Reduced myofibrillar connectivity and increased Z-disk width in nebulin-deficient skeletal muscle

Paola Tonino1,*, Christopher T. Pappas2,*, Bryan D. Hudson1, Siegfried Labeit3, Carol C. Gregorio2 and Henk Granzier1,‡

1Department of Physiology and 2Department of Cell Biology and Sarver Molecular Cardiovascular Research Program, University of Arizona, Tucson, Arizona 85724-5217, USA
3Institute for Integrative Pathophysiology, Universitätsmédizin Mannheim 68167, University of Heidelberg, Germany

*These authors contributed equally to this work
‡Author for correspondence (granzier@email.arizona.edu)

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Summary

A prominent feature of striated muscle is the regular lateral alignment of adjacent sarcomeres. An important intermyofibrillar linking protein is the intermediate filament protein desmin, and based on biochemical and structural studies in primary cultures of myocytes it has been proposed that desmin interacts with the sarcomeric protein nebulin. Here we tested whether nebulin is part of a novel biomechanical linker complex, by using a recently developed nebulin knockout (KO) mouse model and measuring Z-disk displacement in adjacent myofibrils of both extensor digitorum longus (EDL) and soleus muscle. Z-disk displacement increased as sarcomere length (SL) was increased and the increase was significantly larger in KO fibers than in wild-type (WT) fibers; results in 3-day-old and 10-day-old mice were similar. Immunoelectron microscopy revealed reduced levels of desmin in intermyofibrillar spaces adjacent to Z-disks in KO fibers compared with WT fibers. We also performed siRNA knockdown of nebulin and expressed modules within the Z-disk portion of nebulin (M160-M170) in quail myotubes and found that this prevented the mature Z-disk localization of desmin filaments. Combined, these data suggest a model in which desmin attaches to the Z-disk through an interaction with nebulin. Finally, because nebulin has been proposed to play a role in specifying Z-disk width, we also measured Z-disk width in nebulin KO mice. Results show that most Z-disks of KO mice were modestly increased in width (~80 nm in soleus and ~40 nm in EDL fibers) whereas a small subset had severely increased widths (up to ~1 μm) and resembled nemaline rod bodies. In summary, structural studies on a nebulin KO mouse show that in the absence of nebulin, Z-disks are significantly wider and that myofibrils are misaligned. Thus the functional roles of nebulin extend beyond thin filament length regulation and include roles in maintaining physiological Z-disk widths and myofibrillar connectivity.

Key words: Muscle, Cytoskeleton, Contractility, Sarcomeric structure, Nemaline myopathy

Introduction

The sarcomere is the basic contractile unit of striated muscle, and is composed of highly ordered thin (actin) filaments and thick (myosin) filaments (Squire, 1997). The cytoskeleton surrounding this elegant contractile molecular machinery is required as a scaffold to ensure efficiency of contraction. Longitudinally, sarcomeres are connected by Z-disk lattices that anchor thin filaments and transmit force along the myofibril. The strikingly regular lateral alignment of Z-disks between adjacent myofibrils ensures that during contraction myofibrils change length in unison, thus preventing damage to membrane systems that span between sarcomeres (T-tubules, sarcoplasmic reticulum). The mechanisms and proteins responsible for maintaining lateral myofibrillar alignment are incompletely understood. An important intermyofibrillar linking protein is the intermediate filament protein desmin, but the mechanisms that regulate its attachment to the myofibril itself have not been established (Capetanaki et al., 2007; Costa et al., 2004; Wang and Ramirez-Mitchell, 1983). Here we tested whether nebulin is part of the mechanical linkage by using a recently developed nebulin knockout (KO) mouse model (Witt et al., 2006). In the absence of nebulin, Z-disks assemble (Bang et al., 2006; Witt et al., 2006) and, thus, the nebulin KO makes it possible to test the role of nebulin in Z-disk alignment. Although misalignment has been noted in previous work on a nebulin KO (Bang et al., 2006), it has not been studied in detail. These studies were motivated by in vitro studies that suggested that the Z-disk portion of nebulin interacts with desmin (Bang et al., 2002) and more recently, nebulin modules M160-164 were reported to be involved in this interaction (Conover et al., 2009).

