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Various Themes of Myosin Regulation

Sarah M. Heissler[a,*] and **James R. Sellers**[a]

[a]Laboratory of Molecular Physiology, National Heart, Lung, and Blood Institute, National Institutes of Health, 50 South Drive, B50/3529, Bethesda, MD 20892-8015

Abstract

Members of the myosin superfamily are actin-based molecular motors that are indispensable for cellular homeostasis. The vast functional and structural diversity of myosins accounts for the variety and complexity of the underlying allosteric regulatory mechanisms that determine the activation or inhibition of myosin motor activity and enable precise timing and spatial aspects of myosin function at the cellular level.

This review focuses on the molecular basis of posttranslational regulation of eukaryotic myosins from different classes across species by allosteric intrinsic and extrinsic effectors. First, we highlight the impact of heavy and light chain phosphorylation. Second, we outline intramolecular regulatory mechanisms such as autoinhibition and subsequent activation. Third, we discuss diverse extramolecular allosteric mechanisms ranging from actin-linked regulatory mechanisms to myosin:cargo interactions. At last, we briefly outline the allosteric regulation of myosins with synthetic compounds.

Keywords

Actin; Cytoskeleton; Inhibitor; Posttranslational regulation

Introduction

Myosins constitute a large multigene family of actin-based molecular motors in eukaryotes. Family members are widely expressed and tightly integrated in cellular networks of interlinked biochemical pathways where they function as integrators between signaling and the dynamics of cytoskeletal mechanics [1–4]. As highly allosteric enzymes, the stringent regulation is a prerequisite for myosin's function in active transport processes, cytoplasmic contractility, and mechanosensing in both physiological and pathological processes [2–5].

Each mammalian cell has a specific repertoire of more than a dozen members from different myosin classes which are associated with a unique set of functional capacities and

*Address correspondence to: Sarah M Heissler, Laboratory of Molecular Physiology, National Heart, Lung, and Blood Institute, National Institutes of Health, 50 South Drive, B50/3529, Bethesda, MD 20892-8015, USA, Tel.: +1 (301) 496-8010; Fax: +1 (301) 402-1519, sarah.heissler@nih.gov.

limitations. The finely balanced regulation of a specific myosin is strictly required to meet the changing demands of cellular functions. This requires a plethora of regulatory targeting mechanisms to recruit a specific myosin to its site of action, to switch its enzymatic activity on and off and to determine the cargo that may be attached to it. This local myosin regulation can result in global changes of cellular function to produce a coherent cellular response. Defects in the allosteric myosin motor regulation are linked to diseases, including cardiomyopathies, cancer and diabetes and provides a means for the development of myosin-specific drugs.

This review describes the general principles of posttranslational allosteric mechanisms of myosin regulation across different classes and phyla. It focuses on selected, well-characterized myosin classes and is subdivided into sections to reveal reoccurring themes and fascinating variations of intrinsic and extrinsic regulatory features that determine myosin's mechanoenzymology, oligomerization, interactome and cellular localization. As individual members of a myosin class are sometimes synergistically targeted by different regulatory mechanisms, a single myosin class may be discussed in multiple sections of this review. The detailed discussion of individual myosin classes is beyond the current work and can be found in exhaustive recent reviews [3, 4].

Myosin structure, mechanoenzymatic concepts and classification

The diversity of cellular functions in which myosins are involved is attributed to characteristic structural, enzymatic and regulatory properties of individual members of the myosin superfamily. At the level of primary sequence, a myosin heavy chain consists of a motor domain, a neck domain and a tail domain (Fig. 1). The myosin motor domain harbors both a prototypic ATP binding site as well as a binding region for filamentous actin and allosterically links a repeated cycle of ATPase activity to its translocation on actin (Fig. 2) [5]. While the motor domain is the most conserved in terms of amino acid sequence and structural homology, the maximal actin-activated ATPase activity of myosins varies by a factor of 3000 from ~0.13 $s^{-}1$ to ~390 s^{-1}. Large differences are also found in the rate of actin filament sliding in the *in vitro* motility assay that ranges from ~20 $nm{\cdot}s^{-1}$ to ~6000 $nm{\cdot}s^{-1}$ [6–8]. Moreover, some myosins have evolved into pseudoenzymes that do not have an actin-activated ATPase activity [9, 10].

Large structural and functional diversification is found in myosin non-motor domains: The neck domain typically contains one to six IQ motifs in vertebrate myosins which non-covalently associate with a specific set of calmodulin-like light chains [11]. Tail domains are most diverse and constitute a regulatory hotspot of the holoenzyme due to a vast domain complement including coiled-coils that mediate oligomerization, myosin tail homology; fourpoint-one, ezrin, radixin, moesin (MyTH/FERM) and pleckstrin homology (PH) domains that mediate the interaction with binding partners and phosphoinositides (PtdIns). Specifically, these domains confer unique physiological attributes including ligand interaction, thereby defining a requirement for individual regulatory features [5, 12, 13].

Sequence analysis of the conserved motor domain currently divides the eukaryotic myosin superfamily in ~35 phylogenetic classes, out of which 12 are expressed in humans [12]. The

number of myosin classes is expected to increase steadily as more eukaryotic genomes are sequenced. Myosins can also be classified based on their biochemical and mechanical properties into four classes: fast movers, slow/efficient force holders, strain sensors or processive transporters [14]. Members of the myosin classes discussed here and their affiliation with these functional classes is shown in Fig. 3. For example, some dimeric mammalian myosins-5 and -6 are processive transporters, meaning that they can take multiple steps along actin without detaching. Besides structural prerequistites such as two motor domains and a long neck, processivity is facilitated by a high duty ratio, allowing myosin-5 and -6 to spend a long fraction of the kinetic cycle strongly bound to actin. Processivity is a prerequisite to transport cargoes such as melanosomes or endosomes along the actin cytoskeleton over long distances to its cellular destination [15].

In contrast, myosins such as certain class-1 myosins work as monomers and spend only a small fraction of time attached to actin. This feature does not allow them to transport cargoes but instead to function as strain sensors at the cytoskeleton-plasma membrane interface, a function essential to the regulation of membrane tension and cell migration. Other myosins with low duty ratios such as class-2 myosins assemble in bipolar filaments with sizes of 14–300 myosins. Smaller filaments are formed in the cytoplasm of nonmuscle and smooth muscle cells where myosins-2 power cytoplasmic contractility by sliding actin filaments past each other and function as force holders or strain sensors. Sarcomeric class-2 myosins assemble in large filaments made of hundreds of molecules and work together as fast movers to contract skeletal muscle.

Levels of myosin regulation

Myosin regulation occurs at three different levels: The first level includes transcriptional regulation, which dynamically affects the extent of myosin gene expression and alternate splicing in response to intra- and extracellular cues. The second and third levels of regulation includes substrate or effector binding and the regulation by posttranslational modifications, respectively. The latter mechanism is versatile and includes regulation at the myosin active site which is referred to as orthosteric and regulation at sites other than the active site which is referred to as allosteric. Orthosteric and allosteric events include covalent modifications such as phosphorylation and mutations to perturbation sites. Non-covalent targeting mechanisms include autoinhibition, ligand and effector binding [16]. Allosteric perturbations alter myosin’s mechanoenzymology and/or shift a preexisting myosin pool from an active to an inactive state or *vice versa* for example by the local accumulation of kinases and phosphatases or signatures of regulated actin [17].

Myosin regulation can follow either an “all-or-none” or a “modulatory” behavior. A classical example for the first is the phosphorylation-dependent autoinhibition relief of smooth myosin-2 in which the enzymatic activity is turned off in the absence of phosphorylation and is turned on by phosphorylation [18]. In contrast, Mg^{2+} has a modulatory effect on the enzymatic properties of some myosins, changing the activity by less than a factor of ten [19–23]. Moreover, no universal myosin regulation pattern exists: Myosins from one class, which are highly conserved at the levels of primary sequence and tertiary structure between protozoa and vertebrates exhibit multiple and entirely different regulatory aspects. This

paradox underlines that evolution does not create new enzymes within a superfamily but rather new allosteric modes of protein regulation [24]. Fig. 3 summarizes modes of myosin regulation grouped by myosin class which are discussed in this review.

Regulation by phosphorylation

Phosphorylation is a reversible, covalent allosteric regulatory mechanism for myosins and occurs on the heavy chain and the associated light chain. Changes in phosphorylation patterns are rapid and local, induced by the finely balanced interplay of kinases and phosphatases at subcellular compartments and cytoskeletal structures.

