

Interaction of the RhoA Exchange Factor Net1 with Discs Large Homolog 1 Protects It from Proteasome-mediated Degradation and Potentiates Net1 Activity*

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Net1 is a nuclear Rho guanine nucleotide exchange factor that is specific for the RhoA subfamily of small G proteins. Truncated forms of Net1 are transforming in NIH3T3 cells, and this activity requires cytoplasmic localization of Net1 as well as the presence of a COOH-terminal PDZ binding site. We have previously shown that Net1 interacts with PDZ domain-containing proteins within the Discs Large (Dlg) family and relocalizes them to the nucleus. In the present work, we demonstrate that Net1 binds directly to the first two PDZ domains of Dlg1 and that both PDZ domains are required for maximal interaction in cells. Furthermore, we show that Net1 is an unstable protein in MCF7 breast epithelial cells and that interaction with Dlg1 significantly enhances Net1 stability. Stabilization by Dlg1 significantly increases the ability of Net1 to stimulate RhoA activation in cells. The stability of endogenous Net1 is strongly enhanced by cell-cell contact, and this correlates with a dramatic increase in the interaction between Net1 and Dlg1. Importantly, disruption of E-cadherin-mediated cell contacts, either by depletion of external calcium or by treatment with transforming growth factor β , leads to a rapid loss of the interaction between Net1 and Dlg1 and a subsequent increase in the ubiquitylation of Net1. These results indicate that Net1 requires interaction with PDZ domain proteins, such as Dlg1, to protect it from proteasome-mediated degradation and to maximally stimulate RhoA and that this interaction is regulated by cell-cell contact.

Rho family small G proteins control many aspects of cell physiology, including cytoskeletal organization, cell motility, and cell cycle progression (1, 2). They do so by acting as molecular switches, cycling between their active, GTP-bound and inactive, GDP-bound states. Once activated, Rho proteins stimulate signaling in multiple pathways by binding to downstream effector proteins and modulating their activities. Currently, 21 mammalian Rho family GTPases have been identified, with the Rac1, Cdc42, and RhoA proteins being the most thoroughly characterized (3).

Rho protein activation is controlled by a family of enzymes known as Rho guanine nucleotide exchange factors (Rho GEFs)² (4). Net1 (neuroepithelioma transforming gene 1) is a Rho GEF that was cloned as a transforming gene in a screen for novel oncogenes in NIH3T3 cells (5). Two isoforms of Net1 exist in cells, Net1 and Net1A, which are identical except for alternative NH₂-terminal regulatory domains. Both isoforms of Net1 are nuclear proteins that display marked specificities for RhoA as compared with Rac1 or Cdc42 (6, 7). Correspondingly, overexpression of either Net1 isoform in cells profoundly stimulates actin stress fiber formation, which is a hallmark of RhoA activation (8). The mechanism by which Net1 stimulates cell proliferation and transformation is complex. We and others have shown that Net1 must be enzymatically active and localized to the cytoplasm to cause cell transformation (6, 8). In addition, we have observed that Net1-dependent cell transformation requires the presence of a COOH-terminal PDZ domain binding site (8). PDZ domains are protein interaction domains that mediate contact with PDZ domain binding sites typically located at carboxyl termini of target proteins (9). Importantly, the PDZ domain binding site of Net1 is not required for catalytic activity toward RhoA, indicating that interaction with one or more PDZ domain-containing proteins is required only for cell transformation (8).

Using a peptide corresponding to the COOH-terminal PDZ binding site of Net1, Garcia-Mata *et al.* recently identified proteins within the Dlg family as Net1-interacting proteins (10). Dlg1, also known as SAP97, is a member of the membrane-associated guanylate kinase family of scaffolding proteins. It contains three tandem PDZ domains as well as L27, Src homology 3, and guanylate kinase protein interaction domains. In neurons, Dlg1/SAP97 is best known for controlling ion channel clustering within postsynaptic densities. In epithelial cells, Dlg1 controls adherens junction formation and may also function as a tumor suppressor (11–13). Interaction of Dlg1 with Net1 has been shown to redirect Dlg1 to PML nuclear bodies, and in NIH3T3 cells, overexpression of Dlg1 suppresses transformation by an oncogenic form of Net1 (10).

In the present work, we examined whether Net1 interacted directly with Dlg1 and tested the effects of this interaction on Net1 function. We observed that Net1 bound to Dlg1 through

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² The abbreviations used are: GEF, guanine nucleotide exchange factor; shRNA, short hairpin RNA; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RBD, RhoA-binding domain; TGF, transforming growth factor; E3, ubiquitin-protein isopeptide ligase.

Dlg1 Interaction Stimulates Net1 by Blocking Degradation

the first and second PDZ domains of Dlg1 *in vitro* and in cells. Importantly, we also observed that Net1 is a very unstable protein in cells and that interaction with Dlg1 protected Net1 from ubiquitin-mediated degradation. Interaction of Net1 with Dlg1 also significantly enhanced the ability of Net1 to stimulate endogenous RhoA activation. In MCF7 breast cancer cells, the interaction of endogenous Net1 with Dlg1 was dependent on the formation of E-cadherin-mediated cell contacts, and disruption of these contacts, either by removal of extracellular calcium or by treatment with TGF β , caused the dissociation of Net1 from Dlg1 and ubiquitylation of Net1. These data demonstrate that interaction with Dlg1 is a key mechanism for regulating the intracellular stability of Net1 and ultimately its ability to stimulate RhoA activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—HEK293 and MCF7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) plus 100 units/ml penicillin/streptomycin (Invitrogen). MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium/F-12 plus 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. MCF10A cells were grown in Dulbecco's modified Eagle's medium/F-12 plus 5% horse serum (Invitrogen), 20 ng/ml EGF (Calbiochem), 0.5 μ g/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), and 10 μ g/ml insulin (Sigma). HEK293 and MCF7 cells were transfected with Lipofectamine Plus (Invitrogen).

Plasmids, Antibodies, and Recombinant Proteins—For expression in *Escherichia coli*, wild type mouse Net1A was subcloned into pGEX-KG (Amersham Biosciences). The Net1A COOH terminus (residues 448–541), the COOH terminus lacking the PDZ binding site (residues 448–537), and Net1 Δ C4 (residues 1–537) were amplified from mouse Net1A by PCR and subcloned into pGEX-KG. Wild type Net1A was subcloned into pEFmyc. Net1A and Net1 Δ N were contained in pEF_{HA} as described (8). The rat Dlg1 I1B, I3 splice variant was used in all studies (14). For expression in *E. coli*, hexahistidine-tagged, full-length Dlg1 and the Dlg1 PDZ domains 1, 2, 3, and 1–3 were as described (15, 16). Dlg1 was subcloned into pCMV5M to create Myc epitope-tagged Dlg1. Dlg1 was also subcloned into pEGFP-C1 (Clontech) to create enhanced green fluorescent protein-tagged Dlg1. To create inactive PDZ domains within Dlg1, the conserved residues GLGF in PDZ domains 1, 2, and 3 (residues 200–203, 295–298, and 442–445, respectively) were mutated to GRRF by PCR. All PCRs used Pfu Turbo polymerase (Stratagene), and the entire cDNA from each clone was sequenced to confirm correct amplification. Dlg1 shRNA constructs were contained in pGeneClip/Puro (Promega) and used the following human Dlg1 target sequences (accession NM_004087): shRNA 1, nucleotides 803–821, 5'-GAAGATTGCGGGTCAATGA-3'; shRNA 2, nucleotides 79–97, 5'-GACAGACAGCTCA-GAAGTT-3'; shRNA 3, nucleotides 2363–2380, 5'-GAGAG-CAGATGGAAAAAG-3'. Magi-1b contained in pcDNA3.1 and GFP-Scribble were obtained from Addgene.

The following antibodies were used: mouse anti-Dlg1, rabbit anti-Magi-1b, mouse anti-ubiquitin, mouse anti-E-cadherin

clone 67A4, mouse anti-glyceraldehyde-3-phosphate dehydrogenase, mouse anti-SOD-1, rabbit anti-GFP, mouse anti-glutathione *S*-transferase (GST), mouse anti-HA epitope, rabbit anti-Myc epitope, and normal mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-hexahistidine (Bethyl Laboratories); mouse anti- α -tubulin clone DM1 (Sigma); rabbit anti-histone H3 (Cell Signaling). Rabbit anti-Net1 was as described (17).

Recombinant GST, GST-Net1A, GST-Net1 Δ C4, GST-Net1 502–595, and GST-Net1 502–591 were produced as described (8). Purified GST proteins bound to glutathione-agarose were contained in PBS plus 25% glycerol. Hexahistidine-tagged Dlg1 and Dlg1 PDZ domains 1, 2, 3, and 1–3 were expressed in BL21DE3 *E. coli* and purified as described (15). Purified proteins were dialyzed overnight in PBS plus 10% glycerol. All recombinant proteins were stored at -80°C .

