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Oligomerization of the Nrdp1 E3 Ubiquitin Ligase Is Necessary for Efficient Autoubiquitination but Not ErbB3 Ubiquitination*

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Background: Nrdp1 ubiquitinates itself and ErbB3 to facilitate the degradation of both proteins.
Result: Coiled-coil domain deletion abrogates Nrdp1 oligomerization and suppresses Nrdp1 but not ErbB3 ubiquitination and degradation.
Conclusion: Oligomerization is required for efficient Nrdp1-mediated autoubiquitination but not ErbB3 ubiquitination.
Significance: Nrdp1 autoubiquitination and substrate ubiquitination may be functionally separated, allowing a novel treatment strategy for breast cancer patients.

Overexpression of the ErbB3 receptor tyrosine kinase protein in breast and other cancers contributes to tumor malignancy and therapeutic resistance. The RBCC/TRIM family RING finger E3 ubiquitin ligase Nrdp1 mediates the ubiquitination of ErbB3 in normal mammary epithelial cells to facilitate receptor degradation and suppress steady-state receptor levels. Post-transcriptional loss of Nrdp1 in patient breast tumors allows ErbB3 overexpression and receptor contribution to tumor progression, and elevated lability through autoubiquitination contributes to the observed loss of Nrdp1 in tumors relative to normal tissue. To begin to understand the mechanisms underlying Nrdp1 protein self-regulation through lability, we investigated the structural determinants required for efficient autoubiquitination and ErbB3 ubiquitination. Using mutagenesis, chemical cross-linking, size exclusion chromatography, and native polyacrylamide gel electrophoresis, we demonstrate that Nrdp1 self-associates into a stable oligomeric complex in cells. Deletion of its coiled-coil domain abrogates oligomerization but does not affect Nrdp1-mediated ErbB3 ubiquitination or degradation. On the other hand, the presence of the coiled-coil domain is necessary for efficient Nrdp1 autoubiquitination via a trans mechanism, indicating that Nrdp1 ubiquitination of its various targets is functionally separable. Finally, a GFP fusion of the coiled-coil domain stabilizes Nrdp1 and potentiates ErbB3 ubiquitination and degradation. These observations point to a model whereby the coiled-coil domain plays a key role in regulating Nrdp1 lability by promoting its assembly into an oligomeric complex, and raise the possibility that inhibition of ligase oligomerization via its coiled-coil domain could be of therapeutic benefit to breast cancer patients by restoring Nrdp1 protein.

Signaling by the ErbB3 receptor tyrosine kinase is essential for proper embryonic neural and cardiac development (1, 2) and proper ductal morphogenesis of the mammary gland (3, 4) and likely contributes to the maintenance of a variety of tissues in the adult. Overexpression and concomitant aberrant activation of ErbB3 in human tumors contributes to malignancy and therapeutic resistance (4–7); however, the mechanisms underlying ErbB3 overexpression in tumors have not been elucidated. Although amplification of genes encoding the related EGFR and ErbB2 proteins correlates with the overexpression of these proteins in a variety of tumor types (7–9), *erbb3* amplification does not appear to play a significant role in ErbB3 protein overexpression in tumors (7, 10, 11). Importantly, ErbB3 protein levels increase 10–50-fold in tumors relative to surrounding normal tissue in a mouse model of ErbB2 overexpression-induced breast cancer and that difference cannot be accounted for by differences in transcript abundance (12–16). These observations underscore key roles for the dysregulation of post-transcriptional processes such as protein degradation in creating a permissive environment for ErbB3 protein overexpression in breast tumors.

Nrdp1 is a RING finger E3 ubiquitin ligase that physically associates with and ubiquitinates ErbB3 independent of growth factor stimulation (13, 16–21). Overexpression of Nrdp1 cDNA in cultured cells elevates constitutive ErbB3 ubiquitination and suppresses steady-state ErbB3 protein levels, whereas Nrdp1 knockdown or dominant negative markedly stabilizes the receptor. We have observed that although Nrdp1 protein is present in normal mammary tissue from patients and mice, it is suppressed or lost in over half of human breast tumors and is invariably lost in the ErbB2 overexpression mouse model (13). Like ErbB3, Nrdp1 dysregulation in mammary tumors also appears to occur post-transcriptionally, making its transgenic restoration to mouse tumors impossible (15). Interestingly, Nrdp1 is more labile when expressed in human breast tumor cell lines than in nontransformed lines (15), suggesting that accelerated protein degradation could underlie its suppression in tumors. Moreover, we have observed that Nrdp1 protein

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levels in cells are dramatically elevated by mutational abrogation of its E3 ligase activity, strongly suggesting that autoubiquitination may play a key role in regulating its stability in cells.

Nrdp1 is a member of a superfamily of E3 ubiquitin ligases called the RBCC (RING/B-box/coiled-coil) or TRIM (tripartite interaction motif) family, consisting of roughly 80 members in humans. TRIM family members have been implicated in a wide variety of biological processes, including transcriptional regulation, apoptosis, cell cycle regulation, cancer, and viral infection (22–26), and are characterized by a tandem array of three recognizable domains in their amino-terminal regions (27). The RING finger is a zinc-binding domain generally found within 20 residues of the amino terminus of TRIM family members and is thought to be responsible for TRIM protein interaction with E2 ubiquitin-conjugating enzymes (28, 29). This is followed by the B-box, a zinc-binding domain of undefined function (30). The adjacent coiled-coil domain has been implicated in mediating protein-protein interactions, most often homotypic oligomerization events (22, 31). The carboxyl-terminal regions of TRIM proteins vary widely, and it is presumed that these domains are responsible for recruiting target proteins that are to be ubiquitinated to the E2 ubiquitin-conjugating enzyme-E3 ubiquitin ligase complex.

