomos especiais, dos tRNAs e de várias enzimas requeridas para a expressão dos genes mitocondriais.

A membrana interna possui numerosas cristas que aumentam bastante sua área de superfície, contendo as proteínas que conduzem as reações de oxidação na cadeia respiratória, a enzima ATP-sintase e transportadores específicos que regulam a entrada e saída de metabólitos da matriz. Um gradiente eletroquímico de H^+ que direciona a ATP-sintase é estabelecido de forma que ela deve ser impermeável a íons e à maioria de pequenas moléculas.

Já no espaço intermembranas existem várias enzimas que utilizam o ATP proveniente da matriz para fosforilar outros nucleotídeos. Neste espaço também ficam alojadas proteínas como citocromo c e Smac/DIABLO e que, dependendo do estímulo pró-apoptótico e de alterações na membrana externa, podem ser liberadas para o citoplasma, desencadeando a via apoptótica mitocondrial (158).

A membrana externa, por sua vez, possui um tipo de proteína formadora de canais, a VDAC (*voltage-dependent anion-selective channel*), que também é a mais abundante nessa membrana (159). Devido a essa proteína, a membrana externa é permeável a todas as moléculas de até 5 kDa. Há ainda outras proteínas envolvidas na síntese de lipídeos mitocondriais e enzimas que convertem substratos lipídicos que serão metabolizadas na matriz, importadores de proteínas mitocondriais, como o complexo TOM (*translocase of the outer mitochondrial membrane*), e a maquinaria enzimática para a divisão e fusão da organela (160, 161).

VDACs, também conhecidas como porinas mitocondriais, são as mais abundantes proteínas integrais de membrana encontradas na membrana externa da mitocôndria (*MOM – mitochondrial outer membrane*) e consideradas um novo alvo para a ação de drogas anti-câncer. Em mamíferos, três genes homólogos codificam três isoformas de VDAC, nomeadas VDAC1, VDAC2 e VDAC3. As três proteínas possuem massa molecular similar (30–35 kDa), compartilham 70% de identidade e todas as três podem ser encontradas na maioria dos tecidos, sendo a VDAC1 a mais abundante, seguida da VDAC2 e, em menor quantidade, a VDAC3 (162, 163).

O específico papel de cada isoforma de VDAC ainda não é claro, embora evidências indiquem diferentes funções fisiológicas (164). Por exemplo, a ausência de VDAC1 causa múltiplos defeitos na atividade do complexo respiratório em músculo cardíaco e esquelético murino, enquanto que em camundongos deficientes em VDAC3, o defeito fica restrito ao coração (165). Embora diferentes estudos apontem outros problemas em camundongos deficientes para VDAC1 e VDAC3, como a inibição de crescimento e esterilidade em machos, os camundongos ainda são viáveis (166). No entanto, embriões de camundongos com deficiência homozigótica para VDAC2 morrem durante o desenvolvimento (167), indicando que VDAC2 pode estar diretamente envolvida em funções vitais.

Enquanto a necrose é um processo acidental e passivo, a apoptose ou morte celular programada é um processo ativo que requer energia para a ativação de uma maquinaria

intracelular e pode ser dividido em duas vias principais: a via intrínseca ou mitocondrial e a via extrínseca ou citoplasmática (168, 169).

A via intrínseca ou mitocondrial da apoptose é finamente controlada pela família de proteínas Bcl-2. Bak (*Bcl-2 homologous antagonist/killer*) pertence a essa família que pode ser dividida em membros anti-apoptóticos (p.ex., Bcl-2, Bcl-XL) e pró-apoptóticos (p.ex., Bak, Bax, Bid, Bad, Bim) (170). Em células saudáveis, Bax (*Bcl-2-associated X protein*) é inativo e predominantemente citosólico. Em contraste, Bak está constitutivamente integrado na membrana externa da mitocôndria como um monômero inativo ou reprimido pela interação com outras proteínas, como a VDAC2 (167). Em células saudáveis, VDAC2 e Bak estão unidas em um complexo onde Bak se apresenta inativada. Quando ativada, Bak está dissociada de VDAC2, formando homo- ou hetero-oligômeros na MOM. Este mecanismo de formação e dissociação do complexo VDAC2-Bak está intimamente relacionado à via mitocondrial da apoptose (171). As proteínas anti-apoptóticas agem bloqueando a liberação de citocromo c e as pró-apoptóticas agem promovendo a liberação de citocromo c. Seguindo o estímulo apoptótico, Bax e Bak (que está inativo ou associado a VDAC2) sofrem mudanças conformacionais, oligomerizam-se e permitem a liberação de citocromo c e Smac/DIABLO por meio da permeabilização da membrana externa da mitocôndria. No citoplasma, o citocromo c se une a Apaf-1, ATP e caspase 9 formando um complexo chamado de Apoptossomo (172).

Já a via extrínseca é mediada pelos chamados “receptores de morte” os quais encontram-se nas superfícies celulares, transmitindo um sinal de morte para o interior da célula. Receptores de morte incluem o gene da superfamília de receptores para o fator de necrose tumoral (RTNF), incluindo RTNF-1, Fas (CD95), DR-4 e DR-5 (receptores TRAIL). Quando um ligante entra em contato com um Fas inativo, induz a formação de um complexo e recruta proteínas adaptadoras FADD intracelulares, que por sua vez recrutam as pró-caspases 8 e 10, formando um complexo indutor da sinalização de morte (DISC). Isso tem efeito auto-proteolítico nas caspases, tornando-as ativas. Uma vez ativada, a caspase 8 cliva as caspases 3, 6 e 7, que são caspases efetoras. A caspase 8 interage com a via intrínseca ou mitocondrial através da clivagem de Bid, o que resultará na liberação de citocromo c (173).

Em 2010, Lazarou e colaboradores mostraram resultados de estudos sobre mecanismos que regulam a permeabilização da membrana externa da mitocôndria, processo conhecido como MOMP (*mitochondrial outer membrane permeabilization*). Neste trabalho mostraram que, em células saudáveis, Bak está presente na membrana externa da mitocôndria em um complexo de aproximadamente 400 kDa e que a liberação de Bak desse complexo coincide com: a ativação de Bak, a permeabilização da membrana externa da mitocôndria, a liberação de citocromo c e que o complexo com Bak é dependente da presença de VDAC2 (174–177).

Em 2013 e 2014, Ma e colaboradores mostraram que, como Bak, uma pequena população de Bax, que constitutivamente reside na mitocôndria, pode também estar presente no complexo de alto peso molecular envolvendo VDAC2 (171, 178). Respalhando esses dados, outros estudos recentes contestaram a antiga premissa de que Bax é ativamente deslocado para a MOM sob ativação. Em vez disso, Bax se associa constitutivamente com a mitocôndria, mas é constantemente transportado para o citosol. Sob sinalização apoptótica, esse “retrotransporte” é prejudicado levando ao acúmulo de Bax na mitocôndria (179–181).

Embora os últimos estudos tenham contribuído substancialmente no entendimento do mecanismo de regulação de Bak e observado sua presença em um complexo com VDAC2, pouco se sabe sobre as funções exercidas pelo complexo VDAC2-Bak. Plötz e colaboradores (182), por exemplo, observaram que Bcl-x(S) foi capaz de interferir no complexo, deslocando Bak de VDAC2 e induzindo a apoptose em células de melanoma, um tipo de câncer altamente resistente às quimioterapias tradicionais.

3.5.1. Contribuições pretendidas.

Em linhas gerais, essa proposta objetiva a caracterização estrutural, através de crio-microscopia de partículas isoladas (Crio-TEM), da interação entre a porina mitocondrial VDAC2 e a proteína pró-apoptótica Bak, com implicações para o desenvolvimento de novas abordagens terapêuticas em câncer. Foi estabelecido a partir de uma sugestão do Dr. Rodrigo Portugal, pesquisador do Laboratório Nacional de Nanotecnologia (LNNano). Seus esforços estão dedicados no estabelecimento de uma infra-estrutura de equipamentos que permita a formação de uma comunidade interessada em estudos estruturais de proteínas usando Crio-TEM.

Para tanto, pretendemos produzir o complexo estável a partir do ambiente nativo, usando culturas de células humanas estabilizadas, e também recombinante, em modelos de levedura, que se mostraram adequados para os estudos com MPC, acima. A Dra. Sandra Dias coordena as pesquisas nos modelos de células humanas. Uma vez isolado o complexo, como consequência da identificação de detergentes adequados para posterior purificação, este será submetido a estudos de microscopia eletrônica de transmissão, em condições criogênicas, visando a determinação da estrutura tridimensional.

Novamente, esta proposta faz parte de um esforço colaborativo entre pesquisadores do LNBio e LNNano, no sentido de se estabelecer e combinar duas áreas de pesquisa atualmente bastante relevantes e que, provavelmente, representam o futuro da biologia estrutural: proteínas de membrana e criomicroscopia eletrônica de partículas isoladas. Apesar de vários esforços individuais de pesquisadores na região, ambas as áreas ainda estão subdesenvolvidas na América Latina, como um todo. É principalmente neste sentido que pretendemos colaborar.

Como mencionado nas sessões anteriores, ao longo dos últimos dois anos, com financiamento FAPESP, meu laboratório tem adquirido experiência na produção e purificação de proteínas transmembranares, contando principalmente com a colaboração constante da equipe de especialista do *Membrane Protein Laboratory* (MPL). Acredito que a experiência adquirida até então nos permitirá extrair e purificar o complexo VDAC2:Bak em quantidade e condições adequadas para os estudos estruturais.

Atualmente, temos um pós-doutorando desenvolvendo este projeto. Já conseguimos expressar e purificar parcialmente VDAC2 sozinha e também co-expressar, e confirmar a localização mitocondrial de seu complexo com Bak. Mais ainda, recentemente adquirimos a capacidade técnica para a produção de nano-discos, visando posterior reconstituição das proteínas purificadas nestes. Nano-discos constituem uma técnica de fronteira e são cruciais para a remoção completa de detergentes para os experimentos de Crio-TEM. Meu Auxílio regular FAPESP vigente (2017/11766-5) financia estas pesquisas.

3.6. Fator de transcrição MondoA (2013 – presente)

O fator de transcrição MondoA foi inicialmente descrito há pouco mais de 10 anos pelo laboratório do Dr. Donald Ayer, da Universidade de Utah (183), nosso colaborador desse projeto. Seu laboratório está interessado em estudar a habilidade das células em sentir e responder à mudança dos níveis de nutrientes no microambiente celular, em especial com relação ao açúcar glicose.

Sabe-se hoje que dois fatores transcrição, MondoA e seu parálogo ChREBP, agem como os principais biossensores transcripcionais de glicose (184, 185). Estas proteínas são originalmente localizadas no citoplasma das células em condições de baixa glicose, mas acumulam-se no núcleo realizando a transativação de genes alvo em condições de aumento de concentração de glicose (186–188). ChREBP é expresso predominantemente em células hepáticas e regula a expressão de genes envolvidos na conversão de glicose para lipídeos, com fins de armazenamento de energia e crescimento celular (189–191) enquanto MondoA é expressa predominantemente em células musculares esqueléticas e regula a transcrição de genes da via glicolítica (188).

A translocação nuclear de MondoA e ChREBP, e a suas ligações aos elementos responsivos, estão associadas à dimerização obrigatória com o parceiro Mlx, um fator de transcrição da família Max, expresso constitutivamente em todos os tecidos. MondoA, ChREBP e Mlx contém o motivo *basic helix-loop-helix leucine zipper* (bHLH) de ligação a DNA, além do domínio de localização citoplasmática (DCD) que deve ser mascarado anteriormente à translocação nuclear (192, 193). Essas regiões são esquematizadas na figura 12.

Figura 12: As atividade glicose-dependentes de MondoA requerem os domínios MCR e bHLHZip. Adaptado de (193).

Dada a importância de MondoA e ChREBP para o metabolismo de glicose da célula e uma possível relação direta com o fator de transcrição e oncogene Myc, especula-se que MondoA possa cooperar com este último diretamente no processo de adaptação metabólica de tumores dirigidos por Myc (194–197).

Uma vez que MondoA e ChREBP são principalmente proteínas citoplasmáticas, foi surpreendente descobrir que simplesmente prendê-las dentro do núcleo em condições de baixa concentração de glicose não é suficiente para aumentar a expressão de seus genes alvos (184, 185). Em contraste, construções que não possuem a região N-terminal são capazes de constitutivamente transativar genes em meios tanto com baixa quanto alta concentração de glicose (190, 192, 198, 199). Estes resultados indicaram que sequências do domínio N-terminal destas proteínas contribuem para a transativação em resposta à glicose (192, 198). Sabe-se hoje que existem cinco regiões conservadas na porção N-terminal da MondoA, chamadas de *Mondo Conserved Regions* (MCRI-V), mais especificamente entre os resíduos 77 e 324 (que possui um total de 919 aminoácidos). O tamanho, a sequência de aminoácidos e as distâncias entre MCRII, MCRIII e MCRIV em MondoA e ChREBP são bem conservados, o que sugere que estes possam agir como um módulo funcional, enquanto que as regiões MCRI e MCRV variam bastante entre essas duas proteínas (200). MCRII contém um sinal de exportação nuclear (NLS - *Nuclear Export Signal*), quase idêntico ao motivo canônico NLS LxxLFxxLSV, onde x é qualquer aminoácido. Em contraste, MCRIV em ChREBP contém um sinal de localização nuclear (NLS - *Nuclear Location Signal* - bipartido) que medeia sua entrada no núcleo (188, 201). O MCRIII por sua vez contém um motivo de ligação canônico à proteína 14-3-3 a qual está envolvida na retenção

citoplasmática, transativação e ao exporte nuclear de MondoA e ChREBP (190, 192, 202). As funções de MCRI e MCRV não são tão claras, embora MCRI pareça ser necessário para transativação gênica dependente de glicose no ChREBP (203).

Adicionalmente, deleções e mutações demonstram que cada MCR parece ter funções múltiplas e geramente opostas. MCRI é necessário para a resposta à glicose, uma vez que alterações nesta região (MondoA: Δ 1-100, His78Ala/His81Ala/His88Ala ou em ChREBP: Δ 1-58 ou Δ 1-71) são capazes de bloquear transativação na presença de altos níveis de glicose (184, 190, 192, 203). Da mesma forma, a alteração da sequência NES em MCRII (MondoA: Phe130Ala, Met133Ala, Δ 125-137 ou em ChREBP: Leu89Ala, Phe90Ala) aumenta levemente os níveis de transativação, enquanto outras mutações no MCRII (ChREBP: Leu86Ala/Leu93Ala, Tyr85Ala, Leu95Ala, Δ 72-99; MondoA: Leu129Ala) a bloqueia completamente (193, 204, 205). Em MCRIII, interferências no sítio de ligação à 14-3-3 (MondoA: Ile166Ala/Trp167Ala/Arg168Ala; ChREBP: Arg128Ala, Trp130Ala) inibem a transativação. Curiosamente, as mudanças dentro MCRIV têm efeitos ainda mais diversificados. Deleções que eliminam o NLS (ChREBP: Δ 141-197, Δ 158-181) previnem a transativação (188, 200). Em adição, a deleção Δ 144-196 em ChREBP reduz a função de transativação além de eliminar a inibição dependente de glicose (206), enquanto o triplo-mutante Tyr210Asp/Trp211Asp/Lys212A em MondoA aumenta o acúmulo nuclear e transativação (193). Enquanto que a MCRV parece não mostrar efeitos repressivos, a deleção de MCRI-IV (MondoA: Δ 282-324; ChREBP: Tyr275Ala/Val276Ala/Gly277Ala, Leu289Ala/Gln290Ala/Pro291Ala) levam a um aumento em seu acúmulo nuclear de MondoA e consequente aumento dos níveis de transativação (193, 199). Embora as condições celulares, mutações sítio-dirigidas e ensaios com gene repórter variem bastante entre estes estudos, em resumo fica claro que as regiões MCR cooperativamente reprimem e ativam a função de transativação de MondoA e ChREBP em resposta à glicose.

Evidências mais recentes mostram que a ativação da MondoA e ChREBP é dependente da fosforilação de glicose por hexoquinase, a qual metaboliza esse açúcar para formar glicose-6-fosfato-(G6P) (193, 201, 206). Tratamento celular com 2-deoxiglicose (2-DG), que é um análogo da glicose que pode ser fosforilado, porém não mais catabolizado, promove acúmulo nuclear de MondoA, aumenta a ocupação do promotor e recruta histona H3 acetiltransferase, ativando assim a transcrição de genes alvos de MondoA (193). Da mesma forma, experimentos de dose-resposta de 2-DG aumentaram a capacidade de transativação de GAL4-ChREBP, enquanto que tanto o tratamento com o inibidor de hexoquinase d-Manoepulose quanto a superexpressão das enzimas glicolíticas PFK1 e PFK2, as quais metabolizam o G6P gerado pela hexokinase, diminuem os níveis de atividade de ChREBP (206). Isto sugere que a ativação de MondoA e ChREBP é invocada diretamente pela fosforilação de glicose, podendo ocorrer a partir de mecanismo alostérico acionado pela ligação direta do G6P a estas proteínas, ou a partir de alguma cascata de sinalização mediada por G6P, ou ambos. De qualquer maneira, essa seria a primeira comprovação de regulação direta de transcrição gênica através de produtos metabólicos.

3.6.1. Contribuições pretendidas.

No final de final de 2012, por intermédio do Dr. Chi van Dang, nosso colaborador há longo tempo no projeto das glutaminase, fomos apresentados ao Dr. Don Ayer. O Dr. Ayer, descobridor de MondoA e especialista em biologia celular, estava procurando um colaborador em bioquímica para produzir e purificar essa proteína a partir de fonte natural ou recombinante. Seu objetivo era o de confirmar *in vitro* a ligação direta entre MondoA e G6P, o que representaria o primeiro exemplo na literatura de regulação da expressão gênica por um metabólito.

Por comunicação pessoal do Dr. Ayer, de acordo com evidências em célula, sabíamos que a região N-terminal de MondoA (chamada de MCR) seria capaz de se ligar diretamente a G6P,

porém a sequência mínima necessária para tal ainda não estava completamente determinada. O objetivo de nossa colaboração e o que propusemos para um projeto de mestrado FAPESP sob minha orientação, foi o planejamento de uma série de construções abrangendo as regiões MCR I a V, com foco na produção heteróloga, cristalização e posterior resolução estrutural dessa região em complexo com G6P.

Mesmo após um trabalho exaustivo da aluna de mestrado responsável pelo projeto, por quase um ano e meio (entre 2013 e 2014), testando dezenas de construções alternativas, em vários vetores de expressão recombinante em bactéria e levedura, não obtivemos sucesso. TODAS as construções foram sempre insolúveis. Em função desse insucesso, o projeto da aluna foi substituído perante a FAPESP, e se tornou um doutorado direto em glutaminases.

Todavia, não desistimos de MondoA. Em uma das visitas do Dr. Wyatt Yue (SGC, Oxford), ao Brasil, em 2015, acordamos em uma estratégia alternativa. O laboratório do Dr. Yue é especialista em expressão recombinante de proteínas, em larga escala, em sistema de células de insetos e também mamíferos. Entre 2015 e 2016, foram geradas novas construções de MCR de MondoA, que se mostraram adequadamente solúveis e puderam ser purificadas, pelo menos parcialmente.

Com esperanças renovadas, em janeiro de 2017, as novas construções, especialmente aquelas que funcionaram para expressão recombinante em células humanas HEK293, foram encaminhadas ao laboratório do Dr. Ayer, nos EUA, que está conduzindo os ensaios bioquímicos. De acordo com comunicação recente do Dr. Ayer, resultados inéditos devem ser gerados e submetidos para publicação até o final de 2017. Esperamos, de fato, confirmar a interação entre MondoA e G6P. A natureza de tal interação, entre um fator de transcrição e um metabólito, continua inédita, mesmo depois desses anos. Da minha parte, tenho interesse adicional em continuar tais estudos em meu laboratório, através do recrutamento de um novo aluno; principalmente, prevejo o benefício em estabelecer sistemas de expressão recombinante em Sf9 (inseto) e HEK293. Nosso laboratório ainda não domina estas tecnologias.

4. CONSIDERAÇÕES FINAIS

De acordo com o exposto acima, espero ter sistematizado adequadamente a coerência e as motivações da minha linha de pesquisa. Escolhi particularmente estes trabalhos, pois foram (e são) realizados sob minha coordenação direta, em colaboração ativa com colegas pesquisadores (nacionais e internacionais) e estudantes. Todavia, entendo que tão importante quanto as pesquisas propriamente ditas, são as circunstâncias sob as quais estas foram idealizadas e implementadas. Por isso, apresentei um breve histórico destas circunstâncias.

Como descrito anteriormente, meu objetivo como pesquisador é o de procurar estar sempre alinhado com as fronteiras do tema de minha pesquisa e do desenvolvimento tecnológico da área. A instituição na qual atualmente me insiro, o CNPEM, é um centro de pesquisas qualificado pelo Ministério da Ciência, Tecnologia, Inovações e Comunicações. Tem a missão de promover os desenvolvimentos científico e tecnológico, bem como a inovação no país. É de acordo com esta missão que procurei, ao longo desses 8 anos como pesquisador independente, sustentar uma linha de pesquisa atual e relevante. Por isso, devido à minha formação em biologia estrutural, recentemente estabeleci os projetos colaborativos em proteínas de membrana, combinando cristalografia por difração de raios X e crio-microscopia eletrônica. Particularmente, entendo que deva haver um compromisso alinhado da comunidade científica em querer usar ao máximo a infraestrutura que o país oferece, principalmente quando o laboratório síncrotron de quarta geração, Sirius, estiver em pleno funcionamento.

Intrinsecamente associado a esses elementos da pesquisa científica, está a capacidade de captação independente de recursos perante agências de fomento. Até o momento, são quatro Auxílios Regulares FAPESP dentro do tema, além de um Auxílio Universal (faixa A) do CNPq.

Todavia, desde o início, procurei também exercer ativamente atividades de docência, através de credenciamentos como orientador externo junto à programas de pós-graduação da Unicamp. Principalmente, junto ao Instituto de Biologia desta universidade, coordenei e ministrei diversas disciplinas de acordo os calendários e participei de diversas comissões. Principalmente, atuei, e ainda atuo, ativamente na orientação de alunos de pós-graduação. Até o momento, são 18 bolsas da FAPESP, concluídas ou em andamento, sob minha orientação/supervisão. Até o momento, concluí a orientação de duas dissertações de mestrado e três teses de doutorado.

A área de metabolismo tumoral continua cada vez mais relevante. A absorção e o metabolismo “desregulados” de nutrientes se estendem muito além da glicose e da glutamina, embora essas tenham sido as vias mais caracterizadas hoje. A aquisição de lipídios ou ácidos graxos, outros aminoácidos e o acetato extracelular, e sua contribuição e necessidade para a tumorigênese são assuntos de pesquisas recentes (5). As perspectivas são excelentes e, eventualmente, estes podem se tornar novos alvos em nossos estudos.

5. REFERÊNCIAS

1. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of Cancer: The Next Generation. *Cell*. **144**, 646–674
2. Jafri, M. A., Ansari, S. A., Alqahtani, M. H., and Shay, J. W. (2016) Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome Med.* **8**, 69
3. DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., and Thompson, C. B. (2008) The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metab.* **7**, 11–20
4. Warburg, O., Wind, F., and Negelein, E. (1927) The Metabolism of Tumors in the Body. *J. Gen. Physiol.* **8**, 519–30
5. Pavlova, N. N., and Thompson, C. B. (2016) The Emerging Hallmarks of Cancer Metabolism. *Cell Metab.* **23**, 27–47
6. Szablewski, L. (2013) Expression of glucose transporters in cancers. *Biochim. Biophys. Acta - Rev. Cancer.* **1835**, 164–169
7. Kunkel, M., Reichert, T. E., Benz, P., Lehr, H.-A., Jeong, J.-H., Wieand, S., Bartenstein, P., Wagner, W., and Whiteside, T. L. (2003) Overexpression of Glut-1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma. *Cancer.* **97**, 1015–1024
8. Cho, H., Lee, Y. S., Kim, J., Chung, J.-Y., and Kim, J.-H. (2013) Overexpression of Glucose Transporter-1 (GLUT-1) Predicts Poor Prognosis in Epithelial Ovarian Cancer. *Cancer Invest.* **31**, 607–615
9. Younes, M., Juarez, D., Lechago, L. V., and Lerner, S. P. (2001) Glut 1 expression in transitional cell carcinoma of the urinary bladder is associated with poor patient survival. *Anticancer Res.* **21**, 575–578
10. Hay, N. (2016) Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nat. Rev. Cancer.* **16**, 635–649
11. Eagle, H. (1955) The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure. *J. Exp. Med.* **102**, 595–600
12. DeBerardinis, R. J., and Cheng, T. (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene.* **29**, 313–324
13. Krebs, H. A. (1935) Metabolism of amino-acids: The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. *Biochem. J.* **29**, 1951–69
14. Martín-Rufián, M., Tosina, M., Campos-Sandoval, J. A., Manzanares, E., Lobo, C., Segura, J. A., Alonso, F. J., Matés, J. M., and Márquez, J. (2012) Mammalian glutaminase Gls2 gene encodes two functional alternative transcripts by a surrogate promoter usage mechanism. *PLoS One.* **7**, e38380
15. Curthoys, N. P., and Watford, M. (1995) Regulation of glutaminase activity and glutamine metabolism. *Annu. Rev. Nutr.* **15**, 133–159
16. Aledo, J. C., Gómez-Fabre, P. M., Olalla, L., and Márquez, J. (2000) Identification of two human glutaminase loci and tissue-specific expression of the two related genes. *Mamm. Genome.* **11**, 1107–1110
17. Lohmann, R., Souba, W. W., and Bode, B. P. (1999) Rat liver endothelial cell glutamine transporter and glutaminase expression contrast with parenchymal cells. *Am. J. Physiol.* **276**, G743–G750
18. Elgadi, K. M., Meguid, R. A., Qian, M., Souba, W. W., and Abcouwer, S. F. (1999) Cloning and analysis of unique human glutaminase isoforms generated by tissue-specific alternative splicing. *Physiol. Genomics.* **1**, 51–62

19. Gómez-Fabre, P. M., ALEDO, J. C., Del Castillo-Olivares, A., Alonso, F. J., Núñez De Castro, I., Campos, J. A., and Márquez, J. (2000) Molecular cloning, sequencing and expression studies of the human breast cancer cell glutaminase. *Biochem. J.* **345**, 365–375
20. Smith, M., and Watford, M. (1990) Molecular cloning of a cDNA for rat hepatic glutaminase. Sequence similarity to kidney-type glutaminase. *J. Biol. Chem.* **265**, 10631–10636
21. Porter, L. D., Ibrahim, H., Taylor, L., and Curthoys, N. P. (2002) Complexity and species variation of the kidney-type glutaminase gene. *Physiol. Genomics*. **9**, 157–166
22. Pérez-Gómez, C., Matés, J. M., Gómez-Fabre, P. M., del Castillo-Olivares, A., Alonso, F. J., and Márquez, J. (2003) Genomic organization and transcriptional analysis of the human l-glutaminase gene. *Biochem. J.* **370**, 771–784
23. Shapiro, R. A., Farrell, L., Srinivasan, M., and Curthoys, N. P. (1991) Isolation, Characterization, and in Vitro Expression of a cDNA That Encodes the Kidney Isoenzyme of the Mitochondrial Glutaminase. *J. Biol. Chem.* **266**, 18792–18796
24. Olalla, L., Aledo, J. C., Bannenberg, G., and Márquez, J. (2001) The C-terminus of human glutaminase L mediates association with PDZ domain-containing proteins. *FEBS Lett.* **488**, 116–122
25. Colombo, S. L., Palacios-Callender, M., Frakich, N., De Leon, J., Schmitt, C. A., Boorn, L., Davis, N., and Moncada, S. (2010) Anaphase-promoting complex/cyclosome-Cdh1 coordinates glycolysis and glutaminolysis with transition to S phase in human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 18868–73
26. Márquez, J., López de la Oliva, A. R., Matés, J. M., Segura, J. A., and Alonso, F. J. (2006) Glutaminase: A multifaceted protein not only involved in generating glutamate. *Neurochem. Int.* **48**, 465–471
27. Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C. F., Joachimiak, A., Savchenko, A., and Yakunin, A. F. (2008) Functional and Structural Characterization of Four Glutaminases from *Escherichia coli* and *Bacillus subtilis* †. *Biochemistry*. **47**, 5724–5735
28. Curthoys, P., Kuhlenschmidt, T., Weiss, R. F., and Godfrey, S. S. (1976) Phosphate-Dependent Activity Glutaminase from Rat Kidney and Identity with Glutaminase in Response to Acidosis from Other Tissues ′. *Arch. Biochem. Biophys.* **167**, 162–167
29. Cassago, A., Ferreira, A. P. S., Ferreira, I. M., Fornezari, C., Gomes, E. R. M., Greene, K. S., Pereira, H. M., Garratt, R. C., Dias, S. M. G., and Ambrosio, A. L. B. (2012) Mitochondrial localization and structure-based phosphate activation mechanism of Glutaminase C with implications for cancer metabolism. *Proc. Natl. Acad. Sci.* **109**, 1092–1097
30. Campos-Sandoval, J. a. J. A. J. a., Lopez de la Oliva, A. R., Lobo, C., Segura, J. a., Mates, J. M., Alonso, F. J., Marquez, J., López de la Oliva, A. R., Lobo, C., Segura, J. a., Matés, J. M., Alonso, F. J., and Márquez, J. (2007) Expression of functional human glutaminase in baculovirus system: Affinity purification, kinetic and molecular characterization. *Int. J. Biochem. Cell Biol.* **39**, 765–773
31. Kovacevic, Z., and McGivan, J. D. (1983) Mitochondrial metabolism of glutamine and glutamate and its physiological significance. *Physiol. Rev.* **63**, 547–605
32. Shapiro, R. a, Morehouse, R. F., and Curthoys, N. P. (1982) Inhibition by glutamate of phosphate-dependent glutaminase of rat kidney. *Biochem. J.* **207**, 561–6
33. Olsen, B. R., Svenneby, G., Kvamme, E., Tveit, B., and Eskeland, T. (1970) Formation and ultrastructure of enzymically active polymers of pig renal glutaminase. *J. Mol. Biol.* **52**, 239–245
34. Godfrey, S., Kuhlenschmidt, T., and Curthoys, P. (1977) Correlation between activation and dimer formation of rat renal phosphate-dependent glutaminase. *J. Biol. Chem.* **252**, 1927–1931
35. Ferreira, A. P. S., Cassago, A., Gonçalves, K. de A., Dias, M. M., Adamoski, D., Ascensão, C. F. R., Honorato, R. V., de Oliveira, J. F., Ferreira, I. M., Fornezari, C., Bettini, J., Oliveira, P. S. L., Paes

- Leme, A. F., Portugal, R. V., Ambrosio, A. L. B., and Dias, S. M. G. (2013) Active Glutaminase C Self-assembles into a Supratetrameric Oligomer That Can Be Disrupted by an Allosteric Inhibitor. *J. Biol. Chem.* **288**, 28009–28020
36. Robinson, M. M., McBryant, S. J., Tsukamoto, T., Rojas, C., Ferraris, D. V., Hamilton, S. K., Hansen, J. C., and Curthoys, N. P. (2007) Novel mechanism of inhibition of rat kidney-type glutaminase by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES). *Biochem. J.* **406**, 407–14
 37. Thangavelu, K., Pan, C. Q., Karlberg, T., Balaji, G., Uttamchandani, M., Suresh, V., Schöler, H., Low, B. C., and Sivaraman, J. (2012) Structural basis for the allosteric inhibitory mechanism of human kidney-type glutaminase (KGA) and its regulation by Raf-Mek-Erk signaling in cancer cell metabolism. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 7705–10
 38. Delabarre, B., Gross, S., Fang, C., Gao, Y., Jha, A., Jiang, F., Song J., J., Wei, W., Hurov, J. B., Song J, J., Wei, W., and Hurov, J. B. (2011) Full-length human glutaminase in complex with an allosteric inhibitor. *Biochemistry.* **50**, 10764–10770
 39. Bramlett, K. S., Wu, Y., and Burris, T. P. (2001) Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. *Mol Endocrinol.* **15**, 909–22.
 40. Stevens, F. C. (1983) Calmodulin: an introduction. *Can. J. Biochem. cell Biol.* **61**, 906–10
 41. Gomes, A. V., Potter, J. D., and Szczesna-Cordary, D. (2002) The role of troponins in muscle contraction. *IUBMB Life.* **54**, 323–333
 42. Lewit-Bentley, A., and Réty, S. (2000) EF-hand calcium-binding proteins. *Curr. Opin. Struct. Biol.* **10**, 637–643
 43. Pflieger, C. M., and Kirschner, M. W. (2000) The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev.* **14**, 655–65
 44. Kornau, H.-C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) Domain Interaction between NMDA Receptor Subunits and the Postsynaptic Density Protein PSD-95. *Source Sci. New Ser.* **269**, 1737–1740
 45. Márquez, J., Tosina, M., de la Rosa, V., Segura, J. A., Alonso, F. J., Matés, J. M., and Campos-Sandoval, J. A. (2009) New insights into brain glutaminases: Beyond their role on glutamatergic transmission. *Neurochem. Int.* **55**, 64–70
 46. Bergström, J., Fürst, P., Norée, L. O., and Vinnars, E. (1974) Intracellular free amino acid concentration in human muscle tissue. *J. Appl. Physiol.* **36**, 693–7
 47. DeBerardinis, R. J., and Cheng, T. (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene.* **29**, 313–324
 48. Levine, A. J., and Puzio-Kuter, A. M. (2010) The Control of the Metabolic Switch in Cancers by Oncogenes and Tumor Suppressor Genes. *Science (80-).* **330**, 1340–1344
 49. Yelamanchi, S. D., Jayaram, S., Thomas, J. K., Gundimeda, S., Khan, A. A., Singhal, A., Keshava Prasad, T. S., Pandey, A., Somani, B. L., and Gowda, H. (2016) A pathway map of glutamate metabolism. *J. Cell Commun. Signal.* **10**, 69–75
 50. Owen, O. E., Kalhan, S. C., and Hanson, R. W. (2002) The key role of anaplerosis and cataplerosis for citric acid cycle function. *J. Biol. Chem.* **277**, 30409–30412
 51. Altman, B. J., Stine, Z. E., and Dang, C. V (2016) From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat. Rev. Cancer.* **16**, 619–634
 52. Van Den Heuvel, A. P. J., Jing, J., Wooster, R. F., and Bachman, K. E. (2012) Analysis of glutamine dependency in non-small cell lung cancer: GLS1 splice variant GAC is essential for cancer cell growth. *Cancer Biol. Ther.* **13**, 1185–1194
 53. Timmerman, L. A., Holton, T., Yuneva, M., Louie, R. J., Padró, M., Daemen, A., Hu, M., Chan, D. A., Ethier, S. P., van'tVeer, L. J., Polyak, K., McCormick, F., Gray, J. W., Padr??, M., Daemen, A., Hu, M., Chan, D. A., Ethier, S. P., van'tVeer, L. J., Polyak, K., McCormick, F., and Gray, J. W. (2013) Glutamine Sensitivity Analysis Identifies the xCT Antiporter as a Common Triple-Negative Breast

54. Xia, Z., Donehower, L. A., Cooper, T. A., Neilson, J. R., Wheeler, D. A., Wagner, E. J., and Li, W. (2014) Dynamic analyses of alternative polyadenylation from RNA-seq reveal a 3'-UTR landscape across seven tumour types. *Nat. Commun.* **5**, 5274
55. Redis, R. S., Vela, L. E., Lu, W., Ferreira de Oliveira, J., Ivan, C., Rodriguez-Aguayo, C., Adamoski, D., Pasculli, B., Taguchi, A., Chen, Y., Fernandez, A. F., Valledor, L., Van Roosbroeck, K., Chang, S., Shah, M., Kinnebrew, G., Han, L., Atlasi, Y., Cheung, L. H., Huang, G. Y., Monroig, P., Ramirez, M. S., Catela Ivkovic, T., Van, L., Ling, H., Gafà, R., Kapitanovic, S., Lanza, G., Bankson, J. A., Huang, P., Lai, S. Y., Bast, R. C., Rosenblum, M. G., Radovich, M., Ivan, M., Bartholomeusz, G., Liang, H., Fraga, M. F., Widger, W. R., Hanash, S., Berindan-Neagoe, I., Lopez-Berestein, G., Ambrosio, A. L. B. B., Gomes Dias, S. M., and Calin, G. A. (2016) Allele-Specific Reprogramming of Cancer Metabolism by the Long Non-coding RNA CCAT2. *Mol. Cell*. **61**, 520–534
56. Suzuki, S., Tanaka, T., Poyurovsky, M. V., Nagano, H., Mayama, T., Ohkubo, S., Lokshin, M., Hosokawa, H., Nakayama, T., Suzuki, Y., Sugano, S., Sato, E., Nagao, T., Yokote, K., Tatsuno, I., and Prives, C. (2010) Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 7461–7466
57. Hu, W., Zhang, C., Wu, R., Sun, Y., Levine, A., and Feng, Z. (2010) Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 7455–7460
58. Szeliga, M., Bogacińska-Karaś, M., Różycka, A., Hilgier, W., Marquez, J., Albrecht, J., Różycka, A., Hilgier, W., Marquez, J., and Albrecht, J. (2014) Silencing of GLS and overexpression of GLS2 genes cooperate in decreasing the proliferation and viability of glioblastoma cells. *Tumor Biol.* **35**, 1855–1862
59. Szeliga, M., and Albrecht, J. (2015) Opposing roles of glutaminase isoforms in determining glioblastoma cell phenotype. *Neurochem. Int.* **88**, 6–9
60. Liu, J., Zhang, C., Lin, M., Zhu, W., Liang, Y., Hong, X., Zhao, Y., Young, K. H., Hu, W., and Feng, Z. (2014) Glutaminase 2 negatively regulates the PI3K / AKT signaling and shows tumor suppression activity in human hepatocellular carcinoma ABSTRACT : *Oncotarget*. **5**, 2635–2647
61. Zhang, C., Liu, J., Zhao, Y., Yue, X., Zhu, Y., Wang, X., Wu, H., Blanco, F., Li, S., Bhanot, G., Haffty, B. G., Hu, W., and Feng, Z. (2016) Glutaminase 2 is a novel negative regulator of small GTPase Rac1 and mediates p53 function in suppressing metastasis. *Elife*. **5**, 1–20
62. Kuo, T. C., Chen, C. K., Hua, K. T., Yu, P., Lee, W. J., Chen, M. W., Jeng, Y. M., Chien, M. H., Kuo, K. T., Hsiao, M., and Kuo, M. L. (2016) Glutaminase 2 stabilizes Dicer to repress Snail and metastasis in hepatocellular carcinoma cells. *Cancer Lett.* **383**, 1–13
63. Wang, Y., and Shang, Y. (2013) Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. *Exp. Cell Res.* **319**, 160–169
64. Xiang, L., Xie, G., Liu, C., Zhou, J., Chen, J., Yu, S., Li, J., Pang, X., Shi, H., and Liang, H. (2013) Knock-down of glutaminase 2 expression decreases glutathione, NADH, and sensitizes cervical cancer to ionizing radiation. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833**, 2996–3005
65. Qing, G., Li, B., Vu, A., Skuli, N., Walton, Z. E., Liu, X., Mayes, P. A., Wise, D. R., Thompson, C. B., Maris, J. M., Hogarty, M. D., and Simon, M. C. (2012) ATF4 Regulates MYC-Mediated Neuroblastoma Cell Death upon Glutamine Deprivation. *Cancer Cell*. **22**, 631–644
66. Xiao, D., Ren, P., Su, H., Yue, M., Xiu, R., Hu, Y., Liu, H., and Qing, G. (2015) Myc promotes glutaminolysis in human neuroblastoma through direct activation of glutaminase 2. *Oncotarget*. **6**, 40655–66
67. Wise, D. R., DeBerardinis, R. J., Mancuso, A., Sayed, N., Zhang, X.-Y., Pfeiffer, H. K., Nissim, I., Daikhin, E., Yudkoff, M., McMahon, S. B., and Thompson, C. B. (2008) Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 18782–18787

68. Gaglio, D., Metallo, C. M., Gameiro, P. A., Hiller, K., Danna, L. S., Balestrieri, C., Alberghina, L., Stephanopoulos, G., and Chiaradonna, F. (2011) Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. *Mol. Syst. Biol.* **7**, 523
69. Wang, J.-B., Erickson, J. W., Fuji, R., Ramachandran, S., Gao, P., Dinavahi, R., Wilson, K. F., Ambrosio, A. L. B., Dias, S. M. G., Dang, C. V., and Cerione, R. A. (2010) Targeting Mitochondrial Glutaminase Activity Inhibits Oncogenic Transformation. *Cancer Cell.* **18**, 207–219
70. Lee, S. Y., Jeon, H. M., Ju, M. K., Jeong, E. K., Kim, C. H., Park, H. G., Han, S. I., and Kang, H. S. (2016) Dlx-2 and glutaminase upregulate epithelial-mesenchymal transition and glycolytic switch. *Oncotarget.* **7**, 7925–39
71. Garcia-Cao, I., Song, M. S., Hobbs, R. M., Laurent, G., Giorgi, C., De Boer, V. C. J., Anastasiou, D., Ito, K., Sasaki, A. T., Rameh, L., Carracedo, A., Vander Heiden, M. G., Cantley, L. C., Pinton, P., Haigis, M. C., and Pandolfi, P. P. (2012) Systemic elevation of PTEN induces a tumor-suppressive metabolic state. *Cell.* **149**, 49–62
72. Chang, T.-C., Yu, D., Lee, Y.-S., Wentzel, E. A., Arking, D. E., West, K. M., Dang, C. V, Thomas-Tikhonenko, A., and Mendell, J. T. (2008) Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat. Genet.* **40**, 43–50
73. Eilers, M., and Eisenman, R. N. (2008) Myc's broad reach. *Genes Dev.* **22**, 2755–2766
74. Gao, P., Tchernyshyov, I., Chang, T.-C., Lee, Y.-S., Kita, K., Ochi, T., Zeller, K. I., De Marzo, A. M., Van Eyk, J. E., Mendell, J. T., and Dang, C. V (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature.* **458**, 762–5
75. Chhabra, R., Dubey, R., and Saini, N. (2010) Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases. *Mol. Cancer.* **9**, 232
76. Rathore, M. G., Saumet, A., Rossi, J.-F., de Bettignies, C., Tempé, D., Lecellier, C.-H., and Villalba, M. (2012) The NF-κB member p65 controls glutamine metabolism through miR-23a. *Int. J. Biochem. Cell Biol.* **44**, 1448–1456
77. Weinberg, F., Hamanaka, R., Wheaton, W. W., Weinberg, S., Joseph, J., Lopez, M., Kalyanaraman, B., Mutlu, G. M., Budinger, G. R. S., and Chandel, N. S. (2010) Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 8788–93
78. Son, J., Lyssiotis, C. a, Ying, H., Wang, X., Hua, S., Ligorio, M., Perera, R. M., Ferrone, C. R., Mullarky, E., Shyh-Chang, N., Kang, Y., Fleming, J. B., Bardeesy, N., Asara, J. M., Haigis, M. C., DePinho, R. a, Cantley, L. C., and Kimmelman, A. C. (2013) Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature.* **496**, 101–5
79. Lukey, M. J., Greene, K. S., Erickson, J. W., Wilson, K. F., and Cerione, R. A. (2016) The oncogenic transcription factor c-Jun regulates glutaminase expression and sensitizes cells to glutaminase-targeted therapy. *Nat. Commun.* **7**, 11321
80. Pascoal, C. C., Islam, Z., Adamoski, D., Ferreira, I. M., Righetto, R. D., Bettini, J., Portugal, R. V., Yue, W. W., Gonzalez, A., Dias, S. M. G., Ambrosio, A. L. B., Pasquali, C. C., Islam, Z., Adamoski, D., Ferreira, I. M., Righeto, R. D., Bettini, J., Portugal, R. V., Yue, W. W., Gonzalez, A., Dias, S. M. G., and Ambrosio, A. L. B. (2017) The origin and evolution of human glutaminases and their atypical C-terminal ankyrin repeats. *J. Biol. Chem.* **292**, 11572–11585
81. Warburg, O. (1956) On respiratory impairment in cancer cells. *Science.* **124**, 269–70
82. Warburg, O. (1956) On the Origin of Cancer Cells. *Science.* **123**, 309–314
83. Engelman, J. a, Chen, L., Tan, X., Crosby, K., Guimaraes, A. R., Upadhyay, R., Maira, M., McNamara, K., Perera, S. a, Song, Y., Chirieac, L. R., Kaur, R., Lightbown, A., Simendinger, J., Li, T., Padera, R. F., García-Echeverría, C., Weissleder, R., Mahmood, U., Cantley, L. C., and Wong, K.-K. (2008) Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat. Med.* **14**, 1351–6

84. Jun, J. C., Rathore, A., Younas, H., Gilkes, D., and Polotsky, V. Y. (2017) Hypoxia-Inducible Factors and Cancer. *Curr. Sleep Med. Reports*. 10.1007/s40675-017-0062-7
85. Eales, K. L., Hollinshead, K. E. R., and Tennant, D. A. (2016) Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis*. **5**, e190
86. Semenza, G. L., and Wang, G. L. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.* **12**, 5447–54
87. Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer*. **3**, 721–32
88. Liu, W., Shen, S., Zhao, X., and Chen, G. (2012) Targeted genes and interacting proteins of hypoxia inducible factor-1. *Int J Biochem Mol Biol*. **3**, 165–178
89. Semenza, G., Jiang, B. B.-H., Leung, S., Passantino, R., Concordet, J., Maire, P., and Giallongo, A. (1996) Hypoxia Response Elements in the Aldolase A, Enolase 1, and Lactate Dehydrogenase A Gene Promoters Contain Essential Binding Sites for Hypoxia-inducible Factor 1. *J. Biol. Chem.* **271**, 32529–32537
90. Semenza, G. L., Roth, P. H., Fang, H., and Wang, G. L. (1994) Transcriptional Regulation of Genes Encoding Glycolytic. *J. Biol. Chem.*
91. Ebert, B. L., Firth, J.D., Ratcliffe, P.J. (1995) Hypoxia and Mitochondrial Inhibitors Regulate Expression of Glucose Transporter-1 via Distinct Cis-acting Sequences. *J. Biol. Chem.* **270**, 29083–29089
92. Maher, E. R., Neumann, H. P. H., and Richard, S. (2011) von Hippel–Lindau disease: A clinical and scientific review. *Eur. J. Hum. Genet.* **19**, 617–623
93. Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, a. M., Yu, a. Y., and Semenza, G. L. (1998) Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor 1 α . *Genes Dev.* **12**, 149–162
94. Salceda, S. (1997) Hypoxia-inducible Factor 1 α (HIF-1 α) Protein Is Rapidly Degraded by the Ubiquitin-Proteasome System under Normoxic Conditions. ITS STABILIZATION BY HYPOXIA DEPENDS ON REDOX-INDUCED CHANGES. *J. Biol. Chem.* **272**, 22642–22647
95. Taylor, B. L., and Zhulin, I. B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* **63**, 479–506
96. Semenza, G. L. (2013) HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J. Clin. Invest.* 10.1172/JCI67230
97. Bracken, C. P., Whitelaw, M. L., and Peet, D. J. (2003) The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cell. Mol. Life Sci.* **60**, 1376–93
98. Lando, D., Peet, D. J., Whelan, D. a, Gorman, J. J., and Whitelaw, M. L. (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science*. **295**, 858–61
99. Keith, B., Johnson, R. S. R., and Simon, M. C. (2012) HIF1 α and HIF2 α : sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer*. **12**, 9–22
100. Ke, Q., and Costa, M. (2006) Hypoxia-Inducible Factor-1 (HIF-1). **70**, 1469–1480
101. Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, a, and Poellinger, L. (2001) Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature*. **414**, 550–554
102. Li, Q. F., Wang, X. R., Yang, Y. W., and Lin, H. (2006) Hypoxia upregulates hypoxia inducible factor (HIF)-3 α expression in lung epithelial cells: characterization and comparison with HIF-1 α . *Cell Res.* **16**, 548–58
103. Maynard, M. a, Evans, A. J., Hosomi, T., Hara, S., Jewett, M. a S., and Ohh, M. (2005) Human HIF-3 α 4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *FASEB J.* **19**, 1396–406

104. Lindebro, M. C., Poellinger, L., and Whitelaw, M. L. (1995) Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J.* **14**, 3528–39
105. Whitelaw, M., Pongratz, I., Wilhelmsson, a, Gustafsson, J. a, and Poellinger, L. (1993) Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. *Mol. Cell. Biol.* **13**, 2504–14
106. Yamaguchi, Y., and Kuo, M. T. (1995) Functional analysis of aryl hydrocarbon receptor nuclear translocator interactions with aryl hydrocarbon receptor in the yeast two-hybrid system. *Biochem. Pharmacol.* **50**, 1295–302
107. Tanaka, T., Wiesener, M., Bernhardt, W., Eckardt, K.-U., and Warnecke, C. (2009) The human HIF (hypoxia-inducible factor)-3 α gene is a HIF-1 target gene and may modulate hypoxic gene induction. *Biochem. J.* **424**, 143–51
108. Pasanen, A., Heikkilä, M., Rautavuoma, K., Hirsilä, M., Kivirikko, K. I., and Myllyharju, J. (2010) Hypoxia-inducible factor (HIF)-3 α is subject to extensive alternative splicing in human tissues and cancer cells and is regulated by HIF-1 but not HIF-2. *Int. J. Biochem. Cell Biol.* **42**, 1189–1200
109. Maynard, M. a, Qi, H., Chung, J., Lee, E. H. L., Kondo, Y., Hara, S., Conaway, R. C., Conaway, J. W., and Ohh, M. (2003) Multiple splice variants of the human HIF-3 α locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. *J. Biol. Chem.* **278**, 11032–40
110. Ravenna, L., Salvatori, L., and Russo, M. A. (2016) HIF3 α : The little we know. *FEBS J.* **283**, 993–1003
111. Maynard, M. A., Evans, A. J., Shi, W., Kim, W. Y., Liu, F.-F. F., and Ohh, M. (2007) Dominant-negative HIF-3 α 4 suppresses VHL-null renal cell carcinoma progression. *Cell Cycle*. **6**, 2810–6
112. Zhang, P., Yao, Q., Lu, L., Li, Y., Chen, P.-J. J., and Duan, C. (2014) Hypoxia-Inducible Factor 3 Is an Oxygen-Dependent Transcription Activator and Regulates a Distinct Transcriptional Response to Hypoxia. *Cell Rep.* **6**, 1110–1121
113. Hara, S., Hamada, J., Kobayashi, C., Kondo, Y., and Imura, N. (2001) Expression and characterization of hypoxia-inducible factor (HIF)-3 α in human kidney: suppression of HIF-mediated gene expression by HIF-3 α . *Biochem. Biophys. Res. Commun.* **287**, 808–13
114. Huang, Y., Kapere Ochieng, J., Kempen, M. B., Munck, A. B., Swagemakers, S., van Ijcken, W., Grosveld, F., Tibboel, D., and Rottier, R. J. (2013) Hypoxia inducible factor 3 α plays a critical role in alveolarization and distal epithelial cell differentiation during mouse lung development. *PLoS One*. **8**, e57695
115. Chen, S., Xu, Y., Chen, Y., Li, X., Mou, W., Wang, L., Liu, Y., Reisfeld, R. A., Xiang, R., Lv, D., and Li, N. (2012) SOX2 gene regulates the transcriptional network of oncogenes and affects tumorigenesis of human lung cancer cells. *PLoS One*. **7**, 1–12
116. Xue, X., Jungles, K., Onder, G., Samhoun, J., and Györfy, B. (2016) HIF-3 α 1 promotes colorectal tumor cell growth by activation of JAK-STAT3 signaling. *Oncotarget*. 10.18632/oncotarget.7272
117. Möglich, A., Ayers, R. a, and Moffat, K. (2009) Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure*. **17**, 1282–94
118. Henry, J. T., and Crosson, S. (2011) Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu. Rev. Microbiol.* **65**, 261–86
119. Amezcua, C. A., Harper, S. M., Rutter, J., and Gardner, K. H. (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. *Structure*. **10**, 1349–61
120. Taylor, B. L., and Zhulin, I. B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* **63**, 479–506
121. Fala, A. M., Oliveira, J. F., Adamoski, D., Aricetti, J. A., Dias, M. M., Dias, M. V. B., Sforça, M. L., Lopes-de-Oliveira, P. S., Rocco, S. A., Caldana, C., Dias, S. M. G., and Ambrosio, A. L. B. (2015)

