Nebulin-deficient mice exhibit shorter thin filament lengths and reduced contractile function in skeletal muscle

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Nebulin is a giant modular sarcomeric protein that has been proposed to play critical roles in myofibrillogenesis, thin filament length regulation, and muscle contraction. To investigate the functional role of nebulin in vivo, we generated nebulin-deficient mice by using a Cre knock-in strategy. Lineage studies utilizing this mouse model demonstrated that nebulin is expressed uniformly in all skeletal muscles. Nebulin-deficient mice die within 8–11 d after birth, with symptoms including decreased milk intake and muscle weakness. Although myofibrillogenesis had occurred, skeletal muscle thin filament lengths were up to 25% shorter compared with wild type, and thin filaments were uniform in length both within and between muscle types. Ultrastructural studies also demonstrated a critical role for nebulin in the maintenance of sarcomeric structure in skeletal muscle. The functional importance of nebulin in skeletal muscle function was revealed by isometric contractility assays, which demonstrated a dramatic reduction in force production in nebulin-deficient skeletal muscle.

Introduction

Nebulin is a giant modular sarcomeric protein (500–900 kD) in skeletal muscle, where it comprises ~3% of total myofibrillar protein (Wang and Wright, 1988). A critical role for nebulin in skeletal muscle function is demonstrated by human mutations in nebulin, which are causative for nemaline myopathy, a neuromuscular disorder characterized by muscle weakness and the presence of rodlike nemaline bodies in the muscle fibers that contain abnormally arranged Z-line and I-band proteins (Wallgren-Pettersson et al., 1999, 2002). Nebulin has been proposed to be important for multiple aspects of striated muscle form and function (McElhinny et al., 2005). However, its exact role in vivo remains elusive.

Nebulin is encoded by a single gene, and its molecular weight ranges from 500 to 900 kD because of extensive isoform diversity in different muscle types, species, developmental stages, and in disease (Labeit and Kolmerer, 1995; Kazmierski et al., 2003; Donner et al., 2004). Single molecules of nebulin are associated with thin filaments in skeletal muscle and span the entire length of the thin filament with the COOH terminus anchored in the Z-line and the NH2 terminus extending to the pointed end of the thin filaments (Wang and Wright, 1988; Millevi et al., 1998; McElhinny et al., 2001). The molecular size of nebulin correlates with variations in thin filament lengths in different muscle types, suggesting that nebulin may act as a molecular ruler to regulate thin filament length in skeletal muscle (Labeit and Kolmerer, 1995; Wang, 1996). According to the cross-bridge theory of muscle contraction (Huxley and Simmons, 1971), the amount of force that a muscle can exert at different sarcomere lengths is determined by the amount of overlap between thin and thick filaments, which is dependent on thin filament length and the contractile state (Gordon et al., 1966; Granzier et al., 1991). In particular, thin filament lengths are fine tuned in different vertebrate muscles in vivo to overlap with thick filaments by an amount characteristic for each muscle such that the amount of force generated is suited to the physiological requirements for that muscle (Burkholder et al., 1994). Importantly, muscles working beyond their optimal length range, causing an incomplete overlap of thick and thin filaments, results in instability and damage (Morgan and Allen, 1999).
Nebulin might also play a role in myofibrillogenesis, where it appears to participate in the early assembly of precursor I-Z-I bodies, and assembles in a striated pattern before thin filaments attain their mature length (Moncman and Wang, 1996; Shimada et al., 1996; Nwe et al., 1999). This hypothesis is supported by a recent study showing the failure of myofibril assembly in cultured myotubes upon the knockdown of nebulin for 5 d (McElhinny et al., 2005). In addition, nebulin has been proposed to play a role in the regulation of muscle contraction by modulating actomyosin ATPase activity in a Ca²⁺-calmodulin-dependent manner (Root and Wang, 1994, 2001).

Interactions of nebulin with diverse sarcomere-associated proteins suggest potential roles in sarcomeric architecture, signaling, and force transduction. Nebulin is composed mainly of ~35 amino acid modules (M1–185), which are further organized into super-repeats of seven modules within the central region of nebulin (M9–162). Each nebulin module interacts with a single actin monomer, and each super-repeat associates with each tropomyosin–troponin regulatory complex along the length of the thin filament (Jin and Wang, 1991; Pfuhl et al., 1994; Wang, 1996). Within the COOH-terminal region of nebulin, modules M163–170 interact with the intermediate filament desmin in the periphery of the Z-line, suggesting that nebulin might play a role in the lateral registration of sarcomeres and in force transmission (Bang et al., 2002). Nebulin’s extreme COOH-terminal end contains a unique serine-rich domain with several potential phosphorylation sites and a Src homology 3 (SH3) domain, suggesting that nebulin might be involved in signaling pathways at the Z-line. The SH3 domain interacts with myopalladin, which, in turn, binds to α-actinin, thereby tethering nebulin at the Z-line (Bang et al., 2001; McElhinny et al., 2003). In addition, recent evidence suggests that nebulin’s SH3 domain might also bind to the springlike PEVK domain in the I-band region of titin (Ma and Wang, 2002). In nebulin’s NH₂-terminal region, modules M1–3 bind to the thin filament pointed end-capping protein tropomodulin (McElhinny et al., 2001), which is critical for maintaining thin filament length at the pointed ends (Littlefield et al., 2001; McElhinny et al., 2001). The interaction between nebulin and tropomodulin as well as a recent RNA interference study support the proposed function of nebulin as a regulator of thin filament length (McElhinny et al., 2005).

