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The MYO6 interactome: selective motor-cargo complexes for diverse cellular processes.

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Abstract:
Myosins of class VI (MYO6) are unique actin-based motor proteins that move cargo towards the minus ends of actin filaments. As the sole myosin with this directionality, it is critically important in a number of biological processes. Indeed, loss or overexpression of MYO6 in humans is linked to a variety of pathologies including deafness, cardiomyopathy, neurodegenerative diseases as well as cancer. This myosin interacts with a wide variety of direct binding partners such as the selective autophagy receptors optineurin, TAX1BP1 and NDP52 and also Dab2, GIPC, TOM1 and LMTK2, which mediate distinct functions of different MYO6 isoforms along the endocytic pathway. Functional proteomics has recently been used to identify the wider MYO6 interactome including several large functionally-distinct multi-protein complexes, which highlight the importance of this myosin in regulating the actin and septin cytoskeleton. Interestingly, adaptor-binding not only triggers cargo attachment, but also controls the inactive folded conformation of MYO6. Thus, the C-terminal tail domain mediates cargo recognition and binding, but is also crucial for modulating motor activity and regulating cytoskeletal track dynamics.

Introduction:

Myosin motor proteins are powered by ATP hydrolysis to translocate along actin filaments and generate force and movement, which is important for a wide range of dynamic processes. The human genome contains 39 myosin genes; 15 genes code for “conventional” class-II myosins named MYH1 to MYH15. Most of these are expressed in smooth, skeletal or cardiac muscle, but three isoforms (MYH9, MYH10 and MYH14) are selectively expressed in non-muscle cells (non-muscle myosin IIA, B or C). The remaining 24 genes (MYO1 to MYO19 including eight class I myosin genes, MYO1A to MYO1H) encode “unconventional” myosins and the numbering system corresponds to the 12 different myosin classes established by phylogenetic comparison of multiple eukaryotic organisms [1] [2] [3]. Myosins of class II,

also called conventional myosins, were the first to be discovered and are the only class to form bipolar filaments essential for muscle contraction and generation of contractile forces in cells. In contrast, the unconventional myosins fulfil diverse cellular roles, for example, in cell migration, vesicle trafficking or cytokinesis by performing tethering functions or short-range transport. Myosin motor proteins contain a highly conserved motor domain, a neck region varying in length that provides bindings sites for up to six calmodulins or related light chains, and diverse tail domains for cargo selection linked to specialized functions.

All myosin family members move towards the plus-end of actin filaments, except for the unique myosins of class VI (MYO6) [4]. The crystal structure of the MYO6 motor domain reveals the presence of an additional insert (insert-2) in the converter domain that repositions the lever arm into an orientation to allow “backwards” motion [5]. A second unique insert (insert-1) is located near the ATP binding site and modulates ATPase activity [5]. The kinetic and biophysical characteristics of this myosin have been well studied at the bulk and single molecule level *in vitro* and have shown that MYO6 is a relatively slow motor moving actin filaments approximately 110-140 nm/s [6] [7] [8] [9]. The duty cycle and weak gating of this myosin points to its ability to be weakly processive when acting as a dimer [9] and suggest that longer ranges of movement probably require several dimers acting in unison. Furthermore in the presence of load or high ADP MYO6 has been shown to behave more like a molecular anchor than an active transporter [10].

MYO6 performs a wide range of specialized functions for example in membrane ruffles, on early endosomes, on clathrin coated structures at the plasma membrane and at the base of microvilli and stereocilia in the inner ear. In humans and mice MYO6 mutations cause a wide range of pathologies including hypertrophic cardiomyopathy and hearing loss. Furthermore, increased expression of MYO6 has been reported in many different carcinomas [11] [12] [13] [14], making it a potential early marker of cancer development.

The multi-functionality of MYO6 requires interaction with a host of binding partners via its tail domain, which have been identified using a variety of approaches including yeast-2 hybrid screens, native co-immunoprecipitation, affinity chromatography using the MYO6 tail domain or in situ proximity labelling [15] [16] [17] [18]. Binding these adaptor proteins is crucial for precise spatial and temporal targeting of MYO6. In addition, these adaptor proteins may determine whether MYO6 operates in cells as a monomer or a dimer. Purified MYO6 in vitro is a monomer, however, association with different binding partners has been shown to induce dimerization (figure 1 B) [8] [19] [20]. The tail domain contains several unique binding motifs including two adaptor binding domains, two ubiquitin binding regions and one lipid binding motif (see figure 1 D). Although most binding partners have been shown to bind directly to MYO6, recent work has also suggested that binding might involve a variety of ubiquitin chains, which can enhance the affinity and stabilise MYO6-adaptor complexes [21] [22]. MYO6 can also directly interact with membranes through its C-terminal lipid binding motifs. Overall, the cellular positioning and function(s) of MYO6 are likely to involve a combination of lipid and protein binding; sequential or simultaneous binding of different adaptors to distinct sites in the cargo-binding tail, which can either be direct or via

ubiquitin chains (see figure 2). This complex process of motor-cargo recognition requires tight regulation for initiation and eventually deactivation and recycling of MYO6.

In this article, we discuss the molecular interactions between MYO6 and its binding partners and the cellular processes in which they are involved. Moreover, we review the MYO6 domain organization and recent advances in our understanding of MYO6 regulation.

