Supporting Information

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SI Materials and Methods

RNA Isolation, RT-PCR, Sequencing. Total RNA was isolated from primary myocyte cultures using Qiagen RNeasy kit (Qiagen). Total RNA was also isolated from gastrocnemius muscles of C57Bl6, mdx5cv, delta-sarcoglycan null mice using TriReagent (Molecular Research Center, Inc.) as previously described (1). Cardiotoxin (from *Naja mozambica mozambica*) injury was induced via injection of 25 μl of a 50-μM solution into the gastrocnemius of the right limb of 3–4-month-old wild-type C57Bl6 mice; the left muscle was used as contralateral non-injured control. Mice were killed 5 days from the cardiotoxin injection, and the gastrocnemius muscles was isolated and snap-frozen in liquid nitrogen. All procedures on live animals were approved by the Institutional ACUC under protocols A06–11-104R and A06–09-081R. Reverse transcription was performed using the Invitrogen Superscript3 First Strand Synthesis kit for RT-PCR according to the manufacturer's instruction using Oligo-dT for primers. Amplification of full-length murine nephrin mRNA was performed in 25-μl reactions using the following primers at a final concentration of 5 μM: Forward primer, 5′-gtcgccagtccccacctcagcacctc-3′; reverse primer, 5′-gggggcaaatcggacgacaagacg-3′. These primers amplify a 2.5-kb product (bp 525-2970 from sequence AF 191090). Amplification was performed for 40 cycles at an annealing temperature of 62 °C, extension 3 minutes. Nested primers for murine nephrin were as follows: forward, 5′-ggaagccgaagccagggtgaca-3′; and reverse, 5′-gaatggggcgctctgggg tttt-3′. The primers amplify a 1.2-kb product, nucleotides 1050–2253 of AF 191090. Amplification was again performed for 40 cycles at an annealing temperature of 60 °C, extension 3 minutes, 40 cycles. Primers for mouse NEPH1 were as follows: forward, 5′-gaatgaggccatc-cccaatg-3′; and reverse, 5′-gtacctctcgctcagccac-3′. These amplify a product of 546 bp (nucleotides 705–1251 of NEPH1). PCR amplification was performed for 40 cycles at an annealing temperature of 56 °C. Real-time quantitative RT/PCR for human nephrin was performed using the SYBR Green PCR master mix kit (Applied Biosystems) under "default" conditions: 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 40 cycles of amplification at 94 °C for 15 seconds and 60 °C for 1 minute. Nephrin transcript levels were normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) transcript levels. Human nephrin primers were forward 5′-cca aca tcg ttt tca ctt gg-3′ reverse 5′-gggtggtacgacaatccacata-3′; human GAPDH primers were forward 5′ CGACACCCACTCCTCCACCT-3′ and reverse 5′-GAGGTCCACCACCCTGTTGC-3′. Reaction products were separated by gel electrophoresis and the PCR products were extracted and sequenced by the Molecular Genetics Core Facility (MRDDRC) at Children's Hospital Boston using the nested primers (above) as sequencing primers.

Zebrafish Morpholino Experiments. Zebrafish experiments were performed using procedures approved by the Institutional ACUC under protocol A06–11-107R. Morpholino experiments were performed as previously reported (2), using 1.25 and 2.5 ng of nephrin morpholino or mismatched morpholino sequences. MO1 (kindly provided by I. Drummond) was previously described (3). Mismatched MO1 sequence had five mismatches as follows: CGCTcTCgATTACgTTTCAcGCaCC. Approximately 100 fertilized eggs were injected with each morpholino or mismatched morpholino (total ≈200). RNA was isolated at 24 and 96 hpf for RT-PCR analysis to confirm the activity of the morpholino (4). Reverse transcription was performed using the Invitrogen Superscript3 First Strand Synthesis kit for RT-PCR according to the manufacturer's instructions, with Oligo-dT for primers. Amplification was performed in 50-μl reactions using the following primers at a final concentration of 5 μmol: Forward primer, 5′-CAGTCACAGGCCTTAACCCTTCAA-3′; reverse primer, 5′-CGAGGCGTTGATAAGCTCTCTGCT-3′. These primers for MO1 amplify a 398-bp product. Amplification was performed for 35 cycles at an annealing temperature of 58 °C.