- Unsaturated fatty acids as high-affinity ligands of the C-terminal Per-ARNT-Sim domain from the Hypoxia-inducible factor 3 α . *Sci. Rep.* **5**, 12698
122. Halestrap, A. P. (1975) The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J.* **148**, 85–96
 123. Schell, J., and Rutter, J. (2013) The long and winding road to the mitochondrial pyruvate carrier. *Cancer Metab.* **1**, 6
 124. Herzig, S. S., Raemy, E., Montessuit, S., Veuthey, J.-L. J.-L. L., Zamboni, N., Westermann, B., Kunji, E. R. S., and Martinou, J.-C. J.-C. J.-C. C. (2012) Identification and functional expression of the mitochondrial pyruvate carrier. *Science* (80-.). **337**, 93–96
 125. Bricker, D. K., Taylor, E. B., Schell, J. C., Orsak, T., Chen, Y., Cox, J. E., Cardon, C. M., Van, J. G., Dephoure, N., Redin, C., Boudina, S., Gygi, S. P., Thummel, C. S., Rutter, J., Boutron, A., Chen, Y., Cox, J. E., Cardon, C. M., Van Vranken, J. G., Dephoure, N., Redin, C., Boudina, S., Gygi, S. P., Brivet, M., Thummel, C. S., and Rutter, J. (2012) A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, Drosophila, and humans. *Science*. **337**, 96–100
 126. Divakaruni, A. S., Wiley, S. E., Rogers, G. W., Andreyev, A. Y., Petrosyan, S., Loviscach, M., Wall, E. a, Yadava, N., Heuck, A. P., Ferrick, D. a, Henry, R. R., McDonald, W. G., Colca, J. R., Simon, M. I., Ciaraldi, T. P., and Murphy, A. N. (2013) Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 5422–7
 127. Hunte, C., Koepke, J., Lange, C., Roßmanith, T., and Michel, H. (2000) Structure at 2.3 Å resolution of the cytochrome bc₁ complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment. *Structure*. **8**, 669–684
 128. Harrenga, A., and Michel, H. (1999) The Cytochrome c Oxidase from *Paracoccus denitrificans* Does Not Change the Metal Center Ligation upon Reduction. *J. Biol. Chem.* **274**, 33296–33299
 129. Zhou, Y., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001) Chemistry of ion coordination and hydration revealed by a K⁺ channel-Fab complex at 2.0 Å resolution. *Nature*. **414**, 43–8
 130. Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K. I., and Myllyharju, J. (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J. Biol. Chem.* **278**, 30772–30780
 131. Biddlestone, J., Bandarra, D., and Rocha, S. (2015) The role of hypoxia in inflammatory disease (Review). *Int. J. Mol. Med.* **35**, 859–869
 132. Zhang, Q., Gu, J., Li, L., Liu, J., Luo, B., Cheung, H. W., Boehm, J. S., Ni, M., Geisen, C., Root, D. E., Polyak, K., Brown, M., Richardson, A. L., Hahn, W. C., Kaelin, W. G., and Bommi-Reddy, A. (2009) Control of Cyclin D1 and Breast Tumorigenesis by the EglN2 Prolyl Hydroxylase. *Cancer Cell*. **16**, 413–424
 133. Vogel, S., Wottawa, M., Farhat, K., Zieseniss, A., Schnelle, M., Le-Huu, S., Von Ahlen, M., Malz, C., Camenisch, G., and Katschinski, D. M. (2010) Prolyl Hydroxylase Domain (PHD) 2 affects cell migration and F-actin formation via RhoA/Rho-associated kinase-dependent cofilin phosphorylation. *J. Biol. Chem.* **285**, 33756–33763
 134. Lee, D. C., Sohn, H. A., Park, Z. Y., Oh, S., Kang, Y. K., Lee, K. M., Kang, M., Jang, Y. J., Yang, S. J., Hong, Y. K., Noh, H., Kim, J. A., Kim, D. J., Bae, K. H., Kim, D. M., Chung, S. J., Yoo, H. S., Yu, D. Y., Park, K. C., and Yeom, Y. Il (2015) A lactate-induced response to hypoxia. *Cell*. **161**, 595–609
 135. Katschinski, D. M. (2009) In vivo functions of the prolyl-4-hydroxylase domain oxygen sensors: direct route to the treatment of anaemia and the protection of ischaemic tissues. *Acta Physiol.* **195**, 407–414
 136. Takeda, K., Ho, V. C., Takeda, H., Duan, L.-J., Nagy, A., and Fong, G.-H. (2006) Placental but Not Heart Defects Are Associated with Elevated Hypoxia-Inducible Factor Levels in Mice Lacking Prolyl Hydroxylase Domain Protein 2. *Mol. Cell. Biol.* **26**, 8336–8346
 137. Place, T. L., and Domann, F. E. (2013) Prolyl-hydroxylase 3: evolving roles for an ancient signaling

- protein. *Hypoxia*. **2013**, 13
138. Vezina, C. M., Lin, T.-M., and Peterson, R. E. (2009) AHR signaling in prostate growth, morphogenesis, and disease. *Biochem. Pharmacol.* **77**, 566–76
 139. Köditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., Myllyharju, J., Wenger, R. H., and Katschinski, D. M. (2007) Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. *Blood*. **110**, 3610–3617
 140. Luo, W., Lin, B., Wang, Y., Zhong, J., O’Meally, R., Cole, R. N., Pandey, A., Levchenko, A., and Semenza, G. L. (2014) PHD3-mediated prolyl hydroxylation of nonmuscle actin impairs polymerization and cell motility. *Mol. Biol. Cell*. **25**, 2788–96
 141. Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O’Meally, R., Cole, R. N., Pandey, A., and Semenza, G. L. (2011) Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell*. **145**, 732–744
 142. Garvalov, B. K., Foss, F., Henze, A.-T., Bethani, I., Gräf-Höchst, S., Singh, D., Filatova, A., Dopeso, H., Seidel, S., Damm, M., Acker-Palmer, A., and Acker, T. (2014) PHD3 regulates EGFR internalization and signalling in tumours. *Nat. Commun.* **5**, 5577
 143. Koivunen, P., Tiainen, P., Hyvärinen, J., Williams, K. E., Sormunen, R., Klaus, S. J., Kivirikko, K. I., and Myllyharju, J. (2007) An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha. *J. Biol. Chem.* **282**, 30544–52
 144. Bos, R., van der Groep, P., Greijer, A. E., Shvarts, A., Meijer, S., Pinedo, H. M., Semenza, G. L., van Diest, P. J., and van der Wall, E. (2003) Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*. **97**, 1573–81
 145. Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) Differential Function of the Prolyl Hydroxylases PHD1, PHD2, and PHD3 in the Regulation of Hypoxia-inducible Factor. *J. Biol. Chem.* **279**, 38458–38465
 146. Högel, H., Rantanen, K., Jokilehto, T., Grenman, R., and Jaakkola, P. M. (2011) Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma cells. *PLoS One*. **6**, e27112
 147. Chowdhury, R., McDonough, M. a, Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., Domene, C., and Schofield, C. J. (2009) Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. *Structure*. **17**, 981–9
 148. Chowdhury, R., McDonough, M. A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., Domene, C., and Schofield, C. J. (2009) Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases. *Structure*. **17**, 981–989
 149. Gorbunova, V., Hine, C., Tian, X., Abulaeva, J., Gudkov, A. V., Nevo, E., and Seluanov, A. (2012) Cancer resistance in the blind mole rat is mediated by concerted necrotic cell death mechanism. *Proc. Natl. Acad. Sci.* **109**, 19392–19396
 150. Heron, M. (2012) Deaths: leading causes for 2008. *Natl. vital Stat. reports*. **60**, 1–94
 151. Lipman, R., Galecki, A., Burke, D. T., and Miller, R. A. (2004) Genetic loci that influence cause of death in a heterogeneous mouse stock. *J. Gerontol. A. Biol. Sci. Med. Sci.* **59**, 977–83
 152. Nevo, E; Ivanitskaya, E. B. A. (2001) Adaptive Radiation of Blind Subterranean Mole Rats. *Heredity (Edinb)*. **89**, 325–326
 153. Shams, I., Avivi, A., and Nevo, E. (2005) Oxygen and carbon dioxide fluctuations in burrows of subterranean blind mole rats indicate tolerance to hypoxic-hypercapnic stresses. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **142**, 376–382
 154. Avivi, A., Gerlach, F., Joel, A., Reuss, S., Burmester, T., Nevo, E., and Hankeln, T. (2010) Neuroglobin, cytoglobin, and myoglobin contribute to hypoxia adaptation of the subterranean mole rat *Spalax*. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21570–21575
 155. Avivi, a, Ashur-Fabian, O., Joel, a, Trakhtenbrot, L., Adamsky, K., Goldstein, I., Amariglio, N.,

- Rechavi, G., and Nevo, E. (2007) P53 in blind subterranean mole rats--loss-of-function versus gain-of-function activities on newly cloned *Spalax* target genes. *Oncogene*. **26**, 2507–12
156. Scheffler, I. E. (2001) A century of mitochondrial research: Achievements and perspectives. *Mitochondrion*. **1**, 3–31
 157. Dudkina, N. V., Kouřil, R., Peters, K., Braun, H. P., and Boekema, E. J. (2010) Structure and function of mitochondrial supercomplexes. *Biochim. Biophys. Acta - Bioenerg.* **1797**, 664–670
 158. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneider, R., Green, D. R., and Newmeyer, D. D. (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell*. **111**, 331–342
 159. De Pinto, V., Reina, S., Guarino, F., and Messina, A. (2008) Structure of the voltage dependent anion channel: State of the art. *J. Bioenerg. Biomembr.* **40**, 139–147
 160. Nelson, D. L., Lehninger, A. L., and Cox, M. M. (2008) *Lehninger Principles of Biochemistry* (Freeman, W. H. ed)
 161. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2010) *Biologia Molecular da Célula*, 5ª edição, Artmed, Porto Alegre
 162. Shoshan-Barmatz, V., and Mizrahi, D. (2012) VDAC1: from structure to cancer therapy. *Front. Oncol.* **2**, 164
 163. Yagoda, N., von Rechenberg, M., Zaganjor, E., Bauer, A. J., Yang, W. S., Fridman, D. J., Wolpaw, A. J., Smukste, I., Peltier, J. M., Boniface, J. J., Smith, R., Lessnick, S. L., Sahasrabudhe, S., and Stockwell, B. R. (2007) RAS–RAF–MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature*. **447**, 865–869
 164. Wu, S., Sampson, M. J., Decker, W. K., and Craigen, W. J. (1999) Each mammalian mitochondrial outer membrane porin protein is dispensable: Effects on cellular respiration. *Biochim. Biophys. Acta - Mol. Cell Res.* **1452**, 68–78
 165. Anfous-Pharayra, K., Lee, N., Armstrong, D. L., and Craigen, W. J. (2011) VDAC3 has differing mitochondrial functions in two types of striated muscles. *Biochim. Biophys. Acta - Bioenerg.* **1807**, 150–156
 166. Sampson, M. J., Decker, W. K., Beaudet, A. L., Ruitenbeek, W., Armstrong, D., Hicks, M. J., and Craigen, W. J. (2001) Immobile Sperm and Infertility in Mice Lacking Mitochondrial Voltage-dependent Anion Channel Type 3. *J. Biol. Chem.* **276**, 39206–39212
 167. Cheng, E. H. Y., Sheiko, T. V., Fisher, J. K., Craigen, W. J., and Korsmeyer, S. J. (2003) VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science*. **301**, 513–517
 168. Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Millman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-X(L) prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* **274**, 1156–1163
 169. Horvitz, H. R. (2003) Worms, life, and death (Nobel lecture). in *ChemBioChem*, pp. 697–711, **4**, 697–711
 170. Suzuki, M., Youle, R. J., and Tjandra, N. (2000) Structure of Bax. *Cell*. **103**, 645–654
 171. Ma, S., Hockings, C., Anwari, K., Kratina, T., Fennell, S., Lazarou, M., Ryan, M. T., Kluck, R. M., and Dewson, G. (2013) Assembly of the bak apoptotic pore: A critical role for the bak protein $\alpha 6$ helix in the multimerization of homodimers during apoptosis. *J. Biol. Chem.* **288**, 26027–26038
 172. Green, D. R., and Evan, G. I. (2002) A matter of life and death. *Cancer Cell*. **1**, 19–30
 173. Ghobrial, I. M., Witzig, T. E., and Adjei, A. A. (2005) in BASIC SCIENCE Targeting Apoptosis Pathways in Cancer Therapy. *A Cancer J. Clin.* **55**, 178–194
 174. Lazarou, M., Stojanovski, D., Frazier, A. E., Kotevski, A., Dewson, G., Craigen, W. J., Kluck, R. M., Vaux, D. L., and Ryan, M. T. (2010) Inhibition of Bak activation by VDAC2 is dependent on the Bak transmembrane anchor. *J. Biol. Chem.* **285**, 36876–36883

175. Ross, K., Rudel, T., and Kozjak-Pavlovic, V. (2009) TOM-independent complex formation of Bax and Bak in mammalian mitochondria during TNF α -induced apoptosis. *Cell Death Differ.* **16**, 697–707
176. Kozjak-Pavlovic, V., Ross, K., Götz, M., Goosmann, C., and Rudel, T. (2010) A Tag at the Carboxy Terminus Prevents Membrane Integration of VDAC1 in Mammalian Mitochondria. *J. Mol. Biol.* **397**, 219–232
177. Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E., and Pfanner, N. (2001) Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. *J. Cell Biol.* **152**, 289–300
178. Ma, S. B., Nguyen, T. N., Tan, I., Ninnis, R., Iyer, S., Stroud, D. a, Menard, M., Kluck, R. M., Ryan, M. T., and Dewson, G. (2014) Bax targets mitochondria by distinct mechanisms before or during apoptotic cell death: a requirement for VDAC2 or Bak for efficient Bax apoptotic function. *Cell Death Differ.* **21**, 1925–1935
179. Schellenberg, B., Wang, P., Keeble, J. A., Rodriguez-Enriquez, R., Walker, S., Owens, T. W., Foster, F., Tanianis-Hughes, J., Brennan, K., Streuli, C. H., and Gilmore, A. P. (2013) Bax Exists in a Dynamic Equilibrium between the Cytosol and Mitochondria to Control Apoptotic Priming. *Mol. Cell.* **49**, 959–971
180. Edlich, F., Banerjee, S., Suzuki, M., Cleland, M. M., Arnoult, D., Wang, C., Neutzner, A., Tjandra, N., and Youle, R. J. (2011) Bcl-xL retrotranslocates Bax from the mitochondria into the cytosol. *Cell.* **145**, 104–116
181. Dewson, G. (2015) Two roads to death – Bax targets mitochondria by distinct routes before or during apoptotic cell death. *Mol. Cell. Oncol.* **2**, e974460
182. Plötz, M., Gillissen, B., Hossini, a M., Daniel, P. T., and Eberle, J. (2012) Disruption of the VDAC2–Bak interaction by Bcl-xS mediates efficient induction of apoptosis in melanoma cells. *Cell Death Differ.* **19**, 1928–1938
183. Billin, a N., Eilers, a L., Coulter, K. L., Logan, J. S., and Ayer, D. E. (2000) MondoA, a Novel Basic Helix-Loop-Helix-Leucine Zipper Transcriptional Activator That Constitutes a Positive Branch of a Max-Like Network. *Mol. Cell. Biol.* **20**, 8845–8854
184. Davies, M. N., O’Callaghan, B. L., and Towle, H. C. (2008) Glucose Activates ChREBP by Increasing Its Rate of Nuclear Entry and Relieving Repression of Its Transcriptional Activity. *J. Biol. Chem.* **283**, 24029–24038
185. Stoltzman, C. a, Peterson, C. W., Breen, K. T., Muoio, D. M., Billin, A. N., and Ayer, D. E. (2008) Glucose sensing by MondoA: Mlx complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 6912–7
186. Sans, C. L., Satterwhite, D. J., Stoltzman, C. A., Breen, K. T., and Ayer, D. E. (2006) MondoA-Mlx Heterodimers Are Candidate Sensors of Cellular Energy Status: Mitochondrial Localization and Direct Regulation of Glycolysis. *Mol. Cell. Biol.* **26**, 4863–4871
187. Ma, L., Tsatsos, N. G., and Towle, H. C. (2005) Direct role of ChREBP-Mlx in regulating hepatic glucose-responsive genes. *J. Biol. Chem.* **280**, 12019–12027
188. Yamashita, H., Takenoshita, M., Sakurai, M., Bruick, R. K., Henzel, W. J., Shillinglaw, W., Arnot, D., and Uyeda, K. (2001) A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc. Natl. Acad. Sci.* **98**, 9116–9121
189. Burgess, S. C., Iizuka, K., Jeoung, N. H., Harris, R. A., Kashiwaya, Y., Veech, R. L., Kitazume, T., and Uyeda, K. (2008) Carbohydrate-response Element-binding Protein Deletion Alters Substrate Utilization Producing an Energy-deficient Liver. *J. Biol. Chem.* **283**, 1670–1678
190. Li, M. V, Chen, W., Pongvarin, N., Imamura, M., and Chan, L. (2008) Glucose-Mediated Transactivation of Carbohydrate Response Element-Binding Protein Requires Cooperative Actions from Mondo Conserved Regions and Essential Trans -Acting Factor 14-3-3. *Mol.*

191. Tsatsos, N. G., and Towle, H. C. (2006) Glucose activation of ChREBP in hepatocytes occurs via a two-step mechanism. *Biochem. Biophys. Res. Commun.* **340**, 449–456
192. Eilers, A. L., Sundwall, E., Lin, M., Sullivan, A. A., and Ayer, D. E. (2002) A novel heterodimerization domain, CRM1, and 14-3-3 control subcellular localization of the MondoA-Mlx heterocomplex. *Mol Cell Biol.* **22**, 8514–8526
193. Peterson, C. W., Stoltzman, C. A., Sighinolfi, M. P., Han, K. S., and Ayer, D. E. (2010) Glucose Controls Nuclear Accumulation, Promoter Binding, and Transcriptional Activity of the MondoA-Mlx Heterodimer. *Mol. Cell. Biol.* **30**, 2887–2895
194. Collier, J. J., Doan, T.-T. T., Daniels, M. C., Schurr, J. R., Kolls, J. K., and Scott, D. K. (2003) c-Myc Is Required for the Glucose-mediated Induction of Metabolic Enzyme Genes. *J. Biol. Chem.* **278**, 6588–6595
195. Havula, E., and Hietakangas, V. (2012) Glucose sensing by ChREBP/MondoA–Mlx transcription factors. *Semin. Cell Dev. Biol.* **23**, 640–647
196. Sloan, E. J., and Ayer, D. E. (2010) Myc, Mondo, and Metabolism. *Genes Cancer.* **1**, 587–596
197. Zhang, P., Metukuri, M. R., Bindom, S. M., Prochownik, E. V., O’Doherty, R. M., and Scott, D. K. (2010) c-Myc Is Required for the ChREBP-Dependent Activation of Glucose-Responsive Genes. *Mol. Endocrinol.* **24**, 1274–1286
198. Li, M. V., Chang, B., Imamura, M., Pongvarin, N., and Chan, L. (2006) Glucose-Dependent Transcriptional Regulation by an Evolutionarily Conserved Glucose-Sensing Module. *Diabetes.* **55**, 1179–1189
199. Davies, M. N., O’Callaghan, B. L., and Towle, H. C. (2010) Activation and repression of glucose-stimulated ChREBP requires the concerted action of multiple domains within the MondoA conserved region. *Am. J. Physiol. Endocrinol. Metab.* **299**, E665–E674
200. Billin, A. N., and Ayer, D. E. (2006) The Mlx Network: Evidence for a Parallel Max-Like Transcriptional Network That Regulates Energy Metabolism. in *The Myc/Max/Mad Transcription Factor Network*, pp. 255–278, Springer-Verlag, Berlin/Heidelberg, **302**, 255–278
201. Kawaguchi, T., Takenoshita, M., Kabashima, T., and Uyeda, K. (2001) Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. *Proc. Natl. Acad. Sci.* **98**, 13710–13715
202. Merla, G., Howald, C., Antonarakis, S. E., and Reymond, A. (2004) The subcellular localization of the ChoRE-binding protein, encoded by the Williams–Beuren syndrome critical region gene 14, is regulated by 14-3-3. *Hum. Mol. Genet.* **13**, 1505–1514
203. Tsatsos, N. G., Davies, M. N., O’callaghan, B. L., and Towle, H. C. (2008) Identification and function of phosphorylation in the glucose-regulated transcription factor ChREBP. *Biochem. J.* **411**, 261–270
204. Fukasawa, M., Ge, Q., Wynn, R. M., Ishii, S., and Uyeda, K. (2010) Coordinate regulation/localization of the carbohydrate responsive binding protein (ChREBP) by two nuclear export signal sites: Discovery of a new leucine-rich nuclear export signal site. *Biochem. Biophys. Res. Commun.* **391**, 1166–1169
205. Sakiyama, H., Wynn, R. M., Lee, W.-R., Fukasawa, M., Mizuguchi, H., Gardner, K. H., Repa, J. J., and Uyeda, K. (2008) Regulation of Nuclear Import/Export of Carbohydrate Response Element-binding Protein (ChREBP). *J. Biol. Chem.* **283**, 24899–24908
206. Li, M. V., Chen, W., Harmancey, R. N., Nuotio-Antar, A. M., Imamura, M., Saha, P., Taegtmeier, H., and Chan, L. (2010) Glucose-6-phosphate mediates activation of the carbohydrate responsive binding protein (ChREBP). *Biochem. Biophys. Res. Commun.* **395**, 395–400

Mitochondrial localization and structure-based phosphate activation mechanism of Glutaminase C with implications for cancer metabolism

Alexandre Cassago^a, Amanda P. S. Ferreira^a, Igor M. Ferreira^a, Camila Fornezari^a, Emerson R. M. Gomes^a, Kai Su Greene^b, Humberto M. Pereira^c, Richard C. Garratt^c, Sandra M. G. Dias^{a,1,2}, and Andre L. B. Ambrosio^{a,1,2}

^aLaboratório Nacional de Biotecnologia, Centro Nacional de Pesquisa em Energia e Materiais, Campinas-SP 13083-970, Campinas, Brazil; ^bDepartment of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401; and ^cCentro de Biotecnologia Molecular Estrutural, Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos-SP 13560-970, São Carlos, Brazil

Edited by Gregory A. Petsko, Brandeis University, Waltham, MA, and approved November 18, 2011 (received for review August 2, 2011)

Glutamine is an essential nutrient for cancer cell proliferation, especially in the context of citric acid cycle anaplerosis. In this manuscript we present results that collectively demonstrate that, of the three major mammalian glutaminases identified to date, the lesser studied splice variant of the gene *gls*, known as Glutaminase C (GAC), is important for tumor metabolism. We show that, although levels of both the kidney-type isoforms are elevated in tumor vs. normal tissues, GAC is distinctly mitochondrial. GAC is also most responsive to the activator inorganic phosphate, the content of which is supposedly higher in mitochondria subject to hypoxia. Analysis of X-ray crystal structures of GAC in different bound states suggests a mechanism that introduces the tetramerization-induced lifting of a “gating loop” as essential for the phosphate-dependent activation process. Surprisingly, phosphate binds inside the catalytic pocket rather than at the oligomerization interface. Phosphate also mediates substrate entry by competing with glutamate. A greater tendency to oligomerize differentiates GAC from its alternatively spliced isoform and the cycling of phosphate in and out of the active site distinguishes it from the liver-type isozyme, which is known to be less dependent on this ion.

glutamine metabolism | Warburg effect

The highly proliferative phenotype places special demands upon the metabolism of transformed cells. An upscaled glycolytic pathway, even in the presence of oxygen, is used by tumors to supply a great deal of its energetic and biosynthetic demand for cell division. Aerobic glycolysis, or the Warburg effect, as this phenomenon is commonly known, is accepted today as a metabolic hallmark of cancer cells and has been exploited for diagnosis (1, 2). In addition to glucose, tumors are also required to take up great amounts of other extracellular nutrients, such as the amino acid glutamine, and quickly and efficiently metabolize them for shunting into the appropriate pathways (3, 4).

Glutamate production by mitochondrial glutaminase (GA), the first enzyme in glutaminolysis, is a key process for body homeostasis, and a crucial carbon donor for amino acid and lipid synthesis in tumor cells, as reviewed by DeBerardinis, et al. (4). To date, three mammalian GAs have been identified: the Liver-type [LGA or GLS2 (5)], the Kidney-type [KGA (6)] and Glutaminase C [GAC, (7)] a splice variant of KGA (both usually referred to as GLS1). Overall, LGA and KGA present distinct tissue distributions and phosphate dependence for catalysis (8, 9). Recent studies have also stressed the importance of glutaminolysis in maintaining the malignant phenotype, providing indications that glutaminases may be potential therapeutic targets in some cancer cells (4, 10–13). Elevated GAC mRNA levels have been detected in gliomas (14, 15), colorectal carcinomas and adenomas (16), and breast tumor cell lines (7), when compared to normal cells. Gao and coworkers showed that c-Myc induces elevated GAC expression in both prostate cancer and B lymphoma cell lines as well as in prostate cancer tissues vs. normal (17). Importantly,

Wang and colleagues have shown that modulation of the aberrant GA activity via a small molecule, for which GAC was the identified target, inhibits oncogenic transformation (18). This collection of literature raises the suggestion that GAC is the key glutaminase isozyme in tumors. Thus, understanding the molecular basis of GAC's activity and, most importantly, distinguishing it from the KGA and LGA isozymes, will contribute to our understanding of tumor metabolism.

Here we demonstrate that both GAC and KGA protein levels are increased in breast tumors compared to normal healthy tissues. Furthermore a similar pattern is repeated in cases of higher compared to lower grades of invasive ductal breast carcinoma. Cell fractionation of breast, prostate, and lung tumor cell lines, followed by immunoblotting using isoform-specific antibodies indicated that, under the experimental conditions used, GAC but not KGA was found in the mitochondria. Subsequently we show that GAC is the most efficient of the three isozymes in hydrolyzing glutamine in the presence of inorganic phosphate (Pi). Crystal structures of GAC in different states (ligand-free and either bound to phosphate or L-glutamate) established a “gating loop” as the tetramer-driven mechanism of protein activation and show how phosphate enhances catalysis. Our data collectively suggest that GAC is key to the mitochondrial glutaminase metabolism of cancer cells because of its exclusive localization and kinetic properties.

Results

Protein Levels and Localization of the Kidney-Type Isoforms. KGA- and GAC-specific antibodies (Fig. S1 A–C) were employed in immunohistochemical analyses of two human breast cancer tissue arrays. The results show that the expression levels of both isoforms are increased in tumors compared to normal tissues and positively correlate to the tumor grade (Fig. 1A, Fig. S1D). Moreover, the data show that in breast (SKBR3 and MDA-MB231), prostate (PC3 and DU145) and lung (A549) tumor cell lines, KGA is localized in the cytosol but not in the mitochondria

Author contributions: S.M.G.D. and A.L.B.A. designed research; A.C., A.P.S.F., I.M.F., C.F., E.R.M.G., K.S.G., S.M.G.D., and A.L.B.A. performed research; A.C., H.M.P., R.C.G., S.M.G.D., and A.L.B.A. analyzed data; and S.M.G.D. and A.L.B.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank www.pdb.org [PDB ID codes 3ss3 (ligand-free GAC), 3ss4 (phosphate-bound GAC), and 3ss5 (L-glutamate-bound GAC)].

¹S.M.G.D. and A.L.B.A. contributed equally to this work.

²To whom correspondence may be addressed at: Laboratório Nacional de Biotecnologia-LNBio, Centro Nacional de Pesquisa em Energia e Materiais-CNPEM, Rua Giuseppe Máximo Scollaro, 10.000, Pólo II de Alta Tecnologia, Campinas, SP, 13083-100, Brazil. E-mail: andre.ambrosio@lnbio.org.br or sandra.dias@lnbio.org.br.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1112495109/-DCSupplemental.

Fig. 3. The crystal structure of GAC. (A) Orthogonal views of the tetramer in cartoon representation. Circles indicate, in chain A, the two distinct domains comprising the full structure: amino-terminal and glutaminase. Though present in the crystallized construct, the C terminus is heterogeneous in conformation and could not be successfully modeled. Stereographic views of the cationic active site of GAC, represented by electrostatic surface mapping ($\pm 8 k_B T e_c^{-11}$) for the three crystal forms, show the presence of chloride (B), phosphate (C) and L-glutamate (D). $2F_o - F_c$ Fourier electron density maps of the ligands are contoured at 1σ .

The glutaminase domain in GAC is comprised of residues Lys249 to Tyr535, and belongs to the beta-lactamase/transpeptidase-like superfamily, as previously described (*, 23). The active site is located between two tightly associated subdomains: an $\alpha/\beta/\alpha$ sandwich subdomain, including a β -sheet composed of 5 β -strands and a purely α -helical subdomain, arranged into a bifurcated fold. According to the 3V (Voss Volume Voxelator) server (24), the catalytic pocket has a volume of 580 Å³. Electrostatic potential surface mapping shows a very positively charged active site (Fig. 3B), receiving contributions from Lys294 and Lys486 as well as the N-terminal dipole moment of helices spanning residues Ser291 to Leu305 and Asn340 to Ile350. One chloride ion was found inside the active site of each monomer, interacting with the catalytic residue Ser291, as well as Tyr471 and Val489. This very favorable coordination [gain in free energy of -10.6 kcal/mol upon complex formation, according to the PISA (Protein Interfaces, Surfaces and Assemblies) server (25)] explains previous biochemical reports that glutaminase activity is actually impaired under high concentrations of sodium chloride (9), which adds up to 700 mM in the crystallization condition. The majority of the C-terminal portion of GAC is conformationally heterogeneous and therefore could not be modeled. No potential function has been suggested to date for the GAC carboxy-terminal region.

The assembly of the four monomers in the asymmetric unit correlates well in size and shape with the oligomer obtained in solution after size-exclusion purification, either in the absence or presence of 40 mM phosphate, as confirmed by small angle X-ray scattering (SAXS) (Fig. S44). In our structure, chains A and C form one of the dimers and chains B and D the complementary one. The contacts that define the dimers and the tetramers are all within the glutaminase domain. According to the default criteria of the PISA server, 45 residues from each monomer share the dimer interface, making 20 hydrogen bonds (nine salt bridges), resulting in a gain in solvation energy of -11.3 kcal/mol and about $1,600$ Å² of occluded area. The tetramer is then formed by the pairing of chain A with chain D and chain B with chain C. The dimers are twisted by about 25° with respect to each other, along the shortest axis of the oligomer (Fig. S4B). The interfaces result from the stacking of equivalent α -helices extending from Asp391 to Lys401 and are mediated mostly by hydrophobic interactions between Phe394, Ala395, Tyr398, and Tyr399. Each pairing buries on average 460 Å² of area and also favors the formation of four hydrogen bonds (three salt bridges), with an average gain in solvation energy of -7.5 kcal/mol. Oppositely charged residues (Asp391 and Lys401) sit on opposing ends of the

helix, therefore providing proper orientation of the complementary interfaces.

Structures of Phosphate- and L-Glutamate-Bound GAC. To gain insight into the molecular mechanism that drives the activation of GAC, we have also determined its crystal structure bound to Pi at 2.85 Å resolution (R_{factor} and R_{free} of 22.7% and 26.9%, respectively—Table S3). This crystal is isomorphous with the ligand-free form, containing the same tetramer in the asymmetric unit (backbone rmsd of 0.43 Å). Our current knowledge regarding the activation mechanism of GA is related to the correlation between the presence of phosphate and the protein's quaternary structure, in which dimers associate into tetramers, thus establishing the catalysis-competent state of the enzyme (26, 27). This model led us to expect phosphate ions interacting in the surroundings of the tetramerization interface. Surprisingly only one ion was found per monomer, and this was buried inside the highly positive active site where it makes polar contacts with Ser291, Asn340 and Tyr471, and two water molecules (Fig. 3C). On average, the presence of Pi inside the active site results in a gain in free energy of -5.4 kcal/mol. Furthermore, as a consequence of the binding of Pi to Ser291 and Tyr471, the chloride ion found in the previous structure is displaced from the active site.

We subsequently grew crystals of GAC previously incubated with 10 mM sodium L-glutamate. The final model was refined to 2.80 Å resolution (R_{factor} and R_{free} of 20.6% and 24.0%, respectively). The overall structure is highly conserved, with a core rmsd of 0.31 Å and 0.40 Å, when superposed to the ligand-free and the phosphate-bound models, respectively. One molecule of L-glutamate is found bound to the active site of each monomer (Fig. 3D). The amino group in the main chain branch of the ligand is hydrogen-bonded to Glu386, Asn340, Tyr419, and Asn393. On its side chain branch, the carboxylic acid moiety makes polar interactions with Ser291, Tyr471 and the main chain nitrogen of Val489.

Structure-Based Biochemistry. To determine key events for GAC activation, we generated a point mutant by replacing Phe394 from the tetramer interface by a serine (Fig. 4A), termed GAC.F394S. This mutation was enough to break the tetramer into dimers, even in the presence of high phosphate concentrations (Fig. S5A). Differently from the wild-type GAC, the addition of phosphate to GAC.F394S had little effect on its K_{m-app} (14.0 mM and 12.3 mM for 0 mM and 50 mM Pi, respectively), and changed little its already low catalytic efficiency ($k_{cat-app}/K_{m-app}$ of 0.4 mM⁻¹·s⁻¹ and 1.0 mM⁻¹·s⁻¹) when in the absence and presence of 50 mM Pi, respectively (Fig. 4B, Fig. S2, Table S1), reinforcing the importance of tetramerization for protein activation.

As already mentioned, GAC eluted as a tetramer after size-exclusion purification (Fig. S44). By making serial dilutions of pure protein samples and running them in a gel-filtration column,

*Karlberg T, (2008) Manuscript still to be published. Coordinate file and structure factors available at <http://www.thesgc.org/structures/details?pdbid=3CZD> also www.rcsb.org, under code 3czd.

we were able to show that tetramerization is a concentration-dependent event. In Fig. 4C (left box), we observe successive shifts in the population equilibrium, starting at the Stokes radius expected for the tetramer and moving towards smaller species, closer to the radius of the dimer, as the solution is diluted [Stokes radii calculated by Hydropro (28), based on the crystal structure]. Due to experimental sensitivity limitations, the lowest protein concentration measured was 50 nM. We then performed the glutaminase assay in the absence of Pi, at GAC concentrations similarly to those used in the serial dilution experiment (ranging from 25 nM to 200 nM). Interestingly, the results (Fig. 4C, right box, Fig. S2E) readily show a much lower K_m at 25 nM GAC (4.3 mM), when compared to 5 nM GAC (K_m of 18.4 mM, Table S1). As GAC concentration was increased further up to 200 nM, the K_m dropped to 0.9 mM which correlates well with the presence of the heavier species (Fig. 4C, left box). Nonetheless, the turnover rates remained low at all protein concentrations tested, resulting in little change in enzyme efficiency (k_{cat}/K_m going from $0.5 \text{ mM}^{-1} \cdot \text{s}^{-1}$ to $1.1 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 25 nM and 200 nM GAC, respectively), confirming that increases in the turnover rates of GAC depend specifically on Pi and are not solely determined by tetramerization (Fig. 2). This observation can be explained by the crystal structures presented here, which show that phosphate and glutamate compete for the same residues of the cationic active site. Fig. 3C and D show that both anions contact Ser291, the nucleophilic agent of catalysis, and Pi is therefore likely to promote product release during the catalytic cycle and to reduce product accumulation-induced inhibition of GLS1 (29, 30). We then conclude that while protein tetramerization is important for increasing substrate affinity, phosphate guarantees the cycling of the enzyme.

We next focused our attention on the dimer-dimer intermolecular contacts looking for clues for the molecular basis of the oligomerization-driven GAC activation. Particularly noteworthy is the side chain of Phe327, which in all monomers, is part of a hydrophobic cluster located at the tetramer interface, formed by the complementary stacking of Phe327 itself, Ala395, Ile396, Tyr399, in chain A, with its equivalent residues in chain D. Phe327 is the first well structured residue after a consistently disordered

six-residue-long loop found across the three structures (Leu321 to Leu326, Fig. 4D). This observation, combined with its close proximity to the active site region, led us to hypothesize about a possible role in regulating accessibility to the active site, explaining why tetramerization decreases the K_m of the enzyme. The underlying rationale is that, in the GAC dimer, the open form of this loop (hereon referred to as the gating loop), is transient and a closed form must be present in large excess, leading to a low affinity and a limited hydrolytic rate for glutamine. Accompanying tetramer assembly, conformational changes involving Phe327 are triggered, lifting the gating loop and exposing the active site. If this assumption is correct, then artificially disturbing the loop would result in a more substrate accessible glutaminase. Indeed, the point mutant Phe327Ser (GAC.F327S) showed a K_m as low as 8.2 mM, even in the absence of phosphate and at only 5 nM protein concentration (Fig. 4C). The K_m then dropped to 0.8 mM when 50 mM of K_2HPO_4 was added to the assay, thus showing the importance of the gating loop in regulating active site accessibility.

Finally, serial dilution experiments with wild-type GAC, followed by gel-filtration in the presence of 20 mM K_2HPO_4 , showed a shift in the equilibrium of the species towards tetramers and higher oligomers, when compared to the protein in the absence of Pi (Fig. 4C), as already reported (29, 30). Apart from its effect in enzyme cycling, another direct effect of the addition of Pi is the stabilization of tetramers, likely by inducing the opening of the gating loop. Indeed, chloride, which competes with Pi for binding to the catalytic Ser291, at high concentration (500 mM NaCl) can dislodge phosphate from its site and shift the protein equilibrium to lower-order oligomers (Fig. S5B).

GAC and KGA: Same Active Site, Different Catalytic Capabilities. The splicing event that originates GAC occurs at the C-terminal-encoding region of the *gls* gene. Therefore, the N-terminal and glutaminase domains of both KGA and GAC are identical, indicating that the carboxy-terminal region must be crucial for the observed differences in enzymatic behavior (Fig. 2). The great majority of the C-terminal portion of GAC is conformationally heterogeneous in our crystal structures and therefore could not be

Fig. 4. Activation mechanism based on the gating loop and inorganic phosphate. (A) Perpendicular views of the tetramer interface, defined by the symmetric stacking of the helix between residues Asp391 and Lys401 from each monomer. (B) Enzymatic characterization of point and deletion mutants in comparison to the wild-type enzyme show loss-of-function for GAC.F394S and GAC.DelC and gain-of-function for GAC.F327S. (C) Decrease in the K_m for glutamine of GAC (right box) correlates with its protein concentration-dependent oligomerization profile, as determined by size-exclusion chromatography. Introduction of phosphate to the protein solution results in shifts of the equilibrium towards higher molecular weight species. (D) Top view of the active site of GAC and its proximity to the gating loop (dashed line between Gly320 and Phe327). Phe327 is part of the buried interface upon tetramer formation. Fourier $2F_o - F_c$ electron density map (contoured at 1σ) shows that residues flanking the loop are well ordered. Relative positions of Pi, L-glutamate and the chloride ion can be seen inside the active site, as a result of structure superposition.

modeled. Secondary structure prediction using internet tools suggests this region to contain a short α -helix (from Tyr563 to Leu572) and an extended strand spanning residues Thr590 to Glu597, interspersed within random coil. KGA on the other hand, is predicted to have three ankyrin repeats within its longer C-terminal region (Fig. S6).

KGA serial dilution, both in the presence and absence of phosphate (Fig. 5), shows a much slower drift in the equilibrium toward larger species when compared to GAC (Fig. 4C), which is reflected in the kinetic parameters obtained by increasing the KGA concentration (original curves in Fig. S2E). In going from 5 nM to 200 nM KGA, its K_m is reduced only by about 2.5 times, as opposed to 14 times in the case of GAC's (Fig. 4C). Deletion of the stretch between residues Arg539 to Ser 603 of GAC (GAC.DelC), which represents a common denominator between KGA and GAC, especially increased the K_{m-app} of the protein, even in the presence of phosphate, to levels of the dimer mutant (Fig. 4B), suggesting that the unique C terminus of GAC is important for facilitating protein oligomerization, though the specifics are still not clear.

Discussion

GAC Was Found in the Mitochondria and KGA in the Cytoplasm: Implications for Cancer Metabolism. The central role played by glutamine-based intermediary metabolism in malignant cells has been recently highlighted with the observation that previously known oncogenes and signaling pathways tightly regulate glutaminase expression and activity levels. Gao and coworkers have shown that c-Myc induces elevated GA expression in prostate cancer and B lymphoma cell lines (17). Curiously, the GLS1 isoform induced by Myc in mitochondria of P493-6 B cells was GAC. Wang and coworkers demonstrated that transformed fibroblasts and breast cancer cells exhibit elevated GA activity that is dependent on Rho GTPases and NF- κ B activity (18). The authors also present a small molecule that selectively abrogates the growth of transformed cells, by targeting GAC, without impairing the growth of normal cells. Here, working with isoform-specific antibodies, we showed that both KGA and GAC protein levels are enhanced in breast tumor tissues, but only GAC was located in the mitochondria of tumor cell lines.

Named after the discovery of its enzymatic activity (31), mammalian glutaminases turned out to be more complex proteins, with a distinctive combination of additional motifs and functional domains. A consensus sequence of transit peptides that directs the enzyme to the mitochondria can be identified in the first 16 amino acids of GAC and KGA. Therefore, the result presented here demonstrating the localization of KGA in the cytoplasm of the cell lines studied as a likely full-length precursor form of 72–74 kDa, is particularly striking. A possible explanation however, might come from the C-terminal portions, which share only 12% sequence identity. KGA is predicted to contain three ankyrin repeats which exclusively function to mediate protein-protein

interactions and have been found in proteins of diverse function such as cell-cycle regulators, transcriptional initiators, cytoskeletal proteins, ion transporters, and signal transducers (32). Furthermore, aside from the N-terminal NR box motif in all three glutaminase isozymes, as the structure of GAC presented here confirms, the last five amino acids in the KGA sequence (LDGLL) may also serve as a second NR box. Hence, it would come as no surprise if the involvement of KGA in processes taking place outside the mitochondrial boundaries is soon demonstrated. Furthermore, it has already been shown that LGA, which also may contain one NRbox and a C-terminal ankyrin repeat, can localize in the nuclei of neuronal cells (22). Apart from the strong influence on the catalytic activity of GAC, no function can be predicted for its 53 amino acid long C terminus, based on the current knowledge on protein motifs and domains. In this context, GAC might be more relevant for the anaplerosis of the TCA cycle.

GLS1 Mechanism of Activation. Early biochemical studies of the kidney-type isozyme reported that mitochondrial glutaminases are mostly found as inactive dimers in the organelle and that the presence of phosphate correlates with changes leading to tetramerization and enzyme activation (26, 27). We have described here crystal structures of GAC tetramers in different bound states, and shown that the same tetramer is found in solution in the presence or absence of phosphate. Tetramerization, a concentration-dependent event enhanced by phosphate, is the main cause of increasing protein affinity for the substrate due to the opening of a so-called gating loop. Binding of the adjacent Phe327 to the tetramer interface stabilizes the open form of the loop permitting the diffusion of substrate into the active site, thus providing a structural correlation between the cooperative oligomer activation and gating access to the active site. Concomitantly, the presence of phosphate plays the central role in increasing the turnover rates of the enzyme. Enzyme inhibition by glutamate has been reported for the kidney isoform of GLS1 (29). Furthermore, Sayre and Roberts showed in 1958 that glutamate inhibition of glutaminases was found to be competitive with phosphate (33). In this sense, we show with our crystal structures that Pi competes with glutamate for the cationic active site, accelerating product exchange and at the same time avoiding enzyme inhibition as product accumulates. While the described mechanism is shared between KGA and GAC isoforms, as they have the same glutaminase domain, it is possible that direct interactions between the GAC C-terminal region and the active site (which were not captured in our crystal structures) might occur to enhance catalysis.

Inorganic Phosphate Levels as a Sensor for Glutamine-Based Anaplerosis. GAC was first identified and found overexpressed in the human breast carcinoma cell line TSE (7). Subsequent work reported elevated GAC mRNA levels in tumors when compared to normal cell lines (15, 16). These observations, taken together with the work of Gao, et al. (17) and Wang, et al. (18), led us to ask whether this isoform might have intrinsic catalytic properties which allow it to perform better within the cancer microenvironment. Mammalian GA activity has been described as sensitive to inorganic phosphate (Pi), with KGA being activated by it and LGA being less sensitive (8, 9). To evaluate side-by-side the three reported glutaminases, we produced recombinant proteins and performed the activity assays at increasing amounts of Pi, up to 50 mM. We have confirmed that KGA and LGA's individual responses to Pi are quite distinct one from another, but, surprisingly, GAC presented the highest efficiency ($k_{cat-app}/K_{m-app}$) towards L-glutamine at higher concentrations of Pi. To our knowledge there is no data available in the literature concerning the mitochondrial levels of inorganic phosphate in tumor cells. It has been shown, however, that in guinea pig hearts the cellular concentration of Pi changes from 1.7, to 7.1 and 13.9 mM, in going from normal to mildly hypoxic to severely hypoxic conditions, respectively (34). Taking into consideration the changes

Fig. 5. Concentration-dependent oligomerization and activation profile of KGA. This isoform responds much more slowly than GAC to increases in protein concentration regarding shifts in the equilibrium towards species of higher Stokes radius. Furthermore, the addition of phosphate has no effects on the overall profile (left box). The slowed response is also reflected in less significant changes in the enzyme's K_m (right box), when compared to GAC (Fig. 4C).

in mitochondrial homeostasis due to hypoxia for instance, the reduction of F_1F_0 ATPase activity (35), which uses P_i to produce ATP, may lead to local levels of inorganic phosphate reaching even higher concentrations. Surviving hypoxic conditions is one of the main driving forces in the selection that leads to cancer progression. In this sense, we hypothesize that the accumulation of inorganic phosphate in the mitochondria is the trigger for increased GAC-based glutaminase activity. Under these circumstances, GAC may provide for the increased metabolic and biosynthetic needs, thus conferring selective advantage to malignant cells. Therefore, drug-based therapies specifically targeting the mitochondrial metabolism of glutamine seem more likely to succeed if developed to be GAC specific.

Material and Methods

Tissue Array and Cell Culture. Human normal and tumor breast tissue arrays (US Biomax, Inc.) were subjected to immunohistochemical analysis with KGA and GAC antibodies according to the manufacturer's protocol. Five types of human cancer cell lines, from breast (SKBR3 and MDA-MB231), prostate (PC3 and DU145), and lung (A549), were cultured in RPMI medium 1640 (Invitrogen) supplemented with 10% FBS (Invitrogen), at 37 °C with 5% CO_2 . Details can be found in the *SI Text*.

Immunoblot and Immunofluorescence. KGA specific monoclonal antibody targeting to KGA's unique C terminus was acquired from Abnova (clone 5C4). GAC-specific polyclonal purified antibody was custom made by Genscript using the peptide CKLDPRREGDQRHS. Blotting and fluorescence were performed using standard protocols. More details in the *SI Text*.

Heterologous Glutaminases. Rat KGA (Met128-Leu674) cloned in pET15b was previously published by the laboratory of Norman

Curthoys (9). GAC (Met128-Ser603) and LGA (Leu72-Val602) were amplified from a mouse fetal brain tissue cDNA library and cloned into the pET28a plasmid (Novagen). Standard heterologous protein expression and liquid chromatography techniques were used (see *SI Text* for details).

Crystallization and X-Ray Crystallography. Crystallization of the GAC construct is described in detail in the *SI Text*. X-ray diffraction datasets were obtained at beamlines X12-C at NSLS (ligand-free crystals), F-1 at CHESS (phosphate-bound crystals) and W02B-MX2 at LNLS (L-glutamate-bound crystals). Details of data processing, phasing and model refinement can be found in *SI Text*.

Enzyme Assays. The L-Glutamate Dehydrogenase-based (GDH), two-step Glutaminase protocol, as previously published (9), was adapted into a streamlined assay that could be followed in real-time and performed on a plate reader (*SI Text*).

Small Angle X-Ray Scattering. SAXS data for GAC, in the presence and absence of phosphate, was collected at the D01A-SAXS1 beamline at LNLS. Detailed description of data collection parameters and processing can be found in *SI Text*.

ACKNOWLEDGMENTS. We thank Dr. Richard Cerione and his laboratory, at Cornell University, for reagents and useful discussions. We thank Drs. Alessandra Girasol, Marcos Navarro and Andrew Leslie and the staff of the beamlines used in this work. We thank Dr. José Xavier Neto for stimulating discussions and critical reading of the manuscript. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo under grants 2010/05003-0 (A.L.B.A.), 2009/10875-9 (S.M.G.D.), and fellowship 2010/05987-0 (A.C.). We thank LNBio, for financial support and access to all facilities (LPP, LEC, LBE, and Robolab).

