Neogenin Regulates Skeletal Myofiber Size and Focal Adhesion Kinase and Extracellular Signal-regulated Kinase Activities In Vivo and In Vitro

Gyu-Un Bae,^{*†‡} Youn-Joo Yang,^{*†‡} Guoying Jiang,^{*} Mingi Hong,^{*} Hye-Jin Lee,[†] Marc Tessier-Lavigne,[§] Jong-Sun Kang,^{*†} and Robert S. Krauss^{*}

^{*}Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029; [†]Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, South Korea; and [§]Division of Research, Genentech, Inc., South San Francisco, CA 94080

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A variety of signaling pathways participate in the development of skeletal muscle, but the extracellular cues that regulate such pathways in myofiber formation are not well understood. Neogenin is a receptor for ligands of the netrin and repulsive guidance molecule (RGM) families involved in axon guidance. We reported previously that neogenin promoted myotube formation by C2C12 myoblasts in vitro and that the related protein Cdo (also Cdon) was a potential neogenin coreceptor in myoblasts. We report here that mice homozygous for a gene-trap mutation in the *Neo1* locus (encoding neogenin) develop myotomes normally but have small myofibers at embryonic day 18.5 and at 3 wk of age. Similarly, cultured myoblasts derived from such animals form smaller myotubes with fewer nuclei than myoblasts from control animals. These in vivo and in vitro defects are associated with low levels of the activated forms of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK), both known to be involved in myotube formation, and inefficient expression of certain muscle-specific proteins. Recombinant netrin-2 activates FAK and ERK in cultured myoblasts in a neogenin- and Cdo-dependent manner, whereas recombinant RGMc displays lesser ability to activate these kinases. Together, netrin-neogenin signaling is an important extracellular cue in regulation of myogenic differentiation and myofiber size.

INTRODUCTION

Skeletal muscle is the most abundant tissue, by mass, in the vertebrate body. Muscles of the trunk and limbs arise from the somites, with myogenic progenitor cells derived from the dorsal region of the maturing somite, the dermomyotome (Tajbakhsh and Buckingham, 2000; Pownall *et al.*, 2002; Charge and Rudnicki, 2004). In response to signals from the adjacent notochord, neural tube, and surface ectoderm, some dermomyotomal progenitors become committed to the muscle lineage and form the myotome, a set of differentiated muscle cells that underlies the dermomyotome. Subsequent embryonic, fetal, and postnatal stages of myogenesis are thought to involve additional muscle progenitors that migrate from the dermomyotome and ultimately establish the trunk and limb musculature, as well as satellite cells, adult muscle precursor cells (Gros *et al.*, 2005; Kassam-Duchossoy *et al.*, 2005; Relaix *et al.*, 2005).

Somitic progenitor cells are specified to become muscle lineage-committed myoblasts through the action of the myogenic basic helix-loop-helix transcription factors Myf5, MRF4, and MyoD, whereas differentiation of myoblasts is

regulated by myogenin, MyoD, and MRF4 (Tajbakhsh, 2005). These and other transcription factors coordinate the process of differentiation, including cell cycle withdrawal, expression of muscle-specific proteins, cellular elongation, and fusion into multinucleated myofibers. Although transcriptional regulation is at the core of myogenesis, the formation and growth of myofibers is also controlled by a variety of signaling ligands and their receptors, including insulin-like growth factor-1, fibroblast growth factors, Wnts, transforming growth factor- β superfamily members, and others (Tajbakhsh and Buckingham, 2000; Pownall *et al.*, 2002; Charge and Rudnicki, 2004).

Neogenin is a vertebrate member of an evolutionarily conserved subfamily of immunoglobulin (Ig) superfamily receptors (Cole *et al.*, 2007; Wilson and Key, 2007). Other members of this subfamily include, in vertebrates, deleted in colorectal cancer (DCC); in *Drosophila*, Frazzled; and in *Caenorhabditis elegans*, UNC40 (Huber *et al.*, 2003; Round and Stein, 2007). All these receptors contain an ectodomain of four Ig repeats followed by six fibronectin type III repeats, a single pass transmembrane region, and a divergent cytoplasmic tail that lacks known catalytic activity but which harbors short, highly conserved sequences that bind signaling proteins (Huber *et al.*, 2003; Round and Stein, 2007). The members of this family share a role as receptors for netrins, a group of secreted proteins related to laminins that associate with cell membranes and extracellular matrix; the best understood functions of netrins and their DCC/UNC40 family receptors are as regulators of axon guidance (Huber *et al.*, 2003; Round and Stein, 2007).

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[†] These authors contributed equally to this work.

Address correspondence to: Robert S. Krauss (robert.krauss@mssm.edu) or Jong-Sun Kang (jskang@med.skku.ac.kr).

The intracellular regions of DCC and neogenin interact with FAK and the Src family kinase, Fyn, and stimulation of mammalian cortical neurons with netrin-1 in vitro results in tyrosine phosphorylation of both the kinases and the receptors (Li *et al.*, 2004; Liu *et al.*, 2004; Ren *et al.*, 2004). Furthermore, FAK and the activity of Src family kinases (such as Fyn) are required for netrin-mediated turning of axonal growth cones (Li *et al.*, 2004; Liu *et al.*, 2004; Ren *et al.*, 2004). Netrin-1 treatment of neurons also leads to DCC-dependent activation of ERK mitogen-activated protein kinase (MAPK) and inhibition of ERK signaling blocks neurite outgrowth and growth cone turning in response to a netrin gradient in vitro (Forcet *et al.*, 2002; Campbell and Holt, 2003). Additional signaling pathways are also activated by netrin-1 signaling via DCC and neogenin (Xie *et al.*, 2005; Zhu *et al.*, 2007; Li *et al.*, 2008).

