

**Insight into TRAPPII assembly and function through characterization of naturally
occurring *TRAPP* gene variants**

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Abstract

Insight into TRAPP^{II} assembly and function through characterization of naturally occurring TRAPP gene variants

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Proteins and other macromolecules are sorted throughout the cell in membrane bound vesicles in a process known as membrane trafficking. Defects in this process are associated with various human disorders. The transport protein particle (TRAPP) complexes, including TRAPP^{II} and TRAPP^{III}, are highly conserved multi-subunit protein complexes involved in subcellular membrane trafficking pathways.

In my thesis, I assessed the effect of biallelic variants in TRAPPC10 and TRAPPC6B, two TRAPP^{II} components, in individuals with a neurodevelopmental disorder characterized by microcephaly, intellectual disability, and brain atrophy. Here, I present comprehensive genetic, clinical, and functional data to assess the impact of these variants on TRAPP^{II} stability, membrane trafficking, and ciliogenesis.

Molecular studies revealed disruption of protein-protein interactions between mutant TRAPPC10 and its adaptor protein TRAPPC2L, and between mutant TRAPPC6B and TRAPPC3, affecting TRAPP^{II} complex-specific members that lead to the disruption of TRAPP^{II} assembly. Individuals with an alteration in the *TRAPPC10* gene have no functional TRAPPC10 protein, which resulted in a reduction in levels of another TRAPP^{II} component, TRAPPC9. Additionally, *TRAPPC10*^{-/-} knockout cells exhibited a membrane trafficking defect that can be rescued by wild type- but not

mutant TRAPPC10. Similarly, TRAPPC6B patient-derived fibroblasts displayed reduced levels of TRAPPC6B and TRAPPII complex-specific members, suggesting that variants in TRAPPC6B may preferentially affect TRAPPII functions compared to TRAPPIII functions. This observation was consistent with co-immunoprecipitation experiments that revealed significant enrichment of TRAPPC6B in TRAPPII, while its homologue, TRAPPC6A, co-precipitated equally with TRAPPII and TRAPPIII. Additionally, the TRAPPC6B patient-derived fibroblasts displayed reduced trafficking into the Golgi apparatus and Golgi fragmentation. These defects could be rescued by wild type TRAPPC6B. Furthermore, my studies also revealed that *TRAPPC10*^{-/-} knockout and TRAPPC6B patient-derived fibroblasts showed reduced levels of ciliation.

Finally, I demonstrated that translational read-through inducing drugs like Ataluren have the potential to rescue cellular defects attributed to nonsense mutations in the *TRAPPC6B* gene. This promising drug may serve as a viable treatment for TRAPPopathies, a group of neurodevelopmental, skeletal, and muscular disorders collectively associated with genetic variations in *TRAPP* genes.

Overall, my research confirms the crucial role of TRAPPC10 and TRAPPC6B in brain development and function, and provides insights into the potential disease pathomolecular mechanism, which involves the disruption of TRAPPII assembly and functions. The findings suggest that the use of new therapeutic compounds like Ataluren could effectively address these disruptions in a specific patient population.

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As I close, I would like to dedicate this thesis to my friend, Maen. Though he is no longer with us, his memory lives on in my heart, and I am grateful for the inspiration and motivation he has provided throughout my life. I wish you were here with us today.

I miss you, and I dedicate this thesis to you.

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List of Abbreviations

A	Aminoacyl
AAL	Automated Anatomical Labelling
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
Atg	Autophagy Related Protein
Bet3	Blocked early in transport 3
CATCHER	Complexes Associated with Tethering Containing Helical Rods
COG	Conserved Oligomeric Golgi
COP	Coat Protein
CORVET	Class C core Vacuole-Endosome Tethering
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified eagle medium
E	Exit
EARP	Endosome-Associated Retrograde Protein
EEA1	Early Endosome Antigen 1
EEF	Eukaryotic Translation Elongation Factor
EIF	Eukaryotic Translation Initiation Factor
EM	Electron Microscopy
ER	Endoplasmic Reticulum
ERF	Eukaryotic Translation Release Factor
ERGIC	ER-Golgi intermediate compartment
FBS	Fetal Bovine Serum

GAP	GTPase-Activating Protein
GARP	Golgi-Associated Retrograde Protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange actor
GTP	Guanosine Triphosphate
HDL	High-Density Lipoprotein
HOPS	Homotypic Fusion and Vacuole Protein Sorting
HVD	Hypervariable Domain
ID	Intellectual Disability
IKK β	inhibitor of NF- κ B (I κ B) kinase subunit β
IQ	Intelligence Quotient
LCL	Lymphoblastoid Cell Line
LD	Longin Domain
LDL	Low-Density Lipoprotein
LLO	Lipid-linked Oligosaccharide
LoF	Loss of function
MTCs	Multi-subunit Tethering Complexes
NBD	Nucleotide-Binding Domain
NDDs	Neurodevelopmental disorders
NEFAC	Non-Esterified Free Fatty Acids
NIK	NF- κ B-inducing kinase
NMD	Nonsense-Mediated mRNA Decay
OFC	Occipitofrontal Circumference
OFD1	Oral-facial-Digital Syndrome 1

ORF	Open Reading Frames
P	Peptidyl
PBS	Phosphate Buffered Saline Solution
PFA	Paraformaldehyde
PTC	Premature Termination Codon
Rab	Ras-associated binding
RUSH	Retention Using Selective Hooks
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SC	Synthetic Complete
SDS	Standard Deviations
SED1	Spondyloepiphyseal Dysplasia Tarda
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
ST	Sialyl Transferase
TBCD	TBC domain-containing protein
TDC	Typically Developing Children
TGN	Trans-Golgi Network
TRAPP	TRAnsport Protein Particle
TRIDs	Translational Read-through Inducing Drugs
tRNA	Transfer-RNA
Trs	TRAPP Subunit
Ts	Temperature Sensitive

Chapter 1: Introduction to the thesis

1.1 Membrane Trafficking

The endomembrane system of eukaryotic cells includes a number of subcellular compartments such as the endoplasmic reticulum (ER), Golgi and endosomes (Elias, 2010; Field and Dacks, 2009). Transport of proteins and other membrane components between these organelles is known as membrane trafficking (Yarwood et al., 2020). This process is facilitated by vesicular transport. In general it involves the formation and budding of membrane vesicles from a donor membrane, followed by their movement, tethering, docking, and fusion with their target membranes (Cai et al., 2007a).

Vesicle transport allows organelles to communicate and retain their homeostasis. The precise transport of cargo molecules between different compartments of the endomembrane system is crucial for proper functioning of the cell, which is regulated by the activity of various proteins (Yarwood et al., 2020).

1.1.1 Coats, Tethers, SNAREs and Rabs

Membrane trafficking is a tightly regulated process that ensures vesicles generated at a donor compartment are delivered to their correct acceptor compartment. This regulation is achieved through the orchestrated function of various protein families that operate at every step of the vesicular trafficking pathway (Cai et al., 2007a).

In the secretory pathway, two distinct classes of coat proteins (COP), COPI and COPII, are recruited from the cytosol to the donor membrane by small GTPases of the Arf1/Sar1 family to

initiate vesicle budding (Scales et al., 1997). Both types of coat protein complexes participate in cargo selection through the identification of sorting signals of cargo proteins. They also participate in the deformation of the flat membrane into spherical vesicles which leads to vesicle budding at the donor membrane (Barlowe, 1995; Szul and Sztul, 2011; Waters et al., 1991). COPI coated vesicles are involved in the transport of material from the Golgi to the ER and intra-Golgi traffic, while COPII coated vesicles function in the transport of material from the ER to the Golgi (Barlowe, 1995; Béthune et al., 2006).

Clathrin is another type of coat protein, plays a major role in the endocytic pathway. Clathrin coated vesicles are known for their role in receptor-mediated endocytosis, which allows for the internalization of transmembrane receptors at the plasma membrane (Pearse, 1976). they also participates in lysosomal enzyme transport from the Golgi to the lysosome (Heilker et al., 1999; Owen et al., 2004).

In all cases, after budding, the resulting vesicles are transported by motor proteins (kinesin, dynein and myosin) towards their target membranes (Goodson et al., 1997). Prior to fusion, tethering proteins (discussed in section 1.1.2) establish the initial contact and bring the transport vesicle and its target membrane into close proximity to allow interactions between SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins on the vesicles and target membrane to take place. SNAREs are divided into two complementary subsets called Q-SNAREs and R-SNAREs (Fasshauer et al., 1998; Söllner et al., 1993). Three Q-SNAREs reside on the target membrane and one R-SNARE resides on the transport vesicle. Q and R SNAREs recognize each other and form a trans-SNARE complex, and the high stability of the complex (referred to as

docking) helps to bring about the fusion of vesicle and target membranes by overcoming the energy barrier, which is necessary for fusion to happen (Fasshauer et al., 1998).

Ras-related (Rab) GTPases, of which more than 60 have been identified in humans, constitute the largest number of proteins that function at every step of the membrane trafficking pathway. Hence, they are known as the master regulators of intracellular membrane trafficking (Barr and Lambright, 2010). They alternate between active (GTP-bound) and inactive (GDP-bound) forms (Stenmark and Olkkonen, 2001; Touchot et al., 1987). Guanine nucleotide exchange factors (GEFs) promote the intrinsic exchange between GDP and GTP, while GTPase activating proteins (GAPs) inactivate Rab GTPases by increasing their ability to hydrolyse GTP to GDP (Brown and Howe, 1998; Pan et al., 2006; Stenmark and Olkkonen, 2001; Touchot et al., 1987; Wada et al., 1997). Active Rabs interact with functionally diverse effectors including coat, motor, tethering and SNARE proteins to mediate various aspects of vesicle budding, transport, tethering and fusion with target membranes and ensure specificity and spatiotemporal regulation of vesicle trafficking (Stenmark, 2009).

1.1.2 Tethering Factors

The tethering process is crucial for vesicular transport specificity. Previously it was thought to be mediated by SNARE proteins (Söllner et al., 1993; Weber et al., 1998). It is now believed that SNARE proteins play a role in the final stages of vesicle-target membrane recognition and fusion, but it is unlikely that they alone are responsible for the specificity of these interactions. Instead, it is thought that specificity is achieved through combinatorial interactions with other tethering factors (Bhattacharya et al., 2002; Koike and Jahn, 2019; Tsui and Banfield, 2000).

Tethering factors establish the first interaction between vesicles and target membranes before the fusion of the two lipid bilayers (Whyte and Munro, 2002; Yu and Hughson, 2010). Several tethering factors are implicated in this process; coiled-coil tethering factors and multi-subunit tethering factors/complexes (MTCs).

Coiled-coil tethers have a long coiled-coil (helical) domain that interacts with other proteins to form a relatively strong tether that can bridge two membranes over long distance, typically more than 200 nm (Bröcker et al., 2010). Coiled-coil tethers are primarily found in the Golgi; hence many have been named golgins and are implicated in ER–Golgi and intra-Golgi traffic. However, certain coiled-coil tethers such as EEA1 are found at endosomes and are involved in endosomal trafficking (Bröcker et al., 2010; Gillingham and Munro, 2003).

The second group of tethering factors is the MTCs. They function throughout the endomembrane system and at the plasma membrane. These tethers are composed of multiple subunits, suggestive of various functions within one protein complex. MTCs can span short distances (up to 30 nm) which is sufficient to capture vesicles, but much shorter than the distances that coiled-coil tethers can span (Bröcker et al., 2010). MTCs play a role in bringing apposed membranes together before fusion occurs. For some MTCs, it is possible that their involvement in this process is indirect. They appear to link the recognition of membranes through Rab GTPases with the later fusion process facilitated by SNARE proteins (Dubuke and Munson, 2016).

To date, at least nine MTCs have been identified. Based on sequence and structural similarities MTCs are divided into three groups. The first group is the Complexes Associated with Tethering Containing Helical Rods (CATCHER) complexes including Dsl1 complex, the conserved

oligomeric Golgi (COG) complex, the Golgi-associated retrograde protein (GARP) complex, the endosome-associated retrograde protein (EARP) complex and the exocyst (Ungermann and Kümmel, 2019). The subunits of these complexes share a common α -helical compact fold, indicating a similar function despite divergent evolution. These complexes are required for membrane transport in the secretory pathway. The Dsl1 complex is responsible for Golgi-to-ER traffic as well as maintenance of Golgi morphology (Diefenbacher et al., 2011; Spang, 2012). The COG complex is involved in retrograde trafficking at the Golgi (Barlowe et al., 1994; Suvorova et al., 2002; Ungar et al., 2002). The GARP complex is responsible for endosome recycling to the plasma membrane and recycling to the trans-Golgi network (Conibear and Stevens, 2000). The exocyst is responsible for vesicle traffic to the plasma membrane (TerBush et al., 1996). Lastly, the EARP complex is related to the GARP complex and believed to have a role in vesicle recycling between endosomes and the plasma membrane (Schindler et al., 2015).

The second group is the non-CATCHER MTCs which includes the homotypic fusion and vacuole protein sorting (HOPS) complex and the class C core vacuole-endosome tethering (CORVET) complex (Chou et al., 2016). HOPS and CORVET are implicated in endolysosomal transport (Peplowska et al., 2007; Spang, 2016). HOPS and CORVET are believed to work in a sequential manner such that the CORVET complex is required for the tethering of Golgi derived vesicles to the endosome and the HOPS complex is required for tethering at the late endosomes (Balderhaar and Ungermann, 2013).

The TRAPP complexes do not fit into either of the two categories. Despite functioning within the secretory pathway, they lack both sequence and structural similarities with the CATCHR and non-CATCHR complexes. Thus, TRAPP represent a separate third category of MTCs. TRAPP

complexes are multi-subunit GEFs for Rab GTPases that combine coat recognition with tethering. Moreover, their architecture is also distinct and more compact compared to other MTCs (Bagde and Fromme, 2022; Barrowman et al., 2010). TRAPP complexes represent the most well studied MTCs and will be discussed in more details in section 1.2.

1.2 TRAPP Complexes

1.2.1 Yeast TRAPP (yTRAPP)

The TRAPP complexes were first described in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (**Figure 1.1**). Initially it was believed that there was a single complex first identified by co-immunoprecipitation of a number of proteins with blocked early in transport 3 (Bet3) (Rossi et al., 1995; Sacher et al., 1998). Bet3 was found to localize to the cis-Golgi and participate in the late stages of vesicle transport from the ER to the Golgi (Rossi et al., 1995; Sacher et al., 1998). Later, three related complexes named as TRAPPI, TRAPPII and TRAPPIII were identified and found to be conserved among other eukaryotes (Lynch-Day et al., 2010; Sacher et al., 2001, 1998; Scrivens et al., 2011).

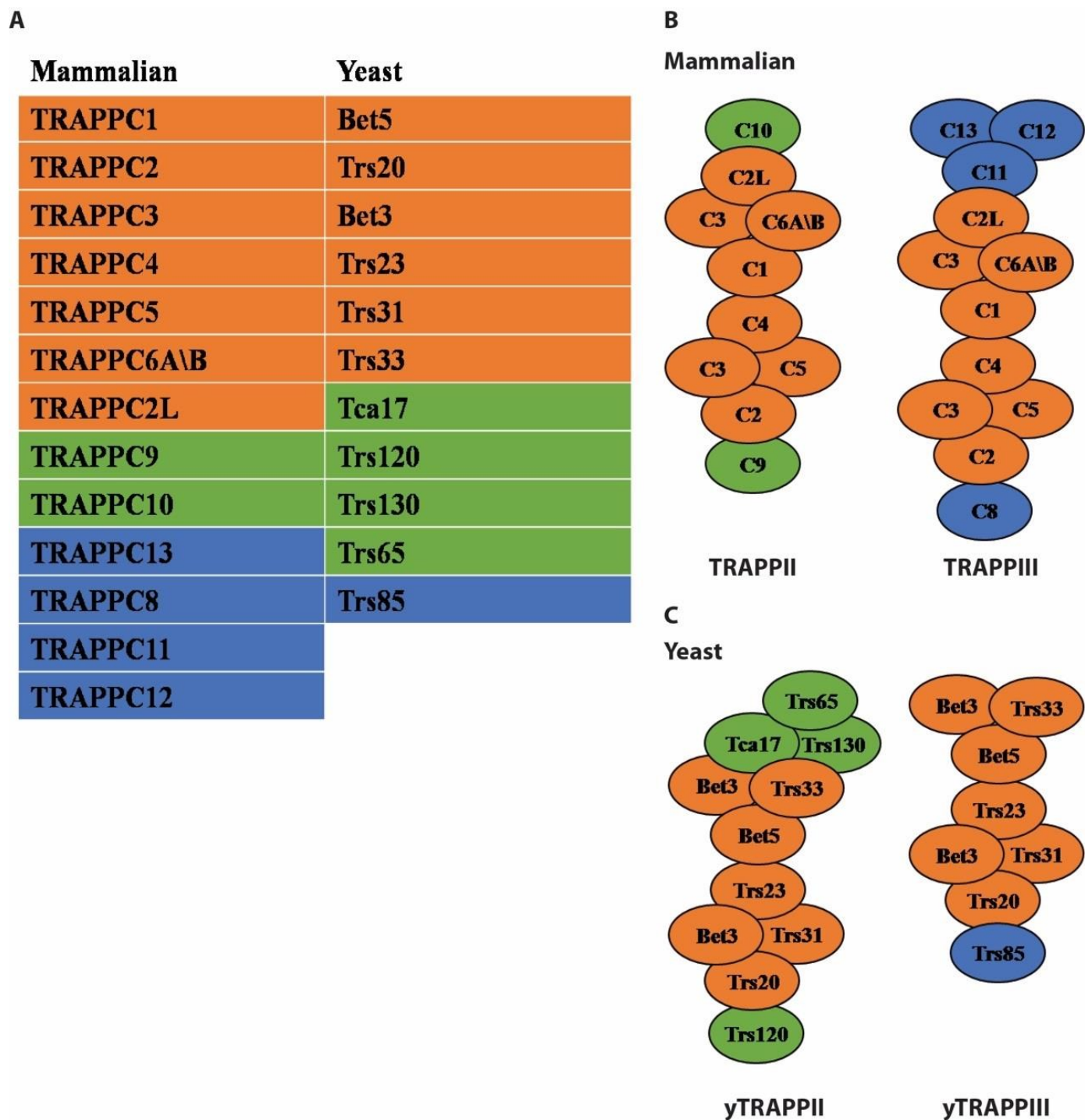


Figure 1.1 Composition of the mammalian and yeast TRAPP complexes.

A. Mammalian TRAPP subunits and their *S. cerevisiae* homologs. B. Cartoons of the mammalian TRAPPII and TRAPPIII. C. Cartoons of the yTRAPPII and yTRAPPIII. The core subunits are colored in orange, TRAPPII-specific subunits are colored in green, TRAPPIII-specific subunits are colored in blue.

1.2.1.1 TRAPPI (The TRAPP core)

The yTRAPPI complex, also referred to as the TRAPP core, was the first TRAPP complex to be identified. It includes Bet3 and is made up of seven polypeptides, including TRAPP subunit 20 (Trs20), Bet5, two copies of Bet3, Trs23, Trs31, and Trs33 (**Figure 1.1A**) (Sacher et al., 1998). These subunits make up the core of all yTRAPP complexes and are essential for its assembly and functioning (Sacher et al., 2008). Four of these subunits, Trs23, Trs31, Bet3 and Bet5, make up the catalytic core that is required for activating the yeast Rabs Ypt1 and Ypt31/32 (Kim et al., 2006). Ypt1 is required for ER - Golgi and intra-Golgi transport while Ypt31 and Ypt32, a pair of related GTPases, are crucial for the departure of proteins from the trans-Golgi (Jones et al., 2000; Wang et al., 2000).

yTRAPPI binds to Ypt1p in its inactive nucleotide-free state, which suggests that yTRAPP may act as a GEF for this Rab GTPase, converting it to its active form (Jones et al., 2000; Wang et al., 2000). This hypothesis has been supported by studies that have shown that all three yTRAPP complexes have GEF activity towards Rab GTPases (Jones et al., 2000; Thomas et al., 2018; Wang et al., 2000). In addition, in ER to Golgi transport, yTRAPPI attaches to COPII-coated vesicles through an interaction between Bet3 and COPII protein Sec23 (Cai et al., 2007b). This interaction allows yTRAPPI to tether the vesicles and marks the first step in the interaction of transport vesicles with their target membrane, suggesting that yTRAPP might also act as a tether (Sacher et al., 2001).

Subsequent work suggested that yTRAPPI may not actually exist in living cells, and that its presence may be due to a purification artifact that occurs under high salt conditions (Brunet et al., 2012). This theory is further supported by the finding that yTRAPPIII, rather than yTRAPPI, is responsible for activating Ypt1 during ER- Golgi trafficking and that yTRAPPI likely does not

exist in vivo either (Thomas et al., 2018). Moreover, no equivalent of mammalian TRAPPI has been identified (Sacher et al., 2019a).

1.2.1.2 Yeast TRAPP^{II} (yTRAPP^{II})

The yTRAPP^{II} complex is composed of the yTRAPP core and four additional subunits known as Tca17, Trs65, Trs120, and Trs130 (**Figure 1.1.A and 1.1C**) TRAPP^{II} plays a role in intra-Golgi transport as well as traffic between the Golgi and early endosomes (Choi et al., 2011; Montpetit and Conibear, 2009; Sacher et al., 2001, 2000). Analysis of *Trs130* and *Trs120* mutants have revealed defects in general secretion and endosome recycling (Cai et al., 2005; Sacher et al., 2001). Additionally, yTRAPP^{II} has been found to interact with COPI coatomer (Cai et al., 2005). Trs120 mutants that are defective in endosomal recycling also display a defect in the localization of COPI, suggesting that yTRAPP^{II} may participate in recycling of COPI vesicles from early endosomes to the Golgi (Cai et al., 2005).

Initially, it was believed that yTRAPP^{II} acted as a GEF for Ypt1 (Cai et al., 2008; Jones et al., 2000); however, later studies have shown that yTRAPP^{II} acts downstream of Ypt1 and only activates Ypt31/Ypt32 (Morozova et al., 2006; Thomas and Fromme, 2016). This discrepancy may be due to the use of methods that rely on purified yTRAPP complexes, which may contain impurities, as well as the use of non-physiological soluble Ypt/Rab substrates, which can lead to incomplete and incorrect conclusions (Bagde and Fromme, 2022).

1.2.1.3 Yeast TRAPP^{III} (yTRAPP^{III})

The yTRAPP^{III} complex is composed of the same yTRAPP core as yTRAPP^I, with a single additional subunit known as Trs85 (**Figure 1.1A and 1.1C**). Trs85 is incorporated into the complex

through the adaptor protein Trs20 (Taussig et al., 2014). While some of the functions of yTRAPPIII were initially believed to be carried out by yTRAPPI, it has since been shown that yTRAPPIII can fulfill the functions of yTRAPPI (Thomas et al., 2018). This complex is localized at the Golgi and plays a role in ER-to-Golgi trafficking (Thomas et al., 2018).

Studies have also linked yTRAPPIII to the cellular process of autophagy, which involves breaking down and recycling cellular components, including organelles and protein aggregates (Kakuta et al., 2012; Lynch-Day et al., 2010). The yTRAPPIII functions in the early secretory pathway and in autophagy are mediated through its GEF activity towards Ypt1 (Lynch-Day et al., 2010; Thomas et al., 2018).

1.2.2 Mammalian TRAPP

The yeast subunits are conserved in mammalian cells. However, mammalian complexes have additional associated proteins not present in yeast. Only two TRAPP complexes, TRAPPII and TRAPPIII, have been identified in mammals (Bassik et al., 2013; Scrivens et al., 2011). Similar to yTRAPPs, these complexes are thought to act as tethers. However, it has become clear that they facilitate the tethering step through their GEF activity towards Rab GTPases and not necessarily through a physical bridging of two apposed membranes (Bagde and Fromme, 2022; Sacher et al., 2019a). TRAPPII is made up of TRAPPC9 and TRAPPC10 subunits, in addition to the core complex composed of TRAPPC1, TRAPPC2, TRAPPC2L, two copies of TRAPPC3, TRAPPC4, TRAPPC5 and TRAPPC6 (**Figure 1.1A and 1.1B**). Similarly, TRAPPIII contains TRAPPC8, TRAPPC11, TRAPPC12 and TRAPPC13 in addition to the core (**Figure 1.1A and 1.1B**) (Bassik et al., 2013; Gavin et al., 2002; Scrivens et al., 2011). TRAPPC14 has been identified as a protein

that associates with the TRAPPII complex. However, it is not essential for the integrity of the TRAPPII complex and does not play a role in its assembly (Cuenca et al., 2019).

1.2.2.1 TRAPPII

The precise roles of TRAPPII in mammals is not yet fully understood. Initially, it was believed to only activate Rab1 (Yamasaki et al., 2009). However, studies in *Drosophila* revealed that TRAPPII also activates Rab11 (Riedel et al., 2018). Rab1 regulates ER to Golgi and intra-Golgi trafficking (Moyer et al., 2001), whereas Rab11 regulates Golgi to plasma membrane and endosome to Golgi traffic (Moyer et al., 2001; Ullrich et al., 1996). Additionally, although not conclusively demonstrated, it is thought that TRAPPII serves as a GEF for Rab18 (Li et al., 2017) as well as Rab43 and its paralog Rab19 (Jenkins et al., 2020). Both Rab43 and Rab19 localize at the Golgi and are evolutionarily similar to Rab1 (Jenkins et al., 2020; Klöpper et al., 2012). Although the notion that TRAPPII acts as a GEF is well supported, the identity of the Rab target remains controversial (Lipatova and Segev, 2019). This controversy may stem from biochemical assays of components purified from cell lysates (Li et al., 2017; Yamasaki et al., 2009) or the use of *in-vitro* GEF assays that lack a true physiological context (Jenkins et al., 2020).

While the TRAPPI core alone can activate Rab1 *in vitro* (Kim et al., 2006; Riedel et al., 2018), TRAPPII specific subunits are required for Rab11 activation both *in vitro* and *in vivo* (Riedel et al., 2018; Thomas et al., 2019). This indicates that the TRAPPII subunits are necessary to switch the GEF activity of the core from Rab1 to Rab11. This was further supported by studies in different model organisms, such as *Aspergillus nidulans* and in *Arabidopsis*, which show that TRAPPII activates Rab11 and not Rab1 (Pinar et al., 2019, 2015; Qi et al., 2011; Qi and Zheng, 2011). The notion that TRAPPII is a Rab11 GEF in metazoans is further supported by the interaction of the

TRAPP^{II} specific subunits (TRAPPC9 and TRAPPC10) with Rab11 *in vivo* (Gillingham et al., 2019). In conclusion, TRAPP^{II} primarily activates Rab11, with limited *in vitro* activity on Rab1 and Rab1 family GTPases.

The TRAPP^{II} complex, similar to γ TRAPP^{II}, interacts with the COPI coat and is involved in early Golgi vesicle trafficking (Cai et al., 2005; Yamasaki et al., 2009). The TRAPPC9 and TRAPPC10 subunits interact with the γ 1COP subunit of the COPI coat, and depletion of TRAPPC10 affects the tethering of COPI coated vesicles to the early Golgi membrane, leading to an accumulation of cargo in this area. TRAPPC10 also colocalizes with Rab1 on cis-Golgi, suggesting that it plays a role in mediating early Golgi traffic through direct interactions with coat proteins and/or its GEF activity towards Rab1 (Yamasaki et al., 2009).

Although TRAPP^{II} was suggested to function as a Rab1 GEF in early Golgi trafficking, more recent work has also identified a role for TRAPP^{II} with Rab11 in late Golgi trafficking during primary cilia assembly (Cuenca et al., 2019; Westlake et al., 2011). Primary cilia are membrane-bound, microtubule-based organelles that extend from the surface of the cell body and are found in most eukaryotic cells. Primary cilia play a crucial role in transmitting chemical and mechanical stimuli from the extracellular environment to the cell interior (Knödler et al., 2010).

TRAPP^{II} in addition to the TRAPP^{II}-associated protein TRAPPC14 and Rab11 target Rabin8, the GEF for Rab8, to the centrosome. Activation of Rab8 is required for the ciliary membrane formation and assembly of primary cilia (Cuenca et al., 2019; Westlake et al., 2011). Additionally, TRAPP^{II} is present on Rab11 positive endosomes. TRAPP^{II} forms a complex with Rab11 and

kalirin, a GEF for the Rho GTPase Rac1. Disruption of this complex impairs endosomal recycling (Wang et al., 2020).

Besides its role in membrane trafficking, TRAPPC9 has been found to exhibit a moonlighting function. Several studies have implicated TRAPPC9 in the activation of the nuclear transcription factor- κ B (NF- κ B) (Bodnar et al., 2020; Hu et al., 2005; Mir et al., 2009a). TRAPPC9 interacts with two critical components of the NF- κ B signaling pathway, the NF- κ B-inducing kinase (NIK) and the inhibitor of NF- κ B (IkB) kinase subunit β (IKK β), leading to the constitutive activation of NF- κ B signaling pathways (Hu et al., 2005). The NF- κ B signaling pathway is involved in cell growth and survival, and higher levels of TRAPPC9 expression have been observed in tumor tissues such as breast and colon cancer (Zhang et al., 2015).

1.2.2.2 TRAPPIII

The TRAPPIII complex differs from yeast in having three extra subunits (TRAPPC11, TRAPPC12 and TRAPPC13) instead of just one (Trs85, the TRAPPC8 homologue) (**Figure 1.1A and 1.1B**) (Scrivens et al., 2011). Despite this difference, TRAPPIII has evolved to perform similar functions as yeast, with added functionality.

TRAPPIII plays a role in ER-to-Golgi trafficking in the early secretory pathway. TRAPPC12 localizes to the ER exit sites and ER-Golgi intermediate compartment (ERGIC), interacts with the Sec13/Sec31A heterotetramer and positively regulates the assembly of COPII during vesicle formation (Scrivens et al., 2011; Zhao et al., 2017). Depletion of TRAPPC8, TRAPPC11 and TRAPPC12 results in altered Golgi morphology and blocks anterograde trafficking at an early stage, either at ER exit sites or ERGIC (Scrivens et al., 2011).

TRAPPIII has been linked to autophagy, a cellular process where a double-membrane known as an autophagosome encapsulates cytoplasmic components. This process begins with the formation and expansion of isolation membranes that eventually form into an autophagosome. This autophagosome then merges with the lysosome to create an autolysosome. Lysosomal enzymes break down the autophagosomal contents, replenishing the cellular nutrient pool (Behrends et al., 2010). All the TRAPPIII-specific subunits (TRAPPC8, TRAPPC11, TRAPPC12, and TRAPPC13) play a role in this process, which requires membrane trafficking and remodeling to form the autophagosome and deliver its contents to lysosomes for degradation (Behrends et al., 2010; Lamb et al., 2016; Ramírez-Peinado et al., 2017; Stanga et al., 2019).

TRAPPC8 was found as part of the human autophagy interaction network through a proteomic study (Behrends et al., 2010). It modulates autophagy through interaction with a TBC domain-containing protein (TBC1D14) (Lamb et al., 2016). TRAPPIII and TBC1D14 regulate trafficking of the autophagy related protein 9 (ATG9) from recycling endosomes to the Golgi. This is crucial for initiating autophagy and formation of isolation membranes (Lamb et al., 2016; Stanga et al., 2019).

Interestingly, TRAPPC11 is not required for the formation of the isolation membrane but acts downstream of this (Stanga et al., 2019). TRAPPC11 recruits ATG2 and its binding partner WIPI4/WDR45 and is required for the sealing of the isolation membrane to form the autophagosome (Stanga et al., 2019). Moreover, TRAPPC12 functions after autophagosome formation and is believed to play a role prior to autolysosome formation (Stanga et al., 2019).

TRAPPC13 is another component of TRAPPIII linked to autophagy. It has been reported that TRAPPC13 is important for autophagic flux under certain stress conditions, but the exact mechanism is yet to be determined (Ramírez-Peinado et al., 2017). It is possible that the role of TRAPPIII in this process is facilitated through Rab1, as TRAPPC13 depletion reduces Rab1 activity and affects autophagic flux (Ramírez-Peinado et al., 2017).

TRAPPIII has a recently discovered role in ciliogenesis, beyond its involvement in the secretory pathway and autophagy. TRAPPC8 and TRAPPC12 have been found at the centrosome and at the base of primary cilia (Schou et al., 2014; C. Zhang et al., 2020). TRAPPC12 and TRAPPC8 interact sequentially with oral-facial-digital syndrome 1 (OFD1), a protein crucial for transporting cargo in and out of the primary cilium (C. Zhang et al., 2020).

In vitro assays have confirmed that TRAPPIII activates Rab1 (Harris et al., 2021; Riedel et al., 2018). Currently, TRAPPIII is the only known physiological Rab1-GEF (Galindo and Munro, 2022). The TRAPP core primarily mediates this GEF activity, though non-core subunits likely interact with Rab1 since the TRAPPIII complex has higher Rab1 exchange activity than the TRAPP core alone (Galindo et al., 2021; Harris et al., 2021).

It is noteworthy that some TRAPPIII-specific subunits might have functions outside the complex. For instance, TRAPPC11 is involved in protein glycosylation as disruption of TRAPPC11 reduces both lipid-linked oligosaccharide (LLO) levels and N-linked protein glycosylation (DeRossi et al., 2016; Milev et al., 2019). TRAPPC12 also functions outside the TRAPPIII

complex, playing a role in mitosis by regulating chromosome congression and kinetochore stability (Milev et al., 2015). It is worth noting that depletion of other TRAPP subunits does not affect glycosylation or mitosis (DeRossi et al., 2016; Milev et al., 2015). Further research is needed to understand the roles of these subunits both individually and within the complex.

1.2.3 The Architecture of the TRAPP complexes

The architecture of the yeast and metazoan TRAPP complexes provides insights into their assembly and function. The crystal structure of TRAPPI subunits and subcomplexes, in addition to cryo-electron microscopy (EM) structures of TRAPPI, TRAPPII and TRAPPIII revealed the mechanism behind the nucleotide exchange activity of all TRAPP complexes and provides insights into their assembly. (Bagde and Fromme, 2022; Galindo et al., 2021; Joiner et al., 2021; Kim et al., 2005, 2006; Kümmel et al., 2005).

In metazoa, the structure of the TRAPPI complex revealed the existence of three sub-complexes: TRAPPC3/TRAPPC6, TRAPPC1/TRAPPC4 and a TRAPPC2/TRAPPC3/TRAPPC5. TRAPPC1, TRAPPC3, TRAPPC4 and TRAPPC5 have been found to be sufficient for activating Rab1 *in vitro* (Kim et al., 2006). The crystal structure of the yeast catalytic core consisting of Bet5, two copies of Bet3, Trs23, and Trs31 in a complex with Ypt1, reveals why multiple subunits are required for the TRAPP GEF activity. Bet3, Bet5, and Trs23 interact directly with the nucleotide-binding domain (NBD) of Ypt1/Ypt31, while Trs31 was believed to be required for the GEF activity without direct interaction with Ypt1/Ypt31. Recent findings indicate that Trs31 may interact with the hypervariable domain (HVD) of Ypt1/Ypt31 (Bagde and Fromme, 2022; Joiner et al., 2021). The C-terminus of Bet3 destabilizes the GDP nucleotide bound to the GTPase by causing a steric clash with the switch I region of Ypt1, promoting nucleotide release. The combined interactions

stabilize Ypt1/Rab1 in a nucleotide-free state, enabling GTP binding to occur (Cai et al., 2008; Kim et al., 2006).

The TRAPP core forms an elongated rod structure with TRAPPC1/TRAPPC4 in the center, flanked by two copies of TRAPPC3. TRAPPC5 and TRAPPC6 interact with TRAPPC3 on either side (Cai et al., 2008; Kim et al., 2005, 2006; Kümmel et al., 2006). TRAPPC2 and TRAPPC2L serve as adapters for TRAPPII and TRAPPIII- specific subunits and are found on opposite ends of the elongated complex (**Figure 1.2 A**) (Milev et al., 2018; Montpetit and Conibear, 2009; Scrivens et al., 2009a).

In TRAPPII, TRAPPC2 binds TRAPPC9 and TRAPPC2L binds TRAPPC10 forming a triangle-like structure (Al-deri et al., 2020; Milev et al., 2018; Pinar et al., 2019; Riedel et al., 2018; Zong et al., 2011). In yTRAPPII, a dimer is formed, requiring the additional subunit Trs65. These dimers resemble the mammalian TRAPPII complex, where Tca17 binds to Trs130 and Trs20 binds to Trs120 (**Figure 1.2 B**) (Bagde and Fromme, 2022; Choi et al., 2011; Mi et al., n.d.; Montpetit and Conibear, 2009; Taussig et al., 2013).

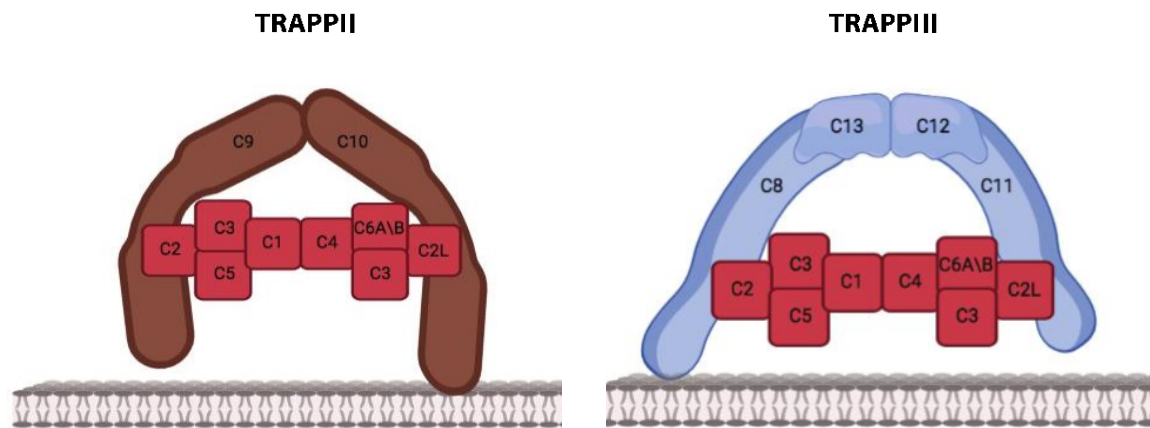
In TRAPPIII, TRAPPC2 recruits TRAPPC8, and TRAPPC2L binds to TRAPPC11, while TRAPPC12 and TRAPPC13 bind to the C-terminus of TRAPPC8/TRAPPC11 (**Figure 1.2 A**) (Galindo et al., 2021; Riedel et al., 2018; Zong et al., 2011). In yeast TRAPPIII, Trs85 is recruited to the complex via Trs20 (**Figure 1.2 B**) (Brunet et al., 2013; Tan et al., 2013; Taussig et al., 2014). Further investigation is necessary to comprehend the mechanism behind the core's selection of either TRAPPII or TRAPPIII subunits for complex formation.

As alluded to above, the TRAPP^{II} and TRAPP^{III} complexes both utilize the same catalytic core to activate Rab11 and Rab1, respectively. This indicates that specific subunits of each complex and/or the unique characteristics of the Rabs determine substrate specificity. A steric gating mechanism was suggested to control the substrate specificity of yTRAPP complexes (Thomas et al., 2019). yTRAPP^{II}-specific subunits (Trs120/TRAPPC9 and Trs130/TRAPPC10) are thought to position the catalytic core of yTRAPP^{II} away from the membrane surface (**Figure 1.2 A**). As a result, only Rab11, which has a longer HVD compared to Rab1, can reach the catalytic core and be activated (Thomas et al., 2019). The recent cryo-EM structure of the yTRAPP^{II}-Ypt31/Rab11 activation intermediate supports this model. Trs130/TRAPPC10 plays a crucial role in this mechanism by extending a leg that positions the active site away from the membrane (Bagde and Fromme, 2022).

In TRAPP^{III}, TRAPPC8 is in close contact with Rab1 and holds the catalytic core with the help of TRAPPC11. This interaction is believed to stabilize Rab1 association with the core and enhance specificity. Rab1 residues that are predicted to interact with TRAPPC8 differ from those in Rab11. Cryo-EM structure of TRAPP^{III} reveals a repulsive interaction between the core and Rab11, which is caused by a negatively charged surface on Rab11 (Galindo et al., 2021). Additionally, in yeast, Trs85 targets the complex and anchors it to anionic membranes through an amphipathic helix, and also interacts with Ypt1 (Joiner et al., 2021). These findings confirm the role played by the non-core subunits in the GTPase specificity.

