

# A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion

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**Skeletal muscle is formed via fusion of myoblasts, a well-studied process in *Drosophila*. In vertebrates however, this process is less well understood, and whether there is evolutionary conservation with the proteins studied in flies is under investigation. *Sticks and stones* (*Sns*), a cell surface protein found on *Drosophila* myoblasts, has structural homology to nephrin. Nephrin is a protein expressed in kidney that is part of the filtration barrier formed by podocytes. No previous study has established any role for nephrin in skeletal muscle. We show, using two models, zebrafish and mice, that the absence of nephrin results in poorly developed muscles and incompletely fused myotubes, respectively. Although nephrin-knockout (*nephrin*<sup>KO</sup>) myoblasts exhibit prolonged activation of MAPK/ERK pathway during myogenic differentiation, expression of myogenin does not seem to be altered. Nevertheless, MAPK pathway blockade does not rescue myoblast fusion. Co-cultures of unaffected human fetal myoblasts with *nephrin*<sup>KO</sup> myoblasts or myotubes restore the formation of mature myotubes; however, the contribution of *nephrin*<sup>KO</sup> myoblasts is minimal. These studies suggest that nephrin plays a role in secondary fusion of myoblasts into nascent myotubes, thus establishing a possible functional conservation with *Drosophila Sns*.**

myoblast fusion | sticks and stones

Vertebrate skeletal muscle is a syncytium formed via two phases of myoblast fusion. During the first phase, myoblasts fuse to generate nascent myotubes that serve as scaffolds for further growth; in the second phase, more myocytes fuse into these nascent myotubes to form mature myotubes (1, 2). Myoblast fusion is a highly regulated process in *Drosophila*, where founder cells serve as the “seeds” in muscle formation and express the membrane protein *Kirre*, also known as *Dumbfounded* (*Duf*) (3, 4). Fusion-competent myoblasts (FCM) express the transmembrane protein *Sticks and stones* (*Sns*) (5) which interacts with *Kirre/Duf* (6), bringing the two muscle cell types together, to initiate a complex intra-cellular program that leads to the fusion of the FCM into the myotubes. There has been sparse evidence demonstrating whether the orthologues of these proteins are conserved in vertebrate myoblasts (7), although recent studies reported a role for *Kirrel* (*Kirre*-like) in developing zebrafish skeletal muscle (8).

Nephrin is a protein the function of which has been associated with maintenance of the kidney filtration barrier (9). Mutations in nephrin cause the congenital nephrotic syndrome of the Finnish type (10). Similarly, nephrin-knockout mice (*nephrin*<sup>KO</sup>) die at day 2 after birth of severe proteinuria (11–13). Despite the structural similarities with *Drosophila Sns*, nephrin expression or function in vertebrate skeletal muscle has not yet been reported. In the current study, nephrin was found expressed in developing mouse skeletal muscle and in human fetal muscle cells undergoing fusion. Nephrin expression is also up-regulated in the skeletal muscle of two murine models of human muscular dystrophies, when myogenic repair is needed. Downregulation of

nephrin expression in developing zebrafish results in fish unable to swim with abnormal myosepta length. Accordingly, muscle cells isolated from *nephrin*<sup>KO</sup> mice exhibit a secondary fusion defect and are unable to form mature myotubes. *Nephrin*<sup>KO</sup> myoblast cultures maintain MAPK/ERK pathway activity during differentiation, but this activation does not affect expression of myogenin, which appears normal. Pharmacological inhibition of the MAPK/ERK pathway does not restore normal myotube formation, suggesting that this pathway is likely not causative of the observed fusion deficiency. In cell mixing experiments, human myoblasts are able to fuse to *nephrin*<sup>KO</sup> nascent myotubes to form hybrid mature myotubes. Conversely, *nephrin*<sup>KO</sup> myoblasts provide little or no contribution to developing human myotubes. These studies suggest a new role for nephrin in skeletal muscle, where its expression appears to be necessary for mononucleated myoblasts to fuse into myotubes. These findings also highlight the possible functional conservation between nephrin and *Drosophila Sns*.

