**Fbw7 Repression by Hes5 Creates a Feedback Loop That Modulates Notch-Mediated Intestinal and Neural Stem Cell Fate Decisions**

Rocio Sancho¹ *, Sophia M. Blake¹ *, Christian Tendeng², Bruce E. Clurman³, Julian Lewis², Axel Behrens¹ *

¹ Mammalian Genetics Laboratory, CR UK London Research Institute, Lincoln’s Inn Fields Laboratories, London, United Kingdom, ² Vertebrate Development Laboratory, CR UK London Research Institute, Lincoln’s Inn Fields Laboratories, London, United Kingdom, ³ University of Washington School of Medicine, Seattle, Washington, United States of America

**Abstract**

FBW7 is a crucial component of an SCF-type E3 ubiquitin ligase, which mediates degradation of an array of different target proteins. The Fbw7 locus comprises three different isoforms, each with its own promoter and each suspected to have a distinct set of substrates. Most FBW7 targets have important functions in developmental processes and oncogenesis, including Notch proteins, which are functionally important substrates of SCF(Fbw7). Notch signalling controls a plethora of cell differentiation decisions in a wide range of species. A prominent role of this signalling pathway is that of mediating lateral inhibition, a process where exchange of signals that repress Notch ligand production amplifies initial differences in Notch activation levels between neighbouring cells, resulting in unequal cell differentiation decisions. Here we show that the downstream Notch signalling effector HE55 directly represses transcription of the E3 ligase Fbw7β, thereby directly bearing on the process of lateral inhibition. Fbw7β/− heterozygous mice showed haploinsufficiency for Notch degradation causing impaired intestinal progenitor cell and neural stem cell differentiation. Notably, concomitant inactivation of Hes5 rescued both phenotypes and restored normal stem cell differentiation potential. In silico modelling suggests that the NICD/HES5/FBW7β positive feedback loop underlies Fbw7 haploinsufficiency. Thus repression of Fbw7β transcription by Notch signalling is an essential mechanism that is coupled to and required for the correct specification of cell fates induced by lateral inhibition.

**Introduction**

FBW7 belongs to the family of SCF (Skp1, Cul1, F-box)-E3 ligases, which degrades several oncoproteins that function in cellular growth and division pathways, including c-MYC, CYCLIN-E, c-JUN, and Notch proteins. Three FBW7 isoforms have been identified (FBW7α, FBW7β, FBW7γ), each with an isoform-specific first exon, linked to 10 shared exons. Each isoform is expressed from its own promoter allowing isoform-specific transcriptional regulation and tissue-specific expression. Whether FBW7 isoforms show preferential degradation of substrates is still controversial, although studies have shown that c-MYC, CYCLIN-E, and PIN1 are degraded specifically by FBW7α [1–3]. FBW7β, however, has remained more enigmatic, partly due to its lower absolute mRNA abundance in several cell lines and tissue, when compared to Fbw7α [2,4]. A further level of complexity of FBW7 function is added by the fact that different substrates are regulated in a tissue-specific manner by FBW7 [4–6].

Intestinal stem cells are located in the crypt base where they produce rapidly proliferating daughter cells, transit amplifying (TA) cells, which fill the crypts and gradually lose their progenitor identity to differentiate into the two main epithelial lineages upon reaching the crypt–villus junction. The absorptive lineage comprises all enterocytes, while the secretory lineage is composed of goblet cells (secreting protective mucins), enteroendocrine cells (secreting hormones like serotonin or secretin), and Paneth cells (secreting bactericidal proteins, and restricted to the bottom of the crypt in the small intestine [7]). TA cells inevitably encounter a binary decision point that will determine whether they differentiate along an absorptive or a secretory pathway [8,9]. The Notch pathway is a key regulator of this choice. RBP-jκ conditional knockout mice or treatment of mice with a γ-secretase inhibitor results in secretory cell expansion [10]. Conversely, in transgenic mice expressing the activated form of Notch1 (NICD1), goblet cells are absent and the proliferative compartment is expanded [11]. FBW7 has proven to be a critical regulator of intestinal stem
Author Summary

