Deficiency of α-actinin-3 is associated with increased susceptibility to contraction-induced damage and skeletal muscle remodeling

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Sarcomeric α-actinins (α-actinin-2 and -3) are a major component of the Z-disk in skeletal muscle, where they crosslink actin and other structural proteins to maintain an ordered myofibrillar array. Homozygosity for the common null polymorphism (R577X) in ACTN3 results in the absence of fast fiber-specific α-actinin-3 in ~20% of the general population. α-Actinin-3 deficiency is associated with decreased force generation and is detrimental to sprint and power performance in elite athletes, suggesting that α-actinin-3 is necessary for optimal forceful repetitive muscle contractions. Since Z-disks are the structures most vulnerable to eccentric damage, we sought to examine the effects of α-actinin-3 deficiency on sarcomeric integrity. Actn3 knockout mouse muscle showed significantly increased force deficits following eccentric contraction at 30% stretch, suggesting that α-actinin-3 deficiency results in an increased susceptibility to muscle damage at the extremes of muscle performance. Microarray analyses demonstrated an increase in muscle remodeling genes, which we confirmed at the protein level. The loss of α-actinin-3 and up-regulation of α-actinin-2 resulted in no significant changes to the total pool of sarcomeric α-actinins, suggesting that alterations in fast fiber Z-disk properties may be related to differences in functional protein interactions between α-actinin-2 and α-actinin-3. In support of this, we demonstrated that the Z-disk proteins, ZASP, titin and vinculin preferentially bind to α-actinin-2. Thus, the loss of α-actinin-3 changes the overall protein composition of fast fiber Z-disks and alters their elastic properties, providing a mechanistic explanation for the loss of force generation and increased susceptibility to eccentric damage in α-actinin-3-deficient individuals.

INTRODUCTION

α-Actinin-3 (ACTN3) and the highly homologous protein α-actinin-2 are major components of mammalian skeletal muscle Z-disks and function as cross-linkers of actin thin filaments (1). In human muscle, α-actinin-2 is expressed in all muscle fibers, while α-actinin-3 has more specialized expression and is restricted to the fast, glycolytic muscle fibers responsible for rapid force generation (2). Interestingly, ~16% of the global human population is completely α-actinin-3 deficient due to homozygosity for a common null polymorphism in ACTN3 (R577X) (3). Consistent with its role as a structural protein in fast fibers, α-actinin-3 deficiency is detrimental to muscle strength and power generation. In elite athletes, the frequency of the ACTN3 577XX genotype (α-actinin-3 deficiency) is significantly lower in sprint and power athletes (4–8), but is higher in endurance athletes (4,9). ACTN3 genotype also influences normal variation in muscle function in non-athletes, with 577XX individuals showing slower baseline sprint times and lower muscle...
power (10–13). Phenotypic analysis of an Actn3 knockout (KO) mouse model has shown that α-actinin-3 deficiency results in a ‘slowing’ of fast fiber characteristics associated with a significant decrease in grip strength, muscle mass and fast 2B fiber size, an increase in muscle aerobic capacity and slowing of muscle contractile properties (14,15). There has been strong positive selection for the 577X allele in European and Asian populations following modern human migration out of Africa (16). The shifts towards ‘slower’ muscle phenotypes with α-actinin-3 deficiency probably conferred a selective advantage due to more efficient metabolism and resistance to famine, at the expense of optimal skeletal muscle power and force generation in fast muscle fibers.

Our studies to date have focused on the metabolic consequences of α-actinin-3 deficiency, while its impact on skeletal muscle structure and sarcomeric integrity remains to be explored. The stability of the Z-disk and the skeletal muscle cytoskeleton is the result of a complex network of interactions (17), and in vitro studies suggest that the C-terminus of sarcomeric α-actinin plays a crucial role in the maintenance of Z-line integrity and myofibrillar organization (18). This region is the site of binding for a number of important interacting proteins, including the Z-line protein myotilin (19). Myotilin acts in concert with the sarcomeric α-actins to cross link actin and regulate the rigidity and strength of the Z-line (20,21). The sarcomeric α-actins also form complexes with dystrophin (22), the calspars (also known as FATZ/myoz- nin) (23–25), γ-filamin (also known as filamin C and ABP-L) (23,26), the PDZ/LIM domain protein family members ZASP (also known as Cypher and Oracle) (27,28) and ALP (29), and the giant ‘sarcomeric ruler’ titin (30,31). Recent RNAi knock-down studies in Drosophila primary muscle cultures and zasp and α-actinin mutant cultures suggested that titin, ZASP and α-actinin act cooperatively to stabilize Z-disks during muscle stress and are necessary for maintenance of the sarcomere (32). α-Actins are also linked to the intermediate filament network, which links the Z-disks to the plasma membrane (33,34).

Although the sarcomeric α-actins have a major structural role in skeletal muscle, α-actinin-3 deficiency does not result in overt disruption of sarcomere formation and structure, probably due to compensatory up-regulation of α-actinin-2 (16). However, the absence of α-actinin-3 expression does result in differences in skeletal muscle function, including lower muscle strength, suggesting that the compensation by α-actinin-2 is not complete at extremes of performance. This could in part result from altered α-actinin protein interactions at the Z-disk, both in dimerization with itself and with other Z-disk proteins. In human muscles, α-actinin-2 and α-actinin-3 form homo- and heterodimers (35); however, only α-actinin-2 homodimers will be present in α-actinin-3-deficient muscles. We therefore hypothesized that the absence of α-actinin-3 would impact on the protein composition of the Z-disk, and lead to increased susceptibility to contraction-induced muscle damage. In this study, we have used the Actn3 KO mouse model to test this hypothesis. We demonstrate that α-actinin-3 deficiency results in greater force deficits post-eccentric contraction, despite up-regulation of α-actinin-2, and maintenance of the total sarcomeric α-actinin levels. By microarray and western blot (WB), we demonstrate that α-actinin-3 deficiency results in the up-regulation of a specific subset of structural Z-disk proteins associated with the maintenance of sarcomeric integrity. We then show that α-actinin-2 has preferential binding for other Z-disk proteins such as ZASP, vinculin and titin, which likely contributes to the altered Z-disk protein composition and the observed changes in the structural integrity of α-actinin-3-deficient muscle.

