Cell

An Ankyrin-Based Mechanism for Functional Organization of Dystrophin and Dystroglycan

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DOI 10.1016/j.cell.2008.10.018

SUMMARY

β-dystroglycan (DG) and the dystrophin-glycoprotein complex (DGC) are localized at costameres and neuromuscular junctions in the sarcolemma of skeletal muscle. We present evidence for an ankyrin-based mechanism for sarcolemmal localization of dystrophin and β-DG. Dystrophin binds ankyrin-B and ankyrin-G, while β-DG binds ankyrin-G. Dystrophin and β-DG require ankyrin-G for retention at costameres but not delivery to the sarcolemma. Dystrophin and β-DG remain intracellular in ankyrin-B-depleted muscle, where β-DG accumulates in a juxta-TGN compartment. The neuromuscular junction requires ankyrin-B for localization of dystrophin/utrophin and β-DG and for maintenance of its postnatal morphology. A Becker muscular dystrophy mutation reduces ankyrin binding and impairs sarcolemmal localization of dystrophin-Dp71. Ankyrin-B also binds to dynactin-4, a dynactin subunit. Dynactin-4 and a subset of microtubules disappear from sarcolemmal sites in ankyrin-B-depleted muscle. Ankyrin-B thus is an adaptor required for sarcolemmal localization of dystrophin, as well as dynactin-4.

INTRODUCTION

The absence of dystrophin in Duchenne muscular dystrophy is accompanied by failure in accumulation of dystroglycan and other components of the dystrophin-glycoprotein complex (DGC) at the sarcolemma (Cohn and Campbell, 2000; Dalkilic and Kunkel, 2003; Ervasti et al., 1990). The DGC is enriched in costameres, which are specialized domains formed at the junction of the plasma membrane and Z discs of peripheral myofibrils (Bloch et al., 2004; Ervasti, 2003; Pardo et al., 1983; Rybakova et al., 2000). Dystrophin and its homolog utrophin form similar complexes that localize to the postsynaptic membrane of the neuromuscular junction (NMJ). β-dystroglycan (β-DG) is the core membrane-spanning component of the DGC, which connects the actin cytoskeleton to the extracellular matrix in skeletal muscle (Lapidos et al., 2004; Rybakova et al., 2000). β-DG also is targeted to NMJs where it links intracellular proteins (dystrophin, utrophin, dystrobrevin, rapsyn) to laminin and agrin in the ECM and is required for postnatal maturation of the postsynaptic membrane (Grady et al., 2000; Sanes and Lichtman, 1999).

The cellular mechanisms underlying the role of dystrophin in localization of the DGC in skeletal muscle remain to be resolved. Dystrophin associates with members of the syntrophin family, which recruit NOS and aquaporin 4 but are not required for association of dystrophin with the sarcolemma (Adams et al., 2001). Several considerations suggest that the ankyrin family of adaptors may play a role in organization of the DGC. Ankyrin-B (ankB) and ankyrin-G (ankG) are localized at costameres (Hopitzan et al., 2005; Nelson and Lazarides, 1984; Tuvia et al., 1999; Williams et al., 2001), while ankG is a component of the neuromuscular junction (Kordeli et al., 1998). Moreover ankB and ankG function in organization of diverse bilayer-spanning proteins in specialized membrane domains (Bennett and Healy, 2008). We report here that ankB and ankG both bind to dystrophin and have distinct roles in organization of dystrophin and β-DG in the sarcolemma. AnkG binds β-DG as well as dystrophin and is required to restrict β-DG and dystrophin at costameres. We demonstrate that ankyrin-dependent organization of sarcolemmal dystrophin and β-DG is required for sarcolemmal integrity during physical exercise. Both dystrophin and β-DG remain intracellular in ankB-depleted muscle. We also demonstrate that ankB binds to dynactin-4 (also known as the p62 subunit of the dynactin complex), which has been proposed to function in connecting the dynactin complex to membrane sites (Eckley et al., 1999), and show that ankB is required for sarcolemmal localization of dynactin-4 as well as a specialized class of microtubules. AnkB thus is an adaptor for dystrophin, as well as dynactin-4.

RESULTS

Loss of Sarcolemmal DGC in Neonatal AnkB$^{-/-}$ Muscle

AnkB$^{-/-}$ mice have elevated serum creatine kinase, suggesting a congenital myopathy (Tuvia et al., 1999). We initially attributed this skeletal muscle myopathy to abnormalities in calcium homeostasis since ankB$^{-/-}$ neonatal cardiomyocytes exhibited

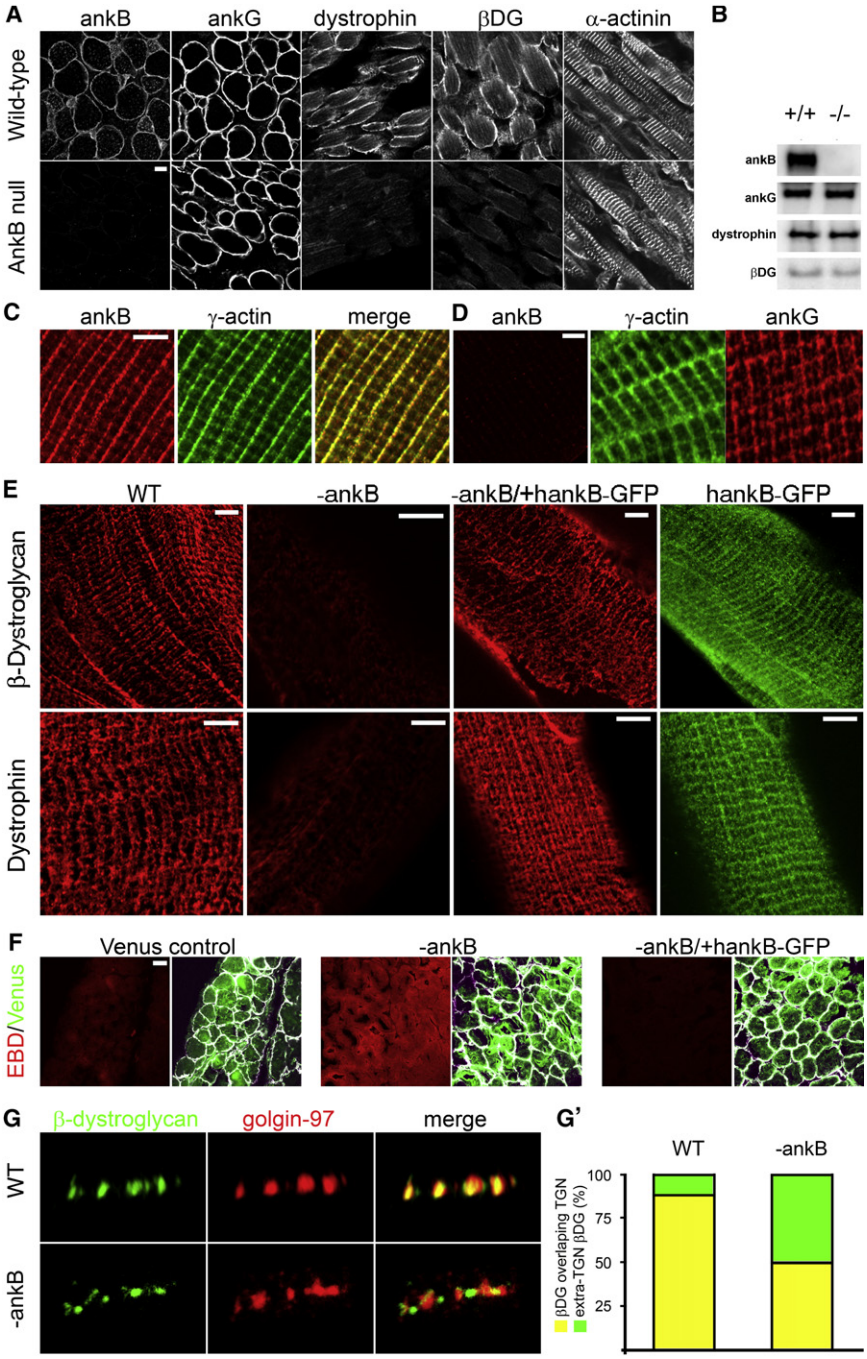

Figure 1. AnkB Is Required for Sarcolemmal Dystrophin and β-DG

(A) DGC proteins are mislocalized in neonatal ankB$^{-/-}$ muscle. Quadriceps cross-sections from P0 wild-type and ankB$^{-/-}$ mice were labeled with antibodies against ankB, ankG, dystrophin, β-DG, and α-actinin. Sarcolemmal staining of dystrophin and β-DG is markedly reduced in ankB$^{-/-}$ fibers while ankG and α-actinin show normal staining. Total of 6–8 pups per genotype, bar = 10 μm.