A

Mammalian



B

Yeast

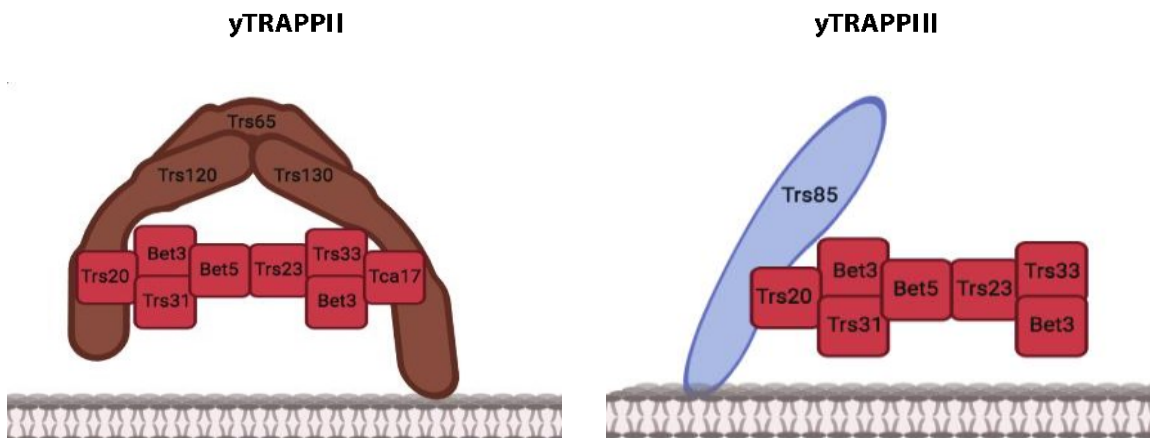


Figure 1.2 Architecture of the metazoan and yeast TRAPP complexes on membranes.

A. Mammalian TRAPPII and TRAPPIII complexes consist of core subunits with large specific subunits bound at both ends. TRAPPC10 and TRAPPC8 anchor TRAPPII and TRAPPIII to membranes, respectively. **B.** Trs130 act as a leg that elevates the catalytic core from the membrane in yTRAPPII, while Trs85 anchors TRAPPIII to membranes without elevating the catalytic core from membranes.

1.2.4 TRAPPopathy

TRAPPopathies comprise a group of disorders associated with genetic alterations encoding defective proteins which result in either improper assembly or function of the TRAPP complex

(Brunet and Sacher, 2014; Sacher et al., 2019a). The TRAPP complexes are involved in the early secretory pathway, endocytic recycling, exocytosis, autophagy, and ciliogenesis, making it crucial for key cellular processes. As a result, it is associated with a range of genetic conditions, including skeletal, muscular, and neurodevelopmental disorders (Stanga et al., 2019; Wang et al., 2020; Westlake et al., 2011; Yamasaki et al., 2009).

Variants in the TRAPPC2 gene are associated to X-linked spondyloepiphyseal dysplasia tarda (SED), a skeletal defect characterized by short stature, a short trunk and a barrel-shaped chest (Gedeon et al., 1999). Notably, TRAPPC2 is the only TRAPP gene linked with skeletal abnormalities, which suggests that TRAPPC2 might play a role outside the TRAPP complex (Gedeon et al., 1999). TRAPPC2L has been identified as a closely related protein to TRAPPC2. However, variants in TRAPPC2L display distinctive phenotypes, including neurodevelopmental disorder (NDD), rhabdomyolysis and encephalopathy (Al-deri et al., 2020; Milev et al., 2018; Scrivens et al., 2009a).

TRAPPC4 has been recently implicated in a recessive NDD condition, intellectual disability (ID), and microcephaly (Ghosh et al., 2021; Kaur et al., 2020; Van Bergen et al., 2020).

TRAPPC6 is another core protein that has been implicated with TRAPPopathy disorders. TRAPPC6A and TRAPPC6B are two isoforms of the TRAPPC6 protein, sharing 56% identity and 72% similarity, and are encoded by two different genes (Kümmel et al., 2008). TRAPPC6A variants have been linked to ID, speech delay, polydactyly and facial dysmorphism. (Mohamoud et al., 2018). On the other hand, several studies have identified variants in TRAPPC6B that result in NDD, ID, microcephaly, autism, epilepsy, and brain malformations (Harripaul et al., 2018;

Marin-Valencia et al., 2018; Nair et al., 2019). The characteristics displayed by these phenotypes are similar to those caused by variations in other components of the TRAPP^{II} complex (see below).

Variants in TRAPP^{II} (TRAPPC9 and TRAPPC10) are associated with NDD, ID, microcephaly and brain abnormalities (Khattak and Mir, 2014; Mir et al., 2009b; Santos-Cortez et al., 2018). Although only one study has pinpointed a single homozygous variant in TRAPPC10 as the cause of an autosomal recessive NDD with ID (Santos-Cortez et al., 2018), numerous studies have implicated variants in TRAPPC9 in pathologies of the central nervous system (Hnoonual et al., 2019; Mir et al., 2009b; Mochida et al., 2009; Radenkovic et al., 2022).

Individuals with TRAPPC9 variants experience moderate to severe non-syndromic autosomal recessive mental retardation. Other phenotypes include microcephaly, speech delay, facial dysmorphism, obesity, thinning of the corpus callosum and a reduction in cerebral white matter volume (Abbasi et al., 2017; Mir et al., 2009b; Mochida et al., 2009; Najmabadi et al., 2007; Philippe et al., 2009). These phenotypes overlap with those of individuals with TRAPPC10 variants. However, while TRAPPC9 has recently been implicated in a congenital disorder of glycosylation, this function of TRAPPC10 remains yet to be determined (Radenkovic et al., 2022).

Additionally, TRAPPC9 has been found to play a vital role in the activation of the NF- κ B signaling pathway (Hu et al., 2005), which is involved in cell growth and survival, and elevated TRAPPC9 expression has been observed in breast and colon cancer tissues where it appears to promote tumorigenesis via NF- κ B signaling pathways (Zhang et al., 2015). However, recent research on a TRAPPC9 knockout mice model has raised questions about the involvement of TRAPPC9 in this pathway in the brain, as no evidence of defective NF- κ B was found (Ke et al., 2020). Further

studies are needed to better understand the role of NF- κ B pathways in brain development and function in TRAPPC9 patients. Overall, these findings underscore the importance of understanding the roles of TRAPPC9 and TRAPPC10 in various physiological processes and disease pathologies, particularly those related to neurodevelopment and cancer.

TRAPPIII variants are associated with a spectrum of phenotypes, some of which overlap with those of TRAPPPII. Individuals with TRAPPC12 variants experience progressive childhood encephalopathy, global developmental delay, hearing loss, and brain atrophy (Aslanger et al., 2020; Milev et al., 2017). On the other hand, individuals with TRAPPC11 variants share some phenotypes with TRAPPC12, such as intellectual disabilities, but also present with distinct phenotypes, such as limb girdle muscular dystrophy, cardiomyopathy and fatty liver formation (Bögershausen et al., 2013; DeRossi et al., 2016; Liang et al., 2015; Milev et al., 2019).

The involvement of different TRAPP subunits in various diseases suggests that individual TRAPP subunits may have independent "moonlighting" roles. Recent findings indicate that some TRAPP proteins play a role in cellular processes outside of membrane trafficking (Milev et al., 2015). The broad range of cellular phenotypes and clinical features associated with TRAPP complexes highlights the significance of these complexes in development and cellular function and implicates many of the subunits in specialized roles.

1.2.5 TRAPPC10

TRAPPC10 shares 22% identity with its yeast orthologue Trs130 (Cox et al., 2007). Genetic screens identified interactions between *ARF1*, *YPT31/32* and *TRS130* in the regulation of vesicle trafficking at the TGN (Zhang et al., 2002). Arf1 is a small GTPase of the Arf family which

recruits cargo adaptors and coat proteins of the COPI vesicles to early Golgi membranes (Serafini et al., 1991). It is believed that Arf1 recruits TRAPPII to anionic membranes of the TGN by interacting with Trs130 (Thomas and Fromme, 2016; Zhang et al., 2002). In GEF reconstitution assays, TRAPPII exhibits stronger substrate specificity for Ypt31/32 than Ypt1 when recruited to anionic membranes by Arf1. However, when prenylated Rab on TGN membranes are used, TRAPPII also activates Ypt1, suggesting that Arf1 and the presence of anionic lipids affects the specificity of TRAPP complexes (Thomas et al., 2019; Thomas and Fromme, 2016).

Trs130 has been implicated in autophagy in a Ypt31/Ypt32-dependent manner. A *trs130* temperature-sensitive (ts) mutant showed defective starvation-induced autophagy and was unable to transport Atg8 and Atg9 to the preautophagosomal structure, which could be rescued by overexpression of Ypt31/Ypt32, but not of Ypt1 (Zou et al., 2013). Additionally, Trs130 along with Ypt31/32 participates in the dissociation of Ypt6, the yeast homolog of Rab6, from late Golgi membranes (Brunet et al., 2016). Trs130 is required for the recruitment of Gyp6p, a GAP for Ypt6p. Ypt6p is involved in ER to Golgi, intra-Golgi, post-Golgi trafficking, and autophagy (Brunet et al., 2016; Suda et al., 2013). It is possible that this function of TRAPPII is mediated by its GEF activity towards Ypt31/32 or that TRAPPII and Ypt31/32 contribute to the recruitment of Gyp6p to the Golgi to regulate Ypt6p dynamics. However, these interactions have not been reported for TRAPPC10 yet (Brunet et al., 2016).

TRAPPC10 functions are not well characterized. As illustrated above, TRAPPC10 localizes on the cis-Golgi and interacts with the COPI coat and regulates early Golgi vesicle trafficking (Yamasaki et al., 2009). It also interacts with ciliary component and Rab11 in late Golgi trafficking during primary ciliogenesis (Cuenca et al., 2019; Westlake et al., 2011). TRAPPC10 is required for the

specificity switch of the core from Rab1 to Rab11. A sequence alignment study predicted a Longin domain (LD), a protein interaction module found in many Rab GEFs, at the N-terminus of human TRAPPC10 (Levine et al., 2013).

TRAPPC10 interacts with the adaptor protein TRAPPC2L and disruption of this interaction impairs TRAPPII assembly and results in a delay of cargo transport in and out of the Golgi (Milev et al., 2018). Prior to this work, no cellular studies on TRAPPC10 variants have been reported. Thus, further research is needed to examine cellular processes affected by dysfunction of this gene product.

1.2.6 TRAPPC6B

In yeast, the TRAPPC6 orthologue Trs33 interact with Bet3 (homologue of TRAPPC3) (Kim et al., 2005). This interaction is conserved in mammalian cells as a TRAPPC6B-TRAPPC3 interaction was shown *in vivo* by co-immunoprecipitation and *in vitro* by pull down of recombinant proteins (Kümmel et al., 2005). The crystal structure of TRAPPC6 suggests it can form heterodimers in association with TRAPPC3 (Kümmel et al., 2005). Subsequent studies identified two isoforms of TRAPPC6 (TRAPPC6A and TRAPPC6B) that co-expressed and form a heterodimer with TRAPPC3. The crystal structure of TRAPPC6A-TRAPPC3 and TRAPPC6B-TRAPPC3 suggest that both isoforms are capable of forming a sub-complex of TRAPP, providing a binding pocket for other TRAPP proteins such as TRAPPC2L (Kim et al., 2005; Kümmel et al., 2006). Binding of TRAPPC2L or its yeast homologue Tca17 is crucial for TRAPPII assembly since it acts as an adaptor for TRAPPC10 and Trs130, respectively (Bagde and Fromme, 2022; Mi et al., n.d.; Milev et al., 2018).

Gene expression profiling of adult human tissues indicates that both TRAPPC6 isoforms are expressed in various tissues, with a higher abundance of TRAPPC6B (Fonseca et al., 2014; Petryszak et al., 2017). However, in *yeast*, *Drosophila* and *Arabidopsis* only Trs33 is present (Garcia et al., 2020; Riedel et al., 2018; Sacher et al., 1998). Trs33 is not essential for yeast growth but becomes essential in the absence of Trs65. Trs65 and Trs33 can substitute for each other and the assembly of TRAPP^{II} requires either Trs33 or Trs65 (Pinar et al., 2019; Tokarev et al., 2009). On the other hand, Trs33 is essential in *Arabidopsis* and *Drosophila*, and both lack a Trs65 homologue (Riedel et al., 2018; J. Zhang et al., 2020). Moreover, TRAPPC6 is not essential for human cell growth possibly due to the presence of the different isoforms (Riedel et al., 2018; Wang et al., 2015). Mammalian TRAPP complexes seem to incorporate both TRAPPC6 isoforms, suggesting that the mammalian paralogs can compensate for each other. *In vitro* studies have shown that TRAPP^{II} and TRAPP^{III} complexes are functional when TRAPPC6A is used for assembly (Harris et al., 2021; Jenkins et al., 2020). However, the specific role of each isoform remains poorly understood. It is possible that each isoform has a unique role outside of the TRAPP complex. TRAPPC6B, but not TRAPPC6A, has been implicated in Shiga toxin intracellular trafficking in renal epithelial cells, and silencing TRAPPC6B prevented the cytotoxic effect of the toxin to the kidneys (Kouzel et al., 2020). Further investigation is needed to understand how cells select and incorporate specific TRAPPC6 isoforms into TRAPP complexes. Despite TRAPPC6B being not essential for cell viability, variants in TRAPPC6B are associated with human pathologies as discussed in section 1.2.4.

1.3 Translational Read-through Inducing Drugs (TRIDs)

Inherited human genetic disorders can be caused by nonsense mutations, which are genetic changes that lead to the creation of a premature termination codon (PTC) or a nonsense codon (UGA, UAG,

or UAA) in the transcribed mRNA (Mort et al., 2008). This "nonsenses" the gene, leading to reduced level of the affected mRNA and degradation by a conserved machinery known as nonsense-mediated mRNA decay (NMD) (Leeds et al., 1991; Losson and Lacroute, 1979; Perlick et al., 1996). While NMD does not degrade all mRNA with PTCs, a small amount of proteins is still produced (Chang et al., 2007). The truncated non-functional proteins produced by nonsense mutations are often structurally impaired and unable to fulfill their cellular role.

Protein synthesis initiation required the coordinated action of several translation initiation factors (eIFs). Among these factors, eIF4G play a critical role in identifying the start codon on the mRNA (Haghighat and Sonenberg, 1997; Joshi et al., 2004; Niedzwiecka et al., 2002; Sonenberg et al., 1978). Meanwhile, eIF3, eIF1, and eIF1A help to bind the mRNA to the 40S ribosomal subunit (Majumdar et al., 2003), and eIF2-GTP facilitates the binding of methionine-charged transfer RNA (tRNA) to the ribosome (Kimball, 1999). Once the eIF2-GTP is hydrolyzed by eIF5, the 60S ribosomal subunit joins the 40S subunit, and the initiation factors are released from the mRNA (Si et al., 1996).

During the elongation phase of protein synthesis, the ribosome moves along the mRNA, and the polypeptide chain is extended through the transfer of amino acids from charged tRNA molecules. This movement is facilitated by the binding of tRNA molecules to three distinct sites on the ribosome. The first tRNA is positioned at the P (peptidyl) site, and the second tRNA is brought to the A (aminoacyl) site by eukaryotic translation elongation factor 1A (eEF1A). As the ribosome moves along the mRNA, three nucleotides are read, and the uncharged tRNA is released through the E (exit) site, making room for the next tRNA to bind to the now-empty A site (Andersen and Nyborg, 2001).

When the ribosome encounters a naturally occurring stop codon (UAA, UAG, or UGA) without a complementary tRNA, mRNA translation terminates. Eukaryotic translation release factor 1 (eRF1) recognizes all three stop codons and together with eRF3 causes the ribosome to hydrolyze the ester bond linking the peptide chain to the tRNA, releasing the newly synthesized protein from the ribosome (Frolova et al., 1996). However, a PTC can occur anywhere within the mRNA. When a PTC enters the ribosomal A site, it often leads to the termination of translation, resulting in truncated protein expression due to activation of the NMD pathway (Chang et al., 2007). Translational read-through of nonsense mutations occurs rarely, with only 0.01-1% of transcripts exhibiting this phenomenon. Near-cognate tRNA (carrying an anticodon that only differs by one base pair from the codon) can be incorporated at the A site despite the presence of a termination codon, leading to the translational read-through of a PTC (Manuvakhova et al., 2000). Enhancing translational read-through of nonsense mutations represents a potential therapeutic target for disorders caused by these mutations (Manuvakhova et al., 2000).

Nonsense mutations underlie a broad spectrum of pathologies, including neurological, metabolic and muscular disorders, in addition to many forms of cancer. While the underlying mechanisms for each of these conditions may vary, the potential for therapies targeting nonsense mutations makes them a compelling area of interest for a significant number of patients (Bushby et al., 2014; Lee and Dougherty, 2012; Liang et al., 2015).

Translational read-through inducing drugs (TRIDs) are used to promote the read-through of PTCs, restoring the expression of full-length functional proteins. TRIDs are classified as aminoglycoside or non-aminoglycoside compounds. Aminoglycosides such as gentamicin, tobramycin and

geneticin (G418) can interfere with ribosome proofreading by facilitating the binding of near-cognate tRNAs, resulting in misreading or suppression of a nonsense codon (Ng et al., 2021). While aminoglycosides exhibit high read-through efficacy, their clinical application is limited due to their capacity to induce read-through of normal stop codons, along with their potential for severe renal, auditory, and vestibular toxicity (Burke and Mogg, 1985; Wilhelm et al., 1978).

Ataluren, also known as PTC124, is a small molecule drug that falls under the non-aminoglycoside category (Welch et al., 2007). It was designed to enhance read-through of PTCs without affecting normal termination codons, and to overcome the side effects of aminoglycosides (Burke and Mogg, 1985; Welch et al., 2007). Ataluren has been shown to successfully promotes the read-through of all three nonsense codons, with the highest levels of efficiency observed at UGA, while avoiding interference with the NMD pathways, meaning it does not affect mRNA levels (Welch et al., 2007). Unlike aminoglycosides, Ataluren does not lead to an increase in the mispairing of near-cognate tRNA with PTCs (Ng et al., 2021). Instead, it binds to the ribosome, competes with eRF1 and eRF3, and inhibits peptidyl-tRNA hydrolysis mediated by eRF1 and eRF3, thus preventing the termination of protein synthesis (Huang et al., 2022; Ng et al., 2021; Welch et al., 2007).

Ataluren is highly selective for PTCs and exhibit low toxicity, even at high concentrations (Welch et al., 2007), it is the only TRID approved by the European Medicines Agency for the treatment of nonsense-mediated Duchenne muscular dystrophy (DMD) in patients (Michorowska, 2021). DMD is a condition characterized by progressive muscle degeneration that leads to loss of ambulation and early death (Campbell et al., 2020). Oral administration of Ataluren (brand name Translarna), for 48 weeks has been found to promotes the production of dystrophin, a protein that protects muscle fibers from breaking down by enzymes, slowing the progression of disease in DMD patients

(Bushby et al., 2014; Campbell et al., 2020; EMA, 2018; McDonald et al., 2017; Michael et al., 2021; Michorowska, 2021).

Enhancing functional protein synthesis can significantly alleviate or eliminate the principal manifestations of disease. Studies on nonsense-mediated cystic fibrosis have shown that promoting the recovery of truncated protein to as little as 5% of normal levels can have a significant impact (Ramalho et al., 2002). Therefore, *in vivo* trials have explored the potential therapeutic benefits of this approach for other conditions such as nonsense-mediated epilepsy, pulmonary arterial hypertension and metabolic disorders (Campbell et al., 2020; Devinsky et al., 2021; Kerem et al., 2014; Peabody Lever et al., 2020; Sarkar et al., 2011).

1.4 Objectives

Objective One: Characterization of pathogenic TRAPPC10 variants and study their effect on TRAPPII-dependent pathways.

While TRAPPII has been implicated in neurodevelopmental disorders, functional studies on TRAPPC10 variants are lacking. One variant of TRAPPC10 has been reported as part of a large group of individuals suffering from autosomal recessive neurodevelopmental disorders with intellectual disability (Santos-Cortez et al., 2018). Recently, we identified multiple individuals from the same family with an autosomal recessive form of neurodegeneration and microcephaly. Whole-exome sequencing has revealed that the affected individuals are homozygous for a variant in TRAPPC10. In this project, I characterize both pathogenic TRAPPC10 variants and investigated their impact on TRAPPII assembly and function. The findings have been published in *PLOS Genetics* and are presented in Chapter two.

Objective Two: Characterization of pathogenic TRAPPC6B variants and assess their effect on TRAPPII-dependent pathways.

TRAPPC6B forms a heterodimer in association with TRAPPC3, and this heterodimer is crucial for TRAPP assembly and function. Homozygous TRAPPC6B variants have been identified in individuals with neurodevelopmental delays (Marin-Valencia et al., 2018; Nair et al., 2019). We identified 29 additional patients with microcephalic neurodevelopmental disorder, speech impairment and movement disorder. My focus for functional studies was on two individuals with homozygous variants: c.454C>T, p.Q152*, nonsense mutation and c.149+2T>A, predicted splice-site variant. I evaluated the impact of these variants on TRAPPII assembly and function. My hypothesis was that TRAPPC6B variants would disrupt the interaction between TRAPPC6B and TRAPPII complex subunits, resulting in the instability and malfunction of the TRAPPII complex. The findings were submitted to *Brain* and are presented in Chapter Three.

Objective Three: Evaluate the ability of TRID (Ataluren) to rescue cellular defects caused by a nonsense mutation in TRAPPC6B patients.

Identifying disease-causing genes is essential for developing gene-specific therapies. One specific variant of TRAPPC6B (c.454C>T, p.Q152*) is a nonsense variant predicted to generate a PTC that would inhibit full-length TRAPPC6B expression, and subsequently inhibition of normal full-length TRAPPC6B expression. In chapter three, I have shown that this variant results in decreased levels of TRAPPC6B and other TRAPPII complex-specific members, as well as a defect in membrane trafficking and altered Golgi morphology. I evaluated the ability of Ataluren to bypass the PTC in the TRAPPC6B (c.454C>T, p.Q152*) variant and to rescue membrane trafficking and the altered

Golgi morphology observed in TRAPPC6B patient cells harboring the nonsense mutation. The findings are presented in Chapter Four.

Chapter 2: Biallelic Variants in TRAPPC10 cause a Microcephalic TRAPPopathy Disorder in Humans and Mice

This Chapter was published as a manuscript in *PLoS Genetics* (Rawlins L and Almousa H et al., 2022). I shared first authorship of this paper.

I generated the results in Figure 2.2 and Figure 2.3.

Figure 2.3A, I prepared the DNA construct for the rescue experiments and Dr. Milev transfected the cells, did the VSVG assay and analyzed the data. Figure 2.3B, Dr. Saint-Dic did the MG132 treatment and the immunoblot, I quantified and analyzed the data. Generation of the *TRAPPC10*^{-/-} cell line in HEK293 cells was done by Ana Maria Hincapie, I tested the sgRNA and guided Ana throughout the process of generating the *TRAPPC10*^{-/-}. All clinical analyses were done by our collaborators.

2.1 Abstract

The highly evolutionarily conserved transport protein particle (TRAPP) complexes (TRAPPII and III) perform fundamental roles in subcellular trafficking pathways. Here we identified biallelic variants in *TRAPPC10*, a component of the TRAPPII complex, in individuals with a severe microcephalic neurodevelopmental disorder. Molecular studies revealed a weakened interaction between mutant TRAPPC10 and its putative adaptor protein TRAPPC2L. Studies of patient lymphoblastoid cells revealed an absence of TRAPPC10 alongside a concomitant absence of TRAPPC9, another key TRAPPII complex component associated with a clinically overlapping neurodevelopmental disorder. The TRAPPC9/10 reduction phenotype was recapitulated in *TRAPPC10*^{-/-} knockout cells, which also displayed a membrane trafficking defect. Notably, both the reduction in TRAPPC9 levels and the trafficking defect in these cells could be rescued by

wild type but not mutant TRAPPC10 gene constructs. Moreover, studies of *Trappc10*^{-/-} knockout mice revealed neuroanatomical brain defects and microcephaly, paralleling findings seen in the human condition as well as in a *Trappc9*^{-/-} mouse model. Together these studies confirm autosomal recessive *TRAPPC10* variants as a cause of human disease and define TRAPP-mediated pathomolecular outcomes of importance to TRAPPC9 and TRAPPC10 mediated neurodevelopmental disorders in humans and mice.

Author summary

Microcephalic neurodevelopmental disorders are a group of conditions that are often inherited in families, involving small head size and abnormal brain development and function. This often results in delayed development of an affected child, affecting their movement, language and/or non-verbal communication and learning, as well as seizures and neuropsychiatric problems. A group of proteins called the transport protein particles (TRAPPs) are important for the transport of cargos inside cells. Alterations within a number of the TRAPP proteins have previously been associated with human inherited diseases called the ‘TRAPPopathies’, which involve neurodevelopmental and skeletal abnormalities. Here we show that *TRAPPC10* gene alterations cause a new TRAPPopathy microcephalic neurodevelopmental disorder, and we provide a detailed clinical description of the condition termed ‘*TRAPPC10*-related disorder’. Our studies in mice lacking the *TRAPPC10* gene identified similar features to those of affected humans, including small brain size and skeletal abnormalities. Our molecular studies showed that an affected individual with an alteration in the *TRAPPC10* gene has no functional TRAPPC10 protein in their cells, which in turn causes a reduction in levels of another important TRAPP molecule, TRAPPC9. Cells lacking TRAPPC10 also display abnormalities in cellular transport processes. Together our

data confirm alterations in *TRAPPC10* as a cause of a microcephalic neurodevelopmental disorder in both humans and mice.

2.1 Introduction

The transport protein particles (TRAPPs) comprise highly evolutionarily conserved multiprotein complexes, originally identified in yeast. Three yeast TRAPP complexes have been described (TRAPP I, II and III) which play important roles in secretory and endocytic subcellular trafficking pathways (Cai et al., 2007b; Sacher et al., 2001). Humans possess only two complexes (TRAPP II and III), both sharing the same core proteins alongside additional complex-specific components, some not identified in yeast. Previous studies have identified diverse cellular roles for the TRAPP II complex including Golgi, COP (coat protein)-I vesicular, lipid droplet, centrosomal and ciliary functions (Li et al., 2017; Westlake et al., 2011; Yamasaki et al., 2009). The TRAPP II complex contains two complex-specific subunits; TRAPPC9 and TRAPPC10 (Sacher et al., 2019a). An additional TRAPP II complex-associated protein has recently been described (TRAPPC14/C7orf43) (Cuenca et al., 2019). However, TRAPPC14 does not appear to be required for TRAPP II complex-associated guanine nucleotide exchange factor activity (Jenkins et al., 2020; Riedel et al., 2018) and lack of the molecule does not affect TRAPP II complex assembly or stability (Cuenca et al., 2019). Thus, further studies are required to clarify the role of TRAPPC14 in TRAPP II. TRAPPC9 has been implicated in COPII vesicle trafficking (Zong et al., 2012) and NF- κ B signalling pathway activation (Hu et al., 2005), with TRAPPC9 depletion associated with increased lipid droplet formation (Li et al., 2017). TRAPPC14 plays a role in Rabin 8 binding to the TRAPP II complex and its tethering of preciliary vesicles to the mother centriole during ciliogenesis (Cuenca et al., 2019). No detailed studies of TRAPPC10 function have been conducted, although it has been shown to interact with TRAPPC2L and may have roles in guanine nucleotide exchange factor (GEF) activity regulation (Milev et al., 2018).

Ten of the 15 TRAPP complex proteins have been associated with human inherited diseases, collectively termed “TRAPPopathies” (Sacher et al., 2019a). This includes TRAPPC2, the only subunit associated with skeletal dysplasia (spondyloepiphyseal dysplasia tarda (SED); OMIM #313400) (Fiedler et al., 2004a), and nine other TRAPP subunits (TRAPPC2L, TRAPPC4, TRAPPC6A, TRAPPC6B, TRAPPC9, TRAPPC10, TRAPPC11, TRAPPC12 and TRAPPC14) associated with neurodevelopmental disorders (Abbasi et al., 2017; Al-deri et al., 2020; Koehler et al., 2017; Liang et al., 2015; Marin-Valencia et al., 2018; Milev et al., 2018, 2017; Mir et al., 2009b; Mohamoud et al., 2018; Mortreux et al., 2018; Perez et al., 2019; Van Bergen et al., 2020). While diverse, the clinical features of these disorders are overlapping and include neurodevelopmental delay, intellectual disability, developmental regression, primary and postnatal-onset microcephaly, epilepsy, hypotonia, craniofacial dysmorphism and structural brain abnormalities including thinning of the corpus callosum and reduced white matter volume (**S2.1 Table**).

Of the TRAPP complex specific subunits, only TRAPPC9 has been robustly associated with human disease, an autosomal recessive neurodevelopmental disorder (OMIM #613192) characterized by postnatal-onset microcephaly with reduced white matter volume and corpus callosum thinning, intellectual disability, dysmorphic features, hypotonia, epilepsy, and raised body mass index (Abbasi et al., 2017; Mir et al., 2009b; Mortreux et al., 2018). TRAPP complex plays important roles in Golgi membrane trafficking. “Golgiopathy” neurodevelopmental disorders including the TRAPPopathies comprise an expansive group of conditions associated with variants in Rab GTPases, conserved oligomeric Golgi (COG) complex, and coat protein (COP) complex proteins among others (Passemar et al., 2017; Rasika et al., 2018) and are characterized by primary or postnatal-onset microcephaly, intellectual disability, seizures and white matter brain abnormalities.

Here, we present comprehensive genetic, clinical, functional and mouse data to define biallelic TRAPPC10 gene variants as a cause of a microcephalic neurodevelopmental disorder, providing important insight into the TRAPPopathy family of disorders.

2.2 Results

2.2.1 Biallelic TRAPPC10 variants are associated with a microcephalic neurodevelopmental disorder

Table 2.1 summarizes the clinical features of affected individuals in this study. Family 1 comprises a large interlinking multi-nuclear Pakistani family, with eight individuals (4.1–18.1 years) affected by a severe microcephalic neurodevelopmental disorder (**Figure 2.1A, Family 1**). All but one individual presented with microcephaly (>3 standard deviations (SDS) below mean), and all displayed short stature (-1.78 to -5.09 SDS). All eight were born at full term, prenatal histories were unremarkable, although antenatal ultrasound scans were not performed. The parents did not report that any affected infant's head was noticeably small (no weight or occipitofrontal circumference (OFC) measurements were recorded). The earliest OFC measurement documented was at 2.5 years (1-IV:4), and at age 4.1 years his microcephaly had not worsened. Affected individuals displayed mild craniofacial dysmorphism (**Figure S2.1A–S2.1C**). Although standardized intelligence quotient (IQ) testing was not possible due to cultural and language barriers, all affected individuals were assessed as severely intellectually impaired (DSM-5 criteria). Developmental trajectories were similar in all, as was the degree of impairment across all developmental domains. Hypotonia in infancy was universal and walking was delayed to between 2.5–4 years. All affected individuals have speech impairment, five have only a few words and three are non-verbal. Behavioural abnormalities including autistic features and aggressive episodes were a consistent feature. A history of seizures from early infancy was reported in four individuals, one has ongoing generalised tonic-clonic seizures (1-IV:2) and the remaining three (1-IV:3, 1-IV:7, 1-IV:13) are seizure free on medication. Neuroimaging was only performed for one case (1-IV:2) due to the remote family location and as sedation/general anaesthesia was deemed necessary. This revealed microcephaly with corpus callosum thinning and no other abnormalities (**Figure 2.1B**

and 2.1C). All had normal systemic examination findings and no evidence of gross skeletal disproportion (**Figure S2.1A**). It was only possible to obtain skeletal radiographs for a single individual (1-IV:12) aged 9 years, these were unremarkable but unfortunately spinal imaging was incomplete.

Assuming homozygosity for a founder variant was responsible, we undertook SNP genotyping (1-IV:2–4, 1-IV:7–8, 1-IV:12–13) in parallel with exome sequencing (1-IV:2) to identify the cause of disease. SNP mapping identified a single notable (>1Mb) homozygous region common to all affected individuals (3.5Mb, rs2839641-rs7278087; chr21:g.43,179,611–46,678,912 [hg38]) as the likely disease locus (**Figure 2.1D**). Concomitant exome sequencing data analysis to identify candidate homozygous and compound heterozygous rare (<0.01 frequency) variants predicted to have a functional impact, identified a single candidate variant within this region which could not be excluded. This frameshift variant (chr21:g.44102823del; NM_003274.4:c.3392del; p.(Gly1131Valfs*19)[hg38]; **Figure 2.1A**) in the penultimate (22/23) exon of *TRAPPC10* located in the chromosome 21q22.12 region, is present within the C-terminal TRAPP domain (**Figure 2.1E**) and is predicted to result in nonsense mediated mRNA decay of the mutant transcript (**Figure 2.1F**). The variant was confirmed in the lymphocyte RNA of an affected individual (**Figure S2.2**), is not listed in publicly available genomic databases (including gnomAD v2.1.1/v3.1.1) and cosegregates appropriately (**Figure 2.1A**). This finding enabled us to revisit the genetic data and more comprehensively phenotype two siblings in an unrelated Pakistani family (**Figure 2.1A, Family 2**) included in a larger study to identify novel candidate neurodevelopmental disease genes ((Santos-Cortez et al., 2018) Family MR107). Both male siblings (2-IV:1, 22 years and 2-IV:2, 25 years), were described to have “severe intellectual disability, aggressive behaviour and poor speech”. A homozygous exon 18 *TRAPPC10* variant (chr21:g.44089849C>T; NM_003274.4:c.2786C>T;

p.(Pro929Leu) [hg38]) (**Figure 2.1A**) was identified as a potential cause of disease (Santos-Cortez et al., 2018). This variant, which co-segregated appropriately, is absent from gnomAD v2.1.1/v3.1.1. Multiple species alignment confirms stringent conservation of Pro929 across vertebrates (**Figure 2.1F**). Additional clinical features noted and not previously reported, included microcephaly/borderline microcephaly (-2.49 SDS, -4.2 SDS), short stature (-3.86, -5.29 SDS) and mild dysmorphism, all consistent with Family 1.

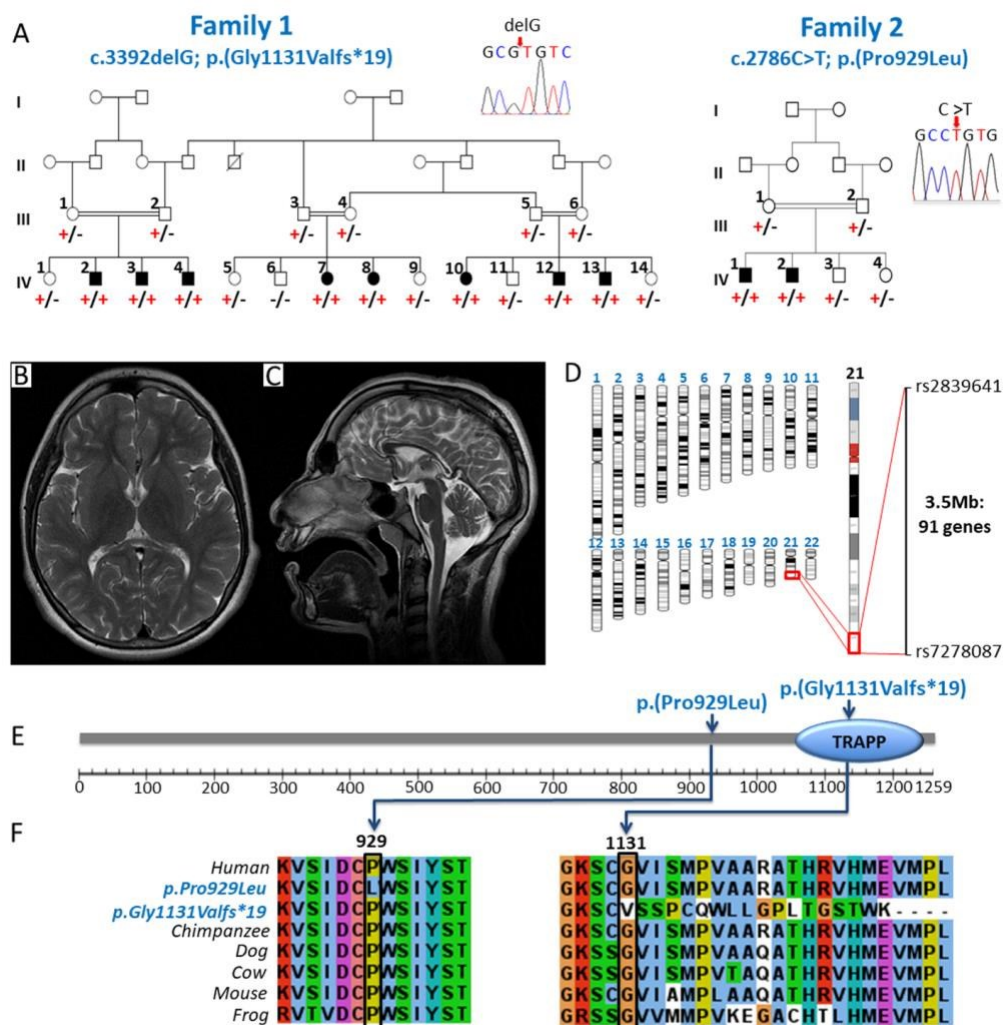


Figure 2.1. Biallelic TRAPPC10 variants identified in two families with individuals affected by a microcephalic neurodevelopmental disorder

(A) Family 1 Extended pedigree comprising three interlinking families and a total of eight affected individuals all of whom are homozygous for the TRAPPC10 c.3392delG; p.(Gly1131Valfs*19)

variant ('+'), with co-segregation confirmed in other family members ('-' indicates the wild type allele). **Inset:** Dideoxy sequence chromatogram of an affected individual homozygous for the TRAPPC10 c.3392delG; p.(Gly1131Valfs*19) variant. **Family 2** Previously reported family (MR107) (Santos-Cortez et al., 2018) showing co-segregation of the TRAPPC10 c.2786C>T; p.(Pro929Leu) variant ('+') in a four generation pedigree ('-' indicates the wild type allele). **Inset:** Dideoxy sequence chromatogram of an affected individual homozygous for the TRAPPC10 c.2786C>T; p.(Pro929Leu) variant. **(B, C)** MRI neuroimaging of individual IV:2 from family 1 demonstrates microcephaly and profound thinning of the corpus callosum with no other abnormalities. **(D)** Genome-wide SNP mapping in seven affected individuals (1-IV:2–4, 1-IV:7–8 and 1-IV:12–13) identified a single (3.5Mb) region of shared homozygosity, containing 91 genes including TRAPPC10. **(E)** Schematic diagram of the TRAPPC10 protein identifying TRAPPC10 sequence variants and TRAPP domain. **(F)** Multi-species alignment showing conservation of the molecular region encompassing the p.Gly1131Valfs*19 and p.Pro929Leu variants.

Table 2.1. A comparison of clinical features of affected individuals with biallelic variants in TRAPPC10.

FAMILY	FAMILY 1								FAMILY 2	
Pedigree reference	IV:2	IV:3	IV:4	IV:7	IV:8	IV:10	IV:12	IV:13	IV:1	IV:2
Genotype	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(G1131 Vfs*19)/ p.(G1131 Vfs*19)	p.(P9 29L)/ p.(P9 29L)	p.(P9 29L)/ p.(P9 29L)
Gender	M	M	M	F	F	F	M	M	M	M
Age at evaluation (years)	18.1	13.8	4.1	14.1	15.6	5.5	9.0	13.7	25	22
GROWTH										
Birth weight kg (SDS)	NK	NK	2.5 (-2.25)	NK	NK	NK	NK	NK	NK	NK
OFC cm (SDS)	55 (-1.32)	50 (-3.49)	47 (-3.59)	46 (-6.64)	49 (-4.56)	43 (-7.46)	43 (-7.05)	47 (-5.28)	50 (-4.24)	53 (-2.49)
Height cm (SDS)	164 (-1.87)	146 (-1.78)	94 (-2.23)	127 (-5.09)	142 (-3.41)	99 (-2.78)	119 (-2.48)	125 (-4.26)	150 (-3.92)	140 (-5.29)
Weight kg (SDS)	52 (-1.96)	40 (-0.99)	18 (0.6)	25 (-4.9)	45 (-1.39)	16 (-1.44)	20 (-2.77)	28 (-3.16)	45 (-3.9)	38 (-4.87)
BMI	19.3	18.7	20.4	15.5	22.3	16.3	14.1	17.9	20	19
DEVELOPMENT										
Intellectual disability	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Severe
Global developmental delay	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Speech impairment	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Walked (years)	<10 words 4y	Non-verbal 4y	<10 words Standing	Non-verbal 3.5y	<10 words 2.5y	Non-verbal 4y	<10 words 4y	<10 words 3y	NK	NK
Hearing	No concerns	Otitis media	No concerns	No concerns	No concerns	No concerns	No concerns	No concerns	No concerns	No concerns
Vision	No concerns	No concerns	No concerns	No concerns	No concerns	No concerns	Strabismus	No concerns	Strabismus	Strabismus
NEUROLOGY										
Childhood hypotonia	✓	✓	✓	✓	✓	✓	✓	✓	NK	NK
Seizures	✓	✓	-	✓	-	-	-	✓	-	-
Gait abnormalities	-	Waddling gait	-	-	-	-	Waddling gait	Waddling gait	NK	NK
Behavioural abnormalities	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Abbreviations: M, male; F, female; NK, not known; SDS, standard deviation score; OFC, occipitofrontal circumference; G, glycine; V, valine; fs, frameshift; (✓), indicates presence of a feature in an affected individual; (-), indicates absence of a feature in an affected individual. Height, weight and OFC Z-scores were calculated using a Microsoft Excel add-in to access growth references based on the LMS method^a using UK 1990 reference population^b, ^a Pan H, Cole TJ.