RESULTS
Deficiency of α-actinin-3 results in increased force deficits following extreme eccentric contractions
To determine whether α-actinin-3-deficient muscle is more susceptible to damage, we compared the effect of lengthening (eccentric) contractions on wild-type (WT) and Actn3 KO extensor digitorum longus (EDL) muscles. We have previously performed this assay on isolated EDL muscles subjected to eccentric contractions at 20% stretch and did not observe a significant eccentric damage or force deficits in WT or KO muscles (both showed ~2% force deficit) (36). This degree of eccentric contractions results in significant force deficit (58%) in muscle from the mdx mouse model of Duchenne muscular dystrophy (37). However, since we are modeling ‘normal variation’ rather than disease, susceptibility to muscle damage may only become apparent at the extremes of physiological stress. We therefore repeated the assay on isolated EDL muscles from WT and KO mice that were stretched 30% above their optimal length and stimulated to fatigue (Fig. 1 and Supplementary Material, Fig. S6). This degree of eccentric contraction resulted in force deficits of up to 15% in WT muscles. Interestingly, force deficits in KO muscles were over 2.5 times greater than WT (P < 0.0001), with KO muscles producing at least 40% less force after eccentric contractions. These results indicate an alteration in the force transmission properties of the Z-disk in the absence of α-actinin-3 expression, and suggest an increased susceptibility to muscle damage.

Electron microscopy (EM) did not reveal any overt signs of muscle damage (such as Z-line streaming) in either the KO or WT eccentrically contracted muscles (data not shown). While this may be due to EM sampling bias, it is also possible to have a force deficit due to eccentric contraction which results in minimal sarcomeric and Z-disk damage (38). Studies in healthy human (38) and WT mouse (39) muscles required a force deficit of >50% to show Z-line streaming and significant muscle damage by EM; Actn3 KO mice showed ~40% force deficit following eccentric contraction.

Gene expression changes in Actn3 KO muscles
To determine global gene expression changes in skeletal muscle associated with the absence of α-actinin-3, we performed a microarray analysis using WT and KO quadriceps muscles. Using R software, we identified the top 250 genes with the highest fold change between the two genotypes. The top 250 genes were then clustered into gene ontology (GO) groups using DAVID (40,41); clusters with an
enrichment score > 1.3 (which correlates to P-value of 0.05) are shown in Table 1.

Analyses revealed the top GO groups as ‘Z-disk’, ‘response to heat’, ‘skeletal muscle development’, ‘negative regulation of cell communication’, ‘polyamine metabolic process’ and ‘cytoskeleton organization’. Within the Z-disk GO cluster, the genes shown to be altered included γ-filamin (Flnc), myotilin (Myot), PDZ and LIM domain 3 (Pdlim3/Alp), heat shock protein 1 (Hspb1), αB-crystallin (Cryab), LIM domain-binding protein 3 (Ldb3/ZASP) and α-actinin-2 (Actn2). Interestingly, all of these Z-disk genes showed higher expression in KO muscles compared with WT. Two genes that associate with γ-filamin (Ky, Cmya1) were also higher in KO muscle. There was also altered expression of genes associated with myogenesis and myotube formation (Myf6, Actc1, Vgll2, Hrta1, Cnmnb1p, Dkk3, Socs2, Smox, Smst), suggesting that the absence of α-actinin-3 is associated with activation of muscle regeneration pathways.

**Z-disk proteins are more highly expressed but normally localized with α-actinin-3 deficiency**

To determine whether the differences in gene expression between the WT and KO mice translated to alterations in Z-disk protein expression, we assessed the expression of γ-filamin, myotilin, desmin, ALP and ZASP. We did not examine αB-crystallin further as we have previously demonstrated up-regulation of αB-crystallin in KO muscles by proteomic analysis (15). By WB, compared with WT, KO muscles demonstrated marked higher expression of desmin, myotilin, γ-filamin and the larger 78 kDa isoforms of ZASP; there was no difference in the expression of ALP between WT and KO (Fig. 2). However, despite the higher expression in KO muscles, immuno-staining with antibodies against myotilin (Fig. 3A) and desmin (Fig. 3B) on stretched longitudinal muscle sections demonstrated normal localization at the Z-disk. This was confirmed by the overlap in staining pattern with α-actinin-2. The absence of α-actinin-3 at the Z-disk thus results in a higher expression level of other Z-disk proteins but has no effect on their localization at the Z-disk.

We further examined the staining patterns of desmin, myotilin and γ-filamin on transverse sections of frozen WT and KO quadriceps muscles (Fig. 4A) by immunohistochemistry. In WT muscles, desmin and myotilin demonstrate a fiber-type-specific staining pattern with higher cytoplasmic staining in non-type 2B fibers. In contrast, desmin and myotilin are expressed in all muscle fibers in KO muscles, suggesting that higher desmin and myotilin expression in the absence of α-actinin-3 is due to higher expression in 2B fibers. Furthermore, the overall staining intensity for desmin, myotilin and γ-filamin in KO muscles is higher compared with WT, consistent with greater protein expression shown by WB. Desmin and myotilin also formed bright accumulations in some KO muscles. Staining with an antibody against MyHC 2B on consecutive muscle sections revealed that aggregates are co-incident in fast 2B fibers in KO muscles, suggesting that aggregate formation reflects higher expression of these proteins as a consequence of α-actinin-3 deficiency. These protein aggregates were also visible by Gomori-trichrome stains as punctate dark blue accumulations (Fig. 4B). The occurrence of protein aggregates varied between muscles, being particularly abundant in the quadriceps and spinalis, but is rarely present in EDL and tibialis anterior muscles (data not shown). We have previously shown by EM that KO quadriceps muscles contain concentric ring-like structures that contain glycogen particles, co-localized with glycogen phosphorylase (15). These inclusions have myofibrillar origins (42) also stain for myotilin (43); thus, they are likely to represent a composite structure containing accumulations of glycogen and Z-line-associated proteins, both of which are up-regulated in KO muscle. We also observed the presence of distinct purple structures in the interior of some KO muscle fibers that contain protein aggregates (Fig. 4B, arrow). Examination by co-staining myotilin with DAPI revealed these to be internalized nuclei in KO mouse muscles fibers, suggesting that these fibers are undergoing regeneration (Fig. 4C, arrow). At baseline, we observed 4.5% of KO muscle fibers to have internalized nuclei compared with 1.1% in WT.