Nebulin is a giant filamentous protein that plays a role in controlling the thin filament length in skeletal muscle (Bang et al., 2006; Castillo et al., 2009; Littlefield and Fowler, 2008; McElhinny et al., 2003; Witt et al., 2006). The N-terminal region of nebulin is located near the pointed end of the thin filament, and the C-terminal region (~50 kDa) is anchored in the Z-disk and contains multiple phosphorylation motifs involved in signaling events (McElhinny et al., 2003). Research into the functions of nebulin has been stimulated by the identification of mutations in the nebulin gene (including in its Z-disk region) that cause nemaline myopathy (NM) in humans. NM is a member of a class of muscle disorders that phenotypically have in common a severe muscle weakness and structural abnormalities of Z-disks (Gurgel-Giannetti et al., 2001; Pelin et al., 1999).

Yeast two-hybrid screens have shown that the C-terminus of nebulin interacts with desmin (Bang et al., 2002), and that the nebulin modules M160-164 are involved in this interaction (Conover et al., 2009). The functional significance of this requires further
study. In the present study we investigated whether nebulin-desmin interaction is important for myofibrillar connectivity, by measuring longitudinal Z-disk displacement between adjacent myofibrils in muscle fibers from wild-type and nebulin knockout mice. We also performed immunoelectron microscopy with anti-desmin antibodies, studied the expression level of desmin, and expressed the C-terminal nebulin repeats M160-M170 in myotubes and studied the effect on desmin localization. Finally, because nebulin has been suggested to play a role in specifying Z-disk width (Millevoi et al., 1998), a role that is likely to be independent of the role of nebulin in laterally linking Z-disks, we also measured Z-disk width and determined the expression level of α-actinin, a major Z-disk actin-linking protein (Sjoblom et al., 2008). Our findings suggest that nebulin plays a role in specifying Z-disk width and in lateral registration of Z-disks in adjacent myofibrils.

Results

The role of nebulin in myofibrillar connectivity was studied using a nebulin KO mouse model (for details, see Witt et al., 2006). We focused on EDL and soleus muscle fibers because they contain predominately fast and a large number of slow twitch fibers (Danieli-Betto et al., 2005), respectively, allowing us to determine whether there are fiber-type-specific changes in myofibrillar connectivity in the nebulin KO mouse. The ultrastructural sarcomeric organization of WT and nebulin KO muscle is shown in Fig. 1. WT myofibrils of both EDL (Fig. 1A,C) and soleus (Fig. 1E,G) muscle had well-aligned sarcomeres (minimal Z-disk displacement in adjacent myofibrils), uniform Z-disk width and an A-band with a visible H-zone (light zone devoid of thin filaments) in the center of the sarcomere. By contrast, a number of abnormalities were observed in the KO fibers. As previously reported for the tibialis cranialis muscle (Witt et al., 2006), sarcomeres of both EDL (Fig. 1B,D) and soleus KO fibers (Fig. 1F,H) did not have H-zones and their Z-disk widths were sometimes irregular with the presence of nemaline rod bodies (rod-shaped inclusion body consisting of Z-disk material, see also below). A difference that we noted, especially in stretched fibers, was that Z-disks of adjacent myofibrils were not well in register but instead were longitudinally displaced. Thus, in addition to the absence of H-zones, which is due to variable thin filament lengths in nebulin KO mice (Witt et al., 2006), Z-disk abnormalities were present as well, including Z-disk misalignment.

