

# Ubiquitination and Degradation of Tal1/SCL Are Induced by Notch Signaling and Depend on Skp2 and CHIP\*

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Notch signaling controls diverse eukaryotic differentiation processes in multiple cell types, thus demanding versatile tools with which Notch triggers downstream events. Ubiquitin-mediated proteolysis has previously been shown to be one such tool with which Notch regulates the turnover of the basic helix-loop-helix transcription factor, E47. Here, we show that Notch signaling also accelerated the degradation of Tal1/SCL (T cell acute leukemia 1/stem cell leukemia) protein, a basic helix-loop-helix protein involved in the development of hematopoietic, vascular, and neuronal tissues. Notch-induced Tal1/SCL degradation was mediated by ubiquitination and proteasomes. The sequence responsible for Tal1 degradation was localized to a region in the C terminus of Tal1, which is evolutionarily conserved, thus suggesting a functional significance. Analogous to the situation for E47, Notch-induced Tal1/SCL degradation not only required Skp2, a substrate-binding subunit of SCF ubiquitin ligase complexes, but also relied on CHIP, a chaperone-binding protein with a ubiquitin ligase activity. In contrast to the fact that the N-terminal tetratricopeptide region (TPR) domain of CHIP is necessary and sufficient for E47 ubiquitination and degradation, CHIP promoted Tal1 degradation with both chaperone binding and ubiquitin ligase activities, which are mediated by its TPR domain and U box, respectively. Although the TPR domain was not involved in Tal1/SCL binding, it was required for enhancing its degradation. Likewise, the ubiquitin ligase activity of CHIP was dispensable for Tal1/SCL binding but essential for degradation. These findings provide both novel mechanistic insights into the operation of cullin-based ubiquitin ligase complexes and potential means by which Notch and Tal1/SCL regulate eukaryotic development.

Signaling through Notch receptors (Notch1–4) plays a pleiotropic role in mammalian development in diverse settings ranging from the neuronal to hematopoietic systems, as well as from stem cell maintenance to terminal differentiation of specific cell types (1–3). Notch signaling is mediated by interaction with their ligands, Delta-like and Jagged, expressed on the surface of adjacent cells, which results in the cleavage of the Notch intra-

cellular domain and its translocation into the nucleus. The intracellular domain of Notch receptors then acts as a transcription coactivator by associating with the DNA-binding subunit, RBP-J $\kappa$  (also referred to as CSL), and stimulates transcription of genes, many of which are still unknown (4–7). Notch signaling is often involved in binary cell fate decisions. Given the variety of cell types and developmental stages where Notch exerts its effect, the collection of genes regulated by these receptors would have to be enormous. Alternatively, Notch signaling could directly or indirectly utilize additional cellular mechanisms to control these developmental processes.

Ubiquitin-mediated proteolysis is a powerful means by which disparate cellular processes are regulated (8, 9). Ubiquitination reactions are catalyzed by a series of enzymes with ubiquitin-activating, -conjugating, and -ligating (E3)<sup>2</sup> activities. Although the variety of ubiquitin-activating and ubiquitin-conjugating enzymes is limited, numerous E3 ligases are present to ensure substrate specificity. We have previously shown (10) that Notch signaling accelerates ubiquitination and degradation of basic helix-loop-helix transcription factors such as E47, encoded by the E2A gene. E47 is ubiquitinated by the SCF<sup>Skp2</sup> ubiquitin E3 ligase complexes in a MAPK-dependent manner. SCF<sup>Skp2</sup> is a multisubunit complex consisting of cullin1, Skp1, ROC, and the substrate-binding F box protein Skp2 (11). Among the well known substrates of SCF<sup>Skp2</sup> are p27, a cyclin-dependent kinase inhibitor, and c-Myc (12–14). In addition, the chaperone-binding protein CHIP (15) has also been found to facilitate E47 ubiquitination, probably by promoting substrate association with the E3 ligase (16). Because down-regulation of RBP-J $\kappa$  by shRNA diminishes the effect of Notch on E47 degradation, it has been postulated that Notch signaling results in transcriptional activation of genes encoding proteins with positive effects on SCF<sup>Skp2</sup> E3 ligase activities (10). Given the diverse functions of Notch in cellular differentiation, it is very unlikely that Notch-stimulated protein ubiquitination and degradation are only reserved for E2A transcription factors. Indeed, we show here that Notch signaling also promotes the degradation of Tal1/SCL protein (called Tal1 hereafter for simplicity) through similar but not identical mechanisms.

Tal1 was originally discovered through analyses of chromosomal translocations in samples from patients with T cell acute lymphoblastic leukemia (17–19). Ectopic expression of Tal1 in T cells leads to leukemogenesis. The physiological function of Tal1 has been delineated by examinations of either germ line or

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<sup>2</sup> The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; TPR, tetratricopeptide region; shRNA, short hairpin RNA; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; N1-IC, Notch1 intracellular domain.

conditional knock-out mice. Germ line disruption of the *Tal1* gene results in embryonic lethality because of failures in production of blood and blood vessels (20). Deletion of the gene specifically in hematopoietic lineages reveals that Tal1 is necessary for short term hematopoietic stem cell function but dispensable for long term stem cell activity (21, 22). This protein is also thought to play an important role in erythroid differentiation, and its overexpression directs multipotent progenitors to develop along the myeloid lineage (23, 24). Interestingly, Notch signaling is also thought to be involved in hematopoietic stem cell maintenance (25). In addition to the hematopoietic system, Tal1 is also expressed in the central nervous system (26–28), where Notch clearly plays a role in its development (29, 30). Taken together, it is possible that post-translational modification of Tal1 induced by Notch signaling is of physiological significance.

Tal1 is a basic helix-loop-helix protein but does not form homodimers (18, 31). It interacts with E47 avidly and binds DNA as heterodimers. We initially intended to address the question whether Notch signaling induces the degradation of E47 heterodimers such as the E47-Tal1 complex. To our surprise, the level of Tal1 protein diminished more dramatically than that of E47 upon Notch activation, which suggested that Notch signaling had an independent effect on Tal1 itself. Further investigation revealed that activation of Notch enhances the ubiquitination and subsequent degradation of Tal1. As in the case of E47, this process involved the SCF<sup>Skp2</sup> E3 ligase complex and depended on CHIP. However, the mechanistic details as to how these proteins acted to ubiquitinate Tal1 differed from those utilized in E47 ubiquitination. Therefore, our findings provide new insights into the molecular mechanisms of action utilized by SCF<sup>Skp2</sup> complexes.