To investigate how the structural phenotypes may be related, we analyzed WT and KO mouse skeletal muscle during early postnatal development (Fig. 5, Supplementary Material, Figs S1, S2 and S5). We compared WT and KO mouse muscle at Day 1, and 1, 2 and 4 weeks of age with our previously reported 8-week-old mouse data (Fig. 5). We have briefly discussed the timing of the onset of protein up-regulation in the KO mouse model in a recent review (43), but present the data here for the first time. In addition, we generated primary mouse myoblast lines from WT and KO mouse muscles to study α-actinin-3 deficiency in an in vitro model, in the absence of the effect of muscle loading.

Over the developmental time course, we observed an up-regulation of α-actinin-2 at 1 week of age preceding the higher expression of desmin and myotilin in KO mouse muscle at 2 weeks of age (Supplementary Material, Fig. S5), which correlates with the time at which α-actinin-3 becomes readily detectable by WB (15). The presence of cytoplasmic...
showed higher levels of desmin and γ-filamin. This suggests that muscle contraction may be required in inclusions in the KO cell lines (Supplementary Material, Fig. S2). This suggests that the higher expression of this specific subset of -actinin-2 is upstream to -actinin-3 deficiency and likely to be responsible for the higher susceptibility to eccentric damage and the structural phenotypes associated with -actinin-3 deficiency. In the absence of -actinin-3, -actinin-2 is the only sarcomeric -actinin present at the Z-line in KO fast muscle fibers.

Interestingly, although the WT primary mouse myoblast cell lines express -actinin-3, we were unable to detect higher levels of structural muscle proteins or the cytoplasmic inclusions in the KO cell lines (Supplementary Material, Fig. S2). This suggests that muscle contraction may be required for the consequences of -actinin-3 deficiency to manifest.

To further explore the time course and factors influencing the formation of cytoplasmic inclusions, we examined the expression levels of Z-disk proteins -actinin-2, desmin and γ-filamin in a transgenic mouse model over-expressing myotilin (TgWT). At age 10 months, myotilin TgWT muscles showed higher levels of desmin and γ-filamin, but not -actinin-2 (44), suggesting that -actinin-2 is upstream to myotilin, desmin and γ-filamin. However, we found no differences in the levels of expression of these proteins at age 2 months (Supplementary Material, Fig. S3), suggesting that alterations in Z-disk-associated protein expression occurs in response to increasing mechanical or aging stress.

Our findings to this point are consistent with the hypothesis that the higher expression of this specific subset of Z-disk-associated structural muscle proteins is a direct effect of -actinin-3 deficiency and likely to be responsible for the higher susceptibility to eccentric damage and the structural phenotypes associated with -actinin-3 deficiency. In the absence of -actinin-3, -actinin-2 is the only sarcomeric -actinin present at the Z-line in KO fast muscle fibers.

### Table 1. Differentially expressed genes between WT and KO at baseline. The top 250 differentially expressed genes by fold change (as analyzed with Limma) were clustered into GO groups using DAVID. Annotation clusters with enrichment scores >1.3 (as shown in brackets) are listed. Fold-change shows gene expression differences in KO relative to WT

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<th>Illumina ID</th>
<th>Accession</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>P-value</th>
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<td>PDZ and LIM domain 3</td>
<td>2.31 × 10⁻⁴</td>
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<td>Heat shock protein 1</td>
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<tr>
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<td>Crystallin alpha B</td>
<td>1.08 × 10⁻⁴</td>
<td>1.26</td>
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<td>Actn2</td>
<td>Actin alpha 6</td>
<td>2.42 × 10⁻³</td>
<td>1.24</td>
</tr>
</tbody>
</table>

| Response to Heat (2.35) |           |             |           |         |             |
| ILMN_1254802 | NM_013558.2 | Hsp40 | Heat shock protein 1B; heat shock protein 1A; heat shock protein 1-like | 6.33 × 10⁻⁷ | 1.62 |
| ILMN_2697797 | NM_009964.2 | Cryab | Crystallin alpha B | 1.08 × 10⁻⁴ | 1.42 |
| ILMN_2532147 | NM_001012401.2 | Hspb6 | Heat shock protein alpha-crystallin-related B6 | 3.87 × 10⁻⁴ | 1.38 |
| ILMN_2515964 | NM_013560 | Hspb1 | Heat shock protein 1 | 6.81 × 10⁻⁵ | 1.37 |
| ILMN_2748205 | NM_019960.1 | Hspb3 | Heat shock protein 3 | 3.11 × 10⁻³ | 1.24 |

