Notch1 modulates timing of $G_1$-S progression by inducing SKP2 transcription and $p27^Kip^1$ degradation

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Cyclin-dependent kinase inhibitors (CKIs) and Notch receptor activation have been shown to influence adult stem cells and progenitors by altering stem cell self-renewal and proliferation. Yet, no interaction between these molecular pathways has been defined. Here we show that ligand-independent and ligand-dependent activation of Notch1 induces transcription of the $S$ phase kinase–associated protein 2 (SKP2), the F-box subunit of the ubiquitin–ligase complex SCF$^\text{SKP2}$ that targets proteins for degradation. Up-regulation of SKP2 by Notch signaling enhances proteasome-mediated degradation of the CKIs, $p27^Kip^1$ and $p21^{Cip1}$, and causes premature entry into $S$ phase. Silencing of SKP2 by RNA interference in $G_S$ stabilizes $p27^{Cip1}$ and $p21^{Cip1}$ and abolishes Notch effect on $G_1$–$S$ progression. Thus, SKP2 serves to link Notch1 activation with the cell cycle machinery. This novel pathway involving Notch/SKP2/CKIs connects a cell surface receptor with proximate mediators of cell cycle activity, and suggests a mechanism by which a known physiologic mediator of cell fate determination interfaces with cell cycle control.

Members of the Notch/Lin12 family are highly conserved transmembrane receptors that influence the cell fate of diverse types of precursor cells in a variety of multicellular organisms (1). Physiologic activation of Notch signaling requires cell–cell contact and occurs through binding of the Notch receptor to one of its ligands (Delta, Serrate/Jagged), followed by proteolytic release of the Notch intracellular domain (Notch$^\text{ICN}$) and its translocation to the nucleus (2). Notch$^\text{ICN}$ interacts with CSL transcription factors (CBF1, Su(H), Lag-1) and converts them from repressors to activators, promoting transcription of downstream genes involved in various differentiation programs (3).

In many cellular systems, Notch activation affects the finely tuned balance between proliferation and differentiation that regulates the stem and progenitor cell pools (1, 4, 5). Regulation of cell differentiation and cell fate decision by Notch is achieved by induction of specific differentiation programs and by an independent regulation of the cell cycle. Notch activation has been shown to induce alterations of the cell cycle kinetics that precede the inhibition of myeloid differentiation in hematopoietic cells (6), and to influence keratinocyte differentiation by two distinct mechanisms that involve induction of cell cycle arrest through $p21^{9p1}$ and transcriptional regulation of specific genes (4).

Regulation of the cell cycle by Notch signaling involves the coordination of different, and sometimes antagonizing, pathways in a highly cell context–dependent manner. For example, Notch activation has been found to induce proliferation of kidney epithelial cells through induction of cyclin D1 (7), and to lead to cell cycle arrest in keratinocytes through induction of $p21^{Cip1}$ (4). These observations indicate the existence of multiple alternative molecular interactions between Notch signaling and the cell cycle machinery, which are...
likely to correlate with the ability of Notch to function as an oncogene or a tumor suppressor (8, 9).

Physiologic regulation of the G₁–S transition is critical during determination of cell fate and is lost during oncogenic transformation. Cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) play key roles in regulating cell cycle progression from G₁ to S phase (10). Absence of the CKIs, p21(Cip1) or p27(Kip1), affect self-renewal of hematopoietic stem cells (11) and the proliferation/differentiation balance of hematopoietic progenitors (12), respectively, and predispose cells to neoplastic transformation (13).

In this study, we explore the role of Notch signaling at the defined G₁–S phase transition of the cell cycle. We found that Notch1 (N1) activation reduces the permanence of the cells in G₁ and accelerates their entry into S phase by promoting transcriptional induction of the F-box protein, SKP2, and in turn, proteasome-mediated degradation of the CKIs, p21(Cip1) and p27(Kip1) (14, 15). Thus, enhancement of SKP2 transcription represents a mechanism by which Notch modulates timing of cell cycle progression and coordinates proliferation and differentiation decisions.

RESULTS

Notch1 activation induces premature cell cycle entry and its effect is enhanced by the lack of p21(Cip1)

We demonstrated previously that N1 activation induces a more rapid G₁–S transition in hematopoietic progenitors (6). To identify the mechanisms that mediate this effect, we determined whether the alterations in cell cycle kinetics caused by N1 activation were enhanced in the absence of the G₁ regulatory molecule, p21(Cip1), which was shown to mediate Notch-induced cell cycle arrest in some cell types (4). WT and p21(Cip1) knock-out (p21⁻/⁻) 3T3 fibroblasts transduced with the retroviral bicistronic construct MSCV-GFP containing the constitutively activated forms of N1, (ICN) intracellular domain of Notch, and ΔE (intracellular with transmembrane domain of Notch) (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050559/DC1) were synchronized by nocodazole block-and-release and screened for alterations in the G₁ to S phase transition. This analysis (Fig. 1, A and B) showed that constitutively active N1 increases the percentage of cells in S phase in WT cells (+10–15%) and that the magnitude of this effect is significantly greater in the absence of p21(Cip1) (+20–30%). Despite the effects of N1 on cell cycle kinetics, Notch activation did not affect the overall cell cycle length and cell proliferation (Fig. 1 C). Preservation of total cell cycle length has been also observed in other 3T3 cells in which the G₁ phase of the cell cycle was shortened (16). Furthermore, overexpression of constitutively active N1 did not induce transformation in WT or p21⁻/⁻ fibroblasts, as measured by soft agar colony assays (unpublished data).