Our previous studies have demonstrated that the RING finger and the carboxyl-terminal ErbB3-binding domains are necessary for Nrdp1 function as a receptor-directed E3 ubiquitin ligase; however, roles for the B-box and coiled-coil domains in Nrdp1 function are not clear. In this study, we demonstrate that the coiled-coil domain is required for Nrdp1 oligomerization, that this domain is required for efficient Nrdp1 autoubiquitination and lability, and that it may be targeted by exogenous agents to restore Nrdp1 to tumor cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK-293T and MCF7 cells were purchased from ATCC and maintained under an atmosphere of 10% CO_2 in DMEM with 10% FCS and 1% penicillin-streptomycin (all medium components from Invitrogen). Antibodies employed in the study include mouse monoclonal anti-FLAG M2 (Stratagene), anti-tubulin (Sigma), anti-ErbB3 Ab-6 (Thermo Scientific), anti-Myc 9E10 (Calbiochem), anti-HA tag (Invitrogen), anti-FLRF/RNF41/Nrdp1 (Bethyl Laboratories), and rabbit polyclonal anti-ErbB3 C-17 (Santa Cruz).

Constructs and Transient Transfection—Cells were transiently transfected using either PolyJet (Signagen) or FuGENE 6 (Promega) according to the manufacturer's instructions. The total amount of DNA transfected was 500 ng/well for 12-well plates and 5 μg/plate for 10-cm dishes. When more than one plasmid was transfected at a time, equal amounts of each plasmid were used. Plasmids encoding ErbB3, wild type Nrdp1-FLAG, Nrdp1–32-FLAG, and Nrpd1-CHSQ-FLAG were described previously (17, 32). HA-labeled ubiquitin has been described elsewhere (33). Nrdp1 constructs lacking the coiled-coil domain were created by excising the regions flanking the coiled-coil domain with EcoRI and XhoI for the amino terminus and XhoI and ApaI for the carboxyl terminus, joining to two halves and leaving an internal XhoI site in the delta coiled-coil clone. Nrdp1–32Δcc was cloned by using the XhoI and ApaI sites of the carboxyl terminus only. Both delta coiled-coil mutants were cloned into pcDNA3.1. The primer pairs used were 5′-GGAATTCGCCATGGGGTATGATGTA-3′/5′-ACTCTCGAGTGGACCGTTATGGTTGGGCAGC-3′, and 5′-GTCCACTCGAGAGTGTCAACCCCAACCTTCAG-3′/5′-GGTGGGCCCTCTAGTCACTTGTCATCG-3′. V5-tagged Nrdp1 was created by the addition of a V5 tag by PCR onto wild type Nrdp1 using the primer pair 5′-GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG-3′/3′-CCATTCGGATAGGGATTGGGAGAGGAGCCAGAGCTAAGATGC-5′. The coiled-coil-GFP fusion protein was created by PCR excising the Nrdp1 coiled-coil region and adding BglII and BamHI sites for cloning into the pEGFP-N1 vector upstream of GFP. The primer pair used for this was 5′-CATCAGATGCGCAGATCTATGATTAAGCACCTGCGCTCAGTGG-3′/5′-CAGCTGCTAAAGGCATACATGCGTGGATCCGCGCATCTGATG-3′. For coiled-coil-GFP fusion experiments, empty pEGFP-N1 vector that expresses GFP was used as a control vector. Wild type Nrdp1 lacking an epitope tag was generated by introducing a stop codon into wild type Nrdp1-FLAG using the primer pair 5′-CGCATGGCGTGGAAGAGATATGAGACTACAAGGACGACGATG3′/5′-CATCGTCGTCCTTGTAGTCTCATATCTCTTCCACGCCATGCG-3′.

Immunoprecipitation, Ubiquitination Assay, and Immunoblotting—HEK-293T cells were co-transfected with HA-tagged ubiquitin, various Nrdp1 constructs, and/or ErbB3, and protein was allowed to express for 48 h. Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-10) with added protease and phosphatase inhibitors (2 μM MG132, 5 mM *N*-ethylmaleimide, 1 mM NaF, 10 mM β-glycerophosphate, 1.5 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and 4 μg/ml each of aprotinin, pepstatin, and leupeptin). Lysates were incubated at 4 °C for 5 min with gentle rocking and microcentrifuged at 13,000 RPM for 10 min at 4 °C, and the supernatant was incubated with 1 μg of primary antibody against either Nrdp1 or ErbB3 for 16 h at 4 °C with rocking to immunoprecipitate the target protein. 30 μl of protein G bead suspension was then added, and the mixture was incubated another 2–4 h at 4 °C with rocking. Subsequently, beads were pelleted at 3,000 rpm for 1 min at 4 °C and washed five times with 1 ml of radioimmune precipitation assay buffer complete with inhibitors. Sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.05% bromphenol blue) was added directly to beads and heated for 5 min at 95 °C. Precipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose (Pall Life Sciences, Pensacola, FL), blocked overnight in TBS/0.05% Tween with 5% dried milk, and then detected with primary antibodies. Chemiluminescence detection of HRP-conjugated secondary antibodies was carried out using SuperSignal reagents (Pierce) and imaged using an Alpha Innotech Digital Imaging Station (Alpha Innotech Corp., San Leandro, CA). Densitometry was performed using ImageJ.

Cross-linking Assay and Co-immunoprecipitation—HEK-293T cells were transfected for 48 h, washed with PBS, and lysed with co-immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% glycerol, 1 mM $MgCl_2$) with inhibitors. Lysates

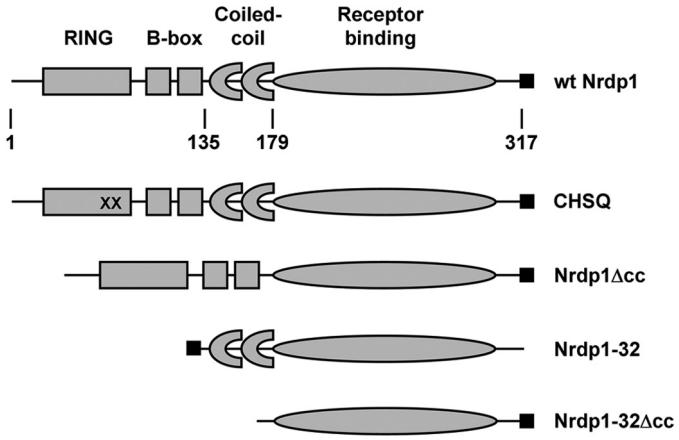

FIGURE 1. **Illustration of the Nrdp1 constructs employed in these studies.** The domain structure of wild type (*wt*) Nrdp1 is depicted, along with the mutants employed in these studies. CSHQ harbors a double point mutation in the Nrdp1 RING finger domain that abrogates ligase activity. *Black squares* indicate the placement of the FLAG, Myc, or V5 epitopes.

