Lifting the Nebula: Novel Insights into Skeletal Muscle Contractility

Nebulin is a giant protein and a constituent of the skeletal muscle sarcomere. The name of this protein refers to its unknown (i.e., nebulous) function. However, recent rapid advances reveal that nebulin plays important roles in the regulation of muscle contraction. When these functions of nebulin are compromised, muscle weakness ensues, as is the case in patients with nemaline myopathy.

Although muscle physiology is a long-existing and well studied field of research, there are still major proteins in the striated-muscle sarcomere whose functions are not well understood, nebulin being a prime example. It is well known that the sarcomere consists of symmetric arrays of thick filaments (mainly comprising of myosin) and thin filaments (mainly comprising of actin) that interdigitate and slide past each other as cross bridges cycle and the muscle contracts. In addition, the sarcomere also contains nebulin, a giant filamentous protein that binds to F-actin. The name of this protein refers to its unknown (i.e., nebulous) function. However, recent rapid advances, due in part to the generation of nebulin KO models, reveal that nebulin plays important roles in the regulation of both thick-thin filament overlap and the regulation of contraction. When these functions of nebulin are absent, muscle weakness ensues, as is the case in patients with nemaline myopathy. Here, we review these exciting new insights into skeletal muscle contractility.

Nebulin Maintains Thin-Filament

The amount of overlap between the thick and thin filaments is, at a given sarcomere length, determined by their length. Whereas thick filament length is a constant 1.6 μm, thin filament lengths are fine-tuned at ~1.0–1.3 μm [depending on species and muscle type (22)] to overlap with thick filaments and to meet the muscle’s physiological requirements (6, 22). The extent of overlap between thick and thin filaments determines the sarcomere’s force-generating capacity: short thin filaments reduce overlap and reduce force (14). Thus thin-filament length is a key aspect of muscle function. For this reason, in recent years, the regulation of thin-filament length in skeletal muscle has gained increased attention. It has been speculated that the thin-filament/titin array may be involved by positioning the thin-filament-pointed end relative to the Z-line (21, 41); alternatively, the existence of a molecular ruler has been an attractive model for determining thin-filament length. Length is not an intrinsic property of actin filaments [actin monomers assemble in vitro to highly variable and very long polymer lengths (37)], and, therefore, thin-filament length is likely to be specified in vivo by an actin-binding protein; for this, nebulin for a long time has been considered a prime candidate (19, 20, 42).

Nebulin is a giant protein (molecular weight of 700–900) expressed at high levels in skeletal muscle. A single nebulin molecule spans the thin filament with its COOH terminus anchored at the Z-disk, and its NH2-terminal region directed toward the thin-filament-pointed end (for a schematic representation, see FIGURE 1) (42). Evidence for a role of nebulin in establishing thin-filament length comes from various multidisciplinary approaches. First, the analysis of nebulin’s cDNA sequence revealed that the bulk of the molecule is comprised of modules with the centrally located modules M9 to M162, each thought to represent individual actin-binding motifs, and organized into seven-module super-repeats that match the repeat of the actin filament (FIGURE 1). This precise arrangement is thought to allow each nebulin module to interact with a single monomer of the actin filament (19, 20) and each nebulin super-repeat to associate with a single tropomyosin (Tm)/tropinin (Tn) complex (16, 26, 31). Nebulin’s extreme NH2-terminal modules M1–M3 (FIGURE 1) contain a high-affinity binding site for the thin-filament-pointed-end capping protein tropomodulin (27). Tropomodulin, in addition to binding nebulin’s NH2 terminus, binds actin and Tm with high affinity and prevents actin filaments from elongating or shortening at the pointed end (10, 11). Furthermore, earlier studies revealed that the electrophoretic mobility of nebulin from different muscle types correlates with thin-filament length (18, 19).