Cellular functions of MYO6

Diverse roles of MYO6 in the endocytic pathway

The process of endocytosis permits the uptake of basic nutrients or cell surface receptors from the plasma membrane. Several parallel uptake routes have been described including clathrin-mediated endocytosis, in which the protein clathrin and associated machinery initiate the formation of vesicles from invaginations in the plasma membrane. After internalisation the receptor-containing vesicles are delivered to early endosomes and sorted either for lysosomal degradation or recycled back to the cell surface. Four splice isoforms of MYO6 are expressed in different cell types and tissues, which perform specialised roles at distinct steps along the endocytic pathway. These splice variants contain a large insert (LI) (adding 21-31 amino acids), a small insert (SI) (adding 9 amino acids), both LI and SI or no insert (NI) (see figure 1).

The MYO6 splice variant containing the large insert (LI) at the beginning of the cargo-binding domain (CBD) is predominantly expressed in polarised epithelial cells, where it associates with clathrin-coated pits or vesicles at the base of microvilli at the apical plasma membrane. Indeed, loss of MYO6 expression in the knock out mice (Snell's waltzer mice) causes defects in endocytosis of the cystic fibrosis transmembrane conductance regulator (CFTR) from the apical domain of enterocytes, as well as in the uptake of the megalin-receptor in the kidney, thereby leading to proteinuria [23] [24] [25]. Interestingly, MYO6 has been shown to facilitate the movement of the Na^+/H^+ exchanger down the microvillus in intestinal cells, as well as the movement of sodium transporters in renal proximal tubule cells to allow clustering in clathrin-coated pits and endocytosis [26] [27]. MYO6 targeting to clathrin-coated structures involves the LI splice variant, which constitutes a clathrin-binding domain [28], and the clathrin adaptor Dab2 (Disabled-2) [15]. The endocytic adaptor protein, Dab2 contains a phosphotyrosine binding (PTB) domain that mediates binding to the cytoplasmic tail of cell surface receptors. In addition, Dab2 features several binding sites for other components of the endocytic machinery. Indeed, in situ proximity labelling using the MYO6 tail domain identified several proteins associated with clathrin-mediated endocytosis such as AP2, ITSN1, SYNJ1, PICALM, EPS15L1 and FCHO2 that are linked to MYO6 through Dab2 [18].

The isoform of MYO6 without any inserts (NI) is present on a subset of peripheral early endosomes, which are positive for the small GTPase Rab5 and the Rab5 effector APPL1 (Adaptor Protein Phosphotyrosine interacting with PH domain and Leucine Zipper 1) [29] [30] [31]. APPL1 is a multifunctional adaptor protein containing multiple domains that mediate phospholipid binding and allow interaction with many signalling receptors. At its C-terminus APPL1 binds to the PDZ domain of GIPC1, which interacts with a large number of cell surface receptors including receptor tyrosine kinases, G protein-coupled receptors and

other transmembrane proteins. GIPC also binds directly to MYO6 and recruits this myosin to APPL1-positive early endosomes [32]. The GIPC1-MYO6 complex has been suggested to translocate early endosomes away from the plasma membrane through the cortical actin network, since expression of a non-functional MYO6 rigor mutant inhibits endosome movement [33] [31] [34]. A crucial role for MYO6 in the spatial organisation of the APPL1-signalling endosomes is also supported by the findings that the depletion of MYO6 leads to the premature maturation and displacement of APPL1 endosomes from the cell cortex in the perinuclear region of the cell [35] and expression of a mutant MYO6 that moves towards the plus end of actin filaments causes extreme cortical clustering of endosomes [36]. Finally, recent in situ proximity experiments identified a tripartite complex of GIPC1, MYO6 and LARG, which is a GEF for RHO GTPases. LARG activation downstream of specific G protein coupled receptors may link Rho signalling to remodelling of the actin cortex to regulate early endosome motility and retrograde movement of internalised receptors [18].

Additional MYO6 adaptor proteins on these early endosomes are TOM1 (Target of Myb protein 1) and the related TOM1L2 (Target Of Myb1 Like 2 Membrane Trafficking Protein). Both proteins contain a ubiquitin-binding GAT domain and a VHS domain, similar to the ESCRT-0 subunits Hrs and STAM. This family of proteins plays an important role in the sorting of ubiquitinated cargo destined for lysosomal degradation into late endocytic intraluminal vesicles [37]. Targeting of TOM1/L2 to endosomes involves binding to TOLLIP (Toll Interacting Protein) and/or endofin, which contains a FYVE domain that binds PI(3)P present on early endosomes. Although the exact function of TOM1 and the MYO6/TOM1 complex is still unclear, TOM1 has been implicated in regulating interleukin-1 and tumour necrosis factor-alpha signalling and trafficking [38] [39].

Another MYO6 adaptor in the endocytic pathway is the transmembrane serine/threonine kinase LMTK2 (Lemur Tyrosine Kinase 2) [40]. This kinase colocalises with MYO6 on Rab5-positive endosomes, where it appears to participate in the sorting of receptors from early endosomes into the recycling compartment [29]. Little is known about the function of this protein compared to other kinases although it has been shown to regulate endocytic trafficking, as well as signalling and apoptosis. Changes in LMTK2 expression and function are linked to neurodegeneration, cancer and defects in spermatogenesis [41] [42] [43]. At present it is not known whether MYO6 activity is regulated through phosphorylation by LMTK2 or whether MYO6 mediates the cellular localisation and trafficking of this kinase.