These results show that the expression of constitutively active N1 in 3T3 fibroblasts accelerates G₁ transition without increasing rates of proliferation, and that this effect is enhanced by the lack of the CKI, p21(Cip1). Furthermore, these observations suggest that additional cell cycle regulators mediate Notch effects on the cell cycle and that p21(Cip1) may play a role in balancing such effects.

Notch1 activation induces p27(Kip1) down-regulation and enhances CDK2 kinase activity

Next, we determined whether N1 activation altered the activity of CDK2 or CDK4 complexes, both critical for S phase entry and progression. WT and p21⁻/⁻ 3T3 cells transduced with constitutively active N1 exhibited a significant increase in CDK2 kinase activity compared with control cells, whereas no significant differences were observed in CDK4 activity (Fig. 2 A). The greater kinase activity was not due to increased cyclin levels because all cell lines expressed similar levels of cyclin D₁, A, and E, as well as CDK2 and CDK4 (Fig. 2 B).

Because CDK2 activity is antagonized strongly by the CKIs of the Cip/Kip family, p21(Cip1) and p27(Kip1), we tested...
the possibility that the greater CDK2 kinase activity observed in the presence of N1 in WT and p21\(^{-/-}\) cells was due to a lower level of CDK2 inhibition by decreased levels of p27\(^{kip1}\). We found that p27\(^{kip1}\) expression was significantly lower in WT and p21\(^{-/-}\) cells transduced with constitutively active N1 (Fig. 2 C and Fig. 3 A). In the WT background, p27\(^{kip1}\) levels in \(\Delta E\) and ICN cells were 80 and 60% of control GFP cells, respectively; in the p21\(^{-/-}\) background, p27\(^{kip1}\) levels in \(\Delta E\) and ICN cells were 50 and 30% of control GFP cells, respectively (Fig. 2 D).

To confirm that p27\(^{kip1}\) down-regulation was a direct consequence of Notch activity, 3T3 cells expressing constitutively active N1 were treated with a \(\gamma\)-secretase inhibitor (GSI). GSIs have been shown to impair activation of full-length Notch and Notch/\(\Delta E\) by blocking the proteolytic intracytoplasmic cleavage that occurs in membrane-tethered forms of Notch (17). Inhibition of this process blocks the generation of the active cleaved form of Notch, Notch\(^{IC}\), and results in the accumulation of an intermediate inactive form of Notch, the metalloprotease cleavage product (\(\Delta E^{*}\); reference 18). Treatment of p21\(^{-/-}\)/\(\Delta E\) cells with GSI caused the inhibition of Notch cleavage and could be observed as an accumulation of \(\Delta E^{*}\) polypeptide. Block of Notch signaling by GSI resulted in the abolishment of Notch-induced p27\(^{kip1}\) down-regulation (Fig. 2 E). Thus, activation of N1 induces a specific down-regulation of p27\(^{kip1}\) in asynchronous WT and p21\(^{-/-}\) cells.

**Notch1 activation promotes p27\(^{kip1}\) ubiquitin-mediated degradation**

To explore the molecular bases for N1-induced p27\(^{kip1}\) down-regulation, we tested whether this effect was dependent on the proteasome-degradation pathway, which is the prevalent mechanism of p27\(^{kip1}\) regulation in many cellular systems (19). Treatment with the 26S proteasome-specific inhibitor, lactacystin, reestablished high levels of p27\(^{kip1}\) in WT and p21\(^{-/-}\) cells overexpressing constitutively active N1 (\(\Delta E\) and ICN; Fig. 3 A); this confirmed the involvement of this pathway in Notch-mediated p27\(^{kip1}\) down-regulation. We further investigated p27\(^{kip1}\) half-life by treating ICN-expressing cells and controls with 10 \(\mu\)M of the ribosomal complex inhibitor, cycloheximide. As shown in Fig. 3 B, p27\(^{kip1}\) disappears more rapidly in ICN cells than in controls, showing a shorter half-life of p27\(^{kip1}\) in the presence of activated N1 (<2 h in ICN cells versus 4 h in controls). Finally, to determine if N1 activation enhances p27\(^{kip1}\) ubiquitination, we evaluated p27\(^{kip1}\) ubiquitinated forms in vivo. Endogenous p27\(^{kip1}\) was immunoprecipitated from extracts of \(\Delta E\) and control cells that had been treated with the proteasome inhibitor, MG132, which is used to stabilize ubiquitinated intermediates. MG132 treatment had the same effects as did lactacystin treatment on GFP and \(\Delta E\) cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050559/DC1). Immunoprecipitates were subjected to immunoblot analysis with anti-p27\(^{kip1}\) antibodies to de-
tect the different migrating forms of p27Kip1 (Fig. 3C). Under these conditions, p27Kip1 showed a basal level of ubiquitination that was increased significantly in the presence of N1 activation. Only p27Kip1 immunoprecipitates from cells overexpressing ΔE treated with MG132 contained detectable levels of slow migrating polyubiquitinated derivatives (inset, Fig. 3C, lanes 3 and 6). Presence of more highly ubiquinated forms could not be detected due to interference with the assay by IgG heavy chain. Together, these findings show that N1 activation results in a rapid decrease of p27Kip1 levels by enhancing proteasome-mediated degradation of the p27Kip1 protein.

