ENERGY REQUIREMENTS IN MEMBRANE TRAFFICKING

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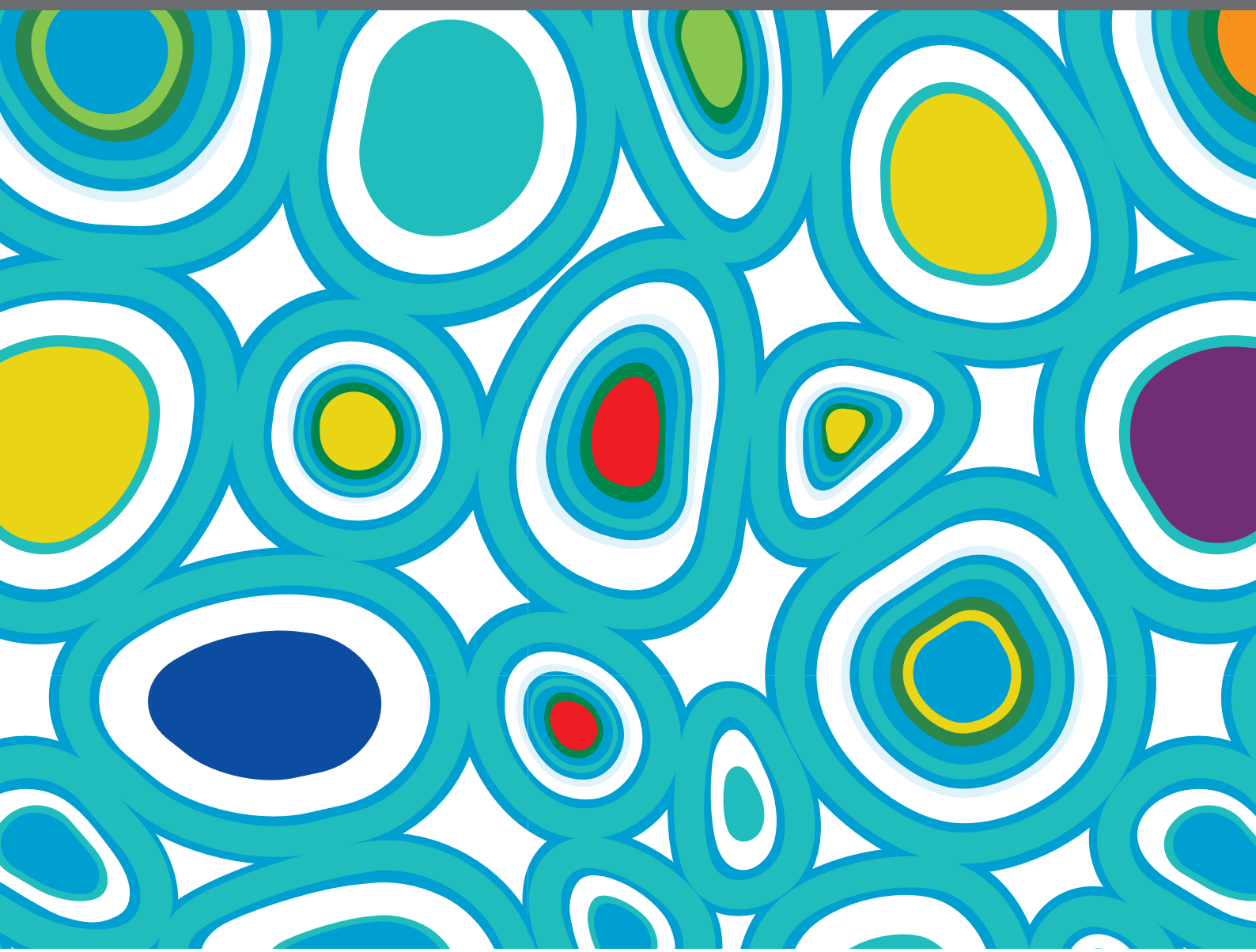

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ENERGY REQUIREMENTS IN MEMBRANE TRAFFICKING

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EDITORIAL
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Editorial: Energy Requirements in Membrane Trafficking

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Editorial on the Research Topic

Energy Requirements in Membrane Trafficking

Whilst studying pancreatic exocrine cells in the 1960's, George Palade discovered that intracellular trafficking events require energy (Jamieson and Palade, 1968), a finding he described as "unexpected" in his Nobel prize lecture (Palade, 1974). The fundamental membrane trafficking components were subsequently determined by the labs of James Rothman, Randy Schekman, and others (Novick and Schekman, 1979; Balch et al., 1984; Kaiser and Schekman, 1990; Perin et al., 1990), inaugurating the modern era of intracellular pathways research. One of those key components was NSF (*N*-ethylmaleimide sensitive factor) initially purified from an *in vitro* reconstitution of Golgi membranes (Block et al., 1988). NSF was later shown to be the ATPase essential for the disassembly of SNARE complexes which mediate membrane fusion (Hata et al., 1993; Söllner et al., 1993), and thus the fundamental principle of subcellular trafficking events requiring energy was established.

One of the most remarkable characteristics of living cells is their ability to adapt and self-organize in response to environmental changes making them out-of-equilibrium systems. This dynamic equilibrium tries to maintain the internal environment of the cell as stable as possible *via* energy transformation. Through intracellular membrane trafficking and complex metabolic pathways, eukaryotic cells have evolved to efficiently transform the energy from their environment into energy storage molecules like ATP, GTP, NADH, and acetyl-CoA. Most of the energy is used to synthesize the biomolecules that provide cells with the tools to survive and interact with their environment (Traut, 1994; Walsh et al., 2018). A great part of the remaining energy produced is used to move the cell around, support cell division and differentiation, incorporate material from the extracellular space, secrete signaling products, assure protein homeostasis, transport cargo along the interior of the cell, and recycle material when necessary (Zala et al., 2017). These processes are therefore exquisitely regulated by events where the limiting step requires the release of energy by ATP or GTP in a controlled manner.

In particular, a number of energy-dependent proteins can be found to be essential for all the steps involved in the intracellular traffic of cargo. This Research Topic contains 10 articles that explore a variety of energy requiring processes in the cell.

At the border of the cell, the intracellular journey of a cargo starts with its internalization by endocytosis. In that context, Matthaeus and Taraska summarize the recent concepts in caveolae trafficking and dynamics with a focus on the ATP and GTP-regulated proteins that control caveolae behavior and their role in the cell metabolism. Once the cargo reaches the endo-lysosomal system, many factors and signals can determine its next destination. Saric and Freeman discuss how

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controlled solute efflux along the endocytic pathway contributes to membrane tension regulation and membrane remodeling with special emphasis on lysosomal solute transport. Wang and Ye review recent exciting studies on novel translocon-associated quality control strategies that cells use for eliminating polypeptides trapped in the endoplasmic reticulum and mitochondria during *de novo* protein synthesis.

Small GTPases act as signaling components that regulate subcellular processes and define sub-cellular compartments. GTP-bound GTPases have the power to recruit effectors that help shape and regulate these organelles and endosomes. Adarska et al. present a summary of the often-overlooked important roles of the ARF1-5 GTPases in trafficking while Fujibayashi and Mima cover a novel role for ARF6 as a membrane tether using chemically defined reconstitution approach with purified proteins and liposomes.

Relatively large cargo (e.g., vesicles, organelles like endosomes, autophagosomes, mitochondria, or molecules such as mRNA-protein complexes) needs to be transported around the cell by the help of motor molecules such as kinesins and dyneins. These molecular machines use the energy of ATP hydrolysis to generate processive and efficient distribution of cargo and their carriers along the microtubule network of the cell. A set of comprehensive reviews from Yadav and Kunwar and Xiang and Qiu summarize our understanding of the effect of temperature on motor molecules and the cargo-mediated activation of cytosolic dynein, respectively, and address important questions whose answers will push forward the research on the growing field of intracellular transport and dynamics.

Finally, this Research Topic includes three insightful articles that put the role of intracellular trafficking in the context of disease. Schiavon et al. discuss previous work on mitochondria intracellular transport and provide an example where impaired mobility of mitochondria may be playing a central role in the pathogenesis of Charcot-Marie-Tooth (CMT) disease, a neurodegenerative disorder. Additionally, Sneeggen et al. describe how the role of intracellular trafficking in cell proliferation, epithelial to mesenchymal transition, and invasion contributes to the regulation of energy consumption and metabolism during cancer progression. Lastly, Tavares et al. delineate the capacity of the HIV-1 virus to hijack key machinery of the intracellular pathway in order to ensure efficient viral replication and survival in the host.

Altogether, these contributions should give the reader an updated view of different aspects of the intracellular traffic field and a glimpse on the future directions of research in each sub-field. We hope the reader can also find the gaps and the many still unanswered questions in the field while navigating through this Research Topic: how is the motor molecules' activity regulated during development and disease progression? What is the next technology that will thrust this field into the next century of discovery? At the molecular level, how do we modulate cargo localization through targeting strategies, and in doing so, influence the adaptive requirements of a cell? How can we prevent future public health crises by manipulating the host-microorganism energy supply and demand flux? How can we generate a more comprehensive understanding of the molecular machinery of protein trafficking? The dysregulation of the machinery involved in controlling intracellular traffic is frequently associated with diseases that include cancer, developmental and degenerative diseases, and multiple immunity disorders, highlighting the urgency of unraveling the mechanisms that regulate the movement of cargo inside the cell.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Cargo-Mediated Activation of Cytoplasmic Dynein *in vivo*

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Cytoplasmic dynein-1 is a minus-end-directed microtubule motor that transports a variety of cargoes including early endosomes, late endosomes and other organelles. In many cell types, dynein accumulates at the microtubule plus end, where it interacts with its cargo to be moved toward the minus end. Dynein binds to its various cargoes via the dynactin complex and specific cargo adapters. Dynactin and some of the coiled-coil-domain-containing cargo adapters not only link dynein to cargo but also activate dynein motility, which implies that dynein is activated by its cellular cargo. Structural studies indicate that a dynein dimer switches between the autoinhibited phi state and an open state; and the binding of dynactin and a cargo adapter to the dynein tails causes the dynein motor domains to have a parallel configuration, allowing dynein to walk processively along a microtubule. Recently, the dynein regulator LIS1 has been shown to be required for dynein activation *in vivo*, and its mechanism of action involves preventing dynein from switching back to the autoinhibited state. In this review, we will discuss our current understanding of dynein activation and point out the gaps of knowledge on the spatial regulation of dynein in live cells. In addition, we will emphasize the importance of studying a complete set of dynein regulators for a better understanding of dynein regulation *in vivo*.

Keywords: dynactin, cargo adapter, LIS1, early endosome, microtubule plus end, fungi, dynein

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INTRODUCTION

In eukaryotic cells, motor proteins such as dyneins, kinesins and myosins are ATPases, and they use the energy from ATP hydrolysis to drive intracellular motility (Hirokawa et al., 2009; Verhey and Hammond, 2009; Dodding and Way, 2011; Hammer and Sellers, 2011; Reck-Peterson et al., 2018; Olenick and Holzbaur, 2019; Scherer et al., 2020). In most mammalian cells, polarized microtubules serve as tracks for long-distance transport: while the plus-end-directed kinesins transport cargoes toward the microtubule plus ends near the cell periphery, the minus end-directed cytoplasmic dynein transports cargoes inward from the cell periphery (Reck-Peterson et al., 2018). Cytoplasmic dynein-1 (called "dynein" hereafter for simplicity) powers the intracellular transport of nuclei/mitotic spindles, Golgi, mitochondria, early endosomes, late endosomes, autophagosomes, proteins, mRNAs and/or virus particles (Dodding and Way, 2011; Reck-Peterson et al., 2018; Olenick and Holzbaur, 2019; Scherer et al., 2020). Deficiencies in dynein and its regulators such as dynactin and LIS1 (Lissencephaly-1) cause devastating neurodegenerative diseases and brain developmental disorders (Wynshaw-Boris, 2007; Maday et al., 2014; Guedes-Dias and Holzbaur, 2019; Markus et al., 2020).

Studies have shown that dynein binds to its various cargoes via the dynactin complex and cargo adapters. Importantly, some cargo adapters not only link dynein to cargos but also activate dynein motility, which implies that the dynein motor is activated by its cargo (Reck-Peterson et al., 2018; Olenick and Holzbaur, 2019; Canty and Yildiz, 2020). While some kinesin and myosin motors are also known to be activated by cargo or specific adapters/scaffolding proteins (Sellers and Knight, 2007; Trybus, 2008; Hirokawa et al., 2009; Verhey and Hammond, 2009; Fu and Holzbaur, 2014; Sweeney and Holzbaur, 2018), the mechanism of dynein activation is distinct due to the unique structure of the dynein motor. In this review, we will cover our current understanding on the mechanism of dynein activation. We will mainly use early endosome transport in filamentous fungi as an example to discuss how dynein activation is spatially regulated *in vivo* and point out unresolved issues that need to be addressed in the future.

DYNACTIN AND CARGO ADAPTER PROTEINS MEDIATE THE DYNEIN-CARGO INTERACTION

Compared to kinesins or myosins, dynein is extremely huge and complex (Höök and Vallee, 2006; Sweeney and Holzbaur, 2018). It is a multi-protein complex of ∼1.4 MDa containing two dynein heavy chains (HCs) as well as other subunits such as intermediate chains (ICs), light intermediate chains (LICs), and light chains (LCs) (Pfister et al., 2005; Reck-Peterson et al., 2018). The HCs form a homodimer, and each HC monomer contains the C-terminal motor head and the N-terminal tail. The motor head is responsible for motility, while the tail is responsible for HC-HC dimerization and also binds other dynein subunits as well as the dynactin complex (King, 2000; Carter et al., 2016; Schmidt and Carter, 2016). The motor head of the dynein HC consists a motor ring with six AAA (ATPases Associated with diverse cellular Activities) domains, a linker (∼10 nm) connecting the motor ring with the tail (Burgess et al., 2003), and a microtubule-binding domain that is connected to the motor ring via a coiled-coil stalk extending out between AAA4 and AAA5 (Gee et al., 1997; Gibbons et al., 2005; Cianfrocco et al., 2015; **Figure 1A**). ATP binding and hydrolysis at AAA1 cause conformational changes in the ring, which can be transmitted via the coiled-coil stalk to the microtubule binding site, driving dynein movement along a microtubule (Roberts et al., 2013; Carter et al., 2016). ATP hydrolysis at AAA3 allows the proper transmission of conformational changes around the ring, allowing dynein to be released from the microtubule when ATP is bound to AAA1 (Bhabha et al., 2014; Dewitt et al., 2015; Nicholas et al., 2015).

The dynactin complex of ∼1 MDa is involved in almost all functions of cytoplasmic dynein (Schroer, 2004). The backbone of the dynactin complex is an Arp1 mini-filament of ∼37 nm (Schafer et al., 1994), which provides the binding sites for dynein tails and cargo adapters (Chowdhury et al., 2015; Urnavicius et al., 2015). The pointed end of the Arp1 mini-filament is occupied by the pointed-end subcomplex containing p25, p27, p62 and Arp11 (Eckley et al., 1999; **Figure 1B**). The barbed end of the

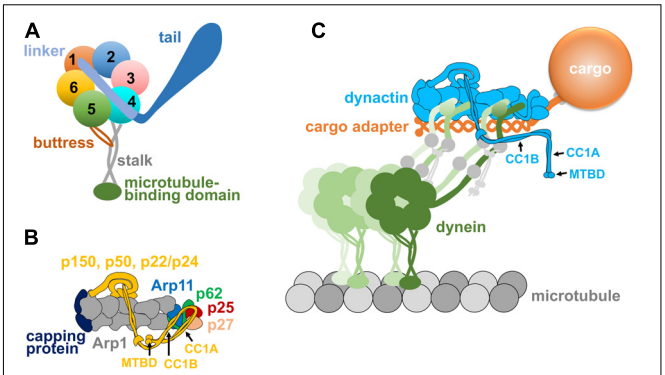

FIGURE 1 | (A) A diagram of the dynein heavy chain with a motor ring containing six AAA domains (1–6), a linker domain connected to the beginning of AAA1 and the N-terminal tail domain connected to the linker. A microtubule-binding domain is connected to the coiled-coil stalk that emerges from a location between AAA4 and AAA5, and the buttress coming from AAA5 supports the stalk. All these domains work together to ensure minus-end-directed motility of dynein as well as other properties of the dynein motor such as tension sensing (Roberts et al., 2013; Carter et al., 2016; Can et al., 2019; Rao et al., 2019). **(B)** A diagram of the dynactin complex with its Arp1 mini-filament, barbed-end capping protein, pointed-end proteins Arp11, p62, p25, and p27, as well as the shoulder/sidearm proteins p150, p22/p24, and p50 (Schroer, 2004; Urnavicius et al., 2015). Note that p150 is depicted as a folded molecule, and its CC1A, CC1B, and microtubule-binding domain (MTBD) are indicated by arrows. **(C)** A diagram of the dynein-dynactin-cargo adapter complex and the cargo linked to the cargo adapter. The cargo adapter, depicted as a homodimer, contains coiled-coil domains. Note that the dynein tails bind to the Arp1 mini-filament of dynactin, as shown by EM structural analyses (Schlager et al., 2014; Chowdhury et al., 2015; Urnavicius et al., 2015). An extended form of p150 is depicted, and its CC1A, CC1B, and microtubule-binding domain (MTBD) are indicated by arrows. Two dynein dimers are depicted, since a fraction of the dynein-dynactin-cargo adapter complexes could contain a second dynein dimer along the Arp1 mini-filament (Grotjahn et al., 2018; Urnavicius et al., 2018). Dynein intermediate chains and dynein light intermediate chains are depicted as gray circles on the dynein tails, and dynein light chains are depicted as small gray circles connected to the dynein intermediate chains.

Arp1 mini-filament is occupied by the actin-capping protein, which also caps the barbed ends of conventional actin filaments (Schafer et al., 1994; Wear and Cooper, 2004). The largest subunit of the dynactin complex is p150^{Glued} (or p150 for simplicity), which contains a microtubule-binding domain (MTBD) at its N-terminus (Holzbaur et al., 1991; Waterman-Storer et al., 1995). Following the MT-binding domain are the coiled-coil domains CC1 and CC2, and CC1 interacts with the N-terminus of dynein IC in biochemical assays (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; King et al., 2003). The CC1 domain of p150 can be further divided into CC1A and CC1B (**Figures 1B,C**), and CC1B contains a dynein-IC-binding domain (McKenney et al., 2011; Loening et al., 2020) whose function may be modulated by the binding of CC1A (Tripathy et al., 2014; Saito et al., 2020). Two other subunit of the dynactin complex, p22/p24 and p50 dynamitin that forms an oligomer (Echeverri et al., 1996; Karki et al., 1998; Melkonian et al., 2007), together with part of the p150 subunit, form a "shoulder/side arm" adjacent to the Arp1 mini-filament, and the p50 oligomer was proposed to function as a template for Arp1 mini-filament assembly (Urnavicius

et al., 2015). Interestingly, in the dynein-dynactin cargo adapter tripartite complex, it is the Arp1 mini-filament rather than the p150 subunit that interacts with the tails of dynein (Schlager et al., 2014; Chowdhury et al., 2015; Urnavicius et al., 2015, 2018; Grotjahn et al., 2018; **Figure 1C**). Future work will be needed to address whether the dynein IC-p150 interaction found by biochemical assays is important for initiating the dynein-dynactin interaction before cargo adapter binding.

The dynactin complex is needed for the dynein-cargo interaction. This function of dynactin was proposed many years ago (Schroer and Sheetz, 1991; Waterman-Storer et al., 1997), but this idea had remained controversial (Haghnia et al., 2007). This subject was revisited in two independent studies on the pointed-end proteins of the Arp1-minifilament, in the filamentous fungus *Aspergillus nidulans* and mammalian cells (Zhang et al., 2011; Yeh et al., 2012). These studies have found that the pointed-end proteins p25 in *A. nidulans* and the p25/p27 heterodimer in mammalian cells play a critical role in the interaction of dynein with its early endosome cargo (Zhang et al., 2011; Yeh et al., 2012). Both p25 and p27 adopt a left-handed beta-helix structure (Parisi et al., 2004; Yeh et al., 2013). Because p25 has many hydrophobic residues, it seemed plausible that p25 may contact membrane directly (Yeh et al., 2012). However, follow-up genetic screens in *A. nidulans* as well as another filamentous fungus *Ustilago maydis* led to the discovery that the FTS-Hook-FHIP (FHF) complex (Xu et al., 2008) functions as an adapter allowing dynein-dynactin to link with early endosomes (Bielska et al., 2014b; Yao et al., 2014; Zhang et al., 2014).

The Hook proteins (three in mammalian cells: Hook1, Hook2 and Hook3) and the FHF complex were initially discovered in higher eukaryotic cells (Krämer and Phistry, 1996; Walenta et al., 2001; Xu et al., 2008). Within the fungal FHF complex, HookA in *A. nidulans* and Hok1 in *U. maydis* use their N-terminal Hook domain (Schroeder and Vale, 2016) and the coiled-coil domains to interact with dynein-dynactin (Bielska et al., 2014b; Zhang et al., 2014; Qiu et al., 2018), and this interaction depends on Arp1 and p25 (Zhang et al., 2014). Structural studies on the mammalian Hook3 protein further demonstrate that a Hook protein binds dynein-dynactin directly via dynein light intermediate chain (Schroeder and Vale, 2016; Lee et al., 2018) and the Arp1 filament (Urnavicius et al., 2018). The interaction of fungal hook proteins with the early endosome depends on FTS and FHIP (Yao et al., 2014; Guo et al., 2016); FHIP makes the closest contact with early endosome (Yao et al., 2014), most likely via its direct interaction with Rab5 (Guo et al., 2016). It is unclear whether FTS and FHIP are involved in targeting mammalian Hook1, Hook2, or Hook3 onto other cargoes or cellular structures, such as the TrkB–BDNF-signaling endosome (Olenick et al., 2019), nuclear envelope (Dwivedi et al., 2019b), centrosome (Szebenyi et al., 2007a), aggresome (Szebenyi et al., 2007b), and the Golgi apparatus (Walenta et al., 2001). It should be pointed out that Hook1 interacts directly with cargo proteins of the recycling endosomes (Maldonado-Báez et al., 2013), and Hook1 and Hook2 bind directly with AP4 (adaptor protein complex 4 of the *trans*-Golgi network), which is responsible for trafficking of the autophagy protein ATG9A (Mattera et al., 2020).

There are several other important dynein adapters (Reck-Peterson et al., 2018; Olenick and Holzbaur, 2019), such as proteins of the Bicaudal D (BICD) family including BicD2 (Hoogenraad and Akhmanova, 2016), Rab11-FIP3 (Horgan et al., 2010) and Spindly (Griffis et al., 2007). The domain organization of these dynein adapter proteins are similar in that they all contain an N-terminal portion including the coiled-coil domains important for binding dynein-dynactin and a C-terminus required for cargo binding (Reck-Peterson et al., 2018; Dwivedi et al., 2019a; Olenick and Holzbaur, 2019). *In vitro* experiments show that the N-terminal portion of the cargo adapters can enhance the interaction between the dynein complex and the dynactin complex (Splinter et al., 2012; McKenney et al., 2014; Olenick et al., 2016). Such an effect was first shown for the N-terminal part of the BicD2 protein (Splinter et al., 2012), a result highly instrumental to the ground-breaking experiments revealing cargo adapters being critical for dynein activation (McKenney et al., 2014; Schlager et al., 2014).

DYNACTIN AND SPECIFIC CARGO ADAPTER PROTEINS ACTIVATE DYNEIN

Mammalian dynein by itself is incapable of moving along the microtubule processively, although the dynein is active in a microtubule-gliding assay (Trokter et al., 2012; McKenney et al., 2014; Schlager et al., 2014). Adding dynactin alone does not seem to help even when dynactin is added in large excess (McKenney et al., 2014; Schlager et al., 2014). Astonishingly, addition of both BicD2 and dynactin enhances the processivity of dynein dramatically (McKenney et al., 2014; Schlager et al., 2014). Importantly, not only the BicD2 N-terminus but also the dynein-dynactin-binding portion of Hook3 and other cargo adapters with a similar domain organization, including Rab11-FIP3 that targets dynein to Rab11-positive vesicles (Horgan et al., 2010) and Spindly that targets dynein to kinetochores (Griffis et al., 2007; Gama et al., 2017), all stimulated dynein processivity via an enhancement of the dynein-dynactin interaction (McKenney et al., 2014). The processivity and velocity of dynein motility activated by the N-terminal Hook1 and Hook3 are even higher than that by the N-terminal BicD2 (Olenick et al., 2016). These coiled-coil domain-containing dynein adapters are considered as "activating adapters," and new dynein activators with this domain signature have continuously been discovered (Redwine et al., 2017; Reck-Peterson et al., 2018; Olenick and Holzbaur, 2019; Wang et al., 2019).

As revealed by a cryo-EM study, dynein activation results from a conformational change of dynein upon binding to dynactin and a cargo adapter (Zhang K. et al., 2017). Specifically, the two dynein heavy chains within the dimer are initially held in an auto-inhibited "phi" conformation with the two heavy chains positioned very close to each other (Amos, 1989; Torisawa et al., 2014; Zhang K. et al., 2017). This conformation can be switched to an "open" state with the two dynein heavy chains separated from each other. Although the "open dynein" has a higher affinity for microtubules, it is still not configured properly to move along a microtubule (Zhang K. et al., 2017). Only

after the dynein tails bind dynactin and a cargo adapter, the two motor domains with the microtubule-binding stalks become parallel, which allows processive movement (Zhang K. et al., 2017). It is important to point out that since dynactin and cargo adapters bind dynein tails (Schlager et al., 2014; Chowdhury et al., 2015; Urnavicius et al., 2015; Zhang K. et al., 2017), the conformational change in the dynein motor domains must be transmitted through the dynein tails. This may explain why changes in the dynein tail, especially subtle mutations that do not affect the dynein-dynactin interaction, can lead to a severe defect in dynein function (Ori-McKenney et al., 2010; Sivagurunathan et al., 2012; Qiu et al., 2013; Hoang et al., 2017; Marzo et al., 2019).

EM-based structural studies have shown that in some dynein-dynactin-cargo adapter complexes, there are two dynein dimers instead of one, and both dimers have their tails positioned along the Arp1 mini-filament (Grotjahn et al., 2018; Urnavicius et al., 2018). Some cargo adapters including Hook3 and BicDR1 have a much stronger tendency to form this type of complexes with an extra dynein compared to other cargo adapters such as BicD2, and the presence of two dynein dimers enhances dynein's speed and force output (Urnavicius et al., 2018). This may be part of the reason why some of the dynein-dynactin-Hook3 complexes move with a higher velocity compared to the dynein-dynactin-BicD2 complexes (Olenick et al., 2016). Recently, the dynein regulator LIS1 has also been shown to enhance the recruitment of the second dynein dimer to the dynein-dynactin-cargo adapter complex *in vitro* (Elshenawy et al., 2020; Htet et al., 2020), further suggesting the importance of the second dynein dimer. However, it still remains to be determined what proportion of cargo-bound dynactin in live cells contain two dynein dimers associated with the Arp1 mini-filament and how different regulators change this proportion.

While the *in vitro* motility studies and structural analysis provided significant insights into the mechanism of dynein activation, knowledges gained from *in vivo* studies further shed light on the spatial regulation of dynein activity. In filamentous fungi including *Aspergillus nidulans* and *Ustilago maydis*, the dynamic microtubule plus ends face the hyphal tip, and both dynein and dynactin are strongly enriched at the microtubule plus ends (Han et al., 2001; Zhang et al., 2003; Lenz et al., 2006). Fungal dynein transports many cargoes and its major cargo is the early endosome, which undergo rapid bi-directional movements (Wedlich-Soldner et al., 2002; Lenz et al., 2006; Abenza et al., 2009; Zekert and Fischer, 2009; Penalva et al., 2017; Hernández-González et al., 2018; Otamendi et al., 2019; Bieger et al., 2020). Early endosome motility not only is coupled to endosome maturation (Abenza et al., 2010, 2012), it also helps distribute hitchhiking cargoes including peroxisomes, ribosomes and RNAs (Baumann et al., 2012; Bielska et al., 2014a; Higuchi et al., 2014; Guimaraes et al., 2015; Pohlmann et al., 2015; Lin et al., 2016; Salogiannis et al., 2016). Early endosomes are moved by kinesin-3 toward the plus ends near the hyphal tip and then delivered to dynein to be moved away from the hyphal tip (Lenz et al., 2006). The accumulation of dynein at the microtubule plus end depends on kinesin-1 and dynactin (especially the microtubule-binding domain of p150) (Xiang et al., 2000; Zhang et al., 2003; Lenz et al., 2006; Egan et al., 2012; Yao et al., 2012), and dynein-mediated early endosome transport depends on kinesin-1, most likely because the accumulation of dynein at the plus end enhances the chance for the dynein-early endosome interaction (Lenz et al., 2006; Zhang et al., 2010).

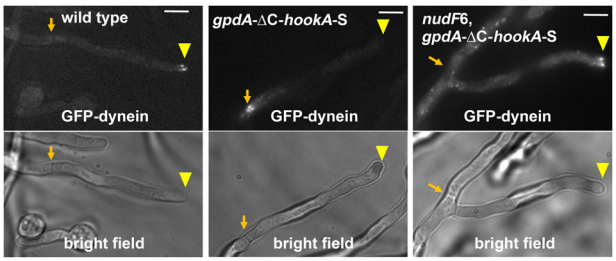

FIGURE 2 | Dynein activation in *A. nidulans* depends on NudF/LIS1. In wild-type cells, dynein is accumulated at the microtubule plus ends, and this accumulation is represented by the comet-like structures formed by GFP-labeled dynein near the hyphal tip (Xiang et al., 2000; Han et al., 2001). Dynein activation, as judged by dynein relocation from the microtubule plus ends at hyphal tip (yellow arrowhead) to the minus ends at septum (Zhang Y. et al., 2017) (brown arrow), is driven by the dynein-dynactin-binding portion of the cargo adapter HookA, ΔC-HookA, overexpressed under the *gpdA* promoter (*gpdA*-ΔC-*hookA*-S, note that "S" indicates S-tag, an affinity tag for biochemical studies) (Qiu et al., 2019). In the *nudF*6 mutant, a NudF/LIS1 loss-of-function mutant, dynein is retained at the microtubule plus ends. Bright-field images are shown below to indicate hyphal shape and septal position. Bars, 5 μm. These images have been published previously in the Journal of Cell Biology (Qiu et al., 2019).

Given the current understanding of dynein's structural change during its activation (Zhang K. et al., 2017), we speculate that fungal dynein is in the autoinhibited phi conformation while being transported by kinesin-1 toward the microtubule plus end. This would prevent a tug-of-war between kinesin-1 and dynein. Conceptually, this is similar to the regulatory mechanism of dynein-2 in intraflagellar transport (IFT) as revealed by cryo-electron tomography: dynein-2 is in an autoinhibited conformation when it is being transported to the plus end by kinesin-2 (Jordan et al., 2018). In *A. nidulans* and *U. maydis*, the microtubule plus end-localized dynein-dynactin interact with early endosomes on which the activating cargo adapter HookA or Hok1 is bound (Bielska et al., 2014b; Zhang et al., 2014). In *A. nidulans*, overexpression of the cytosolic ΔC-HookA drives dynein departure from the microtubule plus ends and causes dynein to accumulate at the minus ends (Qiu et al., 2019; **Figure 2**). This supports not only the idea of cargo adapter-mediated dynein activation emerged from *in vitro* studies (McKenney et al., 2014; Schlager et al., 2014) but also the postulation that the plus-end dynein is activated by its early endosome cargo (Lenz et al., 2006).

The activating function of the cargo adapters has been thought to be involved in the enhancement of the dynein-dynactin interaction. Interestingly, the dynein-dynactin interaction must have occurred to a certain extent before cargo binding in cells. In filamentous fungi, dynactin is required for the plus-end localization of dynein (Xiang et al., 2000; Zhang et al., 2003, 2008; Lenz et al., 2006; Egan et al., 2012; Yao et al., 2012), suggesting that either the two complexes are transported together

by kinesin-1 to the plus end or they associate at the plus end after being transported separately. It seems likely that they are transported together because loss of Arp1 reduced the interaction between dynein and kinesin-1 (Qiu et al., 2018). HookA is able to interact with dynein or dynactin only when both complexes are present (Zhang et al., 2014), although dynein LIC binds the Hook domain directly (Malone et al., 2003; Schroeder and Vale, 2016; Lee et al., 2018). Thus, it is most likely that an early endosome interacts with a plus end dynein-dynactin complex and changes the configuration of the dynein-dynactin interaction to make it productive for minus-end-directed movement. This notion is consistent with previous findings that while the dynein IC in the dynein tail interacts with the p150 subunit of dynactin without cargo adapter (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; King et al., 2003), the dynein tails bind the Arp1 filament in the presence of cargo adapters (Schlager et al., 2014; Chowdhury et al., 2015; Urnavicius et al., 2015, 2018; Zhang K. et al., 2017; Grotjahn et al., 2018).

The spatial regulation of dynein activation appears to be evolutionarily conserved. In Drosophila oocyte, and in *Caenorhabditis elegans* and mammalian neurons, kinesin-1 has been implicated in transporting dynein toward the microtubule plus ends for function (Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002; Palacios and St Johnston, 2002; Yamada et al., 2008, 2010; Arimoto et al., 2011; Twelvetrees et al., 2016). The kinesin-1-dynein interaction has been dissected in detail, and it was found that the dynein intermediate chain interacts directly with the light chains of kinesin-1 in mammalian hippocampal neurons (Ligon et al., 2004; Twelvetrees et al., 2016). In C. *elegans*, however, the interaction between kinesin-1 and dynein is mediated by UNC-16 that binds to the dynein light intermediate chain (Arimoto et al., 2011). In mouse DRG neurons, dynactin and dynein are transported separately by kinesin-1 via mNudC (Yamada et al., 2010). Thus, how kinesin-1 transports dynein may differ in different cell types. What appears to be conserved is the need to get dynein-dynactin to the microtubule plus end using the plus-end-directed kinesin-1, which could enhance the chance of dynein-cargo interaction. In mammalian and Drosophila neurons, the microtubule-binding domain of p150 dynactin is required for enriching dynactin at the distal end of an axon, thereby facilitating the initiation of retrograde transport from the neurite tip or synaptic termini (Lloyd et al., 2012; Moughamian and Holzbaur, 2012). Hook1, which is able to activate dynein (Olenick et al., 2016), is required for transporting signaling endosomes in axons (Olenick et al., 2019).

In the budding yeast, dynein is almost exclusively used for moving nuclei/spindles (Eshel et al., 1993; Li et al., 1993; Winey and Bloom, 2012), and both dynein and dynactin are clearly accumulated at the microtubule plus end (Lee et al., 2003; Sheeman et al., 2003; Moore et al., 2008). Although dynein can be recruited directly from a cytoplasm to the plus end via Bik1/Clip170 and Pac1/LIS1, Kip2 (kinesin-7) also plays an important role in transporting Bik1/Clip170 and dynein to the microtubule plus end (Lee et al., 2003; Sheeman et al., 2003; Carvalho et al., 2004; Markus et al., 2011; Roberts et al., 2014). Dynein accumulated at the microtubule plus end is activated by its cortical anchor Num1, which is a cargo adapter-like molecule containing coiled-coil domains (Farkasovsky and Küntzel, 1995; Lee et al., 2003; Sheeman et al., 2003; Markus and Lee, 2011; Tang et al., 2012; Lammers and Markus, 2015). In the fission yeast, dynein drives the oscillatory movement of meiotic prophase nucleus (Yamamoto et al., 1999), and this function of dynein depends on dynactin and the Num1-like cortical anchor (Niccoli et al., 2004; Yamashita and Yamamoto, 2006). It was found that dynein molecules along microtubules are inactive but activated by the cortical Num1 homolog (Ananthanarayanan et al., 2013). Thus, although yeast dynein is active on its own *in vitro* (Reck-Peterson et al., 2006), yeast dynein's cortical anchor functions as an activating cargo adapter *in vivo*.

LIS1 IS A POSITIVE REGULATOR FOR DYNEIN ACTIVATION

Beside dynactin and cargo adapters, another important protein involved in dynein activation is LIS1 (Lissencephaly-1) (Markus et al., 2020). The mechanism of LIS1 action in the dynein pathway has been controversial, but multiple recent studies suggest that LIS1 promotes the open dynein conformation, thereby facilitating dynein activation (Qiu et al., 2019; Elshenawy et al., 2020; Htet et al., 2020; Marzo et al., 2020; McKenney, 2020). LIS1, a WD40-repeats-containing protein, was initially identified as a causal gene for type 1 lissencephaly, a human brain developmental disorder (Reiner et al., 1993). Its functional connection to dynein was first suggested by genetic studies in fungi and further demonstrated in higher eukaryotic organisms and cell types (Xiang et al., 1995; Geiser et al., 1997; Liu et al., 1999, 2000; Faulkner et al., 2000; Lei and Warrior, 2000; Smith et al., 2000; Markus et al., 2020). In contrast to dynactin that binds to the dynein tail (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; Chowdhury et al., 2015; Urnavicius et al., 2015), LIS1 binds directly to the dynein motor ring at AAA3/AAA4 as shown by cryo-EM studies (Huang et al., 2012; Toropova et al., 2014; Desantis et al., 2017; Htet et al., 2020). LIS1's binding to this site is not compatible with the autoinhibited phi conformation of dynein (Htet et al., 2020; Marzo et al., 2020), which supports a "check valve" (Markus et al., 2020) model of LIS1 mechanism of action: it stabilizes the open dynein conformation and prevents it from switching to the autoinhibited phi state, thereby facilitating cargo-adapter-mediated dynein activation (Qiu et al., 2019; Canty and Yildiz, 2020; Elshenawy et al., 2020; Htet et al., 2020; Markus et al., 2020; Marzo et al., 2020).

In budding yeast and mammalian cells, LIS1 is required for the microtubule plus-end accumulation of dynein and consequently dynein offloading to cortex or cargoes (Lee et al., 2003; Sheeman et al., 2003; Markus and Lee, 2011; Markus et al., 2011; Splinter et al., 2012; Tame et al., 2014). However, dynactin rather than LIS1 plays a critical role in the plus-end accumulation of dynein in filamentous fungi (Zhang et al., 2003; Lenz et al., 2006; Egan et al., 2012). This allows the role of LIS1 in cargo-adapter-mediated dynein activation *in vivo* to be shown clearly in *A. nidulans* (Qiu et al., 2019). Specifically, while overexpression of ΔC-HookA drives almost a complete relocation of dynein from

the microtubule plus ends to the minus ends, dynein remains at the plus ends in the *nudF* (lis1) loss-of-function mutants when ΔC-HookA is overexpressed (Qiu et al., 2019; **Figure 2**). This requirement of LIS1 for HookA-mediated dynein activation *in vivo* is consistent with a role of LIS1 in enhancing the frequency of departure of the dynein-dynactin-cargo adapter complex from the microtubule plus end *in vitro* (Baumbach et al., 2017; Jie et al., 2017). Importantly, a phi mutation that promotes open dynein (Zhang K. et al., 2017) bypasses the requirement for LIS1 to a significant extent (Qiu et al., 2019), suggesting that LIS1 is involved in promoting open dynein, thereby facilitating cargo adapter-mediated dynein activation (Qiu et al., 2019). This main conclusion agrees with three other recent studies (Elshenawy et al., 2020; Htet et al., 2020; Marzo et al., 2020), although LIS1 in *A. nidulans* does not seem to significantly affect the formation of the dynein-dynactin-ΔC-Hook complex (Qiu et al., 2019), while LIS1 enhances the recruitment of the second dynein to the dynein-dynactin-BicD2N complexes *in vitro* (Elshenawy et al., 2020; Htet et al., 2020). Possibly, *A. nidulans* LIS1 still enhances the dynein-dynactin interaction as described in other systems (Dix et al., 2013; Wang et al., 2013), but this effect was not easily detected when the concentration of cytosolic cargo adapters is high enough. We should also point out that LIS1 may play roles beyond stabilizing the open dynein because constitutively opening dynein does not allow the requirement for LIS1 to be completely bypassed (Qiu et al., 2019; Elshenawy et al., 2020; Htet et al., 2020; Marzo et al., 2020).

In filamentous fungi and budding yeast, LIS1 accumulates at the microtubule plus end just like dynein (Han et al., 2001; Lee et al., 2003; Callejas-Negrete et al., 2015). LIS1's plus-end accumulation depends partly on dynein, its binding partner NudE as well as the CLIP170 homolog CLIPA or Bik1 (Zhang et al., 2003; Li et al., 2005; Efimov et al., 2006; Markus et al., 2011). This is consistent with earlier data from mammalian cells and budding yeast indicating a direct interaction between LIS1 and CLIP170/Bik1 (Coquelle et al., 2002; Sheeman et al., 2003). In the budding yeast, the dynein-LIS1-Bik1/CLIP170 complex could be transported by the Kip2 kinesin-7 to the microtubule plus end, or, dynein and LIS1 form a complex before being directly recruited from the cytosol to the plus end via Bik1/CLIP170 (Carvalho et al., 2004; Markus et al., 2011). Thus, the plus-end dynein in yeast is most likely in the open conformation and can interact effectively with dynactin and Num1 (Markus et al., 2020; Marzo et al., 2020). In cultured cells and in reconstituted *in vitro* systems with dynamic microtubules, LIS1 also enhances the plus-end targeting of mammalian dynein, although the plus-end dynein localization also requires dynactin (Splinter et al., 2012; Baumbach et al., 2017; Jha et al., 2017). In the filamentous fungi such as *A. nidulans* and *U. maydis*, the plus-end accumulation of dynein requires kinesin-1 and dynactin but not LIS1 (Zhang et al., 2003; Lenz et al., 2006; Egan et al., 2012). Thus, the plus-end dynein in filamentous fungi could be in the autoinhibited phi conformation before it interacts with LIS1 (**Figure 3**). In *A. nidulans*, dynein localized along microtubules in cells lacking kinesin-1 is still able to be activated by LIS1 and ΔC-HookA (Qiu et al., 2019), suggesting that LIS1 can also bind to dynein not at the plus end. It cannot be excluded that some LIS1 molecules may bind to dynein during plus-end-directed transport mediated by kinesin-1, and in that case, dynein at the plus end is in the open conformation, waiting to be activated by the early endosome cargo.

When bound to dynactin and cargo adapters, dynein carrying LIS1 at its motor ring is able to undergo processive movement toward the microtubule minus end (Baumbach et al., 2017; Gutierrez et al., 2017), which differs from the inhibitory function of LIS1 on dynein alone (Yamada et al., 2008; McKenney et al., 2010; Huang et al., 2012). However, LIS1 tends to dissociate from the motile dynein-dynactin-cargo adapter complex both *in vivo* and *in vitro* (Lenz et al., 2006; Egan et al., 2012; Lammers and Markus, 2015; Jha et al., 2017; Elshenawy et al., 2020; Htet et al., 2020). In the budding yeast, LIS1 has never been observed to co-localize with dynein-dynactin at the cell cortex (Markus et al., 2011). In filamentous fungi such as *U. maydis* and *A. nidulans*, while dynactin remains associated with a motile early endosome after its dynein-mediated movement has been initiated, LIS1 tends to fall off from it (Lenz et al., 2006; Egan et al., 2012). By observing many *A. nidulans* hyphal tip cells of a *nudF*/lis1 deletion mutant in which early endosome motility is rarely observed, it was found that loss of LIS1 does not affect the speed of occasional dynein-mediated transporting events, and thus, LIS1 was considered as an initiation factor (Egan et al., 2012). The idea that LIS1 is only important for transport initiation is consistent with the current results on LIS1 promoting the open dynein state (Qiu et al., 2019; Elshenawy et al., 2020; Htet et al., 2020; Marzo et al., 2020), thereby enhancing the formation of the dynein-dynactin-cargo-adapter complex (Zhang K. et al., 2017). However, the dynein-dynactin-ΔC-HookA complex still forms without LIS1 in *A. nidulans*, suggesting that dynein-dynactin at the microtubule plus end are capable of binding the HookA-linked early endosome but cannot initiate minus-end-directed movement (Qiu et al., 2019). Further studies will be needed to determine the structure of the dynein-dynactin-ΔC-HookA complex isolated from cells with or without NudF/LIS1, as it seems intriguing why such a complex is not capable of leaving the microtubule plus end *in vivo* in the absence of NudF/LIS1. We should also point out that in neurons, while the microtubule-binding domain of the p150 dynactin is only required for transporting initiation from the distal axon containing dynamic MT plus ends (Lloyd et al., 2012; Moughamian and Holzbaur, 2012), LIS1 is additionally required for continued transport in the mid-axon with much more stable MT (Moughamian et al., 2013). Why is LIS1 so critical *in vivo* while the dynein-dynactin-cargo adapter complex can move without LIS1 *in vitro*? We can envision two possibilities. First, the assembly of the dynein-dynactin-cargo adapter complex containing a second dynein, which is promoted by LIS1 (Elshenawy et al., 2020; Htet et al., 2020), leads to a higher force production (Urnavicius et al., 2018; Elshenawy et al., 2020), thereby facilitating the movement of dynein cargoes in a viscous cytoplasm. Second, there could be a negative regulator that keeps dynein at the phi state *in vivo*, which makes LIS1 absolutely necessary to work against such an inhibition. Future studies will be needed to address these possibilities.

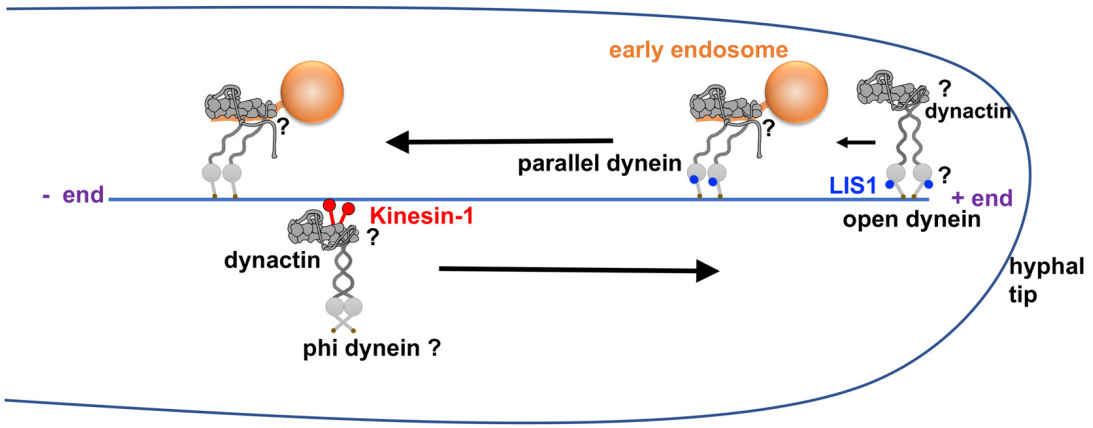

FIGURE 3 | Model of dynein activation in filamentous fungi. This model is based on the structural data on the LIS1-dynein interaction and cargo-adapter-mediated dynein activation (Huang et al., 2012; Zhang K. et al., 2017; Htet et al., 2020), live cell imaging data on dynein, dynactin and LIS1 from filamentous fungi (Han et al., 2001; Zhang et al., 2003; Efimov et al., 2006; Lenz et al., 2006; Egan et al., 2012; Yao et al., 2012) as well as recent data on LIS1 mechanism (Qiu et al., 2019; Elshenawy et al., 2020; Htet et al., 2020; Marzo et al., 2020). In this model, dynein in the autoinhibited phi conformation is transported by kinesin-1 to the microtubule plus end together with dynactin. At the microtubule plus end, the dynein-LIS1 interaction keeps the motor domains of the dynein dimer in an open configuration but still being incapable of processive movement until dynein-dynactin interacts with an early endosome cargo. The cargo adapter (HookA or Hok1) on the early endosome interacts with dynein-dynactin-LIS1 and switches the dynein dimer into a parallel configuration, allowing it to walk toward the microtubule minus end. Note that there is also a conformational change of dynactin after cargo adapter binding so that the p150 protein is in an extended conformation (Urnavicius et al., 2015). Finally, LIS1 dissociates from dynein during the minus-end-directed movement (Lenz et al., 2006; Egan et al., 2012). The questions marks in this figure indicate the speculative nature of the dynein-dynactin configurations at these sites.

OTHER PROTEINS IMPORTANT FOR DYNEIN ACTIVATION *IN VIVO*-NUDE AND P150 OF DYNACTIN

LIS1's binding partner NudE and its homologs are also involved in dynein function (Minke et al., 1999; Efimov and Morris, 2000; Feng et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000; Yan et al., 2003; Liang et al., 2004, 2007; Shu et al., 2004; Li et al., 2005; Guo et al., 2006; Stehman et al., 2007; Ma et al., 2009; Lam et al., 2010; Pandey and Smith, 2011; Wang and Zheng, 2011; Zylkiewicz et al., 2011; Wang et al., 2013; Klinman and Holzbaur, 2015; Reddy et al., 2016; Simões et al., 2018). NudE (homologous to Ro11 in *Neurospora crassa*) (Minke et al., 1999) and its interaction with NudF/LIS1 were first identified in *A. nidulans* and in higher eukaryotic model systems (Efimov and Morris, 2000; Feng et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000). In both *A. nidulans* and budding yeast, loss of NudE causes defects in nuclear migration/spindle orientation, but the defects are much milder compared to that caused by loss of LIS1/NudF/Pac1 (Efimov and Morris, 2000; Li et al., 2005). In *A. nidulans*, mammalian cells, budding yeast and Xenopus egg extract, NudE becomes dispensable if LIS1 concentration is increased (Efimov, 2003; Shu et al., 2004; Li et al., 2005; Wang and Zheng, 2011), consistent with a role of NudE in recruiting LIS1 to dynein (McKenney et al., 2010). In *A. nidulans*, NudE is required for ΔC-HookA-mediated dynein activation, and the requirement of NudE for dynein-mediated early endosome transport can be partially bypassed by constitutively opening dynein using the phi mutations (Qiu et al., 2019). Thus, NudE supports LIS1's function in dynein activation. NudE not only binds LIS1 but also binds dynein (Sasaki et al., 2000; Liang et al., 2004; McKenney et al., 2011; Wang and Zheng, 2011; Zylkiewicz et al., 2011; Nyarko et al., 2012), but intriguingly, NudE and dynactin p150 compete for binding to the N-terminal site of dynein IC (McKenney et al., 2011; Nyarko et al., 2012). Further studies are needed to reveal how these binding events are regulated to allow dynein activation by LIS1, dynactin and cargo adapters.

EM structural analysis suggests that cargo adapter binding to dynactin may change the conformation of p150 dynactin (Urnavicius et al., 2015). Without cargo adapters, p150 proteins can be seen under EM to exist in either folded or more extended conformations (Urnavicius et al., 2015; Saito et al., 2020). In the folded state, p150's CC1A and CC1B domains contact the pointed-end complex of the Arp1 mini-filament (Urnavicius et al., 2015), and its microtubule-binding domain (MTBD) is most likely folded inside rather than being exposed (**Figure 1B**). Although only a minority of dynactin complexes contain folded p150 under EM (Urnavicius et al., 2015), the idea that p150 is mainly in a folded state in the absence of cargo adapters is consistent with the observation that isolated dynactin does not bind microtubules *in vitro* in the presence of dynein without cargo adapters (McKenney et al., 2014). Interestingly, the pointed end proteins including p25 interact with both the cargo adapter and p150's CC1A and CC1B domains (Urnavicius et al., 2015; Qiu et al., 2018), and the binding sites of the dynein cargo adapters BicD2, Hook3, and BicDR1 at the pointed end overlap with the CC1A- and CC1B-binding sites (Urnavicius et al., 2015; Lau et al., 2020). Thus, it seems possible that BicD2, Hook3, BicDR1 or other similar cargo adapters may compete with p150's CC1A and CC1B domains for binding to the pointed end, thereby forcing p150 to open up (Cianfrocco et al., 2015; Lau et al., 2020). The pointed end protein p25 is likely to be critically involved in this

process as it plays a dual role in cargo adapter binding and the regulation of dynactin-microtubule interaction (Qiu et al., 2018). The most recent structural analysis shows that the whole pointed end complex including p25 acts as an interaction hub for cargo adapters and p150 (Lau et al., 2020). Future studies are needed to address whether cargo adapter binding indeed causes p150 to change its conformation, and if so, how this contributes to the process of dynein activation.

FUTURE DIRECTIONS

While the dynein field has made significant progress toward understanding how dynein is activated, some basic questions still remain to be answered. For example, what is the physiological significance of cargo adapter-mediated dynein activation? One obvious purpose of such a regulatory strategy could be to allow inactive dynein to be delivered to the microtubule plus end and stay there to receive its cargo rather than leaving prematurely toward the minus end without the cargo. However, could this regulatory strategy also help save the cellular energy currency ATP and ease the cellular burden of using metabolic pathways to generate ATP, especially when food source is limited? In *A. nidulans*, overexpressing ΔC-HookA in phi mutant cells where dynein is constitutively open produces colonies that are nearly inviable (much sicker than the dynein-null mutant) (Qiu et al., 2019). Could this be related to abnormal ATP consumption, which affects other ATP-utilizing processes or causes the overproduction of unhealthy metabolic products (like lactic acid in a skeletal muscle cell)? Currently, the published dynein ATPase activity (∼200 nmol/min/mg dynein) (Mesngon et al., 2006; McKenney et al., 2010) is likely from inactive dynein, and given the cellular ATP concentration of ∼2 nmol/μl, a fungal tip cell with the volume of $\sim 1 \times 10^{-7}$ μl would need about half a million dynein molecules to consume the total cellular ATP within a minute (if ATP is not generated by metabolic pathways). It does seem unlikely to have so many dynein molecules in a fungal cell to significantly affect the cellular ATP pool. However, if a fully active dynein has a much higher ATPase activity, this number of dynein molecules will become smaller and more reasonable. It is known that the ATPase activity of an activated myosin II motor can be >100× higher than that of an inactive one (Trybus, 1989; Heissler and Sellers, 2016). In this context, it would be worthwhile to measure the ATPase activities of the inactive phi dynein, open dynein and fully activated dynein (with dynactin and cargo adapters).

There are important open questions on the spatial regulation of dynein in live cells. For example, what are the dynein-dynactin conformational states during kinesin-mediated transport to the microtubule plus end and/or at the plus end? While it will be technically challenging to apply structural analysis on live cells to reveal these states, it should be possible to use dynein-dynactin isolated from specific mutants for biochemical, single molecule and structural analyses. For example, in an *A. nidulans* Δ*hookA* mutant without any early endosomal dynein adapters, many dynein and dynactin molecules accumulate at the microtubule plus end, and it will be interesting to determine their conformational states. Similarly, it would be interesting to determine the conformation of dynein-dynactin or dynein-dynactin-cargo-adapter isolated from cells with or without LIS1. Another interesting issue is how kinesin-3 delivers an early endosome to dynein *in vivo* without being a competitor of dynein, given that *U. maydis* Hok1 affects the kinesin-3-early-endosome interaction and the mammalian Hook3 binds both dynein and kinesin-3 (Bielska et al., 2014b; Kendrick et al., 2019; Siddiqui et al., 2019)? In addition, how do the interactions between the microtubule-binding domain of p150 dynactin and differently modified tubulins affect dynein-mediated transport *in vivo* (Barisic and Maiato, 2016; McKenney et al., 2016; Nirschl et al., 2016; Roll-Mecak, 2020)?

One specific question that deserves to be discussed in more detail is how p150 of dynactin is involved in the dynein activation process. The microtubule-binding domain (MTBD) of p150 is needed for the plus-end accumulation of dynactin-dynein (Vaughan et al., 2002; Kim et al., 2007; Yao et al., 2012) and the initiation of minus-end-directed transport, especially in neurons (Lloyd et al., 2012; Moughamian and Holzbaur, 2012), possibly by helping dynein landing on tyrosinated microtubules (McKenney et al., 2016; Nirschl et al., 2016). However, data from multiple labs also suggest that p150 is an allosteric activator of dynein rather than simply a microtubule-tethering factor (Kim et al., 2007; Dixit et al., 2008; Kardon et al., 2009; Tripathy et al., 2014; Feng et al., 2020). The possibility that cargo binding may change p150 conformation is of great interest in this context. Before cargo binding, p150 could be in folded and more extended conformations and these states are in equilibria (Urnavicius et al., 2015; Saito et al., 2020). We speculate that in fungal cells, before the formation of the dynein-dynactin-kinesin-1 complex, the extended p150 allows its MTBD to be exposed to bind microtubule (Yao et al., 2012). This will help recruit dynein to the microtubule, possibly via the p150 (CC1B)-IC interaction (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; King et al., 2003; McKenney et al., 2011). We speculate that the subsequent binding to kinesin-1 changes this interaction mode and promotes a folded state of p150 to prevent its MTBD from interfering with kinesin-1-mediated transport, although it is unclear how dynein binds dynactin with a folded p150 (**Figure 3**). We also speculate that after kinesin-1 is dissociated from dynein-dynactin at the plus end, a transient NudE-IC interaction (Efimov, 2003; McKenney et al., 2011; Wang and Zheng, 2011) may prevent p150's CC1B from binding to IC (McKenney et al., 2011), thereby stabilizing the folded state of p150. Does cargo adapter binding promote the open state of p150 at the microtubule plus end (**Figure 3**) and allow its CC1B domain to bind dynein IC again (Urnavicius et al., 2015)? If so, how does the p150-IC interaction in the presence of the cargo adapter change the configuration of dynein HC tails to position them along the Arp1 filament, which eventually leads to dynein activation (Zhang K. et al., 2017)?

Related to the questions on the conformational states of dynactin p150, another important question is how dynein stays at the microtubule plus end. Since the binding to a cargo adapter is a prerequisite for dynein activation, dynein is expected to remain at the plus end before cargo binding. However, we do not know exactly how dynein interacts with the microtubule plus

end. In the budding yeast, the microtubule-binding domain of dynein HC is not needed for dynein's plus-end accumulation (Lammers and Markus, 2015), and thus, yeast dynein is most likely retained at the plus end via LIS1 that binds the plus end-tracking protein Bik1/CLIP170 (Lin et al., 2001; Sheeman et al., 2003; Markus et al., 2011). However, the plus-end accumulation of dynein in filamentous fungi does not need LIS1 or CLIP170 homologs (Zhang et al., 2003; Efimov et al., 2006; Lenz et al., 2006; Egan et al., 2012), but it needs the MTBD of p150 (Yao et al., 2012). Nevertheless, if p150 is folded at the plus end before cargo binding, its MTBD is unlikely to be exposed and used for anchoring dynein at the plus end. Thus, we hypothesize that in filamentous fungi, an open dynein (with LIS1 bound) contacts the microtubule plus end directly using its own microtubule-binding domains (**Figure 3**), and this open dynein is primed for cargo binding and the subsequent minus-end-directed movement. More data will be needed to either support or refute this hypothesis.

Finally, to gain a full picture of dynein regulation *in vivo*, it will also be important to study the new regulators of dynein. A good example for illustrating this point is the recent progress toward understanding LIS1's mechanism of action, which was possible only after specific cargo adapters were identified and shown to activate dynein. In this context, we should also point out the need of further dissecting how NudE participates in dynein activation (Qiu et al., 2019), as NudE competes with the CC1B domain of p150 for binding to dynein IC (McKenney et al., 2011; Jie et al., 2017). Moreover, is there any negative regulator that helps keep dynein in the autoinhibited conformation *in vivo*? While we still do not know if such a regulator exists for cytoplasmic dynein, a recent study using Tetrahymena has identified a novel axonemal dynein-binding protein, Shulin, as a regulator that keeps axonemal dynein in an inactivate conformation before it is delivered to cilia (Mali et al., 2020). While both biochemical and genetic approaches can be powerful, genetic screens in *A. nidulans* have been highly valuable in identifying new proteins involved in dynein-mediated intracellular transport (Osmani et al., 1990; Xiang et al., 1995; Efimov and Morris, 2000; Yao et al., 2014; Zhang et al., 2014; Salogiannis et al., 2016). Recently, two new proteins, VezA/vezatin and Prp40A/PRPF40A, have been identified in *A. nidulans* as important factors for dynein-mediated early endosome transport (Yao et al., 2015; Qiu et al., 2020). Vezatin was initially identified as a protein involved in stabilizing cell-cell adhesions (Kussel-Andermann et al., 2000), and PRPF40A is homologous to the yeast RNA-splicing factor Prp40 (Kao and Siliciano, 1996). Interestingly, both vezatin and PRPF40A were identified as Arp1-binding proteins in a biochemical pulldown assay (Hein et al., 2015; Qiu et al., 2020). For vezatin, the interaction with Arp1 could be direct as Arp1 (ACTR1A) was identified as a protein in close proximity to vezatin (VEZT) in human cells (Go et al., 2019)[1]. VezA/vezatin in *A. nidulans* is clearly not a cargo adapter like HookA, and intriguingly, it localizes at the hyphal tip in an actin cytoskeleton-dependent fashion (Yao et al., 2015), and how it affects the microtubule plus end-localized dynein-dynactin will need to be addressed. Recently, a forward genetic screen in Drosophila has also identified a vezatin homolog as being important for dynein-mediated axonal transport, and furthermore, a zebrafish vezatin homolog is also involved in a similar dynein-mediated process (Spinner et al., 2020). The mechanisms of actions of these proteins will need to be further studied in different experimental systems.

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REVIEW
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Intracellular Transport in Cancer Metabolic Reprogramming

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Tumor progression is a complex process consisting of several steps characterized by alterations in cellular behavior and morphology. These steps include uncontrolled cell division and proliferation, invasiveness and metastatic ability. Throughout these phases, cancer cells encounter a changing environment and a variety of metabolic stress. To meet their needs for energy while they proliferate and survive in their new environment, tumor cells need to continuously fine-tune their metabolism. The connection between intracellular transport and metabolic reprogramming during cancer progression is emerging as a central process of cellular adaptation to these changes. The trafficking of proteolytic enzymes, surface receptors, but also the regulation of downstream pathways, are all central to cancer progression. In this review, we summarize different hallmarks of cancer with a special focus on the role of intracellular trafficking in cell proliferation, epithelial to mesenchymal transition as well as invasion. We will further emphasize how intracellular trafficking contributes to the regulation of energy consumption and metabolism during these steps of cancer progression.