- Warburg O (1956) On the origin of cancer cells. *Science* 123:309–314.
- Hsu PP, Sabatini DM (2008) Cancer cell metabolism: Warburg and beyond. *Cell* 134:703–707.
- Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg Effect: the metabolic requirements of cell proliferation. *Science* 324:1029–1036.
- DeBerardine RJ, Cheng T (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* 29:313–324.
- Aledo JC, Gómez-Fabre PM, Olalla L, Márquez J (2000) Identification of two human glutaminase loci and tissue-specific expression of the two related genes. *Mamm Genome* 11:1107–1110.
- Curthoys NP, Kuhlenschmidt T, Godfrey SS (1976) Phosphate-dependent glutaminase from rat kidney. Cause of increased activity in response to acidosis and identity with glutaminase from other tissues. *Arch Biochem Biophys* 174:82–89.
- Elgadi KM, et al. (1999) Cloning and analysis of unique human glutaminase isoforms generated by tissue-specific alternative splicing. *Physiol Genomics* 1:51–62.
- Campos-Sandoval JA, et al. (2007) Expression of functional human glutaminase in baculovirus system: affinity purification, kinetic and molecular characterization. *Int J Biochem Cell Biol* 34:765–773.
- Kenny J, et al. (2003) Bacterial expression, purification, and characterization of rat kidney-type mitochondrial glutaminase. *Protein Express Purif* 31:140–148.
- Seltzer MJ, et al. (2010) Inhibition of glutaminase preferentially slows growth of glioma cells with mutant IDH1. *Cancer Res* 70:8981–8987.
- Dang CV (2010) Glutaminolysis: supplying carbon or nitrogen or both for cancer cells? *Cell Cycle* 9:3884–3886.
- Wise DR, Thompson CB (2010) Glutamine addiction: a new therapeutic in cancer. *Trends Biochem Sci* 35:427–433.
- Lobo C, et al. (2000) Inhibition of glutaminase expression by antisense mRNA decreases growth and tumorigenicity of tumour cells. *Biochem J* 348:257–261.
- Szeliga M, et al. (2005) Lack of expression of the liver-type glutaminase (LGA) mRNA in human malignant gliomas. *Neuroscience Lett* 374:171–173.
- Szeliga M, et al. (2008) Relative expression of mRNAs coding for glutaminase isoforms in CNS tissues and CNS tumors. *Neurochem Res* 33:808–813.
- Turner A, McGivan JD (2003) Glutaminase isoform expression in cell lines derived from human colorectal adenomas and carcinomas. *Biochem J* 370:403–408.
- Gao P, et al. (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 458:762–765.
- Wang JB, et al. (2010) Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* 18:207–219.
- Suzuki S, et al. (2010) Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proc Natl Acad Sci USA* 107:7461–7466.
- Hu W, et al. (2010) Glutaminase 2: a novel p53 target gene regulating energy metabolism and antioxidant function. *Proc Natl Acad Sci USA* 107:7455–7460.
- Plevin MJ, Mills MM, Ikura M (2005) The LXXLL motif: a multifunctional binding sequence in transcriptional regulation. *Trends Biochem Sci* 30:66–69.
- Olalla L, et al. (2002) Nuclear localization of L-type glutaminase in mammalian brain. *J Biol Chem* 277:38939–38944.
- Brown G, et al. (2008) Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*. *Biochemistry* 47:5724–5735.
- Voss NR, Gerstein M (2010) 3V: cavity, channel and cleft volume calculator and extractor. *Nucleic Acids Res* 38:W555–W562.
- Krissinel E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372:774–797.
- Godfrey S, Kuhlenschmidt T, Curthoys NP (1977) Correlation between activation and dimer formation of rat renal phosphate dependent glutaminase. *J Biol Chem* 252:1927–1931.
- Morehouse RF, Curthoys NP (1981) Properties of rat renal phosphate-dependent glutaminase coupled to Sepharose. Evidence that dimerization is essential for activation. *Biochem J* 193:709–716.
- García de la Torre J, Huertas ML, Carrasco B (2000) Calculation of hydrodynamic properties of globular proteins from their atomic-level structure. *Biophys J* 78:719–730.
- Curthoys NP, Watford M (1995) Regulation of glutaminase activity and glutamine metabolism. *Annu Rev Nutr* 15:133–159.
- Kovacevic Z, McGivan JD (1983) Mitochondrial metabolism of glutamine and glutamate and its physiological significance. *Physiol Rev* 63:547–605.
- Krebs HA (1935) Metabolism of aminoacids: the synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. *Biochem J* 29:1951–1969.
- Li J, Mahajan A, Tsai MD (2006) Ankyrin repeat: a unique motif mediating protein-protein interactions. *Biochemistry* 45:15168–15178.
- Sayre FW, Roberts E (1958) Preparation and some properties of a phosphate-activated glutaminase from kidneys. *J Biol Chem* 233:1128–1134.
- Gorman MW, He MX, Hal CS, Sparks HV (1997) Inorganic phosphate as regulator of adenosine formation in isolated guinea pig hearts. *Am J Physiol* 272:H913–H920.
- Nguyen T, Johnson JA (2008) Delta protein kinase C interacts with the δ subunit of the F_1F_0 ATPase in neonatal cardiac myocytes exposed to hypoxia or phorbol ester. Implications for F_1F_0 ATPase regulation. *J Biol Chem* 283:29831–29840.

Active Glutaminase C Self-assembles into a Supratetrameric Oligomer That Can Be Disrupted by an Allosteric Inhibitor*

Received for publication, July 14, 2013, and in revised form, August 6, 2013. Published, JBC Papers in Press, August 8, 2013, DOI 10.1074/jbc.M113.501346

Amanda Petrina Scotá Ferreira^{†1}, Alexandre Cassago^{§1}, Kaliandra de Almeida Gonçalves^{†1}, Marília Meira Dias[†], Douglas Adamoski[†], Caroline Fernanda Rodrigues Ascensão[†], Rodrigo Vargas Honorato[†], Juliana Ferreira de Oliveira[†], Igor Monteze Ferreira[†], Camila Fornezari[†], Jefferson Bettini[§], Paulo Sérgio Lopes Oliveira[†], Adriana Franco Paes Leme[†], Rodrigo Villares Portugal[§], Andre Luis Berteli Ambrosio^{†2,3}, and Sandra Martha Gomes Dias^{†2,4}

From the Laboratórios Nacionais de [†]Biociências e [§]Nanotecnologia, Centro Nacional de Pesquisa em Energia e Materiais, Campinas-SP 13083-100, Brazil

Background: GAC supplies for increased metabolic needs of tumors because of exclusive localization and kinetic properties.

Results: Higher than tetramer oligomers are the active form in *in vitro* and in cellular assays. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide disrupts oligomers.

Conclusion: A novel molecular mechanism for GAC activation is proposed.

Significance: The data affect the development of therapies targeting GAC in tumors, with emphasis on allosteric inhibitors.

The phosphate-dependent transition between enzymatically inert dimers into catalytically capable tetramers has long been the accepted mechanism for the glutaminase activation. Here, we demonstrate that activated glutaminase C (GAC) self-assembles into a helical, fiber-like double-stranded oligomer and propose a molecular model consisting of seven tetramer copies per turn per strand interacting via the N-terminal domains. The loop ³²¹LRFNKL³²⁶ is projected as the major regulating element for self-assembly and enzyme activation. Furthermore, the previously identified *in vivo* lysine acetylation (Lys³¹¹ in humans, Lys³¹⁶ in mouse) is here proposed as an important down-regulator of superoligomer assembly and protein activation. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide, a known glutaminase inhibitor, completely disrupted the higher order oligomer, explaining its allosteric mechanism of inhibition via tetramer stabilization. A direct correlation between the tendency to self-assemble and the activity levels of the three mammalian glutaminase isozymes was established, with GAC being the most active enzyme while forming the longest structures. Lastly, the ectopic expression of a fiber-prone superactive GAC mutant in MDA-MB 231 cancer cells provided considerable

proliferative advantages to transformed cells. These findings yield unique implications for the development of GAC-oriented therapeutics targeting tumor metabolism.

Cancer cells have a well established dependence on glutamine metabolism to support their highly proliferative status. Apart from acting as a source for nitrogen as well as reductive power, its anabolic carbon skeletons can be siphoned from the TCA cycle to be used as building blocks for the growing and dividing cells (1, 2). Both Myc and Rho GTPases have been shown to stimulate glutaminase C (GAC),⁵ an isozyme that possesses a distinct cellular localization as well as activation levels purported to provide the aforesaid proliferative advantage to cancer cells (3–5).

We have recently described the structural determinants of the phosphate-dependent activation mechanism of GAC, based on the tetramerization-induced lifting of a so-called gating loop (³²¹LRFNKL³²⁶; NCBI sequence NP_001106854.1), which controls substrate accessibility to the active site. We showed that phosphate binds inside the catalytic pocket, resulting in allosteric stabilization of tetramers and facilitating substrate entry by outcompeting with the product, glutamate, to guarantee enzyme cycling (5). Additionally, recent publications have provided structural insights into glutaminase inhibition by making use of the small molecules BPTES and 968 (6–9). Nevertheless, the precise descriptions of their modes of inhibition are still lacking.

Renewed interest in cancer metabolism has prompted an innovative warfront against metabolic enzymes, aiming at the development of alternative and efficient therapeutic opportunities. Glutaminase C is a key target in this sense (2, 4, 10, 11),

* This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under Grants 2009/10875-9 (to S. M. G. D.) and 2010/05003-0 and 2012/14298-9 (to A. L. B. A.) and Fellowships 2010/05987-0 (to A. P. S. F.), 2010/05987-0 (to A. C.), 2011/06654-7 (to K. A. G.), and 2009/54067-3 (to A. F. P. L.). This work was also supported in part by the Laboratório Nacional de Biociências.

The atomic coordinates and structure factors (code 4jkt) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

[†] These authors contributed equally to this work.

[‡] These authors contributed equally to this work.

[§] To whom correspondence may be addressed: LNBio, CNPEM, Rua Giuseppe Máximo Scolfaro, 10.000, Pólo II de Alta Tecnologia, Campinas, SP, 13083-100, Brazil. Tel.: 55-19-3512-1115; Fax: 55-19-3512-1004; E-mail: andre.ambrosio@lnbio.cnpem.br.

⁴ To whom correspondence may be addressed: LNBio, CNPEM, Rua Giuseppe Máximo Scolfaro, 10.000, Pólo II de Alta Tecnologia, Campinas, SP, 13083-100, Brazil. Tel.: 55-19-3512-1115; Fax: 55-19-3512-1004; E-mail: sandra.dias@lnbio.cnpem.br.

⁵ The abbreviations used are: GAC, glutaminase C; BPTES, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; TEM, transmission electron microscopy; KGA, kidney-type glutaminase; LGA, liver-type glutaminase; DSS, disuccinimidyl suberate.

Supratetrameric Oligomerization-dependent GAC Activation

and the need for new and accurate biochemical and structural information to speed up and improve the development of successful therapies is therefore essential. In this regard, we now provide novel information demonstrating that the assembly of higher order, fiber-like GAC oligomers, henceforth termed the GAC superstructure, is necessary for proper enzyme activation both *in vitro* and in a cancer cell model. First, we demonstrate that the superstructure is mandatorily present when GAC is in the active form, as shown by negatively stained samples analyzed by transmission electron microscopy (TEM). The tendency toward the superstructure correlates well with the activation levels induced by phosphate among GAC and the other two mammalian glutaminase isozymes: the kidney-type glutaminase (KGA) and the liver-type glutaminase (LGA). Although LGA is a synonym for the GLS2 glutaminase, KGA and its splicing variant GAC are both usually indistinguishably referred to as GLS1. Moreover, we observe that the addition of the GLS1 inhibitor BPTES hampers protein polymerization by stabilizing inactive tetramers.

Further research identified a subset of key residues involved in the superstructure formation process. They are located in the gating loop, as well as at the N and C termini, which have been previously shown to be key structural features for enzyme activation (5, 12). One specific gating loop mutant, GAC.K325A, both assembles into the superstructure and shows a 600-fold enhancement in catalytic efficiency toward L-glutamine, even in the absence of phosphate. Conversely, GAC.R322A, also at the gating loop, abrogated protein activation and impeded superstructure formation. A previously identified *in vivo* post-translational modification of human GLS1 (13), the acetylation of Lys³¹⁶ (equivalent to Lys³¹¹ in human), was also studied in this context. We show that the acetylation mimetic GAC.K316Q does not assemble into higher order oligomers, and this modification likely inhibits protein activity in cells. By combining data from point mutants, TEM, MS, and computational biology, we offer a low resolution model for the superstructure assembly. The superstructure is based on a double-stranded helix, with each strand containing tetramers interacting with each other via the N terminus domain. Lastly, we demonstrate that MDA-MB 231 cells silenced for the endogenous GAC expression and stably expressing an ectopic fiber-prone superactive mutant, proliferate more, consume higher amounts of glutamine, and grow bigger than the wild-type and mock transformed cells. Our results shed new light on the molecular mechanism of phosphate-dependent activation of the glutaminases and highlight the importance of the development of allosteric inhibitors when targeting GAC in tumors.

EXPERIMENTAL PROCEDURES

Protein Production, Enzymatic Assay, Size Exclusion Serial Dilution, and Site-directed Mutagenesis—Recombinant protein expression and purification, the streamlined glutaminase activity assay, and the size exclusion analysis of serial dilutions were performed as previously published (5). Point mutants were generated with the QuikChange II site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. The inhibition assays using BPTES (kindly provided by Dr. Chi Van Dang, Abramson Cancer Center, University of Pennsylvania,

Philadelphia, PA) were done with GAC or GAC.K325A at 5 nM and BPTES diluted in Me₂SO, the last always at a 0.5% final concentration. Measurements were done in triplicate and analyzed using GraphPad Prism 5.00 (GraphPad Software) and Origin 8.1 (Originlab). The parameters of the dose-response curve were determined by fitting an error bar-weighted logistic function, in the form of $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p)$, where A_1 is the initial apparent turnover rate (absence of inhibitor), A_2 is the turnover rate at saturating concentrations of inhibitor, x_0 is the half-maximum inhibitory concentration, or IC₅₀, and p is the Hill slope.

Transmission Electron Microscopy—For visualization of negatively stained grids, protein samples, at a concentration ranging from 0.5 to 1 μM, were deposited onto glow-discharged holey carbon-coated grids for 60 s followed by two steps of blotting and staining with 2% uranyl acetate. Images were acquired between −1- and −3-μm defocus at 15,000–80,000× magnification using a Jeol JEM-2100 operating at 200 kV and recorded on a F-416 CMOS camera (Tietz Video and Image Processing Systems).

Crystallization and X-ray Crystallography—Crystallization experiments were performed at 291 K using the conventional sitting drop vapor diffusion technique. Drops were made by mixing three parts of protein (at 3.3 mg/ml) to one of well solution, containing 17% PEG 3350, 0.2 M NaCl, and 0.1 M Bis-Tris, pH 6.5. Clusters of plates were observed after 5 days and used as seeds for a standard streak seeding in a mother liquor solution containing 11% PEG 3350, 0.2 M NaCl, and 0.1 M Bis-Tris, pH 6.5. Before data collection at cryogenic temperature (100 K), harvested crystals were cryoprotected with 10% ethylene glycol added to the mother liquor. X-ray diffraction data were obtained at the D03B-MX1 Beamline at Laboratório Nacional de Luz Síncrotron in Brazil. The data were processed using Mosflm (14) and Scala (15). The first set of phases was obtained by the molecular replacement as implemented in Phaser (16), using the data set from the ligand-free form of GAC, available under Protein Data Bank code 3ss3 (5). Positional and B-factor refinement cycles were carried out with Phenix (17). Manual building of the extra portions and real space refinement, including Fourier electron density map inspection, were performed with Coot (18). The overall stereochemical quality of the final models and the agreements between them and experimental data were assessed by the program Molprobit (19) and the appropriate Coot routines.

Cross-linking Analysis of GAC Complexes by LC-MS/MS—GAC complexes were reduced (5 mM dithiothreitol, 25 min at 56 °C), alkylated (14 mM iodoacetamide, 30 min at room temperature in the dark), and digested with trypsin (Promega). The samples were lyophilized in a vacuum concentrator and reconstituted in 0.1% formic acid. Then 4.5 μl (1 μg) of the resulting peptide was analyzed on an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with LC-MS/MS by an EASY-nLC system (Thermo Fisher Scientific) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–90% acetonitrile gradient in 0.1% formic acid using a pre-column EASY-Column (2 cm × 100-μm inner diameter, 5-μm particle size) and an analytical column PicoFrit Column (20 cm × 75-μm inner diameter, 5-μm particle size; New Objec-

tive) at a flow rate of 300 nl/min over 65 min. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was 275 °C. The instrument methods in LTQ Velos Orbitrap were set up in the data-dependent acquisition mode of higher-energy collisional dissociation fragmentation. The full scan MS spectra (m/z 300–1,600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1×10^6 . The resolution in the Orbitrap system was set to $r = 60,000$, and the five most intense peptide ions with charge states of ≥ 2 were sequentially isolated to a target value of 50,000 and fragmented in HCD with normalized collision energy of 40% with the resolution in the Orbitrap system was set to $r = 7,500$ for MS/MS. The signal threshold for triggering an MS/MS event was set to 80,000 counts, and an activation time of 0.1 ms was used. Dynamic exclusion was enabled with exclusion size list of 400, exclusion duration of 60 s, and repeat count of 2. For cross-linking analysis, the raw data files generated by Xcalibur v.2.1 (Thermo Fisher Scientific) were converted to a peak list format (mgf) using Proteome Discoverer version 1.3 (Thermo Fisher Scientific). The mgf files were analyzed in MassMatrix software to automatically search chemical cross-linkage against database containing the GAC amino acid sequences. The parameters for cross-linking analysis were carbamidomethylation (+57.021460 Da) as fixed modification, oxidation of methionine (+15.99491 Da) as variable modifications, chemical cross-linked with disuccinimidyl suberate-DSS (138.06808 Da) non-cleavable by enzymes, four trypsin missed cleavages, and a tolerance of 10 ppm for precursor and 0.02 Da for fragment ions (20). Potential cross-linked peptides were manually validated. This experiment was independently performed four times for the tetramer form and seven times for the superoligomer form. Unique lysine linkages (underlined in the following peptides) were only observed in the tetramer samples, between residues Lys¹⁸¹ and Lys²⁰⁷ (α -KCVQSNIVLLTQAFR and β -LKECMDMLR), Lys²⁰² and Lys⁵⁷⁸ (α -LTLQTTS DGVM LDKDLFK and β -DTVWKK), and Lys⁴⁰³ and Lys⁵¹² (α -SGVAGGILLVVPNVMGMMCWSPPLDKMGNSVK and β -EKK).

Computational Biology—To obtain a model of the glutaminase fiber, we applied a previously developed rigid body docking algorithm restrained by experimental DSS cross-linking data (20). Initially, we reconstructed both the N- and C-terminal regions of the monomers using a loop reconstruction routine and placed a copy of the tetramer, namely the ligand domain, on the N-terminal of the receptor domain. Using only the exclusive intermonomer linkage that was observed in our reconstructed model (Lys¹⁸¹–Lys²⁰⁷), we selected the decoy that was in the lowest energy range and within 11 Å of lysine pair distance.

Cell Assays—MDA-MB 231 cells were purchased from ATCC (initial passage of 29) and cultivated in RPMI 1640 medium (Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (Cultilab). The pcDNA3.1/V5-His-hGAC (GAC-V5) clone was kindly provided by Dr. Richard Cerione (Cornell University, Ithaca, NY), and the mutation K320A (equivalent to K325A in the mouse gene) performed with the QuikChange II site-directed mutagenesis kit (GAC.K325A-V5) following the manufacturer's instructions. Lipofectamine-transfected cells were G418 selected for stable expression, which was confirmed by Western blotting. The mock control

was obtained by transfecting cells with empty vector. To accomplish the endogenous GAC knockdown, the same cell clones were transduced with lentiviral particles containing pLKO-shGAC plasmid (forward, 5'-CCGGCCTCTGTTCTGTCAGAGTTCTCGAGAACTCTGACAGAACAGAGGTTT-TTG-3', and reverse, 5'-AATTCAAAAACCTCTGTTCTGT-CAGAGTTCTCGAGAACTCTGACAGAACAGAGG-3'), and a pool of cells was selected for puromycin resistance. The cells were lysed with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM NaVO₄, 1 mM β -glycerol phosphate, 11 μ g·ml⁻¹ aprotinin, 11 μ g·ml⁻¹ pepstatin, and 1 mM PMSF. The lysates were resolved by SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated overnight with the primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.1% Tween 20. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies followed by exposure to Pierce SuperSignal West Pico Substrate. We used anti-V5 1:10,000 (Invitrogen), anti-GAC custom made by Genescript as previously described in Ref. 5 at 1 μ g·ml⁻¹, anti-Vinculin 1:1,000 (Cell Signaling) and anti-VDAC 1:1,000 (Cell Signaling). As for the immunofluorescence and glutamine consumption studies, MDA-MB 231 cells were seeded in a 6-well plate (Becton Dickinson) at a density of 2.5×10^5 cells·cm⁻², cultivated for 48 h at 37 °C with 5% of CO₂ and then fixated and permeabilized with 3.7% formaldehyde in PBS, 0.2% Triton X-100. The cells were blocked with 3% BSA in PBS, 0.8% Triton X-100, and incubated at room temperature for 1 h, with anti-V5 1:20,000 diluted in 3% BSA in PBS/0.8% Triton X-100. Goat secondary anti-mouse-Alexa 633 (Invitrogen), diluted in blocking solution (1:200) was used to reveal ectopically expressed V5-tagged GAC. The nuclei and actin were stained with 2.5 μ g·ml⁻¹ DAPI (Sigma) and phalloidin-Alexa 488 (1:40), respectively. Data were collected with the plate reader Fluorescence Microscope Operetta (PerkinElmer Life Sciences), and the cell number and area quantified were using the software Harmony 3.0. Glutamine consumption from the culture medium was assessed using the Bio-Profile Basic 4 (Nova Biomedical). BPTES-treated cells were allowed to attach for 24 h and then had the medium replaced by fresh medium added with 10 μ M of the inhibitor for 48 h. For the whole cell lysate glutaminase activity assay, the cells were lysed by resuspension in 25 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, and 1 $\times$ Protease Inhibition Solution (Qiagen) followed by 20 strokes through an insulin needle. Glutaminase activity assay of the whole cell extracts was performed following the published streamlined assay (5), using 5 μ g of lysate/well and L-glutamine concentration of 7.5 mM. Diameter of detached viable cells was measured by the Countess (Invitrogen) after trypsinization and trypan blue incubation.

In Cell Protein Cross-linking—Approximately 3×10^6 cells were seeded in 10-cm plates and allowed to adhere overnight. The next day, the medium was removed, and the cells were washed twice with PBS and incubated for 24 h with 2 mM L-photo-leucine and 4 mM L-photo-methionine (Pierce) in leucine- and methionine-free DMEM supplemented with 10% PBS-dialyzed FBS. After this incubation, the medium was removed, and cells were washed twice with PBS and then irradiated at 365 nm for 15 min in the Epi Chemi II Darkroom equipment (UVP Laboratory Products). The cells were harvested for lysis, and

Supratetrameric Oligomerization-dependent GAC Activation

FIGURE 1. GAC polymerization is essential for enzymatic activation. *A*, size exclusion chromatography analysis of serial dilutions for wild-type and mutated GAC in the presence or absence of 20 mM phosphate. The green region delimits the expected Stokes radii between GAC dimers (D) and tetramers (T), calculated based on the crystal structure (3ss3) dimensions. The light purple region, delimited by V in the GAC.K325A graph, indicates the void volume of the gel-filtration column used. The asterisk indicates a previously published result for wild-type GAC (5). *B*, the software DigitalMicrograph (Gatan) was used to estimate the two orthogonal size distributions (short and long dimensions) of the wild-type and point mutant glutaminase particles, formed under the diverse conditions described in the main text (absence or presence of 20 mM phosphate and cross-linked versus non-cross-linked particles). *C*, supratetrameric organization of GAC upon the addition of 20 mM phosphate, a well known activator of GLS1 glutaminases. *D*, box plot and scatter representation of the two orthogonal dimensions for GAC wild-type and point mutants, as taken from the TEM micrographs. *E*, chemically cross-linked GAC superoligomers. Some of the observed filaments resemble lateral association between two simple filaments. *F*, GAC.K325A presents a much higher catalytic efficiency, already in the absence of phosphate, when compared with wild-type GAC and the inactive GAC.R322A. *G*, the high enzymatic efficiency of GAC.K325A correlates well with its tendency to self-assemble into rod-like polymers, regardless of the presence of the activator inorganic phosphate (left and middle panels) and DSS. Conversely, the catalytically inactive GAC.R322A protein remains in its tetrameric form, even in the presence of 20 mM phosphate (right panel). The scale bars represent 100 nm.

cross-linked proteins were resolved by 3–15% step gradient SDS-PAGE (3, 9, and 15% SDS-PAGE solutions prepared with 50:1 acrylamide:bisacrylamide mix, poured one after another in volume proportions of 1.5:2:1). The immunoblotting was performed with the anti-V5 1:10,000 (Invitrogen) as described in the preceding section.

Estimation of Intracellular Glutamine Concentration in Breast Tumor Cells—From metabolomics experiments on tumor samples, Yuneva *et al.* (21) reported a relationship of 40 to hundreds of nanomoles of glutamine for each gram of protein. We have found that unattached GAC.K325A-V5, GAC-V5, and mock transformed MD-MBA 231 cells have average diameters of 13.3, 10.9, and 10.6 μm , respectively. Assuming a spherical shape for these cells, the following cell volume can be calculated: 1.2 nl (GAC.K325A-V5 cells) and 0.6 nl (GAC-V5 and mock cells). Using the Pierce BCA protein assay kit (Thermo-Scientific), we quantified an average of 0.23 grams of protein for 2×10^6 cells, sampling from the three cell clones. Based on the lowest detected glutamine amount from Yuneva *et al.* (21) of 40 nmol Gln/g of protein, we estimate that these cells contained between 3.7 and 7.4 mM glutamine.

Quantitative PCR—RNA samples were extracted using the kit RNeasy Mini (Qiagen) following the manufacturer's instructions. cDNA synthesis and PCR amplification were performed with the kits SuperScript III first strand synthesis system for RT-PCR (Invitrogen) and Power SYBR Green PCR

Master Mix (Applied Biosystems), respectively, as instructed by the manufacturers. Samples were run on the Applied Biosystems 7500 real time PCR system and analyzed following the $2^{-\Delta\Delta\text{CT}}$ method. Primers used: (i) both endogenous and ectopic GAC: forward, 5'-GATCAAAGGCATTCCTTTGG-3', and reverse, 5'-TACTACAGTTGTAGAGATGTCC-3'; (ii) ectopic GAC only: forward, 5'-AGAATGGAAAGTCTGGGAGAGAAA-3', and reverse, 5'-CCGAGGAGAGGGTTAGGGATA-3'; (iii) SN2: forward, 5'-GGCTTGGTAGTGTGTTGCCAT-3', and reverse, 5'-GGGCAAAGAGTAAACCCACA-3; (iv) ASCT2: forward, 5'-ATGAAACACTTGGGCTAC-3', and reverse, 5'-ATGACCGAAACAAGGAAA-3'; (v) rRNA 18 S: forward, 5'-ATTCCGATAACGAACGAGAC-3', and reverse, 5'-TCA-CAGACCTGTTATTGCTC-3'.

RESULTS

Active GAC Forms a Rod-like Supratetrameric Structure—We have previously documented that wild-type GAC, when in the presence of its activator P_i , showed a tendency to form molecular species larger than tetramers and that this behavior was counteracted when the concentration of NaCl, a known glutaminase inhibitor, was increased in the protein solution (5, 12). Although the structural nature of the higher order species remained elusive at that time, it was curious to note that there was a significantly major shift toward higher than tetramer olig-

omers in the case of the activity-enhancer mutation F327S (GAC.F327S; Fig. 1A, *top right panel*).

In the course of further studies of the molecular mechanism of activation of mammalian glutaminase, we used TEM to better understand the self-assembly process. First, recombinant GAC (in the absence of P_i) was used to set up negatively stained grids. Image analysis, after sampling ~ 100 randomly chosen particles, allowed the determination of two perpendicular size distributions (exemplified in Fig. 1B): a short dimension, with a mean size of 7 ± 1 nm, and a longer one of 8 ± 1 nm (Fig. 1C, *upper panel*), both close to the expected dimensions for the dimer particle (5). In contrast, particles formed in the presence of 20 mM phosphate had a larger and more heterogeneous size distribution on the grid. Whereas the shorter dimension doubled on average (18 ± 3 nm), there was clearly favored growth across the long dimension, averaging 88 ± 31 nm (Fig. 1C, *lower panel*). The box chart with scatter plot for the size survey is shown in Fig. 1D.

The equilibrium between the GAC oligomeric species depends on protein concentration, phosphate, and NaCl (5). To circumvent the dissociative effects of protein dilution and work at appropriate protein concentrations for TEM characterization, superstructure assembly was stabilized by adding the cross-linking agent DSS in the presence of 20 mM phosphate and then purified by gel filtration. Under the conditions used, the superstructures eluted in the void volume of the column. The appropriate DSS concentration and time of incubation was previously established by a dose-response assay (data not shown). The negative-stained micrographs showed evidence for the formation of elongated, chain-like, nonbranched filaments, heterogeneous in length, that favored side-on adsorption to the carbon film (Fig. 1E). Some of the observed particles, such as those in the *rightmost panels* of Fig. 1E, seem to resemble side by side aggregation of simpler filaments (*left and bottom panels*).

We have previously identified the gating loop as essential for the phosphate-dependent activation process of the GLS1 isoforms (5). By replacing residues in the gating loop by alanine, we identified a point-mutation (GAC.K325A) that conferred a 670-fold increase in catalytic efficiency over that found for the wild-type enzyme, even in the absence of inorganic phosphate (GAC.K325A $k_{\text{cat-app}}/K_{m-app}$ of $281.8 \text{ mM}^{-1}\cdot\text{s}^{-1}$ compared with $0.42 \text{ mM}^{-1}\cdot\text{s}^{-1}$ for GAC) (Fig. 1F). Both negative staining TEM (Fig. 1G, *left panel*) and size exclusion chromatography (Fig. 1A, *bottom left panel*) confirmed a much greater particle size distribution for this mutant, even in non-cross-linked samples. For example, TEM analysis showed dimensions of 17 ± 2 and 107 ± 79 nm, for the short and long dimensions, respectively, in the absence of phosphate (Fig. 1D). More importantly, this mutant assembles into oligomers visually similar to the ones formed with the wild-type protein (Fig. 1E, *rightmost panels*), guaranteeing that the mutation is only enhancing the oligomerization capability. The K325A mutation uncouples the protein dependence on phosphate for activation and further confirms the positive correlation between the superstructure assembly and enzyme activation. On the other hand, a second point mutation within the gating loop, affecting Arg³²² (termed GAC.R322A), was enough to generate a catalytically inactive mutant irre-

FIGURE 2. Glutaminase isozymes versus self-assembly tendency. TEM analysis of the three glutaminase isozymes, cross-linked after incubation with 20 mM phosphate. The average length of the superstructure correlates positively with the previously published glutaminase activity levels (5). GAC is the most active isozyme and forms the longest polymers (*top left panel*), followed by KGA (*top right panel*), with lower activity and shorter structures, and lastly LGA, which is insensitive to phosphate and accordingly does not form filaments (*bottom left panel*). Unless otherwise specified, the scale bars represent 100 nm.

sponsive to phosphate ($k_{\text{cat-app}}/K_{m-app}$ of $0.6 \text{ mM}^{-1}\cdot\text{s}^{-1}$ at 20 mM P_i against $3.6 \text{ mM}^{-1}\cdot\text{s}^{-1}$ for the wild type), which accordingly did not assemble into superoligomers (Fig. 1, *F and G, right panel*). The dimensions observed for particles of GAC.R322A in the presence of 20 mM P_i were very similar to those for the wild-type protein in the absence of P_i (7 ± 1 and 8 ± 2 nm, for the short and long dimensions, respectively) (Fig. 1D).

Superstructure among Glutaminase Isozymes—GAC is the most active of the three known mammalian glutaminase isozymes in response to inorganic phosphate (5). To further investigate the correlation between increased activity and larger oligomeric species, we observed the ability of KGA and LGA (also known as GLS2) to assemble into supratetrameric structures by TEM. Superstructure assembly was induced at 20 mM phosphate, stabilized by cross-linking with DSS, and subsequently gel filtration-purified. A tendency toward longer oligomers correlates well with the activity levels of the three isozymes. More specifically, GAC—the most active glutaminase at 20 mM P_i —forms the longest rod-shape oligomers (Fig. 2, *top left panel*), followed by KGA (Fig. 2, *top right panel*) and then LGA (Fig. 2, *bottom left panel*), which has been previously shown to be enzymatically insensitive to P_i (5, 22) and to not form the superstructure. The mean values for the shortest and longest dimensions of the particles are 16 ± 2 and 181 ± 108 nm for GAC, 14 ± 3 and 74 ± 26 nm for KGA, and 9 ± 2 and 12 ± 3 nm for LGA (Fig. 2, *bottom right panel*).

BPTES Disrupts GAC Superstructure—Counterintuitive to the current dimer to tetramer model of activation for GLS1, the inhibitor BPTES has been shown to lock GLS1 into an inactive yet tetrameric form (6). Interested in deciphering the mode of

Supratetrameric Oligomerization-dependent GAC Activation

FIGURE 3. BPTES inhibits GAC superstructure formation. *A*, dose-response profile of BPTES inhibition on GAC, assayed in the presence of 20 mM phosphate, showing two complementary effects on the apparent turnover rates and the Michaelis constant of the enzyme. *B*, BPTES traps the gating loop (black ribbons) in a rigid open conformation relative to the catalytic pocket of GAC (delimited by a green surface). *C*, stereographic view of a Fourier $2F_{obs} - F_{calc}$ map (at 1σ) confirming the proposed conformation in the refined crystallographic model. *D*, TEM analysis of the effects of BPTES on the formation of GAC superstructure. The filament formation is hindered regardless of whether BPTES is added to the protein solution prior to (middle panel) or after (bottom panel) incubation with 20 mM phosphate. *E* and *F*, conversely, GAC.K325A is catalytically insensitive to BPTES treatment (*E*), and this inhibitor is also incapable of disrupting the non-cross-linked GAC.K325A filaments (*F*).

inhibition by which BPTES exerts its effect and its relationship with the formation of the superstructure, we determined a detailed BPTES dose-response curve for GAC in the presence of 20 mM Pi. This phosphate concentration was chosen because it is closer to the physiological one under hypoxic conditions (23) and substantially stimulates GAC catalytic activity (5). We observed a strong inhibitory effect on GAC, where nanomolar levels of BPTES affected the maximum catalysis rate, $k_{cat-app}$, without changing the K_{m-app} for glutamine (Fig. 3A), a clear indication of an allosteric noncompetitive inhibitor as already published by others (6, 7). Curiously, beyond 200 nM BPTES, a secondary effect is observed and the inhibitor started affecting also the K_{m-app} (a 3-fold increase compared with the absence of BPTES), redefining BPTES as a mixed mode inhibitor (Fig. 3A). By fitting an error bar-weighted logistic sigmoid function to explain the decrease in the turnover rates of the enzyme caused

by the increased presence of inhibitor, we obtained an IC_{50} of 80.4 ± 7.2 nM for BPTES, which is consistent with the value provided by DeLaBarre *et al.* (7). Upper and lower limits for the catalytic rates are 16.4 and $7.9 s^{-1}$, respectively, with a Hill slope of 1.7 ± 0.2 ($R^2 = 0.99$), indicating cooperative binding (Fig. 3A). Concerning the effect on K_{m-app} , the upper and lower limits are 4.6 and 13.8 mM, with an IC_{50} of 629 ± 61 nM for BPTES (Hill slope of 2.0 ± 0.3 , $R^2 = 0.99$).

Concomitant with the studies above, we have also determined the crystal structure of mouse GAC in complex with BPTES (2.77 Å resolution, R_{factor} of 25.3%, and R_{free} of 29.5%; Table 1). Although similar to previously reported BPTES-bound glutaminase structures, with two inhibitor molecules tightly accommodated at the tetramer interface and average backbone root mean square deviations of 0.44 and 0.56 Å when superposed to Protein Data Bank structures 3UO9 and 3VOZ,

TABLE 1

X-ray crystallography data collection parameters and structure refinement statistics of BPTES-bound mouse glutaminase C (Protein Data Bank code 4jkt)

The data for the outer shell are shown in parentheses. LNLS, Laboratório Nacional de Luz Síncrotron.

Data collection	
Beamline	D03B-MX1 at LNLS, Brazil
Wavelength (Å)	1.608
Space group	P2 ₁ 2 ₁ 2 ₁
Cell parameters <i>a</i> , <i>b</i> , <i>c</i> (Å)	100.7, 140.2, 180.9
Resolution range (Å)	38.4–2.77 (2.82–2.77)
Unique reflections	63,967 (2,039)
Multiplicity	3.3 (2.6)
<i>R</i> _{p.i.m.} (%)	11.0 (24.0)
Completeness (%)	95.5 (46.2)
<1/ <i>σ</i> (<i>I</i>)>	4.5 (2.8)
Average mosaicity (°)	1.0
B-factor Wilson Plot (Å ²)	40.5
Monomers/AU	4
Solvent content (%)	59.2
Matthews coefficient (Å ³ /Da)	3.01
Model refinement	
Resolution range (Å)	20.0–2.77
Reflections (cross-validation)	63,892 (5,952)
<i>R</i> _{factor} / <i>R</i> _{free} (%)	25.3/29.5
Average B-factor (Å ²)	
Main chain (no. of residues)	32.9/3.4 (1,563)
Side chain (no. of residues)	32.8/4.3 (1,362)
BPTES (no. of molecules)	44.1/9.2 (2)
Solvent (no. of molecules)	24.4/5.9 (265)
Root mean square deviation from standard geometry	
Bond length (Å)	0.003
Bond angles (°)	0.783
Ramachandran plot	
Most favored (%)	94.5
Allowed (%)	4.8
Outlier (%)	0.7

respectively (7, 8), our crystal form presents a key unique feature. As a consequence of the binding of the small molecule to the tetramer interface, the main chain of the gating loop—consistently poorly ordered across all the GAC crystallographic models—is in a stable open conformation in all four monomers. This is due especially to the contacts made mainly between BPTES and Lys³²⁵, and consequently the loop assumes the conformation of a one-turn helix (Fig. 3, *B* and *C*). Fifteen hydrogen bonds are made between a dimer of GAC and one BPTES molecule (data not shown). Eleven residues from each monomer share the interface with BPTES, occluding a combined total area of 620 Å². Of the total solvent-accessible surface of BPTES (788 Å²), 80% is buried upon interaction with the enzyme.

Next, we assessed the effects of BPTES on the formation of GAC superoligomers. TEM and size exclusion chromatography of wild-type GAC samples prepared in the presence of phosphate and the inhibitor and subsequently cross-linked showed that regardless of whether BPTES is added prior or after protein incubation in 20 mM phosphate; complete disruption of the superstructure is observed at a concentration of 30 μM of BPTES (Fig. 3*D*). The distribution of the disrupted particles on the grids is similar to the wild-type protein in the absence of phosphate and BPTES, as shown in Fig. 1*A* (*top panel*).

Finally, we observed that the mutant GAC.K325A, besides being hyperactive, is also catalytically insensitive to BPTES as determined by a dose-response curve (Fig. 3*E*). Accordingly, BPTES is incapable of disrupting non-cross-linked GAC.K325A

oligomers, assembled in the presence or absence of phosphate (Fig. 3*F*).

Superstructure Characterization—Because of its heterogeneous length distribution, the GAC superstructure is intrinsically inappropriate for crystallization. However, by combining TEM, chemical cross-linking followed by MS analysis, and computational biology, we provide a low resolution model for the superstructure. The extended particles tend to adsorb to the microscopy grids in a very limited number of orientations. However, the best micrographs show a combination of translational and rotational symmetry elements lying parallel to the polymer, thus yielding a helical symmetry along its longitudinal axis. Detailed analysis of low pass filtered images provided its geometrical attributes. The observed spiral polymer, under particular staining conditions, resembles a right-handed double-stranded helix, with a rise per turn of $\sim 53 \pm 2$ nm, a strand inclination of 25°, and an average width for a single strand of 6.6 ± 0.7 nm (Fig. 4*A*).

Simultaneously, size exclusion-purified cross-linked polymers—as well as GAC cross-linked in the tetrameric form (used as a control)—were digested with trypsin, and the resulting peptides were subjected to liquid chromatography coupled to tandem mass spectrometry. The cross-linked peptides were modeled based on the crystal structure of the tetramer, using a spacer arm length for DSS of ~ 11.4 Å as a restraint for maximum distance between the side chain amine groups of linked lysines. This was necessary to distinguish between the different possible inter- and intramolecular cross-links. Unique linkages were only observed in the tetramer samples (spectra shown in Fig. 4*B*), between residues Lys¹⁸¹ and Lys²⁰⁷ (both located in the outer N-terminal portion of the same monomer), Lys²⁰² and Lys⁵⁷⁸ (the latter located at the C terminus), and Lys⁴⁰³ and Lys⁵¹² (both within the glutaminase domains, flanking the active site of two different monomers) as depicted in Fig. 4*C*. The observation that these DSS-mediated links are unable to form upon polymerization suggests occlusion of their respective surfaces from the solvent when in the superstructure form. One lysine residue from each of the cross-linked pairs was then substituted by an oppositely charged glutamate residue, and the individually mutated proteins (termed GAC.K202E, GAC.K207E, and GAC.K512E) were tested for superoligomer-dependent glutaminase activity. Both N-terminal mutants, GAC.K202E and GAC.K207E, resulted in enzymes with enhanced activity (Fig. 4*D*, *left panel*), which coherently formed bigger particles, when compared with wild-type GAC (Fig. 4*D*, *right panel*). Conversely, the glutaminase domain mutant GAC.K512E fully lost the phosphate-dependent enzymatic capability and, accordingly, the ability to self-assemble into the superoligomers. The three point mutations reinforce the need for superoligomer formation for protein enzymatic activity and, most importantly, further show the importance of regions unrelated to and distant from the catalytic pocket for superoligomer formation and enzyme activation.

In the original biochemical characterizations of recombinant GLS1, Kenny *et al.* (12) demonstrated the importance of the GLS1 N-terminal portion for enzyme activity by generating a truncated, catalytically inactive, recombinant construct. Nevertheless, subsequent crystal structures of GAC clearly showed

Supratetrameric Oligomerization-dependent GAC Activation

FIGURE 4. A model of GAC superoligomer assembly. *A*, the removal of high frequencies (strongly associated with noise) from the TEM micrographs highlights the right-handed double-strand nature of the superstructure, allowing the determination of its geometric features. *B*, cross-linked MS/MS spectra were manually validated for *b* and *y* ion series of the α and β chains of cross-linked peptides (between residues Lys¹⁸¹ and Lys²⁰⁷, Lys²⁰² and Lys⁵⁷⁸, and Lys⁴⁰³ and Lys⁵¹²). The ions are indicated by arrows with corresponding *m/z* value. *C*, relative position of DSS-linked lysine pairs in the tetramer, as identified by MS. The boundaries of the active sites are delimited by solid surfaces. †, Lys⁵⁷⁸ is not modeled in the crystal structure. The proposed single strand growth direction is across the boundaries of the active sites, via an end to end interaction between pairs of N-terminal domains. Alternating orange and yellow colors facilitate the identification of the tetramers along the single strand. *D*, one lysine residue from each cross-linked pair was individually substituted by a glutamate and assayed for enzymatic activity (left panel) and superstructure formation (right panel). The N-terminal region mutants (GAC.K202E and GAC.K207E) enhanced both effects. On the other hand, GAC.K512E, within the glutaminase domain, generated a nonfunctional protein, similarly to the acetylation mimetic GAC.K316Q. The green region delimits the expected Stokes radii between GAC dimers (*D*) and tetramers (*T*), calculated based on the crystal structure dimensions. The asterisk indicates a previously published result for wild-type GAC (5). *E*, the double strand was manually modeled (panel *i*), using two entwined copies of the single strand from *C* and following the geometric restrictions of *A*. In panel *ii*, the calculated Fourier F_{calc} map (with amplitudes and phases from the double strand model)—limited to 35 Å maximum resolution—is two-dimensionally projected, and the end result is shown in panel *iii*. All features are comparable to those observed in panel *iv*, concerning the presence of alternating high density narrow regions (indicated by filled triangles) and low density broad, disc-like regions (indicated by open triangles).

no direct participation of residues belonging to the N-terminal portion in catalysis or even their requirement for the proper folding or stabilization of the active site. Based on the dimensions observed in the GAC crystal structures, the average width of 66 Å for a single strand indicates that polymerization occurs along the longest axis of the tetramer, thus suggesting an end

to end interaction via N-terminal regions. The growth is thus consistently favored in one direction and may occur indefinitely, because N-terminal sticky ends will always be available at both termini (computational docking of several tetramers simulating strand growth is shown in Fig. 4C, left panel). Therefore, because activity is dependent on filament assembly and the N-ter-

minal portion is key for such, we provide a plausible explanation for the findings of Kenny *et al.* (12).

Next, to generate a model for the double-stranded filamentous structure, the coordinate files of two single strands were manually coiled around each other, using the helical parameters as restraints (Fig. 4E). The Gaussian low pass filter applied to the reference micrographs remove spatial frequencies higher than $0.028 \text{ \AA}^{-1}$, thus resulting in an estimated $35 \text{ \AA}$ resolution in real space. According to its overall dimensions, both length-wise and angle-wise, the estimated minimum repeating unit (one full helical turn) is composed of seven tetramers in each of the entwined strands. The intrinsic 2-fold dihedral symmetry of the tetramer (5) renders it unnecessary to determine whether the double-stranded helix is of a parallel or anti-parallel nature because each strand is apolar. To validate the model against the experimental data, an F_{calc} Fourier electron density map was generated by the program FFT (24) (to $35 \text{ \AA}$ maximum resolution), using structure factors calculated from the three-dimensional double-stranded coordinate file and subsequently theoretically projected in two-dimensions using the electron microscopy software Imagic (25). The end result is shown in the *right panel* of Fig. 4E and presents features that are fully comparable to those observed in the representative micrographs of Fig. 1E (*bottom panel*), especially concerning the presence of an alternating pattern of narrow, compact regions of high density interspersed with broad, disc-like regions of low density.

Lastly, Lys³¹¹ in human GLS1 glutaminases was found to be a target for *in vivo* acetylation by high resolution mass spectrometry analysis (13). This lysine residue belongs to the glutaminase domain and is located on the opposite surface in relation to the catalytic pocket and the gating loop, with no direct participation in the active site. To evaluate the effects of this reversible, post-translational modification over superstructure formation and therefore protein activation, we have generated a point mutant in the equivalent residue of the mouse protein (GAC.K316Q), replacing it with a glutamine residue, so as to mimic the noncharged nature of acetylated lysine. Interestingly, the newly mutated protein was characterized as less sensitive to the activator phosphate with the concomitant lowered tendency to assemble into the superoligomers, keeping the protein in sizes compatible with only tetramers and dimers (Fig. 4D, *left and right panels*).

GAC Superstructure in Cell Model—To further demonstrate the importance of the superstructure assembly for GAC enzymatic activation, we generated stable clones of the MDA-MB 231 breast cancer cell line expressing either the human V5-tagged wild-type or the GAC.K325A mutant proteins, as well as cells transformed with a mock plasmid. Although in humans the appropriate numbering should be GAC.K320A (NCBI reference sequence AAD47056.1), we have decided to keep GAC.K325A (mouse GAC) for the sake of coherence throughout the paper. Cell clones were selected to present comparable levels of ectopic V5-tagged mRNA and protein while not presenting abnormally higher levels of ectopic expression (Fig. 5, A and B). Proper mitochondrial localization of the ectopic protein was confirmed by cell fractionation and immunoblotting (data not shown). Two distinct phenotypes were readily observed for the GAC.K325A-V5 cells, when com-

FIGURE 5. GAC superstructure in cell models. A and B, endogenous and V5-tagged ectopic proteins expressed to similar levels. C–E, a stable MDA-MB 231 clone selected after GAC.K325A-V5 transfection presented larger and more heterogeneous cell area (C), proliferated more (D), and consumed more glutamine from the culture media (E), all compared with cells bearing the V5-tagged wild-type protein or a mock plasmid. F, relative mRNA levels of ASCT2 and SN2 glutamine transporters, as defined by quantitative PCR using rRNA 18 S as a housekeeping gene, showing that the GAC.K325A-V5 cells do not overexpress these transporters. G, glutaminase activity from whole cell lysate, in the presence of 20 mM phosphate, showing consistently higher turnover rates for the GAC.K325A-V5 samples, against the physiological glutamine levels in tumors. H, *left panel*, step gradient SDS-PAGE (3–15%) followed by immunoblotting (anti-V5) of UV-induced cross-linked intracellular protein with incorporated photo-reactive amino acids, showing the tendency of the GAC.K325A-V5 to form higher molecular weight superstructures within the cells. The UV-induced cross-linking was performed in living, intact cells in culture. *Right panel*, densitometry was performed in conditions of nonsaturated signal, using ImageJ, to evidence the differential cross-linking of bigger species for GAC.K325A-V5. I, similar to what was observed for the recombinant protein, cells expressing the fiber-prone hyperactive GAC.K325 mutant (GAC.K325A-V5) were less sensitive to BPTES treatment, still proliferating more (*left panel*) and consuming more glutamine (*right panel*) than BPTES-treated counterparts. J, the knockdown of endogenous GAC favored the enhancing of the phenotypic differences observed above, better highlighting the outcome from GAC.K325A-V5 expression.

pared with the GAC-V5 and mock counterparts. First, GAC.K325A-V5 cells were, on average, much larger and heterogeneous in size, as based on the measurement of cell area (Fig. 5C). More specifically, whereas the GAC-V5 and the mock transformed cells presented similar, normally distributed cell areas, peaking at 593 and 600 μm^2 , respectively, the GAC.K325A-V5 population was much more heterogeneous in

Supratetrameric Oligomerization-dependent GAC Activation

size (relative standard deviation of 89%) and still $\sim 7\%$ larger, averaging $637 \mu\text{m}^2$. Second, the mutant-transformed cells proliferated $\sim 10\%$ more than the wild-type and mock transfected cells, as determined by the quantification of cell numbers (Fig. 5D). The average diameter of unattached (trypsinized) cells was also assessed to account for the effects of cell spreading on the area measurements. GAC.K325A-V5 cells presented the largest diameters, averaging at $13.3 \mu\text{m}$ ($n = 178$), when compared with the wild-type GAC-V5 ($10.9 \mu\text{m}$, $n = 151$) and mock cells ($10.6 \mu\text{m}$, $n = 174$) (data not shown).