Although neogenin and DCC are similar in their signaling responses to netrin-1 (although not identical, see Xie *et al.*, 2006; Ren *et al.*, 2008), neogenin plays a broader role in development than DCC. First, neogenin, but not DCC, binds to members of a second family of ligands, the GPI-anchored repulsive guidance molecules (RGMa, RGMb, and RGMc) (Rajagopalan *et al.*, 2004; Zhang *et al.*, 2005). Neogenin functions as a receptor for RGMa in axonal chemorepulsion and neuronal survival and differentiation (Matsunaga *et al.*, 2004; Rajagopalan *et al.*, 2004; Matsunaga *et al.*, 2006; Conrad *et al.*, 2007). Furthermore, RGMa-neogenin signaling is required for neural tube closure in *Xenopus* embryos (Kee *et al.*, 2008). RGMc (also known as hemojuvelin [Hjv] or Hfe2) is expressed mainly in liver and skeletal muscle and is involved in iron metabolism (Kuninger *et al.*, 2004; Niederkofler *et al.*, 2004; Oldekamp *et al.*, 2004; Papanikolaou *et al.*, 2004). RGMs also function as bone morphogenetic protein (BMP) coreceptors, and RGMc is required for BMP-mediated induction of hepcidin, a key regulator of iron homeostasis (Babitt *et al.*, 2006; Xia *et al.*, 2008). Neogenin mediates shedding of RGMc from the cell surface, and soluble RGMc inhibits BMP-induced expression of hepcidin (Babitt *et al.*, 2007; Zhang *et al.*, 2007).

Second, although DCC is expressed mainly in the central nervous system (CNS), neogenin is expressed widely during development (Gad *et al.*, 1997). In the developing mammary gland, netrin-1 is expressed in preluminal cells and neogenin is expressed in adjacent cap cells of terminal end buds; mice lacking either factor display cell dissociation and tissue disorganization from loss of netrin-1/neogenin adhesive interactions (Srinivasan *et al.*, 2003). Furthermore, vascular smooth muscle cells are attracted by and adhere to netrin-1 in a neogenin-dependent manner (Park *et al.*, 2004). Neogenin morphant zebrafish display an aberrant neural tube and defects in somitogenesis, suggesting possible roles in convergent extension and/or cell polarity during formation of the rostrocaudal axis (Mawdsley *et al.*, 2004).

Neogenin is expressed throughout the developing mouse embryo but is at high levels in developing skeletal muscle; netrin-1, netrin-3, and RGMc are also expressed in pre-muscle masses of the mouse embryo (Gad *et al.*, 1997; Keeling *et al.*, 1997; Wang *et al.*, 1999; Kuninger *et al.*, 2004; Niederkofler *et al.*, 2004; Fitzgerald *et al.*, 2006; Rodriguez *et al.*, 2007). We recently showed that mouse myoblast cell lines and cultured primary myoblasts derived from satellite cells express netrin-3 and neogenin and that these factors promote formation of multinucleated myotubes (Kang *et al.*, 2004). Furthermore, neogenin forms *cis* complexes with a second promyogenic Ig/fibronectin type III-repeat protein, Cdo (also called Cdon), and Cdo-null myoblasts fail to respond to soluble netrin, suggesting that neogenin-Cdo com-

plexes might be required for some aspects of netrin/neogenin signaling (Kang *et al.*, 2004). We report here that mice homozygous for a gene-trap mutation in the *Neo1* gene (encoding neogenin) develop myotomes normally but have small myofibers at embryonic day (E)18.5 and at 3 wk of age. Similarly, myoblasts derived from such animals fail to form large myotubes in vitro. These defects are associated with low levels of the activated forms of FAK and ERK, and inefficient expression of certain muscle-specific proteins, both in vivo and in vitro. Finally, soluble netrin activates FAK and ERK in cultured myoblasts in a neogenin- and Cdo-dependent manner, whereas soluble RGMc displays lesser ability to activate these kinases. Together, neogenin signaling, probably activated via netrin ligands, is an important extracellular cue in regulation of myogenic differentiation and myofiber size.

MATERIALS AND METHODS

Mice

Mice carrying a secretory gene-trap vector insertion into intron 7 of the *Neo1* gene were constructed previously (Leighton *et al.*, 2001; Mitchell *et al.*, 2001). The embryonic stem (ES) cell line that carried the secretory gene trap from which this mouse line was developed was designated KST265, and we therefore refer to the allele as *Neo1*^{Gt(KST265)Byg} (abbreviated *Neo1*^{Gt}). We mapped the insertion site to nucleotide 58,780,672 (mouse chromosome 9, NCBI Mouse Genome Reference Assembly, Build 37) through a combination of Southern blotting, polymerase chain reaction (PCR), and sequencing. The insertion was accompanied by a deletion of 922 base pairs and an inversion of a 923-base pair sequence immediately adjacent to the insertion site (inverted nucleotides are 58,780,672–58,779,784). This permitted design of a PCR genotyping strategy in which a single oligonucleotide (5'-CAACCTGGGT-CATAGCAAGTGG-3') served as the reverse primer for the wild-type allele and the forward primer for the mutant allele. The forward primer for the wild-type allele was 5'-GTGTGAACAAAGAAGCAGAAAGGC-3' and the reverse primer for the mutant allele was 5'-TAAATGACGACACAGCAGT-GCC-3'. A three-primer PCR produces a 704-base pair fragment for the wild-type allele and a 917-base pair fragment for the mutant allele (Figure 1A). We also genotyped with a previously reported strategy (Burgess *et al.*, 2006) and found the two methods concordant. The insertion is predicted to produce a mutant transcript that encodes a transmembrane protein consisting of the four Ig repeats of neogenin fused in-frame with a vector-encoded transmembrane domain and a cytoplasmic domain consisting of β -geo (β -galactosidase fused to neomycin phosphotransferase). Previous studies suggest that such fusion proteins are targeted to intracellular compartments (Leighton *et al.*, 2001; Mitchell *et al.*, 2001).

All mice and embryos were of a largely C57BL/6 background and were generated from intercrosses of *Neo1*^{+/Gt} animals. Noon of the plug date was designated E0.5.