Keywords: membrane trafficking, cancer cell metabolism, cell proliferation, epithelial to mesenchymal transition, invasion

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INTRODUCTION

During cancer progression, tumor cells go through different stages, which are defined as hallmarks of cancer. One of the main hallmarks is the ability to sustain proliferation. Misregulation of growth-promoting signals stimulates cell survival and energy metabolism, resulting in tumor growth (Hanahan and Weinberg Robert, 2011). As cancer further develops, cells may become able to disseminate from the primary site of origin. This is usually induced by loss of epithelial markers such E-cadherin, and characterized by the transition from an epithelial phenotype to a mesenchymal phenotype, a process known as epithelial to mesenchymal transition (EMT). Genes that in normal tissues express molecules involved in cell-to-cell adhesion and cell-to-extracellular matrix adhesions are altered in highly aggressive carcinomas, typically downregulated (Hanahan and Weinberg Robert, 2011). After losing cell-cell adhesions, cancer cells acquire migratory ability, leading eventually to invasion into neighboring tissues and forming metastatic sites in distant organs (Son and Moon, 2010).

During these transitions, cancer cells undergo metabolic changes, which allow them to satisfy their increased need of energy. The reprogramming of energy metabolism is now recognized as one of the hallmarks of cancer. One of the most known metabolic adaptation in malignant cells is the Warburg effect, that is characterized by increased glucose uptake and lactate production in the presence of oxygen (Potter et al., 2016). To satisfy their high nutritional and energetic requirements, cancer cells exploit intracellular trafficking pathways such as macropinocytosis and autophagy

to scavenge the tumor microenvironment for nutrients and macromolecules. This fuels the cells to sustain proliferation, undergo EMT, as well as drive invasion. In this review, we will highlight the contribution of the intracellular transport for the metabolic adaptations required during the different stages of tumor progression.

INTRACELLULAR TRAFFICKING IN CANCER CELL PROLIFERATION

One of the first challenges that cancer cells overcome during cancer transformation is the ability to sustain chronic proliferation (Hanahan and Weinberg Robert, 2011). Keeping up with a sustained proliferation signal has a cost in term of energy requirement. To be able to proliferate, cells must duplicate their mass. Therefore, they need to reprogram their metabolism to meet the need for larger amount of nutrients to support the synthesis of new macromolecules (Davidson and Vander Heiden, 2017).

Cells that are proliferating rapidly have different metabolic needs than those that are in a resting state. Even though glucose and glutamine have been believed to be the major source of energy, it is now clear that the cells also use nutrients and amino acids available in the environment rather than synthesizing them *de novo*. This is a more convenient strategy as *de novo* synthesis requires more energy compared to reusing already existing nutrients and amino acids (Hosios et al., 2016). Pre-existing nutrients necessitate to be transported either from the extracellular environment or from other cellular compartments inside the cells to lysosomes for their degradation into recyclable building blocks (Davidson and Vander Heiden, 2017; **Figure 1**).

Macropinocytosis and Cancer Cell Proliferation

Cancer cells have an increased need for nutrients and therefore adopt different strategies to access macromolecules from the tumor microenvironment. Macropinocytosis is an effective and rapid way to internalize macromolecules from the environment. It is an actin-dependent endocytic mechanism consisting of non-specific uptake of large amounts of extracellular fluid and nutrients into large vesicles (King and Kay, 2019). This process is crucial for nutrient uptake to support tumor cell fitness and it is associated with cell growth. Indeed, pharmacological inhibition of macropinocytosis suppresses tumor growth and it has been suggested that this process could be a possible target for anticancer therapies (Commisso et al., 2013). Macropinocytosis also boosts intracellular Adenosine triphosphate (ATP) concentration by directly ingesting ATP molecules when they are available in the extracellular environment (**Figure 1**; Qian et al., 2014). Extracellular ATP concentration in tumors is up to 10^4 times higher than in normal tissues (Pellegatti et al., 2008; Falzoni et al., 2013; Qian et al., 2014). This extracellular ATP increases the survival of cancer cells during metabolic stress by protecting against tumor inhibition drugs. It has been suggested that following ATP internalization, the increased intracellular ATP interferes with tumor inhibition drugs that compete with ATP for their anticancer activity (Qian et al., 2014). In line with this, extracellular ATP reduced the function of the cancer drug sunitinib that works as an ATP competitor targeting receptor tyrosine kinases (Papaetis and Syrigos, 2009; Qian et al., 2014).

After internalization, macropinosomes deliver their content to the lysosomes where the internalized macromolecules are broken down. The obtained amino acids provide a carbon source to the central metabolism, and serve as building blocks for protein synthesis during proliferation in conditions lacking free amino acids (Palm, 2019). The small GTPase Rab7a, which regulates the fusion between endosomes and autophagosomes with lysosomes, is enriched in melanoma cells where it also is important for sustaining cell proliferation and cancer progression (Alonso-Curbelo et al., 2014, 2015). In particular, at early stages of melanoma development, Rab7a is upregulated and sustains melanoma cell proliferation controlled by the lineage-specific transcription factor SOX10 and the oncogenic transcription factor MYC, which is activated at early stages of melanoma development (Alonso-Curbelo et al., 2014). Rab7a upregulation at these early stages of melanocyte transformation hyperactivates Rab7a-mediated lysosomal degradation to counteract the enhanced macropinocytic influx associated with oncogene-induced senescence (Alonso-Curbelo et al., 2015). During melanoma progression, Rab7a expression is then downregulated. In highly invasive melanoma cells, this favors invasive phenotypes supporting Rab7a as a risk factor for melanoma metastasis and poor survival (Alonso-Curbelo et al., 2014).

Nutrient scavenging consists in the uptake of macromolecules from the extracellular environment and their degradation to produce ATP or to be used in anabolism (Finicle et al., 2018). Scavenging is controlled by the mechanistic target of rapamycin complex-1 (mTORC1) and AMP kinase (AMPK), which are involved in the regulation of macropinocytosis (Swanson and King, 2019). AMPK activates all forms of scavenging, while mTORC1 represses the effect of scavenging by interfering with the catabolism happening in the lysosomes (Finicle et al., 2018). AMPK regulates diverse metabolic cellular processes, and also endocytic traffic during metabolic stress. When it is activated, it inhibits energy demanding processes and enhances catabolic reaction to generate ATP (Rahmani et al., 2019). AMPK can both suppress but also promote tumor growth (Hardie, 2015). It has been suggested that this depends on the timing of modification, mutation and overexpression of AMPK or of the upstream kinase Liver kinase B1 (LKB1). In the initial steps of cancer, inactivation of this pathway may help cell growth by utilizing anabolic pathways. In later stages, activation of the LKB1–AMPK pathway could protect the tumor cells against oxidative stress by facilitating metabolic adaptations (Jeon, 2016).

mTORC1 is a signaling hub that coordinates nutrient status and cell growth. Activated mTORC1 regulates cellular metabolism and growth by stimulating protein synthesis (Kim and Guan, 2019). The internalization of amino acids in macropinosomes and their delivery to the lysosomes is essential for mTORC1 growth factor-dependent activation (Yoshida et al.,

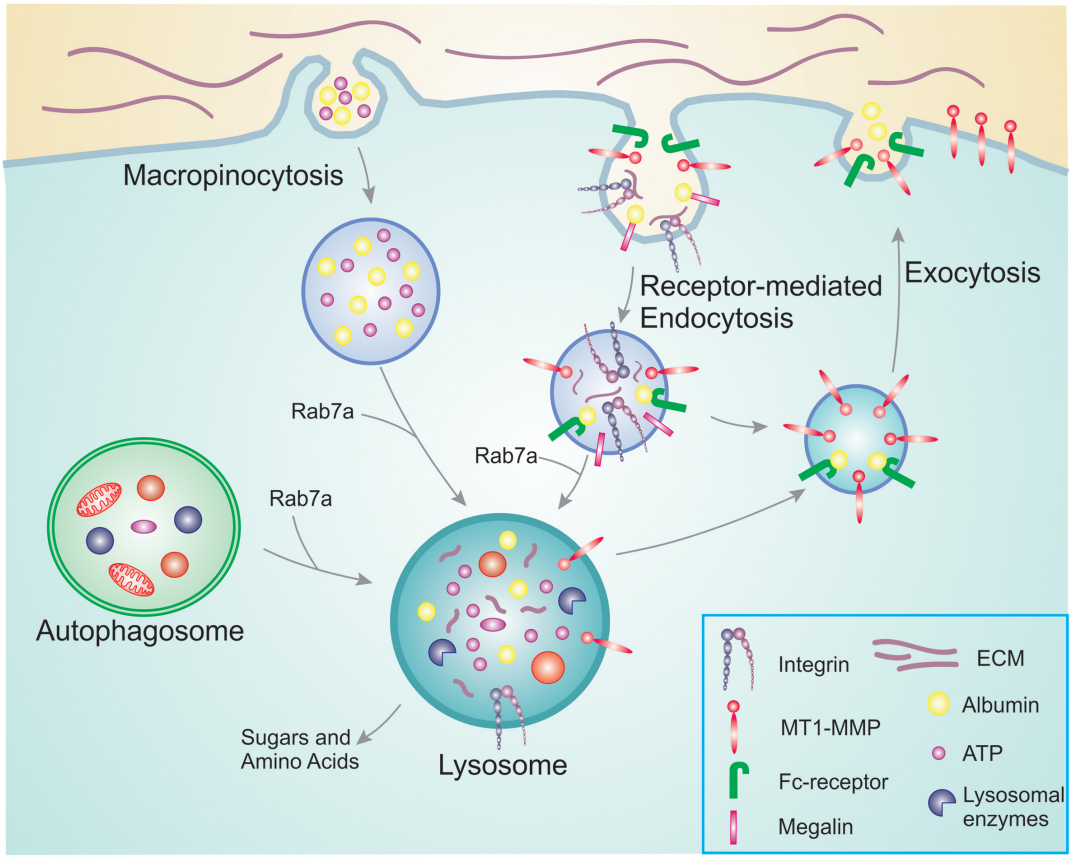

FIGURE 1 | Overview of nutrient scavenging pathways in cancer cells. During cancer progression, cancer cells increase their energy and nutrient requirement to meet the demand of constant proliferation and sustain processes such as migration and invasion. For this, they use membrane trafficking pathways to scavenge nutrients already available. Macropinocytosis allows the bulk internalization of extracellular ATP as well as albumin and other nutrients. In a similar manner, receptor-mediated endocytosis is responsible for the uptake of receptors and their ligands that will be degraded in the lysosomes providing new building blocks to be reused. Examples are integrins that bind to ECM components, and megalin which binds to albumin in the extracellular environment. The internalized albumin can either bind to neonatal Fc receptor inside the endosomes and be recycled back to the plasma membrane, or degraded in the lysosomes. To access the pool of nutrients already available in the cell, cancer cells can hijack autophagy. Engulfed damaged organelles and protein aggregates can thus be broken down and degraded in the lysosomes for reuse. The altered metabolism during cancer progression results in increased MT1-MMP recycling, thus promoting cell invasion.

2015). The control of mTOR signaling is critical for the cells and its dysregulation leads to several diseases such as cancer, diabetes, and metabolic diseases (Yoshida et al., 2015).

Amino acid depletion stimulates macropinocytosis and the scarcity of glutamine drives this process (Lee et al., 2019). The nutrient stress triggered from amino acid depletion enhances epidermal growth factor (EGF) receptor signaling that in turn increases macropinocytosis by regulating membrane ruffling and cytoskeleton dynamics (Lee et al., 2019). The activation of the actin cytoskeleton occurs through the small GTPase Ras (Bloomfield and Kay, 2016; Recouvreux and Commisso, 2017). Ras is frequently mutated in cancer and activated in almost 33% of all human cancer (Bloomfield and Kay, 2016; Lanfredini et al., 2019). Ras-driven cancer cells have a higher rate of macropinocytosis. Over-activation of Ras promotes metabolic rewiring and cell proliferation not only by activation of macropinocytosis to internalize extracellular nutrients and enhancing uptake of glucose, contributing to the Warburg effect, but also by inducing autophagy (Recouvreux and Commisso, 2017; Palm, 2019).

Ras-transformed cancer cells are able to take up albumin through macropinocytosis. Degradation of albumin is a source of glutamine, one of the most deprived nutrients in cancer environments. Hence, the macropinocytic uptake of albumin could serve to sustain the proliferation of oncogenic Ras cells by constituting a source of amino acid supply (Commisso et al., 2013; Ha et al., 2016; Palm, 2019). Glutamine serves indeed as important source of carbon, which in different tumors is utilized for TCA cycle anaplerosis. In proliferating cells, glutamine-dependent anaplerosis is critical for mitochondrial metabolism and essential for cell growth (Cluntun et al., 2017). The internalization of albumin through macropinocytosis and the downstream use of albumin-derived amino acids as a source of energy seems to be a unique property of cancer cells, since normal cells adjacent to a tumor lack this ability (Davidson et al., 2017). The stimulation of macropinocytosis in cancer cells

is, however, not limited to Ras-transformed cells, as activating mutations in Src kinases also drive macropinocytosis (Amyere et al., 2000; Finicle et al., 2018).

Intriguingly, it has been recently demonstrated that under nutrient-limited conditions, cancer cells within pancreatic ductal adenocarcinoma are able to internalize collagen fragments through macropinocytosis. This extracellular matrix protein represents a proline reservoir that is used as a nutrient source in the absence of other fuels. In this way, the collagen-derived proline contributes to promoting cancer cell survival as well as cell proliferation (Olivares et al., 2017). Therefore, it seems that cancer cells have developed an efficient strategy to obtain nutrients from alternative sources through macropinocytosis followed by lysosomal degradation of extracellular proteins. This allows to furnish the energy and nutrient demand for sustained proliferation.

Receptor-Mediated Internalization for Nutrient Scavenging

Nutrient scavenging does not only occur by macropinocytosis. Cancer cells can also utilize receptor-mediated scavenging to sustain their growth and proliferation (**Figure 1**). An example of receptor-mediated scavenging is represented by the integrin-mediated endocytosis of extracellular matrix (ECM) components (Finicle et al., 2018). Integrins are cell surface receptors for ECM components that link the actin cytoskeleton to the ECM. During cancer progression, the trafficking of integrins is often upregulated resulting in the internalization of the receptors but also of the ECM components bound to the integrins. The ECM consists of collagen, laminin, and fibronectin. These extracellular proteins are also heavily glycosylated, thus ECM scavenging yields amino acids and sugars to sustain cell proliferation. Dietary restriction and nutrient deprivation induces laminin scavenging by integrin α6β4-mediated endocytosis. Laminin degradation in the lysosomes enhances mTORC1 signaling, preventing cell death and promoting cell survival (Muranen et al., 2017). Similarly, in ovarian cancer cells, integrin α5β1 binds fibronectin, which is then internalized and degraded in the lysosomes, and the resulting amino acids activate mTORC1 (Rainero et al., 2015). However, the mechanisms for integrin-mediated nutrient scavenging in tumors are still poorly characterized and further studies are required to better understand this process.

It is not only integrins and ECM that are involved in nutrient scavenging by receptor-mediated endocytosis. Also albumin is endocytosed upon binding to megalin or other cell surface scavenger receptors. The internalized albumin can either bind to neonatal Fc receptor (FcRn) in endosomes and be recycled back to the plasma membrane, or degraded in the lysosomes. Degradation of albumin results in increased amino acid and possibly also lipid availability (Finicle et al., 2018).

Autophagy and Cancer Cell Proliferation

Autophagy is another process that provides nutrients and energy to the cell. It is used by cells to recycle their own compartments after sequestering them in a double membrane organelle, the autophagosome. When nutrients are running low, autophagosome formation is initiated to engulf macromolecules, protein aggregates and damaged organelles from the cytosol (Davidson and Vander Heiden, 2017). The autophagosomes then fuse with the lysosomes. Degradation inside the lysosomes provides the cells with new building blocks for protein synthesis (Kimmelman and White, 2017).

Upregulation of autophagy can occur in response to hypoxia or metabolic stress to ensure survival. Mice that lack essential autophagy genes such as *Atg5* and *Atg7*, die from nutrient starvation underlying how essential autophagy is to provide nutrients during metabolic stress (Kuma et al., 2004). Similar to macropinocytosis, autophagy allows the cells to tap into a pool of macromolecules. Both pathways are exploited by cancer cells to obtain nutrients for survival and growth. The main difference is that macropinocytosis internalizes nutrients from the extracellular environment, while autophagy utilizes what is already available inside the cells (Palm, 2019). The importance of autophagy in cancer cell proliferation, therefore, seems to be connected to its role in supporting tumor metabolism. In line with this, mutations in the Ras pathway are often associated with high levels of autophagy required to maintain cancer cell metabolism (Guo et al., 2011; Lock et al., 2011; Yang et al., 2011).

Essential autophagy genes such as *beclin-1*, which is important in the formation of the autophagosome, are upregulated in several types of cancers, including colorectal and gastric cancer (Ahn et al., 2007). Furthermore, Rab escort protein 1 (REP1) is associated with cancer progression by contributing to cell growth and survival through the regulation of mTOR signaling and its downstream pathways (Choi et al., 2017). REP1 is involved in the recruitment of Rab proteins to membranes as well as in the regulation of autophagy (Alexandrov et al., 1994; Choi et al., 2017). Knockdown of REP1 suppresses mTOR activity, blocking autophagy and increasing macropinocytosis. Even though the exact mechanism used by REP1 to regulate autophagy is not known, it is suggested that REP1 modulates the localization of lysosomes and mTOR thereby affecting their activity. It is also reasonable to think that REP1 controls the recruitment of Rab proteins necessary for this process, such as Rab7a (Choi et al., 2017).

The role of autophagy in cancer is quite complex and not fully elucidated yet. It seems to be dependent on several factors such as the type of tumor or the cancer stage. In the initial stages of cancer, autophagy acts as a tumor suppressor through quality control of proteins and removing damaged organelles and protein aggregates (Mathew et al., 2009; Li et al., 2020). By controlling these events, it can prevent sustained proliferation and therefore tumor initiation. However, in the later stages, when a tumor has formed, autophagy can protect cancer cells by helping them cope with cellular stress using the same strategies as in the early phases (Li et al., 2020). Autophagy seems also to contribute to the ability of cancer cells to develop resistance to chemotherapy by protecting them from the stress inflicted by the therapy (Sui et al., 2013; Ma et al., 2014).

EPITHELIAL TO MESENCHYMAL TRANSITION

The ability of cells to change their morphology and phenotype is crucial during embryonic development but also in tissue repair in adults. Transitions from epithelial to mesenchymal cells and back again are known as cellular plasticity (Corallino et al., 2015). Several cancers derive from epithelial cells. These cells are the building blocks of most organs and are organized in tissues by establishing contacts with their neighboring cells. When epithelial cells transition to cancer cells, they lose their epithelial phenotype and acquire a mesenchymal phenotype during a process called epithelial to mesenchymal transition (EMT) (Sciacovelli and Frezza, 2017). During this process, epithelial cells lose their junctions and apical-basal polarity, re-organize their cytoskeleton, change signaling programs and alter gene expression. This results in the loss of contacts with the neighboring cells leading to increased motility of individual cells and in the development of an invasive phenotype. EMT is indeed an essential step in cancer cell progression, which leads to invasion and metastasis (**Figure 2**).

When cells undergo EMT, their metabolic needs become different (Kang et al., 2019). Shaul et al. (2014) found a mesenchymal metabolic signature consisting of 44 upregulated metabolic genes that are essential for EMT but not for cell proliferation. The reprogramming of gene expression has indeed a crucial role during EMT. However, also non-transcriptional changes, including alteration of intracellular trafficking, play a vital part in this process (Le Bras et al., 2012).

In epithelial cells, adherens junctions maintain cell-cell adhesion by connecting transmembrane proteins to the actin cytoskeleton. EMT is characterized by the loss of E-cadherin, one of the major structural components of these junctions. In normal cells, E-cadherin is rapidly internalized from the cell surface and then recycled back to form new cell-cell contacts (Lu et al., 2003; Palacios et al., 2005). However, the endocytic pathway is often dysregulated in cancer, with a shift in the balance between recycling and degradation. In the early phases of EMT, adherens junction dissociation often occurs as result of changes in E-cadherin transport, leading to the internalization of E-cadherin followed by its transportation and degradation into lysosomes (Janda et al., 2006; Ulrich and Heisenberg, 2009; Le Bras et al., 2012). The endocytosis and recycling of E-cadherin is regulated by its interactor NUMB. Loss of NUMB causes E-cadherin to relocate and accumulate at the apical side decreasing cell-cell adhesion, and promoting cell migration (Wang et al., 2009). In line with this, in triple-negative breast cancer, an aggressive type of cancer, reduced NUMB expression is often associated with elevated EMT (Zhang et al., 2016).

During EMT, activated v-Src, a kinase involved in oncogenesis, phosphorylates E-cadherin. After phosphorylation, the E3 ligase Hakai catalyzes the ubiquitination of E-cadherin leading to the trafficking and degradation of E-cadherin to lysosomes (Fujita et al., 2002). Even though the role of Hakai in E-cadherin ubiquitination in physiological conditions remains unclear (Niño et al., 2019), it is intriguing that the expression of this ligase is gradually increased during the different stages of colon cancer progression, which is in line with its suggested role in E-cadherin modulation in EMT (Castosa et al., 2018). Downregulation of E-cadherin facilitates a switch to N-cadherin, which is associated with enhanced migration and invasion (**Figure 2A**; Loh et al., 2019). Recently, it has been demonstrated that E-cadherin impacts cell metabolism as mechanical forces exerted on E-cadherin activates AMPK thereby stimulating actomyosin contractility, glucose uptake and ATP production (Bays et al., 2017).

One of the major EMT inducers is the transforming growth factor beta (TGF-β) (Katsuno et al., 2013; Corallino et al., 2015). Internalization of the TGF-β receptor triggers a series of downstream cascades, which eventually lead to exocytosis of ATP containing vesicles (Cao et al., 2019). The released ATP functions as an extracellular messenger. It binds to and activates the purinergic receptor P2X7, resulting in EMT induction by upregulating mesenchymal markers and downregulating epithelial markers (Cao et al., 2019). The extracellular ATP thus induces metalloproteinase expression, but it also serves as an energy source for cell detachment. This extracellular ATP is indeed internalized by macropinocytosis, providing the energy required to allow morphological changes and movement (Cao et al., 2019).

INTRACELLULAR TRAFFICKING AND ENERGY REQUIREMENT IN CELL INVASION

Cancer cells that have undergone EMT experience changes that include not only the loss of adherens junctions and apical-basal polarity, but also the re-organization of their cytoskeleton and morphology. This leads to the acquisition of migratory ability that can develop in an invasive phenotype.

For efficient cell migration, adhesion molecules such as integrins are rapidly internalized and transported along the endosomal system. To evade lysosomal degradation, integrins are recycled back to the plasma membrane. This replenishes the plasma membrane pool of integrins and promotes their rapid turnover for cell migration (Mosesson et al., 2008; Dozynkiewicz et al., 2012; Sun et al., 2018; Moreno-Layseca et al., 2019). Therefore, it is not surprising that altered integrin trafficking is linked to invasive processes (Hamidi and Ivaska, 2018).

For example, gain-of-function mutant proteins of the tumor suppressor p53, which are often associate with cancer, increase α5β1 integrin recycling (Muller et al., 2009). α5β1, together with Rab-coupling protein (RCP; also known as Rab11-FIP1), recruits receptor tyrosine kinases, regulating their recycling and potentiating downstream signaling via protein kinase B (PKB)/Akt, thus resulting in invasive migration (Caswell et al., 2008; Muller et al., 2009; Jacquemet et al., 2013). RCP-driven endocytic recycling of α5β1 integrin enhances invasive migration of cancer cells by reprogramming the actin cytoskeleton to promote the formation of cell protrusions and actin-related protein 2/3 (Arp2/3) complex-independent cancer cell invasion *in vivo* (Jacquemet et al., 2013; Paul et al., 2015). Furthermore, mutant p53 increases the expression of the motor protein myosin

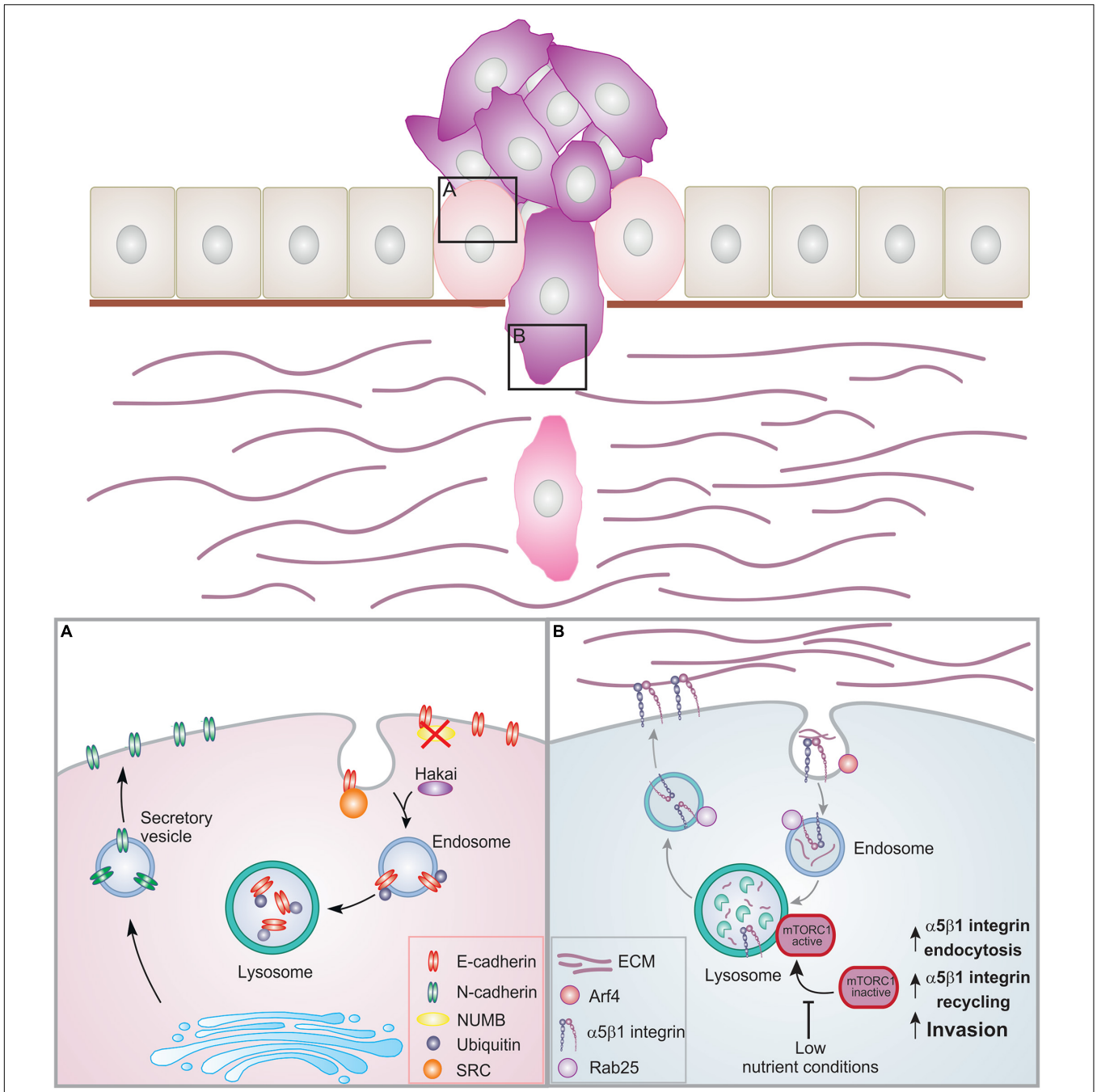

FIGURE 2 | Membrane trafficking events in EMT and cell invasion. Epithelial cells are organized in layers by establishing contacts with neighboring cells as well as the basement membrane. During cancer progression, the cells can lose these contacts leading to EMT and increased proliferation. Cancer cells can then acquire migratory ability, breaching through the basement membrane and invading into the ECM. **(A)** During EMT, E-cadherin is internalized and degraded. NUMB regulates the internalization and recycling of E-cadherin. When NUMB is lost, E-cadherin relocalizes to the apical side. Upon internalization, E-cadherin can be phosphorylated by src leading to ubiquitination by the E3 ligase Hakai. Ubiquitinated E-cadherin is delivered to lysosomes for degradation. This results in the loss of adherens junctions. N-cadherin is then transported to the surface promoting migration. **(B)** Trafficking of α5β1 integrin under low nutrient conditions. Inhibition of the mTORC1 activation and of its recruitment to lysosomes promotes Arf4-dependent endocytosis, α5β1 integrin recycling, and cell invasion.

X, which binds to β1 integrin to mediate its transport to filopodia (Arjonen et al., 2014). It has therefore been suggested that blocking α5β1-integrin might have therapeutic benefit in mutant p53-expressing cancers (Muller et al., 2009).

Gain-of-function p53 mutants, by promoting glucose transporter 1 (GLUT1) translocation to plasma membrane, stimulate glucose uptake, glycolysis and thus, the Warburg effect (Zhang et al., 2013). Hence, mutant p53, by affecting different

intracellular transport pathways, coordinates cell metabolism and integrin recycling to promote cell invasion.

Small GTPases, along with their effectors, control integrin recycling with huge impact on cell invasion. Rab11- and Arf6-dependent recycling of integrins is associated with cancer invasion (Yoon et al., 2005; Das et al., 2018; Moreno-Layseca et al., 2019). A signaling pathway involving phosphorylation of Rab34 inhibits β3 integrin lysosomal degradation mediating its recycling back to the plasma membrane to promote cell migration (Sun et al., 2018). In triple-negative breast cancer cells, Rab5a stimulates Rab4-dependent fast recycling of α5β3 integrin, thus leading to cell invasion (Frittoli et al., 2014; Linder and Scita, 2015). Furthermore, Rab25, which directly associates with integrin α5β1, promotes integrin recycling from late endosomes/lysosomes at the cell front to drive invasion (Caswell et al., 2007; Dozynkiewicz et al., 2012).

Ligand-engaged α5β1 integrin are trafficked under control of Rab25 to late endosomes/lysosomes following Arf4-dependent internalization. This pathway is necessary to maintain lysosomal positioning at the perinuclear region and to recruit and activate the nutrient sensor mTORC1 on lysosomes. Interestingly, in response to low-nutrient status of cancer cells, the recruitment of mTOR to late endosomes/lysosomes is inhibited, further promoting ligand-bound α5β1 internalization and trafficking to lysosomes. This stimulates the degradation of ECM components in the lysosomes as well as Rab25-mediated α5β1 integrin recycling at the plasma membrane (Dozynkiewicz et al., 2012; Rainero et al., 2015). Consequently, this pathway connects nutrient sensing to ECM internalization and integrin recycling to promote cell invasion (**Figure 2B**).

Degradation of ECM components during cancer invasion occurs not only in the lysosomes but also outside the cell. Diverse polarized trafficking pathways converge at the invadopodia, plasma membrane protrusions responsible for ECM degradation and invasion, for the local delivery of proteolytic enzymes, which have pivotal role in defining the malignant features of cancer cells. Indeed, extracellular degradation-mediated cell invasion is carried by proteolytic enzymes, such as cathepsins and matrix metalloproteinases (MMPs), which can be trafficked either through the secretory pathway or via exocytosis of peripheral lysosomes (Bonnans et al., 2014).

Rab5-mediated endocytosis regulates the internalization and delivery of the membrane-associated MT1-MMP (membrane-type 1 matrix metalloproteinase), an important invasion-promoting enzyme, to non-degrading Rab7a-positive endosomal reservoirs before being exocytosed at invadopodia for ECM degradation (Planchon et al., 2018). A further regulation of MT1-MMP recycling to the plasma membrane has been described to be dependent on WDFY2 and Rab4 following a VAMP3-dependent mechanism (Sneeggen et al., 2019). However, while recycling is considered the major route for fast delivery of proteases to the plasma membrane for ECM degradation, MT1-MMP can additionally be delivered to the plasma membrane following Rab8-dependent polarized exocytosis (Bravo-Cordero et al., 2007) or Rab27-dependent exosomal release (Hoshino et al., 2013). High levels of glutamine consumption contribute to cancer aggressiveness by generating a source of extracellular glutamate. This extracellular glutamate activates its receptor GRM3 on the plasma membrane, stimulating Rab27-mediated recycling of MT1-MMP to promote invasiveness (Dornier et al., 2017). This highlights how changes of tumor environment such as the increased extracellular glutamate and low-nutrient status of cancer cells alter cellular metabolism leading to aberrant endosomal recycling to drive cell invasion.

In addition to have a role in catabolic and metabolic signaling, lysosomes can also function as secretory compartments releasing their luminal content in the extracellular space in a calcium-dependent process (Blott and Griffiths, 2002; Xu et al., 2012; Kimmelman and White, 2017; Buratta et al., 2020). The lysosomal calcium-channel TRPML1 is activated by the metabolic stress conditions typical of cancer cells. Its activation promotes mTORC1 activity and ATP release via lysosomal exocytosis (Liu et al., 2012; Takai et al., 2012; Machado et al., 2015; Naegeli et al., 2017; Xu et al., 2019). Extracellular ATP interacts with purinergic receptors on the plasma membrane, and acts as cancer invasion stimulator by activating Rho GTPase-dependent pathways and upregulating the expression of MMPs (Zhang et al., 2010; Li et al., 2013).

Recent evidence demonstrates that the presence of mitochondria at cell protrusions stimulates ATP-driven actin polymerization to drive cell motility and invasion during *Caenorhabditis elegans* development, even in absence of MMPs (Kelley et al., 2019). This indicates special energy requirement in protruding regions. In line with that, a connection between intracellular mitochondrial trafficking and energy gradients has been described, where ATP:ADP ratio changes depending on positioning and density of mitochondria (Altieri, 2017; Schuler et al., 2017). Long-range mitochondrial trafficking relies on microtubule-associated molecular motors kinesins and dyneins as well as on the mitochondrial Rho-GTPase Miro1. In *Miro1*-deficient mouse embryonic fibroblasts (MEFs), mitochondria reposition to the perinuclear area, which correlates with high ATP production in this region. This inhibits energy-demanding processes such as protrusion formation and focal adhesion dynamics at the cell periphery, resulting in decreased cell migration and invasion (Schuler et al., 2017). Conversely, in migrating and invasive cancer cells, mitochondria accumulate at the leading edge (Arismendi-Morillo et al., 2012). Thus, the traffic and dynamics of mitochondria are coupled to the localized energy demand at the protruding cell front for focal adhesion dynamics, cell membrane dynamics and invasion.

ATP consumption at the leading edge promotes mitochondria trafficking with a positive feedback mechanism that depends on the energy sensor AMPK (Cunniff et al., 2016; Furnish and Caino, 2020). This is in agreement with evidence showing that some key glycolytic enzymes are located at the plasma membrane of invasive cells (Attanasio et al., 2011; Havrylov and Park, 2015; James et al., 2020) where the actomyosin machinery used to displace the ECM relies on the readily available supply of ATP (Oser et al., 2009; van Horssen et al., 2009; Kelley et al., 2019).

Interestingly, tumor exposure to inhibitors of the therapeutic target phosphatidylinositol-3-kinase (PI3K) has shown a unique

repositioning of energetically active mitochondria in proximity to focal complexes, which supports membrane dynamics and cytoskeletal remodeling, resulting in increased cell motility and invasion (Caino et al., 2015). Although this response may possibly increase the risk of metastasis, it illustrates the feasibility of targeting mitochondrial reprogramming.

CONCLUDING REMARKS

It is now widely recognized that different metabolic needs are encountered during cancer progression. Therefore, it is of high importance understanding the underlying molecular mechanisms behind this metabolic cancer plasticity for the development of target therapies and also to prevent therapy resistance.

The contribution of intracellular membrane transport to the metabolic rewiring in disease progression is, however, still poorly characterized. Many questions remain unanswered due to the limitations of studying cancer cells in their complex tumor environment. However, studies where nutrient access was restricted by pharmacologically altering membrane trafficking have shown positive results, simultaneously blocking lysosomal degradation of autophagosomes and macropinosomes, and starving cancer cells to death (Kim et al., 2016). In line with this, ongoing studies with agents that target scavenging, macropinocytosis, autophagy or lysosomes seem to be promising (Towers and Thorburn, 2017; Towers et al., 2020). For example, recently developed lysosomal inhibitors for cancer therapy can inhibit multiple lysosomal activities needed for tumor cell survival and growth (Rebecca et al., 2017). Therefore, future research should further explore the molecular mechanisms of intracellular trafficking characterizing tumor initiation, progression and metastasis in relation to the different cellular metabolic needs as these aspects could help to identify new ways or targets for therapy.

Dormant cancer cells are one of the most threatening aspects of cancer and can lead to reoccurrence of metastatic tumors after a long period of latency. Tumor cell dormancy can be induced by nutrient deprivation (Jahanban-Esfahlan et al., 2019) but the mechanism behind the revival of the dormant cells remains mainly elusive. Therefore, further investigation is required to understand whether and how changes in nutrient availability as well as the metabolic adaptation influence this process. Moreover, the role of intracellular trafficking in the re-activation of the dormant cells is still unknown and its characterization may further improve our understanding of tumor dormancy with impact on tumor relapse.

Thus, the tight connection between intracellular trafficking and cell metabolism should be taken into account in the search of novel therapeutic targets for a more integrated cancer therapy.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Endomembrane Tension and Trafficking

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Eukaryotic cells employ diverse uptake mechanisms depending on their specialized functions. While such mechanisms vary widely in their defining criteria: scale, molecular machinery utilized, cargo selection, and cargo destination, to name a few, they all result in the internalization of extracellular solutes and fluid into membrane-bound endosomes. Upon scission from the plasma membrane, this compartment is immediately subjected to extensive remodeling which involves tubulation and vesiculation/budding of the limiting endomembrane. This is followed by a maturation process involving concomitant retrograde transport by microtubule-based motors and graded fusion with late endosomes and lysosomes, organelles that support the degradation of the internalized content. Here we review an important determinant for sorting and trafficking in early endosomes and in lysosomes; the control of tension on the endomembrane. Remodeling of endomembranes is opposed by high tension (caused by high hydrostatic pressure) and supported by the relief of tension. We describe how the timely and coordinated efflux of major solutes along the endocytic pathway affords the cell control over such tension. The channels and transporters that expel the smallest components of the ingested medium from the early endocytic fluid are described in detail as these systems are thought to enable endomembrane deformation by curvature-sensing/generating coat proteins. We also review similar considerations for the lysosome where resident hydrolases liberate building blocks from luminal macromolecules and transporters flux these organic solutes to orchestrate trafficking events. How the cell directs organellar trafficking based on the luminal contents of organelles of the endocytic pathway is not well-understood, however, we propose that the control over membrane tension by solute transport constitutes one means for this to ensue.

Keywords: endocytosis, phagocytosis, macropinocytosis, mTOR, ESCRT, ion transport, V-ATPase, sorting nexin

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INTRODUCTION

The active internalization of extracellular material by eukaryotic cells is key to nutrient acquisition, environment sensing, and maintenance of normal cell physiology. In metazoans, this process is essential for maintaining the specialized functions of tissues and the system as a whole. As such, different cell types engage multiple different uptake pathways including clathrin-dependent and -independent endocytosis, micropinocytosis, and phagocytosis (**Figure 1**). These pathways

operate under distinct mechanisms and scales; receptor-mediated endocytosis internalizes small plasma membrane-derived vesicles, macropinocytosis results in bulk uptake of extracellular fluid, and phagocytosis is employed for the internalization of large (>0.5 μm) particulates. Endocytic pathways are also usurped by a variety of obligate intracellular pathogens as part of their infection cycle including viruses, bacteria, and fungi. In addition, cells undergo autophagy, a process by which cytosolic components are entrapped within a newly generated membrane to form an autophagosome. Despite their differences, these pathways all result in the conception of an intracellular, membrane-bound vesicle bearing cargo (i.e., receptors, ligands), and fluid.

Cargo that is internalized from the extracellular milieu meets one of 3 known fates depending on the cell's needs; (1) receptors/ligands may be recycled back to the plasma membrane, (2) cargo may be routed elsewhere in the cell such as to the *trans*-Golgi network (TGN) via retrograde transport or (3) cargo may be degraded by delivery to lysosomes, organelles that support the enzymatic breakdown of macromolecules (Lawrence and Zoncu, 2019). Regardless of destination, cargo sorting and trafficking from the early (nascent) endosome necessitates an astonishing degree of endomembrane remodeling. This remodeling begins moments after scission of the endosome from the plasma membrane. To retrieve receptors and membrane, fine membrane tubules extend from the endosome and pinch off to form smaller vesicles that recycle the cargo (Yamashiro et al., 1984; Ren et al., 1998; Grant and Donaldson, 2009). These fission events occur even as endosomes undergo homotypic fusion giving rise to a complex balance between membrane addition and removal. After the initial stages of remodeling, the remaining endosome that bears cargo destined for degradation then matures. Maturation can be envisaged as a series of steps including retrograde transport of the organelle, inward budding of the limiting endomembrane, morphing into a multivesicular endosome in the process (Gruenberg, 2020), and finally, graded fusion with (endo)lysosomes (Huotari and Helenius, 2011). Interestingly, these trafficking pathways may be highjacked or arrested by effectors generated by internalized pathogens. For example, bacterial effectors, ejected into the cytosol from their resident vacuoles, can target various steps of endosome maturation, thereby curtailing microbicidal activities of the host (Gruenberg and van der Goot, 2006). Studies on cargo sorting have revealed key protein complexes such as retromer, retriever, and ESCRT that function in the aforementioned pathways of retrograde cargo transport to the TGN, recycling to the plasma membrane and degradation in lysosomes, respectively (Seaman et al., 1998; Raiborg and Stenmark, 2009; McNally et al., 2017).

On the other hand, the fate of the internalized fluid has been relatively unexplored. Recent work suggests that the resolution of volume from the endocytic pathway is not only essential for sorting and trafficking, but may be the initiating event that enables the extensive endomembrane remodeling described above (Freeman et al., 2020). One need only consider the extreme surface-to-volume changes that occur during sorting (Freeman and Grinstein, 2018): A newly formed spherical macropinosome that is anywhere between 0.5 and 5 μm in diameter can project numerous fine tubules that can be several microns long yet contain almost no luminal volume at all (Kerr et al., 2006; Freeman et al., 2020). Such continuous removal of membrane without a parallel loss of volume would quickly limit this system by increasing the hydrostatic pressure within the vacuole, generating a turgid membrane that is refractory to deformation. The solution? Cells rely on a collection of vacuolar channels and transporters for the timely release of solutes from the lumen across the endomembrane. This process ensures that water is forcibly extruded from the vacuole, causing a subsequent drop in its internal hydrostatic pressure and a drop in the membrane tension. The membrane slack afforded by this process results in crenation that permits the recruitment and assembly of curvature sensing/stabilizing proteins, such as BAR domain-containing proteins, to the vacuolar surface (Simunovic and Voth, 2015; Freeman et al., 2020). It is expected therefore that trafficking complexes that associate with highly curved membranes are similarly dependent on membrane tension relief for their recruitment and function. Conversely, the addition of tension to the membrane may be utilized to arrest endomembrane remodeling. Thus, the flux of solutes, and as a consequence, water, to and from the vacuole drives traffic by controlling membrane tension.

Although tremendously understudied, similar mechanisms of membrane tension control may operate at the late stages of endosome maturation as well. As endosomes mature, they ultimately fuse with lysosomes, highly acidic organelles that harbor more than 50 resident acid hydrolases that support the degradation of internalized molecules. And, in addition to endocytosis, autophagy converges on the lysosome for degradation of intracellular cargoes through fusion of autophagosomes with lysosomes to form autolysosomes. While acidification is central to lysosomal function, it comes with osmotic considerations. For example, counter-ion fluxes that are driven by lysosome-resident transporters and channels are key to reaching and maintaining the low pH but have varying osmotic effects on the lysosome. In addition, the liberation of building blocks from enzymatic digestion of the internalized content requires their efflux via transporters of the solute carrier family (SLCs), for use in anabolic cellular processes and to mitigate an osmotic burden that could result in overtly high membrane tension. Thus, the collective activities of numerous lysosomal channels and transporters supports normal lysosomal functions and affords the cell control over endomembrane tension to dictate trafficking. When solute transport mechanisms are impaired, defects in lysosomal function and trafficking ensue, such as those documented in numerous lysosomal storage disorders (LSDs). Moreover, the ability for endocytosed liposomes, exosomes, and enveloped viruses to fuse with the limiting endomembrane are also predicted to be dependent on membrane tension. Understanding how cells utilize solute transport to control endomembrane tension is therefore important to fully appreciate mechanisms that support trafficking, infection, and its dysregulation in storage disorders.

In this review we discuss how cells may be afforded control over membrane tension to regulate endomembrane trafficking,

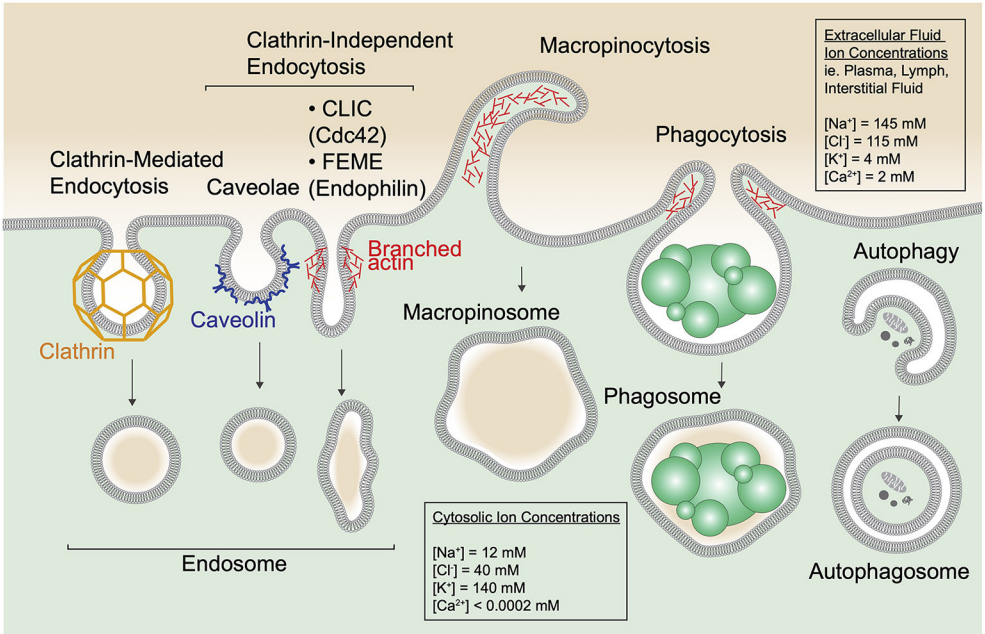

FIGURE 1 | Cells entrap extracellular and cytosolic fluid via diverse mechanisms. Multiple forms of endocytic uptake are depicted along with autophagy. Clathrin-mediated endocytosis occurs when plasma membrane (PM) invaginations are formed by the coat protein clathrin to generate a clathrin-coated pit. The pit dissociates from the PM by scission, induced by the GTPase dynamin (not depicted), and forms an endocytic vesicle. In addition, numerous clathrin-independent endocytic routes are depicted including caveolae, clathrin-independent carriers (CLICs), and fast endophilin-mediated endocytosis (FEME). Caveolae form at cholesterol-rich PM domains called lipid rafts and use the protein caveolin to shape the membrane into flask-like invaginations. CLICs are uncoated tubulovesicular PM invaginations stabilized by the actin cytoskeleton and regulated by the small GTPase Cdc42 that recruits the actin polymerization machinery. FEME relies on a BAR-domain containing protein, endophilin, for curvature of tubular PM invaginations that scission upon dynamin recruitment. In the schematic, one tubular PM invagination represents endocytic uptake via CLICs and FEME. Macropinocytosis and phagocytosis are specialized forms of endocytosis. Macropinocytosis proceeds via polymerization of the cortical actin cytoskeleton to produce PM ruffles that fold back on the cell and fuse, indiscriminantly trapping the surrounding medium into large (up to 5 μm sized) membrane-enclosed vacuoles called macropinosomes. Phagocytosis is a receptor-mediated process by which cells bind and engulf particulates including dead cells (depicted), debris, and pathogens like bacteria and fungi into membrane-enclosed phagosomes. Phagocytosis is also aided by the actin cytoskeleton. Note that extracellular fluid is internalized via all these pathways. In addition, cells use autophagy to sequester protein aggregates, damaged organelles and other cytoplasmic components within a double-membrane structure called an autophagosome.

a view that we and others have proposed (Scott and Gruenberg, 2011; Freeman and Grinstein, 2018). A brief overview of the different endocytic pathways is presented with considerations of plasma membrane tension in these processes and the solute composition of internalized fluid. This is followed by a description of how nascent endosomes flux osmolytes via a suite of channels and transporters to maintain the low endomembrane tension required for the membrane remodeling that accompanies cargo sorting and trafficking. Finally, we consider how lysosomal solute transport via a broad range of channels and transporters may function to fine-tune membrane tension as a mechanism to tightly control membrane trafficking.

MAIN

Endocytosis Mechanisms and the Role of Membrane Tension

Endocytosis, Macropinocytosis, Phagocytosis

Endocytosis, the fundamental process of nutrient uptake and receptor signaling regulation, is utilized by virtually all nucleated cells of the body. The best described is clathrin-mediated endocytosis (CME) that proceeds through invaginations of the plasma membrane (PM) that sequester receptor-ligand complexes into ∼50–200 nm pits coated with the protein clathrin (**Figure 1**) (Ehrlich et al., 2004; McMahon and Boucrot, 2011). These pits ultimately scission with the aid of the GTPase dynamin that constricts the neck of the bud, to form endocytic vesicles bearing cargo such as low-density lipoprotein (LDL), transferrin (Tf), and epidermal growth factor (EGF) bound to their cognate receptors (Kaksonen and Roux, 2018). Thus, while nutrients like lipids and iron are internalized for cell growth, CME also controls receptor signaling by removing receptors from the plasma membrane when needed (Tsao et al., 2001; Goh and Sorkin, 2013). Additionally, numerous clathrin-independent endocytic routes have been described including caveolae, tubulovesicular clathrin-independent carriers (CLICs), and fast endophilin-mediated endocytosis (FEME) (Mayor and Pagano, 2007). These pathways rely on specific biophysical properties for cargo internalization such as distinct membrane lipid compositions (i.e., lipid rafts), the engagement of the actin

cytoskeleton to produce tubular invaginations or non-clathrin membrane shaping proteins (Galbiati et al., 2001; Doherty and McMahon, 2009; Boucrot et al., 2015).

In addition to one or multiple of these pathways, specialized cells also utilize macropinocytosis or phagocytosis to internalize large amounts of extracellular fluid or particulates, respectively. Macropinocytosis involves the rapid polymerization of cortical actin to produce plasma membrane ruffles that capture surrounding fluid and collapse to form large ($>0.2\,\mu m$) internal vacuoles called macropinosomes (Swanson, 2008). Because this process results in the non-discriminant sampling of large amounts of extracellular fluid (Steinman et al., 1976), macropinocytosis is typically utilized by cells of the innate immune system to survey tissues for infection (West et al., 2004), as well as by some cancer cells as a means of sustaining the high nutrient requirements of their altered metabolic states (Commisso et al., 2013). In addition, professional phagocytes like macrophages are tasked with the removal of cell corpses and microbes, and as such utilize phagocytosis, a specialized form of endocytosis that allows for ingestion and degradation of particulates (Aderem and Underhill, 1999; Freeman and Grinstein, 2014).

Membrane Tension and Control of Endocytic Trafficking

It has been known for some time that PM tension regulates endocytic pathways (Gottlieb et al., 1993; Dai and Sheetz, 1995; Bajno et al., 2000). Low membrane tension is permissive of and induces multiple forms of endocytosis (Watanabe et al., 2013; Hirama et al., 2017; Wu et al., 2017; Loh et al., 2019). On the other hand, increases in tension oppose CME (Bucher et al., 2018) and require additional forces exerted by the actin cytoskeleton to complete pit formation and aid scission (Boulant et al., 2011). Yeast that have to invaginate their PM against turgor pressure are entirely dependent on their actin cytoskeleton for endocytosis (Aghamohammadzadeh and Ayscough, 2009). An interesting concept, however, is that cells are able to sense changes in membrane tension and exert control over it in order to govern membrane trafficking. Some of these control mechanisms have been described. For example, caveolae can assemble or disassemble to provide additional membrane as needed (Sinha et al., 2011; Golani et al., 2019), CLICs regulate membrane tension via the mechano-transducer vinculin (Thottacherry et al., 2018), and increases in PM tension during phagocytosis can signal to exocytosis machinery in order to deliver additional membrane as required (Masters et al., 2013). Thus, cells use endocytic machinery like caveolae and CLICs as well as exocytosis as important membrane reservoirs to provide rapid membrane slack when required. Conversely, cells must also have ways to prevent overt membrane slack, since acute decreases in membrane tension dysregulates endocytic uptake (Wu et al., 2017; Loh et al., 2019). Insights gained from studies on PM tension control raise questions regarding intracellular membranes: Do cells sense and regulate membrane tension throughout the endosomal-lysosomal system to direct endomembrane trafficking, and how? One way this could be achieved is by controlled osmotic shifts imposed by the transport of solutes. Numerous endolysosomal resident channels and transporters working in a concerted fashion may achieve such ends.

Immediately upon scission, endosomes containing cargo and fluid undergo rapid recycling of membrane. It should be obvious however, that without a parallel loss of fluid, membrane recycling would abruptly halt as hydrostatic pressure within the organelle would quickly build up to a point where membrane deformation is negated. A critical component of fluid resolution is that it accompanies efflux of osmolytes. While the composition of the extracellular fluid varies depending on the niche, it typically consists of a mix of organic solutes, macromolecules and small inorganic ions. Many of the molecules are in the form of polymers (i.e., polysaccharides, proteins, polynucleotides etc.) and are of relatively low concentrations, thus have a low osmotic contribution. There are some exceptions (including hydrating glucosaminoglycans) but these too are often of low abundance in most tissues. On the other hand, the types and concentrations of inorganic monovalent ions in extracellular fluids are high and kept relatively constant in the body; Na^+ (140 mM) and Cl^- (110 mM) account for the major osmolytes present (**Figure 2**). Given that extracellular concentrations of Na^+ and Cl^- are 3–10 times higher than the cytosol, this gradient favors the efflux of Na^+ and Cl^- from nascent endosomes into the cytosol by channels. Indeed, recent work demonstrates that cells exploit this gradient to drive water out of vacuoles without costing the cell energy to relieve endomembrane tension and enable trafficking from the compartment (Freeman et al., 2020).

Fluid Resolution Lowers Membrane Tension to Enable Early Endocytic Trafficking

Gated Efflux of Na^+ Drives Endomembrane Remodeling and Trafficking

While all forms of endocytosis involve the uptake of fluid (pinocytosis), this is emphasized and can be readily monitored in cell types that *macro*pinocytose. In such cells, the newly formed macropinosomes resolve rapidly (within minutes) by way of shrinkage and extensive tubulation (Racoosin and Swanson, 1993; Steinman and Swanson, 1995). The compartments can also be manipulated by simply performing ion substitutions at the time of their formation and sealing. Monitoring the large endosomes when Na^+ is substituted with non-transportable cations (e.g., N-methyl-D-glucosamine$^+$), demonstrates that a Na^+ gradient is necessary for tubulation and shrinkage of macropinosomes (Freeman et al., 2020). As a result, without Na^+ the endosomes are large and perfectly spherical, as though swollen by trapped water. By inhibiting a panel of endolysosomal channels and transporters pharmacologically, Freeman et al. identified the Na^+-conducting two-pore channels 1 and 2 (Wang et al., 2012; She et al., 2018) (TPC1 and TPC2) as key players in this process: Treatment of cells with the TPC inhibitor tetrandrine resulted in swollen macropinosomes. The resultant, distended macropinosomes, precluded the formation of membrane tubules that are a hallmark of early cargo sorting. Use of single and double knock-out strategies further

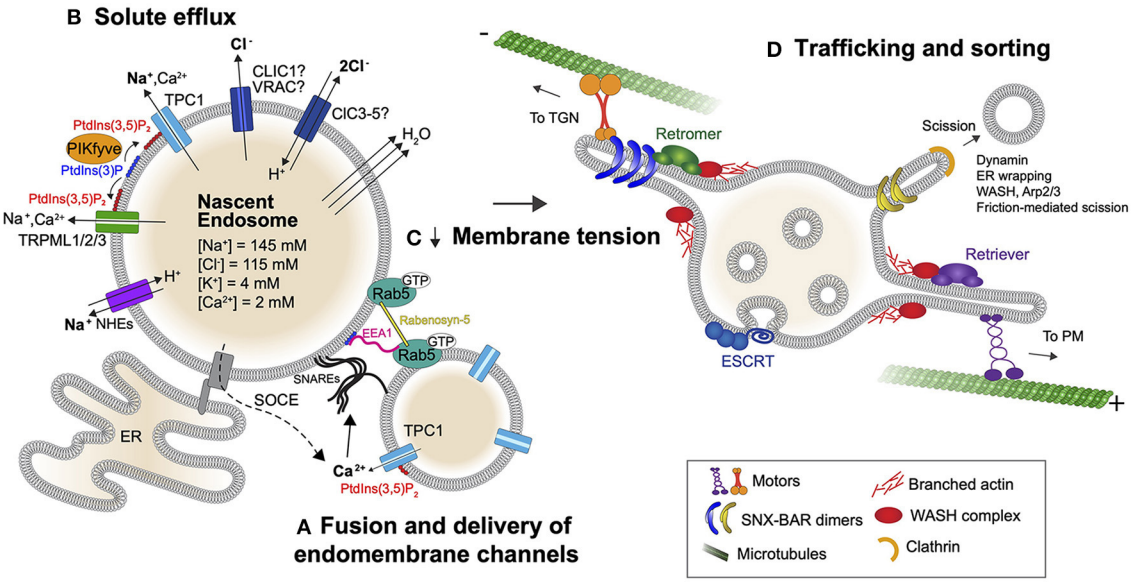

FIGURE 2 | Lipid-gated monovalent ion efflux controls endomembrane tension and trafficking of early endosomes. **(A)** The fusion and delivery of endomembrane-resident channels including TPCs is important for the subsequent efflux of Na^+ from the nascent endosome. Fusion is mediated by SNAREs in a Ca^{2+}-dependent manner. The initial source of Ca^{2+} is not clear but can come from the endocytic fluid or via store operated calcium entry (SOCE). **(B)** Once incorporated into the endosome and activated by PtdIns(3,5)P_2, TPCs and TRPMLs can transport Na^+ down its concentration gradient into the cytosol. A parallel flux of Cl^- is also required, which could be facilitated by CLIC1, ClCs, or VRAC. At the near-neutral pH of early endosomes, ClCs allow for $2Cl^-$ to be transported out in exchange for $1H^+$. ClCs as well as Na^+ proton exchangers (NHEs) may therefore contribute to early acidification of the endosome. Importantly, the efflux of Na^+ and Cl^- from early endosomes drives osmotically obliged water out of the organelle and this fluid resolution reduces membrane tension. Rab5 may sense endomembrane tension as it drives endosome enlargement through effector proteins EEA1 and Rabenosyn-5 that facilitate SNARE-dependent fusion. **(C)** Fluid resolution ultimately lowers membrane tension. **(D)** Low membrane tension fosters the recruitment of membrane remodeling/sorting complexes to the endosome surface to drive early endosomal trafficking.

revealed that TPC1 is primarily responsible for the resolution of volume from macropinosomes (Freeman et al., 2020) (**Figure 2**). Consistent with this finding, TPC1 has been shown to be recruited to macropinosomes immediately upon their formation, while TPC2 is recruited at later stages and operates at lysosomes (Calcraft et al., 2009; Freeman et al., 2020). Not surprisingly, macropinocytic cells like macrophages that handle large amounts of fluid experience pressure for Na^+ efflux, as evidenced by their high expression of TPC1 (Freeman and Grinstein, 2018; Freeman et al., 2020). Nevertheless, $TPC1^{-/-}$;$TPC2^{-/-}$ mice are, for the most part, normal unless pressured to perform high membrane traffic (Grimm et al., 2014; Sakurai et al., 2015; Castonguay et al., 2017), suggesting either redundancy or compensation in Na^+ efflux pathways in these animals.

The failure of macropinosomes to shrink and resolve without a Na^+ gradient is due to the disruption of an efflux that supports the extrusion of osmotically coupled water from the vacuole (**Figure 2**). This loss of water is a critical trafficking prerequisite that lowers endomembrane tension by reducing the hydrostatic pressure within the vacuole, in turn rendering the vacuolar membrane amenable to deformation by trafficking complexes. Indeed, not only is macropinosome resolution impaired when a Na^+ gradient is lost, but canonical receptor recycling pathways are disrupted in fibroblasts and epithelia cell types (Freeman et al., 2020). This suggests that the exploitation of a Na^+ gradient is a universal mechanism used by cells to drive endomembrane trafficking. In principle, sodium proton exchangers (NHEs) could also efflux Na^+ while causing early acidification of vacuoles (Nakamura et al., 2005), a process that would be electroneutral. Thus, while there are likely multiple pathways for vacuolar volume resolution, TPC1-mediated Na^+ efflux represents a prototypical mechanism for relieving hydrostatic pressure and membrane tension to enable trafficking.