Next, glutamine uptake from the culture medium was evaluated after 48 h of plating. We found that although the glutamine consumption levels for the GAC and the mock transformed cells was deemed indistinguishable (0.78 ± 0.09 and 0.81 ± 0.07 nmol/liter per cell, respectively), GAC.K325A-V5 cells consumed 40% more glutamine (1.16 ± 0.07 nmol/liter per cell), as shown in Fig. 5E. In parallel, we showed that GAC.K325-V5 cells do not express more of the glutamine transporters ASCT2 and SN2 (Fig. 5F). To account for the observed increase in glutamine consumption, we assessed the glutaminase activity in a physiological level of L-glutamine (7.5 mM) and observed that the reaction rates for the GAC.K325A-V5 cells are 40–50% higher when compared with the other two clones (Fig. 5G). Faster turnover rates were also consistently observed for the recombinant mutant protein, as shown in the first section.

To inspect for higher than tetramer oligomer formation within the living cells, cells were grown in the presence of photo-reactive amino acids and then exposed to UV light (at 365 nm), followed by immunoblotting against the whole cell extract. The data clearly showed that GAC.K325A-V5 was cross-linked *in cell* into higher molecular weight species than GAC-V5 (Fig. 5H). Although the cross-linked species did not extend as far as those observed for the recombinant protein *in vitro*—likely because of the hetero-oligomerization between the V5-tagged mutant protein and endogenous wild-type GAC inside the mitochondria—the differential cross-linking pattern is evident. We have shown that the enzymatic activity of the recombinant mutant GAC.K325A is unaffected by BPTES treatment (Fig. 3E), which, accordingly, did not disrupt the superstructure (Fig. 3F). As a further confirmation that the superoligomer formation is important for the observed phenotypes, we treated cells with $10 \mu\text{M}$ of BPTES. As expected, GAC.K325A-V5 cells still grew more and consumed higher amounts of glutamine than BPTES-treated control cells (Fig. 5I). BPTES treatment has drastically decreased the glutamine consumption on all the cell clones because it likely disrupted hetero-oligomers (formed by endogenous wild-type GAC and ectopic GAC.K325A-V5) and because it is also inhibits KGA (6). Despite this, the ectopic expression of GAC.K325A was able to improve the proliferation and growth phenotype of the cells.

Lastly, to exclude the possibility that the difference in cell proliferation and growth, as well as Gln uptake for the GAC.K325A-V5 cells were due to higher amounts of endogenous enzyme (in comparison to control cells), as well as to highlight the ectopic protein activity, we knocked down endogenous GAC by using a shRNA target to its mRNA 3'-UTR (Fig. 5B). Not surprisingly, the previously observed phenotypic differences became enhanced. The mutant-transformed cells grew

30% more and were 40% bigger, while consuming ~ 2.2 times more glutamine per cell, than the wild-type-expressing cells (Fig. 5J). Accordingly, the activity levels assessed from the whole cell extract in the presence of 7.5 mM L-glutamine were 75% higher than the control assays (Fig. 5J). Overall, this collection of results directly demonstrated that the phenotypic differences above resulted from the presence of an ectopically expressed, hyperactive, and higher molecular weight prone protein GAC.K325-V5, which presented the same biochemical features and the intrinsic tendency to assemble into a superstructure as the recombinant mutant protein.

DISCUSSION

Previous biochemical studies have established that GLS1 glutaminases are mainly found as inactive dimers and that the presence of phosphate correlates with changes leading to tetramerization and enzyme activation (26, 27). We provide here novel information in that regard and show that the catalytic activation of the GLS1 glutaminases is directly linked to a fiber-like supratetrameric assembly, which correlates well with activation levels of the three glutaminase isozymes. The first observations of this phenomenon were provided ~ 40 years ago, using purified glutaminase from pig renal extract (28, 29). The formation of extended polymers was later used to purify the native enzyme by size exclusion, allowing its first biochemical and kinetic characterization (30), thus implying that our results are not an artifact of recombinantly expressed, truncated protein constructions, or even the chemically induced intermolecular cross-linking. Robinson *et al.* (6) have also documented larger oligomers for recombinant GLS1 in the presence of phosphate. The right-handed double-stranded molecular model that we propose here, when two-dimensionally projected using electron microscopy software, is not only in fully agreement with our experimental micrographs but also with the phosphate-borate induced form and the shadow casting reported by Olsen *et al.* (29) in 1973. Some of our micrographs suggest lateral association between two polymers, as seen, for instance in Figs. 1E (top right and middle panels) and 2 (top left box), as well as those of the GAC.K325A mutant (Figs. 1E and 3F). Regardless of these side by side associations, only the longitudinal growth seems to be connected to an increase in protein activity. Such a feature was also observed by Olsen *et al.* (29). Furthermore, it is also worth mentioning that with regard to the glutaminolytic pathway, polymerization is not exclusive to GLS1 glutaminases. Glutamic dehydrogenase from bovine liver has been shown to self-assemble into long multichain tubular structures under appropriate conditions (31). It is plausible then, that nonpathogenic polymer assembly may be a widespread process for the functioning of other metabolic enzymes.

Robinson *et al.* (6) first demonstrated biochemically that BPTES inhibits the GLS1 isoform (but not the liver-type isozyme, LGA/GLS2) by interfering with its phosphate-dependent allosteric activation and promoting the stabilization of the glutaminase into an inactive tetrameric form, later confirmed structurally (7, 8). Our collection of results indicates that BPTES inhibits GAC by trapping the gating loop at a rigid open conformation, which in turn prevents superoligomer formation. Although the tetramer- and phosphate-induced opening

of this loop is necessary for enzyme activity, its intrinsic flexibility seems to play a major role on the enzymatic process. Indeed, we verified by molecular dynamics simulation on the tetramer that although phosphate binding to the catalytic site increases the gating loop flexibility, both L-glutamate (enzyme inhibition by L-glutamate has been reported for the kidney isoform of GLS1 (32)), and the presence of BPTES freezes the gating loop into a less mobile state (data not shown).

We observed from the kinetic studies with the GAC.K325A mutant that filament formation results in a drastic decrease in the Michaelis constant of the protein, over 100-fold, compared with the wild-type enzyme, in the absence of the activator P_i . A much greater accessibility of the active site to the substrate is suggested upon polymerization, as well as a key role of the gating loop in this process, a phenomenon that is fully reversed when Arg³²² is replaced by alanine. Therefore, the present work unveils a new role for the gating loop, which besides regulating substrate accessibility to active site, also controls the reversible protein polymerization. Because of experimental limitations, which led to a low-resolution 35 Å model, we are unable to describe the specific interactions that hold both the single and the double strands together and result in a fully activated enzyme. However, based on the most diverse effects generated mainly by the individual point mutations, it is plausible to suggest that shape complementarity is the driving force for self-assembly and can only be fully achieved after specific surface charges are enhanced or neutralized, an outcome easily attainable in the presence of a polyanion such as the phosphate ion. This is especially true in the case of the gating loop, where the replacement of two closely positioned, long and positively charged residues (Arg³²² and Lys³²⁵) by alanine had antagonistic effects with regards to protein self-assembly and activation. Concomitantly, major torsions in the relative positions of the monomers inside the tetramer—that are still experimentally undetected, because all GAC crystal structures available to date are virtually identical—may be required for gating loop-mediated superoligomer formation. The binding of BPTES, which shares an extensive area and a large number of hydrogen bonds at the tetramer interface and which also holds the loop into a rigid conformation (besides reducing the flexibility of the tetramer), would also prevent the gating loop from making these contacts. Such torsions in the tetramer cannot be predicted from our manually built model but could possibly be described in the future from higher resolution experimental data, such as by cryoelectron microscopy.

Recently, Katt *et al.* (9) described a potential binding site for the GAC inhibitor 968. In the proposed model, the small molecule docks inside a concave surface region formed at the dimerization interface of two GAC glutaminase domains. The 968 binding region lies very close to the N-terminal portion of GAC, which is predicted here to be involved in the polymerization of the enzyme. The authors further showed that 968 is unable to bind and inhibit a previously phosphate-activated enzyme, which is in full consistency with what we present. Once GAC oligomers are assembled, because of the occlusion of the putative 968-binding site and the possible conformational changes in the N-terminal portion of the enzyme required for self-assembly, they cannot be reversed by the addition of 968,

explaining its limited inhibitory capacity on an already activated enzyme. However, if 968 is bound to free tetramers, then such conformational changes cannot be subsequently achieved, stopping GAC activation via self-assembly. Given the complementary modes of inhibition for BPTES and 968, acting at different stages of enzyme assembly and polymerization, one might envision that a synergy in inhibition would result when both are administered to glutaminase sensitive transformed cell. To our knowledge, this still needs testing.

Our findings also suggest a role for the previously detected *in vivo* acetylation of Lys³¹¹ in human GLS1 glutaminases (13). We propose that this post-translational modification down-regulates the enzymatic levels by antagonizing the formation of the active superoligomers. Given that acetylation is a reversible post-translational modification, it is plausible to suppose that glutaminase superactivation may, at least in part, be regulated by acetyltransferases and deacetylases yet to be identified.

Finally, we need to state that we would not expect that micrometer-long GAC polymers assemble inside the mitochondria. Rather, a more tangible scenario might be the presence of shorter, heterogeneous filaments, similar to those observed in Fig. 1A (*lower panel*). Nonetheless, direct visualization of such in the mitochondria has proven a difficult task. We showed that the superactive mutant GAC.K325A, intrinsically bound to self-assemble, was capable of providing growth and proliferation advantages to cells. However, a more important conclusion that can be drawn from these experiments is that an increased level of GAC in the mitochondria by itself is not sufficient to increase cell proliferation. The protein must be in the active form for such a phenotype to become evident. In cells, phosphate accumulation induced by hypoxia, for instance (23, 33), or even post-translational modifications, such as phosphorylation, could trigger polymerization, suggesting the possibility of distinct therapeutic opportunities for 968-like and BPTES-like inhibitors. Overall, our results reinforce the importance in focusing on the development of allosteric over active site-targeted inhibitors, glutamine analog inhibitors, when targeting GAC in tumors. This would result in preferred isoform-specific inhibitors, because LGA (GLS2), necessary for glutamine metabolism in the liver and brain, does not assemble into superoligomers, as well as avoiding the undesirable cross-inhibition of amidotransferases (21).

Acknowledgments—We thank the Laboratório Nacional de Biociências for access to its facilities (Laboratório de Purificação de Proteínas, Laboratório de Espectroscopia e Calorimetria, Laboratório de Espectrometria de Massas, Laboratório de Bioensaios, Laboratório de Vetores Virais, and Robolab); Laboratório Nacional de Nanotecnologia for access to Laboratório de Microscopia Eletrônica; and Laboratório Nacional de Luz Síncrotron for access to D03B-MX1 Beamline. We thank Dr. Alessandra Girasole for secretarial and technical support of Annelize Aragão and Romênia Domingues for helping with the MS analysis. We thank Dr. Richard C. Garratt (Instituto de Física de São Carlos, Universidade de São Paulo) for critical reading of the manuscript.

REFERENCES

- Gaglio, D., Metallo, C. M., Gameiro, P. A., Hiller, K., Danna, L. S., Balestrieri, C., Alberghina, L., Stephanopoulos, G., and Chiaradonna, F. (2011) Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. *Mol. Syst. Biol.* **7**, 523
- Le, A., Lane, A. N., Hamaker, M., Bose, S., Gouw, A., Barbi, J., Tsukamoto, T., Rojas, C. J., Slusher, B. S., Zhang, H., Zimmerman, L. J., Liebler, D. C., Slebos, R. J., Lorkiewicz, P. K., Higashi, R. M., Fan, T. W., and Dang, C. V. (2012) Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab.* **15**, 110–121
- Gao, P., Tchernyshyov, I., Chang, T. C., Lee, Y. S., Kita, K., Ochi, T., Zeller, K. I., De Marzo, A. M., Van Eyk, J. E., Mendell, J. T., and Dang, C. V. (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* **458**, 762–765
- Wang, J. B., Erickson, J. W., Fuji, R., Ramachandran, S., Gao, P., Dinavahi, R., Wilson, K. F., Ambrosio, A. L., Dias, S. M., Dang, C. V., and Cerione, R. A. (2010) Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* **18**, 207–219
- Cassago, A., Ferreira, A. P., Ferreira, I. M., Fornezari, C., Gomes, E. R., Greene, K. S., Pereira, H. M., Garratt, R. C., Dias, S. M., and Ambrosio, A. L. (2012) Mitochondrial localization and structure-based phosphate activation mechanism of glutaminase C with implications for cancer metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1092–1097
- Robinson, M. M., McBryant, S. J., Tsukamoto, T., Rojas, C., Ferraris, D. V., Hamilton, S. K., Hansen, J. C., and Curthoys, N. P. (2007) Novel mechanism of inhibition of rat kidney-type glutaminase by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES). *Biochem. J.* **406**, 407–414
- DeLaBarre, B., Gross, S., Fang, C., Gao, Y., Jha, A., Jiang, F., Song, J. J., Wei, W., and Hurov, J. B. (2011) Full-length human glutaminase in complex with an allosteric inhibitor. *Biochemistry* **50**, 10764–10770
- Thangavelu, K., Pan, C. Q., Karlberg, T., Balaji, G., Uttamchandani, M., Suresh, V., Schüler, H., Low, B. C., and Sivaraman, J. (2012) Structural basis for the allosteric inhibitory mechanism of human kidney-type glutaminase (KGA) and its regulation by Raf-Mek-Erk signaling in cancer cell metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 7705–7710
- Katt, W. P., Ramachandran, S., Erickson, J. W., and Cerione, R. A. (2012) Dibenzophenanthridines as inhibitors of glutaminase C and cancer cell proliferation. *Mol. Cancer Ther.* **11**, 1269–1278
- Vander Heiden, M. G. (2011) Targeting cancer metabolism. A therapeutic window opens. *Nat. Rev. Drug. Discov.* **10**, 671–684
- Jones, N. P., and Schulze, A. (2012) Targeting cancer metabolism. Aiming at a tumour's sweet-spot. *Drug Discov. Today* **17**, 232–241
- Kenny, J., Bao, Y., Hamm, B., Taylor, L., Toth, A., Wagers, B., and Curthoys, N. P. (2003) Bacterial expression, purification, and characterization of rat kidney-type mitochondrial glutaminase. *Protein Expr. Purif.* **31**, 140–148
- Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets proteins complexes and co-regulates major cellular functions. *Science* **325**, 834–840
- Leslie, A. G. (1992) Recent changes to the MOSFLM package for processing film and image plate data. *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography* **26**, 27–33
- Evans, P. (2006) Scaling and assessment of data quality. *Acta Crystallogr. D* **62**, 72–82
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser Crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674
- Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D* **66**, 213–221
- Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D* **66**, 486–501
- Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D* **66**, 12–21
- Aragão, A. Z., Nogueira, M. L., Granato, D. C., Simabuco, F. M., Honorato, R. V., Hoffman, Z., Yokoo, S., Laurindo, F. R., Squina, F. M., Zeri, A. C., Oliveira, P. S., Sherman, N. E., and Paes Leme, A. F. (2012) Identification of novel interaction between ADAM17 (a disintegrin and metalloprotease 17) and thioredoxin-1. *J. Biol. Chem.* **287**, 43071–43082
- Yuneva, M. O., Fan, T. W., Allen, T. D., Higashi, R. M., Ferraris, D. V., Tsukamoto, T., Matés, J. M., Alonso, F. J., Wang, C., Seo, Y., Chen, X., and Bishop, J. M. (2012) The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type. *Cell Metab.* **15**, 157–170
- Campos-Sandoval, J. A., López de la Oliva, A. R., Lobo, C., Segura, J. A., Matés, J. M., Alonso, F. J., and Márquez, J. (2007) Expression of functional human glutaminase in baculovirus system. Affinity purification, kinetic and molecular characterization. *Int. J. Biochem. Cell Biol.* **39**, 765–773
- Gorman, M. W., He, M. X., Hall, C. S., and Sparks, H. V. (1997) Inorganic phosphate as regulator of adenosine formation in isolated guinea pig hearts. *Am. J. Physiol.* **272**, H913–H920
- Read, R. J., and Schierbeek, A. J. (1988) A phased translation function. *J. Appl. Crystallogr.* **21**, 490–495, DOI 10.1107/S002188988800562X
- Van Heel, M., Portugal, R., Rohou, A., Linnemayr, C., Bebeacua, C., Schmidt, R., Grant, T. R., and Schatz, M. (2012) *Four-dimensional Cryo Electron Microscopy at Quasi Atomic Resolution. IMAGIC 4D in International Tables for Crystallography Volume F. Crystallography of biological macromolecules* (Arnold, E., Himmel, D. M., and Rossmann, M. G., eds) 2nd edition, pp. 624–628, John Wiley & Sons, Inc., Hoboken, NJ
- Godfrey, S., Kuhlenschmidt, T., Curthoys, N. P. (1977) Correlation between activation and dimer formation of rat renal phosphate dependent glutaminase. *J. Biol. Chem.* **252**, 1927–1931
- Morehouse, R. F., and Curthoys, N. P. (1981) Properties of rat renal phosphate-dependent glutaminase coupled to Sepharose. Evidence that dimerization is essential for activation. *Biochem. J.* **193**, 709–716
- Olsen, B. R., Svenneby, G., Kvamme, E., Tveit, B., and Eskeland, T. (1970) Formation and ultrastructure of enzymically active polymers of pig renal glutaminase. *J. Mol. Biol.* **52**, 239–245
- Olsen, B. R., Torgner, I. A., Christensen, T. B., and Kvamme, E. (1973) Ultrastructure of pig renal glutaminase. Evidence for conformational changes during polymer formation. *J. Mol. Biol.* **74**, 239–251
- Curthoys, N. P., Kuhlenschmidt, T., and Godfrey, S. S. (1976) Regulation of renal ammoniogenesis, purification and characterization of phosphate-dependent glutaminase from rat kidney. *Arch. Biochem. Biophys.* **174**, 82–89, DOI 10.1016/0003-9861(76)90326-X
- Josephs, R., and Borisy, G. (1972) Self-assembly of glutamic dehydrogenase into ordered superstructures. Multichain tubes formed by association of single molecules. *J. Mol. Biol.* **65**, 127–155
- Curthoys, N. P., and Watford, M. (1995) Regulation of glutaminase activity and glutamine metabolism. *Annu. Rev. Nutr.* **15**, 133–159
- Fan, T. W., Higashi, R. M., and Macdonald, J. M. (1991) Emergence and recovery response of phosphate metabolites and intracellular pH in intact *Mytilus edulis* as examined *in situ* by *in vivo* ³¹P-NMR. *Biochim. Biophys. Acta* **1092**, 39–47

Allele-Specific Reprogramming of Cancer Metabolism by the Long Non-coding RNA CCAT2

Roxana S. Redis,¹ Luz E. Vela,² Weiqin Lu,^{3,4} Juliana Ferreira de Oliveira,⁵ Cristina Ivan,⁶ Cristian Rodriguez-Aguayo,¹ Douglas Adamoski,⁵ Barbara Pasculli,¹ Ayumu Taguchi,³ Yunyun Chen,⁸ Agustin F. Fernandez,⁹ Luis Valledor,¹⁰ Katrien Van Roosbroeck,¹ Samuel Chang,¹ Maitri Shah,¹ Garrett Kinnebrew,¹¹ Leng Han,¹² Yaser Atlasi,^{13,21} Lawrence H. Cheung,¹ Gilbert Y. Huang,¹ Paloma Monroig,¹ Marc S. Ramirez,¹⁴ Tina Catela Ivkovic,^{1,15} Long Van,² Hui Ling,¹ Roberta Gafà,¹⁶ Sanja Kapitanovic,¹⁵ Giovanni Lanza,¹⁶ James A. Bankson,¹⁴ Peng Huang,³ Stephan Y. Lai,⁸ Robert C. Bast,¹ Michael G. Rosenblum,¹ Milan Radovich,¹¹ Mircea Ivan,¹¹ Geoffrey Bartholomeusz,¹ Han Liang,¹⁷ Mario F. Fraga,¹⁸ William R. Widger,² Samir Hanash,⁷ Ioana Berindan-Neagoe,^{1,19,20} Gabriel Lopez-Berestein,^{1,6} Andre L.B. Ambrosio,⁵ Sandra M. Gomes Dias,⁵ and George A. Calin^{1,6,*}

¹Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

²Department of Biology & Biochemistry, University of Houston, Houston, TX 77204, USA

³Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

⁴Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

⁵Laboratório Nacional de Biociências, Centro Nacional de Pesquisa em Energia e Materiais, Campinas 13083-100, Brazil

⁶Center for RNA Interference and Non-coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

⁷Department of Clinical Cancer Prevention, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

⁸Department of Head & Neck Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

⁹Cancer Epigenetics Laboratory, Institute of Oncology of Asturias (IUOPA), HUCA, Universidad de Oviedo, Oviedo 33006, Spain

¹⁰Department of Organisms and Systems Biology, University of Oviedo, Oviedo 33006, Spain

¹¹Department of Surgery, Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, USA

¹²Department of Biochemistry and Molecular Biology, The University of Texas Health Science Center at Houston McGovern Medical School, Houston, TX 77030, USA

¹³Department of Pathology, Josephine Nefkens Institute, Erasmus Medical Center, Rotterdam 3015, the Netherlands

¹⁴Department of Imaging Physics, Division of Diagnostic Imaging, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

¹⁵Laboratory for Personalized Medicine, Division of Molecular Medicine, Ruder Boskovic Institute, Zagreb 10000, Croatia

¹⁶Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara 44121, Italy

¹⁷Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

¹⁸Nanomaterials and Nanotechnology Research Center (CINN-CSIC), Asturias 33424, Spain

¹⁹Research Center for Functional Genomics, Biomedicine and Translational Medicine, University of Medicine and Pharmacy Iuliu Hatieganu, Cluj-Napoca 400012, Romania

²⁰Department of Functional Genomics, The Oncology Institute, Cluj-Napoca 400015, Romania

²¹Present address: Radboud Institute for Molecular Life Sciences (RIMLS) Department of Molecular Biology, Nijmegen 6525, the Netherlands

*Correspondence: gcalin@mdanderson.org

<http://dx.doi.org/10.1016/j.molcel.2016.01.015>

SUMMARY

Altered energy metabolism is a cancer hallmark as malignant cells tailor their metabolic pathways to meet their energy requirements. Glucose and glutamine are the major nutrients that fuel cellular metabolism, and the pathways utilizing these nutrients are often altered in cancer. Here, we show that the long ncRNA CCAT2, located at the 8q24 amplicon on cancer risk-associated rs6983267 SNP, regulates cancer metabolism in vitro and in vivo in an allele-specific manner by binding the Cleavage Factor I (CFIm) complex with distinct affinities for the two subunits (CFIm25 and CFIm68). The CCAT2 interaction with the CFIm complex fine-tunes the alternative splicing of *Glutaminase* (GLS) by selecting the poly(A) site in intron 14 of the precursor mRNA. These findings uncover a complex, allele-specific regulatory

mechanism of cancer metabolism orchestrated by the two alleles of a long ncRNA.

INTRODUCTION

Long non-coding RNAs (lncRNAs) form the largest part of the mammalian non-coding transcriptome (Mercer et al., 2009) and are generally expressed in a developmental-, tissue-, or disease-stage-specific manner, which makes them attractive therapeutic targets (Ling et al., 2013a). Although the underlying molecular mechanisms are not yet entirely understood, lncRNAs control gene expression at various levels including chromatin modification and transcriptional and post-transcriptional processing (Wilusz et al., 2009).

The revival of Warburg's theory of cancer (Warburg et al., 1924), complemented with novel discoveries in the field, has promoted cellular metabolism as an essential molecular mechanism for driving malignant transformation and progression (Boroughs

and DeBerardinis, 2015). Various studies have exposed the fine interplay between metabolic pathways orchestrated by protein-coding oncogenes and tumor suppressor genes (Chen and Russo, 2012) and, more recently, by ncRNAs (miRNAs and lncRNAs) (Gao et al., 2009; Yang et al., 2014). Glutamine, one of the essential nutrients, is deaminated by *Glutaminase* (GLS) to produce glutamate, which further serves as substrate for a variety of metabolic pathways (e.g., tricarboxylic cycle, TCA). Glutamine metabolism is modulated by MYC via miR-23a/b in prostate cancer and B cell lymphoma (Gao et al., 2009) and by NF- κ B p65 subunit also through miR-23a downregulation in leukemic cells (Rathore et al., 2012; Wang et al., 2010b).

Colon Cancer-Associated Transcript 2 (CCAT2), a lncRNA that spans the highly conserved 8q24 region harboring the rs6983267 SNP (Ling et al., 2013b; Redis et al., 2013), is associated with increased risk for various types of cancer (Tomlinson et al., 2007; Tuupanen et al., 2009) and is specifically overexpressed in the microsatellite stable colorectal cancer (CRC MSS). The two alleles of the rs6983267 SNP present in the general population have been shown to render distinct risks of CRC; namely, the G allele was associated with greater predisposition to CRC than the T allele (Tomlinson et al., 2007). CCAT2 induces chromosomal instability and metastases (Ling et al., 2013b) and regulates the expression levels of MYC oncogene, known to coordinate multiple molecular pathways supporting cell proliferation, metastases and cancer metabolism (Carroll et al., 2015; Stine et al., 2015). However, it is not clear how the two alleles are specifically involved in the malignant process. In this study, we demonstrate that the lncRNA, CCAT2, modulates cellular energy metabolism in an allele-specific manner by interacting with the Cleavage Factor I (CFIm) complex to regulate the alternative splicing of *GLS*.

RESULTS

CCAT2 Modulates Energy Metabolism in an Allele-Specific Manner In Vitro and In Vivo

We observed an unexpected change in the color of the media of in vitro grown cells when modulating the expression of CCAT2 that suggested a possible shift in the energy metabolism consequent to CCAT2 expression. We tested this hypothesis by measuring metabolic parameters in HCT116 colon cancer cells that stably overexpress CCAT2 (OC1 and OC3) (Ling et al., 2013b) versus control cells and observed a significant and reproducible increase in glucose uptake, lactate secretion and oxygen consumption in the CCAT2-overexpressing clones (Figure 1A). These results were further confirmed in KM12SM cells with CCAT2 downregulated expression (Figure 1B). Moreover, we explored whether these metabolic changes were occurring in vivo as well by injecting HCT116 CCAT2-overexpressing cells and control cells subcutaneously into nude mice and subjecting them to hyperpolarized magnetic resonance imaging (HP-MRI). We detected a significant increase in the flux of hyperpolarized [^{1-13}C]pyruvate to [^{1-13}C]lactate for the xenograft tumors derived from the CCAT2-overexpressing cells compared to the tumors derived from control cells (Figures S1A and S1B), consistent with our in vitro findings. These findings confirm that CCAT2 alters metabolism, boosting glycolysis and cellular respiration.

The coexistence of increased glycolysis with increased respiration, in highly proliferative cells, translates into enhanced anaplerotic reactions that replenish the TCA cycle intermediates (Ward and Thompson, 2012). Since glutamine is the main source for replenishing the intermediates of the TCA cycle, we measured the intra- and extracellular glutamate concentration, as well as the glutamine uptake in HCT116 cells with CCAT2 overexpression and control cells. We found higher levels of both intra- and extracellular glutamate corresponding to higher levels of CCAT2 (Figures S1C and S1D), suggesting CCAT2 is boosting glutamine metabolism (glutaminolysis). Surprisingly, the glutamine uptake was not significantly different between the three clones (Figure S1D), implying the higher glutamate is not due to increased glutamine consumption. Therefore, we measured the enzymatic activity of GLS, the rate-limiting enzyme of glutaminolysis, in the whole lysate of the same cells and detected significantly higher activity in the cells with increased CCAT2 expression (Figure S1F). In addition, both metabolic pathways (glycolysis and glutaminolysis) have been shown to be regulated by many factors, including the MYC oncogene (Carroll et al., 2015; Stine et al., 2015), a target of CCAT2 by our previous report (Ling et al., 2013b).

We next explored whether the rs6983267 SNP influences these metabolic changes and assessed the glucose and glutamine uptake, oxygen consumption, lactate secretion, and intra- and extracellular glutamate concentration in HCT116 stably overexpressing either the G or the T allele of CCAT2 and control HCT116 cells. Interestingly, we found on one hand higher glucose uptake and secreted glutamate in both G and T allele cells compared to control cells, while on the other hand we observed significant differences in lactate secretion, oxygen consumption, and intracellular glutamate production between the alleles (Figures 1C and S1E). Moreover, the glutamine consumption was not significantly different between the clones, similar to our previous results (Figure S1E). Consequently, we measured GLS enzymatic activity in these cells and observed that both CCAT2 alleles induced a remarkable increase in activity compared to control, but the cells overexpressing the G allele displayed a significantly higher enzymatic activity compared to the T allele-overexpressing cells (Figure 1D). We also analyzed by mass spectroscopy the metabolites obtained from in vitro culturing of the HCT116 CCAT2 G or T allele and control cells and from in vivo xenografted tumors derived from subcutaneous injection of the same cells. We observed contrasting distribution patterns when performing partial least squares discriminant analysis (PLS-DA) for both the in vitro (Figure 1E) and in vivo analyses (Figure 1F), and similarly for the principal component analysis (PCA) (Figures S1G and S1H). We detected 85.04% (in vitro) and 59.55% (in vivo) of metabolic pathways upregulated by CCAT2 G allele compared to the T allele (Figure S1I, Table S1B). We then compared the pathway analysis for both datasets and identified 40 common pathways for the G allele and five common pathways for the T allele (Figure S1J). For these pathways, metabolic cluster distribution of differentially accumulated compounds revealed a significant overall enhancement of metabolic pathways related to glucose metabolism, TCA cycle, and glutamine metabolism for the G allele cells compared to the T allele cells (Tables S1A and S1B). We evaluated the expression

Figure 1. CCAT2 Regulates Cancer Metabolism In Vitro and In Vivo

(A) Glucose uptake, lactate production, and oxygen consumption assays in HCT116 stable clones (E: empty control vector; OC1 and OC3: CCAT2-overexpressing, GG genotype).

(B) Glucose uptake, lactate production, and oxygen consumption assays in KM12SM cells with CCAT2 downregulation (GT genotype).

(C) Whole-cell lysate *Glutaminase* activity measured in HCT116 CCAT2-overexpressing G or T allele and control cells.

(D) Partial least-squares discriminant analysis (sPLS-DA) of HCT116 cells stably overexpressing CCAT2 with either G or T alleles, and control cells (E, empty vector) in vitro allowed an adequate classification of the different cell lines according to its metabolome. sPLS-DA algorithm allows the classification of the samples based on the different abundances of each metabolite trying to find the maximum covariance between treatments and metabolome, in this way finding the most important metabolites for explaining the different effects of the treatments.

(E) Xenograft tumors derived from the same cell lines were also correctly classified by sPLS-DA analysis.

Results are presented as normalized mean values $\pm$ SD. See also Figure S1 and Table S1.

of MYC between the HCT116 CCAT2 G and T allele cells but did not find impressive differences, consistent with our previous findings (Ling et al., 2013b) (Figure S2A). These results imply that in cells with high CCAT2 expression, MYC activates energy metabolism in a general fashion; however, the fine-tuning of distinct metabolic pathways may occur through MYC-independent, but SNP-dependent, mechanisms. We therefore decided

to direct our efforts toward exploring the CCAT2 MYC-independent mechanism of regulation.

CCAT2 Regulates the Expression of GLS Isoforms

Our group has previously shown that CCAT2 induces chromosomal instability, a process highly reliant on the supply of nucleotides (Bester et al., 2011) and intimately linked to glutamine

metabolism (Jeong et al., 2013). Further supported by the metabolic data, we directed our focus toward pathways metabolizing glutamine. We first assessed the protein expression of the two alternative splicing isoforms of GLS, KGA (glutaminase kidney isoform) and GAC (glutaminase isoform C) as a function of CCAT2. We used specific antibodies for each isoform (recognizing the distinct C termini) (Cassago et al., 2012) and/or a common antibody, recognizing the N terminus shared by the two isoforms, depending on the cell line. Although the two isoforms share the same active site, GAC has a higher catalytic activity than KGA and therefore may be more relevant for replenishing intermediates of the TCA cycle (Cassago et al., 2012; Le et al., 2012). While for GAC we observed an increase in protein expression in the HCT116 CCAT2-overexpressing cells with both antibodies, for KGA the protein expression presented an inconsistent variation with CCAT2 upregulation (Figures 2A, S2B, and S2C). Analogously, the mRNA expression pattern for GAC and KGA reflected the protein expression (Figure 2B). Moreover, downregulation of CCAT2 in KM12SM cells reduced GAC protein expression with 34% and 26%, respectively (Figures 2C, S2D, and S2E), whereas KGA protein expression was either unaltered or slightly increased (Figures 2C, S2D, and S2E). Similar results were obtained when measuring the mRNA expression of the two isoforms in the same cellular model (Figure S2F). Interestingly, when we evaluated the expression of the two isoforms in the HCT116 cells overexpressing the CCAT2 G or T allele and control cells at both the mRNA and protein levels, we found unanimously higher expression of GAC when CCAT2 G allele is upregulated (Figures 2D and S2G–S2I). However, changes in the KGA mRNA expression pattern did not concur with the protein expression (Figures 2D and S2G–S2I). The discordance observed for the KGA isoform is probably due to the regulation by the MYC-miR-23 axis (Gao et al., 2009; additional data available from authors upon request). These results alluded to the idea that CCAT2 may preferentially induce the splicing of the GAC isoform. To determine this, we cloned the intron 14 of GLS precursor mRNA, known to encompass the alternative splicing site, in the RG6 bichromatic fluorescent reporter (Orengo et al., 2006). If the splicing machinery binds to the intron 14, it induces the splicing of a EGFP-tagged protein, which is the equivalent of GAC; otherwise, a dsRED-tagged protein will be produced, which is the equivalent of KGA (Figure S2J). We transfected the HCT116 control and CCAT2-overexpressing cells with the fluorescent reporter and determined the ratio between the expression levels of EGFP and dsRED fluorescence using the VECTRA automated imaging system. Although the EGFP-tagged protein, GAC-equivalent, was predominant in all models, we observed a 50% significantly higher EGFP/dsRED ratio in the CCAT2-overexpressing cells, corresponding to 50% more alternative splicing events (Figure 2E). Similar results were obtained when cells were analyzed by flow cytometry (Table S2). Moreover, when we compared the alternative splicing events occurring in the G and T alleles, using the RG6 reporter, we found significantly higher EGFP/dsRED ratio for the CCAT2 G allele compared to the CCAT2 T allele (Figure 2F and Table S2). Altogether, these data demonstrate that the CCAT2 G allele is more efficient in boosting the alternative splicing of GAC isoform.

CFIm25 Governs the Switch between GLS Splicing Isoforms

To elucidate the underlying mechanism of CCAT2-induced regulation of the GLS alternative splicing, we introduced the MS2 tag (24 repeats) into vectors containing either the CCAT2 G allele or T allele, pulled down the proteins that bind CCAT2, and analyzed them by mass spectrometry (Yoon et al., 2012). Pathway analysis on the QIAGEN platform identified Cleavage and Polyadenylation of Pre-mRNA among the top pathways associated with the G allele (Figure S3A), with CFIm25, the small (25 kDa) subunit of CFIm encoded by the *NUDT21* gene (Elkon et al., 2013; Yang et al., 2011), as the main protein correlated with the pathway. Notably, in the T allele pull down, both CFIm25 and the larger subunit (68 kDa) of the CFIm complex, CFIm68 (encoded by the *CPSF6* gene), were detected (CFIm68 was not detected in the G allele pull down); however, for CFIm25, the area under the peak had a 1.48 higher fold change in the G allele compared to the T allele (Figure S3B). We screened the GLS intron 14 for potential splicing and/or alternative polyadenylation (APA) sites using ASTRA (Alternative Splicing and TRanscription Archives database; <http://dbarchive.biosciencedbc.jp/en/astra/desc.html>) (Nagasaki et al., 2006) and identified a type 2 (skipping exon) poly(A) site (Figure S3C) (Lutz and Moreira, 2011) and multiple conserved binding motifs (UGUA) for CFIm25, consistent with a previous report mapping two poly(A) motifs within the same intron of the GLS pre-mRNA (Tian et al., 2007). Considering that CFIm25 and the CFIm heterotrimer complex have been previously linked to alternative splicing (Millevoi et al., 2006; Zhou et al., 2002), we first downregulated *NUDT21* and assessed the protein levels of GAC and KGA. We noticed a significant decrease in GAC protein expression in both cellular models and a clear increase in the KGA protein expression (Figure 3A). We also measured mRNA expression for the two isoforms and observed a significant reduction of the GAC/KGA mRNA ratio with *NUDT21* knockdown (Figure 3B). In order to evaluate whether the switch in isoform expression is a consequence of CFIm25 binding to the UGUA sequences within intron 14 of GLS pre-mRNA, we designed antisense synthetic oligonucleotides (ASOs) to block the binding of the 25 kDa subunit to these motifs (Figure 3C). Out of the four tested ASOs, we could identify two that were able to reverse the GAC to KGA protein expression ratio similar to the specific downregulation of *NUDT21* in HCT116 OC1 cells (Figure 3C). This result suggested that binding of CFIm25 to intron 14 is responsible for inducing the preferential expression of GAC. To further confirm the direct interaction of CFIm25 with GLS pre-mRNA and CCAT2, we immunoprecipitated the RNA bound to constituting proteins of the CFIm complex, CFIm25 and CFIm68, in HCT116 cells overexpressing either the G or T allele of CCAT2 and control cells, and measured the differences in RNA enrichment between the distinct pull-down lysates by real-time quantitative PCR (qRT-PCR) (Figures 3D–3F and S3D–S3F). We included two lncRNAs as controls: *NEAT1* was chosen to be a positive control as it has been previously shown to interact with the CFIm complex (Naganuma et al., 2012), and *GAS5* was chosen as negative control due to minimal sequence similarity with CCAT2 (Figure S3G). When assessing the fold enrichment of GLS and CCAT2, we detected, respectively, 5.77 and 13.6 times more RNA bound to CFIm25 in the

Figure 2. CCAT2 Induces the Preferential Splicing of GAC

(A) Western blot analysis of GAC, KGA in HCT116 CCAT2-overexpressing cells (OC1 and OC3) and control cells.
 (B) qRT-PCR assessing the mRNA expression of GAC and KGA in HCT116 CCAT2-overexpressing cells (OC1 and OC3) and control cells.
 (C) Western blot analysis of GAC, KGA in KM12SM cells with CCAT2 downregulation.
 (D) Western blot analysis of GAC, KGA in HCT116 stably overexpressing CCAT2 G or T alleles and control cells.
 (E) Fluorescence microscopy images of HCT116 stable clones (E, empty control vector; OC1 and OC3, CCAT2 overexpressing) transfected with the RG6 intron 14 vector and the analysis of the EGFP/dsRED ratio (n = number of analyzed cells).
 (F) Fluorescence microscopy images of HCT116 CCAT2 G allele and T allele transfected with the RG6 intron 14 vector and the analysis of the EGFP/dsRED ratio (n = number of analyzed cells).
 Results are presented as normalized mean values $\pm$ SD. See also Figure S2 and Table S2.

cells overexpressing CCAT2 G allele compared to control cells, while in the cells overexpressing CCAT2 T allele compared to control cells, the fold enrichment ratios were only about half (2.95 and 6.02, respectively) (Figure 3D). Moreover, comparing the fold enrichment in the G-overexpressing cells to the T-overexpressing cells, we observed roughly double fold enrichment in

the G-overexpressing cells for both GLS and CCAT2 (1.95 and 2.26) (Figures 3E and 3F). The positive control (NEAT1) presented 6.3-fold more RNA bound to CFIm25 in the cells overexpressing CCAT2 G allele compared to control cells and 2.79-fold more RNA enrichment in the cells overexpressing CCAT2 T allele compared to control cells (Figures 3D and S3D). The negative

Figure 3. CFIm Protein Complex Binds GLS Pre-mRNA

(A) Western blot analysis of CFIm25, GAC, and KGA in HCT116 OC1 and KM12SM cells transiently transfected with siRNA for NUDT21, GLS (targeting the coding sequence shared by the two isoforms), and siRNA control.

(B) qRT-PCR assessing the GAC/KGA mRNA ratio in HCT116 CCAT2-overexpressing cells (OC1 and OC3) and control cells (E) with modulated CFIm25 expression.

(C) Western blot analysis of GAC and KGA in HCT116 OC1 cells with transient blockage of CFIm25 binding motifs (UGUA) by antisense oligonucleotides (ASOs). Schematic representation of the mechanism is presented below.

(D) qRT-PCR assessing the fold enrichment of GLS, CCAT2, NEAT1, and GAS5 RNA bound to CFIm25 protein (RNA immunoprecipitation). Data are presented as fold enrichment ratios between control HCT116 cells (E) and CCAT2-overexpressing G or T allele.

(E and F) qRT-PCR assessing the fold enrichment of GLS mRNA (E) and CCAT2 (F) bound to CFIm25 and CFIm68 in HCT116 cells, CCAT2-overexpressing G or T alleles, and control cells (E).

(G) Fluorescence microscopy images of KM12SM cells transfected with siNUDT21 and scr, followed by transfection with the RG6 intron 14 vector, and the analysis of the EGFP/dsRED ratio (n = number of analyzed cells).

Results are presented as normalized mean values $\pm$ SD. See also Figure S3 and Table S2.

control (GAS5) revealed only a 1.79-fold increase in the RNA bound to CFIm25 in the cells overexpressing CCAT2 G allele compared to control cells and no difference in RNA enrichment between the overexpressing CCAT2 T allele and control cells (Figures 3D and S3E). Thus, we concluded that, overall, in the cells overexpressing CCAT2, there is an augmentation of interaction between CFIm25 and GLS and CCAT2, with the highest levels in the G-overexpressing cells. The low binding of the RNAs to the CFIm68 was not surprising, as the main function of the protein is merely to enhance RNA binding and facilitate RNA looping, while the 25 kDa subunit has the leading role in binding the RNA via the UGUA elements (Yang et al., 2011) (Figures 3E, 3F, S3D, and S3E). Nonetheless, for our RNAs of interest, we observed an increase in enrichment in the T allele overexpressing cells compared to the G allele overexpressing and control cells, suggesting the secondary structure of CCAT2 T allele may ease the interaction between the 68 kDa subunit and RNA molecules. In addition, we confirmed these results by end-point PCR in both the same cellular model and the KM12SM cell line, with a GT-heterozygous genotype (Figures S4A and S4B). As a further validation, we expressed the RG6 bichromatic fluorescent reporter with the intron 14 in KM12SM cells with knockdown of *NUDT21*. We assessed, by both VECTRA and FACS, the ratio of EGFP to dsRED and found a significantly lower (28%) EGFP/dsRED ratio in the cells with *NUDT21* downregulation, corresponding to fewer splicing events and consequently lower expression of the GAC equivalent (Figure 3G and Table S2).

Alternative Splicing of GLS Is Associated with the Interaction between CCAT2 and the CFIm Complex

We next aimed to investigate in more detail the mechanism leading to the difference in binding affinity of CFIm25 and CFIm68 to the distinct CCAT2 alleles. We first scanned the CCAT2 RNA sequence and identified two CFIm25 binding motifs surrounding the rs6983267, one upstream and the other downstream of the SNP. We performed secondary structure predictions using the RNAfold Webserver (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) and noticed major local structural changes induced near the putative upstream CFIm25 binding sequence, by the single nucleotide variation, especially among the G and T alleles (Figure S4C). Such changes may plausibly translate into distinct tertiary folds that could in principle explain the binding of the G and T alleles with different affinities. To further validate our results and evaluate the contribution of a single nucleotide to the structural changes and binding affinity, we mutated the SNP into an A or C (nucleotides never detected in the human population) and also deleted the 7 nucleotide region encompassing the SNP, which has been reported to have enhancer activity (Tuupainen et al., 2009). We repeated the RNA pull-down assay using different vectors containing the MS2 tags (CCAT2-G, -T, -A, -C, and DEL) and analyzed by western blot the proteins bound to RNAs. When CFIm25 antibody was hybridized on the blot, we detected a strong band of approximately 64 kDa unique to the G allele (band corresponds to the CFIm25 dimer; Figure 4A). When CFIm68 antibody was hybridized on the blot, we distinguished bands of approximately 68 kDa for both the T allele and the allele with the deleted region

(Figure 4A). This implied that the T allele preferentially binds the 68 kDa subunit, but not in the region of the SNP. Notably, neither of the two mutated CCAT2 alleles (A and C) interacted with the CFIm complex, and interestingly, both present secondary structures different from the G and T alleles (Figure S4C).

Additional evidence for the direct interaction between the CCAT2 G and T alleles with the CFIm complex was provided by a His₆-tag pull-down assay using heterologously expressed CFIm68:CFIm25 complex (His₆-tagged CFIm68 subunit; Figure 4B) incubated with in vitro synthesized RNAs. We detected strong affinity of the CFIm complex for the CCAT2 G allele, followed by moderate binding of the CCAT2 T allele. We also identified in the pull down the 600 nt long region of the GLS pre-mRNA intron 14, containing one type 2 poly(A) site, with affinities for the protein complex corresponding to the two alleles (Figure 4B). This suggested that the intron 14 may also interact with CCAT2. To test this, we added a biotin tag to in vitro transcribed CCAT2 RNAs, combined them with the intron 14 fragment and/or CFIm complex, and pulled down the complex with Streptavidin beads. We confirmed not only that the CCAT2 G allele preferentially binds CFIm25, but also that CCAT2 interacts with the intron 14 fragment in a SNP-independent fashion (Figures 4C and S4D). To ensure the specificity of the interaction, we repeated the biotin RNA pull-down assay to include the biotinylated CCAT2 C allele as a negative control. We also added the whole intron 14, previously used for the RG6 splicing assay, to determine if CCAT2 can interact with the entire region. We prepared mixes of CCAT2 (G, T, and C) and the CFIm complex with and without the intron 14 fragment to evaluate how it impacts the interaction between CCAT2 and CFIm complex. We discovered that in the presence of the intron 14, CCAT2 G and T alleles displayed increased binding affinity to the CFIm complex, compared to the C allele (Figures 4D and S4E and Table S3). The G and T alleles presented a remarkable specificity for the CFIm25 dimer in the presence of the intron 14 (both the smaller fragment and the whole intron) with enhanced binding to the G allele (Figure S4E and Table S3), supporting the hypothesis that the secondary structure of CCAT2 influences the interaction with the protein complex. In the absence of the intron, although the specificity of the interaction with the CFIm25 dimer is partly retained, it appeared to be revoked in the case of CFIm complex (Figures 4D and S4E and Table S3). We confirmed that CCAT2 G and T alleles can bind the whole intron 14 as well and aligned the CCAT2 genomic sequence with GLS genomic sequence to determine the extent of the interaction (Figure S4F). We observed multiple short fragments (13–18 nt) of sequence complementarity spanning the entire GLS sequence, present in both introns and exons (Figure S5A). For additional validation, we performed the His₆-tag pull-down assay using the G, T, and C alleles in the presence and absence of the whole intron 14. As expected, when the intron was included in the mix, we could detect only CCAT2 G and T alleles in the pull-down lysates (Figure S4G).

Furthermore, the same mixes of CCAT2 RNA (G/T), CFIm protein complex, and intron 14 RNA were prepared, as well as solutions of individual components, and subjected to atomic force microscopy (AFM) for visualizing the formation of the RNA:protein:RNA quaternary complex (Lyubchenko et al., 2011) (Figures

Figure 4. The rs6983267 SNP Affects the Interaction of CCAT2 with the CFIm Protein Complex

(A) Western blot analysis of the proteins pulled down with the MS2-CCAT2 vectors (G, T, A, C, and DEL) showing the presence of CFIm25 for the G allele and CFIm68 for the T allele.
 (B) Northern blot analysis showing the presence of CCAT2 and intron 14 (600 bp fragment) in the lysate pulled down with TALON resin (upper panel). Western blot analysis showing the presence of the His₆-tagged CFIm complex in the lysate pulled down with the TALON resin (lower panel).
 (C) Northern blot analysis showing the presence of CCAT2 and intron 14 (600 bp fragment) in the lysate pulled down with Streptavidin beads. Lane 1 marked with the star symbol is identical to lane 3 in Figure S4D. Schematic illustration of the interaction of CCAT2 with GLS pre-mRNA (representation is not at scale).
 (D) Northern blot analysis showing the presence of CCAT2 and intron 14 (600 bp fragment) in the lysate pulled down with Streptavidin beads (upper panel). Western blot analysis showing the presence CFIm25, monomer (26 kDa) and dimer (64 kDa), and His₆-tagged CFIm68 (38 kDa) (lower panel).
 (E and F) AFM images of CCAT2:CFIm:intron 14 quaternary complex including either CCAT2 T allele (E) or CCAT2 G allele (F).
 See also Figures S4 and S5 and Table S3.

4E and 4F). Particles of substantial size were detected when scanning the mica surface, suggesting the formation of potential CCAT2:CFIm:intron14 complexes, with higher frequency for the G allele compared to the T allele (Figures 4E and 4F), while the individual components (CFIm protein complex, CCAT2 RNA, and intron 14 RNA) appeared to be evidently smaller (Figures S5B–S5D). Moreover, when measuring the diameter of the complexes (G allele complex, 226.706 nm; T allele complex, 182.844 nm), we found that it corresponds approximately to the sum of the diameter of individual components (CCAT2, 81.865 nm; CFIm, 67.999 nm; intron 14, 48.360 nm).

Collectively, these findings suggested that: (1) GLS pre-mRNA impacts the interaction of CCAT2 with CFIm complex; (2) CCAT2 may be acting as a scaffold or assembly platform, promoting the selection of the poly(A) site in intron 14 of GLS pre-mRNA by

directly binding both the pre-mRNA and the CFIm complex; and (3) the formation of the RNA:RNA:protein complex is dependent on the rs6983267 SNP and the secondary structure of CCAT2.

As an additional layer of regulation, we discovered that MYC is a transcription factor for *NUDT21* (data available from authors upon request).