(B) Expression of ankG, dystrophin, and β-DG in wild-type and ankB$^{-/-}$ is identical in immunoblots of skeletal muscle.

(C) In isolated adult tibialis anterior (TA) fibers, ankB colocalizes with γ-actin in costameres.

(D) Transfection of adult muscle with a plasmid encoding ankB-siRNA knocks down ankB but not ankG or γ-actin.

(E) Dystrophin and β-DG are markedly reduced in the sarcolemma of ankB-depleted muscle fibers but not in fibers cotransfected with plasmids encoding both siRNA and GFP-tagged human ankB (hankB-GFP) (green), which is resistant to mouse siRNA. (N = 4 individual experiments, n = 2 mice per experiment, total of 8 mice. Scale bar, 5 μm.)

(F) EBD (red) penetrates specifically ankB-depleted (-ankB) fibers but not those transfected with Venus vector lacking siRNA in mice subjected to exercise. Fibers transfected with the ankB-siRNA plasmid are identified by Venus expression (green). Muscles cotransfected with plasmids encoding both ankB siRNA and human ankB do not take up EBD. Sarcolemma (white) is marked by ankG staining. N = 4 experiments, n = 3 mice per experiment per genotype (total of 27 mice, 9 per genotype). Bar = 20 μm.

(G) β-DG accumulates in and outside the TGN (marked by golgin 97) in ankB-depleted fibers. Images are 3D reconstructions of Z series comparing control and ankB-depleted fibers for colocalization of β-DG and golgin 97.

(G′) In control fibers, 89% ± 6% of the β-DG overlaps with the TGN. In ankB knockdown fibers, 50% ± 7% of the β-DG overlaps with the TGN while the rest accumulates next to and outside of the TGN (50 individual TGN site were measured for each control and ankB-siRNA in multiple fibers from 3 animals each. Significance of difference between control and ankB-siRNA measurements was calculated using one-way ANOVA, p = 0).

abnormal calcium dynamics (Tuvia et al., 1999). Subsequent studies have revealed that elevated calcium transients in ankB-deficient cardiomyocytes result from loss of a complex of ankB with the Na/Ca exchanger, Na/K ATPase, and IP3R (Mohler et al., 2003, 2005). However, this ankB-based complex is a specific adaptation of cardiomyocytes and is not present in skeletal muscle (Mohler et al., 2005). We therefore tested whether absence of ankB in neonatal mice affected localization of DGC proteins in skeletal muscle. Cross-sections of quadriceps muscles of newborn ankB null mice were immunolabeled for dystrophin and β-DG. Strikingly, labeling for these proteins is markedly reduced in the plasma membrane of ankB$^{-/-}$ muscle fibers (Figure 1A). Note that costameres (only 2 microns apart) appear as a solid line when viewed in these 20-micron-thick tissue sections. Ankyrin-G (ankG) and the Z line protein α-actinin are normally localized in ankB$^{-/-}$ muscle. Protein levels of the above DGC proteins in ankB null muscle are identical to wild-type levels in immunoblots (Figure 1B). Together, these findings suggest a specific defect in either delivery to or maintenance of dystrophin and β-DG in the sarcolemma in ankB$^{-/-}$ muscle.

Loss of Sarcolemmal Dystrophin and β-DG in Adult AnkB-Depleted Muscle

AnkB is expressed in most tissues, including the nervous system, and ankB null mice die soon after birth. These considerations suggested the possibility of an indirect effect of ankB deficiency on neonatal skeletal muscle. In order to study the role of ankB specifically in adult muscle of otherwise healthy animals, we knocked down ankB in skeletal muscle of adult mice using siRNA. We transfected tibialis anterior (TA) muscles of adult mice by electroporation with a bicistronic plasmid encoding a mouse-specific ankB-siRNA and Venus as a fluorescent marker for transfected fibers (Experimental Procedures). Muscle fibers were subsequently isolated and costamere proteins visualized in grazing sections by confocal microscopy. Figure 1C demonstrates sarcolemma-associated ankB in costameres, precisely colocalizing with γ-actin as a marker. Figure 1D shows the effective knockdown of ankB expression following siRNA expression, while γ-actin and ankG remain intact. Control fibers transfected with the Venus vector showed normal expression and distribution of β-DG, dystrophin, ankyrins-B and –G, as well as spectrin and vinculin (data not shown). Strikingly, ankB-depleted fibers exhibited marked reduction of both dystrophin and β-DG in the sarcolemma (Figure 1E). In contrast, γ-actin and ankG (Figure 1D) as well as spectrin and vinculin (data not shown) exhibited an unaltered labeling pattern. AnkB-depleted costameres thus are structurally intact but are selectively missing β-DG and dystrophin.

Loss of β-DG and dystrophin was prevented by cotransfection with cDNA encoding human ankB that was resistant to mouse siRNA (Figure 1E). In rescue experiments, mice were transfected in one leg with an ankB siRNA-expressing plasmid and in the other with ankB siRNA plus cDNA encoding GFP-tagged human ankB. Human ankB-GFP staining, overlaying the diffuse Venus staining (marking siRNA-expressing fibers), is distributed in a costamere pattern indistinguishable from endogenous ankB. Dystrophin and β-DG are clearly present and properly enriched in costameres of rescued fibers. These experiments establish that ankB is required for the presence of β-DG and dystrophin at the sarcolemma of adult muscle.

We next tested whether ankB knockdown results in sarcolemmal fragility. TA muscles were transfected with ankB-siRNA in the bicistronic Venus vector with and without cotransfection with a plasmid encoding human ankB-GFP for rescue as described above. As a control we injected muscles with the Venus vector alone. Mice were exercised on a treadmill and injected IP with Evans Blue Dye (EBD), which penetrates damaged fibers (Straub et al., 1997). To insure that muscle samples evaluated for EBD uptake were representative, we cross-sectioned throughout the entire muscle length at 200 μm intervals (approximately 30 sections per muscle). Fibers expressing only the Venus control plasmid exhibited no EBD uptake (Figure 1F). However, fibers expressing ankB siRNA (about 80% based on coexpression of Venus) exhibit EBD penetration. Cotransfection with plasmids encoding mouse ankB-siRNA and human ankB-GFP prevented EBD uptake. Images in Figure 1F are representative of sections taken from 12 muscles (6 mice) each of control, knockdown, and rescued animals in two different exercise/transfection experiments. In contrast to the MDX mouse and other animal models where EBD dye uptake is variable (Straub et al., 1997), all of the fibers transfected with ankB siRNA also exhibited EBD uptake. A likely difference between acute siRNA-mediated depletion and genetic mouse models is that in the former, upregulation of utrophin and other compensatory mechanisms do not occur. AnkB thus is required for sarcolemmal localization of β-DG and dystrophin in adult skeletal muscle and to maintain structural integrity of muscle challenged by exercise.

We demonstrate below that ankB depletion also has substantial effects on the postnatal NMJ. To determine if the phenotype observed in siRNA knockdown fibers is a result of loss of neuronal input, we evaluated effects of blocking neurotransmission by local injection with fluorescently labeled α-bungarotoxin (αBgt), which irreversibly inactivates nicotinic acetylcholine receptors (nAChR). Individual TA muscles were injected daily with αBgt (0.11 μg/g, half the LD_{50} for the whole mouse; Windholz, 1983) for 3 consecutive days (Supplemental Experimental Procedures available online). We observed no difference between control and αBgt-treated fibers in terms of β-DG localization in costameres, as well as in the general morphology of the NMJ (Figure S1).