Counterion Flux Is Required for Volume Resolution

The efflux of Na^+ by TPCs presents the need for counterion fluxes in order to maintain electroneutrality. It stands to reason that a parallel loss of Cl^- would be similarly necessary for the resolution of nascent endosomes. Indeed, ion substitution experiments in which Cl^- is replaced with the impermeant anion $gluconate^-$ prevents macropinosome shrinkage and tubulation and the organelles again appear swollen, suggesting that water is trapped in the compartment (Freeman et al., 2020). The transport pathway for Cl^- is not known, but endolysosomal organelles bear several Cl^- channels and transporters that could potentially fulfill this role even in the early endosome. To this end, the Cl^- intracellular channel protein 1 may be involved in these counterion fluxes, however, its ion transport activities and mechanisms of action remain controversial (Stauber and Jentsch, 2013). Other endomembrane Cl^- transporters include

members 3–7 of the ClC family of H^+/Cl^- exchange transporters. Some of these transporters are located in early endosomes and can support early acidification by exchanging luminal Cl^- for H^+ (Stauber and Jentsch, 2013), so like organellar NHEs, these could contribute to volume loss. Interestingly, gain of function mutations in members of the ClC family result in volume *increases* in the endocytic pathway (Nicoli et al., 2019), and ClCs are generally outwardly rectifying, so while these transporters have yet to show a role in the rapid process of volume resolution, they may contribute to the osmoregulation of endolysosomes in other ways. In addition, a recent screen identified LRRC8A, a component of the volume-regulated anion channel (VRAC), as necessary for the control of vacuolar volume, since inactivation of the *LRRC8A* gene causes swelling of endolysosomes (Lenk et al., 2019). Whether this effect is directly caused by the loss of VRAC activity from endosomes remains to be formally tested. Should VRAC function along the endocytic pathway (Li et al., 2020), its flux of Cl^- and that of several organic anions (Jentsch, 2016; Kasuya et al., 2018), could yield pleiotropic control over trafficking.

The Role of TRPMLs in Early Endomembrane Trafficking

Among the channels reported to play critical roles in membrane trafficking are the Ca^{2+} conducting transient receptor potential cation channels of the mucolipin subfamily 1-3 (TRPML1-3). Loss of TRPML1 causes severe vacuolation of endolysosomes in restricted cell types including gut epithelial cells (Venugopal et al., 2007) where only the expression of a wild type channel, but not pore-mutants, rescue this phenotype (Dong et al., 2010). TRPML1 is indeed rapidly acquired by nascent endosomes in myeloid cells but $TRPML1^{-/-}$ macrophages do not appear vacuolated or impaired (SF unpublished). It should be noted that Ca^{2+} itself is a minor osmoticant of extracellular and endocytic fluid, with concentrations that change drastically in the endocytic pathway, but never reach >2 mM. The flux of Ca^{2+} from early endosomes may nevertheless be critical for early compartments to fuse with later ones via SNARE-mediated membrane fusion, analogous to proposed methods of secretory vesicle fusion with the plasma membrane (Park and Ryu, 2018). This would ensure that the endomembrane becomes endowed with the necessary channels and transporters that support fluid resolution (**Figure 2**). However, Ca^{2+} in the endocytic fluid is not ostensibly required for the shrinkage of nascent vacuoles (Freeman et al., 2020). This would suggest alternate sources of Ca^{2+} that contribute to fusion beyond that of the early endocytic fluid, potentially from previously formed endosomes. Despite being a minor osmotic contributor, Ca^{2+} indeed exits from early endosomes as its concentration quickly drops from 1 mM to low μM ranges despite volume loss (Scott and Gruenberg, 2011). The efflux of Ca^{2+} may be mediated by TRPML, TPCs, or even store-operated channels stimulated by contacts between the endoplasmic reticulum (ER) and the plasma membrane.

It remains unclear why TRPML1-deficient epithelial cells have enlarged endolysosomes. This could be either because (1) the compartment does not fuse to acquire TPCs or (2) because TRPMLs may be the critical mode of Na^+ efflux in these cells. The former possibility stems from studies demonstrating that endosomal Ca^{2+}, released by TRPML, regulates the fusion of endomembranes (Pryor et al., 2000; Dayam et al., 2015). Since TPCs exist in endomembrane reservoirs and are delivered to newly formed endosomes by fusion (Castonguay et al., 2017), their efficient delivery may require TRPML-mediated Ca^{2+} efflux. The latter is a possibility because TRPMLs are in fact non-selective cation channels, and were shown to be permeable to Na^+ in addition to Ca^{2+} (LaPlante et al., 2002). Thus, TRPMLs may contribute to volume resolution either indirectly by aiding TPC delivery to endosomes or directly by mediating Na^+ efflux along with TPCs. Either way, TRPML activity is necessary for the resolution of vacuolar fluid in select cell types. Interestingly, like some TRP channels in the PM, the gating of TRPML2 is mechanosensitive and activated under hypotonic (high tension) conditions (Chen et al., 2020). This is a remarkable finding, suggesting that channels that support volume loss are activated when biophysical demands present themselves.

A Critical Role for Phosphoinositides in Endomembrane Tension and Trafficking

TPCs and TRPMLs in fact belong to a class of ion channels that are gated by a single, rare lipid species found on the cytoplasmic leaflet of endomembranes—the phosphoinositide $PtdIns(3,5)P_2$ (Dong et al., 2010; Wang et al., 2012; She et al., 2018). $PtdIns(3,5)P_2$ is generated by a single kinase (PIKfyve) which is activated, in part, by osmotic stress (Gary et al., 1998; Bonangelino et al., 2002). This feature then confers the cell with an ability to control Na^+ efflux in space, time and upon changes to ionic strength. Together with some understanding for how monovalent ion effluxes control volume resolution, the connections provide a new perspective on numerous studies reporting endolysosomal defects caused by the loss of $PtdIns(3,5)P_2$. In yeast, it had long been reported that the loss of Fab1, the lipid kinase that synthesizes $PtdIns(3,5)P_2$ by phosphorylating PtdIns(3)P, leads to the formation of abnormally enlarged vacuoles (Yamamoto et al., 1995; Gary et al., 1998). Subsequent studies in mammalian systems further demonstrated extensive vacuolation of endolysosomal compartments upon $PtdIns(3,5)P_2$ depletion (Ikonomov et al., 2001; Rutherford et al., 2006; Chow et al., 2007; Zhang et al., 2007; Jefferies et al., 2008; Zolov et al., 2012; Cai et al., 2013; Sharma et al., 2019). That $PtdIns(3,5)P_2$-deficient vacuoles are large, phase-lucent, highly spherical organelles, suggests that they are fluid-filled and likely experience high membrane tension that precludes the formation of tubules.

Supporting this notion, loss of $PtdIns(3,5)P_2$ blocks endosome fission (Sharma et al., 2019), prevents receptor recycling (Freeman et al., 2020) and trafficking of the proton-pumping vacuolar-type ATPase (V-ATPase) (Buckley et al., 2019), impairs retrograde transport of numerous cargoes to the TGN (Rutherford et al., 2006), and arrests phagosome resolution (Krishna et al., 2016). Importantly, all of these processes proceed through membrane deformation events such as budding, tubulation, and scission. In addition, the acute washout of inhibitors of PIKfyve, promptly results in extensive tubulation, vesiculation, and shrinkage of the engorged endolysosomes

(Sharma et al., 2019; Freeman et al., 2020). Distended macropinosomes in PIKfyve-inhibited cells can be induced to shrink and form tubules when subjected to hypertonic solution (Freeman et al., 2020), a condition that osmotically forces water out the cell and concomitantly, out of the vacuoles. These findings suggest that PtdIns(3,5)P_2 controls the vacuolar efflux of Na^+ through its gating of TPCs and TRPMLs, and that this in turn drives water out of the lumen and triggers membrane remodeling processes by reducing membrane tension. Like all phosphoinositides, the effectors of PtdIns(3,5)P_2 collectively orchestrate a gamut of cellular functions. For example, in yeast, this phosphoinositide can regulate the assembly of the V-ATPase (Li et al., 2014) yet lysosome swelling is prevented when mutating or inhibiting the pump in PtdIns(3,5)P_2-deficient cells (Wilson et al., 2018; Sharma et al., 2019). It seems likely, therefore, that PtdIns(3,5)P_2 may support efflux of Na^+ and Ca^{2+} but also govern ion and specifically proton transport at the lysosome in yet poorly-defined ways.

Endomembrane Tension Sensing

A major outstanding question in the field is how endosomes sense their luminal contents to direct traffic. As previously alluded to, cation channels (e.g., TRPML), PIKfyve, and VRAC/LRRC8 are all responsive to endomembrane tension and/or osmotic stress, suggesting that there are numerous feedback mechanisms for the cell to calibrate ion transport to the tension experienced at organelles (Bonangelino et al., 2002; Chen et al., 2020; Li et al., 2020). Tension on the endomembrane may impact lipid packing or spacing of lipid headgroups and could conceivably recruit or activate signaling complexes that sense these events. In this regard, given that the small GTPases of the Rab family control nearly all aspects of membrane traffic, including vesicle budding, docking, fusion, and transport (Grosshans et al., 2006), they would seem to be likely candidates. Like all GTPases, Rabs function as molecular switches; when GTP-bound they recruit effector proteins with diverse functions in membrane trafficking, whereas they are inactivated by GTPase activating proteins (GAPs) that help convert the GTP to GDP. The cycle repeats when the GDP is removed by a guanine nucleotide-exchange factor (GEF) and the GTPase is loaded with GTP once again. This ability to rapidly cycle between on and off states and their individual specificities for membrane compartments makes Rab proteins well-suited for the task of sensing membrane tension to direct traffic. Rab5 in particular is a possible candidate in as much as its activation can disrupt volume and trafficking of early endosomes. Enlarged endosomes are observed with a constitutively activated mutant of Rab5 (Stenmark et al., 1994; Roberts et al., 2000; Murray et al., 2002; Galperin and Sorkin, 2003), as well as upon ectopic expression of its GEF (Otomo et al., 2003) or mutation of its GAP (Sun et al., 2012). This phenotype is accompanied by impaired transferrin recycling and the retention of transferrin receptor in enlarged endosomes (Stenmark et al., 1994; Sun et al., 2012).

The endosome enlargement under Rab5 activating conditions is attributed to the recruitment of several Rab5 effectors that facilitate SNARE-dependent membrane fusion, including EEA1 and Rabenosyn-5 (Simonsen et al., 1998; Christoforidis et al., 1999; Nielsen et al., 2000). Moreover, Rab5 is required for the endosomal enlargement in PIKfyve inhibited cells (Compton et al., 2016). When considering the extreme size of PtdIns(3,5)P_2-deficient vacuoles, and of those produced by inhibition of TPCs or Na^+ removal, it is clear that while deformation of the turgid membrane is hindered, incoming membrane fusion with this compartment is not impaired. This raises the possibility that Rab5 senses membrane tension to induce fusion, which may be a means to offset some of the tension by providing additional membrane to this stressed system.

Endomembrane Remodeling and Trafficking as a Consequence of Membrane Tension Relief

Upon internalization, various receptors and their ligands are simultaneously sorted into membrane subdomains of the early endosome and trafficked to specific destinations. Transferrin and its receptor are recycled to the plasma membrane (Dautry-Varsat et al., 1983; Dunn et al., 1989) in order to bind and take up more iron (Klausner et al., 1983), acid-hydrolase receptors are retrieved back to the TGN after delivering newly synthesized hydrolases to endosomes (Bonifacino and Rojas, 2006), and cargo such as LDL and EGF bound to its receptor are delivered to lysosomes and degraded (Carpenter and Cohen, 1979; Brown et al., 1983; Dunn et al., 1989). The endomembrane subdomains within which these sorting events occur are morphologically distinct (Mellman, 1996). For example, recycling cargo enters slender membrane tubules that pinch off to form tubular and vesicular transport carriers while remaining cargo is sequestered into inward-budding membrane invaginations that form intralumenal vesicles (ILV) within the endosome. The former, in fact, has also been described as a tubular endosomal network in which multiple interconnected membrane tubules are formed from an endosomal subdomain (Bonifacino and Rojas, 2006).

The formation of tubular subdomains on endosomes is orchestrated by the recruitment and assembly of trafficking complexes, such as retromer that targets cargo to the TGN (**Figure 2**) (Seaman et al., 1998). Retromer is composed of a heterotrimeric cargo selective complex that recognizes cargo in endosomal membranes, as well as a heterodimer of SNX-BAR proteins (combinations of SNX1, SNX2, SNX5, SNX6) that bind and stabilize highly curved membrane tubules (Carlton et al., 2004; Wassmer et al., 2007). Interestingly, the SNX-BAR proteins can also function independently of retromer in cargo trafficking (Kvainickas et al., 2017; Simonetti et al., 2017). SNX5/6 heterodimers and the SNX-BAR protein SNX4 can additionally interact with components of the retrograde microtubule motor protein dynein (Traer et al., 2007; Wassmer et al., 2009) to direct trafficking from endosomes to a perinuclear endosomal recycling compartment (Traer et al., 2007). In addition, endosomal tubules decorated with other SNX-BARs were shown to be affected by perturbations in dynein or the anterograde microtubule motor kinesin (Hunt et al., 2013). Membrane tubule interactions with motors likely contributes to their elongation and scission (Hunt et al., 2013), by their stretching along microtubules.

Retromer can also associate with WASH, which regulates branched actin polymerization on the endosome to facilitate

membrane remodeling, cargo sorting and membrane scission (Derivery et al., 2009; Puthenveedu et al., 2010; Buckley et al., 2016). Furthermore, the recently identified complex, retriever, that shares striking similarity to retromer, also interacts with WASH on endomembranes and directs cargo trafficking toward the plasma membrane (McNally et al., 2017). The membrane tubules that form can be further remodeled into buds, by the recruitment of the coat protein clathrin (Stoorvogel et al., 1996), that eventually scission into smaller cargo-carrying vesicles (Saint-Pol et al., 2004). Membrane scission at these sites was shown to be mediated by the collar-forming GTPase dynamin (Llorente et al., 1998; Nicoziani et al., 2000), contact sites with the endoplasmic reticulum (Rowland et al., 2014) and through friction mediated scission (Simunovic et al., 2017).

Remnant cargo that escapes sorting into tubules may be directed into ILVs as the endosome matures into a multivesicular body. ILVs are formed by the endosomal sorting complex required for trafficking (ESCRT), that assembles as spiral filaments on the surface of endomembranes to drive inward membrane budding and constriction (**Figure 2**) (Pfitzner et al., 2020). ESCRT is made up of 4 subcomplexes: ESCRT-0 mediates initial binding to endomembranes and recruits ESCRT-I, which in turn recruits ESCRT-II, followed by ESCRT-III, the key functional subcomplex that drives membrane deformation and scission along with the ATPase VPS4 (Saksena et al., 2009; Adell et al., 2014; Chiaruttini et al., 2015; Maity et al., 2019). The inward budding events generated by ESCRT-III result in the delivery of membrane receptors into the lumen of the organelle, where they are degraded by subsequent fusion with lysosomes (Katzmann et al., 2002).

All of these membrane remodeling events including tubulation, scission, and invagination that are sculpted by coat proteins and the spiral polymerization of ESCRT involve extreme deformations to the endomembrane. Modeling predictions and solved structures for BAR domain-containing SNXs show preferred binding to tubules of 20–60 nm in diameter (Mim et al., 2012; Simunovic et al., 2015) which is supported by reconstitution experiments (see van Weering et al., 2012 for example). ESCRT-driven invaginations can be even more narrow (Pfitzner et al., 2020). In the case of tubules, the substructures can reach remarkable lengths, even several microns (Kerr et al., 2006). Given the substantive changes in the surface to volume ratios, it follows that to create these features, membrane-deforming proteins require low membrane tension for their assembly while high hydrostatic pressure increases membrane tension which offsets the process altogether (Zimmerberg and Kozlov, 2006; Shi and Baumgart, 2015; Simunovic and Voth, 2015). This notion was empirically tested by exposing liposomes to hypertonic solutions which leads to their crenation and the recruitment of the BAR domain-containing protein BIN1, which in turn induces liposome tubulation. Conversely, swelling the liposomes prevents the BIN1-mediated tubulation (Freeman et al., 2020). And, as expected, ESCRT-III-mediated ILV formation has a similar dependency on low membrane tension (Booth et al., 2019; Mercier et al., 2020) and prefers assembly with curved/crenated membranes (Lee et al., 2015). Thus, membrane tension relief that is accomplished via the efflux of monovalent ions and the concomitant extrusion of water, is a critical event that maintains a low hydrostatic pressure and triggers membrane crenation. This crenation in turn lowers the energy barrier for membrane deformation by proteins like SNX-BAR and ESCRT-III that maintain membrane trafficking.

Failure to relieve endomembrane tension disrupts cargo trafficking and is incapacitating. Treatment of cells with PIKfyve inhibitors or other compounds that cause endosomal swelling impairs retrograde trafficking (Rutherford et al., 2006), recycling of cell surface receptors (Carpentier et al., 2013; Freeman et al., 2020) and leads to cell death (Overmeyer et al., 2011; Martin et al., 2013). In addition, knock-out of TPC1 or inhibition of PIKfyve or TPCs, causes decreased responsiveness of the tumor cell line HT1080 to EGF, and results in delayed growth (Freeman et al., 2020). Disruption of the Na^+ gradient by culturing cells in Na^+-free medium, prevents the recycling of Mac-1 ($\alpha_M\beta_2$-integrin) from endosomes to the plasma membrane and impairs phagocytosis and formation of focal adhesions, processes that require surface integrins (Freeman et al., 2020). The importance of Na^+ efflux was demonstrated *in vivo* as well. Laser ablation of tissue to mimic small injuries such as those that may occur during exercise, normally causes the migration of highly macropinocytic resident tissue macrophages to injury sites to contain the damage (Uderhardt et al., 2019). However, when vacuolar Na^+ efflux is prevented, the macrophages fail to resolve their macropinosomes and are unable to survey their environment and respond to the damage. This results in neutrophil "swarming" of the affected area, an aberrant and inflammatory response (Freeman et al., 2020). Thus, cells exploit a Na^+ gradient to resolve fluid from their endosomes and maintain the low membrane tension required for trafficking and overall cell responsiveness.

Lysosomal Solute Efflux and Membrane Trafficking

Maturing endosomes and autophagosomes ultimately fuse with lysosomes in order to degrade their luminal contents. The fusion with lysosomes confers on these hybrid organelles degradative properties by delivering the V-ATPase to the limiting membrane and soluble acid hydrolases to the endocytic fluid. But (endo)lysosomes are not just terminal compartments where breakdown occurs. Instead, they function in membrane repair, immunity, and are major signaling hubs that posit at the crossroads of nutritional status, transcription/translation, and cellular homeostasis. To meet the demand of such diverse functions, lysosomes are remarkably dynamic organelles. While the steady-state distribution of lysosomes in the cytoplasm is typically perinuclear, with some occupying the peripheral cytosol, they undergo frequent bi-directional transport between these areas (Pu et al., 2016). Lysosomes also undergo fusion, fission and tubulation by coupling to the motor proteins dynein and kinesin (Phaire-Washington et al., 1980; Swanson et al., 1987; Luzio et al., 2007; Mrakovic et al., 2012; Saffi and Botelho, 2019). Indeed, these properties are implicated in many of their functions including encounter and fusion with autophagosomes (Jia et al., 2017), antigen presentation (Vyas et al., 2007; Garg et al., 2011;

Saric et al., 2016), exocytosis (Rodriguez et al., 1997; Tuli et al., 2013; Encarnação et al., 2016), and their reformation following autophagy (Yu et al., 2010). Remarkably, and germane to the principles discussed in this review, at least the signaling pathways known to be initiated at the lysosomal membrane are engaged according to the luminal solutes of the lysosome including amino acids, protons, and we will argue, major osmoticants (Sancak et al., 2008; Zoncu et al., 2011).

Importantly, because multiple pathways converge on lysosomes, this relatively small organelle that normally occupies a fraction of the total cell volume (∼2.5% Steinman et al., 1976) experiences high solute flux. As such, the lysosome is equipped with a suite of channels and transporters that maintain its ionic composition and facilitate the flux of catabolites from the lumen (**Figure 3**). Maintenance of its ionic composition is critical for proper acidification, membrane potential, and in order to remain iso-osmotic with the cytosol (Xu and Ren, 2015). The luminal concentrations of mono- and di-valent inorganic ions have been estimated or determined with ratiometric dyes or by isolating/patch-clamping lysosomes, though values have a wide range owed to challenges with each of these approaches. Na^+ concentrations have been determined as between 20 mM and upwards of 140 mM for example (Steinberg et al., 2010; Morgan et al., 2011; Wang et al., 2012), though many experimental approaches suggest it is the major cation of the lysosome (Morgan et al., 2011). Measurements of lysosomal Cl^- come with similar challenges but largely show a tighter spectrum of estimates ranging from 60 to 110 mM (Sonawane and Verkman, 2003; Morgan et al., 2011; Stauber and Jentsch, 2013; Saha et al., 2015) suggesting Cl^- is the major anion of the compartment. Although it is not known if or how much osmotic pressure lysosomes may experience, dysregulation in Na^+ or Cl^- transport are expected to cause osmotic imbalances and high membrane tension and we describe this in the following subsections. Moreover, well-characterized storage disorders of the lysosome that cause the accumulation of organic osmolytes will also drive such hydrostatic pressure and membrane tension to build up at the lysosome. We therefore also discuss organic solutes and broadly describe osmoregulation of lysosomes in homeostasis and disease.

The V-ATPase and Counterion Fluxes in Endolysosome pH: Osmotic Considerations

Maintaining a low lysosomal pH (4.5-5) aids in enzymatic digestion, is important for lysosomal trafficking, and the steep proton gradient supports lysosomal cotransporters whose activities are H^+-coupled. The low pH is maintained by the V-ATPase, a pump that consumes ATP to constitutively transport protons into the organelle (**Figure 3**). However, this process is electrogenic and the highly positive charge generated within the lumen would quickly inhibit further proton pumping. To circumvent this problem, an ion counter-flux exists to dissipate the electrical potential across the membrane (Fuchs et al., 1989). Theoretically, this can be done either by adding negative charge or removing positive charge from the lumen. From an osmotic standpoint, the addition of negative charge leads to an osmotic gain in the compartment while removal of positive charge does not.

Despite the osmotic consequences, the addition of negative charge is not just theoretical; it is known to be accomplished by Cl^- transporters including the ClC7 H^+/Cl^- exchange transporter that brings $2Cl^-$ in for $1H^+$ out (Stauber and Jentsch, 2013) such that the V-ATPase can bring $3H^+$ in. In the absence of ClC7, osteoclasts cannot remodel bone and mice and patients become severely osteopetrotic (Kornak et al., 2001). Moreover, the loss of ClC7 causes neurodegeneration and lysosomal storage disease (Kasper et al., 2005), so clearly ClC7 is important in pH regulation. It is curious therefore that numerous studies have demonstrated that ClC7 is in fact not required for lysosomes to reach their acidic pH (Kasper et al., 2005; Weinert et al., 2010). The discrepancies between the observed physiological phenotypes and that of single lysosomes in their steady state needs to be rectified. It should be noted, however, that certain circumstances require rapid and deep transitions in the pH of compartments, yet these events are rarely captured experimentally. Examples include host-pathogen interactions where a phagosome has little time to waste in acidifying to become a hostile environment for the microbe. This could equally be the case for the osteoclast lacunae—a large compartment with a seal that evolves along with the substratum being degraded. In the case of the osteoclast lacunae, the extracellular osmotic gain of Cl^- is of no consequence, so using Cl^- efflux to drive the V-ATPase would be advantageous in this setting. For the maturing phagosome, perhaps the tradeoff between contending with a little volume gain and making a steep transition in pH is one the cell is willing to make. Either way, given the osmotic consequence of Cl^- transport, control over ClC7 activity is critical. Indeed, gain-of-function mutations in ClC7 cause massive swelling of lysosomes (Nicoli et al., 2019).

Once a low pH is reached, the steady state is supported by ongoing activity of the V-ATPase coupled to a proton "leak" as mediated by solute transport systems (see below) and alkali cation exchangers (Casey et al., 2010). The latter necessitates the efflux of cations to prevent their accumulation and support electro- and osmo-neutrality (Steinberg et al., 2010). Since lysosomal $[Na^+]$ has been estimated to be high, it is interesting to speculate on the role of TPC2 and Na^+ transport from the lysosome. TPC2 may play a similar role at the lysosome as TPC1 at the endosome—to move Na^+ down its concentration gradient (from the lumen to the cytosol), in this case to promote acidification as well as prevent osmotic swelling that opposes trafficking. In this regard, lysosomes are indeed more alkaline in skeletal muscle cells of $TPC2^{-/-}$ mice compared to wild type animals (Lin et al., 2015). And, interestingly, overexpression of TPC2 alone in HeLa cells causes extensive lysosome tubulation and increases lysosomal motility, whereas overexpression of a pore-mutant prevents tubulation (Freeman et al., 2020). It cannot be disregarded that proton exchangers of lysosomes may equally transport K^+ (Wilson et al., 2018), which would also require a K^+ channel that drives its efflux. This could involve TMEM175, a bidirectional K^+ channel that is essential to maintain normal lysosome biology, especially in neurons (Cang et al., 2015).

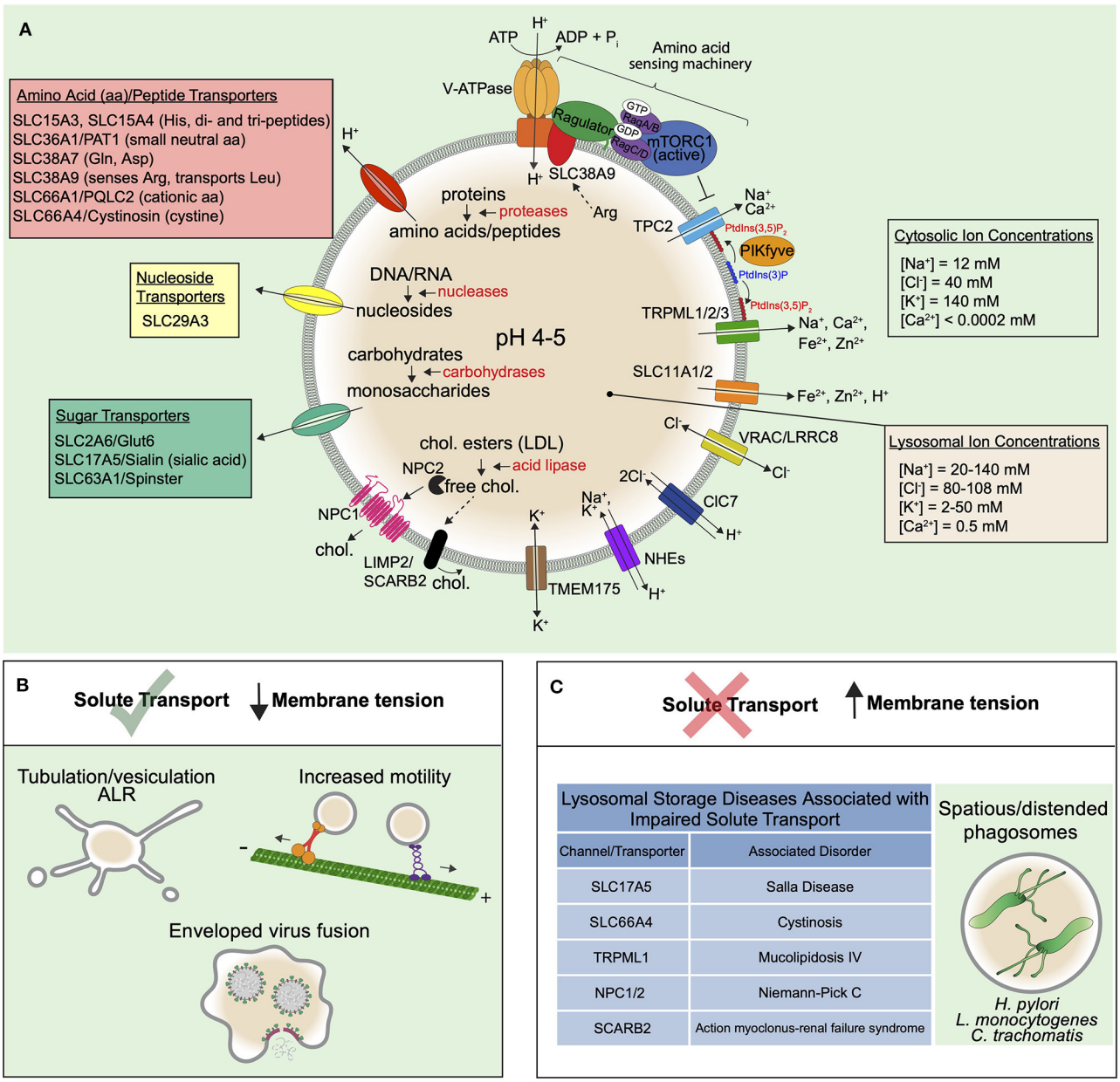

FIGURE 3 | Solute transport controls the trafficking and function of lysosomes. **(A)** Low lysosomal pH (4-5) is maintained by the V-ATPase that uses energy from ATP hydrolysis to constitutively pump H^+ into the lumen. The low pH is supported by a counter-ion flux involving either the efflux of cations or influx of anions. The luminal Na^+ and Cl^- concentrations of the lysosome are higher than that of endosomes, suggesting that both mechanisms may be involved. Cl^- transport by ClC7 is indeed important for maintaining proper lysosomal functions in certain cell types. VRAC/LRRC8 also features in Cl^- transport in lysosomes. Since lysosomal NHEs may exchange either Na^+ *or* K^+ for H^+, the K^+ channel TMEM175 may also be critical for the control of lysosome size, fission, and tension. Indeed, to remodel the limiting membrane of the lysosome, the extrusion of major osmolytes is thought to be a necessary prerequisite and may orchestrate the process altogether. In this regard, it is known that Na^+ efflux, as activated by PtdIns(3,5)P_2, causes the extrusion of water from lysosomes, leading to a lowering of the hydrostatic pressure and subsequent membrane tension. PtdIns(3,5)P_2 also regulates the V-ATPase either directly or indirectly. The low pH of the lysosome maintains the activity of various acid hydrolases (red text) in the luminal fluid that break down the macromolecules that reach lysosomes by various endocytic uptake pathways and autophagy (see **Figure 1**). The building blocks generated from this hydrolysis are exported out of lysosomes via numerous transporters of the SLC family depicted and described in the main text. Known solutes for the SLC transporters are provided in parentheses next to transporter names. The best described example is the amino acid transporter SLC38A9 that senses luminal arginine to activate mTOR on the lysosomal surface via complexes collectively termed the "amino acid sensing machinery." mTOR also binds and inhibits TPCs, providing a link between amino acid sensing and membrane tension control. Cholesterol efflux is also well-described and is exported from lysosomes by NPC1/NPC2 proteins as well as SCARB2. In general, solute efflux is critical for nutrient acquisition but also to prevent an osmotic pressure that could increase lysosomal hydrostatic pressure and membrane tension. **(B)** When solute efflux via lysosomal channels and transporters is functioning optimally, lysosomal membrane tension is controlled and this supports trafficking processes like ALR, that proceeds through lysosome tubulation, lysosomal motor-driven motility, and viral fusion. **(C)** When solute efflux mechanisms are impaired, this leads to lysosomal storage disorders, many of which exhibit distended lysosomes and likely high membrane tension that precludes lysosome tubulation and trafficking. In addition, some bacteria may modulate lysosomal membrane tension for their benefit.

While little is known about the role of Na^+ in the lysosome, Ca^{2+} is much better appreciated. Amongst its many functions, Ca^{2+} is an important second messenger in signal transduction pathways and regulates membrane fusion (Clapham, 2007), thus, it's release from lysosomes must be transient and exquisitely timed. This is nicely reviewed elsewhere (Morgan et al., 2011). From an osmotic view, although the concentration of Ca^{2+} in the lysosome is much higher than that of the cytosol ($[Ca^{2+}]_{lysosome} = 0.5$ mM (Christensen et al., 2002), $[Ca^{2+}]_{cytosol} < 0.0002$ mM), it is of low abundance compared to Na^+ and K^+, and Ca^{2+} efflux also does not appear to play a role in the acidification of lysosomes (Steinberg et al., 2010). Still, the timed release of Ca^{2+} from lysosomes is important for fusion events as mediated by TRPML (Pryor et al., 2000; Dayam et al., 2015). Similar to endosomes, lysosomal TRPML channels and PtdIns(3,5)P_2 likely contribute to the lipid-gated control of Ca^{2+} efflux to support trafficking.

Controlled Lysosomal Solute Efflux Governs Membrane Tension and Trafficking

Maintaining lysosomal acidity is critical for the luminal hydrolases, including proteases like most cathepsins, various carbohydrate-processing enzymes, and nucleases and lipases that function optimally at low pH. Collectively, these enzymes digest macromolecules into their constituent building blocks. These solutes are in turn effluxed from lysosomes via solute transporters, in order to be used by the cell in anabolic reactions that sustain growth. An additional consideration is that in the absence of such efflux, the osmotic burden from enzymatic hydrolysis would be substantial. For example, a single internalized polysaccharide of 100 glucose monomers, when enzymatically processed, generates 100 osmotically active monosaccharides. If solute efflux mechanisms did not exist, the resulting inward flow of water due to a 100-fold higher osmotic gradient would generate hydrostatic pressure against the compartment. Thus, cells continually efflux lysosomal solutes to prevent osmotic swelling and overtly high membrane tension, which in turn supports lysosomal resolution by way of tubulation, vesiculation, and trafficking. Cells likely utilize these pathways to orchestrate endomembrane remodeling events that are coincident with organellar resolution and fission.

A clue to this comes from studies on autophagic lysosome reformation (ALR): Lysosomes that fuse with autophagosomes to form autolysosomes, reform by way of tubulation and fission only upon completion of autophagy (digestion) (Yu et al., 2010). The molecular machinery that facilitates lysosomal membrane remodeling remains to be discovered, however, some coat proteins have been described. For example, the adaptor protein complex AP5 may function with the hereditary spastic paraplegia proteins SPG11 and SPG15 as a novel coat-like complex to retrieve mannose 6-phosphate receptors from late endosomes/lysosomes to the TGN (Hirst et al., 2013, 2018). Consistent with this, enlarged lysosomes are observed in SPG11- or SPG15-depleted cells (Renvoise et al., 2014), and these proteins have been shown to mediate ALR (Chang et al., 2014). Also, clathrin was shown to mediate ALR tubule formation (Rong et al., 2012), suggesting that lysosome trafficking may be similarly dependent on membrane tension as endocytosis and early endosomal sorting. In this regard, it is remarkable that TPC2$^{-/-}$ animals show defects in autophagic flux (Garcia-Rua et al., 2016). Putatively, Na^+ efflux from the autolysosome orchestrates endomembrane remodeling and ALR but how this is timed remains unclear and the $[Na^+]$ of autophagosomes/autolysosomes has not been estimated to our knowledge. It is nevertheless enticing to anticipate that monovalent ion efflux must be choreographed such that organic solutes are fluxed before resolution occurs so as not to defeat the autophagic function.

The Role of the SLC Transporters in Lysosomal Solute Efflux and Membrane Tension Control

The lysosomal efflux of organic solutes is carried out by a suite of transporters belonging to the solute carrier (SLC) protein families (**Figure 3**). When solute efflux is prevented, osmotically-imposed lysosomal swelling occurs, a condition that limits lysosomal transport (Bandyopadhyay et al., 2014). An indication that solute efflux mitigates high membrane tension comes from investigation of sucrosomes. When sucrose, a disaccharide of glucose and fructose that is resistant to cleavage by lysosomal enzymes, is loaded into lysosomes by endocytosis, lysosomes swell (Cohn and Ehrenreich, 1969), suggesting that water is trapped in the compartment. The introduction of invertase, an enzyme that breaks down sucrose into its monosaccharide constituents, induces a prompt shrinkage and, interestingly, extensive tubulation of the lysosome (Swanson et al., 1986; Bright et al., 2016). Since glucose and fructose can be transported out of lysosomes by unknown monosaccharide transporters (Mancini et al., 1990; Lizak et al., 2019), it is likely that efflux of this sugar drives the osmotic extrusion of water from the organelle. Consistent with this, mutations in SLC17A5, a H^+-driven acid sugar transporter, cause Salla disease, a rare lysosomal storage disorder (LSD) in which sialic acid is unable to be exported out of lysosomes (Aula et al., 1979; Renlund et al., 1986; Verheijen et al., 1999; Tarailo-Graovac et al., 2017). Consequently, vacuolated endolysosomes are observed in patient peripheral blood lymphocytes (Aula et al., 1979). In addition, loss of Spinster (Spin), another lysosomal sugar transporter, also causes lysosomal enlargement in cultured NRK cells. Remarkably, the loss of Spin alone blocks lysosome tubulation during ALR (Rong et al., 2011). The requirement for Spinster and other sugar efflux pathways would be especially pronounced in cells undergoing glycophagy or those internalizing polysaccharides from their surrounding fluid. As the efflux of monosaccharides is required to maintain the low membrane tension to support trafficking, cells under these conditions should be investigated with interest.

The efflux of amino acids is equally essential. Numerous lysosomal amino acid transporters have been identified including SLC66A4/Cystinosin, SLC36A1/PAT1, and SLC66A1/PQLC2 that transport cystine (Town et al., 1998), small neutral amino acids (Sagne et al., 2001) and cationic amino acids (Liu et al., 2012). Hematopoietic cells additionally express the proton-coupled transporters SLC15A3 and SLC15A4 that transport histidine as well as di- and tri-peptides. Their activities are particularly important in innate immune responses such as

sensing of bacterial peptides from phagosomes (Nakamura et al., 2014) and mast cell functions (Kobayashi et al., 2017). Indeed, loss of some of these amino acid/oligopeptide transporters was also shown to cause lysosome enlargement (Liu et al., 2012; Kobayashi et al., 2017). In addition, the sodium-coupled neutral amino acid transporters SLC38A7 and SLC38A9 transport glutamate and asparagine (Hagglund et al., 2011; Verdon et al., 2017) and leucine among other amino acids (Wyant et al., 2017). The case of SLC38A9 is particularly intriguing as it not only transports amino acids, but is able to sense luminal arginine and relay this information to activate mTORC1 on the lysosome surface (Zoncu et al., 2011; Rebsamen et al., 2015; Wang et al., 2015). This relay occurs through physical interactions between several proteins and complexes spanning both sides of the lysosomal membrane, that collectively can be termed the "amino acid sensing machinery" (**Figure 3**). And, in addition to SLC38A9, PAT1 can also activate mTOR on lysosomes (Ogmundsdottir et al., 2012). Of note, mTOR interacts with and inhibits TPCs, while nutrient deprivation, a condition that inactivates mTOR and causes its dissociation from lysosomes, constitutively opens the channels (Cang et al., 2013). Given that TPCs can lower membrane tension via Na^+ efflux, and that their activity supports tubulation and trafficking, perhaps mTOR activation via the amino acid sensing machinery simultaneously prevents lysosomal vesiculation and resolution, until sensing is complete. This raises the possibility that mTORC1 could contribute to membrane tension sensing.

Like sugars and amino acids, polynucleotides are another major type of biological polymer that is degraded within lysosomes. Sources of these can come from the DNA and RNA of dead cells that are continually being cleared by phagocytosis (Hochreiter-Hufford and Ravichandran, 2013). Following the breakdown of polynucleotides by lysosomal hydrolases such as acid deoxyribonuclease (Odaka and Mizuochi, 1999), the efflux of their monomers is necessary to prevent excessive osmotically imposed increases in lysosomal membrane tension. To this end, the lysosomal nucleoside/nucleobase transporter SLC29A3 (also known as ENT3), functions to efflux nucleosides in a pH-dependent manner (Rahman et al., 2017). Importantly, splenic macrophages from $ENT3^{-/-}$ mice exhibit swollen lysosomes (Hsu et al., 2012), signifying an osmotic defect that likely precludes lysosome tubulation and trafficking.

Phagocytosed cell corpses (and autophagosomes) are also a significant source of membranes, which are largely composed of phospholipids, glycolipids and cholesterol. These lipids are broken down by various lysosomal enzymes (Schulze et al., 2009) into individual monosaccharides, long chain bases and fatty acids. These products also need to be exported out of lysosomes though the transport pathways involved are poorly described. The one exception is with cholesterol, a significant source of which comes from endocytosed LDL particles, where a single known lysosomal acid lipase converts the LDL-derived cholesteryl esters into free cholesterol. The free cholesterol is transferred to the transmembrane protein Niemann Pick C1 (NPC1) (Infante et al., 2008; Kwon et al., 2009; Li et al., 2016) by the luminal protein NPC2. Cholesterol may also be transported to the limiting membrane by lysosomal integral membrane protein 2 (LIMP2/SCARB2) (Heybrock et al., 2019). Once incorporated into the lysosomal membrane, cholesterol is exported out of lysosomes to reach various other cellular destinations by vesicular and non-vesicular means (Luo et al., 2019) (**Figure 3**). Lysosomes can make direct contacts with the endoplasmic reticulum (ER) to transfer cholesterol down a concentration gradient via transport protein complexes (Hoglinger et al., 2019). Mutations in NPC proteins lead to Niemann Pick type C disease, a severe lysosomal storage disorder characterized by massive cholesterol accumulation within lysosomes (Vanier, 2010). As a result, lysosomes are enlarged (Lim et al., 2019), and, although in this case the enlargement is not attributed to an osmotic effect, the membrane tension may be higher than normal. Interestingly, mTORC1 is constitutively activated in $NPC1^{-/-}$ cells (Lim et al., 2019), a condition that also inhibits TPCs and Na^+ efflux (Cang et al., 2013). Finally, it cannot be ruled out that the direct insertion of lipids into the lysosomal limiting membrane may itself affect membrane tension and lysosomal trafficking: Such a contribution is complex since cholesterol would increase total membrane surface area while also increasing membrane order and effecting lipid packing (Hofsab et al., 2003; Zimmerberg and Kozlov, 2006).

Lysosomal Storage Disorders Are Associated With Impaired Solute Efflux

The inability to transport solutes across the lysosomal membrane results in their accumulation in the lumen and is the cause of numerous lysosomal storage disorders (LSDs), a class of inherited metabolic diseases. At least 70 LSDs have now been identified and we refer the reader to several excellent reviews describing the different types (Futerman and van Meer, 2004; Platt et al., 2018). Majority of LSDs are caused by mutations in genes encoding soluble lysosomal enzymes. This typically leads to lack of or incomplete digestion of their substrates. As a result, transportable products of these macromolecules are not formed and the substrates accumulate in the lumen, as they themselves are unable to be exported from lysosomes. A smaller category of LSD are caused by mutations in genes encoding lysosomal channels and transporters (**Figure 3**). Interestingly, many LSDs exhibit distended lysosomes (Aula et al., 1979; Goldin et al., 1995; Malm and Nilssen, 2008; Arvio and Mononen, 2016), suggesting that they are under high hydrostatic pressure and membrane tension, which likely affects their trafficking. The connections between endomembrane tension and lysosomal trafficking in LSDs is unknown, but recent evidence would at least suggest a role in inward budding and outward tubulation(Freeman et al., 2020; Mercier et al., 2020). A thorough investigation of the osmotic pressure and endomembrane tension incurred in these LSDs would greatly contribute to our understanding of the disease pathology.

The Role of Endomembrane Tension in Infection

Membrane tension is potentially targetable. The first clues come from studies of infections caused by the bacterium *Helicobacter pylori* that colonizes the gastric mucosa, and can cause chronic inflammation, stomach ulcers, and gastric cancer (Suerbaum and Michetti, 2002). This bacterium survives by entering the

endolysosomal system, and causes extreme vacuolation of the late endosomes/lysosomes within which it resides (Leunk et al., 1988; Amieva et al., 2002). The lysosomal vacuolation is largely attributed to a toxin, VacA, produced by the bacterium (Terebiznik et al., 2006), that has been classified as a pore-forming channel able to conduct Cl^-, bicarbonate and small organic anions (Foegeding et al., 2016). Interestingly, TRPML activity is reduced in infected cells and activation of TRPML prevents the VacA-induced lysosomal swelling (Capurro et al., 2019). These findings suggest that the disruption of solute transport causes osmotic swelling of the *H. pylori* vacuole that is likely under high membrane tension. Many bacteria have evolved ways to hijack the host trafficking machinery in order to survive in intracellular compartments (Cossart and Helenius, 2014). *Listeria monocytogenes* and *Chlamydia trachomatis* for example are also known to cause spacious vacuoles to form that accommodate their infection cycle (Van Ooij et al., 1997; Birmingham et al., 2008). In the case of *H. pylori*, *L. monocytogenes* and C. *trachomatis*, manipulation of host endomembrane tension may be a mechanism of arresting traffic to prevent resolution of their niche.

As control of membrane tension is required for membrane bending, it is also not surprising that pathogens that require fusion with host endomembranes are opposed by high hydrostatic pressure and membrane tension. Enveloped viruses that gain entry to the host cytosol by fusing with the (endo)lysosome including Ebola and coronaviruses do so with the use of fusogenic peptides, splayed by proteolytic enzyme processing (White et al., 2008). The fusogens must overcome the glycocalyx of the endosome for their insertion and the hydration force of two opposing bilayers; tension on the host membrane arrests the fusion process altogether (Harrison, 2008; Mercer et al., 2020). In this regard, it is interesting that PIKfyve and TPC inhibitors that prevent the entry of at least some enveloped viruses have been suggested as therapeutics (Kang et al., 2020). The treatment of human macrophages with TPC inhibitors blocks Ebola virus entry and the drug is effective *in vivo* to prevent infection in mice (Sakurai et al., 2015). So, while understanding endomembrane tension in cellular homeostasis is critical to appreciate normal trafficking events, it may also be important from a translational perspective.

CONCLUSION

Studies on membrane trafficking typically focus on cargo (receptors and ligands) sorting and membrane remodeling. However, it is important also to consider the fate of the fluid that is taken up during the course of all endocytic uptake mechanisms. So often we neglect the "black space" of the endocytic pathway. We now know that fluid resolution by solute transport mechanisms is a critical prerequisite for early endosomal trafficking. At the lysosome, a complex balance between enzymatic digestion and solute efflux ensures that membrane tension is controlled to permit lysosomal trafficking. When solute transport is impaired, this balance is perturbed, resulting in numerous LSDs. In addition, endomembrane tension may be exploited in infection.

As we first introduced, membrane tension is a biophysical feature of membranes that has been well-appreciated for the PM and during endocytosis in particular (Popescu et al., 2006; El Alaoui Faris et al., 2009; Dent et al., 2016; Colom et al., 2018). Though we did not include a discussion of the extensive work exploring the impact of tension on reconstituted systems including bilayers and giant unilamellar vesicles (GUVs) in this article, tension has indeed been measured in such experimental set-ups and we direct the reader to the review by Kozlov and Chernomordik for a summary of these details (Kozlov and Chernomordik, 2015). Measurements of endolysosomal membrane tension in particular have been challenging due to numerous limitations. First, these organelles are small, and many are below the resolution of light microscopy. Second, endolysosomes are dynamic organelles that undergo frequent bi-directional transport and membrane remodeling, as described. Third, all biological membranes are subject to continuous changes, such as in the lipid species present, due to ongoing biochemical reactions. Nonetheless, fluorescent probes that intercalate into membranes and display changes in their photophysical properties in response to membrane tension, show promise for endomembrane tension measurement (Colom et al., 2018).

More work is needed to uncover the mechanisms cells use to sense endomembrane tension. For now, at least, there is ample evidence that cells use ion transport as a mechanism to control membrane tension to direct endolysosomal trafficking. Placing priority on understanding how these mechanisms are controlled in space and time would seem to be the logical next step.

AUTHOR CONTRIBUTIONS

AS and SAF wrote and reviewed the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Clearing Traffic Jams During Protein Translocation Across Membranes

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Protein translocation across membranes is a critical facet of protein biogenesis in compartmentalized cells as proteins synthesized in the cytoplasm often need to traverse across lipid bilayers *via* proteinaceous channels to reach their final destinations. It is well established that protein biogenesis is tightly linked to various protein quality control processes, which monitor errors in protein folding, modification, and localization. However, little is known about how cells cope with translocation defective polypeptides that clog translocation channels (translocons) during protein translocation. This review summarizes recent studies, which collectively reveal a set of translocon-associated quality control strategies for eliminating polypeptides stuck in protein-conducting channels in the endoplasmic reticulum and mitochondria.

Keywords: UFM1, ribosome UFMylation, ribosome-associated quality control, ribosome stalling, translocon-associated quality control, translocon clogging, protein translocation, endoplasmic reticulum

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INTRODUCTION

The endoplasmic reticulum (ER) is the entry point of the secretory pathway in eukaryotic cells: Most proteins destined for the endomembrane system or extracellular space are first inserted into the ER membrane or translocated into the ER lumen (Fewell and Brodsky, 2009; Zimmermann et al., 2011). These proteins are folded, assembled, and modified in the ER before reaching their final destinations (Fewell and Brodsky, 2009). Approximately one-third of the eukaryotic proteome is processed in the ER (Chen et al., 2005). Most of these proteins are imported into the ER by the Sec61 translocon *via* either a cotranslational or posttranslational targeting mechanism.

The cotranslational translocation pathway moves nascent polypeptides into the ER while they are being synthesized by ER-bound ribosomes. This process is used by ER clients bearing an amino-terminal signal sequence or a hydrophobic transmembrane domain (TMD), which is recognized by signal recognition particle (SRP) in the cytosol. SRP then targets the ribosome-nascent chain complex to the ER membrane *via* an ER-localized SRP receptor. Nascent chains are handed over to the Sec61 translocon, which uses the energy from translating ribosomes to move polypeptides across the membrane. For membrane proteins, the translocation process is coupled to membrane integration of hydrophobic TMDs through a lateral gate of the translocon (Zimmermann et al., 2011). For a subset of polytopic membrane proteins such as G protein–coupled receptors, the proper engagement of the N-terminal TMD with the translocon and lipid bilayer requires an additional complex named ER membrane protein complex (EMC) (Chitwood et al., 2018; Guna et al., 2018; Chitwood and Hegde, 2019).

A relatively small number of proteins such as those bearing a suboptimal signal sequence or the so-called tail-anchored (TA) proteins (carrying a single membrane targeting TMD at the carboxyl-terminus) use a posttranslational mechanism for ER targeting (Zimmermann et al., 2011;

Ast et al., 2013). In this case, nascent polypeptides are completely synthesized in the cytosol prior to ER targeting. Substrates are kept in an unfolded, translocation-competent state by association with cytosolic chaperons (e.g., Hsp70 or the Bag6-SGTA holdase system) (Ngosuwan et al., 2003; Johnson et al., 2013). For proteins bearing a suboptimal signal sequence, they use a posttranslational translocation machinery containing the accessory proteins Sec62 and Sec63 in addition to the Sec61 translocon (Johnson et al., 2013; Gemmer and Forster, 2020). Recent cryo–electron microscopy (cryo-EM) studies showed that the binding of the Sec62-Sec63 subcomplex to the translocon induces the opening of the lateral gate, priming the channel for insertion of low hydrophobic signal sequences (Itskanov and Park, 2019; Wu X. et al., 2019). While signal sequence is moved into the lateral gate, chaperones dissociate from the remaining polypeptide, which is then threaded into the lumen by luminal ATPase BiP (Matlack et al., 1999; Itskanov and Park, 2019; Wu X. et al., 2019). For TA proteins, Bag6 and SGTA form a chaperone complex, transferring them to a downstream ATPase named TRC40 for membrane insertion (Chio et al., 2017).

THE DIVERSE PROTEIN QUALITY CONTROL SYSTEMS AT THE ER

The biogenesis of membrane and secretory proteins is monitored by various protein quality control (PQC) mechanisms, which safeguard defects in protein folding, assembly, and localization. Deficiencies in ER-associated PQC result in accumulation of malfunctional proteins and trigger ER stress, which contribute to the pathogenesis of many human diseases (Lindholm et al., 2006; Lin et al., 2008; Ozcan and Tabas, 2012).

The best studied ER PQC process is the ER-associated protein degradation (ERAD) pathway (**Figure 1A**). In this pathway, misfolded or misassembled proteins are moved back into the cytoplasm *via* a process termed *retrotranslocation*, which requires a membrane complex and the associated ubiquitination machinery. Polypeptides are subsequently extracted from the membrane by an abundant ATPase complex named Cdc48 in yeast or p97 in mammals (Christianson and Ye, 2014; Ruggiano et al., 2014; Bhattacharya and Qi, 2019). This ATPase complex acts with a few proteasome-associated chaperones such as Bag6 and ubiquilins to transfer the extracted polypeptides to the proteasome for degradation (Lim et al., 2009; Claessen and Ploegh, 2011; Wang et al., 2011). Genetic and proteomic studies have identified most ERAD components (Hampton et al., 1996; Knop et al., 1996; Christianson et al., 2011; Leto et al., 2019), while recent cryo-EM studies have revealed significant mechanistic details on how misfolded proteins are retrotranslocated by the Hrd1 ubiquitin ligase complex (Schoebel et al., 2017; Wu et al., 2020).

When the folding capacity of the ER is mitigated during ER stress, cells can initiate a preemptive quality control (ER-pQC) pathway (Kang et al., 2006; Swanton and High, 2006), which attenuates protein translocation while routing incompletely targeted proteins for degradation in the cytosol (**Figure 1B**) (Kang et al., 2006; Kadowaki et al., 2015). Interestingly, the ER-pQC appears to use an ERAD-like mechanism to dispose of proteins bearing certain types of signal sequences (Kang et al., 2006) as it requires the ERAD components Derlins, p97, Hrd1, and Bag6 (Kadowaki et al., 2015, 2018). The current model suggests that a Derlin family member captures translocation-attenuated nascent chains *via* interactions with SRP and an SRP receptor and reroutes them to an Hrd1-containing retrotranslocon for ubiquitination-mediated degradation (**Figure 1B**).

Protein quality control also eliminates polypeptides mislocalized due to errors in ER targeting. For proteins bearing a signal sequence or TMDs, the exposure of these hydrophobic elements in the cytosol generates a "degron," causing their degradation by the ubiquitin proteasome system *via* a mechanism similar to the well-established cytosolic PQC system (**Figure 1C**) (Buchberger et al., 2010; Chen et al., 2011). For TA proteins mislocalized in the cytosol, prolonged association with the targeting chaperone Bag6 converts Bag6 from a targeting factor to a degradation triaging promoter as it recruits the ubiquitin ligase RNF126 to ubiquitinate the associated TA proteins (**Figure 1D**) (Rodrigo-Brenni et al., 2014).

Although the concept of ER PQC is well established, and many ER PQC processes have been the subject of extensive studies, previous efforts have mainly focused on folding deficiencies either after or prior to ER targeting. It was only until recently that researchers began to appreciate the error-prone facet of membrane translocation: When protein translocation is erroneously halted, the partially translocated polypeptides clog the translocon and disrupt ER homeostasis. How cells cope with clogged translocons has emerged as an intriguing question. Conceptually, several previously established PQC mechanisms could be adopted to resolve a jammed translocon. For instance, a clogged translocon may be viewed as a defective membrane complex and thus might be disposed of by ERAD, which is known for its capacity to degrade misassembled translocon components (Biederer et al., 1996; Needham and Brodsky, 2013). Alternatively, an ERphagy mechanism could allow autophagosomes to engulf damaged ER that contains clogged translocons (Strzyz, 2020). However, recent studies suggest several previously unappreciated translocon-associated quality control (TAQC) strategies that safeguard protein translocation by selectively removing stalled polypeptides.

TRANSLOCON DECLOGGING DURING POSTTRANSLATIONAL TRANSLOCATION AT THE ER

The Sec61 translocon is a highly conserved trimeric complex consisted of Sec61α, Sec61β, and Sec61γ in mammals or Sec61YEG in bacteria. It forms a narrow conduit, accommodating only unfolded linear polypeptides during translocation (Van den Berg et al., 2004). Therefore, translocation clogging could occur during posttranslational translocation if a yet-to-be translocated domain has become folded prior to translocation. This could result from either chaperone deficiency

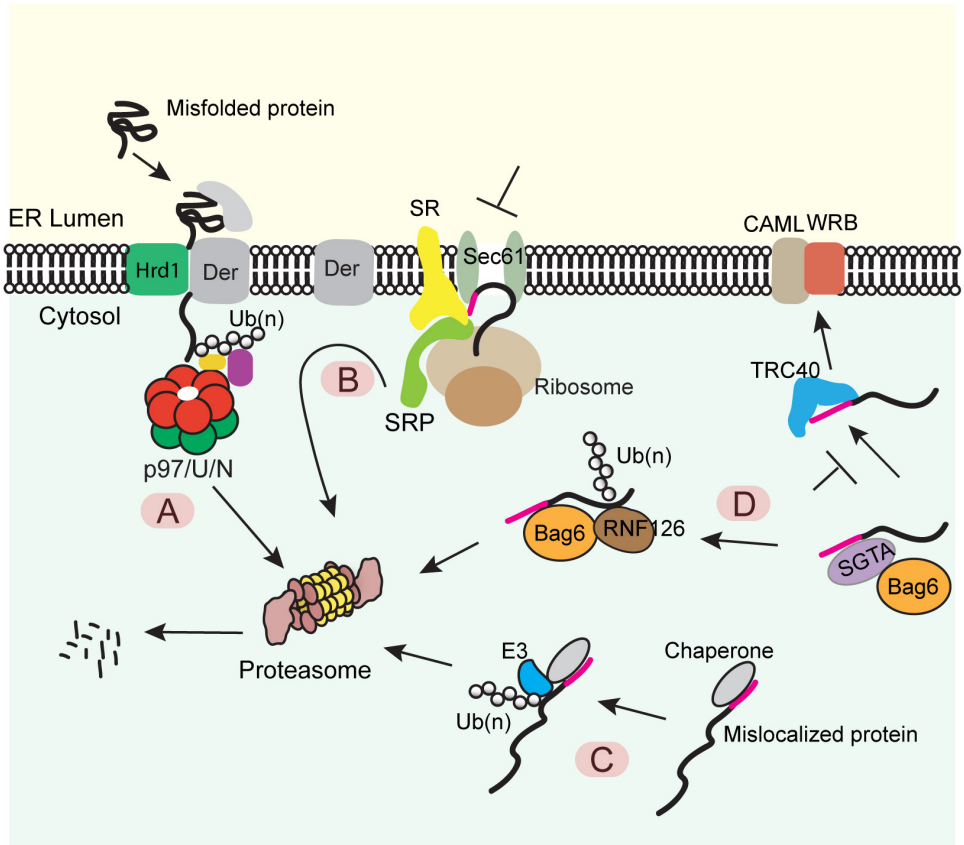

FIGURE 1 | Diverse protein quality control (PQC) mechanisms at the ER membrane **(A)** The ERAD pathway. Misfolded proteins of the ER were chaperoned to a retrotranslocation complex consisting of the membrane-associated ubiquitin ligase Hrd1 and a multitransmembrane protein Derlin (Der) (note that other accessory proteins are not shown). Substrate was retrotranslocated, polyubiquitinated (Ub) by Hrd1, extracted from the membrane by the p97 ATPase and its cofactors Ufd1-Npl4 (U/N), and degraded by the proteasome. **(B)** The pre-emptive quality control pathway. Under ER stress, the SRP-dependent cotranslational translocation was attenuated. The ER-targeted nascent chains are rerouted to the cytosol for proteasome degradation. SR, SRP receptor. **(C)** PQC for mislocalized ER proteins bearing a signal sequence or a TMD. ER proteins mislocalized in the cytosol are identified by cytosolic chaperons, polyubiquitinated by an associated ubiquitin ligase and degraded by the proteasome. **(D)** A triaging pathway for mislocalized TA proteins. The engagement of a TA substrate–SGTA complex with the targeting factor TRC40 results in a quick transfer of the client protein to TRC40, which further targets the TA protein to the WRB-CAML complex for membrane insertion. When this targeting process fails, TA protein can be transferred from SGTA to BAG6. The latter recruits the ubiquitin ligase RNF126, which ubiquitinates the substrate for proteasome degradation.

or premature dissociation of chaperones from polypeptides undergoing translocation.

Ast et al. (2016) recently identified the yeast metalloprotease Ste24 (or ZMPSTE24 in mammals) as a key quality control factor that resolves clogged translocons during posttranslational translocation (**Figure 2A**). Ste24/ZMPSTE24 is an integral membrane protein originally reported as a zinc metalloprotease responsible for the cleavage of prenylated (farnesylated or geranylgeranylated) substrates (Goblirsch and Wiener, 2020). It also processes lamin A precursor to facilitate its maturation (Kilic et al., 1997). To explore how yeast cells cope with "translocon clogging" during posttranslational translocation, Ast and colleagues designed a model substrate containing a rapidly folding, tightly packed domain. They found that the folding of this substrate prior to ER translocation causes translocon clogging, resulting in the recruitment of Ste24 (ZMPSTE24 in mammals) to the clogged translocon and subsequent cleavage of the partially translocated polypeptide by the metalloprotease activity of Ste24 (Ast et al., 2016). Besides Ste24, a recent study demonstrated a role for Hrd1 in degradation of this model substrate, probably *via* an ERAD-like or the aforementioned ER-pQC mechanism (Runnebohm et al., 2020). These mechanisms may collectively resolve clogged translocons to maintain the secretory homeostasis (**Figure 2A**).