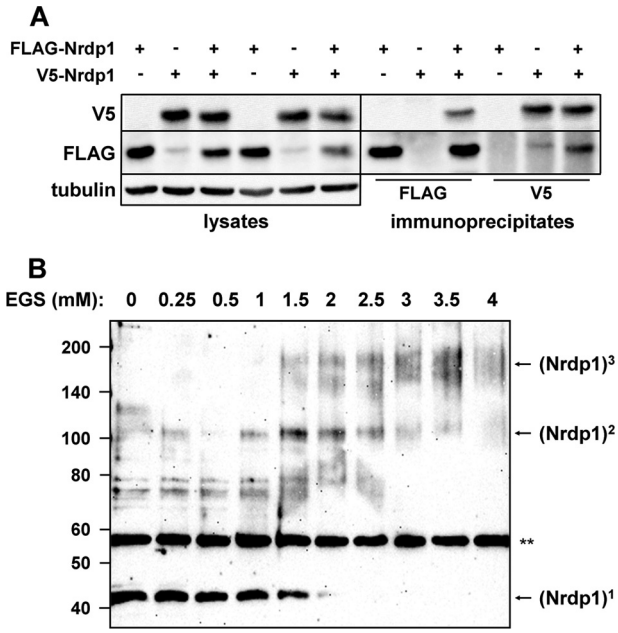

FIGURE 2. **Nrdp1 associates with itself.** *A*, 293T cells were transiently transfected with FLAG-tagged Nrdp1, V5-tagged Nrdp1, or both, as indicated. Lysates were immunoprecipitated with anti-FLAG or anti-V5 antibodies, and precipitates (*right panels*) and lysates (*left panels*) were blotted with FLAG and V5 antibodies. The data are representative of five independent experiments carried out with different epitope tag combinations. *B*, 293T cells were transiently transfected with FLAG-Nrdp1, and lysates were treated with various levels of the chemical cross-linker EGS. Anti-FLAG immunoprecipitates from cross-linked lysates were blotted with anti-FLAG antibodies. *Arrows* indicate cross-linked Nrdp1 bands and estimation of stoichiometry of association. **, heavy chain of immunoprecipitating antibody. The data are representative of at least three independent experiments.

were cleared, and supernatants were titrated with increasing concentrations of ethylglycol *bis*-succinimidyl succinate (EGS)[3] (from 0 to 4 μM) for 30 min at room temperature. Reactions were quenched with 50 mM Tris/HCl, pH 7.4. Anti-FLAG antibody was added, and Nrdp1 was immunoprecipitated as described above. For co-immunoprecipitation experiments, cells were lysed in co-immunoprecipitation buffer with inhibitors and then subjected to the immunoprecipitation protocol.

Size Exclusion Chromatography and Native PAGE—A Superdex 200 10/300 column from GE Healthcare Life Sciences was used with a Waters 1525 HPLC system according to the manufacturer's instructions. The column was calibrated with six molecular mass standards ranging from 670 to 1.3 kDa (Bio-Rad), and a standard curve was created based on the molecular masses of the markers and the elution volume. The relationship $\log(MW) = -0.1817 \times (\text{fraction number}) + 11.298$ was fit to a plot of protein standard log molecular masses *versus* elution fraction, with an $R^2 = 0.9916$. This column was subsequently used to separately analyze lysates from wild type Nrdp1- and Nrdp1Δcc-transfected 293T cells, as well as endogenous Nrdp1 from MCF7 cells. Cells were lysed in co-immunoprecipitation buffer, fifty 0.5-ml fractions were collected for each run, and fractions were subjected to SDS-PAGE. Nrdp1 complex molecular masses were estimated based on the standard curve and the Nrdp1 elution profile. Protein from the above three samples was resolved by 8% native PAGE and immunoblotted as an additional method of Nrdp1 complex size estimation.

RESULTS

The amino-terminal half of the Nrdp1 protein contains each of the domains characteristic of TRIM proteins, including an amino-terminal RING finger domain, followed by a B-box and a coiled-coil domain (Fig. 1). The carboxyl-terminal domain shows no homology to other proteins, exhibits a unique fold (34, 35), and is responsible for ErbB3 binding. To explore the role of the coiled-coil domain in mediating ubiquitin ligase activity, we created several mutant forms of human Nrdp1 (Fig. 1). Nrdp1Δcc lacks the coiled-coil domain but contains the RING finger, B-box, and ErbB3 binding domains. Nrdp1–32 encodes a naturally occurring splice variant lacking the RING finger and B-box domains and acts as a dominant negative to stabilize ErbB3 (13, 16, 17); the Nrdp1–32Δcc derivative also lacks the coiled-coil region. Finally, Nrdp1-CSHQ is a double point mutant in zinc-binding residues 34 and 36 of the RING finger domain that lacks ligase activity (32).

The Coiled-coil Domain Mediates Nrdp1 Oligomerization—Previous studies with a variety of proteins suggest that coiled-coil domains facilitate specific protein-protein interactions and very often mediate protein oligomerization (36, 37). To determine whether the coiled-coil domain of Nrdp1 might be involved in Nrdp1 self-association, we first asked whether V5- and FLAG-tagged Nrdp1 can co-immunoprecipitate after transient co-expression in 293T cells. As illustrated in Fig. 2*A*, V5-tagged Nrdp1 specifically co-precipitates with FLAG-tagged Nrdp1 and vice versa, indicating that Nrdp1 exists in an oligomeric complex in cells.

