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Notch1 activation reduces proliferation in the multipotent hematopoietic progenitor cell line FDCP-mix through a p53-dependent pathway but Notch1 effects on myeloid and erythroid differentiation are independent of p53

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Signaling mediated by activation of the transmembrane receptor Notch influences cell-fate decisions, differentiation, proliferation, and cell survival. Activated Notch reduces proliferation by altering cell-cycle kinetics and promotes differentiation in hematopoietic progenitor cells. Here, we investigated if the G_1 arrest and differentiation induced by activated mNotch1 are dependent on tumor suppressor p53, a critical mediator of cellular growth arrest. Multipotent wild-type p53-expressing ($p53^{wt}$) and p53-deficient ($p53^{null}$) hematopoietic progenitor cell lines (FDCP-mix) carrying an inducible mNotch1 system were used to investigate the effects of proliferation and differentiation upon mNotch1 signaling. While activated Notch reduced proliferation of $p53^{wt}$-cells, no change was observed in $p53^{null}$-cells. Activated Notch upregulated the p53 target $p21^{cip/waf}$ in $p53^{wt}$-cells, but not in $p53^{null}$-cells. Induction of the $p21^{cip/waf}$ gene by activated Notch was mediated by increased binding of p53 to p53-binding sites in the $p21^{cip/waf}$ promoter and was independent of the canonical RBP-J binding site. Re-expression of $p53^{wt}$ in $p53^{null}$ cells restored the inhibition of proliferation by activated Notch. Thus, activated Notch inhibits proliferation of multipotent hematopoietic progenitor cells via a p53-dependent pathway. In contrast, myeloid and erythroid differentiation was similarly induced in $p53^{wt}$ and $p53^{null}$ cells. These data suggest that Notch signaling triggers two distinct pathways, a p53-dependent one leading to a block in proliferation and a p53-independent one promoting differentiation.
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Notch proteins are a family of highly conserved transmembrane receptors that transduce signals involved in the control of cell fate; differentiation, proliferation, and apoptosis in a cell context-dependent manner (reviewed by Wilson and Radtke, Lai, and Hurlbut *et al.*[1–3]). Depending on the cell type and other signals present for the receiving cell, Notch signaling can inhibit or delay differentiation, or promote differentiation. Similarly, Notch activation can induce cell cycle progression thereby increasing cell proliferation, while under different conditions it blocks cell cycle progression leading to growth arrest. Further, Notch activation can influence apoptosis positively or negatively.

Notch receptors are activated by specific transmembrane ligands of the Delta and Serrate/Jagged family. After ligand binding, the intracellular domain of Notch ($Notch^{IC}$) is proteolytically cleaved from the transmembrane region and translocates to the nucleus, where it associates with the transcriptional repressor RBP-J also termed CBF1.[4] After binding of $Notch^{IC}$, RBP-J is converted to a transcriptional activator and in conjunction with chromatin remodeling

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Abbreviations: $p53^{wt}$, wild type p53; $p53^{null}$, p53-deficient; FDCP-mix, factor-dependent cell established at the Paterson Institute with mixed differentiation potential; mNotch1, murine Notch1; $Notch^{IC}$, intracellular domain of Notch; RBP-J, recombination recognition sequence binding protein at the $J\kappa$ site; Hes, hairy and enhancer of split; Hey/Herp, Hes-related repressor; $mN1^{IC}$, intracellular domain of murine Notch1; NERT, $mN1^{IC}$ fused to the hormone binding domain of the human estrogen receptor tamoxifen-sensitive mutant; ERT, estrogen receptor tamoxifen-sensitive mutant; rneo, retroviral control vector carrying the neomyin resistance gene; rNERTneo, retroviral control vector carrying the NERT and neomyin resistance gene; rNERTneo FDCP-mix, FDCP-mix cells expressing an OHT-inducible form of $Notch1^{IC}$; rneo FDCP-mix, FDCP-mix cells carrying the retroviral control vector conferring neomycin resistance; G418, neomycin; OHT, 4-hydroxytamoxifen; CFSE, carboxyfluorescein diacetate succinimidyl ester; p21RBP-Jmut promoter, p21 promoter compromised in RBP-J binding; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum; PE, Phycoerytrin; APC, allophycocyanin; PBS, phosphate-buffered saline; RT, room temperature; p21luc, reporter plasmid carrying a luciferase gene under the control of the p21 promoter; p21Δp53BS1luc, p21 promoter lacking the first p53 binding site; p21Δp53BS2luc, p21 promoter lacking the second p53 binding site; p21Δp53BS1 + 2luc, p21 promoter lacking both p53 binding sites; ChIP, chromatin immunoprecipitation
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enzymes, components of the transcriptional machinery and the activity of other cofactors induces transcription of downstream target genes, including genes of the Hes (Enhancer of Split) and Hey (also called Hes-related repressor Herp, Hesr, Hrt, CHF, gridlock) family.[1,2] Recently, two proteins of the Hes/Hey basic helix-loop-helix transcription factor family, Hes1 and Hey1, were found in a screen for regulators of p53 activity.[5] However, little is known about the biological significance of Hes1- and Hey1-dependent regulation of p53 activity in regard to Notch signaling.

The tumor suppressor protein p53 plays a critical role in maintaining cellular homeostasis both in normal development and following various cellular stresses including DNA damage and oncogene activation.[6] After activation, it initiates a transcriptional program that can result in cell cycle arrest or apoptosis depending on the type and extent of the stimulus. Recently, we reported that activated Notch1 inhibits proliferation of hematopoietic progenitor cells due to cell cycle arrest in G_0/G_1[7] and promotes myeloid differentiation.[8,9] In this study, we examine whether p53 is required for these Notch-mediated effects. Our results show that Notch signaling inhibits proliferation via a p53-dependent mechanism involving upregulation of p21 expression while induction of myeloid and erythroid differentiation is p53 independent.

Results

Generation of p53^{null} FDCP-mix cell lines expressing a conditional activated mNotch1^{IC}. To determine whether p53 has a role in mediating the Notch1-induced cell cycle block and/or myeloid differentiation of hematopoietic progenitor cells, we generated p53^{null} FDCP-mix cells clones expressing an inducible form of the constitutively active intracellular part of murine Notch1. Like the FDCP-mix cells lines A4 or A7, p53^{null} FDCP-mix cells are strictly factor-dependent hematopoietic progenitor cell lines that differentiate in a multilineage response to physiological regulators for differentiation, such as stromal cells or hematopoietic cytokines.[10–12] Both were established from murine long-term cultures in the same way and share many characteristics with primitive hematopoietic progenitor cells.[10,13] First, we confirmed that the p53 pathway was functional in FDCP-mix A7 cells and not responsive in p53^{null} FDCP-mix cells (Figure 1a). Next, p53^{null} FDCP-mix cells were transfected by electroporation with a retroviral vector carrying the mN1^{IC} fused to the hormone binding domain of the human estrogen receptor (NERT[9]), which was used previously to establish A7 FDCP-mix cells with an inducible Notch1.[8] As a control, cells were transfected with a control vector that does not contain the NERT cDNA (rneo). Both vectors confer G418 (neomycin) resistance to transfected cells. To ensure functionality and a tight regulation of the Notch-signaling pathway, clones were then analyzed for the expression of the NERT protein (Figure 1b) and further selected by testing for transactivation of the RBP-J pathway in the presence or absence of 4-hydroxytamoxifen (OHT) (Figure 1c). In the absence of OHT, no transactivation was observed. After addition of OHT, the RBP-J pathway was transactivated in a concentration-dependent manner in all

