Danielle de Paula Moreira

Múltiplas abordagens para determinar os fatores
genéticos que contribuem para o ASD

Multiple approaches to determine ASD genetic
factors

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INTRODUÇÃO GERAL E OBJETIVOS

INTRODUÇÃO

DEFINIÇÃO E CARACTERÍSTICAS

O transtorno do espectro autista (ASD, do inglês, *autism spectrum disorder*), descrito pela primeira vez por Leo Kanner em 1943, é uma doença de neurodesenvolvimento. Os critérios de diagnóstico desse distúrbio têm sido readequados desde sua descrição. Atualmente, o ASD é caracterizado por déficit persistente na comunicação e interação social, incluindo déficit na reciprocidade social, de comportamentos usados na comunicação não verbal para interação social e da habilidade para desenvolver, manter e entender os relacionamentos. Além disso, o diagnóstico do ASD também requer a presença de padrões de comportamentos, interesses e atividades, restritos e repetitivos (AMERICAN PSYCHIATRIC ASSOCIATION, 2013).

Em cerca de 70% dos casos de ASD outras condições médicas, de desenvolvimento e/ou psiquiátricas ocorrem concomitantemente, incluindo: deficiência intelectual (ID, do inglês, *intellectual disability*) (~45%), transtorno de déficit de atenção e hiperatividade (TDAH) (28-44%), epilepsia (~30%), problemas gastro-intestinais (9-70%) e transtorno obsessivo-compulsivo (7-24%) (LAI; LOMBARDO; BARON-COHEN, 2014).

O diagnóstico do ASD é basicamente clínico. Todavia, visto que este distúrbio envolve muitos aspectos clínicos e tem grande variabilidade fenotípica, é muito importante que a conclusão do diagnóstico seja realizada por uma equipe multidisciplinar, o que contribuirá para avaliar e definir se as características compõem, por exemplo, os quadros de síndromes genéticas ou metabólicas. Isto ajuda a evitar equívocos como a falta de diagnóstico nos casos de indivíduos com características clínicas mais brandas, ou o diagnóstico incorreto/inadequado nos casos dos indivíduos com características mais graves que não falam e têm ID (AMERICAN PSYCHIATRIC ASSOCIATION, 2013).

EPIDEMIOLOGIA E FATORES AMBIENTAIS

Desde que foi realizado o primeiro estudo epidemiológico do ASD, a prevalência desta condição aumentou em aproximadamente 25 vezes (LOTTER, 1966; HANSEN; SCHENDEL; PARNER, 2015; LUNDSTRÖM et al., 2015). Embora o primeiro estudo tenha sido bastante restrito em relação ao tamanho amostral e a população estudada, isso nos ajuda a vislumbrar o quanto expandiu o conhecimento sobre este transtorno. Atualmente, a estimativa média da prevalência mundial do ASD é de cerca de 1%, havendo grande variação nos cálculos entre as diferentes populações como, por exemplo, no Brasil a frequência estimada é de quase 0,3%, enquanto nos Estados Unidos da América e Coreia do Sul estudos realizados estimam uma frequência em torno de 2%. Esta variabilidade pode estar associada a uma série de fatores, como, a falta de acesso a profissionais bem treinados para diagnosticar os indivíduos em regiões subdesenvolvidas, a vigilância excessiva aos primeiros sinais sugestivos do diagnóstico e o tamanho amostral (KIM et al., 2011; MATSON; KOZLOWSKI, 2011; PAULA et al., 2011; ELSABBAGH et al., 2012).

Além das diferentes frequências populacionais, foi observado que o ASD afeta de 4 a 5 vezes mais indivíduos do sexo masculino do que do sexo feminino. Este viés tem sido atribuído ao 'efeito protetor específico dos indivíduos do sexo feminino', assim, as meninas precisam apresentar mais fatores etiológicos do que os meninos para atingir as características mínimas para serem diagnosticadas (LAI et al., 2011; DWORZYNSKI et al., 2012; SZATMARI et al., 2012; JACQUEMONT et al., 2014; LAI; LOMBARDO; BARON-COHEN, 2014).

Os fatores etiológicos do ASD envolvem aspectos genéticos, epigenéticos, ambientais e, até mesmo, a combinação de todos estes aspectos. Entre os fatores ambientais estão os poluentes ambientais (i.e. pesticidas, metais pesados, materiais particulados), os suplementos dietéticos (i.e. deficiência de ácidos graxos, vitamina D, ácido fólico e deficiência de minerais), fatores familiares e sociais, como o estresse psicológico e a ordem de nascimento (SOUTH; TEACHING, 2016) e, também, o microbioma (SHARON et al., 2016). Ainda não está muito claro como estes fatores contribuem para determinar o ASD, mas, por exemplo, já foi mostrado que o dióxido de nitrogênio, um poluente ambiental, em ratos pode induzir uma resposta inflamatória sistêmica, levando ao aumento das concentrações de citocinas, que podem ultrapassar a barreira placentária e causar danos no cérebro

dos fetos (HEGAZY; ALI; MAHMOUD, 2015). Neste trabalho, ainda, foi observado que os ratos expostos ao aumento de citocinas, durante a gestação, apresentavam comportamentos que seriam compatíveis com os do ASD (HEGAZY; ALI; MAHMOUD, 2015). Em relação ao microbioma, os trabalhos ainda não mostram padrões claros, mas sim algumas poucas diferenças no microbioma, como o aumento do número e da diversidade de um determinado gênero de bactéria no intestino, além de sutis melhoras do quadro de ASD, como indicado pela diminuição da pontuação no CARS (Childhood Autism Rating Scale – escala que médica aspectos clínicos do ASD) e no SRS (Social Responsiveness Scale – escala que mede comportamentos sociais, hiperatividade, estereotípias e outros), nos casos de terapia de transferência do microbioma de indivíduos saudáveis para aqueles com ASD (FRYE et al., 2015; SHARON et al., 2016; KANG et al., 2017).

Outro fator ambiental que tem sido intensamente discutido é a idade parental, especialmente paterna, que pode conferir um risco, pelo menos, 2 vezes maior para pais com 40 anos ou mais do que pais com menos de 30 anos de idade de terem uma criança com ASD. Este fator pode estar relacionado com as modificações epigenéticas relacionadas com o envelhecimento das células germinativas paternas e com a possibilidade da linhagem de células germinativas de pais mais velhos acumularem mutações novas. (DURKIN et al., 2008; HULTMAN et al., 2010; O'ROAK et al., 2012; PULEO et al., 2012).

ARQUITETURA GENÉTICA DO ASD

A arquitetura genética do ASD é bastante complexa e, embora atualmente haja mais clareza sobre a contribuição de alguns fatores genéticos para esse transtorno, ainda é incerto quais e quantos fatores genéticos contribuem para o ASD.

A herdabilidade do ASD é considerada como moderada a alta. Os trabalhos realizados com irmãos gêmeos mostram que há maior concordância para o ASD entre os gêmeos monozigóticos (96-36%) do que entre os dizigóticos (30-0%). As estimativas de herdabilidade a partir destes dados são elevadas, variando entre 80-90% (MECCA et al., 2011; RONALD; HOEKSTRA, 2011). Contudo, outros trabalhos apresentam valores de herdabilidade menores, entre 38%-78%. Essa grande variabilidade na estimativa da herdabilidade pode estar relacionado aos critérios

clínicos utilizados, métodos de averiguação e tamanho amostral (LIU; ZERUBAVEL; BEARMAN, 2010; HALLMAYER; CLEVELAND; TORRES, 2011; TICK et al., 2016).

De forma geral, o ASD pode ser definido em sindrômico, quando associado a uma condição metabólica e/ou genética, e não sindrômico. O ASD sindrômico, que manifesta o autismo como uma característica secundária, corresponde a cerca de 10% dos casos. Entre as síndromes genéticas conhecidas por terem forte associação com o ASD estão a síndrome do X-frágil (~60% dos indivíduos têm ASD), a síndrome de Phelan-McDermid (>50%), a síndrome Smith-Magenis (~90%) e a síndrome de Williams (~50%) (BETANCUR, 2011). Em grande parte das síndromes a ocorrência do ASD é menos recorrente, como nos casos de Distrofia muscular de Duchenne/Becker (DMD/BMD) onde o ASD ocorre em cerca de 4-20% dos casos (HENDRIKSEN; VLES, 2008; ANAND et al., 2015; RICOTTI et al., 2016). Uma questão que surge a partir dos casos sindrômicos monogênicos, como em DMD/BMD, é se o ASD nestes casos é decorrente da mutação principal ou se dependem de outros fatores genéticos.

Nos casos não sindrômicos, onde o ASD é o diagnóstico primário, cerca de 10-30% dos casos estão relacionados a uma alteração genética principal, que podem ser variantes de nucleotídeo único (SNV, do inglês, single nucleotide variants), inserções e deleções pequenas (indels), variantes de número de cópia (CNV, do inglês, copy number variantion) e alterações cromossômicas microscopicamente visíveis (BOURGERON, 2015; SCHAEFER, 2016). A classificação clínica entre sindrômico e não sindrômico não é simples, particularmente com o número crescente de casos onde se descrevem formas sindrômicas e não sindrômicas na mesma família.

As CNVs, junto com as anormalidades cromossômicas microscopicamente visíveis, são identificadas em 10-15% dos indivíduos afetados e já foram observadas em todos os cromossomos. A maior parte destas alterações são raras ou 'privadas', ou seja, a alteração está presente apenas em uma família. Em mais do que 50% desses casos as CNVs são herdadas de pais saudáveis ou com características subclínicas do ASD ou com outras doenças neurológicas e/ ou psiquiátricas, (NORD et al., 2011; LUO et al., 2012; QIAO et al., 2013). As CNVs recorrentes são poucas e, em geral, cada uma tem uma frequência próxima a 1%. Estão entre as regiões cromossômicas com maior taxa de CNVs nos casos de ASD a 15q11-q13, onde ocorrem os diversos rearranjos estruturais, a 2p16, a 16p11.2, a 17p11.2 e a 17q12

(DEPIENNE et al., 2009; GRISWOLD et al., 2012; WIŚNIEWIECKA-KOWALNIK et al., 2013; MOREIRA et al., 2014, 2016).

Mais recentemente, o sequenciamento completo do exoma ou do genoma tem contribuído significativamente para identificar SNVs e indels potencialmente patogênicos ao ASD em um grande número de genes (mais de 800), como podemos ver listados no banco de dados do *Simons Foundation Autism Research Initiative* (SFARI; <https://sfari.org/resources/sfari-gene>). A maioria dessas mutações são raras e/ou 'privadas'. Em mais de um estudo foram identificadas mutações recorrentes em genes nunca antes correlacionados com o ASD, como por exemplo o *DYRK1A*, o *CHD8*, o *ANK2* e o *TBR1* (O'ROAK et al., 2011; CHAHROUR et al., 2012; IOSSIFOV et al., 2012; NEALE et al., 2012; SANDERS et al., 2012; YUEN et al., 2015). Entre os genes patogênicos também há alguns que já haviam sido associados ao ASD pelos estudos das CNVs, como o *NRXN1*, *SHANK2*, *NLGN1* e o *RELN*. Além disso, muitos dos novos candidatos para o ASD estão localizados dentro de regiões cromossômicas envolvidas por CNVs previamente descritas nos casos de ASD. Isto mostra que, embora haja regiões onde recorrentemente ocorrem alterações genéticas, é ampla a heterogeneidade genética do ASD.

Em muitos indivíduos com ASD são identificadas múltiplas alterações genéticas candidatas. Nestes casos, os modelos genéticos que melhor explicam o fenótipo são aqueles que consideram interações gene(s)-gene, modelos *two (multi)-hits* e oligogênico, ou gene(s)-ambiente, modelo multifatorial (GIRIRAJAN et al., 2010; POOT et al., 2011; LEBLOND et al., 2012).

A maior parte dos dados genômicos (CNVs, SNVs e indels) acima citados foram obtidos a partir dos estudos de casos de ASD esporádicos, isto é, casos únicos de ASD na família. Até o momento, há poucos trabalhos que estudam os padrões genéticos em famílias com múltiplos indivíduos com ASD. Nos casos familiares de ASD é estimado que exista uma contribuição maior dos fatores genéticos do que em casos esporádicos (KLEI et al., 2012; GAUGLER et al., 2014), nos quais, as variantes herdadas, que levam a perda de função do gene, têm um papel principal na etiologia do ASD (TOMA et al., 2013). Diante disso, estudar as variantes genéticas dos casos familiares pode ser uma estratégia interessante para identificação de genes com maior impacto no fenótipo do ASD.

A IMPORTÂNCIA DO ESTUDO FUNCIONAL USANDO MODELOS BIOLÓGICOS

Apesar das análises *in silico* das variantes genéticas identificadas nos casos de ASD estarem predizendo que estas variantes nos genes candidatos para o ASD são danosas e, portanto, tem grande probabilidade de contribuírem para o fenótipo, há ainda pouca informação sobre como esses genes atuam nas vias biológicas e em processos celulares.

Como descrito acima, o número de alterações genéticas relacionadas com o ASD é enorme, porém ainda é questionável se todos os genes candidatos para o ASD realmente contribuem para o fenótipo. Além disso, dentre os genes que são sabidamente patogênicos, há pouco conhecimento sobre o mecanismo molecular e celular que leva aos fenótipos do ASD. Desta forma, é de grande valia adotar diferentes modelos biológicos como ferramenta para detalhar estes diversos mecanismos moleculares e celulares nos quais os genes candidatos para o ASD atuam.

Muitos modelos biológicos têm sido usados para estudar as alterações genéticas relacionadas com o ASD. No presente trabalho, optamos por usar as células-tronco pluripotentes induzidas (iPSC, do inglês, *induced pluripotent stem cell*) e a mosca de fruta (*Drosophila melanogaster*).

Modelagem in vitro de neurônios derivados de iPSC para estudar o ASD

As iPSCs têm características semelhantes às das células tronco embrionárias, incluindo, por exemplo, a morfologia, a capacidade proliferativa, o padrão de expressão gênica, os marcadores de membrana e a capacidade para se diferenciar em células dos três folhetos embrionários. Estas células são geradas a partir da reprogramação de células somáticas por fatores de reprogramação, que primordialmente são os fatores de transcrição *OCT4*, *SOX2*, *cMYC* e *KLF4*, porém outros fatores de reprogramação, atualmente, também são utilizados (TAKAHASHI; YAMANAKA, 2006; TAKAHASHI et al., 2007; LIN; LACHMAN; ZHENG, 2016).

Uma vez que as iPSCs geradas são indivíduo-específico, esta ferramenta permite que as células diferenciadas recapitem os aspectos genéticos e epigenéticos das células doadoras. A utilização desse modelo celular, que é passível de ser diferenciado em células de diferentes tecidos, se tornou de grande valia para

compreender a patofisiologia das mais diversas doenças, em particular, das doenças neurológicas, pois neurônios e outras células do sistema nervoso são de difícil acesso para estudos funcionais (PRILUTSKY et al., 2014).

Dentre os casos síndrômicos de ASD, importantes achados têm sido obtidos com neurônios derivados de iPSCs com mutações em *MECP2*, *SHANK3* e *FMR1*, responsáveis, respectivamente, pela síndrome de Rett, síndrome de Phelan-McDermid e síndrome do X-Frágil. Nas células neuronais com mutações em *MECP2* e *SHANK3* foram observadas deficiências dos processos sinápticos (por exemplo, transmissão sináptica), diminuição do número de espinhos dendríticos e alteração na sinalização por cálcio (MARCHETTO et al., 2010; SHCHEGLOVITOV et al., 2013). Além das alterações em neurônios, também foi observado que o *MECP2* é expresso em astrócitos, que influenciam nas anormalidades morfológicas (neuritos curtos, neurônios pequenos e outros) que ocorrem nos neurônios (WILLIAMS et al., 2014). Os pesquisadores também demonstraram que as alterações de sinalização e morfológicas observados nos neurônios derivados das iPSCs de pacientes com mutação em *MECP2* ou *SHANK3* foram resgatadas com tratamento com *IGF1* (*insulin growth factor 1*) (MARCHETTO et al., 2010; SHCHEGLOVITOV et al., 2013). Nas células com expansão trinucleotídica (CGG) na região 5'UTR do gene *FMR1*, ainda é um grande desafio determinar as alterações fenotípicas nos neurônios derivados de iPSC, devido ao mosaicismismo somático, que faz com que não haja inativação completa do *FMR1* em todas as células e tenham nível de expressão do *FMR1* variável em diferentes clones celulares dos mesmos indivíduos (SHERIDAN et al., 2011; BRYKCZYNSKA et al., 2016; XIE et al., 2016). Estes pesquisadores verificaram que nos neurônios com 50 – 200 repetições CGG, onde há a expressão da FMRP com expansão incompleta, ocorre aumento do número de agregados de inclusão de ubiquitina, os quais são responsáveis pelo fenótipo neurodegenerativo causado pela alteração da expressão do *FMR1* com expansão.

Alguns pesquisadores também têm estudado neurônios derivados de iPSCs de casos de ASD não síndrômico. Wang e colaboradores (2015) demonstraram que haploinsuficiência de *CHD8*, um dos genes recorrentemente mutados no ASD, leva a diminuição da expressão dos *POU3F2* e *AUTS2*, também associados ao ASD, e a super expressão do gene *TCF4*, o qual está em uma via associada com esquizofrenia, mostrando, então, que há uma convergência das vias dos distúrbios de neurodesenvolvimento. Em outro estudo de ASD não síndrômico, realizado por

nosso grupo, foi demonstrado que os neurônios derivados de iPSCs com expressão reduzida do *TRPC6* apresentam defeitos semelhantes aos observados nas células de casos síndrômicos citados acima, tais como, diminuição do influxo de cálcio, diminuição do número dos espinhos dendríticos e das sinapses glutamatérgicas (GRIESI-OLIVEIRA et al., 2015).

Este conjunto de dados, obtidos a partir do estudo das iPSCs, demonstra que adotar este modelo celular tem grande potencial para se avançar no conhecimento dos mecanismos moleculares e celulares pelos quais mutações em diversos genes causam as deficiências neurocomportamentais.

***Drosophila melanogaster* um modelo versátil para o estudo do ASD**

A *D. melanogaster* é amplamente usada nos estudos genéticos e também tem sido usada como ferramenta para o estudo de doenças neurológicas. A adoção deste organismo na investigação dessas doenças é justificada pelo baixo custo para criação e manutenção dessa espécie, rápido tempo das gerações, o genoma já foi sequenciado, vasto arsenal genético e de transgênicos e a conservação no desenvolvimento e função do sistema nervoso (OKRAY; HASSAN, 2013). Mais de 70% dos genes humanos já associados com doenças genéticas tem um respectivo ortólogo nessa mosca, o que reforça a escolha desse organismo como modelo para trazer entendimento dos mecanismos envolvidos no ASD (GATTO; BROADIE, 2011; DOLL; BROADIE, 2014; LEE et al., 2014; NIKITINA; MEDVEDEVA; ZAKHAROV, 2014; YAMAMOTO et al., 2014).

Como uma ferramenta extremamente versátil, diversos métodos têm sido desenvolvidos com *D. melanogaster* e um deles é o sistema GAL4/UAS. O GAL4/UAS é um sistema bipartido, ou seja, composto por duas partes que são mantidas inativas em duas moscas separadas, que se baseia na via da galactosidade de leveduras (BRAND; PERRIMON, 1993; TRAVEN; JELICIC; SOPTA, 2006) e permite a regulação de genes em tecidos específicos. Uma das linhagens, conhecida como *driver*, contém um constructo com a sequência do gene GAL4, que é um ativador da transcrição que se liga a uma sequência específica, a *Upstream Activation Sequence* (UAS), e a montante é inserido o promotor de um gene "X" que tenha expressão forte tecido-específica, possibilitando, assim, a ativação desse constructo apenas no tecido em que esse promotor é ativado. Na

outra linhagem, a *responder*, a jusante a sequência UAS, insere-se um constructo com a sequência que será expressa. Ao cruzar essas duas linhagens de *D. melanogaster* toda a prole, F1, terá o gal4 e a UAS, assim sendo, a gal4 poderá se ligar a UAS, ativando, então, a expressão da sequência de interesse, que no caso do presente trabalho foram moléculas de double-strand RNA (Figura 1). Adicionalmente, na linhagem *driver*, há a proteína fluorescente verde (GFP, do inglês *green fluorescent protein*) que permite a visualização, por microscopia de fluorescência, das células que expressem essa proteína.

Figura 1: Representação do sistema Gal4/UAS. Nessa imagem estão representadas as linhagens parentais (P), driver e responder. A linhagem driver, com inserto contendo o promotor (promoter)/ enhancer, a sequência para a gal4, juntamente com a sequência do GFP, que tem a expressão controlada pelo ligação da gal4 a UAS. A linhagem responder, contém inserto com a UAS e o alvo (*target*) inativo. A linhagem F1, conterá ambos os insertos da driver e da responder e o sistema estará completo, havendo assim a expressão da sequência de interesse (sequência para o dsRNA) Imagem modificada de YAMAMOTO-HINO; GOTO (2013).

Utilizando esse sistema, um crescente número de pesquisas sobre as funções dos diferentes genes associados aos ASD tem sido realizado em *D. melanogaster*. Os trabalhos são diversos e incluem análises comportamentais, análises neuronais e da morfologia de diferentes estruturas. Em modelos para o *FMR1* humano foi mostrado que a desregulação do ortólogo de *D. melanogaster*, o *dfmr1*, pode levar a defeitos da extensão/projeção de neuritos (MORALES et al., 2002), alterações do ciclo celular e apoptose (WAN et al., 2000), anormalidades no ciclo circadiano

(DOCKENDORFF et al., 2002; MORALES et al., 2002) e deficiência na interação social, tais como falta de interesse em manter o comportamento de corte e falhas para realizar gestos motores que levam a interação social (DOCKENDORFF et al., 2002; BOLDUC et al., 2010). Também, usando *D. melanogaster* para estudar os genes das famílias das neurexinas (NRXNs) e neuroliginas (NLGNs), os quais estão relacionados com o ASD, foi mostrado que estes atuam de forma semelhante na reorganização das zonas ativas sinápticas, no crescimento do número dos botões pré e pós-sinápticos e densidade sináptica (ZWEIER et al., 2009; CHEN et al., 2012). Já para genes que são envolvidos por CNVs associadas com o ASD, GRICE e colaboradores (2015) mostraram que os ortólogos em *D. melanogaster* podem ter uma interação sinérgica, como visto com os transheterozigotos *dlg/pak*, *NrxIV/dlg* e *NrxIV/pak*, evidenciando que alguns genes relacionados com ASD podem participar de uma rede de interação.

Estes dados evidenciam versatilidade do modelo, o qual permite um amplo estudo de diversos aspectos relacionados com o ASD. Embora a transposição dos dados obtidos para os genes entre *D. melanogaster* e humanos possa não ser direta, este modelo mostra-se muito eficiente para conhecer os mecanismos moleculares e a forma como os genes atuam para determinar os fenótipos.

OBJETIVOS GERAIS

A) Identificar variantes novas nas regiões codificantes do genoma de casos de ASD;

B) Entender a patofisiologia da perda de função do *TBCK* em neurônios derivados de iPSC;

C) Padronizar o uso de *Drosophila melanogaster* para estudos funcionais de genes candidatos para ASD.

OBJETIVOS ESPECÍFICOS

A.1) Identificar e analisar variantes de perda de função compartilhadas e não compartilhadas entre os indivíduos com ASD da mesma família;

A.2) Estabelecer critérios de priorização de genes candidatos para o ASD;

A.3) Identificar a variante patogênica no gene da distrofina nos casos de DMD com ASD;

A.4) Investigar se nos casos de DMD com ASD da mesma família há outras variantes em outros genes que não o da distrofina que possam contribuir para a manifestação do quadro de ASD;

B.1) Verificar se mutações de perda de função bialélicas no *TBCK* estão associadas a alterações do nível de expressão gênica deste gene e da via mTOR em células progenitoras neurais obtidas a partir de iPSC;

B.2) Verificar se variantes de perda de função bialélicas no *TBCK* causam alterações moleculares e celulares nas células neuronais induzidas a partir de células somáticas dos pacientes em relação a de controles.

