Leiomodin-2 is an antagonist of tropomodulin-1 at the pointed end of the thin filaments in cardiac muscle

Takehiro Tsukada[1,*], Christopher T. Pappas[1,*], Natalia Moroz[2], Parker B. Antin[1], Alla S. Kostyukova[2] and Carol C. Gregorio[1,‡]

[1]Department of Cell Biology and Anatomy, and Sarver Molecular Cardiovascular Research Program, University of Arizona, Tucson, AZ 85724, USA
[2]Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, UMDNJ, Piscataway, NJ 08854, USA
*These authors contributed equally to this work
‡Author for correspondence (gregorio@email.arizona.edu)

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Summary

Regulation of actin filament assembly is essential for efficient contractile activity in striated muscle. Leiomodin is an actin-binding protein and homolog of the pointed-end capping protein, tropomodulin. These proteins are structurally similar, sharing a common domain organization that includes two actin-binding sites. Leiomodin also contains a unique C-terminal extension that has a third actin-binding WH2 domain. Recently, the striated-muscle-specific isoform of leiomodin (Lmod2) was reported to be an actin nucleator in cardiomyocytes. Here, we have identified a function of Lmod2 in the regulation of thin filament lengths. We show that Lmod2 localizes to the pointed ends of thin filaments, where it competes for binding with tropomodulin-1 (Tmod1). Overexpression of Lmod2 results in loss of Tmod1 assembly and elongation of the thin filaments from their pointed ends. The Lmod2 WH2 domain is required for lengthening because its removal results in a molecule that caps the pointed ends similarly to Tmod1. Furthermore, Lmod2 transcripts are first detected in the heart after it has begun to beat, suggesting that the primary function of Lmod2 is to maintain thin filament lengths in the mature heart. Thus, Lmod2 antagonizes the function of Tmod1, and together, these molecules might fine-tune thin filament lengths.

Key words: Leiomodin, Lmod2, Thin filament, Tropomodulin, WH2 domain, Cardiomyocytes

Introduction

Striated muscle cells are composed of dense overlapping arrays of actin-containing thin filaments and myosin-containing thick filaments. Proper contraction requires these two filament systems to appropriately align with one another. Accordingly, their orientations, spacing and lengths are highly regulated. Although the molecular mechanisms underlying this regulation are largely unknown, it is evident that the specification of thin filament lengths requires the coordinated activity of several proteins.

One such protein, tropomyosin, contributes to length regulation by stabilizing filamentous actin. Tropomyosin not only directly prevents actin depolymerization in vitro by binding in a head-to-tail arrangement along the actin filament, but also reduces the depolymerization affects of DNaseI and ADF/cofilin, and severing by gelsolin (Bernstein and Bamburg, 1982; Broschat, 1990; Fattoum et al., 1983; Hitchcock et al., 1976; Weigt et al., 1990). The stabilizing activity of tropomyosin has also been shown in vivo, because a reduction of tropomyosin levels results in disorganized thin filaments in the body wall muscles of *Caenorhabditis elegans* (Ono and Ono, 2002). Tropomyosin is also crucial for development, because homozygous α-tropomyosin-knockout mice die during embryonic days 9.5–13.5 (Blanchard et al., 1997).

Another crucial component for regulation of thin filament length is tropomodulin-1 (Tmod1). Tmod1 is the primary tropomodulin isoform in cardiac myocytes and caps the pointed ends of the actin filaments, preventing actin polymerization and depolymerization in vitro (Almenar-Queralt et al., 1999; Weber et al., 1994). Tmod1 binds the end of the thin filament through one actin-binding and two tropomyosin-binding domains located within its predominantly unstructured N-terminal half, and a second actin-binding domain that resides in a leucine-rich repeat (LRR) domain close to its C-terminal end (Fig. 1A) (Babcock and Fowler, 1994; Fowler et al., 2003; Kostyukova et al., 2000; Kostyukova et al., 2006; Kostyukova et al., 2001). High-affinity capping by Tmod1 requires association with tropomyosin and it is thought that one molecule of Tmod1 simultaneously interacts with two tropomyosin molecules (one from each actin protofilament) (Kostyukova et al., 2006; Weber et al., 1994). Blockade of the C-terminal Tmod1 actin-binding domain results in the loss of its capping ability and lengthening of the actin filaments in cardiomyocytes in culture, although Tmod1 still remains bound to the pointed end of the filament (Gregorio et al., 1995). Conversely, blockade of the first tropomyosin-binding domain results in the depolymerization of the thin filaments (Mudry et al., 2003). Therefore, the individual interacting domains of Tmod1 appear to have very discrete functions in maintaining actin filament stability and length. Proper levels of Tmod1 are also required to maintain thin filament lengths in cultured cardiomyocytes, because a reduction of Tmod1 results in longer thin filaments, whereas Tmod1 overexpression leads to shorter filaments (Littlefield et al., 2001; Sussman et al., 1998a). Regulation of Tmod1 levels is also essential in vivo. Overexpression of Tmod1 in mouse hearts results in dilated cardiomyopathy and degenerating myofibrils (Sussman et al., 1998b). *Tmod1*$^{-/-}$ mice are embryonic lethal because of defects in the myocardium (Chu et al., 2003; Fritz-Six et al., 2003; McKeown et al., 2008). Specifically, abnormalities are first detected at embryonic day 9.5 with very few or no intersomite vasculatures, defective primitive hematopoiesis and mechanically weakened blood cells (Chu et al.,

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2003). The knockout hearts do not loop and are missing the right ventricle. Myofibrils from mouse knockout hearts and from knockout embryonic stem cells that have been differentiated into cardiac myocytes reveal immature Z-discs and thin filaments that do not become well-striated or aligned (Fritz-Six et al., 2003; Ono et al., 2005). Although an extensive amount of evidence indicates that Tmod1 has an important role in heart development and maintenance of thin filament lengths, very little is known regarding how its functional properties are regulated.

Tropomodulin is a member of a family that also contains the leiomodin proteins. There are three leiomodin genes, which encode smooth muscle leiomodin (Lmod1), cardiac leiomodin (Lmod2) and fetal leiomodin (Lmod3). Lmod2 is restricted to skeletal and cardiac muscle (Conley et al., 2001). Since the *LMOD2* gene is located close to the hypertrophic cardiomyopathy locus CMH6 on human chromosome 7q3, it has been hypothesized that Lmod2 is involved in the pathogenesis of hypertrophic cardiomyopathy (Conley et al., 2001). About the first two thirds of Lmod2 are strikingly similar to Tmod1 in domain composition and structure; it contains a tropomyosin- and actin-binding domain at its N-terminus and a LRR domain (Fig. 1A). Interestingly, Lmod2 does not contain a recognizable second tropomyosin-binding domain and has an additional C-terminal extension composed of a proline-rich region and an actin-binding Wiskott–Aldrich syndrome protein homology 2 (WH2) domain (Chereau et al., 2008). Owing to its three actin-binding sites, Lmod2 is a potent nucleator of actin polymerization in vitro (Chereau et al., 2008). Lmod2 is also found at the M-line of rat cardiomyocytes and is required for proper sarcomeric organization (Chereau et al., 2008). Moreover, Lmod2 binds various tropomyosin isoforms with differing affinities (Kostyukova, 2007). Thus, Lmod2 functions might be regulated by its interaction with tropomyosin, which has been demonstrated for tropomodulin (Kimura et al., 1999; Kostyukova and Hitchcock-DeGregori, 2004; Mudry et al., 2003; Weber et al., 1994).

