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Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors $p27^{Kip1}$ and $p57^{Kip2}$

Orbicia Riccio[1], Marielle E. van Gijn[2], April C. Bezdek[1], Luca Pellegrinet[1], Johan H. van Es[2], Ursula Zimber-Strobl[3], Lothar J. Strobl[3], Tasuku Honjo[4], Hans Clevers[2] & Freddy Radtke[1+]

[1]Ecole Polytechnique Fédérale de Lausanne (EPFL), Swiss Institute for Experimental Cancer Research (ISREC), Epalinges, Switzerland, [2]Hubrecht Institute, CT Utrecht, The Netherlands, [3]Institute for Clinical Molecular Biology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Munich, Germany, and [4]Research Institute, Shiga Medical Center, Moriyama-shi, Shiga, Japan

The crucial role of individual Notch receptors and the mechanism by which they maintain intestinal crypt progenitor cells were assessed by using a series of inducible gut-specific Notch mutant mice. We found that Notch1 and Notch2 receptors function redundantly in the gut, as only simultaneous loss of both receptors results in complete conversion of proliferating crypt progenitors into post-mitotic goblet cells. This conversion correlates with the loss of Hes1 expression and derepression of the cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p57^{Kip2}$. We also found that the promoter of both CDK inhibitor genes is occupied by the Notch effector Hes1 in wild-type crypt progenitor cells. Thus, our results indicate that Notch-mediated Hes1 expression contributes to the maintenance of the proliferative crypt compartment of the small intestine by transcriptionally repressing two CDK inhibitors.

Keywords: Notch; intestine; CDKI; growth arrest; differentiation

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INTRODUCTION

The intestinal epithelium is a self-renewing tissue with a high turnover rate and cellular processes such as proliferation, migration and cell death must be highly regulated to ensure homeostasis of the intestinal epithelium. Despite their diversity, these processes are controlled by a relatively small number of signalling pathways. Until recently, the Wnt pathway was thought to be the predominant signalling cascade active in regulating the pool of undifferentiated crypt progenitor cells, as inactivation of Tcf4 (transcription factor 4) or β-catenin (two downstream mediators of Wnt signals), or overexpression of the soluble Wnt inhibitor Dickkopf1, results in the loss of the proliferative crypt compartment (reviewed by Radtke & Clevers, 2005).

Another signalling pathway that has received attention in recent years because of its involvement in gut homeostasis is the Notch cascade. Notch proteins comprise a family of four single transmembrane-bound receptors that influence cell fate decisions and differentiation processes through cell-to-cell signalling in many different organisms (Artavanis-Tsakonas *et al*, 1999). Notch signalling is triggered by ligand binding, inducing a cascade of proteolytic cleavages that release the intracellular domain of Notch receptors (NICD). NICD then translocates to the nucleus, where it binds to the transcription factor Rbpj (recombination signal sequence binding protein for Jk genes, also known as CBF-1 or Csl) and activates target gene transcription. Some of the best-known targets belong to the family of hairy/enhancer of split (Hes) genes, which function as transcriptional repressors.

Expression of several components of the Notch cascade in the murine intestine suggested that Notch signalling might have important functions in the gut (Schroder & Gossler, 2002; Sander & Powell, 2004). Pharmacological studies using γ-secretase inhibitors (Milano *et al*, 2004; Wong *et al*, 2004) and genetic loss-of-function experiments in mouse for *Hes1* (Jensen *et al*, 2000) and *Math1* (Yang *et al*, 2001), or in zebrafish for *DeltaD* (a Notch ligand) and *mindbomb* (Crosnier *et al*, 2005), suggest that Notch signalling might regulate a binary cell fate decision of intestinal progenitors that must choose between the secretory and absorptive lineages.

Furthermore, inducible tissue-specific loss-of-function and gain-of-function studies of Notch signalling components in the

[1]Ecole Polytechnique Fédérale de Lausanne (EPFL), Swiss Institute for Experimental Cancer Research (ISREC), Chemin des Boveresses 155, Epalinges 1066, Switzerland
[2]Hubrecht Institute, Uppsalalaan 8, CT Utrecht 3548, The Netherlands
[3]Institute for Clinical Molecular Biology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Marchioninistrasse 25, 81377 Munich, Germany
[4]Research Institute, Shiga Medical Center, 5-4-30 Moriyama, Moriyama-shi, Shiga 524-8524, Japan
[+]Corresponding author. Tel: +41 21 692 59 64; Fax: +41 21 652 69 33; E-mail: freddy.radtke@epfl.ch

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murine intestine point to an additional role for Notch signalling in the maintenance of proliferative and undifferentiated crypt progenitor compartments. Conditional gut-specific inactivation of Rbpj, which mediates Notch signalling of all four Notch receptors, results in the complete loss of proliferating crypt progenitors followed by their conversion into post-mitotic goblet cells (van Es *et al*, 2005). In reciprocal gain-of-function studies, expression of NICD in the intestine inhibits differentiation of crypt progenitor cells, resulting in a large increase in undifferentiated transient amplifying cells (Fre *et al*, 2005). These complementary loss-of-function and gain-of-function studies indicate an essential role for Notch signalling as a gatekeeper of the gut progenitor compartment.

