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REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF MOTORS

Endosomal sorting sorted – motors, adaptors and lessons from *in vitro* and cellular studies

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ABSTRACT

Motor proteins are key players in exerting spatiotemporal control over the intracellular location of membrane-bound compartments, including endosomes containing cargo. In this Review, we focus on how motors and their cargo adaptors regulate positioning of cargoes from the earliest stages of endocytosis and through the two main intracellular itineraries: (1) degradation at the lysosome or (2) recycling back to the plasma membrane. *In vitro* and cellular (*in vivo*) studies on cargo transport thus far have typically focussed independently on either the motor proteins and adaptors, or membrane trafficking. Here, we will discuss recent studies to highlight what is known about the regulation of endosomal vesicle positioning and transport by motors and cargo adaptors. We also emphasise that *in vitro* and cellular studies are often performed at different scales, from single molecules to whole organelles, with the aim to provide a perspective on the unified principles of motor-driven cargo trafficking in living cells that can be learned from these differing scales.

KEY WORDS: Cytoskeleton, Membrane trafficking, Motor proteins, Dynein, Kinesin, Myosin, Cargo adaptors, Endocytosis

Introduction

Endocytosis controls the uptake of cargoes, such as receptors, nutrients and lipids, which are internalised from the plasma membrane and extracellular milieu into the cell. Upon internalisation, endocytosed cargoes are sorted into two broad endosomal fates – recycling and degradation. Recycling serves to redeliver the endosomal cargo back to the plasma membrane or extracellular environment, whereas degradation results from delivery of the cargo to the lysosome (Redpath et al., 2020). Coupling of endocytosis and endosomal trafficking to the actin and microtubule (MT) cytoskeleton is essential for regulation of cargo sorting for these recycling or degradative fates (Hinze and Boucrot, 2018; Naslavsky and Caplan, 2018).

Firstly, actin branching facilitates endosome extrusion from the plasma membrane, followed by scission to liberate the endosome and enclosed cargo from the plasma membrane. The dense filamentous cortical actin proximal to the plasma membrane provides a physical path for newly formed endosomes to traverse further into the cytoplasm (reviewed by Chakrabarti et al., 2021).

Upon delivery into the cytoplasm, endosomes and their cargo fuse with sorting endosomes, marked by the GTPase Rab5 (which has Rab5a, Rab5b and Rab5c isoforms in mammals) (Naslavsky and Caplan, 2018). Cargoes targeted for recycling via Rab4 (Rab4a and Rab4b)- or Rab11 (Rab11a and Rab11b)-dependent recycling pathways are retrieved from the sorting endosome (Campa et al., 2018; Yudowski et al., 2009). The Rab5-positive sorting endosome matures into a Rab7 (Rab7a and Rab7b)-positive late endosome, with cargoes that are not targeted for Rab4- and Rab11-mediated recycling retained. The late endosome eventually matures into or fuses with the lysosome, exposing the remaining endosomal cargoes to degradative enzymes and ending their endocytic journey (Guerra and Bucci, 2016).

Regardless of endosomal fate, recycling or degradation requires long-range movement of endosomal cargoes along MTs within the cell. Rab11-positive recycling endosomes coalesce in the endocytic recycling compartment in the perinuclear region of the cell (Xie et al., 2016), and degradative lysosomes also cluster around the perinuclear region (Johnson et al., 2016). Indeed, MT depolymerisation has been shown to impair the movement and maturation of Rab5-positive sorting endosomes, inhibiting cargo degradation (Mesaki et al., 2011; Nielsen et al., 1999). MT depolymerisation also prevents the retrieval of recycling cargoes from sorting endosomes (Delevoye et al., 2014), whereas inhibition of stabilised detyrosinated MTs prevents cargo export from the perinuclear recycling endosomal compartment to the plasma membrane (Delevoye et al., 2014; Lin et al., 2002).

Although both endocytic trafficking and motor protein behaviour have been thoroughly described independently in multiple studies over several decades, it is still poorly understood how these distinct processes are coordinated, or how stochastic processes, such as motor binding to cargo, lead to defined cargo movement within cells. It is also not clear how these stochastic processes are spatiotemporally regulated. In this Review, we will follow the journey of a cargo that has just been internalised in an endosome, moving through the cortex and subsequently cytoplasmic MTs, in order to understand how differential sorting, either to the lysosome near the nucleus or back to the plasma membrane, are affected by motor proteins. We will reconcile findings from *in vitro* reconstitution experiments with those from *in vivo* and cellular studies and provide a framework for future studies in this area and to allow for a comparison of observations across different cell and cargo types.

Cytoskeleton and motor proteins work in concert to effect cargo transport

Both F-actin and MTs are polar polymers that self-assemble in energy-dependent active processes; they are both dynamic, that is, they can switch from phases of polymerisation to depolymerisation, and vice versa. Their inherent polarity results in the presence of a plus- and a minus-end of the filament, with the plus-end being highly dynamic [faster (de)polymerisation], and the minus end, less dynamic [slower (de)polymerisation].

There are several excellent reviews that focus on cytoskeletal structure, assembly and disassembly (Brouhard and Rice, 2018;

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Rottner et al., 2017). Here, we reiterate that in non-polar cells (such as HeLa, HEK293 and other cell lines typically used), the polarity of both the cortex and MTs is described as 'plus-end out', meaning the plus-ends are oriented towards the cell periphery. In these cells, MTs emanate radially from a MT-organising centre (MTOC), the centrosome, which is positioned close to the cell centre. MT minus-ends are capped at the centrosome, and therefore exhibit no dynamics. Non-centrosomal MTs are also present in cells and emanate from Golgi compartments.

Motor proteins convert the chemical energy of ATP into mechanical work and are thus able to power the movement of a variety of cargo within living cells. They work in concert with underlying cytoskeletal filaments, F-actin and MTs to enable long-range movement of cargo.

The two main classes of motor proteins are distinguished by their choice of cytoskeletal filaments. Myosin motors are F-actin based, whereas kinesins and dyneins are MT based. Focusing on the motors that participate in endocytic trafficking, myosin V and VI move cargo through the cortex, whereas kinesins and cytoplasmic dynein 1 (hereafter just dynein) are responsible for plus- and minus-end-directed movement on MTs. These motor proteins contain one or more ATPase domains that bind and hydrolyse ATP, and another domain, typically called the tail domain, that interacts either directly or indirectly with the membrane of the cargo-containing endosome. A coiled-coil stalk connects the head to the MT-binding domains (dynein) or to the tail (kinesin and myosin) and a linker connects the head to the stalk. All motor proteins stochastically bind and unbind from cytoskeletal filaments and cargo, and their activity is regulated, for instance via autoinhibition mediated by self-dimerisation of the motor domains (Torisawa et al., 2014), or folding over of the tail domain onto the motor (Belyy et al., 2016). Binding of motor proteins to cargo (typically via an adaptor protein) and in some instances other regulators (such as dynactin for dynein) alleviates this inhibition and enables active movement of the motor.

