UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS

**BIOLOGIA MOLECULAR DE LEVEDURAS E SUA APLICAÇÃO
BIOTECNOLÓGICA PARA ESTUDO DE MECANISMOS
CELULARES DO CÂNCER E SEU TRATAMENTO.**

Gisele Monteiro

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2017

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Texto sistematizado apresentado à Faculdade de Ciências Farmacêuticas da Universidade de São Paulo para obtenção do Título de Professor Livre-Docente junto ao Departamento de Tecnologia Bioquímico-Farmacêutica.

SÃO PAULO

2017

Ficha Catalográfica
Elaborada pela Divisão de Biblioteca e
Documentação do Conjunto das Químicas da USP.

Monteiro, Gisele
G532b Biologia molecular de leveduras e sua aplicação biotecnológica para
estudo de mecanismos celulares do câncer e seu tratamento /
Gisele Monteiro. -- São Paulo, 2017.
90p. + anexos.

Tese (livre-docência) – Faculdade de Ciências Farmacêuticas
da Universidade de São Paulo. Departamento de Tecnologia
Bioquímico-Farmacêutica.

1. Biotecnologia Farmacêutica 2. Estresse oxidativo
I. T.

615.1 CDD

Nothing in life is to be feared, it is only to be understood.

Marie Curie

DEDICATÓRIA

Ao meu filho Gustavo, minha obra mais importante, por me ensinar a redimensionar os problemas e dar valor ao que de fato importa.

À Universidade Estadual de Campinas (UNICAMP) por fazer de mim a profissional que sou, mas principalmente por me transformar num ser humano melhor.

A todos os alunos e professores que contribuíram com seu trabalho, dedicação, apoio e amizade, possibilitando que eu tivesse material para produzir esse texto e para que eu pudesse construir uma história acadêmica.

AGRADECIMENTOS

Aos meus pais, minha irmã, meus sobrinhos. O apoio de vocês me fortalece.

Aos Professores Dr Adalberto Pessoa Junior e Dra Susana M.I. Saad pela amizade e apoio dentro do Departamento e da Faculdade. Vocês me acolheram e tornaram minha jornada mais leve.

Aos meus alunos André de Abreu Cavalheiro, Tallybia Hayrian Trevisan, Gabriel Moretti de Almeida, Iris Munhoz Costa, Lucas de Sousa Cavalcante, Grazielle Fonseca de Sousa, Paula Aryane Huang, Claudia Aznar Alesso, Gisele Cristina Ravaneli, Mariana Silva Moreira Leite, Lucas Fonseca Gonzaga, Henrique Pellin Biasoto, Beatriz de Araújo Bianchi Pedra, SeungHag Lee, Bruna de Souza Divino, Rafaela Coelho Correia, Mariane Augusta Domingues Rodrigues, Melina Cardoso dos Santos, Samarina Rodrigues Wlodarczyk, Marcela Valente Pimenta, Débora Fernandes Custodio, Karen Fulan Discola, Guilherme Meira Lima, Brian Effer Roldán. Vocês fizeram parte e contribuíram de forma essencial para essa etapa da minha vida.

Aos funcionários do FBT e da FCF pelo apoio, compreensão e amizade.

Aos meus amigos e colaboradores Prof Dr Marcos Antonio de Oliveira (UNESP-CLP), Prof Dr Anderson Ferreira da Cunha (UFSCar) e Profa Dra Camila Oresco dos Santos (Cold Spring Harbor Laboratory).

Em especial, à Karen Fulan Discola pelo apoio sempre, pela amizade e pela inestimável ajuda com o memorial. Você me acalma.

À minha irmã e amiga de ciência e da vida Simone Vidigal Alves. Admiro sempre.

Aos meus amigos da FCF/USP Prof Dr Hélio A. Stefani, Profa Dra Sandra H. P. Farsky, Profa Dra Irene da Silva Soares.

Aos amigos Alex Alfredo de Oliveira, Larissa Cristina Bentim, Rafaela Correia Coelho, Bruna de Souza Divino, Valter Feitosa, Talita Camila Nascimento e Tales Alexandre Costa e Silva, queridos pra sempre (PC).

À Profa Dra Carlota de Oliveira Rangel Yagui pela parceria na Pós-Graduação, apoio e orientação para a Livre-Docência.

Aos meus mentores de Ciência Prof Dr Luis E.S. Netto (IB/USP) e Prof Dr Gonçalo A. G. Pereira. Vocês me ensinaram a amar e respeitar a Ciência.

À FAPESP pelo apoio financeiro essencial desde o começo da minha carreira.

À CNPq e CAPES pelo apoio aos Programas e alunos de Pós-Graduação que fazem grande parte da Ciência desse país.

À Universidade de São Paulo que me fascina, move e inspira com essa grandiosidade e força.

RESUMO

MONTEIRO, G. **Biologia molecular de leveduras e sua aplicação biotecnológica para estudo de mecanismos celulares do câncer e seu tratamento.** 2017. 90 fls + anexos. Tese (Livre Docência) – Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, São Paulo, 2017.

Palavras-chave: *Saccharomyces cerevisiae*, *Pichia pastoris*, expressão heteróloga de proteínas, resistência aos antitumorais, estresse oxidativo, biofármaco.

A levedura *Saccharomyces cerevisiae* é o eucarioto mais bem caracterizado quanto às vias metabólicas, celulares e funções de genes. Calcula-se que dos 6.000 genes codificadores de proteínas, pelo menos 3.000 tenham homologia com genes humanos. Além disso, mesmo quando não há homologia direta, os mecanismos moleculares são conservados e em ensaios de complementação de função é possível caracterizar funções análogas em humanos. Leveduras apresentam além de mecanismos conservados, diversas semelhanças metabólicas com células tumorais; talvez a mais marcante delas seja a repressão catabólica por glicose. Esse fenômeno causa uma superativação da via glicolítica e inibição da cadeia respiratória, o que lembra muito o efeito Warburg apresentado pelas células tumorais. Esse comportamento metabólico tem relação estreita com a homeostase redox celular. Estes fatores mostram o quanto à caracterização dessas células fúngicas pode ser aplicada ao entendimento e tratamento do câncer. Mesmo assim, 20 anos após o término do sequenciamento do genoma de *S. cerevisiae*, mais de 1.000 genes continuam anotados como não caracterizados. Além disso, as leveduras têm papel fundamental na produção biotecnológica de insumos farmacêuticos. Nesse texto sistematizado relato as contribuições dos meus estudos, juntamente com os resultados do meu grupo de pesquisa, usando a biologia molecular das leveduras, para responder questões aplicadas à compreensão de funções celulares (com destaque à resposta antioxidante), mecanismos moleculares de resposta aos antitumorais e produção de biofármacos. Além de modelo celular, as leveduras representam excelentes plataformas para expressão heteróloga de proteínas de interesse Biotecnológico-Farmacêutico.

ABSTRACT

MONTEIRO, G. **Molecular biology of yeast and its biotechnological application to study the cellular mechanisms of cancer and its treatment.** 2017. NNN fls. Tese (Livre Docência) – Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, São Paulo, 2017.

Keywords: *Saccharomyces cerevisiae*, *Pichia pastoris*, heterologous protein expression, antitumor resistance, oxidative stress, biopharmaceutical.

The yeast *Saccharomyces cerevisiae* is the most well characterized eukaryote for metabolic, cellular and gene functions. It is estimated that of the 6,000 protein-encoding genes, at least 3,000 have homology to human genes. Moreover, even when there is no direct homology, the molecular mechanisms are conserved and through function complementation assays it is possible to characterize analogous functions in humans. Yeasts present, in addition to conserved mechanisms, diverse metabolic similarities with tumor cells; perhaps the most remarkable of them is the catabolic repression by glucose. This phenomenon causes an over activation of the glycolytic pathway and inhibition of the respiratory chain, which strongly resembles the Warburg effect presented by tumor cells. This metabolic behavior is closely related to cellular redox homeostasis. These factors show how much the characterization of these fungal cells can be applied to the understanding and treatment of cancer. Even so, 20 years after the end of the sequencing of the *S. cerevisiae* genome, more than 1,000 genes remain annotated as uncharacterized. In addition, yeasts play a key role in the biotechnological production of pharmaceutical inputs. In this systematized text I show the contributions of my studies, together with the results of my research group, using molecular biology of yeast to answer questions applied to understanding cellular functions (with emphasis on the antioxidant response), molecular response mechanisms to antitumor drugs and biopharmaceutical production. In addition to cellular model, yeasts represent excellent platforms for the heterologous expression of proteins of Biotechnological-Pharmaceutical interest.

TRABALHOS ANEXADOS (por ordem de exemplificação no texto).

Monteiro G, Horta BB, Pimenta DC, Augusto O, Netto LES. Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C. *Proc Natl Acad Sci U S A* 2007;104:4886-91. doi:10.1073/pnas.0700481104. (ANEXO 1).

Costa IM, Nasser TH, Demasi M, Nascimento RM, Netto LE, Miyamoto S, Prado FM, **Monteiro G**. The promoter of filamentation (*POF1*) protein from *Saccharomyces cerevisiae* is an ATPase involved in the protein quality control process. *BMC Microbiol* 2011;11(268):1-11. doi:10.1186/1471-2180-11-268. (ANEXO 2).

Moretti-Almeida G, Netto LES, **Monteiro G**. The essential gene *YMR134W* from *Saccharomyces cerevisiae* is important for appropriate mitochondrial iron utilization and the ergosterol biosynthetic pathway. *FEBS Lett* 2013;587:3008-13. doi:10.1016/j.febslet.2013.07.024. (ANEXO 3).

Alesso CA, Discola KF, **Monteiro G**. The gene *ICS3* from the yeast *Saccharomyces cerevisiae* is involved in copper homeostasis dependent on extracellular pH. *Fungal Genet Biol* 2015;82:43-50. doi:10.1016/j.fgb.2015.06.007. (ANEXO 4).

Sousa GF, Lima MA, Custodio DF, Freitas VM, **Monteiro G**. Chemogenomic study of Carboplatin in *Saccharomyces cerevisiae*: inhibition of the NEDDylation process overcomes cellular resistance mediated by HuR and Cullin proteins. *Plos One* 2015;10(e0145377):1-18. doi:10.1371/journal.pone.0145377. (ANEXO 5).

Santos JH, Costa IM, Molino JV, Leite MS, Pimenta MV, Coutinho JA, Pessoa Jr A, Ventura SP, Lopes AM, **Monteiro G**. Heterologous expression and purification of active L-asparaginase I of *Saccharomyces cerevisiae* in *Escherichia coli* host. *Biotechnol Prog* 2016 [In press]. doi:10.1002/btpr.2410. (ANEXO 6).

Costa IM, Schultz L, Araujo BPB, Leite, MS, Farsky SH, Oliveira MA, Pessoa Jr A, **Monteiro G**. Recombinant L-asparaginase 1 from *Saccharomyces cerevisiae*: an allosteric enzyme with antineoplastic activity. *Sci Rep* 2016;6(36239):1-11. doi:10.1038/srep36239. (ANEXO 7).

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1. INTRODUÇÃO.

1.1 Levedura como modelo celular para compreensão de mecanismos associados ao câncer e respostas a drogas.

Um dos mais clássicos exemplos da aplicação de leveduras para compreensão de mecanismos celulares conservados ao longo da evolução é a caracterização do ciclo celular. Nas décadas de 60 e 70, Leland Hartwell e Paul Nurse, usando *Saccharomyces cerevisiae* e *Schizosaccharomyces pombe* (respectivamente), obtiveram informações que levaram a um grande avanço na compreensão geral das moléculas e vias metabólicas envolvidas na divisão celular. Isso inclui a descrição das quinases dependentes de ciclinas e a implementação de conceitos como o ponto de inspeção (“checkpoint”) durante o ciclo. Laureados com Prêmio Nobel de Fisiologia e Medicina (2001), seus resultados tiveram impacto na Biologia, Medicina e Farmacologia. Muitos dos estudos de tumorigênese estão embasados nesses conceitos e permitiram o desenvolvimento de drogas para controlar / interromper esse processo.

S. cerevisiae é um dos organismos modelo mais simples, com dezesseis cromossomos e aproximadamente 6.600 ORFs (do inglês “open reading frames” ou quadros abertos de leitura). Seu genoma foi um dos primeiros a ser sequenciado e isso levou essa levedura a ser o eucarionte mais bem estudado. Mesmo com todo o esforço da comunidade científica na caracterização dessa levedura, cerca de 20% de suas ORFs (1.400 genes) continuam sem função biológica definida (<http://www.yeastgenome.org/genomesnapshot>). Para fins comparativos, o genoma humano tem uma estimativa de 20.000 genes codificadores de proteínas, ou seja, pelo menos três vezes mais que a *S. cerevisiae*. Contudo, no genoma humano essas ORFs correspondem a menos de 2% do tamanho do genoma e ainda assim, pelo menos metade desse material genético permanece pouco caracterizada (Galperin & Koonin, 2010).

S. cerevisiae tem sido um excelente modelo para compreensão de fenômenos celulares básicos que se mostraram conservados em organismos de história evolutiva mais recente, o que nos leva a uma possível extrapolação para o entendimento das células humanas. A comunidade científica que estuda essa levedura mantém um repositório de informações muito completo e atualizado, publicamente disponível no site *Saccharomyces Genome Database* – SGD (www.yeastgenome.org). Além disso, esse eucarioto é o único a ter coleções sistematizadas de células *knockouts* para todos os genes não essenciais na forma haploide ou diploide homozigoto e para todos os genes, incluindo os essenciais, na forma diploide heterozigoto. Todas as coleções estão disponíveis comercialmente, num preço acessível. Isso foi possível devido à própria robustez do genoma desse organismo, que se apresenta compacto (praticamente sem regiões intrônicas), com alta taxa de recombinação homóloga (permitindo uma rápida manipulação genética), pequeno tempo de geração e meios de cultivo simples e definidos.

Essas coleções têm sido amplamente aplicadas em estudos de caracterização de respostas celulares nas áreas médica, agrícola, ambiental, farmacêutica e biotecnológica, com uso de ferramentas “ômicas” - genômica, transcriptômica, proteômica, metabolômica e mais recentemente quimiogenômica ou toxicogenômica (Figura 1) (Silberberg et al., 2016 e revisão em Santos et al., 2012).

Figura 1. Diferentes processos celulares e moléculas são estudados e caracterizados pelas ferramentas ômicas aplicadas às células de leveduras. Apesar de ser um eucarioto simples, a maior parte das funções de suas moléculas estão conservadas ao longo da evolução. (1) processo de transcrição – avaliado por transcriptômica; (2) tradução – avaliada por proteômica; (3) interação de proteínas e drogas, bem como (4) interação de drogas com o DNA – avaliados por toxicogenômica. Estão ainda disponíveis a metabolômica – ferramenta para avaliar os metabólitos celulares e a genômica, imprescindível para execução de todas as ferramentas citadas. Fonte: Adaptado de Santos et al., 2012.

Mas como extrapolar mecanismos moleculares de um problema intrinsecamente multicelular como o câncer, a partir de conhecimento obtido de um organismo unicelular como *Saccharomyces cerevisiae*? Além disso, aproximadamente metade dos tumores humanos apresenta algum tipo de mutação na proteína p53, que até o momento não foi encontrada nessa levedura. O ganhador do Prêmio Nobel, Leland Hartwell explica que os mecanismos celulares que podem resultar no câncer são os mesmos que agem para controlar as divisões celulares nas leveduras. Apesar de os eventos acontecerem em organismos muito diferentes, as moléculas efetoras são as mesmas – DNA, RNA, proteínas, carboidratos e lipídeos. Células tumorais apresentam

descontrole na divisão celular, instabilidade genômica (processo não fidedigno de cópia, perda ou ganho do material genético) e perda tanto de mecanismos de reparo de DNA como de parada do ciclo para o reparo. Todos esses processos estão presentes na levedura, em verdade, foram descobertos e caracterizados molecularmente nesse organismo (Hartwell, 2005). Quanto à ausência de p53, apesar de ainda não ter sido encontrada nenhuma molécula homóloga ou de função análoga na levedura, os mecanismos moleculares estão presentes e conservados, pois ao expressar na levedura p53 humana selvagem ou com mutações encontradas nos tumores, essa célula apresenta o mesmo fenótipo observado em células humanas (Brachmann et al., 1996; Inga et al., 1998; Schlichtholz et al., 2004).

O estresse oxidativo e a resposta antioxidante estão relacionados com vários processos patológicos humanos como câncer, doenças neurodegenerativas, envelhecimento, processos inflamatórios, isquemia-reperfusão, infertilidade idiopática, dentre outros. Especialmente para a área de estudos redox e suas relações com o câncer, *S. cerevisiae* é um modelo interessante devido ao fato de ser um organismo preferencialmente fermentador. Isso significa que com a adição de fontes de carbono fermentáveis (como a glicose, frutose) no meio de cultura, mesmo na presença de oxigênio, a levedura irá obter aproximadamente 90% de seu ATP através do processo de fermentação. Como consequência, suas mitocôndrias estarão menos ativas e em menor número, com baixo metabolismo respiratório (revisões em Conrad et al., 2014; Kayikci & Nielsen, 2015). Como a principal fonte endógena de espécies reativas de oxigênio (ROS – *reactive oxygen species*) é o metabolismo mitocondrial, a levedura nos provê uma forma simples de controlar a atividade respiratória celular ao manipularmos apenas seu meio de cultivo. Muitos genes de resposta ao estresse oxidativo têm sua expressão reprimida pela presença de glicose no meio, fenômeno amplamente conhecido em leveduras como repressão catabólica (Monteiro et al., 2002 e 2004).

A repressão catabólica em *S. cerevisiae* é um fenômeno que apresenta muita similaridade com o metabolismo tumoral. É bem aceito pela comunidade científica que tumores, especialmente os sólidos, apresentam uma aceleração da via glicolítica e uma diminuição da atividade respiratória, fenômeno denominado “efeito Warburg”. Esse comportamento metabólico, extremamente semelhante ao de *S. cerevisiae* (Figura 2), tem sido observado em células que apresentam alta taxa de proliferação, sendo relacionado a uma menor produção líquida de ATP, mas de maneira muito mais rápida e independente de oxigênio que favorece o rápido crescimento (Nilsson & Nielsen, 2016). Isso pode ser uma forma de adaptação importante para as células localizadas no interior de tumores sólidos, que possuem menor aporte de oxigênio. A maior utilização da via glicolítica que em leveduras resulta em desejáveis altas concentrações de etanol (utilizado como combustível por nós humanos), em células tumorais gera alta concentração de lactato, o que reflete em outro fenômeno característico que é a acidez do microambiente tumoral. O efeito Warburg tem sido relacionado com a agressividade e o ganho de capacidade metastática do tumor (revisões em Diaz-Ruiz et al., 2011; Tosato et al., 2013; Lu et al., 2015).

A atividade mitocondrial, inibida em tumores e células de levedura crescidas em glicose, exerce uma função extremamente importante na homeostase redox celular. Se por um lado a alta atividade da cadeia respiratória gera mais ROS, o impedimento ou desaceleração da cadeia de transporte de elétrons também. O primeiro caso é intuitivo, pois mais mitocôndrias, maior atividade da cadeia de transporte de elétrons resultam em maior probabilidade de produção de ROS. O segundo tem sido relatado mais recentemente e baseia-se no fato de que a diminuição na velocidade de transporte dos elétrons na cadeia respiratória mitocondrial aumenta a possibilidade de vazamento dos mesmos, levando a uma maior redução parcial do oxigênio. Além disso, a função

primordial das mitocôndrias é produzir cluster ferro-enxofre e heme, essenciais para a viabilidade celular e componente de enzimas antioxidantes como citocromo c peroxidase, catalase e outras (revisão em Liemburg-Apers et al., 2015).

Figura 2: Semelhanças metabólicas do efeito Warburg em células tumorais e a repressão catabólica causada pela glicose em células de leveduras. Ambas as células, mesmo na presença de oxigênio, produzem grande parte da energia na forma de ATP através de ativação da via glicolítica e repressão da atividade respiratória mitocondrial. HK = hexoquinase; PFK = fosfofrutoquinase; LDH = lactato desidrogenase; PDC = piruvato descarboxilase; PDH = complexo piruvato desidrogenase; PDHK = piruvato desidrogenase quinase; SDH = succinato desidrogenase. ↑ = superexpressão; ↓ = inibição da expressão; ⊥ = inibição da atividade; ★ proteína ou complexo superativo. Fonte: Adaptado de Diaz-Ruiz et al., 2011.

A compreensão do metabolismo e da fisiologia dessa levedura pode ter implicações diretas em diversas áreas biotecnológicas e farmacêuticas, já que *S. cerevisiae* é amplamente utilizada nos processos fermentativos industriais (produção de etanol, vinhos, cerveja, pães) e em produção heteróloga de proteínas de interesse industrial e comercial.

1.2 Levedura como fonte de biofármacos.

As principais características que diferem um fármaco sintético de um biofármaco são o tamanho e a complexidade. Apenas para dimensionar essa diferença, enquanto a aspirina (ácido acetilsalicílico) tem massa molar de 180,14 g/mol, o biofármaco asparaginase apresenta 31.731,9 g/mol por monômero (a forma ativa usada é um tetrâmero, ou seja, a asparaginase biofármaco é mais de 700 vezes maior que o fármaco aspirina). Isso implica em enorme complexidade estrutural e de ligações químicas, o que torna inviável a produção sintética industrial de biofármacos. Dessa forma, a biotecnologia farmacêutica apresenta um papel fundamental no suprimento desse tipo de medicamento, produzido em “fábricas vivas”.

De um ponto de vista financeiro, quando nos referimos aos gastos do Sistema Único de Saúde (SUS) brasileiro, observamos que apenas 4% dos medicamentos são da classe biológicos, mas corresponde a 50% dos recursos investidos por essa instituição, devido aos seus altos custos (dados do portal Brasil - <http://www.brasil.gov.br/saude/> informação publicada em 08/12/2016). Nos Estados Unidos da América, uma estimativa do crescimento do mercado biotecnológico entre 2012-2020 mostra a importância econômica desse segmento (Figura 3).

Figura 3: Estimativa de mercado biotecnológico nos Estados Unidos da América por área de aplicação (Fonte: adaptado de <http://www.grandviewresearch.com/industry-analysis/biotechnology-market>).

Em decorrência dessa complexidade, custo de produção e necessidade de fonte constante para obtenção do biofármaco, vários sistemas de produção vêm sendo desenvolvidos ao longo dos anos para a obtenção de medicamentos baseados nas atividades de biomoléculas complexas, sendo as proteínas a principal classe utilizada. Para expressão de proteínas é possível ter sistemas endógenos ou heterólogos, naturais ou recombinantes de produção. Um conjunto de fatores como preço final do produto, forma de administração farmacêutica, produtividade com relação à obtenção da forma ativa da proteína, dentre outros vão reger a escolha da célula produtora. Nesse trabalho iremos centrar as discussões em produção heteróloga e recombinante de proteínas em microrganismos, mais especificamente em bactérias *Escherichia coli* e leveduras e *Pichia pastoris*.

Quando se pretende expressar produtos nos quais mudanças pós-traducionais não sejam necessárias ou desejadas, o sistema de escolha é o procarionte *E. coli*, devido a sua alta produtividade volumétrica e rápido crescimento em meio simples e barato. Por outro lado, em muitos casos, se a proteína for expressa em grandes quantidades ocorre agregação, gerando corpos de inclusão que interferem na atividade e purificação da proteína (Wang et al., 2016; Schmidt, 2004).

No entanto, se modificações pós-traducionais foram essenciais para a atividade da proteína de interesse, o sistema de expressão eucarioto é obrigatório. Em comparação a outros sistemas eucarióticos de expressão heteróloga como células de mamíferos ou de insetos, as leveduras como um sistema microbiano, combinam a facilidade de manipulação genética e crescimento relativamente rápido a altas densidades celulares, em meios relativamente simples (Gellissen, 2000). As células de insetos apresentam bons níveis de expressão de proteínas, mas, ainda menores que a expressão em leveduras; fazem glicosilação diferente das de células de mamíferos e possuem risco potencial de infecção viral, além de necessitar de meios mais complexos para sua manutenção. As células de mamíferos são de alto custo para manutenção, exigem meios de cultivo complexos e nem sempre com componentes bem definidos (normalmente, há necessidade de usar soro fetal bovino nas culturas) e, devido ao crescimento lento em meio rico em nutrientes, são muito vulneráveis à contaminação microbiana. Células de mamífero são de difícil escalonamento industrial, não suportam tensão de cisalhamento por não possuírem parede celular e apresentam alto risco em relação à transmissão viral, devido à proximidade evolutiva com os humanos (Wurm, 2004; Druzinec et al., 2013).

A levedura metilotrófica *P. pastoris* tem várias características que permitem que ela seja uma boa hospedeira para a produção heteróloga de proteínas (Tabela 1). Talvez o mais importante para a indústria seja o fato dessa levedura permitir um processo de purificação mais rápido e eficiente de obtenção do produto devido à baixa concentração de proteínas endógenas que ela normalmente secreta (Weinacker et al., 2014). Assim, ao secretar as proteínas heterólogas *P. pastoris* apresenta duas funções importantes: produz a proteína e secreta-a através de sua membrana e parede celular funcionando como um filtro, o que resulta em um rendimento de 80 a 90% das proteínas totais do meio extracelular sendo representado pelo alvo de interesse; isso facilita e barateia os processos posteriores de purificação (Pennell e Eldin, 1998; Cregg et.al., 2009; Looser et.al., 2015). A secreção celular de proteínas diminui alguns processos unitários *downstream*, como rompimento celular e clarificação. Além disso, essa levedura tem capacidade de alcançar alta densidade celular em meio mínimo salino simples (160 g/L⁻¹ de biomassa seca) (Macauley-Patrick et.al., 2005, Looser et.al., 2015) e possui forte tendência a utilizar rotas aeróbicas e assim, diferentemente da *S. cerevisiae* que é fermentadora, não é acumuladora de etanol, que pode ser danoso à atividade da biomolécula secretada (Spadiut, et.al., 2014).

A primeira parte do texto sistematizado tem o objetivo de mostrar a contribuição científica efetuada usando a biologia molecular de levedura como modelo celular eucariótico para compreensão e caracterização de mecanismos e moléculas celulares envolvidos com o sistema antioxidante, bem como a resistência e sensibilidade celular ao tratamento com drogas antitumorais. Além disso, as leveduras são usadas para produzir biofármacos, também aplicado em tratamento de tumores, assunto e contribuição abordados na segunda parte do texto sistematizado.

Tabela 1: Lista de produtos com aplicação na saúde humana produzidos em *P. pastoris*.

Fonte: Modificado de <http://www.pichia.com/science-center/commercialized-products/>

PRODUTO	EMPRESA	USO
Kalbitor® (Inibidor da caliceína)	Dyax (EUA)	Tratamento de angioedema hereditário
Insugen® (insulina humana)	Biocon (Índia)	Tratamento para diabetes
Medway (albumina humana)	Mitsubishi Tanabe Pharma (Japão)	Regulação do Volume sanguíneo
Shanvac™ (Vacina contra Hepatite B)	Shantha/Sanofi (Índia)	Prevenção de Hepatite B
Shanferon™ (interferon-α 2b)	Shantha/Sanofi (Índia)	Tratamento de Hepatite C e Câncer
Ocriplasmin (microplasmina)	ThromboGenics (Bélgica)	Tratamento de adesão vitreomacular
Nanobody® ALX-0061 (fragmento de anticorpo anti-receptor IL6)	Ablynx (Bélgica)	Tratamento de artrite reumatóide
Nanobody® ALX00171 (fragmento de anticorpo anti - RSV)	Ablynx (Bélgica)	Tratamento de infecção pelo vírus respiratório sincicial
Heparin-binding EGF (fator de crescimento)	Trillium (Canadá)	Tratamento de cistite intersticial
Recombinant collagen (colágeno)	Fibrogen (EUA)	Preenchimento dermatológico

2. BIOLOGIA MOLECULAR DE LEVEDURA - APLICAÇÃO COMO MODELO CELULAR - *SACCHAROMYCES CEREVISIAE*.

2.1 Descoberta de mecanismos enzimáticos conservados.

As peroxirredoxinas (Prxs) são importantes e versáteis enzimas antioxidantes amplamente distribuídas em todos os filos taxonômicos conhecidos. Em relação às outras enzimas antioxidantes, as Prxs foram recentemente descritas e sua função biológica ainda é pouco conhecida e intrigante (Netto et al., 2007).

Prx1 e Prx2, proteoformas citosólicas humanas estão envolvidas em eventos de crescimento e proliferação celular e diferentes trabalhos demonstraram que essas enzimas são superexpressas em tipos distintos de cânceres como de esôfago, pâncreas, tireoide, pulmão e mama. A elevada expressão também relaciona Prx1/Prx2 a um fenótipo mais agressivo de células cancerosas, em razão de maior resistência à quimioterapia e à radioterapia nesses tumores (Kang et al., 2005; Lee et al., 2009; Miki and Funato, 2012).

Em adição aos tumores sólidos, um importante papel de Prx1 e Prx2 tem sido caracterizado em leucemias. Na leucemia mielóide aguda (LMA), a forma de leucemia mais comum entre adultos, ocorre um bloqueio da diferenciação de células sanguíneas em estágios específicos de maturação, impedindo a especialização celular e levando à proliferação descontrolada de células leucêmicas. Acredita-se que essa diferenciação seja mediada por hidroperóxidos, em especial o H_2O_2 e, portanto, regulada por peroxidases. De fato, Liu et al. (2012 e 2013) observaram que a inibição das enzimas Prx1/Prx2 leva à indução da diferenciação de células tumorais de LMA, indicando uma remissão do tumor (Liu et al., 2012 e 2013).

A primeira descrição de Prxs foi feita em *S. cerevisiae* nos grupos dos Drs. Stadtman e Rhee que tentavam purificar a enzima glutamina sintetase (GS). Ao longo dos passos da purificação de GS, esta enzima perdia a atividade em tampões que continham ditioneitol (DTT) ou β -mercaptoetanol, mas era mantida se estivesse na presença de extrato celular de leveduras. Mais tarde, esse grupo de pesquisadores isolou uma proteína antioxidante no extrato proteico de *S. cerevisiae*, cuja propriedade particular era o fato da mesma só proteger GS na presença de tióis (por exemplo, ao se substituir DTT por ascorbato, ela perdia seu efeito protetor) (Kim et al., 1985). Dessa forma, o ensaio de proteção à GS contra danos oxidativos causados pelo sistema $\text{Fe}^{3+}/\text{O}_2$ / DTT tornou-se um método clássico de medida de atividade específica das Prxs. Posteriormente, essa enzima foi caracterizada como uma peroxidase dependente de tiól, sendo conhecida por muitos anos como TSA (do inglês “thiol specific antioxidant”) (Chae et 1994; Netto et al., 1996).

Em *S. cerevisiae* há cinco proteoformas dessas enzimas distribuídas nos mais diversos compartimentos celulares. Dessas, apenas uma pertence à classe das 1-Cys peroxirredoxinas (1-Cys Prxs), a proteoforma mitocondrial – yPrx1, que foi o objeto de estudo do meu doutorado e pós-doutorado. Essa classe de enzimas tem como propriedade particular o fato de ter apenas uma cisteína conservada (a que pertence ao sítio ativo de yPrx1), enquanto as 2-Cys peroxirredoxinas (2-Cys Prxs) possuem, além da cisteína do sítio ativo - ou cisteína peroxidásica, uma outra carboxi-terminal, conhecida como cisteína de resolução (Figura 4).

Figura 4: Esquema representando as diferenças no sítio ativo após a redução de peróxidos entre as classes de peroxirredoxinas 1-Cys e 2-Cys. As 1-Cys Prxs possuem a capacidade de manter a cisteína ácido sulfênico (SOH) como forma oxidada estável. As 2-Cys Prxs típicas formam um dissulfeto intermolecular e as atípicas um intramolecular como forma a ser resolvida - reciclada pelo redutor. O sistema tiorredoxina (composto por tiorredoxina (Trx), tiorredoxina redutase (Trr) e NADPH) tem sido amplamente caracterizado como responsável biológico da redução das 2-Cys Prxs. No caso das 1-Cys Prxs há controvérsias quanto ao redutor biológico. Fonte: autor.

Estudando propriedades enzimáticas de yPrx1, observamos um fato curioso, que de início nos pareceu um artefato: yPrx1 também protegeu GS contra a inativação induzida por $\text{Fe}^{3+}/\text{O}_2$ / ascorbato, um controle negativo usado classicamente nos ensaios de atividade específica de Prxs. As Prxs são conhecidas por sua especificidade quanto ao uso de redutores tiólicos para ciclagem da enzima, porém esses dados indicavam que yPrx1 poderia também utilizar o ascorbato como agente redutor (Figura 5).

Figura 5: Ensaio de proteção às biomoléculas contra danos oxidativos causados pelos sistemas DTT/ Fe^{3+} / O_2 ou ascorbato/ Fe^{3+} / O_2 . (A) Ensaio de proteção à glutamina sintetase (GS). yPrx1 em diferentes concentrações (eixo X em μM) utilizando DTT ou ascorbato (20 mM de concentração final) como agente redutor. (B) Ensaio de proteção ao DNA plasmidial pBR322 (10 ng/μL). DTPA = quelante de metal Ácido Dietileno-Triamino-Pentacético, FeCl_3 1,5 μM, ascorbato ou DTT 10 mM, yPrx1 1 μM. Fonte: Monteiro et al., 2007.

Na Figura 5 observamos que essa enzima é capaz de usar tanto DTT como ascorbato (vitamina C) para suportar sua atividade de proteção às biomoléculas, como a GS e o DNA. Além disso, a atividade se mostrou dose-dependente da concentração de

enzima (Figura 5A). Frente a esses resultados que sempre se repetiam, testamos a dependência da cisteína do sítio ativo de yPrx1 para essa atividade (Figura 6). Isso porque, em 2004 Jang et al descreveram um fenômeno muito interessante: leveduras mutantes para o gene *TSA1* eram sensíveis ao choque térmico, mas quando eram transformadas com a enzima Tsa1-C47S, uma mutante sem a cisteína peroxidásica (ou seja, sem a atividade peroxidásica), recuperavam o fenótipo selvagem. Esses dados sugeriam que a atividade de proteção ao choque térmico era independente da atividade peroxidásica. Isso foi comprovado com outros experimentos e a partir desse trabalho as Prxs citosólicas ficaram conhecidas por terem, além da atividade peroxidásica, uma eficiente atividade chaperona molecular (Jang et al., 2004).

Dessa forma, investigamos a atividade de proteção à GS dependente de ascorbato em proteínas previamente tratadas com o agente alquilante de sulfidrilas *N*-etilmaleimida (NEM) e também usando yPrx1 com mutação sítio-dirigida para sua respectiva cisteína peroxidásica, yPrx1-C95S (Figura 6A). Como a atividade foi abolida em ambos os casos, mesmo usando um excesso de mais de 20 vezes na concentração de proteína, nos perguntamos se essa era uma particularidade de yPrx1 ou se outras Prxs de levedura também apresentariam essa atividade, apesar de ter sido relatado para Tsa1 ausência de capacidade de usar ascorbato. Em nosso laboratório possuíamos vetores de expressão em bactérias para as três proteoformas citosólicas Tsa1, Tsa2 e Ahp1. Após a purificação e teste da atividade, nenhuma delas foi capaz de proteger GS na presença de ascorbato, apenas quando o redutor adicionado no meio reacional era DTT (Figura 6B), como previamente descrito na literatura. A maior diferença entre as proteoformas testadas era a classe ao qual pertenciam, sendo yPrx1 a única 1-Cys Prxs e todas as outras 2-Cys Prxs (Tsa1 e Tsa2 típicas e Ahp1 atípica).

Figura 6. Atividade protetora à GS usando ascorbato é dependente da cisteína peroxidásica de 1-Cys Prxs, mas ausente em 2-Cys Prxs. (A) As enzimas (concentração final de 200 μ M) foram previamente reduzidas com 10 mM de DTT por 1 hora a 25 °C, dialisadas e tratadas com 5 mM de NEM por 30 minutos a 25 °C. Após purificação por coluna de gel filtração (PD-10 desalting - Amersham Pharmacia), foram realizados os ensaios de proteção à GS com 3 μ M ou 70 μ M. (B) A concentração de enzima utilizada foi de 10 μ M (ou excesso de 60 μ M para Tsa1). Concentração de reagentes: Fe^{+3} 3 μ M, DTT ou Ascorbato 10 mM, GS 0,1 μ g/ μ g. Reações foram incubadas a 37 °C por 15 minutos. Fonte: Monteiro et al., 2007.

O uso de ascorbato (vitamina C) por uma Prxs de levedura não era esperado. Assim, para confirmarmos se essa habilidade era exclusiva de yPrx1 ou tratava-se de uma característica das 1-Cys Prxs ainda não relatada, pedimos à diversos pesquisadores de várias partes do mundo vetores de expressão em bactérias de 1-Cys Prxs (gentilmente cedidos), com os quais conseguimos realizar nossos estudos. Caracterizamos as atividades dependentes de tiól e de ascorbato das 1-Cys Prxs de *Mycobacterium tuberculosis*, *Plasmodium falciparum*, *Drosophila melanogaster*, *Arabidopsis thaliana* e *Ratus norvegicus*. A Tabela 2 mostra que, surpreendentemente, o

uso de ascorbato como redutor não é uma particularidade de yPrx1 de *S. cerevisiae*, mas uma propriedade das 1-Cys Prxs amplamente conservada durante a evolução nos mais diversos grupos taxonômicos.

Tabela 2: A atividade peroxidásica dependente de ascorbato é uma propriedade conservada dentro da classe das 1-Cys peroxirredoxinas. Fonte: Monteiro et al., 2007.

Proteína 1 – Cys Prx	Espécie	Atividade com DTT (uM H ₂ O ₂ / 15 min)	Atividade com ascorbato (uM H ₂ O ₂ / 15 min)
MTPxI	<i>S. cerevisiae</i>	280	150
AhpE	<i>M. tuberculosis</i>	292	393
rPfl-Cys-Prx	<i>P. falciparum</i>	330	322
AtPER1	<i>A. thaliana</i>	360	392
Dpx-2540	<i>D. melanogaster</i>	237	296
Dpx –6000	<i>D. melanogaster</i>	333	297
Prdx6	<i>R. norvegicus</i>	300	370

Grande parte das 1-Cys Prxs ainda não possuía substrato fisiológico conhecido. Nossos dados sugerem que o ascorbato pode ser então o doador de elétrons natural dessas enzimas. A principal característica que difere 1-Cys Prxs e 2-Cys-Prxs são os estados oxidados estáveis das enzimas (Figura 4). Sabíamos que 2-Cys Prxs com mutação na cisteína de resolução (no caso da Tsa1-C170S) mantinha o ácido sulfênico estável, assim como as 1-Cys Prxs. Se apenas essa característica fosse suficiente para receber elétrons do ascorbato, essa mutante deveria então apresentar atividade peroxidásica ascorbato-dependente. Testamos essa hipótese e verificamos que Tsa1-C170S não apresentava essa atividade (Figura 7).

Além da forma oxidada em ácido sulfênico, as 1-Cys Prxs apresentam outra característica em comum entre si, a presença de uma histidina no motivo do sítio ativo; 2-Cys Prxs possuem aminoácidos variáveis nessa mesma posição. Assim, realizamos mutações na Tsa1 selvagem, resultando na Tsa1-I40H, e na mutante C170S, resultando na dupla mutante Tsa1- C170S - I40H. A dupla mutação levou essa 2-Cys Prxs a adquirir atividade peroxidásica ascorbato-dependente (Figura 7). Nesse contexto, foi demonstrado que pelo menos duas características de 1-Cys Prxs são importantes para essa atividade relacionada à vitamina C: (1) ausência da cisteína de resolução (mantendo o sulfenato estável) e (2) presença de uma histidina no sítio ativo. Isso pôde ser comprovado pelo fato de uma 2-Cys Prxs típica ter adquirido a capacidade de usar vitamina C como agente redutor através da inserção dessas duas características por mutação sítio dirigida.

Figura 7: Engenharia da enzima Tsa1 2-Cys Prxs para aquisição de atividade ascorbato-dependente de proteção à GS. 50 μ M de cada enzima foi testada nas condições descritas na legenda da Figura 6B. Fonte: Monteiro et al., 2007.

Esses resultados geraram uma quebra de paradigma: as enzimas peroxidases tiól específicas podem usar ascorbato como agente redutor, uma propriedade específica das 1-Cys Prxs e amplamente conservada na história evolutiva dessa classe, uma vez que observamos essa atividade em enzimas advindas de bactérias, mamíferos e plantas (Monteiro et al., 2007 - **ANEXO 1**).

Considero esse trabalho uma contribuição importante para área. Um exemplo disso foi o uso do modelo de par redox ácido sulfênico / ascorbato proposto no trabalho ter explicado um fenótipo observado por um grupo de Cambridge que estava estudando mutações em mamíferos que afetavam a oxidação e atividade das enzimas proteína-dissulfeto isomerases (PDIs). Em leveduras *S. cerevisiae*, esse papel é realizado por uma proteína chamada ERO1, cuja deleção é inviável, mostrando o papel vital dessas enzimas para o metabolismo celular. Dessa forma, esses pesquisadores esperavam que ao deletar os genes codificadores das proteínas EROs de camundongos (ERO1 α e ERO1 β), os embriões também fossem inviáveis. No entanto, três animais nasceram e um deles sobreviveu, mas possuía peso 70% menor que os irmãos da mesma ninhada e suas orelhas e caudas degeneravam com facilidade. Esse animal possuía claro sintoma de escorbuto não-canônico, apesar de ter a mesma dieta dos outros. Ao dosar a quantidade de ascorbato no soro do animal, os pesquisadores notaram que ele possuía metade da dose normal. Além disso, apresentava níveis elevados de proteínas na forma oxidada em ácido sulfênico. Esses dados geraram um modelo no qual a deleção de proteínas EROs leva a um estresse oxidativo intenso, aumentando as proteínas na forma oxidada em ácido sulfênico que deslocam quantidade exacerbada de ascorbato para serem reduzidas e continuarem suas funções biológicas essenciais (Zito et al., 2012). O principal trabalho citado para propor esse modelo foi o publicado durante meu pós-doutoramento (Monteiro et al., 2007).

2.2 Caracterização de funções de genes envolvidos na resposta antioxidante.

As espécies reativas de oxigênio (ROS) e nitrogênio (RNS) apresentam características contrastantes no ambiente celular: em excesso causam danos às biomoléculas resultando em várias patologias; no entanto, em concentrações controladas são essenciais aos processos de sinalização celular, ativando receptores, quinases, fosfatases, realizando assim diversas funções biológicas. Vários estudos têm implicado a produção de ROS com alguns processos patológicos como diabetes, arteriosclerose, doenças neurodegenerativas, câncer e envelhecimento. De fato, há indícios de que ocorre um permanente, embora discreto, aumento de lesões oxidativas em biomoléculas, que se acumulam ao longo dos anos, sendo uma possível explicação para o envelhecimento celular (revisão em Gutteridge & Halliwell 2010).

A todo instante estamos expostos às espécies reativas de oxigênio, subprodutos da respiração celular. Além disso, radiações UV, poluição e vários outros fatores ambientais promovem a formação de espécies pró-oxidantes, que em excesso, levam ao estado de estresse oxidativo. O estresse oxidativo é conhecido por resultar em danos em biomoléculas como DNA, proteínas e lipídeos. Como defesa contra esses danos as células possuem um complexo sistema antioxidante, sendo vários de seus componentes com função biológica pouco compreendida.

Nos últimos anos muito tem sido descoberto sobre diversas propriedades antioxidantes de enzimas e moléculas de baixo peso molecular e suas relações com o câncer (Martinez-Useros et al., 2017; Morry et al., 2017; Kawanishi et al., 2016). Os pró-oxidantes, por sua vez, têm tido papel cada vez mais importante na compreensão da biologia celular, atuando como moléculas sinalizadoras, sendo o exemplo mais clássico

o óxido nítrico. A descoberta das vias de sinalização mediadas por esse radical livre levou ao desenvolvimento de novos medicamentos e significativa melhora na qualidade de vida de muitas pessoas. Esses são exemplos do quanto ainda precisamos estudar os mecanismos redox, pois importantes mediadores desse processo ainda estão sendo descobertos.

Após mais de 20 anos do término do sequenciamento do genoma da *Saccharomyces cerevisiae*, cerca de 1.400 possíveis proteínas não estão caracterizadas. Estudar esse organismo modelo é importante não só para o conhecimento dos mecanismos básicos da biologia celular, como para melhorar diversos processos industriais aumentando a eficiência de crescimento ou de produção da levedura.

Para investigar possíveis proteínas de função desconhecida envolvidas em processos pró / antioxidantes em levedura, utilizamos a ferramenta *Advanced Search* presente no banco de dados *Saccharomyces Genome Database* - SGD (<http://www.yeastgenome.org/>) com os seguintes critérios para busca: uma ORF anotada como não caracterizada e sem introns (facilitando futuras clonagens; de toda forma, levedura possui um número reduzido de regiões intrônicas); a massa molecular da proteína poderia variar entre 5 e 50 kDa; a função biológica e molecular, bem como compartimento celular anotados como desconhecidos. Resultado da busca: 367 hits – presente em nossa coleção de mutantes viáveis YKO haploide (Invitrogen): 217

Iniciamos o projeto testando as 217 linhagens mutantes de leveduras quanto a sua viabilidade para determinarmos genes candidatos que estivessem envolvidos nas respostas antioxidantes. Para essa escolha, as leveduras foram crescidas em YPD (extrato de levedura 1%, peptona 2% e dextrose 2%) até a fase logarítmica ($OD_{600nm} \sim 0.8$) e então plaqueadas (diluição seriada de 5X) em YPD com 4 mM de peróxido de

hidrogênio (H_2O_2) ou 1.5 mM de *t*-butil hidroperóxido (*t*-BOOH), este último um peróxido orgânico, já que algumas enzimas antioxidantes são específicas para um ou outro tipo de peróxido (Munhoz e Netto, 2004). As leveduras mutantes mais sensíveis à exposição aos peróxidos do que a linhagem selvagem foram escolhidas para os estudos. Fizemos o mesmo experimento na fase estacionária de crescimento ($\text{OD}_{600\text{nm}} \sim 6.0$), pois alguns genes não são expressos na fase logarítmica e, portanto, não podem ser selecionados na condição anterior; duas ORFs foram selecionadas (Figura 8). Além disso, meu grupo possuía ainda uma coleção de leveduras mutantes termossensíveis para 57 genes essenciais de *S. cerevisiae*. Essa coleção também foi triada e apenas uma ORF apresentou sensibilidade aos hidroperóxidos (Figura 9).

Esses dados iniciais constituíram a base para meus primeiros projetos e contribuições como chefe de grupo e fazem parte do projeto Jovem Pesquisador FAPESP 2009/01303-1, intitulado “Caracterização de ORFs de função desconhecida envolvidas na resposta antioxidante em *Saccharomyces cerevisiae*”.

Figura 8: Triagens iniciais para escolha de leveduras mutantes viáveis sensíveis ao estresse oxidativo. (A) Experimento de viabilidade de leveduras capazes de crescer em meio YPD com e sem adição de H₂O₂ 4 mM. Resultado representativo de 1 placa com 96 clones, sendo o clone 1A a linhagem selvagem BY4741 (S) isogênica da coleção (linha 1, coluna A, ver esquema da placa abaixo da foto). Os outros clones marcados com círculo branco na foto foram mais sensíveis e selecionados para a confirmação posterior. (B) Refinamento da triagem inicial. Das 217 mutantes totais, sete clones que apresentaram alguma sensibilidade ao peróxido de hidrogênio ou ao *t*-BOOH em YPD foram testadas quanto à especificidade da resposta e ao nível de sensibilidade. Os clones mutantes para os genes *YCL047C* (clone 3) e *YJL077C* (clone 6) foram escolhidos por apresentarem fenótipos claros e marcantes de sensibilidade aos peróxidos. Fonte: autor.

Figura 9: Ensaio de viabilidade e resistência ao estresse oxidativo do mutante termossensível para o gene essencial *YMR134W*. As leveduras foram crescidas em SD-URA⁻ glicose e incubadas a 25 °C ou a 37 °C por 16 horas e posteriormente diluídas e aplicadas em placa de SD-URA⁻ com a respectiva fonte de carbono, contendo ou não peróxido de hidrogênio 0,5 mM ou *t*-butil hidroperóxido 0,75 mM. As placas foram incubadas em estufa a 25°C por três dias. Fonte: Moretti-Almeida et al., 2013.

2.2.1 Estudo da ORF *YCL047C*.

Análises iniciais de alinhamento de sequência de aminoácidos mostraram que a ORF *YCL047C* tinha relativa similaridade com as nucleotidiltransferases, mais especificamente com as citidiltransferases (Figura 10A). Buscando na literatura por funções conhecidas de citidiltransferases de levedura, encontramos um grupo que descreveu sensibilidade ao choque térmico em meio mínimo da mutante para o gene *PCT1* (ORF: *YGR202C* que codifica para a CTP-fosfocolina citidiltransferase) (Howe et al., 2002). Dessa forma, inserimos o gene *YCL047C* no vetor de expressão em levedura pYES 2.1 – TOPO TA (Invitrogen) e transformamos linhagens mutantes para os genes *PCT1* e *YCL047C*, com o intuito de observar reversão do fenótipo pela expressão de *YCL047C* (Figura 10B).

Figura 10: Similaridade de sequência e funcional entre a ORF *YCL047C* e *PCT1*.

(A) Alinhamento das sequências primárias de fosfocolina citidiltransferase (ct) e fosfoetanolamina citidiltransferase (et) de humanos, fosfocolina citidiltransferase de levedura (pct1 yeast) e a proteína de interesse Ycl047cp. Dentro da caixa está evidenciado o possível sítio ativo de Ycl047cp, motivo conservado HxxH. Alinhamento feito usando Clustal W / Megalign – DNASTar. (B) Ensaio de complementação de função por expressão regulada pela presença de galactose de *YCL047C* inserido no vetor pYES2.1-TOPO TA. As células foram crescidas em meio mínimo com galactose (sem uracila) por 8 horas a 30 °C e transferidas para 37 °C por 24 horas. O * significa presença do vetor pYES2.1-TOPO TA+*YCL047C*; as outras linhagens foram transformadas com pYES2.1-TOPO TA vazio. (C) *Western blot* para análise da presença da proteína codificada pelo gene *YCL047C*. O símbolo (*) indica quais linhagens estavam transformadas com o vetor pYES2.1 TOPO TA+*YCL047C*, as demais continham o vetor vazio. Fonte: Costa et al., 2011.

Interessantemente, a expressão de *YCL047C* foi capaz de reverter o fenótipo de sensibilidade ao choque térmico em ambas as linhagens mutantes, o que sugeria uma função metabólica similar (Figura 10B). Além disso, estudos de *Western blot* com anticorpo específico contra a proteína recombinante Ycl047cp mostraram que a mesma tem baixa expressão basal, mas é induzida na linhagem mutante $\Delta pct1$ (Figura 10C).

Assim, realizamos testes de atividade citidiltransferase *in vitro* de Ycl047cp recombinante purificada (Figura 11A).

Figura 11: Ensaio de atividade da proteína Ycl047cp *in vitro*. (A) Gel SDS-PAGE da proteína Ycl047cp superexpressa em *E. coli* e purificada. (B) Testes de atividade *in vitro* de Ycl047cp (renomeada para Pof1 pelo SGD) recombinante pura (1 μ g/ μ L) na presença de fosfoetanolamina 2 mM ou fosforilcolina 1.5 mM, α^{32} CTP ou α^{32} ATP (3 Ci/ μ mol). Os produtos foram separados por cromatografia de camada delgada (TLC), que foi exposta em filme para raio X por 2 minutos (protocolo seguido de Nikawa et al., 1983; Sohal e Cornell, 1990). (C) Quantificação colorimétrica de ATP usando PiperTM Phosphate Assay kit (Invitrogen). Fonte: Costa et al., 2011.

Na Figura 11B fica clara a preferência da enzima em hidrolisar ATP comparado ao CTP. Além disso, a hidrólise acontece independentemente da presença de fosforilcolina ou fosfoetanolamina, indicando que apesar de complementar o fenótipo de *Δpct1* e ter sua expressão induzida nessa mutante, as proteínas não possuem a mesma atividade enzimática.

Pouco antes de o nosso manuscrito ser terminado, a ORF *YCL047C* foi relacionada com o fenômeno de crescimento filamentoso na levedura, sendo assim renomeada para *promoter of filamentation 1* (Pof1) (Fasolo, et al., 2011). Contudo, a função bioquímica ainda não havia sido caracterizada. Nossos ensaios *in vitro* com a proteína Pof1 sugerem que a mesma possui atividade ATPase com eficiência catalítica compatível com sua ocorrência *in vivo* (Figura 11C). Além disso, caracterizamos seu envolvimento no processo de controle de qualidade de proteínas, sua possível localização celular no Golgi e sua interação física com Ubc7p (uma conjugase de ubiquitina) (**ANEXO 2**).

Em conjunto, nossos dados sugerem que Pof1 é uma ATPase que interage com a maquinaria de marcação proteica para degradação pelo sistema ubiquitina-proteassomo. Além disso, Pof1 protege as células do acúmulo tóxico de proteínas desnaturadas causado por estresse térmico, estresse oxidativo ou estresse químico (excesso de agente redutor ou inibidor de glicosilação proteica). Nossos estudos permitiram a classificação da ORF *YCL047C*; esses dados foram publicados no periódico internacional *BMC Microbiology*, fator de impacto 2.58, com 2.717 acessos desde sua publicação.

Considero relevante dizer que o trabalho é citado como uma das principais referências caracterizando essa ORF na página do *Saccharomyces Genome Database* - SGD específica para o gene (<http://www.yeastgenome.org/cgi-bin/locus.fpl?dbid=S000000552>). Esse manuscrito teve boa repercussão internacional, pois fomos convidados pela editora iConcept Press a escrever um artigo expandido intitulado “Revealing a Gene Function in the Cell: The characterization of an unverified ORF from *Saccharomyces cerevisiae*”, que foi publicado como capítulo 13 (páginas 271-292) do livro "Protein Purification and Analysis I – Methods and Applications". O livro está disponível online e o capítulo na íntegra pode ser acessado por meio do endereço <https://www.iconceptpress.com/book/protein-purification-and-analysis-i--methods-and-applications/11000089/1205000518/>.

