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# Myosin light chains: Teaching old dogs new tricks

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**Abbreviations:** CaM, calmodulin; EDTA, ethylenediaminetetraacetic acid; ELC, essential light chain; FRET, Förster resonance energy transfer; MLCK, myosin light chain kinase; PD, phosphorylation domain; PKC, protein kinase C; RLC, regulatory light chain; ROCK, rho kinase; WT, wild type; ZIP kinase, zipper-interacting protein kinase.

The myosin holoenzyme is a multimeric protein complex consisting of heavy chains and light chains. Myosin light chains are calmodulin family members which are crucially involved in the mechanoenzymatic function of the myosin holoenzyme. This review examines the diversity of light chains within the myosin superfamily, discusses interactions between the light chain and the myosin heavy chain as well as regulatory and structural functions of the light chain as a subunit of the myosin holoenzyme. It covers aspects of the myosin light chain in the localization of the myosin holoenzyme, protein-protein interactions and light chain binding to non-myosin binding partners. Finally, this review challenges the dogma that myosin regulatory and essential light chain exclusively associate with conventional myosin heavy chains while unconventional myosin heavy chains usually associate with calmodulin.

## Introduction

Myosins are involved in a myriad of functions such as muscle contraction, cytokinesis, cargo transport, cell adhesion, cell migration and the formation and stabilization of actin-rich structures such as stereocilia.[1] Phylogenetic analysis groups myosins in 35 classes of which 12 are found in humans.[2] The myosin holoenzyme consists of heavy and light chains. The myosin heavy chain is composed of an N-terminal motor domain, a central neck domain and a C-terminal tail domain (**Fig. 1A**). The motor domain mediates the ATP-dependent interaction with the F-actin cytoskeleton. The tail domain determines the oligomeric state of the heavy chain and interaction with binding partners or cellular compartments.

Myosin light chains are members of the calmodulin (CaM) and CaM-related gene families and associate non-covalently with the 20–25 amino acid IQ motif(s) (consensus sequence IQXXXRGXXXR)[3] located in the myosin neck domain (**Fig. 1A and B**).[4] As reviewed previously by Bähler et al., the IQ motif is strictly α-helical and devoid of proline residues.[4] Position 1 of the IQ motif is occupied by a hydrophobic amino acid. The IQ motif may have a partial or no amphipathicity and a basic net charge usually between +2 and +5.[4] IQ motifs often display large hydrophobic residues in conserved positions (1–8–14) which face to one side of the α-helix[5] and usually occur in tandems separated by 20–27 amino acids measuring from the first residue of the motif.[4] IQ motifs of the myosin heavy chain are numbered, starting with IQ1 for the motif in closest proximity to the motor domain. The number of light chains predicted to bind to a single myosin heavy chain can vary between one and 17.[2]

Myosin light chains are required for the structural integrity of the myosin holoenzyme. In addition, they can have regulatory functions on the mechanoenzymatic activity of the protein complex. In this review, we evaluate the interactions between myosin light chains and the myosin heavy chain, summarize recent reports of light chain heterogeneity within the myosin superfamily and discuss how the light chains participate in mechanical and regulatory functions of conventional and unconventional myosins. Further, this review briefly describes implications of myosin light chains in protein-protein interactions and the localization of the myosin holoenzyme and described non-myosin binding partners of myosin light chains.

## Native Light Chain Composition of the Myosin Holoenzyme

Filament-forming class-2 myosins, historically referred to as "conventional," were the first myosins isolated from muscles and later shown to be present in all eukaryotic cells. Biochemical and structural studies reveal that each myosin-2 heavy chain contains 2 IQ motifs and associates exclusively with 2 light chains most commonly referred to as essential light chain (ELC) and regulatory light chain (RLC), though they have been called by many names over the decades (**Table 1**). ELC binds the IQ1 motif closest to the motor domain; the RLC binds to the adjacent IQ2 motif.[6] The origin of the names ELC and RLC is somewhat curious: The term "regulatory" was chosen as this light chain is involved in the regulation of the enzymatic activity of some myosins-2.[7] The term "essential" was chosen because this light chain can only be removed from the myosin-2 heavy chain under harsh conditions in which the enzymatic activity of the holoenzyme is lost.[8] The specificity of the IQ1 and IQ2 motifs in class-2 myosins is high for ELC and RLC respectively, as demonstrated in


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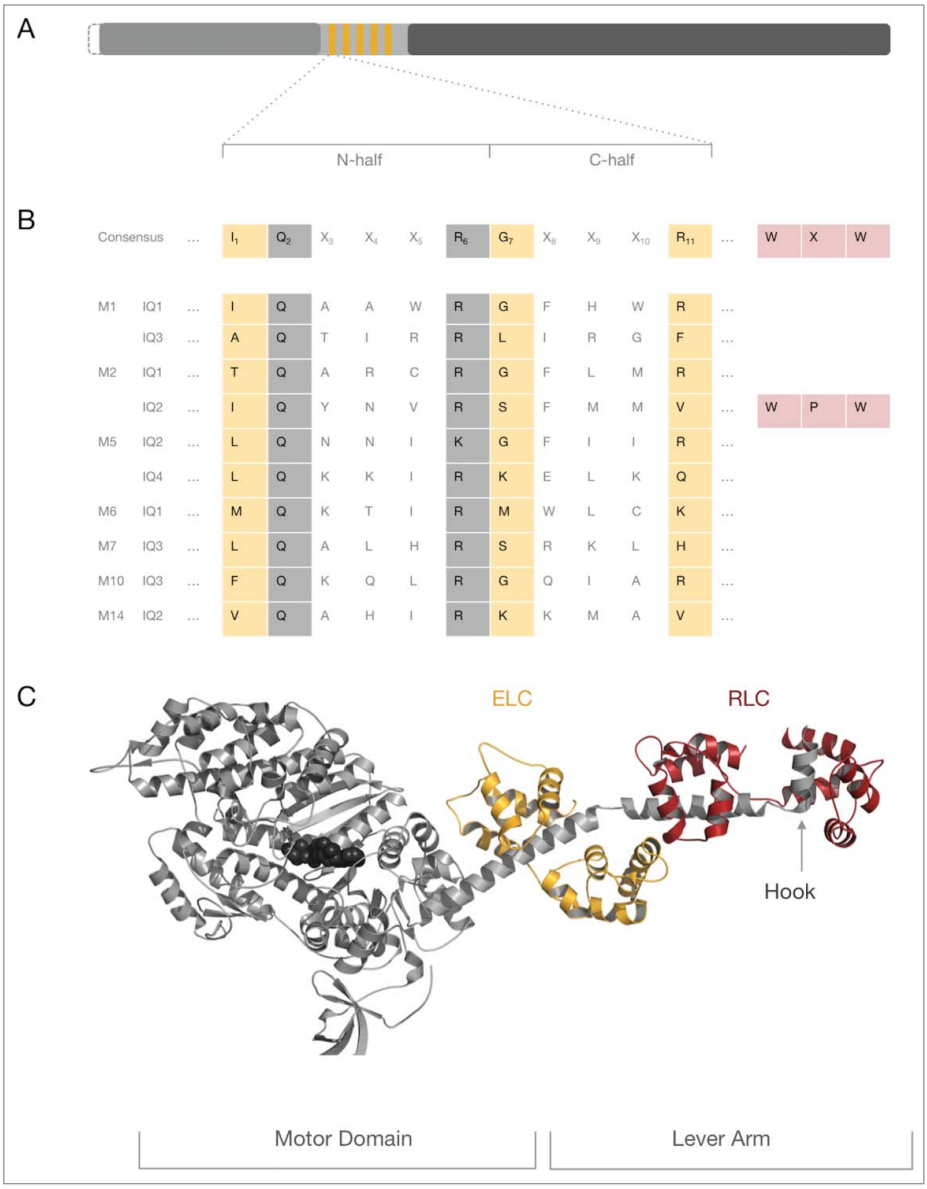


**Figure 1.** The myosin IQ motif in the heavy chain. (**A**) Schematic representation of a generic myosin heavy chain. The N-terminus of the heavy chain represents the catalytic motor domain (gray) that harbors the nucleotide binding site and the F-actin binding region. The central neck domain harbors the IQ motifs (orange). The C-terminal tail domain (dark gray) can contain a vast collection of domains and motifs that determine the oligomeric state of the heavy chain and interaction signatures with binding partners. The length of the myosin motor domain is very conserved, whereas the length of the myosin neck and tail domain varies considerably. Some myosins have an N-terminal extension of variable length prior to the motor domain which is indicated by the dashed line. (**B**) The top line represents the consensus IQ motif found in myosin heavy chains. Position 1 of the 11 amino acid long consensus IQ motif is critically occupied by a hydrophobic residue such as isoleucine (I), leucine (L) or valine (V) though methionine (M), phenylalanine (F) and lysine (K) and threonine (T) are found in some cases. Position 7 is ambiguous for several amino acids. Position 11 is preferentially occupied by a positively charged amino acid (arginine (R), histidine (H) or lysine (K)). Invariant residues are boxed in gray, ambiguous residues in peach. Non-boxed residues are highly variable. The WXW motif found in the hook region of conventional myosins-2 is colored in rose. Of note, the WXW motif is not part of the consensus IQ motif but crucial for the interaction of RLCs with the IQ2 in myosins-2 and therefore depicted for completeness. All other lines shown represent selected IQ motif found in conventional and unconventional myosin heavy chains discussed in this review. The abbreviations used are as follows: M1: IQ1 and IQ3 from human myosin-1C, M2: IQ1 and IQ2 from chicken striated muscle myosin-2; M5: IQ2 and IQ4 from budding yeast Myo2p; M6: IQ1 from pig myosin-6; M7: IQ3 from human myosin-7A; M10: IQ3 from human myosin-10; M14: IQ2 from *P. yoelli*. (**C**) Crystal structure of a prototypic myosin-2–2IQ fragment. Structural overview of the striated myosin-2–2IQ fragment from scallop (PDB ID: 1DFL). The myosin heavy chain is shown in gray cartoon representation, the nucleotide in black spheres. The associated ELC and RLC are represented in orange and cherry color, respectively. ELC and RLC bind distal from the globular motor domain to the IQ motifs of the neck domain. The myosin neck region and the associated light chains form the lever arm. The hook is indicated by a gray arrow.

the bay scallop *Argopecten*. The RLC from this species can be gently removed from IQ2 by treatment with the divalent cation chelator EDTA. The unoccupied IQ2 binds the RLCs from virtually any species presented to it *in vitro*, but does not bind ELCs.[7]

Myosins from all other classes are referred to as "unconventional." Unconventional myosins typically do not form filaments and display a great diversity in their tail domains that determines their localization and interaction with binding partners.[1,9] The number of IQ motifs found in the neck region of unconventional myosin heavy chains is variable and not always conserved within a class.[10]