The Notch signalling pathway is a highly conserved system that controls cell differentiation decisions in a wide range of animal species and cell types, and at different steps during cell lineage progression. An important function of the Notch pathway is in lateral inhibition—an interaction between equal adjacent cells that drives them towards different final states. The basic principle of lateral inhibition is that activation of the Notch cell surface receptor represses production of the Notch ligand (also borne on the cell surface). Consequently, cells expressing less Notch produce more Notch ligand that can activate the Notch pathway in neighboring cells and thereby amplify the differences between these cells. However, the additional regulatory circuits required to fine-tune this delicate process have so far remained elusive. Here we describe the identification of a novel intracellular positive feedback loop that connects Fbw7 (the ubiquitin ligase responsible for targeting Notch for degradation) and Notch itself. We show that Fbw7 reduces the stability of Notch intracellular domain (NICD) protein, as previously established, but also that the fbw7 gene is itself transcriptionally downregulated by the Notch effector Hes5. Thus we conclude that increased Notch activity causes NICD stabilisation. Further, we demonstrate that perturbation of this regulatory loop is responsible for the Fbw7 haploinsufficiency observed for Notch-dependent functions in intestine and brain stem cells.

cell differentiation, as its deletion in the gut significantly increased NICD1 protein levels and reduced goblet cell numbers [5]. Another example demonstrating the importance of Fbw7 in Notch biology and function is that of neural stem cells (NSCs). At the beginning of neurogenesis, neuroepithelial stem cells give rise to radial glial stem cells (RGCs), which represent the majority of NSCs at later stages of embryonic cortex development [12]. Notch activity is very high in RGCs, and needs to be downregulated for neuronal differentiation to occur [13]. Overexpression of NICD1 has been shown to be sufficient to promote radial glial identity during embryogenesis, while abrogation of Notch signalling leads to depletion of RGCs [14,15]. In line with these observations, we have shown that absence of Fbw7 in NSCs causes severely impaired RGC stem cell differentiation, accompanied by accumulation of the Fbw7 substrate NICD1 [4].

The Notch signalling pathway is a highly conserved pathway that is not only involved in the development and stem cell biology of the mammalian intestine and brain, but controls cell differentiation decisions in a wide range of metazoan species, in a broad range of cell types within a single organism, and at different steps during cell lineage progression.

Mammals have 4 Notch receptors (Notch1–4), 3 Delta-like ligands (Dll1, 3, 4), and 2 Serrate-like ligands termed Jagged (Jagged1 and 2). Ligand binding triggers a complex proteolytic cascade involving ADAM proteases and an intramembranous enzyme complex called γ-secretase, which results in the release of the cytoplasmic domain of Notch proteins from the plasma membrane. The Notch intracellular domain (NICD) shuttles all the way from the cell membrane to the nucleus, where it binds to RBP-Jκ and other proteins, and establishes an activator complex, leading to the expression of target genes. In mammals, the best-characterized Notch target genes belong to the Hes (Hairy Enhancer of Split) and Herp/Hey (Hes-related repressor proteins with Y-box) family of basic helix-loop-helix (bHLH) transcriptional repressors [16,17].

An important function of the Notch pathway is in lateral inhibition—an interaction between equal adjacent cells that serves to drive them towards different final states. The basic principle of lateral inhibition is that activation of Notch represses production of the Notch ligand. Consequently, the cell with lower Notch activity produces more ligand, and this activates Notch signalling in the neighbouring cell, which results in reduced ligand production. This in turn enables the cell with lower Notch activity to increase its ligand production even further, because it receives a weakened inhibitory signal back from its neighbours. The effect of this feedback loop is that any initial difference in Notch activity between them, whether stochastic or genetically controlled, is amplified to drive the neighbouring cells into opposite Notch-level status and hence into different developmental pathways [18].

In this manuscript we describe the identification of a novel intracellular positive feedback loop that connects Fbw7 and Notch: Fbw7 not only downregulates stability of NICD protein, as previously established, but is also itself transcriptionally downregulated by NICD (via the action of NICD on Hes5). We demonstrate that Fbw7 is haploinsufficient for Notch-dependent physiological functions, as Fbw7<sup>+/−</sup> heterozygous mice show impaired differentiation of intestinal goblet cells and NSCs. This haploinsufficiency is greatly dependent on the newly identified negative transcriptional regulation of the Fbw7β promoter by Hes5 protein. We can further show for the first time a pronounced isoform-specific function of Fbw7β in driving Notch1 intracellular domain (NICD1) degradation. Genetic rescue experiments and computer modelling of Notch signalling suggest that the Fbw7β/NICD/HES5 feedback loop modulates Notch-dependent cell fate decisions and underlies Fbw7 haploinsufficiency.

