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


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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\(^{\text{Kip1}}\) and p57\(^{\text{Kip2}}\). We also found that the promoter of both CDK inhibitor genes is occupied by the Notch effector Hes1 in wild-type crypt progenitors. 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

**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 (Schröder & 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...
mouse 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 N1 and N2 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 (Schröder & 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<sup>lox/lox</sup>-vill-Cre-ERT2 (N1 KO), Notch2<sup>lox/lox</sup>-vill-Cre-ERT2 (N2 KO), Notch1/Notch2<sup>lox/lox</sup>-vill-Cre-ERT2 (N1N2 dKO), Rbpj<sup>lox/lox</sup>-vill-Cre-ERT2 (Rbpj KO) and corresponding littermate controls lacking the villin-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 lacking the villin-Cre-ERT2 transgene (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 lost in the villin-Cre-ERT2 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 pathways. 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 N1 and N2 receptors in the intestine, and how they maintain the undifferentiated crypt compartment.
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 p21Cip1/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 p21Cip1/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 p21Cip1/Waf1. The expression levels of p21Cip1/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 p21Cip1/Waf1. To confirm genetically that the cell-cycle arrest of Notch-deficient crypt cells is not mediated by p21Cip1/Waf1, p21Cip1/Waf1/C0/C0 mice were intercrossed with Rbpj KO mice. Intestines deficient for both Rbpj and p21Cip1/Waf1 or for p21Cip1/Waf1 alone were compared with those of littermate controls. The intestines of p21Cip1/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 p21Cip1/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 p21Cip1/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 p27Kip1 was performed on RNA from laser capture microdissected crypt cells from control, N1N2 dKO and Rbpj KO mice. The expression levels of p27Kip1 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 p27Kip1 was performed. In littermate controls, p27Kip1 protein expression was found exclusively in the nuclei of the villi, whereas crypt cells were negative (Fig 3B). However, nuclear p27Kip1 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
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 absorptive 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 p27kip1 promoter region including a proximal class C site, but not that of the p27kip1 upstream sequence, was specifically detected in the Hes1 immunoprecipitated DNA complex from formaldehyde-treated crypt cells, indicating Hes1 occupancy of the p27kip1 promoter in vivo. These results show that Notch-induced Hes1 expression contributes to the transcriptional repression of the p27kip1 gene in vivo. Whether other Hes gene family members that are also expressed in the crypt compartment contribute to the repression of p27kip1 remains to be investigated.

To confirm genetically that upregulation of p27kip1 owing to loss of Notch signalling causes crypt progenitor cells to exit the cell cycle and differentiate into post-mitotic goblet cells, p27kip1−/− and control mice were treated with the γ-secretase inhibitor dibenzazepine (DBZ; Milano et al, 2004). Surprisingly, the phenotypes of DBZ-treated p27kip1−/− 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 p27kip1 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 p57kip2, 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 p57kip2 increased 47-fold in Rbpj KO mice (Fig 5A). These mRNA expression studies were confirmed further by immunostaining for p57kip2 in vehicle- and DBZ-treated control and p27kip1−/− mice, respectively. In the crypts of littermate controls and p27kip1−/− mice, p57kip2 staining was found only in Paneth cells (Fig 5H,J, arrowheads), whereas the nuclei of crypt progenitors were negative. By contrast, nuclear p57kip2 staining was found in control and p27kip1−/− 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 p57kip2 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 p57kip2 downstream sequence, is occupied by Hes1 in vivo (Fig 5C), indicating that Hes1 contributes to the transcriptional repression of p57kip2.

The simultaneous upregulation and the redundant function of p27kip1 and p57kip2 in Notch signalling-deficient crypt progenitors might explain why the loss of p27kip1 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 p21cip1/Waf1 (van de Wetering et al, 2002), whereas Notch signalling represses the cell-cycle regulators p27kip1 and p57kip2 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. 
**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.

**Cryopreservation and chromatin immunoprecipitation.** Cryopreparations 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 1h, followed by RNA isolation and quantitative reverse transcription–PCR for p57Kip2. (B) Schematic representation of the p57Kip2 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, 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 p27Kip2−/− mice treated with either vehicle alone (−DBZ (dibenzazepine)) or with the γ-secretase inhibitor DBZ (+ DBZ) using antibodies against p27Kip2 (D–G) or p57Kip2 (H–K). Note that p57Kip2 is expressed only in the nuclei of Paneth cells (arrowheads) when mice are treated with vehicle alone (HJ). By contrast, γ-secretase-mediated blockade of Notch signalling results in nuclear p57Kip2 staining of crypt progenitor cells (L). Scale bars in sections showing p27Kip2 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 p27Kip1 and p57Kip2.

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**FIG S1** Derepression of p57Kip2 expression in the Notch signalling-deficient crypt compartment of the small intestine. (A) Crypt regions of control (blue) and Rbpj KO (purple) were laser microdissected followed by RNA isolation and quantitative reverse transcription–PCR for p57Kip2 (B). Schematic representation of the p57Kip2 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 ‘a’, 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 p27Kip2−/− mice treated with either vehicle alone (−DBZ (dibenzazepine)) or with the γ-secretase inhibitor DBZ (+ DBZ) using antibodies against p27Kip2 (D–G) or p57Kip2 (H–K). Note that p57Kip2 is expressed only in the nuclei of Paneth cells (arrowheads) when mice are treated with vehicle alone (HJ). By contrast, γ-secretase-mediated blockade of Notch signalling results in nuclear p57Kip2 staining of crypt progenitor cells (L). Scale bars in sections showing p27Kip2 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 p27Kip1 and p57Kip2.

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**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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