In Situ Hybridization, β -Galactosidase Staining, Histology, and Immunohistochemistry

For whole-mount RNA in situ hybridization, embryos were prepared essentially as described previously (Mulieri *et al.*, 2002), except that they were treated with 10 μ g/ml proteinase K (QIAGEN, Valencia, CA) in phosphate-buffered saline, 0.1% Tween-20 PBT according to stage (E10.5, 40 min; E11.5, 60 min). Embryos were rinsed, postfixed, and hybridized with digoxigenin-labeled probe in hybridization mix [50% formamide, 1.3 $\times$ SSC, 5 mM EDTA, 50 μ g/ml yeast RNA, 0.2% Tween 20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, and 100 μ g/ml heparin] overnight at 65°C. After washing and blocking, embryos were incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; Roche Diagnostics, Indianapolis, IN) in blocking buffer (2% blocking reagent [Roche Diagnostics]), 20% heat-inactivated sheep serum in 100 mM maleic acid, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 [MABT]. After washes in MABT and 500 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂, and 1% Tween 20, signals were developed using BM Purple AP Substrate (Roche Diagnostics). Dissected embryos were stained for β -galactosidase activity essentially as described previously (Nait-Oumesmar *et al.*, 2002) with the exception that fixation time was dependent on the age of the embryo. All steps except incubation with staining solution were performed at room temperature. Embryos were permeabilized for 2 h in phosphate-buffered saline (PBS), pH 7.0, 2 mM MgCl₂, 0.01% NP-40, and 0.02% sodium deoxycholate. Staining solution was identical to the above permeabilization buffer, supplemented with 17.5 mM each K₃Fe(CN)₆ and K₄Fe(CN)₆ and 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Sigma-Aldrich). Whole-mount embryos were dehydrated into 60% glycerol and PBS for photog-

Figure 1. The *Neo1^{Gt}* allele is variably hypomorphic. (A) Map of the gene-trap insertion site in the *Neo1* locus. The arrow indicates the insertion site of the secretory gene-trap vector in intron 7. The positions of the primers used for genotyping are represented by red and black arrowheads (F, forward primer for wild-type allele; I, reverse primer for wild-type allele and forward primer for mutant allele; R, reverse primer for mutant allele). Lines above primers represent PCR products shown in B. The dotted line in the mutant allele represents a deleted sequence, and the white region in the wild-type and mutant alleles represents an inverted sequence. Within the gene-trap vector, the vertical white rectangle indicates a transmembrane (TM) domain, the blue segment is β -geo, the green segment an internal ribosome entry site (IRES), and the purple segment is an alkaline phosphatase reporter gene (PLAP). (B) Genotype analysis of *Neo1^{+/+}*, *Neo1^{+/Gt}*, and *Neo1^{Gt/Gt}* embryos. A three-primer PCR produces a 704-base pair fragment from the wild-type allele and a 917-base pair fragment from the mutant allele. (C) Western blot analysis of neogenin protein production in embryos and MEFs. Heads from E13.5 embryos of the indicated genotype were removed, and extracts were blotted with antibodies to the neogenin intracellular domain. Extracts from MEFs of the indicated genotype were also analyzed. The ~190-kDa band (arrow) represents full-length neogenin, and the ~60-kDa band (arrowhead) may correspond to a processed form of the neogenin intracellular region. The ~120-kDa band (asterisk) is a nonspecific band recognized in embryonic head, but not MEF, extracts. Extracts were also probed with a pan-Cadherin antibody as a loading control.

raphy. Histological examination of muscle was performed by staining sections with hematoxylin and eosin, as described previously (Zhang *et al.*, 2006). Immunohistochemistry was performed on 8- μ m paraffin sections by standard methods with the following mouse monoclonal antibodies: anti-desmin (Dako North America, Carpinteria, CA), anti-myosin fast (Sigma-Aldrich), anti-myogenin (Dako North America), and anti-phospho-histone H3 (Millipore, Billerica, MA). Slides were incubated in 10% normal goat serum in Tris-buffered saline, pH 7.6, 1% gelatin, and 10% bovine serum albumin fraction V as a blocking solution and diluent for primary antibody, biotinylated goat anti-mouse, and streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections stained for myogenin were pretreated with target unmasking fluid (Zymed Laboratories, South San Francisco, CA) according to the manufacturer's instructions.

Analysis of Dissected E15.5 Limbs by Immunoblotting

Limbs from E15.5 embryos were removed, and muscle and associated connective tissues were dissected away from developing bones and frozen in liquid nitrogen. The tissues were ground and solubilized with lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) containing proteinase inhibitor (Roche Diagnostics), 50 mM NaF, and 1 mM sodium orthovanadate. Lysates were analyzed by immunoblotting as described previously (Kang *et al.*, 1998). Polyvinylidene difluoride membranes were probed with antibodies against Neogenin (Santa Cruz Biotechnology, Santa Cruz, CA), MyoD (Santa Cruz Biotechnology), Myogenin (Santa Cruz Biotechnology), Desmin (Dako North America), myosin heavy chain (MHC) (MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), pan-cadherin (Abcam, Cambridge, MA), ERK (Santa Cruz Biotechnology), ERK2 (Cell Signaling Technology,

Danvers, MA), p90RSK (Cell Signaling Technology), FAK (Cell Signaling Technology), phospho(576/577)-FAK (Cell Signaling Technology), phospho-p90 RSK (Cell Signaling Technology), and phospho-ERK (Cell Signaling Technology).

Analysis of Myofiber Size

Quadriceps muscles of P21 *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice and forelimbs of E18.5 *Neo1^{+/+}* and *Neo1^{Gt/Gt}* embryos were embedded in tragacanth (Sigma-Aldrich), snap-frozen in liquid nitrogen-cooled isopentane, and cryosectioned at 10 μ m. Sections were stained with hematoxylin and eosin, the cross-sectional area of individual myofibers was measured with ImageJ software (National Institutes of Health, Bethesda, MD) and graphically represented with SigmaPlot (Systat Software, San Jose, CA) analysis.

Primary Myoblasts

Primary cultures of myoblasts were obtained from the hind limbs of P21 *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice as described previously (Rando and Blau, 1994; Sabourin *et al.*, 1999). In brief, hind limb muscle tissue was collected, connective tissue and fat were removed, and muscles were dissociated mechanically and enzymatically. Cells were plated on tissue-culture plastic in Ham's F-10 medium with 20% fetal bovine serum (FBS), 10 ng/ml hepatocyte growth factor (HGF), 5 mg/ml heparin, and 2.5 ng/ml basic fibroblast growth factor (bFGF). Twenty-four hours later, floating cells were collected, inoculated on collagen-coated plates, and cultured in the same medium without HGF. Several rounds of selective adhesion were performed until a pure population of myoblasts was obtained. For differentiation studies, cells were at 80% confluence on collagen-coated plates in differentiation medium (DMEM with 2% horse serum). Immunocytochemistry was performed with MHC antibodies, and differentiation and fusion were scored as described previously (Kang *et al.*, 2008). Immunoblotting of cell extracts was performed with antibodies described above and with antibodies against Cdo (Zymed Laboratories), p38 (MAPK) (Santa Cruz Biotechnology), phospho-p38 MAPK (Cell Signaling Technology), and phospho-AKT (Cell Signaling Technology). For cell proliferation assays, myoblasts in growth medium were incubated with 20 mM 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) for 2 h and immunostained with an antibody against BrdU (Millipore Bioscience Research Reagents, Temecula, CA). To monitor cell death, *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts that had been cultured in growth or differentiation medium were fixed, and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed with a kit according to the manufacturer's instructions (Roche Diagnostics). For analysis of activation of FAK or ERK, *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts were treated with 100 ng/ml Netrin-2 (R&D Systems, Minneapolis, MN) or 100 ng/ml RGMc (R&D Systems) in growth condition for indicated times. After stimulation, the cells were rinsed twice with ice-cold phosphate-buffered-saline and extracted in lysis buffer. Samples were analyzed by immunoblotting as described above.