LMS growth, a Microsoft Excel add-in to access growth references based on the LMS method. Version 2.77, <http://www.healthforallchildren.co.uk/;2012>

^b Cole TJ, Freeman JV, Preece MA: British 1990 growth reference centiles for weight, height, body mass index and head circumference fitted by maximum penalized likelihood. *Stat Med* 1998, 17(4):407–4.

2.2.2 TRAPPC10 variants exhibit reduced interaction with TRAPPC2L, destabilize the TRAPP^{II} complex and result in an anterograde trafficking defect

We next sought to determine the molecular consequences of each *TRAPPC10* genetic variant. As we previously determined that TRAPPC10 interacts with TRAPPC2L in the TRAPP^{II} complex (Milev et al., 2018), we used yeast two-hybrid assays to investigate how TRAPPC10 alteration affects TRAPPC2L interactions. When TRAPPC10 was in the bait (pGBKT7) or prey (pGADT7) vector, TRAPPC2L interaction was observed (**Figure 2.2A and 2.2B**). However, both p.Gly1131Valfs*19 and p.Pro929Leu variants showed reduced TRAPPC2L interaction (**Figure 2.2A and 2.2B**), though the proteins were expressed in the yeast cells (not shown). This was confirmed quantitatively by measuring β -galactosidase activity (**Figure 2.2C**), one of the yeast two-hybrid system markers. Given this, we generated a patient lymphoblastoid cell line (LCL) (1-IV:2; *TRAPPC10* p.(Gly1131Valfs*19)) to investigate TRAPP complex outcomes. We examined core TRAPP protein (TRAPPC2, TRAPPC2L, TRAPPC3) levels, as well as TRAPP III-specific (TRAPPC8, TRAPPC12), and TRAPP II-specific (TRAPPC10, TRAPPC9) levels. TRAPPC2L, TRAPPC2 and TRAPPC3 levels were slightly reduced in the p.(Gly1131Valfs*19) lysate compared to control (**Figure 2.2D**). As expected we did not detect TRAPPC10 as the commercially available TRAPPC10 antibody is directed to the molecular region downstream (C-terminal) to the p.(Gly1131Valfs*19) alteration. Intriguingly we also noted an absence of full length TRAPPC9, although in contrast, TRAPP III-specific proteins levels were not affected. We then fractionated p.(Gly1131Valfs*19) and control LCL lysates on a size exclusion column. While TRAPPC10 and

TRAPPC9 fractionated as expected in controls, they were absent from p.(Gly1131Valfs*19) lysate **(Figure 2.2D)** indicating an absence of TRAPPII (peak in fraction 28 of control cells). There was no change in core protein (TRAPPC2, TRAPPC2L, TRAPPC3) fractionation consistent with their presence in TRAPP III, although the portion that co-fractionates with TRAPPII was reduced. Similarly, no change in TRAPPIII proteins (TRAPPC8, TRAPPC12) fractionation was seen, indicating that this complex was not affected in p.(Gly1131Valfs*19) cells.

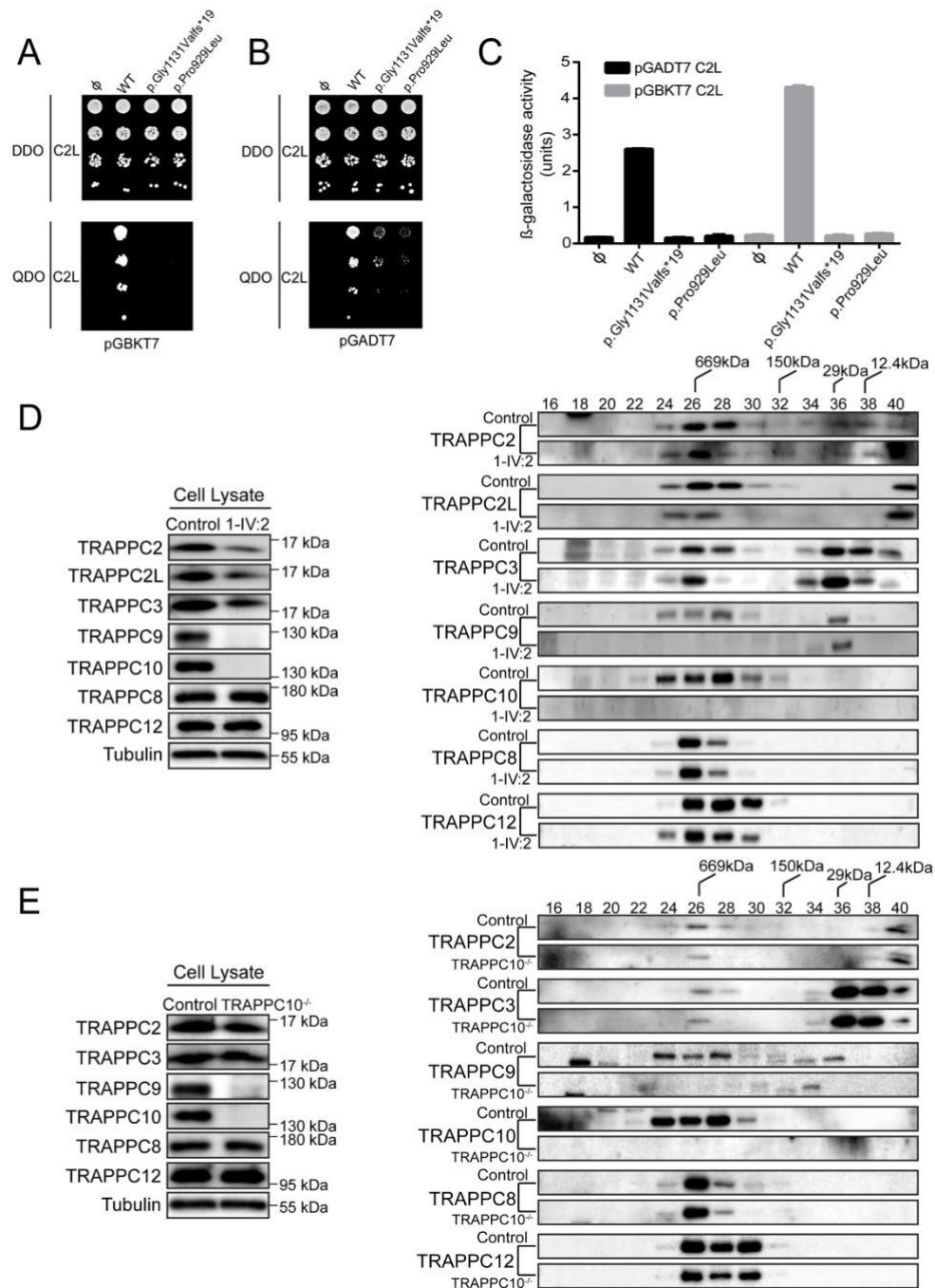


Figure 2.2. TRAPPII complex integrity is affected by TRAPPC10 variants and in TRAPPC10^{-/-} cells

Yeast cells transformed with pGBKT7 or pGBKT7 expressing either wild type TRAPPC10, the p.Gly1131Valfs*19 variant or the p.Pro929Leu variant (A), or pGADT7 or pGADT7 expressing either wild type TRAPPC10, the p.Gly1131Valfs*19 variant or the p.Pro929Leu variant (B) were mated with yeast expressing either pGBKT7-TRAPPC2L (A) or pGADT7-TRAPPC2L (B). Serial dilutions of the diploid cells were grown on SC medium lacking leucine and tryptophan (DDO) or SC medium lacking leucine, tryptophan, histidine and adenine (QDO). (C) Diploid cells from (A) and (B) were quantitatively tested for β-galactosidase activity. Units of activity were calculated

according to the following formula: $(OD_{420} \times 1000)/(OD_{600} \times \text{time in hours})$. Note that western analysis revealed that all forms of TRAPPC10 and the two variants were expressed in the yeast cells. **(D)** Lymphoblastoid cells from control or an individual homozygous for the TRAPPC10 p.(Gly1131Valfs*19) variant were lysed and probed for the TRAPP proteins indicated and for tubulin as a representative loading control. The lysates were fractionated on a Superose 6 size exclusion column. Fractions of 0.5ml were collected. Neighbouring fractions were pooled and fractionated by SDS-PAGE and probed for the indicated TRAPP proteins. **(E)** Wild type HEK293 or TRAPPC10^{-/-} cells were lysed and probed for the TRAPP proteins indicated and for tubulin as a representative loading control. The lysates were fractionated on a Superose 6 size exclusion column. Fractions of 0.5ml were collected. Neighbouring fractions were pooled and fractionated by SDS-PAGE and probed for the indicated TRAPP proteins. The fractionation of molecular size standards are indicated above the top-most panel of the size exclusion portion of **(D)** and **(E)**.

We then examined the consequences of TRAPPC10 loss on membrane trafficking. Since we were unable to perform trafficking assays in smaller LCLs, we generated HEK293 TRAPPC10 knockout cell lines using CRISPR/Cas9. Three different TRAPPC10 knockout lines were generated (**Figure S2.3**), two were examined and behaved identically (TRAPPC10^{-/-} KO1/KO7); as such we present data from one line (KO7, hereafter referred to as TRAPPC10^{-/-}). We first examined TRAPP protein levels in TRAPPC10^{-/-} cells. Consistent with observations in LCLs, we noted a slight reduction in core TRAPP proteins, no effect on TRAPP III-specific proteins (TRAPPC8, TRAPPC12), and the absence of TRAPPC9, consistent with size exclusion chromatography results (**Figure 2.2E**). We then performed the VSVG-GFP ts045 trafficking assay (Scales et al., 1997) in TRAPPC10^{-/-} cells. In this assay, the marker protein VSVG-GFP ts045 is retained in the ER at elevated temperature, but synchronously released at 32°C. The fluorescently-tagged marker protein accumulated in the Golgi region in controls, with peak signal at ~25 minutes (**Figure 2.3A, black curve**). From that point onward the signal was cleared from the Golgi as the protein travelled to the plasma membrane. In contrast, the arrival into, trafficking through and release from the Golgi of the marker protein in TRAPPC10^{-/-} cells was delayed compared to controls (**Figure 2.3A, green curve**). Importantly, the trafficking delay was shown to be TRAPPC10-dependent as cell transfection with wild type (WT) TRAPPC10 resulted in a trafficking defect rescue (**Figure 2.3A, blue curve**).

Although both p.Gly1131Valfs*19 and p.Pro929Leu variants were able to partially rescue these defects, neither rescued anterograde trafficking to the same extent as WT protein (**Figure 2.3A, red/grey curves**). Taken together, these results demonstrate that the *TRAPPC10* variants cause partial functional defects, and that the absence of TRAPPC10 results in a concomitant absence of TRAPPC9.

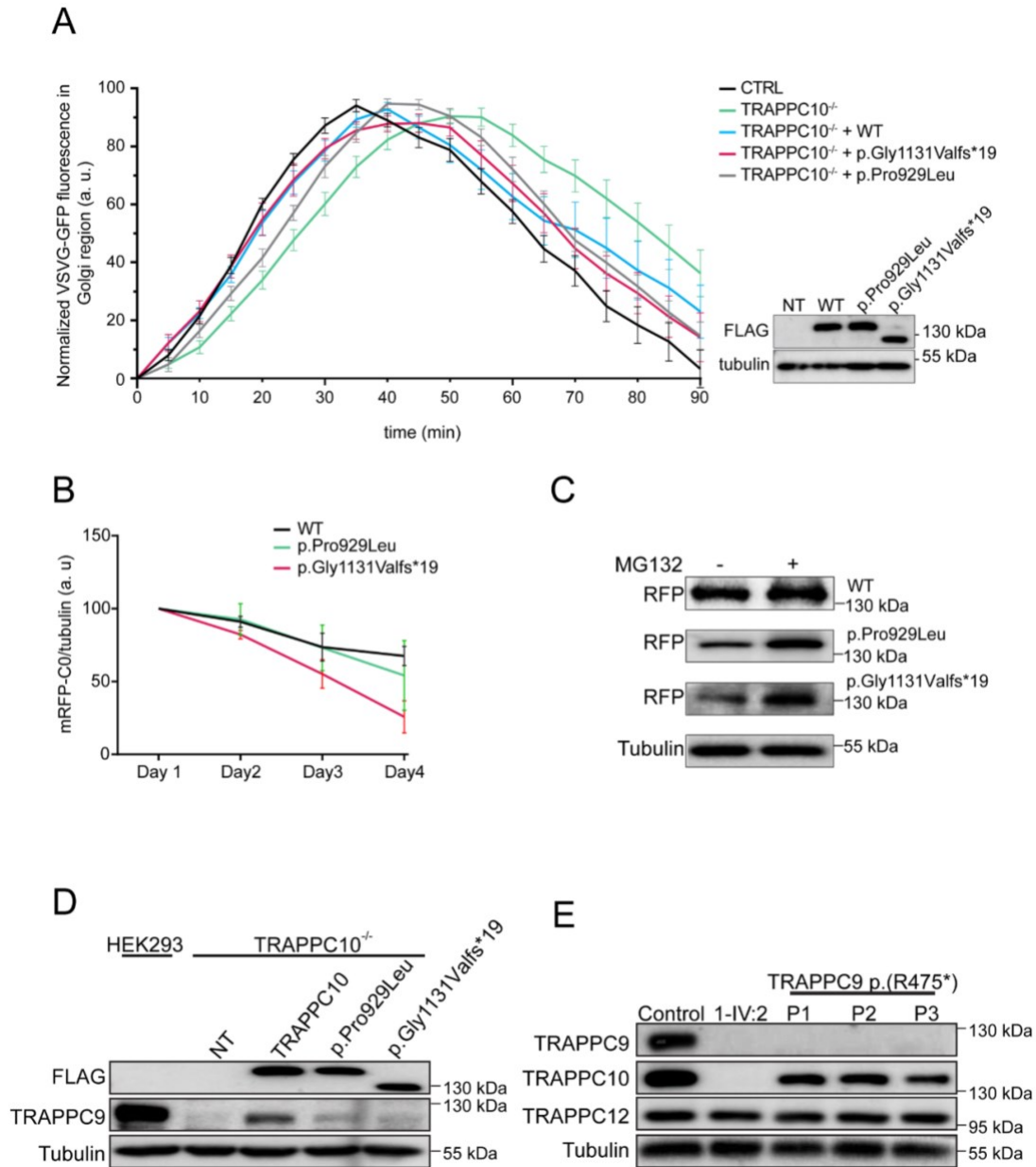


Figure 2.3. Cells devoid of TRAPPC10 display a membrane trafficking defect and lack detectable levels of TRAPPC9

(A) Wild type HEK293 cells or TRAPPC10^{-/-} cells either not transfected or transfected with FLAG-tagged wild type TRAPPC10 or one of the TRAPPC10 variants indicated were infected with VSVG-GFP ts045 4 hours after transfection. After an overnight incubation at 40°C, the cells were shifted to 32°C and imaged every minute. The fluorescence intensity in the Golgi was quantified and plotted versus time as described in the Materials and Methods section. The inset shows a western blot for the transfected proteins probed with anti-FLAG antibody. Representative images are shown in S2.7 Fig. (B) RFP-tagged versions of either wild type TRAPPC10, the p.Gly1131Valfs*19 variant or the p.Pro929Leu variant were transfected into HeLa cells and lysates prepared every day for 4 days. The lysates were fractionated by SDS-PAGE and probed with anti-mCherry antibody to reveal the RFP-tagged construct. Though the trends were consistent,

statistical significance was seen only at day 4. The half-life for each overexpressed protein was found to be approximately 5.4 days, 4.4 days and 3.1 days for the wild type, p.Gly1131Valfs*19 and p.Pro929Leu variants, respectively. (C) RFP-tagged TRAPPC10 and the p.Gly1131Valfs*19 and p.Pro929Leu variants were transfected into HEK293 cells. The cells were either untreated (-) or treated (+) with 50 μ m MG132 for 24 hours on the third day post-transfection. Lysates were then prepared, fractionated by SDS-PAGE and probed with anti-mCherry and tubulin as a loading control. (D) TRAPPC10^{-/-} cells were either untransfected or transfected with FLAG-tagged wild type TRAPPC10, the p.Gly1131Valfs*19 variant or the p.Pro929Leu variant. After 24 hours, lysates were prepared and probed for the FLAG constructs to verify expression, TRAPPC9 and tubulin as a loading control. Parental HEK293 cells were also probed to assess the level of TRAPPC9 in the presence of TRAPPC10. (E) Lysates were prepared from lymphoblastoid cells from either control, an individual homozygous for the TRAPPC10 p.Gly1131Valfs*19 variant or three individuals homozygous for the TRAPPC9 p.(Arg475*) variant and subjected to western analysis to reveal the proteins indicated.

2.2.3 Expression of TRAPPC10 is important for the detection of a cellular pool of

TRAPPC9

As no antibody was available to determine whether mutant TRAPPC10 p.(Gly1131Valfs*19) polypeptide may be expressed in affected individuals, we generated WT and both (p.Gly1131Valfs*19, p.Pro929Leu) constructs to investigate protein stability. The constructs were transfected into HEK293 cells and lysates prepared daily for four days. As shown in (**Figure 2.3B**), WT protein levels on day 4 dropped to ~70% of that on day 1. In contrast, levels of both altered TRAPPC10 proteins (day 4) dropped to 25–50% (day 1), with the drop in the p.Gly1131Valfs*19 protein being most dramatic. The decrease in expression of WT and both p.Gly1131Valfs*19 and p.Pro929Leu TRAPPC10 proteins was blocked by inclusion of proteasome inhibitor MG132 (**Figure 2.3C**). These results suggest that the two mutant proteins are subject to enhanced degradation compared to WT protein. The absence of TRAPPC9 in TRAPPC10 p.(Gly1131Valfs*19) LCLs prompted us to closely examine the inter-relationship between TRAPPC9 and TRAPPC10. We first investigated whether TRAPPC10 expression in TRAPPC10^{-/-} cells would restore TRAPPC9 levels. Upon transfection of WT TRAPPC10 into TRAPPC10^{-/-} cells, the appearance of TRAPPC9 was noted, although not to parental HEK293 cell levels

(**Figure 2.3D**). Overexpression of the p.Pro929Leu variant, and to a much lesser degree p.Gly1131Valfs*19, restored some TRAPPC9 protein although again not to the same extent as WT TRAPPC10. We next investigated whether a reciprocal absence of TRAPPC10 occurred in cells devoid of TRAPPC9. We probed LCL lysates from three individuals with the p.(Arg475*) TRAPPC9 variant (Mir et al., 2009b). As expected TRAPPC9 was not detected in these cells (**Figure 2.3E**), nor was it detected in TRAPPC10 p.(Gly1131Valfs*19) cells (as shown above). Importantly, while TRAPPC10 levels were significantly reduced in p.(Arg475*) TRAPPC9 lysates, the protein was nevertheless present. Collectively, these results suggest that the detection of a cellular pool of TRAPPC9 is strongly dependent upon the expression of full length TRAPPC10.

2.2.4 *Trappc10*^{-/-} mice have similar neurodevelopmental deficits to patients with biallelic *TRAPPC10* variants

We then evaluated tissue sections and datasets from a *Trappc10*^{tm1b(EUCOMM)Wtsi} mouse model, with a focus on the phenotypical components of the human disorder in particular neuroanatomical findings. At weaning age, mouse survival was evaluated from successfully genotyped mice originating from multiple litters derived from a heterozygous-by-heterozygous breeding scheme. We obtained the expected number of WT, heterozygous and homozygous mice. Male and female mice were weighed the same day each week from 4 until 16 weeks of age (**Figure S2.4**).

Using a recently developed robust approach to assess 63 brain parameters across 23 brain regions (Mikhaleva et al., 2016), we analyzed neuroanatomical defects in adult *Trappc10*^{-/-} mice blinded for genotype. To minimize environmental and genetic variation, male mice aged 16 weeks were used. In the homozygous mutant mice, many neuroanatomical parameters were reduced in size when compared to WTs (**Figure 2.4A and 2.4B**). The total brain area parameter was significantly

reduced (14%, $P = 0.0003$), concomitantly with smaller white matter structure size including the genu of the corpus callosum (-25%, $P = 0.026$), hippocampal commissure (-38%, $P = 0.013$), hippocampal fimbria (-17%, $P = 0.0049$), anterior commissure (-28%, $P = 3.8E10^{-8}$) and the internal capsule (-23%, $P = 1.1E10^{-6}$) (**Figure 2.4C and 2.4D**). When combining neuroanatomical parameters from either grey or white matter structures, only white matter structures were reduced in size (-20%, $P = 8E10^{-6}$) (**Figure 2.4E**). Interestingly, the size reductions of the anterior commissure and internal capsule were correlated with loss of myelination whilst oligodendrocyte cell population count and density were unaffected (**Figure 2.4F and 2.4G**). Together these findings suggest that both mouse and human microcephaly stems from defects in white matter structures and myelin biogenesis.

Given the short stature exhibited by patients with *TRAPPC10*-related disorder we also evaluated *Trappc10*^{-/-} mice growth parameters (**S2.1 Text**). While long bone length was marginally reduced in both sexes, it only reached significance for female *Humerus* (**Figure S2.4A**), although body length overall was unaffected (**Figure S2.4B**). While weight was unaffected in *Trappc10*^{-/-} males, female *Trappc10*^{-/-} mice were overweight, showing a rapid weight increase commencing week 10 (**Figure S2.4C**). We investigated the cause of the female obesity phenotype and noted a highly significant fat mass increase ($P = 9E-08$; **Figure S2.4D**), alongside a blood cholesterol increase ($P = 4E-05$) and high-density lipoprotein levels ($P = 0.003$) (**Figure S2.4E**).

We finally sought to further characterize the phenotypical relationship between *Trappc10*^{-/-} mice, and a previously published *Trappc9*^{-/-} mouse model (Liang et al., 2020). Due to the unavailability of live *Trappc9*^{-/-} animals, these evaluations were limited to assessments undertaken on historical brain tissue sections. Overall, the impact of TRAPPC10 loss on brain anatomy was more severe than TRAPPC9 loss (**Figure S2.5**). While there were some overlaps between affected brain regions

(e.g. corpus callosum reduced size), some neuroanatomical phenotypes were specific to *Trappc10*^{-/-} including reduced size of the anterior commissure.

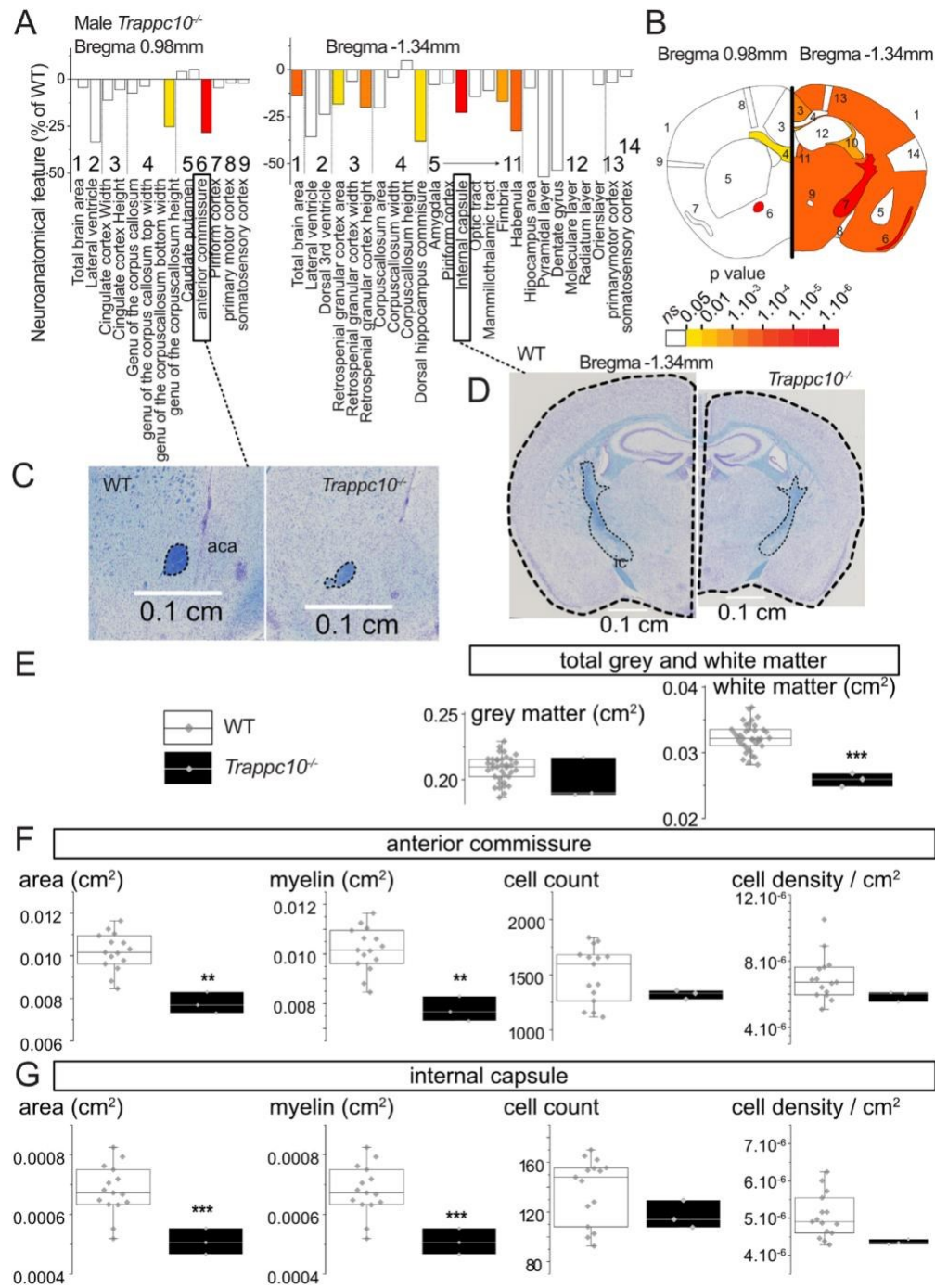


Figure 2.4. Mouse studies implicate TRAPPC10 in the formation of white matter structures
(A) Histograms for three homozygous *Trappc10*^{-/-} mice showing variation (decreased-minus scale or increased-positive scale) in areas and lengths expressed as percentage of 498 WTs together with a color map indicating the significance level (white indicates not significant). The list of

measurements and corresponding numbers are shown below the histograms. **(B)** Schematic representation of a section at Bregma +0.98 mm and Bregma -1.34 mm. Colored regions indicate the presence of at least one significant parameter within the brain region at the 0.05 level. **(C-D)** Nissl-stained sagittal brain sections from *Trappc10^{tm1b/tm1b}* mice, showing the anterior commissure **(C)** and the total brain area and internal capsule size reduction in mutants **(D)**. The corresponding scale is shown in each panel. **(E)** Box plots of combined grey and white matter structures expressed in cm² using a set of 37 local WTs (same genetic background, housing conditions, age, sex, experimental and necropsy dates) compared to three *Trappc10^{tm1b/tm1b}* mice. **(F-G)** Box plots with raw data points detailing the cellular and myelination characterisation of the anterior commissure **(F)** and internal capsule **(G)** using a set of 15 local WTs. Statistical analyses were performed with GraphPad Prism 8.0.2, using two-tailed Student's *t*-tests of equal variances. **p*<0.05 ***p*<0.01 ****p*<0.001.

2.3 Discussion

Here, we define biallelic *TRAPPC10* variants as a cause of a neurodevelopmental TRAPPopathy disorder, with core clinical features including microcephaly, severe global developmental delay and intellectual disability, short stature and pervasive behavioural abnormalities (**Table 2.1**). Additional variable features include hypotonia, gait abnormalities and seizures. The overlapping phenotypical nature of the TRAPPopathies suggests a common pathomolecular basis of disease involving disruption of TRAPP II/III complex functions. The clinical features of *TRAPPC10*-related disorder also show extensive overlap with many other Golgipathy disorders (Passemard et al., 2017; Rasika et al., 2018) indicative of a common disease mechanism relating to disrupted Golgi trafficking processes. We observed notable phenotypic overlap with *Trappc10*^{-/-} mice which display microcephaly, reduced size of white matter brain structures with hypomyelination and skeletal involvement. Brain abnormalities in mice appear to be more severe than those in humans, in which neuroimaging identified profound thinning of the corpus callosum but no other white matter abnormalities or hypomyelination. However as neuroimaging was only available on a single affected individual who appears to display the least severe phenotype of those assessed, it is possible that more extensive abnormalities may be present in other affected individuals who display a greater degree of microcephaly.

Our genetic studies in combination with our functional investigations on both *TRAPPC10*^{-/-} and patient-derived cells indicate that TRAPPC10 loss of function likely underlies the clinical phenotype. Previous subcellular trafficking studies of TRAPPC10 depleted COS-7 (Yamasaki et al., 2009) and HEK293 (Li et al., 2017) cells determined that TRAPPC10 mediates early Golgi (although not endoplasmic reticulum) trafficking, revealing disrupted Golgi architecture and vesicular accumulation indicative of an anterograde trafficking defect. Our cell model membrane

trafficking assays are in complete alignment with such a TRAPPC10 molecular role, defining delayed Golgi trafficking associated with gene KO which importantly could be rescued, though not completely, by WT, and to a lesser extent by mutant TRAPPC10. These findings may in part be explained by differing outcomes of the *TRAPPC10* variants, including the possibility that the protein C-terminus plays a specific though as yet unknown post-Golgi secretory pathway role. Interestingly, in previous studies the yeast homologue of TRAPPC10 (Trs130) was rendered conditionally lethal by short truncations in the C-terminal portion of the protein, indicative of a critical functional role of this region (Sacher et al., 2001). The observed trafficking defects herein may be explained by the loss of GEF activity of TRAPP^{II} towards Rab1 (Yamasaki et al., 2009), Rab11 (Jenkins et al., 2020) and/or Rab18 (Li et al., 2017), which alongside other Rab GTPases are key regulators of cellular trafficking by recruiting specific effector proteins to membranes, including lipid droplet homeostasis. Rab18 has specifically been implicated in secretory pathway regulation (Vazquez-Martinez et al., 2007), and *RAB18* variants are associated with a neurodevelopmental disorder phenotypically overlapping *TRAPPC10*-related disorder. Although the specific molecular role of TRAPPC10 in neurological development and function remains unclear, it is tempting to speculate that the predominantly neurological phenotype seen in individuals affected by *TRAPPC10*-related disorder may be due to the relatively high rates of membrane trafficking shown to be required in the brain for processes such as synapse remodeling (Ethell et al., 2000; Winkle and Gupton, 2016).

An important outcome of our findings was that absence of TRAPPC10 is also associated with a concomitant absence of TRAPPC9. Additionally, although other TRAPP^{III} and core specific components were detected, levels of core TRAPPC2L were reduced suggesting an absence of TRAPP^{II} complex, with no effect on TRAPP^{III} complex. These findings indicate that TRAPPC10

is crucial to TRAPPII complex stability and function. Consistent with our findings, careful examination of the data in (Li et al., 2017), though not commented on, reveals a decrease in the levels of TRAPPC9 protein in TRAPPC10^{-/-} HEK293 cells (see Figure6D in (Li et al., 2017)). However, our studies showed TRAPPC10 was still detectable in LCLs lacking TRAPPC9 p.(Arg475*), suggesting that TRAPPC9 may only incorporate into TRAPPII in the presence of TRAPPC10, while the reciprocal is not true. Our overexpression studies of mutant TRAPPC10 provide further insight into this, and in particular identify the expression of full length TRAPPC10 to be of importance for the detection of TRAPPC9 within the TRAPPII complex. Collectively, our results suggest that the absence of the TRAPPC10 C-terminus results in TRAPPC10 protein degradation, and consequent loss of TRAPPC9 (through protein degradation or some other mechanism), leading to the absence of TRAPP II.

The concomitant loss of TRAPPC9 associated with pathogenic *TRAPPC10* variants is reflected in the close phenotypical parity of human *TRAPPC9*- (OMIM 613192) and *TRAPPC10*-related conditions (**S2.1 Table**) and with both knockout mouse models. Individuals with *TRAPPC9*-related disorder have postnatal-onset microcephaly (Hnoounal et al., 2019; Mochida et al., 2009). While the families reported here did not have any recorded birth OFC measurements, there were no reports of affected children having noticeably small head size at birth suggesting microcephaly associated with *TRAPPC10*-related disorder may be postnatal. Head growth trajectory was only available for one child, with two measurements available (2.5 and 4.1 years), that indicate microcephaly is present from early childhood and is not progressive. However it remains unclear whether the brain growth was initially normal and then slowed, or whether microcephaly was present from/before birth. Identification of further affected families will help clarify the nature of

the microcephaly in *TRAPPC10*-related disorder, as with *TRAPPC9*-related disorder where this was also unclear initially (Mir et al., 2009b).

Biallelic *TRAPPC10* loss of function appears to have a more severe phenotypic impact in mice and humans, compared with biallelic *TRAPPC9* loss. In human *TRAPPC10*-related disorder, microcephaly appears to be a relatively consistent feature, (8/10 individuals), as compared to ~60% of *TRAPPC9* patients (Abbasi et al., 2017; Bodnar et al., 2020). Similarly in mouse models, while the age of onset and progression of microcephaly is unclear, *Trappc10*^{-/-} mice display more extensive brain size reduction and loss of white matter structures than *Trappc9*^{-/-} mice. These findings may be explained by the concomitant loss of *TRAPPC9* observed with *TRAPPC10* absence, possibly acting as a double knockout. Obesity is reported in ~50% of individuals with *TRAPPC9*-related disorder and is the only clinical feature not currently observed in individuals with *TRAPPC10*-related disorder. It is important to note that these differences are based on a relatively small sample size, a limitation of this study. Nevertheless, the *Trappc10*^{-/-} mice share similar phenotypic outcomes compared to individuals with biallelic *TRAPPC10* variants.

Neuroanatomical studies of *Trappc9*^{-/-} mice showed overlapping features with *Trappc10*^{-/-} mice including reduced brain size predominantly of white matter structures (Ke et al., 2020) (**S2.5 Fig**), although findings of enlarged striatal size were not identified in *Trappc10*^{-/-} mice. *TRAPPC9* plays a role in the NF-κB signalling pathway through its interaction with NF-κB inducing kinase (NIK) and the beta subunit of IKK, which both regulate the NF-κB pathway (Hu et al., 2005; Zhang et al., 2015). Given the associated loss of *TRAPPC9* observed with both the *TRAPPC10* variants and *TRAPPC10*^{-/-} cells, it is plausible that *TRAPPC10* loss of function may also impact NF-κB signalling pathways through disruption of *TRAPPC9*, although the involvement of *TRAPPC9* in this pathway in brain has been recently questioned (Ke et al., 2020). Taken together, we define the

genetic, clinical and molecular basis of a novel microcephalic neurodevelopmental disorder associated with biallelic *TRAPPC10* variants in both humans and mice. Our data provides a molecular rationale for the phenotypic overlap between *TRAPPC10*- and *TRAPPC9*-related TRAPPopathy disorders, both involving disruption of TRAPP II-mediated post-Golgi trafficking processes.

2.4 Material and methods

2.4.1 Ethics statement

This study was carried out in accordance with institutional ethics review board-approved research protocols from the Institutional Review Board, International Islamic University (IIU), Islamabad, Pakistan. All individuals (or their families) whose data is included in this study provided written informed consent and where applicable specific written consent for publication of photographs.

2.4.2 Genetic studies

Genomic DNA was extracted from blood/buccal samples using the ReliaPrep kit (Blood gDNA Miniprep System, Promega) and RNA was extracted using the PAXgene blood miRNA kit and converted to cDNA via reverse transcriptase PCR. Single nucleotide polymorphism (SNP) genotyping was performed using Illumina HumanCytoSNP-12 v2.1 beadchip array, as previously described (Rawlins et al., 2019). WES analysis was performed (NextSeq500; Illumina, San Diego, CA, USA) and involved Agilent Sureselect Whole Exome v6 (Agilent Technologies, Santa Clara, CA) targeting, read alignment (BWA-MEM v0.7.17), mate-pairs fixed and duplicates removed (Picard v2.15), InDel realignment and base quality recalibration (GATK v3.7.0), single nucleotide variant and InDel detection (GATK HaplotypeCaller), variant annotation (Alamut batch v1.10 and SnpEff (http://snpeff.sourceforge.net/SnpEff_manual.html))) and read depth assessment (GATK DepthOfCoverage). Copy number variants (CNVs) were detected using SavvyCNV (Laver et al., 2022). Variants with <5 reads and/or an allele frequency >0.01 in public genome databases including the Genome Aggregation Database (gnomAD v2.1.1 and v3.1) and the 1000 Genomes Project were excluded. Homozygous and compound heterozygous variants that were exonic and non-synonymous, synonymous with predicted splicing impact or intronic at ± 6 nucleotides from

splice site were evaluated and cross referenced against the SNP data. Dideoxy sequencing validation of *TRAPPC10* variants was undertaken using standard techniques.

2.4.3 Molecular studies

2.4.3.1 Construction of *TRAPPC10*^{-/-} cell lines

Guide RNA (gRNA) design and cloning (CRISPR plasmid pSPCas9(BB)-2A-Puro (PX459) V2.0, Addgene #62988) was performed as described previously (Ran et al., 2013), with primers: 5'-CACCGCATCTTCGGAGCCCGGCCAT-3' and 5'-AAACATGGCCGGGCTCCGAAGATGC-3'. HEK293 cells were transfected (70% confluency) using JetPRIME with 500ng DNA expressing the gRNA. Following selection (puromycin) and colony expansion, genomic DNA was extracted. The *TRAPPC10* gene region of interest was amplified using oligonucleotide pair: 5'-AGCGTAGTTATGATTTGGGGT-3 and 5'-GCCAAGGAATGAAGGGACAA-3. The resulting PCR product (~540bp, agarose gel electrophoresis) was sequenced. Knockout verification was performed by western analysis (S2.3 Fig).

2.4.3.2 Membrane trafficking assay

Cells were infected with virus expressing VSVG-GFP-tsO45 (one hour, 37°C). In rescue experiments, cells were transfected with either FLAG-TRAPPC10, FLAG-TRAPPC10 p.Gly1131Valfs*19 or FLAG-TRAPPC10 p.Pro929Leu constructs, then infected with VSVG-GFP tsO45 (one hour, 37°C). Cells were maintained at 40°C overnight. Cycloheximide was added (final concentration, 10µg/ml), prior to shifting cells to 32°C. Time-lapse microscopy started three minutes after temperature shift to allow time to select fluorescent cells (Nikon inverted confocal microscope) as previously described (Koehler et al., 2017). Movies used for quantitative fluorescence analysis were not subjected to processing. Integrated fluorescence intensity at the

Golgi region (defined by the region of perinuclear intensity seen 20–40 minutes after temperature shift) and from whole cell was measured using ImageJ (measurements obtained every 5 minutes). The ratio between fluorescent intensities within the Golgi region and whole cell was generated for each time point. The kinetics of VSVG-GFP-tsO45 trafficking represent change in that ratio over time (0 to 90 min).

2.4.3.3 Cell lysis and size exclusion chromatography

Cells were grown in either DMEM (for HEK293) or RPMI 1640 (for lymphoblastoid cells) medium containing 10% fetal bovine serum (FBS). Cells were lysed in a solution containing 50mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% Triton X-100 and protease inhibitor cocktail (EDTA-free; Roche). A total of 2–5 mg of protein was fractionated on a Superose 6 Increase 10/300 GL column at a flow rate of 0.4 ml/min. Fractions of 0.5 ml were collected in wash buffer (50 mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% Triton X-100) and probed with the indicated antibodies.

2.4.3.4 Yeast two hybrid assay

Open reading frames (ORFs) encoding TRAPP proteins were cloned (pGADT7 and pGBKT7 vectors; Clontech). Plasmids were transformed into yeast cells (AH109, Y187). Diploids containing the respective TRAPP ORFs were produced by mating and selecting on synthetic complete medium (SC) lacking leucine and tryptophan. Interactions were assessed on SC medium lacking leucine, tryptophan, histidine and adenine. Serial cell dilutions were spotted onto solid medium. Quantification of interaction was assessed using a β -galactosidase assay employing ONPG and normalized to total protein.

