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The Nebulin Family: an Actin Support Group

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Abstract

Nebulin, a giant actin-binding protein, is the largest member of a family of proteins (including N-RAP, nebulin, lasp-1 and lasp-2) which are assembled in a variety of cytoskeletal structures and expressed in different tissues. For decades nebulin has been thought to act as a molecular ruler, specifying the precise length of actin filaments in skeletal muscle. However, emerging evidence suggests that nebulin should not be viewed as a ruler but as an actin filament stabilizer required for length maintenance. Nebulin has also recently been implicated in an array of regulatory functions independent of its role in actin filament length regulation. In this review, we discuss the current evolutionary, biochemical, and functional data for the nebulin family of proteins - a family whose members, both large and small, function as cytoskeletal scaffolds and stabilizers.

Introduction

The nebulin family of cytoskeletal proteins is composed of members with diverse expression patterns and cellular functions. The founding member of the family is the giant protein nebulin (600-900 kDa) [1]. Nebulin is abundantly expressed in skeletal muscle and plays an important structural role in the force generating machinery of the muscle sarcomere (Glossary) by binding to the actin thin filament and regulating its assembly and function. Nebulin is also present, albeit in low amounts, in vertebrate heart muscle [2,3]. Recently, much progress has been made toward deciphering the mechanisms by which nebulin regulates actin filament architecture. In fact, a decades-old hypothesis that nebulin functions as a molecular ruler for thin filament assembly has recently been challenged and it appears that nebulin does not determine the length of the filament, but instead stabilizes filamentous actin allowing the filament to reach its mature length [4]. The other nebulin family members — N-RAP, nebulin, lasp-1 and lasp-2 — also bind actin, but contain unique combinations of protein domains (e.g., LIM, SH3) that culminate in distinct molecular weights, expression patterns and assembly in specialized cytoskeletal structures (e.g., intercalated discs, focal adhesions). In addition, there is growing evidence implicating nebulin family members in human disease.

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The 5 nebulin family members have molecular weights ranging from 34 to 900 kDa and tissue expression patterns from heart to brain, and at first glance it is difficult to identify a unifying trait other than their capacity to bind actin. However, whether they are interacting with ~1 μ m long actin filaments of striated muscle or the complex focal adhesions of fibroblasts, all members appear to serve as stabilizers and scaffolds for the cytoskeletal structures with which they are associated. In this review, we highlight the diverse cellular functions of the nebulin family including its roles in cytoskeletal stability, cell migration, as protein scaffolds and in disease.

Structure and Evolution of The Nebulin Family: How to Become a Member?

Most nebulin family members are products of different genes; yet, they all contain the defining characteristic of actin-binding domains referred to as “nebulin repeats”. Nebulin repeats are ~35 amino acids in length and contain a conserved SDxxYK motif [5]. Sequence analysis of human nebulin revealed an astonishing 185 tandem repeats (Figure 1) [6]. Within the central region of the molecule (repeats 9-162) groups of seven single repeats are arranged into “super repeats”, which also contain a single conserved motif (WLKGIGW; located at the end of the third repeat within each super repeat) and are thought to interact with troponin/tropomyosin complexes (calcium-mediated regulators of muscle contraction) along the length of the actin filament [6,7]. The N-terminus of nebulin has a unique acidic sequence of unknown structure, while the C-terminus contains a serine-rich domain followed by an SRC homology 3 (SH3) domain [6].

The other members of the nebulin family contain significantly fewer nebulin repeats (Figure 1). N-RAP also contains fewer super repeats than nebulin, while nebulette, lasp-1 and lasp-2 lack super repeats all together. All family members, except N-RAP, have an SH3 domain, while N-RAP, lasp-1 and lasp-2 have a LIM domain not present in nebulin and nebulette. Therefore, all of the nebulin family members are composed of unique combinations of protein motifs, forming multi-domain proteins that interact with filamentous actin through their nebulin repeats.