Since Lmod2 is structurally similar to Tmod1, interacts with actin and tropomyosin and localizes near the pointed ends of the actin filaments in cardiomyocytes in culture, we sought to determine whether Lmod2 has a role in the regulation of thin filament length. Here, we show that the temporal expression patterns of *LMOD2* and *TMOD1* are different. *LMOD2* transcripts are first detected rather late in the development of the heart (Hamburger-Hamilton [HH] stage 14; well after the heart has formed its primary loop and is beating), whereas *TMOD1* is expressed at an earlier stage (HH stage 11). We also demonstrate for the first time that Lmod2 localizes to the pointed ends of the actin filaments in chick cardiomyocytes; an association that was confirmed by biochemical analyses. These data suggest that Lmod2 is not the initial nucleator of thin filament assembly, but might have a role in the assembly or maintenance of thin filaments in the mature heart. Here, we find that excess Lmod2 not only displaces Tmod1 from thin filament pointed ends but also results in the uniform elongation of thin filaments from their pointed ends. In addition, we show that the removal of the Lmod2 actin-binding WH2 domain transforms the molecule into a pointed-end capping protein that is similar to Tmod1. Thus, a combination of biochemical and cellular approaches reveals that Lmod2 antagonizes the function of the actin filament capping protein Tmod1, promoting thin filament elongation, and thereby possibly fine-tuning thin filament lengths.

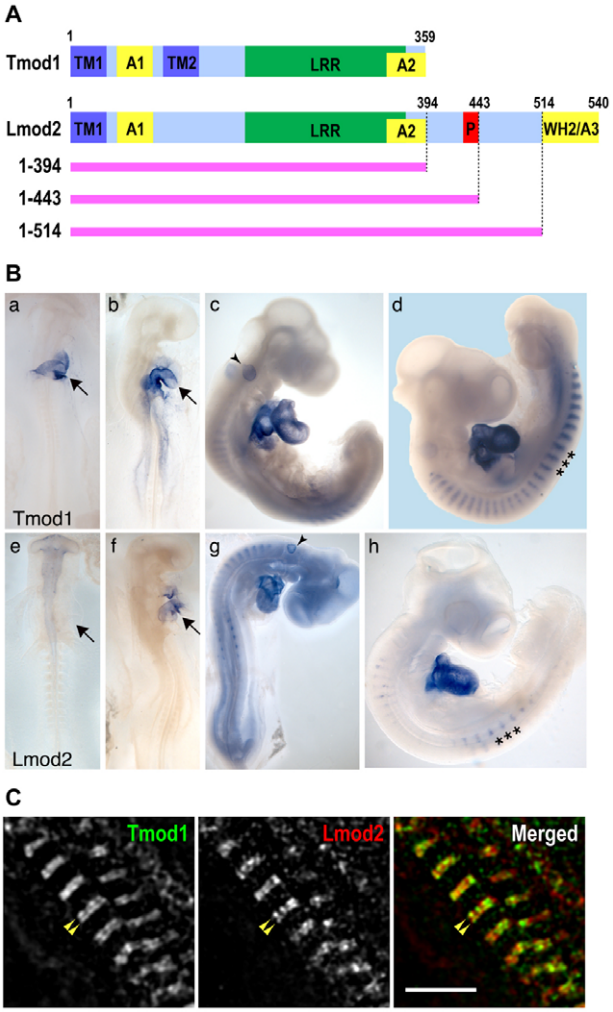

Fig. 1. Structure, expression and localization of chicken Lmod2. (**A**) Domain structure of Tmod1 and Lmod2. Tmod1 domain structure was determined based on biochemical, morphological and structural analysis (Kostyukova, 2008). Lmod2 domain structure was determined based on amino acid sequence homology to Tmod1 (Chereau et al., 2008). Lmod2 and Tmod1 share actin-capping (A1,A2), tropomyosin-binding (TM1) and leucine-rich repeat (LRR) domains. The second tropomyosin-binding domain (TM2) is unique to Tmod1. Lmod2 contains a C-terminal extension, which includes proline-rich (P) and actin-binding Wiskott–Aldrich syndrome protein homology 2 (WH2) domains. Truncated fragments used in this study are indicated by pink lines (residues 1–394, 1–443 and 1–514). (**B**) Whole-mount in situ hybridization localization of *TMOD1* (a–d) and *LMOD2* (e–h) transcripts in developing chicken embryos. At HH stage 11, *TMOD1* expression was detected in the looping heart (a, arrow), whereas expression of *LMOD2* was undetectable (e). By HH stage 14, *TMOD1* and *LMOD2* were both detected in the myocardium (b,f, arrows), although *TMOD1* staining was consistently more intense than that of *LMOD2*. By HH Stage 17 (c,g), *TMOD1* and *LMOD2* transcripts were localized to the myocardium and to the somites. Similar patterns were observed at HH stage 19 (d,h, asterisks). (**C**) Tmod1 (green) and Lmod2 (red) both localized to thin filament pointed ends in cardiomyocytes. Merged image revealed some non-overlap of Tmod1 and Lmod2 staining. Scale bar: 5 µm.

Results

LMOD2 transcripts are expressed later than those of *TMOD1* in chicken development

Since only a predicted nucleotide sequence was available for chicken *LMOD2* (NCBI reference sequence XM_415995.2), we

designed primers based on this sequence and amplified the open reading frame of chick *LMOD2* by PCR from cDNA generated from chick cardiomyocytes in culture. The resulting translated protein sequence contained a smaller proline-rich region (by 12 amino acids) than the predicted chicken Lmod2 sequence (supplementary material Fig. S1).

LMOD2 expression in the human is restricted to the fetal and adult heart and to skeletal muscle (Conley et al., 2001). To determine where and when the *LMOD2* message is expressed in the chicken embryo, whole mount in situ hybridization was performed. *LMOD2* transcripts were first detected in the heart around HH stage 14 (Fig. 1Bf, arrow). At this point in development, the heart has already begun to beat and has formed a primary loop. Stage 11 embryos had no detectable *LMOD2* expression (Fig. 1Be). Faint staining of a subset of somites was also observed at later stages (Fig. 1Bg,h, asterisks). Probing for *TMOD1* at equivalent stages of development revealed that it is expressed earlier than *LMOD2*, with transcripts readily detected at stage 11 (Fig. 1Ba, arrow). *TMOD1* expression was not detected in stage 8 embryos (data not shown). Additionally, *TMOD1* was clearly visible in the somites by stage 17, although unlike *LMOD2*, all of the somites were *TMOD1* positive (Fig. 1Bc,d, asterisks). Both *TMOD1* and *LMOD2* mRNA also seemed to be present in the otic vesicle (Fig. 1Bc,g, arrowheads). The pattern and timing of *TMOD1* expression in the chick embryo was comparable with that of *Tmod1* in the mouse embryo determined by whole-mount X-gal staining of *Tmod1*$^{lacZ+/-}$ embryos (Fritz-Six et al., 2003).

Lmod2 localizes to actin filament pointed ends

Immunofluorescent staining of rat cardiomyocytes with an anti-Lmod2 antibody revealed that Lmod2 colocalized with the M-line marker myomesin (Chereau et al., 2008). The same antibody was used to determine the localization of Lmod2 in chick cardiomyocytes that were relaxed before fixation to allow for better resolution of thin filament pointed ends. Although not all cells were positive for Lmod2 staining, Lmod2 was observed as a single striation at the M-line in the chick, analogous to results previously reported in the rat (Chereau et al., 2008). In some cells, however, a distinct doublet of Lmod2 staining around the M-line was seen, consistent with the prediction that Lmod2 localizes to the pointed ends of the thin filaments (Fig. 1C, middle panel, arrowheads). Interestingly, Lmod2 only partially colocalized with Tmod1, which also assembles at the pointed ends of the thin filaments, and the intensities of the two stains often seemed to have a negative correlation (Fig. 1C, arrowheads; supplementary material Fig. S2). This suggests that these two molecules might not coexist at the ends of the same actin filaments.