C.1) Avaliar a morfologia do grupo de neurônios dorsais e dos grupos de neurônios PDF da medula de *Drosophila melanogaster* com diminuição da expressão dos genes ortólogos aos humanos candidatos para o ASD.

CAPÍTULO 1

Shared and unshared rare loss-of-function variants in ASD-multiplex families

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ABSTRACT

Autism Spectrum Disorder (ASD), a neurocognitive condition, has a strong genetic component with heritability estimated between 50-60%. Most recently, exome/genome sequencing has shown that rare truncating variants, de novo or inherited, strongly contribute to ASD etiology. These alterations are enriched in ASD affected probands as compared to unaffected siblings. However, it is still a challenge to define which of these variants are indeed causative of the phenotype, especially because some of them are inherited, being associated with non-penetrance of the phenotype. Thus, we have been investigating rare loss-of function (rLoF) variants (MAF<0.01) shared and unshared among ASD related individuals with the aim of identifying those that contribute to the phenotype. We performed exome sequencing of 28 ASD-affected individuals belonging to 13 families and selected as likely pathogenic variants those that cause premature stop codon or frameshift located in genes expressed in central nervous system. We identified 55 rLoF variants in 54 genes, which were shared and unshared among affected individuals within a family. Among them, we highlighted 16 genes as major cause of ASD, including DYRK1A, TBCK and AP1S2. We verified that inherited unshared variants were more frequently transmitted from mothers. *In silico* functional analysis of biological process related to these 54 genes revealed an enrichment of terms cellular transport and nervous system development, which are biological functions strongly associated with ASD. Hence, our results demonstrate the inter and intrafamilial heterogeneity of ASD and an overlap of biological processes related to ASD in different works.

Key words: monogenic ASD, neurocognitive, intellectual disability, genetic heterogeneity, TBCK, DYRK1A

INTRODUCTION

Autism spectrum disorder (ASD) is characterized by deficit in social communication and interaction, and persistent repetitive behaviors (AMERICAN PSYCHIATRIC ASSOCIATION, 2013). ASD affects around 1% of the population (ELSABBAGH et al., 2012), and it commonly co-occurs with other neurological conditions such as epilepsy, intellectual disability (ID), and attention deficit and hyperactivity disorder (ADHD) (LAI; LOMBARDO; BARON-COHEN, 2014).

ASD is associated with high heritability (50-90%) (HALLMAYER; CLEVELAND; TORRES, 2011; TICK et al., 2016) and multiple genetic risk factors contribute to its manifestation. These include microscopically discernible chromosomal alterations, copy number variations (CNVs), and rare or common single nucleotide variants (SNVs). Importantly, one major, penetrant genetic alteration can be identified in 10-25% of individuals affected by ASD. The remaining cases are most likely due to a complex pattern of inheritance involving gene-gene and/or gene-environment interactions (i.e. multifactorial, multi- and two-hit, or oligogenic inheritance models). In addition, due to the high heterogeneity and incidence of ASD, affected individuals within a family may not completely share causative variants (JORDE et al., 1991; BOURGERON, 2015; DE RUBEIS; BUXBAUM, 2015).

It has also been shown that the major genetic alterations related to ASD prominently overlap with those that are associated with other neurological disorders, including ID (POOT et al., 2011; LEE; SMITH; PACIORKOWSKI, 2015; ST POURCAIN et al., 2017; ZHAO; NYHOLT, 2017). It is still unclear if the intrafamilial clinical variability commonly observed in families with ASD affected-individuals can be ascribed to genetic heterogeneity or other mechanisms. The molecular investigation of familial ASD cases can contribute to address this question.

In spite of the great number of ASD candidate genes already described, accurate identification of causative variants still poses a significant challenge. Researchers have investigated rare loss-of-function (rLoF) variants, which are more susceptible to the effects of purifying selection than gain-of-function variants and neutral variants (i.e. synonymous variants) (MACARTHUR; BALASUBRAMANIAN; FRANKISH, 2012), and observed a disproportional excess of *de novo* and inherited rLoF mutations in ASD as compared to

controls (TOMA et al., 2013; KENNY et al., 2014; KRUMM et al., 2015). Although rLoF variants alone do not explain all cases of ASD, numerous evidence indicate that this kind of mutation has major impact in this condition manifestation (TOMA et al., 2013; KENNY et al., 2014; SAMOCHA et al., 2014; KRUMM et al., 2015).

Genetic investigation of multiplex ASD families, which represent less than 15% of the cases (CONSTANTINO et al., 2010; RONEMUS et al., 2014; WOODBURY-SMITH et al., 2015), may be a powerful tool to explore the interaction between predisposing genetic components, either inherited or *de novo*, in ASD. Therefore, the aim of this work was to examine rLoF variants in brain-expressed (Bex) genes in families with multiple ASD-affected individuals. We conducted exome sequencing analysis in 13 familial cases and analyzed both shared and unshared variants, to address the contribution of *de novo* and inherited variants to the phenotype. We also investigated the proportion of cases that are caused by a rLoF variant in a major gene, the genetic heterogeneity within families, the intrafamilial common causal variant, and the main biological process predicted to be altered by the candidate genes.

METHODS

Subject recruitment

We selected 13 families with at least two affected individuals ($N_{\text{TOTAL}} = 28$) from our cohort of more than 600 ASD families genetically evaluated at Human Genome and Stem Cell Research Center (HUG-CELL), Universidade de São Paulo. Affected individuals with ASD or ID were previously diagnosed by psychiatrists based on criteria of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), as we referred before (GRIESI-OLIVEIRA et al., 2014; MOREIRA et al., 2014, 2016).

Genomic DNA isolation from blood samples was performed with the Autopure LS automated workstation following manufacturer's procedures (Gentra Systems, Minneapolis, US) after signed consent by patient's caregivers. All affected boys tested negative for Fragile X Syndrome.

This project was approved by the ethics committee of the Institute of Biosciences, Universidade de São Paulo.

Whole-exome sequencing

Except from family F8293 in which we chose 2 out of 6 affected individuals, whole-exome sequencing and bioinformatics analyses were performed in all 28 affected individuals and parents (N=24) at the HUG-CELL sequencing facility. Exome capture was carried out with the TruSeq Exome Library Prep Kit (Illumina, Inc.) or Nextera Rapid Capture Exome kit (Illumina, Inc.), following manufacturer's recommendations. A HiSeq 2500 sequencer (Illumina, Inc) was used for sequencing paired-end reads of approximately 100 x 100bp. The mean read depth for nucleotides with >5 reads was ~67% (between: 50.9 – 81.8%). Reads were aligned with reference genome (GRCh37/hg19) with BWA (Burrows-Wheeler Aligner) (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; LI; HAKONARSON, 2010) was used to annotate variants.

Selection of variants in brain-expressed genes

We adopted the following criteria to filter for possibly pathogenic variants in individuals with ASD: 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 609 elderly Brazilian controls (60+ controls), University of São Paulo (<http://abraom.ib.usp.br/index.php>); 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) inclusion of loss-of-function variants alone: frameshift (small insertions and deletions) and stop codon mutations; e) inclusion of variants in genes expressed in the central nervous system (herein referred to as brain-expressed (Bex) genes) (Supplementary Table 1); f) exclusion of polymorphic genes (FUENTES FAJARDO et al., 2012); and g) exclusion of variants located within the last three amino acids of a protein.

The position of all variants identified were manually converted to GRCh38/hg38, using UCSC website (<https://genome.ucsc.edu/>).

Validation of candidate variants

Sanger sequencing

We validated by Sanger sequencing all shared rLoF variants (N of variants = 12) and those that had a coverage lower than 20 reads or less than 20% of reads with the alternative allele (N total=8; N confirmed = 6). Re-sequencing of individual F5980-4 was not carried out due to insufficient amount of DNA left in our sample bank.

Real-Time quantitative PCR (RT-qPCR) for copy number analysis

We carried out RT-qPCR of *TBCK* in family F6331 to detect a small deletion, as we could not identify any CNVs by array CGH 180K (Agilent). Six primer pairs in the region chr4:107,071,580-107,113,380 and a primer pair for *GAPDH* were designed on Primer-BLAST (NCBI; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Supplementary table 2). Each sample was analyzed in triplicate with the use of Fast SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's recommendations. Assays were run in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). 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).

Gene prioritization and functional enrichment analyses

We selected as best candidate genes for ASD those that fulfilled at least 3 criteria: 1) private mutations (MAF equal to 0 in 1000G, 6500ESP and 60+ controls); 2) *de novo* mutations; 3) intolerance to loss-of-function variation ($pLI \neq 0$ (LEK et al., 2016); $RVIS \leq 25\%$ with a $MAF \leq 1\%$ (PETROVSKI et al., 2013)); 4) gene associated with any neurodevelopmental disorder, based on information available in PUBMED and SFARI database (Simons Foundation Autism Research Initiative; <https://sfari.org/>); and 5) association with a known neurodevelopmental disorder.

Functional enrichment analysis was performed with Gene Ontology (GO; <http://www.geneontology.org/page/go-enrichment-analysis>).

Statistical analysis

We applied the Mann-Whitney U test to compare the mean number of total rLoF variants between cases and controls (609 Brazilian elderly controls) at $p < 0.05$. In these

analyses, we filtered control rLoF variants using the same criteria as individuals with ASD, except that we included only variants with “PASS” in quality control.

We used a chi-square test to compare the parental origin of unshared rLoF variants ($p < 0.05$).

RESULTS

Characterization of ASD families

We included in this analysis 13 ASD families with 2 or more affected individuals: 9 families comprised of 2 or 3 affected sibs and 4 other families in which the affected individuals were more distantly related (first or second cousins) (Figures 1 and 2). All 13 probands were autistic, but the other 15 affected relatives had ASD or ID associated with other clinical signs.

Shared rLoF variants in ASD reveal a recessive pattern inheritance in one family

We performed whole-exome sequencing of the proband from each of the 13 families (13 probands), along with 15 affected relatives and 24 parents. We searched for rLoF variants (nonsense and frameshift mutations) mapped in 3,622 Bex genes (Supplementary Table 1) that were shared among all affected individuals tested within a family. No shared rLoF variant was found in 5 out of the 13 families (affected sibs in families F5502, F5980, and F7935; and affected cousins in families F8293 and F9878). However, we identified 12 shared rLoF variants located in 11 genes, which were distributed in the 8 remaining families (Table 1). In 4 of these 8 families, we detected two shared rLoF variants. Except for *ZNF433*, which presented mutations in two distinct and unrelated families, all other variants were private mutations, that is, in genes specific for each family.

Figure 1: Pedigree of families with ASD and ID affected siblings. A) Siblings did not share likely pathogenic rLoF variants in brain expressed genes. B) Siblings share likely pathogenic rLoF variants in brain expressed genes. Dark symbols indicate individuals with ASD or ID. Propositus are indicated by family identification number with "-1".

Figure 2: Pedigree of families with ASD and ID affected cousins. A) Cousins did not share likely pathogenic rLoF variants in brain expressed genes. B) Cousins share likely pathogenic rLoF variants in brain expressed genes. Dark symbols indicate individuals with ASD or ID. Propositus are indicated by family identification number with “-1”.

Table 1: Rare loss-of-function variants shared among related ASD-affected individuals.

Family ID	Individuals ID	Position (hg38)	Ref allele	Alt allele	Gene	^{&} AA position (alt/ Ntotal)	Inheritance	Brazilian Elderly	ESP6500	1000g
F4482	F4482-1/ F4482-4	12:8932622	C	T	PHC1	389/ 1004	mat/ pat*	0.0033	0	0
F5576	F5576-1/ F5576-4	6:132618079	G	A	TAAR2	43/ 351	pat/ mat*	0	0.0007	0.0005
F5996	F5996-1/ F5996-4	1:26282864	CT	-	UBXN11	384/ 520	mat	0.0025	0.0069	0
		19:12015294	C	A	ZNF433	525/ 673	pat	0.0008	0	0
F6331*	F6331-1/ F6331-4	4:106171200	G	C	TBCK	710/ 893	pat	0	0	0
F7936	F7936-1/ F7936-4	11:6545449	C	T	DNHD1	1504/ 4753	mat	0	0.0006	0
		12:113161904	G	C	DDX54	763/ 882	mat	0	0	0
F8118	F8118-1/ F8118-4	1:52353618	G	-	CC2D1B	826/ 858	pat	0.0025	0	0
		19:12015057	G	A	ZNF433	604/ 673	mat	0.0016	0.0001	0
F8205	F8205-2, F8205-5/ F8205-6	4:152770164	G	A	TIGD4	281/ 512	pat	0.0016	0.0024	0.0014
		11:57740225	-	A	TMX2	253/ 296	pat	0	0	0
F8442	F8442-1/ F8442-4	13:45194567	T	-	KCTD4	1/ 259	father n.a.	0.0025	0.0022	0

* Inheritance follows the order of individuals (ID); n.a. - not available; & - amino acids altered position and total number of amino acids of the longest protein isoform;

We were able to determine the parental origin of 11 shared variants in 7 out of the 8 families (F8402 not included) (Table 1). In families F4482 and F5576, composed by affected cousins (Figure 2A), we observed that shared rLoF variants segregate with the disorder. In the remaining 5 families with affected sibs, 5 variants were transmitted by healthy fathers while 4 variants were transmitted by unaffected mothers (Figure 1B and Table 1).

Apart from the variant identified in family F6331, all other variants were present in a heterozygous state. In family F6331, the premature stop codon variant in exon 23 of *TBCK* (NM_001163435; c. 2130C>G; p.Tyr720*) (Table 1; Figure 1B) was annotated as homozygous. However, Sanger sequencing revealed that only the father carries this variant (Figure 3). Additional investigation showed that the mother and both affected sisters harbor a heterozygous deletion encompassing exon 23 of *TBCK* (chr4: 107,091,846 to 107,094,914; estimated size from 3 kb to 17 kb) (Supplementary table 2, Supplementary figure 1). Therefore, the affected sibs are actually compound heterozygotes for rLoF variants in *TBCK*, thus in accordance with an autosomal recessive inheritance pattern.

Unshared rLoF variants in ASD families show genetic heterogeneity within families

As genetic heterogeneity, multiple-hit hypotheses, incomplete penetrance or phenocopy should be considered in ASD/ID families, we also sought after variants that were exclusive or shared by at least two affected individuals within a family. In this analysis, we excluded family F6331 in which we identified biallelic pathogenic rLoF mutations. We found out 46 heterozygous rLoF variants in 43 Bex genes. Four out of these 46 variants were at *KLRG2* and *ZNF778* (two variants in *cis* in each gene), present in sibs F7935-4 and F7935-5 and in individual F8118-1, respectively (Table 2). It was possible to verify that they were in *cis* as they were in the same sequencing read with an interval smaller than 20bp in each of these genes. For further analysis, we considered them as just one variant/gene. Then, for the next analyses we considered a total of 44 rLoF variants in 43 Bex genes.

Figure 3: Alterations identified in ASD and ID affected individuals from the family F6331. A) Shows the sequencing chromatograms of the region with the identified *TBCK* point mutation (nucleotide marked with blue line). B) Schematic representation of both alteration in *TBCK*, showing that the SNV (red dashed line) is in the region involved by the deletion (blue rectangle).

In families F8205 and F7935 (with three affected individuals each) we observed that F8205-5 and F8205-6, F7935-1 and F7935-4, and F7935-4 and F7935-5 share three (*CYP7B1*, *C19orf71* and *UPK3A*), one (*RGS21*) and one (*KLRG2*) rLoF variants, respectively (Table 2). The remaining 39 variants distributed in 38 genes are exclusive to each affected individual.

Except for two unshared rLoF variants for which we could not determine the parental origin, among the remaining 42, we verified that nine were *de novo*, 22 were maternally transmitted and 11 were paternally transmitted (Table 2). Here, we observed a significant transmission bias for unshared variants of maternal origin (p-value = 0.006769).

Table 2: Rare loss-of-function variants unshared among related ASD-affected individuals.

Family ID	Individuals ID	Position (hg38)	Ref allele	Alt allele	Gene	%AA position (alt/ Ntotal)	Inheritance	Brazilian Elderly	ESP 6500	1000g
F5502	F5502-1	18:77250564	G	-	GALR1	6/ 349	mat	0.0000	0	0
F5576	F5576-1	7:50529209	-	GATCG	DDC	112/ 480	pat	0.0008	0	0
	F5576-1	16:76558669	G	T	CNTNAP4	1232/1308	mat	0.0008	0	0
	F5576-4	2:85597167	AGACG	-	RNF181	131_132/ 153	de novo	0	0.0001	0
	F5576-4	8:23006057	C	T	RHOBTB2	139/ 749	de novo	.	0	0
	F5576-4	10:86665725	-	T	OPN4	471/ 489	de novo	0.0025	0.004	0
F5980	F5980-4	10:24619520	G	T	ARHGAP21	792/ 1958	de novo	0	0	0
	F5980-4	12:50081111	GGGGCCAGATGG GGCTGTTCATC	-	ASIC1	482/ 574	de novo	0	0	0
	F5980-4	15:55869581	C	A	NEDD4	571/ 1319	de novo	0	0	0
	F5980-4	17:3679765	G	A	P2RX5	338/ 422	mat	0.0041	0.003	0.0037
	F5980-4	19:1468850	C	A	APC2	1850/ 2303	de novo	0	0	0
F5996	F5996-4	17:30999078	G	T	RNF135	396/ 432	pat	0.0008	0	0
F7935	F7935-1/ F7935-4	1:192347374	C	-	RGS21	25/ 152	mat	0	0	0
	F7935-4/ F7935-5	7:139483309	CCCCGGGCGC	-	KLRG2**	109_112/ 409	pat	0	0	0
	F7935-4/ F7935-5	7:139483322	-	A	KLRG2**	107/ 409	pat	0	0	0
	F7935-1	21:32631616	-	G	SYNJ1	1320/ 1612	de novo	0	0	0
	F7935-5	2:166405825	CT	-	SCN7A	1601_1602/ 1682	pat	0	0	0
	F7935-5	5:65809527	C	T	NLN	514/ 704	mat	0	0	0
	F7935-5	10:72885820	T	A	MCU	352/ 351	pat	0.0008	0.0025	0.0018
F7936	F7936-4	3:194341066	T	C	CPN2	546/ 546	pat	0	0	0

Table 2 (continued).

F8118	F8118-1	1:236,178,519	CGTCTCTGTG	-	GPR137B	190_193/ 399	mat	0	0	0
	F8118-1	3:169,812,479	-	C	LRRC34	24/ 464	pat	0	0	0
	F8118-1	4:154,305,100	G	A	DCHS2	1343/ 2916	mat	0.0057	0.0018	0.0018
	F8118-1	7:2,712,822	C	T	AMZ1	481/ 498	mat	0.0057	0.0025	0
	F8118-1	11:71,538,327	ATAA	-	KRTAP5-8	91_92/ 187	pat	0	0	0
	F8118-1	16:89,226,945	-	TGGG	ZNF778**	191/ 757	pat	0	0	0
	F8118-1	16:89,226,947	C	-	ZNF778**	192/ 757	pat	0	0	0
	F8118-4	5:141,415,363	C	T	PCDHGA10	730/ 936	mat	0.0082	0.0034	0.0037
	F8118-4	7:45,101,910	-	T	TBRG4	384/631	mat	0	0	0
	F8118-4	11:74,005,744	CT	-	UCP3	176_176/ 312	pat	0	0.0001	0
	F8118-4	17:75,246,568	G	A	GGA3	48/ 723	pat	0	0	0
F8205	F8205-5/ F8205-6	8:64,604,834	G	A	CYP7B1	361/ 506	mat	0	0	0
	F8205-5/ F8205-6	19:3,543,956	C	G	C19orf71	192/ 209	mat	0.0008	0.0013	0
	F8205-5/ F8205-6	22:45,287,223	C	A	UPK3A	87/ 287	mat	0.0041	0.0046	0.0018
	F8205-2	11:113,324,643	C	T	TTC12	95/ 732	mat	0	0	0
	F8205-5	5:141,122,842	C	T	PCDHB4	282/ 795	mat	0	0	0
	F8205-5	5:141,384,657	C	A	PCDHGA7	586/ 932	mat	0	0	0
F8293	F8293-1	5:38,451,332	C	G	EGFLAM	854/1017	mat	0	0	0
	F8293-11	X:15,852,371	G	A	AP1S2	52/ 160	mat	0	0	0
F8442	F8442-1	5:140,850,095	C	T	PCDHA9	534/ 950	mat	0.0025	0.0025	0
	F8442-1	9:137,522,734	CCACC	-	PNPLA7	264_266/ 1342	mat	0	0.004	0
	F8442-1	9:137,542,692	-	G	PNPLA7	181/ 1342	mat	0.0025	0.0007	0
	F8442-1	14:69,424,097	C	T	SLC39A9	34/ 307	father n.a.	0	0	0
	F8442-4	4:38,035,623	ATTG	-	TBC1D1	446_447/ 1168	father n.a.	0.0008	0	0

Table 2 (continued)

	F8442-4	6:44,300,650	C	-	AARS2	952/ 985	mat	0	0	0
F9878	F9878-1	21:37,478,281	-	T	DYRK1A	308_309/ 763	de novo	0	0	0

n.a. - not available; & - amino acid altered position and total number of amino acids of the longest protein isoform;

Family F8293 (Figure 2B) is of particular interest because the individual variant analysis revealed one rLoF variant in *AP1S2* (NM_001272071; c.154C>T; p.Arg52*) in F8293-11 that was not shared by F8293-1, the ASD proband. As *AP1S2* maps to Xp22.2 and the neurological phenotype segregates in an X-linked pattern in this family, we sequenced exon 2 of *AP1S2* in all other affected family members. Apart from individual F8293-1, who inherited from his mother a likely pathogenic mutation in *EGFLAM* (Table 2), we verified that all of them share this variant (Figure 4).

Figure 4: The sequencing chromatograms of the region with the identified *AP1S2* point mutation (nucleotide marked with blue line) in ASD/ ID affected individuals, carrier and not carrier from family F8293.

Another interesting rLoF variant identified through the individual variant analysis was in *DYRK1A* (NM_001396; c.308_309insT; p.T103fs) in F9878-4 (Table 2, Figure 5). We observed that this was *de novo* variant not shared with the affected relative.

Figure 5: The sequencing chromatograms of the region with the identified *DYRK1A* mutation (F9878-4 mutation – black arrow) in members from family F8293.

Burden of rLoF mutations in ASD

It has been shown that the mean number of rLoF variant per ASD-affected individual is greater than in controls (KRUMM et al., 2015). Thus, we tested whether there is enrichment of rLoF variants in Bex genes in individuals with ASD as compared to Brazilian controls. In total, among 26 affected individuals, (except for members of family F6331, as they were not included in the unshared variant analysis), we discovered 55 rLoF variants in Bex genes, that is, 2.12 variants per individual (range = 0 – 8 variants in each affected individual; shared variants were counted only in probands), located in 53 of 3,622 Bex genes, while in 609 Brazilian controls we found 1025 mutations, that is, 1.68 variant per individual (range: 0 – 8 variants in each individual), located in 458 Bex genes.

The comparison of mean number of variants per individual between probands (2.33 ± 2.27 variants/ individual; $N_{\text{probands}} = 12$, $N_{\text{variants}} = 28$, range 0 – 8 variants per individual) and controls (1.68 ± 1.32 variants/ individual; $N_{\text{individuals}} = 609$, $N_{\text{variants}} = 1025$, range = 0 –

8 variants per individual) did not show statistically significant difference ($p=0.209$; Mann Whitney U Test, one-tailed).