PDZ Domain Pull-downs—When testing for interaction of full-length proteins *in vitro*, purified, recombinant GST-Net1A bound to glutathione-agarose and purified, hexahistidine-tagged, full-length Dlg1 (360 and 100 nM, respectively) were incubated overnight at 4°C in Buffer A (20 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 150 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). To measure the interaction of COOH-terminal PDZ binding site of Net1 with the isolated PDZ domains of Dlg1, GST-Net1A 448–541 or 448–537 bound to glutathione-agarose (360 nM each) were mixed with hexahistidine-tagged PDZ domains 1, 2, 3, or 1–3 (100 nM each) in Buffer A overnight at 4°C . In all experiments, bound proteins were pelleted by centrifugation and washed extensively with Buffer A plus 25 mM imidazole. After washing, proteins were solubilized in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to PVDF membrane. The presence of GST-Net1 and PDZ domain proteins were detected by Western blotting using antibodies to GST and hexahistidine, respectively. Western blots were developed using enhanced chemiluminescence or the LI-COR Odyssey Imaging System. When using the LICOR system, membranes were blocked with Odyssey Blocking Buffer and incubated overnight at 4°C with the appropriate antibody. After washing with Tris-buffered saline plus 0.1% Tween 20 (TBST), membranes were incubated with Alexa-Fluor680-conjugated anti-mouse (Invitrogen) or IRDye800-conjugated anti-rabbit (Rockland Immunochemicals) antibodies for 30 min at room temperature. Blots were washed with TBST and scanned at 700 and 800 nm, and band intensities were quantified using Odyssey software.

Dlg1 Knockdown Assays—MCF7 cells were transfected with pGeneClip without insert or with pGeneClip containing one of three different sequences targeting human Dlg1. After 24 h, the transfected cells were enriched by selecting in puromycin (10 μ g/ml) overnight. The puromycin was then removed, and the cells were allowed to grow for an additional 2 days. Cells were then lysed in 2% SDS buffer containing protease and phosphatase inhibitors (18), and protein concentrations were determined by BCA assay (Pierce). Equal amounts of protein were resolved by SDS-PAGE and transferred to PVDF membrane. Levels of endogenous Dlg1, Net1, and α -tubulin were determined by Western blotting.

Net1 Half-life Assays—For [³⁵S]methionine labeling, MCF7 cells were transfected with HA-tagged Net1A alone or plus enhanced green fluorescent protein-tagged wild type Dlg1 or Dlg1 containing inactivating point mutations in PDZ domains 1 and 2. After 2 days, the cells were washed with PBS and incubated at 37 °C for 1 h in methionine-free medium plus 10% fetal bovine serum that was previously dialyzed in PBS, and then 100 μCi of [³⁵S]methionine was added for an additional 4 h. After removal of ³⁵S-containing medium, the cells were washed with PBS and then incubated in normal growth medium plus 1 mM non-radioactive methionine for different periods of time. The cells were then lysed in radioimmune precipitation buffer (18), and the Net1A was immunoprecipitated using an anti-HA antibody and Protein A-Sepharose. Immunoprecipitates were washed multiple times with Buffer B (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), resolved by SDS-PAGE, and transferred to PVDF membrane. Membranes were exposed to x-ray film to detect radioactive HA-Net1A, and the position of the Net1A was confirmed by Western blotting. Levels of radioactivity in the Net1A bands were determined by liquid scintillation after excision from the membrane. When measuring the half-life of endogenous Net1, nontransfected cells were pulse-chased with [³⁵S]methionine as described above, and the endogenous Net1 was immunoprecipitated using rabbit anti-Net1. When Net1 half-life was measured using cyclohexamide, cells were either transfected or not and then treated with the protein translation inhibitor cyclohexamide (10 μg/ml) for different periods of time. The cells were then lysed in 2% SDS buffer, and protein concentrations were determined using a BCA assay. Equal amounts of protein were resolved by SDS-PAGE and transferred to PVDF membrane. Levels of Net1 and other proteins were determined by Western blotting. In all half-life assays, data were plotted using Prism software and fit using a single phase exponential decay curve.

Immunoprecipitations and Cell Fractionation—When testing for interaction between endogenous Net1 and Dlg1 by immunoprecipitation, MCF7 cells were lysed in Triton lysis buffer (8) and incubated on ice for 10 min, and insoluble material was pelleted by centrifugation (16,000 × g, 10 min, 4 °C). Lysates were then cleared by incubation with 2 μg of preimmune serum or nonspecific rabbit IgG (Sigma) plus Protein A-Sepharose (Rockland Immunochemicals) for 1 h at 4 °C. Antibody-Protein A-Sepharose complexes were pelleted by centrifugation, and the supernatants were removed. Net1 or Dlg1 was then immunoprecipitated from these supernatants by incubating with the appropriate antibody plus Protein A-Sepharose for 2 h at 4 °C. Protein A-Sepharose-antibody complexes were pelleted by centrifugation, washed with Buffer C (0.5% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), and resuspended in Laemmli sample buffer. Samples were resolved by SDS-PAGE and transferred to PVDF membrane, and the appropriate proteins were detected by Western blotting. When determining whether extracellular calcium was required for Net1-Dlg1 interaction, cells were washed twice in PBS and incubated at 37 °C in Dulbecco's modified Eagle's medium lacking calcium plus 4 mM EGTA for 30 min prior to lysis. When testing for ubiquitylation of endogenous Net1, MCF7 cells were incubated with a 10 μM concentration of the

proteasome inhibitor MG132 (Calbiochem) for 1 h prior to lysis. MG132 (10 μM) and *N*-ethylmaleimide (1 mM) were also contained in the lysis and wash buffers.

To distinguish between Net1-Dlg1 complexes in the nucleus and elsewhere in the cell, confluent MCF7 cells were lysed in hypotonic lysis buffer (18), using 20 strokes with a Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation (500 × g, 5 min, 4 °C). The pellet was resuspended in hypotonic lysis buffer, and the remaining unbroken cells were lysed with a second round of homogenization. Nuclei were collected by centrifugation and resuspended in Triton lysis buffer, and the DNA was sheared using a 1-ml syringe and 22-gauge needle. Insoluble material was pelleted by centrifugation (16,000 × g, 10 min, 4 °C), and the supernatant was recovered as the nuclear fraction. Triton X-100 was added to the supernatant from the first homogenization to a final concentration of 1%, and after incubation on ice for 10 min the insoluble proteins were pelleted by centrifugation (16,000 × g, 10 min, 4 °C). The soluble fraction was recovered as the extranuclear fraction. Dlg1 was then immunoprecipitated from the nuclear and extranuclear fractions using mouse anti-Dlg1 and resolved by SDS-PAGE. After transfer to PVDF membrane, the presence of Net1 in the immunoprecipitates was determined by Western blotting. Purity of the nuclear and extranuclear fractions was determined by Western blotting for histone H3 and SOD-1, which are present in the nucleus and cytosol, respectively.

Immunofluorescence—MCF7 cells grown on glass coverslips were allowed to reach confluence and then transfected with Myc-Net1A. The cells were fixed and permeabilized as described (8). Cells were then incubated for 1 h at 37 °C with rabbit anti-Myc and either mouse-anti-E-cadherin or mouse anti-Dlg1 antibodies (2 μg/ml) in PBS plus 0.1% Tween 20 (PBST) and 1% bovine serum albumin. After washing in PBST, cells were incubated for 1 h at 37 °C with Cy2-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rabbit antibodies (2.5 μg/ml) (Jackson ImmunoResearch) plus 1 μg/ml DAPI (Sigma). The cells were then washed in PBST, rinsed with water, and mounted on glass slides using Fluormount (Calbiochem). Cells were visualized using a Zeiss Axiovert microscope, and images were captured using Axiovision software (Zeiss).

RESULTS

Identification of PDZ Domains within Dlg1 That Mediate Interaction with Net1A—We showed previously that Net1ΔN requires a COOH-terminal PDZ domain binding site to transform NIH3T3 cells in focus formation assays (8). We subsequently showed that this PDZ binding site mediates interaction with Dlg1 family proteins within cells (10). Dlg1 is a scaffolding protein that contains multiple protein interaction domains, including three tandem PDZ domains. Dlg1 coordinates different aspects of cell signaling by functioning as a platform for multiprotein signaling complexes and may also act as a tumor suppressor (12). To determine whether Net1 isoforms directly interact with Dlg1, we tested whether the recombinant proteins produced in *E. coli* would interact *in vitro*. For these assays, wild type Net1A or Net1A lacking the COOH-terminal PDZ binding

