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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Biologia/Genética.

Orientadora: Maria Rita dos Santos e Passos Bueno

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NOTA DA AUTORA

Esta tese de doutorado compreende um trabalho inédito desenvolvido durante os anos de 2016 a 2021 no Laboratório de Genética do Desenvolvimento do Centro de Estudos sobre o Genoma Humano e Células-tronco (CEGH-CEL), Instituto de Biociências, Universidade de São Paulo.

A apresentação do trabalho seguiu o modelo de divisão em capítulo/artigos, modelo este aceito pelos programas de pós-graduação do Instituto de Biociências da USP. O trabalho começa com uma introdução geral (capítulo I), seguido dos dados que resultaram na elaboração de um artigo científico (capítulo 2), e a discussão geral e conclusões (capítulo 3). Com exceção do artigo científico, redigido em inglês para ressubmissão em revista científica, todas as outras seções foram escritas em português, conforme especificado pelas normas da pós-graduação do IB-USP.

Em anexo a este trabalho, encontra-se artigo científico (já aceito para publicação na revista *Diagnostics*), derivado do trabalho desenvolvido em caráter de emergência, como uma forma de colaborar com o controle da transmissão do vírus da COVID-19.

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RESUMO

O transtorno do espectro autista (TEA) constitui um grupo complexo de doenças do neurodesenvolvimento, caracterizado por déficit na capacidade de comunicação e de interação social, assim como por interesses restritos e padrão de comportamento repetitivo e estereotipado. Estudos genômicos em indivíduos com TEA vem contribuindo com grandes avanços na compreensão da sua arquitetura genética. Neste trabalho, nós descrevemos duas irmãs encaminhadas ao CEGH-CEL com diagnóstico inicial de TEA. Análise de exoma levou à identificação de variantes bialélicas de perda de função em *TBCK*, contribuindo para o diagnóstico da recém descrita encefalopatia, Hipotonia infantil com atraso psicomotor e dismorfismos faciais 3 (IHPRF3). Importante destacar que a avaliação clínica das duas irmãs reforça o autismo como uma característica clínica adicional da síndrome. Embora *TBCK* tenha sido implicado na regulação da via de sinalização mTOR e autofagia em células não neurais, é cada vez mais evidente que a menor ativação dessa via, observada anteriormente em fibroblastos e linfoblastos de pacientes com a síndrome, não é suficiente para explicar toda a complexidade clínica da doença. Dessa forma, com o intuito de investigar as funções de *TBCK* em células neurais, assim como para uma melhor compreensão dos mecanismos moleculares associados à síndrome IHPRF3, nós utilizamos como modelo de estudo células neuroprogenitoras (iNPC) derivadas de células-tronco pluripotentes induzidas (iPSC) das duas irmãs afetadas e três controles. Análises de colocalização entre *TBCK* e marcadores de compartimentos da via secretória em iNPC, são consistentes com papel de *TBCK* sobre a via secretória inicial. Nossos dados sugerem que alterações no transporte de vesículas entre o retículo endoplasmático-Golgi devido à falta da proteína *TBCK* funcional representam potencial mecanismo molecular para prejuízos no transporte de proteínas recém-sintetizadas aos seus locais definitivos, assim como para a biogênese de autofagossomos, progressão do ciclo celular e migração, observados nas células das pacientes. Esses processos são essenciais para a neurogênese, migração neuronal e organização da arquitetura celular do cérebro em desenvolvimento. Dessa forma, alterações no transporte de vesículas entre o retículo e Golgi, devido ao mau funcionamento da proteína *TBCK*, podem contribuir para a manifestação tanto de fenótipos neurodesenvolvimentais, como neurodegenerativos observados em pacientes com IHPRF3. Em concordância com papel de *TBCK* na via secretora inicial, nossa avaliação dos resultados da via mTOR levanta a hipótese de que o papel de *TBCK* sobre essa via de sinalização é mediado pela atividade de *TBCK* sobre outro regulador do transporte de vesícula da via secretória inicial, como as proteínas G ARF1 ou RAB1A. Ainda, embora nós não tenhamos

observado nenhuma evidência de alteração ao longo da via endolisossomal em iNPC derivados das pacientes, nossa análise de colocalização sugere que TBCK pode desempenhar algum papel regulatório nos endossomos primários. Assim, novas análises com outros pacientes com a síndrome e em diferentes contextos serão de grande relevância para o melhor entendimento das funções de TBCK.

Palavras-chave: Autismo sintromico, IHPRF3, TBCK, transporte de vesículas, células progenitoras neurais

ABSTRACT

Autism Spectrum Disorder (ASD) is a complex group of neurodevelopmental disabilities, characterized by deficits in communication and social interaction, as well as restricted interests and repetitive pattern of behavior. Genomic high throughput studies have contributed to the identification of a large and growing number of risk genes. Here, we describe two sisters who were ascertained after initial diagnosis of ASD. Identification of biallelic pathogenic variants in *TBCK* through exome sequencing led to the diagnosis of the recently described encephaloneuropathy Infantile hypotonia with psychomotor retardation and characteristic facies 3 (IHPRF3). Importantly, clinical evaluation of the two sibs highlight autism as an additional clinical feature of the syndrome. Although *TBCK* has been implicated in the regulation of mTOR signaling pathway and autophagy in non-neural cells, it is increasingly evident that reduced mTOR signaling previously observed in IHPRF3 patients' fibroblasts and lymphoblasts is not sufficient to account for all the clinical features of the syndrome. In order to gain more insight into *TBCK*'s function in neural cells and for a better understanding of the molecular mechanisms of IHPRF3 syndrome, we have taken advantage of the study of neuroprogenitor cells (iNPC) derived from induced pluripotent stem cells (iPSC) from the two IHPRF3-affected sibs and three controls. Based on a series of colocalization analysis of *TBCK* protein with markers of secretory compartments, we provide evidence that *TBCK* plays an important role in the early secretory pathway in iNPC. Altered ER-to-Golgi vesicle transport due to the lack of functional *TBCK* protein represents a potential mechanism for the impaired transport of newly synthesized proteins to their final destination, as well as for the impaired autophagosome biogenesis, cell cycle progression and migration observed in patients' cells. These processes are crucial for neurogenesis, neuronal migration and cytoarchitecture organization during brain development. Thus, altered ER-to-Golgi vesicle transport due to hypofunction of *TBCK* protein may represent an important causative mechanism of both neurodevelopmental and neurodegenerative phenotypes observed in IHPRF3 patients. In line with a role of *TBCK* in the early secretory pathway, we hypothesize that *TBCK*'s impact over mTOR signaling is mediated by *TBCK*'s activity over other secretory vesicle transport regulator, such as ARF1 or RAB1A small GTPases. Furthermore, although we did not observe evidence of altered vesicle transport in the endolysosomal system in patients-derived iNPC, our colocalization analysis suggest a potential role for *TBCK* in early endosomes. Thus, additional investigation in other IHPRF3 patients and in different contexts are very important to add new insights into *TBCK*'s functions.

Key words: Syndromic autism, IHPRF3, TBCK, vesicle transport, neuroprogenitor cells

CAPÍTULO 1 – INTRODUÇÃO GERAL E OBJETIVOS

1. ASPECTOS CLÍNICOS E EPIDEMIOLÓGICOS DO TRANSTORNO DO ESPECTRO AUTISTA (TEA)

Autismo, ou Transtorno do Espectro Autista (TEA), constitui um grupo complexo e heterogêneo de doenças do neurodesenvolvimento (DND), caracterizado por déficit na capacidade de comunicação e de interação social, assim como por interesses restritos e por padrão de comportamento repetitivo e estereotipado (American Psychiatric Association, 2013).

O diagnóstico de TEA, definido na maior parte das vezes nos primeiros três anos de vida, é essencialmente clínico, e feito conforme os critérios estabelecidos pela 11ª edição da Classificação Internacional de Doenças (CID-11) e pelo Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM) da Associação Americana de Psiquiatria. Com a publicação da 5ª edição do manual, o DSM-V (2013), houve importantes mudanças com relação ao diagnóstico do TEA, de forma que as categorias Transtorno Autístico, Síndrome de Asperger, Transtorno Global do Desenvolvimento sem outra especificação e Transtorno Desintegrativo da Infância deixaram de existir como condições isoladas e passaram a ser agrupadas sob um termo mais amplo, denominado TEA. Por outro lado, a Síndrome de Rett, uma desordem neuropsiquiátrica de herança ligada ao cromossomo X, causada por mutações no gene *MeCP2*, não faz mais parte desse grupo. Além dessas e outras diretrizes estabelecidas no manual, algumas ferramentas foram desenvolvidas para auxiliar no processo diagnóstico, como os questionários de avaliação respondidos pelos cuidadores (CARS) e o protocolo estruturado de observação do paciente (ADOS).

A definição do diagnóstico na maior parte dos casos é dificultada pela coexistência de uma série de outras manifestações clínicas. Alguns exemplos incluem: deficiência intelectual (DI, 31% dos indivíduos com TEA apresentam quociente de inteligência menor que 70), distúrbios específicos de linguagem (cerca de 1/3 dos indivíduos são não-verbais), transtorno do déficit de atenção e hiperatividade (TDAH, presente em 30-61% dos indivíduos), epilepsia (em 1/3 dos indivíduos), distúrbios do sono (presente em mais da metade das crianças), maior susceptibilidade a complicações gastrointestinais, entre outras (*Autism Speaks*,

<https://www.autismspeaks.org/>). Além disso, em casos brandos os sintomas podem ser encobertos por habilidades cognitivas mais desenvolvidas, o que contribui para o subdiagnóstico do TEA.

Estudos epidemiológicos em diferentes populações estimam que o TEA tenha uma prevalência mundial de aproximadamente 1% (Baxter et al., 2015; De la Torre-Ubieta et al., 2016; Lord et al., 2020), com pequena variação a depender do tamanho do estudo e do tipo de abordagem utilizada. No Brasil, a prevalência do TEA foi estimada em 0,27%. O valor abaixo da estimativa mundial foi atribuído principalmente ao pequeno tamanho amostral e à pouca conscientização tanto da população, como de profissionais da saúde (Paula et al., 2011). O TEA é quatro vezes mais frequente em meninos que em meninas (De la Torre-Ubieta et al., 2016; Lord et al., 2020). Uma possível explicação para tal diferença é o que se tem chamado de “teoria do efeito protetor feminino”, segundo a qual indivíduos do sexo feminino teriam maior tolerância aos fatores de risco, os quais seriam suficientes para a manifestação do fenótipo em meninos. Diversas evidências, principalmente envolvendo estudos em famílias com mais de um indivíduo afetado apoiam essa hipótese, como por exemplo a identificação de maior carga de mutações deletérias em indivíduos do sexo feminino (Levy et al., 2011; Jacquemont et al., 2014; Satterstrom et al., 2020) e a maior taxa de recorrência em famílias cujo indivíduo afetado é do sexo feminino (Robinson et al., 2013; Werling & Geschwind, 2015; Palmer et al., 2017). Há também evidências de que a manifestação clínica do TEA no sexo feminino é diferente da do masculino (Kirkovski et al., 2013; Hull et al., 2020). Por exemplo, meninas apresentam maior capacidade de copiar comportamentos de indivíduos neurotípicos, como a manutenção do contato visual, o que contribui para a camuflagem do quadro de TEA nas meninas. Por outro lado, é importante lembrar que as ferramentas utilizadas para a triagem e diagnóstico de TEA são baseados principalmente em dados coletados a partir da observação do comportamento de indivíduos do sexo masculino, o que também pode contribuir para o subdiagnóstico de mulheres afetadas (Milner et al., 2019).

2. ARQUITETURA GENÉTICA DO TEA

Estudos de recorrência familiar e de concordância entre gêmeos monozigóticos e dizigóticos mostram uma forte contribuição de fatores genéticos na etiologia do TEA. Com base nesses estudos as estimativas de herdabilidade tem variado entre 40-90% (Nakanishi et al., 2019; Lord et al., 2020). No entanto, a arquitetura genética do TEA se mostra bastante complexa e heterogênea, com diferentes modelos de herança.

Em cerca de 10-15% dos casos totais o TEA se manifesta como uma característica secundária a uma síndrome, de padrão de herança mendeliano, sendo denominado TEA sindrômico (Rylaarsdam & Guemez-Gamboa, 2019; Ziats et al., 2021). Dentre as síndromes monogênicas com forte associação com o TEA destacam-se a Síndrome do X-frágil (causada por mutações no gene *FMR1*), cuja prevalência de TEA foi estimada em 46% dos meninos e 16% das meninas; e a Esclerose tuberosa (causada por mutações nos genes *TSC1* ou *TSC2*), com prevalência de TEA estimada entre 26-50% (Leclezio & de Vries, 2015; Kaufmann et al., 2017; Guang et al., 2018; Specchio et al., 2020; Ziats et al., 2021).

A maior parte dos casos de TEA está associada aos modelos de herança multifatorial e poligênico (Klei et al., 2012; Gaugler et al., 2014; Weiner et al., 2017; Grove et al., 2019). Nesses casos, a manifestação do transtorno se dá em decorrência do acúmulo de variantes alélicas comuns (aquelas com frequência maior que 1% na população geral), somadas ou não a fatores ambientais (Brainstorm Consortium et al., 2018). Individualmente essas variantes são de baixo risco para o TEA, mas cada uma confere um risco aditivo para a manifestação do fenótipo (Gaugler et al., 2014; De Rubeis & Buxbaum, 2015; De la Torre-Ubieta et al., 2016). O número mínimo de variantes de risco necessárias para a manifestação clínica é desconhecido, mas acredita-se que diferentes combinações e/ou o número dessas variantes estejam correlacionados com a gravidade do TEA, explicando parte da grande variabilidade clínica observada (De la Torre-Ubieta et al., 2016; Jensen & Girirajan, 2017; Grove et al., 2019).

O aprimoramento das análises genômicas (sequenciamentos de nova geração e a análise genômica comparativa por hibridação ou *array CGH*, do inglês *Array Comparative Genomic Hybridization*) revolucionaram a genética do TEA. Estudos genômicos envolvendo casos de TEA não-sindrômico, representados em sua maior parte como casos únicos na família, têm evidenciado a importância de variantes genéticas raras (aquelas com frequência alélica menor

que 1% na população geral), associadas a um padrão de herança oligogênico (De la Torre-Ubieta et al., 2016; Lord et al., 2020). Ou seja, a manifestação do fenótipo nesses casos depende da combinação de duas ou mais variantes patogênicas raras de efeito moderado a alto (De la Torre-Ubieta et al., 2016; Sánchez-Sánchez et al., 2018). Microalterações cromossômicas estruturais, conhecidas como variações de número de cópia ou CNVs (do inglês, *Copy Number Variations*), variantes *indels* (pequenas deleções e inserções) e variações de nucleotídeo único ou SNVs (do inglês, *Single Nucleotide Variants*) envolvendo genes de grande relevância funcional para o fenótipo vem sendo identificadas na população de TEA e compiladas em bancos de dados, como o SFARI (*Simons Foundation Autism Research Initiative*). Em uma de suas mais recentes atualizações (2019), cerca de 363 genes foram classificados como genes de alta confiabilidade ou como fortes candidatos à etiologia do TEA, e pelo menos outros 470 genes têm evidência sugestiva de contribuição para o quadro clínico (SFARI). Ainda, um crescente número de regiões não-codificantes (regiões regulatórias da expressão gênica, como regiões de *splicing*, regiões que geram micro RNAs não codificadores de proteína e regiões que abrigam elementos regulatórios da transcrição), os quais podem modificar o perfil de expressão de genes fundamentais para o neurodesenvolvimento, vem sendo associado à susceptibilidade ao TEA (Duong et al., 2015; Parikshak et al., 2016; An et al., 2018; Williams et al., 2018; He et al., 2019).

Análises genômicas em TEA também têm contribuído para a identificação de variantes raras em novos genes como causas genéticas únicas, permitindo a caracterização de novas formas sindrômicas do transtorno e para um diagnóstico mais preciso. Por exemplo, variantes de perda de função no gene *CHD8*, seguindo padrão de herança autossômico dominante, são recorrentes na população de TEA e levam a distúrbio do neurodesenvolvimento com quadro clínico que se distingue dos casos típicos pela presença de macrocefalia e dismorfismos faciais, além do TEA (Bernier et al., 2014; Barnard et al., 2015; Hoffmann & Spengler, 2021). Variantes patogênicas nos genes *SHANK3* (responsável pela síndrome de herança autossômica dominante, Phelan McDermid) e *DYRK1A*, associados a altas prevalências de TEA (estimadas em 73-85% e 85-90%, respectivamente), são detectadas em cerca de 0,7% e 0,1-0,5% dos casos totais de TEA (Sooraya et al., 2013; Leblond et al., 2014; van Bon et al., 2016; Earl et al., 2017; Samogy-Costa et al., 2019; Ziats et al., 2021). Mais recentemente (2016), foi descrita a síndrome Hipotonia infantil com atraso psicomotor e dismorfismos faciais ou *IHPRF3* (do inglês,

Infantile hypotonia with psychomotor retardation and characteristic facies 3, MIM#616900), uma encefalopatia de herança autossômica recessiva, causada por mutações bialélicas patogênicas no gene *TBCK*. Caracterizada principalmente por deficiência motora e intelectual e uma série de dismorfismos faciais, a IHPRF3 pode estar associada a outras manifestações clínicas, como epilepsia e alterações estruturais do cérebro sugestivas de processo de neurodegeneração progressiva. No entanto, a associação com o TEA, reportado em apenas um indivíduo (de um total de aproximadamente 40 casos) (Bhoj et al., 2016), ainda não está bem estabelecida.

3. TEA E GENES ENVOLVIDOS NA REGULAÇÃO DO TRÁFEGO DE VESÍCULAS

Dada a grande heterogeneidade na arquitetura genética, identificar vias e mecanismos biológicos comuns, tanto em TEA-sindrômico como não-sindrômico, é fundamental para a investigação de melhores estratégias terapêuticas, ainda limitadas ao tratamento de sintomas específicos.

Diversos genes associados ao TEA codificam proteínas diretamente implicadas na regulação do tráfego intracelular de vesículas. Alguns representam reguladores centrais do tráfego, como os membros da família das proteínas Rab GTPases, *RAB2A* e *RAB39B* (Giannandrea et al., 2010; Sanders et al., 2012; Yuen et al., 2015; Takata et al., 2016; Woodbury-Smith et al., 2017), sendo que variantes em hemizigose no gene *RAB39B*, localizado no cromossomo X, estão associadas à deficiência intelectual, acompanhada ou não de comportamento autístico (MIM#300271) (Russo et al., 2000; Giannandrea et al., 2010).

Diversos outros genes associados ao TEA codificam moléculas efetoras das proteínas Rab-GTPases, como *TRAPPC6B*, *RIMS1*, *RAB11FIP5* e *TRAPPC2L* (Matsunami et al., 2014; Bacaj et al., 2015; Marin-Valencia et al., 2018; Milev et al., 2018; Husson et al., 2020). Interessante notar que mutações bialélicas em alguns desses genes estão associadas a outras doenças do neurodesenvolvimento, com alguma sobreposição clínica ao quadro de TEA. Por exemplo, *TRAPPC6B* e *TRAPPC2L* estão associados a DNDs que incluem epilepsia, ausência de fala e microcefalia em seu quadro clínico (MIM#617862 e MIM#618331, respectivamente) (Kim et al., 2016; Zhao et al., 2017; Milev et al., 2018; Marin-Valencia et al., 2018). Outros componentes

do complexo TRAPP, como *TRAPPC4*, *TRAPPC6A*, *TRAPPC9* e *TRAPPC12*, também foram implicados na etiologia de casos de deficiência intelectual (MIM#613192) e encefalopatia com atraso global do desenvolvimento (MIM#618741, MIM#617669), todos consistentes com padrão de herança autossômico recessivo (Mir et al., 2009; Mochida et al., 2009; Milev et al., 2017; Mohamoud et al., 2018; Rasika et al., 2018; Sacher et al., 2019; Van Bergen et al., 2020). Além das proteínas TRAPP, implicadas na regulação do transporte através das vias secretória inicial (retículo endoplasmático → complexo de Golgi) e autofágica, diversos outros componentes da via secretória já foram implicados em outras DNDs, como GM130 (doença neuromuscular com microcefalia e epilepsia) (Shamseldin et al., 2016); IPORIN (MIM#617773, DI associada à epilepsia) (Alwadei et al., 2016), entre outros (Rasika et al., 2018).