2.4.3.5 Molecular biology techniques and reagents

Standard molecular biological techniques were used to generate FLAG-tagged constructs and the stated *TRAPPC10* variants. Commercially available antibodies used in this study were: anti-TRAPPC2 (Rabbit polyclonal, homemade), anti-TRAPPC2L (mouse monoclonal, Santa Cruz sc-377322), anti-TRAPPC3 (Rabbit polyclonal, homemade), anti-TRAPPC8 (Rabbit polyclonal, Abcam ab122692), anti-TRAPPC9 (Rabbit polyclonal, LS Bio LS-C750497), anti-TRAPPC10 (mouse monoclonal, Santa Cruz sc-101259), anti-TRAPPC12 (Rabbit polyclonal, homemade), anti-FLAG (mouse monoclonal, Sigma F1804), anti- α -Tubulin (mouse monoclonal, Sigma T6199).

2.4.4 *Trappc10* knockout mouse model

The mouse model was generated by homologous recombination in embryonic stem cells using the Knockout-first allele method (Skarnes et al., 2011), adopting a strategy identifying an exon common to all transcripts (exon 14), upstream of which a LacZ cassette was inserted (S2.6 Fig). Exon 14 of the *Trappc10* allele, flanked by *loxP* sequences bilaterally, was deleted using a Cre recombinase that recognizes *loxP* sites, producing the *Trappc10*^{*tm*mb(EUCOMM)Wtsi} knockout allele. Mice were phenotyped by the Mouse Genetics Project (MGP) pipeline at the Wellcome Sanger Institute, UK (S2.5.1 Text).

2.4.5 Mouse neuroanatomical studies

The use of mice in the Wellcome Sanger Institute study was carried out in accordance with UK Home Office regulations (license number 80/2076), UK Animals (Scientific Procedures) Act of 1986. Neuroanatomical studies were carried out using three homozygous *Trappc10*^{-/-} and 498 baseline WT mice on a C57BL/6N pure genetic background at 16-weeks of age as previously

described (Collins et al., 2019; Mikhaleva et al., 2016). We used a recognized statistical model (Gpower) validated for comparison of modest numbers ($n = 3$) of animals to evaluate neuroanatomical defects with an effect size of 10% or more with 80% detection power (Collins et al., 2019). Paraffin embedded brain samples were cut at 5 μ m thickness (sliding microtome, Leica RM 2145) to obtain coronal brain region at Bregma +0.98 mm and Bregma -1.34 mm according to the Allen Mouse Brain Atlas (Sunkin et al., 2013). Sections were stained with 0.1% Luxol Fast Blue (Solvent Blue 38; Sigma-Aldrich) and 0.1% Cresyl violet acetate (Sigma-Aldrich) and scanned (Nanozoomer 2.0HT, C9600 series) at 20 $\times$ resolution. Sixty-three brain parameters made up of area and length measurements as well as cell level features, were taken across the two coronal sections (S2.5.1 Text). Co-variables, for example sample processing dates and usernames, were collected at every step of the procedure and used to identify data drifts. Using in-house ImageJ plugins, an image analysis pipeline was used to standardize measurements of areas and lengths. Images were quality controlled for the accuracy of sectioning relative to the reference atlas and controlled for asymmetries and histological artefacts. All samples were also systematically assessed for cellular ectopia (misplaced neurons). Data were analyzed using a linear mixed model framework to determine whether a brain region is associated with neuroanatomical defect or not.

2.5 Supporting information

2.5.1 Supplementary Methods

2.5.1.1 Mouse Studies

Animal care was as previously described by (White et al., 2013). Mice were fed a standard chow diet using the autoclavable mouse breeder diet 5021 (www.labdiet.com). After weaning, animals were housed three to four mice per cage with WT controls housed separately, in a specific pathogen-free environment in individually ventilated cages under 12/12 light/dark cycle with

temperature-controlled conditions and free access to food and water with hardwood bedding. All animals were regularly monitored for health and welfare concerns and were additionally checked prior to and after procedures. Mice of both genders were weighed between 4 and 16 weeks of age.

2.5.1.2 Mouse neuroanatomical studies

Mice were anaesthetized with Ketamine (100 mg/kg, intraperitoneally) and Xylazine (10 mg/kg, intraperitoneal), blood collected via the retro-orbital route and death confirmed before the brains were dissected and fixed in 4% buffered formalin for 48 hours, then transferred to 70% ethanol. Samples were embedded in paraffin using an automated embedding machine (Sakura Tissue-Tek VIP).

Sixty-three brain parameters made up of area and length measurements as well as cell level features, were taken across the two coronal section regions at Bregma +0.98 mm and Bregma -1.34 mm. Co-variates, for example sample processing dates and usernames, were collected at every step of the procedure and used to identify data drifts. Using in-house ImageJ plugins, an image analysis pipeline was used to standardize measurements of areas and lengths. Each image was quality controlled for the accuracy of sectioning relative to the reference atlas and controlled for asymmetries and histological artefacts. At Bregma +0.98mm brain structures assessed were: 1) the total brain area, 2) the lateral ventricles, 3) the cingulate cortex, 4) the genu of the corpus callosum, 5) the caudate putamen, 6) the anterior commissure, 7) the piriform cortex, 8) the primary motor cortex and 9) the secondary somatosensory cortex. At Bregma -1.34mm, a maximum of 14 brain structures were assessed: 1) the total brain area, 2) the lateral and third ventricles, 3) the retrosplenial granular cortex, 4) the corpus callosum, 5) the amygdala, 6) the piriform cortex, 7) the internal capsule, 8) the optic tract, 9) the mammillothalamic tract, 10) the fimbria of the

hippocampus, 11) the habenular nucleus, 12) the hippocampus, 13) the primary motor cortex and 14) the secondary somatosensory cortex. All samples were also systematically assessed for cellular ectopia (misplaced neurons). For the neuroanatomical assessment of *Trappc9*^{-/-} mice, the brains were cut into halves along the sagittal midline, and embedded separately in paraffin. The section of interest was determined as corresponding to Figure 106 of the Mouse Brain Atlas (Lateral +0.60 mm) (Collins et al., 2018). Brains were sectioned to match the defined section at a thickness of 5µm. The staining, scanning, and quality control steps were identical to the coronal procedure used for the neuroanatomical assessment of *Trappc10*^{-/-} mice. A total of 40 brain morphological parameters including 25 areas, 14 lengths and one number, were measured for each brain. These parameters encompassed 22 distinct brain regions.

2.5.1.3 Mouse body composition and clinical blood chemistry tests

Non-fasted mice at 16 weeks of age were terminally anaesthetised with ketamine/xylazine and blood was collected into lithium/heparin coated tubes via the retro-orbital sinus. Plasma was analysed for the following 27 parameters on an Olympus AU400: 1) for electrolytes: sodium, potassium and chloride; 2) for non-fasted metabolic panel: glucose, fructosamine, triglycerides, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), non-esterified free fatty acids (NEFAC) and glycerol; 3) for thyroid: total thyroxine and adiponectin; 4) pancreatic enzyme: amylase; 5) liver/muscle: alanine aminotransferase, alkaline phosphatase, creatine kinase, aspartate aminotransferase and total bilirubin; 6) protein parameters: total protein and albumin; 7) kidney: creatinine and urea; and 8) minerals and iron: calcium, magnesium, iron and phosphate. For body composition, 14 week mice were imaged on a dual energy X-ray absorptiometry machine (Lunar PIXImus II). This generated an image of the entire mouse and provided bone mineral, body composition and morphometric data.

2.5.2 Supplementary Material

S2.1 Table: Clinical features associated with TRAPPopathy disorders.

TRAPP subunit	Inheritance	OMIM number and clinical features	References
TRAPPC2	XLR	Spondyloepiphyseal dysplasia tarda (OMIM #313400) Short stature, vertebral flattening, kyphosis/scoliosis, small capital femoral epiphyses, mild epiphyseal irregularities	(Fiedler et al., 2004b; Gedeon et al., 2001)
TRAPPC2L	AR	Encephalopathy, progressive, early-onset, with episodic rhabdomyolysis (OMIM #618331) Developmental delay, absent speech, microcephaly, epilepsy, regression, rhabdomyolysis, raised CK, tetraplegia, dystonia, cerebral visual impairment	(Al-deri et al., 2020; Milev et al., 2018)
TRAPPC4	AR	Neurodevelopmental disorder with epilepsy, spasticity, and brain atrophy (OMIM #618741) Early-onset seizures, developmental delay, microcephaly, sensorineural deafness, spastic quadriparesis, progressive cortical and cerebellar atrophy, scoliosis, facial dysmorphism	(Van Bergen et al., 2020)
TRAPPC6A	AR	Intellectual disability, speech delay, facial dysmorphism, polydactyly	(Mohamoud et al., 2018) Single family described
TRAPPC6B	AR	Neurodevelopmental disorder with microcephaly, epilepsy, and brain atrophy (OMIM #617862) Microcephaly, intellectual disability, epilepsy, ataxic gait, cortical atrophy, autistic features, generalised weakness, thin corpus callosum	(Marin-Valencia et al., 2018)
TRAPPC9	AR	Mental retardation, autosomal recessive 13 (OMIM #613192) Microcephaly, intellectual disability, reduced white matter volume, thin corpus callosum, absent speech, hypotonia, dysmorphic features, hyperkinesia, epilepsy, raised BMI	(Abbasi et al., 2017; Mbimba et al., 2018; Mir et al., 2009b; Mortreux et al., 2018)
TRAPPC10	AR	Microcephaly, intellectual disability, developmental delay, speech delay, hypotonia, gait abnormalities, epilepsy, autistic features, facial dysmorphism, short stature, stRabismus, thin corpus callosum, reduced white matter structures	<i>This study</i>
TRAPPC11	AR	Muscular dystrophy, limb-girdle, autosomal recessive 18 (OMIM #615356) Epilepsy, myopathy, microcephaly, developmental delay, ataxia, cerebral atrophy, raised CK, scoliosis, cataracts, hepatomegaly, steatohepatitis, hypotonia, gait abnormalities, short stature, dystonia, spasticity, generalised weakness, achalasia, alacrima	(Bögershausen et al., 2013; Koehler et al., 2017; Larson et al., 2018; Liang et al., 2015; Milev et al., 2019)

TRAPPC12	AR	Encephalopathy, progressive, early-onset, with brain atrophy and spasticity (OMIM #617669) Hypotonia, microcephaly, epilepsy, global developmental delay, brain atrophy, agenesis of the corpus callosum, pons hypoplasia, spasticity, myoclonic jerks, neurogenic bladder	(Milev et al., 2017)
TRAPPC14	AR	?Microcephaly 25, primary, autosomal recessive (OMIM #618351) Short stature, microcephaly, global developmental delay, impaired intellectual development, speech delay, ADHD, decreased white matter and thin corpus callosum	(Perez et al., 2019) Single family described

Abbreviations: TRAPP; transport protein particles. XLR; X-linked recessive, AR; autosomal recessive, BMI; body mass index, CK; creatine kinase.

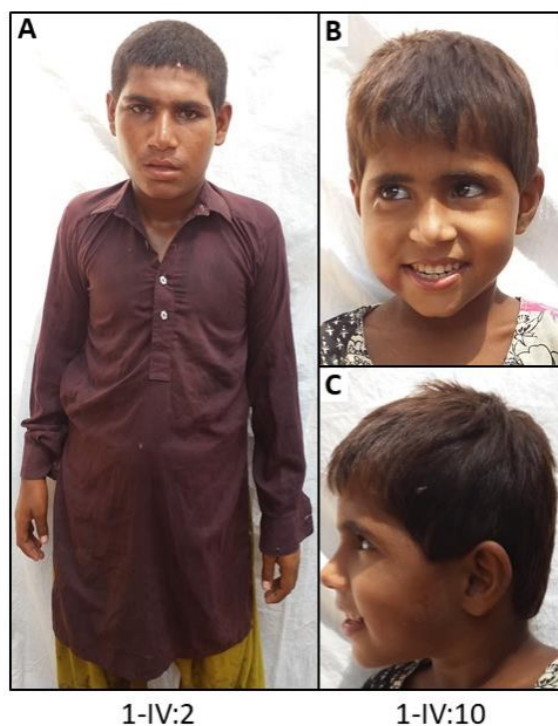


Figure S2.1 Clinical features of individuals with biallelic *TRAPPC10* variants

(A) Individual 1-IV:2 (Family 1) with no evidence of skeletal disproportion. (A-C) Show the facial features of two affected individuals from Family 1 displaying mild craniofacial dysmorphism comprising microcephaly, synophrys and upslanting palpebral fissures

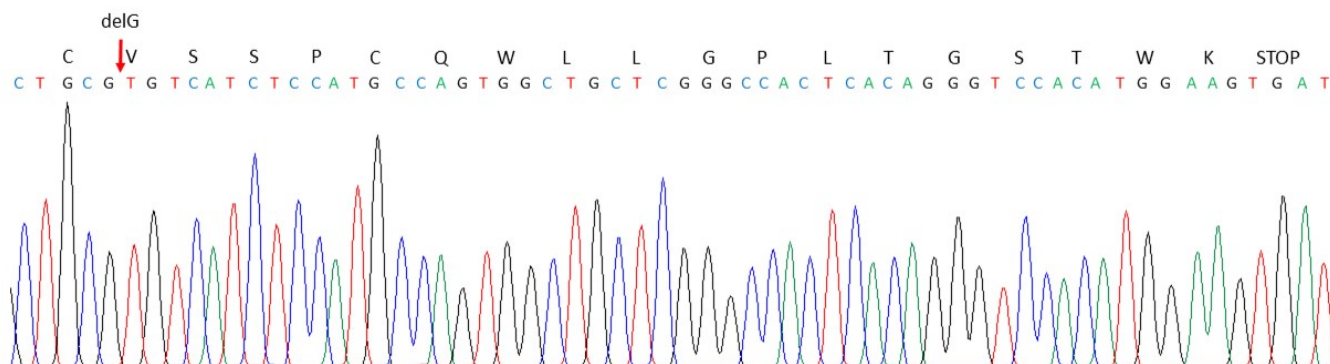


Figure S2.2 Chromatogram of *TRAPPC10* c.3392del rtPCR product

Confirmation of the outcome of the c.3392del; p.(Gly1131Valfs*19) *TRAPPC10* gene variant on the RNA transcript in blood of an affected individual (individual IV:2, Family 1), demonstrating that this frameshift variant results in a premature stop codon 19 codons downstream

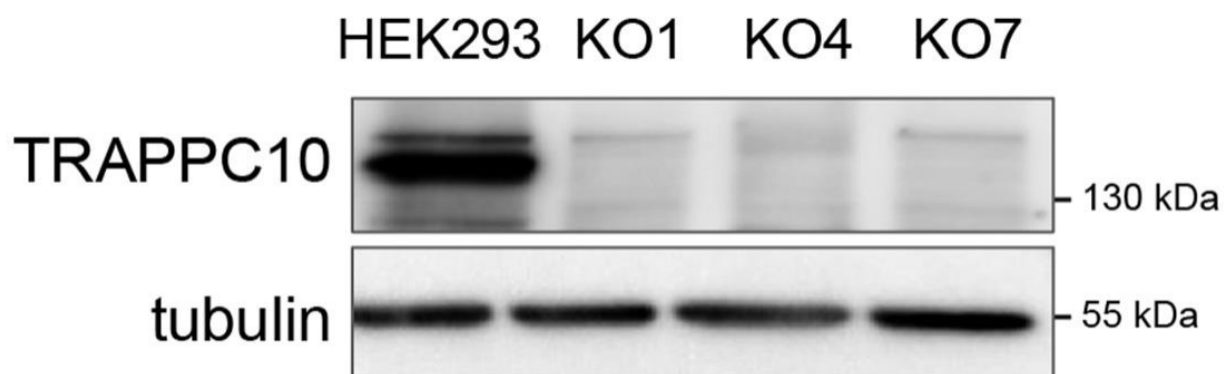


Figure S2.3 Characterization of the *TRAPPC10*^{-/-} cell lines in HEK293 cells

Lysate was prepared from parental HEK293 cells and from 3 clones (KO1, KO3 and KO7) that were treated with sgRNA targeting *TRAPPC10*. The lysates were fractionated by SDS-PAGE and probed for TRAPPC10. Tubulin was included as a loading control.

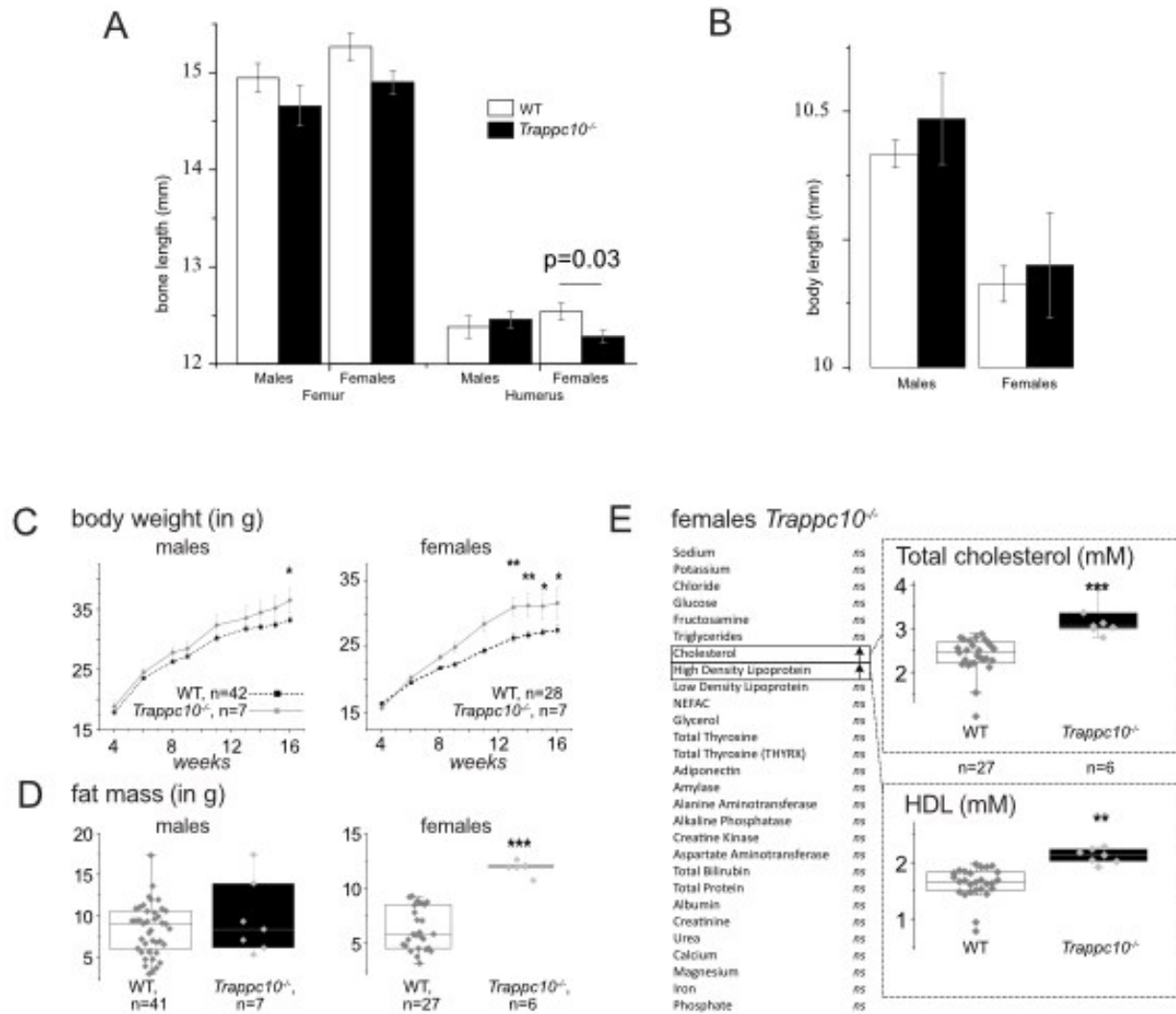


Figure S2.4 Whole-body phenotype analysis of *Trappc10^{-/-}* knockout mouse

(A) Bone length for long bones (Femur and Humerus) in both sexes ($n = 7$ for all groups) (B) Body length for male *Trappc10^{-/-}* ($n = 7$) and WT ($n = 41$) and females *Trappc10^{-/-}* ($n = 7$) and WT ($n = 27$). (C) Body weight curves in grams of male and female *Trappc10^{-/-}* mice, between 4 and 16 weeks of age. (D) Fat body composition in grams of male and female *Trappc10^{-/-}* mice at 16 weeks of age. (E) *Left*: List of 27 assessed clinical blood chemistry parameters and association in female *Trappc10^{-/-}* mice at 16 weeks of age. *Right*: Box plots with raw data points showing results for levels of total cholesterol and high-density lipoprotein (HDL) in millimoles per liter in female *Trappc10^{-/-}* mice at 16 weeks of age. Statistical analyses were performed with GraphPad Prism 8.0.2, using two-tailed Student's *t*-tests of equal variances. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. Arrows indicate directionality of effect and "ns" indicates not significant (p -value > 0.05).

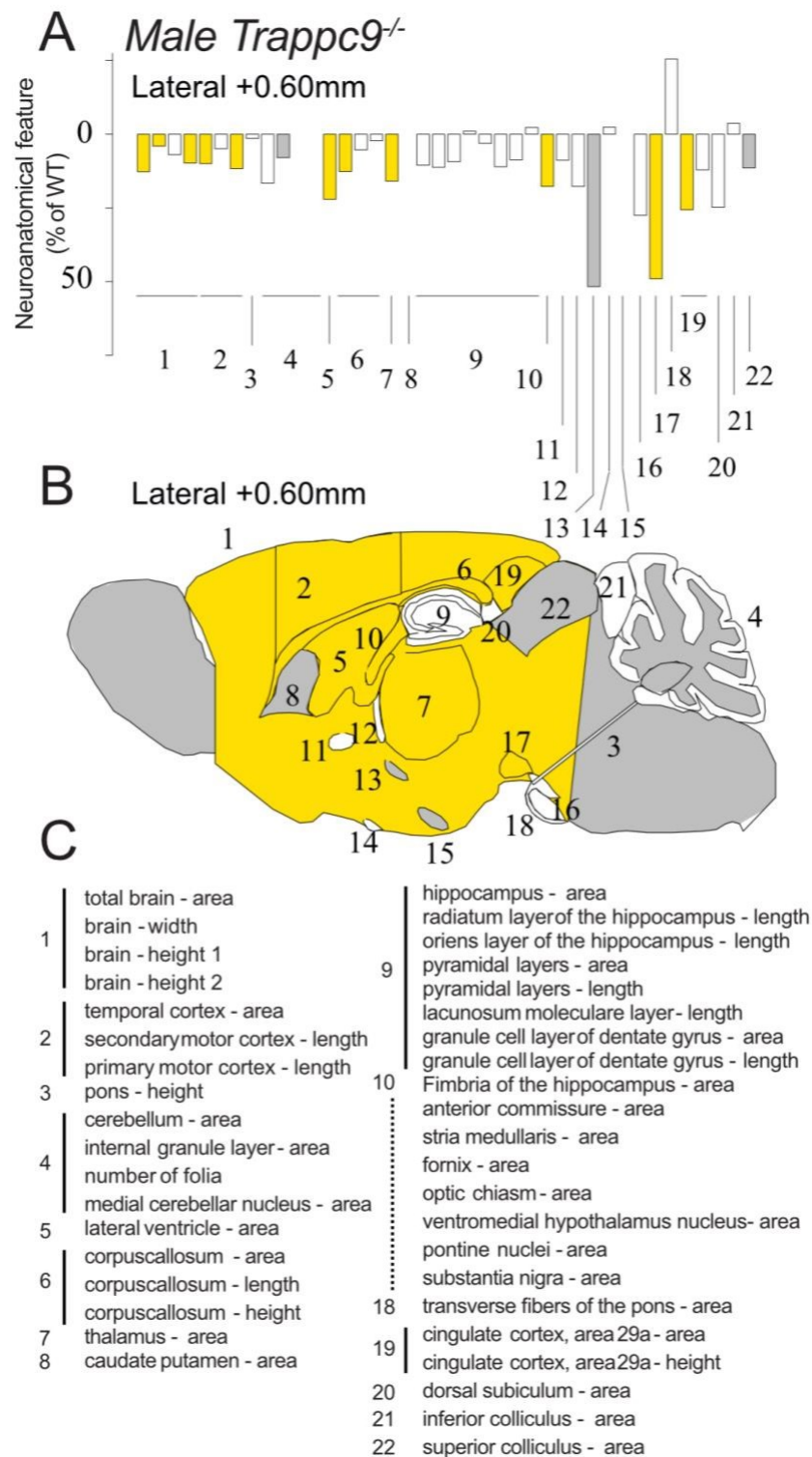


Figure S2.5 Neuroanatomical assessment of *Trappc9* knockout mouse

(A) Histograms of percentage change relative to *Trappc9^{+/+}* (set as 0) for each of the measured parameters. (B) Schematic representation of the 22 brain regions quantified at lateral +0.60 mm on sagittal section from *Trappc9^{+/+}* (n = 4) and *Trappc9^{-/-}* (n = 4) mice. Colored regions indicate the presence of at least one significant parameter within the brain region at the 0.05 level. White

indicates a p-value > 0.05, grey shows not enough data to calculate a p-value. (C) List of assessed brain parameters.



Figure S2.6 Allelic construction showing targeting of critical exon 14 in the *Trappc1*^{tm1b(EUCOMM)Wtsi} mouse.

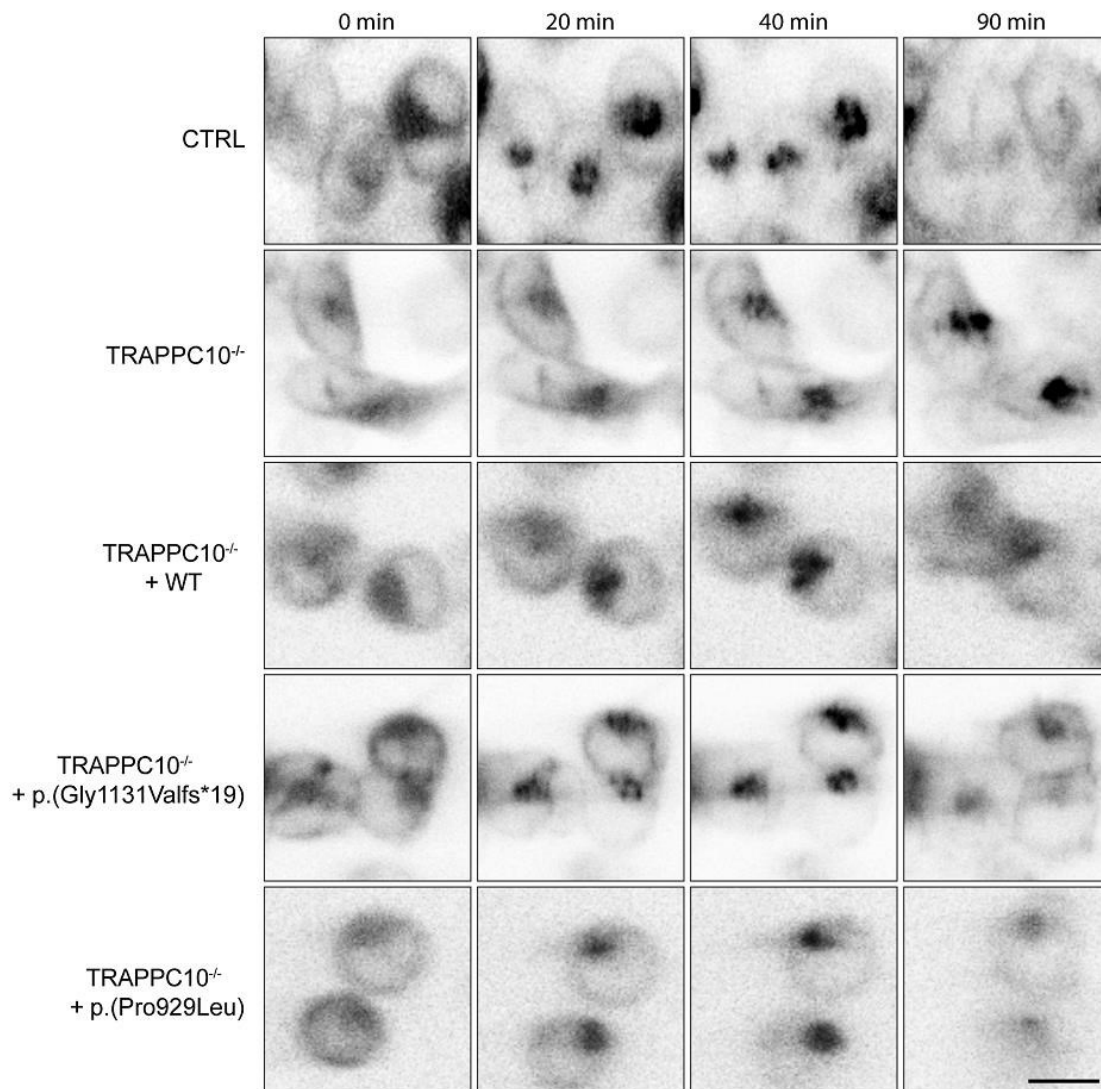


Figure S2.7 Representative images for VSV-G trafficking at 0, 20, 40 and 90 minutes

Wild type HEK293 cells or *TRAPPC10*^{-/-} cells either not transfected or transfected with FLAG-tagged wild type TRAPPC10 or one of the *TRAPPC10* variants indicated were infected with VSVG-GFP ts045 4 hours after transfection. After an overnight incubation at 40°C, the cells were shifted to 32°C and imaged every minute. Representative images at 0, 20, 40 and 90 minutes are shown.

Acknowledgments:

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Chapter 3: TRAPPC6B biallelic variants cause a neurodevelopmental disorder with TRAPPII and trafficking disruptions

This Chapter was submitted as a manuscript to *Brain* (Almoussa et al., 2023). I shared first authorship of this paper. I generated the results in Figure 3.3, Figure 3.4 and Figure 3.5. The clinical analyses were done by our collaborators.

3.1 Abstract

Highly conserved transport protein particle (TRAPP) complexes regulate subcellular trafficking pathways. Accurate protein trafficking has been increasingly recognized to be critically important for normal development, particularly in the nervous system. Variants in most TRAPP complex subunits have been found to lead to neurodevelopmental disorders with diverse but overlapping phenotypes. We expand on limited prior reports on *TRAPPC6B* with detailed clinical and neuroradiologic assessments, and studies on mechanisms of disease, and new types of variants.

We describe 29 additional patients from 18 independent families with biallelic variants in *TRAPPC6B*. We identified 7 homozygous nonsense (n=12 patients) and 8 canonical splice-site variants (n=15 patients). In addition, we identified one patient with compound heterozygous splice-site/missense variants with a milder phenotype and one patient with homozygous missense variants.

Patients displayed non-progressive microcephaly, global developmental delay/intellectual disability, epilepsy, and absent expressive language. Movement disorders including stereotypies, spasticity, and dystonia were also observed. Brain imaging revealed reductions in cortex,

cerebellum, and corpus callosum size with frequent white matter hyperintensity. Volumetric measurements indicated globally diminished volume rather than specific regional losses.

We identified a reduced rate of trafficking into the Golgi apparatus and Golgi fragmentation in patient-derived fibroblasts that was rescued by wild type TRAPPC6B. Molecular studies revealed a weakened interaction between mutant TRAPPC6B (c.454C>T, p.Q152*) and its TRAPP binding partner TRAPPC3. Patient-derived fibroblasts from the TRAPPC6B (c.454C>T, p.Q152*) variant displayed reduced levels of TRAPPC6B as well as other TRAPP II complex-specific members (TRAPPC9 and TRAPPC10). Interestingly, the levels of the TRAPPC6B homologue TRAPPC6A were found to be elevated. Moreover, co-immunoprecipitation experiments showed that TRAPPC6A co-precipitates equally with TRAPP II and TRAPP III, while TRAPPC6B co-precipitates significantly more with TRAPP II, suggesting enrichment of the protein in the TRAPP II complex. This implies that variants in *TRAPPC6B* may preferentially affect TRAPP II functions compared to TRAPP III functions. Finally, we assessed phenotypes in a *Drosophila TRAPPC6B*-deficiency model. Neuronal TRAPPC6B knockdown impaired locomotion and led to wing posture defects, supporting a role for TRAPPC6B in neuromotor function.

Our findings confirm the association of damaging biallelic *TRAPPC6B* variants with microcephaly, intellectual disability, language impairments, and epilepsy. A subset of patients also exhibited dystonia and/or spasticity with impaired ambulation. These features overlap with disorders arising from pathogenic variants in other TRAPP subunits, particularly components of the TRAPP II complex. These findings suggest that TRAPPC6B is essential for brain development and function, and TRAPP II complex activity may be particularly relevant for mediating this function.

3.2 Introduction

Trafficking protein particle (TRAPP) complexes regulate vesicle trafficking via guanine nucleotide exchange factor (GEF) activity of Rab GTPases (Chin et al., 2009) and localization to compartments such as the Golgi apparatus (Riedel et al., 2018). Two TRAPP complexes have been identified in mammals called TRAPP^{II} and TRAPP^{III}. While both complexes activate Rab1 in vitro (Riedel et al., 2018), TRAPP^{II} additionally activates Rab11 (Jenkins et al., 2020; Riedel et al., 2018), a GTPase that also functions in ciliogenesis (Knödler et al., 2010). TRAPP^{II} proteins have also been implicated in ciliogenesis (Westlake et al., 2011). On the other hand, TRAPP^{III} has been implicated in early secretory pathway traffic and autophagy (Scrivens et al., 2011; Stanga et al., 2019). Knockdowns and mutations in both TRAPP II- and III-specific proteins have been shown to affect trafficking into and through the Golgi as well as affecting Golgi morphology (Sacher et al., 2019a). TRAPP-mediated trafficking is crucial for brain development and function, as mutations in multiple TRAPP subunits lead to overlapping neurodevelopmental disorders, with movement disorders, intellectual disability (ID), epilepsy, and neuromuscular features (**Table 3.1**) (Sacher et al., 2019a).

Both TRAPP complexes are built upon a common catalytic core of proteins that include TRAPPC1, TRAPPC2, TRAPPC2L, TRAPPC3, TRAPPC4, TRAPPC5 and TRAPPC6. For the TRAPP^{II} complex, TRAPPC9 and TRAPPC10 are linked to the core while the TRAPP^{III} complex contains the core with TRAPPC8, TRAPPC11 and TRAPPC12 (Bassik et al., 2013; Scrivens et al., 2011, 2009). Two genes encoding the core TRAPPC6 protein have been reported in humans called *TRAPPC6A* and *TRAPPC6B* (Kümmel et al., 2006). *D.melanogaster* and *S.cerevisiae* have a single gene encoding this subunit, *Trs33*. It has been assumed that TRAPPC6A and TRAPPC6B could

incorporate into either TRAPPII or TRAPPIII complex, as both proteins are capable of forming a heterodimer in complex with TRAPPC3 *in vitro* (Kümmel et al., 2008, 2006; Tokarev et al., 2009).

A homozygous nonsense variant in *TRAPPC6B* was recently described in an Iranian family in association with intellectual disability (Harripaul et al., 2018) and a homozygous splice-site founder mutation was found in three Egyptian families in association with microcephaly, intellectual disability, autism, epilepsy and reduced brain volume (Marin-Valencia et al., 2018). A fifth family was then reported (Nair et al., 2019). In this report, we describe 29 individuals from 18 unrelated families with biallelic mutations in *TRAPPC6B* manifesting microcephaly, epilepsy, and intellectual disability with phenotypic expansion to include spasticity and dystonia. We examine variant impact on TRAPPC6B and TRAPPII- versus TRAPPIII-specific protein levels, anterograde protein trafficking, and Golgi apparatus morphology in patient fibroblasts. Finally, we tested whether *TRAPPC6B* can regulate neuromotor function using *Drosophila* locomotor and wing posture assays. Together, the findings expand the *TRAPPC6B*-associated clinical and neuroradiologic phenotype and demonstrate patient variants disrupt TRAPPC6B and TRAPPII protein levels, trafficking, and Golgi morphology. We further find that TRAPPC6B preferentially associates with TRAPPII complex members, which support the observation that TRAPPC6B clinical phenotypes more closely align with TRAPPII complex disorders.

Table 3.1: TRAPP genes with neurological disease connections.

Gene (OMIM#/*) PMID Source	Complex	Disease	Cognitive findings	Epilepsy	Movement disorder	White matter	Brain volume	Language	Dysmorphic features	Visual system	Muscle findings
<i>TRAPPC6B</i> (#617862) this study	II, III	NEDMEBA	ID	Variable	Spasticity, dystonia, stereotypies	Thin corpus callosum, hyperintensity	Brain volume loss, microcephaly	Absent expressive language	Variable	No impairments	Reduced bulk
<i>TRAPPC2L</i> (#618331) (Al-deri et al., 2020; Milev et al., 2018)	II, III	PEERB	ID	Focal seizures and status epilepticus with infection	Tetraplegia; dystonia	Normal-delayed myelination	Variable atrophy	Absent speech	NR	Cerebral visual impairment	Rhabdomyolysis; Nonspecific biopsy
<i>TRAPPC4</i> (#618741) (Ghosh et al., 2021; Saad et al., 2020; Van Bergen et al., 2020)	II, III	NEDESBA	Normal-severe ID; regression	Yes	Spasticity; axial hypotonia; variable dyskinesia	White matter loss	Mild-severe cerebral atrophy; secondary microcephaly	Delay and loss of expressive language	Yes	Visual impairments; variable cataracts	Amyotrophy
<i>TRAPPC6A</i> (#610396) (Mohamoud et al., 2018)	II, III	NDD	ID	NR	NR	NR	NR	Speech delay	Yes	NR	NR
<i>TRAPPC9</i> (#613192) (Bodnar et al., 2020; Kakar et al., 2012)	II	MR T13	Moderate-severe ID	Variable seizures	Hypotonia, stereotypies	Thin corpus callosum, reduced white matter	Postnatal microcephaly	Speech disorder	Yes	NR	NR
<i>TRAPPC10</i> (#602103) (Rawlins et al., 2022; Santos-Cortez et al., 2018)	II	NDD	Severe ID	Variable seizures	Hypotonia, waddling gait	1 patient thin corpus callosum	Microcephaly	Poor speech	NR	Low frequency strabismus	NR
<i>TRAPPC11</i> (#615356) (Wang et al., 2018)	III	LGMD	Normal-severe ID	Variable (generalized seizures)	Some with ataxia and/or choreiform movements	Some reduced volume	Normal-microcephaly; some cerebral atrophy	NR	NR	Variable (amblyopia, cataract)	Limb girdle muscular dystrophy
<i>TRAPPC12</i> (#617669) (Milev et al., 2017)	III	PEBAS	Severe DD and regression	Seizures; myoclonus	Spasticity; truncal hypotonia; dystonia	Corpus callosum agenesis; hyperintensity	Microcephaly; pons hypoplasia; diffuse atrophy	NR	NR	Optic atrophy; cortical visual impairment	NR

phenotype MIM number, * gene/locus MIM number. ID=intellectual disability; LGMD= limb girdle muscular dystrophy; MRT13=mental retardation, autosomal recessive 13; NDD=neurodevelopmental disorder; NEDESBA= Neurodevelopmental disorder with epilepsy,

spasticity, and brain atrophy; NEDMEBA= neurodevelopmental disorder with microcephaly, epilepsy, and brain atrophy; NR =not reported; PEBAS=progressive encephalopathy with brain atrophy and spasticity; PEERB progressive encephalopathy with episodic rhabdomyolysis.

3.3 Methods

3.3.1 Patient recruitment, sequencing and variant calling

All human subject studies were performed in accordance with the ethical standards of the responsible committee on human experimentation according to institutional and national standards. Proper informed consent was obtained for all participants. Clinical phenotypes (Supplementary Clinical Summaries) were abstracted from written records, supplemented by review of available neuroimaging, facial photographs, and laboratory and clinical electrophysiologic data. Patient videos were reviewed by pediatric movement disorder neurologists (J.H. and M.C.K.). Phenograms were created using R (4.1.0). Patients with data not available for a category were not included in that category's calculations. TRAPP disease gene summary was created from OMIM.org, accessed August 2022. Sequencing details and filtering criteria used for each case are provided in the Supplementary Methods.