On the other hand, we observed that the control group (mean = 2.24 ± 2.5 variants per Bex gene; $N_{\text{variants}} = 1,025$; $N_{\text{Bex genes}} = 458$, range = 1 – 19 variants per gene) has statistically more variants per gene than ASD (mean = 1.04 ± 0.19 variants per Bex gene; $N_{\text{variants}} = 55$; $N_{\text{Bex genes}} = 53$; range = 1 – 2 variants per gene) ($p=2.4 \times 10^{-7}$; Mann Whitney U Test, one-tailed). Fifteen out of 53 candidate genes (28,3% of genes) for ASD were also identified in control group, representing 3,28% of total Bex genes (15 out of 458) with rLoF variants in this last group. These results show that the distribution of variants differs between affected individuals and controls, suggesting that a proportion of the rLoF mutations in the affected individuals may be contributing to the phenotype. Also, these data set show genetic heterogeneity of the ASD.

Impact of the genes to the phenotype and their molecular function

One current challenge in ASD studies is to determine if a variant in a gene still unknown to be associated with the disease is indeed causative. In order to prioritize the mutated genes most likely to be pathogenic, we further restricted selection criteria for the 54 genes by considering the following: MAF = 0 in all population databases, gene intolerance to LoF mutations, clear monogenic pattern of inheritance and association with a neurodevelopmental disorder. Sixteen out of all 54 candidate genes identified by the analyses of shared and unshared variants fulfilled at least three of these parameters and were, therefore, classified as the best ASD candidates (Table 3).

Then, we compared the number of rLoF variants per individual in these 16 genes between ASD (mean = 0.57 ± 0.88 variants in the 16 genes/ individual; $N_{\text{individuals}} = 28$; $N_{\text{variants}} = 16$; shared variants counted only in probands) and Brazilian controls (mean = 0.01 ± 0.11 variants in the 16 genes/ individual; $N_{\text{individuals}} = 609$; $N_{\text{variants in 16 genes}} = 4$). This analysis revealed that the mutational burden of variants in these genes is significantly greater in the ASD group ($p<0.0001$, Mann Whitney-U test, one-tailed).

We next evaluated if there was overrepresentation of disease-relevant functions among the 54 mutated genes pointed out in this work. The most significant Gene Ontology

terms and biological functions (GO Slim analysis, without multiple test correction) were related to transport, cellular adhesion, sensory perception and nervous system development (Supplementary Table 3). Accordingly, these same GO terms were also overrepresented among the 16 genes selected as the strongest candidates for ASD.

Table 3: Genes prioritized as most likely to be pathogenic for ASD.

Gene	pLI	RVIS (%)	Neurodevelopmental Disorder	Refs.
<i>TBCK</i>	0	50.50	DI	(ALAZAMI et al., 2015; BHOJ et al., 2016)
<i>DYRK1A</i>	1	25.64	ASD	(EVERS et al., 2017)
<i>ARHGAP21</i>	1	12.78		
<i>APC2</i>	1		Sotos-like Syndrome	(ALMURIEKHI et al., 2015)
<i>ASIC1</i>	0.99	22.36		
<i>AP1S2</i>	0.79	58.00	DI/ ASD	(SAILLOUR et al., 2007; BORCK et al., 2008)
<i>RHOBTB2</i>	0.51	8.90	NDD	(LOPES et al., 2016)
<i>TBRG4</i>	0.78	24.53		
<i>SLC39A9</i>	0.53	66.82		
<i>GGA3</i>	0.51	16.71		
<i>SYNJ1</i>	0.21	79.67		
<i>NEDD4</i>	0	71.71		
<i>TTC12</i>	0	42.25	ADHD	(MOTA et al., 2015)
<i>PCDHB4</i>	0	87.72	NDD	(ALAZAMI et al., 2015)
<i>SCN7A</i>	0	88.57	ASD	(SCHERER; DAWSON, 2011)
<i>ZNF778</i>	0	93.92	ASD	(WILLEMSSEN et al., 2010)

DISCUSSION

Here, the study of ASD families with multiple affected individuals brought a relevant overview about the complex scenario of genetic components associated with this condition. Our analyses revealed a diverse ASD-specific genetic architecture that involved shared and unshared major risk variants as well as multiple variants, corresponding to

multi-hit models. These data also support the relevance of some genes, like *DYRK1A*, and biological processes already related to ASD, as discussed below.

Monogenic inheritance model in ASD

Three of the 13 families had a rLoF variant in a gene related to a neurodevelopmental condition, segregating under distinct inheritance patterns: *TBCK* (family F6331), with autosomal recessive, *DYRK1A* (family F9878) with autosomal dominant and *AP1S2* (family F8293) with X-linked inheritance. In the *TBCK*-mutated family, the two affected sibs harbor biallelic rLoF variants in *TBCK* and the neurodevelopmental phenotype varies among them: while the proband has ASD, her sib has been diagnosed with ID. *TBCK* (TBC1 domain containing kinase) is a protein kinase with highly conserved sequence among organisms (WU et al., 2014; CHONG et al., 2016). It has been shown that *TBCK* regulates mTOR pathway, which is critical for neuronal activity and synapse inputs (HAN et al., 2008; COSTA-MATTIOLI; MONTEGGIA, 2013; ALAZAMI et al., 2015; BHOJ et al., 2016; CHONG et al., 2016; GUERREIRO et al., 2016; MANDEL et al., 2016) and has also been implicated in several neurodevelopmental conditions, including ASD (BHASKAR et al., 2009; MAGRI et al., 2011; BOULTWOOD et al., 2013; COSTA-MATTIOLI; MONTEGGIA, 2013; KA et al., 2014; TANG et al., 2014; SUZUKI et al., 2015).

The *de novo* frameshift mutation in *DYRK1A* (Dual-specificity tyrosine phosphorylation-regulated kinase 1A) (individual F9878-4) was found associated with a syndromic form of ASD. Therefore, even though several works have reported truncated variants in this gene in non-syndromic cases of ASD (IOSSIFOV et al., 2012; KRUMM et al., 2014; RUMP et al., 2016; VAN BON et al., 2016; EVERS et al., 2017), our data further support that heterozygous LoF variants in *DYRK1A* are commonly associated with syndromic autosomal dominant ID (OMIM 614104). *DYRK1A* is located within chromosome 21 in a critical region of Down syndrome (21q22.13), which has ID and ASD as clinical manifestations. Thus, either reduction or increase of *DYRK1A* expression may lead to neurological disturbances, including ASD (FOTAKI et al., 2002; DOWJAT et al., 2012; JI et al., 2015; RABANEDA et al., 2016; RUIZ-MEJIAS et al., 2016).

Finally, the nonsense *AP1S2* mutation was found segregating in a typical X-linked inheritance pattern where the affected individuals presented ASD and a variety of other neuropsychiatric phenotypes, in accordance with previously published observations (TARPEY et al., 2006; BORCK et al., 2008; BALLARATI et al., 2012; CACCIAGLI et al., 2014; TZSCHACH et al., 2015). Although the proband of this family (F8293-1) does not harbor the nonsense mutation in *AP1S2*, he presents ASD and shares similar clinical features with some of the other affected relatives, presenting an undistinguishable phenotype from his affected *AP1S2* mutated-relatives. A pathogenic variant in *EGFLAM* may contribute to his phenotype. Therefore, this represents another family with genetic heterogeneity within affected individuals

In general, our findings suggest that in about 23% of ASD multiplex-families, the condition may be associated with a major mutated allele, and corroborate that rLoF variants in *TBCK*, *DYRK1A* and *AP1S2* cause syndromic and non-syndromic forms of ASD. Further, our data has revealed genetic heterogeneity in both close and more distantly related ASD individuals, an observation that should be considered in genetic counseling.

Multiple genetic risk factors in ASD families

Fifteen out of 26 individuals with ASD here studied carry two or more variants in Bex genes. So, in total, in addition to variants in genes related to monogenic ASD, we identified 53 other variants, in 51 genes out of 3,622 Bex genes, which were either inherited or *de novo* and shared or unshared, as had previously been shown in different cases of disrupting SNVs in ASD (KUMAR et al., 2007; KUMAR, 2010; HALLMAYER; CLEVELAND; TORRES, 2011; O'ROAK et al., 2011; SCHAAF et al., 2011; CHILIAN et al., 2013; LIM et al., 2013; MENASHE; LARSEN; BANERJEE-BASU, 2013; KENNY et al., 2014; GRISWOLD et al., 2015).

Interestingly, we observed bias for maternal transmission of the mutated alleles among unshared rLoF variants, but not for shared variants. This result is intriguing. Even though there are some data suggesting that candidate variants are preferentially transmitted by females (KRUMM et al., 2013, 2015; JACQUEMONT et al., 2014; RISCH

et al., 2014), we did not find any obvious explanation for the female preferential transmission only for the unshared variants.

In order to evaluate if all these variants contribute to the ASD phenotype, we first analyzed the distribution of these mutations in ASD individuals as compared to controls. Possibly because of our small sample size, we did not reach statistical significance for mean number of variants per individual in ASD as compared to control individuals. However, we observed that the average number of rLoF variants per gene, once we assumed that length and mutation rate of all genes do not have great distinction, in ASD group was ~ 1 . These observations suggest that there are an exclusive group of causative genes in ASD individuals within and among families, and, also different pathogenic variants among affected individuals within a family (or genetic heretogeneity within families).

Next, we conducted an analysis to prioritize the most likely pathogenic variants, and 16 strong candidates for ASD were evidenced. rLoF SNVs in these genes were almost 53 times more frequent in ASD individuals than in controls. All criteria used to prioritize these genes have been contributing to the identification of major cause of ASD, as it has been observed that major causative genes of ASD are rare ($MAF < 0.01$) and private (i.e. exclusive of a family/ individual) and overlaps with those related to other neurological condition (TOMA et al., 2013; SAMOCHA et al., 2014; IOSSIFOV et al., 2015; KRUMM et al., 2015; JI et al., 2016; WANG et al., 2016). Therefore, it is very likely that the variants in these genes are indeed contributing to the ASD phenotype. Although there are already enough evidence that rLoF in *DYRK1A*, *AP1S2* and *SCN7A* contribute to ASD (Simons Foundation Autism Research Initiative, <https://sfari.org/>), all remaining 13 genes identified here are being suggested as strong ASD candidates for the first time.

We observed a slight overrepresentation of transport, cellular adhesion, sensory perception and nervous system development GO-terms. Recently, Parikshak et al. (2016), after analyzing the transcriptome of post-mortem samples of cortical and cerebellar regions of individuals with ASD, demonstrated that the modules of downregulated genes were enriched for synaptic function and neuronal genes, including, for example, transport, synaptic transmission and learning and memory, while, upregulated modules were enriched of genes associated with inflammatory process and glial functions. Besides,

recurrent works, which uses different approaches to study ASD, have shown dysregulation of the same biological processes here presented (NISHIMURA et al., 2007; GAI et al., 2011; VOINEAGU et al., 2011; KONOPKA et al., 2012; PINTO et al., 2014). The overlap of the functional modules here reported with those of the literature further support the importance of the candidates here identified to the etiology of ASD.

CONCLUSION

Adopting the analysis of shared and unshared rLoF variants in Bex genes in each one of the 13 ASD-families, we could identify a risk variant in every family, which showed distinct genetic architecture. Therefore, sequencing small number of individuals is feasible and contributes to detection of causal variants to ASD and allowed to identify novel ASD candidate genes. We found a slight excess of rLoF variants in Bex genes and high genetic heterogeneity in ASD, and therefore, genetic testing of all affected members per family should be considered for precise diagnosis and genetic counseling. We highlighted an excess of maternally inherited mutations among familial cases for unshared variants. From the total of 54 mutated genes, three are responsible for major forms of ASD: *TBCK*, *DYRK1A* and *AP1S2*, which along with 13 other candidate genes comprise the set of best candidates for ASD and, probably, for other neurodevelopmental disorders. Finally, we observed that biological processes of most of the risk genes agree with those reported in other works, indicating the redundancy of the genetic factors associated with ASD.

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Capítulo 1: SUPPLEMENTARY INFORMATION

Supp. Table1: List of brain expressed genes used to filter rLoF likely pathogenic variants in the exome of ASD individuals.

AARS	AARS2	AATK	ABAT	ABCA1	ABCA2	ABCA5	ABCA7	ABCA8
ABCB1	ABCB6	ABCB7	ABCC1	ABCC2	ABCC8	ABCC9	ABCD1	ABCG2
ABCG4	ABL1	ABL2	ABLM1	ABR	ABT1	ACAD10	ACAD9	ACAT1
ACE	ACE2	ACHE	ACKR3	ACOT11	ACOT12	ACOT7	ACOT8	ACOT9
ACSBG1	ACSL3	ACSL5	ACSL6	ACSS2	ACTA1	ACTB	ACTG2	ACTL6B
ACTN4	ACVR1	ACVRL1	ADAM10	ADAM17	ADAM19	ADAM22	ADAM23	ADAM32
ADAM8	ADAM9	ADAMTS1	ADAMTS12	ADAMTS13	ADAMTS4	ADAMTS8	ADAP1	ADAR
ADARB1	ADCY1	ADCY2	ADCY3	ADCY4	ADCY5	ADCY6	ADCY7	ADCY8
ADCY9	ADCYAP1	ADCYAP1R1	ADD1	ADD2	ADD3	ADGRA2	ADGRB1	ADGRB2
ADGRB3	ADGRG1	ADGRL1	ADGRL3	ADH1B	ADI1	ADIPOQ	ADK	ADM
ADNP	ADNP2	ADORA1	ADORA2A	ADRA1A	ADRA2A	ADRA2B	ADRA2C	ADRB1
ADRB2	AFF2	AFM	AFP	AGAP2	AGER	AGMAT	AGO2	AGO3
AGRN	AGT	AGTR1	AGTR2	AHCY	AHCYL1	AHCYL2	AHI1	AHNAK
AHR	AHSG	AICDA	AIF1	AIFM1	AIM1L	AIMP2	AIP	AK1
AK4	AK5	AKAP6	AKR1B1	AKR1C1	AKR1C3	AKR7A2	AKT1	AKT1S1
AKT2	AKT3	ALB	ALCAM	ALDH1A1	ALDH1A2	ALDH2	ALDH5A1	ALDH7A1
ALDOA	ALDOB	ALDOC	ALG13	ALK	ALKBH1	ALKBH2	ALOX12	ALOX15
ALOX5AP	ALPL	ALS2	AMBRA1	AMPD2	AMPH	AMT	AMZ1	ANGPT1
ANGPT2	ANGPTL1	ANGPTL4	ANK1	ANK2	ANK3	ANKK1	ANKRD1	ANKRD2 7
ANKS1A	ANKS1B	ANO2	ANOS1	ANP32A	ANP32E	ANXA1	ANXA4	ANXA7
AOC3	AOX1	AOX2P	AP1S2	AP3B2	AP3M2	APAF1	APBA1	APBA2
APBA3	APBB1	APBB2	APC	APC2	APCS	APEX1	APLN	APLNR
APMAP	APOA1	APOA4	APOA5	APOC1	APOD	APOE	APOO	APP
AQP1	AQP4	AQP5	AQP9	AR	ARC	AREG	ARF1	ARF4
ARFGEF1	ARG1	ARHGAP17	ARHGAP21	ARHGAP26	ARHGAP32	ARHGAP33	ARHGAP 35	ARHGDI A
ARHGEF10	ARHGEF2	ARHGEF28	ARHGEF4	ARHGEF40	ARHGEF7	ARID1A	ARID1B	ARL11
ARL13B	ARL4D	ARL6	ARL6IP5	ARMS2	ARNT2	ARNTL	ARNTL2	ARPC2
ARPC5	ARRB1	ARRB2	ARSA	ARSB	ARTN	ARX	ASCL1	ASCL4
ASIC1	ASIC2	ASIC3	ASIC4	ASNS	ASPA	ASPH	ASPM	ASTN1
ASTN2	ATAT1	ATCAY	ATF1	ATF2	ATF4	ATF6	ATL1	ATM
ATN1	ATOH1	ATP10A	ATP13A2	ATP1A1	ATP1A2	ATP1A3	ATP1B2	ATP2B1
ATP2B2	ATP2B4	ATP2C1	ATP5B	ATP6AP2	ATP6V0D1	ATP6V1B2	ATP7A	ATP7B
ATP8A2	ATRX	ATXN1	ATXN10	ATXN3	ATXN7	ATXN8OS	AUNIP	AURKA
AVIL	AVP	AXIN1	AXL	AZIN2	B2M	B3GALNT2	B3GALT1	B3GNT5
B4GAT1	BAALC	BAAT	BACE1	BACE2	BACH1	BAD	BAG3	BAG5
BAG6	BAHD1	BAIAP2	BAIAP2L1	BAIAP2L2	BAIAP3	BAK1	BARD1	BARHL1
BARHL2	BASP1	BASP1P1	BAX	BBS2	BBS4	BBS7	BCAN	BCAS3

BCAT1	BCAT2	BCHE	BCL11A	BCL11B	BCL2	BCL2A1	BCL2L1	BCL2L11
BCL2L2	BCL6	BCR	BCYRN1	BCYRN1P1	BCYRN1P3	BDKRB1	BDKRB2	BDNF
BDNF-AS	BEAN1	BECN1	BEGAIN	BEND3	BEND6	BEX1	BEX2	BEX3
BEX4	BEX5	BHLHB9	BHLHE22	BHLHE23	BHLHE40	BICC1	BICD1	BICDL1
BID	BIN1	BIRC5	BIRC8	BLCAP	BLMH	BLOC1S1	BLOC1S2	BLOC1S3
BLOC1S4	BLOC1S5	BLOC1S6	BMI1	BMP2	BMP4	BMP6	BMP7	BMPR1A
BMPR1B	BMPR2	BNIP1	BNIP3	BOK	BPTF	BRAF	BRCA1	BRCA2
BRD1	BRE	BRI3	BRI3BP	BRI3P1	BRI3P2	BRI3P3	BRINP1	BRINP2
BRINP3	BRMS1	BRPF1	BRPF3	BRS3	BSK1	BSK2	BSCL2	BSG
BSN	BSX	BTBD10	BTBD8	BTG2	BTG4	C11orf63	C14orf1	C14orf16 6
C16orf45	C19orf12	C19ORF71	C1QBP	C1QL1	C1RL	C21orf2	C21orf33	C2CD2L
C2CD3	C3	C4BPA	C5	C5AR1	C5AR2	C5orf42	C7	C8orf37
C9orf72	CA10	CA11	CA12	CA2	CA4	CA7	CA9	CABP1
CABP4	CABYR	CACNA1A	CACNA1B	CACNA1C	CACNA1D	CACNA1E	CACNA1 G	CACNA1 H
CACNG2	CACYBP	CADM1	CADM3	CALB1	CALB2	CALCA	CALHM1	CALM2
CALN1	CALR	CALY	CAMK1	CAMK1D	CAMK1G	CAMK2A	CAMK2B	CAMK2G
CAMK4	CAMKK2	CAMSAP1	CAMSAP2	CAMSAP3	CAMTA1	CANX	CAP1	CAPN1
CAPN2	CAPNS2	CARF	CARTPT	CASK	CASP14	CASP2	CASP3	CASP5
CASP7	CASP8	CASP9	CASR	CAST	CASZ1	CAT	CATSPE R1	CAV1
CBFA2T2	CBL	CBS	CC2D1A	CC2D1B	CC2D2A	CCDC192	CCDC26	CCDC30
CCDC88A	CCDC88B	CCDC88C	CCK	CCKAR	CCKBR	CCL2	CCL21	CCL3
CCL3L1	CCL5	CCM2	CCNA1	CCND1	CCNG2	CCR1	CCR10	CCR2
CCR3	CCR4	CCR5	CCSAP	CD14	CD163	CD200	CD200R1	CD209
CD24	CD248	CD27	CD274	CD33	CD34	CD38	CD4	CD40
CD40LG	CD44	CD47	CD48	CD59	CD68	CD81	CD82	CD9
CD99	CDC14A	CDC34	CDC42	CDCP1	CDH1	CDH10	CDH11	CDH12
CDH13	CDH2	CDH22	CDH4	CDH5	CDH8	CDK1	CDK14	CDK16
CDK18	CDK3	CDK5	CDK5R1	CDK5R2	CDK5RAP1	CDK5RAP2	CDK5RA P3	CDK9
CDKL5	CDKN1A	CDKN1C	CDKN2A	CDKN2B	CDKN2B-AS1	CDNF	CDON	CEACAM 1
CEACAM5	CEBPB	CEBPD	CEL	CELSR1	CELSR3	CEND1	CENPJ	CENPU
CEP126	CEP135	CEP152	CEP290	CEP41	CEP63	CERS1	CES1	CES1P1
CES2	CES3	CES4A	CES5A	CETP	CFAP221	CFB	CFH	CFL1
CFTR	CH25H	CHAF1B	CHAT	CHD5	CHD8	CHEK2	CHGA	CHI3L1
CHI3L2	CHIC1	CHKB	CHL1	CHMP2B	CHMP4A	CHMP4B	CHN1	CHODL
CHP1	CHRM2	CHRNA1	CHRNA2	CHRNA3	CHRNA4	CHRNA5	CHRNA6	CHRNA7
CHRN2	CHRN3	CHRN4	CIB1	CITED1	CIZ1	CKB	CKBP1	CKM
CLCA1	CLCF1	CLCN1	CLCN2	CLDN1	CLDN5	CLEC4E	CLEC4M	CLIC2
CLMN	CLN3	CLN5	CLN8	CLPB	CLSPN	CLTCL1	CLU	CLVS1
CLVS2	CMKLR1	CNDP1	CNKSR2	CNN3	CNNM2	CNP	CNPY2	CNR1
CNR2	CNTF	CNTFR	CNTN1	CNTN2	CNTN3	CNTN4	CNTN5	CNTN6