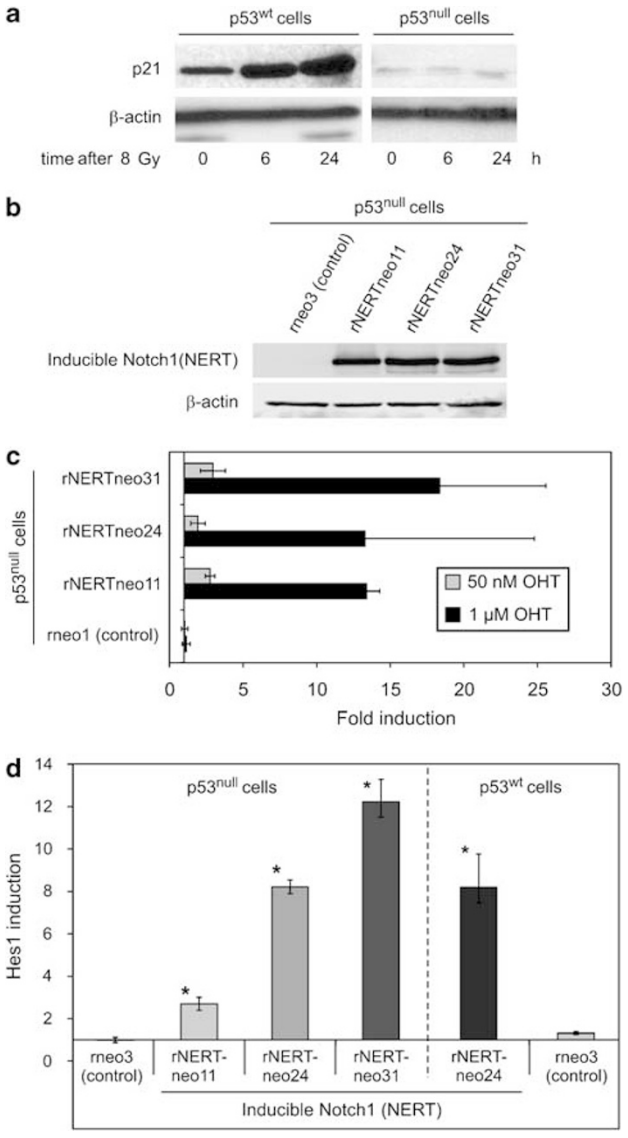

Figure 1 A Notch1 inducible system in p53^{null} FDCP-mix cells. (**a**) p53 is functional in p53^{wt} FDCP-mix cells, but not in p53^{null} FDCP-mix cells. p53^{wt} and p53^{null} FDCP-mix cells were treated with γ-radiation and cell lysates were prepared at indicated time points after irradiation. Western blots were performed using anti-p21 and anti-β-actin antibodies. (**b**) The NERT (inducible Notch1) protein is expressed in p53^{null} rNERTneo clones. p53^{null} FDCP-mix cells were stably-transfected with a retroviral vector carrying the NERT cDNA[9] or an empty control vector (rneo).[9] The correct-sized NERT proteins were detected by Western blotting with an antibody against the estrogen receptor (ERT) moiety. (**c**) Notch signaling is functional and inducible in p53^{null} cell clones expressing the NERT protein. Activation of Notch1^{IC} was determined by transient transfection of rNERTneo and rneo control p53^{null} cells with a reporter plasmid carrying the luciferase gene under the control of 12 RBP-J binding sites. Cells were treated with either 50 nM or 1 μM OHT for 24 h, and luciferase activity in cell lysates was determined. Mean induction values $\pm$ S.E.M. corrected for transfection efficiency are shown, respectively. The experiment was repeated three times with virtually identical results. (**d**) Activated Notch1 induces Hes1 expression in both p53^{null} and p53^{wt} FDCP-mix cells. FDCP-mix cells were cultured in the presence or absence of 500 nM OHT. Total RNA was harvested after 8 h and expression levels of Hes1 RNA were analyzed by real-time PCR. The experiment was repeated three times with virtually identical results. Hes1 is statistically and significantly upregulated in the presence of OHT ($^*P < 0.01$)

clones used in this report (Figure 1c). Control rneo clones did not express the NERT protein and did not transactivate the RBP-J pathway regardless of the addition of OHT (Figure 1b and c). To confirm that the Notch1-signaling pathway is activated by the addition of OHT, we determined the expression of endogenous Notch1 target genes of the Hes and Hey family in response to OHT treatment. In NERT-expressing $p53^{null}$ FDCP-mix cells, Hes1, and Hey1 expression was upregulated after the addition of OHT, whereas in control $p53^{null}$ FDCP-mix cells, the expression of Hes1 and Hey1 remained unaltered (Figure 1d and data not shown)

Reduction of proliferation by activated mNotch1 is dependent on p53. To evaluate if the reduction of proliferation of FDCP-mix cells by activated Notch1 is dependent on p53, cell numbers and [^{3}H] thymidine incorporation into DNA of proliferating wild-type p53 ($p53^{wt}$) and p53-deficient ($p53^{null}$) rNERTneo clones and control rneo clones, respectively, were measured in the presence and absence of OHT. Under conditions optimal for self-renewal and proliferation, that is, in the presence of high IL-3, cell numbers and [^{3}H] thymidine incorporation of rNERTneo cells were virtually identical to that of rneo control cells, for either $p53^{wt}$ FDCP-mix cells or $p53^{null}$ FDCP-mix cells, respectively, although generally $p53^{null}$ cells proliferated at a higher rate as $p53^{wt}$ cells (Figure 2a and data not shown). In contrast to the $p53^{wt}$ cells, however, in which induction of Notch signaling by OHT significantly reduced the proliferation in a concentration-dependent manner,[8] proliferation of $p53^{null}$ cells was not altered by OHT-induced Notch signaling (Figure 2a and data not shown). Proliferation of control rneo $p53^{wt}$ and $p53^{null}$ FDCP-mix cells was unaffected by OHT (Figure 2a and data not shown).

Since with [^{3}H] thymidine incorporation into DNA we can only determine the average proliferation of the whole population, we further assessed the kinetics of the activated Notch1-induced reduction in proliferation by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling.[14] In contrast to DNA labels, all cells in contact with sufficient concentrations of CFSE will become labeled regardless of their stage in the cell cycle. When a CFSE-labeled cell divides, CSFE is apportionated equally between the two daughter cells. Provided that the initial cell labeling and protein distribution between daughter cells are equal, a number of peaks of progressively halving CFSE fluorescence is measured. By using this approach, we confirmed the reduction in proliferation induced by activated Notch1 (Figure 2b).

Expression of $p53^{wt}$ restores reduction in proliferation by activated Notch1. To confirm that the reduction in cell proliferation is dependent on p53, and not caused by another unrelated mutation in the $p53^{null}$ FDCP-mix cells, we attempted to constitutively express $p53^{wt}$ in $p53^{null}$ FDCP-mix cells using several different expression vectors, including a previously described pCAG-based[15] p53 expression construct conferring Puromycin resistance to transfected cells. This proved to be difficult, because directly after re-expression of functional $p53^{wt}$ many cells died due to p53-induced apoptosis on the one hand, and on the other hand