3.3.2 MRI analysis, processing and volumetric quantification

Neuroimaging findings were reviewed and summarized by a board-certified neuroradiologist (P.C.). Several image processing steps were then performed on the T1-weighted brain MRIs (Pagnozzi et al., 2017), including registration to the Colin 27 Average Brain Atlas, correcting image bias using the N4 algorithm, followed by intensity normalisation and image de-noising, using anisotropic diffusion. Skull stripping was performed using an in-house algorithm developed in Python. In this approach, intradural CSF was identified using thresholding and morphological operations, following which the lateral ventricles were isolated based on their spatial location, allowing the volume of the lateral ventricles (in mL) to be extracted. Cerebral brain tissues (grey matter, white matter) were then isolated based on their MR intensities using the Expectation Maximisation (EM)/Markov Random Field (MRF) approach. From the cortical grey matter

segmentation, three measures of cortical shape were measured (cortical thickness, curvature and sulcal depth) to quantify shape abnormalities. Measures were converted to a z-score from healthy cortical shape measures measured from the corresponding cortical region compared to the Child Mind Institute Healthy Brain Network cohort of 564 typically developing children (TDC) (Equation 1), based on cortical regions from the Automated Anatomical Labelling (AAL) atlas.

$$(Eqn\ 1) \quad z - score_{subject} = \frac{(x_{subject} - \mu_{TDC})}{\sigma_{TDC}}$$

3.3.3 MRI statistical analysis

Six participants passed the quality checks for initial MRI data quality and processed segmentations (Families 3,4,6,10). For each participant, z-scores of grey matter volume, white matter volume, ventricle asymmetry (Equation 2), were extracted.

$$(Eqn\ 2) \quad Ventricle\ asymmetry = \frac{(vol_{left} - vol_{right})}{(vol_{left} + vol_{right})}$$

3.3.4 Yeast Two Hybrid Assay

TRAPP open reading frames were cloned into pGADT7 and pGBKT7 plasmids (Clontech, USA). Standard yeast methods were used for transformation of the pGADT7 and pGBKT7 constructs into AH109 and Y187 yeast strains, respectively. Diploid cells were produced by mating on solid YPD media and then selected for on solid synthetic complete (SC) media lacking leucine and tryptophan (DDO). Single colonies of each diploid were then cultured in liquid DDO and then spotted on either

DDO or on SC media lacking leucine, tryptophan and histidine (TDO) to assess for interactions. Plates were grown at 30°C for 72 hours.

3.3.5 Membrane Trafficking Assay

The retention using selective hooks (RUSH) assay was performed as described in (Boncompain et al., 2012). Briefly, fibroblasts grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum were transfected by electroporation with the Golgi-resident enzyme sialyl transferase-GFP (ST-GFP) fused to streptavidin binding protein. The plasmid also expressed KDEL-tagged streptavidin for endoplasmic reticulum (ER) retention. For the rescue experiments, fibroblasts were co-transfected with ST-GFP and TRAPPC6B-RFP. 24 hours after transfection, biotin was added to a final concentration of 60 μ M to release the reporter from the ER hook. Live cells were imaged by fluorescence microscopy every 2 minutes for 60 minutes using a Nikon Livescan sweptfield confocal microscope with a 40X objective lens (NA 0.95). Integrated fluorescence intensity at the Golgi region and from whole cell was measured every 2 minutes using ImageJ v1.53. The ratio between fluorescent intensities within the Golgi and whole cell was generated for each time point. The first time point corresponding to background was subtracted from all time points. These values were then plotted as the mean percentage of maximal intensity.

3.3.6 Golgi Morphology

Fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were washed 2 times with phosphate buffered saline solution (PBS) then fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, quenched with 0.1 M glycine for 10 minutes and permeabilized with 0.1% Triton X-100 for 7 minutes. Blocking was performed in 5%

normal goat serum in PBS for 45 minutes at room temperature. Primary antibodies were prepared in 5% normal goat serum and were added to the cells and incubated overnight at 4°C. Cells were washed 2 times for 5 minutes each with PBS. Secondary antibodies were diluted in 5% normal goat serum in PBS and incubated with the cells at room temperature for one hour and then removed. Hoechst 33342 (Thermo Fisher Scientific) was diluted in PBS (1:2000) and added to stain the nucleus for 2 minutes followed by 2 washes with PBS wash for 10 minutes each. The coverslips were then mounted with Prolong Gold Anti Fade. Images were acquired on an Olympus FV10i confocal laser scanning microscope fitted with a 60X objective, NA 1.35. The number of Golgi fragments per cell were quantified using Imaris software c.9.8.0 (Bitplane, Concord, MA). Golgi structures were identified from the mannosidase II channel using the following surface parameters; surface details = 0.22 μ m and thresholding with a background subtraction of the depicted Golgi spherical structures that have diameter smaller than 0.55 μ m in size. Statistical analyses were carried out using one-way ANOVA corrected for Tukey's multiple comparisons using GraphPad Prism 6.01. A p-value of 0.05 was considered to be statistically significant.

3.3.7 Immunoblotting

Fibroblasts were washed twice with PBS and lysed in a solution containing 50mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% Triton X-100 and protease inhibitor cocktail (EDTA-free; Roche). The lysate was clarified at 13000 $\times$ g for 30 minutes at 4°C. A total of 30-40 μ g of whole cell lysate was loaded and fractionated in either 8% or 15% SDS-polyacrylamide gels (SDS-PAGE). The gels were transferred to nitrocellulose membranes and blocked with 5% skim milk powder in PBS with 0.1% Tween (PBS-T) for 1 hour. Primary antibodies were incubated in PBS-T overnight at 4°C, washed 3 times with PBS-T for 5 minutes each, and secondary antibodies were incubated for 1 hour at room temperature. Membranes were then washed 3 times with PBS-

T for 5 minutes each and incubated with Pierce ECL western blot substrate (Thermo Fisher Scientific) and detected using an Amersham Imager 600 (GE Healthcare).

3.3.8 Immunoprecipitation

HeLa cells were seeded in 10cm dishes 24 hours prior to co-transfecting cells with 5 μ g of DNA expressing either TRAPPC6A-V5/TRAPPC10-FLAG, TRAPPC6A-V5/TRAPPC11-FLAG, TRAPPC6B-V5/TRAPPC10-FLAG, or TRAPPC6B-V5/TRAPPC11-FLAG using Jet Prime (Polyplus). 48 hours after transfection cells were washed twice with ice cold PBS and lysed in a solution containing 50mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% Triton X-100 and protease inhibitor cocktail (EDTA-free; Roche). The lysate was clarified at 13000 $\times$ g for 30 minutes at 4°C. A total of 500 μ g of lysate was incubated with 20 μ l (10 μ l bead volume) anti-Flag M2 affinity beads (Sigma Aldrich) in IP-buffer (50mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100 and protease inhibitor cocktail) for 3 hours at 4°C on an orbital shaker. The beads were collected by centrifugation at 5000 rpm for 30 seconds at 4°C and washed 2 times with IP-buffer and 2 times with IP-buffer without Triton X-100. The beads were resuspended in 20 μ L of 2 $\times$ Laemmli sample buffer containing β -mercaptoethanol and heated at 95°C for 5 minutes to dissociate the immune complexes. The beads were pelleted by centrifugation and SDS-PAGE for protein from input (whole-cell extracts) and immunoprecipitated proteins was performed as described above.

3.3.9 Molecular Biology Techniques

Standard molecular biological techniques were used to generate FLAG-tagged, RFP-tagged and V5 tagged constructs. TRAPPC6B variants were generated by site-directed mutagenesis.

3.3.10 Antibodies

Antibodies used in this study were: anti-TRAPPC2L (1:1000 mouse monoclonal, Santa Cruz sc-377322), anti-TRAPPC3 (1:1000 Rabbit polyclonal, homemade), anti-TRAPPC6A (1:500 mouse monoclonal, Santa Cruz sc-376032), anti-TRAPPC6B (1:1000 Rabbit polyclonal, ABclonal A15561), anti-TRAPPC8 (1:1000 Rabbit polyclonal, Abcam ab122692), anti-TRAPPC9 (1:2000 Rabbit polyclonal, LS Bio LS-C750497), anti-TRAPPC10 (1:500 mouse monoclonal, Santa Cruz sc-101259), anti-TRAPPC12 (1:1000 Rabbit polyclonal, homemade), anti-FLAG (1:5000 mouse monoclonal, Sigma F1804), anti- α -Tubulin (1:5000 mouse monoclonal, Sigma T6199), anti-RFP (1:500 mouse monoclonal, Rockland 200-301-379S), anti-mannosidase II (1:200 kind gift from Dr Kelley Moreman), anti V5 (1:1000 Rabbit monoclonal, Cell Signaling 13202).

3.3.11 Fly rearing and genetics

Drosophila were reared on a standard cornmeal, yeast, sucrose food from the BIO5 media facility, University of Arizona. Stocks for experiments were reared at 25°C, 60-80% relative humidity with 12:12 light/dark cycle. Crosses for controls and mutants and animals selected for locomotor assay were maintained at an elevated temperature of 28.5°C. Fly stocks were obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537). Crosses were performed with *w¹¹¹⁸* for heterozygous studies of genetic controls. P[TRiP.HMJ21139]attP40 was used for expression of UAS-Trs33-RNAi (Ke et al., 2018). Pan-neuronal Gal-4 driver (ELAV, CG4262) was used to direct RNAi expression to post-mitotic neurons during development and throughout adulthood.

3.3.12 Locomotor assays

Naïve, unmated flies collected as pharate adults and controlled for humidity, temperature, and time of day (30% RH, 24°C, 0900-1100) were used. Flies at 14 days post-eclosion were adapted to room

conditions for 1 hour before assaying in groups of 3-20 in coded vials by blinded experimenters (Kim et al., 2016). Distance was determined from still images from video at 3 seconds post-tapping using ImageJ measured distance function from the middle of the fly to the bottom of the vial and averaged between flies for each trial. Graphs and t-test statistic calculations were performed in R (4.1.0). For box and whisker plots, box indicates 75th and 25th percentile with median line; whiskers indicate 10th and 90th percentile.

3.3.13 Erect wing scoring

Each video was manually assessed for flies with erect wing phenotype defined as wings held upright and away from the body (Fernandes and Rao, 2011). The wing must have been held erect during the majority of the video, and upright wing postures limited to before flight or during grooming were excluded. Statistics were determined both as a 3-way and pairwise against both control genotypes using Chi-squared analysis in R (4.1.0).

3.4 Results

Through Genematcher, we identified 18 families with biallelic mutations in *TRAPPC6B* from international collaborators. In our cohort, 27/29 individuals segregated loss of function (LoF) (nonsense and splice-site) homozygous variants, with one individual carrying compound heterozygous splice-site/missense variants and one individual carrying homozygous missense variants. The M:F ratio was 1.6:1 and average age of diagnosis was 7.5 years (range 14 months to 35 years). Affected families were from 11 countries (Iran, Morocco, Pakistan, Tunisia, France, Mali, Gambia, Egypt, Saudi Arabia, Turkey, India) and frequently included multiple affected individuals (**Supplementary Figure. 3.1**). Homozygous variants including 7 homozygous nonsense (n= 12 individuals), 8 canonical splice-site mutations (n=15 individuals), and 1 missense mutation (n=1 individuals) were identified using whole exome sequencing (WES). Additional sequencing information is provided in Supplementary Methods.

We conducted detailed clinical and neuroradiologic assessments. Clinical features are summarized in **Table 3.2** and **Figure. 3.1A** with full descriptions in the Supplementary Clinical Summaries. We noted a constellation of motor features that were not mentioned in a prior report of individuals with *TRAPPC6B* variants (Marin-Valencia et al., 2018). Consistent with prior reports, we found complex neurodevelopmental features including intellectual disability, expressive language defects, autism, seizures or epilepsy, and neurobehavioral features such as aggression and self-injury in some patients. Many patients also exhibited difficulty with walking and some also had issues sitting. Stereotyped movements were evident in most individuals, with dystonia, spasticity, and reduced muscle bulk at lower frequency. Microcephaly was a notable feature in this cohort, with some patients as severe as <6.5 SD. This microcephaly does not appear to be progressive based on serial clinical assessments. Dysmorphic features include a variety of inconsistent facial

features, tapered fingers, and arachnodactyly (**Figure. 3.1B-N**). MRI was assessed for 16 individuals (**Figure. 3.2**) and identified reductions in cortex and cerebellum with increased ventricle size. Foreshortening or thinning of corpus callosum and white matter increased intensity or volume loss were consistent imaging features. Volumetric measures of MRI compared to age-matched large populations (Pagnozzi et al., 2017) did not identify a specific region driving the observed microcephaly (**Figure. 3.2N**). Of note, the individuals with missense variants (patients 7 and 29) exhibited similar phenotypes, suggesting that missense variants could potentially impair protein function and disrupt nervous system development.

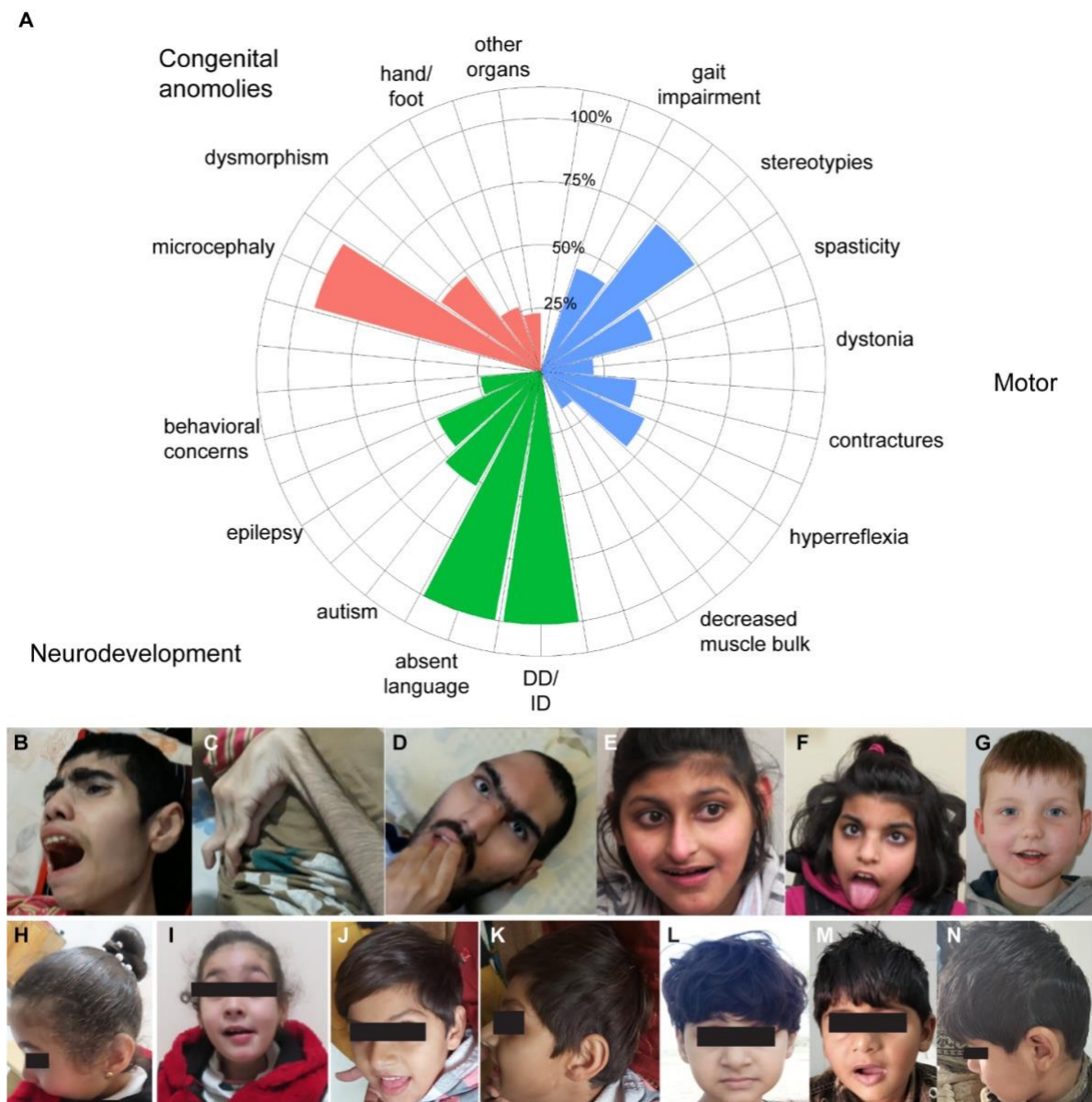


Figure 3.1: Clinical features of patients with TRAPPC6B variants

A: Phenogram of frequency of phenotypic features for 29 new patients and 8 previously reported patients (**Supplementary Table 1**). ID/DD=intellectual disability/developmental disability). Fields with missing data were not included in calculations for that feature. **B-N:** Photos of dysmorphic features. **B-C:** Patient 1 (Family 1) has gingival hypertrophy and swan neck deformity of fingers, representing dystonia. Patient also exhibited spastic-dystonic quadriplegia with contractures in the elbow flexors and plantar flexors, temporal wasting, muscle atrophy, and curling of all her toes (not shown). **C:** Patient 2 (Family 1) has synophrys, bitemporal narrowing, prominent cheekbones, a wide nasal root and bridge, and a papular lesion suspicious for an occult encephalocele. Patient also exhibited spastic-dystonic quadriplegia, brachycephaly, trigonocephaly, temporal wasting and muscle atrophy in his proximal limb muscle (not shown). **E:** Patient 5 (Family 4) has microcephaly, bitemporal narrowing, low anterior hairline, deep-set eyes, prominent ears, long nose with narrow nasal bridge, prognathia. **F:** Patient 6 (Family 4) has microcephaly, bitemporal narrowing, strabismus, positional plagiocephaly, deep set eyes, broad nasal root and narrow nasal bridge, and widely spaced teeth. **G:** Patient 7 (Family 5) showing depressed nasal bridge, upturned nasal tip, and thin upper lip. Patient also exhibited bilateral clinodactyly, tapered fingers, and inverted nipples (not shown). **H-I:** Patient 10 (Family 8) has microcephaly, long face, arched eyebrows, wide spaced eyes, almond shaped eye, straight nasal bridge, broad nose, broad chin, and low set ears. Patient also exhibited arachnodactyly (not shown). **J-K:** Patient 17 (Family 11) has narrow nasal bridge, posteriorly rotated ears. **L:** Patient 18 (Family 11) has upslanting palpebral fissures, square nasal tip. **M-N:** Patient 19 (Family 12) has microcephaly, bulbous nasal tip, and creased earlobe.

Table 3.2: Clinical features of TRAPPC6B patients.

Family	Patient	Walk	Sit	GMFCS	Verbal	Seiz.	Spast.	Contractures	Dystonia	Knee-jerk reflex	Muscle loss	Stereotypies	Microcephaly	Facial feat.	Hand or foot	Other organ	ID/GDD	ASD	Behavioral
1	1	++	++	5	+++	+	+	+	+++	-	+	+	Yes	+	0	0	+	0	0
	2	++	++	5	+++	+	+	+	+	0	+	+	Yes	+	0	0	+	0	0
2	3	++	+	5	+++	+	++	+	0	+	N/A	+	Yes, prog.	+	+	0	+	N/A	0
3	4	+	0	N/A	+++	+	++	+	++	N/A	N/A	+	-3 SD 35y	+	+	0	+	N/A	+
4	5	+	0	4	+	++	0	0	0	+	+	+	-6.5 SD 15y	+	+	0	+	N/A	0
	6	++	+	5	+	0	0	+	0	+	+	+	-6 SD 11y	+	+	+	+	N/A	0
5	7	0	0	3	+++	0	0	0	0	0	N/A	+	No, -1.3 SD 3.5y	+	0	+	+	0	+
6	8	0	0	2	++	0	0	0	0	0	N/A	+	-2.5 SD 5y	+	+	+	+	0	0
7	9	0	0	N/A	+	0	+	0	0	0	+	+	-2 SD birth	+	0	0	+	N/A	0
8	10	+	0	N/A	+	+	Yes	0	0	+	N/A	+	-2 SD birth	+	+	0	+	+	+

9	11	0	0	N/A	++	0	0	0	0	0	N/A	+	-2 SD 4y4m	0	0	+	+	+	+
	12	0	0	N/A	N/A	0	0	0	0	0	N/A	N/A	-2 SD	0	0	0	+	+	+
10	13	0	0	N/A	+++	++	0	0	0	N/A	N/A	N/A	-5.2 SD 11y	+	0	0	+	+	0
	14	0	0	N/A	+	0	0	0	0	N/A	N/A	N/A	-5.7 SD 6y	0	0	0	+	+	0
	15	++	0	N/A	+++	0	0	0	0	N/A	N/A	N/A	-2.4 SD 14m	0	0	0	+	+	0
	16	0	0	N/A	+++	0	0	0	0	N/A	N/A	N/A	N/A	0	0	0	+	0	0
11	17	++	0	N/A	++	0	Yes	+	0	0	N/A	+	N/A	0	0	0	+	N/A	0
	18	0	0	N/A	+	0	0	+	0	0	N/A	+	Yes	0	0	0	+	N/A	0
12	19	0	0	N/A	+	++	Yes	0	0	0	N/A	N/A	Yes	0	0	0	+	+	0
	20	+	0	N/A	++	0	0	0	0	0	N/A	0	Yes	0	0	0	+	N/A	0
13	21	++	0	4	N/A	++	++	N/A	0	N/A	N/A	+	-2 SD birth	+	+	0	+	+	0
14	22	0	0	N/A	N/A	++	Yes	+	0	+	N/A	+	-4.3 SD 5y	0	0	0	+	+	0
15	23	0	0	N/A	+	0	0	0	0	+	N/A	+	No -1.4 SD	0	0	0	+	+	+
	24	0	0	N/A	+	0	0	0	0	+	N/A	0	No -0.5 SD	0	0	+	+	0	0
16	25	0	0	N/A	+	0	+	+	+	+	0	+	-4.8 SD	+	0	+	+	+	+
	26	0	0	N/A	+	0	+	+	+	+	0	+	-4.8 SD	+	0	+	+	+	+
17	27	0	0	N/A	+	++	+	N/A	0	N/A	0	0	-2.4SD 30y	0	0	0	+	0	0
	28	0	0	N/A	+	++	+	N/A	0	N/A	0	0	-2.8 SD 30y	0	0	0	+	0	0
18	29	+	0	4	+++	++	+	0	0	+	N/A	0	Yes, 46 cm	0	0	0	+	0	0

Hand or foot features include syndactyly, tapered, thin, or hypoplastic digits, and arachnodactyly affecting either the hands and/or feet. Other organs include cardiac, genito-urinary, liver, and eye. Behavioral concerns include self-injury, hyperactivity, and aggression. Abbreviations: CC=corpus callosum; WMI=white matter hyperintensity. Symbols describing features: + defects present; 0=no abnormalities; N/A data not available. Walking (0=walks unassisted, 1 = walks assisted, 2=cannot walk) Sitting (0=sits without support, 1=sit with support, 2=cannot sit); Verbal (0=normal speech, 1=single words, 2=babble or vocalize, 3=no speech); Epilepsy (0=no, +=some seizures, ++=epilepsy; Spasticity (0=none, +=lower only,++=upper+lower) Dystonia (0=no, +=upper, ++=lower, +++=both); Knee-jerk reflex (-=absent, 0=normal, +=exaggerated/brisk) Muscle loss (0= none, +=atrophy or reduced bulk with or without hypotonia) Autism (0=no, +=yes. N/A may also refer to inability to assess due to ID severity)

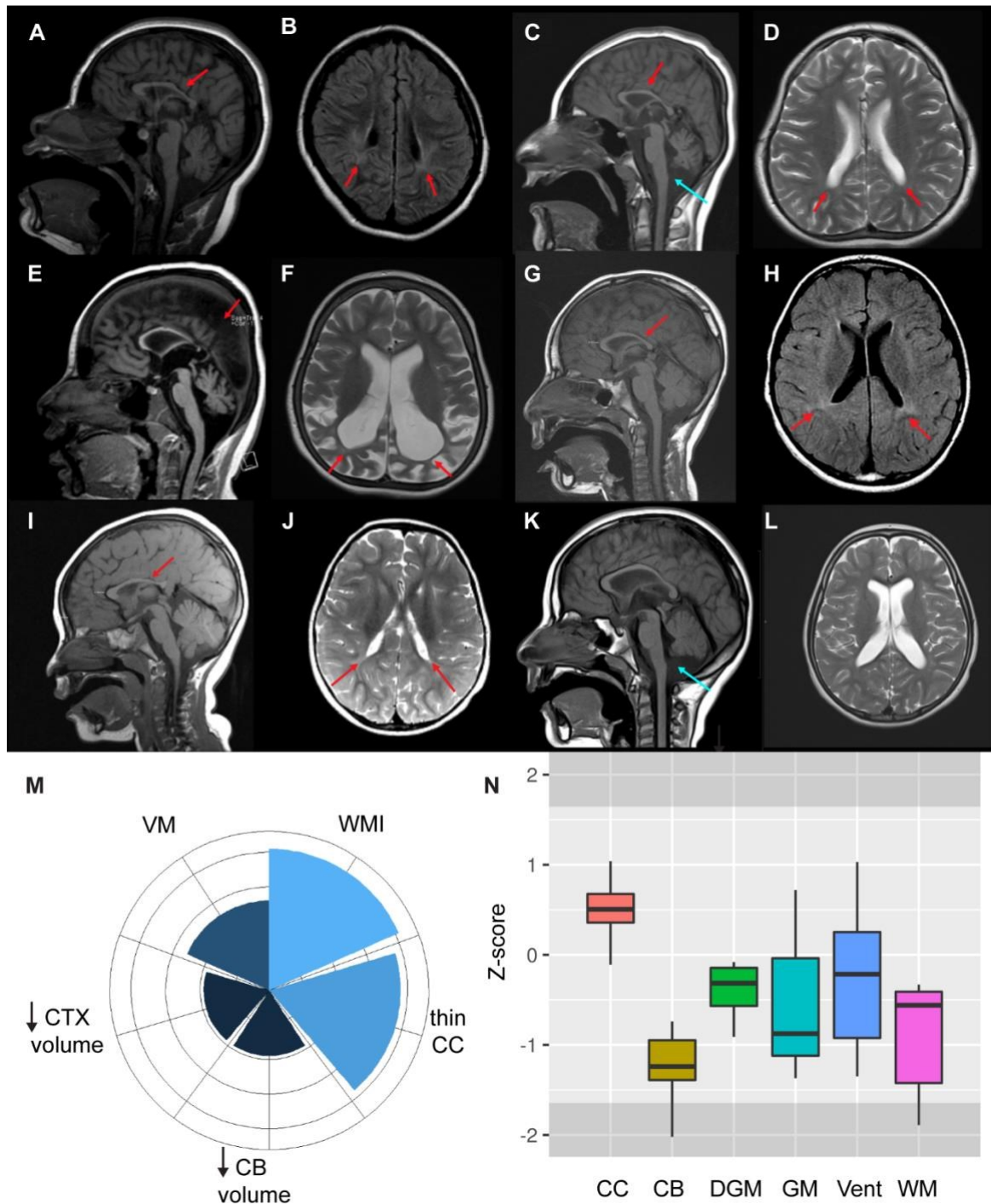


Figure 3.2 Qualitative and quantitative analysis of the MRI images

A, C, E, G, I, K: Sagittal MRI T1 weighted midline images. **B, H:** Axial FLAIR images. **D, F, J, L:** Axial MRI T2 weighted images. **A-B:** Patient 3, Family 2: 13-year-old female. **A.** Small craniofacial ratio, thinning/foreshortening of the corpus callosum with greater involvement of the posterior fibers (red arrow). **B:** Patchy FLAIR signal hyperintensity in the bilateral periventricular white matter (arrows) extending to the centrum semiovale, peri Rolandic and peri atrial regions (not shown). **C-D:** Patient 5, Family 4: 13-year-old female. **C.** Small craniofacial ratio, diffuse thinning/foreshortening of the corpus callosum (red arrow), and brainstem and cerebellum volume loss (cyan arrow). **D:** Abnormal angulation of the posterior margins of the ventricles (arrows). **E-**

F: Patient 6, Family 4: 9-year-old female. **E.** Small craniofacial ratio, thinning/foreshortening of the corpus callosum and posterior greater than anterior parenchymal loss (arrow). **F:** Diffuse parenchymal loss with preferential involvement of the posterior lobes, ex-vacuo dilatation and angulation of the ventricles (arrows). **G-H.** Patient 13, Family 10: 6-year-old male. **G.** Reduced FOD (frontal occipital diameter) diffuse thinning and foreshortening of the corpus callosum (arrow). **H.** Patchy FLAIR signal hyperintensity in peri atrial white matter associated with abnormal square shape of the posterior margins of the lateral ventricles (arrows). **I-J.** Patient 14, Family 10: 2-year-old female. **I.** Reduced FOD, diffuse thinning and foreshortening of the corpus callosum (arrow). **J.** Increased T2 signal in peri atrial white matter associated with abnormal angulation of the posterior margins of the lateral ventricles (arrows). **K-L.** Patient 16, Family 10: 5-year-old female. **K.** Marginal FOD (red arrow) and inferior vermis hypoplasia (cyan arrow). **L.** No signal abnormalities present. **M.** Phenogram of MRI features for 16 new patients and 7 previously reported patients with MRI interpretation. VM=ventriculomegaly. WMI=white matter hyperintensity or abnormalities. CC=corpus callosum. CB=cerebellum. CTX=cortex. **N.** Boxplot of six structural measures quantified from brain MRI volumes of 6 patients, represented as Z scores, in comparison to an age-matched control cohort of typically developing children. A mean -1 SD z-score in cerebellum represents volume loss in that region; there was no consistent reduction in other measurements. CC=corpus callosum, CB=cerebellum, DGM=deep grey matter, GM=grey matter, Vent=ventricle asymmetry, WM=white matter.

3.4.1 TRAPPC6B p.Q152* impairs TRAPPC3 binding and diminishes protein expression for TRAPPC6B and TRAPPII complex members

Given that our previous work suggested that the carboxy-terminus of TRAPPC6A interacts with adaptor subunit TRAPPC2L and both TRAPPC6A and TRAPPC6B interact with TRAPPC2L and TRAPPC3 (Al-Deri et al., 2021), we examined interactions between TRAPPC6B and both TRAPPC2L and TRAPPC3. While an interaction between both TRAPPC2L and TRAPPC3 was seen for the wild type TRAPPC6B irrespective of the vectors used, only the TRAPPC3 interaction was affected by the p.Q152* allele from affected individuals in family 15 (**Figure. 3.3A and 3.3B**). This result suggests that the carboxy-terminus of TRAPPC6B is important for its interaction with TRAPPC3 and that this interaction is diminished in TRAPPC6B p.Q152*. Since the TRAPPC3-TRAPPC6B heterodimer is important for the assembly of the TRAPP complexes, it is not unreasonable to expect that TRAPPC6B variants will decrease the presence of subunits that rely on this heterodimer to associate with TRAPP complexes. Indeed, in p.Q152* fibroblasts, several

TRAPP protein levels were decreased, with the strongest effect seen for the two TRAPP II-specific proteins TRAPPC10 and TRAPPC9 (**Figure. 3.3C**). There was a near complete absence of detectable amounts of TRAPPC6B, suggesting potential nonsense mediated decay or truncated protein instability (**Figure. 3.3C**). It is noteworthy that we consistently saw an increase in the levels of TRAPPC6A in the fibroblasts from the affected individuals, perhaps representing an attempted compensatory response.

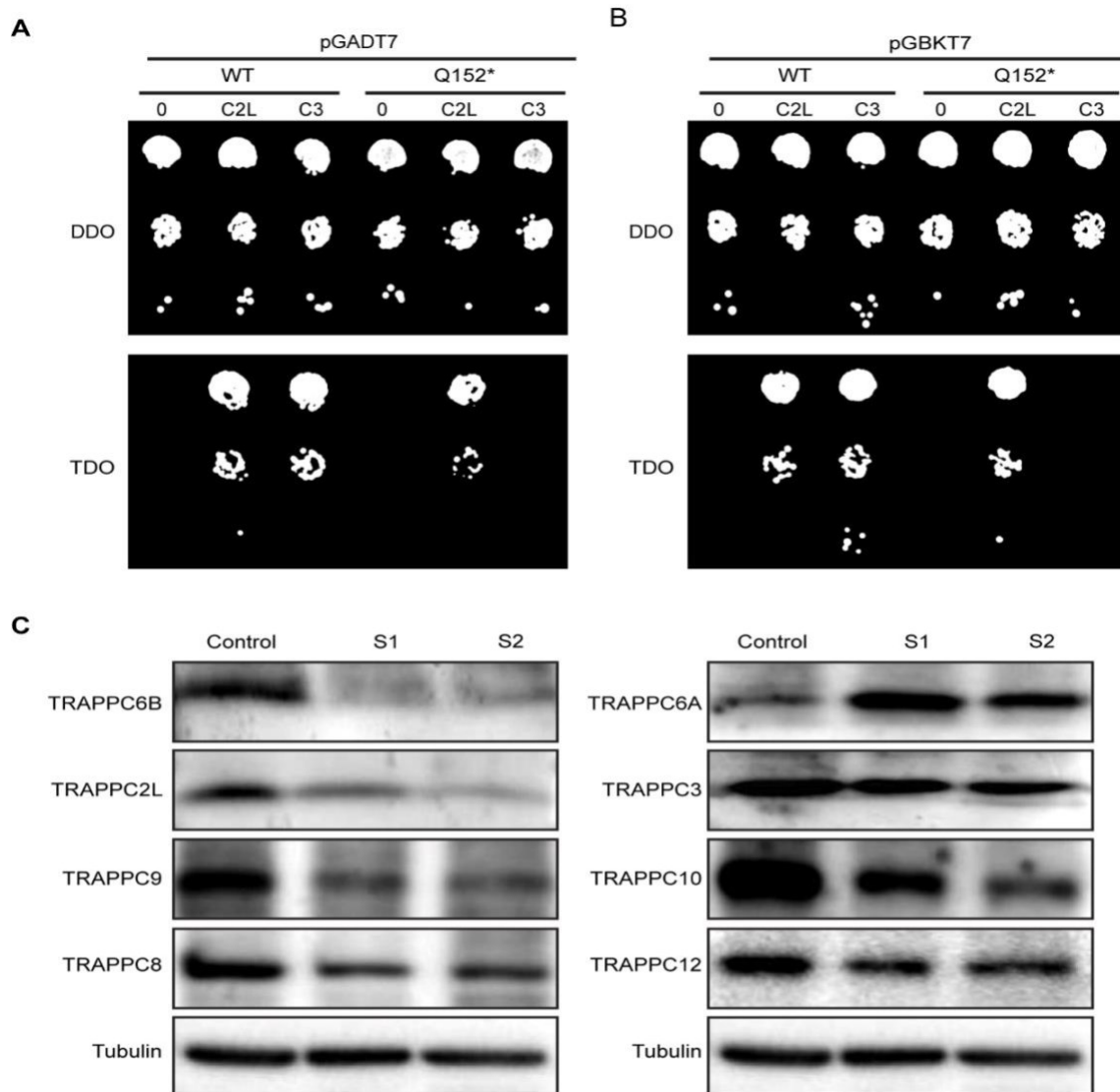


Figure 3.3. TRAPPC6B variants affect the stability of TRAPP II

TRAPPC6B (wild type or the p.Q152* variant), were cloned into either pGADT7 (**A**) or pGBKT7 (**B**), TRAPPC2L and TRAPPC3 were cloned into either pGBKT7 (**A**) or pGADT7 (**B**) and

transformed into haploid yeast cells. In some cases, an empty vector was used (indicated as ϕ). The cells were mated, diploids selected and then spotted as serial dilutions on plates lacking leucine and tryptophan (DDO) or plates lacking leucine, tryptophan and histidine (TDO). C. Fibroblasts from control or affected individuals from family 15 (p.Q152*) were lysed and probed for the indicated TRAPP proteins or for tubulin as a loading control.

3.4.2 TRAPP^{II} complex members associate preferentially with TRAPPC6B

We next examined whether TRAPPC6B was preferentially associated with TRAPP^{II} complexes. To perform this experiment, HeLa cells were mock transfected or co-transfected with TRAPPC6A-V5/TRAPPC10-FLAG, TRAPPC6A-V5/TRAPPC11-FLAG, TRAPPC6B-V5/TRAPPC10-FLAG, or TRAPPC6B-V5/TRAPPC11 and associated proteins were collected on beads harboring anti-FLAG IgG and probed for V5 and FLAG. TRAPPC11 transfections target TRAPP^{III} while the TRAPPC10 transfections target TRAPP^{II}.

Unlike TRAPPC6A, which equally co-precipitated with TRAPPC10 and TRAPPC11, TRAPPC6B was significantly more enriched with TRAPPC10 compared to TRAPPC11 (**Figure. 3.4A and 3.4B**). These results suggest that TRAPP^{II} complexes are enriched in TRAPPC6B compared to TRAPP^{III}.

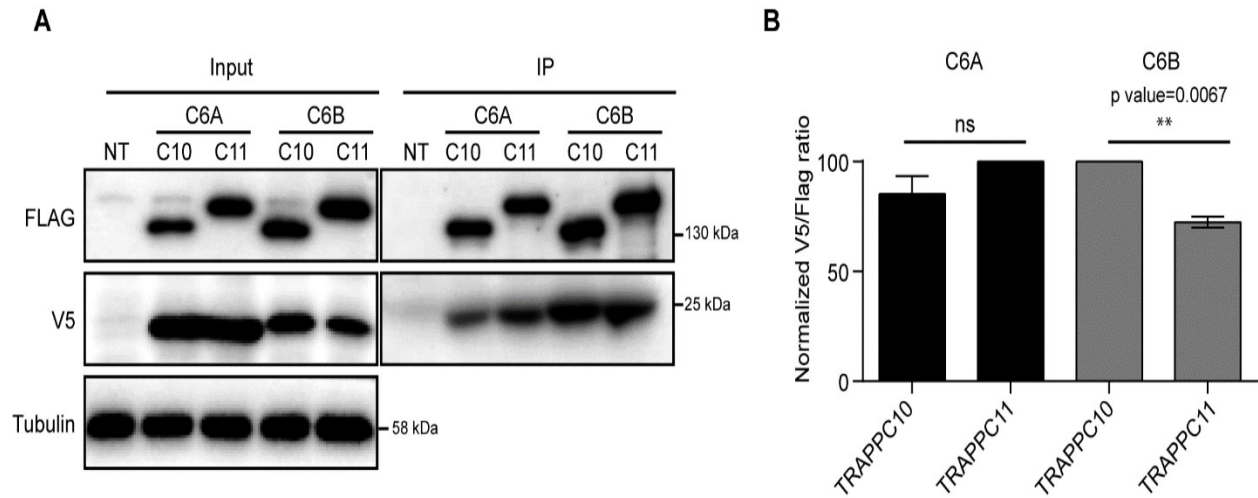


Figure 3.4. TRAPPC6B Preferentially Associates with the TRAPPII Complex

A. HeLa cells were either untransfected or co-transfected with TRAPPC6A-V5/TRAPPC10-FLAG, TRAPPC6A-V5/TRAPPC11-FLAG, TRAPPC6B-V5/TRAPPC10-FLAG, or TRAPPC6B-V5/TRAPPC11. After 48 hours the cells were lysed and treated with anti-FLAG IgG agarose beads. The eluates from the immunoprecipitation were probed for V5, FLAG and Tubulin. The blot is representative of at least three biological replicates. Inputs represent 10% of the sample subjected to immunoprecipitation. **B.** Quantification of the ratio of V5 immunoprecipitated with FLAG from three different experiments.