2.2.2 Estudo da ORF *YMR134W*.

A ORF *YMR134W* faz parte dos genes essenciais de leveduras, assim não é viável obter mutantes *knockouts* para esse gene. Uma forma de possibilitar estudos com genes essenciais é a obtenção de leveduras cujos produtos gênicos sejam sensíveis à temperatura, isto é, com o aumento da temperatura por determinado tempo de exposição, a proteína desnatura e perde a função, permitindo a análise do mutante antes da perda de viabilidade. Havia sido descrito que a proteína Ymr134wp possivelmente participaria de dois processos interligados: a morfogênese mitocondrial e a incorporação de ferro celular, já que mutantes para esse gene apresentam morfologia mitocondrial aberrante e eram incapazes de crescer em meio com carência de ferro (Babcock et al. 1997; Altmann e Westermann, 2005). Como o ferro depois de incorporado é armazenado na mitocôndria, a deficiência do mutante *YMR134W^{ts}* em crescer em meio com pouco ferro poderia ser devida a sua incapacidade de armazená-lo na mitocôndria;

isso levaria a uma carência desse íon nas células, afetando proteínas que possuem ferro como cofator.

Assim, analisamos a incorporação de ferro por essas células (Figura 12). Nossos dados indicam que a linhagem *YMR134W^{ts}* incorpora doze vezes mais ferro do meio de cultivo (e mais rapidamente) do que a linhagem selvagem (Figura 12A). Em adição, ao analisarmos o armazenamento mitocondrial de ferro, observamos que a linhagem mutante armazena menos ferro em suas mitocôndrias do que a linhagem selvagem em temperatura não permissiva, deixando que o ferro captado fique em excesso no citosol (Figura 12 B e C). Esse ferro citosólico pode ser responsável pelo fenótipo de sensibilidade de *YMR134W^{ts}* na presença de peróxido de hidrogênio, pois o excesso poderia participar de reações de Fenton, levando a graves injúrias oxidativas.

Figura 12: Consumo e armazenamento de ferro pelas leveduras selvagem e *YMR134W^{ts}* em temperaturas permissiva e não permissiva. O ferro foi dosado nos diferentes meios e compartimentos celulares usando ensaio colorimétrico por meio do reagente TPZ (2,4,6-tri(2-piridil)-1,3,5-triazine). Fonte: Moretti-Almeida et al., 2013.

Assim, confirmamos a importância de *YMR134W^{ts}* para homeostase celular de ferro. Contudo, isso ainda não explicava o porquê esse fenômeno acontecia. Em 2007, Prof. Dr. Jerry Kaplan – Faculdade de Medicina da Universidade de Utah – EUA- publicou a seguinte comunicação no site do SGD “Erg29p (*YMR134W* product) binds to Erg25p and affects its activity; *erg29* null mutant is non-viable in a respiratory competent background but is viable in a respiratory-defective background; *erg29* mutation is synthetically lethal with *mmt1* and *mmt2* null mutations.” [http://www.yeastgenome.org/cgi-bin/locusHistory.pl?dbid=S000004741]. Com isso, investigamos se as células *YMR134W^{ts}* possuíam quantidades normais de ergosterol. Verificamos que essas mutantes apresentam cerca de 40% menor quantidade total de ergosterol do que as células selvagens em ambas as temperaturas (Figura 13).

Figura 13: Medida de ergosterol total nas células selvagem e *YMR134W^{ts}* em temperaturas permissiva e não permissiva. O ergosterol total extraído foi dosado usando o padrão único de espectro de absorção produzido por esse lipídeo na faixa de 240-300 nm. * Expresso como % de peso seco celular. Fonte: Moretti-Almeida et al., 2013.

Com o resultado da Figura 13 concluímos que *YMR134W* participa da via de síntese de ergosterol, um importante lipídeo constituinte de membranas em leveduras. Por fim, para comprovarmos se o defeito na síntese de ergosterol, afetando o armazenamento de ferro na mitocôndria, de fato gerava mais peróxido de hidrogênio nas células *YMR134W^{ts}*, explicando o primeiro fenótipo caracterizado pelo nosso grupo (a sensibilidade à exposição aos hidroperóxidos), medimos a atividade total catalásica dessas células (Figura 14).

Figura 14: *YMR134W^{ts}* estão em estresse oxidativo. Atividade catalásica total nas células selvagem e *YMR134W^{ts}* em temperaturas permissiva e não permissiva. O produto da reação enzimática da catalase é oxigênio; assim, analisamos o quanto de oxigênio era liberado nos extratos proteicos das diferentes células na presença de H_2O_2 . O oxigênio foi medido usando eletrodo de Clark. Fonte: Moretti-Almeida et al., 2013.

Com os dados apresentados acima atribuímos à ORF *YMR134W* a função biológica de síntese de ergosterol. Vale ressaltar que, mais uma vez, esse manuscrito recebeu destaque como uma das principais referências do SGD na caracterização dessa ORF

(<http://www.yeastgenome.org/locus/S000004741/overview>). O ergosterol é um dos principais lipídeos constituintes das membranas biológicas de *S. cerevisiae*. A classe dos antifúngicos azóis, uma das principais aplicada terapeuticamente no tratamento de doenças causadas por fungos, tem sua ação na interrupção da síntese desse lipídeo. Dessa forma, considero relevante a caracterização feita pelo nosso grupo de um dos membros moleculares dessa via essencial para as leveduras. Esses dados foram publicados no periódico internacional *FEBS Letters*, fator de impacto 3.519 (**ANEXO 3**).

2.2.3 Estudo da ORF *YJL077C*.

De acordo com alguns estudos prévios, *YJL077C* estaria relacionado ao metabolismo do cobre, o que lhe rendeu a denominação de *ICS3* (do inglês “*increased copper sensitivity*” (Entian et al., 1999)). De fato, nossos resultados estão de acordo com os obtidos em larga-escala, pois também verificamos maior sensibilidade da linhagem *Δics3* quando exposta a altas concentrações de cobre. De maneira interessante, esse fenótipo foi específico para o cobre, sendo que as células mutantes *Δics3* não apresentaram diferença na viabilidade em relação à linhagem selvagem quando expostas ao cobalto ou ferro (Figura 15).

Figura 15: Sensibilidade de $\Delta ics3$ a metais. Após exposição por 4 horas (CuSO_4) ou 8 horas (CuSO_4 ou CoCl_2) as células foram serialmente diluídas 5X e plaqueadas em YPD. Fonte: Alesso et al., 2015.

Em 2006, estudos em larga escala sugeriram que *ICS3* estaria envolvido com sensibilidade a acidez do meio (Kawahata et al., 2006). Como a adição de grande quantidade de CuSO_4 poderia alterar o pH do meio, verificamos se essa resposta ao cobre observada era devido ao metal ou devido ao pH do meio de cultivo, acompanhando a curva de crescimento das leveduras em pH 4.0 e 6.0 (Figura 16).

Figura 16: Curvas de crescimento e viabilidade em diferentes pHs e durante a exposição ao Cobre. (A) Células crescidas em pH 4.0; (B) Células crescidas em pH 6.0; (C) Viabilidade em YPD das células de (A) e (B). Fonte: Alesso et al., 2015.

Para nossa surpresa, o fenótipo de sensibilidade ao cobre ocorria apenas em meio ácido, sendo completamente revertido se as células crescessem em meio tamponado pH 6.0. Verificamos assim, se a viabilidade das células Δ ics3 era diminuída em pH 4.0, o que não foi observado (Figura 16C). Assim, o fenótipo de sensibilidade ao cobre era dependente de pH. É amplamente relatado que sulfato de cobre é mais solúvel em meio ácido do que em meio neutro ou alcalino (Gadd and Griffiths, 1977). Assim, verificamos se as células mutantes para *ICS3* acumulavam mais cobre e ferro do que a selvagem (Figura 17). O ferro também foi quantificado, pois é descrito na literatura que o transporte de ferro e cobre é dependente da mesma via inicial de redução e, consequentemente, influenciam o transporte um do outro (Freitas et al., 2003; Rae et al., 1999).

Figura 17: Dosagem de cobre e ferro intracelular. Os metais foram quantificados por espectroscopia de absorção atômica (ICP-AES). Fonte: Alesso et al., 2015.

Na figura 17 observamos que em pH ácido ambas as linhagens possuem a mesma quantidade de cobre (não há diferença estatística significativa); já o ferro, está em menor proporção na linhagem Δ ics3. A menor quantidade de ferro pode impactar nas defesas antioxidantes, pois catalase e citocromo c peroxidase são heme-proteínas. Em pH 6.0, a linhagem selvagem apresenta cerca de três vezes menos cobre e maior quantidade de ferro do que Δ ics3.

Como observado até agora, o efeito do cobre em Δ ics3 é dependente de pH ácido. Assim, investigamos se fatores de transcrição envolvidos com a resposta às variações de pH ambiental (*RIM 101*) e outros de resposta a metais (*MAC1* e *ACE1*) estariam regulando a expressão gênica de *ICS3*. Além disso, verificamos se a expressão do gene *ICS3* poderia estar coordenada com a ATPase tipo P1 transportadora de cobre - *Ccc2*. *ICS3* teve sua expressão aumentada em pH ácido e também em células *Arim101*. Já *CCC2* teve sua expressão induzida nas mutantes Δ ics3 e Δ rim101, apenas em pH ácido (Figura 18).

Figura 18: Expressão do gene *ICS3* em linhagens mutantes para fatores transcricionais de resposta ao pH (RIM101) e metais (ACE1 e MAC1). As células foram crescidas ou não na presença de cobre 0.5 mM. *Northern blot* marcando os genes *ICS3* e *CCC2*. *rRNA* como controle de quantidade total de RNA analisado. Fonte: Alesso et al., 2015.

Nossos dados demonstram que as células $\Delta ics3$ são sensíveis ao cobre apenas no pH ácido extracelular, devido, pelo menos, a dois efetores: Ccc2 e Rim101. A expressão de *CCC2* é aumentada em células $\Delta ics3$, o que pode levar ao aumento de modificação pós-traducional da Metal-oxidase Fet3 que é secretada. Isso aumentaria a quantidade de substrato disponível para a permease transmembrana Ftr1, induzindo a atividade do sistema de transporte de metais de alta afinidade, explicando o acúmulo de metal observado. Além disso, *CCC2* e *ICS3* apresentam perfis de expressão semelhantes em resposta ao pH e ao regulon Rim101. Ccc2p é descrito como um exportador de cobre do citosol e é necessário para a captação de ferro, o que é importante para a homeostase dos metais. Assim, conseguimos explicar o fenótipo observado tanto de sensibilidade ao cobre (que rendeu o nome da ORF), quanto ao estresse oxidativo caracterizado pelo nosso grupo (ANEXO

4). Esses dados foram publicados no periódico *Fungal Genetics and Biology*, fator de impacto 2.933. Ressalto ainda que mais uma vez esse artigo é citado como uma das principais referências na caracterização dessa ORF em *S. cerevisiae* pelo site SGD (<http://www.yeastgenome.org/locus/S000003613/overview>).

2.3 Caracterização de alvos moleculares para suplantar resistência aos antitumorais.

Por ser um organismo eucarioto unicelular e com um genoma pequeno em relação à maioria dos eucariotos superiores, há maior facilidade para a caracterização de vias metabólicas e respostas a estresse e drogas nessa levedura. Desde 1996, a fração dos genes codificadores em *S. cerevisiae* que evoluiu de um entendimento rudimentar de seu papel biológico para função conhecida foi de 30% para 85%. Esta fração é muito maior para a levedura do que para qualquer outro eucarioto descrito até então (Botstein & Fink, 2011).

A facilidade de manipulação genética desse organismo permitiu, por exemplo, a criação de coleções de mutantes com deleções para basicamente todas as suas ORFs (*Open Reading Frames*) individualmente, chamadas YKO (*Yeast Knock-Out*). Além disso, *S. cerevisiae* pode apresentar formas de vida livre tanto como um organismo haploide quanto diploide, o que permitiu a construção de quatro tipos diferentes de coleções de mutantes: homozigoto (diploide, com as duas cópias de cada gene não-essencial deletadas), heterozigoto (diploide, com apenas uma cópia funcional, onde existem inclusive deleções de genes essenciais), e duas haploides (uma com tipo sexual *MATa* e outra *MATα*) (Winzeler et al., 1999), disponíveis comercialmente. Cada um dos

mutantes dessas coleções carrega ainda marcas que possibilitam sua identificação em diferentes circunstâncias, devido às características dos cassetes de deleção utilizados para a interrupção de suas ORFs. Esses cassetes contêm uma marca de resistência (*KanMX4*) ao antibiótico geneticina - G418 flanqueada por duas regiões (tags: UPTAG e DOWNTAG) específicas para cada um dos mutantes, além de adaptadores universais (Figura 19).

Figura 19: Esquema geral do cassete de interrupção de genes usado para construir as coleções YKO. ATG – códon de iniciação da tradução da ORF alvo; TAA – códon de terminação da tradução da ORF alvo; U1 – iniciador universal das ORFs na região à montante; D1 – iniciador universal das ORFs na região à jusante; U2 e D2– iniciadores universais complementares à marca de seleção *KanMX4*. Fonte: Adaptado do site Saccharomyces Genome Deletion Project, no endereço http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html.

Cada cassette foi construído através de duas reações de PCR consecutivas (Giaever et al., 2002). Na primeira delas, iniciadores de 74bp para as regiões UPTAG e DOWNTAG foram utilizados, amplificando o gene *KanMX4* do DNA pFA6-kanMX4. Cada um desses iniciadores consiste em quatro regiões (Figura 19):

- Sequências específicas de 18bp, homólogas em sequência às regiões 5' e 3' que flanqueiam a ORF a ser interrompida;
- Sequências de 18 e 17bp comuns a todos os mutantes (U1: 5'-GATGTCCACGAGGTCTCT-3' ou D1: 5'-CGGTGTCGGTCTCGTAG-3');
- Sequências de 20bp únicas para cada mutante (denominada *barcode* / *tags* da ORF);
- Sequências de 18 e 19bp homólogas ao cassette *KanMX4*, também comuns a todos os mutantes (U2: 5'-CGTACGCTGCAGGTCGAC-3' e D2: 5'-ATCGATGAATTCGAGCTCG-3').

Na segunda reação de PCR, o cassette é estendido utilizando iniciadores de 45bp específicos para a ORF a ser interrompida. Como a interrupção das ORFs é baseada em recombinação homóloga, essa é a forma de ter especificidade em relação ao alvo. Assim, é possível identificar cada linhagem mutante pelo seu código de barras (*barcode*) sequenciando o produto da reação de PCR resultante dos pares de iniciadores U1 e U2 (para a região *UPTAG*) ou D1 e D2 (para a região *DOWNTAG*).

As características desse cassette de deleção permitem diferentes abordagens para o estudo desses mutantes. Eles podem ser diretamente estudados e seu fenótipo analisado através de medidas de crescimento tanto em meio líquido (taxa de crescimento, aptidão ou *fitness*) quanto em meio sólido (unidade formadora de colônia,

viabilidade). É possível, com isso, identificar genes relacionados à resposta a drogas e outros agentes tóxicos; mutantes que apresentam maior sensibilidade ao tratamento carregam interrupções de genes que podem estar associados à resistência celular ao agente, e vice-versa. Um exemplo disso foi a identificação, por meio de estudos de mutantes individuais, de genes associados ao transporte e à sensibilidade a cisplatina, um antitumoral genotóxico utilizado em casos de câncer de testículo, entre outros (Ishida et al., 2002; Schenk et al., 2003; Gaponova et al., 2016). Outro exemplo foi a identificação de genes associados à resistência ao antitumoral imatinibe, um importante inibidor de tirosina quinase, através de uma análise sistemática de toda a coleção YKO haplóide (Santos e Sá-Correia, 2009).

Dessa forma, aplicamos essa coleção para o estudo de alvos de resistência ao antitumoral carboplatina. A carboplatina é um análogo da cisplatina, porém com toxicidade 45 vezes menor e está na lista de medicamentos considerados essenciais pela Organização Mundial da Saúde. Sua mais frequente aplicação é para tratamento de tumor ovariano e a principal causa de interrupção do tratamento com essa droga é a aquisição de resistência pelos tumores nos pacientes (Sousa et al., 2014).

Em 2008, Hillenmeyer et al. publicaram um estudo usando análises de *fitness* das leveduras em ensaios de competição de *pools* da coleção YKO. Nesses estudos, foram realizados 1.144 ensaios diferentes de toxicogenômica, dentre os quais foi testada a carboplatina. Os autores depositaram os dados completos no endereço eletrônico <http://chemogenomics.med.utoronto.ca/fitdb/fitdb.cgi>. Assim, nessa página utilizamos a ferramenta busca rápida por carboplatina e realizamos o *download* dos dados obtidos para a coleção de homozigotos (únicos disponíveis) ao clicar em “*more*”. Seleccionamos os mutantes que de acordo com esse estudo apresentaram maior defeito de *fitness* com a menor dose de carboplatina testada

(250 μ M), usando como critério o $P_{\text{value}} < 0,01$, considerado pelos autores estatisticamente significante.

O resultado foi a seleção de 53 linhagens mutantes de levedura da coleção YKO, as quais foram testadas as viabilidades e curvas de crescimento na presença de 10 mM de carboplatina. Dessas, 19 linhagens mostraram sensibilidade, enquanto 15 delas foram resistentes (Figura 20) e as restantes não responsivas. Essa diferença de fenótipo com relação aos dados iniciais de Hillenmeyer et al. (2008) pode ser explicada pelas diferentes abordagens metodológicas, bem como pela diferente coleção utilizada nos dois trabalhos (nós usamos a coleção haploide e o estudo de Hillenmeyer et al. usou a diploide homozigota).

Como nosso principal interesse era a extrapolação dos dados obtidos para células humanas, verificamos quais ORFs responsivas à carboplatina possuíam ortólogos humanos conhecidos. Assim, das 34 ORFs selecionadas prosseguimos a análise com 21 delas (Tabela 3).

Com essa lista de genes humanos realizamos análises de bioinformática quanto às funções biológicas e moleculares, distribuição cromossômica, vias metabólicas e interações proteína-proteína em busca de alguma característica comum entre eles. De forma interessante, encontramos a proteína HuR (ELAV1 ou ELAVL1) envolvida em interações proteína-proteína com homólogos relacionados à resistência; e culinas 1, 2 e 3 com homólogos relacionados à sensibilidade.

Figura 20: Curvas de crescimento das linhagens selecionadas na presença de 10 mM de carboplatina. Fonte: Sousa et al., 2015.

Tabela 3: Genes homólogos humanos identificados no estudo de resposta da levedura à carboplatina. Fonte: Sousa et al., 2015.

Yeast gene involved in resistance	Human homolog	Yeast gene involved in resistance	Human homolog	Yeast gene involved in sensitivity	Human homolog
AQY1	AQP1	AQY1	MIP	MET30	BTRC
AQY1	AQP2	EAFF3	MORF4L1	DID4	CHMP2A
AQY1	AQP3	EAFF3	MORF4L2	DID4	CHMP2B
AQY1	AQP4	EAFF3	MSL3	COX12	COX6B1
AQY1	AQP5	MUS81	MUS81	COX12	COX6B2
AQY1	AQP7	VPS21	RAB17	YEN1	ERCC5
AQY1	AQP8	VPS21	RAB20	UBX3	FAF1
AQY1	AQP9	VPS21	RAB22A	UBX3	FAF2
AQY1	AQP10	VPS21	RAB31	MET30	FBXW11
AQY1	FAM188B	VPS21	RAB5A	MET30	FBXW2
TRM7	FTSJ1	VPS21	RAB5B	MET30	FBXW7
TRM7	FTSJ2	VPS21	RAB5C	NSG1	INSIG1
RIM101	KLF1	PHO90	SLC13A1	NSG1	INSIG2
RIM101	KLF2	PHO90	SLC13A2	SAS3	KAT5
RIM101	KLF3	PHO90	SLC13A3	SAS3	KAT7
RIM101	KLF4	PHO90	SLC13A4	KTI12	KTI12
RIM101	KLF5	PHO90	SLC13A5	OXR1	NCOA7
RIM101	KLF6	AGC1	SLC25A12	OXR1	OXR1
RIM101	KLF7	AGC1	SLC25A13	PIN4	PABPC1
RIM101	KLF8	AGC1	SLC25A18	PIN4	PABPC1L
RIM101	KLF9	AGC1	SLC25A22	PIN4	PABPC4
RIM101	KLF10	AGC1	SLC25A44	PIN4	PABPC4L
RIM101	KLF11	RIM101	SP5	PIN4	PABPC5
RIM101	KLF12	RIM101	SP6	RPL15B	RPL15
RIM101	KLF13	RIM101	SP7	PIN4	SYNCRIP
RIM101	KLF14	YBL055C	TATDN1	OXR1	TLDC2
RIM101	KLF15	YBL055C	TATDN2	MET30	TRAF7
RIM101	KLF16	YBL055C	TATDN3	UBC11	UBE2C
RIM101	KLF17			UBC11	UBE2U

HuR é primariamente localizada no núcleo, mas durante diversos tipos de estresse é translocada para o citoplasma onde age na estabilidade de mRNAs e na eficiência com que são traduzidos pelos ribossomos. Um ano antes de nossa descoberta, havia sido publicado que carboplatina causava a migração de HuR do núcleo para o citoplasma em células tumorais pancreáticas (Lal et al., 2014). Já as culinas são proteínas importantes para o processo de reconhecimento da maquinaria de degradação proteica mediada pelo proteassomo. Juntos, HuR e culinas regulam 23 diferentes proteínas homólogas humanas selecionadas por meio de 12 diferentes linhagens mutantes de leveduras que responderam à carboplatina (60% dos genes de leveduras que deram origem à Tabela 3). Assim, postulamos um modelo na qual HuR estaria envolvida no aumento da estabilidade das proteínas envolvidas com a resistência e a culinas com o aumento da degradação das proteínas responsáveis pela sensibilidade celular à carboplatina (Figura 21).

Figura 21: Modelo de mecanismos moleculares envolvidos com a sensibilidade e resistência ao antitumoral carboplatina. Fonte: Sousa et al., 2015.

Um processo biológico de regulação tanto da atividade das culinas quanto de HuR é a nedilação, uma modificação pós-traducional, similar à ubiquitinação, mas que não propriamente marca a molécula alvo para degradação. As culinas, por exemplo, são ativadas e HuR é estabilizado, ambos por nedilação (revisão em Zhao et al., 2014). Dessa forma, para validar nosso modelo da Figura 21 analisamos a expressão dos genes envolvidos na resistência em casos clínicos de câncer de ovários, através de dois bancos de dados, o *Gene Expression Omnibus* e o *Oncomine* (Figura 22). A Figura 22 mostra a porcentagem de pacientes com câncer de ovário que mostraram aumento de pelo menos 40% na expressão do respectivo gene com relação ao tecido normal.

Figura 22: Análise da expressão de mRNA dos genes possivelmente estabilizados por HuR durante a resistência tumoral à carboplatina de pacientes com câncer de ovário. N_{TOTAL} representa o número de pacientes analisados para cada gene. Fonte: Sousa et al., 2015.

Um dado impressionante dessa análise foi obtido com relação ao homólogo humano SLC25A44. Esse gene apresentou uma alta frequência de superexpressão, em 96% dos pacientes analisados (929 de 970) e nunca havia sido relacionado ao tumor de ovário. SLC25A44 pertence à família dos carreadores mitocondriais de solutos, cujo gene está localizado no cromossomo 1 e sua função ainda é desconhecida.

Para validarmos o modelo quanto à resposta às culinas, não seria adequado analisar o efeito no mRNA, pois essas são modificadoras de proteínas. Assim, analisamos dois bancos de dados de expressão proteica: *Human Protein Atlas* e o *Database of Differentially Expressed Proteins in Human Cancer* (dbDEPC 2.0); os dados são apresentados na Tabela 4.

Tabela 4: Comparação entre a expressão de proteínas em tecidos normais (control) e câncer de ovário. 12 pacientes analisados e várias linhagens tumorais.

Fonte: Sousa et al., 2015.

PROTEIN EXPRESSION		
	CONTROL	OVARIAN CANCER
CUL1	low expressed	17% tumours presented moderate expression
CUL2	low expressed	67% tumours presented moderate expression
CUL3	moderate expression in stroma	25% tumours presented strong expression
CUL4A	no information	no information
CUL4B	not expressed in stroma	100% tumours presented low expression
UBE2M	not expressed in stroma	strong expression in EFO-21 cell line
NEDD8	moderate expression in stroma	strong expression in EFO-21 cell line
NAE1	moderate expression in stroma	58% tumours presented strong expression
FBXW2	moderate expression	low expressed
PABPC1	not expressed	90% tumours presented low to moderate expression

A nedilação é um processo de adição de uma molécula (NEDD8, semelhante à ubiquitina) em um substrato proteico, levando a uma modulação da atividade do alvo. Na Tabela 4 podemos confirmar que diversas proteínas envolvidas nas vias de nedilação estão superativadas. Interessantemente, a principal enzima envolvida no processo de nedilação, a NAE1 (*NEDD8 activating enzyme*) está superexpressa em quase 60% dos tumores de pacientes de câncer de ovário analisados (Tabela 4).

A molécula MLN4924 é um inibidor específico de NAE1 e seu efeito já foi observado em modelos animais e linhagens celulares em resposta à cisplatina, molécula análoga à carboplatina (Nawrocki et al., 2013; Jazaeri et al., 2013). Dessa forma, testamos o efeito de MLN4924 em reverter o fenótipo de resistência à carboplatina em linhagem de célula tumoral de ovário ES-2 (Figura 23).

Figura 23: Viabilidade das linhagens celulares resistente e não-resistente à carboplatina. Linhagem celular de câncer de ovário ES-2 foi pré-tratada com 50 μ M de carboplatina; as células sobreviventes foram selecionadas como resistentes e usadas nos ensaios de viabilidade. Nos ensaios, as células foram tratadas com 500 μ M de carboplatina, 10 nM de MLN4924 ou ambos por 24 horas. As linhas horizontais indicam os grupos que não mostraram diferença de viabilidade estatisticamente significativa. Os asteriscos representam diferenças estatisticamente significantes entre os grupos comparados. Fonte: Sousa et al., 2015.

Dessa maneira, de acordo com os dados da Figura 23 nosso modelo molecular foi validado, mostrando a importância da nedilação na resposta à carboplatina. Entretanto, é importante ressaltar que não houve efeito sinérgico entre carboplatina e MLN4924 em células não resistentes à carboplatina, sugerindo que a nedilação é um mecanismo de resistência adquirida. Nossos dados mostram o quão útil pode ser a levedura como modelo celular para entender alvos e mecanismos moleculares envolvidos na resposta a drogas. Esses dados foram publicados na revista *PLoS One*, fator de impacto 3.057 (ANEXO 5).

3 BIOLOGIA MOLECULAR DE LEVEDURAS APLICADA NA PRODUÇÃO DE BIOFÁRMACO ASPARAGINASE.

A escolha do tipo de microrganismo mais apropriado para produção de biofármacos é embasada nas prioridades e necessidades apresentadas para obtenção da atividade desejada, em quantidades adequadas de cada proteína heteróloga a ser expressa. Vários critérios devem ser preenchidos para se escolher um sistema microbiano de produção, como alto nível de expressão do produto, estabilidade do material produzido e da cepa, aplicabilidade humana, não ser partícula patogênica nem pirogênica (ou ser possível a purificação do produto de interesse dessas partículas) e preservar a atividade da proteína.

Os microrganismos tais como bactérias e fungos são os sistemas mais utilizados para este fim biotecnológico, por serem de fácil modificação genética, terem elevadas taxas de crescimento alcançando altas densidades celulares e normalmente possuem rendimento elevado de expressão de proteína heteróloga (Schmidt, 2004; Weinacker et al., 2014). A mais clássica expressão heteróloga recombinante é a da insulina humana em bactérias *E. coli*, que foi aprovada para utilização como biofármaco em 1982 pelo órgão regulatório americano *FDA – Food and Drug Administration*. Esse foi o primeiro biofármaco recombinante lançado no mercado farmacêutico e é utilizado até os dias atuais.

Dentre os microrganismos usados para expressar proteínas heterólogas, as leveduras têm se destacado desde os anos 70, sendo a *S. cerevisiae* a mais aplicada (Bill, 2015; Porro et al., 2011; Spadiut et.al., 2014). Isso provavelmente se deve ao fato dessas células terem sido uma das primeiras eucarióticas a ter seu genoma sequenciado, o que facilitou o desenvolvimento de vetores de expressão e de técnicas de transformação, cruciais para a produção heteróloga de proteínas.

A primeira vacina recombinante para uso humano foi produzida em *S. cerevisiae*. O antígeno de superfície do vírus causador da hepatite B foi heterologamente expresso nessa levedura e o título produzido conseguiu suprir a demanda necessária dessa molécula para um efetivo programa de vacinação. Antes da forma recombinante, o antígeno do vírus era obtido através de purificação do mesmo do soro de pacientes infectados, o que representava uma limitação muito grande de fonte do antígeno, além da periculosidade que um processo de purificação falho poderia acarretar na população vacinada, como infecções por vírus adventícios presente nas amostras de soro que deram origem à vacina (McAleer et al., 1984).

Apesar desse exemplo de enorme sucesso, se o biofármaco não se tratar de uma molécula antigênica, *S. cerevisiae* não apresenta o sistema de expressão de proteínas heterólogas mais favorável, por possuir baixa habilidade na secreção dessas proteínas; baixo rendimento na conversão de substrato em biomassa; alta capacidade fermentativa e consequente produção de etanol que pode inativar o produto; hipermanosilação proteica, uma forma de glicosilação específica desse fungo altamente imunogênica aos seres humanos, quando comparada com a de outras leveduras; e aprisionamento da biomolécula-alvo no espaço entre a parede e a membrana celular, o que dificulta os processos de purificação proteica (Damasceno et al., 2012).

Assim, a levedura *Pichia pastoris* ganhou destaque por ser um dos mais úteis e versáteis sistemas de expressão de proteínas heterólogas. Isso se deve a alguns fatores tais como facilidade com a qual são realizadas as técnicas necessárias para a modificação genética (as ferramentas desenvolvidas em *S. cerevisiae* puderam ser aplicadas com sucesso nessa levedura); alta capacidade de produzir proteínas heterólogas intra- ou extracelularmente e de realizar modificações pós-traducionais ausentes nos sistemas bacterianos; disponibilidade do sistema de expressão e de outras

ferramentas de estudo comercialmente. Os vetores utilizados para transformação de *P. pastoris* são integrados ao genoma de forma estável, proporcionando um processo consistente de produção (Cereghino et al., 2002; Gellissen, 2000; Zepeda et al., 2014).

P. pastoris é uma levedura metilotrófica, ou seja, que possui capacidade de utilizar o metanol como única fonte de carbono para seu crescimento em meio de cultura. Para essa habilidade a célula expressa duas enzimas álcool oxidases – Aox1 e Aox2, que catalisam a oxidação de metanol em formaldeído, a primeira reação da via de assimilação celular de metanol. As duas proteoformas apresentam a mesma atividade bioquímica, mas com marcantes diferenças em sua expressão gênica. O promotor *AOX1* é fortemente ativado pela presença de metanol e ausência de glicose e inibido pela presença de etanol no meio de cultura. Já *AOX2* é um promotor fracamente ativado nas mesmas condições (Cereghino et al., 2002). O promotor *AOX1* é sem dúvida o mais utilizado na produção heteróloga de proteínas em *P. pastoris*; assim, a maior parte dos sítios de recombinação homóloga dos vetores de expressão é desenhada para que a mesma ocorra nos *loci* *AOX1*, *AOX2* ou em ambos.

Dessa forma, após a transformação são gerados três possíveis fenótipos recombinantes de *P. pastoris*, relacionados à sua capacidade em utilizar metanol: Mut⁺, Mut^S ou Mut⁻. No fenótipo Mut⁺ os genes *AOX* estão intactos, isto é, a recombinação homóloga durante a inserção do material genético de interesse ocorreu fora desses *loci*, gerando estirpes com crescimento rápido em metanol. O fenótipo Mut^S (s = *slow*; lenta) resulta da perda parcial da atividade de álcool oxidase causada por recombinação apenas no *locus* *AOX1*; o resultado é o crescimento lento em metanol, dependente da baixa expressão de *AOX2*, em comparação com as taxas de crescimento observadas nas estirpes selvagem ou Mut⁺. Por fim, o fenótipo Mut⁻ resulta na incapacidade de utilização de metanol,

nos quais os *loci AOX* são ambos interrompidos durante a recombinação homóloga e a estirpe deixa de produzir álcool oxidase (Cereghino et al., 2002). Esses diferentes fenótipos influenciam diretamente na produção heteróloga específica de cada proteína, com razões ainda não esclarecidas.

3.1 Sistemas de expressão e caracterização de Asparaginases de *S. cerevisiae*.

Tendo por base as informações acima, no ano 2013 iniciamos o projeto Temático FAPESP “Produção de L-Asparaginase extracelular: da bioprospecção à engenharia de um biofármaco antileucêmico”. O objetivo central é a obtenção de um processo industrialmente viável de produção biotecnológica de L-Asparaginase (L-ASNase), biofármaco amplamente utilizado no tratamento de leucemias. Duas grandes frentes são abordadas: a otimização de processos de produção endógena e heteróloga da enzima, com grupos de bioprospecção de fungos dos mais variados biomas; e a engenharia racional de proteínas que utiliza como *scaffold* as L-ASNase de *S. cerevisiae* para estudos comparativos com as proteoformas bacterianas, atualmente empregadas na terapêutica. Esse projeto é coordenado pelo Prof. Dr. Adalberto Pessoa Junior (FBT/FCF/USP) e tem como pesquisadores principais o Prof. Dr. Marcos Antonio de Oliveira (UNESP – CLP) e eu. A parte de estudos envolvendo expressão heteróloga, caracterização das proteínas asparaginases de *S. cerevisiae* e construção de sistemas de produção da L-ASNase secretada para o meio extracelular em *P. pastoris* são de minha responsabilidade.

Para contextualizar, atualmente, a leucemia linfóide aguda (LLA) é o tipo de câncer mais frequente entre crianças e adolescentes e destaca-se, como uma das principais formas de tratamento existentes para o seu combate, a administração de L-ASNase. Esta enzima apresenta diversas formas no mercado, diferindo-se umas das outras principalmente quanto ao organismo de origem (*Escherichia coli* ou *Dickeya chrysanthemi*) e modificação química (nativa ou peguilada). No entanto, esses biofármacos possuem desvantagens, uma vez que podem ocasionar reações imunogênicas e, conseqüentemente, serem rapidamente depuradas do organismo por diferentes mecanismos de eliminação.

Vale ressaltar que após a introdução da L-ASNase nos protocolos de tratamento da LLA, a taxa de remissão completa chegou a 80%. No Brasil, apenas a formulação da enzima nativa provinda de *E. coli* está aprovada pela ANVISA. Contudo, grande parte do mercado mundial usa como primeira linha de tratamento a enzima de *E. coli* peguilada, pois essa causa menos reações alérgicas e possui considerável maior meia-vida. Como resultado, o exportador que supria o Brasil com a L-ASNase perdeu o interesse na comercialização e anunciou em 2011 a interrupção na produção desse biofármaco.

A L-ASNase é uma enzima capaz de degradar o aminoácido asparagina (Asn) em aspartato e amônia (Batool et al, 2016; Lopes et al., 2017). Como Asn é um aminoácido essencial para as células leucêmicas, estas acabam morrendo por apoptose, uma vez que não conseguem sintetizá-lo. A carência de asparagina leva à deficiência na síntese proteica de fatores de sobrevivência como os antiapoptóticos, por exemplo. Por outro lado, as células saudáveis não são afetadas por essa diminuição sérica de Asn, já que são capazes de sintetizar esse aminoácido por vias metabólicas endógenas utilizando a asparagina sintetase, enzima não expressa ou expressa em baixos níveis nas células leucêmicas (Sajitha et al, 2015 e Batool et al, 2016).

Devido à similaridade química e estrutural entre asparagina e glutamina, a L-ASNase pode apresentar uma atividade secundária, de glutaminase, hidrolisando glutamina a ácido glutâmico e amônia. A ausência de glutamina e os produtos da hidrólise desses aminoácidos, especialmente a amônia, são responsáveis pela neurotoxicidade do medicamento (depressão, letargia, fadiga, sonolência, confusão, irritabilidade, tontura, agitação, coma), presente em até 25% dos pacientes (Narta et al., 2007; Raetz e Salzer, 2010). De forma geral, o uso de L-ASNase como medicamento gera respostas de hipersensibilidade no organismo, além de diversas reações alérgicas, podendo promover uma consequente interrupção do tratamento (Pieters et al, 2011).

Além dos efeitos já citados, a L-ASNase pode induzir resposta imune. A produção de anticorpos contra L-ASNase acontece através da degradação da enzima pelas proteases lisossomais humanas catepsina B e asparaginil endopeptidase (AEP), que são produzidas por linfoblastos. A proteólise de L-ASNase resulta na sua inativação, exposição de epítomos e consequente ativação da resposta imune com produção de anticorpos (Offman et al., 2011). As reações de hipersensibilidade devido à produção de anticorpos têm sido observadas em 60% dos pacientes que fazem uso da enzima derivada de *E. coli* (Pieters et al, 2011), sendo uma das principais causas de resistência ao medicamento, culminando na redução de sua atividade e eficácia (Narazaki et al., 2012; Avramis, 2012; Kumar et al., 2014).

Imada et al. (1973) demonstraram que diferentes microrganismos como bactérias, fungos e leveduras, são capazes de produzir L-ASNase. Estudos evolutivos demonstram a homologia entre L-ASNase bacterianas, vegetais e até humanas (Borek e Jaskólsli, 2001; Karamitros e Konrad, 2014). No entanto, para ser considerada uma enzima com potencial efeito antineoplásico, é necessário que a enzima seja altamente ativa em baixas concentrações de substrato e mantenha baixos os níveis séricos de Asn (Broome, 1968).

Em *S. cerevisiae* há a produção de duas L-ASNase, a L-asparaginase 1, expressa pelo gene *ASP1* (*YDR321W*), e a L-asparaginase 2 expressa pelo gene *ASP3*. A proteína ScASNaseI é encontrada no citosol da levedura enquanto a proteoforma codificada pelo gene *ASP3* é periplasmática. Ambas hidrolisam Asn, mas são bioquímica e geneticamente distintas (Dunlop et al., 1978; Kim et al., 1988; Sinclair et al., 1994).

Dessa forma, seria interessante a produção brasileira de uma L-ASNase, com características melhores com relação às proteoformas bacterianas em uso terapêutico. Nossa proposta é usar as enzimas de *S. cerevisiae* como protótipos geneticamente engenheirados de L-ASNase. Contudo, há necessidade de caracterização bioquímica e antineoplásica dessas enzimas para se definir o que precisará ser melhorado. Assim, começamos nossos estudos de produção de sistemas de expressão heteróloga de ScASNase, inicialmente em bactérias *E. coli*. Como já citado, essas bactérias podem representar um sistema heterólogo de expressão rápido, simples e fácil, pelo menos no que concerne aos estudos laboratoriais dessas enzimas.

Iniciamos as análises usando predições bioinformáticas buscando possíveis sítios de glicosilação e a presença ou não de sequencia de endereçamento celular para maquinaria de secreção de proteínas. Os resultados indicaram que ambas as proteínas ScASNaseI e ScASNaseII possuem sítios possíveis de glicosilação. No entanto, para essa modificação pós-traducional ocorrer, a proteína precisa conter um peptídeo sinal que a enderece à maquinaria organelar de secreção proteica. Assim, pudemos concluir que devido à ausência de peptídeo sinal predito, ScASNaseI, mesmo contendo sítios de glicosilação possíveis, provavelmente não é glicosilada. Já ScASNaseII apresenta um claro peptídeo sinal de endereçamento por volta do aminoácido na posição 20 e também possui preditos alguns sítios de glicosilação. De fato, essa enzima é periplasmática na *S. cerevisiae* e quando expressa em sistema heterólogo *P. pastoris* apresenta formas proteicas glicosiladas (Girão et al., 2016).

Construímos os vetores de expressão em bactéria e obtivemos a proteína ScASNaseI solúvel e em grandes quantidades; porém, ScASNaseII foi bastante expressa somente de forma insolúvel nos corpos de inclusão (Figura 24).

Figura 24: SDS-PAGE das proteínas L-ASNase de *S. cerevisiae* expressas em *E. coli*. (A) Diferentes frações da purificação de ScASNaseI por cromatografia de afinidade a metal. Coluna 1- padrão de massa molecular BenchMark™. (B) Frações proteicas de ScASNaseII após a lise celular e clarificação do extrato: solúvel (última coluna à direita) e insolúvel (coluna do meio). Coluna 1 à esquerda - padrão de massa molecular BenchMark™. Fonte: Santos et al., 2016.

Para tentar expressar ScASNaseII na fração proteica solúvel em *E. coli* foram testadas 93 condições diferentes, incluindo vetores, fusões proteicas, posição da cauda de histidina e retirada do peptídeo sinal. Além disso, diferentes linhagens hospedeiras, bem como concentrações de indutor IPTG (isopropiltio- β -D-galactosídeo), temperatura e tempo de indução foram variados buscando obter a proteína solúvel nesse sistema de expressão. Nenhuma dessas condições foi suficiente para obtermos ScASNaseII solúvel em *E. coli*. Postulamos que provavelmente essa proteína necessite da glicosilação para obter sua estrutura terciária / quaternária adequada. Na ausência do sistema eucariótico de expressão e,

consequentemente da glicosilação, essa proteína não foi capaz de adquirir sua conformação espacial correta, agregando em formas inativas nos corpos de inclusão (Figura 24B). Já ScASNaseI foi obtida em grandes quantidades (Figura 24A). Com isso, otimizamos o processo de purificação por afinidade a metais usando *fast protein liquid chromatography* - FPLC. A Tabela 5 mostra os parâmetros de rendimento da purificação obtidos.

Tabela 5: Rendimento da purificação de ScASNaseI usando FPLC. Fonte: Santos et al., 2016.

Amostra	Concentração de proteína (mg/mL)	Atividade enzimática (UI/mL)	Atividade específica (UI/mg)	Fator de purificação o (vezes)	Recuperação da atividade (%)
Extrato celular	0.38 ± 0.01	2.5 ± 0.01	6.52 ± 0.01	1.00	100
Após FPLC	0.02 ± 0.01	2017 ± 0.01	110.06 ± 0.34	16.88	40.5

Em conclusão, não foi possível obter ScASNaseII ativa usando sistema de expressão *E. coli*; contudo, obtivemos alta quantidade e conseguimos estabelecer alto grau de pureza para a proteína ScASNaseI. Esses dados foram publicados no periódico *Biotechnology Progress*, fator de impacto 2.167 (**ANEXO 6**).

Assim, iniciamos a caracterização bioquímica de ScASNase1; essa enzima tem aproximadamente 42 kDa e apresenta múltiplas subunidades (Dunlop et al., 1978; Sinclair et al., 1994). Poucos estudos foram realizados com a proteína ScASNase1; os primeiros e únicos datam da década de 70 por Jones e Mortimer (1973), e

posteriormente por Dunlop et al. (1978). Em ambos os estudos, a proteína ScASNase1 obtida de forma endógena foi submetida diversas vezes a processos de purificação, e ao final não estava suficientemente pura; foi caracterizada com baixa atividade e afinidade pelo substrato. Os autores concluíram que a mesma não tinha potencial para ser usado como biofármaco antileucêmico, mas nenhum grupo havia testado a atividade citotóxica de ScASNase1.

Nossos dados sobre a atividade específica, pH e temperatura ótimos e cinética da enzima, contradizem os primeiros estudos. Jones e Mortimer (1973) encontraram uma atividade específica de 0,06 U/mg e um K_m de $2,5 \times 10^{-4}$ M para asparagina; Dunlop et al. (1978) obtiveram uma atividade de 5,4 U/mg e K_m de $7,4 \times 10^{-4}$ M. Nossos ensaios mostraram uma atividade de 196 U/mg, mais de 30 vezes maior do que o melhor valor relatado anteriormente (Figura 25 A). Além disso, a enzima mostrou uma baixa atividade glutaminásica, cerca de 0,38% da atividade asparaginásica (Figura 25B); esse valor é relatado como 2% para a enzima nativa de *E. coli* usada na terapêutica. ScASNase1 também é promissora em condições fisiológicas humanas, pois em pH 7.4 a enzima apresenta 70% da atividade ótima (Figura 25C) e a temperatura ótima foi de 40 °C (Figura 25D).

Figura 25: Caracterização bioquímica de ScASNase1. (A) Medida da atividade específica de asparaginase. (B) Medida da atividade específica de glutaminase. (C) Atividade específica de asparaginase em diferentes pHs. (D) Atividade específica de asparaginase em diferentes temperaturas. (E) Cinética da atividade asparaginásica. (F) Plote de Hill da atividade asparaginásica. Fonte: Costa et al., 2016.

Contudo, o mais surpreendente foram os parâmetros cinéticos encontrados. ScASNase1 apresentou cinética sigmoidal-alostérica, com cooperação positiva regulada por asparagina e $K_{0.5}$ de 75 μM (Figuras 25E e 25F). As enzimas normalmente são comparadas quanto a sua eficiência catalítica. Enquanto enzimas michaelianas possuem eficiência ditada por k_{cat}/K_m , as enzimas alostéricas são fortemente influenciadas pelo coeficiente de Hill (n_H), tendo sua eficiência regida por $k_{\text{cat}}/[K_{0.5}]^{n_H}$. Sendo assim, a eficiência catalítica da enzima asparaginase 2 de *E. coli* (EcASNase2) quando calculada alcança valor de $10^6 \text{ M}^{-1} \text{ s}^{-1}$, enquanto ScASNase1 possui $10^4 \text{ M}^{-1} \text{ s}^{-1}$, duas ordens de grandeza menor. Contudo, se comparamos com enzimas asparaginases que comprovadamente não apresentam atividade antineoplásica, como a asparaginase 1 de *E. coli* (EcASNase1) que apresenta eficiência catalítica de $10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ (nove ordens de grandeza menor que EcASNase2), ScASNase1 é pouco menos eficiente do que a usada terapeuticamente.

Outro fator interessante que observamos é que a atividade de ScASNase1 é regulada não só pelo próprio substrato Asn, mas pela presença de diferentes íons. Por exemplo, potássio aumenta em 10% a atividade, enquanto cálcio a abole completamente. Outro fator atraente dessa enzima foi observado durante a incubação dela com soro humano. Enquanto a enzima de *E. coli* não sofre alteração em sua atividade específica, ScASNase1 quase dobra sua atividade, mostrando que fatores presentes no soro regulam positivamente essa enzima (Figura 26).

Figura 26: Atividade específica das asparaginases de *E. coli* e *S. cerevisiae* quando incubadas com soro humano. As enzimas foram incubadas a 37 °C por 1 hora com 10% de soro humano antes de a atividade ser dosada. Fonte: Costa et al., 2016.

Por fim, analisamos o efeito citotóxico causado por exposição à ScASNase1 em células leucêmicas das linhagens MOLT-4 e REH. Ambas as linhagens são células de leucemia linfoblástica aguda; a primeira é derivada de linfócitos T e a segunda de linfócitos B. A linhagem REH é considerada resistente, pois possui níveis basais de expressão de asparagina sintetase - ASNS (Hermanova, et al., 2012). Já a MOLT-4 é descrita como uma linhagem sensível a L-ASNase; porém, após seis semanas de tratamento é observada a expressão de ASNS e, conseqüentemente a resistência à enzima (Aslanian, Fletcher e Kilberg, 2001). Como controle, utilizamos a linhagem de células endoteliais saudáveis da veia de cordão umbilical humana HUVEC (www.atcc.org/). Para comparação de efeito, usamos a enzima de *E. coli* – EcASNase2 (Figura 27).

Figura 27: Ensaio de citotoxicidade das asparaginases de *E. coli* e *S. cerevisiae* usando linhagens leucêmicas (MOLT-4 e REH) e normais (HUVEC). Controle = células sem nenhuma adição; Buffer = células incubadas com o tampão de diluição da enzima. 10 U/mL = concentração da respectiva asparaginase adicionada no teste. Todas as incubações foram por 72 horas a 37 °C, em estufa com atmosfera de 5% de CO₂. (A) teste com a enzima de *E. coli* EcASNase2 comercial Prospec-Tany (B) teste com a enzima ScANase1. Fonte: Costa et al., 2016.

A enzima de *E. coli* não apresentou toxicidade para célula saudável HUVEC. Para a linhagem resistente REH foi observada uma mortalidade de aproximadamente 20%. No entanto, para MOLT-4 quase 100% das células foram mortas nessas condições (Figura 27A). Já ScASNase1, assim como a enzima bacteriana, não apresentou toxicidade para linhagem HUVEC, porém, não teve efeito para a linhagem REH. No tratamento com a linhagem sensível, MOLT-4, a enzima mostrou um potencial citotóxico de 85% (Figura 27B). Dessa forma, ao contrário do que foi suposto anteriormente pelos primeiros trabalhos a caracterizar ScASNase1, essa enzima apresenta atividade antineoplásica *in vitro*. Esses dados foram publicados no periódico do grupo Nature, *Scientific Reports* (ANEXO 7), fator de impacto 5.228.

Esses resultados são muito promissores e na verdade inesperados. Quando iniciamos a caracterização dessa enzima acreditávamos obter resultados apenas para ter parâmetros de onde melhorá-la. Para nossa surpresa a enzima já possui características muito interessantes para sua possível aplicação como biofármaco. Considero atualmente essa uma das minhas melhores contribuições científicas desde que me tornei chefe de grupo. Houve uma grande repercussão na mídia sobre esse trabalho, com entrevistas publicadas em diferentes meios de comunicação impresso, eletrônico e rádio. Houve repercussão também na mídia internacional, com a publicação em 02/03/2017 de um editorial (categoria *Research Spotlight, Yeast and Human Disease*) sobre o trabalho publicado na *Scientific Reports*. O editorial é denominado “Feed a Cold, Starve a Cancer?” no site do SGD, ressaltando a importância da descoberta (acesso no endereço <http://www.yeastgenome.org/feed-a-cold-starve-a-cancer>).

3.2 Expressão de ASNases em *P. pastoris*.

Por ser biofármaco injetável de origem bacteriana, a L-ASNase pode induzir resposta imune, produzindo anticorpos antiasparaginase, o que leva à resistência adquirida de pacientes ao medicamento. Essa resistência pode ser sintomática, com sinais de hipersensibilidade clínica, ou assintomática. O uso de profilaxias, como corticoides e anti-histamínicos, não é aconselhável, pois podem mascarar os sintomas de resistência, levando à inativação silenciosa do biofármaco (Narazaki et al, 2012; Avramis, 2012).

A estratégia de conjugação da enzima com polietileno glicol (PEG) pela indústria farmacêutica teve como objetivo limitar a imunogenicidade, com base na observação de que esse polímero pode mascarar epítomos imunogênicos. De fato, a estabilidade da proteína e seu efeito imunogênico aparentemente estão bastante relacionados, pois é observado que a PEG-L-ASNase tem maior meia vida biológica no soro do que a enzima nativa. No entanto, mesmo essas enzimas modificadas podem representar antígenos para o sistema imunológico humano (Armstrong et al., 2007; Nomme et al., 2012).

Desde 1994 a Oncaspar® (PEG-asparaginase) (Salzer et al, 2014), L-ASNase de *E. coli* modificada quimicamente através de ligações covalentes com PEG em sítios não específicos, passou a ser utilizada e hoje é tida como primeira linha de tratamento em diversos países (Holle, 1997; Pieters et al, 2011). Entretanto, o processo de adição de moléculas de PEG durante o seu desenvolvimento ainda não é controlado (Molineux, 2003) e existe a possibilidade de reação do organismo contra esse polímero através da geração de anticorpos contra o PEG, o que de certa forma afetaria a resposta final deste biofármaco (Armstrong et al, 2007).

Dessa forma, o desenvolvimento de uma nova metodologia que emula a peguilação de biomoléculas para fins terapêuticos com a finalidade de minimizar a geração de respostas imunes em seres humanos é interessante. Uma estratégia alternativa à modificação química seria a indução de glicosilação durante a produção recombinante de L-ASNase por um organismo eucarioto, tais como leveduras. A glicosilação é uma modificação pós-traducional complexa, que muitas vezes ocorre em proteínas secretadas ou aquelas associadas com as membranas. Isto envolve a adição de oligossacarídeos em proteínas e estes, assim como o PEG, proporcionam a estabilidade da proteína (Vervecken et al., 2004).

Cabe ressaltar que o mecanismo de glicosilação desempenhado pelas leveduras difere significativamente do existente nas células humanas. Nas leveduras, ocorre maior adição de moléculas de manose quando comparada ao mecanismo que ocorre nas células humanas, o que proporciona maior taxa de degradação da proteína e consequentemente menor atividade (Berlec e Strukelj, 2013). Por isso, nesse projeto, estão sendo usadas linhagens de *P. pastoris* com glicosilação humanizada, o que confere maiores vantagens em relação a outras linhagens. Esta linhagem é conhecida como sistema Glycoswitch® (Biogrammatics) onde o padrão de glicosilação se assemelha com o presente nas células do organismo humano, por apresentar, por exemplo, enzimas da via de glicosilação humana como glicosiltransferases e glicosidases (Laukens et al, 2015).

Além disso, outras vantagens incluem relativa maior uniformidade na glicosilação, expressão de proteínas glicosiladas com maior eficiência, minimização de reações de glicosilação inadequadas, o que pode resultar em maior efeito terapêutico (Pichia, acessado em 09 março 2017 <http://www.pichia.com/glycoswitch/>). Tais vantagens poderiam promover, portanto, uma menor resposta imunogênica e uma maior estabilidade da proteína recombinante.

Realizamos a clonagem e expressão das L-ASNase bacterianas EcASNase2 e de *Dickeya chrysanthemi* EwASNase2. A Figura 28 mostra o SDS-PAGE resultante da expressão extracelular das L-ASNase bacterianas em *P. pastoris*. Como pode ser observado, as proteínas foram expressas nas formas glicosiladas e no meio extracelular. Nosso grupo agora iniciará os estudos de caracterização das atividades bioquímica e antineoplásica dessas enzimas, assim como realizado para ScASNase1. Os pedidos de patente dessas proteínas glicosiladas estão sendo preparados.