Phosphorylation as regulatory targeting mechanism is best studied for class-2 myosins. These myosins uniquely polymerize from a monomeric form into bipolar or side polar filaments by means of a coil-coiled domain in their tail to generate contractile forces and movement by pulling on actin. Phosphorylation of the associated myosin-2 regulatory light chain (RLC) in response to upstream signaling pathways is the major regulatory mechanism of vertebrate smooth and nonmuscle myosins-2, but not the sarcomeric myosins-2 from skeletal and cardiac muscle [25]. Phosphorylation of Ser-19 of the smooth and nonmuscle myosin-2 RLC by kinases such as myosin light chain kinase (MLCK) or Rho-associated protein kinase is associated with increased (>1000 fold) actin-activated ATPase activity and allows myosin-2 to move actin in an *in vitro* motility assay [26–28]. Di-phosphorylation on Ser-19 and Thr-18 further increases the enzymatic activity but not the *in vitro* sliding velocity [29, 30]. RLC phosphorylation is further associated with the adaption of an assembly-competent conformation, contractility and stress-fiber formation, as outlined in greater detail below.

Protein kinase C (PKC) mediated phosphorylation of Ser-1, Ser-2 and Thr-9 on the RLC of smooth and nonmuscle myosin-2 that has already been phosphorylated at Ser-19 by MLCK is associated with decreased enzymatic activity due to decreased actin affinity *in vitro* but the rate of actin translocation in an *in vitro* motility assay is not affected [29, 31]. Phosphorylation at the PKC sites also allosterically renders Ser-19 of the RLC a poorer substrate for MLCK and may result in a decline in phosphorylation levels in cells [32]. Though the effect is evident in *in vitro* studies with purified proteins, PKC phosphorylation of the RLC has not been studied extensively *in vivo.* It is not regarded as major regulatory mechanism for nonmuscle myosin-2 function during cell division of HeLa cells and primary human keratinocytes as well as the assembly of nonmuscle myosin-2a in spreading and resting HeLa cells [33]. However, nonmuscle myosin-2a inhibition after phosphorylation of Ser-1/Ser-2 of the RLC at the leading edge is required for mesenchymal chemotaxis in fibroblasts [34].

In the social amoeba *Dictyostelium*, phosphorylation of the myosin-2 heavy chain is the major regulatory targeting mechanism of the actin-activated ATPase activity as discussed below. However, RLC phosphorylation at Ser-13 by MLCK increases the actin-activated ATPase activity of myosin-2 and the mechanical performance of the motor by a factor of ~5-fold. This modulatory regulation mechanism is dispensable for its *in vivo* function [35, 36].

RLC phosphorylation of mammalian sarcomeric myosins-2 has a minor impact on its enzymatic features but has modulatory effects on the mechanical performance of a muscle fiber. [37]. In cardiac muscle, RLC phosphorylation is associated with increased myocardial performance and enhances myosin motility under loaded conditions *in vitro* [38, 39]. Electron microscopic studies show that the motor domains of unphosphorylated skeletal and cardiac muscle myosin-2 filaments are held in close apposition to the filament backbone with a high degree of order. When the RLC is phosphorylated, the motor domains connected by the proximal coiled-coil regions lift off the surface of the filament and would be more likely to interact with actin [40, 41].

Interestingly, the paradigm that phosphorylation of striated muscle RLC is only modulatory does not extend to invertebrate muscle myosins-2. Myosins-2 from the striated muscle of *Limulus* and *Tarantula* are both regulated in an on/off manner as is seen with vertebrate smooth muscle myosin [42, 43].

Apart from myosin light chain phosphorylation, myosin-2 heavy chain phosphorylation in the tail coiled-coil and the non-helical tailpiece (NHT) is a regulatory targeting mechanism and implicated in the inhibition of filament formation and the interaction with binding partners [44–48].

Myosin-2 heavy chain phosphorylation is best studied in amoeba where multiple Ser and Thr residues in the tails have been identified as phosphorylation sites for myosin heavy chain kinases [49–52]. In *Acanthamoeba*, Ser phosphorylation of the NHT regulates filament assembly by inhibiting the dimerization of myosin monomers and the assembly pathway of dimers into filaments [44]. Recently, phosphorylation of a Ser in an actin-binding surface loop of the motor domain was shown to reduce the steady-state ATPase activity by the allosteric reduction of the rate-limiting P_i release ~ 3-fold (Fig. 2) [53, 54].

In *Dictyostelium*, myosin-2 heavy chain phosphorylation is associated with the disassembly of myosin filaments into monomers and/or small oligomers *in vivo* and *in vitro* [55, 56]. Conversely, the introduction of Ala as unphosphorylatable residues at the respective sites results in myosin overassembly in cells [55]. In contrast, *Drosophila* myosin-2 heavy chain phosphorylation in the NHT by PKC is not a major regulatory mechanism and dispensable for fly viability [57].

The contribution of heavy chain phosphorylation as a regulatory mechanism for mammalian myosin-2 filament formation and regulation is less well understood [48]. Several kinases including PKC and casein kinase 2 (CK2) were shown to phosphorylate isoform-specific Ser and Thr residues in the tail domain of the three mammalian nonmuscle myosin-2 paralogs [47, 58, 59]. Phosphorylation events are associated with the disassembly of existing myosin-2 filaments into monomers or the inhibition of myosin-2 monomers to form filaments *in vitro*, possibly by increasing the critical concentration for filament formation [46, 60]. The exclusive use of myosin-2 tail fragments, which are poor surrogates for full-length myosin-2 in *in vitro* studies, prevents a more detailed model of how heavy chain phosphorylation affects myosin-2 regulation at the molecular level. In cells, nonmuscle myosin-2 filament disassembly caused by phosphomimetic mutations in the tail domain is

associated with increased migration rates and lamellipod extension in breast adenocarcinoma cells, suggesting that nonmuscle myosin-2 filaments act as a drag in biological processes such as cellular motility and chemotaxis [61]. In contrast, studies in osteosarcoma cells demonstrate that nonmuscle myosin-2a phosphorylated at a PKC site in the tail results in increased recruitment of this myosin to focal adhesions [62].

Class-1 myosins are single-headed and dynamically link the actin cytoskeleton to membranes. Phosphorylation of amoeboid myosin-1 molecules at a residue in the solvent exposed surface-loop in the myosin motor domain by STE20 family kinases is required for the actin-activation of their ATPase activity. The site was termed TEDS-site since virtually all myosins contain a Thr (T), Glu (E), Asp (D) or Ser (S) at this position [63].

Phosphorylation of TEDS-site residues in class-1 myosins is required for growth, actin organization and endocytosis in *Dictyostelium* [19, 64–68]. Similar, TEDS-site phosphorylation is indispensible for the localization of Myo1 to actin patches at the plasma membrane and endocytosis to occur in fission yeast. Those results imply increased actin affinity and motor activity of TEDS-site phosphorylated myosins-1 in this organism [69]. Concomitant, phosphomimetic TEDS-site mutants from *Dictyostelium* myosin-1d and -1e are characterized by increased (>20 fold) steady-state ATPase activity and actin affinity when compared to the unphosphorylated motor *in vitro* [19, 65]. For *Dictyostelium* myosin-1e, the speed with which it slides actin in the *in vitro* motility assay positively correlates with TEDS-site phosphorylation [65]. In contrast, the sole kinetic step increased by TEDS-site phosphorylation of *Acanthamoeba* myosin-1c is the rate-limiting phosphate P_i release which does not affect the speed of actin sliding [70].

Compared to amoeboid myosins-1, vertebrate class-1 myosins have a negatively charged amino acid at the TEDS-site which supersedes the phosphorylation requirement [63]. Further, allosteric and non-allosteric myosins can have similar evolutionary origins as seen for class-6 myosins which are phylogenetically closely related to class-1 myosins. Class-6 myosins have a Thr at the TEDS-site and are phosphorylated by PAK kinases *in vitro* [63, 71]. Strikingly, kinetic and functional studies of phosphomimetic mutants exclude TEDS-phosphorylation as a major regulatory mechanism *in vitro,* though cellular studies suggest phosphorylation-dependent myosin-6:actin interactions [72–74]. A possible explanation for discrepancy may be the use of phosphomimetic myosin mutants that do not always mimic *bona fide* phosphorylation [11, 75].

The importance of a negatively charged residue in the TEDS-site is also evident in *in vitro* studies, in which replacement of the endogenous TEDS-site Asp with in nonmuscle myosin-2a with a positive and neutrally charged residue reduces the enzymatic activity 8–10-fold, and the gliding speed of actin filaments in the *in vitro* motility assay 2–3-fold. Binding to actin is marginally affected by the mutations, suggesting that it does not contribute to the formation of a strongly-bound actomyosin complex [76]. This result is supported by recent studies showing that the TEDS-site is not part of the actomyosin interface, as previously speculated [77]. Instead, TEDS-site phosphorylation stabilizes and constrains the TEDS-loop which is crucial for the establishment of the actomyosin interface

and allosterically accelerates the weak-to-strong transition of the myosin kinetic cycle (Fig. 2) [19, 77, 78].