Functions of MYO6 in the exocytic pathway

In addition to its role in the endocytic pathway, MYO6 appears to play a similarly important role in cargo transport in the secretory pathway. In this pathway, proteins synthesized at the endoplasmic reticulum are transported in tubular/vesicular carriers to the Golgi complex, where they are processed and sorted for delivery to the plasma membrane for incorporation or release from the cell. MYO6 localises to vesicles in the perinuclear region around the Golgi complex [44] [45] and to vesicles close to the plasma membrane [16]. Functional studies using fibroblasts isolated from the Snell's waltzer mouse reveal a significant reduction in Golgi size compared to wild type fibroblasts, combined with a defect in constitutive exocytosis [45]. Furthermore, MYO6 is required for the polarised delivery of newly synthesized transmembrane proteins to the basolateral plasma membrane domain in polarised epithelial cells and to the leading edge of migratory cells [46] [47]. The role of

MYO6 in the secretory pathway is closely linked to optineurin and the small GTPase Rab8, an established optineurin binding partner. The functions of optineurin and MYO6 in secretion involve secretory vesicle fusion and possibly fusion pore opening at the plasma membrane [48]. In neurosecretory cells the small insert (SI) isoform of MYO6 tethers secretory granules to the actin cortex regulated via c-Src phosphorylation [49]. MYO6-dependent tethering of these secretory granules to the cortical actin network involves ENA/VASP scaffolding proteins that are involved in actin polymerisation in addition to interactions with other proteins [49, 50].

MYO6 and autophagy

Autophagy is an essential lysosomal degradation pathway for the breakdown and recycling of cytosolic components, which are sequestered in large double-membrane vesicles, called autophagosomes. After autophagosome closure, amphisomes are formed by fusion with early endosomes, which are believed to deliver essential machinery required for autophagosome – lysosome fusion. Autophagy maintains cellular homeostasis and growth under conditions of starvation in a non-selective process. This pathway also targets large cytosolic objects that are too large to be degraded via the proteasome such as protein aggregates, damaged organelles and invading pathogens. These objects are typically marked with a ubiquitin tag, which is recognised by selective autophagy receptors through their ubiquitin binding domain. The autophagy receptors typically interact with LC3 (Microtubule-associated Protein 1A/1B-Light Chain 3), triggering the recruitment of LC3-positive autophagosomal membranes and thereby capturing the ubiquitinated object inside an autophagosome. MYO6 has been shown to bind directly to three of the well-known autophagy receptors, optineurin, NDP52 (Nuclear Dot Protein 52 kDa) and TAX1BP1 (Tax1-Binding Protein 1). In all three autophagy receptors the ubiquitin-binding site exactly overlaps with the MYO6 binding site, however, the binding of TAX1BP1 to MYO6 is stronger than the interaction of the autophagy receptor with ubiquitin [51]. These findings are consistent with a dual function of the autophagy receptors: first in the recognition and capture of ubiquitinated cytosolic objects without MYO6 participation and second in the recruitment of this myosin to the outer surface of autophagosomes by binding simultaneously to MYO6 and autophagosome-associated LC3. The autophagy receptors and TOM1 bind to distinct sites in the MYO6 CBD; pull down experiments have demonstrated that the distance between these two binding domains allows simultaneous binding of the autophagy receptors and the endosome-anchor TOM1 [52]. These results support the model that a ternary complex of MYO6, TOM1 and the autophagy receptors provides a mechanism for the tethering of endosomes to autophagosomes to facilitate endosome-autophagosome fusion and maturation [30].

In addition, MYO6 has a crucial role in xenophagy for the autophagy-dependent clearance of pathogens such as *Salmonella typhimurium* from the cytosol of infected cells [51]. Furthermore, during mitophagy, the pathway for removal of non-functional mitochondria via autophagy, MYO6 is selectively recruited to damaged mitochondria. MYO6 recruitment does not depend on optineurin, TAX1BP1 and NDP52 but requires its ubiquitin-binding domain [53]. MYO6 induces the formation of actin cages around damaged mitochondria thereby isolating dysfunctional mitochondria destined for mitophagy from the healthy network. This novel role for MYO6 is unique to mitophagy, where the actin cages prevent the re-integration of damaged mitochondria into the functional mitochondrial network.