Results

Haploinsufficient Fbw7 Function in Intestinal and NSC Fate Decisions

We have previously used conditional gut-specific knock-out mice allowing for deletion of Fbw7 specifically in the intestinal tissue to investigate Fbw7 function in gut biology and tumourigenesis. Mice harbouring an Fbw7 allele in which exon5 was flanked by two loxP sites were crossed to villin-cre transgenic mice, previously shown to provide efficient gut-specific Cre activity [19]. Deletion of exon 5, which encodes most of the F-box, an essential domain of Fbw7, disrupts the Fbw7 open reading frame and prevents production of detectable Fbw7 protein [20]. Monallelic Fbw7 mutations are frequently observed in human colorectal cancer (CRC) and we described that also in the mouse Fbw7 heterozygosity greatly increased intestinal tumour number in the APC<sup>Min</sup> mouse model [5], indicating that Fbw7 haploinsufficiency in intestinal tumour formation is conserved between mouse and human.

Fbw7<sup>+/−</sup>; villin-cre heterozygous (Fbw7<sup>+/−</sup>/+) mice showed a significant decrease in goblet cell differentiation, suggesting that Fbw7 is a haploinsufficient regulator of goblet cell fate decisions in the gut (Figure 1a,b). Fbw7 controls the stability of several proteins with well-documented functions in the intestine such as NICD [21], N-terminally phosphorylated c-JUN [22], c-MYC [1], and CYCLIN-E [23]. We next determined to what extent protein levels of these substrates were deregulated by heterozygous Fbw7 inactivation. Western blot analysis revealed an increase in NICD1, but the protein levels of N-terminally phosphorylated c-JUN, c-MYC, and CYCLIN-E were less affected in Fbw7<sup>+/−</sup>/+ mice (Figure 1c, Figure S1a). To have a more quantitative measure for NOTCH and c-JUN activity, we performed q-PCR analysis of classical target genes of both transcription factors (Figure 1d,
Figure 1. Fbw7 is haploinsufficient for Notch in the gut and NSCs. (a) H&E and AB/PAS staining in intestinal tissue from Fbw7+/+ or Fbw7ΔG/+ mice. (b) Quantification of goblet (AB-PAS+) cells in intestinal tissue from Fbw7+/+ or Fbw7ΔG/+ mice. (c) Western analysis of protein lysates from intestinal cells isolated from Fbw7+/+ or Fbw7ΔG/+ mice (numbers indicate the fold induction of NICD normalized to actin). (d) Q-PCR analysis of Hes1, Hes5, c-Jun and c-Myc transcripts in Fbw7+/+ intestinal cells compared to Fbw7ΔG/+ (relative fold induction after normalizing to actin ± SEM, n=3 for each genotype). (e) Nestin staining of NSCs isolated from Fbw7+/+ or Fbw7ΔG/+ mice. (f) Quantification of Nestin+ cells in NSCs isolated from Fbw7+/+ or Fbw7ΔG/+ mice (percentage positive cells ± SEM, n=20 for each genotype). (g) Western blot analysis of protein lysates from Fbw7+/+ or Fbw7ΔG/+ NSCs for NICD-1 (numbers indicate the fold induction of NICD normalized to actin). (h) Q-PCR analysis of Hes1, Hes5, c-Jun and c-Myc transcripts in Fbw7+/+ NSCs compared to Fbw7ΔG/+ NSCs (relative fold induction after normalizing to actin ± SEM, n=3 for each genotype).

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Distinct Regulation of the Fbw7β Locus

To understand the haploinsufficiency of FBW7 function, we explored the possibility of feedback regulation and investigated the expression of Fbw7 in Fbw7+/+ control and Fbw7ΔG/+ heterozygous intestine and Fbw7ΔN/+ heterozygous NSCs. The Fbw7 locus encodes three different Fbw7 isoforms (Fbw7α, Fbw7β, Fbw7γ) that are not generated by alternative splicing; rather, each isoform has its unique 5'UTR and is transcribed from an isoform-specific promoter (Figure 2a) [24]. We have previously shown that the α and β Fbw7 isoforms are expressed in the intestine and the brain, whereas the γ isoform was undetectable [4,5]. Using quantitative qPCR analysis we show that the Fbw7α isoform is 170- and 10-fold more abundant than the Fbw7β isoform in the intestine and NSCs, respectively (Figure S2). To circumvent a potential alteration in mRNA stability of the Fbw7α allele, we used Q-PCR primers located in exon3, which is missing in the Fbw7ΔA allele (Figure 2a).