## EXPERIMENTAL PROCEDURES

**Cell Culture, Transient Transfection, and Retroviral Infection**—NIH3T3 and 293T cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Jagged1-expressing and control NIH3T3 cells were gifts from Dr. T. Kadesch (University of Pennsylvania). OP42 stromal cells were kindly provided by Dr. P. Kincade. Jurkat-E (expressing ectopic retroviral receptors) cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50  $\mu$ M  $\beta$ -mercaptoethanol. Cells were cotransfected with desired constructs along with a GFP-expressing plasmid (Invitrogen) by using a calcium phosphate precipitation method. To transduce Jurkat-E cells with retroviruses, cells were spin-infected as described previously (10). Infected Jurkat-E cells were then sorted based on GFP expression.

**Plasmids**—The N-terminal Myc-tagged Tal1 construct was created by inserting a HindIII-XbaI fragment into the pRC/CMV vector as described for the HA-tagged Tal1 (32). This construct was used as the parental vector for all mutants, which were generated by PCR with appropriate primers amplifying the region included in the AscI-XbaI fragment at the 3' end of the cDNA. N1-IC cloned in pcDNA3 and retroviral vectors, as well as the HA-ubiquitin construct, was described previously (10). The HPC4-tagged CHIP and its mutant constructs were

detailed by Huang *et al.* (16). Generation and validation of shRNA constructs were described previously (10, 16).

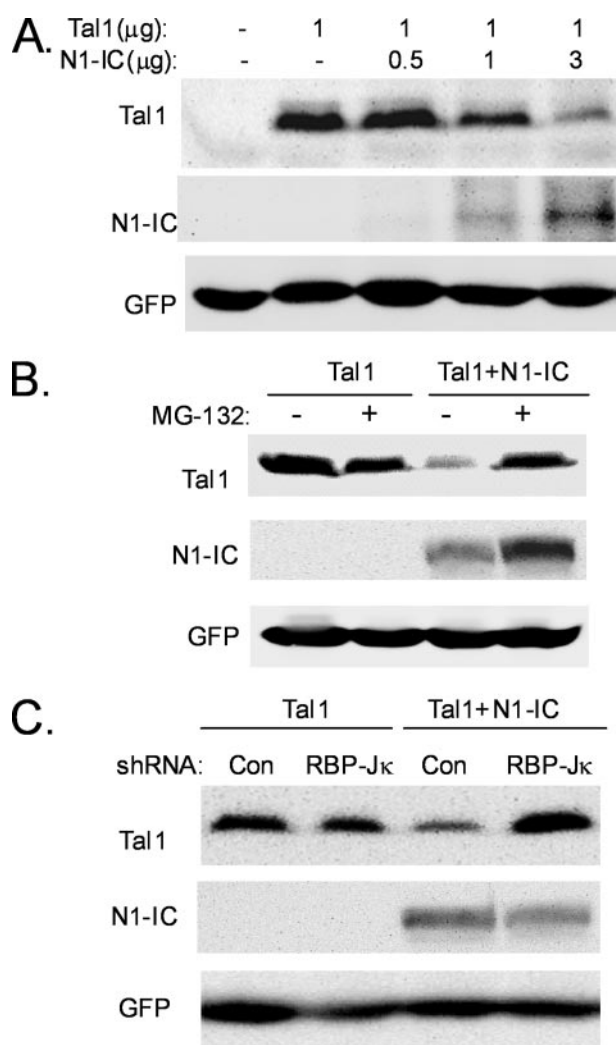
**Immunoblotting and Immunoprecipitation**—For immunoblot analyses, cells were collected 36 h post-transfection and lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline) plus a mixture of protease inhibitors. Samples were subjected to SDS-PAGE. For immunoprecipitation assays, transfected cells or transduced Jurkat cells were lysed in a lysis buffer (150 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 20 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and a mixture of protease inhibitors). The resulting supernatants were pre-cleared with protein A-agarose beads and incubated with 5  $\mu$ g of antibodies against Skp2 or 2  $\mu$ l of anti-Tal1 serum for 4 h. The precipitates were collected with protein A-agarose beads and washed three times. Bound proteins were released from beads by boiling in SDS loading buffer. Antibodies used for immunoblotting analyses for GFP, tubulin, and Skp2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tal1 serum was generated by our laboratory by immunizing rabbits with recombinant Tal1 (33). Monoclonal antibodies against Notch1 and Myc and HA tags were from culture supernatants of hybridoma cell lines, bTAN20, 9E10, and 12CA5.

**In Vivo Ubiquitination Assay**—293T cells were transfected with appropriate constructs. Twenty-four hours later, cells were treated with 10  $\mu$ M MG-132 for 4 h. Retrovirus-transduced Jurkat-E cells were treated similarly. Anti-Tal1 serum was then used for immunoprecipitation, and the precipitates were resolved on 8% SDS-polyacrylamide gel and probed for polyubiquitinated Tal1 with the anti-HA monoclonal antibody. The same membrane was re-probed with the anti-Myc antibody for Tal1.

## RESULTS

**Notch Signaling Induces Ubiquitin-mediated and Proteasome-dependent Degradation of Tal1**—To examine the effect of activated Notch1 receptor on Tal1 degradation, we cotransfected into NIH3T3 cells a construct expressing human Tal1 cDNA with increasing amounts of plasmids producing the N1-IC. A constant amount of GFP-expressing construct was also cotransfected to serve as controls for transfection efficiency in each sample. Immunoblotting assays of whole cell extracts revealed a gradual reduction of Tal1 level as the amount of N1-IC increased (Fig. 1A). However, the level of GFP remained unchanged. This reduction was reversed by addition of the MG-132 proteasome inhibitor to transfected cells, suggesting that Tal1 proteins were degraded by the proteasome in the presence of Notch signaling (Fig. 1B). Furthermore, expression of shRNA against RBP-J $\kappa$  restored the level of Tal1 in cells cotransfected with Tal1 and N1-IC to that in cells expressing Tal1 alone (Fig. 1C). In contrast, a control shRNA construct containing a random sequence had no such effect. Taken together, these results suggest that signaling through Notch pathways induces ubiquitin-mediated and proteasome-dependent degradation of Tal1, likely by transcriptional activation of genes involved in these processes.

