Supplemental Experimental Procedures

Cell Lines and Reagents

C2C12, mouse myoblast cell lines were used for *in vitro* migration assay. To generate stable C2C12;H2B-GFP cell line, a cmv-H2B:GFP plasmid (generously provided by M. Viapiano, Ohio State University) was transfected into C2C12 myoblasts and positive clones were screened with Geneticin (Gibco) selection, and further sorted with a FACS Aria (BD) to enrich for a pure GFP^+ population. Immortalized mouse embryonic fibroblasts (MEFs) were isolated and generated from *p65*$^{+/+}$ and *p65*$^{-/-}$ mice as previously described (Wang et al., 2009). All cell lines were cultured in high glucose DMEM (Gibco) with 10% fetal bovine serum (ATLANTA Biologicals) supplemented with 0.1% Pen Strep (Gibco) at 37°C and 5% CO2 humidified chamber. IL-1β (R&D) at 10 ng/ml was used as an NF-κB inducer on cells grown in 10% DMEM. To differentiate C2C12 cells, differentiation media (DM) containing 2% horse serum (Gibco) and 5μg/ml insulin was used.

Mononuclear Cell Isolation and Flow cytometer Analysis

Mononuclear cell isolation from 5 to 7 P4-P7 *NF-κB*$^{EGFP/+}$ mice were performed based on the method previously described (Ieronimakis et al., 2008). In brief, whole limb muscle was digested with collagenase IV (Worthington) with 1.2 units/ml dispase (gibco) for 45min at 37°C. After inactivating enzyme with 10% DMEM, lysates were filtered through 100μm and 40μm strainers (Millipore) and collected with centrifugation. Pellets were resuspended with PBS containing 0.5% BSA and applied on top of the heat-inactivated horse serum for gradient centrifugation. Mononuclear cells on the bottom were collected for further analysis. For FACS analysis, isolated mononuclear cells were fixed with 4% paraformaldehyde and washed with PBS. Cells were incubated with blocking solution (5% FBS, 0.2% triton X-100 in PBS) on ice for 15min, followed by incubation with primary antibodies: anti-NG2 (Millipore), anti-CD31 (BD Pharmingen), anti-PDGFRα (Santa Cruz), anti-PDGFRβ (Cell Signaling), anti-F4/80 (Caltag Medsystem), anti-ER-TR7 (abCAM), and anti-Alkaline phosphatase (AP) (R&D) in blocking solution for 30min on ice. After washing three times with PBS, fluorescent-conjugated secondary antibodies: anti-Rabbit IgG PE (eBioscience), anti-Rat IgG PE (eBioscience), anti-mouse Alexa647 (Invitrogen), and anti-rabbit Alexa647 (Invitrogen) were incubated for 30min in blocking solution on ice followed by washing with PBS. Around 1×10^6 cells were stained for each cellular marker and for statistical analysis, experiments were performed in triplicate. Stained cells were analyzed with FACSCaliber (BD biosciences) or Amnis (Amnis Corporation) flow cytometers.

$NG2^+$ and $CD31^+$ Cell Isolation

Primary $NG2^+$ and $CD31^+$ cell isolations was performed with streptavidin-coated magnetic beads. Mononuclear cell isolation from P4-P7 wild type mice was performed as described above. For preparation of antibody coated magnetic beads, biotin conjugated CD31 (BD Pharmingen) and NG2 (Millipore) antibodies were incubated with dynabeads (Invitrogen) first, followed by washing and incubation with isolated cells. After washing cell-beads complex, cells were released from magnetic beads and further grown on 0.2% gelatin coated plates for expansion. To enrich for a pure population for each cell type, after cells were collected following 1 or 2 weeks of culturing, and stained with CD31 (BD Pharmingen) or NG2 (Millipore) antibodies for flow sorting (FACSAria BD). For *p65*$^{+/+}$ and *p65*$^{-/-}$ $NG2^+$ cell preparation, whole limb muscles from P2-P5 *p65*$^{+/+}$*;TNFRα*$^{-/-}$ or *p65*$^{-/-}$*;TNFRα*$^{-/-}$ mice were digested with collagenase p (Roche) for 45min with 1.2 units/ml dispase (gibco). After inactivating enzymes with 10% DMEM, cells were spun down and plated on non-coated plates for 2h with 10% DMEM. Cells attached to the plate during this pre-plating were collected for further analysis.