Dlg1 Interaction Stimulates Net1 by Blocking Degradation

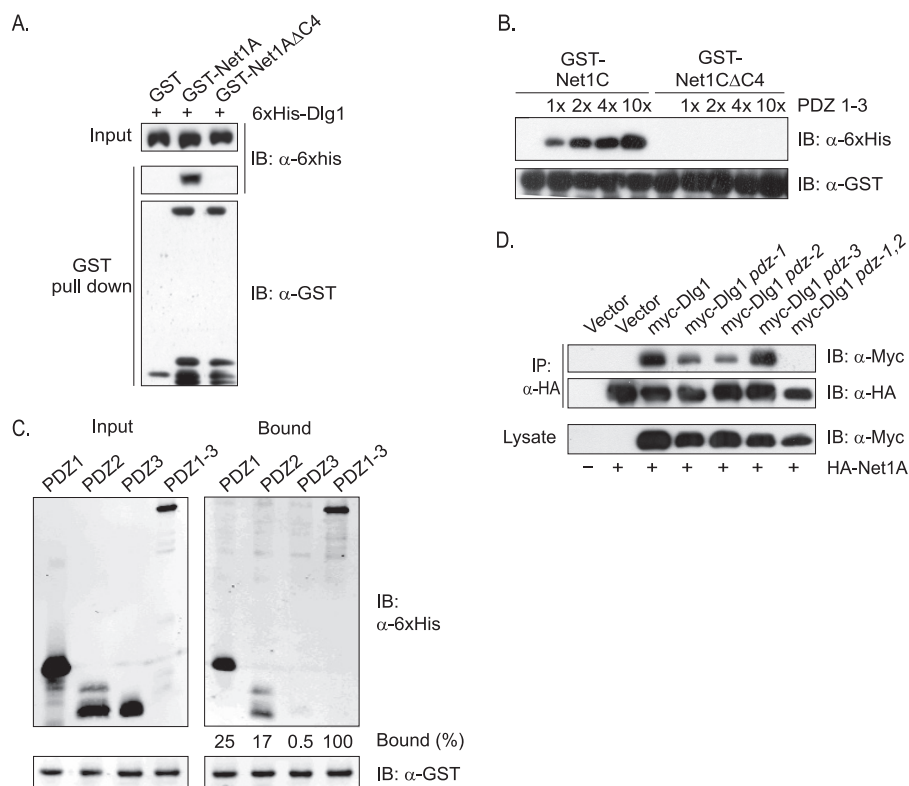


FIGURE 1. Net1 interacts directly with the PDZ1 and PDZ2 domains of Dlg1 *in vitro* and in cells. *A*, equimolar amounts of GST, GST-Net1A, or GST-Net1AΔC4, which lacks the last 4 amino acids corresponding to the PDZ binding site, were prebound to glutathione-agarose and incubated with hexahistidine-tagged, full-length Dlg1. Glutathione-agarose-GST complexes were pelleted by centrifugation, washed, and resolved by SDS-PAGE. GST proteins and hexahistidine-tagged Dlg1 present in the pull-downs were detected by Western blotting (*IB*) using GST and hexahistidine antibodies, respectively. *B*, the GST-Net1 COOH terminus (GST-Net1C) or GST-Net1 COOH terminus ΔC4 (GST-Net1CΔC4) prebound to glutathione-agarose was incubated with increasing amounts of hexahistidine-tagged PDZ domains 1–3 of Dlg1. Bound PDZ1–3 was detected by Western blotting using an anti-hexahistidine tag antibody. *C*, GST-Net1 COOH terminus prebound to glutathione-agarose was incubated with hexahistidine-tagged PDZ1, PDZ2, PDZ3, or PDZ1–3. Interacting PDZ domains were detected by Western blotting. The percentage bound represents the amount of PDZ domain protein bound to GST-Net1C compared with that included in the assay. *D*, HEK293 cells were transfected with HA-Net1A and Myc-wild type Dlg1 or Myc-Dlg1 containing the inactive PDZ domains shown. The HA-Net1A was then immunoprecipitated (*IP*) and tested for co-precipitation of Dlg1 proteins by Western blotting. For *A–D*, at least three independent experiments were performed, with representative experiments shown.

site (Net1AΔC4) were produced as GST fusion proteins and purified using glutathione-agarose. Net1A was chosen for these experiments because this was the isoform used for characterization of Net1-Dlg1 interactions previously, and the COOH-terminal PDZ binding sites of Net1 and Net1A are identical (10). Wild type Dlg1 was produced as a hexahistidine-tagged fusion protein in *E. coli* and purified using Ni²⁺-nitrilotriacetic acid resin. The purified proteins were incubated overnight at 4 °C in the presence of glutathione-agarose, and the GST-Net1A proteins were then precipitated by centrifugation. After washing, co-precipitating Dlg1 was detected by Western blotting. In these assays, we observed that recombinant Dlg1 efficiently co-precipitated with GST-Net1A but not with GST alone or with GST-Net1A lacking the PDZ binding site (Fig. 1*A*, middle lane). Thus, Net1A and Dlg1 directly interact *in vitro*, and this interaction requires the presence of the COOH-terminal PDZ binding site within Net1A.

Dlg1 contains three tandem, type I PDZ domains that display unique specificities for target proteins within cells. To confirm that the PDZ domains of Dlg1 mediate interaction with Net1A,

we examined whether the combined PDZ domains of Dlg1 interacted with the Net1A COOH terminus *in vitro*. We have shown previously that peptides corresponding to the COOH terminus of Net1 are sufficient for interaction with Dlg1 in cell lysates (10). Increasing amounts of recombinant, hexahistidine-tagged Dlg1 PDZ1–3 were incubated with the GST-Net1A COOH terminus (GST-Net1C) or the GST-Net1A COOH terminus lacking the PDZ binding site (GST-Net1CΔC4), and the presence of PDZ1–3 in the precipitates was detected by Western blotting. We observed that PDZ1–3 bound to the full-length Net1A COOH terminus in a dose-dependent manner but not to the Net1A COOH terminus lacking the PDZ binding site (Fig. 1*B*). Thus, the PDZ binding site within the COOH terminus of Net1A interacts directly with one or more of the PDZ domains of Dlg1. We then examined which PDZ domain mediates this interaction. The combined and individual PDZ domains of Dlg1 (PDZ1–3, PDZ1, PDZ2, and PDZ3) were expressed as hexahistidine fusion proteins in *E. coli* and purified. These proteins were then tested for interaction with the Net1A COOH terminus. In these assays, we observed that both the PDZ1 and PDZ2 domains bound to Net1A, whereas the PDZ3 domain

did not interact (Fig. 1*C*, right). Moreover, the PDZ1 and PDZ2 domains bound to the Net1 COOH terminus with similar efficiency, indicating that Net1 isoforms may interact with both PDZ domains in the context of full-length Dlg1.

To determine whether the PDZ1 and PDZ2 domains of Dlg1 mediate binding to full-length Net1A in cells, we examined the ability of Dlg1 containing inactivating mutations in one or more of its PDZ domains to co-immunoprecipitate with Net1A. The PDZ domains were inactivated by substituting the conserved GLGF motif in each domain with GRRF, which precludes interaction with PDZ domain binding sites (19). HEK293 cells were transfected with HA-Net1A and wild type or PDZ mutant Myc-Dlg1 constructs. The HA-Net1A was then immunoprecipitated and tested for the co-precipitation of transfected Dlg1 proteins by Western blotting. As shown in Fig. 1*D*, wild type Dlg1 efficiently co-precipitated with Net1A, as did Dlg1 containing an inactive PDZ3 domain. However, mutation of the PDZ1 domain strongly inhibited the co-precipitation of Dlg1 with Net1A, as did mutation of the PDZ2 domain. Inactivation of both the PDZ1 and PDZ2 domains completely abolished the

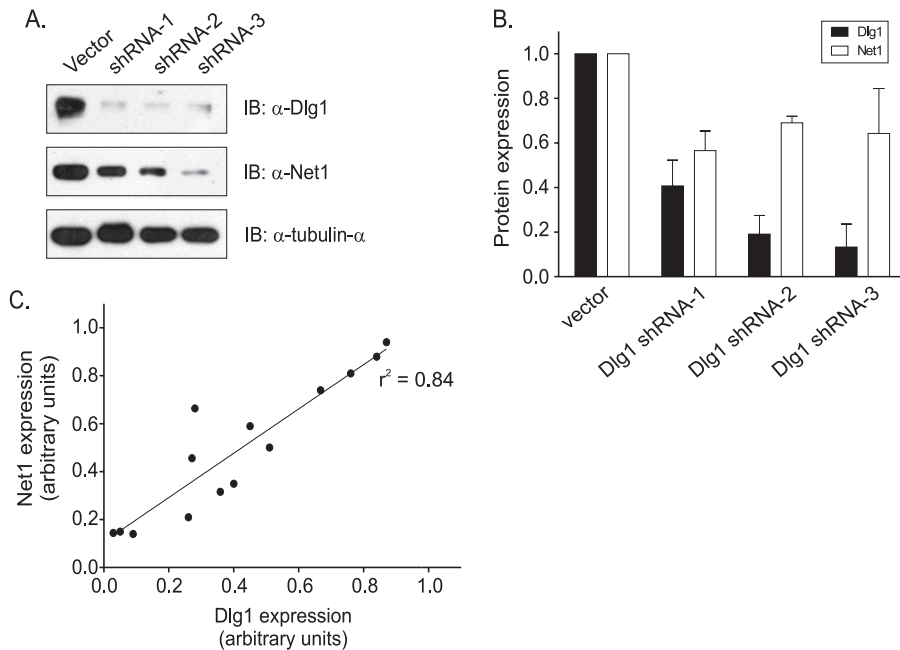


FIGURE 2. shRNA-mediated knockdown of Dlg1 also results in reduced Net1 expression. *A*, MCF7 cells were transfected with a control vector lacking an shRNA insert or one of three shRNA vectors targeting different regions in human Dlg1. Transfected cells were enriched by incubation with puromycin. Four days after transfection, whole cell lysates were isolated, and equal amounts of lysate were resolved by SDS-PAGE. The presence of Dlg1 or Net1 in the lysates was then detected by Western blotting (*B*). The amount of α -tubulin in the lysates was monitored by Western blotting as a loading control. *B*, quantification of the effects of Dlg1 knockdown on Net1 expression. The expression of each protein was normalized to the levels of α -tubulin. Results are from three independent experiments. *Error bars*, S.E. *C*, analysis of Net1 and Dlg1 expression by Western blotting in MCF7 cells transfected with Dlg1 shRNA-1. Results are from 14 independent experiments.

co-precipitation of Dlg1 with Net1A. Thus, these data indicate that Net1A is capable of interacting with the PDZ1 and PDZ2 domains of Dlg1 in cells with approximately equal affinity and that both PDZ domains must be inactivated to block interaction with Net1A.