## Notch Induces Tal1/SCL Degradation



**FIGURE 1. Activation of Notch signaling accelerates Tal1 degradation through ubiquitin-mediated and proteasome-dependent pathway.** A, dose-dependent induction of Tal1 degradation by N1-IC. NIH3T3 cells were transfected with 1  $\mu$ g of Myc-tagged Tal1 and indicated amounts of N1-IC-expressing plasmids along with 0.5  $\mu$ g of a GFP-expressing vector, pEGFP-N1. Tal1 protein levels in whole cell lysates were determined by immunoblotting with the anti-Myc antibody. N1-IC was detected with a monoclonal antibody. Levels of GFP were used as controls of transfection efficiency. B, proteasome-dependent degradation of Tal1. NIH3T3 cells transfected with 1  $\mu$ g of Tal1 and 3  $\mu$ g of N1-IC-expressing plasmids were treated with 12  $\mu$ M MG-132 for 2 h before harvest. Whole cell lysates were probed for the indicated proteins. C, down-regulation of RBP-J $\kappa$  diminishes Notch-induced Tal1 degradation. One microgram of Tal1  $\pm$  3  $\mu$ g of N1-IC constructs were cotransfected with 4  $\mu$ g of RBP-J $\kappa$  or control (Con) shRNA constructs into NIH3T3 cells. Tal1 and GFP proteins were immunoblotted.

Next, we tested the effect of activation of endogenous Notch receptors on levels of Tal1 proteins present in Jurkat-E T cells. When Jurkat-E cells were cocultured with stromal cells expressing a Notch ligand, Jagged1, endogenous Tal1 levels dramatically decreased compared with coculturing on control stromal cells (Fig. 2A). Prevention of Notch activation with a  $\gamma$ -secretase inhibitor significantly increased Tal1 levels in cells treated with Jagged1 but had no effect on control cells. Likewise, expression of N1-IC in Jurkat-E cells caused a marked reduction in the level of endogenous Tal1, which was rescued by the proteasome inhibitor, MG-132 (Fig. 2B). We also performed *in vivo* ubiquitination assay by immunoprecipitating

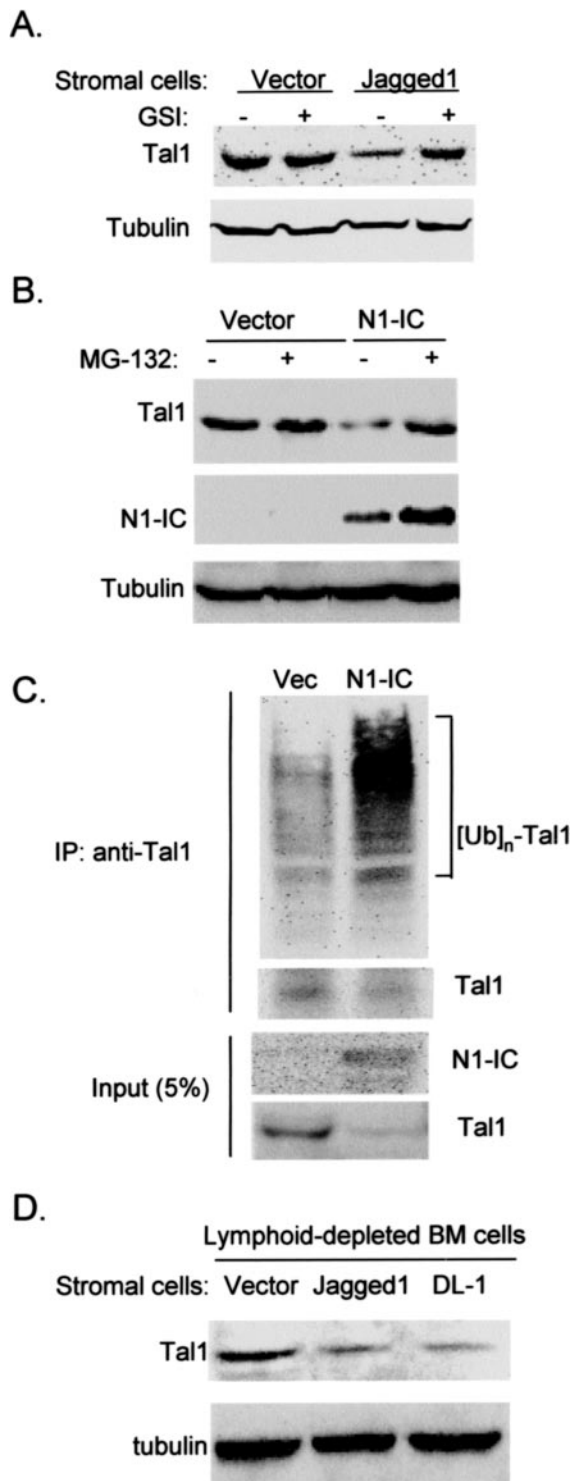
endogenous Tal1 from vector- and N1-IC-transduced Jurkat-E cells. The precipitates were analyzed by immunoblotting for ubiquitin. We found that the levels of ubiquitinated Tal1 in N1-IC-expressing cells were much higher than those in control cells (Fig. 2C). In addition to Jurkat cells, we examined Tal1 levels in primary bone marrow cells enriched for hematopoietic progenitors and myeloid cells obtained by depletion with antibodies against B220, CD19, CD2, and CD8 expressed on lymphocytes. When these cells were then exposed to stromal cells expressing Notch ligands, Jagged1 or Delta-like-1, Tal1 levels decreased obviously compared with those in cells cultured with vector control stromal cells (Fig. 2D). Collectively, these results emphasize the physiological relevance of Notch-induced Tal1 degradation.

**Determining the Sequence in Tal1 Protein Responsible for Its Degradation**—Alignment of Tal1 sequences from various species revealed that the highest sequence conservation resides within the basic helix-loop-helix motif, which is expected because this motif mediates the most important function of Tal1, namely, DNA binding and dimerization. The second region with extensive evolutionary conservation lies in the C terminus of the protein (Fig. 3A), which we suspected of having an important function. To test if this region mediated Tal1 degradation, we initially deleted the entire region to generate the construct, del1, and found that this mutant was completely resistant to Notch-induced degradation when tested in NIH3T3 cells (Fig. 3B).

Refined analyses of the C terminus showed that truncation of the last 23 amino acids (del2) (Fig. 3B), which are less conserved across species, had no effect on the susceptibility of Tal1 to Notch-induced degradation. In contrast, deletion in any part of the highly conserved region from amino acids 277 to 308 abolished Notch-induced degradation (del3 to del5) (Fig. 3B). Computer-aided analyses identified two potential serine phosphorylation sites within this region (34). These sites can be recognized by MAPKs, cyclin-dependent kinases, and protein kinase B. To evaluate whether these sites are important for Tal1 degradation, alanine substitution mutations were introduced to each of the sites to create constructs S284A and S300A. Expression of N1-IC accelerated the degradation of S284A but not of S300A, suggesting that phosphorylation at Ser-300 may be involved in Tal1 degradation (Fig. 3B). In addition, a tyrosine residue is also present in this highly conserved region and can potentially be phosphorylated by various tyrosine kinases. When this residue was replaced with alanine (Y304A), the mutant was resistant to Notch-induced degradation. However, changing it to phenylalanine (Y304F) had no effect. Because the tyrosine to phenylalanine mutation prevents phosphorylation but does not alter the structure of the protein, this result suggests that the tyrosine residue is important for maintaining the proper conformation of the protein necessary for ubiquitination and degradation. This is consistent with the fact that some non-mammalian species have a phenylalanine residue at this position (Fig. 3A).