For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde overnight at 4 °C, then incubated in 100% methanol overnight at −20 °C. After rehydration with methanol series, they were blocked with phosphate-buffered saline (PBS) containing 2% casein and1% bovine serum albumin (BSA) for 1 hour and incubated overnight at 4 °C with anti–β-dystroglycan (DG) antibody (diluted 1:100, Novocastra) or rabbit anti-laminin (diluted 1:100, Sigma) together with mouse anti-MHC (slow fiber) antibody (diluted 1:50, F59, abcam), or rabbit anti–β-catenin antibody (diluted 1:50, abcam). After three washes with PBS containing 0.1% Tween-20, samples were incubated with donkey anti-mouse Alexa 488-conjugated and donkey anti-rabbit Alexa 568-conjugated secondary antibodies (diluted 1:500, Invitrogen) for 1 hour at room temperature, washed in PBS as above, followed by DAPI staining (100 ng/ml in PBS) for 30 minutes at room temperature to visualize nuclei. The length between myosepta was measured with Open lab software (Improvision).

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-knockout neonates as described (5). Myocytes were grown in growth medium (DMEM-high glucose, 4.5 g/L; 20% FBS, 5 ng/ml bFGF, 100 U/ml penicillin, 100 μg/ml streptomycin) on gelatin-coated plates in humidified incubators with 5% CO_2 at 37 °C. Differentiation experiments were performed by first plating the myocytes in a 12 well plate at a density of 8×10^4 cells/well in growth medium; after 2 hours, differentiation was induced by switching to differentiation media (DMEM with low glucose 1 g/L; 2% equine serum, 100 U/ml penicillin, 100 μg/ml streptomycin). The fusion index was determined at 1–4 days later by counting the number of nuclei within myotubes and the total number of nuclei in three to five randomly chosen microscopic fields. The fusion index is the ratio of the number of nuclei within myotubes over the total number of nuclei.

Cell Cycle Analysis. Flow-cytometry analyses of the cell cycle were performed according to a previously described protocol (6). Briefly, $\approx 1 \times 10^6$ wild-type and nephrin-knockout myoblasts were trypsinized, thoroughly resuspended in 0.5 ml PBS, and fixed in 70% cold ethanol for 2 hours at –20 °C. The cells were then pelleted, washed in PBS, and stained with propidium iodide (PI)/Triton-X solution containing RNase A (10 ml of 0.1% Triton X-100 in PBS with 2 mg DNase-free RNase A (Sigma), and 200 μl of 1 mg/ml PI (Sigma). The cells were then analyzed via flow cytometry using a FACS Advantage flow cytometer (Becton Dickinson) by collecting a total of 10,000 events for each culture. Data analyses were subsequently performed using FlowJo version 6.4.7.

Western Blot Analyses. Protein lysates were obtained from 12-well culture dishes. Each well was washed with PBS, trypsinized, and

six wells for each wild-type and knockout myocyte at the specified day were pooled and collected. Each pool was then lysed on ice for 15 minutes in 0.2 ml of lysis buffer (50 mM Tris, pH7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and Halt Protease Inhibitor Mixture (Pierce Biotechnology). Cell lysates were collected and centrifuged at 15,000 *g* for 10 minutes at 4 °C. Protein concentration was determined using the BioRad Bradford assay. Protein lysates were run on the Invitrogen NuPage Novex Bis-Tris 4–12% gradient gels with Mes buffer. Gels were transferred onto nitrocellulose using the Invitrogen NuPage Transfer system. Westerns were performed using the rabbit monoclonal antibodies against phospho-ERK1/2 and non-phosphorylated ERK1/2 (Cell Signaling Technology), both at 1:1000 dilution. Densitometry analyses were performed using Adobe Photoshop software.