More conclusive evidence for a role for nebulin in regulating thin-filament length required studies of muscles that lack nebulin. Such studies were...
They found that, on RNAi knockdown of nebulin transcripts in cultured cardiac myocytes, thin filaments elongated to unrestricted lengths, thus supporting a role for nebulin in regulating thin-filament length. These experiments were carried out in cardiac myocytes where nebulin expression is <1% of the levels found in skeletal muscle (43). To test the role of nebulin in skeletal muscle in vivo, nebulin KO mouse models were generated (3, 43). The first work on these models revealed that, in nebulin-deficient skeletal muscle, the thin filaments are on average shorter, thus supporting a role for nebulin in the in vivo regulation of thin-filament length (3, 43). Witt et al. (43) performed an electron microscopy study and reported that thin-filament lengths in wild-type tibialis cranialis muscle are a constant \( \sim 1.2 \, \mu m \) but in nebulin-deficient muscle are on average \( \sim 0.8 \, \mu m \) and range from \( \sim 0.4 \, \sim 1.2 \, \mu m \) (FIGURE 2A). Such reduction in thin-filament length is expected to greatly affect force production. This can be illustrated by using the obtained range of thin-filament lengths to predict thick-thin filament overlap, and thus force, as a function of sarcomere length, as has been performed by Ottenheijm et al. (34) for both wild-type and nebulin-deficient muscle. As shown in FIGURE 2B, the predicted force-sarcomere length relation of wild-type muscle is characterized by a force plateau reflecting optimal thick-thin filament overlap, followed by a descending limb at higher sarcomere lengths as filament overlap decreases. On the other hand, the shortened thin filaments in nebulin-deficient muscle reduce filament overlap at a given sarcomere length on the descending limb, impairing force production and resulting in a leftward shift of the predicted force-sarcomere length relation (FIGURE 2C). Furthermore, no optimal thick-thin filament overlap exists when thin filaments are non-uniform in length, and therefore the force-sarcomere length relation of nebulin-deficient muscle lacks the characteristic plateau. The predicted force-sarcomere length relations were verified in experiments in which demembranated nebulin-deficient and wild-type muscle fibers were activated at various sarcomere lengths with exogenous calcium [thus ruling out that factors outside of the myofilaments, e.g., calcium handling by the sarcoplasmic reticulum, contribute to changes in force production (32)]. These experiments (34) yielded force-sarcomere length relations that were similar to the predicted relations, thus confirming that shorter thin filaments indeed contribute to the muscle weakness observed in nebulin-deficient skeletal muscle. Consistent with these findings on demembranated muscle, studies

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**FIGURE 1.** Schematic of the structural organization of the skeletal muscle sarcomere

The sarcomere is the smallest contractile unit of striated muscle; it consists of symmetric arrays of thick filaments, mainly comprised of myosin, and actin-based thin filaments that interdigitate and slide past each other as cross bridges cycle and the muscle contracts. In addition, the sarcomere also contains the giant myofilament titin, which connects the thick filament to the z-disk, and nebulin, which spans the length of the thin filament and binds to the thin-filament capping protein tropomodulin. Nebulin has a highly modular structure, with in the central region 7 modular repeats arranged into 22 super-repeats (S1–S22) that match the repeat of the actin filament.
on intact nebulin-deficient muscle from another nebulin knockout model (12), in which muscles were activated at various lengths by electrical stimulation, also revealed a leftward shift of the force-muscle length relation of nebulin-deficient muscle. Thus the force-length relation of nebulin-deficient muscle is altered in a manner that is consistent with the presence of shorter thin filaments.

Work by Bang et al. (3) on their nebulin KO model, using confocal microscopy on 1-day-old mice, indicated that, in the absence of nebulin, thin-filament lengths are reduced from ~1.15–1.3 μm (depending on muscle type) in wild-type muscle to a consistent ~1.0 μm in all muscles types. These findings led to the proposal (22) that a nebulin-independent mechanism specifies uniform thin-filament lengths at ~1.0 μm in all muscle types, whereas nebulin is responsible for specifying longer thin-filament lengths in a muscle-specific manner. Castillo et al. (7) used immunofluorescence microscopy on rabbit muscle and concluded that nebulin specifies the minimum thin-filament length (~1.0 μm), with a nebulin-independent mechanism regulating the final length according to the requirements of a particular muscle. These two studies both used confocal microscopy, and it is possible that their disparate conclusions are related to its limited resolution and inability to detect length gradients. To avoid these limitations, Witt et al. (43) used electron microscopy and decorated thin filaments with gold beads (attached to actin monomers via the actin-binding peptide phalloidin). This made it possible to determine thin-filament length gradients and to show that thin filaments varied in length and were on average shorter than in wild-type muscle (see also FIGURE 2A). Furthermore, a recent analysis using high-resolution protein gels as well as a nebulin exon microarray revealed only small differences in nebulin size between different muscle types that were largely restricted to the Z-disk region of nebulin (5). These studies support the conclusion by Castillo et al. that there is a nebulin-dependent mechanism that sets a minimum thin-filament length. To further our understanding of the role of nebulin in thin-filament length regulation, additional studies on a range of mouse muscle types are needed that measure by electron microscopy thin-filament length and the location of nebulin’s NH₂ terminus. It is clear, however, from the above-referenced studies that nebulin does play a critical role in the regulation of thin-filament length: in its absence, the average thin-filament length is shorter and force is reduced.