**Notch-induced Tal1 Ubiquitination Potentially Involves SCF<sup>Skp2</sup> E3 Ligase Complexes**—Because Notch has been shown to enhance E47 ubiquitination by SCF<sup>Skp2</sup> E3 ligase complexes,





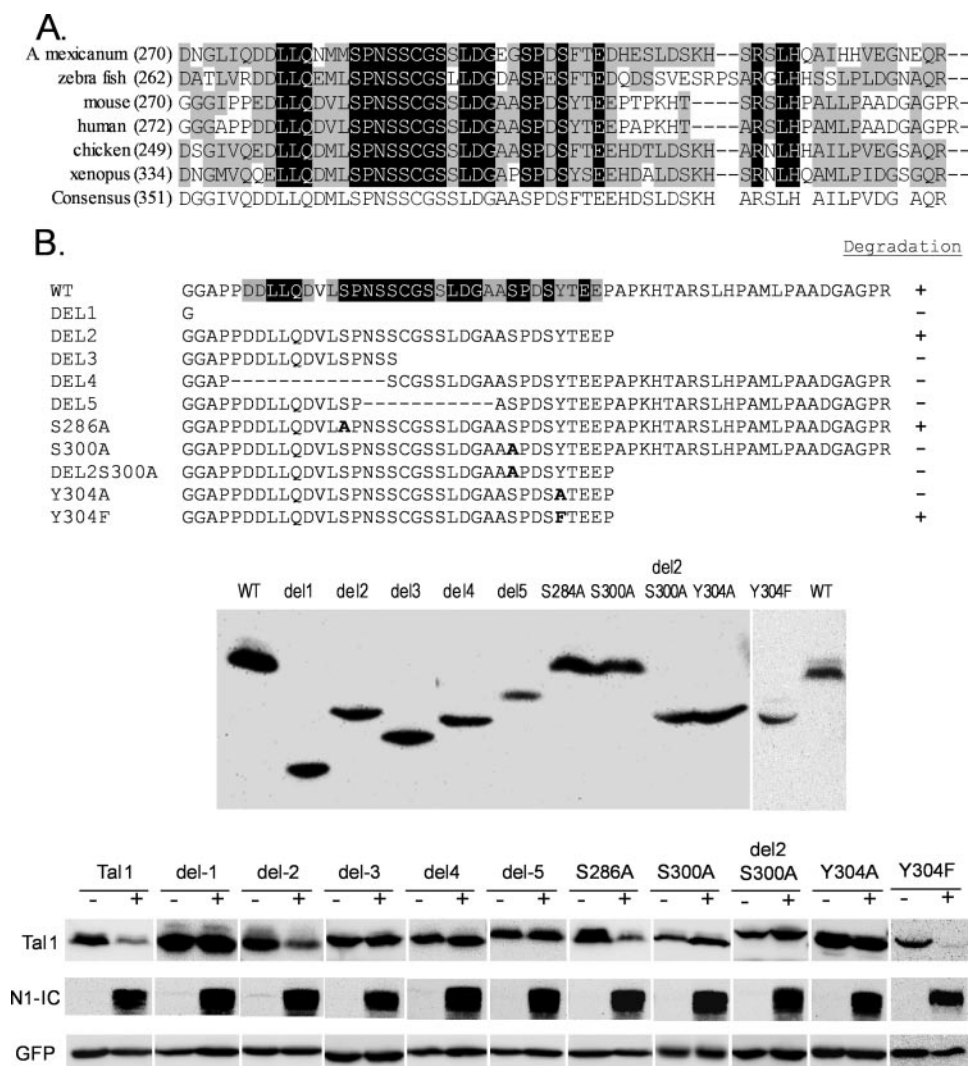
**FIGURE 2. N1-IC enhances endogenous Tal1 degradation.** *A*, Jurkat-E cells were cocultured with vector or Jagged1-expressing OP42 stromal cells for 2 h in the presence or absence of 25  $\mu$ M  $\gamma$ -secretase inhibitor XII (GSI). Immunoblotting was carried out with anti-Tal1 antibodies on whole cell lysates. Levels of tubulin are loading controls. *B*, Jurkat-E cells were infected with N1-IC-expressing or control retroviruses. Infected cells were sorted for GFP fluorescence. Before harvest, cells were treated with or without 10  $\mu$ M MG-132 proteasome inhibitor for 90 min. Immunoblotting was carried out on whole cell lysates with polyclonal antibodies against Tal1. The same membrane was re-probed with antibodies against the indicated proteins. *C*, shown are the results from *in vivo* ubiquitination assay. Immunoprecipitation (IP) was performed with antibodies against Tal1 using whole cell lysates of vector (Vec) or N1-IC-expressing Jurkat-E cells. The precipitates were immunoblotted with

we tested whether Tal1 ubiquitination utilized the same machinery. To determine whether down-regulation of Skp2 expression diminishes the effect of N1-IC, constructs expressing shRNA against Skp2 or control were introduced together with the Tal1-expressing construct into NIH3T3 cells with or without N1-IC. Expression of Skp2 but not control shRNA rescued Tal1 proteins in N1-IC-expressing cells (Fig. 4A). The knockdown effect of Skp2 shRNA was confirmed by cotransfection of the shRNA constructs with a T7-tagged Skp2 construct. Reduction in T7-Skp2 levels by Skp2 shRNA, but not control shRNA, suggested the efficacy of the shRNA construct (Fig. 4A). Furthermore, the Tal1-expressing construct was also cotransfected with the shRNA constructs into NIH3T3 cell lines stably transfected with vector control or Jagged1-expressing constructs. Because Jagged1 is a ligand of Notch receptors, endogenous Notch signaling was achieved by allowing cells to be in close contact. The Skp2 but not control shRNA alleviated the degradation of Tal1 in cells expressing Jagged1 (Fig. 4B). In contrast, it had no effect on control cells. These results suggest that Notch-induced Tal1 degradation involves the function of Skp2, likely through the SCF<sup>Skp2</sup> complex.

