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Thin Filament Length Regulation in Striated Muscle Sarcomeres: Pointed-end dynamics go beyond a nebulin ruler

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The uniformity of actin (thin) filament lengths in striated muscle has been an intriguing problem since 1954 when striated myofibrils were shown to contract by the relative sliding of thin and myosin (thick) filaments [1–3]. Sliding filament theory is the structure-function relationship for striated myofibrils that relates muscle contraction to the molecular organization of proteins within the sarcomere, the basic contractile unit of striated muscle (Fig. 1A–C). Length is not an intrinsic property of actin filaments, as actin monomers assemble *in vitro* and *in vivo* to many allowed polymer lengths [4,5]. Since striated muscle thin filaments all have the same ~1 μm lengths, their lengths must be regulated by actin-binding proteins. How are their lengths specified? At the advent of sliding filament theory, thin filaments were for all intents and purposes thought to be static. Now, we know that actin subunits continuously associate and/or dissociate from the polymer ends despite the uniformity of filament lengths in these and other actin structures [6–8]. Thus, mechanisms to control thin filament length must ultimately rely upon a length-dependent control of actin association and dissociation rates at the polymer ends.

Striated muscle is probably one of the best-studied systems of actin filament length regulation. This is due to the repetitive and hierarchical organization of actin filaments into sarcomeres, myofibrils, and muscle fibers. Alternating stripes of thin filament barbed and pointed ends in sarcomeres are located along the myofibrils (Fig. 1D). The uniform lengths of thin filaments are evidenced by a gap in the middle of the sarcomere (H-zone). The locations of barbed ends at the Z lines, tropomodulin (Tmod) at the pointed ends, and the distance between them (filament length) can be resolved easily by light microscopy, making precise length measurements along with analyses of actin dynamics at filament ends uniquely accessible to experimentation. Nevertheless, challenges arise because the sarcomere is a complex nano-machine [9,10]. Nearly every protein in the sarcomere binds to actin and could in theory affect actin monomer-polymer dynamics and thin filament lengths. Many sarcomeric proteins also regulate one another's interactions, and are regulated by extracellular and cellular signals and mechanics. Obligatory changes in sarcomere length that accompany contraction can interfere with the interpretation of experiments, if not taken into account. Besides being possible candidates for regulating thin filament lengths, most other sarcomere proteins have established roles in contraction or myofibril assembly. Consequently, mutations or knockout approaches often block earlier events during myofibril assembly, such as thin filament alignment, precluding measurements of thin filament lengths.

Since our previous review on thin filament length regulation [10], there has been significant and exciting experimental progress in the field, especially on the roles of pointed-end capping by Tmods and the proposed molecular ruler protein, nebulin. We have organized this review

into five sections: First, we discuss evidence that regulation of actin filament pointed end dynamics by Tmod and other actin regulatory proteins controls thin filament lengths, since this provides the underpinnings of any mechanistic explanation for filament length regulation. Second, we discuss how indirect effects due to perturbation of the actin monomer economy can confound analysis of thin filament length regulation during thin filament assembly and muscle development. Third, we review the nebulin ruler model and possible mechanisms for how it may specify uniform thin filament lengths within a sarcomere, and particular thin filament lengths for different muscles. Fourth, we review recent evidence suggesting that a nebulin-independent mechanism may specify thin filaments in vertebrate muscle. Finally, we discuss the relationship between filament length regulation, sarcomere length and muscle contraction; it is the thesis of this review that mechanisms controlling thin filament lengths are tailored to sarcomere physiology. In this context we suggest that mechanisms specifying thin filament lengths emerge from mechanisms that regulate myofilament overlap.

I. Actin Pointed End Dynamics Controls Thin Filament Lengths

Thin filaments are remarkably stable structures *in vitro*, yet they exhibit unexpected dynamics at their pointed ends *in vivo*. In isolated myofibrils and permeabilized muscle cells, thin filament pointed ends are tightly capped by tropomodulin (Tmod) and do not bind or exchange monomers [11–14]. However, in cultured living chick skeletal myotubes, chick cardiac myocytes, and *Drosophila* primary muscle cells, thin filament pointed ends are transiently capped by Tmod and readily exchange actin subunits with the soluble pool [6,15,16]. Strikingly, unlike actin cytoskeletal structures in non-muscle cells where barbed ends are the predominant assembly ends [17], actin dynamics predominates at thin filament pointed ends in living muscle cells. In chick cardiac myocytes, quantitative comparison of fluorescent-actin subunit exchange shows that the extent of subunit exchange is about 1.6 times greater at the pointed than the barbed ends [6]. In primary muscle cells isolated from *Drosophila* embryos carrying a *Dmefl-Gal4, UAS-GFP-actin* transgene, initial incorporation of GFP-actin also occurred predominantly at thin filament pointed ends [15]. Initial incorporation of epitope-tagged α -tropomyosin (α TmFLAG) also takes place at thin filament pointed ends in adult rat cardiac myocytes, suggesting that actin and tropomyosin subunits may coordinately assemble and disassemble (exchange) at pointed ends [18,19]. In contrast to the rapid incorporation at filament ends (seconds to minutes), incorporation of injected or expressed actin monomers or expressed GFP-tropomyosins along the entire thin filament is much slower (many hours) [6, 15,18,20–22].

