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NET37, a Nuclear Envelope Transmembrane Protein with Glycosidase Homology, Is Involved in Myoblast Differentiation*[S]

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The nuclear lamina and its associated proteins are important for nuclear structure and chromatin organization and also have been implicated in the regulation of cell signaling and gene expression. In this study we demonstrate that the lamina-associated nuclear envelope transmembrane protein NET37 is required for myogenic differentiation of C2C12 cells. NET37, a member of glycosidase family 31, is highly expressed in mouse skeletal muscle and is strongly up-regulated during C2C12 differentiation. By protease mapping we show that its glycosidase homology domain is located in the lumen of the nuclear envelope/endoplasmic reticulum. When NET37 is depleted from proliferating myoblasts by RNAi, myogenic differentiation is significantly impaired, and there is a concomitant delay in up-regulation of the late myogenic transcription factor myogenin. We expressed silencing-resistant NET37 mutated at a conserved residue in the glycosidase domain and found that this predicted catalytically inactive protein is unable to support myogenesis in cells depleted of wild type NET37. Therefore, the enzymatic function of NET37 appears to be important for myogenic differentiation. C2C12 cells depleted of NET37 have reduced activation of Akt after shifting to differentiation medium and are defective in insulin like growth factor-II (IGF-II) secretion, an autocrine/paracrine factor involved in Akt activation. We also observed that pro-IGF-II co-immunoprecipitates with NET37. Based on our results, we propose that NET37 has a role in IGF-II maturation in the secretory pathway during myoblast differentiation. The localization of NET37 at the nuclear envelope raises the possibility that it may coordinate myogenic events between the nuclear interior and the endoplasmic reticulum lumen via transmembrane communication.

The nuclear envelope (NE)[2] forms the barrier between the cytoplasmic and the nuclear compartments (for review, see Ref. 1). It consists of the inner (INM) and outer nuclear membranes joined at the nuclear pore complex. The outer nuclear membrane is connected to the more peripheral endoplasmic reticulum (ER) and functionally overlaps with the latter. The two nuclear membranes are separated by the perinuclear luminal space, which is continuous with the ER lumen. Underlying the INM is the nuclear lamina, a protein meshwork containing nuclear lamins and associated integral membrane proteins of the INM. The lamina is thought to be important for nuclear architecture (for review, see Ref. 2) and for the attachment of the cytoplasmic cytoskeleton to the nucleus (3). It has been linked to DNA replication, chromatin organization, and gene expression. Furthermore, lamins and certain transmembrane proteins of the INM recently have been implicated in regulation of signaling (for review, see Refs. 1 and 2).

The importance of the nuclear lamina is underscored by the fact that to date ~15 different human disorders ("laminopathies") are known to be caused by mutation(s) in lamina proteins (for review, see Refs. 2, 4, and 5), including several dystrophies that affect heart and skeletal muscle. These include Emery-Dreifuss muscular dystrophy, limb girdle muscular dystrophy 1B, and dilated cardiomyopathy, which are caused by mutations in the lamin A gene (6–8). Moreover, an X-linked form of Emery-Dreifuss muscular dystrophy is caused by mutations in gene coding for emerin, a transmembrane protein of the INM (9, 10). Other diseases affecting mesenchymal tissues also are caused by mutations in transmembrane proteins of the INM; MAN1 has been linked to Buschke-Ollendorf syndrome and melorheostosis (11), and lamin B receptor (LBR) has been linked to hydrops-ectopic calcification "moth-eaten"/Greenberg skeletal dysplasia (12). Although the exact molecular basis for lamina-associated diseases remains contested, there is growing evidence that perturbations in signaling pathways are part of the mechanism (for review, see Refs. 13 and 14).

A rapidly growing body of evidence (for review, see Ref. 15) indicates that signaling and its physiological outcomes can be modulated by proteins of the NE/lamina. Some of the best examples are seen with the INM proteins emerin and MAN1 (16, 17). MAN1 has been shown to bind directly to R-Smad proteins and to attenuate transforming growth factor β signaling (18), presumptively by promoting dephosphorylation and nuclear export of R-Smads (for an extensive review, see Refs. 19 and 20). Emerin is thought to help control the activity of the cell cycle regulator Rb and the myogenic factor MyoD, thus influ-

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[2] The abbreviations used are: NE, nuclear envelope; INM, inner nuclear membrane; ER, endoplasmic reticulum; IGF, insulin like growth factor; hNET37, human NET37; BrdUrd, 5-bromo-2′-deoxyuridine; Endo H, endoglycosidase H; MyHC, myosin heavy chain; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; HA, hemagglutinin; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NET, nuclear envelope transmembrane; shRNA, short hairpin RNA; MyHC, myosin heavy chain.

encing the proliferation and differentiation of muscle satellite cells, the stem cell population involved in skeletal muscle regeneration (21). Furthermore, in heart from either emerin null mice or mice with a lamin A mutation causing human Emery-Dreifuss muscular dystrophy, there is altered activation of the Erk1/2 and their downstream targets (22, 23).

NET37 is a recently characterized NE protein (24) that was initially found in a proteomics screen to identify novel integral membrane proteins enriched at the NE (25). NET37 is predicted to have a single transmembrane domain at its N terminus and a family 31 glycosyl hydrolase domain at its C terminus (24). At the transcript level, NET37 was shown to be much more abundant in mouse cardiac and skeletal muscle than in most other tissues and also to be substantially up-regulated during differentiation of C2C12 myoblasts (24).

The murine C2C12 myoblast cell line recapitulates the basic patterns of signaling, gene expression, and differentiation that occur during muscle regeneration from satellite cells (26). This provides a useful model for studying the functions of NE proteins in myogenesis. Using the C2C12 system, we characterize the topology of NET37 and show that its glycosidase domain is located in the NE/ER lumen. We demonstrate that NET37 is required for efficient myoblast differentiation and that this is dependent on its glycosidase catalytic site. Furthermore, we show that NET37 interacts with a pro-form of the autocrine/paracrine factor IGF-II. Moreover, NET37 is needed for IGF-II secretion and, correspondingly, for the activation of Akt during myogenic differentiation that is mediated by IGF-II signaling. Our results show for the first time that the NE is important for secretion of an autocrine/paracrine differentiation factor and underscore its importance for regulation of signaling in myoblast differentiation. Our results raise the possibility that NET37 may mediate transmembrane communication between the ER lumen and the nuclear interior during this process.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Five shRNA sequences were initially screened for their ability to silence the expression of murine NET37, and two were found to be effective. These were NET37sh1, encoded by 5′-GCTACAACGTGACCTCCTTTA-3′, and NET37sh2 encoded by 5′-GCAAATGGCGAAGCTA-CAAGG-3′. Sense and antisense oligonucleotides were annealed and cloned into linearized pENTR/U6 (Invitrogen). Positive clones were confirmed by sequencing from both directions. To provide a control, re-circularized pENTR/U6 was generated.

The human NET37 (hNET37) cDNA was amplified from IMAGE clone 30341915 (Open Biosystems) using PCR with oligonucleotides 5′-CACCATGCTCCAGAACCCTCA-GGA-3′ and 5′-GGGGGACGCCCAGGTAAAGTAGGCG-3′. The 2142-bp PCR product was cloned into expression vector pcDNA3.1/V5-His-TOPO® (Invitrogen) as a C-terminal fusion to V5 tag, and positive clones were verified by sequencing. The hNET37^{D462A}-V5 expression construct with the putative catalytic active site mutated to alanine was created using QuikChange® (Stratagene) according to the manufacturer's instructions. The oligonucleotides used for mutagenesis are 5′-GTGGCTTCCTTCAAGTTCGCCGCG-GGCGAGGTCAGC-3′ and 5′-GCTGACCTCGCCCGC-GGCGAACTTGAAGGAAGCCAC-3′.

The HA-NET37-V5 expression construct (tagged N-terminally with HA and C-terminally with V5) was created by using oligonucleotides 5′-CACCATGTACCCATACGA-TGTTCCAGATTACGCTATGTCCCAGAACCTTCAGG-AGAC-3′ and 5′-GGAAGCCCAGGTGAAATAGG-3′ to PCR-amplify NET37 cDNA from an expression construct previously described (24). The 2183-bp PCR product was cloned into pcDNA3.1/V5-His-TOPO® (Invitrogen) with C-terminal-fused V5 and verified by sequencing.

