Cellular Characterization of Fibroblasts Harboring Variants in TRAPPC2L Linked to Neurodevelopmental Disorders.

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complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract for Masters

Cellular characterization of fibroblasts harboring variants in TRAPPC2L linked to neurodevelopmental disorders.

Noraldin Al-Deri

In this thesis, I focus on TRAPPC2L, a recently discovered core subunit of the TRAnsport Particle Protein (TRAPP) complexes, which is not well studied. I performed biochemical and cell biological functional studies to characterize the cellular phenotype of the first identified homozygous missense variants (p.Asp37Tyr and p.Ala2Gly) in TRAPPC2L linked to neurodevelopmental delays and intellectual disabilities. In this study, I show that only the p.(Ala2Gly) variant, but not the p.(Asp37Tyr) variant, disrupted the interaction between TRAPPC2L and TRAPPC6a, another core TRAPP protein. I also show by using size exclusion chromatography that both TRAPPC2L variants disrupted the assembly and stability of the TRAPP complexes in lysates from fibroblasts harbouring the two variants. In addition, we used two different membrane trafficking assays on fibroblasts from individuals harboring the variants in TRAPPC2L and we found delays in endoplasmic reticulum-to-Golgi and post- Golgi trafficking. In this study, I better characterized the role of TRAPPC2L in the function and assembly of TRAPP and supported the pathogenicity of the two TRAPPC2L variants, p.(Asp37Tyr) and p.(Ala2Gly).

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I would like to acknowledge and thank the patients in this study and for their parents for trusting me to handle this project.

I would also like to acknowledge and thank everyone that contributed to this study by providing data or their valued scientific opinion. Special thanks to Miro for sharing his data and knowledge.

Dedication

I dedicate my thesis work to my family. Starting with my loving parents, Drs. Maher Al-Deri and Camelia Ruso, I couldn't have done this without you. My sisters Mariam and Rania and my brothers Rateb, Nawras, and Nader have never left my side and are very special to me. I also dedicate my work to Princess Jana and Khalil. To my friend James Dhaliwal for being supportive and simply the best, thank you!

I am also dedicating this thesis to two beloved people who mean so much to me. First and foremost, to my paternal grandfather Ratib Al-Deri (1936-2020). Thank you so much "Sedo", I will never forget you. Next, my maternal grandfather George Ruso (1943-2021). I will make sure your memory lives on as long as I shall live. I love you both and miss you beyond words. May Allah (GOD) (SWT) grant you Jannah Firdaws.

I also dedicate this work and special thanks to my hemato-oncologist, Dr. Alain Bestavros, for your help and support during my chemotherapy treatment last year and during the pandemic and beyond. You helped me go through the most challenging times of my life. I am always grateful. Also, special thanks to Professor Dr. Philip Zelazo, for your guidance and support. Thank you for opening my eyes to different possibilities and opportunities. I am looking forward to making a difference together and helping children with intellectual disabilities have a voice.

Contribution of Authors

The manuscript is adapted from: *Al-Deri N, Okur V, Ahimaz P, Milev M, Valivullah Z, Hagen J, Sheng Y, Chung W, Sacher M, Ganapathi M. A novel homozygous variant in TRAPPC2L results in a neurodevelopmental disorder and disrupts TRAPP complex function. J Med Genet. 2020 Aug 25:jmedgenet-2020-107016. doi: 10.1136/jmedgenet-2020-107016. PMID: 32843486*

List of contributions:

- Noraldin Al-Deri performed, analyzed, and organized Figure 1, Figure 3 (A,B,C,D), Figure 4 (A,B,C,D,E), Figure 5 (A,B), and Figure 6 (A inset, C, D). Noraldin Al-Deri also arranged all the figures and graphs in this manuscript.
- Milev M performed and analyzed Figure 6 (A, B).
- Ahimaz P and Chung W provided the clinical data in Figure 2.
- Hagen J and Sheng Y provided and interpreted the haplotype data in Figure 2.

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List of Abbreviations

BioID	Biotin proximity ligation
CD	Circular dichroism
DDO	Double drop-out
ER	Endoplasmic reticulum
GAP	GTPase-activating protein
GEF	Guanine exchange factor
GDP	Guanosine diphosphate
GME	The Greater Middle East
gnomAD	Genome Aggregation Database
GST	Glutathione-S transferase
GTP	Guanosine triphosphate
LD	Longin Domain
SEDT	Spondyloepiphysealdysplasia Tarda
SNARE	Soluble NSF Attachment protein REceptor
TDO	Triple drop-out
TM	Midpoint of the unfolding transition
TOPMed	Trans-Omics for Precision Medicine
TRAPP	TRAnsport Particle Protein

Chapter 1: Introduction

1.1 Membrane trafficking:

The vast majority of proteins in eukaryotic cells are synthesized in the cytosol by the cytosolic ribosomes except for transmembrane and secretory proteins, which are synthesized on ribosomes bound to the endoplasmic reticulum (ER). Upon entering the ER, proteins undergo post-translational modifications such as glycosylation, disulfide bond formation, and phosphorylation to achieve proper folding or attain and modify their protease cleavage site. These proteins then pass from the ER to the Golgi apparatus and eventually traverse to different cellular organelles or are secreted out of the cell. The process by which the cells employ vesicles or membranes to move proteins from one compartment to another is called 'membrane trafficking.'

Membrane trafficking is a fundamental process that ensures the delivery of proteins and lipids to endomembrane compartments. Defects in this process are implicated in various human diseases, including lung diseases such as cystic fibrosis, liver diseases such as hereditary hemochromatosis, and neurological diseases such as fabri disease (Aridor & Hannan, 2000, 2002). In membrane trafficking (also known as vesicular transport), the bidirectional shuttling of lipids and proteins to various organelles and the plasma membrane is mediated by small vesicle carriers that emerge from a donor compartment and then fuse with the appropriate acceptor membrane compartment. The entire process requires the orchestrated activity of several different factors, including; coat proteins that accumulate and mark the donor membrane to encapsulate specific cargo proteins and the budding of vesicles, Rab GTPases that function as molecular switches for vesicle formation and fusion with the target membrane (Béthune & Wieland, 2018) and SNARE proteins and tethering factors that help coordinate vesicle fusion (Bröcker et al., 2010).

1.2 Mammalian TRAPP:

Tethering factors are a group of proteins that mediate the loose bridging (tethering) of transport vesicles with acceptor membranes to ensure correct docking and fusion. This initial tethering is followed by a stable docking interaction that requires SNAREs (Waters & Pfeffer, 1999). One of the best studied tethering factors are the TRAnsport Particle Protein (TRAPP) complexes. In mammalian cells, TRAPP consists of two complexes: TRAPP II and TRAPP III (figure 1) (Bassik et al., 2013). Mammalian TRAPP complexes share a common core consisting of two copies of TRAPPC3 and one copy each of TRAPPC1, TRAPPC2, TRAPPC4, TRAPPC5, and TRAPPC6. The mammalian TRAPP II complex is comprised of two additional subunits, TRAPPC9 and TRAPPC10. TRAPP III, on the other hand, consists of TRAPPC8, TRAPPC13 and two additional metazoan-specific subunits, TRAPPC11 and TRAPPC12 (Scrivens et al., 2011). Mammalian TRAPP II has been implicated in ER-to-Golgi trafficking, while TRAPP III has been implicated in autophagy (Yamasaki et al., 2009; Lamb et al., 2016; Stanga et al., 2019). Mammalian TRAPP II has also been shown to function as a guanine exchange factor (GEF) for two Rab GTPases (Rab11 and Rab1) that both serve as master regulators for membrane trafficking (Riedel et al., 2018).

Variants in genes encoding TRAPP subunits have been linked to a broad spectrum of human disease, confirming the significance of the TRAPP complex in humans (Sacher et al., 2019). Although TRAPP was thought to act as a single unit, variations in genes encoding the TRAPP subunits have resulted in different diseases with overlapping phenotypes. For example, variants in TRAPPC6a, TRAPPC6b, TRAPPC9, TRAPPC11 and TRAPPC12 were all linked to a broad spectrum of neurodevelopmental disorders (Bögershausen et al., 2013; Harripaul et al., 2018; Khattak & Mir, 2014; Milev et al., 2017; Mohamoud et al., 2018). The distinct disorders caused

by those variations suggest different pools of the TRAPP complex or specific functions for the TRAPP subunits outside of the TRAPP complexes.

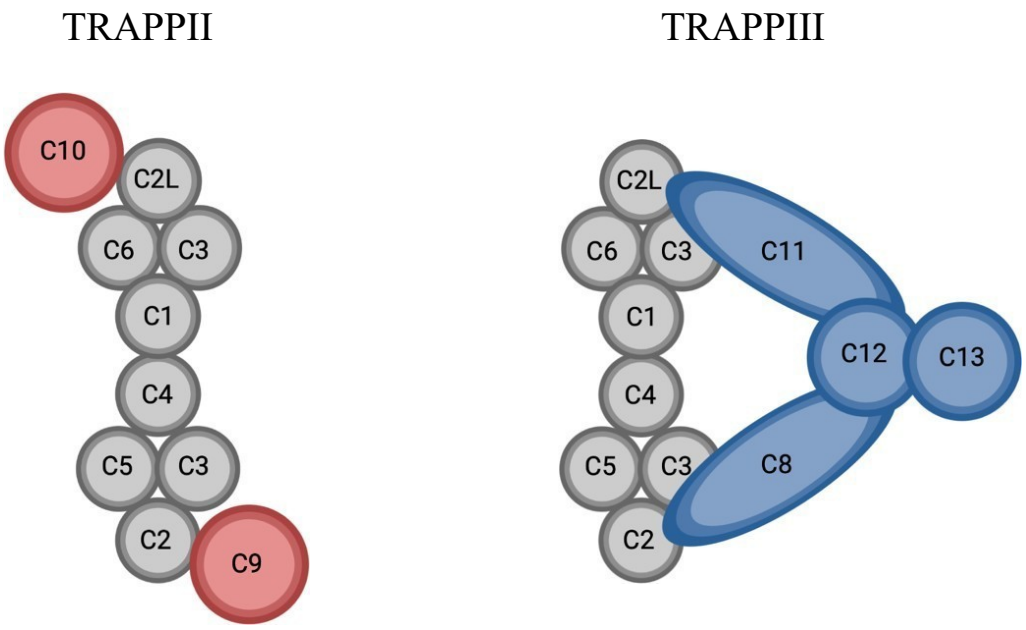


Figure 1. The mammalian TRAPP complexes. Cartoons of the two TRAPP complexes are shown. The core of proteins found in all complexes is colored in grey. The arrangement of the subunits within this core is based on its known organization (Cai et al., 2008; Kim et al., 2006) . The placement of the TRAPPC8 subunit is based on the homology to the yeast protein as well as one study indicating that it interacts with TRAPPC2 (Zong et al., 2011). The placement of TRAPPC10 is based on a study (Milev et al., 2018) that suggests it interacts with TRAPPC2L, and the placement of TRAPPC9 is based on a biochemical study (Zong et al., 2011). The placement of TRAPPC11, TRAPPC12, and TRAPPC13 is based on a recent study (Galindo et al., 2021).

1.3 TRAPP as a tether:

The idea that TRAPP is a tether was based on the accumulation of ER vesicles after TRAPP depletion (Sacher et al., 2001). This effect could be indirect due to its guanine exchange factor activity for Rab1/Ypt1, which is involved in the recruitment of Golgi tethers (Jones et al., 2000). "Tethers" are a class of proteins that function as Rab effectors and bridge two opposing membranes, no to be mistaken with "tethering factors" which are proteins that act as facilitators for membrane trafficking and organize other factors essential for tethering. The interaction of TRAPP with Rab GTPases supported the notion that TRAPP might be a tether. However, unlike other tethers, TRAPP complexes act as GEFs for GTPases and are not downstream effectors.

As a tether, TRAPP is expected to bridge between two membranes. In yeast, TRAPP consists of three complexes (TRAPP I, TRAPP II, and TRAPP III). The two copies of TRAPPC3, situated on opposing sides of the yeast TRAPP I complex were proposed to tether ER-derived vesicles (Cai et al., 2007). However, the distance that TRAPP I can bridge between two membranes cannot exceed 40 Å, which is shorter than a SNARE complex (Kim et al., 2016). Previous studies have suggested that similar to mammalian TRAPP, the yeast TRAPP complex consists of only two complexes, TRAPP II and TRAPP III (Brunet et al., 2012; Thomas et al., 2018). Therefore, evidence that any TRAPP complex acts as a tether is inconclusive, and further studies are needed to elucidate any tethering functions for these complexes. However, TRAPP complexes are still considered tethering factors due to the supporting evidence of their involvement in the tethering process and their GEF activity for Rab GTPases that are essential for tethering (Cai et al., 2007; Sacher et al., 2001; Riedel et al., 2018).