Immunohistochemistry. Mouse or human myocytes were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with PBS containing 1% BSA for 1 hour and incubated overnight at 4 °C with mouse anti-desmin antibody (diluted 1:50, DakoCytomation) or rabbit anti-desmin antibody (diluted 1:100, Santa Cruz) and mouse anti-myogenin antibody (diluted 1:50, DakoCytomation). After three washes with PBS, cells were incubated with donkey anti-mouse TexasRed-conjugated secondary antibody (diluted 1:100, Jackson ImmunoResearch Laboratories) or donkey anti-mouse FITC-conjugated and donkey anti-rabbit TexasRed-conjugated secondary antibodies (diluted 1:100, Jackson ImmunoResearch Laboratories) for 1 hour at room temperature and washed with PBS. Slides were then mounted in Vectashield (Vector Laboratories) supplemented with 100 ng/ml DAPI to visualize nuclei. Cells were visualized using a Nikon Eclipse E-1000 microscope, photographed using a Hamamatsu digital camera, and images were acquired using Openlab software version 3.1.5 (Improvision).

Co-Culturing Experiments of Murine and Human Myoblasts. Murine and human fetal primary muscle cells were isolated and cultured as described (5, 7). Murine myoblasts were stained with 5 μM CellTracker green CMFDA (Molecular Probes) in DMEM for 30 minutes at 37 °C, washed with PBS twice, and incubated with fresh growth medium for additional 30 minutes at 37 °C. Labeled murine and human myoblasts were trypsinized, plated together in equal numbers and allowed to differentiate for 1 and 4 days in differentiation medium (DMEM supplemented with 2% equine serum). Cells were then fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton-X100 and stained with monoclonal anti-human nuclei antibody (Millipore, clone 235–1, MAB 1281), followed by Texas Red-conjugated anti-mouse secondary antibody. Cells were visualized with a Nikon E 1000 microscope, photographed using a CCD camera (Orca ER, Hamamatsu), and processed using Openlab software version 3.1.5 (Improvision). Fusion between mouse and human myoblasts was analyzed in double-stained myotubes. Over 300 nuclei were counted to determine the fusion index and the contribution of mouse and human-derived cells to hybrid myotubes. All nuclei were stained with 100 ng/ml DAPI.

Co-Cultures of Myoblasts with Nascent Myotubes. To form nascent myotubes, murine or human myoblasts at ≈70% confluency were switched to DM for 24 hours. Meanwhile, 20–30% confluent myoblasts were cultured in DM for 24 hours to produce differentiated, mononucleated cells. After 24 hours, mouse myoblasts or myotubes were labeled with 5 μM CellTracker green CMFDA (molecular probes) for 30 minutes at 37 °C, washed twice with PBS, and incubated with fresh growth medium for another 30 minutes at 37 °C. Murine or human cells were trypsinized, plated in 12-well plates at equal cell numbers, and co-cultured for another 24 hours. Cells were then fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton-X100, and stained with monoclonal anti-human nuclei antibody (Millipore), followed by TexasRed anti-mouse secondary antibody. Cells were visualized with a Zeiss LSM 510 meta NLO Confocal microscope and analyzed with LSM software. Fusion between murine myoblasts with human myotubes or murine myotubes with human myoblasts was analyzed in double-stained myotubes with three or more nuclei and totally over 300 nuclei were counted to determine the contribution of mouse and human nuclei in hybrid myotubes. All nuclei were stained with 100 ng/ml DAPI.