Overexpression of Lmod2 results in elongation of thin filaments

To gain insight into the functional properties of Lmod2, GFP-Lmod2 was expressed in chick cardiomyocytes. GFP-Lmod2 localized primarily to the M-line (Fig. 2d,g, yellow arrowheads). GFP-Lmod2 was also associated along the length of the thin filaments and was absent from the Z-disc (and possibly some of the I-band) (Fig. 2d,g). However, extraction of the cytosol before fixation removed the staining along the myofibril, indicating that this association might be nonspecific (data not shown). Interestingly, doublets of GFP-Lmod2 around the M-line were never observed, and correspondingly, staining with fluorescently labeled phalloidin (which only labels filamentous actin) showed a striking elongation

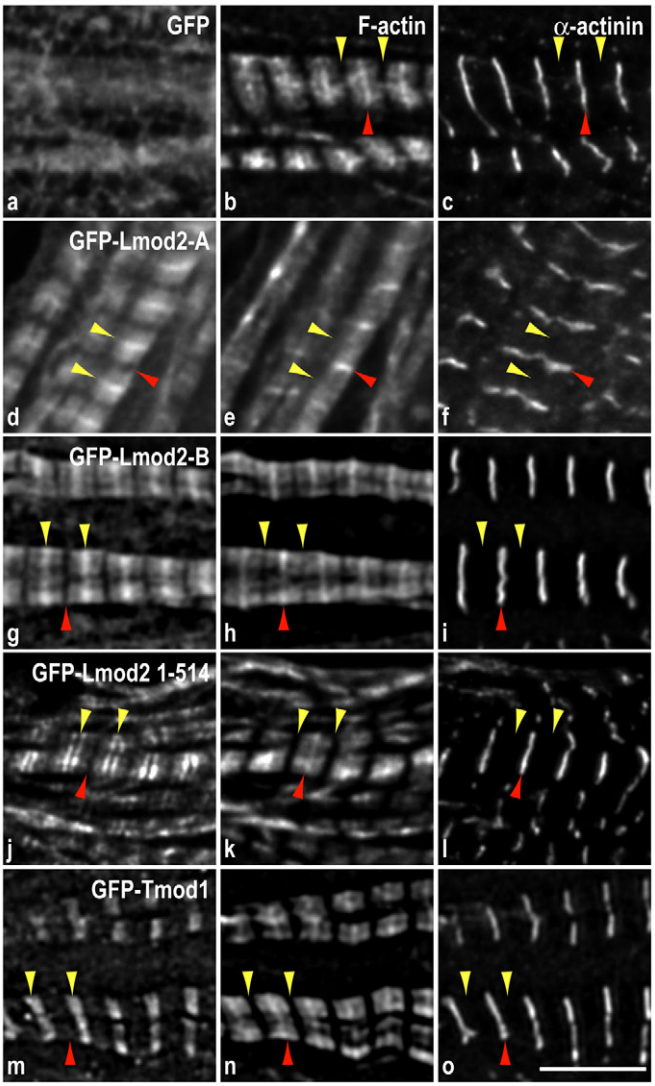

Fig. 2. Expression of full-length Lmod2 results in uniform elongation of actin-thin filaments from their pointed ends; its WH2 domain appears to be required for this effect. Cardiomyocytes expressing GFP alone (a), GFP-Lmod2 (d,g), GFP-Lmod2(1–514) (j), or GFP-Tmod1 (m) were stained with Phalloidin (b,e,h,k,n) and for α-actinin to label the Z-discs (c,f,i,l,o). Expression of full-length GFP-Lmod2 resulted in elongation of thin filament pointed ends as discerned by a narrow gap, or sometimes the absence of a gap, in phalloidin staining in the middle of the sarcomere (e,h). GFP-Lmod2-A is an example of myofibrils with no detectable gap; GFP-Lmod2-B is an example of myofibrils with a discernible narrow gap. Conversely, expression of GFP-Lmod2(1–514), which lacks the actin-binding WH2 domain, resulted in a clear, wide gap of Phalloidin staining in the H-zone, comparable with that seen following the expression of GFP-Tmod1. Doublets of GFP-Lmod2(1–514) were clearly observed at the pointed ends (note: no doublets were observed in the cells expressing full-length GFP-Lmod2). Pointed ends, yellow arrowheads; Z-discs, red arrowheads. Scale bar: 5 μm.

of the actin filaments from their pointed ends. The actin filaments either displayed uniform elongation with a very small gap in Phalloidin staining at the M-line, or no gap at all, giving the filaments a nonstriated appearance (Fig. 2h,e, respectively, yellow arrowheads). By contrast, cells transfected with GFP alone displayed wide gaps in Phalloidin staining (Fig. 2b, yellow arrowheads). Measurements of thin filament lengths confirmed

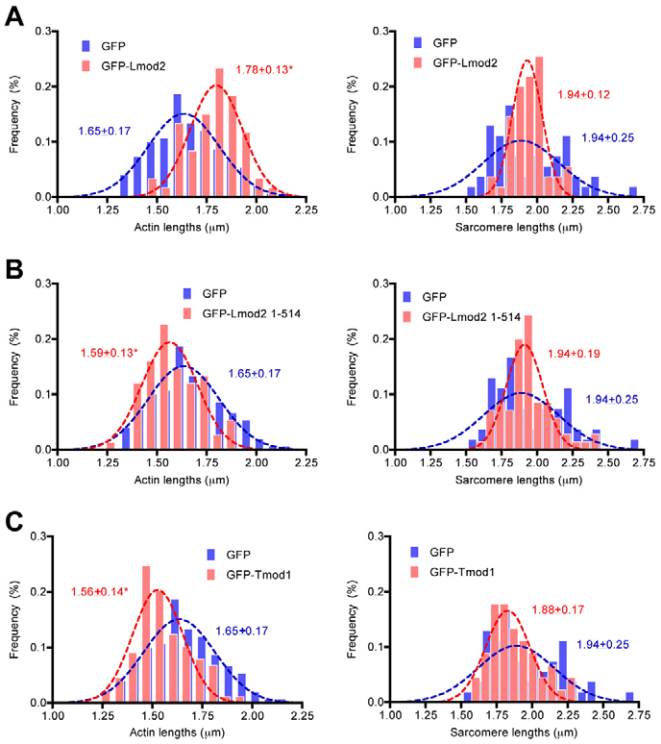

Fig. 3. Expression of full-length Lmod2 results in longer actin filaments, whereas expression of Lmod2(1–514) or Tmod1 results in shorter actin filaments. Cardiomyocytes expressing GFP alone, GFP-Lmod2, GFP-Lmod2(1–514) or GFP-Tmod1 were stained with phalloidin to measure thin filament lengths (from pointed end to pointed end: n=150, 60, 75, 89, respectively) and for α-actinin to measure sarcomere lengths (from Z-disc to Z-disc: n=54, 55, 70, 45, respectively). In the cells expressing full-length GFP-Lmod2, only myofibrils displaying distinguishable F-actin gaps were used for measurements. Data are from 2–5 cultures and the values are presented as a percentage (mean ± s.d.). (**A**) Thin filament lengths were significantly longer in cells expressing GFP-Lmod2 compared with those in cells expressing GFP alone (*P<0.01, Student's t-test). (**B**,**C**) By contrast, thin filament lengths were significantly reduced in cells expressing GFP-Lmod2(1–514) or GFP-Tmod1 (*P<0.01). No significant differences in sarcomere lengths were observed (right panels).