1.4 TRAPPC2L, a novel subunit of TRAPP:

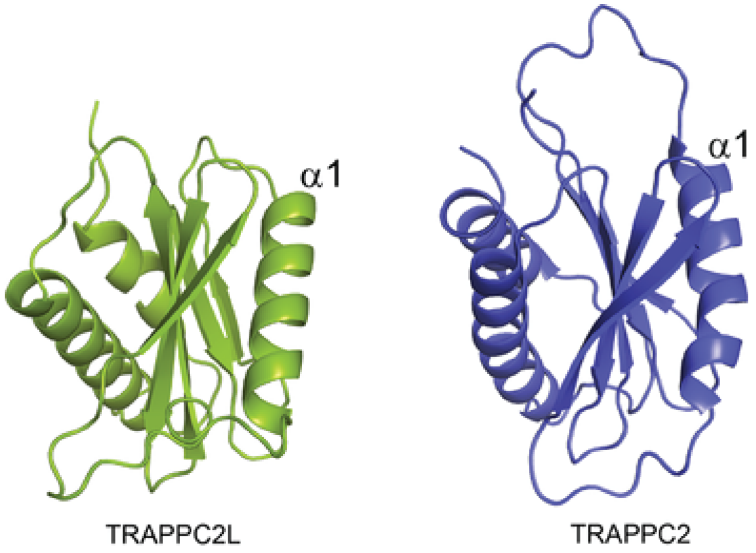
One subunit that will be the focus of my thesis is called TRAPPC2L, a recently discovered component of TRAPP that is not well studied (Scrivens et al., 2009). TRAPPC2L has been suggested to play an essential role in the assembly and function of TRAPP II despite being one of the nonessential TRAPP subunits (Montpetit & Conibear, 2009). Although the structure of TRAPPC2L is not yet determined, it can be modeled using its yeast homologue, Tca17p (PDB ID: 3PR6) (figure 2). TRAPPC2L shares similarities in its structure and sequence to another TRAPP protein called TRAPPC2 (figure 2A) (C. Wang et al., 2014). Both proteins adopt a Longin-Domain (LD) fold composed of a five-stranded β-sheet with one helix on one side and two helices on the other side. Sequence alignment of TRAPPC2 and TRAPPC2L shows that only two residues are 100% conserved between TRAPPC2L and TRAPPC2 (figure 2B). These two residues are predicted to be exposed at the surface of TRAPPC2L suggesting that they are important for the function of TRAPPC2L (figure 2C).

The TRAPPC2 core protein has been shown to function as an adapter by binding to TRAPP-specific subunits of both TRAPP II and III (Zong et al., 2011). An adaptor TRAPP subunit is important for the association of larger TRAPP subunits such as TRAPPC10, TRAPP11, TRAPPC8, TRAPPC9, and TRAPPC12. The yeast homologue of TRAPPC2L called Tca17p was previously suggested to be a TRAPP II- specific subunit playing a role in regulating its stability, assembly, and function (Montpetit & Conibear, 2009; Scrivens et al., 2009). Our recent biotin proximity ligation (BioID) analysis of the protein interactors of TRAPPC11 revealed TRAPPC2L as well as other TRAPP III-specific subunits (TRAPPC12, TRAPPC8, and TRAPPC13) as possible interactors of TRAPPC11 (Stanga et al., 2019). This finding challenges the notion that

TRAPPC2L is a TRAPP II- specific subunit and assigning a potential adaptor function for TRAPPC2L.

Not much is known about TRAPPC2L, yet it may play a crucial role in regulating TRAPP assembly and function. In this study, fibroblasts harbouring single point mutations in TRAPPC2L were obtained. The main objectives of this thesis are to better understand the etiology of the neurodevelopmental disorders linked to the single point mutations in TRAPPC2L and characterize the roles of TRAPPC2L in regulating TRAPP function and assembly.

A



B

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HumTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
PonTrappC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
PanTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
MacTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
RatTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
MusTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
DogTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
CowTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
EquTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
ChkTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
OppTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
XenTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
ZbfTRAPPC2   R-HLNQFIAHAALDLVDEN---MW--LSNN
DroTRAPPC2   R-HLTQFIAHAALDLVDEH---KWKTA---
ElgTRAPPC2   R-HLNHYIGHAALDIVDEH------ALTTS
CerTRS20P    K-ELNPFILHASLDIVEDL---QW-QINPT
PomTrs20p    S-HLNQFIVHSSLDIVD-----QL-QWTSN
PomSedFam    RY---QYLGELSLDVIND--------LVND
CerYEL048C   KY--NV-LSNISLDYFESA-------LVEW
HumTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
PonTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
PanTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
MacTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
RatTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
MusTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
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EquTRAPPC2L  KFH---YMVHTSLDVVDEKISAMGKALVDQ
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OppTRAPPC2L  KFH---YTVHTSLDVVDEKVSAMGKALVDQ
XenTRAPPC2L  KFH---YTVHTSLDVVDEKISAMGKAVMDQ
ZbfTRAPPC2L  KFH---YTVHTSLDVVEEKISGVGKALADQ
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ElgTRAPPC2L  LEIEM-FTFC-SLDIVDEK-S--TKA-SEM
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C

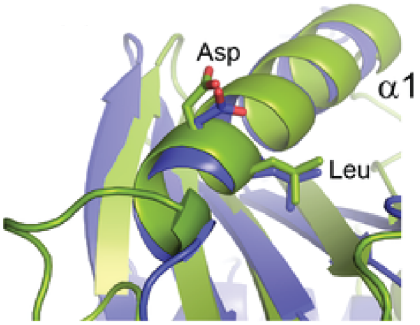


Figure 2. TRAPPC2L is a novel TRAPP subunit that shares similarities with the adaptor TRAPPC2 subunit. (A) The structure of TRAPPC2L was modeled using the structure of its yeast homologue Tca17p (PDB ID: 3PR6). TRAPPC2L consists of a Longin Domain (LD) motif and shares many structural similarities to TRAPPC2 (PDB ID: 1H3Q), shown on the right for comparison (Sacher et al., 2019). (B) Sequence alignment of all identified TRAPPC2 and TRAPPC2L proteins reveal that only two residues show 100% conservation (highlighted in black). Residues highlighted in dark grey show 75% identity between TRAPPC2L and TRAPPC2 and residues highlighted in light grey show 75% similarity between TRAPPC2L and TRAPPC2 (Scrivens et al., 2009). (C) The only residues that show absolute conservation between TRAPPC2L and TRAPPC2 are located on the alpha helix α1 and are located on the surface of the protein suggesting that they are important for the function of TRAPPC2L (Sacher et al., 2019).

Chapter 2: A novel homozygous variant in TRAPPC2L results in a neurodevelopmental disorder and disrupts TRAPP complex function.

Adapted from: Al-Deri N, Okur V, Ahimaz P, Milev M, Valivullah Z, Hagen J, Sheng Y, Chung W, Sacher M, Ganapathi M. A novel homozygous variant in TRAPPC2L results in a neurodevelopmental disorder and disrupts TRAPP complex function. J Med Genet. 2020 Aug 25:jmedgenet-2020-107016. doi: 10.1136/jmedgenet-2020-107016. PMID: 32843486

2.1 Contributions:

- I performed, analyzed, and organized Figure 2, Figure 4 (A,B,C,D), Figure 5 (A,B,C,D,E), Figure 6 (A,B), and Figure 7 (A inset, C, D). I also arranged all the figures and graphs in this manuscript.
- Milev M performed and analyzed Figure 7 (A, B).
- Ahimaz P and Chung W provided the clinical data in Figure 3.
- Hagen J and Sheng Y provided and interpreted the haplotype data in Figure 3.
- All authors reviewed the final manuscript and approved of the contents.

2.2 Abstract

Background: Next-generation sequencing has facilitated the diagnosis of neurodevelopmental disorders with variable and non-specific clinical findings. Recently, a homozygous missense p.(Asp37Tyr) variant in TRAPPC2L, a core subunit of TRAPP complexes which function as tethering factors during membrane trafficking, was reported in two unrelated individuals with neurodevelopmental delay, post-infectious encephalopathy-associated developmental arrest, tetraplegia and accompanying rhabdomyolysis. Methods We performed whole genome sequencing on members of an Ashkenazi Jewish pedigree to identify the underlying genetic aetiology of global developmental delay/intellectual disability in three affected siblings. To assess the effect of the identified TRAPPC2L variant, we performed biochemical and cell biological functional studies on the TRAPPC2L protein.

Results: A rare homozygous predicted deleterious missense variant, p.(Ala2Gly), in TRAPPC2L was identified in the affected siblings and it segregated with the neurodevelopmental phenotype within the family. Using a yeast two-hybrid assay and in vitro binding, we demonstrate that the p.(Ala2Gly) variant, but not the p.(Asp37Tyr) variant, disrupted the interaction between TRAPPC2L and another core TRAPP protein, TRAPPC6a. Size exclusion chromatography suggested that this variant affects the assembly of TRAPP complexes. Employing two different membrane trafficking assays using fibroblasts from one of the affected siblings, we found a delay in traffic into and out of the Golgi. Similar to the p.(Asp37Tyr) variant, the p.(Ala2Gly) variant resulted in an increase in the levels of active Rab11.

Conclusion: Our data fill in a gap in the knowledge of TRAPP architecture with TRAPPC2L interacting with TRAPPC6a, positioning it as a putative adaptor for other TRAPP subunits. Collectively, our findings support the pathogenicity of the TRAPPC2L p.(Ala2Gly) variant.

2.3 Introduction

Exome/genome sequencing has, in addition to identifying new gene–disease associations, identified the underlying genetic causes of neurodevelopmental disorders in many individuals with variable non-specific clinical manifestations. Recently, a homozygous missense p.(Asp37Tyr) variant in TRAPPC2L (MIM:610970) was reported in two unrelated individuals with neurodevelopmental delay, seizures, post-infectious encephalopathy and episodes of rhabdomyolysis (Milev et al., 2018). TRAPPC2L is one of the core subunits of TRAPP (TRAnsport Protein Particle) complexes II and III that play an important role as tethering factors in membrane trafficking (Sacher et al., 2019). Membrane trafficking is a cellular process involved in protein localisation to the various intracellular compartments using membrane-bound vesicles. This process requires numerous proteins and complexes including Rab family GTPases, guanine nucleotide exchange factors, Soluble NSF Attachment protein REceptors (SNAREs) and tethering factors. TRAPP are highly conserved tethering factor complexes in eukaryotes which regulate fusion of vesicles to membranes. These conserved multi-subunit complexes were originally identified in yeast and consist of core and modular subunits. In humans, two TRAPP complexes, TRAPP II and III, have been described. Each has a common core of subunits (TRAPPC1, TRAPPC2, TRAPPC2L, TRAPPC3, TRAPPC4, TRAPPC5 and TRAPPC6) as well as complex-specific proteins (TRAPPC8, TRAPPC9, TRAPPC10, TRAPPC11, TRAPPC12 and TRAPPC13). TRAPPC2L was initially identified based on homology to the TRAPPC2 core subunit and TRAPPC2L complexes may function in a post-Golgi compartment. Many members of the TRAPP complexes have been implicated in neurodevelopmental disorders (TRAPPC6A, TRAPPC6B, TRAPPC9 and TRAPPC12), muscular dystrophies (TRAPPC11) and skeletal dysplasias (TRAPPC2) following autosomal and X-linked recessive patterns of inheritance, respectively

(Bögershausen et al., 2013; Gedeon et al., 1999; Marin-Valencia et al., 2018; Mir et al., 2009; Mochida et al., 2009; Mohamoud et al., 2018).

Here, we report three siblings from an Ashkenazi Jewish family with neurodevelopmental delay/intellectual disabilities in whom we identified a homozygous missense TRAPPC2L variant and performed functional studies to assess the effect of the variant on TRAPPC2L function using human and yeast cells.

2.4 Methods

2.4.1 Patient ascertainment and genomic analysis

Written informed consents were obtained from all participants. We performed wholegenome sequencing on peripheral blood DNA samples from three affected siblings with a similarclinical presentation and clinical course and their unaffected parents. Sequencing methods and variant filtering criteria were as previously described (Okur et al., 2019). The candidate variant and segregation in the family with the neurodevelopmental phenotype were confirmed by Sanger sequencing.