Z-disk misalignment is further highlighted in the sample micrographs shown in Fig. 1D,H. This figure also shows how longitudinal Z-disk displacement (AX) of adjacent myofibrils was quantified. We stretched EDL and soleus muscle fibers from nebulin KO and WT mice to different sarcomere lengths and measured AX. Results are shown in Fig. 2A,B. We used linear regression analysis to determine the relationship between Z-disk displacement and sarcomere length (SL) and compared the slopes by analysis of covariance. Findings in WT fibers indicate that Z-disk displacement is relatively small at SLs of 2.0–2.5 μm, and that displacement increases slightly as sarcomeres are stretched, with similar findings in soleus and EDL fibers. However, slopes were not significantly different from zero. The increase with SL was significantly steeper in KO fibers than in WT fibers [EDL slopes: 0.03 and 0.11 (P=0.002); soleus slopes 0.01 and 0.26 (P=0.003)]. For soleus and EDL KO fibers the slopes were not significantly different from each other, indicating that no major differences exist between myofibrillar connectivity of these muscle types. To address the concern that differences between WT and KO muscles might be due to muscle degeneration, we also studied muscle from WT and KO mice that were only 3 days old. Supplementary material Fig. S1 shows typical electron micrographs, and analyzed results are shown in Fig. 2C,D. Results are similar to those of the 10-day old mice: displacement increased significantly more steeply with SL (P=0.005 in soleus and P=0.01 in EDL) in KO fibers than in WT fibers. We determined (Fig. 3) the mean Z-disk displacement at SLs >2.9 μm (to make our findings comparable to those obtained on desmin KO fibers [see Discussion by Shah et al. (Shah et al. 2002)]. In 10-day-old WT mice the mean displacement of EDL fibers was 0.08±0.05 μm (n=25) and of soleus fibers, 0.07±0.03 μm (n=8). For age-matched KO fibers the values were 0.21±0.09 μm (n=23) and 0.31±0.03 μm (n=14) for EDL and soleus fibers, respectively. The values obtained in 3-day-old mice are also shown in Fig. 3 (gray bars). The mean displacements were again significantly larger in the KO muscles. The effect of postnatal...
day and genotype was assessed by ANOVA with Tukey’s HSD post-hoc comparisons. There were statistically significant differences between all KO and WT group but not within any of the WT and KO groups.

Thus, in our data, Z-disks in EDL and soleus fibers from nebulin KO mice were, at SLs >2.9 μm, approximately three- to four-fold more displaced than in their WT littermates, and this was true for both age groups. Misalignment was not more severe in the 10-day-old than in 3-day-old mice suggesting that the myofibrillar misalignment is not due to a progressive pathology. Taken together these results demonstrate that nebulin is involved in the lateral registration of both fast and slow skeletal muscle myofibrils.

Because it is known that the intermediate filament protein desmin is involved in laterally linking myofibrils (Shah et al., 2004; Shah et al., 2002), we performed immunoelectron microscopy with polyclonal anti-desmin antibodies and gold-conjugated secondary antibodies and studied the localization of desmin in WT and nebulin KO fibers. EDL fibers from WT mice showed an accumulation of gold particles around the Z-disks (Fig. 4A), as well as near the myonuclei (results not shown). In muscle fibers from nebulin KO mice the intermyofibrillar spaces at the level of Z-disks had fewer gold particles (Fig. 4B), whereas near nuclei and costameres enhanced staining was often observed. We quantified the number of gold particles in the Z-disk to Z-disk space (using ImageJ analysis software; Fig. 3C top). The number of gold particle per unit area was significantly reduced in the nebulin KO mice (t-test, P<0.05). Immunogold labeling was also observed throughout the sarcomere, suggesting non-specific labeling. To characterize non-specific labeling, measurements were made in randomly selected areas of the A-band region of the sarcomere. This revealed no difference between WT and KO mice (Fig. 4C, bottom). To determine whether desmin was mislocalized in nebulin KO muscle or, alternatively, had a much reduced expression level, we performed western blot experiments. Analysis revealed an increased desmin expression level in nebulin KO fibers when compared with WT (supplementary material Fig. S1A). Thus, reduced desmin labeling at the Z-disks of KO fibers is not due to a reduced expression level of desmin.

To further study whether nebulin is involved in anchoring desmin to the Z-disk region of the sarcomere we performed cell culture experiments. Our goal was to do this work on cultured muscle fibers (from WT and KO mice) but we were unable to successfully culture these cells and instead performed experiments on quail myoblasts that turned out to be well suited for this work. In order to better understand the assembly of desmin during the differentiation of quail skeletal myotubes in culture, cells were fixed and stained for desmin and α-actinin at various timepoints from 17 hours to 8 days after plating. Based on the staining for...
the well-characterized Z-disk component, α-actinin (e.g. Rhee et al., 1994; Rudy et al., 2001) and the morphological characteristics of the cells, distinct stages of assembly were identified (supplementary material Fig. S2). At early stages of assembly (~17 hours), both α-actinin and desmin were punctate in appearance. Later, (1-2 days) the dots of α-actinin staining had organized into linear arrays, whereas desmin had formed a peri-sarcomeric mesh-like network that was especially evident around the nuclei. At intermediate stages of assembly (3-4 days) α-actinin was assembled into discrete bands, indicating the maturation of the Z-disk, whereas desmin remained as a network. Finally, in mature stages of assembly (4-8 days) desmin also localized to the Z-disk in distinct striations.