Figure 3. N1 activation induces ubiquitin–proteasome–mediated degradation of p27Kip1. (A) Proteasome inhibition. Transduced 3T3 cells were incubated with DMSO (vehicle control) or the proteasome inhibitor, lactacystin. Cell extracts were analyzed by Western blot for p27Kip1 expression. (B) p27Kip1 protein half-life. Transduced 3T3 cells were arrested by nocodazole block. Cycloheximide was added 6 h after release into cell cycle and samples were incubated with cycloheximide for the indicated time points before lysis. p27Kip1 protein levels were determined by Western blot (top panel) and their densitometric values normalized to β-actin, densitometric units (DU) were plotted as line graphs (bottom panel). (C) In vivo detection of ubiquitin conjugates. Cell extracts from p21ΔE GFP and ΔE cells treated with MG132 or DMSO, were immunoprecipitated with anti-p27Kip1 antibodies or with IgG controls, separated by SDS-PAGE and blotted with anti-p27Kip1 antibodies. The inset shows a darker exposure of the blot for detection of slowly migrating forms of p27Kip1.

Notch1 regulates SKP2 and p27Kip1 levels in a cell cycle–dependent manner

Because p27Kip1 degradation is regulated by the SKP1/CUL1/F-box (SCF)SKP2-ubiquitination complex during cell cycle progression (14), we tested the hypothesis that Notch activation decreased p27Kip1 levels by regulating molecules that are involved in its degradation (e.g., the F-box protein, SKP2). We determined whether N1 activation altered the kinetics of SKP2 expression during cell cycle progression. In cells synchronized by nocodazole block-release or by serum starvation-stimulation, entry into the cell cycle was characterized by the induction of SKP2 pro-
tein in both GFP and ΔE cells. However, such induction was more rapid and greater in the presence of activated N1 than in control cells (Fig. 4, A and D). SKP2 levels were consistently two- to threefold higher in ΔE cells than in controls (Figs. 4 B and 5 B), and were especially higher at the early time points of cell cycle entry. The more rapid increase of SKP2 protein in ΔE cells was paralleled remarkably by a more rapid and significant degradation of p27Kip1 in these cells (Fig. 4, A, C, and D; and Fig. 5 B). At 6 h from nocodazole block release, p27Kip1 expression in ΔE cells decreased an average of 70% from the initial level, whereas it decreased only an average of 40% in control cells (Fig. 4 C). Recently, p21Cip1 was reported to be a target of the SCF SKP2 complex (15). Similar to p27Kip1, p21Cip1 was down-regulated faster in ΔE-expressing cells than in control cells (Fig. 4 D; Fig. 5, A and B).

To demonstrate further the role of SKP2 in N1-mediated p27Kip1 degradation, GFP and ΔE cells were transfected with small interfering RNA (siRNA) against SKP2 during serum-induced cell cycle entry. Transfection of the siRNA against SKP2, but not a control siRNA, decreased the level of SKP2 to the limit of detection and prevented N1-induced p27Kip1 and p21Cip1 degradation (Fig. 5, A and B). Accumulation of p27Kip1, consequent to SKP2 depletion, was equivalent in GFP and ΔE cells and resulted in an overall delay of cell cycle entry, as reported by previous studies (20, 21). Evaluation of S phase by Bromodeoxyuridine incorporation indicated that in the presence of equivalent levels of p27Kip1, the effect of activated N1 on cell cycle entry was reduced dramatically (Fig. 5 C). In conclusion, these experiments show that N1 activation has a direct impact on the kinetics of SKP2 expression during cell cycle progression, and that this effect correlates with decreased levels of the CKIs, p27Kip1 and p21Cip1. Furthermore, we demonstrated that SKP2 induction by N1 is required for Notch-induced down-regulation of p27Kip1 (and p21Cip1) and consequent modulation of cell cycle progression.

**Notch1 activation induces CBF-1–dependent transcription of SKP2**

Because the SKP2 promoter is targeted for regulation during cell cycle progression and Notch promotes transcription of several target genes through activation of the CBF-1/RBP-Jκ transcription factor (4, 7), we hypothesized that Notch signaling may regulate SKP2 at a transcriptional level. We performed Northern blot quantification of SKP2 mRNA during cell cycle progression, and we found that SKP2 transcripts appear earlier and are more abundant in cells expressing activated N1 than in controls (Fig. 6, A and B).

To define better the mechanism of induction of SKP2 mRNA by Notch, we searched for possible regulatory elements in the human SKP2 promoter region. We identified and cloned a 3.1-kb region within the SKP2 promoter containing the sequence TGGGAA at position −350 that fully matches the consensus sequence of the high-affinity binding site for CBF-1/RBP-Jκ (22), schematically represented in Fig. 6 C. This consensus sequence is identical to the one present in the murine HES-1 and p21Cip1 promoters (Fig. 6 C), which is transactivated by the Notch1/CBF-1 complex (23). Electrophoretic mobility shift assay (EMSA) analysis showed that the protein–DNA complex could be identified only when the radiolabeled SKP2 WT oligonucleotide was incubated with extracts from 293T cells overexpressing CBF-1 (Fig. 6 D, lanes 2, 3, and 10); this confirmed the ability of CBF-1 to bind to the identified SKP2 sequence. The CBF-1–DNA complex was supershifted specifically by the anti–CBF-1 and anti-N1 antibodies, but not by an unrelated antibody (lane 10); its formation was abolished by excess of unlabeled WT but not mutated SKP2 oligonucleotide (lanes 4, 8 and 5). In the nucleus, N1 physically associates with CBF-1, promoting transcriptional activ-
To determine whether the Notch/CBF-1 complex binds to the endogenous SKP2 promoter, we performed chromatin immunoprecipitation assay with anti–CBF-1 or anti-N1 antibodies, followed by PCR amplification of the SKP2 promoter region. Only DNA coprecipitated with anti–CBF-1 or anti-N1 antibodies generated positive PCR products for the SKP2 promoter region containing the fully conserved CBF-1 binding site (Fig. 6 E). PCR of the Hes1 promoter region containing the CBF-1 site and of the CD11b promoter not containing the CBF-1 site, were performed as positive and negative controls, respectively.

