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REVIEW

SUBJECT COLLECTION: MEMBRANE TRAFFICKING

Dopey proteins are essential but overlooked regulators of membrane trafficking

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ABSTRACT

Dopey family proteins play crucial roles in diverse processes from morphogenesis to neural function and are conserved from yeast to mammals. Understanding the mechanisms behind these critical functions could have major clinical significance, as dysregulation of Dopey proteins has been linked to the cognitive defects in Down syndrome, as well as neurological diseases. Dopey proteins form a complex with the non-essential GEF-like protein Mon2 and an essential lipid flippase from the P4-ATPase family. Different combinations of Dopey, Mon2 and flippases have been linked to regulating membrane remodeling, from endosomal recycling to extracellular vesicle formation, through their interactions with lipids and other membrane trafficking regulators, such as ARL1, SNX3 and the kinesin-1 light chain KLC2. Despite these important functions and their likely clinical significance, Dopey proteins remain understudied and their roles elusive. Here, we review the major scientific discoveries relating to Dopey proteins and detail key open questions regarding their function to draw attention to these fascinating enigmas.

KEY WORDS: Retrograde trafficking, Microtubule motors, Lipid asymmetry, Extracellular vesicle, Morphogenesis, Myelination

Introduction

Dysregulation of Dopey proteins is linked to developmental defects during gastrulation (Guipponi et al., 2000), as well as to neurological conditions and diseases like Down syndrome and Peters anomaly (Rachidi et al., 2009; Darbari et al., 2020). The essential role of Dopey proteins was discovered almost 50 years ago in the fungus *Aspergillus nidulans* (Axelrod et al., 1973; Yager et al., 1982), but Dopey proteins were only molecularly characterized in 2000 (Pascon and Miller, 2000). Bruce L. Miller coined the novel protein family 'Dopey' due to the various morphological defects making the DopA mutant fungi look 'doped' (R. Pascon, Universidade Federal de São Paulo, Brazil, personal communication). Since then, work in multiple model organisms has expanded our knowledge on the physiological functions of Dopey proteins and their molecular interactions.

Dopey proteins are conserved from yeast to man, with many species only expressing a single Dopey (Table 1), suggesting conservation of their functional roles. Dopey proteins are large and form a complex with MON2 and the essential subfamily of P4-ATPases that is thought to remodel membranes (Barbosa et al., 2010; McGough et al., 2018). MON2 is a large non-essential protein related to Arf GTPase guanine nucleotide exchange factors (GEFs), although it does not possess guanine nucleotide exchange activity (Mahajan et al., 2013). The essential P4-ATPases, including yeast Neo1 and worm TAT-5, transport aminophospholipids from the extracellular or luminal face of membrane bilayers to the cytosolic leaflet (Beer et al., 2018; Takar et al., 2016). Together, these proteins are thought to scaffold other proteins and alter lipid localization to modulate membrane tubulation or budding.

Dopey proteins act in multiple cellular processes that involve membrane trafficking. They regulate retrograde recycling of cargoes from endosomes back to and within the Golgi (Gillingham et al., 2006; Zhao et al., 2019), trafficking to multivesicular endosomes (Tanaka et al., 2014; Barbosa et al., 2010) and can interact with microtubule-associated motor proteins to position organelles (Mahajan et al., 2019). They also influence plasma membrane asymmetry and regulate extracellular vesicle formation (Beer and Wehman, 2017). Despite these many essential and clinically relevant functions, Dopey proteins remain something of an enigma, largely due to the limited number of laboratories studying them. In order to increase awareness of these essential regulators, we summarize the current state of knowledge on Dopey proteins and highlight the many open questions on how they function.

Molecular structure and interactions

Dopey proteins are known to associate with membranes (Efe et al., 2005; Pascon and Miller, 2000; Tanaka et al., 2014), although the mechanisms are still under study. Originally, some fungal Dopey proteins were proposed to localize to membranes via a short hydrophobic stretch in the C-terminus that was predicted to be a putative transmembrane domain (Pascon and Miller, 2000). Hydrophobic regions can be identified bioinformatically in other Dopey proteins (Fig. 1A), but it is unlikely that they represent transmembrane domains. Human DOPEY1 (DOP1A) can be extracted in low-detergent conditions, consistent with the absence of any transmembrane domains (Mahajan et al., 2019). Similarly, the Dopey ortholog PAD-1 can be solubilized efficiently after boiling of *Caenorhabditis elegans* extracts, a method that causes aggregation of transmembrane proteins, including the PAD-1 interactor TAT-5 (Beer, 2021). Thus, the experimental evidence so far suggests that Dopey proteins are peripheral membrane proteins, not transmembrane proteins.

Dopey proteins have been shown to associate with membranes by binding to lipids. Human DOPEY1 localizes to the Golgi through an LKRL motif in its C-terminus, which mediates binding to liposomes containing the phospholipid phosphatidylinositol-4-phosphate (PI4P) (Figs 1A,C and 2) (Mahajan et al., 2019). Since PI4P is found on the Golgi, endosomes, lysosomes and plasma membrane (Boutry and Kim, 2021; Moriwaki et al., 2018; Viaud et al., 2016), this PI4P-binding motif is thought to contribute to the localization of Dopey proteins to these structures. The LKRL motif is conserved in flies and nematodes, but not in fungi or in the second human homolog DOPEY2 (DOP1B) (Fig. 1C). Instead, DOPEY2

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Table 1. Dopey proteins and their interactors in relevant model organisms

	Dopey proteins	Mon2	Flippases
Mammals	DOPEY1 DOPEY2	MON2	ATP9A ATP9B
Caenorhabditis elegans	PAD-1	MON-2	TAT-5 TAT-6
Saccharomyces cerevisiae	Dop1	Mon2	Neo1
Aspergillus nidulans	DopA	AN3643	DnfD

contains a LKRQ motif and is unable to bind to PI4P-containing liposomes (Mahajan et al., 2019), despite DOPEY2 localizing to membranous organelles, including the Golgi (Mahajan et al., 2019; Zhao et al., 2020). Furthermore, a C-terminal fragment of human DOPEY2 is not sufficient to localize to the Golgi (Mahajan et al., 2019), suggesting that there are other regions in the protein that regulate its membrane localization. Consistent with this, an internal fragment of yeast Dop1 localizes to the trans-Golgi network (TGN) and endosomes, which includes the region that binds the flippase Neo1 (Barbosa et al., 2010). Therefore, the membrane association of Dopey proteins is likely to be regulated by multiple molecular interactions with both lipids and proteins.