β-DG Accumulates in an Intracellular Compartment in AnkB-Depleted Muscle

We next sought to determine the fate of β-DG and dystrophin in ankB-depleted adult muscle. Immunoblots revealed no reduction of β-DG and dystrophin polypeptides, even though ankB was markedly reduced in these siRNA-treated muscles (data not shown). These results recapitulated the preservation of these proteins in ankB null neonatal muscle (Figure 1B) and suggested that β-DG and/or dystrophin may accumulate in an intracellular compartment. We could not detect dystrophin in ankB-depleted muscle by immunofluorescence under a variety of fixation conditions. However, intracellular β-DG could be visualized in discrete micron-sized loci in both wild-type and ankB-depleted fibers permeabilized with saponin. β-DG-labeled puncta did not colabel with the endosomal marker Rab11 in either wild-type or ankB-depleted fibers. However, β-DG staining in control muscle colocalized with the trans-Golgi network (TGN) marker golgin 97 (Figure 1G). Voxel measurements derived from Z stacks reveal 89% (±6%, n = 50 golgi sites) overlap of β-DG labeling with golgin 97, while the remaining 11% β-DG staining was closely associated with the TGN (Figure 1G′). In contrast, only 50% ± 7% of β-DG staining in ankB-depleted fibers overlaps with the TGN (Figure 1F′), while the remaining 50% of β-DG staining is close to, but distinct from, the TGN. The difference in extra-TGN staining of β-DG of 50% in ankB-depleted fibers compared to 11% in controls was highly significant, with $p = 0$ in one-way ANOVA. Interestingly, the percent overlap between intracellular β-DG and golgin 97 is the same at high magnification and at lower magnification covering multiple sarcomeres for both wild-type and ankB-depleted fibers. Thus intracellular β-DG is primarily associated with or near the TGN, at least under these experimental conditions of fixation and permeabilization. In summary, dystrophin and β-DG are not degraded in ankB-depleted fibers, and β-DG accumulates in a juxta-TGN compartment distinct from rab11-labeled endosomes.

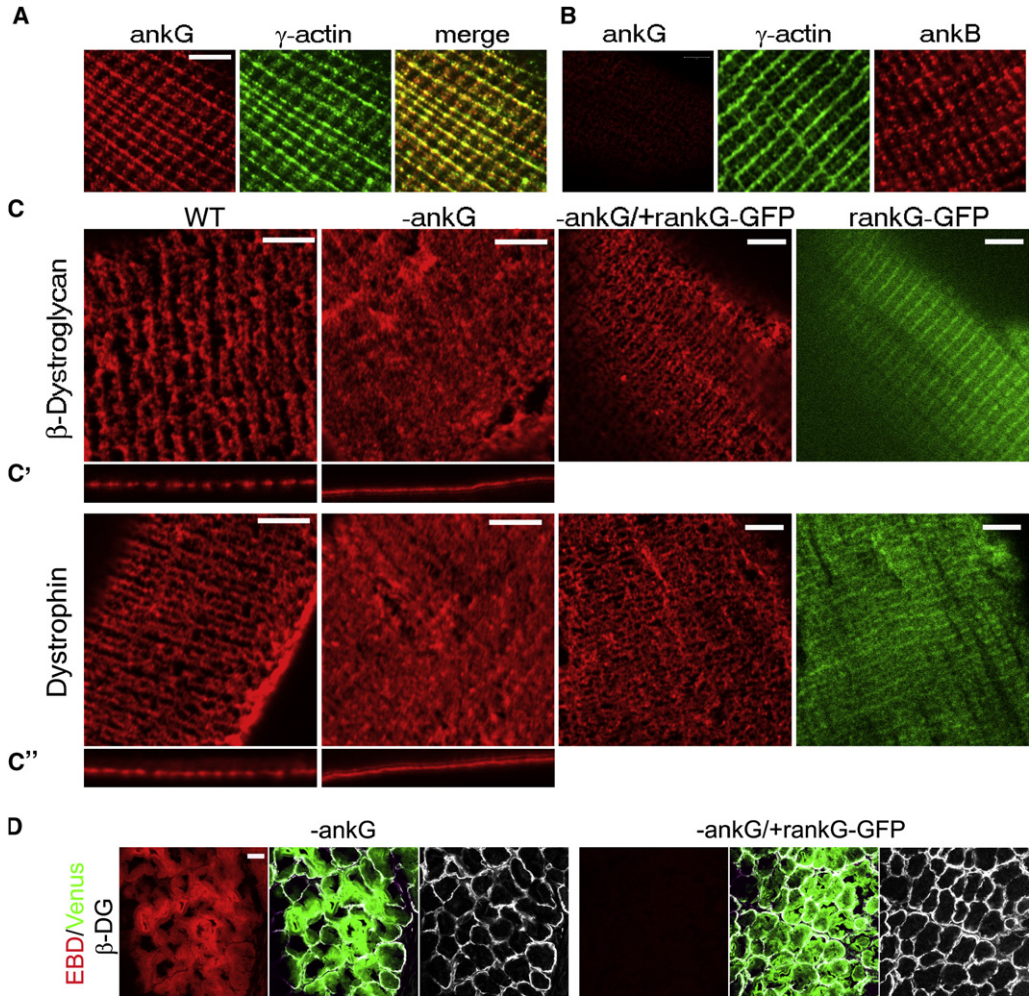

Figure 2. AnkG Is Required for Restriction of Dystrophin and β-DG at Costameres and for Sarcolemmal Integrity

(A) AnkG colocalizes with γ-actin in costameres of isolated muscle fibers.

(B) AnkG-siRNA knocks down ankG but not ankB or γ-actin.

(C) Loss of costamere localization of dystrophin and β-DG in the sarcolemma of ankG-depleted muscle fibers, but not fibers cotransfected with plasmids encoding both ankG siRNA and GFP-tagged rat ankG resistant to mouse siRNA (rankG-GFP).

(C′ and C″) Optical sections through the sarcolemma of β-DG and dystrophin-stained fibers. Punctate costameres are replaced by continuous sarcolemmal labeling following ankG depletion (N = 3, n = 7).

(D) EBD (red) penetrates specifically ankG-depleted (-ankG) fibers but not those cotransfected with plasmids encoding both ankG siRNA and GFP-tagged rat ankG resistant to mouse siRNA (rankG-GFP) in mice subjected to exercise. Sarcolemma marked in white by βDG staining. N = 3, n = 3 mice per experiment per genotype (total 27 mice). Bars = 5 μm.

AnkG Retains β-DG and Dystrophin at Costameres

Ankyrins-B and -G are closely related in their membrane-binding and spectrin-binding domains and are coexpressed in cardiomyocytes as well as other cells. However, these ankyrins are highly divergent in their C-terminal domains, which function in intramolecular regulation, and have distinct cellular roles (Abdi et al., 2006). AnkG is localized in costameres and colocalizes with γ-actin (Figure 2A) (Hopitzan et al., 2005; Williams et al., 2001). We used the same siRNA knockdown strategy as for ankB to test whether ankG has a role in localization of β-DG and dystrophin. Figure 2B demonstrates effective ankG knockdown while ankB and γ-actin remain intact. Localization of ankB and ankG in costameres thus is independent of each other's expression. As shown in Figure 2C, in the absence of ankG, β-DG and dystrophin are present in the sarcolemma. However, their costamere pattern is lost, and they are diffusely distributed throughout the sarcolemma. An optical section along the longitudinal axis through the sarcolemma shows that instead of the punctate costamere pattern in normal fibers, the staining patterns of β-DG and dystrophin in ankG-depleted fibers are continuous (Figures 2C′ and 2C″, respectively). A costamere pattern of dystrophin and β-DG is preserved by introducing GFP-tagged rat ankyrin-G that is resistant to the mouse-specific ankG siRNA (Figure 2C).