As the mechanism by which Notch exerts this function is currently unknown, we used inducible tissue-specific loss-of-function approaches to investigate the role of the Notch1 and Notch2 receptors in the intestine, and how they maintain the undifferentiated crypt compartment.

RESULTS AND DISCUSSION

To investigate which of the Notch receptors mediates the putative gatekeeper function in the crypt progenitor compartment of the small intestine, the expression of *Notch1* (*N1*) and *Notch2* (*N2*) was analysed by using *in situ* hybridization. Both receptors are most prominently expressed in epithelial cells of the crypts (Fig 1A), whereas *Notch3* and *Notch4* are expressed in the villus mesenchyme and endothelial cells, as previously shown (Schroder & Gossler, 2002; Sander & Powell, 2004). These expression studies show that signalling through N1 and/or N2 is important for maintaining the undifferentiated crypt compartment. To investigate directly the roles of both Notch receptors in crypt homeostasis, mice with floxed (loxP sequence containing) alleles of *N1* (Radtke *et al*, 1999), *N2* (Besseyrias *et al*, 2007) or both genes were crossed with transgenic mice expressing Cre-ERT2 under the control of the villin promoter (el Marjou *et al*, 2004). Twelve-day-old *Notch1*$^{lox/lox}$*-vil-Cre-ERT2* (*N1 KO*), *Notch2*$^{lox/lox}$*-vil-Cre-ERT2* (*N2 KO*), *Notch1/Notch2*$^{lox/lox}$*-vil-Cre-ERT2* (*N1N2 dKO*), *Rbpj*$^{lox/lox}$*-vil-Cre-ERT2* (*Rbpj KO*) and corresponding littermate controls lacking the *vil-Cre-ERT2* transgene were injected intraperitoneally with tamoxifen on 5 consecutive days and analysed 12 days after the last injection (the deletion efficiency of the corresponding genes is shown in supplementary Fig 1 online). The body weight of these mice was controlled daily. Littermate control animals and mice with gut-specific inactivation of *N1* or *N2* alone gained weight normally. By contrast, mice in which *N1* and *N2* were simultaneously inactivated did not gain weight, similar to mice with gut-specific inactivation of the *Rbpj* gene (van Es *et al*, 2005; Fig 1B). Histological analysis showed that inactivation of the *N1* or *N2* genes alone did not result in a gut phenotype, as the morphology of the intestine of either gene-targeted mice was comparable to that of littermate controls (supplementary Fig 2 online). However, simultaneous inactivation of both Notch receptors resulted in the same phenotype as loss of *Rbpj* function in the gut. The entire crypt compartment was replaced by post-mitotic goblet cells as shown by haematoxylin/eosin and Alcian blue staining (Fig 2A,B). Ki67 staining (Fig 2C) and 5-bromodeoxyuridine incorporation (Fig 2D) showed a complete lack of proliferating cells in these mice, whereas the proliferative crypt compartment of *N1*- or *N2*-deficient mice appeared to be normal. Paneth cells were present in the crypts of

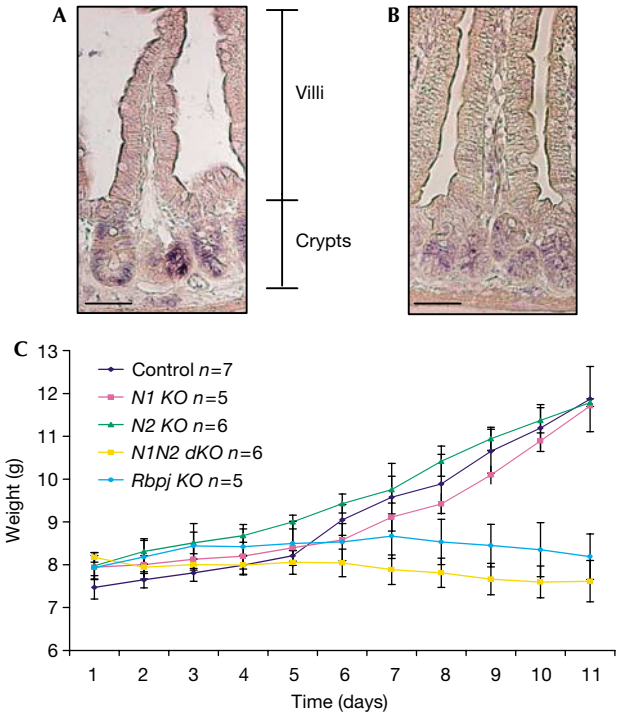

Fig 1 | Expression pattern of Notch receptors in the small intestine of wild-type mice. *In situ* hybridization for (**A**) N1 and (**B**) N2 shows expression of both receptors in the crypts of the small intestine. Scale bars, 50 μm. (**C**) Bodyweight curves of control (*N1/N2*$^{lox/lox}$), *N1 KO* (*Notch1*$^{lox/lox}$*-vil-Cre-ERT2*), *N2 KO* (*Notch2*$^{lox/lox}$*-vil-Cre-ERT2*), *N1N2 dKO* (*Notch1/Notch2*$^{lox/lox}$*-vil-Cre-ERT2*) and *Rbpj KO* (*Rbpj*$^{lox/lox}$*-vil-Cre-ERT2*) post tamoxifen-induced Cre activation. The number of mice analysed is indicated.