In only a few cases has the native light chain composition of unconventional myosins been experimentally determined by co-purification coupled with proteomic or immunological analysis. Among those, myosin-1A from brush border was the first vertebrate unconventional myosin purified to homogeneity. It binds 3 molcules of the ubiquitous $Ca^{2+}$-binding protein CaM as light chains.[11,12] Subsequently, all other vertebrate myosin-1 paralogs purified from various tissues were shown to bind CaM.[13,14,15] In contrast, the light chain composition of lower eukaryotic myosins-1 is more complex, as outlined below. Myosin-5A purified from chicken brain is associated with CaM and ELC, although the stoichiometry and binding order of the light chains to its 6 IQ motifs has not been firmly established.[16] In contrast,

**Table 1.** List of the commonly used historical and recent abbreviation for ELCs, RLCs and CaM in different organisms as found in the NCBI database

| Light chain | Recent abbreviation | Historic abbreviation |
|---|---|---|
| Essential | ELC | LC17 |
| | | Alkali LC |
| | | A1, A3 |
| | | g1, g3 |
| | | MLC1 |
| | | SH-LC |
| Regulatory | RLC | LC20 |
| | | MLC1 |
| | | MRLC |
| | | DTNB LC |
| | | A2 |
| | | g2 |
| | | P-LC |
| Calmodulin | CaM | CDR |

**Table 2.** List of selected myosin heavy chains and identified light chains discussed in this review

| Heavy chain | Organism* | ELC | RLC | CaM | Other | Reference |
|---|---|---|---|---|---|---|
| Myosin-1A | *Gg* | | | X | | 11,12 |
| Myosin-1B | *Rn* | | | X | | 13,15 |
| Myosin-1C | *Hs* | X | | X | | 140,215 |
| | *Mm* | | | X | CaBP1, CIB1 | 34,199 |
| Myosin-2[#] | *All* | X | X | | | |
| Myosin-3 | *Dm, Lp* | | | X | | 18–20 |
| Myosin-5 | *Dm* | X | | X | | 172 |
| Myosin-5A | *Gg* | X | | X | Syntaxin-1A | 16,200 |
| | *Mm* | X | | | | 17 |
| | *Sc* | X | | X | | 36 |
| Myosin-6 | *Dm* | X | | X | Androcam | 170,187,188 |
| | *Ss* | X | | X | | 21,27 |
| Myosin-7A | *Bt, Mm* | | | X | | 22,23 |
| | *Dm* | X | | X | | 170,216 |
| Myosin-9B | *Hs* | | | X | | 24 |
| Myosin-10 | *Bt* | | | X | | 189,190 |
| | *Hs* | | | X | CLP | 191,192 |
| Myosin-14[$] | *Tg* | X | X | | | 169 |
| Myosin-15 | *Mm* | X | X | | | 168 |
| Myosin-18A | *Mm* | X | X | | | 171 |
| | *Hs* | X | | | | 140 |
| | *Ca* | | X | | | 25 |
| Myosin-19 | *Hs* | | | X | | 173 |
| | *Mm* | | X | | | 174 |
| Myosin-21 | *Ld* | | | X | | 167 |

[#]All myosins-2; [$]Myosin-A; *Abbreviations: *Bt*: Bos taurus; *Ca*: Ceropithecus aethiops; *Ld*: Leishmania donovani; *Dm*: Drosophila melanogaster; *Gg*: Gallus gallus; *Hs*: Homo sapiens; *Lp*: Limulus polyphemus; *Mm*: Mus musculus; *Rn*; Rattus norvegicus; *Sc*: Saccharomyces cerevisiae; *Ss*: Sus scrofa; *Tg*: Toxoplasma gondii.

myosin-5A from mouse brain purifies exclusively with CaM as light chain.[17] Based on those early studies, a central dogma was established that conventional myosins bind ELC and RLC, whereas most unconventional myosins bind CaM as light chain.

Subsequently, less stringent methods were used to show that CaM is a light chain of less abundant or more difficult to purify myosins from various sources. These methods include (i) immunoprecipitation of the myosin heavy chain from cell or tissue extracts followed by probing for the presence of CaM in western blots or the detection of $Ca^{2+}$-dependent CaM gel shifts, (ii) CaM overlay assays of blots of cell or tissue extracts which are sometimes partially enriched for the myosin heavy chain by chromatography or (iii) binding of the myosin heavy chain to a CaM-affinity column. All methods demonstrate that CaM can bind to a particular myosin heavy chain, but do not establish that CaM is the sole or preferred light chain since protein gel blots usually were not probed with antibodies to other light chains and gels were usually too lightly loaded for the direct detection of potential light chains by protein gel staining methods. Likewise, most of these studies did not quantify the molar ratio of the light chains to the heavy chain.

By using various combinations of these methods, myosins-3 from *Limulus* and *Drosophila*,[18-20] myosin-6 from a porcine proximal tubule cell line,[21] myosin-7A from mouse and bovine tissues,[22,23] and myosin-9B from human leukocytes[24] were shown to bind CaM as light chain (**Table 2**). More recently, myosin-18A was co-immunoprecipitated with the RLC from COS7 cells[25] and a class-14 myosin, *Toxoplasma* myosin-A, copurified with an RLC (TgMLC1) from recombinant parasites.[26] Notably, the latter myosin does not copurify with CaM.[26]

Finally, there are numerous studies in which a particular unconventional myosin heavy chain or its fragments are recombinantly overproduced with CaM in the baculovirus/*Sf*9 system, despite a lack of information of the native light chain composition. Based on this approach, previously uncharacterized myosins from classes-1, -3, -5, -6 -7, -9, -10, -14, -15, -19 and -21 were shown to be capable of binding CaM (**Table 2**). However, copurification does not mean that CaM is the native light chain for these myosin heavy chains as most reports do not state whether preliminary experiments were pursued to establish that CaM binds competitively with other light chains. Thus, CaM may not preferentially bind to the myosin heavy chain if coproduced along with another or the native light chain.[27] This hypothesis is supported by occasional reports of sub-stoichiometric CaM-binding ratios in preparations of recombinant unconventional myosin heavy chain constructs in which the addition of exogenous CaM increases the saturation of the IQ motifs, the enzymatic activity or the solubility of the myosin holoenzyme at low concentration.[28,29,30] These results led to the realization that CaM might not be a universal light chain for all unconventional myosins.

As outlined in greater detail below, recent reports, primarily utilizing co-expression of heavy chain and light chains genes in the baculovirus/*Sf*9 system, show that RLC may be a physiological subunit of unconventional myosin holoenzymes from classes-14, -15, -18 and -19, ELC a subunit of unconventional myosins from classes-1, -5, -6, -7, -14, -15 and -18 (**Tables 2 and 3**).

## Myosin Light Chains Have a Conserved Structure

Three independent genes, *CALM1, CALM2* and *CALM3* encode 3 identical CaM proteins in humans. CaM is a major

**Table 3.** Compilation of selected myosin and non-myosin binding partners of ELC and RLC. It is of note that not all kinases and potentially phosphatases that interact with the RLC are listed due to space limitations

| Protein | Organism* | Function | Reference |
|---|---|---|---|
| **ELC binding proteins** | | | |
| Myosin-1 | *Hs* | Cytoskeleton | 140 |
| Myosin-2 | *All* | Cytoskeleton | |
| Myosin-5/Myo2p | *Dm, Sc, Gg* | Cytoskeleton | 16,170,172,217 |
| Myosin-6 | *Dm, Ss* | Cytoskeleton | 27,170 |
| Myosin-7A | *Dm* | Cytoskeleton | 170 |
| Myosin-14 (Myosin-A) | *Tg* | Cytoskeleton | 169 |
| Myosin-15A | *Mm* | Cytoskeleton | 168 |
| Myosin-18A | *Mm* | Cytoskeleton | 171 |
| p39 | *Rn* | Signaling | 218 |
| $PI_4$ Kinase | *Sp* | Signaling | 219 |
| SUP45/eRF1p | *Sc* | Translation | 220 |
| Vps27p-like protein | *Sp* | Trafficking | 219 |
| **RLC binding proteins** | | | |
| Myosin-2 | *All* | Cytoskeleton | |
| Myosin-14 (Myosin-A) | *Tg* | Cytoskeleton | 169 |
| Myosin-15A | *Mm* | Cytoskeleton | 168 |
| Myosin-18A | *Mm* | Cytoskeleton | 171 |
| Myosin-19 | *Mm* | Cytoskeleton | 174 |
| MRLC-interacting protein (MIR, MYLIP) | *Mm* | Ubiquitin-protein ligase | 221 |
| Myosin light chain kinase (MLCK) | | Signaling | |
| Myotonic dystrophy kinase-related CDC42-binding kinase (MRCK) | | Signaling | |
| p39 | | Signaling | 218 |
| Protein Kinase C (PKC) | | Signaling | 104 |
| Protein Phosphatase 1 (PP1) | | Signaling | |
| Rho-associated, coiled coil-containing kinase (ROCK) | | Signaling | |

*Abbreviations: Dm: Drosophila melanogaster; Gg: Gallus gallus; Hs: Homo sapiens; Mm: Mus musculus; Rr: Rattus norvegicus; Sc: Saccharomyces cerevisiae; Sp: Saccharomyces pombe; Tg: Toxoplasma gondii.

intracellular $Ca^{2+}$-sensor interacting with more than 3 hundred protein targets.[31] CaM is extremely conserved through phylogeny and has not evolved since the appearance of vertebrates.[31] *CALM1–3 g*ene products have 148 amino acids with a molecular weight of 16 kDa. CaM has a conserved overall dumbbell-shaped structure consisting of an N-terminal (N-lobe) and a C-terminal (C-lobe) globular region separated by a central helix (**Fig. 2A**).[32]

Each CaM lobe contains 2 functional helix-loop-helix motifs termed EF-hands consisting of 2 helices connected by a $Ca^{2+}$-binding loop which provides coordinating oxygen or nitrogen atoms derived from the amino acid side chains or the backbone polypeptide chain. The EF-hands of CaM bind $Ca^{2+}$ with high affinity and specificity compared to other divalent cations. The C-lobe has a 10-fold higher $Ca^{2+}$-binding affinity ($K_d$ ~0.2 μM) than the N-lobe ($K_d$ ~2 μM).[5] $Ca^{2+}$-binding or the interaction with a protein target can alter the conformation of the EF-hands. For example, in the absence of $Ca^{2+}$ (apo), the 2 EF-hand helixes form an antiparallel, "closed" conformation. In the presence of $Ca^{2+}$, the EF-hand helixes are perpendicular, the conformation "open."[33] The EF-hands can also adopt a "semi-open" conformation, which is between the open and the closed conformation. The EF-hand helices are completely separated in the "uncoupled" conformation as recently described.[34] A compilation of EF-hand conformations found in myosin light chains is schematically depicted in **Fig. 2B**. Conformational changes in the CaM EF-hand pair in a CaM lobe exposes a hydrophobic cleft which can be solvent exposed in the "open" conformation, but buried in the "closed" conformation, "semi-open" or "uncoupled." The open, semi-open and uncoupled lobe conformation can interact with a target sequence such as the IQ motif of the myosin heavy chain, whereas the closed lobe conformation does not allow for a tight interaction.

