Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle

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Disruptions in the use of skeletal muscle lead to muscle atrophy. After short periods of disuse, muscle atrophy is reversible, and even after prolonged periods of inactivity, myofiber degeneration is uncommon. The pathways that regulate atrophy, initiated either by peripheral nerve damage, immobilization, aging, catabolic steroids, or cancer cachexia, however, are poorly understood. Previously, we found that Runx1 (AML1), a DNA-binding protein that is homologous to Drosophila Runt and has critical roles in hematopoiesis and leukemogenesis, is poorly expressed in innervated muscle, but strongly induced in innervated muscle shortly after denervation. To determine the function of Runx1 in skeletal muscle, we generated mice in which Runx1 was selectively inactivated in muscle. Here, we show that Runx1 is required to sustain muscle by preventing denervated myofibers from undergoing myofibrillar disorganization and autophagy, structural defects found in a variety of congenital myopathies. We find that only 29 genes, encoding ion channels, signaling molecules, and muscle structural proteins, depend upon Runx1 expression, suggesting that their misregulation causes the dramatic muscle wasting. These findings demonstrate an unexpected role for electrical activity in regulating muscle wasting, and indicate that muscle disuse induces compensatory mechanisms that limit myofiber atrophy. Moreover, these results suggest that reduced muscle activity could cause or contribute to congenital myopathies if Runx1 or its target genes were compromised.

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sible for the defects in myofibrillar organization and severe muscle wasting of denervated runx1 mutant muscle. These findings demonstrate that electrical activity regulates autophagy and muscle wasting by controlling expression of a transcription factor that activates and represses muscle gene expression, thereby compensating, in part, for a loss of innervation and limiting the extent of muscle wasting.

Results

Inactivation of Runx1 selectively in skeletal muscle

Mice lacking Runx1 die during mid-embryogenesis (E12), due to hemorrhaging in the central nervous system (Okuda et al. 1996; Wang et al. 1996). To study the role of Runx1 in skeletal myofibers, we circumvented this embryonic lethality by generating mice that carry a loxp-flanked allele of runx1 and a muscle creatine kinase (MCK)::cre transgene [Fig. 1]. The runx1 allele contains loxp sites that flank exon 4 (Taniuchi et al. 2002), which encodes the major portion of the DNA-binding domain as well as sequences that are essential for interaction with CBFβ, a Runx-interacting protein that is required for Runx1 activity in vivo (de Bruijn and Speck 2004). Splicing from exon 3, the first coding exon in skeletal muscle (data not shown), to exon 5 or exon 6 alters the reading frame, resulting in a truncated protein. Thus, deletion of exon 4 is likely to result in a runx1-null mutation (see below).

Runx1 is expressed in developing muscle, but down-regulated by innervation (Zhu et al. 1994), which begins at E12.5. Because the MCK regulatory elements confer only a low level of Cre recombinase expression in embryonic skeletal muscle and substantially greater expression after birth (Bruning et al. 1998; Nguyen et al. 2003), maximal Cre expression is attained at a time when skeletal muscle runx1 expression has already declined. Since severe defects in myogenesis lead to neonatal lethality, this experimental design minimizes the potential for neonatal lethality if Runx1 were to have a key role during myogenesis.

Indeed, MCK::cre; runx1f/f mice were healthy and fertile and born in expected numbers. We assessed the effectiveness of Cre-mediated recombination by measuring runx1 RNA expression in innervated and denervated muscles from MCK::cre; runx1f/f mice and runx1f/f control adult mice. We used an RNase protection assay, which detects both wild-type and mutant runx1 RNAs, and found that 96% of skeletal muscle runx1 is inactivated in MCK::cre; runx1f/f mice [Fig. 1]. Moreover, these data demonstrate that runx1 expression is not dependent upon autoregulation, as suggested for runx2 (Drissi et al. 2000), as runx1 is induced to the same extent in runx1 mutant and control muscle.