all gene-targeted mice, as shown by Ledrum's phloxine tartazine staining, and the number of synaptophysin-positive enteroendocrine cells was unchanged (data not shown). These results indicate that *N1* and *N2* function redundantly in the intestine, as signalling through only one of the two receptors is sufficient to maintain the crypt compartment. γ-Secretase inhibitors are under development for the treatment of Alzheimer patients, and recently also for treatment of T-cell leukaemia patients to block Notch signalling in cancer cells (Aster, 2005). However, most of these γ-secretase inhibitors show unwanted side effects such as goblet cell metaplasia within the crypt compartment (Milano *et al*, 2004; Wong *et al*, 2004), which is recapitulated in mice with gut-specific inactivation of both *N1* and *N2*. These results indicate that drugs now being developed inhibit Notch signalling mediated by both N1 and N2 receptors, and thus most likely signalling through all Notch receptors. The development of γ-secretase inhibitors that can specifically block N1 but not N2 signalling would certainly attenuate or even fully extinguish the unwanted intestinal side effects, as signalling through N2 alone is sufficient to maintain gut homeostasis.

To gain further mechanistic insights into how Notch signalling maintains the proliferation of crypt progenitors, and with the knowledge that Wnt signalling is also involved in this function, we investigated a potential cross-talk between the two signalling