Although the bulk of the research on nebulin proteins has been in vertebrates, genes of the nebulin family can also be found in invertebrates [8-10]. Genomic analyses suggest that the number of nebulin family members has increased over the course of evolution. Non-chordate invertebrates such as nematodes, fruit flies and sea urchins only have lasp-like genes, which contain a LIM domain, 2-3 nebulin repeats and an SH3 domain [8,10]. However, the chordate invertebrate lancelet only contains a single huge nebulin-like gene. This lancelet nebulin has exons encoding a LIM domain, an extensive number of nebulin repeats (including 2 sets of super repeats), and an SH3 domain [9]. Thus, nebulin may have expanded from the lasp-like gene after chordates diverged. Remarkably, the single lancelet gene includes all the domains necessary to generate any nebulin family member. The invertebrate chordates most closely related to vertebrates, tunicates, have separate nebulin and lasp-like genes [8], while vertebrates display an even greater expansion of the nebulin family, which includes four genes (nebulin, N-RAP, nebulette/lasp-2, lasp-1). Under phylogenetic scrutiny each individual nebulin family member (i.e. the nebulins, the nebulettes, etc.) from various vertebrate species forms a group and collectively they form a super-group, while nebulin from the invertebrate chordates (tunicates and lancelets) forms a group outside of the vertebrate super-group. Thus, one hypothesis is that at some point in the evolution of chordates, the nebulin family expanded from a single common ancestor, possibly lancelet nebulin [9]. One complication to this hypothesis, however, is that sequence analysis of the two sets of super repeats in lancelets indicate that they are not only very different from the super repeats of vertebrate nebulins, but also from each other (including the absence of a WLKGIGW motif) [10]. Thus, it was suggested that the addition of super

repeats occurred independently and multiple times during the course of nebulin's evolution and therefore, lancelet nebulin may not truly represent the common ancestor of the nebulin family in chordates [10].

If the progenitor of the nebulin family indeed resembled lasp, the large number of repeats nebulin contains (e.g., 185 in human) suggests that it has extensively expanded over time. Interestingly, this expansion has predominantly progressed in 7-repeat increments [11], which are the size of a single nebulin super-repeat. This is likely due to constraints placed upon nebulin for proper function. Nebulin super-repeats match the periodicity of, and are thought to interact with, troponin/tropomyosin along the length of the thin filament [6,7]. Therefore, the super-repeat likely represents a functional unit, which was selected for during the course of nebulin's expansion. This could be to elongate the thin filament by adding additional tropomyosin molecules (which have been shown to stabilize the filament) and/or extend some regulatory function (e.g., control of myosin-actin interaction).

Even though no bacterial homolog of nebulin family members has been identified, *Salmonella* invasion protein A (SipA), which promotes entry into a host cell, in part by binding and stabilizing actin filaments, shows remarkable similarity to nebulin repeats in its mechanism of actin binding [12,13]. Thus, SipA and nebulin family members may have convergently evolved to stabilize actin filaments (see functions of nebulin proteins below).

Functions of the Nebulin Family: A Melting-Pot

Members of the nebulin family are involved in the regulation of actin filament architecture and function in a variety of functional contexts. In fact, each family member appears to have importance to its own unique milieu (Figures 2 and 3).

