

## SEL-10 Is an Inhibitor of Notch Signaling That Targets Notch for Ubiquitin-Mediated Protein Degradation

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**Notch receptors and their ligands play important roles in both normal animal development and pathogenesis. We show here that the F-box/WD40 repeat protein SEL-10 negatively regulates Notch receptor activity by targeting the intracellular domain of Notch receptors for ubiquitin-mediated protein degradation. Blocking of endogenous SEL-10 activity was done by expression of a dominant-negative form containing only the WD40 repeats. In the case of Notch1, this block leads to an increase in Notch signaling stimulated by either an activated form of the Notch1 receptor or Jagged1-induced signaling through Notch1. Expression of dominant-negative SEL-10 leads to stabilization of the intracellular domain of Notch1. The Notch4 intracellular domain bound to SEL-10, but its activity was not increased as a result of dominant-negative SEL-10 expression. SEL-10 bound Notch4 via the WD40 repeats and bound preferentially to a phosphorylated form of Notch4 in cells. We mapped the region of Notch4 essential for SEL-10 binding to the C-terminal region downstream of the ankyrin repeats. When this C-terminal fragment of Notch4 was expressed in cells, it was highly labile but could be stabilized by the expression of dominant-negative SEL-10. Ubiquitination of Notch1 and Notch4 intracellular domains in vitro was dependent on SEL-10. Although SEL-10 interacts with the intracellular domains of both Notch1 and Notch4, these proteins respond differently to interference with SEL-10 function. Thus, SEL-10 functions to promote the ubiquitination of Notch proteins; however, the fates of these proteins may differ.**

Notch/LIN-12 receptors regulate cell fate decisions during normal animal development and pathogenesis. For example, in *Caenorhabditis elegans*, LIN-12 ensures that only one of two undifferentiated gonadal cells develops into an anchor cell, while the other cell becomes a ventral uterine precursor cell (8). Notch genes have been linked to several human pathological conditions, including cancer (21), vascular failure (13), and schizophrenia (41).

The Notch/LIN-12 signaling pathway is activated when a ligand-receptor interaction induces a proteolytic cleavage event that releases the intracellular domain of the receptor from the cell membrane (25, 32, 35). Release of the intracellular domain of Notch is dependent on presenilins (5, 35). Mammalian Notch ligands are membrane-tethered proteins referred to as Jagged and Delta-like. The signaling module of a Notch/LIN-12 receptor is the intracellular domain that translocates to the nucleus to modulate gene expression (11, 34). The nuclear activity of Notch/LIN-12 receptors relies on the interaction between the intracellular domain of a Notch/LIN-12 receptor and the transcription factor suppressor of hairless (Su[H]) in *Drosophila*, Lag-1 in *C. elegans* (9, 37, 42), or CBF-1 or RBP-J $\kappa$  in mammals (7, 11). The complex of Su(H) and the Notch/LIN-12 intracellular domain is a transcriptional activator and induces genes with a regulatory sequence recognized by the Su(H) DNA binding domain.

Less is known about the subsequent down-regulation of

Notch signaling. Insight into this aspect of Notch signaling came from identification of the *C. elegans* gene *sel-10*, which was first identified in a genetic screen as a negative regulator of the Notch/LIN-12 signaling pathway (36). SEL-10 is related to the budding yeast protein CDC4 (10). Members of the CDC4 family are characterized by an F-box domain (43) and seven WD40 repeats, both protein-protein interaction motifs. In previous studies, CDC4 family proteins have been shown to mediate target protein ubiquitination and degradation. Specifically, the WD40 repeats bind to the target protein in a phosphorylation-dependent manner, while the F-box domain tethers the protein to the SCF ubiquitin ligase complex via binding to the SKP1 adapter (4, 6, 16, 33). *C. elegans sel-10* was shown to functionally reduce *lin-12* activity, and coimmunoprecipitation studies demonstrated that *C. elegans* SEL-10 protein can associate with LIN-12 or murine Notch4 protein (10). Based on this precedent, we have proposed that SEL-10 is a conserved F-box/WD40 repeat protein that negatively regulates Notch/LIN-12 signaling by targeting the intracellular domain of Notch/LIN-12 receptors for ubiquitin-mediated protein degradation (10).

To elucidate the mechanism by which SEL-10 regulates Notch/LIN-12 signaling, we analyzed the function of a human homologue of *C. elegans sel-10* in mammalian cells. We demonstrate that human SEL-10 (hSEL-10) binds mammalian Notch proteins in a domain-specific manner. We also show that Notch proteins are phosphorylated and that the interaction between SEL-10 and Notch proteins is phosphorylation dependent. Through an in vitro ubiquitination assay, we show that SEL-10 can mediate Notch protein ubiquitination and that Notch proteins are degraded by the 26S proteasome in the cell.

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The proposed role of SEL-10 in Notch ubiquitination and degradation is further supported by data showing that a SEL-10 deletion mutant containing only the WD40 repeats can stabilize Notch proteins by competing with wild-type SEL-10 for binding to Notch. In principle, Notch down-regulation by SEL-10 may be physiologically important for sensitizing cells to incoming signals from Notch ligands; alternatively, SEL-10 may provide a general mechanism for preventing excess Notch signaling.

## MATERIALS AND METHODS

**Cell lines and media.** Bosc23 cells (26) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin. Sf9 insect cells were maintained in Gibco BRL SF900II medium. Hi5 insect cells were maintained in Ex-Cell 400 medium (JRH Biosciences). Bacterial strain DH10Bac was purchased from Gibco BRL.

**Plasmids and vectors.** The following plasmids were constructed by use of pQNCXII (14), a retrovirus vector that drives gene expression under the control of a cytomegalovirus (CMV) promoter. pQNClacZ contains the bacterial *lacZ* gene. pQNCint-3HAHis expresses the entire Int-3 protein (amino acids 1412 to 1964 of the mouse Notch4 protein), whose C terminus is fused to hemagglutinin (HA) and six-His tags. pQNCint-3CHAHis expresses a C-terminal fragment of the mouse Notch4 protein (amino acids 1789 to 1964) with HA and six-His tags at the end. pQNCNotch1CHAHis expresses the rat Notch1 intracellular domain (amino acids 1747 to 2531) with HA and six-His tags at its C terminus.

The following plasmids were constructed by use of pLNCX (24), a retrovirus vector that drives gene expression under the control of a CMV promoter. These plasmids express different regions of the Int-3 protein and have been described previously (40). pLNCint-3HA contains cDNA corresponding to the *Notch4* region expressed in the Int-3 insertion, beginning at amino acid 1411; the Notch4(int-3) protein includes the entire intracellular domain of Notch4 and additional sequences. The entire protein is HA tagged at the C terminus. pLNCint-3ΔNHA expresses an Int-3 protein lacking the region upstream of the CDC10/ankyrin repeats. pLNCint-3ΔCHA expresses an Int-3 protein lacking the region distal to the CDC10/ankyrin repeats. pLNCint-3ΔNΔCHA expresses the CDC10/ankyrin repeat region of Int-3. pLNCint-3ΔCDCHA expresses an Int-3 protein lacking the CDC10/ankyrin repeats. All of the above Int-3 proteins have an in-frame HA tag at the C terminus. pHyTC-Jagged1 is described elsewhere (38) and drives the expression of full-length Jagged1 from the CMV promoter.

The following plasmids were constructed by use of pCS2-MT6 (30), a vector that drives gene expression under the control of a CMV promoter. There are six myc tags upstream of the polyclonal sites. pCS2hSEL-10myc expresses full-length hSEL-10 protein with six myc epitope tags at the N terminus. pCS2hSEL-10WDmyc expresses the WD40 repeat region (amino acids 184 to 540) of hSEL-10 with six myc epitope tags at the N terminus. pCS2hSEL-10Fmyc expresses the F-box region (amino acids 1 to 207) of hSEL-10 with six myc epitope tags at the N terminus.

Plasmid pCS2HA-HsSKP1, which expresses HA-tagged full-length human SKP1, was obtained from Mike Tyers, Mt. Sinai Hospital, Toronto, Canada.

The following plasmids were constructed by use of pFastBac (Gibco BRL), a shuttle vector for making baculoviruses overexpressing proteins in insect cells. pFastBacInt-3HA encodes the entire mouse Notch4(int-3) sequence fused at its C terminus to an HA tag. pFastBacInt-3CHAHis encodes the C-terminal region (amino acids 1789 to 1964) of mouse Notch4(int-3) fused at its C terminus to HA and six-His tags. pFastBacNI1CHAHis encodes the intracellular domain (amino acids 1747 to 2531) of rat Notch1 fused at its C terminus to HA and six-His tags. pFastBachSEL-10myc expresses full-length hSEL-10 with six N-terminal myc epitope tags. pFastBachSEL-10WDmyc expresses the WD40 repeat region (amino acids 184 to 540) of hSEL-10 with six N-terminal myc tags.