RESULTS

The *Neo1^{Gt}* Allele Is Hypomorphic and Results in Incompletely Penetrant Perinatal Lethality

Mice carrying a gene-trap insertion in the *Neo1* gene have been constructed as described previously (Leighton *et al.*, 2001; Mitchell *et al.*, 2001), and homozygous mutants were reported to display perinatal lethality (Srinivasan *et al.*, 2003), but this allele is not well characterized. We refer to the allele here as *Neo1^{Gt(KST265)Byg}* (abbreviated *Neo1^{Gt}*). The insertion site of the gene-trap vector was identified by PCR and sequence analysis, revealing that the vector integrated into intron 7 of the *Neo1* locus (Figure 1A). The insertion was accompanied by both a deletion of 922 base pairs and an inversion of a 923-base pair sequence immediately adjacent (3') to the insertion site. This allowed design of a three-primer PCR genotyping protocol (Figure 1, A and B; see *Materials and Methods*). We intercrossed *Neo1^{+/Gt}* mice and found that at postnatal day (P)10, *Neo1^{Gt/Gt}* mice were present at only ~31% of the expected frequency (Table 1). By P21, additional animals had died, culminating in ~80% lethality by weaning age.

Because gene-trap mutations are sometimes hypomorphic alleles, extracts of heads removed from E13.5 embryos were Western blotted with antibodies directed against the neogenin intracellular region. Eleven *Neo1^{Gt/Gt}* embryos from 6 different litters were compared with a wild-type control, and all 11 expressed detectable full-length neogenin protein

Table 1. Offspring of intercrosses between *Neo1^{+/Gt}* mice

Genotype	P10 (n = 453)	P21 (n = 432)
<i>Neo1^{+/+}</i>	147 (32)	145 (33)
<i>Neo1^{+/Gt}</i>	260 (57)	258 (60)
<i>Neo1^{Gt/Gt}</i>	46 (11)	29 (7)

Values in parentheses are percentages.

(~190 kDa), at low levels that were somewhat variable between animals (Figure 1C). Neogenin protein was not observed by Western blotting at this level of sensitivity in *Neo1^{Gt/Gt}* mouse embryonic fibroblasts (MEFs) (Figure 1C), nor was it seen in dissected E15.5 *Neo1^{Gt/Gt}* limbs or *Neo1^{Gt/Gt}* myoblasts (vide infra). An immunoreactive band of ~60 kDa was also observed in wild-type heads and MEFs, but not in the mutants; this may correspond to a processed form of the neogenin intracellular region (Goldschneider *et al.*, 2008). It is concluded that the *Neo1^{Gt}* allele is hypomorphic in a stage- and/or tissue-dependent manner. All *Neo1^{Gt/Gt}* mice were smaller than wild-type animals and had multiple abnormalities (Srinivasan *et al.*, 2003; Burgess *et al.*, 2006; Metzger *et al.*, 2007). We focus here on skeletal muscle development.

Defective Skeletal Muscle Development in *Neo1^{Gt/Gt}* Mice

Neogenin mRNA and protein are expressed prominently in E14.5 fetal mouse muscle masses and in adult muscle (Keeling *et al.*, 1997; Fitzgerald *et al.*, 2006; Rodriguez *et al.*, 2007). However, little information exists on *Neo1* expression during earlier stages of myogenesis. The gene trap insertion leads to expression of a β -galactosidase reporter from the *Neo1* locus (Leighton *et al.*, 2001; Mitchell *et al.*, 2001) (Figure 1A), and β -galactosidase activity was examined in E9.5, E10.5, and E11.5 embryos. In each case, strong β -galactosidase activity was observed in the dorsal somite, the region of origin of muscle progenitor cells (Figure 2A). To assess the effects of loss of neogenin on early myogenesis, whole-mount RNA in situ hybridization was used to analyze expression of the critical differentiation gene *myogenin* in myotomes of E10.5 and E11.5 *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice (Figure 2B). No difference in *myogenin* expression was observed between the wild-type and homozygous mutant animals at either stage.

To further examine muscle development in *Neo1^{Gt/Gt}* mice, sections from E15.5 embryos were analyzed for expression of muscle-specific markers by immunohistochemistry with antibodies against desmin, myogenin and MHC. Transverse sections of developing intercostal muscles and of the premuscle masses of the trapezius and pectoralis revealed that although muscle patterning seemed largely normal in *Neo1^{Gt/Gt}* embryos, the staining intensity for desmin and myogenin was clearly weaker than that seen in wild-type controls; staining intensity for MHC also seemed reduced, although less dramatically (Figure 3, A–E). To confirm and extend this observation at a biochemical level, extracts of dissected E15.5 hind limbs were analyzed by Western blotting with antibodies against muscle-specific proteins. Neogenin was easily detected in extracts from *Neo1^{+/+}* limbs but, in contrast to E13.5 *Neo1^{Gt/Gt}* heads, was not observed in extracts from E15.5 *Neo1^{Gt/Gt}* limbs (Figure 3F). Similar to what was seen by immunohistochemistry, the levels of myogenin and desmin were strongly reduced in *Neo1^{Gt/Gt}* limb extracts relative to control extracts, whereas

Figure 2. Normal formation of myotomes in *Neo1^{Gt/Gt}* embryos. (A) Analysis of *Neo1* expression by β -gal activity (blue color) in *Neo1^{+/Gt}* embryos of the indicated stage. *Neo1* is expressed ubiquitously but with particularly high levels in dorsal somites (arrows) and the dorsal aspect of the neural tube (arrowheads). (B and C) Whole-mount in situ hybridization of E10.5 (B) and E11.5 (C) embryos of the indicated genotype with a myogenin probe. The red brackets span the length of the myotome, with no significant difference seen between *Neo1^{+/+}* and *Neo1^{Gt/Gt}* embryos.

the levels of MHC were lowered more modestly; MyoD levels were also modestly reduced (Figure 3F).

