Nuclear Import/Export of hRPF1/Nedd4 Regulates the Ubiquitin-dependent Degradation of Its Nuclear Substrates*

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The ubiquitin-protein ligase (E3), hRPF1/Nedd4, is a component of the ubiquitin-proteasome pathway responsible for substrate recognition and specificity. Although previously characterized as a regulator of the stability of cytoplasmic proteins, hRPF1/Nedd4 has also been suggested to have a role in the nucleus. However, in light of the cytoplasmic localization of hRPF1/Nedd4, it is unclear whether bona fide nuclear substrates of hRPF1/Nedd4 exist, and if so, what mechanism may allow a cytoplasmic ubiquitin ligase to manifest nuclear activity. Our search for nuclear substrates led to the identification of the human proline-rich transcript, brain-expressed (hPRTB) protein, the ubiquitination (hPRTB) protein, the ubiquitination and degradation of which is regulated by hRPF1/Nedd4. Interestingly, hPRTB colocalizes with the splicing factor SC35 in nuclear speckles. Finally, we demonstrate that hRPF1/Nedd4 is indeed capable of entering the nucleus; however, the presence of a functional Rev-like nuclear export sequence in hRPF1/Nedd4 ensures a predominant cytoplasmic localization. Cumulatively, these findings highlight a nuclear role for the ubiquitin ligase hRPF1/Nedd4 and underscore cytoplasmic/nuclear localization as an important regulatory component of hRPF1/Nedd4-substrate recognition.

The posttranslational modification of proteins by ubiquitination has been shown to play an important role in the regulation of cell cycle progression, signal transduction, and transcriptional events within the cell. Covalent attachment of the 76-amino acid polypeptide ubiquitin to a substrate protein is a catastrophic signal, targeting the substrate for rapid degradation (1, 2). The specific enzymes involved in this process, E1, E2, and E3, have been studied in great detail (3). A human ubiquitin-activating enzyme (E1) is responsible for the ATP-dependent activation of the ubiquitin polypeptide. Activated ubiquitin is subsequently transferred to a downstream ubiquitin carrier protein (E2), and in many cases to a ubiquitin-protein isopeptide ligase (E3), which mediates the final transfer of activated ubiquitin to a substrate protein. Evidenced by the numerous examples of cellular dysregulation resulting from aberrant ubiquitination (4, 5), this ultimate enzyme-substrate recognition step is crucial for cellular homeostasis. Accordingly, there is of late a heightened level of interest in defining the mechanisms that govern the target specificity of the various E3 ligases and how this event is regulated in target cells.

The experiments that have formed the foundation of our studies have relied on our understanding of the role of E3 ligases were those that describe E6-associated protein and its ability to cooperate with the viral E6 protein in ubiquitinating p53 following human papillomavirus infection (6). This work led to the discovery of a family of proteins with sequence homology to E6-associated protein, the homology to E6-associated protein at the carboxyl terminus (hect) family of proteins (7), and the observation that the amino terminus of hRPF1/Nedd4 places this E3 ligase in the WWhect subclass of ubiquitin ligases. This subclass of enzymes is characterized by 2–4 copies of a WW protein-protein interaction domain (12), followed by a conserved hect domain. The hect domain is the catalytic domain responsible for ubiquitination, an activity that is absolutely dependent upon an invariant cysteine residue located within the active site (7). Similar to many other human WWhect ligases, hRPF1/Nedd4 contains a C2/CaLB domain at the amino terminus, which is responsible for mediating membrane localization in response to calcium (13).