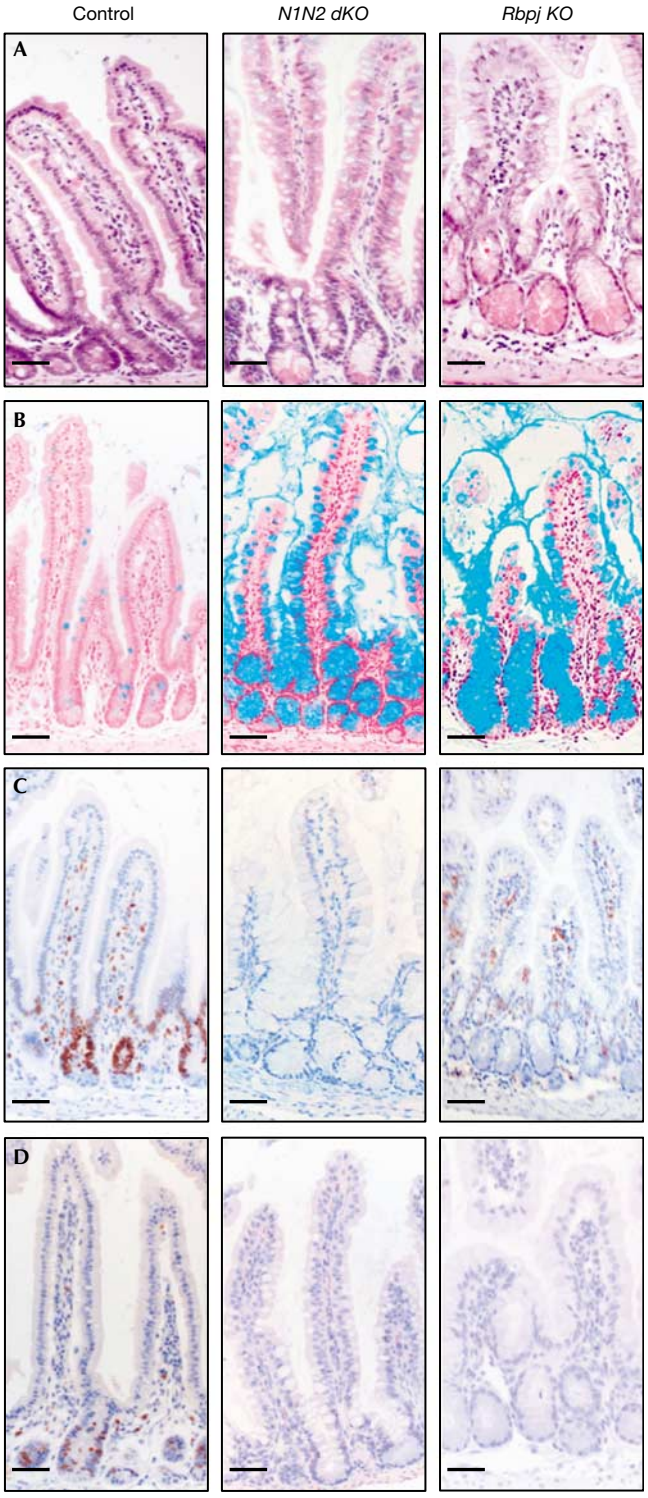

Fig 2 | Redundant function of N1 and N2 signalling in the small intestine. Conversion of the proliferative crypt compartment into post-mitotic goblet cells occurs only after simultaneous inactivation of both N1 and N2. Representative sections of the small intestine from control, *N1N2 dKO* (*Notch1/Notch2*$^{lox/lox}$*-vil-Cre-ERT2*) and *Rbpj KO* (*Rbpj*$^{lox/lox}$*-vil-Cre-ERT2*) stained with (**A**) haematoxylin and eosin, (**B**) Alcian blue for goblet cell identification, and antibodies against (**C**) Ki67 and (**D**) 5-bromodeoxyuridine to identify proliferating crypt progenitors. Scale bars, 50 μm. N1, Notch1; N2, Notch2.

cascades. Proliferating crypt cells show active Wnt signalling, which is characterized by the accumulation of nuclear β-catenin and expression of Wnt target genes such as *CD44* (van de Wetering *et al*, 2002). Expression studies for CD44 and β-catenin performed in all Notch mutant mice indicate that the Wnt cascade is still active even though crypt progenitor cells no longer proliferate in *N1N2 dKO* and *Rbpj*-deficient intestines (supplementary Fig 3 online). Mechanistically, active Wnt signalling was shown to sustain the proliferation of cells in the crypt by β-catenin/Tcf4-dependent upregulation of c-Myc, which subsequently binds to Miz1 and represses the cell-cycle inhibitor p21$^{Cip1/Waf1}$ (van de Wetering *et al*, 2002). As no differences were observed in β-catenin and CD44 expression in Notch-deficient intestines, we investigated whether Notch signalling controls the proliferation of crypt progenitor cells by influencing the expression of p21$^{Cip1/Waf1}$ in a β-catenin-independent manner. Therefore, RNA from crypt sections derived from control, *N1N2 dKO* and *Rbpj KO* mice was isolated by laser capture microdissection to perform quantitative reverse transcription–PCR (RT–PCR) for p21$^{Cip1/Waf1}$. The expression levels of *p21*$^{Cip1/Waf1}$ for control, *N1N2 dKO* and *Rbpj KO* crypts were similar (Fig 3A), indicating that Notch signalling does not regulate proliferation of crypt progenitor cells through the cell-cycle regulator p21$^{Cip1/Waf1}$. To confirm genetically that the cell-cycle arrest of Notch-deficient crypt cells is not mediated by p21$^{Cip1/Waf1}$, *p21*$^{Cip1/Waf1-/-}$ mice were intercrossed with *Rbpj KO* mice. Intestines deficient for both Rbpj and p21$^{Cip1/Waf1}$ or for p21$^{Cip1/Waf1}$ alone were compared with those of littermate controls. The intestines of *p21*$^{Cip1/Waf1}$-deficient and control animals were indistinguishable, as they had normal morphology, goblet cell content and proliferation, as shown by haematoxylin/eosin, periodic acid-Schiff (PAS) and Ki67 staining (supplementary Fig 4 online). Simultaneous loss of Rbpj and p21$^{Cip1/Waf1}$ did not allow crypt progenitor cells to re-enter the cell cycle. The intestines of these mice are identical to *Rbpj KO* mice, as they show complete conversion of crypt progenitor cells into post-mitotic goblet cells, as shown by PAS and Ki67 staining (supplementary Fig 4 online), indicating that the cell-cycle arrest in Notch signalling-deficient crypt cells is not mediated by p21$^{Cip1/Waf1}$. To investigate whether the cell-cycle arrest in Notch signalling-deficient intestines is mediated by another family member of the cyclin-dependent kinase inhibitors, quantitative RT–PCR for *p27*^{Kip1} was performed on RNA from laser capture microdissected crypt cells from control, *N1N2 dKO* and *Rbpj KO* mice. The expression levels of *p27*^{Kip1} increased 400- and 500-fold in *N1N2 dKO* and *Rbpj KO* animals, respectively (Fig 3A). To confirm these messenger RNA expression studies at the protein level, immunostaining for p27^{Kip1} was performed. In littermate controls, p27^{Kip1} protein expression was found exclusively in the nuclei of the villi, whereas crypt cells were negative (Fig 3B). However, nuclear p27^{Kip1} staining was found in crypt cells of *N1N2*- and *Rbpj*-deficient mice (Fig 3C,D). These results show that Notch signalling within the small intestine represses the