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2. Guyon JR, et al. (2003) The dystrophin associated protein complex in zebrafish. *Hum Mol Genet* 12:601–615.
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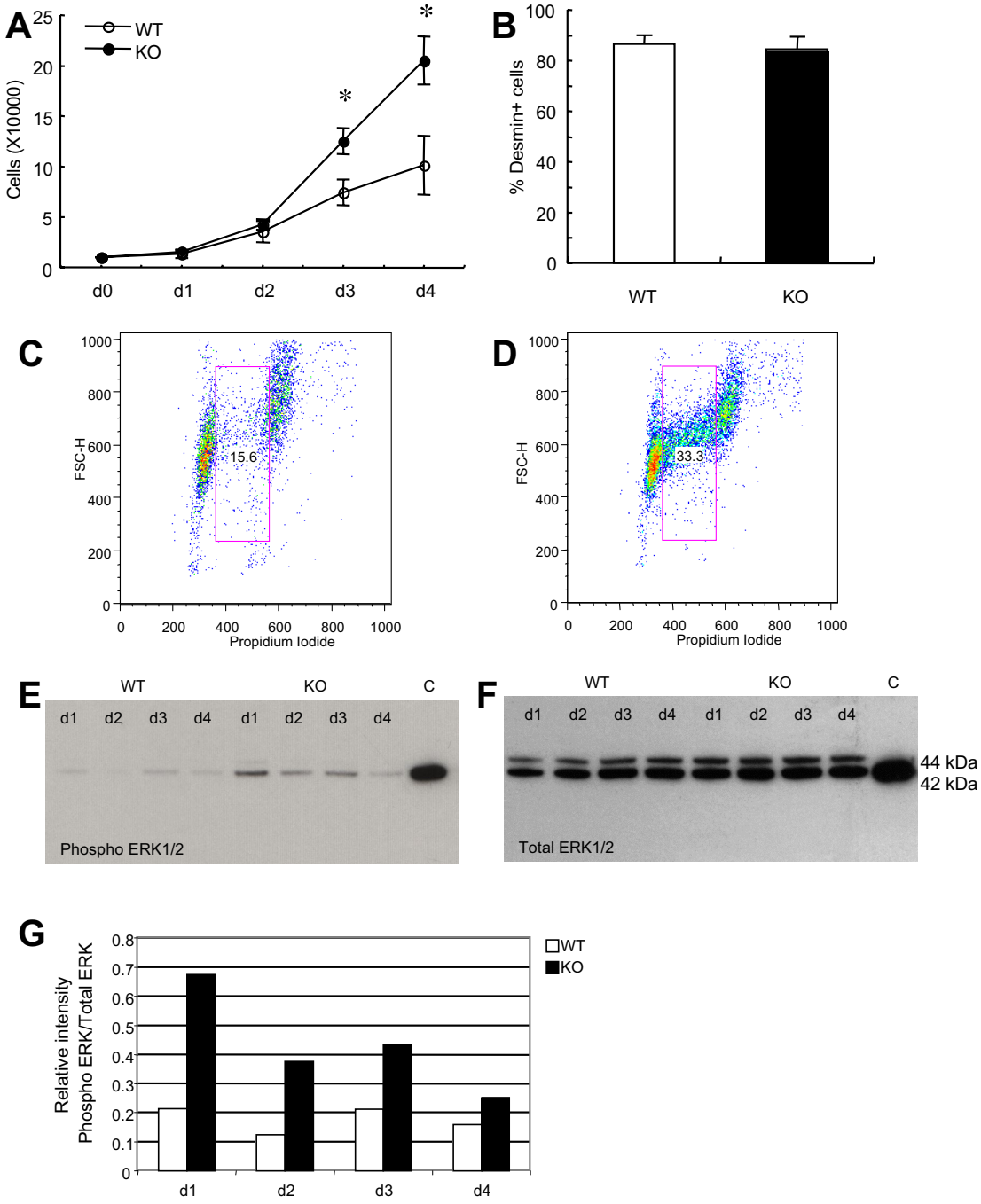

Fig. S1. Nephrin^{KO} myoblasts proliferate faster than wild-type ones and have persistently active MAPK/ERK pathway compared to wild-type cultures. (*A*) Growth curve of wild-type (open circle) and nephrin-knockout (solid circle) myoblasts maintained under proliferation conditions. Cell numbers were counted daily for 4 days. The number of mononuclear cells is significantly greater in knockout cultures compared with wild-type resulting in a 68% and a 100% increase by days 3 and 4, respectively ($n = 3$). (*B*) Samples from the cultures were immunostained at day 4 for desmin and the percentage of positive cells was similar in both cultures, indicating that the nephrin^{KO} primary cultures were myogenic and did not contain high numbers of fast-proliferating fibroblasts. The percentage of desmin-positive cells in wild-type and knockout cultures are similar ($n = 3$). Cell cycle analyses of wild-type (*C*) and nephrin^{KO} myoblast cultures (*D*) with the percentage of cells in the S phase indicated. Control cultures (*C*) contained half the number of cells in S phase compared to nephrin^{KO} cultures (*D*). (*E, F*) Western blot analysis of wild-type and nephrin knockout myoblasts during differentiation with antibodies against phospho-ERK1/2 (*E*), total ERK1/2 for loading control (*F*). Wild-type and nephrin^{KO} myoblasts were plated at equal numbers and differentiated over a period of 4 days. Protein lysates were obtained each day and Western blot analysis was performed using antibodies directed against total unphosphorylated protein as loading controls (*F*) and phospho-p42/44 MAPK (*E*). The p42/44 MAPK pathway appeared persistently active in the nephrin^{KO} samples (*E*). Lanes 1–4: wild-type myocytes; lanes 5–8: knockout myocytes at the indicated days of differentiation; lane C: positive control for antibody. (*G*) Graph of the ratio of phospho-ERK/total ERK determined by densitometry scan using the images shown in (*E*) and (*F*). These studies were repeated on an independent set of cultures and were found to be consistent.