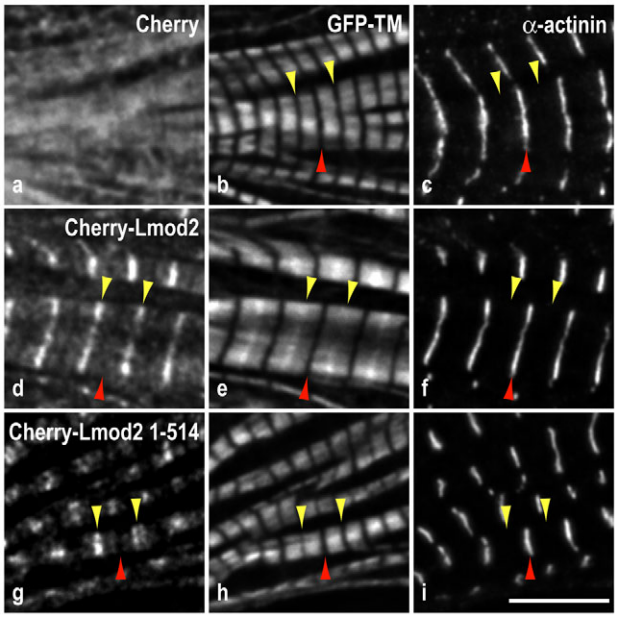

Fig. 4. Expression of Lmod2 also results in longer thin filaments marked by GFP-tropomyosin. Cardiomyocytes expressing GFP-tropomyosin (b,e,h) and mCherry alone (a), mCherry-Lmod2 (d), or mCherry-Lmod2(1–514) (g) were stained for α-actinin (c,f,i). In cells expressing mCherry alone, GFP-tropomyosin assembled in its characteristic pattern along the thin filaments with clear gaps visible at the pointed and barbed ends (b, red and yellow arrowheads, respectively). However, in cells expressing mCherry-Lmod2, the gap in GFP-tropomyosin assembly at the H-zone disappeared indicating the thin filaments had elongated (note, the increase in intensity at the center of the sarcomere indicates overlap of GFP-tropomyosin from opposite sarcomeres; yellow arrowheads). Interestingly, expression of mCherry-Lmod2(1–514) resulted in a clear, wide gap of GFP-tropomyosin assembly at both the H-zone and Z-disc, analogous to expression of mCherry alone. Yellow arrowheads, pointed ends; red arrowheads, Z-discs. Scale bar: 5 μm.

that they were significantly longer in GFP-Lmod2-positive cells (1.78±0.13 mm: note this measurement is an underestimate of thin filament lengthening because only myofibrils with Phalloidin gaps could be measured accurately) than control cells transfected with GFP alone (1.65±0.17 mm) (Fig. 3A). Sarcomere lengths remained unchanged upon expression of GFP-Lmod2 (Fig. 3A), indicating that the lack of a gap (or a small gap) in phalloidin staining at the M-line was not due to contraction or a decrease in sarcomere length. These results are in stark contrast to the overexpression of Tmod1, which results in shorter thin filaments (see below) (Littlefield et al., 2001; Sussman et al., 1998a).

We next examined the effect of the overexpression of Lmod2 on the distribution of tropomyosin, another integral thin filament component. We co-transfected GFP-tropomyosin and mCherry-Lmod2 into cardiomyocytes (Fig. 4). Previous studies have shown that the expression of GFP-tropomyosin does not result in any detectable perturbation of thin filament or sarcomere structure in cultured myocytes (Helfman et al., 1999; McElhinny et al., 2005; Mudry et al., 2003). In control cells transfected with mCherry alone, GFP-tropomyosin associated along the length of the actin filaments and was absent from the H-zone (center of the sarcomere where the thin and thick filaments do not overlap) and the Z-discs (Fig. 4b, yellow and red arrowheads, respectively). However, when full-length mCherry-Lmod2 was expressed, the gap in tropomyosin assembly at the H-zone disappeared (Fig. 4e, yellow arrowheads). Thus, the thin filaments elongated across the H-zone, and sometimes even overlapped, indicated by an increase in signal intensity at the M-line (Fig. 4e, yellow arrowheads).

The Lmod2 WH2 domain is necessary for thin filament elongation

To determine which domains of Lmod2 were required to promote thin filament elongation, we generated Lmod2 truncation mutants (shown in Fig. 1A). The constructs included: GFP-Lmod2(1–394), which comprises the portion of Lmod2 that is analogous to Tmod; GFP-Lmod2(1–443), which also includes the proline-rich domain; and GFP-Lmod2(1–514), which includes the entire molecule except the most C-terminal WH2 domain. Each of these mutants was transfected into cardiomyocytes and their propensities to assemble and resulting effects on thin filament architecture was analyzed. Interestingly, expression of each of the fragments produced identical results, thus, only detailed data regarding GFP-Lmod2(1–514) are shown (Fig. 2j–l; see supplementary material Figs S3 and S4 for

data on the other fragments). GFP-Lmod2(1–514) localized to the pointed ends of the actin filaments, because doublets around the M-line were visible, and also associated along the myofibril (Fig. 2j, yellow arrowheads). Unlike full-length GFP-Lmod2, however, expression of GFP-Lmod2(1–514) did not elongate the thin filaments (Fig. 2k, yellow arrowheads). In fact, measurements of thin filament lengths in cells expressing Lmod2(1–514) indicated that they were significantly shorter (1.59±0.13 mm) than in cells transfected with GFP alone (1.65±0.17 mm) (Fig. 3B). Moreover, overexpression of Lmod2(1–514) reduced thin filament lengths to a similar degree as did the overexpression of Tmod1 (1.56±0.14 mm) (Fig. 2m–o and Fig. 3C). Furthermore, no elongation of GFP-tropomyosin into the H-zone was observed following expression of mCherry-Lmod2(1–514) (Fig. 4h). These results suggest that Lmod2(1–514) functions similarly to Tmod1 with respect to regulation of thin filament lengths. Thus, the removal of its WH2 domain changes Lmod2 from a molecule that promotes thin filament elongation to one that prevents it.

Lmod2 overexpression reduces Tmod1 assembly at thin filament pointed ends

Next, we determined whether the assembly of Tmod1 was perturbed by the overexpression of GFP-Lmod2. A dramatic reduction in Tmod1 staining was observed in myocytes expressing GFP-Lmod2 (Fig. 5Af), suggesting that Lmod2 can compete for the binding of Tmod1 at the pointed ends of the thin filaments. Interestingly, expression of GFP-Lmod2(1–514), the fragment of Lmod2 that results in a reduction of thin filament lengths similarly to Tmod1, was also able to effectively reduce (compete for) the assembly of Tmod1 (Fig. 5Aj). To rule out the possibility that the observed decrease in Tmod1 staining was the result of anti-Tmod1 antibody inaccessibility, two other monoclonal antibodies against epitopes that map to different regions of Tmod1, as well as a polyclonal antibody raised against the entire Tmod1 molecule, were also used; identical results were obtained (data not shown).

We further tested the potential competition of pointed ends between Tmod1 and Lmod2 by co-transfection of both Tmod1 and Lmod2 into chick cardiomyocytes. In these cells, mCherry-Tmod1 prominently localized to the M-line whereas GFP-Lmod2 was found along the myofibril, but was clearly absent from the M-line (Fig. 5B, yellow arrowheads indicate M-line). This indicates that Tmod1 is able to compete with Lmod2 for pointed-end binding and also suggests that Tmod1 has a higher affinity for the pointed ends than Lmod2 in cells.