Cell Culture and Transfection—C2C12 myoblasts (ATCC #CRL-1772) were maintained in proliferation medium: Dulbecco's modified Eagle's medium supplemented with 20% newborn calf serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Differentiation was initiated in confluent myoblast cell populations grown on gelatin-coated dishes by shifting them to differentiation medium: Dulbecco's modified Eagle's medium with antibiotics, glutamine, sodium pyruvate, and 2% horse serum. Myotube formation was monitored for up to 6 days. For transfection, cells were trypsinized, and 1×10^6 cells were mixed with 3 μg of DNA and Optifect™ (Invitrogen) according to the manufacturer's instruction and plated onto 6-well plates (Corning). Cells were maintained for 36–48 h in proliferation medium before inducing differentiation.

To generate stable myoblast cell populations expressing either hNET37V5 or hNET37^{D462A}V5, C2C12 myoblasts were initially transiently transfected with expression constructs. For a control, one population of C2C12 myoblasts was transfected with the empty vector pcDNA3.1/V5-His-TOPO® (Invitrogen). Cells were plated at a 50–70% density 24 h after transfection and selected in the presence of proliferation medium supplemented with Geneticin® (Invitrogen), initially at a concentration of 1 mg/ml for the first 48 h followed by 500 μg/ml for an additional 12 days.

Tissue Samples—Intact brain, liver, heart, and skeletal muscle tissue were harvested from 1-week-old mice. Tissues were washed and resuspended in PBS with Complete Protease Inhibitor Mixture (Roche Diagnostics). To achieve lysis minced tissues were Dounce-homogenized with a tight-fitting pestle followed by sonication. Cell lysates were quantified by Bradford assay (Bio-Rad), and equivalent amounts of cell lysates were combined with SDS loading buffer. Protein samples were separated by SDS-PAGE and subjected to immunoblot analysis.

Reverse Transcription-PCR—RNA samples were prepared from six-well plates using the Qiagen RNeasy Mini kit (Qiagen, Chatsworth, CA). Oligo-dT primers were used to make cDNA using Transcriptor first strand cDNA kit (Roche Diagnostics). For reverse transcription-PCR, primer for IGF-II used was previously described (27). Primers used for GAPDH are 5′-AGGTCGGTGTGAACGGATTTG-3′ (sense) and 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

Antibodies and Reagents—Antibodies to NET37 were generated in rabbits against the entire luminal domain of NET37 (residues 100–716) expressed in *Escherichia coli* as a His-tagged protein from pET28a (Novagen). Antibodies were affinity-purified as described (28) and used at a concentration of 5

μg/ml. The following antibody concentrations were used against: MyoD, 1:100 (Santa Cruz); Myf5, 1:100 (Santa Cruz); myogenin, 1:100 (BD Pharmingen); emerin, 1:3000 (Novocastra); calnexin, 1:1000 (Abcam); HA, 1:100 (Santa Cruz); V5, 1:500 (Invitrogen); myosin heavy chain (MyHC, 1:50; U. S. Biologicals); Alexa Fluor® 594 anti-bromodeoxyuridine (BrdUrd), 1:50 (Molecular Probes); p-Akt$^{Ser-473}$, 1:500 (Cell Signaling); total-Akt, 1:500 (Cell Signaling); p-Erk1/2, 1:250 (Cell Signaling); total-Erk1/2, 1:250 (Cell Signaling); IGF-II, 1:50 (R&D systems); H2B, 1:1000(Abcam); GAPDH, 1:10,000 (Abcam). Recombinant mouse IGF-II was purchased from R&D Systems. Rabbit IgG control antibodies were purchased from Cell Signaling.

Immunofluorescence Microscopy, Differentiation, and Proliferation Assay—Drug-selected C2C12 myoblast populations expressing hNET37-V5 and hNET37^{D462A}V5 were fixed and stained with anti-V5 antibodies as previously described (24). Images were captured using Bio-Rad (Zeiss) Radiance 2100 Rainbow laser-scanning confocal microscope and analyzed using Zeiss LSM Examiner software.

Differentiated C2C12 cells were fixed with 2% formaldehyde in PBS for 10 min at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS and incubated with monoclonal anti-MyHC antibody for 1 h at room temperature. Cells were washed and incubated with Alexa Fluor® 488-conjugated anti-mouse secondary antibody (Invitrogen) for 2 h at room temperature, and before examination, chromosomal DNA was stained using Hoechst 33342 (Invitrogen). Images were obtained using a LEICA DM IRE2 and analyzed using ImageJ. Myogenic index was described as the percent nuclei present in cells expressing myosin heavy chain. Myogenic index was quantified from analyzing a minimum of six separate field of vision covering ~3000 nuclei.

S-phase cells were determined in C2C12 cultures at the indicated time after shift to differentiation medium by incorporation of 25 μM BrdUrd (Invitrogen) for 45 min at 37 °C before fixation with 2% formaldehyde for 10 min. Cells were permeabilized with 0.1% Triton X-100 and stained with Alexa Fluor® 594 conjugated anti-BrdUrd antibodies and counterstained with Hoechst 33342 (Invitrogen). Images were taken using a LEICA DM IRE2 and analyzed using Image J. A minimum of six separate fields of vision covering ~3000 nuclei were quantified, and the fraction of S-phase cells was measured as percent nuclei positive for BrdUrd incorporation.

Subcellular Fractionation—Differentiated C2C12 cells were rinsed with PBS and resuspended in hypotonic buffer (HB: 10 mM Tris-Cl, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol) and allowed to swell for 10 min on ice. Lysis was achieved by 5–8 strokes in a Dounce homogenizer with a tight-fitting pestle. The lysate was brought to a 1.8 M sucrose concentration by the addition of a 2.4 M sucrose solution in HB and overlaid in 12-ml centrifuge tubes with a step gradient of 3 ml of 1.7 M and 4 ml of 1.6 M sucrose solution in HB followed by 1 ml of HB. The gradients were centrifuged at 150,000 × *g* for 2 h at 4 °C in a Beckman SW41Ti rotor. The gradients were fractionated into 1-ml aliquots, and protein samples were precipitated by the addition of trichloroacetic acid to 15%, separated by SDS-PAGE, and subjected to immunoblot analysis.

C2C12 cells transiently expressing HA-NET37-V5 or C2C12 cells allowed to differentiate for 4 days were trypsinized and collected. Cells were rinsed with PBS and permeabilized by treatment with 0.005 or 0.001% digitonin in PBS, respectively, for 5 min on ice. Permeabilized cells were washed twice and finally resuspended in PBS without detergent before the addition of proteinase K. Protease digestion of permeabilized cells was carried out on ice for 30 min at the indicated concentrations of proteinase K. The reactions were inactivated by the addition of SDS loading buffer. Protein samples were separated by SDS-PAGE and subjected to immunoblot analysis.

Immunoprecipitation and Endoglycosidase H (Endo H) Treatment—Differentiated C2C12 cells were rinsed with PBS and resuspended in lysis buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, 10% glycerol, and Complete Protease Inhibitor Mixture (Roche Diagnostics)) and incubated at 4 °C for 20 min. The lysate was clarified by centrifugation at 20,000 × *g* for 10 min at 4 °C. Clarified lysate was incubated with protein G-Sepharose beads for 30 min at 4 °C and further clarified by centrifugation. Lysates representing equivalent cell numbers were incubated with either anti-NET37 antibody or rabbit IgG control overnight at 4 °C. The immune complexes were precipitated using protein G-Sepharose. The immunoprecipitates were washed twice with lysis buffer and six times with ice-cold PBS before elution with 1× SDS loading buffer by heating at 95 °C for 5 min. Equivalent amounts of eluted protein complexes were separated by SDS-PAGE and subjected to immunoblot analysis.

To determine whether NET37 was *N*-glycosylated, the eluted protein complex using anti-NET37 antibody was incubated with 500 units of Endo H (New England Biolabs) for 3 h at 37 °C according to the manufacturer's instructions. As the control, NET37 immunoprecipitate was incubated with buffer alone under the same conditions. Reaction products were separated by SDS-PAGE and subjected to immunoblot analysis.

IGF-II Measurement—Conditioned media was collected from C2C12 myoblasts transfected with NET37sh1, NET37sh2, and control plasmid at day 4 after the initiation of differentiation. Cellular debris was removed by centrifugation, and secreted protein samples were precipitated by the addition of trichloroacetic acid to 30%, separated by SDS-PAGE, and subjected to immunoblot analysis.