Denervated runx1 mutant myofibers are severely atrophied

In wild-type mice, peripheral nerve damage (Zhu et al. 1994), as well as limb immobilization (Bodine et al. 2001), lead to an increase in Runx1 expression and muscle atrophy. To determine whether Runx1 regulates muscle atrophy, we denervated hind-limb muscles in mutant and control adult mice and examined innervated and denervated muscles by light microscopy 2 wk later. Figure 2 shows that denervated, runx1 mutant myofibers are severely and unusually atrophic. In the soleus muscles of control runx1f/f mice and runx1f/f mice, which are heterozygous for the mutant allele, a 2-wk denervation period leads to a ~35% decrease in the cross-sectional area of the myofibers (innervated runx1f/f, 690 ± 236 µm²; mean ± SD, n = 218; denervated runx1f/f, 460 ± 150 µm²; n = 226; innervated runx1f/f, 684 ± 259 µm²; n = 489; denervated runx1f/f, 401 ± 142 µm²; n = 434) and a corresponding decrease in muscle wet weight. In the soleus muscles of mice lacking Runx1 (MCK::cre; runx1f/f), denervation leads to a 10-fold decrease in myofiber size (innervated, 702 ± 252 µm²; n = 877; denervated, 82 ± 29 µm², n = 524) and a similar
The severe wasting of denervated runx1 myofibers is not accompanied by excessive activation of the FoxO-mediated atrophy pathway

The pathways that regulate atrophy are poorly understood, but muscle disuse leads to a reduction in phosphatidylinositol 3-kinase (PI3K)/Akt activities and a decrease in FoxO phosphorylation, triggering nuclear import of FoxO and activation of FoxO target genes (Sandri et al. 2004; Stitt et al. 2004). To determine whether the severe wasting of denervated runx1 mutant myofibers is due to excessive activation of the FoxO-mediated atrophy pathway, we measured expression of a direct target of FoxO1, atrogin-1 [MAFbx; Fbox32] (Bodine et al. 2001; Sandri et al. 2004), an E3-ubiquitin ligase, in innervated and denervated muscles from control and runx1 mutant mice. We found that denervation causes a 5.3 ± 0.4-fold (mean ± SEM, n = 3) increase in atrogin-1 expression in control mice and a 8.6 ± 0.4-fold (mean ± SEM, n = 3) increase in atrogin-1 expression in runx1 mutant muscle (Fig. 3). Thus, the severe wasting of denervated runx1 mutant muscle is unlikely to be due to excessive activation of the FoxO pathway.

Atrophy can be induced by NFκB without inducing expression of atrogin-1 (Cai et al. 2004). Instead, NFκB induces expression of MuRF1, an E3-ubiquitin ligase, which, like atrogin-1, is induced by denervation (Bodine et al. 2001; Cai et al. 2004). To determine whether the severe wasting of denervated runx1 mutant myofibers is due to excessive activation of this NFκB pathway, we measured MuRF1 (Trim63, Rnf28) expression in innervated and denervated muscles from control and runx1 mutant mice. Figure 3 shows that denervation leads to a 4.7 ± 0.7-fold (mean ± SEM, n = 2) increase in MuRF1 expression in control mice and a 9.5 ± 0.1-fold (mean ± SEM, n = 2) increase in MuRF1 expression in runx1 mutant muscle (Fig. 3). Thus, the severe wasting of denervated, runx1 mutant muscle is unlikely to be caused by excessive activation of this NFκB pathway. Although Atrogin-1 and MuRF1 may contribute to wasting of runx1 mutant muscle, other pathways are likely to be responsible for the striking decrease in myofiber size in denervated runx1 mutant muscle.