Next, we assessed the ability of Skp2 to promote Tal1 degradation by coexpressing different amounts of Skp2 together with a constant amount of Tal1. Tal1 levels decreased proportionally to amounts of Skp2 expressed (Fig. 4C). Consistent with this finding, we also detected interaction between Tal1 and Skp2 in coimmunoprecipitation assays. In this experiment, Tal1 was coexpressed with or without Skp2 and N1-IC in 293T cells. Whole cell extracts were immunoprecipitated with antibodies against Skp2 or with rabbit IgG. The precipitates were analyzed by probing for Tal1 or Skp2 (Fig. 4D). In the absence of exogenous Skp2, a small amount of Tal1 coprecipitated with endogenous Skp2. Overexpression of Skp2 increased the amount of Tal1 brought down, but addition of N1-IC did not further increase the amount of Tal1 coprecipitated. These data suggest that Skp2 is in complex with Tal1 but N1-IC does not play a significant role in enhancing the association between Tal1 and Skp2.

**CHIP Is Involved in Notch-induced Tal1 Degradation**—CHIP has previously been shown to facilitate Notch-induced E47 degradation (16). We therefore explored the possibility that CHIP also promotes Tal1 degradation. Coexpression of increasing amounts of CHIP with Tal1 dramatically reduced the Tal1 level to that of N1-IC-expressing cells (Fig. 5A). Conversely, down-regulation of endogenous CHIP with shRNA rescued Tal1 proteins in N1-IC-expressing cells but had no effect on cells transfected with Tal1 alone (Fig. 5B). The control shRNA did not alter Tal1 levels in the absence or presence of N1-IC. As controls for the efficiency of CHIP shRNA, cotransfection experiments were performed with the CHIP expression construct, and CHIP levels were found to be reduced by its shRNA but not

anti-ubiquitin (Ub) and anti-Tal1 antibodies. Input controls were done by immunoblotting for the indicated proteins. *D*, shown is Tal1 degradation in bone marrow cells. Bone marrow cells were enriched for hematopoietic progenitors and myeloid cells by depletion of lymphoid cells with antibodies against CD19, B220, CD2, and CD8 coupled to magnetic beads. The cells were cocultured with stromal cells expressing the indicated Notch ligands for 2 h. Protein levels were analyzed as described above.



**FIGURE 3. Mapping the sequence in Tal1 responsible for Notch-induced degradation.** A, alignment of C-terminal amino acid sequences of Tal1 from the indicated species. Black indicates identical residues and gray shows residues with similar properties. B, shown is analysis of deletion and point mutations of Tal1. Upper panel, C-terminal sequences of wild-type (WT) and mutant constructs shown with their susceptibility to degradation listed on the right. Middle panel, expression of wild-type and mutant Tal1 proteins in NIH3T3 cells analyzed by probing for the Myc tag attached at the N terminus of each construct. Lower panel, susceptibility of each construct to Notch-induced degradation. NIH3T3 cells were transiently transfected with 1  $\mu$ g of Myc-tagged Tal1 or its mutants with or without 3  $\mu$ g of N1-IC expression constructs, along with the GFP control. Tal1 and control protein levels in whole cell lysates were immunoblotted as described in the legend to Fig. 1.

controls. Together, these data established the involvement of CHIP in Notch-induced Tal1 degradation.

To explain how CHIP facilitates Tal1 degradation, we examined the interaction between CHIP and Tal1 in coimmunoprecipitation assays by cotransfecting CHIP with wild-type and mutant Tal1 constructs into 293T cells. Immunoprecipitation was carried out with anti-Tal1 antibodies, and the precipitates were analyzed by immunoblotting for CHIP (Fig. 5C). Wild-type Tal1 bound avidly to CHIP, but the del4 and del5 mutants, which are resistant to Notch-induced degradation, exhibited much lower affinity for CHIP. Interestingly, the S300A mutant, although insensitive to Notch-mediated turnover, was capable of binding to CHIP, suggesting that interaction with CHIP is independent of phosphorylation, which is analogous to the situation found in E47 and CHIP association.

We next evaluated the role of CHIP in Tal1 ubiquitination. Cotransfection of Tal1 with CHIP did not significantly enhance Tal1 ubiquitination (Fig. 5D). However, expression of CHIP dramatically potentiated Notch-induced ubiquitination of Tal1 (Fig. 5D). These results suggest that CHIP, although an E3 ligase itself, facilitates Tal1 ubiquitination mediated by other E3 ligases such as SCF<sup>Skp2</sup>.

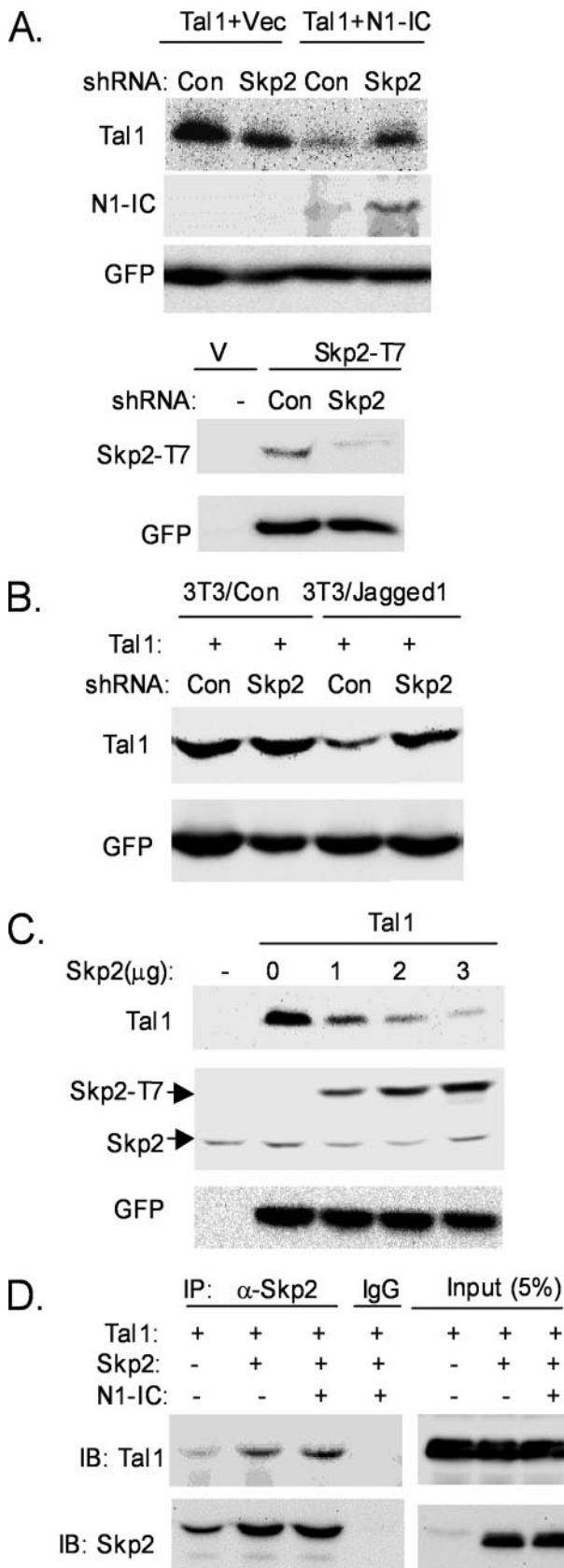
CHIP is a 33-kDa protein consisting of an N-terminal chaperone-binding TPR and C-terminal U box with a ubiquitin ligase activity (35–37). To understand the mechanisms by which CHIP stimulates Tal1 degradation, a series of CHIP mutants were examined for their abilities to promote Tal1 degradation. Constructs containing either the TPR or U box portions of CHIP were incapable of causing Tal1 degradation (Fig. 6A). Furthermore, point mutations K31A and H261A/P270A, which abolish the ability of CHIP to interact with chaperones (36) or the ubiquitin ligase activity (38), respectively, also failed to enhance Tal1 degradation (Fig. 6A), thus suggesting that both functions of CHIP are necessary for Tal1 turnover.