## Results

**Nephrin Is Expressed in Embryonic, Adult Diseased, or Adult Injured Muscle.** If nephrin is important for fusion of myoblasts into myotubes, its expression should be up-regulated when myoblast fusion is occurring. Indeed, nephrin message was present by nested reverse transcription–polymerase chain reaction (RT-PCR) in embryonic murine skeletal muscle (Fig. 1A, E15.5), in the skeletal muscle of young mice with muscular dystrophy, which exhibit spontaneous muscle degeneration/regeneration (14) (Fig. 1A, *mdx* Y (dystrophin null); *Scg* Y ( $\delta$ -sarcoglycan-null), both 3–4 weeks old) and in wild-type muscle after acute injury with cardiotoxin (Fig. 1A, Ctx). No message was detected in uninjured adult wild-type mouse skeletal muscle (Fig. 1A, Wt), in 4-month old *mdx* (Fig. 1A, *mdx* O) or  $\delta$ -sarcoglycan (Fig. 1A, *Scg* O) muscles. In multiple independent PCR reactions followed by sequence analysis, the full nephrin message, including most of exon 1a through the 3'UTR was confirmed to be the same transcript as that found in kidney (data not shown). Nephrin was also transiently up-regulated in human fetal skeletal myoblasts (isolated from 17-week-old fetus) undergoing fusion in culture for 2 days (Fig. 1C–E), after which nephrin expression returned to the low baseline levels (Fig. 1D). Thus,

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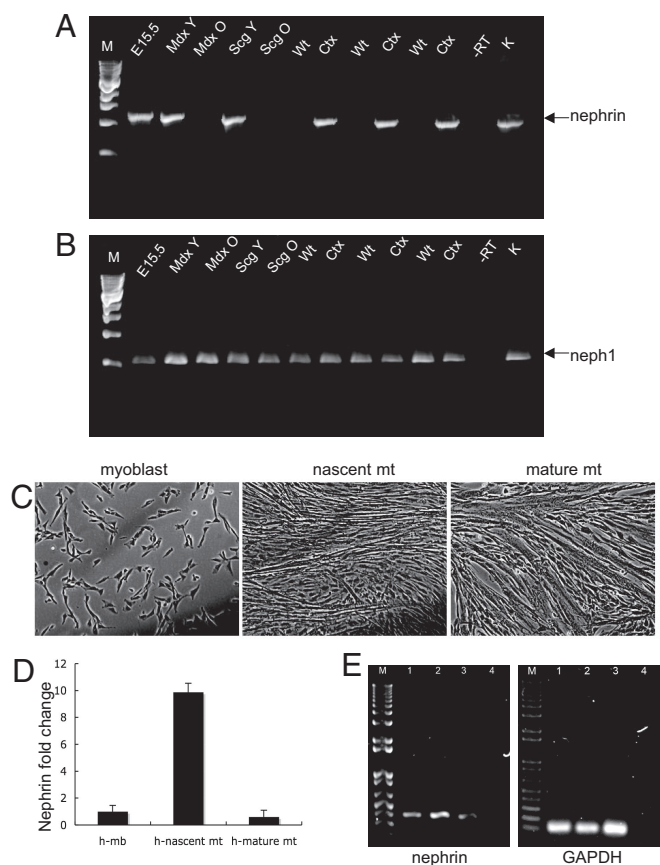
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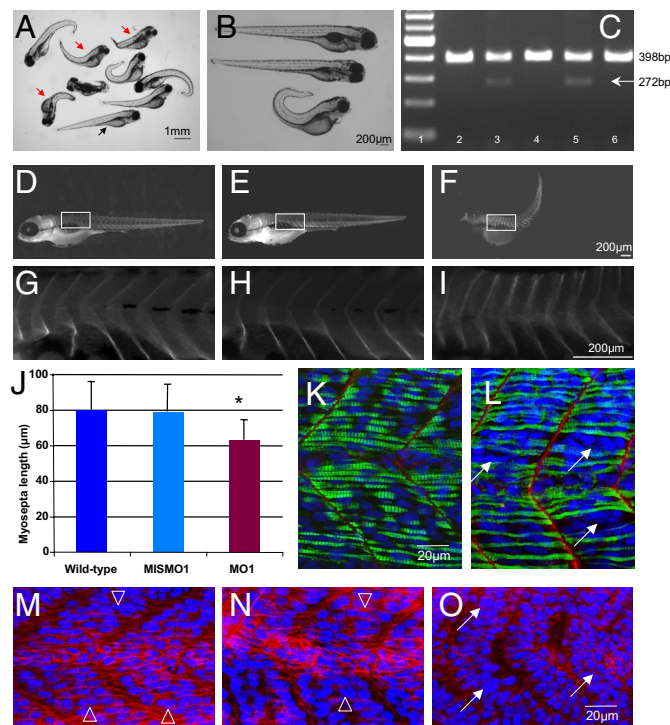
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**Fig. 1.** Nephrin mRNA is present in low quantities in vertebrate skeletal muscle. Nested RT-PCR was performed on RNA isolated from the skeletal muscles of different mice. The expected PCR products (1.2 kb) were gel purified, sequenced and confirmed to be nephrin. (A) RT-PCR product resulting from nested PCR, 1.2 kb (arrow). (B) Neph1, a protein associated with nephrin, is present throughout (expected product is 546 bp). Lanes: M, 1-kb markers; 1, embryonic skeletal muscle, E15.5; 2, mdx skeletal muscle, 3 weeks (Mdx Y); 3, mdx skeletal muscle 4 months old (Mdx O); 4,  $\delta$ -sarcoglycan null 3.5 weeks (Scg Y); 5,  $\delta$ -sarcoglycan null 1 year (Scg O); 6, 8, and 10, WT gastrocnemius, 3 months; 7, 9, and 11, contralateral WT gastrocnemius 5 days after cardiotoxin injection; 12, no RT; 13, kidney. (C) Human fetal myoblasts (17 weeks) at the indicated stages of fusion. (D) Transient up-regulation of nephrin mRNA in nascent myotubes as determined by real-time quantitative PCR analysis. The fold change value in the myoblasts was set as 1, and the fold change values in nascent and mature myotubes were plotted accordingly. GAPDH was used as a reference gene in the amplification. Mb: myoblasts; Mt: myotubes. (E) Agarose gels of end products of qRT-PCR. Lanes: M, 1kb plus markers; 1, myoblasts; 2, nascent myotubes; 3, mature myotubes; 4, negative control.

