Nebulin regulates the assembly and lengths of the thin filaments in striated muscle

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In many tissues, actin monomers polymerize into actin (thin) filaments of precise lengths. Although the exact mechanisms involved remain unresolved, it is proposed that "molecular rulers" dictate the lengths of the actin filaments. The giant nebulin molecule is a prime candidate for specifying thin filament lengths in striated muscle, but this idea has never been proven. To test this hypothesis, we used RNA interference technology in rat cardiac myocytes. Live cell imaging and triple staining revealed a dramatic elongation of the preexisting thin filaments from their pointed ends upon nebulin knockdown, demonstrating its role in length maintenance; the barbed ends were unaffected. When the thin filaments were depolymerized with latrunculin B, myocytes with decreased nebulin levels reassembled them to unrestricted lengths, demonstrating its importance in length specification. Finally, knockdown of nebulin in skeletal myotubes revealed its involvement in myofibrillogenesis. These data are consistent with nebulin functioning as a thin filament ruler and provide insight into mechanisms dictating macromolecular assembly.

Introduction

Sarcomeres, the contractile units of striated muscle, are composed of intricate filament systems that are precisely assembled during development and maintained in register throughout the lifetime of a myocyte. For example, numerous actin monomers and associated proteins polymerize into polar thin filaments whose barbed (fast-growing) ends are aligned and cross-linked by α-actinin in the Z-lines, the borders of sarcomeres. This elaborate structural network contributes to efficient force transmission along the length of the myofibril. The thin filament pointed (slow-growing) ends extend toward the middle of the sarcomere, the M-line, where they interdigitate with the thick filaments to generate contraction. Strikingly, the length distribution of the thin filaments is quite uniform within individual sarcomeres yet varies significantly in different muscle types, likely contributing to the distinct mechanical properties of muscle. The thin filaments are not static in nature but surprisingly dynamic (i.e., their exchange rates are high), especially at their pointed ends (Shimada et al., 1997; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001). Thus, the specification and maintenance of thin filament lengths are tightly regulated processes critical for muscle function. The molecular mechanisms responsible for this precision are just beginning to be elucidated.

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Abbreviations used in this paper: dsRNA, double-stranded RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Lat B, latrunculin B; siRNA, small interfering RNA; Tmod, tropomodulin.

Several studies investigating thin filament length regulation have focused on capping proteins, which prevent depolymerization and elongation of actin filaments in vitro (for review see Littlefield and Fowler, 1998). Capping protein (CapZ) caps the barbed ends of the filaments and likely contributes to their alignment within the Z-discs (Schafer et al., 1995). Tropomodulin (Tmod) is critical for maintaining the lengths of the thin filaments at their pointed ends (Weber et al., 1994; Gregorio et al., 1995), the region of the filament where highly regulated actin dynamics influence their mature lengths (Littlefield et al., 2001). In vitro and cell culture studies indicate that Tmod's interaction with actin prevents the filaments from elongating, whereas its interaction with tropomyosin prevents depolymerization (Gregorio et al., 1995; Mudry et al., 2003). The levels of Tmod present in the cell are critical for thin filament length regulation because its overexpression results in filament shortening, whereas its reduction causes filament elongation across the H zone, in the center of the sarcomere (Sussman et al., 1998a; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001). Interestingly, although it is known that a large portion (~40%) of Tmod is present in a soluble pool (Gregorio and Fowler, 1995), exactly how this molecule's dynamic properties and targeting to the pointed ends are regulated remains unclear.

Although pivotal for understanding thin filament length maintenance and dynamics, investigations into the capping proteins have not revealed how filament lengths are specified. It has long been proposed that molecular templates dictate thin fila-

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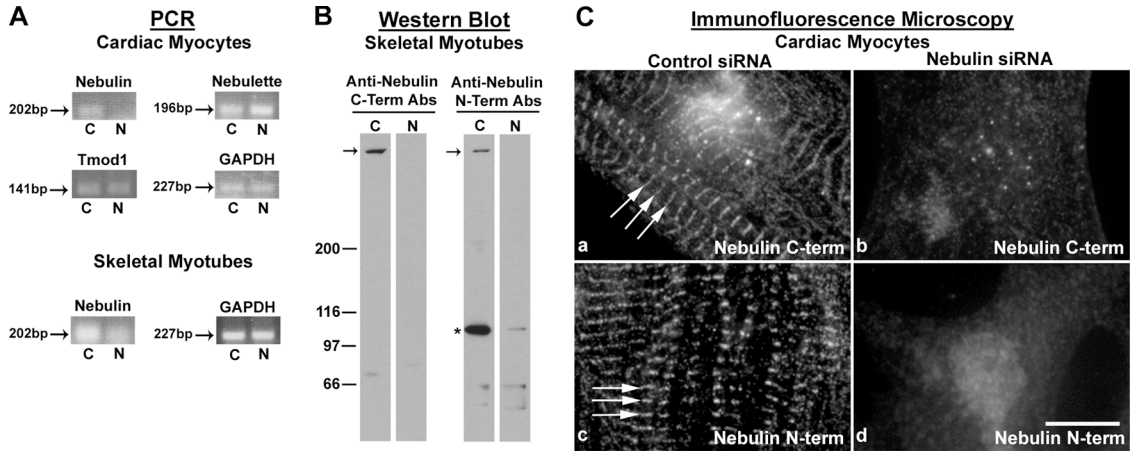

Figure 1. **Treatment of cardiac myocytes with nebulin dsRNA significantly reduces nebulin levels.** (A) A specific 202-bp nebulin product was amplified by RT-PCR from rat cardiomyocyte cDNA; this product was virtually undetectable within 24 h of siRNA treatment. In contrast, transcript levels for nebulette, Tmod1, and GAPDH were not decreased. In rat skeletal myotube cDNA, nebulin transcripts were reduced by ~60% after nebulin siRNA treatment, whereas GAPDH levels remained unchanged. (B) Western blotting revealed a decrease of >90% in nebulin protein levels from rat skeletal myocytes within 48 h of treatment with nebulin siRNA (N) compared with controls (C), using antibodies to the COOH- and NH_2-terminal regions of nebulin. Note that the NH_2-terminal nebulin antibodies also detected a band of ~100 kD in control lysates (asterisk), likely a degradation product from lysate preparation, consistent with a previous study (Kazmierski et al., 2003); this band is not detectable after nebulin siRNA treatment. (C) Immunofluorescence microscopy revealed bright, striated staining for the COOH- and NH_2-terminal regions of nebulin in control myocytes (a and c, arrows). The staining was virtually undetectable in >80% of myocytes treated with siRNAs to nebulin after ~24 h (b and d). Bar, 10 μm.

ment lengths, but their identities have remained elusive. Since its discovery over two decades ago (Wang and Williamson, 1980), the molecular properties of the giant protein nebulin (mol wt = 500–900 kD) have implicated it as a prime candidate to act as a ruler in specifying thin filament lengths in striated muscle. First, single molecules of nebulin span the entire length of the thin filaments: its COOH termini localize to the Z-lines, and its NH_2 termini extend to the thin filament pointed ends (Wang and Wright, 1988; Millevoi et al., 1998; McElhinny et al., 2001). Second, nebulin interacts with thin filament components, including tropomyosin, actin, troponins, and Tmod (Jin and Wang, 1991; Pfuhl et al., 1994; McElhinny et al., 2001). Third, nebulin's modular structure, composed of repeating motifs that are susceptible to alternative splicing events, suggests that its isoforms dictate thin filament architecture (Labeit and Kolmerer, 1995; Wang et al., 1996). Consistent with this idea, the molecular masses of nebulin isoforms correlate with thin filament lengths that differ among muscle fibers, during development, and with disease (for reviews see Trinick, 1994; McElhinny et al., 2003). Finally, many studies have reported that nebulin assembles in a striated pattern before the thin filaments attain their mature, defined lengths during myofibrillogenesis, which is also consistent with the idea that it restricts their lengths (Moncman and Wang, 1996; Shimada et al., 1996; Nwe et al., 1999). Although intriguing, these studies are based on correlative evidence because nebulin's massive size, coupled with the challenges of isolating it in its native form, has precluded definitive testing of its function.