Figura 28: SDS-PAGE da expressão de L-ASNase bacterianas em sistema heterólogo *P. pastoris* com glicosilação humanizada. (A) EwASNase2: 1 – Padrão de massa molecular Precision Plus Protein Dual Extra Standards da BioRad.; 2 – L-ASNase de *D. chrysanthemi* expressa em *E. coli* após purificação; 3 – meio extracelular do clone transformante que foi carregado na coluna de purificação por afinidade a metais - IMAC (Hitrap – GE Healthcare); 4 – proteína eluída após purificação por IMAC; 5 – lavado da coluna durante a purificação por IMAC; 6 – extrato proteico intracelular do clone transformante; 7 - meio extracelular do clone não-transformante; 8 - extrato proteico intracelular do clone não-transformante. (B) EcASNase2: 1 – Padrão de massa molecular. 2 e 4 - meio extracelular concentrado do meio de cultivo de linhagens expressando, respectivamente, L-ASNase com cauda de histidina e L-ASNase sem cauda de histidina. 3 e 5 - mesmo meio extracelular de 2 e 4, mas tratado com a enzima peptídeo N-glicosidase F (PNGase F), que catalisa a quebra dos resíduos glicolíticos presentes nas proteínas glicosiladas. 6 e 7 são as mesmas linhagens, sem e com o tratamento da PNGase, respectivamente, mas transformadas com o vetor vazio. 8 - controle positivo, L-ASNase de *E. coli* pura. A banda menor de aproximadamente 35kD presente nos números (3), (5) e (7) corresponde à PNGase. Setas mais altas: L-ASNase glicosilada; Setas abaixo: L-ASNase não glicosilada.

4. CONCLUSÕES E PERSPECTIVAS.

Esse texto sistematizado teve o objetivo de ressaltar as contribuições científicas que realizei após a obtenção do grau de doutora (em 01/2006). O elo mais forte de todos os trabalhos que publiquei tanto durante minha formação, como agora como chefe de grupo de pesquisa é a aplicação da Biologia Molecular de Leveduras.

No pós-doutorado iniciei estudos de caracterização enzimática de yPrx1, uma 1-Cys Prxs de *S. cerevisiae*, que apresentou uma atividade inesperada durante a execução de um experimento controle – a capacidade antioxidante usando vitamina C como agente redutor. Essa habilidade intrigante e diferente do que havia sido relatado para todas as Prxs até então, mostrou-se parte de uma atividade conservada em enzimas de todos os grupos taxonômicos analisados, representando tanto uma quebra de paradigma que perdurava há mais de 20 anos para as Prxs, como atribuía uma nova função para a vitamina C – a de reciclar peroxidases da família Prxs. Dessa forma, uma enzima de levedura permitiu a descoberta de uma atividade biológica extremamente conservada.

Em relação às contribuições do meu grupo de pesquisa sob minha orientação, foram iniciadas com perguntas pouco palatáveis e cujas respostas nem sempre são muito reconhecidas: para que servem os genes que ainda não foram descritos em levedura? Por que eles estão conservados no genoma desse organismo até os dias de hoje? Quais as implicações dessas funções para a compreensão de mecanismos celulares, que podem estar envolvidos em doenças ou respostas aos fármacos em humanos? Esse foi o tema do meu primeiro projeto de pesquisa (Jovem Pesquisador FAPESP), que contribuiu para compreensão de pelo menos três genes de função desconhecida em leveduras: *YCL047C*, *YJL077C* e *YMR134W*. Todos envolvidos com a

resposta celular antioxidante, que cada vez mais tem mostrado relações causais com o câncer, tiveram um pouco de sua história contada pelo meu grupo de pesquisa. A proteína Ycl047cp (Pof1) foi caracterizada como uma ATPase envolvida na proteção celular contra acúmulo tóxico de proteínas desdobradas. A proteína Yjl077cp (Ics3) foi relacionada com a regulação do acúmulo de metais ferro e cobre em condições ácidas, tendo sua expressão gênica regulada por Rim101 e em concerto com a proteína Ccc2, uma ATPase transportadora de cobre. Por fim, Ymr134wp (Erg29) tem atividade na via metabólica de síntese de ergosterol, influenciando os níveis de ferro mitocondriais.

Usamos a levedura para explicar mecanismos de resposta celular, especialmente resistência, ao antitumoral carboplatina. Esse medicamento é usado principalmente para tratar câncer de ovário e a maior dificuldade em seu uso terapêutico reside na resistência adquirida a ele durante o tratamento. Desvendamos algumas bases moleculares envolvidas na resposta da levedura à carboplatina e esses mecanismos nos permitiram criar um modelo de resposta à droga baseado na regulação por nedilação que foi validada com sucesso em linhagem celular tumoral de ovário ES-2.

Um detalhe da ligação entre esses trabalhos, que considero muito importante: o mecanismo molecular proposto revelou uma relação entre a resposta à carboplatina de 96% dos pacientes e um gene humano, o SLC25A44. Esse gene é *predito* como carreador mitocondrial, mas sua função biológica é ainda desconhecida, assim como eram os genes *YCL047C*, *YJL077C* e *YMR134W*. Pesquisar a função de genes que ainda não foram caracterizados é uma tarefa muito difícil, é como “tatear no escuro” e normalmente os resultados não rendem grande número de citações, pois pouco se entende ainda daquelas moléculas e sua relação com as células humanas. Contudo, como exemplificado nos resultados obtidos com a carboplatina, esses estudos são

necessários e importantes, pois moléculas cruciais ainda estão por ser caracterizadas e compreendidas.

Após o término do JP, iniciei minha participação no grupo temático de estudos do biofármaco L-ASNase. Novamente, aplicando a biologia molecular de leveduras caracterizamos a L-ASNase de *S. cerevisiae* que, ao contrário do esperado, mostrou atividade antineoplásica e um possível biofármaco com potencial para ser desenvolvido. Construímos ainda sistemas de expressão heterólogo de expressão das L-ASNase bacterianas, atualmente empregadas na terapêutica, em *Pichia pastoris* com glicosilação humanizada. Essas proteínas foram expressas no meio extracelular e estão glicosiladas. A glicosilação pode ser uma alternativa de recobrimento de sítios imunogênicos das L-ASNase, emulando a peguilação. A próxima etapa será a caracterização bioquímica e antineoplásica dessas proteínas, além da investigação do efeito da glicosilação sobre estabilidade, meia-vida e alergenicidade.

Usando a biologia molecular de leveduras creio estar contribuindo para o desenvolvimento da área de Biotecnologia Farmacêutica, tanto na caracterização molecular de alvos antitumorais como na produção de biofármacos usados como antitumoral.

5. REFERÊNCIAS.

- Alesso CA, Discola KF, Monteiro G. The gene *ICS3* from the yeast *Saccharomyces cerevisiae* is involved in copper homeostasis dependent on extracellular pH. *Fungal Genet Biol* 2015;82:43-50. doi:10.1016/j.fgb.2015.06.007.
- Altmann K, Westermann B. Role of essential genes in mitochondrial morphogenesis in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2005;16:5410-7. doi:10.1091/mbc.E05-07-0678.
- Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, Garratty G. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* 2007;110:103-11. doi:10.1002/cncr.22739.
- Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. *Biochem J* 2001;357:321-8.
- Avramis VI. Asparaginases: biochemical pharmacology and modes of drug resistance. *Anticancer Res* 2012;32:2423-37.
- Babcock M, Silva D, Oaks R, Davis-Kaplan S, Jiralerspong S, Montermini L, Pandolfo M, Kaplan J. Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science* 1997;276:1709-12.
- Batool T, Makky EA, Jalal M, Yusoff MM. A comprehensive review on L-Asparaginase and its applications. *Appl Biochem Biotechnol* 2016;178:900-23. doi:10.1007/s12010-015-1917-3.
- Berlec A, Strukelj B. Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells. *J Ind Microbiol Biotechnol* 2013;40:257-74. doi:10.1007/s10295-013-1235-0.
- Bill RM. Recombinant protein subunit vaccine synthesis in microbes: a role for yeast? *J Pharm Pharmacol* 2015;67:319-28. doi:10.1111/jphp.12353.
- Borek D, Jaskólski M. Sequence analysis of enzymes with asparaginase activity. *Acta Biochim Pol* 2001;48:893-902.
- Botstein D, Fink GR. Yeast: an experimental organism for 21st century biology. *Genetics* 2011;189:695-704. doi:10.1534/genetics.111.130765.
- Brachmann RK, Vidal M, Boeke JD. Dominant-negative p53 mutations selected in yeast hit cancer hot spots. *Proc Natl Acad Sci U S A* 1996;93:4091-5.

Broome JD. Studies on the mechanism of tumor inhibition by L-asparaginase: effects of the enzyme on asparagine levels in the blood, normal tissues, and 6C3HED lymphomas of mice: differences in asparagine formation and utilization in asparaginase-sensitive and -resistant lymphoma cells. *J Exp Med* 1968;127:1055-72.

Cereghino GP, Cereghino JL, Ilgen C, Cregg JM. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Curr Opin Biotechnol* 2002;13:329-32.

Chae HZ, Chung SJ, Rhee SG. Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 1994;269: 27670-8.

Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Teixeira M, Thevelein JM. Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 2014;38:254–99. doi:10.1111/1574-6976.12065.

Costa IM, Nasser TH, Demasi M, Nascimento RM, Netto LE, Miyamoto S, Prado FM, Monteiro G. The promoter of filamentation (*POF1*) protein from *Saccharomyces cerevisiae* is an ATPase involved in the protein quality control process. *BMC Microbiol* 2011;11(268):1-11. doi:10.1186/1471-2180-11-268.

Costa IM, Schultz L, Araujo BPB, Leite, MS, Farsky SH, Oliveira MA, Pessoa Jr A, Monteiro G. Recombinant L-asparaginase 1 from *Saccharomyces cerevisiae*: an allosteric enzyme with antineoplastic activity. *Sci Rep* 2016;6(36239):1-11. doi:10.1038/srep36239.

Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T. Expression in the yeast *Pichia pastoris*. *Methods Enzymol* 2009;463:169-89. doi:10.1016/S0076-6879(09)63013-5.

Damasceno LM, Huang CJ, Batt CA. Protein secretion in *Pichia pastoris* and advances in protein production. *Appl Microbiol Biotechnol* 2012;93:31-9. doi:10.1007/s00253-011-3654-z.

Diaz-Ruiz R, Rigoulet M, Devin A. The Warburg and crabtree effects: on the origin of cancer cell energy metabolism and of yeast glucose repression. *Biochim Biophys Acta* 2011;1807:568–76. doi:10.1016/j.bbabi.2010.08.010.

Druzinec D, Salzig D, Brix A, Kraume M, Vilcinskas A, Kollwe C, Czermak P. Optimization of insect cell based protein production processes - online monitoring, expression systems, scale up. *Adv Biochem Eng Biotechnol* 2013;136:65-100. doi:10.1007/10_2013_205.

Dunlop PC, Meyer GM, Ban D, Roon RJ. Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. *J Biol Chem* 1978;253:1297-1304.

Entian KD, Schuster T, Hegemann JH, Becher D, Feldmann H, Güldener U, Götz R, Hansen M, Hollenberg CP, Jansen G, Kramer W, Klein S, Kötter P, Kricke J, Launhardt H, Mannhaupt G, Maierl A, Meyer P, Mewes W, Munder T, Niedenthal RK, Ramezani Rad M, Röhmer A, Römer A, Hinnen A, *et al.* Functional analysis of 150 deletion mutants in *Saccharomyces cerevisiae* by a systematic approach. *Mol Gen Genet* 1999;262:683-702.

Fasolo J, Sboner A, Sun MGF, Yu H, Chen R, Sharon D, Snyder M. Diverse protein kinase interactions identified by protein microarrays reveal novel connections between cellular processes. *Genes Dev* 2011;25:767–78. doi:10.1101/gad.1998811.

Freitas J, Wintz H, Kim JH, Poynton H, Fox T, Vulpe C. Yeast, a model organism for iron and copper metabolism studies. *Biometals* 2003;16:185-97. [Review].

Gadd GM, Griffiths AJ. Microorganisms and heavy metal toxicity. *Microb Ecol* 1977;4:303-317. doi:10.1007/BF02013274.

Galperin MY, Koonin EV. From complete genome sequence to “complete” understanding? *Trends Biotechnol* 2010;28:398–406. doi:10.1016/j.tibtech.2010.05.006.

Gaponova AV, Deneka AY, Beck TN, Liu H, Andrianov G, Nikonova AS, Nicolas E, Einarson MB, Golemis EA, Serebriiskii IG. Identification of evolutionarily conserved DNA damage response genes that alter sensitivity to cisplatin. *Oncotarget* 2017;8(12):19156-71. doi:10.18632/oncotarget.13353.

Gellissen G. Heterologous protein production in methylotrophic yeasts. *Appl Microbiol Biotechnol* 2000;54:741-50.

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Véronneau S, Dow S, Lucau-Danila A, Anderson K, André B, Arkin AP, Astromoff A, El-Bakkoury M, Bangham R, Benito R, Brachat S, Campanaro S, Curtiss M, Davis K, Deutschbauer A, Entian KD, Flaherty P, Foury F, Garfinkel DJ, Gerstein M, Gotte D, Güldener U, Hegemann JH, Hempel S, Herman Z, Jaramillo DF, Kelly DE, Kelly SL, Kötter P, LaBonte D, Lamb DC, Lan N, Liang H, Liao H, Liu L, Luo C, Lussier M, Mao R, Menard P, Ooi SL, Revuelta JL, Roberts CJ, Rose M, Ross-Macdonald P, Scherens B, Schimmack G, Shafer B, Shoemaker DD, Sookhai-Mahadeo S, Storms RK, Strathern JN, Valle G, Voet M, Volckaert G, Wang CY, Ward TR, Wilhelmy J, Winzeler EA, Yang Y, Yen G, Youngman E, Yu K, Bussey H, Boeke JD, Snyder M, Philippsen P, Davis RW, Johnston M. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 2002;418:387-91. doi:10.1038/nature00935.

Girão LFC, Rocha SLG, Sobral RS, Ano Bom APD, Sampaio ALF, Silva Jr JG, Ferrara MA, Bon EPS, Perales J. *Saccharomyces cerevisiae* asparaginase II, a potential antileukemic drug: purification and characterization of the enzyme expressed in *Pichia pastoris*. *Protein Expr Purif* 2016;120:118-25. doi:10.1016/j.pep.2015.12.012.

Gutteridge JM, Halliwell B. Antioxidants: molecules, medicines, and myths. *Biochem Biophys Res Commun* 2010;393:561-4. doi:10.1016/j.bbrc.2010.02.071.

Hartwell LH. Yeast and cancer. *Biosc Rep* 2005;24(4/5):523-44. doi:10.1007/s10540-005-2743-6.

Hermanova I, Zaliova M, Trka J, Starkova J. Low expression of asparagine synthetase in lymphoid blasts precludes its role in sensitivity to L-asparaginase. *Exp Hematol* 2012;40:657-65. doi:10.1016/j.exphem.2012.04.005.

Hillenmeyer ME, Fung E, Wildenhain J, Pierce SE, Hoon S, Lee W, Proctor M, St Onge RP, Tyers M, Koller D, Altman RB, Davis RW, Nislow C, Giaever G. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* 2008;320:362-5. doi:10.1126/science.1150021.

Holle LM. Pegaspargase: an alternative? *Ann Pharmacother* 1997;31:616-624. doi:10.1177/106002809703100517.

Howe AG, Zaremborg V, McMaster CR. Cessation of growth to prevent cell death due to inhibition of phosphatidylcholine synthesis is impaired at 37 degrees C in *Saccharomyces cerevisiae*. *J Biol Chem* 2002;277:44100-7. doi:10.1074/jbc.M206643200.

Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of micro-organisms. *J Gen Microbiol* 1973;76:85-99. doi: 10.1099/00221287-76-1-85.

Inga A, Scott G, Monti P, Aprile A, Abbondandolo A, Burns PA, Fronza G. Ultraviolet-light induced p53 mutational spectrum in yeast is indistinguishable from p53 mutations in human skin cancer. *Carcinogenesis* 1998;19:741-6.

Ishida S, Lee J, Thiele DJ, Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci U S A* 2002;99(22):14298-302.

Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 2004;117(5):625-35. doi:10.1016/j.cell.2004.05.002

Jazaeri AA, Shibata E, Park J, Bryant JL, Conaway MR, Modesitt SC, Smith PG, Milhollen MA, Berger AJ, Dutta A. Overcoming platinum resistance in preclinical models of ovarian cancer using the neddylation inhibitor MLN4924. *Mol Cancer Ther* 2013;12(10):1958-67. doi:10.1158/1535-7163.MCT-12-1028.

Jones GE, Mortimer RK. Biochemical properties of yeast L-asparaginase. *Biochem Genet* 1973;9(2):131-46.

- Kang SW, Rhee SG, Chang T-S, Jeong W, Choi MH. 2-Cys peroxiredoxin function in intracellular signal transduction: therapeutic implications. *Trends Mol Med* 2005;11:571–8. doi:10.1016/j.molmed.2005.10.006.
- Karamitros CS, Konrad M. Human 60-kDa lysophospholipase contains an N-terminal L-asparaginase domain that is allosterically regulated by L-asparagine. *J Biol Chem* 2014;289(19):12962–75. doi:10.1074/jbc.M113.545038.
- Kawahata M, Masaki K, Fujii T, Iefuji H. Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Res* 2006;6(6):924–36. doi:10.1111/j.1567-1364.2006.00089.x.
- Kawanishi S, Ohnishi S, Ma N, Hiraku Y, Oikawa S, Murata M. Nitrate and oxidative DNA damage in infection-related carcinogenesis in relation to cancer stem cells. *Genes Environ* 2016;38(26):1–12. doi:10.1186/s41021-016-0070-8.
- Kayikci Ö, Nielsen J. Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2015;15(6):1–8. <http://doi.org/10.1093/femsyr/fov068>.
- Kim K, Rhee SG, Stadtman ER. Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron. *J Biol Chem* 1985;260(29):15394–7.
- Kim KW, Kamerud JQ, Livingston DM, Roon RJ. Asparaginase II of *Saccharomyces cerevisiae*: characterization of the ASP3 gene. *J Biol Chem* 1988;263(24):11948–53.
- Kumar K, Kaur J, Walia S, Pathak T, Aggarwal D. L-asparaginase: an effective agent in the treatment of acute lymphoblastic leukemia. *Leuk Lymphoma* 2014;55(2):256–62. doi:10.3109/10428194.2013.803224.
- Lal S, Burkhart RA, Beeharry N, Bhattacharjee V, Londin ER, Cozzitorto JA, Brody JR. HuR post-transcriptionally regulates WEE1: implications for the DNA damage response in pancreatic cancer cells. *Cancer Res* 2014;74(4):1128–40. <http://doi.org/10.1158/0008-5472.CAN-13-1915>.
- Laukens B, De Wachter C, Callewaert N. Engineering the *Pichia pastoris* N-Glycosylation pathway using the glycoswitch technology. *Methods Mol Biol* 2015;1321:103–22. doi:10.1007/978-1-4939-2760-9_8.
- Lee JY, Jung HJ, Song IS, Williams MS, Choi C, Rhee SG, Kim J, Kang SW. Protective role of cytosolic 2-cys peroxiredoxin in the TNF- α -induced apoptotic death of human cancer cells. *Free Radic Biol Med* 2009;47:1162–71. doi:10.1016/j.freeradbiomed.2009.07.027
- Liemburg-Apers DC, Willems PHGM, Koopman WJH, Grefte S. Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. *Arch Toxicol* 2015;89(8):1209–26. <http://doi.org/10.1007/s00204-015-1520-y>.

Liu C-X, Yin Q-Q, Zhou H-C, Wu Y-L, Pu J-X, Xia L, Liu W, Huang X, Jiang T, Wu M-X, He L-C, Zhao Y-X, Wang X-L, Xiao W-L, Chen H-Z, Zhao Q, Zhou A-W, Wang L-S, Sun H-D, Chen G-Q. Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nat Chem Biol* 2012;8:486–93. doi:10.1038/nchembio.935.

Liu C-X, Zhou H-C, Yin Q-Q, Wu Y-L, Chen G-Q. Targeting peroxiredoxins against leukemia. *Exp Cell Res* 2013;319:170–6. doi:10.1016/j.yexcr.2012.06.013.

Looser V, Bruhlmann B, Bumbak F, Stenger C, Costa M, Camattari A, Fotiadis D, Kovar K Cultivation strategies to enhance productivity of *Pichia pastoris*: a review. *Biotechnol Adv* 2015;33(6 Pt2):1177–93. doi:10.1016/j.biotechadv.2015.05.008.

Lopes AM, Oliveira-Nascimento L, Ribeiro A, Tairum Jr CA, Breyer CA, Oliveira MA, Monteiro G, Souza-Motta CM, Magalhães PO, Avendaño JG, Cavaco-Paulo AM, Mazzola PG, Rangel-Yagui CO, Sette LD, Converti A, Pessoa A. Therapeutic l-asparaginase: upstream, downstream and beyond. *Crit Rev Biotechnol* 2017;37(1):82–99. doi:10.3109/07388551.2015.1120705.

Lu J, Tan M, Cai Q. The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. *Cancer Lett* 2015;356(2):156–64. <http://doi.org/10.1016/j.canlet.2014.04.001>.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 2005;22(4):249–70. doi:10.1002/yea.1208.

Martinez-Useros J, Li W, Cabeza-Morales M, Garcia-Foncillas J. Oxidative stress: a new target for pancreatic cancer prognosis and treatment. *J Clin Med* 2017;6(3 pii:E29):1–16. doi:10.3390/jcm6030029.

McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. *Nature* 1984;307(5947):178–80.

Miki H, Funato Y. Regulation of intracellular signalling through cysteine oxidation by reactive oxygen species. *J Biochem* 2012;151:255–61. doi:10.1093/jb/mvs006.

Molineux G. Pegylation: engineering improved pharmaceuticals for enhanced therapy. *Cancer Treat Rev* 2002;28(suppl A):13–6.

Monteiro G, Pereira GA, Netto LE. Regulation of mitochondrial thioredoxin peroxidase I expression by two different pathways: one dependent on cAMP and the other on heme. *Free Radic Biol Med* 2002;32(3):278–88. doi:10.1016/S0891-5849(01)00801-2.

Monteiro G, Netto LE. Glucose repression of PRX1 expression is mediated by Tor1p and Ras2p through inhibition of Msn2/4p in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* 2004;241(2):221–8. doi:10.1016/j.femsle.2004.10.024.

Monteiro G, Horta BB, Pimenta DC, Augusto O, Netto LES. Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C. *Proc Natl Acad Sci U S A* 2007;104:4886-91. doi:10.1073/pnas.0700481104.

Moretti-Almeida G, Netto LES, Monteiro G. The essential gene *YMR134W* from *Saccharomyces cerevisiae* is important for appropriate mitochondrial iron utilization and the ergosterol biosynthetic pathway. *FEBS Lett* 2013;587:3008-13. doi:10.1016/j.febslet.2013.07.024.

Morry J, Ngamcherdtrakul W, Yantasee W. Oxidative stress in cancer and fibrosis: opportunity for therapeutic intervention with antioxidant compounds, enzymes, and nanoparticles. *Redox Biol* 2017;11:240-53. doi:10.1016/j.redox.2016.12.011.

Munhoz DC, Netto LE. Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide insult: catalases and peroxiredoxins cooperate in the decomposition of H₂O₂ by yeast. *J Biol Chem* 2004;279(34):35219-27. doi:10.1074/jbc.M313773200

Narazaki H, Kaizu K, Miyatake C, Koizumi S, Asano T, Fujino O. Delayed-type hypersensitivity in response to L-asparaginase in a case of acute lymphoblastic leukemia. *J Nippon Med Sch* 2012;79(6):489-93.

Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. *Crit Rev Oncol Hematol* 2007;61(3):208-21. doi:10.1016/j.critrevonc.2006.07.009.

Nawrocki ST, Kelly KR, Smith PG, Espitia CM, Possemato A, Beausoleil SA *et al.* Disrupting protein NEDDylation with MLN4924 is a novel strategy to target cisplatin resistance in ovarian cancer. *Clin Cancer Res* 2013;19(13):3577-90. doi:10.1158/1078-0432.CCR-12-3212.

Netto LES, Chae HZ, Kang SW, Rhee SG, Stadtman ER Removal of hydrogen peroxide by thiol-specific antioxidant enzyme (TSA) is involved with its antioxidant properties: TSA possesses thiol peroxidase activity. *J Biol Chem* 1996;271(26):15315-21.

Netto LE, Oliveira MA, Monteiro G, Demasi AP, Cussiol JR, Discola KF, Demasi M, Silva GM, Alves SV, Faria VG, Horta BB. Reactive cysteine in proteins: protein folding, antioxidant defense, redox signaling and more. *Comp Biochem Physiol C Toxicol Pharmacol* 2007;146(1/2):180-93. doi:10.1016/j.cbpc.2006.07.014.

Nikawa J, Yonemura K, Yamashita S. Yeast mutant with thermolabile CDP-choline synthesis: isolation and characterization of a cholinephosphate cytidyltransferase mutant. *Eur J Biochem* 1983;131(1):223-9.

Nilsson A, Nielsen J. Metabolic trade-offs in yeast are caused by F1F0-ATP synthase. *Sci Rep* 2016;6(22264):1-11. <http://doi.org/10.1038/srep22264>.

Nomme J, Su Y, Konrad M, Lavie A. Structures of apo and product-bound human L-asparaginase: insights into the mechanism of autoproteolysis and substrate hydrolysis. *Biochemistry* 2012;51(34):6816-26. doi:10.1021/bi300870g.

Offman MN, Krol M, Patel N, Krishnan S, Liu J, Saha V, Bates PA. Rational engineering of L-asparaginase reveals importance of dual activity for cancer cell toxicity. *Blood* 2011;117(5):1614-21. doi:10.1182/blood-2010-07-298422.

Pennell CA, Eldin P. *In vitro* production of recombinant antibody fragments in *Pichia pastoris*. *Res Immunol* 1998;149(6):599-603.

Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A, Goekbuget N, Schrappe M, Pui CH. L-asparaginase treatment in acute lymphoblastic leukemia: a focus on *Erwinia asparaginase*. *Cancer* 2011;117(2):238-49. doi:10.1002/cncr.25489.

Porro D, Gasser B, Fossati T, Maurer M, Branduardi P, Sauer M, Mattanovich D. Production of recombinant proteins and metabolites in yeasts: when are these systems better than bacterial production systems? *Appl Microbiol Biotechnol* 2011;89(4):939-48. doi: 10.1007/s00253-010-3019-z.

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* 1999;284(5415):805-8.

Raetz EA, Salzer WL. Tolerability and efficacy of L-asparaginase therapy in pediatric patients with acute lymphoblastic leukemia. *J Pediatr Hematol Oncol* 2010;32(7):554-63. doi:10.1097/MPH.0b013e3181e6f003.

Sajitha S, Vidya J, Varsha K, Binod P, Pandey A. Cloning and expression of L-asparaginase from *E.coli* in eukaryotic expression system. *Biochem Eng J* 2015;102:14-7.

Santos JH, Costa IM, Molino JV, Leite MS, Pimenta MV, Coutinho JA, Pessoa Jr A, Ventura SP, Lopes AM, Monteiro G. Heterologous expression and purification of active L-asparaginase I of *Saccharomyces cerevisiae* in *Escherichia coli* host. *Biotechnol Prog* 2016 [In press]. doi:10.1002/btpr.2410.

Santos SC, Sá-Correia I. Genome-wide identification of genes required for yeast growth under imatinib stress: vacuolar H⁺-ATPase function is an important target of this anticancer drug. *OMICS* 2009;13:185-98. doi:10.1089/omi.2008.0086.

Santos SC, Teixeira MC, Cabrito TR, Sá-Correia I. Yeast toxicogenomics: genome-wide responses to chemical stresses with impact in environmental health, pharmacology, and biotechnology. *Front Genet* 2012;3:63. doi:10.3389/fgene.2012.00063.

Schenk PW, Brok M, Boersma AW, Brandsma JA, Den Dulk H, Burger H, Stoter G, Brouwer J, Nooter K. Anticancer drug resistance induced by disruption of the *Saccharomyces cerevisiae* NPR2 gene: a novel component involved in cisplatin- and doxorubicin-provoked cell kill. *Mol Pharmacol* 2003;64(2):259-68.

Schlichtholz B, Presler M, Matuszewski M. Clinical implications of p53 mutation analysis in bladder cancer tissue and urine sediment by functional assay in yeast. *Carcinogenesis* 2004;25(12):2319-23. doi:10.1093/carcin/bgh256.

Schmidt FR. Recombinant expression systems in the pharmaceutical industry. *Appl Microbiol Biotechnol* 2004;65(4):363-72. doi:10.1007/s00253-004-1656-9.

Silberberg Y, Kupiec M, Sharan R. Utilizing yeast chemogenomic profiles for the prediction of pharmacogenomic associations in humans. *Sci Rep* 2016;6(23703):1-12. <http://doi.org/10.1038/srep23703>.

Sinclair K, Warner JP, Bonthron DT. The ASP1 gene of *Saccharomyces cerevisiae*, encoding the intracellular isozyme of L-asparaginase. *Gene* 1994;144(1):37-43.

Sohal PS, Cornell R B. Sphingosine inhibits the activity of rat liver CTP: phosphocholine cytidyltransferase. *J Biol Chem* 1990;265(20):11746-50.

Sousa GF, Wlodarczyk SR, Monteiro G. Carboplatin: molecular mechanisms of action associated with chemoresistance. *Braz J Pharm Sci* 2014;50:693-701.

Sousa GF, Lima MA, Custodio DF, Freitas VM, Monteiro G. Chemogenomic study of Carboplatin in *Saccharomyces cerevisiae*: inhibition of the NEDDylation process overcomes cellular resistance mediated by HuR and Cullin proteins. *Plos One* 2015;10(e0145377):1-18. doi:10.1371/journal.pone.0145377.

Spadiut O, Capone S, Krainer F, Glieder A, Herwig C. Microbials for the production of monoclonal antibodies and antibody fragments. *Trends Biotechnol* 2014;32(1):54-60. doi:10.1016/j.tibtech.2013.10.002.

Tosato V, Grüning A-M, Breitenbach M, Arnak R, Ralser M, Bruschi CV. Warburg effect and translocation-induced genomic instability: two yeast models for cancer cells. *Front Oncol* 2013;2(212):1-12. doi:org/10.3389/fonc.2012.00212.

Vervecken W, Kaigorodov V, Callewaert N, Geysens S, De Vusser K, Contreras R. *In vivo* synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. *Appl Environ Microbiol* 2004;70(5):2639-46.

Wang M, Jiang S, Wang Y. Recent advances in the production of recombinant subunit vaccines in *Pichia pastoris*. *Bioengineered* 2016;7(3):155-65. doi:10.1080/21655979.2016.1191707.

Weinacker D, Rabert C, Zepeda AB, Figueroa CA, Pessoa Jr A, Farías JG. Applications of recombinant *Pichia pastoris* in the healthcare industry. *Braz J Microbiol* 2014;10;44(4):1043-8.

Winzler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung C, Revuelta JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Véronneau S, Voet M, Volckaert G, Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis RW. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 1999;285(5429):901-6.

Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* 2004;22(11):1393-8. doi:10.1038/nbt1026.

Zepeda AB, Figueroa CA, Abdalla DS, Maranhão AQ, Ulloa PH, Pessoa Jr A, Farías JG. Biomarkers to evaluate the effects of temperature and methanol on recombinant *Pichia pastoris*. *Braz J Microbiol* 2014;45(2):475-83.

Zhao Y, Morgan MA, Sun Y. Targeting neddylation pathways to inactivate cullin-ring ligases for anticancer therapy. *Antioxid Redox Signal* 2014;21(17):2383–400. doi:10.1089/ars.2013.5795

Zito E, Hansen HG, Yeo GSH, Fujii J, Ron D. Endoplasmic reticulum thiol oxidase deficiency leads to ascorbic acid depletion and noncanonical scurvy in mice. *Mol Cell* 2012;48(1):39–51. <http://doi.org/10.1016/j.molcel.2012.08.010>

ANEXOS

ANEXO 1

Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C

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Communicated by E. R. Stadtman, National Institutes of Health, Bethesda, MD, January 20, 2007 (received for review August 17, 2006)

Peroxiredoxins (Prx) are widely distributed peroxidases that can be divided into 1-Cys and 2-Cys Prx groups based on the number of conserved cysteine residues that participate in their catalytic cycle. Prx have been described to be strictly dependent on thiols, but here, we show that ascorbate (vitamin C) also reduces 1-Cys Prx, but not 2-Cys Prx, from several taxonomic groups. Reduction by ascorbate is partly related to the fact that the oxidized form of 1-Cys Prx is a stable sulfenic acid (Cys-SOH) instead of a disulfide. In addition, a histidine residue in the active site is required. In fact, we engineered a 2-Cys Prx with these two features, and it displayed ascorbate peroxidase activity. These data represent a breakthrough in the thiol-specific antioxidant paradigm. Ascorbate may be the long-sought-after biological reductant of 1-Cys Prx. Because ascorbate is present in high amounts in cells, the ascorbate/protein sulfenic acid pair represents an aspect of redox biochemistry that has yet to be explored *in vivo*.

peroxidase | sulfenic acid | cellular redox processes | oxidative stress | ascorbate-dependent activity

Oxygen toxicity is related to the deleterious properties of the so-called reactive oxygen species (ROS) and reactive nitrogen species (RNS). Cellular defenses are comprised of a wide range of components that interact with each other to control the levels of ROS and RNS (1). One of them is vitamin C (ascorbic acid), which is not synthesized by human cells and, therefore, must be consumed in the diet. Some human diseases, such as scurvy, are related to low concentrations of vitamin C (reviewed in ref. 2). In addition to low-molecular-weight compounds, cells also rely on enzymes to cope with oxidative injury.

Peroxiredoxins (Prx) are ubiquitous enzymes that use their highly reactive cysteine residues to decompose peroxides (3). The importance of Prx enzymes is underlined by their high abundance and involvement in multiple cellular processes ranging from antioxidant defense (4, 5), parasite drug resistance (6), and cancer (5, 7, 8) to H₂O₂-mediated cellular signaling (9, 10). In addition to their well known peroxidase activity, some of these enzymes are also efficient molecular chaperones (11). The switch from chaperone to peroxidase activity is regulated by enzymes that reduce sulfenic acids only when they are generated in Prx cysteine residues (12, 13). This redox biochemistry adds a level of versatility to the well established fact that Prx recycle between disulfide/sulfenic acid to the sulfhydryl form (reviewed in ref. 14). These properties may be related to the fact that Prx protect against several stresses (15–17).

Prx can be divided into two main groups, 1-Cys and 2-Cys Prx, based on the number of conserved cysteine residues that participate in the catalytic cycle. Both 1-Cys and 2-Cys Prx contain a reactive cysteine residue, the so-called peroxidatic cysteine (Cys_p-S⁻), that is oxidized to sulfenic acid (Cys_p-SOH) when exposed to peroxides. In the 1-Cys mechanism, Cys_p-SOH is directly reduced, whereas in the 2-Cys catalytic cycle, a second Prx cysteine residue, the resolving cysteine (Cys_r-SH), condenses with the sulfenic acid to form a disulfide. Finally, the 2-Cys disulfide is reduced by another biothiol, particularly thioredoxin, a low-molecular-weight protein with two

vicinal cysteine residues (3). So far, Prx have been described as strictly depending on thiols (RSH) as reducing agents. Indeed, these enzymes have been characterized as peroxidases undergoing cycles of peroxide-dependent oxidation and thiol-dependent reduction during their catalytic activity (14), as shown in Reaction 1:

Although all Prx are assumed to be thiol-specific antioxidant enzymes, the 1-Cys Prx mechanism is not fully understood (reviewed in refs. 3 and 14). An important point to be clarified is the nature of the biological reductant of 1-Cys Prx, which remains unknown in most cases. Here, we present results showing unequivocally that 1-Cys Prx are reduced by ascorbate, and thus advance a change in the thiol-specific antioxidant paradigm. Because ascorbate is present at high-millimolar level in cells, it may be the long-sought-after biological reductant of 1-Cys Prx. Finally, our characterization of the peroxiredoxin (Prx) sulfenic acid (Cys-SOH)/ascorbate pair may open perspectives in the understanding of cellular redox processes.

Results

Ascorbate Peroxidase Activity of Yeast 1-Cys Prx. Although Prx are assumed to be peroxidases strictly dependent on thiols for regeneration of the reduced forms, we observed that a mitochondrial 1-Cys isoform from *Saccharomyces cerevisiae*, yPrx1, protected glutamine synthetase (GS) from inactivation in the presence of ascorbate. Protection was dose-dependent on yPrx1, and ascorbate and DTT were similarly effective as electron donors (Fig. 1A). The enzyme also prevented strand breaks in supercoiled plasmid DNA submitted to a similar oxidizing system (data not shown), indicating that yPrx1 is capable of protecting biomolecules by using ascorbate as reductant. The ability of yPrx1 to consume reducing equivalents from ascorbate was confirmed by the ascorbate oxidase assay, which is based on the ability of this enzyme to catalyze oxygen consumption in amounts proportional to ascorbate concentrations [see supporting information (SI) Fig. 4].

We tested other yeast Prx, all of which were expected to be oxidized to disulfide bonds during their catalytic cycles (2-Cys Prx), but none of them protected GS from inactivation in the presence

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

The authors declare no conflict of interest.

Abbreviations: Prx, peroxiredoxin(s); RSH, thiol; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOH, sulfenic acid; RS⁻, thiolate group; Cys_p, peroxidatic cysteine; Cys_r, resolving cysteine; GS, glutamine synthetase; DTT, dithiothreitol; t-BOOH, *tert*-butyl hydroperoxide; NEM, *N*-ethylmaleimide.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0700481104/DC1.

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Fig. 1. Characterization of yPrx1 ascorbate-dependent peroxidase activity. (A–C) Glutamine synthetase (GS) protection from inactivation by metal-catalyzed oxidation systems containing DTT (10 mM) (gray bars) or ascorbate (10 mM) (white bars) in 15-min incubations at 37°C. GS activity was considered 100% when it was not preincubated with metal-catalyzed oxidation systems. (A) GS protection by increasing yPrx1 concentrations. (B) Inability of other yeast Prx enzymes to use ascorbate. Tsa1, gi: 464970; Tsa2, gi: 2499475; or Ahp1, gi: 1709682. (C) Inhibition of yPrx1 protective effects by inactivation of cysteine residues. yPrx1 was modified by *N*-ethylmaleimide (NEM) and by site-specific mutagenesis as described in *Experimental Procedures*. Afterward, a GS protection assay was performed as described above by using yPrx1 at 3 μ M or in excess of 70 μ M, indicated by *. (D) Deconvoluted MS spectra of yPrx1 in different oxidation states treated with dimedone (140 amu). Samples of reduced yPrx1 (initial), treated with H₂O₂ and dimedone (oxidized) or treated with H₂O₂, ascorbate, and dimedone (ascorbate) were prepared and analyzed as described in *Experimental Procedures*. Molecular masses were calculated by the Transform algorithm of the BioLynx Data Analysis package of MassLynx Software (Micromass). Data were acquired in an ESI-Q-TOF (Micromass) mass spectrometer under positive ionization mode. Calculated molecular masses are indicated at the right side of the corresponding spectra. These spectra were obtained by using a recombinant yPrx1 (100 μ M) without the 40 first amino acids residues and containing only one cysteine residue, which is Cys_p (see supporting information on the PNAS web site for more details). All excess reagents were washed away by ultrafiltration before activity analysis.

of ascorbate (Fig. 1B). Therefore, this ascorbate-dependent property is a particular feature of yPrx1 that is not displayed by any of the tested yeast 2-Cys Prx.

Although the above assays showed that yPrx1 uses ascorbate as reductant, all of them are indirect. Therefore, dehydroascorbate, ascorbate, and peroxide concentrations were determined directly in the presence of DTPA to prevent metal-catalyzed processes. From the data of several experiments summarized in Table 1, it became clear that yPrx1 catalyzed the consumption of 1 mol of peroxide and 1 mol of ascorbate to produce 1 mol of dehydroascorbate. Thus, stoichiometric data (Table 1 and [SI Fig. 5](#)) indicated that yPrx1 catalyzes Reaction 2:

The amount of ascorbyl free radical produced in the incubations was monitored by electron spin resonance (ESR) and found to be in the nM range (SI Fig. 6). This is well below the amount of ascorbate consumed and of dehydroascorbate produced, both

$\approx 100 \mu\text{M}$. Because the ascorbyl free radical has a relatively long lifetime (18), our data indicated that yPrx1 ascorbate peroxidase activity is a two-electron process (SI Figs. 5 and 6) similar to the thiol-dependent activity.

More H₂O₂ than *tert*-butyl hydroperoxide (*t*-BOOH) was removed by the ascorbate-dependent catalysis (Table 1), similar to the yPrx1 thiol-dependent peroxidase activity described by Pedrajas *et al.* (19). Nevertheless, independent of the peroxide nature, yPrx1 was capable of reducing it by consuming a nonthiol reductant. These results led us to verify whether yPrx1 would also accept electrons from other reductants. We tested both thiolic (glutathione, β -mercaptoethanol, and DTT) and nonthiolic reductants (trolox, which is a hydrophilic analogue of vitamin E, and urate). Of all the low-molecular-weight compounds tested, only thiols and ascorbate supported the yPrx1 activity (SI Fig. 7).

Characterization of Amino Acid Residues Involved in 1-Cys Prx Ascorbate-Dependent Catalysis. Because the thiol-dependent per-

Hydrogen Peroxide-Dependent Catalysis. Because the iron-dependent peroxidase activity of yPrx1 relies on a reactive cysteine residue, the

Table 1. Stoichiometry of the yPrx1 ascorbate-dependent peroxidase activity

Reaction time, min	Initial peroxide, 1 mM	Peroxide consumption, μM	Ascorbate consumption, μM	DHA produced, μM	Peroxide/ascorbate	Ascorbate/DHA
15 min	H_2O_2	156 ± 10	150 ± 20	150 ± 20	1.04 ± 0.14	1.0 ± 0.1
45 min	$t\text{-BOOH}$	90 ± 15	98 ± 10	85 ± 25	0.92 ± 0.20	1.2 ± 0.3

The results are average of three independent experiments using HPLC analyses and ascorbate oxidase assay. Peroxides were measured by the FOX2 method. DHA, dehydroascorbate; initial ascorbate, 1 mM; γPrx1, 50 μM.

importance of cysteine residues in ascorbate-dependent GS protection was investigated by alkylating the sulfhydryl groups with *N*-ethylmaleimide (NEM). Protection of GS was inhibited by NEM, implicating yPrx1 cysteine residues as the site of peroxide reduction during the ascorbate-dependent activity. Furthermore, mutation of the Cys_p fully abolished DTT and ascorbate peroxidase activity, indicating that this residue is directly involved in peroxide reduction mediated by both reductants (Fig. 1C).

The results presented so far suggest that protection of biomolecules is due to yPrx1 peroxidase activity that depends on its reactive cysteine residue and ascorbate (Table 1 and Fig. 1A–C). To characterize the mechanism of ascorbate-dependent activity, the redox state of the Cys_p was analyzed by spectrophotometric and mass spectrometry methods in the presence of NBD-Cl (a thiol and sulfenic acid reagent) and dimedone (a specific sulfenic acid reagent), respectively (see also *Experimental Procedures*). The results showed that the catalytic cysteine residue was oxidized to sulfenic acid upon peroxide exposure and was rereduced upon ascorbate treatment (Fig. 1D and SI Fig. 8). To further test the hypothesis that active-site sulfenic acid is reduced by ascorbate, we examined the peroxidase activity of yPrx1 modified with dimedone. Abolishment of the yPrx1 capability to reduce H₂O₂ was observed only when the enzyme was previously oxidized and then incubated with dimedone. Treatment of reduced yPrx1 with dimedone in all other conditions (sample treated with DTT and sample oxidized then treated with DTT or ascorbate) resulted in no activity loss, suggesting that the Cys_p sulfenic acid derivative was reduced by both reductants (SI Fig. 9).

Next, we explored which specific features of 1-Cys Prx would enable them, and not 2-Cys Prx, to use reducing equivalents from ascorbate. One obvious difference between the two main mechanisms of Prx is the presence or absence of the Cys_r (2-Cys and 1-Cys Prx, respectively). The simple absence of Cys_r might predispose the sulfenic acid of 1-Cys Prx to ascorbate reduction, whereas the disulfide bond in 2-Cys Prx would be resistant. To test this hypothesis, we analyzed whether a recombinant yeast 2-Cys Prx (Tsa1-gi: 464970) whose Cys_r was mutated to a serine residue (Tsa1-C170S) acquired the ascorbate-dependent activity. An absent Cys_r provoked the appearance of a small ascorbate peroxidase activity (Fig. 2A), and the sulfenic acid derivative was detectable spectrophotometrically even after ascorbate addition (Fig. 2B). Therefore, the sulfenic acid form of Tsa1-C170S was only partially reduced by ascorbate (Fig. 2B).

Several Prx showing 1-Cys mechanism belong to the PrxVI group, which comprises proteins sharing amino acid sequence similarity (3). PrxVI proteins have a highly conserved histidine residue (SI Fig. 10) in addition to an arginine (fully conserved in all Prx) in the active site (20), probably creating a positively charged environment. This histidine is absent in 2-Cys Prx. We hypothesized that these two features, i.e., the absence of Cys_r and the presence of histidine, would render 1-Cys Prx capable of receiving electrons from ascorbate. The absence of Cys_r should increase the half-life of the sulfenic acid form of the Cys_p, whereas the presence of a positively charged histidine would further improve the sulfenic acid stabilization and favor the interaction with negatively charged molecules such as ascorbate. Therefore, we introduced a histidine residue in a position equivalent to the 1-Cys Prx group. The single addition of histidine in Tsa1 (Tsa1-I40H) did not transform it into an ascorbate peroxidase (Fig. 2A). This is probably related to the fast reaction of sulfenic acid with Cys_r, because no Cys_p-SOH was detected (Fig. 2B). Only when two mutations were introduced into Tsa1 (C170S and I40H) did this 2-Cys Prx acquire an ascorbate-dependent activity comparable with the DTT-dependent one (Fig. 2A). Furthermore, a sulfenic acid derivative was detectable in the double mutant, and it was rereduced by ascorbate (Fig. 2B). The ascorbate peroxidase activity of the Tsa1 double mutant was considerably lower than that of the natural 1-Cys Prx enzyme (yPrx1), indicating that additional structural features are required.

Fig. 2. The 2-Cys Prx engineered for acquiring the ascorbate-dependent peroxidase activity. (A) GS protection assay was used to measure DTT- (gray bars) and ascorbate-dependent (white bars) peroxidase activity of the Tsa1 mutants. Each enzyme (50 μ M) was incubated as described in the Fig. 1 legend. (B) Analysis of the oxidation state of cysteine residues from Tsa1 and the mutants C170S, I40H, and C170S-I40H by the NBD-Cl assay. Treatments and assays were performed as described in *Experimental Procedures*. Sulfhydryl and sulfenic acid adducts peak at 420 nm and 347 nm, respectively, with similar extinction coefficients (46).

Accordingly, the structures available so far in the Protein Data Bank suggest that 1-Cys Prx maintain the secondary structure near the active domain throughout the catalytic cycle, whereas the 2-Cys Prx active sites undergo an unfolding during the oxidation step (SI Fig. 11 and ref. 3). In principle, it is possible that any Prx might acquire ascorbate peroxidase activity if the appropriate amino acid changes are made.

Our results suggest that ascorbate probably supports the peroxidase activity of 1-Cys Prx because it can fully reduce sulfenic acids, but not disulfides, in a positively charged environment. Little is known about the reaction of ascorbate with oxidized forms of thiols to prove or disprove this view. In fact, we found no reported second-order rate constants for ascorbate reacting with disulfides or sulfenic acids. Nevertheless, ascorbate does not reduce the intermolecular disulfide bond of 2-Cys Prx (21) and reduces the low molecular disulfide DTNB only slowly. In our hands, reaction of ascorbate (100 mM) with DTNB (500 μ M) for 24 h at room temperature produced only 15 μ M of reduced DTNB. In contrast, DTNB reacts very fast with thiols (22). Furthermore, reduction of known proteinic and nonproteinic disulfide bonds by ascorbate is thermodynamically unfavorable (see *Thermodynamic Considerations on the Reaction of Ascorbate with Disulfides* in SI Text), but the sulfenic acid formed in glyceraldehyde 3-phosphate dehydrogenase has been reported to be reduced by ascorbate (23).

Ascorbate Peroxidase Is a Conserved Activity in 1-Cys Prx. The identity of most 1-Cys Prx electron donors are unknown, and the yPrx1 studied here is an exception because its peroxidase activity has been shown to be supported by a mitochondrial thioredoxin

Table 2. Comparison of 1-Cys Prx enzymes ascorbate- and DTT-dependent activities

1-Cys Prx protein	Species	Relative peroxidase activity (DTT/ascorbate)
yPrx1	<i>S. cerevisiae</i>	1.9 ± 0.1
Prdx6	<i>R. norvegicus</i>	0.8 ± 0.1
rPf1-Cys-Prx	<i>P. falciparum</i>	1.05 ± 0.15
AtPER1	<i>A. thaliana</i>	0.93 ± 0.21
Dpx-2540	<i>D. melanogaster</i>	0.8 ± 0.14
Dpx-6005	<i>D. melanogaster</i>	1.1 ± 0.17
AhpE	<i>M. tuberculosis</i>	0.73 ± 0.2

The FOX2 assay was used to monitor the peroxidase activity (enzyme concentration, 50 μ M; peroxide, 400 μ M; DTPA, 0.5 mM; Hepes, 50 mM; DTT or Ascorbate, 500 μ M). The results are the average of three independent experiments.

system (19). yPrx1 belongs to the PrxVI group, whose members share structural similarities, with most of them acting by the 1-Cys Prx catalytic mechanism (3). Ascorbate should also support the peroxidase activity of other PrxVI proteins because they are likely to produce stable sulfenic acid intermediates, because of the absence of a Cys_r and the presence of a histidine in the active site. Supporting this scenario, all tested PrxVI from different taxonomic groups displayed ascorbate peroxidase activity (Table 2). Therefore, the ability of ascorbate to support the peroxidase activity of 1-Cys Prx is a highly conserved property. In contrast, tested 2-Cys enzymes from diverse organisms (Ohr from *Xylella fastidiosa*, PDB code 1ZB8; 2-Cys Prx from *Crinipellis perniciosa*, ID DQ813344; and OsmC from *Escherichia coli*, PDB ID 1QW1) did not reduce peroxides in the presence of ascorbate.

We also tested the ascorbate peroxidase activity of AhpE from *Mycobacterium tuberculosis*, which shares moderate sequence identity ($\approx 25\%$) to PrxVI proteins and presents the 1-Cys Prx mechanism (24). AhpE does not possess the PrxVI conserved histidine (SI Fig. 10) but is capable of decomposing peroxides at the expense of ascorbate (Table 2). Interestingly, its active site environment contains several positively charged residues (Arg-53, His-77, Lys-78, Arg-116, Lys-133, and Arg-139) that may play the role attributed to the conserved histidine.

Next, we analyzed the enzymatic activity of Prdx6, a 1-Cys Prx from rat, in more detail by monitoring ascorbate oxidation at 265 nm (Fig. 3). Substrate consumption was continuously monitored, providing more reliable values of reaction rates than the FOX assay. Applying the bisubstrate kinetic approach and assuming a ping-pong mechanism (25), we found no evidence of enzyme saturation by ascorbate in the used range of concentrations (Fig. 3A). Because ascorbate possesses a very high absorption coefficient ($\epsilon_{265} = 14,500 \text{ M}^{-1} \text{ cm}^{-1}$), it could not be added at concentrations $> 80 \mu\text{M}$. H_2O_2 concentrations were then varied under fixed ascorbate concentrations. In this case, a trend of enzyme saturation was observed (Fig. 3B) permitting a nonlinear fit through the Michaelis–Menten equation (Prism 4 for Windows, GraphPad Software, San Diego, CA). Thus, different $V_{\text{max app}}$ values were estimated and plotted against the reciprocal of the ascorbate concentration to provide the kinetic parameters shown in Fig. 3C.

Because the used ascorbate concentrations were well below the $K_{\text{M(ascorbate)}}$ (760 μM) due to experimental constraints, the performed kinetic analysis presents a high degree of uncertainty. Nevertheless, the estimated $K_{\text{M(ascorbate)}}$ value suggests that ascorbate can support Prdx6 activity *in vivo* because its intracellular concentration is in the millimolar range (2). Remarkably, the catalytic efficiency of the ascorbate-dependent activity estimated here [$k_{\text{cat}}/K_{\text{M(ascorbate)}}$ $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{\text{cat}}/K_{\text{M(H}_2\text{O}_2\text{)}}$ $0.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$] is comparable with those previously reported for thiol-dependent processes (26). Thus, the nonthiol peroxidase activity is likely to be a major process. The ascorbate-dependent activity of

Fig. 3. Enzymatic and biological investigations of the ascorbate-dependent peroxidase activity of rat Prdx6. (A–C) Enzymatic activity was monitored by ascorbate consumption at 265 nm in incubations containing Prdx6 (0.5 nM), H_2O_2 and ascorbate at the concentrations specified in the figures. (D) The ability of rat homogenates to support the peroxidase activity of Prdx6 was investigated before and after treatment with ascorbate oxidase as described in *Experimental Procedures*. The 100% value means the amount of H_2O_2 consumed during 15 min at 37°C by each homogenate in the absence of ascorbate oxidase pretreatment. The bars represent the percentage of H_2O_2 consumed when homogenates were pretreated with ascorbate oxidase.

yPrx1 from yeast was also monitored by the $A_{265\text{nm}}$ decay. However, because yPrx1 is less efficient than its mammalian counterpart, it was not possible to determine the individual kinetic parameters with confidence. Nevertheless, it was possible to estimate that the catalytic efficiencies of yPrx1 [$k_{\text{cat}}/K_{\text{M(ascorbate)}}$ and $k_{\text{cat}}/K_{\text{M(H}_2\text{O}_2\text{)}}$] are on the order of 10^4 and $10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively (data not shown). These values are also comparable with those of thiol-dependent processes (19). The detailed characterization of the ascorbate peroxidase activity of 1-Cys Prx will require novel kinetic approaches.

The biological relevance of the 1-Cys Prx activity was also indicated by experiments performed with homogenates of rat organs. As anticipated from the high content of proteinic and nonproteinic reductants in these extracts, they sustained H_2O_2 consumption that depended on Prdx6. Remarkably, however, H_2O_2 consumption consistently decreased when ascorbate was depleted by pretreatment of the homogenates with ascorbate oxidase (Fig. 3D), suggesting a major role for ascorbate in Prdx6 reduction.

Discussion

The 2-Cys Prx are involved in several physiological processes, probably because they modulate the intracellular level of H_2O_2 , an oxidant with signaling properties (14). In comparison with 2-Cys Prx, 1-Cys Prx have been little studied, partly because their biological reductant remains unknown, except for a few described exceptions (19, 26). Indeed, most 1-Cys Prx are not efficiently reduced by thioredoxin, glutaredoxin, or glutathione. Therefore, the data presented here indicated that ascorbate is a strong candidate to support 1-Cys Prx peroxidase activity *in vivo*. Furthermore, our work provides some mechanistic details about 1-Cys Prx catalysis.