RESULTS

NET37 Is Highly Expressed in Muscle and Up-regulated during C2C12 Differentiation—NET37 was first identified in a large-scale proteomic screen involving rodent liver to identify nuclear envelope transmembrane (NET) proteins (25). In a subsequent study, the NET37 transcript was found to be expressed at much higher levels in skeletal muscle and heart than in most other tissues examined and also was found to be up-regulated significantly during C2C12 differentiation (24). Because post-translational mechanisms in addition to transcription are important in gene expression, we examined the levels of NET37 protein in four adult mouse tissues and in C2C12 cultures undergoing myogenic differentiation. Of the four mouse tissues examined, NET37 protein was found to be expressed at the highest level (relative to histone H2B) in skeletal muscle, similar

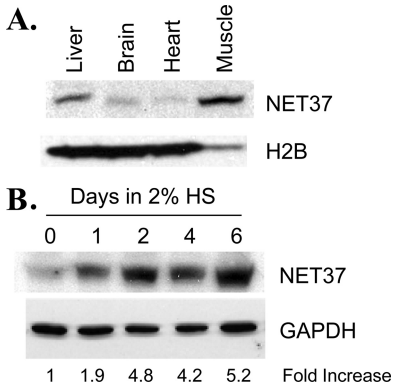

FIGURE 1. **NET37 is highly expressed in skeletal muscle and during C2C12 myoblast differentiation.** *A*, tissue samples were harvested from 1-week-old mice and solubilized. Extracts were separated by SDS-PAGE and subjected to immunoblot analysis using anti-NET37 antibody. To allow normalization to nuclei, extracts were simultaneously probed with anti-H2B antibody. *B*, C2C12 cells were grown to confluence in proliferation medium (20% serum) and then shifted to differentiation medium (2% serum). Cells were harvested at the indicated time points after shift to differentiation medium. Whole cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-NET37 antibody. -Fold induction of NET37 compared with day 0, normalized to the loading control GAPDH, is indicated.

to its transcript (24). It also was detectably expressed in liver, brain, and heart, but surprisingly, the level in heart (Fig. 1*A*) was much lower than predicted by its transcript level in this tissue (24). NET37 expression also increased ~5-fold at the protein level during C2C12 differentiation into myotubes (Fig. 1*B*). Considered together, these results are consistent with a role of NET37 in regulation and/or maintenance of the differentiated state in muscle.

NET37 Is Enriched at the NE in Myotubes and Its Glycosidase Domain Faces the NE/ER Lumen—A previous study from our laboratory showed that ectopically overexpressed NET37 targets to the NE of C2C12 myoblasts and that endogenous NET37 is highly enriched in isolated NEs from liver as compared with a peripheral ER fraction (24). Because of its potential function in myogenesis, we sought to determine the localization of endogenous NET37 in C2C12 myotubes. Because our antibodies to NET37 were not of sufficient quality for immunofluorescence detection of endogenous NET37 (data not shown), we used immunoblotting to determine NET37 levels in the NE *versus* peripheral ER membranes using fractions isolated from C2C12 myotubes on a sucrose gradient (see "Experimental Procedures"). The fractions containing nuclei (and the NE) or peripheral ER membranes were identified by immunoblot analysis using antibodies to emerin, which is localized almost exclusively at the NE, and calnexin, which is present at similar concentrations in NEs and the peripheral ER. The majority of the NET37 cofractionated with emerin in the pellet under the 1.8 M sucrose layer, where nuclei/NE were found (Fig. 2*A*). However, a small fraction of NET37 also was present in the peripheral ER fractions at the 1.6 and 1.8 M sucrose interfaces (containing calnexin but almost no emerin; Fig. 2*A*). These results indicated that in C2C12 myotubes, most of NET37 is localized to the NE, and a minor fraction is present in the peripheral ER.

NET37 is predicted to have a single transmembrane domain near its N terminus (residues 56–78) and a C-terminal domain extending from the membrane toward either the cytosol/nucleoplasm or the ER/NE lumen. The C-terminal domain contains a region homologous to family 31 α-glycosidases (residues 310–716) and an additional region (residues 79–309) with no

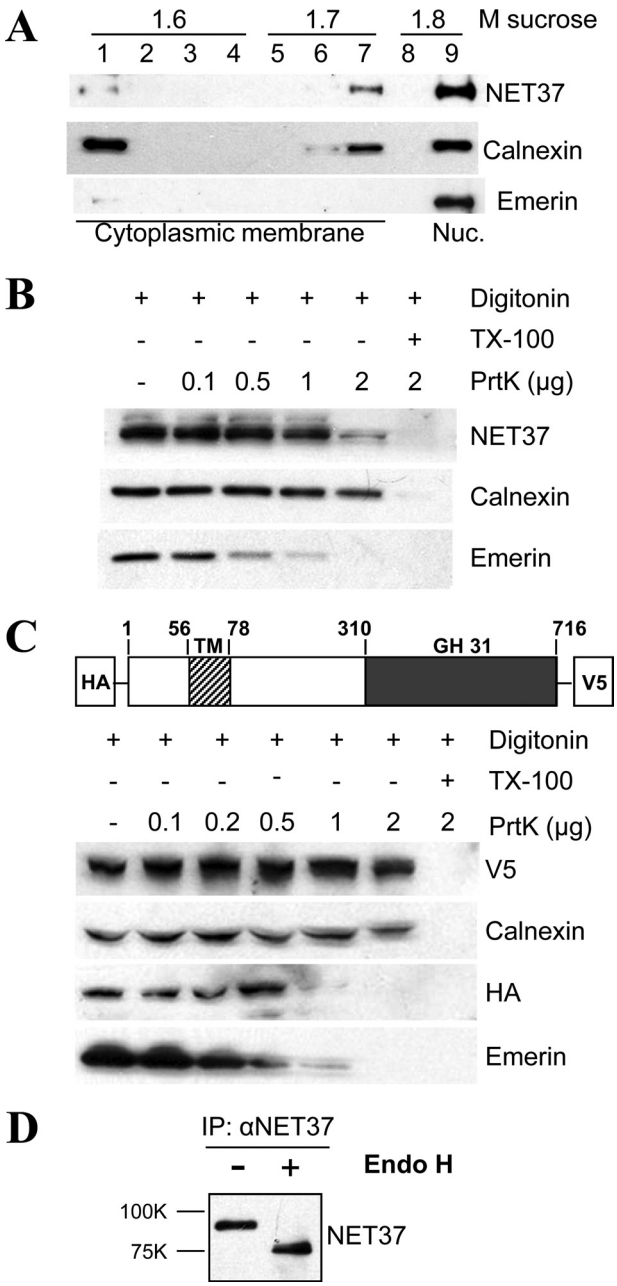

FIGURE 2. **Localization of NET37 to the NE in C2C12 cells.** *A*, homogenates of differentiated C2C12 cells were fractionated on a sucrose step gradient as described under "Experimental Procedures." Gradient fractions were analyzed by immunoblotting with antibodies to NET37, emerin (NE marker), and calnexin (ER marker). Fractions containing nuclei (*Nuc*) and cytoplasmic membranes based on the immunoblot analysis are indicated. *B* and *C*, C2C12 cells allowed to differentiate for 4 days (*B*) or proliferating C2C12 cells expressing HA-NET37-V5 (*C*) were treated with digitonin to selectively permeabilize the plasma membrane and in the lane indicated were subsequently treated with 1% Triton X-100 (*TX-100*) to permeabilize all cellular membranes. Permeabilized cells were incubated with increasing amounts of Proteinase K (*PrtK*). Protease reaction was stopped by the addition of SDS loading buffer, and samples were analyzed by immunoblotting with antibodies against NET37, emerin, calnexin, HA, or V5, as indicated. *TM*, transmembrane domain. *D*, immunoprecipitated (*IP*) NET37 from C2C12 cells allowed to differentiate for 3 days was treated either with buffer only (−) or with Endo H (+). Protein samples were analyzed by SDS-PAGE and immunoblotting using anti-NET37 antibody.