Although Ste24 was suggested as a translocon "unclogger" specifically for posttranslational translocation, a more recent genetic screen in *Saccharomyces cerevisiae* identified it as a suppressor of proteotoxicity induced by amyloid polypeptide (IAPP). IAPP is a signal sequence-containing secretory protein that can clog the translocon by induced oligomerization prior to ER targeting. IAPP oligomerization-associated cytotoxicity in pancreatic β cells is considered a key contributor to type 2 diabetes. Overexpression of an oligomerization-prone IAAP variant in yeast also causes cytotoxicity, which was attributable

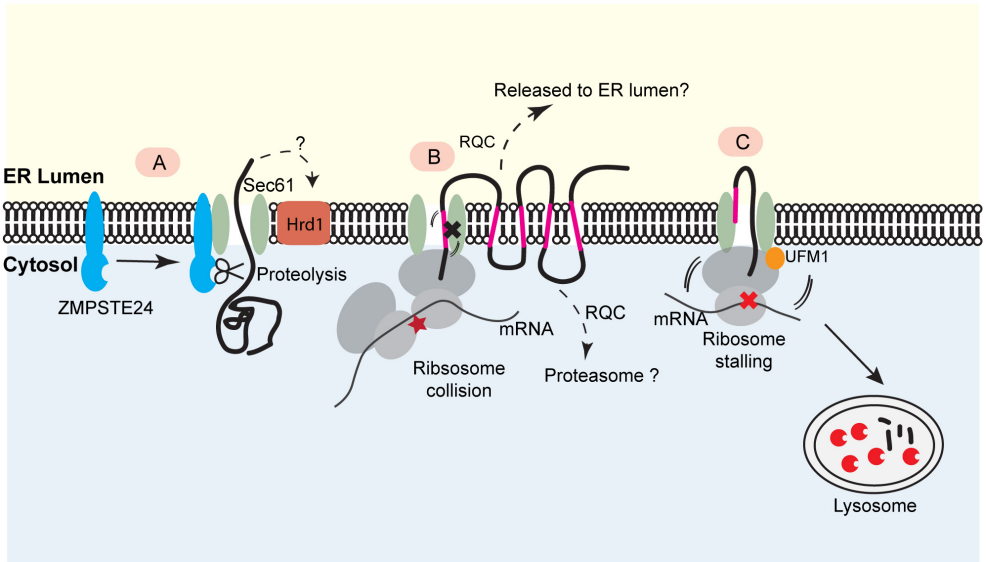

FIGURE 2 | Translocon-associated quality control at the ER. **(A)** For posttranslational protein translocation, translocon clogging occurs when the Sec61 translocon engages a client protein with a folded domain. This causes the recruitment of the ER- bound protease ZMPSTE24 (Ste24 in yeast), which cleaves the protein in the translocon for further degradation. This process may also involve Hrd1. **(B)** The biogenesis of polytopic membrane proteins depends on cotranslational insertion of TMDs into the ER membrane. A defect in TMD membrane integration can stall translocation process, causing ribosome collision. This may trigger a mechanism analogous to ribosome-associated quality control (RQC) to eliminate the faulty nascent proteins. The precise degradation mechanism for stalled membrane proteins (e.g., released into the ER lumen or extracted into the cytosol) is not clear. **(C)** Ribosome UFMylation-dependent trafficking of stalled nascent chains to lysosome. Ribosome stalling during cotranslational protein translocation induces UFMylation of the 60S ribosomal subunit RPL26, which allows the trafficking of stalled nascent chains to lysosomes for degradation.

to a translocation defect because the suppressor function of Ste24 depends on its declogging activity (Kayatekin et al., 2018). Although it is unclear why IAPP is poorly translocated even with an efficient signal sequence, this study underscores a potential detrimental consequence of obstructing ER translocon, which may define a new pathological hallmark for secretory pathway-associated amyloid-like diseases.

How does Ste24 sense translocon clogging? Conceptually, in a clogged translocon, substrate likely engages the translocon in an erroneous way, which may leave a conformational "mark" for Ste24 recognition. Consistent with this hypothesis, a recent study showed that in budding yeast, Ste24 prevents inappropriate engagement of the translocon with substrates lacking signal sequence, which may otherwise generate a clogging-like state (Hosomi et al., 2020).

TRANSLOCON DECLOGGING DURING COTRANSLATIONAL TRANSLOCATION

Scenarios of Translocon Clogging During Cotranslational Protein Translocation

In cotranslational protein translocation, polypeptides are pushed into the Sec61 translocon in a linear way by translating ribosome docked on the translocon. Only until the nascent chain is fully integrated into the ER does the ribosome dissociate from the membrane, allowing downstream ribosomes to engage the translocon for subsequent rounds of protein translocation. As protein translocation is tightly coupled to translation, protein folding, membrane insertion, and protein modifications, this coordinated process can be perturbed by either ribosome stalling or defects in folding, protein modification, and membrane insertion.

Translation of faulty mRNAs bearing no stop codon (NS mRNAs) represents a major trigger of ribosome stalling both in the cytosol and on the ER membrane (Brandman and Hegde, 2016; Joazeiro, 2017, 2019; Sitron and Brandman, 2020). NS mRNAs are frequently generated in eukaryotic cells due to genetic mutations or premature polyadenylation (Arthur et al., 2015), resulting in a poly-A–containing open reading frame that can stall ribosomes during translation (Arthur et al., 2015; Juszkiewicz and Hegde, 2017). Faulty mRNAs bound to the ER may also be generated during ER stress. It is well appreciated that ER stress–activated inositol-requiring enzyme 1 (Ire1) contains both a kinase and an endonuclease activity. The latter can cleave mRNAs encoding membrane and secretory proteins *via* a process dubbed as regulated Ire1-dependent mRNA decay (RIDD) (Hollien and Weissman, 2006; Hollien et al., 2009). This process attenuates the protein flux into the ER to mitigate ER stress, but some truncated mRNAs lacking stop codon might be generated by RIDD, which could cause ribosome stalling.

Unlike ribosome stalling in the cytosol, translation arrest during cotranslational translocation generates a unique proteostasis crisis because the arrested products are embedded in the Sec61 translocon: A significant portion of the substrate

upstream of the stalling sequence is either in the ER lumen or has been integrated into the ER membrane and thus could have participated in cotranslational events such as protein folding, glycosylation, and disulfide bond formation. Consequently, translation arrests not only produce defective nascent polypeptides, but also generate clogged translocons and a blockade in ER import (Izawa et al., 2012; Arakawa et al., 2016). Apparently, stalled ribosomes must be released from the membrane, so the aberrant translocation products can be eliminated.

The Sec61 translocon can also be clogged when a polytopic membrane protein fails to be inserted into the membrane due to defects in TMD specific chaperones. The biogenesis of polytopic membrane proteins can pose a significant challenge to cells because when an amino-terminal amphipathic TMD exits the translocon *via* the lateral gate, it often needs to wait in the hydrophobic lipid environment for assembly with downstream TMDs *via* non-hydrophobic interactions. Recent studies have identified the EMC as a potential TMD-specific chaperone (Chitwood et al., 2018; Guna et al., 2018; Chitwood and Hegde, 2019). Accordingly, defects in EMC-mediated TMD membrane integration are expected to stall the translocation process and cause "translocon clogging."

Ribosome Stalling and Ribosome-Associated Quality Control

Studies on ribosome stalling in the cytosol have established a PQC pathway named ribosome-associated quality control (RQC) (Brandman and Hegde, 2016; Joazeiro, 2017; Sitron and Brandman, 2020). In this process, a collection of cellular factors coordinately sense translation stalling, rescue stalled ribosomes, and degrade arrested polypeptides together with defective mRNAs. Specifically, when a translating ribosome stalls on an mRNA, the following ribosome will collide with the stalled ribosome given the polysome nature of protein translation. This generated a unique "disome" signature (Juszkiewicz and Hegde, 2017; Juszkiewicz et al., 2018; Ikeuchi et al., 2019), which is sensed by the ribosome-associated ubiquitin ligase ZNF598 (hel2 in yeast). ZNF598 ubiquitinates ribosomes at several sites to initiate Dom34/Hbs1-mediated disassembly of ribosomes (Juszkiewicz and Hegde, 2017; Matsuo et al., 2017; Sundaramoorthy et al., 2017), generating a 60S-peptidyl-tRNA subcomplex that further engages downstream RQC factors (Juszkiewicz et al., 2020; Matsuo et al., 2020). Eventually, the linked peptidyl-tRNA was cleaved off from nascent chains by an endonuclease (e.g., ANKZF1) (Kuroha et al., 2018; Verma et al., 2018; Zurita Rendon et al., 2018). The arrested nascent chains are then ubiquitinated by the 60S ribosome-associated ubiquitin ligase Listerin (Ltn1 in yeast) (Bengtson and Joazeiro, 2010), extracted from the ribosome by the Cdc48/p97 ATPase complex and degraded by the proteasome (Brandman and Hegde, 2016; Joazeiro, 2017).

In sharp contrast to the well-documented RQC mechanism, the limited information regarding ribosome stalling on mRNAs encoding secretory or membrane proteins fails to paint a consistent model. Izawa et al. (2012) initially showed that an ER-targeted model substrate bearing no stop codon could be released into the ER lumen following Dom34/Hbs1-mediated ribosome dissociation and that the released substrates appear to escape degradation by the proteasome. However, subsequent studies in yeast suggested that Ltn1-mediated ubiquitination and proteasomal degradation contribute to the cotranslational degradation of arrested secretory and membrane proteins produced by ribosome stalling on the ER membrane (Crowder et al., 2015; Arakawa et al., 2016). Likewise, a recent study showed an ERAD-like mechanism that degrades a translation-arrested product at the ER in mammalian cells (Cesaratto et al., 2019). Furthermore, biochemical fractionation revealed the recruitment of RQC factors such as Listerin and NEMF to the ER membrane in response to ribosome stalling in a cell-free system (von der Malsburg et al., 2015), but given that translocating nascent chains are usually shielded by the translocon and the associated ribosome, the study did not reveal how arrested products could be recognized by Listerin on the cytosolic side of the ER membrane.

The aforementioned contradiction may be resolved if cotranslational TAQC mechanisms are substrate-tailored (**Figures 2B,C**). A conceivable distinction between different TAQC substrates may lie in the state of translocon clogging. Transient clogging due to suboptimal signal sequence may result in the recruitment of ER chaperons to resolve clogged translocon while keeping the nascent chains in the biosynthetic path (Sha Sun, 2020), but aberrant translocon engagement for substrates with either a translocation-impeding domain or deregulated lipid binding (e.g., apoB) may reverse the translocation process, causing the degradation of aberrant polypeptides in the cytosol (Hrizo et al., 2007; Rubenstein et al., 2012; Runnebohm et al., 2020). By contrast, permanent stalling by translation arrest might lock the translocation process in an irreversible state, prompting the release of the stalled polypeptides only toward the ER lumen for clearance by other mechanisms (see below).

TAQC During Polytopic Membrane Protein Biogenesis

In yeast mutants lacking a functional EMC, the biosynthesis of a mutant ABC transporter Yor1ΔF is compromised, which appears to cause ribosome stalling (**Figure 2B**). Lakshminarayan et al. (2020) suggested that when defects in TMD integration arise, the activation of RQC contributes to the translation arresting of Yor1ΔF. In accordance with this interpretation, reducing translation initiation or knockout of upstream RQC factors could partially rescue the biogenesis defects of Yor1ΔF, probably because these perturbations lower the ribosome density on the Yor1ΔF mRNA to avoid ribosome collision and subsequent RQC activation (Lakshminarayan et al., 2020). Additionally, translatome-wide ribosome profiling revealed that polytopic membrane proteins generally have a low translation efficiency in yeast, suggesting an evolution pressure to keep RQC in check in polytopic membrane proteins biogenesis (Lakshminarayan et al., 2020). Another study in human cells identified several multitransmembrane proteins as potential RQC substrates because their degradation is partially mediated by Listerin (Trentini et al., 2020). Although these studies suggested

a link between translocon clogging and certain components of the RQC pathway, the precise fate of the arrested translocation products was not explored. In this regard, a systemic analysis of the involvement of the known cytosolic RQC factors in the degradation of stalled polytopic membrane proteins is warranted. Moreover, the biogenesis of soluble secretory proteins or membrane proteins with low numbers of TMDs does not seem to follow the aforementioned quality control mechanism (Trentini et al., 2020), suggesting the existence of additional mechanism(s) that declogs the translocon during cotranslational ER targeting.

TAQC for Nascent Chains Bearing Ribosome Stalling Sequences

To study how mammalian cells cope with ribosome stalling during cotranslational translocation, we recently generated an ER stalling reporter containing a polyadenine segment downstream of an ER targeting signal sequence. The reporter also contains a GFP-encoding sequence upstream of the stalling site and an RFP-coding sequence downstream. Translation stalling results in a translocon-associated translation product bearing GFP but no RFP. As expected, this translocon-clogging nascent chain is short-lived, but surprisingly, it is not degraded by the proteasome. Instead, it is transported out of the ER and disposed of by lysosomes. Intriguingly, this process requires the modification of the ribosome subunit RPL26 with a ubiquitin-like molecule named UFM1 (ubiquitin-like modifier 1) (**Figure 2C**) (Wang et al., 2020).

Protein modification by UFM1, a process dubbed as UFMylation is a conserved posttranslational modification found in most eukaryotes except in yeast and fungi (Cappadocia and Lima, 2018; Gerakis et al., 2019). In analogous to ubiquitination, UFMylation is mediated by an enzyme cascade involving UBA5 as the activating enzyme (E1), UFC1 as the conjugating enzyme (E2), and an ER-localized trimeric ligase (E3) complex composed of DDRGK1 (also named UFBP1), UFL1, and Cdk5RAP3. The UFMylation system has been tightly linked to ER protein homeostasis: the expression of most UFMylation enzymes is upregulated by ER stress, and conversely, deficiencies in UFMylation enzymes are known to sensitize cells to ER stress–induced apoptosis (Komatsu et al., 2004; Lemaire et al., 2011; Zhang et al., 2015; Wei and Xu, 2016). However, the UFMylation substrate (s) accountable for the deregulation of ER homeostasis in UFMylation-deficient cells has been elusive.

We and other recently identified the 60S ribosomal subunit RPL26 (ul24) as the principal target of UFMylation (Walczak et al., 2019; Wang et al., 2020). Biochemical fractionation demonstrated that UFM1 preferentially modifies ER-bound ribosomes, consistent with the ER localization of the UFM1 ligase complex. UFMylation occurs in two conserved lysine residues (K132 and K134 in human RPL26) in a short C-terminal tail, which appears to be co-evolved with the UFMylation system (Walczak et al., 2019; Wang et al., 2020). Importantly, we showed that translation stalling during cotranslational ER translocation is a specific trigger for ribosome UFMylation. This finding links ribosome UFMylation to TAQC. In support of this notion, we showed that RPL26 UFMylation promotes the ER exit and lysosomal transport of an arrested nascent chain reporter: In cells lacking either the UFMylation system or the modification sites on endogenous RPL26, the polypeptides stalled in the translocon are cleared at a reduced rate, which disrupts ER homeostasis (Wang et al., 2020).

The physiological substrates subjected to UFM1-dependent clearance are currently unknown, but several recent studies echo our findings by demonstrating the requirement of the UFMylation system for lysosomal transport of other defective quality control substrates including an ERphagy reporter (Liang et al., 2020; Stephani et al., 2020), suggesting that UFMylation may regulate the trafficking of a spectrum of defective proteins broader than initially thought. Genetic studies in mice have revealed an indispensable function of UFMylation in cell differentiation and animal development (Cai et al., 2016, 2019; Zhu et al., 2019). Knockout of UFMylation E1 or E3 leads to a severe defect in erythroid lineage differentiation, causing anemia in animals (Tatsumi et al., 2011; Cai et al., 2015). Consistently, in an *in vitro* erythropoiesis model, RPL26 UFMylation is upregulated during erythroid differentiation, which coincides with an increase in the secretory flow. Compromising ribosome UFMylation impairs protein secretion, induces ER stress, and ultimately inhibits hemoglobin production (Wang et al., 2020). Along a similar line, DDRGK1, a key component of the UFM1 ligase complex appears to play a crucial role in the development of antibody-secreting plasma cells (Zhu et al., 2019). These results collectively establish a physiological link between ribosome UFMylation and secretory homeostasis *via* UFMylation dependent TAQC. Intriguingly, genetic mutations in genes encoding UFMylation enzymes are linked to a variety of neurological disorders in humans (Colin et al., 2016; Muona et al., 2016; Nahorski et al., 2018). Whether defective TAQC underlies the molecular driver of these diseases remains to be investigated (Colin et al., 2016; Muona et al., 2016; Mignon-Ravix et al., 2018; Nahorski et al., 2018).

TAQC AT THE MITOCHONDRIA

In eukaryotes, protein translocation machinery is present in a variety of organelles/membranes besides the ER, raising the possibility of additional TAQC pathways in other protein translocation systems. Indeed, recent studies have shed light on several quality surveillance mechanisms that clear stalled precursor proteins from the mitochondrial outer membrane (MOM).

The mitochondrial protein translocation system consists of the Tom20 complex in the outer membrane and two Tim complexes in the inner membrane (Tim22 and Tim23), which import mitochondrial proteins synthesized in the cytosol (Wiedemann et al., 2004; Wickner and Schekman, 2005). The mitochondrial protein translocation-associated degradation (MitoTAD) pathway was recently reported in *S. cerevisiae*, which constantly monitors the TOM machinery under non-stress conditions to prevent channel clogging (Martensson et al., 2019).

A key player is a transmembrane protein named Ubx2, which was previously implicated in ERAD as a membrane adaptor for Cdc48. However, Martensson et al. (2019) showed that a pool of Ubx2 associates with the Tom20 translocase and recruits Cdc48 to remove translocation-arrested precursor proteins. The mitoCPR (mitochondrial compromised protein import response) pathway is another mechanism activated by the stalling of mitochondrial precursor proteins in the Tom translocon, but it primarily acts during stress. Weidberg et al. showed that overexpression of a bipartite signal–containing mitochondrial protein in yeast clogs the Tom translocon, which in turn induces the expression of Cis1 through a transcription factor named Pdr3. Cis1 associates with the clogged Tom translocon, recruiting another ATPase named Msp1 to extract stalled precursor proteins for proteasome degradation (Weidberg and Amon, 2018).

Both the MitoTAD and MitoCPR mechanisms appear to cope with polypeptides stalled during posttranslational translocation, which accounts for most translocation events at the mitochondria. However, recent studies also suggest the existence of localized protein translation in proximity to the MOM, which may result in cotranslational protein import into the mitochondria (Williams et al., 2014; Lesnik et al., 2015). Accordingly, mitochondrial stress due to the loss of membrane potential, etc., can cause ribosome stalling when polypeptides undergo cotranslational translocation *via* the Tom translocon (Wu et al., 2018). This defect activates a quality control mechanism. Specifically, Ltn1 was recruited to stalled nascent chains to facilitate their degradation (Izawa et al., 2017). Intriguingly, stalled nascent chains are released into the mitochondria matrix by Dom34/Ski7 and Vms1 and degraded by matrix resident proteases (Izawa et al., 2012, 2017). This mechanism resembles UFM1-dependent declogging at the ER, which also releases nascent chains into the lumen for transport to lysosomes. Defects in mitochondria-associated TAQC result in protein aggregates in the mitochondria, which sequesters chaperones and proteases to cause cytotoxicity (Izawa et al., 2017; Wu Z. et al., 2019).

Translocation clogging at the MOM might also induce mitophagy, a form of macroautophagy that selectively removes damaged mitochondria to resolve translocon jamming. Wu et al. (2018) showed that loss of MOM potential induces PINK1-dependent recruitment of a ribosome-nascent chain complex containing the mRNA encoding the 30-kD complex-I subunit (c-I30) (Gehrke et al., 2015). Ribosome stalling on c-I30-encoding mRNA triggers the recruitment of RQC factors such as Pelo, ABCE1, and NOT4. As a result of polyubiquitination of ABCE1 by NOT4, mitophagy is initiated, which clears damaged mitochondria including clogged translocons (Wu et al., 2018). Collectively, these studies suggest the use of diverse TAQC strategies to resolve translocon clogging by mitochondria.

PERSPECTIVES

Protein translocation across membranes is critical for cell physiology because it is a fundamental protein biogenesis step for clients destined to secretion or the endomembrane system. While the translocation machineries in different organelles have been extensively studied, the quality control mechanisms dedicated to the surveillance of these translocation processes have just begun to surface. Although TAQC is now being characterized in the ER and mitochondria, similar quality control processes may exist in other organelles such as peroxisomes and chloroplasts.

As a newly emerged field, there are many outstanding questions to be explored for TAQC. What are the physiological substrates of the each TAQC pathway? Are these TAQC mechanisms regulated by stress or under aging conditions in animals? How do cells sense clogged translocons in different TAQC pathways? What are the fates of the clogged substrates in each pathway? More importantly, as TAQC mechanisms are essential for the normal flux of proteins during biogenesis, the declogging function of TAQC may be particularly important for specialized cells with a high demand for protein translocation. Under these circumstances, failure in TAQC may contribute to the pathogenesis of human diseases. For instance, it would be interesting to investigate whether UFMylation-mediated TAQC plays a role in neuronal development and whether the pleiotropic neurological disorders associated with UFMylation deficiencies are caused by defects in TAQC. In short, future studies along these directions will surely reveal the full biological scope for these new PQC pathways, and also shed important insights on various pathophysiological TAQC conditions that are intimately associated with human diseases.

AUTHOR CONTRIBUTIONS

LW and YY conceived the topic and wrote the manuscript together. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Energy and Dynamics of Caveolae Trafficking

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Caveolae are 70–100 nm diameter plasma membrane invaginations found in abundance in adipocytes, endothelial cells, myocytes, and fibroblasts. Their bulb-shaped membrane domain is characterized and formed by specific lipid binding proteins including Caveolins, Cavins, Pacsin2, and EHD2. Likewise, an enrichment of cholesterol and other lipids makes caveolae a distinct membrane environment that supports proteins involved in cell-type specific signaling pathways. Their ability to detach from the plasma membrane and move through the cytosol has been shown to be important for lipid trafficking and metabolism. Here, we review recent concepts in caveolae trafficking and dynamics. Second, we discuss how ATP and GTP-regulated proteins including dynamin and EHD2 control caveolae behavior. Throughout, we summarize the potential physiological and cell biological roles of caveolae internalization and trafficking and highlight open questions in the field and future directions for study.

Keywords: caveolae, caveolin, dynamin, EHD2, membrane trafficking, lipid trafficking

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INTRODUCTION

Caveolae are 70–100 nm diameter sized plasma membrane invaginations that form bulb shape invaginations into the cytosol (**Figure 1**). They are found in a variety of cell types including adipocytes, endothelial cells, muscle cells, fibroblasts, and astrocytes (Cameron et al., 1997; Parton, 2003; Parton and Del Pozo, 2013; Parton et al., 2018; Yan et al., 2019). The plasma membranes of endothelial, muscle, and fat cells are packed with caveolae, suggesting an important role in specialized functions including homeostasis and metabolism. Caveolae also comprise a specific lipid environment containing large amounts of cholesterol, sphingomyelin, and ceramides (Graf et al., 1999; Parton et al., 2020b; Zhou et al., 2020). These lipids accumulate in caveolae, providing a reservoir for these molecules (Hubert et al., 2020b). Therefore, these organelles serve as unique scaffolds for plasma membrane proteins involved in signaling pathways creating unique cell-type specific protein signaling domains. Caveolae are also known to participate in cellular lipid and fatty acid uptake (Pilch and Liu, 2011; Pilch et al., 2011), endothelial transcytosis of large molecules (Frank et al., 2009; Cheng and Nichols, 2016), regulation of the endothelial NO synthase (Förstermann and Sessa, 2012), neurovascular coupling (Chow et al., 2020), viral internalization (Pelkmans et al., 2001; Xing et al., 2020), and pigmentation in melanocytes (Domingues et al., 2020). Furthermore, under some cellular membrane tension regimes caveolae can group into larger clusters at the plasma membrane termed caveolae rosettes (Echarri et al., 2019; Golani et al., 2019). The wide array of structures and actions of caveolae across many different tissues and pathways highlights this small organelle's diverse role in signaling and physiology. Yet, much remains to be uncovered about their regulation, function, and mode of action.

The caveolar coat is minimally composed of several proteins important for forming and stabilizing the bulb-shaped membrane invagination. The key proteins are Caveolin (three orthologous in human, Cav1-3), Cavins (Cavin1-4), the BAR protein domain-containing syndapin/Pacsin2, and the dynamin-related ATPase EHD2 (see **Figure 1**, previously reviewed in the literature; Han et al., 2016b; Lamaze et al., 2017; Parton et al., 2020c). *In vitro* and *in vivo* studies have shown that Caveolin/cholesterol complexes incorporate into the plasma membrane forming elongated and rather shallow invaginations (Fernandez et al., 2002; Hayer et al., 2010a; Ariotti et al., 2015; Han et al., 2020). Caveolin complexes alone do not form the typical bulb shape. Therefore, Cavin coat proteins have been proposed to be essential for generating the classic "cave-like" invaginations. Specifically, Cavins are recruited from the cytosol, oligomerize into trimers, and surround the caveolar membrane resulting in a structured caveolar coat as illustrated in **Figure 1A** (Gambin et al., 2014; Kovtun et al., 2015; Ludwig et al., 2016; Stoeber et al., 2016). Furthermore, it has been shown that Pacsin2 is important for bending the membrane and stabilizing caveolar invaginations at the plasma membrane (Hansen et al., 2011; Senju et al., 2011, 2015; Seemann et al., 2017). A similar function was found for the ATPase EHD2. EHD2 specifically localizes to the neck of caveolae (Morén et al., 2012; Stoeber et al., 2012; Ludwig et al., 2013). Recently, the EHD2 binding partner (EHBP1) was identified as another stabilizer of caveolae in endothelial cells (Webb et al., 2020). Finally, a BAR protein, FBP17, was discovered to be important for the formation of caveolae rosettes at the plasma membrane (Echarri et al., 2019).

Despite the large number of caveolae in muscle and endothelial cells or adipocytes, the loss of caveolae due to Cav1 or Cavin1 deletion is not generally lethal (overview of knockout models reviewed in Le Lay and Kurzchalia, 2005; Hansen et al., 2013; Cheng and Nichols, 2016). However, investigations of various Caveolin or Cavin deficient mouse models has indicated impaired lipid metabolism and lipodystrophy, cardiomyopathies, blood pressure changes, and muscular dystrophy in these animals (Pilch and Liu, 2011; Cheng and Nichols, 2016). It should be noted that some of these phenotypes could be due to non-caveolar functions of Cav1 as recently reviewed (Pol et al., 2020). Additionally, altered Cav1 and Cavin1 expression, mutations in human Caveolin genes, as well as modified caveolae endocytosis and trafficking can be linked to metabolic diseases including obesity and lipodystrophy (Catalán et al., 2008; Kim et al., 2008; Pilch and Liu, 2011; Schrauwen et al., 2015; Matthaeus et al., 2020), cancer (Lee et al., 2002; Martinez-Outschoorn et al., 2015; Ketteler and Klein, 2018) as well as cardiovascular diseases (Cohen et al., 2004a; Han et al., 2016a; Lian et al., 2019) or myopathies (Gazzerro et al., 2010; Dewulf et al., 2019). Therefore, caveolae are currently under investigation as novel therapeutic targets for disease (Carver and Schnitzer, 2003; Navarro et al., 2014).

The process of intracellular membrane traffic, including caveolae endocytosis, transcytosis, transport, and targeting, requires specific signals and regulatory modules to direct movement inside the cell. Nucleoside triphosphates Adenosine-5'-triphosphate (ATP) and Guanosine-5'-triphosphate (GTP) serve as cellular energy resources to drive, localize, and direct these actions. Both nucleosides play essential roles during intracellular trafficking to promote membrane interactions and deformations, protein-protein interactions, and conformational changes in molecular machines. Here, we summarize the state of understanding of caveolae membrane trafficking and highlight the roles of ATP and GTP-dependent processes within these specialized membrane structures.

CAVEOLAE TRAFFICKING

How do caveolae move? Caveolae endocytosis and trafficking has been observed in many cell types although the cellular consequences of these movements are currently not well-understood. The role and even occurrence of caveolae traffic has, indeed, been controversial (Parton and Howes, 2010; Cheng and Nichols, 2016; Parton et al., 2020a). Several studies have shown, however, that caveolae endocytosis supports viral entry and receptor internalization, and caveolae membrane trafficking has been linked to cellular lipid homeostasis and movement. Here, we divide caveolar internalization into 5 steps: (1) caveolae dynamics at the plasma membrane, (2) detachment from the cell membrane, (3) fusion with endosomes followed by accumulation in lysosomes or (4) non-endosomal trafficking to intracellular organelles, and finally (5) recycling of caveolae (see overview in **Figure 2** and the following sections).

With these steps in mind, caveolae internalization and traffic, however, must be distinguished from caveolae flattening and disassembly. Several studies have indicated that increased membrane tension due to osmotic shock and membrane stretch could lead to caveolar membrane flattening. Here, caveolae proteins such as Cavins or EHD2 are proposed to be released into the cytosol and are able to move independently of caveolar membranes (Sinha et al., 2011; Cheng et al., 2015; Garcia et al., 2017; Lim et al., 2017; Torrino et al., 2018). However, this mechano-adaptive caveolae behavior is cargo-independent and therefore different from classical endocytosis and traffic (Del Pozo et al., 2021). Recently, it was shown that cellular stress induced by UV light could also trigger the disassembly of caveolae and the release of caveolar proteins into the cytosol (McMahon et al., 2019). Previous studies further showed that caveolae serve as cellular membrane tension sensors that are coupled to mechano-transduction pathways such as the Hippo system involving the transcriptional regulators YAP and TAZ (Echarri and Del Pozo, 2015; Dewulf et al., 2019; Del Pozo et al., 2021). Here, caveolae are able to sense changes in plasma membrane tension and induce transcriptional changes as well as provide membrane reservoirs to protect the cell from mechanical stress. These behaviors of caveolae are distinct from the classic traffic routes we will focus on in this review.

The study of caveolae trafficking has been complicated by many technical difficulties. To investigate caveolae dependent endocytosis, cholera toxin and simian virus 40 have been the standard cargo. Yet, detailed analysis has shown that both cargos are not exclusively internalized by caveolae. This has led to some amount of conflicting data (Nichols, 2002; Cheng and Nichols,

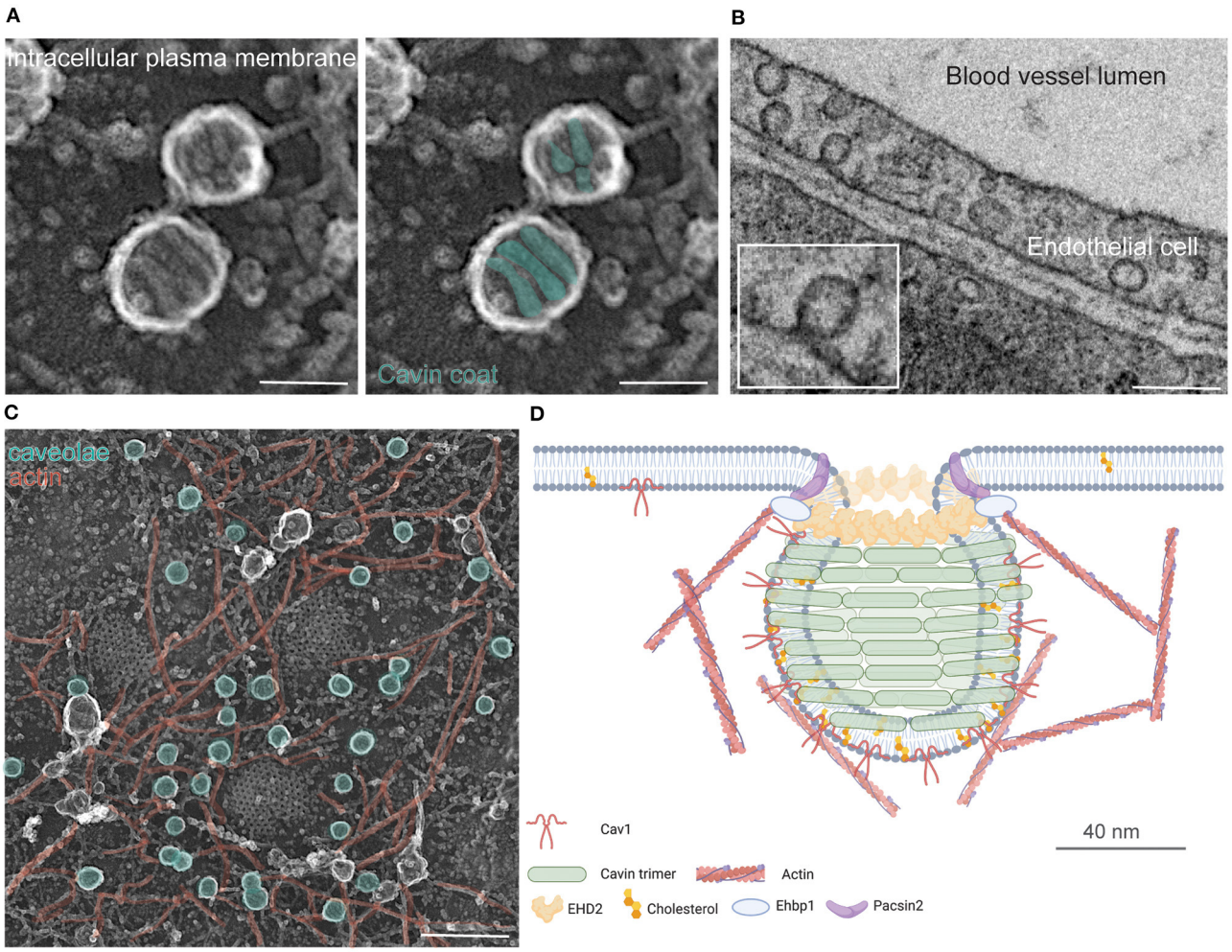

FIGURE 1 | Caveolae structure and components. **(A)** Caveolae imaged in unroofed mouse embryonic fibroblasts (MEF) by platinum replica EM. The typically caveolae coat generated by Cavin protein complexes (colored in green) surround the Cav1 induced plasma membrane invaginations. Scale bar 120 nm. **(B)** 2D TEM image of caveolae in endothelial cells of blood vessels in mouse heart sections. Scale bar 200 nm. **(C)** The MEF plasma membrane contains randomly distributed caveolae (in green) embedded with actin filaments (red). Scale bar 250 nm. **(D)** Schematic overview of caveolae and its components.

2016). To date, no caveolae-specific cargo has been identified (Parton et al., 2020a). This makes it challenging to specifically monitor intracellular caveolae trafficking. Furthermore, non-caveolar Cav and Cavin localization, and overexpression effects of Cav/Cavin proteins, makes it difficult to distinguish between intracellular Cav/Cavin protein migration and caveolae endocytosis (summarized for Cav1 in Pol et al., 2020, non-caveolar Cavin function see Jansa et al., 1998; Liu and Pilch, 2016; McMahon et al., 2019). Additionally, the comparison of Cav1 overexpressing cells with genome-edited Cav1 cells revealed that only a portion of caveolae are actually motile and the majority stay immobile at the plasma membrane (Shvets et al., 2015). In contrast, overexpressed GFP-tagged Cav1 results in many highly mobile Cav1 molecules in cells which may not be assigned to caveolae migration. Therefore, caveolae endocytosis studies should be evaluated with care. Specifically, untangling the movement of coated caveolae membrane-containing vesicles from packets of caveolae proteins without membrane is a challenge. The following section will summarize current caveolae trafficking concepts while highlighting the distinct steps of the process.

Caveolae Detachment From the Plasma Membrane

Caveolae are highly dynamic membrane domains, that are capable of moving laterally in the plasma membrane, similar to lipid rafts (Pelkmans and Zerial, 2005; Boucrot et al., 2011; Shvets et al., 2015). However, it was shown *in vitro* and *in vivo* that EHD2 localizes to caveolar necks. In particular, the observation of an increased number of static caveolae in cells overexpressing EHD2 led to the hypothesis that EHD2 stabilizes caveolae at the plasma membrane (Morén et al., 2012; Stoeber et al., 2012; Ludwig et al., 2013; Matthaeus et al., 2020). Besides EHD2, Pacsin2 is also involved in caveolae plasma

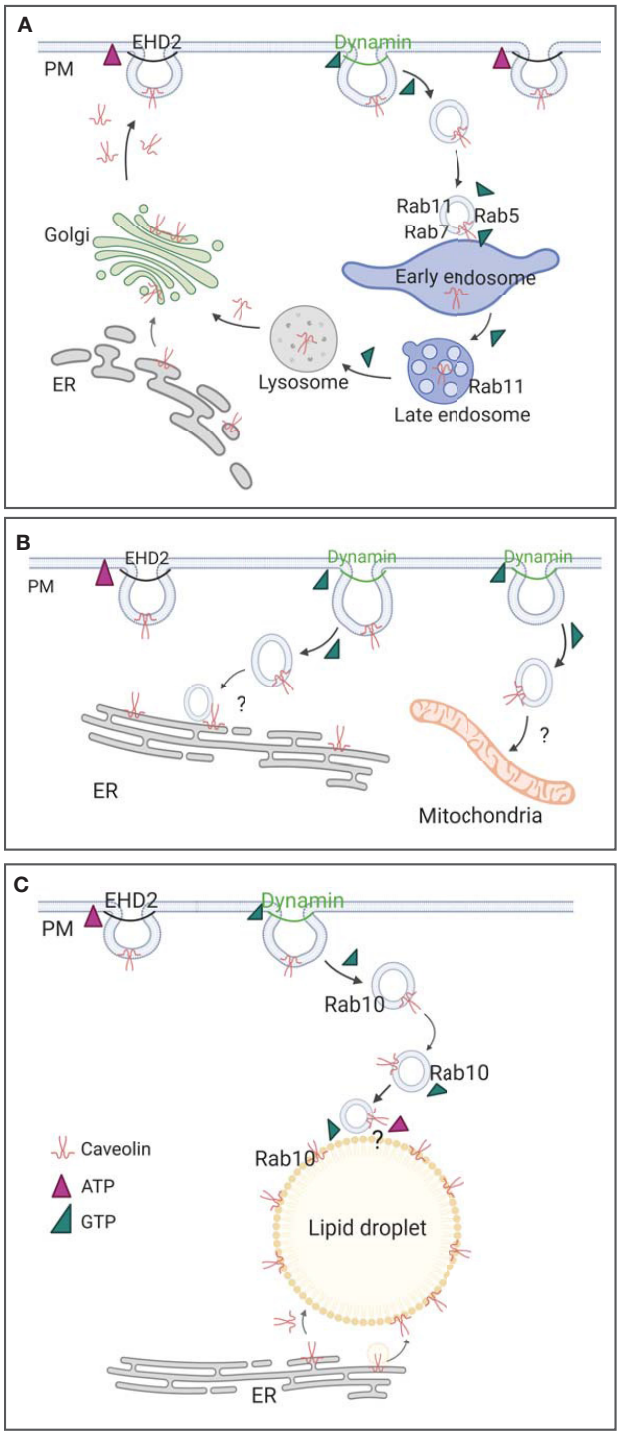

FIGURE 2 | Caveolae endocytosis and trafficking. **(A)** Classic caveolae endocytosis from the plasma membrane to early and late endosomes, followed to lysosomes, occurs in various cell types. Rab5, 7, and 11 are assigned to caveolae trafficking, ATP (pink triangle) and GTP (green triangle) dependent processes are illustrated. **(B)** Caveolae trafficking to endoplasmic reticulum (ER) and mitochondria is suggested based on initial proteomics data and detailed EM images. **(C)** Caveolae trafficking from the plasma membrane to lipid droplets can be observed in adipocytes and fibroblasts. PM, plasma membrane; Dyn, Dynamin; ER, endoplasmic reticulum; question mark indicates unknown processes and proteins.

membrane stabilization (Senju et al., 2011, 2015). Deletion of either EHD2 or Pacsin2 results in increased caveolae mobility and internalization. Most likely, EHD2 removal from caveolar neck promotes caveolae detachment from the plasma membrane followed by intracellular trafficking. How exactly detachment occurs is currently unknown. Dynamin has been proposed to be important for this process (Henley et al., 1998; Oh et al., 1998, 2012). Yet, clear evidence that dynamin acts alone or in combination with other proteins, such as intersectin (Predescu et al., 2003, 2012), is lacking. It is also possible that the fission of caveolae from the plasma membrane is driven by other proteins. Indeed, this is common for dynamin independent endocytosis (e.g., CLICs, Mayor et al., 2014; Sathe et al., 2018) and ESCRT III complex protein driven invaginations (Hanson et al., 2008; Rossman and Lamb, 2013).

To study caveolae scission and trafficking, one needs to differentiate vesicles from membrane bound caveolae. In the past this has been difficult for various endocytic pathways due to their small size and fast dynamics. In fixed cells, however, caveolar vesicles can be distinguished by EM or electron tomography as vesicles containing an enclosed lipid bilayer (Popescu et al., 2006; Hubert et al., 2020b; Matthaeus et al., 2020; Webb et al., 2020). Novel super resolution imaging techniques such as Stochastic Optical Reconstruction microscopy (STORM) and Stimulated Emission Depletion (STED) microscopy with resolution limits up to 40 nm are also able to identify caveolar vesicles (Platonova et al., 2015; Tachikawa et al., 2017; Yeow et al., 2017; Khater et al., 2018; Matthaeus et al., 2019). Of special interest with regard to caveolar vesicle trafficking is the application of STED and structured-illumination microscopy (SIM) to live cells allowing single caveolae to be tracked throughout the cell. Currently, caveolae trafficking is mainly studied with total internal reflection fluorescence (TIRF) microscopy. However, TIRF is diffraction limited in the plane of the cover glass and in the axial plane limited to signals within ∼200 nm of the plasma membrane. Therefore, deeper caveolae events cannot be seen. In summary, rapidly developing imaging techniques will help to further elucidate the exact caveolar detachment process at the plasma membrane and allow single organelles to be monitored as they move through the cytosol. These studies will finally reveal how caveolae move, where they go, and what pathways they participate in within living cells and tissues.

Intracellular Caveolae Trafficking

After detachment from the plasma membrane, caveolae can be internalized and traffic to intracellular organelles. Which signaling events, cargos, or ligands induce caveolae internalization are, however, unclear and has led to some controversy in the field. Thus, the exact role for caveolae traffic has been difficult to generalize. This is in contrast to clathrin mediated endocytosis where defined cargos and trafficking routes are well-established and mainly accepted. Recent data has suggested that high levels of extracellular cholesterol and glycosphingolipids are able to stimulate caveolar dynamics (Hubert et al., 2020b). Also, albumin (Minshall et al., 2000; Botos et al., 2008), okadaic acid and glycosphingolipids (Parton et al., 1994; Shvets et al., 2015), cholesterol and long-chain fatty acids

(Le Lay et al., 2006; Hao et al., 2020), simian virus 40 (Tagawa et al., 2005), and endothelin (Oh et al., 2012) are thought to be potential detachment and internalization triggers in some cell types. Taken together, various tissue and cell specific signaling events or ligands may trigger caveolae internalization. The resulting intracellular caveolae trafficking routes include the conventional endocytic pathway, as well as caveolae migration to the endoplasmic reticulum and lipid droplets (see overview in **Figure 2**, and detailed description below). Likewise, studies have shown that caveolae detachment and return of caveolae vesicles to the plasma membrane can occur (Pelkmans and Zerial, 2005; Hubert et al., 2020a).

Endocytic Caveolae Pathway

When caveolae bud off from the plasma membrane they can fuse with the early endosome. This is followed by maturation of these organelles into late endosomes, multivesicular bodies, and finally degradation of their contents within lysosomes (Hayer et al., 2010b; Shvets et al., 2015). Previous studies showed that Cav1 co-localizes with early and late endosomal markers including Rab5 and Rab7, followed by accumulation into the lysosomes (Pelkmans et al., 2004; Botos et al., 2008; Hayer et al., 2010b; Shvets et al., 2015). Notably, Shvets et al. (2015) determined that caveolae mediated endocytosis in 3T3 fibroblasts comprises a minor fraction of total cellular endocytosis (ca. 5–10% of total endocytic vesicles). By using Cav1 immunogold labeling, Botos et al. (2008) observed an accumulation of Cav1 in multivesicular bodies after detachment from the plasma membrane. Furthermore, polarized epithelial cells contain Cav1 endocytosis and co-localization with the specific apical recycling marker Rab11a (Lapierre et al., 2012). In line with these observations, several proteomics and biochemistry studies showed an enrichment of small Rab GTPases, SNAP molecules, and the vesicle SNARE protein VAMP2 in isolated caveolae fractions (Schnitzer et al., 1995; Aboulaich et al., 2004; McMahon et al., 2006; Wypijewski et al., 2015). These are necessary for a functional membrane fusion machinery needed for the endocytic pathway. In summary, these data illustrate that caveolae are endocytosed. Most likely, viruses such as the simian virus 40 use this caveolar endocytic path way to enter and infect cells (Pelkmans et al., 2001; Tagawa et al., 2005). Furthermore, receptor internalization may be regulated by this pathway, e.g., TGF-beta type 1 (He et al., 2015) or insulin receptor (Fagerholm et al., 2009), although distinct caveolar specific receptors have not been observed.

Caveolae Trafficking to Endoplasmic Reticulum and Mitochondria

Recent advances in imaging and proteomics have uncovered novel caveolae trafficking routes outside the classic endocytic pathway. First, based on the observation of caveolae dependent cholera toxin and autocrine motility factor accumulation in the endoplasmic reticulum (ER) and Golgi, it was suggested that caveolae are able to migrate from the cell surface to the ER (Pelkmans et al., 2001, 2004; Le and Nabi, 2003). Additionally, proteomics of isolated caveolar membrane domains showed an increased amount of ER related proteins (McMahon et al., 2006). Recently, it was shown that Cav1 impairs the formation of ER-mitochondria contact sites and is involved in Drp1 mediated mitochondria fusion (Bravo-Sagua et al., 2019). However, clear evidence of Cav1 and/or caveolae originating from the plasma membrane under these circumstances is lacking. Indeed, the work of Bravo-Sagua et al. (2019) indicates a specific ER related function of Cav1 independent of caveolae endocytosis.

Caveolae have been proposed to form specific ER membrane contact sites. A detailed high resolution EM analysis of rat smooth muscle cells indicated that the majority of caveolae (either located at the plasma membrane or detached) are close to the sarcoplasmic reticulum (SR) (Popescu et al., 2006). The authors further detected electron densities in the caveolae membrane reaching to the corresponding SR membrane and containing potential tethers that establish membrane contact sites. The same observation was found for mitochondria, although caveolae-mitochondria contact sites are less abundant (Popescu et al., 2006). Currently it is not known if caveolae migrate from the plasma membrane to mitochondria. However, previous studies showed that Cav1 is also found in mitochondria (Li et al., 2001; Fridolfsson et al., 2012; Foster et al., 2020).

Caveolae Trafficking to Lipid Droplets

Caveolae trafficking to lipid droplets is of particular interest for lipid homeostasis and metabolism. Initially, Cav1 and independently Cav2, were found at lipid droplets. It was suggested that Cav1 or Cav2 originated from the ER and migrates to lipid droplets because pharmacological inhibition of ER vesicle transport was seen to block Cav translocation (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001, 2004). By overexpressing a Cav1 mutant leading to ER accumulation, Cav1 re-locates to the lipid droplet coat, most likely during lipid droplet formation (Ostermeyer et al., 2004; Pol et al., 2004). The lipid droplet coat consists, in contrast to other membrane-bound organelles, of a phospholipid monolayer, resulting in a unique set of proteins targeted to this area (Walther and Farese, 2009; Kory and Walther, 2016). The recruitment and localization of proteins directly from the cytosol to the lipid droplet coat requires amphipathic helices to ensure correct localization (Kory and Walther, 2016). Caveolins contain amphipathic lipid binding domains and therefore are likely able to bind to lipid droplets (Ariotti et al., 2015; Root et al., 2019).

Cav1 trafficking from the plasma membrane to lipid droplets was first described in 3T3-L1 adipocytes using immunostaining, EM, and biochemistry (Le Lay et al., 2006; Blouin et al., 2010). Blouin et al. (2010) further detected Cavin1, EHD2 and semi-carbazide-sensitive amine oxidase (SSAO, localizes within the adipocyte caveolae domain, Souto et al., 2003) at the lipid droplet coat indicating that not Cav1 alone but caveolae are recruited to lipid droplets. Interestingly, Cav1 (most likely Cav2 as well) is recruited to a specific lipid droplet subpopulation (Storey et al., 2011). The isolation and purification of lipid droplets revealed a Cav1 positive lipid droplet fraction that is also enriched in Perilipin1, a protein protecting stored lipids within the droplets against lipolysis (Sztalryd and Brasaemle, 2017). In contrast, Cav1 negative droplets showed an accumulation of ADRP (adipocyte differentiation-related protein, Storey et al., 2011). Furthermore,

Cav1 and Perilipin1 are able to form a complex, indicating that Cav1 is involved in the regulation of lipolysis of the lipids stored in the lipid droplets (Cohen et al., 2004b; Storey et al., 2011).

These data suggest that there is direct trafficking from the plasma membrane. However, how this is carried out and if Cav1 alone or caveolae vesicles are transferred is currently not understood in mechanistic detail. Recent data from Matthaeus et al. (2020) further supported this idea as increased caveolae mobility and endocytosis resulted in increased lipid droplet size and increased fatty acid uptake. Additionally, lipid accumulation within caveolar membrane domains increased caveolae detachment (Shvets et al., 2015; Hubert et al., 2020b) indicating the importance of this process. Based on these data, we propose the following model. Caveolae may serve as lipid sensors that accumulate specific lipids such as cholesterol, sphingolipids, and fatty acids. By reaching a critical amount of lipids, the stability of membrane-attached caveolae decreases and caveolae detach. This sensing is followed by caveolae traffic to lipid droplets. However, it is currently unclear how caveolae sense the accumulation of lipids within their membrane domains, how EHD2 disassembles, and how this scission is regulated. It is also unclear if caveolae migrate as vesicles to the lipid droplet coat and form membrane contact sites (such as shown for other organelles, reviewed by Olzmann and Carvalho, 2019; Thiam and Dugail, 2019; Henne, 2020), or if Cav1 alone is able to reach them.

Taken together, there is much data illustrating caveolae internalization from the plasma membrane followed by intracellular trafficking. Currently, the conventional pathway is the best studied. Yet, proteomic approaches suggest caveolae target to other organelles and these must be evaluated in detail. Super resolution imaging techniques can help to track the global movements of caveolae throughout cells. Besides the different intracellular caveolae trafficking pathways, the initial steps, ATP dependent EHD2 stabilization at the plasma membrane, and the GTP dependent dynamin-based scission of the caveolar bulbs, are all essential for caveolae internalization. Next, we discuss the function and importance of the energy-dependent enzymes including EHD2 and dynamin in these specific processes.

ATP DEPENDENT EHD2 OLIGOMERIZATION AT THE CAVEOLAR NECK

How is EHD2 oligomerization and its effect on caveolae plasma membrane stabilization and detachment regulated? Recent structural, cellular, and physiological data indicated that the ATP-dependent oligomerization of EHD2 is an important regulator for caveolae traffic. Eps15 homologous domain containing protein 2 (EHD2) and its related EHD proteins belong to the dynamin protein family as they share the same overall domain organization (Daumke et al., 2007). In mammals, four EHD orthologs are found (EHD1-4) that show distinct tissue-specific expression, localization, and functions (Pohl et al., 2000; George et al., 2007). EHD1, 3 and 4 are observed at early and late endosomes and EHD2 is located primarily at the caveolar neck (reviewed in Naslavsky and Caplan, 2011; Bhattacharyya and Pucadyil, 2020). All EHD proteins are able to bind to and bend phospholipid membranes (Daumke et al., 2007; Melo et al., 2017; Deo et al., 2018).

Structurally, EHD proteins share sequence similarity of up to 82% (Pohl et al., 2000) supporting the idea that EHDs could share a common function and lipid binding mechanism. EHD1-4 contain a stalk or helical region that is involved in membrane binding, a G-domain comprising the ATPase and oligomerization domain, and the specific EH domain, a Eps15 homologous protein sequence (**Figure 3A**, Daumke et al., 2007; Shah et al., 2014). EHD proteins are dimers which can be activated after membrane recruitment followed by ATP binding (Daumke et al., 2007; Hoernke et al., 2017; Melo et al., 2017). Mechanistically, the opening of the EHD dimer by repositioning of the EH domains results in the rearrangement of the stalk, freeing it to bind to the lipid bilayer. Detailed studies using EHD2 mutants in liposome binding assays accompanied with *in vivo* analysis showed that residues F322 and K327 are essential for correct membrane binding (Stoeber et al., 2012; Shah et al., 2014). ATP binding induces the oligomerization of EHD proteins resulting in liposome tubes that are decorated with EHD ring-like oligomers (Daumke et al., 2007; Melo et al., 2017). The diameter of these EHD tubes ranges between 20 and 80 nm indicating that EHD2 could form a ring surrounding the caveolar bud neck (**Figure 3C**). Ludwig et al. (2013) clearly localized EHD2 by immunogold labeling in EM section to the caveolar neck (Morén et al., 2012; Ludwig et al., 2013). In support of this, high resolution EM images show a distinct ring-like electron density at the caveolar neck (Popescu et al., 2006; Richter et al., 2008). A correlative imaging approach would be important to show that this density is indeed EHD2.

Upon ATP hydrolysis, the EHD oligomer disassembles and relocates from the caveolae membrane to the cytosol. Importantly, the ATP-dependent oligomerization step of EHD2 is key to stabilizing EHD2 at caveolae. Specifically, EHD2 mutants without the ATPase domain fail to oligomerize, and therefore fail to stabilize caveolae at the plasma membrane (Morén et al., 2012; Stoeber et al., 2012; Matthaeus et al., 2020). Furthermore, it was shown that in 3T3 fibroblasts EHD2 loss can be rescued by other EHD proteins (Yeow et al., 2017). However, a global EHD2 knockout mouse did not exhibit the same observations in adipocytes, fibroblasts, or endothelial cells (Matthaeus et al., 2019, 2020).

This raises the important question, why is EHD2, and in particular the ATP-dependent oligomerization of EHD2, essential for correct caveolae function and behavior? Several cellular studies have shown an increased caveolae mobility, endocytosis, and removal from the plasma membrane when the EHD2 gene is deleted or its ATP function impaired (Morén et al., 2012; Stoeber et al., 2012; Hoernke et al., 2017; Yeow et al., 2017). Endocytosis of transferrin receptor was not impaired in EHD2 knockout or EHD2 overexpressing cells (Pekar et al., 2012), highlighting the specific role of EHD2 in regulating caveolae dependent endocytosis. Additionally, overexpression of EHD2 mutants lacking their lipid binding or ATPase function also resulted in increased caveolae dynamics as a result of decreased

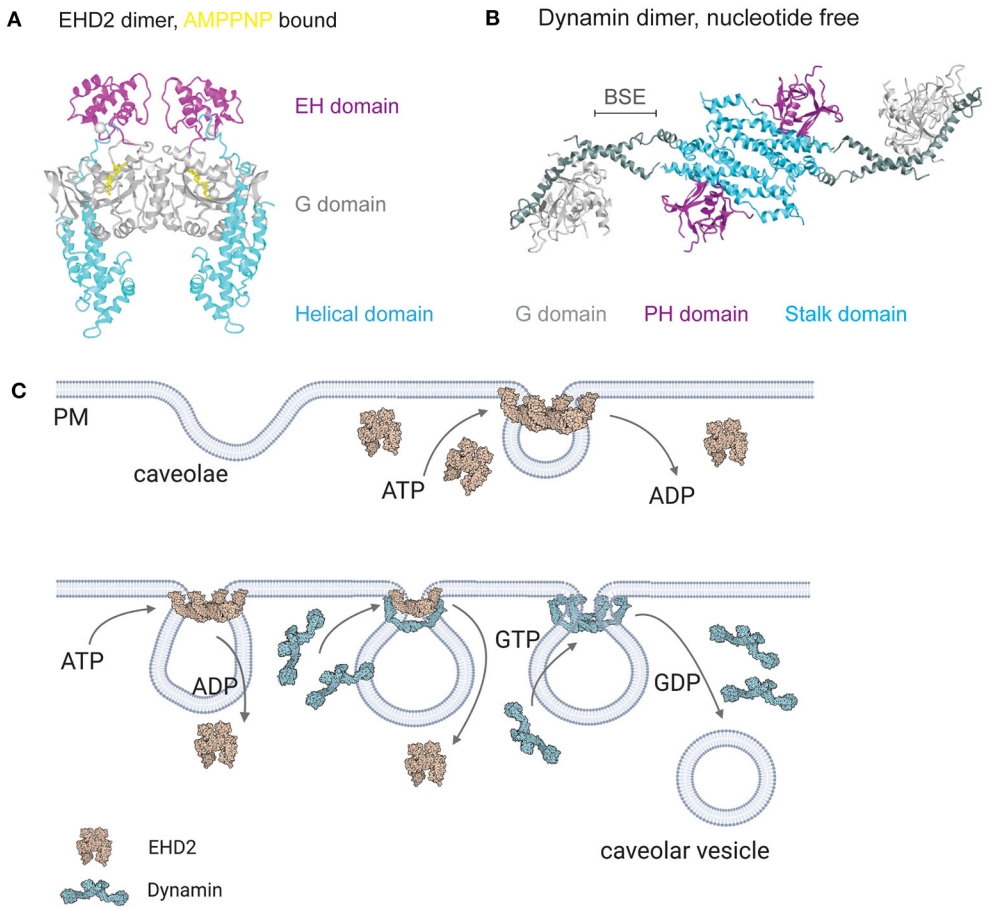

FIGURE 3 | EHD2 and dynamin at the caveolar neck. **(A)** Crystal structure of EHD2 dimer bound with non-hydrolyzing AMPPNP (PDB 2QPT, Daumke et al., 2007). **(B)** Crystal structure of nucleotide free dynamin dimer (PDB 3SNH, Faelber et al., 2011). **(C)** ATP and GTP dependent caveolae stabilization and detachment. After caveolae formation, EHD2 oligomerizes in an ATP dependent manner in large rings at the neck of caveolae. Disassembly of EHD2 oligomers occurs after ATP hydrolyzation, followed by dynamin recruitment to the caveolar neck and GTP dependent membrane scission. Membrane binding of EHD2 and dynamin results in confirmational change in both proteins (membrane bound EHD2 PDB 5MTV, Melo et al., 2017; membrane bound, constricted dynamin PDB 6DLU, Kong et al., 2018).

caveolar membrane stabilization (Stoeber et al., 2012). In the last few years, two cell based studies showed an involvement of EHD2 in lipid accumulation (Li et al., 2016; Morén et al., 2019). Li et al. (2016) revealed an increased lipid droplet size in hepatocytes lacking EHD2. The authors proposed that EHD2 together with Rab10 and Ehbp1 is involved in lipolysis. Contrary, Moren et al. (2019) observed that EHD2 silencing in 3T3-L1 derived adipocytes reduced lipid droplet sizes. Additionally, Yeow et al. (2017) and Torrino et al. (2018) observed increased vulnerability to changes in membrane tension in EHD2 lacking fibroblasts or HeLa cells.

The generation of an EHD2 knockout mouse model helped to determine EHD2's caveolae function *in vivo*. We observed in mice that globally lacked EHD2 increased lipid droplet sizes in various tissue types although the total number of lipid droplets decreased (Matthaeus et al., 2020). Additionally, an increase in fatty acid uptake was detected in EHD2 knockout adipocytes and mouse embryonic fibroblasts. Detailed electron microscopy and tomography further supported the idea that EHD2 is essential for correct membrane stabilization of caveolae as EHD2-lacking tissues contained large numbers of detached caveolae (Matthaeus et al., 2019, 2020; Fan et al., 2020; Webb et al., 2020). Additionally, we observed an increased detachment of caveolae due to EHD2 removal resulting in reduced calcium entry and a resultant lack of activated eNOS and NO generation in endothelial cells. This lead to reduced blood vessel relaxation in EHD2 knockout mice and reduced running wheel endurance (Matthaeus et al., 2019). Taken together, these *in vivo* data clearly indicate that EHD2 oligomerization at the caveolar neck is an essential cell function with severe physiological consequences when EHD2 is missing or its ATPase function is impaired.

GTP DEPENDENT DYNAMIN FACILITATED MEMBRANE SCISSION OF CAVEOLAE

Caveolae detachment has been ascribed to dynamin catalyzed membrane scission. This raises the question of how dynamin drives fission of caveolae and how it is regulated. Dynamin is

a well-known lipid binding GTPase that bends membranes and catalyzes fission. Structurally, it contains the N-terminal GTPase domain (G domain), followed by the central bundle signaling element (BSE), the stalk region, and the pleckstrin homology (PH) domain at the C-terminus (**Figure 3B**, Faelber et al., 2011). The stalk domain mediates dimerization, larger oligomers further include binding between BSE. Dynamin, with its 3 orthologs in mammalians, is expressed in all cell types from early embryonic time points on and is essential in clathrin mediated endocytosis (Ferguson and De Camilli, 2012). In particular, Dyn1 is found in the brain in high levels, Dyn3 in muscle, testis, brain and lung, and Dyn2 is a ubiquitous isoform expressed in many cell types. In clathrin mediated endocytosis, dynamin regulates the scission of clathrin coated membrane pits from the plasma membrane after coat assembly and elongation of the neck. However, dynamin and its related proteins such as interferon-inducible myxovirus resistance (Mx), Optic atrophy type 1 (Opa1) or Dynamin-1 like protein (Dnm1l) are involved in various other cellular processes within different organelles such as mitochondria fusion (Ferguson and De Camilli, 2012; Daumke and Praefcke, 2016).