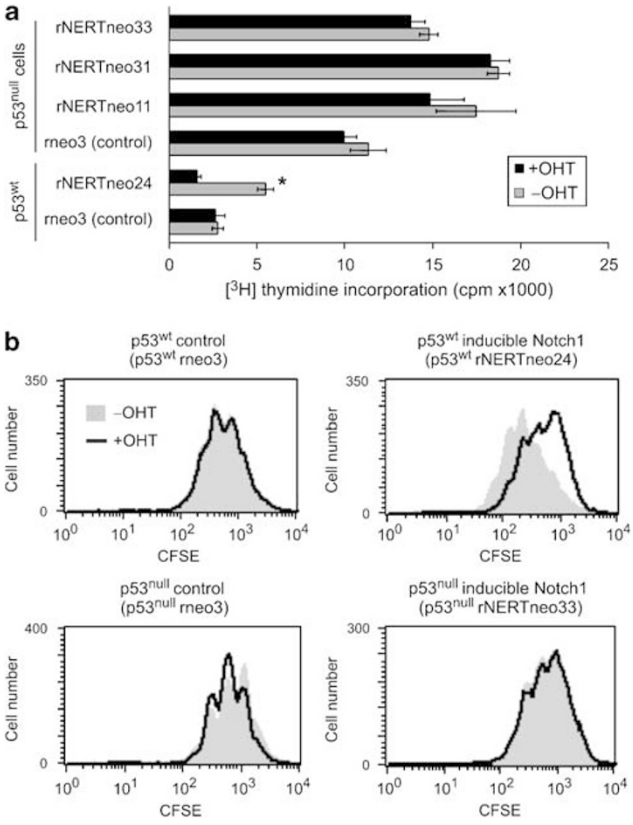

Figure 2 p53 is required for activated $Notch1^{IC}$-induced reduction in proliferation. To analyze the influence of activated Notch1 on the proliferation rate of $p53^{wt}$ and $p53^{null}$ FDCP-mix cells, rNERTneo cell clones were cultured under self-renewal conditions in the absence or presence of 500 nM OHT. (**a**) Cells were plated at 6×10^4 per ml in self-renewal conditions and cultured in the presence or absence of OHT for 24 h. [^{3}H] thymidine incorporation was measured in triplicate cultures as described in *Material and Methods*. The experiment was repeated four times with virtually identical results. Statistically significant reduction of proliferation was only observed in $p53^{wt}$ rNERTneo24 cells ($^*P < 0.001$). (**b**) Cells were labeled with CFSE at a concentration of 5 μM and CFSE intensity was measured after 3 days by FACS analysis. A representative example for each cell line is shown, respectively. The experiment was repeated two times with virtually identical results

the remaining living cell cultures lost functional p53 activity either due to the presence of spontaneous Puromycin resistant $p53^{null}$ cells that have a proliferative advantage because of their more rapid proliferation or due to mutations that lead to loss of p53 activity (data not shown). Nevertheless, we succeeded in achieving transient expression of functional $p53^{wt}$ in $p53^{null}$ FDCP-mix cells for up to 3 weeks (Figure 3a and b). Transfected $p53^{null}$ NERT FDCP-mix cell cultures, which expressed $p53^{wt}$ were then tested for the inhibition of proliferation by activated Notch. As shown in Figure 3c and d, re-expression of $p53^{wt}$ restored the reduction of proliferation of $p53^{null}$ NERT FDCP-mix cells by OHT-induced Notch signalling, while proliferation of control pCAG vector-transfected cells was not affected by OHT.

Activated Notch upregulates p21 expression in dependence of p53. $p21^{WAF/Cip}$ was originally identified as a downstream mediator of p53-induced growth arrest.[16,17] In addition, in keratinocytes, in which Notch signaling also induces growth arrest and differentiation, induction of p21

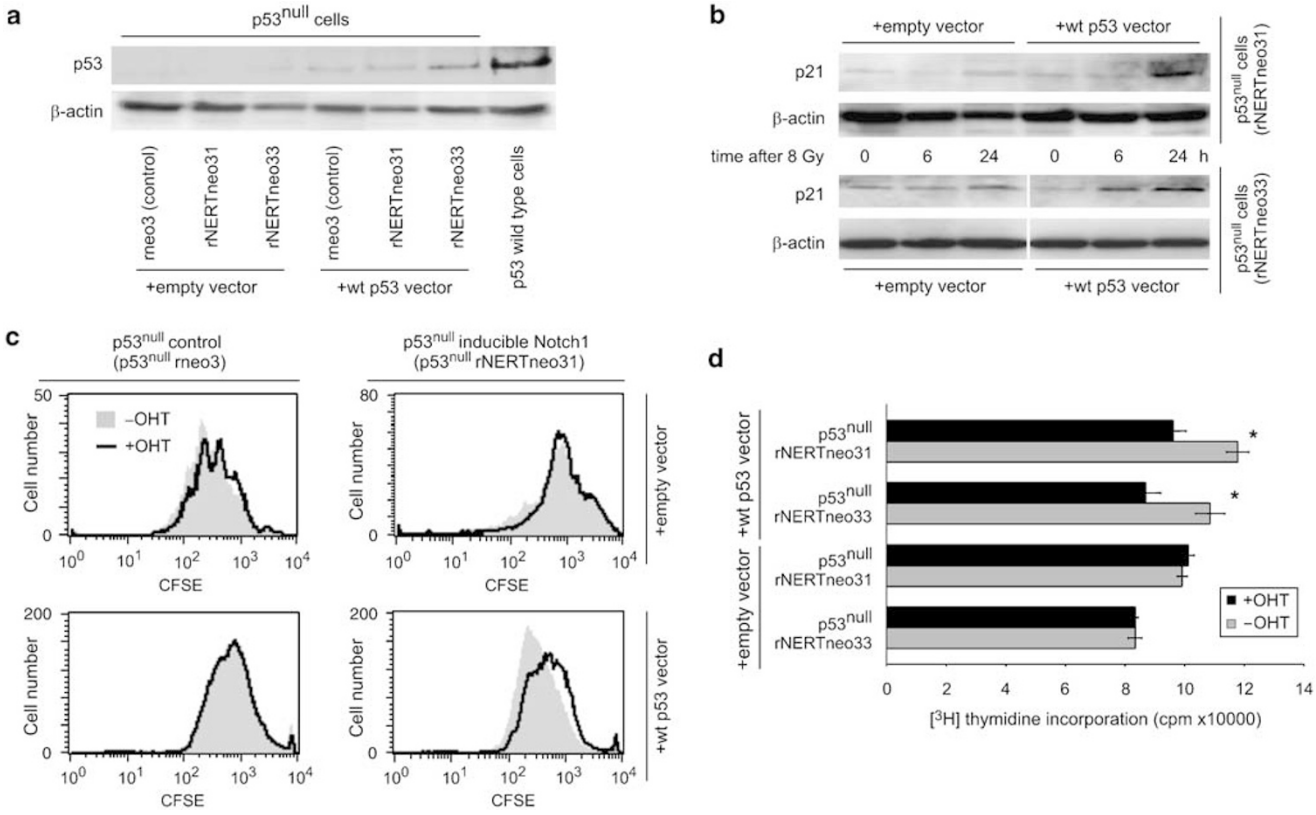

Figure 3 Reconstitution of the p53 status in p53^{null} cells restores Notch1-induced reduction in proliferation. p53^{null} FDCP-mix cells were transfected with a p53^{wt} expressing vector (pCAG-wt p53) or an empty control vector. (**a**) Expression of p53^{wt} protein in transfected p53^{null} FDCP-mix cells. The correct-sized p53 proteins were detected by Western Blotting with an antibody against p53. (**b**) Expression of p53^{wt} protein in transfected p53^{null} FDCP-mix cells restores p21 upregulation after γ-radiation. Transfected cells were treated with eight gray γ-radiation and cell lysates were prepared at indicated time points after irradiation. Western blots were performed using anti-p21 and anti-β-actin antibodies. (**c**) Transfected cells in selection with puromycin were labeled with CFSE at a concentration of 5 μM and CFSE intensity was measured after 3 days by FACS analysis. A representative example for each cell line is shown, respectively. The experiment was repeated three times with virtually identical results. (**d**) FDCP-mix p53^{null} cells were transfected with a p53^{wt} expressing vector (pCAG-wt p53) and puromycin-resistant cells were analyzed 3 weeks after transfection. Cells were plated in self-renewal conditions at 1×10^5 ml and cultured in the presence or absence of 500 nM OHT for 48 h. [^{3}H] thymidine incorporation was measured in triplicate cultures as described in Material and Methods. The experiment was repeated two times with virtually identical results. Reduction in the proliferation rate is significant for both p53^{wt} vector expressing rNERTneo p53^{null} cells (*$P < 0.001$)

expression by activated Notch was described.[18] Thus, a possible underlying mechanism for the Notch1-induced, p53-dependent, cell cycle arrest could be that Notch1 upregulates p21 expression in a p53-dependent manner. First, we determined the protein levels of p21 in p53^{wt} and p53^{null} NERTneo and rneo FDCP-mix clones in the presence or absence of OHT. After addition of OHT, p21 protein expression rapidly increased in p53^{wt} NERTneo FDCP-mix cells, but not in p53^{null} NERTneo FDCP-mix cells (Figure 4a). In control p53^{wt} and p53^{null} rneo FDCP-mix cells, the p21 protein levels remained unchanged by the addition of OHT (Figure 4a). Thus, Notch signaling upregulates p21 expression only in p53^{wt} cells. Nucleotide sequence analysis indicated that the promoter region of p21 contains two p53 binding site and one canonical RBP-J binding site (Figure 5a). To test whether activation of Notch1 signaling could induce the p21 promoter and further, whether this induction would require the presence of p53^{wt}, we determined the transactivation of the p21 promoter containing the p53 and RBP-J-binding sites in p53^{wt} rNERTneo and p53^{null} rNERTneo FDCP-mix cells, respectively, in the presence or absence of OHT. In the absence of OHT, no transactivation of the p21 promoter in either p53^{wt} or p53^{null} rNERTneo FDCP-mix cells was observed. After the addition of OHT, the p21 promoter was only induced in p53^{wt} cells but not in p53^{null} rNERTneo FDCP-mix cells (Figure 4b).