RNA Interference-mediated Inhibition of Dlg1 Expression Reduces Net1 Expression in Breast Cancer Cells—Previously, it has been shown that the Rho GEF Dbl is protected from ubiquitin-mediated degradation by interaction with the chaperone HSC70 (20). In addition, the Rho GEFs Ect2, Vav1, and hPEM-2 are regulated by ubiquitylation (21–23). To determine whether the binding of Dlg1 controlled Net1 expression, we tested the effect of inhibiting Dlg1 expression on the expression of endogenous Net1. MCF7 breast cancer cells were transfected with a control shRNA plasmid or three different shRNA plasmids specific for distinct sequences within human Dlg1. After 24 h, the transfected cells were enriched by incubation overnight with puromycin and then allowed to grow for an additional 2 days. The cells were then lysed and tested for expression of endogenous Dlg1 and Net1. Expression of α -tubulin was monitored as a control. As shown in Fig. 2*A*, each shRNA targeting Dlg1 efficiently reduced its expression (*top*). Importantly, endogenous Net1 expression was also reduced by the knockdown of Dlg1 (*middle*). α -Tubulin expression was not affected (*bottom*). Although the effect on Net1 expression varied between experiments, quantification of the results from three independent experiments indicated that inhibiting Dlg1 expression consistently reduced Net1 expression by at least 35% in

these cells (Fig. 2*B*). Similar results were observed when these experiments were conducted in HeLa cells (data not shown). The reduction in Net1 expression was unlikely to be a nonspecific effect of the shRNAs targeting Dlg1, since each of the three Dlg1-specific shRNAs was targeted to a different region within the Dlg1 mRNA sequence. Because there was a wide range in the change in Net1 expression accompanying Dlg1 knockdown, we performed additional experiments using Dlg1 shRNA-1 to more closely analyze the dependence of Net1 expression on Dlg1. These assays were then combined with the Dlg1 shRNA-1 knockdown data from Fig. 2*B* and plotted to reflect the levels of both Dlg1 and Net1. In a total of 14 experiments, we observed a high degree of correlation between the level of Dlg1 knockdown and the associated reduction in Net1 expression (Fig. 2*C*). Taken as a whole, these results indicate that Net1 expres-

sion is directly dependent upon Dlg1 expression.

Net1A Exhibits a Short Half-life in Breast Cancer Cells That Is Significantly Extended by Co-expression of Dlg1—The effect of Dlg1 knockdown on Net1 expression suggested that the interaction between Net1 and Dlg1 may affect Net1 stability. To determine if this was the case, pulse-chase analysis of Net1 protein half-life was performed. Briefly, MCF7 cells were transfected with HA-tagged Net1A, minus or plus GFP-tagged wild type Dlg1 (GFP-Dlg1) or GFP-Dlg1 containing inactivating mutations in PDZ domains 1 and 2 (Dlg1 mut). The cells were then labeled with [³⁵S]methionine, followed by incubation in the presence unlabeled methionine for periods up to 4 h. After labeling, the cells were lysed in radioimmune precipitation buffer, and the HA-Net1A was immunoprecipitated, separated by SDS-PAGE, transferred to PVDF membrane, and identified by Western blotting. The Net1A bands were excised, and the levels of [³⁵S]methionine labeling were quantified by liquid scintillation. As shown in Fig. 3*A*, HA-Net1A exhibited a relatively short half-life of 0.63 h when expressed alone. However, in the presence of co-expressed GFP-Dlg1, the Net1A was essentially not degraded, exhibiting a half-life to 1.7×10^5 h. This effect clearly required the potential of Dlg1 to interact with Net1A, since Net1A co-expressed with the Dlg1 mutant lacking functional PDZ1 and PDZ2 domains exhibited a half-life of only 0.47 h. Using [³⁵S]methionine labeling, we also tested the stability of endogenous Net1. In these experiments, we observed that the half-life of endogenous Net1 was 0.60 h, similar to that of transfected Net1A (Fig. 3*B*). Thus, these data indicate that

Dlg1 Interaction Stimulates Net1 by Blocking Degradation

co-expression of wild type Dlg1 significantly stabilized Net1A expression and that this required the functionality of the PDZ1 and PDZ2 domains. These data also indicate that endogenous Net1 is an unstable protein in actively growing MCF7 cells.

The Net1-interacting Protein Magi-1b Does Not Efficiently Stabilize Net1A—The PDZ domain-containing scaffolding protein Magi-1b was previously identified as a Net1-interacting protein in yeast two-hybrid assays (24). To determine

whether PDZ domain-containing proteins other than Dlg1 could stabilize Net1, we examined whether Magi-1b associated with Net1A in breast cancer cells and if co-expression of Magi-1b extended the half-life of Net1A in these cells. We first determined whether Net1A interacted with Magi-1b in breast cancer cells. MCF7 cells do not normally express Magi-1b, so the cells were co-transfected with HA-tagged Net1A, minus or plus increasing amounts of Magi-1b. The cells were then lysed, and the HA-Net1A was immunoprecipitated and tested for co-precipitation of Magi-1b by Western blotting. In these experiments, we observed that Magi-1b efficiently co-precipitated with Net1A (Fig. 4A, top). Moreover, high levels of Magi-1b expression effectively blocked the ability of endogenous Dlg1 to co-immunoprecipitate with Net1A, indicating that the affinity of Magi-1b for Net1A is similar to that of Dlg1.

We then examined whether co-expression of Magi-1b extended the half-life of Net1A. MCF7 cells were transfected with HA-tagged Net1A plus GFP, GFP-tagged wild type Dlg1, GFP-Dlg1 containing mutations within PDZ domains 1 and 2 (GFP-Dlg1 mut), or wild type Magi-1b. As

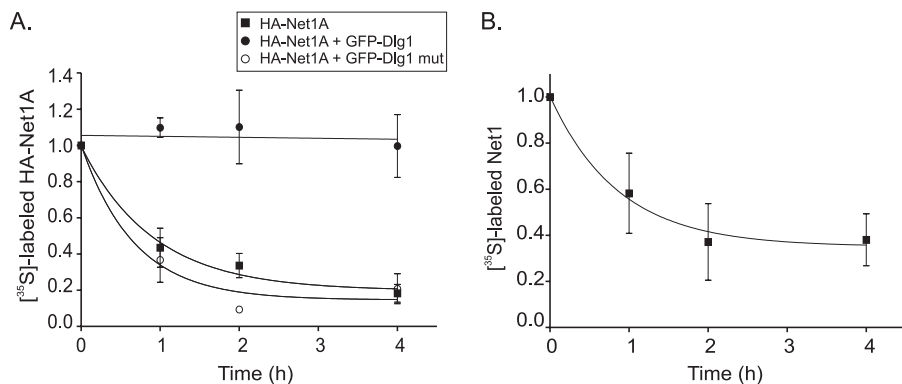


FIGURE 3. Co-expression of Dlg1 dramatically enhances the intracellular stability of Net1A. A, MCF7 cells were transfected with HA-Net1A alone or together with wild type GFP-tagged Dlg1 or Dlg1 containing inactivating point mutations in PDZ domains 1 and 2 (GFP-Dlg1 mut). After 2 days, the cells were labeled with [³⁵S]methionine, followed by incubation in medium lacking radioactive methionine for different periods of time. The cells were then lysed, and the HA-Net1A was immunoprecipitated. After resolving by SDS-PAGE, the amount of HA-Net1A in the precipitates was determined by Western blotting, and the levels of [³⁵S]methionine in the Net1A were determined by scintillation counting. Shown is the average of four independent experiments. Error bars, S.E. B, the half-life of endogenous Net1 in MCF7 cells was determined as described in A. Shown is the average of three independent experiments. Error bars, S.E.