2.4.2 Haplotype analysis

For the haplotype analysis, variant calling was done with Deep- Variant (V.0.8.0), and variants from chromosome 16 (hg38) with a quality score ≥30 were used. The Bcftools (V.1.9) RoH tool along with Ashkenazi Jewish population allele frequency data from Genome Aggregation Database (gnomAD) v3 release (with whole genome sequencing analyses of 1662 Ashkenazi Jewish individuals) were used to identify the stretch of homozygosity flanking the TRAPPC2L variant of interest (Karczewski et al., 2020; Narasimhan et al., 2016; Poplin et al., 2018).

2.4.3 Yeast experiments

Standard yeast methods were used for transformation, strain construction and growth (Guthrie, C. et al., 2004). TRAPP genes were cloned into pGADT7 and pGBKT7 plasmids (Clontech) for yeast two- hybrid analysis. pGADT7 and pGBKT7 constructs were then transformed into AH109 yeast and Y187 yeast, respectively. Mating was performed at 30°C on YPD agar plates overnight. To assess the mating efficiency, plates were then replicated onto double drop-out (DDO) agar plates lacking leucine and tryptophan. To evaluate interactions, plateswere replicated on triple drop-out (TDO) agar plates lacking tryptophan and histidine. Single colonies of each diploid were then cultured in liquid selective media (DDO) for 24 hours, and serial dilutions were prepared. The diploid cells were then spotted on selective media (DDO and TDO) and plates were left for 3 days at 30°C.

2.4.4 Size exclusion chromatography

Fibroblasts were plated in two 15 cm plates. When the cell confluency reached 80%–90%, the cells were washed three times with ice-cold PBS (10 mL each wash), then lysed in a total of 500 µL (250 µL for each 15 cm plate) of lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1.0% Triton and protease inhibitor cocktail, EDTA-free; Roche). The lysate was centrifuged at 16 000×g for 30 min at 4°C then the supernatant was transferred to a fresh tube. Then 5 mg of total protein was loaded on a 24 mL Superose 6 Increase 10/300 GL column (GE Healthcare) and 0.5 mL frac- tions were collected in the wash buffer (50 mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% Triton). The fractions were loaded on two gels for western blotting probed with antibodies recognising TRAPPC8 (monoclonal; Abcam), TRAPPC12

(polyclonal, homemade), TRAPPC2L (mono- clonal; Santa Cruz) or TRAPPC10 (monoclonal; Santa Cruz).

2.4.5 Recombinant protein preparation

Bacterial cultures (500 mL) were grown to an OD600 of 0.5–0.6 at 37°C. Protein production was induced by adding 1 mM IPTG while shaking overnight at 20°C. Cells were then pelleted at 4000 rpm for 10 min and resuspended in 30 mL lysis buffer (50 mM Tris–HCl pH 8.0, 0.4 M NaCl, 1 mM DTT, 5% glycerol, 0.5 mM AEBSF, 0.1% Triton X-100) for glutathione-S transferase(GST) fusion proteins or 35 mL of lysis buffer (0.3 M NaCl, 50 mM Tris–HCl pH 8.5, 10 mM β-mercaptoethanol, 5% glycerol, 0.1% Triton X-100, 0.5 mM AEBSF) for His_6-tagged proteins. Then 3.5 mL of 10% Triton X-100 was added for GST fusion proteins. The cell lysates were sonicated for 2 min by pulsing for 10 s ON and 10 s OFF at an amplitude setting of 25%. The resulting lysates were then cleared by centrifugation at 30 000×g for 30 min.

The crude extracts were incubated with either glutathione sepharose beads (Thermofisher) or Ni-NTA Agarose (Qiagen) for 1 hour at 4°C. The samples were then transferred into a column and washed once with 15 mL of appropriate lysis buffer and twice with 10 mL wash buffer (50 mM Tris–HCl pH 8.0, 0.4 M NaCl, 5% glycerol, 1 mM DTT) for GST- tagged proteins or (50 mM Tris–HCl pH 8.5, 200 mM NaCl, 5 mM β-mercaptanol, 0.5 mM EDTA pH 8.0) for His_6-tagged proteins. GST-tagged proteins were eluted in 1.0 mL fractions of elution buffer (50 mM Tris–HCl pH 8.0; 0.4 M NaCl, 15 mM glutathione). His6-tagged proteins were eluted in 1.0 mL fractions of elution buffer (50 mM Tris pH 8.8, 200 mM NaCl, 50–250 mM imidazole).

2.4.6 In vitro binding assay

In vitro binding assays contained 0.5 μM of the GST fusion proteins (TRAPPC2L, TRAPPC2L A2G or TRAPPC2L D37Y) with increasing amounts (0, 0.1, 0.2, 0.5, 1.0 μM) of the His_6-tagged heterodimeric complex composed of TRAPPC3 and TRAPPC6a. Samples were made up to a total volume of 250 μL with 1× binding assay buffer (25 mM Hepes pH 7.4, 115 mM KOAc, 2.5 mM MgCl2, 1 mM DTT) and left on ice at 4°C overnight. The GST-tagged proteins were collected onto 20 μL of glutathione-agarose resin by incubating on a nutator for 1 hour at 4°C. Samples were washed three times with 250 μL of 1× binding assay buffer. Proteins were then eluted from the beads by heating to 95°C in 25 μL of 1× sample buffer for 3 min. Western analysis used homemade polyclonal antibody recognising TRAPPC3.

2.4.7 UV–visible spectroscopy

The UV–visible spectra (200–500 nm) for TRAPPC2L and the two variants were collected using the Varian Cary 100 Bio UV– Visible Spectrophotometer. The approximate concentration of protein was calculated using the molar extinction coefficient of 15 025 M−1 cm−1 at A280 for TRAPPC2L.

2.4.8 Circular dichroism (CD) spectroscopy

CD spectra in the far-UV region (200–280 nm) were recorded for TRAPPC2L and the two variants in a 0.2 cm cell under a constant nitrogen flow using a Jasco-815 CD spectropolarim- eter. A protein concentration of 0.5 mg/mL in buffer (50 mM Tris pH 8.5, 1 mM DTT, 200NaCl and 1 mM EDTA) was used. The parameters used were as follows: bandwidth of 1 nm, a response time of 0.25 s, a data pitch of 0.2 nm and a scanning speed of 20 nm/min. The spectra obtained were smoothed using the Spectra Analysis Manager program from Jasco.

2.4.9 Thermal denaturation

Thermal denaturation was examined by CD spectroscopy using a protein concentration of 0.5 mg/mL in buffer (50 mM Tris pH 8.5, 1mM DTT, 200mM NaCl and 1mM EDTA) in a 0.2 cm cell. Standard parameters were used (bandwidth of 1 nm, a response time of 0.25 s and a data pitch of 0.2 nm). The midpoint of the unfolding transition (TM) was obtained by measuring the change in ellipticity at 222 nm with standard sensitivity using the variable temperature settings on a Jasco-815 CD spectropolarimeter. The rate used was 60°C/h with a start temperature of 25°C and an end temperature of 65°C.

2.4.10 Fluorescence measurements

Fluorescence of the proteins (0.1 mg/mL) was measured in (50 mM Tris pH 8.5, 1 mM DTT, 200 mM NaCl) buffer using the Varian Cary Eclipse Fluorescence Spectrophotometer with a 1 cm pathlength at room temperature (25°C). The samples were excited at 280 nm and the emission spectra were recorded in the range of 290–500 nm with a scan rate of 600 nm/min and excitation and emission slits of 5 nm. The emission spectra were averaged over 10 separate scans with a 1 nm data interval.

2.4.11 Levels of active Rab11

Monitoring the levels of active Rab11 was performed using the Rab11 Mouse Monoclonal Antibody Kit (Neweast Biosciences) as per the manufacturer's instructions.

2.5 Results

2.5.1 Clinical findings

Three affected individuals in a sibship of five from a nonconsanguineous family of Ashkenazi Jewish ancestry were evaluated for neurodevelopmental delay (figure 3A). Clinical findings of the individuals are given in table 1 along with two previously reported individuals with TRAPPC2L pathogenic variants. Detailed medical histories of each individual are provided in online supplementary notes. Previous genetic testing including chromosome analysis, Fragile-X testing and clinical exome sequencing were non-diagnostic.

2.5.2 Molecular findings

Filtering based on allele frequency (alternate allele frequency <1%) yielded homozygous missense variants in TRAPPC2L and PIEZO1 (MIM:611184) in all three affected siblings (table 2). We assessed the PIEZO1 variant as non-causative since both the human and mice phenotypes associated with PIEZO1/piezo1 were different than the phenotype observed in our patients, leaving the ultra-rare, predicted pathogenic TRAPPC2L missense variant (NM_016209.5:c.5G>C:p.(Ala2Gly)) as the most plausible candidate that could explain the observed phenotype. The population allele frequencies and the computational prediction scores for this variant are given in table 2. Notably, this missense variant was only observed in the Ashkenazi Jewish individuals in gnomAD v2.1.1 release (http://gnomad.broa- dinstitute.org/) with an allele frequency of 0.0002243, and was not observed in gnomAD v3 release containing 71 702 genomes and Trans-Omics for Precision Medicine (TOPMed) Freeze 5 database containing 62 784 genomes (https://bravo.sph.umich.edu/freeze5/hg38/). The variant was confirmed by Sanger sequencing (figure 2B) and segregated with the neurodevelopmental phenotype in the family (figure 2A). The

affected alanine residue is well conserved across phyla except for yeast where an adjacent leucine may act as the hydrophobic residue (figure 3C).

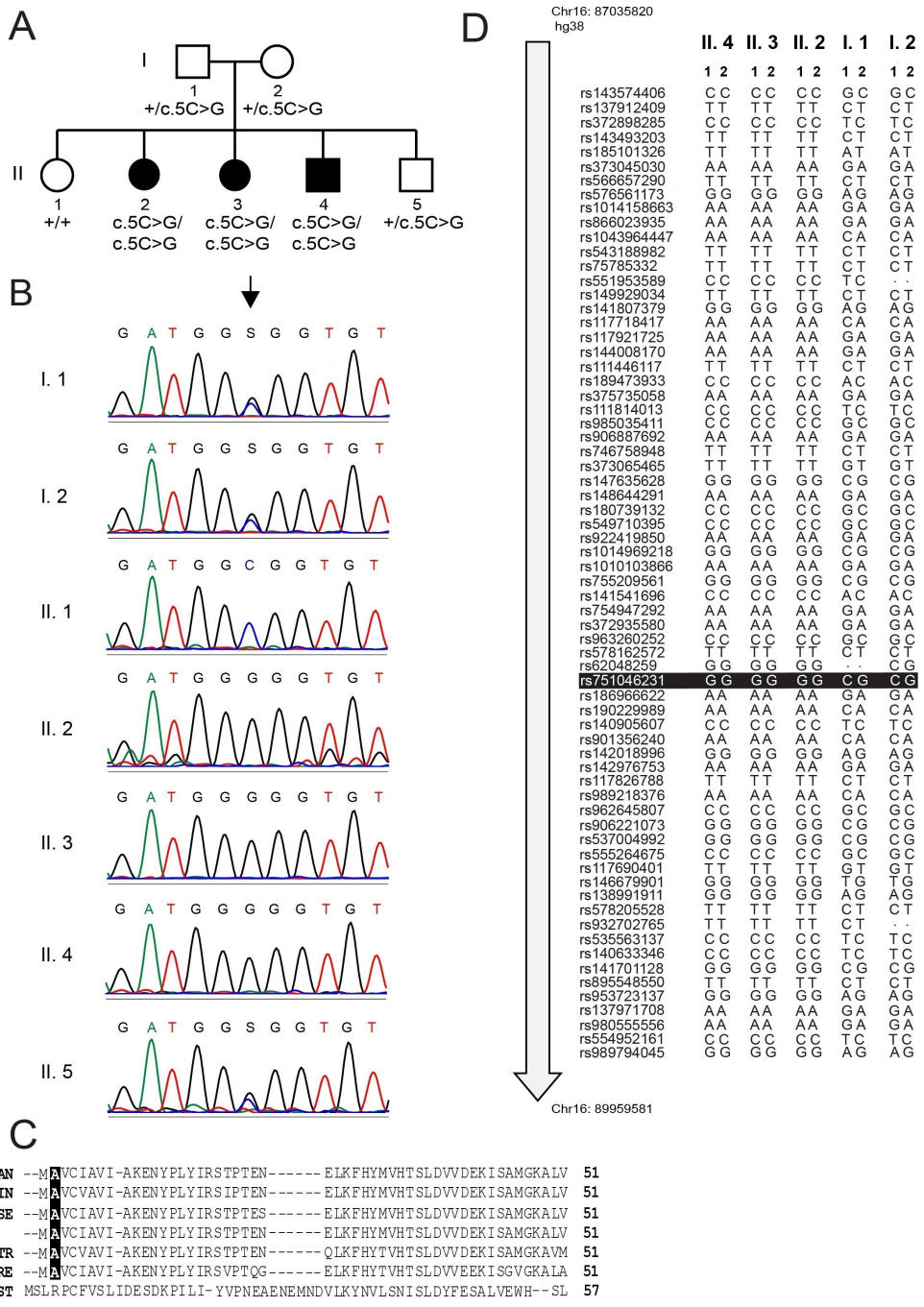
2.5.3 Haplotype analysis

Haplotype analysis in TRAPPC2L and its flanking region using single-nucleotide variants from the genome sequencing data revealed a stretch of homozygosity spanning 3,170,007 base pairs in the three affected siblings, indicating a common ancestral chromosomal region carrying the variant from each parent was transmitted to the affected individuals (figure 3D). The parental SNPs were heterozygous in this region.