recognizable homologies (24). Because family 31 α-glycosidases have diverse carbohydrate substrate specificities, the carbohydrate that might be recognized by NET37 cannot be predicted *a priori*. To gain insight on potential substrates of NET37, we investigated the transmembrane orientation of NET37 glycosidase domain. For this we examined differentiated C2C12 myotubes, where the endogenous protein was analyzed, and C2C12 myoblasts, where transfected HA-NET37-V5 (N-terminal-tagged with HA and C-terminal-tagged with V5) was examined. Cells were treated with a low digitonin concentration that selectively disrupts the plasma membrane while leaving the ER intact, thereby making the nucleoplasmic/cytosolic space, but not the ER/NE lumen, accessible to protease. The resulting permeabilized cells were incubated with increasing concentrations of proteinase K. Calnexin, an ER resident protein facing the lumen, remained protease-protected up to 1–2 μg/ml proteinase K, indicating that the NE/ER membranes remained intact with these conditions (Fig. 2, *B* and *C*). By contrast, emerin (an INM protein), as detected by antibodies directed toward its nucleoplasmic N terminus, was mostly degraded at 0.5 μg/ml proteinase K (Fig. 2, *B* and *C*). The ~85-kDa band corresponding to endogenous NET37, detected with antiserum directed against the entire C terminus (Fig. 2*B*), as well as the C terminus of epitope-tagged NET37, detected with anti-V5 (Fig. 2*C*), remained largely intact at 1–2 μg/ml proteinase K. By contrast, the N terminus of NET37 detected with anti-HA was almost completely degraded at 1 μg/ml, similar to emerin (Fig. 2*B*). When the digitonin-permeabilized cells were further treated with Triton X-100 to permeabilize the ER before treatment with 2 μg/ml proteinase K, the luminal protein calnexin as well as NET37 were completely degraded (Fig. 2, *B* and *C*). These results indicate that NET37 is a type II transmembrane protein, with its N-terminal segment exposed to the nucleoplasm and the C-terminal glycosidase domain facing the NE/ER lumen.

The C-terminal domain of NET37 is predicted to have six consensus *N*-linked glycosylation sites (using the program at the NetNGlyc server). To determine whether NET37 is *N*-glycosylated, immunoprecipitates of endogenous NET37 from differentiated C2C12 cells were treated with Endo H. The apparent molecular mass of endogenous NET37 was decreased ~10 kDa upon treatment with Endo H, consistent with loss of multiple *N*-linked glycans (Fig. 2*D*). Because *N*-linked glycans are present only in the ER lumen, these results support the topology mapping of NET37 determined by protease protection.

NET37 Is Required for C2C12 Myogenesis—C2C12 cells constitutively express the early myogenic transcription factors MyoD and Myf5. Upon serum deprivation to trigger terminal differentiation, they exit the cell cycle and express myogenic transcription factors including MRF4 and myogenin, which control expression of genes for muscle structural proteins such as myosin heavy chain (26). The up-regulation of NET37 after upon serum withdrawal from C2C12 cells (Fig. 1 Ref. 24) suggests that it may play a role in myogenic differentiation. To directly test this possibility, we examined the differentiation capacity of C2C12 cells in which NET37 was silenced by RNAi. Proliferating C2C12 cells were transfected with either of two distinct expression vectors that generate shRNAs targeting NET37 mRNA (NET37sh1 and NET37sh2), and after 48 h, cultures were shifted to differentiation conditions. Examination of cultures 4 days after the medium shift revealed that transfection with the NET37 shRNA vectors led to substantial reduction of NET37 protein as compared with transfection with the empty vector (Fig. 3*B*). The control cultures transfected with empty vector formed a high density of elongated multinucleated myotubes, and nearly half of the nuclei were present in cells expressing MyHC. In contrast, cultures depleted for NET37 had ~50% fewer nuclei in MyHC-positive cells (Fig. 3*A*). Correspondingly, NET-37-depleted cultures were defective in expression of the late myogenic transcription factor myogenin. However, they showed no reduction in the levels of early myogenic transcription factors MyoD and Myf5 (Fig. 3*B*). The defect in myogenic differentiation by two independent shRNAs that depleted NET37, either of which could be rescued by expression of a silencing-resistant form of hNET37 (Fig. 4), demonstrates a specific role for NET37 in myogenesis rather than nonspecific shRNA effects.

A timely exit of C2C12 cells from the cell cycle after serum deprivation is critical for myogenesis (30, 31). To determine whether NET37-silenced cells can accomplish a timely cell cycle exit when triggered to differentiate, the percentage of S-phase cells was measured by BrdUrd incorporation at various time points after shift to differentiation medium. Cells transfected with either NET37sh1 or NET37sh2 showed a similar reduction in the percentage of BrdUrd-positive cells as did control cultures transfected with empty vector at each of the time points examined over 24 h (Fig. 3*C*). This indicated that the cell cycle exit was not measurably affected by NET37 depletion and that NET37 might interfere with myogenesis by some other means (see below). Taken together, these experiments demonstrate that NET37 is required for efficient differentiation of C2C12 cells into myotubes and that silencing it blocks up-regulation of myogenin, which is critical for myogenic differentiation.

Conserved Catalytic Residue in the Glycosidase Homology Domain Is Important for the Myogenic Function of NET37—NET37 is a member of family 31 glycosidases, which are represented in plants, animals, and microorganisms. The general catalytic function of the family involves exo-glycosidic removal of terminal carbohydrates moieties from substrates that range from small disaccharides to large carbohydrate polymers and sugars attached to glycoproteins (32). ER glucosidase II, a well characterized member of the family 31 glycosidase member, is involved in trimming the α-1,3-linked glucoses from *N*-linked oligosaccharide on glycoproteins in the ER lumen (33–35). The catalytic nucleophile in family 31 α-glycosidase has been identified as the aspartic acid residue within the consensus sequence K*X*D*X*GE (32, 36–39), which corresponds to Asp^{462} in murine NET37 (Fig. 4*A*), and mutation of this residue leads to a catalytically inactive protein (32, 39).

To directly investigate whether NET37 has catalytic activity, we examined two recombinant versions of NET37 for the ability to hydrolyze an α-glucosidic bond in the model substrate *p*-nitrophenyl α-D-glucopyranoside. This substrate is used routinely to detect the catalytic activity of various family 31 glycosidases including ER glucosidase II. One recombinant protein