To evaluate the ability of wild-type and mutant CHIP proteins to associate with Tal1, coimmunoprecipitation assays were carried out in 293T cells cotransfected with Tal1 and CHIP constructs (Fig. 6B). Whole cell lysates were immunoprecipitated with antibodies against Tal1 or with control IgG. Precipitates were analyzed for CHIP and

Tal1. Tal1 was found to associate with wild-type CHIP, U box, K31A, and H261A/P270A constructs but not the TPR construct, which includes the N-terminal 191 amino acids. Therefore, the Tal1 interacting domain was localized to the C-terminal 113 amino acids. However, mutations abolishing the U box ubiquitin ligase activity did not interfere with Tal1 binding, even though they eliminated the ability of CHIP to accelerate Tal1 degradation. Because CHIP did not stimulate Tal1 ubiquitination by itself (Fig. 5D), its ubiquitin ligase activity may be necessary for other functions.

## DISCUSSION

We report here that activation of Notch signaling pathways accelerates Tal1 degradation through ubiquitin-mediated and proteasome-dependent pathways. This finding



**FIGURE 4. Involvement of SCF<sup>Skp2</sup> in Notch-induced Tal1 degradation.** A, down-regulation of Skp2 rescues Tal1 degradation induced by N1-IC over-expression. NIH3T3 cells were cotransfected with 1 μg of Tal1 ± 3 μg of N1-IC constructs together with 4 μg of Skp2 or control (Con) shRNA constructs. Whole cell lysates were analyzed 36 h later. To control for the efficiency of

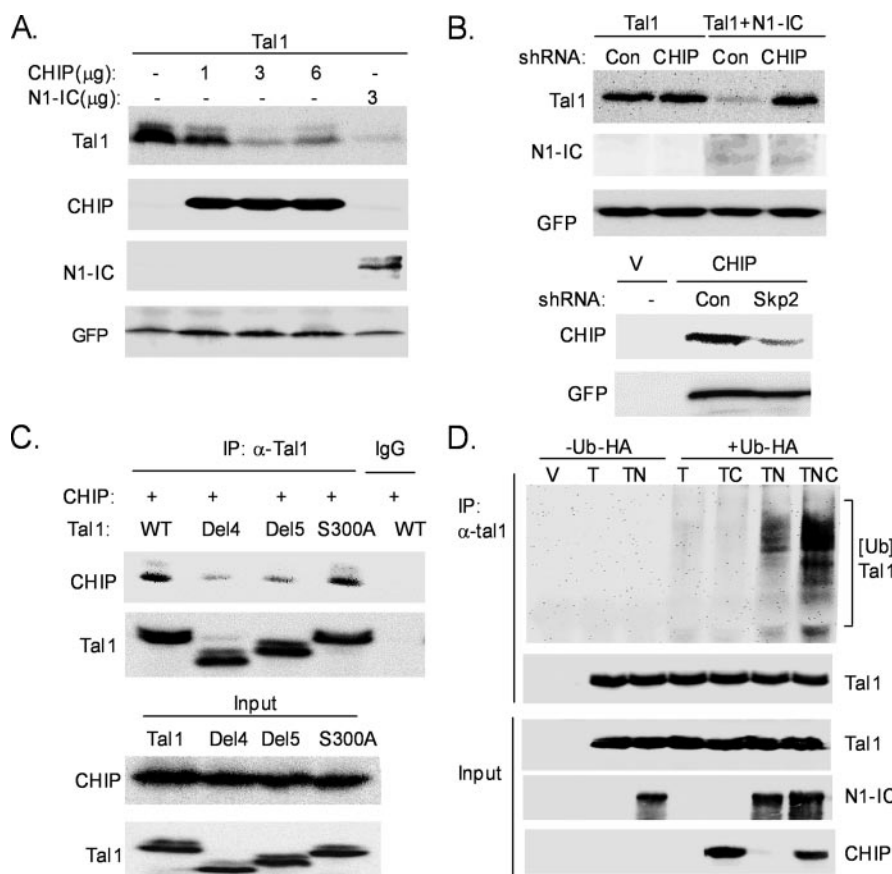
adds Tal1 to the list of proteins whose turnover can be stimulated by Notch, which includes E47, p27, and Jak proteins (10).<sup>3</sup> How Notch enhances the ubiquitination and degradation of these proteins is an important issue to address. We have evidence to suggest that Notch-induced degradation of these proteins depends on its ability to activate transcription of downstream target genes because shRNA-mediated down-regulation of RBP-Jκ, the DNA-binding partner of Notch intracellular domains, diminishes the effect of Notch (Fig. 1C) (10). Although such downstream genes have not yet been identified, they likely encode products that influence ubiquitination reactions controlled by common mechanisms shared by these disparate substrates. Cullin-based E3 ubiquitin ligases are involved in the ubiquitination of all the substrates mentioned above even though different substrate-binding subunits and different cullin molecules may be present in the E3 ligase complexes (10, 12, 13, 39, 40). Therefore, it seems reasonable for Notch-induced genes to intervene at this point. Data obtained using shRNA against CHIP suggest that Notch-induced Tal1 degradation depends on CHIP, but Notch signaling does not cause transcriptional activation of CHIP expression (data not shown).