The physiological relevance of the ascorbate-dependent peroxidase activity in the yeast *S. cerevisiae* may be questionable. As mentioned before, this microorganism possesses a mitochondrial thioredoxin system capable of reducing yPrx1 (19). Furthermore, *S. cerevisiae* does not synthesize ascorbate; it synthesizes an analog, erythroascorbate, whose antioxidant role remains controversial (27). At this point, it is difficult to compare the relevance of erythroascorbate with the thioredoxin system because their relative

abundance in yeast has yet to be determined. In any case, our results show that ascorbate and thioredoxin have similar catalytic efficiencies [$k_{\text{cat}}/K_{\text{M}}(\text{ascorbate}) \approx 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{\text{cat}}/K_{\text{M}}(\text{H}_2\text{O}_2) \approx 10^5 \text{ M}^{-1} \text{ s}^{-1}$].

Although it is premature to reach a conclusion about the biological relevance of the ascorbate-dependent peroxidase activity in yeast, the results obtained with mammalian enzymes favor such relevance. Despite the limitations of the assays used to monitor enzyme activity, it was possible to conclude that the ascorbate-dependent peroxidase activity of Prdx6 is comparable with the thiol-dependent process (Fig. 3 A–C). Furthermore, ascorbate depletion considerably decreased the ability of rat homogenates to support Prdx6 peroxidase activity (Fig. 3D). This is consistent with the fact that ascorbate is present at millimolar levels in most mammalian tissues (reviewed in ref. 2). It has been recently shown that 1-Cys Prx from mammals can heterodimerize with π -glutathione transferase and accept electrons from glutathione (reviewed in ref. 26). Our results indicate that ascorbate is also a significant source of reducing power for these enzymes (Fig. 3). Further studies are necessary to establish the relative importance of ascorbate and glutathione as physiological electron donors for mammalian 1-Cys Prx. Interestingly, ascorbate depletion in the rat homogenates resulted in different levels of a drop in Prdx6 activity (Fig. 3D). Among other reasons, these differences may be due to the endogenous ascorbate content of each organ, either intrinsic or that depend on the level of redox-active transition metal ions capable of promoting ascorbate oxidation (2).

It is also significant that ascorbate in mammalian tissues, especially in blood plasma, may serve as reductant for 1-Cys Prx from pathogens. Several pathogens have to cope with oxidative stress generated by phagocytic cells in blood. Diverse microorganisms such as *Plasmodium falciparum* and *Trypanosoma cruzi* possess no catalase or selenocysteine-dependent glutathione peroxidase and, therefore, rely on Prx to decompose peroxides (28).

An ascorbate-dependent peroxidase activity of 1-Cys Prx should be also relevant for plants (Table 2) because these organisms present very high levels of vitamin C in all subcellular compartments, particularly in chloroplasts, where 50 mM concentration can be attained (29). Consistent with these findings, intracellular redox homeostasis in plants is governed not only by GSH/GSSG ratios but also by ascorbate levels (30).

The product of the ascorbate-supported peroxidase activity of 1-Cys Prx, dehydroascorbate, is immediately reduced to ascorbate in the intracellular media, where it accumulates at millimolar levels (reviewed in ref. 2). Several mechanisms have been proposed to account for this fast reduction, such as chemical reaction with biothiols (31, 32) and enzymatic processes that depend on NADPH and glutathione (2). Glutaredoxin has often been considered an important dehydroascorbate reductase whose catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) ranges from 10^4 to $10^7 \text{ M}^{-1} \text{ s}^{-1}$ (33, 34). However, other enzymes have also been considered to be relevant (35–39). Most likely, intracellular reduction of dehydroascorbate is a very fast reaction because many of these pathways operate together.

In conclusion, our studies extended previous observations about the redox versatility of Prx. All previously reported Prx reductants are thiol groups of low-molecular-weight substances or of proteins (thioredoxins and sulfiredoxins). Thus, the ascorbate-mediated reduction of protein sulfenic acids reported here represents a modification of the peroxiredoxin-thiol-specific antioxidant paradigm. In addition, our results reveal a previously uncharacterized antioxidant function for ascorbate. Of note, both sulfenic acids and ascorbate have been proposed in cell signaling (29, 40). The ascorbate/sulfenic acid pair may operate in the case of other proteins, representing an aspect of redox biochemistry that has yet to be explored *in vivo*. Thus, our results open perspectives in the understanding of cellular redox processes.

Experimental Procedures

Standard Reaction Buffer and Sample Preparation. The standard mixture for most experiments was Hepes (50 mM), pH 7.5, containing azide (1 mM) and DTPA (0.5 mM). All enzymes were previously reduced with DTT (100 mM) overnight at 4°C. Proteins were subsequently gel filtered (PD10; Amersham Pharmacia, Piscataway, NJ) to eliminate excess DTT. This procedure was performed immediately before the experiments to obtain homogeneously reduced samples.

GS Protection Assay. GS activity was measured by the γ -glutamyl-transferase assay essentially as described (41). GS protection was evaluated by the amount of γ -glutamyltransferase activity remaining in solution after metal-catalyzed oxidation. GS oxidative inactivation was carried out as described (42, 43). The reagent concentrations used were DTT or ascorbate (10 mM), GS (0.2 mg/ml), and FeCl_3 (3 μM) in Hepes (50 mM), pH 7.5, containing azide (1 mM). The incubation conditions were 15 min at 37°C. Remaining GS activity was measured by adding 1 ml of assay solution containing ADP (0.4 mM), glutamine (150 mM), Na_2HAsO_4 (10 mM), NH_2OH (20 mM), MnCl_2 (0.4 mM), and Hepes (0.1 M), pH 7.4. The reaction was incubated for 15 min at 37°C and then stopped by addition of 0.2 ml of FeCl_3 (55 mg/ml) in HCl (6 M). Absorbance of the γ -glutamylhydroxamate- Fe^{3+} complex was spectrophotometrically measured at 540 nm. The concentrations of tested enzymes are described in the corresponding figure legends. GS activity was considered 100% when the enzyme was preincubated in the absence of metal-catalyzed oxidation systems. “No Prx” is the negative control establishing the extent to which the metal-catalyzed oxidation system inactivated the GS without the antioxidant enzymes.

Mass Spectrometry. Accurate molecular mass determination was performed by mass spectrometry. Measurements were taken in positive ionization mode on a Q-TOF Ultima API fitted with an electrospray ion source (Micromass, Manchester, U.K.). The protein was solubilized in NH_4HCOOH (50 mM) and directly injected into the instrument by using a Rheodyne 7010 sample loop coupled to a LC-10A VP Shimadzu pump at a constant flow rate (20 $\mu\text{l}/\text{min}$). Instrument control and data acquisition were conducted by a MassLynx 4.0 data system (Micromass), and the experiments were performed by scanning from a mass-to-charge ratio (m/z) of 50–1,800 by using a scan time of 2 seconds applied during the entire infusion. The mass spectra corresponding to the total ion current (TIC) chromatogram were averaged, allowing an accurate molecular mass determination. External calibration of the mass scale was performed with NaI.

Ferrous Oxidation Xylenol Orange (FOX2) Assay. Peroxide concentration was determined by this method as described (44). Reaction mixtures containing standard reaction buffer with various enzyme concentrations (indicated in the legend or abscissa axis of the figures) and peroxide (500 μM) (H_2O_2 or *tert*-butyl hydroperoxide), ascorbate or DTT (1 mM) were incubated at 37°C for the indicated time. Reactions were stopped by addition of HCl at a final concentration of 0.2 M. Samples containing ascorbate were incubated for an additional 15 min with 1 unit of ascorbate oxidase (Sigma) at room temperature before ferrous xylenol orange solution addition. This was required because an excess of ascorbate reduces Fe^{+3} , interfering with peroxide quantitation. Absorbance due to the nonenzymatic reaction, i.e., standard reaction buffer plus peroxide and reductant, was subtracted from the absorbance corresponding to the whole reaction mixture. Furthermore, appropriate controls were performed in each experiment (positive control, standard reaction buffer plus peroxide; negative Prx controls, standard reaction buffer plus peroxide and Prx without addition of any reductant).

NBD-Cl Assay. The 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reacts with both sulfhydryl (-SH) and sulfenic acid (-SOH) to generate products that can be distinguished by the UV-visible absorption spectra. Sulfhydryl adducts of NBD-Cl peak at 420 nm ($\epsilon = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$) (45). Sulfenic acid of NBD-Cl peaks at 347 nm, with a similar extinction coefficient (46).

Modification of Proteins with Thiol or Sulfenic Acid Reagents. The proteins were previously reduced with DTT (100 mM) overnight in standard reaction buffer. Excess DTT was removed by gel filtration through a PD-10 column (Amersham Pharmacia). An aliquot, named "initial," was taken from the reduced protein and added to 10 equimolar NBD-Cl or 1,000 equimolar 5,5-dimethyl-1,3-cyclohexanedione (dimedone). Then, the reduced protein was subsequently treated with 1.5 equimolar of H_2O_2 for 30 min at room temperature under anaerobic conditions (in the standard reaction buffer). An aliquot of this sample was treated with 10 equimolar NBD-Cl or 1,000 equimolar dimedone and named "oxidized." Finally, the residual oxidized protein was treated with ascorbate (10 mM) or DTT (10 mM) for 1 h at room temperature; the excess reductant was washed away by ultrafiltration, and the protein was incubated with a 10-fold excess of NBD-Cl or 1,000-fold excess of dimedone. This final sample was named "ascorbate." In all samples, excess NBD-Cl or dimedone were removed by ultrafiltration. Other modification was performed by incubation of reduced yPrx1 with a 100-fold excess of NEM for 3 h at room temperature. The excess NEM was washed away by ultrafiltration (by using a Microcon YM10; Millipore).

Protein Expression and Purification. yPrx1 and all other Prx used in this work were purified from bacteria by specific affinity chromatography as described by the respective researcher who kindly provided us with each one of the 1-Cys Prx overexpression vectors (described in *Experimental Procedures in SI Text*): yPrx1 (*S. cerevisiae*), yeast recombinant enzyme in fusion with T7-tag for immunoaffinity purification; AhpE (*M. tuberculosis*), recombinant enzyme in fusion with histidine tag for nickel-affinity purification; ArPer 1 (*A. thaliana*), recombinant enzyme in fusion with biotin; rPfl-Cys-Prx (*P. falciparum*), recombinant enzyme in fusion with GST-tag, purified by using a GST-Glutathione Affinity System with the AKTA Prime Liquid Chromatography system (Amersham Biosciences); Dpx-2540 and Dpx-6005 (*D. melanogaster*), recombinant proteins in fusion with histidine tags for nickel-affinity chro-

matography purification; and Prdx6 (*Rattus norvegicus*), native protein purified by anion exchange (mono Q; Amersham Biosciences) followed by cation exchange (carboxymethyl Sepharose fast flow column; Amersham Biosciences). All the enzymes were analyzed by SDS/PAGE to determine their purity and integrity.

Enzymatic Kinetic Parameters. Ascorbate peroxidase activities from yPrx1 (1 nM) and Prdx6 (0.5 nM) were assayed in a standard reaction mixture. The reaction was started with addition of H_2O_2 (200 μM) when ascorbate concentration varied from 10 to 80 μM . Ascorbate oxidation was monitored spectrophotometrically ($\epsilon_{265} = 14,500 \text{ M}^{-1} \text{ cm}^{-1}$) at 37°C.

Peroxide Consumption by Prdx6 in the Presence of Rat Homogenates. The soluble fraction of rat kidney, pancreas, liver, and brain (*R. norvegicus*, strain: Sprague-Dawley) were obtained through potter homogenization of the organs in cold PBS. The homogenates were centrifuged for 45 min at 4°C and $16,000 \times g$. Purified Prdx6 (10 μM) was incubated with H_2O_2 (300 μM), and the rat organ homogenates (90 μg of protein per ml) were used as reductants. Consumption of H_2O_2 ($\approx 150 \mu\text{M}$) by Prdx6 was measured by FOX assay during 15 min at 37°C. In identical incubation conditions, homogenates were treated with ascorbate oxidase (1 unit) to deplete the reducing power derived from ascorbate, and these treated extracts were used as reductants of Prdx6. The effects of ascorbate oxidase or of extracts alone were analyzed as controls, and, in these conditions, minimal consumption was detected.

We thank S. Kawazu (Research Institute, International Medical Center of Japan, Tokyo, Japan), A.B. Fisher (University of Pennsylvania School of Medicine, Philadelphia, PA), R. B. Aalen (University of Oslo, Oslo, Norway), B. G. Guimarães (Brazilian Synchrotron Light Laboratory, São Paulo, Brazil), and W. C. Orr (Southern Methodist University, Dallas, TX) for kindly providing expression clones; Earl R. Stadtman and Barbara Berlett (National Heart, Lung, and Blood Institute, National Institutes of Health) who kindly provided glutamine synthetase purified in their laboratory; Walter S. Sheppard, Marilene Demasi, and Maria C. Arias for helpful suggestions and critical reading of this manuscript; Dr. M. A. de Oliveira, Dr. E. Linares, Dr. A. J. Kowaltowski, and C. C. Caldeira for technical advice and assistance; S. Marana for helpful discussions about enzymatic procedures; and J. Hesson for revision of English grammar. This research was supported by grants from Fundação de Amparo a Pesquisa no Estado de São Paulo and Conselho Nacional de Pesquisas, as part of the Instituto do Milênio Redoxoma.

1. Imlay JA (2003) *Annu Rev Microbiol* 57:395–418.
2. Rumsey SC, Levine M (1998) *J Nutr Biochem* 9:116–130.
3. Wood ZA, Schroder E, Harris JR, Poole LB (2003) *Trends Biochem Sci* 28:32–40.
4. Konig J, Baier M, Horling F, Kahmann U, Harris G, Schurmann P, Dietz KJ (2002) *Proc Natl Acad Sci USA* 99:5738–5743.
5. Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA (2003) *Nature* 424:561–565.
6. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE, III, Stover CK (1996) *Science* 272:1641–1643.
7. Park SH, Chung YM, Lee YS, Kim HJ, Kim JS, Chae HZ, Yoo YD (2000) *Clin Cancer Res* 6:4915–4920.
8. Chung YM, Yoo YD, Park JK, Kim YT, Kim HJ (2001) *Anticancer Res* 21:1129–1133.
9. Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, Park HS, Kim KY, Lee JS, Choi C, et al. (2005) *Nature* 435:347–353.
10. Vivanco AP, Castillo EA, Biteau B, Nicot C, Ayte J, Toledano MB, Hidalgo E (2005) *Proc Natl Acad Sci USA* 102:8875–8880.
11. Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, et al. (2004) *Cell* 117:625–635.
12. Biteau B, Labarre J, Toledano MB (2003) *Nature* 425:980–984.
13. Budanov AV, Sablina AA, Feinstein E, Koonin EV, Chumakov PM (2004) *Science* 304:596–600.
14. Rhee SG, Chae HZ, Kim K (2005) *Free Radical Biol Med* 38:1543–1552.
15. Demasi AP, Pereira GAG, Netto LES (2006) *FEBS J* 273:805–816.
16. Huang ME, Rio AG, Nicolas A, Kolodner RD (2003) *Proc Natl Acad Sci USA* 100:11529–11534.
17. Rand JD, Grant CM (2006) *Mol Biol Cell* 17:387–401.
18. Zee JVD, Broek PJA (1998) *Free Radical Biol Med* 25:282–286.
19. Pedrajas JR, Miranda-Vizuet A, Javanmard N, Gustafsson JA, Spyrou G (2000) *J Biol Chem* 275:16296–16301.
20. Choi HJ, Kang SW, Yang CH, Rhee SG, Ryu SE (1998) *Nat Struct Biol* 5:400–406.
21. Chae HZ, Chung SJ, Rhee SG (1994) *J Biol Chem* 269:27670–27678.
22. Ellman GL (1959) *Arch Biochem Biophys* 82:70–77.
23. You K-S, Benitez LV, McConachie WA, Allison WS (1975) *Biochim Biophys Acta* 384:317–330.
24. Li S, Peterson NA, Kim MY, Kim CY, Hung LW, Yu M, Lekin T, Segelke BW, Lott JS, Baker EN (2005) *J Mol Biol* 346:1035–1046.
25. Segel HI (1975) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (Wiley, New York), pp 606–621.
26. Manevich Y, Fisher AB (2005) *Free Radical Biol Med* 38:1422–1432.
27. Hancock RD, Galpin JR, Viola R (2000) *FEMS Microbiol Lett* 186:245–250.
28. Wilkinson SR, Kelly JM (2003) *Biol Chem* 384:517–525.
29. Smirnov N (2000) *Curr Opin Plant Biol* 3:229–235.
30. Pignocchi C, Foyer CH (2003) *Curr Opin Plant Biol* 6:379–389.
31. Guaiquil VH, Farber CM, Golde DW, Vera JC (1997) *J Biol Chem* 272:9915–9921.
32. Krauth-Siegel RL, Ludemann H (1996) *Mol Biochem Parasitol* 80:203–208.
33. Washburn MP, Wells WW (1999) *Biochem Biophys Res Commun* 257:567–571.
34. Shimaoka T, Miyake C, Yokota A (2003) *Eur J Biochem* 270:921–928.
35. Del Bello B, Maellaro E, Sugherini L, Santucci A, Comporti M, Casini AF (1994) *Biochem J* 304:385–390.
36. Ishikawa T, Casini AF, Nishikimi M (1998) *J Biol Chem* 273:28708–28712.
37. May JM, Mendiratta S, Hill KE, Burk RF (1997) *J Biol Chem* 272:22607–22610.
38. Wells WW, Xu DP, Yang YF, Roque PA (1990) *J Biol Chem* 265:15361–15364.
39. Whitbread AK, Masoumi A, Tetlow N, Schmuck E, Coggan M, Board PG (2005) *Methods Enzymol* 401:78–99.
40. Poole LB, Karplus PA, Claiborne A (2004) *Annu Rev Pharmacol Toxicol* 44:325–347.
41. Stadtman ER, Smyrniotis PZ, Davis JN, Wittenberger ME (1979) *Anal Biochem* 95:275–285.
42. Kim K, Rhee SG, Stadtman ER (1985) *J Biol Chem* 260:15394–15397.
43. Levine RL (1983) *J Biol Chem* 258:11828–11833.
44. Jiang ZY, Hunt JV, Wolff SP (1992) *Anal Biochem* 202:384–389.
45. Birkett DJ, Price NC, Radda GK, Salmon AG (1970) *FEBS Lett* 6:346–348.
46. Ellis HR, Poole LB (1997) *Biochemistry* 36:15013–15018.

ANEXO 2

RESEARCH ARTICLE

Open Access

The promoter of filamentation (POF1) protein from *Saccharomyces cerevisiae* is an ATPase involved in the protein quality control process

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Abstract

Background: The gene *YCL047C*, which has been renamed promoter of filamentation gene (*POF1*), has recently been described as a cell component involved in yeast filamentous growth. The objective of this work is to understand the molecular and biological function of this gene.

Results: Here, we report that the protein encoded by the *POF1* gene, Pof1p, is an ATPase that may be part of the *Saccharomyces cerevisiae* protein quality control pathway. According to the results, $\Delta pof1$ cells showed increased sensitivity to hydrogen peroxide, *tert*-butyl hydroperoxide, heat shock and protein unfolding agents, such as dithiothreitol and tunicamycin. Besides, the overexpression of *POF1* suppressed the sensitivity of $\Delta pct1$, a strain that lacks a gene that encodes a phosphocholine cytidyltransferase, to heat shock. *In vitro* analysis showed, however, that the purified Pof1p enzyme had no cytidyltransferase activity but does have ATPase activity, with catalytic efficiency comparable to other ATPases involved in endoplasmic reticulum-associated degradation of proteins (ERAD). Supporting these findings, *co-immunoprecipitation* experiments showed a physical interaction between Pof1p and Ubc7p (an ubiquitin conjugating enzyme) *in vivo*.

Conclusions: Taken together, the results strongly suggest that the biological function of Pof1p is related to the regulation of protein degradation.

Keywords: unfolded protein response, endoplasmic reticulum stress, antioxidant response

Background

Cells possess several mechanisms to control the quality of their components, such as proteins [1]. One of these mechanisms ensures proper folding and function of proteins, sending misfolded proteins to be degraded by the ubiquitin-proteasome system and represents the best characterized protein quality control process [2-4]. In the lumen of endoplasmic reticulum (ER), one relevant protein quality control mechanism operates, where misfolded proteins are recognized by ER chaperones and some of them are eventually translocated to the cytosol, in the interface with the ER membrane. Finally, the degradation of non-functional proteins can take place by

the ubiquitin-proteasome system in a process known as ER-associated degradation (ERAD) [2-4].

The importance of protein quality control mechanisms is evident if it is taken into account that as much as 30% of all nascent polypeptides are misfolded [5,6]. E3 ubiquitin ligases are associated with ribosomes to degrade proteins with aberrant folds, which mean that several proteins can be degraded during translation [7]. Therefore, it is not surprising that several mutants of genes encoding critical proteasome subunits are lethal. Remarkably, accumulation of misfolded proteins is implicated with several human diseases, especially neurodegenerative illnesses that are associated with protein aggregates [8-10].

Proteins that enter the secretory pathway are directed to the ER, where their folding and post-translational modifications occur. However, when the processing capacity of the ER is overwhelmed, misfolded proteins accumulate in

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this compartment, which triggers a cell defense mechanism known as the unfolded protein response (UPR). The UPR is mediated by the Ire1p, an RNase, which is activated when misfolded proteins accumulate in the ER lumen. Activated Ire1p removes an inhibitory intron from the *HAC1* mRNA, which, in turn, is efficiently translated. Hac1p is a transcription factor responsible for activating genes related to ERAD. To accommodate the accumulation of misfolded proteins until their degradation or their homeostatic recovery, the transcription factors Opi1p and Opi3p (*overproducer of inositol 1 and 3 proteins*) are responsible for controlling the expression of genes involved in expansion of the ER membrane, especially genes encoding proteins that are involved in lipid synthesis [11-14].

Three well-characterized ERAD pathways are present in yeast: ERAD-L, -M and -C, depending on the site of the misfolded lesion. Proteins whose misfolded domains are located in the ER lumen are targeted to ERAD-L, whereas proteins with misfolded membrane domains are directed to ERAD-M and proteins with defective domains on the cytoplasmic side of the ER membrane are degraded by the ERAD-C pathway. Therefore, when a protein is misfolded in the ER lumen or membrane, it is transported to the cytoplasm, polyubiquitinated and subsequently degraded by the proteasome (for a review on this process, see [15]).

The ERAD-C pathway is mainly composed by the E3 ubiquitin ligase Doa10p and its associated protein complex. The Doa10p complex is small when compared to the other two ERAD pathway complexes [2]. In addition to Doa10p (the scaffold membrane protein), the Doa10p complex contains Ubc7p (an E2 ubiquitin conjugating enzyme), its anchoring protein Cue1p and the ATPase complex Cdc48, which is composed of the AAA-ATPase Cdc48p, the cofactors Ufd1p and Npl4p and the complex anchorage protein Ubx2p [2].

Some studies describe a post-ER system of protein quality control, which would occur at the Golgi compartment. This system was suggested to be used in addition to the ERAD pathway upon saturation of the ERAD system by misfolded proteins [16,17]. Only recently, Wang and Ng (2010) characterized a substrate dependent on post-ER Golgi quality control, the protein Wsc1p, which is a transmembrane protein that functions as a sensor of plasma membrane/cell wall integrity [18]. Thus, the description of this quality control process and determination of its specific substrates represented a breakthrough since a novel biological function, *i.e.* degradation of proteins, was revealed.

Here, we show that Pof1p, a protein that was recently reported as a filamentation promoter protein [19], is an ATPase that is likely involved in the protein degradation pathway. The expression of *POF1* gene was able to

suppress the sensitivity of Δ *pct1* strain (mutant for a phosphocholine cytidyltransferase enzyme) to heat shock; however, the Pof1p enzyme possesses no cytidyltransferase activity but does have ATPase activity. Some studies have related membrane lipid biosynthesis with the ERAD pathway ([20]; reviewed by [21]) and wide-scale studies of protein-protein physical interactions found Pof1p in complex with Doa10p [22], Ubc7p (Database of Interacting Proteins (DIP), 2010) and Nas2p [23]. Doa10p and Ubc7p are components of the ERAD-C pathway [1], and Nas2p is a protein involved in proteasome assembly [24]. Taken together, the data suggest that the biological function of Pof1p is related to protein quality control.

Results

We were interested to identify deletion mutant strains for genes with unknown functions that might be sensitive to oxidative stress. Therefore, several yeast strains were exposed to hydrogen peroxide (H₂O₂) or *tert*-butyl hydroperoxide (*t*-BOOH). Among them, Δ *pof1* (*YCL047C* ORF was named *POF1* due to its involvement in yeast filamentation process [19]) was highly sensitive to these oxidants (Figure 1).

To get insights on the involvement of Pof1 in the antioxidant cell response, a series of bioinformatics analysis were performed (Protein Information Resource (PIR) site, the UniProt Consortium <http://pir.georgetown.edu/cgi-bin/ipcEntry?id=S19376>, and the Munich Information Center for Protein Sequences (MIPS) site <http://mips.helmholtz-muenchen.de/genre/proj/yeast/searchEntryAction.do?text=YCL047C>, indicating that the *POF1* gene may belong to the cytidyltransferase family. Therefore, the primary sequence of *POF1* was aligned with the amino acid sequence of the most studied phosphocholine cytidyltransferase protein in yeast, *PCT1*, the rate-limiting enzyme in the phosphatidylcholine synthesis pathway, which is a major membrane lipid component. Also, human isoforms of choline (ct human) or ethanolamine (et human) cytidyltransferases amino acid sequences were aligned with *POF1* (Figure 2A). Although the overall similarity among sequences was low (around 10%), the conserved motif HxxH [25], which is characteristic of the active site of the cytidyltransferase family, was present in the predicted primary sequence of *POF1*.

Accordingly, to investigate the hypothesis that Pof1p is a cytidyltransferase, the biological complementation assay of the *PCT1* mutant strain was performed by overexpressing *POF1* in cells challenged with heat shock stress because Δ *pct1* is sensitive to this stress [26]. Overexpression of *POF1* was able to reverse the heat shock sensitivity of the Δ *pct1* strain (Figure 2B), suggesting that Pof1p and Pct1p share a common function. Indeed, as Δ *pct1* cells, the Δ *pof1* strain was highly sensitive to heat

Figure 1 *Δpof1* cells are sensitive to oxidative stress. A representative viability assay showing cells exposed to hydrogen peroxide (H_2O_2) or *tert*-butyl hydroperoxide (*t*-BOOH) on rich solid media (YPD). The cells (collected at stationary phase) were diluted to $OD_{600\text{ nm}} = 0.2$, followed by 4 serial dilutions of 5X. A total of 5 μ L of each dilution were spotted on the plates, which were incubated at 30°C for 48 h and photographed.

shock. Moreover, overexpression of *POF1* also partially rescued the wild type phenotype in *Δpof1* strain.

Pure, recombinant Pof1p was obtained in the soluble fraction (Figure 3A), and Pof1p was assayed for phosphocholine or phosphoethanolamine cytidyltransferase activities. Intriguingly, *POF1* did not hydrolyse CTP as analyzed by thin layer chromatography (TLC), but instead it displayed ATPase activity (Figure 3B). The ATPase activity was independent of the presence of phospholipid precursors in the reaction media, indicating that Pof1p was not interacting with these substrates, at least when hydrolyzing ATP. The reaction products were also analyzed by mass spectrometry, but no CDP-choline

or CDP-ethanolamine could be detected (data not shown).

Since the ability of Pof1p to complement Pct1p function during heat shock is not related to CDP-choline activity, the hypothesis that Pof1p participates in some protein quality control was tested. Cells were submitted to ER stress, by exposing them to high concentrations of dithiothreitol (DTT) and tunicamycin (a protein glycosylation inhibitor). Both agents are well known to provoke accumulation of unfolded proteins in the ER. *Δpof1* cells displayed higher sensitivity to ER stress agents than wild-type cells and *Δubc7* cells (mutant strain which lacks *UBC7* gene which encodes ubiquitin conjugating enzyme

involved in ERAD, a control cell line [27]) (Figure 4), suggesting that Pof1p is involved in UPR. Besides, Pof1p presented an ATPase-specific activity of 5 nmol of released phosphate per hour per μM enzyme (Figure 5A). This activity is comparable to the ATPase activity of the ERAD-associated protein Kar2p (approximately 4 nmol of phosphate release per hour per μM of Kar2p in complex with both the nucleotide exchanger Lhs1p and the DnaJ-like partner) [28], indicating that this enzymatic process is relevant *in vivo*.

Interestingly, using the bioinformatics tool PIPE 2 (Protein-Protein Interaction Prediction Engine, freely available at <http://pipe.cgmlab.org/>), with the default cutoff of 0.06 (sensitivity = 57% and specificity = 89%), we could predict an interaction between the Kar2p ATPase and Pof1p [29]. At a lower cutoff of 0.04 (sensitivity = 70% and specificity = 83%), an interaction between Pof1p and Cdc48p was predicted, which is the ATPase present in all types of ERAD pathways [2,29]. As a positive control, Pof1p and Kss1p interaction was predicted using the default cutoff of 0.06. This is in agreement with experimental data showing through transcriptome data that this mitogen-activated

protein kinase (MAPK) (involved in signal transduction pathways that control filamentous growth and pheromone response) interacted with *POF1* [19]. As a negative control, the ATPase from vacuole VMA10 was not predicted to be an interacting partner with Pof1p, even using a lower cutoff of 0.01 (sensitivity = 92% and specificity = 47%).

To validate these *in silico* protein-protein interactions predictions, anti-Pof1p rabbit polyclonal antibodies were produced. The interactions between Pof1p with Doa10p and with Ubc7p, two components of the ERAD pathway, were investigated, since these complexes were previously described [22]. The physical interaction between Nas2p and Pof1p could not be investigated because there is no commercially available Nas2p antibody. Doa10p and Pof1p did not co-immunoprecipitate under the cell growth conditions tested (data not shown); however, we did observe a physical interaction between Ubc7p and Pof1p *in vivo* (Figure 5B). The migration of the band relative to the Ubc7p and Pof1p corresponded to the sum of the molecular mass of the two proteins. Since *Western blot* was performed in denaturing conditions (after SDS-PAGE) the

Figure 3 Pof1p purification and activity analyses. (A) SDS-PAGE showing the purification of recombinant Pof1p obtained through metal affinity chromatography. Lane 1: molecular weight standard; subsequent lanes were different fractions obtained during the elution process. (B) Thin layer chromatography analyses to observe Pof1p ATP transferase activity; the controls were included to assay for alterations in CTP and ATP. See the Materials and Methods section for details.

band depicted with asterisk might be observed due to the formation of a mixed disulfide bond between Pof1p and Ubc7p. Pof1p possesses six cysteine residues. Probably the concentrations of DTT (1 mM) employed were too low to reduce the mixed disulfide between Pof1p and Ubc7p.

Taking advantage of the anti-Pof1p antibody, the Pof1p sub-cellular distribution was studied. A punctuated Pof1p distribution was observed in wild-type cells (Figure 6), which was more evident in $\Delta pct1$ cells. This is in agreement with higher protein expression of Pof1p in $\Delta pct1$ cells, which was also observed by Western blotting (data not shown), suggesting a compensatory response. Based on previous immunocytochemistry studies [30], we speculate that Pof1p localizes to the Golgi compartment.

Discussion

The first suggestion that the *POF1* gene was related to the protein quality control response arose from wide-scale studies about the relationship between the ERAD and UPR systems [20]. Indeed, mRNA levels of *POF1* gene were significantly increased in cells that were treated with ER stress agents (DTT and tunicamycin), and this induction was dependent on both Ire1p and Hac1p. In addition, a proteasome inhibitor (PS-341) provoked a four-fold induction of *POF1* gene expression [31].

Furthermore, the expression of *POF1* gene is repressed in the $\Delta opi1$ strain [20], suggesting an involvement of Pof1p with membrane and protein metabolism. The viability data presented here are in agreement with this idea, especially when considering the fact that all stressful conditions tested (oxidative, heat shock, and ER stress in Figures 1, 2 and 4) are well known to provoke protein misfolding. Yet, oxidative stress and heat shock (Figures 1 and 2) caused the most severe phenotypes in $\Delta pof1$ cells, which is likely due to the fact that these stresses damage both membrane and protein homeostasis [32,33]. The fact that *POF1* overexpression was able to complement the function of *PCT1* in $\Delta pct1$ cells during heat shock (Figure 2) and its expression levels by Opi1p [20] suggests the involvement of Pof1p in membrane lipid metabolism. In addition, the levels of Pof1p are augmented in $\Delta pct1$ cells (Figure 6 and western blot analyses - data not shown), which indicated that Pof1p might at least partially backing up Pct1p.

However, the molecular function of Pof1p could not be directly related to membrane lipid synthesis although the protein displayed ATPase activity (Figure 3). Pof1p ATPase activity was efficient enough to occur *in vivo*, and its specific activity was comparable to Kar2p, an Hsp70 chaperone from the ER that reaches its maximum activity (approximately 4 nmol of released phosphate per

Figure 4 $\Delta pof1$ cells are sensitive to protein unfolding. The cells from the stationary phase of growth were diluted to $\text{OD}_{600 \text{ nm}} = 0.2$, followed by 4 serial dilutions of 5X. A total of 5 μL of each dilution were spotted on SD complete media plates containing no unfolding agent, DTT or tunicamycin. The plates were incubated at 30°C for 48 h and photographed.

hour per μM of Kar2p) when in complex with co-chaperones Sec63p (a soluble protein that contains a J-domain) and Lhs1p [28]. Pof1p ATPase activity was also comparable with p97, the mammal homolog of yeast Cdc48p, which is the main ERAD ATPase [34,35]. As indicated by PIPE 2 bioinformatics analyses Pof1p is predicted to

interact with others proteins involved in ERAD, such as Kar2p and Cdc48p.

In addition to viability and activity results indicating that Pof1p is involved in protein quality control, protein-protein interactions studies in wide-genome scale indicated the participation of Pof1p as a component of

Figure 5 ATPase activity of Pof1p and its physical interaction with Ubc7 (ubiquitin conjugase 7) protein. (A) Hydrolysis of ATP as measured by the PiPer™ Phosphate Assay Kit (Invitrogen). (-) ATP represents the result of the reaction in the absence of ATP; (-) POF1 represents the result of the spontaneous ATP hydrolysis in the absence of Pof1p; the lane labeled "Pof1p + ATP" contains the complete reaction. The assays were performed at 37°C for 1 h. (B) Western blot analyses of Ubc7p using the commercial antibody Ube2G2 (Abcam). The fractions were obtained from co-immunoprecipitation assays using Pof1p polyclonal antibody from the following protein extract: WT = wild type; Δ pct1 and Δ pof1. The asterisk shows the Pof1p-Ubc7p complex. CE = total soluble wild type cell extract; IgG LC = IgG light chain. The arrow points Ubc7p dimer.

the ubiquitin-proteasome pathway. Hesselberth *et al.* (2006) described the Doa10p-Pof1p complex using protein microarray technology, whereas The DIP site and Genemania Fast Gene Function Predictions tool (September 2nd, 2010 database update) reported the Ubc7p-Pof1p interaction. Under our growth conditions of stationary growth phase and galactose-containing medium, we did not observe Doa10p-Pof1p co-immunoprecipitation (data not shown); however, under the same growth conditions, we detected an Ubc7p-Pof1p interaction (Figure 5B).

Still taking advantage of a polyclonal Pof1p antibody produced in this study, a punctuated Pof1p cell distribution was observed (Figure 6) that is very similar to proteins localized in the Golgi compartment [30]. Although these results are preliminary, the immunocytochemical data clearly showed that Pof1p is not uniformly distributed in the cytoplasm and does not co-localize with the nucleus or mitochondria where DNA is stained with DAPI (see merged figure, Figure 6). Since ER protein distribution is expected to be perinuclear, Pof1p probably was not located in this organelle.

The post-ER Golgi protein quality control pathway has already been reported, and at least one specific substrate of this system has been characterized [36]. Taken together, the results suggest that Pof1p is an ATPase that interacts with the ubiquitin conjugating protein (an E2) Ubc7p and protects cells from accumulating misfolded proteins caused by oxidative, heat, reductive or chemically (tunicamycin) stressful conditions. A possible explanation for the functional relationship between Pct1p and Pof1p could be due to the participation of Pof1p in protein quality control. For instance, the autophagy system controls the turnover of the majority of stable proteins and coordinates degradation through the engulfment of these polypeptides into a double-lipid bilayer - the autophagosome - which fuses with a lysosome/vacuole in which degradation occurs [37]. Given that Δ pct1 cells have deficient membrane lipid turnover [38], which probably results in lower membrane repositioning during autophagy, the ER expansion would be impaired. In this situation, an increase in Pof1p levels, together with several other proteins, would improve the proteasomal degradation process.

The results presented here, together with previously published data, allow us to speculate that Pof1p is part of the ERAD pathway as an E3 ubiquitin ligase. Pof1p may be involved in substrate recognition during ubiquitin marking because it interacts physically with an E2 ubiquitin conjugating enzyme, Ubc7p, and it is important in the unfolded protein response. $\Delta pof1$ cells were more sensitive to reductive stress than the $\Delta ubc7$ cells (cells in which Ubc7p is absent), this last a well-characterized protein that participates in the ERAD-C pathway. A possible substrate would be the MAP kinase molecule Kss1p, which interacts physically with Pof1p [19]. As mentioned above, Kss1p is a kinase involved in the control of filamentous growth and the pheromone response.

Fasolo *et al.* (2011) observed that $\Delta pof1$ cells are defective in invasive growth and pseudohyphal growth. We hypothesize that the phenotype observed in $\Delta pof1$ cells is due to the absence of stability regulation of Kss1p exerted by Pof1p.

Therefore, the results described here showed that a protein involved in the yeast-to-hyphal transition [19] possesses ATPase activity and is important in the response of yeast to various stresses. A study on gene expression modulation during yeast filamentous-form growth showed an enriched number of genes involved in protein quality control, such as N-linked glycosylation, ubiquitin-dependent protein catabolism and ER to Golgi transport. Moreover, this study pinpointed the

26S proteasome as an important component in the regulation of *S. cerevisiae* filamentous growth [39]. The yeast-to-hyphal transition is a response of several fungi to stressful conditions. For the majority of pathogenic fungi, this transformation is an essential step in their infectious process, and modifications in plasma membrane and cell wall constituents have been implicated [40,41]. The mechanisms that trigger the transition to filamentous growth in *S. cerevisiae* are associated with carbon or nitrogen stresses [39,42]. The interplay between the filamentation process and protein quality control may be an important feature that deserves to be further investigated.

Conclusions

This study characterized the molecular function of Pof1p as an ATPase involved in protein quality control. Pof1p was important to yeast defense against oxidative, heat shock and chemically induced stress. Several protein quality control components are still poorly described, despite their importance in neurological diseases. The molecular characterization of the components in yeast can be useful to understand the function of conserved human proteins.

Methods

Chemicals: t-BOOH, tunicamycin and DTT were purchased from Sigma Chemical Company (St. Louis, MO, USA). The other chemicals used were analytical grade or better. H₂O₂ (30%) was obtained from Merck.

Yeast strains and growth conditions: The yeast strains used here were obtained from the Yeast Deletion Clones repository (Invitrogen - Carlsbad, CA, USA). The wild-type strain was BY4741 (*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*); the mutant strains were Δ pof1 (*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YCL047C::kanMX4*); Δ pct1 (*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGR202C::kanMX4*); Δ ubc7 (*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR022W::kanMX4*). The yeast cells were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose), YPGAL (1% yeast extract, 2% peptone and 2% galactose) or complete synthetic medium (0.17% yeast nitrogen base (YNB), 0.5% ammonium sulfate, all required amino acids plus 2% glucose). SD = synthetic dextrose medium. For most analyses, when yeast strains were grown on glucose or galactose, the cells were harvested by centrifugation at stationary phase, which corresponds to an OD_{600 nm} between 2.0 and 5.0.

Viability assays: The tolerance of yeast cells to H₂O₂ or to t-BOOH was determined by the spot test, as described below. Inoculates were obtained from cells that were grown overnight in YPD or complete synthetic media with 2% glucose (indicated in the figures). Inoculates were diluted to OD_{600 nm} = 0.2, and yeasts were grown until cell density reached stationary phase

(around 16 h). Finally, the cell cultures were diluted again to OD_{600 nm} = 0.2, and then four subsequent 1:5 dilutions of these cell suspensions were performed. A 5 μ L droplet of each dilution was plated onto YPD or complete synthetic medium (SD) plus agar with the stress agent. Peroxides were added to plates at the concentrations indicated in the figures. DTT or tunicamycin was spread onto the plates just before use. To test cell viability under heat shock conditions, the strains were grown until cell density reached OD_{600 nm} = 0.8, and they were divided into two aliquots, which were incubated at 30°C (control) or 37°C. The serial dilutions (starting from OD_{600 nm} = 0.2) were spotted onto YPD agar plates, and the plates were incubated for 48 h at 30°C.

Construction of yeast overexpression vector pYES-TOPO + *POF1*: The coding region of *POF1* gene was cloned from yeast genomic DNA using the following specific primers: *POF1* forward 5'TGCTGTCACATATGAA-GAAGAC and *POF1* reverse 5'TAAACGGATCCTCAA TCAAATATTG, which contain *NdeI* or *BamHI* restriction enzyme sites adaptors, respectively (underlined sequences). This PCR-isolated DNA fragment was purified with the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Uppsala, Sweden) and ligated into the pYES-TOPO backbone to form pYES-TOPO + *POF1* for yeast expression (controlled by *GAL1* promoter) and into the pET15b vector to generate pET15b + *POF1* for bacterial expression (controlled by T7 promoter). The *POF1* gene was added to pYES2.1-TOPO TA (Invitrogen) reaction media according to the manufacturer. The ligation product was transformed into *Escherichia coli* DH5 α bacteria strain by electroporation. The transformed clones were grown in LB + ampicillin (100 μ g/mL), and the plasmids were isolated with the Illustra plasmidPrep Mini Spin Kit (GE Healthcare). The clones were analyzed for proper directionality of the insertion by PCR using the TOPO *GAL* forward primer and the *POF1* reverse primer. The correct plasmids were sequenced and transformed into the respective yeast strains by electroporation [43].

Heterologous expression and purification of recombinant Pof1p: Recombinant Pof1p, which possesses an N-terminal His-tag, was expressed in the *E. coli* BL21 (*DE3*) strain that was transformed with the pET15b-Pof1p plasmid (the *POF1* coding region was cloned into the expression vector pET15b from Novagen using the *NdeI* and *BamHI* restriction sites). The cells were cultured (50 mL) overnight in LB + ampicillin (100 μ g/mL), transferred to 1 L of fresh LB + ampicillin medium and cultured further until the OD_{600 nm} reached 0.6-0.8. IPTG was added to a final concentration of 1 mM. After 3 h of incubation at 37°C, the cells were harvested by centrifugation. The pellet was washed and suspended in the start buffer composed of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl and 20 mM

imidazole. The cells were sonicated twice for 45 s (40% amplitude), followed by 30 s on ice between sonications using a Branson Cell Disruptor. The cell extracts were kept on ice during streptomycin sulfate treatment (1% for 20 min), and the suspension was centrifuged at 16,000 g for 30 min to remove nucleic acid precipitates and cell debris. Finally, the extracts were applied to a Hi-trap nickel-affinity column (Life Technologies). The conditions for protein purification were optimized using the gradient procedure for imidazole concentration described by the manufacturer.

Thin Layer Chromatography (TLC) analyses: The assays were performed as previously described [44]. Briefly, the reaction media contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 20 μM phosphatidylcholine:oleate vesicles, 10 mM DTT, 1.5 mM phosphocholine (or 2 mM phosphoethanolamine), 1 μg/μL (20 μM) Pof1p and 200 μCi/μmol of [α-³²P]CTP or [α-³²P]ATP. The reactions were incubated at 37°C overnight in the presence of [α-³²P]CTP or 2 h in the presence of [α-³²P]ATP. Controls were subjected to the same conditions in the absence of Pof1p. The reactions were analyzed by TLC at room temperature using silica gel plates (Merck) with a solvent system composed of ethanol/NH₄OH (1:1). The plates were autoradiographed, and the resulting bands were compared with [α-³²P]CTP or [α-³²P]ATP without any incubation or addition of enzyme.

ATPase activity. The reactions containing 1 mM ATP, 1 μM Pof1p, 5 mM MgCl₂ and 100 mM Tris-HCl (pH 7.5) were incubated at 37°C for 1 h. Subsequently, the reactions were boiled for 5 min and centrifuged for 10 min at 16,000 g. The P_iPer Phosphate assay mix was added to the supernatant according to the manufacturer's instructions (Molecular Probes - Invitrogen). The reactions were incubated at 37°C for an additional 1 h in the dark. The absorbance of resorufin, the Amplex Red reagent reaction product, was detected by its absorbance at 565 nm. A calibration curve with known concentrations of inorganic phosphate was used to quantify the P_i released during the ATPase reactions. Controls without Pof1p and without substrate (ATP) were subjected to the same conditions.

Co-immunoprecipitation assays: Wild type, *Δpct1* and *Δpof1* cells were grown until stationary phase in synthetic galactose complete medium. The cells were centrifuged and washed with 1X phosphate-buffered saline (PBS). The cells were lysed using glass beads in lysis buffer (50 mM Hepes (pH 7.5), 5 mM EDTA, 150 mM NaCl, 300 mM KCl, 1% Triton X-100, 2 mM PMSF, 5% glycerol and 20 mM β-mercaptoethanol). The insoluble fraction was separated by centrifugation at 16,000 g for 30 min and 4°C. The soluble fraction was incubated with a Dynabead-anti-Pof1p complex overnight at room temperature under gentle agitation. The complexed

proteins were washed three times using the washing buffer provided by the Dynabeads Protein G kit (Invitrogen), and the samples were eluted using 20 μL of elution buffer (provided in the kit), incubated for 10 min at 70°C in 10 μL of 5X protein SDS-PAGE loading buffer and 1 mM DTT (recommended 10 mM). One-third of each sample was subjected to western blot analyses.

Western blot analyses: Immunoblot analyses were performed using rabbit polyclonal antibodies against Pof1p produced in this study by immunization with pure recombinant Pof1p. The commercial antibodies from Abcam were used to study Doa10p (mouse monoclonal antibody to MARCH6 (ab56594)) and Ubc7p (rabbit polyclonal antibody to Ube2G2 (ab97279)). Proteins were transferred to nitrocellulose, and the processing of nitrocellulose blots was performed using the BioRad system. The HRP and luminol-based reagent from ECL (Amersham GE Healthcare) was used as a detection system. The membranes were autoradiographed using Amersham Hyperfilm and photo-documented.

Acknowledgements

We would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

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Authors' contributions

IMC, THTN performed the majority of the experiments. SM and FMP carried out TLC and mass spectrometry analyses. MD and RMPN executed the antibody production and immunocytochemistry studies. GM and LESN have made substantial contributions to conception and design, analysis and interpretation of data. All authors have been involved in drafting the manuscript or revising it critically for important intellectual content.

Competing interests

The authors declare that they have no competing interests.

Received: 31 August 2011 Accepted: 28 December 2011

Published: 28 December 2011

References

- Leidhold C, Voos W: Chaperones and proteases—guardians of protein integrity in eukaryotic organelles. *Ann N Y Acad Sci* 2007, **1113**: 72-86.
- Carvalho P, Goder V, Rapoport TA: Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 2006, **126**:361-373.
- Denic V, Quan EM, Weissman JS: A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell* 2006, **126**:349-359.
- Carvalho P, Stanley AM, Rapoport TA: Retrotranslocation of a misfolded luminal ER protein by ubiquitin-ligase Hrd1p. *Cell* 2010, **143**:579-591.
- Turner GC, Varshavsky A: Detecting and measuring cotranslational protein degradation *in vivo*. *Science* 2000, **289**:2117-2120.

6. Schubert U, Antón LC, Gibbs J, Norbury CC, Yewdell JW, Binnik JR: *Nature* 2000, **404**:770-774.
7. Bengtson MH, Joazeiro CAP: **Role of a ribosome-associated E3 ubiquitin ligase in protein quality control.** *Nature* 2010, **467**:470-473.
8. Tsai YC, Weissman AM: **The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer.** *Genes Cancer* 2010, **1**:764-778.
9. Douglas PM, Dillin A: **Protein homeostasis and aging in neurodegeneration.** *J Cell Biol* 2010, **190**:719-729.
10. Bedford L, Lowe J, Dick LR, Mayer RJ, Brownell JE: **Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets.** *Nat Rev Drug Discov* 2011, **10**:29-46.
11. Gardner RG, Shearer AG, Hampton RY: **In vivo action of the HRD ubiquitin ligase complex: mechanisms of endoplasmic reticulum quality control and sterol regulation.** *Mol Cell Biol* 2001, **21**:4276-4291.
12. Schuck S, Prinz WA, Thorn KS, Voss C, Walter P: **Membrane expansion alleviates endoplasmic reticulum stress independently of the unfolded protein response.** *J Cell Biol* 2009, **187**:525-536.
13. Wilson JD, Thompson SL, Barlowe C: **Yet1p-Yet3p interacts with Scs2p-Opi1p to regulate ER localization of the Opi1p repressor.** *Mol Biol Cell* 2011, **22**:1430-1439.
14. Rubio C, Pincus D, Korennykh A, Schuck S, El-Samad H, Walter P: **Homeostatic adaptation to endoplasmic reticulum stress depends on Ire1 kinase activity.** *J Cell Biol* 2011, **193**:171-184.
15. Ismail N, Ng DT: **Have you HRD? Understanding ERAD is DOAble!** *Cell* 2006, **126**:237-239.
16. Haynes CM, Caldwell S, Cooper AA: **An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport.** *J Cell Biol* 2002, **158**:91-101.
17. Spear ED, Ng DT: **Stress tolerance of misfolded carboxypeptidase Y requires maintenance of protein trafficking and degradative pathways.** *Mol Biol Cell* 2003, **14**:2756-2767.
18. Philip B, Levin DE: **Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1.** *Mol Cell Biol* 2001, **21**:271-280.
19. Fasolo J, Sboner A, Sun MG, Yu H, Chen R, Sharon D, Kim PM, Gerstein M, Snyder M: **Diverse protein kinase interactions identified by protein microarrays reveal novel connections between cellular processes.** *Genes Dev* 2011, **25**:767-778.
20. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P: **Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation.** *Cell* 2000, **101**:249-258.
21. Pineau L, Ferreira T: **Lipid-induced ER stress in yeast and β cells: parallel trails to a common fate.** *FEMS Yeast Res* 2010, **10**:1035-1045.
22. Hesselberth JR, Miller JP, Golob A, Stajich JE, Michaud GA, Fields S: **Comparative analysis of *Saccharomyces cerevisiae* WW domains and their interacting proteins.** *Genome Biol* 2006, **7**:R30.
23. Hart GT, Lee I, Marcotte ER: **A high-accuracy consensus map of yeast protein complexes reveals modular nature of gene essentiality.** *BMC Bioinformatics* 2007, **8**:236.
24. Saeki Y, Kudo T, Kawamura H, Tanaka K: **Multiple proteasome-interacting proteins assist the assembly of the yeast 19S regulatory particle.** *Cell* 2009, **137**:900-913.
25. Veitch DP, Gilham D, Cornell RB: **The role of histidine residues in the HXGH site of CTP:phosphocholine cytidyltransferase in CTP binding and catalysis.** *Eur J Biochem* 1998, **255**:227-234.
26. Howe AG, Zarembek V, McMaster CR: **Cessation of growth to prevent cell death due to inhibition of phosphatidylcholine synthesis is impaired at 37 degrees C in *Saccharomyces cerevisiae*.** *J Biol Chem* 2002, **277**:44100-44107.
27. Biederer T, Volkwein C, Sommer T: **Role of Cue1p in ubiquitination and degradation at the ER surface.** *Science* 1997, **278**:1806-1809.
28. Steel GJ, Fullerton DM, Tyson JR, Stirling CJ: **Coordinated activation of Hsp70 chaperones.** *Science* 2004, **303**:98-101.
29. Pitre S, Dehne F, Chan A, Cheetham J, Duong A, Emili A, Gebbia M, Greenblatt J, Jessulat M, Krogan N, Luo X, Golshani A: **PIPE: a protein-protein interaction prediction engine based on the re-occurring short polypeptide sequences between known interacting protein pairs.** *BMC Bioinformatics* 2006, **7**:365.
30. Michelsen K, Schmid V, Metz J, Heusser K, Liebel U, Schwede T, Spang A, Schwappach B: **Novel cargo-binding site in the beta and delta subunits of coatomer.** *J Cell Biol* 2007, **179**:209-217.
31. Fleming JA, Lightcap ES, Sadis S, Thoroddsen V, Bulawa CE, Blackman RK: **Complementary whole-genome technologies reveal the cellular response to proteasome inhibition by PS-341.** *Proc Natl Acad Sci USA* 2002, **99**:1461-1466.
32. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, Kalyanaram B, Mutlu GM, Budinger GR, Chandel NS: **Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity.** *Proc Natl Acad Sci USA* 2010, **107**:8788-8793.
33. Hamanaka RB, Chandel NS: **Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes.** *Trends Biochem Sci* 2010, **35**:505-513.
34. Meyer HH, Kondo H, Warren G: **The p47 co-factor regulates the ATPase activity of the membrane fusion protein, p97.** *FEBS Lett* 1998, **437**:255-257.
35. Ye Y, Meyer HH, Rapoport TA: **Function of p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains.** *J Cell Biol* 2003, **162**:71-84.
36. Wang S, Ng DT: **Evasion of endoplasmic reticulum surveillance makes Wsc1p an obligate substrate of Golgi quality control.** *Mol Biol Cell* 2010, **21**:1153-1165.
37. Behrends C, Sowa ME, Gygi SP, Harper JW: **Network organization of the human autophagy system.** *Nature* 2010, **466**:68-76.
38. Dowd SR, Bier ME, Patton-Vogt JL: **Turnover of phosphatidylcholine in *Saccharomyces cerevisiae*. The role of the CDP-choline pathway.** *J Biol Chem* 2001, **276**:3756-3763.
39. Prinz S, Avila-Campillo I, Aldridge C, Srinivasan A, Dimitrov K, Siegel AF, Galitski T: **Control of yeast filamentous-form growth by modules in an integrated molecular network.** *Genome Res* 2004, **14**:380-390.
40. Rida PC, Nishikawa A, Won GY, Dean N: **Yeast-to-hyphal transition triggers formin-dependent Golgi localization to the growing tip in *Candida albicans*.** *Mol Biol Cell* 2006, **17**:4364-4378.
41. Wimalasena TT, Enjalbert B, Guillemette T, Plumridge A, Budge S, Yin Z, Brown AJ, Archer DB: **Impact of the unfolded protein response upon genome-wide expression patterns, and the role of Hac1 in the polarized growth, of *Candida albicans*.** *Fungal Genet Biol* 2008, **45**:1235-1247.
42. Colomina N, Ferrezuelo F, Vergés E, Aldea M, Garí E: **Whi3 regulates morphogenesis in budding yeast by enhancing Cdk functions in apical growth.** *Cell Cycle* 2009, **8**:1912-1920.
43. Ausubel FM, Brent R, Moore DD, Seidman JA, Smith JA, Struhl K: *Current Protocols in Molecular Biology* John Wiley & Sons, Inc., New York, NY; 1998, 13.0.3-13.13.7.
44. Sohal PS, Cornell RB: **Sphingosine inhibits the activity of rat liver CTP:phosphocholine cytidyltransferase.** *J Biol Chem* 1990, **265**:11746-11750.

doi:10.1186/1471-2180-11-268

Cite this article as: Costa et al.: The promoter of filamentation (POF1) protein from *Saccharomyces cerevisiae* is an ATPase involved in the protein quality control process. *BMC Microbiology* 2011 **11**:268.