Although hRPF1/Nedd4 contains a hect domain, implying that it is involved in ubiquitination, many studies performed thus far with putative targets have not been illuminating with respect to the function of the enzyme or how the activity is regulated. To date, the best characterized substrate of Nedd4 is the rat sodium epithelial channel (14–16). These studies, which demonstrate that Nedd4 can interact with and regulate the turnover of the sodium epithelial channel, imply a nonnuclear function of this enzyme. This contention is supported by additional work that indicates that Nedd4 and the cytoplasmic adapter protein mGrb10 interact (17). However, recent studies from our laboratories have demonstrated that hRPF1/
Nedd4 may have roles in the nucleus. Specifically, it has been shown that 1) a Nedd4 homolog, yeast RSP5, is responsible for the ubiquitination of the large subunit of RNA polymerase II in response to DNA damage (18), and 2) overexpression of hRPF1/Nedd4 alters the transcriptional activity of the progesterone receptor (19). In addition, an erythroid-specific transcription factor, NF-E2, has been shown to physically associate with hRPF1/Nedd4 (20), although the significance of this interaction remains to be determined. Cumulatively, these data suggest that hRPF1/Nedd4 can modulate target protein ubiquitination in both the cytoplasm and the nucleus. However, because hRPF1/Nedd4 is localized predominantly in the cytoplasm (21), the physiological significance of the interaction between hRPF1/Nedd4 and nuclear proteins is unclear. In an effort to evaluate the potential roles of hRPF1/Nedd4 in the nucleus and how these activities are manifest, we have undertaken a strategy to identify additional nuclear substrates of hRPF1/Nedd4 with a view to (a) confirming that this enzyme can interact with and regulate the stability of nuclear proteins and (b) defining a mechanism by which this cytoplasmic ubiquitin ligase can exert its activity in the nucleus.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Splicing factor SC35 monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All horseradish peroxidase-conjugated secondary antibodies and ECL reagents were obtained from Amersham Pharmacia Biotech. Anti-mouse Texas Red-conjugated secondary antibodies, horse serum, and VectaShield with DAPI (4,6-diamidino-2-phenylindole) were purchased from Vector Laboratories (Burlingame, CA).

**Plasmids**—The complete 5′ coding sequence of hRPF1/Nedd4 (aa 1–900) was obtained using 5′ rapid amplification of cDNA ends, polymerase chain reaction (PCR) amplification, and subcloned into an incomplete RFP1/Nedd4 cDNA (derived from pBK-c-hRPF1 (19) using standard subcloning procedures. hRPF1/Nedd4 deletion constructs encoding C2 (aa 1–192), WW (aa 173–563), and AA (aa 293–900) were cloned by polymerase chain reaction from full-length hRPF1/Nedd4 constructs, and all sequences were verified by sequence analysis. To create the hRPF1/Nedd4-C867A mutant, we utilized the Sall site just upstream of ampicillin resistance for construct preparation. Briefly, a primer was designed (5′-ggcagcgaatctcagcttttagttg-3′) that allowed polymerase chain reaction amplification of amino acids 862–900, resulting in the incorporation of a two-base substitution that changed cysteine 867 to alanine. The SacI-NcoI fragment containing the C867A amino acid change was incorporated into the context of the full-length hRPF1/Nedd4 using standard subcloning procedures. Site-directed mutagenesis was used to introduce the PY motif in hRPF1/Nedd4-C867A. Primers used were as follows: PRT-PYmut, 5′-cggatctcgagctcgtctgctcgcgtcg-3′ (sense) and 5′-ggagtgcggggcgactctggctggc-3′ (antisense); hRPF1/Nedd4-C-867A-ntutNES, 5′-gaattaagagcagagccagttattaatcgcg-3′ (sense) and 5′-cgctgagttttaacgaagtctctgctcgcgt-3′ (antisense). The integrity of all constructs was verified by sequencing.

Enhanced green fluorescent protein (EGFP) fusion constructs were the result of subcloning hPRTB (from the library vector pGADGH) into the EcoRI-BamHI sites of pEGFP-C1 (CLONTECH). To create Myc-tagged fusions, we inserted a BamHI-BamHI fragment of hPRTB cDNA (containing extra 5′ BamHI-EcoRI linker sequence: ggatccggt) into the BamHI site of pcDNA3–5′-Myc vector.

**Cell Culture and Transfections**—HeLa cells were cultured in minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate and maintained in a humidified incubator at 37 °C, 5% CO2. Cells were transiently transfected using Lipofectin (Life Technologies, Inc.) for 4 h and allowed to recover for 24–48 h prior to harvest and analysis.

**Yeast Two Hybrid Screen**—A cDNA encoding the amino terminus of hRPF1/Nedd4 (NW, aa 26–506) was subcloned into the vector pGBT9 (CLONTECH) and introduced by standard lithium acetate protocol into HF7c cells (CLONTECH). A HeLa Matchmaker library was sequentially transformed, and colonies were grown on selective plates containing 1 mM 3-aminotriazole. A HeLa cervical carcinoma cDNA library was chosen based upon previous observations (19) and immunodetection of endogenous hRPF1/Nedd4 protein product in HeLa cells. His+ clones were isolated after 10 days of growth at 30 °C, restreaked onto selective plates (1 mM 3-aminotriazole), and grown for 3 days prior to β-galactosidase filter lift assays. Library and bait plasmids were subsequently sequenced by subcloning into Y190 yeast vectors to verify identity and modulate interaction using a liquid β-galactosidase assay. Briefly, a mid-logarithm phase culture (A600 = 0.5–0.8) was pelleted, washed, and subjected to two cycles of freeze-thaw lysis. The β-galactosidase activity of the lysate was measured at A420, and quantitated using CPRG (chlorophenol red-β-p-galactopyranoside) as a substrate.