Genes fundamentais para a dinâmica do transporte através da via endolisossomal (rota de degradação de biomoléculas) e de reciclagem (dos endossomos de volta à membrana plasmática) também foram associados a DNDs, como *SLC9A6* e *SLC9A9*, implicados tanto em TEA, como DI e TDAH (Garbern et al., 2010; Ouyang et al., 2013; Schuurs-Hoeijmakers et al., 2013; Piton et al., 2013; Kondapalli et al., 2014; Cardon et al., 2016; Zhang-James et al., 2019); *AP2M1*, implicado em casos de DI de herança autossômica dominante (MIM#618587), frequentemente associada à presença de comportamento autístico (Helbig et al., 2019), *AGAP1* (Iossifov et al., 2014; Arnold et al., 2016), entre outros.

4. TRÁFEGO DE VESÍCULAS NO DESENVOLVIMENTO NEURONAL

O sistema de tráfego de vesículas, orquestrado pela família das proteínas Rab-GTPases, transporta proteínas específicas de um compartimento celular a outro através das rotas endossomais, secretória e autofágica para regular múltiplos processos celulares. Pelo menos 60 Rab GTPases foram identificadas em humanos, como um reflexo da ampla complexidade desse sistema, em que cada Rab é recrutada a membranas intracelulares específicas, onde coordena processos como o brotamento e formação das vesículas na membrana doadora, a seleção de cargas, a determinação da rota, assim como o recrutamento de toda a maquinaria necessária para o transporte ao longo do citoesqueleto e o reconhecimento e fusão com a membrana alvo (Pylypenko et al., 2018). Enquanto cada uma dessas etapas é regulada pelo

recrutamento espaço-temporal de proteínas efetoras específicas, a interação com tais moléculas é regulada pelas proteínas Rab GEF (*Guanine nucleotide Exchange Factor*) e Rab GAP (*GTPase Activating Protein*), as quais atuam respectivamente, convertendo as proteínas Rab GTPases à sua forma ativa, ligada a GTP, e à forma inativa, ligada a GDP, a qual se dissocia das moléculas efetoras, levando à interrupção de suas atividades (Ishida et al., 2016).

Estudos têm mostrado um papel crucial do sistema de transporte de vesículas na localização espaço-temporal das moléculas sinalizadoras que regulam a intensidade e duração da atividade das vias de sinalização que coordenam diversos aspectos do desenvolvimento e funcionamento neuronal. Um exemplo bem conhecido ocorre cedo no desenvolvimento cerebral: a divisão assimétrica de células progenitoras neurais durante a neurogênese, regulada pela via de sinalização Notch. Nesse processo, diferentes rotas de tráfego de vesículas através do sistema endossomal (reciclagem ou degradação) determinam o destino dos receptores Notch, e consequentemente a atividade da via de sinalização e o destino da célula-filha. Isto é, a reciclagem dos receptores de volta à membrana plasmática mantém a via de sinalização Notch ativa e, portanto, a identidade de célula proliferativa. Em contrapartida, a degradação dos receptores nos lisossomos suprimem a atividade da via de sinalização Notch, induzindo a diferenciação das células progenitoras neurais em neurônios (Mestres et al., 2017; Shao et al., 2017). Utilizando-se desse tipo de estratégia, ou seja, a dinâmica remoção e (re)inserção ou degradação espaço-temporal de moléculas sinalizadoras em resposta aos estímulos celulares, a maquinaria do transporte de vesículas também coordena, de forma bastante sofisticada, as vias de sinalização que regulam outros processos neurodesenvolvimentais, como a migração neuronal, neuritogênese, e ainda o crescimento e direcionamento axonal (Winkle & Gupton, 2016; Yadaw et al., 2019).

No contexto da especificação tecidual durante o desenvolvimento do córtex cerebral, é cada vez mais evidente que há um enriquecimento na expressão (dependente de *splicing* alternativo) de isoformas específicas de diversos componentes da maquinaria do transporte de vesículas, os quais contribuem para a diferenciação celular e identidade tecidual (Calarco et al., 2009; Voineagu et al., 2011; Irimia et al., 2014; Zhang et al., 2016; Blue et al., 2018; Porter et al., 2018). Em concordância com esses achados, pesquisa em TEA tem mostrado que indivíduos com TEA frequentemente apresentam alterações de regulação de *splicing* e perda de

identidade entre córtex frontal e temporal (Calarco et al., 2009; Voineagu et al., 2011; Quesnel-Vallières et al., 2019).

No aspecto funcional, estudos em camundongos, *Drosophila melanogaster* e *C. elegans* mostraram que o transporte anterógrado (a partir da região somatodendrítica até o sítio pré-sináptico, por meio dos microtúbulos axonais) de vesículas sinápticas e vesículas fundamentais para o estabelecimento do sítio de liberação de neurotransmissores (também conhecido como zona ativa) são essenciais para a biogênese da região pré-sináptica e funcionamento sináptico (Klassen et al., 2010; Maeder et al., 2014; Neupert et al., 2015; Vukoja et al. 2018). Defeitos nesse transporte levam ao acúmulo de moléculas pré-sinápticas na região do corpo celular dos neurônios, contribuindo para grandes prejuízos no funcionamento sináptico. Ainda, a exocitose, assim como a reciclagem e degradação de vesículas sinápticas através do sistema endolisossomal são fundamentais para a formação e manutenção das sinapses (Lou, 2018; Chanaday et al., 2019).

Portanto, o desenvolvimento e funcionamento neuronal são altamente dependentes do tráfego intracelular de vesículas, e sensíveis a alterações na dinâmica desse transporte. Como evidenciado no tópico anterior, mutações que levam a alterações nessa dinâmica estão associadas a diversos distúrbios do neurodesenvolvimento, incluindo o autismo (Quesnel-Vallières et al., 2019; Satterstrom et al., 2020, Griesi-Oliveira et al., 2020). Apesar dos grandes avanços no conhecimento dos mecanismos moleculares e celulares associados ao sistema de tráfego de vesículas que levam às alterações de neurodesenvolvimento e funcionamento sináptico, ainda há muito a ser explorado, principalmente no âmbito da pesquisa em TEA.

5. TBCK

Estudo genômico de casos familiares de TEA realizado pelo nosso grupo levou à identificação de duas irmãs apresentando variantes bialélicas de perda de função no gene *TBCK* (Moreira, 2017), associado à encefalopatia de herança autossômica recessiva IHPRF3, já mencionada e foco deste estudo.

Identificado há menos de 20 anos no projeto *Mammalian Gene Collection Program* (Ota et al., 2004; Gerhard et al., 2004; MGC Project Team et al., 2009), *TBCK* foi associado a um

papel regulatório sobre uma via de sinalização frequentemente alterada em transtornos do neurodesenvolvimento, a via mTOR, em células não-neurais (Liu et al., 2010). Redução na atividade dessa via contribuiria para um prejuízo no fluxo autofágico e no processo de degradação de biomoléculas, culminando em um quadro de neurodegeneração (Bhoj et al., 2016; Moreira et al., 2017; Ortiz-González et al., 2018). A análise preliminar da atividade desta via em células progenitoras neurais (iNPC) e neurônios derivados de células-tronco pluripotentes induzidas de uma das irmãs afetadas, identificadas pelo nosso grupo, mostrou redução da atividade desta via em iNPC, mas não em neurônios (Moreira et al., 2017), sugerindo que alterações nessa via são insuficientes para explicar a complexidade clínica da doença.

Desde sua identificação pouco ainda se conhece sobre os mecanismos pelos quais TBCK atua. Com um domínio TBC (Ter-2/Bub2/Cdc16), altamente conservado, TBCK é previsto como uma proteína com atividade GAP contra proteínas Rab GTPases específicas, ou seja, que catalisa a hidrólise de GTP de proteínas Rab GTPases, contribuindo para a conversão de Rab-GTP à sua forma inativa, ligada a GDP e consequentemente, à interrupção de suas atividades (Liu et al., 2010; Fukuda, 2011). Uma das primeiras evidências que apoiam um papel regulatório de TBCK sobre o tráfego intracelular de vesículas foi descrito por Collinet e colaboradores (2010), que observaram que a depleção de TBCK leva a alterações no processo de endocitose. Mais tarde, estudos de triagem de interação proteína:proteína envolvendo moléculas que compõem a maquinaria do tráfego intracelular de vesículas, tanto em camundongos, como *Drosophila melanogaster* e células humanas sugeriram uma possível interação de TBCK com duas moléculas (Perini, 2012; Havugimana et al., 2012; Gillingham et al., 2014; Gillingham et al., 2019): 1) RAB5 GTPase, um regulador central da endocitose e biogênese dos endossomos; e 2) a molécula adaptadora STAM, a qual apresenta localização tanto nos endossomos primários, como um componente da maquinaria ESCRT (responsável pela seleção de moléculas ubiquitinadas para que sejam degradadas nos lisossomos), como em vesículas COPII que medeiam o transporte de proteínas recém sintetizadas do retículo endoplasmático (RE) ao aparelho de Golgi. No entanto, até o momento estudos funcionais que comprovam tais interações ainda não foram publicados, tampouco estudos que comprovem a atividade GAP de TBCK.

6. MODELAGEM *IN VITRO* DE CÉLULAS PROGENITORAS NEURAIS DERIVADAS DE CÉLULAS-TRONCO PLURIPOTENTES INDUZIDAS PARA O ESTUDOS DE DNDs

Modelos animais, assim como tecidos cerebrais *post mortem* humanos têm contribuído fortemente na caracterização das alterações neuronais associadas às diferentes desordens neuropsiquiátricas. No entanto, a menor complexidade neural dos modelos *in vivo* em relação aos humanos, assim como o difícil acesso e a análise limitada a um estágio avançado da doença quando utilizando tecidos cerebrais *post mortem*, representam fatores limitantes importantes para o estudo dos mecanismos patofisiológicos associados às DNDs.

A técnica de reprogramação de células somáticas adultas para um estado pluripotente, por meio da superexpressão de genes específicos (Takahashi et al., 2007; Yu et al., 2007), surgiu como uma alternativa interessante para a modelagem *in vitro* de doenças neuropsiquiátricas. Além de serem relativamente simples de serem obtidas, as células-tronco pluripotentes induzidas ou iPSC (do inglês, *Induced Pluripotent Stem Cells*), como são conhecidas, são isogênicas à célula somática doadora, e portanto, apresentam o componente genético que confere a predisposição ao desenvolvimento da doença de interesse. Ainda, as iPSC tem o potencial de se diferenciar em células progenitoras neurais e neurônios específicos, possibilitando a recapitulação do desenvolvimento neuronal *in vitro* e o melhor entendimento dos mecanismos moleculares e celulares que levam às alterações de trajetória no desenvolvimento neural específicas de cada condição (Griesi-Oliveira et al., 2021).

Uma das primeiras contribuições da tecnologia de iPSC no estudo de DNDs foi descrita por Marchetto e colaboradores (2010), que investigaram as implicações de mutações patogênicas no gene *MeCP2* para o fenótipo neuronal dos indivíduos com a Síndrome de Rett. Em concordância com os achados em modelos animais da síndrome (Chao et al., 2007; Wang et al., 2013; Nerli et al., 2020), os neurônios diferenciados a partir de iPSC de pacientes apresentaram alterações morfológicas importantes, como diminuição do soma neuronal e alteração na densidade dos espinhos dendríticos, quando comparados aos controles, características que indicam menor maturidade e complexidade que neurônios saudáveis (Ardhanareeswaran et al., 2017; Nerli et al., 2020). Funcionalmente, esses neurônios derivados de iPSC apresentaram redução no número de sinapses excitatórias e defeitos na sinalização por cálcio, contribuindo para alterações de amplitude e frequência do potencial de ação. Estudos

subsequentes em neurônios e astrócitos derivados de iPSC de outros pacientes com a síndrome corroboram essas observações, e contribuíram com novos achados, como alteração de sinalização GABA (inibitório) e alteração de transporte de vesículas dependentes de microtúbulos (Williams et al., 2014; Delépine et al., 2016). O tratamento dos neurônios em cultura com IGF-1 (fator de crescimento que modula maturação e função sináptica) ou epotilona D (para estabilização de microtúbulos) contribuíram para o resgate parcial dos fenótipos alterados (Marchetto et al., 2010; Williams et al., 2014; Delépine et al., 2016).

Importantes achados também têm sido obtidos em neurônios derivados de iPSC de casos síndrômicos de TEA, como a Síndrome do X-frágil, Síndrome de Timothy e Síndrome de Phelan-McDermid, causadas por mutações patogênicas nos genes *FMR1*, *CACNA1C* e *SHANK3*, respectivamente (Ardhanareeswaran et al., 2017; Vitrac & Cloëz-Tayarani, 2018). A modelagem da Síndrome do X-frágil utilizando a tecnologia de iPSC contribuiu para a observação de importantes alterações de diferenciação neuronal, como menor crescimento e maturação de neuritos em neurônios derivados de iPSC de pacientes (Sheridan et al., 2011; Doers et al., 2014). Tais características foram atribuídas a alterações em etapas do neurodesenvolvimento que precedem a sinaptogênese, no entanto, mais estudos são necessários para a confirmação de tal hipótese. Diferente abordagem foi utilizada para o estudo da Síndrome de Timothy. Análises transcriptômicas de células progenitoras neurais e neurônios derivados de iPSC de pacientes com mutação no gene *CACNA1C* (codifica subunidade alfa-1 de canais de cálcio dependentes de voltagem) levaram a identificação de alterações em módulos de expressão associados à diferenciação neuronal e despolarização neuronal, os quais seriam co-regulados por uma série de fatores de transcrição dependentes da sinalização por cálcio (Paşca et al., 2011; Tian et al., 2014). Os pesquisadores também observaram que genes diferencialmente expressos em pacientes estão enriquecidos com genes associados ao TEA e DI, evidenciando a relevância de estudos funcionais em casos síndrômicos, com causas genéticas únicas, para o melhor entendimento dos mecanismos moleculares e celulares que levam às alterações de funcionamento neuronal em casos de TEA não-sindrômico.

A tecnologia de iPSC também tem sido fundamental para o estudo de casos não-sindrômicos de TEA, como aqueles associados ao gene *TRPC6*, no qual alterações morfológicas (menor arborização dos neuritos e menor densidade de espinhos dendríticos) e funcionais (alterações de influxo de cálcio) foram observadas em neurônios diferenciados a partir de iPSC

do paciente (Griesi-Oliveira et al., 2015). Estudos transcriptômicos em células neurais derivadas de iPSC de casos não-sindrômicos também têm mostrado que apesar da grande heterogeneidade na arquitetura genética do TEA, alterações em módulos de co-expressão envolvendo genes sinápticos representam características patofisiológicas comuns dentre os indivíduos afetados (Mariani et al., 2015; DeRosa et al., 2018; Schafer et al., 2019; Griesi-Oliveira et al., 2021).

Ainda, na última década, grande esforço tem sido feito para o aprimoramento dos protocolos de formação de organoides cerebrais (cultura de células neurais em 3D) a partir de iPSC para a modelagem de DNDs. Um exemplo importante foi descrito por Mariani e colaboradores (2015), que desenvolveram organoides telencefálicos (o telencéfalo dá origem ao córtex cerebral, hipocampo e bulbo olfatório, e contém tanto neurônios corticais glutamatérgicos, como GABAérgicos) de casos idiopáticos de TEA com macrocefalia. Os organoides derivados desses pacientes apresentaram ciclo celular mais rápido e aumento na produção de neurônios inibitórios GABAérgicos. Em concordância com esses achados, a análise transcriptômica desses organoides revelou um aumento na expressão de genes envolvidos em processos como a proliferação e diferenciação neuronal, o que foi atribuído ao aumento na expressão do fator de transcrição FOXG1.

Esses exemplos mostram a relevância das células neurais derivadas de iPSC como modelo *in vitro* tanto para a recapitulação de importantes aspectos do desenvolvimento da doença, complementando os achados em modelos *in vivo* e tecidos cerebrais *post mortem*, como para os testes de potenciais estratégias terapêuticas.

7. OBJETIVOS

Desde sua identificação pouco ainda se conhece sobre as funções de TBCK, principalmente no âmbito do tráfego de vesículas, e tampouco sobre os mecanismos patofisiológicos pelos quais mutações de perda de função nesse gene levam a alterações de fenótipo neuronal. Além disso, relatos da literatura, principalmente associados ao câncer, apontam para diferenças funcionais de TBCK em diferentes tipos celulares (Wu et al. 2021), sugerindo a importância de células neurais para o estudo da patofisiologia da IHPRF3. Dessa

forma, o objetivo principal deste trabalho foi investigar os mecanismos patofisiológicos que contribuem para alterações de fenótipo neural na IHPRF3, com foco no papel de TBCK sobre o transporte de vesículas, utilizando como modelo de estudo culturas de células progenitoras neurais (iNPC) derivadas de células-tronco pluripotentes induzidas (iPSC) de pacientes com a síndrome de IHPRF3 (com deficiência de TBCK) e respectivos controles. Para atingir esta meta, nossas propostas foram:

- a.** Analisar o perfil de ativação da via de sinalização mTOR em iNPC com deficiência de TBCK;
- b.** Caracterizar a localização intracelular de TBCK em iNPC, por meio da colocalização com marcadores específicos de compartimentos das rotas endolisossomal (vesículas endocíticas e endossomos primários) e secretória (vesículas COPII e aparelho de Golgi);
- c.** Verificar se iNPC com deficiência de TBCK apresentam comprometimento das rotas endolisossomal e secretória;
- d.** Para avaliar as implicações funcionais das variantes de perda de função no gene *TBCK* no contexto do desenvolvimento neural, nós também propomos investigar se iNPC das pacientes apresentam alterações de proliferação e migração celular, dois processos altamente dependentes do tráfego intracelular de vesículas.

CAPÍTULO 2

Neuroprogenitor cells derived from patients with biallelic variants in *TBCK* show altered intracellular vesicle transport

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ABSTRACT

Biallelic pathogenic variants in *TBCK* cause the recently described encephaloneuropathy, Infantile hypotonia with psychomotor retardation and characteristic facies 3 (IHPRF3). The molecular mechanisms underlying IHPRF3 neuronal phenotype are largely unexplored. Here, we report two sisters, initially diagnosed with syndromic autism, who harbor biallelic pathogenic variants in *TBCK*, and met diagnostic criteria for IHPRF3. Our data suggest that reduced *mTOR* signaling is insufficient to account for all the clinical features of the syndrome. Alternatively, we provide evidence that TBCK plays an important role in the early secretory pathway in neuroprogenitor cells (iNPC) differentiated from induced pluripotent stem cells (iPSC) of IHPRF3 patients and controls. Lack of functional TBCK protein is associated with impaired endoplasmic reticulum (ER)-to-Golgi transport and autophagosome biogenesis in IHPRF3 patients. Patients-derived iNPC also presented with altered cell cycle progression and severe impairment in the capacity of migration, which are crucial for neurogenesis, neuronal migration and cytoarchitecture organization during brain development. Whether reduced mTOR signaling is secondary to impaired TBCK function over other secretory vesicle transport regulators, such as Arf1 or Rab1A small GTPases, still needs further investigation. Altogether, we propose that altered ER-to-Golgi vesicle transport due to hypofunction of TBCK protein is an important causative mechanism of both neurodevelopmental and neurodegenerative phenotypes observed in IHPRF3.