The production of phosphorylated forms of signaling proteins that lie downstream of neogenin was also analyzed. The levels of the phosphorylated forms of ERK and the ERK substrate p90RSK were both lower in *Neo1^{Gt/Gt}* limbs than in control limbs, but total ERK and p90RSK levels were unchanged (Figure 3F). Similarly, there was a strong reduction in the concentration of FAK phosphorylated on Tyr576/577, Src family kinase sites required for maximal FAK kinase activity that are stimulated by netrin-1 in cultured cells (Calalb *et al.*, 1995; Li *et al.*, 2004; Liu *et al.*, 2004; Ren *et al.*, 2004; Mitra *et al.*, 2005) (Figure 3F). Therefore, although muscle development was not grossly defective at E15.5 in *Neo1^{Gt/Gt}* mice, these animals displayed diminished expression of both muscle-specific proteins and markers of activity of signaling pathways downstream of neogenin. Cell proliferation and apoptosis were also examined in sections of E15.5 embryos by immunostaining for phospho-histone H3 and TUNEL assay, respectively; significant differences between *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice were not observed.

To assess muscle development at later stages, sections through E18.5 forelimbs of *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice and through the quadriceps of P21 *Neo1^{+/+}* and surviving *Neo1^{Gt/Gt}* mice were stained with hematoxylin and eosin and compared. Analysis of E18.5 sections revealed proper organization of muscle groups in *Neo1^{Gt/Gt}* mice, although they were somewhat smaller than those of *Neo1^{+/+}* controls (Figure 4A). However, the average cross-sectional area of *Neo1^{Gt/Gt}* forelimb myofibers was reduced by ~28% as compared with those of *Neo1^{+/+}* mice (Figure 4, B and C). In contrast, the diameter of the radius and ulna was not significantly different between E18.5 *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice (Figure 4A). This phenotype was exacerbated in surviving P21 *Neo1^{Gt/Gt}* animals, where average cross-sectional area of myofibers in the quadriceps was reduced by ~50% compared with controls (Figure 4, D and E).

Defective Differentiation of *Neo1^{Gt/Gt}* Myoblasts In Vitro

Primary myoblasts were isolated from surviving P21 *Neo1^{+/+}* and *Neo1^{Gt/Gt}* mice and analyzed for their ability to

differentiate in culture, as assessed by the formation of multinucleated myotubes and expression of muscle-specific proteins. When differentiated cultures were stained with an antibody against MHC, ~70% of both *Neo1^{+/+}* and *Neo1^{Gt/Gt}* cells were positive, but visual inspection revealed that *Neo1^{Gt/Gt}* cells formed smaller myotubes than *Neo1^{+/+}* cells (Figure 5A). The smaller size of *Neo1^{Gt/Gt}* myotubes was further assessed by quantifying both the average number of nuclei per myotube, and the distribution range of numbers of nuclei per myotube. *Neo1^{+/+}* myotubes had an average of 6.2 ± 1.1 nuclei, whereas *Neo1^{Gt/Gt}* myotubes had an average of 2.6 ± 0.6 nuclei. In addition, *Neo1^{Gt/Gt}* cultures produced ~60% more singly nucleated MHC⁺ cells and only ~25% the number of large myotubes (those with ≥ 6 nuclei) seen with *Neo1^{+/+}* cultures (Figure 5B). Several independent isolates of *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts displayed similar characteristics; there was some variability between different isolates of *Neo1^{Gt/Gt}* cells in their ability to form myotubes but all were at least as strongly defective as those shown in Figure 5.

The possibility that the differences in the size of myotubes formed by *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts was due to alterations in cell proliferation or apoptosis was examined by measuring BrdU incorporation and by TUNEL assay, respectively. *Neo1^{+/+}* and *Neo1^{Gt/Gt}* cells cultured in growth medium (15% FBS plus bFGF) displayed similar levels of BrdU incorporation (Figure 5, C and D). Apoptosis was negligible in cultures of either genotype in growth medium but 48 h after switching to differentiation medium, *Neo1^{Gt/Gt}* cultures displayed more TUNEL-positive cells than did *Neo1^{+/+}* cultures (13.7 vs. 6.9%, respectively; Figure 5, E and F). Although this ~7% increase in apoptosis may contribute to the partially defective differentiation of *Neo1^{Gt/Gt}* myoblasts, it is insufficient to explain the quantitatively much stronger effects on myotube formation as assessed by the percentage of nuclei in multinucleated cells or number of nuclei per myotube.

We therefore examined expression of muscle-specific and signaling proteins by Western blot analyses of differentiating cultures of *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts. As was observed with E15.5 limbs, neogenin protein levels were

Figure 3. Reduced expression of muscle-specific proteins and diminished concentration of phospho-FAK and phospho-ERK in premuscle masses of E15.5 *Neo1*^{Gt/Gt} embryos. (A–E) Transverse sections through developing intercostal muscles (A and E), premuscle mass of the pectoralis (B and C) and premuscle mass of the trapezius (D) stained with antibodies against the indicated proteins. Within each panel, the lower micrographs are higher magnification views of the ones directly above and are delineated by the boxed areas in A and D. (F) Western blot analysis of production of muscle-specific and signaling proteins in extracts of dissected E15.5 hind limbs. Bars, 0.5 mm (A, top), 50 mm (A, bottom), and 50 mm (B–E).