**GloTox™ GST-Pulldown Interaction Assays**—hPRTB was subcloned into pcDNAs (Invitrogen), in vitro translated, and radiolabeled in a rabbit reticulocyte lysate system (TNT, Promega). hRPF1/Nedd4 or deletions thereof (C2, aa 1–192; WW, aa 173–563; AA, aa 293–900) were fused to GST, expressed, and purified from bacteria. Purified GST fusion proteins were bound to glutathione-Sepharose (Amersham Pharmacia Biotech) and incubated with radiolabeled hPRTB in NETN-A (25 mM Tris 8.0, 75 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA) overnight at 4 °C. Bound proteins were washed in NETN-B (150 mM NaCl), eluted, and analyzed by SDS-PAGE by autoradiography.

**In Vitro Ubiquitination Assays**—Assays were performed essentially as described previously (18). Briefly, an in vitro translated, radiolabeled substrate was incubated with BL21 bacterial extracts and E1 and an E2 (UbH5B). Reaction mixtures contained 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM dithiothreitol, 2 mM ATP, 2 mM MgCl2, 2 μg of bovine ubiquitin (Sigma), and 500 ng of purified E3 enzyme (either yRSP5 or hRPF1/Nedd4-whect). In some cases, an ATP regeneration system was also included, consisting of 0.1 mM phosphocreatine, 3.5 units/ml creatine phosphokinase, and 0.6 units/ml inorganic pyrophosphatase. After incubation at 30 °C for 1 h, reactions were terminated with 3 × SDS-PAGE sample buffer, resolved, and detected by SDS-PAGE followed by autoradiography.

**Detection of in Vivo Ubiquitin Conjugates**—Following a previously published procedure (15), HeLa cells were transiently transfected with mammalian expression plasmids for His-tagged ubiquitin and a Myc-tagged substrate (hRPF1 or PY mutant). Thirty-six hours after transfection, cells were harvested in lysis buffer (PBS, 1% Triton X-100, 10% glycerol). The insoluble fraction was removed by a high speed spin, and the clarified supernatant was denatured by addition of 2% SDS followed by boiling for 5 min. Denatured extract was diluted with 12 volumes of lysis buffer and incubated with 50 μl of nickel-nitrilotriacetic acid resin (Qiagen) for 4 h at 4 °C. After thorough washing with lysis buffer containing 1 M NaCl, the resin was washed with 0.5 M NaCl for 10 min, and His-tagged proteins were eluted with 3 × SDS sample buffer and analyzed by Western blot analysis using an anti-Myc antibody, 9E10.

**Pulse-Chase Analysis**—HeLa cells (~70–80% confluency) that had been plated in 60 mM dishes were transiently transfected with 2.7 μg of a Myc-hPRTB or Myc-hRPF1/PYmut expression plasmid and 20 ng of the internal control Myc-EGFP. In experiments assaying the effect of Nedd4, cells were cotransfected with a 1 μg expression plasmid consisting of pc:RPF1 or pc:RPF1-C867A (or an equimolar amount of empty pcDNA3 vector) cotransfected as well. Twenty-four hours posttransfection, cells were washed with PBS and incubated in methionine- and cysteine-free medium for 30 min. A radioactive mixture of methionine and cysteine (175 μCi of Tran^35S-Label, ICN) was used to metabolically label cells for 2 h, after which the medium was removed and replaced with cold medium containing an excess of methionine and cysteine (2 and 1 mM, respectively) to chase for indicated time points. At each time point, cells were harvested in PBS, 0.5% Triton X-100 plus protease inhibitors, flash-frozen, and subsequently immunoprecipitated with an antibody directed against the Myc tag (9E10, Santa Cruz Biotechnology). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography and quantitated using a phosphorimage.