MYO6 adaptor proteins regulate actin track dynamics

Accumulating evidence indicates that myosin motors do not simply translocate along pre-existing actin filaments, but also actively induce actin tracks as required. For example, class IX myosins (MYO9) have RhoGAP domains in their tails, which regulate RhoGTPase-dependent modulation of actin dynamics; class V myosins (MYO5) have recently been shown to coordinate motor targeting with assembly of actin tracks by binding to the actin nucleator SPIRE2, and myosins of class I can interact directly with proteins that regulate actin patch formation at the plasma membrane [54] [55] [56]. The recent identification of MYO6-associated RhoGEF complexes, which link to different actin modulatory pathways, builds significantly on this regulatory theme and highlights the extent to which motor activity is synchronised with actin track assembly. *In situ* proximity labelling identified LRCH1 and LRCH3 of the LRCH (Leucine Rich repeats Calponin Homology domain) family of proteins in a complex with MYO6. These proteins comprise a series of N-terminal leucine rich repeats (LRRs), involved in protein-protein interactions, and a C-terminal calponin homology domain, which can directly bind to actin. LRCH1 and LRCH3 associate directly with MYO6 and DOCK7 (Dedicator Of CytoKinesis 7) thus constituting the linker between MYO6 and DOCK7 [57]. DOCK7 belongs to the DOCK180 family of guanine nucleotide exchange factors (GEFs), which catalyse the loading of GTP onto Rho GTPases through a DOCK homology region (DHR)-2 domain and thereby activating them [58]. In the case of DOCK7 it appears to act as a GEF towards prenylated membrane localised RAC1 (Ras-related C3 botulinum toxin substrate 1) and CDC42 (Cell division control protein 42), two major regulators of the actin cytoskeleton [59]. Functionally, DOCK7 acts predominantly in the nervous system and has been implicated in Schwann cell migration, myelination and neurite outgrowth [60] [61]. MYO6, LRCH3 and DOCK7 form a tripartite complex, the DISP (DOCK7-Induced Septin disPlacement) complex [18]. BioID data revealed possible links between LRCH3 and septins (SEPT), in particular SEPT7, but also SEPT8, SEPT9, and SEPT10. Septins are a large family of GTPases with phospholipid binding activity that assemble into filamentous or ring-like structures, which associate with distinct subsets of actin filaments and microtubules, as well as membranes of specific curvature and lipid composition [62] [63]. Interestingly, overexpression of components of the DISP complex displaces the septins from actin filaments and induces the formation of cytosolic actin rings. Dynamic actin filaments in membrane ruffles for example are devoid of septin structures, therefore, the DISP complex might regulate the removal of septin structures from actin filaments to enable actin filament reorganisation through the DOCK7-stimulated RAC1 and CDC42 GTPase activity [18].

The second RhoGEF associated with MYO6 is LARG (Leukemia-associated RhoGEF), also known as ARHGEF12, which is a RhoA-specific RhoGEF first identified as a fusion product with MLL in acute myeloid leukaemia [64]. LARG is among a family of RhoGEFs activated by the $G\alpha_{12/13}$ G protein subunits and thus mediates RhoA activation downstream of a number of receptors.

MYO6 adaptors for specialised functions

MYO6 also appears to have a number of distinct tissue-specific functions. The interaction between MYO6 and SAP97 (Synapse associated Protein 97) has only been reported in neuronal tissues so far [65]. Indeed, MYO6 has been identified in a complex with SAP97 and the AMPA receptor subunits, GluA1 and GluA2, and functionally appears to regulate the

delivery or endocytosis of the receptors to or from the cell surface in an activity-dependent fashion [66] [67] [65].

In inner ear hair cells, MYO6 binds to otoferlin, a transmembrane protein and putative Ca^{2+} sensor of synaptic exocytosis. The otoferlin-MYO6 complex has been suggested to participate in targeting neurotransmitter vesicles to the specialised ribbon synapses at the basolateral membrane of these hair cells for exocytosis and signal transmission to ganglia neurons [68] [69]. Since mutations in both MYO6 and otoferlin cause deafness in humans, this suggests a complementary role for these proteins in normal hair cell function in the ear [70] [71].

A yeast two-hybrid screen has identified phospholipase C (PLCδ3), an important enzyme in phosphoinositide metabolism, as a MYO6 binding partner [72]. Although PLCδ3 is also expressed in inner ear hair cells, no hearing abnormalities were observed in the PLCδ3 KO mouse. The expression of MYO6, however, is decreased in the intestine of the PLCδ3 KO mouse and loss of the enzyme decreases the number of microvilli in the small intestine. Thus, a functional complex of PLCδ3 and MYO6 may be required for maintenance of apical microvilli in polarised epithelial cells [72].

Finally, MYO6 has also been suggested to function together with the putative transcription co-activator NDP52 in the nucleus and to regulate RNA polymerase II-dependent transcription [73] [74].

MYO6 Molecular interactions

A limited data set of structural information is available on the tail domain with or without their binding partners [20] [21] [75]. To date, cryo-EM has been successfully applied to the motor domain bound to actin filaments [76], but this technique has yet to be used on the flexible tail domain.

The cargo-binding tail domain contains protein-binding motifs, ubiquitin-binding sites and a phospholipid binding domain (see figure 1 D and figure 2). The first MYO6 protein-binding motif, the adaptor binding domain 1 (ABD1), encompasses the $RRL^{1116-1118}$ motif (positions refer to canonical MYO6 sequence Q9UM54-3 uniprot) and the second protein-binding domain, the adaptor binding domain 2 (ABD2) contains the $WWY^{1201-1203}$ motif [20] [21] [75]. Point mutations in the RRL motif inhibit MYO6 binding to GIPC, NDP52, TAX1BP1 and optineurin [16] [77] [78]. Dab2, LMTK and TOM1 proteins all interact with MYO6 via ABD2, which includes the WWY motif [15] [29] [30]. The tail also contains two ubiquitin binding sites, a Motif Interacting with Ubiquitin $(MIU)^{990-1014}$ and a Myosin VI Ubiquitin-Binding Domain $(MyUb)^{998-1025}$, which overlaps with the $ABD1^{1084-1128}$ and the lipid binding $domain^{1122-1131}$ (PIP2) [20] [78, 79] [21]. Through the ABD1/MyUb domain MYO6 can interact directly with adaptor proteins such as GIPC, TAX1BP1, NDP52 and optineurin [52] [16] [51]. In addition, the MyUb may allow indirect binding via ubiquitin chains to other proteins. This may involve direct binding of MYO6 to, for example, ubiquitinated proteins on the outer mitochondrial membrane or indirect binding via free ubiquitin chains, which may enhance binding to MYO6 adaptor proteins that contain a ubiquitin binding domain, such as