Key words: STAM, early secretory pathway, vesicle trafficking, autophagy, GM130, Clathrin, mTOR, iPSC-neurodevelopmental disease modeling

INTRODUCTION

Genomic high throughput studies in cohorts of individuals with neurodevelopmental disorders, involving autism spectrum disorder (ASD), epilepsy and intellectual disability (ID), have recognized several novel rare genetic syndromes. Among them, biallelic pathogenic variants in *TBCK* (TBC1 Domain containing kinase) have been shown to cause Infantile hypotonia with psychomotor retardation and characteristic-facies 3 (IHRPF3, OMIM#616900), a severe early-onset encephaloneuropathy mainly characterized by developmental delay, hypotonia and facial dysmorphisms (Bhoj et al., 2016; Chong et al., 2016; Sumathipala et al., 2019). The number of reported cases are still limited to about 40 cases, and the description of novel cases can contribute to a better characterization of the spectrum of clinical variability of this syndrome.

TBCK, a member of the TBC family of proteins, contains a predicted active TBC Rab-GAP domain, which usually functions as GAP for members of the Rab family of small GTPases (also known as small G-proteins), flanked by an N-terminal catalytically inactive kinase domain and a C-terminal rhodanese homology domain (RHOD) (Komurov et al., 2010; Gabernet-Castello et al., 2013). *TBCK* has been proposed to act on cell proliferation and autophagy through *mTOR* signaling pathway in non-neural studies (Liu et al., 2013; Ortiz-González et al., 2018). However, to date little is known about *TBCK*'s mechanism of action, being unknown whether disruption of *mTOR* signaling is a primary or secondary effect of loss of *TBCK*. In addition, depending on cell type, *TBCK* has been associated with both tumor-promoting and tumor suppressive function (Liu et al., 2013; Wu et al., 2014; Wu & Lu, 2021), suggesting variable regulation according to cell type. Furthermore, although biallelic loss-of-function variants in *TBCK* are associated with several neural clinical phenotype, functional studies of *TBCK* in neural cells are still lacking.

In this work, we describe two new IHRPF3-affected sibs that present autism as an additional clinical feature of the syndrome and are compound heterozygotes for loss-of-function variants in *TBCK*. In order to gain more insights into *TBCK*'s mechanisms of action in neural cells and to better understand the pathophysiology of IHRPF3 syndrome, we have taken advantage of the study of neuroprogenitor cells (iNPC) derived from induced pluripotent stem cells (iPSC) from the two IHRPF3-affected sibs. Based on a series of colocalization analysis of *TBCK* protein

with early secretory pathway markers, our results suggest altered ER-to-Golgi vesicle transport as a possible mechanism for the impaired autophagosome biogenesis, cell cycle progression and migration observed in patients' cells. Our studies also showed that patients' iNPC present decreased *mTOR* signaling, which may be secondary to impaired regulation of TBCK-mutated protein over other early secretory vesicle transport regulators.

METHODS

Subjects

The two affected sisters (F6331-1 and F6331-4) were ascertained after initial diagnosis of Autism Spectrum Disorder (ASD) and following referral to the genetic counseling service at Centro de Estudos do Genoma Humano e Células-Tronco (CEGH-CEL), Universidade of São Paulo (USP). Patients were routinely diagnosed based on DSM-5 (American Psychiatric Association, 2013) and CARS evaluation.

Control individuals (F7007-1, F8799-1 and F10006-1; one male and two females, respectively aged 31, 29, 33) are healthy and unrelated to the affected individuals.

The study was approved by the National Ethics Committee (Comissão Nacional de Ética em Pesquisa no Brasil, Process no. CAAE43559314.0.0000.5464). Blood from all the individuals were collected after a signed written informed consent by the participants of the study or their legal representatives.

Pathogenic variants investigation

DNA extraction

Genomic DNA extraction from whole blood was performed using the QIAasympphony automated workstation, following manufacturer's instructions (Qiagen, US).

Whole exome sequencing and variants selection

Whole-exome sequencing (WES) and bioinformatics analysis were performed at the CEGH-CEL sequencing facility. Exome capture was carried out with the TruSeq Exome Library Prep Kit (Illumina, Inc., USA) following manufacturer's recommendations. HiSeq 2500 sequencer (Illumina) was used for sequencing paired-end reads of approximately 100x100 bp. Reads were aligned with BWA (Burrows-Wheeler Aligner), against the hg19 reference genome (Li & Durbin, 2009). Data processing and variant calling were carried out on Picard and GATK (Genome Analysis Tool Kit) (McKenna et al., 2010). ANNOVAR (Wang et al., 2010) was used to annotate variants. Candidate pathogenic variants were filtered according to the following criteria: a) exclusion of low quality variants; b) inclusion of rare variants with minor allele frequency (MAF) < 0.01 in reference databases (i.e. 1000 Genomes Project (1000G), National Institutes of Health; 6500 Exome Sequencing Project (6500ESP), Washington University; and ABraOM, University of São Paulo (Naslavsky et al., 2017); c) inclusion of variants with frequency < 0.05 in internal control samples (i.e. DNA samples that were sequenced and processed in the same batch); d) exclusion of polymorphic genes (Fajardo et al., 2012); and e) exclusion of variants located within the last three amino acids of a protein. We prioritized homozygous or compound-heterozygous loss-of-function (LoF) variants shared between the two affected siblings. The position of the candidate LoF variant identified was manually converted to GRCh38/hg38, using the UCSC website (<https://genome.ucsc.edu/>).

Sanger sequencing

Sanger sequencing of genomic DNA and cDNA were performed for the validation of the candidate variant and segregation analysis. Primers for PCR amplification and sequencing were designed on Primer-BLAST (NCBI; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Supplementary Material Table 1).

aCGH and real-time quantitative PCR (RT-qPCR) for copy number analysis

Array comparative genomic hybridization (aCGH, 180K, Agilent, USA) was performed in both patients according to the manufacturer's recommendations. The analysis was carried out using Agilent GenomicWorkbench 7.0 (Agilent Technologies, Santa Clara, CA, USA).

RT-qPCR of *TBCK* was performed to detect the predicted small deletion in the region chr4:107,071,580-107,113,380. Six primer pairs were designed on Primer-BLAST

(Supplementary Material Table 2). Relative quantification was carried out by normalization to *GAPDH*, and quantification data were calibrated relative to a control without any known CNV in *TBCK* (D'haene et al., 2010).

iPSC reprogramming

Induced pluripotent stem cells (iPSC) were reprogrammed from peripheral blood mononuclear cells (PBMC), using a non-viral method with non-integrating plasmids (Okita et al., 2013), which was established at HUG-CELL with minor modifications (Griesi-Oliveira et al., 2015; Miller et al., 2017). AMAXA nucleoporator (Lonza, Basel, Switzerland) was used for the transfection of plasmids containing the transcription factors *OCT-4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28*. Each reprogrammed cell line was co-cultivated with irradiated murine embryonic fibroblasts (MEF, Millipore) in DMEM/F12 medium supplemented with 2mM GlutaMAX-I, 0.1 mM nonessential amino acids, 55 μ M 2-mercaptoethanol, 30 ng/mL FGF-2, and 20% of knockout serum replacement (KSR, Life Technologies). iPSC colonies with typical morphology were then transferred to Matrigel (BD-Bioscience) coated plates and fed with E8 medium (Life Technologies). All iPSC colonies were tested for plasmid integration into the host genome and excluded for the presence of aneuploidies with SALSA MLPA P070 Human Telomere-5 probe mix (MRC-Holland). Cell pluripotency was evaluated through stem cell markers prior to differentiation to iNPC.

iNPC differentiation

Differentiation of iPSC into iNPC was carried out using a protocol previously established at HUG-CELL (Griesi-Oliveira et al., 2015). Briefly, iPSC were cultivated in 0.5X NB medium ($\frac{1}{2}$ DMEM/F12 : $\frac{1}{2}$ Neurobasal media with 0.5X N-2 supplement (100X) (Thermo Fisher Scientific) and 0.5X B27 minus vitamin A supplement (50X) (Thermo Fisher Scientific) supplemented with Dorsomorphin, 1 μ M (Tocris) for 2 days. Then, iPSC were harvested with accutase treatment and cell clumps were manually transferred into low-attachment plates (Corning) on 0.5X NB medium supplemented with 1 μ M Dorsomorphin. The next day, the medium was replaced with 0.5X NB medium supplemented with 20 ng/ml FGF-2 and 20 ng/ml EGF (Thermo Fisher Scientific) and allowed to grow in suspension for 7 days. The resulting embryoid bodies were then lightly dissociated with accutase and plated on matrigel-coated plates, from which

rosettes start to form within 4-7 days. The selected rosettes were manually collected and plated in poly-L-ornithine (10 µg/mL; Sigma) and natural mouse laminin (5 µg/mL; Invitrogen) coated plates for iNPC expansion. All iNPC were characterized through neural progenitor markers.

Except in cell cycle and BrdU incorporation assays, for which we used iNPC only from patient F6331-1, in each experiment performed in this work we used iNPC from all individuals (patients: F6331-1 and F6331-4; and controls F7007-1, F8799-1 and F10006-1).

Neurosphere formation (3D model)

25 µl drops of cell suspension containing a total of 4×10^4 iNPC (N = 5, in passage number ranging between 6 to 8) in 0.5X NB medium containing 20 ng/ml FGF-2 and 20 ng/ml EGF were placed onto the lid of a 10cm dish and set to rest for 48 hours at 37°C and 5% CO₂. After this period the cells aggregated into homogenous neurosphere size were placed into 6 wells low attachment plates in 0.5X NB medium supplemented with 20 ng/ml FGF-2 and 20 ng/ml EGF.

Cell culture conditions

mTOR signaling activity and autophagy in iNPC were evaluated in normal culture condition and in three stress-inducing conditions: i) absence of EGF and FGF-2 for 24 hours; ii) absence of EGF and FGF-2 and concomitant incubation with *mTOR* inhibitor, rapamycin (100 nM) for 24 hours; and iii) absence of EGF and FGF-2 for 24 hours and subsequent addition of brefeldin A (BFA, 4 µM), which induce Golgi complex fragmentation, for 3 hours.

RNA extraction and RT-qPCR

Total RNA from all iPSC and iNPC cultures were isolated using NucleoSpin RNA II kit (Macherey-Nagel), and cDNA was synthesized by reverse transcription using SuperScript IV (Thermo Fisher Scientific).

Primer pairs for *TBCK* (Supplementary Material Table 3) were designed at Primer-BLAST, while pluripotency markers' (*OCT3/4* and *NANOG*), neural markers' (*PAX6* and *SOX1*) and housekeeping genes' (*GAPDH* and *TBP*) primers were adopted from the literature (Miller et al., 2017; Ishiy et al., 2015). Each sample was analyzed in triplicate with the use of Fast SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's recommendations. The reactions were run in a QuantStudio® 5 Real-Time PCR system (Applied Biosystems). The

expression of each gene was normalized to *GAPDH* or *TBP* housekeeping gene, and the results are shown as the mean fold change of the normalized gene expression relative to a calibrator sample. For pluripotency or neural markers, another cell type with no expression of the gene tested was used as negative control.

Cell Lysis and Western blot (WB)

Cells were homogenized in RIPA buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitor cocktails (Sigma). Lysates were incubated on ice for 10 minutes and then centrifuged at $8,000 \times g$ for 15 minutes at 4°C , and the supernatants of total cell lysates were collected. Protein concentrations were determined with a BCA microprotein assay kit (BioAgency). 20 μg of protein from each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked with 5% bovine serum albumin (BSA) for 1 hour and then incubated with primary antibodies overnight at 4°C . Detection was performed using horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies (1:2000, Cell Signaling Technology), ECL substrate (GE Healthcare), and ImageQuant LAS-4000 (GE Healthcare). The intensity of the bands was determined by densitometry using NIH ImageJ software (<http://rsbweb.nih.gov/ij/>). The following primary antibodies were used: anti-TBCK^{ab1} (1:50, #PA5-34789, Thermo Fisher Scientific); anti-TBCK^{ab2} (1:50, #sc-81865, Santa Cruz Biotechnology), anti-pRPS6^{240/244} (1:5000, #5364), anti-LC3A/B (1:1000, #12741), anti-BECN1 (1:1000, #3738), anti-p62/SQSTM1 (1:1000, #8025) from Cell Signaling Technology; anti-Cathepsin D (1:2000, #ab75852), anti-Vinculin (1:5000, #ab18058) from Abcam; PP1gamma (1:10000, #MA-17250, Invitrogen) and anti- β -actin (1:15000, A2228 Sigma) antibodies. All target protein levels were quantified and normalized to the corresponding β -actin or Vinculin levels.

***AKT/mTOR* multiplex assay**

mTOR signaling activity from iNPC cultured in the absence of EGF and FGF-2 for 24 hours were also assayed using the MILLIPLEX[®] MAP *Akt/mTOR* Phosphoprotein Magnetic Bead 11-Plex panel (#48-611MAG) and the MILLIPLEX MAP *AKT/mTOR* 11-plex panel (#48-612MAG) (Millipore) with 10 μg of total protein extracts of each sample, according to the manufacturer's instructions.

Immunofluorescence

iPSC and iNPC grown on coverslips were fixed in 4% PFA, permeabilized in 0.2% Triton X-100 in PBS for 30 minutes at 4°C, and blocked for one hour in PBS containing 5% BSA, and then incubated overnight at 4°C with primary antibodies diluted in the same blocking buffer. Primary antibodies used: anti-SOX1 (1:200, #4194S), anti-RAB7 (1:200, #9367), anti-LC3 (1:200, #3868) from Cell Signaling Technology; anti-SOX2 (1:100, #ab171380), anti-GM130 (1:50, #ab52649) from Abcam; anti-TBCK (1:50, #PA5-34789), anti-COPII (1:200, #PA1-069A) from Thermo Fisher Scientific; anti-TBCK (1:50, #sc-81865), anti-RAB5A (1:100, #sc-309); anti-clathrin (1:200, #sc-6579), anti-caveolin (1:200, #sc-894) from Santa Cruz Biotechnology; anti-STAM (1:200, #12434-1-AP, Proteintech). Cells for anti-LC3 labeling were fixed with methanol and proceeded with the same protocol described. Cells were then washed 3 times with PBS and incubated with secondary antibodies conjugated with Alexa fluor 488 or Alexa fluor 680 for one hour at room temperature. After another washing step, the cells were mounted in a Vectashield mounting medium with DAPI (1 µg/mL, Vector Labs). Samples were analyzed by a confocal laser scanning microscope, LSM800 (Zeiss), using immersion lens (63X/1.40 Oil). Each channel was imaged separately. The acquired fluorescent images were converted to tiff format and further processed using Fiji software. Background from all the images was subtracted using Fiji background subtraction algorithm (rolling ball radius = 50 pixels). To quantify TBCK co-localization with Caveolin, Clathrin, RAB5A, STAM, GM130/GOLGA2 and COPII, Manders method of correlation (MCC) was performed using ImageJ/Fiji-Coloc2 plugin. For the analysis of LC3 puncta the binary images generated after background subtraction were filtered for particles with Feret's diameter larger than 100 nm. The number of puncta was calculated by dividing the total number per number of cell nuclei labeled in a given image. We then calculated the mean and standard deviation for puncta number and puncta size (Kjos et al., 2017) of each sample.

Cell cycle and cell proliferation analysis

A total of 5×10^5 iNPC from each sample (in similar passage number, ranging between 5 and 6) were seeded in duplicate into 6-well plates (Corning) in 0.5X NB medium supplemented with 20 ng/ml EGF and 20 ng/ml FGF-2. The day after seeding, EGF and FGF-2 were removed from 0.5X NB medium during 24 hours for cell cycle synchronization at G0/G1 (T0)

(Mazemondet et al., 2011). After this period, the medium was replaced with 0.5X NB medium supplemented with EGF and FGF-2 to induce cell cycle progression and cell proliferation. Cells were collected at T0 (right after 24 hours of EGF and FGF-2 deprivation), 24 and 40 hours after growth factors supplementation (T24 and T40, respectively).

Three hours before collection of the cells, in each time point, 20 μ M BrdU was added (Sigma-Aldrich). Then, iNPC were rinsed twice with PBS, harvested with trypsin incubation and fixed in 70% EtOH overnight at -20°C. After complete fixation, cells were double-stained with propidium iodide (PI) and anti-BrdU (BrdU Monoclonal Antibody, FITC, Invitrogen), in order to ascertain the cell distribution through cell cycle stages (G0/G1, S, G2/M) and cell proliferation, respectively. Appropriate assay controls were used in the assay (unstained sample; PI-stained- and anti-BrdU-stained-only samples), and at least 5000 events were acquired. Data were analyzed with Guava Express PRO software (Millipore) and gated to remove debris and cell clumps.

Apoptosis analysis

A total of 2×10^5 iNPC were seeded into a 12-well plate (Corning), in duplicate. In the following day, cells were incubated with 20 μ l of CellEvent® Caspase-3/7 Green ReadyProbes® Reagent (Thermo Fisher Scientific) for 1 hour, at 37°C and 5% CO₂. Then, iNPC were rinsed with PBS, fixed with 4% PFA and the nuclei stained with Vectashield mounting medium with DAPI. Images were captured on a Nikon fluorescence microscope (Nikon Eclipse Ti-E, Nikon). The quantification of caspase marked cells was performed on ImageJ software from three different fields from each duplicate. We determined the total number of cells by DAPI nuclear staining.

Cell migration

For radial migration assay, 25 μ l drops of cell suspension containing a total of 5×10^4 iNPC (in passage number ranging between 5 to 6) in 0.5X NB medium containing 20 ng/ml FGF-2 and 20 ng/ml EGF were placed onto the lid of a 10cm dish and set to rest for 48 hours at 37°C and 5% CO₂. After this period the cells aggregate into homogenous neurosphere size. A total of 4 neurospheres per sample were then transferred to poly-L-ornithine (10 μ g/mL; Sigma) and natural mouse laminin (5 μ g/mL; Invitrogen) coated plates in 0.5X NB medium containing 20 ng/ml FGF-2 and 20 ng/ml EGF. Images were captured at 4, 8 and 24 hours after adhesion to

the coated plates. The pictures were acquired using EVOS Cell Imaging System (Thermo Fisher Scientific). Cell migration was analyzed using ImageJ software. For each time point we measured the neurosphere outer edges. Cell migration was estimated by the outer diameter of neurosphere migration normalized to inner neurosphere diameter (μm) (Ge et al., 2016).

Co-immunoprecipitation (Co-IP) and mass spectrometry (MS)

Co-immunoprecipitation assays were performed using a Pierce Co-immunoprecipitation kit (Thermo Fisher Scientific) with 500 μg of total protein extract from iNPC cultured under normal growth conditions (presence of EGF and FGF-2), and 8 μg of TBCK antibody ab1 (#PA5-34789) was used, as previously described (Smith et al., 2017). The protein of interest and the proteins bound to it were eluted from the resin and ran through an *Acquity M-Class* (Waters, Co) ultra performance liquid chromatography (UPLC) system, coupled online to a Synapt G2-Si Mass Spectrometer (Waters, Co). MS/MS data were collected and subsequently analyzed with Progenesis QI software for Proteomics (version 4.0.6403). Individual samples' liquid chromatography profiles were automatically aligned by the software to create an *in silico* aggregate map containing every peptide in the complete sample set, allowing consistent detection and improved comparative quantitation of features across the samples. Potential TBCK interactors were identified according to the following parameters: maximum ion charge of +8, maximum protein mass of 600 kDa, up to 1 missed tryptic cleavage allowed, fixed modifications by carbamidomethyl of cysteine (C) and variable oxidation of methionine (M), a minimum of 1 fragment per peptide, 3 fragments per protein, and 1 peptide per protein. A false discovery rate (FDR) value of 4% was used, calculated by a reverse databank created 'on-the-fly' by Progenesis. The Uniprot *Homo sapiens* revised database (obtained in April, 2019) was used. Relative quantitation of each identified protein was performed by the HiN method (based on the 3 most abundant peptides), incorporated in Progenesis QIP. Data of protein/peptide/ion identifications with a mass error of > 20 ppm were excluded. The resultant list of peptides was converted into a list of Uniprot accession numbers of the respective proteins. Protein:protein interaction (PPI) network of the selected proteins was generated at STRING V11.0 (<https://string-db.org/>) and functional enrichment analysis was performed with Gene Ontology (GO) database. An FDR of 5% was used (default).