NEBULIN

Over 20 years ago nebulin was proposed to function as a “molecular ruler” that specifies the exact lengths of actin-thin filaments in skeletal muscle (Box 1). Since nebulin is notoriously difficult to work with (i.e., large and susceptible to proteolysis), direct evidence that it is responsible for regulating thin filaments lengths has only been gathered in the last few years. The first report implicating nebulin in length regulation in live cells was in primary cultures of cardiomyocytes in which the knockdown of nebulin via small interfering RNAs (siRNAs) resulted in the growth of thin filaments to abnormally long lengths [14]. Conversely, the removal of nebulin in two independent nebulin knockout mouse models, and in skeletal myocytes in culture, resulted in shorter thin filaments [3,4,15,16]. Why the perturbation of nebulin levels results in different effects on thin filament lengths is unclear, but may indicate different roles for nebulin in skeletal muscle versus heart, where much lower levels of nebulin are expressed [2,3]. The proper assembly of nebulin was also found to be important for its actin regulatory functions in cultured myocytes since displacement of Z disc (C-terminal) nebulin resulted in altered thin filament lengths [17]. While these studies demonstrate that nebulin is involved in dictating the final lengths of the thin filaments, they do not provide a mechanism by which it functions. A strict ruler mechanism predicts that the absence of nebulin would result in either the complete lack of actin filaments or the formation of filaments with a broad length distribution. Intriguingly, shorter but uniform thin filaments are seen in nebulin knockout mice [3] suggesting that a nebulin-independent mechanism controls immature actin filament lengths, while nebulin is responsible for specifying mature lengths. Contrastingly, another study [15] reported shorter, non-uniform thin filaments in their nebulin knockout mice, an observation consistent with a ruler mechanism. One possible explanation of this discrepancy is that actin filament non-uniformity could arise from muscle use over time, since the mice in the latter study were

analyzed at a later stage of development (10-15 days old) compared to the former study (1 day old).

BOX 1

What is a molecular ruler?

What constitutes a molecular ruler is highly debatable. By the strictest definition, it is a molecule that is necessary and sufficient to specify the length of another structure by providing a template for that structure. Only a few examples of molecular rulers in the natural world have been described. gpH and gp29 are two proteins which define the tail lengths of bacteriophages lamda and T4, respectively [66,67]. Also, YscP regulates the length of the needle of the type-III secretion system (injectosome) of the bacteria *Yersinia* [68]. Lastly, an extensive amount of correlative evidence has been collected suggesting that nebulin also functions as a molecular ruler controlling the lengths of thin filaments within skeletal muscle [69-71]. Nebulin extends along the entire length of the thin filament and is able to interact with many thin filament components (e.g., actin, tropomyosin, and troponins) (Figure 2) [7,72-74]. Furthermore, nebulin is abundantly expressed in skeletal muscle (2-4 molecules per thin filament) and its length, which can vary due to alternative splicing, correlates with thin filament lengths in various muscle types [5,75,76]. Analysis of nebulin's sequence and structure suggests that it is helical upon actin binding and may interact along the main groove between the two actin protofilaments [77]. However, it does not have any domains that are recognized to function as actin cappers and is therefore unlikely to possess the ability to inhibit actin polymerization. Without a way to restrict actin growth it was unclear how nebulin could function as a molecular ruler. This puzzle was thought to be resolved when high affinity binding sites for the barbed and pointed end actin capping proteins, CapZ and Tmod respectively, were discovered at the ends of nebulin [15,16,19]. Therefore, for the last few years, one model of nebulin's function was that it specifies the length of the thin filament by measuring/organizing a specific number of actin and tropomyosin/troponin molecules, and then directs the capping proteins to the ends of the filament to restrict polymerization to that defined length [78]. Consequently, nebulin does not meet the criteria of a strict molecular ruler (i.e., both necessary and sufficient) but regulates actin filament length in conjunction with other factors as a component of a ruler complex.

More recently, the "molecular ruler" hypothesis was directly challenged by constructing a novel, small synthetic version of nebulin (mini-nebulin) [4]. Mini-nebulin contains both the unique N- and C-terminal regions of human nebulin but is missing 18 (out of 22) super-repeats in the central portion of the molecule. When expressed in cultured skeletal myocytes, mini-nebulin extends ~200 nm out of the Z-disc versus ~1 μ m for endogenous nebulin. When endogenous nebulin was replaced with mini-nebulin, actin filament assembly was not restricted to the size of mini-nebulin, an observation that is inconsistent with a strict ruler function. However, when the actin filaments were depolymerized, the filaments that remained, remarkably, either matched or were longer than the length of mini-nebulin, indicating that mini-nebulin was able to stabilize the actin filaments at its own length, as well as at lengths longer than itself. Furthermore, the knockdown of nebulin resulted in more dynamic populations of thin filament components (actin, tropomyosin and tropomodulin). Taken together, these results reveal that nebulin regulates thin filament lengths by stabilizing the filaments and preventing depolymerization, not by a traditional ruler mechanism (Figure 4).