A substantial body of data demonstrates that thin filament lengths are controlled by inhibition of actin assembly at pointed ends by Tmods. Tmods are a conserved family of pointed end capping proteins that are associated stoichiometrically with thin filament pointed ends in all striated muscles of vertebrates, flies and worms (Fig. 1) [for reviews, see refs. 23,24,25]. Over-expression of Tmod1 inhibits actin incorporation specifically at thin filament pointed ends, shortening thin filaments in cultured cardiomyocytes [6,26], and resulting in dilated cardiomyopathy in mouse hearts [27]. Conversely, thin filaments lengthen in response to reduced Tmod1 levels or capping activity in cultured cardiomyocytes [26,28], and in developing skeletal muscle in a Tmod1 null mouse (C.R. McKeown, R.B. Nowak, R.S. Littlefield, V.M. Fowler, unpublished data). In contrast, inhibition of actin assembly at barbed ends by cytochalasin D has no effect on thin filament lengths in cardiomyocytes, and treadmilling of actin monomers from barbed to pointed ends is not observed (R.S. Littlefield, V.M. Fowler, unpublished data)[6]. Inhibition of CapZ capping in chick skeletal myotubes also had little effect on thin filaments of mature myofibrils, but led to impairment and delays in myofibril assembly, indicating that barbed end polymerization does not regulate filament lengths [29]. Instead, CapZ may organize actin filaments at Z lines early during myofibrillogenesis, prior to thin filament length regulation (Section II).

Tmod capping may affect thin filament lengths by several mechanisms. First, by transiently binding to pointed ends (dynamic capping) and reducing the rate of monomer addition below the rates of nucleotide hydrolysis and phosphate release, Tmod can increase the proportion of low-affinity ADP-actin pointed ends, resulting in shorter filaments at steady state [19]. This implies that Tmod may be more effective at inhibiting polymerization (elongation) as compared to depolymerization (shortening) of thin filaments, which could be significant in the case of dynamic actin polymer extensions at thin filament pointed ends in some muscles (Section III). Second, by binding directly to tropomyosin at the pointed end, Tmod may prevent dissociation of the terminal tropomyosin molecules and promote filament stability [30,31]. Indeed, disruption of Tmod1 binding to α -tropomyosin by microinjection of an inhibitory monoclonal antibody into cultured cardiomyocytes dissociates α -tropomyosin from pointed ends leading to actin disassembly and thin filament shortening [32]. Whether Tmod regulates lengths through one or both of these mechanisms may depend on the relative association and dissociation rates of actin, tropomyosin and Tmod at pointed ends, resulting in different proportions of filament ends with or without tropomyosin [for a discussion, see ref. 23]. The precise relationship between Tmod capping affinity, actin dynamics at pointed ends and filament lengths remains to be directly measured in living muscle cells with altered Tmod levels or functional interactions.

Additional evidence that thin filament lengths are controlled by direct regulation of elongation at pointed ends comes from studies of myofibril assembly in *Drosophila*. In arthropods, short sarcomeres with regulated thin filament lengths form early in muscle development [16,33, 34]. For example, in *Drosophila* indirect flight muscle (IFM) the first myofibrils formed are striated, and the thin filaments elongate during development from $\sim 0.8\mu\text{m}$ to $\sim 1.5\mu\text{m}$, concomitant with both sarcomere and thick filament elongation (Fig. 1C) [16,34]. Transient over-expression of Tmod from an *Hsp-90* promoter during this process prevents thin filament elongation from pointed ends and impairs flight [16]. Sarcomere elongation is unaffected by Tmod overexpression, indicating that thin filament elongation can be uncoupled from sarcomere elongation in the IFM. In cultured primary muscle cells derived from *Drosophila* embryos carrying *UAS-Tmod* and *Dmef2/Gal4* transgenes, constitutive over-expression of Tmod also leads to $\sim 30\%$ shorter thin filaments [15]. In this case, sarcomeres are also shorter in the presence of excess Tmod, indicating that thin filament and sarcomere elongation appear to be coupled in these primary cultured muscle cells (Section V). Endogenous Tmod is associated with pointed ends of the elongating thin filaments [15,16], indicating that Tmod capping is dynamic and does not preclude thin filament elongation. How dynamic Tmod capping is regulated to permit thin filament elongation during myofibril assembly, yet terminate filament elongation in mature myofibrils is not understood.