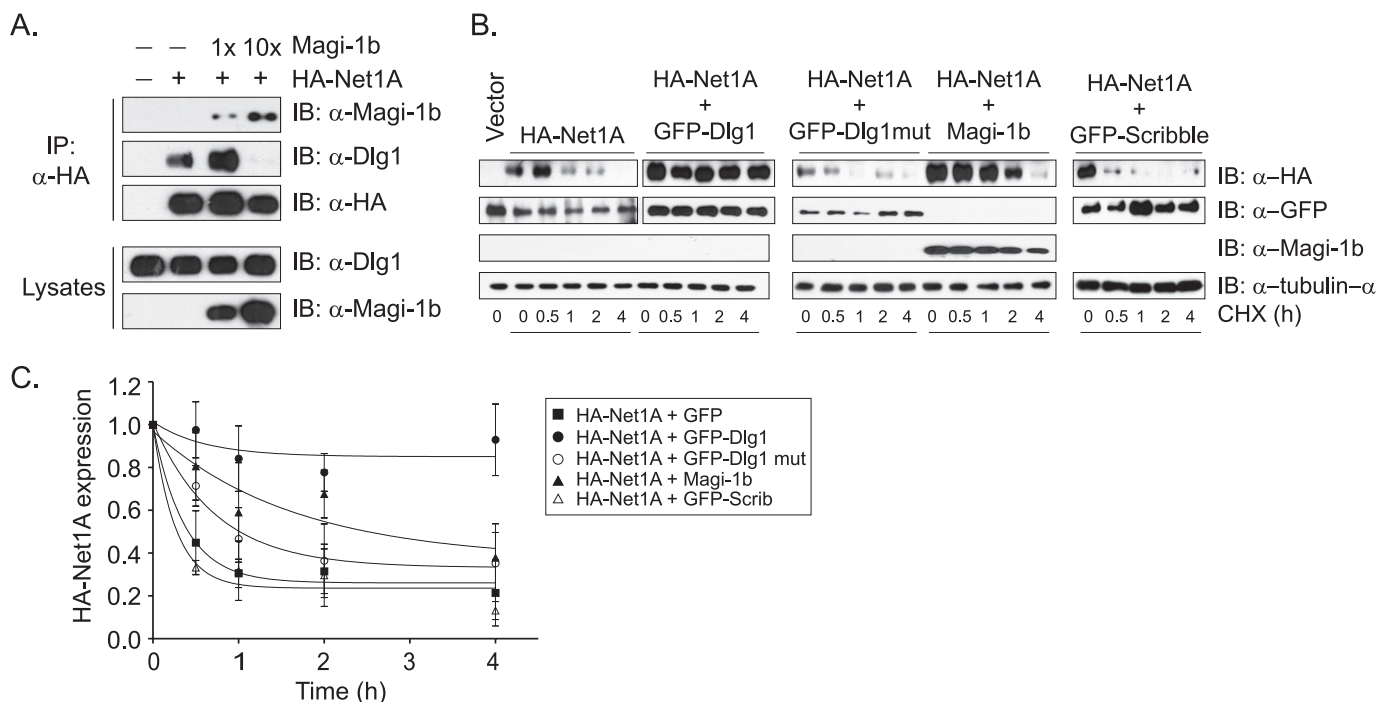


FIGURE 4. The Net1-interacting protein Magi-1b does not significantly enhance Net1 stability. A, MCF7 cells were transfected with HA-Net1A minus or plus different amounts of Magi-1b. The HA-Net1A was then immunoprecipitated (IP) and tested for the co-precipitation of endogenous Dlg1 and transfected Magi-1b by Western blotting (IB). Shown is a representative experiment from three independent experiments. B, MCF7 cells were transfected with HA-Net1A plus GFP, GFP-Dlg1, GFP-Dlg1 PDZ1/2 mutant, Magi-1b, or GFP-Scribble. The protein translation inhibitor cyclohexamide (CHX) was then added for the times shown, and whole cell lysates were produced. Equal amounts of lysate were resolved by SDS-PAGE, and the levels of each transfected protein as well as endogenous α -tubulin were detected by Western blotting. Shown is a representative experiment. C, quantification of effects on HA-Net1A half-life. Shown are the results of four independent experiments. Error bars, S.E.

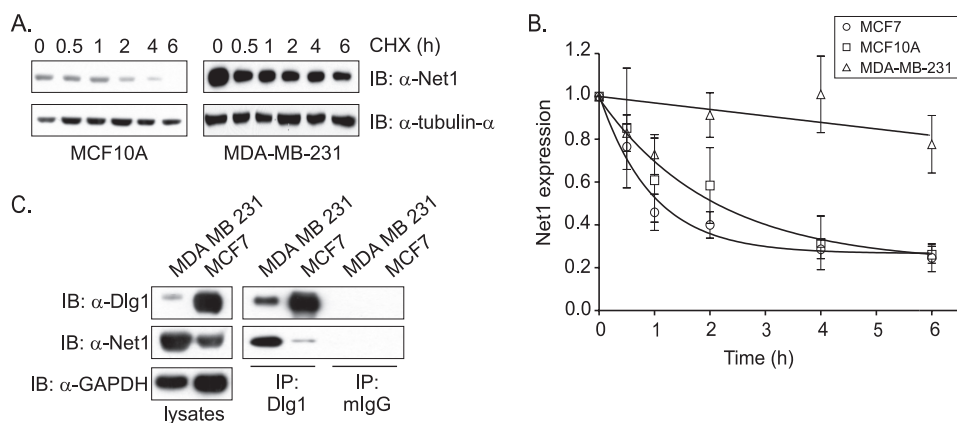


FIGURE 5. Analysis of Net1 stability in human breast cell lines. A, actively growing MCF10A and MDA-MB-231 cells were treated with cyclohexamide for the times shown, and whole cell lysates were produced. Levels of endogenous Net1 and α -tubulin were examined by Western blotting (IB). B, quantification of Net1 half-life in MCF7, MCF10A, and MDA-MB-231 cells. Results are the average of three independent experiments. Error bars, S.E. C, actively growing MCF7 and MDA-MB-231 cells were lysed and used for immunoprecipitation (IP) with control mouse IgG (mlgG) or mouse anti-Dlg1. Co-immunoprecipitation of endogenous Net1 was examined by Western blotting. Shown is a representative experiment from three independent experiments.

a control, we also co-expressed GFP-Scribble, which is a PDZ domain-containing protein that does not interact with Net1A.³ Protein translation was then inhibited for different periods of time using cyclohexamide, following which the cells were lysed, and levels of the transfected proteins were determined by Western blotting. As a control, we also measured the levels of α -tubulin, which has a half-life of greater than 40 h (25). In these assays, we observed that Net1A expressed together with GFP exhibited a half-life of 0.35 h (Fig. 4, B and C). This value correlated with the half-life of both transfected HA-Net1A and endogenous Net1, as determined using [³⁵S]methionine pulse-chase analysis (Fig. 3). Similarly, co-expression of wild type Dlg1 extended the half-life of Net1A to 1×10^5 h, whereas Net1A in the presence of the Dlg1 PDZ1/2 domain mutant exhibited a half-life of 0.50 h. Thus, blocking protein translation with cyclohexamide provided essentially the same results as [³⁵S]methionine pulse-chase analysis. Importantly, in these assays, we observed that co-expression of Magi-1b only marginally stabilized Net1A, extending its half-life to 1.14 h. This inability to stabilize Net1A may have been due to the short half-life of Magi-1b in these cells, which was 1.79 h, or to a specific inability of Magi-1b to protect Net1A from degradation. Co-expression of GFP-Scribble did not affect Net1A stability, consistent with its lack of interaction with Net1A. Taken together, these data indicate that the ability of Dlg1 to extend the half-life of Net1A is not shared among all PDZ domain-containing proteins that interact with Net1A.

Net1 Stability and Interaction with Dlg1 Varies between Human Breast Cell Lines—We then examined whether Net1 stability was similarly regulated in other human breast cell lines. For these experiments, we analyzed MCF10A cells, which are immortal breast epithelial cells, and MDA-MB-231 cells, which are highly metastatic breast cancer cells. Actively growing cells were treated with cyclohexamide for different periods of time and then lysed and tested for endogenous Net1 and α -tubulin expression. As shown in Figs. 5, A and B, there was a significant

difference in Net1 stability between these cell lines. MCF10A cells displayed a Net1 half-life of 1.4 h, whereas the half-life of Net1 in MDA-MB-231 cells was 16.5 h. To determine whether the increased half-life of Net1 in the MDA-MB-231 cells correlated with an increased interaction with Dlg1, we compared the ability of endogenous Net1 to co-immunoprecipitate with endogenous Dlg1 from MDA-MB-231 and MCF7 cells. Equal amounts of lysate from each cell line were used for this assay. Importantly, we observed that more Net1 co-immunoprecipitated with Dlg1 from these cells than from MCF7 cells, although there was far less Dlg1 in the MDA-MB-231 cells (Fig. 5C).

There was also an elevated level of Net1 expressed in these cells, in agreement with its longer half-life. Taken together, these data indicate that the half-life of Net1 and its association with Dlg1 are co-regulated in multiple human breast cell lines. They also demonstrate that Net1 is highly stable in the metastatic breast cancer cell line MDA-MB-231.

The Interaction of Net1A with Dlg1 Potentiates Its Ability to Activate Endogenous RhoA—Previously, we had shown that RhoA activation by transfected Net1 Δ N was not enhanced by co-expression of Dlg1 (10). However, it is possible that Net1 Δ N expression is not regulated in the same way as full-length Net1A. Indeed, the stability and Rho activating activity of truncated forms of the Rho GEF Dbl are significantly enhanced compared with wild type Dbl (20). Thus, we first examined whether the half-life of Net1 Δ N was significantly different than that of full-length Net1A. MCF7 cells were transfected with HA-Net1A or HA-Net1 Δ N, and then protein translation was inhibited for different periods of time using cyclohexamide. In these assays, we observed that Net1 Δ N was essentially not degraded during the course of the assay (Figs. 6, A and B). Thus, deletion of the amino terminus leads to a profound stabilization of Net1A. We then examined whether the enhanced stability of Net1 Δ N was due to an increased interaction with Dlg1. MCF7 cells were transfected with HA-Net1A or HA-Net1 Δ N, and then the HA-tagged proteins were immunoprecipitated and tested for the co-precipitation of endogenous Dlg1. As shown in Fig. 6C, Net1A was at least as efficient at interacting with endogenous Dlg1 as Net1 Δ N. Thus, the enhanced stability of Net1 Δ N must be due to the removal of its amino terminus.

Because Dlg1 selectively enhanced the stability of Net1A, we then tested whether the co-expression of Dlg1 also potentiated the ability of Net1A to stimulate endogenous RhoA activation. MCF7 cells were transfected with Net1A alone or with GFP-wild type Dlg1 or GFP-Dlg1 containing mutations within the PDZ1 and PDZ2 domains. As a positive control, another group of cells were transfected with Net1 Δ N. The activation of endogenous RhoA was then measured in pull-down assays using the GST-rhotekin RhoA-binding domain (GST-RBD) (26). In these

³ H. S. Carr and J. A. Frost, unpublished observations.