To further assess Nrdp1 self-association, we examined the Nrdp1 species created after adding a covalent chemical cross-linker to cell lysates. In the experiment shown in Fig. 2*B*, 293T cells were transiently transfected with FLAG-tagged Nrdp1, cleared cell lysates were prepared and treated with increasing concentrations of the chemical cross-linker EGS, and Nrdp1 was immunoprecipitated with anti-FLAG. Blotting of precipitates revealed a progressive increase in Nrdp1 apparent molecular masses with increasing cross-linker concentration, con-

[3] The abbreviation used is: EGS, ethylglycol *bis*-succinimidyl succinate.

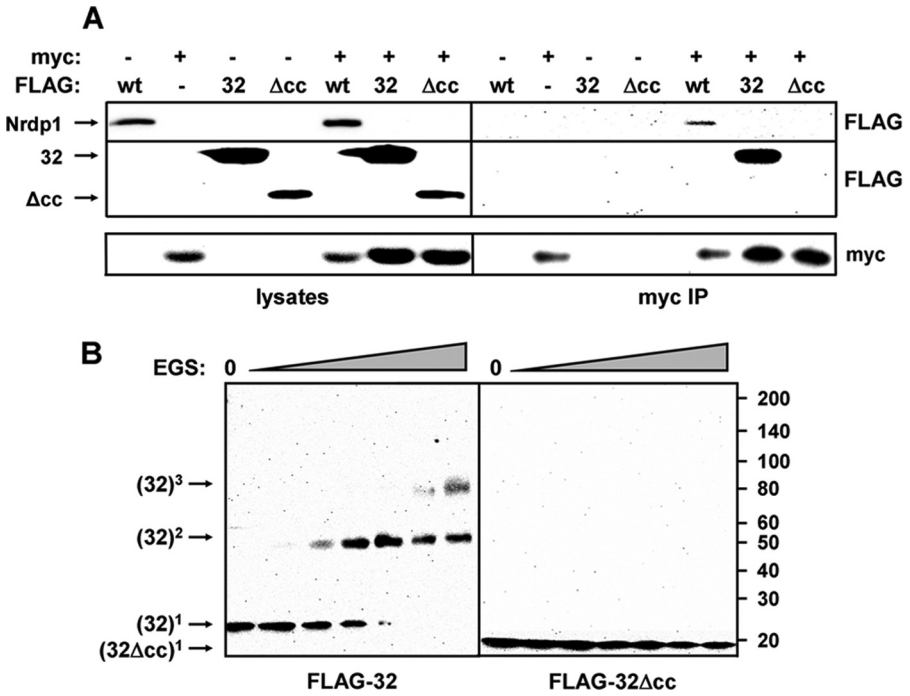

FIGURE 3. **The coiled-coil domain of Nrdp1 mediates self-association.** *A*, 293T cells were transiently transfected with FLAG-tagged Nrdp1 (*wt*), Nrdp1–32 (*32*), or Nrdp1–32Δcc (Δ*cc*), without or with Myc-tagged Nrdp1, as indicated. Lysates (*left panels*) and anti-Myc immunoprecipitates (*right panels*, *myc IP*) were immunoblotted with anti-FLAG (*upper panels*) and anti-Myc (*lower panels*). *B*, cells were transfected with FLAG-tagged Nrdp1–32 or Nrdp1–32Δcc, proteins in lysates were cross-linked with EGS, and immunoprecipitated proteins were blotted with anti-FLAG. The data in each panel are representative of at least four independent experiments.

sistent with the trapping of dimeric and trimeric forms of the protein.

To assess the involvement of the characteristic TRIM domains in Nrdp1 oligomerization, we examined the self-association of the Nrdp1–32 and Nrdp1–32Δcc mutants. In the experiment depicted in Fig. 3*A*, 293T cells were co-transfected with Myc-tagged Nrdp1 and/or FLAG-tagged Nrdp1–32 or Nrdp1–32Δcc. Immunoprecipitation with anti-Myc revealed that Nrdp1–32 is able to associate with wild type Nrdp1, whereas Nrdp1–32Δcc is not. Moreover, Nrdp1–32 could be shifted into dimeric and trimeric species with the addition of the EGS cross-linker, whereas Nrdp1–32Δcc could not (Fig. 3*B*). These observations indicate that the RING finger and B-box domains of Nrdp1 are dispensable for oligomerization, whereas the coiled-coil domain is required.

Finally, to confirm the existence of endogenous Nrdp1 within an oligomeric complex, we carried out size exclusion chromatography using a Superdex 200 10/300 column. In the experiment depicted in Fig. 4 (*A* and *B*), we transiently expressed FLAG-tagged versions of either wild type Nrdp1 or Nrdp1Δcc in 293T cells and subjected cell lysates to gel filtration chromatography alongside lysates from MCF7 cells. Fractions were blotted for FLAG or endogenous Nrdp1 (Fig. 4*A*), the signal was quantified, and the peaks were normalized to 1 (Fig. 4*B*). When compared with known molecular mass standards, wild type Nrdp1 eluted from the column in fraction 34 as a ~132-kDa complex, whereas Nrdp1Δcc eluted in fraction 36/37 as a ~46-kDa monomer. Endogenous Nrdp1 from MCF7 cells eluted in fraction 35, similar to that observed for transfected wild type FLAG-tagged Nrdp1. Native polyacrylamide gel electrophoresis revealed that the mobility of endogenous