**Luciferase reporter assays.** Transient transfections were performed by calcium phosphate precipitation. For assessment of activated Notch signaling, HeLa cells ( $1.2 \times 10^5$ ) plated 1 day earlier in six-well plates were transfected in triplicate with 50 ng of pQNCNI1CHA or 50 ng of pLNCX in combination with 670 ng of luciferase vector (pGA981-6) (15) and 160 ng of pLNClacZ (control for transfection efficiency) and with or without 500 ng of pCS2hSEL-10WDmyc. To determine ligand-induced Notch signaling, coculture assays were performed using HeLa and Bosc23 cells. HeLa cells ( $1.2 \times 10^6$ ) plated 1 day earlier in 10-cm plates were transfected with 7.5 μg of pBOS Notch1 (20), 4 μg of pGA981-6, and 1 μg of pLNClacZ and with or without 1.5 μg of pCS2hSEL-10WDmyc. Bosc23 cells ( $3 \times 10^6$ ) plated 1 day earlier in 10-cm plates were transfected with either

25 μg of pHyTCJagged1 (38) or 25 μg of pLNCX. One day after transfection, the HeLa and Bosc23 cells were cocultured in triplicate (1:1) on six-well plates for 24 h. Luciferase activity was determined 2 days posttransfection using an enhanced luciferase assay kit (BD Pharmingen), and β-galactosidase activity was determined using a Galacto-Light Plus kit (PE Biosystems) and a Berthold dual-injection luminometer.

**Transfection, immunoprecipitation, and Western blot analysis.** For transient transfection, a confluent plate of Bosc23 cells was split 1:3 on the day prior to transfection. For each 60-mm plate of cells, 4 μg of each plasmid DNA was transfected using the calcium phosphate precipitation method. The total amount of DNA was kept constant by supplementation with *lacZ*-containing plasmids.

Two days after transfection, cells were harvested and lysed in TENT buffer (50 mM Tris-Cl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (2 μg of aprotinin/ml, 2 μg of leupeptin/ml, 2 μg of pepstatin/ml, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). Lysates were clarified by centrifugation at  $10,000 \times g$  for 10 min, and protein content was determined using a Bio-Rad protein assay kit. Equal amounts of extracts from samples were precleared with Sepharose CL-4B beads, incubated with antibodies (50 μl of 12CA5 anti-HA supernatant, 200 μl of 9E10 anti-myc supernatant, or 2 μl of anti-FLAG antibody) for 2 h at 4°C, and then incubated with 40 μl of a 50% slurry of protein A-Sepharose for 1 h at 4°C. The protein A-Sepharose beads were washed with TENT buffer three times by vortexing for 5 min each time. The beads were boiled in 30 μl of protein gel loading buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% [wt/vol] glycerol), electrophoresed on an SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane.

A Western blot was blocked overnight at 4°C with TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.2% Tween 20) containing 1% bovine serum albumin (BSA). The blot was then incubated with primary antibody diluted (1:50 for 12CA5; 1:10 for 9E10; 1:2,000 for anti-FLAG) in TBST-BSA for 1 h, washed three times for 5 min each time with TBST, and incubated with secondary antibody (anti-mouse immunoglobulin-horse radish peroxidase, 1:10,000; Amersham) in TBST-BSA for 1 h. After three washes, the signal was visualized by chemiluminescence (Amersham ECL kit).

12CA5 anti-HA antibody was obtained from BabCo., Richmond, Calif. 9E10 anti-myc antibody was prepared from culture supernatants of the 9E10 hybridoma (18). Anti-FLAG antibody was purchased from Sigma.

**Dephosphorylation of proteins with CIP.** Immunoprecipitation was carried out to obtain the protein to be treated with calf intestinal phosphatase (CIP). At the end of the immunoprecipitation, the protein A-Sepharose beads were thoroughly washed with TENT buffer, and the solution was completely removed by aspiration. The beads were then suspended in 30 μl of buffer containing 2 μl of CIP (New England Biolabs) and incubated at 37°C for 2 h. Ten microliters of 4× protein gel loading buffer was added to the reaction mixture. The sample was boiled, loaded onto an SDS-polyacrylamide gel, and subjected to Western blot analysis.

**Pulse-chase labeling assay.** Bosc23 cells were transfected with plasmid DNA as described above. Two days after transfection, the cells were washed and incubated in DMEM lacking methionine (Met) and cysteine (Cys) for 0.5 h to deplete Met and Cys. Cells were then incubated for 0.5 h in labeling DMEM, containing <sup>35</sup>S-labeled Met and Cys at 0.5 mCi/ml. The labeling medium was then replaced with regular DMEM. Cells were harvested every 0.5 h during the chase period for up to 2.5 h. Lactacystin (10 μM) was added to both pulse-labeling and pulse-chase media.

The harvested cells were lysed and immunoprecipitated as described above. The precipitates were separated on an SDS-polyacrylamide gel. The gel was fixed for 30 min in isopropanol-water-acetic acid (25:65:10), stained for 30 min with Amplify (Amersham), vacuum dried, and exposed to X-ray film to visualize and quantitate the signal.

**Generation of baculoviruses and in vitro ubiquitination assays.** Baculoviruses used in the in vitro ubiquitination assays were generated with the Gibco BRL FastBac system by following the manufacturer's protocols. Hi5 insect cell lysates were prepared 48 h postinfection from cells coinfecting with baculoviruses that expressed SEL-10myc or SEL-10WDmyc plus hCUL1, hSKP1, and hHRT1. Cell lysis was achieved by incubating cells in buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 50 mM NaF, 60 mM β-glycerophosphate, 0.3% Triton X-100, 100 μM *N*-acetyl-Leu-Leu-norleucinal (LLnL), and protease inhibitor cocktail (Sigma). Crude Hi5 cell lysates (500 μg) were incubated with 10 μl of anti-myc beads for 2 h at 4°C to allow binding of myc-tagged SEL-10 subunits. Beads were washed three times with lysis buffer and incubated with 500 μg of crude lysates prepared from Hi5 cells infected with baculoviruses that expressed either Notch4(int-3)HA, Notch4(int-3)CHAHis6, or NI1CHAHis6 to allow substrate binding to SCF. Beads were washed three times with lysis buffer, washed two

times with 20 mM HEPES (pH 7.4)–100 mM potassium acetate–1 mM dithiothreitol, and incubated with the following ubiquitination reaction components: 50 ng of His6yUBA1, 500 ng of hCDC34, 1  $\mu$ l of 10 $\times$  ATP-regenerating system, 1  $\mu$ l of 10 $\times$  reaction buffer (6), and 5  $\mu$ g of either ubiquitin or methylubiquitin (Boston Biochem). Ubiquitination reactions were carried out for 60 min at 30°C and terminated by the addition of protein gel loading buffer. The samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and Notch proteins were visualized by Western blotting with anti-HA antibodies.