Runx1 induction is required to prevent disused myofibers from undergoing autophagy, myofibrillar disorganization, and severe muscle wasting

In wild-type mice, disused myofibers have a reduced capacity to generate force, due to their smaller cross-sectional area, but these atrophic myofibers retain most structural features that are characteristic of innervated muscle. We examined runx1 mutant and control soleus...
muscles by electron microscopy to determine whether denervated runx1 mutant muscles display additional signs of muscle wasting. We found that denervated runx1 mutant myofibers have structural aberrations that are not evident in atrophic denervated control myofibers, but are reminiscent of structural abnormalities found in a variety of congenital myopathies (Fig. 4; Engel 1999; Nishino 2003; Selcen et al. 2004). First, in denervated runx1 mutant muscles, the Z-discs are misaligned, fragmented, and irregularly spaced. Second, the distinctive A- and I-bands, readily evident in innervated and denervated muscles of control mice, are not apparent in denervated runx1 mutant muscles. Instead, the myofibrils appear to contain only thin filaments, presumably composed of actin, and to lack thick filaments composed of myosin. Third, the sarcoplasmic reticulum in denervated runx1 mutant muscle is severely dilated and extends from the Z-disc into the space normally occupied by myofibrils. Fourth, scattered throughout denervated runx1 mutant myofibers are double- or multilayered vacuoles, which enclose heterogeneous contents including mitochondria and additional membrane-enclosed structures [Fig. 5]. These features define these organelles as autophagic vacuoles (Gozuacik and Kimchi 2004), demonstrating that Runx1 is required to prevent denervated myofibers from undergoing autophagy, and indicating that excessive autophagy is responsible for the severe wasting of denervated runx1 mutant myofibers.

Twenty-nine genes, encoding channels, signaling molecules, structural proteins, but not transcription factors, are misexpressed in denervated runx1 mutant muscle

A loss of muscle activity leads to a reduction in signaling that promotes muscle growth (Murgia et al. 2000), but disused muscle does not undergo autophagy. Taken together, these findings suggest four potential mechanisms by which Runx1 prevents autophagy. First, Runx1 may partially counter-balance a loss of muscle activity by inducing the same genes that are induced by muscle activity. Second, Runx1 may induce a different set of genes that compensate for the loss of muscle activity and likewise promote muscle growth/maintenance, thereby preventing autophagy. Third, Runx1 may induce a different set of genes that compensate for the loss of muscle activity and likewise promote muscle growth/maintenance, thereby preventing autophagy. Fourth, Runx1 may induce genes that encode for structural components of the myofiber, and incomplete or partially assembled structures may lead to a stress response that provokes autophagy. To identify genes that are dependent upon Runx1 expression and regulate muscle structure, we screened oligonucleotide microarrays with RNA from innervated and denervated muscles from wild-type and runx1 mutant mice (Fig. 6). To increase the probability of identifying direct targets of Runx1, we probed microar-

Figure 4. Z-discs are disorganized, thick filaments are absent, and the sarcoplasmic reticulum is dilated in runx1 mutant denervated myofibers. [a,b] In denervated (14-d) muscle of control runx1f/f mice, the Z-discs (arrow) are aligned, the actin-only I-bands (I) and actin + myosin A-bands (A) are readily apparent, and the sarcoplasmic reticulum (SR) and transverse-tubules (T) are situated between the myofibrils. [c,d] In denervated (14-d) MCK::cre, runx1f/f myofibers, the Z-discs are misaligned and fragmented (arrow in c), distinct I- and A-bands are not discernible, and the SR is severely dilated and extends from the Z-disc into the myofibril. Myofibers that had been denervated for 1 wk were similarly, though less severely affected. Bars: a,c, 0.5 µm; b,d, 0.12 µm.

Figure 5. Autophagic vacuoles are prominent in runx1 mutant denervated myofibers. The vacuoles in runx1 mutant denervated (14-d) muscle (MCK::cre, runx1f/f) are enclosed by two bilayers (arrows) that enclose heterogeneous contents, including mitochondria [a], myofibrils [b], and other membrane-enclosed structures [c,d], hallmark features of autophagic vacuoles (Gozuacik and Kimchi 2004). (*) Autophagic vacuoles, [M] mitochondrion. Bar, 0.12 µm.
rays with RNA from skeletal muscle that had been de-
nervated for 3 d, 1 d after runx1 expression is maximal in
wild-type mice, but prior to overt structural changes in
denervated runx1 mutant muscle. We validated the mi-
croarray data by measuring expression of a subset of
genes, identified as misregulated in the microarray
screen, by RNase protection (Fig. 6).