The C-terminus of Dopey proteins also contains a conserved series of leucine zippers that play important roles in Dopey function. A point mutation (I1695R) in one of the leucine zippers of DopA disrupts fungal morphogenesis (Fig. 1A) (Pascon and Miller, 2000). The leucine zipper region includes a coiled-coil region (Käll et al., 2004), and both motifs are typically involved in protein–protein interaction and dimerization (Landschulz et al., 1988). Indeed, isolated yeast Dop1 can form oligomers, with the C-terminus showing a homotypic interaction (Barbosa et al., 2010). However, isolated mammalian DOPEY1 protein appears to be monomeric (Mahajan et al., 2019). Therefore, it will be important to determine which other proteins interact with this conserved C-terminal region.

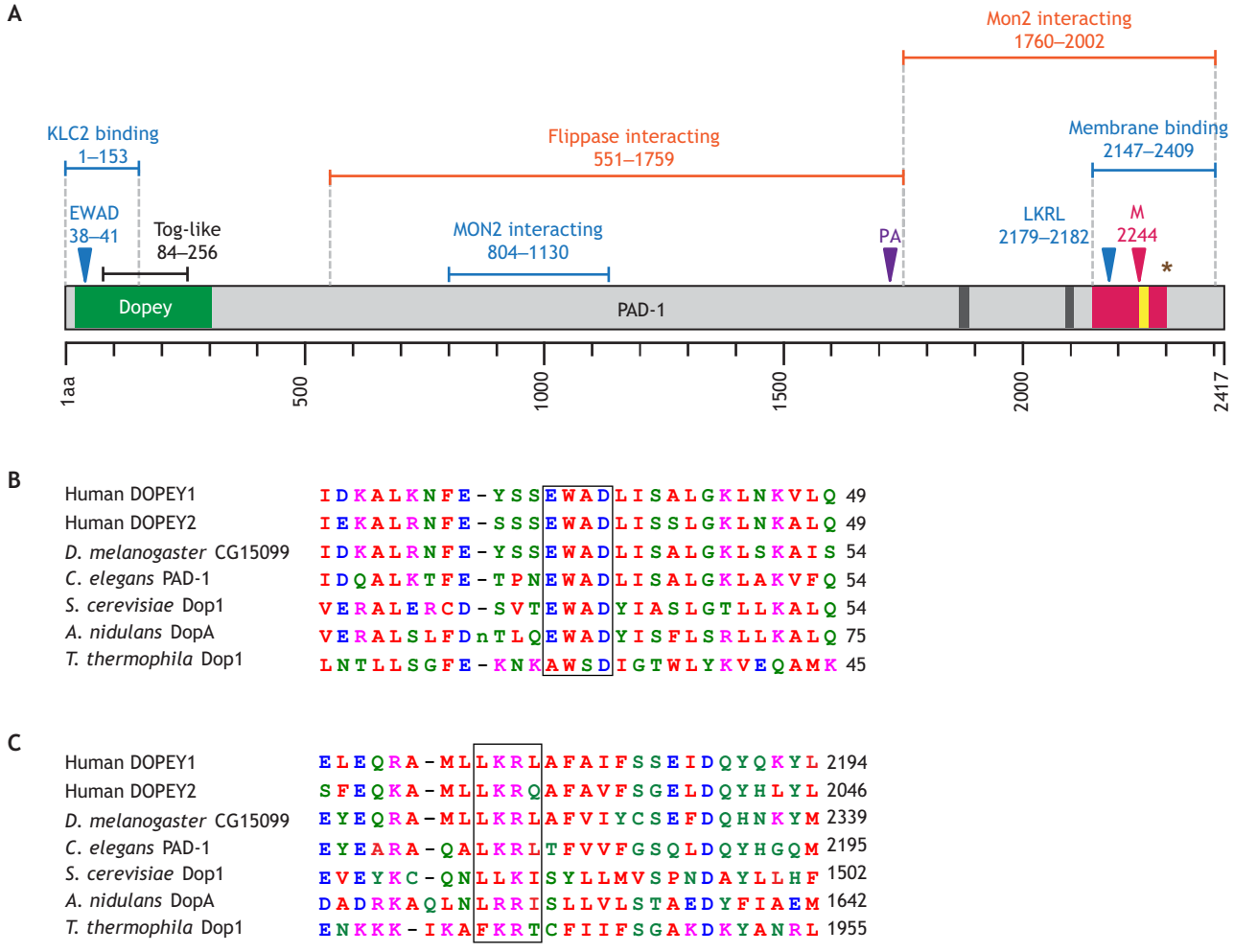

Fig. 1. Dopey protein domains. (A) The Dopey protein PAD-1 contains a highly conserved Dopey domain at the N-terminus [amino acids (aa) 16–301], based on InterPro (Mitchell et al., 2019). The Dopey domain includes a region predicted to fold like a Tog domain, based on Phyre2 models (Kelley et al., 2015), and the conserved EWAD motif required for KLC2 binding. Central regions of the protein are predicted to bind to Mon2 and essential lipid flippases, although the C-terminus has also been shown to bind to Mon2. The DOPEY2 V1660I mutation is implicated in Peters anomaly. The conserved LKRL motif in the C-terminal membrane-binding region of DOPEY1 is required for binding PI4P-containing membranes. The DopA I1695R mutation, corresponding to M2244 in PAD-1, disrupts a conserved region of eight putative leucine zippers (red, aa 2223–2300). Two hydrophobic regions or potential transmembrane domains (dark grey, aa 1870–1891 and aa 2087–2104) and a coiled-coil structure (yellow, aa 2245–2265) within the leucine zipper region are predicted by Phobius (Käll et al., 2004). The vacuole formation (VF) rat mutant has a premature stop codon at aa 2305 (brown *). The corresponding fragments of Dopey orthologs are indicated: DopA (red), Dop1 (orange), DOPEY1 (blue), and DOPEY2 (purple). (B,C) Multiple alignment of Dopey proteins highlighting the EWAD (B) and LKRL (C) motifs. Aligned and colored using Clustal Omega (McWilliam et al., 2013). Red, small/hydrophobic; blue, acidic; magenta, basic; green, hydroxyl/sulfhydryl/amine/G.

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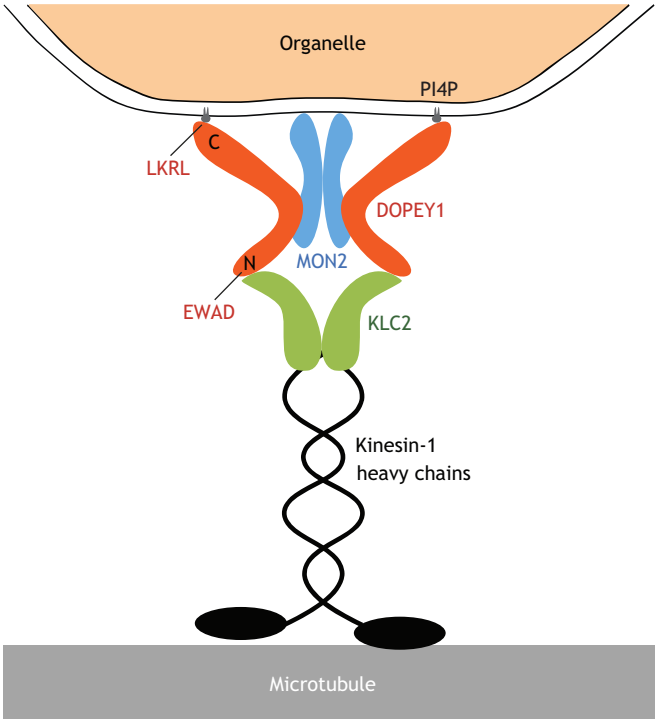

Fig. 2. Proposed interaction between DOPEY1, MON2 and kinesin-1. DOPEY1 interacts with the kinesin-1 light chain KLC2 through its EWAD motif and docks to PI4P-containing organelle membranes through the LKRL motif, connecting organelles to microtubule motors. Mammalian MON2 promotes dimerization of DOPEY1, possibly by forming a heterotetrameric complex with DOPEY1.