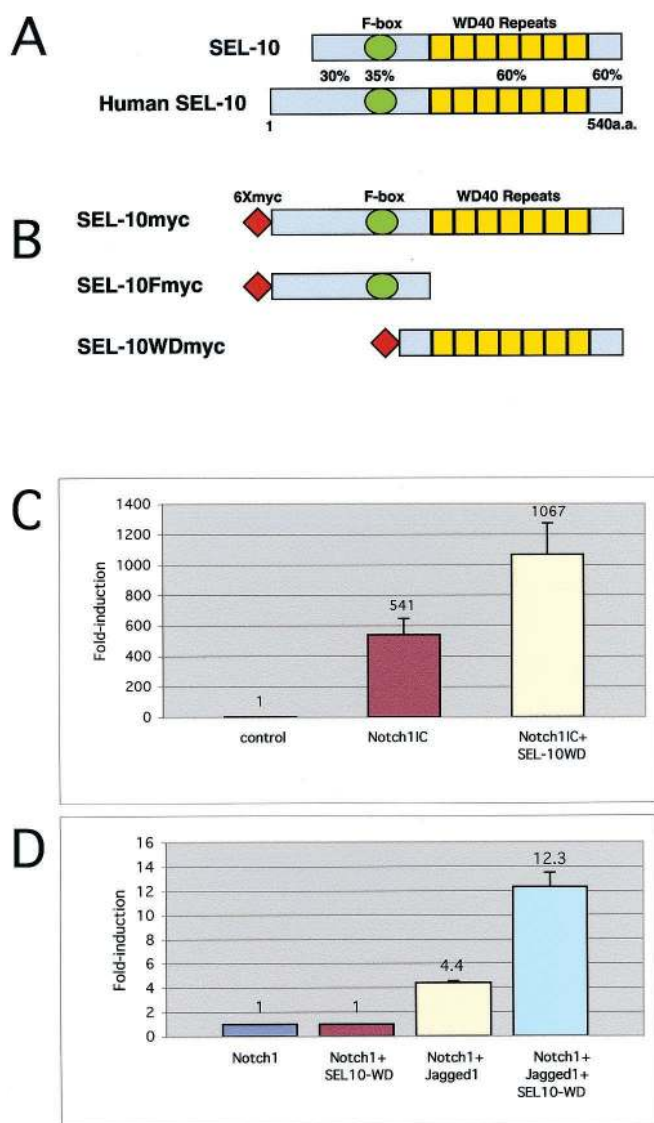
## RESULTS

**hSEL-10 is an F-box/WD40 protein that inhibits Notch signaling.** Figure 1A presents a schematic of the identified domains of the hSEL-10 protein. The full coding sequence for human *SEL-10* translates into a 540-amino-acid protein (GenBank accession no. AY008274). Like its homologue in *C. elegans*, hSEL-10 contains an N-terminal F-box domain followed by seven WD40 repeats. The predicted protein sequences of *C. elegans* Sel-10 and hSEL-10 show 47.6% identity and approximately 57% similarity. Higher conservation is observed in the WD40 repeats (60% identity) than in the N terminus and F-box domains (30 and 35% identities, respectively). The human Sel-10 gene reported here is similar to FLJ117071 of the New Energy and Industrial Development Organization (NEDO) human cDNA sequencing project and is also referred to as FBXW6.

To explore the activity and binding potential of hSEL-10, the coding sequence for six consecutive myc epitope tags was added to the 5' end of *SEL-10* (Fig. 1B). We also generated two variants of SEL-10; SEL-10Fmyc contains the N terminus through the F-box and terminates just prior to the first WD40 repeat, and SEL-10WDmyc contains the WD40 repeats but not the F-box or any sequences upstream of the F-box (Fig. 1B). By analogy to mammalian F-box/WD40 repeat family members, we predict that the F-box mediates association with the ubiquitination machinery and that the WD40 repeats provide a binding domain for substrates.

We were interested in how SEL-10 might interact, both physically and functionally, with a classic Notch intracellular domain protein, Notch1IC, and an oncogenic form of the Notch4 protein. We used a variant of the Notch4 protein, originally referred to as int-3, and we refer to this variant as Notch4(int-3) (39). It contains a short region of the extracellular sequence, the transmembrane domain, and the intracellular domain of mouse Notch4 (see Fig. 3) but does not contain a signal sequence. Notch4(int-3) behaves as a gain-of-function mutant of Notch4 (12, 38, 40) and promotes mammary tumorigenesis in mice.

Engineered versions of F-box/WD40 proteins, such as  $\beta$ -TRCP, which regulates  $\beta$ -catenin stability, that contain only the WD40 repeats function as dominant-negative proteins (17, 19, 46). We asked whether a variant of SEL-10 containing only the WD40 repeats (SEL-10WDmyc; Fig. 1B) could inhibit endogenous SEL-10 function. Using an in vitro culture system, we examined how the overexpression of SEL-10WDmyc influences Notch receptor signaling. Receptor activation was assessed with a luciferase reporter assay that responds to the transcriptional activation of a downstream Notch signaling component, RBP-J $\kappa$ . The expression of an activated Notch1 receptor (Notch1IC) in HeLa cells induced reporter expression greater than 500-fold relative to that in control HeLa cells



**FIG. 1.** hSEL-10 and its effects on Notch signaling. (A) Comparison of *C. elegans* SEL-10 and hSEL-10 proteins. Percentages indicate amino acid identities between homologous domains of the two proteins. (B) Epitope-tagged hSEL-10 proteins used in biochemical studies. Six myc epitope tags (6Xmyc) were engineered to fuse in frame to three SEL-10 variants at their N termini. (C and D) A SEL-10 variant expressing only the WD40 repeats upregulates signaling of activated Notch1 (C) and Jagged1-induced signaling of full-length Notch1 (D). Notch signaling was assessed by a luciferase assay using a reporter construct containing the RBP-J $\kappa$  binding sites. In panel C, an activated Notch1 construct (Notch1IC) and the reporter construct were transfected with or without SEL-10WDmyc into HeLa cells. Reporter gene transactivation was measured 2 days posttransfection, and the fold induction of luciferase activity was calculated relative to that in HeLa cells that were transfected with an empty vector (pLNCX) and the reporter construct. In panel D, Jagged1 or pLNCX (control) was transiently expressed in Bosc23 cells. These cells were cocultured with HeLa cells transiently expressing the full-length Notch 1 receptor, luciferase reporter, and SEL-10WDmyc. Luciferase activity was measured 1 day after coculturing. Each bar represents the mean from experiments done in triplicate. Error bars represent standard deviations.



transfected with the reporter and an empty vector (Fig. 1C). Coexpression of the SEL-10WDMyc construct with activated Notch1 increased reporter expression by approximately two-fold (Fig. 1C), indicative of enhanced Notch signaling. Notch4(int-3) expression induced reporter expression greater than 400-fold; however, coexpression of the SEL-10WDMyc construct with Notch4(int-3) only minimally increased reporter activity (data not shown). Thus, Notch1- and Notch4-induced signaling responded differently to interference with endogenous SEL-10 function. We also overexpressed full-length SEL-10 with Notch proteins and did not find a significant effect on signaling via either Notch1IC or Notch4(int-3) (data not shown), suggesting that sufficient SEL-10 activity is present within cells to mediate its activity.

We determined whether the SEL-10WDMyc construct could augment ligand-induced activation of Notch1 signaling. For these experiments, we used a coculture reporter assay with one cell type expressing the Notch ligand, Jagged1, and another cell type expressing full-length Notch1 and a reporter. Bosc23 cells (a derivative of human HEK 293 cells) expressing either Jagged1 or an empty vector were cocultured with HeLa cells expressing full-length Notch1, the reporter, and SEL-10WDMyc (Fig. 1D). We observed approximately a fourfold induction in reporter activation in cocultures of cells expressing Jagged1 and Notch1 relative to that in cocultures in which Notch1 was expressed without exogenous Jagged1. In cocultures of cells expressing Jagged1 with cells expressing both Notch1 and SEL-10WDMyc, a further 2.5-fold increase in reporter induction was observed. These results demonstrate that with activated Notch1 signaling and with Jagged1-induced Notch1 signaling, expression of the SEL-10WDMyc protein enhanced Notch signaling. Thus, the truncated SEL-10 protein acts in a dominant-negative manner, potentially by competing with endogenous SEL-10 for binding to Notch. We postulate that the augmented Notch1 signaling that we observed is due to a blockade of SEL-10-mediated degradation of the Notch1 protein.

**SEL-10 binds Notch through the WD40 domain and SKP1 through the F-box domain.** To investigate whether hSEL-10 is involved in Notch protein ubiquitination and degradation, we first studied the physical interaction between hSEL-10 and mouse Notch4 proteins using coimmunoprecipitation assays. Previous reports suggested that detecting F-box/WD40-target protein interactions is difficult due to the extreme lability of the target protein after ubiquitination. In initial studies we found that hSEL-10 binding to the oncogenic Notch4(int-3) variant could be detected, and we thus focused our analysis on defining this interaction in detail. Binding assays were conducted after coexpression of the myc-tagged variants of hSEL-10 and HA-tagged Notch4(int-3) (40). Bosc23 cells were used for transient transfections with various expression constructs. Cell extracts were prepared and immunoprecipitated with either anti-HA or anti-myc antibodies. Western blotting of the immunoprecipitates demonstrated that Notch4(int-3)HA (Fig. 2A, bottom panel) and all three SEL-10 variants (Fig. 2A, second panel from the top) were recovered at comparable levels. By probing the anti-myc immunoprecipitates with anti-HA antibody, we demonstrated that Notch4(int-3)HA associated with either full-length hSEL-10 (Fig. 2A, top panel, lane 6) or the WD40 domain (Fig. 2A, top panel, lane 8) but not with the F-box

domain (Fig. 2A, top panel, lane 7). We confirmed the interaction between Notch4(int-3) and SEL-10 proteins by immunoprecipitating Notch4(int-3)HA with anti-HA antibody and then probing for myc-tagged SEL-10 proteins. As shown in Fig. 2A (third panel from the top), full-length hSEL-10 (lane 6) but not the F-box domain (lane 7) complexes with Notch4(int-3)HA; SEL-10WDMyc (lane 8) comigrated with the heavy chain of immunoglobulin and therefore could not be visualized in this experiment. In conclusion, hSEL-10 physically associates with mouse Notch4(int-3) through the WD40 domain, whereas the F-box domain is not required for this interaction.