| Striated muscle tissue development (1.84) |           |             |           |         |             |
| ILMN_2598916 | NM_009608.3 | Act1 | Actin alpha cardiac muscle 1; similar to alpha-actin (AA 27–375) | 2.09 × 10⁻⁵ | 0.49 |
| ILMN_1222215 | NM_024291 | Kv | Kv | 2.09 × 10⁻³ | 1.41 |
| ILMN_1235230 | NM_0167978.3 | Pdlim3 | PDZ and LIM domain 3 | 2.32 × 10⁻⁴ | 1.34 |
| ILMN_268415 | NM_019684.1 | SrpK | Serine/arginine-rich protein specific kinase 3 | 8.87 × 10⁻⁵ | 1.29 |
| ILMN_2429680 | NM_153786.1 | Vgl2 | Vestigial like 2 homolog (Drosophila) | 9.45 × 10⁻⁶ | 1.26 |
| ILMN_1231765 | NM_993589.1 | Cymal1 | X-in actin-binding repeat containing 1 | 6.74 × 10⁻⁵ | 1.26 |
| ILMN_1213941 | NM_008657.2 | Myof | Myogenic factor 6 | 2.15 × 10⁻⁵ | 1.25 |

| Negative regulation of cell communication (1.65) |           |             |           |         |             |
| ILMN_1254016 | NM_001008533.2 | Adora1 | Adenosine A1 receptor | 6.71 × 10⁻³ | 1.45 |
| ILMN_1224173 | NM_015814.2 | Dkk3 | Dickkopf homolog 3 (Xenopus laevis) | 7.12 × 10⁻⁵ | 0.42 |
| ILMN_2695819 | NM_030143.3 | Ddit4L | DNA-damage-inducible transcript 4-like | 4.07 × 10⁻³ | 0.63 |
| ILMN_2628178 | NM_007706.3 | Socs2 | Suppressor of cytokine signaling 2; predicted gene 8000 | 1.59 × 10⁻⁴ | 0.69 |
| ILMN_1215446 | NM_007702.1 | Cidea | Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A | 6.11 × 10⁻⁵ | 0.71 |
| ILMN_1252918 | AK044878 | Npc1 | Niemann Pick type C1 | 5.62 × 10⁻⁶ | 0.72 |
| ILMN_2746738 | NM_019564.2 | Htra1 | HtrA serine peptidase 1 | 3.22 × 10⁻⁵ | 0.77 |
| ILMN_1232182 | NM_023465.3 | Ctnnbip1 | Catenin beta interacting protein 1 | 2.73 × 10⁻⁵ | 0.81 |

| Polymine metabolic process (1.51) |           |             |           |         |             |
| ILMN_2766409 | NM_145531.3 | Smox | Spermine oxidase | 6.85 × 10⁻⁶ | 0.50 |
| ILMN_1217803 | NM_009214.3 | Smu | Predicted gene 7270; predicted gene 14680; spermine synthase | 5.44 × 10⁻⁴ | 0.54 |
| ILMN_2676074 | NM_001479434.1 | LOC100047905 | S-adenosylmethionine decarboxylase pseudogene | 1.30 × 10⁻⁵ | 0.76 |

| Cytoskeleton organization (1.37) |           |             |           |         |             |
| ILMN_2598916 | NM_009608.3 | Act1 | Actin, alpha cardiac muscle 1; similar to alpha-actin (AA 27–375) | 2.09 × 10⁻⁵ | 0.49 |
| ILMN_1235070 | NM_198059.2 | Nrap | Nebulin-related anchoring protein | 2.78 × 10⁻⁴ | 1.35 |
| ILMN_1235230 | NM_0167978.3 | Pdlim3 | PDZ and LIM domain 3 | 2.32 × 10⁻⁴ | 1.34 |
| ILMN_1229878 | NM_026481.2 | Tcp3 | Tubulin polymerization-promoting protein family member 3 | 1.12 × 10⁻³ | 1.32 |
| ILMN_2777491 | NM_177699 | Fhod1 | Formin homology 2 domain containing 1 | 2.01 × 10⁻⁴ | 1.32 |
| ILMN_2487292 | NM_017379 | Tuba8 | Tubulin, alpha 8 | 3.03 × 10⁻⁴ | 0.74 |
| ILMN_1231765 | NM_993589.1 | Cymal1 | X-in actin-binding repeat containing 1 | 6.74 × 10⁻² | 1.26 |
There are two possible mechanisms to explain the change in Z-disk properties: (i) \( \alpha \)-actinin-3 deficiency results in a lower total pool of sarcomeric \( \alpha \)-actinins available to incorporate into the Z-disk of fast muscle fibers (i.e. difference in the ‘dosage’); (ii) the phenotypic effects of \( \alpha \)-actinin-3 deficiency are due primarily to functional differences between \( \alpha \)-actinin-3 and \( \alpha \)-actinin-2 such as affinity for binding Z-disk-associated proteins. We next explored these two possibilities.

\( \alpha \)-Actinin-2 is expressed more highly to compensate for \( \alpha \)-actinin-3 deficiency without a significant change in the total actinin pool

We first determined the relative and total amount of sarcomeric \( \alpha \)-actinins in WT and KO muscles, relative to total muscle protein, using serial dilutions of known quantities of purified recombinant \( \alpha \)-actinin-2 and \( \alpha \)-actinin-3 proteins to construct standard curves (Fig. 6). Compared with WT muscles, KO muscles showed a trend for higher total sarcomeric \( \alpha \)-actinin content, although this did not reach formal statistical significance \((P = 0.40)\). In WT muscles, \( \alpha \)-actinin-2 and \( \alpha \)-actinin-3 are expressed in roughly equal proportions. This corresponds to previously published data on \( \alpha \)-actinin-2 and \( \alpha \)-actinin-3 expression levels relative to fiber types (2).

ZASP, but not myotilin, preferentially binds to \( \alpha \)-actinin-2

The protein–protein interactions at the Z-disk determine the stability and rigidity of the Z-disk. Although \( \alpha \)-actinin-2 and \( \alpha \)-actinin-3 share 88% sequence similarity (2), the differences in the two sarcomeric \( \alpha \)-actinins (which determines their functional specialization) (45) could result in altered Z-disk protein composition, especially when compounded with the absence of \( \alpha \)-actinin-3 and the compensatory higher expression of \( \alpha \)-actinin-2. Therefore, preferential binding of Z-disk proteins to \( \alpha \)-actinin-2 or -3 may influence the composition of the Z-disk and underlie the increased force deficits in KO muscles post-eccentric contraction. Myotilin and ZASP directly associate with the sarcomeric \( \alpha \)-actinins and show higher expression in the absence of \( \alpha \)-actinin-3; up-regulation of myotilin has been associated with an increased rigidity of the Z-disk (21). On this basis, we examined the binding affinity of these proteins to \( \alpha \)-actinin-2 and \( \alpha \)-actinin-3 by yeast-two hybrid and β-galactosidase assays. We further sought to identify the \( \alpha \)-actinin domains associated with any differential binding interactions.