A recently discovered actin binding protein, *sarcomere length short* (SALS), appears to regulate Tmod-dependent control of thin filament lengths in *Drosophila*. SALS promotes thin filament and sarcomere elongation during myofibril assembly in primary *Drosophila* muscle cells [15] and in the IFM (J. Bai, personal communication). RNAi treatment with SALS leads to dramatically shorter thin filaments and sarcomeres, while over-expression of SALS leads to longer thin filaments and sarcomeres. Genetic analysis shows that SALS antagonizes the activity of Tmod in regulating thin filament lengths. However, the exact mechanism by which SALS promotes thin filament elongation at pointed ends is uncertain. The ~ 150 kD isoform of SALS expressed in embryonic muscle has two WH2 domains downstream of a Pro-rich domain, remarkably similar to actin nucleating proteins such as N-WASP and WIP [35], and to barbed end-elongation promoting proteins such as Ena/VASP [36,37]. Surprisingly, SALS inhibited rates of pointed end elongation *in vitro*, similar to Tmods, and opposite to expectations, suggesting that other factors may regulate SALS activity *in vivo*. Interestingly, SALS was localized near thin filament pointed ends in assembling myofibrils but near Z lines in mature myofibrils. One could speculate that SALS may function as an anti-capping protein

at pointed ends for thin filament elongation during myofibril assembly. This would be analogous to the mechanism of Ena/VASP in promoting actin assembly at barbed ends for leading edge protrusion or Listeria movement, respectively [36,38].

In conclusion, regulation of actin assembly and disassembly at pointed ends clearly plays an important role in thin filament length control, both in vertebrate and invertebrate myofibrils. Elucidation of the roles for Tmod and SALS in actin pointed end dynamics and thin filament length regulation is based on utilization of experimental systems where striated myofibrils are already assembled so that thin filament lengths can be measured directly, based on the distance from Z line to the pointed ends at the edge of the H zone. However, in systems when protein functions are perturbed as myofibrils assemble *de novo*, these and other actin regulatory proteins may have dramatic global effects on actin filament assembly and/or stability, precluding conclusions as to specific roles in thin filament length control mechanisms. In the next section, we discuss how perturbing the actin monomer economy during thin filament assembly affects additional processes that prevent the assessment of any specific effects on lengths.

II. The actin economy, thin filament assembly and stability

Thin filament assembly and length regulation takes place in the cellular context of an actin monomer 'economy' [17], coordinated and balanced by a plethora of actin-binding proteins that regulate the amount and availability of monomers, barbed, and pointed filament ends. Notably, during myofibril assembly in vertebrate muscles; many components acquire a striated sarcomeric organization while actin filaments remain overlapping with variable lengths, exhibiting continuous (nonstriated) actin staining along the immature myofibrils [12,39,40]. Moreover, in myofibrils with nonstriated actin organization, actin filaments are considerably more dynamic than in mature striated myofibrils with regulated thin filament lengths (as evidenced by a gap at the H zone) [21,41–44]. Thus, it is expected that perturbations in actin regulatory proteins that control the actin monomer-polymer economy can have consequences for early events of thin filament assembly and turnover during myofibrillogenesis, prior to the late events of thin filament length control *per se*.

Therefore, since Tmods affect the actin monomer economy [23], it is not surprising that Tmods appear to play an early role in controlling actin filament polymerization into sarcomeres during *de novo* myofibril assembly in cardiac muscle cells. The absence of Tmod1 in a *Tmod1* null transgenic mouse results in failure of myofibril assembly in the E7.5–9.5 embryonic heart, leading to aborted cardiac development and embryonic lethality at E9.5 [45,46]. Instead of myofibrils, large aggregates of actin filaments and rod-like α -actinin/F-actin-containing structures, as well as disordered filament bundles with immature Z bodies, are present in the cardiomyocytes of the embryonic heart. Tmod1 may be required to stabilize the actin filaments in I-Z-I complexes at early stages of myofibrillogenesis, before thin filaments are organized into identifiable sarcomeres [46,47]. The large actin filament bundles may be due to depolymerization of unstable filaments from their pointed ends in absence of Tmod1, releasing monomers and causing excess actin nucleation and/or barbed end polymerization [for more discussion, see 23]. Similarly, in mouse ES cells differentiated *in vitro* to cardiomyocytes, actin filament aggregates and disorganized structures are predominant [48]. Curiously, in these cells the absence of Tmod1 does not preclude occasional myofibril assembly, and both sarcomere and thin filament lengths in the rare myofibrils that do assemble in the Tmod1 null ES cells are indistinguishable from myofibrils in wild-type cells [48]. The reason for differences in myofibril assembly between Tmod1 null ES cells differentiated to cardiomyocytes *in vitro* and Tmod1 null cardiomyocytes in the embryonic heart are not clear, but may reflect differences in the actin monomer economies or in the mechanical environments in these two systems.