Given the orientation of F-actin at the cortex and MTs in a typical non-polar cell, the minus-end-directed myosin VI is the first motor to encounter and traffic endosomes through the cortex. The endosome is then handed over to the minus-end-directed MT motor dynein (Watanabe and Higuchi, 2007). Early endosomes have been described to move bidirectionally on MTs, relying on kinesin-1 motors for runs towards the plus-end. If the cargo is destined for lysosomal degradation, dynein continues to move the cargo-containing endosome to interior compartments of the cell. In contrast, if the cargo is destined to be recycled back to the plasma membrane, the respective recycling endosomes are first moved by kinesin motors, and then handed over to myosin V for crossing the dense cortex before reaching the membrane; see extensive reviews on these motors for further details (Batters and Veigel, 2016; Magistrati and Polo, 2021; Reck-Peterson et al., 2018; Wang et al., 2015).

Navigating the dense cortex with myosin VI

The cortex presents a dense barrier through which newly formed endocytic vesicles must navigate before they can engage with MTs for long-range movement throughout the cell. The cortex is organised with the barbed (or plus) end of short actin filaments oriented toward the plasma membrane and the pointed (or minus) end toward the cytoplasm (Chakrabarti et al., 2021). Although multiple myosin motors are involved in the scission of endosomes from the plasma membrane, myosin VI is unique in that it is not only involved in scission (Buss et al., 2001), but also mediates the inward movement of endosomes from the cell periphery through the actin cortex (Aschenbrenner et al., 2003), being the only minus-end directed myosin motor (Wells et al., 1999). Myosin VI functions exclusively in clathrin-dependent endocytosis (Puri, 2009, 2010), yet multiple clathrin-independent endocytic mechanisms exist in the cell. Our understanding of how motors and their adaptors regulate these clathrin-independent processes is beginning to be uncovered by recent studies (Ferreira et al., 2021; Jiang et al., 2010; Schink et al., 2021; Feng and Yu, 2021; Williamson and Donaldson, 2019; Renard et al., 2020; Wayt et al., 2021; Tyckaert et al., 2022), and detailed in Box 1.

The myosin VI cargo adaptor proteins DAB adaptor protein 2 (DAB2) and GAIP-interacting protein C-terminus (GIPC; also known as GIPC1) confer scission or actin movement functionalities on myosin VI, respectively. DAB2 regulates the interaction of myosin VI with clathrin-coated pits at the plasma membrane (Dance et al., 2004; Spudich et al., 2007), whereas GIPC regulates the interaction of myosin VI with endocytic vesicles following clathrin uncoating and their subsequent transport along actin filaments (Naccache et al., 2006; Rai et al., 2022) (Fig. 1A; Fig. 2A,B).

Box 1. Motors in clathrin-independent endocytosis

Recent advances have begun to uncover motors and adaptors involved in endosomal movement in clathrin-independent endocytosis (CIE). We detail these below, highlighting remaining unknown elements in CIE motor transport.

Fast endophilin-mediated endocytosis

In fast endophilin-mediated endocytosis (FEME), Bin1/amphiphysin II interacts with FEME mediators, and in turn recruits dynein to the FEME endosome. Bin1 knockout reduces dynein association with FEME endosomes, but not endocytosis of FEME cargoes, indicating that as-yet-unidentified actin-based motor might still be required for endocytic uptake and cortex clearance (Ferreira et al., 2021).

Macropinocytosis

Macropinocytosis, a bulk, fluid-phase endocytic mechanism, requires myosin IE, myosin IIA and myosin IIB for the closure of the macropinosome (Jiang et al., 2010; Schink et al., 2021). Following closure, dynein and its adaptors JIP3 and JIP4 are posited to be required for macropinosome transport along MTs, and dynein inhibition and JIP3 or JIP4 knockdown inhibit macropinocytic uptake, perhaps indicating a close coupling of macropinosome formation and coupling to the MTs (Williamson and Donaldson, 2019).

Invadopodium endocytosis

Integrin-β3 endocytosis in the invadopodium occurs via membrane tubulation in the absence of clathrin. The dynein activating adaptor Hook1 and dynactin subunits p150 and Arp1 localise to the cytoplasmic bud of this membrane tubule prior to scission from the plasma membrane, although dynein itself has not yet been identified on this endocytic structure (Feng and Yu, 2021).

Endophilin A3-dependent endocytosis

CIE of CD166 is regulated by endophilin A3 and galectin-8, and is distinct from FEME (Renard et al., 2020). Interestingly, depletion of myosin-II and minus-end-directed kinesin-14 family members, but not dynein, inhibits CD166 endocytosis, indicating that a tight coordination between CD166 endosome formation, cortex tension and MT association regulates this process (Tyckaert et al., 2022).

MHC-I and CD59 endocytosis

Myosin-II has also been identified as regulating the uptake of the CIE cargoes MHC-I and CD59. Myosin-II depletion results in MHC-I and CD59 endosomes being trapped in the cortex, but only minimally affects uptake of the CME cargo transferrin. Myosin-II is posited to regulate CIE by modulating cortex tension; however, how MHC-I and CD59 traverse the cortex is still unknown (Wayt et al., 2021).