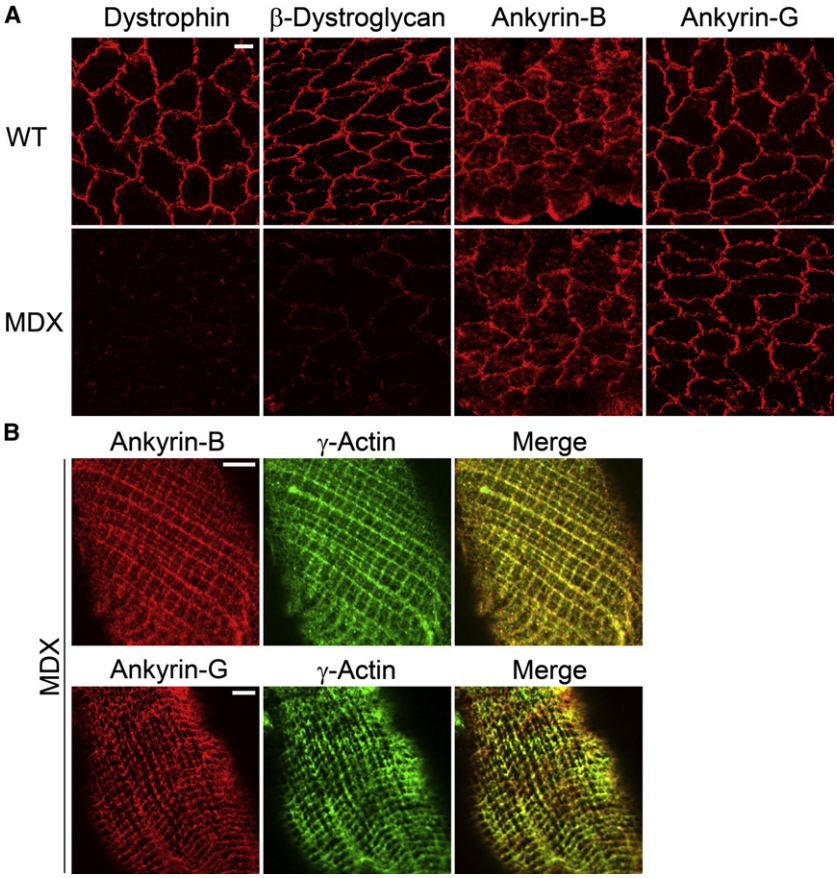

Figure 3. Costamere Localization of AnkB and AnkG Is Independent of Dystrophin and β-DG

(A) Cross-sections of 4- to 6-week-old wild-type and mdx TA muscles were stained and compared for presence of dystrophin (antibody also crossreacts with utrophin), β-DG, ankB, and ankG using identical image acquisition settings. While dystrophin/utrophin and β-DG show no or residual sarcolemmal staining, both ankyrins label mdx sarcolemma as they do in wild-type muscle (bar = 20 μm).

(B) Grazing optical sections of isolated mdx fibers verify not only that ankyrins-B and -G are expressed in mdx sarcolemma, but also that they are properly localized at costameres, colocalizing with γ-actin (bar = 5 μm).

We repeated the exercise experiment with mice transfected with ankG siRNA, both with and without rescue with rat ankG-GFP (same experimental procedure as described above for ankB-siRNA). AnkG-depleted muscle fibers exhibit uptake of EBD when subjected to physical exercise and can be rescued by introducing rat ankG-GFP (Figure 2D). Thus muscle fiber integrity not only depends on the presence of β-DG and dystrophin in the sarcolemma but may also require that these proteins are specifically retained at costameres by ankG.

Ankyrins Act upstream of Dystrophin

We next asked whether localization of ankyrins-B and-G at costameres is dependent on dystrophin and β-DG. We evaluated wild-type and dystrophin-deficient *mdx* mice that were 4 to 6 weeks old. *Mdx* mice at this age have previously been reported to express only residual levels of utrophin and β-DG in skeletal muscle (Roma et al., 2004). In Figure 3A we show in cross-sections that dystrophin/utrophin (the dystrophin antibody used crossreacts with utrophin) are absent and β-DG is missing from the *mdx* sarcolemma. However, ankyrins-B and -G show sarcolemmal labeling in cross-sections that is indistinguishable from wild-type muscles. Figure 3B further demonstrates that ankyrins-B and -G properly localize at costameres in isolated fibers from *mdx* muscle and colocalize with γ-actin. AnkB and ankG therefore localize at costameres independently from dystrophin and dystroglycan.

Loss of Dystrophin and β-DG in AnkB-Depleted Postnatal NMJs

The neonatal neuromuscular junction develops extensive branches and in-foldings during the first few weeks after birth (Sanes and Lichtman, 1999). β-DG, dystrophin, and utrophin are enriched in the postsynaptic membrane of the NMJ (Grady et al., 2000; Sanes and Lichtman, 1999) and are required for postnatal differentiation of the NMJ (Cote et al., 1999; Grady et al., 2000). In Figure 4A we show that ankB localizes at adult NMJs, where it forms a "foot print" layer underneath the NMJ boundaries marked by fluorescently labeled α-bungarotoxin (αBgt). We therefore evaluated the role of ankB in localization of β-DG, dystrophin, and utrophin at neonatal and adult NMJs.

In ankB$^{-/-}$ neonatal NMJs, β-DG is still present at the NMJ despite the absence of staining for β-DG in the rest of the sarcolemma (Figure S2A) and lack of NMJ staining for dystrophin/utrophin (Figure S2B). Evidently, accumulation of β-DG at the neonatal NMJ does not require either ankB or dystrophin/utrophin. At this neonatal stage, the general morphology of the neonatal NMJ, as revealed by αBgt, is identical in wild-type and ankB$^{-/-}$ mice (determined by 3D reconstruction of Z series of NMJs taken on isolated fibers, not shown).

We next assessed localization of β-DG and dystrophin/utrophin at the NMJ in muscles of adult mice treated with ankB siRNA, as described above. The NMJ of ankB-siRNA-treated muscles exhibit dramatic loss of both β-DG (Figure 4B) and dystrophin/utrophin (Figure 4C). Moreover, the typical pattern of nAChRs marked by αBgt is replaced by an amorphous blur (Figures 4B and 4C) resembling the diminished NMJs observed in dystroglycan-deficient mice (Cote et al., 1999). Three-dimensional images show the dramatic deformation and neonatal appearance of the adult NMJ morphology following ankB depletion (Figure 4D). We were unable to deplete ankG from the NMJ using the same protocol that was effective in knocking down ankG at costameres. AnkG apparently is too stable at the NMJ for this type of siRNA-based knockdown. In summary, in the absence of ankB, dystrophin, utrophin, and β-DG are missing from the

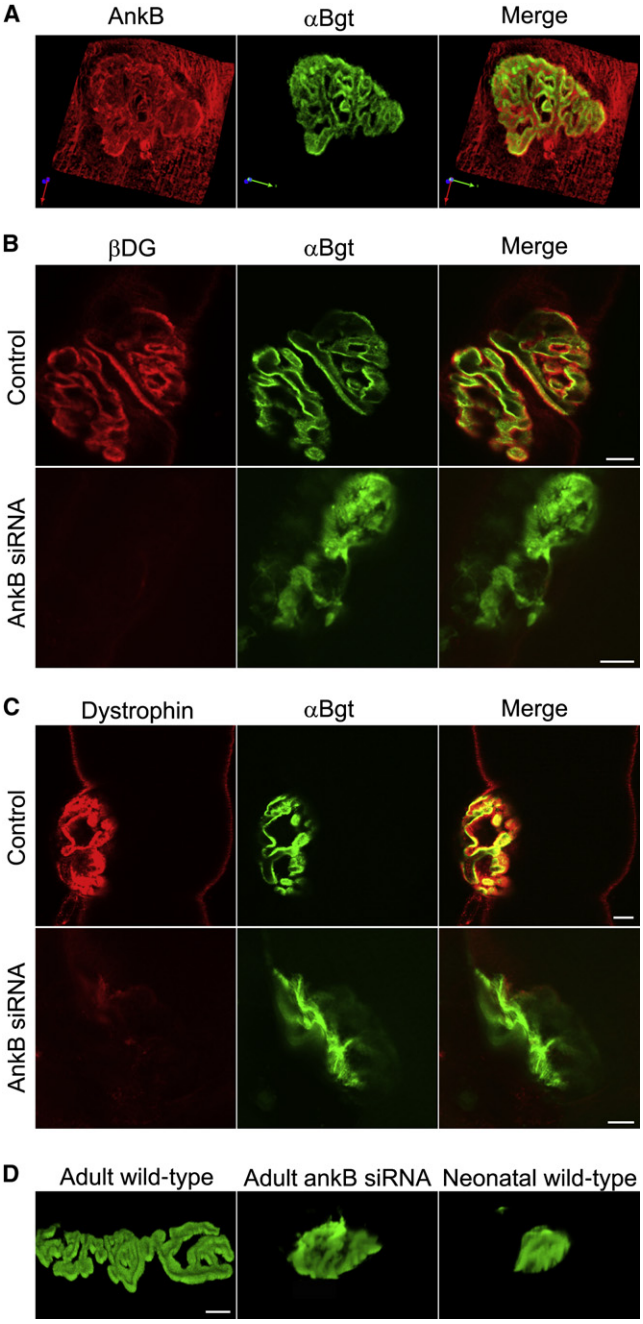

Figure 4. Loss of Dystrophin and β-DG in AnkB-Depleted Postnatal NMJs

(A) AnkB (red) is localized at the postnatal NMJ marked by αBgt (green); 3D image is shown (Experimental Procedures).