CNTNAP1	CNTNAP2	CNTNAP3	CNTNAP4	COASY	COL17A1	COL18A1	COL1A2	COL25A1
COL3A1	COL4A1	COL6A1	COL6A2	COL6A3	COMT	COPA	COPS2	COPS4
COPS5	CORIN	CORO1A	COX17	COX2	CP	CPA4	CPA6	CPB2
CPEB1	CPEB2	CPEB3	CPEB4	CPLX1	CPM	CPN2	CPNE1	CPNE3
CPNE5	CPNE6	CPT1C	CPT2	CR1	CR2	CRACR2A	CRB1	CRB2
CRBN	CREB1	CREM	CRH	CRHR1	CRIM1	CRLF1	CRLF2	CRMP1
CRP	CRTC1	CRY1	CRYAB	CRYM	CSF1R	CSF2	CSF2RA	CSF3
CSF3R	CSK	CSMD2	CSNK1D	CSNK1E	CSNK2A1	CSNK2A2	CSPG4	CSPG5
CST3	CST6	CTAGE1	CTBP1	CTDSP1	CTF1	CTGF	CTH	CTNNA2
CTNNA3	CTNNB1	CTNND1	CTNND2	CTNS	CTSB	CTSC	CTSD	CTSK
CTSL	CTSS	CTSV	CTTN	CTTNBP2	CTXN3	CUL4B	CUL5	CUTA
CX3CL1	CX3CR1	CXADR	CXCL1	CXCL10	CXCL11	CXCL12	CXCL13	CXCL14
CXCL16	CXCL2	CXCL6	CXCL8	CXCL9	CXCR2	CXCR3	CXCR4	CYB5D2
CYBB	CYCS	CYCSP14	CYFIP1	CYFIP2	CYGB	CYP11A1	CYP19A1	CYP1A1
CYP1B1	CYP26B1	CYP27A1	CYP27B1	CYP2A6	CYP2C19	CYP2D6	CYP2R1	CYP2U1
CYP3A4	CYP46A1	CYP4X1	CYP7B1	D2HGDH	DAB1	DAB2IP	DAG1	DAGLA
DAO	DAOA	DAPK1	DAPK3	DARS	DARS2	DAXX	DAZAP2	DBI
DBN1	DBNL	DBX1	DBX2	DCAF17	DCC	DCDC1	DCDC2	DCHS2
DCK	DCLK1	DCLK2	DCTN1	DCX	DDC	DDHD1	DDIT3	DDIT4
DDR1	DDX20	DDX54	DDX56	DDX6	DEF6	DEFB4A	DEPDC5	DGKG
DGKH	DGUOK	DHCR24	DHX9	DIABLO	DICER1	DIO2	DIO3	DIP2B
DIRAS2	DISC1	DISC1FP1	DISP3	DIXDC1	DKK1	DKK4	DLD	DLG1
DLG4	DLGAP2	DLL1	DLX1	DLX2	DLX5	DMBT1	DMBT1P1	DMBX1
DMD	DMRTA2	DNAJB2	DNAJC15	DNAJC5	DNAL4	DNER	DNHD1	DNM1L
DNM2	DNMT1	DNMT3A	DNMT3B	DOC2A	DOCK6	DOCK7	DOPEY2	DPP4
DPYD	DPYSL2	DPYSL3	DPYSL4	DRAXIN	DRD1	DRD2	DRD3	DRD4
DRD5	DRGX	DRP2	DSC1	DSC3	DSCAM	DSCAML1	DTNB	DTNBP1
DTX1	DUOXA1	DUSP1	DUSP4	DUSP5	DUSP8	DVL1	DYM	DYNC1H1
DYNC2H1	DYNLT1	DYRK1A	DYX1C1	E2F1	EBP	ECE1	ECE2	ECT2
EDN1	EDN2	EDN3	EDNRB	EEF1A1	EEF1A2	EEF2	EEF2K	EFEMP1
EFHC1	EFHD1	EFNA1	EFNA3	EFNA4	EFNB3	EGF	EGFL6	EGFLAM
EGFR	EGLN1	EGLN2	EGLN3	EGR1	EGR2	EGR3	EHD1	EHF
EIF2AK4	EIF2B5	EIF2S1	EIF4A3	EIF4E	EIF4EBP1	EIF4EBP2	EIF4ENIF1	EIF4G1
ELANE	ELAVL2	ELAVL3	ELAVL4	ELK1	ELOVL2	ELP3	ELP4	ELSPBP1
EML1	EMP1	EMX1	EMX2	EN1	EN2	ENAH	ENC1	ENDOG
ENG	ENKD1	ENO1	ENO2	ENPEP	ENPP6	EOMES	EPAS1	EPB41L1
EPB41L3	EPCAM	EPHA2	EPHA3	EPHA4	EPHA5	EPHA7	EPHA8	EPHB1
EPHB2	EPHB3	EPHB4	EPHX2	EPM2A	EPM2AIP1	EPN2	EPO	EPOP
EPOR	ERBB2	ERBB3	ERBB4	ERCC1	ERCC6	ERCC6L2	ERLIN2	ERMARD
ERMN	ERVW-1	ESCO2	ESM1	ESR1	ESR2	ETHE1	ETS1	ETV1

ETV4	ETV5	EYA1	EYA4	EZH2	EZR	F11R	F13A1	F2
F2R	F2RL1	F3	F5	FA2H	FAAH	FABP3	FABP4	FABP5
FABP7	FABP7P1	FABP7P2	FADD	FADS1	FAIM2	FAM107A	FAM120C	FAM126A
FAM134B	FAM134C	FAM150A	FAM150B	FAM162A	FAM19A1	FAM19A2	FAM19A3	FAM19A4
FAM19A5	FANCD2	FARP2	FAS	FASLG	FBL	FBXO38	FBXO45	FBXO7
FBXW7	FCGR1A	FCGR3A	FCN3	FES	FEV	FEZ1	FEZF1	FEZF2
FGD1	FGF10	FGF13	FGF17	FGF19	FGF2	FGF20	FGF21	FGF23
FGF5	FGF7	FGF8	FGF9	FGFR1	FGFR2	FGFRL1	FGGY	FH
FHIT	FHL5	FIG4	FILIP1	FKBP1A	FKBP4	FKBP5	FKRP	FKTN
FLNA	FLRT1	FLRT2	FLRT3	FLT1	FLT3LG	FLVCR2	FMO1	FMR1
FN1	FNDC5	FOLH1	FOLR1	FOS	FOSB	FOSL1	FOXA1	FOXA2
FOXB1	FOXC1	FOXF2	FOXG1	FOXH1	FOXJ1	FOXMI	FOXO3	FOXP1
FOXP2	FOXP3	FPGS	FRAT1	FRMD7	FRRS1L	FRY	FRYL	FSHR
FST	FSTL3	FSTL4	FTH1	FTL	FTO	FUOM	FURIN	FUS
FUT4	FXN	FYN	FZD1	FZD10	FZD2	FZD3	FZD4	FZD5
FZD7	FZD8	FZD9	G6PC	G6PD	GAB2	GABBR1	GABBR2	GABPB1
GABRA1	GABRA2	GABRA3	GABRA4	GABRA5	GABRA6	GABRB1	GABRB2	GABRB3
GABRD	GABRG2	GABRG3	GABRR1	GABRR2	GABRR3	GAD1	GAD2	GADD45A
GAK	GALNT9	GALR1	GALR2	GALT	GAMT	GAN	GAP43	GAPDH
GAR1	GAREM1	GARS	GAS6	GAS7	GATA2	GATA3	GATA4	GATA6
GATM	GBA	GBA2	GBE1	GBP2	GBP4	GBX1	GBX2	GCG
GCH1	GCK	GCLC	GCLM	GCSH	GDF11	GDF5	GDF6	GDF7
GDI1	GDI2	GNDF	GDPD5	GEM	GEMIN2	GFAP	GF11	GF11B
GFRA1	GFRA2	GFRA3	GFRAL	GGA3	GGT5	GH1	GHR	GHRH
GHRL	GHSR	GIGYF2	GIT1	GJA1	GJB2	GJC2	GLCE	GLDC
GLG1	GLI2	GLI3	GLIPR2	GLO1	GLP1R	GLRA1	GLRX2	GLRX3
GLS	GLS2	GLTP	GLUD1	GLUD2	GLUL	GMDS	GMFB	GMFBP1
GMFG	GMPPB	GNA13	GNAI2	GNAL	GNAO1	GNAQ	GNAS	GNL3
GNMT	GNRH1	GNRHR	GP1BB	GPATCH1	GPC1	GPC2	GPC3	GPC4
GPHN	GPI	GPM6A	GPM6B	GPMB	GPR1	GPR132	GPR137B	GPR139
GPR148	GPR149	GPR162	GPR173	GPR27	GPR37	GPR37L1	GPR4	GPR61
GPR63	GPR75	GPR85	GPRASP1	GPRC5B	GPRC6A	GPRIN1	GPS2	GPSM2
GPT	GPX1	GRB10	GRHL2	GRIA1	GRIA2	GRIA3	GRIA4	GRID1
GRID2	GRIK1	GRIK2	GRIK3	GRIK5	GRIN1	GRIN2A	GRIN2B	GRIN2C
GRIN2D	GRIN3A	GRIP1	GRK1	GRK2	GRK3	GRM1	GRM2	GRM3
GRM4	GRM5	GRM6	GRM7	GRM8	GRN	GRP	GRPR	GSK3A
GSK3B	GSN	GSTM1	GSTM3	GSTM3P1	GSTO1	GSTO2	GSTP1	GSTT1
GSX1	GSX2	GUCY1B3	GUSB	GYPC	GZMB	H2AFY2	H3F3A	H3F3B
HAAO	HACE1	HAMP	HAND2	HAP1	HAPLN2	HAPLN4	HAVCR1	HBA2
HBB	HCFC1	HCN1	HCN2	HCN4	HCRT	HDAC1	HDAC11	HDAC2
HDAC4	HDAC5	HDAC6	HDAC9	HDGFRP3	HELT	HEPH	HERC1	HES1

HES3	HES5	HESX1	HEXA	HEXB	HEY1	HEYL	HFE	HGF
HGH1	HGS	HID1	HIF1A	HINT1	HIP1	HIPK1	HIPK2	HIST3H3
HK1	HK2	HLA-A	HLA-B	HLA-DQB1	HLA-DRA	HLA-DRB1	HLA-DRB5	HMCES
HMG20A	HMG20B	HMGB1	HMGCS1	HMOX1	HMP19	HMX2	HMX3	HN1
HNMT	HNRNPA1	HNRNPA2B1	HNRNPA3	HOMER1	HOPX	HOXA2	HOXC10	HOXC8
HOXD1	HOXD10	HOXD3	HOXD9	HP	HPCA	HPCAL1	HPRT1	HPSE
HPX	HRAS	HRH1	HRH3	HS3ST2	HS3ST4	HS3ST5	HS6ST1	HSD11B1
HSD11B1L	HSD17B10	HSD17B4	HSF1	HSP90AA1	HSP90AB1	HSP90B1	HSPA12A	HSPA1A
HSPA4	HSPA5	HSPA8	HSPA9	HSPB1	HSPB11	HSPB2	HSPBAP1	HSPD1
HSPG2	HTR1A	HTR1B	HTR2A	HTR2B	HTR2C	HTR3A	HTR3B	HTR6
HTR7	HTRA1	HTRA2	HTT	HVCN1	HYAL2	HYDIN	HYMAI	HYOU1
IAPP	ICAM1	ICAM5	ID1	ID2	ID3	ID4	IDE	IDH1
IDH2	IDO1	IDUA	IER2	IER3IP1	IFI30	IFNA1	IFNA2	IFNAR1
IFNB1	IFNG	IFT172	IGF1	IGF1R	IGF2	IGFBP1	IGFBP2	IGFBP3
IGFBP6	IGFBP7	IGFL1	IGSF11	IKBKKG	IL10	IL12A	IL12B	IL13
IL13RA2	IL15	IL15RA	IL17A	IL17C	IL18	IL1A	IL1B	IL1R1
IL1RAPL1	IL1RL1	IL1RN	IL2	IL20	IL2RA	IL3	IL34	IL4
IL6	IL6R	IL7	ILK	IMMP2L	IMMT	IMPA2	IMPACT	ING4
INHA	INHBA	INHBC	INPP5E	INPP5J	INPP5K	INS	INSM1	INSR
IQGAP1	IRAK1BP1	IRAK4	IRF2BPL	IRF3	IRS2	IRS4	IRX3	IRX5
ISCA1	ISG15	ISL1	ISL2	ISPD	ITGA1	ITGA2	ITGA3	ITGA4
ITGA5	ITGA6	ITGA7	ITGA8	ITGAE	ITGAM	ITGAV	ITGAX	ITGB1
ITGB2	ITGB3	ITGB4	ITGB5	ITGB6	ITGB8	ITM2B	ITM2C	ITPKB
ITSN1	JAG1	JAK2	JAM2	JAM3	JMY	JUN	KANK1	KAT6B
KATNB1	KCNA3	KCNA6	KCNAB1	KCNB1	KCNC1	KCNC2	KCNC4	KCNE2
KCNE4	KCNF1	KCNG3	KCNG4	KCNH1	KCNH2	KCNH3	KCNH4	KCNH5
KCNIP3	KCNIP4	KCNJ10	KCNJ11	KCNJ13	KCNJ15	KCNJ2	KCNJ3	KCNJ6
KCNK10	KCNK18	KCNK2	KCNK3	KCNK9	KCNMA1	KCNN1	KCNN2	KCNN3
KCNN4	KCNQ1	KCNQ2	KCNQ3	KCNQ5	KCNS3	KCNV1	KCP	KCTD11
KCTD12	KCTD4	KCTD8	KDM1A	KDM2B	KDM4A	KDR	KDSR	KIAA0319
KIAA0408	KIAA1191	KIAA2022	KIDINS220	KIF14	KIF16B	KIF1B	KIF20B	KIF2A
KIF3A	KIF5A	KIF5B	KIF5C	KIF7	KIFAP3	KIFC1	KIRREL3	KISS1
KIT	KITLG	KL	KLC1	KLC3	KLF4	KLF6	KLHL1	KLHL14
KLHL17	KLHL41	KLK11	KLK6	KLK8	KLRG2	KLRK1	KNL1	KPNA1
KRAS	KRIT1	KRT18	KRT19	KRT20	KRT5	KRT75	KRTAP5-8	KYAT1
KYNU	L1CAM	L2HGDH	L3MBTL1	L3MBTL2	L3MBTL3	L3MBTL4	LAMA1	LAMA2
LAMA4	LAMB1	LAMB2	LAMB3	LAMC1	LAMP2	LAMP5	LARGE1	LARS2
LBP	LBX1	LCN2	LDB1	LDHA	LDLR	LEF1	LEP	LEPR
LETM1	LGALS1	LGALS3	LGI1	LGI3	LGI4	LGMN	LGR5	LHPP
LHX1	LHX3	LHX4	LHX5	LHX8	LHX9	LIF	LIG4	LIMK1

LIMS1	LIN28A	LIN7A	LIN7B	LIN7C	LINC00271	LINC00299	LINGO1	LINS1
LIPG	LIX1	LMNA	LMNB1	LMO3	LMO4	LMTK2	LMX1A	LMX1B
LOC100131395	LOC100132090	LOC100287944	LOC100506023	LOC100507140	LOC101927525	LOC400927-CSNK1E	LOC642862	LOC642969
LOC643387	LOC643576	LOC654338	LOC729800	LPAR1	LPAR2	LPCAT4	LPIN1	LPL
LRG1	LRP1	LRP12	LRP4	LRP6	LRRC15	LRRC34	LRRC4	LRRC7
LRRK2	LRRTM1	LSAMP	LSM1	LSM4	LSR	LTBP1	LTBP2	LTF
LTK	LUC7L2	LUZP2	LY75	LYN	MACROD2	MAFB	MAG	MAGEA3
MAGED4B	MAGEE1	MAGI1	MAGI2	MALAT1	MALRD1	MAML3	MANF	MAOA
MAOB	MAP1A	MAP1B	MAP1LC3A	MAP1S	MAP2	MAP2K1	MAP2K4	MAP3K11
MAP3K12	MAP3K5	MAP4	MAP7D2	MAPK1	MAPK10	MAPK14	MAPK3	MAPK8
MAPK8IP1	MAPK8IP2	MAPK8IP3	MAPK9	MAPT	MARK1	MARK2	MARK3	MARK4
MARVELD2	MATK	MATN2	MATR3	MAX	MBD1	MBD3	MBL2	MBNL2
MBP	MC1R	MC4R	MCAM	MCL1	MCM3	MCM3AP	MCPH1	MCU
MDGA1	MDGA2	MDK	MDM2	MECP2	MED1	MED12	MED13L	MED17
MED20	MEF2C	MEGF10	MEGF8	MEIS1	MEIS2	MELK	MEN1	MEOX2
MEP1B	MET	MFN2	MFSD2A	MGAT5B	MGLL	MGMT	MIB1	MICALL1
MICALL2	MICU1	MID1	MIF	MIR107	MIR10B	MIR124-1	MIR125A	MIR125B1
MIR132	MIR134	MIR137	MIR144	MIR145	MIR146A	MIR149	MIR155	MIR181C
MIR191	MIR196A2	MIR197	MIR200B	MIR203A	MIR21	MIR210	MIR218-1	MIR218-2
MIR27A	MIR30A	MIR30C1	MIR30C2	MIR320A	MIR328	MIR339	MIR33A	MIR34A
MIR34B	MIR34C	MIR378A	MIR4669	MIR499A	MIR509-1	MIR511	MIR7-1	MIR9-2
MIR92B	MIR93	MIR95	MKKS	MKL1	MKL2	MKS1	MLC1	MLH1
MLIP	MMD	MMD2	MME	MMEL1	MMP1	MMP10	MMP12	MMP14
MMP19	MMP2	MMP3	MMP7	MMP9	MNX1	MOB2	MOG	MORC1
MOXD1	MPC1	MPC1L	MPC2	MPG	MPL	MPO	MPPED1	MPPED2
MPST	MRC1	MRGPRE	MRGPRX1	MRGPRX3	MRGPRX4	MRI1	MSH2	MSH6
MSN	MSR1	MSRB2	MST1	MST1L	MST1R	MSTN	MT3	MT-CYB
MTDH	MTHFD1L	MTHFR	MTMR2	MT-ND5	MTNR1A	MTNR1B	MTOR	MTPN
MTRR	MT-TK	MTUS1	MUC1	MUC16	MUSK	MUT	MVP	MX1
MXRA8	MYC	MYCN	MYD88	MYEF2	MYH10	MYH2	MYL1	MYL10
MYL12A	MYL12B	MYL2	MYL3	MYL4	MYL5	MYL6	MYL6B	MYL7
MYL9	MYLIP	MYLK	MYO10	MYO1D	MYO5A	MYOC	MYOCD	MYT1
NAAA	NAALAD2	NAB2	NACA	NAE1	NAGLU	NAIP	NAMPT	NANOG
NANOS1	NAP1L2	NAPA	NAT2	NAT8L	NAT9	NAV1	NAV2	NAV3
NBEA	NBEAL1	NBL1	NCAM1	NCAM2	NCAN	NCAPD3	NCDN	NCF1
NCK1	NCKAP5	NCKIPSD	NCOA1	NCOA2	NCOA3	NCOA6	NCR1	NCS1
NCSTN	NDE1	NDEL1	NDN	NDNF	NDRG1	NDRG2	NDRG3	NDRG4
NDUFS4	NDUFS7	NEB	NECAB3	NECTIN1	NEDD4	NEDD4L	NEFH	NEFL
NEFM	NEGR1	NEK3	NELL2	NENF	NEPRO	NES	NETO1	NETO2
NEURL1	NEURL1B	NEUROD1	NEUROD2	NEUROD4	NEUROD6	NEUROG1	NEUROG2	NEUROG3

NF1	NF2	NFASC	NFE2L2	NFIA	NFIB	NFKB1	NFKBIA	NFKBIZ
NGB	NGF	NGFR	NGRN	NHLRC1	NHS	NICN1	NIF3L1	NINJ2
NIPBL	NKX2-1	NKX2-2	NKX2-5	NKX6-1	NKX6-2	NLGN1	NLGN2	NLGN3
NLGN4X	NLGN4Y	NLN	NLRP1	NLRP5	NLRP8	NMB	NME1	NME2
NMI	NMNAT2	NNAT	NNMT	NNT	NODAL	NOG	NONO	NOP56
NOS1	NOS2	NOS3	NOTCH1	NOTCH2	NOTCH3	NOTCH4	NOVA1	NOVA2
NOX4	NPAP1	NPAS3	NPAS4	NPBWR1	NPBWR2	NPC1	NPEPPS	NPHP1
NPM1	NPPA	NPPB	NPR1	NPR3	NPSR1	NPTN	NPW	NPY
NPY5R	NQO1	NQO2	NR1H2	NR1H3	NR1I2	NR1I3	NR2E1	NR2F1
NR2F2	NR2F6	NR3C1	NR3C2	NR4A1	NR4A2	NR4A3	NRAS	NRBP2
NRCAM	NRDC	NREP	NRG1	NRG3	NRGN	NRN1L	NRP1	NRP2
NRSN1	NRTN	NRXN1	NRXN2	NRXN3	NSD1	NSDHL	NSF	NSG1
NSMF	NT5E	NTF3	NTF4	NTM	NTN1	NTN4	NTNG1	NTNG2
NTRK1	NTRK2	NTRK3	NTS	NTSR1	NTSR2	NUAK2	NYAP1	NYAP2
OCLN	OCRL	OFD1	OGDH	OGG1	OLFM1	OLFM3	OLIG1	OLIG2
OLIG3	OLR1	ONECUT2	OPA1	OPCML	OPHN1	OPN3	OPN4	OPN5
OPRD1	OPRK1	OPRL1	OPRM1	OPTN	ORAI1	ORM1	OSBPL1 A	OSMR
OTOF	OTP	OTX1	OTX2	OXCT1	OXR1	OXT	OXTR	P2RX1
P2RX4	P2RX5	P2RX7	P2RY1	P2RY11	P2RY12	P2RY2	P2RY4	P2RY6
P4HB	PABPC1	PACRG	PACSIN1	PADI2	PAFAH1B1	PAFAH1B2	PAFAH1 B3	PAK1
PAK3	PAK5	PAM	PANK2	PANX2	PAPD4	PAPSS1	PAQR3	PAQR8
PARK2	PARK7	PARP1	PATZ1	PAX2	PAX5	PAX6	PAX7	PBX1
PBX3	PBXIP1	PCDH10	PCDH11X	PCDH11Y	PCDH12	PCDH15	PCDH17	PCDH18
PCDH19	PCDH20	PCDH7	PCDHA@	PCDHA1	PCDHA10	PCDHA11	PCDHA1 2	PCDHA1 3
PCDHA2	PCDHA3	PCDHA4	PCDHA5	PCDHA6	PCDHA7	PCDHA8	PCDHA9	PCDHAC 1
PCDHAC2	PCDHB11	PCDHB4	PCDHG@	PCDHGA1	PCDHGA10	PCDHGA11	PCDHGA 12	PCDHGA 2
PCDHGA3	PCDHGA4	PCDHGA5	PCDHGA6	PCDHGA7	PCDHGA8	PCDHGA9	PCDHGB 1	PCDHGB 2
PCDHGB3	PCDHGB4	PCDHGB5	PCDHGB6	PCDHGB7	PCDHGC3	PCDHGC4	PCDHGC 5	PCK1
PCLO	PCM1	PCP4	PCSK1	PCSK2	PCSK5	PCSK6	PCSK7	PCSK9
PDCD1	PDCD10	PDCD1LG2	PDE10A	PDE2A	PDE4A	PDE4B	PDE7A	PDGFA
PDGFB	PDGFD	PDGFRA	PDGFRB	PDIA3	PDK1	PDLIM5	PDPK1	PDXK
PDXP	PDYN	PEA15	PECAM1	PELP1	PENK	PER1	PER2	PER3
PEX13	PEX7	PFKFB3	PFKFB4	PFN1	PGAM1	PGAM1P8	PGAM2	PGBD1
PGF	PGR	PHACTR3	PHC1	PHF20	PHF20L1	PHF21A	PHF8	PHGDH
PHOX2A	PHOX2B	PHPT1	PI4K2A	PIAS3	PICALM	PICK1	PIGT	PIK3C2A
PIK3C2B	PIK3C2G	PIK3C3	PIK3CA	PIK3CB	PIK3CD	PIK3CG	PIK3R1	PIK3R2
PIM1	PIN1	PINK1	PIP5K1C	PISD	PITPNM1	PITRM1	PITX2	PITX3
PJA1	PKIA	PKIB	PKM	PLA1A	PLA2G1B	PLA2G2A	PLA2G4A	PLA2G6
PLA2G7	PLAGL1	PLAT	PLAU	PLAUR	PLBD1	PLCB1	PLCD3	PLCG1
PLCH2	PLCL1	PLD1	PLEKHA5	PLEKHB1	PLEKHG5	PLG	PLK1	PLK5