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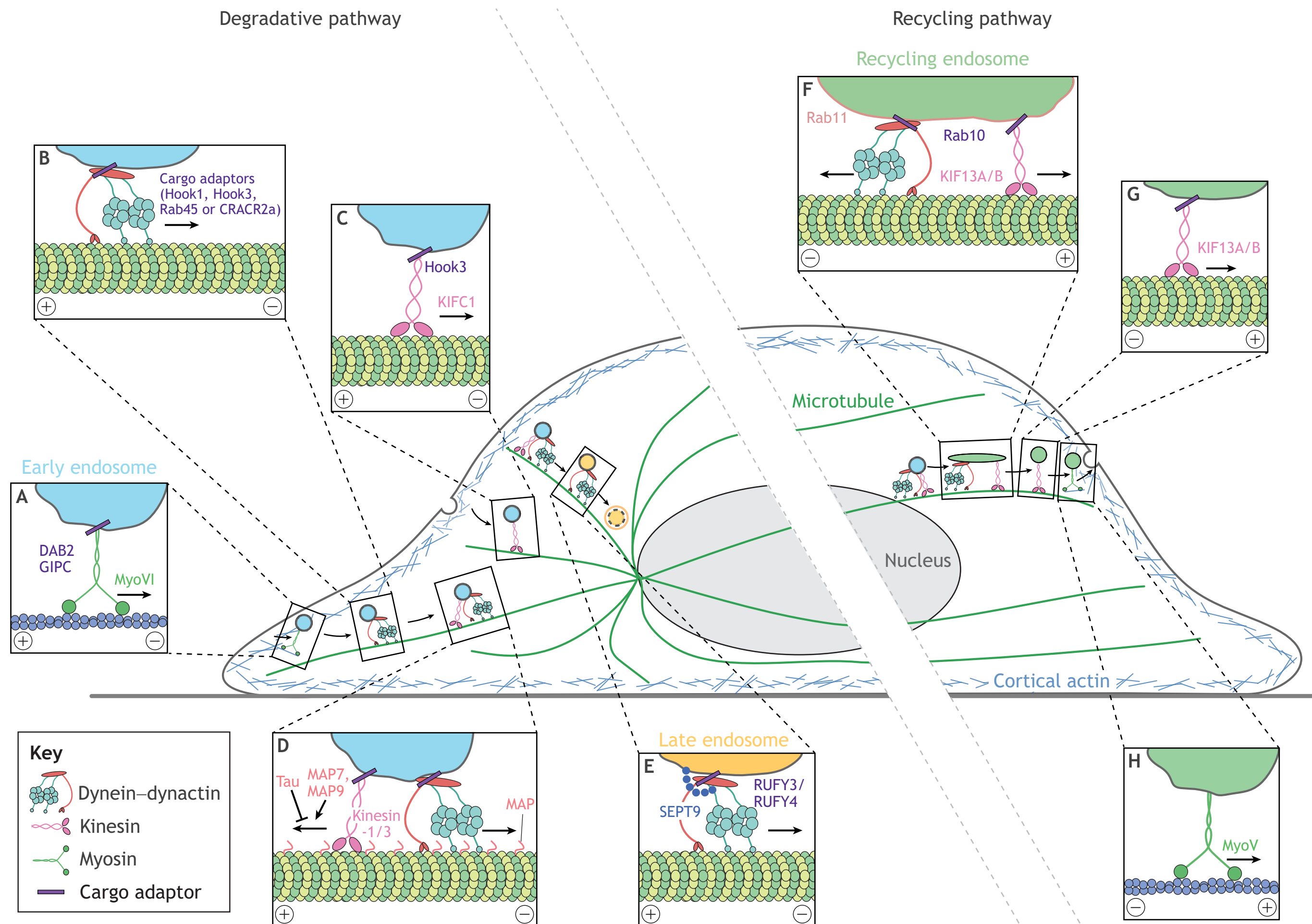

Fig. 1. Overview of motor-driven endosomal trafficking in the degradative pathway and the recycling pathway. The degradative pathway is shown on the left and the recycling pathway on the right. (A) Upon cargo endocytosis, the actin motor myosin VI engages the adaptors DAB2 or GIPC and the endosomal cargo, driving endosome scission and movement through the actin cortex. (B) Cargo adaptors, such as Hook1 and Hook3, Rab45 and CRACR2A, and the dynein–dynactin complex engage with the endosome, driving the early endosome towards the minus-end of the MT. (C) For specific endosomal cargoes, minus-end directed kinesins can drive retrograde MT movement once engaged by the adaptor Hook3. (D) MT-localised early endosomes can engage both plus-end-directed kinesins and minus-end-directed dynein simultaneously, leading to a 'tug-of-war'. MT binding proteins, such as tau and MAPs, can enhance or inhibit plus-end directed movement, influencing the directionality of endosomal transport. (E) Late endosomes engage specifically the dynein adaptors RUFY3 and RUFY4 and the septin SEPT9, which help drive endosomal maturation, leading to cargo delivery to the lysosome and its subsequent degradation. (F) For endosomal cargoes targeted for recycling, Rab11 endosomes bud off the early endosome and are delivered to the perinuclear region of the cell by dynein; here, the putative cargo adaptor Rab10 recruits the kinesin KIF13A and KIF13B (KIF13A/B). (G) KIF13A/B transports the recycling endosome towards the plus-end of the MT at the cell periphery. (H) Myosin V transports the recycling cargo back through the actin cortex, leading to endosome fusion with the plasma membrane and cargo recycling.

Role of DAB2 in endocytosis versus actin-based transport

Whether DAB2 in complex with myosin VI regulates endocytosis specifically or also participates in actin-based endosomal transport remains unclear. DAB2 has a well-established role in regulating clathrin-mediated endocytosis (CME) of a wide range of receptors by interacting with clathrin-binding motifs on multiple receptors, phosphoinositide(4,5)bisphosphate [PI(4,5)P_2], which is enriched at clathrin-coated structures, and the clathrin adaptor AP-2 (reviewed in Finkielstein and Capelluto, 2016). DAB2 overexpression recruits myosin VI to clathrin-coated vesicles (Dance et al., 2004), whereas, conversely, mutation of this site prevents myosin VI recruitment to clathrin-coated vesicles (Spudich et al., 2007).

Myosin VI is non-processive as a monomer, with DAB2 binding allowing its dimerisation and activation (Dos Santos et al., 2022; Phichith et al., 2009; Spudich et al., 2007), driving its processive movement along actin filaments (Rai et al., 2021) *in vitro*. However, when DAB2 and myosin VI were added to an isolated cortical actin network from keratocytes, myosin VI was less motile, and paused more often and for longer than compared to a constitutive myosin VI dimer in the absence of DAB2 (Rai et al., 2021), indicating that in a more complex, cell-like environment, DAB2 might not drive myosin VI movement. Rather, results from cellular and *in vivo* studies suggest DAB2 and myosin function primarily by regulating endosome scission from the plasma membrane, allowing cargo uptake (Fig. 2A). DAB2 and myosin VI both associate with clathrin-coated pits in live cells (Bond et al., 2012). Indeed, clathrin-coated pit formation and receptor endocytosis is impaired in cells isolated from myosin VI-deficient mice, and a wide range of cargoes have perturbed endocytic uptake when DAB2 is depleted *in vivo* (reviewed in Tao et al., 2016).