The 2.4 kb region of the p21 promoter contains a sequence that fully matches the consensus binding site of RBP-J[19] and two conserved p53 binding sites.[17] To test whether activated Notch1 transactivates the p21 promoter via the canonical RBP-J-binding site, luciferase reporter constructs containing either the functional RBP-J binding site of the p21 promoter or a mutated sequence (Figure 5a) that does not bind RBP-J[19] were transiently transfected into p53^{wt} rNERTneo and rneo FDCP-mix cells and luciferase activity was determined in the presence or absence of OHT. Induction of Notch signaling by the addition of OHT resulted in the transactivation of the construct containing the canonical RBP-J binding site after induction (Figure 5b). Addition of OHT to control rneo FDCP-mix cells did not activate transcription (Figure 5b). Unexpectedly, the mutant p21RBP-Jmut promoter compromised in

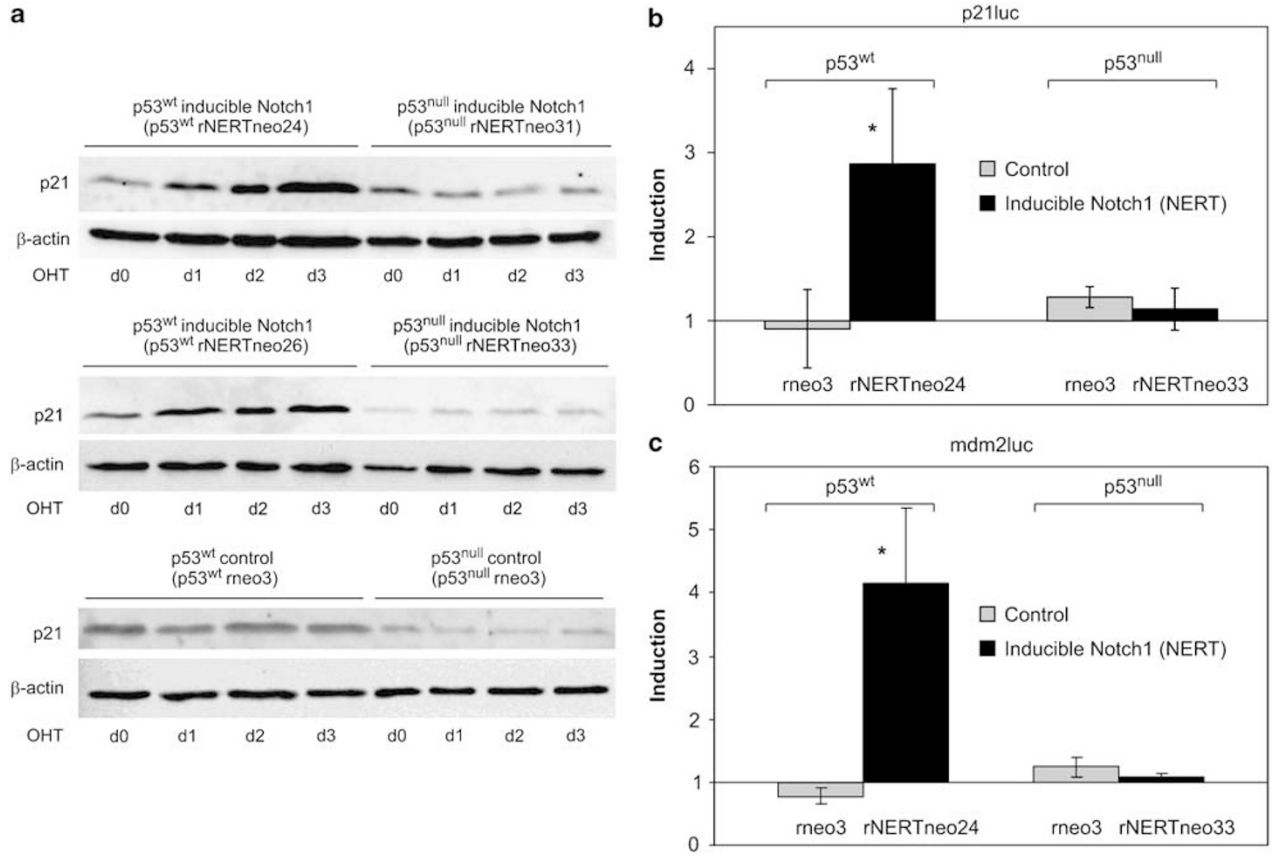

Figure 4 p21 expression is only activated by Notch1 signaling in $p53^{wt}$ cells. (**a**) Activated $Notch1^{IC}$ increases the level of p21 protein in $p53^{wt}$, but not in $p53^{null}$ FDCP-mix cells. $p53^{wt}$ rneo control, $p53^{null}$ rneo control, $p53^{wt}$ rNERTneo and $p53^{null}$ rNERTneo clones were treated with 500 nM OHT for up to 3 days (three independent experiments). Cell lysates were prepared at indicated time points after induction. Western blots were carried out using antibodies against p21 and actin. A representative Western blot analysis is shown. (**b**) Activated Notch1 induces p21 promoter activity in $p53^{wt}$ FDCP-mix cells, but not in $p53^{null}$ FDCP-mix cells. $p53^{wt}$ rneo control, $p53^{null}$ rneo control, $p53^{wt}$ rNERTneo and $p53^{null}$ rNERTneo clones were transiently transfected in triplicates with a reporter plasmid (p21luc) carrying a luciferase gene under the control of a 2.3 kb fragment of the p21 promoter shown in Figure 5a. Cells were cultured under self-renewal conditions in the absence or presence of 250 nM OHT for 16 h, and luciferase activity was measured and normalized to the activity of renilla luciferase. The means ± S.D. corrected for transfection efficiency are shown. The experiment was repeated three times with virtually identical results. Statistically significant upregulation of p21 promoter activity was only observed in $p53^{wt}$ rNERTneo24 cells ($^*P<0.02$). (**c**) Activated Notch1 induces MDM2 promoter activity in $p53^{wt}$ FDCP-mix cells, but not in $p53^{null}$ FDCP-mix cells. $p53^{wt}$ rneo control, $p53^{null}$ rneo control, $p53^{wt}$ rNERTneo and $p53^{null}$ rNERTneo clones were transiently transfected in triplicates with a reporter plasmid (mdm2luc) carrying a luciferase gene under the control of a 0.4 kb fragment of the mdm2 promoter. Cells were cultured under self-renewal conditions in the absence or presence of 250 nM OHT for 16 h, and luciferase activity was measured and normalized to the activity of renilla luciferase. The means ± S.D. corrected for transfection efficiency are shown. The experiment was repeated three times with virtually identical results. Statistically significant upregulation of p21 promoter activity was only observed in $p53^{wt}$ rNERTneo24 cells ($^*P<0.001$)

RBP-J binding was transactivated by induction of Notch1 signaling to a similar extent as the construct containing the canonical RBP-J binding site (Figure 5b). These data suggest that the canonical RBP-J site is not involved in mediating the Notch1-dependent transactivation of the p21 promoter. Next, we tested whether the transactivation of the p21 promoter by activated Notch1 would depend on the p53 binding sites. As shown in Figure 5c, deletion of either one of the p53 binding site led to a reduction in Notch1-dependent transactivation of the p21 promoter and deletion of both p53 binding sites completely abolished Notch1-induced transactivation of the p21 promoter. In line with these results, activated Notch1 also transactivated the mdm2 promoter, which does contain two p53 binding sites but no canonical RBP-J binding site (Figure 4c).