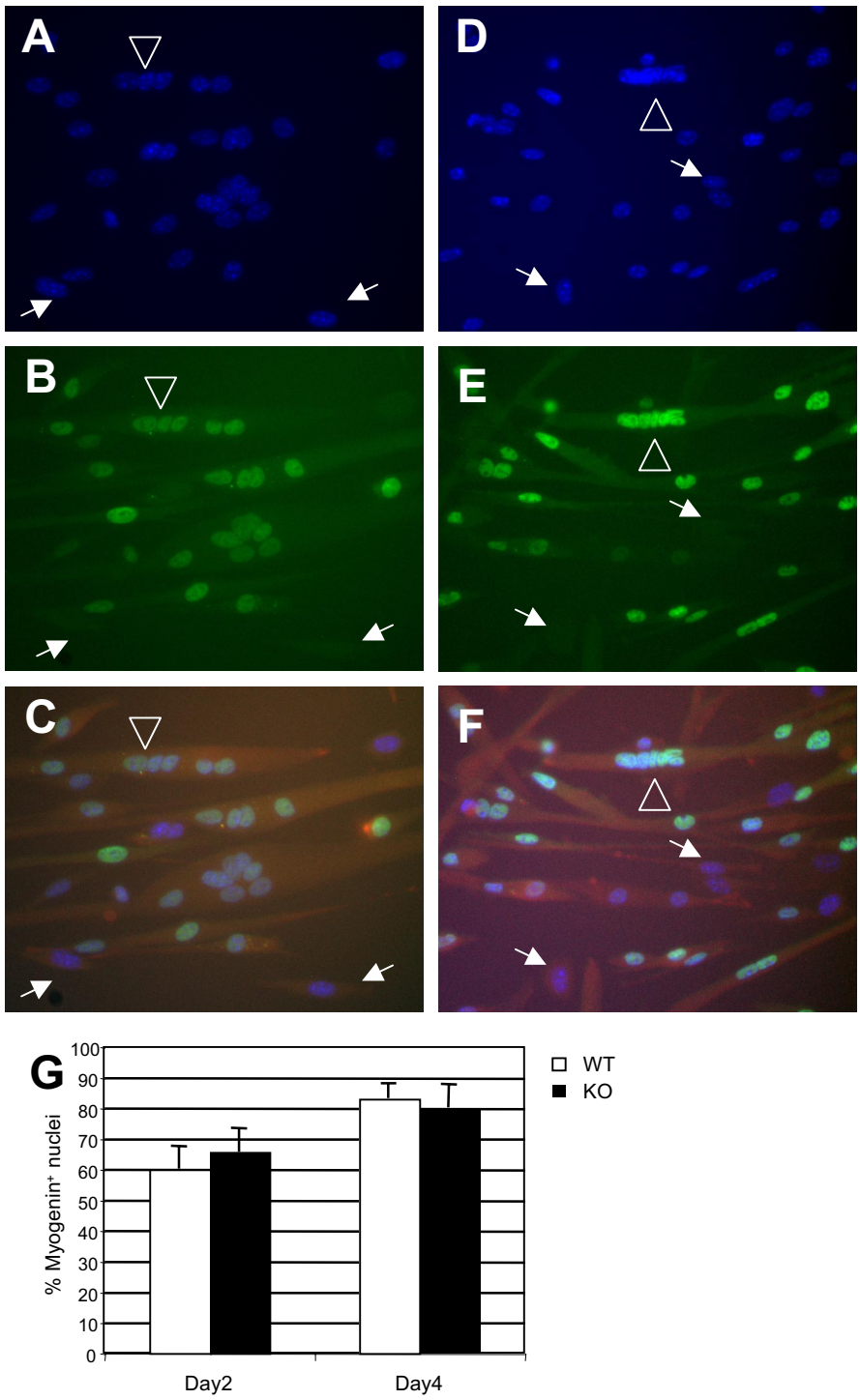

Fig. S2. Early myogenic differentiation does not seem to be affected in nephrin^{KO} myoblasts compared with control. Myoblast cultures from wild-type (*A–C*) and nephrin^{KO} (*D–F*) animals were differentiated for 4 days to promote formation of myotubes. At days 2 and 4, immunostaining for myogenin (*B, E*, green signal) was performed in conjunction with desmin detection (*C, F,* red signal) to visualize all myogenic cells and myotubes. Nuclei were stained in blue with DAPI (*A, D*). (*A–C*) and (*D–E*) are images of the same microscopic fields; (*C*) and (*F*) are merged images. Open arrowheads point to myogenin-positive nuclei, whereas arrows point to myogenin-negative nuclei in cells that express desmin. No significant differences in the percentage of myogenin-positive nuclei were observed at day 2 or 4 between wild-type and nephrin^{KO} cultures (*G*).