Dlg1 Interaction Stimulates Net1 by Blocking Degradation

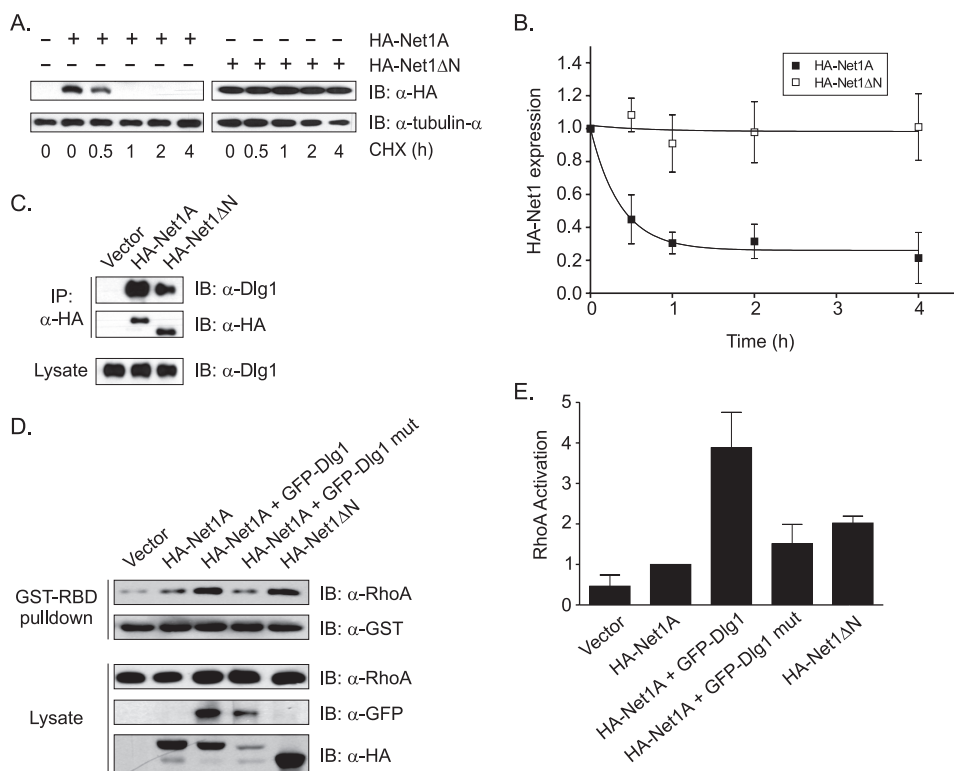


FIGURE 6. Interaction with Dlg1 potentiates RhoA activation by Net1A. *A*, MCF7 cells were transfected with HA-Net1A or HA-Net1 Δ N and then treated with cycloheximide for the times shown. The levels of HA-tagged Net1 proteins and α -tubulin were determined by Western blotting (*IB*). Shown is a representative experiment. *B*, quantification of the stability of HA-Net1A and HA-Net1 Δ N in MCF7 cells. Results are the average of three independent experiments. *Error bars*, S.E. *C*, MCF7 cells were transfected with HA-Net1A or HA-Net1 Δ N. HA-Net1 proteins were immunoprecipitated (*IP*) and tested for the co-precipitation of endogenous Dlg1 by Western blotting. Shown is a representative experiment from three independent experiments. *D*, MCF7 cells were transfected with HA-Net1A or HA-Net1 Δ N, alone or plus wild type GFP-Dlg1 or the GFP-Dlg1 PDZ1/2 mutant. The cells were then lysed, and amounts of active RhoA were determined in RBD pull-down assays. Shown is a representative experiment. *E*, quantification of the effects of Dlg1 co-expression on RhoA activation by Net1A. RhoA activation is calculated as the amount of active RhoA in the RBD pull-down divided by the amount of RhoA in the lysate. All results are compared with the degree of RhoA activation in cells transfected with HA-Net1A alone. Results are the average of four independent experiments. *Error bars*, S.E.

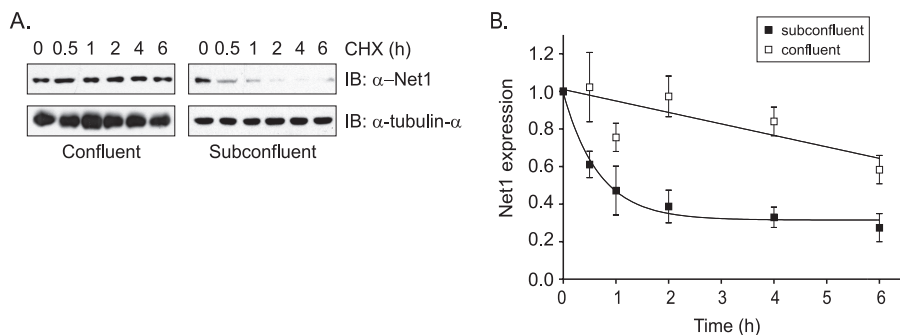


FIGURE 7. Endogenous Net1 stability is enhanced in confluent MCF7 cells. *A*, confluent and subconfluent MCF7 cells were treated with cycloheximide for the times shown. Levels of endogenous Net1 and α -tubulin were determined by Western blotting (*IB*). Shown is a representative experiment. *B*, quantification of the stability of endogenous Net1 in confluent and subconfluent MCF7 cells. Results are the average of four independent experiments. *Error bars*, S.E.

assays, we observed that Net1A alone was not as efficient as Net1 Δ N at activating endogenous RhoA (Fig. 6, *D* and *E*). However, co-expression of wild type Dlg1, but not the Dlg1 mutant incapable of interacting with Net1A, significantly enhanced the ability of Net1A to stimulate RhoA activation, such that Net1A was better than Net1 Δ N at activating RhoA. Thus, these experiments show that stabilization of Net1A by Dlg1 leads to a sig-

nificant enhancement of its ability to activate cellular RhoA. In addition, since Net1 Δ N is already stable in the absence of Dlg1 interaction, these experiments explain why we previously were unable to observe an effect of the interaction between Net1 Δ N and Dlg1 on RhoA activation (27).

Net1 Stability and Binding to Dlg1 Are Regulated by Cell-Cell Interaction—While testing for Net1A half-life in MCF7 cells, we noticed that there was a trend toward increased stability of transfected Net1A in more confluent cells. Thus, we examined more precisely what effect cell confluence had on Net1 stability. To analyze this, actively growing MCF7 cells that were less than 30% confluent were compared with those that were allowed to grow 1 day past 100% confluence. Each group of cells was then treated with cycloheximide for different periods of time, and levels of endogenous Net1 were determined by Western blotting. Importantly, we observed that the degree of cell confluence had a profound effect on the stability of endogenous Net1. In confluent cells, endogenous Net1 exhibited a half-life of \sim 10 h, whereas in the subconfluent cells, its half-life was only 0.47 h (Fig. 7, *A* and *B*). This represents a greater than 20-fold difference in Net1 stability.

Because we observed that interaction with Dlg1 stabilized Net1, we then examined whether the interaction between endogenous Net1 and Dlg1 was similarly affected by the degree of cell confluence. Subconfluent and confluent MCF7 cells were lysed, and the endogenous Dlg1 was immunoprecipitated. Co-precipitation of endogenous Net1 was determined by Western blotting. We observed that endogenous Net1 only co-precipitated with Dlg1

in confluent cells (Fig. 8*A*, *middle*). Taken together, these experiments indicate that cell-cell interaction drives the association of endogenous Net1 with Dlg1, thus leading to Net1 stabilization.

A consequence of cell-cell interaction in epithelial cells is the generation of apical-basal polarity. Cell polarization is supported by the formation of adherens junctions on the basolat-

Dlg1 Interaction Stimulates Net1 by Blocking Degradation

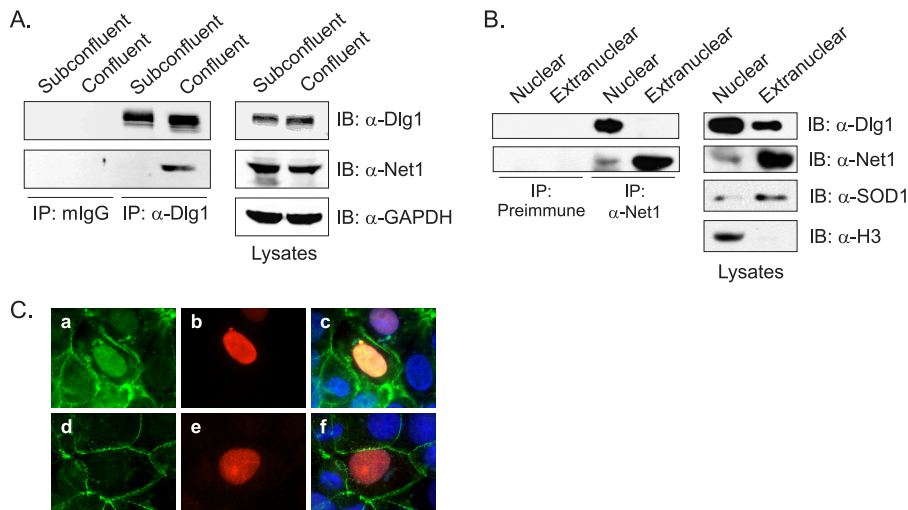


FIGURE 8. Cell confluence promotes the accumulation of Net1-Dlg1 complexes in the nucleus. *A*, subconfluent or confluent MCF7 cells were lysed and used for immunoprecipitation (IP) with control mouse IgG or mouse anti-Dlg1. Co-immunoprecipitation of endogenous Net1 was tested by Western blotting (IB). Shown is a representative experiment from three independent experiments. *B*, confluent MCF7 cells were lysed and separated into nuclear and extranuclear fractions. Cell fractions were then used for immunoprecipitation with preimmune serum or rabbit anti-Net1. Co-immunoprecipitation of endogenous Dlg1 was tested by Western blotting. The purity of cell fractions was determined by Western blotting for the cytoplasmic and nuclear proteins SOD-1 and histone H3 (H3), respectively. Shown is a representative experiment from three independent experiments. *C*, confluent MCF7 cells were transfected with HA-Net1A and then fixed and stained for endogenous Dlg1 (*a* and *c*), endogenous E-cadherin (*d* and *f*), and transfected HA-Net1A (*b*, *e*, and *f*). Shown is a representative experiment from three independent experiments.