In *C. elegans*, *Unc-94* encodes a Tmod (*tmd-1*) that is localized to thin filament pointed ends in the obliquely striated body wall muscle [49,50]. *Unc-94/tmd-1* mutants have defective muscle function and exhibit large accumulations of actin filaments near the muscle cell-to-cell boundaries [49–51]. Strikingly, in other regions of less severely affected muscle cells, myofibril organization and thin filaments can appear relatively normal; although actin filament lengths were not measured and small length changes would not have been detected. The actin disorganization phenotype of *unc-94/tmd-1* mutants appeared to be more pronounced when combined with mutants for proteins that enhance actin disassembly and filament turnover, such as *unc-60B* (ADF/cofilin), *unc-78* (actin interacting protein 1 (AIP1) and *pfn-2* and *pfn-3* (profilin-2 and -3) [50]. However, since striated myofibrils do not assemble at all in severely affected *unc-60B* or *unc-78* mutants [52,53], it is difficult to ascribe a direct role for these components in thin filament length regulation *per se*, as no sarcomeric thin filaments are present whose length is to be regulated.

Larger, Tmod-related ~70 kD proteins, termed Lmods are also expressed in striated muscle [23,54]. Lmods have an N-terminal portion with tropomyosin binding and actin capping domains homologous to Tmods, and a C-terminal extension with a Pro-rich region as well as a WH2 domain [54–56]. Lmod1 is mainly expressed in smooth muscle, but also in a subset of striated fibers in tongue and extraocular muscle where it colocalizes with slow myosin staining in the A band [57,58]. Lmod2 expression is restricted to striated muscle [54] and was reported recently to localize in the middle of the sarcomere in cultured neonatal rat cardiomyocytes, similar to Tmod1 [55]. Lmod2 is proposed to nucleate assembly of thin filaments into sarcomeres, based on the ability of recombinant Lmod2 to nucleate actin filament assembly *in vitro*, and the disorganized actin filaments observed after Lmod2 knockdown in neonatal rat cardiomyocytes [55]. These observations suggest that, like Tmods, Lmods may play a role in thin filament assembly and turnover during myofibrillogenesis, but also do not rule out additional roles for Lmods in later processes of thin filament length regulation in striated myofibrils.

In conclusion, these and other actin regulatory proteins may, in effect, act to regulate the balance of the actin monomer-polymer economy upstream of specific sarcomeric thin filament length control mechanisms. This would make sense, as thin filaments must be assembled from actin monomers using basic cellular actin polymerization mechanisms [10,59]. In future, it will be interesting to design experiments to test the roles of these and other actin regulatory proteins in thin filament length regulation by selectively interfering with the functions of actin regulatory proteins after mature striated myofibrils have assembled, using inducible expression systems or using acute perturbations via microinjection of function-blocking antibodies or protein domains.

III. Nebulin ruler mechanisms

A molecular ruler mechanism has been a favorite, long-standing mechanism proposed to determine thin filament lengths in vertebrate striated muscle sarcomeres, based on the structure and the properties of the giant molecule, nebulin [10,60–62]. Nebulin (Fig. 1A) and nebullette (Fig. 1B) are homologous modular proteins that are associated with Z-lines and thin filaments in striated muscles [63–66]. Both proteins have a C-terminal SH3 domain and adjacent serine-rich linker anchored to the Z-line, and a unique N-terminal domain associated with the thin filament. Between these end regions, both proteins are composed of a tandem series of actin-binding (nebulin) modules, each of which forms a single ~35 residue alpha-helix that spans the distance between adjacent actin subunits (5.5 nm), allowing both proteins to extend away from the Z-line along the thin filament. Based on sequence analysis, groups of seven nebulin modules can be organized into super-repeats that correspond to the 38.5 nm tropomyosin repeat along the thin filament. In humans, nebulin contains ~185 modules (including 22 super-repeats)

and nebulin contains ~22 modules (but with no super-repeats) allowing nebulin and nebulin to extend ~1 μm and ~150 nm away from the Z-line, respectively. The complete sequences of the human and mouse nebulin genes have been determined and show that differential expression of additional modules and super-repeats could permit nebulin to extend even further than 1 μm away from the Z-line in some muscles [67,68]. While nebulin appears to be long enough to function as a molecular ruler that regulates thin filament lengths, the shorter nebulin molecule must have other 'non-ruler' functions, such as organizing Z-lines during assembly [64–66,69].