2.5.4 TRAPPC2L missense variant p.(Ala2Gly) fails to interact with TRAPPC6a and TRAPPC12.

To assess the effects of the missense p.(Ala2Gly) variant on the interactions of TRAPPC2L with other TRAPP subunits, a yeast two-hybrid assay was performed (figure 4A) using the subunits that were previously shown to interact with TRAPPC2L by this method (Milev et al., 2018). This variant disrupted the interactions with TRAPPC6a (MIM: 610396) and the TRAPP III-specific subunit TRAPPC12 (MIM: 614139). Interestingly, unlike the previously reported TRAPPC2L missense variant p.(Asp37Tyr), the inter- action with the TRAPP II-specific subunit TRAPPC10 (MIM: 602103) was not affected. It is noteworthy that the interaction with TRAPPC6a was affected regardless of whether TRAPPC2L was in the pGADT7 or pGBKT7 vector. In contrast, an inter- action with TRAPPC12 was only seen with TRAPPC2L in the pGADT7 vector.

We next employed the previously established yeast system to study the functionality of the TRAPPC2L variant by using the conditionally lethal tca17Δ TRS130-HA yeast strain (Milev et al., 2018). The expression of only the wild-type TRAPPC2L, but not the p.(Ala2Gly) variant, could


A
I
1
+/c.5C>G
2
+/c.5C>G
II
1
+/+
2
c.5C>G/
c.5C>G
3
c.5C>G/
c.5C>G
4
c.5C>G/
c.5C>G
5
+/c.5C>G
B
I. 1
I. 2
II. 1
II. 2
II. 3
II. 4
II. 5
G A T G G S G G T G T
G A T G G C G G T G T
G A T G G G G G T G T
C
HUMAN --MAVCIAVI-AKENYPLYIRSTPTEN------ELKFHYMVHTSLDVVDEKISAMGKALV 51
BOVIN --MAVCVAVI-AKENYPLYIRSIPTEN------ELKFHYMVHTSLDVVDEKISAMGKALV 51
MOUSE --MAVCIAVI-AKENYPLYIRSTPTES------ELKFHYMVHTSLDVVDEKISAMGKALV 51
RAT --MAVCIAVI-AKENYPLYIRSTPTEN------ELKFHYMVHTSLDVVDEKISAMGKALV 51
XENTR --MAVCVAVI-AKENYPLYIRSTPTEN------QLKFHYTVHTSLDVVDEKISAMGKAVM 51
DANRE --MAVCIAVI-AKENYPLYIRSVPTQG------ELKFHYTVHTSLDVVEEKISGVGKALA 51
YEAST MSLRPCFVSLIDESDKPILI-YVPNEAENEMNDVLKYNVLSNISLDYFESALVEWH--SL 57
D
Chr16: 87035820
hg38
II. 4 II. 3 II. 2 I. 1 I. 2
1 2 1 2 1 2 1 2 1 2
rs143574406 C C C C C C G C G C
rs137912409 T T T T T T C T C T
rs372898285 C C C C C C T C T C
rs143493203 T T T T T T C T C T
rs185101326 T T T T T T A T A T
rs373045030 A A A A A A G A G A
rs566657290 T T T T T T C T C T
rs576561173 G G G G G G A G A G
rs1014158663 A A A A A A G A G A
rs866023935 A A A A A A G A G A
rs1043964447 A A A A A A C A C A
rs543188982 T T T T T T C T C T
rs75785332 T T T T T T C T C T
rs551953589 C C C C C C T C . .
rs149929034 T T T T T T C T C T
rs141807379 G G G G G G A G A G
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rs117921725 A A A A A A G A G A
rs144008170 A A A A A A G A G A
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rs189473933 C C C C C C A C A C
rs375735058 A A A A A A G A G A
rs111814013 C C C C C C T C T C
rs985035411 C C C C C C G C G C
rs906887692 A A A A A A G A G A
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rs373065465 T T T T T T G T G T
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rs148644291 A A A A A A G A G A
rs180739132 C C C C C C G C G C
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rs922419850 A A A A A A G A G A
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rs755209561 G G G G G G C G C G
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rs578162572 T T T T T T C T C T
rs62048259 G G G G G G . . C G
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rs138991911 G G G G G G A G A G
rs578205528 T T T T T T C T C T
rs932702765 T T T T T T C T . .
rs535563137 C C C C C C T C T C
rs140633346 C C C C C C T C T C
rs141701128 G G G G G G C G C G
rs895548550 T T T T T T C T C T
rs953723137 G G G G G G A G A G
rs137971708 A A A A A A G A G A
rs980555556 A A A A A A G A G A
rs554952161 C C C C C C T C T C
rs989794045 G G G G G G A G A G
Chr16: 89959581

Figure 3 Molecular genetic analysis of individuals with a TRAPPC2L variant. (A) Pedigree of the family presented in this study. (B) Sanger sequencing of all seven individuals in the pedigree. The arrow points to the affected nucleotide (c.5C in wild type). (C) Multiple sequence alignment of TRAPPC2L from various species indicating the highly conserved alanine at residue 2 in the proteins. (D) Analysis of the SNPs on chromosome 16 revealed a contiguous stretch of homozygosity (Chr16:86948799–90118806, 3.17 Mb, hg38) around the TRAPPC2L gene in the 3 affected individuals of this family. In this region, rare variants with at least one alternate allele at the genomic position in the parents and with allele frequency ≤0.01 in the Ashkenazi Jewish population (gnomAD v3) were filtered (n=68) to plot the figure. For each individual, the two alleles are represented as 1 and 2. The TRAPPC2L gene variant seen in this family is highlighted in black (Chr16:88857155-C-G, hg38; rs751046231). Dots in the figure represent genomic positions in the sample where the genotype call could not be assessed or did not cross the filtering criteria threshold.

Table 1 Clinical findings of individuals with homozygous *TRAPPC2L* variants

	This study			Milev *et al*, 2018[1]	
Individual	**II.2**	**II.3**	**II.4**	**S1**	**S2**
Age/sex	F/38	F/36	M/33 yo	F/3 yo	F/16 yo
Ancestry	Ashkenazi Jewish			Italian	Austrian
Variant	c.5G>C:p.(Ala2Gly)			r)	
Prenatal and perinatal	Unremarkable			Perinatal distress	Perinatal distress, preterm labour
Presenting symptom (age of onset)	Developmental delay (12 months)	Developmental delay (4 months)	Developmental delay (6 months)	Developmental delay (9 months)	Developmental regression (9 months)
Illness provoked regression	No			Yes	Yes
Development prior to illness	NA			Delayed	Normal
CK during illness	Unknown			Up to 16 000	Up to 5500
Intermittent CK levels	Unknown			Normal to 1000	Normal range
Brain MRI before the first event and after the first event	Not performed		Normal at 23 years old	Delayed myelination at 10 months and follow-up MRI acute encephalopathy with posterior oedema (16 months) Progressive brain atrophy (18 and 30 months)	Delayed myelination at 10 months and no
Developmental delay	Yes				
Motor milestones	Sat independently at 12 months never crawled, walked at 2 years old	Very mild motor delay, walked at 15 months old	Sat at 12 months, crawled at 18 months, walked at 2–3 years old	Could sit alone at 11 months but subsequently lost this ability, never achieved independent walking	Could sit unsupported at 7 months but subsequently lostthis ability, never achieved independent walking
Speech	Severe expressive language delays			Non-verbal	Non-verbal

Tetraplegia		No		Yes	Yes
Dystonia	No	No	Yes	Yes	Yes
Seizure	No	No	No	Yes	Yes
Vision		No reported problems		Cerebral visual impairment	Clinical suspicion of cerebral visual impairment
Other		None		Protein-losing enteropathy at 39 months	Vitamin B12 and folic acid deficiency, cholelithiasis, recurrent pneumonia

suppress the conditional lethality of the yeast (figure 4B), consistent with the previous report showing that p.(Asp37Tyr) was also incapable of supporting growth. Together with our previous data (Milev et al., 2018), we suggest that TRAPPC2L functions as an adaptor protein to aid in the assembly of TRAPP complexes most likely through an interaction with the core TRAPP protein TRAPPC6a.

2.5.5 TRAPPC2L binds directly to the TRAPPC6a-TRAPPC3 heterodimer

TRAPPC2L was previously suggested to bind to the side of the TRAPP complex opposite to that of TRAPPC2 (Duarte et al., 2011; Scrivens et al., 2009; Wang et al., 2014). Our yeast two-hybrid assay above supports this notion. To test this hypothesis, we performed an in vitro protein binding assay to investigate the association of TRAPPC2L with TRAPPC6a. Since TRAPPC6a forms a tight complex with TRAPPC3 (M.-S. Kim et al., 2005; Kümmel et al., 2006), we purified a heterodimer of the latter two proteins for these studies and assessed binding to GST-tagged TRAPPC2L wild type or to either the p.(Ala2Gly) or the previously reported p.(Asp37Tyr) variants (figure 5A) by western analysis using anti- TRAPPC3. As shown in figure 5B and quantified in figure 5C, while TRAPPC2L and the p.(Asp37Tyr) variant both showed a concentration-dependent increase in binding to TRAPPC6a- TRAPPC3, the p.(Ala2Gly) variant was severely impaired in its ability to bind to this heterodimer. This was not due to gross changes

to the three-dimensional structure of the protein since CD spectroscopy (figure 5D), thermal denaturation temperature (figure 6A) and tyrosine fluorescence (figure 6B) were similar among TRAPPC2L wild type and the two missense variants. Collectively, our results suggest that the Ala2 residue, but not Asp37, is necessary for the association of TRAPPC2L with the TRAPP core.

We then mapped the region on TRAPPC6a that was important for this interaction. A yeast two-hybrid assay was employed by examining residues 1–83 and 84–159 individually. This effectively separates the protein into a region that is involved in interaction with the TRAPP core (1–83) and a region that is theoretically exposed and available for non-core interactions (84–159) (figure 4C). As shown in figure 3D, none of the TRAPPC2L constructs interacted with TRAPPC6a (1–83). Interestingly, while wild-type TRAPPC2L and the p.(Asp37Tyr) variant interacted with TRAPPC6a (84–159), the p.(Ala2Gly) variant did not. These results further support that notion that Ala2 is critical for the interaction between TRAPPC2L and TRAPPC6a and that this interaction takes place on a region of TRAPPC6a that is not involved in its interaction with the TRAPP core subunits.