To ascertain the role of nebulin in the assembly of desmin, small interfering RNA (siRNA) was used to reduce the levels of nebulin in quail skeletal myotubes (supplementary material Fig. S3). Quail myotubes were then co-stained with anti-desmin and anti-α-actinin antibodies 4-8 days after siRNA treatment. The reduction of nebulin levels resulted in a marked perturbation of desmin assembly at the Z-disk. The majority of cells lacked any observable desmin Z-disk assembly (as in Fig. 6, bottom two rows); example of less typical cell type in which broad and faint Z-disk labeling of desmin was seen.)

Desmin assembly at the Z-disk was also reduced following the expression of nebulin C-terminal modules M160-170, which contain binding sites for desmin (Bang et al., 2002). Nebulin M160-170 fused to mCherry localized to the Z-disk and along the periphery of the myofibrils, whereas mCherry alone associated with the myofibrils but lacked the distinctive Z-disk distribution (Fig. 6). A significant decrease in the Z-disk localization of desmin was observed in myocytes expressing nebulin M160-170, in comparison to cells expressing mCherry alone (Fig. 6, desmin panels). In three independent experiments we counted the number of cells that were devoid of distinct Z-disk desmin labeling (as in Fig. 6, bottom row). Of all cells transfected with mCherry alone 19.0±1.1% of the cells had diffuse and non-striated desmin labeling; this value was significantly (P<0.001) increased in cultures transfected with nebulin M160-170 fused to mCherry. Thus, both knockdown of nebulin with siRNA and overexpression of M160-M170 did not interfere with the formation of normal striation patterns of α-actinin but did prevent the mature Z-disk localization of desmin.

Our studies also provided an opportunity to study the Z-disk structure. Previous work has suggested that nebulin plays a role in specifying Z-disk width (Millevoi et al., 1998) and the Z-disk width is thus expected to be dysregulated in nebulin-deficient muscle. (Note that the role of nebulin in Z-disk width specification is likely to be independent of its role in anchoring desmin.) As noted above, the Z-disk morphology of nebulin KO fibers was also altered, and this phenomenon is further highlighted in Fig. 7. Some Z-disks were unusually wide with an irregular or rounded shape (Fig. 7B,D), similar to the typical structure of nemaline rod bodies (Gurgel-
Giannetti et al., 2001). These nemaline rod bodies were found throughout the fibers but had a tendency to be more prominent in areas close to the myonuclei. Consistent with the work of Wallgren-Pettersson and colleagues (Wallgren-Pettersson et al., 1995) we were unable to detect immunogold particles in the IEM experiments described above, indicating that the presence of desmin in rod bodies is unlikely. Previous work by others has shown that the rods contain α-actinin (Wallgren-Pettersson et al., 1995) and we consider it probably that this is the case in the nebulin KO as well, consistent with the increased levels of α-actinin that we found in our western blot experiments (see below). In other cases, the Z-disk exhibited interruptions and a zigzag shape that appeared to be more pronounced at long sarcomere lengths, whereas in severely damaged myofibrils a disintegration of the Z-disk was also observed (which was independent of stretch). Histograms of Z-disk widths are shown in Fig. 8. The average width of the Z-disk in EDL muscle from WT mice was 127±21 nm (range 96-175 nm; Fig. 8A) and in nebulin KO muscle 271±165 nm (range 100-950 nm; Fig. 8B). The average width was significantly larger in soleus muscle from WT mice: 145±20 nm (range 83-200 nm; Fig. 8C), and nebulin KO mice: 465±422 nm (range 100-2075 nm; Fig. 8D). Consistent with wider Z-disks, we found that α-actinin, a major constituent of Z-disks, was upregulated several-fold in KO fibers (supplementary material Fig. S4).