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ANEXO 3

The essential gene *YMR134W* from *Saccharomyces cerevisiae* is important for appropriate mitochondrial iron utilization and the ergosterol biosynthetic pathway

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ARTICLE INFO

Article history:

Received 17 May 2013

Revised 4 July 2013

Accepted 9 July 2013

Available online 23 July 2013

Edited by Francesc Posas

Keywords:

Ergosterol biosynthesis

Iron homeostasis

Mitochondrial function

Antioxidant response

Essential gene

Uncharacterized gene

ABSTRACT

A thermosensitive strain (*YMR134W^{ts}*) of the essential gene *YMR134W* presented up to 40% less ergosterol, threefold lower oxygen consumption and impaired growth on respiratory conditions. The iron content in the mitochondrial fraction of *YMR134W^{ts}* cells was considerably low, despite these cells uptake and accumulate more iron from the culture media than wild-type cells. *YMR134W^{ts}* cells were also more susceptible to oxidative stress. The results suggest that *Ymr134wp* is essential to aerobic growth due to its function in ergosterol biosynthesis, playing a role in maintaining mitochondrial and plasma membrane integrity and consequently impacting the iron homeostasis, respiratory metabolism and antioxidant response.

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1. Introduction

In 1997, applying a random mutagenesis approach, Babcock et al. isolated a mutant that was named *bm-8*, which was unable to grow under low-iron-containing media. The expression of two genes, *YFH1* or *YMR134W*, in the *bm-8* background resulted in the complementation of the mutant phenotype back to the wild-type state. The group described that the gene responsible for allelic complementation of the low iron growth defect was *YMR134W*; however, they focused their studies on the *YFH1* gene (able to restore the wild-type state of *bm-8* only when over-expressed) because of its homology to the human frataxin gene [1]. Later on, several studies confirmed the hypothesis that yeast frataxin (*Yfh1p*) is a mitochondrial protein involved in iron homeostasis and it is now accepted as one of the components of the mitochondrial machinery involved in [Fe–S] cluster synthesis, acting either as an iron donor or as a chaperone interacting with desulfurase/

scaffold proteins involved in cluster [Fe–S] assembly [2]. In contrast, the other gene, *YMR134W*, was not further studied, but the *bm-8* mutant phenotype suggested that *YMR134W* may also be involved in iron metabolism.

Previously, genome-wide analyses produced more data about *YMR134W*: *Ymr134wp* is localized at the endoplasmic reticulum (ER) [3] and it is involved in mitochondria morphogenesis [4]. Later on, Snoek and Steensma showed that the gene *YMR134W* is essential when yeast is grown under aerobic conditions, but not in anaerobic ones [5]. Finally, Jerry Kaplan (2007), in a communication posted at the *Saccharomyces* Genome Database, renamed the gene *YMR134W* as *ERG29*, explaining that “Erg29p (*YMR134W* product) binds to Erg25p and affects its activity; *erg29* null mutant is non-viable in a respiratory competent background but is viable in a respiratory-defective background; *erg29* mutation is synthetically lethal with *mmt1* and *mmt2* null mutations.” [<http://www.yeastgenome.org/cgi-bin/locusHistory.pl?dbid=5000004741>]. Erg25p is a C-4 sterol methyl oxidase, responsible for catalyzing the conversion of 4,4-dimethylzymosterol to zymosterol, a precursor of ergosterol [6]. *MMT1* and *MMT2* are mitochondrial metal transporters [7], which further indicate the involvement of *YMR134W* in mitochondrial and iron metabolism. In the following year, Tarassov et al. in a genomic-wide study using a Protein-fragment

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Fig. 1. Ymr134wp is involved in ergosterol biosynthesis. Total ergosterol was extracted and quantified using the Sterol Quantitation Method (SQM) exactly as described by Arthington-Skaggs et al. (1999). *Expressed as a percentage of the wet weight of the cell (grown on YPD media). The graph represents the average and standard deviation ($\pm$ S.D.) from three independent experiments. The difference between mutant and wild-type was statistically significant for p -value < 0.01 (determined from paired t test) at both temperatures.

Fig. 2. *YMR134W^{ts}* cells have impaired growth on a respiratory carbon source (glycerol). SD-URA⁻ plates with indicated carbon source. The first spot on the left in each lane corresponds to 5 μ l of the cell suspension at $A_{600\text{ nm}} = 0.2$. Four serial 1:5 dilutions were performed from left to right. Figure representative of at least three independent experiments.

Complementation Assay, also observed a physical interaction between Erg25p and Ymr134wp [8], reinforcing the suggestion that the last protein could be involved in ergosterol synthesis.

Ergosterol is a major lipid component in yeast membranes that is associated with structural features such as fluidity and permeability, which are relevant for proper functioning of membrane proteins. Several steps in the ergosterol biosynthetic pathway are essential to yeast cell viability, and because of this, the major class of antifungal—the azoles—target their components [9]. Ergosterol and iron have a strict relationship because several key enzymes in ergosterol biosynthesis are iron-centered proteins. In particular, Erg25p is an oxo-diiron enzyme, essential to aerobic growth of yeast [6]. In addition, ergosterol synthesis is strictly dependent on aerobic growth conditions because twelve oxygen molecules are required for this process [10].

Seventeen years after the completion of the yeast genome sequence [11], more than 1600 genes do not have a function clearly assigned. One of them is *YMR134W* which is annotated as uncharacterized. In the present study, we took advantage of a thermosensitive strain (*YMR134W^{ts}*) to study the role of *YMR134W*, an essential gene in yeast. Here we show that Ymr134wp is involved in ergosterol biosynthesis and its decreased activity in a thermosensitive strain leads to the formation of functionally impaired mitochondria affecting the growth of yeast in a respiratory carbon

source (such as glycerol) and the oxygen consumption by the cells. The mutation also increased the iron uptake of the cells from the culture media, but it diminished the concentration of iron inside the mitochondria. Besides, *YMR134W^{ts}* cells were more sensitive to oxidative stress and presented increased catalase activity. Together, our data suggest that the phenotypes relating *YMR134W* to iron metabolism and mitochondria morphology and function are due to its role in ergosterol biosynthesis.

2. Materials and methods

2.1. Materials

2.1.1. Thermosensitive strain *YMR134W^{ts}* genotype

(*MATa*, *ura3 Δ 0*, *leu2 Δ 0*, *his3 Δ 1*, *lys2 Δ 0*, *met15 Δ 0*, *can1 Δ ::LEU2*, *MFA1pr::His3*, *ymr134w-ts::URA3*). The thermosensitive collection was kindly provided by Shay Ben-Aroya and Philip Hieter from the University of British Columbia, Canada [12]. The wild-type strain BY4741 (*MATa*, *ura3 Δ 0*, *leu2 Δ 0*, *his3 Δ 1*, *met15 Δ 0*) was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA) and it was transformed with empty vector pYES.2.1 TOPO-TA (Invitrogen) to confer to the wild-type strain the capacity to grow on media without uracil, such as *YMR134W^{ts}* cells.

2.1.2. Culture media

YPD (yeast extract 1%, peptone 2% and dextrose 2%), YEPG (yeast extract 1%, ethanol 2%, peptone 2% and glycerol 2%), YPGAL (yeast extract 1%, peptone 2% and galactose 2%) or SD-URA⁻ (Yeast Nitrogen Base without amino acids 0.7% + ammonium sulfate 0.5%, glucose 2% or glycerol 2%, DROPOUT 0.12% (mix of all amino acids, except leucine, uracil, tryptophan, and histidine), histidine 0.8%, leucine 0.8%, tryptophan 0.8%. Agar (2%) was added to solid media.

Hydrogen peroxide was obtained from Merck (Darmstadt, Germany); *tert*-butyl hydroperoxide and most of other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.1.3. Viability assay conditions

YMR134W^{ts} and wild-type cells growth analyses on different carbon sources or exposed to oxidative agents. Cells were initially inoculated in a 5 ml SD-glucose URA⁻ at 25 °C, overnight, at 180 rpm (Innova 42R NEW BRUNSWICK SCIENTIFIC, Eppendorf, Hamburg, Germany). Then cells were diluted on 50 ml fresh media (YPD, YEPG or SD-glucose URA⁻, specifically indicated in the figure legends) at an optical density ($OD_{600\text{ nm}}$) equal to 0.2 and then incubated in the permissive temperature (25 °C) for 4 h. The cells were then divided into two equal aliquots and incubated further at 25 °C or at non-permissive temperature (37 °C) overnight (precisely 16 h). The $OD_{600\text{ nm}}$ was corrected to 0.2 in sterile water and an additional four serial dilutions of fivefold were carried out. An aliquot of 5 μ l of each cell dilution was dropped into solid SD-URA⁻ media (glucose 2% or glycerol 2% + glucose 0.1% as carbon source) containing or not (control) hydrogen peroxide (H_2O_2 0.5 mM) or *tert*-butyl hydroperoxide (0.75 mM). The plates were incubated at permissive temperature for 3 days and photographed (Major Science-UVDI-312; Saratoga, CA, USA).

2.1.4. Quantitation of ergosterol

The amount of ergosterol in the cells was spectrophotometrically determined, exactly as described by Arthington-Skaggs et al. The method takes advantage of the unique spectral absorption pattern produced by sterols in the range 240–300 nm [13].

2.1.5. Cellular iron uptake measurement

Cells were grown as described above in YPD media. The total iron of the media was obtained from YPD before cell inoculation. The iron content was measured colorimetrically using a TPTZ re-

Fig. 3. Impaired oxygen consumption in *YMR134W^{ts}* cells. Cells were grown on YEPG media for 16 h harvested by centrifugation, resuspended in buffer containing sucrose/ethanol as respiratory substrates, and the oxygen uptake was measured using Clark electrodes for at least 30 min. The graph represents the average and standard deviation ($\pm$ S.D.) from three independent experiments. The difference between mutant and wild-type was statistically significant for p -value <0.01 (determined from paired t test) at non-permissive temperature.

Fig. 4. *YMR134W^{ts}* cells possess low iron concentration in their mitochondria at non-permissive temperature. Cells were grown on YPGAL to avoid glucose repression in mitochondria proliferation. The graph represents the average and standard deviation ($\pm$ S.D.) from three independent experiments. The difference between mutant and wild-type was statistically significant for p -value <0.01 (determined from paired t test) only at non-permissive temperature.

agent (2,4,6-tri(2-pyridyl)-1,3,5-triazine) which strongly binds reduced iron and this complex possesses an extinction coefficient of $21,600 \text{ M}^{-1} \text{ cm}^{-1}$ at 593 nm (Hach, Loveland, CO, USA). Detection limits are from 0.01 to 1.80 mg/L. The components of the kit include ascorbic acid in high concentration to reduce iron content, so the values represent total iron (reduced and oxidized). After growth, cells were harvested and supernatant (culture media) was used to measure the remaining iron content. A standard curve with known concentrations of FeCl_3 was used to calculate iron concentrations of the samples.

2.1.6. Cellular iron content measurement

Cells were grown as described above in YPD media and collected by centrifugation after 16 h at permissive or non-permissive temperature. The cell pellets were washed twice in distilled cold

water and were disrupted in 1 volume of glass beads and 2 volumes of 3.5% sulfosalicylic acid. The suspension was vortexed for 20 min at 4 °C in a multifold vortex and harvested at 16,000 g. This procedure was repeated twice and the supernatants were combined. The pH of the samples was adjusted to pH 4.0 with NaOH, because TPTZ reagent only produces color between pH 3.0–5.0. Aliquots of 50 μl were used to react with TPTZ, as described above. The pellets of disrupted cells were homogenized with Tris-HCl pH 7.5 buffered guanidine 5 M overnight and an aliquot of each sample was used to measure total protein content. Protein concentration was determined with the Bradford reagent from BIORAD (Hercules, CA, USA), using bovine serum albumin (Sigma) as a standard.

2.1.7. Mitochondrial iron content

The mitochondrially enriched fraction was obtained from cells grown on YPGAL, and following the protocol exactly as described earlier [14]. The mitochondria were then lysed in sterile water and an aliquot possessing 200 μg of protein (previously determined with Bradford reagent) of this homogenate was used to measure the iron content as described above.

2.1.8. Oxygen uptake measurements

Experiments were carried out using a Clarke-type electrode in a glass cuvette equipped with magnetic stirring. To adjust the uptake for cell concentration, the cell dry weight was measured until three consecutive constant values. The cells were incubated at 30 °C in buffer containing HEPES 10 mM pH 7.2, sucrose 250 mM, KCl 65 mM and ethanol 2.5 mM for at least 30 min [14].

2.1.9. Catalase activity assay

Oxygen-release measurements were carried out using a Clarke-type electrode in a glass cuvette equipped with magnetic stirring. To measure oxygen release, corresponding to catalase activity, the cells (grown on YPD) were disrupted in a vortex using glass beads in phosphate buffer saline 1 $\times$ with 1 mM of PMSF added to inhibit proteolysis. The protein extract was harvested at 16,000g for 30 min at 4 °C, the supernatant was collected and the total protein amount was quantified by Bradford assay [15]. Oxygen release due to catalase activity ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$) in cell extracts was specifically determined using a Clark electrode at 30 °C (Yellow Springs Instrument Co.). The saturating oxygen concentration, which corresponds to the full scale of the electrode, was taken to be 0.225 mM [15]. The initial peroxide concentration added was 1 mM in 50 mM HEPES and 100 mM NaCl. The activity observed was completely abolished when 1 mM azide (inhibitor of catalase) was added previously to peroxide in the reaction media.

3. Results and discussion

The data published about phenotypes (aberrant mitochondrial morphology [4] and inability to growth in iron deficient media [1]) and about protein physical interactions (with Erg25p [8], a key enzyme in ergosterol biosynthesis) lead us to hypothesize that Ymr134wp is involved in ergosterol biosynthesis in yeast. To test this hypothesis, the content of total ergosterol was measured in wild-type and *YMR134W^{ts}* cells (Fig. 1). *YMR134W^{ts}* cells showed almost 40% less ergosterol compared with the wild-type strain at non-permissive temperature, suggesting a significant importance of Ymr134wp to the synthesis of this lipid. Corroborating this idea, Ymr134wp is localized in the ER [3], where most of the components of the superpathway of ergosterol biosynthesis are present. In addition, ergosterol mutant strains and *ymr134w* mutant cells share several common phenotypes. Mutation of *erg25* (LT06) [6], *erg3* and *erg6* [16] renders cells unable to grow under low iron

Fig. 5. Consumption and accumulation of iron is increased in *YMR134W^{ts}* cells. (A) The residual iron content on YPD media was measured at 0, 8 and 16 h after cell inoculation. The total initial iron content on YPD media was 1.8 mg/L. (B) Total cellular iron content was measured at 16 h after cell inoculation as described in “Section 2”. The graphs represent the average and standard deviation ($\pm$ S.D.) from three independent experiments. The difference between mutant and wild-type was statistically significant for p -value <0.01 (determined from paired t test) at both temperatures in (A) and only at permissive temperature in (B).

Fig. 6. *YMR134W^{ts}* cells are sensitive to oxidative stress. SD-URA⁻ plates in the absence (control) or presence of the oxidant agents, hydrogen peroxide (H₂O₂), and *tert*-butyl hydroperoxide (*t*-BOOH). Serial dilution performed as described in the legend of Fig. 2. Figure representative of at least three independent experiments.

conditions, such as observed in the *ymr134w* (*bm-8*) mutant cells [1]. Strains whose expression of *YMR134W* or ergosterol biosynthetic genes was strongly inhibited by doxycycline addition (through the regulation of a TetO₇ promoter) produced aberrant mitochondrial morphology profile [4]. In this regard, the ergosterol deficiency observed in *YMR134W^{ts}* cells (Fig. 1) is in agreement with these previous results and it can explain them, implicating Ymr134wp as a key component of ergosterol biosynthesis.

Ymr134wp was firstly related to iron metabolism [1] and several studies have suggested the relationship between mitochondrial function (mainly oxygen consumption) and iron metabolism [17–21]. It is well accepted that heme (an iron cofactor synthesized into mitochondria) is the oxygen sensor molecule in yeast and that this pathway is mediated by the Heme Activator Protein (Hap1p) [22]. In particular, Hap1p induces the expression of *ROX1* repressor that, in turn, inhibits the expression of the ergosterol import system from extracellular media in the presence of oxygen [23]. So, ergosterol supplementation under aerobic conditions is not effective in reverting phenotype of mutant genes involved in ergosterol

biosynthesis because the absence of its importer system. However, the supplementation of media with ergosterol under anaerobic conditions renders reversal of lethal phenotype of *Δymr134w* [5] what reinforce our data involving this gene in ergosterol biosynthesis. Besides, the ergosterol biosynthesis links iron and oxygen cell-requirements because this anabolic pathway requires several iron-dependent enzymes (heme and oxo-diiron) and twelve oxygen molecules as substrate to generate ergosterol [10]. Remarkably, it has been suggested that the ability of organisms, such as *S. cerevisiae*, to grow aerobically or anaerobically is due to the presence of sterol importers that are only active in the absence of oxygen [5].

Since depletion of any gene involved in ergosterol biosynthetic pathway leads to mitochondrial morphology aberration [4], it would be expected that *YMR134W^{ts}* cells possessed impaired mitochondrial function and deficient growth in a respiratory carbon source, such as glycerol. In fact, *YMR134W^{ts}* cells grew poorly in the presence of this carbon source (Fig. 2). In addition, oxygen consumption was also analyzed to assess mitochondrial function and a

Fig. 7. *YMR134W^{ts}* cells are in oxidative stress. Cells were grown on YPD. Catalase activity measured by the release of molecular oxygen, one of the reaction products. The graph represents the average and standard deviation ($\pm$ S.D.) from three independent experiments. The difference between mutant and wild-type was statistically significant for p -value <0.01 (determined from paired t test) at both temperatures.

considerable decrease in oxygen uptake was observed at the non-permissive temperature in *YMR134W^{ts}* cells (Fig. 3), suggesting that the mitochondrial function was affected.

Due to the ability of *YFH1* gene overexpression to rescue *bm-8* phenotype to wild-type state, it was expected that *YMR134W^{ts}* cells would present similar phenotype in respect to the mitochondrial iron homeostasis. *yfh1* mutant cells accumulate high amounts of iron into mitochondria compartment, but this metal is not available to biological processes, such as [Fe-S] cluster biogenesis, because it is precipitated with phosphate [21]. Unexpectedly, *YMR134W^{ts}* cells at the non-permissive temperature possessed almost half of the concentration of iron in the mitochondria (Fig. 4). Therefore, in this aspect, inhibition of the function of the *YMR134W* and the *YFH1* genes resulted in opposite outcomes (depletion or accumulation of mitochondrial iron content, respectively). Although the overexpression of *Yfh1p* was able to complement *bm-8* growth defects, this protein has no relation to ergosterol biosynthesis, since the levels of ergosterol in *yfh1* mutants are normal [24]. In contrast to *Yfh1p*, *Ymr134wp* is not localized in mitochondria but in the ER [3], features which taken together could be related to these distinct phenotypes.

Next, the ability of *YMR134W^{ts}* cells to uptake iron from the culture medium and accumulate this metal inside the cell was investigated. Remarkably, *YMR134W^{ts}* cells consumed high amounts of iron from the culture media and accumulate it (Fig. 5). After 16 h of growth at permissive or non-permissive temperatures, *YMR134W^{ts}* cells almost completely exhausted the iron content of the media (Fig. 5A). Interesting, in wild-type strain the non-permissive temperature increased the iron accumulation in the cells (Fig. 5B). At least to our knowledge, this phenomenon has not yet been reported to yeast, and deserves further studies. In any case, the data described in Fig. 5 suggests that *YMR134W^{ts}* cell iron uptake is deregulated and cells probably accumulate excessive iron in the cytosol due to the fact that delivery of iron to mitochondria in these cells is impaired (Fig. 4), suggesting a possible condition of oxidative stress [20,25].

Therefore, the response of *YMR134W^{ts}* cells to oxidative stress was investigated. Accordingly, *YMR134W^{ts}* cells presented high

sensitivity not only to hydrogen but also to *tert*-butyl hydroperoxide insult (Fig. 6). In fact, at both temperatures (25 or 37 °C), *YMR134W^{ts}* cells suffered a significant decrease in their viability in the presence of peroxide. In addition, the catalase activity was measured in both strains, since this enzyme participates in the antioxidant response. *YMR134W^{ts}* cells displayed double the catalase activity at the non-permissive temperature when compared with wild-type (Fig. 7). However, these cells presented lower catalase activity at permissive temperature, which is in agreement with cell sensitivity to oxidant insult in this growth condition. It is noteworthy to observe that, also at the permissive temperature, *YMR134W^{ts}* cells consumed and accumulated excessive iron, explaining this sensitivity to hydroperoxides (Fig. 5).

In agreement with these results, Folmer et al. [26] showed that during adaptation to hydrogen peroxide the plasma membrane suffers modulation of their biophysical properties, resulting in decreased diffusion rate of hydrogen peroxide. Interestingly, this modulation was dependent on ergosterol biosynthesis, since Δ *erg3* or Δ *erg6* cells, displays increased permeability to hydrogen peroxide [26]. So, it is expected that in addition to excess of iron, *YMR134W^{ts}* cells are also more permeable to peroxides, both factors probably contributing to lower viability of this strain when exposed to these oxidants (Fig. 6). In addition, there is a relationship between the progression in the pathway from ergosterol precursors toward ergosterol and the ability of the lipid to protect membrane against oxidation, influencing the cell resistance to oxidative stress [27]. Considering that *Ymr134wp* is important to *Erg25p* function, the ergosterol pathway was disrupted early in *YMR134W^{ts}* cells, leading to formation of a less protective structural lipid against oxidative stress.

The data suggest that *Ymr134wp* is a key component in ergosterol biosynthesis, leading the mitochondria of *YMR134W^{ts}* cells to present problems in their membrane composition, probably resulting in deficiency of iron transport from the cytosol to the mitochondria. The critical transporters involved in mitochondrial iron import are still poorly understood. Among the probable proteins involved in this process are *Mmt1p*, *Mmt2p* and *Mrs3p*, *Mrs4p*. *Mmt1p* and *Mmt2p* (mitochondrial metal transporters) are putative mitochondrial iron transporters since their over-expression increases iron accumulation into mitochondria by 2- to 5-fold when compared to the wild-type [7]. Bioinformatics' predictions suggest that both *Mmt1p* and *Mmt2p* are integral to the membrane (TMHMM tool, an application available at The Center for Biological Sequence Analysis at the Technical University of Denmark DTU). However, the role of these transporters is controversial since the double mutant Δ *mmt1* Δ *mmt2* does not show decreased mitochondrial iron content [28]. The best candidates for mitochondrial iron importers are *Mrs3* and *Mrs4* proteins ("Mitochondrial RNA Splicing") whose double deletion causes impairment in mitochondrial iron accumulation [28,29]. It is expected that mutations damaging ergosterol biosynthesis result in membrane fluidity alterations, impairing the proper function of integral membrane protein transporters. Consequently, mutations in iron transporters to the mitochondria in *ymr134w* mutant cells could cause a severe iron deficiency in the organelles of these cells, leading to lethality. This hypothesis is in agreement with the observation of Jerry Kaplan (2007) that "*erg29* mutation is synthetically lethal with *mmt1* and *mmt2* null mutations". The lower intra-mitochondrial iron concentration in *YMR134W^{ts}* cells (Fig. 5) could be related to the inappropriate function of mitochondrial iron transport process.

The relationship of the ergosterol content and the mitochondria profile is known for a long time (Jollow et al.). When cells are cultivated in anaerobic conditions and limiting the levels of the lipids (ergosterol, and unsaturated fatty acids) mitochondrial and vacuole morphology are profoundly altered [30]. In agreement with this data, the results presented here show that low ergosterol content

in *YMR134W^Δ* cells probably affects mitochondrial membrane composition, resulting in low iron concentration inside the organelle, low oxygen consumption, and consequently less ability to grow in a respiratory carbon source. In consequence, the disturbed iron homeostasis and altered plasma membrane peroxide permeability prone cells to oxidative stress.

4. Conclusions

In conjunction, the data suggests the involvement of the *YMR134W* gene in the ergosterol biosynthesis, which affects mitochondrial and plasma membrane composition, and, in turn, has implications for iron homeostasis, mitochondria function, respiratory growth and antioxidant cell protection.

Acknowledgments

We thank Dr. Marilene Demasi for helpful comments and discussion of the results. We also thank to São Paulo Research Foundation FAPESP (Grant 2009/01,303-1) and CNPq for financial support.

References

- [1] Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M. and Kaplan, J. (1997) Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science* 276, 1709–1712.
- [2] Li, H., Gakh, O., Smith 4th, D.Y. and Isaya, G. (2009) Oligomeric yeast frataxin drives assembly of core machinery for mitochondrial iron-sulfur cluster synthesis. *J. Biol. Chem.* 284, 21971–21980.
- [3] Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S. and O'Shea, E.K. (2003) Global analysis of protein localization in budding yeast. *Nature* 425, 686–691.
- [4] Altmann, K. and Westermann, B. (2005) Role of essential genes in mitochondrial morphogenesis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 16, 5410–5417.
- [5] Snoek, I.S. and Steensma, H.Y. (2006) Why does *Kluyveromyces lactis* not grow under anaerobic conditions? Comparison of essential anaerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces lactis* genome. *FEMS Yeast Res.* 6, 393–403.
- [6] Li, L. and Kaplan, J. (1996) Characterization of yeast methyl sterol oxidase (ERG25) and identification of a human homologue. *J. Biol. Chem.* 271, 16927–16933.
- [7] Li, L. and Kaplan, J. (1997) Characterization of two homologous yeast genes that encode mitochondrial iron transporters. *J. Biol. Chem.* 272, 28485–28493.
- [8] Tarassov, K., Messier, V., Landry, C.R., Radinovic, S. and Serna Molina, M.M. (2008) An in vivo map of the yeast protein interactome. *Science* 320, 1465–1470.
- [9] Kristan, K. and Rizner, T.L. (2012) Steroid-transforming enzymes in fungi. *J. Ster. Biochem. Mol. Biol.* 129, 79–91.
- [10] Maczek, J., Junne, S., Nowak, P. and Goetz, P. (2006) Metabolic flux analysis of the sterol pathway in the yeast *Saccharomyces cerevisiae*. *Bioprocess Biosyst. Eng.* 29, 241–252.
- [11] Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., et al. (1996) Life with 6000 genes. *Science* 274, 563–567.
- [12] Ben-Aroya, S., Coombes, C., Kwok, T., O'Donnell, K.A., Boeke, J.D. and Hieter, P. (2008) Toward a comprehensive temperature-sensitive mutant repository of the essential genes of *Saccharomyces cerevisiae*. *Mol. Cell* 30, 248–258.
- [13] Arthington-Skaggs, B.A., Jradi, H., Desai, T. and Morrison, C.J. (1999) Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J. Clin. Microbiol.* 37, 3332–3337.
- [14] Monteiro, G., Kowaltowski, A.J., Barros, M.H. and Netto, L.E. (2004) Glutathione and thioredoxin peroxidases mediate susceptibility of yeast mitochondria to Ca(2+)-induced damage. *Arch. Biochem. Biophys.* 425, 14–24.
- [15] Munhoz, D.C. and Netto, L.E. (2004) Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide insult: catalases and peroxiredoxins cooperate in the decomposition of H₂O₂ by yeast. *J. Biol. Chem.* 279, 35219–35227.
- [16] Minear, S., O'Donnell, A.F., Ballew, A., Giaever, G., Nislow, C., Stearns, T. and Cyert, M.S. (2011) Curcumin inhibits growth of *Saccharomyces cerevisiae* through iron chelation. *Eukaryot. Cell* 10, 1574–1581.
- [17] Li, L. and Kaplan, J. (2004) A mitochondrial-vacuolar signaling pathway in yeast that affects iron and copper metabolism. *J. Biol. Chem.* 279, 33653–33661.
- [18] Hausmann, A., Samans, B., Lill, R. and Mühlenhoff, U. (2008) Cellular and mitochondrial remodeling upon defects in iron-sulfur proteinogenesis. *J. Biol. Chem.* 283, 8318–8330.
- [19] Shakoury-Elizeh, M., Protchenko, O., Berger, A., Cox, J., Gable, K., Dunn, T.M., Prinz, W.A., Bard, M. and Philpott, C.C. (2010) Metabolic response to iron deficiency in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 285, 14823–14833.
- [20] Lin, H., Li, L., Jia, X., Ward, D.M. and Kaplan, J. (2011) Genetic and biochemical analysis of high iron toxicity in yeast: iron toxicity is due to the accumulation of cytosolic iron and occurs under both aerobic and anaerobic conditions. *J. Biol. Chem.* 286, 3851–3862.
- [21] Seguin, A., Santos, R., Pain, D., Dancis, A., Camadro, J.M. and Lesuisse, E. (2011) Co-precipitation of phosphate and iron limits mitochondrial phosphate availability in *Saccharomyces cerevisiae* lacking the yeast frataxin homologue (YFH1). *J. Biol. Chem.* 286, 6071–6079.
- [22] Kwast, K.E., Burke, P.V., Staahl, B.T. and Poyton, R.O. (1999) Oxygen sensing in yeast: evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. *Proc. Natl. Acad. Sci. USA* 96, 5446–5451.
- [23] Crowley, J.H., Leak Jr, F.W., Shianna, K.V., Tove, S. and Parks, L.W. (1998) A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. *J. Bacteriol.* 180, 4177–4183.
- [24] Chen, O.S., Crisp, R.J., Valachovic, M., Bard, M., Winge, D.R. and Kaplan, J. (2004) Transcription of the yeast iron regulon does not respond directly to iron but rather to iron-sulfur cluster biosynthesis. *J. Biol. Chem.* 279, 29513–29518.
- [25] Chen, O.S., Hemenway, S. and Kaplan, J. (2002) Genetic analysis of iron citrate toxicity in yeast: implications for mammalian iron homeostasis. *Proc. Natl. Acad. Sci. USA* 99, 16922–16927.
- [26] Folmer, V., Pedroso, N., Matias, A.C., Lopes, S.C., Antunes, F., Cyrne, L. and Marinho, H.S. (2008) H₂O₂ induces rapid biophysical and permeability changes in the plasma membrane of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1778, 1141–1147.
- [27] Dupont, S., Lemetals, G., Ferreira, T., Cayot, P., Gervais, P. and Beney, L. (2012) Ergosterol biosynthesis: a fungal pathway for life on land? *Evolution* 66, 2961–2968.
- [28] Foury, F. and Roganti, T. (2002) Deletion of the mitochondrial carrier genes MRS3 and MRS4 suppresses mitochondrial iron accumulation in a yeast frataxin-deficient strain. *J. Biol. Chem.* 277, 24475–24483.
- [29] Mühlenhoff, U., Stadler, J.A., Richhardt, N., Seubert, A., Eickhorst, T., Schweyen, R.J., Lill, R. and Wiesenberger, G. (2003) A specific role of the yeast mitochondrial carriers MRS3/4p in mitochondrial iron acquisition under iron-limiting conditions. *J. Biol. Chem.* 278, 40612–40620.
- [30] Jollow, D., Kellerman, G.M. and Linnane, A.W. (1968) The biogenesis of mitochondria: 3. The lipid composition of aerobically and anaerobically grown *Saccharomyces cerevisiae* as related to the membrane systems of the cells. *J. Cell Biol.* 37, 221–230.

ANEXO 4

Regular Articles

The gene *ICS3* from the yeast *Saccharomyces cerevisiae* is involved in copper homeostasis dependent on extracellular pH

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ARTICLE INFO

Article history:

Received 9 February 2015

Revised 8 June 2015

Accepted 10 June 2015

Available online 27 June 2015

Keywords:

Copper homeostasis

Acidic conditions

Copper accumulation

pH dependent response

ABSTRACT

In the yeast *Saccharomyces cerevisiae*, many genes are involved in the uptake, transport, storage and detoxification of copper. Large scale studies have noted that deletion of the gene *ICS3* increases sensitivity to copper, Sortin 2 and acid exposure. Here, we report a study on the Δ *ics3* strain, in which *ICS3* is related to copper homeostasis, affecting the intracellular accumulation of this metal. This strain is sensitive to hydrogen peroxide and copper exposure, but not to other tested transition metals. At pH 6.0, the Δ *ics3* strain accumulates a larger amount of intracellular copper than the wild-type strain, explaining the sensitivity to oxidants in this condition. Unexpectedly, sensitivity to copper exposure only occurs in acidic conditions. This can be explained by the fact that the exposure of Δ *ics3* cells to high copper concentrations at pH 4.0 results in over-accumulation of copper and iron. Moreover, the expression of *ICS3* increases in acidic pH, and this is correlated with *CCC2* gene expression, since both genes are regulated by Rim101 from the pH regulon. *CCC2* is also upregulated in Δ *ics3* in acidic pH. Together, these data indicate that *ICS3* is involved in copper homeostasis and is dependent on extracellular pH.

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1. Introduction

Copper and other transition metals are essential micronutrients, because of their catalytic and structural functions as prosthetic groups in metalloproteins. A variety of enzymes require copper as a cofactor for electron transfer reactions (Freitas et al., 2003); however, when in excess, metal ions produce toxic effects. Free metal ions can catalyze superoxide anion radical (O_2^-) formation, which leads to the formation of hydrogen peroxide (H_2O_2). Hydrogen peroxide reacts with free iron or copper to generate the highly reactive hydroxyl radical ($HO^\bullet$) through the Fenton

reaction (Farrugia and Balzan, 2012). All of these reactive oxygen species can damage bio-molecules including lipids, nucleic acids and proteins, and there is no enzymatic protection against $HO^\bullet$ (Farrugia and Balzan, 2012). Furthermore, copper can be toxic even in anaerobic conditions, since it can damage iron–sulphur clusters that are exposed in the structure of proteins, inactivating them (Macomber and Imlay, 2009; Foster et al., 2014).

Organisms have developed complex regulatory mechanisms to maintain a tightly controlled homeostasis of metal ions through the concerted action of transporters, chaperones, sequestering and storage molecules, and metal-responsive transcriptional regulators (Eide, 1998; Ma et al., 2009; O'Halloran and Culotta, 2000). These components allow the organisms to obtain metals ions from different sources under a variety of conditions, distribute the ions to appropriate targets and maintain very low levels of unbound metals in cellular compartments (Eide, 1998; Rae et al., 1999). Disruption of this balance is related to many human diseases, such as Menkes syndrome and Wilson disease, in which the distribution of copper in cells and tissues is impaired (Bleackley and MacGillivray, 2011).

In the yeast *Saccharomyces cerevisiae*, in metal-deficient growth conditions, metal uptake is performed by high-affinity systems, mediated by the copper-responsive transcription factor Mac1 (Keller et al., 2005). On the other hand, in metal-replete conditions,

Abbreviations: Ace1, transcriptional activator in copper-replete cells; Ccc2, copper transporting P-type ATPase; CcO, cytochrome c oxidase; Ccs1, copper chaperone for SOD; Cox1/Cox2, cytochrome c oxidase subunits; Ctr, copper transporter; Fet, ferrous transport; Fre, ferric and cupric reductase; Ftr1, iron permease; ICS, increased copper sensitivity; Mac1, transcriptional activator in copper-deficient cells; MTs, metallothioneins; Rim101, alkaline responsive gene repressor; SOD, copper–zinc superoxide dismutase; YPD, yeast extract peptone dextrose.

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metal uptake is regulated by low affinity systems, under control of the transcriptional activator Ace1 (Keller et al., 2005). In the presence of low copper levels, the cell surface iron/copper reductases Fre1 and Fre2 reduce extracellular Cu^{2+} to Cu^+ , which is then transported across the plasma membrane by the high-affinity copper transporter proteins Ctr1 and Ctr3 (Hassett and Kosman, 1995; Georgatsou et al., 1997; Dancis et al., 1994). High-affinity copper uptake is specific for Cu^+ over other metals, and it is an energy dependent process which is coupled to K^+ antiport (Dancis et al., 1994; Lin and Kosman, 1990; De Rome and Gadd, 1987). In presence of high copper levels, copper is transported by the low-affinity copper/iron Fet4 transporter and Smf1, which can also transport Mn^{2+} , Fe^{2+} , Cu^{2+} , and Zn^{2+} (Hassett et al., 2000; Cohen et al., 2000).

Inside the cell, copper binds to intracellular chaperones responsible for delivering the metal to proteins that use copper as a cofactor. In *S. cerevisiae*, copper is a cofactor in at least three proteins: Fet3 oxidase, cytochrome c oxidase (CcO), and Cu/Zn superoxide dismutase (SOD) (Freitas et al., 2003; O'Halloran and Culotta, 2000). The Ccc2 P1-type ATPase is responsible for the transport of copper into Golgi vesicles, where the Fet3 oxidase is copper-metallated (Robinson and Winge, 2010). Chaperones of the Cox family (Cox11 and Cox17) are involved in the delivery of copper to CcO, whereas Ccs1 is the metallochaperone for SOD (Robinson and Winge, 2010). In high copper conditions, the expression of the genes encoding copper-binding metallothioneins (MTs) is upregulated to control the excess of intracellular metal (Thiele, 1988). MTs are low molecular weight cysteine-rich polypeptides, commonly found in eukaryotes, which bind metal ions and are important for their primary storage, transport and detoxification (Hamer et al., 1985; Adamo et al., 2012).

In yeast, most of the excess metal ions are stored in the vacuole, a dynamic organelle that is critical for the maintenance of intracellular pH, and functions as a storage compartment for amino acids, metal ions, and other molecules (Klionsky et al., 1990). It has been proposed that the vacuole is as important as MTs for copper detoxification (Eide et al., 1993). In copper deprivation conditions Fre6 and Ctr2, present in the vacuolar membrane, mediate the reduction and transfer, respectively, of copper from the lumen of the vacuole to the cytosol (Cyert and Philpott, 2013).

Copper and iron homeostasis are both influenced by environmental pH, since the maintenance of a proton gradient through the plasma membrane H-ATPase, which extrudes protons, is important for the uptake of nutrients and cations (Serrano et al., 2004). Many genes involved in iron and copper uptake and transport (FET3, FRE1, FRE3, FRE4, FET4, CTR1, CTR3) are upregulated in alkaline pH stress (Arino, 2010). Iron and copper are limiting factors for growth in pH stress. In fact, the addition of these metals in micromolar concentrations improves growth at alkaline pH (Serrano et al., 2004). In *S. cerevisiae*, Rim101 is a transcriptional repressor in alkaline pH stress, inhibiting the expression of other transcriptional repressor genes, such as *NRG1*, which is related to ion homeostasis and alkaline pH protection, and *SMP1*, related to sporulation and invasive growth (Lamb and Mitchell, 2003).

The gene *YJL077C* was denoted as *ICS3* (for “increased copper sensitivity”) in a high throughput study performed with 150 deletion mutants for genes of unknown function (Entian et al., 1999). *ICS3* has also been indirectly implicated in the process of vacuolar homeostasis. It was identified in a screen study as a gene related to sensitivity to Sortin 2, a synthetic compound that affects protein targeting to the vacuole in *S. cerevisiae* (Norambuena et al., 2008). Additionally, the Δ ics3 strain is sensitive to hydrochloric and acetic acids (Kawahata et al., 2006), and possesses abnormal vacuole morphology (Michaillat and Mayer, 2013). Our group identified *ICS3* in a screen for genes of unknown function which were sensitive to oxidative stress (Costa and Monteiro, 2014).

The objective of the present study is to explain the oxidant and copper sensitivities of the mutant strain for the *ICS3* gene. Here, we show that the Δ ics3 strain is sensitive to oxidative stress because these cells have copper concentration three times higher than the wild-type strain in optimal growth conditions. High copper concentration exposure in acidic conditions also leads these cells to accumulate intracellular copper and iron. Furthermore, Northern blot analyses indicate that *ICS3* is regulated by Rim101 from the pH-dependent regulon, rather than by the copper-dependent transcription factors Mac1 and Ace1. Together, the data indicate that the *ICS3* gene is involved in copper homeostasis in a pH-dependent manner, impacting the antioxidant response.

2. Material and methods

2.1. Yeast strains

S. cerevisiae wild-type strain BY4741 (MATa *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*), Δ ics3 (MATa *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 ICS3::KANMX4*), Δ ics1 (MATa *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 ICS1::KANMX4*), Δ ace1 (MATa *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 ACE1::KANMX4*), Δ rim101 (MATa *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 RIM101::KANMX4*), and Δ mac1 (MATa *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 MAC1::KANMX4*) were obtained from Yeast Knockout (YKO) Deletion Collection (Invitrogen, Carlsbad – CA, USA).

2.2. Sensitivity to hydrogen peroxide

Cells were pre-inoculated in YPD culture media (yeast extract 1%, peptone 2% and glucose 2%) and grown at 30 °C overnight. The cultures were diluted to an initial OD₆₀₀ of 0.2 and grown in fresh YPD until logarithmic phase (OD₆₀₀ 0.6–1.0). An aliquot of cells was diluted in water to OD₆₀₀ of 0.2, and four subsequent 1:5 dilutions were performed; 5 μ L drops of each dilution were spotted onto YPD plates containing no (control) or 3 mM hydrogen peroxide (Merck, Millipore, Billerica – MA, USA). Growth was recorded after 24 h incubation at 30 °C. The figure shown (Fig. 1) is representative of at least three independent experiments.

2.3. Sensitivity to metal ions

Cells pre-inoculated in YPD were grown at 30 °C overnight. The cultures were diluted to OD₆₀₀ of 0.2 in fresh liquid YPD containing no (control), 7 mM or 10 mM copper sulphate and grown at 30 °C for the time indicated in the figure legends. Cells were then diluted in water to OD₆₀₀ of 0.2, and four subsequent 1:5 dilutions were performed; 5 μ L drops of each dilution were spotted onto YPD plates. Tests with cobalt chloride and iron sulphate were performed similarly. When necessary, the pH of the culture media was adjusted to 4.0 with the addition of HCl, or to 6.0 with the addition of NaOH immediately before the first dilution to OD₆₀₀ of 0.2 in fresh liquid media. In all sensitivity tests, growth was recorded after 24 h of incubation at 30 °C. The figures are representative of at least three independent experiments.

2.4. Growth curves

Cells were grown at 30 °C in YPD overnight, and then diluted to OD₆₀₀ of 0.1. After dilution, the pH of the media was adjusted to 4.0 or 6.0 when necessary and 10 mM copper sulphate was added or not (control). After these additions the final pH of the culture was measured. The growth was followed spectrophotometrically (UNICO SQ4802 Double Beam Spectrophotometer – NJ – USA) for 75 h by measuring the OD₆₀₀ at different time intervals. The curve

Fig. 1. Sensitivity to hydrogen peroxide. The wild-type strain BY4741 and the mutant Δ ics3 were grown in YPD medium pH 6.0 at 30 °C for 24 h. The cultures were then diluted to an OD₆₀₀ of 0.2, and 5 times serial dilutions were grown in YPD plates pH 6.0 containing no or 3 mM of hydrogen peroxide. Growth was monitored after 24 h at 30 °C. This image represents at least three independent experiments.

represents the mean (with standard deviation bars) of three biological replicates. Curve fitting and statistical analyses were performed with software GraphPad Prism version 6.05.

2.5. Metal content

The experiments were performed following the protocol described in Schuller et al. (2013) with modifications (Schuller et al., 2013). Briefly, cells were grown at 30 °C in YPD for 20 h, and then diluted to OD₆₀₀ of 0.6. Copper sulphate was added or not to the YPD medium at 10 mM concentration and the pH was adjusted to 4.0 with HCl, or to 6.0 with NaOH. After growth in the absence or presence of copper for 20 h at 30 °C, the cells were harvested by centrifugation, the cell pellet was washed with deionized sterile water and the supernatant was discarded (repeated twice). The remaining pellets were dried under vacuum for 20 h at 30 °C in a Speed Vac Concentrator plus (Eppendorf, Hamburg – Germany). After weighing, the samples were digested with concentrated nitric acid in a block digester until complete solid dissolution. The iron and copper content of each sample was analysed at the Analytical Centre of the Institute of Chemistry at the University of São Paulo in an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) Spectro Ciros (Kleve, Germany), model Arcos, CCD equipment. Iron was analysed at 259.941 nm, and copper at 324.754 nm. The amounts given in the figures are the means and standard deviations of three independent experiments. Statistical analyses were performed using software GraphPad Prism version 6.05, through unpaired *t*-student test, with *p* < 0.05.

2.6. ICS3 regulation

The wild-type strain BY4741 and the mutants Δ ics3, Δ rim101, Δ ace1 and Δ mac1 were grown in YPD media overnight at 30 °C. The cultures were diluted to OD₆₀₀ 0.2 in fresh YPD media at pH 4.0 and 6.0 in the presence or absence of 0.5 mM of copper sulphate, and grown until mid-logarithmic phase (OD₆₀₀ between 0.8 and 1.4). Cells were washed with 50 mM EDTA in deionized sterile water, and harvested by centrifugation. Total RNA of each sample was obtained by the hot acid phenol method (Ausubel et al., 1994), and separated through electrophoresis formaldehyde-agarose gels. The separated RNAs were transferred to a positively charged membrane (Amersham Hybond™ – N⁺) by capillary blotting and fixed by UV exposure for 2 min. Pre-hybridization, ICS3 and CCC2 DNA labelling, hybridization and membrane washing were performed with the Amersham Gene Images AlkPhos Direct Labelling and Detection System kit, according to manufacturer's manual instructions without modifications. Detection was carried out using the NBT/BCIP Reagent Kit from Invitrogen for 24 h of incubation.

3. Results

In order to identify the still uncharacterised viable mutants (total of 217 selected strains), whose genes could confer resistance to oxidative stress in *S. cerevisiae*, the cell viability of the knockout strains from the YKO Deletion Collection was tested in the presence of hydrogen peroxide (Costa and Monteiro, 2014). Among the mutants tested, six strains were found to have higher sensitivity to hydrogen peroxide; one was the knockout for the gene *YJL077C/ICS3* (Δ ics3). This experiment was reproduced focusing on the Δ ics3 mutant, and the sensitivity of these cells to oxidative stress induced by hydrogen peroxide at 3 mM concentration was confirmed (Fig. 1).

Previously, the mutant Δ ics3 was described as sensitive to copper in a screen of 150 deletion mutants for genes of unknown function (Entian et al., 1999). To characterise the sensitivity of the Δ ics3 strain to metals, we performed viability tests in the presence of copper, cobalt and iron (Fig. 2). Δ ics3 cells were not sensitive to cobalt chloride or iron sulphate at either of the concentrations tested (7 mM and 10 mM). At 7 mM copper sulphate, both wild-type and Δ ics3 strains behaved similarly. However, at 10 mM concentration, Δ ics3 cells were more sensitive to copper exposure than the wild-type strain (Fig. 2). Furthermore, incubation in the presence of copper for only 4 h resulted in lower viability of Δ ics3 cells, whereas 8 h of incubation with cobalt and iron did not induce sensitivity. This indicates that, of all the metals tested, ICS3 is likely to be specifically related to copper homeostasis.

It has also been suggested that Δ ics3 cells are sensitive to hydrochloric and acetic acids (Kawahata et al., 2006). Considering that the addition of copper sulphate could change the pH of the culture medium, the pH of all media was measured before cell inoculation. YPD medium initially presented pH 6.0, however, the addition of 10 mM copper sulphate decreased the pH to around 4.0. This raises the possibility that the sensitivity of Δ ics3 cells in the presence of copper sulphate was due to the acidic pH, rather than to the metal.

To clarify this issue, we constructed growth curves for the wild-type and Δ ics3 strains in the absence and presence of copper (10 mM) at pH 4.0 (Fig. 3A) and 6.0 (Fig. 3B). In these experiments, pH 4.0 was reached either with the addition of 10 mM copper sulphate or with the addition of HCl in the absence of copper sulphate. In cultures with copper sulphate, pH 6.0 was reached with the addition of NaOH. At pH 4.0, the growth curve of wild-type and Δ ics3 cells was similar in the absence of copper (Fig. 3A). In the presence of 10 mM of copper sulphate at pH 4.0, both wild-type and Δ ics3 strains grew at a slower rate when compared to the curve in the absence of copper. Interestingly, after 48 h, the growth curve of the wild-type and Δ ics3 strains diverged significantly: the wild-type strain recovered and began to grow faster, while the Δ ics3 mutant continued to grow slowly. Indeed, we also observed

Fig. 2. Sensitivity to metals. The wild-type strain BY4741 and the mutant Δ ics3 were grown in YPD medium in the absence (control) or, presence of metals at 7 mM or 10 mM concentration, as indicated. After 4 h (CuSO_4) or 8 h (CoCl_2 and FeSO_4) of incubation at 30 °C (the pH of the media was not determined), the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. Growth was monitored after 24 h at 30 °C and the image is representative of three replicates.

that the wild-type strain recovered its cell viability, forming colonies in solid fresh media, while the Δ ics3 strain did not (data not shown). Unexpectedly, when the pH was adjusted to 6.0, the presence or absence of copper did not affect the growth of either the wild-type or the Δ ics3 strains. Both grew at similar rates as in the absence of copper at pH 4.0 (Fig. 3B).

Fig. 3. Growth curves for the wild-type strain BY4741 and the mutant Δ ics3 in the absence or presence of copper (10 mM) at pH 4.0 (A) and pH 6.0 (B). The cells were grown in YPD pH 6.0 at 30 °C. Mean and standard deviation of three experiments performed independently.

To better characterise this pH dependence, we also carried out viability tests in which we challenged cells with separate stressors, at pH 4.0 in the absence of copper (Fig. 4A), and at pH 6.0 in the presence of 10 mM of copper sulphate (Fig. 4B). In the absence of copper, the acidic pH did not impair the growth of either wild-type or Δ ics3 strains (Fig. 4A). Additionally, in the presence of 10 mM of copper sulphate, when the pH was adjusted to 6.0, both wild-type and Δ ics3 strains grew at the same rate as in the control without copper sulphate (Fig. 4B). These results indicate that the cellular response to copper depends on low pH, since the sensitivity of the Δ ics3 mutant was not observed in pH 6.0. Despite the suggestion that Δ ics3 cells are sensitive to hydrochloric and acetic acids, the present data indicate they are not sensitive to pH 4.0 in the absence of copper. One possible explanation for this apparent inconsistency is that previous experiments reached lower pH values (2.6 with hydrochloric acid and 3.3 with acetic acid) (Kawahata et al., 2006).

To investigate the dependence of copper sensitivity on pH, the knockout for the gene *YIR004W/ICS1* (Δ ics1) was also analysed, since this strain had been shown to be even more sensitive to copper than the Δ ics3 mutant (Entian et al., 1999). We performed viability tests for Δ ics1 cells after 8 h of incubation in the presence of 7 mM or 10 mM of copper sulphate at pH 4.0 (Fig. 5). The Δ ics1 strain was significantly more sensitive to copper at pH 4.0 than Δ ics3, since it suffered even with 7 mM of copper sulphate, whereas Δ ics3 suffered only at 10 mM concentration. When the pH was adjusted to 6.0, however, the Δ ics1 strain behaved identically to the wild-type (Fig. 5). These results indicate that both Δ ics1 and Δ ics3 strains are copper-sensitive only in acidic conditions.

One possible explanation for the sensitivity of the Δ ics3 strain to copper and oxidant exposure could be an increase in the accumulation of intracellular copper. Higher levels of copper could increase the ratio of free metals that can catalyse the generation of toxic levels of reactive oxygen species through Fenton's reaction, or impair the activity of proteins dependent on iron-sulphur clusters. Additionally, copper accumulation may depend on pH, since acidic conditions have been shown to induce genes involved in metal metabolism and homeostasis in yeast (Kawahata et al., 2006).

To analyse the concentration of copper in wild-type and Δ ics3 cells, we performed atomic emission spectroscopy experiments with cells previously incubated with 10 mM of copper sulphate and with control cells. The copper content in wild-type cells in control conditions (grown only in YPD) was significantly different at pH 4.0 and pH 6.0 (Fig. 6A), with three times more copper in acidic pH. The difference in copper content for Δ ics3 cells at pH 4.0 and 6.0 was not significant (Table 1). However, Δ ics3 cells at both pH values accumulate the same amount of copper as wild-type cells at pH 4.0 (Fig. 6A), indicating that the Δ ics3 strain has abnormal copper homeostasis even in neutral conditions. This threefold greater amount of copper at pH 6.0 for Δ ics3 cells in comparison to wild-type cells is consistent with their sensitivity to oxidant exposure in optimal growth conditions (Fig. 1).

When the cells were incubated with 10 mM of copper sulphate before metal content determination (Fig. 6B), the levels of copper for both wild-type and Δ ics3 strains were similar at pH 6.0, whereas at pH 4.0, the levels of copper for the Δ ics3 strain were higher than those of the wild-type (Fig. 6B, Table 1). These results are in agreement with those from the viability tests, since the bioaccumulation of copper was greater at lower pH, especially for Δ ics3 cells, leading to copper sensitivity.