For bait, we used short \( \alpha \)-actinin-2 (A2) and \( \alpha \)-actinin-3 (A3) constructs containing the actin-binding domain (A2A, A3A), the rod domain containing the spectrin-like repeats (A2R, A3R) and the EF-hand domain (A2E, A3E), as well as full-length constructs (A2F, A3F). \( \alpha \)-Actinin constructs were sub-cloned into vectors containing the Gal4-binding domain (pGBT9-based), while the proteins of interest were cloned into pGAD10-based vectors containing the Gal4 activation domain, and vice versa. Positive and differential interactions indicated by growth on minimal media were subsequently quantitated by liquid β-galactosidase assays.

Yeast-two hybrid assays showed similar levels of growth on minimal media for the myotilin interactions with the full-length constructs, A2F and A3F, suggesting that myotilin does not have preferential binding for either \( \alpha \)-actinin-2 or \( \alpha \)-actinin-3 and interacts strongly with both sarcomeric \( \alpha \)-actinins (Supplementary Material, Fig. S4). In contrast, by both yeast-two hybrid plate growth and liquid assays, ZASP showed a stronger interaction with full-length \( \alpha \)-actinin-2 (A2F) and \( \alpha \)-actinin-2 EF-hand domain (A2E) than full-length \( \alpha \)-actinin-3 (A3F) or \( \alpha \)-actinin-3 EF-hand domain (A3E) (Fig. 7A, Supplementary Material, Fig. S4). These results
suggest that ZASP exhibits a higher binding affinity to α-actinin-2 compared with α-actinin-3, and the preferred site of interaction is the α-actinin-2 EF hand domain. We confirmed this by performing a domain-swap experiment where the α-actinin-2 EF hand domain was sub-cloned into the α-actinin-3 construct (A332), which restored binding of ZASP to this chimeric construct, with increased β-galactosidase activity compared with A3F (Fig. 7A). The interaction of α-actinin-3 with myotilin and ZASP had not previously been demonstrated.

Z-disk proteins titin and vinculin preferentially bind to α-actinin-2

Since the Z-disk protein ZASP showed preferential binding to α-actinin-2, we further examined the binding affinity of other sarcomeric α-actinin-binding partners to α-actinin-2 and α-actinin-3. Of the vast numbers of sarcomeric α-actinin interaction partners, we specifically focused on titin and vinculin because of their known functions at the Z-disk. Titin is the principle passive force-bearing element in the sarcomere, and is thought to have an important role in muscle extensibility and elasticity (46). Similarly, the interaction between vinculin and sarcomeric α-actinins is thought to be involved in the modulation of the width and ultrastructure of the Z-disk, and in turn the capacity of the muscle fibers to withstand loading (47). Since titin is the largest known protein, with a molecular weight of nearly ~4 MDa, we specifically cloned the identified binding region of titin to α-actinins, the Z-repeats of titin. We also specifically cloned the head domain of vinculin, since it is the known site of interaction with the α-actinins (48). By the yeast-two hybrid assay, both

Figure 3. Myotilin and desmin showed normal localization at the Z-disk in KO muscles. (A) Myotilin and (B) desmin co-localized with α-actinin-2 in both WT- and KO-stretched muscles, suggesting normal localization of these Z-disk proteins at the Z-disk. Images were taken at ×400 magnification.
titin and vinculin demonstrated stronger binding to α-actinin-2 than to α-actinin-3 (Fig. 7B and C, Supplementary Material, Fig. S4). The interaction of α-actinin-3 with titin and vinculin has not previously been demonstrated. In the absence of α-actinin-3, higher binding affinity of these Z-disk proteins to α-actinin-2 could alter the functional and structural properties of the Z-disk and its capacity to withstand eccentric stress in α-actinin-3-deficient muscles.

DISCUSSION

α-Actinin-3-deficient muscle is more susceptible to damage

The sarcomeric α-actinins are major structural components of the Z-disk in skeletal muscle and α-actinin-3 deficiency in the general population is detrimental to muscle strength and athletic ability in power sports, suggesting that it affects the properties of the Z-disk and the sarcomere. The skeletal muscle Z-disk is thought to be the most vulnerable structure to eccentric contraction-induced injury (49), and here we present several lines of evidence to demonstrate that α-actinin-3 deficiency directly results in an increased susceptibility to eccentric damage. Force deficit is an indirect but reproducible measure of the magnitude of contraction-induced damage that reflects both fatigue and injury (50,51), and is also influenced by fiber size and fiber type (with increased susceptibility in larger type 2 fibers) (49,52,53). In this study, the increased force deficit in KO muscle is unlikely to be due to greater fatigue. Our protocol prescribed a 30–60 min recovery period prior to assessment of post-eccentric contraction force generation, which provides ample time for recovery from fatigue and we have previously shown that KO muscles...
exhibit greater and faster force recovery post-fatigue (14). Fiber typing was also unlikely to play a role in increased force deficits in KO since there is no difference in fiber-type proportions in WT and KO muscles (14). Finally, 2B fibers are smaller in Actn3 KO mice and reduced fiber size would be ‘protective’ against mechanical damage, resulting in lower, rather than higher post-eccentric contraction force deficits. Thus, the higher force deficits post-eccentric contraction probably reflect greater susceptibility of the sarcomere to damage associated with the absence of α-actinin-3 expression.