How does dynamin facilitate membrane scission at caveolae? The scission of a phospholipid membrane requires the transition of the chemical energy gained during GTP hydrolysis into mechanical constriction to merge the lipid bilayers of the vesicle neck (Daumke and Praefcke, 2016). It is proposed that the hydrolysis of a few GTP molecules can provide the necessary energy for scission (Morlot et al., 2012). Structurally, the stalk region of dynamin forms ring-like oligomers surrounding the membrane (Kong et al., 2018). Following GTP binding, the GTPase domains dimerize, and GTP hydrolysis occurs resulting in a conformational change within the dynamin oligomer. This "power-stroke" is thought to pull the adjacent dynamin filaments along each other and thereby constricting the underlying membrane (Antonny et al., 2016; Daumke and Praefcke, 2016). Indeed, several *in vivo* studies showed the GTP dependent dynamin oligomerization and membrane scission at clathrin coated pits (Takei et al., 1995; Iversen et al., 2003; Grassart et al., 2014). Based on these results, it was concluded that caveolae detachment from the plasma membrane is also driven by dynamin. First, Schnitzer et al. (1996) showed a GTP dependent caveolae scission in a cell free assay indicating the involvement of a GTP handling enzyme (Schnitzer et al., 1996). Independently, Oh et al. (1998) and Henley et al. (1998) then described the GTP dependent caveolae internalization is mediated by dynamin (Henley et al., 1998; Oh et al., 1998). Furthermore, the involvement of dynamin was also observed in caveolae dependent albumin transcytosis (Shajahan et al., 2004). Importantly, overexpression of the non-GTP hydrolyzing dynamin mutant (Dyn2-K44A) abolished caveolae mobility, detachment, and trafficking from the plasma membrane in several cell types (Pelkmans et al., 2001; Senju et al., 2011; Oh et al., 2012). Taken together, these data indicated the involvement of dynamin in caveolae scission and detachment.

Some previous studies struggled to clearly localize dynamin to caveolar invaginations in different cell types. Using EM immunogold labeling, however, Dyn2 localization in caveolae was shown in kidney cells (Yao et al., 2005), and Dyn2 overexpressing MEFs or endothelial cells also showed co-localization with Cav1 (Shajahan et al., 2004; Matthaeus et al., 2020). The general difficulty of localizing dynamin could be caused by the fact that dynamin might only assembles at the caveolar neck shortly before internalization, followed by a fast re-location into the cytosol. To overcome this issue, the accumulation of non-hydrolyzing dynamin mutants at the caveolar neck was used to visualize dynamin with STORM microscopy (Platonova et al., 2015). Yao et al. (2005) could further identify a specific binding interaction between Cav1 and Dyn2. The exact role of dynamin at caveola is, however, still unclear.

Importantly, oligomerization of dynamin at the plasma membrane can only occur when thin membrane tubes are present. As previously described, EHD2 oligomerizes in rings around lipid bilayers in diameters ranging from 20 to 80 nm. Therefore, at the caveolar neck, we propose that EHD2 is key to create the necessary membrane structure for correct dynamin assembly and subsequent fission of caveolar invaginations (see model in **Figure 3C**). It was proposed previously, that EHD proteins are able to recruit dynamin to membrane tubes (Jakobsson et al., 2011), indicating an interaction between these two enzymes. However, based on the steric hindrance at the caveolar neck due to the large EHD2 oligomer, we suggest that before dynamin locates to the caveolae, EHD2 must start to disassemble (after ATP hydrolyzation) and relocate to the cytosol. Then, dynamin would be recruited to caveolae, followed by its oligomerization around the caveolar neck. The GTP dependent power stroke of dynamin would result in membrane scission. Later, detached caveolae would migrate to other intracellular organelles and dynamin would relocate to the cytosol. Further experiments, especially live cell high resolution imaging, are needed to test the temporal sequence of these events. Taken together, recent data shows the importance of GTP-dependent dynamin regulation during caveolae detachment. However, how these spatial and temporal mechanisms operate at the caveolar neck is still unknown and requires further study. Cleary, the interplay of EHD2 and dynamin at the membrane neck of caveolae is of particular interest for future work.

OTHER REGULATORS FOR CAVEOLAE TRAFFICKING

Rab GTPases in Caveolae Internalization and Trafficking

Besides dynamin and EHD2, several other GTP/ATP dependent proteins were assigned roles in caveolae trafficking. Specifically, Rab proteins have been linked with caveolae endocytosis. Rab proteins are small Ras-like GTPases that switch between the "on," GTP bound—and the "off"—GDP bound—state and therefore temporally and spatially regulate the recruitment of various effector proteins such as vesicle coat proteins, membrane fusion complexes or motor proteins (see review Stenmark, 2009). The relatively high cytosolic GTP concentration (∼0.5 mM; Traut, 1994) allows a very fast exchange of GDP

with GTP securing the fast switching between the "off" and "on" state. It can be ventured that local changes in the GTP concentration might influence the activation of certain Rab molecules and their binding affinity to corresponding effector proteins. As Rab molecules are essential vesicle trafficking regulators it was expected to detect Rabs also at caveolar vesicles. Indeed, several studies focusing on caveolae endocytosis revealed that caveolae trafficking depends on Rab5, Rab7, and Rab11 (Pelkmans et al., 2004; Botos et al., 2008; Hayer et al., 2010b; Shvets et al., 2015). There, the Rab proteins direct the traffic of caveolae vesicles to early and late endosome and lysosome. However, to date, no caveolar specific Rab molecules were found which makes it challenging to study. Novel techniques analyzing protein-protein interaction such as SplitAPEX2 (Han et al., 2019) or fluorescence resonance energy transfer (FRET) could help to detect caveolae specific Rab molecules within distinct trafficking routes or organelles. Additionally, caveolae specific Rabs would help to determine the cellular implications for controlled caveolae trafficking at specific organelles.

Regulation of Caveolae Internalization by Tyrosine Kinases

The characteristic lipid environment of caveolar invaginations leads to a specific set of membrane proteins including ATP handling enzymes localized to the caveolar membrane. One example is the distribution of Na/K-ATPase within different plasma membrane domains. A non-pumping Na/K-ATPase subpopulation is found in caveolae (Wang et al., 2004; Liang et al., 2007). Further, a study revealed the loss of Cav1 and caveolae at the plasma membrane after knockdown of Na/K-ATPase (Cai et al., 2008). Additional binding studies between purified Na/K-ATPase and Cav1 as well as cross-linking experiments demonstrated a direct interaction although within a low molar stoichiometry (Yosef et al., 2016; Nie et al., 2020). Knockdown of Na/K-ATPase also resulted in increased Src levels at the plasma membrane (Cai et al., 2008). It was shown previously that the tyrosine kinase Src is able to bind Cav1 leading to phosphorylation of the Cav1 residue tyrosine 14. After phosphorylation, Cav1 disassembles from the plasma membrane and caveolae internalization occurs (Parton et al., 1994; Lee et al., 2001). Several studies identified Cav1 as a substrate for Src which can be activated by various ligands and molecules, e.g., insulin or okadaic acid (Kiss and Botos, 2009), indicating that the phosphorylation of Cav1 plays an important role in caveolae internalization.

Besides Src, the tyrosine kinase Abl is also able to phosphorylate Cav1 tyrosine 14 in response to oxidative or tension stress (Sanguinetti and Corley Mastick, 2003). Interestingly, Abl is involved in the crosstalk between caveolae and stress fibers. Thereby, Abl together with the stress fiber regulator mDia1 (formin homology protein) and the F-BAR protein FBP17 regulates the formation and stabilization of caveolae at the plasma membrane (Echarri et al., 2012, 2019). Replica EM showing the cytosolic side of the plasma membrane (such as in **Figure 1**) illustrate that caveolae regularly locate in close proximity to actin filaments. Indeed, Filamin A was found to connect Cav1 with actin (Stahlhut and Van Deurs, 2000). Several studies showed that actin filaments are important for Cav1 and caveolae internalization, and that upon disruption of actin polymerization intracellular Cav1 trafficking is impaired (see detailed review, Echarri and Del Pozo, 2015). Additional, Pacsin2 and EHBP1 contain actin binding domains, and thereby both proteins are able to closely connect actin to caveolar membrane invaginations (Guilherme et al., 2004; Kostan et al., 2014). Of note, both proteins locate at the neck of caveolae suggesting that their actin binding motif might be an important regulator for caveolae mobility.

CONCLUSION AND OUTLOOK

Caveolae internalization is found in many cells and tissues. Here, we summarized current concepts in caveolae trafficking and its role in physiology. As discussed above, essential steps during the internalization and migration are dependent on ATP or GTP (see also summary **Figure 2**). In the past, the small size of caveolae made it difficult to monitor their intracellular movements. Therefore, the application of recently developed high resolution live imaging methods will enable the full elucidation of intracellular caveolae trafficking throughout the cell. Novel imaging techniques such as super resolution light imaging and its combination with electron microscopy and tomography will allow for future detailed investigations of the caveolae life cycle and trafficking routes (Taraska, 2019). Also focus-ion beam scanning electron microscopy (FIB-SEM) could help to further dissect intracellular caveolae movement as this technique allows to visualize complete cell volumes in the highest resolution. A correlative approach to identify Caveolin and Cavin positive membranes would help to identify caveolar vesicles in the FIB-SEM stacks.

Of particular interest is the development or identification of caveolae specific cargos and receptors that are only internalized via caveolar endocytosis. This would allow caveolae-specific intracellular routes to be monitored and further identify novel factors involved in these pathways. New techniques for identifying the protein-protein interactions will provide additional insights in caveolae contact sites between various organelles. Finding these caveolae contact sites will help to determine how caveolae are involved in lipid metabolism and diseases. Another largely unexplored aspect in caveolae dynamics is the influence of local ATP/GTP concentrations. These changes can occur due to hypoxia. Indeed, cells growing under low oxygen levels show altered caveolae behavior. In adipocytes, it was observed that loss of oxygen results in decreased Cav1 expression and consequently reduced caveolae number (Regazzetti et al., 2015; Varela-Guruceaga et al., 2018). However, hypoxia in cancer cells and in the colon of the mouse intestine led to increased Cav1 expression (Wang et al.,

2012; Xie et al., 2014; Bourseau-Guilmain et al., 2016). The oxygen sensitive transcription factor hypoxia-inducible factor (HIF1) was assigned to cause the changes in Cav1 gene expression (Wang et al., 2012; Xie et al., 2014; Bourseau-Guilmain et al., 2016; Varela-Guruceaga et al., 2018). This raises the possibility that caveolae could be sensitive to local ATP/GTP levels due to oxygen and nutrient deficiencies. The impact of low ATP levels on caveolae in cancer cells are of particular interest as caveolae are proposed to be involved in cancer progression. Taken together, energy requirements in caveolae trafficking are an important regulator for cellular metabolism and physiology. The extent of these mechanisms are not yet fully understood. Future work aimed at unraveling these questions will lead to a deeper understanding of the role these small plasma membrane organelles play in both health and disease.

AUTHOR CONTRIBUTIONS

CM wrote the manuscript and prepared figures. JT commented on and edited the manuscript.

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BRIEF RESEARCH REPORT
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The Small GTPase Arf6 Functions as a Membrane Tether in a Chemically-Defined Reconstitution System

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Arf-family small GTPases are essential protein components for membrane trafficking in all eukaryotic endomembrane systems, particularly during the formation of membrane-bound, coat protein complex-coated transport carriers. In addition to their roles in the transport carrier formation, a number of Arf-family GTPases have been reported to physically associate with coiled-coil tethering proteins and multisubunit tethering complexes, which are responsible for membrane tethering, a process of the initial contact between transport carriers and their target subcellular compartments. Nevertheless, whether and how indeed Arf GTPases are involved in the tethering process remain unclear. Here, using a chemically-defined reconstitution approach with purified proteins of two representative Arf isoforms in humans (Arf1, Arf6) and synthetic liposomes for model membranes, we discovered that Arf6 can function as a bona fide membrane tether, directly and physically linking two distinct lipid bilayers even in the absence of any other tethering factors, whereas Arf1 retained little potency to trigger membrane tethering under the current experimental conditions. Arf6-mediated membrane tethering reactions require *trans*-assembly of membrane-anchored Arf6 proteins and can be reversibly controlled by the membrane attachment and detachment cycle of Arf6. The intrinsic membrane tethering activity of Arf6 was further found to be significantly inhibited by the presence of membrane-anchored Arf1, suggesting that the tethering-competent Arf6-Arf6 assembly in *trans* can be prevented by the heterotypic Arf1-Arf6 association in a *cis* configuration. Taken together, these findings lead us to postulate that self-assemblies of Arf-family small GTPases on lipid bilayers contribute to driving and regulating the tethering events of intracellular membrane trafficking.

Keywords: Arf GTPase, Arf6, small GTPase, membrane tethering, membrane trafficking, reconstitution, liposome

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INTRODUCTION

Small GTPases of the Arf (ADP-ribosylation factor) family, belonging to the Ras superfamily, are known to be essential protein components for intracellular membrane trafficking in all eukaryotic endomembrane systems and, in general, to function through cooperation with their specific interactors, termed Arf effectors (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011; Jackson and Bouvet, 2014; Sztul et al., 2019). In particular, a large body of prior studies on Arf-family small GTPases have established their pivotal functions during the formation of

membrane-bound, coat protein complex-coated transport carriers (e.g., secretory and endocytic transport vesicles) at the donor membranes of subcellular compartments (D'Souza-Schorey and Chavrier, 2006; Sztul et al., 2019): Arf1 recruits the COPI (coat protein complex I) subunits for initiating vesicle formation in the retrograde Golgi-to-endoplasmic reticulum (ER) pathway (Spang et al., 1998; Bremser et al., 1999); Arf6 binds to the AP-2 adaptor complex in the clathrin-mediated endocytic pathway (Paleotti et al., 2005); and Sar1p assembles with Sec23/24p and Sec13/31p to form the COPII (coat protein complex II) coat and thereby promote cargo sorting and vesicle budding in the anterograde ER-Golgi trafficking pathway (Matsuoka et al., 1998; Sato and Nakano, 2004, 2005). In addition to their roles in the transport carrier formation above, it should also be noted that a number of Arf-family small GTPases, including Arl (Arf-like) GTPases, have been reported to physically interact with various long coiled-coil tethering proteins and multisubunit tethering complexes as a non-coat Arf effector (Donaldson and Jackson, 2011; Sztul et al., 2019), which include golgin GMAP-210 for Arf1 (Drin et al., 2008), the exocyst complex for Arf6 (Prigent et al., 2003), Golgin-97 and Golgin-245 for Arl1 (Lu and Hong, 2003), the GARP complex for Arl5 (Rosa-Ferreira et al., 2015), and the HOPS complex for Arl8 (Khatter et al., 2015). All of these tethering proteins or tethering complexes are thought to be directly involved in membrane tethering, vesicle tethering, or vesicle capture, a process of the initial physical contact of membrane-bound transport carriers with their target subcellular membrane compartments (Waters and Pfeffer, 1999; Yu and Hughson, 2010; Gillingham and Munro, 2019). This tethering process is vital for determining the spatiotemporal specificity of membrane trafficking (Waters and Pfeffer, 1999; Yu and Hughson, 2010; Gillingham and Munro, 2019), before the following SNARE-mediated membrane docking and fusion steps, another critical layers for conferring the fidelity of membrane trafficking (McNew et al., 2000; Parlati et al., 2002; Furukawa and Mima, 2014). Nevertheless, Arf-family small GTPases have not been recognized as the so-called tethering factors (Waters and Pfeffer, 1999; Yu and Hughson, 2010; Gillingham and Munro, 2019), and thus, whether and how indeed Arf-family proteins contribute to the tethering process remain unclear. In this study, to thoroughly explore the molecular functions of Arf-family proteins in membrane tethering, we have employed reconstituted membrane tethering assays with Arf-anchored proteoliposomes, which were prepared from purified human Arf1 and Arf6 proteins and synthetic liposomes bearing defined lipid species. Using the chemically-defined reconstitution approach, here we report that Arf6, but not Arf1, can directly and physically tether two distinct lipid bilayers in the absence of any other tethering factors or Arf effectors.

MATERIALS AND METHODS

Protein Expression and Purification

Bacterial expression vectors for human Arf1 (UniProtKB: P84077) and Arf6 (UniProtKB: P62330) were constructed using a pET-41 Ek/LIC vector kit (Novagen), as described for protein expression of human Rab-family small GTPases (Tamura and Mima, 2014; Inoshita and Mima, 2017; Segawa et al., 2019; Ueda et al., 2020). DNA fragments encoding these two Arf-family proteins with the additional sequences for a human rhinovirus (HRV) 3C protease-cleavage site and a His12-tag at the N-terminus were amplified by PCR using KOD-Plus-Neo polymerase (Toyobo) and Human Universal QUICK-Clone cDNA II (Clontech) as a template cDNA and then cloned into a pET-41 Ek/LIC vector (Novagen). The GST-His6-tagged forms of His12-Arf1 and His12-Arf6 proteins were expressed in *Escherichia coli* BL(DE3) (Novagen) cells harboring the pET-41-based expression vectors constructed. After inducing expression in the presence of IPTG (final 0.3 mM) at 25°C for 4 h, cells were harvested by centrifugation, resuspended in RB150 (20 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 10% glycerol) containing 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin A, freeze-thawed in liquid nitrogen and a water bath at 30°C, lysed by sonication, and ultracentrifuged with a 70 Ti rotor (Beckman Coulter; 50,000 rpm, 75 min, 4°C). The supernatants were mixed with COSMOGEL GST-Accept beads (Nacalai Tesque) and incubated with agitation (4°C, 5 h), followed by washing the protein-bound beads in RB150 with 5 mM $MgCl_2$ and 1 mM DTT. The washed beads were resuspended in the same buffer containing HRV 3C protease (8 units/ml in final; Novagen) and incubated without agitation (4°C, 16 h) to cleave off and elute His12-Arf proteins from the beads. His12-Arf1 and His12-Arf6 proteins eluted were harvested by centrifugation (15,300 × g, 10 min, 4°C). Human Rab5a-His12 was purified as described (Segawa et al., 2019; Ueda et al., 2020). Protein concentrations of purified His12-Arf1, His12-Arf6, and Rab5a-His12 were determined using Protein Assay CBB Solution (Nacalai Tesque) and BSA for a standard protein. Nucleotide loading of human Arf-family small GTPases was performed by incubating purified Arf proteins in the presence of GTP or GDP (1 mM), EDTA (2 mM), and $MgCl_2$ (1 mM), as described previously (Antonny et al., 1997; Drin et al., 2008).

GTPase Activity Assay

GTP hydrolysis activities of purified His12-Arf proteins were assayed by quantitating free phosphate molecules released in the hydrolytic reactions, using the Malachite Green-based reagent Biomol Green (Enzo Life Sciences) as described for the activity assays with Rab-family small GTPases (Inoshita and Mima, 2017; Segawa et al., 2019). Purified His12-Arf1 and His12-Arf6 proteins (2 μM final) were mixed with GTP or GTPγS (1 mM final) in RB150 containing 5 mM $MgCl_2$ and 1 mM DTT (100 μl each), incubated (30°C, 1 h), and then supplemented with the Malachite Green reagent (100 μl each). After incubation with the reagent (30°C, 30 min), the absorbance at 620 nm (A620) of the reactions (200 μl each) was measured with a DU720 spectrophotometer (Beckman Coulter). The raw A620 data were corrected by subtracting the A620 values of the control reactions without His12-Arf proteins. Concentrations of phosphate molecules released in the reactions were calculated using the corrected A620 data and the A620 values of phosphate standards (Enzo Life Sciences). Means and standard deviations of

the specific GTPase activities (μM phosphate/min/μM protein) were determined from three independent experiments.

Liposome Preparation

All of the non-fluorescent lipids for liposome preparation, including POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), POPE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine), liver PI (phosphatidylinositol), POPS (1-palmitoyl-2-oleoyl-phosphatidylserine), cholesterol, and DOGS-NTA (1,2-dioleoyl-sn-glycero-3-{[N-(5-amino-1-carboxypentyl) iminodiacetic acid]-succinyl}), were from Avanti Polar Lipids. The two fluorescence-labeled lipids, Rh-PE (rhodamine-PE) and FL-PE (fluorescein-PE), were from Molecular Probes. Lipid mixes used were prepared in chloroform with the lipid compositions of 41% (mol/mol) POPC, 17% POPE, 10% liver PI, 5% POPS, 20% cholesterol, 6% DOGS-NTA, and 1% Rh-PE or FL-PE, dried up by evaporating chloroform with a stream of nitrogen gas, and subsequently resuspended in RB150 containing 5 mM $MgCl_2$ and 1 mM DTT (final 8 mM lipids) by vortexing vigorously and incubating with agitation (37°C, 1 h). After freeze-thawing in liquid nitrogen and a water bath at 30°C, lipid suspensions were extruded 25 times through polycarbonate filters (pore diameters, 200 nm; Avanti Polar Lipids) in a mini-extruder (Avanti Polar Lipids) pre-heated at 40°C. The liposome solutions prepared were stored at 4°C and used within a week for liposome turbidity assays and fluorescence microscopy.

Liposome Turbidity Assay

The intrinsic membrane tethering activities of human Arf-family small GTPases were assayed by monitoring turbidity changes of liposome solutions in the presence of purified Arf proteins, as described for the turbidity assays with human Rab-family small GTPases (Tamura and Mima, 2014; Inoshita and Mima, 2017; Mima, 2018; Segawa et al., 2019; Ueda et al., 2020) and Atg8-family proteins (Taniguchi et al., 2020). Purified His12-Arf1, His12-Arf6, or Rab5a-His12 (0.5–3 μM final) and DOGS-NTA-bearing liposomes (200-nm diameter; final 1 mM total lipids), which had been separately pre-incubated (30°C, 10 min), were mixed in RB150 containing 5 mM $MgCl_2$ and 1 mM DTT (160 μl each), applied to a 10-mm path-length cell (105.201-QS, Hellma Analytics), and immediately subjected to monitoring the turbidity changes with measuring the optical density changes at 400 nm (ΔOD400) for 5 min with 10-s intervals in a DU720 spectrophotometer at room temperature. The ΔOD400 values obtained from the kinetic turbidity assays were further quantitatively analyzed by curve fitting using ImageJ2 (National Institutes of Health) and the logistic function formula, $y = a/[1 + b \times \exp(-c \times x)]$, in which y is the ΔOD400 value and x is the time (min), as described (Segawa et al., 2019; Taniguchi et al., 2020; Ueda et al., 2020). The maximum tethering capacities of Arf and Rab proteins were defined as the maximum ΔOD400 values of the fitted sigmoidal curves at $t = \infty$, and they can be calculated as "a" from the logistic formula. The initial tethering velocities were defined as the maximum slopes of the fitted curves, and they can be calculated as "$c \times a/4$" from the logistic formula. Means and standard deviations of the maximum capacities (ΔOD400) and the initial velocities (ΔOD400/min) were determined from three independent experiments. All of the kinetic turbidity data shown were obtained from one experiment and typical of those from three independent experiments.

Fluorescence Microscopy

Fluorescence microscopy assays of Arf-mediated membrane tethering were employed using a LUNA-FL fluorescence cell counter (Logos Biosystems) and LUNA cell-counting slides (L12001, Logos Biosystems), as described (Segawa et al., 2019; Taniguchi et al., 2020; Ueda et al., 2020). Purified His12-Arf1 or His12-Arf6 (1 or 2 μM final) and extruded liposomes bearing DOGS-NTA and fluorescence-labeled Rh-PE or FL-PE lipids (200-nm diameter; final 2 mM lipids) were preincubated separately (30°C, 10 min), mixed in RB150 containing 5 mM $MgCl_2$ and 1 mM DTT (80 μl each), and further incubated (30°C, 1 h). The liposome suspensions incubated with Arf proteins were applied to the cell-counting slides (15 μl/well) and subjected to microscopic assays using the LUNA-FL fluorescence cell counter to obtain the bright field images and rhodamine- and fluorescein-fluorescence images. Particle sizes of liposome clusters in the rhodamine-fluorescence images were analyzed using ImageJ2 with setting the lower intensity threshold level to 150, the upper intensity threshold level to 255, and the minimum particle size to 10 pixels^2, which corresponds to ∼10 μm^2.

RESULTS AND DISCUSSION

Among twenty-nine members of the Arf small GTPase family in humans, including five Arf GTPases, twenty Arl GTPases, and two Sar1 GTPases (Sztul et al., 2019), in the current reconstitution studies on membrane tethering, we selected the two representative Arf isoforms, Arf1 and Arf6, both of which have been well-known to play key roles in regulating the secretory and endocytic trafficking pathways (D'Souza-Schorey and Chavrier, 2006; Sztul et al., 2019) and interact with putative membrane tethers or tethering factors such as coiled-coil tethering proteins and multisubunit tethering complexes (Prigent et al., 2003; Drin et al., 2008; Donaldson and Jackson, 2011). These two Arf-family proteins (Arf1 and Arf6), which share over 65% sequence identity (**Figure 1A**), and most of the other Arf isoforms are small monomeric globular proteins (20-25 kDa) consisting of the amphipathic helices at the N-terminus (around 10 residues; the helix α1 in **Figure 1A**) and the following conserved Ras-superfamily GTPase domains (G-domains; 160–170 residues; **Figure 1B**), and typically, they are further post-translationally modified by a myristoyl group at the N-terminus, which functions as a lipid anchor required for their stable attachment on the membrane surface (Donaldson and Jackson, 2011; Sztul et al., 2019). To mimic the native membrane-bound state of myristoylated Arf proteins in the present chemically-defined reconstitution systems, we prepared recombinant Arf1 and Arf6 proteins as the N-terminal polyhistidine-tagged forms (His12-Arf proteins; **Figures 1B,C**) that can be efficiently and specifically bound to DOGS-NTA lipids incorporated into synthetic liposomal membranes used. Purified His12-Arf1 and His12-Arf6 proteins (**Figure 1C**) retained their intrinsic GTP-hydrolysis activities, specifically converting GTP to GDP and

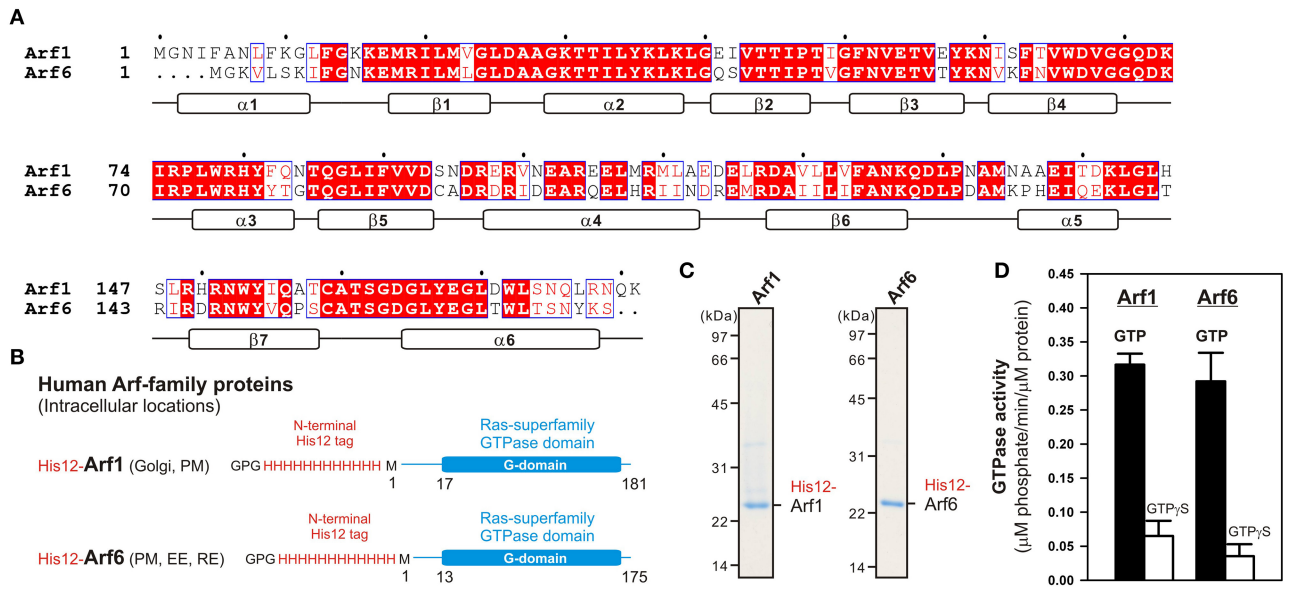

FIGURE 1 | Human Arf-family small GTPases used in the current reconstitution studies. **(A)** Sequence alignment of human Arf1 and Arf6 proteins. Amino acid sequences of these two Arf isoforms were obtained from UniProtKB (https://www.uniprot.org/), aligned using ClustalW (https://www.genome.jp/tools-bin/clustalw), and rendered by ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/). Identical and similar amino acid residues in the sequence alignment are highlighted in red boxes and in red characters, respectively. Secondary structures determined by the crystal structure of human Arf1 containing the residues 2–181 (PDB code, 1HUR), including six α-helices (α1–α6) and seven β-strands (β1–β7), are indicated at the bottom of the alignment. **(B)** Schematic representation of recombinant N-terminally His12-tagged human Arf1 (His12-Arf1) and Arf6 (His12-Arf6) proteins used in the current studies, which contain three extra residues (Gly-Pro-Gly) at the extreme N-terminus, an N-terminal His12-tag, and the Met1 – Lys181 residues for Arf1 or the Met1–Ser175 residues for Arf6. The intracellular locations of Arf1 and Arf6 indicated include the Golgi apparatus (Golgi), plasma membrane (PM), early endosome (EE), and recycling endosome (RE). **(C)** Coomassie blue-stained gels of purified His12-Arf1 and His12-Arf6 proteins, which were tested in the reconstituted liposome tethering assays in **Figures 2**–**4**. **(D)** GTP hydrolysis activities of purified recombinant His12-Arf1 and His12-Arf6 small GTPases. Purified Arf proteins (2 μM final) were incubated with 1 mM GTP or GTPγS (30°C, 1 h) in RB150 containing 5 mM $MgCl_2$ and 1 mM DTT and then assayed for the specific GTPase activities by measuring the free phosphate molecules released in the hydrolytic reactions.

a free phosphate (**Figure 1D**), which were comparable to those of Rab5a-His12 used as the control of a tethering-active small GTPase (**Figure 2**) and the other human Rab-family proteins tested in our prior studies on reconstituted membrane tethering (Inoshita and Mima, 2017; Segawa et al., 2019). Thus, these results confirmed that His12-Arf1 and His12-Arf6 in the current preparations were correctly folded and a functional protein with a native-like active G-domain.

Using purified His12-Arf1 and His12-Arf6 proteins (**Figure 1**) and extruded 200-nm protein-free liposomes bearing DOGS-NTA lipids and the complex lipid composition (**Figure 2A**), which roughly mimics the compositions of subcellular membrane compartments in mammalian cells (van Meer et al., 2008; Vance, 2015; Yang et al., 2018), we first examined whether these two Arf isoforms can exhibit their intrinsic potency to directly and physically tether two distinct lipid bilayers by employing kinetic liposome turbidity assays (**Figures 2B–D**), as established for Rab-mediated membrane tethering in our earlier studies (Tamura and Mima, 2014; Inoshita and Mima, 2017; Mima, 2018; Segawa et al., 2019; Ueda et al., 2020). Strikingly, the kinetic turbidity assays revealed that Arf6 triggered rapid and efficient tethering of liposomes when tested at the Arf protein concentrations ranging from 0.5 to 2 μM, which correspond to the Arf-to-lipid molar ratios (mol/mol) of 1:2,000 to 1:500 (red open circles and red solid lines, **Figure 2B**). It should be noted that these Arf-to-lipid molar ratios are similar to the protein-to-lipid ratios tested for evaluating the tethering activities of putative membrane tethers or tethering factors in prior reconstitution experiments, which include the typical ratios of 1:400 for golgin GMAP-210 (Drin et al., 2008), 1:330 for Vps21p (Lo et al., 2012), 1:2,000 for HOPS (Ho and Stroupe, 2015, 2016), 1:100–1:5,000 for Rab-family small GTPases (Segawa et al., 2019; Ueda et al., 2020), 1:800 for Atg8p (Nair et al., 2011), and 1:100–1:5,000 for LC3B and GATE-16 (Taniguchi et al., 2020). By contrast, the other Arf isoform, Arf1, exhibited little or no tethering activity under the current experimental conditions tested at 2 μM Arf proteins with the 200-nm liposomes (blue open circles, **Figure 2B**), consistent with our prior results showing no significant tethering activity of Arf1 with larger 400-nm liposomes (Segawa et al., 2019). To further evaluate the novel tethering activity of Arf6 found here, we also tested the non-Arf, Rab-family small GTPase, Rab5a, which has been recognized as the most tethering-active Rab isoform (Segawa et al., 2019), in the same kinetic turbidity assays and compared the tethering activities of Arf6 and Rab5a (**Figure 2B**). Although Rab5a retained its decent tethering activity for the 200-nm liposomes (black open circles, **Figure 2B**), Arf6 exhibited about 2.5-fold higher tethering capacity and over 10-fold higher initial

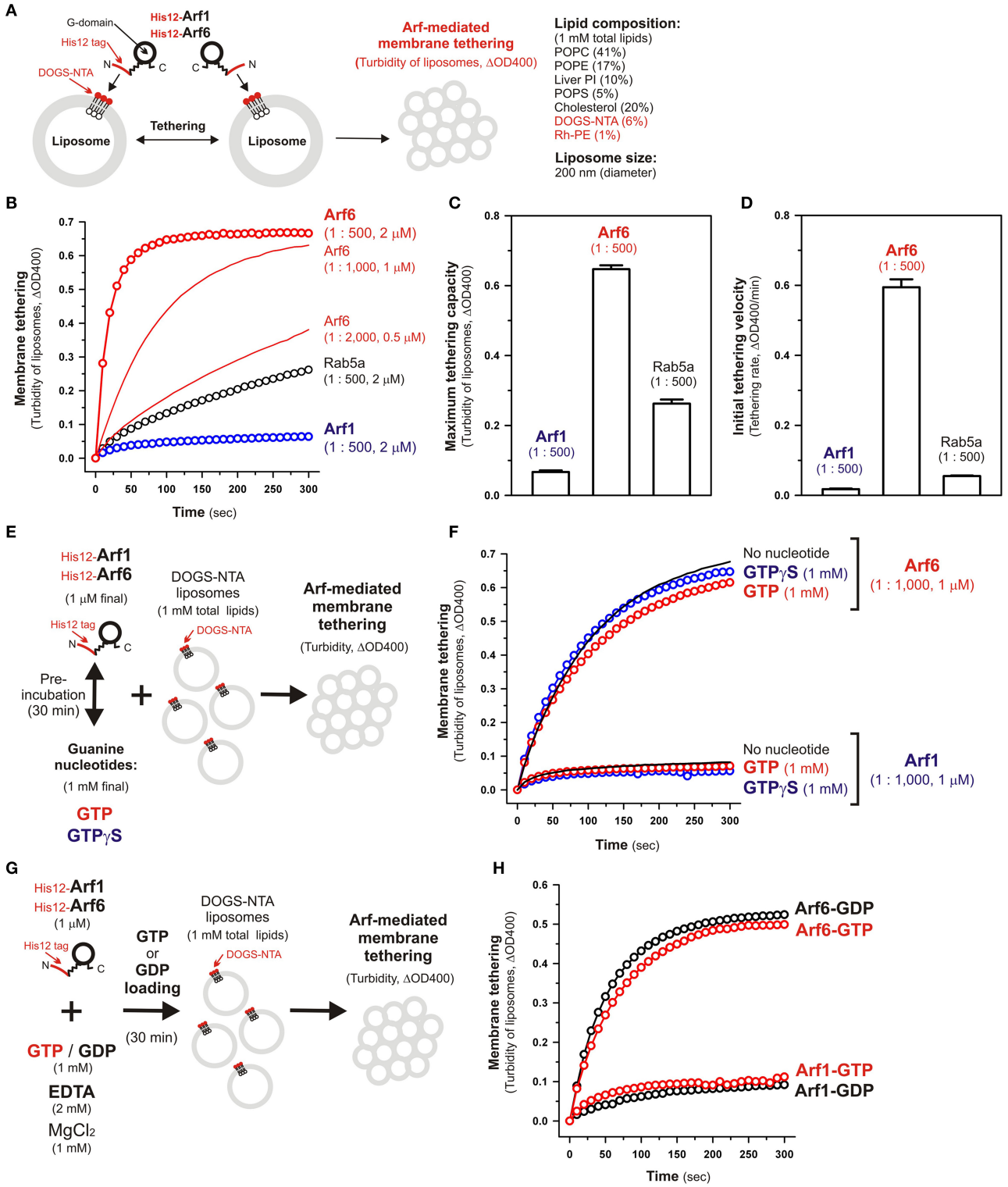

FIGURE 2 | Human Arf6, but not Arf1, can directly mediate membrane tethering in a chemically-defined reconstitution system. **(A)** Schematic representation of liposome turbidity assays for testing the intrinsic membrane tethering potency of human Arf-family proteins. **(B–D)** The intrinsic membrane tethering activities of human Arf1 and Arf6 proteins in kinetic liposome turbidity assays. Purified His12-Arf1, His12-Arf6, and Rab5a-His12 for the control of a tethering-competent small GTPase (0.5, 1, or 2 μM in final) were mixed with DOGS-NTA-bearing liposomes (200-nm diameter; 1 mM total lipids in final), immediately followed by assaying the turbidity changes by measuring the optical density changes at 400 nm (ΔOD400) for 5 min with 10-s intervals **(B)**. Maximum tethering capacities (ΔOD400) **(C)** and

(Continued)

FIGURE 2 | initial tethering velocities (ΔOD400/min) **(D)** of the turbidity reactions at 2 μM His12-Arf1, His12-Arf6, and Rab5a-His12 proteins were determined using a sigmoidal curve fitting method. **(E)** Schematic representation of liposomes turbidity assays with His12-Arf proteins in the presence of guanine nucleotides. **(F)** Addition of guanine nucleotides, GTP and GTPγS, has little or no effect on the intrinsic tethering activities of Arf1 and Arf6. After pre-incubating His12-Arf1 and His12-Arf6 proteins (1 μM final) with GTP or GTPγS (1 mM final) or without any guanine nucleotides (30°C, 30 min), kinetic liposome turbidity assays were employed as in **(B)**. **(G)** Schematic representation of liposomes turbidity assays with GTP-loaded or GDP-loaded Arf proteins. **(H)** Nucleotide-loaded state has little or no effect on the tethering activities of Arf1 and Arf6. After incubating His12-Arf1 and His12-Arf6 proteins (1 μM) with GTP or GDP (1 mM), EDTA (2 mM), and $MgCl_2$ (1 mM) at 30°C for 30 min, kinetic liposome turbidity assays for the nucleotide-loaded forms of Arf1 (Arf1-GTP, Arf1-GDP) and Arf6 (Arf6-GTP, Arf6-GDP) were employed as in **(B)**. The protein-to-lipid molar ratios used, ranging from 1:500 to 1:2,000 (mol/mol), are indicated **(B–D,F)**. Error bars, SD.

tethering velocity than those values of Rab5a (**Figures 2C,D**). These results established that Arf6 can function as a more potent membrane tether than the highly-active Rab-family tether, Rab5a, for the 200-nm liposomes at least.

Since Ras-superfamily small GTPases, including Arf and Rab GTPases, are generally thought to function in the active GTP-bound forms on membranes (Rojas et al., 2012), we next examined the intrinsic tethering activities of Arf1 and Arf6 in the presence of guanine nucleotides (**Figures 2E,F**). His12-Arf1 and His12-Arf6 were pre-incubated with GTP or the non-hydrolyzable analog GTPγS under the conditions similar to those used in the GTPase activity assays in **Figure 1D** (final 1 μM Arf proteins and 1 mM guanine nucleotides, 30°C, 30 min; **Figure 2E**) and then subjected to the kinetic turbidity assays as in **Figures 2B,F**. With respect to both of the Arf isoforms, Arf1 and Arf6, the presence of either GTP or GTPγS had little or no effect on their tethering capacities and initial tethering velocities in the turbidity assays (**Figure 2F**), establishing that Arf-mediated membrane tethering is achieved in a guanine nucleotide-independent manner. This consistent with our earlier experiments for reconstituted Rab-mediated tethering, in which the tethering activities of human Rab-family proteins were totally insensitive to the addition of guanine nucleotides (Tamura and Mima, 2014). In addition, nucleotide-independent Arf-mediated tethering was further demonstrated by reconstituted tethering assays with nucleotide-loaded Arf proteins, in which GTP-loaded Arf6 (Arf6-GTP) and Arf1 (Arf1-GTP) proteins had tethering capacities and kinetics almost identical to those of their GDP-loaded forms (**Figures 2G,H**). Considering that the Rab11a effectors, class V myosins, can specifically stimulate membrane tethering mediated by the cognate Rab11a in a GTP-dependent manner (Inoshita and Mima, 2017), perhaps specific Arf effectors can confer the nucleotide-dependence of Arf-driven tethering reactions.

Although the intrinsic tethering potency of human Arf small GTPases was not able to be controlled by the nucleotide-bound states in the current reconstitution systems using His12-tagged Arf proteins and DOGS-NTA-bearing liposomes (**Figures 2E–H**), it should be noted that, in living cells, native myristoylated Arf-family proteins are soluble and inactive in the GDP-bound state at the cytoplasm, but they can be functionally active in the GTP-bound state on subcellular membranes through inserting their N-terminal myristoyl lipid anchors and amphipathic helices to membrane surfaces (Antonny et al., 1997; Liu et al., 2009, 2010). Liposome co-sedimentation assays for recombinant Arf6 proteins with or without a His12 tag indicated that their stable membrane association was not achieved by the amphipathic helices alone even in the GTP-loaded states (**Supplementary Figure S1**). Thus, this suggests that the artificial membrane anchoring via an N-terminal His12 tag and a DOGS-NTA lipid can bypass the GTP requirement for membrane binding of Arf proteins and their following tethering functions in a reconstitution system.

To further strengthen the experimental evidence for the intrinsic membrane tethering potency of the Arf-family GTPase, Arf6, we next performed fluorescence microscopic imaging assays to analyze particle sizes of liposome clusters induced by Arf6-mediated tethering (**Figure 3**). When incubated Arf proteins with fluorescence-labeled 200-nm liposomes bearing Rh-PE (Arf-to-lipid molar ratios of 1:1,000, 30°C, 1 h; **Figure 3A**), Arf6 induced efficiently the formation of massive liposome clusters, yielding the total particle area of 410,000 ± 38,000 μm^2 and the average particle size of 1,300 ± 530 μm^2, whereas Arf1 exhibited little potency to induce large liposome clusters that were detectable by the current imaging assay (**Figures 3B,C**). In addition, Arf6-induced liposome clusters were able to be dissociated into undetectable small particles by incubating in the presence of imidazole (250 mM) that blocks the association of His12-Arf6 with a DOGS-NTA lipid on liposomes (**Figures 3D,E**). This establishes that Arf6-mediated tethering is a non-fusogenic, reversible tethering reaction, and it can be strictly controlled by the membrane attachment and detachment cycle of Arf6 on lipid bilayers. Using the same microscopic imaging assay but with two types of the fluorescence-labeled liposomes bearing DOGS-NTA and either Rh-PE or FL-PE (**Figures 3F–H**), we also asked whether Arf6-mediated membrane tethering requires *trans*-assembly between membrane-anchored Arf6 proteins on two opposing membranes to be tethered, as established for reconstituted membrane tethering reactions mediated by human Rab-family small GTPases (Tamura and Mima, 2014; Inoshita and Mima, 2017; Segawa et al., 2019; Ueda et al., 2020) and by Atg8-family proteins (Taniguchi et al., 2020). When DOGS-NTA lipids were present in both of the Rh-bearing and FL-bearing liposomes (**Figure 3F**), Arf6 retained the tethering capacity to induce massive clusters containing the two fluorescence-labeled liposomes, yielding the total particle area of 430,000 ± 23,000 μm^2 (**Figure 3F**). However, by omitting a DOGS-NTA lipid from the FL-labeled liposomes (**Figure 3G**), Arf6 lost its ability to tether them, forming only Rh-labeled large liposome clusters (the total particle area of 180,000 ± 13,000 μm^2; middle panel, **Figure 3G**). Moreover, as expected, the tethering activity of Arf6 was completely abrogated when DOGS-NTA lipids were

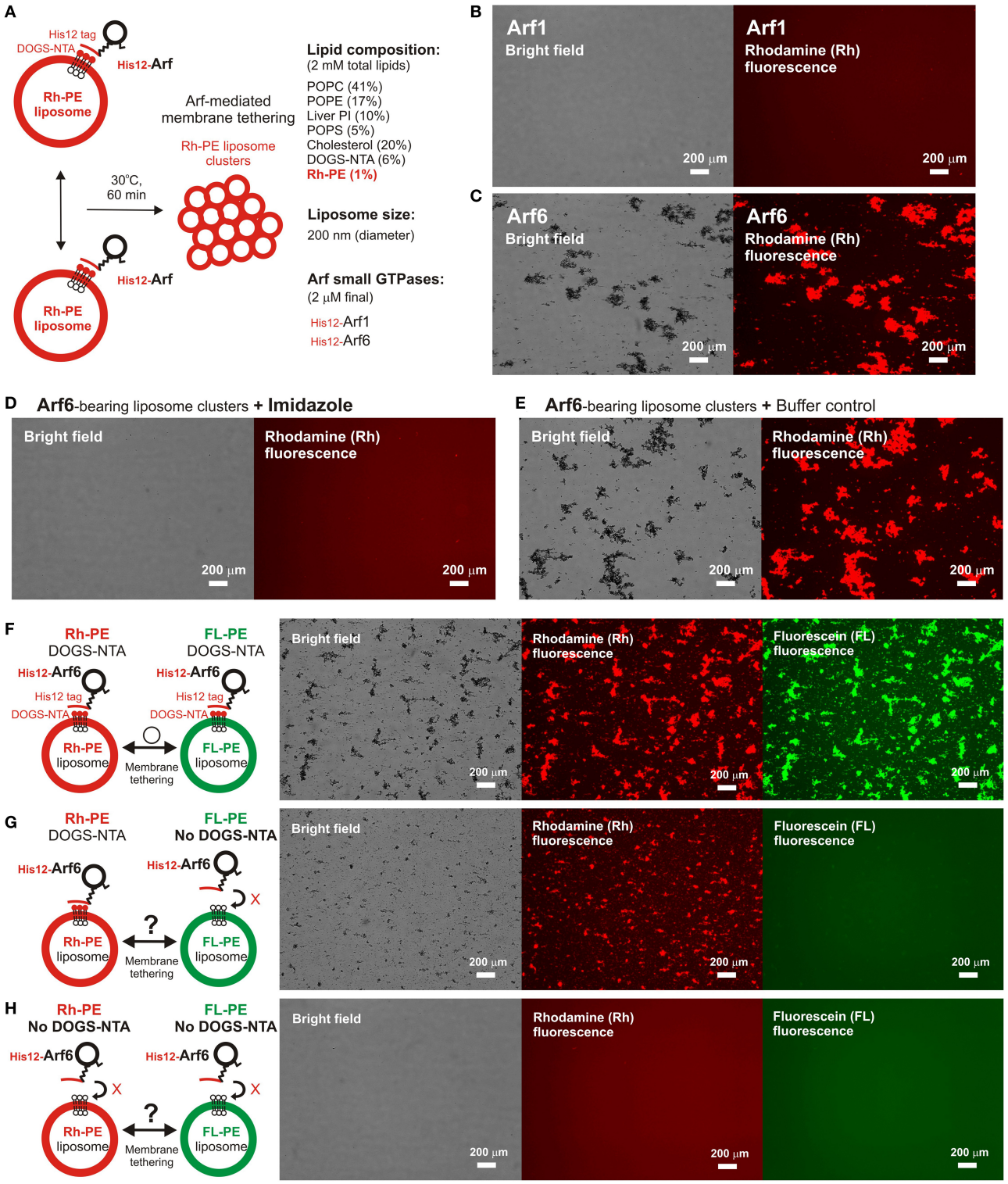

FIGURE 3 | Fluorescence microscopic analysis of human Arf6-mediated membrane tethering. **(A)** Schematic representation of fluorescence microscopy-based tethering assays with human Arf-family small GTPases. **(B,C)** Purified His12-Arf1 **(B)** and His12-Arf6 **(C)** proteins (final 2 μM) were mixed with fluorescence-labeled liposomes bearing Rh-PE and DOGS-NTA (200-nm diameter; final 2 mM lipids), incubated (30°C, 1 h), and subjected to fluorescence microscopy. **(D,E)** Reversibility of Arf6-mediated membrane tethering. Purified His12–Arf6 was incubated with Rh-PE/DOGS-NTA-bearing liposomes as in **(C)** (30°C, 1 h), supplemented with imidazole (final 250 mM) which causes the dissociation of His12-Arf6 from the DOGS-NTA-containing liposomes **(D)** or with the buffer control **(E)**, further incubated (30°C, 1 h),

(Continued)

FIGURE 3 | and subjected to fluorescence microscopy. **(F–H)** Requirement of *trans*-assembly between membrane-anchored Arf proteins for Arf6-mediated membrane tethering. Purified His12-Arf6 (final 2 μM) was incubated with two types of fluorescence-labeled liposomes, Rh-PE-bearing liposomes and FL–PE-bearing liposomes (200-nm diameter; final 1 mM lipids for each), as in **(C)** (30°C, 1 h) and subjected to fluorescence microscopy. DOGS-NTA lipids were present in both of the fluorescence-labeled liposomes **(F)**, only in the Rh-PE liposomes **(G)**, or not present in either of the liposomes **(H)**. Scale bars, 200 μm.

not present in either of the two fluorescence-labeled liposomes (**Figure 3H**). The requirement of membrane-anchored Arf6 proteins for membrane tethering was further established by liposome turbidity assays in the presence of soluble Arf6 proteins lacking an N-terminal His12 tag, showing that a vast molar excess of untagged Arf6 had no potency to competitively inhibit membrane tethering mediated by membrane-anchored Arf6 proteins (**Supplementary Figure S2**). Therefore, these results demonstrate that Arf6-mediated tethering is selectively driven by *trans*-assembly of the membrane-anchored form of Arf6 proteins between two apposing lipid bilayers.

We previously reported that membrane tethering mediated by Rab-family small GTPases can be triggered not only by "homotypic" Rab-Rab assembly in *trans* (e.g., Rab5a-Rab5a, Rab6a-Rab6a, and Rab7a-Rab7a; Tamura and Mima, 2014; Inoshita and Mima, 2017; Segawa et al., 2019), but also by "heterotypic" *trans*-assembly between two different Rab isoforms, including Rab1a-Rab6a, Rab1a-Rab9a, and Rab1a-Rab33b (Segawa et al., 2019). Therefore, although Arf1 had little tethering potency in a homotypic fashion (**Figures 2B**, **3B**), next we employed reconstituted tethering assays in the presence of both Arf1 and Arf6, to test whether the inactive isoform Arf1 can be functional in the tethering reactions through interacting with Arf6 on membranes (**Figure 4A**). Nevertheless, in the kinetic turbidity assays (**Figure 4B**), the tethering activity of Arf6 was substantially diminished by adding a 2-fold molar excess of Arf1 (red open circles, **Figure 4B**), exhibiting an ∼6–8-fold decrease in the maximum tethering capacity (**Figure 4C**) and in the initial tethering velocity (**Figure 4D**), whereas the addition of extra Arf6, instead of Arf1, significantly enhanced the rate of Arf6-mediated liposome tethering (black solid line, **Figure 4B**). This specific inhibitory effect on Arf6-mediated tethering requires the membrane-anchored form of Arf1, as the tethering activity of Arf6 was unable to be inhibited by either soluble untagged Arf1 or a His12 peptide (**Supplementary Figure S3**). Fluorescence microscopy assays also demonstrated that the formation of massive liposome clusters induced by Arf6-mediated tethering was totally inhibited by the presence of membrane-anchored Arf1 on liposomes (**Figures 4E,F**). Thus, these results suggest that Arf1 can interact with Arf6 on lipid bilayers, not in *trans* for initiating the tethering events of two distinct membranes, but in a *cis* configuration, thereby preventing the self-assembly of Arf6 proteins in *trans*. We further investigated guanine nucleotide dependence of the heterotypic *cis*-interactions between Arf6 and Arf1 on lipid bilayers by employing turbidity assays with the GTP/GDP-loaded forms of Arf6/Arf1 proteins (**Figures 4G,H**). Arf1-GTP retained the potency to substantially inhibit membrane tethering mediated by Arf6-GTP (red open circles, **Figure 4G**), consistent with the prior results obtained in the absence of guanine nucleotides (**Figure 4B**). Interestingly, the GDP-loaded form of Arf1 exhibited relatively moderate inhibitory effect on Arf6-GDP-mediated tethering (black open circles, **Figure 4G**). This may reflect that *cis*- and *trans*-assemblies of Arf-family proteins on membranes are driven by distinct modes of the protein-protein interactions.

Taken together, using a chemically-defined reconstitution approach with purified proteins of Arf1 and Arf6 in humans and synthetic liposomal membranes, we uncovered that Arf6 can directly mediate reversible membrane tethering reactions through the self-assembly in *trans* between two opposing lipid bilayers (**Figures 2**, **3**). Despite the fact that Arf6 functions as a bona fide membrane tether in the reconstituted system, the other isoform Arf1, which shares over 65% sequence identity with Arf6 and also retains the GTP hydrolysis activity comparable to that of Arf6 (**Figures 1A,D**), exhibited little or no tethering activity under the same experimental conditions (**Figures 2**, **3**). In addition, the current tethering assays in the presence of both these two Arf isoforms revealed that membrane-anchored Arf1 can significantly inhibit the tethering activity of Arf6 (**Figure 4**), reflecting the heterotypic Arf1-Arf6-assembly in *cis* on lipid bilayers. These findings on the novel molecular functions of Arf6 and Arf1 in membrane tethering lead us to postulate that self-assemblies of Arf-family small GTPases in *trans* and in *cis* can directly contribute to driving and regulating the tethering events of membrane trafficking in eukaryotic cells, perhaps through collaborating with their specific effectors including coiled-coil tethering proteins and multisubunit tethering complexes. Future studies will need to be focused on mechanistic details of Arf-mediated membrane tethering, particularly determining the dimer interface required for *trans*-assembly of Arf molecules on lipid bilayers. Recent experimental and computational works on the membrane-associated dimers of K-Ras and H-Ras small GTPases have proposed that their putative dimer interfaces are located at the C-terminal allosteric lobes in the G-domains, not within the N-terminal effector lobes which contain the switch I, switch II, and inter-switch regions (Muratcioglu et al., 2015; Prakash et al., 2017; Spencer-Smith et al., 2017; Abankwa and Gorfe, 2020). Although it remains totally unknown whether the dimer interfaces of K-Ras and H-Ras are conserved through all of the members belonging to the Ras superfamily, we speculate that the C-terminal allosteric lobes of Arf small GTPases, as well as Rab-family GTPases, are involved in their *trans*-assemblies on membranes, as reconstituted membrane tethering reactions mediated by Arf and Rab proteins are found to be completely insensitive to the guanine nucleotide-bound states.

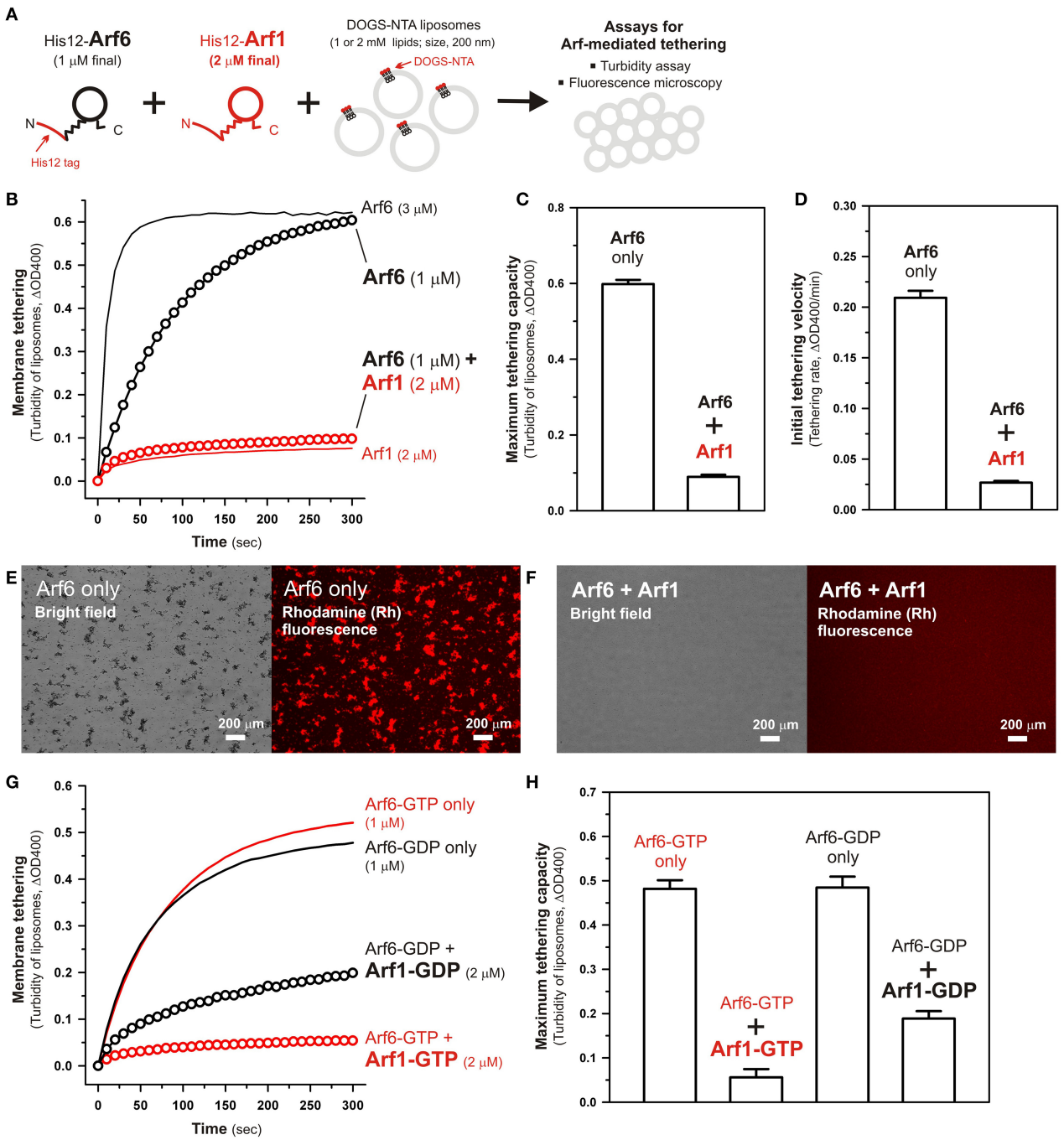

FIGURE 4 | Arf-mediated membrane tethering in the presence of the heterotypic pair of Arf6 and Arf1. **(A)** Schematic representation of reconstituted liposome tethering assays for testing membrane tethering mediated by the heterotypic combination of Arf6 and Arf1. **(B–D)** Kinetic liposome turbidity assays. Purified His12-Arf6 (final 1 or 3 μM), His12–Arf1 (final 2 μM), or both (1 μM Arf6 and 2 μM Arf1 in final) were mixed with DOGS-NTA-bearing liposomes (200-nm diameter; final 1 mM lipids) and immediately assayed for the turbidity changes **(B)**, as in **Figure 2B**. Maximum tethering capacities **(C)** and initial tethering velocities **(D)** were determined for the turbidity reactions with Arf6 only (final 1 μM) and the reactions with both Arf6 and Arf1, as in **Figures 2C,D**. **(E,F)** Fluorescence microscopy. Purified His12-Arf6 (final 1 μM) was incubated (30°C, 10 min) in the absence **(E)** or the presence **(F)** of purified His12-Arf1 (final 2 μM), mixed with Rh-PE/DOGS-NTA-bearing liposomes (200-nm diameter; final 2 mM lipids), further incubated (30°C, 1 h), and subjected to fluorescence microscopy, as in **Figures 3B,C**. **(G,H)** Kinetic liposome turbidity assays for nucleotide-loaded Arf proteins. GTP-loaded or GDP-loaded Arf6 (Arf6-GTP or Arf6-GDP; 1 μM) was incubated with Arf1-GTP or Arf1-GDP (2 μM), mixed with DOGS-NTA-bearing liposomes, and assayed for the turbidity changes **(G)**, as in **(B)**. Maximum tethering capacities **(H)** were determined for the turbidity reactions, as in **(C)**. Scale bars, 200 μm.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JM designed the research and wrote the manuscript. JM and KF performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.628910/full#supplementary-material

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Impaired Mitochondrial Mobility in Charcot-Marie-Tooth Disease

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Charcot-Marie-Tooth (CMT) disease is a progressive, peripheral neuropathy and the most commonly inherited neurological disorder. Clinical manifestations of CMT mutations are typically limited to peripheral neurons, the longest cells in the body. Currently, mutations in at least 80 different genes are associated with CMT and new mutations are regularly being discovered. A large portion of the proteins mutated in axonal CMT have documented roles in mitochondrial mobility, suggesting that organelle trafficking defects may be a common underlying disease mechanism. This review will focus on the potential role of altered mitochondrial mobility in the pathogenesis of axonal CMT, highlighting the conceptional challenges and potential experimental and therapeutic opportunities presented by this "impaired mobility" model of the disease.

Keywords: organelle transport, axonal transport deficiency, neurodegeneration, cytoskeleton, mitochondria, Charcot-Marie-Tooth (CMT) disease

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INTRODUCTION

Charcot-Marie-Tooth (CMT) disease is the most commonly inherited neurological disorder, affecting ∼1 in 5000 people (Skre, 1974; Barreto et al., 2016). It is a peripheral neuropathy defined by progressive deterioration of the peripheral nerves in the distal parts of the body, specifically the feet, hands, and lower extremities. This typically results in both motor and sensory loss in the affected areas. Unraveling the pathogenic mechanism(s) underlying CMT is somewhat complicated by the fact that CMT is both genetically and clinically heterogeneous. CMT has many subtypes including, demyelinating (affecting mainly Schwann cells), axonal, and intermediate (affecting both axons and Schwann cells). Herein, we are focusing on perturbations of mitochondrial mobility that might underlie the pathogenesis of axonal CMT.