In addition to its proposed role as a thin filament ruler, several investigations using recombinant nebulin fragments suggest the intriguing possibility that it may have other functions (for review see McElhinny et al., 2003). Nebulin may contribute to actin nucleation and enhance filament stability (Chen et al., 1993; Gonsior et al., 1998). Biochemical evidence also suggests that it regulates the interaction of actin with myosin in response to calcium levels, perhaps in cooperation with the tropomyosin–troponin complex (Root and Wang, 1994, 2001). Finally, although speculative, nebulin may function in myofibril-based signaling events because it contains an SH3 domain and Ser-rich domain in its COOH-terminal, Z-line region (Labeit and Kolmerer, 1995; Wang et al., 1996).

Given the fact that nebulin may have these critical functions, a conundrum that existed in the field for years was that nebulin was reportedly expressed in skeletal muscle but not in heart or other tissues (Stedman et al., 1988; Labeit and Kolmerer, 1995; Zhang et al., 1996). Cardiac muscle contains nebulette, a smaller (~107 kD) protein that has nebulin-like motifs but is encoded by a separate gene (Moncman and Wang, 1995; Millevoi et al., 1998). If extended, nebulette would span ~25% of the lengths of the mature thin filaments and would not be expected to function in length specification. The apparent absence of nebulin in cardiac muscle was puzzling because although the thin filament lengths are more variable than those in skeletal muscle (Robinson and Winegrad, 1979), the length distributions in both tissues are Gaussian, not exponential, as actin filaments in vitro have been observed to be (for review see Littlefield and Fowler, 1998). In resolution to this conundrum, nebulin recently was detected in cardiac muscle in a molecular layout identical to skeletal muscle nebulin, although at lower levels (Fock and Hinssen, 2002; Kazmierski et al., 2003; Donner et al., 2004).

We investigated the functional properties of nebulin in living myocytes. Using RNA interference technology, we found that the preexisting thin filaments in rat cardiomyocytes dramatically elongated from their pointed ends immediately upon nebulin knockdown. Other myofibril components, including the barbed end marker α-actinin, were unaffected. When the thin filaments were depolymerized by latrunculin B (Lat B),

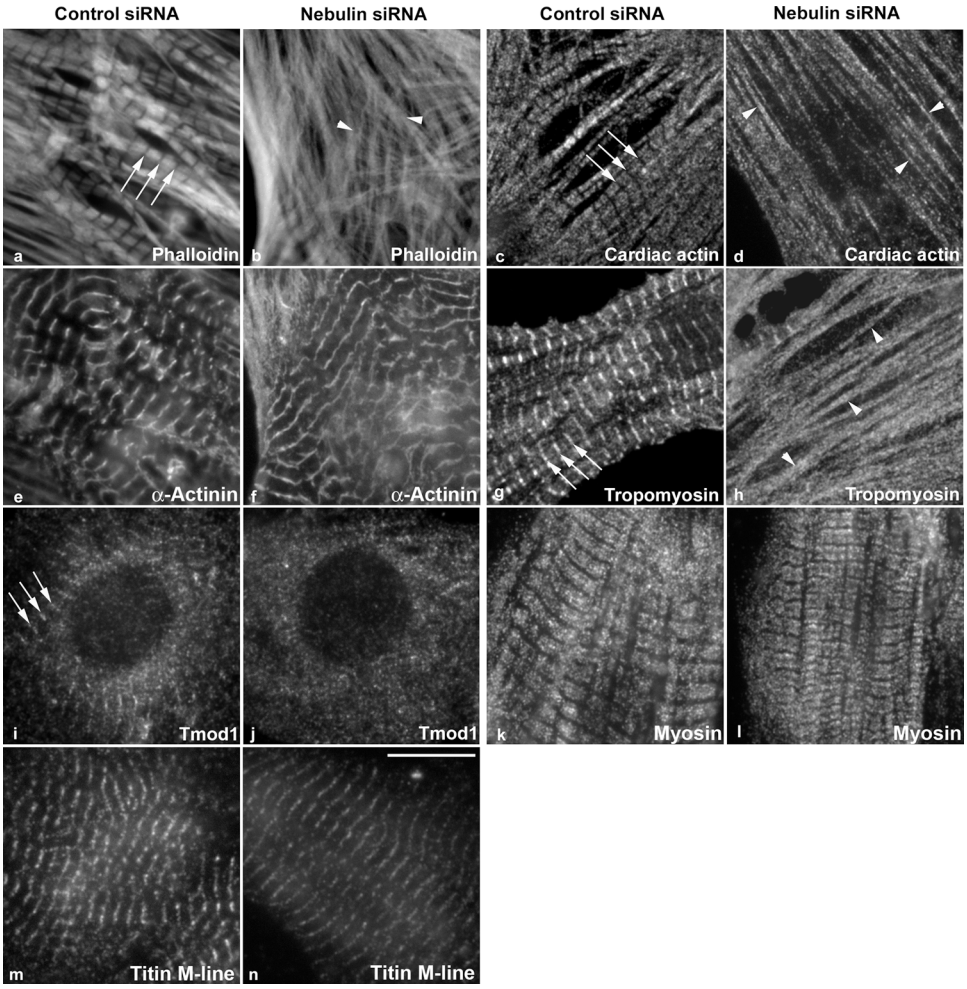

Figure 2. **Nebulin is critical for maintaining thin filament lengths from their pointed ends in cardiomyocytes.** After 24 h of siRNA treatment, cardiomyocytes were stained with phalloidin to label F-actin (a and b) and with antibodies to cardiac actin (c and d), α-actinin (e and f), tropomyosin (g and h), Tmod1 (i and j), myosin (k and l), and the M-line region of titin (m and n). Note that the staining patterns with both cardiac actin and tropomyosin antibodies are typical in control myocytes. Thin filament components appeared nonstriated in myocytes with reduced nebulin levels (b, d, and h), yet the barbed ends appeared unaffected (f), suggesting that the thin filaments had elongated from their pointed ends across the H zone. Note that myocytes were double stained with phalloidin and for α-actinin (a, e, b, and f). In contrast, the thin filaments in control siRNA–treated myocytes demonstrated regular, striated staining patterns (a, c, and g). Tmod1 staining was absent from the myofibrils in nebulin siRNA–treated myocytes (j). In control and nebulin siRNA–treated myocytes, thick filaments (k and l) and titin (m and n) appeared unaffected 24 h after treatment. Arrows mark striated thin filament components, and arrowheads mark nonstriated thin filament components. Bar, 10 μm.

they reassembled to unrestricted lengths in the absence of nebulin. Finally, knockdown of nebulin in skeletal myocytes delayed myofibril assembly. Thus, a primary function for nebulin is in thin filament length regulation and assembly, and our data support the hypothesis that this giant molecule acts as a ruler to specify thin filament lengths.