Discussion

Linkage of myofibrils allows for lateral force transmission and 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. 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 (Capetanaki et al., 2007; Costa et al., 2004; Lazarides and Granger, 1978; Wang and
Ramirez-Mitchell, 1983). The subunit proteins of desmin filaments are elongated coiled-coils with extensive intermolecular ionic and hydrophobic interactions between individual subunits, giving rise to filaments with high tensile strength as well as plasticity (Costa et al., 2004). That desmin tethers adjacent Z-disks is supported by work on a desmin KO mouse in which Z-disk misalignment was shown to occur in stretched muscle (Shah et al., 2002). Previous in vitro work suggested that desmin binds to the sarcomeric protein nebulin (Bang et al., 2002), and in the present study using a nebulin KO mouse model we examined this possibility. We found that myofibrils translocate in muscle devoid of nebulin to a much higher degree than in WT muscle (for definition of translocation, see 1D,H for results, see Fig. 2). Our work also shows that desmin is present in muscle devoid of nebulin but that it is reduced in the intermyofibrillar spaces that surround the Z-disks (Fig. 4C, top). Consistent with this, we found that both knockdown of nebulin with siRNA and overexpression of M160-M170 did not interfere with the formation of normal striation patterns but did prevent desmin localization at the mature Z-disk (Figs 5 and 6). It is unclear why two distinct phenotypes were observed; that is, lack of detectable Z-disk staining and broader and less defined Z-disk staining. We predict that the broad Z-disk staining might reflect displaced Z-disk desmin. Combined, our knockdown and overexpression studies suggest a model in which desmin attaches to the Z-disk through an interaction with nebulin. This model is consistent with a previous yeast two-hybrid study that found that nebulin modules M163-M183 interact with a 19-kDa central coiled-coil domain (known as coil 1B) of desmin (Bang et al., 2002) and a more recent study in which M160-M164 was found to interact with desmin (Conover et al., 2009). In the absence of nebulin, desmin is unable to bind to these sites on the myofibril and as a result myofibrils differentially translocate when passively stretched. In our work we studied both soleus and EDL muscles, examples of predominately slow- and fast-fiber-containing muscles (Danieli-Betto et al., 2005; Girgenrath et al., 2005), respectively. Essentially the same findings were obtained (Fig. 2) suggesting that the model (desmin binding to nebulin) applies equally to different muscle types. In summary our work on a nebulin KO mouse suggests that myofibrils are laterally linked at the level of the Z-disk by relatively stiff desmin filaments that bind to nebulin.

It is currently unknown whether in addition to Z-disks other linkage sites exist between adjacent myofibrils that are sufficiently stiff to prevent translocation of myofibrils. A comparison of results obtained with the nebulin KO and desmin KO models might shed light on this issue. If the Z-disk is the only site of importance, largely identical Z-disk displacements are expected in the two models. Shah et al. (Shah et al., 2002) reported that in desmin KO fibers with SLs >2.90 μm there was a Z-disk displacement of 0.49±0.10 μm, larger that the values that we found in nebulin KO fibers [−0.2 μm (EDL) and −0.3 μm (soleus)]. However, Z-disk displacement of WT fibers in the Shah et al. study was, at 0.25 μm, also much larger than in our WT fibers (~0.08 μm). This might indicate that forces were much higher in the Shah et al. study than in ours (we stretched the tendons very slowly and in small increment to keep passive force relatively low). It has been shown in single molecule studies (Krepelak et al., 2008) that at high force, desmin filaments can be elongated several-fold, presumably because the α-helical coiled-coil dimer can be converted into β-sheet-type structures upon stretching. The finding that Z-disk displacement in WT fibers is approximately constant at ~0.08 μm (Fig. 2) suggests that in our study forces were insufficient to cause filament elongation. Because the increase in Z-disk displacement above that of WT fibers is similar in our study to that of Shah et al. (Shah et al., 2002) we consider it likely that the desmin-nebulin linkage site is most important for myofibrillar connectivity. Thus although it is possible that other desmin-Z-disk linkages exist that do not involve nebulin [as suggested by the remaining Z-disk labeling of desmin in nebulin KO mice (see Bang et al., 2006)] the desmin-nebulin linkage is likely to be very important for establishing myofibrillar connectivity. Our similar findings in soleus and EDL muscles, representatives of slow and fast muscle types, respectively, suggest that nebulin-based myofibrillar connectivity is important in different muscle types, irrespective of their activity patterns. The importance of this linkage is also suggested by the upregulation of desmin, which might be viewed as a response to correct compromised myofibrillar connectivity. Finally, it is possible that in addition to desmin and nebulin other proteins also play a role in linking myofibrils. A good candidate is plectin, an ~500 kDa linking protein with isoforms 1d and 1f binding to desmin and Z-disks (Konieczny et al., 2008). It is unknown to which Z-disk protein(s) plectin binds and it is of importance to determine whether plectin is arranged in series or in parallel with the desmin-nebulin linkage.