Co-immunoprecipitation assay and mass spectrometry were performed in collaboration with Dr. Daniel Martins de Souza's research group and guidance of MSc. Bradley Joseph Smith (Institute of Biology, Unicamp).

Statistical analysis

Data were analyzed by the non-parametric unpaired two-tailed Wilcoxon-Mann-Whitney-test or one-way analysis of variance (ANOVA) followed by the recommended correction test, using Graphpad 7.0 (Graphpad Software Inc., La Jolla, CA). A regression via decision tree was used to analyze cell-cycle progression, using the R package (R Core Team 2019). For functional enrichment analysis of proteomic data Fisher's exact test was applied. *P*-values < 0.05 were considered statistically significant. In the figures, statistical significances are indicated as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Data are expressed as mean ± SD.

RESULTS

Patients' clinical presentation

The two affected sisters here included were born from a non-consanguineous healthy couple (Figure 1A). The proband (Patient F6331-1), first evaluated at the age of 6 years, was born by vaginal delivery at week 38 of pregnancy with normal anthropometric measurements (Table 1). At the age of 3 months, the parents noticed that she had low muscle tone. She was able to sit at about 12 months, to walk unassisted at about 32 months, and she spoke the first words at 24 months. When she was 3 years old, she was diagnosed with autism and intellectual disability. At the age of 7 years, she had a severe psychotic episode. Clinical evaluation showed several dysmorphic features (Table 1). Prominent digit pads were also observed. She also developed dyslipidemia and hypothyroidism. Brain magnetic resonance image (MRI) showed white matter volumetric reduction and T2 hyperintensity (Supplementary Figure 1A). Electrocardiogram and EEG were normal. Currently at 14 years old, she studies in a special school and only says a few words and short sentences and presents inattention and lack of motivation to engage in educational activities.

The affected sister of the proband (Patient F6331-4), first evaluated at 3 years of age, was born at 38 weeks of gestation with normal anthropometric measurements. She sat unaided at about 6 months and could walk unassisted at about 14 months. At around 12 months, she was diagnosed with autism and global developmental delay. She had the first seizure episode at the age of 4 years and was diagnosed with focal and generalized epilepsy at about 6 years of age. Clinical evaluation at the age of 7 years showed similar dysmorphic features to her sister F6331-1 (Table 1). Other clinical features included prominent digit pads, brachydactyly of the fourth and fifth toes and hypoplastic nails. The patient also developed dyslipidemia and hypothyroidism. She is currently 10 years old, and presents progressive weakness in her lower limbs, first noticed approximately at the age of 8 years. Brain MRI showed volumetric reduction of the white matter (Supplementary Figure 1B). EEG demonstrated diffuse bilateral slow waves with multifocal epileptiform discharges. **Electrocardiogram was normal.**

Table 1: IHPRF3 patients clinical features.		
F6331-1 14, Female	F6331-4 10, Female	Clinical data Age (yr), Sex
3990g (p90)	3850g (p50)	Birth evaluation Birth Weight (centile)
51cm (p75)	48cm (p50)	Birth Length (centile)
-	-	Head circumference (centile)
10/ 10	8/ 9	Apgar Scores (1st/5th min)
53.5kg (p90)	33.5kg (p75)	Last evaluation Weight (centile)
164cm (p>97)	133cm (p50)	Height (centile)
55cm (p96)	53.5cm (p<96)	Head circumference (centile)
-	+	Development Regression
-	+	Seizures
Few words	Non-verbal	Speech
Severe delay	Severe delay	Neuropsychomotor development
Severe delay	Severe delay	Cognitive
+	+	ASD
+	+	ID
+	-	Hypotonia
Motor delay	Motor dyspraxia	Motor
-	Diffuse bilateral slow waves with multifocal epileptiform discharges	EEG
Volumetric reduction and abnormal T2 signal hyperintensity in white matter	Volumetric reduction and abnormal T2 signal hyperintensity in white matter	MRI findings
Deep-set eyes, prominent nasal bridge, accentuated Cupid's bow of the upper lip, high palate, widely spaced teeth; bitemporal narrowing	Deep-set eyes, prominent nasal bridge, accentuated Cupid's bow of the upper lip, high palate, widely spaced teeth, bitemporal narrowing	Facial dysmorphism
Prominent digit pads	Prominent digit pads, brachydactyly of the 4th and 5th toes and hypoplastic nails	Other features
Dyslipidemia and hypothyroidism	Dyslipidemia and hypothyroidism	Metabolic alterations

+ Presense, - absence of the corresponding clinical feature

Biallelic loss-of-function (LoF) variants in *TBCK*

To investigate the genomic cause of patients' clinical phenotypes, we performed array comparative genomic hybridization (aCGH), whole exome sequencing (WES) and subsequent

site specific quantification of genomic DNA by RT-qPCR. WES and RT-qPCR analyses revealed that the sisters share rare LoF variants in exon 23 of the *TBCK* gene: a stopgain variant (NM_001163435: c.2130C>G; p.Tyr710*) and a microdeletion (NM_001163435: c.2060_2235del; p.Glu687Valfs9*) (Figures 1B-C; Supplementary Figures 2A-D). Both variants were validated by conventional PCR and Sanger sequencing, using either genomic DNA or cDNA isolated from iNPC. cDNA sequencing revealed that the microdeletion leads to loss of exon 23 and juxtaposition of exons 22 and 24, resulting in a predicted premature termination codon (PTC) in exon 24 (Figures 1B-C, Supplementary Figure 2A-C). Segregation analysis in the family showed that these variants are in *trans* in the two affected sibs, consistent with an autosomal recessive inheritance pattern, in which the stopgain variant was inherited from their father and the microdeletion from their mother (Supplementary Figures 2B-D). The unaffected sib did not inherit any of these variants. Both variants were considered pathogenic according to the ACMG guidelines (Richards et al., 2015). No additional disrupting variants following an autosomal recessive inheritance model were identified by WES or aCGH.

Figure 1: IHPRF3 patients harbouring biallelic pathogenic variants in *TBCK* and relative quantification of mRNA and protein expression levels in iNPC.

A) Pedigree of family F6331. Circle – female; square – male; symbol filled – affected patients; symbol empty – not affected sister; circle half filled – harbors the heterozygous microdeletion; square half filled – harbors the heterozygous stopgain variant.

B) Chromatograms of cDNA sequence showing a base change in Band 1, in the left image (expected base G changed to a C in reverse strand); and the junction of exon 22 and 24 (excision of exon 23) in Band 2, in the right image. Band 1 and 2 can be seen in Supplementary Figure 2A.

C) Schematic representation of *TBCK* transcript and protein with the two identified variants' sites and the antibodies target regions. The pathogenic variants create a putative premature

termination codon (PTC) in exon 23 and 24, located within the C-terminal region of the protein, upstream of the RHOD domain.

D) *TBCK* mRNA expression shows reduced transcript levels in patients, compared to controls.

E and F) Western blot shows *TBCK* protein levels with two distinct antibodies, ab1 (polyclonal) and ab2 (monoclonal). The data reveal similar levels of *TBCK*-mutated protein, compared to controls.

p*-value < 0.05, **p*-value < 0.001, n.s. – not significant. Patients: F6331-1 and F6331-4; controls: F7007-1, F8799-1, F10006-1

TBCK protein is expressed in patients-derived iNPC

To explore the functional consequences of the identified variants in *TBCK*, we generated iPSC from the 2 IHPRF3 patients and from 3 unrelated controls and differentiated them into iNPC. All iPSC colonies were positive for pluripotency markers (OCT3/4 and NANOG) (Supplementary Figures 3A-B), and the iNPC were positive for neural stem cell markers (SOX1, SOX2 and PAX6) (Supplementary Figures 3C-E), suggesting successful cell reprogramming and differentiation.

Next, we investigated whether mRNA and protein expression of *TBCK* were abnormal in the affected sisters' iNPC. We observed that patients' cells exhibit over 2-fold decrease in *TBCK* transcript levels compared to controls (Figure 1D). cDNA sequencing revealed that both alleles were expressed (Figure 1B, Supplementary Figure 2A). Quantification of *TBCK* protein by western blot with two different antibodies revealed different results: while normal protein levels were observed in patients-derived iNPC with ab1 (polyclonal antibody targeting amino acids aa457-694 on the TBC Rab-GAP domain), compared to controls, very low levels were observed in patients' cells with ab2 (monoclonal antibody targeting a more distal region of the protein, aa601-701) (Figure 1E-F). More recently, we found out that the polyclonal ab1 antibody cross-reacts with other proteins (Bhoj E, personal communication). Therefore, we only considered the results with ab2 antibody that suggests a significant reduction in *TBCK* protein levels in patients' iNPC.

Patients-derived iNPC under stress-inducing conditions exhibit downregulation of *mTOR* signaling activity

In order to investigate whether iNPC from IHPRF3 patients show abnormal *mTOR* signaling activity, we first analyzed by western blot the phosphorylation levels of the ribosomal protein RPS6, a downstream target of *mTORC1* signaling pathway. Phosphorylated RPS6 (p-RPS6^{S240/244}) was quantified in iNPC cultured in normal growth conditions (under the presence of EGF and FGF-2 growth factors) and in three stress-inducing conditions. Under normal growth conditions, we did not detect any difference in the phosphorylation levels of RPS6 between patients-derived and controls-derived iNPC (Figure 2A-C, Supplementary Figure 4A,C,D). Intracellular stress caused by the withdrawal of EGF/FGF-2 alone significantly decreased RPS6 activation in patient's iNPC in comparison to controls (p-RPS6^{S240/244} protein level: patients - 0.55 ± 0.11 versus controls - 0.96 ± 0.05 ; Mann-Whitney test, two-tailed, p -value = 0.0238), and the same was observed under EGF/FGF-2 withdrawal combined with treatment with mTORC1 complex inhibitor rapamycin (p-RPS6^{S240/244} protein level: patients - 0.46 ± 0.09 versus controls - 0.73 ± 0.13 ; Mann-Whitney test, two-tailed, p -value = 0.0286) (Figure 2B, Supplementary Figure 4B,C). Conversely, inhibition of *mTOR* signaling through EGF/FGF-2 deprivation for 24 hours and subsequent addition of brefeldin A (BFA, interferes with glutamine induced mTOR's lysosomal localization and activation - Jewell et al., 2015; Meng et al., 2020) led to similar RPS6 phosphorylation in both groups (Figure 2C, Supplementary Figure 4D). Using a multiplex assay, we also measured the phosphorylation status of other *AKT-mTOR* pathway components in cells cultured in the absence of EGF and FGF-2. In line with the RPS6 findings, patients' cells showed a trend towards decreased activation of other *mTOR* pathway components ($p > 0.05$) (Figure 2D). Together, these results show that *mTOR* signaling in patients-derived iNPC is altered only under specific stress-inducing conditions and suggest that TBCK acts through an indirect mechanism over *mTOR* signaling.

Figure 2: Under stress inducing conditions, patients-derived iNPC exhibit reduced RPS6 phosphorylation. A-C) Protein level of phospho-RPS6 (residues S240-244) from iNPC cultivated under normal growth condition and under stress inducing conditions. iNPC without EGF and FGF-2 for 24 hours only (A), without EGF/FGF-2 and concomitant treatment with rapamycin (100 nM) for 24 hours (B), or without EGF/FGF-2 and subsequent treatment with BFA (4 μM) for 3 hours (C).

D) Ratio of phosphorylated/total protein levels of *AKT-mTOR* signaling pathway components from iNPC cultivated without EGF and FGF-2 for 24 hours.

**p*-value < 0.05. WB experiments were replicated twice. Data are shown as mean ± SD of technical replicates and biological samples. Patients: F6331-1 and F6331-4; controls: F7007-1, F8799-1, F10006-1

TBCK colocalizes with proteins involved in both endocytic and secretory pathways

Previously, a few evidences have suggested a potential role of TBCK on intracellular vesicle transport (Collinet et al., 2010; Perini, 2012; Havugimana et al., 2012; Gillingham et al., 2014; Gillingham et al., 2019). However, functional studies that confirm these evidences are still lacking.

In order to investigate whether TBCK localizes to intracellular vesicle transport machinery in iNPC, we analyzed by immunofluorescence the colocalization levels of TBCK with several proteins involved in endocytic (caveolin, RAB5A) and/or secretory (COPII, GM130/GOLGA2), in addition to Clathrin and STAM implicated in both pathways in patients' and controls' iNPC growing under normal culture conditions. We observed that TBCK protein was diffusely distributed in the cytosol of control and patients' iNPC, with a more condensed perinuclear localization, suggesting that TBCK-mutated protein is not retained in any particular cellular compartment in patients' iNPC (Supplementary Figure 5A-F). Subsequent colocalization analysis showed that wild type TBCK exhibits modest colocalization with caveolin (MCC mean value: 0.32 ± 0.15) and GM130/GOLGA2 (MCC mean value: 0.37 ± 0.13), and high colocalization levels with STAM, RAB5A, COPII and clathrin (MCC mean values: 0.76 ± 0.13 , 0.74 ± 0.28 , 0.79 ± 0.17 and 0.8 ± 0.13 , respectively), consistent with an important role for TBCK in both endocytic and early secretory pathways (Figures 3A-F, Supplementary Figures 5A-F). Importantly, whereas a similar colocalization pattern was observed between TBCK-mutated protein and caveolin, COPII and RAB5A (MCC mean values: 0.29 ± 0.23 , 0.74 ± 0.26 and 0.67 ± 0.35 , respectively) (Figures 3A-C and Supplementary Figures 5A-C), TBCK-mutated protein showed decreased colocalization with clathrin (MCC mean value: 0.73 ± 0.14 ; $p < 0.00001$) (Figure 3D and Supplementary Figure 5D) and increased colocalization with STAM (MCC mean value: 0.83 ± 0.1 ; $p < 0.0001$) (Figure 3E and Supplementary Figure 5E) and GM130/GOLGA2 (MCC mean value: 0.49 ± 0.14 ; $p < 0.0001$) (Figure 3F and Supplementary Figure 5F). Decreased colocalization with clathrin was not attributed to reduced clathrin protein levels, since western blot analysis in iNPC under normal growth conditions showed no difference between the two groups (Supplementary Figure 5G).

We also examined the effect of lack of functional TBCK protein over Clathrin and STAM transcripts and protein levels in iNPC grown as neurospheres (3D model system). RT-qPCR

showed that clathrin heavy chain and STAM1/2 transcripts are significantly reduced in patients' neurospheres, compared to controls (Figure 3G-I). Interestingly, clathrin protein levels showed to be increased in patient F6331-1 only (protein levels: F6331-1: 0.79; F6331-4: 0.23; controls - 0.275 ± 0.08), while a trend towards increased STAM protein levels was observed in both patients (STAM protein levels: patients - 1.155 ± 0.21 versus controls - 0.613 ± 0.25 , Mann-Whitney test, two-tailed, p -value = 0.0714) (Figure 3G'-H'). Together, these results further suggest altered intracellular membrane trafficking along the endolysosomal and/or the early secretory pathways in patients-derived iNPC.

Figure 3: Patients-derived iNPC show altered colocalization levels with endocytic and early secretory pathway markers.

(A-F) Representative images of confocal microscopy of iNPC coimmunostained for endogenous TBCK and specific vesicle transport machinery components: A) Caveolin (Cav); B) COPII, C) RAB5A, D) Clathrin, E) STAM and F) GM130/GOLGA2. For more detailed images see Supplementary Figure 5.

(A'-F') Graphs of Manders coefficient colocalization (MCC) of TBCK with each target protein. Measurements were performed using ImageJ/Fiji-Coloc2 plugin. Error bars indicate S.D. (each dot represents the MCC in each cell; data represent the analysis of $n > 150$ cells / individual).

G-I) Clathrin heavy chain and STAM1/2 mRNA expression show reduced transcript levels in patients' neurospheres, compared to controls'.

G'-H') Clathrin and STAM immunoblots densitometries reveal a tendency to increased protein levels in patients' neurospheres, compared to controls'.

J-K) Analysis of large COPII carrier ($> 1 \mu\text{m}$) average diameter (G) and number per cell (H).

*** p -value < 0.001 , **** p -value < 0.0001 . Unpaired t-test, two-tailed. Data are shown as mean $\pm$ SD of biological replicates. DAPI (blue) marks cell nuclei. Scale bar = $10 \mu\text{M}$. Patients: F6331-1 and F6331-4; controls: F7007-1, F8799-1, F10006-1

Patients-derived iNPC does not show evidence of altered endosome maturation along the endolysosomal system

As part of the ESCRT sorting machinery, STAM together with clathrin contributes to the recognition and sorting of ubiquitinated cargos at early endosomes, which mature to late endosomes prior to targeting ubiquitinated proteins for degradation at lysosomes (Vietri et al., 2020). Thus, to investigate whether altered colocalization of TBCK-mutated protein with STAM and clathrin is associated with defects in the endolysosomal system, we quantified the immunofluorescence intensities of RAB7A, a late-endosome marker. RAB7A fluorescence intensities did not differ between patients and controls (Supplementary Figure 6), suggesting that expression of TBCK-mutated protein does not disrupt late endosome maturation in cells under normal growth condition.

Lack of functional TBCK protein may alter early secretory pathway and autophagosome membrane recruitment in basal autophagy

Next, we sought to verify if patients-derived cells show any sign of impaired early secretory pathway. STAM has been implicated in the deubiquitination of SEC31A (component of COPII outer layer), modulating the formation of large COPII carriers that mediate the transport of macromolecules (such as procollagen) from endoplasmic reticulum (ER)-to-Golgi (Rismanchi et al., 2009; Kawaguchi et al., 2018). Thus, we first investigated whether patients-derived iNPC present abnormal large COPII-carriers formation ($> 1 \mu\text{m}$). Measurement of the diameter of COPII puncta showed that patients' iNPC had a smaller average diameter compared to controls (large COPII carriers' average diameter - patients: $1.89 \pm 1.01 \mu\text{m}$ and controls: $2.31 \pm 1.23 \mu\text{m}$; $p < 0.0001$) (Figure 3G) and a tendency to have less large COPII carriers per cell (Figure 3H).

COPII-coated vesicles have also been shown to act as templates for LC3 lipidation (conversion of cytoplasmic LC3I to membrane-bound LC3II) during autophagy, playing an important role as membrane source for autophagosome biogenesis (Ge et al., 2014; Farhan et al., 2017; Shima et al., 2019). Thus, we also evaluated whether patients' iNPC show evidence of defective autophagosome biogenesis, by quantifying the protein levels of the autophagosome marker LC3 under normal culture condition (basal autophagy) and the 3 stress inducing conditions (absence of EGF/FGF-2 only, and absence of EGF/FGF-2 with treatment with either rapamycin or BFA). Western blot analysis of cells in all conditions showed a modest decrease in LC3II/I ratio in patients-derived iNPC, compared to controls, but not statistically significant (Figure 4A-B; Supplementary Figure 4E-H). Several other autophagy markers, including BECN1, p62/SQSTM1 and cathepsin D have also been evaluated in cells deprived of the growth factors only, which showed to be similar between the two groups (Supplementary Figure 4F and Supplementary Figure 7). Despite lack of statistical significance, LC3II/I ratio always shows a trend to be decreased in patients' iNPC. Further confocal microscope analysis of the number of LC3B puncta per cell and the diameter of LC3B puncta in cells under normal growth condition showed that LC3B puncta's number is also trended to be reduced in patients' cells, while the average diameter of LC3B puncta showed to be significantly smaller, compared to controls (LC3B average puncta diameter - patients: $528.7 \pm 280.6 \text{ nm}$ and controls: $621 \pm$

341.2 nm; $p < 0.001$) (Figures 4C-E). These results suggest altered autophagosome formation in basal autophagy in patients-derived iNPC.

Figure 4: Autophagosome biogenesis analysis: LC3B puncta diameter is reduced in patients-derived iNPC.