PLP1	PLPPR1	PLPPR4	PLPPR5	PLTP	PLVAP	PLXNA1	PLXNA2	PLXNA3
PLXNA4	PLXNB1	PLXNB2	PLXNB3	PLXNC1	PLXND1	PM20D1	PMAIP1	PMCH
PML	PMM1	PMP22	PMS1	PMS2	PNKD	PNMA1	PNMA3	PNPLA2
PNPLA6	PNPLA7	POLB	POLG	POLR2A	POLR3A	POLR3B	POMGNT1	POMGNT2
POMK	POMT1	POMT2	PON1	POSTN	POU3F2	POU3F3	POU3F4	POU4F1
POU4F2	POU4F3	POU5F1	POU6F1	PPA1	PPARA	PPARG	PPARGC1A	PPEF1
PPFIA1	PPFIA2	PPID	PPIL4	PPM1E	PPM1K	PPP1CA	PPP1CC	PPP1R12A
PPP1R12B	PPP1R13B	PPP1R14C	PPP1R14D	PPP1R15A	PPP1R1B	PPP1R9A	PPP1R9B	PPP2CA
PPP2R2A	PPP2R2B	PPP2R2C	PPP2R3A	PPP2R5B	PPP3CA	PPP3CC	PPP5C	PPT1
PPY	PQBP1	PRAF2	PRCP	PRDM12	PRDX1	PRDX5	PRDX6	PREP
PRICKLE1	PRICKLE2	PRKACA	PRKACB	PRKACG	PRKAR2B	PRKCA	PRKCB	PRKCDBP
PRKCE	PRKCG	PRKCH	PRKCI	PRKCZ	PRKD1	PRKDC	PRKG1	PRL
PRLHR	PRMT1	PRMT6	PRNP	PROC	PROCR	PRODH	PROM1	PROS1
PROX1	PROX2	PRPF19	PRR12	PRRT2	PRRX1	PRSS1	PRSS12	PRSS22
PRSS3	PRSS33	PSEN1	PSEN2	PSENE	PSMC1	PSPH	PTBP1	PTBP2
PTCH1	PTEN	PTF1A	PTGDR2	PTGDS	PTGES3	PTGS1	PTGS2	PTH2
PTH2R	PTK2	PTK2B	PTK6	PTK7	PTN	PTPA	PTPN11	PTPN13
PTPN18	PTPN2	PTPN23	PTPN5	PTPRB	PTPRD	PTPRF	PTPRG	PTPRH
PTPRK	PTPRM	PTPRN2	PTPRO	PTPRS	PTPRT	PTTG1	PTX3	PURA
PWARSN	PWRN1	PXK	PYGB	PYGL	PYGM	PYGO2	PYY	QARS
QDPR	QKI	QPCT	QPRT	QRFP	RAB11A	RAB13	RAB18	RAB29
RAB35	RAB39B	RAB3B	RAB3C	RAB3GAP1	RAB3GAP2	RAB43	RAB44	RAB5A
RAB6B	RAB7B	RAC1	RAC2	RAC3	RACK1	RAD51	RAF1	RALBP1
RAP1A	RAP1GAP	RAP1GAP2	RAPGEF1	RAPGEF2	RAPGEF3	RAPGEF4	RAPSN	RARA
RARB	RASA1	RASEF	RASGRF1	RASGRF2	RASGRP2	RASSF6	RB1	RB1CC1
RBBP8	RBFOX2	RBFOX3	RBL1	RBM38	RBM4	RBM45	RCAN1	RCOR1
RECQL	REEP1	REG3A	REL	RELA	RELN	REN	RERE	REST
RET	RETN	RFX4	RFX7	RGN	RGS17	RGS2	RGS21	RGS4
RHBDD1	RHEB	RHEBL1	RHEBP1	RHEBP3	RHOA	RHOBTB2	RHOC	RIBC2
RIC3	RIC8B	RIDA	RILPL1	RIT1	RIT2	RLN2	RLN3	RNASEL
RND1	RNF112	RNF135	RNF165	RNF17	RNF181	RNF213	RNF40	RNLS
ROBO1	ROBO2	ROCK1	ROGDI	RORA	RORB	ROS1	RP2	RPGRIP1L
RPH3A	RPL10	RPP25	RPS15	RPS6	RPS6KA2	RPS6KA3	RPS6KB1	RPSA
RRAS2	RRN3	RSPH14	RSPO2	RTEL1	RTKN	RTN4	RTN4R	RUNDC3B
RUNX1	RUNX2	RUNX3	RXRA	RXRG	RYK	RYR3	S100A1	S100A10
S100A4	S100A5	S100A7	S100B	S1PR1	S1PR5	SACS	SAMD12	SAMHD1
SARM1	SAT1	SATB2	SCARB2	SCARF1	SCG2	SCGN	SCML2	SCN11A
SCN1A	SCN1B	SCN2A	SCN3A	SCN7A	SCN8A	SCN9A	SCNN1D	SCRG1
SCRT1	SCRT2	SCT	SDCBP	SDCCAG8	SEBOX	SECISBP2	SEL1L3	SELENBP1
SELENOM	SELENON	SELENOP	SELL	SELPLG	SEMA3A	SEMA3E	SEMA3F	SEMA5B

SEMA6A	SEPT2	SEPT4	SERAC1	SERP1	SERPINA1	SERPINA3	SERPINB3	SERPINB5
SERPINE1	SERPINE2	SERPINF1	SERPINF2	SERPINI1	SET	SETDB1	SETX	SEZ6L2
SF3A2	SFMBT1	SFRP1	SFRP2	SGCE	SGK1	SGMS1	SH3BP1	SH3GL2
SH3KBP1	SHANK1	SHANK2	SHANK3	SHARPIN	SHBG	SHC3	SHH	SHROOM2
SHROOM4	SHTN1	SIAH1	SIAH2	SIGLEC11	SIGMAR1	SIM1	SIN3A	SIPA1L1
SIRPA	SIRT1	SIX1	SIX3	SIX4	SIX6	SKI	SKOR1	SKOR2
SLC10A4	SLC11A2	SLC12A2	SLC12A5	SLC12A6	SLC13A3	SLC16A1	SLC16A12	SLC16A2
SLC17A6	SLC17A7	SLC17A8	SLC18A1	SLC18A2	SLC19A3	SLC1A1	SLC1A2	SLC1A3
SLC1A4	SLC1A6	SLC20A2	SLC22A1	SLC22A15	SLC22A16	SLC22A17	SLC22A18A5	SLC22A2
SLC22A3	SLC23A1	SLC23A2	SLC24A2	SLC24A4	SLC25A13	SLC25A14	SLC25A14P1	SLC25A15
SLC25A23	SLC25A27	SLC26A1	SLC29A1	SLC29A4	SLC2A1	SLC2A13	SLC2A2	SLC2A3
SLC2A4	SLC30A1	SLC30A10	SLC30A3	SLC30A6	SLC32A1	SLC37A4	SLC38A10	SLC38A2
SLC38A3	SLC39A1	SLC39A12	SLC39A14	SLC39A8	SLC39A9	SLC40A1	SLC46A1	SLC4A10
SLC4A3	SLC4A4	SLC4A7	SLC4A8	SLC51B	SLC52A2	SLC5A5	SLC5A6	SLC5A7
SLC6A1	SLC6A11	SLC6A12	SLC6A13	SLC6A15	SLC6A17	SLC6A2	SLC6A3	SLC6A4
SLC6A7	SLC6A8	SLC6A9	SLC7A11	SLC8A1	SLC8A3	SLC9A1	SLC9A6	SLCO1A2
SLCO1B1	SLCO1C1	SLCO2A1	SLCO3A1	SLIT1	SLIT2	SMAD1	SMAD2	SMAD4
SMAD6	SMARCA1	SMARCA1	SMARCB1	SMARCC2	SMG1	SMG9	SMN1	SMN2
SMNDC1	SMNP	SMO	SMOC1	SMPD1	SMS	SMU1	SMURF1	SMURF2
SNAP23	SNAP25	SNAPIN	SNCA	SNCAIP	SNCB	SND1-IT1	SNORD109A	SNORD109B
SNORD115-1	SNORD118	SNORD64	SNPH	SNRNP70	SNRPB	SNRPB2	SNRPD1	SNRPD2
SNRPD3	SNRPE	SNRPF	SNRPG	SNRPN	SNTG1	SNX12	SNX3	SOAT1
SOCS1	SOCS3	SOCS7	SOD1	SOD2	SOD3	SORBS1	SORCS3	SORL1
SORT1	SOX1	SOX10	SOX11	SOX14	SOX17	SOX2	SOX3	SOX4
SOX8	SOX9	SP1	SP100	SPAG9	SPARC	SPARCL1	SPAST	SPATA5
SPDEF	SPEF1	SPEF2	SPEN	SPG11	SPG7	SPHK1	SPHK2	SPINT1
SPINT2	SPOCK1	SPOCK2	SPP1	SPTA1	SPTAN1	SPTBN1	SPTBN2	SPTBN4
SPTBN5	SRC	SREBF2	SREK1	SREK1IP1	SRF	SRGAP2	SRGAP2B	SRGAP2C
SRGAP2D	SRI	SRPK1	SRPK2	SRPX2	SRR	SRRD	SRSF1	SS18L1
SST	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	SSX4	ST14	ST3GAL3
ST5	ST6GAL2	ST8SIA5	STAMPB	STAR	STARD13	STAT1	STAT3	STAT5A
STAT6	STC1	STEAP4	STIL	STIM1	STIM2	STIP1	STK11	STK31
STK32C	STK36	STK39	STMN1	STMN2	STMN3	STMN4	STON2	STOX1
STS	STUB1	STX1A	STX1B	STX3	STXBP1	STXBP3	SUB1	SUCLA2
SUCLG2	SUCNR1	SUFU	SULT1A1	SULT1A3	SULT1C4	SULT4A1	SUMO1	SUOX
SURF1	SUV39H1	SV2A	SV2B	SV2C	SYK	SYN1	SYN2	SYN3
SYNGAP1	SYNGR1	SYNJ1	SYNPR	SYP	SYT1	SYT11	SYT4	SYT5
SYVN1	SZT2	TAAR1	TAAR2	TAAR3	TAAR4P	TAAR5	TAAR6	TAAR7P
TAAR8	TAAR9	TAB2	TAC1	TAC3	TACR1	TAF1	TAGLN3	TAL1
TAP1	TAP2	TARBP1	TARDBP	TAS2R20	TBATA	TBC1D1	TBC1D15	TBC1D24

TBC1D4	TBCE	TBCK	TBK1	TBP	TBR1	TBRG4	TBX1	TBX20
TBX6	TC2N	TCEAL1	TCEAL2	TCEAL3	TCEAL4	TCEAL5	TCEAL6	TCEAL7
TCEAL8	TCEAL9	TCF12	TCF3	TCF4	TCF7L2	TCTN1	TCTN2	TCTN3
TDP2	TDRD1	TDRD12	TDRD15	TDRD3	TDRD5	TDRD6	TDRD7	TDRD9
TDRKH	TENM1	TENM2	TENM3	TENM4	TERF1	TERT	TF	TFAM
TFAP2A	TFAP2B	TFAP2D	TFRC	TGFB1	TGFB2	TGFB3	TGFB1	TGFB1
TGFB2	TGIF1	TGIF2	TGM1	TGM2	TH	THAP1	THBD	THBS2
THBS4	THOC2	THOP1	THRB	THY1	TIA1	TIAL1	TIAM1	TIAM2
TIGAR	TIGD4	TIMP1	TIMP2	TJP1	TJP2	TK1	TLE1	TLE6
TLL1	TLN2	TLR2	TLR3	TLR4	TLR7	TLR8	TLR9	TLX3
TM2D1	TM4SF20	TMBIM6	TMEFF1	TMEFF2	TMEM106B	TMEM119	TMEM132D	TMEM138
TMEM158	TMEM18	TMEM212	TMEM216	TMEM231	TMEM237	TMEM240	TMEM257	TMEM259
TMEM30A	TMEM47	TMEM5	TMEM57	TMEM59	TMEM59L	TMEM67	TMEM87B	TMOD2
TMX2	TNC	TNF	TNFAIP3	TNFAIP6	TNFRSF10A	TNFRSF10B	TNFRSF10C	TNFRSF10D
TNFRSF11A	TNFRSF12A	TNFRSF1A	TNFRSF1B	TNFRSF21	TNFRSF6B	TNFRSF9	TNFSF10	TNFSF12
TNFSF13	TNFSF13B	TNFSF9	TNIK	TNNI3	TNNT2	TNR	TOMM40	TOP2B
TOPBP1	TOR1A	TOX3	TP53	TP53BP2	TP63	TP73	TPH1	TPH2
TPM1	TPP1	TPPP	TPPP3	TPSAB1	TRA	TRA2B	TRAF2	TRAF3
TRAF3IP2	TRANK1	TRAPPC9	TREM2	TREX1	TRIM2	TRIM27	TRIM3	TRIM32
TRIM37	TRIM67	TRIM9	TRPC3	TRPC5	TRPC6	TRPM2	TRPM3	TRPV1
TRPV3	TRPV4	TRRAP	TSC1	TSC2	TSGA10	TSN	TSNAX	TSPAN12
TSPAN2	TSPO	TTBK1	TTC12	TTC21B	TTPA	TTR	TTYH1	TTYH2
TUBA1A	TUBA8	TUBB	TUBB2A	TUBB2B	TUBB3	TUBG1	TUG1	TULP3
TUNAR	TUSC3	TWF2	TWIST1	TWINK	TXN	TXNL4A	TYMP	TYRO3
TYROBP	UBA1	UBB	UBC	UBE2D1	UBE2M	UBE2V2	UBE3A	UBE4B
UBQLN1	UBQLN2	UBXN11	UCHL1	UCN	UCN2	UCN3	UCP2	UCP3
UFL1	UGCG	UGT8	UHMK1	ULK1	ULK4	UNC13A	UNC5A	UNC5B
UNC5C	UNC5D	UNK	UPK3A	UQCRQ	USF1	USP11	USP2	USP21
USP9X	UTP11	UTP3	UTRN	UTS2	UTS2R	VAMP1	VAMP2	VAMP3
VAMP7	VANGL2	VAPA	VAPB	VASP	VAX1	VCAM1	VCP	VCX
VCX2	VCX3A	VCX3B	VCY	VCY1B	VDAC1	VDR	VEGFA	VEGFC
VEGFD	VGf	VHL	VHLL	VIM	VIP	VIT	VLDLR	VPS35
VSNL1	VSTM2L	VSX1	VTI1A	VTN	VWA3B	VWC2	VWC2L	VWF
WAS	WASHC2A	WASHC2C	WASL	WDR45	WDR5	WDR6	WDR62	WDR73
WDR81	WDR83	WEE1	WFDC2	WFS1	WHSC1	WNK1	WNK2	WNK3
WNT1	WNT10A	WNT10B	WNT11	WNT16	WNT2	WNT2B	WNT3	WNT3A
WNT4	WNT5A	WNT5B	WNT6	WNT7A	WNT7B	WNT8A	WNT8B	WNT9A
WNT9B	WRB	WRN	WT1	WWC1	WWOX	XBP1	XDH	XIAP
XPR1	XRCC1	XRCC2	XRCC3	XRCC4	XRCC5	XRCC6	XRN2	YBX1
YBX3	YEATS4	YWHAB	YWHAE	YWHAG	YWHAH	YWHAQ	YWHAZ	ZAN

ZBTB18	ZC3H12A	ZC4H2	ZEB1	ZEB2	ZFH3	ZFP36	ZFP57	ZFP64
ZFYVE27	ZGPAT	ZHX2	ZIC1	ZIC2	ZIC5	ZKSCAN1	ZMYM3	ZMYND1 1
ZMYND19	ZNF175	ZNF207	ZNF24	ZNF250	ZNF284	ZNF335	ZNF33A	ZNF354C
ZNF365	ZNF417	ZNF433	ZNF468	ZNF521	ZNF536	ZNF568	ZNF746	ZNF778
ZNF804A	ZPR1	ZSWIM6	ZYX					

Supp. Table 2: Primers used for real-time quantitative PCR experiments with *TBCK* (NM_001163435) on genomic DNA.

	Forward	Reverse
TBCK_qPCR_mut_1	TGTTGAAACACCTACATGGCATAG	ACTCCTCCAGGTAGCAAGTTTTG
TBCK_qPCR_2	CCCAAATACATTCCGTTCAAAAAC	AGTGCCAAAGTCTTTCCCATCAG
TBCK_qPCR_3	GACCAAGGAGTGAGGGTCAGGG	GCTTCGGTTATTAGCTCCAGCCC
TBCK_qPCR_4	GCAGTCCTTCAATCACTGGCATTGG	TGTGCTTTGGAAGGGTTTTGAGATG
TBCK_qPCR_5	GACACCTTCAAGTTGACAGCAC	GAAAGAGAGCAGCTGTCCAGGG
TBCK_qPCR_6	GCAGGAATCCTTTCACAAACCAG	TGTAACGTGATTCTAGGAAGCTG
GAPDH_qPCR	TGCTCACATATTCTGGAGGAGC	TGTAAACCTGGGGGAATACGTG
HPRT_qPCR	TGCTGAGGATTTGGAAAGGGTG	TTGTTCTGGTCCCTACAGAGTC

Supp. figure 1: Relative quantification of *TBCK* in ASD and ID affected individuals and their parents from the family F6331. Real-time PCR was used to quantify the copy number of a genomic DNA of *TBCK*. Results were normalized to the gene *GAPDH*, which has normal (2N) copy number. Six primers pairs were designed in the region chr4:107,071,580-107,113,380. Grey bars – segment with reduced copy number (1N). Two independent experiments with three replicates were performed.

Supp. table 3: Gene ontology terms enrichment.

GO terms	GO number
protein ubiquitination	GO:0016567
protein ubiquitination involved in ubiquitin-dependent protein catabolic process	GO:0042787
protein polyubiquitination	GO:0000209
transport	GO:0006810
regulation of transport	GO:0051049
regulation of macromolecule metabolic process	GO:0060255
cation transport	GO:0006812
ion transport	GO:0006811
organic cyclic compound metabolic process	GO:1901360
heterocycle metabolic process	GO:0046483
cellular aromatic compound metabolic process	GO:0006725
positive regulation of catabolic process	GO:0009896
regulation of catabolic process	GO:0009894
cation transmembrane transport	GO:0098655
metal ion transport	GO:0030001
intracellular protein transport	GO:0006886
nucleic acid metabolic process	GO:0090304
nucleobase-containing compound metabolic process	GO:0006139
positive regulation of protein catabolic process	GO:0045732
divalent metal ion transport	GO:0070838
divalent inorganic cation transport	GO:0072511
regulation of protein catabolic process	GO:0042176
regulation of vesicle-mediated transport	GO:0060627
negative regulation of transport	GO:0051051
ubiquitin-dependent protein catabolic process	GO:0006511
modification-dependent protein catabolic process	GO:0019941
calcium ion transmembrane transport	GO:0070588
mitochondrial transport	GO:0006839
calcium ion transport	GO:0006816
proteasome-mediated ubiquitin-dependent protein catabolic process	GO:0043161
modulation of synaptic transmission	GO:0050804
proteasomal protein catabolic process	GO:0010498
calcium ion transmembrane import into mitochondrion	GO:0036444
mitochondrial calcium ion transport	GO:0006851
mitochondrial transmembrane transport	GO:1990542
calcium ion import	GO:0070509
organophosphate ester transport	GO:0015748
sodium ion transmembrane transport	GO:0035725
regulation of lipid transport	GO:0032368
regulation of synapse organization	GO:0050807
regulation of synapse structure or activity	GO:0050803
regulation of cellular protein metabolic process	GO:0032268

CAPÍTULO 2

Disrupting variants in *DPYSL4* and *OPALIN* in a family with DMD/ASD-affected individuals

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ABSTRACT

Autism spectrum disorders (ASD) are a genetically complex group of neurodevelopmental disorders that have been reported either as an isolated condition or in association with some syndromes. It has been shown that about 4% of patients with Duchenne /Becker muscular dystrophy (DMD/BMD) have ASD. Here we report three ASD-affected non-identical twins, of which two were also diagnosed with DMD. The ASD diagnosis was made according the DMS-IV-TR criteria. Exome sequencing of the two ASD/DMD patients revealed that they carry a 22–base-pair deletion (c.64_69+16delTCTAAGGTAAGAATGGTTTGT) in dystrophin gene, maternally inherited. In order to verify if they harbor other pathogenic variants that could contribute to the ASD phenotype, we filtered in exome data nonsense variants in brain expressed genes. We highlight in the two ASD/DMD brothers two variants which, in addition to the dystrophin mutation, might contribute to the ASD phenotype: a heterozygous stop codon mutation in *OPALIN* and a frameshift mutation in *DPYSL4*, which were not found in 600 Brazilian controls. Dystrophin, a cytoskeleton protein essential for neuron survival, has already been associated with neurodevelopmental disorders. *OPALIN*, a transmembrane glycoprotein present in oligodendrocytes that is related to myelination, and *DPYSL4*, a regulatory protein of neuronal differentiation and death and of neurite outgrowth, up to now have not been reported in individuals with a neurodevelopmental condition. Our preliminary findings suggest that the ASD phenotype in these boys might depend on at least two hits. Moreover, as the three sibs are twins, we must consider that environmental factors are also predisposing to ASD.

Key words: dystrophinopathy, *CRMP3*, syndromic ASD, neurodevelopment

INTRODUCTION

Autism spectrum disorder (ASD) is a genetically complex group of neurodevelopmental disorders that have been reported either as an isolated condition or as part of a syndrome as, for example, Duchenne/ Becker Muscular Dystrophy (DMD/ BMD) (BETANCUR, 2011; AMERICAN PSYCHIATRIC ASSOCIATION, 2013). The frequency of ASD in the general population is around 1%, and recurrence rate in second and third affected-sibs is ~15% and ~25%, respectively. These findings together with high heritability estimates (50-90%) have suggested the contribution of inherited genetic factors to the ASD phenotype (CONSTANTINO et al., 2010; ELSABBAGH et al., 2012). Genomic analysis has shown that rare loss-of-function (LoF) mutations, as a major genetic event, contribute to about 10-25% of the cases (BOURGERON, 2015).

Syndromic cases of ASD represent about 10% of the total. In many of these syndromes, ASD is the main symptom and seems to be caused by mutation in a major gene. In these syndromic forms of ASD, the recurrence rate is based on the inheritance model of each syndrome (BOURGERON, 2015).

DMD/ BMD are X-linked recessive neuromuscular conditions caused by LoF mutations in dystrophin, which has been shown that around 4 - 20% DMD/ BMD cases also meet the diagnostic criteria for ASD (HENDRIKSEN; VLES, 2008; SNOW; ANDERSON; JAKOBSON, 2013; RICOTTI et al., 2016). Although the frequency of ASD in DMD/BMD is greater than in the general population, ASD is not a typical trait of DMD/BMD. Thus, it has been suggested that both genetic and environmental factors may influence the manifestation of ASD in individuals with DMD/BMD.

Up to now, few studies have been performed to understand the genetic aspects that determine ASD phenotype in individuals with DMD. Further, it is still unknown if the primary cause of ASD is related to lack of dystrophin in the brain or if ASD depends on additional genetic or environmental factors in these specific cases. In order to address these questions, we have taken the advantage of a rare family constituted of 3 non-identical ASD twins, from which two of them are also affected by DMD. To achieve our goals, through exome sequencing analysis, we searched for disrupting variants in the two brothers with DMD out of three ASD-affected non-identical twins in dystrophin and other brain expressed genes.

METHODS

Subjects and DNA samples

The three ASD-affected non-identical twin patients (Figure 1) were initially referred to the Human Genome and Stem Cell Research Center (HUG-CELL) for evaluation by the multidisciplinary neuromuscular disorders group and later by the autism group because of the ASD symptoms. Informed consents were obtained for the three children and their parents. Peripheral blood samples were collected for DNA extraction. DNA samples were stored in the biorepository at HUG-CELL.

ASD diagnosis was made according to the DMS-IV-TR criteria. Autism severity was assessed under the Childhood Autism Rating Scale (CARS) and information about adaptive behavior was also collected, under the Vineland scale (Vineland Adaptive Behavior Scales).

Figure 1: Family F8402 pedigree.