Results

Nebulin levels are significantly reduced after treatment with small interfering RNAs (siRNAs)

To investigate the function of nebulin, we designed four independent, synthetic double-stranded RNAs (dsRNAs) to specifically target the rat nebulin sequence, three of which significantly reduced its levels in both cardiac and skeletal myocytes (Fig. 1). RT-PCR studies from rat cardiac myocyte cDNA determined that nebulin mRNA transcripts had decreased by >90% within 24 h of treatment, compared with control dsRNA–treated myocytes (Fig. 1 A). The nebulin mRNA reduction was specific because other transcripts examined, including nebulette, Tmod1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were not decreased (Fig. 1 A). To visualize the reduction of nebulin protein levels on an individual cardiomyocyte basis, we used three independent, well-characterized antinebulin antibodies in immunofluorescence microscopy studies. We determined that the staining for nebulin was virtually undetectable in >80% of cardiomyocytes ~24 h after treatment with nebulin dsRNAs, compared with myocytes treated with control dsRNAs that exhibited bright, striated nebulin staining (Fig. 1 C; cells were double stained with antisarcomeric α-actinin antibodies to distinguish myocytes [not depicted]). Unfortunately, our attempts to quantify nebulin protein levels by Western blot analysis in the cardiac myocytes proved unsuccessful. This was likely the re-

sult of several factors, including nebulin's susceptibility to proteolysis and the difficulty of transferring it to membranes (Granzier and Wang, 1993), combined with the lower levels of nebulin expressed in cardiac relative to skeletal myocytes (Kazmierski et al., 2003), and the variable number of contaminating fibroblasts in the cultures. We were, however, successful in determining that skeletal myocytes treated with the same nebulin dsRNAs exhibited a >90% reduction in nebulin protein levels by Western blot analysis (Fig. 1 B; see section Nebulin is involved in de novo myofibrillogenesis). Together, our data demonstrate that the nebulin dsRNAs significantly reduced nebulin gene expression by acting as siRNAs.

Nebulin is required to maintain thin filament lengths in cardiac myocytes

After 24 h of treatment of cardiomyocytes with nebulin siRNA, we relaxed and fixed the cells and performed immunofluorescence microscopy to examine the thin filaments and other myofibril components. These studies revealed the striking appearance of a vast majority of thin filaments that contained no distinguishable gaps at the H zone, suggesting that their lengths were unrestricted at their pointed ends after nebulin knockdown (Fig. 2). In contrast, control myocytes contained typically striated actin filaments with clearly distinguishable pointed ends and H zones, indicating that they were of defined lengths. These data were revealed by staining with phalloidin, which labels F-actin, and confirmed with antibodies to cardiac actin and tropomyosin (Fig. 2, a–d, g, and h). Importantly, double and triple staining protocols that included staining for α-actinin, a marker of thin filament barbed ends in the Z-lines (Littlefield et al., 2001), revealed its normal, striated organization in the identical myofibrils containing elongated thin filaments (Fig. 2 f). These data suggest that the filaments had elongated from their pointed ends. Consistent with this observation, the pointed end capping protein Tmod1 was absent from the myofibrils in nebulin siRNA–treated myocytes (Fig. 2 j) despite normal expression levels (Fig. 1 and not depicted). Thus, nebulin is required for the physiological localization of Tmod1, and the two proteins likely function coordinately in thin filament length regulation (see Discussion). Finally, double and triple staining revealed that the thick and titin filaments appeared in their striated organizations in myocytes containing reduced nebulin levels and elongated thin filaments (Fig. 2, k–n; and not depicted), revealing that nebulin is not required for thick filament or titin architecture. Together, these data indicate that nebulin is specific and essential for maintaining the lengths of the thin filaments.

To analyze this phenotype further, we cotransfected siRNA-treated cardiac myocytes with GFP-tropomyosin, which assembles along the entire length of the thin filaments. This strategy allowed us to track thin filaments within individual myofibrils in real time, before and during nebulin knockdown. Within 16–19 h of treatment, myocytes treated with nebulin siRNA contained myofibrils with typically striated GFP-tropomyosin (Fig. 3 c). However, within 21 h, the thin filaments began to elongate from their pointed ends in many of the identical myofibrils in treated cells (Fig. 3 d). Importantly, observation of myocytes treated with nebulin siRNA revealed that their contractile activity

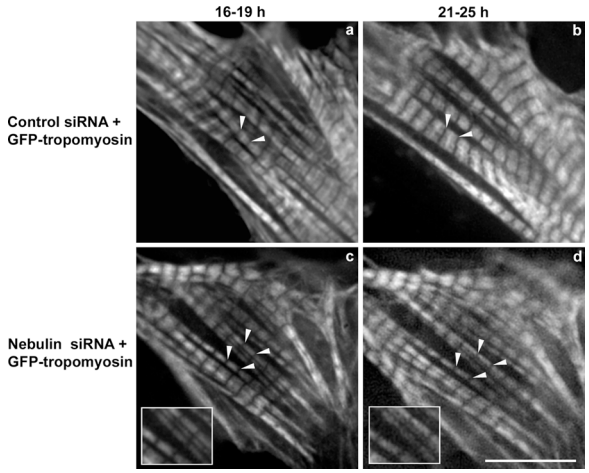

Figure 3. **Real-time imaging using GFP-tropomyosin revealed thin filament elongation after nebulin siRNA treatment.** (A) Cardiomyocytes were cotransfected with siRNAs and GFP-tropomyosin and imaged in real time. Within ~21 h, some GFP-tropomyosin–labeled thin filaments appeared to elongate from their pointed ends in nebulin siRNA–treated myocytes (d). In contrast, the thin filaments did not appear to change in myocytes treated with control siRNA (a and b). Arrowheads mark the pointed ends of the thin filaments, and the inset is a magnified view of those filaments. Bar, 10 μm.

was reduced once GFP-tropomyosin began to lose its striations, whereas control myocytes beat normally (unpublished data).

We next relaxed and fixed siRNA-treated cells to quantify thin filament alterations at the time points determined from the live cell experiments. Within ~21 h of treatment, we first observed that the majority of nebulin siRNA–treated myocytes exhibited a reduction in nebulin staining and contained a mixture of three types of thin filaments (Fig. 4 A). Filaments with distinct gaps at the H zones ("gaps;" Fig. 4 A, d, long arrow) and those with barely discernible gaps ("slight gaps;" Fig. 4 A, d, short arrow) were associated with dim staining for nebulin. Thin filaments lacking detectable gaps at the H zone ("no gaps;" Fig. 4 A, d, arrowhead) were devoid of nebulin staining. Strikingly, all three types of filaments contained regularly striated barbed ends (Fig. 4 A, f), again suggesting that the filaments elongated from their pointed ends.

To confirm these observations, the pixel intensities of phalloidin staining along the filaments were quantified (Fig. 4 B). Nebulin siRNA–treated filaments with distinct gaps (Fig. 4 B, right, blue; mean = 0.88 μm) and slight gaps (red; mean = 0.91 μm) were measurably longer from their Z-lines to their pointed ends, compared with controls (Fig. 4 B, left; mean = 0.68 μm). Thin filaments with no distinguishable gaps (Fig. 4 B, right, green) had elongated so that they overlapped with thin filaments from the opposing sarcomeres (note the peak in pixel intensity at the H zone). These results were not attributable to hypercontraction of the myofibrils because the Z-to-Z-line distances in all the nebulin siRNA–treated groups of filaments were similar or in fact longer (mean = 1.97 μm for gaps, 2.04 μm for slight gaps, and 2.00 μm for no gaps) than the controls (mean = 1.80 μm). Finally, most thin filaments contained no distinguishable gaps >24 h after transfection, corresponding to when nebulin staining levels were virtually undetectable (Fig. 1 C and Fig. 2 b).