In addition, if nebulin worked in concert with actin filament capping proteins to define the precise lengths of thin filaments (i.e., was part of a ruler complex, Box 1) then the ends of nebulin would be predicted to colocalize with the capping proteins. Comparisons of the

location of the N-terminus of nebulin and the pointed end actin capping protein tropomodulin (Tmod) by immunofluorescence microscopy in various rabbit skeletal muscles imply that Tmod, and presumably the end of the actin filament, extend beyond the end of nebulin [18]. Furthermore, Tmod was never observed to localize to the end of mini-nebulin in skeletal myocytes in culture [4]. This is puzzling because the N-terminus of nebulin was found to contain a high affinity Tmod binding site by *in vitro* assays [19,20]. Perhaps the interaction of nebulin with Tmod is transient and plays an important developmental role at a specific stage in myofibrillogenesis. In any case, these results suggest there is some form of nebulin-independent mechanism to control actin filaments that have grown beyond the length of nebulin, which is consistent with nebulin functioning as an actin filament stabilizer.

On the opposite end of the filament, the barbed end actin capping protein, CapZ, colocalizes with the C-terminus of nebulin within the Z-disc and they have been shown to interact *in vitro* [15,16]. However, the manner in which these two proteins interact *in vivo* is unclear since the precise layout nebulin adopts within the Z-disc is unknown. The original model, based on immunoelectron microscopy, suggested that the C-terminal end of nebulin extends only partially into the Z-disc [21]. However, this would position the binding sites of multiple, integral Z-disc proteins within nebulin outside the Z-disc. Thus, a new model that incorporates significant binding data [16,17,22] has recently been proposed, which places a significant amount of nebulin within the Z-disc where it crosses from one thin filament to the next connecting adjacent sarcomeres [16] (Figure 4). Nebulin could stabilize actin filaments either directly by providing actin monomers with additional molecular contacts and preventing their dissociation from the filament and/or indirectly by stabilizing tropomyosin, which itself has been shown to prevent actin depolymerization [23]. A third possibility, recently proposed [24], is that nebulin could stabilize the thin filament by exerting a compressive force. Analysis of single nebulin molecules by atomic force microscopy suggests that nebulin is quite compliant and when fully extended, may apply a significant restorative force to the thin filament [24]. This force could mechanically protect the filament from contractile-induced strain and prevent actin depolymerization.

Besides contributing to actin filament length regulation, nebulin has recently been shown to function in a host of other capacities. Multiple lines of evidence suggest nebulin is necessary for proper Z-disc structure. Nebulin knockout mice have wider Z-discs and electron dense structures reminiscent of nemaline rod bodies, which are aggregates of thin filament and Z-disc proteins found in the muscles of patients with nemaline myopathy (Box 2) [3,15,25]. Also, the knockdown of nebulin in skeletal myocytes resulted in reduced assembly of CapZ and the non-uniform alignment of actin filament barbed ends [16]. Nebulin not only maintains the structure of the Z-disc but also may specify its width. In the mouse soleus muscle, the number of C-terminal nebulin exons expressed increases during development and correlates with an increase in Z-disc width [26]. Nebulin is also essential for the proper contractile function of skeletal muscle; the muscles of nebulin knockout mice produce significantly less force than their wild-type counterparts, which results in death shortly after birth [3,15,27]. This is likely the result of the loss of multiple nebulin-associated functions. For example, decreased actin filament lengths as observed in knockout mice leads to a decreased potential for actin-myosin cross-bridge formation. Additionally, nebulin directly affects contractility by promoting the interaction of myosin and actin [28,29]. Finally, nebulin also plays a role in calcium handling; therefore its loss indirectly influences contractility [30].