The original hypothesis proposed that nebulin was a static ruler that acted to *precisely* specify thin filament lengths during skeletal muscle myofibril assembly by binding along each thin filament in an extended conformation from the Z-line towards the pointed end (Fig. 2A). Nebulin would then direct high-affinity Tmod capping to terminate filament growth only when the thin filament was the same length as nebulin, while longer or shorter thin filaments would remain dynamic until they had attained the specified length and were capped. After myofibril assembly, high-affinity Tmod capping would maintain uniform thin filament lengths. Thus, the physical length of the nebulin molecule would specify a unique thin filament length, yet different thin filament lengths could be achieved by expressing nebulin isoforms with different numbers of nebulin modules (Fig. 2A). In support, the apparent molecular weights of nebulin positively correlate with thin filament lengths in different muscles [70,71], a construct of three nebulin modules (M1M2M3) located at the N-terminal end of nebulin has been reported to bind Tmod *in vitro* [72], and stoichiometric amounts of nebulin are present in vertebrate skeletal muscles [73]. However, in most vertebrate cardiac muscles (Fig. 1B), stoichiometric amounts of nebulin are not present [67,68,74,75]. In addition, the presence of actin pointed-end dynamics in living skeletal and cardiac muscle cells indicates that thin filament lengths are not maintained by high-affinity Tmod capping, but that actin association and dissociation at filament ends must be continually regulated for ongoing specification of thin filament lengths [6].

Modifications of the nebulin ruler mechanism have been proposed to account for actin dynamics and to specify thin filament lengths when there are insufficient amounts of nebulin to bind to each thin filament ('Dynamic ruler' mechanisms, Fig. 2B–D). As compared to skeletal muscle, sub-stoichiometric nebulin amounts in cardiac muscle have been associated with their relatively broad, yet regulated, distributions of thin filament lengths (Fig. 1B), ranging from 0.6 – 1.1 μm in rat atria, 0.9 – 1.1 μm in rat ventricles, and 0.8 – 1.3 μm in frog atria [76, 77]. By contrast, lamprey cardiac muscles, which have stoichiometric nebulin amounts (1–2 molecules per thin filament), have uniform thin filament lengths [74]. Fowler et al [78] suggested a 'Cap Locator' mechanism (Fig. 2B) where nebulin could recruit a soluble pool of Tmod that would be located (relative to the Z-line) at the length specified by nebulin (L_{nebulin}), yet could diffuse away and transiently cap thin filaments that were either longer or shorter than L_{nebulin} . This mechanism predicts at steady-state that L_{nebulin} should specify the average thin filament length, yet individual length variations (i.e. the breadth of the length distribution) should depend on the association and dissociation rates of Tmod and actin monomers at pointed ends, Tmod levels and diffusion rates, etc. With this mechanism, stoichiometric amounts of nebulin in skeletal muscle would result in only minor thin filament length variations, yet permit actin exchange to occur at pointed ends; while sub-stoichiometric amounts in cardiac muscle would recruit less Tmod, resulting in broadly specified thin filament length distributions.

For some dynamic ruler mechanisms, pointed-end dynamics may, by skewing the resulting steady-state thin filament length distribution away from L_{nebulin} , affect the average thin filament length in addition to the breadth of the distribution. For example, Marshall [60] suggested a 'Progressive Capping' mechanism (Fig. 2C) where Tmod, recruited by nebulin at

L_{nebulin} , transiently caps and progressively reduces the actin monomer assembly rate at pointed ends as thin filaments assemble up to a maximum length equal to L_{nebulin} . Alternatively, in a ‘Stabilizing’ mechanism (Fig. 2D), nebulin may stabilize the thin filament by preventing monomer dissociation at the pointed end [79], effectively specifying a minimum length equal to L_{nebulin} . At steady-state, dynamic Tmod capping (Section I) would allow short actin ‘pointed-end extensions’ to polymerize and increase the average thin filament length beyond L_{nebulin} . In both the ‘Progressive Capping’ and ‘Stabilizing’ mechanisms, increasing Tmod expression levels would tend to shorten thin filaments while decreasing Tmod expression levels would tend to lengthen thin filaments, as observed (Section I). These dynamic ruler mechanisms may explain how siRNA knockdown of nebulin in cultured neonatal rat cardiomyocytes leads to uniform thin filament elongation [78,80] and how ~100 nm variations in thin filament lengths observed within individual skeletal muscles [81] could result from cell-to-cell variations in Tmod levels or the actin monomer economy [60].

By accounting for all the observations, these dynamic mechanisms strengthen the nebulin ruler model, however they also reveal the model’s Achilles heel. As long as pointed-end dynamics are unregulated, then nebulin is the only component that specifies length; however, if pointed-end dynamics are differentially regulated to produce distinct thin filament lengths in different muscles, then regulation of pointed-end dynamics specifies length in these cases, not nebulin (Fig. 2E). To distinguish these possibilities, it is important to determine whether thin filament lengths remain specified in nebulin’s absence or differ with respect to either nebulin lengths or pointed-end dynamics in different muscles.