Mammalian class-3 myosins are both, *bona fide* actin-based molecular motors and active STE20 Ser/Thr kinases and are best known for their function as cargo transporters in hair-cell stereocilia. Kinase activity is conferred by an N-terminal kinase domain prior to the myosin motor domain which is dependent on autophosphorylation. Phosphorylation of residues in an actin-binding surface loop of the myosin motor domain of human myosin-3a decreases the actin affinity under steady-state conditions > 100 fold along with the duty ratio of the motor [79, 80]. Deletion of the kinase domain increases the ATPase activity, actin affinity and duty ratio of human myosin-3a, implying that autophosphorylation reduces motor activity [81]. The kinetic data support a model for myosin-3a function in actin-based projections such as filopodia or stereocilia suggesting that the reduced motor activity of the phosphoprotein decreases the formation and number of filopodial protrusions and myosin accumulation to filopodial tips [81, 82].

Regulation by autoinhibition

Autoinhibition is a non-covalent allosteric mechanism and a reoccurring theme of myosin regulation at the single protein level for myosins from classes-2, -5, -7 and -10 which are subsequently discussed [83–86]. A mutual feature of autoinhibition is the adaption of a compact myosin conformer which is associated with reduced catalytic activity. Modes of autoinhibition at the structural level as well as mechanism of autoinhibition relief are myosin-specific.

In vitro, smooth and nonmuscle myosins-2 exist in a three-state equilibrium between a compact, autoinhibited conformation (10S, the sedimentation rate during ultracentrifugation assays), an extended conformation (6S), and filaments [83, 87]. In cells, the filamentous state likely represent the primary functional state of myosin [83, 88]. Electron micrographs of myosins-2 in the 10S conformation show that the two motor domains interact in an asymmetric manner and the tails are sharply bent at two locations to allow it to interact with and stabilize the motor-motor interaction [87, 89, 90]. The conformational restriction imposed by the motor:tail interaction allosterically shifts the myosin into a kinetically inert and assembly-incompetent state by trapping the motor in the weak actin-binding M.ADP·P_i state (Fig. 2) [91]. The motor-motor interaction required for the off state explains why S1 fragments of these myosins or asymmetric HMM molecules which contain only one motor domain are constitutively active in the absence of RLC phosphorylation [92, 93].

RLC phosphorylation at Ser-19 relieves autoinhibition, promotes filament formation and ATPase activity thereby triggering contractile forces on actin, as outlined above [87, 89]. Effects of RLC dephosphorylation are opposing. This widely accepted 10S-6S-filament three-state-equilibrium model is supported by cell biological studies demonstrating that smooth muscle myosin-2 exists in two functionally distinct cytoplasmic pools: A diffuse soluble 10S conformation and a filamentous one [94]. Interconversion can be exogenously induced and suggests a functional storage pool of monomeric 10S that could be readily

mobilized to assemble *via* the transient 6S intermediate into filaments upon phosphorylation to meet changing demands of the cell [94].

The ability to adopt the asymmetric motor-motor autoinhibited conformation is conserved in myosin-2 molecules throughout evolution, as the motor domains of myosins-2 in relaxed *Tarantula*, *Limulus* and scallop thick filaments are in the same conformation as the motor domains of mammalian smooth and nonmuscle myosins-2 [95–97].

Recent studies suggest that the three-state equilibrium model for the regulation of nonmuscle myosin-2 has to be extended by a fourth state, as the three mammalian nonmuscle myosin-2 paralogs can form heterotypic filaments by co-polymerizing with each other. In addition, they form mixed heterotypic filaments with the enzymatically inactive pseudoenzyme myosin-18a *in vitro* and *in vivo* [98–100]. The physiological significance of heterotypic and mixed heterotypic filaments remains elusive but is hypothesized to regulate filament size and the collective mechanoenzymatic properties of the filament, thereby affecting force output. Other possible functional consequences of the formation of heterotypic and mixed heterotypic filaments are, besides altered cellular localizations, different interaction signatures with binding partners [99]. Moreover, this increased complexity and diversity in the myosin superfamily imposes different regulatory means which may contribute to the plethora of functions nonmuscle myosins-2 are involved in.

The dimeric cargo transporter myosin-5a is in a two-state equilibrium between an extended (14S) and an autoinhibited conformation (11S) [85, 101, 102]. Electron micrographs show a triangular structure of the autoinhibited myosin-5a holoenzyme with the two motor domains docked against the globular tail domains (GTD) [103, 104]. This intramolecular allosteric interaction reduces the kinetic competence of the motor domain which is characterized by a very low ATPase activity due to a reduced actin affinity [105]. A Ca^{2+}-dependent autoinhibition relief is observed *in vitro* but is unlikely to be physiologically relevant as outlined in greater detail below [85]. A physiological cargo-dependent activation model suggests that interactions between the myosin GTD and binding partners such as melanophilin shift the equilibrium towards the catalytically active conformer while constituting a feedback mechanism that prevents futile ATP consumption under cargo-free conditions [106, 107].

Monomeric MyTH/FERM domain class-7 and -10 myosins adopt an autoinhibited conformation in which the GTD intramolecularly docks against the motor domain to form a tightly folded molecule with low actin-activated ATPase activity. Structural and biochemical studies indicate interactions between the second MyTH4 domain of the myosin-7 GTD, the motor and neck region [108, 109]. MyTH4 and PH domains of the myosin-10 tail also interact with the motor domain, respectively. Motor-tail crosstalk correlates with kinetically inertness in both myosins [84]. Ca^{2+} counteracts the tail-mediated inhibition of vertebrate class-7 but not class-10 myosins, where PtdIns(3,4,5)P_3 relieves autoinhibition and renders the motor into a catalytically active dimer with cargo-transport function [84]. The presence of MyTH/FERM domains in the tail domain of class-15 myosins suggest autoinhibition as a possible regulatory mechanism.

Regulation by divalent cations

Divalent cations are second messengers, master regulators and integrators of myosin function: Transient Ca^{2+} gradients affect local cytoskeletal dynamics though the overall cytosolic cation concentration is tightly controlled and retains unchanged with a 1000-fold excess of Mg^{2+} over Ca^{2+} under resting conditions in mammalian cells [110, 111]. The effect of both, free Ca^{2+} and Mg^{2+} on the myosin holoenzyme is multimodal and either direct or indirect *via* interactions with the myosin heavy chain or the associated light chains [11]. Most identified myosin light chains have 4 EF-hand domains that may bind Ca^{2+} and/or Mg^{2+}. For example MlcD, the native light chain of *Dictyostelium* myosin-1d, binds Mg^{2+} under physiological conditions. In contrast the versatile Ca^{2+} receptor calmodulin has four Ca^{2+}-binding sites, two of which bind Ca^{2+} with high affinity even in the presence of excess Mg^{2+} [112]. Ca^{2+}:calmodulin interactions trigger conformational rearrangements that may either result in the dissociation of a calmodulin subset from calcium-sensitive IQ-domains in the neck of some unconventional myosins from classes-1 and -5, or result in higher affinity for particular IQ motifs in some class-1 myosins [102, 113–116].

Ca^{2+}-binding to the calmodulin occupying the first IQ motif of myosin-1c is associated with a conformational change that directly effects some transient kinetic parameters but not the steady-state ATPase activity [117]. Ca^{2+}-bound myosin-1c has a lower duty ratio when compared to Ca^{2+}-free myosin-1c which may be of physiological significance in the adaptation response of the inner ear [117]. A similar direct effect of Ca^{2+} has also been reported for mammalian myosin-1b [118].

In contrast to the direct effect on the enzymatic properties of some class-1 myosins, Ca^{2+}-induced calmodulin dissociation and the associated rigidity changes alter the mechanochemical compliance of some myosin motors. For example, at least one calmodulin is released under physiological Ca^{2+} *in vitro* from myosins-1a and -1c [116, 119–123]. The *in vitro* sliding velocity decreased for both myosins, yet was restorable by the addition of exogenous calmodulin. The clutch-model suggests that calmodulin integrates force transduction from the motor to the tail domain in load-sensitive class-1 myosins [124]. Calmodulin is bound to the myosin neck in the absence of Ca^{2+} and provides mechanical stiffness: The clutch is engaged, the motor compliant. Ca^{2+}-induced calmodulin dissociation from the first IQ-motif renders the neck floppy and disengages the clutch which results in idling of the myosin motor. Decreased neck stiffness prevents the motor domain to be restrained during the catalytic cycle, triggering increased catalytic activity. Noncompliance of the neck might be a regulatory mechanism to release myosin-1 from actin, a possible physiological scenario in Ca^{2+}-dependent hair cell adaptation [124].