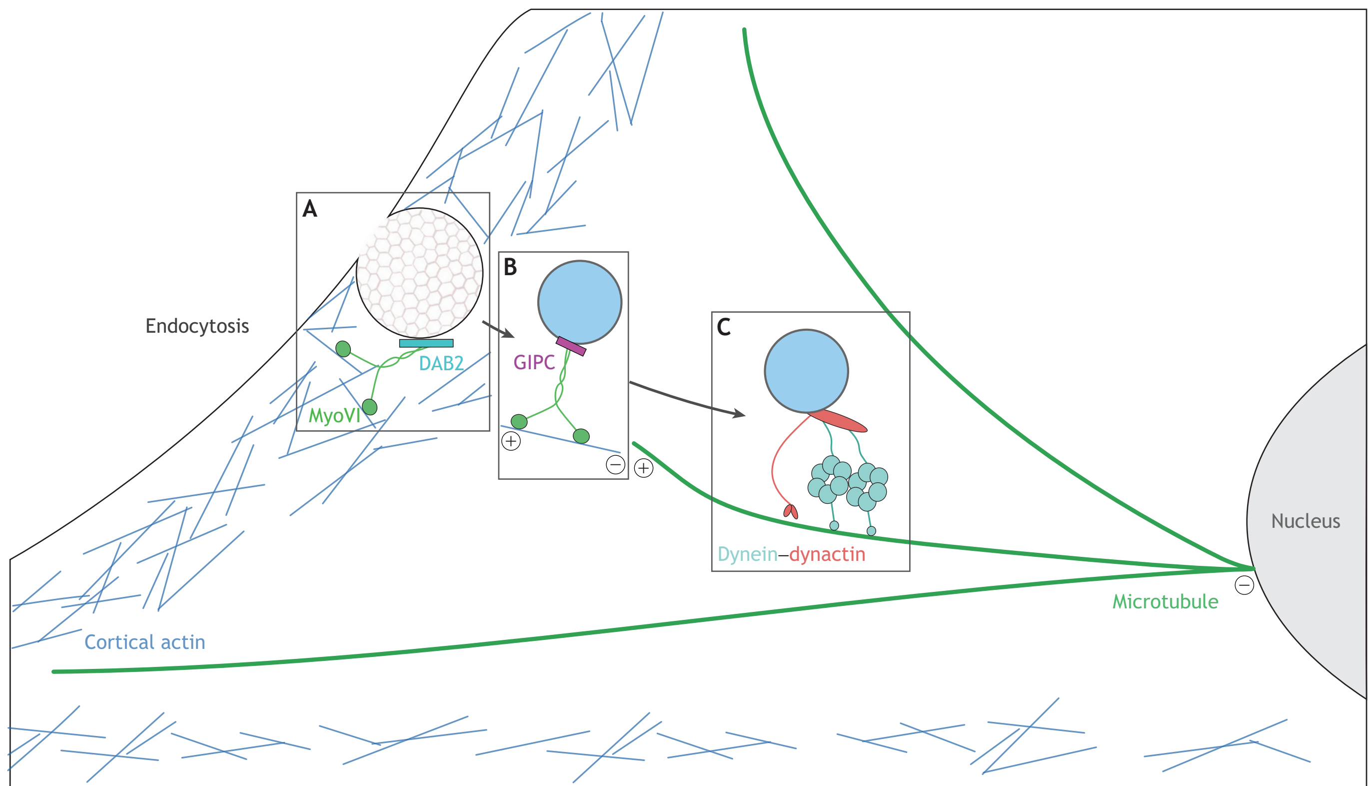

Fig. 2. Detailed view of myosin VI functions in endocytosis. (A) The myosin VI adaptor DAP2 interacts directly with the clathrin-coated pit containing the cargo to be endocytosed. Myosin VI binds DAB2 and drives scission of the clathrin-coated pit, resulting in cargo endocytosis. (B) Upon formation of the endosome, clathrin dissociates and the adaptor GIPC binds to the endosomal cargo. GIPC recruits and activates myosin VI for processive movement, driving minus-end-directed movement through the actin cortex and delivering the endosome to the cytoplasm. (C) Once transported through the cortex, MT-based transport can take over, which is driven by dynein.

GIPC regulates myosin VI-based endosomal transport

Unlike DAB2, GIPC is not required to recruit myosin VI to clathrin-coated pits (Spudich et al., 2007), but it instead interacts with myosin VI on newly formed endosomes after uncoating of the clathrin coat and remains associated with those until their delivery to Rab5-positive sorting endosomes (Aschenbrenner et al., 2003). In PC12 cells, GIPC knockdown results in TrkA receptor-containing endosomes being retained in the cell periphery, as opposed to trafficked to the perinuclear region in control cells (Varsano et al., 2006). In HEK293 cells, GIPC is recruited to luteinising hormone receptor-containing endosomes immediately following clathrin dissociation, as quantified by TIRF live-cell microscopy, and knockdown of GIPC resulted in reduced cAMP production upon luteinising hormone treatment (Jean-Alphonse et al., 2014). Recent work demonstrated that *in vitro*, GIPC increased the run speed of myosin VI-conjugated DNA origami cargo on F-actin, but not run length or the number of myosin VI motors moving (Rai et al., 2022). However, on the reconstituted keratocyte cortical actin networks mentioned above, DNA origami cargoes moved faster, had longer run lengths and shorter pause times in the presence of GIPC and myosin VI when compared to that seen with myosin VI only (Rai et al., 2022). GIPC induces myosin VI dimerisation following the release of autoinhibition upon receptor binding *in vitro* (Shang et al., 2017), and myosin VI becomes a processive dimer upon binding to newly formed endosomes in the cell (Altman et al., 2007). Disruption of myosin VI dimerisation abolishes its processivity *in vitro* and perturbs endocytosis in cellular experiments (Mukherjea et al., 2014). Together, cellular and *in vitro* studies strongly indicate that the GIPC–myosin VI interaction with newly formed endosomes is responsible for endosomal egress from the actin cortex (Fig. 2B,C).

The interaction of GIPC with endocytic cargoes and myosin VI is important in regulating the endosomal interaction with signalling effectors. APPL1-positive endosomes represent a specialised endosomal signalling compartment for multiple receptors (Jean-Alphonse et al., 2014; Sposini et al., 2017). Knockout and knockdown of myosin VI (Masters et al., 2017), or GIPC knockdown (Varsano et al., 2006), increased the perinuclear localisation of APPL1-positive endosomes, as well as abrogating signalling in cell models. Hence, myosin VI and GIPC not only drive endosomal movement through the cortex cellular, but also maintain the positioning of signalling endosomes.

Switch from myosin VI to dynein on endosomes

The role of dynein in endocytic trafficking is well established (Aniento et al., 1993). However, questions still remain regarding how and where dynein is loaded onto endosomal cargo. On its own, mammalian dynein is poorly motile and does not undergo processive minus-end-directed movement (Trokter et al., 2012). *In vitro* reconstitution assays with purified and recombinant mammalian dynein have established that dynein additionally requires its well-known regulator dynactin, as well as a cargo adaptor, such as BICD2, Hook1 and Hook3 (which serves to link dynein to the cargo) for its activation and directed movement (McKenney et al., 2014; Schlager et al., 2014).