3.4.3 Patient-derived fibroblasts have ER-Golgi trafficking defects and altered Golgi morphology

morphology

Previous studies have shown an anterograde trafficking defect for variants in most TRAPP-associated genes (Al-Deri et al., 2021; Rawlins et al., 2022; Sacher et al., 2019a; Van Bergen et al., 2020). Therefore, we subjected fibroblasts derived from families 4 (c.149+2 T>A splice-site; patients 5 and 6) and 15 (p.Q152*; patients 23 and 24) to a membrane trafficking assay (Boncompain et al., 2012). In all four cases, upon biotin-mediated release of an ER-retained protein there was a delay in transport to the Golgi compared to control (**Figure. 3.5A and 3.5B**). This was determined to be TRAPPC6B-related since transfection of RFP-tagged TRAPPC6B resulted in a noticeable rescue of this trafficking defect. Golgi fragmentation is also commonly seen in cells harboring TRAPP gene mutations as well as in cells with mutations in other membrane trafficking-related proteins. (Makhoul et al., 2019). When the fibroblasts from affected individuals were

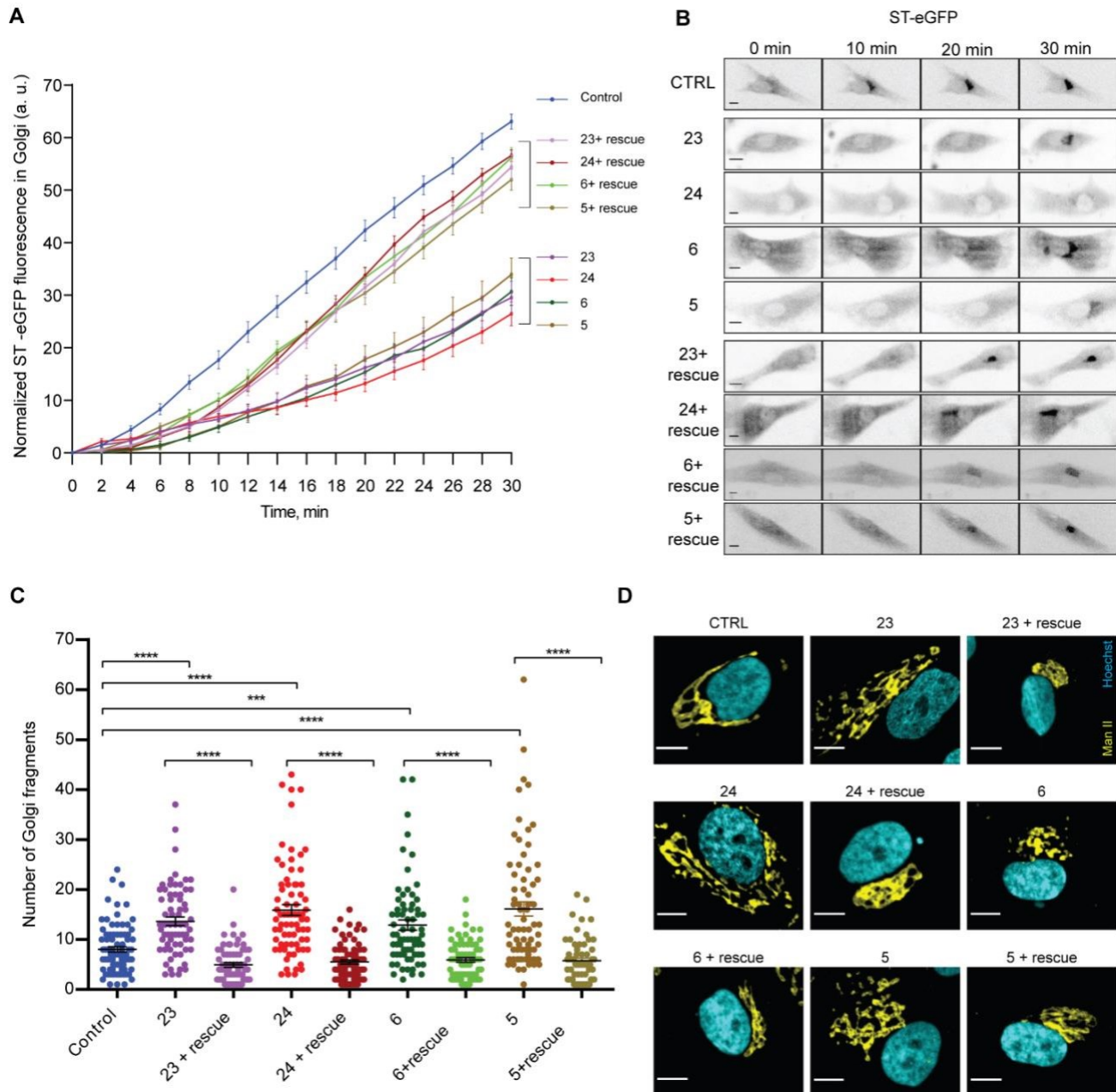


Figure 3.5. Fibroblasts from individuals with TRAPPC6B variants display membrane trafficking defects and have fragmented Golgi

A. Fibroblasts from families 4 (c.149+2 T>A splice; Patients 5 and 6) and 15 (p.Q152*; Patients 23 and 24) were transfected with ST-GFP and incubated overnight. The next day, cells were treated with 60 μ M biotin and imaged every 2 minutes. Golgi-associated fluorescence was quantified and plotted as a function of time. In some cases, TRAPPC6B-RFP was co-transfected to verify a rescue of the trafficking defect. **B.** Representative images used for quantification of the RUSH trafficking assay at 0, 10, 20 and 30 minutes. N values ranged from 48-61 and come from at least three biological replicates. The scale bar represents 10 μ m. **C.** Fibroblasts were either un-transfected or transfected with TRAPPC6B-RFP, fixed and stained for mannosidase II as a Golgi marker. The mannosidase II-positive structures were quantified as described in the methods section. N values ranged from 65-82 and come from at least three biological replicates. **D.** Representative images used for the quantifying the number of Golgi fragments. The scale bar represents 10 μ m.

3.3.4 Loss of neuronal TRAPPC6B impairs locomotion and wing posture in *Drosophila*

We examined whether TRAPPC6B could regulate neuromotor function in a *Drosophila* model. The *Drosophila* ortholog, Trs33, has excellent conservation with TRAPPC6B (DIOPT score=15/15) with 72% similarity and 54% identity (flyrnai.org). As *Trs33* is an essential gene for development, we utilized an RNAi knockdown approach specifically in neurons under the ELAV-Gal4 driver. Neuronal expression of Trs33 shRNA decreased distance traveled in a locomotor assay compared to the heterozygous controls of the Gal4 and UAS lines alone (**Figure. 3.6A**). Additionally, an abnormality of wing posture was noted in 10.6% of these animals, suggesting defects in the innervation of the indirect flight muscles (**Figure. 3.6B**). Together, this suggests TRAPPC6B is crucial for motor function.

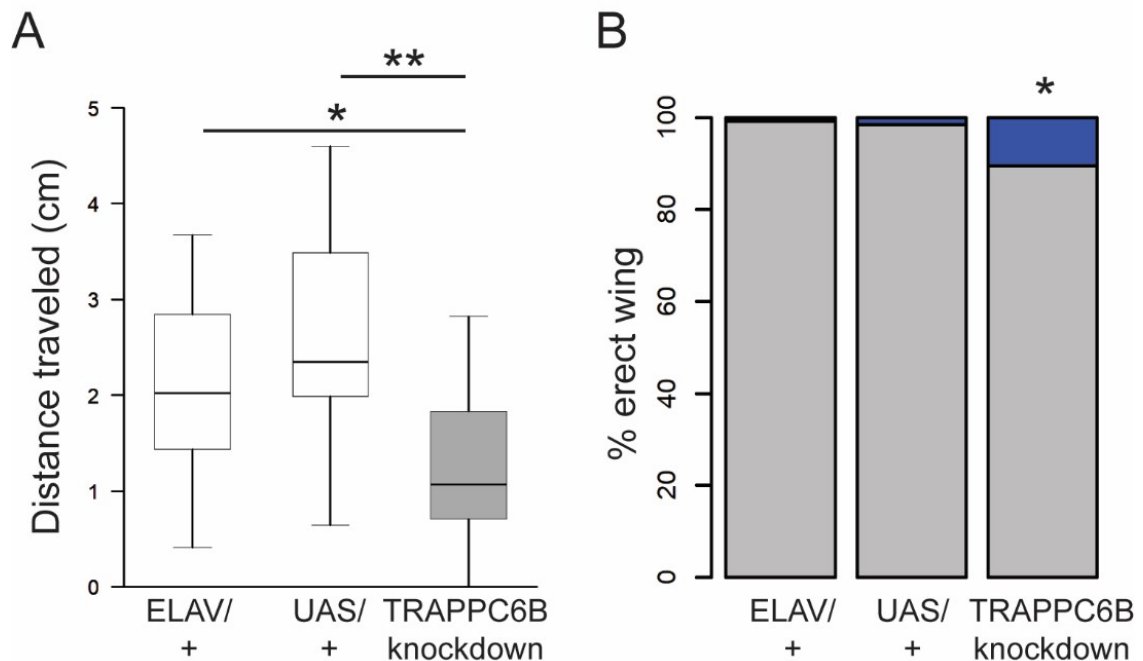


Figure 3.6. Neuronal TRAPPC6B knockdown impairments in a *Drosophila* model

A. Box and whisker plot of distance traveled in 3 seconds in negative geotaxis assay comparing Gal4 and UAS driver heterozygotes and shRNA neuronal-driven expression for TRAPPC6B knockdown. Statistics determined using paired t-test (n=16 trials). * p<0.01, ** p<0.001. **B.** Erect wing phenotype is significantly increased in TRAPPC6B knockdown compared to heterozygous controls. (n=113-137 flies/genotype). Statistics determined both as a 3-way and pairwise against individual controls using Chi-squared analysis. * p<0.01.

3.5 Discussion

Marin-Valencia et al. recently reported a founder *TRAPPC6B* homozygous splice-site variant segregating with disease in 3 Egyptian families and found that morpholino treatment against *Danio* TrappC6B led to reduced head size and increased spontaneous neuronal activity in zebrafish (Marin-Valencia et al., 2018). We present here 29 individuals from 18 families with biallelic mutations in *TRAPPC6B*. These individuals share a syndromic phenotype consisting of primary microcephaly, global developmental delay/intellectual disability, stereotypies and impaired expressive language. Brain MRI findings include consistent thin corpus callosum and white matter intensity and volume changes. Variable features include epilepsy, spasticity/dystonia, and dysmorphic features. Our findings thus provide an independent validation for biallelic *TRAPPC6B* variants as a cause of a complex neurodevelopmental disorder and expand on the clinical features.

Notably, two individuals in our series harbored missense *TRAPPC6B* variants (patients 7 and 29). The individual with compound heterozygous variants (patient 7) had a milder phenotype, gaining the ability to use limited expressive language, falling short of a formal diagnosis of microcephaly, and achieving independent walking by age 3. The homozygous missense variant (p.G124V) is predicted to be pathogenic with a CADD score of 32 and is absent from gnomAD. This individual (patient 29) has clinical features that match the remaining cohort, including microcephaly, spasticity, epilepsy, and a thin corpus callosum. This suggests that missense variants can contribute to a *TRAPPC6B* genetic disorder, with similar but potentially milder manifestations. The remaining 27 individuals in the present cohort and those reported by Marin-Valencia all had homozygous splice-site and early stop mutations. We confirmed diminished protein expression in a family with a homozygous early stop variant (p.Q152*), consistent with a loss of function mutation that may

manifest with decreased protein abundance or the loss of an as-yet unidentified motif in the carboxy-terminus important for subcellular localization or protein-protein interactions.

Our biochemical studies suggest that variants in TRAPPC6B such as p.Q152* likely impair TRAPPII complex stability by a weakened interaction with TRAPPC3 and decreasing protein expression for TRAPPC6B and TRAPPII complex members. TRAPPC6B appears to preferentially associate with TRAPPII compared to TRAPPIII. It is interesting that TRAPPC6A levels increase in the absence of TRAPPC6B as seen in affected individuals of family 15. It is possible that TRAPPC6A can partially compensate for the loss of TRAPPC6B. This would be consistent with the studies of TRAPP guanine nucleotide exchange factor (GEF) activity towards Rab for recombinant *Drosophila* and human complexes (Harris et al., 2021; Jenkins et al., 2020). In *Drosophila*, only a single (TRAPPC6B) homologue is found, while humans have both TRAPPC6A and TRAPPC6B. Therefore, *Drosophila* TRAPP complexes do not distinguish between the two TRAPPC6 human orthologues and TRAPPIII is functional with TRAPPC6B. In the human recombinant complexes, TRAPPC6A was used for assembling both TRAPPII and III, and both complexes were functional (Harris et al., 2021; Jenkins et al., 2020). It remains possible that had TRAPPC6B been used in the recombinant TRAPPII complex, stronger or additional GEF activity would have been seen. Nevertheless, our present study suggests a bias for TRAPPC6B in TRAPPII and implies the cell has a mechanism to distinguish and incorporate specific TRAPPC6 orthologues into specific TRAPP complexes. It is presently unclear how this would be accomplished.

Disruption of TRAPPII stability has been shown previously to affect anterograde trafficking (Rawlins et al., 2022). Biallelic variants of *TRAPPC6B* show similar cellular features, such as an ER- to -Golgi trafficking defect and altered Golgi morphology.

We found that neuronal loss of TRAPPC6B in a *Drosophila* model impaired locomotion and wing posture. The erect wing phenotype has been linked to impaired synaptic growth (Hausmann et al., 2008) and disrupted central nervous system commissures and longitudinal tracts innervating the indirect flight muscles which regulate wing posture (DeSimone et al., 1996). Knocking down Trs33 in *Drosophila* with the Mef2 muscle and neuron expressing Gal4-driver was reported to impair flight ability, but without obvious changes to the flight muscles, myofibrils, or sarcomeres themselves (Schnorrer et al., 2010). Together with our ELAV-mediated neuronal knockdown, this suggests a neuronal origin for the previously reported flight muscle weakness that has similarities to the postural/locomotor impairments in *TRAPPC6B*-affected individuals.

TRAPPC6B-associated phenotypes overlap substantially with variants in genes encoding other TRAPP subunits. These phenotypes include intellectual disability, movement disorders, white matter involvement, cerebellar volume loss, microcephaly, and muscle involvement (**Table 3.1**) (Sacher et al., 2019a). Many TRAPP-related disorders, particularly those affecting TRAPPIII, identified a progressive loss of cerebral volume over time (**Table 3.1**). However, we did not find evidence that loss of *TRAPPC6B* function causes neurodegeneration. Treating clinicians did not note progressive microcephaly and available OFC measurements seem to start low at birth and maintain the same course. Consistent with the biochemical studies, we found *TRAPPC6B* phenotypes overlapped more closely for genes exclusively localized to TRAPP II (*TRAPPC9* (#613192) and *TRAPPC10* (*602103)). In contrast, individuals with variants in genes exclusively encoding autophagy-associated TRAPPIII components (*TRAPPC11* and *TRAPPC12*) are notably different from the *TRAPPC6B* patients. *TRAPPC11* variants lead to a muscular dystrophy with visual system impairments and individuals with *TRAPPC12* display a progressive encephalopathy.

This supports the conclusion that *TRAPPC6B* is important for TRAPPII complex function with impairments resulting in a distinct and recognizable TRAPPopathy.

3.6 Supplementary Material

3.6.1 Supplementary Figure

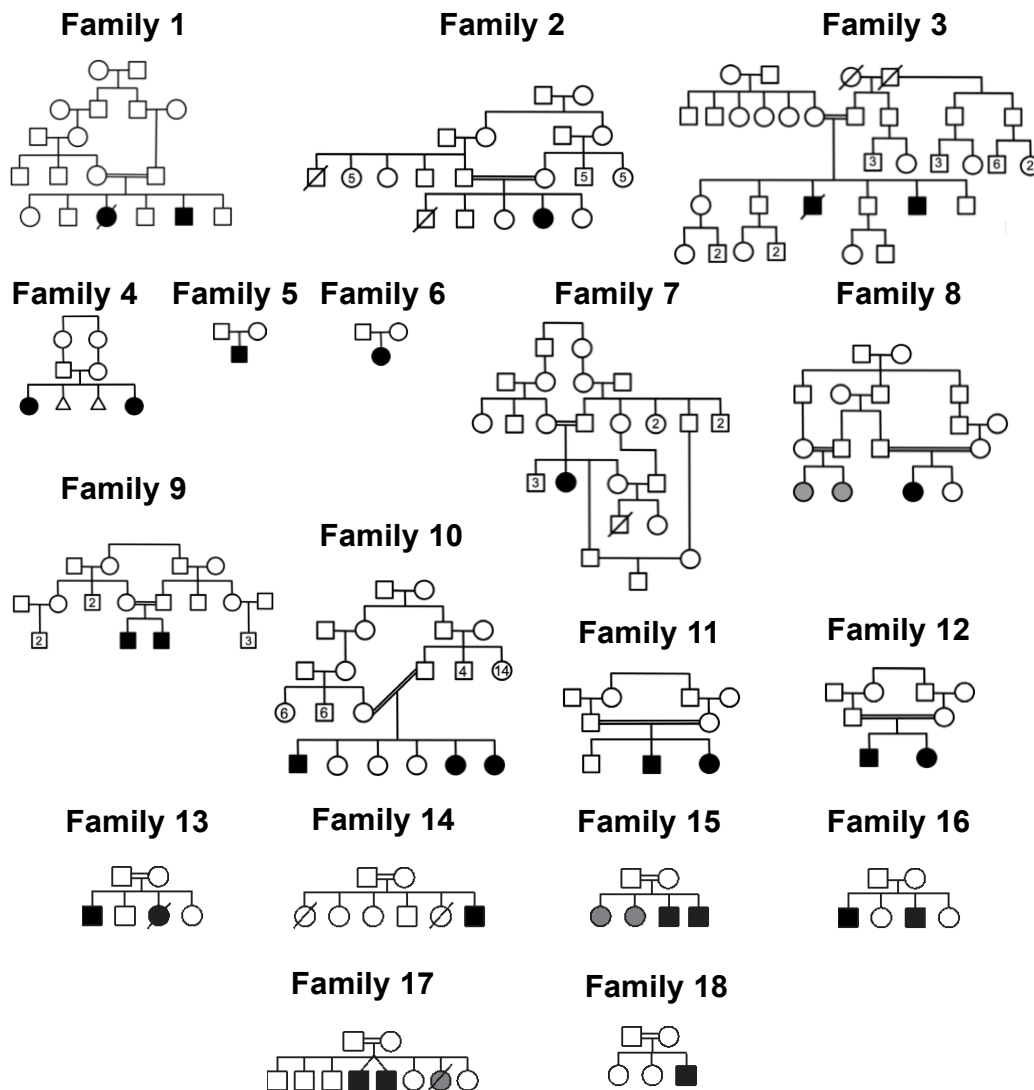


Figure S3.1 Pedigrees of 18 families with TRAPPC6B variants included in study

3.6.2 Supplementary Clinical Summaries

Patient 1: This patient is a female born in Iran to consanguineous parents (first cousins) including a G3P2 mother via spontaneous vaginal delivery at 40 weeks gestational age. There were no obstetric complications reported. No developmental concerns existed until age 3 years, when she experienced a regression and loss of verbal communication and ambulation skills. She has not experienced further clinical regression. She developed seizures at age 3, but these became well-controlled with anticonvulsant medication. At the time of last contact (age 30), the patient was non-verbal with severe-profound intellectual disability, non-ambulatory (GMFCS level V), and completely dependent for all activities of daily living. She has an older brother (Patient 2) with similar features (see full description below). The family history was otherwise unremarkable.

Physical examination revealed temporal wasting and diffuse muscle atrophy, gingival hypertrophy (without history of phenytoin use) (**Figure. 3.1B-C**). There was minimal interaction with her environment. Eye contact was diminished, and there was a paucity of volitional movement. She exhibited repetitive, stereotyped mouth movements (alternate opening and closing). The patient was unable to sit independently. Mixed spastic-dystonic quadriplegia with contractures (especially prominent in the elbow flexors and plantar flexors bilaterally) was evident, with swan neck deformity of her fingers and curling of all her toes. Deep tendon reflexes and plantar responses could not be reliably elicited (Supplementary Video P1). Brain MRI has never been performed. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.124C>T; p.Arg42*).

Patient 2: This patient, the older brother of patient 1, was born in Iran to a G1P0 mother (herself part of a consanguineous union) via spontaneous vaginal delivery at 40 weeks gestational age

without obstetric complications. This patient also developed relatively normal until age 3 years, when he too experienced a regression and loss of verbal communication and ambulation. He also began having seizures at age 3 years (current well-controlled with anti-epileptic medication). At the time of last contact (age 32), the patient was non-verbal with severe-profound intellectual disability, non-ambulatory (GMFCS level V), unable to sit independently, and completely dependent for all of his care.

His examination revealed brachycephaly and trigonocephaly, with temporal wasting and has diffuse muscle atrophy, especially prominent in his proximal limb muscles. Dysmorphic features included prominent cheekbones, a wide nasal bridge, and a papular lesion suspicious for an occult encephalocele (**Figure. 3.1C**). He was visually attentive to his environment and blinked to visual threat. He exhibited spontaneous voluntary movements of his bilateral upper extremities, bringing his hands to his mouth, but does have moderate spasticity and dystonia in a quadriplegic distribution, with spasticity especially prominent in the elbow and plantar flexors with relative sparing of the hands. Pyramidal tract signs were not elicited. Contractures were noted in his upper extremities and at his knees bilaterally. He also displayed repetitive, stereotyped hand (tapping his mouth with his fingers) and head movements (turning side-to-side) (Supplementary Video P2). A CT of the head performed as a school-aged child was reportedly normal and MRI was never obtained. Whole exome sequenced revealed a homozygous nonsense mutation in *TRAPPC6B* shared by his affected sister (NM_001079537.1 c.124C>T; p.Arg42*).

Patient 3: This girl was born in Morocco to a G4P2 mother (mother had one prior late-term miscarriage) at 40 weeks 3 days gestational age via spontaneous vaginal delivery without complications with the pregnancy or delivery. The parents are first cousins. Birthweight was 3 kg. Concerns first arose during infancy with failure to meet language and motor milestones. As a

toddler, she experienced two unprovoked seizures, but these did not recur. Treating physicians identified progressive microcephaly with diminishing head circumference. At the age of last contact (16 years old) she exhibited severe-profound intellectual disability and had never been able to sit without support.

Physical examination was remarkable for dysmorphic features (microcephaly, mild mandibular prognathia, arachnodactyly, foot digit 2-3 syndactyly and hypoplastic 4th digit on one foot). She was non-verbal, and visually attentive for only brief periods of time. She did exhibit some voluntary movement and was able to sit in a wheelchair and to reach for and grasp objects. She displayed repetitive, stereotyped movements of her hands, and hyperkinetic movements. Spastic quadriplegia with pyramidal tract signs, kyphoscoliosis and limb contractures were noted. Brain MRI was remarkable for enlarged extra-axial fluid spaces with diminished cortical and cerebellar volume with extensive white matter changes in the centrum semiovale bilaterally (**Figure. 3.2A-B**). Family history was significant for early infantile epilepsy in a paternal aunt and uncle who died at ages 4 months and 1 year, respectively. Her younger sister has a history of venous sinus thrombosis of the vein of Galen, and electrical status epilepticus of slow-wave sleep (ESES). Whole exome sequencing revealed a homozygous premature stop mutation in *TRAPPC6B* (NM_001079537.1; c.91C>T; p.Arg31*).

Patient 4: A consanguineous male born in Tunisia at full term via spontaneous vaginal delivery to a G6P5 mother, this child weighed 2.9 kg (12th percentile) with length of 38 cm (15th percentile) and head circumference of 34 cm (30th percentile). He re-presented to medical attention at age 35 years after a long interval without medical care for evaluation of microcephaly (-3 SD) and developmental disability. This visit was also the time of last contact. Parents reported that the patient been globally delayed in attaining his early milestones. He was not able to stand

independently until age 27 months (at this age microcephaly was first noted (-2 SD). He never acquired speech, and remained under his parents' care at age 35, dependent on care-givers for all activities of daily living. Parents did not report an obvious history of developmental regression or loss of previously acquired skills. Physical examination disclosed dysmorphic features, including thin upper lip, wide mouth, low anterior hairline, mandibular prognathia, short philtrum, and deep-set eyes. Kyphoscoliosis and koilonychia were evident, and the patient frequently assumed a crouched position, leading to prominent calluses on his knees. Neurological examination revealed intellectual disability, mixed spasticity-dystonic diplegia, and hand stereotypies. He could ambulate only with a walker and took amitriptyline for anxiety. The family indicated that he had never experienced seizures. Brain MRI was not performed. He has 2 brothers with intellectual disability. One brother had moderate intellectual disability and was non-ambulatory, non-verbal, and died at age 28 years. The other brother has mild intellectual disability (WAIS-III) and infertility. *TRAPPC6B* sequencing was not able to be performed for these brothers. Whole exome sequencing revealed the same homozygous mutation in *TRAPPC6B* (NM_001079537.1; c.91C>T; p.Arg31*) seen in patient 3.

Patient 5: This girl was the product of a first cousin union and was born in Pakistan at full term to a G1P0 mother via spontaneous vaginal delivery after an uncomplicated pregnancy. The patient was 15 years old at the time of genetic diagnosis (16 years old at last contact). On physical exam, she displayed severe microcephaly (head circumference 47 cm, -6.5 SD) and failure to thrive (weight 29.4 kg, -3.5 SD). Dysmorphic features included deep-set eyes, prominent ears, a long thin nose, prognathia, thin fingers and toes, prominent heels, and a large irregular, hypopigmented patch on her torso (**Figure. 3.1E**). She had reduced muscle bulk. Neurological assessment indicated mild hypotonia, brisk reflexes in her lower limbs and sustained clonus of both ankles (Supplementary

Video P5). She was able to reach for and grasp objects. Stereotypies included clapping, shaking her head, vocalizing and grinding her teeth.

She was globally delayed in her attainment of early milestones. She walked for the first time at age 6 years with assistance and functioned at a GMFCS level IV. She began babbling at the age of 5 years, but never acquired language. She demonstrated an appropriate social smile and laugh and appeared to recognize familiar individuals and enjoyed being in a social setting. She had epilepsy, with seizure onset at 5 months of age. Typical seizures were tonic, affecting all four limbs. EEG between events was non-epileptiform. Seizures were occurring every 2-3 days on a combination of levetiracetam and valproic acid. MRI revealed microcephaly (**Figure. 3.2C-D**). Whole exome sequencing disclosed a homozygous splice site variant in *TRAPPC6B* (NM_001079537.1; c.149+2T>A). Family history was remarkable for a younger sister with similar symptoms (patient 6) who segregated the same homozygous canonical splice site variant in *TRAPPC6B*.

Patient 6: This girl, the sister of patient 5, was also born in Pakistan at term via spontaneous vaginal delivery after an uncomplicated pregnancy. This patient was 11 years old at the time of genetic diagnosis (13 years old at last contact). On examination, she displayed microcephaly (OFC 46 cm; -6 SD) with positional plagiocephaly and her weight was 23 kg (-3 SD). Dysmorphic features included deep set eyes, prominent ears, high-arched palate, and widely spaced teeth (**Figure. 3.1F**). Examination of her hands revealed thin, tapered fingers, and her toes were long and thin with overriding 2nd and 3rd toes on the left foot. Muscle bulk was diffusely decreased, and elbow, hip, and knee flexion contractures were evident bilaterally. Past medical history included peptic ulcer disease associated with hematemesis. Neurologically, the patient did not fix or follow. StRabismus was noted. She had diffuse hypotonia with brisk reflexes but no clear pyramidal tract signs. She was able to lift her head and torso off the bed, but is unable to roll, sit unsupported or ambulate

(GMFCS level V) (Supplementary Video P6). She did not spontaneously reach for or grasp objects. She vocalized but did not babble or use spoken words. She smiled appropriately and laughed when spoken to but did not appear to recognize familiar individuals. Stereotypies included head banging, hand flapping, mouth opening, and vocalizing. She was delayed in achieving all early milestones but did not experience any developmental regression. Hearing and vision assessments had been normal. She had a non-epileptiform EEG, a dilated aortic root (Z-score = 2.3), and chronic subluxation of the left hip. MRI of the brain revealed a hypoplastic corpus callosum, diminished cerebral and cerebellar volumes and T2 hyperintensity of the parietal, occipital and temporal lobes (**Figure. 3.2E-F**). She shares a homozygous splice site variant (NM_001079537.1; c.149+2T>A) with her older sister, reported as patient 5 above.

Patient 7: This young boy was born in France to unrelated French parents after a 38-week gestation to a G1P0 mother via spontaneous vaginal delivery. There were no complications with the pregnancy or the delivery. His birth weight was 2815 g, birth length was 48 cm, and birth head circumference was 33 cm. Physical examination at the time of last contact revealed a depressed nasal bridge, thin upper lip, and inverted nipples (**Figure. 3.1G**). The last measured head circumference was 49 cm at age 3.5 years (-1.3 SD). Neurological evaluation indicated intellectual disability, hyperactivity, and self-injurious behaviors. His eye contact was appropriate. Stereotypies of the head and hands were evident. There was no history of seizures and no other movement disorders were appreciated.

Developmental history revealed a global delay in milestone attainment. He was first able to sit independently at age 13 months and was able to walk independently by age 3 years. He was first able to speak 2 syllable words at age 3 years. Past medical history was significant for left-sided pyelo-ureteral surgery at age 14 months for ureteral reflux. Brain MRI showed periventricular and

deep white matter signal abnormalities associated with ex vacuo ventriculomegaly and hypoplastic corpus callosum (not shown). Family history was unremarkable. Whole exome sequencing revealed compound heterozygous mutations (NM_001079537.1; c.26_28delinsCTT (predicted to alter the 3' splice site of intron 3) and c.268-2_268-1del; (p.Leu9Leu10delinsProPhe).

Patient 8: This patient is a female born in Mali to a G6P5 mother via spontaneous vaginal delivery at gestational age 39 weeks (full term). The pregnancy and delivery were uncomplicated, and the patient was a singleton birth (not a twin). The birthweight was 2410 g, birth length was 45 cm, birth head circumference was 32 cm. Parents are consanguineous. There is no known family history of any neurologic disorders.

Patient presented to medical attention for the first time at age 5 years (age at last contact was 5 years old). She first walked independently at age 19 months. She is able to speak a few single words. She has some visible dysmorphic features, including short philtrum, tapering fingers, and obesity. She also has mild liver enlargement. She does not have appreciable spasticity or dystonia. Her disability score is GMFCS II. She displays hand stereotypies and echolalia but has not received a formal diagnosis of autism spectrum disorder. She has moderate intellectual disability. She does not have epilepsy and has had no lifetime seizures. She does not have any psychiatric diagnoses. She does not have any known visual impairment and is noted to make good eye contact. At the time of her last visit, she was noted to have obesity and microcephaly (weight 30 kg (+4 SD), height 115 cm (+3 SD), HC 47.5 cm (-2.5 SD)). Patellar reflexes are noted to be normal on exam. She does not have any contractures on exam. Whole exome sequencing revealed a homozygous splice site variant (NM_001079537.1; c.150-1G>A).

Patient 9: This patient is a male born in the Gambia to a G6P5 mother via spontaneous vaginal delivery at gestational age 38 weeks (full term). The pregnancy and delivery were uncomplicated,

and the patient was a singleton birth (not a twin). The birthweight was 2670 g, birth length was 47 cm, birth head circumference was 32.5 cm, Apgar scores reported as 10 and 10. Parents are consanguineous. There is no known family history of any neurologic disorders.

Patient presented to medical attention for the first time at age 12 years (age at last contact was 12 years old). He first walked independently at age 20 months. He is non-verbal. He has some visible dysmorphic features, including microcephaly, brachycephaly, and muscle atrophy of one lower extremity. He does not appear to have any other organ system pathology outside the nervous system. He has spasticity of one lower extremity, but no appreciable dystonia. He displays hand stereotypies. He has severe intellectual disability. He does not have epilepsy and has had no lifetime seizures. He does not have any known visual impairment and is noted to make good eye contact. Patellar reflexes are noted to be normal on exam. He does not have any contractures on exam. He has not received any known therapies to treat these symptoms. MRI brain imaging is remarkable for cerebral atrophy, cerebellar atrophy, and hypoplastic corpus callosum (not shown). Whole exome sequencing disclosed a homozygous splicing variant (NM_001079537.1; c.150-1G>A)

Patient 10: Female patient aged 8 years and 7 months old derived from unaffected first cousin parents. There is a healthy younger sister and no family history of similar condition; however, 2 paternal side cousins with phenylketonuria were recorded. The pregnancy and delivery histories were unremarkable. Birth weight was 2.9kg, length 48cm and head circumference 32cm. Milestones of development were delayed; she was able to sit at 1 year and 3 months and walk with support at the age of examination after operation of tendon repair at age 7 years old due shortening of tendons and spasticity. She could follow objects, respond to her name but couldn't obey simple orders. Masturbation like movement developed at the age 4 and is fairly controlled. Patient

experienced seizures at 2y old in the form of generalized and tonic controlled with valproate and oxcarbamazepine. On examination, autistic behavior included hand flapping and stereotypies; hyperactivity was also present. No speech recorded except few double syllable words and was non-functional. Current measurements are weight 19kg (-1.2SD), height 114cm (-2.2SD) and head circumference 46cm (-4.5SD). Dysmorphic features are present as small head, long face, arched eyebrows, wide spaced eyes, almond shaped eye, straight nasal bridge, broad nose, broad chin, low set ears and arachnodactyly (**Figure. 3.1H-I**). General examination was unremarkable and neurological examination showed mild hypertonia and brisk reflexes especially in lower limbs. Patient had normal karyotyping, metabolic screening, acylcarnitine profile, organic acid in urine, fundus examination, and auditory brain stem evoked potential. Electroencephalogram showed focal right frontotemporal epileptogenic discharges.

Neuroimaging revealed mild cortical atrophy, wide interhemispheric fissure, thin dysplastic corpus callosum, mild central atrophy, deep white matter signal at T2W around the frontal horn and body of lateral ventricles, mild vermian atrophy (**not shown**).

Whole exome sequencing revealed a homozygous splicing variant in *TRAPPC6B* (NM_001079537.1; c.352-2 A>G).

Patient 11: This patient is a 7-year-old boy. He was first presented to the neurologist at 4 years 4 months of age because of cognitive and speech delay.

Though at 2.5 years he was repeating few words (echolalia), this stopped and now he is nonverbal. Nevertheless, he recognizes the family names and that of common things in his surroundings and obeys many simple one step orders.

He has autistic features, acts as deaf, has some stereotype movements and his imaginative play and imitation has improved a bit lately. The parents were complaining of severe hyperactivity though

he was tried on antipsychotics, but they failed to help. His gross motor function is appropriate for age, but his fine hand functions are a bit delayed and he recently started to hold a spoon. He was born at full term by elective CS with smooth antenatal and postnatal periods. He is the first offspring of first cousin parents with a younger male similarly affected sibling. There is no other family member with a similar condition. Mom aborted three times before.

The boy is the first offspring of cousin parents with a similarly affected younger male sibling. When first seen, his OFC was below -2SD and his height at 50th centile. He had no definite dysmorphic features or neurocutaneous stigmata. His neurologic examination, chest and abdomen was unremarkable. However, of note that he was very hyperactive and repeating some stereotype movements relentlessly. MRI brain (April 2018) showed mild defective white matter in the posterior deep periventricular regions with peritrigonal widening and subvolumia of corpus callosum (**not shown**). VEP and ERG showed bilateral mild peripheral retinal dysfunction, moderately impaired retinocortical transmission and bilateral moderate to severe optic atrophy. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.283 C>T; p.Q95*).

Patient 12: This patient is the younger brother of patient 11 and is currently 5-years-old. Like his sibling, he has marked cognitive and speech delay. He used to produce few intended words, but these stopped at around the age of 2.5 years. He showed similar cognitive delay as his sibling. He understands few simple orders, but many times acts as deaf and cannot perform imaginative play or imitates and is very aggressive to people.

Unlike his brother he had mild motor delay; walked at 21 months but he caught up. The fine motor functions were delayed as well.

His OFC is at -2SD and height at 15th centile and like his sibling he is very hyperactive and aggressive. He had no definite dysmorphic features or neurocutaneous stigmata. Examination of chest and abdomen was free of abnormalities.

His MRI showed similarly periventricular increased signal of white matter in T2 in the occipital and posterior parietal periventricular regions (**not shown**).

Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.283 C>T; p.Q95*).

Patient 13: This patient is a 12.5-year-old boy with a very rare condition known as NEDMEBA (Neurodevelopmental disorder with microcephaly, epilepsy, and brain atrophy) caused by a homozygous pathogenic variant in *TRAPPC6B* (NM_001079537.1:c.268-1delAG) as reported in PMID: 31130284. He was born to a healthy 22-year-old G1P0 mother following uneventful FT pregnancy and through normal spontaneous vaginal delivery (NSVD). Floppiness was apparent at 4m of age, and this was the harbinger of the global developmental delay that ensued. He sat at 14m and walked at 4yrs. His IQ was estimated at 20-30 with very limited speech. Epilepsy started nearly 1 year ago and is well controlled on antiepileptics although he had at least one breakthrough seizure. His past medical history is also notable for right inguinal hernia s/p repair at age 2m and adenoidectomy and tonsillectomy at age 6yrs. Family history is notable for half first cousin parents who have two other similarly affected children. Physical examination revealed severe microcephaly as well as short stature and underweight. He had subtle dysmorphism in the form of upslanting palpebral fissures, thin upper lip with underdeveloped vermilion border, pointed chin, cautery marks in the scalp. Brain MRI at 6y revealed decreased frontal occipital diameter, diffuse thinning and foreshortening of the corpus callosum. Also noted was patchy, increased FLAIR

signal in the peritrial white matter that was associated with the abnormal square shape of the posterior margins of the lateral ventricles (**Figure. 3.2G-H**).

Patient 14: This patient, the younger sister of patient 13, is a 4-year-old girl with a very rare condition described as NEDMEBA (Neurodevelopmental disorder with microcephaly, epilepsy, and brain atrophy) reported in PMID: 31130284. This patient and homozygous pathogenic variant in TRAPPC6B (NM_001079537.1:c.268-1delAG) have been previously described and are shared with her older brother as. She was born to a healthy 30yr old G6P4AB1 mother following uneventful full term pregnancy and delivery was normal spontaneous vaginal delivery. Floppiness was apparent at 6m of age, and this was the harbinger of the global developmental delay that ensued. She sat at 12m, walked at 30m and only says mama and dada. No history of seizures. Family history is notable for half first cousin parents who have two other similarly affected children. Brain MRI showed partial agenesis of corpus callosum and partial agenesis and atrophy of the vermis and abnormal white matter signal. Physical examination revealed severe microcephaly as well as short stature and underweight.

Patient 15: This patient, the younger sister of patient 13, is a 2.5yr old girl with a very rare condition known as NEDMEBA caused by a homozygous pathogenic variant in TRAPPC6B (NM_001079537.1:c.268-1delAG) shared with her older brother as reported in PMID: 31130284. She was born to a healthy 32yr old G7P5AB1 mother following uneventful FT pregnancy and delivery was NSVD. Floppiness was apparent at 6m of age and this was the harbinger of the global developmental delay that ensued. She sat at 12m and still can't walk or say any word. There is no history of seizures. Physical examination revealed severe microcephaly as well as short stature

and underweight. Brain MRI showed partial agenesis of corpus callosum and partial agenesis and atrophy of the vermis and abnormal white matter signal.

Patient 16: This patient is a female cousin of patient 13, born at 37 weeks gestation via normal spontaneous vaginal delivery. Toxemia was noted during pregnancy. The patient was diagnosed at 1.5 years old. She has global developmental delay. There are not indicators of autism, or psychiatric conditions. There is no history of epilepsy or seizures. She was able to sit at 3 years and is able to walk. No contractures have been noted. She is unable to speak. There has been no evidence of progression or regression. The age at last contact was 11 years. Brain MRI at 5 y showed reduced frontal occipital diameter, short corpus callosum, mildly prominent lateral and third ventricles, and a hypoplastic inferior vermis (**Figure. 3.2 K-L**). Whole exome sequencing identified a homozygous pathogenic variant in TRAPPC6B (NM_001079537.1:c.268-1delAG).

Patient 17: This patient is a male (brother of patient 18) born in Pakistan to a G2P1 mother via spontaneous vaginal delivery at full term gestational age. Birthweight was 3.2 kg. There were no obstetric complications reported. Parents are consanguineous (mother and father are first cousins). At the time of diagnosis, patient was age 5 years. (Age at last contact was 5 years). On physical examination, no dysmorphic features were reported (**Figure. 3.1J-K**), and there was no other organ involvement outside the nervous system. Patient displayed spasticity and motor stereotypies (hand flapping and clapping behaviors), but no dystonia or other movement disorders. Patellar reflex is reportedly normal bilaterally. There are contractures of bilateral lower limbs. Patient was non-ambulatory, but able to sit independently. The patient vocalizes but is non-verbal. Patient has intellectual disability. The patient does not have epilepsy or seizures. At the time of the last clinic visit, patient was taking risperidone for sleep. MRI brain results are not available. Whole exome

sequencing revealed a homozygous splicing mutation in *TRAPPC6B* (NM_001079537.1; c.149+2T>A).

Patient 18: This patient is a female (sister of patient 17) born in Pakistan to a G3P2 mother via spontaneous vaginal delivery at 37 weeks gestational age. Birthweight was 3.5 kg. There were no obstetric complications reported. Parents are consanguineous (mother and father are first cousins). At the time of diagnosis, patient was age 4 years. (Age at last contact was 4 years). On physical examination, patient had microcephaly (**Figure. 3.1L**), but no other dysmorphic features were reported, and there was no other organ involvement outside the nervous system. Patient displays a motor stereotypy (head nodding), but no spasticity, dystonia or other movement disorders. Patellar reflex is reportedly normal bilaterally. There are contractures of bilateral wrists. Patient was able to sit and ambulate independently. The patient vocalizes but is non-verbal. Patient displays some autistic features (poor eye contact, sensory integration problems), and has intellectual disability. The patient does not have epilepsy or seizures. At the time of the last clinic visit, patient was taking risperidone for sleep. MRI brain results are not available.

Whole exome sequencing revealed a homozygous splicing mutation in *TRAPPC6B* (NM_001079537.1; c.149+2T>A).