Motility defects, uncoupling of ATPase activity and lever arm mechanics induced by Ca^{2+} are also described for myosin-5a: Submicromolar Ca^{2+} concentrations allosterically relieves autoinhibition and potently activates the steady-state ATPase activity of full length myosin-5a *in vitro* [85]. This is likely due to the calmodulin-deficient myosin not being able to adopt the folded off state since Ca^{2+} does not affect the actin-activated ATPase activity of a two-headed fragment of myosin-5a which is missing the GTD [85]. Other means to prevent the formation of the myosin-5a off state include (i) the artificial increase or decrease

in the neck length or tail, and (ii) single point mutations in the N-terminus of the myosin motor domain or the GTD [125, 126]. Caveats associated with the significance of Ca^{2+} activation as physiological regulatory mechanism are emerging: Ca^{2+} binding to the myosin-5a holoenzyme results in the dissociation of a calmodulin subset from the neck domain, as described for class-1 myosins. Calmodulin dissociation results in decreased mechanical stiffness of the neck region, which is paralleled by the impairment of a communication path that couples the conformation of the neck to the myosin motor domain [127]. Loss of mechanical compliance is reflected in motility defects in the presence of Ca^{2+} which can be restored in the presence of excess calmodulin in *in vitro* assays [127, 128]. That Ca^{2+} has no direct effect on the mechanoenzymatic properties of the myosin-5a other than through calmodulin dissociation is evident from experiments with calmodulin mutants that remain associated with the myosin heavy chain in the presence of Ca^{2+} and render the motor Ca^{2+}-insensitive in the *in vitro* motility assay [127].

A Ca^{2+}-dependent autoinhibition relief is likely to be a physiological regulatory mechanism for mammalian class-7 myosins which contain Ca^{2+}-insensitive IQ domains. Ca^{2+}:calmodulin interactions are proposed to induce conformational changes in the heavy chain bound calmodulin at the distal IQ motif which attenuate interactions with the tail, thus abolishing the tail-induced inhibition of myosin motor function [108].

Ca^{2+} also influences the enzymatic activity of conventional myosin-2s in a myosin-specific manner: In the absence of Ca^{2+}, the two motor domains of molluscan muscle myosin-2 adopt a similar motor-motor autoinhibited state on the surface of the myosin filament as is seen in the monomeric state of unphosphorylated smooth and nonmuscle myosin-2. Ca^{2+} binding to the myosin essential light chain (ELC) activates the enzymatic activity, motility and tension generation of molluscan smooth muscle myosin-2. Structural studies indicated that Ca^{2+} mediates interactions between the ELC and the RLC. The Ca^{2+}-bound ELC:RLC complex renders the neck into a rigid structure which does not allow the myosin motor domains to adopt an autoinhibited conformation with compromised enzymatic activity [129, 130].

Striated muscle myosins-2 in contrast are inherently unregulated but their activity is exogenously regulated by actin-linked Ca^{2+}:troponin:tropomyosin interactions as reviewed in-depth elsewhere [131, 132]. The vertebrate skeletal muscle RLC can bind both Mg^{2+} or Ca^{2+}, but preferentially binds Mg^{2+} under physiological conditions with high affinity. The Mg^{2+} dissociation rate is too slow to allow for transient binding of Ca^{2+} during the course of a muscle contraction [133].

The action of free Mg^{2+} on myosin is dual – cofactor-like in MgATP and allosteric. *In vitro* studies revealed that elevated levels of free Mg^{2+} reduce the actin-activated ADP release from Mg^{2+}-sensitive class-1, -2, -5, -6 and -7 myosins *in vitro* (Fig. 2) [19–22, 65]. Effects of free Mg^{2+} differ depending on myosin's mechanochemistry: High duty ratio motors such as myosin-5, -6 and -7a with a rate limiting actin-activated ADP release display reduced steady-state ATPase activity as the free Mg^{2+} concentration is increased [21–23]. Low duty ratio motors such as class-1 myosins and nonmuscle myosins-2 have a Mg^{2+}-insensitive ATPase activities but display strong changes in their duty ratio paralleled with changes in

free Mg^{2+} [19, 20, 65]. Universal characteristics of Mg^{2+}-sensitive myosins are increased ADP affinity and reduced motility with increasing free Mg^{2+} concentration. Concomitantly, an Mg^{2+}-dependent increase in run length of an artificially dimerized myosin-6 construct has been reported [134]. Allosteric regulatory targeting mechanisms can act simultaneously on a myosin as described for *Dictyostelium* myosin-1d where a high free Mg^{2+} concentration and TEDS-site dephosphorylation act synergistically and result in a > 40 fold reduction in motile activity [19].

As a cofoactor for ATP, magnesium binds together with the nucleotide as MgATP to the myosin active site. A structural model of Mg^{2+}-sensitivity implies that interactions between active site residues with the bound nucleotide and Mg^{2+} need to be broken before the hydrolysis products can be sequentially released: P_i exits the nucleotide binding pocket first, followed by Mg^{2+} and ADP (Fig. 2) [23]. Changes in the free Mg^{2+} concentration allosterically shift the equilibrium between Mg^{2+}-free and Mg^{2+}-bound states by mass action. Accordingly, elevated free Mg^{2+} favors and stabilizes the tension and load-bearing A·M·ADP state and hinders the motor's ability to produce rapid movement [19, 65]. Structural and functional conservation within the myosin motor domain suggests Mg^{2+}-sensitivity to be a general regulatory mechanism. However, *Dictyostelium* myosins-1b, myosins-2 from *Dictyostelium* and *Drosophila*, as well as rabbit skeletal muscle myosin-2 are Mg^{2+}-insensitive in the physiological concentration range *in vitro*, indicating different allosteric targeting mechanisms and intramolecular communication pathways [135, 136].

The effect of Mg^{2+} on the kinetic and functional activity of several myosins has been demonstrated *in vitro*, however, the physiological significance of Mg^{2+} as regulatory targeting mechanism has not been elucidated in detail in cells.

Actin-linked regulation

Six actins, more than forty tropomyosins and numerous other actin-binding proteins diversify and dynamically compartmentalize the mammalian actin cytoskeleton. Specifically, actin-binding proteins determine the speed of actin polymerization, depolymerization, and the length of actin filaments. Moreover, some actin-binding proteins induce the formation of higher-order actin structures such as bundles in response to a plethora of signal transduction cascades. This causes spatiotemporal patterning and segregation of actin-populations with different signatures which directly influence myosin's track-selectivity and mechanochemisty to meet changing requirements of cellular functions [17]. Signatures of actin such as age and higher-order actin structures such as bundles are believed to guide myosin to its correct cellular destination, an important feature for example in active transport where misdelivery of a cargo or an interruption of a supply chain can result in pathologies.

Human nonmuscle myosin-2 isoforms and myosin-7a distinctly interact with isoactins [137]. *In vitro*, the enzymatic activity of nonmuscle myosin-2a and -2b is 4-fold greater with cytoplasmic β- and γ-actin than with skeletal muscle α-actin, as is the mechanical competence [137]. The paralog nonmuscle myosin-2c shows the greatest stimulation of its *in vitro* ATPase activity in the presence of β-actin, as does the myosin-7a:γ-actin pairing [137]. In a physiological context, intracellular isoactin:myosin cognate pairs are predicted to

locally fine-tune the mechanochemical properties and target the motor to areas with a specialized actin composition such as stress fibers or filopodia. It was further shown that not only the isoactin but also its age, determined by its nucleotide state, is involved in myosin sorting: The transporter myosin-5a has a longer run length on “young” actin which is the ADP·Pi state, concomitant with the site of actin polymerization at the plus-end at the cell periphery. Myosin-6, a minus-end directed motor takes longer runs on “old” actin in the ADP state, consistent with its movement to the center of the cell [138]. Sorting mechanism of the actin cytoskeleton result in regions that are enriched for actin signatures that either promote or exclude the interaction with a specific myosin class are cell type specific and dependent on cellular needs [139]. Regional selective motility of unconventional myosins has been observed in detergent-extracted, permeabilized cells, underlining that the actin cytoskeleton fine-tunes myosin motor function and localization [139, 140].

Actin cross-linking proteins increase the complexity of cytoskeletal architecture by organizing actin into higher-order structures such as bundles with different spacing, register and polarity. Though the current data are controversial, myosins such as the cargo transporter myosin-10 may recognize these structural differences, adapt their motile behavior and move selectively on a specific population in the actin cytoskeleton. Myosin-10 was initially proposed to move processively only on actin bundles [141], but other studies demonstrate that it also moves processively along single actin filaments [142–144]. High resolution measurements of the myosin-10 stepping mechanism along fascin-bundled actin filaments shows that while it most commonly steps from one actin monomer to another in the same filament, it also take side steps to an adjacent actin filament [142]. Speculatively, bundle-selectivity might also be an actin-linked regulatory mechanism for members of the cargo transporting class-3, -6, -7 and -15 myosins which are commonly found in actin protrusions and sensory hair cell stereocilia [145].

Mammalian skeletal muscle myosin-2 is inherently unregulated and the cyclical interaction with actin is instead regulated by the Ca^{2+}-sensitive tropomyosin-troponin regulatory complex as reviewed elsewhere [146, 147]. By contrast, the interaction between nonsarcomeric myosins-2 and regulated actin filaments is strikingly different due to the lack of troponin. The actin binding protein caldesmon which binds to actin filaments in the absence, but not the presence of Ca^{2+}-calmodulin, might play a similar role as troponin in nonmuscle and smooth muscle cells but its regulatory effect on the actomyosin interaction has not been elucidated in detail [148].