To test whether activated Notch would enhance binding of p53 to the endogenous promoter, chromatin preparations from $p53^{wt}$ rNERTneo FDCP-mix cells in the presence or absence of OHT were crosslinked to cellular DNA and immunoprecipitated with anti-p53 antibodies, followed by PCR amplifications of specific p21 promoter regions. As shown in Figure 6, the two fragments of the mouse p21 promoter containing the p53 binding sites, in particular binding site 2, were of higher abundance in the immunoprecipitates from rNERTneo FDCP-mix cells treated with OHT compared to rNERTneo FDCP-mix cells kept without OHT. As expected, no difference was observed after OHT treatment in the amount of immunoprecipitated fragments of a p21 promoter proximal control region that does not harbor p53 binding sites (Figure 6). These results were also confirmed by real-time PCR (data not shown). Taken together, these results provide evidence that Notch-signaling upregulates p21 expression by a p53-dependent mechanism involving enhanced binding of p53 to the p21 promoter.

Activated Notch promotes myeloid and erythroid differentiation independently of p53. Recently, we have shown that activated Notch1 induced myeloid and erythroid differentiation in p53^{wt} rNERTneo FDCP-mix cells.[8,20] Therefore, we asked whether differentiation into myeloid and erythroid cells by the induction of activated Notch1 was similarly accelerated in p53^{null} rNERTneo FDCP-mix cells in the presence of lineage-affiliated cytokines. In the presence of GM-CSF and G-CSF (myeloid differentiation conditions) differentiation is directed along the myeloid lineage and in the presence of erythropoietin and low amounts of IL-3 along the erythroid lineage. Thus, p53^{null} rNERTneo FDCP-mix cells, p53^{wt} rNERTneo FDCP-mix cells, p53^{null} control rneo FDCP-mix cells and p53^{wt} control rneo FDCP-mix cells were cultured under conditions that promote either myeloid differentiation or erythroid differentiation in the presence or absence of OHT and were monitored for changes in morphology and cell surface phenotype. As shown recently for p53^{wt} rNERTneo FDCP-mix cells,[8,20] differentiation along the myeloid as well as erythroid lineages was accelerated in the p53^{null} rNERTneo FDCP-mix cells (Tables 1 and 2). Differentiation of p53^{wt} and p53^{null} rneo control cells was unaltered by the addition of OHT (Tables 1 and 2). Since activated Notch1 accelerated myeloid and erythroid differentiation in both p53^{wt} and p53^{null} rNERTneo FDCP-mix cells, we analyzed whether activated Notch1 alters proliferation in differentiation conditions. While the activation of Notch1-signaling considerably reduced proliferation of p53^{wt} rNERTneo FDCP-mix cells (Table 3) in differentiation conditions,[8] Notch1 signaling did not alter proliferation during the first 4 days in differentiation medium in p53^{null} cells (Table 3). Only with the appearance of terminally differentiated cells, proliferation also started to decrease in p53^{null} rNERTneo FDCP-mix cells to a similar extent as in p53^{null} rneo FDCP-mix cells (data not shown). Taken together, our data show that activated Notch1 promotes differentiation of p53^{wt} and p53^{null} FDCP-mix cells along the myeloid and erythroid lineages, but activated Notch1 reduces cell proliferation only in p53^{wt} FDCP-mix cells and not in p53^{null} FDCP-mix cells.

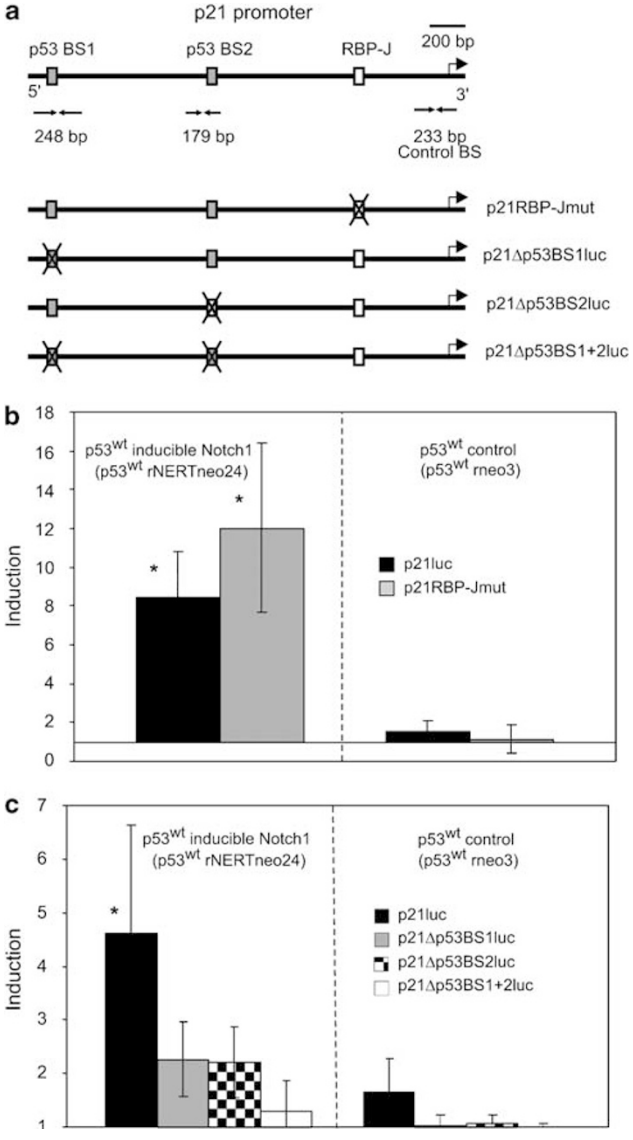

Figure 5 Activated Notch1 induces p21 promoter activity in p53^{wt} FDCP-mix cells in a p53-dependent manner. (**a**) The diagram shows the schematic p21 promoter used in this study with the known p53 binding sites and the consensus binding site for RBP-J. Amplicons for investigation of these sites and a control binding site in the ChIP assay (see Figure 6) are indicated by head to head arrows. Below are the luciferase constructs used in this assay: p21RBP-Jmut contains the p21 promoter with a mutated and non-functional RBP-J-binding site. The other plasmids used lack either the first p53 binding site (p21Δp53BS1luc), the second p53 binding site (p21Δp53BS2luc) or both p53 binding sites (p21Δp53BS1 + 2luc) of the p21 promoter. (**b**) Induction of the p21 promoter by activated Notch1 is not dependent on binding of RBP-J. The p53^{wt} control clone rneo3 and the p53^{wt} clone rNERTneo24 were transiently transfected in triplicates with a reporter plasmid carrying a luciferase gene either under the control of the p21 promoter (p21luc) or under a p21 promoter lacking a functional RBP-J binding site (p21RBP-Jmut). Cells were cultured under self-renewal conditions in the absence or presence of 250 nM OHT for 16 h, and luciferase activity was measured and normalized to the activity of renilla luciferase. The means ± S.D. corrected for transfection efficiency are shown. The experiment was repeated three times with virtually identical results. Statistically significant upregulation (asterisk) of p21 promoter activity was observed in p53^{wt} rNERTneo24 cells for both reporter constructs ($^*P<0.02$). (**c**) Induction of the p21 promoter by activated Notch1 is dependent on binding of p53. The p53^{wt} control clone rneo3 and the p53^{wt} clone rNERTneo24 were transiently transfected in triplicates with a reporter plasmid carrying a luciferase gene under the control of the p21 promoter (p21luc) or a luciferase gene under the control of a p21 promoter lacking either the first p53 binding site (p21Δp53BS1luc), the second p53 binding site (p21Δp53BS2luc) or both p53 binding sites (p21Δp53BS1 + 2luc). The cells were cultured under self-renewal conditions in the absence or presence of 250 nM OHT for 16 h, and luciferase activity was measured and normalized to the activity of renilla luciferase. The means ± S.D. corrected for transfection efficiency are shown. The experiment was repeated three times with virtually identical results. Statistically significant upregulation of p21 promoter activity was only observed in p53^{wt} rNERTneo24 cells for the construct p21luc ($^*P<0.02$)