Figure 4. *Neo1*^{Gt/Gt} mice form small myofibers. (A) Cross-sections through the hind limbs of E18.5 *Neo1*^{+/+} and *Neo1*^{Gt/Gt} mice stained with hematoxylin and eosin. Note that overall muscle patterning is not different between wild-type and mutant animals. R, radius; U, ulna. (B) Cross-sections through the hind limbs of E18.5 *Neo1*^{+/+} and *Neo1*^{Gt/Gt} mice stained with hematoxylin and eosin. (C) Quantification of myofiber cross-sectional area (CSA) from B. Asterisk indicates different from control, *p* < 0.0001 by Student's *t* test. (D) Cross-sections through the hind limbs of P21 *Neo1*^{+/+} and *Neo1*^{Gt/Gt} mice stained with hematoxylin and eosin. (E) Quantification of myofiber CSA from D. Asterisk indicates different from control, *p* < 0.0001 by Student's *t* test. Bars, 0.5 mm (A) and 50 μm (B and D).

high in *Neo1*^{+/+} cells but not detected in *Neo1*^{Gt/Gt} cells (Figure 5G). Levels of MyoD and Cdo, both of which are expressed in proliferating and differentiating myoblasts (Kang *et al.*, 1998; Sabourin *et al.*, 1999; Cole *et al.*, 2004), were similar in wild-type and *Neo1*^{Gt/Gt} cells. However, as seen with developing *Neo1*^{Gt/Gt} muscle masses in vivo, *Neo1*^{Gt/Gt} cells had lower levels of both myogenin and MHC compared with *Neo1*^{+/+} cells. Also, similar to the in vivo situation, the amounts of the phosphorylated forms of ERK and of its substrate, p90RSK, were lower in *Neo1*^{Gt/Gt} cells than *Neo1*^{+/+} cells (Figure 5G). However, the inefficient activation of ERK signaling was specific, in that the levels of the phosphorylated forms of two other kinases involved in myogenesis, p38 MAPK and AKT, were not substantially different between *Neo1*^{+/+} and *Neo1*^{Gt/Gt} cells. The concentration of FAK phosphorylated on Tyr576/577 was also lower in *Neo1*^{Gt/Gt} cells than *Neo1*^{+/+} cells, but the difference was not as pronounced in vitro as was seen in vivo (compare Figures 3F and 5G). Lack of neogenin therefore resulted in similar myogenic phenotypes in vivo and in vitro: production of small myofibers/myotubes with reduced expression of muscle-specific proteins and diminished ERK and FAK activation.

Defective Response to Netrin Signaling in *Neo1*^{Gt/Gt} and *Cdo*^{-/-} Myoblasts

The low concentrations of the phosphorylated, active forms of FAK and ERK in developing *Neo1*^{Gt/Gt} muscles in vivo and differentiating myoblasts in vitro suggest a loss of li-

gand-initiated, neogenin-dependent signaling. Specific netrin and RGM family members function as ligands for neogenin (Cole *et al.*, 2007; Wilson and Key, 2007). Netrin-1 and netrin-3 are expressed in developing mouse skeletal muscle, and cultured murine myoblasts express netrin-3 but little or no netrin-1 (Wang *et al.*, 1999; Kang *et al.*, 2004). We have shown previously that recombinant chicken netrin-2 promotes myotube formation by C2C12 myoblasts in a neogenin-dependent manner (Kang *et al.*, 2004) (note that mouse netrin-3 is related to chicken netrin-2 and that there is no netrin-3 in chicken and no netrin-2 in mice; netrin-2, unlike netrin-3, can be obtained in soluble form and is therefore used in our studies; Kang *et al.*, 2004). RGMc, but not RGMa or RGMb, is expressed in developing muscle and is induced during myoblast differentiation in vitro (Kuninger *et al.*, 2004, 2006; Niederkofler *et al.*, 2004; Oldekamp *et al.*, 2004). Although RGMa has been demonstrated to be a ligand for neogenin in the developing CNS (Matsunaga *et al.*, 2004, 2006; Rajagopalan *et al.*, 2004; Conrad *et al.*, 2007; Kee *et al.*, 2008), it is not clear that RGMc has a similar function in muscle. We therefore assessed whether soluble netrin-2 and/or RGMc were capable of inducing FAK and ERK phosphorylation in myoblasts and whether neogenin was required for such activity through analysis of *Neo1*^{+/+} and *Neo1*^{Gt/Gt} myoblasts. Cdo is an Ig superfamily member that binds in *cis* to neogenin and may function as a neogenin coreceptor (Kang *et al.*, 2004); *Cdo*^{+/+} and *Cdo*^{-/-} myoblasts were therefore also examined.

Figure 5. Defective differentiation of *Neo1*^{Gt/Gt} myoblasts in vitro. (A) Photomicrographs of *Neo1*^{+/+} and *Neo1*^{Gt/Gt} myoblasts cultured in differentiation medium, and fixed and stained with an antibody to MHC. (B) Quantification of myotube formation by cell lines shown in A. Values represent means of triplicate determinations $\pm$ 1 SD. The experiment was repeated three times with similar results and with multiple, independent isolates of the cells. Asterisks indicate different from control, $p < 0.01$ by Student's *t* test. (C) BrdU incorporation into proliferating *Neo1*^{+/+} and *Neo1*^{Gt/Gt} myoblasts. Cultures were exposed to BrdU for 2 h, fixed, and stained with an antibody to BrdU. (D) Quantification of BrdU incorporation shown in C. (E) Photomicrographs of *Neo1*^{+/+} and *Neo1*^{Gt/Gt} myoblasts cultured in growth medium (GM) or differentiation medium (DM) for 48 h and analyzed for apoptotic cells by TUNEL assay (green). Cultures were also stained with 4,6-diamidino-2-phenylindole to reveal nuclei (blue). (F) Quantification of myotube formation by cell lines shown in E. Values represent means of triplicate determinations $\pm$ 1 SD. Asterisk indicates different from *Neo1*^{+/+} control, $p < 0.01$ by Student's *t* test. (G) Western blot analysis of production of muscle-specific and signaling proteins in extracts of differentiating *Neo1*^{+/+} and *Neo1*^{Gt/Gt} myoblasts.