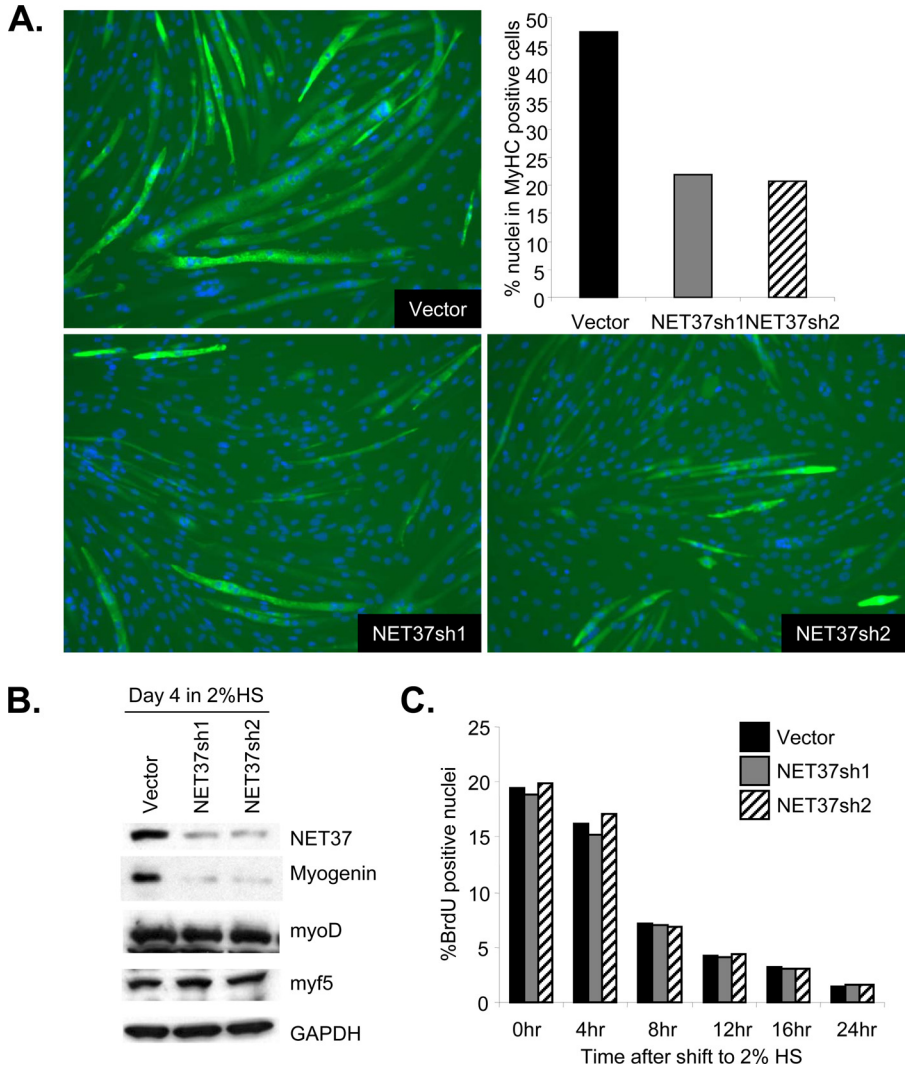

FIGURE 3. **NET37 depletion leads to reduction in myoblast differentiation.** *A*, C2C12 cells were transfected with empty vector or with two independent shRNAs and allowed to differentiate for 4 days. Subsequently cells were stained for immunofluorescence to detect MyHC. *Left*, representative images of cells stained with antibodies to MyHC and counterstained with Hoechst 33342 are shown. *Right*, quantification of myogenic index, *i.e.* percent nuclei present in cells expressing myosin heavy chain. Shown are data from a representative experiment of three independent trials. *B*, samples were transfected and differentiated as in *A*. Whole cell lysates were separated by SDS-PAGE and subjected to immunoblot analysis to detect NET37, myogenin, MyoD, and Myf5. As a loading control samples were also probed with antibodies to GAPDH. *C*, C2C12 cells were either transfected with empty vector or with two independent shRNAs and allowed to differentiate. At the indicated time points after the switch to differentiation medium, cells were labeled with BrdUrd. Cells were fixed and stained with anti-BrdUrd antibodies and counterstained with Hoechst 33342. Images were quantified, and the fraction of S-phase was measured as percent nuclei positive for BrdUrd incorporation. Shown is quantification of images from a representative experiment of two essentially identical trials. *HS*, horse serum.

contained the C-terminal glycosidase domain of NET37 (residues 300–716) fused to maltose-binding protein, expressed, and affinity-purified from *E. coli*. A second version was full-length NET37 with an additional V5 epitope tag, expressed in and immunoprecipitated from HEK293T cells. Incubation of each recombinant protein preparation with *p*-nitrophenyl α-D-glucopyranoside yielded no detectable α-glucosidic activity, as normally detected by the release of *p*-nitrophenyl (data not shown). Although we could detect no glycosidase activity for NET37 by these methods, it is possible that it does have enzymatic activity that is not detectable with the substrate analyzed or when NET37 is removed from its native biological milieu by solubilization.

To investigate whether the putative catalytic residue Asp^{462} is important for the myogenic function of NET37, we assayed a predicted catalytically inactive mutant ($NET37^{D462A}$) for its ability to complement the myogenic defect achieved with NET37 depletion. For this, we derived C2C12 cell populations that were stably transfected with expression constructs for human NET37, which is predicted to be resistant to silencing by the shRNAs that target the mouse protein. We obtained one population expressing wild type NET37 (hNET37-V5), a second population expressing the catalytic site mutant ($hNET37^{D462A}$-V5), and a third (control) population transfected with empty expression vector. The NE localization and expression level of the mutant and wild type human proteins were similar (Figs. 4, *B* and *C*, and 5*A*). These three cell populations were transiently transfected with NET37sh1 or NET37sh2, shifted to differentiation medium for 4 days, and then analyzed. We found that stable expression of wild type human NET37 fully complemented the silencing of murine NET37 by either NET37sh1 or NET37sh2, as seen by myogenin expression and MyHC expression (Fig. 4, *C* and *D*). By contrast, stable expression of human $NET37^{D462A}$ did not complement loss of the mouse protein (Fig. 4, *C* and *D*), indicating that the putative catalytic residue of NET37 is needed for its myogenic function.

Although none of the three stably transfected cell populations described in Fig. 4 spontaneously differentiated in high serum-containing proliferation medium (data not shown), C2C12 cells stably transfected with hNET37-V5 showed more rapid kinetics of myogenin up-regulation when shifted to differentiation medium as compared with control cells (Fig. 5*A*) and showed an increased percentage of MyHC-positive cells at an early time point (*Day 2*, Fig. 5*B*). In contrast, C2C12 cells stably transfected with $hNET37^{D462A}$-V5 had kinetics of myogenin and MyHC expression that were comparable with the control cultures (Fig. 5, *A* and *B*). However, the difference in MyHC-positive cells between hNET37-V5-transfected cultures and control cultures was less pronounced on day 4, and on day 6 all cultures showed the same extent of differentiation (Fig. 5*B* and supplemental Fig. S1). This indi-