A-B) Graphs show the densitometries of immunoblots shown in Supplementary Figure 4. Patients are represented in gray ray bars and controls in black bars. Graphs show LC3A-B II/I ratio from iNPC cultivated under normal growth condition and under stress inducing conditions: absence of EGF and FGF-2 for 24 hours and A) concomitant treatment with rapamycin (100 nM) for 24 hours; and B) subsequent treatment with BFA (4 μ M) for 3 hours. β -actin was used as a loading control. Graphs are represented in arbitrary units.

C-E) Immunostaining analysis of endogenous LC3B by confocal microscopy. Representative images are presented in C); average LC3B puncta diameter (nm) in D); and average number of LC3B puncta per cell in E).

** p -value < 0.01; n.s. – not significant. Data are shown as mean values $\pm$ SD of biological replicates. Patients: F6331-1 and F6331-4; controls: F7007-1, F8799-1, F10006-1

iNPC expressing TBCK-mutated protein show altered cell cycle progression and severe impairment in the capacity of cell migration

Next, we sought to verify whether expression of TBCK-mutated protein is associated with aberrant cell proliferation and migration in the context of neural development. Flow cytometry analysis showed a higher percentage of cells arrested at G0/G1 in patient-derived iNPC at 24h and 40h of recovery from growth factors withdrawal compared to control iNPC (p -value < 0.01) (Figure 5A, Supplementary Figure 8). Detection of S-phase cells via BrdU incorporation showed that BrdU-positive cells in the control population increased on average 4.5% and 6.7% after 24h and 40h of medium supplementation respectively, while BrdU-positive cells in the *TBCK*-mutated population decreased 12.6% and 6.8% after 24h and 40h of medium supplementation, respectively (Figure 5B, Supplementary Figure 8). These alterations were not attributed to an increase in apoptosis since activation of caspase 3/7 did not differ between patient and control cells (Supplementary Material Table 4). Taken together, these results suggest that patient-derived iNPC show reduced cell proliferation due to delayed cell cycle progression.

We also evaluated the migration rates of iNPC from IHPRF3 patients and controls by quantifying the distance of cell migration outwards from the edge of neurospheres at 4, 8 and 24 hours after adhesion. While iNPC from control neurospheres showed robust migration rates after 24 hours, we observed that patients-derived iNPC showed dramatically reduced capacity of migration (Figures 5C-D).

Figure 5: iNPC expressing TBCK-mutated protein show altered cell cycle progression, cell proliferation and cell migration.

A) G0/G1-phase analyses at 3 time points, showing an increased percentage of patient's cells arrested at G0/G1 cell cycle stage. Cell cycle experiments were replicated twice.

B) BrdU-positive cells showing that patient's iNPC had a lower incorporation of BrdU. Data is shown as percentages. BrdU incorporation experiment was performed once for each sample. Patient: F6331-1; controls: F7007-1, F8799-1, F10006-1.

C-D) iNPC migration from neurospheres, measured as the distance of the longest outer diameter of the cells that migrated from the neurosphere, normalized to the inner neurosphere edge.

D) Representative images of migration assay. Red dashed line – inner diameter; black dashed line – outer diameter. Scale bar 400 μm. Patients: F6331-1 and F6331-4; controls: F7007-1, F8799-1, F10006-1; n = 4 neurospheres per individual; ***p-value < 0.0001.

Data are shown as mean values ± SD of patients and controls biological replicates.

Altered phenotypes observed in patients-derived iNPC may be associated with altered microtubule organization

Finally, in order to gain more insights into TBCK's mechanism of action and IHPRF3's pathogenesis we performed co-immunoprecipitation assay against polyclonal TBCK antibody (ab1), followed by mass spectrometry in iNPC cultured under normal growth condition. From the proteomic analysis we identified a total of 15 potential interactors for TBCK, of which only CALM1 (highlighted in yellow in table 2), one of the major calcium sensors in eukaryotes, showed reduced interaction with TBCK-mutated protein, as compared to the wild type protein (Mann-Whitney test, p -value = 0.019). Two other molecules (PPP1CC and SKP1, also highlighted in table 2) were found to interact with wild type TBCK, but not with patients' mutant protein. In support of altered PPP1CC interaction in patients-derived iNPC, protein level quantification of PPP1CC by western blot in iNPC grown as neurospheres showed a trend towards increased levels in patients, compared to controls (PPP1CC protein level: patients - 1.09 ± 0.05 versus controls - 0.43 ± 0.14), but not statistically significant (Figure 6A). Western blot for CALM1 and SKP1 could not be evaluated due to antibody failure. Protein:protein interaction (PPI) network created by STRING database (V11.0) with the three molecules identified, together with other potential protein interactors (suggested as potential interactors by previous studies, including STAM, RAB5A, RAB33A/B and MAD2L1) (Lee et al., 2008; Perini, 2012; Havugimana et al., 2012; Gillingham et al., 2014; Chong et al., 2016; Gillingham et al., 2019) as input list suggest that all the molecules are biologically connected (Figure 6B). Gene ontology (GO) enrichment analyses with the same input list and addition of other molecules here evidenced to be altered in patients' iNPC provide additional support for a regulatory role of TBCK in protein transport (p -value < 0.001) (Table 3). Intracellular enrichment at microtubules (MT) and mitotic spindle (p -value < 0.001) (Table 3) further suggest potential impairment in MT-dependent processes in patients-derived iNPC. Importantly, given the potential nonspecificity of polyclonal antibody, further validation of these interactions using TBCK monoclonal antibody (ab2) shall still be performed.

Table 2: List of proteins identified by liquid chromatography and mass spectrometry.

Accession Number	Gene name	FC Patient/Control	Log2 FC
P01011	SERPINA3	1,5427	0,625475346
P05109	S100A8	1,6837	0,751620419
P05387	RPLP2	0,9171	-0,124783338
P06702	S100A9	1,2064	0,270704865
P08697	SERPINF2	1,4400	0,526037385
P08708	RPS17	1,1706	0,227239129
P0DP23	CALM1	0,2785	-1,843996584
P12273	PIP	1,2532	0,325613413
P39019	RPS19	0,7765	-0,36498988
P62269	RPS18	0,8662	-0,207252368
P62851	RPS25	0,5235	-0,933684902
Q9NYL9	TMOD3	0,5020	-0,994336732
Q9Y224	RTRAF	0,9868	-0,01911492
P36873	PPP1CC	CONTROL "EXCLUSIVE"	N/A
P63208	SKP1	CONTROL "EXCLUSIVE"	N/A

Figure 6: Deciphering TBCK's mechanism of action and IHPRF3's pathophysiology.

A) PPP1CC protein quantification by western blot shows a trend to increased levels in patients' neurospheres, compared to controls.

B) Protein:protein interaction (PPI) network, obtained from STRING database, shows that TBCK's potential partners are closely connected. The red node represents the protein of interest, while white nodes represent its potential interactors. Line thickness indicates the strength of data support.

C) Illustrative scheme of TBCK localization and hypothesized IHPRF3's molecular mechanism in the 2 affected sibs. Our data suggest that TBCK is recruited to COPII vesicles and thus, predicted to mediate the transport of newly synthesized proteins from ER-to-Golgi. Expression of TBCK-mutated protein impairs both, the transport of newly synthesized proteins from the *trans*-Golgi network (TGN) to their final destination and recruitment of COPII membranes to

autophagosome biogenesis. Red filaments represent microtubules (MT) which organization may be impaired in patients-derived iNPC.

Table 3: Gene ontology (GO) enrichment analyses

Cellular Component	GO_Term description	Count in network	Fold Enrichment	Raw P-value	FDR	GO_Input list
GO:0072686	mitotic spindle	4 of 137	46.83	1.36E-06	1.37E-03	TBCK
GO:0000922	spindle pole	3 of 167	28.81	1.43E-04	2.39E-02	CALM1
GO:0030658	transport vesicle membrane	3 of 215	22.38	2.98E-04	3.73E-02	PPP1CC
GO:0005874	microtubule	4 of 441	14.55	1.25E-04	2.28E-02	SKP1
GO:0005768	endosome	5 of 991	8.09	2.29E-04	3.06E-02	STAM
						RAB5A
						MAD2L1
						RP56
						CLTC
						GOLGA2
						MAP1LC3B
						RAB33A
						RAB33B

Biological Process	GO_Term description	Count in network	Fold Enrichment	Raw P-value	FDR
GO:0150093	amyloid-beta clearance by transcytosis	2 of 7	> 100	1.29E-05	1.13E-02
GO:0032482	Rab protein signal transduction	2 of 14	> 100	4.28E-05	2.42E-02
GO:1904668	positive regulation of ubiquitin protein ligase activity	2 of 14	> 100	4.28E-05	2.34E-02
GO:0017157	regulation of exocytosis	4 of 203	31.60	6.27E-06	7.66E-03
GO:0006914	autophagy	4 of 306	20.97	3.07E-05	1.88E-02
GO:0016055	Wnt signaling pathway	4 of 346	18.54	4.94E-05	2.37E-02
GO:1903047	mitotic cell cycle process	6 of 680	14.15	1.74E-06	3.45E-03
GO:0033365	protein localization to organelle	5 of 779	10.29	7.40E-05	3.36E-02
GO:0009057	macromolecule catabolic process	6 of 1060	9.08	2.21E-05	1.52E-02
GO:0015031	protein transport	7 of 1509	7.44	1.22E-05	1.14E-02
GO:0051649	establishment of localization in cell	8 of 2378	5.40	2.19E-05	1.65E-02
GO:0044267	cellular protein metabolic process	10 of 3822	4.20	7.17E-06	7.58E-03
GO:0006996	organelle organization	9 of 3586	4.03	4.86E-05	2.48E-02

Proteins highlighted in yellow in the input list represent in silico predicted interactions with TBCK (Lee et al., 2008; Chong et al., 2016)

DISCUSSION

In the present work, we describe two sisters who were referred to the CEGH-CEL at the age of 3 and 6 years because of suspected ASD. Detailed clinical investigation and follow up for 7 years revealed several facial dysmorphic features and progressive muscle weakness and psychomotor developmental delay. These clinical symptoms along with the biallelic LoF variants in *TBCK* led to the diagnosis of IHPRF3 (OMIM#616900), an autosomal recessive condition with approximately 40 cases reported so far. Several cases of IHPRF3 show premature death before early adulthood. Importantly, in keeping with another patient described by Bhoj and colleagues (2016), we highlight autism as an additional clinical feature of the syndrome.

Our patients are compound heterozygous for pathogenic LoF variants in *TBCK*: a stopgain variant (p.Tyr710*) in exon 23, and a microdeletion (p.Glu687Valfs9*) predicted to create a premature stop codon in exon 24 that has been previously identified in homozygosity in another IHPRF3 patient (Sumathipala et al., 2019). While most pathogenic variants reported in IHPRF3 patients locates at the region encompassing the kinase and TBC domains (Zapata-Aldana et al., 2019), the variants here identified, together with two variants reported elsewhere

(Bhoj et al., 2016; Sumathipala et al., 2019) locate in a more distal region, downstream of the TBC domain.

The biallelic disrupting variants in *TBCK* here reported led to reduced mRNA and protein expression levels in patients-derived iNPC in comparison to controls, in agreement with previous studies on fibroblasts and lymphoblastoid cells from IHPRF3 patients, harboring mutations upstream of the TBC domain (Bhoj et al., 2016; Sumathipala et al., 2019). However, high number of TBCK-positive puncta could be observed through confocal microscope immunofluorescence labling in patients' iNPC. These results may suggest that *TBCK* in patients-derived iNPC partly escapes from both, nonsense mediated-mRNA decay and protein quality control pathways (Giannandrea et al., 2013; Supek et al., 2021). Nevertheless, even though the clinical progression of the syndrome in our patients are milder than most of the patients reported so far (Beck-Wödl et al., 2018; Sumathipala et al., 2019; Zapata-Aldana et al., 2019), they present the clinical hallmarks of the syndrome (OMIM#616900).

In accordance with previous data on *TBCK* depleted cells and fibroblasts and lymphoblastoid cells from IHPRF3 patients (Liu et al., 2013; Bhoj et al., 2016), iNPC from our patients showed evidence of altered *mTOR* signaling activity, compared to controls. Reduced RPS6 phosphorylation was not observed in cells under normal growth condition, but rather in cells submitted to the withdrawal of EGF and FGF-2 growth factors, either with or without rapamycin treatment. In the absence of the growth factors, *mTOR* signaling can still be activated by amino acids, suggesting that altered *mTOR* signaling may not be induced by defective growth factors receptors signaling in patients' iNPC, but rather induced by impaired amino acid stimuli. Additional treatment with mTORC1's complex direct inhibitor, rapamycin in growth factors deprived cells seems to have affected *mTOR* signaling similarly to the iNPC under EGF/FGF-2 withdrawal only. Interestingly, addition of BFA, which interferes with glutamine induced mTORC1's lysosomal localization and activation (Jewell et al., 2015; Meng et al., 2020), led to similar phosphorylation levels between patients and controls. That is, BFA seemed to have affected controls' iNPC in a more pronounced way than patients' iNPC (if we take into consideration the RPS6 phosphorylation pattern in iNPC of patients and controls under EGF/FGF-2 withdrawal only). These results may suggest that TBCK plays a role on the same pathway to *mTOR* signaling as BFA, and preexisting disruption of this signaling in patients' iNPC makes patients cells less sensitive to the inhibitory effect of BFA, compared to controls. Thus,

understanding BFA's mechanism of action is critical to gain new insights into TBCK's mechanism over *mTOR* signaling. In line with previous data showing that mTORC1 activation induced by amino acids requires additional small GTPases other than Rheb, to anchor mTORC1 in close proximity to its activator, BFA targets the intra-Golgi vesicle transport regulator ARF1 GTPase's GEF, preventing the conversion of ARF1 from its GDP-bound state to the active GTP-bound state. Importantly, ARF1's activity towards *mTOR* signaling in turn, has been shown to be induced only by specific subset of amino acids, including arginine and glutamine, with the later being highly enriched in NB medium. Thus, reduced RPS6 activation in iNPC expressing TBCK-mutated protein may be due to impaired ARF1 activation, compared to controls, making patients-derived iNPC less sensitive to BFA's inhibitory effect. Conversely, the fact that in Bhoj's (2016) and Ortiz-González's (2018) reports, leucine treatment contributed to partial rescue of the reduced RPS6 phosphorylation and the accumulation of aberrant oligosaccharides in patients' non-neural cells (fibroblasts and lymphoblastic cells) suggests that RagA/B GTPase dependent leucine-induced (and probably other amino acids that have been shown to depend on RagA/B GTPases) activation of *mTOR* signaling is not impaired in IHPRF3 patients. It is also worth mentioning that besides ARF1, another small GTPase has been implicated in mTORC1 activity induced by amino acids in a Rag complex independent manner. It has been shown that amino acids can promote GTP loading of RAB1A GTPase which in turn stimulate the interaction of mTORC1 with Rheb at Golgi apparatus, in a lysosome independent mechanism (Thomas et al., 2014). Like ARF1, RAB1A is involved in the regulation of vesicle transport at early secretory compartments and has been shown to activate ARF1-GTPase through ARF1's GEF recruitment (Dumaresq-Doiron et al., 2010; Sastre et al., 2021). Thus, investigating whether early secretory traffic is affected in IHPRF3 patients is an important matter.

In this study, we show that in iNPC under normal growth conditions, TBCK protein seems to localize to the endosomes (e.g. RAB5A⁺ compartment) and the early secretory pathway (e.g. COPII vesicles), corroborating previous suggestions from protein interaction studies (Rismanchi et al., 2009; Collinet et al., 2010; Perini, 2012; Gillingham et al., 2014; Farhan et al., 2017; Gillingham et al., 2019). Our results provide support for the interaction between TBCK and STAM (Havugimana et al., 2012), an adaptor protein first associated with the ESCRT sorting machinery at early endosomes, and also found to directly regulate the formation of COPII vesicles and Golgi morphogenesis in the secretory pathway (Rismanchi et al., 2009;

Havugimana et al., 2012; Kawaguchi et al., 2018). While evidence of altered endolysosomal transport was not observed in patients-derived iNPC, increased colocalization of TBCK-mutated protein with STAM and GM130/GOLGA2 (a *cis*-Golgi tethering factor that facilitates ER-derived vesicle fusion) and decreased colocalization with clathrin observed in patients' cells, suggest altered early secretory transport and impaired clathrin coated vesicle-dependent post Golgi transport of proteins. In agreement with this view, patients-derived iNPC showed smaller average diameter of COPII carriers, compared to controls, suggesting impaired macromolecule transport from ER to Golgi. Also, consistent with early secretory defects, patients' iNPC showed smaller LC3B puncta, and despite the lack of statistical significance, which may be due to the limited detection capability of WB and/or autophagy rates in neuroprogenitor cells, the average LC3II/I ratio tends to be decreased in patients' iNPC, in both basal and stress-induced autophagy. Thus, despite further work is needed, our findings strengthen the hypothesis that TBCK acts in ER-to-Golgi vesicle trafficking and that lack of functional TBCK protein impairs both the transport of newly synthesized proteins to their final destination and autophagosome biogenesis due to altered early secretory transport (Figure 6C). It is important to note that unlike Ortiz-González and colleagues observations (2018), reduced *mTOR* signaling in patients iNPC treated with the well established autophagy inducer, rapamycin, was not associated with an increased level of autophagosome formation, compared to controls. This apparent contradictory data may be associated with previous suggestions that TBCK seems to present functional differences according to cell type, and emphasize the relevance of studying neural cells to investigate the pathophysiological mechanisms of IHPRF3's neuronal phenotypes. Furthermore, the discordance between *mTOR* signaling activity and autophagosome formation observed in our patients' cells may also suggest that dysregulated *mTOR* signaling alone may be insufficient to account for all the clinical features of the syndrome.

Furthermore, in this study we observed that patients-derived iNPC showed G0/G1 cell cycle arrest and delayed S-phase progression, as well as a significantly impaired capacity of migration. Impaired cell cycle progression has also been observed by Wu and colleagues (2014). Importantly, in line with our preliminary proteomic data, these authors together with Liu and colleagues (2013) observed an overlapped localization between TBCK and α - and γ -tubulin, respectively, suggesting a potential role of TBCK on microtubule nucleation. In this context, studies have shown that Golgi apparatus can function as an important microtubule organizing

center (MTOC) in many cell types, and unlike centrosomal MTOC, it can give rise to polarized MTs important for a number of cellular processes, including Golgi reassembly after mitosis (Maia et al., 2013) and polarized transport of post-Golgi carriers that are important for cell migration (Vinogradova et al., 2009; Hurtado et al., 2011; Vinogradova et al., 2012; Wu et al., 2016). In neuronal cell types Golgi-anchored MTs has also been implicated in neurite outgrowth and branching (Oddoux et al., 2013, Ori-McKenney et al., 2012, Yalgin et al., 2015). Thus, it is tempting to speculate that impaired activity of TBCK mutant protein over other Golgi resident vesicle transport regulator impact MT nucleation and Golgi-derived MT dependent processes that are essential for normal brain development and structural organization, such as cell division, migration and neuronal morphogenesis (Ori-McKenney et al., 2012; Etienne-Manneville, 2013; Roccoio et al., 2013; Borrell et al., 2014; Maizels & Gerlitz, 2015; Garcin & Straube, 2019; Shokrollahi & Mekhail, 2021). Impairment in these processes may further contribute to abnormal brain structures, like microcephaly and cortical atrophy presented by IHPRF3 patients. Whether these altered phenotypes are associated with calcium/calmodulin, SKP1 and/or PP1 phosphatase, identified as potential interactors of TBCK in our preliminary proteomic study, still needs further investigation.