nephrin expression is exquisitely controlled and is present when myoblast fusion is expected to occur. It was also confirmed that one nephrin-associated protein, Neph1 (kirrel), is ubiquitously present in all of the muscles tested, regardless of age, disease, or injury (Fig. 1B).

**Downregulation of Nephrin Expression in Developing Zebrafish.** Zebrafish nephrin has been localized to the pronephros as in mammalian kidneys (15). Loss of nephrin by morpholino knock-down results in altered podocyte morphology and nephrosis at 96 hpf. Whether the muscles were affected was not indicated. Nephrin morpholino experiments were therefore conducted. Two morpholinos, one directed against nephrin (MO1) and a mismatched morpholino (MIS-MO1), were injected into fertilized eggs. The morpholino MO1 (kindly provided by I. Drummond) is directed against the transmembrane domain of nephrin



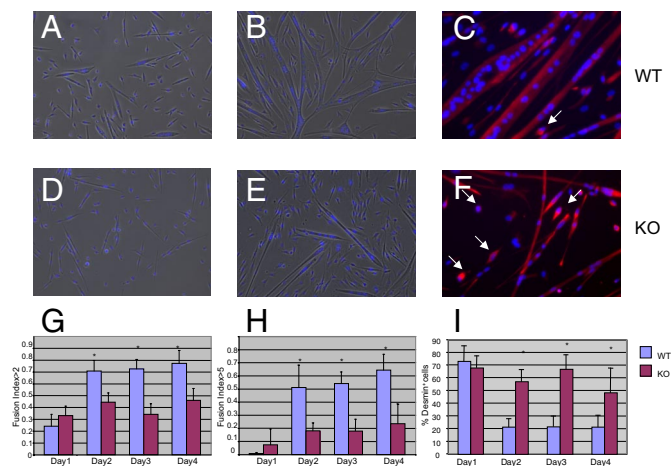
**Fig. 2.** Nephrin morpholino experiments in zebrafish reveal smaller muscles. (A) Light micrograph of variable phenotypes noted with nephrin morpholino in zebrafish embryos at 4 days post fertilization (dpf). Many of the embryos appear curved and shorter (red arrows) compared with control (black arrow). (B) Light micrograph of wild-type (top) mismatched (middle) and nephrin morpholino embryos (MO1) at 4 dpf. (C) RT-PCR analysis of mismatched and nephrin morpholino-injected embryos confirms the expected splicing defect leading to an in-frame deletion of the transmembrane domain, rendering a protein no longer able to anchor at the membrane; this is manifest by a decrease in RT-PCR product size (from 398 bp to 272 bp). Lane 1, molecular weight markers; lane 2, uninjected wild-type embryo; lane 3, 1.25 ng nephrin morpholino MO1; lane 4, 1.25 ng mismatched morpholino; lane 5, 2.5 ng nephrin morpholino MO1; lane 6, 2.5 ng mismatched morpholino. (D–I) Morphometric analyses performed on whole-mount embryos stained with anti  $\beta$ -dystroglycan antibody confirm that the myosepta to myosepta distance of embryos injected with nephrin morpholino MO1 (F, I) are smaller than those injected with mismatched morpholino (E, H) or wild-type uninjected embryo (D, G). (J) Bar graph of myosepta length measurements from wild-type, mismatched MO1 and nephrin morpholino MO1 injected embryos. Asterisk denotes a statistically significant *P* value by *t* test ( $P \leq 0.00000004$ ). Individual measurements are listed in Table S1. Whole-mount control (K) and nephrin morpholino (L) embryos stained with anti-laminin (red) and anti-myosin heavy chain antibody (green); nuclei are stained in blue with DAPI. Nephrin morpholinos displayed shorter myosepta and presence of clustered nuclei (white arrows). (M–O) Whole-mount 1 dpf embryos are stained with anti- $\beta$ -catenin antibody (red), and nuclei are stained in blue with DAPI. (M) Wild-type control; (N) control mismatched morpholino (MISMO1); (O) nephrin morpholino (MO1). Open arrows in (M) and (N) point to nuclei that appear regularly aligned, whereas arrows in (O) point to clustered nuclei.