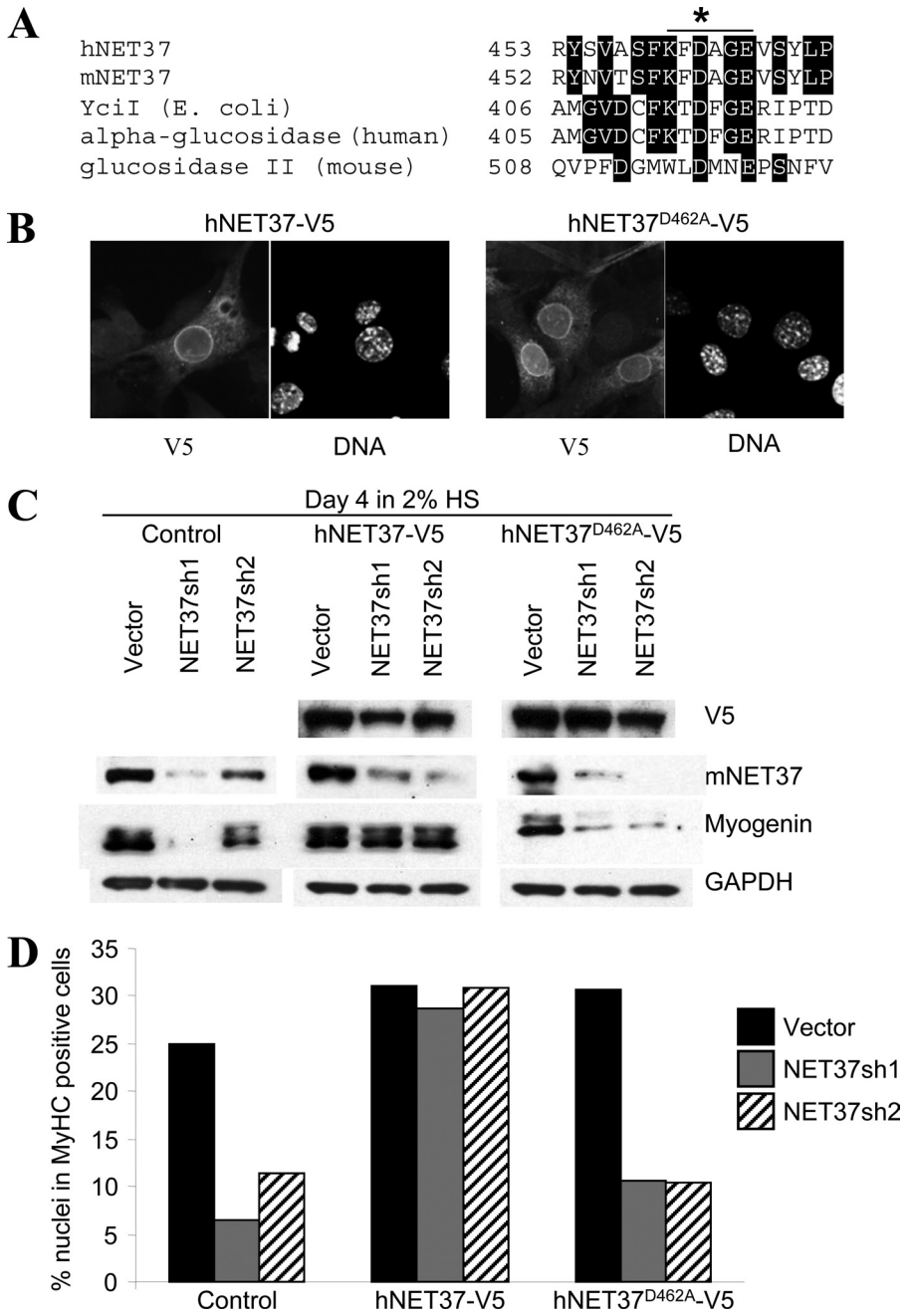

FIGURE 4. **Predicted glycosidase catalytic residue of NET37 is important for myoblast differentiation.** *A*, amino acid sequence alignment of the active site of various family 31 glycosidases. Shown are hNET37 (*Homo sapiens*), mNET37 (*Mus musculus*), YciI (*E. coli*), α-glucosidase (*H. sapiens*), and glucosidase II (*M. musculus*). Conserved residues are *highlighted in black*, and the conserved catalytic aspartate residue is marked with an *asterisk*. *B*, C2C12 cell populations stably expressing hNET37V5 or hNET37^{D462A}-V5 were fixed and stained for immunofluorescence with antibodies to V5 and counterstained with Hoechst 33342. Shown are representative images. *C*, drug-selected control C2C12 cell populations (empty expression vector pcDNA3.1/V5-His-TOPO® (Invitrogen)) and C2C12 cell populations stably expressing hNET37V5 or hNET37^{D462A}-V5 were transfected with empty vector or with two independent shRNAs and allowed to differentiate for 4 days. Whole cell extracts were separated by SDS-PAGE and subjected to immunoblot analysis to detect hNET37V5 (anti-V5), NET37, and myogenin. As a loading control samples were probed with antibodies to GAPDH. *HS*, horse serum. *D*, myogenic index, *i.e.* percent nuclei present in cells expressing myosin heavy chain, was calculated from images of MyHC-stained cells. A representative experiment of two essentially identical independent trials is shown.

cates that the kinetic but not the maximal degree of differentiation was influenced by overexpression of NET37. Together these experiments provide additional evidence that the predicted catalytic residue Asp^{462} in NET37 is important for the myogenic activity of this protein. The requirement for the predicted catalytic residue Asp^{462} to support myogenic function strongly suggests that NET37 is indeed an enzymatically active glycosidase *in vivo*, even though we could not detect enzymatic activity with our methods.

NET37 Is Required for Proper Akt Activation during C2C12 Myogenesis—During muscle differentiation, several signaling pathways are important for the myogenic program. These include the phosphatidylinositol 3-kinase/Akt pathway, which promotes expression of late MRFs (40, 41), and the p38 MAPK pathway, which promotes muscle-specific gene expression via activation of MRFs (42, 43). Conversely, aberrant activation of Erk1/2 occurred during the first 20 min after initiation of differentiation in C2C12 cells depleted of a select group of LEM domain INM proteins, causing inhibition of myotube formation.[3]

We examined if the NET37 role in myogenesis involves control of the phosphatidylinositol 3-kinase/Akt or MAPK signaling pathways. For this, NET37 was silenced in C2C12 myoblasts using NET37sh1 or NET37sh2, and the activation of MAPKs and Akt at various times after initiation of differentiation was determined with antibodies recognizing the activated (phosphorylated) forms of the kinases. Depletion of NET37 protein led to a 3–5-fold decrease in levels of phospho-Akt$^{Ser-473}$ at 2 and 4 days after shift to differentiation medium, without significant alteration of total Akt levels (Fig. 6*A*). However, depletion of NET37 did not reproducibly alter the levels of phospho-Akt$^{Ser-473}$ or Erk1/2 and p38 MAP kinase during the first hour after initiation of differentiation (Fig. 6*B* and data not shown).

NET37 Interacts with Pro-IGF-II and Is Required for IGF-II Secretion during C2C12 Myogenesis—The IGF-I and IGF-II are critical for skeletal muscle development and adult muscle regeneration (44–46). During the initial

[3] M. Huber and L. Gerace, manuscript in preparation.

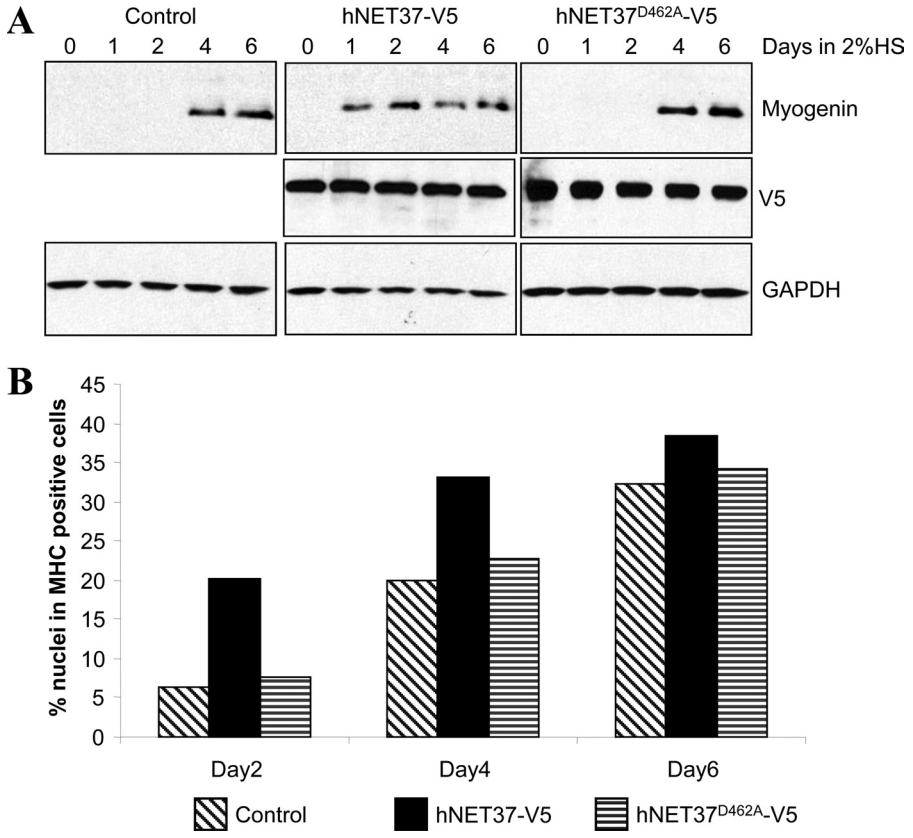

FIGURE 5. **Predicted catalytic residue of NET37 is needed for acceleration of C2C12 differentiation by human NET37.** *A*, drug-selected control C2C12 cell populations (empty expression vector pcDNA3.1/V5-His-TOPO® (Invitrogen)) and C2C12 cell populations stably expressing hNET37V5 or hNET37^{D462A}-V5 were seeded at equal density and allowed to differentiate after reaching confluence. Whole cell extracts were separated by SDS-PAGE and subjected to immunoblot analysis to detect hNET37V5 and myogenin using protein-specific antibodies. As a loading control, samples were probed with antibodies to GAPDH. *B*, the myogenic index, *i.e.* percent nuclei present in cells expressing myosin heavy chain (*MHC*), was calculated from images of MyHC-stained cells from the experiment in *A*. Data are from a representative experiment of two essentially identical independent trials is shown.

stages of myotube formation from cultured myoblasts, the phosphatidylinositol 3-kinase/Akt signaling pathway is activated by the autocrine/paracrine action of secreted IGF-II, which binds to the IGF-I receptor to induce myogenin expression (47–51). IGF-II production during myotube formation is controlled at the level of transcription, translation, and post-translational modifications (52–55). IGF-II is synthesized as a precursor pro-IGF-II. The IGF-II gene in mouse (ENSMUSG00000048583) is predicted to give rise to six alternatively spliced mRNA transcripts. All are predicted to encode protein products comprising 180 amino acids with the exception of transcript IGF-II-002 (ENSMUST00000121128), which is predicted to encode an alternate isoform containing an additional 11-amino acid extension of at the N terminus. Pro-IGF-II undergoes *O*-glycosylation on multiple residues in the ER/Golgi to give rise to multiple isoforms that migrate between ~17–26 kDa (54, 55). There is no evidence for *N*-glycosylation of pro-IGF-II in published studies, consistent with our finding that the mobility of the pro-IGF-II isoform does not change upon incubation with Endo H (data not shown). Glycosylated pro-IGF-II undergoes proteolytic cleavage in the Golgi to release mature IGF-II, which is subsequently secreted (55).

