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Four Things to Know about Myosin Light Chains as Reporters for Nonmuscle Myosin-2 Dynamics in Live Cells

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Abstract

The interplay between nonmuscle myosins-2 and filamentous actin results in cytoplasmic contractility which is essential for eukaryotic life. Concomitantly, there is tremendous interest in elucidating the physiological function and temporal localization of nonmuscle myosin-2 in cells. A commonly used method to study the function and localization of nonmuscle myosin-2 is to overexpress a fluorescent protein (FP)-tagged version of the regulatory light chain (RLC) which binds to the myosin-2 heavy chain by mass action. Caveats about this approach include findings from recent studies indicating that the RLC does not bind exclusively to the nonmuscle myosin-2 heavy chain. Rather, it can also associate with the myosin heavy chains of several other classes as well as other targets than myosin. In addition, the presence of the FP moiety may compromise myosin's enzymatic and mechanical performance. This and other factors to be discussed in this commentary raise questions about the possible complications in using FP-RLC as a marker for the dynamic localization and regulatory aspects of nonmuscle myosin-2 motor functions in cell biological experiments.

Introduction

Bipolar filament forming nonmuscle myosin-2 holoenzymes isolated from nonmuscle cells of vertebrates, invertebrates as well as from amoeboid sources are composed of two heavy chains and a set of two light chains. For historical reasons the nonmuscle myosin-2 associated light chains are now collectively referred to as essential light chain (ELC) and regulatory light chain (RLC). In mammals, three genes (*MYH9*, *MYH10*, *MYH14*) encode for three nonmuscle myosin-2 isoforms referred to as -2A, -2B and -2C, respectively. Most cells simultaneously express two or three nonmuscle myosin-2 paralogs and their splice variants in a strictly regulated manner. Each mammalian nonmuscle myosin-2 heavy chain associates with one ELC encoded by one of two genes (*MYL6*, *MYL6B*) and with one RLC encoded by either *MYL9*, *MYL12A* or *MYL12B*. There has not been a systematic examination of the cellular and subcellular localizations of these RLCs at the protein level and so the precise subunit composition of the nonmuscle myosin-2 holoenzyme in cells and tissues is largely unknown.

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The nonmuscle myosins-2 holoenzyme oligomerizes into discrete ~310 nm long bipolar filaments which interact with F-actin in an ATP-dependent manner and drive cytoplasmic contractility [Billington et al., 2013]. There is tremendous interest in the roles nonmuscle myosins-2 play in cytokinesis, cell motility, cell adhesion and maintenance of cell shape. Mutations in several nonmuscle myosin-2 heavy chains are associated with diseases such as squamous cell carcinomas in mice and humans (*MYH9*) [Schramek et al., 2014], platelet disorders and other associated syndromic issues such as glomerulosclerosis and deafness (*MYH9*, *MYH14*) [Ma and Adelstein, 2014b].

Nonmuscle myosin-2 filaments can be visualized as puncta inside cells when the nonmuscle myosin-2 heavy chain is tagged with a fluorescent reporter protein or via immunofluorescence microscopy [Beach et al., 2014; Burnette et al., 2011]. Many endeavors have been made to study the dynamics of the actomyosin cytoskeleton, the discrete localization patterns and physiological functions of the individual nonmuscle myosin-2 isoforms in cells and tissues. Specific antibodies for the nonmuscle myosin-2 light chains and the heavy chains allow the study of the distribution of nonmuscle myosin-2 in fixed cells and tissues. Knock-in animal models allow the expression of FP-tagged myosin heavy chain genes under control of the native promoter and give valuable insights in the dynamics of the actomyosin cytoskeleton and localization patterns of individual nonmuscle myosin-2 motors in health and disease [Ma and Adelstein, 2014b]. Besides knock-in and knock-out animal models, cell biological studies address the question about the dynamics of the actomyosin cytoskeleton with various techniques, including the si/shRNA mediated knock-down of the expression of the individual myosins, the transient expression of FP-tagged nonmuscle myosin-2 heavy chains or combinations of both techniques. Another popular technique that has been widely used is to overexpress cDNA which encodes for a FP-labeled light chain, in most cases a green fluorescent protein (GFP)-labeled RLC in which the tag is either fused to the N- or C-terminus of the RLC. The FP- RLC replaces the endogenous RLC by mass action in order to follow the localization of nonmuscle myosin-2 in live cells [Beach et al., 2011; Komatsu et al., 2000; Peterson et al., 2004].

The advantage of the transient incorporation of the fluorescent light chain into the myosin holoenzyme is the small size of the DNA to be manipulated, the high degree of replacement of endogenous LCs (between 80–95% efficient [Beach et al., 2011]) and the rapid readout of the experiment compared to the time to make transgenic animals or stable cell lines. However, underlying is the explicit assumption that an FP-tagged light chain binds exclusively to nonmuscle myosin-2 isoforms and does not adversely affect myosin's regulation or enzymatic activity.