When bound to the myosin heavy chain, the overall structure of CaM can be either compact ("canonical") or extended (**Fig. 2A**). In the compact conformation, the CaM N- and C-lobe interact with the myosin heavy chain whereas only the CaM C-lobe interacts strongly with the IQ motif when bound in the extended conformation. The extended light chain conformation results in one free light chain lobe, which is predicted to recruit binding partners in some cases and potentially determines the localization of the ternary complex.[26,35-38]

The human RLC gene family includes *MYL2, MYL5, MYL7, MYL9, MYL10, MYL11, MYL12A, MYL12B*, the ELC gene family *MYL1, MYL3, MYL4, MYL6* and *MYL6B*. RLC gene products have a high structural similarity to CaM, 172–226 amino acids and molecular weights ranging from 18.8–25.3 kDa.[10] RLCs are comprised of 4 EF-hand motifs, although some may be degenerated since they lack the requisite number of $Ca^{2+}$-coordinating ligands. Degenerated EF-hand motifs have partially or entirely lost their $Ca^{2+}$-binding properties or bind divalent cations in general with little selectivity toward $Ca^{2+}$.

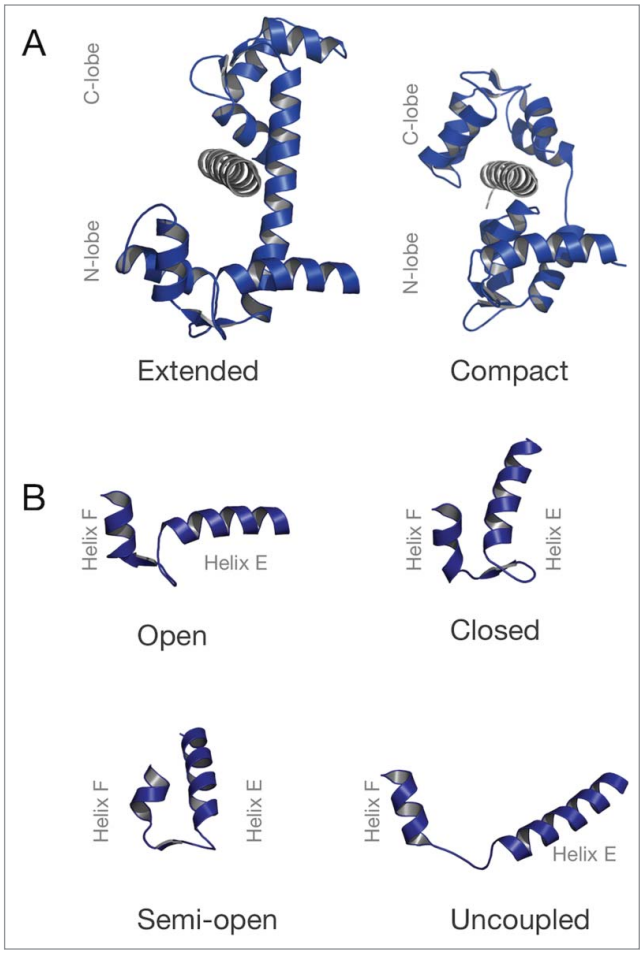


**Figure 2.** Structure of CaM and its EF-hand motifs in various conformations. (**A**) Structures of CaM (blue) bound to a target sequence (gray) in the extended (PDB ID: 3CLN) and compact conformation (PDB ID: 2IX7). (**B**) EF-hand conformations found in CaM. Top row: EF-hand in an open (PDB ID: 1CLL) or closed (PDB ID: 1CFC) conformation. Bottom row: The semi-open (PDB ID 2IX7) and the uncoupled conformation of the EF-hand motif (PDB ID 4R8G). Figure 2B is adapted from Lu et al.[34]

ELCs have 4 EF-hand motifs with some being degenerated, 150–208 amino acids and molecular weights ranging from 16.9–22.8 kDa.[10] Expression of ELCs from genes *MYL1* and *MYL3* as well as RLC from genes *MYL2* and *MYL7* is confined to cardiac tissue in human adults, *MYL4* expression to the embryonic heart. Major expression of the RLCs from genes *MYL5* and *MYL11* is described in skeletal muscle. *MYL10* encodes for an RLC that might be a lymphocyte specific precursor. Light chains believed to be nonmuscle myosin-2 specific are gene products of *MYL6* and *MYL6B* as well as *MYL9, MYL12A, MYL12B* and have a vast, however isoform-specific tissue distribution, as described below. How a light chain recognizes the appropriate myosin heavy chains is not well understood. Based on double-epitope tagging competition experiments, Komiyama et al. propose that the ELC N-lobe is responsible for intracompartmental and isoform-specific sorting.[39] More specifically, another study using ELC chimeras added evidence that the second EF-hand motif of the N-lobe determines isoform-specificity for the myosin heavy chain.[40]

Alternate splicing of the ELC genes *MYL1, MYL3, MYL4, MYL6, MYL6B* and the RLC gene *MYL9* is reported and increases light chain diversity at the protein level. The significance of alternate splicing events of myosin light chain pre-mRNA and differences in isoform composition are largely unknown but are associated with pathological disease states in some cases, as discussed later. Further, alternate splicing events and resulting changes in ELC expression levels can be age-dependent as seen in skeletal muscle.[41]

In fast skeletal muscle and the myocardium, *MYL3* and *MYL4*, are alternatively spliced at the N-terminus which results in an ~40 amino acid long extension. This extension contains a cluster of positively charged residues which interact with acidic residues on F-actin, thereby bridging the latter and the myosin-2 motor domain.[42,43] This may be responsible for the differences in the mechanoenzymatic activity of myosins-2 containing one or the other ELC which is only seen in the presence of F-actin and crucial for the alignment of the sarcomeric myosin-2 heavy chain on F-actin for optimal force production.[44,45] The long N-terminal extension of some invertebrate RLCs is thought to have similar functions.[46] Truncation of the N-terminal extension from *Drosophila* indirect flight muscle myosin regulatory light chain (DMLC2) reduces flight ability, wing beat frequency and the frequency of maximum power output.[47,48] Further, DMLC mutant males generate a courtship song with altered song parameters and exhibit impaired mating behavior when compared to control males.[47]

The regulatory function of the N-terminal ELC extension on muscle performance is vertebrates is myosin-specific: Removal of the N-terminal ELC extension in the cardiac myosin-2 holoenzyme affects cross-bridge prepositioning which negatively affects contractility by generating lower force and a pathological phenotype in a mouse model.[45] In contrast, an artificial increase of the ELC splice variant lacking the N-terminal extension in aged rat skeletal muscle is associated with increased contractility of single muscle fibers.[41] Time-resolved FRET studies add evidence that the extent of the force-generating power stroke of fast skeletal muscle myosin-2 strongly depends on the ELC splice variant in its lever arm.[49] The extent of the myosin power stroke is smaller in the presence of an ELC splice variant that lacks the N-terminal extension when compared to myosins that associate with the ELC that harbors the N-terminal extension.[49] The results provide a structural explanation for the effect of the ELC splice variant on the contractile function of muscle and supports the hypothesis that the N-terminal ELC extension enhances the isometric force while slowing the speed of shortening.[49]

## Disease States Associated with ELC and RLC

Familial hypertrophic cardiomyopathy is the most common inherited heart disease and linked to mutations in genes encoding many sarcomeric proteins including the β-cardiac myosin-2 heavy chain *(MYH7)*, ELC (*MYL3*) and RLC (*MYL2*).[50] Eight

mutations in the RLC and 5 mutations in the ELC have been linked to the disease in humans. ELC mutations cluster around 2 of the EF-hand motifs in the ventricular ELC. Their pathological phenotypes vary significantly in severity and mostly result in sudden cardiac death at young age.[51] *In vitro* studies suggest that RLC mutations R58Q and N47K reduce the isometric force and power output. The load at which peak power is achieved is shifted toward lower loads due to changes in the strain-dependence of β-cardiac myosin-2.[52] The effect of light chain mutations on cardiac myosin-2 and muscle properties have been well studied and the reader is directed to 2 recent reviews for an in depth discussion.[51,53] *MYL3* mutations are further linked to a rare myopathy of skeletal muscle.[54]

Besides mutations in light chain genes, altered splicing events or intracellular levels of light chain proteins are associated with human disease states. An example for the former includes aberrant splicing of *MYL6* pre-mRNA which is causative for altered contractility of smooth muscle myosin-2 in the *corpus cavernosum* in patients with erectile dysfunction.[55] An example for the latter is the post-transcriptional down-regulation of *MYL7* in chromosome 21 chimeric mice and neonatal hearts from humans with Down syndrome which is associated with developmental abnormalities in early stages of cardiogenesis. Altered *MYL7* expression and the associated downregulation of the protein level plays a key role in congenital heart disease observed in Down syndrome patients.[56] Down-regulation of *MYL9* expression is associated with the development and metastasis of non-small cell lung cancer.[57]

## Structural Aspects of the Myosin Heavy Chain: Light Chain Interaction

The crystal structure of chicken skeletal muscle myosin-2–2IQ reveals that the N-terminus of the myosin heavy chain forms a globular motor domain and results in an extended α-helical neck domain. It harbors the 2 IQ motifs which bind the ELC and the RLC.[6] This sequence of domains is conserved in other myosin-2 molcules as is illustrated in the structure of scallop myosin-2 (**Fig. 1C**). The neck domain with the bound light chains is also referred to as the lever arm. The myosin light chain: heavy chain interaction is critical at several levels. First, the 2 light chains are strictly required for maintenance of the structural integrity of the myosin holoenzyme and are critical to myosin function. Ablation or the reduction of the RLC expression levels leads to intracellular aggregation of the nonmuscle myosin-2 heavy chain in *Drosophila*.[58,59] Second, the myosin neck region with the bound light chains acts as a rigid lever arm that amplifies movements within the myosin motor domain into a large mechanical stroke that directionally propels the myosin along the actin filament. Evidence for this comes from *in vitro* motility and single molecule optical trapping studies with recombinant mutant myosins where the length of the lever arm is artificially extended, shortened or replaced with artificial lever arms.[60-63] Removal of a light chain from the myosin heavy chain exposes the hydrophobic IQ region to the solvent and likely results in a collapse of the α-helical neck domain. Myosins lacking the appropriate number of light chains to saturate the IQ motifs typically have a compromised power stroke but do exhibit actin-activated ATPase activity.[28,64,65] To circumvent this problem in functional and kinetic studies of conventional and unconventional myosins in which the native light chain composition has not been firmly established or substoichiometric light chain binding is observed, an artificial lever arm that replaces the native neck region can be used as powerful surrogate.[66,67] Note as will be discussed later, there is evidence that the light chain bound to IQ1 may interact with structural elements of the motor domain and influences the kinetic or mechanical properties of some myosins.[68] Thus caution must be exercised in truncating the myosin heavy chain, in selecting a light chain or in the use of artificial lever arms.[29,44,68]

Third, light chains bound to the neck region of some myosins-2 are intimately involved in the regulation of its enzymatic and mechanical output as discussed later.