Only 29 genes are misregulated (greater than or equal
to threefold) in denervated muscle lacking Runx1, sug-
gesting that only a few genes are responsible for the dra-
matic muscle wasting observed in runx1 mutant mice.

To assess the reliability of the microarray data, we mea-
sured expression of six misregulated genes in innervated
and denervated muscle from wild-type and runx1 mu-

tant muscle by an RNase protection assay. In each case,
the RNase protection data corroborated the results from
the microarray analysis, confirming the reliability of the
microarray data (Fig. 6).

As most genes are unaffected by Runx1 expression,
including genes that are known to be dependent upon
innervation including myogenin, MaSK, and the acetyl-
choline receptor α and δ subunit genes, it is unlikely that
Runx1 simply compensates for a loss of innervation. The
29 misregulated genes encode ion channels [five genes],
signaling molecules [14 genes], and structural proteins
[four genes], but not transcription factors, indicating that
the identified genes are good candidates for direct targets
of Runx1 [Fig. 6]. Sixteen genes are not appropriately
up-regulated or maintained in runx1 mutant denervated
muscle, suggesting that Runx1 activates their expres-
sion. Thirteen genes are expressed at unusually high lev-

eels in denervated muscle lacking Runx1, suggesting that
Runx1 represses their expression. These findings are
consistent with other studies showing that Runx family
members can activate or repress target genes [Kramer et
al. 1999]. Further studies of these Runx1 target genes
should lead to a better understanding of their roles in
muscle wasting.

Discussion

Disruptions in myofiber electrical activity, including de-
nervation, lead to muscle atrophy [Jagoe and Goldberg
2001; Glass 2003]. Although innervation provides tro-
phic signals that are critical for muscle differentiation
and growth, atrophic myofibers neither degenerate nor
undergo apoptosis, but retain most of the structural fea-
tures that are characteristic of normal muscle. These re-

sults suggest that myofiber size and differentiation are
only partially dependent upon innervation or that dener-
vation induces compensatory mechanisms that limit the
extent of muscle atrophy and wasting. Our results
demonstrate that Runx1 induction is required to prevent
myofibrillar disorganization and severe muscle wasting,
indicating that muscle disuse indeed induces pathways
that compensate for a loss innervation, thereby prevent-
ing myofibrillar disorganization and limiting the extent
of muscle atrophy and wasting [Fig. 7]. Moreover, our
experiments demonstrate that these compensatory path-
ways depend upon Runx1 induction.
Despite the importance of Runx1 in leukemogenesis, only CD4 has been identified as a bona-fide target of Runx1 in hematopoietic cells (Taniuchi et al. 2002). Among the Runx1-dependent genes identified in our microarray screen, we identified several genes, including acetylcholine receptor α9 subunit, osteopontin, orosomucoid 2, lipocalin 2, and amiloride-sensitive cation channel 1, that were not known to be expressed in skeletal muscle. In addition, the microarray screen identified multiple genes, including aquaporin 4, keratin 2–6, and keratin 1–18, that were known to be expressed in muscle, but not known to be regulated by innervation. Moreover, the screen identified multiple genes, including embryonic myosin heavy chain, sodium channel type V, and acetylcholine receptor γ subunit genes, that were known to be regulated by electrical activity, but by unknown transcriptional mechanisms.

Similarly, very few target genes for Runx2 and Runx3 have been identified. Nonetheless, Runx2 activates osteopontin expression in bone and cartilage [Ducey et al. 1997; Komori et al. 1997]. Because we find that osteopontin is a target of Runx1 in skeletal muscle, these results demonstrate that the same gene can be a target for different Runx family members in different cell types. Thus, other Runx1-target genes, identified in our screen of skeletal muscle, may be targets for Runx family members in other cell types.