Alkaline Phosphatase Activity Staining

To measure alkaline phosphatase activity on muscle sections, a Red Alkaline Phosphatase Substrate kit (VECTOR laboratories) was used according to manufacturer's protocol. In brief, muscle sections were dried at room temperature before staining. Substrate solutions were prepared with 100mM Tris-HCl, pH 8.2 buffer and applied to sections for 25min at room temperature. Sections were washed with 100mM Tris-HCl, pH 8.2 buffer for 5min followed by rinsing with water. Sections were stained with DAPI and mounted with Fluorogel (Electron Microscopy Sciences). The images were obtained with either an Olympus FV 1000 Spectral or filter confocal laser microscope and analyzed by Image J and FV10-ASW 4.0 viewer.

Immunofluorescence Staining with Antigen Retrieval

Sections were air-dried and incubated with 4% paraformaldehyde for fixation at room temperature, followed by incubation with 95-100°C Sodium Citrate antigen retrieval buffer for 30min. After incubating slides in a blocking buffer at 4°C for 4h, primary antibodies: anti-Ki67 (Dako), anti-MANDYS1 (3B7-IgG2a, MDA Monoclonal

Antibody Resource) (Nguyen thi et al., 1990; Nguyen and Morris, 1993), anti-α laminin (Sigma), and anti-Pax7 (DSHB) were diluted in a blocking buffer and incubated for overnight at 4°C. After washing three times with PBS, fluorescent-conjugated secondary antibodies (Invitrogen) were incubated for 1h at room temperature. The slides were rinsed with PBS and stained with DAPI solutions. The images were obtained with an Olympus FV 1000 Spectral confocal laser microscope.

***In vitro* Migration Assays**

Boyden chamber assays were performed with an 8.0μm culture plate insert in a 12mm diameter Millicell (Millipore). Cells were seeded as illustrated in figure 1F and C2C12-H2B:GFP cells were allowed to migrate for 24h in freshly changed DM media. For treatment-induced migration experiments, C2C12 myoblasts were seeded on the inserts and next day, 1 μg/ml Fc-clustered recombinant ephrins (R&D) or 1-3 μM SNP (Sigma-Aldrich) were treated in DM media. After 2h of migration, inserts were harvested and fixed in pre-chilled methanol at -20°C for 10min. After washing with PBS, 2% crystal violet solutions were used for staining at room temperature for 15min. After wiping out the inside of the membrane, the chambers were air-dried and membranes were isolated incubated with 10% SDS for 10min at 55°C to release the crystal violet stain. Absorbance was measured at 590nm with a multiwall plate reader. For two-chamber migration assays, two-chamber inserts (ibidi) were attached to 35mm culture plate and 1.2×10^4 C2C12 cells were seeded on one side and differentiated for 3 days in DM prior to the addition of 0.6×10^4 of each C2C12-H2B:GFP and $NG2^+$ cells, or MEFs transfected with selected siRNAs to the other side of the two-chamber system. The next day, the chamber was removed and the 35mm plate was washed with PBS and incubated with DM media for 24h to allow the migration of C2C12-H2B:GFP myoblasts for 24h. Plates were then fixed with 4% paraformaldehyde after washing with PBS and further stained with troponin T (sigma) and DAPI as described above. The numbers of C2C12-H2B:GFP myoblasts present in the gap area were scored by selecting three random fields. For statistical analysis, all migration assays were performed in triplicate.

Quantitative Real Time PCR

RNA was isolated with Trizol (Invitrogen) and 1μg of RNA were used for synthesizing cDNA using M-MuLV Reverse transcriptase (NEB) and oligo dT (IDTdna). All Real-time PCR reactions were performed using SYBR Green (Roche) with designated primers sets in a Step-One PLUS Real-time PCR reaction (Applied Bioscience). For normalization, the housekeeping gene, *gapdh,* was used and the ΔΔCt method was applied for all real-time PCR analysis.

***siRNA* Transfections**

EFNA5 (Dharmacon) and *EFNB1* (Dharmacon) siRNA pools were purchased from Thermo Scientific and 100pmole of each siRNA were transfected into MEFs plated on 35mm dishes with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After 48h, half of the cells were used for RNA extraction to check for siRNA efficiency while remaining cells were used for a two chamber migration assay as described above. Scrambled siRNA (Dharmacon) was used as a control.