Higher expression of Z-disk proteins in KO muscles reflects greater muscle remodeling

The basis for increased force deficit and susceptibility to mechanical damage induced by stretch is generally attributed to changes in the force generating or force transmitting structures of the myofibrils (51), and in particular changes in the cytoskeletal proteins located at the level of the Z-disk (54). In α-actinin-3-deficient muscle, we observed higher expression of α-actinin-2 and the Z-disk-associated proteins γ-filamin, myotilin, desmin, αB-crystallin and ZASP, as well as genes associated with muscle growth and regeneration. Up-regulation of α-actinin-2, desmin and myotilin in response to eccentric exercise damage (55–57) and exercise training (58–61) are thought to be involved in adaptive remodeling of myofibrils. Although we did not observe overt sarcomeric damage in KO muscles, the higher expression of these proteins, and an increase of internalized nuclei at baseline suggests that α-actinin-3-deficient muscles are susceptible to ongoing damage that requires remodeling and regeneration.

It is interesting to note that dominant mutations in γ-filamin, myotilin, desmin, αB-crystallin and ZASP result in the late adult-onset muscle disease myofibrillar myopathy (MFM). This disorder is characterized by disruptions to the Z-disk, myofibrillar disorganization and abnormal accumulations of sarcomeric proteins within muscle (62), suggesting that the proteins associated with MFM play a vital role in the maintenance of the structural integrity of the Z-line. Intriguingly, the formation of protein accumulations is also a consequence of excessive Z-disk protein expression (44), and we have shown that the higher expression of Z-disk proteins precedes the formation of protein inclusions in KO skeletal muscle. Stress or load-bearing may also be necessary for aggregate formation. Protein aggregates occurred most frequently in weight-bearing muscles in areas with high fast 2B fiber composition, such as vastus lateralis white and spinalis muscles, but were not observed in the smaller ankle and foot muscles such as the tibialis anterior and the EDL, despite the presence of higher levels of Z-disk proteins in these muscles (data not shown). The greater force deficit in KO EDL muscles is thus unrelated to the presence of protein accumulations but is likely due to alterations in Z-disk composition.
Desmin and Up-regulation of myotilin leads to increased expression associated with the absence of a-
Greater Z-disk susceptibility to damage is directly associated with the absence of α-actinin-3

Up-regulation of myotilin leads to increased expression of desmin and γ-filamin (but not α-actinin-2) (44) and results in increased cross-linking of actin and increased rigidity of the Z-disk (21). Surprisingly, over-expression of myotilin in the transgenic (TgWT) mouse model did not result in greater force deficits compared with WT after eccentric contraction at 30% stretch above optimal sarcomere length (44). Thus, the susceptibility to eccentric damage seen in the Actn3 KO mouse cannot be attributed to the higher expression of these proteins alone, suggesting that the absence of α-actinin-3 and/or the subsequent greater expression of α-actinin-2 are necessary to alter Z-disk stability.

Differences in fast fiber Z-disk structural properties suggest a potential mechanism to explain the structural phenotypes associated with α-actinin-3 deficiency

Increased force deficits post-eccentric contraction in α-actinin-3-deficient muscle indicate that the up-regulation of α-actinin-2 (and maintenance of the total pool of sarcomeric α-actinin) is not sufficient to fully compensate for the absence of α-actinin-3 in skeletal muscle Z-disks. Thus, the structural phenotype associated with α-actinin-3 deficiency is more likely due to functional differences between α-actinin-2 and α-actinin-3 which, in part, are attributable to differences in their interactions with other Z-disc-associated proteins. The critical role of ZASP, titin and α-actinin-2 in Z-disc maintenance during muscle stress (32) suggests that variation in the interaction of these proteins could influence sarcomeric stability during muscle contraction. We have demonstrated in vitro that ZASP, titin and vinculin preferentially bind to α-actinin-2. In α-actinin-3-deficient muscle, the presence of α-actinin-2 would result in altered interactions with these proteins in fast 2B fibers (63). ZASP has a role in the maintenance of the structural integrity of the Z-disk and is more highly expressed in α-actinin-3-deficient muscle. The ablation of ZASP results in contraction-induced structural disorganization at the Z-disk (64) and missense mutations that include α-actinin interaction sites on ZASP result in Z-disk streaming and protein accumulations (65). The interaction between α-actinin-2 and vinculin has been associated with changes in Z-disk width (47). Similarly, the number of Z-repeats in titin determines Z-disk width (66), and different titin isoforms of variable sizes are major determinants of passive stiffness or tension of the sarcomere, particularly in fast muscles (46,67–69). Therefore, our finding that these proteins preferentially bind to α-actinin-2 would likely result in the altered structural and elastic properties of the Z-disk associated with α-actinin-3 deficiency. Our findings provide a mechanistic explanation for the lower force generation and higher susceptibility to damage with α-actinin-3 deficiency. Although there is an established correlation between binding affinity determined by yeast-two hybrid assays and other biochemical techniques (70), we plan to explore this further in future studies using surface plasmon resonance to fully characterize and accurately quantify the nature of preferential interactions with α-actinin-2, compared with α-actinin-3.

Since α-actinin-3 is absent in ~16% of people worldwide, these findings have broad relevance to the normal variations in skeletal muscle function as well as potential implications for inherited and acquired muscle disorders. ACTN3 genotype has already been shown to modify exercise capacity in patients with the metabolic myopathy, McArdles disease (71). ACTN3 is an excellent candidate disease-modifying gene due to the frequency of the null polymorphism and the effect of α-actinin-3 deficiency on skeletal muscle performance.

MATERIALS AND METHODS

Ethics statement

This study was approved by our local Animal Care and Ethics Committee.

Mouse models and tissue collection

All mice [Actn3 KO (16) and transgenic over-expressing WT myotilin (44)] were given food and water ad libitum, and were maintained on a 12:12 h light and dark cycle. Mice were euthanized by cervical dislocation immediately prior to tissue collection. Muscles and organs were removed and either placed in RNAlater (Applied Biosystems) or immediately covered in cryo-preservation medium (Tissue-Tek, ProSci-Tech) and snap frozen in partially thawed isopentane. Tissues were stored in liquid nitrogen until use.