Nebulin was long thought to be absent from cardiac muscle, where the homologous but smaller protein nebulette is expressed (Moncman and Wang, 1995). However, recent studies have shown that nebulin is expressed in cardiac muscle and is localized in a layout identical to that in skeletal muscle, although at lower levels (Fock and Hinssen, 1999; Kazmierski et al., 2003; Donner et al., 2004; Joo et al., 2004). In addition, an RNA interference study in cardiomyocytes has suggested that nebulin is involved in thin filament length regulation in both cardiac and skeletal muscle (McElhinny et al., 2005). Based on this study, two distinct models have been proposed to explain how nebulin functions to regulate thin filament lengths in the heart (Fowler et al., 2006; Horowits, 2006).

As discussed above, multiple roles have been suggested for nebulin, including the regulation of thin filament length.
myofibrillogenesis, signal transduction, regulation of muscle contraction, and myofibrillar force generation. However, requirements for nebulin in vivo have not yet been addressed. To study the functional role of nebulin in vivo, we generated nebulin-deficient mice by using a Cre knockin approach. This strategy also allowed us to study the endogenous expression pattern of nebulin by crossing these mice with Rosa26 lineage reporter mice (Soriano, 1999). Our lineage studies revealed that nebulin is expressed in all skeletal muscle myocytes. Nebulin expression was also identified in the heart. However, nebulin was shown to be expressed mainly in the atria, where it was expressed heterogeneously in ~50% of atrial cardiomyocytes, whereas it was expressed only in a minor percentage of ventricular cardiomyocytes. These results suggest that nebulin may have a more critical role in skeletal muscle than in cardiac muscle.

Nebulin-deficient mice die within 8–11 d after birth, with symptoms including decreased milk intake and muscle weakness. Transmission EM (TEM) and immunostaining analyses demonstrated that nebulin is not important for the normal assembly of sarcomeres. However, nebulin-deficient mice had skeletal muscle thin filaments that were decreased in length by up to 25% compared with wild type. Also, thin filaments were uniform in length both within and between muscle types. This is in contrast to a recent study (McElhinny et al., 2005) in cultured cardiomyocytes in which the RNA interference knockdown of nebulin resulted in a 30% increase in thin filament lengths. Our analyses further indicate a critical role for nebulin in the maintenance of sarcomere structure and demonstrate a dramatic reduction in force production by nebulin-deficient skeletal muscle.

Results

Generation of nebulin-deficient mice

To study the function and expression pattern of nebulin in vivo, we generated nebulin-deficient mice by gene targeting. Exon 1 was deleted and replaced by Cre recombinase cDNA as well as the neomycin resistance gene flanked by frt sites (Fig. 1 A). After electroporation of the targeting vector into R1 embryonic stem (ES) cells, one clone was identified that had undergone homologous recombination (Fig. 1 B). The clone was injected into blastocysts from C57/B6 mice and gave rise to chimera mice that were then bred with Black Swiss mice to generate germ line–transmitted heterozygous nebulin+/− mice. These mice were subsequently mated to generate homozygous nebulin−/− mice. To verify that homozygous knockout mice were null mutants, we performed Southern blot analysis on DNA isolated from nebulin−/− and nebulin+/− mice (Fig. 1 C) as well as Western blot analyses for nebulin protein using a polyclonal antibody raised against domain M161–165. As shown in Fig. 1 D, no nebulin protein was detected in nebulin−/− mice. In addition to the high molecular mass band at nebulin’s expected size, lower molecular mass bands of ~130 and 60 kD were detected that were also absent in nebulin−/− mice. This could either be alternative splice isoforms of nebulin or degradation products from the lysate preparation, which has also been seen in other studies using various antibodies (Kazmierski et al., 2003; McElhinny et al., 2005). We also performed RT-PCR analysis on RNA isolated from gastrocnemius muscle using primers located in exons 2, 3, 98–102, 163, and 164 (mouse nebulin has 165 exons; Kazmierski et al., 2003), which suggested that the entire nebulin was successfully knocked out (Fig. 1 E).