$NG2^+$ cell staining for whole mount EDL

Whole P14 EDL muscles were harvested and incubated at 4% paraformaldehyde overnight. After washing with PBS three times, 0.3% Triton X-100 was used for permeabilization for 15min followed by an incubation with blocking solution (5% FBS, 0.2% triton X-100 in PBS). Anti-NG2 (Millipore) and anti-laminin (Sigma) antibodies were incubated overnight at 4°C. Next day, after washing three times with PBS, fluorescent-conjugated secondary antibodies (Invitrogen) were incubated for 1h at room temperature. The slides were rinsed with PBS and stained with DAPI solutions. The images were obtained with either an Olympus FV 1000 Spectral or filter confocal laser microscope.

BrdU Injection and Staining of EDL Muscles

BrdU (20 mg/pup) was subcutaneously injected to P2 mice along with tamoxifen, and EDL muscles were subsequently harvested at P30. Frozen EDL muscles were prepared as described above and ~200 serial sections, 10μm in thickness, were prepared starting from the end of EDL to the midline of the muscle. We defined the midline of the EDL as approximately 1200 ~ 1800μm from the end of the muscle. All $BrdU^+$ myonuclei were counted and normalized to fiber numbers from at least three sections. Approximately, 60 to 120 myonuclei from 40 to 80 fibers were analyzed per section. For BrdU immunostaining, sections were dried at room temperature and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, sections were permeabilized with 0.3% Triton X-100 for 15min. Freshly made 2N HCl was applied to sections to denature DNA at 37°C for 30min followed

by 0.1M borate buffer for neutralization. After washing with PBS, blocking solution containing 0.5% Tween-20 and 2% goat serum was applied for 30min. Sections were incubated with primary anti-BrdU (AbD Serotec) and anti-α laminin (Sigma) antibodies for 1h followed by PBS washing and designated fluorescence conjugated secondary antibodies (Invitrogen). The slides were then rinsed and stained with DAPI for mounting with coverslips. For BrdU staining of whole-mount EDL muscles, P14 and P21 EDL were incubated in 4% paraformaldehyde for overnight followed by the same procedure described above. Images were taken with a confocal laser microscopy and analyzed by Image J and FV10-ASW 4.0 viewer. For statistical analysis, at least three mice were used for each genotype and time point.

ChIP Assays

MEF cells or primary $NG2^+$ cells were treated with IL-1β for 1h in 10% DMEM and fixed with 1% formaldehyde for 10min at 37°C. After adding glycin to inactivate formaldehyde for 5min at room temperature, cells were lysed with SDS lysis buffer and further sonicated using a Bio-Roptor (diagenode) at 4°C with 10-13 cycles of 30s ON/OFF. After pre-clearing DNA bound protein complexes with protein A magnetic beads (Invitrogen) and tRNA, the complexes were immunoprecipitated with anti-p65 antibody (Millipore) and IgG (Sigma) for overnight at 4°C. Next day, immunocomplexes were immobilized with 40μl of protein A magnetic beads (Invitrogen) for 2-3h at 4°C followed by stringent washes and elution. Decrosslinking of eluted DNA was performed with Proteinase K (Denville) at 65°C overnight. The next day, recovered DNA was further purified with a PCR purification kit (Thermo) and used for quantitative real-time PCR using SYBR green (Roche). Each NF-κB binding site was amplified with primer sets of -2969, -1460, and +163 as shown below. Relative p65 binding was calculated as fold increase from IgG binding after normalization to Input.

Time-lapse Live Cell Imaging

All time-lapse live cell imaging was conducted in 37°C and 5% CO2 conditions. Primary isolated $CD31^+$ and $NG2^+$ cells or $p65^{+/+}$ and $p65^{-/-}$ $NG2^+$ cells were plated with C2C12-H2B:GFP myoblasts separately at a 1:1 ratio in non-coated 6 well or 12 well plates. Next day, media was changed with DM and time-lapse live cell imaging was conducted with IncuCyte Zoom (Essen Bioscience) for 10h by collecting images every 30 or 45min. Each image was collected and analyzed using MTrackJ software (provided by Image J), tracking C2C12-H2B:GFP myoblasts following their contact with $CD31^+$ or $p65^{+/+}$ and $p65^{-/-}$ $NG2^+$ cells. Each dot shown in Figures 1E and 2A represent single cell movements collected from duplicated live cell imaging experiments. This information was transferred and further analyzed to generate trajectory graphs and velocity plots using Chemotaxis software. 3 to 10 cells from duplicated co-culture experiments were tracked and average velocities were calculated for each co-culture condition. To generate live cell images for two-chamber migration assays, a two-chamber insert (ibidi) was prepared as described earlier. $NG2^+$ cells were tracked with Cell Tracker Orange CMTMR (Invitrogen), and images were collected immediately after the removal of the chamber.