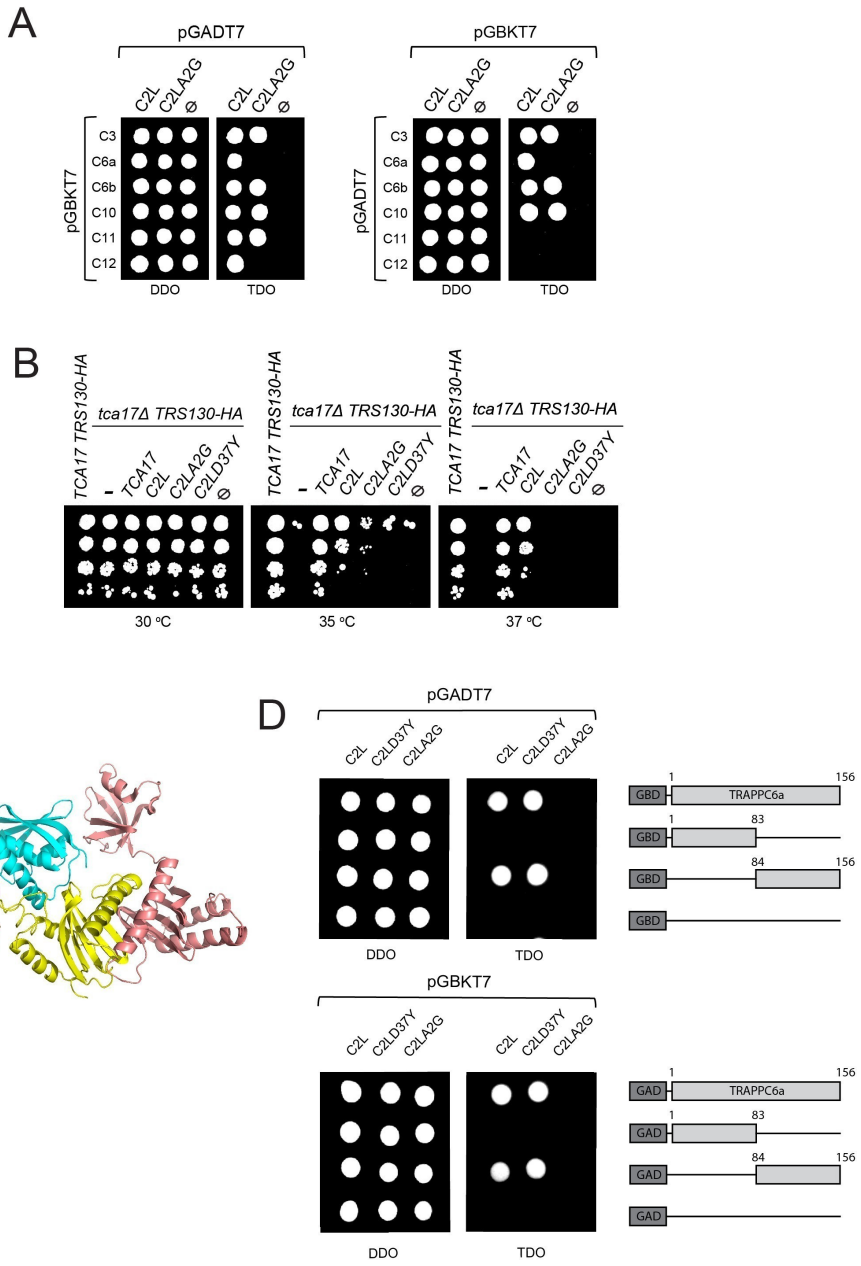

A
pGADT7
C2L
C2LA2G
∅
pGBKT7
C3
C6a
C6b
C10
C11
C12
DDO
TDO
pGBKT7
pGADT7
B
TCA17 TRS130-HA
tca17Δ TRS130-HA
-
TCA17
C2L
C2LA2G
C2LD37Y
30 °C
35 °C
37 °C
C
D
pGADT7
C2L
C2LD37Y
C2LA2G
DDO
TDO
GBD
TRAPPC6a
1
156
83
84
pGBKT7
GAD

Figure 4 TRAPPC2L p.(Ala2Gly) has reduced function compared with wild type. (A) The TRAPP subunits indicated were cloned into either pGBKT7 or pGADT7. TRAPPC2L and the two variants p.(Ala2Gly) and p.(Aspr37Tyr) were also cloned into these same vectors. A yeast two-hybrid assay was performed whereby growth on DDO (double drop-out medium lacking leucine and tryptophan) indicates the presence of the indicated plasmids, while growth on TDO (triple drop-out medium lacking leucine, tryptophan and histidine) indicates an interaction. (B) A yeast strain harbouring a deletion of the TRAPPC2L homologue TCA17 and an HA-tagged TRS130 gene (TRS130 is the yeast homologue of TRAPPC10) is temperature sensitive for growth at 35°C and 37°C. The growth sensitivity is rescued by wild-type TCA17 and partially rescued by wild-type TRAPPC2L. The two TRAPPC2L variants p.(Ala2Gly) and p.(Asp37Tyr) do not rescue the growth defect. (C) A model of a portion of the TRAPP core complex that shows TRAPPC4 (salmon coloured), TRAPPC1 (yellow coloured), TRAPPC3 (cyan coloured) and TRAPPC6a (residues 1–83 coloured in pink and residues 84–159 coloured in orange). (D) The yeast two-hybrid assay was repeated using TRAPPC2L and the two variants. The second plasmid expressed TRAPPC6a residues 1–83 or residues 84–159 as indicated.

Table 2 Homozygous *TRAPPC2L* variants identified in affected individuals with relevant population frequencies and *in silico* prediction scores

Genomic coordinates on chr16 (hg19 hg38)	HGVS (NM_016209.5)		Population frequency (*n*/MAF/hom)		*In silico* predictions			
	cDNA	Protein	gnomAD (v2.1.1; v3)	TOPMed (Freeze 5)	CADD	REVEL	PROVEAN	SIFT
88923563 C-G 88857155 C-G	c.5C>G	p.(Ala2Gly)	2/9.6e−6/No; 0/0/No	0/0/No	28.3	0.68	D (−3.16)	T (0.117)
88925102 G-T* 88858694 G-T	c.109G>T	p.(Asp37Ty)	4/1.6e−5/No; No	3/0.0024/ 0/0/No	29.5	0.981	D (−8.75)	D (0)

CADD v1.3 scores: https://cadd.gs.washington.edu/snv
REVEL scores:
https://rothsj06.u.hpc.mssm.edu/revel/revel_segments/
https://rothsj06.u.hpc.mssm.edu/revel/revel_segments/ SIFT,
Provean scores for the variant were added from
http://provean.jcvi.org/genome_submit_2.php?species=human https://gnomad.broadinstitute.org/
https://bravo.sph.umich.edu/freeze5/hg38/(TOPMed)
**TRAPPC2L* variant reported in *J Med Genet*. 2018 Nov;55(11):753–764.
MAF, minor allele frequency; No, no homozygotes were seen; D, damaging (SIFT)/deleterious (Provean); T, tolerated.

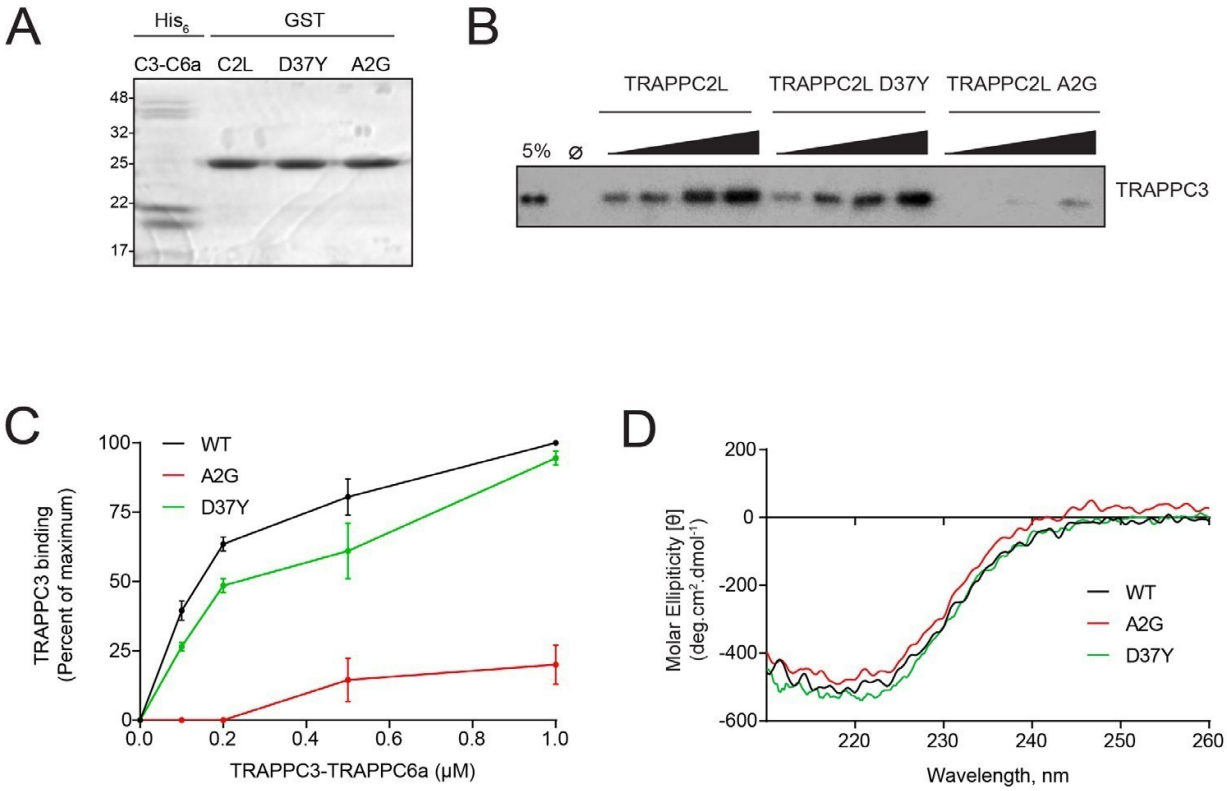
2.5.6 TRAPPC2L missense variant p.(Ala2Gly) disrupts the assembly of TRAPP

Our results thus far suggest that the p.(Ala2Gly) variant has impaired association with the TRAPP core. As an adaptor for other complex-specific proteins, this might affect the assembly of TRAPP complexes. To test this, we followed the fractionation of TRAPP complexes by size exclusion chromatography in lysates from fibroblasts derived from an affected individual with the p.(Ala2Gly) variant (II.3). We used TRAPPC8 and TRAPPC12 as markers for TRAPP III, and TRAPPC10 as a marker for TRAPP II. In all cases, the proteins were shifted to a smaller molecular size fraction in lysates from fibroblasts harbouring the p.(Ala2Gly) variant (figure 5E), suggesting that this variant affects the assembly or stability of TRAPP complexes.

2.5.7 TRAPPC2L missense variant p.(Ala2Gly) affects membrane trafficking in fibroblasts but not autophagic flux

Since the TRAPP complexes appear to be destabilised in the presence of the p.(Ala2Gly) variant, and these complexes are important for membrane trafficking in cells (J. J. Kim et al., 2016), we hypothesised that membrane trafficking would be affected in fibroblasts derived from the individual with the p.(Ala2Gly) variant (II.3). To test this notion, we employed the RUSH assay (Boncompain et al., 2012) to examine trafficking between the endoplasmic reticulum (ER) and Golgi. As shown in figure 7A, there was a noticeable delay in arrival of the marker protein in the Golgi, similar to what was seen for the p.(Asp37Tyr) variant (S1). This was not due to an absence of the TRAPPC2L protein since the protein was present at a similar level as the wild-type protein in fibroblasts from an unaffected individual (figure 7A inset). The delay in trafficking was rescued to near wild-type levels upon expression of an RFP-tagged wild-type TRAPPC2L, suggesting that the delay in membrane trafficking was due to a dysfunctional TRAPPC2L protein. We also examined VSVG-GFP ts045, a cargo whose traffic through the entire biosynthetic pathway can be followed by fluorescence microscopy and demonstrated a similar delay in traffic into and out of the Golgi in fibroblasts from both affected individuals (figure 7B). Collectively, our data suggest that the p.(Ala2Gly) variant affects assembly of TRAPP complexes resulting in membrane trafficking defects in cells.