BOX 2

Nebulin family's impact on human disease

NEBULIN

Mutations in nebulin are the leading cause of the human muscle disorder nemaline myopathy and have also been shown to result in distal and core-rod myopathies [79-81]. Individuals with nemaline myopathy present with muscle weakness, which can be severe leading to neonatal lethality. Rod-like “nemaline” bodies, composed of Z-disc and thin filament proteins, are prevalent in the muscle fibers of nemaline myopathy patients. To date, 64 separate mutations have been identified in nebulin that result in nemaline myopathy [82]. Most mutations are deletions or insertions producing frameshifts that are predicted to result in the early termination of the protein, but some are point mutations that may affect splicing or result in a missense mutation. Excitingly, Granzier and colleagues have recently directly linked a nebulin mutation that results in nemaline myopathy to improper assembly of the thin filament and impaired contractility [83,84]. This mutation (deletion of exon 55) results in decreased nebulin levels, which in turn leads to decreased force production due to shorter thin filaments and reduced cross-bridge formation.

NEBULETTE

Several human polymorphisms in the nebulin gene have been associated with dilated cardiomyopathy (DCM), a cardiac disease characterized by dilation of the left ventricular cavity and systolic dysfunction [85,86]. Transgenic mice expressing these same human nebulin polymorphisms also exhibit signs of DCM, with some of the mutations leading to severe heart failure, changes in sarcomeric proteins and mislocalization of nebulin [85].

N-RAP

N-RAP is highly upregulated in two mouse models of DCM [87,88]. These mice exhibit an early increase in N-RAP expression before the manifestation of DCM. The striking upregulation of N-RAP might be an adaptive response to strengthen the link between the myofibrils and the membrane at intercalated discs [88].

LASP-1

Lasp-1 is overexpressed in breast, ovarian and liver cancer [49,50,52]. Due to this overexpression, it has been proposed that lasp-1 may be involved in the increase in migration of cancer cells and invasion into surrounding tissue. Contrary to this conclusion, however, are data from the lasp-1 knockout mouse that show loss of lasp-1 actually increases cell migration and results in an increased number of tumors. Furthermore, some studies have shown that overexpression of lasp-1 in cancer cell lines actually reduces cell migration and invasion [44,49]. These data suggest that the severe upregulation of lasp-1 found in human cancer might be linked to a compensatory mechanism rather than being detrimental.

Myofibrils of striated muscle are connected to one another by a network of desmin intermediate filaments [31]. Desmin localizes primarily to the Z-disc and interacts with Z-disc nebulin modules [17,22,32]. In the nebulin knockout mice, loss of myofibril lateral alignment and a reduction in desmin assembly at the Z-disc was observed indicating that these two molecules work together to correctly position myofibrils [3,25]. In conclusion, it is becoming increasingly clear that nebulin has a diverse array of functions, the sum of which is required for proper muscle function.

NEBULETTE

Nebulette is the only cardiac-specific nebulin family member. This led to early reports that speculated nebulin could be the functional counterpart of nebulin in heart muscle [33].

Since nebulin is around a sixth the size of nebulin and only localizes to the Z-disc (Figure 2) [33], it is difficult to imagine that this protein could function to stabilize the entire thin filament in the same manner as nebulin. However, reduction of endogenous nebulin levels decreases the lengths of thin filaments in cardiomyocytes [34], a phenotype similar to that observed in the skeletal muscles of nebulin-deficient mice. Therefore, nebulin may be able to stabilize actin filaments longer than itself, analogous to what was seen following the addition of mini-nebulin to nebulin-depleted skeletal myocytes [4]. This suggests that the function of nebulin somewhat overlaps with nebulin. Additionally, reduction of endogenous nebulin impairs the beating of cultured cardiomyocytes and results in loss of tropomyosin (a nebulin binding partner) and troponin from the thin filament [34,35]. Thus, the nebulin-tropomyosin interaction is likely important for thin filament stability in cardiac muscle.