Based on previous studies of F-box/WD40 proteins, we predict that the F-box domain of SEL-10 should interact with other components of the ubiquitination machinery, such as SKP1. We tested this possibility with coimmunoprecipitation assays using HA-tagged full-length human SKP1 and myc-tagged SEL-10 proteins. As shown in Fig. 2B (top panel, lanes 6, 7, and 8), SKP1HA interacted with full-length hSEL-10 and the F-box domain but not with the WD40 domain. Consistent with this observation, we were able to detect full-length SEL-10 and the F-box domain in the immunoprecipitates of SKP1 (Fig. 2B, third panel from the top, lanes 6 and 7). This observed interaction implies that SEL-10, like other F-box/WD40 family proteins, is part of an E3 ubiquitin ligase that mediates the ubiquitination and degradation of target proteins.

**The Notch4 C-terminal domain binds to hSEL-10.** To map the domains of Notch4(int-3) that are required for the physical interaction between Notch4 and SEL-10, we tested a series of Notch4(int-3) deletion variants (schematized in Fig. 3A) for their ability to complex with full-length hSEL-10. We chose the Notch4(int-3) proteins to map domains of interaction because of the ease of detection of Notch-SEL-10 complexes.

Coexpression of myc-tagged SEL-10 and HA-tagged Notch4(int-3) variants was monitored by immunoprecipitation of SEL-10 with anti-myc antibody; the immunoprecipitates were then probed with anti-HA antibody. This coimmunoprecipitation assay revealed that N4(int-3), N4(int3) $\Delta$ N, and N4(int-3) $\Delta$ CDC proteins interacted with SEL-10 (Fig. 3B, top panel, lanes 8, 9, and 12). When the coimmunoprecipitation was conducted in a complementary fashion by immunoprecipitating with anti-HA antibody for Notch4 proteins and then probing with anti-myc antibody for SEL-10, we detected SEL-10 in the immune complexes of N4(int-3) and N4(int-3) $\Delta$ N (Fig. 3B, third panel from the top, lanes 8 and 9). All three of the Notch4(int-3) variants that interact with SEL-10 contain the C-terminal domain, and removal of this domain abolishes the interaction between Notch4(int-3) and SEL-10. These results suggest that the C-terminal domain of Notch4(int-3), distal to the CDC10/ankyrin repeats, binds SEL-10.

We found that the C-terminal domain of Notch4(int-3) alone was sufficient to bind SEL-10 in coimmunoprecipitation assays using full-length hSEL-10 and a Notch4(int-3) fragment containing only the C-terminal domain, N4(int-3)C. N4(int-3)C could be detected after expression in Bosc23 cells (Fig. 3C, middle panel, lanes 2, 4, 6, and 8). However, when coimmunoprecipitations were carried out under standard conditions, as described above, we failed to detect a complex between Notch4(int-3)C and hSEL-10. To prevent ubiquitin-mediated turnover, we treated cells with a specific proteasome inhibitor,

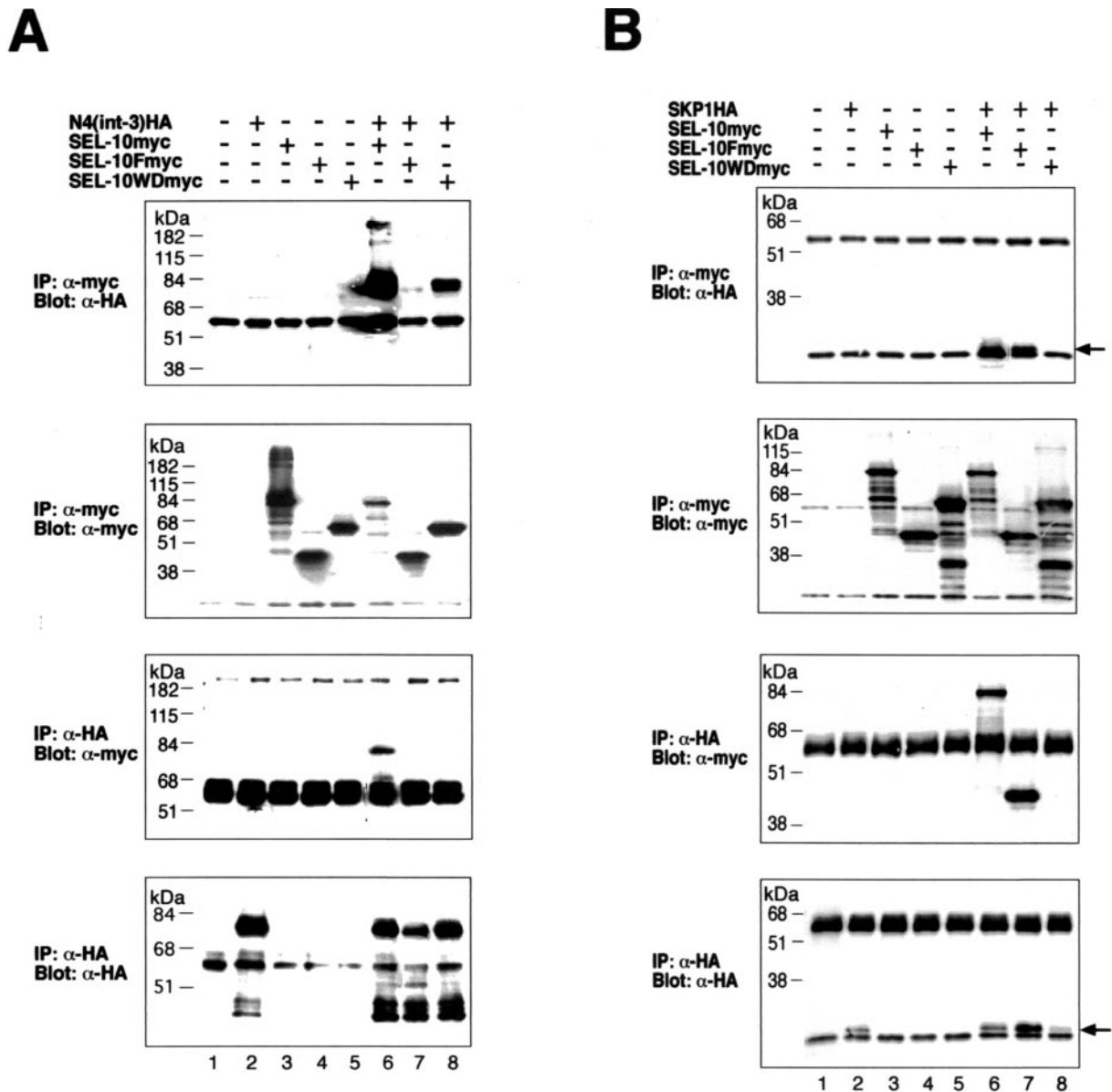
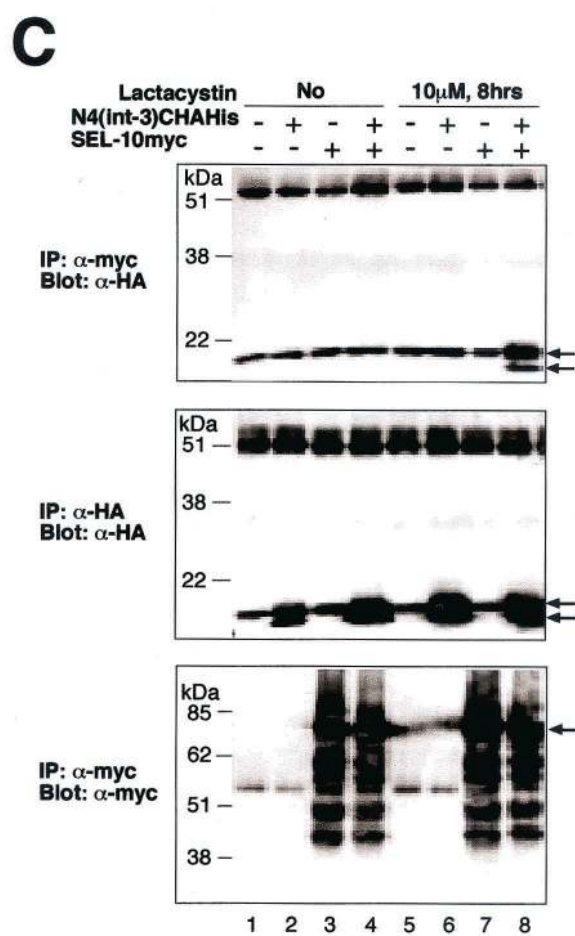
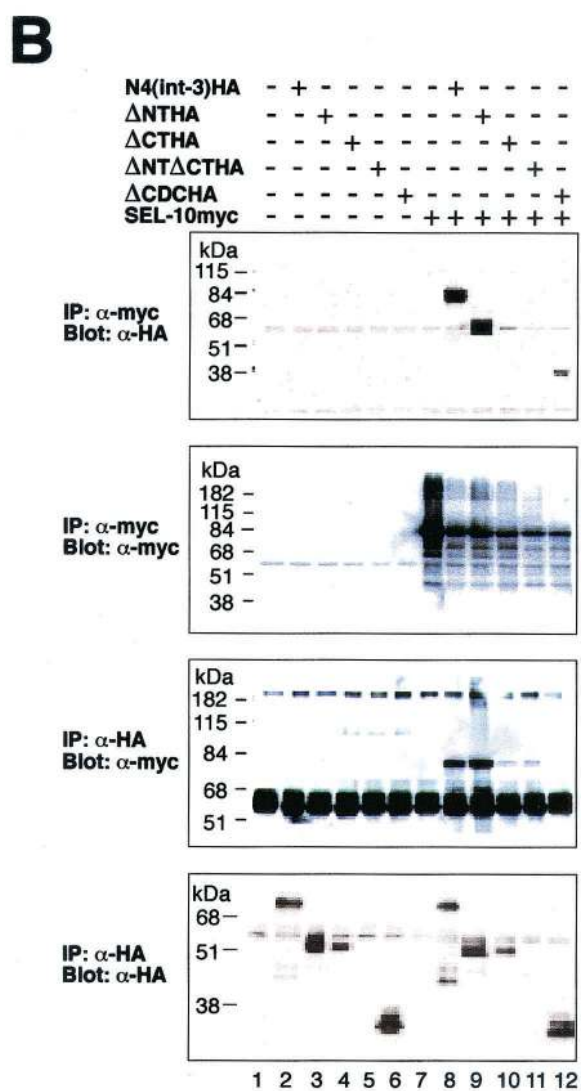
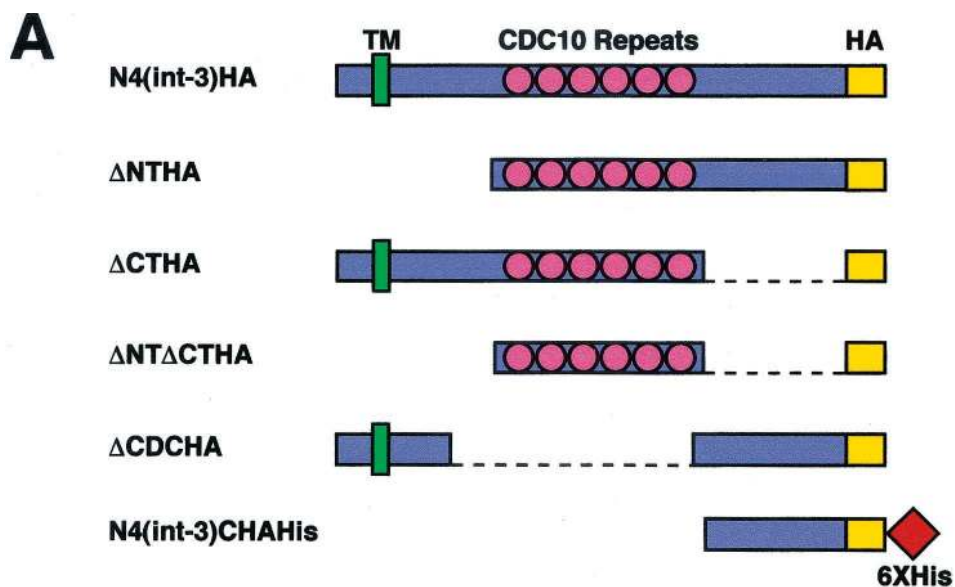