Nebulin is uniformly expressed in skeletal muscle

Nebulin−/− mice were viable and indistinguishable from their wild-type littermates. To determine the expression pattern of endogenous nebulin in vivo, nebulin−/− mice were crossed with the Rosa26 lineage reporter mouse (Soriano, 1999). In the progeny of this cross, Cre-mediated excision at the Rosa26 locus results in the expression of the β-galactosidase (lacZ) gene under the control of the ubiquitously expressed Rosa26 locus, thus allowing lineage analysis of nebulin-expressing cells. Various tissues from 10-wk-old nebulin−/− Rosa26 mice were fixed and stained with X-galactosidase. As expected, strong β-galactosidase

Figure 2. Nebulin is expressed in skeletal and cardiac muscle. X-galactosidase staining of tissue from nebulin−/− Rosa26 mice. (A) X-galactosidase staining of heart sections reveals nebulin expression mainly in atrial cardiomyocytes and only in a small percentage of ventricular cardiomyocytes. Enlarged images of different areas from the heart are shown (arrows refer to the enlarged areas). (B) X-galactosidase staining of heart sections reveals nebulin expression mainly in atrial cardiomyocytes and only in a small percentage of ventricular cardiomyocytes. (Bottom right) LacZ-positive cardiomyocyte. (C and D) X-galactosidase staining was also positive for the liver (C) and aorta (D). Bars (C and D), 1 mm.
activity was detected homogeneously in all skeletal muscle types (Fig. 2 A). In addition, β-galactosidase expression was detected in the heart, aorta, and liver (Fig. 2, B–D). No β-galactosidase activity was detected in other tissues. Because the presence of nebulin in the heart has only recently been reported (Fock and Hinssen, 1999; Kazmierski et al., 2003; Donner et al., 2004; Joo et al., 2004) and has not yet been thoroughly characterized, we further examined its expression pattern in the heart. X-galactosidase staining of frozen sections from the heart revealed that nebulin is expressed mainly in the left and right atria (~50% of cardiomyocytes), whereas in the ventricles, nebulin is expressed only in a small percentage of cardiomyocytes (Fig. 2 B).

**Nebulin-deficient mice die at postnatal day 8–11**

Nebulin−/− mice were born in Mendelian ratios with a similar body weight compared with their wild-type littermates. Nebulin−/− mice were able to breathe and move their legs and were indistinguishable from their wild-type littermates except that nebulin−/− mice had little or no detectable milk in their stomachs, presumably reflecting an inability to suckle. Consistent with this observation, nebulin−/− mice barely increased in weight after birth and exhibited minimal subcutaneous fat. Nebulin−/− mice died ~8–11 d after birth with a weight ~25% of that of their wild-type littermates, which was most likely the result of decreased milk intake caused by muscle weakness (Fig. 3, A and B). Because nebulin−/− mice were able to survive up until 11 d, we assumed that they were able to drink some milk. Therefore, we tested whether nebulin−/− mice would be able to survive longer in the absence of competition from wild-type littermates by removing wild-type littermate control mice from postnatal day 1–11 when nebulin−/− mice die. No statistical differences between wild-type and nebulin−/− pups were observed. Numbers above the bars for nebulin−/− mice indicate the number of remaining viable mice. Error bars represent SEM.

Histological analysis by hematoxylin and eosin staining of frozen sections of skeletal and heart muscle from postnatal day 1 mice showed no obvious differences between nebulin−/− and wild-type mice (unpublished data). No fibrosis was detected by trichrome staining of either skeletal or heart muscle from nebulin−/− mice (unpublished data). We also analyzed myosin heavy chain composition in several distinct skeletal muscles by gel electrophoretic analyses and observed no differences between mutant and wild-type mice (unpublished data). Both had prominent neonatal and embryonic myosin heavy chains bands that were consistent with previous findings (Agbulut et al., 2003).

**Nebulin−/− mice have normal myofibrillogenesis but exhibit sarcomeric misalignment and disorganization after muscle usage**

To determine the structure of skeletal muscle in more detail, TEM analysis was performed on tibialis anterior (TA) muscle from 1-d-old nebulin−/− and littermate control mice. Postnatal day 1 was chosen because nebulin−/− and wild-type littermate mice were still similar in size at this stage, thus facilitating a valid comparison. To allow us to study the ultrastructure of TA muscle at different sarcomere lengths, legs were fixed with the knee joint at 90° and with the ankle at 90° (neutral) or 180° (fully plantarf lexed), as plantarflexion of the ankle joint causes stretching of the TA muscle.

At resting length, muscles from nebulin−/− mice had relatively normal sarcomeric structure with wide myofibrils, distinct A-bands, and narrow, uniformly spaced Z-lines (Fig. 4, A and B), suggesting that sarcomere assembly and organization were preserved in the absence of nebulin. However, the misalignment of myofibrils was often observed. In moderately stretched muscle, myofibrillar misalignment was more pronounced (Fig. 4, C–F), and myofibril splitting frequently appeared. In addition, fragmented Z-lines were apparent, whereas M-lines appeared unaffected. Similar results were obtained by TEM of muscle tissue from nebulin−/− mice at postnatal day 6 (unpublished data).