Dynein–dynactin clustering at MT plus-ends as a mechanism of cargo capture

Dynein-mediated transport of Rab5-positive early endosomes is essential for their movement towards the cell interior (Fig. 1B) (Driskell et al., 2007). Dynactin, the multisubunit regulator of dynein, accumulates at the growing plus-ends of MTs at the cell periphery (Vaughan et al., 1999). The p150^{glued} subunit (also known as DCTN1) of dynactin contains a CAP-Gly domain that is able to bind to the MT plus-tip-tracking end-binding (EB) proteins, such as EB1 and EB3 (also known as MAPRE1 and MAPRE3, respectively) (Ligon et al., 2003; Moughamian et al., 2013; Tirumala and Ananthanarayanan, 2020). Given that dynactin associates with dynein, both dynactin and dynein were demonstrated to cluster at MT plus-ends at the cell periphery, poised to receive incoming endosomes due to their proximity to the membrane (Vaughan et al., 2002). In a recent study, characterisation of the endoplasmic reticulum transmembrane protein STIM1 in primary venous endothelial cells revealed that STIM1 has the ability to promote EB1–dynactin interaction (Villari et al., 2020). As a result, STIM1 enabled recruitment of dynein to MT plus-ends and subsequent minus-end-directed movement of early endosomes in these cells. This study additionally identified the kinesin 14 protein KIFC1 as mediating minus end-directed trafficking of early endosomes (Fig. 1C).

Accumulation of dynein at MT plus ends is dispensable for early endosomal transport

Surprisingly, in HeLa cells, deletion of p150^{glued} does not result in an abrogation of early endosomal trafficking (Watson and Stephens, 2006), indicating that clustering of dynein at MT plus-ends might not be a universal mechanism for cargo trafficking. Indeed, our recent study in HeLa cells, where we visualised single dynein motors reinforced this idea, as we found that endogenous p150 localised not only to growing MT plus tips, but also along the length of the entire MT lattice (Tirumala et al., 2021 preprint). Although dynein transiently bound to and unbound from MTs, the dynactin complex remained associated with MTs and early endosomes. Stochastic attachment of dynein to one such dynactin–cargo unit results in a short run of the cargo towards the minus-end, and such short runs in succession might give rise to long-range movement (Tirumala et al., 2021 preprint). Similar run-and-pause events have previously been described for early endosomal movement in HeLa cells (Flores-Rodriguez et al., 2011) and in Arpe-19 cells (Zajac et al., 2013).

Cargo adaptors in the spatiotemporal regulation of dynein recruitment

In addition to forming an integral part of the active minus-end transport complex, cargo adaptors play a crucial role in recruiting dynein to the correct subset of membrane-bound compartments. BICD2 was the one of the first dynein adaptors proteins to be identified; it was found to enhance interaction between dynein and dynactin and enable the activation of dynein as a processive motor (Splinter et al., 2012). Subsequent work confirmed BICD2 as an activating adaptor for dynein, and additionally established others, such as Hook1, Hook3, BICD1, Rab11FIP3 and Spindly (McKenney et al., 2014; Schlager et al., 2014).

Hook1, Hook2 and Hook3 are highly conserved proteins that act as adaptors between endosomal cargo and dynein (Bielska et al., 2014). Hook proteins are a part of a tripartite FTS (also known as AKTIP)–Hook–FHIP (FHF) complex that links dynein–dynactin to cargo. The Hook proteins interact with the motor, and FHIPs link to cargo, with the FHIP isoform (i.e. FHIP1A, FHIP1B, FHIP2A or FHIP2B) likely conferring specificity (Christensen et al., 2021). Recently, the motor-adaptor pairs dynein–Hook1 and KIFC1–Hook3 were characterised as carrying out early endosomal transport in primary venous endothelial cells (Fig. 1C) (Villari et al., 2020). Likewise, the trafficking of brain derived neurotrophic factor (BDNF)-containing Rab5-positive and Rab7-positive endosomes is dependent on dynein–Hook1 in primary rat hippocampal neurons (Olenick et al., 2019). Although in that study, Hook3 depletion was found to not affect BDNF uptake or maturation, FHF complexes comprising FTS–Hook1 or Hook1–FHIP1B associate with Rab5-positive early endosomes in human cell lines (HEK293 and U2OS) through a direct interaction between FHIP1B and Rab5 (Christensen et al., 2021). Hook3 could additionally simultaneously bind dynein and the kinesin-3 KIF1C, with Hook3 binding relieving the autoinhibited state of KIF1C (Siddiqui et al., 2019). These studies highlight the differential roles of cargo adapters in cell type- and process-dependent fashion.

Rab45 and CRACR2a (also known as RASEF and Rab46, respectively) are two recent additions to the growing list of dynein-activating adaptors, but uniquely also contain Rab GTPase domains (Wang et al., 2019). Interestingly, both Rab45 and CRACR2a also contain EF-hands, which can respond to intracellular Ca^{2+} levels. In primary T-cells and Jurkat cells, CRACR2a has been implicated in early endosomal uptake of the cell surface protein CD47 (Wang et al., 2019). Another study identified CRACR2a as being required for the centrosomal clustering of Weibel–Palade bodies (WPBs), endothelial cell-specific organelles that are involved in cellular response to vascular injury (Miteva et al., 2019).

Bidirectionality of early endosomes

Bidirectional movement is an established feature of early endosomes (Loubéry et al., 2008; Nielsen et al., 1999) and is thought to be a result of concomitant association of plus-end-directed kinesin motors and minus-end-directed dynein motors (Fig. 1D) (Soppina et al., 2009). Such bidirectional movement could be important for endosomal maturation and to allow for time to decide between degradation or recycling of the cargo within the early endosome. An individual early endosome commonly contains cargo that is both destined for degradation (e.g. EGF), and for recycling (e.g. transferrin). Indeed, transferrin-containing vesicles have been observed to bud out of early endosomes that contain other cargo and subsequently fuse with lysosomes (Driskell et al., 2007). Thus, removing the bidirectionality might result in quick transport of endosomes to either MT minus- or plus-ends, but might also lead to a mis-sorting of these cargoes.

Force production and binding rates of motors predict directionality

Typically, a single kinesin motor is thought to produce more force than a single dynein motor [measured stall forces of 5–6 pN (Svoboda and Block, 1994) versus 1 pN (Mallik et al., 2004), respectively], and therefore a single kinesin motor can participate in a tug-of-war with groups of dynein motors bound to the same cargo. However, in the presence of high loads, the kinesin motor on Rab7-positive late phagosomes with engulfed latex beads was more likely to detach due to a 'catch bond' behaviour adopted by the dynein group under high load (Rai et al., 2013). This catch-bond behaviour of dynein under high load could thus promote the net minus-end-directed movement of endosomes. However, in experiments where a DNA scaffold was used to attach an active dynein–dynactin–BICD2 (DDB) complex to constitutively active kinesin-1, -2 or -3, all three kinesins were able to out-compete DDB (Gicking et al., 2022). This

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is thought to be due to kinesins compensating for their load-dependent detachment with faster re-binding kinetics (Gicking et al., 2022). Additionally, more recent measurements of stall force of dynein indicate a higher force production of the active DDB complex at ∼4.6 pN (Belyy et al., 2016), therefore predicting that a single dynein could be capable of participating in a tug-of-war with a kinesin motor.