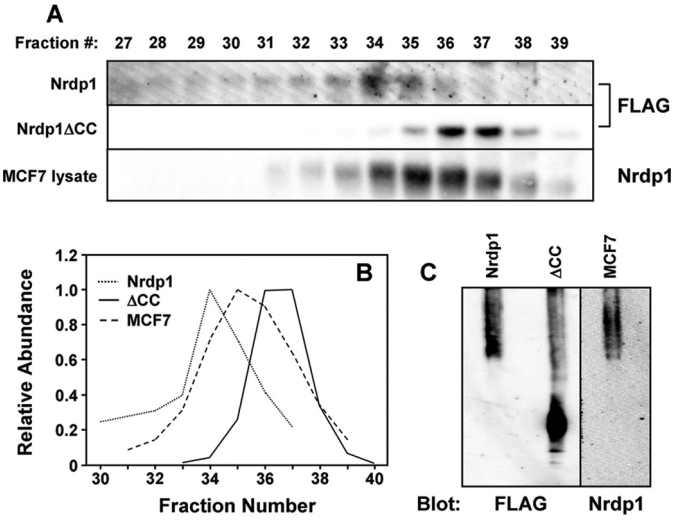

FIGURE 4. **Nrdp1 forms a trimer.** *A*, lysates from 293T cells transiently transfected with FLAG-tagged wild type Nrdp1 (*upper panel*) or Nrdp1Δcc (*middle panel*), as well as lysates from MCF7 cells (*lower panel*), were subjected to size exclusion chromatography using a Superdex 200 10/300 column. Eluted fractions were immunoblotted with anti-FLAG or anti-Nrdp1, as indicated. *B*, blots from *A* were quantified, and relative Nrdp1 levels were plotted. *C*, lysates were subjected to 8% native PAGE and immunoblotted with anti-FLAG or anti-Nrdp1. The data in each panel are representative of three independent experiments.

Nrdp1 from MCF7 cells is essentially identical to that of transfected wild type FLAG-tagged Nrdp1 and dramatically slower than that of Nrdp1Δcc (Fig. 4*C*). Collectively, our co-immunoprecipitation, gel filtration, native PAGE, and cross-linking data indicate that the coiled-coil domain of Nrdp1 mediates its self-association and that oligomeric Nrdp1 likely exists in a trimeric state.

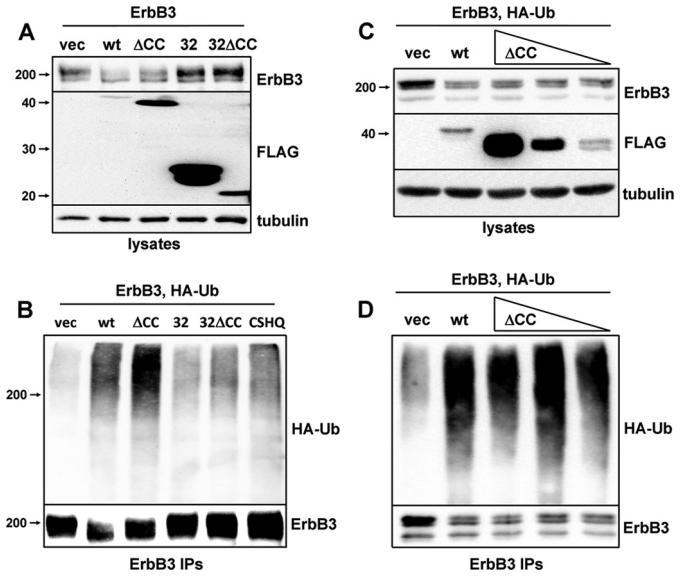

FIGURE 5. **The coiled-coil domain is not necessary for Nrdp1-mediated ErbB3 ubiquitination and degradation.** *A*, 293T cells were transfected with ErbB3 along with vector control (*vec*) or the indicated Nrdp1 constructs, and lysates were immunoblotted with antibodies to ErbB3, FLAG, and tubulin. *B*, cells were co-transfected with HA-tagged ubiquitin, ErbB3, and the indicated Nrdp1 constructs; lysates were immunoprecipitated with anti-ErbB3; and precipitates were blotted for HA and ErbB3. *C* and *D*, cells were transfected with ErbB3 and HA-ubiquitin and co-transfected with vector control, wild type Nrdp1, or various quantities of Nrdp1Δcc. Lysates (*C*) and ErbB3 immunoprecipitates (*D*) were blotted with the indicated antibodies. The data in each panel are representative of at least three independent experiments.

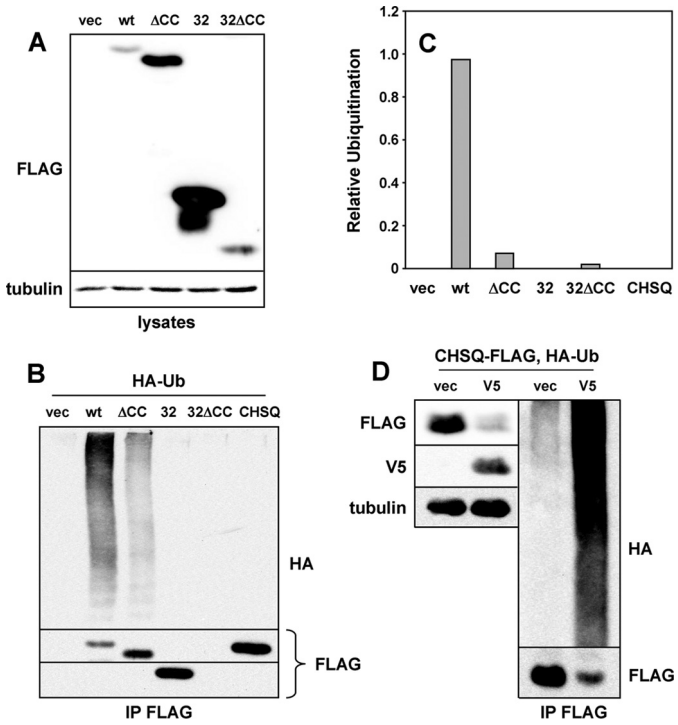

FIGURE 6. **The coiled-coil domain is necessary for efficient Nrdp1 autoubiquitination via intermolecular ubiquitin transfer.** 293T cells were transiently co-transfected with HA-tagged ubiquitin along with vector control (*vec*), wild type Nrdp1 (*wt*), Nrdp1Δcc (Δ*CC*), Nrdp1–32 (*32*), Nrdp1–32ΔCC (*32ΔCC*), or ligase-null Nrdp1-CSHQ, as indicated. *A*, lysates were blotted with antibodies to FLAG epitope and tubulin. *B*, anti-FLAG immunoprecipitates (*IP*) were blotted with antibodies to HA or FLAG. *C*, the bands in *B* were quantified, and the relative extent of Nrdp1 ubiquitination (HA to FLAG ratio) was plotted for each Nrdp1 construct. *D*, 293T cells were transiently transfected with HA-tagged ubiquitin and the FLAG-tagged ligase-inactive Nrdp1-CHSQ along with either vector control or wild type V5-tagged Nrdp1 (*V5*). Lysates (*left panels*) were immunoprecipitated with anti-FLAG (*right panels*) and blotted with the indicated antibodies. The data in each panel are representative of at least three independent experiments.