Discussion

In the present work, we provide evidence that p53 is a critical determinant for Notch-mediated inhibition of proliferation: depending on the presence of functional p53, proliferation of multipotent hematopoietic progenitor cells is inhibited by activated Notch1. Recently, we showed that this inhibition of proliferation is related to a G_0/G_1 cell cycle arrest.[7] Induction of p21 expression is one of the earliest cell cycle regulatory

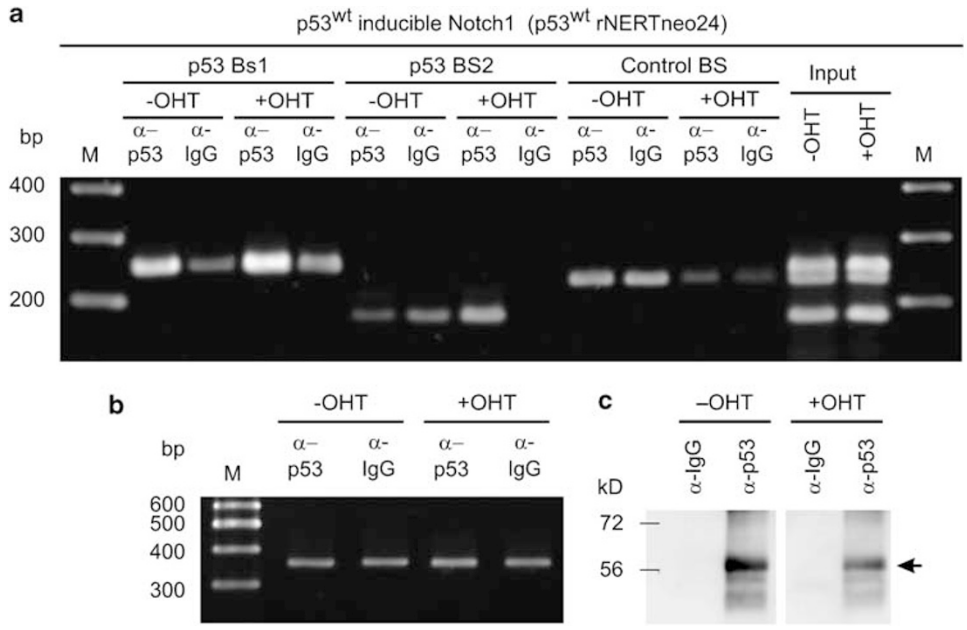

Figure 6 Notch1 signaling induces increased binding of the p53 protein to the endogenous p21 promoter. p53^{wt} rNERTneo24 cells were cultured under self-renewal conditions in the presence or absence of 500 nM OHT for 8 h. ChIP assays were performed as described in Material and Methods with antibodies specific for p53 or nonimmune IgG control followed by PCR amplifications of the p53 binding site 1 (BS1) and 2 (BS2) and an additional control binding site (no p53 binding) of the p21 promoter as indicated in Figure 5a. (**a**) Analysis of the BS1, BS2 and control binding site by semi-quantitative PCR. Additionally, 0.8% input DNA was analyzed in the multiplex reaction for all three-primer pairs. (**b**) Even DNA recovery of the different immunoprecipitated DNAs. Recovery was checked by spiking samples with external pUC18 DNA and semi-quantitative PCR. (**c**) p53 protein could be detected in the immunoprecipitated fractions. 1/12th of the beads bound fraction was analyzed by Western blotting with an antibody against p53

Table 1 Activated Notch1^{IC} accelerates myeloid differentiation of p53^{null} cells

p53^{null} clone	Culture conditions	% of cells after 4 days		
		BI	EG	LG
rneo1	−OHT	94	4	2
	+OHT	91	5	4
rNERTneo11	−OHT	94	2	4
	+OHT	78	5	17
rNERTneo24	−OHT	91	5	4
	+OHT	65	12	23
rNERTneo25	−OHT	96	4	0
	+OHT	79	11	10
rNERTneo31	−OHT	95	5	0
	+OHT	37	23	40

Cells were cultured under cytokine conditions that promote myeloid differentiation with or without 1 μM OHT. Differentiation of the cultures was followed by scoring the morphology of May–Grünwald–Giemsa stained cytospin preparations of the cells. BI, undifferentiated blasts; EG, early granulocytes (promyelocytes and myelocytes); LG, late granulocytes (metamyelocytes and segmented neutrophils). Data from a representative experiment are shown. The experiment was repeated two times with virtually identical results. The increase in myeloid differentiation by activated Notch^{IC} is statistically significant for all rNERTneo clones analyzed (clones 11, 24, 25, and 31, $P<0.001$)

Table 2 Activated Notch1^{IC} accelerates erythroid differentiation of p53^{null} cells

p53^{null} clone	Culture conditions	Ter119^{+} cells (%)
rneo 3	−OHT	22
	+OHT	21
rNERTneo11	−OHT	27
	+OHT	39
rNERTneo33	−OHT	20
	+OHT	25

Cells were cultured under cytokine conditions that promote erythroid differentiation with or without 1 μM OHT. Differentiation of the cultures was followed by FACS analyses of living cells using an antibody against Ter119. Data from a representative experiment are shown. The experiment was repeated two times with virtually identical results. The increase in erythroid differentiation by activated Notch^{IC} is statistically significant for all rNERTneo clones analyzed (clones 11 and 33, $P<0.001$)

events underlying growth arrest. We have shown here that Notch1 signaling upregulates p21 expression. This upregulation required the presence of p53. Because p53 can also cause cell cycle arrest by transcriptionally upregulating p21,[21] one possibility could be that activated Notch1 upregulates p53 expression. In several mammalian cell lines including human hepatocellular carcinoma cells, mouse neural progenitor cells, and human cervical cancer cells, expression of activated Notch1^{IC} elevated the levels of nuclear p53 and its target gene transcription.[22–24] In addition, two well known direct Notch target genes, Hes1 and Hey1, upregulate p53 activity through repression of HDM2 transcription.[5] However, since we did not see an upregulation of p53 mRNA and protein in p53^{wt} FDCP-mix cells after activation of Notch signaling (data not shown), it seems unlikely that the Notch1-induced proliferation arrest is mediated by an upregulation of p53 expression.

The p21 promoter used in this study contains two p53[17] and one RBP-J binding site. Although binding of the RBP-J protein to this RBP-J site as well as an involvement of RBP-J in Notch-dependent transactivation was recently shown in keratinocytes,[18] transactivation of the p21 promoter by

Table 3 Activated Notch1^{IC} does not affect proliferation of p53^{null} cells in differentiation conditions

	Cell number $\times 10^5$	
Cell clone	**−OHT**	**+OHT**
p53^{null}		
rneo1	1.8 ± 0.3	1.7 ± 0.4
rNERTneo11	1.6 ± 0.3	1.9 ± 0.4
rNERTneo24	1.5 ± 0.3	1.2 ± 0.4
rNERTneo25	1.6 ± 0.3	1.6 ± 0.4
rNERTneo31	1.5 ± 0.3	1.2 ± 0.4
p53^{wt}		
A7rneo1	1.3 ± 0.4	1.2 ± 0.3
A7rNERTneo24	1.3 ± 0.3	0.6 ± 0.2
A7rNERTneo25	0.7 ± 0	0.1 ± 0
A7rNERTneo26	1.2 ± 0.4	0.4 ± 0.1

p53^{null} and p53^{wt} FDCP-mix cells were cultured under cytokine conditions that promote myeloid differentiation with or without 1 μM OHT. Cell numbers were determined after 3 days (p53^{wt} cells) or 4 days (p53^{null} cells). Data from a representative experiment are shown. The experiment was repeated two times with virtually identical results. The reduction in proliferation is statistically significant for p53^{wt} A7rNERTneo cells (all p53^{wt} A7rNERTneo clones analyzed (clones 24, 25 and 26, $P<0.001$) but not for p53^{null} rNERTneo cells. The slight reduction of cell numbers in p53^{null} rNERTneo24 and p53^{null} rNERTneo31 clones results from terminally differentiated cells in the presence of OHT (compare Table 1)

activated Notch was not mediated via this RBP-J-binding site in hematopoietic progenitor cells. A recent report has indicated that Notch activity on RBP-J-responsive promoters critically depends on protein modules and promoter context.[25] Thus, one possibility to explain this discrepancy could be that activated Notch1 transactivates the p21 promoter via the RBP-J binding site in keratinocytes, but not in hematopoietic progenitor cells due to the lack of other essential cofactors in hematopoietic cells.