(B and C) β-DG (B, red) and dystrophin/utrophin (C, red) are absent from the NMJ in ankB-siRNA-treated fibers. αBgt (green) labels AChRs. Note disrupted morphology of the mature NMJ.

(D) Three-dimensional images of αBgt-labeled NMJs of wild-type adult (left), ankB-depleted adult (middle), and neonatal wild-type muscle. All images in (D) are to scale. Bars = 5 μm.

postsynaptic NMJ membrane, and the whole structure reverts to a neonatal morphology.

Dystrophin-Dp71 Binds AnkB and AnkG while β-DG Binds AnkG

Next we addressed potential protein interactions of ankyrins-B and -G underlying localization of dystrophin and β-DG. Neither ankyrin was effectively solubilized following published protocols for DGC isolation (data not shown), which explains why no ankyrin was noted as stoichiometric components of the DGC isolated from skeletal muscle (Campbell and Kahl, 1989). Identification of ankyrin partners by coimmunoprecipitation therefore was not feasible. We adopted a candidate gene approach and performed in vitro binding assays evaluating interactions of ankB and ankG with either dystrophin-Dp71 or the cytoplasmic tail of β-DG, both fused to GST and immobilized on glutathione-sepharose beads. Dystrophin-Dp71 is a naturally occurring dystrophin variant lacking the first 62 exons encoding the actin-binding and rod domain but retaining the cysteine-rich/C-terminal domain as well as activity in targeting the DGC to costameres (Cox et al., 1994).

Both ankB and ankG bind directly to GST-dystrophin-Dp71 (Figure 5A). Moreover, ankG also associates with the GST-tagged cytoplasmic tail of β-DG, although ankB does not (Figure 5A). Controls included no binding to GST alone and no binding of Dp71 and the cytoplasmic tail of β-DG to ankyrins denatured by diethyl pyrocarbonate (data not shown). To identify the ankyrin-binding region in dystrophin-Dp71, we tested the N′ and the C′ termini halves of Dp71 for ankyrin binding in vitro. The junction between amino acids Q359/L360 of Dp71 was chosen based on a prediction of a transition point between a structured N-terminal region and an unstructured C-terminal region (http://iupred.enzim.hu/Theory.html; see Experimental Procedures). In Figure 5B we show that ankB binds only the N-terminal portion of Dp71, corresponding to exons 63–72 of the full-length dystrophin gene (Dp427) and containing the cysteine-rich domain. The same result was obtained for ankG (data not shown).

We next mapped the ankG-binding site of β-DG. We tested a series of deletion mutants in the cytoplasmic domain of β-DG (DGcyto), described in Figure 5C, for binding to ankG in vitro. As described in Figure 5D, a 33 amino acid membrane-proximal region, DGcytoΔ810-895, bound ankG to the same extent as the whole cytoplasmic domain. A deletion mutant, DGcytoΔ776-809, lacking the membrane-proximal residues, did not bind ankG. Point mutations in the proximal region of the full-length cytoplasmic domain further defined the binding site. Two mutations, IIF798AAA and DE802AA, show loss of binding to ankG down to almost background levels, while a third mutant, DD805AA, shows a major reduction. Other mutants exhibited normal binding. The ankG-binding site of β-DG thus is localized to 10 amino acids located adjacent to the membrane-bilayer-spanning region. The ankG-binding site is distinct from the reported binding site for dystrophin located at the C terminus of β-DG (Jung et al., 1995).

We next evaluated whether β-DG could bind to Dp71 in the presence of ankB or ankG. Assays with GST-tagged Dp71 revealed no effect of either ankB or ankG on binding of the β-DG cytoplasmic domain (GST removed by procission protease)

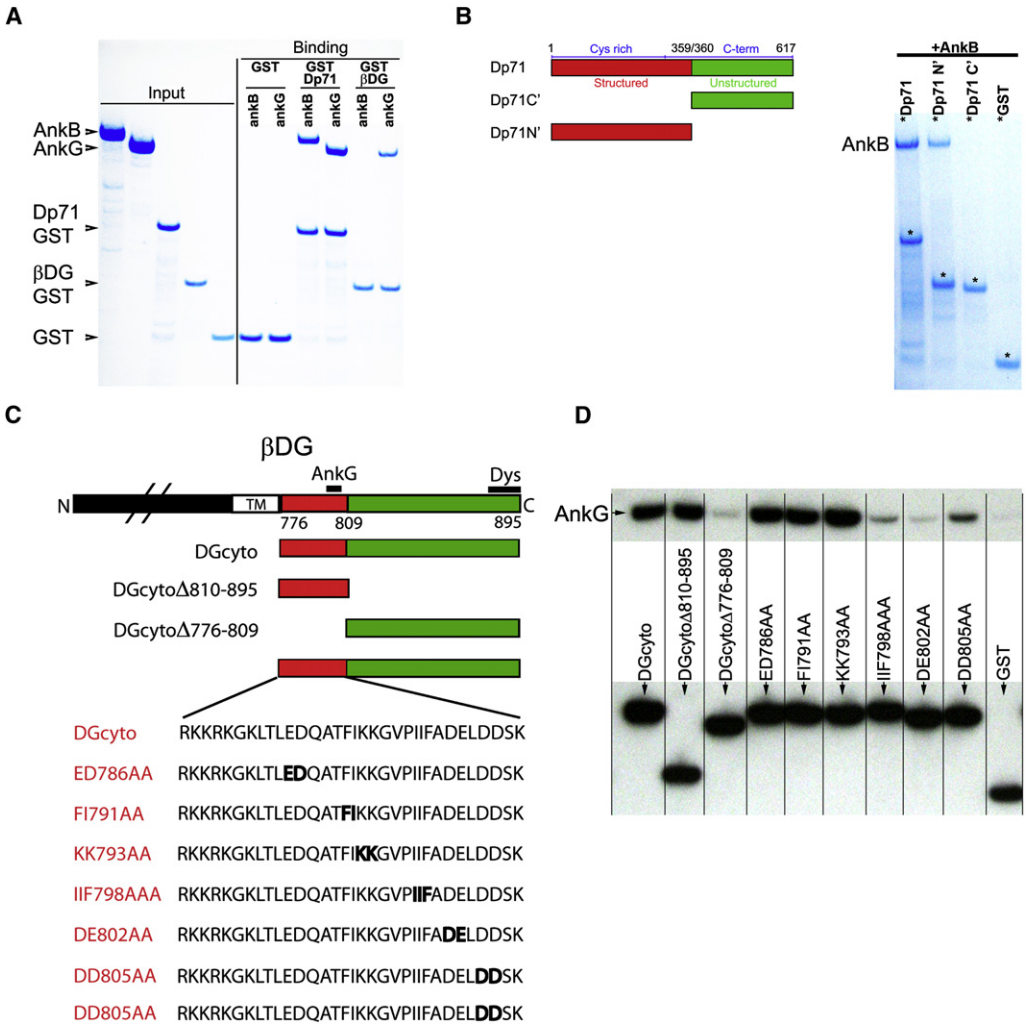

Figure 5. Dp71 Binds AnkB and AnkG, and β-DG Binds AnkG In Vitro

(A) Coomassie blue gel shows (left) input of purified proteins. On right, binding assays show that immobilized GST-Dp71 binds ankyrins-B and-G. GST-β-DG cytoplasmic domain binds only ankG.