FIG. 2. hSEL-10 interacts with Notch through the WD40 repeats and SKP1 through the F-box. (A) Physical interactions between hSEL-10 and mouse Notch4. HA-tagged Notch4(int-3) protein was coexpressed with three six-myc-epitope-tagged SEL-10 variants in Bosc23 cells by transient transfection. Immunoprecipitation (IP) and Western blotting were performed using either anti-HA or anti-myc antibody to demonstrate that the proteins are well expressed and properly immunoprecipitated (second panel from the top and bottom panel). Anti-HA antibody was then used to probe the anti-myc antibody immunoprecipitates to reveal Notch4(int-3)HA protein in immune complexes of either SEL-10myc or SEL-10WDMyc (top panel). Similarly, anti-myc antibody was used to probe anti-HA antibody immunoprecipitates to reveal SEL-10 proteins associated with Notch4(int-3)HA (third panel from the top). (B) Physical interactions between hSEL-10 and human SKP1. HA-tagged SKP1 was coexpressed with three SEL-10 variants in Bosc23 cells. Immunoprecipitation followed by Western blotting using anti-HA or anti-myc antibody shows that the proteins are well expressed and precipitated efficiently (second panel from the top and bottom panel). Anti-myc antibody immunoprecipitates were probed with anti-HA antibody (top panel) to reveal SKP1HA associated with SEL-10 proteins, and anti-myc antibody was used to detect SEL-10 proteins in immunoprecipitates of SKP1HA (third panel from the top). Arrows indicate the signal of SKP1HA, which comigrates with the light chain of the antibody.

lactacystin, before harvest. After lactacystin treatment, we detected N4(int-3)C proteins in the immunoprecipitates of hSEL-10 (Fig. 3C, top panel, lane 8). Thus, under normal conditions, the interaction between N4(int-3)C and hSEL-10 is probably transient and difficult to detect due to proteasome-dependent turnover of SEL-10-bound Notch4(int-3)C.

**SEL-10 binds to phosphorylated forms of Notch4(int-3) proteins.** Upon Western blot analysis of Notch4(int-3) variants, we noted that some of the Notch4(int-3) proteins appeared as multiple bands (Fig. 3B, bottom panel, lanes 9 and 12), which might represent Notch protein modification by phosphorylation. To address this question, we determined if the pattern of



migration could be altered by phosphatase treatment of immunoprecipitated Notch4(int-3) proteins. We focused on the N4(int-3) $\Delta$ N variant, which contains the CDC10 repeats and the C-terminal region (Fig. 3A). SEL-10myc and N4(int-3) $\Delta$ NHA were coexpressed in Bosc23 cells. When immune complexes containing N4(int-3) $\Delta$ NHA were probed with anti-HA antibody, three bands, ranging from 48 to 51 kDa, were detected (Fig. 4, lane 1). After treatment with CIP, the top two bands were significantly diminished, indicating that they had become dephosphorylated (Fig. 4, lane 2).

We next determined which forms of N4(int-3) $\Delta$ N were associated with SEL-10. The same cell lysate was immunoprecipitated with anti-myc antibody to isolate SEL-10myc and then probed with anti-HA antibody. The slowest migrating form of N4(int-3) $\Delta$ NHA protein was the predominant form associated with SEL-10 (Fig. 4, lane 3). This immune complex was treated with CIP, and the slower migrating forms were diminished at the expense of the faster migrating forms (Fig. 4, lane 4). This observation suggests that hSEL-10 preferentially binds to phosphorylated forms of Notch4(int-3) and is consistent with the behavior of CDC4-like proteins, which bind to phosphorylated target proteins. The bulk population of N4(int-3) $\Delta$ N molecules was efficiently dephosphorylated by CIP, whereas those bound to SEL-10 were not (Fig. 4, compare lanes 2 and 4). We speculate that the direct interaction of SEL-10 with phosphate residues on N4(int-3) $\Delta$ N may have shielded N4(int-3) $\Delta$ N from access to CIP. We also noted that Notch4 variants that contain the C terminus [N4(int-3) $\Delta$ N, N4(int-3) $\Delta$ CDC, and N4(int-3)C] typically migrated as several species. These other Notch4(int-3) variants were also tested in this assay, and the slower migrating forms of these proteins were also diminished after phosphatase treatment (data not shown). On the basis of these results and the results shown in Fig. 3B and C, we conclude that the C terminus of Notch4(int-3) is a site of phosphorylation and is the domain required for the Notch4–SEL-10 interaction.