MT-associated proteins differentially regulate motor recruitment

MT-associated proteins (MAPs), such as tau, MAP2, MAP7 and MAP9, promote preferential activity or recruitment of motors (Monroy et al., 2020). For instance, tau was found to inhibit the movement of kinesin-1 and kinesin-3 (Fig. 1D), where it only led to a slightly slowing down with dynein–dynactin (Chaudhary et al., 2018; Dixit et al., 2008; Monroy et al., 2018; Tan et al., 2019). In contrast, MAP7 directly recruits kinesin-1 to MTs without altering dynein movement (Chaudhary et al., 2019; Métivier et al., 2019), whereas MAP9 does not affect the processivity of kinesin-3 on MTs, but does inhibit that of dynein (Monroy et al., 2020). Within cells, differential recruitment of certain MAPs to MTs could mediate biased transport of endosomes, for instance, endosomes destined for degradation being perhaps loaded on to tau-decorated MTs.

Dynein in the maturation of early endosomes into late endosomes

Phopshoinositide(3)phosphate [PI(3)P] generation on Rab5 endosomes leads to two divergent outcomes: recruitment of Rab7 and displacement of Rab5 and maturation into a late endosome (Rink et al., 2005), or recruitment of the kinesin family member KIF16B, which sequesters cargoes from Rab5-positive early endosomes for delivery to Rab11-positive recycling endosomes (Hoepfner et al., 2005). The complex control of endosomal maturation and endosomal recycling by PI(3)P is reviewed in detail in Redpath et al. (2020).

Following maturation into a Rab7-positive late endosome, the minus end-directed dynein motor has been shown in several studies to be essential for fusion of Rab7-positive late endosomes with lysosomes and for lysosomal positioning (Fig. 1E) (Cabukusta and Neefjes, 2018; Cason et al., 2021; Johansson et al., 2007; Jordens et al., 2001). Although lysosomes can be localised throughout the cytoplasm, perinuclear lysosomes have been demonstrated to exhibit a higher degradative capacity. The maturation from early to late endosomal compartments correlates with a switch to a more biased, unidirectional (minus-end-directed) movement; latex bead-containing phagosomes isolated from macrophages move in a highly unidirectional fashion due to cholesterol-mediated clustering of dynein motors (Rai et al., 2016). The new studies discussed below exemplify other strategies used by cells to recruit dynein to late endosomal and lysosomal compartments.

Novel regulators of dynein recruitment to late endosomes and lysosomes

Septins are GTP-binding proteins that can form polymers that interact with cell membranes and cytoskeletal filaments (Mostowy and Cossart, 2012). Septins have been recognised for their role in lysosomal delivery (Dolat and Spiliotis, 2016), including that of bacteria (Krokowski et al., 2018). In a recent study, the septin SEPT9 was discovered to recruit dynein to promote perinuclear lysosomal localisation in a Rab7-independent manner (Fig. 1E) (Kesisova et al., 2021). Here, the N-terminus of SEPT9 interacts with dynactin. In contrast to Rab7, which relies on GTP binding to associate with lysosomes, SEPT9 associated with the dynein intermediate chain and assembles into multimeric complexes when GDP bound. This difference in nucleotide state was proposed to be critical for SEPT9-dependent lysosomal redistribution during acute oxidative stress (Kesisova et al., 2021).

The small G-protein Arl8b has known roles in lysosomal positioning (Khatter et al., 2015). RUFY3 (Keren-Kaplan et al., 2022; Kumar et al., 2022) and RUFY4 (Keren-Kaplan et al., 2022) have recently been identified to recruit dynein-dynactin to preferentially regulate the perinuclear localisation of lysosomes (Fig. 1E). Accordingly, depletion of RUFY3 diminishes the size and typical number of lysosomes, in addition to reducing the degradative capacity of lysosomes (Kumar et al., 2022).

Interaction of kinesins and myosin V with recycling endosomes

Endosomal recycling is crucial for fine-tuning receptor signalling as it maintains the plasma membrane levels of receptors and regulates the levels of circulating proteins, as well as preserving the overall plasma membrane (Goldenring, 2015). Endosomal recycling predominantly occurs through Rab11-positive recycling endosomes, which are mostly localised to the MTOC in the perinuclear region of the cell (Naslavsky and Caplan, 2018). Transport from Rab5-positive sorting endosome to these perinuclear recycling endosomes from is mediated by dynein (Fig. 3A); these are then delivered to the plasma membrane by kinesin-like protein 13A (KIF13A) and KIF13B initially along MTs, followed by myosin-V, then through the actin cortex, finally resulting in fusion with the plasma membrane (Fig. 3).

Dynein interactors drive cargo delivery to the perinuclear region

Rab11 endosomes bud from Rab5-positive sorting endosomes, thus sequestering cargoes from the endolysosomal pathway for their recycling (Campa et al., 2018). Their scission involves the FERARI complex, which among other proteins, contains sorting nexin 4 (SNX4) and Rab11 family interacting protein 2 (Rab11FIP2) (Solinger et al., 2020) (Fig. 3A). SNX4 has previously been shown to interact with dynein, and its knockdown in cells prevents the delivery of the Rab11-recycled cargo transferrin to the perinuclear region, instead resulting in its lysosomal degradation (Traer et al., 2007). Rab11FIP3, another Rab11 family interacting protein, is an established activating adaptor of dynein *in vitro* (McKenney et al., 2014), and overexpression of mutants incapable of binding the dynein intermediate light chain 1 prevent transferrin trafficking to the perinuclear region, sequestering it in the cell periphery (Horgan et al., 2010), further confirming a role for Rab11FIP3 in dynein-mediated recycling endosome transport.