**Indirect Immunolocalization**—HeLa cells were transiently transfected with an expression plasmid for an EGFP-hPRTB (or PY mutant) fusion protein and plated onto 25-mm round glass coverslips. Cells were fixed using 4% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 for 10 min. Samples were blocked in PBS containing 10% BSA and then incubated with anti-SC35 (Sigma) at a dilution of 1:2000 in PBS/0.2% horse serum, followed by incubation with anti-mouse Texas Red-conjugated secondary antibody at a 1:75 dilution in PBS/0.2% horse serum. Coverslips were mounted in VectorShield plus DAPI (4′,6-diamidino-2-phenylindole). Localization of transfected Myc-hRPF1/Nedd4 deletion or mutation constructs was performed essentially as described above, only using the anti-c-Myc antibody (9E10) at a 1:2000 dilution. Indicated samples were treated with 20 ng/ml of leptomycin B.
with full-length hRPF1/Nedd4, we assayed the ability of hPRTB is its proline-rich composition (18%).

amino acid sequence does not share significant sequence homology with that of other proteins, the most notable feature of hPRTB (Fig. 1B), based upon its high amino acid identity with hRPF1/Nedd4, we next sought to determine the subcellular localization of the potential ubiquitination substrate hPRTB. To this end, we fused hPRTB to EGFP and analyzed its subcellular localization using fluorescence confocal microscopy. EGFP-hPRTB and the corresponding PY mutant (P40A/Y42A) have been shown to be ubiquitinated by hRPF1/Nedd4 (Fig. 1C, D). It is interesting to note that the WWhect ligase, Smurf1, similarly binds to a PPAY motif in its substrate, Smad1 (9). With the prediction that this conserved motif may mediate the interaction of hPRTB with hRPF1/Nedd4, we substituted the second proline and subsequent tyrosine with alanine and assayed for the ability of this PY mutant to interact with hRPF1/Nedd4. The two-amino acid substitution within the PPAY motif in hPRTB was able to disrupt the ability of hPRTB to bind to hRPF1/Nedd4 (Fig. 1D), further demonstrating that the WW domains of hRPF1/Nedd4 directly interact with the PPAY motif of hPRTB. The direct association of a WW domain-containing enzyme and a substrate with a PPXY motif is ideal for an enzyme-substrate interaction in that WW domains typically bind with high specificity rather than high affinity (26), a property that may explain our inability to isolate hPRTB-hRPF1/Nedd4 complexes from cellular extracts (data not shown).

To independently verify that hPRTB does indeed interact with full-length hRPF1/Nedd4, we assayed the ability of in vitro translated, [35S]methionine-labeled Myc-hPRTB to interact with recombinant GST fusions of hRPF1/Nedd4 (see schematic in Fig. 1C). Myc-hPRTB was able to interact with full-length hRPF1/Nedd4 or yRSP5, a yeast homolog that has a domain structure similar to that of hRPF1 (containing only three WW domains). The WW domains of hRPF1/Nedd4 were sufficient for interaction with hPRTB; it is important to note that the greater hPRTB signal associated with GST-WW (Fig. 1D) is likely a reflection of the greater molar amount of GST-WW used. Neither the hect domain nor the C2 domain resulted in any detectable interaction (Fig. 1D). WW domains are predicted to interact with proline-containing consensus sequences, which are either PPXY, PPLP, or PGM (12, 23–25).

Examination of the proline-rich regions within hPRTB revealed a consensus PPAY motif located in the central portion of the protein (Fig. 1B). It is interesting to note that the WWhect ligase, Smurf1, similarly binds to a PPAY motif in its substrate, Smad1 (9). With the prediction that this conserved motif may mediate the interaction of hPRTB with hRPF1/Nedd4, we substituted the second proline and subsequent tyrosine with alanine and assayed for the ability of this PY mutant to interact with hRPF1/Nedd4. The two-amino acid substitution within the PPAY motif in hPRTB was able to disrupt the ability of hPRTB to bind to hRPF1/Nedd4 (Fig. 1D), further demonstrating that the WW domains of hRPF1/Nedd4 directly interact with the PPAY motif of hPRTB. The direct association of a WW domain-containing enzyme and a substrate with a PPXY motif is ideal for an enzyme-substrate interaction in that WW domains typically bind with high specificity rather than high affinity (26), a property that may explain our inability to isolate hPRTB-hRPF1/Nedd4 complexes from cellular extracts (data not shown).

hPRTB Colocalizes with Splicing Factors in Nuclear Speckles—Given our interest in a possible nuclear function of hRPF1/Nedd4, we next sought to determine the subcellular localization of the potential ubiquitination substrate hPRTB. To this end, we fused hPRTB to EGFP and analyzed its subcellular localization using fluorescence confocal microscopy. EGFP-hPRTB and the corresponding PY mutant (P40A/Y42A) have identical fluorescence patterns and are localized to the nucleus in a discrete speckled pattern (Fig. 2). We were intrigued by our observation of the localization of hPRTB to spots in the nucleus, reminiscent of “nuclear speckles,” which are enriched in
splicing factors and contain a population of hyperphosphorylated RNA polymerase II (27, 28). Using fluorescent confocal microscopy, we demonstrated that both hPRTB and the PY mutant indeed colocalize with the splicing factor, SC35, in nuclear speckles (Fig. 2), suggesting that hPRTB may have a role in the transcription and/or splicing of RNA transcripts. Thus, in addition to identifying a potential nuclear substrate of hRPF1/Nedd4, we have localized hPRTB to splicing-factor-rich speckles, a subnuclear localization where a population of RNA polymerase II, another WWhect E3 substrate, is known to reside.