Patient 19: This patient is a male (brother of patient 20) born in Pakistan to a G1P0 mother via caesarian section (due to non-progression of labor) at 37 weeks gestational age. Birthweight was 3.0 kg. There were no obstetric complications reported. Parents are consanguineous (mother and father are first cousins).

Patient's age at diagnosis was 2.5 years, (Age at last contact was 2.5 years). On physical examination, patient had microcephaly, bulbous nasal tip, and creased earlobe (**Figure.3.1M-N**),

but no other dysmorphic features were reported, and there was no other organ involvement outside the nervous system. Patient displayed spasticity, cogwheel rigidity, and sensory seeking/self-harm behavior (head banging), but no dystonia or other movement disorders. Upper extremity reflexes are noted to be brisk, but patellar reflex is reportedly normal bilaterally. Plantar response is flexor bilaterally. There are no contractures present. Patient was able to sit independently and ambulate independently with a broad-based, spastic gait. Patient noted to have global developmental delay, possible autism spectrum disorder, and intellectual disability. The patient vocalizes but is non-verbal. Patient has epilepsy and takes anti-seizure medication. MRI brain results are not available. Whole exome sequencing revealed a homozygous splicing mutation in *TRAPPC6B* (NM_001079537.1; c.149+2T>A).

Patient 20: This patient is a female (sister of patient 19) born in Pakistan to a G2P1 mother via caesarian section delivery at 37 weeks gestational age. Birthweight was 3.0 kg. There were no obstetric complications reported. Parents are consanguineous (mother and father are first cousins). Patient's age at diagnosis was 14 months. (Age at last contact was 14 months). On physical examination, patient had microcephaly, but no other dysmorphic features were reported, and there was no other organ involvement outside the nervous system. Patient did not display motor stereotypy, spasticity, dystonia, or other movement disorders. Patellar reflex is reportedly normal bilaterally. There are no contractures present. Patient was able to sit independently and ambulate with support. The patient vocalizes but is non-verbal. Patient has intellectual disability. The patient does not have epilepsy or seizures. The patient does not take any medications. MRI brain results are not available.

Whole exome sequencing revealed a homozygous splicing mutation in *TRAPPC6B* (NM_001079537.1; c.149+2T>A).

Patient 21: This boy was born to gravida 4, para 2 Iranian mother as the product of a consanguineous union. The pregnancy was complicated by bleeding at 8 weeks gestation, but the pregnancy proceeded to term and the infant was born via spontaneous vaginal delivery at 40 weeks estimated gestation. At birth, length was 48 cm, weight was 2800 grams, and orbitofrontal circumference was 32 centimeters. Apgars were 5 at 1 minute and 7 at 5 minutes. Developmental delay was evident early, and he experienced multiple febrile seizures. At the time of ascertainment at age 7 years, microcephaly was evident and dysmorphic features included mandibular prognathism, depressed nasal bridge, and tapering fingers. Spastic tetraplegia was evident, and the patient exhibited stereotypies as well. Concurrent neurodevelopmental disorders included severe intellectual disability, autism spectrum disorder, and generalized epilepsy with tonic seizures. Brain MRI findings included periventricular leukomalacia, thin corpus callosum, T2 hyperintensities in the posterior white matter and centrum semiovale. At age 7, the time of last contact, he was able to sit unassisted but could not stand independently or take steps without assistance. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.37 G>T; p.E13*).

Patient 22: This patient is a Turkish male born by Cesarean section at 40 weeks gestation. The pregnancy was normal, but fetal distress and cardiac arrest occurred during delivery. His birth weight was 3450 grams, and Apgars were 1 at 1 minute and 3 at 5 minutes. The age of diagnosis was 3.5 years. Microcephaly was noted at 2 years, with a head circumference of 45 measured at 5 years. No dysmorphic features or other organ involvement were noted. Spasticity is present despite receiving physical therapy. No dystonia was noted. He has autism with stereotypical movements

and inability to make eye contact, and intellectual disability. He speaks only 2-3 words and is unable to form a sentence. Drug-resistant epilepsy involving spasm and tonic seizures is also present. He is able to sit without support. Contractures are present. The age at last contact was 5.5 years. Brain MRI findings indicate cerebral atrophy and thin corpus callosum, as well as sequelae changes in parietooccipital white matter at 2.5 years. EMG and echocardiography were normal. A 1.5 y sibling exhibits hypotonicity with an unknown diagnosis. Another sibling died at 4 m due to leukemia. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.47 C>G; p.Y19*).

Patient 23: This patient is a Moroccan male (brother of patient 24) born by Cesarean section at 39 weeks gestation. The pregnancy and delivery was normal. His birth weight was 3000 grams. The age at genetic diagnosis was 12 years. Head circumference is -1.4 SD, weight + 0.6 SD, and height -1.7 SD. He shows signs of autism as indicated by stereotypies of the hands and behavioral problems but no formal diagnosis has been established. . He has intellectual disability. He can speak a few words and can form a few sentences. Gross neurological examination was unremarkable. No dysmorphic features, spasticity, dystonia, contractures or other organ involvement have been noted. The patient does not have epilepsy or seizures. Brain MRI findings show a high signal in white matter on T2 images around occipital horns of lateral ventricles. . The age at last contact was 12 years. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.454 C>T; p.Q152*).

Patient 24: This patient is a Moroccan male (brother of patient 23) born at 39 weeks gestation. The pregnancy and delivery were normal. His birth weight was 3500 grams. The age at genetic diagnosis was 10 y. Head circumference is -0.5 SD, weight +3.6 SD, and height +0.9 SD. He has intellectual disability. He can speak a few words. Neurological examination was normal except for brisk knee-jerk reflexes. No dysmorphic features, spasticity, dystonia or contractures were noted. His medical history showed cryptorchidism and hypermetropia ODS with astigmatism ODS. The patient does not have epilepsy or seizures. The age at last contact was 10 years. Brain MRI revealed high signal in white matter on T2 images around occipital horns of lateral ventricles and decreased myelinization in the frontal subcortical region. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.454 C>T; p.Q152*).

Patients 25 and 26: These patients are male siblings diagnosed at age 29 years (patient 25) and 34 years (patient 26). Dysmorphic facial features include thin-long nose, eyes close together and short stature. Microcephaly was also noted in both siblings. GI bleeding is also present in patient 25, and constipation in patient 26. They have spasticity, dystonia, contractures. Autism and stereotypies were noted, as well as psychiatric and behavioral issues, and intellectual disability. Contractures are also noted. These siblings do not have epilepsy. They are able to sit with support, and walks with a broad-based gait with small steps with upper body bent forward and knees bent. Whole exome sequencing revealed a homozygous nonsense mutation in *TRAPPC6B* (NM_001079537.1; c.283 C>T; p.Q95*).

Patients 27 and 28: Twin boys were born full-term to a gravida 7 Iranian mother. Parents are consanguineous (mother and father are first cousins). The pregnancy and delivery was normal. Age of diagnosis was 4 years and concordant features were seen for both brothers. Microcephaly (51.5 cm and 50 cm) was noted. They have spasticity and tonic-clonic epilepsy that is managed with phenobarbital. They are able to walk and sit unassisted. No autistic features or abnormal eye contact was present. Issues with insomnia are reported. No other organ system involvement was noted. They were last seen at age 30 and some regression was noted. Whole exome sequencing found a homozygous splicing mutation in *TRAPPC6B* (NM_001079537.1; c.445+5G>T)

Patient 29: This patient is an Indian male born at full term to parents who are first cousins. Family history includes 2 unaffected sisters. The pregnancy and delivery were normal. Microcephaly was noted. The patient has spasticity. No dystonia or other movement disorders have been noted. He has intellectual disability and is unable to speak. No signs of autism or psychiatric disorders are present. He can make good eye contact. This patient has tonic-clonic epilepsy, which has effectively been treated with Divalproex Sodium and Clobazam. He is able to sit when made to sit and can stand with support and tries to take steps. No contractures have been noted. The age at last contact was 3.5 years.

MRI findings indicates diffuse periventricular white matter loss and thinning of the corpus callosum.

Whole exome sequencing revealed a homozygous missense mutation in *TRAPPC6B* (NM_001079537.1; c.317 G>T; p.G124V).

3.6.3 Supplementary Methods

3.6.3.1 Sequencing and Variant Calling

Family 1: Illumina reads were aligned to hg19 using BWA-mem (v 0.7.13) followed by indel realignment and base recalibration using GATK (v 3.2.2) and variant calling by Unified Genotyper (GATK). Variants were called in the standard VCF format (v 4.1) and annotated by ANNOVAR, Alamut (v 1.4.4 Interactive Biosoftware, Rouen, France) and EDGC Annotator (v 1.0.0 EoneDiagnomics Genome Center, Songdo Ichon, South Korea).

SOLiD sequencing was aligned to the hg19 reference genome using Shrimp2. Once alignment files were generated, variant calling and annotation were performed as previously described. Proton data was aligned using the TMAP algorithm and variants were called using the Ion Proton Variant Caller as part of the Torrent Server analysis pipeline (ThermoFisher Scientific, Waltham, MA, US). Ion Reporter was used in addition to the annotation software mentioned above for Ion Proton VCF files (ThermoFisher Scientific, Waltham, MA, US).

Variants were filtered computationally and manually given the HBD regions calculated by autozygosity mapping using FSuite and HomozygosityMapper. The variants were filtered based on the sequencing quality, zygoty and type of mutation. The truncating mutations (stop and frameshift loss of function mutations) were given a higher priority to the missense mutations and inframe indels. Missense mutations were ranked based on the SIFT and Polyphen scores. Only rare variants with $MAF < 10^{-4}$ were reported. The variants that passed the filters were checked against the Exome Aggregation Consortium (ExAC), Cambridge, MA (URL:

<http://exac.broadinstitute.org>) accessed 03, 2016, OMIM and Genecards to determine expression level in tissues and other functional characteristics.

Family 2: After referral for routine diagnostic exome sequencing, the exome of child was enriched using the SureSelect XT Human All Exon V5 kit (Agilent) and sequenced in rapid run mode on the HiSeq2500 sequencing system (Illumina) at a mean target depth of ~90X. The target was defined as all coding exons of UCSC and Ensembl +/- 20bp intron flanks. At this depth >95% of the target is covered at least 15X. Reads were aligned to hg19 using BWA-MEM (v0.7.5a) and variants were called using the GATK HaplotypeCaller (v2.7-2). Detected variants were annotated, filtered, and prioritized using the Bench NGS Lab platform (Cartagenia, Leuven, Belgium). Analysis was based upon a tiered analysis approach. In the first tier known intellectual disability genes were analyzed. This first tier did not lead to a diagnosis. The second tier, which filters for homozygous, compound heterozygous or hemizygous variants, resulted in the detection of a homozygous TRAPPC6B nonsense variant.

Family 3: Three micrograms of genomic DNA per individual was subjected to whole-exome capture and sequencing using the SureSelect Human All Exon V5 kit (Agilent). The resulting libraries were sequenced on a HiSeq 2000 (Illumina) as paired-end 76 bp reads. BAM files were aligned to a human genome reference sequence (GRCh37/hg19) using BWA (Burrows-Wheeler Aligner; v0.7.15). All aligned read data were subject to the following steps: (i) duplicate paired-end reads were removed by Picard 2.4.1, (ii) indel realignment and (iii) base quality score recalibration were done on the Genome Analysis Toolkit (GATK v3.7). Variants with a quality score >30 and an alignment quality score >20 were annotated with SeattleSeq SNP Annotation. Rare variants present at a frequency above 1% in GnomAD Browser, ExAC Browser and the

NHLBI GO Exome Sequencing Project or present in 100 local exomes of unaffected individuals were excluded. We focused on genes with homozygous variants in this consanguineous family. Homozygosity mapping was performed on the raw VCF file using homozygosity mapper with the following parameters: minimum coverage of 10 reads for all variants mapping to RefSeq, only homozygous blocks longer than 100 pb were detected.

Candidate variants were confirmed by Sanger sequencing. Genomic DNA was amplified by Polymerase Chain Reaction (PCR) using HotStarTaq PCR kit (Qiagen) according to the manufacturer's protocol. PCR products were purified by the Agencourt CleanSEQ system (Beckman Coulter) and sequenced with the BigDye Terminator Cycle Sequencing kit, v3.1 (Applied Biosystems) in ABI 3730 sequencer (Applied Biosystems). Sequence data were analyzed using Mutation Surveyor v4.0.9 (Softgenomics).

Family 4: Total genomic DNA was extracted from the biological sample using a spin column method. DNA quality and quantity were assessed through gel electrophoresis and fluorometric analysis, respectively. DNA sample was then shipped to BGI where whole exome capture and sequencing were performed. After assessment of DNA quality at BGI, qualified genomic DNA sample was randomly fragmented by Covaris technology, and the size of the library fragments was mainly distributed between 200bp and 300bp. Then adapters were ligated to both ends of the resulting fragments. Extracted DNA was amplified by ligation-mediated PCR (LM-PCR), purified, and hybridized to the exome array for enrichment (Agilent SureSelect Human All Exon V6). Non-hybridized fragments were then washed out. Captured LM-PCR products were subjected to Agilent 2100 Bioanalyzer, and quantitative PCR was used to estimate the magnitude of enrichment. Each qualified captured library was then loaded on Illumina HiSeq platforms, and high-throughput sequencing was performed for each captured library while ensuring that each sample met the

desired average sequencing coverage ($>100\times$). Sequencing-derived raw image files were processed by Illumina base calling software for base-calling with default parameters and the sequence data of each individual was generated as paired-end reads, which was defined as "raw data" and stored in FASTQ format. The bioinformatics analysis began with quality control of raw sequence reads. Clean sequence reads of each sample was mapped to the human reference genome (GRCh37/hg19). Burrows-Wheeler Aligner (BWA) software was used to do the alignment. Local realignment around indels and base quality score recalibration were performed using GATK, with duplicate reads removed by Picard tools. The sequencing depth and coverage for each individual were calculated based on the alignments.

After generation of exome sequencing data at BGI, data was delivered by a secure connection to Blueprint Genetics. Each exome batch was subjected to thorough quality control measures, after which raw sequence reads are transformed into variants by a proprietary bioinformatics pipeline. The pathogenic potential of variants was predicted by taking into account the predicted consequence, biochemical properties of the codon change, the degree of evolutionary conservation as well as allelic frequencies from large population studies, including e.g., data from the 1000 Genomes project, the ExAC consortium and ClinVar archive, as well as an in-house database of previously reported variants. The clinical association of genes was determined using our curated set of clinically associated genes included in Blueprint Genetics diagnostic panels, and in addition information from Clinical Genomics Database (<https://research.nhgri.nih.gov/CGD>) and The Developmental Disorders Genotype-Phenotype Database (DDG2P) (<https://decipher.sanger.ac.uk/info/ddg2p>) was used. The exome variant data of the family was filtered based on all possible modes of inheritance of the disorder. The clinical evaluation team assessed the pathogenicity of the identified variants by evaluating the information in the patient

referral, reviewing the relevant literature, and manually inspecting the sequencing data. Variant(s) classified as pathogenic or likely pathogenic were confirmed using Sanger sequencing. Reporting was carried out using HGNC-approved gene nomenclature and mutation nomenclature following the HGVS guidelines.

Family 5: Library preparation, exome capture, sequencing and data analysis were performed by IntegraGen SA, Evry, France. Genomic DNA was captured using Twist Human Core Exome Enrichment System (Twist Bioscience) and Custom IntegraGen, followed by paired-end 75 bases massively parallel sequencing on Illumina HiSeq4000. (Gnirke et al., 2009).

Sequence capture, enrichment and elution were performed according to the manufacturer's instruction and protocols (Twist Bioscience) without modification except for library preparation performed with NEBNext® Ultra II kit (New England Biolabs®). For library preparation 150 ng of each genomic DNA were fragmented by sonication and purified to yield fragments of 150-200 bp. Paired-end adaptor oligonucleotides from the NEB kit were ligated on repaired, a-tailed fragments then purified and enriched by 7 PCR cycles. 500ng of these purified Libraries were then hybridized to the Twist oligo probe capture library for 16 hr in a singleplex reaction. After hybridization, washing, and elution, the eluted fraction is PCR-amplified with 8 cycles, purified and quantified by QPCR to obtain sufficient DNA template for downstream applications. Each eluted-enriched DNA sample was then sequenced on an Illumina HiSeq4000 as Paired End 75 reads. Image analysis and base calling was performed using Illumina Real Time Analysis (2.7.7) with default parameters.

Base calling was performed using the Real-Time Analysis software sequence pipeline (v.2.7.7) with the default parameters. Sequence reads were mapped to the Human genome build (hg38) using

the Burrows-Wheeler Aligner (BWA) tool. The duplicated reads were removed (sambamba tools). Variant calling was performed via the Broad Institute's GATK Haplotype Caller GVCF tool (v.3.7). Annotation and variants filtering were performed using Cartagenia Lab Bench NGS platform (Agilent Technologies). Only rare variants (allele frequency <1% and absence of homozygotes in GnomAD database), with a potential effect on proteins (non-synonym, and synonym or intronic variants within 5 bp of a splice site), or already described in the HGMD database (Qiagen) were kept for analysis. The variants with possible Mendelian inheritance in this family (*de novo*, homozygous, compound heterozygous, inherited for imprinted genes) were included.

Family 6: Trio-based exome sequencing was performed for this family. The samples were processed with the Roche MedExome prep kit on the Illumina NextSeq 500 sequencer, with a 2x150 high output kit. The bioinformatic method summary is as follows: Raw reads (FASTQ) were mapped to human reference genome hg19/GRCh37 using BWA-MEM to obtain standard BAM files. Read duplicates were flagged in the BAM files using Picard Tools. Broad Institute's GATK HaplotypeCaller was used to perform short variant calling for SNV (single variant nucleotides) and INDEL (short insertions and deletions, <100bp) to obtain the standard VCF files. The variants in this VCF were annotated using Ensembl VEP for functional annotation, gnomAD (reference set of allele frequencies), Clinvar, HGMD, OMIM, pathogenicity prediction scores such as CADD, and splicing variant effect prediction such as MaxEntScan, dbcsnv. Filtering was performed based on the quality metrics (base quality, mapping quality, genotype quality), annotations and familial pedigree. Sanger sequencing was performed on the parents and proband samples.

Family 7: Trio exome sequencing was performed on the affected child and his parents. Sequencing libraries were constructed using the Roche MedExome prep kit. Sequencing was performed on the Illumina NextSeq 500 sequencer, with a 2x150 high output kit. The Basespace cloud computing platform (with BWA 2.1 and GATK Unified Genotyper 1.6) and the Variant Studio v.3.0 software provided by Illumina was used for mapping the raw reads to human reference genome hg19/GRCh37, variant calling, and annotation. DNA variants were prioritized according to the following criteria: high quality score (Q Phred score ≥ 30) and coverage ≥ 20 X, minor allele frequency (MAF) < 0.005 in database gnomAD), conservation and predicted impact on coding and noncoding sequence, presence in ClinVar. Filtered variants were analyzed using the interactive biosoftware ALAMUT V2.10 which uses several computational (*in silico*) predictive programs that makes an overall prediction of potential pathogenic variants, notably for non-synonymous variants (SIFT, MutationTaster, and PolyPhen-2), for splice variants (GeneSplicer, MaxEntScan, NNSplice and SpliceSiteFinder-like), and evolutionary conservation of the residues across vertebrates and invertebrates (PhastCons and PhyloP) (<http://www.interactive-biosoftware.com/>). We added the analysis of missense variants using the Combined Annotation Dependent Depletion (CADD) software (<https://cadd.gs.washington.edu/>). Missense variants were excluded if they were predicted benign by more than one of the three software.

Family 8: After informed consent was obtained prior to genetic testing from the family, genomic DNA was extracted from peripheral blood samples according to standard procedures of phenol chloroform extraction. WES on the proband was performed as described elsewhere (Makrythanasis et al., 2014) in Macrogen, Korea. Briefly, target enrichment was performed with 2 μ g genomic DNA using the SureSelectXT Human All Exon Kit version 6 (Agilent Technologies, Santa Clara, CA, USA) to generate barcoded whole-exome sequencing libraries. Libraries were sequenced on

the HiSeqX platform (Illumina, San Diego, CA, USA) with 50x coverage. Quality assessment of the sequence reads was performed by generating QC statistics with FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>). Our bioinformatics filtering strategy included screening for only exonic and donor/acceptor splicing variants. In accordance with the pedigree and phenotype, priority was given to rare variants (<0.01% in public databases, including 1,000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, Iranomw, and GME Variome, and gnomAD as well as the in-house database consisting of 14000 exomes) that were fitting a recessive (homozygous or compound heterozygous) model and/or variants in genes previously linked to developmental delay, intellectual disability, and other neurological disorders. The candidate variants were validated by Sanger sequencing in all family members and tested for segregation. Autozygosity analysis on all affected family members was performed as described elsewhere (Papić et al., 2011). The multi-sample VCF was then uploaded to www.homozygositymapper.org and analyzed with the requirement for genetic homogeneity.

Family 9: The proband and the affected sibling were analyzed at Centogene, Germany. Double stranded DNA capture baits against approximately 36.5 Mb of the human coding exome (targeting >98% of the coding RefSeq from the human genome build GRCh37/hg19) were used to enrich target regions from fragmented genomic DNA with the Twist Human Core Exome Plus kit. The generated library was sequenced on an Illumina platform to obtain at least 20x coverage depth for >98% of the targeted bases. An inhouse bioinformatics pipeline, including read alignment to GRCh37/hg19 genome assembly, variant calling, annotation and comprehensive variant filtering was applied. All variants with minor allele frequency (MAF) of less than 1% in gnomAD database, and disease-causing variants reported in HGMD®, ClinVar or CentoMD® were considered. The investigation for relevant variants was focused on coding exons and flanking +/-20 intronic

nucleotides of genes with a clear gene-phenotype evidence (based on OMIM® information). All potential modes of inheritance patterns were considered. In addition, provided family history and clinical information were used to evaluate identified variants with respect to their pathogenicity and causality. Variants were categorized into five classes (pathogenic; likely pathogenic; VUS; likely benign; benign). All variants related to the phenotype of the patient were reported. Centogene has established stringent quality criteria and validation processes for variants detected by NGS. Variants with low quality and/or unclear zygosity were confirmed by orthogonal methods. Consequently, a specificity of >99.9% for all reported variants was warranted.

Family 10: Patients 13, 14, and 15 from this family have been previously described (Monies et al., 2019). An affected cousin, patient 16, was sequenced using the same approach (Monies et al., 2017). WES was performed using an Agilent Sureselect All Exons V5 (50 Mb) capture kit (Agilent Technologies; Santa Clara, CA, USA) for library preparation. Briefly, DNA was sheared mechanically after which targeted fragments were captured by probe hybridization and amplified before sequencing. An Illumina HiSeq 2500 (Illumina Inc; San Diego, CA, USA) was used for paired-end 100nt sequencing. Sequence alignment, indexing of the reference genome (hg19), variant calling and annotation used a pipeline based on BWA, Samtools, GATK (<https://software.broadinstitute.org/gatk/>) and Annovar, respectively.

Families 11-14 and Families 17-18: Genomic DNA was extracted from peripheral blood samples according to standard procedures of phenol chloroform extraction. WES on each proband was performed as described elsewhere (Mencacci et al., 2016) in Macrogen, Korea. Briefly, target enrichment was performed with 2 µg genomic DNA using the SureSelectXT Human All Exon Kit version 6 (Agilent Technologies, Santa Clara, CA, USA) to generate barcoded whole-exome

sequencing libraries. Libraries were sequenced on the HiSeqX platform (Illumina, San Diego, CA, USA) with 50x coverage. Quality assessment of the sequence reads was performed by generating QC statistics with FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>).

The bioinformatics filtering strategy included screening for only exonic and donor/acceptor splicing variants. In accordance with the pedigree and phenotype, priority was given to rare variants (<0.01% in public databases, including 1,000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, and Exome Aggregation Consortium [ExAC v0.2]) that were fitting a recessive (homozygous or compound heterozygous) or a de novo model and/or variants in genes previously linked to developmental delay, intellectual disability, and other neurological disorders. The families were collected as part of the SYNAPS Study Group collaboration funded by The Wellcome Trust and strategic award (Synaptopathies) funding (WT093205 MA and WT104033AIA). This research was conducted as part of the Queen Square Genomics group at University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. The affected siblings and parents were whole exome sequenced on the Illumina Hiseq 4000 platform, with pair-end 150 bp sequencing. The TRAPPC6B variant was confirmed by Sanger sequencing.

Family 15: DNA was extracted from peripheral blood samples from the two affected brothers and healthy parents. Trio exome capturing was carried out using Agilent SureSelect Target Enrichment Clinical Research Exome V2 (Agilent Technologies, Santa Clara, CA, USA). Sequencing (paired-end 150bp) was performed by the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA). Data was demultiplexed by Illumina Software CASAVA. Reads are mapped to the genome (build hg19/GRCh37) with the program BWA (reference: <http://bio-bwa.sourceforge.net/>). Variants are detected with the Genome Analysis Toolkit (reference: <http://www.broadinstitute.org/gatk/>).

Subsequently, variants were filtered with the Alissa Interpret software package (Agilent technologies) on quality (read depth ≥ 10), frequency in databases ($\leq 1\%$ in 200 alleles in dbSNP, ESP6500, the 1000 Genome project or the ExAC database) and location (within an exon or first/last 10 bp of introns). Variants were further selected based on three inheritance models (de novo autosomal dominant, autosomal recessive and X-linked recessive).

Family 16: The proband and affected sibling were sequenced at the Yale Center for Genome Analysis, as previously described (Jin et al., 2020). To summarize, Genomic DNA from venous blood was captured using the Roche Exome V2 capture kit, followed by Illumina DNA sequencing with 40x coverage. WES data was processed independently in Phoenix Children's Hospital (PCH) and Washington University in St. Louis. The sequenced reads were aligned to the reference genome, GRCh37, using BWA-MEM and further processed using GATK Best Practice workflows, which include duplication marking, indel realignment and base quality recalibration, as previously described. Single-nucleotide variants and small indels were called with GATK HaplotypeCaller and annotated using ANNOVAR, dbSNP(v138), 1000 Genomes (August 2015), NHLBI Exome Variant Server (EVS) and the Exome Aggregation Consortium v3 (ExAC). MetaSVM and CADD (v1.3) algorithms were used to predict deleteriousness of missense variants (D-Mis, defined as MetaSVM-deleterious or CADD ≥ 20). Inferred LoF variants consist of stop-gain, stop-loss, frameshift insertions/deletions, canonical splice sites and start-loss. LoF + D-Mis mutations were considered 'damaging'.

The analysis was focused on homozygous variants since the father's sample was not available.

The homozygous variants were filtered for rare (MAF $\leq 10^{-3}$ across all samples in 1000 Genomes, EVS and ExAC) variants that exhibited high-quality sequence reads (pass GATK variant

score quality recalibration) and had a minimum of 8 total reads for the proband. Only LoF variants (stop-gain, stop-loss, canonical splice-site, frameshift indels and start-loss), D-Mis (MetaSVM = D or CADD $\geq$ 20) and non-frameshift indels were considered potentially damaging to protein function.

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Chapter 4: Targeting Premature Termination Codons with Ataluren: Potential Therapeutic Strategy for TRAPPC6B-Related Neurodevelopmental Disorder

4.1 Abstract

Premature termination codons (PTCs) caused by nonsense mutations can lead to the production of truncated and dysfunctional protein products, which are responsible for various hereditary disorders. Translational read-through inducing drugs such as Ataluren can stimulate the ribosomal machinery to bypass the PTC in mRNA, leading to the synthesis of full-length and functional protein. TRAPPC6B is part of TRANsport Protein particle (TRAPP) complexes that function in membrane trafficking. Genetic variations in TRAPP genes have been associated with a group of neurodevelopmental, skeletal and muscular disorders collectively known as “TRAPPopathies”. Nonsense variants in *TRAPPC6B* have been associated with a microcephalic neurodevelopmental disorder, intellectual disability and brain atrophies. Currently, no treatment exists for TRAPPopathies. Here, we investigated the effectiveness of Ataluren in inducing read-through of a *TRAPPC6B* nonsense variant (c.454C>T, p.Q152*) and compared it to a splice-site variant (c.149+2 T>A) as a control. Our findings demonstrate that Ataluren can rescue membrane trafficking and Golgi morphology defects caused by the TRAPPC6B nonsense variant. Using Ataluren to induce read-through of TRAPPC6B may offer a potential therapeutic strategy for nonsense mutations in various *TRAPP* genes responsible for the TRAPPopathy disorders.

4.2 Introduction

Neurodevelopmental disorders (NDDs), refer to a group of conditions that affect brain development and function, including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), intellectual disability (ID), childhood epilepsy disorders along with several other rare genetic disorders (Lord et al., 2018; Park et al., 2015; Shea, 2012; Tripp and Wickens, 2009; Zablotsky et al., 2019). These conditions can cause a range of impairments, from mild to severe delayed development, and can affect various abilities related to learning, memory, emotion, movement, language and/or non-verbal communication (Zablotsky et al., 2019).

Membrane trafficking is vital for normal cell function, and ultimately brain function. Processes such as neuronal growth and migration, synaptic transmission, and myelin sheath production rely heavily on the efficient movement of proteins and lipids between organelles of the secretory pathway. Defects in this process are known to be involved in a variety of human neurological disorders (Ghosh et al., 2021; Kitagishi et al., 2015; Passemard et al., 2017; Rasika et al., 2018; Tang, 2021).

TRAnsport Protein Particle complexes (TRAPP^{II} and TRAPP^{III}) are multi-subunit complexes that perform fundamental roles in subcellular trafficking pathways (Bassik et al., 2013; Scrivens et al., 2011). Both TRAPP^{II} and TRAPP^{III} have a common core composed of several subunits including TRAPPC1, TRAPPC2, TRAPPC2L, two copies of TRAPPC3, TRAPPC4, TRAPPC5 and TRAPPC6 (Sacher et al., 2019b; Scrivens et al., 2011). TRAPP^{II} has two additional subunits, TRAPPC9 and TRAPPC10, and functions as a guanine nucleotide exchange factor (GEF) for Rab11 (Bagde and Fromme, 2022; Riedel et al., 2018). On the other hand TRAPP^{III} acts as a GEF

for Rab1 and has four additional subunits, namely TRAPPC8, TRAPPC11, TRAPPC12 and TRAPPC13 (Galindo et al., 2021).

Several *TRAPP* genes have been implicated in NDDs. Genetic variation in *TRAPPC2L*, *TRAPPC4*, *TRAPPC6*, *TRAPPC9*, *TRAPPC10*, *TRAPPC11*, and *TRAPPC12* give rise to diverse but overlapping traits including but not limited to microcephalic neurodevelopmental delay, ID/mental retardation, ADHD, epilepsy, autistic features, facial dysmorphism, speech delay, and brain atrophy including decreased white matter and thin corpus callosum (Abbasi et al., 2017; Al-deri et al., 2020; Koehler et al., 2017; Marin-Valencia et al., 2018; Milev et al., 2018, 2017; Mohamoud et al., 2018; Rawlins et al., 2022; Van Bergen et al., 2020).

Two homozygous nonsense variants in *TRAPPC6B* were reported in two separate families of Lebanese and Iranian origin (Harripaul et al., 2018; Nair et al., 2019). Previously, we identified 7 homozygous nonsense variants in *TRAPPC6B* individuals with microcephalic neurodevelopmental disorder, epilepsy, intellectual disability, movement disorders and brain atrophy (Almousa et al., 2023). Nonsense mutations creates premature termination codons (PTCs), which often results in reduced mRNA levels due to degradation by nonsense-mediated mRNA decay. In addition, the mutation can cause premature termination of translation, resulting in the production of a truncated, non-functional protein (Chang et al., 2007).

The TRAPPC6B nonsense variant (c.454C>T, p.Q152*) resulted in reduced levels of TRAPPC6B, in addition to reduced rate of trafficking into the Golgi and Golgi fragmentation in fibroblasts derived from patients (Almousa et al., 2023).

Currently, there is no pharmacologic therapy available to specifically target NDDs or to improve the condition of affected individuals. However, there is growing interest in exploring new avenues to address the underlying genetic defect caused by nonsense mutations. In this context, translational read-through inducing drugs (TRIDs) have emerged as promising therapeutic options. TRIDs, like Ataluren, have shown potential in promoting translational read-through of nonsense mutations, which can help to restore the function of the mutated proteins (Bushby et al., 2014; Devinsky et al., 2021; Drake et al., 2013; Welch et al., 2007).

Ataluren, also known as PTC124, is a small molecule drug that promotes the read-through of PTCs during protein synthesis (Welch et al., 2007). It works by competing with eukaryotic release factors 1 and 3 (eRF1 and eRF3), which would normally terminate translation at a PTC (Huang et al., 2022). Instead, Ataluren prevents the release of the growing polypeptide from the ribosome, allowing the ribosomal machinery to continue translating beyond the PTC. This results in the production of a full-length, functional protein (Ng et al., 2021; Welch et al., 2007). As a result, Ataluren has been used to treat several genetic disorders caused by nonsense mutations, including Duchenne muscular dystrophy (DMD) and cystic fibrosis (Bushby et al., 2014; Campbell et al., 2020; Kerem et al., 2014). Ataluren was approved by the European Medicines Agency for the treatment of nonsense-mediated DMD (Michorowska, 2021) and is currently being investigated in clinical trials for other conditions, such as epilepsy, lysosomal storage disorders, and certain forms of inherited retinal diseases (Beryozkin et al., 2022; Devinsky et al., 2021; Leung et al., 2022; Wang et al., 2022).

Here, we evaluate the ability of Ataluren to rescue cellular defects caused by a nonsense mutation in TRAPPC6B. We show that Ataluren can rescue membrane trafficking and Golgi morphology

defects in fibroblasts derived from individuals with a TRAPPC6B PTC variant but not a splice-site variant. These findings highlight the potential of protein synthesis modulation as a promising therapeutic approach for treating this debilitating condition.

4.3 Materials and Methods

4.3.1 Cell Cultures

Primary fibroblasts from individuals carrying the c.454C>T, p.Q152* nonsense variant or the c.149+2 T>A predicted splice-site variant were grown in DMEM supplemented with 10% FBS. Once cells reached 70-80% confluence, they were incubated in the presence/absence of 5 µg/mL Ataluren (Selleckchem, Canada) dissolved in dimethyl sulfoxide (DMSO) for 48 hours.

4.3.2 Membrane Trafficking Assay

The retention using selective hooks (RUSH) assay was performed as described in Boncompain et al., 2012. Briefly, fibroblasts were transfected by electroporation with the Golgi-resident enzyme sialyl transferase-GFP (ST-GFP) fused to streptavidin binding protein. The plasmid also expressed KDEL-tagged streptavidin for endoplasmic reticulum (ER) retention. 24 hours after transfection, biotin was added to a final concentration of 60 µM to release the reporter from the ER hook. Live cells were imaged by fluorescence microscopy every 2 minutes for 60 minutes using a Nikon Livescan sweptfield confocal microscope with a 40X objective lens (NA 0.95). Integrated fluorescence intensity at the Golgi region and from the whole cell was measured every 2 minutes using ImageJ v1.53. The ratio between fluorescent intensities within the Golgi and whole cell was generated for each time point. The first time point corresponding to background was subtracted from all time points. These values were then plotted as the mean percentage of maximal intensity.

4.3.3 Golgi Morphology

Fibroblasts were washed 2 times with phosphate buffered saline solution (PBS) then fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, quenched with 0.1 M glycine for 10 minutes and permeabilized with 0.1% Triton X-100 for 7 minutes. Blocking was performed in 5%

normal goat serum in PBS for 45 minutes at room temperature. Primary antibodies were prepared in 5% normal goat serum and were added to the cells and incubated overnight at 4°C. Cells were washed 2 times for 5 minutes each with PBS. Secondary antibodies were diluted in 5% normal goat serum in PBS and incubated with the cells at room temperature for one hour and then removed. Hoechst 33342 (Thermo Fisher Scientific) was diluted in PBS (1:2000) and added to stain the nucleus for 2 minutes followed by 2 washes with PBS for 10 minutes each. The coverslips were then mounted with Prolong Gold Anti Fade. Images were acquired on an Olympus FV10i confocal laser scanning microscope fitted with a 60X objective (NA 1.35). The number of Golgi fragments per cell was quantified using Imaris software c.9.8.0 (Bitplane, Concord, MA). Golgi structures were identified from the mannosidase II channel using the following surface parameters; surface details = 0.22 μ m and thresholding with a background subtraction of the depicted Golgi spherical structures that have diameter smaller than 0.55 μ m in size. Statistical analyses were carried out using one-way ANOVA corrected for Tukey's multiple comparisons using GraphPad Prism 6.01. A p-value of 0.05 was considered to be statistically significant.

4.3.4 Immunoblotting

Fibroblasts were washed twice with PBS and lysed in a solution containing 50mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% Triton X-100 and protease inhibitor cocktail (EDTA-free; Roche). The lysate was clarified at 13000 $\times$ g for 30 minutes at 4°C. A total of 30-40 μ g of whole cell lysate was fractionated in either 8% or 15% SDS-polyacrylamide gels (SDS-PAGE). The gels were transferred to nitrocellulose membranes and blocked with 5% skim milk powder in PBS with 0.1% Tween (PBS-T) for 1 hour. Primary antibodies were incubated in PBS-T overnight at 4°C, washed 3 times with PBS-T for 5 minutes each, and secondary antibodies were incubated for 1 hour at room temperature. Membranes were then washed 3 times with PBS-T for

5 minutes each and incubated with Pierce ECL western blot substrate (Thermo Fisher Scientific) and detected using an Amersham Imager 600 (GE Healthcare).

4.3.5 Antibodies

Antibodies used in this study were: anti-TRAPPC6B (1:1000 Rabbit polyclonal, ABclonal A15561), anti- α -Tubulin (1:5000 mouse monoclonal, Sigma T6199), anti-mannosidase II (Man II) (1:200 kind gift from Dr Kelley Moreman).

4.4 Results

We previously described several individuals from unrelated families who have NDD, ID and microcephaly associated with biallelic *TRAPPC6B* variants (Almoussa et al., 2023). Here we examined the ability of Ataluren in suppressing a PTC associated with the *TRAPPC6B* nonsense variant in Subject 1 (S1), who is of Moroccan descent and carries a homozygous nonsense variant (c.454C>T, p.Q152*) in *TRAPPC6B*. Subject 2 (S2), of Pakistani origins, has a predicted splice-site variant (c.149+2 T>A) in *TRAPPC6B* and was used as a non-PTC control. Clinical characteristics of both subjects are summarized in (Table 4.1).

Table 4.1: Clinical Features of TRAPPC6B patients.

Patient	Variant	cDNA NM_001079537.1	Protein	Sex	Age at diagnose	Walk	Sit	Verbal	Muscle loss	Microcephal y	ID\ GDD	ASD
S1	HMZ Stop	c.454C>T	p.Q152*	M	10y	0	0	+	N/A	No -0.5 SD	+	0
S2	HMZ Splice-Site	c.149+2 T>A	-	F	11y	++	+	+	+	-6 SD 11y	+	N/A

HMZ Homozygous. Symbols describing features: + defects present; 0=no abnormalities; N/A data not available.

Walking (0=walks unassisted, ++=cannot walk) Sitting (0=sits without support, +=cannot sit); Verbal (0=normal speech, +=babble or vocalize); Muscle loss (+=atrophy or reduced bulk with or without hypotonia).

4.4.1 Ataluren rescues the membrane trafficking defect in the c.454C>T, p.Q152* fibroblasts

Ataluren has been shown to rescue cellular defects caused by nonsense mutations (Welch et al., 2007; Wu et al., 2022). Fibroblasts from both subjects displayed membrane trafficking defects manifested as reduced rate of trafficking into the Golgi (Figure 4.1 A and Almoussa et al., 2023).

To investigate whether Ataluren could rescue these defects, we employed the RUSH assay by

transfecting a fluorescently tagged Golgi protein (sialyl transferase (ST)-eGFP) into fibroblasts and treating them with Ataluren (5 μ g/ml) for 48 hours. Biotin was added to release the ST-eGFP from the ER and arrival in the Golgi was monitored. As shown in **Figure 4.1**, S1 and S2 fibroblasts (non-treated) have reduced rate of transport of the cargo protein (ST-eGFP) from the ER to the Golgi. After Ataluren treatment, the rate of transport in S1 fibroblasts was greatly increased to near control levels, while neither control nor S2 fibroblasts showed any changes following Ataluren treatment (**Figure 4.1**). These findings support previous research indicating that Ataluren can only rescue cellular defects caused by nonsense mutations.