Nebulin’s role in thin-filament length regulation provides a mechanism for the first time to explain severe muscle weakness in patients with nemaline myopathy, a debilitating disease frequently caused by nebulin gene mutations (35, 36). Indeed, muscle fibers from patients suffering from severe muscle weakness in patients with nemaline myopathy, a debilitating disease frequently caused by nebulin gene mutations (35, 36). Indeed, muscle fibers from patients suffering from

![FIGURE 2](http://physiologyonline.physiology.org.org/)

**FIGURE 2.** The effect of nebulin on thin-filament length and the sarcomere length dependence of force

A: sarcomere schematic illustrating the range of thin-filament lengths found in nebulin-deficient muscle fibers and how this affects thick-thin-filament overlap. B: predicted force-sarcomere length relation using the measured range of thin-filament lengths in TC muscle. Note that the predicted force-sarcomere length relation of murine wild-type muscle fibers has a characteristic force plateau followed by a descending limb. The predicted relation of nebulin-deficient fibers is shifted leftward compared with wild-type fibers, and the force plateau is absent (see also Ref. 34).
Nemaline myopathy, due to nebulin mutations that cause nebulin deficiency, show remarkable phenotypic similarities to fibers from nebulin KO mice, i.e., shorter and non-uniform thin-filament lengths and significantly impaired force-generating capacity (34). Thus loss of thin-filament length regulation appears to be an important contributor to muscle weakness in patients with nemaline myopathy.

**Nebulin Maintains Myofibrillar Alignment**

In addition to regulating thin-filament length, nebulin also plays an important role in the transverse linking of myofibrils. Myofibrils are not independent of each other but instead are laterally connected at the level of their Z-disks. This limits the degree to which adjacent myofibrils translocate relative to each other during active contraction or passive stretch, thereby preventing damage to intermyofibrillar membrane systems, such as T-tubules and the sarcoplasmic reticulum. An important protein involved in linking adjacent Z-disks is the intermediate filament protein desmin, which forms a network of filaments that surrounds myofibrils at the level of the Z-disk (39). The attachment site within the sarcomere has been unknown so far. In vitro work, using a yeast two-hybrid approach, suggests that desmin binds to the COOH-terminal region of nebulin (2, 9). This role for nebulin in intermyofibrillar connectivity was tested recently in nebulin-deficient mouse muscle (25). In these studies, it was found that, on stretch, myofibrils devoid of nebulin translocate to a much higher degree than wild-type myofibrils, resulting in a much larger Z-disk displacement. Although desmin is present in muscle devoid of nebulin, it is reduced in the intermyofibrillar spaces that surround the Z-disks, suggesting that nebulin is required for proper localization of desmin at the Z-disk (25). Consistent with this, both knockdown of nebulin with siRNA and overexpression of nebulin’s COOH terminus prevents desmin localization at the mature Z-disk (25). Thus nebulin is required to localize and connect desmin to the Z-disk, and nebulin prevents structural damage due to myofibrillar misalignment.

**Nebulin as a Regulator of Contraction**

Recent work shows that nebulin’s role is not merely structural but that nebulin also regulates contraction. Muscle contraction is driven by the cyclic interaction between the myosin-based cross bridges and actin, and the level of force a muscle generates is proportional to the force generated per cross bridge and the number of cross bridges in the force-generating state. It is generally accepted that this interaction between actin and myosin is regulated through a steric hindrance mechanism in which $T_m$ and $T_n$ control the conversion between interaction permissive and non-permissive states (13). This view needs to be extended since two independent studies (1, 8) recently identified that nebulin contributes to the regulation of cross-bridge cycling kinetics, with one of the two (8) also identifying a role for nebulin in the calcium sensitivity of force generation.