hPRTB Is a Substrate of WW Hect E3 Ubiquitin Ligases in Vitro—Given that hPRTB specifically binds to hRPF1/Nedd4, we next wanted to determine whether it could serve as a substrate for the E3 ubiquitin ligase activity of this enzyme. A recombinant hRPF1/Nedd4 (wheel) derivative lacking the amino-terminal C2 domain was used in enzymatic assays, as full-length protein is not active under the conditions tested (18). Efficient multi-ubiquitination of hPRTB was observed when assayed in the presence of purified hRPF1/Nedd4 (wheel) or yRSF5, but not the hect E3 ligase E6-AP, which lacks WW domains in its amino terminus (Fig. 3A). Additionally, no ubiquitination was observed when either the E2 (UbcH5B) or E3 (RPF1/Nedd4-wheel) enzyme was omitted from the reaction mixture (data not shown and Fig. 3A). With the prediction that substrate binding is necessary for E3 ligase activity, we next tested the hypothesis that the hPRTB-PYmut would not be ubiquitinated in this assay. Indeed, mutation of two key residues within the PPAY motif of hPRTB results in a 3.5-fold increase in the half-life of hPRTB (2 h to 7 h) (Fig. 4B). Cumulatively, these observations provide compelling evidence that hPRTB is a physiological substrate of an endogenous WW-hect E3 ubiquitin ligase, such as hRPF1/Nedd4.

hRPF1 Regulates the Stability of hPRTB in Cells—It has previously been shown that nuclear proteins such as the large subunit of RNA polymerase II and the transcription factor NF-E2 bind to or are able to be ubiquitinated by mammalian Nedd4 family members in vitro (18, 20, 29); however, as of yet, there is no evidence that such proteins are physiologically regulated by a particular mammalian E3 ubiquitin ligase within cells. Given our data thus far, we suspected that the WW-hect E3 ubiquitin ligase responsible for the ubiquitin-dependent degradation of PRTB was hRPF1/Nedd4. Thus, we sought to establish that exogenous hRPF1/Nedd4 was able to alter the ubiquitin-dependent degradation of hPRTB. Using pulse-chase analysis, we demonstrated that the degradation of hPRTB was accelerated in samples containing transiently transfected hRPF1/Nedd4 but not in samples transfected with the catalytic mutant hRPF1/Nedd4-C867A or an empty expression plasmid (Fig. 5A). Specifically, the half-life of hPRTB in the presence of overexpressed hRPF1/Nedd4 was 70 min, compared with a t1/2 of 150 min in samples containing either hRPF1/Nedd4 or hRPF1/Nedd4-C867A or no exogenous hRPF1/Nedd4 (Fig. 5B). Western blot analysis confirmed that the total amount of hRPF1/Nedd4 or hRPF1/Nedd4-C867A protein in transfected cells was at least 2–3 times the amount normally present within HeLa cells (Fig. 5C). Thus, we have identified a nuclear speckle-associated protein, hPRTB, as a substrate of the E3 WW-hect ubiquitin ligase hRPF1/Nedd4 within cells. These data provide direct evidence that hRPF1/Nedd4 can interact with, ubiquitinate, and regulate the stability of a confirmed nuclear protein in intact cells.

hRPF1/Nedd4 Contains a Rev-like Nuclear Export Sequence—To further substantiate our observations that hRPF1/Nedd4 is able to target the nuclear protein hPRTB for ubiquitination and degradation, we finally sought to understand the mechanism by which a primarily cytoplasmic E3 enzyme, hRPF1/Nedd4, is able to modify the nuclear protein hPRTB.

hRPF1/Nedd4 has been reported to contain a bipartite nuclear localization signal between amino acids 534–550; how-
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FIG. 3. hPRTB is an in vitro and in vivo ubiquitination substrate. A, hRPF1/Nedd4 ubiquitates hPRTB in vitro. 35S-Labeled Myc-hPRTB was incubated with purified hRPF1/Nedd4 (21, 30). Myc-hPRTB or Myc-hPRTB-PYmut, containing a two- amino acid substitution (P40A/Y42A) of the PPAY motif, were assayed in a standard ubiquitination assay using E1, E2 (UbH5B), and hRPF1/Nedd4 (wt) as the E3 enzyme. B, His-ubiquitin conjugates were detected by Western blot and immunofluorescence analysis using an antibody directed against c-Myc (9E10). C, His-ubiquitin conjugates of hPRTB isolated from HeLa cells. HeLa cells were transfected with expression plasmids for His-ubiquitin and Myc-hPRTB or Myc-hPRTB-PYmut. Lysates were prepared (lanes 1–3), and His-conjugates were purified on nickel resin (lanes 4–6). Myc-hPRTB or Myc-hPRTB His-conjugates were detected by Western immunoblot analysis using an antibody directed against c-Myc (9E10). WT, wild type.