The Nrdp1 Coiled-coil Domain Does Not Influence ErbB3 Ubiquitination—We have previously observed that overexpression of wild type Nrdp1 suppresses ErbB3 levels by promoting receptor ubiquitination and degradation, whereas overexpression of the 32 variant markedly augments receptor levels by interfering with endogenous Nrdp1 activity (13, 17). To examine the impact of the Nrdp1 coiled-coil domain on ErbB3 stability, we co-transfected 293T cells with ErbB3 and the Nrdp1 constructs depicted in Fig. 1 and examined cell lysates for ErbB3 protein levels by immunoblotting (Fig. 5*A*). As expected, we observed that Nrdp1 and 32 suppressed and augmented, respectively, ErbB3 levels in transfected cells. Interestingly, deletion of the coiled-coil domain from either full-length Nrdp1 or the 32 variant did not significantly affect the ability of either form to act on ErbB3. To examine the impact of coiled-coil deletion on ErbB3 ubiquitination, we co-transfected ErbB3 with HA-tagged ubiquitin and the various Nrdp1 constructs, immunoprecipitated ErbB3, and examined the Nrdp1-induced ubiquitin content of the receptor (Fig. 5*B*). As we have previously observed (21), the presence of Nrdp1 markedly augments the amount of ubiquitin associated with ErbB3, whereas the 32 variant and the ligase-deficient CSHQ double point mutant do not significantly affect ErbB3 ubiquitination. Quantification of the degree of ErbB3 ubiquitination indicates that Nrdp1Δcc induced similar levels of ErbB3 ubiquitination as does wild type Nrdp1 (not shown). Importantly, titration of Nrdp1Δcc indicated that it elicits a similar level of ErbB3 ubiquitination (Fig. 5*D*) and degradation (Fig. 5, *C* and *D*) as does wild type Nrdp1 when present at similar levels. These observations indicate that the Nrdp1 coiled-coil domain plays little role in targeting the ErbB3 substrate for ubiquitination and degradation.

The Nrdp1 Coiled-coil Domain Is Necessary for Efficient Nrdp1 Autoubiquitination—In addition to its activity toward ErbB3, Nrdp1 harbors a potent autoubiquitination activity that leads to its constitutive degradation and short half-life in transformed cells (15, 32). Indeed, the CSHQ mutant that is unable to undergo autoubiquitination is significantly more stable in cells than wild type Nrdp1. Likewise, we observed that Nrdp1Δcc is more stable than wild type Nrdp1 (*e.g.*, see Figs. 5*A* and 6*A*), suggesting that the presence of the coiled-coil domain may facilitate autoubiquitination. To test this, we co-transfected HA-tagged ubiquitin with FLAG-tagged Nrdp1 mutants, immunoprecipitated Nrdp1 with FLAG antibodies, and assessed the extent of Nrdp1 ubiquitination by blotting precipitates with antibodies to HA and FLAG (Fig. 6*B*). We observed that although the CSHQ mutant contained little if any detectable ubiquitin, wild type Nrdp1 ubiquitination was robust. Importantly, even though its levels were significantly higher, the amount of ubiquitin associated with Nrdp1Δcc was substantially decreased relative to wild type Nrdp1; quantification and normalization to the amount of Nrdp1 in the samples (Fig. 6*C*) revealed that Nrdp1Δcc contained only ~5% of the ubiquitin contained by the wild type ligase. Finally, when the FLAG-tagged ligase-inactive CHSQ mutant was co-expressed with

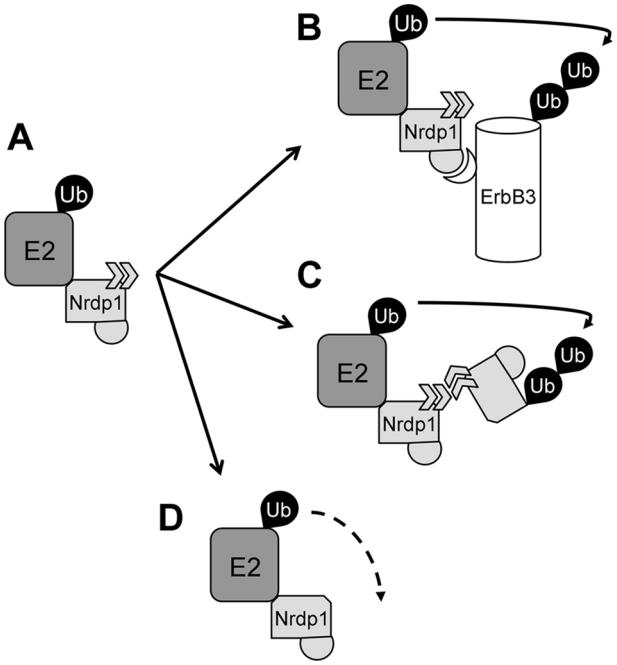

FIGURE 7. **Model depicting oligomerization involvement in Nrdp1-mediated ubiquitination.** *A*, we propose that different domains of Nrdp1 function to bring different substrates to a complex containing a RING domain-associated E2 ubiquitin-conjugating enzyme. *B*, in the case of ErbB3, the carboxyl-terminal receptor binding domain of Nrdp1 mediates the recruitment of the receptor to the ubiquitin-charged E2 to facilitate its efficient ubiquitination. *C*, in the case of Nrdp1, efficient intermolecular autoubiquitination is facilitated by coiled-coil domain-mediated oligomerization. *D*, in the absence of oligomerization, Nrdp1 experiences inefficient intramolecular autoubiquitination.