Diversification of the decoration status of actin with tropomyosins is a major regulatory mechanism of the mechanochemistry and recruitment of myosin motors in nonmuscle cells. Tropomyosin is a coiled-coil forming protein that binds along the entire length of the actin filament. Conventional smooth and nonmuscle myosins-2 show a tropomyosin-dependent actin-activated ATPase activity and actin gliding *in vitro* as well as tropomyosin-dependent cellular localization to stress fibers, which result in decreased cell migration [29, 31, 149, 150]. *In vitro*, tropomyosin isoforms have differential effects on the actin-activated ATPase activity and motile properties of nonmuscle myosins-2a, -2b and -2c [149].

Tropomyosin also promotes the motor activity and increases the actin affinity of class-5 myosins from fission yeast and switches myosin-5 from budding yeast, Myo2p, from a nonprocessive to a processive motion by increasing the duty ratio [73, 151]. Strikingly, Myo2p exhibits processive movement with slower speeds but increased run length on tropomyosin decorated actin bundles when compared to single actin-tropomyosin filaments [151]. Processivity, run length and frequency are tropomyosin-isoform-dependent and isoactin-independent [151]. In contrast, *in vitro* reconstitution of the yeast Myo4p messenger ribonucleoprotein complex (mRNP), an essential molecular transport complex important for mRNA localization, shows a significant increased run length and run frequencies on parallel fascin-actin-tropomyosin bundles over single actin-tropomyosin filaments without speed changes [152]. Processive movement of Myo4p is tropomyosin-independent [152]. Strikingly, mammalian myosin-5a displays no track-selectivity for single actin filaments or bundles [142].

Tropomyosin is a negative effector of the mechanoenzymatic properties of the monomeric strain sensor myosin-1b, which is excluded from tropomyosin-decorated actin and regulated actin structures such as stress-fibers and bundles [153, 154]. In agreement, tropomyosin-decorated actin does not support *in vitro* motility of brush border myosin-1a and inhibits its steady-state ATPase activity [116]. The actin-crosslinking protein fimbrin relieves the tropomyosin-mediated inhibition of Myo1p in fission yeast by displacement of tropomyosin from the actin filament [155]. A model for the tropomyosin-linked myosin regulation suggests that by virtue of actin binding characteristics and terminal amino acid composition, different tropomyosin isoforms control the accessibility of myosin to the respective binding site on actin and directly regulate myosin mechanochemistry and localization.

Actin-linked regulatory mechanisms challenge the notion that the tail solely determines myosin's spatial distribution since the motor domain discriminates the actin tracks that lead the myosin to its correct cellular destination. In line with this interpretation, a structural model for the tropomyosin-based regulation of myosin binding to regulated actin suggest direct myosin:tropomyosin interactions [77]. The surface loop-4 in the myosin motor domain is a central element of the actomyosin interface and contributes to non-covalent interactions with actin and tropomyosin [77]. Loop-4 sterically interferes with myosin binding to regulated actin in tropomyosin-sensitive class-1 myosins [78]. Concomitantly, mutational analysis reveals that truncation of loop-4 or the exchange of the loop with a corresponding loop from a tropomyosin-insensitive donor myosin promotes the interaction with regulated actin *in vitro* but is not sufficient to target class-1 myosins to different actin-tropomyosin structures in the cell [156].

Regulation by PtdIns

PtdIns recruit and anchor some myosins at cytoskeleton-membrane interfaces. On-site, the bilateral interactions impact myosin's mechanochemistry which *vice versa* regulates membrane tension in biological processes including endocytosis, exocytosis and cell migration that require membrane deformability [157]. Myosin:PtdIns interactions are mediated by PH domains and clusters of polybasic residues in the myosin tail [158–160]. The balancing act to function as a divalent crosslinker between the actin cytoskeleton and

the membrane impacts the mechoenzymatic properties of some class-1 myosins. For example, myosin-1c powers actin gliding on fluid lipid bilayers *in vitro*. The mechanical activity decreases parallel to the content of PtdIns(4,5)P_2 in the bilayer while the membrane affinity increases [161]. Moreover, myosin-1c, but not myosins-1a and -1b, drives asymmetric, counterclockwise motility on lipid bilayers, suggesting a nonaxial component to the powerstrokes of some class-1 myosins. The physiological significance remains elusive but suggest myosin-1 isoforms to be involved in symmetry breaking events *in vivo* [161].

The myosin-6 heavy chain contains a lever arm extension that adopts a stable and monomeric three-helix bundle in solution [162, 163]. Exposure to lipid membranes triggers a reversible conformational change of this compact three-helix bundle to a rod-shaped structure. A proposed kiss-and-run model suggest the synergistic action of adaptor-regulated dimerization and cargo-vesicle induced unfolding of the three-helix bundle to render monomeric myosin-6 into a dimeric, processive transporter with large and variable step size [162, 164]. In line with this finding, structural changes in the myosin-6 tail are also induced by Ca^{2+}-dependent PtdIns(4,5)P_2-binding [165]. Crosslinking experiments suggest a lipid-mediated dimerization of two myosin tail fragments. Mutational studies indicate that both, PtdIns$(4,5)_2$ and the binding partner disabled-2 (Dab2) are required for cellular positioning and the functional role of myosin-6 in clathrin-coated structures in the early endocytic pathway [165].

The monomeric transporter myosin-10 directly binds PtdIns(3,4,5)P_3 through its tail PH domains which is required for its localization, the frequency of filopodia formation and function in macrophage phagocytosis [84, 166, 167]. PtdIns(3,4,5)P_3 binding to myosin-10 activates the steady-state ATPase activity by relieving the tail-induced autoinhibition and promotes the motile activity of myosin-10 in the *in vitro* actin gliding assay [84]. Further, PtdIns(3,4,5)P_3-mediates myosin-10 dimerization which is crucial for processive motion *in vitro* and *in vivo* and its function as intracellular transporter that shuttles components required for the filopodial growth, maintenance and *bona fide* function to the filopodial tip [142, 144, 168, 169].

Regulation by binding partners and cargos

Many non-filamentous unconventional myosins from classes-3, -5, -6 and -7 and -10 are essential intracellular transporters that actively shuttle their cargo between different cellular compartments. Myosin cargoes are structurally and functionally diverse and include proteins, organelles, mRNA and membrane compartments [152, 170–173]. Cargo-tethering to the myosin tail domain is mediated and specified by adaptors which provide a functional link between the molecular motor and its cargo. Myosin:cargo interactions determine the mechanochemistry, oligomerization and localization of the motor complex. Pertinent questions including the processes of cargo recognition, selection, loading and unloading are still elusive for most myosin:cargo complexes.

The well-studied class-5 myosins are best known for the transport of membranous structures such as melanosomes, recycling endosomes and the endoplasmic reticulum along the actin cytoskeleton [172–175]. For example, the myosin-5a GTD binds the adaptor melanophilin

which couples the motor protein to the Rab27a-melanosome. The myosin-5a:melanophilin complex exhibits slower movement, enhanced run length and uniform processive stepping when compared to cargo-free myosin-5a in single molecule assays *in vitro* [173, 176, 177]. Melanophilin is an active component of the transport complex and dually functions as myosin-5a adaptor protein and tether that links the motor:cargo-complex to the actin track *via* its actin binding site [27]. Although the receptor that links the melanosome to *Xenopus* myosin-5 is unknown, phosphorylation of the myosin GTD by CK2 triggers cargo unloading in a cell cycle-dependent manner [178].

The monomeric class-5 myosin Myo4p is involved in posttranslational events of gene expression by transporting non-membranous mRNP into the emerging bud tip in budding yeast [170]. To accomplish active transport function with a single motor domain, Myo4p forms a heterodimer with the adaptor protein She3p and couples to a cargo mRNA *via* the tetrameric mRNA-binding protein She2p. *In vitro* reconstitution of the mRNP demonstrates cargo-linked myosin regulation since cargo mRNA is required for the formation of a functional, processive transport complex whereas cargo-free Myo4p is immotile [152]. Artificially increasing the number of localizing elements on the mRNA and multiple motors recruitment to the mRNP increases the frequency and supports longer processive runs [152]. In yeast, cargo unloading from Myo2 has is triggered through adaptor protein degradation and subsequent proteolytic degradation or GTP-hydrolysis within the myosin:cargo complex [179, 180].

Different from the high duty ratio motor myosin-5a which dimerizes *via* an extended coiled-coil region in the tail and exhibits processive movement along actin, both monomeric high duty ratio motors myosin-6 and -7a require external dimerization factors to function as cargo transporters [21, 22, 72]. The endocytic clathrin cargo adaptor Dab2 for example induces the regulated dimerization of the cargo-binding domain (CBD) of two myosins-6 in anterograde trafficking pathways [164, 165]. Adaptor-induced dimerization converts the non-processive myosin monomer into a processive dimer and is required for the localization of myosin-6 to clathrin-coated vesicles [164, 181]. A mutation within the myosin-6 motor domain that disrupts the necessary communication between the motor domains of the dimer is correlated with a strongly perturbed mechanoenzymatic regulation which renders the myosin to a nonfunctional transporter. Physiological consequences are the disrupted transport of nascent uncoated vesicles to the early endosome and impaired myosin-6 motor function in stereocilia resulting in deafness in mice [182].