Kinesin and myosin V induce cargo recycling from the perinuclear region

Following dynein-mediated delivery to the perinuclear compartment, KIF13A and KIF13B facilitate cargo delivery to the plasma membrane (reviewed in Thankachan and Setty, 2022) (Fig. 1F,G; Fig. 3). The small GTPase Rab10 was recently discovered to be required for the formation of tubular recycling endosomes in HeLa cells in conjunction with KIF13A and KIF13B (Etoh and Fukuda, 2019). Rab10 knockout results in accumulation of the LDL receptor and transferrin cargoes in perinuclear Rab11 and Rab4 recycling compartments (Khan et al., 2022). KIF13A was previously implicated in the tubulation of GTP-Rab11-positive recycling endosomes, which is requisite for cargo redelivery to the plasma membrane (Delevoye et al., 2014); together these results

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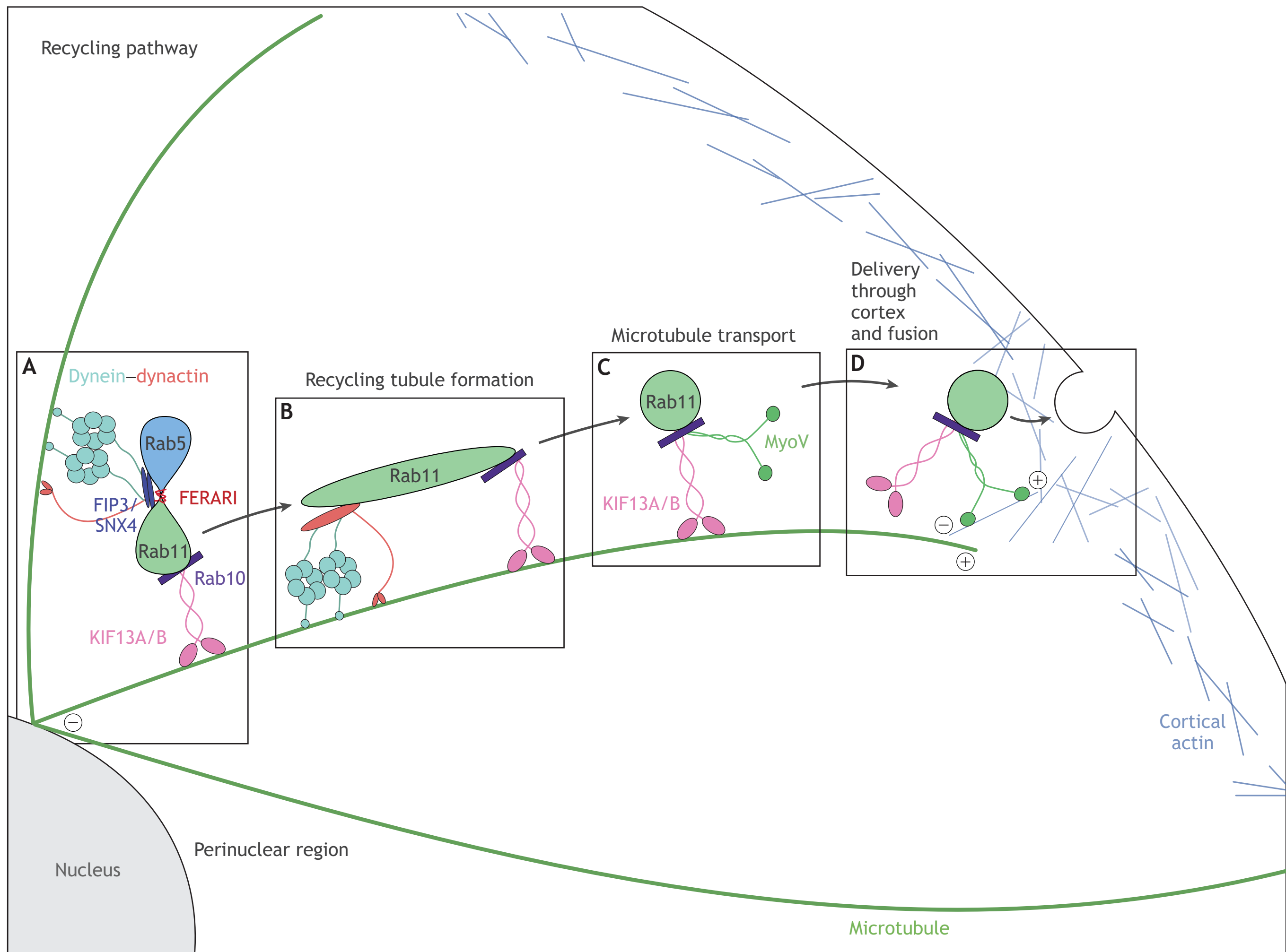

Fig. 3. Detailed view of dynein and kinesin in endosomal cargo recycling. (A) Dynein facilitates scission of Rab11-positive domains from Rab5-positive early endosomes via interaction with the FERARI complex, Rab11FIP3 (FIP3) and SNX4, resulting in their delivery to the perinuclear region of the cell. Rab10 acts as a putative adaptor for kinesins KIF13A and KIF13B (KIF13A/B) and recruits them to the Rab11-positive endosome. (B) Opposing action of dynein and kinesins on Rab11-positive recycling endosomes leads to the formation of tubular recycling endosomes. (C) From these tubular endosomes, recycling vesicles bud. Myosin V interacts with kinesins on these vesicles, which are then delivered to the plus-end of the MT. (D) Upon arrival at the MT plus-end, myosin V mediates the transport of recycling vesicles through the actin cortex, delivering them into proximity to the plasma membrane, allowing fusion and cargo recycling to occur.

implicate Rab10 and KIF13A and B in being crucial mediators of cargo egress from the Rab11-positive perinuclear region for redelivery to the plasma membrane, suggesting that Rab10 could be considered a putative kinesin cargo adaptor (Fig. 3B).

The final step in cargo recycling is navigation through the cortex to allow fusion of recycling endosomes the plasma membrane, and this is mediated by myosin V, which moves cargoes from the minus-ends of actin filaments, which are directed towards the cell interior, to the plus end located just underneath the plasma membrane (reviewed in Hammer and Sellers, 2011) (Figs 1H and 3C,D). Although an actin-based motor, myosin V appears to be involved in the movement of recycling endosomes from when they leave the perinuclear region, potentially acting synergistically with kinesin. Overexpression of the myosin Vb tail inhibits myosin V transport and induces striking accumulation of cargo and Rab11-positive endosomes in the perinuclear region in both non-polarised and polarised cells (Hales et al., 2002; Lapierre et al., 2001; Xie et al., 2016), indicating that without myosin V activity recycling cargoes do not engage with MTs. Consistent with this, when mouse myosin V and *Drosophila* kinesin are incubated together *in vitro*, myosin V undertakes longer runs on actin, and kinesin undertakes longer runs on MTs than either motor alone (Ali et al., 2008). Synergistic action of myosin V and kinesin has not yet been demonstrated in cells. However, myosin V and kinesin can interact (Ali et al., 2008), and perturbation of either results in cargo accumulation in the perinuclear region of the cell (Hales et al., 2002; Lapierre et al., 2001; Xie et al., 2016). Both motors potentially interact with the same recycling cargoes to facilitate efficient cargo transfer from MT to actin-based tracks (Fig. 3C).