Multiple proteins have been shown to interact with Dopey proteins through a large central fragment with no predicted domains (Fig. 1A), including mammalian MON2 and an essential lipid flippase that has different names in different organisms (Table 1). Biochemical studies from yeast first showed that the three proteins can form a complex, with a large central fragment of Dop1 needed for interaction with the flippase Neo1 and the C-terminus of Dop1 showing the strongest interaction with Mon2 (Barbosa et al., 2010) (Fig. 1A). This complex was later confirmed in HeLa cells, where DOPEY2, MON2 and the flippase ATP9A localized to the same organelles and were able to co-immunoprecipitate each other (McGough et al., 2018). However, human DOPEY1 was shown to interact with MON2 through a central fragment (Mahajan et al., 2019) (Fig. 1A), while a central fragment of yeast Dop1 only weakly interacted with Mon2 (Barbosa et al., 2010), leaving the protein–protein interactions that define this conserved membrane remodeling complex in question.

In vertebrates, the two Dopey homologs may have diverged in their interactions with Mon2. Mon2 is required for the Golgi localization of Dop1 in yeast (Gillingham et al., 2006), but only for the Golgi localization of DOPEY1 in HeLa cells (Mahajan et al., 2019). One study found that MON2 immunoprecipitated DOPEY1 and promoted DOPEY1 dimerization in HEK293T cell lysate but did not find an interaction between MON2 and DOPEY2 (Mahajan et al., 2019). However, another study found that DOPEY2 co-precipitated with MON2, as well as ATP9A, in HEK293 cells (McGough et al., 2018). Similarly, a third study found that both DOPEY1 and DOPEY2 could immunoprecipitate with MON2 in HEK293 cell lysate and that MON2 overexpression was able to relocalize both DOPEY1 and DOPEY2 (Zhao et al., 2020). Although these conflicting results could be due to technical differences such as antibody-binding sites, they could also hint that DOPEY1 and DOPEY2 differ in their affinity for MON2.

Sequence comparisons between Dopey orthologs revealed that the highest degree of sequence similarity lies within the N-terminal region (Pascon and Miller, 2000; Barbosa et al., 2010), often referred to as the Dopey domain (Fig. 1A). An N-terminal fragment of the Dopey domain binds to KLC2, a light chain of the kinesin-1 microtubule motor (Mahajan et al., 2019). KLC2 binding is mediated by a highly conserved tryptophan flanked by acidic residues (EWAD) (Figs 1A,B and 2). This interaction with KLC2 was originally proposed to be unique to DOPEY1 (Mahajan et al., 2019), even though DOPEY2 also bears the EWAD motif. However, a recent study found that both DOPEY1 and DOPEY2 interact with KLC2 (Zhao et al., 2020). Together with the C-terminal membrane-binding motif, DOPEY1 is proposed to facilitate the docking of kinesin-1 to organelle membranes (Fig. 2).