We found that both soleus and EDL muscles of nebulin KO mice contain electron-dense Z-disk-like bodies that are similar to nemaline rod bodies that are a hallmark of nemaline myopathy in humans (Wallgren-Pettersson et al., 2004). Furthermore, histograms of Z-disk width (Fig. 6) show that the majority of all Z-disks in both WT and nebulin KO sarcomeres can be fit with a Gaussian curve that is shifted to higher values in KO than in WT fibers (by an average of −80 nm in soleus and −40 nm in EDL fibers). We conclude that absence of nebulin affects most Z-disks by widening them by a modest amount and that it affects a small subset of sarcomeres more severely (nemaline rod bodies). Previously titin has been suggested to play a role in Z-disk assembly (Gautel et al., 1996) and our present work indicates that nebulin also contributes to establishing and/or maintaining the Z-disk structure. The Z-disk region of titin contains a family of differentially expressed repeats, the titin Z-repeats (Gautel et al., 1996). These Z-repeats are a family of α-actinin-binding motifs, which are differentially expressed in a tissue- and developmental-stage-specific fashion (Gautel et al., 1996). As previously pointed out, it is unlikely that the differential expression of the titin Z-repeats alone can determine the Z-disk width, because too few isoforms exist to account for the wide range of different Z-disk widths (Millevoi et al., 1998). Our present work supports a model in which titin and nebulin together specify Z-disk width, with titin constructing the central region of the Z-disk, including the number and positions where α-actinin cross-links the thin and the titin filaments. Nebulin determines the ending of the Z-disk structure and its transition to the I-band, i.e. nebulin functions as a Z-disk terminator.

The mechanism by which nebulin terminates the Z-disk might involve interaction between nebulin and Z-disk-localized proteins such as, titin and CapZ. CapZ is a barbed-end actin capping protein that binds near the C-terminus of nebulin (Pappas et al., 2008). In muscle fibers devoid of nebulin [nebulin KO (Witt et al., 2006)] or siRNA knockdown of nebulin in myotubes (Pappas et al., 2008), CapZ does not localize properly, allowing the barbed ends of Z-disks to continue to grow beyond the Z-disk resulting in widened Z-disks. It is unclear why most Z-disks only widen by a modest amount and others develop into nemaline rod bodies. Because similar results were obtained in soleus and EDL, a mainly slow twitch posture muscle and fast twitch intermittently used muscle,
respectively, differences in mechanical load might not be the main explanation. Instead we speculate that gradients in protein levels in the muscle fiber play an important role. Clearly further research is required to resolve this question.

In summary, structural studies on a nebulin KO mouse show that in the absence of nebulin, Z-disks are significantly wider and that myofibrils are misaligned. Thus the functional roles of nebulin extend beyond thin filament length regulation and include maintaining physiological Z-disc widths and myofibrillar connectivity. It is interesting to note that the Z-disc functions of nebulin might be performed in cardiac muscle (where nebulin levels are very low or absent) by nebulette, a ~100 kDa nebulin-like protein that shares extensive similarity with the C-terminal region of nebulin (Millevoi et al., 1998). Thus thin filament length regulation might not be the main function of nebulin (because cardiac muscle can do without) but instead the Z-disc functions of nebulin might be most crucial.