GAC Promotes Metastases and Cell Proliferation

We proceeded with evaluating the contribution of GAC to aggressiveness of CRC. Considering that we have previously demonstrated that CCAT2 promotes metastases (Ling et al., 2013b), we first assessed the in vitro migration potential of HCT116 CCAT2-overexpressing cells treated with either the allosteric inhibitor 968 or siRNA for GLS (Katt et al., 2012). We

Figure 5. GLS Promotes In Vivo Metastases and In Vitro Cell Proliferation and Migration

(A) Migration of HCT116 OC1 cells (GG genotype) treated with the inhibitor 968 and DMSO (left panel) and with siGLS and scrambled siRNA (right panel). (B) Migration of KM12SM cells (GT genotype) with stable downregulation of GAC. KM12SM shGFP cells represent the control cells. (C) Number of the lung micrometastases for two groups (shGFP, 4 mice; shGAC, 4 mice) assessed by IHC (left panel). IHC images showing micrometastases in the three groups and images showing the presence or absence of lung metastases for mice injected in the tail vein with KM12SM shGFP and shGAC cells, respectively (right panel). (D and E) Growth curves for HCT116 OC1 and control cells (GG genotype) treated with DMSO (control) or the GLS allosteric inhibitor 968 (10 μM) (D) and KM12SM cells (GT genotype) with stable downregulation of GAC (E). (F and G) Glutamine and glutamate concentration in the media relative to the empty well 24 hr after seeding HCT116 CCAT2-overexpressing cells (OC1, GG genotype) transfected with siRNA against *NUDT21* and scrambled (F) and ASOs for inhibiting the binding sites of CFIm25 (G). Results are presented as normalized mean values ± SD. See also Figure S6 and Table S4.

observed a reduction by half of the migration in the cells where GLS was either inhibited or downregulated (Figure 5A). We next assessed the individual contribution of GAC to the migration, in KM12SM cells with stable downregulation of the isoform (Figure S6A), and found that 60% fewer cells migrated when GAC expression was reduced (Figure 5B). We then injected the KM12SM stable clones in the tail vein of nude mice, sacrificed the mice 8 weeks after injections, and evaluated the in-lung macro- and micrometastases. Supporting our in vitro results, the incidence of metastases to the lung was 50% higher in the mice injected with the control cells (shGFP) compared to the shGAC group (Figure 5C). Moreover, when

we assessed the proliferation of HCT116 control and CCAT2-overexpressing cells treated with the GLS inhibitor 968, we found that cells overexpressing CCAT2 were more sensitive to GLS (GAC in this case) inhibition, implying that cells with high CCAT2 expression are dependent on GAC for survival (Figure 5D) (Katt et al., 2012; Wang et al., 2010a). Similar results were obtained when using KM12SM cells with GAC downregulation compared to control cells in vitro (Figure 5E). Additional confirmation of the higher dependency of the CCAT2 G allele on GAC was provided by the colony formation assay for NIH 3T3 cells transfected with the CCAT2-overexpressing vectors (Figures S6B and S6C).

We next sought to determine if the shift in *GLS* isoform expression and CFIm25 is responsible for the marked metabolic changes observed in cells overexpressing *CCAT2*. We therefore modulated the expression of the isoforms using the ASOs and downregulated *NUDT21* in HCT116 *CCAT2*-overexpressing cell line (OC1) and measured the extracellular lactate concentration and glutamine metabolism. We observed that downregulation of *NUDT21* significantly decreased lactate and glutamate secretion and glutamine consumption similarly to the effects of *CCAT2* overexpression in the same cell line (Figures S6D and 5F). However, these metabolic changes appeared to only partly mirror the metabolic effects of *CCAT2* overexpression, suggesting there are additional layers of regulations independent of *NUDT21*, most likely through the bona fide cancer metabolism modulator and *CCAT2* target, *MYC*. On the other hand, the switch in *GLS* isoforms reflected in lower secretion of glutamate with the glutamine consumption remaining relatively constant but had a modest effect on the extracellular levels of lactate (Figures 5G and S6E). These findings suggested that *GLS* is not the only contributor to *CCAT2*-induced metabolic profile. We hypothesized that other metabolic targets might be regulated by *CCAT2* via the same mechanism; thus, we performed Affymetrix HTA 2.0 array to compare the whole transcriptome splicing pattern in HCT116 *CCAT2*-overexpressing G and T allele cells. Pathway analysis revealed that several genes associated with two major metabolic pathways, “Metabolism of Carbohydrates” and “Fructose and Mannose Metabolism,” are significantly spliced between the *CCAT2* G and T alleles (Table S4). Moreover, validating our previous results, we identified a negative splicing index (SI = −1.19) for *GLS* for the exclusion junction connecting exon 14 and exon 15, suggesting higher signal for the exclusion junction in the T allele-expressing cells. This translates into less GAC being spliced in the T allele cells compared to the G allele cells, supportive of our findings (Figure S6F).

Collectively, these data demonstrate that GAC adds to the CRC phenotype; however, it is not solely responsible for the metabolic phenotype observed in cells overexpressing *CCAT2*.

CCAT2-CFIm-GLS Regulation Axis in CRC Tumors

We continued with evaluating the expression pattern of *CCAT2*, *GLS*, *NUDT21*, and *CPSF6* in colon tumors by analyzing the publicly available TCGA database of colon cancer (<http://cancergenome.nih.gov/>). We first compared 18 normal samples to 193 tumor samples and identified an enrichment of the GAC isoform, as well as *NUDT21* and *CPSF6* in tumor tissue, whereas *KGA* showed the opposite pattern (Figures 6A–6D). We further analyzed the associations between *CCAT2*, *NUDT21*, *CPSF6*, *GAC*, and *KGA* in the TCGA dataset of colon cancer samples (Cancer Genome Atlas Network, 2012) and detected direct correlations of *CCAT2*, *CPSF6*, and *NUDT21* with *GAC* ($r_s = 0.26$, $p = 0.0006$; $r_s = 0.68$, $p < 0.0001$; and $r_s = 0.72$, $p < 0.0001$, respectively; Figure S7A) and inverse correlations between *CCAT2*, *CPSF6*, *NUDT21*, and *KGA* ($r_s = -0.17$, $p = 0.0271$; $r_s = -0.47$, $p < 0.0001$; and $r_s = -0.60$, $p < 0.0001$; Figure S7A), as well as between *KGA* and *GAC* ($r_s = -0.590$, $p < 0.0001$) (Figure S7A). We also obtained significant direct associations of *CCAT2* with *CPSF6* and *NUDT21* ($r_s = 0.20$, $p = 0.0082$ and $r_s = 0.26$, $p =$

0.0007; Figure S7A). We did not find any significant correlations between the expression of *MYC* and *NUDT21*, *GAC*, or *KGA*. Since our in vitro findings advanced the concept that *CCAT2* G allele is promoting the expression of *GAC*, we compared the levels of *GAC* and *KGA* between patients having GG, GT, and TT genotypes. We observed a significant association with the genotypes for *GAC*, having the highest expression in patients with GG genotype, but not for *KGA* (Figures 6E and S7B). Similarly, we did not find any association of *NUDT21* or *MYC* with the genotypes (Figures S7C and S7D). These results suggested the molecular mechanism uncovered in vitro is present in CRC patients. Moreover, we analyzed the correlation between the expression of the two isoforms and the overall survival of patients from the TCGA dataset and observed a significant association of high *GAC* expression combined with low *KGA* expression with shorter overall survival (Figure S7E). This suggested that the *GAC* isoform may accelerate the progression of cancer. In addition, we interrogated the TCGA colon cancer dataset for genes that significantly correlate with the lncRNA and performed gene set enrichment analysis (GSEA) and ingenuity pathway analysis (IPA) (QIAGEN) to identify the *CCAT2* gene signatures. Various metabolically relevant pathways were found significantly associated with *CCAT2* expression by both analyses (Figures 7A and 7B). We further inquired as to whether the genes that were found significantly correlated with *CCAT2* expression were also associated with the genotypes of rs6983267 SNP. We repeated the GSEA and screened for pathways that correlated with either of the genotypes (FDR q value < 0.25 and nominal p value < 0.05). We identified various metabolic and RNA processing pathways that were associated with certain genotypes (Figure 7C). Interestingly, several of the pathways were related to the ones identified by the Affymetrix HTA 2.0 array (highlighted in green in Figure 7C), suggesting that *CCAT2* may modulate cellular metabolism in CRC patients through a network of metabolic genes regulated most likely via the same mechanism of alternative splicing (see Table S4).

To assess the protein expression of CFIm68, CFIm25, *GAC*, and *KGA*, we performed western blot analysis on paired normal mucosae and CRC samples (patient cohort #1) and identified the same high protein levels of CFIm25 and *GAC* in tumor tissue compared to normal mucosae for 61.5% (8/13) of the pairs (Figure 6F). As for the *KGA* isoform, protein levels were mostly lower in tumor samples or comparable to the levels in normal samples (Figure 6F). The 68 kDa subunit of the CFIm complex was either very low or not expressed in approximately half of the paired samples (7/13), while in the rest of the pairs it was clearly overexpressed in tumors (Figure S7F). We also measured *CCAT2* expression by qRT-PCR in the same set of samples and confirmed a positive correlation between *CCAT2*, CFIm25, and *GAC* for 69.2% of the samples (Figures 6F, 6G, S7G, and S7H). Moreover, the samples that had elevated levels of *CCAT2* displayed a high *GAC/KGA* ratio ($GAC/KGA = 2$ –5.7) (Figure S7I). We repeated the measurements in a second set of paired normal mucosae and CRC samples (patient cohort #2) and obtained similar results (Figures S7J and S7K). Both CFIm25 and *GAC* proteins were overexpressed in 60% (3/5) of tumor samples. In all samples, elevated protein levels of CFIm25 and *GAC* matched the increased RNA levels of

Figure 6. CCAT2, NUDT21, CPSF6, and GLS Expression Pattern in TCGA Dataset and CRC Patient Samples

(A–D) Analysis of *NUDT21* (A), *CPSF6* (B), *GAC* (C), and *KGA* (D) mRNA expression in TCGA RNA-seq colon cancer sample set. (E) Association of *GAC* mRNA expression with the genotypes (GG, GT, and TT) of the rs6983267 SNP for CRC patients (TCGA RNA-seq dataset). (F) Western blot analysis of CFIm25, *GAC*, and *KGA* expression in paired CRC samples (patient cohort #1). (G) qRT-PCR analysis for *CCAT2* in the same paired CRC samples (patient cohort #1). Results are presented as normalized mean values $\pm$ SD. See also Figure S7.

CCAT2. Additionally, we have genotyped the tumors from patient cohort #1 and found that the association of GG genotype with higher *GAC* and CFIm25 protein expression (samples marked in red) was consistent for 75% of patients (3/4), while for the GT genotype the association was present in only 62.5% of patients (5/8) (Figure 6G). No conclusion can be drawn for the TT genotype due to the limited representation of the genotype in this cohort (one patient). Altogether, this mechanism of *GLS* regulation was detected in the majority of the analyzed CRC cases (61%, 11/18).

DISCUSSION

Our study demonstrates that the rs6983267 SNP (G/T) induces changes in the secondary structure of the lncRNA, *CCAT2*, initiating a domino effect mechanism, which leads to allele-specific

reprogramming of cellular energy metabolism. The consequence of the allele-specific interaction between *CCAT2*, CFIm, and *GLS* pre-mRNA appears to be the selection of the poly(A) site within intron 14 of *GLS*, resulting in the preferential splicing to the *GAC* isoform, the more catalytically active of the two *GLS* isoforms (Cassago et al., 2012). Although a recent study has described the negative regulation of *GLS* by CFIm25 in glioblastoma via 3' UTR processing mechanisms (Masamha et al., 2014), suggesting a tumor-suppressive role for CFIm25, in our model, neither of the *GLS* isoforms is subjected to 3' UTR shortening (data not shown). In the context of these findings and considering that *CCAT2* is not expressed in glioblastoma (data not shown), our results reveal an intriguing aspect of lncRNA mechanism of action, namely the ability of an lncRNA to alter the function of the partner RNA-binding protein/complex. The enrichment on metabolites related to TCA cycle that we

Figure 7. CCAT2 Gene Signature in Colon Cancer Patients—TCGA Dataset

(A and B) Genes associated with CCAT2 were analyzed by gene set enrichment analysis (GSEA) (A) and ingenuity pathway analysis (IPA, QIAGEN) (B). Relevant examples for each analysis are presented in (A) and (B).

(C) Table containing the pathways significantly associated with CCAT2 expression and rs6983267 genotype (FDR q value < 0.25 and nominal p value < 0.05). Pathways that were positively correlated are marked with red, and the ones that are negatively correlated are marked with blue. Highlighted in green are the pathways found common between GSEA analysis and the Affymetrix HTA 2.0 pathways analysis.

See also Figure S7 and Table S4.

observed for tumors derived from HCT116 cells overexpressing CCAT2 the G allele are supported by Kaldma and colleagues' findings describing that CRC tumors are not purely glycolytic, but rather dependent on OXPHOS for ATP production (Kaldma et al., 2014).

The aberrant expression of GLS has been reported in many types of cancer, including CRC (Huang et al., 2014). Furthermore, various studies have described GAC as the more abundant isoform in lung adenocarcinoma, head and neck squamous

cell carcinoma, kidney renal clear cell carcinoma, and AML (Jacque et al., 2015; van den Heuvel et al., 2012; Xia et al., 2014). Glutamine metabolism has also been associated with genomic instability (Jeong et al., 2013), commonly encountered in CRC and previously shown to be promoted by CCAT2 (Ling et al., 2013b), and appears to be endorsed by the G risk allele. However, it must be stated that CCAT2 is modulating energy metabolism in a general fashion via MYC and in an allele-specific manner via GLS and other metabolic enzymes and/or

metabolites, whose expression is finely regulated by the interaction of *CCAT2* with CFIm. Although the differences in regulation between the G and T alleles may not be impressive, the variation in expression of multiple enzymes/metabolites may have an additive effect towards a clear phenotypical change. The complex mechanism presented in this manuscript encompassing lncRNA, protein complexes, oncogenes, and transcription factors opens several windows for targeted therapy. The metabolic enzyme *GLS* is already considered a therapeutic target for cancer (Vander Heiden, 2011); however, our work introduces the opportunity of targeting the cancer-specific GAC isoform in particular.

Lastly, our study reveals the complexity and refinement of the interaction networks among the alleles of a non-coding RNA, the components of a protein complex, and the splicing isoforms of a metabolic enzyme that contribute to the malignant transformation and progression of CRC.

EXPERIMENTAL PROCEDURES

Full details of the [Experimental Procedures](#) are presented in the [Supplemental Experimental Procedures](#). Primer sequences and information regarding the antibodies used in this study can be found in [Tables S5](#) and [S6](#).

Patient Samples

18 paired samples, normal colon mucosa and colon tumor, were used in this study. The samples were obtained from two different sources: The Ruder Boskovic Institute, Croatia (15 paired samples) and University of Ferrara, Italy (5 paired samples). Tissue samples were obtained from fresh surgical specimens frozen in liquid nitrogen and stored at -80°C . All the samples were obtained with the patients' informed consent and under the approval and supervision of the institutional review boards. The samples were histologically confirmed prior to use.

In Vivo Models and Tissue Processing

70 male athymic nude mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD) and were cared for according to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Glucose Uptake Assay

Cells were plated in 96-well plates (25,000 cells/well) 16 hr before performing the assay. The medium was removed and cells were washed twice with PBS. To the wells containing the blanks, 50 μl of PBS was added, while for the wells with the samples 50 μl of 2-NBDG (100 μM) (Sigma) was added and the mixture was incubated for 10 min at 37°C and 5% CO_2 . After incubation, cells were washed twice with ice-cold PBS to stop the reaction, and 200 μl PBS was added to each well. Fluorescence measurements were performed at 485/520 nm with the PHERAstar FS (BMG Labtech).

Lactate Production Assay

To measure lactate production, cells that were 80% confluent were replenished with fresh medium. Aliquots of the medium were removed at the indicated time points (24 or 48 hr) for measurement of lactate using an Accutrend lactate analyzer (Roche). At each time point, cell numbers were also counted for normalization of lactate generation.

Intracellular Glutamate Assay

The glutamate concentration in cell lysates was measured using the Glutamate Colorimetric Assay Kit (Biovision) following the manufacturer's protocol.

Briefly, 1×10^6 cells per tested sample were homogenized in 100 μl of assay buffer and centrifuged to remove insoluble material. 100 μl of reaction mix was added to the supernatant, standards, and background control samples, and after 30 min incubation at 37°C , absorbance was measured at 450 nm with a SpectraMax Plus384 MicroPlate Reader (Molecular Devices). The experiment was performed in quadruplicate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2016.01.015>.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: R.S.R., L.E.V., W.L., J.F.O., A.F.F., M.S.R., M.I., G.B., M.F.F., S.H., W.R.W., A.L.B.A., S.M.G.D., and G.A.C. Performed the experiments: R.S.R., L.E.V., W.L., J.F.O., C.R.-A., D.A., B.P., A.T., Y.C., K.V.R., S.C., M.S., Y.A., L.H.C., G.Y.H., P.M., M.S.R., T.C.I., L.V., and H.L. Analyzed the data: R.S.R., L.E.V., W.L., J.F.O., C.I., C.R.-A., D.A., A.T., A.F.F., L.V., G.K., J.A.B., M.R., A.L.B.A., S.M.G.D., and G.A.C. Contributed reagents/materials/analysis tools: L.H., S.K., R.G., G.L., S.Y.L., R.C.B., P.H., M.G.R., I.B.-N., and G.L.-B. Wrote the first draft of the manuscript: R.S.R., S.M.G.D., A.L.B.A., and G.A.C. Contributed to the writing of the manuscript: R.S.R., S.M.G.D., A.L.B.A., and G.A.C. Agree with manuscript results and conclusions: all authors. Critical revision of the manuscript for important intellectual content: all authors. Statistical analysis: C.I., L.H., G.K., M.R., and H.L. Study supervision: S.M.G.D. and G.A.C.

ACKNOWLEDGMENTS

G.A.C. is The Alan M. Gewirtz Leukemia & Lymphoma Society Scholar. Work in G.A.C.'s laboratory is supported in part by the NIH/NCI grants 1UH2TR00943-01 and 1 R01 CA182905-01, the UT MD Anderson Cancer Center SPORE in Melanoma grant from NCI (P50 CA093459), Aim at Melanoma Foundation and the Miriam and Jim Mulva research funds, the Brain SPORE (2P50CA127001), the Center for Radiation Oncology Research Project, the Center for Cancer Epigenetics Pilot project, a 2014 Knowledge GAP MDACC grant, a CLL Moonshot pilot project, the UT MD Anderson Cancer Center Duncan Family Institute for Cancer Prevention and Risk Assessment, a SINP grant in colon cancer, the Laura and John Arnold Foundation, the RGK Foundation, and the Estate of C.G. Johnson, Jr. I.B.-N. was financed by a grant entitled Non-Invasive Intelligent Systems for Colorectal Cancer Diagnosis and Prognosis Based on circulating miRNAs Integrated in the Clinical Workflow – INTELCO. S.M.G.D., A.L.B.A., and D.A. are supported by the São Paulo Research Foundation FAPESP under grants 2014/15968-3, 2014/20673-2, and 2014/17820-3, respectively. W.L. was partly supported by grants from The University of Texas MD Anderson Cancer Center Sheikh Ahmed Bin Zayed Al Nahyan Center for Pancreatic Cancer Research. J.A.B. was supported by the Cancer Center Support Grant (P30 CA016672), and the HP imaging program of the Small Animal Facility (SAIF) was supported by the Cancer Prevention and Research Institutes of Texas grant RP-101243P5. H.L. was supported by NIH/NCI grant R01CA175486, a grant (RP140462) from the Cancer Prevention and Research Institute of Texas, and the R. Lee Clark Fellow Award from The Jeanne F. Shelby Scholarship Fund. We would like to thank Dr. Riccardo Fodde (Erasmus Medical Center) for the scientific support and advice, Dr. Sylvie Doublet for the generous gift of CFIm25 and CFIm68 plasmids, Dr. Riccardo Spizzo for generating the HCT116 *CCAT2* stable clones, and Dr. Thomas A. Cooper for the generous gift of RG6 plasmid. We would also like to thank the IM Bioscope II-UT core facility and Dr. Ana Maria Zaske for performing the AFM imaging. Additionally, we thank the members of Flow Cytometry & Cellular Imaging Core Facility (Department of Leukemia) and Dr. Jared Burks for performing the VECTRA imaging and analysis and Drs. Liuqing Yang (MDACC), Yibin Zhou (IBT), Clifford Stephan (IBT), Shawn Bratton (MDACC), Charles V. Kingsley (MDACC), Jorge Delacerda (MDACC), Iva Maestri (University of Ferrara), and Linda Ulazzi (University of Ferrara) for the technical

support. We also would like to thank the Biological Imaging Facility and the Protein Purification and Bioassay Laboratories (LNBio/CNPq/Brazil) for access to the equipment.

Received: April 3, 2015
 Revised: October 23, 2015
 Accepted: January 8, 2016
 Published: February 4, 2016

REFERENCES

- Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., and Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell* **145**, 435–446.
- Boroughs, L.K., and DeBerardinis, R.J. (2015). Metabolic pathways promoting cancer cell survival and growth. *Nat. Cell Biol.* **17**, 351–359.
- Cancer Genome Atlas Network (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487**, 330–337.
- Carroll, P.A., Diolaiti, D., McFerrin, L., Gu, H., Djukovic, D., Du, J., Cheng, P.F., Anderson, S., Ulrich, M., Hurley, J.B., et al. (2015). Deregulated Myc requires MondoA/Mlx for metabolic reprogramming and tumorigenesis. *Cancer Cell* **27**, 271–285.
- Cassago, A., Ferreira, A.P., Ferreira, I.M., Fornezari, C., Gomes, E.R., Greene, K.S., Pereira, H.M., Garratt, R.C., Dias, S.M., and Ambrosio, A.L. (2012). Mitochondrial localization and structure-based phosphate activation mechanism of Glutaminase C with implications for cancer metabolism. *Proc. Natl. Acad. Sci. USA* **109**, 1092–1097.
- Chen, J.Q., and Russo, J. (2012). Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. *Biochim. Biophys. Acta* **1826**, 370–384.
- Elkon, R., Ugalde, A.P., and Agami, R. (2013). Alternative cleavage and polyadenylation: extent, regulation and function. *Nat. Rev. Genet.* **14**, 496–506.
- Gao, P., Tchernyshyov, I., Chang, T.C., Lee, Y.S., Kita, K., Ochi, T., Zeller, K.I., De Marzo, A.M., Van Eyk, J.E., Mendell, J.T., and Dang, C.V. (2009). c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* **458**, 762–765.
- Huang, F., Zhang, Q., Ma, H., Lv, Q., and Zhang, T. (2014). Expression of glutaminase is upregulated in colorectal cancer and of clinical significance. *Int. J. Clin. Exp. Pathol.* **7**, 1093–1100.
- Jacque, N., Ronchetti, A.M., Larrue, C., Meunier, G., Birsén, R., Willems, L., Saland, E., Decroocq, J., Thiago, T.T., Lambert, M., et al. (2015). Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. *Blood* **126**, 1346–1356.
- Jeong, S.M., Xiao, C., Finley, L.W., Lahusen, T., Souza, A.L., Pierce, K., Li, Y.H., Wang, X., Laurent, G., German, N.J., et al. (2013). SIRT4 has tumor-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting mitochondrial glutamine metabolism. *Cancer Cell* **23**, 450–463.
- Kaldma, A., Klepinin, A., Chekulayev, V., Mado, K., Shevchuk, I., Timohina, N., Tepp, K., Kandashvili, M., Varikmaa, M., Koit, A., et al. (2014). An in situ study of bioenergetic properties of human colorectal cancer: the regulation of mitochondrial respiration and distribution of flux control among the components of ATP synthasome. *Int. J. Biochem. Cell Biol.* **55**, 171–186.
- Katt, W.P., Ramachandran, S., Erickson, J.W., and Cerione, R.A. (2012). Dibenzophenanthridines as inhibitors of glutaminase C and cancer cell proliferation. *Mol. Cancer Ther.* **11**, 1269–1278.
- Le, A., Lane, A.N., Hamaker, M., Bose, S., Gouw, A., Barbi, J., Tsukamoto, T., Rojas, C.J., Slusher, B.S., Zhang, H., et al. (2012). Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab.* **15**, 110–121.
- Ling, H., Fabbri, M., and Calin, G.A. (2013a). MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat. Rev. Drug Discov.* **12**, 847–865.
- Ling, H., Spizzo, R., Atlasi, Y., Nicoloso, M., Shimizu, M., Redis, R.S., Nishida, N., Gafà, R., Song, J., Guo, Z., et al. (2013b). CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. *Genome Res.* **23**, 1446–1461.
- Lutz, C.S., and Moreira, A. (2011). Alternative mRNA polyadenylation in eukaryotes: an effective regulator of gene expression. *Wiley Interdiscip. Rev. RNA* **2**, 23–31.
- Lyubchenko, Y.L., Shlyakhtenko, L.S., and Ando, T. (2011). Imaging of nucleic acids with atomic force microscopy. *Methods* **54**, 274–283.
- Masamha, C.P., Xia, Z., Yang, J., Albrecht, T.R., Li, M., Shyu, A.B., Li, W., and Wagner, E.J. (2014). CFIm25 links alternative polyadenylation to glioblastoma tumour suppression. *Nature* **510**, 412–416.
- Mercer, T.R., Dinger, M.E., and Mattick, J.S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* **10**, 155–159.
- Millevoi, S., Loulergue, C., Dettwiler, S., Karaa, S.Z., Keller, W., Antoniou, M., and Vagner, S. (2006). An interaction between U2AF 65 and CF Im links the splicing and 3' end processing machineries. *EMBO J.* **25**, 4854–4864.
- Naganuma, T., Nakagawa, S., Tanigawa, A., Sasaki, Y.F., Goshima, N., and Hirose, T. (2012). Alternative 3'-end processing of long noncoding RNA initiates construction of nuclear paraspeckles. *EMBO J.* **31**, 4020–4034.
- Nagasaki, H., Arita, M., Nishizawa, T., Suwa, M., and Gotoh, O. (2006). Automated classification of alternative splicing and transcriptional initiation and construction of visual database of classified patterns. *Bioinformatics* **22**, 1211–1216.
- Orengo, J.P., Bundman, D., and Cooper, T.A. (2006). A bichromatic fluorescent reporter for cell-based screens of alternative splicing. *Nucleic Acids Res.* **34**, e148.
- Rathore, M.G., Saumet, A., Rossi, J.F., de Bettignies, C., Tempé, D., Lecellier, C.H., and Villalba, M. (2012). The NF- κ B member p65 controls glutamine metabolism through miR-23a. *Int. J. Biochem. Cell Biol.* **44**, 1448–1456.
- Redis, R.S., Sieuwerts, A.M., Look, M.P., Tudoran, O., Ivan, C., Spizzo, R., Zhang, X., de Weerd, V., Shimizu, M., Ling, H., et al. (2013). CCAT2, a novel long non-coding RNA in breast cancer: expression study and clinical correlations. *Oncotarget* **4**, 1748–1762.
- Stine, Z.E., Walton, Z.E., Altman, B.J., Hsieh, A.L., and Dang, C.V. (2015). MYC, Metabolism, and Cancer. *Cancer Discov.* **5**, 1024–1039.
- Tian, B., Pan, Z., and Lee, J.Y. (2007). Widespread mRNA polyadenylation events in introns indicate dynamic interplay between polyadenylation and splicing. *Genome Res.* **17**, 156–165.
- Tomlinson, I., Webb, E., Carvajal-Carmona, L., Broderick, P., Kemp, Z., Spain, S., Penegar, S., Chandler, I., Gorman, M., Wood, W., et al.; CORGI Consortium (2007). A genome-wide association scan of tag SNPs identifies a susceptibility variant for colorectal cancer at 8q24.21. *Nat. Genet.* **39**, 984–988.
- Tuupaniemi, S., Turunen, M., Lehtonen, R., Hallikas, O., Vanharanta, S., Kivioja, T., Björklund, M., Wei, G., Yan, J., Niittymäki, I., et al. (2009). The common colorectal cancer predisposition SNP rs6983267 at chromosome 8q24 confers potential to enhanced Wnt signaling. *Nat. Genet.* **41**, 885–890.
- van den Heuvel, A.P., Jing, J., Wooster, R.F., and Bachman, K.E. (2012). Analysis of glutamine dependency in non-small cell lung cancer: GLS1 splice variant GAC is essential for cancer cell growth. *Cancer Biol. Ther.* **13**, 1185–1194.
- Vander Heiden, M.G. (2011). Targeting cancer metabolism: a therapeutic window opens. *Nat. Rev. Drug Discov.* **10**, 671–684.
- Wang, J., Liu, X., Wu, H., Ni, P., Gu, Z., Qiao, Y., Chen, N., Sun, F., and Fan, Q. (2010a). CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. *Nucleic Acids Res.* **38**, 5366–5383.
- Wang, J.B., Erickson, J.W., Fuji, R., Ramachandran, S., Gao, P., Dinavahi, R., Wilson, K.F., Ambrosio, A.L., Dias, S.M., Dang, C.V., and Cerione, R.A. (2010b). Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* **18**, 207–219.
- Warburg, O., Posener, K., and Negelein, E. (1924). Über den Stoffwechsel der Carcinomzelle. *Biochem. Zeitschr* **152**, 309–344.

- Ward, P.S., and Thompson, C.B. (2012). Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 21, 297–308.
- Wilusz, J.E., Sunwoo, H., and Spector, D.L. (2009). Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev.* 23, 1494–1504.
- Xia, Z., Donehower, L.A., Cooper, T.A., Neilson, J.R., Wheeler, D.A., Wagner, E.J., and Li, W. (2014). Dynamic analyses of alternative polyadenylation from RNA-seq reveal a 3'-UTR landscape across seven tumour types. *Nat. Commun.* 5, 5274.
- Yang, Q., Gilmartin, G.M., and Doublé, S. (2011). The structure of human cleavage factor I(m) hints at functions beyond UGUA-specific RNA binding: a role in alternative polyadenylation and a potential link to 5' capping and splicing. *RNA Biol.* 8, 748–753.
- Yang, F., Zhang, H., Mei, Y., and Wu, M. (2014). Reciprocal regulation of HIF-1 α and lincRNA-p21 modulates the Warburg effect. *Mol. Cell* 53, 88–100.
- Yoon, J.H., Srikantan, S., and Gorospe, M. (2012). MS2-TRAP (MS2-tagged RNA affinity purification): tagging RNA to identify associated miRNAs. *Methods* 58, 81–87.
- Zhou, Z., Licklider, L.J., Gygi, S.P., and Reed, R. (2002). Comprehensive proteomic analysis of the human spliceosome. *Nature* 419, 182–185.

The origin and evolution of human glutaminases and their atypical C-terminal ankyrin repeats

Received for publication, March 21, 2017, and in revised form, May 11, 2017. Published, Papers in Press, May 19, 2017, DOI 10.1074/jbc.M117.787291

Camila Cristina Pasquali^{†1}, Zeyaul Islam^{†1}, Douglas Adamoski^{†1}, Igor Monteze Ferreira^{†5}, Ricardo Diogo Righeto^{¶1,2}, Jefferson Bettini[¶], Rodrigo Villares Portugal[¶], Wyatt Wai-yin Yue[§], Ana Gonzalez^{**}, Sandra Martha Gomes Dias^{†3}, and Andre Luis Berteli Ambrosio^{†4}

From the [†]Laboratório Nacional de Biociências, Centro Nacional de Pesquisa em Energia e Materiais, Campinas, São Paulo 13083-970, Brazil, the [§]Structural Genomics Consortium, University of Oxford, Oxford OX3 7DQ, United Kingdom, the [¶]Laboratório Nacional de Nanotecnologia, Centro Nacional de Pesquisa em Energia e Materiais, Campinas, São Paulo 13083-970, Brazil, the ^{||}School of Electrical and Computer Engineering, University of Campinas, São Paulo 13083-852, Brazil, and the ^{**}Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, Menlo Park, California 94025

Edited by Norma Allewell

On the basis of tissue-specific enzyme activity and inhibition by catalytic products, Hans Krebs first demonstrated the existence of multiple glutaminases in mammals. Currently, two human genes are known to encode at least four glutaminase isoforms. However, the phylogeny of these medically relevant enzymes remains unclear, prompting us to investigate their origin and evolution. Using prokaryotic and eukaryotic glutaminase sequences, we built a phylogenetic tree whose topology suggested that the multidomain architecture was inherited from bacterial ancestors, probably simultaneously with the hosting of the proto-mitochondrion endosymbiont. We propose an evolutionary model wherein the appearance of the most active enzyme isoform, glutaminase C (GAC), which is expressed in many cancers, was a late retrotransposition event that occurred in fishes from the Chondrichthyes class. The ankyrin (ANK) repeats in the glutaminases were acquired early in their evolution. To obtain information on ANK folding, we solved two high-resolution structures of the ANK repeat-containing C termini of both kidney-type glutaminase (KGA) and GLS2 isoforms (glutaminase B and liver-type glutaminase). We found that the glutaminase ANK repeats form unique intramolecular contacts through two highly conserved motifs; curiously, this arrangement occludes a region usually involved in ANK-mediated protein-protein interactions. We also solved the crystal structure of full-length KGA and present a small-angle X-ray scattering model for full-

length GLS2. These structures explain these proteins' compromised ability to assemble into catalytically active supra-tetrameric filaments, as previously shown for GAC. Collectively, these results provide information about glutaminases that may aid in the design of isoform-specific glutaminase inhibitors.

Glutamine, the most abundant amino acid in human plasma and muscles (1), is consumed by rapidly proliferating tumor cells to meet their increased energy and biosynthetic precursor demands (2, 3). The enzyme glutaminase converts glutamine into glutamate, which is further catabolized to produce ATP, nucleotides, amino acids, lipids, and glutathione. In this regard, glutaminase is a well established target for the inhibition of cell transformation (4). Accordingly, several oncogenes and tumor suppressors have already been described to be involved in glutaminase-dependent glutamine breakdown (4). Notably, c-Myc stimulates glutamine uptake and degradation by GLS (specifically the product of the *GLS* gene) to sustain cellular viability and Krebs cycle anaplerosis (5). Similarly, nuclear factor- κ B (NF- κ B) enhances glutamine metabolism by decreasing the expression of miR-23a, which targets the *GLS* mRNA (6); Rho GTPases, ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C)-Cdh1, and c-Jun also regulate glutamine metabolism by acting on *GLS* and consequently supporting cancer growth (7–9). However, a second gene encodes another glutaminase enzyme, named *GLS2*. In contrast to *GLS*, *GLS2* is regulated by the tumor suppressor p53 and has tumor suppressor features toward gliomas, glioblastomas, lung cancer, and hepatocarcinomas (10–14).

The existence of multiple glutaminases in mammals was first reported by Hans Krebs (15), on the basis of the detection of tissue-specific kinetic parameters and their susceptibility to inhibition by their catalytic products, when he probed the conversion of glutamine into glutamate in tissue extracts. In addition to being expressed by two different genes, at least four glutaminase isoforms have been described in mammals. The kidney-type isoforms, kidney-type glutaminase (KGA)⁵ and

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo Grant 2014/20673–2 (to A. L. B. A.), Grant 2015/25832–4 (to S. M. G. D.), Scholarship 2014/19518–2 (to C. C. P.), Fellowship 2014/12663–7 (to Z. I.), Scholarship 2014/17820–3 (to D. A.), and Scholarship 2013/05668–0 (to I. M. F.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supplemental Table S1 and Fig. S1.

The atomic coordinates and structure factors (codes 5u0i, 5u0j, 5u0k, and 5uqe) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

¹ These authors contributed equally to this work.

² Present address: Center for Cellular Imaging and NanoAnalytics (C-CINA), Biozentrum, University of Basel, Basel, CH-4058, Switzerland.

³ To whom correspondence may be addressed. E-mail: sandra.dias@lnbio.cnpem.br.

⁴ To whom correspondence may be addressed: Laboratório Nacional de Biociências, LNBio, CNPEM, Rua Giuseppe Máximo Scolaro, 10.000, Pólo II de Alta Tecnologia, Campinas, São Paulo 13083-970, Brazil. Tel.: 55-19-3512-1115; Fax: 55-19-3512-1004; E-mail: andre.ambrosio@lnbio.cnpem.br.

⁵ The abbreviations used are: KGA, kidney-type glutaminase; LGA, liver-type glutaminase; GAB, glutaminase B; ANK, ankyrin; STAS, sulfate transporter and anti- σ factor antagonist; EH, Eps15 homology; SAD, single-wavelength anomalous dispersion; r.m.s., root mean square; BPTES, (bis-2[5-phenylac-

glutaminase C (GAC), are generated by alternative splicing of the *GLS* gene (2q32.2). Both isoforms are activated by inorganic phosphate and inhibited by glutamate (16–18). In contrast, the liver-type isozymes, liver-type glutaminase (LGA) and glutaminase B (GAB), originate from the *GLS2* gene (12q13.3) through the use of alternative transcription initiation sites (19). The recombinant construct that spans a common region between the *GLS2* isoforms responds poorly to phosphate (20) and is not inhibited by glutamate (19, 21).

Although both the kidney isozymes and GAB are expected to be localized in the mitochondrial matrix because of the transit peptide sequences at their N termini, KGA and LGA can also localize to the cytosol and nuclei (20, 22). However, GAC has been shown to be exclusively located in the mitochondrion (20). Moreover, GAC provides key growth advantages to cancer cells (20). KGA, LGA, and GAB share a glutaminase domain that is well conserved in sequence and structurally similar to bacterial glutaminases, and it is flanked by a long N-terminal domain folded in an EF-hand-like four-helix bundle and a C-terminal domain with three putative ANK repeats (20). In contrast, GAC has a shorter, 48-amino acid-long C terminus with no canonical motif or domain. GAC is also unique because it assembles into highly active, long double-stranded helical filaments in the presence of inorganic phosphate, whereas KGA forms shorter and less active structures under the same conditions (23).

Intrigued by the diversity in numbers, architecture, mechanism of activation, and enzymatic capabilities of the human glutaminases, we felt motivated to investigate the origin, evolution, and possible functions of such complex features. First, by identifying homologous prokaryotic and eukaryotic sequences and building a phylogenetic tree, we propose that the multidomain architecture of the mammalian glutaminases GLS and *GLS2* is a feature inherited from bacterial ancestors, probably simultaneously with the hosting of the proto-mitochondrion endosymbiont. From this phylogenetic tree, we also propose an evolutionary model wherein a *GLS*-like predecessor gene in tunicates gave rise to the currently known human isozymes through exon remodeling, gene duplication, and retrotransposition events. In addition, we solved high-resolution crystallographic structures of the C-terminal ANK repeats from both human KGA and GAB/LGA. These structures display a unique type of dimerization for ANK domains, governed by two short amino acid motifs that are highly conserved from bacteria to higher eukaryotes. Finally, we present the first C terminus-containing crystallographic structure of human KGA, which explains its inability to assemble into the catalytically active supra-tetrameric filaments, as previously shown for GAC. A similar model for human *GLS2* is also proposed on the basis of small-angle X-ray scattering data and cryo-electron microscopy. Collectively, these results contribute to the general knowledge on the many human glutaminase isoforms as well as explain the mechanistic diversity that poses challenges that still must be overcome to

increase the chances of successfully inhibiting the enzyme in a clinical setting.

Results

The multidomain origin of glutaminases

An evolutionary analysis of 2,796 bacterial glutaminase protein sequences and 789 eukaryotic sequences selected from a BLAST search was performed using the glutaminase domain from human KGA (Ile²²¹-Arg⁵⁴⁴) as the query sequence (*E* value ≤ 0.0001). Particularly, no glutaminase homologs were identified in archaea. The bacterial sequence sizes presented three normal distributions. The first and major distribution ($\sim 85\%$ of the sequences) was centered at 313 ± 10 amino acids (Fig. 1A), thus suggesting that the proteins predominantly contained only the catalytic domain (24). The second distribution was smaller (10% of the sequences) and centered at 429 ± 16 amino acids. Detailed analysis suggested that this group possesses a sulfate transporter and anti- σ factor antagonist (STAS) domain located C-terminal to the glutaminase domain (Fig. 1A). Finally, 4% (109) of bacterial glutaminases clustered in a third distribution centered at 613 amino acids. These sequences, in addition to the STAS domain, have an extra cyclic nucleotide-binding domain located N-terminal to the glutaminase domain. Notably, three cyanobacteria sequences from this group possess a glutaminase domain flanked by an Eps15 homology-like domain (EH-like) at the N terminus and two clusters of ANK repeats at the C terminus (Fig. 1A). The Eps15 homology domain is a pair of EF-hand motifs that recognize and bind to proteins containing Asn-Pro-Phe (NPF) sequences (25). Aside from these three-domain cyanobacteria sequences, 17 proteo- and actinobacteria sequences situated between the second and third normal distributions (Fig. 1A, *blue oval*) also exhibit a glutaminase domain flanked N-terminal by an EH-like domain and C-terminal by only one cluster of ANK repeats.

Eukaryotic glutaminases, which primarily belong to the kingdoms Animalia and Fungi, are consistently longer and present a single Gaussian size distribution averaging 631 amino acids in length (mode at 615), with a skewed standard deviation of 45 amino acids toward shorter sequences and 26 amino acids toward longer sequences (Fig. 1B). The left-most end of the distribution contained the fungal glutaminases, which are 450 to 500 amino acids long and present a unique architecture, wherein the glutaminase domain is followed by a long C terminus (156 ± 7 amino acids) with an unknown fold (Fig. 1B, *pink bar*). The remaining eukaryotic glutaminases that are longer than 500 amino acids in length mostly belong to the Animalia kingdom and have extended N-terminal sequences of varying lengths (Fig. 1B). The N-terminal region immediately adjacent to the glutaminase domain is more than 40% similar to the EF-hand-like four-helix bundle, which is structurally similar to the EH-like domain found in bacteria. Indeed, the X-ray structures of human GLS containing this domain have an EH-like structure (20).

Putative ANK repeats are predicted for about 550 of the longer eukaryotic glutaminases and are located C-terminal to the glutaminase domain with no exception. Another 76 sequences,

etamido-1,2,4-thiadiazol-2-yl]ethyl sulfide; BIS-TRIS propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; ISAC, iterative stable alignment and clustering; PDB, Protein Data Bank; Rh, hydrodynamic radius.

The multidomain architecture of human glutaminases

Figure 1. Sequence and architecture analysis of bacterial and eukaryotic glutaminases. The amino acid sequence corresponding to the glutaminase domain of the *GLS* gene (Ile²²¹-Arg⁵⁴⁴) was used as input of blastp search against the non-redundant protein sequences (nr) database (E value ≤ 0.0001). The sequences obtained were analyzed by number of amino acids (aa); the normal distribution of the sequence length for bacterial and eukaryote glutaminases are, respectively, represented in A and B, with the respective architectures indicated in rectangles; in parentheses, the number of sequences containing the corresponding architecture/the total number of sequences contained in the referred region of the distribution plot. C, cladogram based on architectural organization obtained from a maximum likelihood phylogenetic reconstruction approach. D, the generated phylogenetic tree and respective alignment were used to reconstruct ancestral protein sequences for specific nodes (ancestral chordates (1); nematodes (2); arthropodes (3); and fungi (4)) using maximum parsimony. The obtained ancestral sequences were aligned with the human *GLS* (KGA and GAC) and *GLS2* (GAB and LGA) and the sequence similarity displayed as a heatmap of pairwise distances constructed in SDT using MUSCLE alignment. The obtained result suggests that *GLS* is the most primitive gene, which has been further duplicated to generate *GLS2*. cNMP, cyclic nucleotide-binding domain; SRPBCC, START/RHO α C/PITP/Bet v1/CoxG/CalC.

all of mammalian origin, have a shorter C terminus that closely resembles human GAC in sequence (Fig. 1B). Finally, a few glutaminase sequences (11 in total) belonging to the major haptophyte and heterokont lines of eukaryotes were identified. These sequences are much longer, ranging from 800 to 1250 amino acids in length, and the predicted architectures of some of them consist of two complete tandemly associated ANK-containing glutaminases (data not shown).

To detect evolutionary relationships based on the architectural organization, we next reconstructed an unrooted cladogram using the analyzed sequences (Fig. 1C). The derived tree structure has a taxa organization similar to that found in rRNA-based phylogenetic trees (26). Many unresolved nodes (polytomy) were observed, particularly for the bacterial glutaminases. We believe that such observation stems from the fact that many bacterial species possibly have more than one glutaminase, often as a result of the combination of a single protein with a multidomain isozyme. Except for fungal glutaminases, which clustered closer to the bacterial homologs, the eukaryotic proteins had a better-resolved tree. Protists, nematodes, and arthropods form a polytomic group with chordates, as well as with ANK-containing proteo- and cyanobacteria; this grouping suggests that the appearance of this multidomain feature was

an early event, concomitant with the symbiotic association related to the appearance of eukaryotes, because archaea does not carry any glutaminase-like coding gene. A subsequent lineage split linked to the appearance of multiple variations around the same basic architecture (EH-like + glutaminase + ANK) suggests that the gene duplication event that produced the *GLS* and *GLS2* genes and isoform differentiation (with the appearance of GAC-like isoforms; Fig. 1B, dark blue oval) appeared with chordates. However, interestingly, birds have completely lost the *GLS2* gene.

Finally, based on a theoretical reconstruction of the ancestral glutaminases from each of the main eukaryotic branches (chordata, arthropods, nematodes, and fungi), the most primitive glutaminase acquired from a bacterial ancestor was most probably the kidney-type glutaminase (KGA-like), as shown in Fig. 1D. The general organization EH-like + glutaminase + ANK is preserved in all four reconstructed ancestral sequences. Comparison of the sequence identity of human genes with the reconstructed common ancestor of *GLS* and *GLS2* from Gnathostomata suggested that *GLS* was restrained during evolution to maintain the same function, whereas *GLS2* exhibited more changes, probably acquiring new functions and/or regulatory mechanisms.

The origin of glutaminase isoforms

Having established a plausible origin for the multidomain architecture of the glutaminases, we next asked how the different variants of the enzyme, currently observed in humans, might have evolved. We first performed a “top-down” search for glutaminase genes in phylum Chordata using the complete genomic sequence of the human *GLS* and *GLS2* genes as templates. Second, we identified regions homologous to exon 14 of *GLS* (after which the *GLS* splicing event occurs) and exon 15 (the “GAC exon”).

In the lower chordates, we verified that the genome of the tunicate sea squirt (27) (*Ciona intestinalis*) has a single *GLS*-like gene containing 12 exons. A region similar to human exon 14 (70% identity, red bar in the tunicate branch, Fig. 2A) is inserted in exon 11. The Cephalochordate amphioxus (28) (*Branchiostoma floridae*) also has a single glutaminase gene, which, interestingly, has only two exons. In this gene, the region homologous to the human exon 14 is appropriately located at the 3' boundary of exon 1; however, no homology to the GAC exon was identified in the downstream sequence. A gene duplication event was observed further upstream in lampreys (29) (*Pekinus marinus*, Hyperortia), which generated distinct sequences related to the human *GLS* and *GLS2* genes.

Next, the Chondrichthyes *Callorhynchus milii* (elephant shark (30)) also has two glutaminases that are phylogenetically related to the *GLS* and *GLS2* genes without annotated splicing regulation. However, in the *GLS* gene, a region homologous to the human GAC exon is for the first time observed and located downstream of an exon 14-equivalent sequence, thereby suggesting an origin for the splicing variant GAC. Zebrafish (*Danio rerio* (31)) and other fish species possess 5 glutaminase genes, thus suggesting subsequent autapomorphic duplication events for both *GLS* and *GLS2*. Interestingly, the GAC isoform was maintained in both copies of the *GLS* gene, thus supporting the hypothesis that the GAC exon appeared before this duplication; a new *GLS*-like (*GLSL*) sequence was identified in species within this branch, such as zebrafish, elephant shark, and others.

Of note, the amphibian *Xenopus tropicalis* (Western clawed frog (32)), with only one copy of both *GLS* and *GLS2*, points to a compaction of the *GLS2* introns and exons against the spread of the *GLS* across a larger genomic region. Finally, the human glutaminase gene structures retained the same architecture observed in the amphibian genes, although the sequence lengths were smaller.

The identification of several transposable elements such as Alu and L2 (Fig. 2B), within the human *GLS* intron 15, strongly suggests that the insertion of the GAC exon was due to an early retrotransposition event; however, no consensus matching elements were found in the rapid evolving intron 14. Nevertheless, a thorough tblastn analysis failed to identify a region homologous to the GAC exon (exon 15) within the human genome, preventing us from proposing an original location for this sequence.

Crystal structures of the human glutaminase ankyrin repeats

We next determined the crystal structures of the ANK-containing C-terminal regions of both human KGA (KGA.ANK: Val⁵⁵¹-Leu⁶⁶⁹) and GLS2 (GLS2.ANK: Lys⁴⁸⁵-Val⁶⁰²). Both

proteins were subjected to limited proteolysis with trypsin (for KGA.ANK) or chymotrypsin (for GLS2.ANK) during purification to remove the likely intrinsically flexible regions and obtain well diffracting crystals. X-ray diffraction datasets were obtained for KGA.ANK crystals belonging to two different space groups, namely the tetragonal P₄₃2₁2 and a monoclinic P1₂₁1. Sulfur single-wavelength anomalous dispersion (SAD) phasing was used to produce a higher resolution molecular model (at 1.41 Å maximum) from the tetragonal crystals. The obtained structure was then used as a search model for molecular replacement of the monoclinic crystals, which diffracted up to 1.74-Å maximum resolution. Finally, the structure, which was collected at 2.55-Å maximum resolution, of the GLS2.ANK hexagonal crystals (space group P6₅) was solved by molecular replacement using the KGA.ANK structure. The statistics of the data collection, processing, phasing, and model refinement data for all the structures are shown in Table 1. Because of the high structural similarity observed for both KGA.ANK structures (backbone r.m.s. deviations of 0.4 Å), only the higher resolution model was used for the subsequent detailed analysis and comparison to the GLS2.ANK structure.

Overall, the C-terminal domains of KGA and GLS2 each contain three ANK repeats (labeled ANK1 to ANK3) and are very similar, both in sequence composition (80% identical) and overall structure (r.m.s. deviation of 0.5 Å over 89 equivalent Cα positions; Fig. 3A). The largest structural variation (a shift between 1.3 and 1.8 Å of the Cα atom positions) occurs in the β-hairpin that links ANK1 to ANK2 (β-hairpin 2), whereas the minimal deviations (~0.1 Å) are observed in the inner and outer α-helices of ANK2. The primary sequences of ANK1 and ANK3 repeats in both KGA and GLS2 diverge from the canonical ANK repeats at the TPLH tetrapeptide motif, which is located at the beginning of the inner α-helices and is responsible for the stabilization of ANK repeats through reciprocal hydrogen bonding between the threonine hydroxyl and histidine imidazole groups (33, 34) (Fig. 3B). The ANK1 inner helix of both KGA and GLS2 is a half-turn longer and lacks the β1 turn compared with the typical inner helix observed in ANK repeats (Fig. 3B), whereas in the ANK3 inner helix, the histidine is replaced with an aspartate, thus preventing the formation of the conserved hydroxyl-imidazole hydrogen bond. Moreover, in both structures, ANK2 contains an extra residue, a surface-exposed lysine (Lys⁶¹¹ at KGA and Lys⁵⁴³ at GLS2, indicated by a plus sign in Fig. 3B), at the C terminus of the outer helix. Finally, the GLS2 ANK3 outer helix is one turn longer than the canonical ANK repeats (Fig. 3B).