Copper and iron homeostasis are linked: in the cell membrane, both iron and copper are reduced by iron/copper reductases before uptake, and the high-affinity iron uptake system requires the copper containing oxidase Fet3 (Freitas et al., 2003; Rae et al., 1999). In order to verify whether *ICS3* deletion influences iron homeostasis,

Fig. 4. Sensitivity to acidic pH. (A) Wild-type and Δ ics3 strains were grown in YPD medium with pH adjusted to 4.0 with HCl. After 8 h, the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. (B) Wild-type and Δ ics3 strains were grown in YPD medium in the absence (control) or presence of 10 mM of CuSO_4 . The pH of the medium was adjusted to 6.0 with NaOH. After 8 h of incubation at 30 °C, the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. The image is representative of at least three independent experiments.

we also determined the iron content. Iron levels were always higher at pH 4.0 than at pH 6.0 (Fig. 6C and D), however, the ability to accumulate iron in acidic pH was impaired in Δ ics3 in control conditions (Fig. 6C – Table 2). Interestingly, the iron accumulation of the Δ ics3 strain with respect to the wild-type varied in different pH conditions. At pH 6.0, the Δ ics3 strain had less than half of the content of the wild-type after copper treatment (Fig. 6C and D). At pH 4.0, on the other hand, the iron concentration of Δ ics3 treated cells was almost 30% greater than the wild-type (Fig. 6D). These results indicate that the role of *ICS3* in copper homeostasis is dependent on the pH; this may directly or indirectly influence iron homeostasis. All these features together make mutant cells sensitive to oxidant agents.

Because *ICS3* appears to be involved in copper homeostasis in a pH-dependent manner, we carried out a Northern blot analysis to

Fig. 5. Sensitivity to copper is dependent on pH. The wild-type and Δ ics1 strains were grown in YPD media in the absence (control), or presence of 7 mM or 10 mM of CuSO_4 , as indicated. In the last two lines at the bottom of the figure, the pH of the medium was adjusted to 6.0 with the addition of NaOH. After 8 h of incubation at 30 °C, the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. The images are representative of at least three independent experiments.

determine whether the expression of *ICS3* is regulated by copper-dependent transcription factors, such as Ace1 or Mac1, or by Rim101, which is involved in the response to alkaline pH stress (Mira et al., 2009). *ICS3* expression is induced at acidic pH in wild-type, and this increase is more pronounced in Δ rim101. In relation to copper exposure, we did not observe a clear effect of the copper-related transcriptional factors Ace1 and Mac1 (Fig. 7).

Because the pH and copper affect the vacuole, and previous results have shown *ICS3* has a role in protein trafficking Golgi–endo some–vacuole (Norambuena et al., 2008; Copic et al., 2009), we also studied the expression of the gene encoding the copper transporting P1-type ATPase, CCC2. CCC2 gene expression was increased in Δ ics3 and Δ rim101 cells only at pH 4.0. Interestingly, CCC2 expression in Δ ics3 at pH 4.0 is slightly higher in the presence of copper than in its absence. This suggests a close relationship between *ICS3* and CCC2 expression.

4. Discussion

Although the genome of *S. cerevisiae* was sequenced 19 years ago, the biological and molecular functions of more than 20% of its ORFs (“open reading frames”) remain unknown. The *ICS3* gene was identified in an initiative to characterise the still unknown oxidative stress responsive genes in yeast (Costa and Monteiro, 2014). Prior to this study, the function of *ICS3* was poorly characterised in wide scale genes studies (Entian et al., 1999; Norambuena et al., 2008; Kawahata et al., 2006; Copic et al., 2009). This paper presents a more detailed characterisation of the function of *ICS3* and an investigation of the increased sensitivity of the mutant strain to copper exposure.

We first observed that Δ ics3 cells show increased sensitivity to hydrogen peroxide (Fig. 1). Our data also indicate that *ICS3* is involved in copper homeostasis, and more specifically, that Δ ics3 cells are sensitive to copper only in acidic conditions. We also observed pH-dependent copper sensitivity in wild-type and Δ ics1 strains, suggesting that this is a common yeast cell phenomenon. However, it is important to consider that in pH 4.0, copper sulphate solubility is higher than in pH 6.0, turning the copper availability increased to yeast in acidic culture media (Gadd and Griffiths, 1978). This can explain the data observed in wild-type strain (and perhaps Δ ics1, but it was not analysed), however Δ ics3 cells accumulate more copper even in the lowest availability condition (without pre-treatment with copper and in pH 6.0, Fig. 6A).

In this regard, it was observed that the accumulation of both copper and iron is greater in acidic conditions. This result agrees with a recent study aimed at identifying genes related to copper homeostasis, which showed enrichment for the Gene Ontology (GO) processes “Golgi to vacuole transport and vacuolar acidification” (Schlecht et al., 2014). These authors also observed that when respiratory growth is impaired by alkaline media, the growth ratio can be rescued by the addition of copper sulphate (Schlecht et al., 2014). Together, the data suggest that the relationship of the copper response to pH may be mediated by vacuolar processes.

We note that deletion of the *ICS3* gene leads to an impairment in copper homeostasis and in the ability of yeast to increase iron to wild-type levels in acidic conditions (Fig. 6 and Table 2). At pH 6.0, Δ ics3 cells possess a threefold higher endogenous copper content than wild-type cells. This explains why these cells are more sensitive to oxidative exposure, since there is more copper available to participate in Fenton’s reaction (Farrugia and Balzan, 2012) and to damage iron–sulphur clusters in proteins (Macomber and Imlay, 2009; Foster et al., 2014).

Under exposure to high copper concentrations at pH 4.0, Δ ics3 cells accumulated higher copper levels than the wild-type. At pH 6.0, conversely, the levels of copper were the same for both

Fig. 6. Atomic emission spectroscopy analysis of copper (A and B) and iron (C and D) contents of wild-type and Δ ics3 strains at pH 4.0 or 6.0, previously incubated in the absence (A and C) or presence of copper sulphate (B and D) for 20 h. Mean and standard deviation of three experiments performed independently.

Table 1
Statistical analyses of the copper content difference between the strains tested.

	Unpaired test <i>t</i> significant	<i>p</i> < 0.05
Control conditions	pH 4.0	pH 6.0
WT × WT	Y, <i>p</i> = 0.00062	
Δ ics3 × Δ ics3	N	
WT × Δ ics3	N	Y, <i>p</i> = 0.0004
Copper treated		
WT × WT	Y, <i>p</i> < 0.0001	
Δ ics3 × Δ ics3	Y, <i>p</i> < 0.0001	
WT × Δ ics3	Y, <i>p</i> < 0.0001	N

Y = yes, N = no.

wild-type and Δ ics3 strains after incubation with copper. We observed that the viability and the growth ratio of Δ ics3 cells

Table 2
Statistical analyses of the iron content difference between the strains tested.

	Unpaired test <i>t</i> significant, <i>p</i> < 0.05	
Control conditions	pH 4.0	pH 6.0
WT × WT	Y, <i>p</i> < 0.0001	
Δ ics3 × Δ ics3	Y, <i>p</i> < 0.0001	
WT × Δ ics3	Y, <i>p</i> < 0.0001	Y, <i>p</i> < 0.0001
Copper treated		
WT × WT	Y, <i>p</i> = 0.0139	
Δ ics3 × Δ ics3	Y, <i>p</i> < 0.0001	
WT × Δ ics3	Y, <i>p</i> = 0.0014	Y, <i>p</i> = 0.0012

Y = yes, N = no.

decreased only in acidic conditions, which is consistent with the metal accumulation we reported. It is important to note that the vacuole uptake of metal ions is carried out by H⁺/ion antiport systems (Klionsky et al., 1990), suggesting that when H⁺ concentration is high, the vacuole contains abundant protons that impair the antiport of copper, resulting in less vacuolar copper storage. The vacuole morphology of Δ ics3 cells has already been characterised as abnormal (Michaillat and Mayer, 2013).

Copper is known to be required for iron homeostasis in yeast, plants and mammals (Freitas et al., 2003). *S. cerevisiae* takes up iron from the environment through multiple mechanisms. One of these,

Fig. 7. *ICS3* regulation. Northern blot analysis of *ICS3* and *CCC2* in cells grown in YPD at pH 4.0 or pH 6.0, in the presence (+) or absence (–) of 0.5 mM of CuSO₄. The image shows ribosomal RNA (rRNA) as loading reference.

the high-affinity iron uptake system, is active in iron deprivation conditions and requires the copper-containing oxidase Fet3, which oxidises Fe^{2+} to Fe^{3+} which, in turn, is transported through the plasma membrane by the Ftr1 permease (Askwith et al., 1994; Stearman et al., 1996). Our results show that at pH 6.0, non-treated Δics3 cells accumulate more copper but less iron than wild-type cells. At pH 6.0, both wild-type and Δics3 cells incubated with copper accumulate more copper, but, again, less iron than in non-treated cells, especially for the Δics3 strain (Fig. 6). One possible explanation for the lower iron levels under copper-replete conditions is the competition between copper and iron ions for the low-affinity iron transport protein Fet4, which may also transport metals other than iron (Dix et al., 1994). Additionally, iron and copper are both substrates for the reductases Fre1 and Fre2 in *S. cerevisiae*, which reduce Fe^{3+} and Cu^{2+} before they are transported through the plasma membrane by their respective transporters (Freitas et al., 2003). Nevertheless, metals in excess impair metalloenzyme functions by competing with and replacing the optimal metal cofactors (Argüello et al., 2012). There should be a balance: copper is necessary for iron uptake, but if the copper concentration is too high, there should be competition between copper and iron for Fre1/Fre2 and Fet4 proteins, decreasing iron uptake.

In acidic conditions, the profile is different from that described for neutral conditions. Non-treated wild-type cells accumulate more copper and iron at pH 4.0 than Δics3 cells. However, when incubated with copper at pH 4.0, Δics3 treated cells accumulate more copper and iron than wild-type cells. The higher metal levels observed in acidic conditions are possibly related to a higher electrochemical potential in the vacuolar membrane at lower pH (Cyert and Philpott, 2013). It was shown that yeast cells with mutations in genes essential for vacuolar structure or acidification have defects in copper detoxification, and that normal vacuolar function and integrity are necessary to retain normal iron and copper homeostasis in yeast (Szczyńska et al., 1997).

Our data show that *ICS3* copper response depends directly on pH. Therefore, we also examined the transcriptional regulation of *ICS3* in response to alterations in copper concentration and pH (Fig. 7). We observed that pH is the main determinant of the transcriptional response, since the expression of *ICS3* was induced in acidic pH in wild-type cells and enhanced with deletion of *RIM101*, the alkaline-responsive transcriptional repressor. We did not observe changes in transcription with 0.5 mM of copper in rich medium. Lower doses of copper were used to preserve cell viability for mRNA analyses. We also tested the transcriptional activators responsive to copper Ace1 and Mac1, but we did not observe a clear effect of these factors on *ICS3* expression. In summary, the extracellular pH is a critical factor regulating the expression of this gene.

The description of the *ICS3* gene in *Saccharomyces Genome Database* (SGD: <http://www.yeastgenome.org/locus/S000003613/overview>), based on Copic et al. (2009) and Norambuena et al. (2008), suggests that the protein encoded by this gene is involved in secretory protein trafficking (Golgi-endosome-vacuole). A potential candidate to connect copper and pH to protein vacuolar sorting is the Ccc2 P1-type ATPase which uses copper, increased at acidic pH (Fig. 6A), to metallate the multicopper oxidase Fet3 into Golgi apparatus, rendering its secretion to extracellular media. For this reason, we also analysed the expression pattern of the *CCC2* gene (Fig. 7). Interestingly, *CCC2* gene expression increases in Δics3 cells, suggesting a close relationship between their metabolic functions. *CCC2* and *ICS3* expression patterns are similar in response to *RIM101*, since both are induced in Δrim101 cells only at acidic pH. Again, the presence of copper was not a critical regulator of *CCC2* expression, although it seems slightly increased in Δics3 and Δace1 cells exposed to copper at pH 4.0.

The present data demonstrate that Δics3 cells are sensitive to copper only in acidic extracellular pH, due to, at least, two important effectors: Ccc2 and Rim101. Ccc2 is augmented in Δics3 cells, which can lead to enhanced post-translational modification of the metal oxidase Fet3 that is secreted. This increases the amount of substrate available to the transmembrane permease Ftr1, increasing the activity of the high affinity metal transport system, leading to the observed metal accumulation. Furthermore, *CCC2* and *ICS3* present similar expression profiles in response to pH and to Rim101 regulon. Ccc2p is described as a copper exporter from the cytosol and is necessary for uptake of iron, which is important for metal homeostasis (Fu et al., 1995).

In conclusion, the data presented suggest an intersection between Ccc2 and Ics3 pathways. Additional experiments, such as the investigation of the sensitivity to copper in the double mutant $\Delta\text{ics3}\Delta\text{ccc2}$, or complementation of function between these genes, could be helpful to elucidate this hypothesis. Clearly, a complex cellular mechanism involving vacuolar conditions, pH, and *ICS3* is critical for maintaining the interconnected homeostasis of iron and copper in yeast.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank to Prof Dr Luiz Carlos Martins das Neves for technical support and advice. Financial support was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Grant 2009/01303-1) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

- Adamo, G.M., Lotti, M., Tamás, M.J., Brocca, S., 2012. Microbiology 158, 2325–2335.
- Argüello, J.M., Raimunda, D., González-Guerrero, M., 2012. J. Biol. Chem. 287, 13510–13517.
- Arino, J., 2010. J. Integr. Biol. 14, 517–523.
- Askwith, C., Eide, D., Van Ho, A., Bernard, P.S., Li, L., Davis-Kaplan, S., Sipe, D.M., Kaplan, J., 1994. Cell 76, 403–410.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.A., Smith, J.A., Struhl, K., 1994. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York, 2.5.1–2.6.7, 2.9.1–2.10.8, 3.0.3–3.1.6, 3.4.1–3.16.10, 13.0.3–13.13.7.
- Bleackley, M.R., MacGillivray, R.T.A., 2011. Biometals 24, 785–809.
- Cohen, A., Nelson, H., Nelson, N., 2000. J. Biol. Chem. 275, 33388–33394.
- Copic, A., Dorrington, M., Pagant, S., Barry, J., Lee, M.C.S., Singh, I., Hartman, J.L., Miller, E.A., 2009. Genetics 182, 757–769.
- Costa, I.M., Monteiro, G., 2014. Protein Purification and Analysis I – Methods and Applications, first ed., 1. iConcept Press, Hong Kong, 271–292.
- Cyert, M.S., Philpott, C.C., 2013. Genetics 193, 677–713.
- Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., Klausner, R.D., 1994. Cell 76, 393–402.
- De Rome, L., Gadd, G.M., 1987. FEMS Microbiol. Lett. 43, 283–287.
- Dix, D.R., Bridgman, J.T., Broderius, M.A., Byersdorfer, C.A., Eide, D.J., 1994. J. Biol. Chem. 269, 26092–26099.
- Eide, D.J., 1998. Annu. Rev. Nutr. 18, 441–469.
- Eide, D.J., Bridgman, J.T., Zhao, Z., Mattoon, J.R., 1993. Mol. Gen. Genet. 241, 447–456.
- Entian, K.D., Schuster, T., Hegemann, J.H., Becher, D., Feldmann, H., Guldener, U., Gotz, R., Hansen, M., Hollenberg, C.P., Jansen, G., Kramer, W., Klein, S., Kotter, P., Kricke, J., Launhardt, H., Mannhaupt, G., Maierl, A., Meyer, P., Mewes, W., Munder, T., Niedenthal, R.K., Ramezani Rad, M., Röhrer, A., Römer, A., Rose, M., Schafer, B., Siegler, M.L., Vetter, J., Wilhelm, N., Wolf, K., Zimmermann, F.K., Zollner, A., Hinnen, A., 1999. Mol. Gen. Genet. 262, 683–702.
- Farrugia, G., Balzan, R., 2012. Front. Oncol. 2. <http://dx.doi.org/10.3389/fonc.2012.00064>.
- Foster, A.W., Dainty, S.J., Patterson, C.J., Pohl, E., Blackburn, H., Wilson, C., Hess, C.R., Rutherford, J.C., Quaranta, L., Corran, A., Robinson, N.J., 2014. Mol. Microbiol. 93, 317–330.
- Freitas, J., Wintz, H., Kim, J.H., Poynton, H., Fox, T., Vulpe, C., 2003. Biometals 16, 185–197.
- Fu, D., Beeler, T.J., Dunn, T.M., 1995. Yeast 11, 283–292.
- Gadd, G.M., Griffiths, A.J., 1978. Microb. Ecol. 4, 303–317.

- Georgatsou, E., Mavrogiannis, L.A., Fragiadakis, G.S., Alexandraki, D., 1997. *J. Biol. Chem.* 272, 13786–13792.
- Hamer, D.H., Theile, D.J., Lemontt, J.E., 1985. *Science* 228, 685–690.
- Hassett, R., Kosman, D.J., 1995. *J. Biol. Chem.* 270, 128–134.
- Hassett, R., Dix, D.R., Eide, D.J., Kosman, D.J., 2000. *Biochem. J.* 351, 477–484.
- Kawahata, M., Masaki, K., Fujii, T., Iefuji, H., 2006. *FEMS Yeast Res.* 6, 924–936.
- Keller, G., Bird, A., Winge, D.R., 2005. *Eukaryot. Cell* 4, 1863–1871.
- Klionsky, D.J., Herman, P.K., Emr, S.D., 1990. *Microbiol. Rev.* 54, 266–292.
- Lamb, T.M., Mitchell, A.P., 2003. *Mol. Cell. Biol.* 23, 677–686.
- Lin, C.M., Kosman, D.J., 1990. *J. Biol. Chem.* 265, 9194–9200.
- Ma, Z., Jacobsen, F.E., Giedroc, D.P., 2009. *Chem. Rev.* 109, 4644–4681.
- Macomber, L., Imlay, J.A., 2009. *Proc. Natl. Acad. Sci. USA* 106, 8344–8349.
- Michaillat, L., Mayer, A., 2013. *PLoS One* 8, e54160.
- Mira, N.P., Lourenço, A.B., Fernandes, A.R., Becker, J.D., Sa-Correia, I., 2009. *FEMS Yeast Res.* 9, 202–216.
- Norambuena, L., Zouhar, J., Hicks, G.R., Raikhel, N.V., 2008. *BMC Chem. Biol.* 8, 1. <http://dx.doi.org/10.1186/1472-6769-8-1>.
- O'Halloran, T.V., Culotta, V.C., 2000. *J. Biol. Chem.* 275, 25057–25060.
- Rae, T.D., Schmidt, P.J., Pufahl, R.A., Culotta, V.C., O'Halloran, T.V., 1999. *Science* 284, 805–808.
- Robinson, N.J., Winge, D.R., 2010. *Annu. Rev. Biochem.* 79, 537–562.
- Schlecht, U., Suresh, S., Xu, W., Aparicio, A.M., Chu, A., Proctor, M.J., Davis, R.W., Scharfe, C., St Onge, R.P., 2014. *BMC Genomics* 15, 263.
- Schuller, A., Auffermann, G., Zoschke, K., Schmidt, U., Ostermann, K., Rödel, G., 2013. *Yeast* 30, 201–218.
- Serrano, R., Bernal, D., Simon, E., Arino, J., 2004. *J. Biol. Chem.* 279, 19698–19704.
- Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D., Dancis, A., 1996. *Science* 271, 1552–1557.
- Szczyпка, M.S., Zhu, Z., Silar, P., Thiele, D.J., 1997. *Yeast* 13, 1423–1435.
- Thiele, D.J., 1988. *Mol. Cell. Biol.* 8, 2745–2752.

ANEXO 5

RESEARCH ARTICLE

Chemogenomic Study of Carboplatin in *Saccharomyces cerevisiae*: Inhibition of the NEDDylation Process Overcomes Cellular Resistance Mediated by HuR and Cullin Proteins

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Citation: de Sousa GF, Lima MdA, Custodio DF, Freitas VM, Monteiro G (2015) Chemogenomic Study of Carboplatin in *Saccharomyces cerevisiae*: Inhibition of the NEDDylation Process Overcomes Cellular Resistance Mediated by HuR and Cullin Proteins. PLoS ONE 10(12): e0145377. doi:10.1371/journal.pone.0145377

Editor: Yoshikazu Ohya, University of Tokyo, JAPAN

Received: July 28, 2015

Accepted: December 1, 2015

Published: December 21, 2015

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by GFS received grant by Fundação de Amparo à Pesquisa do Estado de São Paulo - grant number 2011/04173-1 to GFS, and by Fundação de Amparo à Pesquisa do Estado de São Paulo - grant numbers 2009/01303-1 and 2015/07749-2 to GM. DFC, MAL and VMF received no specific funding for this work.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

The use of carboplatin in cancer chemotherapy is limited by the emergence of drug resistance. To understand the molecular basis for this resistance, a chemogenomic screen was performed in 53 yeast mutants that had previously presented strong sensitivity to this widely used anticancer agent. Thirty-four mutants were responsive to carboplatin, and from these, 21 genes were selected for further studies because they have human homologues. Sixty percent of these yeast genes possessed human homologues which encoded proteins that interact with cullin scaffolds of ubiquitin ligases, or whose mRNA are under the regulation of Human antigen R (HuR) protein. Both HuR and cullin proteins are regulated through NEDDylation post-translational modification, and so our results indicate that inhibition of this process should sensitise resistant tumour cells to carboplatin. We showed that treatment of a tumour cell line with MLN4924, a NEDDylation inhibitor, overcame the resistance to carboplatin. Our data suggest that inhibition of NEDDylation may be a useful strategy to resensitise tumour cells in patients that have acquired carboplatin resistance.

Introduction

Platinum-based drugs, such as cisplatin, oxaliplatin and carboplatin, are used in the treatment of many of the more aggressive and hard to treat cancers, including those of the lung (non-small and small cell cancers), breast, bowel, oesophagus, testes, cervix and ovaries, as well as non-Hodgkin's lymphoma. Carboplatin is the least toxic of the platinum-based drugs, but like all promoters of DNA damage, its effectiveness reduces on a patient-by-patient basis over multiple chemotherapy cycles due to the emergence of resistance. In this context, understanding

the molecular basis of drug resistance could lead to the clinical ability to overcome acquired resistance in tumours using a resensitising molecule, resulting in an improved treatment outcome.

However, studying these processes directly in human cells is expensive and difficult, and reducing the number of animals used in pre-clinical tests is increasingly demanded, requiring previous directional studies to provide a smaller number of targets to be validated *in vivo* and finally in humans. Yeast chemogenomic studies have been of considerable value in identifying biomarkers indicative of possible human cell responses upon drug exposure [1]. Hillenmeyer *et al.* (2008) [2] published a “yeast genomic portrait”, where more than 1100 chemogenomic assays were used to test the fitness of yeast knockout collections (YKO) when exposed to compound libraries of small molecules, including carboplatin. The entire dataset is in the public domain (<http://chemogenomics.med.utoronto.ca/fitdb/fitdb.cgi>), and so we used this large-scale fitness assay as the preliminary data to select 53 mutant strains that showed the strongest phenotype in response to exposure to the lowest tested concentration of carboplatin.

The growth behaviour and viability of these cells when challenged with carboplatin was analysed in detail. Thirty-four yeast strains (S1 Table) were responsive in the first 24 hours of exposure and were classified in relation to the response of wild-type strain as sensitive or resistant. Among the yeast genes found, 21 possessed human homologues, and these were selected for further systematic analyses. Using bioinformatics tools, it was found 15 human homologue genes whose mRNAs are under the regulation of Human antigen R (HuR; also known as ELAVL1) protein. Additionally, 10 human homologue gene products interact with cullin 1, 2 or 3. The connection between HuR and cullin proteins in the cellular response to carboplatin could be the NEDDylation pathway, since both are regulated by it. NEDDylation is a post-translational modification that modulates the target's activity/function, through the attachment of the NEDD8 molecule. In agreement with this hypothesis, several cancers have shown increased NEDD8 expression and over-activated cullin-RING ligases (CRLs), whose activation is dependent on NEDD8 attachment, suggesting that NEDDylation is closely related to tumorigenesis [3]. We tested this hypothesis by treating an ovarian tumour cell line with MLN4924 –a NEDD8-activating enzyme (NAE) inhibitor–and carboplatin. Our results suggest that the inhibition of NEDDylation could be a promising way to overcome carboplatin resistance.

Materials and Methods

Source and maintenance of yeast cells

Wild-type *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the yeast *Knockout* (YKO) *deletion collection* (Invitrogen, Carlsbad, CA, USA) of non-essential gene knockouts, which is isogenic to BY4741, were used in this study. Cells were grown in YPD medium (2% w/v glucose, 2% w/v peptone, 1% w/v yeast extract, 2% w/v agar added in the case of solid media) or SD complete synthetic medium (0.67% w/v yeast nitrogen base, 0.12% w/v dropout mixture of amino acids, excluding leucine, tryptophan and histidine, 2% w/v glucose, 60 µg/ml leucine, 20 µg/ml histidine, 40 µg/ml tryptophan, 20 µg/ml uracil, pH 6.5). Carboplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and MLN4924 from Merck (Merck-Millipore, Billerica, MA, USA).

Criteria of mutant selection in Hillenmeyer's dataset

Using the data from the “yeast genomic portrait” (<http://chemogenomics.med.utoronto.ca/fitdb/fitdb.cgi>), the yeast mutant strains that presented a fitness defect when exposed to 250 µM carboplatin (the lowest dose tested) were selected [2]. This criterion was imposed so that the most drug-sensitive strains would be chosen. The authors considered a significant

fitness defect when the P-value was lower than 0.01, which means a confidence of 99%. After screening the dataset using these parameters, 53 mutant strains in candidate genes were isolated whose deletions would confer the highest response to carboplatin exposure (S1 Table). The mutant strains selected were organised individually in a 96-well plate. It should be noted that the present study was performed with the YKO haploid collection.

Treatment of yeast cells with carboplatin

The yeast mutant cells were grown in SD medium supplemented with 200 µg/ml geneticin G-418 (USB Affymetrix Brand, Santa Clara, CA, USA) at 30°C for 72 hours with 200 rpm shaking. Cells were diluted to an initial optic density of $OD_{600nm} = 0.0625$ in 96-well plates in SD medium containing or not (control) 10 mM carboplatin. This high drug concentration was chosen because we aimed for a loss of viability and not only a fitness defect. The wild-type strain was grown under the same conditions, but in the absence of G-418. The growth ratio of cells was determined spectrophotometrically using a SpectraMax plate reader at 600 nm (Molecular Devices, Sunnyvale, CA, USA), collecting data every 2 hours for 24 hours. The growth ratios (increase or decrease due to carboplatin exposure) were calculated by obtaining the slope of the OD_{600nm} value versus time curve, during the logarithmic phase, in comparison to the growth ratio of YKO mutant cells and wild-type cells in the absence of carboplatin. The viability of cells after the experiment was determined by sub-culturing 5 µl of each yeast sample for 2 days at 30°C on YPD agar. All the measurements were performed in biological triplicates, and the data were examined using analysis of variance with multiple comparisons (ANOVA) with the statistical software Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA, USA).

Bioinformatics analyses

The selected genes were analysed using the *Saccharomyces* Genome Database (SGD) tool, *YeastMine*–Batch Analysis (<http://yeastmine.yeastgenome.org/yeastmine/>). Using the “Analyse” tab, the list of all genes whose deletion caused some response to carboplatin was inputted. Through the “Homologues” tab, it was possible to retrieve human homologue(s) of yeast gene(s) and any of their associated OMIM (*Online Mendelian Inheritance in Man*) disease phenotypes. Finally, the list of human homologue genes involved in resistance to carboplatin and (separately) the genes involved in sensitivity to carboplatin were inputted into the *Metabolic-Mine* tool (<http://www.metabolicmine.org/>). Each tool presents an internal statistical measurement that is cited when necessary. When the data were compiled and analysed further, a one-sample *t*-test was used (GraphPad Software). Gene Expression Omnibus (GEO) and Oncomine datasets were used to confirm the differential expression of mRNAs thought to be stabilised by HuR in ovarian cancer in human patients. The analyses were performed by comparing Normal x Cancer, specifically for ovarian cancer. Only patients whose mRNA levels presented an increase of $\geq 40\%$ compared to normal tissue levels were considered overexpressers. In addition, the Cancer Genome Atlas study (TCGA 2) present on Oncomine was analysed to observe differences in the DNA copy number of the genes. The Human Protein Atlas and Database of Differentially Expressed Proteins in Human Cancer (dbDEPC 2.0) were used to analyse the expression of cullins, NEDD8, NAE1 and UBE2M proteins.

Ovarian tumour cell line challenge with carboplatin

The cell line ES-2 was developed from a clear-cell carcinoma of the ovary; it has a fusiform morphology. Cells were obtained from the collection of the Rio de Janeiro Cell Bank. ES-2 cells were cultured in Dulbecco’s Modified Eagle’s Medium-F12 (DMEM-F12, Sigma Chemical Co.,

St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil). Cells were maintained in 75-cm² flasks in a humidified atmosphere of 5% CO₂ at 37°C. The ES-2 cell line was exposed to 50 µM carboplatin once a week for two consecutive weeks to create the chemoresistant phenotype.

Treatment of the tumour cell line with carboplatin and MLN4924

ES-2 cells presenting a chemoresistant phenotype or ES-2 control cells (untreated) were plated in 24-well plates (2x10⁵ cells/well). Cells were cultured in DMEM-F12 supplemented with 10% charcoal-stripped FBS for 24 hours. After that, the cells were treated with 500 µM carboplatin (Sigma), 10 nM MLN4924 (Merck) or both substances diluted in phenol red-free DMEM/F12 with 10% FBS for 24 hours. Cells without treatment with carboplatin or MLN4924 served as the control. After 24 hours, the cells were released from the plate with trypsin and counted in a Neubauer chamber; the results are presented as the percentage of viable cells. All the measurements were performed in biological triplicates, and the data were analysed using ANOVA with multiple comparisons with the statistical software GraphPad Prism version 6.05.

Results and Discussion

We tested the growth ratio and cell viability of the 53 selected yeast mutants and observed that 19 were sensitive and 15 resistant after 24 hours of exposure to 10 mM carboplatin (Fig 1). The increase or decrease in the growth ratio caused by carboplatin exposure of each individual strain is quantitatively shown in the lower panels (Fig 1). The difference in the phenotypes observed in this study compared to Hillenmeyer's data—since according to the fitness results, all the strains should be highly sensitive—may be due to variations in the type of yeast collection used, as well as the time and dose of carboplatin exposure applied. The final classification as sensitive or resistant mutant cells was also based on the viability loss, which was measured by the ability of the each individual yeast strain to form colonies in fresh rich solid medium (YPD) after the different periods of drug exposure during growth (Fig 2). Table 1 shows the 34 yeast genes whose deletion caused some phenotypic response to carboplatin exposure, their standard name and the presence or absence of known human homologues (Table 1).

Because our aim was to investigate the human drug response, of the 34 yeast strains, we selected 21 candidate genes that presented human homologues (Table 1). We next used the SGD tool (<http://yeastmine.yeastgenome.org/yeastmine/>) to correlate human diseases to the set of yeast genes responsive to carboplatin and found two human homologue genes that were previously associated with cancer: Kruppel-like factor 6 (KLF6, selected because it is one of the homologues of the yeast gene *RIM101*) and Excision Repair Cross Complementing Group 5 (ERCC5, selected because it is the homologue of the yeast gene *YEN1*) (Table 2). The $\Delta rim101$ (gene *YHL027W/RIM101*) mutant was one of the most sensitive strains observed in this study and lost its viability after only 2 hours of exposure to carboplatin (Fig 2). The KLF6 human homologue of the yeast gene *RIM101* has been implicated in gastric and prostate cancer, tumours that are usually treated with carboplatin. The second yeast mutant possessed the *YEN1* deletion and was resistant to carboplatin. This result is in agreement with the suggestion that the correspondent human homologue ERCC5 is involved in DNA repair and can be used as a biomarker of survival in patients with ovarian cancer treated with carboplatin [4]. Also, specific ERCC5 promoter polymorphisms have been shown to increase the platinum chemotherapy response in patients with advanced non-small-cell lung cancer (NSCLC) [5]. These data help to validate our results obtained using yeast as a primary model cell to identify human genes involved in cancer chemoresistance.

Fig 1. Growth rates of the yeast mutants in response to carboplatin exposure. The cells were thawed and grown in synthetic dextrose minimum media (SD) at 30°C for 72 hours in the presence of the antibiotic geneticin (G418), except for the wild-type strain. These reactivated cells were then normalised to an initial OD_{600nm} of 0.0625 in fresh SD media in the presence (carboplatin treated) or absence (control) of carboplatin 10 mM and the growth rates were spectrophotometrically analysed during 24 hours (see [Material and Methods](#)). These values were quantitatively represented as the percent decrease

(sensitive) or increase (resistant) in the growth ratio in relation to the untreated cell response. The results are presented as mean and SD of three independent experiments, analysed by one-way ANOVA with multiple comparisons in relation to the wild-type strain, $p = 0.0001$, using GraphPad software.

doi:10.1371/journal.pone.0145377.g001

We continued the analysis of the 21 yeast genes and observed that they possess 88 homologous genes in humans (Table 3): 57 human homologues to yeast genes related to resistance and 31 human homologues to yeast genes related to sensitivity. It is important to note that yeast genes whose disruptions led to sensitivity are probably responsible for resistance to carboplatin (nine genes identified: *YBL055C*; *TRM7*; *MUS81*; *RIM101*; *PHO90*; *VPS21*; *AGC1*; *AQY1*), and yeast genes whose disruptions led to resistance may be related to sensitivity to this drug (12 genes identified: *PIN4*; *SAS3*; *UBX3*; *YEN1*; *NSG1*; *MET30*; *DID4*; *KTI12*; *COX12*; *RPL15*; *UBC11*; *OXR1*). This nomenclature will be used hereafter, including for classifying human homologues. Human homologue genes were analysed regarding their chromosome localisation, metabolic pathway, molecular function and interactions using the *MetabolicMine* tool (<http://www.metabolicmine.org/>).

Fig 2. Cell viability analyses during different hours of carboplatin exposure. At the indicated times, aliquots of the yeast mutant cultures in the presence (carboplatin) or absence (control), whose growth are shown in Fig 1, were withdrawn and dripped with a replicator onto a YPD plate, which was incubated at 30°C for 2 days and photographed. These images are representative of at least three biological replicates.

doi:10.1371/journal.pone.0145377.g002

Table 1. Identification of the yeast set selected.

Systematic Name	Standard Name	Human Homolog	Systematic Name	Standard Name	Human Homolog
YAR023C		No	YDL091C	UBX3	Yes
YAR028W		No	YDR386W	MUS81	Yes
YBL051C	PIN4	Yes	YDR494W	RSM28	No
YBL052C	SAS3	Yes	YJL159W	HSP150	No
YBL053W		No	YJL198W	PHO90	Yes
YBL054W	TOD6	No	YLR038C	COX12	Yes
YBL055C		Yes	YLR232W		No
YBR061C	TRM7	Yes	YMR121C	RPL15B	Yes
YER041W	YEN1	Yes	YOL070C	NBA1	No
YHL027W	RIM101	Yes	YOL108C	INO4	No
YHR133C	NSG1	Yes	YOR089C	VPS21	Yes
YIL024C		No	YOR339C	UBC11	Yes
YIL046W	MET30	Yes	YPL196W	OXR1	Yes
YIL055C		No	YPL241C	CIN2	No
YKL002W	DID4	Yes	YPR021C	AGC1	Yes
YKL110C	KTI12	Yes	YPR023C	EEF3	Yes
YKR073C		No	YPR192W	AQY1	Yes

Yeast set selected for further characterisation in this work, previously involved in the cellular response to carboplatin, according to the fitness defect showed in the presence of carboplatin. Total number of genes analysed = 34; genes that possess human homologs = 21 (62%); genes that do not possess human homologs = 13 (38%).

doi:10.1371/journal.pone.0145377.t001

The chromosome distribution of human homologues of genes involved in the carboplatin response is shown in Fig 3. We calculated the mean distribution of genes involved in the carboplatin response in each chromosome. The number of genes presented on the y-axis corresponds to the number of different mutant yeast strains related to the carboplatin response that possess at least one human homologue in the chromosome depicted on the x-axis. The mean distribution of genes related to the carboplatin response was 2.96 genes in each chromosome, taking into account that in some chromosomes the frequency found was zero (i.e. chromosomes 14, 21 and 23). There was a significantly higher frequency of these homologues on chromosomes 1, 2, 3, 7, 8, 17, 20 and X using one-sample *t*-test statistical analysis. This result is in good agreement with previous studies characterising the high-resolution profiling of carboplatin resistance in ovarian cancer cells [6, 7]. In these reports, the authors associated chromosomes 1, 8, 17 and X with the carboplatin response in ovarian cancer. Interestingly, resistance to carboplatin was mainly associated with chromosome 1, and our results show that this chromosome presented the highest number of genes related to the carboplatin response. In addition, genes on human chromosome 1 have previously been associated with the tumour response to platinum-based drug treatment. For example, genes on chromosome 1 have been related to cisplatin resistance in a human neuroblastoma and to sensitivity in human lymphoblastoid cell lines [8, 9]. This supports the ability of yeast cells to indicate good molecular candidates in the characterisation of the cellular response to carboplatin.

The human homologues of yeast carboplatin-response genes were next annotated for biological and molecular functions (Tables 4 and 5). Table 4 shows the genes that were most likely to be involved in the cellular resistance to carboplatin. The main biological process associated by the Gene Ontology term was transport (29 human homologue genes, derived from the following yeast mutant strains: *Δaqy1*; *Δrim101*; *Δvps21*; *Δagc1*; *Δpho90*). The main biochemical

Table 2. Human diseases related to genes involved in the cellular response to carboplatin.

Yeast standard name	Human homolog standard name	Diseases	Datasets
<i>AGC1</i>	SLC25A12	Hypomyelination, global cerebral	Ensembl, Panther, TreeFam
<i>AGC1</i>	SLC25A13	Citrullinemia, typell, neonatal-onset and adult onset—CTLN2	Ensembl, Panther, TreeFam
<i>AGC1</i>	SLC25A22	Epileptic encephalopathy early infantile - 3EIEE3	Ensembl, Panther, TreeFam
<i>AQY1</i>	AQP1	Blood group—Colton CO	Ensembl, Panther
<i>AQY1</i>	AQP2	Diabetes insipidus, Nephrogenic, autosomal	Ensembl, Panther, TreeFam
<i>AQY1</i>	AQP3	Gill blood group	Panther
<i>AQY1</i>	AQP5	Palmoplantar keratoderma, Bothnian type PPKB	HomoloGene, TreeFam
<i>AQY1</i>	AQP7	Glycerol quantitative trait locus—GLYCQTL	Panther
<i>AQY1</i>	MIP	Cataract 15, Multiple types—CTRCT15	Ensembl, Panther, TreeFam
<i>DID4</i>	CHMP2B	Frontotemporal dementia, chromosome 3-linked FTD3 and amyotrophic lateral sclerosis 17 ALS17	Ensembl
<i>RIM101</i>	KLF1	Blood group- Lutheran inhibitor INLU; anemia, dyserythropoietic congenital, type IV—CDAN4; and fetal hemoglobin quantitative trait locus 6—HBFQTL6	Panther
<i>RIM101</i>	KLF6	Gastric cancer, intestinal included and prostate cancer	Panther
<i>RIM101</i>	SP7	Osteogenesis imperfecta, type XII—OI12	Panther
<i>RPL15B</i>	RPL15	Diamond–Blackfan anemia 12 DBA12	Ensembl, HomoloGene, Panther, TreeFam
<i>TRM7</i>	FTSJ1	Mental retardation, X-linked 9 -MRX9	Ensembl, HomoloGene, Panther
<i>YEN1</i>	ERCC5	Xeroderma pigmentosum, complement group GXP G	Panther

The analysis was performed using the tool YeastMine: Advanced Search—HOMOLOGY—Yeast gene— OMIM human homolog(s) — OMIM Disease Phenotype(s) in the SGD site www.yeastgenome.org.

doi:10.1371/journal.pone.0145377.t002

pathway found 11 genes associated with transmembrane transport of small molecules. The main molecular function of 16 genes was substrate-specific transmembrane transporter activity. Not surprisingly, these data suggest that cell resistance to carboplatin is related to import/export of the drug, which is in agreement with a review of the literature on the effects of ion channels and aquaporin expression in ovarian cancer [10]. Xuejun *et al.* [11] demonstrated that when the ovarian cancer cell line SKOV3 is treated with mercuric chloride, which blocks aquaporins, the cells become more sensitive to cisplatin, thereby implicating aquaporins in resistance to platinum-based drugs, similarly to the results observed here (Table 4).

Otherwise, Table 5 shows the genes that are most likely to be involved in cell sensitivity to carboplatin. The results indicate that two main biological processes appear to be involved in cell sensitivity: the sterol regulatory element-binding protein (SREBP) signalling pathway (related to the function of three human homologue genes, derived from the following yeast strains: *Ans1*; *Δmet30*) and the cellular macromolecule catabolic process (involving nine human homologue genes, derived from the following yeast mutant strains: *Apin4*; *Δmet30*; *Δyen1*; *Δubx3*; *Δrpl15b*; *Δubc11*). No significant correlation was found with molecular function or regulatory systems. However, the main pathway related to sensitivity was ubiquitin-mediated proteolysis, which is reinforced by the observation that an increased burden of quality-control systems has been associated with aberrant DNA-copy number in yeast and mammals

Table 3. Human homologues identified in the study of yeast carboplatin response.

Yeast gene involved in resistance	Human homolog	Yeast gene involved in resistance	Human homolog	Yeast gene involved in sensitivity	Human homolog
AQY1	AQP1	AQY1	MIP	MET30	BTRC
AQY1	AQP2	EAF3	MORF4L1	DID4	CHMP2A
AQY1	AQP3	EAF3	MORF4L2	DID4	CHMP2B
AQY1	AQP4	EAF3	MSL3	COX12	COX6B1
AQY1	AQP5	MUS81	MUS81	COX12	COX6B2
AQY1	AQP7	VPS21	RAB17	YEN1	ERCC5
AQY1	AQP8	VPS21	RAB20	UBX3	FAF1
AQY1	AQP9	VPS21	RAB22A	UBX3	FAF2
AQY1	AQP10	VPS21	RAB31	MET30	FBXW11
AQY1	FAM188B	VPS21	RAB5A	MET30	FBXW2
TRM7	FTSJ1	VPS21	RAB5B	MET30	FBXW7
TRM7	FTSJ2	VPS21	RAB5C	NSG1	INSIG1
RIM101	KLF1	PHO90	SLC13A1	NSG1	INSIG2
RIM101	KLF2	PHO90	SLC13A2	SAS3	KAT5
RIM101	KLF3	PHO90	SLC13A3	SAS3	KAT7
RIM101	KLF4	PHO90	SLC13A4	KT112	KT112
RIM101	KLF5	PHO90	SLC13A5	OXR1	NCOA7
RIM101	KLF6	AGC1	SLC25A12	OXR1	OXR1
RIM101	KLF7	AGC1	SLC25A13	PIN4	PABPC1
RIM101	KLF8	AGC1	SLC25A18	PIN4	PABPC1L
RIM101	KLF9	AGC1	SLC25A22	PIN4	PABPC4
RIM101	KLF10	AGC1	SLC25A44	PIN4	PABPC4L
RIM101	KLF11	RIM101	SP5	PIN4	PABPC5
RIM101	KLF12	RIM101	SP6	RPL15B	RPL15
RIM101	KLF13	RIM101	SP7	PIN4	SYNCRIP
RIM101	KLF14	YBL055C	TATDN1	OXR1	TLDC2
RIM101	KLF15	YBL055C	TATDN2	MET30	TRAF7
RIM101	KLF16	YBL055C	TATDN3	UBC11	UBE2C
RIM101	KLF17			UBC11	UBE2U

doi:10.1371/journal.pone.0145377.t003

[12], indicating that this pathway is intimately related to tumour biology and, in particular, the response to carboplatin.

Finally, the analyses of physical interactions between molecules provided interesting results; it was intriguing to note that the HuR protein was implicated in both resistance and sensitivity responses to carboplatin exposure (Tables 4 and 5). HuR is a member of the embryonic lethal abnormal vision 1 (ELAV1 or ELAVL1) family and is primarily localised in the nucleus, but under several types of stress is also translocated to the cytoplasm. In the cytoplasm, HuR binds to the 3'-untranslated region of target mRNAs, resulting in improved translation and stability. HuR activation has been observed in response to several cancer-related stressors, such as exposure to gemcitabine, tamoxifen, staurosporine, UV light and actinomycin D, indicating that the HuR protein primarily functions as a cytoprotective mechanism in tumour cells [13]. Recently, Lal *et al.* [14] observed that tumour cell treatment with carboplatin causes HuR translocation from the nucleus to the cytoplasm, showing that this drug modulates HuR activity and suggesting a tumour pro-survival function of HuR during carboplatin exposure.

Fig 3. Chromosome distribution of human homologues predicted to be involved in carboplatin response. The graph was constructed using human chromosome localisation (retrieved from *metabolicmine* analysis) of each gene that presented different yeast gene deletions that caused the phenotype, i.e. chromosome 1 possesses at least eight different human genes corresponding to eight different yeast mutant strains. The arithmetic mean distribution of genes was calculated by the sum of total human genes that possess different yeast homologues divided by the number of chromosomes. The mean distribution was 2.958 genes per chromosome. We calculated the chromosomes that presented an accumulation of genes responsive to carboplatin considering statistical significance in relation to the mean of $p = 0.0047$ using a one-sample *t*-test.

doi:10.1371/journal.pone.0145377.g003

In addition, a genome-wide screen that used systematic overexpression of 12,200 pooled human ORFs in the human cell line HEK293 was carried out to understand drug mode-of-action and resistance mechanisms. The authors found that four main human ORFs that were overexpressed provide protection against cisplatin cytotoxicity: MAPK kinase 1 interacting

Table 4. Metabolic pathways of human homologue genes predicted to be involved in the cellular resistance to carboplatin exposure.

Biological Process		
GO term	P value	MATCHES
transport [GO:0006810]	0.026101	AQP1; AQP2; AQP3; AQP4; AQP5; AQP7; AQP8; AQP9; AQP10; MIP; SLC25A12; SLC25A13; SLC25A22; RAB5A; RAB5B; RAB5C; RAB17; RAB20; RAB22A; RAB31; KLF13; KLF14; KLF15; KLF16; SLC13A1; SLC13A2; SLC13A3; SLC13A4; SLC13A5
Pathway Enrichment		
Pathway	P value	MATCHES
Transmembrane transport of small molecules	0.003694	AQP1; AQP2; AQP3; AQP4; AQP7; AQP9; SLC13A1; SLC13A2; SLC13A3; SLC13A4; SLC13A5
Molecular Function		
substrate-specific transmembrane transporter activity [GO:0022891]	1.799845e-4	AQP1; AQP2; AQP3; AQP4; AQP5; AQP7; AQP8; AQP9; MIP; SLC13A1; SLC13A3; SLC13A4; SLC13A5; SLC25A12; SLC25A13; SLC25A22
Interactions		
Physical interaction	ELAVL1 protein	AQP3; KLF2; KLF5; KLF9; KLF11; MORF4L1; RAB5A; RAB5B; RAB22A; RAB31; SLC25A44

Source *MetabolicMine*. The P-value was calculated using the Hypergeometric distribution.

doi:10.1371/journal.pone.0145377.t004

Table 5. Metabolic pathways of human homologue genes predicted to be involved in the cellular sensitivity to carboplatin exposure.

Biological Process		
GO term	P value	MATCHES
SREBP signaling pathway [GO:0032933]	0.002740	INSIG1; INSIG2; FBXW7
cellular macromolecule catabolic process [GO:0044265]	0.005467	BTRC; ERCC5; FAF1; FBXW7; FBXW11; PABPC1; PABPC4; RPL15; UBE2C
Pathway Enrichment		
Pathway	P value	MATCHES
Ubiquitin mediated proteolysis	0.017076	UBE2C; UBE2U; FBXW7; FBXW11; BTRC
Molecular Function		
NO ENRICHMENT		
Interactions		
Physical interaction	CULLIN (S) 1, 2 and 3	SYNCRIP; FAF1; FAF2; FBXW11; FBXW2; FBXW7; PABPC1; PABPC4; RPL15; BTRC
Physical interaction	ELAVL1 protein	UBE2C; FAF2; PABPC1; PABPC4; INSIG1

Source *MetabolicMine*. The P-value was calculated using the Hypergeometric distribution.

doi:10.1371/journal.pone.0145377.t005

protein 1—MAP2K1IP1, monocyte to macrophage differentiation-associated protein—MMD, protein tyrosine phosphatase, non-receptor type 2—PTPN2 and Rhox homeobox family, member 2—RHOXF2 [15]. Analysing the interactors of these ORFs that are implicated in platinum-based drug resistance, by a completely different approach, using the *MetabolicMine* tool, it was found that MAP2K1IP1 and MMD mRNAs interacted with HuR, reinforcing the present hypothesis that HuR mediates platinum-drug resistance.

If HuR is important in ovarian cancer survival and the carboplatin response, the mRNAs shown in Table 4 should be stabilised by HuR and consequently show overexpression in clinical cases of ovarian cancer. To test this hypothesis, we used two large datasets of human gene expression—Gene Expression Omnibus and Oncomine—to analyse the mRNA levels of the SLC25A44, RAB22A, RAB5A, RAB5B, AQP3, KLF2 and KLF5; the mRNAs of these genes have been observed in physical interaction with the HuR protein (S2 Table). Fig 4 shows the percentage of patients with ovarian cancer that presented at least a 40% increase in the mRNA level when compared to the level in the respective normal tissue. It was possible to analyse 970 or 998 patients with ovarian cancer using these datasets; markedly, four genes showed a high frequency of overexpression: SLC25A44, RAB5B, AQP3 and KLF5. RAB5B and KLF5 have been previously characterised as being in the top 4% and 9%, respectively, of genes overexpressed in ovarian cancer [16, 17]. In addition, AQP3, also called aquaglyceroporin-3, has been recently related to the estrogenic response driving cell migration and cell invasion in breast cancer [18]. In a study comparing patients who were either sensitive or chemoresistant to carboplatin, our results were again reinforced with the overexpression found for AQP3, KLF5, RAB5A and RAB22A in chemoresistant patients [19]. Finally, an interesting gene not previously involved in ovarian cancer, SLC25A44, presented a higher frequency of overexpression in 96% of the 970 patients with analysed ovarian tumours. SLC25A44 belongs to the mitochondrial solute carrier family; the gene is localised on chromosome 1 and its molecular function is unknown (UniProtKB—Q96H78).

Otherwise, ten human homologues of yeast carboplatin-responsive genes were annotated as interacting physically with cullin proteins (Table 5 and S2 Table). Cullins are scaffold proteins that bind to an adaptor protein, a substrate receptor protein and a RING protein, assembling

Fig 4. mRNA expression analyses of genes supposed to be stabilised by HuR. The bioinformatics analysis was performed using datasets present in Gene Expression Omnibus and Oncomine. It was considered as overexpression in ovarian cancer just increasing in mRNA level $\geq 40\%$ of expression level in normal tissue (control). The number (N) depicted above each bar is the total of patients that presented overexpression of each gene; the N_{TOTAL} represents the total number of patients studied for each gene.

doi:10.1371/journal.pone.0145377.g004

cullin-RING ligases (CRLs)—the largest family of E3 ubiquitin ligases. E3 ubiquitin ligases are important and diverse protein complexes that contribute to the specificity of proteasome-

Fig 5. Model of proposed cellular responses involved in carboplatin resistance and susceptibility. A NEDDylation-associated pathway controls nuclear localisation and stabilises HuR and activates cullins 1/2/3 proteins involved in the regulation of 23 human homologues related to the carboplatin response.

doi:10.1371/journal.pone.0145377.g005

mediated protein degradation [20]. Together, HuR and cullins 1/2/3 regulate 23 different human homologues, which were originated from 12 different yeast genes whose deletion led to a carboplatin response (almost 60% of yeast genes selected; Tables 4 and 5). We propose that, in conjunction, cullins and HuR lead to a loss of proteins responsible for carboplatin sensitivity through ubiquitylation and proteasome-mediated degradation, in addition to increasing the mRNA stabilisation and translation efficiency of proteins related to tumour resistance (Fig 5).

If our hypothesis is correct, the proteins related in Table 5 should be found at decreased levels in ovarian cancer. We first analysed their mRNA expression and no significant difference was observed (data not shown), which was expected since our hypothesis is that cullins (that modulate proteins and not mRNA) were responsible for diminished levels of related proteins. Furthermore, we analysed the mRNA expression of cullins 1, 2, 3, 4A, 4B, 5 and 7. Only cullin 4B and cullin 7 presented overexpression in 77% and 90%, respectively, of 998 patients analysed. This was interesting, but does not explain our results since cullins 1, 2 and 3 found interacting physically with proteins related in Table 5 did not present changes in expression.

Next, we analysed the TCGA2 dataset (The Cancer Genome Atlas 2, present in Oncomine) to assess changes in DNA copy number. Fig 6 shows that, except for cullin 4A, in the 607 patients with ovarian cancer analysed, the median DNA copy number of cullin encoding genes was increased when compared to normal tissues. In addition, NEDD8 and UBA3 (the E1-NEDD8 activating enzyme that forms a heterodimer with NAE1 [3]) also presented high DNA copy number in ovarian cancer. This is an intriguing fact since these proteins are known to be key components of the NEDDylation pathway [3], and the DNA copy number of NAE1 (the E1-NEDD8 activating enzyme) and UBE2M (E2-NEDD8 ligase) presented a decrease in ovarian cancer patients. However, there are some data which suggest that this decrease in DNA copy number does not necessarily result in less NAE1 and UBE2M in ovarian cancer. Bonome *et al.* [16] characterised UBE2M as being in the top 10% of genes overexpressed in their study on ovarian carcinoma, involving 185 patients. In addition, in the comparison between patients sensitive and chemoresistant to carboplatin (three patients of each group analysed in

Fig 6. Analysis of DNA copy number aberration of genes involved in NEDDylation pathway in ovarian tumour patients. 1,168 samples were analysed, composed of 431 normal blood, 130 normal ovary tissue and 607 ovarian serous cystadenocarcinoma, present in the Cancer Genome Atlas 2 (TCGA2)- Ovarian Serous Cystadenocarcinoma DNA Copy Number Data. The analysis was performed with OncoPrint tool gene analysis using the filters: Cancer x Normal, Cancer type: ovarian. The number depicted in y axis represents the median number of DNA copy in patients analysed. The normal median frequency of DNA copy number was 0.005, almost unchangeable in control samples.

doi:10.1371/journal.pone.0145377.g006

triplicate), up to a 47% increase in NAE1 expression was observed in ovarian cancer from chemoresistant patients [19].