Comparison between the mechanisms involved in Tal1 and E47 ubiquitination reveals several similarities as well as differences. Like E47, Tal1 is found to associate with Skp2, and its ubiquitination probably involves SCF<sup>Skp2</sup>. Furthermore, CHIP not only facilitates E47 but also Tal1 degradation. However, the interacting domains in CHIP are different for E47 and Tal1. Previous studies (16) showed that the TPR domain is sufficient for CHIP to interact with E47 and promote its degradation. In contrast, the TPR domain was not involved in association with Tal1 but was necessary for its turnover (Fig. 6). Instead, the C-terminal portion including the U box was responsible for Tal1 binding. Point mutations that abolish the ubiquitin ligase activity elicited by the U box (38) did not interfere with its association with Tal1, suggesting that the interaction was independent of the ubiquitin ligase activity of CHIP. Likewise, a point mutation disrupting the interaction between CHIP and chaperones like Hsc70 (36) also allows Tal1 binding, which led to the conclusion that CHIP and Tal1 association was not mediated by chap-

<sup>3</sup> L. Nie and X.-H. Sun, unpublished data.

Skp2 knockdown, constructs expressing T7-tagged Skp2, GFP, and the indicated shRNA were cotransfected into NIH3T3 cells. Immunoblotting was performed with anti-T7 and anti-GFP antibodies. Vec and V, vector. B, down-regulation of Skp2 rescues Tal1 degradation induced by endogenous Notch signaling. Jagged1-expressing and control NIH3T3 cells were transiently transfected with 1 μg of Tal1 and 4 μg of Skp2 or control shRNA constructs. Transfected cells were harvested 12 h after they reached confluence and immunoblotted for indicated proteins. C, overexpression of Skp2 accelerates Tal1 degradation. NIH3T3 cells were cotransfected with 1 μg of Tal1 and the indicated amounts of Skp2-expressing constructs along with 0.5 μg of GFP control. Tal1 and control protein levels were immunoblotted as described in the legend to Fig. 1. D, interaction between Tal1 and Skp2. 293T cells were transfected with 4 μg each of Skp2 and Myc-tagged Tal1 constructs with or without 8 μg of N1-IC-expressing plasmids and incubated for 24 h. Coimmunoprecipitation assay was performed with polyclonal antibodies against Tal1 or control IgG. Tal1 and Skp2 in the precipitates were probed with monoclonal antibodies against the Myc tag on Tal1 and Skp2. IP, immunoprecipitation; IB, immunoblot.





**FIGURE 5. CHIP is required for Notch-induced Tal1 degradation.** *A*, expression of CHIP induces Tal1 degradation. NIH3T3 cells were transfected with 1  $\mu$ g of Tal1 and the indicated amounts of HPC4-tagged CHIP constructs plus 0.5  $\mu$ g of GFP construct. Cotransfection with the N1-IC-expressing construct was performed in parallel as a positive control. Protein levels in whole cell lysates were detected with antibodies against the Myc tag on Tal1, HPC4 tag on CHIP, N1-IC, and GFP. *B*, down-regulation of CHIP blocks Notch-induced Tal1 degradation. Tal1 with or without N1-IC-expressing constructs was cotransfected with the indicated shRNA constructs into NIH3T3 cells. Levels of the indicated proteins were determined by immunoblotting. To control for the efficiency of CHIP knockdown, constructs expressing HPC4-tagged CHIP, GFP, and the indicated shRNA were cotransfected into NIH3T3 cells. Immunoblotting was performed with anti-HPC4 and anti-GFP antibodies. *Con*, control. *C*, interaction between CHIP and Tal1 mutants. Four micrograms of HPC4-tagged CHIP construct was cotransfected with 4  $\mu$ g of wild-type (WT) or mutant Myc-tagged Tal1 constructs. Coimmunoprecipitation assay was performed by using antibodies against Tal1, followed by immunoblotting with antibodies against the HPC4 and Myc tags. *IP*, immunoprecipitation. *D*, shown are the results from *in vivo* ubiquitination assay. Three micrograms of the Myc-tagged Tal1 (T)  $\pm$  9  $\mu$ g of the HA-ubiquitin (Ub) constructs were cotransfected into 293T cells with or without 6  $\mu$ g of the N1-IC (N) and CHIP (C) expression plasmids. A vector (V) control was also included. Transfected cells were treated with MG-231 at a final concentration of 12  $\mu$ M for 4 h before harvest. Immunoprecipitation was performed with antibodies against Tal1. The precipitates were immunoblotted with anti-HA and anti-Myc monoclonal antibodies. Input controls were done by immunoblotting for indicated proteins.

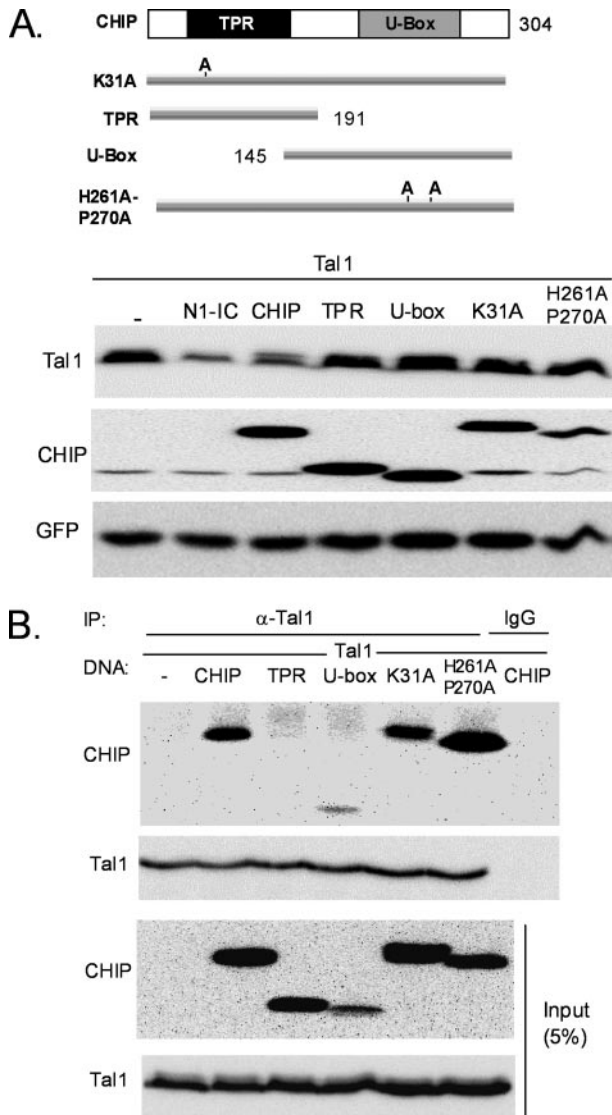
erones. Despite the abilities of these point mutants of CHIP to interact with Tal1, they were not able to promote Tal1 degradation, suggesting that both chaperone binding and U box-mediated ubiquitin ligase activity are required. This notion is consistent with the previous data that CHIP binds to Skp2 via Hsc70 (16). Therefore, failure to interact with Hsc70 could prevent CHIP from bringing Tal1 to Skp2. CHIP has also been shown to directly associate with cullin1 (16), which is useful for recruiting a pre-ubiquitination complex consisting of Skp2, Hsc70, CHIP, and Tal1 to the remainder of the SCF complexes (Fig. 7). The ubiquitin ligase activity of CHIP also appears to be crucial for Tal1 degradation. Based on the data shown in Fig. 5D, it is unlikely that CHIP directly initiates Tal1 ubiquitination as it does to other molecules (37, 41, 42). Rather, it may cooperate with

other E3 ligases in the polyubiquitination reactions, which is sometimes considered to be mediated by the function of E4 ligases (43). Alternatively, CHIP could attach ubiquitin to other proteins involved in Tal1 ubiquitination and enhance their activity. Thus, CHIP likely plays an integral role in the ubiquitination reactions mediated by SCF<sup>Skp2</sup> complexes, creating a scenario where multiple ubiquitin ligases are present in cullin-based E3 complexes.