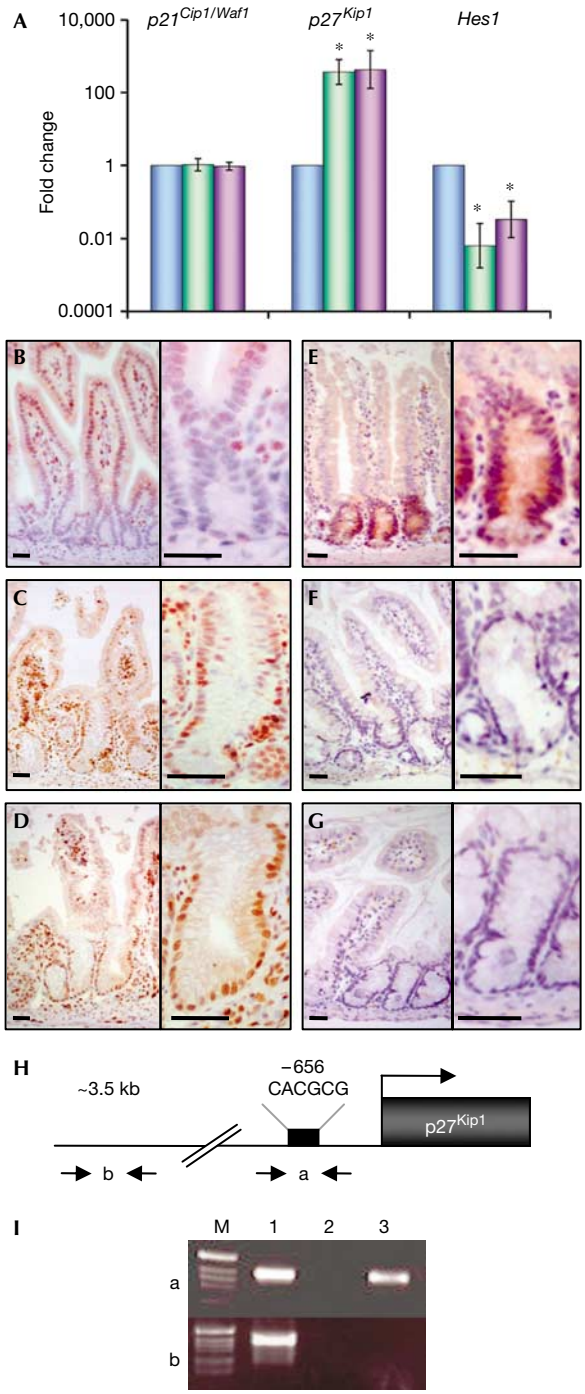

Fig 3 | Loss of Notch signalling in the crypt compartment results in loss of Hes1 expression and derepression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. (**A**) Crypt regions of control (blue), *N1N2 dKO* (*Notch1/Notch2*$^{lox/lox}$*-vil-Cre-ERT2*; green) and *Rbpj KO* (*Rbpj*$^{lox/lox}$*-vil-Cre-ERT2*; purple) mice were laser microdissected and RNA isolated for quantitative reverse transcription–PCR. Relative levels of messenger RNA of *p21*$^{Cip1/Waf1}$, *p27*^{Kip1} and *Hes1* genes normalized with glyceraldehyde phosphate dehydrogenase are shown (logarithmic scale, $*P<0.05$; results represent the average of four independent experiments). (**B–D**) Immunohistochemical analysis of sections of the small intestine from (**B**) control, (**C**) *N1N2 dKO* and (**D**) *Rbpj KO* stained with an antibody against $p27^{Kip1}$ bars. (**E–G**) Immunostaining for Hes1 on sections of the small intestine derived from (**E**) control, (**F**) *N1N2 dKO* and (**G**) *Rbpj KO*. The right-hand panels show a higher magnification of crypts from the left-hand panels. Scale bars, 50 μm. (**H**) Schematic representation of the *p27*^{Kip1} promoter indicating the primers used (a and b) for the chromatin immunoprecipitation (ChIP) analysis. The black rectangle corresponds to a class C Hes1-binding site (CACGCG; Murata *et al*, 2005). Primers indicated by 'b', 3 kb upstream of the ATG, were used for control PCR. (**I**) ChIP assay. Epithelial cells from crypt enrichment preparations were processed for ChIP with an antibody against Hes1 (lane 3) and purified rabbit IgGs as a control (lane 2). PCRs of two distinct regions indicated by 'a', containing the class C Hes1-binding site, and by 'b', an Hes1-binding-site-free region, which was used as a negative control, are shown. Lane 1 corresponds to the input DNA.

cyclin-dependent kinase inhibitor *p27*^{Kip1} to maintain proliferating crypt progenitor cells.