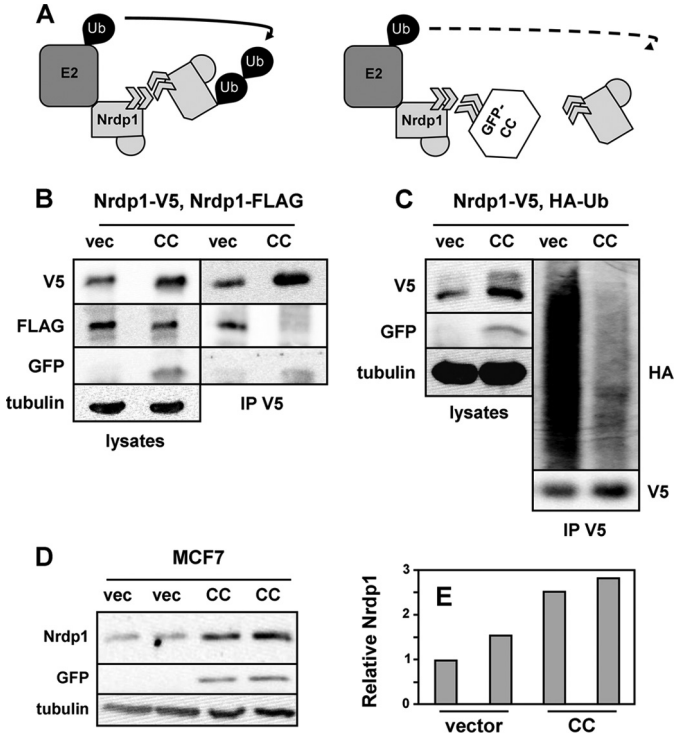

FIGURE 8. **A coiled-coil-GFP fusion disrupts Nrdp1 oligomerization to prevent auto-ubiquitination and degradation.** *A*, we predict that a GFP fusion of the coiled-coil domain would interfere with Nrdp1 ubiquitination to promote ligase stabilization. *B*, 239T cells were co-transfected with Nrdp1-FLAG, Nrdp1-V5, and either GFP vector (*vec*) or a coiled-coil-GFP fusion construct (*CC*). Lysates (*left panels*) were immunoprecipitated anti-V5 (*right panels*, *IP*) and blotted with the indicated antibodies. *C*, 293T cells were transfected with Nrdp1-V5, Ub-HA, and either GFP vector or the coiled-coil-GFP fusion protein construct. Lysates were subjected to immunoprecipitation with anti-V5 and blotted with anti-HA for ubiquitin. *D*, MCF7 breast cancer cells were transiently transfected in duplicate with control vector or GFP-coiled-coil, and lysates were blotted for endogenous Nrdp1, GFP, and tubulin. *E*, bands in *D* were quantified, and relative Nrdp1 levels were plotted. The data in each panel are representative of at least three independent experiments.

wild type V5-tagged Nrdp1, CHSQ levels were markedly suppressed, and the ligase-inactive protein was robustly ubiquitinated, indicating that Nrdp1 autoubiquitination is mediated by a trans reaction (Fig. 6*D*).

Collectively, our observations are consistent with the model illustrated in Fig. 7, where Nrdp1 mediates the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme bound to its RING finger domain to substrates bound to its other domains. Binding of ErbB3 to the carboxyl-terminal domain of Nrdp1 allows receptor ubiquitination (Fig. 7*B*), whereas Nrdp1 oligomerization through its coiled-coil region facilitates autoubiquitination in *trans* (Fig. 7*C*). Deletion of the coiled-coil domain abrogates the ability of Nrdp1 to self-associate and undergo efficient autoubiquitination (Fig. 7*D*).

A GFP Fusion Protein of the Coiled-coil Domain Stabilizes Nrdp1—One prediction of the model depicted in Fig. 7 is that overexpression of the Nrdp1 coiled-coil domain would block the autoubiquitination and stabilization of full-length Nrdp1 by interfering with its oligomerization (Fig. 8*A*). Because Nrdp1 loss in breast tumors results from accelerated autoubiquitination and protein degradation in tumors relative to normal tissue (15), it is possible that introduction of exogenous coiled-coil domain to breast tumor cells could restore endogenous Nrdp1 protein. To test this possibility, we created a GFP fusion with the 45-amino acid Nrdp1 coiled-coil domain and found that its presence interferes with the co-immunoprecipitation of FLAG-tagged Nrdp1 with V5-tagged Nrdp1 (Fig. 8*B*). GFP coiled-coil also interferes with Nrdp1 ubiquitination (Fig. 8*C*) and stabilizes endogenous Nrdp1 in MCF7 cells (Fig. 8, *D* and *E*). Importantly, co-expression of the GFP-CC stabilizes co-expressed Nrdp1 (Fig. 9*A*), resulting in the augmentation of ErbB3 ubiquitination (Fig. 9, *B* and *C*) and the suppression of ErbB3 receptor levels (Fig. 9*A*). These observations provide proof for the principle that the coiled-coil domain may be used to restore Nrdp1 to breast tumor cells to regulate ErbB3 levels.