To determine whether activated Notch induces CBF-1-dependent SKP2 transcription, we performed luciferase reporter assays on 3T3 fibroblasts following ΔE or ICN transduction. Constitutive activation of N1 correlated with significant transactivation of HES-1 and SKP2 promoters (Fig. 6 F). SKP2 promoter activity increased ~4- to 10-fold in cells overexpressing activated N1 (ΔE and ICN) compared with controls and paralleled HES-1 promoter activity, which was used as a positive control for CBF-1-dependent activity. Deletion of the CBF-1 binding motif resulted in the abrogation of transcriptional activation (Fig. 6 G). In conclusion, these data demonstrate that N1 activation promotes CBF-1-dependent transcription of SKP2.

To confirm the physiologic relevance of our findings, we determined whether SKP2 expression could be induced by ligand-dependent stimulation of endogenous Notch in hematopoietic cells. To this end, we generated murine stromal MS5 cell lines (25) overexpressing each of the Notch ligands (J1, J2, Dll1, and Dll4) and screened their effect on HL-60 cells. This analysis indicated that Dll4 induces the strongest inhibitory effect on HL60 cell differentiation, which is recapitulated by a recombinant Dll4-Fc fusion protein (unpublished data). Next, we determined whether Dll4-dependent stimulation of endogenous N1 induced SKP2 expression and p27Kip1 down-regulation in HL-60 cells (Fig. 7 A). Incubation with Dll4 ligand induced strong activation of Notch signaling, confirmed by the induction of the cleaved active form of N1, NotchC (Fig. 7 B). N1 activation upon

**Figure 5.** SKP2 is required for Notch effect on p27<sup>Kip1</sup>. (A) WT-GFP and WT-ΔE were transfected with siRNA against SKP2 or GFP (negative control) during the serum starvation-stimulation procedure. Cell extracts were obtained at each indicated time point and analyzed by Western blot. The white lines indicate that intervening lanes have been spliced out. (B) Histogram summarizes multiple experiments at 12 h after serum stimulation. SKP2, p27<sup>Kip1</sup>, and p21<sup>Cip1</sup> protein levels were quantified from Western blot bands and normalized with β-actin values. Bars represent average densitometric units of four independent observations for GFP and ΔE cells, and two independent observations for ΔE cells in the presence of siSKP2. Statistically significant differences between ΔE and GFP cells: SKP2 P = 0.015; p27<sup>Kip1</sup> P = 0.033; p21<sup>Cip1</sup> P < 0.003. (C) Cells stimulated with serum in the absence or presence of SKP2 siRNA were pulsed with bromodeoxyuridine at 8 h and 12 h after stimulation. The difference between percentages of cells in S phase in ΔE and GFP cells was calculated. Bars represent averages of the differences expressed as percentage of S phase increment.
Figure 6. N1 activation induces CBF-1–dependent SKP2 transcription. (A) Kinetics of SKP2 mRNA induction in 3T3 cells synchronized by nocodazole block-release by Northern blot (left panel); the line graph represents percentages of SKP2 expression normalized to β-actin over time (right panel). (B) Transduced 3T3 cells were synchronized by serum starvation and stimulation and were analyzed by Northern blot; values represent percentages of SKP2 expression normalized to β-actin. (C) Identification of CBF-1 binding site in the SKP2 promoter. Top panel: representation of the human SKP2 promoter region containing CBF-1. Bottom panel: comparison of known CBF-1 binding sites in different gene promoters. (D) EMSA analysis was performed by incubating radiolabeled oligonucleotide with no extract (−) or lysates from 293T transfected with GFP or CBF-1. Radiolabeled WT oligonucleotide was incubated with lysates preincubated with 50-fold excess of unlabeled WT (wt) or mutated (mut) oligonucleotide for competition experiments (c.c.), and with unrelated antibody (nr), anti-N1, or anti–CBF-1 antibodies for supershift experiments. (+) and ++ indicate that 2.5 and 5 μl of ab were used. Specific CBF1-DNA complex is indicated as CBF1. (*) indicates the supershifted band obtained when anti–CBF-1 or anti-N1 antibodies were present in the binding mixture. (E) Binding of N1 and CBF1 to the endogenous SKP2 promoter by chromatin IP analysis. SUPT1 cells, which express high levels of activated N1, were processed for chromatin immunoprecipitation with antibodies against CBF-1, N1, or affinity purified IgGs. Input DNA and recovered DNA were analyzed by PCR using specific primers for the indicated promoters. In the Hes1 PCR, the input DNA lane is substituted by a nonimmune IP sample. Positive PCR products were generated from the input DNA. (F) Increased SKP2 and HES-1 promoter activity in the presence of activated N1. Transduced 3T3 cells were transfected with the luciferase reporter plasmid containing the 3.1-kb SKP2 promoter (SKP2-luc) or the site specific deletion in the HES promoter (Δ-CBF1) that abrogate CBF-1/N1–dependent transcription. Cells were harvested after 40 h and cell extracts were prepared for the luciferase reporter assays. Values represent the average percentage of luciferase relative units obtained using the Δ-CBF1 construct relative to SKP2 construct (100%).
Figure 7. Stimulation of endogenous N1 by Dll4 ligand promotes SKP2 induction in vivo. (A) N1, Notch1, SKP2, and p27kip1 expression in undifferentiated HL-60 cells. (B) Left panel: HL-60 cells were seeded in wells coated with IgG1-Fc control fragment (Fc) or recombinant Dll4-Fc fusion protein (Dll4) and cultured in the presence of ATRA. Cell extracts obtained from cells at 48 h and 72 h of culture with Fc or Dll4 and ATRA were analyzed by Western blot using specific antibodies directed to Val 1744-cleaved form of Notch (Notch1), SKP2, p27kip1, and \( \beta \)-actin. Right panel: HL-60 cells cultured for 72 h on Fc or Dll4 were incubated with GSI. At the indicated days, cells were analyzed for the expression of the differentiation marker, CD11b, by FACS. Values indicate an average of three independent experiments.