To determine whether the observed ultrastructural abnormalities in nebulin−/− muscle resulted from abnormal sarcomere assembly or an inability to maintain myofibrillar integrity during muscle contraction, we compared embryonic and postnatal diaphragm muscle in nebulin−/− mice with littermate controls. Diaphragm muscle remains inactive during embryogenesis, thus allowing a determination of whether nebulin is required for myofibrillogenesis before the onset of contraction. After birth,
the diaphragm is continuously contracting and, therefore, is useful for studying the requirement for nebulin in actively contracting muscle.

At embryonic day 18.5, nebulin−/− diaphragm muscle had well-aligned sarcomeres that were virtually indistinguishable from those of wild-type muscle (Fig. 5, A and B). At postnatal...
day 1, the misalignment of myofibrils and moderate thickening of Z-lines were observed in diaphragm muscle from nebulin−/− mice (Fig. 5 D). At postnatal day 9, nebulin−/− diaphragm muscle exhibited a severe disruption of myofibrillar structure, including a lack of well-defined A- and I-bands (Fig. 5, F–H). Z-lines exhibited various types of morphologies. Some Z-lines were extremely thick, rounded, and highly electron dense, resembling nemaline rod bodies (Fig. 5 F; Wallgren-Pettersson et al., 1999, 2002), whereas others were punctate and appeared fragmented (Fig. 5, F–H). Moreover, some fibers exhibited a complete lack of organization with Z-lines that appeared to be dissolving (Fig. 5 H). Another interesting observation was the abnormal accumulation of mitochondria within myofibers (Fig. 5 H). These observations suggest that nebulin is not required for myofibrillogenesis but is important for the maintenance of myofibrillar integrity during muscle contraction.

Nebulin−/− mice have normally assembled sarcomeric components

To determine how the ablation of nebulin affected other cytoskeletal proteins, we stained TA and extensor digitorum longus (EDL) muscles from 1-d-old nebulin−/− mice and littermate controls for α-actinin 2, α-myosin, actin (phalloidin), tropomodulin, tropomyosin, desmin, and palladin (Fig. 6), many

Table I. Thin filament measurements in skeletal muscle from nebulin−/− and wild-type mice

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Probe</th>
<th>Wild type</th>
<th>nebulin−/−</th>
<th>Wild-type nebulin−/−</th>
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<tr>
<td></td>
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<td>Length</td>
<td>Range</td>
<td>n</td>
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<tr>
<td>Gast</td>
<td>Tmod</td>
<td>1.27 ± 0.06</td>
<td>1.11–1.35</td>
<td>26</td>
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<tr>
<td>VL</td>
<td>Tmod</td>
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<td>1.19–1.38</td>
<td>13</td>
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<tr>
<td>TA</td>
<td>Tmod</td>
<td>1.29 ± 0.05</td>
<td>1.19–1.39</td>
<td>32</td>
</tr>
<tr>
<td>EDL</td>
<td>Tmod</td>
<td>1.16 ± 0.06</td>
<td>1.01–1.31</td>
<td>60</td>
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<tr>
<td>TA</td>
<td>Phall</td>
<td>1.17 ± 0.02</td>
<td>1.14–1.20</td>
<td>4</td>
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<tr>
<td>Total</td>
<td></td>
<td>135</td>
<td>131</td>
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Tmod, tropomodulin; Phall, phalloidin; Gast, gastrocnemius. Note that calculations based on phalloidin staining generally resulted in shorter thin filament lengths in both wild-type and nebulin−/− mice, which is likely to be the result of systematically shorter thin filament lengths estimated by the Gaussian distribution that was used to estimate the microscope point spread function (Littlefield et al., 2001). However, in TA muscle stained with phalloidin, distributed deconvolution analysis revealed a 0.22-μm decrease in thin filament length in the absence of nebulin, which is similar to the observed decrease of 0.21 μm that was measured based on α-actinin and tropomodulin costainings.
of which have been shown to interact with nebulin. Although nebulin−/− sarcomeric structure was less well organized compared with wild-type control mice, no obvious differences in the localization of any of these proteins were found. We also assessed the localization of these proteins at postnatal day 8 before the death of nebulin−/− mice and found no significant changes (unpublished data). Thus, nebulin is not critical for normal sarcomere assembly or the localization of several sarcomeric proteins.

**Nebulin−/− mice have reduced thin filament lengths compared with wild type**

Because nebulin has been proposed to play a role in the regulation of thin filament length, we examined thin filament lengths in nebulin−/− mice. In nebulin−/− mice, tropomodulin was localized in its typical striated pattern at the pointed ends of the thin filaments and was expressed in similar amounts compared with wild type as assessed by Western blot analysis (unpublished data). Thus, we measured thin filament lengths either by double immunostaining for α-actinin and tropomodulin, which stain Z-lines and thin filament pointed ends, respectively, or by fluorescently labeled phalloidin to directly localize thin filaments. Stained samples were analyzed by distributed deconvolution, which determines the location, brightness, and dimensions of each thin filament array (I-Z-I body) along a myofibril at sub-pixel resolution and is independent of sarcomere length differences (Littlefield and Fowler, 2002).