Since the assembly and/or stability of TRAPP III was affected in vitro in cells harbouring the p.(Ala2Gly) variant, and since TRAPP III functions in autophagy (Stanga et al., 2019), we examined if autophagic flux was affected in the presence of this variant. As shown in figure 7C, there was no significant difference in the appearance of LC3-II, an autophagy marker, during starvation of the cells. This is similar to what was reported for the p.(Asp37Tyr) variant (Milev et al., 2018).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C3-C6a
C2L
D37Y
A2G
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32
25
22
17
B
TRAPPC2L
TRAPPC2L D37Y
TRAPPC2L A2G
5%
Ø
TRAPPC3
C
WT
A2G
D37Y
TRAPPC3 binding
(Percent of maximum)
100
75
50
25
0
0.0
0.2
0.4
0.6
0.8
1.0
TRAPPC3-TRAPPC6a (μM)
D
Molar Ellipticity [θ]
(deg.cm².dmol⁻¹)
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-600
220
230
240
250
260
Wavelength, nm
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A2G
D37Y

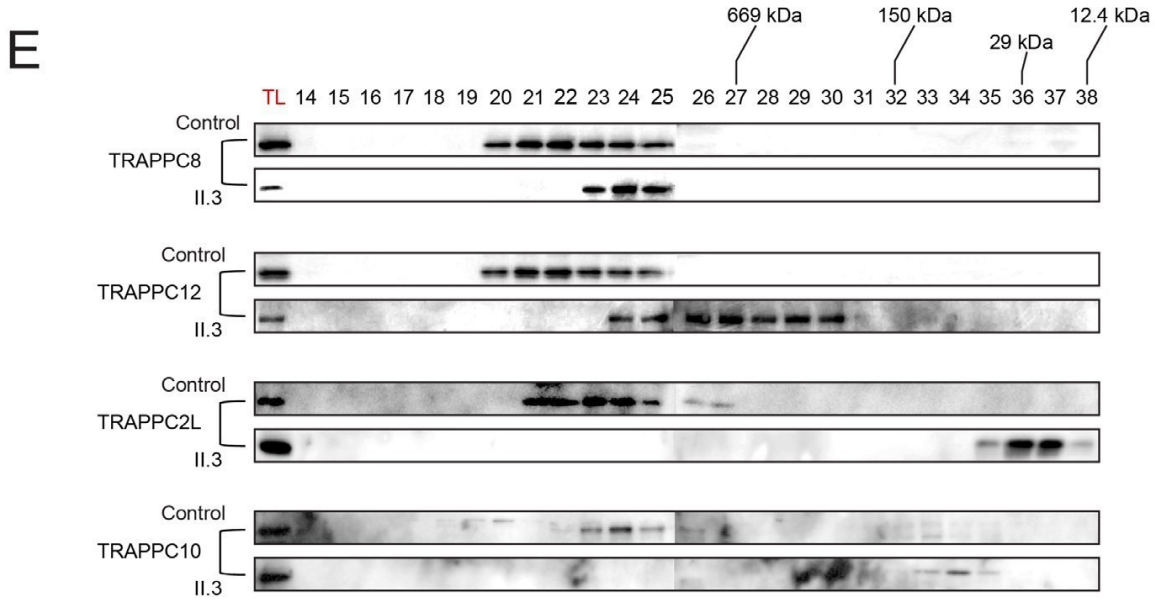
E
669 kDa
150 kDa
29 kDa
12.4 kDa
TL 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
Control
TRAPPC8
II.3
Control
TRAPPC12
II.3
Control
TRAPPC2L
II.3
Control
TRAPPC10
II.3

Figure 5 TRAPPC2L p.(Ala2Gly) has a weakened interaction with TRAPPC6a. (A) SDS–polyacrylamide gel of His-tagged recombinant TRAPPC3-TRAPPC6a heterodimer and GST-tagged TRAPPC2L or the TRAPPC2L variants p.(Ala2Gly) and p.(Asp37Tyr). Molecular size standards (kDa) are displayed on the left. (B) The GST-tagged proteins from panel (A) were incubated with increasing concentrations of the TRAPPC3-TRAPPC6a heterodimer. Following binding, the samples were probed by western analysis using anti-TRAPPC3 IgG. A sample representing 5% of the maximum amount of the heterodimer is shown to the left of the panel. (C) The TRAPPC3 signal from panel (B) was quantified and plotted vs the concentration of the heterodimer added to the reaction. (D) A circular dichroism curve for His-tagged recombinant TRAPPC2L or the two variants was performed. (E) Lysates from control fibroblasts (control) and fibroblasts derived from the individual harbouring the p.(Ala2Gly) variant (Figure 2A, II.3) were prepared and subjected to size exclusion chromatography. Total cell lysate (TL) and fractions (indicated above the panels) from the column were analysed by western blot analysis for the indicated TRAPP proteins.

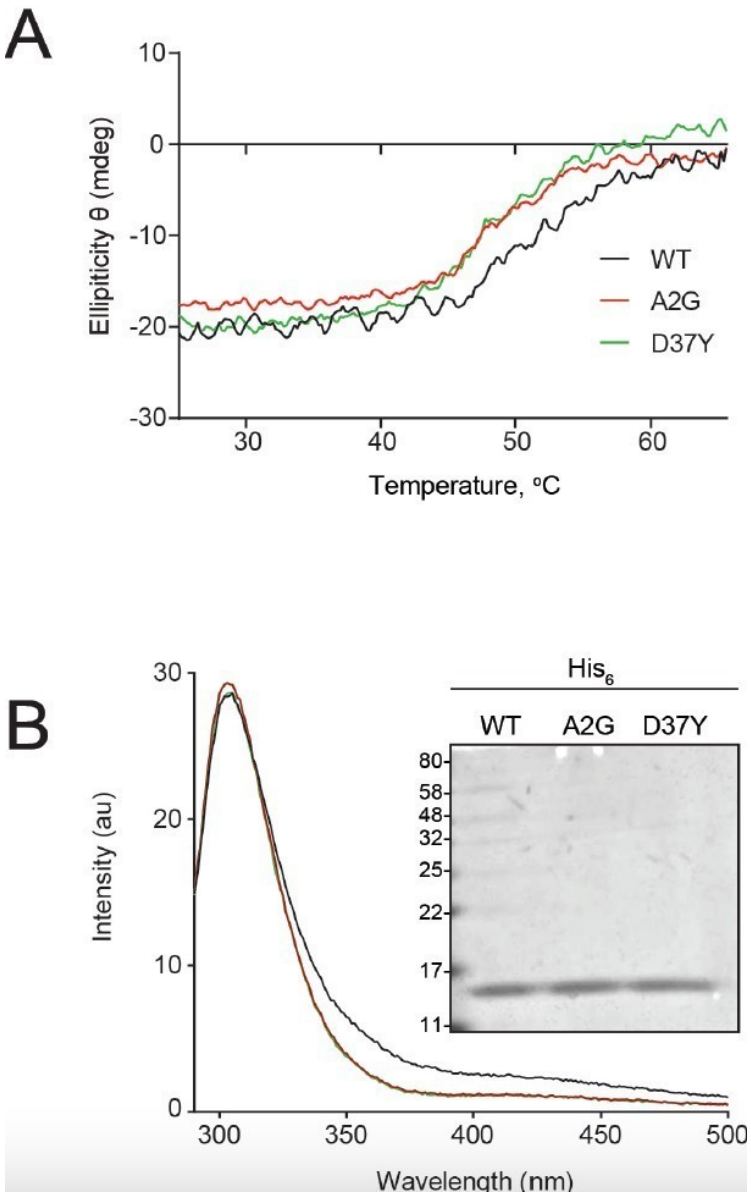

Figure 6 Variants in TRAPPC2L do not affect the stability nor folding of the protein. A novel homozygous variant in TRAPPC2L results in a neurodevelopmental disorder and disrupts TRAPP complex function. (A) Thermal denaturation of recombinant purified wild type TRAPPC2L and the two variants showed that the variants didn't affect the stability of the protein. (B) Fluorescence spectroscopy of the recombinant purified wild type TRAPPC2L and the two variants showed that the variants didn't affect the folding of the protein.

2.5.8 TRAPPC2L missense variant p.(Ala2Gly) affects the levels of active Rab11

We previously demonstrated that fibroblasts derived from an individual with the p.(Asp37Tyr) variant had elevated levels of active Rab11 (Milev et al., 2018), suggesting a role for TRAPP in Rab11 function. Previous reports have indicated that the yeast and Drosophila TRAPP II complexes are guanine nucleotide exchange factors for the Rab11 homologues (Jones et al., 2000; Riedel et al., 2018; Thomas & Fromme, 2016). Given that TRAPP II is destabilised in fibroblasts harbouring the p.(Ala2Gly) variant, we asked whether this might result in elevated levels of active Rab11. Using a conformation-specific antibody, we found that the p.(Ala2Gly) variant resulted in a small but significant increase in active Rab11 (figure 7D). The increase, however, was not as high as that seen for the p.(Asp37Tyr) variant, suggesting that the former variant may not affect TRAPP II assembly and/or stability in vivo as severely as the latter variant.

2.6 Discussion

We assessed a homozygous rare missense variant p.(Ala2Gly) in TRAPPC2L in three affected siblings with a neurodevelopmental disorder. The family is of Ashkenazi Jewish (AJ) ethnicity, and sequence analysis showed that the haplotype surrounding this variant (AJ allele frequency is 0.0002243) is likely from a common ancestor. Recently, another missense homozygous variant p.(Asp37Tyr) in TRAPPC2L was reported in two unrelated individuals with post-infectious encephalopathy, spastic quadriplegia, and intermittent rhabdomyolysis. Haplotype analysis suggested a common founder for those individuals. Strengthening the evidence that the p.(Ala2Gly) and p.(Asp37Tyr) variants are causative of disease is the fact that non-synonymous variants of p.Ala2 reported in gnomAD are of low allele frequency, are predicted to be pathogenic and that neither p.(Ala2Gly) and p.(Asp37Tyr) variants have been observed in a homozygous state

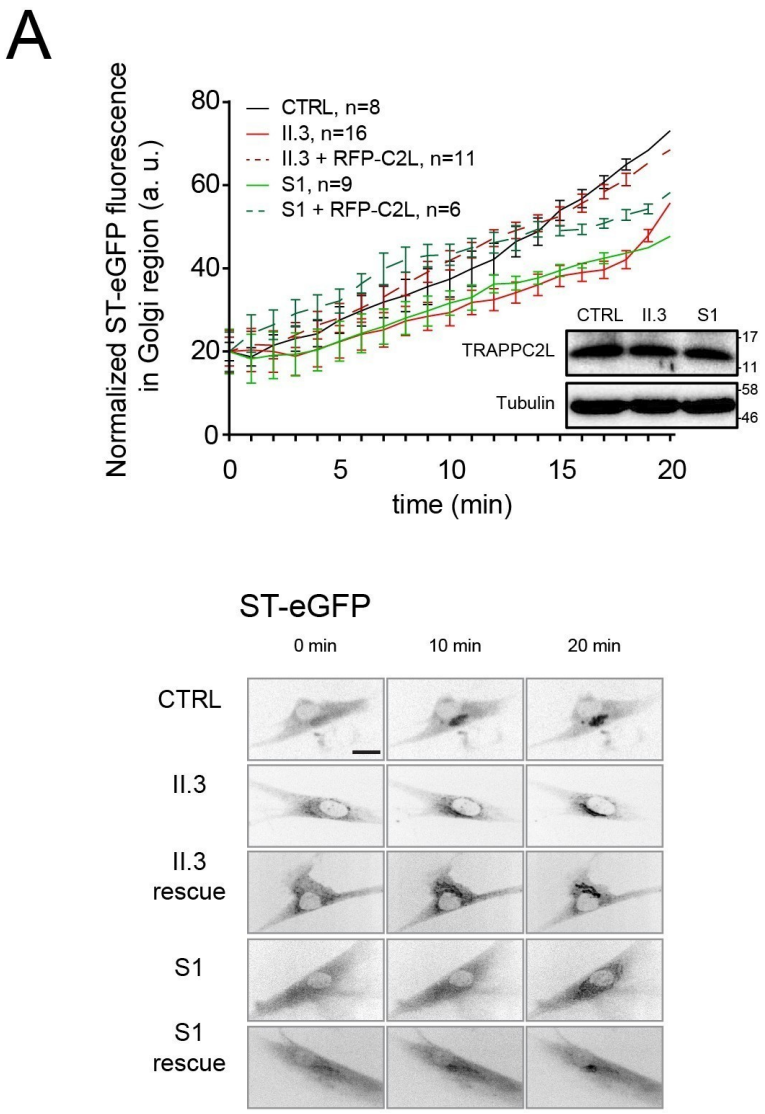

Figure 7 Functional studies reveal a membrane trafficking defect and increased active Rab11 in fibroblasts derived from an individual with TRAPPC2L p.(Ala2Gly). (A) The RUSH assay measuring traffic between the endoplasmic reticulum and the Golgi was performed and quantified for control (CTRL), p.(Ala2Gly) (II.3), and p.(Asp37Tyr) (S1) fibroblasts using the cargo protein ST-eGFP. The inset shows a western blot analysis for TRAPPC2L and tubulin from the cells that were analysed, with molecular size standards on the right. Representative images used for the quantification are shown to the right of the graph.