IV. Nebulin-dependent and independent thin filament length regulation

A key test of all nebulin ruler mechanisms is that the absence or elimination of nebulin from muscles would be expected to lead to failure of thin filament regulation, with mis-specified and more variable length filaments. Human nebulin mutations are associated with nemaline myopathy (NM), a progressive muscle disease characterized by muscle weakness and *nemaline bodies* that seem to arise from unregulated, wide Z-lines; but thin filament lengths have not been measured [82]. Recently, nebulin-deficient knockout mice were generated in two labs [75,83]. These mice developed normally and survived past birth, yet developed severe, NM-like muscle weakness and pathology and died within two weeks. Light and electron microscope analyses of skeletal muscle myofibril structure showed that thin filament lengths were mis-regulated in nebulin-deficient mice: in 1-day-old nebulin-deficient mice, thin filaments were uniform in length, yet ~15 – 25% shorter than wildtype mice [75]; in 10-day-old nebulin-deficient mice, thin filaments were non-uniform in length [83]. These studies were the first to show that nebulin directly regulates thin filament lengths in vivo, yet their results are unexpected considering a putative ruler function for nebulin. First, the uniform thin filament lengths present in skeletal muscles of 1-day-old nebulin-deficient mice indicate that a nebulin-independent mechanism can specify thin filament lengths when nebulin is absent. Second, nebulin appears necessary to maintain uniform thin filament lengths after birth, during muscle use. Third, the absence of nebulin did not affect Tmod association with thin filament pointed ends, indicating that thin filament length is not specified by a unique high-affinity capping site as proposed in the original static nebulin ruler mechanism (Fig. 2A). Instead, the NM phenotypes of the nebulin-deficient mice suggest that nebulin may have important non-ruler functions within skeletal muscle [75,83]. For example, nebulin has been shown to stabilize the association of TM with actin [21,22] and regulate actomyosin interactions [84,85]. Additional studies on these mice will be necessary to fully understand how uniform thin filament lengths are specified in nebulin’s absence and how nebulin affects the initial specification and subsequent maintenance of thin filament lengths during muscle use.

Recent measurements of thin filament lengths can potentially reconcile the disparate nebulin models and explain both how thin filament lengths depend on nebulin (Section III), yet are controlled by regulation of pointed-end dynamics independent of nebulin (Section I). In one study, nebulin size and layout along the thin filament were compared with thin filament lengths in different rabbit muscles [86]. Thin filament lengths were measured in isolated myofibrils using indirect immunofluorescence staining and image analysis to determine the locations of Tmod and the nebulin M1M2M3 epitope at the N-terminal end of nebulin with respect to their distances from the Z line. Surprisingly, thin filament lengths varied from 1.13 μm in rabbit psoas, up to 1.32 μm in rabbit diaphragm, yet the N-terminal end of nebulin was invariably located 1.0 μm from the Z-line in all the muscles. The lengths of the nebulin molecules *in situ* were consistent with previous epitope mapping studies and estimated molecular weights of rabbit nebulin, confirming that nebulin is in an extended conformation along the thin filament [87]. However, despite an apparent ~50 kDa size difference for nebulin in some muscles [88], the expected 70 nm difference in length was not detected in the position of the N-terminal end of nebulin with respect to the Z line [86]. Thus, the apparent size differences may be accounted for by differences in the position of the C-terminal end of nebulin, or by post-translational modifications. Importantly, variations in thin filament lengths were detected in each individual muscle, such that the minimum thin filament length of ~1 μm corresponded to the distance of the nebulin N-terminal end from the Z line. These observations suggest that nebulin could stabilize a large ‘core’ region [79] that specifies a ~1 μm minimum thin filament length, consistent with a dynamic ruler ‘Stabilizing’ model (Fig. 2D). Notably, differences in nebulin size and length did not account for the changes in thin filament lengths between these rabbit skeletal muscles [86,88]. Instead, variations in the lengths of the pointed-end extensions accounted for the different thin filament lengths independent of nebulin.

Together with the phenotypes of the nebulin-deficient mice, these data lead us to suggest that nebulin cooperates with and modifies a nebulin-independent mechanism to regulate thin filament lengths in vertebrate muscle (Fig. 2E). In this ‘Composite’ model, the nebulin-independent mechanism would regulate pointed-end dynamics, and the specified lengths would be sensitive to Tmod capping activity, as observed (Sections I and II). The nebulin-independent mechanism would normally specify a wide range of individual thin filament lengths, some of which are shorter than the molecular length of nebulin [75,83]. However, nebulin binding along skeletal muscle thin filaments would stabilize a long core region and specify a minimum thin filament length equal to the molecular length of nebulin. This serves to modify the lengths specified by the nebulin-independent mechanism by preventing thin filaments from shortening below this minimum length, yet allowing pointed ends to extend beyond this minimum length, resulting in a narrow distribution of long thin filaments [86]. Similar to nebulin, nebulin would stabilize a core region and a minimum thin filament length in cardiac muscle. However, the short length of nebulin would permit the nebulin-independent mechanism to specify a wide range of individual thin filament lengths in cardiac muscle, as observed [76,77]. Consistent with this, cardiac muscle structure and function is unaffected in nebulin-deficient mice [75,83]; yet, interestingly, thin filaments shorten when dominant-negative nebulin constructs are expressed in cardiomyocyte cultures [89]. The important distinction between this ‘Composite’ model and a dynamic ruler model is that (skeletal) muscle-specific changes in thin filament lengths *in vivo* are predominantly generated by regulating pointed-end dynamics through the nebulin-independent mechanism, not by changing the length of the nebulin core region (e.g. by alternative splicing of nebulin) [86]. In the next section, we discuss how the nebulin-independent mechanism could cooperate with sarcomere lengths to tune pointed-end dynamics and optimize thin filament lengths for different muscle physiologies.