Luciferase Assays

For luciferase assays, a 580 bp from the *EFNA5* promoter that included the +163 NF-κB binding site was cloned into a pGL2-sv40 promoter plasmid (Promega). For construction of a mutant reporter construct, the +163 site was mutated to GCCGATCCAC by amplifying the *EFNA5* promoter with primers containing mutated sequences. 0.75×10^5 MEFs were plated on 12 well plates and the next day 250ng of wild type or mutant plasmid was transfected using Lipofectamine (Invitrogen) according to manufacturer's protocol. Transfection efficiency was controlled with transfections including an sv40-β-gal construct. 48h after transfection, cells were lysed with mammalian cell protein isolation buffer (Thermo scientific) and luciferase activity was measured by incubating the lysates with luciferin (Sigma-Aldrich), according to manufacturer's protocol. Relative luciferase activity was calculated by normalizing to β-gal activity measured from 2% of total lysates incubated with *ortho*-Nitrophenyl-β-galactoside (ONPG) (Sigma-Aldrich).

Microarray and RNA Seq

RNA were prepared from whole hindlimb muscle of P2, P7, and P14 wild type mice with Trizol (Invitrogen) according to manufacturer's protocol and RNA was further purified using an RNA easy kit (Quiagen). RNA concentration was determined using the RNA 6000 Nano assay on the Agilent Bioanalyzer (Agilent Technology). Affymetrix GeneChIP Mouse Gene 2.0 ST (Affymetrix) arrays were used for microarray. Probe intensity (CEL) files were obtained and used for the array analysis. Gene summary expression estimates were retrieved using Robust multi-array average (RMA) method from probe level data after back ground correction and quantile normalization

with Partek software (http://www.r-project.org/). RNAseq was done by BGI using the Hiseq2000 Illumina platform (Illumina). Raw reads were mapped to the reference genome of mouse from the latest GRCm[38]/mm[10]. To quantify the normalized counts, Cufflinks software from the tuxedo suite (http://cufflinks.cbcb.umd.edu/) was utilized and differential expression analysis was performed to analyze fold changes among different time points.

Primers used in this study

Real-time PCR primers		5' to 3'
Ephrin a1	F	AGCTACTACTACATCTCCAAACCT
	R	ATCTGCTTGGAGTCTCTTCTCT
Ephrin a2	F	CCAACTCTTCACCCCCTTTT
	R	GCTCTGGAGCCTCATACAGG
Ephrin a3	F	GCACTGGAAGTGTCTGAGGAT
	R	TCAAAGTCTTCCAACACGTT
Ephrin a4	F	CTTTGAGTTCTTGCCTGGA
	R	AGCTGACTCATGTGATGA
Ephrin a5	F	GCTACGCCGTCTACTGGAAC
	R	CTTGAACCCTTTGGAGGTGT
Ephrin b1	F	TTACATCAACGTCCAATGGGA
	R	AGTGTTGTCTGACTCCTTGCT
Ephrin b2	F	GAGCTAGAAGCTGGTACAAATG
	R	ATAAGGCCACTTCGGAAC
Ephrin b3	F	ATGTGACCGGCCAGACCT
	R	TTGCAAGCTCTCAAGGCCTT
ChIP assay primers		5' to 3'
EFNA5 -2969	F	GTCTGGACGCTTCCAAATTTCTC
	R	TCCTCTCAACACTGCGTCTTTTC
EFNA5 -1480	F	ATGACCTGGGCCGCGGAGG
	R	TTAATGACCTGCGTGTAAAGCCG
EFNA5 +163	F	TCGCCCCTTCATCGTATTTA
	R	CCTGGCTGAACACACACATC

Supplemental References

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