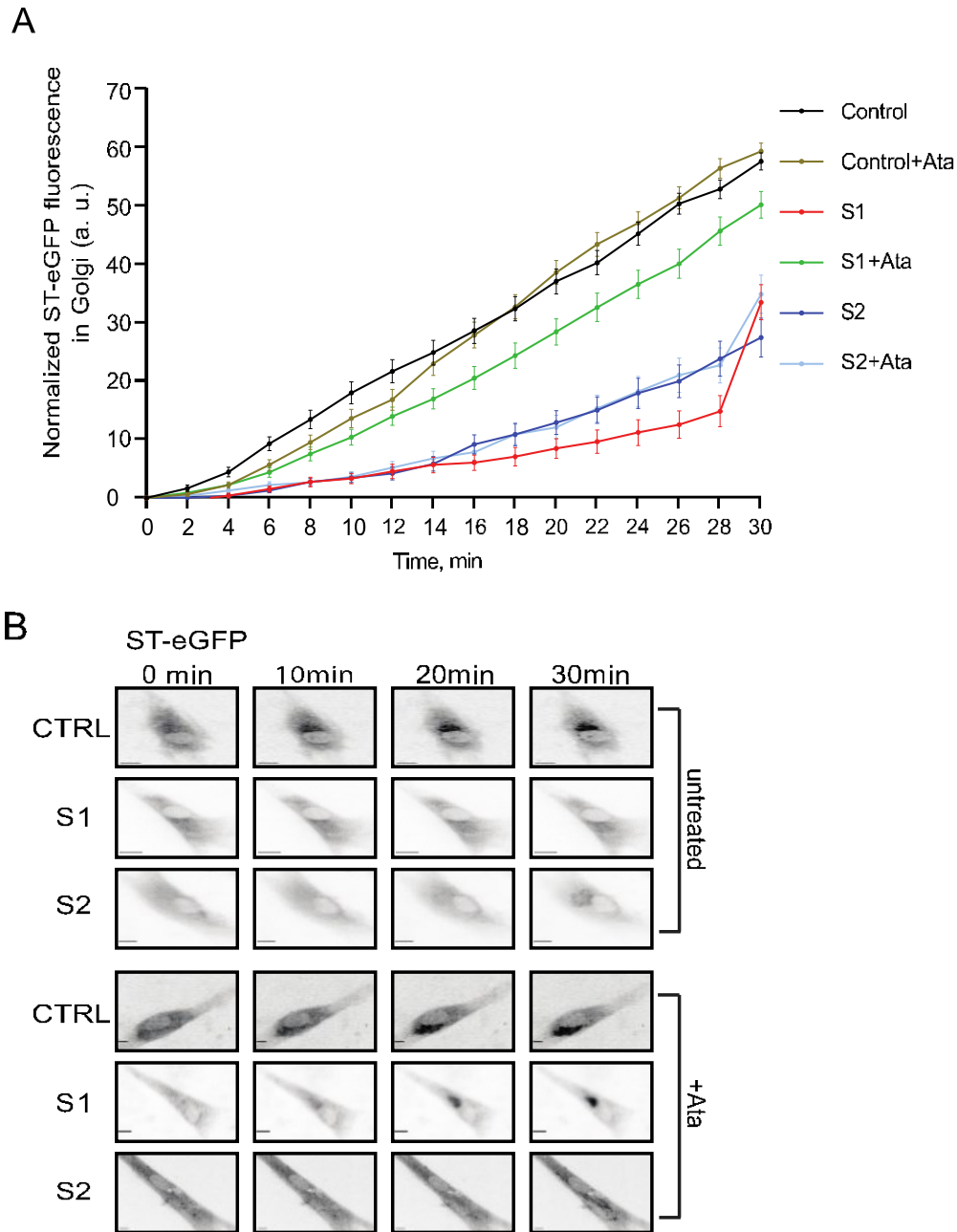


Figure 4.1 Ataluren treatment rescues ER to Golgi transport in human fibroblasts with a *TRAPPC6B* nonsense variant.

A. Fibroblasts were untreated or treated with Ataluren (5 μ g/ml). 24h later cells were transfected with ST-GFP and incubated overnight. The next day, cells were treated with 60 μ M biotin and imaged every 2 minutes. Golgi-associated fluorescence was quantified and plotted as a function of time. (N values ranged from 45 – 60) and come from three biological replicates. **B.** Representative images used for quantification of the RUSH trafficking assay at 0, 10, 20 and 30 minutes. The scale bar represents 10 μ m.

4.4.2 Ataluren rescues the fragmented Golgi morphology defect in the c.454C>T, p.Q152* fibroblasts.

Our previous study (Almoussa et al., 2023) revealed that *TRAPPC6B* variants lead to dispersal of Golgi compartments throughout the cytoplasm. This altered Golgi morphology can be rescued with wild type *TRAPPC6B*. To further investigate the ability of Ataluren to restore normal Golgi morphology, we stained fibroblasts with the cis-Golgi marker mannosidase II (Man II). Consistent with our previous findings, fibroblasts from both subjects exhibited Golgi morphological alterations characterized by dispersed Golgi membranes (**Figure 4.2**). However, after Ataluren treatment, Golgi morphological alterations in S1 fibroblasts were rescued, while neither control nor S2 fibroblasts display any changes following treatments (**Figure 4.2**). These results are consistent with the ability of Ataluren to exclusively rescue cellular defects caused by nonsense mutations.

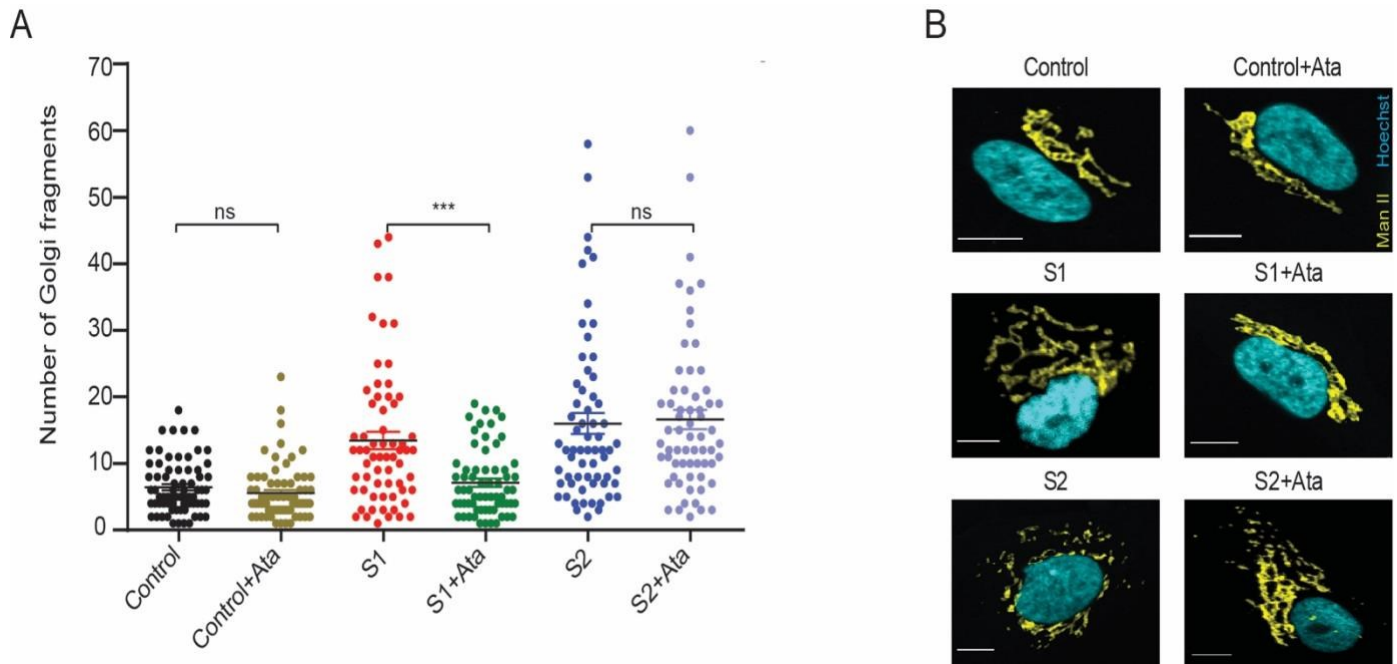


Figure 4.2 Ataluren treatment rescues the altered Golgi morphology in human fibroblasts with a *TRAPPC6B* nonsense variant.

A. Fibroblasts were either treated or untreated with Ataluren (5μg/ml) for 48h, fixed and stained for mannosidase II as a Golgi marker. Golgi fragments per cell were quantified from mannosidase II-positive structures. Statistical analyses were carried out using one-way ANOVA corrected for Tukey's multiple comparisons. *** $p < 0.001$. N values ranged from 64-70 and come from three biological replicates. **B.** Representative images used for quantifying the number of Golgi fragments. The scale bar represents 10 μm.

4.4.3 Ataluren does not induce observable read-through in lysate from the c.454C>T, p.Q152* fibroblasts.

The p.Q152* variant causes a significant reduction in detectable levels of TRAPPC6B, possibly due to nonsense mediated decay or production of an unstable truncated polypeptide. Ataluren facilitates the production of a fully functional protein by preventing the release of the polypeptide from the ribosomal machinery when it encounters the PTC site, thereby promoting read-through of PTCs. Our results above suggest that Ataluren could overcome the PTC in the *TRAPPC6B* c.454C>T, p.Q152* variants and enable translation of the full length TRAPPC6B protein. To this end, we treated fibroblasts derived from subject S1 as well as control cells with Ataluren (5μg/ml)

for 48h. However, western blot analysis revealed no observable increase in TRAPPC6B protein levels in the treated group (**Figure 4.3**). These results suggest that Ataluren may induce a marginal increase in TRAPPC6B levels, enough to rescue the cellular defects but not substantial enough to be detected through immunoblotting.

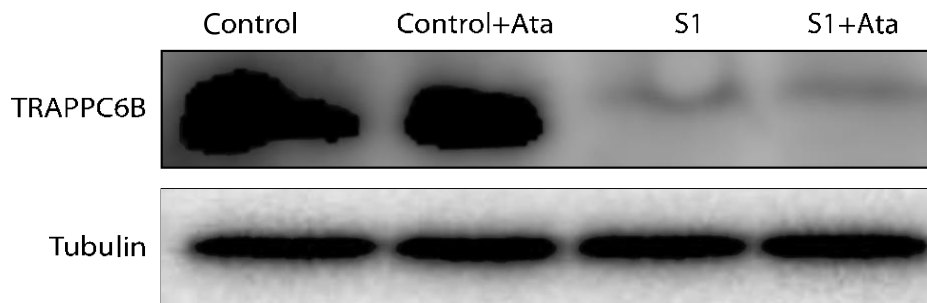


Figure 4.3 Ataluren treatment does not alter TRAPPC6B levels in fibroblasts with the c.454C>T, p.Q152* variant.

Control and S1 patient fibroblasts were untreated or treated with (5µg/ml) Ataluren for 48h, Lysate was prepared and fractionated by SDS-PAGE and probed for the TRAPPC6B protein. Tubulin was included as a loading control.

4.5 Discussion

NDDs are complex and heterogeneous conditions with considerable clinical overlap. Despite the pressing need for effective treatments, options are currently limited (Tripp and Wickens, 2009; Zablotsky et al., 2019). However, TRIDs such as Ataluren offer a promising new approach to these challenging disorders, particularly in cases where NDDs are caused by nonsense mutations leading to PTCs (Welch et al., 2007). In this study, we investigated the potential of Ataluren to rescue cellular defects in patients with a *TRAPPC6B* nonsense variant who suffer from NDD and ID.

Our results demonstrate that Ataluren treatment restored normal cargo movement between ER and Golgi, as well as rescued altered Golgi morphology caused by the *TRAPPC6B* nonsense variant c.454C>T, p.Q152*. As expected, the treatment was ineffective against the *TRAPPC6B* predicted splice-site variant c.149+2 T>A. Several TRAPP genes have been associated with NDDs and genetic variation of these genes have been shown to have membrane trafficking defects and altered Golgi morphology (Milev et al., 2019, 2018, 2017; Rawlins et al., 2022). Membrane trafficking is essential for proper functioning of the cells, especially in neuronal cells that have a high demand for cellular trafficking (Kitagishi et al., 2015; Passemard et al., 2017). The Golgi apparatus is responsible for protein and lipid trafficking, sorting, and modification, and disruptions to its normal morphology can impact membrane trafficking homeostasis. Structural and functional changes to the Golgi can affect the central or peripheral nervous system, highlighting the critical importance of the Golgi for neural function (Liu et al., 2021; Makhoul et al., 2019). Individuals with *TRAPPC6B* variants share similarities with those who have defects in genes encoding Golgi-associated proteins involved in membrane trafficking. These disorders are often characterized by ID, microcephaly and white matter defects (Passemard et al., 2017; Rasika et al., 2018). Therefore, using Ataluren to restore normal Golgi morphology and membrane trafficking in *TRAPPC6B* cells has the potential for restoring normal cell functioning.

Although Ataluren treatment successfully rescued membrane trafficking and Golgi morphology in fibroblasts with the PTC in TRAPPC6B, our immunoblotting assay did not detect observable read-through of the UAG termination codon caused by the c.454C>T, mutation. It is possible that the mRNA degradation by NMD led to low read-through of the UAG PTC. However, studies on nonsense-mediated cystic fibrosis have revealed that a significant reduction in the severity of the disease can be achieved by boosting functional protein synthesis to as little as 5% of normal levels (Ramalho et al., 2002). Therefore, it is plausible that the rescue of cellular defects in the TRAPPC6B fibroblasts with the PTC variant may result from boosting TRAPPC6B protein levels by small amounts that are undetectable by immunoblotting. To address this issue, the use of NMD inhibitors in combination with TRIDs may increase the amount of functional full-length protein (Gonzalez-Hilarion et al., 2012). Research has demonstrated that reactivating neuronal plasticity in the adult brain through pharmacotherapy can lead to significant improvements in various aspects of behavior (Castrén et al., 2012). Mouse models of NDDs further support the notion that certain molecular and behavioral deficits such as synaptic maturation deficits, seizures, hyperactivity, learning deficits, and anxiety problems, associated with these disorders can be reversed in adults through pharmacological interventions (de Vrij et al., 2008; McBride et al., 2005). The safe use of Ataluren and its ability to rescue cellular defects caused by the TRAPPC6B PTC variant offers new possibility for using TRIDs in combination with rehabilitation to alleviate or reverse the symptoms associated with nonsense mutations in NDDs. With this promising potential, a minor improvement in patient performance could yield significant contributions to their overall health and wellbeing.

Chapter 5: Discussion

5.1 Overview

The TRAPPII complex is a multi-subunit complex that is highly conserved and functions as a GEF for Rab11 (Bagde and Fromme, 2022; Riedel et al., 2018). In yeast, the complex has been shown to be involved in protein secretion, endosomal trafficking, and autophagy (Cai et al., 2005; Zou et al., 2013). However, the exact functions of TRAPPII in mammalian cells are not yet fully understood. While metazoan TRAPPII shares several features with yTRAPPII, there are some notable differences in their composition and function. For instance, the Trs65 homolog TRAPPC13 is a part of the TRAPPIII complex rather than TRAPPII, and its presence is necessary for the dimerization of the yeast complex. Unlike in yeast, TRAPPII in metazoans is monomeric (Bassik et al., 2013; Tokarev et al., 2009). These distinctions underlie functional differences between fungal and metazoan TRAPPs.

Here, I have identified biallelic variants in TRAPPC10 and TRAPPC6B as a cause of a severe NDD characterized by microcephaly and ID. Furthermore, I have observed that in cells derived from individuals with mutations in TRAPPC10 and TRAPPC6B, components of the TRAPPII complex are affected, while components of the TRAPPIII complex are unaffected in individuals with mutations in TRAPPC10 and slightly affected in individuals with mutations in TRAPPC6B. Additionally, I observed a delay in membrane trafficking between the ER and Golgi. These observations, coupled with the overlapping phenotypes of the TRAPPC10 and TRAPPC6B mutations, suggests a common molecular pathway leading to disease involving disruption of TRAPPII complex functions.

In addition to identifying the genetic and the molecular basis of the NDD associated with TRAPPC10 and TRAPPC6B mutations, I have also shown a possible therapeutic strategy for the treatment of such conditions. Specifically, I investigated the effect of Ataluren, a small molecule that allows the read-through of PTCs in nonsense mutations. I showed that Ataluren was able to rescue cellular defects associated with the TRAPPC6B nonsense variant, providing evidence for its potential therapeutic effect in individuals with this particular variant. These results have important implications for understanding the underlying molecular pathology of TRAPP II gene variants and developing effective treatment strategies for affected individuals.

5.2 Pathogenic TRAPPC10 and TRAPPC6B variants impact the stability of the TRAPP II complex

The study of genetic variations helps understand protein functions. Pathogenic protein variants cause phenotypic changes as they can alter the shape, stability, binding properties and ultimately the function of the protein. In chapter 2, I showed that TRAPPC10 variants affect the stability of the protein and impacted its binding capabilities. Both p.Gly1131Valfs*19 and p.Pro929Leu variants of TRAPPC10 failed to interact with the adaptor protein TRAPPC2L which led to disruption of TRAPP II complex stability. Similarly, in chapter 3, I showed that p.Q152* variants in TRAPPC6B affected the stability of the protein and impacted its binding properties. Specifically, TRAPPC6B p.Q152* disrupted the interaction with TRAPPC3, an interaction that is crucial for the complex stability as the TRAPPC6-TRAPPC3 heterodimer forms a subcomplex essential for TRAPP II assembly (Kümmel et al., 2006, 2005). Despite the fact that TRAPPC6B is supposedly a component of both TRAPP II and TRAPP III, truncation of the C-terminus in the p.Q152* seemed to affect TRAPP II (TRAPPC10 and TRAPPC9) more than TRAPP III (TRAPPC8 and TRAPPC12). Additionally, I showed that unlike TRAPPC6A which equally associated with both

TRAPP^{II} and TRAPP^{III}, TRAPPC6B preferentially associated with TRAPP^{II}. This suggests that TRAPPC6B plays its essential physiological role within TRAPP^{II}.

TRAPP^{II}-specific subunits switch the GEF activity of the core of TRAPP from Rab1 to Rab11 (Morozova et al., 2006). Genetic disruptions of either *Trs120* and *Trs130* are lethal in *Aspergillus nidulans* and this lethality is rescued by a constitutively active form of Rab11 but not Rab1 (Pinar et al., 2019). Similarly, the double *trs33Δ trs65Δ* mutation in *A. nidulans* is lethal and can only be rescued by an active form of Rab11 and not active Rab1 (Pinar et al., 2019). This further supports the notion that the physiological function of TRAPPC6B resides within TRAPP^{II}.

In chapter 3, I also showed that the p.Q152* variant affected the stability of the TRAPPC6B protein and there was a corresponding increase in the levels of TRAPPC6A (Figure 3.4). The two TRAPPC6 homologs seem to compensate for each other to some extent. This is clearly shown in the mammalian recombinant TRAPP^{II} complex that functions as a GEF for Rab GTPases since one study only used the TRAPPC6A protein (Jenkins et al., 2020). It remains a possibility that studying the GEF activity of the complex in the presence of TRAPPC6B might reveal different outcomes. It is worth mentioning that attempts to precipitate endogenous TRAPPC6A with either TRAPPC10 or TRAPPC11 were not successful in HeLa cells despite detecting the protein in whole cell extracts (data not shown). This could be explained by the low expression levels of TRAPPC6A compared to TRAPPC6B (**Figure 5.1**). Even though RNA transcript levels do not necessarily reflect protein levels, the data suggest a low abundance of TRAPPC6A in different tissues and a higher abundance of TRAPPC6B, with the highest being detected in the cerebellum. Interestingly, this was the brain region which featured decreased volume detected by MRI in *TRAPPC6B*-affected individuals (**see Figure 3.2C**). It is unclear how cells distinguish between the two

TRAPPC6 homologs. To address this, one future direction is to examine the subcellular localization of TRAPPC6A and TRAPPC6B. Employing endogenous labeling might reveal distinct subcellular localization, varying abundances and different association partners of the two homologs. Additionally, To better understand how the paralogues are incorporated into their respective complexes, it is very important to study the consequence of knocking out each paralogue on the stability of the TRAPPII and TRAPPIII complexes. This approach will help to understand the assembly process of TRAPP complexes.

The study of natural variants of proteins is a valuable approach to understand their function, as it allows us to identify crucial protein domains or residues important for their activity. In chapters 2 and 3, I showed that the C-terminus of both TRAPPC10 and TRAPPC6B is essential for the stability of TRAPPII. In the case of TRAPPC10, the loss of the C-terminus seems to have a more dramatic effect on TRAPPII, resulting in a complete absence of a major TRAPPII component, TRAPPC9. This suggests that TRAPPC10 is required for TRAPPC9 incorporation into the TRAPPII complex. A recent cryo-EM structure of the γ TRAPPII further highlight the importance of TRAPPC10 for the function of TRAPPII (Bagde and Fromme, 2022). TRAPPC10 provides a leg that positions the complex on membranes and is also important for the counter selection of Rab1 by TRAPPII. This leg lifts the active site away from the membrane, preventing the shorter hypervariable domain of Rab1 from allowing the GTP-binding pocket of the Rab to access the active site. This highlights the importance of TRAPPC10 for the function of the complex and provides an explanation to the more severe phenotypes associated with TRAPPC10 loss of function compared to TRAPPC9 loss of function, but does not explain the complete absence of TRAPPC9. Further research should investigate the mechanism that leads to loss of TRAPPC9. Overall, these findings suggest that TRAPPC10 is indispensable for the stability of the TRAPPII complex while

TRAPPC6A can compensate for the loss of TRAPPC6B. Gel filtration experiments can provide a better understanding of the impact of TRAPPC6B variants on the stability of the TRAPPII and TRAPPIII complexes. Any alterations in the stability of the complexes due to the TRAPPC6B variants can be determined by subjecting cell lysate from patient cells harboring TRAPPC6B variants to gel filtration chromatography.

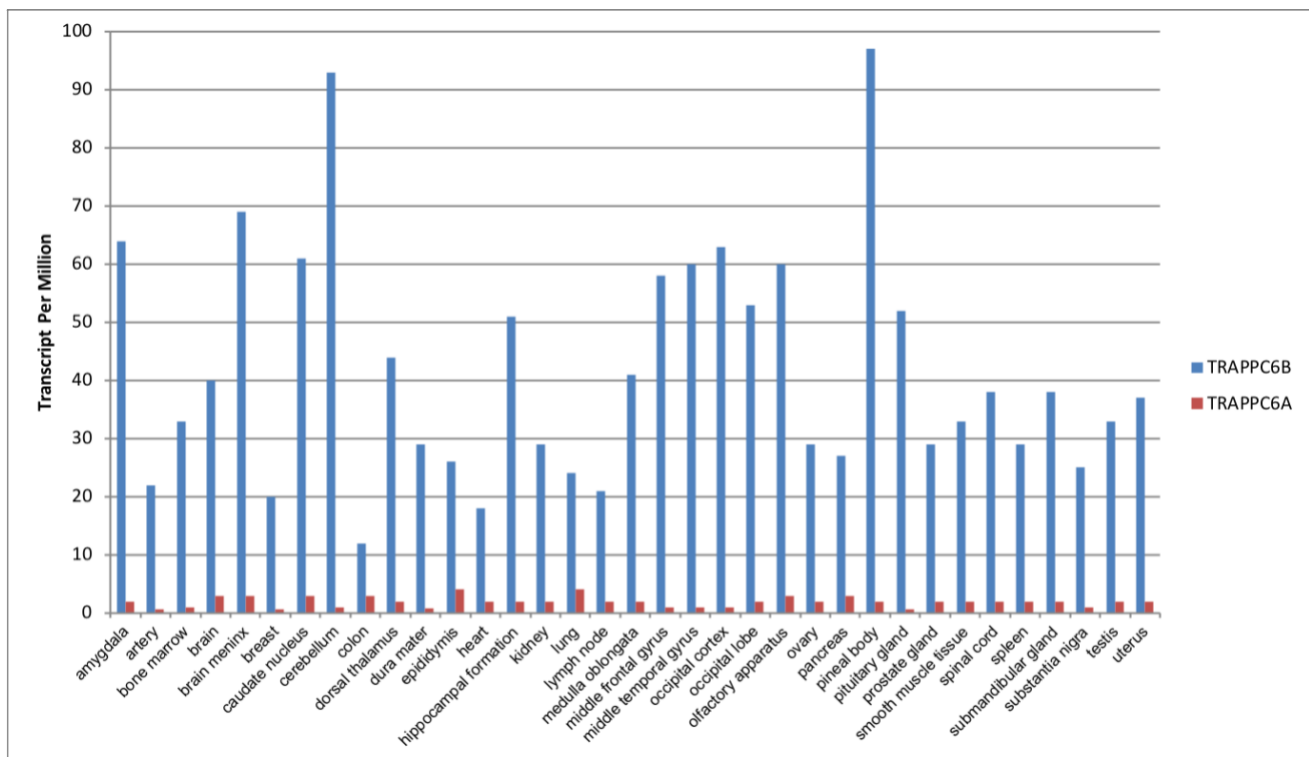


Figure 5.1 *TRAPPC6B* and *TRAPPC6A* gene expression profile of adult human tissue. RNA-seq data from the expression atlas tool (<https://www.ebi.ac.uk/gxa>).

5.3 Pathogenic TRAPPC10 and TRAPPC6B variants disrupt TRAPPII complex and impaired membrane trafficking

As alluded to above, pathogenic variants in TRAPPC10 and TRAPPC6B impact the stability of the TRAPPII complex, without significant impact on the TRAPPIII complex, indicating the latter

complex was not affected and any cellular defect is a consequence of TRAPP^{II} disruption in these cells. Membrane trafficking assays in chapters 2 and 3 revealed similar delayed anterograde trafficking defects as a result of *TRAPPC10* and *TRAPPC6B* disruption.

TRAPP^{II} has been implicated in early Golgi trafficking, TRAPPC10 interacts with COPI and its depletion results in altered Golgi morphology and accumulation of cargo in the Golgi (Yamasaki et al., 2009). Our membrane trafficking assay in fibroblasts harboring a TRAPPC10 variant are consistent with cargo accumulation in the Golgi. Similarly, the RUSH assay in the TRAPPC6B-defective fibroblasts revealed accumulation of cargo molecules at the Golgi as well as altered Golgi morphology. Further investigation is required to explore the mechanisms by which TRAPP^{II} affects the Golgi morphology and membrane traffic into the Golgi. One possibility is the lack of GEF activity of TRAPP^{II} towards Rab11, which mediates intra-Golgi, post-Golgi and endocytic recycling transport (Chen et al., 1998; Ullrich et al., 1996; Urbé et al., 1993).

GEFs are responsible for Rab GTPase recruitment from the cytosol to the correct membrane, and Rabs are mistargeted when their GEFs are disrupted or artificially relocated to a different membrane (Blümer et al., 2013). One future direction is to examine the localization of Rab11 in TRAPPC10- and TRAPPC6B-defective cells and test whether the expression of constitutively-active Rab11 can rescue membrane trafficking defects. Additionally, it is also important to examine the levels of active Rab11 in cells from individuals harboring the TRAPPC10 and TRAPPC6B variants compared to unaffected healthy controls. Furthermore, to explore the potential involvement of Rab1 in the TRAPPopathy disorder associated with TRAPPC10 and TRAPPC6B, the localization of Rab1 should be examined in these cells.

Given the complex interplay between the ER and the Golgi, the ER to Golgi trafficking delay in TRAPPC10- and TRAPPC6B-defective cells is most likely an indirect effect, with cargo accumulation in the Golgi resulting in traffic jam effect that disrupts ER to Golgi transport. Rab1, which regulates ER to Golgi transport, is activated by TRAPPIII and not TRAPPPII (Galindo et al., 2021; Plutner et al., 1991). The trafficking assays in chapters 2 and 3 indicate that the cargo molecules are reaching the Golgi in both normal and patient cells, ruling out a complete blockage in the ER- Golgi transport. To identify the specific stage (intra-Golgi or late-Golgi) at which TRAPPPII affects membrane trafficking, it is important to include specific Golgi markers in the membrane trafficking assays described in chapters 2 and 3, localizing the transported cargo molecule with respective Golgi markers (cis, medial or trans). This will provide better insight into the accumulation of a cargo molecule and reveal the specific stage at which membrane trafficking is defective.

The altered Golgi morphology seen in TRAPPC6B could be a result of a membrane trafficking defect, since a block in ER to Golgi or intra-Golgi transport has been shown to alter Golgi morphology, possibly due to loss of Golgi resident enzymes (Petrosyan, 2019). I did not evaluate Golgi morphology in the p.(Gly1131Valfs*19) or in the TRAPPC10 knockout cells, but given the general disruption of TRAPPPII in these cells, I would expect a similar outcome.

Defects observed in individuals with defects in TRAPPPII proteins raise the question of how they translate into neurological defects. Here I present two possible explanations. The first is related to the high demand for membrane trafficking during the development of neuronal cells, which is crucial for the continued supply of vesicles to achieve rapid cell growth, maintain synaptic transmission and dendritic growth (Pfenninger, 2009; Winkle and Gupton, 2016). Trafficking

defects have been found to suppress dendritic growth in hippocampal neurons (Ye et al., 2007), indicating the crucial role of this pathway. However, a major challenge in understanding the neurological disorder linked to TRAPP II variants is the lack of human neuronal cells for study. Cellular reprogramming of patient cells into patient-derived induced pluripotent stem cells (iPSCs) that can be induced to differentiate into specific neural cells can overcome this challenge and provide better insight in the etiology of NDDs associated with TRAPP II variants (Takahashi et al., 2007). A second explanation is discussed in section 5.4.

5.4 TRAPP II disruption is associated with ciliogenesis defects

TRAPP II has been implicated in ciliogenesis (Cuenca et al., 2019; Westlake et al., 2011) and this cellular process was not investigated in chapters 2 and 3. To investigate the effect of TRAPPC10 deletion on ciliogenesis, we generated a TRAPPC10 knockout in RPE1 cells using CRISPR-Cas9 (**Figure 5.2**). Consistent with the TRAPPC10^{-/-} knockout in HEK293 presented in chapter 2, we noted complete absence of TRAPPC9 with no effect on TRAPP III-specific protein (TRAPPC12) (**Figure 5.2**). My preliminary data showed that upon serum starvation, ciliation was observed in $59.23 \pm 4.27\%$ (mean $\pm$ S.E.M.) of control RPE1 cells. Ciliogenesis was reduced in RPE1 cells lacking TRAPPC10 ($21.41 \pm 1.57\%$) (**Figure 5.3 A-B**). This indicates that TRAPPC10 is important for ciliogenesis in RPE1 cells. Similarly, the percentage of ciliated cells in lymphoblasts derived from an individual with the p.Q152* variant showed a reduction in the percentage of ciliated cells (68.8 %), compared to (85.8 %) in the control cells (**Figure 5.3 C-D**). Although less severe than the defect observed upon TRAPPC10 deletion, this suggests that TRAPPC6B is important for ciliogenesis in human cells.

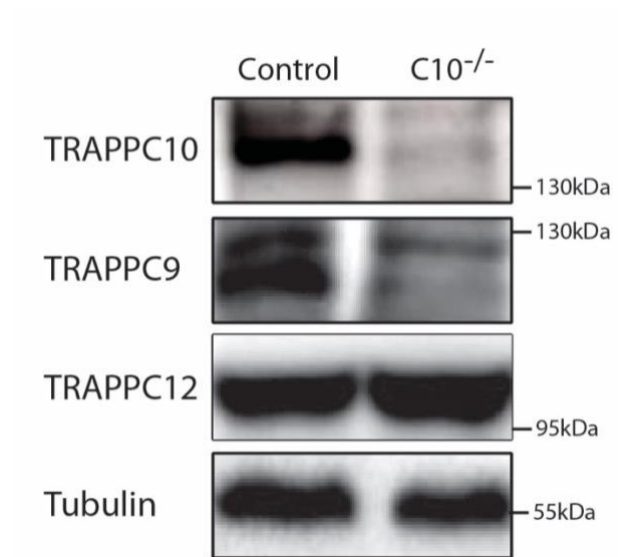


Figure 5.2 Characterization of the *TRAPPC10*^{-/-} cell lines in RPE1 cells

Lysate was prepared from parental RPE1 cells and from *TRAPPC10*^{-/-} that was treated with sgRNA targeting *TRAPPC10*. The lysates were fractionated by SDS-PAGE and probed for the indicated proteins. A representative tubulin blot was included as a loading control.

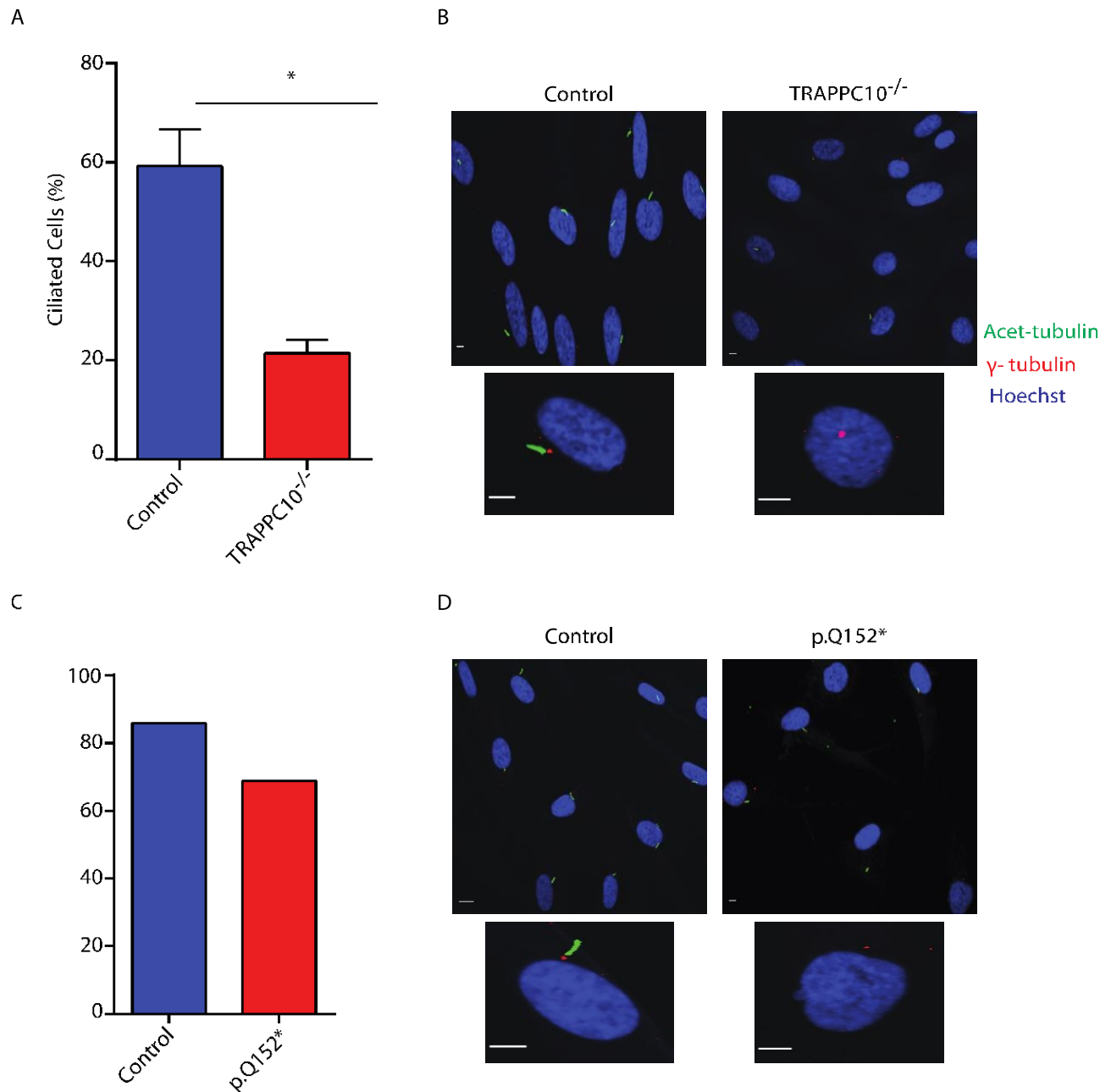


Figure 5.3 TRAPPII disruption impairs cilia formation.

A and **C**. Quantification of ciliation in RPE1 cells (**A**) and human lymphoblasts (**C**), cells were grown in serum starved medium for 48 hours before fixing the cells and staining with anti-acetylated tubulin (green) to reveal cilia, and anti-γ-tubulin (red) to reveal the associated centrosomes and imaged by the Fluoview FV10i confocal microscope. Cells were counted for RPE1, control (n= 1281) and *TRAPPC10*^{-/-} (n= 851) **A**. Cells were counted for human lymphoblasts control (n= 176) and p.Q152* (n= 141) **C**. The percentage of ciliated cells in RPE1 was determined from three different experiments and from one experiment in human lymphoblasts. For (**A**) statistical significance was determined using a paired t-test. * p<0.05. **B** and **D**. Representative images used for quantifying the percentage of ciliated cells. The scale bar represents 5 μm.

The TRAPP-associated protein TRAPPC14 mediates interaction between Rabin8 and the TRAPPII complex. Rabin8 is a GEF for Rab8 and is required for the accumulation of preciliary vesicles at the centrosome, while active Rab11 associates with the Rabin8 C- terminus and is required for Rabin8 preciliary trafficking to the centrosome. The ciliogenesis defects resulting from TRAPPC10 disruption could be attributed to the lack of GEF activity of TRAPPII towards Rab11 or to a defective interaction with Rabin8. In contrast, the TRAPPC6B p.Q152* variant appears to have less severe impact on TRAPPII, which could explain the moderate effect of the TRAPPC6B variant on ciliogenesis.

The primary cilium is a membrane-bound microtubule-based organelle that extends from the surface of the cell body of most eukaryotic cells. it plays a crucial role in early embryonic development by transmitting chemical and mechanical stimuli from the extracellular environment to the cell interior (Anvarian et al., 2019). However, defects in genes involved in the assembly and functioning of cilia can result in ciliopathies, a group of disorders affecting multiple organ systems including the nervous system (Reiter and Leroux, 2017). Primary cilia are dynamically expressed in cycling cells where they are formed in quiescent cells and resorbed during cell cycle re-entry (Avasthi and Marshall, 2012). This highlights the role of cilia in regulating cell division. Similar to mutations in TRAPPII, one study identified TRAPPC14 mutations in individuals with primary microcephaly (Perez et al., 2019), a condition characterized by a small head circumference. Microcephaly has also been attributed to mutations in genes that regulate ciliogenesis, cell proliferation, and survival during development (Reilly et al., 2019; Waters et al., 2015; Willsey et al., 2020). The connection between TRAPPII, ciliogenesis and microcephaly could offer a possible explanation for NDD associated with TRAPPII mutations. Further research is required to understand the precise role of TRAPPII in ciliogenesis during early developmental stages.

5.5 Nonsense mutations in *TRAPP* genes can benefit from TRIDs.

Next-generation sequencing has enabled the identification of new mutations in *TRAPP* genes. The frequency of mutations in the *TRAPP* genes has steadily increased over the past decade (Sacher et al., 2019). TRAPP gene defects are associated with a diverse group of disorders collectively known as TRAPPopathies. Unfortunately, no treatments for these disorders currently exist. In chapter 4, I showed that Ataluren treatment can rescue membrane trafficking defects and altered Golgi morphology caused by a PTC in *TRAPPC6B*. Given the urgent need to develop effective therapeutic approaches for the treatment of the TRAPPopathy disorders, this finding holds great promise. To further investigate the therapeutic potential of Ataluren, it is crucial to assess its effects on different TRAPP mutations, as different TRAPP variants can lead to diverse phenotypes. It is also important to investigate the effectiveness of Ataluren in rescuing other cellular defects related to TRAPP gene defects, such as ciliogenesis. Such research could lead to significant improvements in the health conditions of many patients suffering from TRAPPopathy disorders.

5.6 Conclusion

TRAPPC10 and TRAPPC6B are both part of the TRAPPII complex, a GEF for Rab11 and a tethering factor that mediates early and late Golgi transport. The cellular functions of TRAPPII can be inferred from cellular defects caused by disruptions in its components. In my thesis, I define the genetic and molecular basis of NDD associated with biallelic variants in *TRAPPC10* and *TRAPPC6B*. The data presented in chapters 2 and 3 provide insight into the molecular mechanisms underlying the phenotypic overlap between TRAPPC10- and TRAPPC6B-related disorders, which involves the disruption of TRAPPII and pathways mediated by this protein complex. These findings support the conclusion that TRAPPC10, and to a lesser extent TRAPPC6B are important

for the stability and function of TRAPP^{II}, and that impairments in these proteins can lead to a distinct and recognizable TRAPPopathy caused by either a membrane trafficking defect essential for proper brain functioning or by a ciliogenesis defect crucial for brain development in the early developmental stages. The association of these variants with disease highlights the importance of TRAPPC10 and TRAPPC6B in normal cell physiology and pathology.

Taken together, my findings suggest that mutations in TRAPPC10 and TRAPPC6B cause severe neurodevelopmental disorders by disrupting the TRAPP^{II} complex function, and that this dysfunction can be targeted by a novel therapeutic compound.

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