Structural plasticity about the central helix of myosin light chains allows them to adopt several binding conformations on different IQ motifs. For example, ELC in a compact conformation wraps around the IQ1 of myosins-2. The C-lobe is in the semi-open conformation and interacts with the N-terminal half of the IQ1. The ELC N-lobe is in the closed conformation and interacts with the C-terminal half of the IQ1 (**Fig. 1C**).[6,69,70] The comparison between chicken skeletal muscle and smooth muscle myosin-2 crystal structures shows that the C-lobe of the ELC further interacts with the very N-terminus of the heavy chain or with a surface loop of the myosin motor domain depending on its nucleotide state, suggesting a communication between both subunits of the holoenzyme. This interaction could modulate the enzymatic activity by favoring or disfavoring conformations of the myosin motor domain during its ATPase cycle.[6,70] The ELC also establishes extensive interactions between its C-lobe and the myosin motor domain of the heavy chain that persists in all conformational states of the lever arm.[6,69,71]

The RLC interacts in an extended conformation with the IQ2 of conventional myosins where the C-lobe of the light chain interacts strongly with the N-terminal half of the IQ2 motif in the semi-open conformation.[6] The RLC N-lobe is in the open conformation and interacts with a conserved WXW motif immediately C-terminal to the IQ2, which is also termed the "hook" region (**Fig. 1B and 1C**).[69,72] All myosin-2 heavy chains bend sharply in the hook region. In crystal structures of myosin-2 from the sea scallop *Placopecten*, the heavy chains adopt different angles about the hook region which involve a 10 Å variation in the length of the light chain binding region.[73] The mechanical relationship between the angle of the hook region and the RLC conformation might account for some of the proposed compliance in the myosin molecule related to its force generating capabilities and be important for the regulation of myosin-2 function.[73]

Crystal structures of unconventional myosins-1, -5, -6 highlight different interaction modes between the myosin heavy chain and the associated light chain which is usually CaM.[35,68,69,74] In most CaM:IQ interactions, CaM adopts a canonical

conformation in which the C-lobe is in the semi-open conformation and the N-lobe remains closed and interacts weakly with a hydrophobic residue in the 8 position of the IQ motif.

A myosin-1B-1IQ structure demonstrates an interaction between CaM and the very N-terminus of the myosin heavy chain that crucially tunes the enzymatic activity and force sensitivity of the holoenzyme.[68] An identical fragment of myosin-1C reveals an interaction between the CaM C-lobe and a portion of the myosin motor domain involved in the communication with the nucleotide binding site. $Ca^{2+}$-binding to CaM may alter this interaction and account for the $Ca^{2+}$-dependence of some transient-kinetic parameters of myosin-1C's ATPase cycle which could have important implications for its role in the adaptation of the auditory system.[76,77] The crystal structure of a myosin-1C fragment containing its 3 IQ motifs and the remainder of the tail reveals an unprecedented mode of CaM:heavy chain interaction. The CaM C-lobe interacts with IQ3 in a semi-open conformation while the N-lobe interacts with a downstream region termed the "post-IQ" motif.[34] This interaction greatly distorts the CaM N-lobe compared to its canonical conformation and results in a strong bending of the structure of the tail at this region. In this sense, the CaM:heavy chain interaction resembles the interaction of the RLC with the IQ2 motif and the hook region of myosins-2.[69] However, the CaM bound to IQ3 of myosin-1C has a uniquely uncoupled EF-hand motif (**Fig. 2B**), raising the possibility that CaM and other $Ca^{2+}$-binding EF-hand proteins may recognize additional targets via uncoupled EF-hands.[34] $Ca^{2+}$:CaM binding to IQ3 and the post-IQ induces a flexibility change in the myosin-1C tail which is predicted to alter the mechanoenzymatic properties of the motor.[34] In contrast, CaM interacts with the first 2 IQ motifs in myosin-1C in the canonical, compact conformation: the N-lobe is closed, the C-lobe semi-open.[34]

For chicken myosin-5A it is still not known which IQ motif binds ELC. A crystal structure of the motor domain and the IQ1 motif in complex with a truncated ELC shows that IQ1 is capable of binding an ELC,[74] however another study showed that IQ1 is also capable of binding CaM.[29] Likewise, a proteolysis study of tissue purified chicken brain myosin-5A indicates that the ELC does not bind to either IQ1 or IQ2.[78] Notably, the chicken myosin-5A-1IQ:ELC and the myosin-5A-1IQ:CaM complex display slightly different kinetic parameters.[29] A subsequent structure of a peptide corresponding to IQ1 and IQ2 of mouse myosin-5A complexed with CaM reveals that IQ1 can bind CaM as well.[79] Structural comparison indicates that ELC and CaM adopt a very similar, semi-open conformation of the C-lobe that allows gripping of the first portion of the IQ1 motif in the absence of $Ca^{2+}$. The N-lobe is closed and interacts weakly with the last portion of the IQ1 motif. Interestingly in the IQ1-IQ2 peptide, the fine structures of the 2 CaM molecules are strongly influenced by the non-conserved residues of the IQ motif. Treatment of myosin-5A with physiological and higher concentrations of $Ca^{2+}$ results in dissociation of at least one CaM per heavy chain. This mechanism has been used by many investigators to insert a fluorescently labeled CaM into the lever arm for single molecule motility studies.[62,80] Several studies provide evidence that the IQ2 motif is most likely to release its CaM in the presence of $Ca^{2+}$.[78,81]

Of peripheral interest, alternate splicing of the myosin-5A tail domain results in melanocyte- and brain-specific isoforms. The brain-specific isoform binds the low molecular weight (8 kDa) dynein light chain (DLC8, DYNLL1) which has also been shown to interact with other proteins, notably dynein.[16] DLC8 is not a CaM gene family member, does not bind to an IQ motif and should not be mistaken for a myosin light chain.[82]

Two studies examined the interaction of IQ motifs from a yeast myosin-5 (Myo2p) with the ELC (Mlcp1). Mlcp1 binds to IQ2 or to a peptide corresponding to IQ2-IQ3 in a compact conformation in which each of the light chain lobes interacts with the heavy chain: The C-lobe interacts with the N-terminal half of the IQ motif, the N-lobe with the C-terminal half of the IQ motif.[35] In contrast, Mlcp1 interacts with IQ4 in an extended conformation where only the C-lobe of the light chain interacts with the IQ motif and the N-lobe is free.[35] These 2 disparate interaction modes of Mlcp1 with 2 different IQ motifs from yeast Myo2p coupled with the compact binding of ELC to IQ1 of myosins-2 demonstrates that the sequence of the IQ motif plays a significant role in determining the light chain binding mode.

Whether or not a light chain adopts a compact or extended conformation may depend on the amino acid presence at position 7 of the consensus IQ motif (**Fig. 1B**). A glycine residue in this position is predicted to favor the compact light chain conformation whereas the presence of a residue with a more bulky side chain such as arginine, lysine or methionine favors the extended conformation.[36] This is consistent with the observation that IQ4 from Myo2p and the IQ2 from all conventional myosins bind Mlcp1 or RLC in an extended conformation, respectively. Based on the crystallographic data, Terrak et al. propose a model for the yeast Myo2p neck domain which binds 2 of the 6 light chains in the extended conformation, and predict from sequence analysis that vertebrate myosin-5A would also have a subset of its light chains bound in the extended conformation.[36] However, a cryo-electron microscopy structure of the myosin-5A lever arm was best fit with crystal structures of light chains bound in the compact conformation to the IQ motifs.[83]

Myosin-6 is unique in that it moves in the opposite direction on F-actin than any other myosin.[84] Direction reversal is mediated by an unconventionally bound CaM to a unique sequence insertion ("insert-2") in between the motor domain and its only IQ1 motif.[75] The unconventional heavy chain:CaM interaction involves 4 hydrophobic residues from the heavy chain spaced at a 1–6–14 amino acid interval. This interaction effectively repositions the lever arm so that the power stroke moves in the opposite direction. The CaM associated with insert-2 tightly binds 4 $Ca^{2+}$ ions.[27] The sole canonical IQ1 motif in the myosin-6 heavy chain binds apo-CaM.[27,75] Recent reports present evidence for an unfolding event of a 3-helix bundle located downstream of IQ1 into a long α-helix which exposes an additional CaM binding site. Association of CaM with this site efficiently stabilizes and extends the myosin-6 lever arm.[75,85]

Taken together, the crystallographic findings suggest that sequence variations in both, the target IQ motif and sequences up- and downstream of it determine light chain selectivity, specificity and binding mode.[35] Whether the light chain binding mode in individual myosins is related to mechanoenzymatic properties remains mostly elusive.

## Involvement of ELC and RLC in the Regulation of Conventional Myosins

Intrinsically regulated myosin-2 holoenzymes are responsive to RLC phosphorylation or $Ca^{2+}$-binding to the ELC.[86] In cells, the level of smooth and nonmuscle myosin-2 RLC phosphorylation is controlled by a complex equilibrium between kinases that phosphorylate the RLC such as myosin light chain kinase (MLCK), rho-associated coiled coil-containing kinase (ROCK), zipper-interacting protein kinase (ZIP), protein kinase C (PKC) and myosin phosphatase which dephosphorylates the RLC. Myosin kinases and phosphatase are downstream effectors of various signal transduction pathways which tightly regulate RLC phosphorylation levels.[87,88]

Phosphorylation of the RLC at S19 is a major regulatory mechanism of vertebrate smooth and nonmuscle myosins-2. It serves as an on/off-switch of the actin-activated ATPase activity of the holoenzyme and regulates filament assembly. Myosin-2 is enzymatically inactive in the absence of RLC phosphorylation.[89,90] In the presence of ATP, it assumes a conformation in which the 2 heads make an asymmetric interaction and the tail folds back upon itself in 2 places to form a compact conformation that inhibits myosin-2 filament formation (**Fig. 3**).[91,92] RLC S19 phosphorylation is associated with the adoption of an extended conformation and an increase in the actin-activated ATPase activity of the holoenzyme, the ability to translocate F-actin in the *in vitro* motility assay and the formation of filaments, as reviewed previously.[87,93] The significance of the regulation of filament assembly is clear in motile nonmuscle cells or in dividing cells in which the cytoskeleton must be continually remodeled. In contrast, it is controversial whether filament assembly is regulated by RLC phosphorylation in smooth muscle tissue.[94]

Di-phosphorylation of the RLC at T18 and S19 further increases the actin-activated ATPase activity via an effect on the F-actin affinity and results in increased filament formation of the myosin-2 holoenzyme when compared to S19 mono-phosphorylation of the RLC.[95-97]