TOM1, TAX1BP1, optineurin and NDP52 [53] [21]. The MYO6 CBD contains two phosphorylation sites TINT$^{1096\text{-}1100}$ and DYD$^{1145\text{-}1147}$. Phosphomimetic mutations of the TINT to EINE inhibited the binding of MYO6 to optineurin, suggesting that phosphorylation of MYO6 at this site might regulate binding of adaptor proteins to ABD1 [16]. The different splice variants of MYO6 allow additional regulatory mechanisms by providing extra regulatory phosphorylation motifs or adaptor protein binding sites. For example, the DYD motif is a potential c-src-kinase phosphorylation site only present in the MYO6 SI isoform, which regulates tethering of secretory granules to the actin cortex in PC-12 cells [49]. The LI isoform is located prior to the ABD1/MyUB domain and forms a unique regulatory amphipathic α-helix that contains a clathrin-binding site [28]. This helix also provides steric hindrance for binding of the adaptor proteins GIPC, NDP52, TAX1BP1 and optineurin to the ABD1 [28]. Between the ABD1 and ABD2 in the tail there is a large unstructured region, which may increase flexibility for cargo-binding to allow different adaptor proteins to bind simultaneously. Furthermore, protein binding itself may induce unique structural re-organisation, as binding of MYO6 to lipids leads to an increased presence of α-helices in the tail domain [78]. In the next section, we will focus on the interaction of MYO6 with various cargo adaptors and the effect on its oligomerization state which is important for its function as an anchor or transporter. Upon purification MYO6 appears to be a monomer *in vitro* [8], however, the interaction with different cargo-adaptor proteins may induce dimerization or even oligomerization (see figure 1B) [75] [19] [20], and thus determine the detailed cellular roles and functions of MYO6 as a monomer or dimer.

MYO6 ABD1 and MyUb domain encompassing the RRL motif

GIPC binds directly to the ABD1 [78]. In its inactive form GIPC exists as an autoinhibited dimer, which opens up upon cargo binding to interact with the RRL motif in MYO6 [20]. The GIPC-MYO6 complex can assemble into linear higher-order oligomers, in which GIPC binds via its C-terminal GIPC-homology 2 (GH2) domain to the RRL motif, which is embedded in the middle of the second α-helix in MYO6 ABD2$^{1084\text{-}1128}$. The GH2 of the first GIPC molecule interacts with the two arginines (RR^{1116,1117}) of the RRL motif and an extended region to allow hydrophobic interactions [20]. The GH2 domain of the second GIPC molecule binds to the opposite side of the MYO6 CBD covering the leucine (L^{1118}) of the RRL motif allowing the formation of five, or even longer, linear GIPC-MYO6 oligomers. *In vivo* observations support the importance of MYO6 oligomerization induced by GIPC through a mutant MYO6+ that moves towards the plus end of actin filaments. MYO6+ induced filopodia formation only occurs in the presence of GIPC, which accumulates together with MYO6+ at the tips [36]. Interestingly, an artificially dimerised MYO6 without the CBD, can rescue this phenotype, which supports the importance of GIPC-mediated dimerization/oligomerisation for MYO6 functions in vivo.

Direct binding of GIPC, but also the other adaptor proteins to the ABD1 not only requires the RRL motif but also the neighbouring extended hydrophobic surface$^{1123\text{-}1131}$ [21]. Interestingly, this region is dispensable for ubiquitin binding, but essential for direct adaptor binding. Furthermore, the presence of the extra α-helix introduced by the LI isoform inhibits binding of these four direct binding partners to MYO6, as it excludes their binding to the second arginine and leucine (RL^{1117,1118}) of the RRL motif [28]. In contrast to GIPC, NDP52, TAX1BP1 and optineurin not only interact directly with MYO6 [52] [51], but potentially also bind indirectly via ubiquitin chains to the MYO6 MyUb domain [21] [22]. MYO6 interacts

with K48, K63, K29 and K11 linked ubiquitin chains, with a clear preference for the latter three [21]. Both MYO6 and TAX1BP1 have been shown to bind to K63 Ub^4 chains simultaneous, thus forming a MYO6-ubiquitin-TAX1BP1 complex [22]. Therefore, the interaction of MYO6 with the autophagy receptors may have extra regulation via ubiquitin chains.

Finally, MYO6 can also directly interact with ubiquitinated proteins via the MyUb or the MIU binding motif in the absence of any of the known cargo-adaptor proteins. MYO6 MyUb recognizes and directly interacts with ubiquitinated mitochondria marked for degradation by Parkin *in vivo* [22]. The MIU binding motif shows no affinity or preference towards ubiquitin linkages; however, in cooperation with the MyUb domain it seems to specifically support K11-linked ubiquitin binding.