Charcot-Marie-Tooth variants were originally classified based purely on clinical data. However, a recent explosion in genetic data can be mined to generate some compelling hypotheses. To date, over 100 mutations across more than 40 different proteins have been implicated in axonal and intermediate CMT. A large fraction of CMT-associated proteins has been shown or is predicted to affect the mobility of mitochondria or other organelles (**Table 1**). In this review, we will focus on those that affect mitochondrial mobility and hypothesize that defects in this process might begin to explain why CMT mutations mainly affect peripheral neurons. At the same time, we highlight important limitations to this "impaired mobility" model. Our belief is that the insights gained from studying the effects of CMT mutations in peripheral neurons will inform the role of mitochondrial mobility in other types of neurons and neurodegenerative disorders, including those associated with aging.

CMT IS A PROGRESSIVE DISORDER THAT AFFECTS PREDOMINANTLY THE LONGEST NEURONS

Charcot-Marie-Tooth usually affects only the feet, hands, and lower extremities. The axons leading to these distal sites can be as long as a meter in some individuals. There have been some reports of central nervous system involvement but these instances are rare (Pareyson and Marchesi, 2009; Lee et al., 2017). Some CMT mutations also cause optic atrophy, and the optical nerve notably consists of relatively long axons (∼50 mm). Patients are usually born unaffected, but typically by age 10 display major losses of function. However, the range of age can be from the toddler years to the 5th decade of life (Skre, 1974; Verhoeven et al., 2003; Zuchner et al., 2004, 2006; Chung et al., 2006; Engelfried et al., 2006; Cho et al., 2007; Calvo et al., 2009; Braathen et al., 2010; Boyer et al., 2011b). The severity of the disease is directly correlated with the age of onset (Chung et al., 2006; Verhoeven et al., 2006), and the longer axons (i.e., the feet) invariably degenerate before the shorter axons (i.e., the hands). In cases where CMT patients also display optic atrophy, this occurs after loss of function in the hands (Verhoeven et al., 2006; Zuchner et al., 2006). Together, these observations indicate a disease that directly correlates the length of the axon with the speed of onset, and the speed of onset with the magnitude of the pathology.

CMT MUTATIONS LARGELY AFFECT MITOCHONDRIAL MOBILITY

As mentioned above, a unique peripheral nerve characteristic is their extreme length, which suggests these cells are uniquely sensitive to impaired long-distance transport. Put simply, a decrease in mobility would have a greater impact on longer distance commutes than shorter ones. In support of this theory, 23 out of the 48 genes mutated in axonal or intermediate CMT encode proteins that play roles in mitochondrial function, often impacting mitochondrial mobility (**Table 1**).

The majority of axonal CMT studies have centered on Mitofusin 2 (MFN2) mutations, which consistently result in reduced axonal mitochondrial mobility. This phenotype has been reproduced in mouse models and in patient cell lines and tissues. Neurons expressing MFN2 CMT mutants and neurons from MFN2 CMT mouse models also show reduced axonal mitochondrial mobility (Baloh et al., 2007; Vallat et al., 2008; Rocha et al., 2018). MFN2 is also implicated in mitochondrial fusion dynamics, and MFN2 CMT mutations cause clustering of improperly fused mitochondria (Baloh et al., 2007; Detmer and Chan, 2007; Vallat et al., 2008; Rocha et al., 2018). Thus, it is possible that this mitochondrial clustering contributes to reduced mitochondrial mobility. Although several MFN2 CMT mutants cause mitochondrial fragmentation suggesting a disruption of its fusogenic activity, there are other MFN2 CMT mutants that do not alter mitochondrial morphology or, seemingly paradoxically, even cause mitochondrial elongation (Detmer and Chan, 2007; Codron et al., 2016; Rocha et al., 2018). MFN2 is also implicated in mitophagy, lipid transfer, lipid droplet-mitochondria contacts, and endoplasmic reticulum (ER)-mitochondria contacts, although whether MFN2 increases or decreases ER-mitochondria contacts is still under debate (de Brito and Scorrano, 2008; Chen and Dorn, 2013; Sugiura et al., 2013; Gong et al., 2015; Leal et al., 2016; Naon et al., 2016, 2017; Boutant et al., 2017; Filadi et al., 2017; Basso et al., 2018; McLelland et al., 2018; Hernández-Alvarez et al., 2019). While MFN2 CMT mutants reduce ER-mitochondria contacts (Bernard-Marissal et al., 2019; Larrea et al., 2019), it is unclear whether these changes affect mitochondrial mobility.

How alterations in mitochondrial motility impact mitochondrial function, particularly in the context of CMT, remains poorly understood. Despite clear defects in mitochondrial mobility, some studies have concluded CMT mutations do not alter readouts of mitochondrial OXPHOS function such as mitochondrial membrane potential, oxygen consumption, and ATP production, or impair cellular calcium levels which mitochondria are involved in controlling (Baloh et al., 2007; Larrea et al., 2019). However, other studies have demonstrated that CMT mutations cause defects in these readouts (Loiseau et al., 2007; Guillet et al., 2010; Barneo-Munoz et al., 2015; Saporta et al., 2015; Rocha et al., 2018; Almutawa et al., 2019; Bernard-Marissal et al., 2019). And, another study demonstrated that bioenergetic efficiency and viability in a fly model can be rescued with only minor alterations in mitochondrial distribution (Trevisan et al., 2018). These discrepancies may be at least partially explained by differences in the model systems and experimental conditions used. There are now a wide variety of tools to study CMT including mouse and fly (*Drosophila melanogaster*) genetic models and iPSC-derived motor neurons (Saporta et al., 2015; Yamaguchi and Takashima, 2018; Juneja et al., 2019).

There is also clear evidence for a role of organelle-organelle contacts affecting mitochondrial mobility in CMT caused by mutations in the endo-lysosomal protein RAB7A. Wong et al. demonstrated reduced mitochondrial mobility due to prolonged inter-mitochondrial contacts in HeLa cells expressing CMT-mutant MFN2, RAB7A, or TRPV4 (Transient Receptor Potential Cation Channel Subfamily V Member 4) (Wong et al., 2019). RAB7A CMT mutations also increase tethering between mitochondria and endolysosomes, leading to changes in mitochondrial morphology and reduced mitochondrial mobility (Wong et al., 2018, 2019; Cioni et al., 2019). There is also evidence pointing towards an interaction between RAB7A and MFN2 (Zhao T. et al., 2012). Together, these findings suggest that interpretations of RAB7A mutations causing CMT based solely on defects in its endo-lysosomal function may be too simplistic. In the same vein, a recent study found that CMT-causing GDAP1 (Ganglioside Induced Differentiation Associated Protein 1) mutations result in defective mitochondria-lysosome contacts (Cantarero et al., 2020). That mitochondria-organelle contacts can affect mitochondrial mobility and dynamics highlights the limitations of evaluating protein and organelle dysfunction in isolation.

TABLE 1 | Genes mutated in axonal and intermediate CMT.

Gene/CMT subtype/OMIM Code	Function	References
AARS1/CMT2N/613287	Catalyzes the attachment of alanine to tRNA.	McLaughlin et al., 2012; Zhao Z. et al., 2012; Bansagi et al., 2015; Dohrn et al., 2017; Weterman et al., 2018
ATP1A1/CMT2DD/618036	Catalyzes the hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane.	Lassuthova et al., 2018
BAG3/CMT2*	Acts as a nucleotide-exchange factor promoting the release of ADP from the HSP70 and HSC70 proteins thereby triggering client/substrate protein release. Has anti-apoptotic activity. Plays a role in cytoskeletal proteostasis and dynamics.	Kim et al., 2018; Shy et al., 2018
BSCL2/CMT2/619112*	Plays a crucial role in the formation of lipid droplets. Mediates the formation and/or stabilization of endoplasmic reticulum-lipid droplet contacts. Binds anionic phospholipids including phosphatidic acid.	Chaudhry et al., 2013
DCTN2/CMT2*	Component of a large macromolecular complex required for the cytoplasmic dynein-driven movement of organelles along microtubules. Pays a role in prometaphase chromosome alignment and spindle organization during mitosis.	Braathen et al., 2016
DGAT2/CMT2	Required for synthesis and storage of intracellular triglycerides.	Hong et al., 2016
DHTKD1/CMT2Q/615025	Catalyzes the overall conversion of 2-oxoglutarate to succinyl-CoA and CO_2.	Baets et al., 2014; Dohrn et al., 2017; Zhao et al., 2019
DNAJB2/CMT2T/604139	Functions as a co-chaperone, activating the ATPase activity of chaperones of the HSP70/heat shock protein 70 family. Contributes to the ubiquitin-dependent proteasomal degradation of misfolded proteins.	Gess et al., 2014; Lupo et al., 2016
DNM2/CMT2M/602378; CMTDIB/606482*	Plays an important role in vesicular trafficking processes, in particular endocytosis. Involved in producing microtubule bundles. Involved in cytokinesis.	Echaniz-Laguna et al., 2007; Bitoun et al., 2008; Haberlová et al., 2011; González-Jamett et al., 2014; Saghira et al., 2018
DYNC1H1/CMT2O/614228*	Acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules. Plays a role in mitotic spindle assembly and metaphase plate congression.	Weedon et al., 2011
GARS1/CMT2D/601472	Catalyzes the attachment of glycine to tRNA	James et al., 2006; Xie et al., 2006; Hamaguchi et al., 2010; Motley et al., 2011; Morelli et al., 2017
GDAP1/CMT2K/607831; CMTRIA608340/; CMT4A/214400*	Regulates the mitochondrial network by promoting mitochondrial fission. Proposed roles in mitochondrial transport, redox processes, calcium homeostasis, and energy production.	Baxter et al., 2002; Cuesta et al., 2002; Ammar et al., 2003; Senderek et al., 2003; Di Maria et al., 2004; Zhang et al., 2004; Baránková et al., 2007; Kabzińska et al., 2007; Auer-Grumbach et al., 2008; Rougeot et al., 2008; Xin et al., 2008; Moroni et al., 2009; Sahin-Calapoglu et al., 2009a,b; Cassereau et al., 2011; Fusco et al., 2011; Zimoń et al., 2011, 2015; Vital et al., 2012; Auranen et al., 2013; Manganelli et al., 2015; Martin et al., 2015; Dohrn et al., 2017; Ho et al., 2017; Martí et al., 2017; Yoshimura et al., 2017; He et al., 2018; Masingue et al., 2018; Rzepnikowska and Kochański, 2018; Mai et al., 2019; Qin et al., 2019
HARS1/CMT2W/616625	Catalyzes the attachment of histidine to tRNA.	Baets et al., 2014
HINT1/CMT2/137200	Hydrolyzes purine nucleotide phosphoramidates with a single phosphate group.	Baets et al., 2014; Laššuthová et al., 2015; Zimoń et al., 2015; Dohrn et al., 2017
HSPB1/CMT2F/606595*	Functions as a molecular chaperone maintaining denatured proteins in a folding-competent state. Plays a role in stress resistance and actin organization. Regulates numerous biological processes including phosphorylation and axonal transport of neurofilament proteins.	Liu et al., 2005; Tang et al., 2005; Chung et al., 2008; Houlden et al., 2008; Solla et al., 2010; Amornvit et al., 2017; Dohrn et al., 2017; Ho et al., 2017; Weeks et al., 2018
HSPB8/CMT2L/608673*	Displays temperature-dependent chaperone activity. Forms complex with BAG3.	Nicholson et al., 2009
IGHMBP2/CMT2S/616155	5′ to 3′ helicase that unwinds RNA and DNA duplices. Acts as a transcription regulator.	Cottenie et al., 2014; Wagner et al., 2015; Dohrn et al., 2017; Yuan et al., 2017
JPH1/CMT2K/607831	Contributes to the formation of junctional membrane complexes which link the plasma membrane with the endoplasmic or sarcoplasmic reticulum in excitable cells. Provides a structural foundation for functional cross-talk between the cell surface and intracellular calcium release channels.	Pla-Martín et al., 2015; Kanwal and Perveen, 2019
KIF1B/CMT2A1/118210*	Motor for anterograde transport of mitochondria.	Nakagawa and Takashima, 2003; Bissar-Tadmouri et al., 2004
KIF5A/CMT2*	Microtubule-dependent motor required for slow axonal transport of neurofilament proteins. Contributes to the vesicular transport of VAPA, VAPB, SURF4, RAB11A, RAB11B and RTN3 proteins in neurons.	Dohrn et al., 2017
LMNA/CMT2B1/605588	Plays an important role in nuclear assembly, chromatin organization, nuclear membrane and telomere dynamics.	De Sandre-Giovannoli et al., 2002; Chaouch et al., 2003; Hamadouche et al., 2008; Zhang et al., 2010; Liang et al., 2016; Dohrn et al., 2017

(Continued)

TABLE 1 | Continued

Gene/CMT subtype/OMIM Code	Function	References
LRSAM1/CMT2G/614436	E3 ubiquitin-protein ligase. Bacterial recognition protein that defends the cytoplasm from invasive pathogens. Potential role in mitophagy?	Guernsey et al., 2010; Nicolaou et al., 2013; Dohrn et al., 2017
MARS1/CMT2U/616280	Catalyzes the attachment of methionine to tRNA.	Baets et al., 2014; Sun et al., 2017
MED25/CMT2B2/605589	A coactivator involved in the regulated transcription of nearly all RNA polymerase II-dependent genes.	Leal et al., 2009, 2018; Tazir et al., 2013
MFN2/CMT2A2/609260*	Mitochondrial outer membrane GTPase that mediates mitochondrial clustering and fusion. Involved in the clearance of damaged mitochondria via mitophagy. Potential roles in mitochondria-ER contacts and mitochondrial transport.	Kijima et al., 2005; Lawson et al., 2005; Engelfried et al., 2006; Verhoeven et al., 2006; Zuchner et al., 2006; Loiseau et al., 2007; Muglia et al., 2007; Neusch et al., 2007; Del Bo et al., 2008; Calvo et al., 2009; Cartoni and Martinou, 2009; Braathen et al., 2010; Ouvrier and Grew, 2010; Feely et al., 2011; McCorquodale et al., 2011; Park et al., 2012; Vital et al., 2012; Brožková et al., 2013; Chapman et al., 2013; Kotruchow et al., 2013; Lv et al., 2013, 2015; Vielhaber et al., 2013; Bergamin et al., 2014; Choi B. O. et al., 2015; Wang et al., 2015; Bannerman et al., 2016; Di Meglio et al., 2016; Neupauerová et al., 2016; Rudnik-Schöneborn et al., 2016; Tan et al., 2016; Werheid et al., 2016; Xie et al., 2016; Ando et al., 2017; Beręsewicz et al., 2017; Dohrn et al., 2017; El Fissi et al., 2018; Finsterer et al., 2018; Iapadre et al., 2018; Milley et al., 2018; Larrea et al., 2019; Xu et al., 2019
MME/CMT2T/617017	Biologically important in the destruction of opioid peptides. Able to cleave angiotensin. Involved in the degradation of atrial natriuretic factor.	Auer-Grumbach et al., 2016; Fujisawa et al., 2017
MORC2/CMT2Z/616688	Essential for epigenetic silencing by the HUSH complex.	Albulym et al., 2016
MPV17/CMT2EE/618400*	Non-selective channel that modulates membrane potential under normal conditions and oxidative stress, and is involved in mitochondrial homeostasis. Involved in mitochondrial deoxynucleoside triphosphates pool homeostasis and mitochondrial DNA maintenance.	Choi Y. R. et al., 2015
MPZ/CMT2J/607736; CMTDID/607791; CMT1B/118200	Mediates adhesion between adjacent myelin wraps and ultimately drives myelin compaction.	Hayasaka et al., 1993; Pham-Dinh et al., 1993; Nelis et al., 1994; Latour et al., 1995; Blanquet-Grossard et al., 1996; Roa et al., 1996; Silander et al., 1996; Bissar-Tadmouri et al., 1999; De Jonghe et al., 1999; Lagueny et al., 1999; Quattrini et al., 1999; Senderek et al., 2000; Kochański et al., 2004; Kurihara et al., 2004; Bienfait et al., 2006; Sabet et al., 2006; Laurà et al., 2007; Lee et al., 2008; Mazzeo et al., 2008; Gallardo et al., 2009; Avila et al., 2010; Brozková et al., 2010; Kleffner et al., 2010; Choi et al., 2011; Høyer et al., 2011, 2014; Chavada et al., 2012; Maeda et al., 2012; Marttila et al., 2012; Rosberg et al., 2013; Speevak and Farrell, 2013; Bergamin et al., 2014; Leal et al., 2014; Sanmaneechai et al., 2015; Tokuda et al., 2015; Wang et al., 2015; Rudnik-Schöneborn et al., 2016; Werheid et al., 2016; Dohrn et al., 2017; He et al., 2018; Milley et al., 2018; Xu et al., 2019
MT-ATP6/CMT2*	Mitochondrial membrane ATP synthase. Key component of the proton channel.	Pitceathly et al., 2012
MYH14/CMT2/614369*	Conventional non-muscle myosin. Actin-dependent motor protein. Mediates mitochondrial fission.	Almutawa et al., 2019
NAGLU/CMT2V/616491	Involved in the degradation of heparan sulfate.	Tétreault et al., 2015
NEFH/CMT2CC/616924*	Component of neurofilaments, the most abundant cytoskeletal component of myelinated axons.	Bian et al., 2018
NEFL/CMT2E/607684*	Component of neurofilaments, the most abundant cytoskeletal component of myelinated axons. Regulates mitochondrial morphology.	Lupski, 2000; Luo et al., 2003; Fabrizi et al., 2007; Miltenberger-Miltenyi et al., 2007; Shin et al., 2008; Bhagavati et al., 2009; Berciano et al., 2016; Werheid et al., 2016; Dohrn et al., 2017; Horga et al., 2017; Fu and Yuan, 2018; Xu et al., 2019
PNKP/CMT2B2/605589	Plays a key role in the repair of DNA damage, functioning as part of both the non-homologous end-joining and base excision repair pathways.	Leal et al., 2018
RAB7A/CMT2B/600882*	Key regulator in endo-lysosomal trafficking. Plays roles in growth-factor-mediated cell signaling, nutrient-transporter mediated nutrient uptake, neurotrophin transport in the axons of neurons and lipid metabolism. Regulates mitochondrial fission, mitophagy, and mitochondria-lysosome tethering.	Meggouh et al., 2006; Zhang et al., 2010; Manganelli et al., 2015
SPG11/CMT2X/616668*	Plays a role in neurite plasticity by maintaining cytoskeleton stability and regulating synaptic vesicle transport.	Montecchiani et al., 2016

(Continued)

TABLE 1 | Continued

Gene/CMT subtype/OMIM Code	Function	References
TRIM2/CMT2R/615490*	E3 ubiquitin-protein ligase that mediates the ubiquitination of NEFL and of phosphorylated BCL2L11.	Baets et al., 2014; Pehlivan et al., 2015
TRPV4/CMT2C/606071*	Non-selective calcium permeant cation channel involved in osmotic sensitivity and mechanosensitivity. Some data supporting a role in regulating mitochondrial motility.	Klein et al., 2003; Deng et al., 2010; Manganelli et al., 2015; Dohrn et al., 2017
VCP/CMT2Y/616687*	Necessary for the fragmentation of Golgi stacks during mitosis and for their reassembly after mitosis. Involved in the formation of the transitional endoplasmic reticulum. Plays a role in the regulation of stress granule clearance. Involved in DNA damage response. Essential for the maturation of ubiquitin-containing autophagosomes and the clearance of ubiquitinated protein by autophagy and mitophagy.	Gonzalez et al., 2014
C1ORF194/CMTDI	May affect intracellular Ca^{2+} homeostasis.	Sun et al., 2019
GNB4/CMTDIF/615185	Modulator/transducer in various transmembrane signaling systems.	Soong et al., 2013; Baets et al., 2014; Miura et al., 2017
INF2/CMTDIE/614455*	Mediates actin polymerization at ER-mitochondria contact sites. Regulates mitochondrial morphology and motility.	Boyer et al., 2011a,b; Mademan et al., 2013; Rodriguez et al., 2013; Vallat et al., 2013; Caridi et al., 2014; Park et al., 2014; Jin et al., 2015; Werheid et al., 2016; Dohrn et al., 2017; Echaniz-Laguna and Latour, 2019; Fu et al., 2019
SLC12A6/CMTDI/218000	Mediates electroneutral potassium-chloride cotransport.	Lupo et al., 2016
YARS1/CMTDIC/608323	Catalyzes the attachment of tyrosine to its corresponding tRNA	Jordanova et al., 2006; Xie et al., 2007
COX6A1/CMTRI/616039*	A subunit of the cytochrome c oxidase complex	Tamiya et al., 2014
KARS1/CMTRIB/613641	Catalyzes the aminoacylation of tRNA-Lys in the cytoplasm and mitochondria	McLaughlin et al., 2010
PLEKHG5/CMTRIC/615376	Activates the nuclear factor kappa B (NFKB1) signaling pathway. Also implicated in distal spinal muscular atrophy.	Kim et al., 2013

Genes that play a role in mitochondrial function and/or motility are marked with an asterisk.

Recently, a screen for RAB7A binding partners found that another CMT protein, INF2 (Inverted Formin 2), is one of several actin-binding candidate interaction partners for RAB7A (Pan et al., 2020). This is particularly relevant to our discussion on CMT, inter-organelle contacts, and mitochondrial mobility for multiple reasons. First, a splice isoform of INF2 is tail-anchored to the ER. Second, dominant active mutations in ER-anchored INF2 that mimic INF2 CMT mutations have been shown to increase actin-dependent mitochondrial fragmentation and decrease mitochondrial mobility (Korobova et al., 2013; Chakrabarti et al., 2018). Together, these data point towards an important role in ER-mitochondria inter-organelle contacts in somehow regulating mitochondrial mobility via the actin cytoskeleton. That INF2 also potentially interacts with RAB7A suggests that mitochondria, endo-lysosomes, and ER all contact one another via CMT-associated proteins.

All INF2 CMT mutations are predicted or have been shown to increase actin assembly (Bayraktar et al., 2020). While some actin-binding motor proteins likely facilitate microtubule-independent mitochondrial transport, numerous studies have shown that long-range microtubule-based mobility of mitochondria is antagonized by actin and actin-binding motor proteins (Chada and Hollenbeck, 2004; Quintero et al., 2009; Pathak et al., 2010; Venkatesh et al., 2019; Cardanho-Ramos et al., 2020). Thus, while the effects of INF2 CMT mutations have yet to be studied in neurons, it is reasonable to expect that INF2 CMT mutations will cause an actin-dependent decrease in mitochondrial mobility in axons. Furthermore, since the ER regularly contacts many other organelles, and even appears to drive actin-assembly at ER-organelle contact sites (Korobova et al., 2013, 2014; Manor et al., 2015; Chakrabarti et al., 2018; Yang and Svitkina, 2019; Schiavon et al., 2020), it is quite possible INF2 CMT mutations cause aberrant actin assembly on other organelles, reducing their mobility as well (**Figure 1**).

Together with the newly uncovered role for RAB7A in (indirectly) modulating actin assembly (Pan et al., 2020), these observations point towards a role for multiple CMT mutations causing aberrant organelle-organelle and organelle-actin contacts, all of which cause decreased mitochondrial mobility. Whether some (or all) CMT mutations also cause decreased mobility of other organelles remains an important open question.

The focus of the role of mitochondria in CMT has been primarily on MFN2 and GDAP1, and to a lesser extent on associated motor proteins (KIF1B – Kinesin Family Member 1B, KIF5A – Kinesin Family Member 5A, DYNC1H1 – Dynein Cytoplasmic 1 Heavy Chain 1, DCTN2 – Dynactin Subunit 2) and some cytoskeletal proteins (NEFL – Neurofilament Light). Here, we have highlighted INF2 and RAB7A as CMT-associated proteins likely involved in mitochondrial mobility and dynamics. However, we propose that the proteins mutated in CMT that play roles in mitochondrial function, dynamics and mobility likely extend well beyond just these two (see **Table 1** for a full list).

WHY DO MOBILITY DEFECTS USUALLY ONLY AFFECT PERIPHERAL NEURONS IN CMT PATIENTS?

Hopefully, we have provided a convincing argument that many CMT mutations likely reduce mitochondrial mobility. Given the extreme lengths of peripheral axons, it is tempting to

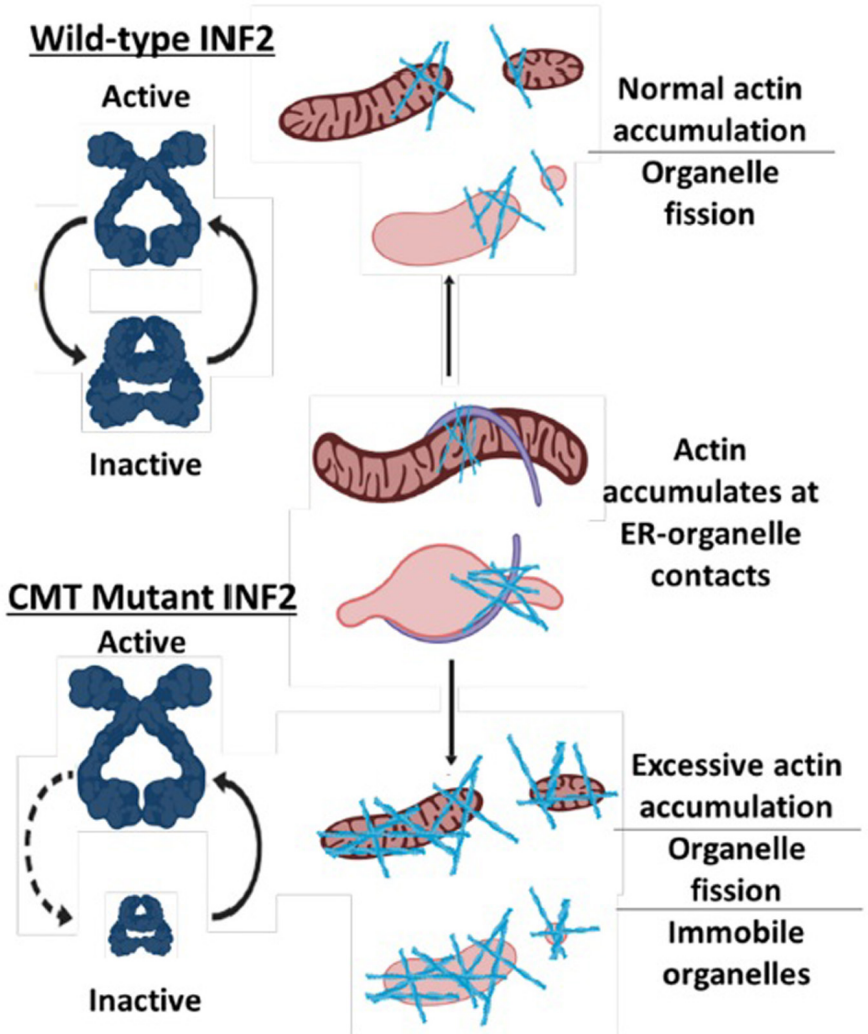

FIGURE 1 | Model for how CMT mutations in INF2 reduce mitochondrial mobility. Normal INF2 is autoinhibited as indicated by open conformation compared to the closed conformation and thus has regulated actin-assembly activity. CMT mutations in INF2 reduce autoinhibition, resulting in excessive actin assembly on mitochondria and potentially other organelles, which in turn reduces their mobility.

conclude that a reduction in mobility due to CMT mutations simply affects longer axons more severely (the "impaired mobility model" of CMT). One can easily reconcile two key features of CMT using the impaired mobility model: The progressive nature of the disease: This suggests that dysfunction must accumulate over time. One can imagine this more severely affects longer axons, due to reduced turnover of damaged mitochondria resulting from reduced mitochondrial mobility. Interestingly, one could imagine that reduced mobility of other organelles associated with turnover (e.g., lysosomes) could also cause increased accumulation of damaged mitochondria in longer axons. The longest peripheral axons (i.e., the feet) progressively degenerate prior to the next-longest axons (i.e., the hands): This further supports the impaired mobility model, wherein damage accumulates first in the longest axons due to the more demanding, "longer commute" resulting in faster accumulation of damaged mitochondria.

Unfortunately, while this model is compelling, it appears to be overly simplistic. The weakness in relying on mobility alone as an explanation can best be highlighted by comparing the lengths of different axons both within and between species. For example, the longest axon in mice is approximately 2 cm, whereas in humans the longest axon is ∼60 times longer. Just as striking, some unaffected axons in the human CNS may be longer than the mouse's longest axon. The very same mutation in humans and mice can cause CMT, yet no defects are found in the brains of human CMT patients. Meanwhile, the motor proteins, cytoskeletal tracks, and mitochondria of mice and humans are all roughly the same size, and all possess roughly the same biophysical properties (e.g., velocity, force generation, etc.) when transporting their organelles across long distances (**Figure 2**).

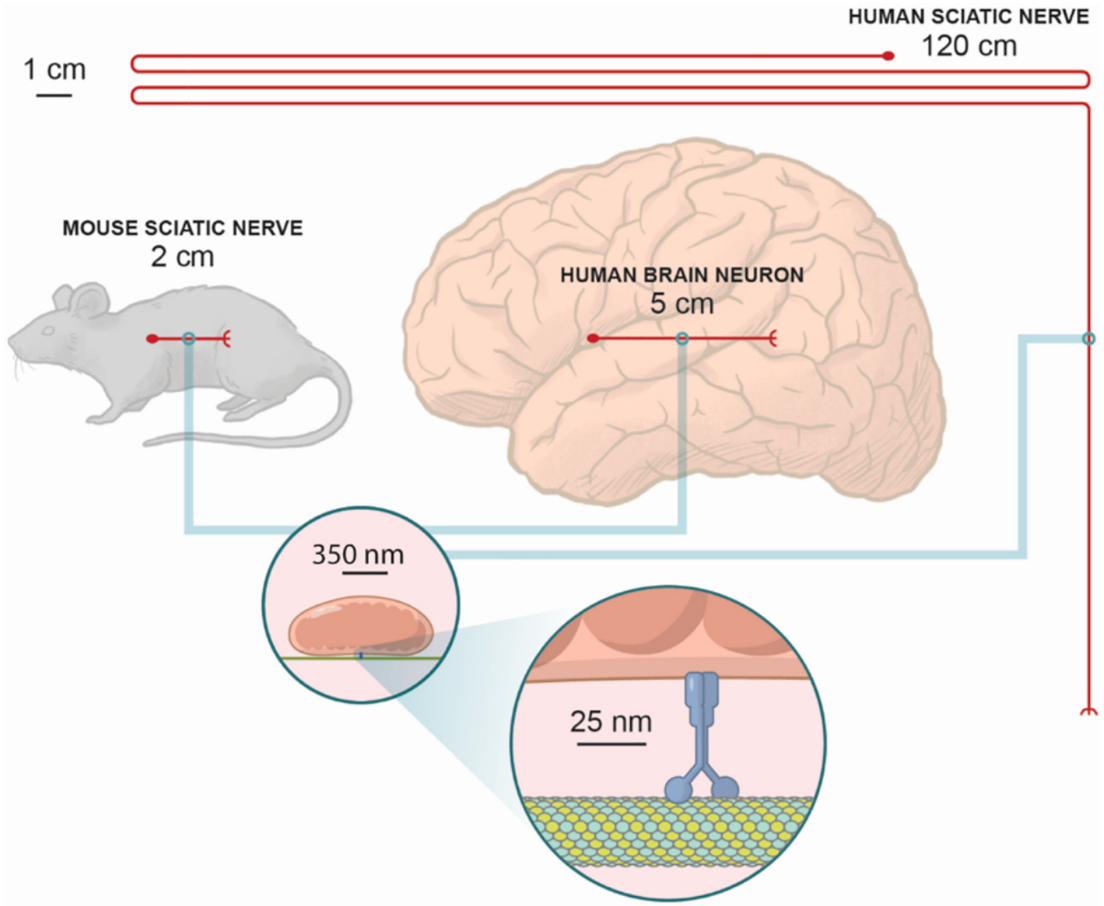

FIGURE 2 | The relative scales of axons in humans versus mice. While microtubules, motor proteins, and mitochondria have nearly identical sizes in mice and men, the length of the axons can be very different. At the same time, axons in the mouse sciatic nerve are not as long as some of the longer axons within the human brain. For example, it is known in the macaque there are direct connections from the frontal eye fields in the anterior bank of the arcuate sulcus to the primary visual cortex, which in the macaque is ∼5 cm, and probably as long as 12.5 cm in humans. Notably, for most CMT patients the pathology is often constrained to only peripheral neurons. Since the same mutations can cause CMT in mice and men, the distances mitochondria must travel can only provide a partial explanation for the physiopathology of these mutations.

Any analysis of mobility must consider not just distance but also time. Most laboratory mice only live for ∼2 years, while human CMT patients may not even experience symptoms until adolescence or adulthood. Thus, there is clearly a "missing variable" that underlies differences in lifespan and disease susceptibility between species (e.g., differences in metabolism or oxidative stress). Thus, CMT may serve as a "model disease" to better understand age-related neurodegeneration. It is well established mitochondria play myriad roles at the pre-synapse, including ATP production, intra- and intercellular signaling (e.g., calcium signaling and signaling via reactive oxygen species), and the biosynthesis of signaling molecules (e.g., lipids, hormones, and neurotransmitter intermediates) (Devine and Kittler, 2018). Perhaps perturbed transport of mitochondria to the pre-synapse of peripheral neurons in CMT provides an opportunity to better understand other neurodegenerative disorders associated with defective presynaptic mitochondria, including Alzheimer's, ALS, Parkinson's, Friedreich's Ataxia, and Hereditary Spastic Paraplegia (Devine and Kittler, 2018).

But even considering the impaired mobility model for CMT within a single organism has some issues. It is difficult to imagine how reductions in mobility as high as 100% (Baloh et al., 2007; Rocha et al., 2018) could have a severe effect only on the longest axons, but not other axons that, while shorter than the peripheral neurons, are still very long compared to the ∼25 nm step size of a motor protein.

When considering these conundrums, it is helpful to consider alternative mechanisms for replenishing mitochondria in neurons, many of which are reviewed elsewhere (Misgeld and Schwarz, 2017; Yu and Pekkurnaz, 2018). Briefly, mitochondrial rejuvenation is speculated to be at least partially mediated via local translation in the axon. Interestingly, multiple CMT mutations affect local translation machinery (**Table 1**). More recent work showed that mitochondria serve as a stable compartment for mediating biogenesis by serving as an energy

source for synaptic translation (Rangaraju et al., 2019). This raises a chicken vs. egg question: Does reduced mitochondrial mobility impair local translation needed for synaptic and therefore neuronal health and maintenance? Or does impaired local translation lead to dysfunctional mitochondria that cannot be replaced without sufficient mobility? That CMT is caused by mutations disrupting both mobility and local translation indicates these two processes have a unique relationship in long axons.

CONCLUSION AND OPEN QUESTIONS

One open question is how the overall distribution of mitochondria is altered in CMT neurons, and how this relates to axonal maintenance. A recent study showed mitochondria tend to distribute along the length of axons with regular spacing, and that inter-mitochondrial feedback mediates their positioning and movement (Matsumoto et al., 2020). Is this feedback-based positioning altered in CMT? Do mutations affecting mobility result in CMT via a "domino effect" caused by defects in relatively local repositioning between axonal mitochondria, which then cascades with increasing defects as a function of increasing axonal length? How much longer does it take mitochondria in CMT patients to traverse the entire length of an axon? Defective mitophagy has been implicated in other neurodegenerative disorders and some studies have linked CMT to alterations in autophagy (Colecchia et al., 2018; Gautam et al., 2019). Is there a reduction in the turnover rate of mitochondria in CMT patients? Mitochondria are increasingly being implicated as important players in adaptive and innate immune responses and inflammatory pathology, including neurodegeneration (West, 2017; Newman and Shadel, 2018). Could "mitoflammation" contribute to the pathophysiology of CMT? How do any and all of these factors affect mitochondria at the pre-synapse of CMT peripheral neurons, likely the most important subpopulation of mitochondria in these cells? These are surprisingly open questions we expect to be addressed in the coming years using animal and cell models of CMT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Temperature-Dependent Activity of Motor Proteins: Energetics and Their Implications for Collective Behavior

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Molecular motor proteins are an extremely important component of the cellular transport system that harness chemical energy derived from ATP hydrolysis to carry out directed mechanical motion inside the cells. Transport properties of these motors such as processivity, velocity, and their load dependence have been well established through single-molecule experiments. Temperature dependent biophysical properties of molecular motors are now being probed using single-molecule experiments. Additionally, the temperature dependent biochemical properties of motors (ATPase activity) are probed to understand the underlying mechanisms and their possible implications on the enzymatic activity of motor proteins. These experiments in turn have revealed their activation energies and how they compare with the thermal energy available from the surrounding medium. In this review, we summarize such temperature dependent biophysical and biochemical properties of linear and rotary motor proteins and their implications for collective function during intracellular transport and cellular movement, respectively.

Keywords: temperature, kinesin, dynein, myosin, arrhenius, molecular motor, intra-cellular transport

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INTRODUCTION

Intracellular transportation system utilizes molecular motor proteins along with their cofactors to haul cargos along cytoskeletal filaments from one location to another. Intra-cellular transport by molecular motor proteins is essential for cell growth, cell division, and cell survival. Most living organisms on earth reside in varying environmental conditions, and some even survive under extreme weather conditions, from boiling water swamps to polar ice desert. Every place on earth is home to different life forms starting from unicellular bacteria and fungi to multicellular vertebrates. Hence, intracellular transport in these organisms is tuned to cater to their survival in adverse and extreme weather conditions. Many biophysical properties of motor proteins are now known to be temperature dependent. Investigations to understand these temperature-dependent properties of motor proteins were initiated from different studies such as understanding of hibernation from the molecular to organism levels under unfavorable environmental conditions (Carey et al., 2003) and temperature-dependent changes in axonal transport (Ochs and Smith, 1971; Grafstein et al., 1972; Heslop and Howes, 1972; Edström and Hanson, 1973; Gross, 1973; Brimijoin, 1974, 1975; Brimijoin and Helland, 1976; Cosens et al., 1976). These studies are the foundational mark to imply that motor properties change due to variation in temperature leading to observed changes in cellular transport, direction, velocity, and other parameters.

The study of thermal properties of a molecular motor initiated from different studies of the cellular phenomenon, for example, a study was conducted on the maintenance of intracellular transport due to unfavorable temperature conditions. They report the attribute of hibernation in mammals follows extended bouts of torpor, during which minimal body temperature (Tb) can fall as low as −2.9°C and metabolism can be reduced to 1% of euthermic rates. It lists down the repetition of survival cycles of torpor and arousal during hibernation period (Truhlar and Kohen, 2001; Carey et al., 2003).

Similarly, studies involving axonal transport inside different animal cells have been done to understand their temperature dependent behavior. Such studies involving rabbits and bullfrogs' sciatic nerves reported an exponential increase in transport velocity of Dopamine-beta-hydroxylase (DBH) with temperature (**Figure 1A**; Brimijoin, 1974, 1975; Brimijoin and Helland, 1976; Cosens et al., 1976). DBH is a neuronal protein involved in the production of dopamine. Cosens also reported that microtubule density remained mostly stable and decreased only at lower temperatures (∼30%), i.e., below 13°C in rabbit nerves and 10°C in frog nerves (Edström and Hanson, 1973; Cosens et al., 1976). This implies that increase in microtubule density does not significantly affect the transport of DBH along with temperature change. This further implies that exponential increase in velocity is due to changes in inherent properties of molecular motors, which thereafter reflects toward the major conformational change as an underlying mechanism. A separate study involving frog sciatic nerves defines the average transport rate in axonal transport. They report that the transport rate in these cells varies from 32 to 290 mm/day in the temperature range of 5.5–28°C. The temperature coefficient was observed to decrease in this temperature range (3.4–2.3) (Edström and Hanson, 1973). A common finding of these studies reports Arrhenius dependence of transport rates of cellular entities (tagged proteins, macromolecules, etc.) with respect to the temperature inside cells. Arrhenius equation links rates of temperature dependent chemical or physical reactions with respect to Arrhenius constant and Activation energy. Arrhenius constant is the frequency of collisions and depends on the types of molecules involved in the reaction, whereas Activation energy is the energy barrier required by substrates to undergo the reaction. This Arrhenius relation is expressed as-

$$k_T = k_o * \exp(-E_a/RT)$$

where k_T is kinetic reaction rate, k_o is Arrhenius constant, E_a is the activation energy, R is the universal gas constant and T is the absolute temperature (Ashter, 2013). Additionally, some motors exhibit piecewise Arrhenius trend which points toward change in the enzymatic cycle (preferably rate-limiting step) of the motor and are popularly referred to as "Arrhenius Breaks." Arrhenius Breaks have been observed in various studies on temperature dependent biochemical and biophysical properties of molecular motors. In this review, we first briefly discuss earlier works on temperature dependent transport properties of a tagged protein or cellular entity that were done prior to the classification of motor proteins. These studies used sciatic nerves, olfactory nerve, muscular cells, etc., to study transport rates with different temperature ranges (Yeatman et al., 1969; Gross, 1973; Cosens et al., 1976). The temperature dependence of these rates was evaluated using temperature coefficient (Q_{10}) of rates at different temperatures. Q_{10} is a measure of rate of a biological process with temperature difference of 10°. Thereafter, we discuss studies on biochemical properties such as ATPase activity and biophysical properties such as velocity, processivity, and stall force due to variation in temperature for linear motors, respectively. These sections contain studies of Arrhenius dependence and breaks in Arrhenius relationship for Kinesin, Dynein, Myosin, and F1-ATPase motor proteins (**Figure 2**). Additionally, we discuss studies that hint at the universality of Arrhenius breaks as they have been reported for all kinds of molecular motors (Doval et al., 2020).

EARLIER STUDIES ON TEMPERATURE DEPENDENCE OF CELLULAR TRANSPORT

Cellular transport system of different cell types has roughly the same components, i.e., cargo, motors, and their regulators. Before the discovery of different types of molecular motors, numerous studies on thermal and biochemical properties of cellular environment and transport have been done, which include rates of aggregation, rates of chemical catalysis, average velocity, processivity, etc. These studies date back to the time when cellular motors and their types were unknown; however, they report Arrhenius dependence on temperature indicating toward the role of motors underlying in these observations (Truhlar and Kohen, 2001).

Studies of thermal properties of cellular transport were initially done using sciatic nerves of rabbits, bullfrogs, rats, garfish, etc., and their axonal transport was studied *in vitro* using temperature coefficient Q_{10} (Kawai et al., 2000; Humphries et al., 2003; Grove et al., 2005). Guenter W. Gross in 1973 estimated the temperature dependence of axoplasmic transport rates in the olfactory nerve of garfish through simple distance-time graphs for 10–28°C. His results showed linear dependence of transport rate on temperature with values 53 ± 3.8, 249 ± 5.2, and 410 ± 30 mm/day at 10, 25, and 37°C, respectively (Gross, 1973). In a separate study, rabbit sciatic nerves were observed *in vitro* for axonal transport of dopamine-β-hydroxylase (DBH) activity. DBH was accumulated due to cooling of nerves to 2°C and when this accumulated DBH was subjected to different temperature ranges between 13 and 42°C, the velocity increased exponentially given by relation $V = 0.546\ (1.09)^T \mathbf{V} = \mathbf{0.5}46(\mathbf{1.09})^{\mathbf{T}}$, where V is velocity of transport in mm/h and T is temperature in degree Celsius. The velocity showed Arrhenius dependence on temperature and varies from 0.4 ± 0.1 at 10°C to 12.8 ± 0.6 at 37°C, which again dropped to 1.5 ± 0.9 mm/h at 47°C due to degradation of proteins at such higher temperatures. The temperature coefficient Q_{10} was observed to be 2.33 and activation energy to be 14.8 kcal (Cosens et al., 1976). A study of *in vitro* axonal transport of tagged leucine molecule was observed in frog sciatic nerves from 5.5 to 28°C. The rate of biophysical transport, namely velocity was calculated based on the distance

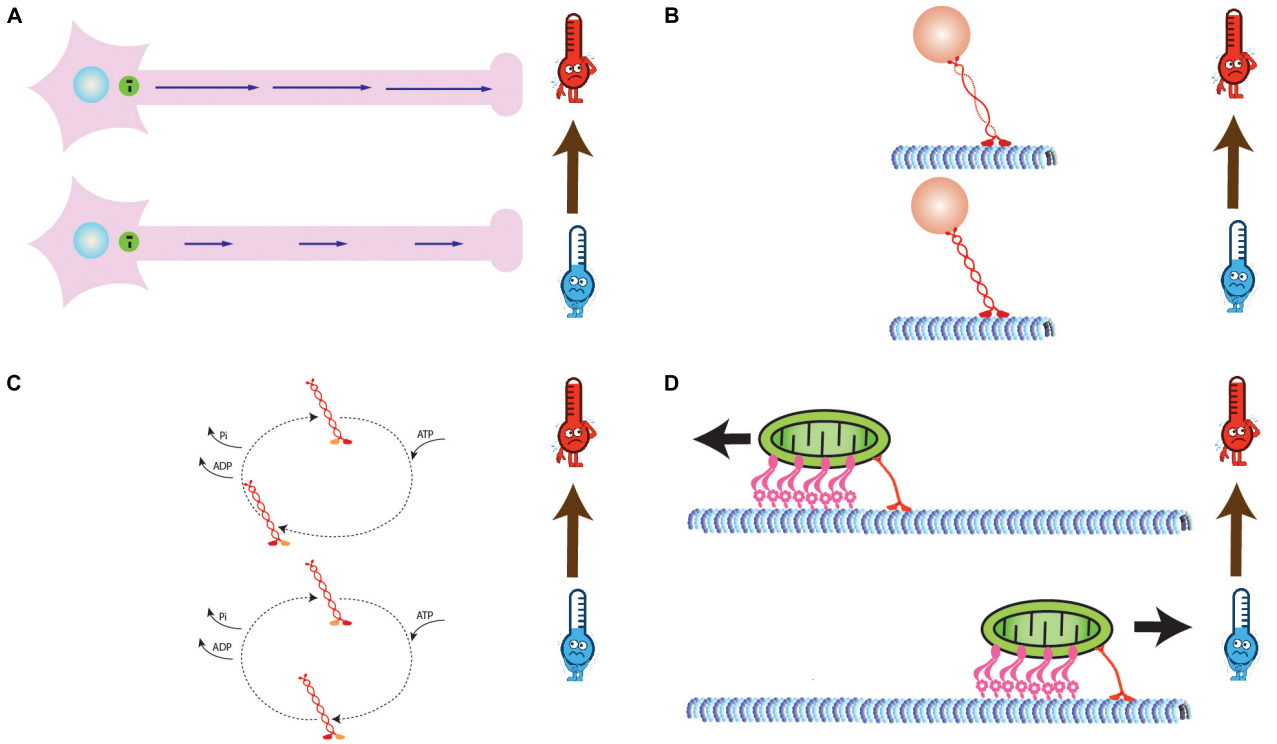

FIGURE 1 | (A) Intracellular transport velocity increases with increase in temperature. The length of blue arrows is proportional to magnitude of transport velocities. **(B)** Decrease in helicity of stalk domain of motor proteins with increase in temperature **(C)** Modified biochemical properties of motor proteins (Adenosine triphosphate (ATP) binding, Hydrolysis, and ADP + Pi release rates) due to change in temperature. The size of dashed arrows is proportional to time required for reactions. **(D)** Reversal of direction of bidirectional transport due to change in temperature dependent biophysical properties of a set of antagonistic motors (Kinesin-1 and Dynein).

traveled by tagged leucine during the incubation time. They found the rate to increase non-linearly from 32 mm/day at 5.5°C to 290 mm/day at 28°C. Experimentally random values of rates were observed due to instability of protein and its surrounding. However, curve extrapolation yielded a rate to be 400 mm/day approximately. It was suggested that increased electrical activity in the nervous system caused enzymatic changes which alters the metabolic rates in cells (Edström and Hanson, 1973). Mammalian muscle contraction and ATPase cycle rate were studied in rat and mouse muscles' myofibrils from 8 to 38°C. ATPase rates, rate of increase in tension and maximum velocity had similar Arrhenius temperature dependence with energy of ∼66 kJ/mol. The temperature coefficient (Q_{10}) of tension relaxation had a value of 2.5, which means that its rate constant increased 2.5 times with ΔT of 10°C (Stein et al., 1982; Rall and Woledge, 1990). Another study on rat skeletal muscle between 20 and 35°C showed Arrhenius dependence with activation energies to be 40–45 and 60–80 kJ for shortening and relaxation, respectively (Ranatunga, 1982; Roots and Ranatunga, 2008). A study that dates back to 1984 examined force-velocity relationship of fast and slow-twitch muscles in rats in the range of 10–35°C. The curve of force-velocity increased with cooling for both muscles with Q_{10} decreasing by factors of 1.8 from 35 to 25°C and 2.4 below 20°C (Elmubarak and Ranatunga, 1984). Later, the emergence of fluorescence microscopy to study temperature dependent properties of Myosin proteins helped in *in vitro* temperature dependent properties of velocity and processivity of a single motor protein (Anson, 1991; Pierce and Vale, 1998).

Earlier reports on temperature dependent cellular transport were mostly done in terms of transport rate, temperature coefficient Q_{10} and velocity of tagged protein or vesicles transported inside cells. These studies show Arrhenius or exponential dependence of the above listed parameters with temperature. Additionally, temperature coefficient is indicative of underlying transition in their behavior and may be structural or conformational properties too. These properties have been explored in the later studies which we will be discussing in the sections to follow.

TEMPERATURE-DEPENDENT MOTOR PROPERTIES: ARRHENIUS RELATIONSHIP AND ARRHENIUS BREAKS

Temperature-Dependent Biochemical Properties of Molecular Motors

ATPase activity is the ability of motor proteins to catalyze the decomposition of ATP. This process is known as ATP hydrolysis

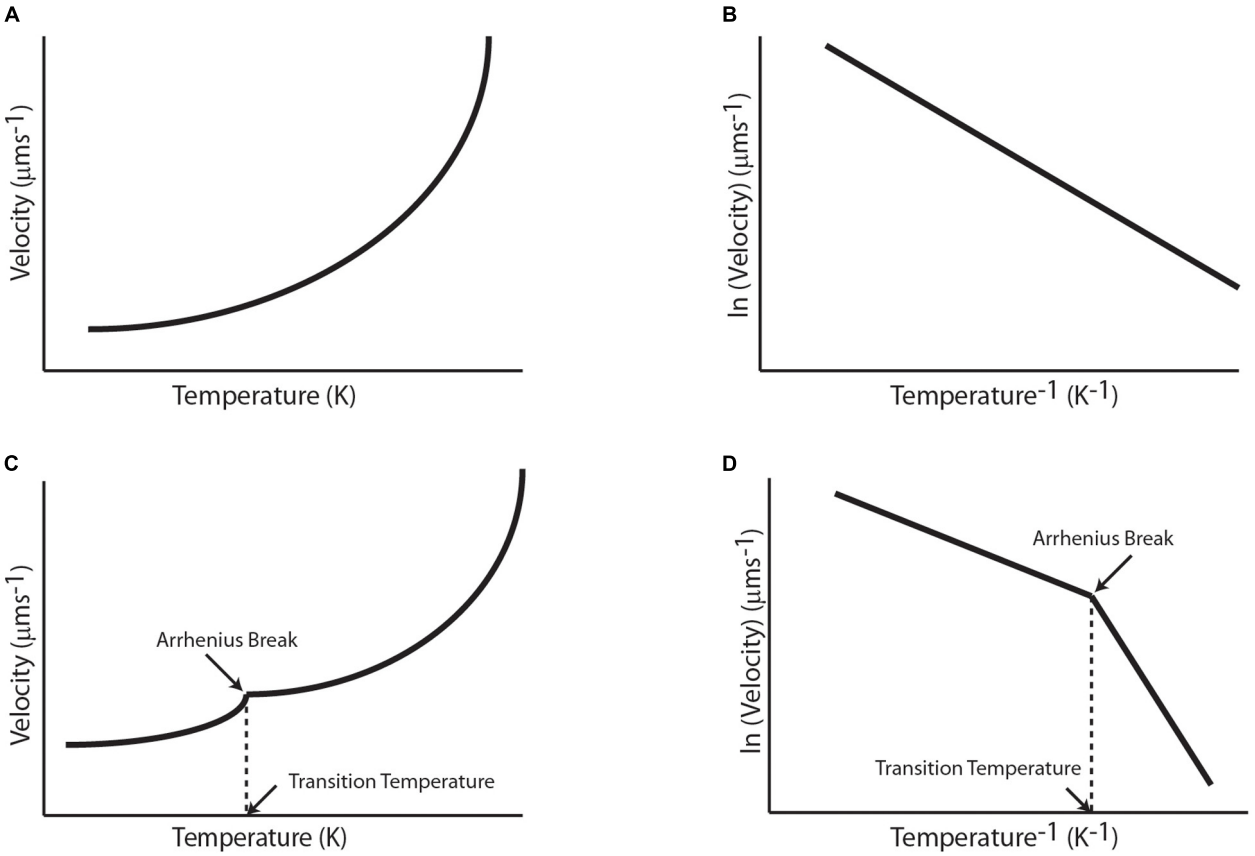

FIGURE 2 | Qualitative graphical representation of reported temperature dependence of biophysical and biochemical properties of molecular motors with temperature for **(A)** A simple Arrhenius enzyme, **(B)** corresponding Arrhenius plot of a simple Arrhenius enzyme, **(C)** A complex Arrhenius enzyme, and **(D)** corresponding Arrhenius plot of a complex Arrhenius enzyme.

and releases the energy which is used for mechanical motion by molecular motors. Temperature dependence of their ATPase activity determines the catalytic efficiency of motor proteins and points towards their confirmational modification as an underlying molecular mechanism as observed in various studies; we will be discussing for both Linear as well as Rotary in subsequent sections.

Temperature Dependent ATPase Activity of Linear Motors

Molecular motors derive their energy by catalyzing the decomposition of Adenosine triphosphate (ATP), known as the energy molecule of a living cell. Roughly, a kinesin's energy efficiency based on the maximum load it can bear (6 pN) is around ∼60%, which is quite higher than any conventional man-made motor. It has been a subject of researchers' curiosity as to how these motors function in different temperature ranges of cold-blooded or hot-blooded animals and their catalytic efficiency. Temperature dependence of their ATPase activity indicates conformational modification in their head domains or stalk fragment. Hence, these studies focus on either structural modification or rates of catalysis. Temperature dependent *in vitro* studies of different motors reveal Arrhenius-like catalytic behavior of these motors (de Cuevas et al., 1992; Böhm et al., 2000).

Circular Dichroism (CD) based structural analysis of Kinesin from *Drosophila melanogaster* reveals that its stalk fragment is ∼55–60% α-helical at 25°C and 85–90% α-helical at 4°C and 0% β-sheets at either temperature ranges, hence suggesting that stalk fragments of these proteins melt in solution while approaching physiological temperature ranges (de Cuevas et al., 1992). Further, they report that coil 1 of Kinesin stalk is thermally more stable as compared to coil 2 (**Figure 1B**). This might regulate the binding properties of kinesin. However, based on their observation, they conclude that stalk may regulate force production *in vivo*, but it does not affect the *in vitro* motility of Kinesin and Myosin motor (Toyoshima et al., 1987; Lovell et al., 1988; de Cuevas et al., 1992). Additionally, they report that Kinesin motor protein undergoes conformational modification which breaks its Arrhenius dependence into two major temperature ranges, i.e., 25–30 and 45–50°C. Hence, it indirectly reflects that conformational change might be the underlying molecular mechanism for piecewise Arrhenius trend for molecular motors. It can be inferred from these observations that conformational changes lead to a change in enzymatic reaction times (rate-limiting step) and rates

(**Figure 1C**), hence the trend (de Cuevas et al., 1992). However, this study did not evaluate temperature dependent transport properties of Kinesin motor protein (no subtypes of Kinesin were discovered back them). A study involving gliding motility assay of kinesin for temperatures between 5 and 37°C explored the effect of conformational changes on kinesin gliding velocity and ATPase activity. Both, the gliding velocity and ATPase activity showed Arrhenius dependence on temperature with a break at 27°C recorded for both and activation energies to be 79 and 5 kJ/mol for temperature range above and below 27°C. Gliding velocity and ATPase activity have a similar profile and are linearly correlated and increased up to 37°C. However, beyond this range, MTs detach from the surface rigorously, which leads to decrease in gliding velocity and ATPase activity. A linear correlation between gliding velocity and ATPase activity in this study suggests an efficient motor system utilizing chemical energy for mechanical force generation. Through this study, the authors studied correlation between ATPase activity and motor processivity to know if a step in ATPase cycle is rate-limiting and responsible for similar thermal dependence of velocity. The correlation plot revealed linear curve between velocity and ATPase activity. This curve can be used to qualitatively estimate the efficiency of conversion of chemical energy to mechanical energy by Kinesin (Böhm et al., 2000).

Another study on kinesin-3 motor protein derived from *T. lanuginosis* estimates its ATP hydrolysis rate with respect to temperature and ATP concentration and was reported to follow Michaelis-Menten kinetics with change in ATP concentration, while it was found to be constant, i.e., $\sim$1 s^{-1} (non-coupling between ATPase rate and gliding velocity) with temperature. Authors claim that this is due to turnover rate of the reaction to be below signal-to-noise threshold of the assay since gliding motility assay show Arrhenius behavior in this temperature range. Hydrolysis rate displayed a sharp increase from 45 to 65°C with maximum values in the range of 60–65°C ($\sim$6 s^{-1}) (Rivera et al., 2007).

Temperature-Dependent ATPase Activity of Rotary Motors

F_1-ATPase is a rotary motor protein which couples ATP hydrolysis to mechanical rotations. They are membrane bound motors which either use NADH coupled energy to make ATP energy molecules (mitochondria) or use ATP hydrolysis for rotational motion and act as channels of uptake or release (cell membrane). They make rotations in steps of 120°. Temperature dependence of their ATPase activity and torque generation has been speculated upon with different approaches. An experimental study of mitochondrial membrane composition on ATPase activity was done in *Saccharomyces cerevisiae* containing different concentrations of ergosterol (7.3 mg/g protein) showed not only Arrhenius dependence on temperature but breaks in this behavior too with transition temperature decreasing from 34 to 18°C as the sterol content increased from 7.3 to 105 mg of ergosterol/g protein. This early study reflects that it can be used to tune the ATPase activity of mitochondrial F_1-ATPase motor protein (Cobon and Haslam, 1973). Another study involving F_1 rotary motor shows Arrhenius Break at 17°C with the increase in Activation Energy and points toward its conformational change with temperature (Baracca et al., 1986). Analysis of Bovine-heart F1-ATPase using circular dichroism showed conformational changes in the protein which resulted in a break in the Arrhenius plot with 2.7-fold increase in ATP hydrolysis activation energy. Earlier studies along with the present study show temperature dependence of ATP hydrolysis activity and the same also being the rate determining step for ATPase activity of F_1 rotary motor. Although, transition temperature may vary for motors derived from different sources, the conformational change was observed in all of them nevertheless (Baracca et al., 1989). However, these studies do not report reaction intermediate and rate-limiting step in the ATPase activity of F_1-ATPase. In the subsequent work, the analyses of F1-ATPase rotation at different temperature values (2, 4, 9, and 23°C) showed ADP release to be an intermediate step and also temperature dependent. Hence, it would competitively suppress the rate of ADP release through negative feedback by regulating the concentration of free ADP in the solution. Additionally, they reported an unusually high Q_{10} factor of 19. This could be due to high conformational change in the protein with an increase in temperature (Watanabe et al., 2008). Another study on beef mitochondrial F1 (thermophilic F1) shows that it remains active in the range of 0–90°C with ADP release as the intermediate temperature dependent step.

F1-ATPase is a mixture of an active and an inhibited domain. After the addition of nucleotide-free F_1, most of the F1 molecules are active. However, at higher temperatures, comparatively fewer F1 molecules are active with 35°C becoming an equilibrium mixture of active and inhibited populations. This is self-explanatory as its physiological role is ATP synthesis and not ATP hydrolysis or its consumption and thermophilic F1 is stable with its ATPase activity peak at 70°C. The rates of F1 rotation increase with temperature which is expected for any protein until it denatures (Furuike et al., 2008).

All the linear or rotary motors follow Arrhenius dependence on temperature of their catalytic behavior and have been speculatively suggested to undergo structural modifications or conformational changes. This modification is more evident around the transition temperature which results in the change in activation energy causing Arrhenius Breaks. Some *in vitro* studies focus on attaining thermal stability of motors and study different domains to understand which of them plays a crucial role in their function and processivity. Studies done so far reveal that the stalk fragments or the coils of motors are temperature sensitive and undergo major modifications. It might be due to their maximum exposure to the surface in 3D conformation of the motor.

Temperature-Dependent Biophysical Properties of Motors

Molecular motors harness chemical energy from ATP energy packets to perform mechanical motion such as translation, rotation, or sliding. Motors entail certain characteristic properties that govern the mechanical behavior of motors. These are categorized as biophysical properties of motors. Processive or linear motors carry cellular bodies from one place to another

in a directed manner by trailing on cytoskeletal filaments (actin and microtubules). Their biophysical properties include rates of attachment, stepping, and detachment on and from their tracks, processivity, and velocity. Processivity is the total distance traveled by a motor before detaching from its track. On the other hand, rotary motors' rates of rotation are defined in terms of the rotation steps of 120°. Rotary motors are embedded in the bi-layer membranes of cells and membranous organelles of the cells. F1-ATPase is a rotary motor that drives the electron pump to generate ATP energy molecules.

Temperature-Dependent Biophysical Properties of Linear Motors

Motor proteins are involved in specific transport mechanism inside cells, for example, cytoplasmic Dynein and Kinesin motors working in groups inside cells, Myosin II involved in muscle contraction and relaxation (Howard, 1996; Hirokawa, 1998; Kato et al., 1999). Hence, all the motors have unique biophysical properties according to their function. Additionally, Kinesin and Dynein being antagonistic pair of motors constantly undergo tug-of-war to haul cargo (bodies that are transported by motors inside cells such as mitochondria) to their desired locations. Some studies have also reported sliding mechanism as a means of cargo transport. This majorly happens for non-processive motors which are tethered and the cargo body slides over them (Stepp et al., 2017).