Although no structural studies on Dopey proteins have yet been published, modeling the Dopey domain in Phyre2 predicts a structural similarity to a microtubule-binding Tog domain (Fig. 1A) (Kelley et al., 2015). This raises the possibility that the Dopey domain also coordinates the interaction between kinesin-1 and tubulin, which could provide an additional molecular explanation for their trafficking roles. However, this computational prediction awaits experimental confirmation.

There are also studies linking Dopey proteins to the Arf-like small GTPase Arl1, although where and how they molecularly interact is unclear. This interaction was discovered through genetic experiments in yeast, where Arl1 overexpression could partially rescue the neomycin sensitivity of a temperature-sensitive *dop1* allele, while Neo1 overexpression could fully rescue the phenotype (Barbosa et al., 2010). Arl1 overexpression also leads to increased Dop1 expression in *mon2* mutants, similar to what is seen upon Neo1 overexpression. Arl1 has also been shown to stabilize the Dop1–Mon2 interaction (Dalton et al., 2017), raising the possibility that additional proteins such as Arl1 modify the complex interactions of Dopey proteins.

Dopey proteins control retrograde endosomal trafficking

One of the best-established roles of Dopey proteins lies in the regulation of endosomal trafficking, especially retrograde trafficking, which transports proteins from endosomes back to the Golgi (Lu and Hong, 2014; Shafaq-Zadah et al., 2020). Subcellularly, Dopey proteins reside in endosomes and the TGN in yeast and mammalian cells (Gillingham et al., 2006; Tanaka et al., 2014; Barbosa et al., 2010), consistent with a role in retrograde trafficking. Yeast Dop1 is required for the recycling of the v-SNARE Snc1 and t-SNARE Sso1 from sorting endosomes to the TGN (Gillingham et al., 2006), as well as the retrograde transport of Golgi-resident glycosyltransferases such as Och1 from the TGN to the Golgi (Fig. 3) (Zhao et al., 2019). Disrupting Dop1 thereby results in broad defects in N- and O-glycosylation (Zhao et al., 2019), making it more difficult to establish the direct trafficking roles of Dopey proteins after chronic depletion or disruption.

Dopey proteins, together with MON2, were recently proposed to recycle Wntless as part of the SNX3–retromer retrograde trafficking pathway (Fig. 3). Human DOPEY2 and MON2 have been found in the SNX3 interactome, and the lipid flippase ATP9A can rescue the SNX3 cargo Wntless from lysosomal degradation in RPE-1 cells (McGough et al., 2018). Similarly, knocking down MON-2 or the *C. elegans* Dopey protein PAD-1 causes a decrease in the protein levels of the Wntless ortholog MIG-14. When PAD-1 or MON-2

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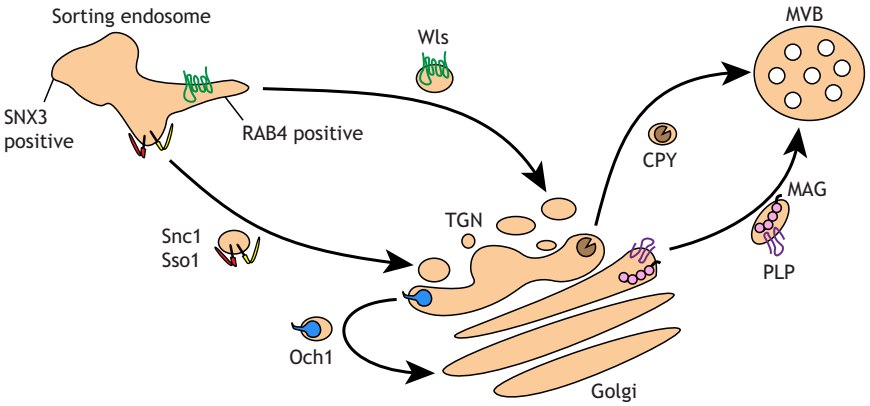

Fig. 3. Retrograde recycling and endolysosomal trafficking pathways regulated by Dopey proteins. DOPEY2 and Dop1 regulate retrograde trafficking of Wntless (Wls) and Snc1–Sso1, respectively, from sorting endosomes back to the Golgi. Dop1 also regulates retrograde trafficking of Och1 from the TGN to the Golgi. Dop1 and DOPEY1 are required for trafficking of CPY and the myelin membrane proteins PLP and MAG, respectively, to multivesicular bodies (MVB).

knockdown was further combined with a sensitizing mutation in a retromer subunit, Q-cell migration, an *in vivo* model used to study Wnt secretion, was disrupted (McGough et al., 2018). Depletion of PAD-1, MON-2 or the flippase TAT-5 can also rescue the polarized localization of the β-catenin WRM-1 in a phospholipase A mutant, similar to what is found with depletion of retromer and sorting nexin mutants (Kanamori et al., 2008). These data demonstrate that Dopey proteins play important roles in trafficking factors for Wnt signaling in animals but leave open whether Dopey and MON2 are part of the SNX3 retrograde pathway or define an overlapping endosomal recycling pathway.

There is growing evidence that Dopey and MON2 are not part of the sorting nexin or retromer-linked retrograde trafficking pathways. The lipid flippases TAT-5 and Neo1 are cargoes of the SNX3 recycling pathway and are mislocalized in *snx-3* mutants in *C. elegans* (Beer et al., 2018) and yeast (Dalton et al., 2017). However, the Dopey protein PAD-1 and MON-2 are not required for TAT-5 localization in *C. elegans* (Beer et al., 2018), indicating that PAD-1 and MON-2 are not an essential part of the SNX-3 pathway. Furthermore, PAD-1 and MON-2 redundantly regulate plasma membrane budding with SNX-3, as well as with the SNX-1–SNX-6 complex, suggesting that MON-2 and PAD-1 are not part of either the SNX-3 or the SNX-1–SNX-6 retrograde trafficking pathways. Thus, PAD-1 and MON-2 work independently of SNX-3, SNX-1 and SNX-6 on sorting endosomes but act on partially overlapping cargoes.