**Proteasome inhibitors and a dominant-negative form of hSEL-10 stabilize Notch proteins.** To study whether Notch proteins are degraded by the ubiquitin/proteasome pathway, cells expressing Notch proteins were treated with proteasome inhibitors. After treatment, Western blot analysis was used to measure the changes in the steady-state levels of these proteins. A protein containing the C-terminal tail of Notch4(int-3) was expressed poorly in transfected Bosc23 cells (Fig. 5A, zero-hour time point), but the levels of this protein were increased after lactacystin treatment (Fig. 5A), indicating turnover by the proteasome. Notch4(int-3) proteins were expressed well in transfected Bosc23 cells (Fig. 2A, bottom panel), and

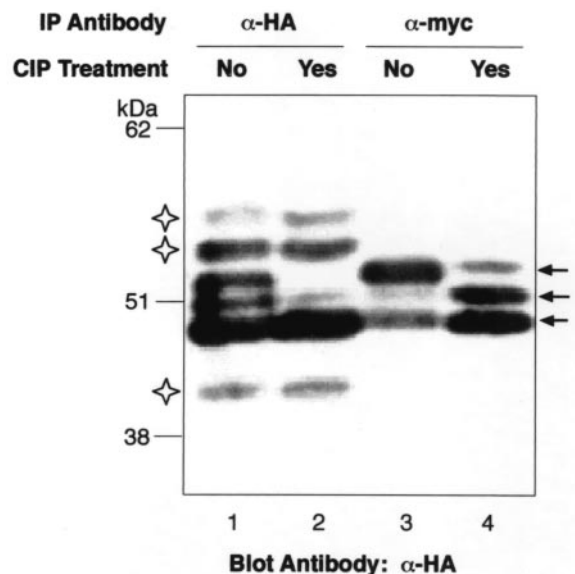


FIG. 4. SEL-10 binds to phosphorylated Notch. Notch4(int-3) $\Delta$ NTHA and SEL-10myc were coexpressed in Bosc23 cells by transient transfection. Immunoprecipitation (IP) was performed with either anti-HA or anti-myc antibody. Each precipitate was divided into two tubes; one was treated with CIP, and one was left untreated. The blot was probed with anti-HA antibody to visualize Notch4(int-3) $\Delta$ NTHA precipitated either directly by anti-HA antibody or indirectly by anti-myc antibody. Arrows indicate the three species of Notch4(int-3) $\Delta$ NTHA. Stars indicate the heavy and light chains of immunoglobulin.

the levels of these proteins were not significantly increased after lactacystin treatment (data not shown). Thus, the Notch4(int-3) protein was not efficiently processed by the proteasome, whereas a fragment containing the C-terminal tail of Notch4(int-3) was. A protein containing the entire intracellular domain of murine Notch1, N1CHAHis, was stabilized by treatment with another specific proteasome inhibitor, MG132 (Fig. 5B). Steady-state levels of several intracellular Notch proteins increased upon treatment with proteasome inhibitors, indicating that they are targeted for degradation via the proteasome pathway.

To determine whether the increased steady-state levels of Notch proteins were due to increased stability, we carried out pulse-chase analysis to assess the half-life of the N4(int-3)C protein, the C terminus of N4(int-3). Transfected cells were pulse-labeled, cell extracts were prepared and immunoprecipitated, and the intensities of immunoprecipitated protein bands were quantified to compare the relative Notch protein levels.

FIG. 3. The C-terminal domain of Notch4 mediates the interaction of Notch4 and SEL-10. (A) Epitope-tagged Notch4(int-3) deletion variants. The HA tag is labeled in yellow, and six-His tags are labeled in red. (B) Physical interactions between SEL-10 and Notch4 deletion variants. Six-myc-epitope-tagged full-length hSEL-10 was coexpressed with HA-tagged Notch4(int-3) deletion variants in Bosc23 cells. All the proteins were well expressed (second panel from the top and bottom panel), except for  $\Delta$ NT $\Delta$ CTHA (bottom panel, lanes 5 and 11). Anti-HA antibody was used to detect Notch4 proteins in the immunoprecipitates of SEL-10myc (top panel), and anti-myc antibody was used to detect SEL-10myc in the immunoprecipitates of Notch4 proteins (third panel from the top). (C) A C-terminal fragment of Notch4 complexes with SEL-10 in the presence of a proteasome inhibitor. Notch4(int-3)CHAHis, a fragment of Notch4 containing only the C-terminal domain, was tested for its ability to coimmunoprecipitate with SEL-10. Before the coimmunoprecipitation assays, cells were either treated with lactacystin, a specific proteasome inhibitor, or left untreated. The bottom two panels show that both Notch4(int-3)C and SEL-10 are well expressed. Notch4(int-3)C can be detected by anti-HA antibody in the immune complex of SEL-10myc (top panel). Arrows in the top two panels indicate the signal of Notch4(int-3)CHAHis, and the arrow in the bottom panel indicates the major species of SEL-10myc protein. IP, immunoprecipitation.



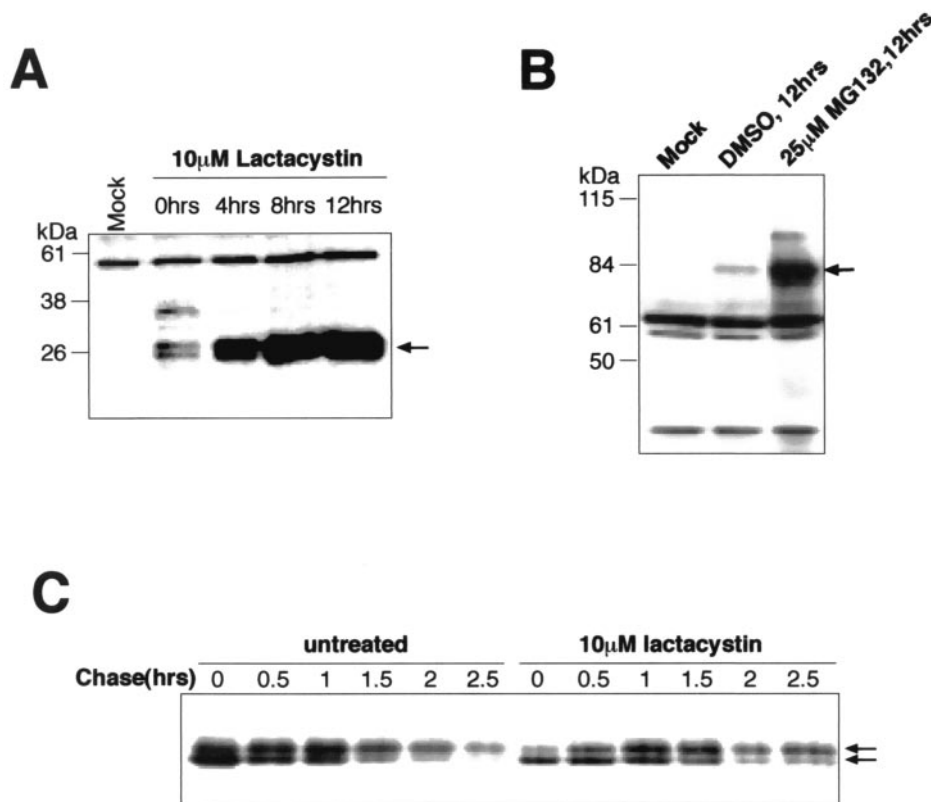


FIG. 5. Notch proteins are stabilized by proteasome inhibitor treatment. (A) Two micrograms of a plasmid expressing Notch4(int-3)CHAHis was transiently transfected into Bosc23 cells on a 60-mm plate. The cells were treated with 10  $\mu$ M lactacystin for the indicated number of hours before harvest. Western blotting using anti-HA antibody was carried out to assess the steady-state levels of expression of Notch4(int-3)CHAHis (arrow). (B) A Notch1 fragment containing the intracellular domain tagged with HA and hexahistidine was expressed in Bosc23 cells by transient transfection. Cells were treated with a proteasome inhibitor, MG132, for 12 h before harvest. Dimethyl sulfoxide (DMSO) was used as a negative control. The arrow indicates the Notch1 protein revealed by Western blotting using anti-HA antibody. (C) Proteasome inhibitor treatment leads to a longer half-life for the Notch protein. Bosc23 cells were transfected to express Notch4(int-3)CHAHis. Two days after transfection, cells were pulse-labeled with  $^{35}$ S-labeled methionine and cysteine for 30 min and chased with regular DMEM for up to 2.5 h. Samples were harvested every 0.5 h and then immunoprecipitated using anti-HA antibody. The immunoprecipitates were separated by SDS-PAGE and autoradiographed to reveal the amount of labeled Notch4(int-3)CHAHis. For cells treated with 10  $\mu$ M lactacystin, a proteasome inhibitor was added to both pulse-labeling and pulse-chase media. Arrows indicate the two bands representing Notch4(int-3)CHAHis.

Figure 5C shows that in the absence of lactacystin, more than half of the N4(int-3)C protein was turned over after approximately 1.5 h of chase. In contrast, after treatment with 10  $\mu$ M lactacystin, the amount of Notch4(int-3)C was diminished only slightly throughout the 2.5-h chase. Based on these results, the increase in Notch4(int-3)C levels after lactacystin treatment (Fig. 5A) was likely due to decreased degradation by the proteasome pathway.