Because of the observed effect of NET37 depletion on Akt activation, we examined whether its function in myogenesis is connected to IGF-II. Silencing of NET37 in C2C12 myoblasts with either NET37sh1 or NET37sh2 led to a strong decrease in the level of IGF-II in the culture medium 4 days after initiation of differentiation (Fig. 7*A*). In addition, C2C12 myoblast populations expressing hNET37, which showed accelerated differentiation kinetics in comparison to control populations (Fig. 5), had an increased level of secreted IGF-II in the culture medium 2 days after initiation of differentiation (data not shown). Furthermore, the addition of exogenous IGF-II at the time of shift to differentiation medium restored MyHC expression in C2C12 cultures depleted of NET37 with either NET37sh1 or NET37sh2 (Fig. 7*B* and supplemental Fig. S2). This rescue of myogenesis was not because of simple restoration of NET37 levels but, rather, because of restoration of levels of phospho-Akt$^{Ser\text{-}473}$ and myogenin levels (Fig. 7*C*, *right panel*), indicating that NET37 functions upstream of IGF-II autocrine/paracrine signaling. To further delineate the role of NET37 in IGF-II regulation, we examined if NET37 silencing results in a decrease in IGF-II expression. Depletion of NET37 with either NET37sh1 or NET37sh2 did not alter the level of IGF-II mRNA and pro-IGF-II levels (Fig. 7*C*, *left*), indicating NET37 is required for IGF-II secretion. To determine whether there is physical association between pro-IGF-II and NET37, C2C12 cells were allowed to differentiate for 3 days to achieve peak NET37 expression. Detergent lysates were generated, NET37 with associated proteins was immunoprecipitated, and the immunoprecipitate was probed for the presence of pro-IGF-II. The most slowly migrating isoform of pro-IGF-II with an apparent molecular weight of ~26 kDa was found specifically in the NET37 immunoprecipitate (Fig. 7*D*). Although we did not detect the more major, faster migrating isoforms of pro-IGF-II in the immunoprecipitate, these also might interact with NET37 in cells but might have a lower affinity and be dissociated from NET37 under the detergent/salt conditions used for immunoprecipitation.

DISCUSSION

In recent years there has been growing evidence that lamins and lamina-associated transmembrane proteins are involved in regulation of signaling and gene expression (for review, see Ref. 15). A role for NET37 in muscle biology was previously suggested by its strong transcriptional up-regulation during myoblast differentiation and by its high level of expression in muscle

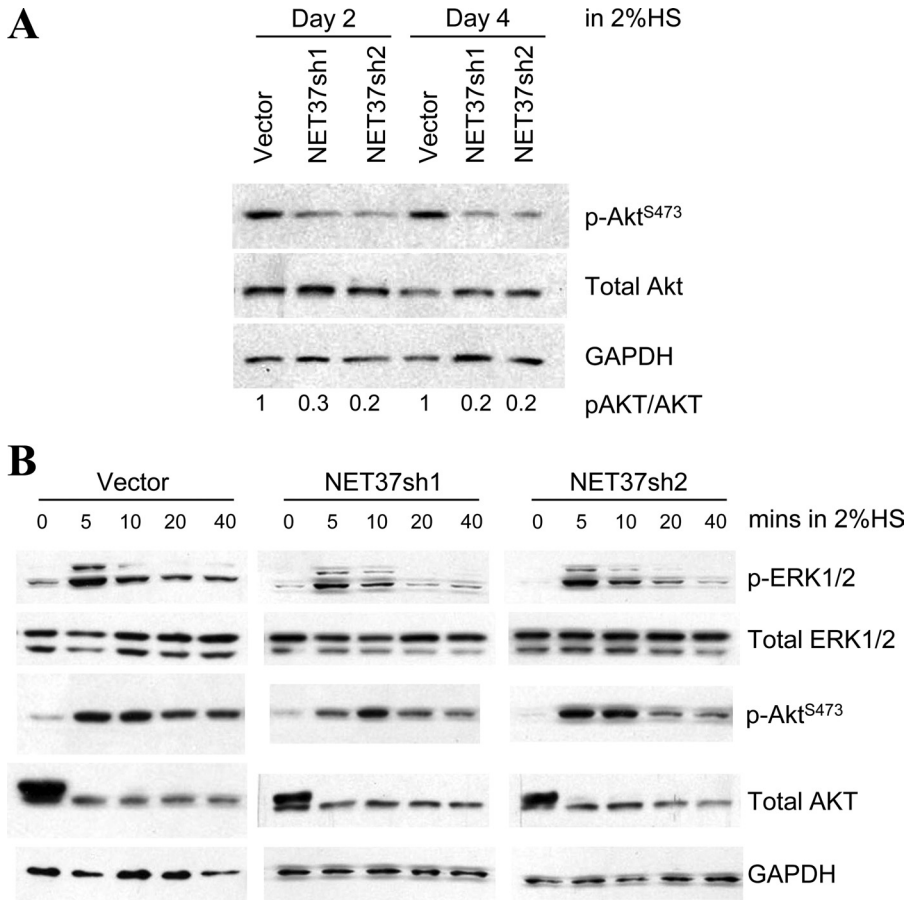

FIGURE 6. **NET37 is required for appropriate Akt activation during myogenic differentiation.** C2C12 cells were either transfected with empty vector or with two independent shRNAs and allowed to differentiate for the indicated time points. Whole cell extracts from 2- and 4-day differentiated cultures (*A*) or from samples taken 0–40 min after shift to differentiation medium (*B*) were separated by SDS-PAGE and subjected to immunoblot analysis with antibodies to p-Akt$^{Ser-473}$, total-Akt, p-Erk1/2, and total-Erk1/2. As a loading control, samples were probed with antibodies to GAPDH. The ratio of phospho-Akt$^{Ser-473}$ to total-Akt is indicated *below panel A*, with the vector control normalized to a ratio of 1 for the 2- and 4-day samples. *HS*, horse serum.

(24). In this study we have investigated whether NET37 might be involved in signaling during myogenesis.

We show that NET37 is highly expressed at the protein level in muscle and is required for myogenic differentiation of C2C12 cells. Depletion of NET37 by expression of shRNAs in myoblasts leads to a substantial reduction in the number of MyHC-expressing cells that are obtained after a shift to differentiation medium. Correspondingly, the NET37-silenced cells fail to up-regulate myogenin, a transcription factor that is critical for differentiation. Moreover, depletion of NET37 leads to a decrease in activation of Akt during the 2–4 days after shift to differentiation medium without altering the initial cell cycle exit kinetics. Because activation of phosphatidylinositol 3-kinase/Akt during myotube formation is required for sustained activation of MyoD at the myogenin promoter (51, 56), the loss of Akt activation can account for the reduced myogenin levels in NET37-depleted myotubes and the corresponding myogenic defect. Activation of phosphatidylinositol 3-kinase/Akt during myotube formation requires the autocrine/paracrine action of secreted IGF-II (40, 41, 49, 50, 56). Significantly, we found that IGF-II secretion was strongly reduced in NET37-silenced cultures even though the amount of IGF-II mRNA and the level of cell-associated pro-IGF-II were unchanged. The complete rescue of myogenesis in NET37-depleted cultures by the addition of purified IGF-II to the medium argues that the primary myogenic defect arising from NET37 involves IGF-II secretion. It is interesting to note that NET39, another NE transmembrane protein we have implicated in myogenesis, also targets the IGF-II axis, in this case by modulating mTOR-regulated transcription/translation of IGF-II.[4]

Clues on how NET37 might affect IGF-II secretion come from our analysis of the NET37 region homologous to family 31 glycosyl hydrolases. Using epitope tagging and protease protection strategies, we found that this domain faces the NE/ER lumen. Thus, it is predicted to act on substrates located in the ER lumen/secretory pathway. Furthermore, our mutational analysis suggests that that this region of NET37 may have enzymatic activity that is required for its myogenic function. Complementation assays showed that, unlike expression of the wild type hNET37, a human NET37 allele in which the conserved family 31 glycosidase catalytic nucleophile Asp^{462} was mutated to alanine (hNET37^{D462A}) (32, 36–39) was unable to rescue the myogenic defect induced by depletion of endogenous wild type NET37. In addition, ectopic overexpression of hNET37^{D462A} does not accelerate the expression of myogenin or increase the number of MyHC-expressing cells, unlike overexpression of wild type hNET37. Although this provides evidence that NET37 has a glycosidase activity important for myogenesis, we have not been able to detect *in vitro* glycosidase activity for recombinant NET37 with a model substrate. Thus, it cannot be excluded that glycosidase homology domain of NET37 is involved only in sugar recognition analogous to a lectin rather than in sugar hydrolysis.