As outlined in greater detail above, myosin-6 binds lipids *via* a three-helix bundle immediately preceding the CBD [162]. A proposed kiss-and-run model suggests that lipid binding of the three-helix bundle to cargo vesicles increases the local pool of monomeric myosin-6 at the membrane, thereby facilitating the cargo-induced dimerization [164]. Further, optical trapping experiments with artificially dimerized myosin-6 show that under low load, the myosin-6 functions as an efficient transporter while increasing loads render the motor to a dynamic actin tether - a prerequisite for its function as stereocilia cargo transporter and anchor of the apical cell membrane to the stereocilia base [183].

Artificially dimerized myosin-7a from *Drosophila* moves processively in single molecule *in vitro* assays, a requirement for its function as intracellular cargo transporter [86, 184]. In agreement, cell biological studies indicate that solely dimerized and not monomeric myosin-7a transports the MyRIP/Rab27 complex to the filopodial tip of retinal cells [184]. The model for the myosin-7a–dependent cargo transport suggests that the motor associates with the adaptor protein MyRIP which binds Rab27 thereby promoting the association with membrane vesicles such as melanosomes. Lateral movements on the membrane results in clustering of ternary myosin-7a/MyRIP/Rab27 complexes and crowding. The increase in the local concentration of monomeric myosin-7a on membrane vesicles might be sufficient to transport the cargo to filopodial tips. Alternatively, crowding-induced self-dimerization of myosin-7a could promote processive movement along the actin cytoskeleton [184]. Regulated cargo- or clustering-induced dimerization of two myosins is a conceptually similar regulatory mechanism for class-10 myosins [84, 144, 167].

Nonmuscle myosin-2 isoforms operate as teams in small bipolar ensembles with characteristic filament and functional signatures [185]. S1004A, a small Ca^{2+} binding protein associates with the myosin tail domain and promotes myosin filament disassembly and preventing filament assembly [186]. The nonmuscle myosin-2a:S100A4 interaction has physiological significance in metastasis-associated cellular motility [187]. Structurally, shifting of the monomer-filament equilibrium is triggered by partial unwinding of the myosin coiled-coil upon S100A4 binding and steric constraints [188]. The S100A4 interaction is inhibited by myosin tail phosphorylation by CK2 [46]. In contrast, the actin binding protein fesselin stabilizes smooth muscle myosin-2 filaments and increases filament length and thickness. Fesselin also decreases the rate of actomyosin dissociation in the presence of ATP and inhibits the actin-activation of the myosin ATPase activity in a concentration-dependent and tropomyosinindependent manner with an IC_{50} of 0.6 μM [189, 190].

Mammalian class-3 myosins challenge the existing dogma that monomeric myosins can’t exhibit effective transport functions. Different from the monomeric high duty ratio motors myosin-6 and -7a, myosin-3 exhibits dimerization-independent transport functions. An additional ATP-insensitive actin binding site in the myosin-3a tail which minimizes diffusion of the motor off the track is proposed to allow the motor:cargo complete inchworm to the tip of actin protrusions. Association with the cargo such as the actin-binding protein espin-1 actively enhances the length of actin rich protrusions such as filopodia and stereocilia [171, 191]. Myosin-3b lacks the tail actin binding site but utilizes a actin binding site in its cargo protein espin-1 to target the protrusion tip. Utilization of the biochemical properties of the cargo is indispensable for myosin-3b tip-localization to actin protrusions such as stereocilia and filopodia [191]. The cargo-assisted motility of the myosin-3b:espin-1 complex is another example of cooperative myosin regulation.

Regulation by small molecules

Myosins are druggable and promising targets for therapeutic intervention due to their implications in normal and pathological physiology [5]. Small-molecule druggability of the myosin motor domain gives valuable insight in myosin’s chemomechanical cycle (Fig. 2) at

the molecular level and pharmacological block and release approaches have fundamentally advanced our understanding of myosin function in cells.

Allosteric extrinsic myosin regulation with covalent and non-covalent myosin inhibitors and activators has been described. The P_i-analog vanadate (V_i) is a nonspecific, competitive myosin inhibitor in the presence of ADP. Incorporation of ADP.V_i in the nucleotide binding pocket of the myosin motor domain leads to the formation of the kinetically inert ternary M.ADP.V_i state, which has been widely used in x-ray crystallography to trap myosin in a state that resembles the ATP bound conformation [192].

Mutual features of all other small molecule effectors are a non-competitive mode of action on the myosin motor domain. 2,3-butanedione monoxime (BDM), *N*-benzyl-p-toluenesulphonamide (BTS) and blebbistatin are inhibitors of class-2 myosins [193–198]. The inhibitory effect of the low-affinity compound BDM is skeletal-muscle myosin-2 specific (IC_{50} ~5 mM) and associated with the allosteric inhibition of P_i release [197]. BDM has many reported off targets and it is not recommended for use in cell biological experiments [197]. The inhibitory effect of BTS is specific for fast-twitch skeletal muscle myosin-2 (IC_{50} ~ 5 μM) caused by the reduction in the release rates of the ATP hydrolysis products [195].

Primary myosin targets of the small molecule compound blebbistatin are nonmuscle and skeletal muscle myosins-2 with isoform-dependent IC_{50} between ~ 0.5 and 5 μM [199]. Blebbistatin does not inhibit *Drosophila* nonmuscle myosin-2 and human unconventional myosins-1b, -5a and -10 [136, 198, 199]. Mechanistically, blebbistatin binds with high affinity to the M·ADP·P_i state and inhibits the P_i release (Fig. 2). The M·ADP·blebbistatin complex has a very low actin affinity and adopts a structural state that resembles the starting point of the powerstroke [193, 196, 200]. Structural studies show the blebbistatin binding site in the apex of the myosin cleft [201]. Blebbistatin is widely used in *in vitro* and cell biological studies to dissect processes which depend of the contractility of the actin cytoskeleton in nonmuscle cells such as cytokinesis and cell migration. The major disadvantages, which limit the use of blebbistatin in live cell experiments to study dynamic aspects of nonmuscle myosin-2 motor function, are its blue-light sensitivity and cytotoxic side effects which precludes its use with GFP-tagged myosins [198, 202, 203]. To circumvent this problem, blebbistatin-derivatives have been synthesized. Among those, para-nitroblebbistatin is the most latest described non-cytotoxic and photostable blebbistain derivative that displays the same inhibitory potency to myosins-2 as blebbistatin and allows studies of GFP-nonmuscle myosin-2 inhibition in live cells [204]. Another blebbistatin derivative, photoreactive azidoblebbistatin, covalently crosslinks to the myosin-2 motor domain after UV-irradiation [205]. This photoreactivity improves the *in vitro* and *in vivo* effectiveness when compared to blebbistatin but its binding to the myosin-2 motor domain is irreverible [205].

Halogenated pseudilins bind to an allosteric site near the tip of the 50 kDa subdomain and reversibly inhibit the enzymatic activity of unconventional myosins. Pentabromopseudilin (PBP) potently reduced the steady-state ATPase activity (>50-fold) and motile activity (>50-fold) of eukaryotic class-5 myosins (IC_{50} ~ 0.4 μM) and exhibits less potency for class-1 and

-2 myosin [206]. Mechanistically, PBP interferes with ATP binding, hydrolysis and ADP release (Fig. 2) and alters the myosin-5a dependent distribution of mitochondria between mother and daughter cells during cytokinesis in yeast [206]. The pyrazolopyrimidine compound MyoVin-1 also inhibits the actin-activated ADP release of myosin-5 but with a weak IC_{50} ~ 6 μM *in vitro* [207]. Its inhibitory potency to inhibit myosin-5a function in cells has not been firmly established yet.

Another pseudilin derivative, pentachloropseudilin (PClP), is a class-1 myosin specific inhibitor (IC_{50} ~ 1–5 μM) with drastically reduced or no affinity for class-2, -5, -6 and -7 myosins (IC_{50} ≥ 100 μM) [208]. PClP allosterically lowers the coupling efficiency (> 30-fold) between the nucleotide binding site and the actin binding region and the motile behavior of class-1 myosins [208]. PClP-treatment of HeLa cells results in changes in lysosome morphology and distribution – an effect similar to that observed after siRNA-mediated myosin-1c depletion [208].

The motor activity of the closely related myosin-6 is inhibited ~ 3-fold by the small molecule 2,4,6-triiodophenol (TIP) [22]. In the cell, TIP-mediated myosin-6 inhibition is associated with defects of vesicle fusion at the plasma membrane in the final stages of the secretory pathways [22].