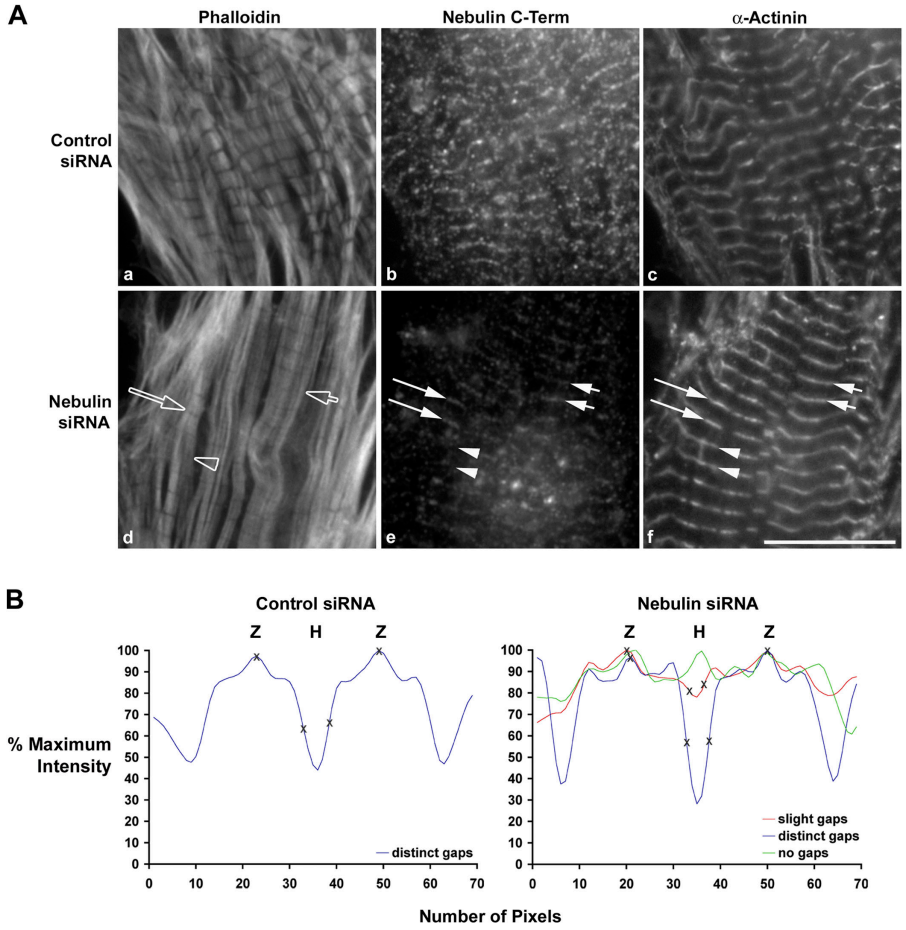

Figure 4. **Analysis at ~21 h after siRNA treatment revealed that thin filament elongation correlates with a reduction in nebulin staining intensity.** (A) Myocytes were relaxed, fixed, and triple stained with phalloidin (a and d) for the COOH-terminal region of nebulin (b and e), and for α-actinin (c and f). Control myocytes contained normal, striated thin filaments (a), bright nebulin staining (b), and normal α-actinin (c). In contrast, the majority of nebulin siRNA–treated myocytes exhibited dimmer or undetectable nebulin staining (e) and contained a mixture of thin filaments differing in their extent of elongation from their pointed ends (d): the barbed ends of the filaments in the identical myofibrils remained unaffected (f). (d) The thin filaments contained no visible H zones (no gaps; arrowhead), narrow H zones (slight gaps; short arrow), or more distinct H zones (gaps; long arrow). The thin filaments with no gaps were devoid of detectable nebulin staining (e, arrowheads) but contained normal α-actinin staining in the identical sarcomeres (f, arrowheads), whereas those with slight or distinct gaps contained dim nebulin staining compared with controls (e, long and short arrows). The long arrows, short arrows, and arrowheads mark identical myofibrils, including the Z-line and M-line regions. (B) Plotting the pixel intensities along the lengths of the three different thin filament arrays (via phalloidin staining) revealed quantitative differences in their distributions. Nebulin siRNA–treated thin filaments containing distinct gaps were measurably longer (right, blue; from Z-line to pointed end = 0.87 μm) compared with controls (left; from Z-line to pointed end = 0.68 μm), whereas those with only slight gaps (right, red) were even more elongated (from Z-line to pointed end = 0.92 μm). Finally, the filaments with no visible gaps (right, green) elongated so that they overlapped with the opposing thin filaments in the H zone (note the peak in pixel intensities). The pixel intensities were averaged and plotted as a percentage of the maximum intensity (the Z-lines). The x's mark values that equal the mean half-maximum thin filament lengths. Bar, 10 μm.

Together, these studies indicate that nebulin knockdown and thin filament elongation did not occur synchronously within individual myofibrils; i.e., thin filaments with detectable nebulin staining began to elongate from their pointed ends, whereas filaments lacking detectable nebulin were unrestricted in their lengths. Thus, proper nebulin levels are needed to maintain the mature lengths of the thin filaments in cardiac myocytes.

Nebulin is required for thin filament reassembly after depolymerization

So far, the results have indicated that nebulin is required for thin filament length maintenance because the filaments were already in their normal, striated pattern before nebulin was knocked down (unpublished data). Therefore, we aimed to determine whether nebulin is also involved in thin filament assembly. We first treated myocytes with nebulin siRNA for 18 h and then added Lat B for 4 h to specifically depolymerize the thin filaments. At this time, only remnants of total cellular actin filaments were visible by phalloidin staining, whereas cardiac actin, nebulin, and Tmod1 staining were diffuse throughout the cytoplasm in both control and nebulin siRNA–treated myocytes (Fig. 5, a–h). Not surprisingly, in >70% of both populations of myocytes, α-actinin was severely perturbed (Fig. 5, i and j), indicating that the thin filaments had completely depolymerized (i.e., both barbed and pointed ends were affected). In contrast, no discernible effects on titin or thick filament integrity were detected (Fig. 5, k–n), indicating that the thin filaments specifically were depolymerized by Lat B treatment. It also should be noted that no difference in the rates of depolymerization were observed between the nebulin and control siRNA–treated myocytes, suggesting that nebulin expression is not immediately essential for thin filament stability.

4 h after Lat B was removed to allow for thin filament reassembly, double and triple staining revealed that cardiac myocytes with reduced levels of nebulin contained thin filaments that were dramatically elongated (with no distinguishable pointed ends) as observed by staining with phalloidin and for cardiac actin (Fig. 6, b and d). The thin filaments also lacked assembled Tmod1 (Fig. 6 h) and were not beating (not depicted); this persisted for >24 h after wash out. In contrast, most myocytes treated with control siRNA assembled thin filaments to defined lengths, as exhibited by the striated staining patterns for nebulin, phalloidin, cardiac actin, and Tmod1 (Fig. 6, a, c, e, and g), and were beating normally. Furthermore, 4 h after wash, nebulin siRNA–treated myocytes did not reassemble α-actinin into its normal striated organization (Fig. 6 j and Fig. 7 d), yet double staining for the NH_2-terminal titin Z1-Z2 modules remained rela-