Hes gene family members are among the best-characterized target genes of Notch signalling (Kageyama *et al*, 2005). Furthermore, the fetal intestines of *Hes1*$^{-/-}$ mice show increased numbers of goblet cells at the expense of adsorptive enterocytes (Jensen *et al*, 2000), indicating that the phenotype observed in Notch signalling-deficient mice might be, at least in part, mediated by the loss of *Hes1* expression. To investigate this possibility further, we performed quantitative RT–PCR followed by immunostaining for Hes1. Hes1 mRNA levels showed a 50- and 40-fold decrease in *N1N2*- and *Rbpj*-deficient animals, respectively, compared with controls (Fig 3A). Hes1 immunostaining was confined to the crypt compartment in control animals (Fig 3E), whereas it was not detected in N1N2- and Rbpj-deficient crypts (Fig 3F,G), confirming the role of *Hes1* as a target gene of *N1* and/or *N2* in the small intestine.

Protein levels of the cell-cycle regulator $p27^{Kip1}$ in cycling cells are regulated at the transcriptional and post-transcriptional levels by protein degradation through the ubiquitin–proteasome system (Hengst & Reed, 1996). Notch signalling has been linked to both of these regulatory mechanisms. One study indicates that $p27^{Kip1}$ expression is repressed by Hes1 at the transcriptional level (Murata *et al*, 2005), whereas another report suggests that N1 controls expression of the S-phase kinase-associated protein 2 (Skp2)—the F-box subunit of the ubiquitin-ligase complex SCF^{Skp2} that targets proteins for degradation (Sarmento *et al*, 2005). We investigated the expression of Skp2 by quantitative RT–PCR and immunohistochemistry. However, we were unable to detect Skp2 expression at either mRNA or protein levels on gut sections derived from the different gene-targeted mice. Therefore, we focused our studies on the possibility that Hes1 might directly bind to, and thereby repress, the *p27*^{Kip1} promoter, as previously suggested (Murata *et al*, 2005). Promoter analysis identified one important Hes1-binding site (class C site) within the *p27*^{Kip1} promoter that conveys transcriptional repression (Murata *et al*, 2005). Therefore, we performed a chromatin immunoprecipitation (ChIP) assay on isolated crypts from wild-type mice to investigate whether Hes1 binds to the *p27*^{Kip1} promoter in crypt progenitor

cells. DNA of the *p27^{Kip1}* promoter region including a proximal class C site, but not that of the *p27^{Kip1}* upstream sequence, was specifically detected in the Hes1 immunoprecipitated DNA complex from formaldehyde-treated crypt cells, indicating Hes1 occupancy of the p27^{Kip1} promoter *in vivo*. These results show that Notch-induced *Hes1* expression contributes to the transcriptional repression of the *p27^{Kip1}* gene *in vivo*. Whether other *Hes* gene family members that are also expressed in the crypt compartment contribute to the repression of *p27^{Kip1}* remains to be investigated.

To confirm genetically that upregulation of p27^{Kip1} owing to loss of Notch signalling causes crypt progenitor cells to exit the cell cycle and differentiate into post-mitotic goblet cells, *p27$^{Kip1-/-}$* and control mice were treated with the γ-secretase inhibitor dibenzazepine (DBZ; Milano *et al*, 2004). Surprisingly, the phenotypes of DBZ-treated *p27$^{Kip1-/-}$* and control mice were indistinguishable and were characterized by the complete conversion of proliferating crypt progenitor cells into post-mitotic goblet cells, as shown by Ki67 and PAS staining (Fig 4A–C). Although the loss of Notch signalling clearly leads to upregulation of p27^{Kip1} in crypt progenitor cells (Figs 3C,D,5E), its loss is not sufficient to allow N1N2-deficient crypt progenitors to re-enter the cell cycle, indicating that additional inhibitory cell-cycle regulators are active. To investigate this further, quantitative RT–PCR for additional cyclin-dependent kinase inhibitors was performed on RNA from laser microdissected crypts from control and *Rbpj*-deficient mice. We chose to study Cdkn2a and p57^{Kip2}, the latter having been shown to be transcriptionally regulated by Hes1 in the pancreas (Georgia *et al*, 2006). Expression of Cdkn2a was not detected, whereas the expression levels of p57^{Kip2} increased 47-fold in *Rbpj KO* mice (Fig 5A). These mRNA expression studies were confirmed further by immunostaining for p57^{Kip2} in vehicle- and DBZ-treated control and *p27$^{Kip1-/-}$* mice, respectively. In the crypts of littermate controls and *p27$^{Kip1-/-}$* mice, p57^{Kip2} staining was found only in Paneth cells (Fig 5H,J, arrowheads), whereas the nuclei of crypt progenitors were negative. By contrast, nuclear p57^{Kip2} staining was found in control and *p27$^{Kip1-/-}$* animals in which Notch signalling was blocked by γ-secretase treatment (Fig 5I,K). This indicates that the loss of Notch signalling results in upregulation of two cyclin-dependent kinase inhibitors of the Kip family. Promoter analysis of *p57^{Kip2}* led to the identification of a potential class C Hes1-binding site (Fig 5B), which might convey transcriptional repression similar to that observed for the *p27* promoter (Fig 3). ChIP experiments performed on isolated crypt cells from wild-type mice show that this particular Hes1-binding site, but not that of an unrelated *p57^{Kip2}* downstream sequence, is occupied by Hes1 *in vivo* (Fig 5C), indicating that Hes1 contributes to the transcriptional repression of p57^{Kip2}.