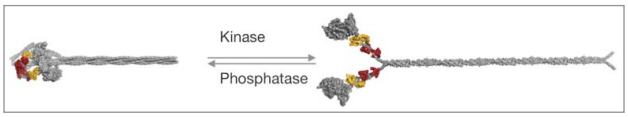


**Figure 3.** Structural consequences of RLC phosphorylation on the conformation of smooth and nonmuscle myosins-2. RLC phosphorylation at S19 promotes a conformational change from a compact (left) to an extended conformation of the myosin holoenzyme (left), which readily assembles into higher order bipolar filaments (not shown). The structural transition from the compact to the expended conformation is accompanied with an increase in the mechanoenzymatic activity. RLC dephosphorylation by myosin phosphatase reverses the conformational change. Figure adapted from Heissler and Manstein.[87]

The first 24 amino acids of the N-terminus of the RLC have been termed the phosphorylation domain (PD). The PD is rich in positively charged amino acids[98] and modeling studies assume that its structure is either fully α-helical or consists of 2 α-helices.[98,99] Molecular interactions between the PD, the heavy chain and ELC required to adopt the off-state of the myosin holoenzyme are not well characterized, in part because the PD is not resolved in any crystal structure. Smooth muscle myosin-2 that binds a truncated RLC that lacks the first 16 amino acids of the PD adopts the off-state, suggesting that the N-terminal portion of the PD is required for the activation of the myosin holoenzyme.[100] In line with this finding, several positively charged residues, in particular K11 and K12, have been shown to be important for the activation of smooth muscle myosin-2s ATPase activity following S19 phosphorylation.[100,101]

Time resolved FRET studies suggest that the PD exists in 2 states – "open" or "closed" – characterized by the positions of probes placed in the N-terminal PD and the C-lobe of the RLC.[98] These studies demonstrate that the probe separation distance increased by 2 nm upon RLC phosphorylation. Molecular dynamic simulations of RLC phosphorylated smooth muscle myosin-2 predict the formation of a salt bridge between the phosphoryl group of S19 and R16 in the RLC that may stabilize the open, active conformation of the PD.[98,99] However, mutation of R16 to a neutral or acidic amino acid still allowed RLC phosphorylated smooth muscle myosin-2 to be activated.[100]

A recent study demonstrates a prominent role of the ELC in the regulation the mechanoenzymatic properties of smooth muscle myosin-2. Charge reversal mutations E10K and E13R of the ELC have an inhibitory effect on the actin-activated ATPase activity and motile behavior of RLC phosphorylated smooth muscle myosin-2.[99] This study suggests that interactions between this region of the ELC and the positively charged residues of the RLC PD are essential for stabilization of the active myosin-2 conformation. Another study pointed out the importance of the interaction between the 2 light chains as well as the heavy chain for regulation.[102]

The RLC of vertebrate smooth and nonmuscle myosins-2 is also a substrate for PKC which phosphorylates S1 or S2 *in vivo*. *In vitro*, PKC phosphorylates these 2 residues in addition to T9, though T9 phosphorylation of the RLC is not observed *in vivo*.[103,104] *In vitro* studies show that PKC phosphorylation of the RLC is inhibitory toward myosin-2 activation in 2 ways. First, myosin-2 RLC phosphorylated at the PKC sites as well as at S19 has the same maximal actin-activated ATPase activity, but requires more F-actin for half maximal activity. Second, myosin-2 pre-phosphorylated at the PKC sites is a poorer substrate for MLCK which might result in an overall decrease in RLC S19 phosphorylation in cells – under the assumption that phosphatase activity is unaffected. Notably, most *in vitro* studies were carried out on myosin-2 with the RLC phosphorylated at both the S1/S2 site and the T9 site. How T9 phosphorylation affects these kinetic parameters is not fully known, although one study suggests that T9 and not S1/S2 phosphorylation is inhibitory toward RLC S19

phosphorylation MLCK.[105] *In vivo*, S1/S2/T9 phosphorylation does not seem to be a substantial regulatory mechanism of nonmuscle myosin-2 function in HeLa cells and human primary keratinocytes, though a recent paper links the ability of fibroblasts to chemotact to phosphorylation at the PKC sites.[106,107]

The RLCs of vertebrate skeletal and cardiac muscle myosins-2 are also phosphorylated at their N-termini by MLCK, but phosphorylation is only modulatory with respect to the enzymatic and motile activities of the holoenzymes *in vitro* and their mechanical properties in muscle.[108-110] Vertebrate skeletal muscle myosin-2 RLC phosphorylation increases the post-tetanic twitch potentiation, increases $Ca^{2+}$-sensitivity of contraction and enhances the transition from a nonforce to a force-producing state.[110-112] However, RLC phosphorylation does not alter the maximum velocity of shortening or maximal isometric force.[113] Electron microscopy studies show that the heads of unphosphorylated skeletal muscle myosin-2 thick filaments are highly ordered with the heads held close to the filament backbone. Following RLC phosphorylation, the heads release from the thick filament backbone.[114] Vertebrate cardiac myosin-2 RLC phosphorylation is modulatory and associated with a 3-fold increase in force production and a 7-fold increase in peak power output.[115] RLC phosphorylation is reduced in hypertrophic hearts.[116,117] Interestingly, overexpression of the skeletal muscle MLCK gene in mouse cardiac tissue attenuates the isopreternol-induced hypertrophy. This finding makes small molecules that target the RLC phosphorylation pathways in cardiac tissue attractive for the treatment of hypertrophic cardiomyopathy.[118]

Similarly, the RLC of myosin-2 from the lower eukaryote *Dictyostelium* is also phosphorylated at the N-terminus, but this phosphorylation is only modulatory with the primary regulation of ATPase activity being associated with phosphorylation of residues in the tail domain.[119] The enzymatic activity of myosin-2 from fission yeast *Schizosaccharomyces* is independent from RLC phosphorylation *in vivo* and *in vivo*.[120,121]

RLC phosphorylation in striated muscle myosins-2 from the horseshoe crab *Limulus* as well as *Tarantula* is associated with regulation of the ATPase activity in an on/off manner, similar to the effect of RLC phosphorylation of vertebrate smooth and nonmuscle myosins-2.[122-126] The structure of native unphosphorylated *Tarantula* thick filaments in the off-state demonstrates that the myosin heads are held in close apposition to the thick filament backbone and form the same asymmetric head-head interaction observed in the off-state of smooth and nonmuscle myosin-2.[125]

$Ca^{2+}$ regulates the ATPase activity of molluscan myosin-2.[127] *In vitro*, RLC dissociation from scallop muscle myosin-2 is associated with a loss of $Ca^{2+}$-dependent regulation and $Ca^{2+}$-binding. However, the ATPase activity of the holoenzyme is elevated even in the absence of $Ca^{2+}$, indicating that the RLC is necessary for the off-state.[128] The crystal structure of the scallop myosin-2 "regulatory domain," containing both IQ motifs and light chains, revealed a surprise as $Ca^{2+}$ does not bind to the RLC, but rather to the first EF-hand of the ELC.[129] This degenerated EF-hand is not predicted to bind $Ca^{2+}$ on its own based on the lack of appropriate numbers of coordinating ligands. However, when the ELC is bound to the myosin heavy chain, the conformation of its EF-hand is altered via a direct interaction between F20 and R24 of the ELC with G117 of the RLC creating a functional $Ca^{2+}$-binding site on the ELC by rotating a carbonyl oxygen in the first EF-hand into a $Ca^{2+}$-coordinating position.[69,129] The importance of this interaction is confirmed by mutagenesis of G117 of the RLC which abolishes $Ca^{2+}$-binding at this site.[130]

Crystal structures of the scallop myosin-2 regulatory domain provide valuable mechanistic insights into the interaction between $Ca^{2+}$ and the ELC and the function of the $Ca^{2+}$:ELC interaction in the activation of the myosin holoenzyme. Further, the structures allow for speculations about similarities in the activation mechanisms of myosins-2 that are intrinsically regulated by $Ca^{2+}$-binding or RLC phosphorylation.[131] It is likely that profound structural similarities between the regulatory domains of scallop and smooth muscle myosins-2 exist since it was shown that the ATPase activity of a chimeric myosin containing the scallop myosin-2 heavy chain and ELC with a vertebrate smooth muscle RLC could be activated either by $Ca^{2+}$ binding or by phosphorylation of the RLC.[132] In both smooth muscle and scallop myosin-2 RLCs, several C-terminal residues, in particular K149 in scallop and K163 in smooth muscle, are required for the intrinsic regulation.[133-135] A lysine at equivalent positions is exclusively present in RLCs from intrinsically regulated myosins-2, but is not found in RLCs from myosins-2 that are not regulated by RLC phosphorylation such as the skeletal muscle myosin-2 RLCs. K149 forms a hydrogen bond with T83 in the $Ca^{2+}$-bound scallop myosin-2 regulatory domain structure which, when broken in the $Ca^{2+}$-free state, may help reduce the rigidity of the regulatory domain. It is also observed that fewer light chain:heavy chain interactions are present in the $Ca^{2+}$-free state.[131] The more flexible regulatory domain may be more conducive to bending in order to adopt the off-state. In this regard, it is of note that the lever arm of solely one of the 2 heads of smooth muscle myosin-2 in the off-state shows a strong bend between the ELC and the RLC. This bend is not found in the other head or in the structure of the phosphorylated state.[136] Such bending may be necessary for the adoption of the head-head interaction observed in the myosin-2 off-state (**Fig. 3**).[91]

Interestingly, the conformation of the off-states of vertebrate smooth and nonmuscle myosins-2, which are regulated by RLC phosphorylation, and the $Ca^{2+}$-regulated molluscan myosins-2 are similar and involve the asymmetric intramolecular interaction between the 2 myosin heads and folding of the long tail domain into thirds.[137] Similarly, the asymmetric head-head interactions in the off-state is preserved in vertebrate smooth and nonmuscle myosins-2 as well as myosins-2 from molluscs, *Tarantula* and *Limulus*. This suggests that this conformational change is an ancient mechanism for regulating conventional myosins.

## Nonmuscle Myosin-2 Heavy Chain Specificity and Tissue Distribution of ELC and RLC

The presence of 3 nonmuscle myosin-2 heavy chain genes (*MYH9, MYH10, MYH14* – out of which *MYH10* and *MYH14* can be alternatively spliced at multiple loci) and 5 nonmuscle

myosin-2 light chain genes in mammals create the potential for dozens of nonmuscle myosin heavy-2 chain:light chain combinations. The physiological subunit composition of the nonmuscle myosin-2 holoenzyme in most tissues and subcellular compartments remains elusive because of the lack of information on expression profiles of the myosin heavy chain and light chain subunits at the protein level.