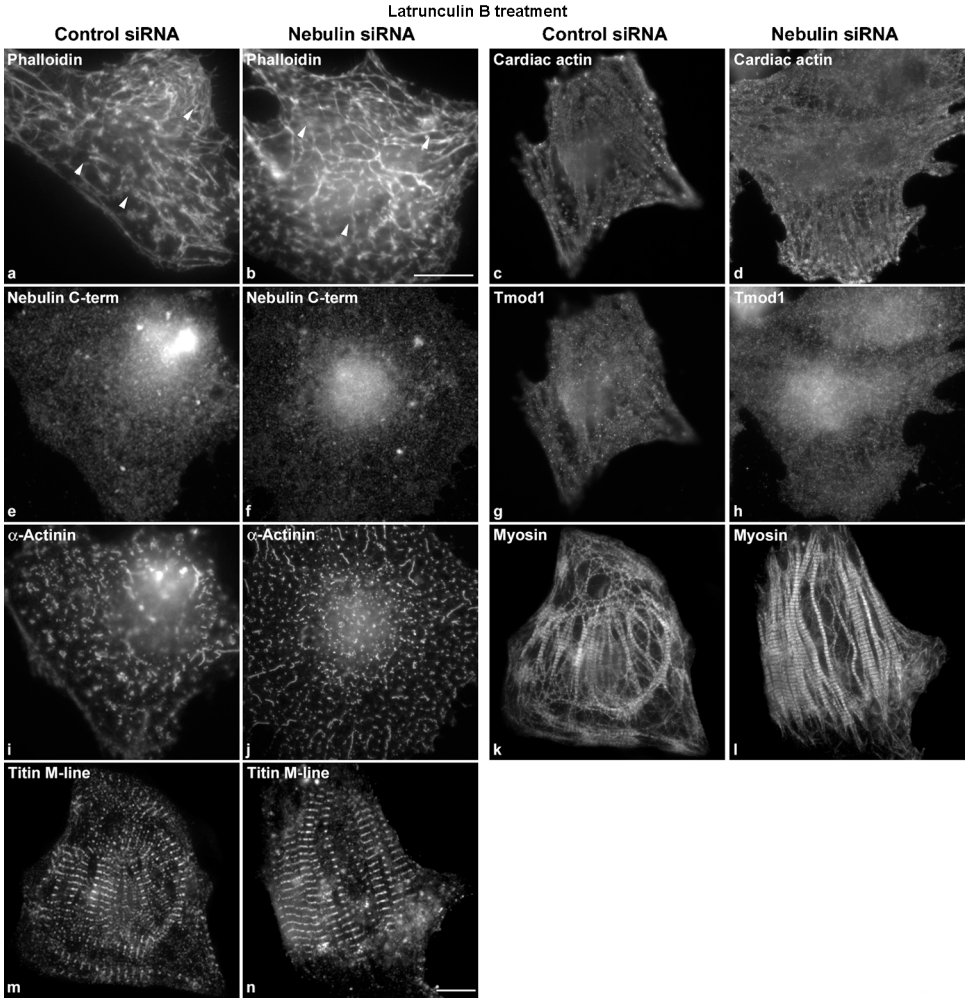

Figure 5. **Thin filaments in cardiomyocytes are specifically depolymerized by Lat B treatment.** Myocytes were treated with control or nebulin siRNA for ~18 h, followed by Lat B for 4 h. Lat B depolymerized thin filament components in both populations of myocytes, as observed by remnants of F-actin by phalloidin staining (a and b, arrowheads) and diffuse staining for cardiac actin (c and d), nebulin (e and f), and Tmod1 (g and h). α-Actinin also appeared severely perturbed in the majority of myocytes (i and j), indicating that the thin filaments were completely depolymerized at the barbed ends, whereas titin and myosin appear unaffected in Lat B–treated myocytes (k–n). Note that the following myocytes were double stained: c and g; d and h; e and i; f and j; k and m; l and n. Bars, 10 μm.

tively organized in many of the identical myofibrils (Fig. 7 b). These data indicate that the lack of nebulin expression after Lat B treatment did not globally perturb Z-line structure but specifically affected the barbed ends of the thin filaments. (It should also be noted that nebulin siRNA–treated myocytes not treated with Lat B displayed well-organized α-actinin yet contained elongated thin filaments at this time point, as in Fig. 2 [b and f].) Finally, in both the control and nebulin siRNA–treated myocytes, the M-line region of titin and the thick filaments remained striated (Fig. 6, k–n). Together, these data indicate that nebulin expression is required specifically for thin filament components to assemble both in their proper organization at their barbed ends and to their proper lengths at the pointed ends.

Nebulin is involved in de novo myofibrillogenesis

Our final studies were designed to investigate the role of nebulin during de novo myofibrillogenesis. We used primary cultures of fetal rat skeletal myocytes, derived from muscle satellite cells that withdraw from the cell cycle, fuse, and differentiate into myotubes. Within 48 h after nebulin siRNA treatment of fusing myoblasts, significant reductions in nebulin mRNA (~60%) and proteins levels (~95%) were observed by RT-PCR and Western blot analysis, respectively (Fig. 1, A and B), which is consistent with a reduction in nebulin staining intensity (Fig. 8, e and f).

Strikingly, throughout the culture period (~5 d), myotubes with reduced nebulin levels remained less mature than control myotubes (e.g., by day 5, nebulin siRNA–treated myotubes contained <15 nuclei, whereas control myotubes contained >30 nuclei). Double staining with phalloidin and for α-actinin, myosin, and titin revealed that myotubes with reduced nebulin staining did not assemble striated thin filaments, Z-lines, titin, or thick filaments during the 5 d in culture (Fig. 8, b, d, h, and j). In contrast, control myotubes contained thin filaments of defined lengths, and regularly striated Z-lines, thick filaments, and titin staining (Fig. 8, a, c, g, and i). Together, our data indicate that nebulin plays a role in thin filament length regulation and is involved in myofibril assembly.