LASP-2 (LIM and SH3 Protein-2)

Lasp-2 (LIM-nebulin), a shorter splice variant of nebulin, is the most recently identified member of the nebulin family [36,37]. Interestingly, although lasp-2 shares a gene with nebulin, it contains 4 unique exons and is not transcribed from a muscle-specific promoter [38], which gives lasp-2 multiple tissue expression patterns. The strongest lasp-2 expression appears to be in brain; it is localized to actin filament bundles in lamellipodia of cultured neuroblastoma cells [37]. *In vitro*, lasp-2 crosslinks actin filaments [39] and its overexpression in fibroblasts increases cell spreading [40]. Lasp-2 also appears to be multifunctional since it localizes to different cytoskeletal structures in cardiomyocytes including the Z-disc, cell-cell contacts (intercalated discs) and focal adhesions (Figures 2 and 3) [39,41]. Based on its numerous assembly patterns, lasp-2 probably has several cellular roles. It has been speculated that lasp-2 acts as a molecular scaffold that is important for actin filament and focal adhesion stabilization and organization [39,40,42,43].

LASP-1 (LIM and SH3 Protein-1)

Lasp-1 and lasp-2 have similar domain structures (Figure 1) but while at the moment the role of lasp-2 seems to be more structural, lasp-1 is involved in various cellular processes such as cell signaling, migration and proliferation [44-48]. However, the precise role of lasp-1 in these processes remains unclear. Initial studies using cultured cells concluded that lasp-1 depletion results in decreased cell migration and proliferation [44,47,49,50]. Contrarily, studies of fibroblasts from lasp-1 knockout mice revealed that loss of lasp-1 results in increased rates of migration, adhesion and focal adhesion turnover [51]. These seemingly opposite effects of lasp-1 on cell migration might be reconciled by the fact that the lasp-1 depletion studies represent transient, incomplete reduction of lasp-1 whereas the lasp-1 knockout mouse is a chronic complete loss of lasp-1. Interestingly, lasp-1 is the only two-subcellular compartment nebulin family member. It predominantly localizes to the cytoplasm and binds to focal adhesion proteins, but can also be found in nuclear preparations (e.g., in cancer cells) (Figure 3) [52]. In fact, the degree of nuclear localization of lasp-2 appears to be correlated with tumor size and patient survival [52,53]. The role of nuclear lasp-1 may be to modulate cell proliferation, cell-cycle control and to bind/affect the localization of known nuclear-shuttling proteins like zyxin [50]. Gene expression profiling of lasp-1 knockout fibroblasts also implicated lasp-1 as having transcriptional control over several key genes involved in cell adhesion, matrix organization, signal transduction and regulation of transcription [51]. More work needs to be done to determine if the other nebulin family members can also shuttle to the nucleus and are involved in gene regulation. While lasp-1 might seem the most distantly related nebulin family member since it does not appear to have a role in striated muscle, lasp-1 still participates in the stabilization of cytoskeletal structures like actin filaments and focal adhesions [43].

N-RAP (Nebulin-related anchoring protein)

N-RAP is another member of the nebulin family that is exclusively expressed in striated muscle. N-RAP promotes distinct steps of myofibril assembly [54-57] which is reflected in the protein's changing localization: at early stages of myofibril differentiation N-RAP is found in Z-disc precursors while in differentiated cells it is at the intercalated discs of heart muscle and myotendinous junctions of skeletal muscle (Figure 2) [58,59]. Furthermore, knockdown of N-RAP in cultured cardiomyocytes results in disrupted myofibril assembly [55]. Thus, N-RAP is a nebulin family member that acts as an organizing center for the initial recruitment and assembly of sarcomeric actin filaments and Z-discs.