Using distributed convolution on α-actinin- and tropomodulin-stained muscle sections, we measured thin filament lengths in four different muscles from wild-type and nebulin−/− mice: gastrocnemius, TA, vastus lateralis (VL), and EDL (Table I). In addition, our results were independently verified by phalloidin staining for TA muscle. A total of three wild-type and three nebulin−/− mice were used for the analysis. Examples of the analysis on TA and gastrocnemius muscle are shown in Fig. 7 and as supplemental data (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200603119/DC1), respectively. As shown in Table I, the absence of nebulin resulted in a significant shortening of thin filament lengths by 0.07–0.28 μm in all four muscle types examined. Intriguingly, the absence of nebulin resulted in similar thin filament lengths of ~0.95–1.07 μm in all four muscles (Table I), whereas in wild-type muscle, thin filaments lengths were 1.27 ± 0.06, 1.29 ± 0.05, 1.29 ± 0.05, and 1.16 ± 0.06 μm in gastrocnemius, VL, TA, and EDL muscle, respectively. Thus, gastrocnemius, VL, and TA thin filaments were shortened the most (0.28, 0.25, and 0.21 μm, respectively), whereas EDL thin filaments, which were the shortest in wild-type mice, only shortened by 0.07 μm in the nebulin−/− mice.

**Nebulin−/− skeletal muscle generates significantly lower stress compared with wild type**

To determine the functional effects of the ultrastructural changes in the nebulin−/− mice, we measured force production. At postnatal day 1, TA muscle in nebulin−/− mice were grossly indistinguishable from wild-type littermates (Fig. 8 A, inset). However, the neonatal tendon was translucent and mechanically fragile, making it difficult to test contractile force in nebulin−/− mice using traditional techniques. As a result, isometric contractile testing of the isolated TA bone–tendon–muscle–tendon–bone complex was performed using high resolution force transducers to yield a signal to noise ratio of >20 (Fig. 8 A). A total of 16 TA muscles from wild-type and 9 from nebulin−/− mice were used for the analysis. Electrophysiological threshold contractile kinetics were similar between nebulin−/− and wild-type mice, which suggested that excitation/contraction coupling was not significantly altered in nebulin−/− mice (unpublished data). However,
the magnitude of the stress generated by nebulin<sup>−/−</sup> mice was <50% of the wild-type value (Fig. 8 B). Overall stress generated by neonatal muscle was 5–10% that of adult mammalian muscle, probably because of the fact that the myofibrils were less dense (Fig. 3). It is important to note that the low stress was not simply because nebulin<sup>−/−</sup> TA muscles were smaller since there was no significant difference between the physiological cross-sectional area (PCSA; Fig. 8 C) of TA muscles. Thus, the force transmission efficiency of the contractile complex of the nebulin<sup>−/−</sup> mouse was critically compromised even though the contractile machinery appeared to be of normal morphology (Fig. 5) and fiber size between the two genotypes was similar (not depicted).

**Discussion**

To determine the functional role of nebulin in vivo, we generated mice deficient for nebulin. Nebulin exon 1 was replaced by a Cre-neo cassette, thereby knocking out nebulin. Successful ablation of the entire nebulin was confirmed by DNA, RNA, and protein analysis. The presence of CRE in the targeting allele allowed us to examine cells in which nebulin is/has been expressed by crossing nebulin<sup>+/−</sup> mice with the Rosa26 reporter line. As expected, this revealed a strong and uniform expression of nebulin in all types of skeletal muscle. In addition, we confirmed the recently reported nebulin expression in cardiac muscle (Fock and Hinssen, 2002; Kazmierski et al., 2003; Donner et al., 2004; Joo et al., 2004). However, nebulin was primarily expressed in the atria (~50% of atrial cardiomyocytes) and only in a small percentage of ventricular cardiomyocytes, predominantly in the inner layer of the myocardium. Further studies are needed to elucidate the potential functions of nebulin in the heart as well as in other sites of expression.

**Nebulin is critical for survival in mice**

Our study shows that nebulin is critical for mouse survival because the genetic ablation of nebulin caused death with complete penetration in neonatal mice from postnatal day 8–11. Lethality may be caused by several factors. The reduced milk intake and impaired growth observed in nebulin<sup>−/−</sup> mice suggest that these mice may have difficulties in sucking, presumably as a result of decreased muscle function. This is supported by our ultrastructural and biophysical studies demonstrating abnormal sarcomeric structure and severely reduced force generation in skeletal muscle of nebulin<sup>−/−</sup> mice. Thus, starvation as a result of reduced sucking may contribute to death. Because severely disorganized diaphragm muscle structure was also observed in postnatal nebulin<sup>−/−</sup> mice, impaired breathing caused by defective diaphragm muscle may also cause lethality.