In addition to increases in DNA copy number and mRNA expression, to provide indirect evidence of increases in encoded protein, we analysed two proteomic databases: the Human Protein Atlas and the Database of Differentially Expressed Proteins in Human Cancer (dbDEPC 2.0). We found that, at the protein level, although proteomic information is much scarcer than mRNA in terms of clinical patient samples available to be analysed, our hypothesis that the NEDDylation pathway is overactive in ovarian cancer was confirmed (Table 6). In the Human Protein Atlas, we found this observation in 12 patient samples and several tumour cell lines; of these, we chose the EFO-21 cell line, an ovarian cystadenocarcinoma, to present the results.

Cullins are activated by NEDDylation, thereby increasing protein degradation mediated by the ubiquitin-proteasome system (reviewed by Zhao, Morgan and Sun [3]). However, it has only recently been demonstrated that HuR is stabilised by NEDDylation. This stabilisation is mediated by the murine double minute 2 protein (Mdm2) and the levels of these two proteins are correlated in human hepatocellular carcinoma and colon cancers [21]. Therefore, we also suggest that there may be a common feature regulating the resistance or sensitivity of cells to carboplatin—the NEDDylation pathway (Fig 5).

Protein NEDDylation involves the attachment of NEDD8 (an ubiquitin-like molecule) onto a substrate protein. This modification does not lead to degradation itself, but to the modulation of target protein activity and function. In contrast to ubiquitylation, protein NEDDylation is a more restricted event biochemically, with no more than 20 substrates described in the literature [reviewed in 3]. These substrates include key proteins in the regulation of DNA damage

Table 6. Comparison of protein expression in ovarian cancer and normal tissue.

PROTEIN EXPRESSION		
	CONTROL	OVARIAN CANCER
CUL1	low expressed	17% tumours presented moderate expression
CUL2	low expressed	67% tumours presented moderate expression
CUL3	moderate expression in stroma	25% tumours presented strong expression
CUL4A	no information	no information
CUL4B	not expressed in stroma	100% tumours presented low expression
UBE2M	not expressed in stroma	strong expression in EFO-21 cell line
NEDD8	moderate expression in stroma	strong expression in EFO-21 cell line
NAE1	moderate expression in stroma	58% tumours presented strong expression
FBXW2	moderate expression	low expressed
PABPC1	not expressed	90% tumours presented low to moderate expression

doi:10.1371/journal.pone.0145377.t006

responses, such as p53, which is NEDDylated by the same protein that regulates HuR, Mdm2 (reviewed in Zhao, Morgan and Sun [3]).

A small molecule designated MLN4924 is an inhibitor of NEDDylation and is listed as a drug candidate on the NCI/NIH Drug Directory (<http://www.cancer.gov/drugdictionary?cdrid=596795>). MLN4924 specifically inhibits NEDDylation by acting on NEDD8-activating enzyme (NAE1) and is currently undergoing several Phase 1b clinical trials (<http://www.cancer.gov/clinicaltrials/search/results?protocolsearchid=13020760>). MLN4924 has also been demonstrated to overcome cisplatin resistance in ovarian tumour cell lines and in a mouse ovarian tumour xenograft model [22]. MLN4924 is synergistic when used in combination with cisplatin; this synergy is dependent on cullin 3, since siRNA knockdown of CUL3 produced a 45% increase in cisplatin cytotoxicity. The authors affirmed that the majority of their results were similar to those with carboplatin [23].

We tested the effect of NEDDylation inhibition with MLN4924 in ES-2 cancer cell lines non-resistant and chemoresistant to carboplatin (Fig 7). In carboplatin non-resistant cells, the viability decrease was similar in cells treated with carboplatin, MLN4924 or a combination of both. The synergy of MLN4924 with cisplatin observed previously [23] was not observed in combination with carboplatin. This can be attributed to the lower dose of MLN4924 (up to 100-fold) or the different culture media used in the present study to obtain the same ratio of tumour cell cytotoxicity. However, although carboplatin chemoresistant cells were not responsive to carboplatin or MLN4924, the combination of drugs was able to overcome resistance, rescuing the sensitivity of these cells to non-resistant levels. These results were also not in agreement with the results with cisplatin resistant cells, since they did not acquire resistance to MLN4924 and the combination with cisplatin was not significantly better than the effect of NEDDylation inhibition alone [23]. Our results suggest that it is not recommended to use a NEDDylation inhibitor at the beginning of patient treatment, because there was no statistical significant improvement in the carboplatin response. However, it was clear that, in the case of tumour cells that acquired resistance during carboplatin treatment, NEDDylation inhibition with MLN4924 rescued the sensitivity of ovarian tumour cells. Interestingly, although the vast majority of literature considers the platinum-based drugs redundant, our results suggest a specific tumour response to carboplatin when compared to cisplatin, mainly concerning the application of a NEDDylation inhibitor. In conclusion, our results indicate that preventing NEDDylation of cullins and HuR may provide an adjunct therapy to overcome tumour acquired resistance during carboplatin chemotherapy.

Fig 7. Viability assay of non-treated and chemoresistant ovarian cancer cell lines challenged with carboplatin, NEDDylation inhibitor MLN4924 or both. The ES-2 ovarian cancer cell line was pre-treated with 50 μ M carboplatin and the surviving cells were selected as chemoresistant to carboplatin. The chemoresistant and non-treated cells were challenged with 500 μ M carboplatin, 10 nM MLN4924 or a combination of both for 24 hours. The results are presented as mean and SD, analysed by one-way ANOVA—multiple comparisons, with statistical significant $p = 0.0001$, using GraphPad software. The capped lines represent the group of samples that were not statistically different between each other, and the asterisks represent a statistically significant difference between the groups that were compared.

doi:10.1371/journal.pone.0145377.g007

Conclusion

Carboplatin is an anticancer drug that is on the World Health Organisation's List of Essential Medicines, which categorises it as one of the most important antineoplastic drugs needed in a basic health system. It is the least toxic platinum-based drug, and is widely used in cancer treatment, especially in ovarian tumours, where it is the first-line choice of treatment. However, some patients acquire resistance to the drug during treatment, leading to clinical complications. Here, we show a potential way to sensitise carboplatin-resistant tumours through inhibition of the signalling NEDDylation pathway. Our results have implications on clinical outcomes, since we showed that inhibition of this molecular pathway in non-chemoresistant cells is not useful, and the effect was not synergistic, such as the effect previously shown with cisplatin. However, inhibition of NEDDylation was highly efficient in sensitising carboplatin-resistant cells to the drug to the same levels as those before resistance, thus allowing the continuation of this line of treatment.

Supporting Information

S1 Table. Selected mutants that presented fitness defect when exposed to the lower dose of carboplatin (250 μ M). Tested by Hillenmeyer *et al.* (2008) in a chemogenomic study of more than 1000 different assay conditions. The P-value of <0.01 was chosen because, according to the authors, this was the minimum P-value to be considered as statistically significant. The entire dataset is available at <http://chemogenomics.med.utoronto.ca/fitdb/fitdb.cgi>. (XLSX)

S2 Table. Human homologues of yeast carboplatin-responsive genes annotated as interacting with cullins and ELAVL1 proteins and the respective reference that observed the physical interaction. (XLSX)

Acknowledgments

The authors thank Dr PF Long from King's College London and Faculdade de Ciências Farmacêuticas, Universidade de São Paulo for critically reviewing the data and manuscript.

Author Contributions

Conceived and designed the experiments: GM VMF. Performed the experiments: GFS MAL DFC. Analyzed the data: GM GFS MAL DFC VMF. Contributed reagents/materials/analysis tools: GM VMF. Wrote the paper: GM VMF.

References

1. Navarro FJ, Weston L, Nurse P (2012) Global control of cell growth in fission yeast and its coordination with the cell cycle. *Curr Opin Cell Biol* 24 (6): 833–7. doi: [10.1016/j.ceb.2012.10.015](https://doi.org/10.1016/j.ceb.2012.10.015) PMID: [23182517](https://pubmed.ncbi.nlm.nih.gov/23182517/)
2. Hillenmeyer ME, Fung E, Wildenhain J, Pierce SE, Hoon S, Lee W *et al.* (2008) The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* 320 (5874): 362–5. doi: [10.1126/science.1150021](https://doi.org/10.1126/science.1150021) PMID: [18420932](https://pubmed.ncbi.nlm.nih.gov/18420932/)
3. Zhao Y, Morgan MA, Sun Y (2014) Targeting neddylation pathways to inactivate cullin-ring ligases for anticancer therapy. *Antioxid Redox Signal* 21(17):2383–400. doi: [10.1089/ars.2013.5795](https://doi.org/10.1089/ars.2013.5795) PMID: [24410571](https://pubmed.ncbi.nlm.nih.gov/24410571/)
4. Walsh CS, Ogawa S, Karahashi H, Scoles DR, Pavelka JC, Tran H *et al.* (2008) ERCC5 is a novel biomarker of ovarian cancer prognosis. *J Clin Oncol* 26 (18): 2952–8. doi: [10.1200/JCO.2007.13.5806](https://doi.org/10.1200/JCO.2007.13.5806) PMID: [18565881](https://pubmed.ncbi.nlm.nih.gov/18565881/)
5. He C, Duan Z, Li P, Xu Q, Yuan Y (2013) Role of ERCC5 promoter polymorphisms in response to platinum-based chemotherapy in patients with advanced non-small-cell lung cancer. *Anticancer Drugs* 24 (3): 300–5. doi: [10.1097/CAD.0b013e32835bd6ce](https://doi.org/10.1097/CAD.0b013e32835bd6ce) PMID: [23211354](https://pubmed.ncbi.nlm.nih.gov/23211354/)
6. Osterberg L, Levan K, Partheen K, Helou K, Horvath G (2005) Cytogenetic analysis of carboplatin resistance in early-stage epithelial ovarian carcinoma. *Cancer Genet Cytogenet* 163 (2): 144–50. PMID: [16337857](https://pubmed.ncbi.nlm.nih.gov/16337857/)
7. Osterberg L, Levan K, Partheen K, Staaf J, Sundfeldt K, Horvath G (2009) High-resolution genomic profiling of carboplatin resistance in early-stage epithelial ovarian carcinoma. *Cytogenet Genome Res* 125 (1): 8–18. doi: [10.1159/000218744](https://doi.org/10.1159/000218744) PMID: [19617691](https://pubmed.ncbi.nlm.nih.gov/19617691/)
8. Yasuno T, Matsumura T, Shikata T, Inazawa J, Sakabe T, Tsuchida S *et al.* (1999) Establishment and characterization of a cisplatin-resistant human neuroblastoma cell line. *Anticancer Res* 19 (5B): 4049–57. PMID: [10628353](https://pubmed.ncbi.nlm.nih.gov/10628353/)
9. Dolan ME, Newbold KG, Nagasubramanian R, Wu X, Ratain MJ, Cook EH Jr *et al.* (2004) Heritability and linkage analysis of sensitivity to cisplatin-induced cytotoxicity. *Cancer Res* 64 (12): 4353–6. PMID: [15205351](https://pubmed.ncbi.nlm.nih.gov/15205351/)
10. Frede J, Fraser SP, Oskay-Özcelik G, Hong Y, Ioana Braicu E, Sehouli J *et al.* (2013) Ovarian cancer: Ion channel and aquaporin expression as novel targets of clinical potential. *Eur J Cancer* 49 (10): 2331–44. doi: [10.1016/j.ejca.2013.03.016](https://doi.org/10.1016/j.ejca.2013.03.016) PMID: [23683551](https://pubmed.ncbi.nlm.nih.gov/23683551/)

11. Xuejun C, Weimin C, Xiaoyan D, Wei Z, Qiong Z, Jianhua Y (2014) Effects of aquaporins on chemosensitivity to cisplatin in ovarian cancer cells. *Arch Gynecol Obstet*, 290(3):525–32. doi: [10.1007/s00404-014-3216-6](https://doi.org/10.1007/s00404-014-3216-6) PMID: [24695904](https://pubmed.ncbi.nlm.nih.gov/24695904/)
12. Tang YC, Amon A (2013) Gene copy-number alterations: a cost-benefit analysis. *Cell* 152(3): 394–405. doi: [10.1016/j.cell.2012.11.043](https://doi.org/10.1016/j.cell.2012.11.043) PMID: [23374337](https://pubmed.ncbi.nlm.nih.gov/23374337/)
13. Brody JR, Gonye GE (2011) HuR's role in gemcitabine efficacy: an exception or opportunity? *Wiley Interdiscip Rev RNA* 2 (3): 435–44. doi: [10.1002/wrna.62](https://doi.org/10.1002/wrna.62) PMID: [21957028](https://pubmed.ncbi.nlm.nih.gov/21957028/)
14. Lal S, Burkhart RA, Beeharay N, Bhattacharjee V, Londin ER, Cozzitorto JA et al. (2014) HuR posttranscriptionally regulates WEE1: implications for the DNA damage response in pancreatic cancer cells. *Cancer Res* 74 (4): 1128–40. doi: [10.1158/0008-5472.CAN-13-1915](https://doi.org/10.1158/0008-5472.CAN-13-1915) PMID: [24536047](https://pubmed.ncbi.nlm.nih.gov/24536047/)
15. Arnoldo A, Kittanakom S, Heisler LE, Mak AB, Shukalyuk AI, Torti D et al. (2014) A genome scale over-expression screen to reveal drug activity in human cells. *Genome Medicine* 6:32. doi: [10.1186/gm549](https://doi.org/10.1186/gm549) PMID: [24944581](https://pubmed.ncbi.nlm.nih.gov/24944581/)
16. Bonome T, Levine DA, Shih J, Randonovich M, Pise-Masison CA, Bogomolnii F, Ozbun L, Brady J, Barrett JC, Boyd J, Birrer MJ (2008) A gene signature predicting for survival in suboptimally debulked patients with ovarian cancer. *Cancer Res* 68 (13): 5478–86. doi: [10.1158/0008-5472.CAN-07-6595](https://doi.org/10.1158/0008-5472.CAN-07-6595) PMID: [18593951](https://pubmed.ncbi.nlm.nih.gov/18593951/)
17. Yoshihara K, Tajima A, Komata D, Yamamoto T, Kodama S, Fujiwara H, Suzuki M, Onishi Y, Hatae M, Sueyoshi K, Fujiwara H, Kudo Y, Inoue I, Tanaka K (2009) Gene expression profiling of advanced-stage serous ovarian cancers distinguishes novel subclasses and implicates ZEB2 in tumor progression and prognosis. *Cancer Sci* 100 (8): 1421–8. doi: [10.1111/j.1349-7006.2009.01204.x](https://doi.org/10.1111/j.1349-7006.2009.01204.x) PMID: [19486012](https://pubmed.ncbi.nlm.nih.gov/19486012/)
18. Huang YT, Zhou J, Shi S, Xu HY, Qu F, Zhang D, Chen YD, Yang J, Huang HF, Sheng JZ (2015) Identification of Estrogen Response Element in Aquaporin-3 Gene that Mediates Estrogen-induced Cell Migration and Invasion in Estrogen Receptor-positive Breast Cancer. *Sci Rep* 5:12484. doi: [10.1038/srep12484](https://doi.org/10.1038/srep12484) PMID: [26219409](https://pubmed.ncbi.nlm.nih.gov/26219409/)
19. Peters D, Freund J, Ochs RL. (2005) Genome-wide transcriptional analysis of carboplatin response in chemosensitive and chemoresistant ovarian cancer cells. *Mol Cancer Ther* 4 (10): 1605–16. PMID: [16227411](https://pubmed.ncbi.nlm.nih.gov/16227411/)
20. Sarikas A, Hartmann T, Pan ZQ (2011) The cullin protein family. *Genome Biol* 12 (4): 220. doi: [10.1186/gb-2011-12-4-220](https://doi.org/10.1186/gb-2011-12-4-220) PMID: [21554755](https://pubmed.ncbi.nlm.nih.gov/21554755/)
21. Embade N, Fernández-Ramos D, Varela-Rey M, Beraza N, Sini M, Gutiérrez JV et al. (2012) Murine double minute 2 regulates Hu antigen R stability in human liver and colon cancer through NEDDylation. *Hepatology* 55 (4): 1237–48. doi: [10.1002/hep.24795](https://doi.org/10.1002/hep.24795) PMID: [22095636](https://pubmed.ncbi.nlm.nih.gov/22095636/)
22. Nawrocki ST, Kelly KR, Smith PG, Espitia CM, Possemato A, Beausoleil SA et al. (2013) Disrupting protein NEDDylation with MLN4924 is a novel strategy to target cisplatin resistance in ovarian cancer. *Clin Cancer Res* 19 (13): 3577–90. doi: [10.1158/1078-0432.CCR-12-3212](https://doi.org/10.1158/1078-0432.CCR-12-3212) PMID: [23633453](https://pubmed.ncbi.nlm.nih.gov/23633453/)
23. Jazaeri AA, Shibata E, Park J, Bryant JL, Conaway MR, Modesitt SC et al. (2013) Overcoming platinum resistance in preclinical models of ovarian cancer using the neddylation inhibitor MLN4924. *Mol Cancer Ther* 12 (10): 1958–67. doi: [10.1158/1535-7163.MCT-12-1028](https://doi.org/10.1158/1535-7163.MCT-12-1028) PMID: [23939375](https://pubmed.ncbi.nlm.nih.gov/23939375/)

ANEXO 6

Heterologous Expression and Purification of Active L-Asparaginase I of *Saccharomyces cerevisiae* in *Escherichia coli* Host

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DOI 10.1002/btpr.2410

Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com)

L-asparaginase (ASNase) is a biopharmaceutical widely used to treat child leukemia. However, it presents some side effects, and in order to provide an alternative biopharmaceutical, in this work, the genes encoding ASNase from Saccharomyces cerevisiae (Sc ASNaseI and Sc ASNaseII) were cloned in the prokaryotic expression system Escherichia coli. In the 93 different expression conditions tested, the Sc ASNaseII protein was always obtained as an insoluble and inactive form. However, the Sc ASNaseI (His)₆-tagged recombinant protein was produced in large amounts in the soluble fraction of the protein extract. Affinity chromatography was performed on a Fast Protein Liquid Chromatography (FPLC) system using Ni²⁺-charged, HiTrap Immobilized Metal ion Affinity Chromatography (IMAC) FF in order to purify active Sc ASNaseI recombinant protein. The results suggest that the strategy for the expression and purification of this potential new biopharmaceutical protein with lower side effects was efficient since high amounts of soluble Sc ASNaseI with high specific activity (110.1 ± 0.3 IU mg⁻¹) were obtained. In addition, the use of FPLC-IMAC proved to be

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Additional Supporting Information may be found in the online version of this article.

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an efficient tool in the purification of this enzyme, since a good recovery ($40.50 \pm 0.01\%$) was achieved with a purification factor of 17-fold. © 2016 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2016

Keywords: biopharmaceutical, fast protein liquid chromatography, protein purification, recombinant DNA, enzyme technology

Introduction

The L-asparaginase (ASNase), L-asparagine amidohydrolase [EC 3.5.1.1] is an enzyme which catalyzes the hydrolysis of the amino group of L-asparagine, forming L-aspartic acid and ammonia. The discovery of this enzyme started with Kidd's¹ remarkable observation that Guinea pig serum had anti-tumor activity against several types of lymphoma in mice. Broome² elucidated this anti-leukemic activity attributing it to ASNase action. Nevertheless, only in 1967, the ASNase was isolated in enough quantity to prove its anti-tumoral activity in clinical trials.³ Since then, the demand for the proper and efficient isolation and purification techniques for ASNase has increased, due to its pharmacological importance and proved application.

ASNase was shown to be very useful in treating acute lymphocytic leukemia (ALL),^{4–6} acute myeloid leukemia⁷ and lymphomas.⁸ ALL is a blood and bone marrow-related cancer whose prevalence is higher in childhood.^{9,10} Nowadays, ASNase based drugs are available in different preparations/formulations as described in Table 1.^{11–16} However, there are some factors limiting its use as a chemotherapeutic agent, namely (i) the daily dose demand ($10\text{--}200\text{ IU}\cdot\text{kg}^{-1}$ per day), for different periods of time, which may last for 21 days, and (ii) the toxic side effects that can occur during therapy, *i.e.*, hyperglycemia, decreased serum albumin, lipoproteins and fibrinogen, increased liver fat, some mild brain dysfunctions,^{6,11,17–20} and (iii) the development of hypersensitivity to treatment. The hypersensitivity reactions ranging from a small allergy in the drug injection area, bronchospasm and even anaphylactic shock, occurs in 5–50% of the treated patients. Nowadays, the researchers are focused in the partial or total elimination of these problems, regarding some protein structural modification approaches, through genetic engineering, gene enhancement and/or by ASNase conjugation with biopolymers (*e.g.*, polyethylene glycol (PEG), dextran, albumin and heparin)^{5,11,21–23} or by changing the expression host systems.²⁴

In this work, the genes codifying ASNase were isolated from the *Saccharomyces cerevisiae* BY4741 (Invitrogen), a microorganism able to synthesize two distinct forms of ASNase: *Sc*_ASNaseI, an intracellular constitutive enzyme and *Sc*_ASNaseII, a periplasmic enzyme which is secreted when the microorganism is exposed to some stress conditions, in particular to nitrogen starvation. Both enzymes are poorly characterized; Ferrara et al.²⁵ obtained a better endogenous amount of *Sc*_ASNaseII using the double mutant strain $\Delta ure2\Delta dal80$. However, the posterior yield of *Sc*_ASNaseII heterologous expression in *Pichia pastoris* was 7-fold higher than in the mutant *S. cerevisiae*²⁶ and the protein has been successfully expressed and purified in its active form, using this host cell.²⁷

In this work, the intention was to analyze the expression of the *Sc*_ASNases, eukaryotic forms of the biopharmaceutical ASNase, by applying the well-characterized, simple, fast and cheap host heterologous *E. coli* expression system. In

order to access this system capacity, a bioinformatics analysis was conducted to evaluate both enzymes requirements as for its post-translational modifications in the native host, followed by the expression of the enzymes and evaluation of their activities. Moreover, the Fast Protein Liquid Chromatography (FPLC) purification of *Sc*_ASNaseI was applied as a downstream methodology platform. Actually, the use of the FPLC purification in proteins has been previously explored,^{28–30} thus being considered a well-established technique in different biotechnological industries.

Materials and Methods

E. coli expression vector construction

In order to amplify the *ASP1* and *ASP3* genes, the genomic DNA of *S. cerevisiae* extracted with PureLink® Genomic DNA Purification Kit (Invitrogen®, Carlsbad, CA, USA) was used. Also, the *ASP3* synthetic gene with optimized codons to express protein in *E. coli* was purchased from GenScript Corporation. Primers to amplify the target yeast genes were commissioned to the company *Exxtend* (Paulínea, SP, Brazil) (sequences described in Table 2). *Sc*_ASNase genes were cloned into expression vectors described in Table 3. Restriction enzymes *NdeI*, *BamHI* and *XhoI* were purchased from New England Biolabs (NEB, Ipswich, UK). T4 DNA ligase was obtained from Promega (Madison, WI, USA). The bacteria transformation was carried out by electroporation in a MicroPulser Electroporator (BioRad®, Hercules, CA, USA). Luria Bertani—L.B. (0.5% NaCl, 0.5% yeast extract, and 1% triptone) solid medium (2% agar added) with added carbenicillin ($50\text{ }\mu\text{g}\cdot\mu\text{L}^{-1}$) was used to select transformants which contain pET15b or pET22b vectors or added of kanamycin ($15\text{ }\mu\text{g}\cdot\mu\text{L}^{-1}$) to select transformants with pET28a + SUMO construction. The recovery of the plasmids from bacteria was performed using a QIAprep Spin Miniprep Kit (Qiagen®, Hilden, Germany), according to the manufacturer's instructions. The correct insert of *Sc*_ASNase genes into the pET vectors were confirmed by DNA sequencing, for all expression vectors described in Table 3.

Heterologous expression of *Sc*_ASNaseI

The *E. coli* BL21 (DE3) strain was grown in LB liquid media, supplemented with the appropriate antibiotics, at 37°C until $\text{OD}_{600\text{nm}} = 0.6\text{--}0.7$. When the bacteria were in the log-phase, 1 mM of isopropylthio- β -D-galactoside (IPTG) was added, and the solution was incubated for 3 h at 37°C to induce the transcription and, consequently, to allow the production of *Sc*_ASNaseI. The sample pre-treatment was conducted by a chemical lysis procedure; herein cell pellets from genetically transformed *E. coli* BL21 (DE3) were suspended in 10 mL of BugBuster Master Mix (Merck-Millipore™, Billerica, MA, USA) per g of cell, mixed gently for 20 min and centrifuged at $16,000 \times g$ for 20 min at 4°C; the pellet was discarded and the supernatant was subjected to

Table 1. Commercially Approved ASNase^{11–16}

Drug Name	Production Source	Type	Manufacturer
Elspar [®]	<i>Escherichia coli</i>	Native enzyme	Lundbeck Inc
Kidrolase [®]	<i>Escherichia coli</i>	Native enzyme	EUSA Pharma S.A.
Oncaspar [®] (PEG-asparaginase)	<i>Escherichia coli</i>	PEGylated native enzyme	Sigma Tau Pharmaceuticals
Graspa [®]	<i>Escherichia coli</i>	Native enzyme encapsulated in erythrocytes	ERYTECH Pharma
Leunase [®]	<i>Escherichia coli</i>	Modified <i>E.coli</i> HAP strain	Kyowa Hakko Kirin Co. Ltd.
Spectrila [®]	<i>Escherichia coli</i>	Recombinant Enzyme	Medac Gesellschaft fuer klinische Spezialpraeparate mbH
Erwinase [®]	<i>Erwinia chrysanthemi</i>	Native enzyme	EUSA Pharma
Crisantaspace [®]	<i>Erwinia chrysanthemi</i>	Native enzyme	Ohara Pharmaceutical

Table 2. Sequence of Primers Used to Carry Out the Constructions of the *Sc*_ASNases Overexpression Vectors

Primer Name	Sequence (5'→3')	Restriction Enzyme Site
ASP1-fw	GGGAATTCATATGAAAAGCGATTCAGTTGAAATCA	<i>NdeI</i>
ASP1-rv	CGCGGATCCTCACCACCATAGAC	<i>BamHI</i>
ASP3-1-fw	GGGAATTCATATGAGATCTTTAAATACCC	<i>NdeI</i>
ASP3-26-fw	CGGGATCCGAGAGAAGAATTCTTC	<i>BamHI</i>
ASP3-32-fw	GGGAATTCATATGTTGCCATCAATCAAAATTTTGG	<i>NdeI</i>
ASP3-26opt-fw	GGGAATTCATATGGAAGAAAAAATAGCTC	<i>NdeI</i>
ASP3-BamHI-rv	CGGGATCCTTAACCACCGTAGACG	<i>BamHI</i>
ASP3-XhoI -rv	CCGCTCGAGACCACCGTAG	<i>XhoI</i>
ASP3-opt-rv	CATTGGATCCTCAACCGCC	<i>BamHI</i>

Table 3. Description of the Primers Combination, Type of DNA Templates and Expression Vectors Used in this Work

Forward Primer	Reverse Primer	DNA Template	Vector	Final Construction
ASP1-fw	ASP1-rv	<i>S. cerevisiae</i> genomic DNA	pET15b	ASP1+pET15b
ASP3-1-fw	ASP3-BamHI-rv	<i>S. cerevisiae</i> genomic DNA	pET15b	ASP3_1-362+pET15b*
ASP3-32-fw	ASP3-BamHI-rv	<i>S. cerevisiae</i> genomic DNA	pET15b	ASP3_32-362+pET15b [†]
ASP3-26-fw	ASP3-XhoI -rv	<i>S. cerevisiae</i> genomic DNA	pET22b	ASP3_26-362+pET22b [‡]
ASP3-26opt-fw	ASP3-opt-rv	<i>ASP3</i> optimized gene	pET15b	ASP3opt_26-362_pET15b [§]
ASP3-26-fw	ASP3-XhoI -rv	<i>S. cerevisiae</i> genomic DNA	pET28b+SUMO	ASP3_26-362+ pET28b+SUMO

*Complete *Sc*_ASNaseII fused with N-terminal His₆-tag and expressed in cytoplasm in *E. coli*.

[†]*Sc*_ASNaseII without the first 32 amino acids—sequence of protein that aligns with bacterial *Ec*_ASNaseII- fused with N-terminal His₆-tag and expressed in cytoplasm in *E. coli*.

[‡]Mature *Sc*_ASNaseII without the 26 first amino acids involved in the intermembrane signalling secretion in yeast *S. cerevisiae*. Protein fused with C-terminal His₆-tag and expressed in periplasmic space in *E. coli*.

[§]Mature *Sc*_ASNaseII without the 26 first amino acids involved in the intermembrane signalling secretion in yeast *S. cerevisiae*. The gene was synthesised with optimal codons to express in *E. coli*; expressed with N-terminal His₆-tag and in cytoplasm.

^{||}Mature *Sc*_ASNaseII without the 26 first amino acids involved in the intermembrane signalling secretion in yeast *S. cerevisiae* fused with N-terminal high solubilizing protein SUMO, expressed in cytoplasm.

vacuum filter membrane (0.45 μ m, Merck-Millipore); to the filtered sample, the final concentration of 20 mM imidazole was added.

The affinity chromatography was performed on a FPLCTM System (ÄKTA purifier, GE Healthcare) using a Ni²⁺-charged, 5 mL HiTrap IMAC FF column (GE Healthcare). A linear gradient, running from 0 to 500 mM of imidazole at 5.0 mL min⁻¹ was carried in order to be possible to identify the lowest imidazole concentration of the elution buffer required to extract the highest amount of the purified *Sc*_ASNaseI. This concentration range was further used in an imidazole gradient step, in which two-step concentrations of imidazole (160 and 272 mM) were applied. The initial sample volume applied was 5.0 mL. A binding buffer (20 mM sodium phosphate buffer at pH 8.0 and 500 mM NaCl) and an elution buffer (20 mM sodium phosphate buffer at pH 8.0, 500 mM NaCl and 500 mM imidazole) were used in the purification process. The samples were previously centrifuged 4,000 $\times$ g for 20 min at 4°C and desalted through an exclusion chromatography step in a PD-10 column (GE Healthcare Life Sciences) with Tris-HCl buffer (20 mM) at pH 8.6.

Screening of conditions for *Sc*_ASNaseII expression

The *E. coli* strains (Supporting Information Table A.1 at ESI[†]) were genetically transformed with the chosen construction containing the *ASP3* gene (Table 3). The screening of conditions for *Sc*_ASNaseII protein expression was carried out in 10 mL of LB medium plus the selective antibiotic, starting with a cell optical density of OD_{600nm} = 0.2, at 37°C and 180 rpm until a cell density of OD_{600nm} = 0.6–0.7. At this point, an IPTG solution was added at the described concentrations (0.01, 0.1, 0.5 and 1.0 mM) and incubated for 3 h at 37°C or overnight at 20°C to induce the production of *Sc*_ASNaseII using the conditions, vectors and gene combinations described in Supporting Information Table A.2. At the end of this procedure, the cells were pelleted, the supernatant discarded and 1.5 mL of BugBuster Master Mix (Merck-Millipore) was added, gently mixed for 20 min and centrifuged at 16,000 $\times$ g for 20 min. The pellet containing the cellular debris and inclusion bodies (containing insoluble proteins), was separated from the supernatant (with the soluble proteins) and both were analyzed by SDS-PAGE 12%, under reducing conditions, in accordance with the Laemmli' method.³¹ The electrophoresis was performed at 120 V using

a vertical BioRad® system. The protein markers of different molecular weights were acquired from BioRad®. The volume of the samples added to the gel was 10 µL with a protein concentration ranging from 0.01 to 3 mg/mL. The gels were silver stained for the protein bands detection, using the Silver Staining Kit (Amersham Pharmacia Biotech®) and following the manufacturer's instructions.

Nessler activity assay and purification parameters calculation

The determination of the enzymatic activity was performed by the Nessler assay (expressed in IU mL⁻¹).³² The sample volume of 0.1 mL was incubated at 37°C for 30 min in Tris-HCl buffer (50 mM, pH 8.6) with L-asparagine (9 mM). Then, the reaction was stopped with the addition of trichloroacetic acid (TCA). The Nessler reagent (Merck) was mixed by inversion for 1 min and then the reactions were measured in a microplate reader at $\lambda_{436\text{nm}}$ (Spectramax Plus384, Molecular Devices). A standard curve was prepared using ammonium sulfate. In order to remove the possible interferences of quantification, two blanks composed by Tris buffer + L-asparagine + TCA were made for each sample. Moreover, at least three samples of each enzymatic activity measurement were prepared, being the results described, the average of the three replicas.

The total protein concentration was measured through absorbance assay, measuring the absorbance at $\lambda_{280\text{nm}}$, by UV-Vis spectroscopy. A calibration curve with bovine serum albumin (BSA) (lyophilized powder, ≥ 96 wt%, Sigma-Aldrich, St. Louis, MO, USA) was used to determine the protein concentration in each sample.

Computational analysis of recombinant Sc_ASNases

The possible N-glycosylation sites were predicted using NetNGlyc 1.0 program.³³ The presence and location of signal peptide cleavage sites in the amino acid sequence was determined using the SignalP 4.1 program.³⁴ The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models.

Results and Discussion

Bioinformatics analysis of Sc_ASNases

A computational analysis of the Sc_ASNases using bioinformatics tools was carried out. This analysis included the prediction of glycosylation sites using sequence information (NetNGlyc 1.0) and the presence, prediction and location of signal peptide cleavage sites in the ASNase amino acid sequences (SignalP 4.1).

The bioinformatics results obtained predicted that the Sc_ASNaseI could be expressed in its active form in *E. coli*, despite its prokaryotic expression nature. These data (NetNGlyc 1.0 and SignalP 4.1) resulted in three possible glycosylation sites (Figure 1A), even in the absence of a signal peptide (Figure 2A). This observation suggests that the glycosylation, if present in its native host, is not essential for the enzyme activity, which is supported by the high value of the enzyme specific activity (SA) found for the purified Sc_ASNaseI (see below). This is an important result, since *E. coli* is a robust expression system, largely adopted by biopharmaceutical companies due its high titers outcomes.

Figure 1. Prediction results of N-glycosylation in the Sc_ASNaseI (A) and Sc_ASNaseII (B), using the NetNGlyc 1.0 software.

The sequence position represents the protein amino-acids position. The graph depicts the possible sites of N-glycosylation in the Sc_ASNases sequence (— green lines), represented by the green bars that rise vertically and pass the program threshold (— red line).

In contrast, Sc_ASNaseII presents three possible glycosylation sites (Figure 1B) and a signal peptide to direct this protein to the endoplasmic reticulum (Figure 2B); therefore, it is highly probable that this protein undergoes glycosylation. In fact, endogenously produced Sc_ASNaseII was observed with a high molecular weight due to hypermannosylation.³⁵ Moreover, Castro-Girão et al.²⁷ have described that heterologous produced Sc_ASNaseII produced by *P. pastoris* undergoes glycosylation, since after the purification process, two bands were observed by electrophoresis, which were then turned into one, just after treatment of Sc_ASNaseII with PNGase F (a deglycosylation protein). In addition, as previously discussed, these authors observed the specific activity of Sc_ASNaseII produced by *P. pastoris* in the soluble fraction. Summing up, glycosylation seems to play an essential role for this enzyme activity and a prokaryotic system might be inappropriate to produce Sc_ASNaseII.

Expression and purification of Sc_ASNaseI

Recombinant protein expression in industrial scale demands careful evaluation of all process production steps. The choice of an appropriate host lies as a major step to this goal, moreover in the production of biopharmaceuticals. The proper protein folding, activity, purity and yield are dependent on the host ability to translate, fold and keep high amounts of the desirable protein in its soluble form. Sajitha et al.²⁴ successfully expressed the ASNase from *E. coli* into the eukaryotic host *P. pastoris*. The authors obtained a soluble and active ASNase in the supernatant and speculate that this protein might undergo eukaryotic glycosylation conferring more stability to the protein and fewer side effects.

In an attempt to obtain a cost effective, fast and efficient Sc_ASNase expression system, the gene *ASP1* was cloned into pET15b vector between *NdeI* and *BamHI* restriction

Figure 2. Presence and location predictions of signal peptide and signal peptide cleavage sites in the *Sc_ASINaseI* (A) and *Sc_ASINaseII* (B) sequences by the SignalP-4.1 software.

C-score represents the raw cleavage site score generated by the software. S-score: signal peptide score generated by the software. Y-score: combined cleavage site score generated by the software. If a signal peptide cleavage site is predicted, a C-score above the threshold (dashed purple line) would be present for some of the amino-acids. In our specific case, a signal peptide cleavage site and signal peptide itself is not predicted.

sites, which results in a *Sc_ASINaseI* N-terminal His-tag fusion protein. The native protein is predicted to present a molecular weight of 41,385.6 Da, but the recombinant protein with His-tag and thrombin recognition site, presents a molecular weight of 43,139.0 Da. As depicted in Figure 3, the prokaryotic *E. coli* cell was able to express high amounts of soluble *Sc_ASINaseI*. However, the protein obtained in this first test presented a considerable number of contaminant proteins, especially in the fraction of higher *Sc_ASINaseI* concentration (Figure 3, lane 6).

Taking into account that the administration of this biopharmaceutical (ASINase) will be potentially carried by its direct injection into the human blood, the high purity of ASINase is certainly a crucial demand for it to be considered as a promising therapeutic alternative. In this sense, an FPLC purification was performed considering two main tasks; the first one using a linear gradient of imidazole in order to identify the lowest imidazole concentration able to elute the highest amount of *Sc_ASINaseI*, and the second one using the optimized imidazole concentration to develop a purification protocol in which the same buffer was used to

Figure 3. SDS-PAGE stained with Coomassie-Blue representing the first attempt of *Sc_ASINaseI* purification by IMAC. In the left are indicated the molecular protein weights of the highlight bands of the BenchMark protein ladder (lane 1).

Lanes 2-6: sequential eluted *Sc_ASINaseI* from a nickel column purification.

Table 4. FPLC Purification With an Imidazole Linear Gradient (0–500 mM) Step at 5.0 mL min⁻¹

Sample	Protein Concentration (mg mL ⁻¹)	Enzymatic Activity (IU mL ⁻¹)	Specific Activity (IU mg ⁻¹)
Fractions 4–5	3.52 ± 0.03	0.06 ± 0.01	0.02 ± 0.01
Fractions 14–18	0.05 ± 0.01	0.03 ± 0.01	0.49 ± 0.01
Fractions 20–31	0.02 ± 0.01	0.11 ± 0.01	6.61 ± 0.08

Fractions eluted with *Sc_ASINaseI* activity are shown, along with the protein concentration (mg mL⁻¹), enzymatic activity (IU mL⁻¹) and specific activity (IU mg⁻¹) values.

perform a two-step purification procedure. Both approaches were applied and from the general results (Table 4), it was concluded that, while fractions 4–5 contain the expression of host contaminant proteins resulting in a very low specific *Sc_ASINaseI* activity (0.02 ± 0.01 IU mg⁻¹), the purified fractions 20–31 exhibited the highest specific activity (6.61 ± 0.08 IU mg⁻¹). Considering the results obtained, the imidazole concentrations chosen for the stepwise purification were 160 and 272 mM, which correspond to fractions 23 and 30, respectively. Table 5 summarizes the results obtained, correlating the fractions collected with the respective *Sc_ASINaseI* activity results, along with the protein concentration, enzymatic activity (EA), specific activity (SA), purification factor (PF), and recovery (%Rec) parameters.

From the set of results obtained (Figure 4 and Table 5), we conclude that the most pure fraction exhibited a SA of 110.1 IU mg⁻¹ comparable to the commercially available ASINase formulations, like Erwinaze® (from *E. chrysanthemi*) and Elspar® (from recombinant *E. coli*) containing 225 IU mg⁻¹ (information displayed in the Product Data-sheet—Active Asparaginase full-length protein ab73439). In what concerns the application of the purification step (FPLC purification), even higher specific activities would be reached, these similar to the values described for the commercial ASINase. Moreover, not only the enzyme activity is maintained but also the yields of purification of ASINase obtained were considerable (enzyme yield circa 1,001 IU.g

Table 5. FPLC Purification With the Two-Step Concentration Methodology of the Initial Imidazole Concentration (160 and 272 mM), Applied at 5.0 mL min⁻¹

Sample	Protein concentration (mg mL ⁻¹)	Enzymatic activity (IU mL ⁻¹)	Specific activity (IU mg ⁻¹)	Purification factor (fold)	Recovery Activity (%)
Cell extract	0.38 ± 0.01	2.50 ± 0.01	6.52 ± 0.01	1.00	100.00
Stepwise FPLC purification (Fractions 38-42)	0.02 ± 0.01	2.017 ± 0.01	110.06 ± 0.34	16.88	40.50

Fractions eluted with *Sc*_ASNaseI activity are shown, along with protein concentration (mg mL⁻¹), enzymatic activity (IU mL⁻¹), specific activity (IU mg⁻¹), purification factor (fold) and recovery (%) parameters.

Figure 4. Polyacrylamide gel electrophoresis combined with silver staining, showing the purified samples from the two-step FPLC purification.

The brown box stresses the (His)₆-tagged ASNase bands (~45.0 kDa). The different lanes represented the eluted fractions (5, 6 and 7: washout unbound proteins; 26-40: purified *Sc*_ASNase I and 48-57: non-purified *Sc*_ASNase I) and PM means Unstained Protein Molecular weight marker.

cell⁻¹). It should be highlighted that *Sc*_ASNaseI was remarkably purified at 15% of total cell dry weight, which is a sizable piece of total cellular protein.

Taking into account the results obtained and the demands of future applications, further optimization studies should be performed to the FPLC purification protocol, namely evaluating the effect of the fraction volume, pump pressure, initial volume injected, elution and binding buffer formulations in order to further improve the purifying yields. Other authors have reported some attempts to purify *Sc*_ASNaseI in the same line of work, namely Dunlop and collaborators.³⁵ These authors used several steps of purification to obtain the endogenous *Sc*_ASNaseI from *S. cerevisiae*; being the best specific activity found around 5 IU mg⁻¹, which is almost 20-fold lower than the obtained in the present work. In addition, the same authors commented on the difficulty to separate both isoforms endogenously expressed *Sc*_ASNaseI from *Sc*_ASNaseII,³⁵ a difficulty surpassed in this work by the use of an heterologous strategy and just one chromatographic purification step, which allowed the purification of *Sc*_ASNaseI resulting in a much higher specific activity than that obtained from endogenous expression.

For a method/process to be of industrial relevance, the polishing of the target macromolecule being produced and purified is a crucial step. In this case, and considering the potential application of the biopharmaceutical in the ALL treatment, the elimination of the (His)₆-tag from the biopharmaceutical³⁶ can be achieved through the use of the aminopeptidase dipeptidyl peptidase I (DPPI), either alone or in combination with glutamine cyclotransferase (GCT) and pyroglutamyl aminopeptidase (PGAP). In both cases, the (His)₆-tag is cleaved off by DPPI, which catalyses a stepwise

Figure 5. SDS-PAGE stained with Coomassie-blue representing the expression of *Sc*_ASNaseII in the insoluble fraction of the *E. coli* cell lysate.

Lane 1: BenchMark™ Protein Ladder; Lane 2: insoluble fraction pelleted after clarification of the cell lysate by centrifugation. Lane 3: supernatant of cell lysate, representing the protein soluble fraction.

excision of a wide range of dipeptides from the N-terminus of a peptide chain.³⁷ Other major concern considering the potential use of the biopharmaceutical in the ALL treatment is related to metal leaching from the column during purification³⁸. Although additional costs may be inserted for validation, new technologies have been developed in a lab scale in this context, in particular the treatment of the final sample with EDTA (GE Healthcare's excel³⁹ and Roche's cOmplete⁴⁰). Albeit, large-scale application of this system still needs to be fully evaluated.

Expression and purification of *Sc*_ASNaseII

Initially, the same expression and purification procedures described for *Sc*_ASNaseI were applied to express *Sc*_ASNaseII. Figure 5 depicts the high level of *Sc*_ASNaseII expression in the *E. coli* host cells. However, the protein was completely concentrated in the insoluble fraction of the cell lysate (Figure 5, lane 2). From the results of the lower growth temperature test, the conclusion was that this test was not improving the insoluble fraction presence as suggested elsewhere.⁴¹ In this context, several growth conditions including temperature of induction, concentration of (the inducer) IPTG and the time of induction were tested, since the optimization of these parameters is being recognized as a good strategy to overexpress soluble recombinant proteins in bacterial cells (as reviewed by Papaneophytou and Kontopidis⁴²). None of these conditions were able to produce soluble *Sc*_ASNaseII in the *E. coli* host cell. It was hypothesized that the native gene from the amino-acids 1 to 362, i.e., with the yeast periplasmic signal, should enhance the solubility of

Figure 6. SDS-PAGE stained with Coomassie-blue representing the expression of *Sc_ASNaseII* in the soluble fraction of the *E. coli* cell lysate and the specific activity of this protein.

The construction pET15b + ASP3₃₂₋₃₆₂ was expressed in the bacterial host strain CodonPlus (DE3) (Merck™); cells were pre inoculated in LB media overnight at a 37°C and 250 rpm. The inoculum was initially diluted to OD_{600nm} 0.2 in fresh culture media, grown at the same conditions until OD_{600nm} 0.4 when 0.1mM of IPTG was added and culture temperature was changed to 12°C. This *Sc_ASNaseII* induction was performed for 24 hours. In this expression vector construction, the protein starts at the amino-acid residue number 32, without the predicted yeast periplasmic signal. Fractions of nickel affinity purification. Lane 1: Total supernatant after cell lysis; Lane 2: BenchMark™ Protein Ladder; Lanes 3 and 4: Fractions from column elution with 500 mM of imidazole.

the protein; however, this expression also resulted in an insoluble protein. Then, several genetic attempts to obtain soluble *Sc_ASNaseII* were carried out, namely (i) the synthetic ASP3 gene was expressed with optimized codons to the *E. coli* host, (ii) by changing the expression vector modifying the position of His-tag from the N- to C-terminal, and (iii) constructing a vector that expresses *Sc_ASNaseII* in fusion with the SUMO protein (a highly soluble protein). Moreover, eight different types of *E. coli* strains were also tested (Supporting Information Table A.1 in ESI[†]). In conclusion, 93 different expression conditions (described in Supporting Information Table A.2 in ESI[†]) were tested without any success in the production of soluble *Sc_ASNaseII*. The only condition that resulted in low amounts of soluble *Sc_ASNaseII* (described briefly in Figure 6) produced a protein with a low specific activity (3.8 IU mg⁻¹ of protein) after the Immobilized Metal ion Affinity Chromatography (IMAC) purification step. These results are in accordance with the bioinformatics analysis, since *Sc_ASNaseII* was predicted to be glycosylated in the native host. The absence of glycosylation, caused by *E. coli* lack of glycosylation machinery, seems to be a key factor in the *Sc_ASNaseII* activity. Dunlop et al.³⁵ also purified *Sc_ASNaseII* endogenously produced in *S. cerevisiae*; the authors obtained a maximum SA of 60 IU mg⁻¹ of protein. However, this protein was characterized as hypermannosylated, which means that endogenous obtained *Sc_ASNaseII* is highly immunogenic and cannot be used as a biopharmaceutical from this source, because human immune system cells possess a receptor for mannose.⁴³ In addition, *Sc_ASNaseII* has been described as possessing 204 IU mg⁻¹ of protein, when expressed in *P. pastoris* host, a 3-fold higher activity than the endogenous source.²⁷ From the main results, it is suggested that, in contrast with *Sc_ASNaseI*, which is soluble

and active when expressed in *E. coli* host cells, the *Sc_ASNaseII* cannot be properly produced in this host cell, independent of the growth conditions used.

Conclusions

The optimized two-step FPLC purification method allowed the successful purification of *Sc_ASNaseI* from *S. cerevisiae* expressed in *E. coli* BL21 (DE3). Moreover, the bioinformatics analysis together with the enzyme activity results obtained suggested that N-glycosylation is not essential for *Sc_ASNaseI* activity. Antitumor activity and biochemical characterization of *Sc_ASNaseI* are being now conducted to evaluate the potential of this enzyme in acute lymphocytic leukemia treatment. By contrast, the results suggest that *Sc_ASNaseII* requires glycosylation, and therefore a eukaryotic expression host system to produce soluble and active enzyme.

Acknowledgments

This research was supported by grants from the Coordination for Higher Level Graduate Improvements (CAPES/Brazil), National Council for Scientific and Technological Development (CNPq/Brazil) and State of São Paulo Research Foundation (FAPESP/Brazil, process numbers 2013/08617-7 and 2015/07749-2). This work was developed in the scope of the project CICECO-Aveiro Institute of Materials (Ref. FCT UID/CTM/50011/2013), financed by national funds through the FCT/MEC. The authors also thank FCT for the doctoral SFRH/BD/102915/2014 and post-doctoral grants SFRH/BPD/79263/2011 of J.H.P.M. Santos and S.P.M. Ventura, respectively. JS doctoral grant and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. Moreover, S.P.M. Ventura acknowledges the FCT/MEC (Portugal) for a contract under Investigador FCT 2015 contract number IF/00402/2015.

Notation

ALL = acute lymphocytic leukemia
 ASNase = L-asparaginase
 BSA = bovine serum albumin
 FPLC = fast protein liquid chromatography
 IMAC FF = immobilized metal ion affinity chromatography fast flow
 IPTG = isopropylthio-β-D-galactoside
 IU = International unit
 L.B. = Luria Bertani
 OD = optical density
 PEG = polyethylene glycol
 PNGase F = peptide-N-glycosidase F
Sc_ASNaseI and = genes encoding ASNase from *Saccharomyces cerevisiae* were cloned in the prokaryotic expression system *Escherichia coli*
 TCA = trichloroacetic acid

Authors' Contributions

JS, IC, ML and MP constructed vectors and expressed ASNase, carried out enzyme activity assay and purification process, and wrote the manuscript drafting, JM participated in data analysis and carried out the computational analysis of recombinant enzyme, JC, PJ and SV participated in the design of the study analysed data, and wrote the manuscript,

AL and GM conceived the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors confirm they have read BioMed Central's guidance on competing interests and declare none of the authors have any competing interests.

Ethics Statement

The manuscript does not contain any data collected from humans, animals or plants.

Literature Cited

- Kidd JG. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum, I: course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. *J Exp Med* 1953;98:565–582.
- Broome JD. Studies on the mechanism of tumor inhibition by L-asparaginase. *J Exp Med* 1968;127:1055–1072.
- Wade HE, Robinson HK, Phillips BW. Asparaginase and glutaminase activities of bacteria. *J Gen Microbiol* 1971;69:299–312.
- Piatkowska-Jakubas B, Krawczyk-Kuliś M, Giebel S, Adamczyk-Cioch M, Czyz A, Lech Marañón E, Paluszewska M, Pałynyczko G, Piszcz J, Hołowiecki J. Polish Adult Leukemia Group. Use of L-asparaginase in acute lymphoblastic leukemia: recommendations of the Polish Adult Leukemia Group. *Pol Arch Med Wewn* 2008;118:664–669.
- Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A, Goekbuget N, Schrappe M, Pui CH. L-asparaginase treatment in acute lymphoblastic leukemia: a focus on *Erwinia* asparaginase. *Cancer* 2011;117:238–249.
- Rizzari C, Conter V, Starý J, Colombini A, Moericke A, Schrappe M. Optimizing asparaginase therapy for acute lymphoblastic leukemia. *Curr Opin Oncol* 2013;25:S1–S9.
- Takahashi H, Koh K, Kato M, Kishimoto H, Oguma E, Hanada R. Acute myeloid leukemia with mediastinal myeloid sarcoma refractory to acute myeloid leukemia therapy but responsive to L-asparaginase. *Int J Hematol* 2012;96:136–140.
- Beard ME, Crowther D, Galton DA, Guyer RJ, Fairley GH, Kay HE, Knapton PJ, Malpas JS, Scott RB. L-asparaginase in treatment of acute leukaemia and lymphosarcoma. *Br Med J* 1970;1:191–195.
- Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Eng J Med* 2004;350:1535–1548.
- Pui CH, Robison LL, Look AT. Acute lymphoblastic leukemia. *Lancet* 2008;371:1030–1043.
- Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. *Cr Rev Oncol-Hem* 2007;61:208–221.
- Guo QL, Wu MS, Chen Z. Comparison of antitumor effect of recombinant L-asparaginase with wild type one *in vitro* and *in vivo*. *Acta Pharm Sinic* 2002;23:946–951.
- Soares AL, Guimarães GM, Polakiewicz B, Pitombo RNDM, Abrahão-Neto J. Effects of polyethylene glycol attachment on physicochemical and biological stability of *E. coli* L-asparaginase. *Int J Pharm* 2002;237:163–170.
- EMA, European Medicines Agency; 2016. Available at: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002661/human_med_001954.jsp&mid=WC0b01ac058001d124. Accessed 04/11/2016.
- Cortijo-Cascajares S, Jiménez-Cerezo MJ, Tejada AHD. Revisión de las reacciones de hipersensibilidad a antineoplásicos. *Farm Hosp* 2012;36:148–158.
- Pieters R, Appel I, Kuehnel HJ, Tetzlaff-Fohr I, Pichlmeier U, van der Vaart I, Visser E, Stigter R. Pharmacokinetics, pharmacodynamics, efficacy, and safety of a new recombinant asparaginase preparation in children with previously untreated acute lymphoblastic leukemia: a randomized phase 2 clinical trial. *Blood* 2008;112:4832–4838.
- Billett AL, Carls A, Gelber RD, Sallan SE. Allergic reactions to *Erwinia* asparaginase in children with acute lymphoblastic leukemia who had previous allergic reactions to *Escherichia coli* asparaginase. *Cancer* 1992;70:201–206.
- Knoderer HM, Robarge J, Flockhart DA. Predicting asparaginase-associated pancreatitis. *Pediatr Blood Cancer* 2007;49:634–639.
- Raja RA, Schmiegelow K, Frandsen TL. Asparaginase-associated pancreatitis in children. *Br J Haematol* 2012;159:18–27.
- Schleis SE, Rizzo SA, Phillips JC, LeBlanc AK. Asparaginase-associated pancreatitis in a dog. *Can Vet J* 2011;52:1009–1012.
- Van den Berg H. Asparaginase revisited. *Leuk Lymph* 2011;52:168–178.
- Fu CH, Sakamoto KM. PEG-asparaginase. *Exp Opin Pharmacol* 2007;8:1977–1984.
- Keating MJ, Holmes R, Lerner S, Ho DH. L-asparaginase and PEG asparaginase—past, present, and future. *Leukemia Lymphoma* 1993;10:153–157.
- Sajitha S, Vidya J, Varsha K, Binod P, Pandey A. Cloning and expression of l-asparaginase from *E. coli* in eukaryotic expression system. *Biochem Eng J* 2015;102:14–17.
- Ferrara MA, Mattoso JMV, Bon EPS, Pereira N. Jr Kinetics of Asparaginase II fermentation in *Saccharomyces cerevisiae* ure2dal80 mutant—effect of nitrogen nutrition and pH. *Appl Biochem Biotech* 2004;113–116:299–305.
- Ferrara MA, Severino NMB, Mansure JJ, Martins AS, Oliveira EMM, Siani AC, Pereira N, Torres FAG, Bon EPS. Asparaginase production by a recombinant *Pichia pastoris* strain harbouring *Saccharomyces cerevisiae* ASP3 gene. *Enzyme Microb Tech* 2006;39:1457–1463.
- Castro-Girão LF, Rocha SLG, Teixeira RS, Ferrara MA, Perales J, Bon EPS. Purification and characterization of asparaginase II from *Saccharomyces cerevisiae* cloned in *Pichia pastoris*: a study on a possible anti-leukemic drug. *BMC Proc* 2014;8:78.
- Dorrestein E, Ferreira RB, Laureano O, Teixeira AR. Electrophoretic and FPLC analysis of soluble proteins in four Portuguese wines. *Am J Enol Vitic* 1995;46:235–242.
- Sharma A, Ng TB, Wong JH, Lin P. Purification and characterization of a lectin from *Phaseolus vulgaris* cv. (Anasazi beans). *J Biomed Biotechnol* 2009;2009:929568.
- Pattenden LK, Thomas WG. Amylose affinity chromatography of maltose-binding protein: purification by both native and novel matrix-assisted dialysis refolding methods. *Methods Mol Biol* 2008;421:169–189.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of micro-organisms. *J Gen Microbiol* 1973;76:85–89.
- Zielinska DF, Gnad F, Schropp K, Wiśniewski JR, Mann M. Mapping N-glycosylation sites across seven evolutionarily distant species reveals a divergent substrate proteome despite a common core machinery. *Mol Cell* 2012;46:542–548.
- Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* 2011;8:785–786.
- Dunlop PC, Meyer GM, Ban D, Roon RJ. Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. *J BiolChem* 1978;253:1297–1304.
- Kinna A, Tolner B, Rota EM, Titchener-Hooker N, Nesbeth D, Chester K. IMAC capture of recombinant protein from unclarified mammalian cell feed streams. *Biotechnol Bioeng* 2016;113:130–140.
- Pedersen J, Lauritzen C, Madsen MT, Weis Dahl S. Removal of N-terminal polyhistidine tags from recombinant proteins using engineered aminopeptidases. *Protein Exp Purif* 1999;15:389–400.
- Crowe J, Döbeli H, Gentz R, Hochuli E, Stüber D, Henco K. 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol Biol* 1994;31:371–387.