However, it has been suggested in the past that Nedd4 is primarily a cytoplasmic protein (21, 30). Indeed, when Myc-tagged RPF1 is expressed in HeLa or NIH3T3 cells, we have shown that it is primarily cytoplasmic (data not shown). In an attempt to artificially place hRPF1/Nedd4 into the nuclear compartment of cells, we fused a strong SV40 nuclear localization signal to the amino terminus of hRPF1/Nedd4. When cellular localization of SV40 nuclear localization signal-RPF1/Nedd4 was assayed, little to no nuclear staining was detected despite significant cytoplasmic staining (data not shown), raising the possibility that hRPF1/Nedd4 is a protein that is constitutively exported from the nucleus.

Many nuclear proteins undergo nuclear export via the CRM1-dependent nuclear export pathway (31–33). To determine whether the WWhect E3 ligase hRPF1/Nedd4 might be a substrate of this nuclear export system, we evaluated hRPF1/Nedd4 localization in the presence of the drug leptomycin B, a specific inhibitor of CRM1-dependent export (34). Myc-hRPF1/Nedd4-expressing cells or Myc-hRPF1/Nedd4-C867A-expressing cells were treated with 20 ng/ml leptomycin B, and the subcellular localization of hRPF1/Nedd4 was analyzed by immunofluorescence with an antibody directed against the Myc tag. The results of this analysis (Fig. 6A) indicate that a population of both wild type and catalytically inactive hRPF1/Nedd4 were localized within the nucleus after treatment with leptomycin B, indicating that this WWhect E3 ubiquitin ligase, or a complex containing this protein, is a substrate of CRM1-dependent nuclear export.

Given the data of others that a hRPF1/Nedd4 derivative encoding aa 404–900 is localized primarily within the nucleus (21), whereas full-length constructs are cytoplasmic, we next used a series of amino-terminal deletion constructs to map the region of hRPF1/Nedd4 responsible for its cytoplasmic localization. Full-length hRPF1/Nedd4, as well as aa 293–900, localized primarily to the cytoplasm, but proteins encoding aa 309–900 and 404–900 were present in both the cytoplasm and the nucleus (data not shown), suggesting that a sequence between amino acids 293 and 309 of hRPF1/Nedd4 is responsible for its steady state cytoplasmic localization. Therefore, we compared amino acids 293–309 of hRPF1/Nedd4 with the leucine-rich consensus for Rev-like nuclear export. Indeed, amino acids 297–307 of hRPF1/Nedd4 contain sequence identity with this NES consensus and share significant homology with the NESs found in other proteins, such as PKI, HIVrev, human p53, and Rex (Fig. 6B) (31, 35–37). To prove that this sequence within hRPF1/Nedd4 is able to act as an NES, we substituted conserved residues with alanine (L305A and I307A) and assayed for the cellular localization of this putative NES mutant. Mutation of these two conserved amino acids within the export sequence significantly increased the amount of hRPF1/Nedd4 protein that was detected in the nucleus, as compared with the primarily cytoplasmic localization of Myc-hRPF1/Nedd4-C867A (Fig. 6C) or Myc-RPF1 (data not shown). Interestingly, when the NES mutant was created in the context of a wild type hRPF1/Nedd4, extremely low quantities of exogenous protein were detected by Western blot and immunofluorescence analysis. Nonetheless, these observations cumulatively demonstrate that amino acids 297–307 mediate the CRM1-dependent nuclear export of hRPF1/Nedd4.

DISCUSSION

Presented in this study is evidence that hPRTB, a proline-rich protein that colocalizes with splicing machinery in nuclear speckles, is a bona fide nuclear substrate of the WW hect E3 ubiquitin ligase, hRPF1/Nedd4. This identification of a nuclear substrate extends the role of the hRPF1/Nedd4 ubiquitin ligase to nuclear proteins. Furthermore, deletion and mutational analyses have led to our subsequent identification of a leucine-rich rev-like nuclear export sequence within hRPF1/Nedd4. Thus, we propose that nuclear import/export is an important component of the regulation between the primarily cytoplasmic E3 enzyme, hRPF1/Nedd4, and its nuclear substrate, hPRTB.