In summary, we described a novel IHPRF3 family, adding autism as a clinical feature of this syndrome. Using iNPC from IHPRF3 patients and control individuals, we pinpoint a role for TBCK in regulating early secretory pathway, and suggest that the impairment in *mTOR* signaling and autophagosome biogenesis observed in *TBCK*-mutated cells might be related to impaired signaling towards other early secretory transport regulators, such as RAB1A or ARF1 small GTPases. It is of note that pathogenic mutations in other members of the TBC family of proteins (TBC1D20, TBC1D23, TBC1D24), which also mediate intracellular membrane transport, such as endosome-to-golgi and ER-to-golgi trafficking or autophagy, have also been implicated in neurodevelopmental syndromes (OMIM#615663, OMIM#617695 and OMIM#220500, respectively) with high clinical overlap with IHPRF3 (Falace et al., 2014; Sidjanin et al., 2016; Ivanova et al., 2017; Shin et al., 2017; Aprile et al., 2019), reinforcing the importance of vesicle trafficking kinetics in neurodevelopment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Material Table 1: Primer pairs for <i>TBCK</i> genomic DNA and cDNA sequencing.		
Pimer-pair-name	Sense	Antisense
TBCK-seq-gDNA	GCAAACTTGCTACCTGGAGGA	TTCACTTAGGAGAGATGGTGTTT
TBCK-seq-cDNA	ATCTCTATGCCATCCCTTGGTT	ACACAAGTCAATCAGGTCCTCT

Supplementary Material Table 2: Primer pairs in <i>TBCK</i> (to identify intragenic CNV) and GAPDH (normalizing control).		
Primer Pair	Sense	Antisense
TBCK_P1	TGTTGAAACACCTACATGGCATAG	ACTCCTCCAGGTAGCAAGTTTTG
TBCK_P2	CCCAAATACATTCCGTTCAAAAAC	AGTGCCAAAGTCTTTCCCATCAG
TBCK_P3	GACCAAGGAGTGAGGGTCAGGG	GCTTCCGGTTATTAGCTCCAGCCC
TBCK_P4	GCAGTCCTTCAATCACTGGCATTGG	TGTGCTTTGGAAGGGTTTTGAGATG
TBCK_P5	GACACCTTCAAGTTGACAGCAC	GAAAGAGAGCAGCTGTCCAGGG
TBCK_P6	GCAGGAATCCTTTCACAAACCAG	TGTAACGTGATTCTAGGAAGCTG
GAPDH	TGCTCACATATTCTGGAGGAGC	TGTAAACCTGGGGGAATACGTG

Supplementary Material Table 3: Primer pairs for <i>TBCK</i> mRNA quantification (RT-qPCR).		
Target	Sense	Antisense
TBCK-mRNA-1	CTGCTCTTCTGGGAGTTGAGGGAG	CAGGATGAGACACTACCCAGGCTT
TBCK-mRNA-2	GTCTTTGTGCTTGCCTGCGTCC	GCTCCCATTTTCAGCGTCCTTCAG
TBCK-mRNA-3	GGTCATTGTCATCGTGGGGCAT	GGCAGTCACACTCTTGTTCTTCA
TBCK-mRNA-4	TCCTGTAGCAAAGACGAATGGC	TGTAAAGCAATGAGAGAAGGTAATGAGT

Supplementary Figure 3: iPSC and iNPC characterization.

A-D) mRNA expression levels of pluripotency markers, OCT3/4 and NANOG (A-B), and neural markers, PAX6 and SOX1 (C-D);

E) Immunostaining of two neural cell markers (SOX1 and SOX2).

Supplementary Figure 4: Western blot for p-RPS6 and autophagosome marker LC3, in iNPC.

A-D) Blots of phospho-RPS6 of iNPC cultured in normal and stress inducing conditions;

E-H) Blots of LC3A-BII/I of iNPC cultured in normal and stress inducing conditions.

Stress conditions were induced through: removal of EGF and FGF-2 for 24 hours only; removal of EGF and FGF-2 for 24 hours and addition of either rapamycin (RAPA – 100 nM) for 24 hours or brefeldin A (BFA – 4 μ M) for 3 hours. β -actin was used as a loading control. The molecular mass is indicated in kDa.

Supplementary Figure 5A

E

Supplementary Figure 5: Immunostaining and western blot of intracellular vesicle trafficking components in iNPC.

Colocalization of *TBCK* with: A) Caveolin; B) COPII, C) RAB5A, D) Clathrin; E) STAM; and F) GM130/GOLGA2. DAPI (blue) marks cell nuclei. Scale bar = 10 μ M.

G) Blots of Clathrin and the endogenous control β -actin in iNPC cultured under normal growth conditions show no difference between patients and controls.

Supplementary Figure 6: Fluorescence immunostaining of RAB7A.

A) Graph showing quantification of RAB7A immunofluorescence intensities (arbitrary unit, AU);
B) Representative images showing RAB7A in iNPC. DAPI (blue) marks cell nuclei. Scale bar = 10 μ M.

Supplementary Figure 7: Western blot for autophago-lysosomal markers in iNPC cultured in the absence of EGF/FGF-2 for 24 hours.

A, A') Blots of BECN1, showing two bands (band I and II);
 B, B') Blots of p62/SQSTM1;
 C, C') Blots of cathepsin D, showing cathepsin and pro-cathepsin.
 β -actin was used as a loading control. The molecular mass is indicated in kDa.

Supplementary Figure 8: Analysis of cell cycle phases in iNPC.

Graphs show the percentage of cells in S (A) and G2/M (B) phases of the cell cycle.

The analyses were performed at 3 time points (0, 24 and 48 hours). * p -value < 0.05; *** p -value < 0.0001. Cell cycle experiments were replicated twice. Data are shown as percentages of cells in patient's and controls' biological replicates.

Supplementary Material Table 4: Caspase 3 staining in *TBCK* -mutated NPC.

Sample	Average nuclei	SD_nuclei	average Casp	SD_Casp	P value (t-test)
F6331-1	452,5	45,96194078	4,5	0,707106781	
F7007-1	362	42,42640687	1	1,414213562	0,178178823
F8799-1	329	49,49747468	1	1,414213562	0,12332093
F10006-1	334	1,414213562	3,5	3,535533906	0,17011571

CAPÍTULO 3 – DISCUSSÃO GERAL E CONCLUSÕES

Estudos genômicos vem contribuindo com grandes avanços na área da genética do TEA e com frequência, como no presente trabalho que envolveu o estudo de duas irmãs com forma sindrômica de TEA, para um diagnóstico mais preciso. Ainda, a avaliação clínica das duas irmãs reforça o autismo como mais uma característica clínica associada à síndrome IHPRF3. No entanto, ainda há muito a ser explorado com relação aos mecanismos moleculares e celulares pelos quais tais genes levam a alterações de funcionamento neuronal, fundamentais para o desenvolvimento de melhores estratégias terapêuticas. Um aspecto ainda pouco explorado na pesquisa em TEA é o papel do tráfego intracelular de vesículas em sua patogênese. Dessa forma, selecionamos para um estudo funcional as irmãs diagnosticadas com a síndrome IHPRF3, cujo gene responsável TBCK, tem sido estudado principalmente com relação à regulação da via de sinalização mTOR. No entanto, é cada vez mais evidente que alterações nessa via em indivíduos com IHPRF3 representam um efeito indireto à falta de TBCK (Moreira et al., 2017). Previsto como um regulador de proteínas Rab-GTPases, estudos que comprovam o papel regulatório de TBCK sobre o tráfego de vesículas ainda é escasso, principalmente em células neurais. Dessa forma, propomos investigar, pela primeira vez em iNPC das próprias pacientes, os mecanismos patofisiológicos pelos quais a falta de TBCK funcional leva a alterações de fenótipo neural, focando no papel de TBCK sobre o transporte de vesículas.

Nossa análise de colocalização entre TBCK e componentes da maquinaria do transporte de vesículas reforça um papel regulatório de TBCK, tanto sobre a via secretória inicial, como a via endocítica/endolisossomal. Uma particularidade deste estudo é o fato da proteína TBCK estar expressa em células progenitoras neurais de nossas pacientes, o que nos permitiu comparar os níveis de colocalização com marcadores de compartimentos de ambas as vias em células de pacientes com células controles e propor novos mecanismos celulares e moleculares associados à síndrome.

Neste estudo, iNPC das pacientes não mostraram prejuízos na maturação dos endossomos primários (via endolisossomal), tampouco na manutenção da sinalização mTOR em condições normais de cultura, sugerindo que a via de reciclagem dos receptores dos fatores de crescimento endocitados, de volta à membrana plasmática, também não está comprometida nessas condições. No entanto, avaliar a via endocítica/endolisossomal em

outros pacientes com IHPRF3 é fundamental para confirmar estes resultados. Ainda, assim como o complexo de Golgi, os endossomos primários são compartimentos celulares onde ocorre intenso *sorting* de proteínas, e abrigam diferentes complexos protéicos responsáveis pela seleção de cargos a serem degradados nos lisossomos (maquinaria ESCRT, o qual STAM faz parte), ou para serem reciclados de volta à membrana plasmática, ou ainda para o aparelho de Golgi (Jovic et al., 2010; Naslavsky et al., 2018). Portanto, alterações de localização espaço-temporal de proteínas específicas em diferentes contextos também devem ser exploradas.

De maior destaque, neste trabalho, alterações de colocalização entre TBCK mutante e STAM, GM130/GOLGA2 e clatrina e análises complementares (medição do tamanho de vesículas COPII e de punctas do marcador de autofagossomos, LC3B) nas células das pacientes, são compatíveis com prejuízos na função de TBCK sobre algum componente da maquinaria do transporte de vesículas residente no complexo de Golgi. Prejuízos na atividade de um regulador do transporte de vesículas localizado no Golgi também parecem estar envolvidos na menor ativação de mTOR em iNPC das pacientes. Nesse contexto, diferente da análise de mTOR em dois outros casos de IHPRF3 realizada por Bhoj e colaboradores (2016), nossa estratégia de investigação em diferentes condições de cultura, as quais interferem na ativação de mTOR em diferentes pontos, permitiu uma avaliação mais profunda sobre o potencial mecanismo molecular pelo qual TBCK regula a via de sinalização. Análise dos diferentes perfis de ativação de mTOR em iNPC das pacientes em relação aos controles, somada aos dados da literatura (Jewell et al., 2015; Nguyen et al., 2017; Meng et al., 2020), sugerem a proteína ARF1-GTPase como um dos principais candidatos alvos de TBCK. A ação de TBCK sobre ARF1 pode ser direta (pelo menos 2 exemplos descrevendo a atuação de proteínas TBC sobre proteínas da família Arf já foram descritos na literatura, incluindo a interação de Arf6 com TBC1D24 e TRE-17 - Martinu et al., 2004; Falace et al., 2010). Ação sobre ARF1 também pode ser indireta, possivelmente via RAB1A (Dumaresq-Doiron et al., 2010), a qual também foi sugerida como outro potencial candidato alvo de TBCK pelas análises de ativação da via mTOR. Assim como ARF1, RAB1A atua no transporte da via secretória, mais especificamente entre o RE e Golgi. Na face *cis*, RAB1 interage com diversas proteínas *tethering*, como p115, GM130/GOLGA2 e GRASP65, favorecendo o reconhecimento e fusão de vesículas derivadas do RE, assim como a manutenção da integridade do Golgi (Goud et al., 2018; Sastre et al., 2021). Mais recentemente, Cavieres e colaboradores (2020) mostraram que RAB1-GTP também interage

com uma proteína da face *trans*-Golgi, GOLPH3, no entanto, o papel biológico dessa interação ainda não foi definido.

RAB1A ativa ARF1 por meio do recrutamento de sua proteína GEF, GBF1 (Dumaresq-Doiron et al., 2010; Sastre et al., 2021). Assim como as proteínas Rab-GTPases, proteínas da família Arf também atuam por meio da ciclagem entre a forma ativa, ligada a GTP, e a forma inativa ligada a GDP. ARF1-GTP ocupa toda a estrutura do complexo de Golgi. Em sua face *cis*, voltada para o RE, ARF1 interage com GMAP-210, uma proteína *tethering* que junto com GM130/GOLGA2 garante o reconhecimento e facilita a fusão das vesículas derivadas do retículo à membrana do Golgi (Wong & Muno, 2014; Roboti et al., 2015; Magdeleine et al., 2016). Na face *trans*, ARF1-GTP é essencial para o recrutamento das moléculas adaptadoras AP-1, AP-3, AP-4 e GGA1-3 à membrana, onde selecionam seus respectivos cargos para serem transportados, em vesículas revestidas de clatrina, até os endossomos, lisossomos ou à membrana plasmática (Sauvageau et al., 2017; Jackson, 2018; Tan & Gleeson, 2019). Além da regulação do transporte de vesículas, ARF1-GTP também se liga a Golgin-160/GOLGA3 na face *cis*. Golgin-160/GOLGA3 por sua vez, se liga à proteína motora dineína, que se move em direção à extremidade menos dos microtúbulos. Dessa forma, além de contribuir para a manutenção da estrutura do Golgi, a interação com Golgin-160/GOLGA3 também é fundamental para o posicionamento do Golgi próximo ao centróssomo (o centro organizador de microtúbulos), o qual confere polaridade intracelular fundamental para a migração celular (Yadav et al., 2012; Jackson et al., 2018). A dissociação de Golgin-160/GOLGA3 da membrana do Golgi por meio da inativação de ARF1, leva à fragmentação do Golgi, uma etapa essencial para a fase mitótica do ciclo celular (Yadav et al., 2012). Falha na redução dos níveis de ARF1 ativo na transição entre as fases G2/M, impede a dissociação de Golgin-160/GOLGA3 e a fragmentação do Golgi, e consequentemente leva ao bloqueio da divisão celular (Yadav et al., 2012; Valente & Colanzi, 2015; Jackson, 2018). Embora a importância de ARF1-GTP durante a intérfase não seja clara, estudos mostram que ao fim da mitose, as atividades de ARF1 e sua GEF são alvos de regulação (Altan-Bonnet et al., 2003; Altan-Bonnet et al., 2006; Magliozzi et al., 2018), possivelmente para a recomposição do complexo de Golgi e início de novo ciclo celular.

Tanto ARF1 como RAB1A interagem com a proteína C9ORF72, associada à doença neurodegenerativa de herança autossômica dominante, Demência frontotemporal e/ou esclerose lateral amiotrófica (MIM#105550). C9ORF72 foi implicada na iniciação da formação

dos autofagossomos (Webster et al., 2016; Yang et al., 2016). Nesse contexto, foi demonstrado que C9ORF72 se liga a RAB1A-GTP para regular o transporte do complexo ULK1, central para a indução da autofagia, à membrana onde se dará a formação dos autofagossomos (Webster et al., 2016). Já a relevância da interação com ARF1 para a autofagia não é clara, uma vez que C9ORF72 nesse caso, parece atuar em conjunto com SMCR8, para inativar ARF1 (Su et al., 2021).

Assim sendo, este estudo cumpriu com os objetivos propostos:

- a.** Analisar o perfil de ativação da via de sinalização mTOR:
 - a’.** Sinalização reduzida somente em condições específicas de estresse nas pacientes confirma alteração da via em célula neural em pacientes com a síndrome IHPRF3, e sugere uma regulação indireta de TBCK sobre a via mTOR: dependente de proteína G, a qual atua em compartimentos da rota secretória inicial (entre retículo endoplasmático e Golgi). Tanto ARF1 como RAB1A GTPases representam potenciais moléculas intermediárias para a atuação de TBCK sobre a via;
- b.** Caracterizar a localização intracelular de TBCK:
 - b’.** TBCK em iNPC está localizada tanto em compartimento da rota endossomal (RAB5⁺), como secretória (vesículas COPII⁺);
- c.** Verificar se iNPC das pacientes apresentam comprometimento das rotas endolisossomal e secretória:
 - c’.** Nossos dados indicam que a falta de TBCK funcional em iNPC compromete a dinâmica da via secretória inicial, com implicações tanto no transporte a partir do Golgi, como na formação de autofagossomos. Não observamos alteração na rota endolisossomal nas condições investigadas, mas novas análises devem ser realizadas para confirmar esses resultados.
- d.** Avaliar as implicações funcionais das variantes de perda de função em *TBCK* no contexto do neurodesenvolvimento:
 - d’.** iNPC das pacientes apresentam alterações de proliferação e migração celular, assim como no fluxo autofágico, processos esses altamente dependentes da maquinaria do tráfego intracelular de vesículas.

Com este trabalho, novos questionamentos foram levantados e suas respostas poderão contribuir para um melhor esclarecimento da síndrome, bem como para o desenvolvimento de estratégias terapêuticas direcionadas à base molecular da doença.

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ANEXO

Article

A novel saliva RT-LAMP workflow for rapid identification of COVID-19 cases and restraining viral spread

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ABSTRACT: Rapid diagnostics is pivotal to curb SARS-CoV-2 transmission, and saliva has emerged as a practical alternative to naso/oropharyngeal (NOP) specimens. We aimed to develop a direct RT-LAMP (reverse transcription loop-mediated isothermal amplification) workflow for viral detection in saliva, and to provide more information regarding its potential in curbing COVID-19 transmission. Clinical and contrived specimens were used to optimize formulations and sample processing protocols. Salivary viral load was determined in symptomatic patients to evaluate clinical performance of the test and to characterize saliva based on age, gender and time from onset of symptoms. Our workflow achieved an overall sensitivity of 77.2% (n = 90), with 93.2% sensitivity, 97% specificity, and 0.895 Kappa for specimens containing >10² copies/μL (n = 77). Further analyses in saliva showed that viral load peaks in the first days of symptoms and decreases afterwards, and that viral load is ~10 times lower in females compared to males, and declines following symptom onset. NOP RT-PCR data did not yield relevant associations. This work suggests that saliva better reflects transmission dynamics than NOP specimens, and reveals gender differences that may reflect higher transmission by males. This saliva RT-LAMP workflow can be applied to track viral spread and, to maximize detection, testing should be done immediately after symptoms are presented, especially in females.

Keywords: LAMP assay; 2019 Novel Coronavirus; saliva; viral diagnostics

INTRODUCTION

Molecular diagnostics of the novel coronavirus (SARS-CoV-2) amidst the COVID-19 pandemic has been crucial to monitor infection dynamics and prevent spread of the disease. The gold standard has been Real-Time Polymerase Chain Reaction (RT-PCR), which is performed on nasopharyngeal and oropharyngeal (NOP) specimens that pose discomfort to patients and require specialized materials and trained healthcare professionals for collection. In addition, RT-PCR typically requires viral inactivation followed by a lengthy RNA extraction/isolation step, further complicating diagnostic

workflows and increasing turnaround time for reporting results. Faster and simplified protocols for viral detection are desirable to curb transmission, especially in point-of-care settings and places that lack infrastructure, access to material, or financial means. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) has emerged as a viable, affordable alternative to RT-PCR, since it allows rapid and direct detection of pathogens without nucleic acid extraction and sophisticated equipment (1).

Although SARS-CoV-2 is transmitted via infected saliva droplets and aerosols, attention has been focused on the upper airway tract rather than the oral cavity. Recently, viral shedding has been observed in the salivary glands and oral mucosa, further implicating saliva in infection and transmission (2). Saliva has been considered a suitable alternative specimen for COVID-19 molecular diagnostics and to monitor viral spread, as it is easily accessible and can be collected by unsupervised patients into simple airtight vessels, diminishing costs and risk of transmission (3). Implementation of salivary diagnostics must account for some issues, such as poor understanding of the viral biology in the oral cavity and viral load dynamics across individuals, which is important to determine the optimal test window to detect SARS-CoV-2. Furthermore, development of robust protocols for viral detection in saliva may be useful for diagnosis of other respiratory pathogens.

Here, we describe a novel workflow for RT-LAMP-based detection of SARS-CoV-2 that includes a stabilization solution to prepare saliva specimens without RNA isolation. This workflow stabilizes viral RNA, allows sample manipulation without biosafety rooms and cabins, and shows 93.2% sensitivity for viral loads above 10^2 μ L of saliva, 97% specificity, and 0.895 Kappa coefficient. We also provide insights into viral load differences between saliva and NOP swab specimens and relate them to gender, age, and time from onset of symptoms, further elucidating the diagnostic capabilities of saliva and bringing forth recommendations to maximize chances of detection. This rapid and efficient workflow is suitable for COVID-19 diagnostics in both centralized or point-of-care settings, and may be of particular value for use in places lacking sophisticated infrastructure.