Dll4 stimulation was accompanied by higher levels of SKP2 and failure to accumulate p27kip1 (Fig. 7 B, lanes 3 and 4), whereas in the control, response to ATRA treatment was characterized by SKP2 down-regulation and p27kip1 accumulation (Fig. 7 B, lanes 1 and 2), as documented (26). SKP2 transcripts increased after physiologic stimulation of endogenous N1 by Dll4 (Fig. 7 C). Dll4 stimulation of Notch signaling was associated with resistance of HL-60 cells to arrest in G1 and to differentiate (Fig. 7 D)—as we previously reported—with J2 stimulation (6). To prove that SKP2 up-regulation was directly due to Notch activation upon Dll4 stimulation, we treated stimulated HL-60 cells with the Notch inhibitor, GSI. GSI treatment induced a complete inhibition of N1 cleavage, prevented Dll4-mediated induction of SKP2 and which resulted in p27kip1 accumulation (Fig. 7 B, lanes 5 and 6), and promoted cell differentiation (Fig. 7 D). Similarly, expression of a dominant negative form of SKP2 (SKP2ΔF) that prevents p27kip1 degradation (14), increased rates of differentiation in HL-60 cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050559/DC1) which indicated a direct impact of SKP2 on cell differentiation. These experiments demonstrate that ligand-dependent stimulation of endogenous N1 leads to SKP2 induction and p27kip1 down-regulation, and affects cell differentiation.

Next, we examined whether the loss of N1 had an impact on SKP2 expression and differentiation in vivo. To address this question we used mice transgenic for an N1 antisense (N1AS+/−) driven by the mouse mammary tumor virus LTR promoter, which specifically suppresses N1 transcripts in a variety of cell types, including hematopoietic precursors (27, 28). Reduced N1 expression in N1AS+/− correlated with a modest, but reproducible, decrease in SKP2 transcripts (Fig. S4 A) and protein levels (not depicted), when compared with controls (N1AS− and N1AS+/−). Analysis of the hematopoietic subsets in N1AS+/− and control mice did not show significant differences in the representation of primitive Lin− Sca−1 and myeloid mature Gr−/Mac−1 subsets in the BM and in the spleen (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20050559/DC1), which is consistent with recent observations made in an inducible knock-out model (29). These data suggest that although N1 activation may have a significant effect on SKP2 induction in adult hematopoietic cells, its absence likely is compensated by other Notch receptors. Evaluation of the Notch/SKP2 pathway should provide more useful information in a model where Notch receptors signaling are abrogated completely.

Finally, we evaluated the effects of Notch stimulation and inhibition in primary hematopoietic cells. BM Lin− cells (enriched in progenitors) were sorted from control mice and were stimulated with Dll4 in the absence or presence of GSI. Dll4 stimulation resulted in induction of SKP2, in a higher fraction of cells in S phase and in a significant higher maintenance of the more primitive subsets Lin+Kit+ (Fig. 8 B–D). All of these effects were abolished by the presence of the Notch inhibitor GSI. Taken together, these data show that abrogation of N1 in adult cells results in mild effects, because it is compensated readily by other Notch receptors, whereas an increase in Notch signaling can induce SKP2 and affect cell cycle and differentiation in vivo. Given that Notch ligands can be up-regulated in the BM stroma by hormones, such as parathyroid hormone (30), and by inflammatory cytokines, such as TNF-α (unpublished data), we hypothesize that this mechanism could be of physiologic relevance for hematopoietic progenitors’ expansion during BM response to stress conditions.

DISCUSSION

Regulation of the cell cycle, and in particular, of the G1 checkpoint is one of the fundamental mechanisms underlying determination of cell fate (31). Absence of the critical G1 regulators CKIs, p21cip1 or p27kip1 or activation of the Notch receptor, a well-known cell fate regulator (1), induce similar alterations in the self-renewal/proliferation/differentiation balance of stem and progenitor cells (4, 5, 11, 12). However, no interaction between these molecular pathways
has been defined. The present study provides the first demonstration that the F-box protein, SKP2, links Notch signaling with p27Kip1 and p21Cip1 regulation.