and results in an in-frame deletion that prevents localization of nephrin to the membrane (15). The injected embryos were examined at 1 and 4 dpf (days postfertilization). Morpholino disruption of nephrin expression resulted in shorter, slightly curved embryos that were unable to swim properly and that sank to the bottom of the dish (Fig. 2A and B). Analysis of 120 embryos at 1 dpf injected with the nephrin morpholino MO1 demonstrated that 55% of the embryos were curved, short, and poor swimmers, whereas no embryos displayed this phenotype when injected with the control MIS-MO1. At 4 dpf, 37% of the embryos injected with nephrin morpholino MO1 had died, and 10% were curved and short. The embryos injected with MIS-

MO1 appeared to be normal (wild-type) (Fig. 2*B*). RT-PCR analysis confirmed the splicing defect in the embryos injected with the nephrin morpholino MO1 (Fig. 2*C*). To study whether the abnormal length of MO1-injected embryos was caused by shorter individual myosepta, the distances between myosepta was determined by microscopic analysis on whole-mount embryos at 96 hpf stained for alpha-dystroglycan (Fig. 2*D–I*). Ten embryos each were analyzed for myosepta length in non-injected control, MIS-MO1, and nephrin MO1-injected embryos. Within each embryo, five myosepta were measured starting from the same anatomical position (Fig. 2*D–I*, [supporting information (SI) Table S1]). Compared with MIS-MO1-injected embryos, the nephrin MO1-injected zebrafish displayed shorter myoseptum to myoseptum distance, consistent with a muscle defect (Fig. 2*J*;  $P < 4E-08$  via *t* test). Comparison using a *t* test of the myoseptum to myoseptum distance in wild-type compared with MO1-injected embryos was also significant ( $P < 2.08E-08$ ), whereas no significant difference was observed by comparing wild-type and MIS-MO1-injected embryos ( $P = 0.75$ ). Finally, whole-mount control, MIS-MO1-injected, or MO1-injected zebrafish embryos were stained at 1dpf using antibodies directed to laminin and myosin heavy chain (MHC, F59) (Fig. 2*K* and *L*) or with anti- $\beta$ -catenin (Fig. 2*M–O*). Wild-type (Fig. 2*K* and *M*) and MIS-MO1-injected embryos (Fig. 2*N*) displayed regularly aligned nuclei (Fig. 2*M* and *N* open arrowheads) and compacted myofibers (Fig. 2*K*). In contrast, zebrafish embryos injected with nephrin morpholino MO1 exhibited a less organized structure in several (but not all) myosepta, with compacted clusters of nuclei (Fig. 2*L* and *O*, arrows) that were not observed in wild-type or MIS-MO1-injected embryos. Therefore, downregulation of nephrin expression in developing zebrafish results in a phenotype consistent with muscle defects.