Results from a recent study instead suggest that DOPEY2 and MON2 could regulate RAB4-dependent trafficking (Zhao et al., 2020). RAB4 is a small GTPase important for fission and tubulation of sorting endosomes (D'Souza et al., 2014), but RAB4 orthologs are missing in *C. elegans* and *S. cerevisiae* (Diekmann et al., 2011). Wntless colocalizes predominantly with MON2, RAB4A and RAB4B rather than SNX3 in HEK293 cells (Zhao et al., 2020), suggesting it is trafficked in RAB4-positive membranes from sorting endosomes to the Golgi. Indeed, MON2 knockdown leads to an aggregation of both Wntless and RAB4B at sorting endosomes (Zhao et al., 2020). The Dopey interactors Mon2, Neo1 and Arl1 act together in the recruitment of the clathrin adaptor Gga2 (Singer-Krüger et al., 2008), a crucial step in RAB4-dependent cargo exit (D'Souza et al., 2014). However, instead of RAB4B accumulating at endosomes, as in MON2-knockdown cells, RAB4B accumulated at the Golgi after DOPEY2 knockdown (Zhao et al., 2020). Furthermore, DOPEY1 knockdown had little effect on RAB4B localization. These different effects on RAB4 localization suggest a more complex regulation of Dopey and Mon2, requiring further investigation.

Together, these studies leave it unclear whether Dopey proteins and MON2 recycle specific cargoes, control a bulk recycling pathway with no cargo specificity or promote recycling by multiple pathways. A recent study in mammalian cells showed that DOPEY1 and MON2 link membranes to the minus-end-directed motor protein kinesin-1 (Mahajan et al., 2019). DOPEY1 and DOPEY2 have also been shown to interact with the dynactin DCTN1 (Zhao et al., 2020), which is an essential cofactor for plus-end-directed dynein motors (Schroer, 2004). These motor proteins guide the bidirectional movement of organelles and vesicles along microtubules and can also help to extend endosomal tubules to create recycling vesicles (Freeman et al., 2014; Delevoye et al., 2014; Gomez and Billadeau, 2009). Dopey proteins may thereby also promote tubulation in general by connecting organelle membranes to microtubules.

Dopey proteins impact endolysosomal trafficking and morphology

In addition to retrograde trafficking, Dopey proteins influence vacuolar trafficking and morphology. In mammals, DOPEY1 helps traffic the myelin membrane proteins proteolipid protein (PLP, also known as PLP1) and myelin-associated glycoprotein (MAG) to the degradative pathway (Fig. 3) (Tanaka et al., 2014). In oligodendrocytes, PLP and MAG accumulate in multivesicular bodies and lysosomes before myelination (Bakhti et al., 2011). However, in the vacuole formation (VF) mutant rat strain that has a premature stop codon in the C-terminus of DOPEY1, PLP and MAG accumulate in the dilated Golgi of oligodendrocytes (Tanaka et al., 2014). Yeast Dop1 together with Mon2 and the flippase Neo1 are also required for the trafficking of a vacuolar hydrolase carboxypeptidase Y (CPY) (Fig. 3) (Barbosa et al., 2010). CPY is normally transported from the Golgi to multivesicular endosomes to vacuoles (Li and Kane, 2009), but in *dop1* and *neo1* mutants, CPY is instead released extracellularly (Barbosa et al., 2010; Wicky et al., 2004). *Dop1*, *mon2* and *neo1* mutants also have fragmented and hyperacidified vacuoles that accumulate tubular structures in their lumen (Gillingham et al., 2006; Hua and Graham, 2003; Wicky et al., 2004; Brett et al., 2011; Efe et al., 2005; Barbosa et al., 2010; Jochum et al., 2002). However, given the importance of protein glycosylation in targeting cargo to lysosomes (Coutinho et al., 2012), these vacuolar defects might also be secondary to Dop1-mediated recycling of Golgi glycosyltransferases (Zhao et al., 2019).

Dopey proteins are also important for endolysosomal morphology. In *C. elegans*, multivesicular late endosomes are enlarged after disruption of PAD-1 or MON-2 (Beer et al., 2018). Enlarged vacuoles are also seen in *Tetrahymena thermophila* after knockdown of Dop1 (Cheng et al., 2016) and in brains of *DOPEY1*-mutant VF rats (Nakane et al., 2002). However,

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enlarged multivesicular endosomes or vacuoles could be secondary to a decrease in recycling and increased degradative flux. Indeed, PAD-1 and MON-2 can rescue transmembrane proteins, such as TAT-5, from being sorted into the degradative pathway in sorting nexin mutants, as TAT-5 only accumulated in late endolysosomes when both the MON-2–PAD-1 and SNX recycling pathways were defective (Beer et al., 2018). Taken together, Dopey proteins impact endolysosomal trafficking and morphology, although it is still unclear whether they act directly or indirectly through other trafficking roles.

DOPEY1 promotes centrifugal trafficking to position organelles

Dopey proteins also regulate organelle positioning in cells, likely through their ability to bind both membranes and microtubule motors (Fig. 2). Depletion of DOPEY1 and MON2 causes endoplasmic reticulum (ER) exit sites and the Golgi to cluster more compactly in the center of HeLa cells (Mahajan et al., 2019). Various markers for endolysosomal structures also clustered in the cell center, although it is unclear whether that was due to organelle positioning or defective trafficking of these transmembrane proteins. *DOPEY2* knockdown showed a minor defect in the positioning of ER exit sites, but no role has yet been tested for the flippases ATP9A or ATP9B. These defects are consistent with Dopey proteins linking kinesin-1 to membrane-bound organelles (Fig. 2), as this motor drives centrifugal movement along the oriented microtubule network. In a gain-of-function experiment, mislocalizing DOPEY1 to peroxisomes was sufficient to increase their bidirectional movement (Mahajan et al., 2019), suggesting interactions with both kinesin-1 and dynein motors could be involved. Together, these findings demonstrate that Dopey localization to organelles can influence organelle positioning along microtubules.