METHODS

Subjects

This project was approved by the Ethics Committee of Instituto de Biociências, Universidade de São Paulo, Brazil (accession number 31655320.0.0000.5464), and involved the collaboration with several groups in order to obtain anonymized clinical samples from individuals with respiratory symptoms.

Crude saliva samples from 26 symptomatic individuals were collected in August 2020 by two clinical laboratories (Instituto de Ensino e Pesquisa Prevent Senior and Grupo Fleury) and one research group (Instituto de Medicina Tropical, Universidade de São Paulo - IMT-USP), and sent to our laboratory in ice on the same day. These individuals have been tested positive for SARS-CoV-2 via RT-PCR on nasopharyngeal/oropharyngeal (NOP) specimens by those institutions, and cycle threshold (Ct) values were shared whenever necessary.

Crude saliva and NOP samples from 131 symptomatic individuals were collected in January-February 2021 at Universidade Municipal de São Caetano do Sul/IMT-USP (4) (51 individuals for characterization of the diagnostic yield of saliva compared to NOP swabs - 29 females and 22 males - and 80 for the blind study - 48 females and 32 males). Saliva samples were aliquoted locally and transported to our laboratory in dry ice, while NOP specimens were tested for SARS-CoV-2 by RT-PCR in each institution, which shared Ct results and clinical data whenever necessary.

Saliva collection

Briefly, individuals were asked not to eat for at least 30 minutes before collection of 3 mL of saliva in sterile, nuclease-free 15 mL conical tubes. Immediately post-collection, saliva was heat-inactivated by incubation at 95°C.

DGS preparation

We developed a solution (detailed information is described in the 'Results' section), named as 'DGS' (DTT/GuHCl solution), that prevents degradation of viral RNA and provides a better stabilization of the viral genome for RT-LAMP reactions. DGS contains 30 mM Tris-HCl pH 8.0, 600 mM GuHCl and 200 mM DTT, diluted in nuclease-free ddH₂O. Inactivated saliva samples are mixed with this solution and incubated at 55°C for 5 minutes prior to RT-LAMP reactions.

RT-LAMP and rtRT-LAMP reactions

RT-LAMP reactions (12.5 µL total volume) contained 1x WarmStart® Colorimetric LAMP Master Mix (New England Biolabs, #M1800L), a primer set composed of 1.6 µM FIP/BIP internal primers, 0.4 µM LF/LB loop primers and 0.2 µM F3/B3 external primers, and 1.25 µL of DGS:saliva mixture in 1:1 ratio, previously centrifuged at 1,000 × g for 30 seconds. Previously published primer sets targeting different regions of SARS-CoV-2 genome and the human gene *ACTB* were used (Table S1). All primer sets targeting SARS-CoV-2 have already been tested for specificity against other respiratory pathogens (5–9). RT-LAMP reactions were carried out at 65°C for 30 to 40 minutes.

For real time analysis of RT-LAMP (rtRT-LAMP), the above reaction was supplemented with 1 µM SYTO®-9 DNA binding dye (Thermo Fisher, #S34854) and 0.125 µL Low ROX reference dye (New England Biolabs, #E7638A), and incubated in a QuantStudio 5 qPCR machine (ThermoFisher) at 65°C for 30 or 40 minutes (fluorescence signal acquisition at 15-second intervals), followed by a dissociation curve stage from 95°C to 60°C with temperature change rate of 0.1°C/second.

To improve color discrimination, the reaction protocols above was further adjusted to contain 1.32x WarmStart® Colorimetric LAMP Master Mix and 1 µL of DGS:saliva mixture in 2:1 ratio. Time to threshold (Tt) of positivity was defined at threshold = 0.8 relative fluorescence units. Each batch of reactions included positive controls with 1000 and 500 copies of SARS-CoV-2 RNA per reaction. Analysis of amplification plots and melting temperatures (Tm) were used to discriminate non-specific from specific amplifications (specific Tm = median Tm_{positive control} ± 1°C).

RNA isolation and RT-PCR reactions on saliva samples

SARS-CoV-2 RNA was isolated from crude saliva samples using QIAamp Viral RNA Mini Kit (QIAGEN, #52906), following manufacturer's recommendations. RT-PCR was performed based on CDC's protocol, which targets SARS-CoV-2 gene N (Centre for Disease Control and Prevention, 2020). RT-PCR reactions (12 µL total volume) contained TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher, #4444432) and a primer/probe mix (500 nM forward primer, 500 nM reverse primer, 125 nM probe; 2019-nCoV_N1, IDT #10006600), and were cycled in a QuantStudio 5 qPCR machine as per manufacturer's recommendations (Thermo Fisher). Absolute quantification of viral RNA copies was performed via standard curve assays with 2019-nCoV_N_Positive Control (IDT, #10006625), in triplicates.

Simulation of saliva positive controls with SARS-CoV-2 RNA

SARS-CoV-2 RNA was isolated from cell culture pellets (kindly provided by collaborators from Instituto de Ciências Biomédicas, USP), and viral titer was determined via absolute quantification with RT-PCR, as described above. Quantified SARS-CoV-2 RNA was spiked into saliva from a healthy donor (previously processed with 2:1 ratio of

DGS:saliva and tested negative for SARS-CoV-2) to produce simulated specimens, and then stored in 10- μ L aliquots at -80°C for further use in RT-LAMP reactions.

Statistical Analyses

D'Agostino-Pearson normality test was applied to evaluate data distribution and to select appropriate statistical analyses to compare groups. Graphing and analyses were carried out with Graphpad Prism. Statistical significance was set at $p < 0.05$.

RESULTS

Stabilization of SARS-CoV-2 RNA in saliva

Searching for a solution capable of stabilizing SARS-CoV-2 RNA in saliva, we initially screened 6 formulations containing Proteinase K (namely PK1-6). AVL, a commercial guanidine-based buffer recommended for SARS-CoV-2 inactivation (10), was used as the experimental control, without any heat treatment. Since unprotected viral RNA is rapidly degraded in crude saliva, we simulated samples by first mixing saliva from a healthy donor with the solutions (1:1, v/v) and heating at 65°C for 15 minutes followed by a step at 95°C for 2 minutes, before spiking in SARS-CoV-2 RNA. After sample processing, RNA was isolated and RT-PCR targeting the N gene was performed. We observed that PK6 led to similar Ct values to AVL (23.9 and 23.15, respectively), while no amplification was detected for the remaining PK formulations. Notably, PK6 and AVL were the only solutions that contained guanidine hydrochloride (GuHCl), suggesting that GuHCl is important for stabilizing viral RNA. PK6 was composed of 800 mM GuHCl, 400 μ g/mL PK, 10% Tween 20 (T20) and 30 mM Tris-HCl pH 8.0 (Table 1).

Table 1. Solutions used in the initial screening and Ct values after processing.

	Composition	Ct
AVL	Guanidinium thiocyanate (50-70%)	23.15
PK1	PK (2 mg/mL)	ND
PK2	PK (0.4 mg/mL)	ND
PK3	PK (0.4 mg/mL) + T20 (10%)	ND
PK4	PK (0.4 mg/mL) + T20 (10%) + Tris-HCl pH 8.0 (30 mM)	ND
PK5	PK (0.4 mg/mL) + Tris-HCl pH 8.0 (30 mM)	ND
PK6	PK (0.4 mg/mL) + T20 (10%) + GuHCl (800 mM) + Tris-HCl pH 8.0 (30 mM)	23.9

PK: Proteinase K; T20: Tween 20; GuHCl: guanidine hydrochloride; ND: not detected.

Aiming towards a minimal solution that enables RNA stabilization, several modifications were made to the PK6 formula to reduce the amount of components. The removal of T20 and PK increased the Ct value in relation to AVL (Δ Ct = 1.433), while no amplification was detected when substituting GuHCl with PK at a higher concentration (4 mg/mL), RNase OUT (11) or varying amounts of DTT (see below), further indicating that GuHCl is necessary to stabilize SARS-CoV-2 RNA in saliva (Fig. 1A). By varying the amount of GuHCl in PK6, we observed that viral RNA could be detected only in concentrations above 400 mM, and that 800 mM was sufficient for RNA stabilization (Δ Ct $\leq$ 0.104) in the presence of T20 and PK (Fig. 1B). PK and T20 were then replaced with varying amounts of DTT (50 mM to 200 mM) in combination with lower concentrations of GuHCl (400 mM and 600 mM). Only the combination of 600 mM GuHCl and 200 mM DTT resulted in a negligible Ct increment in comparison to AVL (Δ Ct = 0.148) (Fig. 1C). Furthermore, saliva samples processed with this formulation presented the smallest Ct rise after storage at 8°C for 24 hours (Δ Ct = 0.428) (Fig. 1D). Together, these results indicate that heating saliva in this solution (30 mM Tris-HCl pH 8.0, 600 mM GuHCl, 200 mM DTT)

at 65°C/15 minutes plus 95°C/2 minutes protects viral RNA from degradation. This DTT/GuHCl solution is hereafter referred to as ‘DGS’.

Figure 1. Modifications to the PK6 formula. A) Removal of PK and T20 increases Ct value, while substituting GuHCl with PK (4mg/mL), RNase OUT or DTT results in no amplification. B) 800 mM GuHCl is sufficient to stabilize RNA in the presence of PK and T20. (*) The solution containing RNase OUT was buffered by Tris-EDTA pH 8.0 instead of Tris-HCl (6). C, D) Optimization of the stabilization solution. C) RNA stabilization is achieved by replacing PK and T20 with 200 mM DTT and reducing GuHCl to 600 mM, and (D) this is maintained after storage of processed specimens for 24 hours at 8°C. n.d. = not detected.

Compatibility of DGS with direct RT-LAMP in saliva

Next, we examined whether DGS is compatible with direct RT-LAMP reactions. For this, we used saliva specimens from 3 individuals confirmed positive for COVID-19 via NOP swab RT-PCR by an external laboratory. In this test, specimens were heated with DGS under a slightly different protocol (55°C/15min followed by 95°C/2min) modified from previous work (8). RT-LAMP was performed with the previously reported primer set N (Table S1) (9) and 10% volume of DGS:saliva (1:1) mix, resulting in a 20-fold dilution of the DGS formulation (Table 2). These three samples amplified specifically and changed color from pink to yellow within 30-40 minutes of reaction at 65°C, while the no-template controls (NTCs) remained pink (Fig. 2A), showing that DGS is compatible with colorimetric readouts.

Table 2. DGS constituents and carryover into RT-LAMP.

Component	DGS	DGS:saliva (1:1)	Carryover into RT-LAMP (1.25 µL input)
GuHCl	600 mM	300 mM	30 mM
DTT	200 mM	100 mM	10 mM
Tris-HCl pH 8.0	30 mM	15 mM	1.5 mM
Saliva	-	100 µL	0.625 µL
Total volume	100 µL	200 µL	12.5 µL

To characterize the effects of the DGS heat treatment on RT-LAMP, we performed direct real-time RT-LAMP (rtRT-LAMP) with additional three published primer sets targeting different regions of the SARS-CoV-2 genome (orf1ab, N1, E ;Table S1) (9,12,13). Standard curves were made with serially diluted RNA spiked into DGS-processed saliva or H₂O.. Compared to RNA in H₂O, DGS-simulated saliva showed higher reaction speed and amplification efficiency for all primer sets, revealed by reduction in time to threshold (Tt) and doubling time (DT) values, respectively (Fig. 2B). DGS processing also enabled formation of specific LAMP products and color change to yellow, in opposition to nonspecific and failed amplifications, which remained pink (Fig. 2C) and were clearly distinguishable from specific amplifications by dissociation analysis or agarose gel electrophoresis (Fig. D, D'). Altogether, these results show that sample processing with DGS improves direct RT-LAMP reactions and allows SARS-CoV-2 detection either via endpoint analysis of color output or analysis of amplification and melting curves in rtRT-LAMP.

Figure 2. Effect of DGS heating on direct RT-LAMP reactions. A) Compatibility between direct RT-LAMP and DGS-processed saliva. After 40-minute incubation at 65°C, colors were visibly distinguishable between the pink NTC and the yellow positive samples. Agarose gel electrophoresis confirmed specific amplification, as band patterns of individuals 13717, 13713 and 13813 matched those of the positive control (10⁴ RNA copies). Nonspecific amplification was observed in NTC after longer incubation (up to 60 minutes). B–F) Compatibility with direct rRT-LAMP. B) SARS-CoV-2 RNA serially spiked in DGS-simulated saliva or in H₂O was used to assemble standard curves for primer sets orf1ab, N, N1 and E, in duplicate reactions. Nonspecific and failed amplifications were observed at 0–120 copies/reaction in some reactions (not shown). Doubling time (DT) values were calculated to assess amplification speed/efficiency (14) for each primer set. Determination coefficients (r²) point to high linearity (>0.939) between RNA input and T_t in DGS-simulated saliva, except for primer set N (r² = 0.8488). C) Representative color output after rRT-LAMP (primer set E is shown). D) Representative dissociation analysis and gel electrophoresis showing non-specific LAMP products at 0 and 12 copies/reaction (primer set E is shown). NTC (no-template control) reactions were performed with H₂O.

Optimization of the DGS workflow

The aforementioned protocols require mixing infected patient samples with DGS before viral inactivation, demanding high biosafety requirements. To overcome this issue, we sought to optimize sample processing to include a heat inactivation step before collection tubes were uncapped. We initially tested three saliva specimens heat-inactivated at 95°C/20 minutes (protocol A) in the absence of DGS (no-DGS control), resulting in failed amplifications across reaction replicates in rRT-LAMP. In contrast, subjecting these heat-inactivated specimens to a second heating step with DGS at 55°C/15 minutes (protocol B) improves detection, leading to specific amplification in all replicates. This was also observed when specimens were mixed with DGS and single-heated at 95°C/20 minutes (protocol C) or under the original protocol (55°C/15 minutes; 95°C/2

minutes; protocol D), suggesting that 2-step heating (protocol B) does not overly affect stabilization of SARS-CoV-2 RNA in saliva (Fig. 3A).

We further characterized DGS protocols B, C and D in comparison to the no-DGS control (protocol A). Three saliva specimens were processed under these protocols in two parallel experiments. rtRT-LAMP showed no differences in T_t values across protocols B, C, and D. However, we observed less specific amplification events under protocol A (4 out of 6 replicates), while protocols B, C and D achieved specific detection in all replicates, except for one under protocol D (Fig. 3B). Similarly, DGS protocols B, C and D enabled detection in simulated specimens (10^4 , 10^3 and 10^2 copies/reaction) without differences in average T_t values, while no amplification was detected under protocol A, indicating that single heat inactivation in the absence of DGS is insufficient to counteract salivary ribonuclease activity (Fig. 3C). To further compare RNA stabilization across protocols, the processed specimens (Fig. 3B) were then incubated at 8°C or 30°C for up to 48 hours. After 24 and 48 hours of incubation at either temperature, the number of specific amplifications under protocol A further decreased, while greater detection rates (≥ 5 out of 6 replicates) were maintained with DGS protocols B, C and D (Fig. 3D). Moreover, these 3 DGS protocols showed no appreciable shifts in average T_t values after 24 hours at 8°C (Fig. 3E), while at 30°C, this was only observed for protocol B (Fig. 3E'). Together, these results suggest that the 2-step protocol B is suitable to process clinical specimens, as it improves detection of SARS-CoV-2 compared to the no-DGS controls, and shows the most robust RNA stabilization effects.

To further assess its performance in rtRT-LAMP, protocol B was applied to 11 additional clinical samples and compared to single heat inactivation without DGS (protocol A). rtRT-LAMP was performed in duplicates with primer set N1 and the human-specific primer set *ACTB* (Table S1) (13). In both methods, specific amplifications for *ACTB* were observed throughout all replicates, while specific amplifications for N1 were observed in at least 1 out of 2 replicates in 9 samples. Differences in reaction speed (ΔT_t) were calculated as average $T_{t\text{Protocol B}} - T_{t\text{Protocol A}}$. In 4 of these 9 samples, an important reduction in T_t values was observed for protocol B, varying from 3.5 to 9 minutes, while *ACTB* showed no significant T_t fluctuations (Fig. 3F). This indicates that protocol B improves detection rates (Fig. 3A) and reaction speed (Fig. 3F) in clinical saliva, and suggests that the latter effect may be specific to SARS-CoV-2 detection, as *ACTB* showed low ΔT_t variability. Finally, viral RNA was extracted from these 11 specimens and RT-PCR C_t values were used to estimate viral load. We observed that SARS-CoV-2 RNA was detected in the majority of specimens with $C_t < 30$ (8/8), suggesting robustness for high viral loads (Fig. 3G).

Figure 3. Assessment of different DGS heating protocols via rtRT-LAMP. A) rtRT-LAMP results for clinical specimens heat-inactivated (no-DGS control) or processed under 4 DGS protocols (Pt.A-D). Reactions were performed in different days. B) rtRT-LAMP results for clinical specimens after protocols A-D, in two parallel experiments. Data points above the dashed line are nonspecific amplifications. rtRT-LAMP was performed in a single reaction plate. C) Reaction output of simulated samples spiked with SARS-CoV-2 RNA (10^2 to 10^4 copies/reaction). RNAs spiked in H₂O were used as positive controls. Lines indicate mean Tt values. D) Detection rate of the 3 DGS-processed clinical specimens incubated for 48 hours at 8°C and 30°C ($n = 6$). E, E') rtRT-LAMP results after storage at 8°C (E) and 30°C (E'). Data points above the dashed line are nonspecific amplifications. Results shown in D/E/E' were plotted with data from B ($t = 0$ hours). F-G) Assessment of protocol B in 11 additional specimens via rtRT-LAMP for primer sets N1 and ACTB. F) Changes in reaction speed of protocol B compared to A were plotted as ΔTt values; negative values indicate gain in reaction speed. Nonspecific and failed amplifications were not included. G) Saliva RT-PCR Ct values were plotted against Tt values used in F. Experiments in A-E were performed with $n = 3$ biological samples (P220031, P220032, and P220041 or P220051). All rtRT-LAMP reactions were carried out in duplicates. Since GuHCl was recently shown to improve speed and sensitivity of RT-LAMP (13), all rtRT-LAMP performed on no-DGS controls (protocol A) were supplemented with 40 mM GuHCl to allow more precise evaluation of the DGS protocols.

Optimization of color discrimination and sample processing time

We observed that up to 15% of specimens showed discordant color output and amplification results after RT-LAMP followed by agarose gel electrophoresis (data not shown). This could be explained by pH variation of saliva or excess of salivary inhibitors impairing amplification efficiency. To ensure appropriate color-based analysis, volumetric adjustments were implemented in the DGS and RT-LAMP protocols. The DGS:saliva ratio was increased to 2:1 to enhance inactivation of salivary nucleases by DTT/GuHCl, while sample input into RT-LAMP reactions decreased from 1.25 μ L to 1 μ L and the volume of Colorimetric Master Mix increased from 6.25 μ L to 8.25 μ L (Table 3).

These modifications resulted in better color discrimination after RT-LAMP, as color change and viral RNA amplification showed no discrepancies (Fig. 4A). These adjustments were associated with a >95% limit of detection at 750 viral copies/ μ L in simulated specimens (Fig. 4B).

To accelerate sample processing, we tested saliva samples confirmed positive via RT-PCR, as better detailed in the next item. We simultaneously processed 29 positive specimens with the previously devised protocol (heat inactivation for 20 minutes at 95°C and DGS stabilization for 15 minutes at 55°C) and under shorter processing times (5 minutes at 95°C and 5 minutes at 55°C). Here, primer set N1 was replaced by E1, which is more sensitive (15) (Table S1). Shortening both heat inactivation and DGS stabilization to 5 minutes led to the largest improvement in detection rate, enabling detection in 100% (14/14) of samples containing over 10^3 viral copies/ μ L and 67% (10/15) of samples below that cutoff, a 10-fold improvement over the unmodified protocol (Fig. 4C). These results show that reducing sample processing time improves direct RT-LAMP sensitivity, particularly for low viral loads in saliva.

Table 3. DGS constituents and carryover into RT-LAMP after volumetric adjustments.