All nonmuscle myosin-2 heavy chain genes have a vast distribution which is tissue-specific and regulated in a developmentally dependent manner.[138,139] RT-PCR and northern blot analysis in mice indicates the same for the 3 nonmuscle myosin-2 RLCs: *Myl12a* and *Myl12b* are abundantly expressed in most tissues but are absent in the brain (*Myl12a*) and striated muscle (*Myl12b*).[139] *Myl9* is widely expressed with the highest transcription rates in smooth muscle and bladder tissue. *Myl12a, Myl12b* and *Myl9* can interact with all conventional myosin-2s at the protein level but have a strong preference for the nonmuscle myosin-2 heavy chain.[139] This is seen for example in immunofluorescence studies on cardiomyocytes where all nonmuscle myosin-2 RLCs colocalize to F-actin stress fibers and the z-lines but do not colocalize with the α- and β-cardiac myosin heavy chains.[139] Immunoprecipitation studies indicate that all RLCs form a complex with nonmuscle myosins-2A and -2B in various cell types.[139] Similar results were obtained from affinity-purification mass spectrometry assays of human cell lines, indicating the heteromeric interaction between nonmuscle myosins-2A and -2B with the closely related myosin-18A and the association of the aforementioned myosins with both RLCs, MYL12A and MYL12B.[140] Nonmuscle myosins-2A and -2B interact with both ELC isoforms (MYL6 and MYL6B), as does myosin-18A. In contrast, nonmuscle myosin-2C, smooth muscle myosin-2 and unconventional myosin-1C interact exclusively with the ELC isoform MYL6.[140] Those data imply complementary and combinatorial expression patterns for the nonmuscle myosin-2 heavy and light chains. The physiological significance of the variations in light chain subunits of the nonmuscle myosin-2 heavy chain remains largely elusive.

## CaM as Subunit of Unconventional Myosin Holoenzymes

Many unconventional myosins bind CaM as light chain(s). The binding affinity between the myosin heavy chain IQ motif and CaM can be either strengthened or weakened by the presence of $Ca^{2+}$ and, in the extreme, result in the dissociation of the complex. *In vitro*, this may regulate myosin's mechanical properties since it could affect the stiffness of the lever arm. As a caveat, it is not clear yet whether the $Ca^{2+}$-induced dissociation of CaM from the myosin heavy chain is a significant regulatory mechanism for the mechanoenzymatic properties of the holoenzyme *in vivo*.

$Ca^{2+}$-regulation has been most critically studied in mammalian myosin-5A, a motor that moves processively along actin filaments. Myosin-5A has 6 IQ motifs in its neck which allows it to take 36 nm steps along F-actin.[141] In the absence of $Ca^{2+}$, myosin adopts a folded off-state in which the 2 motor domains contact the 2 lobes of the globular tail domain.[83,142] This conformation of myosin-5A has a low affinity for F-actin and exhibits a low ATPase activity.[143] In the presence of $Ca^{2+}$, one or more CaMs dissociate and the myosin extends, freeing the motor domains to interact with F-actin and potently activates the ATPase activity.[144-146] As a consequence of the $Ca^{2+}$-mediated CaM-dissociation from the myosin-5A heavy chain, the molecule is mechanically incompetent since the unoccupied IQ motif does not allow for a force producing power stroke likely due to a "floppy" heavy chain segment.[81,147] Thus, it is unlikely that $Ca^{2+}$ is a direct cellular activator of myosin-5A activity. Instead myosin-5A has been shown to be activated by the cargo adaptor protein melanophilin, which links myosin-5A to Rab27a and to melanosomes in melanocytes.[148,149,150] Several studies have shown that the CaM bound to IQ2 of chicken myosin-5A most easily dissociates in the presence of $Ca^{2+}$.[78,151] Those studies support the hypothesis that the affinity of $Ca^{2+}$-CaM for an IQ motif is dependent on the neighboring IQ motif as bridging may occur, where the 2 CaM domains interact with 2 adjacent IQ motifs.[152,153]

$Ca^{2+}$-regulation has also been observed for some vertebrate class-1 myosins. The neck region of myosin-1B is subject to alternative splicing events which result in isoforms with 4 to 6 IQ motifs. Laakso et al. added evidence to the concept that the myosin-1B light chain binding region is indeed a lever arm, by showing that the power stroke size measured by optical trapping increased with the lever arm length.[154] Biochemical studies show that myosin-1B splice variants have different kinetic, motile and force-sensing properties. The actin-activated ATPase activity of myosin-1B is activated several fold by $Ca^{2+}$ whereas some transient kinetic parameters are reduced due to an effect on the IQ1:CaM interaction.[155-158] The interaction of myosin-1B with F-actin is very force dependent and its interaction time increases 50-fold when the working stroke is opposed by forces in the pN range.[154,159] $Ca^{2+}$ dramatically shortens the force-dependent attachement lifetimes of myosin-1B.[158] The affinity of CaM for the myosin-1B IQ motifs varies significantly from 0.2 μM to 5 μM. This suggests that a subset of its IQ motifs might not be associated with CaM under physiological conditions and might be free or bind to other light chains or proteins.[28,154]

In line with this finding, Cyr et al. show that CaM interacts very weakly with the IQ4 of unconventional myosin-1C, suggesting that only a fraction of the molecules is associated with CaM *in vivo*.[160] In contrast, IQ1, IQ2 and 1Q3 have stronger CaM affinities.[160] At a low intracellular $Ca^{2+}$ concentration, IQ1 and IQ3 are fully saturated with CaM whereas CaM dissociates from IQ2. The unoccupied IQ2 and potentially CaM-bound IQ1 and IQ3 form a binding site in the myosin-1C heavy chain which allows the interaction with stereociliary receptors.[161] A drastic increase in $Ca^{2+}$ results in a loss of all CaM molecules and abolishes the myosin-1C:receptor interaction which was initially suggested as a mechanism for the adaptation of hair-cell mechanoelectrical transduction important for hearing.[161] However, subsequent work shows that the rate of CaM dissociation from myosin-1C is too slow to account for the adaptation rate in

stereocelia.[162] While $Ca^{2+}$ has only modest effects on the steady-state actin-activated ATPase activity of myosin-1C, transient kinetic analysis shows that it dramatically decreases the rate of ATP hydrolysis and increases the rate of ADP dissociation from actomyosin.[77] $Ca^{2+}$ also increases the size of the working stroke of myosin-1C perhaps by stiffening or effectively increasing the length of the lever arm.[163] It should be noted that $Ca^{2+}$ has no effect on the working stroke of myosin-1B or myosin-5.[147,158]

The myosin-9B lever arm contains 4 to 6 IQ motifs that bind CaM when purified from human leukocytes or recombinantly overproduced in *Sf*9 insect cells.[24,164] Myosin-9B contains an additional CaM binding site that resides in an actin-binding surface loop in the motor domain that is highly conserved and unique to class-9 myosins.[164] The potential regulatory effect of $Ca^{2+}$ on the enzymatic activity of myosin-9B is difficult to decipher since a myosin-9B-4IQ construct has distinct mechanoenzymatic properties from a motor domain construct that lacks the IQ motifs with respect to the steady-state ATPase activity and *in vitro* motile behavior.[164] Interestingly, a point mutation in the third IQ motif of human unconventional myosin-9B predisposes to inflammatory bowel disease and might affect the interaction with CaM.[165] Similar, a loss of the myosin heavy chain:CaM interaction or impaired CaM binding due to a missense mutation in the fifth IQ motif of unconventional myosin-7A represents a pathomechanism for genetic hearing loss.[166]

Apart from regulatory functions on the myosin holoenzyme, CaM also determines the oligomeric state of myosin-21 from the parasite *Leishmania*. CaM-binding to its IQ motif prevents dimerization of 2 myosin-21 heavy chains via a coiled-coil forming sequence. *Vice versa*, myosin-21 dimerizes in the absence of CaM. Notably, monomeric myosin-21 is mechanoenzymatically active, the dimer inactive.[167]

## Unconventional Myosins as Targets of ELC and RLC

Recent biochemical reports on unconventional myosin heavy chains recombinantly overproduced along with various light chains in the baculovirus/*Sf*9 expression system, immunoprecipitation and proteomic studies strongly contradict the dogma of the myosin-2 heavy chain specificity of ELC and RLC. ELC and/or RLC may be subunits of the unconventional myosin holoenzyme of classes-1, -5, -6, -7, -14, -15, -18, and -19 (**Tables 2 and 3**).[16,27,140,168-171] The interaction signatures can be identical or distinct from those reported for conventional myosin-2s: ELC and RLC can bind together, independently from each other or in conjunction with CaM and potentially other light chains in various sequential orders to the IQ motifs in the unconventional myosin heavy chain.

As described above, tissue purified myosin-5A from chicken brain is associated with CaM and ELC. *In vitro* studies with recombinant chicken myosin-5A-IQ1 fragments show that it can bind the ELCs MYL6, MYL6B or CaM, but not RLC.[29] *In vitro* studies show no differences in kinetic parameters under steady-state conditions in the presence of different ELCs. The presence of CaM has subtle effects on the ATPase activity.[29] Pre-steady-state kinetics indicate that most parameters are independent from the light chain bound to the IQ motifs, making it unlikely that the light chain isoform determines the functional properties of myosin-5A under physiological conditions as long as the IQ motifs are saturated.[29] Notably, mouse myosin-5A fragments do not appreciably bind ELC when overproduced in *Sf*9 insect cells and native myosin-5A purified from mouse brain does not bind ELC but copurifies with CaM.[17] In contrast, *Drosophila* myosin-5 copurifies with ELC and CaM when recombinantly overproduced with the 2 light chains, however the binding order is unexplored.[172] Those findings suggest species-(and tissue)-specific associations of a myosin heavy chain and its light chain subunits, as described above for nonmuscle myosins-2.

The dimeric pseudoenzyme myosin-18A, a close relative of conventional nonmuscle myosins-2, forms a complex with both ELC and RLC when coproduced in the baculovirus/*Sf*9 system.[171] Myosin-18A also co-immunoprecipitates with RLC from COS7 cells.[25] The binding sites for the ELC and RLC on myosin-18A are believed to occur in identical ("conventional") order to those on the myosin-2 heavy chain with the ELC binding to the IQ1 and RLC to IQ2, based on sequence analysis of the 2 IQ motifs.[171] Similar, recombinant overproduction of *Toxoplasma* myosin-A with RLC (TgMLC1) and ELC (TgELC) reveals the formation of a ternary complex. Sequence analysis of the IQ motifs suggests that ELC and RLC bind in conventional order to the myosin heavy chain.[169]

Recombinant myosin-15A also binds both ELC and RLC. However, the binding order, as determined by expression of differential length fragments, is reversed when compared to myosins-2: RLC binds to IQ1, ELC binds to IQ2.[168]

Porcine myosin-6–1IQ copurifies in complex with 2 CaMs from *Sf*9 insect cells when it is solely overproduced with CaM: One CaM binds to the unique insert-2, the second CaM to the adjacent IQ1 motif.[27] Myosin-6–1IQ forms a ternary complex with CaM and ELC when coproduced along with both light chains: CaM binds to the unique insert-2, ELC to the IQ1 motif.[27] Interestingly, when only the myosin heavy chain is overproduced in the *Sf*9 cells, myosin-6–1IQ copurifies with endogenous insect cell CaM,[27] supporting the hypothesis that the association of a light chain with the IQ motif is driven by affinity and mass action.