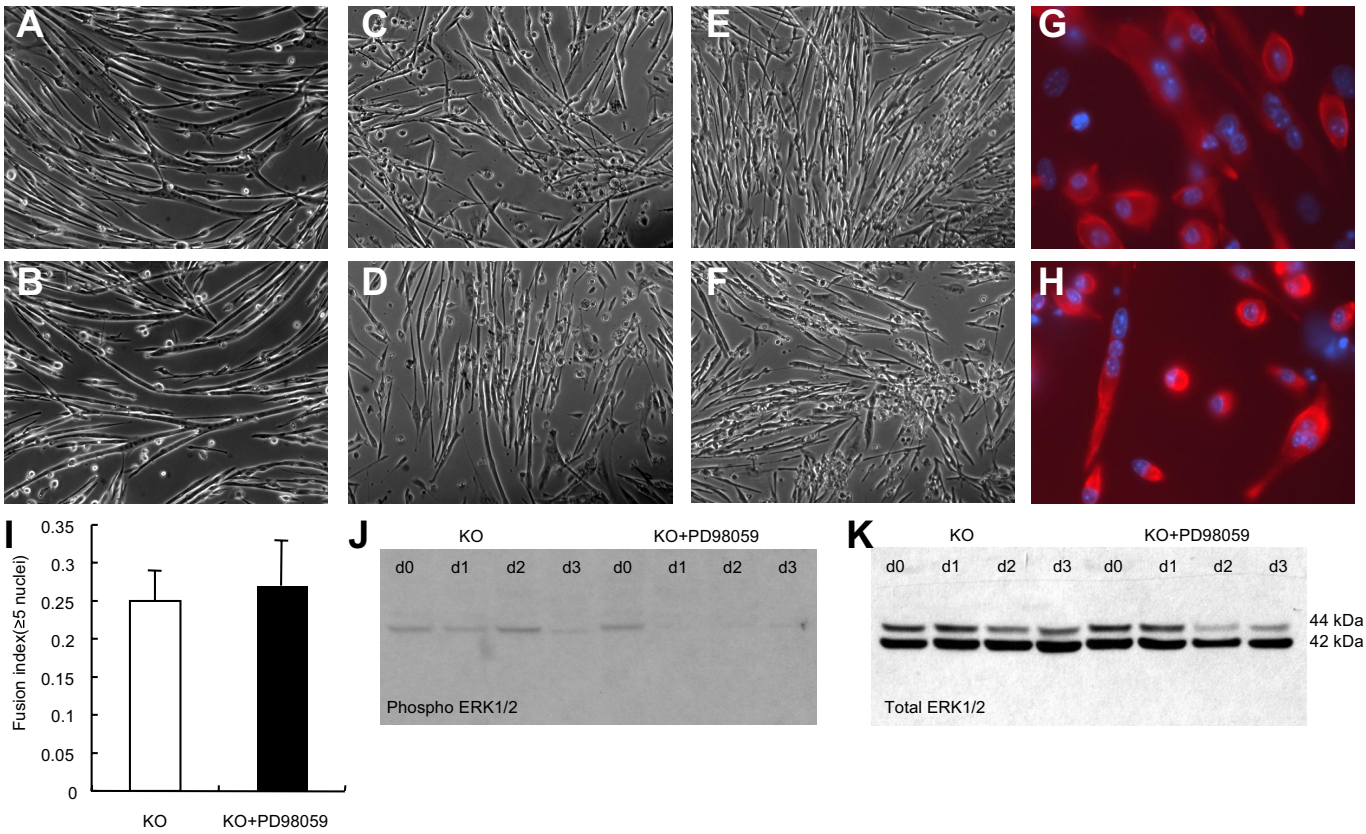

Fig. S3. Inhibition of MAPK/ERK pathway does not restore formation of mature myotubes in nephrin^{KO} cultures. Phase contrast images of wild-type (*A, B*) and nephrin^{KO} (*C–F*) myocytes cultured in differentiation medium (DM) (*A, C*) or DM supplemented with 50 μM PD98059 for 3 days (*B, D*). Nephrin^{KO} myocytes were cultured in DM for 2 days, then treated with (*F*) or without (*E*) 50 μM PD98059 for another 2 days. In none of the tested conditions did nephrin^{KO} cultures exhibit an increased fusion index compared with nontreated culture. (*G, H*) Nephrin^{KO} myoblasts cultured in DM or DM plus 50 μM PD98059 for 3 days and stained with anti-desmin antibody. Nuclei were counterstained with DAPI. (*I*) Fusion index for nephrin^{KO} myotubes containing five or more nuclei did not increase upon treatment with PD98059 inhibitor, with fusion indices of 0.27 $\pm$ 0.06 and 0.25 $\pm$ 0.04, respectively. (*J, K*) Nephrin^{KO} myoblasts were induced to differentiate for up to 3 days in the absence (lanes 1–4) or presence (lanes 5–8) of 50 μM MAPK inhibitor PD98059 and subjected to immunoblotting with phospho-ERK1/2 (J) and total ERK1/2 (K), to prove that ERK1/2 activity was downregulated by the inhibitor. Treatment of nephrin^{KO} cells with 50 μM PD98059 markedly blocked the phosphorylation of ERK1/2 (*J*).