**Assessment of Myocyte Fusion in Nephrin-Knockout Myoblast Cultures.** To assess whether nephrin plays a role in myoblast fusion and determine when its expression is necessary, we turned to a murine model. Methods for propagation and differentiation of muscle cells are well established for mouse compared with zebrafish tissue (16–19). Skeletal myoblasts were isolated from neonate nephrin<sup>KO</sup> and wild-type mice, as nephrin<sup>KO</sup> mice die within the first 2 days of life because of kidney failure (11). The fusion ability of nephrin<sup>KO</sup> and wild-type cultures were assessed *in vitro*. For each culture, equal numbers of mononuclear cells were plated, allowed to differentiate and the fusion indices (FI; number of nuclei in myotubes divided by total number of nuclei) were calculated over 4 successive days. Wild-type and nephrin<sup>KO</sup> myoblasts began to fuse from day 1 (Fig. 3*A* and *D*), but by day 2 clear differences arose: more of the knockout myocytes remained mononuclear and the overall myotube size was small (Fig. 3*E* and *F*), whereas myotubes in the wild-type cultures were branching and twitching (Fig. 3*B* and *C*, twitching not shown). The nephrin<sup>KO</sup> myocytes had lower fusion indices (Fig. 3*G*) and were also inefficient in forming large myotubes (containing five or more nuclei) compared with wild-type myocytes (Fig. 3*H*). Immunohistochemistry with an antibody against desmin, a myogenic specific marker, demonstrated that the mononuclear cells in the knockout cultures were indeed myogenic, even if they were not fusing (Fig. 3*F*, arrows). Moreover, the number of desmin-positive mononuclear cells was significantly higher in knockout cultures compared with wild-type cultures at days 2, 3 and 4 in differentiation medium (Fig. 3*I*). These findings raised the question as to whether nephrin<sup>KO</sup> mononuclear cells had impaired differentiation potential because of persistent proliferative activity.

**Nephrin<sup>KO</sup> Myocytes Have Constitutively Activated MAPK/ERK Pathway, but This Does Not Seem to Affect Myogenin Expression.** Wild-type and knockout myoblasts were plated at equal numbers and



**Fig. 3.** Myoblasts isolated from nephrin<sup>KO</sup> neonatal mice are unable to form mature myotubes. Light micrographs of wild-type (*A*, *B*) and knockout (*D*, *E*) myocytes after 1 day (*A*, *D*) and 4 days (*B*, *E*) in differentiation medium. Immunofluorescence staining for desmin of wild-type (*C*) and knockout (*F*) cultures demonstrates that the mononuclear cells in the nephrin<sup>KO</sup> cultures are myogenic (white arrows). (*G*, *H*) Quantification of the fusion indices for control and nephrin<sup>KO</sup> myoblasts. The fusion index (FI) is determined by dividing the number of nuclei found within myotubes by the total number of nuclei in a microscopic field (three to four microscopic fields per sample; three independent experiments). (*G*) Overall FI, counting nuclei in myotubes containing two or more nuclei. (*H*) FI for myotubes containing five or more nuclei. (*I*) Percentage of desmin-positive mononuclear cells decreases in wild-type as the FI increases, whereas it remains the same in the nephrin<sup>KO</sup> cultures. Asterisk indicates significant differences between wild-type (lilac) and knockout (crimson),  $P < 0.05$ .

cultured under proliferation conditions. Cell numbers were counted daily for 4 days and although there was no significant difference during the first 2 days, nephrin<sup>KO</sup> cells subsequently grew much faster than wild-type cells (Fig. S1*A*). Samples from the cultures were immunostained at day 4 for desmin and the percentage of positive cells was similar in both cultures (Fig. S1*B*). Cell cycle analyses were also performed using flow cytometry, and control cultures were found to contain half the number of cells in S phase compared with nephrin<sup>KO</sup> cultures (Fig. S1*C* and *D*).