During the cross-bridge cycle, unbound non-force-generating cross bridges move to an actin-bound force-generating state followed by ATP-driven cross-bridge release back to the non-force-generating state (15, 24). Brenner et al. (4) proposed an analytical framework in which this transition between force and non-force-generating cross-bridge states can be described by two apparent rate constants; one for cross-bridge attachment ($f_{app}$) and one for cross-bridge detachment ($g_{app}$). These two rate constants determine the fraction of force-generating cross bridges during activation, and a change in one or both will affect this fraction and thus force generation.
production. \( g_{\text{app}} \) is directly proportional to the ATP consumption rate normalized to tension generation (i.e., tension cost) and can therefore be estimated from the simultaneous determination of ATP consumption rate and tension in activated muscle fibers. Such studies on nebulin-deficient muscle (8) revealed significantly higher tension cost in nebulin-deficient muscle (see Figure 3A), thus indicating a faster \( g_{\text{app}} \) and cross-bridge detachment rate when nebulin is absent. Likewise, studies on another nebulin KO mouse model (1) reported higher velocity of unloaded shortening in nebulin-deficient muscle, also suggesting that \( g_{\text{app}} \) is higher when nebulin is absent. It is also important to highlight that these findings are consistent with results of in vitro motility assays in which nebulin fragments were found to reduce the sliding velocity of F-actin over myosin (38).

In Brenner’s framework (4), the rate constant of force redevelopment (\( K_{\text{tr}} \)) is proportional to \( f_{\text{app}} + g_{\text{app}} \), and the fraction of force-generating cross bridges to \( f_{\text{app}}/(f_{\text{app}} + g_{\text{app}}) \). The rate constant of force redevelopment can be estimated by imposing a rapid release-restretch protocol on an activated fiber, mechanically disengaging all bound cross bridges so that force drops to zero and then measuring force redevelopment. Such experiments (see Figure 3B) revealed that force redevelopment is slower in nebulin-deficient muscle (1, 8). Thus the decrease in \( K_{\text{tr}} \) of nebulin-deficient muscle, together with the notion that \( g_{\text{app}} \) is increased, indicates that \( f_{\text{app}} \) must be reduced and that the reduction must be larger than the increase in \( g_{\text{app}} \) (for a schematic, see Figure 3C). Combined, this leads to the conclusion that the fraction of force-generating cross bridges \( f_{\text{app}}/(f_{\text{app}} + g_{\text{app}}) \) is reduced in nebulin-deficient muscle. Furthermore, stiffness measurements indicated that the force per cross bridge was not affected by the absence of nebulin (1, 8). In summary, recent studies suggest that nebulin increases the rate of cross-bridge attachment and reduces the rate of cross-bridge detachment and that, as a result, the number of force-generating cross bridges is increased. Although the mechanism by which nebulin affects cross-bridge cycling needs further investigation, previous work (38) has shown that nebulin associates with the actin NH\(_2\) terminus in subdomain 1, where also the myosin cross bridge binds. Thus the presence of nebulin at or near the S1 binding site might enhance the binding of cross bridges and slow their detachment. Chandra et al. estimated that the effect of nebulin on cross-bridge kinetics enhances a muscle’s force-generating capacity by \( \sim 50\% \) and increases the economy of contraction by \( \sim 35\% \) (8). These estimations are in line with findings reported by Bang et al. (1), and they can largely account for the more pronounced leftward shift of the measured force-sarcomere length relation of nebulin-deficient muscle compared with the predicted relation based on only thin-filament length measurements. Clearly, nebulin is a major factor in determining the level of force and the energetic cost of force production in skeletal muscle. In line with this role of nebulin in the regulation of cross-bridge cycling kinetics, recent studies on muscle fibers from patients with nemaline myopathy with severely reduced nebulin protein levels revealed that, in addition to altered thin-filament length, changes in cross-bridge cycling kinetics contribute to the muscle weakness observed in these patients (33).