In this commentary, we would like to point out that there are some serious complications inherent to the approach to utilize FP-RLC as an indirect marker for the localization of nonmuscle myosins-2 and deliver a caution to the cell biology community. First, FP-RLC will likely bind to all three nonmuscle myosin-2 paralogs expressed in a cell which will blur the individual functions and localizations of these myosins. Secondly, RLC does not bind exclusively to nonmuscle myosin-2 paralogs; there are other myosin and non-myosin targets. Thirdly, the GFP-RLC containing nonmuscle myosin-2 holoenzyme may have impaired enzymatic and mechanical properties and regulation of its activity in the cell may

also be impaired. In addition, widely used phosphomimetic versions of the RLC do not appear to mimic the effect of phosphorylation very faithfully.

Caveat 1: RLC Has No Nonmuscle Myosin-2 Heavy Chain Specificity

First, expression of FP-RLC to follow the spatiotemporal localization of nonmuscle myosin-2 blurs the details of the function of individual nonmuscle myosins-2 since FP-RLC will likely bind to all nonmuscle myosin-2 paralogs present in a given cell type. Relative affinities of the FP-RLC for a given nonmuscle myosin-2 heavy chain have not been measured and it is not known whether the myosin holoenzyme is assembled cotranslationally. Another complication is that the above mentioned products of the three RLC genes are expressed in various combinations in different cells and there is little information on the relative association of these with the three nonmuscle myosin-2 heavy chain. These light chains are highly conserved and there is no information in the literature to decide whether they are functionally redundant with respect to their effect on the mechanical properties of nonmuscle myosin-2 paralogs.

Animal models and immunofluorescent staining of cells with antibodies specific for the three mammalian nonmuscle myosin-2 paralogs show that there are regions of unique expression as well as regions where the expression of two or more of the nonmuscle myosin-2 paralogs overlap [Golomb et al., 2004; Ma et al., 2010; Maupin et al., 1994]. Various studies demonstrate that the three nonmuscle myosins-2 are not functionally redundant and that they can only compensate for each other in very rare cases, although recent studies have shown that nonmuscle myosin-2 isoforms can co-polymerize to form heterotypic filaments intracellularly [Beach et al., 2014; Ma and Adelstein, 2014b; Shutova et al., 2014].

Caveat 2: RLCs and ELCs Are Promiscuous

The assumption explicitly made is that the FP-RLC will uniquely report the localization of the total nonmuscle myosin-2 pool within a cell, but recent reports question the exclusive association of both ELC and RLC to the nonmuscle myosin-2 heavy chain. RLC has been shown to associate with the heavy chains of unconventional myosins from classes -14, -15, -18 and -19, and ELC with the heavy chains of unconventional myosin members of classes -1, -5, -6, -7, -14, -15 and -18 [Bird et al., 2014; Bookwalter et al., 2014; Espindola et al., 2000; Franke et al., 2006; Guzik-Lendrum et al., 2013; Lu et al., 2014; Maliga et al., 2013].

Furthermore, many non-myosin off targets of ELC and RLC involved in various physiological functions have been described and characterized [Bajaj et al., 2009; Chan et al., 2005; Franke et al., 2006; Ledee et al., 2007; Szymanski and Goyal, 1999; Valouev et al., 2004; Weissbach et al., 1998]. For example, synaptic N-methyl-D-aspartate receptor subunits bind the isolated RLC, but not the nonmuscle myosin-2B associated light chain. Interestingly, the MLCK phosphorylated RLC does not bind to its receptor target [Bajaj et al., 2009].

Those studies clearly contradict the dogma of the exclusive association of ELC and RLC with the nonmuscle myosin-2 heavy chain and expand the light chain interactome

tremendously to proteins involved for example in intracellular signaling and trafficking. There are reports of independent localization patterns of GFP-RLC from endogenous RLC, the nonmuscle myosin-2 heavy chain or phosphorylated RLC, for example to the mitotic spindle or the midbody [Beach et al., 2011; Kondo et al., 2012]. Thus, it is unlikely that FP-RLC will uniquely report the localization of nonmuscle myosin-2 paralogs in cells.

There is little information on the relative amounts of the various myosin superfamily members inside cells. In some cases, such as for myosin-18A, the expression level is on the order of 1% of that of the nonmuscle myosin-2 paralogs [Tan et al., 2008]. This may help mitigate the problem with promiscuity of the FP-RLC localization, but researchers should verify any suspicious or novel localization using antibodies against the three nonmuscle myosin-2 heavy chains. Though the exchange of the endogenous RLC with FP-RLC is very efficient, up to 20% of the nonmuscle myosin-2 has the endogenous RLC bound and is *bona fide* regulated by upstream signaling cascades [Beach et al., 2011]. This is especially of importance when FP-RLC phosphomimetics are used, as outlined in greater detail below.