The simultaneous upregulation and the redundant function of p27^{Kip1} and p57^{Kip2} in Notch signalling-deficient crypt progenitors might explain why the loss of p27^{Kip1} is not sufficient to maintain proliferation of crypt progenitors.

Together, our results show that N1 and N2 function redundantly in the small intestine. Unlike in the skin, in which Notch and Wnt signalling have opposing functions (Nicolas *et al*, 2003), both signalling cascades work together to preserve the proliferative crypt compartment of the small intestine. Wnt signalling results in repression of the cyclin-dependent kinase inhibitor *p21$^{Cip1/Waf1}$* (van de Wetering *et al*, 2002), whereas Notch signalling represses the cell-cycle regulators *p27^{Kip1}* and *p57^{Kip2}* at least in part through its target gene *Hes1* (Fig 5L). Although the Wnt and the Notch pathways do not show any apparent cross-talk in crypt progenitor cells, both show similar functions by inhibiting cyclin-dependent kinases.

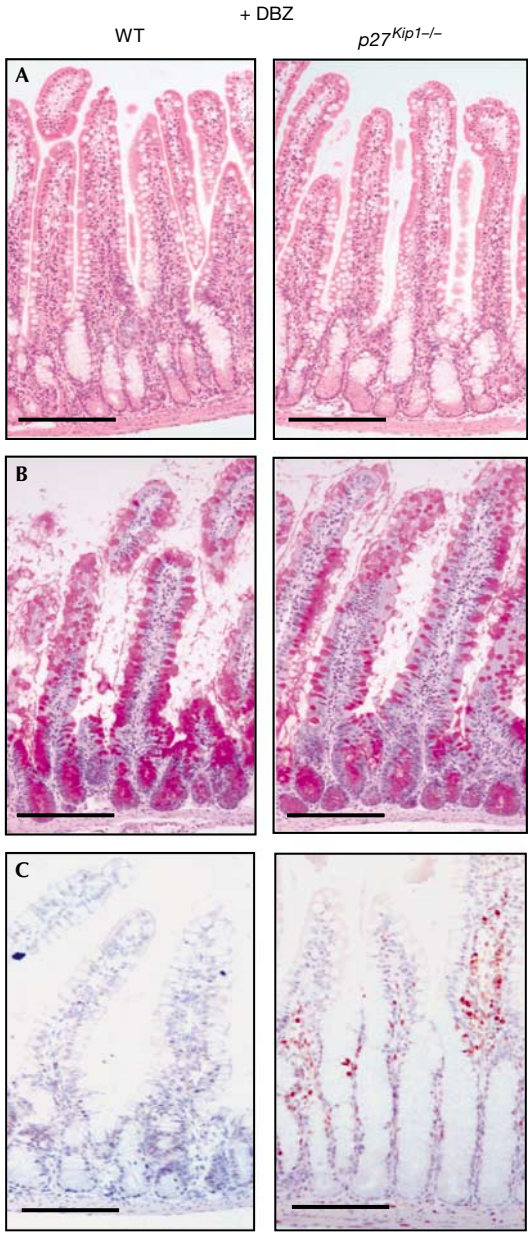

Fig 4 | Loss of p27^{Kip1} in Notch signalling-inhibited progenitors is not sufficient to maintain their proliferative and undifferentiated phenotype. Treatment of wild type (WT; left) and *p27$^{Kip1-/-}$* mice (right) with the γ-secretase inhibitor dibenzazepine (DBZ) followed by morphological and immunohistochemical analysis is shown. Representative small intestine sections stained with (**A**) haematoxylin and eosin, and (**B**) periodic acid-Schiff or (**C**) with an antibody against Ki67 are shown. Scale bars, 100 μm.