Glutaminase ankyrin repeats assemble into atypical dimers

A search for the biologically relevant assembly of the KGA and GLS2 ANK domains was performed using the PISA (protein interfaces, surfaces and assemblies) server (35). The program identified a thermodynamically stable interface involving a crystallographic homodimer, with a solvation free energy gain of -5.4 kcal/M upon formation (Fig. 3C). The identified interface occludes an area of 800 Å² and corresponds to a symmetric groove-to-groove interaction in both structures. Twelve hydrogen bonds and 12 salt bridges are formed between the short loops present in the β-hairpins 1 (linking ANK1 to ANK2) and

The multidomain architecture of human glutaminases

Figure 2. Evolution of glutaminase intron-exon structure. A, protein sequence corresponding to human *GLS* exon 14 was used as a query of translated blast (tblastn) searches against available genomes from *C. intestinalis* (Tunicate), *B. floridae* (Cephalochordate), *P. marinus* (Cyclostomata), *C. milii* (Chondrichthyes), *D. rerio* (Actinopterygii), and *X. tropicalis* (Amphibia). After an initial Reciprocal Best Hit region was found, the correct position of the exon was determined by pairwise LALIGN between sequences. Region downstream genomic sequence homologous to exon 14 were also evaluated by LALIGN against human GAC-exclusive exon (exon 15). As both *P. marinus* glutaminases are incomplete, available exons were found in contig using LALIGN approach. The human genes are represented at the top branch (Amniota). The thin red bars represent the homologous region to the *GLS* exon 14 and the light blue boxes indicate homologous regions to the exon 15 of human *GLS*. The introns, represented as dashed lines, are not drawn to scale and the omitted sections are indicated by two bars (||). The asterisk (*) indicates that the deposited sequences are incomplete. B, a search for retrotransposition evidences in the genomic region comprehending exon 15 and introns 14 and 15 of human *GLS* using TranspoGene resulted in the identification of various transposable elements in the intron 15; however, no consensus matching elements were found in the intron 14.

Table 1**X-ray crystallography data collection parameters and structure refinement statistics**

Data for the outer shell are shown in parentheses.

Data collection and processing				
Beamline	12–2 at SSRL, USA	12–2 at SSRL, USA	12–2 at SSRL, USA	I03 at DLS, UK
Wavelength (Å)	0.9795	0.9795	0.9795	0.9762
Space group	P 43 21 2	P 1 21 1	P 65	P 42 21 2
Cell parameters (Å)	$a = b = 75.3, c = 88.3$	$a = 76.5, b = 47.2, c = 115.7$ ($\beta = 90.1^\circ$)	$a = b = 85.2, c = 336.8$	$a = b = 144.1, c = 615.2$
Resolution range (Å)	75.0 – 1.42 (1.45 – 1.42)	64.0 – 1.72 (1.75 – 1.72)	84.0 – 2.55 (2.64 – 2.55)	50.0 – 3.6 (3.67 – 3.60)
Unique reflections	48,262 (2,342)	82,659 (4,303)	45,005 (4,413)	76,934 (4,463)
Multiplicity	22.7 (23.2)	6.4 (6.3)	8.1 (8.1)	23.4 (20.8)
R_{pim} (%)	2.6 (30.9)	2.4 (31.8)	3.5 (25.2)	7.4 (49.4)
CC (%)	0.994 (0.441)	0.999 (0.902)	0.999 (0.860)	0.998 (0.716)
Completeness (%)	100 (100)	94.1 (92.1)	99.9 (99.8)	100 (100)
$\langle I/\sigma(I) \rangle$	17.1 (2.9)	15.7 (2.1)	14.1 (3.4)	8.8 (1.9)
Average mosaicity ($^\circ$)	0.62	0.55	0.49	0.30
Wilson B-factor ($\text{\AA}^2$)	13.6	25.7	48.5	104.6
Monomers/AU	2	4*	10	5
Solvent content (%)	46.7	36.4*	53.0	77.1
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.32	1.93 ^a	2.62	5.37
Model refinement				
Resolution range (Å)	57.3 – 1.42	64.0 – 1.72	73.8 – 2.55	50.0 – 3.6
Test set reflections (%)	2500 (5.2)	4285 (5.2)	2366 (5.3)	7160 (5.0)
$R_{\text{factor}}/R_{\text{free}}$ (%)	14.4/17.9	18.3/22.2	21.8/25.7	27.4/31.2
Clashscore	2	2	5	3
Side chain outliers (%)	0.6	0.7	2.4	2.6
RSRZ outliers (%)	1.6	0.6	2.8	5.7
Average B, all atoms ($\text{\AA}^2$)	22.0	34.0	53.0	137.0
Number of protein atoms	1,506	2,943	6,860	18,905
Solvent molecules	285	640	110	
Inorganic atoms	1 Cl^- , 3 Na^+	6 Na^+		
Ligand molecules				3 BPTES
R.m.s. deviations from standard geometry				
Bond length (Å)	0.015	0.019	0.002	0.003
Bond angles ($^\circ$)	1.313	1.454	0.479	0.566
Ramachandran plot (%)				
Most favorable	98.0	98.6	95.9	87.5
Outliers	0	0	0.2	2.1
PDB ID	5u0i	5u0j	5u0k	5uqe

^a Translational non-crystallographic symmetry was detected for this dataset.

2 (linking ANK2 to ANK3, Fig. 3*B*) in chain A with their respective equivalents in chain B (Fig. 3*C*, *left panel*, for β -hairpin 1 loop interactions and *right panel* for β -hairpin 2 loop interactions). The short loops of β -hairpins 1 and 2 contain the motifs Asp-Tyr-Asp (DYD, residues 583–585 in KGA and 516–518 in GLS2) and Asp-Arg-Trp (DRW, residues 617–619 in KGA and 550–552 in GLS2), respectively (Fig. 3*C*). Despite the overall relatively low sequence identity of the eukaryotic and bacterial glutaminase ANK repeats, both the DYD and DRW motifs are highly conserved (Fig. 3, *D* and *E*, respectively). Finally, additional polar interactions are observed between the β -hairpin 1 loop and the ANK3 inner helix and between the β -hairpin 2 loop and the ANK2 inner helix, complementing the identified symmetrical interface (data not shown).

Crystal structure of full-length human KGA

In addition to the determination of the crystal structures of the isolated C-terminal ANK dimers, we also solved the novel crystal structure of KGA containing the C terminus, which was bound to the inhibitor bis-2[5-phenylacetamido-1,2,4-thiadiazol-2-yl]ethyl sulfide (BPTES) (36). The diffraction dataset was collected at a 3.6-Å maximum resolution (space group $P4_22_12$) with lengthy cell parameters and a high solvent content of ~78% (Table 1). Phasing was achieved by molecular replacement using the coordinates of the previously solved N-terminal and glutaminase domains from GAC bound to BPTES (PDB code 4jkt) and the KGA.ANK dimer described here as

search models. The $P4_22_12$ asymmetric unit contains five KGA monomers; accordingly, one canonical tetramer (a dimer of dimers) is formed within the asymmetric unit by four monomers and, the fifth monomer is related to another tetramer by the crystal 2-fold screw axis. The final model obtained (Fig. 4*A*) was refined to R_{factor} and R_{free} of 27.3 and 31.8%, respectively.

The ANK dimers in the KGA structure (*green surface* in Fig. 4*B*) are spatially located between the N-terminal EF-hand-like domains (*blue surfaces* in Fig. 4*B*). Notably, both ANK dimers of a tetramer lie on the same side of a sectional plane defined by the two longest axes of the protein (Fig. 4*B*), breaking the previously established 2-fold dihedral symmetry of the glutaminase structures lacking the C terminus (20).

The low-resolution model did not allow us to provide a detailed description of the side chain interactions between the ANK dimers and the N-terminal domain. However, the close contact between the ANK1 outer helix of chain A with the EF-hand-like domain helix H1 of chain B is unmistakable (Fig. 4*C*). The interface contains large, exposed polar residues, such as glutamic acid and arginine, which are likely to be responsible for the contact. An equivalent arrangement was also observed for the ANK1 outer helix of chain C with the EF-hand-like domain helix H1 of chain D (data not shown). The majority of the C-terminal region of KGA adjacent to the ANK repeats (residues Thr⁶⁴⁷-Leu⁶⁶⁹) is likely heterogeneous in conforma-

The multidomain architecture of human glutaminases

Figure 3. Overall structural features of ANK domain of glutaminases. A, schematic representation of three ANK repeats (ANK1 to ANK3) present as a C-terminal domain of KGA and GLS2. Both ANK repeats are very similar in sequence as well as in the overall structure. B, alignment of the glutaminase ANK repeats and the consensus sequence (shown on top), proposed in Ref. 39. Highly conserved residues are capitalized and in red, semiconserved residues are colored cyan and not capitalized. Residues involved in the dimer interface are indicated by a bar. ANK2 in both KGA and GLS2 contains an extra surface-exposed lysine, which is indicated by the + signal. On the right, the superposition of ANK1, ANK2, and ANK3 is represented. C, dimer interface and associated interaction of ANK repeats. The side chains of the motifs DYD (left) and DRW (right) are represented in sticks. D, representation of sequence conservation of glutaminase ANK from eukaryotes and E, bacteria. The size of the letters is proportional to the degree of conservation of residues. The motifs DYD and DRW are highlighted. The residues in red show the 100% conservation throughout the alignment. Residues are numbered according to human KGA.

tion and therefore could not be modeled. According to secondary structure predictions by the JPred4 server (37), this region is mostly unstructured. This information, added to the fact that such region is present in a KEN box involved on KGA degradation (8), suggest that this segment is likely functionally independent from the ankyrin domain.

As shown in our previous study (23), the phosphate-dependent enzymatic activity of the GLS isoforms is directly related to their ability of self-assembling into supra-tetrameric helical filaments; GAC is the most active isoform and forms longer filaments. Moreover, we previously demonstrated that this

assembly was generated by end-to-end associations via the N-terminal domains; the KGA assembly was always shorter, although it formed the same structure. Based on the KGA crystal structure presented here, we hypothesized that the ANK repeats located between the N-terminal domains prevent KGA from forming longer higher active filaments due to the fact that it destabilizes the polymerization. More specifically, the ANK dimers limit the association of the KGA tetramers into long polymers (23) because they limit the formation and availability of the filamentation interface (Fig. 4D). This interface is the groove region by which the single strand filaments of the GAC

isoform grows indefinitely, via end to end interaction between pairs of N-terminal domains (23).

To further confirm this hypothesis, we generated a C terminally truncated GLS construct containing the common sequence between KGA and GAC (N terminus and glutaminase domains, called ΔC), but lacking the ANK repeats. Accordingly, the construct had a larger Stokes radius in comparison to KGA and GAC and was more heterogeneous in size (Fig. 4E). Moreover, as expected, the ΔC construct was as active as GAC, possibly due to its increased ability to form extended filaments (Fig. 4F).

Low resolution structure of GLS2

Last, the availability of the crystal structure of individual domains of GAB/LGA, such as the C-terminal ANK dimers reported here and the glutaminase domain tetramer (PDB code 4bqm), allowed for a proposition of the full-length structure based on small-angle X-ray scattering data (Fig. 5, A and B) for the multidomain portion common to both isoforms. The C-terminal ANK domains are expected to be spatially located between the N-terminal domains, in an organization similar to that observed for KGA. Consequently, this protein is also expected not to assemble in filament-like higher order oligomers, being stable at the tetramer form. This has been previously observed by our group (23) and is here corroborated by measurements on cryo-electron microscopy images (Fig. 5C).

Discussion

The human genome has two copies of glutaminase-coding genes, which are known to produce at least four isoforms through alternative splicing or transcription at alternative initiation sites (4). Genetic evolutionary models often predict an increase in the gene copy number prior to the specialization or gain of new functions, with a momentary intermediate state of specialization with an overlap of functions (38). In addition, this apparent redundancy in glutaminase isoforms can be explained by differential tissue expression patterns, as well as the cell proliferation state. In this regard, KGA is expressed in kidney tubule epithelial cells, in which ammonia production is key to renal acid-base regulation (16); LGA is expressed in periportal hepatocytes, where it participates in urea synthesis (17). In addition, although GAC meets the glutamine-dependent metabolic demands of several types of cancer (20), GAB has tumor-suppressing activity in hepatocarcinomas and gliomas (40).

Despite this functional diversity, the human glutaminase isoforms converge into a conserved multidomain structure. Based on a combination of computational predictions and experimental evidence mainly produced by X-ray crystallography studies, we found that all four isoforms contain an N-terminal EF-hand-like domain, followed by a glutaminase domain in the middle and three ANK repeats at the C terminus. The only exception is GAC, in which the C terminus is a short

The multidomain architecture of human glutaminases

Figure 5. Low-resolution solution model for full-length GLS2 (GAB/LGA). Intensity (left panel) and pair-distance (right panel) distribution functions, obtained for GAB/LGA subject to SAXS experiments. *B*, orthogonal views of the superposition between the SAXS envelope and the collection of X-ray crystal structures from GLS2 glutaminase domain (PDB code 4BQM), N-terminal portion of human GLS (PDB code 3CZD) and GAB/LGA.ANK. *C*, box plot of the two perpendicular dimensions for GAB/LGA, large (L) and short (S) as taken from the cryo-EM micrographs (inset). KGA dimer and tetramer crystal structures projections were used as a reference for the dimensions of these two oligomeric states.

unstructured region, because of alternative splicing of the *GLS* gene.

In this work, the multidomain architecture was shown to be shared by all chordates, including fishes, reptiles, birds, and amphibians, as well as by arthropods and nematodes. By surveying the database-deposited glutaminase protein sequences, we observed similar organizational characteristics in free-living proteo- and cyanobacteria, but not fungi. From our data, we propose that the multidomain glutaminase structure has a very ancient origin. In addition, we verified that multiple *GLS* genes and isoforms are present in a wide range of vertebrates. By further analyzing the genomes of representative species of the phylum Chordata, we were able to identify early events of exon repositioning among tunicates and cephalochordates, followed by gene duplication in Hyperoartia and exon retrotransposition in Chondrichthyes, concomitant with the change from a simple kidney structure pronephros to a more complex mesonephros with tubules (41). Together, all these features are likely the basis of the isozyme diversity observed in vertebrates. We also identified a glutaminase ancestor that was more similar to the human kidney-type glutaminase and gave rise to the liver-type glutaminase, whereas the appearance of GAC-like glutaminases, with shorter C termini, was a late event in glutaminase evolution. GAC is the most active isoform compared with KGA and LGA, a feature linked to its capacity to assemble into long filament-like superstructures (23). In the present study, we found that this feature is possible only because GAC lacks the original C-terminal bulky ANK repeats of parental KGA, because the Δ C-terminal construct assembles in longer fila-

ments and is as active as GAC. Therefore, we propose that filament formation is a gain-of-function characteristic of vertebrate glutaminases, which has been positively selected to create a more active enzyme due to the substrate channeling effect.

Our group and others have previously published the structure of the N-terminal EF-hand-like and glutaminase domains of GLS (20, 42), but the structure of the C-terminal ANK repeats remained unsolved. Here, we provide novel crystal structures for the ANK domains of KGA and GLS2, which surprisingly form an atypical dimer. ANK repeats usually mediate the interaction of a protein with a different partner. However, KGA.ANK and GLS2.ANK mediated the formation of homooligomers, a rare feature. A survey of structures in the Protein Data Bank revealed 68 unique crystal structures of ANK-containing proteins, containing between 2 to 24 ANK repeats (supplemental Table S1). Of these crystal structures, only 5 displayed an ANK-to-ANK association (supplemental Fig. S1). However, none contained an interface that resembles the glutaminase ANK dimer (contact between the DYD and DRW motifs). Therefore, we propose that the ANK dimer association described here is structurally unique to glutaminases and was selected as a conserved feature throughout glutaminase evolution. The explanation for this positive selection remains elusive.

The short regions located immediately after the ANK repeats in KGA and GLS2 (KEN and ESMV motif, respectively) are involved in E3 ubiquitin ligase-dependent degradation (8) and direct interactions with PDZ domain-containing proteins (43), respectively. An analysis of protein complexes including ANK-containing proteins and their interaction partners (44–47)

showed that most of the contacts necessary for ANK-to-ANK interaction involves the ANK groove (the concave surface). Although most of the known cases involve the concave surface, some ANK repeats mediate protein-protein contacts through the convex face. One example is the human oncoprotein g-ankyrin, which was crystallized in complex with an antibody fragment. In this structure, the interaction occurred through the outer α -helices of the ANK4-ANK6 repeats (48). The vaccinia virus K1 protein consists entirely of ANK repeats that are involved in interactions mediated by the convex surface (49). In addition, the VPS9-domain ANK repeat protein binds to Rab32 through the convex side of its ANK repeat (50).

In this regard, although the concave faces of the ANK repeats of KGA and GLS2 make contacts within the glutaminase, we predict that the ANK repeats may still mediate protein-protein interactions through the convex surface. Recently, GAB has been shown to bind and inhibit the small GTPase Rac1 by preventing its interaction with a guanine-nucleotide exchange factor, an interaction that involves the C terminus of GLS2 (residues 464 to 602) (40). Because the region contains the ANK repeats of GAB, we predict that the contact involves the ANK concave face. Considering the long list of partners that were shown to interact with glutaminases using mass spectrometry-based approaches (51), further investigations are required to confirm the mechanism by which the ANK repeats in glutaminases mediate protein-protein interactions.

Last, although at low resolution, the novel ANK-containing structure of human KGA confirms our previous hypothesis that the long-range propagation of filaments for this isoform was thwarted by the presence of the ankyrin repeats themselves (23). As observed in the crystallographic model, the ANK dimer occludes the formation of the filamentation groove, by which the single strand filament of the GAC isoform grows indefinitely, via end to end interaction between pairs of N-terminal domains. Because GAC possesses a shorter unstructured C-terminal sequence, this hypothesis is confirmed when the ANK domain is completely deleted in a mutant construct, which can form longer polymeric species, and therefore, resulting in a more active protein.

Experimental procedures

Phylogenetic reconstruction

726 Glutaminase protein sequences obtained from GenBankTM (52), by performing a BLAST search with human glutaminase domain sequence from *GLS* as the query, were aligned using kalign (53). Maximum likelihood phylogenetic reconstruction was performed with RaxML (54) with 260 bootstrap pseudoreplicates and using automated model search. Replicates were summarized with sumtrees from DendroPy (55). The generated phylogenetic tree and respective alignment were used to reconstruct ancestral states for specific nodes using maximum parsimony, as implemented in phangorn (56). Heatmaps from pairwise distances were constructed in SDT (57) using MUSCLE (58) alignment.

Evolution of glutaminase exon structure

Protein sequence corresponding to human *GLS* exon 14 was used as input of translated blast (tblastn) searches against avail-

able genomes from *C. intestinalis* (27), *B. floridae* (28), *P. marinus* (29), *C. milii* (30), *D. rerio* (31), and *X. tropicalis* (32). After an initial Reciprocal Best Hit (59) region was found, the correct position of the exon was determined by pairwise LALIGN between sequences (60). Regions downstream from the genomic sequence homologous to exon 14 were also evaluated by LALIGN against human GAC-exclusive exon (exon 15). As both *P. marinus* glutaminases are incomplete, available exons were found in contig using the LALIGN approach. The *GLS* region comprising intron 14, exon 15, and intron 15 was used as input of TranspoGene (61) retrotransposon searching.

Protein expression and purification

The constructs of human KGA Val¹²⁴-Leu⁶⁶⁹ and Val⁵⁵¹-Leu⁶⁶⁹ were amplified from the pcDNA3.1/hKGA-V5 clone, kindly provided by Dr. Richard Cerione (Cornell University, Ithaca, NY), and subcloned into the pET28a plasmid (Novagen) using the NdeI and XhoI restriction sites with N-terminal His₆ tag. KGA Val¹²⁴-Arg⁵⁵⁰ was generated from the pET28a KGA Val¹²⁴-Leu⁶⁶⁹ construct by site-directed mutagenesis of the Val⁵⁵¹ residue into a stop codon (TAA) using the QuikChange II XL Site-directed Mutagenesis Kit (Agilent Technologies). The construct of human GAB Lys⁴⁸⁵-Val⁶⁰² was cloned into pNIC28-Bsa4 plasmid. The construct of GAC Met¹²⁸-Ser⁶⁰³ was amplified from a mouse fetal brain tissue cDNA library and cloned into the pET28a plasmid. The four constructs were transformed into *Escherichia coli* Rosetta-2 thermocompetent cells (Merck). Overnight cultures, grown in LB medium in the presence of 50 $\mu\text{g ml}^{-1}$ of kanamycin and 50 $\mu\text{g ml}^{-1}$ of chloramphenicol, were inoculated in a ratio of 1:100 in 3-liter cultures supplemented with the same antibiotics and left shaking at 200 rpm for 5 h at 37 °C. The cultures were then down-tempered to 18 °C for 1 h before induction with 200 nM isopropyl β -D-1-thiogalactopyranoside for 16 h at 18 °C. Cells were collected by rapid centrifugation and resuspended in 500 mM NaCl, 50 mM Tris HCl, pH 8.5 (or 50 mM HEPES, pH 7.5, for GAB construct), 10% glycerol, and 2 mM β -mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride). Cell lysis was performed chemically, by incubation with hen egg white lysozyme, DNase I, and deoxycholate (all three reagents from Sigma) for about 1 h, incubated on ice. The soluble fractions were separated from the debris by high speed centrifugation and subsequently loaded, by gravity and in a cold room, on immobilized metal affinity column, Co²⁺-charged TALON (Clontech) for the GLS constructs and nickel-nitrilotriacetic acid Superflow (Qiagen) for the GAB construct, previously equilibrated with the running buffer 10 mM NaCl and 50 mM Tris-HCl, pH 8.5, HEPES, pH 7.5. The constructs were eluted stepwise using running buffer to which up to 500 mM imidazole (v/v) had been added. For the GLS constructs, the tag was removed by overnight digestion with bovine thrombin (Sigma) and the samples loaded into a HiTrap Q HP anion exchange chromatography column (GE Healthcare). Elution was done by performing a linear gradient with a buffer containing 1 M NaCl, 50 mM Tris-HCl, pH 8.5, and 2 mM β -mercaptoethanol. The fractions containing the GLS constructs were loaded in a HiLoad 16/600 Superdex 200 (for KGA Val¹²⁴-Leu⁶⁶⁹, KGA Val¹²⁴-Arg⁵⁵⁰, and GAC Met¹²⁸-Ser⁶⁰³ constructs) or 75 pg (GE

The multidomain architecture of human glutaminases

Healthcare) (for KGA Val⁵⁵¹-Leu⁶⁶⁹). The final buffer conditions were 150 mM NaCl, 30 mM HEPES, pH 8, and 0.5 mM tris(2-carboxyethyl)phosphine). For the GAB construct, the eluate from the affinity chromatography was directly loaded in a HiLoad 16/600 75 pg. Protein concentration was determined by UV 280 nm using calculated extinction coefficients. The hydrodynamic parameters (Stokes radii, R_h) of human KGA, GAC, and the deletion mutant ΔC were determined by gel filtration chromatography using a prepacked Superdex 200 HR 10/30 column (GE Healthcare) in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine). For each purified protein, $\sim 1 \text{ mg ml}^{-1}$ concentration in a 500- μl volume was injected into the column. To induce the formation of the higher-order oligomeric species, 20 mM K_2HPO_4 as a final concentration was added in the protein solution before loading it onto the column. The gel filtration buffer was also supplemented with 20 mM K_2HPO_4 . The flow rate was maintained at 0.5 ml min^{-1} . To establish the hydrodynamic radius (R_h) and elution volume relationship of the protein, proteins of known R_h were run on the same column. The proteins used as standards were ferritin (440 kDa, $R_h = 60.8 \text{ \AA}$), aldolase (158 kDa, $R_h = 48.1 \text{ \AA}$), conalbumin (75 kDa, $R_h = 36.4 \text{ \AA}$), ovalbumin (44 kDa, $R_h = 30.5 \text{ \AA}$), and ribonuclease A (13.7 kDa, $R_h = 15.9 \text{ \AA}$).

Crystallization

Following size-exclusion purification, KGA Val¹²⁴-Leu⁶⁶⁹ was concentrated using an Amicon 30-kDa cutoff concentrator (Millipore) to a final concentration of $\sim 7.5 \text{ mg ml}^{-1}$. Crystallization experiments were performed at 277 K using the conventional sitting drop vapor diffusion technique. Drops were made by mixing two parts of protein previously incubated with 1.2 mM BPTES to one part of the well solution, containing 1.8 M sodium formate, 0.5 M NaCl, and 0.1 M BIS-TRIS propane, pH 6.8. Before data collection at cryogenic temperature (100 K), harvested crystals were cryoprotected with 10% ethylene glycol added to the mother liquor.

For large-scale limited proteolysis, KGA Val⁵⁵¹-Leu⁶⁶⁹ after a size-exclusion purification step was incubated with 1:100 trypsin (Sigma) at 23 °C for 20 min. The proteolysis was stopped by the addition of 1.5 mM PMSF. For limited proteolysis of GAB Lys⁴⁸⁵-Val⁶⁰², purified protein was incubated overnight with 1:1000 α -chymotrypsin at 23 °C. The digested fragments were immediately purified by size-exclusion chromatography. For crystallization trials, digested KGA Val⁵⁵¹-Leu⁶⁶⁹ and GAB Lys⁴⁸⁵-Val⁶⁰² were concentrated, respectively, to 25 and 50 mg ml^{-1} using Amicon 10-kDa cutoff concentrators.

Both constructs were crystallized by sitting drop vapor diffusion, by mixing equal parts of protein solution and mother liquor. KGA Val⁵⁵¹-Leu⁶⁶⁹ crystals were grown in (a) 3.2 M NaCl, 0.1 M Tris, pH 8.5 and (b) 3.8 M NH_4Cl , 0.1 M BIS-TRIS propane, pH 7. GAB Lys⁴⁸⁵-Val⁶⁰² crystals were grown in 1.1 M trisodium citrate, 0.1 M imidazole, pH 8, 20 mM L-glutamine.

X-ray crystallography

Diffraction data were collected at beamlines I03 at the Diamond Light Source (UK) and 12-2 at the Stanford Synchrotron Radiation Lightsource, respectively, for KGA Val¹²⁴-Leu⁶⁶⁹ and

for KGA/GAB ANK crystals. Datasets were integrated using Mosflm (62) (for native datasets) and XDS (63) (for sulfur-SAD dataset) and scaled with Aimless (64). The first set of phases of KGA Val¹²⁴-Leu⁶⁶⁹ was obtained by the molecular replacement technique as implemented in the program Phaser (65), using the coordinates of the mouse GAC isoform (PDB code 3SS3). KGA ANK was solved by sulfur-SAD using SHELX (66) and the model was refined using higher resolution native datasets. The model obtained for KGA was employed as a search model for solving the GAB ANK structure by molecular replacement (Table 1). Positional and B -factor refinement cycles, as well as solvent modeling, were performed with Refmac (67) and Phenix (68), followed by visual inspection using COOT (69).

Glutaminase activity assay

To obtain the kinetic parameters for KGA Val¹²⁴-Leu⁶⁶⁹, KGA Val¹²⁴-Arg⁵⁵⁰, and GAC Met¹²⁸-Ser⁶⁰³, a mixture containing 10 nM glutaminase, 50 mM Tris acetate, pH 8.6, 3 units of bovine L-glutamate dehydrogenase (Sigma), 2 mM NAD (Sigma) was pipetted into 96-well plates previously filled with 6 or 12 serial dilutions of L-glutamine, to achieve a range of concentrations from 60 to 0.15 mM. K_2HPO_4 (2 M stock, pH 9.4) was added to the mixture at a final concentration of 20 mM. The formation of NADH was tracked by absorbance readings at 340 nm, for up to five consecutive minutes, at room temperature. Measurements were done in triplicate. The initial velocities, in picomoles of NADH produced per second, were calculated using an extinction coefficient for NADH of $6,220 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm and 0.5 cm of path length. The total volume per reaction was 200 μl . Plate-reader used was an EnSpire (PerkinElmer Life Sciences). Measurements were done in triplicates and analyzed using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA).

Small-angle X-ray scattering

Scattering data were collected at $\lambda = 1.488 \text{ \AA}$, for sample-detector distances of 1.1 m covering the momentum transfer ranges $0.015 < s < 0.442 \text{ \AA}^{-1}$ ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). The data were normalized to the intensity of the incident beam and corrected for the detector response using an in-house program. Two frames of 250 s were collected and compared for radiation damage using the program PRIMUS (70). The same program was used to average the frames and subtract the buffer. The different protein concentrations were evaluated for aggregation by following increases in the measured R_g (radius of gyration) as calculated by auto R_g . The R_g was confirmed by using the indirect Fourier transform program GNOM (71), which was also used to calculate the distribution function $P(r)$ and D_{max} . The data were analyzed and processed, including *ab initio* construction and model averaging, using the programs contained in the ATSAS package (72).

Cryo-electron microscopy

For visualization of cryogrids, purified GAB samples were frozen onto a Gatan 626 sample holder, prepared with FEI Vitro-robot Mark II (force of -5 for 2s). Images were acquired using a JEM 2100 (200 kV) electron microscope with a LaB6 filament Gatan 4k $\times$ 4k slow scan CCD camera (US4000). Exposure time

was 1 s/frame, with a dose of $20 \text{ e} \text{ \AA}^{-2} \text{ s}^{-1}$. The micrographs were processed using IMAGIC (73) and EMAN 2.1 (74). Iterative stable alignment and clustering (ISAC) (75) was used to generate reference-free class-averages from both the IMAGIC stack (25,000 particles) and the EMAN2.1 stack (33,608 particles). Using IMAGIC and UCSF Chimera (76), 10,000 projection images in random orientations of the GAC crystallographic model (PDB code 3SS3) were generated, in both dimer and tetramer configurations. The bounding rectangle dimensions of 200 particles randomly extracted from the IMAGIC, EMAN2.1, ISAC/IMAGIC, and ISAC/EMAN2.1 datasets (50 from each) were classified according to longer (L) and shorter (S) dimensions. Images were low-pass filtered to reduce noise. Only the particles contained in the 50 “best” class averages generated by each program were considered (total: 200 particles). Measurements were performed in Digital Micrograph based on the integration profile across each perpendicular direction, as shown in Fig. 5C. Measurements were taken as the distance, in pixels, between the valleys confining the particle signal. For comparison, we also measured 200 randomly selected projections of the GAC atomic model in dimer and also in tetramer configuration.

Author contributions—S. M. G. D. and A. L. B. A. conceived and initiated the research; C. C. P., Z. I., D. A., I. M. F., R. D. R., J. B., R. V. P., and A. G. conducted the research; all authors analyzed and discussed the data; C. C. P., Z. I., D. A., S. M. G. D., and A. L. B. A. wrote the manuscript. All authors revised and approved the final manuscript.

Acknowledgments—We thank LNBio for accessibility to core facilities as well as for financial support. We are very grateful to Dr. Alessandra Girasole for expert technical support. We thank the Biotechnology group at the SGC Oxford for cloning and expression testing of glutaminase constructs. We thank Dr. Artur T. Cordeiro and Américo T. Ranzani for help with crystallographic data collection. We thank Dr. Wah Chiu for access to the cryo-electron microscopy facility at Baylor College of Medicine. We acknowledge the staff of all the beamlines used in this work. The KGA.ANK and GLS2.ANK data were collected during the RapiData 2015 Course for Data Collection and Structure Solving. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the United States Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and National Institutes of Health, National Institute of General Medical Sciences Grant P41GM103393.

References

- Bergström, J., Fürst, P., Norée, L. O., and Vinnars, E. (1974) Intracellular free amino acid concentration in human muscle tissue. *J. Appl. Physiol.* **36**, 693–697
- DeBerardinis, R. J., and Cheng, T. (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* **29**, 313–324
- Levine, A. J., and Puzio-Kuter, A. M. (2010) The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* **330**, 1340–1344
- Altman, B. J., Stine, Z. E., and Dang, C. V. (2016) From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat. Rev. Cancer* **16**, 619–634
- Wise, D. R., DeBerardinis, R. J., Mancuso, A., Sayed, N., Zhang, X.-Y., Pfeiffer, H. K., Nissim, I., Daikhin, E., Yudkoff, M., McMahon, S. B., and Thompson, C. B. (2008) Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 18782–18787
- Rathore, M. G., Saumet, A., Rossi, J.-F., de Bettignies, C., Tempé, D., Lecellier, C.-H., and Villalba, M. (2012) The NF- κ B member p65 controls glutamine metabolism through miR-23a. *Int. J. Biochem. Cell Biol.* **44**, 1448–1456
- Wang, J. B., Erickson, J. W., Fuji, R., Ramachandran, S., Gao, P., Dinavahi, R., Wilson, K. F., Ambrosio, A. L., Dias, S. M., Dang, C. V., and Cerione, R. A. (2010) Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* **18**, 207–219
- Colombo, S. L., Palacios-Callender, M., Frakich, N., De Leon, J., Schmitt, C. A., Boorn, L., Davis, N., and Moncada, S. (2010) Anaphase-promoting complex/cyclosome-Cdh1 coordinates glycolysis and glutaminolysis with transition to S phase in human T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 18868–18873
- Lukey, M. J., Greene, K. S., Erickson, J. W., Wilson, K. F., and Cerione, R. A. (2016) The oncogenic transcription factor c-Jun regulates glutaminase expression and sensitizes cells to glutaminase-targeted therapy. *Nat. Commun.* **7**, 11321
- Suzuki, S., Tanaka, T., Poyurovsky, M. V., Nagano, H., Mayama, T., Ohkubo, S., Lokshin, M., Hosokawa, H., Nakayama, T., Suzuki, Y., Sugano, S., Sato, E., Nagao, T., Yokote, K., Tatsuno, I., and Prives, C. (2010) Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 7461–7466
- Hu, W., Zhang, C., Wu, R., Sun, Y., Levine, A., and Feng, Z. (2010) Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 7455–7460
- Szeliga, M., and Albrecht, J. (2015) Opposing roles of glutaminase isoforms in determining glioblastoma cell phenotype. *Neurochem. Int.* **88**, 6–9
- Szeliga, M., Bogacińska-Karaś, M., Różycka, A., Hilgier, W., Marquez, J., and Albrecht, J. (2014) Silencing of GLS and overexpression of GLS2 genes cooperate in decreasing the proliferation and viability of glioblastoma cells. *Tumour Biol.* **35**, 1855–1862
- Liu, J., Zhang, C., Lin, M., Zhu, W., Liang, Y., Hong, X., Zhao, Y., Young, K. H., Hu, W., and Feng, Z. (2014) Glutaminase 2 negatively regulates the PI3K/AKT signaling and shows tumor suppression activity in human hepatocellular carcinoma. *Oncotarget* **5**, 2635–2647
- Krebs, H. (1935) Metabolism of amino-acids: The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. *Biochem. J.* **29**, 1951–1969
- Curthoys, N. P., Kuhlenschmidt, T., Weiss, R. F., and Godfrey, S. S. (1976) Phosphate-dependent activity glutaminase from rat kidney and identity with glutaminase in response to acidosis from other tissues. *Arch. Biochem. Biophys.* **172**, 162–167
- Curthoys, N. P., and Watford, M. (1995) Regulation of glutaminase activity and glutamine metabolism. *Annu. Rev. Nutr.* **15**, 133–159
- Elgadi, K. M., Meguid, R. A., Qian, M., Souba, W. W., and Abcouwer, S. F. (1999) Cloning and analysis of unique human glutaminase isoforms generated by tissue-specific alternative splicing. *Physiol. Genomics* **1**, 51–62
- Martín-Rufián, M., Tosina, M., Campos-Sandoval, J. A., Manzanares, E., Lobo, C., Segura, J. A., Alonso, F. J., Matés, J. M., and Márquez, J. (2012) Mammalian glutaminase *Gls2* gene encodes two functional alternative transcripts by a surrogate promoter usage mechanism. *PLoS ONE* **7**, e38380
- Cassago, A., Ferreira, A. P., Ferreira, I. M., Fornezari, C., Gomes, E. R., Greene, K. S., Pereira, H. M., Garratt, R. C., Dias, S. M., and Ambrosio, A. L. (2012) Mitochondrial localization and structure-based phosphate activation mechanism of glutaminase C with implications for cancer metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1092–1097
- Campos-Sandoval, J. A., López de la Oliva, A. R., Lobo, C., Segura, J. A., Matés, J. M., Alonso, F. J., and Márquez, J. (2007) Expression of functional human glutaminase in baculovirus system: affinity purification, kinetic and molecular characterization. *Int. J. Biochem. Cell Biol.* **39**, 765–773

The multidomain architecture of human glutaminases

22. Olalla, L., Gutiérrez, A., Campos, J. A., Khan, Z. U., Alonso, F. J., Segura, J. A., Márquez, J., and Aledo, J. C. (2002) Nuclear localization of L-type glutaminase in mammalian brain. *J. Biol. Chem.* **277**, 38939–38944
23. Ferreira, A. P., Cassago, A., Gonçalves Kde, A., Dias, M. M., Adamoski, D., Ascensão, C. F., Honorato, R. V., de Oliveira, J. F., Ferreira, I. M., Fornezari, C., Bettini, J., Oliveira, P. S., Paes Leme, A. F., Portugal, R. V., Ambrosio, A. L., and Dias, S. M. (2013) Active glutaminase C self-assembles into a supratetrameric oligomer that can be disrupted by an allosteric inhibitor. *J. Biol. Chem.* **288**, 28009–28020
24. Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C. F., Joachimski, A., Savchenko, A., and Yakunin, A. F. (2008) Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*. *Biochemistry* **47**, 5724–5735
25. de Beer, T., Carter, R. E., Lobel-Rice, K. E., Sorkin, A., and Overduin, M. (1998) Structure and Asn-Pro-Phe binding pocket of the Eps15 homology domain. *Science* **281**, 1357–1360
26. Pace, N. R. (2009) Mapping the tree of life: progress and prospects. *Microbiol. Mol. Biol. Rev.* **73**, 565–576
27. Dehal, P., Satou, Y., Campbell, R. K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D. M., Harafuji, N., Hastings, K. E., Ho, I., Hotta, K., Huang, W., et al. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* **298**, 2157–2167
28. Putnam, N. H., Butts, T., Ferrier, D. E., Furlong, R. F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J.-K., Benito-Gutiérrez, E. L., Dubchak, I., Garcia-Fernández, J., Gibson-Brown, J. J., Grigoriev, I. V., et al. (2008) The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453**, 1064–1071
29. Smith, J. J., Kuraku, S., Holt, C., Sauka-Spengler, T., Jiang, N., Campbell, M. S., Yandell, M. D., Manousaki, T., Meyer, A., Bloom, O. E., Morgan, J. R., Buxbaum, J. D., Sachidanandam, R., Sims, C., Garruss, A. S., et al. (2013) Sequencing of the sea lamprey (*Petromyzon marinus*) genome provides insights into vertebrate evolution. *Nat. Genet.* **45**, 415–421
30. Venkatesh, B., Lee, A. P., Ravi, V., Maurya, A. K., Lian, M. M., Swann, J. B., Ohta, Y., Flajnik, M. F., Sutoh, Y., Kasahara, M., Hoon, S., Gangu, V., Roy, S. W., Irimia, M., Korzh, V., et al. (2014) Elephant shark genome provides unique insights into gnathostome evolution. *Nature* **505**, 174–179
31. Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., et al. (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**, 498–503
32. Hellsten, U., Harland, R. M., Gilchrist, M. J., Hendrix, D., Jurka, J., Kapitonov, V., Ovcharenko, I., Putnam, N. H., Shu, S., Taher, L., Blitz, I. L., Blumberg, B., Dichmann, D. S., Dubchak, I., Amaya, E., et al. (2010) The genome of the Western clawed frog *Xenopus tropicalis*. *Science* **328**, 633–636
33. Sedgwick, S. G. and Smerdon, S. J. (1999) The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem. Sci.* **24**, 311–316
34. Mosavi, L. K., Cammett, T. J., Desrosiers, D. C., and Peng, Z.-Y. (2004) The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci.* **13**, 1435–1448
35. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* **372**, 774–797
36. Robinson, M. M., McBryant, S. J., Tsukamoto, T., Rojas, C., Ferraris, D. V., Hamilton, S. K., Hansen, L. C., and Curthoys, N. P. (2007) Novel mechanism of inhibition of rat kidney-type glutaminase by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES). *Biochem. J.* **406**, 407–414
37. Drozdetskiy, A., Cole, C., Procter, J., and Barton, G. J. (2015) JPred4: a protein secondary structure prediction server. *Nucleic Acids Res.* **43**, W389–W394
38. Bergthorsson, U., Andersson, D. I., and Roth, J. R. (2007) Ohno's dilemma: evolution of new genes under continuous selection. *Proc. Natl. Acad. Sci.* **104**, 17004–17009
39. Mosavi, L. K., Minor, D. L., Jr., and Peng, Z.-Y. (2002) Consensus-derived structural determinants of the ankyrin repeat motif. *Proc. Natl. Acad. Sci.* **99**, 16029–16034
40. Zhang, C., Liu, J., Zhao, Y., Yue, X., Zhu, Y., Wang, X., Wu, H., Blanco, F., Li, S., Haffty, B. G., Hu, W., and Feng, Z. (2016) Glutaminase 2 is a novel negative regulator of small GTPase Rac1 and mediates p53 function in suppressing metastasis. *Elife* **5**, e10727
41. Scott, A. P. (1987) Reproductive endocrinology of fish. In *Fundamentals of Comparative Vertebrate Endocrinology* (Chester-Jones, I., Ingleton, P. M., Philips, and J. G., eds), pp. 223–256, Springer US, New York, NY
42. DeLaBarre, B., Gross, S., Fang, C., Gao, Y., Jha, A., Jiang, F., Song, J. J., Wei, W., and Hurov, J. B. (2011) Full-length human glutaminase in complex with an allosteric inhibitor. *Biochemistry* **50**, 10764–10770
43. Olalla, L., Gutiérrez, A., Jiménez, A. J., López-Téllez, J. F., Khan, Z. U., Pérez, J., Alonso, F. J., de la Rosa, V., Campos-Sandoval, J. A., Segura, J. A., Aledo, J. C., and Márquez, J. (2008) Expression of the scaffolding PDZ protein glutaminase-interacting protein in mammalian brain. *J. Neurosci. Res.* **86**, 281–292
44. Russo, A. A., Tong, L., Lee, J.-O., Jeffrey, P. D., and Pavletich, N. P. (1998) Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. *Nature* **395**, 237–243
45. Chiswell, B. P., Zhang, R., Murphy, J. W., Boggon, T. J., and Calderwood, D. A. (2008) The structural basis of integrin-linked kinase-PINCH interactions. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 20677–20682
46. Takeda, S., Minakata, S., Koike, R., Kawahata, I., Narita, A., Kitazawa, M., Ota, M., Yamakuni, T., Maeda, Y., and Nitani, Y. (2010) Two distinct mechanisms for actin capping protein regulation: steric and allosteric inhibition. *PLoS Biol.* **8**, e1000416
47. Holzer, S., Ban, N., and Klinge, S. (2013) Crystal structure of the yeast ribosomal protein rpS3 in complex with its chaperone Yarl1. *J. Mol. Biol.* **425**, 4154–4160
48. Robin, G., Sato, Y., Desplancq, D., Rochel, N., Weiss, E., and Martineau, P. (2014) Restricted diversity of antigen binding residues of antibodies revealed by computational alanine scanning of 227 antibody–antigen complexes. *J. Mol. Biol.* **426**, 3729–3743
49. Li, Y., Meng, X., Xiang, Y., and Deng, J. (2010) Structure function studies of vaccinia virus host range protein k1 reveal a novel functional surface for ankyrin repeat proteins. *J. Virol.* **84**, 3331–3338
50. Hesketh, G. G., Pérez-Dorado, I., Jackson, L. P., Wartosch, L., Schäfer, I. B., Gray, S. R., McCoy, A. J., Zeldin, O. B., Garman, E. F., Harbour, M. E., Evans, P. R., Seaman, M. N., Luzio, J. P., and Owen, D. J. (2014) VARP is recruited on to endosomes by direct interaction with retromer, where together they function in export to the cell surface. *Dev. Cell.* **29**, 591–606
51. Huttlin, E. L., Ting, L., Bruckner, R. J., Gebreab, F., Gygi, M. P., Szpyt, J., Tam, S., Zarraga, G., Colby, G., Baltier, K., Dong, R., Guarani, V., Vaites, L. P., Ordureau, A., Rad, R., et al. (2015) The BioPlex network: a systematic exploration of the human interactome. *Cell* **162**, 425–440
52. Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. (2016) GenBank. *Nucleic Acids Res.* **44**, D67–D72
53. Lassmann, T., and Sonnhammer, E. L. (2005) Kalign: an accurate and fast multiple sequence alignment algorithm. *BMC Bioinformatics* **6**, 298
54. Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313
55. Sukumaran, J., and Holder, M. T. (2010) DendroPy: a Python library for phylogenetic computing. *Bioinformatics* **26**, 1569–1571
56. Schliep, K. P. (2011) phangorn: phylogenetic analysis in R. *Bioinformatics* **27**, 592–593
57. Muhire, B. M., Varsani, A., and Martin, D. P. (2014) SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS ONE* **9**, e108277
58. Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797
59. Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997) A genomic perspective on protein families. *Science* **278**, 631–637
60. Huang, X., and Miller, W. (1991) A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math.* **12**, 337–357

61. Levy, A., Sela, N., and Ast, G. (2008) TranspoGene and microTranspoGene: transposed elements influence on the transcriptome of seven vertebrates and invertebrates. *Nucleic Acids Res.* **36**, D47–D52
62. Leslie, A. G. W., and Powell H. R. (2007) Processing diffraction data with mosflm. In *Evolving methods for macromolecular crystallography* (Read, R. J., and Sussman, J. L., eds), pp. 41–51, Springer, Dordrecht, Netherlands
63. Kabsch, W. (2010) XDS. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 125–132
64. Evans, P. R., and Murshudov, G. N. (2013) How good are my data and what is the resolution? *Acta Crystallogr. D Biol. Crystallogr.* **69**, 1204–1214
65. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674
66. Sheldrick, G. M. (2010) Experimental phasing with SHELXC/D/E: combining chain tracing with density modification. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 479–485
67. Vagin, A. A., Steiner, R. A., Lebedev, A. A., Potterton, L., McNicholas, S., Long, F., and Murshudov, G. N. (2004) REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2184–2195
68. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L.-W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221
69. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501
70. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* **36**, 1277–1282
71. Svergun, D. I. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Crystallogr.* **25**, 495–503
72. Petoukhov, M. V., Konarev, P. V., Kikhney, A. G., and Svergun, D. I. (2007) ATSAS 2.1: towards automated and web-supported small-angle scattering data analysis. *J. Appl. Crystallogr.* **40**, s223–s228
73. van Heel, M., Portugal, R., Rohou, A., Linnemayr, C., Bebeacua, C., Schmidt, R., Grant, T., and Schatz, M. (2012) Four-dimensional cryo-electron microscopy at quasi-atomic resolution: IMAGIC 4D. *Int. Tables. Acta Crystallogr.* **Vol. F**, ch. 19.9, 624–628
74. Tang, G., Peng, L., Baldwin, P. R., Mann, D. S., Jiang, W., Rees, I., and Ludtke, S. J. (2007) EMAN2: An extensible image processing suite for electron microscopy. *J. Struct. Biol.* **157**, 38–46
75. Yang, Z., Fang, J., Chittuluru, J., Asturias, F. J., and Penczek, P. A. (2012) Iterative stable alignment and clustering of 2D transmission electron microscope images. *Structure* **20**, 237–247
76. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera: a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612

SCIENTIFIC REPORTS

OPEN

Unsaturated fatty acids as high-affinity ligands of the C-terminal Per-ARNT-Sim domain from the Hypoxia-inducible factor 3 α

Received: 26 March 2015

Accepted: 07 July 2015

Published: 03 August 2015

Angela M. Fala^{1,*}, Juliana F. Oliveira^{1,*}, Douglas Adamoski¹, Juliana A. Aricetti², Marília M. Dias¹, Marcio V. B. Dias^{1,†}, Maurício L. Sforça¹, Paulo S. Lopes-de-Oliveira¹, Silvana A. Rocco¹, Camila Caldana^{2,3}, Sandra M. G. Dias^{1,†} & Andre L. B. Ambrosio^{1,†}

Hypoxia-inducible transcription factors (HIF) form heterodimeric complexes that mediate cell responses to hypoxia. The oxygen-dependent stability and activity of the HIF- α subunits is traditionally associated to post-translational modifications such as hydroxylation, acetylation, ubiquitination, and phosphorylation. Here we report novel evidence showing that unsaturated fatty acids are naturally occurring, non-covalent structural ligands of HIF-3 α , thus providing the initial framework for exploring its exceptional role as a lipid sensor under hypoxia.

HIF, closely linked to disorders of the circulatory system and cancer progression¹, consists of two multidomain subunits including the constitutively expressed HIF-1 β and an oxygen-labile HIF-1 α (or paralogs HIF-2 α and -3 α). The gene encoding for HIF-3 α (*HIF3A*) is exclusively susceptible to post-transcriptional processing in humans, with six isoforms shown to exist at mRNA levels and three already confirmed as functional protein^{2,3}. HIF-3 α 9, the longest (669 aa) and canonical isoform³, is a potent oxygen-labile transcription factor, tightly regulating the expression of a unique set of genes⁴. HIF-3 α 4 (363 aa), on the other hand, while insensitive to intracellular oxygen levels and incapable of transactivation, is a dominant-negative regulator of HIF-1 α ⁵. Due to the use of different transcription initiation sites and a combination of splicing events in *HIF3A*, the C-terminal Per-Arnt-Sim sensor domain (PASb) is the only common architectural feature among the HIF-3 α isoforms³. The PASb domains in both HIF- α and - β subunits have a crucial role in forming active HIF heterodimers and recruiting co-regulators^{6,7}.

PAS members are versatile sensory modules of stimuli such as oxygen tension, redox state and light intensity through binding to cofactors such as heme, flavin adenine dinucleotide and even divalent metal ions⁸. In the case of the HIF proteins, PAS domains-mediated protein-protein interactions have been shown independently of a cofactor and simply based on the presence of the partner PAS^{9,10}. However, recently, the identification of a 290 Å³ polar cavity in HIF-2 α PASb domain led to the proposition that endogenous ligands may exist¹¹; this cavity was later exploited for the selection of artificial small molecules that disrupt the full-length HIF-2 heterodimer^{10–12}.