Whole-exome sequencing and variant analysis

Whole-exome sequencing was performed in the two individuals affected by DMD and ASD. Exome capture was carried out using the TruSeq Exome Library Prep Kit (Illumina, Inc.), following manufacturer's instructions. HiSeq 2500 sequencer (Illumina, Inc) was used for sequencing of paired-end reads of approximately 100 x 100bp. Reads were aligned to the reference genome (GRCh37/hg19) by BWA (Burrow-Wheeler Aligner) (LI; DURBIN, 2009) and PCR duplicates were removed with the Picard toolkit. Variant calling were carried out on GATK (Genome Analysis Tool Kit) (MCKENNA et al., 2010). ANNOVAR was used to annotate variants (WANG; LI; HAKONARSON, 2010).

First, we looked for variants in the DMD gene (MIM 300377). Subsequently, we searched for loss-of-function variants (frameshift and nonsense variants), filtering according to the following criteria: (a) excluded low quality variants; (b) included rare variants, with minor allele frequency (MAF) ≤ 0.01 in reference databases 1000 Genomes (1000G) Project National Institutes of Health, 6500 Exome Sequencing Project (6500ESP) Washington University and ABraOM (Online Archive of Brazilian Mutations) (NASLAVSKY et al., 2017); (c) included variants with frequency < 0.05 in internal control samples (i.e. DNA samples that were sequenced and data processed in the same set); (d) excluded variants within polymorphic genes (FUENTES FAJARDO et al., 2012); and, (e) looked for genes that were expressed in brain. All candidate variants were converted to reference genome GRCh38/hg38 on UCSC website (<https://genome.ucsc.edu/>)

Candidate variants were sequenced by Sanger methods, in all available individuals of the family, using BigDye Terminator v3.1 Cycle Sequencing Kit. Amplicons were sequenced in an ABI 3730 DNA Analyzer (Thermo Fisher Scientific) and data analyzed with the Codon Code Aligner v.6.0.2 software.

CASE REPORT

We first clinically evaluated the three twin boys (patients referred to as F8402-1, F8402-4 and F8402-5), conceived by *in vitro* fertilization (IVF), at the age of 3-years old due to neurodevelopmental delay. They were born by cesarean section at the 34th gestational week and stayed in the hospital for around a month. Their birth features are described in table 1. Their parents are healthy and unrelated. The age of the mother at pregnancy was 40 years.

Table 1: Birth features of ASD-affected individuals from family F8402

	Weight (centile)	Length (centile)	Apgar 1st/5th min	HC (centile)
F8402-1	1625g (p=3rd)	41cm (p=3rd)	9/ 9	29.5cm (p>10th)
F8402-4	1745g (p<10th)	43cm (p=10th)	9/ 10	29cm (p=10th)
F8402-5	1920g (p=10th)	44cm (50th>p>10th)	9/ 9	31cm (50th>p>10th)

Motor development

Individuals F8402-1 and F8402-4 started walking at 18 months of age, and very frequent falls were reported. At 3 years of age, hypothesis of DMD was raised due to global hypotonia, pseudohypertrophy of the calf muscles, flat foot and hyperlordosis. Creatine kinase (CK) serum test revealed a value of 6014 U/L (normal range: 24 – 162 U/L) and muscle biopsy with dystrophic pattern showed reduced dystrophin (data not shown). The clinical and biochemical parameters were very suggestive of DMD.

F8402-5 began walking at 15 months of age. CK serum test showed normal levels, so DMD diagnosis was excluded.

Language and behavior

All three children by the age of 3 years did not develop verbal language and did not use any nonverbal language to interact; they were only able to indicate things that they wanted. They also typically displayed repetitive and stereotyped behavior. For the whole period of school, they have been kept in different rooms along with an auxiliary teacher that helps them with the activities.

The two DMD boys, F8402-1 and F8402-4, were scored with 32.5 and 31, respectively by the CARS autism scale, which is compatible with ASD diagnosis. Vineland score were 44 and 45, respectively, indicating low adaptive behavior. The non-DMD individual (F8402-5) fulfilled the ASD criteria with low adaptive behavior when evaluated at 7 years of age (CARS score = 32,5; and Vineland score = 50).

RESULTS

The two individuals with DMD/ASD (F8402-1 and F8402-4) harbor a 22–base-pair deletion, comprising six base pairs in exon 2 of *DMD* (NM_000109: chrX:33,020,123-33,020,144; c.64_69+16delTCTAAGGTAAGAATGGTTTGTGTT; p.lys22_Ser23del). Selection of variants enabled the identification of two rLoF mutations (Table 2): a heterozygous frameshift variant located in exon 9 of *DPYSL4* (NM_006426: c.918delT: p.Val307Serfs*22) (Figure 2) and a heterozygous stop codon variant located in exon 4 of *OPALIN* (NM_001040102: c.C94T: p.Arg32*) (Figure 3).

Except for the variant in *DMD*, both *DPYSL4* and *OPALIN* variants were transmitted by their mother, who inherited them from their maternal grandmother. The autistic non-DMD boy (F8402-5) also inherited the variant in *OPALIN*.

Figure 2: Family F8402 sequence chromatograms of exon 9 of *DPYSL4*, showing the variant, c.918delT (black arrows), in three members of the family.

DISCUSSION

In this work, we searched for genetic factors that can be influencing ASD manifestation in three non-identical twins, discordant for DMD but concordant for ASD. Analysis of twins offers an opportunity to evaluate the influence of genetic and non-genetic causes to a specific phenotype.

The identified variant in *DMD* is exclusive to this family, and it alters the transcription of the longest isoforms of DMD, which are expressed in muscle (Dp427m) and brain (Dp427c). It has been suggested that ASD and Intellectual disability (ID), which have together a prevalence of approximately 20% in individuals with DMD (HENDRIKSEN; VLES, 2008; CHAMOVA et al., 2013; MILIC RASIC et al., 2014; ANAND et al., 2015; HENDRIKSEN et al., 2016; RICOTTI et al., 2016), are mainly caused by dystrophin deficiency in the brain. Dystrophin gene has at least 17 different isoforms with tissue-specific expression patterns. Among all isoforms, depletion of dp71 and dp140, which have high expression in brain, are reported as having correlation with lower results in cognitive scales in DMD individuals. Therefore, absence of these isoforms is considered to be the main cause of neuropsychiatric conditions manifested by DMD individuals (JAKOBSSON et al., 2008; ERTURK et al., 2010; PAGNAMENTA et al., 2011; CHAMOVA et al., 2013; SNOW; ANDERSON; JAKOBSON, 2013; MILIC RASIC et al., 2014; RICOTTI et al., 2016). In the present work, the pathogenic variant in *DMD* does not affect these two dystrophin isoforms cited above. It is also noted that the non-

DMD boy also has ASD, implying a role for additional factors in the etiology of the ASD in this family.

In the two DMD/ASD-affected brothers, we showed two disruptive risk variants, one in *DPYSL4* (Dihydropyrimidinase like 4) and the other in *OPALIN* (Oligodendrocytic myelin paranodal and inner loop protein) genes, that were transmitted from the maternal grandmother. Only the variant in *OPALIN* was shared with the third ASD brother. Additional molecular alterations in individuals with DMD and ASD have been rarely investigated. For example, Karaca and colleagues (KARACA et al., 2015) reported two affected siblings diagnosed with DMD and Smith-Lemli-Opitz syndrome, who harbored a hemizygous deletion in *DMD* and homozygous missense mutations in *DHCR7*. Pagnamenta and colleagues (PAGNAMENTA et al., 2011) reported a case of a 5-year-old boy with ASD, but not DMD, who also carried an intragenic in-frame duplication in the dystrophin gene and a heterozygous disrupting deletion in *TRPM3*, which is a calcium channel that act in neuron and oligodendrocytes during myelination (PAPANIKOLAOU; LEWIS; BUTT, 2017). Pagnamenta and colleagues did not reject the possibility that this individual could manifest BMD later (PAGNAMENTA et al., 2011). Both *DPYSL4* and *OPALIN* regulate important functions of the central nervous system (CNS). *DPYSL4*, also named *CRMP3* (Collapsin mediator protein 3), is important to neurite outgrowth and, consequently, exhibit function in neuronal polarization and plasticity (QUACH et al., 2011, 2013), while *OPALIN* is highly expressed at postnatal oligodendrocytes that myelinate axons in CNS (YOSHIKAWA et al., 2008, 2016; SATO et al., 2014). The dysregulation of these process controlled by *OPALIN* and *DPYSL4* are reported as responsible for ASD manifestation (HASHIMOTO et al., 2015; PETRELLI; PUCCI; BEZZI, 2016). These observations, together with our results suggest that the ASD phenotype in DMD may be triggered by additional genetic hits and their understanding will contribute to improve diagnosis and genetic counseling.

CONCLUSION

In summary, we described genetic variants in DMD/ASD-affected individuals that confer risk for these two conditions. We detected a new deletion in dystrophin, which is the cause of the DMD phenotype, and also two disrupting variants in *DPYSL4* and *OPALIN* that may contribute to ASD. Our findings suggest that the ASD phenotype

in individuals with DMD arises from a combination of genetic alterations.

Figure 3: Family F8402 sequence chromatograms of exon 4 of *OPALIN*, showing the point variant, c.C94T (dotted rectangle), in the three brothers (F8402-1, F8402-4 and F8402-5) and their mother (F8402-2).

Table 2: Rare loss-of-function variants identified in DMD/ASD individuals

Gene	Position (hg38)	Alteration	dbSNP (148)	Population Frequency				*pLI	#RVIS (%)
				1000g	6500 ESP	ExAC	60+ Brazilian controls		
DMD	chrX:33,020,123 - 33,020,144	AACAAACCATTCTTACCTTAGA/ -	.	0	0	0	0	1.00	97.78
DPYSL4	chr10:132,200,461 - 132,200,462	CT/ -	rs375554103	0.0006	0.0002	0.0001	0	0.00	4.38
OPALIN	chr10:96,349,736 - 96,349,736	G/ A	rs550798688	0.0002	0	0.00003	0	0.06	82.04

*pLI - probability of being loss-of-function intolerant (LEK et al., 2016); #RVIS - residual variation intolerance score, %Exac RVIS (PETROVSKI et al., 2013)

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CAPÍTULO 3

Biallelic loss-of-function mutations in *TBCK* lead to *mTOR* dysregulation in neuronal cells

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ABSTRACT

A small fraction of Autism Spectrum Disorder (ASD) is caused by one single major mutation (monogenic inheritance). Recently, we identified two sisters with ASD and intellectual disability (ID) carrying biallelic loss-of-function (LoF) mutations in *TBCK*. Functional studies involving *TBCK* are still lacking, especially in neuronal cell types. Here we investigate the effects of *TBCK* loss-of-function mutations in neural progenitor cells and neurons *in vitro* as well as if leucine rescue the *TBCK* derived neuronal phenotypes. We observed in NPCs that loss of *TBCK* reduces *mTOR* signaling, increased levels of LC3B-II (an autophagy biomarker), which caused alteration cell cycle progression and cell proliferation. We also verified that *TBCK* deficient NPCs lead to reduced growth rates (1.27 ± 0.01) compared to controls (1.65 ± 0.16), as well as reduced activation of $\text{rpS6}^{\text{S240/244}}$. Finally, in *TBCK* deficient neurosphere, we showed that L-leucine treatment induced an increasing of growth rate compared to non-supplemented culture. Here, we showed that depletion of *TBCK* leads to reduction in *mTOR* signaling, accompanied by altered cell cycle progression and cell proliferation of NPC and reduced neurosphere size. Interestingly, L-leucine supplementation was able to rescue neurosphere decreased size. It was already shown reduced *mTOR* activity in post mortem brains tissue of idiopathic autistic individuals, suggesting that the dysregulation of this pathway is an important causative mechanism of ASD. Thus, a subset of ASD individuals can benefit from leucine supplementation. These results suggest a potential therapeutic approach not only for patients with *TBCK* loss-of-function mutations, but for a subset of patients that presents decreased *mTOR* signaling.

Key words: autism, cell cycle deficiency, autophagy increasing, neurodevelopment, neurospheres

INTRODUCTION

Since the first genetic studies of autism spectrum disorder (ASD), evidence for underlying genetic complexity and heterogeneity has become increasingly clear. The genetic architecture of ASD is composed of monogenic forms with one locus as a major trigger for the neurocognitive features, and multigenic and multifactorial forms determined by two or more genetic abnormalities and, possibly, environmental factors (POOT et al., 2011; DEVLIN; SCHERER, 2012; KLEI et al., 2012; BOURGERON, 2015). A fraction of ASD cases is largely associated with monogenic syndromes, such as the recently identified autosomal recessive *TBCK*-related syndrome (POOT et al., 2011; ALAZAMI et al., 2015; BHOJ et al., 2016; CHONG et al., 2016).

The *TBCK*-related syndrome is caused by biallelic loss-of-function mutations in *TBCK*. Up to now, 23 affected individuals distributed in 15 families have been described in the literature. Their main clinical features include intellectual disability (ID), ASD, developmental delay, hypotonia, epilepsy and brain abnormalities such as cerebral atrophy (ALAZAMI et al., 2015; BHOJ et al., 2016; CHONG et al., 2016; GUERREIRO et al., 2016; MANDEL et al., 2016).

TBCK (TBC1 domain-containing kinase) is acts upon *mTOR* pathway components at a post-transcriptional level, regulating cellular proliferation and other biological processes (LIU; YAN; ZHOU, 2013; WU et al., 2014); however, it remains unclear how *TBCK* modulates neurocognitive function. The *mTOR* pathway is known for playing major roles in maintaining nervous system homeostasis, and disturbances in this pathway may lead to neurocognitive phenotypes (LIPTON; SAHIN, 2014). Therefore, clarifying *TBCK* – *mTOR* interaction in brain models will aid in understanding the neurological phenotypes of *TBCK*-associated disorders.

To disentangle the role of *TBCK* defects on cognitive function, we used iPSC-derived neuronal cells from an individual with biallelic mutations in *TBCK* to examine *mTOR* pathway signaling and *mTOR*-associated biological mechanisms. In addition, we also explored the l-leucine effect in *TBCK*-depleted cells.

METHODS

Subjects

Patient: the ASD-individual (F6331-1) is an eight year-old girl with biallelic LoF mutation in *TBCK* (a nonsense mutation and a deletion >3kb), which was identified by our group (Moreira et al, in preparation – Capítulo 1). This child has a sister that also harbor the same mutations in *TBCK* and was diagnosed with intellectual disability (ID). F6331-1 was ascertained for ASD at the HUG-CELL and at “Irmandade da Santa Casa de Misericórdia de São Paulo Centro de Atenção Integrada à Saúde Mental” through a group of physician, psychiatrist and psychologist, which diagnosed her based on DSM-V criteria (AMERICAN PSYCHIATRIC ASSOCIATION, 2013) and other auxiliary instruments.

Controls: control individuals (F7007-1, F8799-1 and F10006-1) are healthy and unrelated to ASD-affected individual.

Blood from ASD-affected and control individuals were collected at the Human Genome and Stem Cell Research Center (HUG-CELL). This project was approved by the ethic committee of the institute at which the study was conducted. After a complete description of the study, written informed consent was provided by ASD-affected individual and controls parents' when they were lower age and by controls by themselves when adulthood.

iPSC reprogramming

Induced pluripotent stem cells (iPSC) were reprogrammed from peripheral blood mononuclear cells (PBMCs), using non-integrating plasmids following a protocol suggested by (OKITA et al., 2011) with minor modifications, which was established at the HUG-CELL. Reprogramming was performed with nucleoporation, AMAXA nucleoporator (Lonza, Basel, Switzerland), of non-integrating plasmids containing transcription factors *OCT-4*, *SOX2*, *KLF4*, *L-MYK* and *LIN28* (TAKAHASHI et al., 2007). Each reprogrammed cell line was co-cultivated with irradiated murine embryonic fibroblast (MEF, Millipore) in DMEM/F12 medium supplemented with 2mM GlutaMAX-I, 0.1mM nonessential amino acids, 55uM 2-mercaptoethanol, 30 ng/mL FGF, and 20% of knockout serum replacement (Life Technologies). iPSCs colonies with typical morphology were transferred to Matrigel (BD-Bioscience) coated plates and fed with E8 medium (Life Technologies). Accutase (STEMCELL Technologies) was used to enzymatically dissociate iPSCs into single cells. To promote cell survival during

enzymatic passaging, cells were passaged with the 10mM of p160-Rho-associated coiled-coil kinase (ROCK) inhibitor Y-27632 (Sigma-Aldrich). All iPSC colonies were tested for plasmid integration and it did not integrate into host genome. We investigate aneuploidies with SALSA MLPA P070 Human Telomere-5 probe mix (MRC-Holland). Cellular pluripotency was evaluated through stem cell markers (Figure 1A).

This project was approved by the human research ethical committee of the Institute of Bioscience, USP.

NPC and neuron differentiation

Differentiation of iPSC into neural progenitor cells (NPC) and neurons was carried out using a protocol that was previously established in HUG-CELL (GRIESI-OLIVEIRA et al., 2014). Briefly, iPSCs were cultivated in 0,5xNB medium (1:1 DMEM/F12:Neurobasal media with 0.5x N2 (marca) and 1x B27 (marca)) supplemented with Dorsomorphin, 1 μ M (Tocris) for 2 days. Then, iPSCs were harvested by accutase treatment and clumps were manually transferred on two wells of low-attachment plates (Corning) on 0.5x NB medium supplemented with 1 μ M Dorsomorphin. In the next day, the medium was replaced for 0.5x NB medium + 20ng/ml *FGF* + 20ng/ml *EGF* and allowed to grow in suspension for 7 days. The resulting EBs were plated on matrigel-coated plate, from which formed visible rosettes that were manually collected and plated in poly-L-ornithine (10 μ g/mL; Sigma) and natural mouse laminin (5 μ g/mL; Invitrogen) coated plates. From these rosettes derived NPCs that formed a homogenous population after a couple of passages. To obtain mature neurons, NPC were cultivated in 0.5x NB medium supplemented with 1 μ M Retinoic Acid (RA, Sigma-Aldrich) and 200nM Ascorbic Acid (AA, Sigma-Aldrich) and maintained for at least 30 days, replacing media every other day.

Neurosphere growth measurement

To assess neurospheres growth rate, we harvest NPCs by accutase treatment and transferred to a 6-well low-attachment plate (Corning) and cultivated them in suspension under 0.5x NB medium supplemented with 1 μ M RA for 10 days. Replacing medium every other day. Neurosphere sizes was measured on day 2 and 10. We photographed neurospheres using a light

microscope (EVOS XL Core Cell Imaging System, Thermo Fisher) and measured using ImageJ software. Growth rate was determined for each sample based on the mean value of the area (μm^2) of 50 neurospheres

L-leucine treatment

NPCs were seeded (density: 4×10^4 cell/ cm^2) in a 6-well plate (Corning) in 0,5x NB medium supplemented with *EGF*, *FGF-2* and L-Leucine (L-Leu, 20mM, Sigma, cat# L8912). Two days later, we replaced for a neuron induction medium, 0,5x NB medium supplemented with RA, AA and L-Leu. Medium was replaced each other day. To obtain mature neurons we maintained the culture for >30 days.

Neurosphere were cultivated in 0,5x NB medium supplemented with L-leucine (20 mM supplementation, Sigma-Aldrich) during the 10 days of differentiation, replacing medium every other day.

RNA extraction and qRT-PCR

RNA samples were isolated from iPSC, NPC and neuros using NucleoSpin RNA II kit (Macherey-Nagel), following manufacturer's protocol. Sample concentrations and quality were evaluated using a Nanodrop 1000 (Thermo Fisher Sc) and gel electrophoresis.

cDNA was synthesized by reverse transcription using SuperScript IV (GibcoBRL, Life Technologies), following procedure detailed in the kit.

Primer pairs for *TBCK* (Supplementary table 1) were designed on Primer-BLAST (NCBI; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), while pluripotency markers (*OCT3/4* and *NANOG*), neuronal markers (*CDH2*, *PAX6* and *SOX1*) and housekeeping genes (*GAPDH* and *TBP*) were adopted from literature (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. Assays were run in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Relative quantification was carried out by normalization to *GAPDH*, and quantification data were calibrated relative to a control. For pluripotency or neural markers, we used as control a cell line known to do not express the marker.

Cell cycle and cell proliferation analysis

A total of 5×10^5 NPCs were seeded into 6-well plated (Corning) in 0,5x NB medium supplemented with 20ng/ml *EGF* and 20ng/ml *FGF-2*. NPCs were plated in duplicate to evaluate the cell cycle and cellular proliferation in each time point, T0, T24 and T40. The day after seeding, it was removed *EGF* and *FGF-2* from 0,5x NB medium, which are factors that *in vitro* contribute to keep NPC in undifferentiated state (CICCOLINI; SVENDSEN, 1998) and, consequently, their removing also drive cell cycle synchronization. Twenty-four hours after replacing medium, we collected cells at T0 point and replaced the medium of the NPC for 0,5x NB medium supplemented with *EGF* and *FGF-2* and, then, followed the incubation for 24 and 40 hours, at 37°C/5% CO₂, to collect cells at T24 and T40 points, respectively.

Three hours before collect NPCs, in each time point, we added BrdU (20uM, Sigma-Aldrich). Then, NPCs were rinsed twice with phosphate-buffered saline washing buffer (PBS), harvested by 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 cell cycle distribution and cellular 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 NPCs were seeded into 12-well plate (Corning), in duplicate. In the following day, we replaced the 0,5x NB medium supplemented with *EGF* and *FGF-2* and incubated cells with 20ul of CellEvent® Caspase-3/7 Green ReadyProbes® Reagent (Thermo Fisher) for 1 hour, 37°C/ 5% CO₂. Then, NPCs were rinsed with PBS, fixed with 4% paraformaldehyde (PFA) and stained nuclei with Vectashield mounting medium with DAPI (1µg/mL, Vector Labs). Images were captured on a Nikon fluorescence microscope (Nikon Eclipse Ti-E, Nikon). The quantification of caspase marked cells was performed by analyzing on ImageJ three different fields derived from duplicates. The total number of cells were referred by DAPI marked nuclei.

Cell Lysis and Immunoblotting

Cells were homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). Lysates were incubated on ice for 10 min and then centrifuged at 8,000×g for 15 min at 4°C, and the supernatants of total cell lysates were collected. Protein concentrations were determined with a BCA microprotein assay kit (BioAgency). Equal amounts of proteins (20ug) were separated by SDS-PAGE and transferred to nitrocellulose membranes by western blot. Membranes were blocked 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 conventional developing using x-ray films. 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-p*mTORS*²⁴⁴⁸ (1:1000, #2971 Cell Signaling Technology), anti-prp*S6S*^{240/244} (1:5000, #5364 Cell Signaling Technology), anti-LC3A/B (1:1000, #12741 Cell Signaling Technology) and anti-βactin (1:15000, A2228 Sigma) antibodies. p-mTOR, p-rpS6 and LCA3/B protein levels were quantified and normalized to the corresponding β-actin levels.

Statistical analysis

Student's t-test was used to compare patient's data versus control. The differences were considered significant when $p < 0.05$.

RESULTS

Cell characterization and TBCK evaluation

iPSCs were derived from an ASD-affected individual (F6331-1) with biallelic alterations in *TBCK* (compound heterozygote: nonsense mutation in one allele, NM_001163435; c. 2130C>G; p.Tyr720*; and a small frameshift deletion in the other, chr4: 107,091,846 to 107,094,914) (Moreira, in preparation – Capítulo 1) and three controls (F7007-1, F8799-1 and F10006-1). These iPSCs were differentiated into NPCs and neurons. We observed that pluripotency markers *OXT3/4* and *NANOG*, and neural markers *CDH2*, *SOX1* and *PAX6* were

appropriately expressed in these cells according to their differentiation state (Figures 1 and 2). Next, we investigated mRNA expression levels of *TBCK* in NPCs, and a reduction of at least 2-fold was observed in cells from the affected individual as compared to control cells (Figure 1).