V. Coordination of thin filament length with sarcomere length

Thin filament lengths are the primary determinant of the force-sarcomere length relationship for muscles [3,90]. The optimal sarcomere length for maximal force generation occurs at twice the length of the thin filaments when the thick and thin filaments completely overlap and the thin filament pointed ends are located at the M-line in the middle of the bare zone. Ideally, mechanisms regulating thin filament length should be coordinated with sarcomere length to optimize sarcomere physiology. Indeed, for most skeletal muscles, thin filament lengths appear to be matched to the *in vivo* (working) range of sarcomere lengths [91,92]. The working range of sarcomere lengths in vertebrates (up to ~20% of resting sarcomere length) is likely controlled by alternative splicing in the spring-like I-band region of titin (Fig. 1A), which connects the ends of the thick filaments to Z-lines and is responsible for generating muscle-specific passive force characteristics [93–96]. In addition, the wide range of individual thin filament lengths in the atria and ventricles appears to be matched with passive muscle stiffness to optimize force production [77]. Moreover, the working range of arthropod muscles (~1–3% of resting sarcomere length) is limited by the stiffness of the hard cuticle, yet thin filaments elongate from their pointed ends (Section I) to remain continuously matched with sarcomere length during growth (Fig. 1C).

Strikingly, the recent analysis of thin filament lengths in rabbit muscles demonstrates that average thin filament lengths and the variations in thin filament lengths are significantly correlated with titin isoform size in these muscles, matching resting (half-)sarcomere lengths [86,88]. Because the rabbit thin filament lengths varied independently of nebulin, we therefore suggest that the working range of sarcomere lengths coordinates how the vertebrate nebulin-independent mechanism regulates pointed-end dynamics to optimize thin filament lengths in different muscle. The absence of a long nebulin core region in cardiac muscle may allow the working range of sarcomere lengths to tune pointed-end dynamics to generate an optimal distribution of individual thin filament lengths, as observed [77]. By contrast, if nebulin were to solely specify thin filament lengths through a static or dynamic ruler mechanism (Fig. 2A–D), then alternative splicing of titin and nebulin genes would need to be coordinated to optimize myofibril overlap [94], which seems unlikely [88]. Finally, considering that pointed-end dynamics is coordinated with sarcomere lengths in invertebrate muscles [15,16,34], we suggest that this invertebrate nebulin-independent mechanism is similar or identical to the vertebrate nebulin-independent mechanism proposed for the ‘Composite’ model (Section IV) (Fig. 2E). Tmod is likely to be a key component of this nebulin-independent mechanism based on its ability to regulate pointed end dynamics and control thin filament lengths in both vertebrate and invertebrate muscles (Section I). Because of the limited sarcomere length working range, nebulin may not be required for maintaining uniform thin filament lengths in arthropods, unlike vertebrates [83].

In conclusion, the evidence suggests that there are two types of thin filament length regulation mechanisms (a nebulin-dependent mechanism and a nebulin-independent mechanism) that cooperate with each other to dynamically regulate thin filament lengths. An ancient nebulin-independent mechanism common to invertebrates and vertebrates appears to regulate pointed-end dynamics in coordination with sarcomere length to primarily specify thin filament lengths [10,15,16,86]. The lengths it specifies are sensitive to alterations in Tmod capping activity (Section I) and the actin monomer economy (Section II), as well as to the working range of sarcomere lengths (Section V). The nebulin-dependent mechanism does not appear to directly regulate pointed-end dynamics through a ruler mechanism [75], but specifies a long, stable core region in skeletal muscle thin filaments [86]. This stable core region appears to prevent disassembly from pointed ends and maintain a narrow thin filament length distribution at short working sarcomere lengths (Section IV). Thus, nebulin may specify a short, stable core region

in cardiac muscle thin filaments that allows a wide distribution of individual thin filament lengths to be generated in coordination with sarcomere working lengths [77].

This unified model for regulating thin filament lengths in both vertebrates and invertebrates suggests that comparative studies of different muscles and species will become increasingly useful and relevant. In this regard, it will be particularly interesting to find whether homologs of SALS are expressed in vertebrate muscles [15]. Future studies on nebulin function and isoform variation in vivo will be necessary to further understand its role in specifying thin filament lengths. However, by excluding a specific ruler function from nebulin's proposed repertoire [75,86], we believe that an additional focus on putative non-ruler functions, such as organizing Z-lines and regulating contraction [64,75,83,97,98], will provide the most insight. Finally, much work is needed to identify how pointed-end dynamics are regulated and coordinated with working sarcomere lengths independent of nebulin. The general properties of a titin/myosin scaffold mechanism where thick filament interactions continually regulate pointed-end dynamics to target thin filament pointed ends to the thick filament bare zone [10] are consistent with the qualities of the nebulin-independent mechanism proposed above. In this fast-moving field, we anticipate additional insights and perhaps more surprises into this fascinating and complex biological process will soon be at hand.