MYO6-adaptor interaction at the WWY binding motif

The interaction of MYO6 with the clathrin adaptor Dab2 for example, has been suggested to induce dimerization of MYO6 [75]. Targeting of MYO6 to clathrin-coated structures at the plasma membrane involves Dab2-binding via the WWY and $IWE^{1261-1263}$ protein-interaction motifs within the ABD2, and binding to PtdIns(4,5)P_2 via the phospholipid $domain^{1122-1131}$ (PIP_2) [75] [80] [81] [52]. In addition, MYO6 may interact directly with clathrin through a binding region that has been identified in the α2-linker helix of the LI splice isoform [28]. The MYO6 CBD contains two discrete spatially separated Dab2 binding sites, site I (including the WWY motif) and site II (including the IWE motif). Structural data suggests that Dab2 may induce dimerization of MYO6 by binding to the WWY motif on one MYO6 monomer and the IWE motif of another [75]. The remaining two binding sites on the two MYO6 monomers are occupied by a second Dab2 in the reverse orientation thereby stabilising the dimer. Site I is the major binding site, allowing hydrophobic interactions between Dab2 and MYO6, whereas binding site II interacts with Dab2 through both charge and hydrophobic interactions thereby increasing the binding affinity [75]. Point mutations of the tryptophan of either binding motif, W^{1202} of WWY or W^{1262} of IWE, disrupt Dab2 binding to MYO6 and abolish cellular localization to clathrin-coated structures at the plasma membrane *in vivo* [75] [52]. The importance of site I was confirmed by *in situ* proximity labelling, which demonstrated the loss of Dab2 and other proteins associated with clathrin-mediated endocytosis upon mutation of the WWY site [18].

TOM1 also binds to the WWY motif in the ABD2, as determined by pull downs and mammalian 2-hybrid in vitro [30]. MYO6 may simultaneously interact, through its ABD1 with the LC3-binding autophagy receptors (NDP52, TAX1BP1 and optineurin) and through the WWY motif in ABD2 with TOM1-positive endocytic vesicles [82]. Indeed, *in vitro* experiments using purified proteins have shown that two adaptor proteins such as TOM1 and NDP52 can bind simultaneously directly to the MYO6 ABD1 and the ABD2 [52]. Interestingly, in situ proximity labelling of MYO6 NI CBD has indicated that the mutation of the RRL motif also leads to the loss of TOM1, and its partner TOLLIP [18]. These results suggest that although the interaction between TOM1 and MYO6 occurs directly via the WWY motif, *in vivo* the binding between MYO6 and TOM1 may also be indirect via ubiquitin using the ubiquitin-binding TOM1 VHS domain and the MYO6 MyUb domain. A detailed structural and biochemical analysis is required, to distinguish whether the MYO6-TOM1 interaction requires ubiquitin chains or occurs by direct binding.

Potential mechanisms for regulation of MYO6 motor activity

All myosin motors, with the exception of class XVIII (MYO18) [83], are actin-activated ATPases, and this prevents futile ATP hydrolysis when the motor is not attached to actin filaments. To further control random non-productive translocation along the dense cellular actin network, tight regulation of myosin mobilisation is required. Several layers of regulation are present in most myosins, some being unique and other being universal across the myosin family to coordinate the timing of cargo binding with activation of ATP hydrolysis and force production.

Actin track composition

The actin filament track composition provides the first layer of regulation as myosin activity is responsive to the actin isoform involved, the actin binding proteins present and the nucleotide-state of the actin monomer.

Actin filaments are highly dynamic structures that assemble by the addition of new ATP-actin monomers to the plus-end of filaments at the plasma membrane or the surface of internal membranes. Through the constant addition of new ATP-actin the filaments undergo retrograde flow and sequential ATP-hydrolysis, referred to as filament ageing. Whereas ATP-actin and ADP-Pi actin filaments have very similar properties, the ADP-actin accumulating at the minus end of the filament is less rigid and stable. Interestingly, *in vitro* MYO5 shows increased run lengths on ADP+Pi-rich filaments, whereas MYO6 which prefers 'old' ADP-rich filaments [6]. Recent cryo-EM structures confirm minor conformational changes between nucleotide states of F-actin [84], as well as within the actin binding interface of the motor domain of non-muscle myosin class IIc (MYH14) and MYO6. [85] [76].

From the six different actin isoforms expressed in mammalian cells, four isoforms are only present in muscle cells. The two cytoplasmic actin isoforms (β-cyto and γ-cyto) are found in most cell types and tissues. Although these two cytoplasmic actins only differ by four residues in their N-terminal sequence, they can assemble into distinct actin structures in non-muscle cells and preferentially associate with different classes of myosins. At present it is not known whether MYO6 is differentially activated by different actin isoforms, however, non-muscle myosin IIc (MYH14) shows increased kinetics when interacting with cytoplasmic β-actin whereas ATPase activity of MYO7A results in greater stimulation by γ-actin [86].

Activation of select myosin motors is not only influenced by the actin isoforms that make up a filament, but is also regulated by a large number of actin binding proteins. In this respect the most important one is probably tropomyosin, which has been well-characterised together with troponin for its role in Ca^{2+} regulation of sarcomeric myosins of class II in striated muscle. In non-muscle cells 40 tropomyosin (Tm) isoforms have been identified, which decorate spatially and temporally distinct actin filament assemblies [87] [88]. The presence of these tropomyosin isoforms on different actin structures modulates the affinity of myosin motors and leads to the select recruitment of specialised myosins to functionally distinct actin filaments. For example, myosin class Ic (MYO1C) does not bind to actin filaments containing Tm2, whereas MYO5A selectively engages with Tm3.1 –decorated actin filaments [89] [90]. Future studies will show whether MYO6 preferentially binds to different actin filament populations containing distinct tropomyosin isoforms.