Force-velocity relationship defines the load-bearing capacity of a motor (Svoboda and Block, 1994). Kinesin motors have been most extensively studied motors due to their stability in different *in vitro* experimental conditions. Kawaguchi in 2000 used gliding assay to estimate velocity and run-length, and bead assay to estimate the velocity of a single kinesin (Sakowicz et al., 1999). Gliding assay and bead assay are two *in vitro* assays to study different properties of a single motor under a microscope (Howard et al., 1989; Block et al., 1990). In bead assay, stabilized microtubules are tethered to glass surfaces and motors are in the solution, where as in gliding assay, motors are tethered to the glass while microtubules slide over them (Yamashita et al., 1994). Kinesin motor's velocity was reported to increase with Arrhenius activation energy of 50 kJ/mol and average run-length of the motor increased qualitatively from ∼0.7 to ∼1.5 μm between 15 and 35°C. Increased processivity implies decreased possibility of detachment of kinesin with increasing temperature. However, to author's surprise, generated force remained unchanged thermally with an average value of ∼7 pN. Based on the findings, force generation was coupled with nucleotide-binding state or conformational change in kinesin, whereas velocity was coupled with temperature-sensitive ATPase activity. Hence, both the properties are coupled with different steps of mechanochemical cycle of kinesin motor. They also studied the force-velocity relation at different temperatures and the curve was linear for all temperature ranges. Thermal tuning of velocity without change in its force generation capacity determines the efficiency of motors to utilize the chemical energy derived from ATP molecules in their mechanical motion (Svoboda and Block, 1994; Kawaguchi and Ishiwata, 2000). However, Shin'ichi Ishiwata in his repeated studies for different temperature ranges did not report any breaks in the temperature dependent Arrhenius relation of Kinesin motor protein. They estimated temperature dependent biophysical properties between 15 and 35°C for kinesin in 2000. Later in 2001, they used temperature pulse microscopy (TPM) which helps to elevate temperatures till the boiling temperatures to analyze the same properties at higher temperatures, as the proteins used to degrade at higher temperatures earlier. It uses illuminating thin metal layer, evaporating it on the glass surface with infrared laser beam. The illumination is shut off and on periodically and heat dissipates into the surrounding medium in 10 ms. It allows to record motor activity at elevated temperature by activating it thermally. TPM was used by Kawaguchi to estimate the gliding velocities of MTs at temperatures higher than 35°C up to 53°C. The average velocities were obtained from 3.65 μm/s at 50°C to 0.48 μm/s at 20°C. However, repeated thermal activation damaged kinesin molecules and acted as an internal load for their movement. They obtained the velocity values within the temperature range of 15 and 35°C and the curve fitting was done with no Arrhenius breaks, activation energy being 50 kJ/mol (Kawaguchi and Ishiwata, 2001). They did not record any Arrhenius breaks in this study too (Kawaguchi and Ishiwata, 2000, 2001). However, in 2006, a quantitative estimation of run-length, duration, and velocity was done using single kinesin isolated from bovine brain between 20 and 40°C. This group recorded deviation of linear Arrhenius velocity curve above 30°C with activation energy to be 48 kJ/mol, which is quite close to the earlier recorded values, i.e., transition temperature to be 27°C and activation energy 50°C. However, they base these findings on the deterioration of proteins at elevated temperatures, and hence, the less steep slopes with slower rates of ATP hydrolysis and decreased velocity. Additionally, detachment probability was calculated indirectly from one cycle of kinesin stepping (8 nm). Average run-length was reported to increase from 0.6 to 1.3 μm and average duration decreased from 1.5 to 0.7 s at 20 and 40°C, respectively. On the other hand, velocity followed a Gaussian distribution at each temperature, increasing exponentially following Arrhenius relation with temperature. The activation energy was found to be 48 kJ/mol (in accordance with the previous value, i.e., 50 kJ/mol). Detachment probability decreased with temperature from 0.0143 at 20°C to 0.0062 at 40°C. Here, temperature dependence of run-length is weaker than velocity as duration is inversely proportional to temperature and run-length is the dependent parameter among all three of them (Nara and Ishiwata, 2006). However, all of these studies have been done for dimeric kinesin. Thermal biochemical and biophysical properties of monomeric protein, i.e., Kinesin-3 were studied by the research group of George D. Bachand. They studied temperature dependent gliding motility rates and ATP hydrolysis rate for kinesin-3 (TKIN) isolated from *Thermomyces lanuginosus* (fungus). Apart from temperature dependence, gliding velocity was observed with ATP concentration and followed Michaelis-Menten kinetics with the change in ATP concentration. The gliding velocity displayed Arrhenius dependence on temperature with maximum velocity of 5.5 μm/s at 45°C. MTs depolymerized beyond

this temperature range and failed to bind to tethered kinesin molecules. The Arrhenius energy (∼103 kJ mol^{-1}) is quite high and almost twice than those reported for Eg5 or ncd-like kinesin (Crevel et al., 1997). Hence, authors conclusively state that TKIN belongs to thermostable enzymes as they display increased Arrhenius energies. Additionally, they reported the break both for ATP hydrolysis as well as gliding velocity for Kinesin-3 *in vitro* at 27°C. Their recorded range is as high as 65°C for ATP hydrolysis, whereas 45°C for velocity (Rivera et al., 2007). Beyond this range, microtubules become highly unstable and do not bind to kinesin-coated surfaces which results in a sharp decrease in velocity. Interestingly, in this study both the activities follow similar Arrhenius behavior above 20°C (Aquilanti et al., 2010). Below this range, ATP hydrolysis curve goes flat. This might be due to turnover rate being in the range of noise as stated earlier. Surprisingly, the Kinesin-3 seems to be the fastest motor at physiological temperature range (Rivera et al., 2007; Hong et al., 2016) with the velocity of 5.5 μm/s. Despite this, its turnover rate is low. This discrepancy may be due to motors being less processive and non-cooperative to haul cargo (Rivera et al., 2007).

Apart from the Kinesin superfamily, cytoplasmic Dynein is also a processive motor that transports cellular entities from cell periphery to the nucleus. Additionally, these motors are also a part of the flagellum in cells and enable their beating to help the cells move in a defined gradient of a micronutrient. An *in vitro* single molecule-based study was conducted on the velocity of mammalian Kinesin-1, mammalian cytoplasmic Dynein and yeast cytoplasmic Dynein. Mammalian cytoplasmic Dynein showed the break at 15°C, yeast Dynein showed the break at ∼8°C, whereas Kinesin-1 showed no breaks. This implies that Dynein motors are thermally sensitive motors, hence more tunable. This study emphasized on the tuning of the net direction of cargo transport by varying number of Dynein and Kinesin motors, respectively. Since transport inside cells is carried out by a team of different kinds of similar as well as antagonistic motors, hence this sheds light at more realistic picture of intracellular transport. The net transport direction of a cargo is determined by the biophysical properties of each type of engaged motors in the team. Additionally, tuning of these rates can be done by varying the number or physiological conditions of the surrounding environment. This can help change the net direction of transport, run-length as well as velocity of both unidirectional and bidirectional transport. In the temperature-dependent study of Kinesin-1 and Dynein, the velocity and detachment rate of Kinesin-1 and mammalian cytoplasmic Dynein were found to follow Arrhenius relation with temperature. The velocity of Dynein exhibited a break from simple Arrhenius relation at 15°C, whereas Kinesin-1 showed no such behavior. On the other hand, yeast cytoplasmic Dynein showed this break at ∼8°C. Additionally, they put forth temperature dependent mathematical expression of detachment rate, velocity, and run length of single Kinesin-1 and mammalian Dynein motors. Extremely high activation energy below 15°C for Dynein means that Dynein is more sensitive to temperature, shuts down at low temperatures, and hence it is thermally more tunable, whereas Kinesin-1 is a relatively thermostable motor. Hence, in bidirectional transport governed by Dynein and Kinesin-1 motors in the ratio of 4:1, cargo tends to move in the negative direction of MTs at higher temperatures (>15°C), whereas at lower temperature range, it moves in the positive direction (**Figure 1D**; Hong et al., 2016). Hence, Kinesin-1 dominates in lower temperature ranges. Study of temperature dependent biophysical properties of Kinesin-1 motor protein *in vitro* has always been limited below 30°C due to its degradation. However, such is not the case inside a cell as it functions well at temperatures close to 40°C.

Next, there is a class of axonemal Dynein motors present in beating flagellum which are responsible for their periodic beating with microtubules sliding over the tethered motors. This environment is similar to *in vitro* gliding assay, i.e., Dynein motors inside flagellum function in a similar set-up. This explains the Arrhenius dependence of flagellum's oscillation frequency in the observed temperature range of 5–35°C (Machemer, 1972; Coakley and Holwill, 1974; Mitchell, 2007; Warren et al., 2010). Flagellum oscillation frequencies showed breaks at 17°C. These locomotive organelles contain tethered Dynein motors which slide microtubules in a periodic manner and facilitate the beating of the flagellum. Hence, Dynein motors are responsible for spontaneous oscillations (SO) and the underlying drivers for piecewise Arrhenius dependence. Corresponding activation energies are 30 and 43 kJ mol^{-1} above and below 17°C (Warren et al., 2010). The SO frequency ($w(T)$) was expressed as a function of temperature as $\ln(w(T)) = \frac{-E}{RT} + \ln(K)$, where T is the temperature, E is activation energy, K is pre-exponential factor, and R is the gas constant.

On the other hand, the study of F-actin sliding on skeletal myosin *in vitro* between 3 and 42°C shows change in its velocity from 11 nms^{-1} at 3°C to 12 μms^{-1} at 42°C. Additionally, their experiments showed the same curve for both cooling and heating indicating reversibility of the temperature dependent rates. However, Arrhenius break at 15.4°C and activation energies are reported to be 50 and 289 kJ/mol for temperatures >15and <15°C, respectively. The energy for lower temperature range is abnormally higher. This indicates that Myosin might have a different rate-limiting step unlike other molecular motors due to drastic difference in activation energies of both the ranges. Additionally, the temperature dependence of ATP hydrolysis and velocity of Myosin motors above 20°C are similar but different at lower temperatures (Anson, 1991; Higuchi and Goldman, 1991). Detection of transport velocity of rhodamine phalloidin-labeled F-actin moving *in vitro* on rabbit skeletal myosin revealed a non-linear Arrhenius plot by M. Anson in 1992 (Anson, 1991). The curve was fitted to follow a cubic relation with temperature. Additionally, the author report same profile for both cooling and heating which indicates reversibility in the rates of enzymatic behavior of motors. However, the curve was divided in two linear ranges with activation energies to be 50 kJ/mol above 15°C and 289 kJ/mol below it with corresponding temperature coefficients (Q_{10}) to be 1.9 and 76.5. This study reports three orders of magnitude increase in actin velocity. However, results differ at lower temperatures, i.e., the curve follows a linear relation roughly (Anson, 1991). Hence, this study provides evidence

that temperature dependence of velocity and ATP hydrolysis might be uncoupled.

Briefly, so far temperature-based studies of motors recorded breaks from Arrhenius activity for Dynein and Myosin motors from different sources, namely, Drosophila, mammals, rat brain sciatic nerves, etc. (Edström and Hanson, 1973; Gross, 1973; Cosens et al., 1976; Highsmith, 1977; Stein et al., 1982; Rall and Woledge, 1990). However, for Kinesin motor proteins, studies contradicted each other in reporting deviations or breaks. It has been reported that Kinesin motors obtained from different sources show deviations at different temperatures. *Drosophila* Kinesin-1 shows transition at 17°C, whereas bovine brain Kinesin-1 reported this transition at 27°C (Crevel et al., 1997; Böhm et al., 2000). Research group of M. Vershinin introduced TMAO as the heat-protecting agent to prevent degradation of Kinesin-1 *in vitro*. 200 mM TMAO successfully stabilized the kinesin till 50°C (Chase et al., 2017). Although, it has been long known that velocity follows a Gaussian distribution with peak at the mean velocity at a fixed temperature, the same was demonstrated for Kinesin-1 and Kinesin-3 (KIF1A) (Chase et al., 2017; Doval et al., 2020). Furthermore, single-molecule *in vitro* experiments on Kinesin-1 and Kinesin-3 motility reveal Arrhenius dependence of velocity on temperature both in the presence of ATP as well as GTP. However, average velocity decreased with the use of GTP as an energy molecule and the Activation energy for GTP was higher (Doval et al., 2020). The main focus of this work was to study Arrhenius breaks for Kinesin motor proteins. They report break for Kinesin-1 at 4.7°C and for Kinesin-3 at 10.5°C. These transition temperature values clarify the disputed breaks for Kinesin motor proteins, i.e., they fall outside the physiological range and hence were uncovered in the temperature regimes taken before. Additionally, they observed that these transition temperatures can be varied from 10.5 to 16°C for Kinesin-3 by using 200 mM TMAO *in vitro* (Ma et al., 2014; Doval et al., 2020). A review done in 2003 focused on the maintenance of intracellular transport due to unfavorable temperature conditions. They report the attribute of hibernation in mammals follows extended bouts of torpor, during which minimal body temperature (Tb) can fall as low as −2.9°C and metabolism can be reduced to 1% of euthermic rates (Carey et al., 2003).

Transport and motor properties discussed above often showed Arrhenius dependence on temperature and sometimes a piecewise Arrhenius-trend, i.e., the linearity of Arrhenius plot breaks at a transition temperature which divides the curve in two range of temperatures with distinct activation energies.

Temperature-Dependent Biophysical Properties of Rotary Motors

As discussed earlier, rotary motors are known to undergo drastic conformational modification which alters their ATPase activity. Additionally, these studies explore their rotational motion as a function of ATP concentration as well as temperature. The research group of Hiroyuki Noji in 2008 reported that rotational rate followed Michaelis–Menten relation with ATP concentration at 23 and 4°C. Similarly, the rates increased slightly with temperature from 4 to 23°C (Watanabe et al., 2008). In a different study, rotations of Beef heart F1-ATPase were recorded in the temperature range of 4–40°C. The rates were observed in distinct steps of 120° at saturating ATP concentrations (2 mM) and were observed to increase with temperature with Arrhenius dependence. Apparently, they did not observe any breaks in this behavior and ADP release was observed to be rate-limiting step (Furuike et al., 2008). Another study by Hiroyuki Noji focused exclusively on biophysical properties of F1 rotary motion and used magnetic tweezers to understand temperature dependence of rotational torque by analyzing temperature sensitive (TS) reaction (intermediate reaction of ADP release) and hydrolysis dwell times. They studied both k^{on} and k^{off} rates of TS reaction signifying forward and reverse reaction. K^{on} increased exponentially by approximately 6.2-fold per 20°C, whereas k^{off} remained constant at 0.3 s^{-1}. This implies strong temperature sensitivity of rotation at all temperature ranges (Watanabe and Noji, 2014). Quite recently, the effects of temperature on the angular velocity profile of the 120°F_1-ATPase power stroke were resolved at a time resolution of 10 μs (Martin et al., 2018). The angular velocity of F_1-ATPase changed inversely with temperature during phase 1 (0°–60°) with a parabolic dependence resulting in negative activation energy values. This directly indicates that it was powered by elastic energy of a torsional spring consistent with unwinding the γ-subunit coiled-coil. In contrast, Phase 2 of the power stroke had an enthalpic component suggesting that additional energy input was required to enable the γ-subunit to overcome energy stored by the spring after rotating beyond its equilibrium position. The correlation between the probability distribution of ATP binding to the empty catalytic site and the negative activation values of the power stroke during phase 1 indicates that this additional energy is derived from the binding of ATP to the empty catalytic site. Based on these observations, an elastic coupling mechanism was proposed that uses the coiled-coil domain of the γ-subunit rotor as a torsion spring during phase 1, and as a crankshaft which powered by ATP-binding–dependent conformational changes during phase 2 to drive the power stroke (Martin et al., 2018).

The indirect implication of studies discussed above on linear and rotary motors is that not only Arrhenius relationship but also break in this profile is a characteristic behavior of molecular motors as they undergo conformational change. However, the scale of this modification can vary over different motor families or source organisms determining the transition temperature, thermal stability, or sensitivity of these biomolecules.

DISCUSSION

In this review, we briefly list and discuss the major findings of temperature dependent kinetics of molecular motors and the energies involved, the abruptness or the abnormal behavior observed and the underlying molecular mechanisms. We started with preliminary studies of pre-motor era when different types of molecular motor proteins and their possible roles were not known. These research publications focused straightaway on successfully varying surrounding temperature of the sample and studying the transport rates of tagged proteins (DBH, leucine,

etc.) inside the cells (Gross, 1973; Cosens et al., 1976). Others focused on understanding the rates of tension (extension and contraction) in muscle cells *in vitro* (Stein et al., 1982; Rall and Woledge, 1990). Additionally, the temperature dependence of rates was evaluated in terms of temperature coefficient Q_{10} which revealed the ratio of rates at the difference of 10°C.

Temperature change tends to modify conformation of both linear as well as rotary motor proteins. These modifications have been presumed to be responsible for change in enzymatic activity of motors, hence changing their rates of ATPase activity in ATP hydrolysis. On the other hand, it is perceived that modified rates of ATP hydrolysis reflect in the duration of each step in the motor stepping cycle. Hence, influencing the properties of velocity, runlength, force production, etc. Therefore, both mechanochemical and biophysical properties of motors have been studied with variation in temperature. With the study of structure of kinesin motor protein, it was found that one of the two domains of its stalk fragment are less stable with temperature which leads to the change in binding properties of kinesin with temperature. In the next series of studies, attempts were being made to unwind any hidden correlation between Arrhenius relation of ATPase activity and velocity of kinesin. However, different temperature regimes were followed in these studies. One of the studies reported linear curve of correlation between the two phenomena (27–35°C), whereas the other study reported deviation of ATP-hydrolysis curve from linear Arrhenius behavior below 20°C. This was, however, attributed to lower turnover rate of the studied kinesin-3 motor protein and different kinesin proteins used in these studies (Crevel et al., 1997). Rotary motors on the other hand show temperature dependence due to ADP release step which is said to be its temperature-sensitive (TS) reaction. Similar to linear motors, they also follow Arrhenius relationship with temperature but their Q_{10} factor is unusually high (17) due to high conformational change. However, this is only speculative theory and has not been subjected to experimental validation or *in silico* simulation studies on 3-D protein conformation.

The advancement of single-molecule experiments, optical trap, temperature pulse microscopy (TPM) and Fluorescence Total internal reflection microscopy (FTIR) facilitated *in vitro* studies of biophysical properties of molecular motors and their subtypes. Bead assay and gliding assay are two kinds of *in vitro* methods to study transport properties of motors under different physiological conditions. Hence, temperature dependent biophysical parameters, i.e., Force generation, run-length, velocity, and rate of detachment are studied using these assays (Mao et al., 2005). A series of *in vitro* studies focused on the behavior of motor velocity, its run-length and force production capacity with different ranges of temperatures. Interestingly, velocity and run-length increased exponentially following Arrhenius relation with temperature, but force generation capacity of motors remained unchanged (average force ∼7 pN for Kinesin). This discrepancy is attributed to force generation being dependent on temperature-independent nucleotide-binding step whereas velocity being dependent on temperature-dependent ATP hydrolysis cycle. Force-velocity curve followed linear relation at different temperatures (Kawaguchi and Ishiwata, 2000). The ability of motor to translate the generated force into its processivity shows its efficiency. Hence, the slope of force-velocity curve at different temperatures qualitatively reflects the efficiency of that motor. Studies of kinesin motors revealed maximum efficiency of 60% for these nanomachines in the physiological temperature range. Molecular motors are also present in beating organelle-flagellum of a cell. Spontaneous oscillation (SO) frequencies of flagellum were reported to show Arrhenius dependence on temperature. This is reflective of temperature sensitivity of Dynein motors present in these organelles (Warren et al., 2010). Furthermore, this study also explicitly recorded the break in temperature dependent curve at 17°C. However, it cannot be conclusively stated that this is due to property of engaged Dynein motors in flagellum. But later, in a follow up, *in vitro* single-molecule experiment of mammalian and yeast Dynein revealed break at ∼15 and 8°C (Hong et al., 2016). Kinesin-1 on the other hand did not show any breaks in the observed temperature range. However, they not only studied temperature dependence of biophysical properties of single motor molecules *in vitro*. They also put forth theoretical expressions for temperature dependence of detachment rate, stepping rate, velocity, and run-length for Dynein and Kinesin-1 motor protein. Furthermore, they also explained physical effects of these properties on bidirectional transport by the group of these antagonistic motors. Dynein is thermally more sensitive whereas Kinesin-1 is the thermally stable motor. Thus, Dynein motors in the ratio of 4:1 with Kinesin-1 leads the cargo in negative direction with increase in temperature, whereas Kinesin-1 motors dominate the transport at lower temperature ranges and haul the cargo in positive direction of microtubules (**Figure 1D**). These observations are reflective of the concentration, roles, and positioning of these motors in cellular transport of organisms with different body temperatures and requirements (warm-blooded mammals, cold-blooded reptiles, ectotherms, etc.). In another study, gliding of F-actins over Myosin proteins had shown the break at 5°C. Hence until recently, it was assumed that the property of Arrhenius breaks was limited to Dynein and Myosin motors, but Arrhenius breaks observed for Kinesin-1 and Kinesin-3 at ∼4 and 10°C show that motors of Kinesin superfamily follow piecewise Arrhenius trend too. Thus, it can be stated that Arrhenius breaks are universal to the behavior of molecular motors and can be tuned by varying the chemical concentration of motor proteins and energy molecules (Chase et al., 2017; Doval et al., 2020).

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SY and AK wrote the manuscript together. Both authors contributed to the article and approved the submitted version.

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REVIEW
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HIV-1 Hijacking of Host ATPases and GTPases That Control Protein Trafficking

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The human immunodeficiency virus (HIV-1) modifies the host cell environment to ensure efficient and sustained viral replication. Key to these processes is the capacity of the virus to hijack ATPases, GTPases and the associated proteins that control intracellular protein trafficking. The functions of these energy-harnessing enzymes can be seized by HIV-1 to allow the intracellular transport of viral components within the host cell or to change the subcellular distribution of antiviral factors, leading to immune evasion. Here, we summarize how energy-related proteins deviate from their normal functions in host protein trafficking to aid the virus in different phases of its replicative cycle. Recent discoveries regarding the interplay among HIV-1 and host ATPases and GTPases may shed light on potential targets for pharmacological intervention.

Keywords: HIV-1, GTPases, ATPases, HIV-1 accessory proteins, HIV-1 pathogenesis, HIV-1 trafficking

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; CCR5, C–C chemokine receptor type 5; CXCR4, C-X-C chemokine receptor type 4; AIDS, acquired immunodeficiency syndrome; HAART, highly active antiretroviral therapy; PIC, pre-integration complex; PM, plasma membrane; GDP, guanosine diphosphate; GTP, guanosine triphosfate; ATP, adenosine triphosphate; GAPs, GTPase-activating proteins; GEFs, guanine nucleotide exchange factors; ABC, ATP-binding cassette; Env, HIV envelope glycoprotein (precursor gp160, surface gp120, and transmembrane/cytosolic gp41); Gp41C, cytosolic tail of gp41; Nef, negative factor; Gag, group-specific antigen; NC, nucleocapsid domain of Gag; MA, matrix domain of Gag; Pol, polymerase; Tat, *trans*-activator of transcription; Vpu, viral Protein U; Vif, viral infectivity factor; Vpr, viral protein R; Rev, regulator of virion expression; RREs, rev response elements; CME, clathrin-mediated endocytosis; Ran, Ras-related nuclear; TRN-SR2/TNPO3, transportin-SR2; VLPs, virus-like particles; ARFs, ADP-ribosylation factors; GGA, golgi-localized, γ-ear containing, ARF-binding; AP, adaptor protein complex; RNAi, RNA interference; Rab, Ras-related in brain; BST2/Tetherin, bone marrow stromal cell antigen 2; DCs, dendritic cells; VSs, virological synapses; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; MPRs, mannose 6-phosphate receptors; TIP47, tail-interacting protein of 47 kDa; ABCE1, ATP-binding cassette sub-family E member 1; TULA, T-cell ubiquitin ligand; FIP1C, Rab11-family interacting protein 1C; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; PI(3,4,5)P3, phosphatidylinositol (3,4,5)-triphosphate; PI4KIIα, phosphatidylinositol 4-kinase type 2 α; Cdc42, cell division control protein 42 homolog; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog family member A; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin; LARG, leukemia-associated Rho guanine nucleotide exchange factor (Rho GEF); Tiam1, T-lymphoma invasion and metastasis-inducing protein 1 (Rac GEF); ESCRT, endosomal sorting complex required for transport; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; VPS4, vacuolar protein sorting 4; ILVs, intraluminal vesicles; MVBs, multivesicular bodies; TSG101, tumor susceptibility gene 101; ALIX/AIP1/PDCD6IP, programmed cell death 6-interacting protein; CHMP, charged multivesicular body protein; ABCA1, phospholipid-transporting ATPase ABCA1; HDL, high-density lipoprotein; apoA-I, apolipoproteinA-1; ERAD, endoplasmic-reticulum-associated protein degradation; CCV, clathrin-coated vesicle; MHC-I, major histocompatibility complex I; PI3K, class I phosphatidylinositol 3-kinase; ARNO, ARF nucleotide-binding site opener.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of acquired immunodeficiency syndrome (AIDS) and the cause of one of the longest and most devastating viral pandemics in human history. Although highly active antiretroviral therapy (HAART) inhibits the spread of HIV-1, the currently available treatments do not eradicate the virus from infected individuals, and viral mutations may confer resistance to the available drugs. Therefore, research into additional therapeutic strategies against HIV-1 is of high importance. In almost 40 years of intensive study, much has been learned about how HIV-1 manipulates the molecular machinery of the host cell to its own benefit. HIV-1 hijacks many host proteins to ensure an efficient replication cycle and to evade the immune response, leading to pathogenesis.

The HIV-1 replicative cycle in a host cell can be divided into early and late phases. The early phase (**Figure 1**) extends from virus entry to the integration of the provirus into the host cell genome and includes events such as the uncoating of the viral capsid, the reverse transcription of viral RNA to cDNA, the formation of the preintegration complex (PIC) and the nuclear import of the PIC. Although the order of these events is under debate (previously reviewed in Toccafondi et al., 2021). The late phase (**Figure 2**) starts with the transcription of the viral RNAs and comprises their subsequent nuclear export to the cytoplasm, the translation of the viral proteins, the trafficking of structural proteins to virus assembly sites, and the assembly, budding and maturation of the viral particle (previously reviewed in Freed, 2015).

The efficient transport of virus-derived proteins and nucleic acids to and from specific membrane-bound compartments within the host cell is critical in several steps of the HIV-1 replicative cycle. These translocation processes require specific transport and membrane remodeling machinery and a considerable amount of chemical energy provided by the host cell through ATP and GTP hydrolysis. Therefore, it is not surprising that HIV-1 co-opts several ATPases, GTPases and their regulators and effectors during infection and that these proteins are essential to virus replication and pathogenesis. Especially relevant among the GTPases are the small GTPases of the Ras superfamily, which are known to control critical processes implicated in intracellular trafficking. These include actin network dynamics, membrane specification, transport vesicle formation, translocation across the cytosol, and tethering to acceptor membranes. These monomeric proteins are found in GDP- or GTP-bound forms switching between inactive and active states in a cycle controlled by GAPs (GTPase-activating proteins) and GEFs (nucleotide exchange factors), respectively (previously reviewed in Itzen and Goody, 2011). When in their GTP-bound active state, these GTPases are membrane-associated and may interact with specific effector molecules.

This review presents examples and discusses data regarding the interplay between HIV-1 and host ATPases and GTPases (**Table 1**) involved in the intracellular trafficking of macromolecules and membrane modification. We will also discuss cases in which the subcellular localization of transmembrane ATPases themselves is altered by HIV-1 to aid virus replication and spread. Host restriction factors with ATPase and GTPase activity acting against HIV will not be covered here, and we refer to recent reviews (Ghimire et al., 2018; Staeheli and Haller, 2018).

HIV-1 CELL ENTRY

HIV-1 enters cells mainly by fusing its envelope membrane to the plasma membrane (PM) of target cells (**Figure 1**). This process requires physical interactions between the gp120 subunit of the virus envelope (Env) protein and specific host proteins at the cell surface that function as the main receptor (CD4) (Maddon et al., 1986; Mcdougal et al., 1986) and as coreceptors (either CXCR4 or CCR5) (Alkhatib et al., 1996; Dragic et al., 1996; Feng et al., 1996) for the virus. These interactions expose a fusion domain in the gp41 subunit of Env, leading to membrane fusion and the delivery of the capsid containing the viral genome into the cytoplasm (Wilen et al., 2012).

There is also evidence, obtained mainly from non-canonical HIV-1 target cells, indicating that the virus may enter cells via endocytosis (Fackler and Peterlin, 2000; Fredericksen et al., 2002; Daecke et al., 2005). Consistent with this model, the impairment of endosome acidification by drug treatments was shown to compromise the infection of polarized trophoblasts with HIV, suggesting that incoming viruses are delivered to endosomes, where the acidic pH would facilitate membrane fusion (Vidricaire and Tremblay, 2005). In fact, the expression of small GTPases Rab5 or Rab7 mutants, known to impair endosome maturation, also inhibited HIV-1 infection in those cells (Vidricaire and Tremblay, 2005). Recently, Marin et al. (2019) found that the knockdown of Rab5, Rab11A or the Rab effector protein RABEP1 decrease HIV-1 fusion with endosomes in CD4+ T cells, highlighting the importance of the endocytic machinery in the HIV-1 entry process.

Regardless of the exact pathway, evidence from independent studies indicates that the GTPase Dynamin participates in the HIV-1 entry process (Daecke et al., 2005; Miyauchi et al., 2009; de la Vega et al., 2011; Jones et al., 2017). Dynamin is a GTPase best known for catalyzing membrane fission during clathrin-mediated endocytosis (CME). Based on this well-characterized role, it was originally proposed that Dynamin is involved in HIV-1 entry by endocytosis, possibly via CME (Daecke et al., 2005; Miyauchi et al., 2009). The authors of this study showed that pharmacological inhibition of Dynamin or overexpression of a Dynamin dominant-negative (K44) mutant impaired HIV-1 infection (Miyauchi et al., 2009). However, the exact molecular connection between HIV entry and Dynamin was not elucidated.

A study by de la Vega and collaborators provided a hint on the mechanism by which Dynamin assists on HIV entry. They showed that Dynamin activity, which was inhibited with the drug dynasore, is required for the efficient fusion of the virus with target cell membranes and the release of the viral content into the cytosol (de la Vega et al., 2011). The authors proposed that in addition to playing a role in HIV-1 endocytosis, Dynamin may also facilitate virus fusion with the endosome membrane. Importantly, under dynasore treatment virus fusion with the PM was also impaired (de la Vega et al., 2011), most likely also

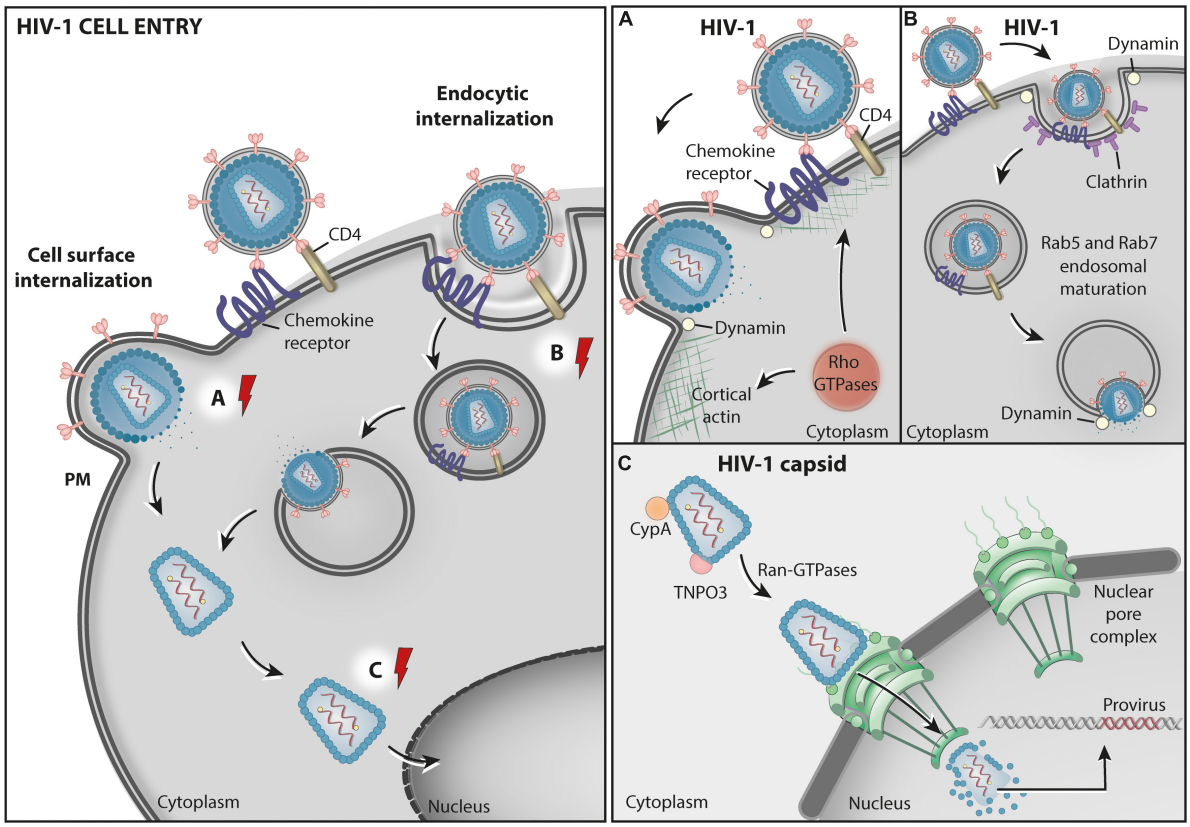

FIGURE 1 | HIV1 cell entry. Schematic representation of two proposed pathways for HIV-1 entry into cells. The cell surface internalization pathway involves the fusion between the viral envelope and the cell PM and is believed to be the main route for HIV entry into permissive CD4 expressing cells **(A)** – Rho GTPases: RhoA, Rac1, and Cdc42 participate in HIV-1 entry by promoting fusion complex stabilization, and fusion pore formation and expansion at the host cell surface through actin cytoskeleton remodeling. The HIV-1 entry pore is also stabilized by Dynamin GTPase activity, facilitating the release of the capsid containing the virus genome into the cytosol. In an alternative, poorly characterized, endocytic internalization entry pathway, HIV-1 particles are endocytosed via clathrin-coated vesicles (CCVs) and delivered to endosomes. **(B)** Dynamin GTPase activity is involved in both the formation of CCVs and the fusion between the virus and the endosome membrane. This pathway also involves Rab5 and Rab7 acting in endosomal maturation. **(C)** The viral capsid in the cytosol is transported to the nuclear pore by CypA, TNPO3, and the nuclear transporter GTPase Ran-GDP while the RNA is reverse transcribed into cDNA. At the nucleoplasm, the HIV-1 provirus is integrated into the host chromatin. The red electric ray symbols represent critical steps in the transport of viral factors that require energy.

contributing to inhibit viral entry in the experimental setup. Indeed, new data show that Dynamin participates in HIV-1 entry via a CME-independent mechanism (Aggarwal et al., 2017; Jones et al., 2017). In this process, Dynamin molecules associate to form tetramers that stabilize the HIV entry pore, facilitating the release of viral nucleocapsids into the cytosol through the actin cortex at the cell surface (Jones et al., 2017). The authors proposed that Dynamin may indirectly control this process by recruiting effector proteins. In support of this model, a previous report from Taylor et al. (2012) revealed that Dynamin proteins work cooperatively with actin and N-terminal containing BAR (BIN/Amphiphysin/RVS) domain proteins at sites of membrane remodeling at the cell surface. The roles of actin cytoskeleton dynamics in HIV entry will be discussed later in this review (see section "Rho GTPases").

Finally, the fluidity of the PM, regulated by the presence of the lipid phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2], was shown to be crucial for HIV-1 entry in lymphocytes (Barrero-Villar et al., 2008). The small GTPase ADP ribosylation factor 6 (ARF-6) plays a significant role in this process by regulating the PI(4,5)P2 enrichment at the inner leaflet of the PM. García-Expósito et al. (2011) showed that expression of ARF-6 mutants defective on GTP/GDP cycle caused the accumulation of PI(4,5)P2-associated structures at the cell surface impeding CD4-dependent HIV-1 entry and infection, but without affecting CD4-viral attachment (García-Expósito et al., 2011). These results indicate that efficient early HIV-1 infection of permissive cells requires ARF6-mediated PM dynamics, possibly affecting pore formation.

HIV-1 TRANSPORT TO THE NUCLEUS AND TRANSLOCATION TO THE NUCLEOPLASM

The fusion of the HIV-1 envelope with the target cell membrane results in the delivery of the viral capsid containing the viral

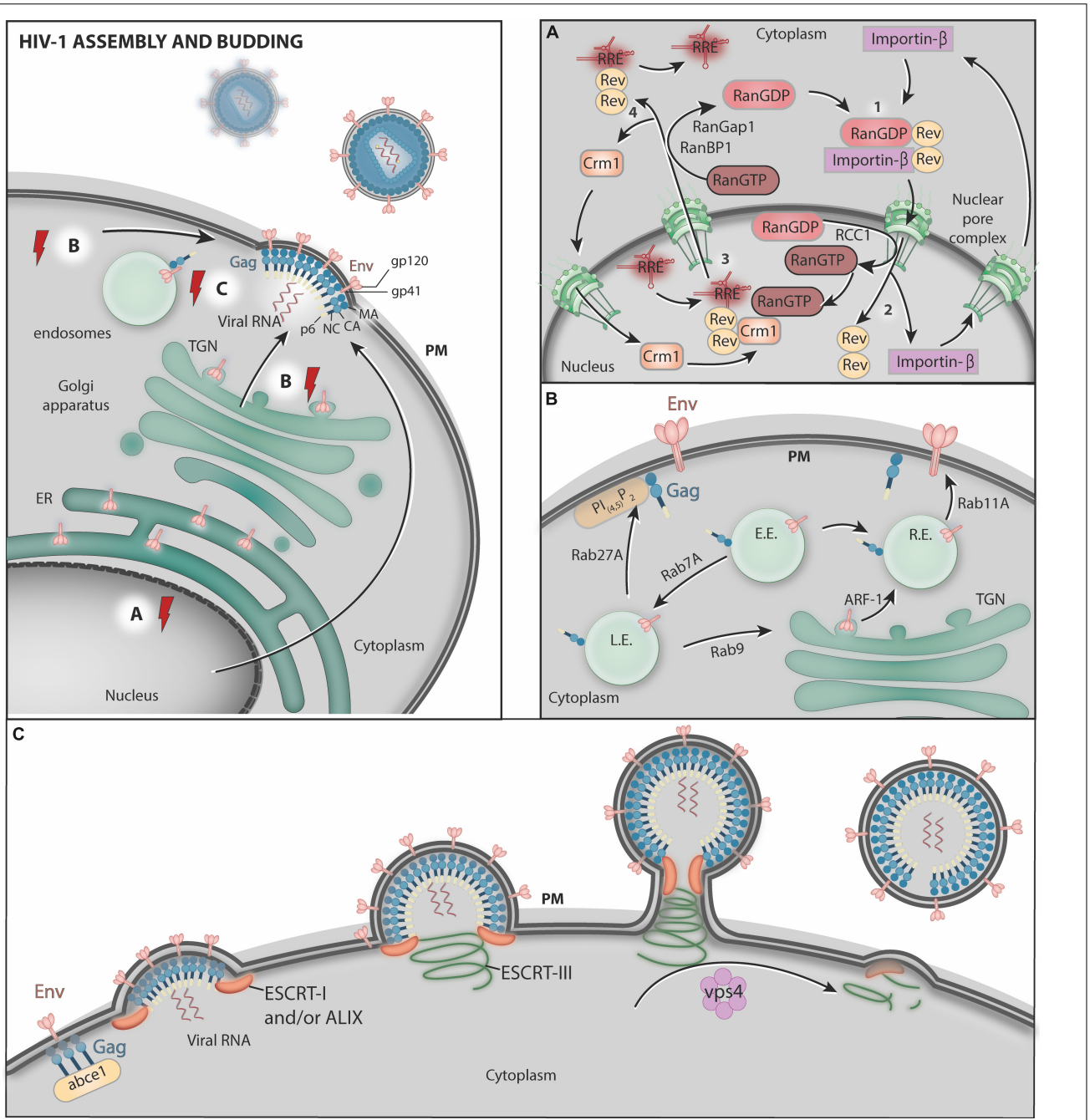

FIGURE 2 | HIV-1 assembly and budding. **(A)** The host transcriptional machinery transcribes the viral genes. After synthesis in the cytoplasm, Rev associates with RanGDP and Importin-β, forming the complex Rev-Importin-β-RanGDP which is imported into nucleus through nuclear pores (step 1). In the nucleus, the conversion of RanGDP to RanGTP mediated by RRC1 disassemble Rev from Importin-β, allowing Importin-β to be exported back to the cytoplasm (step 2). Rev then binds RRE (present in viral RNA molecules), RanGTP, and Crm1 and the Crm1-RanGTP-Rev-RRE complex exits the nucleus through the nuclear pores (step 3). After the translocation to the cytoplasm, RanGTP is converted to RanGDP by RanGAP1 and RanBP1, allowing the disassembly of the complex, and Crm1 is imported again to the nucleus while transported viral RNAs are free to be translated (step 4). **(B)** Env is synthesized in association with the ER membrane and transported to the Golgi complex. After reaching the Golgi, Env is transported to the PM, where new HIV-1 particles are formed. This trafficking occurs via transport vesicles that require ARF-1 GTPase. Rab GTPases control several steps of HIV-1 structural protein trafficking in endosomes. Rab7A is required for mature Env incorporation into nascent virus particles. Rab9 was proposed to Env and Gag from the endolysosomal pathway back to the Golgi complex. This process reroutes HIV-1 proteins to virus assembly sites at the PM. Rab11 controls the pathway that recycles Env from the cell surface to endosomes and back to the PM. Rab27a activity helps to target Gag to virus assembly sites at the PM. Moreover, Rab8 and Rab7L1 (Rab29) activity are exploited by HIV-1 during *trans*-infection from dendritic cells (DCs) to target T cells via virological synapses (not shown). L.E., late endosomes; E.E., early endosomes; R.E., recycling endosomes. **(C)** Finally, ABCE1 facilitates Gag organization at the cell surface, where the ESCRT machinery and the AAA-ATPase VPS4 facilitate virus particle release. The red electric ray symbols represent critical steps in the transport of viral factors that require energy.

TABLE 1 | ATPases and GTPases co-opted by HIV-1 during its replication cycle are presented in the order that they appear in the text.

ATPase/GTPase	Process in the HIV-1 replicative cycle	References
Dynamin	• Viral membrane fusion with the host cell. • Stabilization of the HIV-1 entry pore. • Work together with Actin and Bar domain proteins to facilitate the release of viral nucleocapsid into the cytosol.	Miyauchi et al., 2009; de la Vega et al., 2011; Taylor et al., 2012; Aggarwal et al., 2017; Jones et al., 2017
Rab5 and Rab7	• Involved in endocytic entry of HIV-1	Vidricaire and Tremblay, 2005; Marin et al., 2019
Ran	• HIV PIC nuclear import. • Interacts with TRN-SR2 to release HIV PIC in the nucleoplasm. • Nuclear egress of Crm1-Rev-RRE-cargo complex. • Ran GDP, together with Importin-β, promotes Rev nuclear import.	Malim et al., 1989a; Fornerod et al., 1997; Henderson and Percipalle, 1997; Neville et al., 1997; Askjaer et al., 1998; Christ et al., 2008; König et al., 2008; Dong et al., 2009; Monecke et al., 2009; Güttler et al., 2010; Krishnan et al., 2010; De Iaco and Luban, 2011; Zhou et al., 2011; Larue et al., 2012; Valle-Casuso et al., 2012; Taltynov et al., 2013; Behrens et al., 2017
ABCE1	• HIV-1 assembly.	Lingappa et al., 1997, 2006; Zimmerman et al., 2002; Dooher and Lingappa, 2004; Dooher et al., 2007; Klein et al., 2011; Reed et al., 2018
ARF-1	• Viral particle production. • *Trans*-infection at virological synapse.	Joshi et al., 2008, 2009b; Bayliss et al., 2020
ARF-3	• Viral particle production.	Joshi et al., 2008
ARF-5	• Viral particle production.	Joshi et al., 2008
ARF-6	• Regulates CD4-dependent HIV-1 entry and infection by controlling PI(4,5)P2 dynamics at PM.	García-Expósito et al., 2011
Rab7A	• Env processing. • Env incorporation into virions. • Release of the viral particle.	Caillet et al., 2011
Rab7L1 (Rab29)	• *Trans*-infection at virological synapse.	Bayliss et al., 2020
Rab8A	• *Trans*-infection at virological synapse.	Bayliss et al., 2020
Rab9	• Viral particle production. • Gag transport to HIV-1 assembly site. • Together with TIP47, help the retrograde transport of Env from endosome to TGN.	Blot et al., 2003; Murray et al., 2005
Rab11A	• Control the levels of cellular and viral particle-associated Env. • FIP1C (Rab11 effector) reroutes Env to recycling endosomes.	Qi et al., 2013; Kirschman et al., 2018
Rab27A	• Participates in Gag, PI4KIIα and $PI_{(4,5)}P_2$ levels at cell surface. • HIV-1 assembly. • *Trans*-infection at virological synapse.	Gerber et al., 2015
Rac1	• Promote pore formation and expansion during HIV-1 entry • control Gag levels at the cell surface and VLP release. • Gag expression activates Rac1 and increases F-actin content.	Pontow et al., 2004, 2007; Harmon et al., 2010; Vorster et al., 2011; Thomas et al., 2015
RhoA	• HIV-1 entry via stabilization of the fusion complex. • gp41 binds p115-RhoGEF which play a role in viral replication. • Control monocyte migration and adhesion, which may affect HIV-1 dissemination. • Activation of a RhoA–ROCK–LIMK–Cofilin signaling cascade mediated by Filamin-A facilitates HIV-1 entry	Zhang et al., 1999; Wang L. et al., 2000; Hodges et al., 2007; Jiménez-Baranda et al., 2007; Lucera et al., 2017
Cdc42	• Promotes plasma membrane expansions that facilitate viral transfer from DCs to T cells • Promotes HIV-1 T cell infection.	Nikolic et al., 2011; Shrivastava et al., 2015; Lucera et al., 2017
VPS4	• ESCRT-dependent HIV-1 assembly and budding.	Baumgärtel et al., 2011; Jouvenet et al., 2011; Bleck et al., 2014; Van Engelenburg et al., 2014; Johnson et al., 2018

genomic RNA (gRNA) into the cytoplasm (**Figure 1**). The HIV-1 capsid core comprises a conical structure made of capsid (CA) protein, which contains the gRNA and copies of the viral reverse transcriptase and integrase enzymes. In addition to protecting the viral genome from host restriction factors and innate immunity recognition, the capsid provides an optimized environment for reverse transcription, leading to the synthesis of viral DNA that is, ultimately, integrated into the host cell chromatin to form a provirus (**Figure 1**).

Viral capsid uncoating/disassembly is required for integration and productive infection. However, the timing and location of capsid disassembly is still a matter of debate, with models proposing that it occurs either at the cytoplasm, at the nuclear pore, or within the nucleus (previously reviewed in Toccafondi et al., 2021). Several recent studies favor a model in which capsid disassembly occurs after complete nuclear translocation by providing evidence that reverse transcription finalizes within the nucleus and that the capsids enter the nucleus and remain intact (or nearly intact) until they uncoat near the integration sites, just minutes before integration (Burdick et al., 2020; Dharan et al., 2020; Selyutina et al., 2020; Li et al., 2021). The recent direct visualization of apparently intact, cone-shaped HIV-1 capsids

being imported through nuclear pores in infected T cells provides definite proof that capsid uncoating may occur after nuclear transport is complete (Zila et al., 2021).

The nuclear pore complexes and the soluble transport receptors of the karyopherin family of proteins mediate the transport of macromolecules across the nuclear envelope. This process requires an input of energy derived from GTP hydrolysis by Ras-related nuclear (Ran) small GTPases, which provide selectivity and directionality to the nuclear translocation process. Interaction between GTP-bound Ran (Ran-GTP) and nuclear transport receptors directs the binding and release of cargo, and the enrichment of Ran-GDP in the cytoplasm and Ran-GTP in the nucleoplasm forms a gradient that controls the bidirectional flow of molecules in and out of the nucleus (Koyama and Matsuura, 2010).

Several components of the nuclear transport machinery are required for HIV infection, including the β-karyopherin Transportin-SR2 (TRN-SR2, also known as TNPO3) (Brass et al., 2008; Christ et al., 2008; König et al., 2008). Consistent with a model in which HIV-1 hijacks the Ran-GTPase system to invade the nucleus, Taltynov et al. (2013) observed that TNPO3 associates with Ran-GTP, which may facilitate the release of viral material in the nucleoplasm. TNPO3 requirement during HIV-1 and other lentivirus infection was correlated to capsid binding and proposed to occur after nuclear entry (Krishnan et al., 2010; De Iaco and Luban, 2011; Valle-Casuso et al., 2012). Interestingly, recombinant TNPO3 stimulates the uncoating of HIV-1 cores *in vitro*, a property that is inhibited by the CA-binding host protein cyclophilin A (CypA) (Shah et al., 2013). This relationship between TNPO3 and CypA suggests that these proteins coordinate proper disassembly of the viral capsid in target cells, with TNPO3 favoring capsid disassembly within the nucleus.

Although TNPO3 was shown initially to bind integrase (Christ et al., 2008), the role of this interaction in HIV-1 infection is presently not clear (Cribier et al., 2011) and, in light of new data discussed above, may occur within the nucleus. Nevertheless, a library with more than 25,000 small molecules was recently screened for inhibitors of the HIV-1 integrase-TNPO3 interaction, and new compounds that significantly reduce HIV-1 integration were identified (Demeulemeester et al., 2018). These compounds may represent potential future drugs to treat HIV infection. Despite these advances, much remains to be learned about the process by which HIV-1 material is imported into the nucleus, as other host factors are likely to be required for this process. The emerging candidates include importin α, importin β, and importin 7, but divergent data indicate a complex process, and other proteins may also be involved (previously reviewed in Matreyek and Engelman, 2013).

NUCLEAR EXIT OF HIV PRODUCTS

The late phase of the HIV-1 replication cycle starts with provirus gene expression (**Figure 2**). The transcription of the HIV-1 genome is mediated by host cell RNA polymerase II and initiates from the U3 promoter region within the proviral 5′LTR. To achieve maximum production, this process is enhanced by the viral regulatory protein Tat (transactivator of transcription) (Siekevitz et al., 1987). HIV-1 RNA synthesis and downstream processing result in the production of a variety of RNA species, including completely spliced mRNA molecules [encoding the Tat, regulator of expression of virion proteins (Rev), and negative factor (Nef) proteins], partially spliced mRNAs (encoding the structural proteins Gag, Pol and Env), and unspliced gRNA (previously reviewed in Cullen, 2003), which need to reach the cytosol for protein synthesis and/or virion assembly.

HIV-1 RNA export from the nucleus is mainly mediated by the regulatory protein Rev, which facilitates the nuclear export and cytoplasmic build-up of singly spliced and unspliced viral RNA molecules (Malim et al., 1989b) through an energy-consuming process. Rev binds and oligomerizes to Rev response elements (RREs) present in viral RNA molecules, allowing the formation of ribonucleoproteins that are competent for nuclear export. This process evades the host cell quality control mechanisms that prevent the nuclear export of incompletely spliced RNA molecules (Malim et al., 1989a). The Rev–RRE ribonucleoprotein complex interacts with host export factor Crm1, an karyopherin family member, also known as exportin 1 (Xpo1) (Fornerod et al., 1997; Güttler et al., 2010).

The nuclear egress of the Crm1-Rev-RRE-cargo complex through nuclear pores is an energy-dependent process. This event is controlled by Ran GTPase, which enables the formation of the Crm1-RanGTP-Rev-RRE-cargo complex. In fact, Ran in its active (GTP-bound) form mediates the interaction between Crm1 and Rev through a surface-exposed hydrophobic pocket on Crm1 (Dong et al., 2009; Monecke et al., 2009; Güttler et al., 2010). Recently, Behrens et al. (2017) have shown that Rev tolerates several nuclear export signals, even those that bind Crm1 in a Ran-GTP-independent manner. Additionally, interactions between Rev proteins may mask their nuclear export signals and favor the nuclear accumulation of Rev (Behrens et al., 2017).

Once in the cytoplasm, the Crm1-RanGTP-Rev-RRE-cargo complex is dissociated as a consequence of GTP hydrolysis induced by RanGAP1 and RanBP1, which releases Cmr1 and RanGDP from the Rev-RRE-cargo complex (Neville et al., 1997; Askjaer et al., 1998). Thereafter, the RRE disassociates from Rev and the RRE-RNA is translated, whereas Crm1 is re-imported into the nucleus. In the cytoplasm, an importin-β binds to the nuclear localization signal of Rev and, combined with RanGDP, promotes the nuclear import of Rev via the nuclear pores (previously reviewed in Meyer and Malim, 1994; Truant and Cullen, 1999; Suhasini and Reddy, 2009). In the nucleus, the disassembly of the Rev-RanGDP-Importin-β complex is triggered by the conversion of RanGDP to RanGTP, resulting in the release of Rev to facilitate further viral RNA nuclear export (previously reviewed in Meyer and Malim, 1994; Henderson and Percipalle, 1997; Suhasini and Reddy, 2009).

HIV-1 ASSEMBLY AND BUDDING

The HIV-1 structural proteins Gag and Env are synthesized in the cytosol and in association with the endoplasmic reticulum

(ER) membrane, respectively, and their correct targeting to viral assembly sites is crucial in HIV-1 replication (**Figure 2**). Although Gag protein is sufficient for the assembly and budding of virus-like particles (VLPs) (Gheysen et al., 1989) as well as for the recruitment and packaging of the viral genome into VLPs (Shields et al., 1978; Sakalian et al., 1994), the proper incorporation of Env glycoproteins into the nascent virion is essential to the production of infectious particles. Depending on the cell type, HIV-1 assembly has been proposed to take place at the inner leaflet of the PM (Ono et al., 2000; Ono and Freed, 2004), at the intraluminal vesicles (ILVs) of late endosomes/multivesicular bodies (MVBs) (Nydegger et al., 2003; Sherer et al., 2003; Joshi et al., 2009a) or at intracellular compartments connected to the cell surface by tubules (Bennett et al., 2009). However, how Env and Gag reach the sites of viral assembly in each case is not fully understood. Intense study on this subject has revealed a number of host ATPases and GTPases involved in HIV-1 particle morphogenesis and budding.

Small GTPases Regulating the Intracellular Trafficking and Subcellular Distribution of HIV-1 Structural Proteins

ARF GTPases

The ADP-ribosylation factors (ARFs) form a protein family within the Ras superfamily of small GTPases (previously reviewed in D'Souza-Schorey and Chavrier, 2006) and play important roles in intracellular vesicle trafficking, actin remodeling and phospholipid metabolism (previously reviewed in Kahn et al., 2005; D'Souza-Schorey and Chavrier, 2006; Gillingham and Munro, 2007; Randazzo et al., 2007; Kahn, 2009). In the context of HIV-1 infection, cellular depletion of ARF-1, ARF-3 or ARF-5 levels by RNAi or the expression of dominant-active forms of these molecules was shown to impair HIV-1 particle production (Joshi et al., 2008).

ARF molecules may initiate the formation of transport vesicles by recruiting vesicle coat components to specific donor membranes. Among the vesicle coat proteins recruited by ARFs are the monomeric Golgi-localized, γ-ear containing, ARF-binding (GGA) adaptors (Boman et al., 2000; Dell'Angelica et al., 2000) and members of the adaptor protein (AP) complex family (Stamnes and Rothman, 1993; Traub et al., 1993). GGAs and APs are sorting adaptors involved in protein trafficking in the late secretory pathway and have been implicated in the intracellular transport of Gag and Env. Specifically, GGA1-3 (Joshi et al., 2008, 2009b), AP-1 (Camus et al., 2007), AP-2 (Batonick et al., 2005), AP-3 (Dong et al., 2005; Garcia et al., 2008; Alford et al., 2016), and AP-5 (Alford et al., 2016) have been described to play key roles in the correct targeting of Gag to viral assembly sites. GGA overexpression was shown to compromise viral particle production, and mutation of the ARF-binding sites in GGAs abrogated this phenotype (Joshi et al., 2008, 2009b). The authors of this study suggested that GGA overexpression hinders viral production by sequestering free ARF-1 molecules and thus impairing its activity.

The HIV-1 Env polyprotein precursor gp160 is processed in the Golgi complex to produce gp120 (surface glycoprotein) and gp41 (hydrophobic transmembrane glycoprotein), which are bound to each other in a non-covalent manner (McCune et al., 1988; Freed et al., 1989; Stein and Engleman, 1990; Hallenberger et al., 1992). Importantly, gp160 processing is essential for efficient HIV-1 membrane fusion and infectivity (McCune et al., 1988; Freed et al., 1989; Bosch and Pawlita, 1990; Guo et al., 1990; Dubay et al., 1995). From the Golgi, gp120-gp41 heterodimers are transported to the cell surface, possibly via endosomes, in an ARF-1/AP-1-dependent manner (Berlioz-Torrent et al., 1999; Wyss et al., 2001).

Recently, an RNAi-based screen of membrane trafficking regulators showed that ARF-1 depletion in dendritic cells (DCs) reduced the transfer of viral particles to T cells by rerouting Gag molecules away from the sites of virological synapsis, leading to virus particles accumulation in small vesicle structures at the donor cell periphery (Bayliss et al., 2020). However, this inhibitory effect in virus transfer may not be merely due to altered Gag trafficking, because ARF-1 depletion also affected the delivery of virological synapse (VSs) structural proteins to the PM, such as the tetraspanin CD81 (Bayliss et al., 2020). Therefore, it is not currently possible to conclude whether ARF-1 plays a direct role in Gag trafficking and HIV-1 cell-to-cell transfer.

Rab GTPases

The Rab (Ras-related in brain) proteins form another family of small GTPases involved in the trafficking of HIV-1 Gag and Env. In general, Rabs provide identity and function to the membranes of secretory pathway compartments with multiple functions in vesicle transport (previously reviewed in Lamber et al., 2019; Homma et al., 2021). The roles of Rab proteins in viral replication have been recently reviewed (Spearman, 2018). Regarding HIV, a study by Caillet et al. (2011) implicated the activity of several Rab proteins (Rab4A, Rab6A, Rab7A, Rab8A, Rab9A, and Rab11A) in viral replication. Among these Rabs, the function of Rab7A (an endosome-associated Rab) was described as being the most important for efficient HIV-1 particle production (Caillet et al., 2011). The authors showed that the depletion of Rab7A impairs both Env processing and the incorporation of mature Env into viral particles, compromising viral infectivity. Moreover, Rab7A depletion hampers the release of HIV-1 progeny, which accumulates at the cell surface (Caillet et al., 2011). Interestingly, this phenotype was dependent on the expression of the host restriction factor BST2/Tetherin, which is normally antagonized by the HIV-1 accessory protein viral protein U (Vpu) (Neil et al., 2008); this process is discussed later in this review (see section "Vpu"). Furthermore, a recent study showed that trafficking pathways controlled by Rab8A and Rab7L1 (also known as Rab29) are exploited by HIV-1 during *trans*-infection from DCs to target T cells via VSs. The authors showed that the depletion of Rab8A and Rab29 in DCs leads to a reduction in HIV-1 localization at VSs and a consequent accumulation of Gag and CD81, a host tetraspanin that is normally recruited to VSs, in intracellular compartments (Bayliss et al., 2020).

Another Rab potentially involved in HIV-1 replication is Rab9 (Murray et al., 2005). Rab9 was originally linked to protein transport between endosomes and the TGN (Lombardi et al., 1993; Shapiro et al., 1993; Riederer et al., 1994) and more recently

to lysosomes and lysosome-related organelles biogenesis and autophagy (Riederer et al., 1994; Ganley et al., 2004; Nishida et al., 2009; Kloer et al., 2010). Using gene-trap insertional mutagenesis and RNAi assays, it was found that interfering with Rab9 activity causes the rerouting of Gag to lysosomes and a decrease in HIV-1 particle production (Murray et al., 2005). Interestingly, TIP47 (also known as Perlipin-3), a Rab9 interacting protein originally implicated in protein trafficking (Díaz and Pfeffer, 1998; Hanna et al., 2002) and more recently in lipid droplet biogenesis (Wolins et al., 2001; Bulankina et al., 2009), was proposed to play a role in HIV-1 particle biogenesis (Blot et al., 2003; Lopez-Vergès et al., 2006). A study showed that TIP47/Perlipin-3 binds Env via a di-aromatic $Y_{802}W_{803}$ motif in gp41 that is required for proper retrograde transport of Env from endosomes to the TGN and for Env incorporation into virions (Blot et al., 2003). TIP47 also interacts with Gag via the matrix (MA) domain, and a study show that Env incorporation was inhibited by TIP47 depletion or by the disruption of the Gag-TIP47 interaction (Lopez-Vergès et al., 2006). The authors proposed that TIP47 may function as a connector between Env and Gag, controlling proper Env incorporation during viral particle assembly. However, this notion was challenged by a more recent study (Checkley et al., 2013). Although TIP47 interaction with MA was confirmed, the authors of this other study did not observe changes in Env incorporation, virus release, infectivity, or replication upon TIP47 depletion in either HeLa cells or Jurkat T cell lines. Therefore, the mechanism by which Rab9 and TIP47 affect HIV-1 replication remains unclear.