(B) Ankyrin-B binds the structured N′-terminal half of dystrophin-Dp71. Junction between the N′-terminal Dp71 section (Dp71N′, red) and the C′-terminal section (Dp71C′, green) was designed based on a prediction of transition from a structured to an unstructured section of Dp71 (see Results for details). Purified GST-fusion proteins of either full-length Dp71, Dp71N′, or Dp71C′ were tested for binding to ankyrin-B (shown) and ankyrin-G (not shown). Coomassie blue gel shows that only the N′-terminal region of Dp71 binds ankyrin-B.

(C and D) GST-tagged β-DG cytoplasmic domain binds ankG in a juxta-membrane region. (C) Truncations and mutations tested for identification of ankG-binding region. (D) AnkG binding to constructs described in (C). All constructs are His tagged and visualized using anti-His antibody. All bands are from one original immunoblot but cropped for clearer presentation.

(Figure S3). Therefore, dystrophin can form a ternary complex with β-DG and either ankG or ankB.

A Becker MD Mutation Impairs AnkB Binding and Localization of Dp71

We identified in the Leiden Muscular Dystrophy (MD) database nine missense mutations located in the N-terminal ankyrin-binding domain of dystrophin-Dp71 that have been reported to cause either Duchenne or Becker muscular dystrophies in human patients (Aartsma-Rus et al., 2006). We introduced eight of these mutations into full-length dystrophin-Dp71 and assessed their effect on binding to ankyrins-B and -G. The mutation Asp3335Asn, which was reported in Becker MD (equivalent to Asp267Asn in dystrophin-Dp71), reduces the binding of dystrophin-Dp71 to both ankB (Figures 6A and 6B) and ankG (data not shown) by 50%.

We next evaluated the effect of the Asp267Asn mutation of Dp71 on its localization in skeletal muscle. We transfected TA muscles in vivo with either an HA-tagged Dp71 or the HA-tagged Dp71 Asp267Asn. Both wild-type and mutant Dp71 were expressed at equivalent levels based on immunoblots of transfected muscle using HA antibody (data not shown). Transfected fibers were identified by cotransfection with a Venus plasmid in a 1:10 ratio in favor of Dp71, and only Venus-expressing muscle

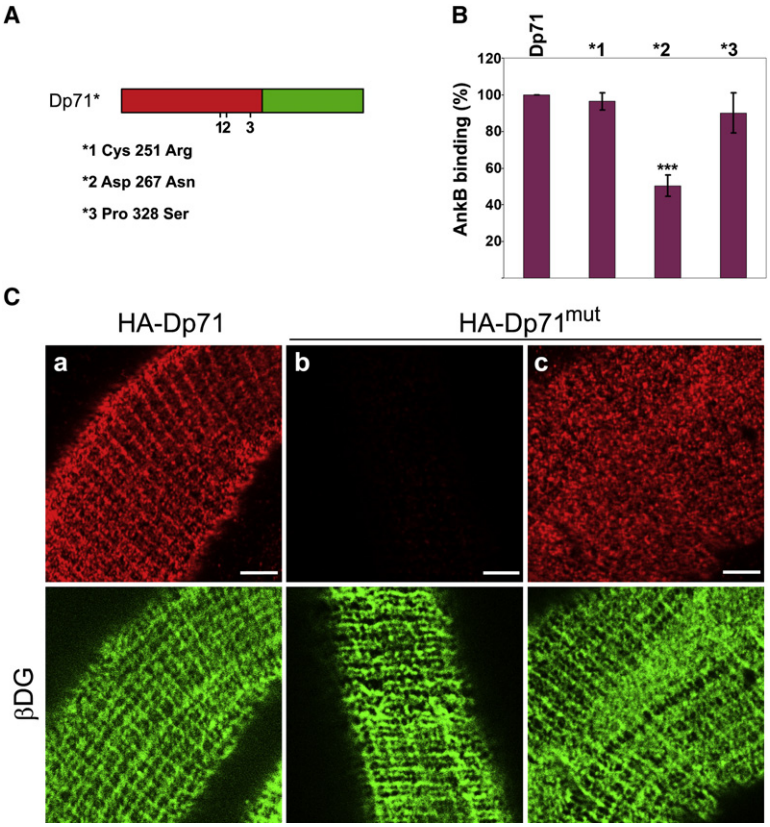

Figure 6. A Becker MD Mutation (Asp267Asn) in Dystrophin Dp71 Impairs Ankyrin Binding and Dystrophin Dp71 Localization in Skeletal Muscle

(A) Mutations *1, *2, *3 in positions 251, 267, 328 of Dp71 correspond to human MD mutations Cys3319Arg (T9955C), Asp3335Asn (G10003A), Pro3396Ser (C10186T) in full-length dystrophin Dp427, respectively.

(B) GST-Dp71 Asp267Asn (Dp71^{mut}) has 50% reduction in binding to ankB (shown) and ankG (not shown) (Experimental Procedures). Molar ratios of ankB bound to GST-Dp71 proteins were determined in six separate experiments presented in the bar graph (***p = 0, Student's t test). Bars, from left to right: mutation *1: 96.3 ± 4.6; mutation *2: 50.3 ± 5.9; mutation *3: 90.0 ± 10.9.

(C) Mislocalization of Dp71^{mut} in transfected muscle fibers. (Ca) HA-tagged wild-type Dp71 (detected with HA antibody) (red) localizes to costameres overlapping the endogenous β-DG (green). (Cb) Most fibers transfected with HA-Dp71^{mut} (red) (*2 panels A and B) show no membrane localization. (Cc) In a minority of fibers, HA-Dp71^{mut} does localize to the sarcolemma but fails to localize at costameres (marked by endogenous β-DG). Venus expression identified the transfected fibers. Scale bars, 5 μm.

fibers were examined. In Figure 6Ca, we demonstrate using antibody against the HA epitope that HA-tagged wild-type Dp71 does target to costameres. In contrast, mutated Dp71 (Dp71^{mut}) exhibited two abnormal localization phenotypes. Approximately 70%–80% of the transfected fibers did not show any sarcolemmal labeling for HA-Dp71^{mut} (Figure 6Cb). In the minority of fibers that did show sarcolemmal staining, the Dp71^{mut} distribution is amorphous, while endogenous β-DG in the same fibers shows normal costamere localization (Figure 6Cc). These results are consistent with a requirement for Dp71 interaction with ankB for its localization at the sarcolemma and with ankG for retention of Dp71 at costameres.

Costamere-Associated Microtubules Require AnkB

Muscle contains a subset of microtubules associated with the sarcolemma and aligned with costameres (Boudriau et al., 1993). Moreover, ankB binds to microtubules in vitro (Bennett and Davis, 1981; Davis and Bennett, 1984). We therefore wondered if costamere-associated microtubules depend on ankB. Colabeling for ankB and α-tubulin in control fibers reveals that α-tubulin overlaps at costameres with ankB (Figure 7A). Strikingly, costamere-associated microtubules disappear in ankB-depleted fibers, while longitudinal microtubules are unaffected (Figure 7B). Three-dimensional images further resolve the costamere-associated subset of microtubules, which form transverse rings exclusively at the circumference of the fiber (Figure 7A′). Deeper intracellular microtubules are parallel to the fiber axis, running along the myofibrils (Figure 7A″). AnkB-depleted fibers entirely lose the costamere-associated microtubules while retaining intracellular longitudinal microtubules (Figures 7B′ and 7B″). In contrast to ankB, ankG-depleted fibers exhibit normal staining of both microtubule populations (data not shown). A network of microtubules surrounding mature NMJs is closely associated with the plasma membrane (Figure S4A). In Figure 4 we show that ankyrin-B underlies the NMJ. In Figure S4B we show that the NMJ-associated microtubules are lost in ankB-siRNA treated fibers, while in the same fiber the deeper longitudinal microtubules remain (Figure 7B′). These experiments demonstrate that ankB is required for formation and/or stability of a special subset of microtubules associated with costameres and neuromuscular junctions.