Concluding remarks and future perspectives

Mysteries of the roles of the multi-functional protein nebulin and its relatives are closer than ever to being elucidated. Immense progress has been made in this field in just the last few years due to the generation of nebulin knockout mice and the applications of state-of-the-art cell biological and biophysical techniques. Furthermore, analysis of muscle tissue and/or individual myocytes expressing mutations within the nebulin family that lead to human disease complements the use of animal models and *in vitro* assays in understanding the mechanisms by which these proteins function (Box 2). It is now clear that nebulin on its own is not sufficient to regulate actin filament length (i.e., nebulin is not a strict molecular ruler) but perhaps should be viewed as a stabilizer of filamentous actin that works in concert with many other molecules like Tmod, tropomyosin and CapZ to regulate thin filament architecture. It is also clear that nebulin functions beyond length regulation. It has been implicated in Z-disc structure, myofibril inter-connectivity, calcium handling and optimizing actin-myosin interactions. Likewise, the other members of the nebulin protein family play essential physiological roles highlighted by their connection to human diseases. Even though all nebulin family members likely bind actin filaments in a similar way due to their highly conserved nebulin repeats, they regulate actin filament architectures in remarkably diverse ways due to the presence of other protein domains, which allows for unique complexes of binding partners. Nevertheless, all the family members likely evolved from a single gene, suggesting that analogous functions may unite them as a family. Certainly, at the most basic level, nebulin and the nebulin family members stabilize and contribute to the assembly/organization of various cytoskeletal structures: thin filaments, intercalated discs, Z-discs and focal adhesions.

The recent construction of mini-nebulin and ability to purify full-length nebulin opens the door to a whole range of experiments that were previously not possible [24,60]. For example, nebulin's potential role as a stabilizer can be analyzed in more detail using biochemical techniques that require isolated nebulin protein. In particular, *in vitro* actin polymerization and depolymerization experiments in the presence of nebulin would certainly provide novel insights into its actin filament regulatory roles. Furthermore, we can now attempt to identify the minimal components necessary to construct actin thin filaments of a defined length. Is thin filament length regulation autonomous (i.e., can combining only purified thin filament components, actin, nebulin, tropomyosin and Tmod produce actin filaments that match the length of thin filaments *in vivo*) or are other components such as the thick filaments, titin and/or signaling molecules required?

Although we have a fundamental understanding of the nebulin family members including expression patterns, binding partners, basic functions and some limited clues into their links to human disease, many of the family members remain understudied, with just about a dozen laboratories investigating their functions. No doubt future studies of these proteins would benefit greatly from additional *in vivo* models to learn more about the precise function(s) of the nebulin family members.

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Glossary

Sarcomere	The sarcomere is the basic contractile unit of skeletal and cardiac muscle. It is defined as the region between two Z-discs (see below). The sarcomere is composed of arrays of precisely interwoven thin and thick filaments, predominantly composed of actin and myosin, respectively. The thin filaments are anchored within the Z-disc, and extend out towards the middle of the sarcomere where they overlap with the thick filaments. Myosin binds actin and pulls the thin filaments toward the middle of the sarcomere causing the sarcomere to shorten. The combined shortening of multiple sarcomeres results in contraction of the entire muscle cell [61].

Z-discs

The Z-disc is a multiprotein structure that defines the lateral boundaries of the sarcomere and plays an important role in striated muscle formation, maintenance, structure and function. Within the Z-disc, actin filaments from adjacent sarcomeres overlap and are cross-linked by α -actinin. This physically links, and is responsible for transmitting force from, one sarcomere to the next along the length of the myofibril during contraction. Additionally, Z-discs provide a structural backbone for the insertion of the giant proteins titin and nebulin [62,63].

Focal adhesions

Focal adhesions are protein rich attachments between actin filaments and the extracellular matrix. Focal adhesions are large, dynamic protein complexes that consist of a wide variety of cytoskeletal and signaling proteins through which both mechanical force and regulatory signals are transmitted. The number of identified focal adhesion proteins is expanding (~ 70 have been described to date) and the complex relationships between all the components are involved in cell migration, attachment and communication. The composition of focal adhesions is dynamic, as proteins must associate and dissociate frequently to adapt to the changing needs of the cellular environment [64,65].