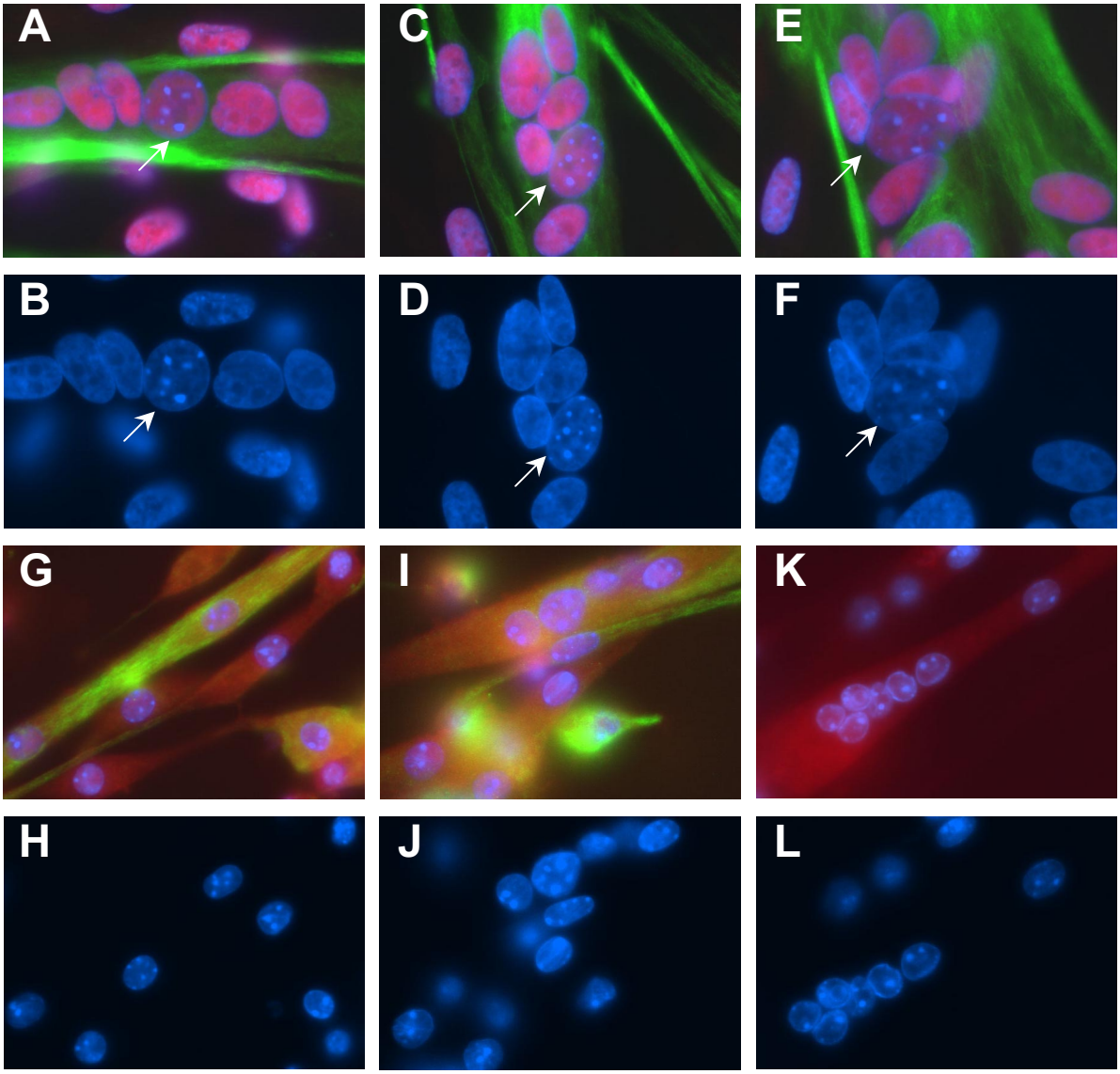

Fig. S4. DAPI staining alone does not exclusively discern mouse and human-derived nuclei. Human (*A, C, E*) and mouse (*G, I*) myotubes are immunostained in green for desmin, whereas the red signal represents specific immunoreactivity using an anti human-nuclei antibody. Nuclei are stained in blue with DAPI. Images in (*B), (D), (F), (H), (J), (L)* represent DAPI staining of (*A), (C), (E), (G), (I), (K),* respectively. White arrows indicate dotted nuclei within human cultures, demonstrating that DAPI staining alone is not reliable to distinguish mouse-derived from human-derived nuclei, whereas the immunostaining with anti-human nuclei antibody is human specific. Note that, in (*G), (I),* and (*K),* the non-specific red fluorescence detected in the cytoplasm of the myotubes and not on the nuclei. (*K, L*) are mouse-derived myotubes stained with the secondary antibody alone.