An in Vivo Nuclear Substrate of hRPF1/Nedd4—It has been well established that Nedd4 can interact with and modulate...
the level of several cytoplasmic proteins (14, 15, 17). In this study, we have explored an additional role for Nedd4 with the identification and characterization of substrate proteins that are localized within the nucleus. In every aspect examined, the proline-rich nuclear protein, hPRTB, is a characteristic substrate for hRPF1/Nedd4 ubiquitination and degradation within the cell. However, given the lack of known determinants of in vivo WWhect specificity, we cannot exclude the possibility that there may be other human WWhect E3 ubiquitin ligases that are able to ubiquitinate this novel nuclear substrate expressed within cells. Indeed, there are several human WWhect E3 ubiquitin ligases with similar domain structure to hRPF1/Nedd4, including Smurf1, a human WWhect ligase containing two WW domains, which targets Smad1 and Smad5 for ubiquitin-dependent degradation (9). However, our examination of Smurf1 amino acid sequence failed to identify either a bipartite nuclear localization sequence or rev-like export sequence similar to those in hRPF1/Nedd4, suggesting that certain WWhect enzymes, such as Smurf1, may specifically target substrates in the cytoplasm. Although our work strongly suggests a nuclear function for hRPF1/Nedd4, additional research into the in vivo substrate specificity of this and other potentially nuclear human WWhect proteins is necessary to address precise questions of overlapping enzyme/substrate choice.

The localization of a ubiquitination substrate such as hPRTB in nuclear speckles is not surprising, given observations that cellular proteins modified by the ubiquitin-like protein SUMO-1 are targeted to precise subnuclear localizations. SUMO-1-modified promyelocytic leukemia gene product (PML) localizes to nuclear bodies (38); similarly, the sumoylation of the homeodomain-interacting protein kinase 2 results in localization to nuclear speckles (dots) that are distinct from either splicing factor-rich speckles or PML bodies (39). The localization of hPRTB in nuclear speckles is unaffected by a mutation that blocks ubiquitination, suggesting that the covalent attachment of a ubiquitin moiety is not required for nuclear speckle localization. However, in addition to playing a significant role in subnuclear localization, SUMO-1 modification also antagonizes the ubiquitin-dependent degradation of proteins such as IκB, leading to an increase in protein stability (40). We acknowledge the possibility that hPRTB may also be targeted (by a distinct enzyme) for sumoylation, a ubiquitin-like modification, which could either direct its localization to nuclear speckles or antagonize its ubiquitin-dependent degradation. Further studies are needed to address whether hPRTB may be modified by such a ubiquitin-like protein.

Although the precise function of hPRTB remains unknown, its localization in nuclear speckles may offer clues to a possible

![Image](https://example.com/image.png)
evidence that Nedd4 can indeed access both cytoplasmic and nuclear compartments within a cell. However, it is apparent that hRFP1/Nedd4 protein that enters the nucleus has a very strong constitutive export sequence, resulting in little time spent resident within the nucleus. For example, exogenous hRFP1/Nedd4 targeted to the nucleus by a strong SV40 nuclear localization signal or a mutated nuclear export sequence is not tolerated by the cell, and protein does not accumulate (data not shown). Thus, although a population of hRFP1/Nedd4 is able to enter the nucleus, its presence appears to be transient, with the cell having a strong preference to return it to the cytoplasm. This transient nuclear localization of hRFP1/Nedd4 offers an additional explanation for our inability to isolate and immunoprecipitate what are presumably nuclear hRFP1/Nedd4-hPRTB complexes (data not shown).