The effect of Lmod2 on actin filament pointed ends in vitro

As shown previously by Chereau and colleagues (Chereau et al., 2008), we demonstrated that Lmod2 was highly effective at nucleating actin polymerization in vitro (Fig. 6; supplementary material Fig. S5). However, our myocyte data presented above suggest that Lmod2 interacts with, and affects actin polymerization from, the pointed ends of the actin filaments. To test the influence of Lmod2 on preformed actin filaments, actin seeds (short actin filaments capped at their barbed ends by gelsolin) were prepared by polymerization of 3 μM actin in the presence of 30 nM gelsolin. The seeds were then diluted fivefold in G-actin solution. Actin

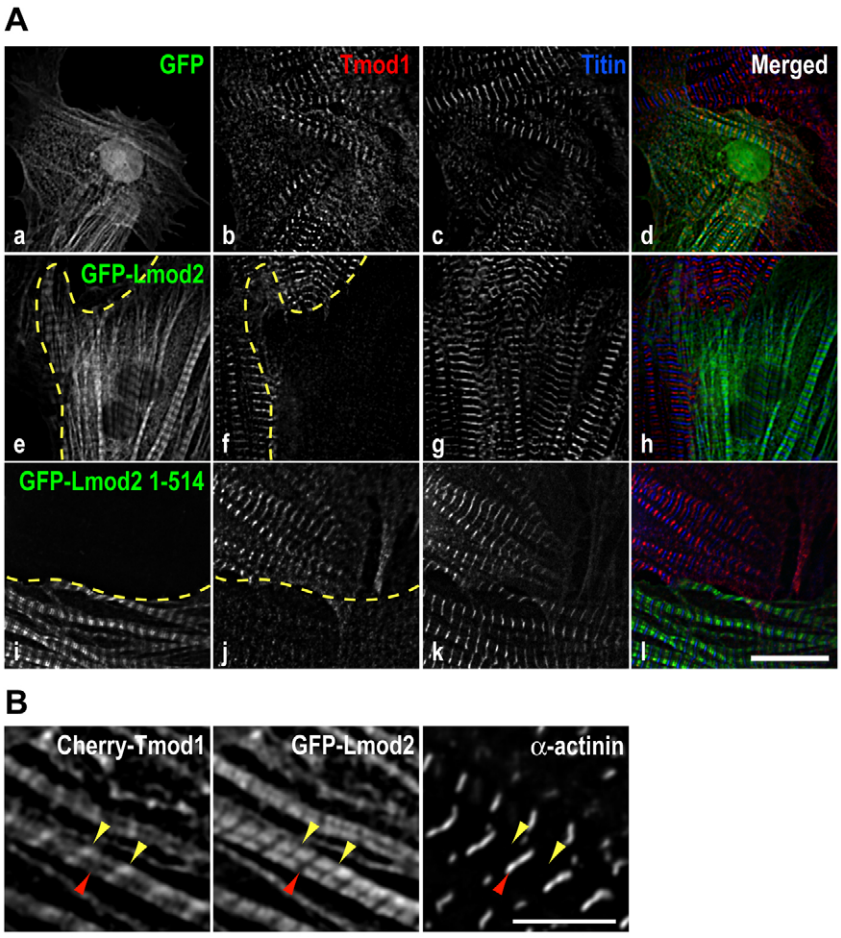

Fig. 5. Lmod2 and Lmod2(1–514) can displace Tmod1 from the pointed ends. (**A**) Cardiomyocytes expressing GFP alone (a) GFP-Lmod2 (e), or GFP-Lmod2(1–514) (i) were stained for Tmod1 (red, b,f,j) and Z-disc titin (blue, c,g,k). Little or no detectable Tmod1 localization was observed in cells expressing full-length GFP-Lmod2 or GFP-Lmod2(1–514) (f,j). This suggests that both proteins can compete with Tmod1 for pointed-end association. Yellow dashed lines indicate boundaries between cells. Scale bar: 15 μm. (**B**) Pointed-end assembly of GFP-Lmod2 was not observed in cardiomyocytes co-expressing mCherry-Tmod1 (left) and GFP-Lmod2 (center). These data suggest that Tmod1 can outcompete Lmod2 for pointed-end binding. Yellow arrowheads, pointed ends; red arrowheads, Z-discs. Scale bar: 5 μm.

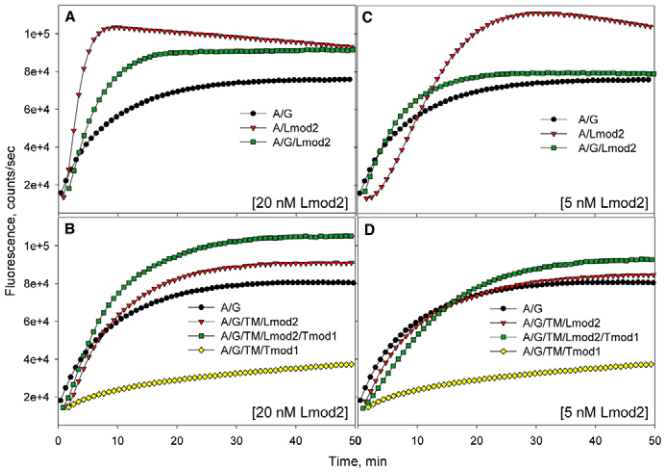

Fig. 6. Lmod2 binds to, but does not cap, the pointed ends of preformed actin filaments; tropomyosin enhances this binding. Pyrene fluorescence, which measures actin polymerization, is plotted vs. time. Initial protein concentrations were 6 nM gelsolin-capped filaments and 1.1 µM G-actin. (**A**) Lmod2 (20 nM) was able to nucleate G-actin (red triangles); however, its addition to gelsolin-capped actin seeds reduced this ability, indicating that it bound the seeds (i.e. was not available to nucleate) (green squares). (**B**) The addition of stTM (1 µM) enhanced the binding of Lmod2 (red triangles). Lmod2 association with the gelsolin-capped seeds could be partially removed by adding 20 nM Tmod1 (green squares), whereas the addition of Tmod1 (20 nM) to the seeds without Lmod2 resulted in a significant decrease in polymerization (yellow diamonds). (**C**) At a molar concentration that was lower than the concentration of the seeds (6 nM), Lmod2 (5 nM) mostly bound to the seeds (green squares). Note that 5 nM Lmod2 could still nucleate G-actin in the absence of seeds (red triangles). (**D**) At 5 nM, Lmod2 completely bound to the seeds in the presence of tropomyosin (1 µM) (red triangles). As before, Lmod2 association with the seeds could be partially removed by adding 20 nM Tmod1 (green squares). Control, A/G, is actin plus gelsolin-capped actin filaments (red circles).

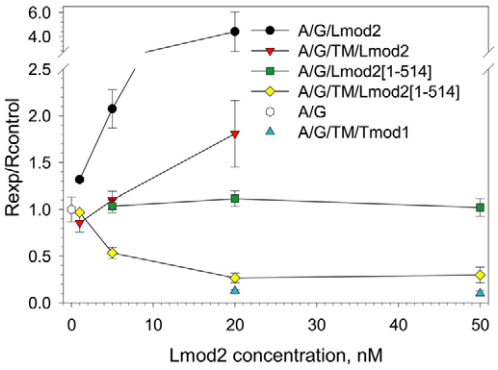

Fig. 7. Tropomyosin increases the affinity of Lmod2 for actin seeds and enhances the capping activity of Lmod2(1–514). The polymerization data were fitted to a single exponential equation and initial polymerization rates (R_{exp}) were calculated. R_{exp} values were then normalized by the initial rate obtained for polymerization of actin on gelsolin-capped actin seeds ($R_{control}$) (open hexagon). Initial protein concentrations were 6 nM gelsolin-capped filaments and 1.1 µM G-actin. In the presence of tropomyosin (1 µM), Lmod2 decreased the rate of actin polymerization (red triangles). This suggests that the affinity of Lmod2 for the seeds increased in the presence of tropomyosin. At 5, 20 and 50 nM, tropomyosin-dependent capping activity of Lmod2(1–514) was observed (yellow diamonds), although no actin capping activity was seen in the absence of tropomyosin (green squares). Although not dramatically different, the capping activity of Lmod2(1–514) is lower than that of Tmod1 (blue triangles).

polymerized on the gelsolin-capped seeds from their pointed ends only; this was faster than polymerization with actin alone and had no lag period. However, the final fluorescence values were lower because the critical concentrations for the pointed and barbed ends differ, 0.6 and 0.12 µM, respectively (Pollard, 1986). When G-actin was added to the seeds mixed with 20 nM Lmod2 (Fig. 6A, green squares), it polymerized more slowly than without the seeds (Fig. 6A, red triangles), although still faster and at higher fluorescence values than without Lmod2 (Fig. 6A, black circles). This might be explained by the binding of Lmod2 to the filaments. As a result of binding, fewer molecules of Lmod2 are available to nucleate G-actin, which results in a slower polymerization rate because fewer free barbed ends form.