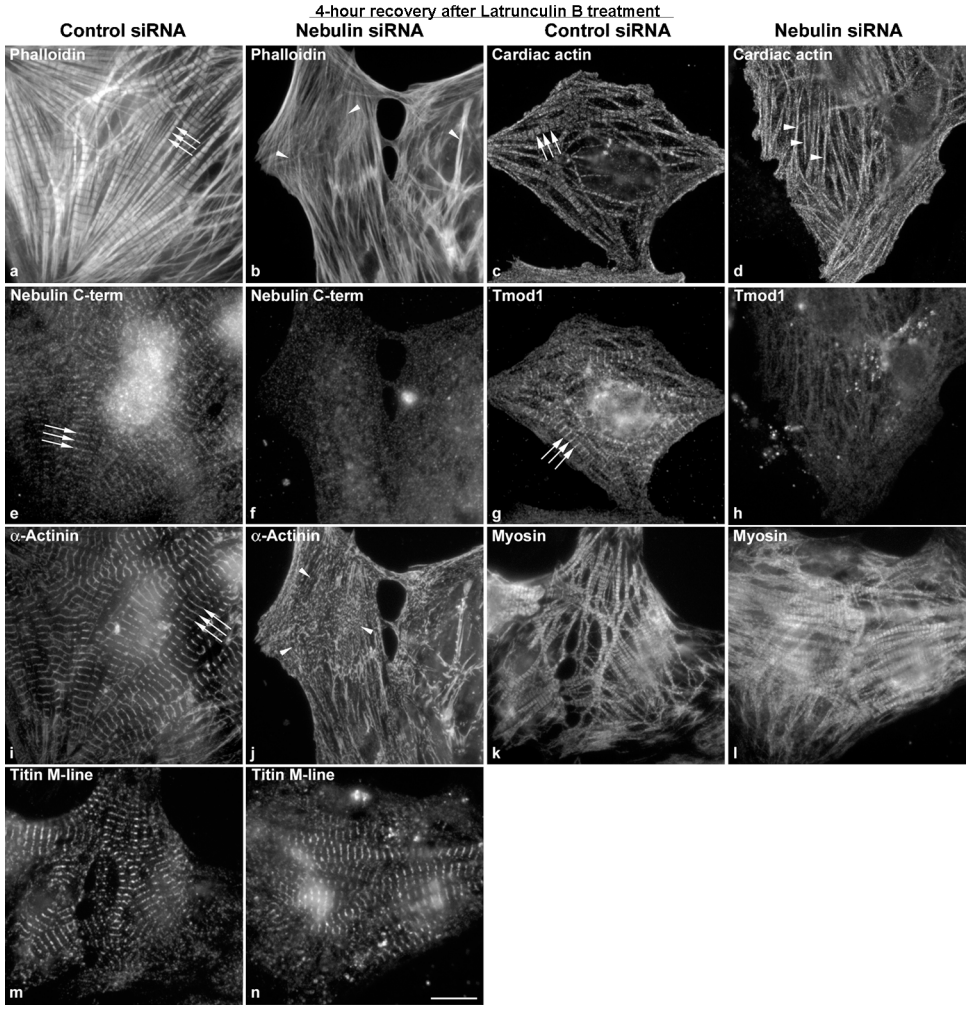

Figure 6. **Nebulin is required for proper thin filament assembly after depolymerization.** After depolymerization, Lat B was washed out and the thin filaments were allowed to reassemble in the presence (control siRNA) or absence (nebulin siRNA) of physiological nebulin levels. Within 4 h of wash out, control thin filaments repolymerized to their mature lengths as shown by striated phalloidin (a), cardiac actin (c), nebulin (e), and Tmod1 (g) staining, and they contained normal α-actinin organization (i, arrows). In contrast, nebulin siRNA–treated myocytes contained reduced nebulin (f), nonstriated phalloidin (b), and cardiac actin (d) staining; an absence of striated Tmod1 (h); and severely perturbed α-actinin (j, arrowheads). Titin and myosin remained organized in their striated distribution pattern (k–n). Note that some myocytes were triple stained (a, e, and i; b, f, and j) and some were double stained (c and g; d and h; k and m; l and n). Bar, 10 μm.

Discussion

Nebulin is required for the proper assembly of thin filaments, maintenance of their lengths, and contractile function in cardiac myocytes

In this study, we determined that nebulin has critical roles in thin filament length regulation in cardiomyocytes. Specifically, nebulin is required for proper thin filament assembly and length maintenance. These observations are consistent with nebulin's long proposed role as a ruler that dictates thin filament architecture.

Our data reveal that although thin filament components polymerize in the absence of nebulin, the giant molecule is required for restricting the thin filaments to their proper lengths at the pointed ends. Importantly, it is the pointed ends of the filament that undergo dynamic exchange of the actin monomers Tmod1 and tropomyosin (Suzuki et al., 1998; Michele et al., 1999; Littlefield et al., 2001). Not surprisingly, then, the pointed ends are where the variability in mature thin filament lengths is apparent and where mechanisms for length determination and regulation have been indicated (Robinson and Winegrad, 1979; Littlefield et al., 2001; Luther et al., 2003). Based on data to date, we propose that one important mechanism by which nebulin participates in thin filament length regulation is that its NH_2-terminal region, which contains a high affinity binding site for Tmod1 (McElhinny et al., 2001), targets Tmod1 to the pointed ends. Thus, immediately upon nebulin knockdown, the thin filaments became permanently "uncapped" at their pointed ends and elongate across the H zone. In fact, thin filament elongation across the H zone is known to occur upon inhibition of Tmod's capping activity (Gregorio et al., 1995) and upon reduction of Tmod levels (Sussman et al., 1998b). It is likely that nebulin specifies thin filament lengths and then regulates Tmod dynamics for maintaining and "fine-tuning" the lengths of the filaments. In this regard, mounting evidence has led to the proposal

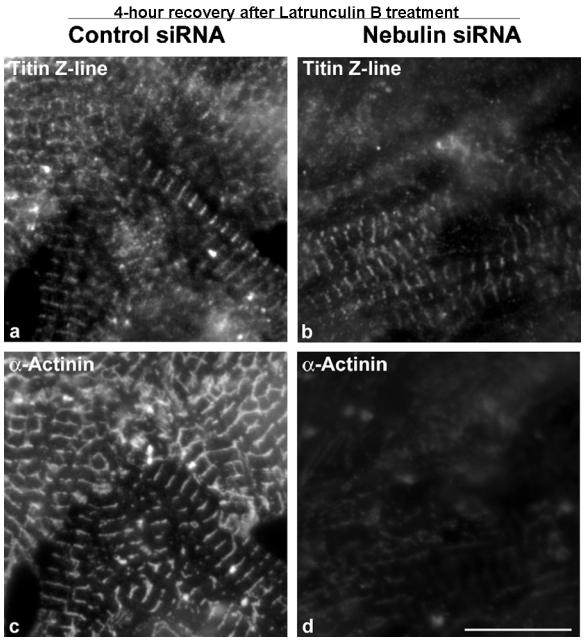

Figure 7. **The thin filament barbed ends are specifically perturbed in the absence of nebulin after Lat B treatment.** Cardiac myocytes were double stained with antibodies against α-actinin, a marker of the thin filament barbed ends, and titin's Z-line region, 4 h after wash out of Lat B. Although nebulin siRNA–treated myocytes contained severely perturbed barbed ends (d), titin's Z-line region remained intact in many of the same myofibrils within the identical myocytes (b), indicating that nebulin is specifically required for thin filament barbed end reassembly. Bar, 10 μm.

that thin filament length specification is not simply a one-time event (Littlefield et al., 2001). Instead, the ongoing, coordinated activity of caps (Tmod and CapZ), a ruler (nebulin), and potentially other proteins that regulate thin filament dynamics are likely involved in linking length specification with maintenance during development and throughout the lifetime of the cell.

Our studies using Lat B to depolymerize the preexisting thin filaments also implicate nebulin as an essential regulator of thin filament assembly. In the absence of proper nebulin levels, depolymerized thin filament components were unable to reassemble to their uniform lengths and lacked assembled Tmod1. These data are also consistent with the idea of nebulin's role in specification of their mature lengths and targeting Tmod1 to their pointed ends for maintenance. However, our studies also indicate that nebulin plays a critical role in the assembly and organization of thin filament barbed ends because nebulin siRNA–treated myocytes were unable to reorganize α-actinin into its normal, striated pattern after Lat B treatment. This important finding raises many questions concerning nebulin's mechanism of action in the Z-lines.

Why is nebulin required for barbed end reassembly but appears to be nonessential for barbed end structural maintenance in the preexisting thin filaments? One idea is that nebulin is critical to the initial assembly of I-Z-I bodies (precursor Z-line and thin filament structures; Ojima et al., 2000). It has been hypothesized that these structures are more dynamic and compliant (i.e., contain unsaturated binding sites) than the tightly packed, mature Z-lines (Schroeter et al., 1996; Ojima et al., 2000). Thus, the thin filament components depolymerized by Lat B may have been rendered to a configuration similar to their organization in I-Z-I bodies (consistent with their staining patterns; Fig. 6 j and Fig. 7 d), and may require nebulin's binding sites and/or potential signaling capabilities in its COOH terminus to attain their mature structure. This is particularly interesting given the fact that proteins containing SH3 and Ser domains are implicated in the assembly of specialized cytoskeletal structures (for reviews see Mayer, 2001; Kioka et al., 2002) and these domains in nebulin's Z-line region have long been proposed to function in assembly (for review see McElhinny et al., 2003).