Rates of G1-S progression depend on p27Kip1 and p21Cip1 removal from the CDK2 complexes, either by sequestration by cyclin D-CDK4 complexes or by protein degradation (10). Previous observations correlated Notch activation with lower levels of the CDK inhibitor, p27Kip1 (32); however, the direct link to Notch signaling and the mechanism involved have not been investigated. Here, we provide evidence that the Notch signaling pathway regulates the specific degradation of p21Cip1 and p27Kip1 by directly inducing the transcription of SKP2, the F-box subunit of the ubiquitin-ligase SCFSKP2 that targets these CKIs for degradation (14). Notch activation recently was associated with enhanced proteasome-mediated degradation of E47, a molecule involved in lymphoid cell differentiation (33), suggesting that modulation of protein degradation could represent a general mechanism through which Notch signaling modulates cellular processes.

We identified a highly conserved CBF-1 binding motif within the SKP2 promoter region and demonstrated that Notch activation drives SKP2 transcription directly through a CBF-1–dependent mechanism. Notch-mediated SKP2 up-regulation resulted in a more rapid p21Cip1 and p27Kip1 down-regulation, increased CDK2 activity, and accelerated cell cycle entry. Furthermore, lack of p21Cip1 resulted in a more pronounced effect on CDK2 activity and cell cycle progression in the presence of Notch, perhaps by cooperating with decreased levels of p27Kip1 in relieving CDK2 inhibition. Depletion of SKP2 by siRNA abrogated Notch-induced p27Kip1 down-regulation, and showed that SKP2 is required for Notch-induced p21Cip1 and p27Kip1 degradation in 3T3 cells. Elimination of the SKP2/CKI pathway reversed the Notch effect on cell cycle entry and resulted in a significant reduction of cells in S phase. However, the effect of Notch on cell cycle entry was not abolished completely in the absence of SKP2; this reinforces the present view that Notch signaling regulates the cell cycle through multiple molecular interactions, as is emerging from several recent reports (34, 35). Although no study has addressed the integrity of the Notch signaling pathway in the SKP2 null cells, SKP2 null mice do not present the obvious abnormalities of the nervous and lymphoid systems derived by altered Notch signaling (20). It is possible that the specific defect in the Notch/SKP2 pathway is compensated by alternative pathways or emerges only under conditions of stress. Similarly, loss of N1 did not result in marked alterations of adult stem and progenitor cells, which suggests a compensatory effect of other Notch receptors.

The physiologic relevance of our findings is confirmed by the demonstration that stimulation of endogenous Notch by its cognate ligand, Dll4, is capable of inducing up-regulation of SKP2 in the hematopoietic cell line HL-60 and in primary progenitor cells. In both models, activation of the Notch/SKP2/CKI pathway correlated with inhibition of progenitor differentiation and with maintenance of more primitive precursors, as described previously (24). It is possible that the Notch/SKP2/CKIs pathway fine tunes the balance of proliferation and differentiation by accelerating entry into cell cycle

Figure 8. Physiologic relevance of the Notch/SKP2 pathway in vivo. (A) SKP2 expression in the absence of N1. RNA obtained from BM and spleen cells of N1AS+/− and controls was used for RT and PCR amplification for the indicated gene products. All samples were positive for the low molecular size PCR product of the housekeeping gene, GSα, which confirmed the absence of genomic DNA. Noticeable SKP2 expression was found in the spleen cells (left). Bar graph (right) represents densitometric values of SKP2 normalized with GSα (densitometric units, DU); values indicate averages of three controls and four N1AS+/− samples (right; P = 0.064). (B–D) BM Lin− cells sorted from control mice were exposed to MS5 cells overexpressing vector alone (Co) or Dll4+/− GSI. At day 3, cells were harvested and processed for the following: (B) PCR for SKP2 and GSα and (C) Bromodeoxyuridine incorporation. Bars indicate average percentage of cells in S phase in three independent experiments; difference between control/GSI and Dll4/GSI is not statistically significant. P = 0.1. (D) Immunophenotyping: cells were stained with anti–c-Kit antibody and analyzed by flow cytometry. Bars indicate average percentage of Lin−c-Kit+ cells in three independent experiments. *P = 0.03.
and shortening the time spent by cells in G\textsubscript{1}, the temporal window where decisions regarding cell cycle withdrawal and differentiation are made. In support of this view, the CKI \(p21^{Cip1}\) has been shown to be the mediator of the quiescent status of hematopoietic stem cells, as loss of \(p21^{Cip1}\) results in a temporary stem cell expansion followed by long-term stem cell exhaustion in vivo (11). Considering that Notch has been shown to induce \(p21^{Cip1}\) transcription in some cellular systems (4, 36), and that, in contrast, we observed that it can cause \(p21^{Cip1}\) down-regulation, we hypothesized that Notch may regulate hematopoietic stem cell self-renewal through a fine-tuned balance of these two mechanisms. The Notch/\(SKP2/CKI\) pathway may have a more defined role in the expansion of hematopoietic progenitors, where \(p27^{kip1}\) plays a critical regulatory function. Notch activation (5, 6, 37) and the loss of \(p27^{kip1}\) (12) promote hematopoietic progenitor expansion. Our studies provide evidence that Notch signaling promotes \(p27^{kip1}\) degradation and suggest that this may be an important regulatory mechanism of hematopoietic progenitors expansion linking microenvironmental cues with the cell cycle effectors.

Neither constitutive loss of \(p27^{kip1}\) nor constitutive activation of Notch results in myeloid cell transformation or myeloid cell leukemia, suggesting the presence of other negative regulators in the myeloid lineage. In our experimental model, Notch activity alone did not bypass the mitogen requirement for \(SKP2\) induction; this suggests that activation of Notch “per se” is not sufficient to lead to uncontrolled proliferation. Based on these observations, we propose that Notch signaling “primes” \(SKP2\) transcriptional activation during G\textsubscript{1} and S phase entry but needs the presence of conserved cell cycle regulatory mechanisms to accomplish this function successfully. Thus, by anticipating and reinforcing \(SKP2\) induction in the presence of mitogens, Notch signaling accelerates entry into the cell cycle (Fig. 9).