Another distinction between Notch-induced E47 and Tal1 degradation is the dependence on MAPK activities. Inhibitors of MAPK/extracellular signal-related kinase kinase-1 (MEK1) inhibit Notch-induced E47 (10, 44) but not Tal1 degradation (data not shown). However, this does not mean that phosphorylation of Tal1 is not required for its degradation. The S300A mutation, which replaces a serine residue in a potential phosphorylation site with alanine, renders the protein resistant to Notch-induced degradation (Fig. 3). This would suggest that phosphorylation at this site is important for Tal1 degradation. Incidentally, no Skp2 substrate has been found to be ubiquitinated without phosphorylation. Because multiple kinases, including cyclin-dependent kinase, p38, and extracellular signal-regulated kinase (ERK), can all recognize this site, it is not surprising that incubation with inhibitors of any one kinase did not prevent Notch-induced

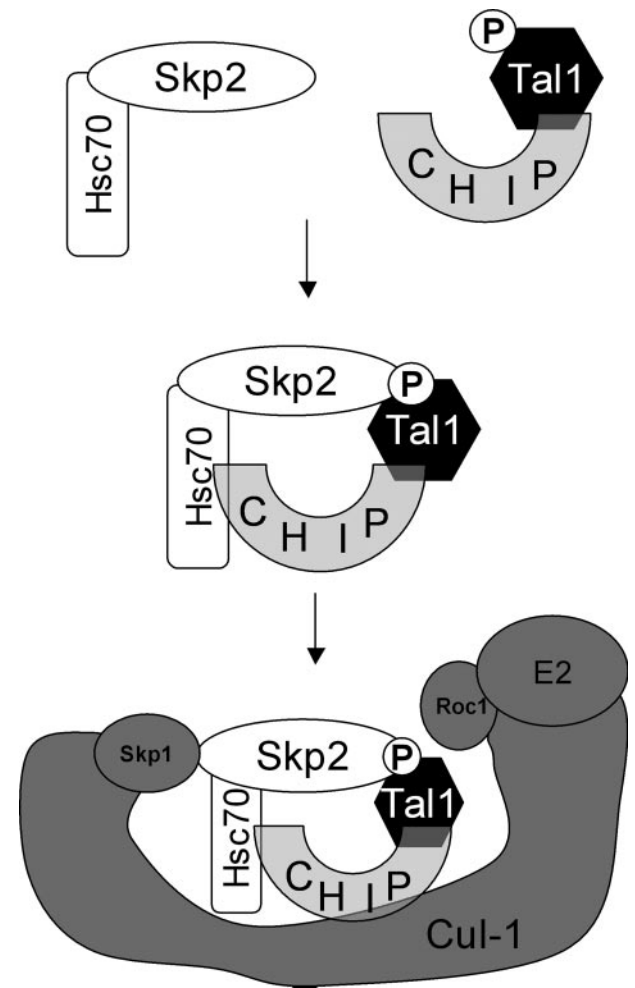
Tal1 degradation (data not shown).

The Tal1 sequence responsible for its degradation shares no significant similarity with those for E47 and p27 degradation. This is probably due to the different interfaces utilized to bind to molecules facilitating their ubiquitination. For example, the TPR domain of CHIP is important for associating with E47, whereas the U box region interacts with Tal1. Likewise, Cks1 is known to assist p27 binding to Skp2, but it has no effect on Tal1 degradation (data not shown). Despite this disparity, the sequence for Tal1 degradation is highly conserved across species from amphibian to man. The length of the conserved sequence and degree of homology, although somewhat shorter and lower, are comparable with those of the basic helix-loop-helix domain responsible for DNA binding and dimerization, thus arguing for a biological sig-



**FIGURE 6. Functional domains of CHIP necessary for Tal1 degradation.** A, examination of CHIP mutants. Upper panel, schematic representation of CHIP and its mutants. Each construct contains an HPC4 tag at the N terminus. Lower panel, determination of effects of CHIP mutants on Tal1 degradation. NIH3T3 cells were cotransfected with 1  $\mu$ g of Tal1 and 3  $\mu$ g of wild-type or mutant CHIP constructs along with 0.5  $\mu$ g of GFP construct. Immunoblotting was carried out as described in the legend to Fig. 5. B, interaction between Tal1 and CHIP. Four micrograms of Myc-tagged Tal1 construct was cotransfected with 4  $\mu$ g of wild-type or mutant CHIP constructs. Coimmunoprecipitation assay was performed by using antibodies against Tal1, followed by immunoblotting with antibodies against the HPC4 and Myc tags. IP, immunoprecipitation.

nificance of this degradation-responsive sequence. Although the scenario where Notch-induced Tal1 degradation plays a biological role has not been identified, plenty of situations exist where both Notch and Tal1 are involved. For example, both Notch and Tal1 regulate the development of hematopoietic, vascular, and neuronal tissues (2, 3, 25, 30). However, detailed investigation is necessary to precisely determine the cell type and stage in development where Notch and Tal1 coexist and evaluate the consequence of Notch-induced Tal1 degradation in these settings. Nevertheless, given the essential functions both Notch and Tal1 have in eukaryotic development, Notch-induced Tal1 degradation is



**FIGURE 7. A hypothesis for the assembly of cullin-based E3 ligase complex for Tal1 ubiquitination.** The ability of CHIP to interact with Tal1 and Hsc70 brings Skp2 to a pre-ubiquitination complex, which then joins with the remainder of the SCF<sup>Skp2</sup> complex via the interaction between CHIP and Cul-1, as well as Skp2 and Skp1.

likely an important mechanism by which cellular differentiation programs are coordinated.

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# Ubiquitination and Degradation of Tal1/SCL Are Induced by Notch Signaling and Depend on Skp2 and CHIP

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