**Nebulin<sup>−/−</sup> is not required for myofibrillogenesis but is essential for the maintenance of myofibrillar integrity during muscle contraction**

Our ultrastructural observations in embryonic day 18.5 diaphragm muscle demonstrated that nebulin is not essential for myofibrillogenesis. In addition, immunostaining analyses demonstrated that nebulin is not required for the association of several sarcomeric proteins with the sarcomere, including tropomodulin. Whereas nebulin<sup>−/−</sup> diaphragm muscle displayed a mild misalignment of myofibrils and moderate thickening of Z-lines at postnatal day 1 when the diaphragm had recently started to contract, diaphragm muscle was severely disorganized at postnatal day 9. This suggests that nebulin is essential for the maintenance of muscle integrity during contraction. Compared with TA muscle at postnatal days 1 and 6, diaphragm muscle was much more severely affected, likely because of the constant stress during the continuous contraction of diaphragm muscle. Interestingly, the thickening of Z-lines and the presence of rod-like nemaline bodies in diaphragm muscle at postnatal day 9 is reminiscent of observations in patients with nemaline myopathy (Wallgren-Pettersson et al., 1999, 2002). We are not sure of the composition of these enlarged Z-lines, but based on the characterization of nemaline rod bodies from nemaline myopathy patients, we predict that they may represent an accumulation of...
Z- and I-band components (Wallgren-Pettersson et al., 2002). Several of the observed abnormalities at postnatal day 9 are likely to be secondary to prolonged muscle dysfunction, which appear to initiate with myofibrillar misalignment and loss of Z-line integrity and eventually lead to the dissolution of muscle structure.

**Nebulin is required to specify thin filament length in a muscle-specific manner**

Because nebulin has long been proposed to function as a ruler for specifying thin filament length and fit into many of the criteria that could be expected from a molecular ruler, one of our main aims was to determine the effect of nebulin deficiency on thin filament length in skeletal muscle. Intriguingly, individual muscles in nebulin<sup>−/−</sup> mice had thin filaments that were uniform in length but up to 25% shorter compared with wild type. Also, whereas in wild-type mice, thin filament lengths varied between different muscle types, nebulin<sup>−/−</sup> mice had almost uniform thin filament lengths (~0.95–1.07 μm) among the four muscle types examined.

In contrast to our findings, a recent siRNA nebulin knockdown study performed in cultured cardiomyocytes resulted in the absence of localized tropomodulin and a 30% increase in thin filament lengths (McElhinny et al., 2005). Discrepancies between our findings and those of McElhinny et al. (2005) could reflect differences in the systems being used: cardiac versus skeletal, rat versus mouse, or in vivo versus in vitro. Because nebulin is only expressed in a small percentage of mouse ventricular myocytes, whereas it is uniformly expressed in all skeletal muscle cells, we performed our thin filament length measurements in skeletal muscle.

Nebulin-deficient mice had uniform thin filament lengths of ~1 μm in all muscles tested, so a nebulin-independent mechanism must be responsible for specifying thin filament length in the absence of nebulin (Littlefield and Fowler, 1998). This supports a model in which a nebulin-independent mechanism defines uniform thin filament lengths of ~1 μm in all muscle types, whereas nebulin is responsible for specifying longer thin filament lengths in a muscle-specific manner, thereby allowing individual muscles to fulfill specific physiological requirements. This is consistent with previous studies showing a correlation between the molecular size of nebulin and thin filament length (Labeit and Kolmerer, 1995; Wang, 1996). Thus, the expression of distinct nebulin isoforms in different muscles may be correlated with thin filament lengths in individual skeletal muscle types.

**Nebulin is essential for efficient force transmission**

TEM of nebulin<sup>−/−</sup> TA and diaphragm muscle revealed sarcomeric misalignment and various degrees of Z-line streaming and fragmentation, which was apparent from postnatal day 1 and increased with age. Although our immunofluorescence data showed that desmin is localized in its normal pattern in the periphery of the Z-line (Fig. 6), a possible explanation for the observed myofibrillar misalignment and Z-line streaming could be the missing linkage between desmin and the Z-line via nebulin. Disruption of the attachment of desmin in the Z-line could result in the loss of the lateral alignment of sarcomeres and less efficient force transmission. Intriguingly, active stress generation in nebulin<sup>−/−</sup> mice was <50% compared with wild-type controls. Similarly, stress generation in desmin-deficient mice was shown to be reduced by ~20% (Sam et al., 2000). Thus, the mechanical relationship between nebulin and desmin might have dramatic consequences for force transmission in muscle, and nebulin is likely to play a role in transmitting the force from the thin filament to the intermediate filament system via desmin.

The reduction in thin filament lengths would also be expected to reduce force generation as a result of decreased overlap between thin and thick filaments (Gordon et al., 1966) and/or limit the working range of the muscle (Burkholder et al., 1994). In addition to reduced force transmission, a possible mechanism explanation for decreased muscle stress could be the increased sarcomeric heterogeneity and misalignment observed in nebulin<sup>−/−</sup> mice. The significant change in active stress is likely to be a reflection of several of these factors.