To determine whether endogenous SEL-10 was required to target Notch proteins for turnover, we used the dominant-negative form of SEL-10, which interferes with endogenous SEL-10 function (SEL-10WDmyc; Fig. 1B). We tested the ability of SEL-10WDmyc, which expresses only the WD40 repeats and not the F-box, to interfere with endogenous SEL-10 function by coexpressing SEL-10WDmyc with either Notch4(int-3)C or N1ICHA in Bosc23 cells. Two days after transfection, cells were harvested and the steady-state levels of Notch proteins were examined by Western blot analysis. The expression of SEL-10WDmyc resulted in increased steady-state levels of N4(int-3)C (Fig. 6A) and of Notch1IC (Fig. 6B). The increased levels of these proteins were apparent after

equivalent levels of the plasmids were used in transfection, and the levels increased in a dosage-dependent manner as more SEL-10WDmyc plasmid was used. The increased expression of N4(int-3)C upon coexpression of SEL-10WDmyc was reflected in increased stability of the protein after pulse-chase analysis and quantitation (Fig. 6C). In contrast, the expression of full-length SEL-10 did not have a significant effect on steady-state levels of Notch1IC (data not shown). Thus, the WD40 repeat region of SEL-10 functions as a dominant-negative form of SEL-10, and the expression of this form results in decreased turnover of Notch proteins. In contrast, expression of the SEL-10WDmyc plasmid did not significantly alter the levels of oncogenic Notch4(int-3) protein (data not shown). This result is consistent with the lack of an appreciable increase in signaling mediated by Notch4(int-3) when coexpressed with SEL-10WDmyc (data not shown).

**SEL-10 mediates Notch protein ubiquitination in vitro.** To examine whether SEL-10 functions as part of an SCF ubiquitin ligase that can target Notch proteins for ubiquitin-dependent degradation, we first tested whether SEL-10myc and SEL-10WDmyc assembled into SCF complexes. Both proteins were



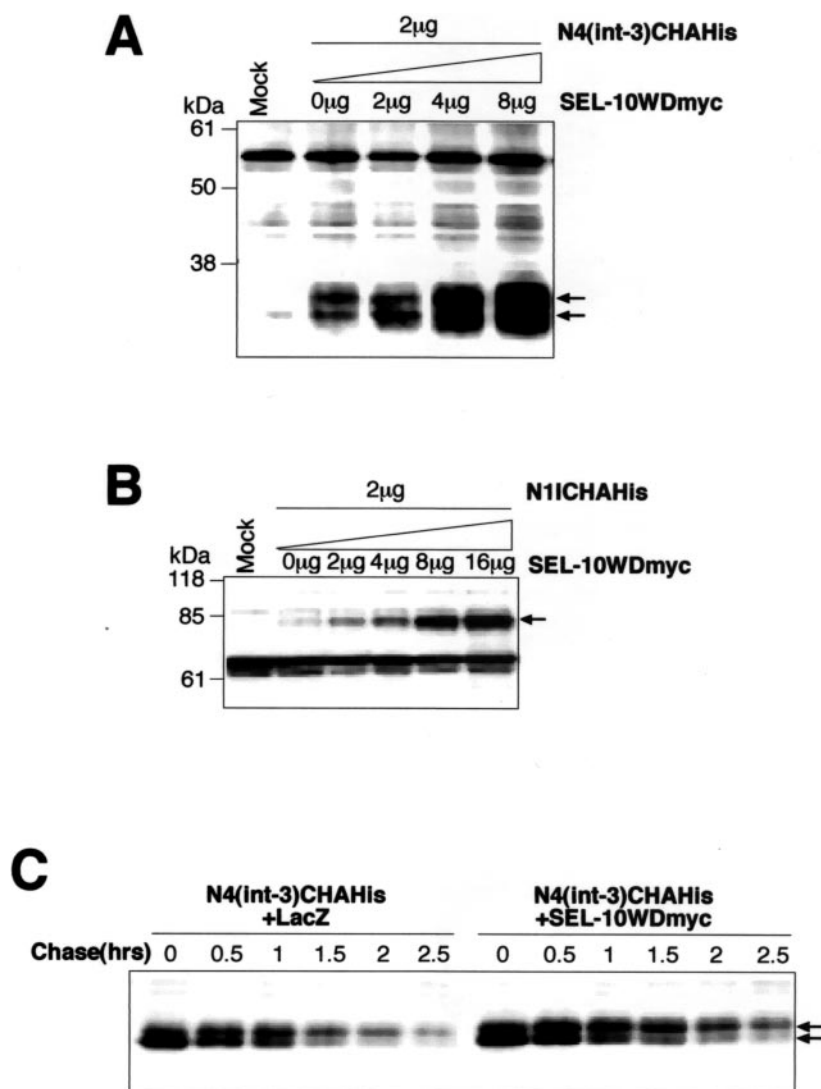


FIG. 6. Expression of the WD40 repeats of SEL-10 stabilizes Notch proteins. (A) Plasmids expressing Notch4(int-3)CHAHis and SEL-10WDmyc were cotransfected into Bosc23 cells. The amounts of plasmids used are indicated at the top of the panel. Two days after transfection, cells were harvested and subjected to Western blotting. The steady-state levels of the HA-tagged Notch protein are indicated by the arrows. (B) SEL-10WDmyc was coexpressed with N1ICHAHis, a Notch1 fragment containing the intracellular domain. Two days later, the steady-state levels of expression of N1ICHAHis (arrow) were assessed by Western blotting. (C) Overexpression of the WD40 repeats of SEL-10 results in an increased half-life for Notch4(int-3)CHAHis. A pulse-chase labeling experiment was done in the absence or presence of SEL-10WDmyc to determine the half-life of Notch4(int-3)CHAHis. Metabolically labeled Notch4(int-3)CHAHis (arrows) was visualized by immunoprecipitation followed by autoradiography.

coexpressed in insect cells with hCUL1, hSKP1, and hHRT1, all components of the SCF complex. SEL-10 protein complexes were retrieved by immunoprecipitation with anti-myc antibody beads. Full-length SEL-10 efficiently coprecipitated hCUL1, hSKP1, and hHRT1, but the WD40 repeat domain by itself was unable to recruit the other SCF subunits (Fig. 7A); these results provide an explanation for the observed dominant-negative effect of SEL-10WDmyc in transfected cells (Fig. 1C and E and Fig. 6).

We next tested whether Notch proteins were ubiquitinated by recombinant SCF<sup>SEL-10</sup>. As shown in Fig. 7B, high-molecular-weight (HMW) forms of coprecipitated Notch4(int-3), Notch4(int-3)CHAHis, and N1ICHAHis proteins were gener-

ated in the presence of SEL-10myc immunoprecipitates that contained all subunits of SCF but not in the presence of SEL-10WDmyc immunoprecipitates. Formation of the HMW forms of Notch4 proteins was dependent on the presence of ubiquitin, confirming that Notch4 was ubiquitinated in the presence of SCF<sup>SEL-10</sup>. We next examined in more detail N1ICHAHis ubiquitination in the presence or absence of ubiquitin and its chain-terminating derivative, methyl-ubiquitin. In the presence of ubiquitin and SCF<sup>SEL-10</sup>, SCF-bound N1ICHAHis was completely converted into HMW conjugates. These conjugates migrated as an apparent band instead of as a more characteristic smear, because they were compressed at the interface of the stacking and running gels. Substituting ubiquitin with methyl-



ubiquitin dramatically reduced the apparent size of ubiquitinated N1ICHAHis, whereas the omission of ubiquitin from the reaction completely abolished the formation of HMW conjugates. These results confirm that the HMW forms observed were ubiquitinated forms of N1ICHAHis. As expected, immunoprecipitates that contained full-length SEL-10 but not those that contained SEL-10WD retained N1ICHAHis ubiquitination *in vitro*.

## DISCUSSION

Although the mechanisms of Notch/LIN-12 signal transmission are being defined through genetic and biochemical analyses, little is known about the mechanisms involved in down-regulating the Notch signal. Notch signaling mediates numerous key developmental decisions in both vertebrates and invertebrates. As such, mechanisms to down-regulate Notch signaling are likely critical to maintain proper developmental programs or to prevent oncogenic functions of Notch proteins. *sel-10* was originally identified genetically in *C. elegans* as a negative regulator of *lin-12* activity (10). The fact that the SEL-10 protein is related to the F-box/WD40 repeat family of proteins suggested that SEL-10 down-regulates Notch/LIN-12 signaling by targeting these proteins for ubiquitin-mediated protein turnover (10). This proposed function of SEL-10 would represent a key mechanism by which Notch signaling is reduced in physiological settings. Based on the paradigm established by analysis of budding yeast Cdc4, F-box/WD40 proteins are predicted to bind their target proteins in a phosphorylation-dependent fashion.