The biologic effects that are induced by Notch signaling are cell context-dependent. In contrast to what is seen in hematopoietic stem and progenitor cells, N1 activation severely compromises T cell development and promotes T cell leukemias (8, 38). In addition, \(SKP2\) was shown to function as an oncprotein in some cellular contexts (39) and its overexpression is common in leukemias and lymphomas (40). Therefore, it is possible that the capacity of Notch to induce \(SKP2\) and to down-regulate \(p27^{kip1}\) expression may constitute the basis of its oncogenic potential in T lymphoid cells.

In conclusion, we propose that the ability of Notch to modulate a key cell cycle regulator, such as \(SKP2\), plays a role in balancing proliferation/differentiation in the hematopoietic system, and may prove to be critical in cooperating with additional genetic and epigenetic alterations toward the acquisition of a malignant phenotype.

**MATERIALS AND METHODS**

cDNA expression constructs and retroviral transduction. N1 ICN and \(\Delta E\) cDNAs were subcloned into the retroviral vector MSCV-GFP and used for retroviral infections as described (6). GFP-positive cells were sorted by fluorescence-activated cell sorting (FACSVantage; Becton Dickinson). Each individual experiment was performed within 1–2 wk of the transduction and with pools of GFP-expressing cells to avoid the selection bias. Each set of experiments was confirmed by multiple independent transductions.

**Cell lines and cell culture.** Early passage (20–40) 3T3 fibroblasts were derived from WT and p21\(^{-}\) mouse embryo as described (16). 293T, 3T3, HL-60, SUP-T1, and MSS cells were maintained as described (6, 16, 23, 25). Transduced 3T3 cells were treated for 12 h with MG132 (10 \(\mu\)M; Sigma-Aldrich), lactacystin (20 \(\mu\)M; AG Scientific, Fluorochem), vehicle DMSO (1 \(\mu\)l/ml; Sigma-Aldrich); or cycloheximide (Sigma-Aldrich, 50 \(\mu\)g/ml final concentration). 3T3 and HL-60 cells were treated with GSI DFPAA (100 nM; provided by J. Aster, Brigham and Women’s Hospital, Boston, MA) or vehicle DMSO (1 \(\mu\)l/ml; Sigma-Aldrich) for 12 and 24 h. HL-60 cells were seeded at \(3 \times 10^5\) cells/ml in 6-well plate wells coated with the Fc portion of human IgG (Fc, Jackson ImmunoResearch Laboratories), or with recombinant Dll4 fusion protein consisting of the Dll4 extracellular domain fused to the Fc portion of human IgG (Dll4-Fc) and cultured in RPMI 1640 10% FBS supplemented with 2 \(\mu\)M ATRA (Sigma-Aldrich). Dll4-Fc (10 \(\mu\)g/ml) or Fc (10 \(\mu\)g/ml) was immobilized on culture wells in 1 ml of PBS (Sigma-Aldrich) overnight at 4°C. MSS-vascular and MSS-Dll4 cell lines were obtained by transducing MSS cells with the retroviral vector MSCV-GFP alone or containing the cDNA for Dll4 as described (6).

**Mice and primary cultures.** C57BL/6 N1AS\(^{+/+}\) were generated using a 1164-bp N1AS construct encompassing the S\(^{+}\) coding region of mouse N1 expressed under the control of the mouse mammary tumor virus LTR promoter as described (27, 28). Hemizygous transgenic mice N1AS\(^{+/+}\) were bred to each other, selecting homozygous transgenic N1AS\(^{+/+}\) as the progenitor cell line. The transgenic mice were backcrossed to C57BL/6 and C57BL/6N1AS\(^{+/+}\) mice. Transgene integration was confirmed by PCR and transgene expression and function were confirmed by RT-PCR and Western blot analysis for N1. Age-matched (>16 wk) adult female mice were used. Primary BM cells from C57BL/6 control mice were harvested from femurs and labeled with antibodies directed to lineage markers (Gr1, Mac1, CD3, CD4, CD8, B220, NK, Ter 199). Lin\(^{-}\) enriched for hematopoietic progenitor cells) were isolated by FACS and seeded on MSS-vector and MSS-Dll4 feeder layers. Lin\(^{-}\)cells were cocultured in IMDM 10% FBS supplemented with 100 nM GSI (Calbiochem); at day 3 of coculture, cells were harvested and processed for analysis of differentiation, cell cycle, and gene expression.