In contrast, in the preexisting thin filaments, the barbed ends may have been securely "bolted down" via interactions with other sarcomeric components in mature Z-lines. Thus, nebulin may not be immediately required for maintaining their stability. For example, nebulette, which is anchored in the Z-lines (Moncman and Wang, 1995), may have compensated for the loss of nebulin. Consistent with this idea, both nebulin and nebulette SH3 domains bind myopalladin, which also interacts with α-actinin, perhaps forming a docking site for the barbed ends (Bang et al., 2001). In fact, SH3 domains are known to exhibit broad binding specificity for several target proteins (Buday, 1999; Mayer, 2001). It should be emphasized, however, that the barbed ends maintained their structure in the absence of nebulin for only a few hours after elongation from their pointed ends was first observed, eventually widening and then becoming less defined (unpublished data). Thus, we cannot rule out that a reduction of nebulin influenced barbed end dynamics or stability in the preexisting thin filaments, even though α-actinin organization was not immediately affected. Finally, it is important to note that nebulin knockdown perturbed thin filament reassembly at both barbed and pointed ends after Lat B treatment yet did not affect the integrity of the thick filaments or titin's Z-line region (Figs. 6 and 7). This is consistent with studies demonstrating that the integrity of titin and thick and thin filaments are not dependent on each other in cardiomyocytes (Linke et al., 1999; McElhinny et al., 2002; Moncman and Wang, 2002).

Is nebulin a ruler for thin filament length regulation?

Our studies support the long-proposed hypothesis that nebulin is a thin filament ruler to specify the uniform thin filament lengths in muscle. We favor the following model for nebulin's role in thin filament length regulation: (a) Nebulin assembles early in myofibrillogenesis (Moncman and Wang, 1996; Shimada et al., 1996) and aids in aligning and organizing thin filament components at their barbed ends. This may occur in cooperation with CapZ and α-actinin. (b) Nebulin isoform sizes specify the precise lengths of the thin filaments. (c) Nebulin's NH_2-terminal modules target Tmod to the pointed ends of mature thin filaments, where it functions as a dynamic cap to maintain their specified lengths. (d) Nebulin and Tmod1 work together to maintain (and perhaps fine-tune) the lengths at the pointed ends throughout the lifetime of the myocyte.

This model incorporates a myriad of biochemical and structural data suggesting that nebulin's modules dictate the

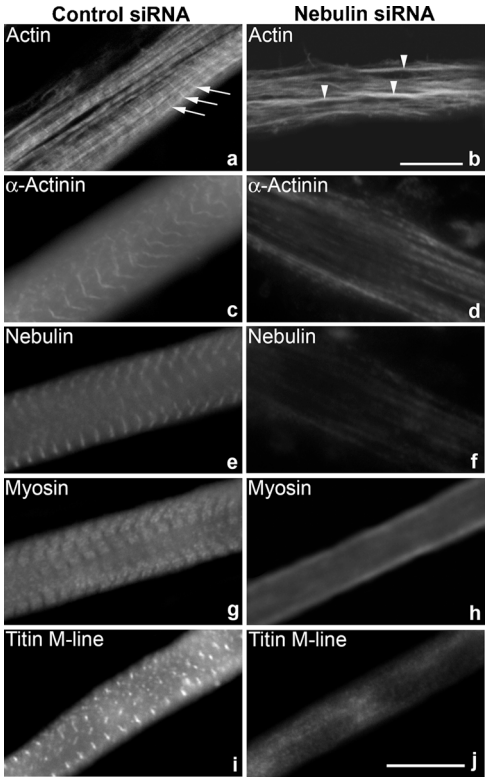

Figure 8. **Nebulin is involved in de novo myofibrillogenesis in rat skeletal myotubes.** Nebulin staining was significantly reduced in nebulin siRNA–treated myotubes (f), compared with control myotubes (e). By 5 d, control myotubes contained thin filaments (arrows) of defined lengths with clear H zones (a), whereas myotubes with reduced nebulin levels contained nonstriated thin filaments (b, arrowheads). Additionally, myotubes with reduced nebulin levels did not assemble Z-lines (d) and remained thinner and shorter in comparison to control myotubes (c). Myotubes with reduced nebulin also demonstrated overall delayed myofibril assembly because the distribution of myosin (h) and titin (j) never organized into striated myofibrils, whereas control myotubes that contained myosin (g) and titin (i) organized into striated patterns within 5 d. Bars, 10 μm.

number of actin monomers per thin filament. However, we cannot rule out the possibility that nebulin functions in thin filament assembly and length regulation via another mechanism. For example, nebulin may mediate the activities or expression of downstream molecules required for actin filament length regulation. Another possibility is that perturbation of nebulin and/or its associated proteins may alter the critical concentration for actin polymerization, which would change thin filament lengths via thermodynamic and kinetic considerations. Definitively proving a ruler function requires complex, future studies using model systems expressing mutant nebulins, both larger and smaller.

Important questions remain concerning our model and the results from our investigations. First, how are nebulin isoform sizes controlled during development and in different muscle tissues? Perhaps chronic demands placed on muscles modulate nebulin's splicing machinery via nebulin-interacting proteins proposed to affect gene expression. Additionally, what is the mechanism by which the contractile activity of cardiomyocytes was severely inhibited upon nebulin knockdown? We hypothesize that this resulted from the elongated thin filaments, which crossed the H zone and thus were of opposite polarity with respect to the thick filaments from the opposing half-sarcomere. This idea is consistent with studies suggesting that misregulated thin filament lengths affect contractile activity (Gregorio et al., 1995; Sussman et al., 1998a; Mudry et al., 2003). Another possibility is that absence of nebulin disrupted the positioning of the tropomyosin–troponin complex along the thin filament, an idea based on the proposal that nebulin dictates the architecture of the regulatory complex (Labeit and Kolmerer, 1995; Wang et al., 1996). On the other hand, nebulin may participate in contractile regulation more directly. For example, fragments of nebulin affect actin sliding over myosin filaments in vitro (Root and Wang, 1994, 2001). Clearly, further investigations into nebulin's role in contractile regulation are warranted.

Nebulin is involved in myofibrillogenesis

Our final experiments revealed that, upon nebulin knockdown during myofibrillogenesis in skeletal myotubes, no components examined assembled into mature, striated patterns during the 5 d in culture. Therefore, nebulin is involved in the formation of myofibrils during differentiation. Given the reduction of the I-Z-I complexes in myocytes with reduced nebulin, as demonstrated by staining for α-actinin and other thin filament components (Fig. 8), it is not surprising that myofibril assembly was abrogated (Ojima et al., 2000). Our data is consistent with studies revealing that perturbation of key sarcomeric components is deleterious to normal muscle differentiation. For example, the indirect flight muscles of flies defective in myosin lack thick filaments, and their myofibrils are perturbed (Mogami et al., 1986; O'Donnell and Bernstein, 1988). Furthermore, a functional knockout of titin impairs thick filament formation and blocks myofibrillogenesis (van der Ven et al., 2000).

However, other reports suggest that thick and thin filament assembly occur independently in skeletal myocytes. For example, perturbation of CapZ function in myotubes delays thin filament formation but does not affect thick filament assembly (Schafer et al., 1995). Additionally, *Drosophila melanogaster* actin mutants lack thin filaments and Z-lines but have normal thick filaments, and α-actinin mutants have normal myofibrils (Beall et al., 1989; Roulier et al., 1992). Thus, it appears that during muscle development, perturbation of some sarcomeric components is deleterious to myofibril assembly, whereas disruption of others has more subtle consequences. In summary, our data indicate that nebulin is involved in myofibrillogenesis. However, it remains to be determined whether thin filament assembly and length regulation are required processes for differentiation and whether nebulin is a multifunctional giant, perhaps involved in gene expression and signaling.

In conclusion, our results demonstrate that more than two decades after its discovery, a primary function for nebulin is indeed thin filament length regulation and assembly. An exciting implication is that the precise lengths of actin filaments in numerous cellular structures are regulated by molecular templates. Thus, there may be an entire nebulin family in cells that contain actin filaments of defined lengths, an idea supported by an in situ hybridization study that detected nebulin in other tissues (Kazmierski et al., 2003), as well as a study identifying nebulin-like molecules in many species (for review

see Clark et al., 2002). Future investigations into ruler proteins can serve as a precedent for investigating molecular length regulation and macromolecular assembly in many types of biological systems and will provide insight into actin dynamics in most, if not every, cell type.