Materials and Methods

Muscle specimens

The nebulin KO mouse model (BL6) including its genotyping has been described previously (for details, see Witt et al., 2006). Mice die at a relatively early age as a result of muscle weakness and respiratory failure and, therefore, animals in this study were used at 3 days and 10 days of age. At day 10, the KO mice weighed 2.3±0.2 g (n=8) and the WT mice 7.2±0.8 g (n=6). The values at day 3 were 2.0±0.3 g (KO, n=6) and 2.4±0.3 g (WT, n=5). Mice were killed and the skin from both hindlimbs was rapidly removed before immersing the hindlimbs in relaxing solution (40 mM BES, 10 mM EGTA, 6.5 mM MgCl2, 5.88 mM Na-ATP; 1 mM DTT, 46.35 mM potassium proprionate, 15 mM creatine phosphate) containing protease inhibitors [0.005 mM leupeptin, 0.1 mM E64, 0.2 mM phenylmethylsulphonyl fluoride (PMSF) from a stock in 100% ethanol, pH 7.0] and 1% Triton X-100 (w/v). After skimming overnight, the hindlimbs were washed with relaxing solution; EDL and soleus muscles were quickly removed and one end of their tendons was pinned down with a minute pin (Fine Science Tools) to a Sylgard elastomer base (Dow Corning, Midland, MI) in a small Petri dish filled with relaxing solution. The muscles were then passively stretched in small increments (in ~30 seconds) to a final length that exceeded the slack length by ~20%, 40%, 60% and 80%, followed by pinning the other end of the muscle tendon to the extensor dissection base. The muscles were kept in this stretched state for 10 minutes at 4°C, and were then processed for electron microscopy (see below). We typically studied ~20 fibers per muscle. All experiments were conducted and approved by the University of Arizona Institutional Animal Care and Use Committee, following the guidelines ‘Using Animals in Intramural Research’ of the National Institutes of Health.

Transmission electron microscopy

For electron microscopy, stretched EDL and soleus muscle from WT and nebulin KO mice were fixed with 3% paraformaldehyde in PBS for 30 minutes. The muscles were then rinsed for 15 minutes in PBS containing protease inhibitors (Granzier et al., 1997). A secondary fixation was performed in 3% glutaraldehyde containing 2% potassium propionate, 15 mM creatine phosphate) containing protease inhibitors. The bundles were rinsed with PBS and PBS containing protease inhibitors. Blocking was performed with 1% BSA in PBS containing protease inhibitors for 1 hour at 4°C, followed by incubation with polyclonal anti-desmin antibody (rabbit whole serum, Sigma-Aldrich, D8281) diluted 1:10 in PBS containing protease inhibitors for 4 hours at 4°C. (Note that long labeling durations are commonly used in IEM on skinned muscle.) After rinsing in PBS, the fiber bundles were incubated for 48 hours at 4°C with secondary anti-rabbit Nanogold goat antibody (1.4 nm, Nanoprobe) at a dilution of 1:10 in PBS containing protease inhibitors. The bundles were rinsed with PBS and then fixed in 3% glutaraldehyde containing 0.01% tannic acid in PBS for 30 minutes at 4°C. After rinsing in PBS, aldehydes were quenched with 50 mM glycine in PBS for 15 minutes at 4°C, followed by gold enhancement (Nanoprobe; as per the manufacturer’s instructions) for 3-5 minutes depending on fiber bundle thickness. Bundles were washed with PBS and postfixed with 1% OsO4 in PBS for 15 minutes at 4°C. Fiber bundles were washed with distilled water and dehydrated in a graded series of ethanol (50%, 75%, 95%, 100%). Then the bundles were infiltrated with propylene oxide for 15 minutes, then in a mixture of 1:1 propylene oxide:Araldite followed by Araldite alone. Bundles were cut into small fragments and then prepared for mounting, embedding and polymerization for 48 hours at 60°C. Sections were only lightly contrasted in lead citrate, to ensure that gold particles stood out well from the sarcomeric structures, and the sections were examined using either a Jeol 1200EX or a Philips CM12 microscope operated at 100 kV.