Although hRFP1/Nedd4 is able to enter the nucleus, it is intriguing that neither leptomycin B treatment nor mutation of the NES is sufficient to “trap” all of the hRFP1/Nedd4 protein within the nucleus. Presumably the remaining cytoplasmic hRFP1/Nedd4 population has not received a signal for nuclear entry but instead may be poised to act upon known cytoplasmic Nedd4 targets, such as the sodium epithelial channel. Accordingly, one logical question raised is what stimulus or signal targets cytoplasmic hRFP1/Nedd4 to the nucleus? hRFP1/Nedd4 is cleaved in cells in response to apoptotic stimuli (41); however, a truncated Nedd4 protein corresponding to the caspase cleavage product retains a primarily cytoplasmic localization (data not shown), suggesting that removal of the first 200 amino acids is not a sufficient signal for nuclear import. Although other factors such as phosphorylation, acetylation, or a conformational change may signal hRFP1/Nedd4 nuclear import, regulation of Nedd4 localization may also occur at the level of nuclear export. For example, the sequences flanking the NES of hRFP1/Nedd4 could be modified or change conformation to specifically block accessibility of the NES to the export receptor, preventing efficient export. Such NES masking has been proposed to play a role in the blocking of p53 nuclear export upon p53 tetramerization (37). We have not yet observed a set of conditions under which hRFP1/Nedd4 is exclusively nuclear, but we remain interested in identifying factors that regulate such nuclear import and/or export. Experiments aimed at identifying developmental stages, cell types, or conditions under which hRFP1/Nedd4 may be localized within the nucleus will likely offer insight into the important role of nuclear/cytoplasmic localization in substrate recognition.

Given that hRFP1/Nedd4 may spend only a short time in the nucleus, it is likely that nuclear import of hRFP1/Nedd4 is the limiting step in enzyme/substrate recognition and catalysis. For example, an activating signal could effect the transient nuclear localization of hRFP1/Nedd4, resulting in ubiquitination of a substrate within the nucleus, followed by rapid export of hRFP1/Nedd4 to the cytoplasm. Alternatively, nuclear import of hRFP1/Nedd4 could be the limiting step if transient enzyme entry was needed to allow the enzyme to bind a nuclear substrate and transport it to the cytoplasm, where ubiquitination and degradation might occur. Such a piggyback mechanism is one explanation for the complex regulation of p53 by its RING-domain E3 ubiquitin ligase, MDM2, a protein demonstrated to shuttle to and from the nucleus (42, 43). Additional studies aimed at elucidating the location in the cell at which hRFP1/Nedd4 substrate binding and catalysis occur are needed to further our understanding of the localization constraints that affect in vivo enzyme/substrate regulation.

Cumulatively, this work establishes a novel role for Nedd4 within the nucleus, expanding our understanding of this E3 ubiquitin ligase as a regulator of both cytoplasmic and nuclear targets. Specifically, we have identified and characterized a function. Nedd4 was first described as a transcript that is dramatically down-regulated upon the maturation of neural precursor cells (11). Conversely, PRTB is a transcript that is highly expressed in the developing mouse inner ear and is present in high amounts in the adult brain (22). These reports of temporal and spatial expression of the Nedd4 and hPRTB transcripts are provocative given the results of this study. Given its colocalization with splicing factor-rich nuclear speckles, could hPRTB function as a developmental specific splicing factor? Initial attempts to demonstrate colocalization of core Sm proteins with hPRTB immunoprecipitates or alteration of a splice site choice in vitro by hPRTB (data not shown) were not successful. Although its cellular function remains unknown, it nonetheless remains possible that hPRTB may modulate a splicing or RNA processing event within cells.

Nuclear Import and Export of hRFP1/Nedd4—With our identification of a Rev-like NES within hRFP1/Nedd4, we provide evidence that Nedd4 can indeed access both cytoplasmic and nuclear compartments within a cell. However, it is apparent that hRFP1/Nedd4 protein that enters the nucleus has a very strong constitutive export sequence, resulting in little time spent resident within the nucleus. For example, exogenous hRFP1/Nedd4 targeted to the nucleus by a strong SV40 nuclear localization signal or a mutated nuclear export sequence is not tolerated by the cell, and protein does not accumulate (data not shown). Thus, although a population of hRFP1/Nedd4 is able to enter the nucleus, its presence appears to be transient, with the cell having a strong preference to return it to the cytoplasm. This transient nuclear localization of hRFP1/Nedd4 offers an additional explanation for our inability to isolate and immunoprecipitate what are presumably nuclear hRFP1/Nedd4-hPRTB complexes (data not shown).

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nuclear speckle-associated protein, hPRTB, as a substrate of hRPF1/Nedd4. Importantly, the identification of a nuclear export sequence within hRPF1/Nedd4 offers a mechanism by which a predominantly cytoplasmic enzyme accesses a nuclear substrate. Despite significant research efforts to date, there remains a paucity of knowledge of the in vivo determinants of substrate specificity for the WWhect E3 ubiquitin ligase family. With the identification of a nuclear import/export mechanism by which Nedd4 accesses a nuclear substrate, we propose that subcellular localization is an important component of in vivo WWhect enzyme/substrate recognition and regulation.

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Nuclear Import/Export of hRPF1/Nedd4 Regulates the Ubiquitin-dependent Degradation of Its Nuclear Substrates

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