Perspectives and outstanding questions

We have come a long way in our understanding of how endosomal trafficking occurs in cells and the essential role of motor proteins in this process (see Fig. 1). However, there are still some unresolved questions, in part owing to experimental choice and analysis techniques, that need to be addressed in order to understand how *in vitro* motor and adaptor behaviour translated to the *in vivo* behaviour in cells and allow their full comparison as outlined below.

Factors causing discrepancies between *in vitro* and cellular experiments

Although *in vitro* reconstitution of motor behaviour has proven indispensable for investigating motor-driven trafficking, live-cell imaging and the ability to visualise single motors in cells, has shown that the behaviour of motors in cells and *in vitro* behaviour is not always comparable. For instance, activated dynein and kinesin motors walk processivity over several tens of micrometres on MTs in *in vitro* assays (McKenney et al., 2014; Schlager et al., 2014), but neither motor has been documented to do so in living cells at the single-molecule level (Cai et al., 2009a; Tirumala et al., 2021 preprint; Tirumala and Ananthanarayanan, 2023). Similarly, reconstitution of endosomal transport using beads attached to motors through biotin-streptavidin linkage abolishes motor unbinding from the cargo, which is an inherent motor property. Similarly, motors pulled down on phagosomes attached to latex beads also do not participate in dynamic binding-unbinding (Rai et al., 2016). Therefore, the complexity of cargo binding/unbinding and its role in transport is typically absent in *in vitro* assays.

Another central feature of the intracellular environment that is poorly captured in *in vitro* experiments is crowding. The cytoplasm is filled with proteins, ribosomes, organelles, cytoskeletal filaments, and is estimated to have a viscosity three-fold greater than that of water (Swaminathan et al., 1997). This parameter is rarely considered in *in vitro* experiments, with such assays generally performed in buffers with viscosities similar to that of water. Additionally, cytoskeletal filaments such as MTs are occupied by MAPs and interact with membrane-bound compartments, and it is therefore not surprising that uninterrupted long-range movements on MTs are rare in a cell. Further, the ionic strength of the buffer, the concentration of reactants and temperature at which *in vitro* assays are carried out also vary from physiological values and may contribute to the differences in motor and/or endosome behaviour between *in vitro* and *in cellulo* measurements.

Standards for visualisation and analysis of endosomal movement

Another important point is to recognise that the experimental parameters and analysis methods chosen to quantify endosomal movement vary between studies. Indeed, even experiments carried out with identical cargo, in the same cell line and experimental condition could give rise to different outcomes. Therefore, we believe that the field has to establish standards for reporting endosomal movement and motor function. Reporting on and comparing across the following features would be key in ensuring reproducibility across different labs:

(i) if visualising ligand-mediated internalisation of a receptor, the concentration of ligand used; (ii) parameters used for fluorescent visualisation of endosomal movement, such as time interval between consecutive images, 3D imaging or 2D, duration of visualisation; and (iii) most importantly, the analysis parameters employed. This includes the analysis routine used for tracking endosomes, tracking of all visible endosomes rather than only motile subsets, reporting the instantaneous and net displacements and velocities of all endosomes.

A unified framework for motor-driven endosomal transport?

Finally, we end this Review with proposing a potential mechanism motor proteins might employ to enable long-range transport of endosomal cargoes in a variety of cell types. Increasing the number of motors bound to cargo could potentially enhance run length of endosomes by ensuring at least one motor contacts the MT and engages in active movement at any given time. While groups of motors are typically thought to be required for long-range movement of cargo (Gicking et al., 2022; Hendricks et al., 2010; Rai et al., 2013), in our recent work (Tirumala et al., 2021 preprint), we illustrate that the repetitive binding, followed by short runs and then unbinding of dynein motors is sufficient to mediate cargo trafficking across large length scales of the order of tens of micrometres within cells. So too, increasing kinesin-1 motor numbers of cargo *in vivo* did not result in increased run lengths or movement (Shubeita et al., 2008).

There is also little evidence from endosomal and single-molecule tracking experiments performed in cells to suggest that large motor numbers occur or bind on endosomes. First, if motors indeed occurred in large numbers, imaging of fluorescently labelled motors should reveal clusters of motors (presumably bound to endosomes) on MT tracks. In live-cell imaging, these clusters would processively move with the endosome. To the best of our knowledge, neither have been observed to date. Theoretical calculations based on the size and binding–unbinding kinetics of kinesin motors to supported lipid bilayers mimicking vesicles predicted that in order to travel a distance of 10 µm uninterrupted, a 500 nm diameter vesicle would need to have 800 kinesin motors bound to it, so as to effectively have three kinesin motors in contact with a MT to engage in active motion (Jiang et al., 2019). The authors thus concluded motor clustering to be an inefficient and unlikely mode of long-range movement (Jiang et al., 2019).

Second, single-molecule visualisation of motors would reveal the presence of multiple motors on cargo. Although dynein has been visualised to cluster in a cholesterol-dependent manner on late phagosomes pulled down from cells (Rai et al., 2016), neither dynein (Shin et al., 2019; Tirumala et al., 2021 preprint) nor kinesin-1, -2 or -3 occur in clusters in cells (Cai et al., 2009b; Guedes-Dias et al., 2019; Norris et al., 2014; Siddiqui et al., 2019). Likewise, the run lengths and run times of individual dynein and kinesin motors in living cells are similar and short, of the order of 1 s or less (Cai et al., 2009a; Tirumala et al., 2021 preprint), and they correlate with the characteristic 'stop-and-go' motion that has been described for endosomal movement (Chaudhary et al., 2018; Shin et al., 2019; Tirumala et al., 2021 preprint; Villari et al., 2020). Therefore, the picture that emerges is that the binding and unbinding of a single motor molecule to and from endosomes are sufficient to mediate their transport in cells. Therefore, motor binding rates are a key regulator of the net velocity of cargo transport, with faster reattachment of motor to the cargo resulting in faster cargo transport (Jiang et al., 2019; Tirumala et al., 2021 preprint). Finally, modulation of motor binding rates could be fine-tuned by cells to achieve appropriate cargo movement. Indeed, recent *in vitro* reconstitution of kinesin-1 movement on supported lipid bilayers and subsequent calculations also concluded that motor binding kinetics is the primary determinant for long-range transport (Jiang et al., 2019).

Conclusion

In vitro studies of myosin, dynein and kinesin motors have revealed a wealth of information about their function, especially with regard to the interplay between cargoes, motor adaptors and processive motor movement. However, with our increasing ability to visualise motor function in the native environment of the live cell, it is clear that *in vivo*, motor dynamics, and in some cases adaptor function, does not match the motor parameters determined *in vitro*. With the continuing advances in live-cell imaging, it is important we adopt a standardised set of experimental parameters to resolve motor function in cells to ensure we capture the true behaviour and

function of motors and their adaptors during endosomal movement throughout the cell.

Competing interests
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