Materials and methods

Cell culture, siRNA, and Lat B treatment

Rat fetal cardiac myocytes were isolated by J. Bahl and Y. Wu (University of Arizona, Tucson, AZ) and maintained as described previously (Bang et al., 2001; Kazmierski et al., 2003). 2 nM dsRNAs were incubated with 6 μl of Cytofectene (Bio-Rad Laboratories) in 100 μl OptiMEM for 15 min at 37°C and added to myocytes on day 3. Transfection efficiency was >80%, and >50 cultures were analyzed. Four dsRNAs to the rat nebulin sequence (19 mers + 5′ AA overhangs) were synthesized, using Ambion's website and Silencer Construction kit, and blasted to ensure no significant homology to nebulette or other known sequences in the National Center for Biotechnology Information database. Three of the four dsRNAs significantly knocked down nebulin in cardiac myocytes (target cDNA: 5′-GGAGACGGTGTACGAGGAG-3′; 5′-CCACAACTACAAGGACATC-3′; and 5′-CAAGGACATCTGACTATGA-3′), using concentrations titrated from 0.5–10 nM, with 2 nM being the lowest effective dosage. Control siRNAs included GAPDH (Ambion), a scrambled nebulin siRNA, and the three siRNAs to nebulin containing 6-bp changes in their centers. Nebulin reduction was confirmed by immunofluorescence with three independent antibodies (Millevoi et al., 1998; McElhinny et al., 2001; Kazmierski et al., 2003). For live cell imaging, cells were cotransfected with GFP-tropomyosin (a gift from J.-C. Perriard, Swiss Federal Institute of Technology, Zurich, Switzerland) and imaged as described previously (Mudry et al., 2003). To depolymerize thin filaments, we added 20 μM Lat B (Invitrogen) to cells for 4 h (8 h after siRNA treatment), and then washed them out and let them recover for 4 h.

For skeletal myotube cultures, myoblasts were isolated from rat hind limbs. The deboned tissue was suspended in 10 ml trypsin/EDTA (Life Technologies) and incubated for 10 min at 37°C. After centrifugation, the pellets were resuspended in MEM + 15% chick embryo extract + 10% horse serum, and cells were preplated at 37°C for 1 h on 100-mm dishes. Collected cells were plated at 4×10^5 cells/ml in 35-mm culture dishes containing 12-mm round coverslips coated with rat-tail collagen (Sigma-Aldrich). Cells were transfected with siRNA 24 h later, except this time using 10 nM siRNA. After 3 d, cells were treated with 1 μg/ml 1-[β-D-arabinofuranosyl]-cytosine (Ara-; Sigma-Aldrich) in MEM with 2.5% chick embryo extract and 10% horse serum and fixed on days 5 and 6.

RT-PCR

Total RNA was harvested from cardiac myocytes within 24 h of siRNA treatment, or from skeletal myotubes within 48 h, using the RNeasy Mini kit (QIAGEN), or with Trizol (Sigma-Aldrich), and quantified using a Biophotometer (Eppendorf). cDNA was synthesized from 1–3 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen). 0.5–3 μl of template were used per 25 μl PCR reaction. Reagents were from the TripleMaster Taq kit (Eppendorf). For nebulin amplification, at least 28–30 cycles were performed (with the threshold of detection being ~30 cycles in cDNA from cardiac myocytes and ~27 from skeletal myotubes), and for other transcripts, 26–30 cycles were used. Primers used included rat nebulin (forward, 5′-ACTGTCTTCCATCCCGTCAC-3′, and reverse, 5′-GCCATACATCCAGCCTTCAT-3′, to amplify a 202-bp product; rat nebulette (forward, 5′-ATTGGGAAGGGCTACAGCTT-3′, and reverse, 5′-GAAGCCTCTTCCCTTCGTCT-3′ to amplify a 196-bp product); rat GAPDH (forward, 5′-CCAGTATGATTCTACCCACGGC-3′, and reverse, 5′-CGGAGATGATGACCCTTTTGGC-3′, to amplify a 227-bp product); and Tmod1 (forward, 5′-ACTGTAAGGCCATGGACAGC-3′, and reverse, 5′-GCTGCAGTTGTGTTTCAAGG-3′, to amplify a 141-bp product). All PCR products were sequenced.

Western blotting

Myotube lysates were solubilized in SDS sample buffer, sonicated, and incubated at 70°C for 5 min before loading onto a 4–20% gradient SDS-PAGE gel. After transfer to nitrocellulose, strips were probed with anti–COOH- and anti–NH_2-terminal nebulin antibodies (~1 μg/ml), followed by anti–rabbit IgG-conjugated HRP (1:25,000; Jackson ImmunoResearch Laboratories). After incubation in SuperSignal chemiluminescent substrate (Pierce Chemical Co.), the strips were exposed to BioMax MR film (Eastman Kodak Co.), and band intensity was quantified using National Institutes of Health image.

Immunofluorescence microscopy

Cells were stained as described previously (Gregorio and Fowler, 1995). All cultures were double or triple stained to distinguish myocytes from fibroblasts and/or to evaluate the intensity of nebulin staining. Myocytes were treated with relaxing buffer (150 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.4, 1 mM EGTA, and 4 mM ATP) and fixed in 3% PFA for 15 min. Cells were incubated with affinity purified rabbit anti–NH_2 and anti–COOH-terminal nebulin antibodies (~5 μg/ml), monoclonal antimyosin F59 antibodies (1:10 of culture supernatant; provided by F. Stockdale, Stanford University, Stanford, CA), rabbit antititin antibodies (A168-170 at 1:500; Z1-Z1 at 1:100; Centner et al., 2000), monoclonal antisarcomeric α-actinin antibodies (1:1,500; EA-53; Sigma-Aldrich), affinity-purified rabbit anti–human Tmod1 antibodies (10 μg/ml), monoclonal anticardiac actin antibodies (1:10; Ac1-20.4.2; American Research Products, Inc.), and monoclonal antitropomyosin CH1 antibodies (1 μg/ml; Developmental Studies Hybridoma Bank). The following secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and Invitrogen: goat anti–mouse AlexaFluor 488 (1:1,000), goat anti–mouse Texas red (1:600), donkey anti–rabbit Texas red (1:600), goat anti–rabbit AlexaFluor 350 (1:300), and goat anti–mouse AlexaFluor 350 (1:200) IgG. AlexaFluor phalloidin 488 (Invitrogen) or Texas red phalloidin (Sigma-Aldrich) labeled thin filaments, and DAPI (5 μg/ml; Sigma-Aldrich) labeled myotube nuclei. Coverslips were mounted using Aqua Poly/Mount (Polysciences, Inc.) and analyzed on a microscope (Axiovert; Carl Zeiss MicroImaging, Inc.) using a 63 (NA 1.4) or 100× (NA 1.25) objective, and micrographs were collected as digital images on a camera (Orca-ER; Hamamatsu) using OpenLab software (Improvision). Imaging was also performed using a microscope (DeltaVision Deconvolution model D-OL; Olympus) with a 100× objective (1.3 NA) using a charge-coupled device camera (series 300; Photometrics), and on a multiphoton microscope (model 510; Carl Zeiss MicroImaging, Inc.) using a 100× objective (1.4 NA). For pixel intensity plots, images of phalloidin-stained thin filaments were oriented parallel to the long axis in Adobe Photoshop, and intensity levels along the lengths of two adjacent sarcomeres were quantified using boxes drawn to encompass 69 × 6 total pixels (SoftWorx Data Inspector). Images were processed using Adobe Photoshop 7.0, and statistical analyses were performed using Microsoft Excel.

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