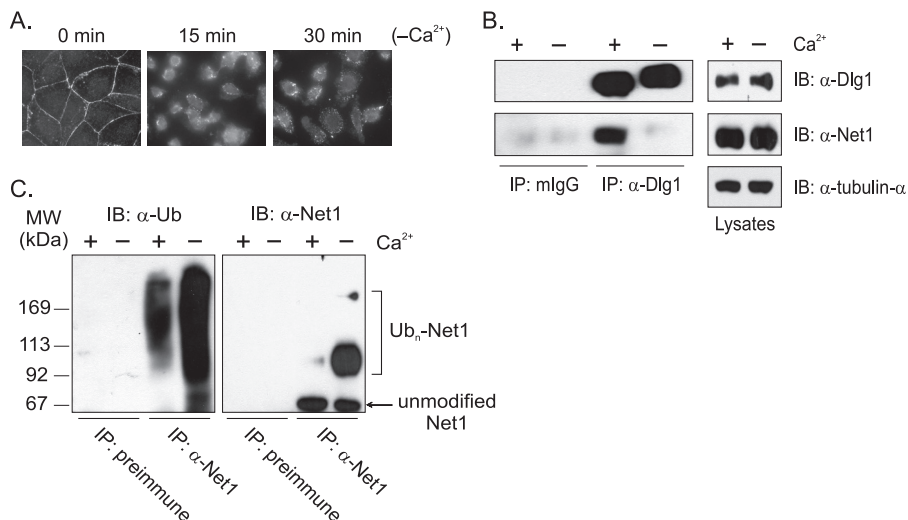


FIGURE 9. Disruption of E-cadherin-mediated cell contacts causes dissociation of endogenous Net1 and Dlg1 and results in Net1 ubiquitylation. *A*, confluent MCF7 cells were switched to calcium-free medium plus EGTA ($-Ca^{2+}$) for the times shown. The cells were then fixed and tested for E-cadherin localization by indirect immunofluorescence. Shown is a representative experiment. *B*, confluent MCF7 cells were left in normal growth medium or switched to calcium-free medium plus EGTA for 30 min. The cells were lysed and subjected to immunoprecipitation (IP) with control mouse IgG or mouse anti-Dlg1. Co-immunoprecipitation of endogenous Net1 was tested by Western blotting (IB). Shown is a representative experiment from three independent experiments. *C*, confluent MCF7 cells were incubated with the proteasome inhibitor MG132 for 1 h and then either left in normal growth medium or switched to calcium-free medium plus EGTA for 30 min. The cells were lysed and subjected to immunoprecipitation with preimmune serum or rabbit anti-Net1. Immunoprecipitated Net1 was tested for ubiquitylation by Western blotting (α -Ub). Shown is a representative example from three independent experiments.

eral membrane and tight junctions at the apical surface. Since Dlg1 function is required for the formation of adherens junctions and Dlg1 localizes to these structures in polarized cells, we examined whether Net1 interacts with Dlg1 within this domain. MCF7 cells were allowed to grow 1 day past confluence and then harvested and separated into nuclear and extranuclear

fractions. The Dlg1 was then immunoprecipitated from each fraction and tested for co-precipitation of Net1. Surprisingly, Net1 only co-precipitated with Dlg1 from the nuclear fraction, indicating that in maturely polarized MCF7 cells, Net1 and Dlg1 stably interact only in the nucleus (Fig. 8*B*). To confirm this result, we transfected HA-tagged Net1A into confluent MCF7 cells and examined the subcellular localization of HA-Net1A and endogenous Dlg1. In these experiments, the Net1A was transfected, because antibodies suitable for detection of endogenous Net1 by immunofluorescence are not available. As shown in Fig. 8*C*, all of the cells contained Dlg1 at the cell margins, consistent with its localization at adherens junctions. Importantly, in the cells overexpressing Net1A, the endogenous Dlg1 was also clearly recruited to the nucleus (Fig. 8*C*, *a* and *c*). In these cells, the HA-Net1A was not detectable at the cell margins (*b* and *c*). To determine whether the recruitment of Dlg1 to the nucleus was specific, we also tested for the localization of endogenous E-cadherin in the Net1A-transfected cells. We observed that the E-cadherin remained at the cell membrane and was not recruited to the nucleus (Fig. 8*C*, *d* and *f*). Thus, these experiments indicate that Dlg1 and Net1 interact in the nuclei of confluent, polarized MCF7 cells.

Disruption of E-cadherin Contacts Causes Dissociation of Endogenous Net1 and Dlg1 and Stimulates Net1 Ubiquitylation—Contacts between neighboring epithelial cells are stabilized by calcium-dependent, homophilic interactions between E-cadherin family molecules (28). Thus, we examined whether cell-cell contact regulated the interaction between Net1 and Dlg1 via E-cadherin. MCF7 cells were allowed to grow 1 day past confluence

and then switched to calcium-free medium plus EGTA for 30 min. We confirmed that this treatment completely disrupted E-cadherin-mediated cell contacts (Fig. 9*A*). The cells were then lysed, and the endogenous Dlg1 was immunoprecipitated. Co-precipitation of endogenous Net1 was detected by Western blotting. We observed that disruption of

Dlg1 Interaction Stimulates Net1 by Blocking Degradation

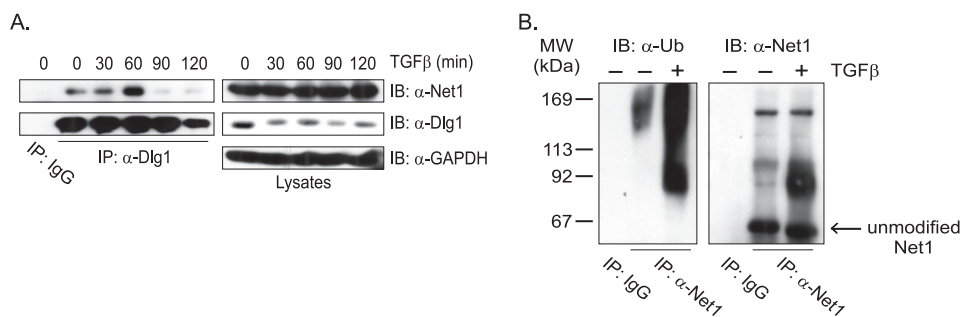


FIGURE 10. Treatment with TGF β stimulates Net1 dissociation from Dlg1 and results in Net1 ubiquitylation. *A*, confluent MCF7 cells were treated with TGF β for the times shown. The cells were then lysed, and endogenous Dlg1 was immunoprecipitated (IP). Co-immunoprecipitation of endogenous Net1 was tested by Western blotting (IB). Shown is a representative experiment from three independent experiments. *B*, confluent MCF7 cells were pretreated with MG132 for 1 h and then left in growth medium or treated with TGF β for 90 min. The cells were then lysed, and the endogenous Net1 was immunoprecipitated. Ubiquitylation of Net1 was tested by Western blotting (α -Ub). Shown is a representative experiment from three independent experiments.

E-cadherin-mediated contacts almost completely disrupted the interaction between Dlg1 and Net1 (Fig. 9B). In separate experiments, we observed that loss of Dlg1-Net1 interaction was extremely fast, with a significant effect as soon as 15 min after the removal of extracellular calcium (data not shown). We then examined whether the loss of interaction between Net1 and Dlg1 resulted in ubiquitylation of endogenous Net1. Confluent MCF7 cells were pretreated with the proteasome inhibitor MG132 for 1 h. The cells were then switched to calcium-free medium for 30 min and lysed, and endogenous Net1 was immunoprecipitated. The immunoprecipitated Net1 was then tested for ubiquitylation by Western blotting. As shown in Fig. 9C, ubiquitylation of Net1 was dramatically enhanced by removal of extracellular calcium (*left*). Reprobing the blot with an antibody to Net1 confirmed the presence of slower migrating forms of Net1, consistent with its ubiquitylation. Detection of the ubiquitylated Net1 was strictly dependent on inclusion of proteasome inhibitor in the cell lysate, suggesting that the ubiquitylated Net1 was targeted for degradation. Thus, these data indicate that maintenance of E-cadherin-mediated contacts drives the interaction between Net1 and Dlg1 and protects Net1 from proteasome-mediated degradation.