Regulation of MYO6 through phosphorylation

In addition to the regulation of motor activity through crosstalk with the actin filament track, post-translational modifications of the myosin heavy chain or associated light chains are another important regulatory mechanism. For example, non-muscle MYO2s are regulated by phosphorylation of the regulatory light chains by a variety of kinases, which trigger dramatic structural changes of the myosin from a folded inactive state to an unfolded active state [91]. In *Acanthamoeba,* several of the myosin class I family members (myosin Ia, b and c) have been shown to be regulated by phosphorylation at a single serine or threonine in the motor which enhances the actin activated ATPase [92]. This site obeys the TEDS rule (it must either be a Thr, Glu, Asp or Ser) for myosin motor domain phosphorylation postulated by Bement and Mooseker [93]. MYO6 is also phosphorylated at Thr405 in the cardiomyopathy loop in the actin binding region, and this phosphorylation event affects the K_{ATPASE} but not the V_{max} [9]. Phosphorylation in the MYO6 motor domain occurs downstream of growth factor receptor activation and is accompanied by its recruitment to membrane ruffles [44]. Moreover, Salmonella virulence effectors, such as SopE, have been suggested to activate MYO6 through PAK-dependent phosphorylation at Thr405 [94].

Backfolding regulates ATPase kinetics

Backfolding as a way to inactivate ATP hydrolysis is a recurring theme in the myosin family (II, V, VI, VII and X) [95] [96] [97]. MYO5 has been shown to take a compact conformation, where the tail back-folds onto the motor domain, in this conformation the ATPase activity is reduced 50-fold [98]. In the case of MYO5 this backfolding is regulated by calcium binding to, and changing the conformation of calmodulin bound to the neck domain.

MYO6 has two calmodulins bound at the neck domain. The first calmodulin is a structural calmodulin that is 'locked' into the molecule. The second calmodulin has been shown to change conformation depending on the number of Ca^{2+} ions bound. Upon binding Ca^{2+} one lobe detaches from the heavy chain and rebinds to a higher affinity binding site present in the 3-helix bundle at the beginning of the tail [97]. This has the effect of "breaking" the lever arm, so the molecule cannot translocate cargo, simultaneously the tail domain enters into a "primed" position making it more readily able to bind cargo [97]. In the absence of cargo, and in a return to the low Ca^{2+} condition the molecule will retake a compact backfolded conformation, if cargo is bound the restored lever arm can now facilitate active translocation. The backfolded molecule can also unfold in the presence of high concentrations of binding partners similar to MYO5 [99], this may simply be a matter of shifting equilibriums between the inactive and active state, however, on its own this is probably not an effective method of regulation within the cell.

Conclusion and Perspectives

The unique mechanical and biochemical characteristics of MYO6 make it a motor that cannot easily be replaced in the cell. MYO6 binds a large range of cargoes and acts as a hub in a number of protein networks linked to different cellular pathways. With recent advances in functional proteomics we believe that an even greater number of transient, short-lived interactions will be discovered, further highlighting that MYO6 is the linchpin in many cellular functions.

Although some progress has been made with the understanding of the regulation of MYO6, the mechanisms which constrict the selection, substitution and release of cargo are still not understood. Answering these questions as well as further structural details, in particular of the cargo binding domain, are crucial for using MYO6 as a future therapeutic target to improve human health.

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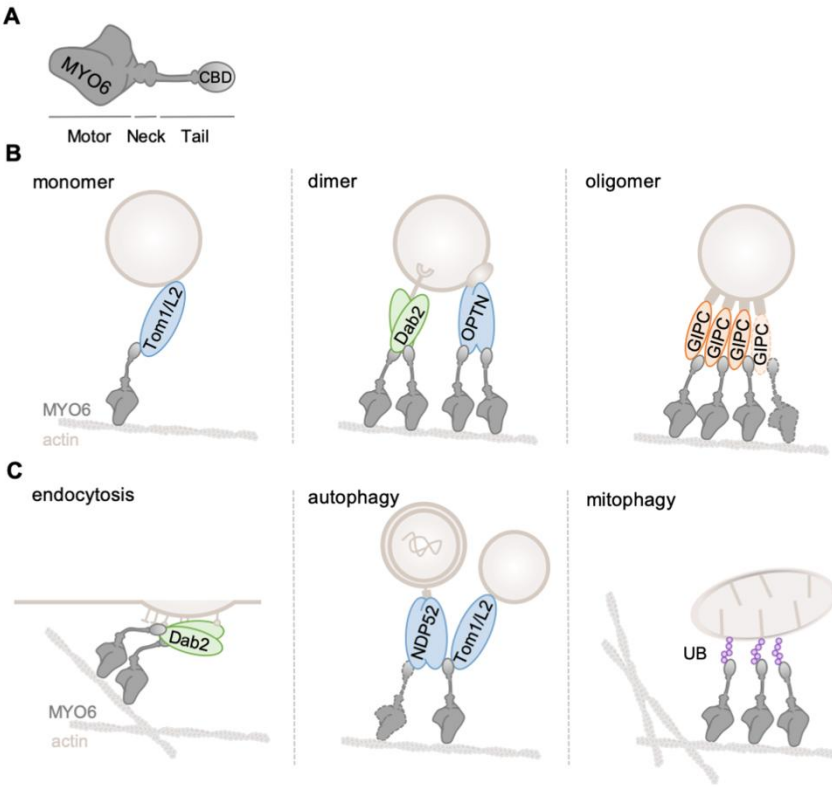
Figure 1: MYO6 structure and interaction with binding partners. A. Cartoon of MYO6 structure, denoting its cargo binding domain (CBD). B-C. MYO6 functions during transport and tethering with various bindings partners and oligomerization states. B. Monomeric TOM1 may interact with one MYO6 monomer; two Dab2 monomers may dimerize MYO6 or one optineurin dimer can bind two MYO6 monomers; native dimer GIPC may oligomerize MYO6. C. MYO6 interacts with PIP2, clathrin and Dab2 in clathrin-mediated endocytosis; MYO6 may interact simultaneously with TOM1 and NDP52 or TAX1BP1 and optineurin to drive autophagosome maturation; MYO6 interacts with ubiquitin (UB) on mitochondria during mitophagy. D. MYO6 amino acid sequences based on the human no insert (NI), small (SI) or large insert (LI) isoform. Protein binding domains and isoform-specific inserts are