**Conclusions**

In conclusion, our studies have demonstrated that nebulin is not required for myofibrillogenesis. However, nebulin is important for muscle stability, organization, and force generation in vivo. In addition, we have demonstrated that nebulin is required to specify distinct thin filament lengths in individual skeletal muscle types, suggesting that the specificity of thin filament length within individual muscle types may be dictated by the expression of specific nebulin isoforms. Variable thin filament lengths between different muscle types may reflect intrinsic functional requirements. Intriguingly, thin filament lengths in the absence of nebulin were uniform within and between individual muscle types and were consistently shorter in nebulin<sup>−/−</sup> mice, suggesting that thin filament length must be specified by another mechanism in the absence of nebulin. This is also indicated by the absence of nebulin in invertebrate striated muscle. The identification of this alternative mechanism for thin filament length regulation will be an interesting subject for further studies.

**Materials and methods**

**Gene targeting and generation of nebulin-deficient mice**

Nebulin genomic DNA was isolated from a 129SVJ mouse genomic library (Stratagene) and was used to construct a nebulin-targeting construct for the replacement of nebulin exon 1 by the Cre recombinase cDNA and the neomycin resistance gene. The construct was generated in the pBluescript II KS<sup>+</sup> vector, and the 5′ arm of homology consisted of a 4.096-kb Nol–Sall fragment upstream of nebulin exon 1 fused in frame with Cre followed by neomycin flanked by FRT sites. The 3′ arm of homology was a 3.7364-kb Sall–Xhol fragment located downstream of exon 2 (Fig. 1 A). The targeting construct was verified by sequencing and linearized with NotI before electroporation into R1 ES cells at the Transgenic Core Facility at the University of California, San Diego. 1,000 G418-resistant ES clones were screened for homologous recombination by Southern blot analysis as described in the next section.

**Southern blot analysis**

Genomic DNA was extracted from G418-resistant ES cell clones and mouse tails as previously described (Chen et al., 1998). ES cell DNA was digested with SacI and analyzed by Southern blot analysis. A 507-bp fragment corresponding to 2,676–3,182 kb of the target vector [indicated in Fig. 1 A] was generated by PCR using mouse genomic DNA and specific nebulin primers [forward, GCTGTGAGACATTGAGTIT; reverse, AGTG- CATCACAGGGTAAAGC]. The PCR product was subsequently radiolabeled...
using α-[32P]dATP by random priming (Invitrogen). DNA blots were hybridized with the radiolabeled probe and visualized by autoradiography. The wild-type allele is represented by a band of 8.977 kb, whereas a band of 6.982 kb represents the correctly targeted mutant allele.

**Generation and genotyping of mice**

One independent homologous recombinant ES clone was microinjected into blastocysts from C57/B6 mice at the Transgenic Core Facility at the University of California, San Diego. Male chimeras were inbred with female Black Swiss mice to generate germ line–transmitted heterozygous mice (nebulin+/−). Nebulin−/− mice were subsequently intercrossed to generate mice, which were homozygous null mutant mice (nebulin−/−).

Expression intercrosses were genotyped by PCR analysis using mouse tail DNA and wildtype (forward, AGGGCATAATGAAAGTTGTAAGGT; reverse, AACATGAAACTGCTTTCTTTGA) and mutant allele-specific primers (forward, GTTCCGAAAGCTTACGTGACACA; reverse, CTAGACCTTGTGGACCCGT). Total protein extracts were prepared [Chen et al., 1998] from postnatal day 1 skeletal muscle and separated on a 3–8% SDSPAGE and electroblotted onto Hybond-C-extra membranes (Amersham Biosciences). Membranes were incubated in TBS-T (100 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and blocked with 5% nonfat dry milk (NFDM) for 1 h at room temperature. Membranes were then incubated overnight with a primary antibody in TBS-T containing 5% NFDM. After washing with TBS-T, membranes were incubated with a secondary antibody conjugated to HRP for 1 h at room temperature. Membranes were then washed and exposed to X-ray film for autoradiography. Immunoblotting signals were quantified using densitometry analysis.

**RT-PCR analysis**

Total RNA was isolated from skeletal muscle and heart tissue from 1-d-old neonatal mice by using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed with the random primer and Superscript kit (Invitrogen). The cDNA was used as a PCR template to perform PCR by standard protocols. Specific primers for nebulin exons 2 and 3 (forward, CTTGGTCTACATACACAGGGA; reverse, ACAATGTCGGCGACGACATAGGAC) were used for nebulin PCR. The PCR products were separated on a 1.5% agarose gel.

**X-galactosidase staining**

Fate mapping of nebulin-expressing cells was assessed by crossing nebulin−/− heterozygous mice with the reporter Rosa26 mouse line to generate double heterozygous nebulin−/−/Rosa26 mice in which lacZ is expressed from the Rosa26 promoter after Cre-mediated recombination (Soriano, 1999). Tissue was stained for β-galactosidase activity according to the standard procedure. Tissue was fixed for 30 min at room temperature in 4% PFA in PBS and washed in PBS. Subsequently, tissue was stained at room temperature overnight with 3.7 μl of X-galactosidase solution (5 mM potassium ferricyanide, 2 mM MgCl2, 0.4% X-galactosidase in PBS) and rinsed twice in PBS. For frozen sections, tissue was fixed in optimal cutting temperature (OCT) compound before freezing in OCT. 10-μm sections were cut on a microtome and mounted in glycerol.