When long muscle α-tropomyosin (stTM) was added to the seeds (at saturation, 1 µM) in addition to 20 nM Lmod2, a further decrease of fluorescence was observed (Fig. 6B, red triangles). However, fluorescence was still higher than in the control (actin plus seeds alone; Fig. 6B, black circles). To determine the initial rates of polymerization (R_{exp}), nonlinear regression curves were fitted to the polymerization data using a single exponential equation, then normalized by dividing by the initial rate obtained for polymerization of actin alone on the seeds ($R_{control}$). Addition of tropomyosin decreased the initial rate of polymerization on seeds plus Lmod2 by more than 50% (Fig. 7, red triangles and black circles). Therefore, the presence of tropomyosin seemed to enhance the ability of Lmod2 to bind to the seeds.

To establish whether Lmod2 had any effect on the elongation of actin filaments from their pointed ends, experiments were repeated at concentrations of Lmod2 lower than the concentration of the pointed ends. In these experiments, the concentration of the pointed ends was not more than 6 nM, which is the concentration of gelsolin used for seed formation after the fivefold dilution. When 5 nM Lmod2 was added to the gelsolin-capped actin filaments (Fig. 6C, green squares) there was only a slight increase of fluorescence compared with the control (Fig. 6C, black circles), although the initial rate was still over twofold higher than the control (Fig. 7, red triangles and black circles at 5 nM). In the presence of tropomyosin, however, there was no detectable increase in either fluorescence or the initial rate of polymerization (Fig. 6D; Fig. 7, red triangles). Therefore, we conclude that at this concentration, all of the Lmod2 bound to the stTM decorated seeds, probably at their pointed ends. Supporting this conclusion, the extent of Lmod2 nucleation correlated with the number of pointed ends (i.e. the higher the number of pointed ends, the greater the reduction of Lmod2-nucleating activity) (supplementary material Fig. S6). These data also suggest that the bound Lmod2 did not cap the pointed ends because there was no decrease in fluorescence or initial rates of actin polymerization (Fig. 6D, Fig. 7 and supplementary material Fig. S6). Conversely, 20 nM Tmod1, a pointed-end capping protein, effectively inhibited polymerization (Fig. 6B,D, yellow diamonds, and Fig. 7, blue triangles).

Next, experiments were performed to determine how the addition of Tmod1 would affect pointed-end elongation in the presence of Lmod2. Lmod2 (5 or 20 nM) followed by Tmod1 (20 nM) was added to seeds covered by stTM (1 µM). An increase of fluorescence was observed following the addition of Tmod1 to

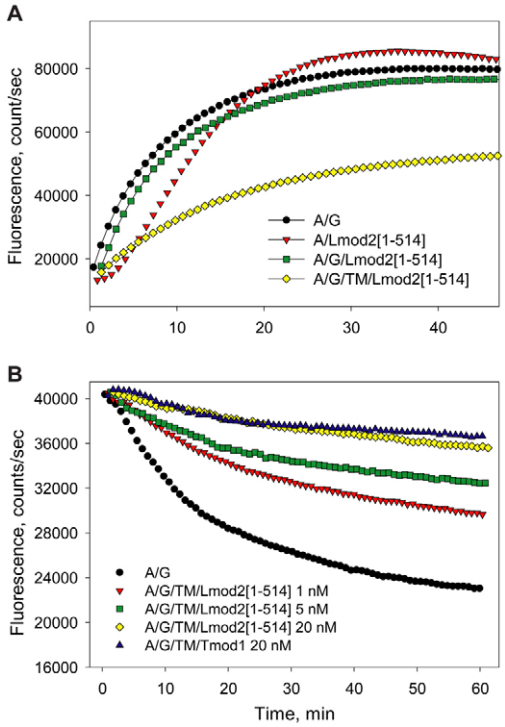

Fig. 8. Lmod2(1–514), similarly to Tmod1, can inhibit actin polymerization and depolymerization in the presence of tropomyosin. (**A**) Lmod2(1–514) (20 nM) had weak nucleation activity when added to actin alone (red triangles). When added to stTM-decorated filaments, Lmod2(1–514) (20 nM) remarkably prevented actin polymerization (yellow diamonds), whereas addition to filaments without stTM only resulted in a slight reduction of polymerization (green squares). Initial protein concentrations were 6 nM gelsolin-capped filaments, 1.1 μM G-actin, and 1 μM stTM. Control, A/G, is actin plus gelsolin-capped actin filaments. (**B**) Lmod2(1–514) prevented depolymerization from the pointed ends in a dose-dependent manner (downward red triangles: 1 nM, green squares: 5 nM, yellow diamonds: 20 nM). At 20 nM Lmod(1–514) inhibited polymerization similarly to 20 nM Tmod1 (upward blue triangles). The graph shows the depolymerization of actin at the pointed ends of gelsolin-capped actin filaments (0.6 μM F-actin, 6 nM gelsolin) in the presence of 0.75 μM stTM, 20 nM Tmod1 or different concentrations of Lmod2(1–514).

seeds with both 5 and 20 nM Lmod2 (Fig. 6B,D, green squares), implying that Lmod2 molecules become free to initiate barbed end polymerization by nucleation. Therefore, we conclude that Tmod1 is able to replace Lmod2 at the pointed ends of the actin filaments. However, as fluorescence did not reach the same values as actin alone plus Lmod2, it is possible that it was just a partial replacement.

Lmod2(1–514) blocks elongation of actin filaments at the pointed ends

Lmod2(1–514) was also able to nucleate actin polymerisation, although not as effectively as full-length Lmod2, because its nucleation activity at 50 nM was close to that of 1 nM full-length Lmod2 (supplementary material Fig. S5). When G-actin was added to gelsolin-capped seeds mixed with 20 nM Lmod2(1–514) (Fig. 8A, green squares), no difference in fluorescence was observed compared with the seeds alone (Fig. 8A, black circles). Furthermore, there was also no difference in initial polymerization rate when G-actin was added to gelsolin-capped seeds mixed with 5, 20 or 50 nM Lmod2(1–514) (Fig. 7, green squares). Interestingly, when stTM (1 μM) was added to the gelsolin-capped seeds, we observed a remarkable concentration-dependent decrease of polymerization rate in the presence of Lmod2(1–514) (Fig. 7, Fig. 8A, yellow diamonds); therefore, we conclude that Lmod2(1–514) inhibited elongation of actin filaments at the pointed end similarly to Tmod1.

To ascertain whether Lmod2(1–514) is able to block (cap) pointed ends, depolymerization experiments were also performed. In these experiments gelsolin-capped seeds were diluted fivefold with P-buffer. When Lmod2(1–514) was added in the presence of stTM it inhibited actin depolymerization from the pointed ends in a dose-dependent manner (Fig. 8B). These results confirmed that Lmod2(1–514) effectively caps the pointed ends and prevents both polymerization and depolymerization in a tropomyosin-dependent manner.

Discussion

Here, we demonstrate that Lmod2 and Tmod1 appear to have opposite roles in maintaining thin filament lengths. Both proteins have similar domain compositions and interact with the pointed ends of the thin filaments. However, Lmod2 has a C-terminal extension, which includes a WH2 domain that is responsible for abrogating the capping ability of this molecule. Thus Lmod2 is able to associate with the pointed ends of the thin filaments but does not cap them, which effectively antagonizes the function of Tmod1. The loss of the Tmod1 pointed-end capping activity then results in an extension of the actin filaments.