Interestingly, we found that the slowest migrating isoform of pro-IGF-II is specifically associated with NET37 in immunoprecipitates from differentiating C2C12 cultures. It is likely that this interaction involves one or more of the multiple *O*-linked sugars on pro-IGF-II. Although we did not detect the majority of pro-IGF-II isoforms in the immunoprecipitates, these also might interact with NET37 *in vivo*, but their associations might be weaker and not withstand the stringent detergent/salt conditions needed to solubilize NET37 for immunoprecipitation. Regardless, the interaction between at least one form of pro-

[4] G. Liu, T. Guan, K. Datta, J. Coppinger, J. Yates III, and L. Gerace, manuscript in preparation.

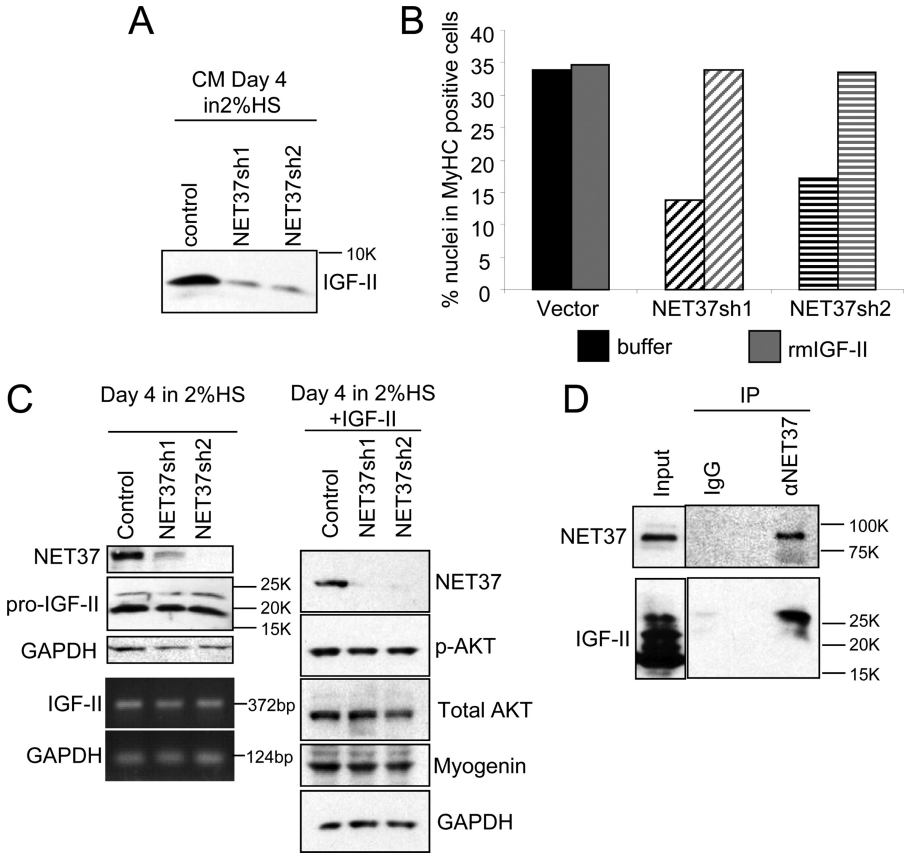

FIGURE 7. **NET37 is required for IGF-II secretion during myogenic differentiation and interacts with pro-IGF-II.** *A*, C2C12 cells were either transfected with empty vector or with two independent shRNAs and allowed to differentiate for the indicated time points. Proteins from conditioned media were trichloroacetic acid-precipitated, separated on by SDS-PAGE, and subjected to immunoblot analysis to detect secreted IGF-II. A representative blot of two identical independent trials is shown. C2C12 cells were either transfected with empty vector or with two independent shRNAs and allowed to differentiate for 4 days either in the absence or presence of 150 ng/ml of IGF-II. *HS*, horse serum; *mIGF-II*, mouse IGF-II. *B*, myogenic index *i.e.* percent nuclei present in cells expressing myosin heavy chain was calculated from images of MyHC-stained cells. A representative experiment of two essentially identical independent trials is shown. *C*, whole cell extracts were separated by SDS-PAGE and subjected to immunoblot analysis to detect NET37, pro-IGF-II, p-Akt$^{Ser\text{-}473}$, total-Akt, and myogenin. As a loading control, samples were probed with antibody to GAPDH. RNA samples at the indicated time point were prepared and subjected to reverse transcription-PCR using primer specific for IGF-II mRNA. As a control, primers specific for GAPDH were used. *D*, detergent lysates from C2C12 cells allowed to differentiate for 3 days were subjected to immunoprecipitation with antibodies specific to NET37. As a control, lysates were incubated with control rabbit IgG. Eluates and 10% of input material were separated by SDS-PAGE and subjected to immunoblot analysis to detect NET37 and pro-IGF-II. In *panel D*, to visualize the 26-kDa IGF-II isoform in whole cell extracts, a longer exposure had to be used than in *panel C*.

IGF-II and NET37 raises the attractive possibility that the presumptive glycosidase and/or carbohydrate recognition activity of NET37 is required for pro-IGF-II folding and/or egress from the ER compartment.

A potentially analogous situation involves the processing of *N*-linked carbohydrate on secretory/membrane proteins in the ER, which is required for proper folding and ER export of these proteins. *N*-Glycosylation is initiated in the NE/ER lumen by the addition of high mannose oligosaccharide ($Glc_3Man_9GlcNAc_2$). Sequential removal of two of the terminal glucose molecules from the high mannose oligosaccharide by glucosidase I followed by glucosidase II (which is also a family 31 glycosidase member (57, 58)) allows nascent glycoproteins to be recognized by calnexin/calreticulin via the remaining glucose and to continue with their folding. The third glucose molecule is trimmed by glucosidase II, and if proteins are correctly folded, they are exported from the ER for secretion. Conversely, incorrectly folded proteins are re-glucosylated by glucosyltransferase, thus directing them to re-associate with calnexin/calreticulin and giving them the chance to reach their correct folding conformation (for review, see Refs. 59 and 60). Thus, ER glucosidase II, acting in conjugation with the lectin-like chaperones calnexin/calreticulin, serves to establish a checkpoint for protein folding and quality control in the ER. By analogy, NET37 could be part of a similar machinery involved in the folding and ER export of pro-IGF-II. Because NET37 is expressed highly in only a few tissues, it may act on a limited range of client substrates, similar to other tissue-specific folding cofactors such as those involved in adiponectin secretion (29, 61). Furthermore, in addition to recognizing carbohydrate residues, NET37 also may interact with specific polypeptide motifs of substrates, possibly through the sequence in its luminal domain (residues 79–309) preceding the glycosidase homology domain. In these respects NET37 may differ from the machinery for processing and recognition of *N*-linked sugars, which is globally expressed and does not discriminate on the basis of polypeptide sequences of client substrates.

We found that NET37 is highly concentrated at the NE as compared with the peripheral ER in liver (24) as well as in myotubes (this study). This raises the question of why a potential modifying enzyme/folding chaperone for a secretory protein would be restricted to the NE rather than occur throughout the ER, as seen for other folding chaperones. One interesting possibility is that the NE localization of NET37 allows signaling across the INM between the nucleoplasm and the NE lumen. In one scenario, it is conceivable that the NET37 N terminus senses a "pro-myogenic" state in the nuclear interior and relays this information across the INM to its C-terminal NE/ER luminal domain to modulate NET37 activity and/or interactions with substrates. Conversely, and not mutually exclusively, association of the luminal domain of NET37 with interacting partners could transmit a transmembrane signal to the nucleoplasmic domain of NET37, which could positively influence myogenic signaling in the nuclear compartment. In this manner, transmembrane signaling across the INM could provide a mechanism to coordinate myogenic transcription in the nucleus with secretion of pro-

myogenic factors from the ER lumen. The short N-terminal nucleoplasmic domain of NET37 contains two consensus phosphorylation motifs for protein kinase C and one SH3 domain binding motif (SH3-Hunter), consistent with a role in signaling-related functions. Future work will be aimed at examining these questions and further elucidating the specific molecular targets of NET37 in myogenesis.

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