Dynactin-4 Binds AnkB and Requires AnkB to Localize at Costameres

A yeast two-hybrid screen using a heart cDNA library identified dynactin-4 (dyn4/p62) as a potential ankB-interacting protein (data not shown). Dyn4/p62 is associated with the pointed end of the dynactin complex and has been predicted to connect dynein to membrane proteins (Schroer, 2004). To test whether ankB and dyn4/p62 associate in solution, we expressed dyn4/p62 as a GST-fusion protein and performed binding assays with purified ankB. In Figure 7C we show that ankB binds to GST-dyn4/p62 with high affinity with a dissociation constant (K_D) of 50 nM. We generated a specific dyn4/p62 antibody to visualize dyn4/p62 in muscle fibers. In Figure 7D, we show that in adult muscle fibers, dyn4/p62 is localized in a punctate manner along costamere lines overlapping with costamere ankB (71% overlap). p50 and p150^{Glued}, two additional subunits of the dynactin complex, also show identical costamere-associated punctate staining as dyn4/p62 (data not shown). Strikingly, costamere-associated dyn4/p62 disappears in ankB-depleted fibers (Figure 7D). In summary, ankB is required for the costamere localization of

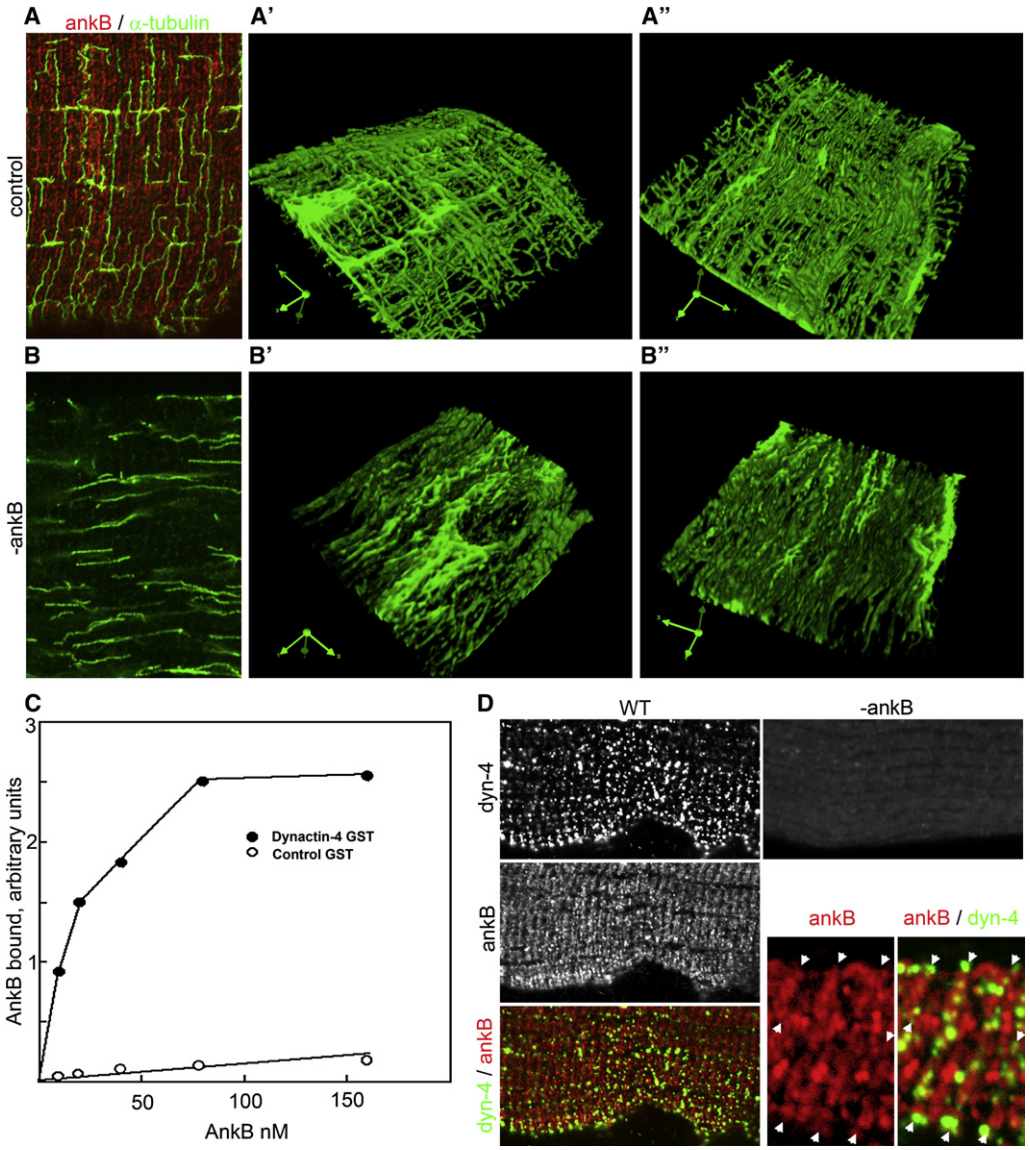

Figure 7. Costamere-Associated Microtubules and dyn4 Disappear in AnkB-Depleted Skeletal Muscle

(A) Tubulin staining (green) overlaps with costamere ankB (red).

(B) In ankB-depleted fibers transverse microtubules disappear while longitudinal microtubules remain.

(A′ and B′) Three-dimensional images show transverse costamere-associated microtubules at the circumference of the fiber of wild-type (A′) that are missing in ankB-depleted fibers (B′).

(A″ and B″) Same 3D images rotated 180° along their long axis to reveal deeper longitudinal microtubules, parallel to the axis of the fiber present in both wild-type and ankB-depleted fibers.

(C) Curve of purified proteins ankB and GST-dyn4 binding in vitro demonstrate a K_D of 50 nM.

(D) In control fibers dyn4 demonstrates a punctate distribution along costameres, marked by ankB (both low and high magnification). In ankB-depleted fibers dyn4 staining is lost.

dyn4/p62 and is a candidate to mediate interactions of dyn4/p62 with membrane cargo as well as to stabilize the fast-growing ends of specialized microtubules associated with costameres.

DISCUSSION

We present evidence for an ankyrin-based pathway required for sarcolemmal localization of dystrophin and β-DG and for resistance of skeletal muscle to exercise-induced injury as well as maintenance of the postnatal NMJ. The dystrophin cysteine-rich domain binds ankB and ankG, while the cytoplasmic domain of β-DG binds to ankG through residues distinct from its dystrophin-binding site. Moreover, dystrophin and β-DG require ankB for their sarcolemmal localization and require ankG for their retention at costameres. Skeletal muscle depleted of either ankB or ankG by siRNA exhibited increased fragility following

exercise, and this was prevented by cotransfection of siRNA-resistant versions of the corresponding ankyrins. The NMJ also requires ankB for localization of dystrophin/utrophin and β-DG as well as for maintenance of its postnatal morphology. Interestingly, ankB depletion and dystroglycan depletion both had similar effects on the postnatal NMJ (Cote et al., 1999). We identified in the Leiden MD database a Becker muscular dystrophy mutation that reduces binding of dystrophin-Dp71 to ankB and ankG and impairs sarcolemmal localization of dystrophin-Dp71. These observations establish that ankB and ankG together are essential for localization and function of dystrophin and β-DG in skeletal muscle.

Dystrophin and utrophin complexes have been implicated in postnatal maturation of the NMJ, which develops elaborate branching in the weeks after birth. One explanation for localization of DGC proteins at the NMJ has been regulated transcription at the subsynaptic nuclei (Sanes and Lichtman, 1999; Schaeffer et al., 2001) and postsynaptic localization of specific mRNAs (Joe and Chakkalakal, 2003). Our results indicate that despite the close proximity between the NMJ and the transcription\-translation machinery, an ankB-based pathway is required for accumulation of β-DG and dystrophin/utrophin at the postnatal NMJ. Moreover, in the absence of ankB, the postnatal NMJ resembled an immature neonatal-stage structure. Interestingly, neonatal NMJs were morphologically normal in ankB null skeletal muscle. Thus ankB, dystrophin/utrophin, and β-DG all are required for the maturation of the NMJ rather than in earlier developmental stages.