Hereby, we present the crystal structure of the recombinant human HIF-3 α PASb, or PASb-3 α , at 1.15 Å maximum resolution (Table 1 and Supplementary Fig. 1). Although with a backbone virtually identical to that of the PASb domains of both HIF-1 α ⁹ and HIF-2 α ¹⁰ (Fig. 1a), PASb-3 α has a unique extensive C-shaped hydrophobic cavity of 510 Å³ (Fig. 1b). We identified a 1-(11Z-octadecenoyl)-sn-

¹Laboratório Nacional de Biociências and ²Laboratório Nacional de Ciência e Tecnologia do Bioetanol, Centro Nacional de Pesquisa em Energia e Materiais, Campinas, SP, Brazil, 13083-100. ³Max Plack-partner group at the Laboratório Nacional de Ciência e Tecnologia do Bioetanol. ⁴Current address: Institute of Biomedical Science, University of São Paulo, São Paulo, SP, 05508-000, Brazil. ^{*}These authors contributed equally to this work. [†]These authors jointly supervised this work. Correspondence and requests for materials should be addressed to S.M.G.D. (email: sandra.dias@lnbio.cnpem.br) or A.L.B.A. (email: andre.ambrosio@lnbio.cnpem.br)

The C-terminal Per-Arnt-Sim (PASb) domain of human HIF-3 α bound to 18:1-1-monoacylglycerol, PDB ID 4wn5			
Data Collection		Model Refinement	
Beamline	P13 at PETRA III	Resolution range (Å)	28.5–1.15
Wavelength (Å)	0.9770	Reflections (cross-validation)	68777 (5952)
Space group	P2 ₁ 2 ₂ 1	R _{factor} /R _{free} (%)	12.2/14.1
Cell parameters a, b, c (Å)	52.7, 53.9, 67.8	Average B-factor (Å ²)	
Resolution range (Å)	28.5 – 1.15 (1.17 – 1.15)	main chain (no. of atoms)	8.8 (870)
Unique reflections	68827 (3381)	side chain (no. of atoms)	13.2 (812)
Multiplicity	9.8 (9.1)	ligands (no. of atoms)	19.9 (155)
R _{pim} (%)	1.9 (5.2)	solvent (no. of atoms)	28.0 (295)
CC (½)	0.998 (0.987)		
Completeness (%)	99.3 (99.4)	R.m.s.d. from standard geometry	
<I/ σ (I)>	22.8 (11.5)	bond length (Å)	0.01
Average mosaicity (°)	0.5	bond angles (°)	1.36
B-factor Wilson Plot (Å ²)	6.7		
Monomers/AU	2	Ramachandran plot,	
Solvent content (%)	36.1	favoured (%)	100
Matthews coeff. (Å ³ /Da)	1.92		

Table 1. X-ray crystallography data collection parameters and structure refinement statistics. Data for outer shell are shown in parentheses.

glycerol molecule of bacterial nature buried in this cavity, as clearly suggested by the size and geometrical features of the electron density map (Fig. 1c, left panel). As such, the aliphatic tail of the fatty acid extended to eighteen carbons in length (maximum length limited by the cavity size), with a *cis* double bond between C11 and C12, interspersed by four gauche configurations (at positions C5, C7, C9 and C13, (Fig. 1c, right panel)). The ester group at the glycerol sn-1 position makes polar contact with the main chain carboxyl group of His339 and main chain amino group of Leu340. The sn-2 position is not substituted, also sharing a hydrogen bond with the Leu340 main chain amino group (Fig. 1d). Based solely on the electron density map, we could not tell whether the glycerol sn-3 position was substituted, due to absence of well-structured atoms beyond its remaining hydroxyl group. However, the presence of a well-coordinate sulfate ion (abundant in the crystallization solution), 6 Å apart from the glycerol sn-3 hydroxyl, suggested that PASb-3 α could also bind to phospholipids (Fig. 1d).

Aiming at experimental confirmation of the identity of the proposed monoacylglycerol, the hydrophobic fraction was carefully isolated from pure protein samples in solution and analyzed by thin-layer chromatography (TLC). By applying two mobile phases distinct in polarity, as well as dual staining to allow for differential detection of neutral and phosphorous-containing lipids¹³, we surprisingly observed the dominant presence of two neutral lipids and two phospholipids as ligands of PASb-3 α (Fig. 2a).

Accordingly, mass spectrometry analysis on the isolated hydrophobic fraction readily identified two differential peaks with *m/z* of 478.40 and 509.36 (Supplementary Fig. 2a,b), subsequently determined to be the lyso-phospholipids 1-(11Z-octadecenyl)-2-hydroxy-sn-glycero-3-phosphoethanolamine, or 18:1-LPE, and 1-(11Z-octadecenyl)-2-hydroxy-sn-glycero-3-phospho-(1'-sn-glycerol), or 18:1-LPG, respectively (Fig. 2a), in accordance with the TLC analysis. Experimental limitations related to the ionization of the neutral lipids kept us from identifying the precise nature of such.

Collectively, all complex lipids identified as PASb-3 α ligands include the 18-carbon unsaturated 11Z-octadecenoic acid (or *cis*-vaccenic acid; 18:1, *cis*-11) and an unmodified sn-2 glycerol position as common structural denominators. Given the heterologous nature of the expression system used for human PASb-3 α production, it is key to mention that *cis*-vaccenic is the sole 18-carbon unsaturated fatty acid found in *E. coli*¹⁴.

Next, in order to quantitatively check whether the degree of unsaturation influenced the binding of 18-carbon fatty acids to PASb-3 α , we performed a fluorescence-based assay in the presence of the hydrophobic probe 1,8-ANS, upon the titration of fatty acids to the delipidated protein. Intriguingly, upon fatty acid titration, no clear dose-response curve was observed for stearic acid (18:0), while sigmoid curves could be fitted in response to both oleic (18:1; *cis*-9) and linoleic acids (18:2; *cis*-9,12) (Fig. 2b). An apparent *K_d* of 39 nM for the monounsaturated oleic acid was determined, which improved 50-fold (up to 0.8 nM) for the polyunsaturated linoleic acid. Therefore, in addition to showing that 18-carbon fatty acids that occur in human cells, such as oleic and linoleic acids, are high-affinity ligands of PASb-3 α , we also establish that unsaturation is a required feature for fatty acid binding and the greater the degree of unsaturation, the stronger the interaction with the protein becomes.

Figure 1. Structure of lipid-bound PASb-3α. (a) High structural identity between the main chain tracing of PASb-3α and the PASb domains of both HIF-1α and HIF-2α (similar core r.m.s.d. of 0.6 Å). (b) However, a 510 Å³ hydrophobic cavity was exclusively identified in the core of PASb-3α (delimited by gray surface), and (c) found to enclose a monoacylglycerol molecule. (d) Extensive network of polar contacts (yellow dashed lines) shared by specific residues in PASb-3α with the lipid and a neighbor sulfate ion, suggesting that minor rearrangements in the protein may allow for binding of phospholipids.

The mechanism by which the lipid molecule enters the PASb-3α pocket is unclear. However, the outcomes of the binding assay above can be reasoned from the perspective of the free fatty acids, since the ligand conformations in the bound state are dependent on the frequency of such microstates as part of the unbound ensemble in vacuum (thus simulating lipid desolvation). Upon ensemble recapitulation, it is curious to observe that, while the putative bound conformation of stearic acid has the lowest conformational enthalpy inside the pocket (−3 kcal/mol), such a conformation has very low probability of happening upon desolvation (Fig. 2c). On the other hand, the bound conformations of oleic and linoleic acids, although with higher enthalpies (4 and 37 kcal/mol, respectively), are more frequent and easily achieved since the estimated ensemble entropies are significantly smaller than that of stearic acid (Fig. 2c).

NMR and fluorescence polarization data showed that purified, lipid-associated PASb-3α, has stronger association with HIF-1β PASb, albeit with slower binding kinetics, when compared to the well-characterized HIF-1α:HIF-1β PASb dimer (Supplementary Fig. 3a–e). In hopes of characterizing exactly how the absence of lipids could affect the heterodimerization of PASb-3α to HIF-1β PASb, we performed extensive attempts to remove the accompanying bacterial lipids from the purified protein by conventional hydrophobic interaction chromatography, reversible unfolding and detergents, all of which failed. Limited success was attained after treatment with the LIPIDEX-1000 matrix allowing, however, the recovery of only minor amounts of protein¹⁵. This observation may point to the role of the lipids as a structural cofactors for PASb-3α, which is not without precedents, since some ligand-free PASb have already been shown to be predominantly unstructured in solution and prone to precipitation^{16,17}. A fitting example is the association between the ligand-free PASb domain of the Aryl Hydrocarbon Receptor (AHR), with a predicted similarly large hydrophobic cavity (500 Å³) used for ligand binding, and the chaperone *Heat shock protein 90* (Hsp90). The presence of compounds known to activate the AHR response pathway displaces Hsp90 from this complex, suggesting that these chemicals stabilize a folded form of the PASb domain¹⁸. Therefore, we suggest that the binding of lipids to PASb is mandatory for the proper folding and stability of HIF-3α, inducing its translocation to the nucleus and subsequent activation of HIF function. This hypothesis is strongly supported by the direct observation that HIF-3α

Figure 2. Characterization of the lipids bound to PASb-3 α . (a) Two neutral lipids (yellow bands on the iodine vapor stained plate) and two phosphorous-containing lipids (blue bands over white background) were carried over by the purified protein samples. The two phospholipids were identified as 18:1-lysophospholipids. (b) Free oleic and linoleic acids bind with low-nanomolar affinity to delipidated PASb-3 α , suggesting that unsaturation is a mandatory feature for the partner fatty acid and, as unsaturation accumulates, the higher the affinity for the protein. (c) Boltzmann distributions representing the canonical ensembles of fatty acid desolvation. The estimated entropies decrease as the degree of unsaturation increase. Crossed-circles over the curves point out the frequency of putative bound-state enthalpy into the distribution.

indeed co-precipitates with HSP90 in cell models, likely via its PASb domain¹⁹. However, the dependence of this interaction on the presence of lipids has yet to be tested.

Curiously, the splicing event that gives rise to the HIF-3 α 4 isoform completely changes the amino acid sequence for the second half of its PASb domain³ (Supplementary Fig. 4a). *Ab initio* homology modelling of PASb-3 α 4 strongly suggest that amino acid changes relative to PASb-3 α (Supplementary Fig. 4a), result in substantial deformation of the topography of the hydrophobic cavity as a consequence of the presence of longer, bulkier and polar side chains inside the cavity (Supplementary Fig. 4b). These observations strongly suggest that, out of all HIF-3 α isoforms, the splicing variant HIF-3 α 4 - which is uniquely insensitive to cellular oxygen levels⁵ - may as well be exclusively insensitive to lipids. Of note, HIF-3 α 4 is a negative regulator of HIF-1⁵. Extensive attempts to produce PASb-3 α 4 domain in bacterial system only resulted in insoluble protein products, hindering the testing of our hypothesis.

With all the possible scenarios described above several outstanding questions arise. These include the identification of which lipids may in fact regulate HIF-3 α function, for instance in cancer (Supplementary Figs. 5a–b and 6a–d) or obesity²⁰, under what exact circumstances lipids must be sensed and what are the possible outcomes of lipid binding. However, by defining PASb-3 α as a lipid-binding domain, our findings lay the groundwork to explore the role of HIF-3 α as a link coupling lipid sensing under hypoxia, thus adding an unanticipated layer of complexity to the already intricate mechanism for HIF-mediated gene expression. Finally, similarly to HIF-2 α , the description of an exclusive hydrophobic pocket yield unique implications for the future development of small molecule-oriented therapeutics targeting specifically HIF-3 α .

Collectively, our structural and biochemical observations also carry implications for the overall molecular aspects of the PAS fold. For the first time, we document that a eukaryotic PAS domain may have lipids as high-affinity cofactors, thus introducing a new class of PAS ligands and expanding its already vast functional portfolio⁸.

Methods

Cloning. PASb-1 α (Asp238-His348, UniProt Q16665), PASb-1 β (Pro349-Ser467, UniProt P27540) and PASb-3 α (Gly237-Thr345, UniProt Q9Y2N7-1) coding sequences were amplified by Polymerase Chain Reaction from a cDNA library from the breast cancer cell line SKBR3. PASb-1 α and PASb-3 α constructs were cloned into the pET28a plasmid (Novagen) using the *NdeI* and *XhoI* restriction sites. PASb-1 β was cloned into a modified version of pETSUMO vector (Invitrogen) using *BamHI* and *HindIII*

restriction sites. The following pairs of oligonucleotides were used: PASb-1 α – Forward: 5'-AGTCTCA TATGGATAGCAAGACTTTCCTCAGTCGA-3' and Reverse: 5'-CGTACCTCGAGTTAGTGCTGAATA ATACCACTCAC-3'; PASb-1 β – Forward: 5'-TTGGTGGATCCCCCACTGTACAGACATGAGTAAT-3' and Reverse: 5'-GCCGCAAGCTTTTAAGAGTTCTTCACATTGGTGT-3'; PASb-3 α – Forward: 5'-AGTCTCATATGGGCCGAGGGGCCTTCCTCAGCCGC-3' and Reverse: 5'-CGTACCTCGAGTTAGG TCTCTTCCACCTGGCTGAT-3'.

Expression and purification of recombinant proteins. The following expression and purification protocol was applied to the three Pasb constructs, transformed into *Escherichia coli* Rosetta-2 thermocompetent cells (Merck). Overnight cultures, grown in LB medium supplemented by 50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ chloramphenicol, were inoculated in a ratio of 1:100 in 5 liter cultures supplemented with the same antibiotics and left shaking at 200 rpm for 5 hours at 37°C. The cultures were then down-tempered to 18°C for 1 hour before induction with 0.2 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) for additional 16 hours at 18°C. Cells were harvested by centrifugation (5,000 $\times g$) for 10 min at 4°C and resuspended in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM BME (β -mercaptoethanol) and 1 mM phenylmethylsulfonyl fluoride. Cell lysis was performed chemically by incubation with hen egg-white lysozyme, DNase I and deoxycholate (all three reagents from Sigma-Aldrich) for about 1 hour, incubated on ice. The soluble fractions were separated from the debris by high speed centrifugation (23,000 $\times g$) and subsequently loaded, by gravity, on Co²⁺-charged resin TALON (Clontech), previously equilibrated with the running buffer 50 mM Tris-HCl pH 7.5, 30 mM NaCl and 2 mM BME. The constructs, bearing a 6xHis-tag fused to their N-terminus, were eluted step-wise using running buffer to which up to 500 mM imidazole (v/v) was added. The tags were removed by overnight digestion with either bovine thrombin (Sigma-Aldrich) or ULP-1 protease and the samples loaded into a MonoQ 5/50 GL anion exchange chromatography column (GE Healthcare). Elution was done by performing a linear gradient with a buffer containing 1 M NaCl, 30 mM Tris-HCl pH 7.5 and 2 mM BME. The fractions containing the protein of interest were loaded into a HiLoad 16/600 Superdex 75 size-exclusion column (GE Healthcare), equilibrated with 30 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.5 mM TCEP (tris(2-carboxyethyl)phosphine). Protein concentrations were determined by UV 280 nm using calculated extinction coefficients.

Crystallization. Size-exclusion purified PASb-3 α was concentrated down with an Amicon ultrafiltration device (10 KDa cutoff; Millipore) to a final concentration of approximately 300 μM . Crystallization experiments were performed at 291 K using the conventional sitting drop vapor diffusion technique. Drops were made by mixing equal parts of protein and well solution, containing 3% PEG 400, 1.9 M Amonium Sulphate, 0.1 M Sodium Acetate pH 5.7. Small bar-shaped crystals started growing two days after the setting up of the crystallization drops, reaching about 50 μM in longest dimension on average usually after 5 days. Before data collection at cryogenic temperature (100 K), harvested crystals were cryoprotected with 10% ethylene glycol added to the mother liquor (v/v).

X-ray crystallography. X-ray diffraction data sets were obtained at the P13 beamline at PETRA III, Hamburg, Germany. Data was processed using Mosflm²¹ and merged and scaled with Aimless²². The first set of phases was obtained by the molecular replacement as implemented in the program Phaser²³, using the PASb domain of HIF-1 α (PDB ID 4h6j⁹) as a search model. Positional and anisotropic B-factor refinement cycles were carried out with Phenix²⁴. Manual building of the extra portions and real space refinement, including Fourier electron density map inspection, were performed with Coot²⁵. Solvent water molecules, treated as oxygen atoms, were added using the appropriate Coot routine. The survey for cavities was done using KVFinder²⁶. During model refinement, the inspection of Fourier difference maps indicated the presence of very strong non-protein electron densities (over 3 σ in height in the $F_{\text{obs}} - F_{\text{calc}}$ Fourier maps) that were interpreted - due to their sizes and shapes and in respect with the crystallization condition - as two 1-11Z-vaccenoyl-sn-glycerol molecules, five PEG 400 molecules and two sulfate ions, all subsequently refined as such. Two additional strong electron density distributions also resembling PEG400 molecules were found on the vicinities of Tyr264 in chain A and Trp315 in chain B. The refinement of such molecules, however, did not converge after many modeling configurations and the densities were then left as unexplained. The overall stereochemical quality of the final models and the agreements between them and experimental data were assessed by the program Molprobity²⁷ and the appropriate Phaser and Coot routines.

Thin-layer chromatography. Chromatographic separations were carried out on 4 $\times$ 2 cm TLC Silica gel 60 F₂₅₄ aluminum plates (Merck). The complex lipid mixture was applied on the edge of the plates (at the sample spotting line) using a glass capillary. The samples were allowed to run for 4 cm in a 8 $\times$ 4 cm glass chamber, previously saturated with the mobile phase. Two distinct mobile phases were used - cyclohexane:ethyl acetate (4:1) and chloroform:methanol:water (75:25:2.5) - which respectively allow for the separation of neutral lipids and the separation of phospholipids by head group polarity. The plates were then left exposed to air to allow for evaporation of the mobile phase residual. For differential detection of neutral lipids and phosphorous-containing lipids, iodine vapor and a modified Dittmer-Lester reagent¹³ were employed for dual staining. The plates were scanned immediately after drying.

Mass spectrometry. Lipids were extracted from 400 μL of purified protein ($\sim 1 \text{ mg} \cdot \text{mL}^{-1}$) using 1 mL of a precooled (-15°C) mixture of MTBE: methanol: water 3:1:1 (v/v/v) as previously described^{28,29}. Five hundred microliters of the upper phase, containing the lipids, were transferred to a fresh 1.5 mL Eppendorf tube and dried down in a speed-vac. The extracts that were reconstituted in acetonitrile: isopropanol 7:3 (v/v) (final concentration $25 \mu\text{g} \cdot \text{mL}^{-1}$) were gradient-eluted on an UPLC-system (Accela, Thermo) using a reverse phase C8 column ACQUITY UPLC BEH $1.7 \mu\text{m}$, $2.1 \times 150 \text{ mm}$ (Waters, 186003377). The gradient of separation was done at $400 \mu\text{L} \cdot \text{min}^{-1}$ using (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in methanol (60% B during 4 min, 60% B to 80% B in 4 min, 80–99% B in 0.5 min, 99% B for 3.5 min). The samples were measured in negative ion mode and mass spectra were acquired using an LTQXL mass spectrometer (MS) (ThermoFisher Corporation) with electrospray ionization (ESI). The MS interface capillary was maintained at 325°C , with a sheath gas flow of 45 (arbitrary units) and auxiliary gas flow of 2 (arbitrary units) for negative injections. The spray voltage ion injection was 4 kV, and the instrument scanned 350–600 m/z. MS/MS normalized collision energy was set to 41, activation Q 0.25, activation time 30 ms, and a 2 m/z isolation window. MS/MS scans were collected using dynamic exclusion with an exclusion time of 30 s. Obtained raw-chromatograms were further processed using Excalibur software version 2.10 (Thermo Fisher). The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the standard library spectrum from the Lipid Mass Database^{30,31}.

Fluorescence polarization. Purified PASb-1 β was labeled with the FITC fluorophore (fluorescein isothiocyanate, Thermo Scientific) by mixing 1 mg of protein with 20-fold molar excess of FITC in 50 mM borate buffer pH 8.5 for 1 hour at room temperature on the dark. The excess FITC was removed by overnight dialysis at 4°C with 30 mM Hepes pH 7.5, 150 mM NaCl and 0.5 mM TCEP. Unlabeled PASb-1 α and PASb-3 α , initially at a concentration of approximately 1 mM, were serially diluted 1:2 nineteen consecutive times into buffer 30 mM Hepes pH 7.5, 150 mM NaCl and 0.5 mM TCEP. Then, 18.75 μL from each protein dilution was mixed with 6.25 μL of PASb-1 β labeled with FITC at 80 nM (final concentration of labeled protein was fixed at 20 nM). A final reaction volume of 25 μL was pipetted into a 384-well black-walled microplate (Greiner Bio-one), in triplicate. Protein:protein complex samples were incubated at room temperature in the dark and fluorescence polarization (FP) was measured in a CLARIOstar microplate reader (BMG Labtech) with excitation and emission wavelengths of 480 nm and 520 nm, respectively. Experimental values were output as mean $\pm$ standard deviation and logistic sigmoidal curves fitted to determine dissociation constants (K_d) for each protein:protein complex interaction.

Lipid binding assay. Removal of bacterial lipids from purified PASb-3 α was reached by protein incubation (1.7 mL at 10 μM) with 100 mg of LIPIDEX 1000 (Sigma Aldrich) for 1 h at 37°C in buffer containing 30 mM Tris pH 7.5, 150 mM NaCl and 0.5 mM TCEP, for three to five consecutive times. After delipidation the protein was incubated with different fatty acids for binding assay using the fluorescent probe 8-Anilino-1-naphthalenesulfonic acid (1,8-ANS). The final protein concentration used in the measurements was around 1 μM , with 10 μM of ANS. The fatty acid solutions, at initial concentration of 33 μM in pure ethanol, were diluted in ethanol by serially diluted 1:3 twelve consecutive times. The concentration of ethanol in the final reaction never exceeded 1%. The fluorescence measurements were recorded on an EnSpire multimode plate reader (Perkin Elmer) using a 96-well black-walled plate (Perkin Elmer), with excitation wavelength of 380 nm and emission wavelength range of 400–600 nm, at 25°C .

Isotopic labeling of PASb-1 β . To prepare ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labelled PASb-1 β , *Escherichia coli* Rosetta II cells were allowed to grow in a modified M9 minimal medium³² supplemented with 1 g $\cdot \text{L}^{-1}$ $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories) combined with 4 g $\cdot \text{L}^{-1}$ D(+)-glucose (Sigma) to obtain only ^{15}N -labelled proteins or with [^{13}C]-D(+)-glucose (Cambridge Isotope Laboratories) to obtain double-labeled proteins. Expression and purification of labeled PASb-1 β was performed using the same procedure as described for the unlabeled protein.

NMR spectroscopy. ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled PASb-1 β was produced and purified in a final buffer containing 30 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ pH 7.2, 70 mM NaCl, 0.5 mM TCEP and 10% D_2O . NMR experiments were performed using an Agilent Inova spectrometer, operating at a ^1H Larmor frequency of 599.887 MHz and temperature of 298 K. The spectrometer is equipped with a triple resonance cryogenic probe and a Z pulse-field gradient unit. The spectra were processed with NMRPipe/NMRDraw³³ and analyzed with NMRView³⁴. The ^{15}N HSQC spectra of PASb-1 β was collected and analyzed based on a previously deposited set of assigned chemical shifts into a public database at BioMagResBank (BMRB Entry 6597) and confirmed using the three-dimensional experiments HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO^{35–38}. PASb-1 α and PASb-3 α , both initially at 900 μM , were titrated in ^{15}N -labeled PASb-1 β (200 μM), followed by the recording of the two-dimensional ^{15}N HSQC spectra. Binding of proteins was characterized by changes in ^{15}N HSQC signals, intensities and chemical shift values, as a function of the concentration of unlabeled protein. The chemical shift perturbation of the ^{15}N -HSQC spectra was normalized according to the equation $\Delta\delta(^{15}\text{N} + ^1\text{H}) = [(\Delta\delta^{15}\text{N}/10)^2 + (\Delta\delta^1\text{H})^2]^{1/2}$, in ppm units³⁹ and the relative peak intensity changes calculated using the equation $(I - I_0)/I_0$, where I is the peak intensity

measured in the presence of the maximum amount of unlabeled protein and I_0 is the peak intensity measured in the free labeled protein.

Molecular Modelling of PASb-3 α 4. *Ab initio* homology modelling of PASb domain of HIF-3 α 4, or PASb-3 α 4 (Gly237-Pro351, Uniprot Q9Y2N7-4) was done using methodology implemented by I-TASSER⁴⁰. The top output model was used for comparison against the crystallographic model of lipid-bound PASb-3 α .

Tissue array. Human normal and tumor tissue arrays containing 78 normal tissue spots (FDA805-1, U.S. Biomax) or 18 normal tissues and 54 tumors (FDA805-2, U.S. Biomax) from multiple organs were subjected to immunohistochemical analysis with Anti-HIF3 alpha antibody (ab10134, Abcam). The tissue array slides were deparaffinized by two xylene washes (10 min each) and then rehydrated in ethanol series. After washing with water, the slides were microwaved for 10 rounds of 1 min in 10 mM sodium citrate buffer (pH 6.0) and allowed to cool to room temperature to retrieve antigens. Endogenous peroxidase activity was extinguished after 30 min incubation in 0.3% H₂O₂ in PBS. The slides were then blocked with Avidin and Biotin blocking solutions (Avidin/Biotin Blocking Kit; Vector Labs) and 1% goat serum in PBS (Vectastain Elite ABC Kit, Vector Labs). The tissue arrays were incubated overnight with the primary antibody (1:100 dilution in 1X PBS, 0.2% BSA, 0.02% Triton X-100, 10 mM Glycine), washed in same buffer, incubated with biotinylated secondary antibody 1:200 and treated for 30 min with the Elite ABC reagent (Universal R.T.U. Vectastain Elite ABC Kit; Vector Labs). After washing with PBS, the slides were processed with chromogen solution (ImmPACT DAB Substrate; Vector Labs). The slides were then dehydrated by ethanol series and mounted using Entellan New (Merck). The resulting staining obtained were captured using Operetta (PerkinElmer) 2X magnification objective, stitched by Harmony software (PerkinElmer) and quantified using ImageJ software. To allow comparison between slides, all data was normalized first to Skin Malignant melanoma spot (present in both slides). A second normalization was done to the highest expression in each specific tissue type. Samples were selected based on $n \geq 2$ and the simultaneous presence of both normal and cancer representatives. Statistical significance was tested using Two-sample t-test, considering $p < 0.1$.

TCGA data analysis. All the gene-expression data derived from RNA-seq were downloaded from The Cancer Genome Atlas data portal (<http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>). For the evaluation of HIF-3 α 9 expression levels across tumor types, the normalized RSEM count from uc002peh.2 were used. For tumors with mean expression above 5, the 2-component Gaussian mixture distribution model with fixed variance for definition of expressing and non-expressing patients^{41,42} was implemented using mclust⁴³ package (R statistical software⁴⁴) and fitted independently for each tumor dataset using the uc002peh.2 expression levels. Patients were categorized in each class and gene differential expression analysis was performed using EBSeq⁴⁵ package (R statistical software) using as input raw RSEM values and performing a 75h quantile normalization⁴⁶. Genes up- (>1) and down- regulated (<1) with posterior probability of being differentially expressed above 0.95 were used as input for KOBAS2.0⁴⁷ pathway enrichment analysis for REACTOME database. The Venn plot were made with VennDiagram⁴⁸ package (R statistical software).

Canonical ensemble simulation of lipid in vacuum. A Metropolis-Monte Carlo simulation was performed to recapitulate a canonical ensemble for the free stearic, oleic and linoleic acids. One-million-steps simulation, as implemented in Python, was performed, with temperature set to 300 K. The associated energies of randomly generated microstates was calculated using YASARA's function Energy("All")⁴⁹ and the AMBER03 force field⁵⁰ for the parametrization of the lipid molecules. Probability of micro-states $P(s)$ were calculated by discretization of Boltzmann distributions using $\text{hist}(\text{Energy})/\text{density}$ function of the statistical software R⁴⁴. Entropy was estimated with $S(\text{lipid, vacuum}) = -P(s) \cdot \log(P(s))$.

References

1. Semenza, G. L. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol* **9**, 47–71 (2014).
2. Maynard, M. A. *et al.* Multiple splice variants of the human HIF-3 α locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. *J Biol Chem* **278**, 11032–11040 (2003).
3. Pasanen, A. *et al.* Hypoxia-inducible factor (HIF)-3 α is subject to extensive alternative splicing in human tissues and cancer cells and is regulated by HIF-1 but not HIF-2. *Int J Biochem Cell Biol* **42**, 1189–1200 (2010).
4. Zhang, P. *et al.* Hypoxia-inducible factor 3 is an oxygen-dependent transcription activator and regulates a distinct transcriptional response to hypoxia. *Cell Rep* **6**, 1110–1121 (2014).
5. Maynard, M. A. *et al.* Human HIF-3 α 4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *FASEB J* **19**, 1396–1406 (2005).
6. Partch, C. L., Card, P. B., Amezcua, C. A. & Gardner, K. H. Molecular basis of coiled coil coactivator recruitment by the aryl hydrocarbon receptor nuclear translocator (ARNT). *J Biol Chem* **284**, 15184–15192 (2009).
7. Partch, C. L. & Gardner, K. H. Coactivators necessary for transcriptional output of the hypoxia inducible factor, HIF, are directly recruited by ARNT PAS-B. *Proc Natl Acad Sci USA* **108**, 7739–7744 (2011).
8. Henry, J. T. & Crosson, S. Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu Rev Microbiol* **65**, 261–286 (2011).
9. Cardoso, R. *et al.* Identification of Cys255 in HIF-1 α as a novel site for development of covalent inhibitors of HIF-1 α /ARNT PasB domain protein-protein interaction. *Protein Sci* **21**, 1885–1896 (2012).

10. Scheuermann, T. H. *et al.* Artificial ligand binding within the HIF2alpha PAS-B domain of the HIF2 transcription factor. *Proc Natl Acad Sci USA* **106**, 450–455 (2009).
11. Rogers, J. L. *et al.* Development of inhibitors of the PAS-B domain of the HIF-2 α transcription factor. *J. Med. Chem.* **56**, 1739–1747 (2013).
12. Scheuermann, T. H. *et al.* Allosteric inhibition of hypoxia inducible factor-2 with small molecules. *Nat Chem Biol* **9**, 271–276 (2013).
13. Dynska-Kukulska, K., Ciesielski, W. & Zakrzewski, R. The use of a new, modified Dittmer-Lester spray reagent for phospholipid determination by the TLC image analysis technique. *Biomed Chromatogr* **27**, 458–465 (2013).
14. Cronan, J. E. & Vagelos, P. R. Metabolism and function of the membrane phospholipids of *Escherichia coli*. *Biochim Biophys Acta* **265**, 25–60 (1972).
15. Glatz, J. F. & Veerkamp, J. H. Removal of fatty acids from serum albumin by Lipidex 1000 chromatography. *J Biochem Biophys Methods* **8**, 57–61 (1983).
16. Hoff, W. D. *et al.* Global conformational changes upon receptor stimulation in photoactive yellow protein. *Biochemistry* **38**, 1009–1017 (1999).
17. Kroon, A. R. *et al.* Spectral tuning, fluorescence, and photoactivity in hybrids of photoactive yellow protein, reconstituted with native or modified chromophores. *J Biol Chem* **271**, 31949–31956 (1996).
18. Pandini, A., Denison, M. S., Song, Y., Soshilov, A. A. & Bonati, L. Structural and functional characterization of the aryl hydrocarbon receptor ligand binding domain by homology modeling and mutational analysis. *Biochemistry* **46**, 696–708 (2007).
19. Katschinski, D. M. *et al.* Interaction of the PAS B domain with HSP90 accelerates hypoxia-inducible factor-1 α stabilization. *Cell Physiol Biochem* **15**, 351–360 (2004).
20. Dick, K. J. *et al.* DNA methylation and body-mass index: a genome-wide analysis. *Lancet* **383**, 1990–1998 (2014).
21. Leslie, A. G. Recent changes to the MOSFLM package for processing film and image plate data. Joint CCP4+ESF-EAMCB Newsletter on Protein Crystallography **26**, 27–33 (1992).
22. Evans, P. R. & Murshudov, G. N. How good are my data and what is the resolution? *Acta Crystallogr D Biol Crystallogr* **69**, 1204–1214 (2013).
23. McCoy, A. J. *et al.* Phaser Crystallographic software. *J Appl Cryst* **40**, 658–674 (2007).
24. Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213–221 (2010).
25. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. P. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486–501 (2010).
26. Oliveira, S. H. *et al.* KVFinder: steered identification of protein cavities as a PyMOL plugin. *BMC Bioinformatics* Jun **15**, 197 (2014).
27. Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* **66**, 12–21 (2010).
28. Giavalisco, P. *et al.* Elemental formula annotation of polar and lipophilic metabolites using (13)C, (15)N and (34)S isotope labelling, in combination with high-resolution mass spectrometry. *Plant J* **68**, 364–376 (2011).
29. Hummel, J. *et al.* Ultra performance liquid chromatography and high resolution mass spectrometry for the analysis of plant lipids. *Front Plant Sci* **2**, 54 (2011).
30. Fahy, E., Sud, M., Cotter, D. & Subramaniam S. LIPID MAPS online tools for lipid research. *Nucleic Acids Res* **35**, W606–12 (2007).
31. Fahy, E. *et al.* LIPID MAPS comprehensive classification system for lipids. *J Lipid Res* **50**, S9–S14 (2009).
32. Li, M. X., Corson, D. C. & Sykes B. D. Structure determination by NMR: isotope labeling. *Methods Mol Biol* **173**, 255–265 (2002).
33. Delaglio, F. *et al.* NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**, 277–293 (1995).
34. Johnson, B. & Blevins, R. NMR View: A computer program for the visualization and analysis of NMR data. *J Biomol NMR* **4**, 603–614 (1994).
35. Ikura, M., Kay, L. & Bax, A. A novel approach for sequential assignment of 1H, 13C, and 15N spectra of larger proteins: Heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry* **29**, 4659–4667 (1990).
36. Grzesiek, S. & Bax, A. Amino acid type determination in the sequential assignment procedure of uniformly 13C/15N-enriched proteins. *J Biomol NMR* **3**, 185–204 (1993).
37. Wittekind, M. & Mueller, L. HNCACB, a high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with the alpha- and beta-carbon resonances in proteins. *J Magn Reson Ser B* **101**, 201–205 (1993).
38. Yamazaki, T., Lee, W., Arrowsmith, C., Muhandiram, D. R. & Kay, L. E. A suite of triple resonance NMR experiments for the backbone assignment of 15N, 13C, 2H labeled proteins with high sensitivity. *J Am Chem Soc* **116**, 11655–11666 (1994).
39. Cavanagh, J., Fairbrother, W., Palmer, A., III, Rance, M. & Skelton, N. *Protein NMR Spectroscopy: Principles and Practice* (Academic Press, San Diego, 2007).
40. Roy, A., Kucukural, A. & Zhang, Y. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Prot* **5**, 725–738 (2010).
41. Lehmann, B. D. *et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* **121**, 2750–2767 (2011).
42. Curtis, C. *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346–52 (2012).
43. Fraley, C. & Raftery, A. E. Model-based Clustering, Discriminant Analysis and Density Estimation. *J Am Stat Assoc* **97**, 611–631 (2002).
44. Ihaka, R. & Gentleman, R. R: A language for data analysis and graphics. *J Comput Graph Stat* **5**, 299–314 (1996).
45. Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106 (2010) *Genome Biol* **11**, R106 (2010).
46. Bullard, J. H., Purdom, E., Hansen, K. D. & Dudoit, S. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC Bioinformatics* **11**, 94 (2010). *BMC Bioinformatics* **11**, 94 (2010).
47. Xie, C. *et al.* KOBAS 2.0: A web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* **39**, 316–322 (2011).
48. Chen, H. & Boutros, P. C. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* **12**, 35 (2011).
49. Krieger, E., Darden, T., Nabuurs, S., Finkelstein, A. & Vriend, G. Making optimal use of empirical energy functions: force-field parameterization in crystal space. *Proteins* **57**, 678–683 (2004).
50. Duan, Y. *et al.* Point-Charge Force Field for Molecular Mechanics Simulations of Proteins. *J Comput Chem* **24**, 1999–2012 (2003).

Acknowledgements

This work was supported by the São Paulo State Research Foundation, FAPESP, under grants 2012/14298-9 and 2014/20673-2 (to ALBA) and 2014/15968-3 (to SMGD), and fellowships 2010/13739-6 and 2014/04927-4 (to AMF). We thank LNBio for financial support and access to facilities (LPP, MAS, LBE, NMR and Robolab). We thank Dr. Alessandra Girasole for expert technical and secretarial support. We are very grateful to Dr. Chi Van Dang and Zandra Walton (Abramson Cancer Center, University of Pennsylvania, USA) for expert comments and suggestions as well as stimulating discussions. We are also grateful to Dr. Richard Charles Garratt (IFSC/USP, Brazil) for detailed and critical reading of the manuscript. The results published here are part based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>.

Author Contributions

A.M.F., J.F.O., S.M.G.D. and A.L.B.A. designed research; A.M.F., J.F.O., D.A., J.A., S.A.R., M.M.D., M.V.B.D., M.L.S. and P.S.L.O. performed research; A.M.F., J.F.O., D.A., J.A., S.A.R., M.M.D., M.V.B.D., M.L.S., P.S.L.O., C.C., S.M.G.D. and A.L.B.A. analyzed and discussed data; S.M.G.D. and A.L.B.A. wrote the paper.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Fala, A.M. *et al.* Unsaturated fatty acids as high-affinity ligands of the C-terminal Per-ARNT-Sim domain from the Hypoxia-inducible factor 3α. *Sci. Rep.* 5, 12698; doi: 10.1038/srep12698 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

Human mitochondrial pyruvate carrier 2 as an autonomous and selective transporter

Raghavendra Sashi Krishna Nagampalli^{1,*}, José Edwin Neciosup Quesñay^{1,*}, Douglas Adamoski^{1,*}, Zeyaul Islam¹, James Birch^{2,3}, Heitor Gobbi Sebinelli⁴, Caroline Fernanda Rodrigues Ascensão¹, Angela Maria Fala¹, Bianca Alves Pauletti¹, Sílvia Roberto Consonni^{1,‡}, Juliana Ferreira Oliveira¹, Amanda Cristina Teixeira Silva¹, Kleber Gomes Franchini¹, Adriana Franco Paes Leme¹, Pietro Ciancaglini⁴, Isabel Moraes^{2,3}, Sandra Martha Gomes Dias^{1,†} & Andre Luis Berteli Ambrosio^{1,†}

From the ¹Laboratório Nacional de Biociências, Centro Nacional de Pesquisa em Energia e Materiais, Campinas, SP, 13083-970, Brazil; the ²Membrane Protein Laboratory, Diamond Light Source, Harwell Science and Innovation Campus, Didcot, Oxfordshire OX11 0DE, England; ³Research Complex at Harwell, Rutherford Appleton Laboratory, Harwell, Didcot, Oxfordshire OX11 0FA, England; the ⁴Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, 14040-901, Brazil.

*RSKN, JENQ and DA contributed equally to this work.

‡current address: Departamento de Bioquímica e Biologia Tecidual, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, 13083-862, Brazil.

†To whom correspondence may be addressed: Sandra MG Dias or Andre LB Ambrosio, Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Rua Giuseppe Máximo Scolfaro, 10.000, Pólo II de Alta Tecnologia, Campinas, SP, 13083-970, Brazil. Tel: +55 19 3512 1115/Fax: +55 19 3512 1004; e-mail: andre.ambrosio@lnbio.cnpem.br

KEYWORDS: membrane protein, mitochondria, transporter, pyruvate carrier, MPC1, MPC2.

The active transport of glycolytic pyruvate across the human inner mitochondrial membrane is thought to involve two mitochondrial pyruvate carrier subunits, MPC1 and MPC2, assembled as a 150 kDa oligomeric heterotypic structure. Here, the recombinant production of human MPC subunits through a co-expression strategy is described; however, substantial complex formation was not observed, and predominantly individual subunits were purified. In contrast to MPC1, which co-purifies with a host chaperone, we demonstrated that MPC2 promotes efficient pyruvate transport into reconstituted proteoliposomes. The derived functional requirements, kinetics and inhibition features of MPC2 resemble those previously demonstrated for pyruvate transport in mitochondrial extracts. Importantly, the ectopic expression of human MPC2 in yeast lacking endogenous MPC stimulated growth in culture media lacking

valine and increased the consumption of oxygen, thus validating an autonomous role for MPC2 in yeast cells. The presence of multiple oligomeric species of MPC2 in mitochondrial isolates, purified protein and in artificial lipid bilayers suggest functional dimers or high-order complexes. Significant changes in the secondary structure content of MPC2, as probed by synchrotron radiation circular dichroism, further supports the interaction between the protein and ligands. Our results provide the initial framework for exploring the independent role of MPC2 in homeostasis and diseases related to dysregulated pyruvate metabolism.

Nearly four decades after the demonstration of the protein-mediated transport of pyruvate across the inner mitochondrial membrane (IMM) (1), two concurrent studies identified the oligomeric complex formed by MPC1 and MPC2 as necessary and sufficient for this task (2, 3); MPC1 and MPC2 were proposed to function

together via the formation of an oligomeric structure of approximately 150 kDa (2). These original findings inspired many subsequent investigations that have further characterized MPC-dependent pyruvate transport in the cellular context (4–6), with multiple groups showing that either the loss of MPC1 or MPC2 in mitochondria is sufficient to confer similar loss of function phenotypes (6–10). However, to date, the isolation and reconstitution of MPC1:MPC2 into proteoliposomes is still considered necessary to perform the ultimate proof-of-concept experiment to measure pyruvate transport (10–12).

In this context, we report the first successful, large-scale, recombinant production and functional reconstitution of this family of carriers in an artificial lipid bilayer. We provide an unprecedented *in vitro* demonstration that human MPC2 functions independently of MPC1 to induce pyruvate transport, possibly as high-order oligomers. In cells, the ectopic expression of human MPC2 improved oxygen consumption and stimulated growth compared to yeast cells lacking endogenous MPC. Most importantly, these observations are consistent with those of early and recent publications, suggesting that mitochondrial pyruvate transport by MPC is a rapid and specific process that depends on co-proton import and redox balance and is sensitive to inhibition by a small molecule (13–16). Our findings open a discussion concerning pyruvate import regulation by at least two different molecular entities in human mitochondria: heterotypic MPC1:MPC2 and homotypic MPC2:MPC2. Our work also has immediate implications for the development of small-molecule-oriented therapeutics that specifically target MPC2 in pyruvate-related diseases such as cancer, Alzheimer's disease, and diabetes (10, 12, 17–20).

RESULTS

Purification of recombinant MPC. Human MPC1 and MPC2 proteins were co-expressed from codon-optimized genes in a heterologous yeast system (*Saccharomyces cerevisiae* JRY472) that was modified to lack endogenous MPC ($\Delta mpc1/2/3$). This mutant strain is hereafter referred to as 3 Δ (2). A bi-directional expression plasmid was initially engineered to contain MPC1 fused to an 8xHis tag

and MPC2 linked to a cleavable monomeric GFP (Fig. 1A). This system enabled the detection of inducible expression levels and cellular compartmentalization as well as subsequent purification via cobalt-based immobilized metal affinity chromatography (Co-IMAC) of the target complex. Controlled expression (Supp. Fig. 1A) and proper mitochondrial localization of MPC were confirmed (Fig. 1A, left panel and Supp. Fig. 1B). n-Dodecyl- β -D-maltoside (DDM, at 1 %) was identified as a suitable detergent for the extraction of proteins from membrane pellets. The target proteins were purified via Co-IMAC, followed by gel-filtration chromatography (GF), both of which were carried out in the presence of 0.03 % DDM (Fig. 1A, center panel). A sharp monodisperse peak was obtained after GF, which corresponded to a molecular weight of 64 kDa for the protein-detergent complex (Fig. 1A, right panel). Surprisingly, the peak sample derived from human MPC1 was strongly associated with the yeast 60S ribosomal protein L28 (both proteins were further identified using mass spectrometry, Supplementary Table 1). In addition, traces of co-purified, GFP-fused MPC2 were confirmed via silver staining and in-gel fluorescence (Fig. 1A, inset).

Intrigued by the trace amounts of human MPC2 isolated during the complete purification of MPC1, as well as the lower-than-expected molecular weight, we generated an alternative plasmid construct. In this new construct, the poly-histidine tag from MPC1 was transferred to the C-terminus of the MPC2-GFP fusion (Fig. 1B), similar to a previously successful approach employed for other eukaryotic membrane proteins expressed in yeast (21). Upon confirmation of proper mitochondrial localization of the new construct (Fig. 1B, left panel), the recombinant proteins were successfully extracted with 1 % C12E8 (octaethylene glycol monododecyl ether) and purified in the presence of 0.03 % DDM via Co-IMAC, followed by GFP-10xHis removal and GF (Fig. 1B, center panel). Again, a single monodisperse protein-detergent peak was obtained, which corresponded to a molecular weight of 70 kDa (Fig. 1B, right panel). This sample was shown to exclusively contain MPC2, which was then purified to homogeneity (Fig. 1B, center panel).

Functional reconstitution of MPC in lipid vesicles.

To evaluate the pyruvate transport activity of recombinant purified MPC *in vitro*, we reconstituted both purified protein products, individually and in combination, into asolectin liposomes (Supp. Fig. 2A-B) (22). Substrate uptake was quantified based on the observed decrease in extraliposomal pyruvate content, as indicated by the enzymatic activity of lactate dehydrogenase A (LDHA) (Supp. Fig. 2C). A pH gradient-dependent assay ($\Delta\text{pH} = 1.5$) was derived in accordance with the chemical conditions recently established for mouse MPC (13). Notably, successful pyruvate transport activity was not observed for proteoliposomes reconstituted with MPC1 co-purified with RPL28 and traces of MPC2 (termed MPC1*, Fig. 2A). By contrast, compared to protein-free liposomes, which were used as a control, proteoliposomes containing MPC2 alone showed significant pyruvate uptake ($47 \pm 2\%$) (Fig. 2A). Similarly, vesicles co-reconstituted with comparable levels of MPC1* and MPC2 (Supp. Fig. 2D) also induced the uptake of extraliposomal pyruvate; however, the corresponding transport levels were similar ($45 \pm 2\%$) to those obtained with MPC2 alone (Fig. 2A).

Most importantly, the self-sufficient role of human MPC2 in pyruvate transport was further supported when this protein was overexpressed and purified from a plasmid construct lacking the MPC1 coding sequence (Fig. 1C). As such, when reconstituted into proteoliposomes, this new construct (termed MPC2**) induced pyruvate transport across the bilayer at levels statistically similar ($44 \pm 2\%$; Fig. 2A) to those mentioned above.

In vitro pyruvate transport is dependent on time and electrochemical gradient. Within a pH gradient of 1.5 units, MPC2 proteoliposomes induced rapid pyruvate transport. Pyruvate intake, which exhibited saturation kinetics, was proficient during the first 5 min of incubation, presenting half-maximum efficiency after 1.3 min (Fig. 2B). Upon saturation, approximately $53 \pm 2\%$ of the external substrate was incorporated into the proteoliposomes (Fig. 2B, given by the asymptote of the exponential decay model in blue).

The dependence of MPC2 activity on a proton electrochemical gradient was further

confirmed upon proper calibration of LDHA activity (Supp. Fig. 2C). In a shallower pH gradient (ΔpH 0.8 units), pyruvate transport reached $34 \pm 2\%$ (Fig. 2C) compared with the $53 \pm 1\%$ transported at a ΔpH of 1.5 units. However, when the pH gradient collapsed ($\Delta\text{pH} = 0$), $14 \pm 4\%$ pyruvate transport was still measured (Fig. 2C), likely reflecting the chemical potential of pyruvate moving down its own concentration gradient.

In vitro pyruvate transport is sensitive to chemical inhibition, redox modification and substrate type.

The previous landmark demonstration of the carrier-dependent transport of pyruvate across the IMM involved the identification of monocarboxylate mimetic cinnamates as effective and specific MPC inhibitors (1). In the present study, compared to the vehicle control (DMSO, $50 \pm 2\%$), UK-5099, a potent cyanocinnamate ($K_i \approx 10$ nM in mitochondrial extracts (23)), did not exert any significant inhibitory effect on *in vitro* MPC2-dependent pyruvate transport ($47 \pm 2\%$) when used at a final concentration of 50 μM , as shown in Fig. 3A.

Nevertheless, the inhibitory mechanism of cinnamates was originally shown to rely on a reversible Michael addition to a cysteine thiol group in MPC; loss of conjugation was achieved by the addition of reducing agents such as β -mercaptoethanol (14, 24). Here, although the complete elimination of TCEP (TRIS(2-carboxyethyl)phosphine) from the early steps of membrane preparation up to proteoliposome reconstitution still permitted the standard purification of MPC2 (Supp. Fig. 3A), complete functional inactivation was observed (Fig. 3B). Re-incubating MPC2 with TCEP at the stage of proteoliposome reconstitution resulted in the recovery of approximately three quarters ($32 \pm 2\%$) of the original activity ($48 \pm 2\%$, Fig. 3B).

To further explore the importance of redox modification on human MPC2 function, we generated a point mutant by replacing the single cysteine residue at position 54 with serine (Supp. Fig. 3B). Although the overall activity of the isosteric mutant (termed MPC2.C54S) was compromised ($31 \pm 2\%$ total transport), it was distinctly insensitive to TCEP ($32 \pm 2\%$ total transport; Fig. 3C). Due to the lack of detectable MPC2 activity in the absence of TCEP ($2 \pm 2\%$

total transport; Fig. 3B), combined with the observation that UK-5099 had no significant effect on the activity of proteoliposomes supplemented with TCEP (Fig. 3A), the true *in vitro* effects of cinnamates on MPC2 activity cannot be conclusively established. Noteworthy, MPC1, but not MPC2, was recently demonstrated to be the true target of cinnamates (2, 16). Together, however, these findings suggest that competent pyruvate transport also relies on a sulfhydryl-sensitive site on MPC2.

Thiazolidinedione (TZD) insulin sensitizers, such as rosiglitazone and pioglitazone, are commonly used to treat type 2 diabetes mellitus. This class of small molecules was also recently shown to inhibit pyruvate transport in cells at clinically relevant concentrations by directly and specifically targeting the MPC2 subunit (16, 18). In the present study, when compared to the vehicle control (DMSO, $50 \pm 2\%$), rosiglitazone, at $50 \mu\text{M}$, significantly decreased the MPC2-dependent internalization of pyruvate down to $28 \pm 2\%$, as shown in Fig. 3A. The finding that a TZD derivative can decrease MPC2 activity has been substantiated by previous studies, further confirming the protein-mediated component of *in vitro* pyruvate transport (16, 18).

Finally, among all of the many carboxylates metabolized in the cell, lactate and pyruvate are directly linked and structurally similar. However, MPC2 is selective for pyruvate over lactate, as no detectable transport of the latter was observed over the range of concentrations tested and under the chemical conditions shown to be successful for pyruvate (Fig. 3D).