Figure 1. Schematic representation of the structure of nebulin and nebulin family members
The unifying element in each nebulin family member protein is the nebulin repeat (orange square: ~35 amino acids in length and contains a conserved SDxxYK motif). In nebulin and N-RAP, groups of 7 repeats form super repeats that also contain a conserved motif (WLKGIGW) (rectangular box surrounding 7 squares). In addition to the presence of nebulin repeats and/or super repeats, all of the nebulin family members contain a unique blend of other protein motifs: LIM domain (diamond), SH3 domain (oval), serine-rich regions (grey rectangle) and acidic regions (trapezoid). These distinctive domain organizations and compositions lead to extraordinarily different molecular weights, from the giant ~750-900 kDa nebulin protein to the merely 34 kDa lasp-2 protein. Furthermore, with the exception of nebulette and lasp-2, the family members are all produced from different genes with unique tissue expression patterns. It should be noted that even though nebulette and lasp-2 are splice variants of one another, they are transcribed from different promoters with different tissue specificity.

Figure 2. Location of nebulin family members in striated muscle

The two types of striated muscle (cardiac and skeletal) are both illustrated. Structures and proteins common to both are labeled in the center while those specific to cardiac muscle are off to the side. Nebulin is abundantly expressed in skeletal muscle (2-4 molecules per thin filament), with significantly lower levels found in cardiac muscle. Nebulin incorporates along the thin filament spanning nearly half the sarcomere. Nebulette is the only family member that is specifically expressed in cardiac muscle and is localized to the Z-disc. N-RAP is localized to intercalated discs of cardiomyocytes, as well as myotendinous junctions (not depicted here) of skeletal muscle. Finally, lasp-2 is localized at the Z-disc and cell-cell contacts in striated muscle. The inset depicts a general striated muscle Z-disc which

represents an amalgamation of both cardiac and skeletal muscle Z-discs (note: only some components are shown). The bottom left micrograph is a cultured cardiomyocyte expressing mCherry-lasp-2 (red) costained with DAPI (blue). Image published with permission from [89]. The micrograph on the right is a cultured skeletal myocyte stained with antibodies to both N-terminal (orange) and C-terminal (yellow) nebulin. Bar, 10 μ m.

Figure 3. Location of nebulin family members in fibroblasts

Lasp-1 and lasp-2 are localized to focal adhesions in fibroblasts where they interact with a variety of structural and signaling molecules including actin, α -actinin and zyxin. Note, only some components are shown. The micrograph on the bottom is a C2C12 myoblast expressing mCherry-lasp-1 (red), which was costained with DAPI to label the nucleus (blue). Bar, 10 μ m.

Figure 4. Current model of nebulin's function as a stabilizer in thin filament length regulation in skeletal muscle

Nebulin molecules span the length of the thin filament and interact with the thin filament components actin, tropomyosin, Tmod and CapZ. However, Tmod, and the pointed end of the actin filament, are often observed to extend farther from the Z-disc than the end of nebulin. The question mark indicates that the exact conformation nebulin adopts within the Z-disc remains unclear. In the current model, based on its binding to CapZ and other integral Z-disc proteins, nebulin extends into the Z-disc and connects two thin filaments from adjoining sarcomeres (depicted here). Thin filaments are stable but not static structures since actin continually exchanges at both ends. When nebulin is depleted, actin, tropomyosin and Tmod are all more dynamic, indicating a reduction in the stability of the thin filament. This loss of stability likely explains why the thin filaments are shorter when nebulin is removed. Furthermore, the loss of nebulin results in abnormally wide Z-discs, reduced CapZ assembly and the failure of barbed ends of the actin filaments to uniformly align within the Z-disc.