Neo1^{+/+} and *Neo1*^{Gt/Gt} myoblasts were treated with recombinant netrin-2 or RGMc, and cell lysates collected over a 3-h time course. Production of phospho(576/577)-FAK, total FAK, phospho-ERK, and total ERK2 was then analyzed by Western blotting. A low level of phospho(576/577)-FAK was present in both *Neo1*^{+/+} and *Neo1*^{Gt/Gt} cells before any

treatment (time 0). Treatment with netrin-2 elevated phospho(576/577)-FAK concentration in *Neo1*^{+/+} cells within 30 min, and this increase was sustained for at least 1 h (Figure 6A). In contrast, netrin-2 did not induce phospho(576/577)-FAK in *Neo1*^{Gt/Gt} cells. Similar results were obtained with *Cdo*^{+/+} and *Cdo*^{-/-} myoblasts: netrin-2 enhanced phos-

Figure 6. Defective response to netrin signaling in *Neo1^{Gt/Gt}* and *Cdo^{-/-}* myoblasts. (A) Western blot analysis of phospho-FAK (p-FAK) and total FAK levels in *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts treated with netrin-2 for the indicated times. Lysates were also probed with an antibody to neogenin. (B) Western blot analysis of p-FAK and total FAK levels in *Cdo^{+/+}* and *Cdo^{-/-}* myoblasts treated with netrin-2 for the indicated times. Lysates were also probed with an antibody to Cdo. (C) Western blot analysis of p-FAK and total FAK levels in *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts treated with RGMc for the indicated times. (D) Western blot analysis of p-FAK and total FAK levels in *Cdo^{+/+}* and *Cdo^{-/-}* myoblasts treated with RGMc for the indicated times. (E) Western blot analysis of phospho-ERK2 (p-ERK2) and total ERK2 levels in *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts treated with RGMc for the indicated times. (F) Western blot analysis of p-ERK2 and total ERK2 levels in *Cdo^{+/+}* and *Cdo^{-/-}* myoblasts treated with RGMc for the indicated times. (G) Western blot analysis of p-ERK2 and total ERK2 levels in *Neo1^{+/+}* and *Neo1^{Gt/Gt}* myoblasts treated with RGMc for the indicated times. (H) Western blot analysis of p-ERK2 and total ERK2 levels in *Cdo^{+/+}* and *Cdo^{-/-}* myoblasts treated with RGMc for the indicated times.

pho(576/577)-FAK levels in the control cells but this induction was largely absent in *Cdo^{-/-}* cells (Figure 6B). RGMc treatment also induced phospho(576/577)-FAK in the control *Neo1^{+/+}* and *Cdo^{+/+}* cells, but this seemed to be less robust than that seen with netrin-2 and was observed only at the 30 min time point (Figure 6, C and D). A slight induction was retained by *Neo1^{Gt/Gt}* myoblasts but not by *Cdo^{-/-}* cells. Similar results to those obtained with FAK phosphorylation were seen with ERK phosphorylation. Phospho-ERK was induced in control myoblasts 30 min to 1 h after treatment with netrin-2 and was maintained for at least 3 h, and both *Neo1^{Gt/Gt}* and *Cdo^{-/-}* cells did not respond to this factor (Figure 6, E and F). In contrast, RGMc failed to enhance phospho-ERK levels above background in control or mutant myoblasts, though the levels of phospho-ERK in the presence of RGMc were generally higher in control than in mutant cells (Figure 6, G and H). Based on these results, it seems most likely that netrin-3 is the major neogenin ligand of relevance in skeletal myogenesis, although an auxiliary role for RGMc cannot be excluded.

DISCUSSION

A variety of signal transduction pathways participate in the development of skeletal muscle, but the extracellular cues that regulate such pathways in myofiber formation are not well understood (Pownall *et al.*, 2002; Charge and Rudnicki, 2004). We reported previously that netrin-3 and neogenin promoted myotube formation by C2C12 myoblasts (Kang *et al.*, 2004). In this report, it is demonstrated that mice homozygous for a gene-trap mutation (*Neo1^{Gt}*) in the *Neo1* locus develop small myofibers. Furthermore, myoblasts derived from such mice display a similar phenotype in vitro, forming smaller myotubes with fewer nuclei than myoblasts from control animals. In both cases, the phenotype is associated with inefficient expression of certain muscle-specific proteins, including myogenin, which is required for myoblast differentiation (Pownall *et al.*, 2002), and MHC, a marker of terminal differentiation. The diminished expression of these muscle-specific proteins indicates that the differentiation process occurs inefficiently in the absence of

neogenin, and it is likely that myofibers fail to attain a normal size as a consequence of this defect. This phenotype is similar but not quite as strong as the one observed with the C2C12 cell line, where RNA interference (RNAi)-mediated knockdown of neogenin resulted in a sharply reduced ability to form myotubes (Kang *et al.*, 2004). It is possible that during the process of immortalization, C2C12 cells acquired changes that rendered them more dependent on neogenin for differentiation than primary cells or developing muscles *in vivo*. *Neo1^{Gt/Gt}* mice and myoblasts also had reduced levels of the active, phosphorylated forms of FAK and ERK, both shown previously to be activated by neogenin signaling in cultured neurons (Li *et al.*, 2004; Liu *et al.*, 2004; Ren *et al.*, 2004); this is the first study to demonstrate that these kinases are downstream of neogenin *in vivo*.

Neo1 is expressed in fetal premuscle masses and adult muscle (Keeling *et al.*, 1997; Fitzgerald *et al.*, 2006; Rodriguez *et al.*, 2007) and, as shown here, developing somites. However, although *Neo1^{Gt/Gt}* mice displayed an obvious defect in the formation of fully sized myofibers, they did not show a defect in the formation of myotomes. Neogenin's role may therefore be restricted to later stages of myogenesis. It should be noted, however, that the *Neo1^{Gt}* allele used in these studies is clearly hypomorphic, as demonstrated by the detection of full-length neogenin protein in extracts of E13.5 *Neo1^{Gt/Gt}* heads. In contrast, extracts of dissected E15.5 *Neo1^{Gt/Gt}* limb muscle and cultured *Neo1^{Gt/Gt}* myoblasts and MEFs did not reveal detectable neogenin protein at a similar level of sensitivity, indicating that the splicing events that allow bypass of the intronic gene-trap vector may occur in a tissue- and/or embryonic stage-dependent manner. Thus, although the phenotype observed in *Neo1^{Gt/Gt}* mice may be similar to that achieved with a true null allele, a final conclusion on the full role of neogenin in myogenesis must await the generation of a targeted, null mutation. Morpholino-mediated knockdown of neogenin in zebrafish and *Xenopus* embryos resulted in defective neural tube development, including failure of cavitation or closure, respectively (Mawdsley *et al.*, 2004; Kee *et al.*, 2008). These phenotypes were not seen in *Neo1^{Gt/Gt}* mouse embryos (Hong and Krauss, unpublished data). The lack of early neural tube phenotypes in *Neo1^{Gt/Gt}* mice may also be due to the hypomorphic nature of this allele, as the morpholino knockdown may be more effective at depleting neogenin. Alternatively, neogenin may play a less important role in these processes in mice than in fish and amphibians.