Studies by others have suggested that the p42/44 (Erk1/Erk2) MAPK pathway is necessary for the activation and proliferation of satellite cells upon injury (20, 21). To address whether the decreased fusion ability of nephrin<sup>KO</sup> myoblasts could be caused by a persistent activation of Erk1/Erk2, wild-type and nephrin<sup>KO</sup> myoblasts differentiated cultures were analyzed by Western blot using antibodies directed against total and phospho-p42/44 MAPK (Fig. S1*E–G*). Whereas the p42/44 MAPK pathway appeared nearly inactive during differentiation in control cultures, it was persistently active in the nephrin<sup>KO</sup> samples (Fig. S1*E*).

To address whether the constitutive activation of the MAPK pathway could result in slowed myogenic differentiation and, consequently, fusion of nephrin<sup>KO</sup> myocytes, wild-type and nephrin<sup>KO</sup> myoblast cultures were differentiated over the course of 4 days, and myogenin expression was monitored (Fig. S2). It was found that myogenin expression was similar in wild-type and nephrin<sup>KO</sup> cultures, suggesting that early myogenic differentiation is not altered by the persistent activation of MAPK/ERK in nephrin<sup>KO</sup> myoblasts. Next, the MAPK inhibitor PD98059 was applied during myoblast differentiation. Treatment of nephrin<sup>KO</sup> cells with 50  $\mu$ M PD98059 markedly blocked the phosphorylation of ERK1/2; however nephrin<sup>KO</sup> myotubes treated with the inhibitor remained small (Fig. S3), suggesting that the persistent





nephrin. Studies suggest that nephrin expression can be increased in the kidney by PPAR- $\gamma$  agonists, such as pioglitazone, a drug for diabetes (36). Perhaps similar treatment with myoblasts can also increase nephrin expression and thereby increase myoblast fusion. Thus, with more knowledge of the regulation of nephrin in the skeletal muscle, it may become possible to improve the efficiency of cell-based therapies for skeletal muscle diseases such as Duchenne Muscular Dystrophy.

## Materials and Methods

**RNA Isolation and RT-PCR Amplification of Nephrin mRNA.** Total RNA was isolated from primary myocyte cultures using Qiagen RNeasy kit (Qiagen) and from gastrocnemius muscles of C57Bl6, mdx5cv, delta-sarcoglycan null mice as previously described (37). Detailed methods, including cardiotoxin injury, primer sequences, and RT/PCR amplification conditions can be found in the [SI Materials and Methods](#).

**Zebrafish Morpholino Experiments.** Zebrafish experiments were performed using previously described procedures (38), with 1.25 and 2.5 ng of nephrin morpholino or mismatched morpholino oligonucleotides. Morpholino sequences, RT-PCR, and whole-mount immunostaining analyses are detailed in [SI Materials and Methods](#).

**Primary Muscle Cell Culture and Fusion Experiments.** Primary muscle cultures were derived from the limb muscles of 1–2-day-old wild-type and nephrin<sup>KO</sup> neonates as described (18). Differentiation experiments were performed by plating the myocytes at a density of  $8 \times 10^4$  cells/well in 12-well plates. Detailed information on culture conditions and determination of the fusion indices is described in [SI Materials and Methods](#).

**Cell Cycle Analysis.** Flow-cytometry analyses of the cell cycle were performed according to a previously described protocol (39). Additional details including

Western blot analyses of ERK1/2 proteins can be found in [SI Materials and Methods](#).

**Co-Culturing Experiments of Murine and Human Myoblasts.** Murine myoblasts were stained with 5  $\mu$ M CellTracker green CMFDA (Molecular Probes). Labeled mouse and human myoblasts were plated together in equal numbers and allowed to differentiate for 1 and 4 days in differentiation medium. Cells were then stained with anti-human nuclei antibody (Millipore, clone 235-1, MAB 1281). Additional information can be found in [SI Materials and Methods](#).

**Co-Cultures of Myoblasts with Nascent Myotubes.** To form nascent myotubes, mouse or human myoblasts at  $\approx 70\%$  confluency were switched to differentiation medium (DM) for 24 hours. Meanwhile, 20–30% confluent myoblasts were cultured in DM for 24 hours to produce differentiated, mononucleated cells. After 24 hours, murine myoblasts or myotubes were labeled with 5  $\mu$ M CellTracker green CMFDA. Murine and human cells were trypsinized, plated in 12-well plates at equal cell numbers, and co-cultured for another 24 hours. Cells were then fixed, permeabilized, and stained with anti-human nuclei antibody (Millipore) as detailed in [SI Materials and Methods](#).

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