The genes that are misregulated in runx1 mutant muscle provide clues to the causes for the profound structural changes. Notably, expression of myosin heavy-chain IIA [Myh2] is not maintained [sixfold decrease] and embryonic myosin heavy chain [Myh3] fails to be induced [19-fold decrease] following denervation of runx1 mutant muscle. This reduction in myosin expression may therefore explain the absence of thick filaments and distinct A- and I-bands in runx1 mutant denervated muscle. Two keratin genes (Krt1–18, Krt2–8), which encode subunits of a heterodimer, fail to be appropriately induced [five- and ninefold decrease, respectively] in denervated runx1 mutant muscle. As these keratins are thought to link Z-discs and M-lines with costameres [Ursitti et al. 2004], plasma membrane structures proposed to anchor myofibrils, a reduction in Krt1–18 and Krt2–8 expression may account for the fragmentation and misalignment of Z-discs. Moreover, these findings raise the possibility that a failure to induce myosin and keratin expression not only leads to myofibrillar disorganization, but by leading to structural perturbations, may also trigger a stress response that stimulates autophagy. Alternatively, muscle wasting may occur independently from the myofibrillar defects. As myofiber size can be regulated by signaling proteins, such as IGF-1 [Florini et al. 1991; Goldspink 1999; Musaro et al. 1999; Rommel et al. 2001], the inappropriate expression of genes encoding secreted signaling molecules may contribute to wasting of runx1 mutant denervated muscle. The failure to induce osteopontin and thrombospondin I [31-fold and fourfold decrease, respectively] raises the possibility that their induction may be required to counter-balance the loss of innervation-dependent growth signals and promote muscle growth/maintenance. In addition, overexpression of orosomucoid 2 and lipocalin 2 [seven- and fivefold increase, respectively] in runx1 mutant denervated muscle is consistent with the possibility that their anomalous expression promotes muscle wasting. Further, increased expression of genes that regulate metabolism, such as resistin-like α or cytosolic acyl-CoA-thioesterase [10-fold and fivefold increase, respectively] may stimulate autophagy and contribute to muscle wasting. Given the small number of Runx1 target genes, further studies of these genes should lead to a detailed understanding of the mechanisms that regulate skeletal muscle wasting and may allow for a rational strategy to control autophagy in diseased muscle.

Mammalian Runx proteins contain motifs that allow Runx to associate with coactivators and corepressors, and Drosophila Runx proteins can indeed activate and repress transcription in vivo [Kramer et al. 1999]. Although several studies have shown that mammalian Runx proteins can repress transcription in vivo, the evidence for transcriptional activation in vivo is less compelling [Durst and Hiebert 2004; Taniuchi and Littman 2004]. We show that Runx1 is required to activate and repress gene expression in mammalian skeletal muscle, and our data indicate that these Runx1-dependent genes are direct targets for Runx1. Thus, these data indicate that mammalian Runx proteins, like their Drosophila counterparts, can activate and repress target genes in vivo.

Autophagy is responsible for the normal bulk degradation of long-lived proteins and organelles, but the program can be overactivated by a variety of stress stimuli,
presumably to facilitate cell survival during periods of acute stress (Klionsky and Emr 2000). The pathway for constructing autophagic vacuoles and delivering vacuoles to lysosomes requires the sequential action of a set of genes, identified in yeast and conserved in mammalian cells. The products of these “autophagy genes”, mTOR, class III PI3K, two ubiquitin-like, protein conjugation systems, and a cysteine protease, act in a concerted series of post-translational modification steps to form double-membrane autophagosomes and to transfer autophagic vacuoles and their contents to lysosomes (Klionsky and Emr 2000; Gozuacik and Kimchi 2004). The transcriptional changes that initiate and attenuate this autophagy program are poorly understood. Our findings indicate that Runx1 has a role in restraining pathways leading to autophagy, as a failure to up-regulate Runx1 in denervated muscle results in severe muscle wasting accompanied by hallmarks of autophagy.