Solely one myosin activator is described: Omecamtiv mecarbil (OM) specifically targets cardiac myosin-2 (EC_{50} ~ 1.6 μM) and enhances the efficiency of myocyte contractility independent from Ca^{2+} *in vitro* and *in vivo* [209, 210]. The drug binds in a cleft between the 25 kDa and the lower 50 kDa subdomain of the myosin motor domain and allosterically increases the actin-activated steady-state ATPase activity of cardiac myosin-2 in a dose-dependent manner by increasing the rates of both, the ATP hydrolysis the rate-limiting P_i release *in vitro* (Fig. 2) [209] [211]. An activating effect of omecamtiv mecarbil is not reported for other muscular myosins-2 [209].

The small molecule EMD 57033 is predicted to bind to the same region as omecamtiv mercarbil in cardiac myosin-2. EMD 57033 is not a direct activator of cardiac myosin-2 and rather acts as pharmacological chaperone. By binding to and refolding of stress-induced misfolded protein, EMD57033 can restore the enzymatic activity of the motor, a function that may improve therapeutic approaches of protein-misfolding diseases including myosinopathies [212].

Conclusion and Perspectives

One myosin - numerous regulation signatures. Posttranslational allosteric myosin regulation provides a mechanism for increased protein diversity and reveals fascinating insights in basic mechanisms that influence the mechanoenzymatic properties of a motor *in vitro* and *in vivo*. The awareness of multifaceted modes of myosin regulation drastically expands the knowledge of how myosin motors are functioning within a cell and how they are integrated in complex signaling networks. At present, no comprehensive information is available on how much the levels of distinct myosin motors are spatially and temporally distributed to cellular subcompartments and tissues, their regulatory state and how they fluctuate over time

in living cells. Is there a correlation between myosin dysregulation and pathogenic events? Exploring these emerging challenges with the innovative integration of experimental and computational approaches will relate myosin's mechanoenzymology and interactome to its cellular function and address the effect of myosin regulation on the systems-level. Future research will focus on posttranslational regulation signatures of myosins to address the 'who', 'what', 'when', 'where', and 'how' does it work.

To summarize how multifaceted the regulation of a given myosin can be, we will revisit the global regulation of nonmuscle myosin-2b. The primary regulation of this myosin is via phosphorylation of its RLC. In the absence of phosphorylation of nonmuscle myosin-2b is inactive and adopts a monomeric conformation where the two heads make an asymmetric interaction and where the tail folds in hairpins at two locations to give rise to a compact conformation lacking actin-activated ATPase activity and the ability to form filaments. Phosphorylation of Ser-19 on the RLC disrupts this conformation, allowing the myosin to assemble into bipolar filaments where the myosin motor domains can interact productively with actin that result in stimulation of the ATPase activity and translocation of actin filaments. Several kinases which are targets of different signal transduction pathways can affect this phosphorylation including Rho-associated protein kinase and MLCK, which is Ca^{2+}-calmodulin dependent. The RLC can also be phosphorylated at Thr-18 which is further stimulatory and at Ser-1/2 and Thr-9 which are inhibitory to a certain extent. The activity of individual myosin-2b filaments can be modulated by co-polymerization with nonmuscle myosins-2a and -2c or with the pseudoenzyme myosin-18a. The monomer-filament equilibrium can further be shifted for example by heavy chain phosphorylation and binding partners. The nonmuscle myosin-2b heavy chain can be alternatively spliced at two locations in the motor domain which either slightly increase or significantly decreases motor activity depending on the location. The activity may also be modulated by the isoform of actin, the actin age, by the tropomyosin isoform bound to the actin or higher-order actin structures. All those regulatory mechanisms follow spatiotemporal patterns, suggesting that each nonmuscle myosin-2 filament is constantly exposed to different regulatory mechanism that fine-tune it mechanoenzymatic activity which in the context of a cell results in a coherent response.

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Abbreviations

CBD	Cargo-binding Domain
CK2	Casein Kinase 2
EC_{50}	half maximal effective concentration
ELC	Essential Light Chain
GTD	Globular Tail Domain

HMM	heavy meromyosin
IC_{50}	half maximal inhibitory concentration
mRNP	messenger ribonucleoprotein complex
NHT	non-helical tailpiece
PKC	protein kinase C
PtdIns	Phosphatidylinsositol
RLC	Regulatory Light Chain
S	Svedberg
S1	Myosin subfragment-1
TEDS	Thr-Glu-Asp-Ser-site

Glossary

Allostery	The communication of distinct sites in a protein that results in a functional change of its activity.
Duty ratio	The fraction of time during the ATPase cycle a myosin spends strongly bound to actin.
Processivity	The ability of a myosin to undergo subsequent steps on actin without detaching.

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Highlights

- Numerous intrinsic and extrinsic allosteric mechanisms regulate myosin activity
- Regulatory properties are myosin-specific
- Multiple regulatory mechanism can synergistically act on one myosin

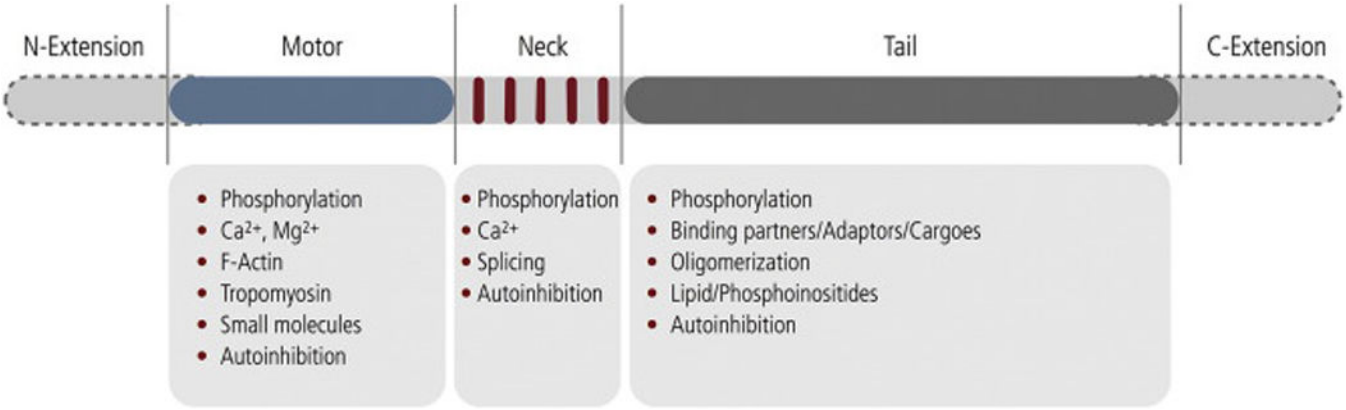


Fig. 1.

Basic building blocks of the myosin heavy chain: A myosin heavy chain consists of a prototypic motor domain (blue) which comprises the ATP binding site and an actin binding region, a light chain binding neck region containing multiple IQ-motifs (red), and a tail region (grey). N- and C-terminal extensions (grey dashed line) for the heavy chain are reported that may include N-terminal kinase domains, ATP-insensitive actin binding sites. Myosin light chains noncovalently associate with the myosin heavy chain to form the myosin holoenzyme. Moreover, myosin light chains stabilize and prime the neck region to competently function as a rigid lever that rotates relative to the myosin motor domain during the force generating power stroke that translocates myosin along actin [5]. Allosteric feedback mechanisms discussed in the present review target the motor domain, the neck domain and the tail region of the heavy chain and are listed below the respective domain. The N-terminal fraction of the molecule including the neck region and the associated light chains is referred to as S1. A longer fragment which additionally includes a portion of the tail domain is referred to as HMM. S1 fragments are monomeric whereas HMM fragments are dimeric. S1 is inherently active and serves as a powerful surrogate to study the transient-kinetic properties of the isolated myosin motor domain.