PAD-1 controls lipid asymmetry and extracellular vesicle formation

The *C. elegans* Dopey ortholog PAD-1 plays an important role in regulating lipid asymmetry and inhibiting extracellular vesicle (EV) formation (Beer and Wehman, 2017). Microvesicles (also called ectosomes) are a subclass of EVs that form by direct budding of the plasma membrane into the extracellular space, also known as ectocytosis (van Niel et al., 2018). After RNAi-mediated knockdown of *pad-1,* there is a 100-fold increase in the number of EVs released by embryonic cells (Beer et al., 2018; Fazeli et al., 2020), similar to the result of losing the ATPase activity of the lipid flippase TAT-5 (Wehman et al., 2011). TAT-5 maintains phosphatidylethanolamine (PE) asymmetry in the plasma membrane and inhibits ectocytosis (Fig. 4). When TAT-5 lipid flippase activity is lost, the normally cytofacial lipid PE becomes externalized, the plasma membrane recruits the membrane-sculpting endosomal sorting complexes required for transport (ESCRT) and overproduces extracellular vesicles (Wehman et al., 2011). Intriguingly, PAD-1 is also required to maintain PE asymmetry and inhibit ESCRT recruitment to the plasma membrane in *C. elegans* (Beer et al., 2018; Beer, 2021). As PAD-1 is a peripheral membrane protein with no domains associated with lipid transport (Pascon and Miller, 2000; Jensen et al., 2017), it is unlikely that PAD-1 can promote PE translocation across membrane bilayers autonomously. In *C. elegans*, PAD-1 localizes to subdomains of the plasma membrane, in addition to cytosolic vesicles (Beer et al., 2018), and could therefore interact with TAT-5 at the plasma membrane (Wehman et al., 2011). Given that PAD-1 is not needed for TAT-5 trafficking to the plasma membrane (Beer et al., 2018), these data led to the proposal that PAD-1 is required for the PE-flipping activity of TAT-5 (Fig. 4), although how their molecular interaction would regulate TAT-5 ATPase activity is unknown. Whether Dopey proteins influence lipid asymmetry or EV release in other species is untested, but knocking down the flippase ATP9A also increased EV release from human cells (Naik et al., 2019).

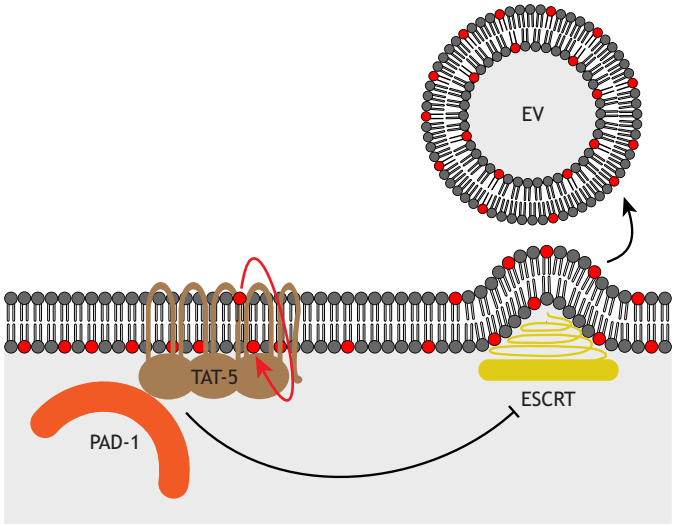

Fig. 4. The Dopey protein PAD-1 regulates lipid asymmetry and EV release through the flippase TAT-5. PAD-1 is required for TAT-5 flippase activity through an unknown mechanism. TAT-5 hydrolyzes ATP to flip the lipid phosphatidylethanolamine (PE, red) from the extracellular to the cytofacial leaflet of the plasma membrane. In the absence of TAT-5 activity, PE exposure on the outer leaflet is correlated with ESCRT recruitment to the plasma membrane and ectocytosis to release EVs.

Interestingly, although Mon2 and Dopey proteins have been shown to act together during most endosomal trafficking events, MON-2 is not required for either PE lipid asymmetry or EV release in *C. elegans* (Beer et al., 2018). This suggests separable, context-dependent roles for different subcomplexes of the Dopey–Mon2–flippase complex. Furthermore, the duramycin staining used to detect PE externalization was significantly higher after *pad-1* knockdown compared to after *tat-5* knockdown, raising the possibility that PAD-1 may be required for the activity of another PE flippase (e.g. the TAT-5 paralog TAT-6) and/or inhibit an unidentified lipid scramblase that causes PE externalization.

One major unanswered question is under which physiological conditions Dopey proteins and flippases regulate PE asymmetry and/or EV release. One hypothesis is that flipping PE could regulate endosomal tubulation and vesicle budding through the initiation of positive membrane curvature, thus impacting a variety of endosomal trafficking processes (Sebastian et al., 2012). Additionally, PE asymmetry is lost during cytokinesis and needs to be re-established for the final step of abscission (Emoto et al., 2005). EV release is also seen at the intercellular bridge in both *C. elegans* and mammalian cells (Elia et al., 2011; König et al., 2017), which could help to sculpt membranes to thin the intercellular bridge during abscission. Thus, Dopey proteins and flippases might need to be regulated for both intracellular and extracellular vesicle formation, including at the intercellular bridge for animal cells to separate after cytokinesis.