Gel electrophoresis and western blotting analysis

Gastrocnemius muscle from 10-day-old WT and nebulin KO mice (n=3) were quick-frozen in liquid nitrogen, pulverized and rapidly solubilized as previously described (Granzier and Irving, 1995). Samples were run on 12% SDS-PAGE gels, and transferred to polivinylidene difluoride membranes. Blots were probed with anti-desmin polyclonal (Sigma-Aldrich) and sarcomeric anti-α-actinin monoclonal (clone 567H; NeoMarkers, Fremont, CA) antibodies. A secondary fixation was performed in 3% glutaraldehyde containing 0.01% tannic acid in PBS for 30 minutes at 4°C, followed by gold enhancement (Nanoprobe; as per the manufacturer’s instructions) for 3-5 minutes depending on fiber bundle thickness. Bundles were washed with PBS and postfixed with 1% OsO4 in PBS for 15 minutes at 4°C. Fiber bundles were washed with distilled water and dehydrated in a graded series of ethanol (50%, 75%, 95%, 100%). Then the bundles were infiltrated with propylene oxide for 15 minutes, then in a mixture of 1:1 propylene oxide:Araldite followed by Araldite alone. Bundles were cut into small fragments and then prepared for mounting, embedding and polymerization for 48 hours at 60°C. Sections were only lightly contrasted in lead citrate, to ensure that gold particles stood out well from the sarcomeric structures, and the sections were examined using either a Jeol 1200EX or a Philips CM12 microscope operated at 100 kV.

Cell culture, transfections and siRNA treatment

Primary cultures of quail skeletal myotubes were prepared as described (Almenar-Queralt et al., 1999). A nebulin-specific siRNA was previously designed, validated and purchased from Ambion (Austin, Texas; target cDNA sequence: 5’-TGATGCTAGCTCTCAATTA-3’), western blot analysis demonstrated that siRNA treatment reduced endogenous nebulin levels by >90% (Pappas et al., 2008). As a control, a random siRNA was also generated (target cDNA sequence: 5’-CTCGACTAGCTCTCGTCTCA-3’). Nebulin antibodies were fixed in 3% glutaraldehyde containing 0.01% tannic acid in PBS for 30 minutes at 4°C, followed by gold enhancement (Nanoprobe; as per the manufacturer’s instructions) for 3-5 minutes depending on fiber bundle thickness. Bundles were washed with PBS and postfixed with 1% OsO4 in PBS for 15 minutes at 4°C. Fiber bundles were washed with distilled water and dehydrated in a graded series of ethanol (50%, 75%, 95%, 100%). Then the bundles were infiltrated with propylene oxide for 15 minutes, then in a mixture of 1:1 propylene oxide:Araldite followed by Araldite alone. Bundles were cut into small fragments and then prepared for mounting, embedding and polymerization for 48 hours at 60°C. Sections were only lightly contrasted in lead citrate, to ensure that gold particles stood out well from the sarcomeric structures, and the sections were examined using either a Jeol 1200EX or a Philips CM12 microscope operated at 100 kV.

Immunofluorescence microscopy

To observe sarcomeric components, cells were stained as described previously (Pappas et al., 2008). The fixed cells were permeabilized in 0.2% Triton X-100 in PBS, blocked with 2% BSA plus 1% normal donkey serum in PBS, and incubated for 1 hour with primary antibodies diluted in PBS. The primary antibodies consisted of a polyclonal anti-N-terminal nebulin antibody [3.5 μg/ml (McElhinny et al., 2001)], a monoclonal anti-α-actinin antibody (1:15,000; Sigma, A7811) and a polyclonal anti-desmin antibody (1:2; Biomedra, Foster City, CA; 213M). The cells were then washed with PBS for 15 minutes at room temperature and then incubated with Alexa Fluor 594-conjugated donkey anti-rabbit (1:10,000) or Alexa Fluor 488-conjugated donkey anti-mouse (1:1000) and imaged using a Leica confocal microscope. Alexa Fluor 594-conjugated donkey anti-rabbit (1:10,000) or Alexa Fluor 488-conjugated donkey anti-mouse (1:1000) and imaged using a Leica confocal microscope. Alexa Fluor 594-conjugated donkey anti-rabbit (1:10,000) or Alexa Fluor 488-conjugated donkey anti-mouse (1:1000) and imaged using a Leica confocal microscope.
Statistical analysis
Data were expressed as mean ± s.d. Unpaired t-tests were used to determine whether Z-disc displacements of EDL and soleus muscles were significantly different, with P<0.05 regarded as statistically significance. The slopes for displacement versus SL data from muscles of wild-type and KO mice were compared by analysis of covariance (ANCOVA) using StatView software (v. 5.01, SAS Institute).

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