**Cell cycle and cell division analysis.** 3T3 cells were synchronized in G\textsubscript{0}/M, by treatment with 50 ng/ml of nocodazole (Sigma-Aldrich) for 12 h, and at the G\textsubscript{0}/G\textsubscript{1} boundary by serum starvation (0.1% FCS) for 96 h, fol-

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**Figure 9.** Notch/\(SKP2/CDK\) pathway model. (A) In basal conditions, mitogen stimulation promotes reentry into the cell cycle by inducing \(SKP2\) expression, which in turn, mediates \(p21^{Cip1}\) and \(p27^{kip1}\) degradation. (B) Activation of Notch by its physiologic ligands synergies with mitogen stimulation and enforces \(SKP2\) transcription, leading to increased degradation of \(p21^{Cip1}\) and \(p27^{kip1}\) and to enhanced G\textsubscript{1}-S transition.
lowed by the readout of secretion (10% FCS). The cell cycle was evaluated by bromodeoxyuridine (Sigma-Aldrich) and propidium iodide (Sigma-Aldrich) incorporation followed by analysis with the FACSCaliber flow cytometer and the Cell Quest software (BectonDickinson). To document cell division history, 3T3 cells labeled with the red fluorescent membrane dye, PKH26-GL (Sigma-Aldrich), were seeded at low density in multiple equivalent wells and each well was harvested every 24 h for 4 d. Collected samples were analyzed by FACS. Cell division profiles were obtained by evaluating the dilution of the dye's mean intensity of fluorescence that occurs at each cell division.

**Immunologic reagents and procedures.** Immunoprecipitation (IP) and Western blots were performed as described (16). Gel loading was normalized to protein concentration and confirmed by β-actin probing. Signals were quantified using Molecular Dynamics scanner and ImageQuant analysis software. Antibodies used included the intracytoplasmic region of N1 (6); cyclin D1 and CDK4 (for IP, provided by E. Harlow); murine p21(0/1) (provided by C. Schneider, Laboratoire Nationale Biotechnologie, Trinite, Italy); CBF-1-R3BP.Je (provided by E. Kieff, Brigham and Women's Hospital, Boston, MA; reference 41; Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20050559/DC1); p27Kip1 (K25020; Transduction Laboratories); and Notch6 (Val 1744; 2421, Cell Signal); and the following: cyclin A (H-432), cyclin E (M-20), CDK2 (M2), CDK4 (C-22), SKP2 (H-435), and β-actin (I-19), obtained from Santa Cruz Biotechnology, Inc. Detection of in vivo p27Kip1-ubiquitin conjugates was performed as described (21). Lysates (1.5 mg total protein) were IP with anti-p27Kip1 antibody (C-19, Santa Cruz Biotechnology, Inc.) and p27Kip1 forms were detected by immunoblotting (K25020; Transduction Laboratories).

Silencing by small interfering RNA. 3T3 cells seeded at a 30–40% density were starved for 96 h before serum (10% FCS) readdition. The siRNA duplexes that were used for silencing SKP2 or GFP (negative control) were 21-bp synthetic oligonucleotides synthesized by Dharmacon Research (SMARTpool). Two rounds of transfections were carried on for 12 h using lipofectamine 2000 reagent (Invitrogen) as recommended.

**SKP2 promoter cloning and luciferase assays.** The human SKP2 promoter region was identified by analysis of the human genome database and analyzed for regulatory motifs using the Genome Exploring and Modelling Software (GEMS) by Genomatrix. A region from −3136 to +154, including the first exon (GenBank sequence accession no. U 33761), was amplified by PCR using genomic DNA of HL-60 cells and cloned into the pGEM-T easy vector (Promega). A 3074-bp fragment was subcloned in the SmaI site of the pGL2-basic vector (Promega) containing the luciferase gene. The following primers were used to amplify the 3′-end of the first exon (GenBank sequence accession no. U 33761), was amplified by PCR using genomic DNA of HL-60 cells and cloned into the pGEM-T easy vector (Promega). A 3074-bp fragment was subcloned in the SmaI site of the pGL2-basic vector (Promega) containing the luciferase gene. The following primers were used to amplified DNA were performed to optimize PCR conditions. The following primers were used to amplified promoter regions for: SKP2 (200 bp) F: 5′-GATCCAGGCTCAGAGAGC-3′; R: 5′-CTCTTCCCTGGAGCTTTACC-3′; Hes1 (120 bp) F: 5′-CCCAAGAAGAGCTGAGAC-3′; R: 5′-GAAACGAGTGACTGCGAGTG-3′; and CD11b (100 bp) F: 5′-GACAGGCAAGGCG-3′; TATGT-3′; R: 5′-AAAGCAAAGAAGGCGAAGAG-3′. RNA from BM, spleen, and sorted Lin− cells was extracted using Trizol (Invitrogen). RNA treated with DNase (Ambion) was used for RT reaction. The primers were used: N1: F: 5′-CGGTTAGGGGAAGAGACAATGTCATGCATG-3′; R: 5′-GAATGTCCGGGCCAGCGCCACC-3′; SKp2: F: 5′-GACACAACTTTCAAAGGTC-3′; R: 5′-AATCTCGGCCAACATCATGTC-3′; Hes1: F: 5′-AACACGACACCGCGGACAAC-3′; R: 5′-GAATGGCCGGGAACGTACTTCT-3′. The primers for the housekeeping gene GSt (44). F: 5′-GCTGCTGTCACCGACAGAAT-3′; R: 5′-GTGACACGGCAGGCTACT-3′, allow discrimination of contaminating genomic DNA by identification of a high molecular product containing an intervening intron. PCR conditions: 95° for 30′; 95° 1′; 64° 1′; 72° 1′) × 32 cycles; 72° 10′.

Statistical analysis. Equality of distributions for matched pairs of observations was tested using the t test.

Online supplemental materials. Fig. S1 shows retroviral transduction. Fig. S2 shows effects of proteasome inhibition by MG132. Figs. S3 shows effect of SKP2 inhibition on cell differentiation. Fig. S4 shows hematopoietic subsets in the absence of Notch1. Fig. S5 shows specificity of the antibody directed to CBF1/RBP-Jk. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050559/DC1.

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