Lmod2 has been proposed to be the long sought-after muscle actin nucleator because it: (1) is only expressed in striated muscle; (2) efficiently nucleates actin filaments in vitro; and (3) is required for proper sarcomere assembly in cultured cardiomyocytes (Chereau et al., 2008; Conley, 2001). If Lmod2 were indeed the primary nucleator of thin filaments in the heart, it would be expected to be present very early in development, just as the first thin filaments are forming. In chick, however, *LMOD2* transcripts were only detected in the heart well after beating had begun and primary looping had been completed (HH stage 14, Fig. 1Bf). This suggests that Lmod2 functions in the mature heart. One possibility is that only a small quantity of *LMOD2* (with mRNA below the level of detection by whole mount in situ hybridization) is required to nucleate initial actin filaments and larger quantities of Lmod2 are required later in myofibril assembly to add additional actin filaments and/or contribute to the dynamic turnover of existing filaments. Another possibility is that primary nucleation is performed by the barbed-end capping protein CapZ, or some other unidentified nucleator, perhaps the fetal isoform of leiomodin, which has not been characterized (Lmod3; NCBI database, http://www.ncbi.nlm.nih.gov/) (Caldwell et al., 1989). Finally, a third possibility is that Lmod2 has a primary function unrelated to its nucleating ability. Our approach, using cell biological and biochemical techniques strongly support the third possibility. We revealed a function of Lmod2 in cardiac myocytes; Lmod2 antagonizes the function of the pointed-end capping protein Tmod1 and promotes elongation of the thin filaments from their pointed ends.

A model of how Lmod2 might regulate thin filament lengths, based on our data, is shown in Fig. 9. During the early stages of heart development, after thin filaments and myofibrils have formed, only Tmod1 is expressed and the thin filaments are short (Fig. 9A).

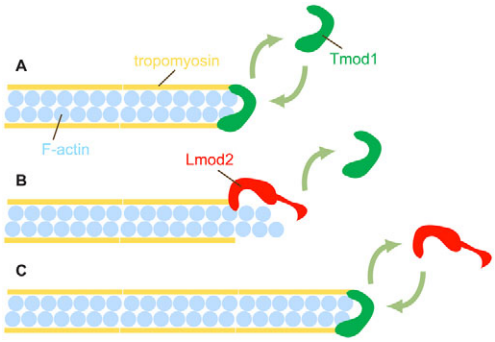

Fig. 9. Model of the function of Lmod2 at thin filament pointed ends. (**A**) During early stages of heart development when the myofibrils have formed, only Tmod1 is expressed, resulting in short thin filaments. (**B**) At a later stage of development, Lmod2 is expressed. Lmod2 assembles at the pointed ends of the thin filaments and competes for binding with Tmod1. Since Lmod2 is unable to cap, the thin filaments elongate. (**C**) Interactions between Tmod1, Lmod2, tropomyosin, and actin at the pointed ends probably contribute to maintaining the final lengths of the thin filaments.

Once expressed, Lmod2 competes with and displaces Tmod1 without capping the end of the actin filaments (Fig. 9B). We propose that the loss of Tmod1 capping results in longer thin filaments because both a reduction of Tmod1 levels and blocking its capping activity has previously been shown to lengthen thin filaments (Fig. 9C) (Gregorio et al., 1995; Sussman et al., 1998a). Since Lmod2 and Tmod1 cannot coexist at the pointed ends of the same filaments, a myocyte expressing both proteins would be predicted to contain two populations of thin filaments, a more stable population associated with Tmod1 and a more dynamic population associated with Lmod2. This model is consistent with the observation that thin filaments uniformly lengthen in chick cardiac explants as de novo myofibrillogenesis progresses (D. Rudy and C.C.G., unpublished data). Although we favor a competition model, we cannot rule out the possibility that overexpression of Lmod2 downregulates Tmod1 transcript or protein levels, which in turn results in the elongation of the thin filaments.

It is unclear how Lmod2 is able to bind to actin filament pointed ends without capping. One possibility is that Lmod2 associates and dissociates with the end so quickly that it cannot effectively cap. Another possibility is that the conformation of Lmod2 (e.g. via the presence of the WH2 domain, see below) or association with molecules other than actin (e.g. tropomyosin) positions it in such a way that it is ineffective at blocking actin exchange at the end of the filament. Although we can only speculate as to how Lmod2 can bind the pointed end without capping, our data clearly showed that the WH2 domain is involved in this phenomenon. Removal of only 26 amino acids that comprise the WH2 domain results in a molecule [Lmod2(1–514)] that can still displace Tmod1, but is also able to cap the end of the filament, resulting in a decrease in actin filament lengths in the cardiomyocytes (Figs 2–4). In vitro actin polymerization assays confirmed that the removal of the WH2 domain decreased the nucleation ability of Lmod2 (supplementary material Fig. S5), and inhibited actin polymerization and depolymerization in the presence of tropomyosin. Since removal of the WH2 domain alters the fundamental functional properties of Lmod2, it appears that the WH2 domain either changes the conformation of the entire molecule, thereby disrupting capping, or physically interferes with capping by binding F- or G-actin. The WH2 domain is predicted to form two short helices. However, preliminary analysis of the structure of Lmod2 by circular dichroism indicates that the removal of the WH2 domain results in a drastic decrease in helical content (larger than that predicted for the loss of the WH2 domain only) and an increase of random coil content (A.S.K., unpublished results). Thus, the helices that comprise the WH2 domain might form a tertiary structure with other secondary structure elements of full-length Lmod2.

Another remaining question is: what is responsible for mediating the competition between Tmod1 and Lmod2? One attractive candidate is tropomyosin, because full-length Lmod2, which cannot cap the pointed ends of the actin filaments, and therefore might bind actin differently or ineffectively, is still able to displace Tmod1. Although it is known that Lmod2 binds to various tropomyosin isoforms (Kostyukova, 2007), our data further show that Lmod2 binding affinity to the actin seeds increased in the presence of tropomyosin (Fig. 7) and that the actin-capping activity of Lmod2(1–514) is tropomyosin dependent (Fig. 8A). Therefore, like with Tmod1, the interaction of Lmod2 with tropomyosin is important for its assembly at the pointed end of the thin filaments.

Although the myocyte data clearly showed that Lmod2 associated with, and elongated, the thin filaments from their pointed ends, biochemical confirmation of the influence Lmod2 has on actin filament assembly at the pointed ends proved difficult because of the potent ability of Lmod2 to nucleate the polymerization of new actin filaments. Nevertheless, the preponderance of biochemical evidence indicates that Lmod2 can bind the pointed ends of actin filaments without capping them. First, the addition of Lmod2 to preformed actin filaments (gelsolin-capped seeds) resulted in a decrease of polymerization compared with the addition of Lmod2 to actin alone, suggesting that Lmod2 binds to the filaments. Second, the nucleating activity of Lmod2 was (1) inversely correlated with the number of preformed actin filaments that were present, and (2) only lost when Lmod2 was added at a molar concentration that was less than that of the preformed actin filaments both with and without tropomyosin. This suggests that Lmod2 either binds to the barbed (gelsolin) or pointed ends of the filaments. In addition, Lmod2 binding to these filaments did not decrease polymerization, suggesting that, if Lmod2 bound to the pointed ends, it lacked capping activity. This result is consistent with a previous report that indicated Lmod2 does not influence the rate of actin filament elongation at either the barbed or pointed ends (Chereau et al., 2008). Finally, addition of Tmod1 (which only interacts with the pointed ends of the actin filaments) was able to displace Lmod2 associated with the preformed filaments, confirming that Lmod2 interacts with the pointed ends of the actin filaments.

The function proposed for Lmod2 in this study is nearly identical to that described for the *Drosophila* protein sarcomere length short (SALS) (Bai et al., 2007). Although not a homolog of Lmod2, SALS contains two WH2 domains and a proline-rich region. It is only expressed in muscle tissue at a late stage of embryogenesis. SALS can bind G-actin and also associates with the pointed ends of the actin filaments. A knockdown or null mutation of SALS results in short thin filaments, whereas overexpression of this protein produces longer thin filaments from their pointed ends. Additionally, overexpression of tropomodulin in a null SALS background results in even shorter thin filaments than either the overexpression or null alone indicating that SALS antagonizes the

function of tropomodulin. It is also clear that SALS regulates the lengths of existing actin filaments rather than functioning in their initial assembly, because short thin filaments still form in the *sals*-null mutant. Therefore, Lmod2 might be a functional analog of SALS in vertebrates, which promotes thin filament elongation by antagonizing the capping activity of Tmod1.