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Dopey proteins and flippases are required for cell and tissue morphogenesis

Dopey family members are involved in morphogenesis and patterning in several species. This connection was first observed in *A. nidulans* with the original Dopey protein DopA (Axelrod et al., 1973; Yager et al., 1982). DopA is required for all aspects of fungal morphogenesis; *dopA* mutants are characterized by abnormal cell morphology and growth, asynchronous development and loss of the reproductive cycle (Pascon and Miller, 2000). Deletion of the flippase ortholog *dnfD* yields similar phenotypes to those seen in *dopA* mutants (Schultzhaus et al., 2019), suggesting they regulate cell and tissue morphogenesis together.

In the budding yeast *Saccharomyces cerevisiae*, overexpression of the N-terminal Dopey domain caused cell morphological defects (Pascon and Miller, 2000). The yeast cells were larger and showed abnormal elongation or pear shapes while budding. This suggests that expression of the Dopey domain is tightly regulated and both under- and over-expression can lead to morphological defects. Although no changes to cell shape have been reported for classical loss-of-function alleles, both *dop1* and *neo1* flippase mutants are lethal in *S. cerevisiae* (Gillingham et al., 2006; Hua et al., 2002), suggesting that they share essential roles.

In *C. elegans*, depletion of the Dopey ortholog *pad-1* by RNAi results in embryonic lethality due to defects in cell shape and cell movement (Guipponi et al., 2000; Beer et al., 2018). Embryos fail to undergo morphogenesis during gastrulation, resulting in misshapen and mispositioned cells. Thus the *C. elegans* ortholog was named *pad-1,* for patterning defective (Guipponi et al., 2000). Depleting the flippase TAT-5 resulted in similar gastrulation defects and embryonic lethality (Wehman et al., 2011). Intriguingly, reducing EV overproduction in *tat-5* mutants was able to ameliorate cell shape defects, suggesting that the accumulation of intercellular EVs in *pad-1* mutants could compromise cell adhesion and thereby disrupt the cell shape changes and cell movements that lead to tissue morphogenesis.

Although Dopey and flippase orthologs seem to function together in their regulation of morphogenesis, there is currently no evidence that Mon2 orthologs are required for cell or tissue morphogenesis in *A. nidulans, S. cerevisiae* or *C. elegans* (Schultzhaus et al., 2019; Gillingham et al., 2006; Kanamori et al., 2008). However, both MON-2 and PAD-1 are required for lifespan extension in long-lived *C. elegans* mutants (Jung et al., 2021), confirming that Dopey–Mon2 complexes have physiologically relevant roles apart from morphogenesis. MON-2-dependent lifespan extension is thought to occur through autophagy regulation, as *mon-2* mutants show fewer autophagosomes after autophagy-inducing stimuli (Jung et al., 2021). A similar role for Dopey proteins in autophagy has not yet been tested. This emphasizes that different parts of the Dopey–Mon2–flippase complex or different subcomplexes regulate distinct membrane trafficking pathways that influence distinct physiological functions.

Dopey proteins play essential roles in the nervous system

Dopey proteins influence brain development, and alterations in Dopey proteins are associated with neurological disorders in mammals. A premature stop codon occurred spontaneously in the C-terminus of DOPEY1 in VF rats, resulting in tremors, decreased myelination, and reduced oligodendrocyte maturation and size, as well as abnormal, enlarged vacuoles in certain neural tissues (Nakane et al., 2002; Tanaka et al., 2012). The premature stop codon leads to decreased DOPEY1 mRNA levels and no detectable DOPEY1 protein (Tanaka et al., 2014), making this likely to be a strong loss-of-function or null allele. The decreased myelination in VF rats is thought to be caused by defective trafficking of the transmembrane proteins PLP and MAG (Fig. 5), crucial components for myelinogenesis, which accumulate in the dilated Golgi of VF oligodendrocytes (Tanaka et al., 2014). However, DOPEY1 disruption is also likely to alter the trafficking of other proteins important for nervous system function. Indeed, the disruption of oligodendrocyte maturation and morphological differentiation is another likely cause of decreased myelination in VF rats (Tanaka et al., 2012). Mature oligodendrocytes release EVs to inhibit myelination and the morphological differentiation of nearby oligodendrocytes (Bakhti et al., 2011), raising the possibility that DOPEY1 also influences myelination through EV release. As the VF rat studies were performed on complex brain tissue and DOPEY1 is broadly expressed in neurons, oligodendrocytes and astrocytes (Tanaka et al., 2014), among other tissues (Uhlén et al., 2015), it will be important to determine the specifics underlying defective myelination using cell-based or conditional knockout studies.

Dysfunction of DOPEY2 is also implicated in neurological function. DOPEY2 (human DOP1B) is linked to a congenital eye disorder, Peters anomaly, which results in blurred vision due to thinning and clouding of the cornea (Darbari et al., 2020). A homozygous variation in DOPEY2, V1660I, was found as a likely cause of the impaired eye development in three related individuals (Darbari et al., 2020). Although this mutation maps to one edge of the large fragment shown to interact with lipid flippases (Fig. 2), this sequence is not conserved in DOPEY1, PAD-1 or Dop1, making it