Ectopic human MPC2 expression stimulated growth and induced glucose-dependent respiration in yeast. The yeast strain JRY472 (3Δ) used in the present study to produce recombinant human MPC, carries a mutation in the *leu2* gene and is therefore dependent on external leucine to grow. When cultured on synthetic dropout medium lacking valine but supplemented with leucine and threonine, this mutant strain grows with a doubling time of 53 ± 1 min (Fig. 4A, filled green circles). Ectopic expression of human MPC2 in this strain, however, decreased the doubling time necessary by 16% (45 ± 1 min; Fig. 4A, filled blue squares).

Accordingly, expression of the MPC2 isosteric mutant (C54S), with expected intermediary transport activity compared with wild-type MPC2, showed a 7% decrease in the doubling time of the same strain (50 ± 1 min; filled red triangles in Fig. 4A). When cultured in synthetic medium supplemented with external valine, cells from all three groups (shown as open shapes in Fig. 4A) grew at similar levels to those for 3Δ +MPC2 in the absence of valine. Therefore, the improved growth rates of 3Δ cells expressing human MPC2 might reflect the use of glycolytic pyruvate for biosynthetic purposes in the mitochondria, particularly for the production of valine.

The ability of human MPC2 to stimulate pyruvate-dependent respiration in yeast cells was also evaluated by quantifying oxygen consumption in response to the addition of 0.08% glucose to basal media. Whereas the mutant strain 3Δ responded to glucose addition with a slight, 10% increase in oxygen consumption over basal respiration (1.1 ± 0.1 -fold; filled green circles in Fig. 4), the same cells expressing human MPC2 responded to glucose addition with a 60% increase in oxygen consumption over basal respiration (1.6 ± 0.1 -fold; filled blue squares in Fig. 4). Again, as expected, the ectopic expression of MPC2.C54S led to a reduced increase in oxygen consumption over basal levels (1.3 ± 0.1 -fold) in response to glucose addition (filled red triangles in Fig. 4). To provide further evidence that all the added glucose was being oxidized via respiration, ethanol addition, which blocks residual fermentation, produced nearly the same response under all three test conditions (Fig. 4, open shapes). Taken together, these results further support the *in vitro* findings using artificial vesicles and, most importantly, confirm an autonomous role for MPC2 in promoting pyruvate transport.

Oligomeric nature and secondary structure composition of human MPC2. The lower-than-expected molecular weight of the purified protein-detergent complex that was obtained above (Fig. 1B-C), led us to further investigate the oligomeric assembly of the functional MPC2. First, by isolating the mitochondrial extracts (25), followed

by short-range chemical cross-linking using disuccinimidyl suberate (DSS, 11.4 Å linker), we observed the formation of MPC2 oligomers in its native lipid environment, starting from dimers up to higher-order molecular species, according to electrophoretic separation (Fig. 5A, left panel). When such experiment was performed with purified protein, only the presence of dimeric species was resolved, probably due to the association between the protein and the detergent (Fig. 5A, middle panel) (26). However, the subsequent reconstitution of purified MPC2 into proteoliposomes displayed the similar high-order, multi-species pattern that was observed in the mitochondrial extracts (Fig. 5A, right panel). This suggests that recombinant MPC2 in artificial lipid bilayer, which was proven functional for pyruvate transport, shares oligomeric similarities to the native mitochondrial membrane environment.

Lastly, in order to investigate the secondary structure composition of human MPC2, and to gauge the conformational changes due to binding of substrate and inhibitor, we performed synchrotron radiation circular dichroism (SRCD) analysis. SRCD indicated that this protein, in the absence of any ligands, is predominantly helical in nature (Fig. 5B, top left panel). The binding of pyruvate leads to improved structural stability, since the helical content is increased from 44 % in the ligand-free control sample up to 56 % when the substrate is present (Fig. 5B, top right panel); this might be due to the fact that pyruvate needs to be transported across the channel of MPC2, by means of opening up or rearranging the structure. Conversely, the absence of TCEP lead to an overall increased disorder (Fig. 5B, bottom left panel), which is in agreement with the lack of *in vitro* transport activity under a similar condition (Fig. 3B). Notably, the addition of rosiglitazone had minor impact on the secondary structure composition of MPC2 (45 % versus 44 % helices, respectively; Fig. 5B, bottom right panel), which could probably be associated with weak binding and thereby explaining its limited inhibitory effects on *in vitro* transport (Fig. 3B).

DISCUSSION

A rich body of evidence has established MPC1 and MPC2 as mandatory components for the mitochondrial uptake of pyruvate in both intact cells and mitochondrial isolates (2, 3, 5, 6, 9, 10, 18). Co-immunoprecipitation from purified mitochondria, followed by electrophoretic separation, suggested that MPC1 and MPC2 form an oligomeric complex of approximately 150 kDa (2). Here, we developed an experimental setup aimed at the co-expression and purification of recombinant human MPC as a heterocomplex. To this end, we used a bi-directional expression plasmid with inducible promoters that respond to galactose, such that both isoforms could be expressed simultaneously at high and similar levels. Moreover, we used a mutant yeast strain lacking the endogenous *mpc1*, *mpc2* and *mpc3* genes (2) to avoid cross-contamination with yeast MPC.

Regardless, only the human MPC2 subunit was purified to homogeneity and shown to function in both *in vitro* and *in vivo* pyruvate transport. This finding was further confirmed when MPC2 was expressed without MPC1 and shown to be similarly functional. MPC1 co-purified with the yeast 60S RPL28 protein and only traces of MPC2. In contrast to MPC2, MPC1 associated with 60S RPL28 was not active. These results suggest that yeast may lack some critical and yet unidentified protein component for stable complex formation of human MPC1:MPC2, as previously suggested (11). The existence of novel and direct interacting partners for MPC has been experimentally postulated (27, 28); moreover, a host non-membrane protein contaminant (60S RPL28) may act as a chaperone for human MPC1, potentially preventing detectable *in vitro* transport activity.

Collectively, the functional observations described above are consistent with previous and recent reports, suggesting that mitochondrial pyruvate transport by MPC is a rapid and specific process that depends on co-proton import and redox balance (13–15). Here, the finding that the *in vitro* MPC2 activity can be inhibited by a TZD derivative, albeit partially, is also substantiated by previous studies (16, 18). Of note, these functional parameters, which were proven essential for the demonstration of the protein-dependent pyruvate transport, are further reinforced by the circular dichroism studies using synchrotron radiation.

However, the same purification and functional findings that indicate an autonomous role for MPC2 in pyruvate transport raise major questions about the need for a mandatory two-component heterotypic interaction for efficient pyruvate transport, as previously proposed. Notably, the dynamic stoichiometry of MPC subunits was recently shown to shift between hetero- and homo-oligomers in cells due to variations in both metabolic and tissue-related conditions (6, 10, 29).

Here, the study of the oligomeric composition of recombinant MPC2 by chemical cross-linking in native (mitochondrial extracts) and native-like (asolectin vesicles) environments, further support the formation of large homotypic assemblies as the functional components, ranging hundreds of kilodaltons, as previously proposed for the heterotypic MPC1:MPC2 complex (2). The true size of the MPC2 oligomers, however, are yet to be determined.

Given all the potential scenarios described above, a major question remains: which physiological conditions promote MPC2-mediated pyruvate transport? Recently, MPC2 was shown to be strictly regulated by acetylation, which can critically determine the active or inactive states of this protein (7, 30–32); indeed, acetylation has been directly linked to decreased cellular oxygen consumption rates and deficient pyruvate transport activity (30). Deletion of MPC2 causes embryonic lethality in mice, indicating that this carrier subunit is particularly important for cell survival (33). We showed that human MPC2 alone can increase oxygen consumption in yeast cells driven by glucose addition and can rescue cell growth when valine is absent, suggesting that, in cells, human MPC2 facilitates mitochondrial pyruvate intake. This finding was further confirmed using the isosteric mutant. Curiously, MPC2 significantly increased oxygen consumption in the 3Δ mutant strain by 60 %; yet, compared with the control, MPC2 stimulated cell growth rates (measured as doubling time) by 16 % in this strain. These results suggest that MPC2 may play a particular role in the distinct energetic and biosynthetic fates of pyruvate, which should be further investigated, perhaps in association to regulation by post-translational modifications.

In conclusion, although the heterotypic MPC1:MPC2 component for transport was proposed based on a co-immunoprecipitation assay and yeast-based functional complementation assays (2), and despite the rapid increase in the literature regarding MPC pyruvate transport in the last 4-5 years, evidence regarding the direct interaction of these molecules has been lacking at the level of the isolated protein. Our work provides the first demonstration of the heterologous expression and purification of human MPC1 and MPC2. MPC2, but not MPC1, was purified to homogeneity and found to be active and sufficient for pyruvate transport in artificial liposomes and in cells.

EXPERIMENTAL PROCEDURES

Construct design and cloning. pBEVY-GU (34) (bi-directional expression vector for yeast, galactose inducible, URA3 selection) was a generous gift from Graham D. Pavitt (University of Manchester, UK). Standard molecular biology techniques were used to modify the original pBEVY-GU plasmid to add (i) the coding sequence for an 8xHis tag (with a four-amino acid spacer, VDGS) to the 3' end of multiple cloning site 1 and (ii) a PreScission Protease recognition sequence, followed by EGFP at the 3' end of multiple cloning site 2. Codon-optimized, synthetic genes encoding human MPC1 (NCBI Reference Sequence: NP_057182.1) and MPC2 (NCBI Reference Sequence: NP_001137146.1) were acquired from GenScript, USA. The restriction sites Sall/BamHI and XmaI/EcoRI were used to subclone *MPC1* and *MPC2*, respectively. Standard techniques were also used to transfer the poly-histidine tag from the C-terminus of MPC1 to the C-terminus of EGFP. The additional constructs MPC1-mTFP+MPC2-GFP-10xHis, MPC2 alone (plasmid construct devoid of the coding sequence for MPC1), and MPC2.C54S were generated using standard molecular biology techniques. The plasmids were propagated in TOP10 chemically competent bacteria (Thermo Fisher, USA).

Preparation of competent cells and transformation into yeast. The expression plasmids were transformed into a triple deletion mutant yeast strain (JRY2242, W303 *mpc1Δmpc2Δmpc3Δ*, *his3 lys2 met15 trp1 ura3*) (2), kindly provided by Jared Rutter (University of Utah, USA). The procedure

for transformation was adapted from a previously published protocol (35). Briefly, one yeast colony was inoculated into 5 mL of yeast peptone dextrose medium (YPD) and cultured overnight in an orbital shaker at 200 rpm, 30 °C. The following day, 0.8 mL of overnight culture was diluted into 30 mL of YPD in a 250-mL flask and grown as above until reaching an OD₆₀₀ of 0.8. The cells were centrifuged at 4,000 g for 10 min at 4 °C, and the pellet was resuspended in 25 mL of sterile water. The centrifugation process was repeated, and the competent cells were initially resuspended in 1 mL and then 0.4 mL of 100 mM lithium acetate. Subsequently, transformation was performed on ice, unless otherwise mentioned. To the 50 µL of competent cells 240 µL of 50 % (w/v) PEG 3350 was added for each transformation. A total of 25 µL of 2 mg.mL⁻¹ single-stranded carrier DNA was added to each tube and incubated for 5 s. Subsequently, 50 µL of DNA encoding human pyruvate carrier (100-150 ng) diluted in sterile water was added to each tube and incubated for 5 s, followed by heat shock for 45 min at 42 °C. The cells were centrifuged at 8,000 g for 1 min at RT, and the pellet was resuspended in 100 µL of sterile water. The cell suspension was plated onto SD-URA plates and incubated for 3 days at 30 °C.

Protein expression and purification. Typically, for large-scale overexpression of recombinant MPC, colonies from the SD-URA plates with the highest fluorescence levels were selected and used as pre-inoculum, with large-scale cultures (typically 4 L) grown in shake flasks. Two hundred-milliliter cell cultures (in 500 mL flasks) transformed with MPC plasmids were grown overnight in YPD (2 % glucose) with shaking at 200 rpm at 30 °C. The following morning, 10 mL of overnight culture was added to 500 mL of YPD (0.2 % glucose) in a 2 L flask and cultured as above until the OD₆₀₀ reached 0.8. Then, 2 % galactose (from a 20 % stock) was added to the culture. After 22 h, the cells were harvested via centrifugation at 4,000 g for 10 min. The pellets were resuspended in 400 mL of buffer containing 1X PBS, supplemented with 150 mM NaCl, 10 % glycerol, 2 mM β-mercaptoethanol and a protease inhibitor mix. Cell lysis was performed using a benchtop cell disruptor (Constant Systems) at increasing pressures (25, 28, 30, 32, 35 and 38 kPsi). After lysis, the cell suspension was centrifuged at 10,000 g for 10 min at 4 °C. The

supernatant was retained and further ultra-centrifuged at 150,000 g for 2 h to pellet the membranes. Isolated membranes of MPC1-8xHis or MPC2-10xHis were resuspended in similar solubilization buffer (SB) containing 1X PBS, pH 8.0, and 150 mM NaCl; 0.5 mM TCEP; 1 % (w/v) C12E8 was used for MPC2-10xHis, and 1 % (w/v) DDM was used for MPC1-8xHis. Membrane-detergent mixtures at a 2:1 ratio were rotated for 1 h at 4 °C. The suspension was cleared via ultra-centrifugation at 100,000 g for 45 min, and 10 mM imidazole was added to the solubilized solution. The solubilized samples were incubated for 3 h at 4 °C with TALON beads (Clontech) pre-equilibrated with SB (supplemented with 0.1 % (w/v) C12E8 + 0.1 % (w/v) DDM for MPC2-GFP-10xHis and with 0.1 % (w/v) DDM for MPC1-8xHis). Following incubation, the sample was loaded onto a glass column (Bio-Rad) and washed with 20 column volumes (CVs) of SB containing 0.1 % (w/v) DDM and 15 mM imidazole. The column was subsequently washed with another 20 CVs of the same buffer containing 30 mM imidazole. Bound proteins were eluted with SB containing 0.1 % (w/v) DDM and 300 mM imidazole. A similar procedure of column washing and elution was followed for both MPC2-10xHis and MPC1-8xHis. The eluted protein from the MPC1-8xHis construct was collected, concentrated to 5 mg.mL⁻¹ and immediately loaded onto a Superdex 200 PG 16/30 column (GE Healthcare, Sweden) pre-equilibrated in 20 mM TRIS, pH 8.0, 150 mM NaCl, 0.5 mM TCEP and 0.03 % DDM. The protein eluted from MPC2-10xHis construct was dialyzed overnight against 20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % glycerol, 0.05 mM TCEP and 0.03 % DDM in a 6,000-Da MWCO dialysis bag (Spectrum Laboratories, USA) in the presence of 10:1 (GFP fused pyruvate carrier: protease) GST-tagged PreScission Protease at 4 °C. After dialysis, the digest was passed through TALON (Clontech, USA) and GST (GE Healthcare, USA) beads equilibrated in dialysis buffer. The flow-through containing the membrane protein was collected and concentrated to 5 mg.mL⁻¹ and loaded directly onto a Superdex 200 PG 16/30 column previously equilibrated with 20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % glycerol, 0.05 mM TCEP and 0.03 % DDM. MPC2-10xHis in the absence of TCEP, and additional constructs, such as MPC2

alone (plasmid construct devoid of the coding sequence for MPC1) and the MPC2.C54S mutant, were similarly purified as described above. For all MPC constructs used in the present study, a typical purification preparation starting with inducible expression from four liters of yeast culture, resulted in approximately 1 mg of pure protein sample after full purification. Electrophoretic analysis was performed using Tricine-SDS-PAGE.

Confocal Microscopy. The Mito-mCherry plasmid for yeast expression used for mitochondrial labeling was kindly provided by Jean-Claude Martinou (Université de Genève, Switzerland). Yeast co-transformed with either of the MPC-expression plasmids or Mito-mCherry were cultured under the same conditions described above to facilitate the expression of GFP-labeled MPC. Subsequently, 10 mL of 22-h induced cells (at OD₆₀₀ of 1) was centrifuged at 250 rpm for 5 min. The pellet was resuspended in 1 mL of YPD. From this, 7 µL of cells were added to a slide and sealed with a cover slip. Samples were examined in the Biological Imaging Facility (LIB) at LNBio, using a Leica TCS SP8 confocal on a Leica DMI 6000 equipped with an HCX PL APO 63x/1.40 CS2 objective.

Quantitative PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen Corporation), and 5 µg of total RNA was used for retrotranscription using GoScript™ Reverse Transcription System (Promega). Real-time quantitative PCR for *MPC1* and *MPC2* was performed using the SYBR Green PCR Master Mix (Applied Biosystems). The threshold cycle (CT) values of the target genes were normalized to the actin gene, and relative expression ratios were calculated using the 2-ΔΔCT Ct method (36). Next, the readings were normalized to the URA3 transcripts to calculate the levels of transformed plasmids. The primer sequences were as follows: (1) Actin: ACT1.qPCR.For: 5' – ATTCTGAGGTTGCTGCTTTGG – 3' and ACT1.qPCR.Rev: 5' – TGTCTTGGTCTACCGACGATAG – 3'; (2) URA3: URA3.qPCR.For: 5' – TGGCAGCAACAGGACTAGGATG – 3' and URA3.qPCR.Rev: 5' – CGAACAGAAGGAAGAACGAAGGAAG – 3'; (3) Human MPC1: hMPC1.qPCR.For: 5' – TGACCTTTGCTTTGTGTTGC – 3' and hMPC1.qPCR.Rev: 5' – CACCTTGGATCAATTGTGCT – 3'; (4) Human

MPC2: hMPC2.qPCR.For: 5' – CGATATGGCTAGACCAGCAG – 3' and hMPC2.qPCR.Rev: 5' – ACAGCGAACAAGACCAATTT – 3'.

Fluorescence size-exclusion chromatography (FSEC). The appropriate detergent for protein extraction from membrane pellets was determined by FSEC analysis (35). A total of 250 µL of solubilized membranes containing the overexpressed EGFP-fused pyruvate carrier in DDM (Anatrace, OH, USA) for MPC1 and C12E8 (Anatrace, OH, USA) for MPC2 was loaded onto a Superdex 200 PG 16/30 column. The column was pre-equilibrated with 20 mM TRIS, pH 8.0, 150 mM NaCl, 0.5 mM TCEP and 0.03 % DDM for MPC1-8xHis and 20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % glycerol, 0.5 mM TCEP and 0.03 % DDM for MPC2- and connected to an AKTA start FPLC system at 4 °C. The flow rate was set at 1 mL.min⁻¹. Fractions of 200 µL were drawn from the elution into 96-well optical plate. GFP emission was measured at 512 nm on bottom read, with an excitation wavelength of 488 nm, using an EnSpire microplate reader (Perkin Elmer, Massachusetts, USA).

Calibration of gel filtration columns. Standard proteins from the low and high molecular weight Gel Filtration calibration kits (GE Healthcare, USA) were used. Protein resuspension buffer and running buffer were 20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % Glycerol, 0.5 mM TCEP and 0.03 % DDM.

Mass spectrometry. The protein bands from gel-filtration-purified samples were extracted from tricine-SDS-PAGE gels. The gels were first stained with Coomassie dye for band identification and excision. Bands were then isolated and destained in methanol (50 %)/acetic acid (2.5 %) solution, and the samples were reduced, alkylated and digested with trypsin (37). Peptides (4.5 µL) were separated by a C18 (75 µm x 100 mm, 1.7 µm particle size) nanoUPLC (nanoAcquity, Waters) coupled with a Q-ToF Premier mass spectrometer (Waters) with a nanoelectrospray source at a flow rate of 0.6 µL.min⁻¹. The gradient was 2-35 % acetonitrile in 0.1 % formic acid over 31.6 min for the digested proteins. The nanoelectrospray voltage was set to 3.5 kV, with a cone voltage of 30 V, and the source temperature was 80 °C. The instrument was

operated in the ‘top three’ mode, in which one MS spectrum is acquired, followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on an exclusion list for 60 s. The spectra were acquired using MassLynx v.4.1 software, and the raw data files were converted to a peak list format (mgf) using Mascot Distiller v.2.4.0.0 software 2011 (Matrix Science Ltd.) and were searched against the Human UniProt-SwissProt database (92,180 sequences; 36,693,332 residues; release date March 2016) and the Yeast UniProt-SwissProt database (550,299 sequences; 196,347,838 residues; release date March 2016), with *Saccharomyces cerevisiae* taxonomy (7,743 sequences) using Mascot engine v.2.3.2 (Matrix Science Ltd.) with carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions.

Preparation of liposomes and proteoliposomes. The purified MPC protein samples were reconstituted into liposomes using previously described procedures (38, 39) with minor modifications. Briefly, asolectin from soybean extract (Sigma Aldrich) was dissolved in chloroform and dried into a thin film by blowing gaseous N₂. This film was then resuspended to a final concentration of 4 mg.mL⁻¹ in buffer containing 20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % glycerol, 0.5 mM TCEP and 0.03 % (w/v) DDM and heated at 70 °C for 1 h with vortexing every 10 min. This suspension was sonicated in a water-bath sonicator at 40 kHz for 1 min to obtain the homogenized lipid-detergent solution. The purified proteins were added to the solution at a lipid-to-protein ratio of 10:1 (w/w), along with 0.05 % (w/v) DDM to avoid protein denaturation. The ternary protein-lipid-detergent mixture was freeze-thawed three times for full reconstitution and then sonicated for 1 min under the aforementioned conditions. To remove the detergent and allow proteoliposome formation, Bio-Beads SM-2 resin (Bio-Rad) was added at a ratio of 1:0.2 (w/w) and incubated at 4 °C with exchange after 5, 10 and 40 min, followed by ultra-centrifugation of the suspension at 100,000 g for 45 min, and the final proteoliposome pellet was resuspended in 20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % glycerol, and 0.5 mM TCEP. Protein-free liposomes (small unilamellar vesicles, or

SUVs) were prepared following a similar procedure, except that the protein solution was replaced with the gel filtration buffer. Purified MPC2-10xHis in the absence of TCEP, as well as the purified proteins from the additional constructs, such as MPC2 alone (plasmid construct devoid of the coding sequence for MPC1) and the MPC2.C54S mutant, were reconstituted into liposomes following the above protocol. The size and homogeneity of the liposomes and proteoliposomes were assessed via dynamic light scattering and Nanoparticle Tracking Analysis (NTA, Supp. Fig. 2A) as described below. To check for unwanted bursting or swelling of the liposomes and proteoliposomes upon incubation with the pyruvate solution, dynamic light scattering analysis was performed (Supp. Fig. 2B).

Nanoparticle Tracking Analysis. The size, homogeneity and concentration of the liposomes and proteoliposomes were assessed via NTA, as available on the NanoSight instrument (Malvern) Dynamic light scattering measurements were performed on a NanoSight NS300 (Malvern Instruments Ltd., United Kingdom). Fifty microliters of total proteoliposomes was diluted in 1 mL of filtered buffer (20 mM TRIS, pH 8.0, 300 mM NaCl, 5 % glycerol, 0.5 mM TCEP); 500 µL of this suspension was injected into the sample chamber using a sterile syringe. All measurements were performed at 18 °C, and the average of quintuple readings was determined.

In vitro pyruvate transport. Transport analysis was performed at room temperature and initiated by mixing 50 µL of the liposome/proteoliposome samples with 100 µL of 50 µM sodium pyruvate in a 96-well flat bottom Greiner plate. To induce a pH gradient of 1.5 units between the inner and outer vesicle environments, sodium pyruvate (Sigma, USA) was resuspended at the desired concentration in transport buffer containing 120 mM KCl and 100 mM MES, pH 6.5. Upon incubation for the desired times (1, 3, 5, 15 and 30 min) at room temperature, 50 µL of stop buffer (2 nM LDHA, 0.5 mM NADH, 50 mM TRIS, pH 8.0) was added. The mixture was immediately subjected to quantification of absorbance at 340 nm on an EnSpire plate reader (Perkin Elmer, USA) to track the oxidation of NADH. In addition, the necessity of various proton electrochemical gradients was measured as described above with minor modifications. Briefly,

sodium pyruvate was also dissolved in transport buffers containing 120 mM KCl, 100 mM HEPES, pH 7.2 (to achieve Δ pH of 0.8) and 120 mM KCl, 100 mM TRIS, pH 8.0 (for Δ pH = 0). Raw absorption readings were converted to amounts of NADH using an extinction coefficient of $6,220 \text{ M}^{-1}\text{cm}^{-1}$. Initial velocities for LDHA under all conditions were obtained using linear regression, keeping unrestrained intercepts. When appropriate, analysis of covariance (ANCOVA) was performed to determine whether the difference between two similar slopes was statistically non-significant (indicated as n.s. in the figures and respective legends). Velocities were rounded up to the first significant digit. The obtained enzyme velocities for each transport condition were compared with an LDHA standard curve (Supp. Fig. 2C) generated under the same chemical conditions used in the assay but in the absence of liposomes or proteoliposomes, termed Ctrl (-L/PL). Spontaneous oxidation of NADH was shown not to occur during the experimental timeframe (Supp. Fig. 2E). The plasmid for bacterial expression of human LDHA was a kind gift from Anne Le (Johns Hopkins Medicine, USA). The experiments were repeated four to six times, and the means $\pm$ standard deviations obtained were graphically represented.

Inhibition of MPC2-dependent in vitro pyruvate transport. Experiments to demonstrate the MPC2 pyruvate transport inhibition were performed at room temperature. Fifty microliters of liposome/proteoliposome samples was pre-incubated for 30 min with UK5099 and rosiglitazone dissolved in 0.1 % DMSO at a final concentration of 50 μM . As a control, liposome/proteoliposome samples were also similarly incubated with 0.1 % DMSO. After incubation for 30 min, 100 μL of 50 μM sodium pyruvate was added to the inhibitor-treated liposome/proteoliposome samples in a 96-well flat-bottom Greiner plate. Upon incubation for an additional 30 min at room temperature, 50 μL of stop buffer (2 nM LDHA, 0.5 mM NADH, 50 mM TRIS, pH 8.0) was added. The mixture was immediately subjected to quantification of absorbance at 340 nm on an EnSpire plate reader (Perkin Elmer, USA) to track the oxidation of NADH. The obtained enzyme velocities for transport in the presence of inhibitor were compared with an LDHA standard curve (Supp.

Fig. 2C) generated under the same conditions used in the assay, but in the absence of liposomes or proteoliposomes, termed Ctrl (-L/PL). Data processing and analysis were performed as described above. The experiments were repeated four to six times, and the means $\pm$ standard deviations obtained were graphically represented.

Substrate specificity assay. Lactate was assessed using the L-lactate colorimetric assay kit from Abcam, Inc. (ab65331) according to the manufacturer's instructions, with minor modifications. Substrate specificity analysis was performed at room temperature and initiated by mixing 50 μL of the liposome/proteoliposome samples with 100 μL of 50 μM sodium lactate (Sigma, USA) in a 96-well flat-bottom Greiner plate. Sodium lactate (Sigma, USA) was suspended to the desired final concentrations of 30, 60 and 120 μM in transport buffer containing 120 mM KCl, 100 mM MES, pH 6.5, which was also used to dissolve sodium pyruvate. Upon incubation for 30 min at room temperature, 50 μL of the lactate enzyme mix provided with the kit was added, and the plate was measured at 450 nm. Data processing and analysis were performed as described above for pyruvate transport. The obtained enzyme velocities for each transport condition were compared with a lactate standard curve obtained in a vesicle-free solution, termed Ctrl (-L/PL). The experiments were repeated four to six times, and the means $\pm$ standard deviations obtained were graphically represented.

Chemical cross-linking of MPC2. Cross-linking of MPC2 in mitochondrial extracts was performed according as previously published (6), but using DSS instead. For crosslinking of purified protein and protein reconstituted in proteoliposomes, MPC2 was prepared as described above, except that HEPES was used instead of TRIS in the final gel filtration buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, 5 % glycerol, 0.05 mM TCEP and 0.03 % DDM). Approximately 10 μg of protein was used in each reaction and incubated with DSS at 0.2, 0.4, 2 and 4 mM for 30 min at room temperature. The reactions were stopped using 50 mM TRIS, pH 8.0.

Growth assays. Yeast cells were grown overnight and inoculated in 5 mL of SD-URA medium supplemented with 0.2 % glucose for 22 h at 30 $^{\circ}\text{C}$, 200 rpm. Cells were recovered by centrifugation for

5 min at 3000 g, 4 °C, washed twice and then resuspended in 2.5 mL of SD-URA-VAL supplemented with 0.2 % glucose. OD₆₀₀ was adjusted to 0.034 (approximately 0.23 at 10 mm path-length) for all conditions using an EnSpire Plate Reader (Perkin-Elmer). Then, 10 µL of each cell culture was added to a 96-well plate containing 200 µL of SD-URA (supplemented with 0.2 % glucose) in the presence and absence of valine. Optical density was measured every 5 min, while cells were left to grow inside the EnSpire Plate Reader, with shaking at 200 rpm, at 30 °C.

Yeast cells were grown overnight and inoculated in 10 mL of SD-URA medium supplemented with 0.2 % glucose for 22 h at 30 °C, 200 rpm. Cells were recovered by centrifugation for 5 min at 3000 g, 4 °C, and resuspended in 1 mL of SD-URA-VAL. OD₆₀₀ was adjusted to 0.3 for all conditions using an EnSpire Plate Reader (Perkin-Elmer), and cells were centrifuged and resuspended again in the same culture medium.

Oxygen consumption measurements. Yeast cells were grown overnight and inoculated on 10 mL of SD-URA medium supplemented with 0.2 % glucose for 22 h at 30 °C, 200 rpm. Cells were recovered via centrifugation for 5 min, 3,000 g at 4 °C and resuspended in 1 mL of 25 mM HEPES-Buffered DMEM, pH 7.1 (Sigma, D5030). OD₆₀₀ was adjusted to 0.034 (approximately 0.23 at 10-mm path-length) for all conditions using an EnSpire

Plate Reader (Perkin-Elmer), and cells were centrifuged and resuspended again in the same buffer to remove any residual glucose. Measurements were performed using an Oxytherm (Hansatech, Norfolk, UK). Briefly, 970 µL of buffered DMEM was pre-warmed to 30 °C with shaking at 100 rpm in a reaction chamber. Next, 30 µL of OD-standardized cells was added, and the chamber was equilibrated open for 1 min. The chamber was then closed and incubated for 8 min to measure the basal respiration, followed by another 8 min for glucose (0.08 % final concentration to induce mitochondrial oxidation of pyruvate and not fermentation to ethanol (40)), and the final 8 min for ethanol (2 % final concentration to inhibit any residual fermentation (41, 42)). The raw oxygen concentration in the chamber collected every second was used to calculate multiple linear regressions of 150 s in length and average the oxygen consumption rate using the R software package.

Circular Dichroism. Synchrotron Radiation Circular Dichroism experiments were performed using a nitrogen-flushed Module X end-station spectrophotometer at B23 Beamline at the Diamond Light Source, Oxfordshire, UK (43), under proposal SM16289-2.

ACKNOWLEDGEMENTS

This work was supported by FAPESP under grants 2014/20673-2 (to ALBA) and 2015/25832-4 (to SMGD), fellowships 2014/06954-9 and 2017/02391-8 (to RSKN), 2014/12663-7 (to ZI), and scholarships 2015/02734-7 (to JENQ) and 2014/17820-3 (to DA). We thank LNBio for accessibility to core facilities as well as for financial support. We are very grateful to Dr. Alessandra Girasole for expert technical support and to Prof. Eneida de Paula (Unicamp, Brazil) for help in devising the *in vitro* pyruvate transport assay. The authors are also grateful for the use of the Membrane Protein Laboratory funded by the Wellcome Trust (099165/Z/12/Z and 202892/Z/16/Z) at the Diamond Light Source, UK. We acknowledge the support by the staff of the B23 Synchrotron Radiation Circular Dichroism beamline at the Diamond Light Source, UK.

COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.M.G.D. and A.L.B.A. conceived and initiated the research; R.S.K.N, J.E.N.Q, D.A., Z.I., J.B., H.G.S., C.F.R.A., A.M.F., B.A.P., S.R.C., J.F.O., A.C.T.S. and A.L.B.A. conducted the research; R.S.K.N, J.E.N.Q, D.A., Z.I., J.B., H.G.S., C.F.R.A., A.M.F., B.A.P., S.R.C., K.G.F., A.F.P.L., P.C., I.M., S.M.G.D and A.L.B.A. discussed and analyzed the data; R.S.K.N, J.E.N.Q, D.A, Z.I., S.M.G.D and A.L.B.A. wrote the manuscript. All authors revised and approved the final manuscript.

REFERENCES

1. Halestrap, A. P., and Denton, R. M. (1974) Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by alpha-cyano-4-hydroxycinnamate. *Biochem. J.* **138**, 313–316
2. Bricker, D. K., Taylor, E. B., Schell, J. C., Orsak, T., Boutron, A., Chen, Y.-C., Cox, J. E., Cardon, C. M., Van Vranken, J. G., Dephoure, N., Redin, C., Boudina, S., Gygi, S. P., Brivet, M., Thummel, C. S., and Rutter, J. (2012) A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, *Drosophila*, and humans. *Science*. **337**, 96–100
3. Herzig, S., Raemy, E., Montessuit, S., Veuthey, J.-L., Zamboni, N., Westermann, B., Kunji, E. R. S., and Martinou, J.-C. (2012) Identification and functional expression of the mitochondrial pyruvate carrier. *Science*. **337**, 93–96
4. Schell, J. C., Olson, K. A., Jiang, L., Hawkins, A. J., Van Vranken, J. G., Xie, J., Egnatchik, R. A., Earl, E. G., DeBerardinis, R. J., and Rutter, J. (2014) A Role for the Mitochondrial Pyruvate Carrier as a Repressor of the Warburg Effect and Colon Cancer Cell Growth. *Mol. Cell*. **56**, 400–413
5. Vacanti, N. M., Divakaruni, A. S., Green, C. R., Parker, S. J., Henry, R. R., Ciaraldi, T. P., Murphy, A. N., and Metallo, C. M. (2014) Regulation of Substrate Utilization by the Mitochondrial Pyruvate Carrier. *Mol. Cell*. **56**, 425–435
6. Bender, T., Pena, G., and Martinou, J. J.-C. (2015) Regulation of mitochondrial pyruvate uptake by alternative pyruvate carrier complexes. *EMBO J.* **34**, 1–14
7. Liang, L., Li, Q., Huang, L., Li, D., and Li, X. (2015) Sirt3 binds to and deacetylates mitochondrial pyruvate carrier 1 to enhance its activity. *Biochem. Biophys. Res. Commun.* **468**, 807–812
8. Kortüm, G., Vogel, W., and Andrussow, K. (1960) Dissociation constants of organic acids in aqueous solution. *Pure Appl. Chem.* **1**, 187–536
9. Compan, V., Pierredon, S., Vanderperre, B., Krznar, P., Marchiq, I., Zamboni, N., Pouyssegur, J., and Martinou, J. C. (2015) Monitoring Mitochondrial Pyruvate Carrier Activity in Real Time Using a BRET-Based Biosensor: Investigation of the Warburg Effect. *Mol. Cell*. **59**, 491–501
10. Vanderperre, B., Bender, T., Kunji, E. R. S., and Martinou, J. C. (2015) Mitochondrial pyruvate import and its effects on homeostasis. *Curr. Opin. Cell Biol.* **33**, 35–41
11. Halestrap, A. P. (2012) The mitochondrial pyruvate carrier: Has it been unearthed at last? *Cell Metab.* **16**, 141–143
12. McCommis, K. S., and Finck, B. N. (2015) Mitochondrial pyruvate transport: a historical perspective and future research directions. *Biochem. J.* **466**, 443–454
13. Gray, L. R., Rauckhorst, A. J., and Taylor, E. B. (2016) A Method for Multiplexed Measurement of Mitochondrial Pyruvate Carrier Activity. *J. Biol. Chem.* **291**, jbc.M115.711663

14. Halestrap, A. P. (1975) The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J.* **148**, 85–96
15. Halestrap, A. P. (1978) Pyruvate and Ketone-Body Transport across the Mitochondrial Membrane. *Biochem. J.* **172**, 377–387
16. Colca, J. R., McDonald, W. G., Cavey, G. S., Cole, S. L., Holewa, D. D., Brightwell-Conrad, A. S., Wolfe, C. L., Wheeler, J. S., Coulter, K. R., Kilkuskie, P. M., Gracheva, E., Korshunova, Y., Trusgnich, M., Karr, R., Wiley, S. E., Divakaruni, A. S., Murphy, A. N., Vigueira, P. A., Finck, B. N., and Kletzien, R. F. (2013) Identification of a Mitochondrial Target of Thiazolidinedione Insulin Sensitizers (mTOT)-Relationship to Newly Identified Mitochondrial Pyruvate Carrier Proteins. *PLoS One.* **8**, 1–10
17. Divakaruni, A. S., Wallace, M., Buren, C., Martyniuk, K., Andreyev, A. Y., Li, E., Fields, J. A., Cordes, T., Reynolds, I. J., Bloodgood, B. L., Raymond, L. A., Metallo, C. M., and Murphy, A. N. (2017) Inhibition of the mitochondrial pyruvate carrier protects from excitotoxic neuronal death. *J. Cell Biol.* **216**, 1091–1105
18. Divakaruni, A. S., Wiley, S. E., Rogers, G. W., Andreyev, A. Y., Petrosyan, S., Loviscach, M., Wall, E. a, Yadava, N., Heuck, A. P., Ferrick, D. a, Henry, R. R., McDonald, W. G., Colca, J. R., Simon, M. I., Ciaraldi, T. P., and Murphy, A. N. (2013) Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 5422–7
19. Patterson, J. N., Cousteils, K., Lou, J. W., Fox, J. E. M., MacDonald, P. E., and Joseph, J. W. (2014) Mitochondrial metabolism of pyruvate is essential for regulating glucose-stimulated insulin secretion. *J. Biol. Chem.* **289**, 13335–13346
20. Rampelt, H., and Laan, M. Van Der (2015) Metabolic remodeling : a pyruvate transport affair. *EMBO J.* **34**, 835–7
21. Newstead, S., Kim, H., von Heijne, G., Iwata, S., and Drew, D. (2007) High-throughput fluorescent-based optimization of eukaryotic membrane protein overexpression and purification in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 13936–13941
22. Nałecz, M. J., Nałecz, K. a, Broger, C., Bolli, R., Wojtczak, L., and Azzi, a (1986) Extraction, partial purification and functional reconstitution of two mitochondrial carriers transporting keto acids: 2-oxoglutarate and pyruvate. *FEBS Lett.* **196**, 331–336
23. Hildyard, J. C. W., Ämmälä, C., Dukes, I. D., Thomson, S. A., and Halestrap, A. P. (2005) Identification and characterisation of a new class of highly specific and potent inhibitors of the mitochondrial pyruvate carrier. *Biochim. Biophys. Acta - Bioenerg.* **1707**, 221–230
24. Halestrap, A. P. (1976) The mechanism of the inhibition of the mitochondrial pyruvate transportater by alpha-cyanocinnamate derivatives. *Biochem. J.* **156**, 181–3
25. Diekert, K., de Kroon, a I., Kispal, G., and Lill, R. (2001) Isolation and subfractionation of mitochondria from the yeast *Saccharomyces cerevisiae*. *Methods Cell Biol.* **65**, 37–51
26. Seddon, A. M., Curnow, P., and Booth, P. J. (2004) Membrane proteins, lipids and detergents: not just a soap opera. *Biochim. Biophys. Acta.* **1666**, 105–17
27. Floyd, B. J., Wilkerson, E. M., Veling, M. T., Minogue, C. E., Xia, C., Beebe, E. T., Wrobel, R. L., Cho, H., Kremer, L. S., Alston, C. L., Gromek, K. A., Dolan, B. K., Ulbrich, A., Stefely, J. A., Bohl, S. L., Werner, K. M., Jochem, A., Westphall, M. S., Rensvold, J. W., Taylor, R. W., Prokisch, H., Kim, J. J. P., Coon, J. J., and Pagliarini, D. J. (2016) Mitochondrial Protein Interaction Mapping Identifies Regulators of Respiratory Chain Function. *Mol. Cell.* **63**, 621–632
28. Huttlin, E. L., Ting, L., Bruckner, R. J., Gebreab, F., Gygi, M. P., Szpyt, J., Tam, S., Zarraga, G.,

- Colby, G., Baltier, K., Dong, R., Guarani, V., Vaites, L. P., Ordureau, A., Rad, R., Erickson, B. K., Wühr, M., Chick, J., Zhai, B., Kolippakkam, D., Mintseris, J., Obar, R. A., Harris, T., Artavanis-Tsakonas, S., Sowa, M. E., De Camilli, P., Paulo, J. A., Harper, J. W., and Gygi, S. P. (2015) The BioPlex Network: A Systematic Exploration of the Human Interactome. *Cell*. **162**, 425–440
29. Timoñ-Gómez, A., Proft, M., and Pascual-Ahuir, A. (2013) Differential regulation of mitochondrial pyruvate carrier genes modulates respiratory capacity and stress tolerance in yeast. *PLoS One*. **8**, 1–9
30. Vadvalkar, S. S., Matsuzaki, S., Eyser, C. A., Giorgione, J. R., Bockus, L. B., Kinter, C. S., Kinter, M., and Humphries, K. M. (2017) Decreased Mitochondrial Pyruvate Transport Activity in the Diabetic Heart: Role of Mitochondrial Pyruvate Carrier 2 (MPC2) Acetylation. *J. Biol. Chem.* **292**, 4423–4433
31. Lundby, A., Lage, K., Weinert, B. T., Bekker-Jensen, D. B., Secher, A., Skovgaard, T., Kelstrup, C. D., Dmytriiev, A., Choudhary, C., Lundby, C., and Olsen, J. V. (2012) Proteomic Analysis of Lysine Acetylation Sites in Rat Tissues Reveals Organ Specificity and Subcellular Patterns. *Cell Rep.* **2**, 419–431
32. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res.* **43**, D512–D520
33. Vigueira, P. A., McCommis, K. S., Schweitzer, G. G., Remedi, M. S., Chambers, K. T., Fu, X., McDonald, W. G., Cole, S. L., Colca, J. R., Kletzien, R. F., Burgess, S. C., and Finck, B. N. (2014) Mitochondrial Pyruvate Carrier 2 Hypomorphism in Mice Leads to Defects in Glucose-Stimulated Insulin Secretion. *Cell Rep.* **7**, 2042–2053
34. Miller, C. A., Martinat, M. A., and Hyman, L. E. (1998) Assessment of aryl hydrocarbon receptor complex interactions using pBEVY plasmids: expression vectors with bi-directional promoters for use in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **26**, 3577–83
35. Drew, D., Newstead, S., Sonoda, Y., Kim, H., von Heijne, G., and Iwata, S. (2008) GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. *Nat. Protoc.* **3**, 784–798
36. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and. *Methods*. **25**, 402–408
37. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–8
38. Xu, Y., Tao, Y., Cheung, L. S., Fan, C., Chen, L.-Q., Xu, S., Perry, K., Frommer, W. B., and Feng, L. (2014) Structures of bacterial homologues of SWEET transporters in two distinct conformations. *Nature*. **515**, 448–452
39. Yoneda, J. S., Rigos, C. F., De Lourenço, T. F. A., Sebinelli, H. G., and Ciancaglini, P. (2014) Na,K-ATPase reconstituted in ternary liposome: The presence of cholesterol affects protein activity and thermal stability. *Arch. Biochem. Biophys.* **564**, 136–141
40. Takeda, K., Starzynski, C., Mori, A., and Yanagida, M. (2015) The critical glucose concentration for respiration-independent proliferation of fission yeast, *Schizosaccharomyces pombe*. *Mitochondrion*. **22**, 91–95
41. Fernandes, L., Côrte-Real, M., Loureiro, V., Loureiro-Dias, M. C., and Leão, C. (1997) Glucose respiration and fermentation in *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* express different sensitivity patterns to ethanol and acetic acid. *Lett. Appl. Microbiol.* **25**, 249–253
42. Brown, S. W., Oliver, S. G., Harrison, D. E. F., and Righelato, R. C. (1981) Ethanol inhibition of

- yeast growth and fermentation: Differences in the magnitude and complexity of the effect. *Eur. J. Appl. Microbiol. Biotechnol.* **11**, 151–155
43. Hussain, R., Jávorfí, T., and Siligardi, G. (2012) Circular dichroism beamline B23 at the Diamond Light Source. *J. Synchrotron Radiat.* **19**, 132–5

FIGURE LEGENDS

Fig. 1. Recombinant expression and purification of human MPC. (A) A schematic of the initial human MPC protein constructs expressed in yeast (co-expression set 1). Left panel: Proper localization to the mitochondria was confirmed via confocal microscopy. DIC: Differential interference contrast. Middle panel: Electrophoretic analysis of MPC1-8xHis samples purified using Co-IMAC and GF. *N-terminally truncated portion of 60S RPL28. The inset displays the silver staining and in-gel fluorescence detection of trace amounts of MPC2-GFP, which co-purified with MPC1. Right panel: The corresponding GF trace showed a predominant protein-detergent monodisperse peak at approximately 64 kDa. (B) Diagram of the alternative MPC constructs (co-expression set 2) and corresponding confocal microscopy (left panel). Middle panel: Electrophoretic (Tricine-SDS-PAGE) analysis of representative chromatography steps. PP: PreScission Protease. Right panel: GF peak analysis revealed that pure monodisperse MPC2 (associated with DDM) was obtained at an equivalent molecular weight of 70 kDa. (C) Diagram of the single expressed MPC2 construct and corresponding confocal microscopy (left panel). Middle panel: Electrophoretic (Tricine-SDS-PAGE) analysis of representative chromatography steps. PP: PreScission Protease. Right panel: GF peak analysis revealed that pure monodisperse MPC2 (associated with DDM) was obtained at an equivalent molecular weight of 70 kDa. In all GF profiles above, v_0 indicates void volume, and v_t indicates the total liquid volume of the GF column. The corresponding elution volumes for calibration standards are shown in red.

Fig. 2. *In vitro* MPC activity and its dependence on time and electrochemical gradient. (A) Quantification of extraliposomal pyruvate, as detected based on LDHA activity, in liposomes (L) and proteoliposomes (PL) reconstituted with MPC1 and MPC2 and a control condition free of lipid vesicles (Ctrl (-L/PL)). The inset indicates the pH gradient across the outer and inner vesicle environments. Left panel: Initial velocities of LDHA for which the differences are not statistically significant (n.s.; ANCOVA $P > 0.01$;) are indicated. An asterisk (*) indicates that MPC1 co-purified with yeast RPL28. MPC2 expressed and purified in the complete absence of MPC1 is indicated with two asterisks (**). Right panel: Using the experimental Michaelian parameters for LDHA (Supp. Fig. 2C), the corresponding external pyruvate concentrations were calculated and plotted for each condition. The extent of transport for a given proteoliposome sample was quantified based on the loss of pyruvate from the extraliposomal medium with respect to the protein-free liposome and expressed as percentages. (B) Quantification of external pyruvate in the liposome and MPC2-proteoliposome as a function of different incubation times with a Δ pH of 1.5 units. An exponential decay model curve was fitted (solid blue line, $R^2 = 0.96$). (C) First to third panels depict the quantification of external pyruvate after 30 min of incubation for Δ pH = 1.5, 0.8 and 0. Right panel: Using the appropriate experimental Michaelian parameters for LDHA for each external pH applied (Supp. Fig. 2C), the corresponding external pyruvate concentrations were calculated and plotted for each condition to demonstrate the dependence of pyruvate transport on the electrochemical gradient according to the respective insets. In all bar graphs above, the protein components of proteoliposomes are indicated inside the respective bars.

Fig. 3. Evaluation of the effect of small-molecule inhibition, redox balance and substrate type on MPC2-dependent transport. (A) Left panel: Quantification of extraliposomal pyruvate in liposomes and MPC2-proteoliposomes in the presence of DMSO (vehicle control) and the well-established MPC inhibitors UK-5099 and rosiglitazone (Rgz). The inset indicates the pH gradient across the outer and inner vesicle environments. Statistically comparable initial velocities are indicated (n.s.; ANCOVA $P > 0.01$). Right panel: The corresponding extraliposomal substrate levels were calculated based on corresponding LDHA activity. The extent of transport for a given proteoliposome sample was quantified based on the loss of pyruvate from the extraliposomal medium with respect to the protein-free liposome and expressed as percentages. (B) Left panel: Quantification of extraliposomal pyruvate in proteoliposomes reconstituted with wild-type MPC2 in the presence and absence of TCEP as well as supplemented TCEP. The inset indicates the pH gradient across the outer and inner vesicle environments. The comparison between the blue and black curves indicates that LDHA activity was not affected by the absence of TCEP. Right panel:

Quantification of external pyruvate and corresponding transport levels (as percentages). **(C)** Similar analyses performed for the point mutant MPC2.C54S. **(D)** Under the same chemical conditions used for pyruvate transport, MPC2 displays selectivity for this substrate, as no apparent transport of lactate was observed over a range of extraliposomal concentrations (30, 60 and 120 μ M). As such, the enzymatic quantification of lactate at the beginning of the reaction and after a 30-min incubation (at equilibrium) are statistically indistinguishable (n.s.) between liposomes (L, gray) and proteoliposomes (PL, light gray) compared with a liposome/proteoliposome-free control (black, Ctrl (-L/PL)).

Figure 4. Autonomous transport function of MPC2 *in vivo*. **(A)** Differential growth rates of 3 Δ cells or JRY472 (green circles) expressing either wild-type MPC2 (blues squares) or MPC2.C54S in the absence and presence of external valine. **(B)** Differential respiration rates of 3 Δ cells or JRY472 (green circles) expressing either wild-type MPC2 (blues squares) or MPC2.C54S when exposed to glucose (filled geometric shapes) and ethanol (empty geometric shapes). Lines indicate the mean $\pm$ standard error. The inset displays the statistical significance of the differences according to the Benjamini-Hochberg multiple testing correction.

Fig. 5. Oligomeric state and secondary structure composition of MPC2. **(A)** Chemical cross-linking suggested that MPC2 assembled into higher oligomers in a lipid environment. Left panel: Cross-linking of human MPC2 from isolated yeast mitochondrial extracts by 0.2 mM DSS. Middle panel: Cross-linking, using increasing DSS concentrations (0.2 – 4 mM), of purified MPC2-detergent (DDM) complex. Right panel: Cross-linking, using increasing DSS concentrations (0.2 – 4 mM), of purified MPC2 reconstituted in asolectin-derived lipid vesicles. **(B)** Secondary structure analysis of MPC2 in presence and absence of ligands, as probed by Circular Dichroism. Far-UV CD spectra, as well as calculated percentage of secondary structure content in the form of pie-chart, for MPC2 alone (Ctrl; top left), in the presence of 25 μ M pyruvate (+Pyr; top right), in the absence of TCEP (-TCEP; bottom left), and in the presence of 50 μ M rosiglitazone (+Rgz; bottom right).

FIGURE 1.

FIGURE 2.

FIGURE 3.

FIGURE 4.

FIGURE 5.