Here, we demonstrate that Notch1 signaling is negatively regulated by SEL-10. Interference with SEL-10 function by expression of the WD repeat region enhances steady-state levels of Notch1 proteins by reducing the rate of turnover, thus increasing Notch1-mediated signaling, indicating that SEL-10 is directly involved in mediating Notch ubiquitination and degradation. We also found the Notch4 proteins interact efficiently with SEL-10 but that the levels and activity of the intracellular domain of Notch4, Notch4(int-3), are relatively resilient to interference with SEL-10 function. Thus, these two Notch proteins behave differently in response to blocking of SEL-10 function.

The ease with which we could detect Notch4(int-3)-SEL-10 protein complexes prompted us to choose Notch4(int-3) proteins as a focus for detailed binding studies. This proved an effective way of dissecting the biochemical interactions in greater detail than could be achieved with substrates that would be tremendously labile when complexed to SEL-10. We demonstrated that the WD40 repeats of SEL-10 bind to the C-terminal domain of Notch4, a domain important for Notch4 phosphorylation. Moreover, SEL-10 binds preferentially to phosphorylated forms of Notch4 and shields the phosphate groups from nonspecific dephosphorylation by CIP, suggesting that the interaction is directly mediated by phosphorylated amino acids within the C-terminal domain of Notch4. We also found that several forms of Notch proteins, Notch1ICD and Notch4(int-3)C, are very unstable as a result of rapid turnover via the proteasome pathway. However, full-length SEL-10 expression did not have an appreciable effect on Notch protein levels or activities (data not shown). This result may indicate

that sufficient SEL-10 activity exists in these cells to mediate Notch protein turnover.

Finally, recombinant SEL-10 assembles into SCF ubiquitin ligase complexes in insect cells. These complexes bind coexpressed Notch and mediate highly processive (and efficient) ubiquitination of bound Notch proteins. Given that other SCF complexes (including SCF<sup>Cdc4</sup>, SCF<sup>Grr1</sup>, SCF<sup>Skp2</sup>, and SCF<sup>β-TRCP</sup>) have been shown to be extremely selective for phosphorylated substrates, we presume that recombinant Notch proteins are targeted to SCF<sup>SEL-10</sup> by an endogenous protein kinase in insect cells. Although the exact mechanism by which Notch proteins are targeted for ubiquitination remains unclear, it is evident from the *in vitro* experiments that Notch proteins can serve as excellent substrates for SCF<sup>SEL-10</sup>. Given the extraordinary substrate specificity that is evinced by all other SCF ubiquitin ligases that have been evaluated to date, the efficient and highly processive *in vitro* ubiquitination that we observed (Fig. 6C) indicates that Notch is a physiological substrate for SCF<sup>SEL-10</sup>. Based on all of the data, the most parsimonious hypothesis is that the phosphorylation of Notch by an unidentified protein kinase targets it to SCF<sup>SEL-10</sup>, which in turn extensively ubiquitinates Notch as a prelude to its degradation. Conclusive proof of this hypothesis *in vivo* will ultimately require the mapping of phosphorylation sites and the construction of nonphosphorylatable point mutant versions of Notch.

We conclude that the C-terminal domain of Notch4 distal to the CDC10/ankyrin repeats is a negative regulatory domain because it is responsible for interactions with SEL-10. This notion is consistent with the fact that the C-terminal domain contains a PEST sequence, which is characteristic of many short-lived proteins and which is thought to be a target for phosphorylation and ubiquitination (28). It has also been observed that a C-terminal deletion can activate GLP-1, a *C. elegans* Notch protein (22). The C-terminal domain of Notch proteins is also where some other regulatory proteins bind. For example, *Drosophila* protein Dishevelled has been reported to bind to this region and may thus mediate the interaction between the Wingless and Notch signaling pathways (2). Our results predict that Notch levels and activity may be controlled by a kinase(s) that phosphorylates the C terminus of Notch proteins. This phosphorylation would mediate SEL-10 binding and thus ubiquitination and degradation by the 26S proteasome. Little is known about kinases that phosphorylate and regulate Notch, but one would predict that the kinase that phosphorylates the C terminus has a negative regulatory function in Notch signaling.

An interesting observation is that Notch4(int-3) proteins show strong and specific interactions with SEL-10 but do not seem to be readily degraded by the proteasome pathway, in contrast to Notch1IC. Overexpression of the WD40 repeat region or treatment by lactacystin failed to increase the steady-state levels of Notch4(int-3) (data not shown). Pulse-chase analysis indicated that Notch4(int-3) has a much longer half-life in cells than does Notch4(int-3)C, the C-terminal domain of Notch4(int-3) (data not shown). However, Notch4(int-3) still seems to be ubiquitinated in cells because a Western blot of Notch4(int-3) often displays a very high-molecular-weight smear in addition to the main Notch4(int-3) signal at the predicted molecular weight (unpublished observations). This smear can be seen even without proteasome inhibitor treat-



ment and is very typical of proteins that are ubiquitinated. Notch4(int-3) also serves as a substrate for SEL-10-dependent *in vitro* ubiquitination (Fig. 7A). The fact that Notch4(int-3) protein levels are relatively unaffected by interference with SEL-10 activity is consistent with the fact that in signaling assays, Notch4(int-3) activity was not elevated by coexpression with a dominant-negative form of SEL-10 (data not shown). One possible explanation for these observations is that the extracellular sequence, the transmembrane domain, or the ankyrin repeats in Notch4(int-3) can function to prevent the protein from being degraded by the proteasome even after ubiquitination. The resultant increased stability of Notch4(int-3) may also contribute to the potent oncogenic activity of this variant of Notch4, whose gene was originally defined as a mammary oncogene (12, 29). In contrast, our studies show that Notch4(int-3)C and Notch1IC, both lacking a transmembrane domain and extracellular sequence, can be readily stabilized by proteasome inhibitors or overexpression of the WD40 repeat region of SEL-10. This issue can be further addressed by biochemical studies using a Notch4(int-3) fragment containing only the intracellular domain or possibly chimeric proteins of Notch4(int-3) and Notch1IC.

Other reports also suggest that Notch proteins are likely turned over by ubiquitination. For example, it has been reported that the steady-state level of the Notch1 intracellular domain can be elevated by lactacystin, a proteasome inhibitor (32). In addition, *Notchless*, a novel *Drosophila* gene identified as a modulator of *Notch* activity, encodes a WD40 repeat-containing protein that binds to the intracellular domain of Notch (31). However, the function of *Notchless* is not clear because both loss-of-function mutations and overexpression of the gene lead to increased Notch activity. These results, once again, suggest that regulation of the Notch pathway is very complex. A recent report suggests that Notch proteins are targets for ubiquitination and provides biochemical evidence that the Itch protein may participate in mediating Notch ubiquitination (27). However, this study did not establish that Itch is responsible for or participates in the ubiquitination of Notch *in vivo* or that Notch ubiquitination, stability, or activity is altered in mice with the *Itch* mutation.

Ubiquitin-mediated protein degradation is a highly regulated and selective process used to down-regulate several signaling pathways (1, 3). F-box/WD40 family proteins can bind to multiple target proteins (23, 44, 46). We report that Notch signaling also utilizes ubiquitin-mediated protein turnover to down-regulate the Notch/LIN-12 signal. This is evident both in *C. elegans* (10) and in Notch signaling in mammalian cells (Fig. 1C and D). Thus, clear evidence from sequence homology, functional studies, and binding studies points to the high level of conserved function of SEL-10 as a negative regulator of Notch signaling from worms to humans. SEL-10 also interacts genetically and physically with the *C. elegans* presenilin, SEL-12 (45), and may target both Notch and presenilin for degradation. The data presented here suggest that hSEL-10 may serve a similar role in the down-regulation of presenilin function as in the down-regulation of Notch. Presenilin is required for the activation of Notch, probably by mediating the proteolytic cleavage of the transmembrane domain of Notch, enabling the nuclear access of the Notch intracellular domain (5, 35, 47). It is not clear whether SEL-10 has effects on the

presenilin-Notch interaction or whether it targets each protein separately. It will be interesting to define how SEL-10 is directed to distinct targets, such as Notch and presenilins. Understanding how Notch proteins are regulated by SEL-10 may also provide new approaches to controlling Notch activity. For example, as constitutive activation of Notch can lead to tumorigenesis, SEL-10 activity could be used to reverse Notch activity in these circumstances.

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