β-DG is localized in a polarized pattern in nonmuscle tissues including brain astrocytes where it is targeted to the glia limitans at the cerebral spinal fluid-brain interface (Moore et al., 2002). Interestingly, ankyrin-B$^{-/-}$ mice have CNS defects, including degeneration of the optic nerve and aplasia of the corpus callosum (Scotland et al., 1998), that are similar to those observed in muscle-eye-brain disease, which is associated with defective glycosylation of dystroglycan (Brancaccio, 2005; Moore et al., 2002). We found that the continuous linear compact staining characteristic of β-DG in glia limitans in wild-type sections is replaced in ankyrin-B$^{-/-}$ brain by diffuse staining throughout the astrocytes (Figures S5A and S5B). Loss of localization of β-DG to astrocyte endfeet is accompanied by infiltration of cell bodies into cortical layer 1, where cell bodies are normally sparse (Figure S5C). Moreover GFAP labeling reveals astrocytes throughout the depth of the cortex, which are missing in wild-type brain (Figure S5D). A similar marked increase in cortical GFAP staining was described for brain β-DG knockout mice (Moore et al., 2002). AnkB therefore is required for normal localization of β-DG in astrocyte glia limitans as well as muscle costameres and NMJ.

We next addressed how ankyrins function as adaptors for dystrophin and β-DG. Steady-state levels of dystrophin and β-DG are maintained in ankB null neonatal skeletal muscle as well as adult muscle depleted of ankB by siRNA. We did not measure turnover of these proteins, so it is possible that increased degradation is balanced by increased synthesis. However, a parsimonious interpretation is that the principal defect in ankB-depleted muscle is in either delivery and/or retention of dystrophin and β-DG at their sarcolemmal sites. We could not identify dystrophin in ankB-depleted muscle by immunofluorescence. We did find that β-DG accumulates in a juxta-TGN compartment in ankB-depleted muscle. Biochemical data indicate that dystrophin can associate with both ankB and β-DG. However, we were not able to identify colocalization of these proteins in an intracellular compartment. A major caveat in interpreting these negative findings is that interactions may either be transient and/or not preserved under our fixation conditions.

We observed a subpopulation of microtubules associated with costameres and the NMJ that disappeared in ankB-depleted muscle. Interestingly, ankyrin-2 in *Drosophila* also has been reported to stabilize presynaptic microtubules (Pielage et al., 2008; Koch et al., 2008). These observations suggest that ankB and ankyrin-2 determine long-range organization of microtubules in addition to their established interactions with membrane-associated proteins. Dystrophin-deficient *mdx* muscle fibers also exhibit loss of costamere-associated microtubules (Percival et al., 2007). Thus ankB and dystrophin, perhaps as a complex, may both contribute to costamere-associated microtubules.

A clue as to how ankB could regulate microtubules comes from our finding that ankB is a partner for dynactin-4 (dyn4/p62). Dyn4/p62 is localized at the pointed end of dynactin arp1 filaments and has been proposed to link the microtubule motor dynein to membrane cargo (Eckley et al., 1999; Schroer, 2004). Dynactin is required for dynein function and promotes bidirectional transport along microtubules (Ross et al., 2006; Schroer, 2004). Dynactin also functions in stabilizing the fast-growing ends of microtubules at the plasma membrane (Shaw et al., 2007). The coordinated loss of costamere-associated dyn4/p62 and microtubules in ankB-depleted fibers suggest ankB may be a membrane receptor for dynactin, which in turn stabilizes microtubules.

Interestingly, β-spectrins also associate with dynactin through arp1 (Holleran et al., 1996, 2001; Muresan et al., 2001). β-spectrins are well-established partners of ankyrins (Bennett and Baines, 2001; Kizhatil et al., 2007) and also are components of skeletal muscle costameres (Craig and Pardo, 1983; Nelson and Lazarides, 1984; Vybiral et al., 1992). Moreover, β-spectrin knockout in *C. elegans* causes major muscle breakdown (Moorthy et al., 2000). It is possible that ankB and β-2-spectrin collaborate in coordinating dynactin interactions with membrane cargoes and/or docking sites for the fast-growing ends of microtubules.

We hypothesize that ankB-dependent microtubules associated with costameres and the NMJ could provide the transport route for newly synthesized β-DG, perhaps associated with dystrophin, exiting from the trans-Golgi network. A similar direct microtubule-based targeting has recently been suggested for delivery of gap junction subunits to cadherin-based adhesion sites in cardiomyocytes, where the ends of microtubules are captured through interactions with the dynactin-complex subunit p150^{glued} (Shaw et al., 2007). This hypothesis immediately raises questions for future studies regarding how ankB-based cargo is sorted to the appropriate microtubule track, whether ankB accompanies cargo during transport, and, most importantly, how ankB itself is targeted to costameres.

In summary, we present the evidence for a novel ankyrin-dependent pathway for localizing the DGC to costameres and

the postnatal NMJ in skeletal muscle and to the glia limitans in brain astrocytes. We propose a mechanism where an ankB-dystrophin interaction is critical for targeting dystroglycan to the sarcolemma by transport along costamere-associated microtubules that are stabilized by ankB through its binding to dyn4/p62 of the dynactin complex. We also show that ankG binds to dystrophin and β-DG, and that ankG is required for retention of these proteins at costameres. Further, we speculate based on the phenotype of the ankB null mouse that a class of muscle-eye-brain diseases in humans may result from failure in this pathway for dystroglycan localization.

EXPERIMENTAL PROCEDURES

Molecular Biology

See detailed description of plasmids encoding siRNA, β-DG, dystrophin Dp71, dynactin-4, ankB, and ankG as well as mutagenesis in the Supplemental Data.

Antibodies

Affinity-purified antibodies against ankB and ankG were previously described (Kizhatil and Bennett, 2004; Scotland et al., 1998, respectively). AnkB antibody was pre-absorbed on a purified ankG column and ankG antibody was pre-absorbed on a purified ankB column to eliminate crossreactivity. β-DG rabbit antibody (AP83) was a generous gift from Dr. Kevin P. Campbell, University of Iowa. Affinity-purified rabbit and goat antibodies were prepared against the β-DG cytoplasmic domain. An affinity-purified rabbit antibody was raised against dynactin-4. For a list of all antibodies, see the Supplemental Data.

Muscle Tissue Preparations

AnkB knockout mice and their genotyping were described previously (Scotland et al., 1998). Muscles were snap-frozen in OCT in liquid nitrogen chilled iso-pentane. Cross sections were fixed in 2% PFA, blocked (PBS, 4% BSA, 0.2% Tween 20), and permeabilized (blocking solution with 0.1% Triton X-100). Sections were incubated at 4°C overnight in primary antibodies and 2 hr with secondary antibodies.

In Vivo Transfections and Fiber Preparation

Briefly, tibialis anterior (TA) muscles of 3- to 4-week-old C57B\6 mice were injected with 15U hyaluronidase (30 μl in saline) (Worthington; Lakewood, NJ, USA) 2 hr prior to transfection (McMahon et al., 2001). TA muscles were then injected with either siRNA or cDNA constructs depending on the experiment and electroporated. For a detailed description, see the Supplemental Data.

Exercise and EBD Experiments

Mice were treadmill exercised (LE 8706 Letica; Barcelona, Spain) twice a day for 3 days (30 min, 10° uphill angle, 30 cm\s). Twelve hours before final exercise, EBD (sigma; St. Louis, MO, USA) was injected IP (1 mg per 0.1 ml per 10 g body weight). After the last running session TA muscles were removed for sectioning.

Microscopy and Imaging

Microscopy was performed with a Zeiss LSM510 Meta confocal microscope. Three-dimensional reconstructions of Z stack images and voxel overlap measurements were done with Velocity software (Improvision; Coventry, England). EBD uptake in muscle sections was visualized using a 568 channel. Statistical analysis was done with Origin 6.1 software (OriginLab; Northampton, MA, USA).

Binding Assays and Immunoblots

See detailed description in Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and five figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01309-3.

ACKNOWLEDGMENTS

We thank K. Kizhatil for the plasmid-siRNA constructs, K.P. Campbell for his gift of β-DG antibody, and L. Davis, J. Hostettler, and J. Hoffman for technical assistance. We also acknowledge P. Mohler and K. Abdi for performing the yeast two-hybrid screen that revealed ankB binding by dyn4/p62. This study was funded in part by the Muscular Dystrophy Association. Author contributions: G.A.: Figures 1–7, Figures S1–S5; J.Q.D.: Figures 5 and 6, Figure S3; P.B.S.: Figure 5; G.A. and V.B. wrote the paper.

Received: June 27, 2007
Revised: August 11, 2008
Accepted: October 7, 2008
Published: December 24, 2008

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