Antibodies

Antibodies to the α-actinins were a gift from A. Beggs (Children’s Hospital Boston). α-Actinin-2 was analyzed using the

Figure 6. WT and KO muscles show similar levels of total sarcomeric α-actinin content. Known concentrations of recombinant α-actinin-2 and α-actinin-3 proteins were serially diluted and loaded on a 3–8% tris-acetate gel to generate a standard curve. Total protein concentration of muscle lysates from WT and KO quadriceps was determined; the amount of KO muscle lysates loaded was halved that of WT muscles to ensure that lysates from WT and KO quadriceps was determined; the amount of KO

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rabbit antibody 4B3 at 1:1000 for immunohistochemistry (IHC) and at 1:200 000 for WB, and α-actinin-3 was analyzed using the rabbit antibody 5B3 at 1:800 for IHC and 1:12 000 for WB. Antibodies to desmin (NCL-L-DES-DERII, Novoceastra Laboratories, 1:50 for IHC, 1:1500 for WB) and myotilin (NCL-MYOTILIN, Novoceastra Laboratories, 1:20 for IHC, 1:2000 for WB) were also used. The antibody to γ-filamin (FLN2-A2, rabbit antibody, WB 1:2500) was a gift from T. Thompson and L. Kunkel (Harvard Medical School). For secondary antibodies, we used Cy3-conjugated affiniPure goat anti-mouse IgG (1:250 dilution; Jackson ImmunoResearch), Alexa Fluor 555 goat anti-mouse IgM (1:250 dilution; Molecular Probes), Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution; Molecular Probes), sheep anti-mouse IgG-HRP conjugates (1:2000 dilution; GE Healthcare) and donkey anti-rabbit IgG-HRP conjugates (1:2000 dilution; GE Healthcare).

Immunohistochemistry

Transverse 8 μm sections were cut from the mid-section of frozen quadriceps and spinalis muscles. All sections were blocked with AffiniPure Fab fragment goat anti-mouse IgG (1:25 dilution; Jackson ImmunoResearch) for 1 h to prevent cross-reaction with endogenous mouse antibodies whenever mouse primary IgG antibodies were applied. Muscle sections were incubated for 1 h at room temperature with primary antibodies, then washed twice with 1× PBS and re-blocked with 2% BSA for 10 min prior to incubation with appropriate secondary antibodies for 1 h at room temperature. All samples

Figure 7. ZASP, titin and vinculin preferentially binds to α-actinin-2. Both β-galactosidase assay and growth on minimal media results show a preference for (A) ZASP (aa 1–203) and (B) titin to interact with α-actinin-2. The ZASP interaction site at the EF-hand domain may be mediating this preference indicated by the EF-hand results and the restoration of binding when the EF-hand of α-actinin-3 is replaced by that of α-actinin-2. The titin interaction site at the rod domain may be mediating this preference. (C) The growth on minimal media as a dilution series also shows a preference for vinculin (aa 1–350) to interact with α-actinin-2. This interaction was weak or transient interaction so could not be quantitated using a β-galactosidase assay. A2F, full-length α-actinin-2; A3F, full-length α-actinin-3; A2E, α-actinin-2 EF-hand; A3E, α-actinin-3 EF-hand; A332, full-length α-actinin-3 with α-actinin-2 EF-hand; A2R, α-actinin-2 rod domain; A3R, α-actinin-3 rod domain; A2AR, α-actinin-2 actin-binding domain and rod domain; A3AR, α-actinin-3 actin-binding domain and rod domain. ‘-’ indicates no growth, while number of ‘+’ indicates strength of growth.
were washed twice with 1 × PBS prior to mounting to remove non-specific binding of secondary antibodies.

**Immunoblotting**

Equal sample loading was evaluated using intensity of myosin and actin bands on pre-cast mini-gels (Invitrogen) stained with Coomassie Blue Brilliant (Sigma-Aldrich). Samples adjusted for loading were separated by SDS–PAGE on pre-cast mini-gels, transferred to polyvinylidene fluoride membranes (Millipore), which was then blocked with 5% skim milk/1 × PBS/0.1% Tween-20, then probed with indicated antibodies and developed with ECL chemiluminescent reagents (Amersham Biosciences). Primary antibodies used included desmin (1:1500), myotilin (1:2000), α-actinin-2 (1:500 000), α-actinin-3 (1:12 000), γ-filamin (1:2500) and ZASP (1:200 000). α-Sarcomeric actin (SC5; 1:2000; Sigma) was also examined. Following probing, PVDF membranes were stained with Coomassie Blue Brilliant (Sigma-Aldrich) to generate final myosin loading controls.

**Muscle preparation**

The EDL muscle was dissected from the hindlimb and tied by its tendons to a force transducer (World Precision Instruments, Fort 10) at one end and a High Speed length controller at the other, using silk suture (Deknatel 6.0). The muscle was placed in a bath continuously superfused with Krebs solution, with composition (mM): 4.75 KCl, 118 NaCl, 1.18 KH2PO4, 1.18 MgSO4, 24.8 NaHCO3, 2.5 CaCl2 and 10 glucose, with 0.1% fetal calf serum and continuously bubbled with 95% O2–5% CO2 to maintain pH 7.4. The muscle was stimulated by supramaximal pulses of a current booster to provide sufficient current to maximally activate the muscle. At the start of the experiment, the muscle was set to its optimum length L0 by finding the length that produced maximum twitch force. All experiments were conducted at room temperature (~ 22 to 24°C).