Materials and Methods

Construct preparation

Chicken *LMOD2* was cloned from cDNA generated from chick cardiomyocyte cultures (supplementary material Fig. S1). For overexpression experiments, full-length and truncated *LMOD2* were inserted into pEGFP-C2 (Clontech) using *Xho*I and *Eco*RI restriction sites. These constructs were also inserted into a modified pEGFP-C2 in which EGFP was replaced with mCherry. For protein expression, constructs were made essentially as described (Chereau et al., 2008). Using PCR, a His tag (CATCATCATCATCATCAC) was added to the N-terminus, and a single alanine (GCA) to the C-terminus of Lmod2 (for efficient DTT-induced cleavage), and inserted between *Nco*I and *Sap*I within pTYB3 (New England BioLabs), which fuses an intein-tag to the C-terminus of Lmod2. All sequences were confirmed by DNA sequencing. GFP-tropomyosin was a generous gift from Jean-Claude Perriard (Helfman et al., 1999).

Protein expression and purification

Full-length Lmod2 and truncated Lmod2(1–514) were expressed and purified as described (Chereau et al., 2008). Following purification, proteins were exchanged into 20 mM Tris-HCl, pH 7.4, for biochemical analysis. Chicken pectoral muscle G-actin was purified from acetone powder and labeled with pyrenyl-iodoacetamide (Kostyukova and Hitchcock-DeGregori, 2004). Tmod1 was expressed in *Escherichia coli* BL21 (DE3) pLysS and purified according to published methods (Kostyukova et al., 2000). Protein purity was evaluated using SDS-PAGE (Laemmli, 1970). The concentrations were determined by using a BCA assay kit (Thermo Fisher Scientific) or by measuring their difference spectra in 6 M guanidine-HCl between pH 12.5 and pH 7.0 (Edelhoch, 1967), using the extinction coefficients of 2357 for tyrosine and 830 for tryptophan (Fasman, 1989).

Recombinant human gelsolin was a generous gift from John Hartwig (Brigham and Women's Hospital, Boston, MA). N-acetylated striated muscle α-TM, stTM (TM1a9a), was a generous gift from Sarah Hitchcock-DeGregori (RWJMS, Piscataway, NJ).

Fluorescence measurements

The rates of actin polymerization and depolymerization were measured as the change in pyrene actin fluorescence using a PTI fluorimeter (excitation, 366 nm, and emission, 387 nm, with a 3 nm slit), as described (Kostyukova and Hitchcock-DeGregori, 2004). To measure polymerization of actin at the pointed end, seeds (short filaments capped at the barbed ends with gelsolin) were prepared by polymerization of 3 μM G-actin in the presence of 30 nM gelsolin. Polymerization was monitored by the increase in fluorescence when the seeds were diluted fivefold with G-actin (10% pyrenylactin) in 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM NaN_3, 10 mM imidazole, pH 7.0. The initial concentrations of F- and G-actin after dilution were 0.6 μM and 1.1 μM respectively. Lmod2 (1–50 nM), Tmod1 (20 nM) and tropomyosin (1 μM) were added to the seeds before dilution with G-actin solution. For depolymerization experiments the seeds were diluted fivefold with P-buffer (100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole, pH 7.0). Tmod1 (20 nM), Lmod2 (1–20 nM) and/or tropomyosin (1 μM for polymerization experiments and 0.75 μM for depolymerization experiments) were added 5 minutes before dilution. Gelsolin-capped seeds were prepared in sets of four and fluorescence measurements were carried out in parallel in a four-cuvette holder with actin alone as a control in each set. Exponential curves were fitted to the polymerization or depolymerization data using SigmaPlot, and initial rates (R) were calculated as the first derivatives at time zero. At the end of each measurement, actin filaments polymerized in the presence of tropomyosin were pelleted at 60,000 r.p.m. (TLA-100.2, Beckman) for 20 minutes at 20°C, and washed with P-buffer. The pellet and supernatant compositions were analyzed by SDS-PAGE. Coomassie Blue (R250)-stained gels were scanned and the tropomyosin to actin ratio was calculated for each sample to determine whether the amount of tropomyosin was sufficient for saturation of actin filaments.

Cell culture and transfection

Cardiac myocytes were isolated from day 6 embryonic chick hearts, as described (Gregorio and Fowler, 1995). Isolated cells were plated on 12-mm-diameter coverslips for staining (1×10^6 cells/35mm dish). Myocytes were transfected with 0.1 μg plasmid DNA using Effectene (Qiagen). Transfection efficiencies were 20–50%.

Immunofluorescence microscopy

3–5 days after transfection, cells were incubated in relaxing buffer (150 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.4, 1 mM EGTA, and 4 mM ATP) for 15 minutes and fixed with 2% paraformaldehyde in relaxing buffer for 15 minutes. When indicated, the cells were extracted in cytoskeleton-stabilization buffer (10 mM PIPES, pH 6.8, 100 mM KCl, 300 mM sucrose, 2.5 mM $MgCl_2$, 0.5% Triton X-100 plus protease inhibitors) before fixation. Transfected myocytes were stained as described (Pappas et al., 2008). The primary antibodies included polyclonal anti-leiomodin2 (8 μg/ml) (Chereau et al., 2008), monoclonal anti-sarcomeric α-actinin (1:3000) (EA-53; Sigma), monoclonal anti-tropomodulin1 (m95, 5 μg/ml), and polyclonal anti-titin (Z1Z2, 4 μg/ml). Texas-Red- or Alexa-Fluor-488-conjugated Phalloidin (1:50) was used to stain F-actin. The secondary antibodies included Alexa Fluor 488 goat anti-mouse IgG (1:1000), Alexa Fluor 350 goat anti-mouse IgG (1:350), Alexa Fluor 350 goat anti-rabbit IgG (1:350) and Texas Red donkey anti-rabbit IgG (1:600). All coverslips were analyzed on a Deltavision RT system with 100× (1.3 NA) and 60× (1.4 NA) objectives and a CoolSnap HQ charge-coupled device camera (Photometrics) using softWoRx 3.5.1 software. Images were prepared for presentation using Photoshop® CS (Adobe Systems). Actin filament lengths were measured from images of cells using ImageJ 1.41 software (NIH).

In situ hybridization

Fertile chicken eggs were incubated in a humidified incubator at 38.5°C for 2–5 days. Embryos were collected into chilled saline (123 mM NaCl), removed from the vitelline membrane and cleaned of yolk. Extra-embryonic membranes and large body cavities (brain vesicles, atria, allantois, eye) were opened to minimize trapping of the reagents. Embryos were fixed overnight at 4°C in fresh 4% paraformaldehyde, rinsed in PBS, then in PBS plus 0.1% Tween-20 (PBT), and dehydrated (25, 50, 75, 100, 100%) into methanol before being cooled to –20°C overnight (or up to 10 days). Rehydration reversed this series. Embryos were rinsed in PBS and older embryos were treated with proteinase K: stages 11–13 and 14–18 at 10 μg/ml proteinase K for 10 and 20 minutes, respectively; stages 19 and older at 20 μg/ml proteinase K for 20 minutes. Embryos were rinsed in PBT to stop the digestion, and were then transferred to prehybridization solution. Antisense probe preparation and in situ hybridization was carried out as described (Nieto et al., 1996; Baker and Antin, 2003). Additional images showing Tmod1 and Lmod2 expression were deposited in http://geisha.arizona.edu.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/18/3136/DC1

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