annotated above the sequences. Predicted secondary structures are shown on top of the sequences; grey lines, zigzags and arrows represent unstructured regions, α-helices and β-sheets respectively (modified from [28] [75] [81]). MYO6 interaction regions are shown for GIPC (orange), Dab2 (green) and K_{63}-linked diubiquitin (purple) (based on structural data of [75] [20] [21]. Small (grey) dots refer to every 10^{th} amino acid.

Figure 2: Cartoon highlighting binding sites in the MYO6 CBD. Schematic of the cellular processes in which MYO6 is implicated through direct (blue) and indirect (grey) binding partners. In autophagy, MYO6 interacts directly with autophagy receptors TAX1BP, NDP52 and optineurin through its RRL domain (orange) or indirectly by binding to ubiquitin via its MyUb domain (purple dotted lines). Ubiquitin (UB) binding is also required during mitophagy (purple). A second ubiquitin-binding domain (MIU) allows binding to selective ubiquitin chains. In early stages of endocytosis, MYO6 interacts with LMTK2, TOM1/L2 and Dab2 via its WWY motif (green). Dab2-mediated dimerization also involves the IWE motif (green dotted line). MYO6 binds to lipids through its PIP_2 domain and to clathrin via the clathrin-binding region present in the LI isoform. MYO6 is recruited via GIPC to APPL1-positive signalling endosomes, or linked via GIPC to LARG and SHBP4 potentially regulating GPCR signalling. MYO6 binding to LRCH3 provides a link to regulation of actin and septin organisation via DOCK7.



D

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                    MIU domain                                                  Large insert 21-31aa
MYO6 NI  EESQQQAVLEQERRDRELALRIAQSEAELISDEAQADLALRR----------------------------------G 1037
MYO6 SI  EESQQQAVLEQERRDRELALRIAQSEAELISDEAQADLALRR----------------------------------G 1037
MYO6 LI  EESQQQAVLEQERRDRELALRIAQSEAELISDEAQADLALRRSLDSYPVSKNDGTRPKMTPEQMAKEMSEFLSRG 1060

                                MyUb domain / Adaptor binding domain 1          PIP2 domain
MYO6 NI  PAVLATKAAAGTKKYDLSKWKYAELRDTINTSCDIELLAACREEFHRRLKVYHAWKSKNKKRNTETEQRAPKSVT 1112
MYO6 SI  PAVLATKAAAGTKKYDLSKWKYAELRDTINTSCDIELLAACREEFHRRLKVYHAWKSKNKKRNTETEQRAPKSVT 1112
MYO6 LI  PAVLATKAAAGTKKYDLSKWKYAELRDTINTSCDIELLAACREEFHRRLKVYHAWKSKNKKRNTETEQRAPKSVT 1165
MyUb                GTKKYDLSKWKYAELRDTINTSCDIELLAACREEFHRRLKVYH
ABD1                     YDLSKWKYAELRDTINTSCDIELLAACREEFHRRLKVYHAWKSKN

                Small insert 9aa                                    Adaptor binding domain 2
MYO6 NI  DY---------PQQNPAAQIPARQREIEMNRQQRFFRIPFIRPADQYKDPQSKKKGWWYAHFDGPWIARQMELH 1177
MYO6 SI  DYDFAPFLNNSPQQNPAAQIPARQREIEMNRQQRFFRIPFIRPADQYKDPQSKKKGWWYAHFDGPWIARQMELH 1186
MYO6 LI  DY---------PQQNPAAQIPARQREIEMNRQQRFFRIPFIRPADQYKDPQSKKKGWWYAHFDGPWIARQMELH 1209

                    Adaptor binding domain 2
MYO6 NI  PDKPPILLVAGKDDMEMCELNLEETGLTRKRGAEILPRQFEEIWERCGGIQYLQNAIESRQARPTYATAMLQSLLK 1253
MYO6 SI  PDKPPILLVAGKDDMEMCELNLEETGLTRKRGAEILPRQFEEIWERCGGIQYLQNAIESRQARPTYATAMLQSLLK 1262
MYO6 LI  PDKPPILLVAGKDDMEMCELNLEETGLTRKRGAEILPRQFEEIWERCGGIQYLQNAIESRQARPTYATAMLQSLLK 1285
```

Key: 1. Inserts or Domains: ——

2. Motifs: **RRL, WWY, IWE**

3. Interaction residues: **ubiquitin GIPC Dab2**