**Thin filament length analysis**

Thin filament length analysis was performed by distributed deconvolution using a custom plug-in written for ImageJ (National Institutes of Health [NIH]; http://rsb.info.nih.gov/ij/). This method determines the best fit of a probe-specific intensity [model] distribution to 1D myofibril fluorescence intensity profiles (line scans) by estimating the spread of light along the line scan and the positions and intensities of each probe with adjustable intensity distribution models for the thin filament array (β-Z body; Littlefield and Fowler, 2002). Image regions containing at least three stretched or hypercontracted sarcomeres were identified based on the appearance of tropomodulin doublets or phalloidin gaps (Z regions). Regions were not analyzed if tropomodulin doublets or phalloidin doublets were not visible. Background-corrected line scans were calculated by averaging the intensity across the width of the myofibril for each point along the selected myofibril length by subtracting the minimum intensity above and/or below the myofibril from the mean intensity across the myofibril width. Line scans were analyzed by distributed deconvolution using model distributions for α-actinin, phalloidin, and tropomodulin as described in Littlefield and Fowler (2002). Thin filament length was defined as half the distance between tropomodulin peaks or half the width of the phalloidin bands. Based on Z-line positions in muscles contained with α-actinin and tropomodulin and corrected for possible image registration, we estimated the positional error associated with the method to ~30 nm. For example, for gastrocnemius muscles from nebulin−/− mice, there was an estimated error of 66 nm in Z-line measures lengths and 44 nm in Z-line positions, which could not result from image misregistration.

**Neonatal TA contractile testing**

To provide the best estimate of muscle mechanical properties that did not reflect the progressive deterioration of the pups from days 1–11 (Fig. 3), mechanical testing experiments were performed on 1-d-old pups. Animals were killed by decapitation, and hind limbs were dissected at the midfemur, immediately placed into a mammalian Ringer’s solution (137 mM NaCl, 5 mM KCl, 24 mM NaHCO3, 1 mM NaH2PO4, 2 mM MgCl2, 1.1 mM MgSO4, 11 mM glucose, and 10 mg/L curare), and kept on ice until testing. Subsequent experiments revealed that the neonatal tetrad was extraordinarily fragile and, thus, could not be secured directly to the testing apparatus. Therefore, we tested the muscle–tendon–bone unit associated with the TA muscle. Distal to the TA muscle, the tarsal bone was secured to the lever arm of a servomotor (305B; Aurora Scientific). Proximal to the TA muscle, an anodized stainless steel minuten pin (0.2-mm diameter; Fine Science Tools) was bent to a 90° angle, and the sharp end was driven down the shaft, perpendicular to the femur from the front of the foot. The bone at the heel was then secured with a set screw to an XYZ translator (Newport Corporation) and adjusted until both the ankle and knee joints were at 90° and the tibia was perpendicular to the foot. Once the leg was fixed in this position, the plantar flexors were severed at the Achilles tendon and dissected away. The length of the TA muscle was measured using a stereomicroscope (Leica MZ16; Leitz Instruments) fitted with an eyepiece crosshair reticle, translating the chamber under the field of view from origin to insertion of the
muscle using a digital micrometer (350-712-30; Mitutoyo), and taking the mean of three measurements. Data acquisition was performed with a custom-written LabVIEW program (National Instruments) to trigger the 6-bp stimulator (Pulsar; FHC) and record force from the servomotor using a data acquisition board (PC6040; National Instruments) sampling at 4,000 Hz. Maximum isotonic tension was measured by stimulating dorsiflexors via platinum plate electrodes with a bipolar 400-ms train of 0.3-ms pulses delivered at 100 Hz. Two measurements were taken 2 min apart and averaged. After mechanical testing, the legs were removed from the chamber, and the TA and EDL muscles were dissected and weighed. The force-time records were then analyzed by a computer algorithm written in Matlab (The MathWorks) to calculate isometric force.

Architectural measurements on neonatal TA muscle
To determine the specific force generated by each muscle, raw force was normalized by PCSA, the only anatomical value, which has been shown to be proportional to muscle force generation (Lieber and Friden, 2000). PCSA takes into account both fiber length and fiber pennation angle to be proportional to muscle force generation (Lieber and Friden, 2000). normalized by PCSA, the only anatomical value, which has been shown to be proportional to muscle force generation (Lieber and Friden, 2000). Muscle-specific tension was then calculated as muscle force/PCSA. Because normal mammalian muscle generates a specific tension of ~250 kPa, this method also allowed us to compare these neonatal muscles with their adult counterparts.

Online supplemental material
Fig S1 shows an example of the thin filament length analysis in gastrocnemius muscle using distributed convolution. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603119/DC1.

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References