**Eccentric contraction protocol**

The eccentric contraction protocol was as follows: at time = 0 ms, the muscle was stimulated by supramaximal pulses of 1 ms duration and 100 Hz frequency. At time = 750 ms, it had attained its maximum isometric force, the muscle was stretched (High speed length controller, Aura Scientific Inc Canada L4G3V7) at a speed of 10 mm/s until it was 30% longer than its optimum length, held at this length for 2 s, then returned at the same speed to its original position. A strain of 30% was chosen because previous experiments indicated that a smaller strain of 20% strain did not damage KO muscles. The electrical stimulus was stopped at time = 5000 ms. This eccentric contraction was performed three times, at intervals of 5 min. The muscle was allowed to recover for 30 min before the final force was measured; this was to exclude any force deficits due to fatigue. See Supplementary Material, Figure S6 for an example of the raw data.

**Microarray analysis**

Quadriiceps muscles from six WT and six KO male 2-month-old mice were harvested and stored in RNA Later (Applied Biosystems) prior to synthesis of biotin-labeled aRNA (antisense amplified RNA) using the MessageAmp II kit from Ambion as per manufacturer’s instructions. Concentration of aRNA was determined with a NanoPhotometer (Implen). To perform the microarray comparison, aRNA was hybridized to Illumina mouse WG-6 version 1 expression BeadChips, stained with streptavidin-Cy3 conjugate and scanned using the Illumina BeadArray reader. Results were extracted using Illumina BeadStudio software, and the top 250 differentially expressed genes were determined using the Limma package (72). GO clustering was performed using the functional annotation clustering tool from DAVID (40,41).

cDNA cloning and sequencing

All PCR inserts for yeast-two hybrid analysis were amplified from the human skeletal muscle cDNA and subcloned into pGBT9- and pGAD10-based vectors (Clontech). Primer design for ZASP (NM_007078), myotilin (NM_006790), vinculin (NM_003373), α-actinin-2 (NM_001103) and α-actinin-3 (NM_007078) were based on their respective Genbank template sequences indicated in brackets. Sequences of the completed constructs were confirmed by DNA sequencing.

**Yeast-two hybrid analysis**

**Yeast transformation.** Yeast transformation was performed using the method as in Yeast Protocols Handbook (Clontech). Various pGAD10-α-actinin constructs were co-transformed with various pGBT9 constructs containing ZASP (aa 1–203), vinculin (aa 1–350), titin (aa 644–990) or myotilin (aa 1–498) inserts. Transformants were plated and incubated for 3 days at 30°C on minimum yeast medium lacking leucine and tryptophan. Transformants were then streaked on minimum yeast medium lacking leucine, tryptophan and histidine and incubated at 30°C. The plates were observed over 5 days to assess the strength of growth.

**Liquid β-galactosidase assay.** Liquid β-galactosidase assay was performed using the method as in Yeast Protocols Handbook (Clontech). Transformants were grown in 5 ml of selective liquid media overnight at 30°C. Two milliliters of culture were transferred to 8 ml of YPD media and incubated for a further 5 h. An aliquot of 1.5 ml of each culture was spun, and the pellet was washed and then resuspended in 100 μl of β-galactosidase buffer 1 (23.8 mg/ml HEPES, 9 mg/ml NaCl, 0.65 mg/ml l-aspartate, 10 mg/ml BSA, 0.05% Tween 20 v/v, pH 7.25). Cell were lysed by five rounds of freeze–thawing in liquid nitrogen followed by the addition of 700 μl of buffer 1 containing chlororphenol red-β-D-galactopyranoside (1.36 mg/ml) and the mixture was incubated at 30°C until the samples turned red. The reaction was stopped by the addition of 500 μl of 3 mM ZnCl2 and absorbance was read at 570 nm.
For each sample, arbitrary units were calculated to correct for culture OD and incubation time.

Protein expression and purification
The inserts for full-length α-actinin-2 and α-actinin-3 were subcloned into the pGEX-2T vector from the pGEXT9 construct. Expression of soluble GST-tagged recombinant protein was then induced in E. coli BL21 (GE Healthcare) using 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma). The purification was carried using a Glutathione Sepharose 4B microspin column (GE Healthcare) with thrombin to cleave recombinant proteins from the column. Protein purity was assessed on a Gelcode (GE Healthcare) stained SDS–PAGE gel. Protein concentration was quantified using NanoOrange protein quantification kit as per manufacturer’s instructions (Invitrogen).

Total sarcomeric α-actinin protein quantification
A purified protein standard curve of known concentration was generated from the above recombinant α-actinin-2 and α-actinin-3 proteins. WT and KO total muscle protein levels were determined by BCA kit (Pierce). Less KO total protein (625 ng) compared with WT (1.25 μg) was loaded on SDS–PAGE pre-cast mini-gels to take into account α-actinin-2 up-regulation in KO muscles (16) and to ensure that the resultant bands will lie within the standard curve. Western blotting was carried out as described above. Protein expression of α-actinin-2 and α-actinin-3 in WT and KO was quantified by densitometry using Quantity-One software (Biorad) and used to determine the level of sarcomeric α-actinin relative to total muscle protein.

Statistics
Pair-wise comparisons were performed using the non-parametric Mann–Whitney U test. Differences were considered as significant at \( P < 0.05 \).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

AUTHORS’ CONTRIBUTIONS
J.T.S., D.G.M., N.Y. and K.N.N. designed the study; S.I.H. performed the eccentric damage analyses; J.T.S. performed the histology, immunohistochemistry and western blots; K.G.R.Q. performed the developmental studies; M.L. and X.F.Z. synthesized the recombinant proteins; M.L., K.G.R.Q. and X.F.Z. performed the yeast-two hybrid and liquid assays; J.T.S. and P.J.H. quantified the total sarcomeric α-actinin content; D.G.M., J.M.R and N.Y. performed the microarray; J.T.S. and M.L. analyzed the results from the microarray; J.T.S and F.G quantified the internalized nuclei in muscle fibers; S.M.G. and M.A.H. supplied the myotilin transgenic mice and provided intellectual input; J.T.S. interpreted the overall results; J.T.S. and K.N.N. wrote the paper.

Conflict of Interest statement. None declared

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