The unconventional myosin-19 heavy chain associates with both CaM and RLC subunits.[173,174] Interestingly, the myosin-19:RLC holoenzyme binds MYL9 and MYL12B in a 2:1 ratio to its 3 IQ motifs, suggesting differential affinities which determine light chain selectivity and specificity of the IQ motif.[174] Independent of subunit composition, both myosin-19 holoenzymes are functional and only display subtle enzymatic differences suggesting that light chain heterogeneity does not automatically imply changes in mechanoenzymatic properties.[173,174]

The RLCs bound to unconventional myosins are in some cases phosphorylatable at the T18/S19 activation sites by MLCK, but to date no effect of this phosphorylation event on the enzymatic activity of the myosin holoenzyme has been demonstrated.[171,174] This suggests that phosphorylation is – so far –

an exclusive regulatory mechanism for the aforementioned conventional myosins. Moreover, the presence of $Ca^{2+}$ does not influence the mechanoenzymatic properties of characterized myosins from classes-14 and -18 which bind ELC and RLC in "conventional" order.[169,171]

## Non-Myosin Binding Partners of ELC and RLC

An increasing number of non-myosin binding partners of ELC and RLC have been discovered in the past years. The interaction partners do not share common features or obvious similarities between IQ motifs. ELC and RLC non-myosin binding partners are involved in cellular functions such as translation, signaling, cell division and the regulation of the actomyosin cytoskeleton; their localizations are cytosolic or membrane-bound. A compilation of selected binding partners can be found in **Table 3**. It remains mostly elusive if the interaction between a non-myosin binding partner occurs between a heavy chain associated or isolated light chain. A pool of free RLC has been demonstrated in cell biological experiments showing that T18/S19 di-phosphorylated RLC localizes independently from S19 mono-phosphorylated RLC and the nonmuscle myosin-2 heavy chain to the midzone during cytokinesis.[175] A pool of free light chains or light chains bound to non-myosin binding partners is also proposed after extensive immunoprecipitation studies of the nonmuscle myosin-2 heavy chain in *Drosophila*.[170] The NMDA-type glutamate receptor for example favorably binds free RLC with nanomolar affinity, but does not bind the nonmuscle myosin-2 heavy chain associated RLC. This suggests that the NMDA:RLC interaction is not a mechanism for localization of nonmuscle myosin-2. RLC phosphorylation disrupts the interaction with NMDA-type glutamate receptor. Disruption of the NMDA-type glutamate receptor:RLC interaction results in a delay in delivery of the receptors to the cell membrane, indicating trafficking functions.[176]

If non-myosin binding partners interact with the myosin heavy chain bound light chain, then 2 different scenarios are plausible: Transient ternary complexes can be formed with the conventional or unconventional myosin holoenzyme, similar to the interaction of the nonmuscle myosin-2 holoenzyme with kinases and phosphatases. An example for such a ternary complex is the CaM-mediated interaction between the G-protein RalA and the unconventional myosin-1C heavy chain during the insulin-stimulated glucose uptake.[37]

As discussed above, light chains can bind in an extended conformation to the IQ motif which could allow the free N-lobe to simultaneously interact with another protein, thereby forming a ternary complex. A CaM-mediated dimerization has for example been shown for the $Ca^{2+}$-activated $K^+$ channel.[177] In the case of myosin light chains, such an interaction would result either in a ternary complex formed by a myosin holoenzyme and a non-myosin binding partners or potentially the exclusive light chain-mediated dimerization of 2 myosins or 2 non-myosin binding partners (**Fig. 4**). An example for the former is the potential RLC mediated ternary complex between the nonmuscle myosin-2 heavy chain and the bile salt export protein BSEP that is required for BSEP trafficking and delivery to the apical membrane.[178] Also, a ternary complex of yeast ELC with the myosin-2 heavy chain and another, so far unknown binding partner, is suggested.[179,180]

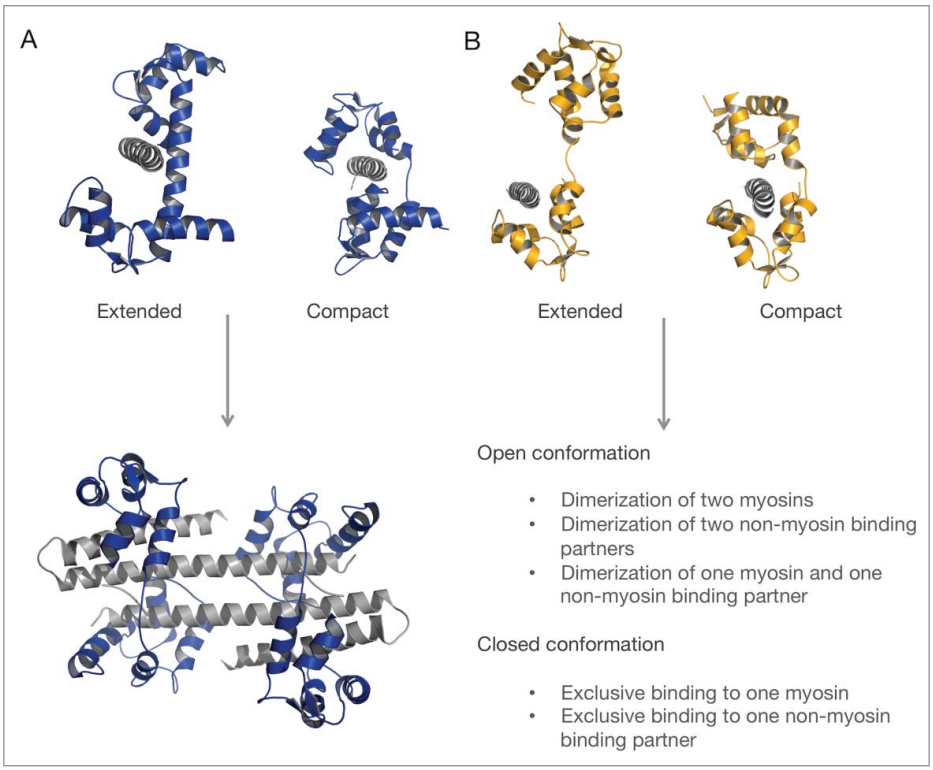


**Figure 4.** Consequences of different binding modes of myosin light chains. (**A**) Schematic representation of the open and closed conformation of CaM (blue) bound to an exclusive target IQ-motif (gray). CaM-dependent dimerization of 2 domains of the $Ca^{2+}$-activated $K^+$ channel (gray), each bound to one lobe of CaM (PDB ID: 1G4Y). (**B**) Extended and compact light chain conformation exemplarily shown for the ELC (orange) on the IQ motifs (gray) of the yeast myosin-5 (PDB IDs: 1M45, 1M46). Potential functional consequences of the extended and compact light chain conformation are valid for ELC, RLC and CaM.

## Other Myosin Light Chains and Potential Light Chains

Besides ELC, RLC and CaM, other EF-hand proteins are described as potential myosin light chains. As demonstrated by Kollmar, numerous CaM-

related $Ca^{2+}$-binding proteins from the centrin/caltractin, troponin C, frequinin/hippocalcin, and calcineurin families and other unconventional proteins might potentially function as myosin light chains.[181] Experimentally verified examples include the lower eukaryotic light chains MIMLC, MlcB, MlcC and MlcD that associate with the IQ motifs of *Acanthamoeba* myosin-1C and *Dictyostelium* myosins-1B, -1C and -1D.[182-184] *Dictyostelium* MlcD is a CaM-like protein with a molecular weight of 16.5 kDa. MlcD has 4 EF-hands, though all of them have lost high affinity $Ca^{2+}$-binding sites.[182] In contrast, MlcB and MlcC are low molecular (8.3–8.6 kDa), single-lobe light chains.[185,186] MlcC EF-hands have lost the ability to bind $Ca^{2+}$.[186] In contrast, MlcB can bind $Ca^{2+}$ and undergoes a $Ca^{2+}$-dependent conformational change, however its tight, submicromolar affinity for the myosin-1B IQ motif is $Ca^{2+}$-independent suggesting that structural plasticity is not critical for the MlcB:IQ interaction.[184,185] A recent NMR-structure of the globular, 4-helix bundle MlcB indicates a significantly different IQ motif recognition mode when compared to bi-lobed light chains that involves extensive hydrophobic and electrostatic interactions.[185] The structure of single-lobed MlcB is most reminiscent to the closed conformation of the CaM C-lobe under $Ca^{2+}$-free conditions.[185]

Androcam is a CaM-like protein expressed in limited tissues in *Drosophila* which associates with the myosin-6 heavy chain in testes as determined by immunolocalization, co-immunoprecipitation, yeast-2-hybrid and peptide studies.[187] The myosin-6: androcam complex localizes to F-actin cones and does not co-localize with CaM by immunofluorescence microscopy, strongly suggesting that androcam is the physiological light chain for myosin-6 in fly testes. Recent studies demonstrate that androcam has an unusual structure: Its N-lobe noncanonically binds a single $Ca^{2+}$ weakly through a site created from both EF-hands.[188] The site remains in the closed position regardless of whether $Ca^{2+}$ is present. The C-lobe binds 2 $Ca^{2+}$ with high affinity and may be saturated with $Ca^{2+}$ over the entire physiological concentration range. Androcam binds to peptides from both, the insert-2 region and the canonical IQ motif of the fly myosin-6 heavy chain. Binding of androcam to the insert-2 peptide occurs only via the C-lobe and thus differs from the binding mode of CaM to the insert-2 region in the mammalian myosin-6 heavy chain.[188] Notably, peptides of the insert-2 region and the canonical IQ motif also bind *Drosophila* CaM, which is probably the physiological myosin-6 light chain in most other tissues.[188]

The native light chain composition of unconventional myosin-10 is unknown, though recombinant heavy chain interacts with CaM.[189,190] CaM-like protein (CLP) is expressed in epithelial tissues where it associates with the 3 IQ motifs of myosin-10.[191] The binding of CLP to myosin-10 was first shown by CLP overlays of cell extracts and subsequent yeast-2-hybrid studies and confirmed by co-immunoprecipitation of overproduced proteins in mammalian cells and later by direct binding studies using bacterially produced IQ motif fragments. Direct binding studies with IQ1-IQ2 and IQ1-IQ2-IQ3 peptides suggest that IQ3 preferentially associates with CLP in the presence of $Ca^{2+}$, though subsequent studies using the individual IQ motifs do not confirm the CLP specificity for IQ3 in the presence of $Ca^{2+}$, but do confirm the strong effect of $Ca^{2+}$ on the CLP:IQ interaction.[192]

As mentioned above, apicomplexan-specific class-14 myosins interact with their light chains via strongly degenerated IQ motifs. The association between myosin heavy and light chains is critically dependent for parasite motility and invasion.[26] *T. gondii* has a repertoire of 7 putative myosin light chains (TgMLC1–7) which all contain 4 degenerated EF-hand domains.[38] Six out of the 7 light chains contain a long N-terminal extension when compared to CaM which in case of TgMLC3 is predicted to form a coiled-coil forming sequence.[38]