39. Ni Sepharose excel—GE Healthcare Life Sciences; 2016. Available at: <http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeSciences-br/17371202>. Accessed 04/11/2016.
40. cOmplete His-Tag Purification Resin—Roche Life Sciences; 2016. Available at: www.sigmaaldrich.com/catalog/product/roche/cohisrro. Accessed 04/11/2016.
41. Vasina JA, Baneyx F. Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the *Escherichia coli* cspA and tac Promoter systems. *Protein Exp Purif* 1997;9:211–218.
42. Papaneophytou CP, Kontopidis G. Statistical approaches to maximize recombinant protein expression in *Escherichia coli*: a general review. *Protein Exp Purif* 2014;94:22–32.
43. Lee SJ, Evers S, Roeder D, Parlow AF, Risteli J, Risteli L, Lee YC, Feizi T, Langen H, Nussenzweig MC. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science* 2002;295:1898–1901.

Manuscript received Apr. 5, 2016, and revision received Nov. 4, 2016.

ANEXO 7

SCIENTIFIC REPORTS

OPEN

Recombinant L-asparaginase 1 from *Saccharomyces cerevisiae*: an allosteric enzyme with antineoplastic activity

Received: 30 April 2016

Accepted: 12 October 2016

Published: 08 November 2016

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L-asparaginase (L-ASNase) (EC 3.5.1.1) is an important enzyme for the treatment of acute lymphoblastic leukaemia. Currently, the enzyme is obtained from bacteria, *Escherichia coli* and *Erwinia chrysanthemi*. The bacterial enzymes family is subdivided in type I and type II; nevertheless, only type II have been employed in therapeutic proceedings. However, bacterial enzymes are susceptible to induce immune responses, leading to a high incidence of adverse effects compromising the effectiveness of the treatment. Therefore, alternative sources of L-ASNase may be useful to reduce toxicity and enhance efficacy. The yeast *Saccharomyces cerevisiae* has the *ASP1* gene responsible for encoding L-asparaginase 1 (ScASNase1), an enzyme predicted as type II, like bacterial therapeutic isoforms, but it has been poorly studied. Here we characterised ScASNase1 using a recombinant enzyme purified by affinity chromatography. ScASNase1 has specific activity of 196.2 U/mg and allosteric behaviour, like type I enzymes, but with a low $K_{0.5} = 75 \mu\text{M}$ like therapeutic type II. We showed through site-directed mutagenesis that the T64-Y78-T141-K215 residues are involved in catalysis. Furthermore, ScASNase1 showed cytotoxicity for the MOLT-4 leukemic cell lineage. Our data show that ScASNase1 has characteristics described for the two subfamilies of L-asparaginase, types I and II, and may have promising antineoplastic properties.

L-asparaginase amidohydrolase (L-ASNase) (EC 3.5.1.1) catalyses the hydrolysis of L-asparagine (L-Asn) in aspartic acid and ammonia. L-ASNases are classified in three families: the plant-type, the *Rhizobium etili*-type and the bacteria-type. The bacterial family is subdivided into type I and type II¹; type I enzymes are cytosolic, expressed constitutively and have low affinity for L-Asn, resulting in non-therapeutic applications, while type II enzymes are restricted in the periplasmic space, with induced expression during anaerobiosis, have high affinity for L-Asn and present antitumor activity^{2,3}.

L-ASNase type II from *Escherichia coli* (EcASNase2) and *Erwinia chrysanthemi* (EwASNase2), native and PEGylated forms, are used for the treatment of acute lymphoblastic leukaemia (ALL) due to the fact that leukemic cells need extracellular L-Asn for protein synthesis, and L-ASNase depletes L-Asn and L-glutamine (L-Gln) from serum causing death by starvation and the absence of anti-apoptotic proteins^{4–6}. Interestingly, only leukaemia cells are sensitive to L-ASNase, as they frequently present asparagine synthetase (ASNS) genes that have been silenced by epigenetic mechanisms, while normal blood cells do not^{7,8}.

However, during treatment with bacterial L-ASNases, patients show a high incidence of adverse effects, such as neurotoxicity caused by the hydrolysis of L-Gln, hypersensitivity and allergic reactions that can lead to anaphylactic shock, and the formation of antibody anti-asparaginase^{4,9–12}. Nowadays, new L-ASNases have been identified in eukaryotic sources, in an effort to find new promising biopharmaceuticals with fewer side effects¹³.

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The yeast *Saccharomyces cerevisiae* constitutively expresses the gene *ASP1*, producing an intracellular cytoplasmic enzyme L-asparaginase 1 (ScASNase1). However, it has high molecular similarity to bacterial enzymes used in therapy^{14,15} and is classified in the bacterial type II family^{1,16}. In the 1970s, the few studies performed with ScASNase1 demonstrated low activity and affinity to L-Asn^{14,17}. Since then, no studies about this enzyme have been reported.

Therefore, we here describe the structural and biochemical characterization of the recombinant ScASNase1. Our results revealed that ScASNase1 presents allosteric behaviour similar to that of type I enzymes. Using site-directed mutagenesis approach to substitute important residues used in catalysis in bacterial enzymes, which are conserved in ScASNase1, we show that substitutions abrogated the enzyme activity but do not exert significant effects on the secondary structure of the enzyme. Moreover, we have demonstrated that ScASNase1 has antineoplastic potential in the MOLT-4 leukemic cell lineage, similarly to that observed for type II bacterial enzymes.

Results

Determining specific activity, optimum pH, temperature and kinetic parameters of ScASNase1.

The *ASP1* gene from *S. cerevisiae* has 1,146 bp and was obtained by PCR from genomic DNA, then cloned into the expression vector pET15b. The ScASNase1 was expressed in cytosol in the soluble fraction of proteins from the *E. coli* BL21 (DE3) strain. After purification, the enzyme was analysed by SDS–PAGE. The molecular mass of ScASNase1 is 41.4 kDa, and the His-tag addition resulted in a recombinant enzyme of approximately 45 kDa (<http://web.expasy.org/protparam/>). The gel showed a homogenous and pure protein with the expected molecular weight (see supplementary Fig. S1).

Using the purified protein, we determined the specific activity for L-Asn with Nessler's reagent and by coupled assay with NADH oxidation for L-Gln. The specific activity was calculated by the initial velocity of L-Asn or L-Gln hydrolysis as a function of enzyme concentration (Fig. 1A,B). ScASNase1 presented high specific activity for L-Asn of 196.2 ± 5.8 U/mg and low for L-Gln of 0.4 ± 0.02 U/mg; this last represents 0.38% of the L-asparaginase activity, considering the determination of the activity by the same method for both substrates (see supplementary Fig. S2). The pH effect on the optimum activity of the enzyme was measured in the range from 4.0 to 12.0. ScASNase1 was active in the range from pH 5.0 to 11.0, and its optimal activity was at pH 8.6 (Fig. 1C). The optimum temperature was determined by measuring enzyme activity in a temperature range from 20 °C to 65 °C for 20 minutes, and the higher value observed was at 40 °C (Fig. 1D).

The kinetic parameters were determined by coupled assay with NADH oxidation. Different concentrations of L-Asn were used to create a plot of initial velocity as a function of L-Asn concentration; the initial velocity was determined through linear regression. The graph revealed a sigmoidal profile and allosteric behaviour (Fig. 1E) and affinity for the substrate in the μ M range with positive cooperativity, as indicated by the Hill plot (Fig. 1F). The Hill coefficient measures the deviation of the Michaelis–Menten kinetics and describes quantitatively the degree of enzyme cooperativity¹⁸. The kinetic parameters determined are presented in Table 1. Statistical analysis of the kinetic models (Michaelis–Menten and sigmoidal allosteric), using the F-test under the null hypothesis that the enzyme has a Michaelis–Menten kinetic profile, revealed that the best kinetic model for ScASNase1 is the allosteric sigmoidal model with $R^2 = 0.9726$ and $p < 0.001$. The commercial EcASNase2 enzyme used as the control showed michaelian behaviour with $R^2 = 0.9797$ and K_m in the μ M range (Table 1), in agreement with the described in the literature, and specific activity of 224 ± 7 U/mg (see supplementary Fig. S3).

EcASNase2 has recently been described as possessing profile with allosteric positive cooperativity ($n_H = 1.5$) regulated by L-Gln¹⁹. At submicromolar concentrations of L-Asn, as occurs in the blood for the treatment of ALL, L-Gln is the most abundant substrate competing for the active site, and so the enzyme begins to present $n_H = 1.0$, which features Michaelis–Menten kinetics. ScASNase1 showed allosteric behaviour using only L-Asn as the substrate. To evaluate whether L-Gln could regulate the degree of cooperativity for ScASNase1, we also determined the kinetic parameters in the presence of L-Asn and L-Gln at a ratio of 1: 16 for each concentration of L-Asn. However, the addition of L-Gln did not alter the kinetic profile of the enzyme, different of observed to EcASNase2¹⁹ (Fig. 2A,B, Table 1).

Moreover, allosteric enzymes have the characteristic of being inhibited by the product of the reaction^{20,21}; the specific activity of ScASNase1 in the presence of 20 mM L-aspartate showed that the product is not able to interfere with enzyme activity (Fig. 2C). We also tested whether certain ions can act as enzyme–cofactors. The addition of Zn^{2+} and Ca^{2+} resulted in the decrease of ScASNase1 activity. No difference was detected when Mg^{2+} was used, whereas K^+ increased the level of enzymatic activity (Table 2). Since Zn^{2+} and Ca^{2+} diminished the activity of ScASNase1 *in vitro*, we evaluated the effect of the enzymes incubation with human serum over the specific activity of ScASNase1 and EcASNase2; we observed an increase in the activity and no effect, respectively (Fig. 2D), suggesting that in human serum ScASNase1 can be up stimulated and not repressed.

Site-directed mutagenesis of ScASNase1. ScASNase1 shares 38% sequence identity with bacterial type II counterparts, and amino acids already determined as essential to enzyme catalysis are strictly conserved between bacterial enzymes and ScASNase1 (Fig. 3A,B). To verify the importance of conserved catalytic residues to the mechanism of ScASNase1, four residues were replaced individually with alanine by site-directed mutagenesis (highlighted in Fig. 3A). The mutants T64A, Y78A, T141A and K215A were expressed intracellularly and soluble in the BL21 (DE3) *E. coli* strain. The isoforms were purified and analysed by SDS–PAGE. The molecular mass of the isoforms was approximately 45 kDa with the His-tag, and the enzymes obtained were homogeneous and pure (see supplementary Fig. S4). The activities of isoforms were measured by Nessler's reagent in the same conditions as the wild-type enzyme, and a 99.9% loss of activity was observed for all mutants (Fig. 3C). Statistical analysis of the specific activities of the T64A, K215A and T141A mutants showed no significant difference between them. The Y78A mutant presented a significant difference when compared to others, $p \leq 0.05$.

Figure 1. Determination of specific activity, optimum reaction conditions and kinetic characterisation for ScASNase1 enzyme. (A) Specific activity of ScASNase1 to hydrolyse L-Asn: Plot of the reaction velocities (V_0) of L-Asn hydrolysis as a function of mg of purified ScASNase1 as measured by Nessler's reagent. (B) Specific activity of ScASNase1 for L-Gln as measured by coupled assay with NADH oxidation: Plot of the reaction velocities (V_0) of L-Gln hydrolysis as a function of mg of purified ScASNase1. (C) The effect of pH was determined in different buffers (acetate pH 4.0; sodium phosphate pH 6.0, 7.0 and 12.0; Tris-HCl pH 8.0 and 9.0; sodium bicarbonate pH 10.0). (D) Optimum temperature was determined by measuring the specific activity in the range from 25 $^{\circ}\text{C}$ to 50 $^{\circ}\text{C}$. (E) ScASNase1 kinetics, activity dependence on a substrate concentration plot. The inset shows the sigmoidal profile of the enzyme at lower substrate concentrations. (F) Hill plot of the data. Points in the graph represent means $\pm$ SD ($n = 3$).

Recently, similar mutations were performed to the L-asparaginase from guinea pig AspG (amino acids T19A, T116A and K188M, equivalent to the ScASNase1 T64A, T141A and K215A) that also decreased or abrogated the AspG activity²². Additionally, the determination of crystallographic structure of the AspG mutants revealed that no significant differences were observed among the mutants and the wild-type enzyme, indicating that mutations do not exert significant effects on the protein structure but only over the enzyme activity²². To investigate if the amino acids substitutions also exerted influence over ScASNase1 structure we carried out structural approaches using circular dichroism spectroscopy. The analysis of secondary structure revealed that all mutant enzymes presented structural content slightly different to the wild type enzyme (Fig. 3C; Table 3), similar to the observed to AspG and similar to other mutants using a different approach²².

Figure 2. Influence of L-Asp and L-Gln on the parameters of ScASNase1. (A) ScASNase1 kinetic parameters using L-Asn and L-Gln together as substrates at the ratio of 1:16; activity dependence on a substrate concentration plot. (B) Hill plot of the data. Points represent means $\pm$ SD ($n = 3$). (C) Specific activity of ScASNase1 with added L-aspartate. 20 mM L-asparagine (L-Asn, 20 mM) and 10 mM L-asparagine + 20 mM L-aspartate (20 mM L-Asn + 10 mM L-Asp). (D) Specific activity of ScASNase1 and EcASNase2 after incubation at 37°C for 60 minutes with 10% human serum. The graphs of specific activities and kinetic parameters were obtained with GraphPad Prism 6.05 software. The specific activity was quantified by Nessler's reagent. Error bars were calculated from the mean $\pm$ SD ($n = 3$).

EcASNase2	K_m (μ M)	V_{max} (μ mol/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	n_H
L-asparagine	23 ± 5	0.032 ± 0.00061	134 ± 2	5.8×10^6	1.1 ± 0.1
ScASNase1	$K_{0.5}$ (μ M)	V_{max} (μ mol/min)	k_{cat} (s^{-1})	$k_{cat}/[K_{0.5}]^{n_H}$ ($M^{-1} \cdot s^{-1}$)	n_H
L-asparagine	75 ± 27	0.042 ± 0.0011	217 ± 14	1.6×10^4	2.2 ± 0.3
L-asparagine + L-glutamine	122 ± 31	0.097 ± 0.0023	523 ± 34	1.3×10^4	2.2 ± 0.2
EcASNase1 ^a					
L-asparagine	400 ± 50	—	7.4 ± 0.3	5.8×10^{-3}	3.5 ± 0.3

Table 1. Kinetic parameters of ScASNase1 to hydrolyse of L-asparagine and comparison with EcASNases. ^aKinetics data from Karamitros and Konrad, 2014 – using the same NAD⁺ coupled assay²⁹.

Effector	Concentration	Relative activity %
K ⁺	5.0 mM	109.7%
Mg ²⁺	10.0 mM	100.5%
Zn ²⁺	0.5 mM	7.3%
Ca ²⁺	0.5 mM	0%

Table 2. Relative activity of ScASNase1 in the presence of different effectors.

We also assessed the thermal stability of wild-type ScASNase1 using structure circular dichroism spectroscopy. The thermal profile of ScASNase1 revealed that the enzyme is very thermoresistant, presenting a melting temperature of 68.25 ± 0.21 °C (Fig. 3E), which may be related to the high cysteine content present in the enzyme, which in turn may form structural disulphides (Fig. 3A).

Figure 3. Structural and mutational analysis of ScASNase1. (A) Alignment of L-asparaginase amino acid sequences of *S. cerevisiae* (ScASNase1) (Uniprot number P38986), *E. coli* (EcASNase2) (Uniprot number P00805) and *E. chrysanthemi* (EwASNase2) (Uniprot number P06608). The amino acids are shaded in grey tones according to physico-chemical similarities among the three isoforms. The black boxes denote conserved amino acids involved in catalytic activity. The alignment was produced using Clustal Ω (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), and the graphical representation was generated using JalView (<http://www.jalview.org>). (B) Crystallographic structure of *E. coli* (PDB code = 3ECA), highlighting the position of conserved residues and a ligand molecule. (C) Specific activity of the active site residues mutated in ScASNase1 by an alanine. The error bar represents means $\pm$ SD ($n=3$). (D) Circular dichroism spectra of the ScASNase1 (black) and the mutant enzymes T64A (green), Y78A (red), K125A (grey) and T141A (blue). The protein concentrations used in experiments were $5\mu\text{M}$ in Tris buffer 10 mM (pH 7.4). All spectra were recorded at 25°C and corrected against the buffer. The graphical representations are averages from eight consecutive scans. (E) The thermal melting profile for ScASNase1 was recorded at 222 nm, and the temperature was increased from 20 to 90°C . The protein concentrations used in experiments were $5\mu\text{M}$ in Tris buffer 10 mM (pH 7.4). The melting temperature obtained was $68.25 \pm 0.21^\circ\text{C}$.

	ScASNase1	T64A	Y78A	T141A	K215A
Helix	16.50%	16.36%	16.12%	14.30%	16.93%
Antiparallel	26.65%	26.10%	26.82%	28.21%	26.21%
Parallel	9.25%	9.37%	9.31%	9.60%	9.22%
Beta-turn	16.32%	16.26%	16.23%	15.67%	16.44%
Random Coil	31.28%	31.91%	31.48%	32.22%	31.22%

Table 3. Secondary structure analyzed by circular dichroism spectroscopy. The secondary structure contents were obtained using CDNN software.

Figure 4. Cytotoxicity assay using HUVEC, REH and MOLT-4 cell lines. (A) Relative survival using EcASNase2. (B) Relative survival using ScASNase1. Cells were incubated with 10 U/mL of ScASNase1 or EcASNase2 (Prospec-Tany, Israel) for 72 h. The control is represented by the cell culture containing only RPMI 1640 medium with 10% foetal bovine serum. The enzyme buffer was Tris-HCl 50 mM pH 8.8 to ScASNase1 or Tris-HCl 50 mM pH 7.4 to EcASNase2. Error bars were calculated from the mean $\pm$ SD (n = 3). Statistical analysis using the ANOVA with Bonferroni test performed using GraphPad Prism 6.05 software showed $p < 0.001$ versus respective control and buffer.

Cytotoxicity of ScASNase1. The cytotoxic potential of ScASNase1 was analysed *in vitro* in three different cell types: HUVEC, as healthy cells that cover the blood vessel wall and are therefore in contact with circulating drugs; REH, considered resistant to treatment with L-ASNase because it has high ASNS levels of expression²³; and MOLT-4, described as a sensitive L-ASNase lineage. Nevertheless, MOLT-4 evolves resistance six weeks after L-ASNase treatment, through the augmented expression of ASNS²⁴.

Cells were incubated with 10 U/mL of ScASNase1 or EcASNase2. Controls were incubated with only RPMI medium or protein buffer for 72 hours (Fig. 4). As expected, the *E. coli* enzyme did not cause toxicity to normal healthy cells HUVEC. However, EcASNase2 treatment killed almost 100% of MOLT-4 cells and 20% of the resistant lineage, REH. Similarly to treatment with EcASNase2, treatment with ScASNase1 did not cause toxicity to normal cells HUVEC and caused death about to 85% of MOLT-4 cells, but REH cells were resistant to ScASNase1 (Fig. 4). Statistical analysis of the data, using ANOVA with the Bonferroni model, demonstrated that the mortality of the REH cells treated with EcASNase2 and MOLT-4 cells in the presence of EcASNase2 or ScASNase1 was significant at a level of $p < 0.001$, compared with the respective controls.

Discussion

To our knowledge, we describe here for the first time the characterization of biochemical and antileukemic activity of the ASNase1 from *S. cerevisiae*, providing evidence of its biopharmaceutical potential. Moreover, the heterologous expression of ScASNase1 was obtained with high purity (see supplementary Fig. 1) through one chromatographic step with specific activity of 196.2 U/mg for L-Asn (Fig. 1A). Only Jones and Mortimer¹⁷ and Dunlop *et al.*¹⁴ had found specific activity of 0.06 U/mg and 5.4 U/mg, respectively, to ScASNase1^{14,16}. However, these authors obtained the enzyme by purification of the native protein direct from *S. cerevisiae* through multiple

chromatographic steps. The specific activity found in our study is similar to that reported for type II L-ASNase from *E. coli* (EcASNase2), as confirmed here using commercial enzyme as control (223 ± 7 U/mg) (see supplementary Fig. S3).

EcASNase2 and EwASNase2 also show specific activity for L-Gln with values corresponding to a range of 2% and 10% of their L-ASNase activities^{4,5}. According to our results, ScASNase1 presents glutaminase activity that is 0.38% of L-ASNase activity (Fig. 1B), which is approximately twenty-five times smaller than that of the commercial enzyme EcASNase2 tested in our laboratory (~10%) (see supplementary Fig. S3).

Glutaminase activity has been demonstrated to be important for effective treatment in ASNS-positive leukemic lines (which express asparagine synthetase), while for the ASNS-negative line (which does not express asparagine synthetase), only L-ASNase activity would be required²⁵. However, another study showed that glutaminase activity in ASNS-negative lines is important to increase the death of leukemic cells, and this effect can be associated with the expression of ASNS by subclones, or with the achievement of L-Asn from other cells²⁶. According to Anishkin *et al.*¹⁹, L-Gln is responsible for ensuring the asparaginase activity of the enzyme at sub-micromolar concentrations of L-Asn, concentrations that are reached in the serum during ALL treatment. L-Gln was a positive modulator, because in these very low concentrations, EcASNase2 would be unable to recognize the substrate, increasing the affinity of the enzyme for L-Asn¹⁹. This mechanism of regulation justifies the need for L-glutaminase activity for the effective treatment ASNS-negative cells. Nevertheless, this activity is responsible for enhancing adverse effects such as neurotoxicity and hyperammonemia^{4,11}. Thus, an enzyme with low glutaminase activity and dependence, such as ScASNase1, may be of utility in reducing adverse effects in the treatment of ALL in an ASNS-negative patient.

Dunlop *et al.*¹⁴ reported a K_m of $740 \mu\text{M}$ and suggested that ScASNase1 could not deplete low levels of L-Asn. We demonstrated allosteric behaviour (Fig. 1E,F) with a $K_{0.5}$ of $75 \mu\text{M}$ and positive cooperativity, as indicated by n_H of 2.2 (Table 1). Allosteric and cytoplasmic L-ASNs are classified as bacterial type I enzymes, such as L-ASNase1 from *E. coli* (EcASNase1), which has n_H of 3.5 and $K_{0.5} = 0.4 \text{ mM}$ ^{27,28}, and human L-ASNase1 (HsASNase1), which has n_H of 3.9 and $K_{0.5} = 11.5 \text{ mM}$ ²⁹. In the same way as for type I enzymes, ScASNase1 is regulated by the substrate to catalyse the hydrolysis of L-Asn but shows high affinity for the substrate and no inhibition by product (Fig. 2C). However, the catalytic efficiency of ScASNase1 is affected by allosteric behaviour and Hill coefficient^{30,31}. While michaelian EcASNase2 has an efficiency dictated by k_{cat}/K_m of $10^6 \text{ M}^{-1} \text{ s}^{-1}$, ScASNase1 efficiency is defined by $k_{\text{cat}}/[K_{0.5}]^{n_H}$ reaching a value of 10^4 . This is two orders of magnitude lower than EcASNase2, but it is seven orders higher comparing with EcASNase1 (see Table 1).

Because of allosteric behaviour, the effect of ions was tested. The presence of Mg^{2+} did not change the activity and Zn^{2+} and Ca^{2+} decreased the activity (Table 2); this result is indicative that ScASNase1 is not a metalloprotein²⁹. Furthermore, the enzymatic inhibition caused by metal ions and the presence of 10 cysteines in ScASNase1 (Fig. 3A) suggest that certain vicinal sulfhydryl groups are important for protein stabilization, a property described for other L-ASNs^{32,33}. It is noteworthy to cite that the incubation of EcASNase2 with human serum did not affect the activity, while ScASNase1 presented a significant augment of specific activity (Fig. 2D), suggesting that other serum factors can contribute to positive allosteric regulation of the enzyme.

EcASNase2 and EwASNase2 have high affinity for the substrate, with K_m in the μM range^{34–37}. Even with a michaelian profile, EcASNase2 has a degree of cooperativity at submicromolar concentrations of L-Asn, which is modulated by L-Gln¹⁹. The addition of L-Gln during the reaction did not affect the affinity for the substrate or the degree of cooperativity for ScASNase1 (Table 1 and Fig. 2A,B), which is different from that recently described for EcASNase2 and reveals distinct functional characteristics of the yeast enzyme.

Despite structural differences, type I and type II L-ASNs present similarity in their amino acid sequences, and their functional active site residues are conserved throughout evolution^{1,16,38}. The mechanism of action proposed for L-ASNs relies on EcASNase2 observations, in which a first nucleophilic attack of T12 on the substrate is followed by the release of ammonia and formation of the acyl-enzyme complex. In the subsequent step, with K162 and T89, this intermediate undergoes a second nucleophilic attack by a water molecule, resulting in hydrolysis of the acyl-enzyme intermediate, producing the aspartic acid, leaving the free enzyme³⁹. The hydrogen bonds formed between Y25 and T12 promote the correct orientation of T12 for catalysis⁴⁰. A comparison of the primary structure of EcASNase2 and EwASNase2 with ScASNase1 revealed that the active site residues involved in catalysis are conserved in ScASNase1 (Fig. 3A,B). Single substitutions of the T64, Y78, T141 and K215 residues to alanine revealed that all mutants have significant loss of activity compared to the wild-type enzyme (Fig. 3C). Structural analysis of secondary structure content revealed that all mutant enzymes presented similar α -helix and β -sheet content relative to the wild-type ScASNase1 indicating that the amino acids substitutions performed in this work did not affect the integrity of the enzyme structure (Table 3).

Additional analyses aiming to evaluate enzyme thermostability showed that ScASNase1 possesses high thermostability ($\sim 68^\circ\text{C}$ – Fig. 3E), in agreement with kinetic thermal analysis, which revealed that the enzyme retains >60% of its asparaginase activity at 50°C (Fig. 1D).

The main characteristic of bacterial type II enzymes is their antineoplastic potential. The first characterization of ScASNase1 demonstrated low specific activity and a K_m of $740 \mu\text{M}$ for L-Asn¹⁴; this affinity would not allow the depletion of human serum levels of L-Asn, since an enzyme has to be very active at low substrate concentrations to present antineoplastic potential³⁶. Yun *et al.*²⁷ demonstrated the allosteric profile of EcASNase1 and proposed that L-ASNs type I cannot be considered for therapeutics because it does not deplete low levels of L-Asn²⁷. Considering that, asparagine concentration in circulating human serum is $50 \mu\text{M}$ ⁴¹, using the equation 1, equal amount of enzymes and kinetic values of Table 1, we can compare the velocity of reactions in physiological conditions among EcASNase1, EcASNase2 and ScASNase1.

$$v/[E] = k_{\text{cat}} \times [S]^{n_H} / [S]^{n_H} + [K_{0.5}]^{n_H} \quad (1)$$

Equation 1: where v is initial velocity of reaction; $[E]$ is total enzyme concentration; k_{cat} is the turnover constant; $[S]$ is the substrate concentration; $K_{0.5}$ is the concentration of substrate in which enzyme reaches half of maximal velocity and n_H is the Hill coefficient³¹.

In this prediction, EcASNase2 exerts its antileukemic activity with $v = 92 \mu\text{M/s}$; ScASNase1 would present $v = 63 \mu\text{M/s}$, while EcASNase1 would show $v = 0.005 \mu\text{M/s}$. This strongly suggests that, at least in catalytic kinetic terms, ScASNase1 potentially exerts asparaginase activity compatible with antileukemic effect.

In 2013, a study with encapsulated ScASNase1 showed that this enzyme can induce the death of leukemic cell lines *in vitro*⁴². Here, we corroborated this data and also showed that ScASNase1 is active in physiological conditions (pH 7.4 at 37 °C), with optimum activity even at pH 8.6 and 40 °C (Fig. 1C,D) and kills 85% of human acute lymphoblastic leukaemia MOLT-4 cells *in vitro* (Fig. 4). MOLT-4 is an important cell line that has been used to investigate the effect of other L-ASNases, such as L-ASNase from *Erwinia carotovora* and *Helicobacter pylori*. In these cases, 10 U/mL of *Erwinia carotovora* or *Helicobacter pylori* caused mortality of 83% and 30%, respectively, in MOLT-4 cells^{43,44}. Using the same concentration of EcASNase2, the mortality of MOLT-4 cells was 95%⁴³. The REH cell line uses L-Gln pathway asparagine synthetase for the synthesis of L-Asn²³. The low glutaminase activity of ScASNase1 prevents the mortality of this lineage. However, the antileukemic potential of ScASNase1 demonstrated here was superior to that observed for type II enzymes from *Erwinia carotovora* and *Helicobacter pylori* and is equivalent to that caused by EcASNase2 in MOLT-4 cells. It is noteworthy that the non-bacterial origin of ScASNase1 may present an additional advantage in the antileukemic treatment of sensitive lines, reducing the adverse effects due to low glutaminase activity.

In conclusion, our results show that ScASNase1 has specific activity of 196.2 U/mg, which is compatible with the enzymes used in the treatment of ALL and almost forty-fold higher than previously characterized. In addition, it has allosteric behaviour with a $K_{0.5}$ of 75 μM , showing a high affinity for the substrate L-Asn. Residues T64-Y78-T141-K215 play important roles in enzyme catalysis. We suggest that ScASNase1 may have interesting antineoplastic properties, because ScASNase1 shows toxicity for leukemic cells but not to normal healthy cells (HUVCEC), can reduce adverse effects due to low glutaminase activity and may be less immunogenic than bacterial enzymes. ScASNase1 is classified as an enzyme belonging to bacterial type II, has high affinity for the substrate and exhibits antineoplastic properties. However, ScASNase1 also presents characteristics of bacterial type I enzyme, such as allosteric behaviour and cytoplasmic localization in yeast. Additional structural and antileukemic studies that are being conducted by our research group could help to elucidate the active site microenvironment and unique properties of ScASNase1.

Methods

Gene cloning, protein expression and purification of ScASNase1. The *ASP1* gene was isolated from genomic DNA from the BY4741 strain of *S. cerevisiae* by polymerase chain reaction, using the oligonucleotides SC_Asp1F 5' GGGAAATTCATATGTTACCAAGAATCAAATCTTGGG 3' and SC_Asp1R 5' CGCGGATCCTCACCACCATAGAC 3' with restriction site adaptors to *Nde* I and *Bam* HI (Exxtend Biotecnologia São Paulo, Brazil). The PCR product was cloned into *Nde* I and *Bam* HI restriction sites of the pET15b vector (Novagen – Merck Millipore). pET15b-*ASP1* was used to transform *E. coli* DH5 α . Single colonies were selected; their plasmids were extracted and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit using the ABI 3730 DNA Analyser (Thermo Scientific) automatic sequencer to confirm gene integrity. The correct constructions were used to transform *E. coli* BL21 (DE3) (Novagen – Merck Millipore) strain by electroporation.

Transformed *E. coli* BL21 (DE3) containing pET15b-*ASP1* were grown overnight at 37 °C in 100 mL of medium LB (10% tryptone, 5% NaCl and 5% yeast extract) containing 50 $\mu\text{g/mL}$ of carbenicillin and then transferred to 1 L of fresh LB containing carbenicillin (50 $\mu\text{g/mL}$) and grown to $\text{OD}_{600\text{nm}} = 0.6\text{--}0.8$. Protein expression was induced by IPTG addition to a final concentration of 1 mM for 3 hours at 37 °C. The cells were harvested by centrifugation at $4,000 \times g/4^\circ\text{C}/20\text{ min}$, and the pellet was suspended with start buffer (20 mM sodium phosphate pH 7.4; 300 mM NaCl; 20 mM imidazole) and added to PMSF 1 mM. Cell disruptions were performed by sonication with 30% amplitude, sonicating for 24 cycles of 5 seconds and resting for 15 seconds in ice. The cellular lysate was treated with 1% sulfate streptomycin in ice for 20 min. The suspension was clarified by centrifugation at $16,000 \times g/4^\circ\text{C}/30\text{ min}$ and homogenised by filtration using a 45- μm membrane (Merck–Millipore) and applied to a Hi-Trap nickel-affinity column (GE Healthcare) for purification. The column was equilibrated with 10 volumes of start buffer (20 mM sodium phosphate pH 7.4; 300 mM NaCl; 20 mM imidazole); the protein extract was applied, and the unbound proteins were washed with 5 column volumes using a stepwise gradient of imidazole (20 mM sodium phosphate pH 7.4; 300 mM NaCl; initializing with 50 mM, followed by 100 mM and 150 mM imidazole); ScASNase1 was eluted with 20 mM sodium phosphate pH 7.4; 300 mM NaCl; 500 mM imidazole.

Protein purification and integrity were evaluated by SDS–PAGE according to the method of Laemmli (1970). The ScASNase1 was applied to a PD-10 Desalting Column (GE Healthcare), using 20 mM Tris–HCl pH 8.8 buffer. The protein concentration was measured by spectrophotometry at $\lambda = 280\text{ nm}$, with an extinction coefficient of ScASNase1 ($\epsilon = 25955\text{ M}^{-1}\text{ cm}^{-1}$) obtained using the ProtParam tool (<http://web.expasy.org/protparam/>).

Mutagenesis of ScASNase1. Mutants T64A, Y78A, T141A and K215A were obtained using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) with pET15b-*ASP1* as the template and the following primers: ASP1T64A_F 5'GGGTACCGGTGGTGCG ATTGCATCGAAAGC 3' and ASP1T64A_R 5'GCTTTCGATGCAATC GCACCACCGGTACCC 3'; ASP1Y78A_F 5'AAACTG CCGGCGCGCATGTTGACCTGACC 3' and ASP1Y78A_R 5'GGTCAGGTCAACATGCGCGCCGGCAGTTT 3'; ASP1T141A_F 5'ATTACCCATGGGGCCGATACGCTAT 3' and ASP1T141A_R 5' ATAGCGTATC GGCCCCATGGGTAAT 3'; ASP1 K215A_F 5' TCTGGTTACTACATTACTGCCACGAATGCAAATAGTTTGG 3' and ASP1 K215A_R 5' CCAAACATTTGCATTCGTGGCAGTAATGTAGTAACCAGA 3'. The products of

the reaction were treated with *Dpn* I to remove the original methylated plasmids. The *E. coli* XL1-Blue strain was used as host in the transformations. The plasmids were sequenced, as described before, to confirm the codon substitutions, and positive ones were used to transform *E. coli* strain BL21 (DE3) (Novagen – Merck Millipore) by electroporation. The recombinant enzymes were expressed and purified as described for the wild-type enzyme.

Enzyme activity assay. To determine the level of L-ASNase-specific activity, we measured the ammonia produced by the hydrolysis of L-Asn (Sigma–Aldrich/USA) catalysed by the enzyme through Nessler's reagent (Merck–Millipore). The protocol used was adapted to a microplate reader according to the manufacturer's instructions. The reaction containing 50 mM Tris–HCl (pH 8.8), 20 mM L-Asn and 36–540 ng of enzyme was incubated at 37 °C for 20 min; the reaction was stopped with trichloroacetic acid (TCA) 1.5 M and then diluted in water (10×) followed by the addition of Nessler's reagent. The same assay was used to determine the interference of L-Aspartate (L-Asp) in the reaction, using 10 mM L-Asn + 20 mM L-Asp. For the mutant isoforms T64A, Y78A, T141A and K215A, 0.03–0.27 mg protein were used with 20 mM L-asparagine. The reactions were incubated for 1 hour at 37 °C. The results were recorded spectrophotometrically at $\lambda = 440_{\text{nm}}$ using the SpectraMax Microplate Reader (Molecular Devices). The standard curve was constructed with ammonium sulfate using concentrations ranging from 0 to 20 $\mu\text{mol/mL}$ ⁴⁵.

Glutaminase activity was estimated spectrophotometrically using an NADH oxidation coupled assay adapted from Balcão, *et al.*⁴⁶. The L-glutamate (L-Glu) produced in the hydrolysis of L-Gln by L-ASNase is used for the synthesis of α -ketoglutarate in the presence of L-glutamate dehydrogenase (GDH) (Sigma–Aldrich/USA) and β -nicotinamide adenine dinucleotide (NADH) (Sigma–Aldrich/USA). The reactions were performed using microplates as follows: 50 mM Tris–HCl pH 8.0, 20 mM L-Gln, 0.13 mM NADH, 0.5 U GDH (diluted in 50 mM sodium phosphate pH 7.4; 50% glycerol) and 0 mg to 0.09 mg of ScASNase1 enzyme or the commercial enzyme EcASNase2 (Prospec–Tany, Israel), which was used as a positive control. The absorbance was measured at $\lambda = 340_{\text{nm}}$ at 37 °C, and the extinction coefficient used was $\epsilon = 0.85 \mu\text{mol}^{-1} \text{cm}^{-1}$.

Effect of pH and temperature on ScASNase1. To determine the optimum pH for ScASNase1, enzyme activity was measured at 37 °C in different buffers: acetate pH 4.0; sodium phosphate pH 6.0, 7.0 and 12.0; Tris–HCl pH 8.0 and 9.0; sodium bicarbonate pH 10.0. To determine the optimum temperature, enzyme activity was measured from 25 °C to 65 °C. After 20 min of reaction, the ammonium generated was measured by Nessler's reagent as described above.

Determination of specific activity of ScASNase1 in the presence of Zn^{2+} , K^+ , Mg^{2+} , Ca^{2+} and human serum. The final concentrations of reagents were used in all assays: L-asparaginase (1–30 nM), Tris–HCl buffer pH 8.8 50 mM, 20 mM L-asparagine. The enzyme was pre-incubated at 37 °C for 30 minutes with each compound at concentrations of 0.5 or 10 mM from Zn^{2+} , K^+ , Mg^{2+} or Ca^{2+} . To assay with human serum, ScASNase1 and EcASNase2 were pre-incubated at 37 °C for 60 minutes with 10% human serum (Agilent Technologies).

The reactions were incubated at 37 °C for 20 minutes with L-asparagine. The ammonium release was measured using Nessler's reagent as described above.

Kinetic analysis. The kinetic parameters of purified ScASNase1 to hydrolysis of L-Asn were determined by spectrophotometry through NADH-consumption-coupled assay as adapted from Balcão, *et al.*⁴⁶. The ammonia released is used in the production of L-Glu in the presence of GDH and reduced β -nicotinamide adenine dinucleotide (β -NADH) (Sigma). NADH oxidation was measured spectrophotometrically at $\lambda = 340_{\text{nm}}$ at 37 °C, and the extinction coefficient used was $\epsilon = 0.85 \mu\text{mol}^{-1} \text{cm}^{-1}$. Microplates received 50 mM Tris–HCl pH 8.0; 0.07 mM, 0.1 mM, 0.3 mM, 0.5 mM, 0.7 mM, 1.5 mM, 2.0 mM and 2.5 mM of L-Asn, 0.13 mM β -NADH, 1 mM α -ketoglutarate 0.5 U GDH (diluted in 50 mM sodium phosphate pH 7.4; 50% glycerol) and 180 ng of ScASNase1. A second assay was conducted at the same concentrations of L-Asn and ScASNase1 with added L-Gln at a ratio of 1:16 for each concentration of L-Asn. The concentrations of L-Gln used were 1.12 mM, 1.6 mM, 4.8 mM, 8.0 mM, 11.2 mM, 24 mM, 32 mM and 40 mM. The substrate affinity and turnover number were determined using non-linear regression analysis of experimental steady-state data using GraphPad Prism version 6.05 software. Kinetic parameters were determined under the same conditions described above for commercial enzyme EcASNase2 (Prospec–Tany, Israel).

Circular dichroism spectroscopy of ScASNase1. The CD spectra of ScASNase1 native and reduced proteins were obtained using a 0.1 cm path length cuvette containing 10 μM of protein sample in 5 mM Tris buffer (pH 7.4). The assays were carried out at 25 °C in a Jasco J-815 spectropolarimeter (Jasco Inc.). The spectra are representative of an average of eight scans recorded from 190 to 260 nm. The content of secondary structures in each protein (wild-type and mutants) was estimated using CDNN 2.1 software⁴⁷. Melting profiles at a constant wavelength of 222 nm, θ_{222} , were recorded while the sample was heated from 20 to 95 °C, with a 1 °C increment/min.

Cells culture and cytotoxicity assay. The leukaemia human cell lineage MOLT-4 and REH, as well as the Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from the Banco de Células do Rio de Janeiro (RJ/Brazil). Cells were maintained in RPMI 1640 medium with 10% foetal bovine serum (v/v) and incubated at 37 °C in a 5% CO_2 incubator. These procedures followed the ATCC instructions (<http://www.atcc.org/>). After reaching confluence, cell lineages were centrifuged at $600 \times g$ at 4 °C for 10 min and suspended in fresh RPMI medium. MOLT-4, REH or HUVEC, at 1.0×10^4 cells/well, were incubated in 24-well microplates with and without 10.0 U/mL of ScASNase1 or EcASNase2. As a control, the cells were incubated with RPMI medium and with protein buffer (50 mM Tris–HCl pH 8.8 for ScASNase1 and 50 mM Tris–HCl pH 7.4 for EcASNase2) for 72 hours at 37 °C in a 5% CO_2 incubator. Cells were centrifuged at $600 \times g$ at 4 °C for 10 min, then stained with Trypan blue

(Sigma–Aldrich/USA) to select dead cells. Cells were counted in a Neubauer chamber to determine cell viability, which is expressed as a percentage of living cells.

Statistical Analysis. All analyses were performed, at least, in triplicate. Results were represented as mean $\pm$ standard deviation (SD). The statistical analysis was performed using ANOVA, considering $p \leq 0.05$, and using GraphPad Prism version 6.05 software.

References

- Borek, D. & Jaskólski, M. Sequence analysis of enzymes with asparaginase activity. *Acta Bioch. Pol.* **48**, 893–902 (2001).
- Schwartz, J. H., Reeves, J. Y. & Broome, J. D. Two L-Asparaginases from *E. coli* and their action against tumor. *Proc. Natl. Acad. Sci. USA* **56**, 1516–1519 (1966).
- Cedar, H. & Schwartz, J. H. Localization of the two L-Asparaginases in anaerobically grown *Escherichia coli*. *J. Biol. Chem.* **242**, 3753–3755 (1967).
- Narta, U. K., Kanwar, S. S. & Asmi, W. Pharmacological and clinical evaluation of l-asparaginase in the treatment of leukaemia. *Crit. Rev. Oncol. Hematol.* **61**, 208–221 (2007).
- Avramis, V. I. Asparaginases: Biochemical pharmacology and modes of drug resistance. *Anticancer Res.* **32**, 2423–2438 (2012).
- Emadi, A., Zokaee, H. & Sausville, E. A. Asparaginase in the treatment of non ALL hematologic malignancies. *Cancer Chemother. Pharmacol.* **73**, 875–883 (2014).
- Akagi, T. *et al.* Methylation analysis of asparagine synthetase gene in acute lymphoblastic leukemia cells. *Leukemia* **20**, 1303–1306 (2006).
- Pastorczyk, A. *et al.* Asparagine synthetase (ASNS) gene polymorphism is associated with the outcome of childhood acute lymphoblastic leukemia by affecting early response to treatment. *Leuk. Res.* **38**, 180–183 (2014).
- Narazaki, H. *et al.* Delayed-type hypersensitivity in response to L-asparaginase in a case of acute lymphoblastic leukemia. *J. Nippon Med.* **79**, 489–493 (2012).
- Pieters, R. *et al.* L-asparaginase treatment in acute lymphoblastic leukemia: a focus on *Erwinia* asparaginase. *Cancer* **117**, 238–249 (2011).
- Raetz, E. A. & Salzer, W. L. Tolerability and efficacy of L-asparaginase therapy in pediatric patients with acute lymphoblastic leukemia. *J. Pediatr. Hematol. Oncol.* **32**, 554–563 (2010).
- Rizzari, C. *et al.* Optimizing asparaginase therapy for acute lymphoblastic leukemia. *Co-oncology* **25**, S1–S9 (2013).
- Shrivastava, A. *et al.* Recent developments in l-asparaginase discovery and its potential as anticancer agent. *Crit. Rev. Oncol. Hematol.* **100**, 1–10 (2016).
- Dunlop, P. C., Meyer, G. M., Ban, D. & Roon, R. J. Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **253**, 1297–1304 (1978).
- Sinclair, K., Warner, J. P. & Bonthron, D. T. The *ASP1* gene *Saccharomyces cerevisiae*, encoding the intracellular isozyme of L-asparaginase. *Gene* **144**, 37–43 (1994).
- Bonthon, D. T. & Jaskólski, M. Why a “benign” mutation kills enzyme activity. Structure-based analysis of the A176V mutant of *Saccharomyces cerevisiae* L-asparaginase I. *Acta Biochimica Polonica* **44**, 491–504 (1997).
- Jones, G. E. & Mortimer, R. K. Biochemical Properties of Yeast L-Asparaginase. *Biochemical Genetics* **9**, 131–14 (1973).
- Ricard, J. & Cornish-Bowden, A. Co-operative and allosteric enzymes: 20 years on. *Eur. J. Biochem.* **166**, 255–272 (1987).
- Anishkin, A. *et al.* Catalytic role of the substrate defines specificity of therapeutic L-asparaginase. *J. Mol. Biol.* **427**, 1–19 (2015).
- Gerhart, J. From feedback inhibition to allostery: the enduring example of aspartate transcarbamoylase. *FEBS Journal* **218**, 612–620 (2014).
- Cornish-Bowden, A. Understanding allosteric and cooperative interactions in enzymes. *FEBS Journal* **281**, 621–632 (2014).
- Schalk, A. M., Antansijevic, A., Caffrey, M. & Lavie, A. Experimental Data in Support of a Direct Displacement Mechanism for Type I/II L-Asparaginases. *J. Biol. Chem.* **291**, 5088–5100 (2016).
- Hermanova, I., Zaliova, M., Trka, J. & Starkova, J. Low expression of asparagine synthetase in lymphoid blasts precludes its role in sensitivity to L-asparaginase. *Exp. Hematol.* **40**, 657–665 (2012).
- Aslanian, A. M., Fletcher, B. S. & Kilberg, M. S. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. *Biochem. J.* **357**, 321–328 (2001).
- Chan, W. K. *et al.* The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. *Blood* **123**, 3596–3606 (2014).
- Parmentier, J. H. *et al.* Glutaminase activity determines cytotoxicity of l-asparaginases on most leukemia cell lines. *Leuk. Res.* **39**, 757–762 (2015).
- Yun, M., Nourse, A., White, S. W., Rock, C. O. & Heath, R. J. Crystal structure and allosteric regulation of the cytoplasmic *Escherichia coli* L-asparaginase I. *J. Mol. Biol.* **369**, 794–811 (2007).
- Karamitros, C. S. & Konrad, M. Human 60-kDa Lysophospholipase contains an N-terminal L-asparaginase domain that is allosterically regulated by L-Asparagine. *J. Biol. Chem.* **289**, 12662–12975 (2014).
- Borek D., Kozak M., Pei J. & Jaskolski M. Crystal structure of active site mutant of antileukemic L-asparaginase reveals conserved zinc-binding site. *FEBS J.* **281**, 4097–4111 (2014).
- Cornish-Bowden, A. & Cárdenas, M. L. Specificity of Non-Michaelis–Menten Enzymes: Necessary Information for Analyzing Metabolic Pathway. *J. Phys. Chem. B.* **49**, 16209–16213 (2011).
- Kuo, Y. M., Henry, R. A. & Andrews, A. J. Measuring specificity in multi-substrate/product systems as a tool to investigate selectivity *in vivo*. *Biochim Biophys Acta* **1**, 70–76 (2016).
- Kumar, K., Kaur, J., Walia, S., Pathak, T. & Aggarwal, D. L. l-asparaginase: an effective agent in the treatment of acute lymphoblastic leukemia. *Leukemia and Lymphoma* **55**, 256–262 (2014).
- Warangkar, S. C. & Khobragade, C. N. Purification, characterization, and effect of thiol compounds on activity of the *Erwinia carotovora* L-asparaginase. *Enzyme Res.* 1–10 (2010).
- Kotzia, G. A. & Labrou, N. E. L-Asparaginase from *Erwinia Chrysanthemi* 3937: Cloning, expression and characterization. *Journal of Biotechnology* **127**, 657–669 (2007).
- Gervais, D. & Foote, N. Recombinant deamidated mutants of *Erwinia chrysanthemi* L-asparaginase have similar or increased activity compared to wild-type enzyme. *Mol. Biotechnol.* **56**, 865–877 (2014).
- Broome, J. D. Factors which may influence the effectiveness of L-asparaginase as tumor inhibitors. *British Journal of Cancer* **22**, 595–602 (1968).
- Derst, C., Hensling, J. & Röhm, K. Engineering the substrate specificity of *Escherichia coli* asparaginase II. Selective reduction of glutaminase activity by amino acid replacements at position 248. *Protein Sci.* **9**, 2009–2017 (2000).
- Lopes, A. M. *et al.* Therapeutic l-asparaginase: upstream, downstream and beyond. *Crit Rev Biotechnol.* 1–18 (2015).
- Palm, G. J. *et al.* A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: Crystal structure of a Thr-89-Val mutant. *FEBS Journal* **390**, 211–216 (1996).

40. Aung, H. P., Bocola, M., Schleper, S. & Röhm, K. H. Dynamics of a mobile loop at the active site of *Escherichia coli* asparaginase. *Biochim Biophys Acta*. **1481**, 349–359 (2000).
41. Rifai, K. *et al.* Changes in plasma amino acids during extracorporeal liver support by fractionated plasma separation and adsorption. *Artif. Organs* **34**, 166–170 (2010).
42. Karamitros, C. S., Yashchenok, A. M., Möhwald, H., Skirtach, A. G. & Konrad, M. Preserving catalytic activity and enhancing biochemical stability of the therapeutic enzyme asparaginase by biocompatible multilayered polyelectrolyte microcapsules. *Biomacromolecules* **14**, 4398–4406 (2013).
43. Papageorgiou, A. C., Posypanova, G. A., Andersson, C. S., Sokolov, N. N. & Krasotkina, J. Structural and functional insights into *Erwinia carotovora* L-asparaginase. *FEBS Journal* **275**, 4306–4316 (2008).
44. Cappelletti, D. *et al.* *Helicobacter pylori* L-asparaginase: A promising chemotherapeutic agent. *Biochem. Bioph. Res. Comm.* **377**, 1222–1226 (2008).
45. Orsonneau, J. L. *et al.* Automated kinetic assay of plasmatic L-asparaginase activity undergoing therapy for acute lymphoblastic leukemia. *Annales de Biologie Clinique* **62**, 568–572 (2004).
46. Balcão, V. M., Mateo, C., Fernández-Lafuente, R., Malcará, A. X. & Guisán, J. M. Structural and functional stabilization of L-Asparaginase via multisubunit immobilization onto highly activated supports. *Biotechnol.* **17**, 537–542 (2001).
47. Böhm, G., Murh, R. & Jaenicke, R. Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng.* **5**, 191–195 (1992).

Acknowledgements

This research was supported by grants from State of São Paulo Research Foundation (FAPESP/Brazil), process number 2013/16685-2, 2015/07749-2, 2015/03477-8 and 2013/08617-7. We thank technician Paula Gabriela da Cunha for her assistance with the cytotoxicity assay.

Author Contributions

I.M.C., G.M., M.A.d.O., S.H.P.F. and A.P. designed the experiments and wrote the manuscript; I.M.C., L.S., M.S.M.L. and B.d.A.B.P. performed the experiments and analysed data.

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: Costa, I. M. *et al.* Recombinant L-asparaginase 1 from *Saccharomyces cerevisiae*: an allosteric enzyme with antineoplastic activity. *Sci. Rep.* **6**, 36239; doi: 10.1038/srep36239 (2016).

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