Strong evidence for the ability of HIV-1 proteins to co-opt Rabs and Rab effectors in recycling endosome-mediated pathways has also come to light. Rab11 is one of the main regulators of membrane recycling in the late secretory pathway (Ullrich et al., 1996; Casanova et al., 1999; Wang X. et al., 2000; Hales et al., 2001). Although Rab11A depletion does not alter Env incorporation into virions, the expression of a Rab11A active (GTP-bound) form decreases the levels of cellular and particle-associated Env (Qi et al., 2013). Consistent with this result, Env expression modifies the localization of the Rab11 effector FIP1C from recycling endosomes to the cell periphery, and FIP1C depletion reduces the levels of Env in cells and nascent viral particles, delaying HIV-1 replication (Qi et al., 2013). Furthermore, the expression of a C-terminal fragment of FIP1C ($FIP1C_{560-649}$) reroutes Env to recycling endosomes and diminishes the levels of Env on the cell surface and the incorporation of Env into virions (Kirschman et al., 2018). The function of Rab11/FIP1C may also involve Rab14 because the expression of a FIP1C mutant ($S_{580}N/S_{582}L$) that does not bind Rab14 similarly depletes Env from the viral particles (Qi et al., 2013), a phenotype recapitulated by the expression of an inactive GDP-bound Rab14 ($Rab14S_{25}N$) (Qi et al., 2013).

Importantly, it is known that the lipid phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2] present in the inner leaflet of the PM plays an essential role in Gag localization at the PM (Ono et al., 2000; Ono and Freed, 2004). In a process called the "myristoyl switch," the binding of the negatively charged inositol headgroup of PI(4,5)P2 to the MA domain of Gag exposes the N-terminal myristoyl group present in the MA domain, which mediates the anchorage and stabilization of Gag at the PM (Ono et al., 2000; Ono and Freed, 2004; Brügger et al., 2006; Saad et al., 2006; Shkriabai et al., 2006). Additionally, Gag traps PI(4,5)P2 and cholesterol, suppressing their mobility at the T cell PM and creating an efficient microdomain platform for virus assembly (Favard et al., 2019). Taking these findings into consideration, Gerber et al. (2015) carried out a study that revealed that Rab27A controlled PI(4,5)P2 levels at HIV-1 assembly sites by directing the enzyme PI4KIIα (phosphatidylinositol 4-kinase type 2 α) from late endosomes to the PM, where PI4KIIα produces phosphatidylinositol 4-phosphate [PI(4)P], a precursor of PI(4,5)P2 (Rameh et al., 1997; Doughman et al., 2003). Therefore, the depletion of Rab27A reduced the pools of PI4KIIα and PI(4,5)P2 at the cell surface, reducing Gag association with the PM and HIV-1 assembly/production (Gerber et al., 2015). Although the trafficking and processing of Env were not affected in Rab27A-silenced cells, Rab27A ablation impaired HIV-1 cell-to-cell spread either through free viral particles or by *trans*-infection at the VSs (Gerber et al., 2015). Finally, expression of the Rab27A effector proteins Slac2b, Slp2a, and Slp3 was also shown to be required for Gag association with the PM and efficient HIV-1 particle production (Gerber et al., 2015). Altogether, these results demonstrate an important role for Rab27A and its effector proteins in HIV-1 production and spread through a PI4KIIα trafficking mechanism.

Rho GTPases

The Rho GTPase family (including Cdc42, Rac1, and RhoA) regulates multiple cellular processes involving the activation of signaling pathways, such as cell adhesion, migration, survival, differentiation, and proliferation (Tybulewicz and Henderson, 2009). Given the intricate manipulation of host cell signaling by HIV-1, it is not surprising that several studies have reported that Rho GTPases are involved in various events in the HIV-1 replication cycle. Rho GTPases are especially relevant in the host actin cytoskeleton subversion by HIV-1, a recently reviewed subject (Stella and Turville, 2018). There is strong evidence that incoming HIV-1 particles interfere with actin cytoskeleton dynamics at the target cell cortex by triggering the activation of signaling cascades mediated by distinct Rho GTPases (**Figure 1**).

Jiménez-Baranda et al. (2007) showed that the interaction of Env with CD4 and the chemokine coreceptors (CCR5 and CXCR4) promote F-actin stabilization via phosphorylation (and inactivation) of Cofilin, an F-actin depolymerizing factor (Jiménez-Baranda et al., 2007). The LIM domain kinase 1 (LIMK1), a protein that phosphorylates Cofilin is activated by RhoA or Rac1 effectors ROCK and PAK-1, respectively (Maekawa et al., 1999; Vadlamudi et al., 2002; Jiménez-Baranda et al., 2007). Interestingly, Filamin-A, an actin cross-linking protein that binds ROCK and PAK-1 (Ohta et al., 2006), also interacts with CD4, CCR5, and CXCR4 (Jiménez-Baranda et al., 2007), and this interaction was shown to be required for Env-mediated RhoA activation, Cofilin phosphorylation, and efficient HIV-1 infection. Since HIV-1 infection was impaired by a ROCK inhibitor, and not by disruption of Rac1 or PAK-1 activity, it was proposed that Filamin-A mediates

activation of a RhoA–ROCK–LIMK–Cofilin signaling cascade that facilitates HIV-1 entry via stabilization of the fusion complex (Jiménez-Baranda et al., 2007).

In contrast, work from other groups support a crucial role for Rac1 activation in promoting HIV infectivity. Vorster et al. (2011) showed that HIV-1 infection, or gp120 treatment, activates Rac1 and induces PAK-mediated activation of LIMK1, leading to phosphorylation and inactivation of Cofilin in resting CD4 T cells (Vorster et al., 2011). In fact, the interaction of Env with CD4 and CCR5 or CXCR4 was shown to trigger a defined Gαq-mediated signaling cascade activating Rac1 to promote actin polymerization events at the cell cortex that facilitates fusion (Pontow et al., 2004; Harmon and Ratner, 2008). Further studies into this mechanism show that this Env-induced signaling cascade activates the tyrosine kinase Abl that stimulates the Rac GEF Tiam-1 to activate Rac1. Subsequently, Abl and Rac1-GTP activate the Wave2 complex, which stimulates actin polymerization via the Arp2/3 complex (Harmon et al., 2010). Because pharmacological inhibition of Abl was shown to arrest Env-mediated membrane fusion at the hemifusion step, the authors of this study proposed that actin remodeling mediated by Abl and Rac1 promote pore formation and expansion during HIV-1 entry (Harmon et al., 2010).

Binding of Env to target cells was also shown to activate Cdc42 and contribute to viral spreading. Env-binding to the DC-SIGN protein in the surface of DCs, triggers Cdc42 activation via a c-Src cascade leading to filopodia formation via the Arp2/3 complex and Diaphanous 2. These PM expansions in DC cells where shown to facilitate viral transfer to T cells (Nikolic et al., 2011; Shrivastava et al., 2015). Moreover, DC-SIGN stimulation by HIV-1 in monocyte-derived DCs results in a complex formation containing DC-SIGN, Rho and a Rho GEF called leukemia-associated Rho guanine nucleotide exchange factor (Rho GEF) (LARG) (Hodges et al., 2007). The Rho activation mediated by LARG via DC-SIGN is essential for optimal HIV-1 replication and VS formation (Hodges et al., 2007). The extensive manipulation of Rho GTPases pathways during HIV entry was nicely illustrated in a recent study by Lucera et al. (2017). Using a phosphoproteomics approach, the authors confirmed that HIV binding to CD4 and CCR5 activates Rac1 and Cdc42 leading to dramatic changes in the phosphorylation status of proteins associates to GTPase signaling (Lucera et al., 2017).

While the importance of Rho GTPases activity in HIV entry and spreading seems to be a consensus in the field, the relative importance of the family members (either Cdc42, Rac1, or RhoA) in the specific process has been a matter of controversy. This difficulty is partially due to the great degree of overlap and crosstalk among the Rho GTPases signaling pathways with shared regulators and downstream effectors. For instance, the LIMK-Cofilin pathway unites all three major Rho GTPases. The increasing availability of specific inhibitors targeting the GTPases themselves will help clarify the individual functions of Rho GTPases in HIV replication processes and provide valuable tools to fight infection.

Besides their role in virus entry, there is a well-documented interplay between Rho GTPases and the HIV-1 structural proteins during viral assembly/replication. It has been demonstrated that the cytosolic tail of HIV-1 gp41 (gp41C) binds the regulatory domain of p115 (Zhang et al., 1999), a RhoA GEF (Hart et al., 1996). This interaction is functionally relevant since the disruption of the p115-RhoGEF binding site in gp41 (Zhang et al., 1999) or the overexpression of a p115-RhoGEF activator (Wang L. et al., 2000) were shown to inhibit HIV-1 replication. Moreover, gp41C expression inhibited p115-RhoGEF-mediated actin stress fiber formation (Zhang et al., 1999), suggesting that HIV-1 regulates RhoA activity via its GEF. Because RhoA activity was shown to normally regulate the cell survival (Galandrini et al., 1997) and migration (Aepfelbacher et al., 1996) of HIV-1 target cells, the gp41C-p115-RhoGEF interaction and RhoA activity are likely to affect HIV-1 dissemination and pathogenesis (Zhang et al., 1999; Wang L. et al., 2000).

Finally, the trafficking of Gag to HIV-1 assembly sites was shown to be dependent on Rac1, where the levels of Gag at the PM and the release of HIV-1 Gag VLPs were inhibited in Rac1-depleted T cells (Thomas et al., 2015). In fact, the efficient production of HIV-1 Gag VLPs requires a functional Rac1-Wave2-IRSp53-Arp2/3 pathway in T cells (Thomas et al., 2015). Although, the HIV-1 accessory protein Nef potently induces Rac1 activation (as discussed later in this review, see section "Nef") and may contribute to the role of Rac1 in virus release, Gag expression alone activates Rac1 and increases intracellular F-actin content (Thomas et al., 2015).

ATPases Involved in HIV Budding

The ATPAse ABCE1

The membrane budding process driven by the HIV-1 Gag protein requires ATP, given that it is inefficient in ATP-depleted cells (Lingappa et al., 1997; Tritel and Resh, 2001). Since HIV-1 Gag does not itself interact with ATP and it is unable to harness its stored chemical energy, researchers sought to identify a host protein that could serve as an adaptor in this process. Using coimmunoprecipitation assays, Lingappa's lab identified the interaction of Gag with ABCE1 (also called HP68 or RNase L inhibitor) (Zimmerman et al., 2002), a member of subfamily E of the ATP-binding cassette (ABC) ATPases (Dean et al., 2001). The authors showed that ABCE1 interacts with capsid assembly intermediates and is essential for immature capsid formation in a cell-free system, a notion confirmed by expressing an ABCE1 dominant-negative protein in cells (Zimmerman et al., 2002). Because ABCE1 binding appears to promote conformational changes in nascent capsid structure (Zimmerman et al., 2002), this ATPase may act as a Gag chaperone during oligomerization to facilitate viral particle assembly. Indeed, the function of ABCE1 in the capsid assembly pathway is energy-dependent and occurs in a stepwise manner, involving its progressive association to assembly intermediates (Dooher and Lingappa, 2004). Moreover, the requirement of ABCE1 is conserved for several primate lentivirus capsids, such as HIV-1, HIV-2, and simian immunodeficiency viruses (SIV_{mac239} and SIV_{agm}) (Dooher and Lingappa, 2004) and non-primate lentiviruses, such as the feline immunodeficiency virus (FIV) (Reed et al., 2018).

A basic amino acid residue within the nucleocapsid (NC) domain of Gag is important for the recruitment of ABCE1, and Gag molecules carrying mutations in this residue fail to form fully assembled capsids (Lingappa et al., 2006). Indeed, double immunogold labeling followed by cryo-EM revealed that ABCE1 is redirected to the Gag assembly site depending on the integrity of the critical basic residue in NC (Dooher et al., 2007). Interestingly, further research showed that ABCE1 does not bind NC directly. Instead, Gag dimerization promoted by NC exposes an ABCE1-binding domain located outside the NC (Klein et al., 2011). Moreover, ABCE1 interacts with TULA (T-cell Ubiquitin Ligand) and recruits it to HIV-1 Gag assembly sites, where TULA acts in late steps of the HIV-1 replication cycle (Smirnova et al., 2008).

Evidence for the relevance of ABCE1 in HIV-1-positive patients has been reported. Resequencing analysis of the *ABCE1* gene and genomic comparisons revealed an excess of rare genetic variations in the *ABCE1* gene among HIV-1-positive African–American individuals compared to those among populations of European descent, suggesting positive selection through *ABCE1* and the surrounding genomic regions (Crawford et al., 2009; previously reviewed in Tian et al., 2012). Indeed, by using $CD4^+$ T cells from healthy donors and an *ex vivo* $CD4^+$ T cell HIV-1 infection system, the authors found that an insertion/deletion variant (rs9333571) in the *ABCE1* gene decreased HIV-1 permissiveness (Bleiber et al., 2005; previously reviewed in Tian et al., 2012). These findings are suggestive of ABCE1 importance in HIV-1 infection/pathogenesis. However, additional studies are necessary to correlate the findings *in vitro* regarding the role of ABCE1 in particle assembly with a possible function of this ATPase in infection *in vivo*.

The AAA ATPase VPS4

The ESCRT (Endosomal Sorting Complex Required for Transport) machinery comprises four distinct multimeric complexes (ESCRT-0 to ESCRT-III) that work sequentially to coordinate the formation of the ILVs of late endosomes/MVBs and the selection of ubiquitinated and non-ubiquitinated cargo proteins (daSilva et al., 2009; Dores et al., 2012a,b; Amorim et al., 2014; previously reviewed in Hurley et al., 2020). The selection of the cargo begins in subdomains of early endosomes enriched in clathrin and HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), one of the components of the ESCRT-0 complex (Raiborg et al., 2002). HRS interacts with both the cargo and ESCRT-I, which also binds ESCRT-II. The ESCRT-III complex is subsequently recruited and mediates the invagination of the endosome limiting membrane and the fission of ILVs. VPS4 (vacuolar protein sorting 4), an AAA-ATPase, binds ESCRT-III subunits and catalyzes the disassembly of ESCRTs upon ATP hydrolysis. This latter process facilitates ESCRT machinery recycling and is essential for sustained ILV biogenesis (Lata et al., 2008).

In addition to ILV formation, the ESCRT machinery components function in other cellular processes requiring membrane remodeling, such as in the abscission phase of cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007; Elia et al., 2011; Guizetti et al., 2011; Peel et al., 2011), and in the regeneration of the nuclear envelope during mitosis (Olmos et al., 2015, 2016; Raab et al., 2016; Ventimiglia et al., 2018; von Appen et al., 2020). HIV and other retroviruses hijack ESCRT machinery components to perform membrane fission events that are topologically equivalent to ILV biogenesis in MVBs, i.e., facing away from the cytosol (Hurley and Hanson, 2010; Peel et al., 2011; Sundquist and Kräusslich, 2012; Freed, 2015). At HIV-1 assembly sites, Gag recruits the ESCRT-I subunit TSG101 (tumor susceptibility gene 101) via the P(T/S)AP motif present in the Gag p6 domain (Huang et al., 1995; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001; Demirov et al., 2002). In an alternative budding pathway, Gag recruits the ESCRT-related protein ALIX (also known AIP1 or PDCD6IP) via an YPX_nL motif that is also present in the p6 domain (Strack et al., 2003; Fujii et al., 2009; Usami et al., 2009; Eekels et al., 2011). In addition to ESCRT-I and ALIX, subunits of ESCRT-II were also shown to be important for HIV-1 budding (previously reviewed in Carlson and Hurley, 2012). Notably, the EAP45 protein, a component of the ESCRT-II complex, is required for the late stages of HIV-1 replication via the YPX_nL-ALIX pathway (Meng et al., 2020).

As a result of Gag interactions with ESCRT-I/ALIX and ESCRT-II, ESCRT-III and, subsequently, VPS4 are also recruited to HIV-1 assembly sites to accomplish membrane scission and viral particle release (Wollert et al., 2009; Bleck et al., 2014; Van Engelenburg et al., 2014). Interestingly, only a subset of the ESCRT-III subunits (the CHMP2 and CHMP4 families) involved in ILV formation are required for HIV-1 budding (Morita et al., 2011).

Polymerization of the CHMP4 and CHMP2 proteins (ESCRT-III subunits) is thought to drive the closure of the membrane neck of a budding virus (Hanson et al., 2008), and lead to exposure of the helical sequence domain in the CHMP2 C-terminus, which in turn binds the MIT domain of the N-terminal region of VPS4. This process allows VPS4 to remove ESCRT-III subunits from the viral membrane neck, culminating in viral membrane fission (Obita et al., 2007; Stuchell-Brereton et al., 2007; Baumgärtel et al., 2011). This VPS4 activity is coupled to ATP hydrolysis, which converts chemical energy into mechanical force to trigger the constriction and cleavage of ESCRT-III polymer ring (Monroe et al., 2017; Schöneberg et al., 2018; Maity et al., 2019). Therefore, the energy required for ESCRT-dependent HIV-1 assembly/budding comes from ATP hydrolysis mediated by VPS4 (previously reviewed in Sundquist and Kräusslich, 2012).

HIV-1 ACCESSORY PROTEINS

The HIV-1 accessory proteins [Nef, viral protein R (Vpr), viral infectivity factor (Vif), and Vpu] are not essential for virus replication *in vitro*, but they are decisive in creating an intracellular environment that allows efficient viral particle production and spread *in vivo* (previously reviewed in Sauter and Kirchhoff, 2018). In this section, we discuss how the HIV-1 accessory proteins Nef and Vpu co-opt GTPases, ATPases and

their regulators and effectors to assist in virus replication and to evade host defenses.

Negative Factor (Nef)

The HIV negative factor (Nef) protein is a critical determinant of viral pathogenesis (Deacon et al., 1995; Gulizia et al., 1997; Oelrichs et al., 1998; Learmont et al., 1999; Gorry et al., 2007). Most of Nef's functions in infection rely on its ability to modify the trafficking of membrane proteins in host cells (previously reviewed in Pereira and daSilva, 2016; Buffalo et al., 2019). Among the targets of Nef is the ATPase ABCA1, an ABC transporter family member that mediates lipid efflux from cells and contributes to the biogenesis of HDL (high-density lipoprotein) by transferring phospholipids and cholesterol to extracellular apoA-I (apolipoproteinA-1) molecules (Santamarina-Fojo et al., 2001; Neufeld et al., 2004; Fitzgerald et al., 2010; Kang et al., 2010).

As previously mentioned, HIV-1 assembly platforms at the cell surface are enriched in PI(4,5)P2 and lipid rafts, which contain high levels of cholesterol and sphingolipids (Nguyen and Hildreth, 2000; Ono and Freed, 2004; Hogue et al., 2011). There is evidence that Nef contributes to the enrichment of cholesterol on lipid rafts at the PM by impeding ABCA1-mediated cholesterol efflux (Zheng et al., 2003; Mujawar et al., 2006; Fitzgerald et al., 2010; Cui et al., 2012). There are at least two proposed mechanisms underlying Nef's antagonism to ABCA1 (**Figure 3**). First, Nef was shown to retain ABCA1 in the ER and stimulate ABCA1 proteasome-mediated degradation (Mujawar et al., 2010). Although the binding of Nef to ABCA1 (via its C-terminal domain – $ABCA1_{2225-2231aa}$) was demonstrated, an ABCA1 mutant that does not interact with Nef also failed to exit the ER and was degraded in response to Nef expression (Mujawar et al., 2010). Rather than acting directly on ABCA1, Nef appears to disrupt the association between newly synthesized ABCA1 and the ER chaperone calnexin, which leads to the enhanced degradation of ABCA1 via the proteasomal/endoplasmic-reticulum-associated protein degradation (ERAD) pathway (Jennelle et al., 2014).

There is also evidence that Nef targets surface ABCA1 for lysosomal degradation (Cui et al., 2012). In support of this alternative model, the authors of one study show that in Nef-expressing cells, ABCA1 was relocated from the PM to lysosomes for degradation (Cui et al., 2012). Interestingly, the effects of Nef on ABCA1 during HIV-1 infection may not be restricted to infected cells, as exogenous Nef taken up by cells was also shown to be active in ABCA1 downregulation and cholesterol efflux reduction (Mukhamedova et al., 2019) (**Figure 3**). Nef antagonism of ABCA1 is likely relevant in HIV-1 pathogenesis, as ABCA1 downregulation and low HDL cholesterol levels were found in HIV-1 treatment-naïve patients, an effect that was reverted by HAART (Feeney et al., 2013; Lo et al., 2014; Thangavel et al., 2018).

Another ATPase that interacts with Nef is the vacuolar H^+ ATPase (also known as V-ATPase or V_1V_0-ATPase), a multimeric complex that mediates the acidification of cellular compartments, such as the Golgi complex, endosomes and lysosomes, by pumping protons into their lumen (Oot et al., 2017). Nef interaction with V-ATPase is thought to occur via its regulatory subunit H (V1H), also known as Nef binding protein 1 (NBP1), relying on the C-terminal flexible loop of Nef (Lu et al., 1998). As the depletion of NBP1, or the disruption of NBP-1-Nef interaction, impaired the Nef-mediated reduction of cell-surface CD4 levels, the authors suggested that Nef interaction with V-ATPase plays a role in CD4 downregulation (Lu et al., 1998). Furthermore, V_1H binds the μ2 subunit of the endocytic clathrin adaptor AP-2 (AP-2μ2), via the $133–363_{aa}$ domain of V_1H and the N-terminal ($1–145_{aa}$) domain of AP-2μ2, which led to the proposal of a refined model in which the V-ATPase connects Nef with the endocytic machinery (Geyer et al., 2002). Nef was later shown to bind AP-2 directly (Chaudhuri et al., 2007; Ren et al., 2014) and to bridge AP-2 to CD4 (Januário and daSilva, 2020; Kwon et al., 2020); therefore, the relative importance of V_1H in this process remains an open question. Moreover, the fact that V_1H overexpression inhibits the Nef-mediated increase in HIV-1 infectivity (Geyer et al., 2002) indicates that this host factor may be involved in additional Nef actions.

In a different role, Nef changes protein trafficking by usurping the vesicle-sorting machinery regulated by the GTPase ARF-1 (**Figure 3**). This strategy is applied, for instance, to prevent viral antigen presentation by major histocompatibility complex I (MHC-I) molecules, a process that contributes to HIV-1 immune evasion (Cohen et al., 1999; Specht et al., 2008; previously reviewed in Pereira and daSilva, 2016). Nef precludes the delivery of newly synthesized MHC-I molecules to the cell surface, redirecting their transport from the TGN to the endolysosomal system for degradation (previously reviewed in Pereira and daSilva, 2016). As previously mentioned, the clathrin adaptor AP-1 is recruited by ARF-1 to TGN membranes to form clathrin-coated vesicles (CCVs) that are destined for endosomes. In fact, MHC-I downregulation by Nef was shown to require AP-1 (Williams et al., 2002; Roeth et al., 2004; Lubben et al., 2007; Schaefer et al., 2008; Leonard et al., 2011; Jia et al., 2012; Tavares et al., 2020) and ARF-1 activity (Wonderlich et al., 2011). Structural analysis revealed that ARF-1-GTP binding to AP-1 triggers conformational changes in AP-1, which acquires an 'unlocked' state that is compatible with vesicle-cargo binding (Ren et al., 2013). Intensive research into the mechanism of MHC-I downregulation by Nef revealed that the viral protein links the cytosolic tail of MHC-I to AP-1. Specifically, in the presence of the MHC-I cytosolic tail and ARF-1, Nef induces the trimerization of unlocked AP-1 in an "open" conformation that promotes CCV biogenesis (Shen et al., 2015).

Strikingly, a very similar strategy is used by Nef to prevent the cell surface delivery of the host restriction factor BST2 (also known as Tetherin) through retention at the TGN. In the presence of the cytosolic tail of BST2, Nef changes ARF-1-mediated AP-1 trimerization to a "closed" conformation that appears to be incompatible with CCV assembly (Shen et al., 2015). The structural basis for the cargo-dependent diversity of Nef-induced AP-1 trimerization was recently elucidated, and it was also revealed that it is finely tuned by the phosphorylation state of Nef (daSilva and Mardones, 2018; Morris et al., 2018).

The hijacking of ARF-1-mediated trafficking pathways by Nef may also occur beyond the TGN. It has been reported that

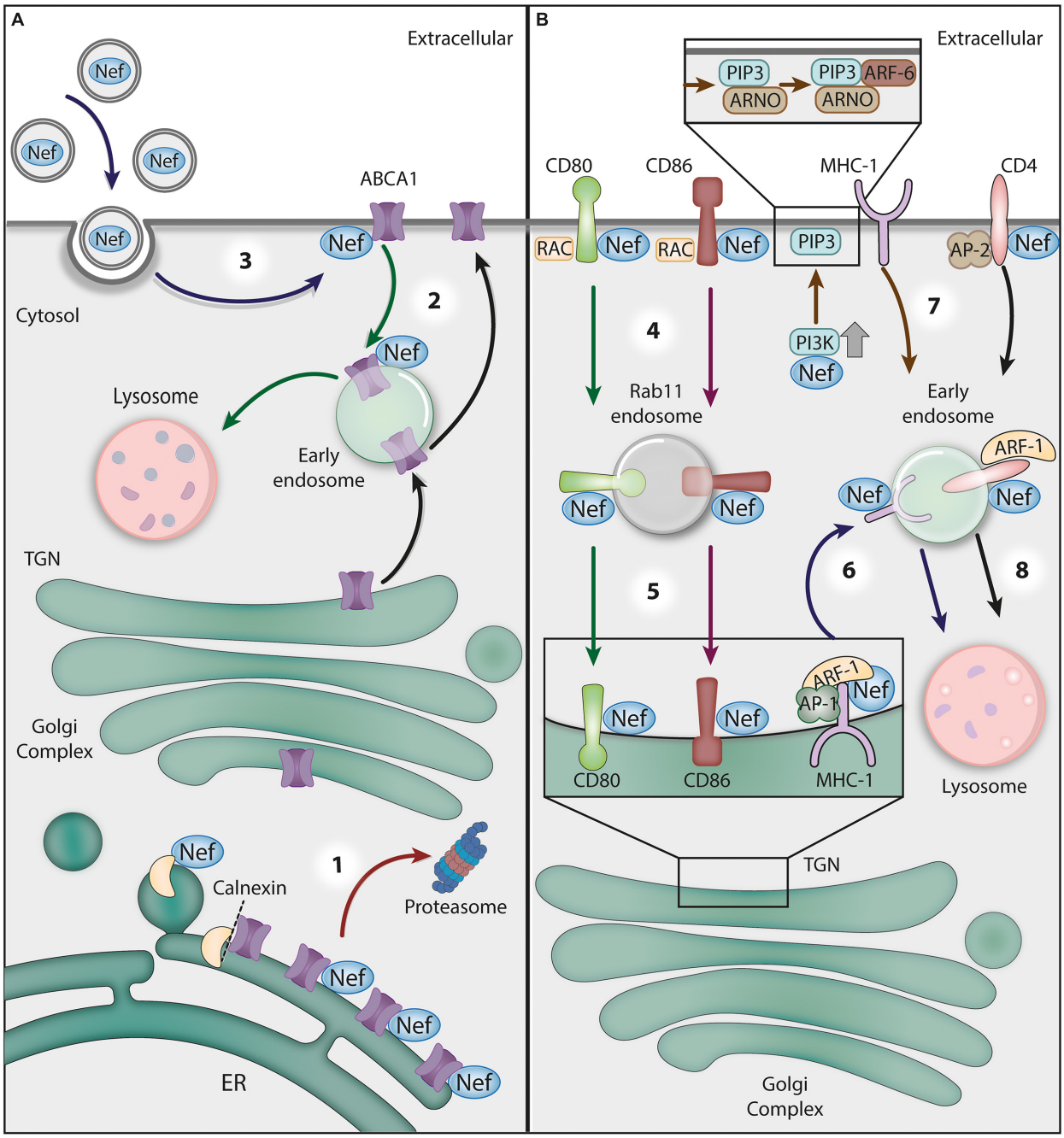

FIGURE 3 | The crosstalk between HIV-1 Nef and GTPases and ATPases in protein trafficking. **(A)** The Nef-mediated downregulation of ABCA1 ATPase. (1) Nef disrupts the interaction between newly synthesized ABCA1 and the ER chaperone calnexin, leading to the targeting of ABCA1 to the ERAD/proteasomal pathway (red arrow). (2) Additionally, Nef directs ABCA1 molecules that reach the plasma membrane (PM) to lysosomes for degradation (green arrows). (3) Recently, it has been proposed that Nef obtained from extracellular vesicles taken up by bystander cells may also downregulate ABCA1 (blue arrows). **(B)** Nef hijacks GTPase activities for receptor downregulation. (4) Nef interacts with the cytosolic tails of the surface proteins CD80 and CD86 to target them for Rac1 GTPase-dependent endocytosis. Nef stimulates the translocation of Src kinase to the PM, where it activates a Rac GEF (TIAM), which in turn activates Rac1, resulting in actin polymerization nucleation sites at the cell surface. (5) After endocytosis, Nef recruits Rab11- to CD80/CD86-positive vesicles to target them to the Golgi complex (red and green arrows). (6) To prevent MHC-I delivery to the PM, Nef links the MHC-1 CT to AP-1 and induces the ARF-1 GTPase-dependent trimerization of AP-1 into an 'open' conformation. This promotes the recruitment of MHC-I to forming clathrin-coated vesicles destined for endosomes (dark blue arrows). (7) Alternatively, Nef activates PI3K at the PM, leading to PIP3 accumulation, which favors the recruitment of PIP3-binding ARF-6 GEF (ARNO) and the subsequent ARF-6-dependent endocytosis of MHC-I (brown arrow). (8) Nef also induces the endocytosis of CD4 via AP-2/clathrin vesicles and then targets this receptor to lysosomes for degradation through an ARF-1-dependent mechanism (black arrow).

ARF-1 activity stabilizes Nef in endosome membranes and may facilitate Nef-mediated targeting of CD4 to this compartment for transport to lysosomes for degradation (Fauré et al., 2004). In fact, the expression of an ARF-1 dominant-negative form inhibited Nef-CD4 complex migration toward the lysosome (Fauré et al., 2004), highlighting the importance of ARF-1 in other receptor downregulation strategies utilized by Nef. Interestingly, it has been shown that Nef uses a variant of AP-1 (AP-1-γ2) to retain CD4 (Tavares et al., 2017) and MHC-I (Tavares et al., 2020) in endosomes for lysosomal delivery.

Nef-induced endocytosis via ARF-6 was also proposed to contribute to MHC-I downregulation. Specifically, Nef was shown to activate class I phosphatidylinositol 3-kinases (PI3K), leading to the accumulation of phosphatidylinositol (3,4,5)-triphosphate [PI(3,4,5)P3] at the inner leaflet of the PM. This was proposed to favor PM recruitment of the PI3P-binding protein ARF nucleotide-binding site opener (ARNO), an ARF-6 GEF, which stimulates the ARF-6-dependent endocytosis of MHC-I (Blagoveshchenskaya et al., 2002; Hung et al., 2007). Indeed, the pharmacological inhibition of class I PI3K or the overexpression of inactive ARNO was shown to compromise efficient MHC-I downregulation by Nef (Blagoveshchenskaya et al., 2002; Hung et al., 2007).

In addition to MHC-I, Nef also removes the immune costimulatory molecules CD80 and CD86 from the surface in antigen-presenting cells (**Figure 3**), such as DCs and macrophages, thus interfering with T cell priming (Chaudhry et al., 2007, 2008). The mechanism differs from that of MHC-I downregulation and involves the activity of Rac1, a small GTPase within the Rho family mentioned earlier in this review. Nef was shown to promote the translocation of Src kinase to the cell periphery, where Src promotes Rac GEF (TIAM) activation, which in turn activates Rac1 (Chaudhry et al., 2007). At the cell surface, Nef interacts with the CD80 and CD86 cytosolic tails and targets these molecules to actin polymerization nucleation sites at the PM that support endocytosis, possibly via Nef co-interaction with Rac1 itself, facilitating CD80/CD86 internalization (Chaudhry et al., 2007). Additionally, Nef was proposed to recruit Rab11 to vesicles containing internalized CD80/CD86 to return them to the Golgi complex (Chaudhry et al., 2008) (**Figure 3**).

Viral Protein U (Vpu)

The viral protein U (Vpu) encoded by the HIV-1 genome, is a type I transmembrane protein that contributes to viral immune evasion by antagonizing host proteins that are detrimental to virus replication and dissemination. Vpu acts by modifying the intracellular distribution of its targets, frequently directing them to a degradative pathway. Among the targets of Vpu are CD4, HLA-C, Tim-3, BST2 (Tetherin), CD1d and NTB-A (Strebel, 2014; previously reviewed in Sauter and Kirchhoff, 2018; Prévost et al., 2020). In many cases, the mechanisms underlying the actions of Vpu depend on the participation of GTPases.

Among the most intensively studied functions of Vpu is its antagonism to BST2, an interferon-induced host restriction factor that attenuates viral transmission by impairing the release of HIV-1 and other enveloped viruses from infected cells (Neil et al., 2008; Van Damme et al., 2008; Perez-Caballero et al., 2009). Vpu is thought to remove BST2 from HIV budding sites at the PM mainly by preventing naturally internalized and newly synthesized BST2 molecules from reaching the cell surface, eventually leading to its downregulation (Schmidt et al., 2011). Similar to the downregulation of MHC-I by Nef, Vpu also hijacks the ARF-1/AP-1 sorting machinery to antagonize BST2. Specifically, Vpu was shown to form a tripartite complex with BST2 and AP-1 at the TGN that is thought to load BST2 into CCVs destined for endosomes and to block the resupply of BST2 to the PM (Jia et al., 2012).

Vpu was also shown to target BST2 to lysosomes for degradation (Mitchell et al., 2009; Dubé et al., 2010). In this regard, the GTPase Rab7A plays a key role in the constitutive turnover of BST2, and its depletion was shown to compromise BST2 endolysosomal degradation induced by Vpu (Caillet et al., 2011). HRS, a component of the ESCRT-0 machinery, has also been shown to participate in Vpu-mediated BST2 downregulation by recognizing ubiquitinated BST2 and targeting it for lysosomal degradation via the MVB pathway (Janvier et al., 2011). Therefore, another energy-harnessing molecule likely involved in the antagonism of BST2 by Vpu is the AAA-ATPase VPS4, whose activity, as previously discussed, is essential for ESCRT function at MVBs.

FINAL CONSIDERATIONS AND FUTURE DIRECTIONS

The spatiotemporal control of viral and host proteins distribution in infected cells is key to several steps in the HIV-1 replication cycle. These processes require specialized transport machinery and demand chemical energy, which is supplied by the host cells through ATP and GTP hydrolysis. With the exception of the reverse transcriptase and the integrase, other HIV-1 proteins do not possess known ATP or GTP binding properties and are incapable of directly harnessing energy from these molecules. Instead, HIV-1 factors repurpose host GTPases (such as Dynamin and small GTPases – Ran, Rab, ARF, and Rho family members) and ATPases (such as ABCE1, ABCA1 and VPS4) to regulate: (1) the subcellular distribution of viral components, (2) the subcellular distribution of host proteins that affect virus replication and infectivity and, (3) membrane remodeling reactions required for viral entry and assembly/egress. Several examples of these strategies are discussed here, and many others are likely to arrive.

Despite the outstanding effort in understating the interplay between HIV-1 and energy-related proteins, questions issues remain unsolved. These include, but are not limited to: (1) Why the function of Dynamin in endocytosis, Rab5, and Rab7 is relevant in HIV-1 entry in only some cell types? (2) Which is the precise role of ABCE1 in HIV-1 infection *in vivo*? (3) What are the mechanisms used by the HIV-1 to activate Rho GTPases and their downstream effectors to modulate the actin

cytoskeleton? (4) How can ARF-1, Rab8A, and Rab29-mediated transport pathways influence Gag targeting tetraspanin-enriched microdomains during the VSs? (5) Which is the primary model that explains ABCA1 downregulation by Nef? (6) Why is the binding of Nef to the vacuolar H+ ATPase important in CD4 downregulation if Nef can directly bridge CD4 to AP-2?

To address these questions, efforts should be directed to using cellular models that are physiologically relevant for HIV infection and validating the findings using primary human cells and animal models when possible. RNAi and knockout library screening techniques, the several novel high-resolution imaging approaches, and the multiple structural and biochemical methods that became available in recent years will help shed light on these unsolved issues.

Besides contributing to viral fitness, deviating these energy-related molecules from their normal cell function may have broader cellular physiology effects, which likely influences HIV-1 pathogenesis. In this respect, drugs that interfere with specific GTPases or ATPases function may represent potential new anti-HIV agents candidates. Moreover, efforts to discover specific interactions between HIV-1 factors and energy-related molecules may offer new targets for small molecule inhibitors to develop additional anti-HIV therapies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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ARF GTPases and Their Ubiquitous Role in Intracellular Trafficking Beyond the Golgi

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Molecular switches of the ADP-ribosylation factor (ARF) GTPase family coordinate intracellular trafficking at all sorting stations along the secretory pathway, from the ER-Golgi-intermediate compartment (ERGIC) to the plasma membrane (PM). Their GDP-GTP switch is essential to trigger numerous processes, including membrane deformation, cargo sorting and recruitment of downstream coat proteins and effectors, such as lipid modifying enzymes. While ARFs (in particular ARF1) had mainly been studied in the context of coat protein recruitment at the Golgi, COPI/clathrin-independent roles have emerged in the last decade. Here we review the roles of human ARF1-5 GTPases in cellular trafficking with a particular emphasis on their roles in post-Golgi secretory trafficking and in sorting in the endo-lysosomal system.

Keywords: membrane trafficking, Golgi, adaptors, ARF GTPase, TGN, endosomes, clathrin, COPI

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INTRODUCTION

ADP-ribosylation factor GTPases are major regulators of intracellular trafficking. Based on sequence similarities, they have been classified into type I (ARF1-3; humans lacking ARF2), type II (ARF4 and ARF5) and type III with the only member being the PM-localized ARF6. ARFs on/off switch is tightly regulated by guanine nucleotide exchange factors (GEFs), that turn ARFs on and GTPase activating proteins (GAPs), that trigger GTP hydrolysis, returning ARFs to the off state (Sztul et al., 2019). Hence, GEFs and GAPs establish the duration of signaling driven by specific ARFs. Activation of ARFs by GEFs triggers exposure of a N-terminal amphipathic helix that is important for membrane association and has an active role in membrane curvature generation (Beck et al., 2008). GTP binding and the downstream conformational changes in ARFs are crucial for effector recruitment and to trigger a specific biological response. Importantly, while numerous ARF GEFs and GAPs have been identified, not much is known about which GEF/GAP/ARF coordinates which specific cellular function (for a more in-depth review regarding the function and localization of the ARF GEFs and GAPs see Sztul et al., 2019). Intriguingly, although the ARF-GTP conformation is referred to as the "active" state, ARF proteins can also interact with effectors in the "inactive" ARF-GDP bound state. This is the case of ARF6-GDP, which engages with regulators of other small G proteins, raising the interesting possibility that GDP and GTP bound ARF6 would lead to activation of alternate signaling pathways depending on the bound nucleotide [reviewed in Donaldson and Jackson (2011)]. Turning off ARFs is also crucial for their function as failure to inactivate ARFs can block downstream trafficking. For example, a GTP-locked ARF6 mutant blocked membrane recycling between the PM and endosomes (Eyster et al., 2009) and the

GTP-locked form of ARF1 prevented cargo loading into COPI vesicles (Malsam et al., 1999), highlighting the importance of GTP hydrolysis beyond being a mere turning off switch.

ADP-ribosylation factors are highly conserved in sequence and structure, with type I ARFs >96% identical, type II ARFs 90% identical to each other and 80% identical to type I ARFs and ARF6 >65% identical to type I and II ARFs. ARFs possess two switch regions (switch I and II), which change conformation upon GTP binding and mediate the interaction with effectors and regulators. In all ARF family members, switch I and II are almost identical (Goldberg, 1998; Pasqualato et al., 2001). Due to the very high sequence and structural similarities, it is still unclear what drives ARFs association with different intracellular membranes and how effectors are differentially recruited to trigger a specific cellular response. Interestingly, GTP-bound, but not GDP-bound, ARF1 and ARF6 are structurally very similar, suggesting ARFs may discriminate between effectors in their inactive form (e.g., binding to different GEFs for activation). The structures of GDP-bound ARF6 and ARF1 show that differences in sequence outside of the switch regions result in conformational differences within the switch regions, possibly driving the specificity for regulators in living cells (Menetrey et al., 2000). While structural differences have been highlighted for ARF1 and ARF6, which are the most divergent ARFs in sequence, structural data is not available for the other type I and II ARFs with a higher degree of sequence similarity. Recruitment of ARFs to intracellular membranes was also shown to be driven by the interaction of ARFs with specific membrane localized receptors. A specific 16 amino acid sequence in ARF1 is responsible for its recruitment to early Golgi cisternae via the SNARE membrin (Honda et al., 2005). ARF1-GDP is additionally recruited to the Golgi via binding to p23, a member of the p24 family of transmembrane proteins via its C-terminus. The *trans*-Golgi network (TGN) localization of ARF3 is also determined by its C-terminus, making it unlikely that membrane association is driven by the interaction with the activating GEF, as the surface interacting with the Sec7 domain lies on the opposite side of the protein. This suggests the presence of a yet to be identified receptor for ARF3 recruitment to the TGN membranes (Manolea et al., 2010).

In terms of cellular functions, the role of ARF1 in the formation of COPI vesicles at the Golgi has been extensively studied through *in vitro* reconstitution or hybrid approaches using COPI budding assays in semi-permeabilized cells and purified Golgi membranes (Orci et al., 1986; Reinhard et al., 2003; Adolf et al., 2013). These studies led to a detailed molecular understanding of the ARF on/off switch. However, in the test tube, the specificity of action driven by the specific recruitment of a GTPase and its regulators to a sub-cellular membrane is lost. Studies of ARF function had initially been primarily focused on understanding how ARF1 regulates COPI coat recruitment. In the last decade, various publications have highlighted the roles of type I and II ARFs in post-Golgi trafficking steps, including exocytosis, endo-lysosomal trafficking and also coat-independent mechanisms of action. In this short review, we will focus on what is known about ARFs in terms of localization as well as function in post-Golgi secretory and endosomal trafficking.

ARF PROTEINS SHOW DISTINCT AS WELL AS OVERLAPPING DISTRIBUTION THROUGHOUT THE CELL

Types I and II ARFs have all been localized to the Golgi apparatus, while the sole type III member ARF6 is the only non-Golgi associated ARF and localizes to the PM and endosomes (**Figure 1**; Sztul et al., 2019). When dissecting the intra-Golgi localization, ARF1, ARF4 and ARF5 localize to the *cis* cisternae and the TGN, whereas ARF3 localization is limited specifically to the TGN due to its two unique C-terminal determinants (A174 and K180), which are conserved among ARF3 homologs across species (Claude et al., 1999; Kawamoto et al., 2002; Deretic et al., 2005; Honda et al., 2005; Mazelova et al., 2009; Manolea et al., 2010; Sadakata et al., 2010). Golgi-associated ARFs are responsible for COPI recruitment, however, it is unclear which ARF contributes to COPI vesicles formation in living cells. The double knockdown (KD) of ARF1 and ARF4 was reported to trigger dissociation of COPI from the Golgi (Volpicelli-Daley et al., 2005) and ARF1, ARF4, and ARF5 all support COPI vesicle formation of *in vitro* generated vesicles from Golgi membranes (Popoff et al., 2011). Moreover, COPI vesicles generated in semi-permeabilized cells with type I or type II ARFs show similar content (Adolf et al., 2019).

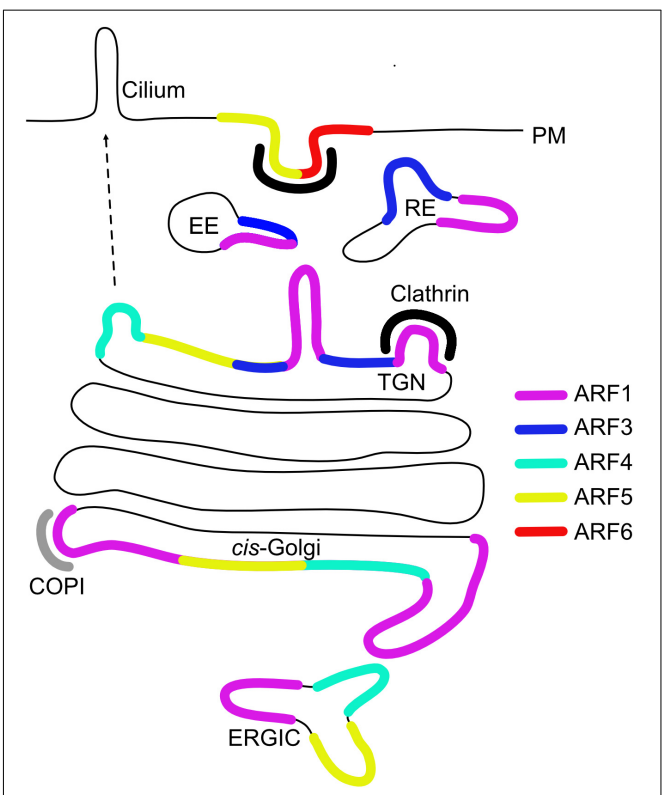

FIGURE 1 | Intracellular localization of ARF GTPases. Early endosome (EE), recycling endosome (RE), *trans*-Golgi network (TGN), ER-Golgi intermediate compartment (ERGIC), and plasma membrane (PM).

Although predominantly at the Golgi, fluorescently tagged ARF1 and type II ARFs were also reported to localize to peripheral ERGICs in living cells (Chun et al., 2008). Tubulation of the ERGIC after simultaneous depletion of ARF1 and ARF4 already hinted to an involvement of both type I and type II ARFs in sorting at the ERGIC a few years earlier (Volpicelli-Daley et al., 2005). However, type I and II ARFs show differential membrane association dynamics, suggesting a functional difference (Duijsings et al., 2009).

GFP-tagged ARF1 and ARF3 have been shown to localize to recycling endosomal compartments containing endocytosed transferrin (Tfn) and their simultaneous depletion inhibited PM recycling of Tfn (Kondo et al., 2012). Furthermore, ARF1 and ARF3 have been found on Rab4-positive endosomal membranes (D'Souza et al., 2014).

At the PM and endosomes, ARF6 is involved in clathrin-mediated endocytosis, clathrin-independent endocytosis and recycling pathways (D'Souza-Schorey et al., 1995; Peters et al., 1995; Donaldson, 2003). Interestingly, while ARF6-GTP localization is restricted to clathrin coated pits and endocytic vesicles, its role may be downstream of endocytosis in facilitating fast PM recycling (Montagnac et al., 2011). Aside from its role at the Golgi, ARF5 has also been found to act at the PM. Here, ARF5 controlled integrin endocytosis and was localized to clathrin-coated pits together with IQSEC1/BRAG2, a GEF known for activating ARF6 (Moravec et al., 2012). Recently, ARF5 and IQSEC1 were also implicated in Ca^{2+}-dependent disassembly of focal adhesions during cell migration (D'Souza et al., 2020). Further supporting a role for ARF5 in migration, the GTPase was shown to be recruited to vesicular structures in cell protrusions via IQSEC1 and to promote invasion and metastatic cancer by controlling phosphoinositide metabolism (Nacke et al., 2021).

In conclusion, ARFs show overlapping yet distinct distribution and functions throughout the secretory and endocytic pathways. This complexity makes the investigation of their diverse functions and the identification of specific regulators and effectors especially challenging. In particular, because of the high sequence similarity, the endogenous localization of all ARFs is not yet known and localization studies have relied on the overexpression of tagged GTPases until very recently. A promising technique that will help define the roles of ARFs in living cells is gene editing, which has been instrumental in highlighting the multiple roles of ARF1 at the Golgi (Bottanelli et al., 2017).

THE ROLE OF ARFs IN ADAPTOR RECRUITMENT

At the TGN, cargoes are sorted into distinct classes of vesicles destined either to the endo-lysosomal system or to the PM for secretion. Interestingly, direct, as well as indirect, secretory pathways that detour via endosomal compartments have been characterized (Stalder and Gershlick, 2020). At the TGN and in downstream endosomal compartments, ARFs have a role in recruiting cargo adaptor proteins, such as the oligomeric adaptor protein (AP) complexes and the monomeric Golgi-localized, γ-ear-containing, ARF-binding proteins (GGAs).

AP1-4 are known to function at the TGN and/or downstream endosomes (Tan and Gleeson, 2019). Still, the exact cellular function of AP complexes is not fully understood - which in particular extends to their interaction with different ARFs. The best studied case is the ARF1-dependent recruitment of AP-1 to the TGN. TGN-associated GTP-bound ARF1 recruits cytosolic AP-1 and changes the conformation of AP1 from closed to open, thus "unlocking" AP-1 and allowing binding to the sorting motifs of cargoes (Stamnes and Rothman, 1993; Ren et al., 2013). Membrane curvature is then induced by AP interaction partners, such as coat (e.g., clathrin) and accessory proteins, initiating the formation of vesicles. The crystal structures in Ren et al. (2013) highlight the structural change of AP-1 upon ARF1 activation and demonstrate that ARF1 binds the β1 and γ subunits of AP-1 via its switch I and II motifs. However, the switch I and II residues are highly conserved among ARFs. Therefore, the observed recruitment of AP-1 to the membrane could be also driven by other ARFs, which have been localized to late Golgi compartments (like ARF3). Additionally, AP-3 and AP-4 are structurally similar to AP-1 (Ren et al., 2013) and have also been shown to be recruited to membranes via interaction with ARF1 (Ooi et al., 1998; Boehm et al., 2001). AP-3 has a role in transport to late endosomes (LEs) and lysosomes (Peden et al., 2004), while AP-4 has recently been implicated in autophagosomal membrane formation from the TGN (Mattera et al., 2017). It would be interesting to explore whether different AP complexes are recruited through different membrane-localized ARFs. A better understanding of the localization of ARFs in the endo-lysosomal system will be necessary to understand which ARF recruits which AP on a specific cellular membrane.

Monomeric adaptors of the GGA family are also recruited to the TGN by binding to ARFs via their GAT domain (Collins et al., 2003). Three human GGA proteins (GGA1-3) have been identified and have all been localized to the TGN, where it is unclear whether they have distinct or overlapping roles in the transport to the lysosome (Ghosh and Kornfeld, 2004). Interestingly, the TGN association of GGAs relies on coincidence detection via ARF1 and phosphatidylinositol-4-phosphate (PI4P) (Dell'Angelica et al., 2000; Puertollano et al., 2001; Wang et al., 2007; Kametaka et al., 2010), as for AP-1 (Wang et al., 2003). Thus, PI4P plays a general role in ensuring the specificity of recruitment of various machinery to the TGN, as adaptors and other ARF effectors, like four-phosphate-adaptor protein (FAPP), require the concomitant presence of phosphoinositide (PI) species and specific ARFs to associate with membranes. PI4P is predominantly enriched at the TGN and regulated by phosphatidylinositol-4-OH kinases (PI4Ks), specifically PI4KIIIβ and PI4KIIα (Makowski et al., 2020). It has been reported that ARF1 mediates PI4KIIIβ activation and recruitment to the Golgi membrane (Godi et al., 1999; Haynes et al., 2005), where PI4KIIIβ can then stimulate PI4P synthesis (Godi et al., 1999). Although this is an interesting model that puts ARF1 functionally upstream of other PI4P-binding proteins, there are also other proteins that may mediate PI4KIIIβ membrane association (Waugh, 2019). Additionally, membrane contact sites between the TGN

and the ER have been shown to have a role in regulation of TGN PI4P levels (Mesmin et al., 2013; Capasso et al., 2017; Wakana et al., 2021).

In conclusion, various ARFs, together with PI4P, may provide the molecular specificity for cargo selection mediated by adaptors. While ARF1 is again the best studied member, investigating the role of the other ARFs at the TGN may bring some interesting insights.

THE DIVERSE FUNCTIONS OF ARFs AT THE TGN

ARF1 is responsible for the recruitment of the adaptor AP-1 and clathrin to the TGN. AP-1 has a role in diverse sorting steps, including constitutive secretion, basolateral sorting in polarized cells, as well as bi-directional communication between TGN and endosomes (Stamnes and Rothman, 1993; Folsch et al., 1999; Wang et al., 2003; Chi et al., 2008). It is unclear whether ARF1 is the sole player in the recruitment of AP-1 to different membranes. In fact, depletion of both ARF1 and ARF4 was necessary to trigger dissociation of AP-1 from Golgi membranes (Nakai et al., 2013). Whether ARF1 and ARF4 act in concert or have redundant functions remains to be investigated. The Golgi localization of ARF3 is confined to the TGN, making it a potential candidate for adaptor recruitment for post-Golgi trafficking. However, the high sequence similarity between the type I ARFs (ARF1 and ARF3) and the lack of known differential interactors has made it very hard to pin-point any functional differences between the two members. Interestingly, exit of secretory cargoes from the TGN is inhibited at 20°C and ARF3 dissociates from the membrane at this temperature. However, membrane dissociation of ARF3 does not seem to be the cause of the blockage of secretory trafficking as ARF3 KD does not affect AP-1 and PI4P levels (Manolea et al., 2010). The Brefeldin A-inhibited guanine nucleotide exchange factors 1 and 2 (BIG1 and BIG2) have been shown to activate both ARF1 and ARF3 for the downstream recruitment of AP-1, suggesting a role of ARF3, in addition to ARF1, in AP-1-dependent pathways (Lowery et al., 2013). Further, ARF3 regulates trafficking of Toll-like receptor 9 (TLR9) to endo-lysosomes, possibly by facilitating TGN export (Wu and Kuo, 2015). Even though ARF1, ARF3, and ARF4 have all been implicated in TGN export, it has been difficult to directly pin-point the exact place of action of these ARFs using functional trafficking assays. Diffraction-limited light microscopy does not provide sufficient resolution to clearly separate Golgi cisternae and ERGIC elements, making it hard to identify the exact place where trafficking is impaired upon ARF depletion. While single ARFs were shown not to have an effect on secretory trafficking, in particularly on TGN export of vesicular stomatitis virus G (VSV G) protein (Volpicelli-Daley et al., 2005), it was recently reported that KD of ARF1 and ARF4 inhibit TGN export of the β-secretase BACE1 but not amyloid precursor protein (APP) (Tan et al., 2020). The latter study suggests that specific ARFs may be required for the trafficking of specific cargoes via recruitment of different adaptors.

Another recently highlighted role for ARF1 is the formation of tubular vesicular trafficking intermediates containing secretory cargoes at the TGN. Interestingly, these tubules contain discrete patches of clathrin but it is unclear whether clathrin has a functional role or if adaptors are involved in this process (Bottanelli et al., 2017). ARF1 interacts with effectors, like the PH-domain containing protein FAPP, which may facilitate membrane deformation and aid tubulation (for more details, read Stalder and Gershlick, 2020). In addition, ARF1 tubules do not fuse with the PM, suggesting they either lose ARF1 prior to fusion or an intermediate sorting station may be present (Bottanelli et al., 2017).

ARFs also seem to possess specialized secretory functions in specific cell types. ARF4 and ARF5 have been shown to regulate biogenesis/trafficking of dense core vesicles (DCVs) in rat PC12 cells (Sadakata et al., 2010). DCVs are important carriers, which transport cargoes like neuropeptides to the nerve terminal in neurons. Interestingly, here GDP-bound ARFs are recruited to the TGN by calcium-dependent activator protein for secretion 1 (CAPS1). ARF4 was also shown to specifically bind the C-terminal ciliary target signal on rhodopsin for its transport to the primary cilium (Deretic et al., 2005) and the various GEFs and GAPs involved in ARF4-dependent ciliary transport have also been identified (Deretic et al., 2021).

In summary, all four type I and II ARFs are implicated in TGN export, AP-1 dependent pathways or secretion in specialized cells. However, it is unclear which ARF (if any) is responsible for constitutive secretion and direct TGN-to-PM pathways. Additionally, more specialized functions of ARFs in different cell types may have yet to be discovered.

THE ROLE OF ARFs IN TRAFFICKING IN THE ENDO-LYSOSOMAL SYSTEM

ARFs and their effectors are emerging as key components of the molecular machinery mediating cargo sorting in endosomal pathways. Recycling endosomes (REs) are involved in recycling of cargoes back to the PM after endocytosis and from endosomes back to the TGN. Volpicelli-Daley et al. (2005) were among the first to investigate the role of ARFs in endosomal recycling pathways. Their approach consisted of monitoring the morphology of endosomes, as well as various membrane trafficking steps upon depletion of human ARF1-5 in HeLa cells. As observed for other trafficking steps, KD of individual ARFs was not sufficient to alter the morphology of Tfn-positive REs. However, simultaneous KD of ARF1 and ARF3 caused REs tubulation (Volpicelli-Daley et al., 2005; Kondo et al., 2012) and inhibited endocytic recycling of the Tfn receptor (Volpicelli-Daley et al., 2005). This is in agreement with the observation that the ARF GEF BIG2 has a role in the maintenance of the structural identity of REs through activation of ARF1 and ARF3 (Shin et al., 2004). Additionally, ARF1 and ARF4 double KD also resulted in tubulation of REs and inhibited retrograde endosome-to-TGN transport (Nakai et al., 2013), without affecting endocytic recycling (Volpicelli-Daley et al., 2005; Nakai et al., 2013). ARF4 has been recently identified as an

interactor of active GTP-bound Rab30, which also plays a role in the same recycling pathway (Zulkefli et al., 2021). Taken together, ARF1 may differentially regulate export from REs to either the PM (together with ARF3) or the TGN (with ARF4).

ARFs have also been implicated in sorting processes at early endosomes (EEs). EEs are sorting hubs that can either sort proteins to the PM, to the TGN or to lysosomes for degradation. Tubular domains on EEs are thought to be responsible for retrograde EE-to-TGN transport and cargo recruitment is mediated via various adaptors (GGAs, AP-1, and AP-3). D'Souza et al. (2014) reported that ARF1 KD, but not ARF3 KD, was sufficient to induce the formation of long tubules emanating from Rab4-positive EEs and to trigger membrane dissociation of AP-1 and AP-3. KD of either ARF1 or ARF3, however, resulted in dissociation of GGA3 from EEs, suggesting that ARF1 and ARF3 may have overlapping (GGA3 recruitment) yet distinct (AP-1 recruitment) sorting roles on EEs.

Regulators of ARF function have also been extensively studied and provide, albeit indirectly, proof of the importance of ARF-mediated sorting in the endosomal system. The GEFs BIG1 and BIG2 localize to the TGN and endosomes, where they activate ARFs resulting in recruitment of adaptors (Shinotsuka et al., 2002; Zhao et al., 2002; D'Souza et al., 2014). Interestingly, catalytically inactive BIG2 induces tubulation of REs (Shin et al., 2004), similar to what was observed when depleting BIG2 (but not BIG1) via small interfering RNA (Ishizaki et al., 2008). In particular, downregulation of BIG2 affected protein association with the REs, whereas simultaneous depletion of BIG1 and BIG2 resulted in the dissociation of TGN and RE proteins, as well as impaired retrograde trafficking from the LEs to the TGN (Ishizaki et al., 2008). This suggests a model in which BIG2 functions at the REs, while BIG1 and BIG2 may cooperate to regulate retrograde transport from LEs back to the TGN.

Next, various ARF GAPs have also been implicated in the regulation of ARFs beyond the Golgi. AGAP1 reportedly interacts with AP-3, regulating trafficking from the TGN to the lysosomes (Nie et al., 2003) and AGAP2 was shown to interact with AP-1 (Nie et al., 2005). A gene mutated in amyotrophic lateral sclerosis (ALS) and frontal temporal degeneration (FTD), called C9orf72, was recently identified as a GAP for ARF1. Intriguingly, C9orf72 localization is restricted to lysosomes under amino acid starvation conditions (Su et al., 2020) and has been suggested to regulate ARF1 in *trans* via interaction of lysosomes and ARF1-positive membranes.

Like ARFs themselves, their GEFs and GAPs may be recruited to multiple locations where they have different functions. Pinpointing the function of distinct ARFs, as well as their GEFs and GAPs is a challenging task, further complicated by the lack of a complete understanding of the sorting pathways emerging from the TGN. Additionally, it should be also considered that regulatory networks of the various ARFs may be closely interconnected via ARF cascades, explaining why multiple GTPases have been found to interact with the same ARF regulator (Stalder and Antonny, 2013).

OUTLOOK

Untangling the cellular ARF code will be a challenge due to the complexity of ARF synergies, their differential functions and the lack of knowledge about the specificity of each ARF for GAPs and GEFs *in vivo*. Thus, an interesting challenge ahead is to investigate the specific interplay between GEFs/GAPs and ARFs as well as their nanoscale localization in an unperturbed cellular environment. ARF GTPases are the master regulators of intracellular trafficking. While we know a lot about the molecular mechanisms governing their GTPase cycle, it has been incredibly hard to study their function in living cells due to their high sequence and structural similarity and possible functional redundancy. The fact that concomitant depletion of two ARFs is often necessary to observe a cellular phenotype raises the interesting possibility that ARFs may be acting in pairs. Work from the Weiland lab has shown that ARF1-GTP dimers form *in vitro* and may be responsible for the scission of vesicles (Beck et al., 2008, 2011). A recent publication shows that ARF-GEF dimers recruit two closely spaced ARF1-GTP to the membranes, promoting vesicles formation (Brumm et al., 2020), supporting a functional role for ARF dimers in living cells. Further work will be required to test whether different ARF heterodimers are responsible for selective recruitment of various downstream effectors and cargoes via differential adaptor recruitment.

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