In addition to the RBP-J-dependent mechanism, Mammucari *et al*.[26] identified a second Calcineurin-dependent mechanism for transactivation of p21 by activated Notch1 in keratinocyte growth and differentiation control, acting on the p21 TATA box proximal region. While transactivation of the p21 promoter by increased Calcineurin/NFAT activity induced by Notch signaling required RBP-J and implies a crosstalk between these two signaling pathways, we show here a novel, RBP-J independent mechanism for transactivation of the p21 promoter by activated Notch1 that critically depends on the p53 binding sites of the p21 promoter. Deletion of both p53 binding sites of the p21 promoter completely abolished transactivation, suggesting that p53 is required for Notch1-mediated transactivation of p21. The activated Notch1-induced, increased binding of p53 to these sites further suggests that enhanced binding of p53 to the p21 promoter after activation of Notch1 signaling is involved in the activation of p21 transcription, thereby inducing a cell cycle arrest.

Post-translational modifications of the p53 protein enhance the ability of p53 to activate transcription.[27] Phosphorylation of p53 at various sites can lead to either stabilization of p53, activation of p53 activity or recruitment of CBP/p300 or PCAF and p53 acetylation. p53 acetylation can be involved in enhancing p53 DNA binding, activating p53 transcriptional activities and in p53 stabilization. Furthermore, specific combinations of post-transcriptional modifications generate distinct p53 cassettes that direct p53 towards precise cellular functions.[28] While acetylation at K320 suppresses the apoptotic program and activates promoters such as p21 leading to a temporary cell cycle arrest, acetylation at K373 together with phosphorylation at S46 and S15 stabilizes the interaction with p300 and activates promoters of proapoptotic genes.[28] Recently, it was shown that Notch1 interacts with p53 and inhibits its phosphorylation at S15 and S46[29] sites which were implicated in the study by Knights *et al*.[28] in directing p53 to promoters for proapoptotic genes thereby activating the proapoptotic program. In line with the lack of p53 modifications,[28] activated Notch1-inhibited p53-dependent transactivation and suppressed p53-dependent apoptosis of human colon cancer cells.[29] In addition, several other reports have indicated that Notch signaling may negatively regulate p53 function and inhibit apoptosis.[30–32] These data seem to be in contrast to other studies[18,22–24] and our data shown here and previously[7,8] that activated Notch1 reduces proliferation of hematopoietic progenitor cells through a p53-dependent pathway by blocking the cell cycle in G_0/G_1 but does not influence apoptosis. However, Notch effects as well as p53 modifications depend largely on the cell type, and considering the direct interaction of Notch with p53, it is tempting to speculate, that activated Notch may increase levels of p53 acetylated at K320, thereby blocking the cell cycle via p21.

Irrespective of the presence or absence of p53, Notch1-signaling promoted myeloid and erythroid differentiation of FDCP-mix cells as described previously.[8,20] This suggests that the Notch1-induced cell-cycle block and the induction of differentiation are unlinked events and most likely mediated by distinct biochemical mechanisms. Consistent with its function in temporarily halting the cell cycle under stress conditions, mice with a deficiency in p53 as well as mice with a deficiency in p21 have increased numbers of cycling hematopoietic stem cells under stress conditions, while hematopoietic differentiation is entirely normal.[33–35] Thus, activated Notch1 may reduce proliferation in dependence of p53 via p21 upregulation, however, the induction of myeloid and erythroid differentiation by increased Notch1-signaling occurs by a different p53-independent pathway. Recently, we have shown that activated Notch1 directly and specifically upregulates the hematopoietic transcription factor PU.1 in correlation with its induction of myeloid differentiation.[8] Along this line, we have identified β-globin as a direct Notch1 target gene involved in erythroid maturation.[20] This suggests that the induction of differentiation by Notch1 signaling involves hematopoietic transcription factors as well as functional proteins but not cell cycle regulators. The role of Notch signaling for self renewal, maintenance, and proliferation of adult hematopoietic stem and progenitor cells is controversial.[1] This may reflect the fact that the hematopoietic stem and progenitor compartment consists of several diverse cell types with different molecular signatures that are likely to respond in a different way to Notch signals. In the present study, we have used the multipotent cell line FDCP-mix that, although being an immortal cell line, shares many characteristics with primary common myeloid progenitor cells. It will be of considerable interest to determine, which of the hematopoietic stem and progenitor cell types respond in a similar way to Notch and p53 signaling

as the FDCP-mix cells. In line with our work with FDCP-mix cells, Notch-signaling triggers two distinct pathways in keratinocytes, involving a p21 and a RBP-J independent mechanism, respectively, leading to growth arrest and differentiation.[18] It remains to be tested whether p53 plays also a role for the Notch-induced growth arrest in keratinocytes.

Materials and Methods

Cell culture. FDCP-mix cells were maintained (self renewal conditions) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% pretested horse serum and mouse IL-3 conditioned medium at a concentration that stimulated optimal cell growth, which corresponds to 100 U rIL-3 per ml. FDCP-mix cells were kept in a density between 6×10^4 to 10^6 cells/ml and were carefully controlled for normal growth rates, factor dependency and differentiation. For activation of the OHT-inducible $mN1^{IC}$ FDCP-mix cells, 4-Hydroxy-Tamoxifen (OHT, RBI, USA) was added to the medium at the concentrations indicated. All cells were regularly checked to be free of mycoplasma contamination using a Mycoplasma PCR Elisa Kit (Roche, Germany).

Erythroid differentiation of cells was induced by washing the cells once in IMDM and plating 1×10^5 cells/ml in IMDM containing 20% pretested fetal calf serum (FCS), 5 U/ml erythropoietin (Roche, Germany) and 5 U/ml IL-3 (Roche, Germany). Myeloid differentiation of cells was induced by washing the cells once in IMDM and plating 1×10^5 cells/ml in IMDM containing 20% pretested FCS, 1000 U/ml G-CSF (Neupogen, Amgen, USA), 2 U/ml IL-3 (Roche, Germany) and 250 U recombinant murine GM-CSF per ml.[36] Aliquots were removed for analysis at time points indicated. To ensure optimal growth and differentiation conditions, the cells were split to constant density and fed with fresh differentiation medium every fourth day. Viable cells were counted by trypan blue dye exclusion. Differentiation of FDCP-mix cells was monitored by FACS analyses and by morphological scoring of May-Grünwald-Giemsa and *O*-Dianiside stained cytospin preparations. Considerable care was taken to validate accurate differential counts. All differential counts were done on 100–200 cells in a blinded fashion by TS and UJ.