Caveat 3: Kinetic, Mechanical and Regulatory Consequences

Both myosin light chains play at least two important roles in the function of myosin-2. First, they are a structurally important part of the myosin holoenzyme since they bind to and stabilize an elongated helical segment of the heavy chain. Thus ELC and RLC serve to form the “neck” or “lever arm” of the myosin which rotates about a fixed point in the myosin motor domain to give rise to the power stroke that propels F-actin. [Rayment et al., 1993]. The structural importance of the myosin light chains is evident in *Drosophila* where ablation of the RLC gene results in the aggregation of the nonmuscle myosin-2 heavy chain [Jordan and Karess, 1997].

Second, the RLC is intimately involved in the regulation of mammalian nonmuscle myosins-2 ATPase activity via its phosphorylation on a serine residue near the N-terminus, as reviewed previously [Sellers, 1991]. The primary phosphorylation site on the RLC is S19, but T18 can also be phosphorylated in addition to S19 at a much slower rate [Ikebe, 1989]. S19 phosphorylation activates the ATPase activity of nonmuscle myosin-2 in the presence of F-actin and double phosphorylation of S19 and T18 further increases the ATP turnover. Activation of the enzymatic activity is accompanied by a conformational change of the nonmuscle myosin-2 heavy chain: In the absence of RLC phosphorylation, the nonmuscle myosin-2 adopts a folded, compact conformation where the two myosin motor domains make an asymmetric interaction that abolishes F-actin activation of the ATPase activity [Jung et al., 2008; Scholey et al., 1980]. This interaction is broken when the RLC is phosphorylated resulting in an activation of the enzymatic activity by F-actin (for review, see [Bresnick, 1999; Heissler and Manstein, 2013; Sellers, 1991; Vicente-Manzanares et al., 2009]).

One must ask the question of whether the FP-RLC bound to the nonmuscle myosin-2 holoenzyme adversely affects its regulation, filament formation, localization, enzymatic activity or mechanical properties. There are only a few studies that have actually addressed these questions with recombinant myosins *in vitro*. Komatsu *et al.* exchanged a RLC fused

to a C-terminal GFP into smooth muscle myosin-2 and demonstrate that its actin-activated ATPase activity is regulated by phosphorylation [Komatsu et al., 2000]. However, the paper lacks a crucial control experiment to determine the ATPase activity of a myosin that had been exchanged with a wild type RLC and thus, it is unknown whether the RLC-GFP restores full enzymatic activity to the myosin. Kengyel *et al.* fused a GFP to the N-terminus of the RLC (GFP-RLC) and overproduced it along with nonmuscle myosin-2A heavy meromyosin fragment in the baculovirus/Sf9 system. The ATPase activity of the GFP-RLC-containing myosin in the presence of F-actin is low when the light chain was unphosphorylated and increases dramatically upon its phosphorylation with Ca^{2+} /CaM-dependent myosin light chain kinase (MLCK), indicating that the GFP fusion did not prevent myosin from adopting the off state. Though the steady-state ATPase activity is increased upon phosphorylation of the GFP-RLC by MLCK, the $V_{\max}$ is only about 50% of that of the control. Furthermore, the rate of F-actin gliding in the *in vitro* motility assay is slightly reduced [Kengyel et al., 2010]. A subsequent study using full length nonmuscle myosin-2B demonstrated that the GFP-RLC does not prevent myosin from forming filaments and that these filaments could move as a processive unit along F-actin *in vitro* [Nagy et al., 2013]. Neither of these *in vitro* studies nor any *in vivo* study make a systematic comparison between N- and C-terminal GFP fusions of the RLC.

Intriguingly, Kengyel *et al.* also report that the *in vitro* phosphorylation rate of GFP-RLC bound to the nonmuscle myosin-2A heavy chain by MLCK is reduced [Kengyel et al., 2010]. Intracellularly, the activity of actomyosin driven contractility is strictly regulated by the interplay between kinases and phosphatases, with nonmuscle myosin-2 being a downstream effector, as reviewed previously [Heissler and Manstein, 2013]. The *in vitro* studies suggests a lower overall RLC phosphorylation level inside cells, although the corresponding effect of the GFP-fusion on phosphatase activity was not tested *in vitro*, nor was the activity of other kinases that phosphorylate the RLC such as ROCK. A compromised or temporally delayed phosphorylation of GFP-RLC may impact nonmuscle myosin-2's spatiotemporal performance, a critical determinant of cellular homeostasis. Malfunction of any of the parameters can result in aberrant cellular performance and pathologies.