Although it is currently unclear whether excessive autophagy promotes or prevents cell damage and leads to improvement or worsening of disease outcome, the presence of autophagic vacuoles is a prominent and characteristic structural feature in a variety of congenital myopathies [Engel 1999; Nishino 2003; Selcen et al. 2004], neurodegenerative disorders [Shintani and Klionsky 2004, and cancer (Gozuacik and Kimchi 2004; Shintani and Klionsky 2004). Myofibrillar myopathies, characterized by pathological defects in myofibrillar organization and accumulation of autophagic vacuoles, can be caused by mutations in Z-disc proteins, desmin [Goldfarb et al. 1998; Munoz-Marmol et al. 1998], αB-crystallin [Vicart et al. 1998; Selcen and Engel 2003], or myotilin [Selcen and Engel 2004]. Moreover, Danon’s disease, which is also typified by the presence of autophagic vacuoles, is caused by mutations in LAMP-2, a lysosomal membrane protein [Nishino et al. 2000; Tanaka et al. 2000]. The genes responsible for most congenital myopathies, however, are not yet known. Our findings demonstrate an unexpected role for electrical activity in regulating autophagy and raise the possibility that reductions in muscle activity could cause or contribute to these static muscle-wasting diseases if expression of Runx1 or Runx1-target genes were compromised. Moreover, these findings raise the intriguing possibility that congenital myopathies, which do not follow simple Mendelian inheritance or become evident only late in life, may require two initiating events: a decrease in muscle activity due to trauma, aging, or immobilization, as well as a mutation in Runx1 or its target genes.

Materials and methods

Hind-limb muscles of adult mice were denervated by cutting the sciatic nerve. Mice were perfused with fixative (1% glutaraldehyde, 4% formaldehyde in 0.1M sodium phosphate at pH 7.3), and dissected muscles were immersion-fixed for a further 1 h. The fixed muscles were treated with 1% osmium for 1 h, stained en bloc with saturated aqueous uranyl acetate for 1 h, dehydrated, and embedded in Epon. For light microscopy, cross-sections of 2 wk-denervated muscles were stained with toluidine blue; images were captured on a Sony DRC-500 camera, and the cross-sectional area of individual myofibers was measured using NIH Image. We measured muscle wet weight and myofiber size from three mutant and control mice. For electron microscopy, longitudinal sections of 2 wk-denervated muscle were stained with uranyl acetate and lead citrate.

Runx1 RNA expression in innervated and denervated gastrocnemius muscles was measured by RNase protection [Zhu et al. 1994], a uniformly labeled RNA probe, complimentary to the sequences encoded by exons 4, 5, and 18 bp from exon 3, was hybridized to RNA isolated from innervated or 4-d-denervated muscle of MCK::cre, runx1+/− mice, or runx1+/− control mice. Runx1+/− mice were generated by crossing runx1+/− mice with CMV::cre mice. Expression of MuRF1 (XM_354614, nucleotides 2104–2372), Aqp4 (NM_009700, nucleotides 1406–1672), and Gapd (XM_009604, nucleotides 172–471) were measured by RNase protection (Blagden et al. 2004).

Affymetrix mouse genome 430 2.0 microarrays were probed with cDNA copied from RNA isolated from innervated and 3-d-denervated tibialis anterior muscles from runx1+/− control and MCK::cre, runx1+/− male mice. Experiments were performed on three separate microarrays for each of the four experimental conditions. The chips were scanned with an Affymetrix GeneChip Scanner 3000, and the raw data (http://arrayconsortium.tgen.org/np2/viewProject.do?action=viewProject&projectId=64764) were processed with Affymetrix GCOS software. The signals were normalized and analyzed by dChip [Parmigiani 2003]. We calculated the ratio of expression in denervated/innervated muscle for runx1+/− control and MCK::cre, runx1+/− mice.

Results from the microarray screen were validated by measuring expression of selected target genes using an RNase protection assay: Myh2 [NM_144961, nucleotides 1650–1910], Myh3 [XM_354614, nucleotides 2104–2372], Aqp4 [NM_009700, nucleotides 445–895], Accn1 [NM_007384, nucleotides 1406–1672], Tbias1 [NM_011580, nucleotides 298–548], Clarg [NM_009604, nucleotides 696–1244], and Scn5a [NM_021544, nucleotides 76–611].

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