Cell-specific dysregulation of mTOR components in TBCK-deficient cells

As *TBCK* alters *mTOR* pathway (LIU; YAN; ZHOU, 2013), we examined phosphorylation of *mTOR* pathway components rpS6 and *mTOR* (phospho-rpS6^{S240/244} and phospho-*mTOR*²⁴⁸⁸, respectively), in iPSCs, NPCs and neurons. We observed that phospho-*mTOR* and phospho-rpS6 show dysregulation patterns according to the differentiation stage of the *TBCK*-deficient cells, in comparison with control cells: in the patient's cells, we detected decreased phospho-*mTOR* only in iPSCs while phospho-rpS6 was reduced in iPSCs and NPCs. None of these *mTOR* components was altered in neurons (Figure 2).

Abnormal cell cycle progression and proliferation in TBCK-deficient NPCs

Next, in NPC, we analyzed cell cycle, cell proliferation and apoptosis. Cell cycle and cell proliferation were analyzed at 24h after cell starvation (T0) (removing *EGF* and *FGF-2*) and 24h and 40h after supplementing medium with *EGF* and *FGF-2*. Cell proliferation was evaluated with BrdU pulse. We observed deficiency in cell cycle progression of *TBCK*-deficient NPCs: 24h and 40h after supplementation (Figure 3A). BrdU-positive (S-phase) cells in control NPCs increased in average 4.5% and 6.7%, respectively, while the fraction of BrdU-positive cells in *TBCK*-deficient NPCs reduced in 12.6% and 6.7% (Figure 3B). Apoptosis assay to detect activated Caspase 3/7 did not exhibit any distinction between control and *TBCK*-deficient NPCs (Table 1 and Figure 4).

Autophagy is another process regulated by *mTOR* signaling (TANG et al., 2014). Thus, we further investigated NPC autophagy, through LC3A/B-II biomarker analysis. We detected an increase in LC3A/B-II protein expression in *TBCK*-deficient NPCs (F6331-1_{Mean LC3 A/B I}: 0.58 ± 0.12) in relation to controls (F7007-1_{Mean LC3 A/B I}: 0.24 ± 0.03; F8799-1_{Mean LC3 A/B I}: 0.18 ± 0.05; F1006-1_{Mean LC3 A/B I}: 0.19 ± 0.08) (Figure 5).

Figure 1: Cellular characterization. A) Relative mRNA expression of pluripotency markers (*OCT3/4* and *NANOG*) and C) neuronal markers (*CDH2*, *PAX6* and *SOX1*) of controls and ASD-affected individual cells (iPSC and NPC). B) Relative mRNA expression of *TBCK* (Primer pair 3) in NPCs. Results were normalized to the housekeeping gene TBP.

Figure 2: Reduction mTOR pathway markers in iPSC, NPC, but not in neurons of an individuals with biallelic mutations in TBCK (F6331-1). The expression level of phosphorylated mTOR and S6 in iPSC (A and D), NPC (B and E) and neuron (C and F). Protein levels were normalized to β -actin. Gray bar indicates affected individual and black bars control individuals.

Figure 3: Deficient cell cycle progression and BrdU incorporation in *TBCK*-depleted NPCs. A) Quantification of the percentage of each cell cycle stage (G0/G1, S and G2) in NPC cultures without EGF/FGF supplementation for 24h and with EGF/FGF supplementation for 24 and 40h. B) Quantification of the percentage of BrdU positive NPCs in culture conditions explained in A. Results are the average of 2 independent experiments.

Table 1: Apoptosis analysis in NPCs through Caspase3/7 assay.

	Mean N DAPI marked	(SD)	Mean N Casp 3/7 marked	(SD)	pvalue - t-test
F6331-1	452.5	(45.96)	4.5	(0.71)	
F7007-1	362	(42.43)	1	(1.41)	0.178179
F8799-1	329	(49.50)	1	(1.41)	0.123321
F10006-1	334	(1.41)	3.5	(3.54)	0.170116

Figure 4: Apoptosis is not altered in *TBCK*-depleted NPCs. The apoptosis quantification was performed with Caspase3/7 marker. DAPI was used to mark nuclei. Results are the average of 2 independent experiments.

Figure 5: Increased autophagy process in *TBCK*-depleted NPCs. A) Western blot analyses of the LC3A/BII in NPCs. B) Mean and standard deviation of LC3A/BII protein levels of each individual. Protein levels LC3A/BII were normalized to β -actin levels. Black bar – affected-individual; gray bars - control individuals. Results are the average of 2 independent experiments.

Reduced growth rate and deficiency of S6 marker in TBCK-deficient neurospheres

To further investigate whether *TBCK* alters cellular proliferation and *mTOR* pathway signaling during neuronal development, we proceeded to analyze these parameters in neurospheres. We evaluated the level of phospho-rpS6 (residue S240/244) in *TBCK*-deficient neurospheres and observed that protein level was decreased in relation to control (Figure 6).

Then, we investigated if the growth rate of *TBCK*-deficient neurospheres would be altered. The neurosphere growth rate of each cell line (controls and *TBCK*-deficient) was estimated as a mean area (μm^2) ratio on day 2 and again on day 10 after plating cells in suspension. *TBCK*-deficient neurospheres grew, in average 1.27 ± 0.01 times, while controls grew 1.65 ± 0.16 times, validating the results of cell cycle and cell proliferation (Figure 6).

L-leucine treatment rescues growth rate in TBCK-depleted neurospheres but not in neurons

L-leucine (L-leu) supplementation has been shown to activate *mTOR* pathway (AVEROUS et al., 2014). To verify if L-leucine can rescue *mTOR* signaling during neuronal differentiation of *TBCK*-deficient cells, we analyzed the *mTOR* components before and after L-leu supplementation (20mM) in NPC-derived neurons. We did not detect any difference in the phosphorylated rpS6

and *mTOR* protein levels between *TBCK*-depleted neurons and control neurons with and without L-leu treatment. Nevertheless, the treatment with L-leu induced a slight increase of protein levels in neurons (average increase phospho-rpS6 = 0.25; average increase phospho-*mTOR* = 0.19) (Figure 7A and B).

Subsequently, we analyzed the effect of L-leu (20mM) supplementation in *TBCK*-depleted neurospheres. We observed an increase of >15 times from day 2 to 10 after plating cells in suspension, in L-leu-supplemented neurospheres as compared to non-supplemented ones (Figure 7C).

Figure 6: *TBCK*- depleted neurospheres exhibit reduced *mTOR* signaling and growth. A) The expression level of phosphorylated rpS6. B) and C) Neurospheres growth rate on days 2 (up) and 10 (bottom). Patient's clones 1 and 2 (gray bars) growth rates compared to control neurospheres (black bars) after 10 days of *in vitro* differentiation. Growth rate was determined for each sample based on the mean value of the area of 50 neurospheres.

DISCUSSION

Here, we provide the first data on the cellular effects due to *TBCK* depletion in neuronal cell types (NPC, neurons and neurospheres) *in vitro*.

Through the investigation of functional effect of *TBCK* biallelic loss-of-function mutations in different neural differentiation stages, aside from decreased *TBCK* expression, we have shown their role in *mTOR* signaling pathway and *mTOR*-coupled biological processes.

Figure 7: L-leucine increases mTOR signaling in neurons and neurospheres. The expression level of phosphorylated mTOR A) and rpS6 B) in neurons with and without L-leucine treatment (20mM). C) Neurospheres growth rate on day 2 (grey bars) and 10 (blue bar) with L-leucine treatment. Growth rate was determined for each sample based on the mean value of the area of 50 neurospheres.

During differentiation (iPSCs to NPCs, NPCs to neurons, and NPCs to neurospheres), we observed that activation of *mTOR* pathway components was decreased in *TBCK*-deficient cells. It has been previously shown that *TBCK* knockdown inhibits *mTOR* signaling pathway in HEK293 and Hela cells, and this same dysregulation was also observed in lymphoblastic cells from individuals with biallelic mutations in *TBCK* (BHOJ et al., 2016; LIU; YAN; ZHOU, 2013; WU

et al., 2014). Thus, our results show that *TBCK* loss of function reduces *mTOR* signaling pathway in neuronal cells, expanding the cell types evaluated for the effect of decreased *TBCK*.

It is well established that *mTOR* pathway modulates many major cellular processes, including cell cycle, proliferation and growth, and autophagy (LIPTON; SAHIN, 2014). *TBCK*-depleted NPC showed alterations in cell cycle progression. Moreover, cellular proliferation was also impaired in such cells, as shown here by decreased BrdU incorporation in *TBCK*-depleted cells. We could also verify a reduction in growth rate in *TBCK*-depleted neurospheres in relation to controls. These interconnected processes (cell cycle, proliferation and growth) are shown to be reduced in other cellular models with decreased activity of *mTOR* signaling pathway (CLOËTTA et al., 2013; KA et al., 2014). CLOËTTA and collaborators (2013) and KA and collaborators (2014), reported that mouse models deficient for *mTOR* signaling show that reduction of these cellular process causes reduction in brain size and cortical thickness. Our data, along with these previous observations, suggest that *TBCK* is important for brain development and associate brain/neurocognitive abnormalities with loss of *TBCK*, as observed in individuals harboring loss-of-function mutations in this gene (BHOJ et al., 2016; CHONG et al., 2016).

Another cellular process altered in *TBCK*-depleted NPCs was autophagy, as demonstrated by increased expression of the autophagy biomarker LC3-II. Autophagy contributes to homeostatic balance between cell death and cell cycle regulation of neural cells during neurodevelopment (CECCONI et al., 2007). Thus, dysregulation of these processes may disrupt balance between NPC proliferation/ neuronal differentiation (CECCONI et al., 2007; SARKAR et al., 2014), leading to defective neurodevelopment, a common finding in individuals with biallelic loss-of-function mutations in *TBCK*. Dysregulation of cellular autophagy is a common phenotype in several other syndromes, such as in tuberous sclerosis, Roberts syndrome and Cowden syndrome, in which alterations in *mTOR* signaling are also at play (COSTA-MATTIOLI; MONTEGGIA, 2013; XU et al., 2013, 2016; TANG et al., 2014).

In addition to investigating the role of *TBCK* in neuronal cells, it is relevant to search for drug candidates to develop therapeutic strategies for affected individuals. We showed that treatment with the amino acid L-leucine increases

mTOR signaling in control and *TBCK*-deficient neurons, leading to a prominent increase in neurosphere growth rate. Currently, L-leucine is known to activate the *mTOR* pathway, specifically *mTORC1*, via the RAG complex (AVEROUS *et al.*, 2014) and has been used to correct phenotypes in models for Roberts syndrome (BONFILS *et al.*, 2012; HAN *et al.*, 2012; XU *et al.*, 2013, 2016). Accordingly, our data point out L-leucine as a possible molecule to treat syndromes caused by deficiency in this signaling pathway.

In summary, we have shown that *mTOR* pathway is dysregulated in *TBCK*-deficient neuronal cells, leading to disturbances in the NPC proliferation during neuronal differentiation, which in turn likely contribute to the impaired cognitive phenotype of individuals with biallelic loss-of-function mutations in *TBCK*. Although some of the main effects of *TBCK*-depletion in neuronal cells have been known, it is still worth noting that many roles of *TBCK* are still unclear. Moreover, the prospect of applying L-leucine to treat affected individuals is promising, and it will be important to further investigate its effects in *TBCK*-deficient neurodevelopmental models.

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Capítulo 3: SUPPLEMENTARY INFORMATION

Supp Table1: Primers used for real-time quantitative PCR experiments with TBCK (NM_001163435).

	Forward primer	Reverse primer
TBCK_P1	CTGCTCTTCTGGGAGTTGAGGGAG	CAGGATGAGACACTACCCAGGCTT
TBCK_P2	GTCTTTGTGCTTGCCTGCGTCC	GCTCCCATTTCAGCGTCCTTCAG
TBCK_P3	GGTCATTGTCATCGTGGGGCAT	GGCAGTCACACTCTTGTTCTTCA
TBCK_P4	TCCTGTAGCAAAGACGAATGGC	TGTAAAGCAATGAGAGAAGGTAATGAGT

Supp. figure 1: Relative expression of *TBCK* in NPCs of controls (black bars) and patient (F6331-1, gray bar). Real-time PCR was used to quantify expression of *TBCK* in NPCs from control and autistic individual. Results were normalized to the housekeeping gene TBP. Expression of the TBP gene was not changed in any of the groups. Expression of the *TBCK* gene was reduced in F6331-1. *TBCK* – P1 and P2 targeted before *TBCK* mutations; *TBCK* – P4 targeted after mutations.

CAPÍTULO 4

Dysregulation of autism spectrum disorder candidate genes in *Drosophila melanogaster* leads to abnormalities in neuronal morphology

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ABSTRACT

Several hundreds of candidate genes have been implicated in autism spectrum disorder (ASD), but the functional relevance of most of them remains unclear. In the present work, we used *Drosophila melanogaster* to investigate the contribution of four ASD candidate genes identified by our group. We analyzed the DCN and PDFme morphology in *D. melanogaster* larvae (L3) with reduced expression of four ASD-candidate genes: *trpy*, *mahjong*, *dys* and *crmp*, which are the orthologs of *TRPC6*, *VPRBP*, *DMD* and *CRMP3*, respectively. We verified that *trpy* and *mahjong* had higher frequency of axon defasciculation than controls, while *dys* and *crmp* presented aberrant form of ipsilateral-contralateral neurite angles, in addition to axon defasciculation and shortened ipsilateral neurite, more often than in controls. These results indicate that *D. melanogaster* is a suitable model to investigate ASD, and that decreased expression of *trpy*, *mahjong*, *dys* and *crmp* leads to neuronal abnormalities.

Keywords: *trpy*, *mahjong*, *dys*, *crmp*, *drosophila brain*, *neurodevelopment*

INTRODUCTION

Autism spectrum disorder (ASD) is a neuropsychiatric condition characterized by deficit in the use of communication for social interaction, and stereotyped and repetitive behavior (AMERICAN PSYCHIATRIC ASSOCIATION, 2013). ASD affects about 1% of the general population (ELSABBAGH et al., 2012), and its heritability has been estimated as 50-90%, highlighting an important genetic component. (HALLMAYER; CLEVELAND; TORRES, 2011; RONALD; HOEKSTRA, 2011). Genomic studies have revealed several genetic alterations in ASD (POULTNEY et al., 2013; QIAO et al., 2013), but few of them are recurrent, such as CNVs within 15q11-q13, *CHD8* point mutations, and *SHANK3* deletion (MOESSNER et al., 2007; MOREIRA et al., 2014; COTNEY et al., 2015). This small fraction of ASD candidate genes recurrently mutated are the most functionally investigated in regard to their biological and molecular roles. The role of remaining ASD candidate genes is still poorly explored, and it is difficult to establish which genetic variants are indeed causative. Moreover, establishing whether one or more genetic alterations are contributing to ASD in a given individual remains challenging.

Our group has identified two novel and intriguing ASD cases. In the first case, an ASD individual harbored a balanced translocation between chromosomes 3 and 11, disrupting the genes *TRPC6* and *VPRBP*. Griesi-Oliveira and collaborators (2014) showed that reduced expression of *TRPC6* due to gene disruption leads to abnormalities in neuron function, but this first study did not explore the possible neuronal alterations related to *VPRBP*. Thus, questions about the contribution of *VPRBP* to ASD and how *TRPC6* and *VPRBP* interact to determine cognitive phenotypes remained unanswered. In the second case, we studied three non-identical twin brothers with ASD, and two of them also have Duchenne Muscular Dystrophy (DMD). It has been suggested that loss-of-function mutations in the dystrophin gene, the causative mechanism of DMD, are also responsible for ASD characteristics in these patients. As models that involve two- and multi-hits are considered common mechanisms in ASD, we asked whether the DMD/ASD phenotype would also depend on the occurrence of at least two genetic hits. In this context, a recent research by our group has identified that these DMD/ASD children carry nonsense mutations in *OPALIN* and *DPYSL4* (*CRMP3*), genes important for neuron organization.

Considering the large number of ASD-related candidate genes and the high cost entailed by most mammalian model organisms and cell culture systems for functional studies, *Drosophila melanogaster* has been explored as a cost-effective alternative model to investigate ASD and other neuropsychiatric conditions. Neural development of *D. melanogaster* is very well characterized and shows great similarity to humans (SÁNCHEZ-SORIANO et al., 2007). This organism has been successfully used in the understanding of several human neurodevelopmental conditions, such as Fragile X syndrome (MORALES et al., 2002; REEVE et al., 2005; LI; BASSELL; SASAKI, 2009; CALLAN et al., 2010) (BOLDUC et al., 2010)(MONYAK et al., 2016) and other autism-related conditions, including those with mutations in *NRXN1*, *CNTNAP2*, *NLGN3* and *CYFIP1* (SCHENCK et al., 2003; ZWEIER et al., 2009; HAHN et al., 2013; LEE et al., 2014; GRICE; LIU; WEBBER, 2015; GUPTA et al., 2016; PARK et al., 2016). These research reinforce the relevance of this model organism into the study of ASD and other neurodevelopmental disorders.

Thus, defining if ASD candidate genes determine *in vivo* neuronal dysfunction is crucial for understanding its pathophysiology and selecting which are the true causative mutations of this phenotype. In this study, we evaluated the effect of loss-of-function mutations of 4 candidate ASD genes characterized by our group: *TRPC6*, *VPRBP*, *DMD* and *CRMP3*. We used RNAi molecules to knockdown expression of their respective orthologs in *D. melanogaster*, in the dorsal cluster neurons (DCN) and pigment-dispersing factor medulla (PDFme) neurons of larvae. We focused on morphological analyses, including type and frequency of DCN alterations in the mutated flies as compared to controls.

METHODS

Identification of orthologous genes

First, to obtain *D. melanogaster* orthologous genes, we searched previous publications in database. For genes without information in public databases, we performed an *in silico* analysis. Human protein sequences were used as queries for sequences with higher identity in *D. melanogaster*, using the tools BLAST/BLAT (<http://www.ensembl.org/Multi/blastview>) and ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). *D. melanogaster* lines are listed on table 1. No ortholog was identified for the human *OPALIN* gene.

Table 1: Driver and responder lineages of *D. melanogaster* lines

Lineage	Hs gene	Dm gene	Identity (%)	# stock	Genotype	Ref.
ato.gal4	.	.	.	.	w5UAS-GFP; ato.GAL4_UAS-lacZ/TM6c	
pdf.gal4	.	.	.	.	UAS.GFP;pdf.gal4/CyO; UAS-dcr2/MKRS	
RNAi	<i>DMD</i>	<i>dys</i>	54	BL55641	y1 sc* v1; P{TRiP.HMC03789}attP40	(GREENER; ROBERTS, 2000)
RNAi	<i>DMD</i>	<i>dys</i>		BL31553	y1 v1; P{TRiP.JF01118}attP2	
RNAi	<i>CRMP3</i>	<i>crmp</i>	49	BL53354	y1 sc* v1; P{TRiP.HMC03583}attP40	
RNAi	<i>TRPC6</i>	<i>trpy</i>	42	BL31299	y1 v1; P{TRiP.JF01244}attP2	
RNAi	<i>VPRBP</i>	<i>mahjong</i>	38	BL34912	y1 sc* v1; P{TRiP.HMS01260}attP2	(TAMORI et al., 2010)

Dm - *Drosophila melanogaster*; Hs - *Homo sapiens*

Fly stocks

Drosophila stocks: Responder lines that contained RNAi-inducing sequences used in our study were: Upstream Activating Sequence (UAS)-trpy (BL 31299), UAS-mahjong (BL 34912), UAS-dysBL31553 (BL 31553), UAS-dysBL55641 BL 55641) and UAS-crmp (BL 53354). They were obtained from the Bloomington Stock Center (Bloomington, IN, USA). Driver lines ato.gal4 and pdf.gal4 were provided by Dr. John Ewer. Besides the gal4 sequence, these driver lines contain a UAS-GFP (Green fluorescent protein) sequence and were used to induce neuron-specific knockdown via dsRNA molecules, based on the well-established GAL4-UAS system (BRAND; PERRIMON, 1993), that are visualized due to the GFP.

Husbandry: All fly stocks were maintained raised in a standard media at 18°C. During crosses of driver and responder lines the stock lines were maintained in 25°C as well as the F1 from such crosses. Flies in incubators with both temperatures were entrained to 12–12h light:dark (L:D) conditions. We used as control the driver lines (ato.gal4 and pdf.gal4).

Brain dissection and imaging

Third instar larvae (L3) of F1 lineage and parental driver (ato.gal4 and pdf.gal4) were dissected in cold 1X Phosphate-buffered saline (PBS) under the stereomicroscope SMZ800 (Nikon, NY, USA) and, immediately after dissection,

brains were placed to a microtube containing 4% paraformaldehyde (PFA) on ice, where they were kept for at least 1h. Brains were rinsed once in PBST (1X PBS containing 0,03% of Triton-100) and twice in 1X PBS. Samples were mounted on slides in Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA) and covered with a glass coverslip.

Larval brains were imaged on a laser scanning confocal microscope LSM800 (Zeiss Multifóton Microscope, Carl Zeiss, Germany) under 20x or 40x magnification. Z-stack images were scanned at 0.6-mm section intervals with a resolution of 1024×1024 pixels. Images were analyzed in Zen 2 imaging software (Blue edition, 2012, Zeiss).

Morphology of cluster of neurons in third instar larvae

Morphological and qualitative analyses of two clusters of neurons were performed using as reference the traits described in cited papers below. At least, two researchers carried out blind and independent analyses of these clusters of neurons and, then, results were compared.

Dorsal cluster neurons (DCN) exhibit a stereotypical axonal structure composed of two clusters of 20-40 cells located in the dorso-lateral regions, disposed in both brain sides and proximal to the optic lobe. These neuron clusters extend dendrites ipsilaterally, in a dorso-ventral direction, and axons contralaterally, forming a bundle over the dorsal commissure between brain hemispheres (HASSAN et al., 2000; LANGEN et al., 2013).

PDFme: Pigment-dispersing factor (PDF) neurons form distinct clusters of neurons; however, in this work we analyzed PDF-medulla (PDFme) neurons, which in larvae consist of a group of four cells close to the optic neuropil that extends axonal projections that run dorsolaterally in each brain hemisphere (HELFRICH-FÖRSTER, 1997).

Statistical analysis

Fisher's exact test (significance set at $p < 0.05$) was used to estimate if control (ato>ato.gal4) and test groups (RNAi group) were statistically different.

RESULTS:

To investigate the relevance of *trpy*, *mahjong*, *dys* and *crmp* to neurodevelopment, we analyzed the morphology of PDFme and DCN in third-instar (L3) larvae expressing dsRNA molecules for the *D. melanogaster* genes of interest. DCNs and PDFme were labeled with GFP driven by *ato.gal4* and imaged. Stringent criteria for morphological traits were adopted to avoid misinterpretation, as described in the methodology. Although PDFme has been reported to show a typical pattern of organization, here this structure showed great morphological variation across individuals within the same line, including controls (Supplementary Figure 1), so we deemed this structure unfit for analysis. We observed that DCN, which form a stereotyped structure, exhibited less morphological variability, so we opted to concentrate our analyses on this structure (Figure 1A and 2A, Table 2).

Table 2: Frequency of DCN alterations in *D. melanogaster* larval brains.

F1	Altered	(%)	Normal	Total	P-value*
ato>ato.gal4	2	18.2	9	11	.
ato>mahjong BL34912	10	66.6	5	15	0.021416
ato>trpy BL31299	17	94.4	1	18	<0.0001
ato>crmp BL53354	4	80.0	1	5	0.0357
ato>dys BL31553	13	92.9	1	14	0.0002
ato>dys BL55641	8	61.5	5	13	0.0472

*Fisher's exact test.