It is reassuring that the presence of the GFP-RLC does not block the ability of the myosin to form the off conformation, but the question arises to how much should we be concerned by the lowered enzymatic activity brought about by the GFP-RLC? In animal models where the enzymatic activity of either nonmuscle myosin-2A or 2B was lowered through mutations in the heavy chains, in some cases to mimic mutations known to cause human diseases, strong phenotypes are observed in the animals [Ma and Adelstein, 2014a; Ma et al., 2004]. More strikingly, many of the cardiac myosin-2 mutations associated with hypertrophic and dilated cardiomyopathies have only very minor effects on steady-state ATPase and *in vitro* motility activities, but cause major heart diseases, sometimes associated with mortality both in humans or when engineered into animal models [Bloemink et al., 2014; Sommese et al., 2013]. Thus even subtle changes in myosin's enzymatic activity can have strong consequences within a lifetime of an animal. Therefore, it is possible that even the subtly compromised mechanoenzymatic properties of nonmuscle myosin-2 containing GFP-RLC

holoenzyme might adversely affect its cellular performance in ways that might be difficult to detect.

Caveat 4: Phosphomimetics May Not Mimic!

Several studies have mutated the S19/T18 residues to aspartic acid or glutamic acid to create what is hoped to be a phosphomimetic of the phosphorylated RLC. However, phosphomimetic RLC mutants bound to nonmuscle myosin-2 do not phenocopy the phosphorylated RLC in *in vitro* biochemical and cell biological studies. Two *in vitro* studies examined the effect of incorporation of phosphomimetic RLCs into myosin, both using smooth muscle myosin-2 which is regulated in a manner very similarly to the nonmuscle myosin-2 paralogs. The results of these studies were not completely clear, however, in no case does a single replacement of S19 with either aspartic or glutamic acid fully mimic the phosphorylated RLC state [Kamisoyama et al., 1994; Sweeney et al., 1994]. In one case, a glutamic acid replacement of S19 gives no activation of the ATPase activity by F-actin and no *in vitro* motility; in the other case less than 20% of the maximal activity is found [Kamisoyama et al., 1994; Sweeney et al., 1994]. Replacement of both S19 and T18 gives somewhat better results, though the double mutant also does not fully phenocopy the kinetic and regulatory features of the native, RLC phosphorylated myosin holoenzyme [Kamisoyama et al., 1994; Sweeney et al., 1994]. Presumably, based on the Kengyel *et al.* study, the effect of coupling a FP moiety to the phosphomimetic RLC might additionally compromise the mechanokinetic activity of the myosin [Kengyel et al., 2010]. Further, phosphomimetic mutations on the RLC also uncouple nonmuscle myosin-2 from its regulatory upstream signaling cascades, whereas the residual native nonmuscle myosin-2 is still regulated.

In *Drosophila*, introduction of some phosphomimetic RLCs results in nonmuscle myosin-2 aggregation, as does ablation of the RLC [Jordan and Karess, 1997; Vasquez et al., 2014]. Phosphomimetic GFP-RLC expressed near endogenous concentrations results in a down-regulation of the endogenous nonmuscle myosin-2 heavy chain and light chain in *Drosophila* embryos and presumably mammalian cells and tissues [Vasquez et al., 2014]. Such a compensatory mechanism adds another caveat to cell biological aspects since the total amount of the relative subunits of the nonmuscle myosin-2 holoenzyme might be changed compared to native cells and tissues. Decreased expression levels of the nonmuscle-2 heavy chain results in faster rates of cell migration observed for example in tumor cells and tissues as reviewed previously [Heissler and Manstein, 2013].

Conclusion and Perspective

Studies of the spatiodynamic aspects of nonmuscle myosin-2 localization and motor function are extremely challenging. Expression of GFP-RLC certainly allows investigators to follow the localization of nonmuscle myosin-2 in cells, but the experimenter must be cognizant that the fluorescent light chain is not uniquely specific to nonmuscle myosin-2. Therefore, any unexpected localization should be confirmed by nonmuscle myosin-2 heavy chain antibodies. In addition, one must be aware that the FP-RLC-complexed nonmuscle myosin-2 is likely to have somewhat compromised enzymatic properties which must be

considered in interpreting the results of these studies. How the FP-RLC binding to unconventional myosins and non-myosin targets affect their function is also unknown and could possibly give rise to “off target” effects that are common in pharmacological studies.

Strategies to avoid overexpression-induced artifacts include the use of plasmids in which the majority of the CMV promoter is deleted (delCMV) or single cell microinjection of fluorescently labeled recombinant light chains [Post et al., 1995; Watanabe and Mitchison, 2002]. The former drastically reduces the amount of expressed FP-RLC, a technique successfully used in speckle microscopy, the latter delivers a defined protein dose of protein and is advantageous for transduction-challenged cells [Zhang and Yu, 2008].

Regardless of which experimental setup is chosen: Bottom line when conducting overexpression studies with FP-RLC...*caveat emptor!*

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