An important question is which ligand or ligands work with neogenin in myogenesis. Mice homozygous for strong hypomorphic *Ntn1* (encoding netrin-1) alleles are not reported to have defects in muscle development (Serafini *et al.*, 1996; Salminen *et al.*, 2000), and *Hfe2* (encoding RGMc)-null mice are reported to have normal muscle development (Huang *et al.*, 2005; Niederkofler *et al.*, 2005). Mice lacking netrin-3 have not been generated. Netrin-2 (the chick family member most closely related to mammalian netrin-3) promoted C2C12 myotube formation in a neogenin-dependent manner, whereas stable overexpression of RGMc in C2C12 cells was reported to have no effect on myotube formation (Huang *et al.*, 2005; Niederkofler *et al.*, 2005; Kuninger *et al.*, 2006). Furthermore, netrin-2 stimulated FAK and ERK activation in *Neo1^{+/+}*, but not *Neo1^{Gt/Gt}*, myoblasts *in vitro*; in contrast, RGMc displayed lesser ability to do so, though the levels of phospho-FAK and phospho-ERK were generally a bit higher in wild-type than mutant cells in the presence of RGMc. Because developing muscles in *Neo1^{Gt/Gt}* embryos and *Neo1^{Gt/Gt}* myoblasts had low concentrations of the activated forms of FAK and ERK, and *Ntn1* and *Hfe2* mice lack

gross muscle phenotypes, we speculate that netrin-3 is the major ligand for neogenin in the context of myogenesis, perhaps redundantly with netrin-1. Obviously, *Ntn3* mutant mice will be required to prove this point. It also is possible that unidentified neogenin ligands exist and play a role in myogenesis. Finally, it should be mentioned that we have used soluble forms of netrin-2 and RGMc, and these factors may be presented *in vivo* in a membrane- and/or matrix-bound manner, and this may also affect activity.

We reported previously that Cdo might function as a coreceptor for neogenin in myoblasts (Kang *et al.*, 2004). Consistent with this possibility, netrin-2 treatment did not stimulate FAK or ERK phosphorylation in *Cdo^{-/-}* myoblasts, similar to the lack of response seen with *Neo1^{Gt/Gt}* myoblasts. We have not observed obvious binding of netrins to Cdo (unpublished data), so Cdo may be a nonligand-binding coreceptor for neogenin within specific contexts. A similar situation exists with neogenin and the cell surface receptor UNC5b. UNC5b associates with neogenin as a coreceptor for RGMa in signaling growth cone collapse but does not bind RGMa directly (Hata *et al.*, 2009). It is possible that neogenin associates in a *cis*-manner with a variety of coreceptors in a context-dependent manner to provide appropriate signaling capabilities to receptor complexes. Interestingly, UNC5b is a netrin-1 receptor, both independently and as part of a complex with DCC, that modulates short- and long-distance axon repulsion (Barallobre *et al.*, 2005; Round and Stein, 2007). It is likely, therefore, that the specific component composition of axon guidance receptor complexes regulates responses to multiple ligands in a spatiotemporal manner; this may serve as a paradigm for many types of receptor complexes during development in general, including neogenin:Cdo complexes. It should be noted, however, that *Neo1^{Gt/Gt}* and *Cdo^{-/-}* mice and myoblasts do not have identical phenotypes (Cole *et al.*, 2004; Takaesu *et al.*, 2006; this study). *Cdo^{-/-}* mice display delayed myogenesis and form somewhat smaller muscles than controls, whereas myofiber size in *Neo1^{Gt/Gt}* animals is more severely affected. Conversely, *Cdo^{-/-}* myoblasts have a more strongly disrupted differentiation program *in vitro* than *Neo1^{Gt/Gt}* myoblasts. It may be that some but not all of these two proteins' functions during myogenesis are interdependent.

Several signaling pathways are activated by netrin signaling through DCC/UNC40 family members, although neogenin is understudied compared with its mammalian paralogue DCC (Forcet *et al.*, 2002; Campbell and Holt, 2003; Li *et al.*, 2004, 2008; Liu *et al.*, 2004; Ren *et al.*, 2004; Xie *et al.*, 2005; Zhu *et al.*, 2007). Both FAK and ERK are activated by, and required for, netrin-1/DCC signaling in axon guidance (Forcet *et al.*, 2002; Campbell and Holt, 2003; Li *et al.*, 2004; Liu *et al.*, 2004; Ren *et al.*, 2004). FAK and ERK play complex roles in myogenesis. FAK activity is required for efficient myotube formation during muscle regeneration in response to injury, in fusion and costameric development of primary mouse myoblasts, and in survival of proliferating C2C12 myoblasts and their differentiation into myotubes (Clemente *et al.*, 2005; Quach and Rando, 2006; Quach *et al.*, 2009). ERK activity has both positive and negative roles in C2C12 cell differentiation (Wu *et al.*, 2000; Li and Johnson, 2006; Cho *et al.*, 2007; Yokoyama *et al.*, 2007); it is also implicated in fiber type identity *in vivo* (Murgia *et al.*, 2000; Shi *et al.*, 2008). The ERK substrate p90RSK has also been implicated in C2C12 cell differentiation (Cho *et al.*, 2007). Given the lower than normal levels of the phosphorylated forms of these kinases in *Neo1^{Gt/Gt}* embryos and myoblasts, it is logical to suggest that they are involved in neogenin's promyogenic function. However, FAK, ERK, and p90RSK activities are regulated by

several different stimuli, including various soluble factors and adhesion proteins (Mitra *et al.*, 2005; Ramos, 2008), and the approaches taken to explore their various functions in muscle cell differentiation generally affect their overall activity without regard to initiating stimulus (e.g., overexpression of wild-type or mutant proteins, RNAi-mediated depletion, pharmacological agents). Neogenin-dependent FAK, ERK, and p90RSK activities would presumably be regulated in concert with these kinases' activities initiated by other promyogenic stimuli, such as integrins. Furthermore, they would also probably occur as part of a broader network of signaling events initiated by neogenin, as occurs in DCC-mediated turning of growth cones (Huber *et al.*, 2003; Round and Stein, 2007). Further studies will be aimed at deciphering such networks.

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