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Figure 1. Thin filament organization in striated sarcomeres

(A–C) Diagrams of vertebrate skeletal (A), vertebrate cardiac (B) and invertebrate (C) striated muscle sarcomeres showing the location of actin-binding proteins and the organization of thin and thick filaments. Elastic titin filaments (gray) extend an entire half-sarcomere from Z-line (Z) to M-line (M) to form a stable, yet flexible myofibril scaffold. Actin (thin) filaments (green) are precisely aligned with their fast-growing (barbed, B) ends at the Z-line where they are capped by capZ (cyan) and crosslinked by α -actinin (blue) to thin filaments in adjacent sarcomeres. The non-crosslinked (free) portions of the thin filaments extend $\sim 1\ \mu\text{m}$ away from the Z-line, overlap with the $1.6\ \mu\text{m}$ bipolar myosin (thick) filaments (purple), and their slow-growing (pointed, P) ends are capped by tropomodulin (Tmod, red). Tropomyosin/troponin

(TM/Tn) complexes copolymerize along the entire free portions of the thin filaments where they regulate myosin motor binding and bind Tmod at the pointed filament end (not shown). Nebulin (gold, A) is anchored at the Z-line and extends $\sim 1 \mu\text{m}$ along skeletal muscle thin filaments. Nebulette (gold, B), a nebulin homolog, extends $\sim 150 \text{ nm}$ along cardiac muscle thin filaments. Invertebrate (arthropod) muscles (C, shown at a reduced scale) have titin-like proteins that connect thick filaments to Z-lines (gray) and do not have nebulin homologs. Thin and thick filaments elongate to maintain overlap during sarcomere growth. (D) Thin filaments (green, phalloidin) are readily observed by light microscopy. Barbed (B) and pointed (P) ends are visualized using α -actinin (blue) and Tmod (red) antibodies, respectively. A gap in phalloidin staining (H-zone, H) indicates that thin filaments are regulated in length. Narrow Tmod striations indicate that thin filament lengths are uniform. Narrow α -actinin striations indicate that thin filament barbed ends are precisely aligned at Z-lines. The distance between Tmod and associated Z-lines (L) is equal to thin filament length [81].

Figure 2. Models for determining thin filament lengths

A, 'Static' ruler mechanism: nebulin (red line) extends along each thin filament, binds actin monomers (green circles), and determines where Tmod (blue trapezoid) is located. In this idealized model, thin filaments remain capped for their entire lifetime and there are no actin dynamics. Except for measurement error, the distance from the Z line for Tmod (L_{Tmod}) is equal to and specified by the length specified by nebulin (L_{nebulin}). Individual thin filament lengths do not vary. B–D, Dynamic ruler mechanisms. B, 'Cap Locator' mechanism: dynamic capping by Tmod, recruited to L_{nebulin} by nebulin, allows thin filament lengths to increase or decrease. L_{Tmod} is equal to and specified by L_{nebulin} . Individual thin filament lengths vary depending on pointed end dynamics. Sub-stoichiometric nebulin amounts in cardiac muscle

reduce how much T_{mod} is recruited to $L_{nebulin}$, resulting in a broader length distribution. C, 'Progressive Capping' mechanism: dynamic T_{mod} capping progressively slows elongation from pointed ends up to a maximum thin filament length equal to and specified by $L_{nebulin}$. However, L_{Tmod} is less than $L_{nebulin}$ because dynamic T_{mod} caps at shorter lengths. Individual thin filament lengths vary depending on pointed end dynamics. D, 'Stabilizing' mechanism: dynamic T_{mod} capping allows pointed ends to extend beyond $L_{nebulin}$, yet nebulin prevents filament shortening by stabilizing the thin filament, thereby specifying a minimum length equal to $L_{nebulin}$. L_{Tmod} is greater than $L_{nebulin}$ because dynamic T_{mod} caps at longer lengths. Individual thin filament lengths vary depending on pointed end dynamics. E, A Pointed-end regulation ('Composite') mechanism is similar to the stabilizing mechanism except that thin filament pointed end dynamics are regulated independently of nebulin in different muscles to control the length of the pointed-end extensions (L_{ext}). L_{Tmod} is specified by $L_{nebulin}$ and L_{ext} in each muscle. Individual thin filament lengths vary for each muscle depending on pointed end dynamics which may account for L_{ext} in different muscles. For clarity, only one strand of actin subunits in the filament is shown.