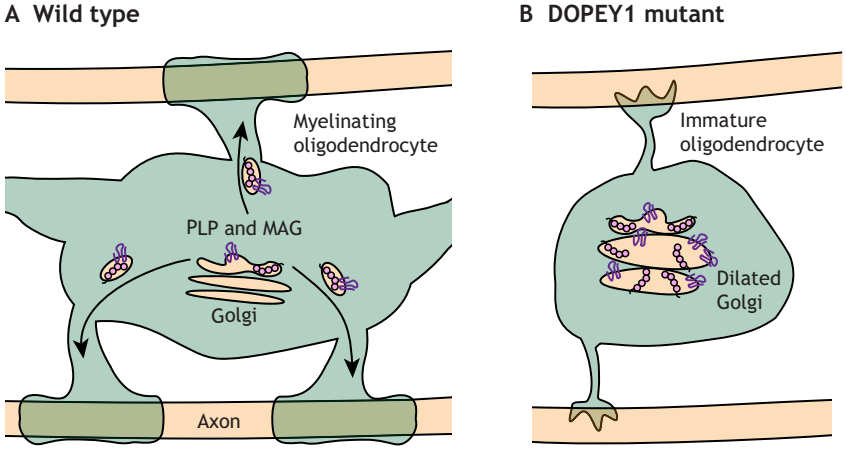

Fig. 5. DOPEY1 is required for myelin protein trafficking, oligodendrocyte maturation, and myelination. Mature oligodendrocytes transport the myelin membrane proteins PLP and MAG to myelinate surrounding axons. A premature stop codon in DOPEY1 in vacuole formation (VF) rats results in small immature oligodendrocytes with reduced processes and a dilated Golgi containing PLP and MAG, resulting in impaired myelination.

unclear how this variation would translate into altered DOPEY2 function.

Overexpression of DOPEY2 has also been proposed to contribute to the cognitive defects of Down syndrome and Alzheimer's disease patients. DOPEY2 is found in the Down syndrome critical region (DSCR) on chromosome 21 (Korenberg et al., 1994) and its expression is increased in brain tissues of Down syndrome fetuses with chromosome 21 trisomy (Rachidi et al., 2009). In support of this hypothesis, mice overexpressing human DOPEY2 show mild cognitive defects in learning and memory (Rachidi et al., 2005; Lopes et al., 2003). These mice also have increased density of DOPEY2-expressing cortical cells, which could correlate with the abnormal cortical lamination pattern observed in Down syndrome patients (Golden and Hyman, 1994). Interferon hypersensitivity, another Down syndrome phenotype, may be linked to DOPEY2 as well. The *DOPEY2* gene contains four interferon response enhancer elements (Jagadeesh et al., 2020), although further research is needed to establish whether *DOPEY2* is overexpressed in response to interferons. Duplication of *DOPEY2* or its N-terminus has also been found in the brain tissue of seven late-onset Alzheimer's disease patients (Swaminathan et al., 2011, 2012a,b), raising the possibility that an extra copy of *DOPEY2* or its N-terminus can lead to cognitive defects in Alzheimer's disease, as well as in Down syndrome patients.

Conclusions

By regulating membrane trafficking, Dopey proteins hold fundamental importance in proper cell function and development. The molecular mechanisms by which Dopey proteins and their interactors regulate endosomal trafficking are still up for debate, in part because there is currently no structural information on Dopey or its complexes. It seems unlikely that Dopey proteins are part of specific trafficking pathways such as sorting nexin recycling, as they act in parallel to multiple pathways and are involved in trafficking a variety of cargoes to different organelles. A common denominator for these trafficking events is the budding of cargo vesicles from organelle membranes. If Dopey proteins are directly involved in budding or tubulation, their disruption would result in a wide range of trafficking defects. It has been recently identified that Dopey proteins interact with microtubule motor proteins (Mahajan et al., 2019; Zhao et al., 2020), and this interaction could facilitate budding by helping to extend tubules to create vesicles (Freeman et al., 2014; Delevoye et al., 2014; Gomez and Billadeau, 2009). Indeed, MON2 is required for the tubulation of RAB4B-positive endosomes (Zhao et al., 2020), presumably together with Dopey proteins. Furthermore, associated flippases could flip lipids to the cytofacial leaflet and thereby induce positive membrane curvature to facilitate vesicle budding (McGough et al., 2018). Thus, Dopey proteins appear to connect lipid movement with microtubule motors to promote vesicle budding and trafficking. Therefore, structural analysis and *in vitro* tubulation assays with purified proteins will be crucial to establish how Dopey and its interactors regulate membrane trafficking.

A key open question is under which conditions Dopey subcomplexes perform distinct roles. Mon2 and Dopey proteins act together to regulate the trafficking of many cargoes (Fig. 3), while the less well-studied flippases are at least involved in CPY and Wntless trafficking (McGough et al., 2018; Barbosa et al., 2010). In contrast, Mon2 proteins are not involved in the regulation of lipid asymmetry, EV budding, or morphogenesis, while Dopey proteins and the essential flippases play similar roles in these processes. Thus, Dopey can act in both Mon2-dependent and Mon2-independent ways, but it is unclear whether Dopey always requires a lipid flippase. Furthermore, which proteins act with Dopey during myelination or other neural processes is unknown. Defining when and how Dopey, Mon2, flippases and other factors associate will be key to understanding the diverse functions of these different subcomplexes.

To understand the role of Dopey proteins in human disease, it will also be critical to resolve the molecular differences between DOPEY1 and DOPEY2. Both DOPEY1 and DOPEY2 are broadly expressed (Uhlén et al., 2015), but show distinct localization and localize to the Golgi differently, with DOPEY2 lacking the conserved LKRL PI4P-binding motif. Furthermore, DOPEY1 and DOPEY2 appear to act in distinct trafficking pathways, with DOPEY2 but not DOPEY1 controlling Wntless trafficking and RAB4B localization (Zhao et al., 2020), whereas only DOPEY1 and not DOPEY2 controls the localization of endolysosomal proteins, such as LAMP1 and transferrin receptors (Mahajan et al., 2019). Furthermore, alternative splicing of DOPEY1 has been identified as a biomarker for breast cancer (Lend et al., 2015), while a DOPEY2 circular RNA has been implicated as a functional biomarker in esophageal carcinoma (Liu et al., 2021), raising the possibility that different Dopey transcripts, isoforms, and paralogs regulate distinct molecular interactions and trafficking functions. We have much to learn about this 'dope' family of membrane trafficking regulators.

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Competing interests

The authors declare no competing or financial interests.

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