Because calcium depletion can have unintended effects on cells, we also examined whether treatment of polarized MCF7 cells with a physiologically relevant ligand that disrupts cell-cell adhesion also caused the release of Net1 from Dlg1 and its subsequent ubiquitylation. For these experiments, we used TGF β , which is known to stimulate epithelial to mesenchymal transition and disrupt cell-cell contacts (29, 30). Confluent MCF7 cells were treated with TGF β for different periods of time, after which they were lysed, and the endogenous Dlg1 was immunoprecipitated. Co-precipitating Net1 was monitored by Western blotting. As shown in Fig. 10A, treatment of MCF7 cells with TGF β for 90 min or greater blocked the co-precipitation of Net1 with Dlg1. We then examined whether treatment with TGF β caused ubiquitylation of endogenous Net1. MCF7 cells were pretreated with MG132 for 1 h and then exposed to TGF β for 90 min. The cells were lysed, and endogenous Net1 was immunoprecipitated and tested for ubiquitylation by Western blotting. We observed that TGF β treatment also caused a significant increase in the ubiquitylation of endogenous Net1 (Fig. 10B).

Thus, these experiments demonstrate that exposure to TGF β , which is known to disrupt epithelial polarization, also blocks the interaction of Net1 with Dlg1 and targets it for proteasome-mediated degradation.

DISCUSSION

We have shown previously that the ability of oncogenic Net1 to transform cells is dependent upon the presence of a COOH-terminal PDZ domain binding site and that this site mediates interaction with Dlg1 family proteins (8, 10). In the present work, we have demonstrated that the interaction between

Net1A and Dlg1 is direct and is dependent upon the PDZ1 and PDZ2 domains of Dlg1. Importantly, interaction with Dlg1 protects Net1 from proteasome-mediated degradation and potentiates the ability of Net1A to stimulate endogenous RhoA activation. Furthermore, the interaction between Net1 and Dlg1 is regulated by E-cadherin-mediated cell-cell interaction. Disruption of these contacts, either by removal of extracellular calcium or by treatment with TGF β , causes dissociation of Net1 from Dlg1 and results in Net1 ubiquitylation. Thus, these data demonstrate that regulation of Net1 stability is tightly controlled within the cell and is a critical factor in determining its ability to activate RhoA.

Identification of the PDZ domains that mediate interaction between Dlg1 and Net1 is an important step in understanding the regulatory mechanisms that control their interaction. We observed that Net1 binds directly to the PDZ1 and PDZ2 domains of Dlg1 *in vitro* and in cells. In this regard, there are a number of proteins expressed in epithelial cells that interact with the PDZ1 and PDZ2 domains of Dlg1 that may compete with Net1 for access. For example, the adenomatous polyposis coli protein, the serine/threonine protein kinase TOPK/PBK, and pro-TGF α have each been reported to bind to the PDZ2 domain of Dlg1 (31–33). Similarly, the MAPK p38 γ has been shown to interact with both the PDZ1 and PDZ3 domains of Dlg1, whereas TACE has been shown to interact exclusively with the PDZ3 domain (34, 35). Thus, the ability of Net1 to interact with Dlg1 may be modulated, in part, by competition from other proteins that require access to Dlg1. The interaction of any one of these proteins with Dlg1 may be also regulated by post-translation modification. For instance, the PDZ1 domain of Dlg1 is phosphorylated by CamKII in neuronal cells, and this blocks the association of the NR2A subunit of the NMDA receptor with Dlg1/SAP97 (36). Similarly, the ability of PTEN to interact with the PDZ domain protein Magi-2 is negatively regulated by phosphorylation of the COOH-terminal PDZ binding site of PTEN (37). Thus, an important area for future studies will be to discern the complex regulation of interaction between Net1 and Dlg1.

Our observation that the half-life of Net1 varies between human breast cell lines may provide a powerful avenue for understanding how the interaction between Net1 and Dlg1 is

regulated as well as its phenotypic consequences. Specifically, Net1 was highly stable in the metastatic breast cancer cell line MDA-MB-231, and this correlated with a higher degree of association with Dlg1. Interestingly, the Dlg1 in these cells migrated more slowly than in MCF7 cells, which may indicate differences in post-translational modification or in the expression of particular splice variants of Dlg1. Because RhoA activity is required for cell motility, it is tempting to speculate that the enhanced stability of Net1 in these cells and/or its association with Dlg1 contributes to their metastatic behavior. Future work will be required to determine the relative contributions of Net1 and Dlg1 to the invasive behavior of these cells.

The ability of Dlg1 to stabilize Net1 is apparently not shared among all PDZ domain-containing proteins that bind to Net1A, since Magi-1b, which interacted with Net1A and competed with Dlg1 for binding, did not significantly stabilize Net1A. This may indicate that merely interacting with the PDZ binding site of Net1 is not sufficient to protect it from degradation. Since Dlg1 is a scaffolding protein with multiple protein interaction domains, it may be that binding to Dlg1 brings Net1 into proximity with additional proteins that prevent its ubiquitylation. This may be through direct interaction with one or more deubiquitylating enzymes or through a more indirect mechanism. Alternatively, interaction with Dlg1 may prevent binding to one or more E3 ligases or may relocalize Net1 to a subcellular domain where it is protected from ubiquitylation. In this regard, we observed that Net1-Dlg1 complexes accumulate in the nucleus of confluent MCF7 cells, so it may be that nuclear pools of Net1 are not subject to ubiquitylation. We are currently working to identify the protein interaction domains within Dlg1 that are required to stabilize Net1 and to ascertain whether the stability of Net1 differs between subcellular localizations.

An important finding of this work is that the interaction between Net1 and Dlg1 is regulated by cell-cell interaction in MCF7 cells. These cells are epithelial in origin and will establish E-cadherin-mediated contacts and apical-basal polarity when allowed to reach confluence. Importantly, we observed that endogenous Net1 and Dlg1 only interacted in confluent MCF7 cells and that disruption of E-cadherin function by chelating extracellular calcium caused a rapid dissociation of Net1 from Dlg1 and allowed for Net1 ubiquitylation. We also demonstrated that short term treatment with TGF β , which causes the disruption of epithelial polarization and loss of E-cadherin contacts, also caused a loss of Net1-Dlg1 interaction and the subsequent ubiquitylation of Net1. Thus, by two distinct methods, we have shown that E-cadherin-mediated cell-cell interactions are likely to be a powerful mechanism for controlling Net1 stability via its interaction with Dlg1. Because Dlg1 is an important regulator of adherens junction formation and epithelial polarity (13) and because RhoA activation is required for the formation of adherens junctions (38), we expected to find that Net1 and Dlg1 interacted mainly at the plasma membrane. However, we were surprised to find that in fully polarized cells, Net1-Dlg1 complexes were present mainly in the nucleus. The ultimate function of such complexes is not clear. It may be that Net1 has an unrecognized role in controlling the activation of nuclear pools of RhoA and that this is regulated by association with

Dlg1. Alternatively, it is possible that Net1 is being stabilized in the nucleus to prevent it from accessing the plasma membrane until RhoA activation in that cellular domain is required. The requirement for RhoA activation differs between fully polarized cells and those that are just beginning this process (39), and it is possible that Dlg1 recruits Net1 to the cell membrane when cells are initially making contact.

The ability of TGF β to stimulate Net1 ubiquitylation may shed light on the identity of the ubiquitin E3 ligases that ultimately control Net1 stability. TGF β treatment stimulates the ubiquitylation of many of proteins involved in this signaling pathway, including each of the Smads, TGF β -RI, and the transcriptional repressors SnoN and c-Ski. The best characterized E3 ligases in these responses are Smurf1 and Smurf2, although nine additional E3 ligases have been implicated in regulating TGF β signaling (40). Thus, one or more of these enzymes are good candidates to control Net1 ubiquitylation. The difference in stability between wild type Net1A and the NH₂-terminal truncation mutant Net1 Δ N indicates that the NH₂-terminal regulatory domain of Net1 controls its stability. This may indicate that one or more E3 ligases directly interact with this domain or that the NH₂ terminus contains the residues that are subject to ubiquitylation. We are currently working to determine the sites of ubiquitylation on Net1 and to identify the E3 ligases that control Net1 stability.

The magnitude of the effect of Dlg1 on Net1 stability indicates that regulation of Net1 degradation will be an important mode of controlling Net1 activity within the cell. Thus, our present work adds significantly to our understanding of the regulatory mechanisms controlling the cellular activity of Net1. It has previously been shown that Net1 transcription is stimulated by cell cycle progression in primary T cells and by TGF β treatment in fibroblasts (41, 42). Additionally, the ability of expressed Net1 to stimulate RhoA activation is negatively regulated by nuclear sequestration and by phosphorylation by the protein kinase Pak1 (7, 43). Thus, it appears critically important in many cell types to tightly control the activity of Net1. Our finding that E-cadherin-mediated cell-cell adhesion controls the interaction of Net1 with Dlg1 and thus regulates its intracellular stability suggests that Net1 may play an important role in the establishment or maintenance of apical-basal polarity in epithelial cells. Indeed, Net1 activity is required for maintenance of the basement membrane in chicken embryos, and loss of Net1 expression is necessary for the epithelial to mesenchymal transition that occurs during gastrulation (44). Our observation that treatment of polarized MCF7 cells with TGF β , which causes epithelial to mesenchymal transition, also leads to the dissolution of Net1-Dlg1 complexes and Net1 degradation, supports such a role in mammalian cells. Future work will be required to understand the function of Net1 in initiating and maintaining breast epithelial cell polarization.

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