VPRBP (mahjong) and TRPC6 (trpy) induce formation of additional branch of DCN axon

Morphological analyses of larval DCN under decreased expression of *trpy* (N of ato>trpy BL31299 = 18) and *mahjong* (N of ato>mahjong BL34912 = 15) showed additional branches of DCN axons in one or both brain sides in over 50% of the brains (N of ato>trpy BL31299 = 12 out of 18; ato>mahjong BL34912 = 10 out of 15), while in 9.1% controls exhibited the same type of alteration (N of ato>ato.gal4 = 1 out of 11) (Figure1 B-E). Forty percent of mahjong (N = 6 out of 15) and 22% of trpy (N =

4 out of 18) larvae had axonal defasciculation/ neurite misrouting (Table 3 and Figure1 B-E) not observed in any of the controls.

Dystrophin (dys) and CRMP3 (crmp) knockdown leads to decreased angle between contralateral and ipsilateral DCN axons

D. melanogaster L3 under *dys* knockdown (N of *ato>dys* BL31553 = 14; N of *ato>dys* BL55641 = 13) and *crmp* (N of *ato>crmp* BL53354 = 5) displayed abnormalities in cell bodies and axonal position, such as decreased ipsilateral/contralateral axonal angle ($<90^\circ$), shortened ipsilateral axon, and axonal defasciculation (Table 3). Only *dys* knockdown lines (*ato>dys*BL31553 and *ato>dys*BL55641) showed a curvature of ipsilateral axons over contralateral axons (Table 3 and Figure 2). *crmp* lines also showed additional branching in both brain sides (Table 3 and Figures 2).

Table 3: Frequency of structural alterations in DCN of *D. melanogaster* larval brain.

	Additional branch	(%)	Short neurite	(%)	Curved ipsi/ contralateral connection	(%)	Neurite angle $<45^\circ$	(%)
<i>ato>ato.gal4</i>	1/11	(9,1)	1/11	(9,1)	0/11	(0)	0/11	(0)
<i>ato>mahjong</i> BL34912	10/15	(66,7)	0/6	(0)	0/15	(0)	0/15	(0)
<i>ato>trpy</i> BL31299	12/18	(66,7)	1/18	(5,6)	0/18	(0)	0/18	(0)
<i>ato>crmp</i> BL53354	1/5	(20)	2/5	(40)	2/5	(40)	2/5	(40)
<i>ato>dys</i> BL31553	1/14	(7,1)	8/14	(57,1)	0/14	(0)	4/14	(28,6)
<i>ato>dys</i> BL55641	2/13	(15,4)	2/13	(15,4)	1/13	(7,7)	5/13	(38,5)

Table 3 (Continued)

	Curved termination of neurite	(%)	CB/ neurite defasciculated	(%)	CB out of neurite axis	(%)
<i>ato>ato.gal4</i>	0/11	(0)	0/11	(0)	0/11	(0)
<i>ato>mahjong</i> BL34912	0/15	(0)	6/15	(40)	0/15	(0)
<i>ato>trpy</i> BL31299	0/18	(0)	4/18	(22,2)	0/18	(0)
<i>ato>crmp</i> BL53354	2/5	(40)	1/5	(20)	0/5	(0)
<i>ato>dys</i> BL31553	0/14	(0)	5/14	(35,8)	4/14	(28,6)
<i>ato>dys</i> BL55641	0/13	(0)	2/13	(15,4)	5/13	(38,5)

CB – cell body; xxxxx

Figure 1: *crmp* and *dys* decreased expression cause neurons morphological alterations. *ato>crmp BL53354* (B and C), *ato>dys BL31553* (D and E) and *ato>dys BL55641* exhibit and defasciculation of the axons (white head arrow), shorten axon (green double head arrow) and abnormal position in neurites (white arrow).

Discussion:

In this study, we investigated the neurodevelopmental effects elicited by knockdown of 4 ASD-associated genes in *D. melanogaster*, aiming to model loss-of-function mutations found in ASD individuals. The DCN architecture displays typically symmetrical and stereotyped morphology (HASSAN et al., 2000; SRAHNA et al., 2006; LANGEN et al., 2013). We showed that decreasing expression of ASD-associated genes leads to DCN morphological alterations across different mutant lines of *D. melanogaster* larvae more frequently than in control lines (Table 2). The lack of abnormal phenotypes in some specimens can be explained by variation in RNAi efficiency.

D. melanogaster lines with reduced expression of *trpy* and *mahjong*, which are orthologous to *TRPC6* and *VPRBP*, respectively, exhibited both neuronal defasciculation and additional branches of neurons. *Trpy*, a light-sensitive Ca^{2+} -dependent TRP channel (XU et al., 2000; JÖRS et al., 2006; AKITAKE et al., 2015), has been implied in neurodegeneration through dysregulation of *dPDK1* (NELSON et al., 2005), and reduced lifespan (NAKAYAMA et al., 2014). *Trpy* is supposed to alter cell specification during development, and it may influence cell physiology and function at various stages of the fly life cycle (NAKAYAMA et al., 2014). *Mahjong* has been mainly explored for its role in regulating an E3 ubiquitin ligase and has been implicated in cell cycle progression, apoptosis and in cell competition (MCCALL et al., 2008; TAMORI et al., 2010; KIM et al., 2013; NAKAGAWA; MONDAL; SWANSON, 2013). Importantly, inhibition of *mahjong* suppresses cellular proliferation in neuronal cancer cells via modulation of Merlin/NF2 pathway (HUANG; CHEN, 2008; LI et al., 2010; SHINGLETON, 2010).

In *crmp* and *dys* knockdown lines, we observed abnormalities in axonal contralateral and ipsilateral connection, and in *dys* lines only, we detected alteration in the position of cell bodies, which were curved over ipsilateral dendrites. Both of these genes are well conserved between human and *D. melanogaster* and are components of cytoskeleton, acting in its organization (SHCHERBATA et al., 2007; MARRONE et al., 2011; MIRANDA et al., 2015). In *dys*-depleted lines, the altered axon pathfinding can be related to deficient cell migration (KUCHERENKO et al., 2008). Neuronal *crmp* is involved in neuronal migration, growth cone guidance and

axon outgrowth, all process related to cytoskeleton formation (MORRIS *et al.*, 2012; QUACH, TAM *et al.*, 2013; QUACH, *et al.*, 2011).

These data indicate that decreasing expression of ASD candidate genes (*mahjong*, *trpy*, *crmp* and *dys*) in *D. melanogaster* alters larval brain and can be used as a model to explore their role in neuronal cells and social behavior. To validate our findings, we will expand the number of mutated animals and controls. Because some genes have specific spatio-temporal expression patterns, evaluating more phenotypes will be worthwhile. Additionally, we will explore the morphology of DCN in adult flies, compound eyes, which is considered a simple nervous system, and head size of adult *D. melanogaster* specimens. Besides, the analysis of social fly behavior and circadian rhythm of flies with might add important information about the relevance of such genes to the neurological phenotypes.

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Capítulo 4: SUPPLEMENTARY INFORMATION

Supplementary Figure 1: A-D) Variable structure of PDFme neurons (pdf.gal4), exhibiting different positions of cell bodies (grey circle) and axons (grey arrows) direction.

DISCUSSÃO GERAL E CONCLUSÕES

DISCUSSÃO GERAL

Neste trabalho, os objetivos principais foram identificar os fatores genéticos causativos do ASD e investigar como estes podem contribuir para as características deste distúrbio. Para atingir os objetivos, nós analisamos as variantes raras de perda de função nas regiões codificadoras de genes expressos no sistema nervoso central (SNC) compartilhadas e não compartilhadas entre indivíduos com ASD aparentados. Outra abordagem escolhida foi a análise das células neuronais derivadas de iPSC de um indivíduo com mutação bialélica no *TBCK*, onde avaliamos se a expressão reduzida deste gene causa alterações de processos celulares e moleculares; e, por fim, investigamos as alterações morfológicas das estruturas neuronais de linhagens de *D. melanogaster* com diminuição da expressão do *trpy*, *mahjong*, *crmp* e *dys*.

A análise das variantes de perda de função raras em genes expressos no sistema nervoso central, realizada em 13 famílias sem suspeita clínica revelou 55 variantes em 54 genes, onde 12 destas variantes foram compartilhadas entre os afetados da mesma família e 44 não foram compartilhadas. Dentre os 54 genes mutados, 16 genes foram considerados de maior risco para o ASD, destes, o *AP1S2*, o *DYRK1A* e o *TBCK* se destacaram por determinarem padrões monogênicos de herança. Além disso, 13 dos 16 genes estão sendo sugeridos pela primeira vez como fortes candidatos para o ASD. Esta heterogeneidade dos padrões genéticos tem sido mostrada nos variados trabalhos do ASD (CASEY *et al.*, 2012; CHAHROUR *et al.*, 2012; CHAPMAN *et al.*, 2015; CHO *et al.*, 2009; CUKIER *et al.*, 2014; GLESSNER *et al.*, 2009; IOSSIFOV *et al.*, 2012; MICHAELSON *et al.*, 2012; MOREIRA *et al.*, 2016; NEALE *et al.*, 2012; O'ROAK *et al.*, 2011, 2012; POULTNEY *et al.*, 2013; SANDERS *et al.*, 2012; TOMA *et al.*, 2013; WOODBURY-SMITH *et al.*, 2015; YU *et al.*, 2013; YUEN *et al.*, 2015). Somado aos três genes acima citados, outros, como o *TTC12*, associado a transtorno de déficit de atenção e hiperatividade, e o *APC2*, relacionado a síndrome de Sotos, têm sido identificados em outras doenças de neurodesenvolvimento (ALMURIEKHI *et al.*, 2015; MOTA *et al.*, 2015). Os dados da literatura corroboram os nossos achados, uma vez que mostram a

sobreposição de causas genéticas das condições neurológicas e evidenciam a heterogeneidade genética do ASD.

Ainda, observamos que dentre as variantes de risco herdadas e não compartilhadas, elas foram preferencialmente transmitidas pelas mães de indivíduos com ASD. Adicionalmente, por análise de enriquecimento de termos do Gene Ontology, mostramos que para os genes mutados nos casos familiares houve uma super-representação de termos relacionados com transporte celular de moléculas e desenvolvimento do sistema nervoso. O enriquecimento destes processos biológicos nos casos de ASD é frequentemente descrito (GAI *et al.*, 2011; KONOPKA *et al.*, 2012; NISHIMURA *et al.*, 2007; PARIKSHAK *et al.*, 2013; PINTO *et al.*, 2014; VOINEAGU *et al.*, 2011), indicando, dessa maneira, que devem ser fundamentais para o ASD. Portanto a presente pesquisa, contribuiu para identificação de novos genes candidatos e confirma que, nos casos familiares, todos os indivíduos devem ser analisados de forma conjunta e também de forma independente.

A importância de estudos de casos familiares de ASD foi novamente ilustrada ao investigarmos a causa molecular do ASD em uma família composta por três irmãos gêmeos não idênticos com ASD, dos quais dois foram também diagnosticados com DMD. Nos dois indivíduos com ASD/DMD identificamos uma pequena deleção no gene da distrofina. Apesar da distrofina ter isoformas que se expressam no SNC, o ASD não é típico de todos casos de DMD. Visando esclarecer se outras variantes poderiam ser responsáveis por este fenótipo neuropsiquiátrico, buscamos por variantes raras de perda de função em genes expressos no SNC e detectamos duas variantes: uma no *DPYSL4* e *OPALIN*, os quais nunca foram anteriormente associados ao ASD. O *DPYSL4* atua na regulação do citoesqueleto, enquanto o *OPALIN* age na mielinização nos oligodendrócitos (QUACH, TAM T *et al.*, 2013; QUACH, T T *et al.*, 2011; SATO *et al.*, 2014; YOSHIKAWA *et al.*, 2008, 2016). Embora a investigação da contribuição de outros genes para o ASD nos casos de DMD seja um grande desafio, com esta família pudemos investigar a intrincada estrutura genética destes fenótipos e verificar que outros genes podem estar interferindo nas características cognitivas dos indivíduos com DMD.

O nosso estudo genômico de duas irmãs revelou mutações bialélicas no *TBCK*, que são certamente patogênicas considerando o tipo da mutação bem como os dados da literatura (ALAZAMI *et al.*, 2015; BHOJ *et al.*, 2016; CHONG *et al.*, 2016). Neste trabalho, utilizando células neuronais derivadas de iPSC, confirmamos

que o *TBCK* atua na via *mTOR*, e que apresenta importante papel na regulação do ciclo celular, na proliferação e crescimento celular e na autofagia de células neuronais.

Uma perspectiva futura é estabelecer tratamento dos indivíduos com ASD. Para isso, é importante entender como as moléculas atuam no resgate dos fenótipos moleculares e celulares. Assim, investigamos a capacidade da L-leucina no resgate dos fenótipos alterados das NPCs com deficiência do *TBCK* e, assim, verificamos o aumento do crescimento celular e da sinalização da via *mTOR*. A via *mTOR* é uma das centrais na regulação de diversos processos celulares e tem sido muito relacionada com distúrbios de neurodesenvolvimento (CECCONI *et al.*, 2007; LIPTON; SAHIN, 2014; SATO *et al.*, 2014; YOSHIKAWA *et al.*, 2008, 2016). Entretanto, a atuação do *TBCK* nessa via e em qualquer outra é ainda pouco compreendida. Apesar de termos contribuído para compreender a função deste gene em células neuronais, ainda faltam estudos para mostrar os outros mecanismos pelo qual o *TBCK* pode levar a alterações neurológicas.

Por fim, exploramos o uso de *D. melanogaster* como modelo para investigarmos a contribuição de alguns dos genes identificados como candidatos para o ASD a partir de nossos estudos, em particular os genes *DMD* e *DPYSL4* identificados na família com estes dois fenótipos e os genes *TRPC6* e *VPRBP*, detectados em um paciente com ASD em um estudo anterior (GRIESI-OLIVEIRA *et al.*, 2014). Os resultados mostraram-se promissores, pois observamos um número maior de alterações nas estruturas neuronais das linhagens de *D. melanogaster* com diminuição da expressão dos ortólogos de *DPYSL4*, *DMD*, *TRPC6* e *VPRBP* em relação aos controles. Diante disso, a nossa perspectiva futura é investigar se a diminuição da expressão dos *DPYSL4:DMD* e *TRPC6:VPRBP* conjuntamente poderiam ter um efeito sinérgico ou aditivo para, então, conseguirmos elucidar melhor a causa do ASD nestes indivíduos. Já os estudos comportamentais estão sendo realizados em colaboração com John Ewer, e, assim que possível, os dados serão cruzados obtermos as conclusões sobre as alterações nos genes citados.

CONSIDERAÇÕES GERAIS E CONCLUSÕES

A partir de abordagens complementares, análise genômica e estudo funcional utilizando diferentes modelos, o presente trabalho nos possibilitou chegar às seguintes conclusões:

Estudo genômico:

1. Em cerca de 23% dos casos familiares, o ASD foi devido a variantes genéticas em um gene principal e as variantes de risco podem ou não ser compartilhadas entre os afetados;
2. Cada família de ASD tende a ter variantes genéticas de risco únicas;
3. Entre as variantes não compartilhadas entre os indivíduos com ASD da mesma família, há um excesso de variantes de origem materna;
4. Dezesseis dos 54 genes candidatos para o ASD compreendem os candidatos com maior probabilidade de serem causativos dos fenótipos neuropsiquiátricos;
5. Há uma redundância dos processos biológicos alterados nos casos de ASD;
6. Pelas análises *in silico* realizadas é possível predizer que as variantes que rompem *DPYSL4* e *OPALIN* podem contribuir para o fenótipo de ASD.

Estudos funcionais:

1. A redução dos níveis de *TBCK* nas células neuronais causa alterações no ciclo, proliferação e crescimento celular e também leva ao aumento da autofagia;
2. O tratamento com L-leucina das células neuronais com reduzida expressão do *TBCK* tem melhoras dos fenótipos avaliados;
3. A diminuição da expressão de *mahjong*, *trpgamma*, *crmp* and *dys*, ortólogos dos genes humanos *VPRBP*, *TRPC6*, *DPYSL4* e *DMD*, respectivamente, levam a alterações na morfologia dos neurônios de *D. melanogaster*.

RESUMO

O transtorno do espectro autista (ASD, do inglês, autism spectrum disorder) é uma condição neuropsiquiátrica de início precoce, caracterizado por déficit do uso da comunicação para socialização e presença de padrões de comportamentos restritos e repetitivos. A herdabilidade do ASD tem sido estimada em 50-90%. O ASD pode se apresentar como uma condição sindrômica, considerando, nesses casos, síndromes genéticas, como a distrofia muscular de Duchenne (DMD), e como uma condição não sindrômica. Além disso, o ASD apresenta uma grande heterogeneidade genética e centenas de genes têm sido relatados como candidatos. As variantes com alta patogenicidade para o ASD são mais comumente raras, *de novo* e levam ao truncamento da proteína. A função da maioria dos genes candidatos para o ASD ainda é desconhecida e pode explorá-la pode trazer grande conhecimento sobre essa condição. O estudo genômico de casos familiares de ASD pode facilitar a identificação de fatores genéticos possivelmente patogênicos para o ASD, uma vez que esses casos podem estar enriquecidos de fatores genéticos e têm sido pouco explorados. Então, uma estratégia para identificar e validar genes candidatos para o ASD é investigar variantes que levam ao truncamento das proteínas nos casos familiares de ASD. Além disso, entender as alterações dos processos moleculares e celulares desregulados pelos genes candidatos para o ASD pode nos ajudar a compreender melhor a relevância desses genes na manutenção da homeostasia do sistema nervoso central e, também, como esses genes podem causar o fenótipo do ASD. Dessa forma, primeiramente, nós investigamos variantes exômicas raras de perda de função (rLoF, do inglês, rare loss-of-function) ($MAF < 0,01$) potencialmente patogênicas compartilhadas e não compartilhadas por indivíduos aparentados afetados pelo ASD em 13 famílias não relacionadas. Ademais, analisamos se outras variantes rLoF poderiam contribuir para o ASD em dois irmãos que também são afetados por DMD. A partir disso, identificamos 56 variantes rLoF em 54 genes, as quais foram compartilhadas (12 variantes em 11 genes) e não compartilhadas (44 variantes em 43 genes) entre os indivíduos afetados das famílias. Desse total de 54 genes, foi possível destacar 16 genes como principais causas do ASD, nos quais as mutações observadas foram tanto herdadas quanto *de novo*. Nos indivíduos com ASD/DMD, detectamos uma deleção no gene da distrofina, a qual explica o fenótipo de DMD, e outras duas variantes possivelmente patogênicas no *DPYSL4* e no *OPALIN* que podem contribuir para o ASD. Em uma das famílias estudadas, identificamos

mutações bialélicas de perda de função no *TBCK*, assim estudamos as células neuronais derivadas de iPSC de um indivíduo com rompimento do *TBCK*. A compreensão da função desse gene pode auxiliar no entendimento das vias de sinalização e assim na busca de tratamentos para os fenótipos neurológicos. No presente estudos, mostramos que a depleção do *TBCK* nas células neuronais causa alterações no ciclo e proliferação celular, além de desregulação da via *mTOR*. O tratamento com a L-leucina, um aminoácido que sinaliza na via *mTOR*, das células neuronais com diminuição de *TBCK* resgatou a sinalização da via *mTOR*, bem como, aumentou a proliferação celular. Assim, os nossos resultados sugerem que a L-leucina pode resgatar os fenótipos causados pela redução da expressão do *TBCK*, os quais abrem novas perspectivas de tratamento de crianças com mutações nesse gene. Somado a isso, nós exploramos o uso da *Drosophila melanogaster* para realizar estudos funcionais para outros genes candidatos para o ASD. Nós analisamos a morfologia neuronal nas larvas dessa mosca com expressão reduzida do *trpy*, *mahjong*, *dys* e *crmp*, os quais são, respectivamente, os ortólogos dos genes humanos candidatos para o ASD: *TRPC6*, *VPRBP*, *DMD* e *DPYSL4* (*CRMP3*). Nas linhagens com diminuição da expressão do *trpy*, *mahjong*, *dys* e *crmp*, nós observamos várias alterações morfológicas nas estruturas neuronais das larvas, tais como, defasciculação axonal e anormalidade no formato do ângulo nos neuritos ipsilaterais-contralaterais, em uma frequência maior do que nos controles. Assim sendo, este trabalho evidenciou a heterogeneidade genética do ASD em famílias brasileiras, permitiu a validação e identificação de genes candidatos adicionais para o ASD, contribuiu para a melhor compreensão do papel de alguns genes, em particular, o *TBCK*, e para o estabelecimento do uso da *D. melanogaster* para estudar os genes candidatos para o ASD no nosso laboratório.

ABSTRACT

Autism Spectrum Disorder (ASD) is a neuropsychiatric condition of early onset, characterized by deficit in social communication and repetitive and restrict behavior. Its heritability has been estimated between 50-90%. The ASD cases can be related to both syndromic conditions, considering, in these cases, genetic syndromes like Duchenne muscular dystrophy (DMD), and non-syndromic conditions. Moreover, this disorder presents high genetic heterogeneity, and several hundreds of genes have reported as candidates. Variants associated with high pathogenicity in ASD are most usually rare, *de novo* and lead to protein truncation. The role of most of the ASD candidate genes is still under unclear and explore it could bring important knowledge about this condition. The genomic study of familial cases of ASD could aid the identification of likely pathogenic genetic factors, as it might be enriched by genetic factors and have not been largely explored. Therefore, a potential approach to validate and identify additional ASD candidate variants would be to investigate if truncating variants would explain the ASD phenotype in familial cases. Besides, understanding the molecular and cellular process altered by ASD candidate genes could clarify the relevance of these genes on keeping central nervous system homeostasis as well as how the deficiency of these genes would cause the ASD phenotype. Thus, firstly, we investigated rare loss-of-function (rLoF) variants (MAF<0.01) shared and unshared among ASD related individuals from 13 unrelated families with the aim of identifying those that contribute to the phenotype. Also, we tested if additional rLoF variants would contribute to the ASD phenotype in DMD brothers. We identified 56 rLoF variants in 54 genes, which were shared (12 variants in 11 genes) and unshared (44 variants in 43 genes) among affected individuals within a family. We pinpointed 16 genes out of 54 as major cause of ASD, which included both inherited and *de novo* mutations. In ASD/DMD individuals, we detected a deletion in dystrophin gene, which explains the DMD phenotype, and other two likely pathogenic variants in *DPYSL4* and *OPALIN* that can contribute to ASD. In one of the families, we identified biallelic loss-of-function mutations in *TBCK*. Thus, we studied the phenotypes of iPSC-derived neuronal cells of an individual with disruption of the *TBCK*. The comprehension of the gene function can lead us to look for treatments for the neurological phenotypes. In the present study, we show that the depletion of *TBCK* in neuronal cells cause cell cycle and proliferation abnormalities and *mTOR* dysregulation. The treatment of *TBCK*-depleted neuronal cells with L-leucine improved mTOR signaling, as well as increased cell proliferation. Thus, our results suggest that L-leucine could rescue the neuronal phenotypes caused by reduced expression of *TBCK*, which can open new perspectives on the treatment of children with mutations in this

gene. Additionally, we explored the use of *Drosophila melanogaster* to conduct functional studies of ASD candidate genes. We analyzed neuronal morphology in flies' larvae with reduced expression of *trpy*, *mahjong*, *dys* and *crmp*, which are, respectively, the orthologs of the ASD candidate human genes TRPC6, VPRBP, DMD and DPYSL4 (CRMP3). In lines with reduced expression of *trpy*, *mahjong*, *dys* and *crmp*, we observed several morphological alterations in the neuronal structure, like axonal defasciculation and aberrant form of ipsilateral-contralateral neurite angles, more frequently than in controls. Hence, this work pointed the genetic heterogeneity of ASD in Brazilian families, allowed the validation and identification of additional gene targets for ASD, contributed to a better understanding of the role of some ASD genes, most particularly of *TBCK*, and, we could set up the use of *D. melanogaster* to explore ASD candidate genes in our laboratory.

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