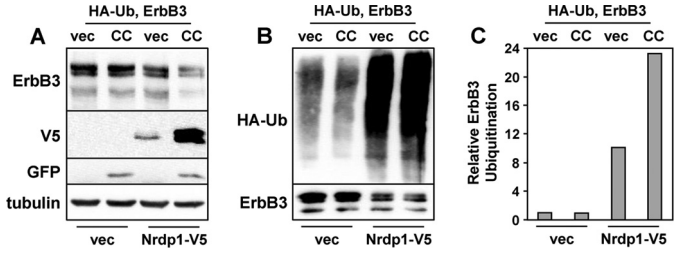

FIGURE 9. **GFP-CC potentiates Nrdp1-mediated ErbB3 ubiquitination and degradation.** 293T cells were transfected with ErbB3 and HA-tagged ubiquitin and co-transfected with vector controls (*vec*), V5-Nrdp1 and GFP-coiled-coil (*CC*), as indicated. *A* and *B*, lysates (*A*) and ErbB3 immunoprecipitates (*B*) were blotted with the indicated antibodies. *C*, bands from *B* were quantified, and the relative ErbB3 ubiquitination (ubiquitin/ErbB3) was plotted for each condition. The data in each panel are representative of three independent experiments.

DISCUSSION

In addition to its impact on ErbB3, Nrdp1 is believed to mediate the ubiquitination of several other proteins including ErbB4 (17), several type 1 cytokine receptors (38–40), the Toll-like receptor signaling adapter protein MyD88 (41), the inhibitor of apoptosis domain-containing protein BRUCE (42), the nuclear factors retinoic acid receptor (38) and C/EBPβ (43), and the E3 ligase Parkin (44). This breadth of substrates targeted by Nrdp1 suggests that the ligase is a key regulator of critical signaling proteins and gives Nrdp1 potential roles in prostate cancer (45), cardiac disease (46, 47), and Parkinson disease (44) in addition to breast cancer. Thus, an understanding of the mechanisms by which Nrdp1 is regulated could shed light on the control of a variety of physiological and pathological processes.

Our observations point to two key functions for the Nrdp1 coiled-coil domain in modulating its activity. The first function is in mediating Nrdp1 self-association into an oligomeric complex. Chemical cross-linking and size exclusion chromatography allowed us to arrive at the conclusion that full-length Nrdp1 likely assembles into trimeric structures. Both methods are characterized by specific strengths and drawbacks. Cross-linking with EGS allows the direct visualization of the oligomeric state of the cross-linked products by SDS-PAGE but requires the presence of multiple reactive amine residues within 16 Å of each other on each of the associated protomers within the oligomeric complex. If sufficient reactive amines are not present, this method could underestimate the stoichiometry of the assembled complex. On the other hand, size exclusion chromatography is not dependent on the chemical properties of the particle but can be sensitive to particle shape; non-spheroid species are more efficiently excluded from resins and elute at higher apparent molecular masses. This phenomenon likely accounts for the elution of Nrdp1Δcc at an apparent mass of 46 kDa, somewhat larger than its 36–38-kDa migration on SDS-PAGE. Finally, endogenous Nrdp1 migrates identically in native PAGE as does transfected Nrdp1-FLAG but not transfected Nrdp1-Δcc-FLAG, confirming that endogenous Nrdp1 exists in an oligomeric state.

The second function of the coiled-coil domain is in mediating Nrdp1 autoubiquitination but not exogenous substrate ubiquitination. We envisage a model where different domains of Nrdp1 are responsible for the recruitment of different substrates to the ubiquitin-charged, RING finger-mediated E2-Nrdp1 complex (Fig. 7). The receptor-binding and coiled-coil domains bring heterologous and homologous substrates, respectively, into the complex for transubiquitination. The carboxyl-terminal receptor-binding domain is necessary (17) and sufficient (35) to bind ErbB3, such that multiple Nrdp1 molecules are not necessary to mediate its recruitment into the ubiquitination complex (Fig. 7*B*). On the other hand, Nrdp1 autoubiquitination occurs by transubiquitination of Nrdp1 molecules within the oligomeric complex (Fig. 7*C*), leading to the marked instability of oligomeric Nrdp1. As expected, disruption of Nrdp1 oligomerization by coiled-coil domain overexpression significantly impairs Nrdp1 autoubiquitination and degradation. Future studies will assess whether Nrdp1 oligomerization is regulated in cells. In addition, future studies will be directed toward identifying the E2 ubiquitin-conjugating enzyme(s) responsible for ErbB3 and Nrdp1 ubiquitination, as well as the nature of the ubiquitin linkages that become attached to each protein.

Curiously, the results of Fig. 6*B* indicate that a low level of residual Nrdp1 ubiquitin is associated with Nrdp1Δcc and that this ubiquitination is likely dependent on its ligase activity because the CSHQ mutant harbors essentially no detectable ubiquitin. These observations raise the possibility that Nrdp1 can undergo a true intramolecular autoubiquitination event, in contrast with the canonical view that E3 ligases generally autoubiquitinate through an intermolecular mechanism (28). However, we have not ruled out that Nrdp1Δcc might experience a low level of oligomerization beneath that detectable by our assays.

It has been suggested that the B-box of TRIM proteins is necessary for the proper orientation of the coiled-coil domain to achieve proper self-association and substrate interaction (27, 48). Our observations suggest that the B-box region of Nrdp1 is dispensable for oligomerization because the clone 32 variant can efficiently oligomerize with itself and with full-length Nrdp1. Thus, the function of the B-box region of Nrdp1 remains obscure.

Although our studies focus on the behavior of Nrdp1 in cultured cells, a recent publication highlights the importance of TRIM protein self-association for polyubiquitin chain formation *in vitro* (49). The authors of this study demonstrated that oligomerization of the RING domain of TRIM32 is necessary to drive autoubiquitination, although they did not directly implicate the coiled-coil domain in the process of oligomerization. Nevertheless, in their studies the fusion of dimeric GST to the RING domain of TRIM32 was sufficient to induce autoubiquitination of the isolated RING domain. In addition to implicating the coiled-coil domain in self-association, we have demonstrated that disruption of this domain with an exogenous agent could disrupt Nrdp1 ubiquitination in cells and stabilize the endogenous protein. Together these findings underscore the importance of self-association in the process of autoubiquitination from both *in vitro* and cellular contexts. Moreover, our observations suggest that exogenous agents that affect TRIM protein oligomerization through disruption of coiled-coil-mediated self-association could be employed to regulate ligase levels and function, which may have application to a variety of clinical situations.

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