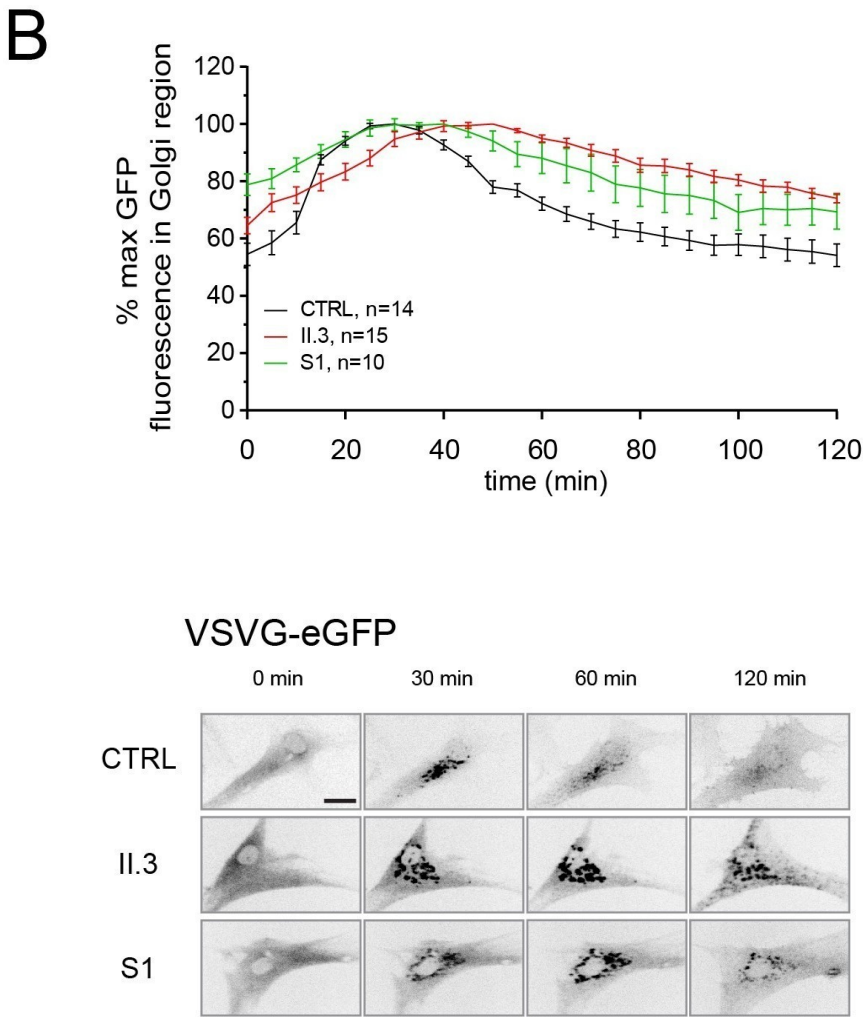

Figure 7 Functional studies reveal a membrane trafficking defect and increased active Rab11 in fibroblasts derived from an individual with TRAPPC2L p.(Ala2Gly). (B) The transport of VSVG-GFP ts045 was performed on control (CTRL), p.(Ala2Gly) (II.3) and p.(Asp37Tyr) (S1) fibroblasts, and quantified. Representative images used for the quantification are shown to the right of the graph.

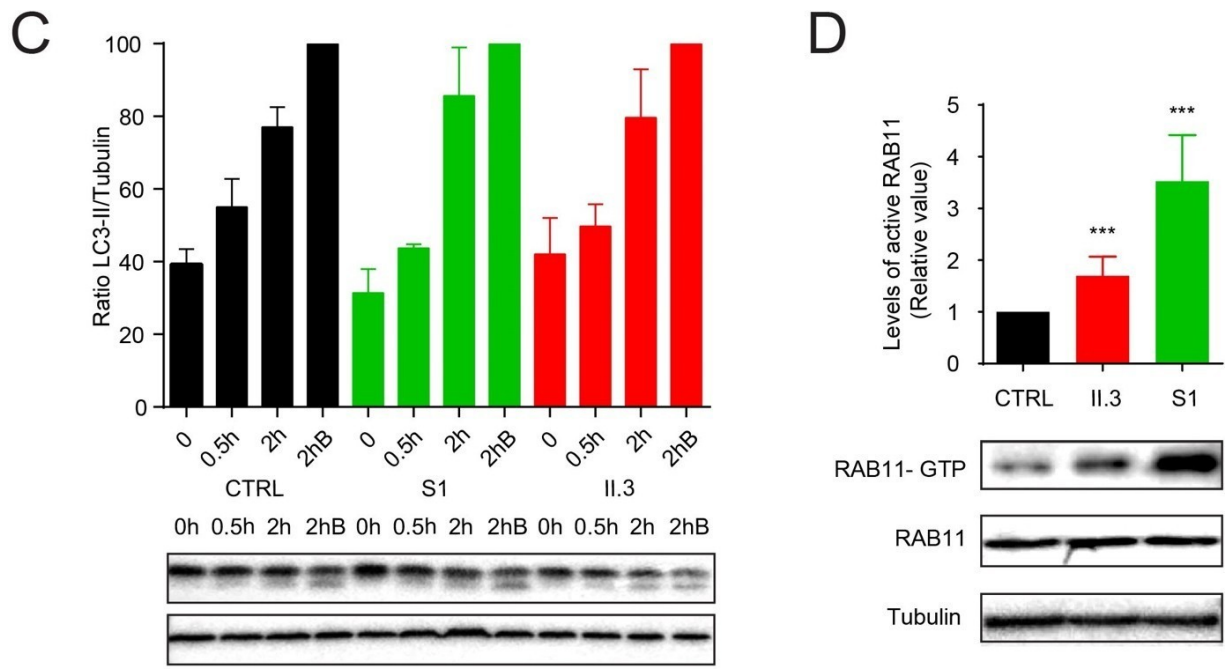

Figure 7 Functional studies reveal a membrane trafficking defect and increased active Rab11 in fibroblasts derived from an individual with TRAPPC2L p.(Ala2Gly). (C) Cells from control (CTRL), p.(Ala2Gly) (II.3), and p.(Asp37Tyr) (S1) fibroblasts were left in nutrient-rich medium (0) or starved for 0.5 hour or 2 hours. Some cells were starved for 2 hours in the presence of bafilomycin A1 which prevents formation of autolysosomes (2hB). Lysates were prepared and analysed by western blot analysis for the autophagy marker LC3-II as well as tubulin. The normalised LC3-II:tubulin ratio was determined. A sample western blot is shown beneath the graph. (D) Lysates from control (CTRL), p.(Ala2Gly) (II.3), and p.(Asp37Tyr) (S1) fibroblasts were treated with IgG recognising active (GTP-bound) Rab11. The precipitated Rab11 was then revealed by western blot analysis using anti-Rab11 IgG. The immunoprecipitated protein is shownin the top panel while the lysates are in the lower two panels. The signal was quantified and plotted as relative levels. Statistical significance was assessed using a one-way ANOVA. ***$p<0.001$.

in gnomAD (v2.1.1 and v3.0), TOPMed Freeze 5 and The Greater Middle East (GME) Variome Project, which is enriched for consanguineous populations. Also, the recently available Trappc2l homozygous knock-out mice (https://www.mousephe-notype.org/) show significant nervous system phenotypes that are in parallel to the neurodevelopmental phenotype observed in the reported individuals.

While neurodevelopmental delay and intellectual disabilities are the common clinical findings between our family and the previously reported individuals,1 there are also differences in the clinical findings, severity and clinical course (table 1). While cerebral visual impairment, dystonia and seizures were reported in both individuals in the previous study, dystonia (lower extremities, progressive dystonia) was observed in only one out of three affected siblings in our study and seizures have not been documented. In addition, in contrast to the patients reported herein, the two previously reported individuals experienced post- infectious encephalopathy with rhabdomyolysis, which may have complicated the clinical spectrum and resulted in additional findings such as spastic paraplegia. In our family, one of the affected individuals (II.3) had viral meningitis at 5–6 weeks of life, though without any obvious acute neurological sequelae. Neither sibling has had their serum creatine kinase levels measured nor have there been manifestations of a myopathy. Furthermore, in one of the previously reported individuals, developmental delay and delayed myelination had already been noted at 9 and 10 months of age, respectively, before the first infectious event took place at 16 months of age.1 Brain MRI of one of the affected siblings in the present study at 23 years of age was normal. The variability in clinical manifestations might be due to differences in the molecular effects of the two missense variants. Future case reports and longitudinal follow-up studies are warranted to delineate the complete clinical spectrum and

natural history of the TRAPPC2L- associated neurodevelopmental disorder and to determine if infectious insults contribute to the neurological course.

TRAPPC2L is a member of the highly conserved tethering factor complexes TRAPP II and III that share a common core of subunits (TRAPPC1, TRAPPC2, TRAPPC2L, TRAPPC3, TRAPPC4, TRAPPC5, TRAPPC6) as well as complex-specific proteins (TRAPPC9 and TRAPPC10 for TRAPP II; TRAPPC8, TRAPPC11, TRAPPC12 and TRAPPC13 for TRAPP III). TRAPP complexes play an important role in membrane trafficking between the ER and Golgi (TRAPP III) and at later stages of the secretory pathway (TRAPP II), and the genes encoding both core and complex-specific subunits are associated with Mendelian disorders (Bögershausen et al., 2013; Gedeon et al., 1999; Marin-Valencia et al., 2018; Mir et al., 2009; Mochida et al., 2009; Mohamoud et al., 2018).

Although clinically there are some differences between the individuals in the present study and those reported previously,1 at the cellular level there are a number of similarities including a TRAPPC2L-dependent delay in ER-to-Golgi traffic, no effect on autophagic flux and a rise in the levels of active Rab11. One interesting difference is the impaired interaction between TRAPPC2L and TRAPPC6a in the presence of the p.(Ala2Gly) variant compared with the previously reported p.(Asp37Tyr) variant. It has previously been suggested that TRAPPC2L binds to the complex close to the TRAPPC3-C6 interface (Duarte et al., 2011; Scrivens et al., 2009; C. Wang et al., 2014). The data we now present are consistent with such a model and further delineates the region of TRAPPC6a that is important for this interaction. There are presently no homozygous or compound heterozygous TRAPPC6a variants in affected individuals that fall within the region of the protein (residues 84–159) that we now show to be important for its interaction with TRAPPC2L except for one study which identified an individual with a homozygous p.(Tyr107Asn) variant

(Mohamoud et al., 2018). That study also identified four other homozygous variants in the affected individual, making comparison with the affected individuals in the present study complicated.\

Unlike TRAPPC2L p.(Ala2Gly), the p.(Asp37Tyr) variant did not affect the interactions with TRAPPC6a/TRAPPC12 subunits in the yeast two-hybrid assay (Milev et al., 2018), but rather disrupted the interaction with TRAPPC10, a TRAPP II-specific subunit. Furthermore, the p.(Ala2Gly) variant resulted in a modest increase in the levels of active Rab11 in fibroblasts from an affected individual, whereas the levels increased substantially for the p.(Asp37Tyr) variant as was shown previously (Milev et al., 2018). Rab11A GTPase is a member of the RAS oncogene superfamily, which cycles between an inactive GDP-bound form and an active GTP-bound form and regulates intracellular membrane trafficking. The active form recruits downstream effectors to membranes and is directly responsible for vesicle formation, movement, tethering and fusion. These data along with the yeast two-hybrid assay suggest that the two TRAPPC2L missense variants may result in slightly different cellular effects on membrane trafficking. It is note- worthy that both TRAPP II and III have been reported to have nucleotide exchange factor activity towards Rab11 and Rab1 in Drosophila (Riedel et al., 2018). Thus, it is possible that the levels of active Rab1 are also affected in individuals with TRAPPC2L variants.

The lack of an effect on autophagic flux was surprising given the shift in TRAPPC12 seen by size exclusion chromatography, which indicates a change in TRAPP III. The subunits of this complex, including TRAPPC11 and TRAPPC12, have been implicated in autophagy (Stanga et al., 2019). While a role for autophagy in other TRAPP gene mutations has not been reported, we cannot rule out a subtle defect in autophagy as being involved in the presently reported individuals, perhaps consistent with a relatively mild phenotype.

The core clinical findings of individuals with pathogenic TRAPPC2L variants show significant overlap with individuals carrying pathogenic variants in other TRAPP subunit genes (TRAPPC6A, TRAPPC6B, TRAPPC9, TRAPPC11) (reviewed in (Sacher et al., 2019)); the majority of these individuals have neurodevelopmental delay with severe speech difficulties, microcephaly, seizures and extrapyramidal symptoms like dystonia (Bögershausen et al., 2013; Gedeon et al., 1999; Marin-Valencia et al., 2018; Mir et al., 2009; Mochida et al., 2009; Mohamoud et al., 2018). A possible neurodegenerative course was also reported for some individuals with TRAPPC6B variants, and zebrafish models were consistent with the microcephaly and neuronal hyperexcitability seen in humans (Marin-Valencia et al., 2018).