The studies discussed above were carried out at a maximal activating calcium level. Chandra et al. (8) also measured active force at a range of calcium levels, and the obtained force-pCa relations were markedly shifted to the right in nebulin-deficient muscle fibers, with a 0.16-unit reduction in pCa\(_{50}\) (pCa that gives the half-maximal force level). To rule out a possible difference in the Tn complex (isomorph composition and posttranslational modification) between wild-type and KO fibers, these studies were carried out on wild-type and KO fibers that had been reconstituted by the same recombinant Tn complex. An analysis of expression levels of Tm, myosin heavy chain, and myosin light chain did not reveal a significant difference in these proteins between the fibers, suggesting that the absence of nebulin is the most likely explanation for the lower calcium sensitivity of the KO fibers. Interestingly, the studies by Witt et al. (43) and Bang et al. (1) found no difference in calcium sensitivity. It is possible that the discrepancy is due to the fact that those studies did not carry out a Tn exchange and that differences in the Tn complex between wild-type and KO fibers could have ne-
gated nebulin’s effect on calcium sensitivity. An alternative explanation involves the difference in sarcomere length between the studies. The two studies that did not detect a difference in calcium sensitivity were carried out at long sarcomere lengths (∼2.5 μm (43) and ∼2.6 μm (1)), whereas the study that did show a difference (8) was performed at ∼2.0 μm. The implication is that nebulin plays a role in the length dependence of activation with a much larger ΔpCa0 in the nebulin KO fibers than in wild-type fibers. It is a well known phenomenon that as sarcomere length increases muscle becomes more calcium sensitive. This length dependence of activation is most prominent in cardiac muscle (and is thought to underlie the Frank-Starling law of the heart) but is much less pronounced in skeletal muscle (17). The presence of nebulin provides an explanation for why skeletal muscle has less length dependence: the presence of nebulin increases calcium sensitivity at short length. Thus nebulin is an important player in a wide range of skeletal muscle characteristics.

The structure and protein-binding properties of nebulin support a role in thin-filament activation. Nebulin contains ∼200 domains of ∼35 amino acids that are characterized by the actin-binding sequence SDXXVYK; these domains make up seven domain super-repeats characterized by the Tm/Tn binding motif WLKGIGW (26). Biochemical studies have shown that a single nebulin module interacts with a single actin monomer and that each nebulin super-repeat interacts with a Tm/Tn complex of the thin filament (31), binding characteristics that support that nebulin follows the helical path of F-actin. It is interesting that, similar to Tm, nebulin appears to have different binding sites on F-actin with one site in close proximity to both the strong binding site for myosin and the blocked state of Tm (23). This leads to the intriguing possibility that nebulin acts in concert with Tm and that it promotes the transition of contractile regulatory units (Tm/Tn) from the nonpermissive to the permissive states, thereby increasing myofilament calcium sensitivity.

It is striking that nebulin-deficient skeletal muscle shares the low calcium sensitivity and low maximal active force with cardiac muscle, where stoichiometric levels of nebulin are absent (3, 43). Cardiac muscle contains the nebulin-homolog nebullette (30). However, nebullette is much smaller than nebulin (∼100 vs. ∼800 kDa for nebulin), and its location is restricted to the Z-disk and the near Z-disk I-band region (29). Thus the thin filament in cardiac muscle is largely nebullette-free, making it unlikely that nebullette is involved in thin-filament activation. Cardiac muscle has multiple mechanisms for enhancing thin-filament activation, such as enhanced length-dependent activation and the presence of multiple cardiac-specific phosphorylation sites in various thin- and thick-filament-based protein sites (40), which allow cardiac muscle to grade its force response to different loading conditions. Since skeletal muscle lacks the aforementioned features unique to cardiac muscle, nebulin might be essential for tuning thin-filament activation for optimal skeletal muscle function.

In summary, recent studies on nebulin-deficient skeletal muscle provide evidence that nebulin significantly increases force generation in skeletal muscle by 1) specifying thin-filament length to optimize thin-thick filament overlap, 2) increasing thin-filament activation, and 3) regulating crossbridge cycling kinetics to increase the number of strongly attached cross bridges (FIGURE 4). These findings provide important novel insights in skeletal muscle contraction and provide new avenues for understanding and combating nemaline myopathy, a debilitating disease that is frequently caused by nebulin deficiency and that is characterized by severe muscle weakness. Thus the nebulsa is lifting, and exciting novel roles for nebulin in skeletal muscle function are beginning to emerge.

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