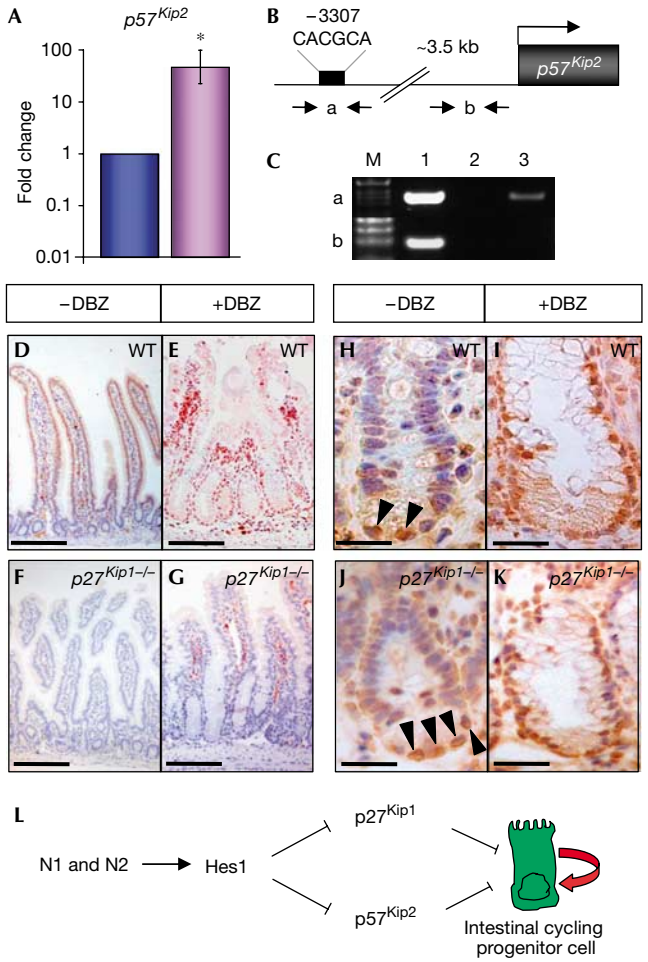

Fig 5 | Derepression of $p57^{Kip2}$ expression in the Notch signalling-deficient crypt compartment of the small intestine. (**A**) Crypt regions of control (blue) and *Rbpj KO* (purple) mice were laser microdissected followed by RNA isolation and quantitative reverse transcription–PCR for $p57^{Kip2}$. (**B**) Schematic representation of the $p57^{Kip2}$ promoter indicating the primers used (a and b) for the chromatin immunoprecipitation (ChIP) analysis. The black rectangle corresponds to a class C Hes1-binding site. Primers indicated by 'b', localizing about 1 kb upstream of the ATG, were used for the control PCR. (**C**) ChIP assay. Epithelial cells from crypt enrichment preparations were processed for ChIP with an antibody against Hes1 (lane 3) and purified rabbit IgGs as control (lane 2). PCRs of two distinct regions, indicated by 'a', containing the class C Hes1-binding site, and by 'b', an unrelated downstream sequence within the p57 promoter used as negative control, are shown. Lane 1 corresponds to input DNA. (**D–K**) Immunohistochemical analysis of small intestine sections from wild-type (WT) and $p27^{Kip1-/-}$ mice treated with either vehicle alone (−DBZ (dibenzazepine)) or with the γ-secretase inhibitor DBZ (+DBZ) using antibodies against $p27^{Kip1}$ (**D–G**) or $p57^{Kip2}$ (**H–K**). Note that $p57^{Kip2}$ is expressed only in the nuclei of Paneth cells (arrowheads) when mice are treated with vehicle alone (**H,J**). By contrast, γ-secretase-mediated blockage of Notch signalling results in nuclear $p57^{Kip2}$ staining of crypt progenitor cells (**I,K**). Scale bars in sections showing $p27^{Kip1}$ immunostaining correspond to 100 μm, whereas in sections showing p57Kip2 staining, they correspond to 50 μm. (**L**) Model indicating a possible mechanism by which the Notch signalling pathway promotes the cycling of progenitor cells. Signalling through Notch1 (N1) and Notch2 (N2) in crypt progenitor cells represses the two cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p57^{Kip2}$.

METHODS

Animals. Gene-targeted and transgenic mice are described in the supplementary information online.

Immunohistochemistry, antibodies and *in situ* hybridization. Intestinal tissue preparation and immunohistochemistry were performed as described previously (van Es *et al*, 2005). Antibodies, laser capture microdissection and quantitative RT–PCR are described in the supplementary information online.

Crypt enrichment preparation and chromatin immunoprecipitation. Crypts from wild-type mice were prepared as described previously (Kobayashi *et al*, 2005). ChIP analysis was performed in three independent experiments by using the ChIP assay kit (Millipore/Upstate, Zug, Switzerland) following the manufacturer's instructions. The Hes1 antibody was incubated at 4 °C for 24 h. An IgG antibody was used as a negative control. The mouse primer set for identification of the Hes1 C-box-binding site of the $p27^{Kip1}$ gene was sense 5′-CGGCCGTTTGGCTAGTTTGTTTGT-3′ and antisense 5′-CAAGGTTTGGAGAGGGGCTGCGTT-3′ (Murata *et al*, 2005) and for control, sense 5′-CTTCGATTCTATCG CTAAACTC-3′ and antisense 5′-GTGAAGAAGGAGGCGGAGAT CAG-3′. The primer set for the C-box of the $p57^{Kip2}$ promoter was sense 5′-AGGGGAGACAAGTCTCAACTCTAC-3′ and antisense 5′-AAGTCAGTTTAGTTTCCCAGAACG-3′ and for control, sense 5′-CTCTGCAGGGCCTTTCAAGTATGT-3′ and antisense 5′-TTGGC TGGAAGTAGTTATGCTAGA-3′.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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