Table S1. Length of myosepta (in μm) from wild-type (uninjected), mismatched, and nephrin morpholino–injected zebrafish embryos

Fish	Wild-type						Mismatched morpholino				Nephrin morpholino			
1	57.3	52.3	52.6	56.3	55.4	59.7	52.8	58.2	59.1	54.3	47.4	42.2	49.2	47.1	43.6
2	63.3	66.3	52.3	65.3	59.7	58.3	58.7	66.5	52.1	63.2	55.4	53.1	50.8	44.2	43.3
3	66.5	72.2	53.9	65.4	66.2	61.3	64.8	69.1	60.5	74.6	62.8	54.2	59.9	60.4	55.9
4	74.7	77.4	68.6	75.3	76.1	67.2	68.2	79.4	70.1	79.1	60.1	64.8	60.2	56.5	55.5
5	80.6	80.7	70.4	75.4	82.4	77.6	76.2	76.1	70.2	79.1	75.5	61.1	69.9	64.7	63.5
6	89.5	83.3	75.5	85	88.2	87.2	79.2	85.7	72.2	89.6	69.6	62.3	57.2	74.3	62.4
7	97.2	87.6	78.5	88.3	94.3	85	82.1	90.6	82.2	90.7	77.9	61.2	69.1	70.2	63.6
8	101.2	96.3	83.2	95.1	100.3	91.2	92.5	94.6	88.9	94.1	83.9	60.8	67.4	77.7	67.1
9	102.4	98.7	92.3	96.3	100.3	91.6	95.5	101.2	96.5	101.4	88.4	61.6	65.2	73.2	70.2
10	102.4	99.4	95.8	99.2	102.2	98.4	100.1	102.3	98.7	102.3	92.9	61.2	61.5	78.3	73.6