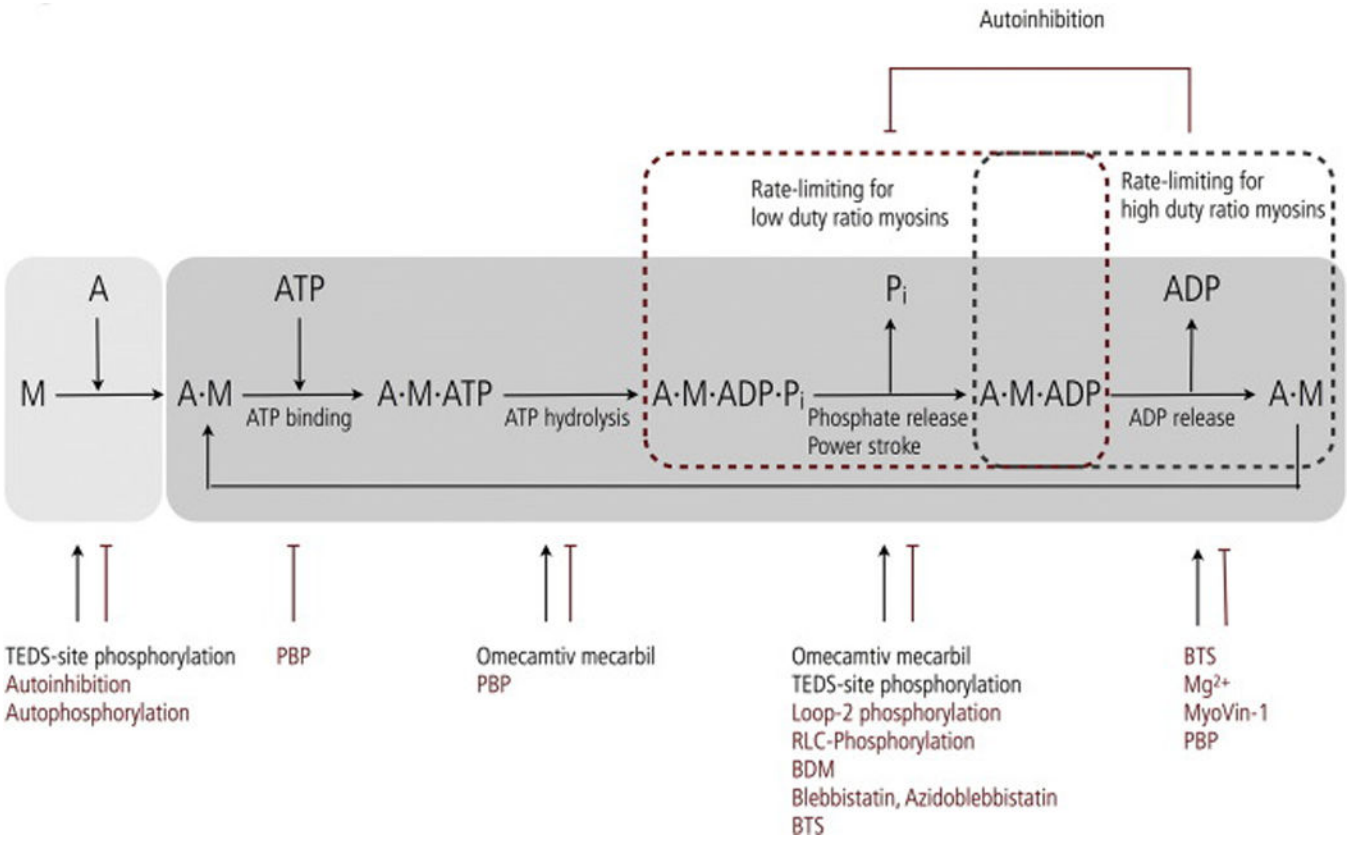
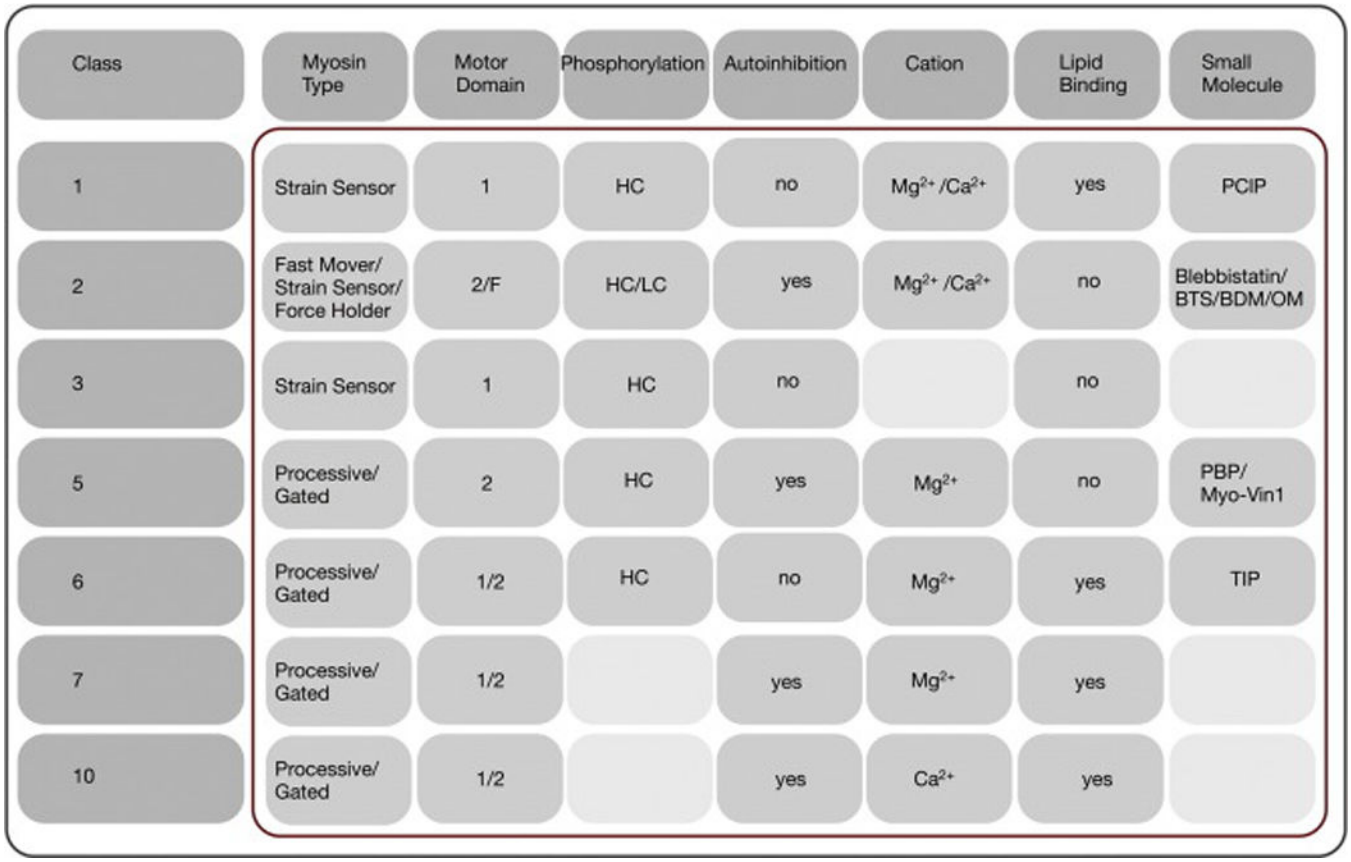


Fig. 2.

Simplified model of the basic actomyosin ATPase cycle and the kinetic steps altered by endogenous and exogenous regulatory mechanisms. P_i release (red dashed line) and ADP release (black dashed line) are entry and exit point to states during the ATP hydrolysis cycle in which the myosin motor domain is strongly bound to actin. The fraction of time of the ATP hydrolysis cycle myosin stays attached to actin is referred to as its duty ratio. P_i- and ADP release are commonly associated with the rate-limiting steps of low and high duty ratio myosins. The release of ADP also determines the speed myosins translocate on actin. Allosteric targeting mechanisms affecting key steps in the chemomechanical cycle are indicated. Black arrows and font color indicate activation; red blunt arrows and font color indicate inhibition. The abbreviations used are as follows: M = myosin, A = actin, P_i = inorganic phosphate.



Class	Myosin Type	Motor Domain	Phosphorylation	Autoinhibition	Cation	Lipid Binding	Small Molecule
1	Strain Sensor	1	HC	no	Mg^{2+} /Ca^{2+}	yes	PCIP
2	Fast Mover/ Strain Sensor/ Force Holder	2/F	HC/LC	yes	Mg^{2+} /Ca^{2+}	no	Blebbistatin/ BTS/BDM/OM
3	Strain Sensor	1	HC	no		no	
5	Processive/ Gated	2	HC	yes	Mg^{2+}	no	PBP/ Myo-Vin1
6	Processive/ Gated	1/2	HC	no	Mg^{2+}	yes	TIP
7	Processive/ Gated	1/2		yes	Mg^{2+}	yes	
10	Processive/ Gated	1/2		yes	Ca^{2+}	yes	

Fig. 3.

Selected myosin classes, their regulatory targeting mechanisms and cellular function. It is of note that not each of the regulatory mechanisms listed is applicable to each member in a given myosin class. The reader is redirected to comprehensive reviews for the detailed physiological function of individual members of a given myosin class [3, 4]. Motors indicate the number of myosin motor domains in the minimum functional unit of the protein. The notation 1/2 indicates that the myosin is a monomer but requires dimerization to become processive. The notation 2/F indicates that class-2 myosins are dimeric but assemble into filaments under physiological conditions inside cells. The column “small molecule” includes both, myosin activators and inhibitors. Light grey color indicated that regulatory events of phosphorylation, cations, and small molecule modulators have not be reported.
Abbreviations are as follows: HC: heavy chain; LC: light chain.