Although a larger cohort of TRAPPC2L mutations is required to draw conclusions regarding genotype–phenotype correlations, TRAPPC2, TRAPPC11 and TRAPPC9 cohorts are the largest groups for which such correlations may be drawn. TRAPPC2 mutations have been exclusively linked to a skeletal disorder (SEDT) to the exclusion of other TRAPP proteins (Sacher et al., 2019). Mutations include missense, nonsense and splicing variants. To date, no other features have been reported in such individuals including those seen in other TRAPPopathies (eg, neurodevelopmental, microcephaly, muscular). TRAPPC9 mutations all share common features which include non-syndromic autosomal recessive intellectual disability and microcephaly (Sacher et al., 2019). Some phenotypic features have been suggested to be due to impaired NF-κB signalling as TRAPPC9 was identified as a regulator of NIK, a kinase that functions in this pathway (Hu et al., 2005). Mutations in TRAPPC11 result in a spectrum of phenotypes including muscular dystrophy (LGMD2S or congenital muscular dystrophy), intellectual deficit, ocular and hepatic involvement as well as cerebral atrophy (Sacher et al., 2019). Although this protein has been implicated in a number of different cellular processes including membrane trafficking

(Scrivens et al., 2011), protein glycosylation (DeRossi et al., 2016), endocytosis (Bassik et al., 2013) and autophagy (Stanga et al., 2019), it is unclear which impaired function results in which phenotype.

Collectively, these data suggest that the TRAPPC2L p.(Ala2Gly) variant leads to reduced interaction with the core subunit TRAPPC6a, affects membrane trafficking and supports the pathogenicity of the p.(Ala2Gly) variant.

Chapter 3: Discussion

In my thesis, I focused on the highly conserved transport protein particle (TRAPP) multisubunit complexes. The TRAPP complexes are one of the best-studied tethering factors which maintain the fusion efficiency during membrane trafficking, a fundamental process that ensures the delivery of proteins and lipids to the plasma membrane and specific organelles. Variants in genes encoding TRAPP subunits have been linked to a wide spectrum of human disease which confirms the significance of the TRAPP complex in humans (Sacher et al., 2019). Neurodevelopmental disorders are clinical phenotypes commonly manifested by variants in TRAPP genes. Neurodevelopmental disorders have a very diverse etiology, and their causes can involve genetic, environmental factors, traumatic injuries, and prenatal events. Such developmental phenotypes are observed in individuals with variants in TRAPP-encoding genes including TRAPPC9, TRAPPC4, TRAPPC6, TRAPPC11, TRAPPC10, and TRAPPC12. In

addition, we have recently identified the first human variants in TRAPPC2L, a component of TRAPP linked to neurodevelopmental disorders and intellectual disabilities (Milev et al., 2018; Al-Deri et al., 2020). Neurodevelopmental disorders are disabilities related mainly to defects in the functioning of the neurological system and central nervous system. Examples of neurodevelopmental disorders include attention-deficit/hyperactivity disorder, autism, learning disabilities, mental retardation, conduct disorders, cerebral palsy, and impairments in vision and hearing (Thapar et al, 2017). Children with neurodevelopmental disabilities commonly face difficulties with language and speech, motor skills, behavior, memory, learning, or other neurological and psychological functions. Although genetics usually plays a major role in many neurodevelopmental disorders, such as learning disabilities, most neurodevelopmental disorders are caused by a combination of factors rather than one clear cause.

The two TRAPPC2L residues Ala2 and Asp37 are important for TRAPPC2L function.

Although the structure of TRAPPC2L is not yet determined, it can be modeled using its yeast homologue, Tca17p (PDB ID: 3PR6). Based on the structure analysis of this model and the structure of Tca17, the N-terminus is buried in the protein suggesting that the Ala2 residue is not exposed. In contrast, the Asp37 residue is predicted to be exposed at the surface of TRAPPC2L. TRAPPC2L acts as an adaptor for the larger TRAPP subunits similar to TRAPPC2. The two TRAPPC2L variants disrupted the assembly of the TRAPP complexes and the association of the TRAPP-specific subunits TRAPPC12 and TRAPPC10 with the core TRAPP complex. A model for the assembly of the TRAPP complexes and the effects of these variants is presented in Figure 8. Unlike TRAPPC2, TRAPPC2L has not yet been shown to be a TRAPP core subunit. Core subunits are TRAPP subunits that form a subcomplex called the "TRAPP core". On the other hand, adaptor subunits are TRAPP subunits that are important for the association of the larger TRAPP subunits. Both TRAPPC2 and TRAPPC2L function as adaptors for specific TRAPP subunits but only TRAPPC2 has been shown to be both a core-TRAPP subunit and a TRAPP adaptor. Further analysis is needed to assesswhether TRAPPC2L is a core-TRAPP subunit or simply a TRAPP adaptor.

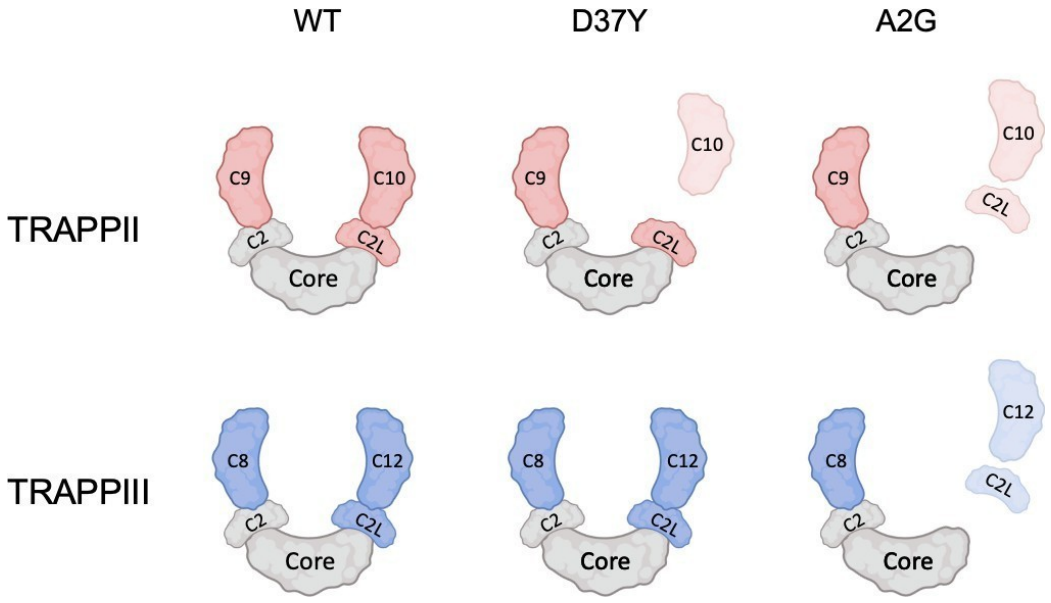

Figure 8. The TRAPPC2L variants disrupt the assembly of TRAPP complexes. The predicted assembly of the TRAPP complexes in fibroblasts harboring the TRAPPC2L variants is based on their cellular characterization presented in this thesis. The p. (Asp37Tyr) variant disrupts the assembly of TRAPP II only. In contrast, the p. (Ala2Gly) variant disrupts the assembly of TRAPP II and TRAPP III.

The two variants affected the interactions of TRAPPC2L with the other TRAPP subunits differently. The p.(Ala2Gly) variant disrupted the interactions of TRAPPC2L with TRAPPC6a and TRAPPC12 while the p. (Asp37Tyr) variant disrupted the interaction of TRAPPC2L with TRAPPC10. The interactions of TRAPPC2L with TRAPPC10 and TRAPPC6a was seen regardless of whether TRAPPC2L was in the pGADT7 or pGBKT7 vector which suggests that these interactions are "real" interactions. In contrast, since the interaction of TRAPPC2L with TRAPPC12 was only seen when TRAPPC2L was in the pGADT7 vector, suggests that this could be due to a false negative in the other orientation or due to the possibility that the protein encoded by the cloned sequence activates transcription in the absence of two-hybrid-interacting partner protein when fused with the binding domain. That is why it is crucial to assess the protein-protein interactions in both pGADT7 and pGBKT7 orientations to eliminate false negatives or false positives in the yeast two hybrid analysis and screen for all possible interactors of the protein.

Variants in TRAPPC2L all manifested in a similar clinical phenotype specific to the nervous system as summarized in **Table 1**. One question that remains unanswered is why the variants manifest with a clinical phenotype confined to the nervous system. I propose two possible explanations for this question**.** One explanation would be that the nervous system is more susceptible to ER-to-Golgi membrane trafficking defects and disruption compared to other organ systems (Jepson et al., 2019). Variants in genes encoding TRAPP subunits were linked to ER-to-Golgi trafficking delays making this cellular process of interest in understanding the etiology of neurodevelopmental diseases linked to TRAPP. Plasmalemmal expansion is one role of the secretory pathway in neurons that may be of particular relevance to TRAPPC2L. Compared to other types of cells, the plasmalemmal surface area of neurons is much greater (Pfenninger, 2009). The phospholipids required to drive neuronal plasmalemmal growth are synthesised and transported through the ER and Golgi apparatus within the cell body (Pfenninger & Johnson,

1983). Neuronal growth relies heavily on membrane trafficking leading to the hypothesis that neurons may be highly sensitive to disruptions in the secretory pathway (Pfenninger, 2009). Variants in TRAPPC2L are clinically relevant confirmations of this hypothesis. Membrane trafficking assays revealed that fibroblasts harboring variants in TRAPPC2L have delayed ER-to-Golgi and post-Golgi trafficking. Interestingly, inhibition of post-Golgi and ER-to-Golgi trafficking suppressed dendritic growth in hippocampal neurons (Horton et al., 2005). This finding suggests that neuronal dendritic growth relies heavily on ER-to-Golgi trafficking. Membrane trafficking delays and disruptions seem to have less effect on axonal growth compared to dendrites (Jareb & Banker, 1997). In *Drosophila*, mutations in genes encoding proteins involved in COPII vesicle budding or subsequent anchoring to the cis-Golgi membrane, such as Sar1, Sec23 and Rab1, have been linked to reduced neuronal dendritic growth but normal axonal growth (Ye et al., 2007). These findings suggest that reducing vesicle fusion can substantially impact dendritic but not axonal growth. This, in turn, may have substantial effects for the nervous system of TRAPPC2L patients.

Another explanation for the observed clinical phenotype in patients with variants in TRAPPC2L would be that neurons have highly dynamic cellular processes and rely heavily on regulation of protein synthesis and degradation for proper functioning. Therefore, the autophagic pathway could play a crucial role in neuron survival and function under both physiological and pathological conditions. It has been previously shown that autophagy is induced in several diseases in the nervous system to remove toxic substrates in the cell and protect the neurons from cell death (Todde et al., 2009). Although all cell types have autophagic pathways, there is a consensus that the importance of autophagy is dependent on the cell type (Mizushima et al., 2004), which could potentially explain why mutations in TRAPPC2L lead to neurological dysfunctions rather than a system-wide dysfunction. To explain the increased sensitivity of neurons to autophagy, three-key

differences between neurons and other cell types must be noted. First, as previously mentioned, unlike other cell types, neurons have axons and dendrites in which many molecules such as proteins and RNA are synthesized, degraded and transported for proper synaptic growth and function. Secondly, neurons are non-dividing cells and are therefore more sensitive to accumulation of toxins. Lastly, the brain has a higher energy demand compared to other cell types and requires and highly regulated mechanism like autophagy for supplying energy under extreme stressful conditions such as starvation (Boland et al., 2008). Therefore, the tight control of autophagy under both physiological and pathological conditions is crucial for survival of neurons and regulation of their particular functions. Interestingly, fibroblasts harboring variants in TRAPPC2L did not have defects in autophagy suggesting that this pathway is not linked to the neurodevelopmental disorders found in these patients.

Variants in genes encoding the TRAPP subunits TRAPPC2L, TRAPPC4, TRAPPC6, TRAPPC9, TRAPP10, TRAPPC11 and TRAPPC12 were linked to neurodevelopmental disorders and intellectual disabilities (Al-Deri et al., 2020; Bögershausen et al., 2013; Harripaul et al., 2018; Khattak & Mir, 2014; Milev et al., 2017; Mohamoud et al., 2018). Cells from patients harboring variants in these genes were shown to have delays in membrane trafficking making this cellular process of interest in understanding the etiology of neurological dysfunctions and intellectual disabilities. Membrane trafficking is not only important for the morphology of neurons but also for synaptic neurotransmission which has been linked to different neurological dysfunctions (Zoghbi & Bear, 2012; B. Wang et al., 2020). To date, most identified variants in TRAPP genes have been linked to neurodevelopmental disorders and intellectual disabilities connected to membrane trafficking delays. As exceptions, variants in TRAPPC11 and TRAPPC4

were linked to both membrane trafficking delays and defects in the autophagic pathway, and variants in TRAPPC2 were linked to a skeletal disorder due to the special function of the protein in trafficking collagen. As shown in this thesis, not all intellectual disabilities linked to TRAPP involve defects in the autophagic pathway. Collectively, these findings support the notion that the nervous system is more vulnerable to membrane trafficking defects compared to other organ systems because of their specific functions and characteristics providing a better insight into the etiology of these neurological diseases.

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