Component	DGS	DGS:saliva (2:1)	Carryover into RT-LAMP (1 μ L input)
GuHCl	600 mM	400 mM	32 mM
DTT	200 mM	133.3 mM	10.66 mM
Tris-HCl pH 8.0	30 mM	20 mM	1.6 mM
Saliva	-	100 μ L	0.33 μ L
Total volume	200 μ L	300 μ L	12.5 μ L

To assess conservation of diagnostic properties, 8 of these DGS:saliva mixtures were stored at -80°C, thawed and analyzed via direct rtRT-LAMP, without consistent increments in Tt values. Considering that the processed specimens were freeze-thawed twice before this test, this suggests that DGS-processed saliva withstand repeated freeze-thaw cycles (Fig. 4D). Following this, DGS:saliva mixtures were incubated at 8°C and 30°C for 36 hours. At 8°C, no consistent Tt increments were observed, while at 30°C, all samples showed increased Tt values, and one returned negative, suggesting that refrigeration is necessary to maintain diagnostic properties of specimens after processing (Fig. 4D').

Figure 4. Direct rtRT-LAMP output after volumetric adjustments and reduction of processing time. A, B) Output after volumetric adjustments. A) Representative color output before and after rtRT-LAMP performed on 4 clinical specimens and on 3000 and 1500 simulated viral copies/μL. B) Sensitivity using simulated specimens (20 replicates each). No-template control (NTC) reactions had H₂O as input. C) Reduction of heat inactivation (HI) and DGS incubation times with respective rtRT-LAMP readout and sensitivity (stratified at 10³ copies/μL; dashed line). D, D') Before-after plots for 8 specimens processed with HI for 5 minutes and DGS for 5 minutes. D) rtRT-LAMP Tt profiles after 1 freeze-thaw cycle. D') Tt profiles after storage for 36 hours at 8°C and 30°C. cp/μL = viral copies per μL of saliva.

Characterization of the diagnostic properties of saliva compared to NOP swabs

To evaluate the diagnostic performance of saliva, we analyzed 51 saliva samples collected concomitantly with NOP swabs from symptomatic individuals upon hospital admission. These cases were confirmed by an external laboratory via NOP RT-PCR targeting genes N and RdRp. We quantified viral copies in the paired saliva specimens via RT-PCR for the N gene, wherein 48 specimens returned positive (94% agreement). These samples showed viral loads between 1.6 and 10⁷ copies/μL of saliva (0.2–7 log₁₀ copies/μL; mean = 3.51, SD = 1.51) (Fig. 5A; Table S2). No correlation between NOP swab Ct values and viral copies in saliva was observed (Fig. 5B). On average, Ct values in saliva were lower compared to NOP swabs, indicating higher viral load in saliva (Fig. 5C).

Next, we analyzed viral load stratified by gender, time from onset of symptoms, and age (Table S2). These clinical data were available for 49 of the 51 individuals. Interestingly, males showed lower mean Ct values in saliva compared to NOP swabs, which was not observed for females (Fig. 5C'). Furthermore, males showed significantly higher viral loads in saliva than females (mean difference = 1.03 log₁₀ copies/μL; p = 0.032, Student's t-test) (Fig. 5D, D'), while no gender differences were found for NOP Ct values. In both male and female saliva, viral load peaked during the initial 2 days of symptoms and was

negatively correlated with time (range = 2 to 10 days from onset of symptoms) (Fig. 5E). Linear regression analysis showed that salivary viral load falls below 10^3 copies/ μ L at around 5 days after symptom onset ($x = 4.92$ days [95%CI: 3.92 - 6.86]), which in females occurs earlier than in males ($x_{\text{females}} = 3.74$ days [95%CI: 2.42 - 5.24]; $x_{\text{males}} = 6.35$ days [95%CI: 4.71 - 15.38]) (Fig. 5F). Further, viral load in saliva was positively correlated with age in females, but not in males (range = 14-88 years) (Fig. 5G). Notably, among younger patients (aged 14-38), females showed lower viral load than males, while no differences were observed in older individuals (aged 42-88), suggesting that the observed gender differences in salivary viral load decrease with age (Fig 5G'). In NOP swabs, no relationship was detected between viral load (Ct values) and age, gender or days since symptom onset (Fig. 5H-K). Together, these results show that viral load in saliva peaks in the early days of symptoms and is depleted with time, with females displaying lower viral load compared to males.

Figure 5. Analysis of saliva as a diagnostic specimen for SARS-CoV-2 detection via direct RT-LAMP. A) Distribution of viral load in saliva from 51 COVID-19 patients. B) Pearson's correlation between viral load in saliva (gene N) and Ct values of NOP swab specimens (genes N and RdRp). C) Comparison of mean Ct values between saliva and NOP swab specimens; and C') the same analysis was performed for each gender. One-way ANOVA with Dunnet's post-tests; * $p < 0.05$; ** $p < 0.001$. D) Cumulative frequency distribution and D') comparison of mean viral loads between males and females; Student's t-test. E) Pearson's correlation between salivary viral load and days since onset of symptoms and F) the same analysis stratified by gender. Dashed lines or gray shading indicate 95% confidence intervals of the linear trends for significant correlations. G) Pearson's correlation between viral load and age for each gender. G') Comparison of mean viral loads between genders for younger (aged 14–38) and older (aged 42–88) individuals; Two-way ANOVA with Bonferroni post-tests. H–K') Comparisons performed for saliva (D', E, F, G) were applied to NOP swabs using Ct values from RT-PCR targeting N and RdRp (H, I, J/J', K/K').

Direct RT-LAMP in 80 saliva samples from symptomatic individuals

Without prior knowledge of NOP RT-PCR results, we tested 80 additional saliva samples from symptomatic individuals collected concomitantly with NOP swabs. Specimens were processed with the established DGS protocol. Direct rtRT-LAMP was carried out with primer sets N1, E1 and As1e, which was also reported to be more sensitive than N1 (Table S1) (15), in addition to the human-specific primer set *ACTB*. All specimens showed successful amplification for *ACTB*, confirming adequate sample quality (data not shown). In preliminary analyses, we observed that E1 was more sensitive than N1 and As1e (data not shown), so reactions using E1 were performed in duplicates to increase sensitivity and to minimize false negatives. Results were considered positive upon detection in at least one of them, while single reactions were carried out with N1 and As1e. Nonspecific and failed amplifications were considered negative. RT-PCR in NOP specimens reported 51 positives (NOP⁺) and 29 negatives (NOP⁻).

Among the 51 NOP⁺ individuals, saliva rtRT-LAMP returned positive in 40 with primer set E1, 35 with primer set As1e, and 31 with primer set N1, which was excluded from subsequent analyses due to reduced sensitivity and excessive nonspecific amplifications (Fig. 6A). Joint analysis considering either E1 or As1e resulted in 41 positives and 10 negatives. RT-PCR on saliva from these 10 discordant NOP⁺ individuals revealed either viral loads below 10^2 copies/ μ L or absence of SARS-CoV-2 (Fig. 6B).

Among the 29 NOP⁻ individuals, joint E1/As1e analysis returned 4 positive saliva specimens, which were confirmed in an independent rtRT-LAMP assay (data not shown). Of note, RT-PCR on saliva from these NOP⁻ individuals detected SARS-CoV-2 at $>10^2$ copies/ μ L in 3 of the 4 E1/As1e positives (~6%; 3/51). The remaining patients, negative in both NOP RT-PCR and saliva rtRT-LAMP, showed either undetectable (23/25) or low salivary viral load ($<10^1$ copies/ μ L; 2/25) (Fig. 6B'). These results further highlight viral load disparities between NOP and saliva specimens, and indicate that a fraction of infected patients escape detection via NOP RT-PCR.

detection via rtRT-LAMP and B') in saliva from the 29 NOP⁻ individuals, including the 4 discordant rtRT-LAMP positives. Undet. = undetermined. C) rtRT-LAMP with primer sets E1 (in duplicates) and As1e for saliva specimens in which viral titer was determined via RT-PCR (shown in B/B' and in Fig. 5). D) Assessment of sensitivity, specificity and Kappa values of rtRT-LAMP considering primer set E1 alone or E1/As1e for specimens containing at least 10² viral copies/μL (dashed line). E) Representative comparisons between visual color interpretation and rtRT-LAMP results (Tt values). Over 97.6% of specimens were correctly classified as positive (P, yellow) or negative (N, pink) for E1 and As1e, and the remaining was classified as inconclusive (I, orange-shaded). F) Gender comparison of average Tt values in saliva from the NOP⁺ cases ascertained in 'A' (n = 41 rtRT-LAMP positives). Results for primer set E1 were used. Mann Whitney test was used to compare medians. F') Gender comparison of Ct values from NOP RT-PCR for genes N and RdRp. Student's t-test was used to compare means. n.s. = not significant. G) Comparison of joint E1/As1e detection rates between genders for 100 saliva specimens ascertained herein via rtRT-LAMP. G') Stratified analysis of detection rates between genders, based on days from onset of symptoms.

Clinical performance of the direct RT-LAMP workflow

To evaluate the diagnostic capabilities of our workflow, we tested a total of 91 saliva specimens in which viral load was herein determined via RT-PCR, comprising 58 specimens confirmed positive and 33 specimens confirmed negative for SARS-CoV-2. These specimens had been processed with DGS and stored at -80°C. rtRT-LAMP reactions were carried out using E1 in duplicates and As1e in single reactions, as previously described. One positive sample failed to amplify *ACTB* likely due to low quality, and was excluded from the analysis (data not shown). rtRT-LAMP correctly identified 38/57 positive specimens with As1e and 44/57 in at least one of the E1 replicates, with the majority of specimens that escaped detection displaying viral loads below 10² copies/μL (Fig. 6C). Notably, since all 38 As1e-positives were covered by E1, joint E1/As1e analysis also returned 44/57 positives, suggesting little benefit from pairing E1 and As1e as presented. Among the 33 saliva specimens negative for SARS-CoV-2, we observed one positive result by rtRT-LAMP. Considering all 90 specimens and the full range of viral loads represented here, this results in 77.2% overall sensitivity and 97% specificity with primer set E1 or joint E1/As1e. Importantly, stratification at 10² copies/μL resulted in 93.2% sensitivity (41/44 positives) and Kappa = 0.895 for viral loads above this cutoff, demonstrating high sensitivity, specificity and reliability of the test (Fig. 6D).

Next, we sought to determine the efficiency of the RT-LAMP color readout upon visual examination. Without prior knowledge of the results, the color output of 131 samples tested herein via rtRT-LAMP was visually classified as positive (yellow), negative (pink) or inconclusive (orange-shaded), for primer sets E1 and As1e. All yellow- and pink-colored reactions were correctly classified as positive and negative, respectively, resulting in 97.6% agreement for primer set E1 (206/211 reactions) and 97.7% agreement for primer set As1e (128/131 reactions). The orange-shaded output of the remaining reactions resulted from nonspecific or specific amplifications mostly with late Tt values in rtRT-LAMP, indicating absence of SARS-CoV-2 or low viral loads, respectively (Fig. 6E). This demonstrates high agreement between endpoint color output and rtRT-LAMP analysis.

Finally, to confirm our findings regarding viral load differences between males and females, we analyzed E1 Tt values from rtRT-LAMP performed on saliva from the above NOP⁺ cases (n = 41 out of 51). Albeit less sensitive than RT-PCR, this approach evidenced lower Tt values in male saliva, indicating higher viral load (Fig. 6F), while no differences were observed between NOP RT-PCR Ct values (Fig. 6F'). Moreover, analysis of rtRT-LAMP results from 100 cases ascertained herein (Table S3) revealed a lower detection rate in women compared to men (79.7% and 89.5%, respectively; Fig. 6G). Stratification based on days from onset of symptoms showed increased detection rate in women tested up to the 3rd day (88.9%), and decreased detection in women tested from the 4th day onward

(74.3%). Compared to females, males consistently show high detection rates up to the 3rd day and beyond (82.4% and 95.3%, respectively). These observations are in line with females showing lower viral load and faster viral clearance in saliva compared to males, which may adversely impact salivary diagnostics (Fig. 6G').

Discussion

In this work, we developed a DTT/GuHCl-based RNA stabilization solution (DGS) and a workflow that enables robust detection of SARS-CoV-2 in saliva via direct RT-LAMP, with either colorimetric or real-time fluorescence readout. We showed that heat treatment of saliva mixed with DGS protects SARS-CoV-2 RNA from degradation, while improving efficiency, reaction speed and detection rates during subsequent analysis by RT-LAMP. We also characterized saliva and NOP swab specimens according to viral load, gender, age, and time from symptom onset, providing more insight into the advantages and limitations of SARS-CoV-2 salivary diagnostics.

In simulated saliva, we observed that heat treatment with PK-based formulations is insufficient to stabilize viral RNA, contrasting previous reports using samples containing SARS-CoV-2 virions (8,16–18). This discrepancy could be explained by the fact that the spiked RNA in our simulated samples is not protected by the nucleocapsid and other structural viral proteins, being readily digested by any active nucleases left after sample processing. This indicates that PK did not sufficiently inactivate salivary nucleases under the conditions examined here. Thus, care must be taken when using PK and other agents to process specimens, especially when combined with additional viral lysis methods, which may expose viral RNA to digestion. Accordingly, although SARS-CoV-2 remains stable in crude saliva (19), diagnostic sensitivity may drop depending on the method employed to inactivate/process samples, such as heat inactivation, inclusion of detergents, and other factors (20,21), making the RNA protection provided by DGS nevertheless critical.

In clinical saliva, DGS heat treatment improves detection of SARS-CoV-2 compared to heating without DGS. The active ingredients in DGS act by reducing and denaturing salivary extracellular ribonucleases and other inhibitors, and may also facilitate access of RT-LAMP enzymes to the SARS-CoV-2 genome through reduction and denaturation of viral proteins. GuHCl was recently reported to improve speed and sensitivity when added to RT-LAMP (13), so carryover of DGS into reactions may cooperate to increase SARS-CoV-2 detection by modulating reaction chemistry as well. Importantly, this carryover is not solely responsible for the rise in detection rates and reaction speed in clinical specimens, because these effects were observed in relation to no-DGS controls in which RT-LAMP was supplemented with GuHCl (40 mM). Thus, DGS improves SARS-CoV-2 detection both via stabilization of the viral genome and increasing RT-LAMP speed/sensitivity. Furthermore, DTT/GuHCl are also mucolytic agents (22), and therefore ameliorate pipetting of viscous specimens.

Of the DGS protocols investigated here, the best overall performance was achieved with a 2-step method that includes heat inactivation of the saliva in the collection vessel before further manipulation (protocol B). This protocol improved direct RT-LAMP speed and detection rates compared to the no-DGS control, and elicited the strongest RNA stabilization effects compared to the other methods we examined. The volumetric adjustments to the DGS:saliva ratio and RT-LAMP mastermix improved endpoint color interpretation, while shortening the heat inactivation and DGS steps to 5 minutes each improved sensitivity for specimens containing less than 10^3 copies/ μ L, likely owing to greater RNA stability. These steps should suffice to inactivate SARS-CoV-2, since complete viral inactivation has been reported for periods as short as 3 minutes at 95°C (23,24). Furthermore, the final sample processing protocol is amenable to repeated freeze-thaw cycles and allows storage at 8°C for up to 36 hours without adversely affecting diagnostic output, which is important if RT-LAMP must be performed or repeated later.

Finally, shortening sample processing further reduced diagnostic turnaround time. This 2-step protocol reduces risk of infection by healthcare specialists and clinicians since the collection vessel remains sealed until heat inactivation of the virus, thus facilitating diagnostic workflows and alleviating biosafety concerns in point-of-care settings and in test sites lacking in sophisticated infrastructure.

After these modifications, RT-LAMP achieved 77.2% overall clinical sensitivity and 97% specificity, with 93.2% sensitivity in saliva containing at least 10^2 viral copies/ μ L (Kappa = 0.895), which is on par or more sensitive compared to most of the direct RT-LAMP approaches reported so far (8,15,25–33). Moreover, the high agreement (>97.6%) observed between color interpretation and specific amplification plots in rtRT-LAMP demonstrates reliability on end-point color readout if real-time analysis is not possible. Although only 23% of specimens containing less than 10^2 viral copies/ μ L were detected by the present method, the gain in exam turnaround time may be desirable at the cost of sensitivity, since lower viral loads are associated with lower transmissibility of SARS-CoV-2 (34). Likewise, it has been proposed that effective COVID-19 surveillance depends on test frequency and turnaround time rather than on test sensitivity (35), especially for identifying nonsymptomatic carriers. Therefore, periodic testing using a faster, cheaper and noninvasive saliva protocol may be preferable to more lengthy and costly methods, such as standard RT-PCR workflows. Still, if test sensitivity poses an issue in SARS-CoV-2 detection efficiency, this may be mitigated if subjects are tested when salivary viral load is highest, as discussed below.

Using RT-PCR, we detected SARS-CoV-2 in 94% of the saliva specimens from symptomatic patients, with Ct comparisons suggesting that viral load in saliva may be higher than in NOP specimens, particularly in males. The observed absence of correlation between NOP and salivary viral loads reflects distinct viral shedding dynamics in these tissues (2). Our results confirm that saliva is a suitable diagnostic specimen compared to NOP swabs, especially in cases where SARS-CoV-2 is detectable only in saliva, as ascertained in 3 patients herein. Male sex and old age are risk factors for developing severe COVID-19 (36,37). Here, males showed mean viral load around 10 times higher than females in saliva but not in NOP swabs, which was confirmed in a second cohort with the use of RT-LAMP Tt values to estimate viral load. Since airborne transmission is related to the amount of virions per saliva droplet, this indicates that males could be more likely to spread the virus than females. Notably, compared to age-matched males, young females (< 38 years old) showed lower salivary viral load that increases with age, while no clear age-related effects were found in males. Clinical severity and immunological profiles seem to better correlate with viral load in saliva than in NOP swabs (38,39), so our findings could be attributed to distinct immune responses between genders leading to higher viral shedding and disease severity in males (40), and could also explain the higher proportion of asymptomatic females in couples positive for SARS-CoV-2 (41). These observations further suggest that salivary viral load together with older age, male gender and other risk factors could be important to predict disease duration, severity and mortality (39,42). Contrasting the lack of correlation between viral load and time in NOP swabs, viral load in saliva peaked in the first days of symptoms and declined within 10 days, agreeing with recent estimates that show peak transmissibility 1.8 days before onset of symptoms and low chance of transmission beyond 9.5 days after onset (43). Importantly, we estimate that the sensitivity threshold of 10^3 copies/ μ L employed by several RT-LAMP approaches is crossed around the 5th day of symptoms, with males showing delayed virus clearance compared to females. Accordingly, we observed a lower detection rate via RT-LAMP in females on the 4th day of symptoms and beyond, compared to females tested earlier. Based on these observations, we suggest that salivary diagnostics in symptomatic individuals should be performed as soon as possible after symptom onset to increase chances of detection and to overcome limitations in sensitivity, especially for females.

In summary, we report a simple and rapid RT-LAMP diagnostic workflow that obviates RNA extraction and specialized equipment, providing a cost- and time-efficient

alternative to standard RT-PCR diagnostics. The use of DGS to process specimens and modifications to the RT-LAMP reaction resulted in high sensitivity and specificity to detect SARS-CoV-2 in saliva via real-time or endpoint colorimetric analysis, thus qualifying it for point-of-care testing. This work not only provides recommendations to optimize viral detection, but it also indicates that saliva may provide clues to clarify the biological determinants of SARS-CoV-2 infection and transmission. Recent reports show that saliva has higher sensitivity than nasal/nasopharyngeal swabs to identify asymptomatic cases (41), and that viral load distribution is equivalent in saliva from symptomatic and nonsymptomatic individuals (42). Considering that a fraction of infected patients escape detection via NOP RT-PCR, and since saliva better correlates with transmissibility and clinical outcomes, rapid salivary diagnostics stands as an invaluable opportunity to efficiently tackle the COVID-19 pandemic. We reinforce that rapid saliva tests should be prioritized in screenings to suppress viral spread, which may be extended to other respiratory pathogens.

Declaration of Competing Interest: The authors declare no conflict of interest.

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