Plasmids, stable transduction and selection procedures. To obtain clones of the $p53^{null}$ multipotent hematopoietic progenitor cell line,[11] in which translocation of the constitutive active intracellular domain of mNotch1 into the nucleus and RBP-J-dependent transactivation of target genes can be regulated, $p53^{null}$ FDCP-mix cells were transfected by electroporation with a retroviral vector carrying an intracellular domain of the murine Notch1 fused to the hormone-binding domain of the human estrogen receptor (rNERTneo[9]) and stable cell lines were established by G418 selection. As a control, cells were transfected with the retroviral vector alone (rneo[9]). Cell clones derived from three independent transfections were used in this study. To obtain $p53^{null}$ NERTneo cells, which express functional $p53^{wt}$, a DNA fragment containing the $p53^{wt}$ gene was subcloned into the pCAG-expression vector carrying the puromycin resistance gene,[15] and cells were transfected by electroporation or nucleofection (Amaxa, Germany). In brief, 5×10^6 cells were either electroporated as described previously[9] or nucleofected with 1 μg pCAG-p53 in solution R with program W-01. Mass cultures were selected with 0.5–2.0 μg Puromycin for 3–21 days and clonal cell lines were established after 3 weeks by cloning in soft agar in the presence of 2.0 μg Puromycin. Mass cultures were analyzed 3 days (CFSE staining) or 3 weeks ([^{3}H] thymidine incorporation) after transfection.

FACS analyses. Phycoerytrin (PE)- and Allophycocyanin (APC)-conjugated monoclonal antibodies directed against Ly76 (clone Ter119) and CD11b (Mac-1, clone M1/70), respectively, or their respective isotype controls were used (all Pharmingen, Europe). Cells were harvested by centrifugation and resuspended in PBS containing 3% FCS. Fc-Block (#01241D, Pharmingen, Europe) was added at a dilution of 1 : 100 for 5 min at room temperature (RT). Subsequently, antibodies were added at a dilution of 1 : 100. After an incubation for 20 min at RT in the dark, cells were washed and resuspended in PBS containing 1% FCS and 1 μg/ml propidium iodide or 7AAD for dead cell exclusion. FACS analysis was done with a Becton Dickinson FACSCalibur machine and Cell Quest software, or a Becton Dickinson FACSCanto machine and FACSDiva software using standard procedures.

Reporter constructs, transient transfections and luciferase assays. Cells (5×10^6) were transiently transfected by electroporation[9] and luciferase reporter assays[8] were done as described. The following constructs were used: For control of Notch activation: 1 μg of (RBP-J RE)12-Luc (pGa981-6, firefly luciferase reading frame under the control of a minimal β-globin promoter and 12 RBP-J-binding sites[37]); for transactivation of the p21 promoter: 18 μg of p21luc, which carries a firefly luciferase gene under the control of the -2.2 kb human *p21* promoter;[38] for transactivation of the mdm promoter: 18 μg of mdm2luc, which carries a firefly luciferase gene under control of the -350 bp human mdm2 promoter;[38] and either 0.1 μg phRL-CMV plasmids or 3 μg pTK-renilla plasmids (constitutive expression of Renilla-luciferase for transfection efficiency control), respectively. Cells were treated with different concentrations of OHT as indicated and measurements of luciferase activities were performed using the Dual Luciferase Kit (Promega) according to manufacturers instructions.

Mutation of the RBP-J binding site. Mutation of the RBP-J binding site within the p21-promoter (p21RBP-Jmut) was performed by a three-step site-directed PCR mutagenesis using the primers p21mut for (5′-gagggatcacacc gtctagaggtgatattgtggg-3′), p21mut rev (5′-tcacctctagacggtgtgatccctcactaggtca-3′), *Nhe*I for (5′-cggtcccggaacctcgcgtgctgcagagg-3′) and *Pst*I rev (5′-tacgcgtgctagccc gggctc-3′) and the plasmid p21luc as a template. Deletion of the p53 binding sites was performed by the same method using the primers p53BS1 for (5′-ggccattagagctctggcatagaagag-3′), p53BS1 rev (5′-gccagagctctaatggccagaaag cca-3′), *Xho*I for (5′-tcttacgcgtgctagcccgggctcgagatc-3′), and *Ava*III rev (5′-caag cacacatgcatcagatcc-3′) for deletion of the first p53 binding site and primers p53BS2 for (5′-agaggaagagagatttccagactctga-3′), p53BS2 rev (5′-ggaaatctctcttcctctaacgca gct-3′), *Pst*I rev and *Nhe*I for for deletion of the second p53 binding site. Mutation of the RBP-J binding site and deletion of the p53 binding sites were confirmed by sequencing.

CFSE-assay. Cells (5×10^6) were incubated with CFSE (Sigma, Munich) at a final concentration of 5 μM in 1 ml PBS containing 0.1% BSA for 5 min at 37°C. The reaction was stopped by adding 10 ml cold-medium containing 20% horse serum and incubation on ice for 5 min. The cells were washed two times and resuspended at a concentration of 2×10^5 cells/ml in self-renewal medium in the presence or absence of 500 nM OHT. Proliferation was analyzed after 3 days by FACS analysis.

[^{3}H]-thymidine incorporation. Cells were plated in self-renewal medium at either 6×10^4 or 1×10^5 per ml in the absence or presence of 500 nM OHT. After 24 or 48 h, [^{3}H] thymidine incorporation was measured as described previously.[7] Data are expressed as mean counts per minute of triplicate wells ± S.D.

ChIP assay. The ChIP was performed as described previously[39] with slight modifications. Briefly, 6.5×10^7 FDCP-mix cells (A7rNERTneo24 cells) were induced or not induced for 8 h with 500 nM OHT, washed with PBS and resuspended in 1.5 ml SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) plus Complete protease inhibitor (Roche). To confirm the induction of the Notch-signaling pathway, the upregulation of Hes1 and Hey1 transcripts was checked by real-time PCR as described previously[40] (data not shown). 2×400 μl of the lysate were sonified and precleared with 70 μl Protein A (Upstate, Charlottesville, VA, USA)/Protein G (Active Motif, Carlsbad, CA, USA) agarose beads mix (1 : 1, Salmon Sperm saturated) for 1 h at 4°C. 10 μg of anti-p53 antibody (Pab240) or 10 μg anti-mouse IgG (Active Motif) were added as a control, incubated for 1 h at 4°C, and 150 μl Protein A/G slurry mix were added overnight. Additionally, samples were spiked with 100 ng of pUC18 vector DNA as an external control for recovery. The beads were washed and crosslinks were reversed as described in the Upstate ChIP protocol. DNA was purified using the ChIP-IT purification columns (Active Motif) and eluted in a 100 μl H_2O. Five microliter of this DNA were measured either in a semi-quantitative PCR using the Multiplex PCR Kit-100 (Qiagen, Hilden, Germany) in 50 μl or in a real-time PCR using FastStart Universal SYBR Green Master Mix (Rox) (Roche Diagnostics, Mannheim, Germany) in a 10 μl volume. Primers for p53-binding site within the p21 promoter at a final concentration of 100 nM were: BS1 forward: 5′-cccttggatttccttttctatcag-3′; BS1 reverse: 5′-gtagttgggtatcatcaggtctcc-3′; BS2 forward: 5′-tgtgtttctgaacaggatgagg-3′; BS2 reverse: 5′-tgagttctgacatctgctctcc-3′; control BS (no p53 binding) forward: 5′-gttcatagatgtatgtggctctgc-3′; control BS reverse: 5′-gtcgagctgcctccttatagc-3′; pUC forward: 5′-aagttggccgcagtgttatc-3′; pUC reverse: 5′-tttgccttcctgtttttgct-3′. For semi-quantitative PCR BS1, BS2, and control BS were cycled 36 times and pUC was cycled 28 times (protocol on request).

Western Blotting. Cell were cultured under self-renewal conditions and in the presence or absence of 500 nM OHT. Harvesting, electrophoresis and Western Blotting of protein extracts were performed as described previously.[9] Ten micrograms of protein was separated per lane. Antibodies specific for p21 (sx-118, Pharmingen/BectonDickinson, Heidelberg, Germany), β-actin (C2, Santa Cruz Biotechnology, CA, USA) or estrogen receptor (SC-8002, Santa Cruz) were used. The detected proteins were visualized by the ECL system (Amersham Pharmacia Biotec, UK). For control of the ChIP assay 25 μl of the Protein A/G slurry were incubated in SDS loading buffer and half of it were used for a Western blot. Pab240 anti-p53 antibody (1 ng/ml) was used to detect the precipitated p53.

Statistical analysis. Statistical differences were assessed using the Student's *t*-test for paired data.

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