*Toxoplasma* myosin-A copurifies with TgMLC1 from parasites. The heavy chain:light chain interaction is unique in that TgMLC1 anchors the myosin holoenzyme to the plasma membrane.[26] Similarly, the N-terminal extension of TgMLC2 is required for the localization of the myosin-D holoenzyme to the parasite pellicle and anchoring to the plasma membrane, thereby substituting for the function of the lacking tail domain to determine the localization of the myosin-D holoenzyme.[38] The association between TgMLC2 and the plasma membrane is predicted to be mediated by putative palmitoylation sites in the N-terminal domain of the light chain, whereas the C-terminal CaM-like domain of TgMLC2 is sufficient for the interaction with the myosin-D heavy chain. The small molecule tachypleginA and its analogs covalently bind to a cysteine residue (C58) in the N-terminus of TgMLC1. The covalent light chain modification inhibits the enzymatic activity of the holoenzyme, parasite motility and invasion and might be a first step toward the design of antiparasitic drugs.[193-195]

In *Plasmodium* species, light chain MLC1 is also called myosin-A tail-interacting protein (MTIP). MTIP localizes the heavy chain in complex with other proteins of the host cell invasion machinery to the inner membrane complex. *P. knowlesi* MTIP interacts with the myosin-A tail of *P. yoelli* in an extended conformation in which the C-lobe makes extensive hydrophobic contacts with the tail domain.[196] *P. falciparum* MTIP interacts in a compact conformation with the *P. yoelli* tail sequence.[197] The C-lobe still establishes hydrophobic interactions with the tail domain but the central helix is kinked, which allows the N-lobe to interact with the myosin-A tail helix. The compact conformation of MTIP is reminiscent to the structure of the ELC bound to scallop myosin-2.[69] Intriguingly, lysine K813 at the seventh position of the myosin-A IQ motif is crucially involved in the formation of the compact conformation.[197] This is counter to the results from studies on the *P. knowlesi* myosin-A and yeast myosin-5 which predict that a lysine at this position would interfere with binding of the N-lobe to the IQ motif, thereby preventing the formation of a compact conformation, as discussed above.[36,196] This exception to the rule indicates impressively the structural plasticity of the light chain:heavy chain interaction which – in the case of *P. falciparum* – is crucially involved in complex formation, as suggested by yeast-2-hybrid studies.[197]

*Plasmodium* myosin-B localization and function differs substantially from myosin-A. Myosin-B does not associate with MTIP but binds the 78.7 kDa MLC-B, the largest myosin light chain identified in any species.[198] MLC-B harbors a CaM-like

C-terminus and an extended α-helical N-terminus which is predicted to form a dimeric or trimeric coiled-coil.[198] Those findings support the idea that class-14 myosins compensate for the lack of a tail domain by a light chain that localizes the holoenzyme to its intracellular destination. Further, the extended N-terminus of MLC-B and its ability to form a coiled-coiled hypothetically results in a light chain mediated dimerization or oligomerization of 2 or more monomeric myosin-B heavy chains.

CaM competes with the CaM-like proteins calcium-binding protein 1 (CaBP1) and the calcium- and integrin-binding-protein-1 (CIB1) for the IQ motifs of mammalian myosin-1C.[199] In myosin-5A, the t-SNARE syntaxin-1A competes with CaM for the first IQ-motif at high $Ca^{2+}$, an interaction required for tethering the motor complex to the plasma membrane during the early stages of exocytosis.[200] The effects of light chain substitutions on myosin's mechanochemical activity are not yet elucidated.

## Caveats for the Use of Fluorescent Protein (FP) Tagged RLC and Phosphomimetics in Cell Biological Studies

Because of the supposed exclusive association to conventional myosins, FP fusion to the N- or C-terminus of RLC and the use of RLC phosphomimetics is a popular tool to study nonmuscle myosin-2 localization, function and regulation in live cells.[106,201-207] As reviewed recently, there are a few caveats associated with it.[208] The first major caveat to this approach – besides the widely accepted FP-driven mislocalization and aggregation of its fusion protein – are the above mentioned numerous unconventional myosin and non-myosin binding partners of ELC and RLC (**Tables 2 and 3**). Further, FP-labeled light chains will bind to the 3 nonmuscle myosin-2 isoforms and their splice variants in virtually all mammalian cell types and tissues which excludes a strict distinction of any functional diversity that may exists among these myosins.

The second major caveat is that FP-RLC adversely affects nonmuscle myosin-2 regulation and enzymology. A recombinant nonmuscle myosin-2 with a GFP fused to the RLC N-terminus is regulated by light chain phosphorylation, however, the maximum catalytic activity of the holoenzyme under steady-state conditions is about half of the control and the *in vitro* motility is reduced by a smaller amount.[209] These effects are relatively small in terms of absolute rates, but are large compared to the effect of disease-associated point mutations in cardiac myosin-2 where the effects of potentially fatal mutations are subtle when probed enzymatically.[210,211] Therefore, it is possible that even the small effects seen for nonmuscle myosin-2 might be adversely affecting its performance in cells in ways that might be difficult to interpret. The *in vitro* phosphorylation rate of GFP-RLC bound to the nonmuscle myosin-2A heavy chain by MLCK is reduced, which suggests a lower overall phosphorylation level inside cells with unpredictable consequences for cellular homeostasis.[209]

The third caveat is perhaps the most serious. To mimic the constitutively active, phosphorylated state of nonmuscle myosin-2, RLC T18/S19 are often replaced with aspartate/glutamate residues – despite a considerable chemical difference between the phosphorylated amino acid and the side chain of the surrogate, including size and charge at neutral pH. Strikingly, phosphomimetics do not fully phenocopy the phosphorylated state of the RLC *in vivo*.[58,59] Further, T18/S19 phosphomimetic RLCs interact differently with nonmuscle myosin-2A and -2B. Whereas phosphomimetic RLC for the mono- and di-phosphorylated RLC regulate the assembly and stability of nonmuscle myosin-2B filaments, nonmuscle myosin-2A filaments are not impacted.[212] In *Drosophila*, some phosphomimetic RLC mutants accumulate nonmuscle myosin-2 in aggregates, which reduce in size when the native RLC is present at endogenous levels.[59] Nonmuscle myosin-2 aggregation is also observed in hypomorphic flies that have a 90% reduced RLC level.[213] The cause for nonmuscle myosin-2 aggregation is unknown but it is suggested that the aggregates, that do not contain F-actin, could result from constitutive nonmuscle myosin-2 filament assembly.[59]

*In vitro*, biochemical experiments show that a single replacement of either T18 or S19 with a charged amino acid does not fully activate smooth muscle myosin-2 in the absence of T18 or T19 phosphorylation. RLC T18D/E and S19D/E myosin holoenzymes have less than 10% of the actin-activated ATPase activity of wild type (WT) phosphorylated myosins and the S19E mutant does not move actin filaments in the *in vitro* motility assay in the absence of phosphorylation by MLCK. In contrast, the unphosphorylated S19E myosin exhibits 94% of the filament forming ability of the WT phosphorylated smooth muscle myosin-2 in the presence of ATP, indicating an uncoupling of the regulation of filament formation and enzymatic activation. Even simultaneous replacement of both, RLC S19 and T18, with charged amino acids only results in 16–30% of the ATPase activity and 45–60% of the actin sliding speed of phosphorylated WT myosin. This T18E/S19E double mutant forms filaments nearly as well as WT phosphorylated smooth muscle myosin-2.[96,101] Note that a recent molecular dynamics simulation of the effect of phosphomimetic substitutions of the light chain on its conformation also support the idea that single phosphomimetic amino acid replacement of T18 or S19 does not activate, whereas the double substitution might be partially activating the myosin-2 holoenzyme.[214]

To mimic the dephosphorylated RLC state, alanine is sometimes used as a surrogate for T18/S19. Biochemical studies with smooth muscle-2 indicate that a single alanine replacement at S19 or at T18 does not drastically affect regulation or activity since MLCK will phosphorylate the remaining phosphorylatable residue. These mutant smooth muscle myosins-2 are inactive when unphosphorylated and have WT-like activities when phosphorylated.[96,101]

There are no *in vitro* data to address the question of whether phosphomimetics of the S1/S2 PKC sites actually mimic the phosphorylation.

In summary, none of the biochemical studies supports the hypothesis that RLC phosphomimetics faithfully mimic the phosphorylated or dephosphorylated RLC state and could *bona fide* regulate the activity of the myosin-2 holoenzyme. Further,

RLC mutants that simultaneously mimic the phosphorylated or dephosphorylated state on both, T18 and S19 are completely uncoupled from upstream signaling pathways whereas RLC mutants carrying solely amino acid surrogate at either T18 or S19 are still integrated. This may lead – apart from the predicted misregulation - to myosin-2 mislocalization.

## Summary and Perspectives

Myosin light chains are as diverse as the associated heavy chains. They are more than passive subunits of the myosin holoenzyme and have structural and regulatory functions. Light chains are themselves regulated by cation binding or phosphorylation and allosterically communicate with the myosin heavy chain. Further, light chains can mediate the association of a myosin heavy chain with a binding partner to form a ternary complex or determine the intracellular localization of a myosin heavy chain. Mutations of some myosin light chain genes, alternate splicing events of pre-mRNA and aberrant expression levels are disease causing in humans. As a target for small molecules, myosin light chains are druggable and represent promising targets for example for the design of anti-parasitic drugs.

The historically strict categorization of ELC and RLC as exclusive light chains for conventional myosins and CaM as the light chain for unconventional myosins is obsolete. We are beginning to understand that ELC and RLC – as CaM – can exist as free light chains in the cytosol, bind with nanomolar affinity to the heavy chain of unconventional myosins and non-myosin binding partners in both, a phosphorylation dependent and independent manner. Therefore, the term "myosin holoenzyme" *per se* is needs to be re-defined, given the subunit heterogeneity for both, the heavy and the light chains. A challenge in the future will be to delineate the native heavy and light chain composition of conventional and unconventional myosins, identify how myosin heavy chains select their light chain in a spatiotemporal manner, and research on the physiological significance the heavy:light chain composition in health and disease.

As an editorial comment, we would like to point out that many of the publications, including ones from our own laboratory, have often used myosin heavy and light chain genes from different species when recombinantly overexpressing myosin fragments and holoenzymes in *Sf*9 insect cells. Likewise, the same mix-and-match strategy is frequently used in cell biological experiments where further the species of the host cell can be different. It is very tedious and sometimes impossible to determine the exact origin and isoform of the genes used for *in vitro* and *in vivo* studies. For better comparison of the results in the "post-genomic" era, it would be extremely useful for authors to provide the accession numbers for the myosin heavy and light chain genes to create their myosin holoenzyme of interest in biochemical and cell biological studies.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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Searching only the terms "myosin essential light chain" and "myosin regulatory light chain" on PubMed results in ~1000 hits, which is a great underestimation given the light chain diversity within the myosin superfamily. Many more excellent publications deserve the inclusion in this review but could not be cited due to space limitations.

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