Thus Fbw7 is haploinsufficient for Notch degradation during both goblet cell and NSC differentiation.
Figure 2. Fbw7 is transcriptionally regulated by Hes5. (a) Schematic representation of the Fbw7 genomic locus (adapted from [24]). mRNA transcripts for the different Fbw7 isoforms and Q-PCR primers used to detect Fbw7 isoforms are depicted in the figure. (b) Q-PCR analysis of Fbw7α, Fbw7β, and Fbw7(exon5) in intestinal cells and NSCs isolated from Fbw7+/+ or Fbw7DG/+ mice and Fbw7f/+ or Fbw7DN/+ NSCs, respectively (relative fold induction after normalizing to actin ± SEM, n=3 for each genotype). (c) Q-PCR analysis of Fbw7α and Fbw7β in intestinal tumours isolated from APCmin/−; Fbw7f/+ or APCmin/−; Fbw7DG/+ mice (relative fold induction after normalizing to actin ± SEM, n=3 for each genotype). (d) Schematic representation of Fbw7α, Fbw7β, and Ngn3 promoter regions. Red boxes denote consensus N-box sites. Green arrows indicate primers used for ChIP. (e) ChIP was performed using HCT116 cells transfected with p-Dest-flag or p-Dest-Hes5-flag. Flag binding to the consensus sites in Fbw7α, Fbw7β, and Notch in Lateral Inhibition.
Ngn3 promoters was determined by Q-PCR. Data were represented as fold activation of percentage input versus the p-DEST-Flag samples. Red line denotes background-binding activity. (l) Schematic representation of the different Fbw7β-luciferase constructs generated. Red rectangles represent putative N-boxes. Crossed red rectangles represent mutated N-boxes. (g) HCT116 cells were transfected with Fbw7β-N1, Fbw7β-N2, Fbw7β-N1-mut, pGL3–Fbw7β–N2-mut together with p-Dest-Hes5–Flag overexpression vector or p-Dest-Flag as a control. Data represent luciferase activity relative to Fbw7β–N1–pDest-Flag transfected cells.

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intestines as well as in F boa 7α/α* NSCs. In contrast, expression of F boa 7β mRNA was greatly reduced in F boa 7α/α* intestines and F boa 7α/α+ NSCs (Figure 2b). Mono-allelic (i.e., heterozygous) F BW 7 mutations are frequently observed in human CRC, and F boa 7 heterozygosity greatly increases intestinal tumour number in the APC(α/α*) mouse model [5]. Similarly, a reduction in F boa 7β mRNA was observed in tumours from APC(α/α) mice compared to APCΔmin/+; F boa 7α/α+ mice (Figure 2c).

To gain insights into the mechanism of F boa 7 transcriptional regulation, we performed an in silico transcription factor binding site analysis of the genomic F boa 7 locus. This revealed the presence of putative N-box sites, the consensual binding element recognized by HES transcription factors, in the promoters of both the N1 site was unaffected. Mutation of the N1 N-box (Figure 2d). Our attempts to perform Chromatin immuno-precipitation (ChIP) analysis on endogenous HES5 failed as we were unable to identity a suitable Hes5-specific antibody (Figure S3). For this reason Flag–HES5 was overexpressed in HCT116 colon cancer cells and ChIP performed using Flag antibody. This revealed binding of HES5 protein to the N-box in the neunogranin3 promoter (Ngn3), a known HES target gene [25], which served as a positive control. However, we also observed some unspecific DNA binding of FLAG-HES5 relative to control vector transfected cells at the GAPDH, β-ACTIN, and CYCLIND1 promoters, which all served as negative controls. HESS5 bound to predicted N-box elements present in the FBW 7α and FBW 7β promoters to a similar extent to Ngn3, but did not bind significantly to a putative N-box in exon1 of FBW 7β (Figure 2e).

When inserted into a luciferase reporter construct, the FBW 7β promoter fragment including the functional (N1) Hes5 binding site (FBW 7β N1-luc) was repressed by HES5 overexpression, whereas an FBW 7β fragment covering exon1 (FBW 7β-N2-luc) and lacking the N1 site was unaffected. Mutation of the N1 N-box (FBW 7β N1-mut-luc) rendered the FBW 7β promoter fragment unresponsive to HES5 (Figure 2f,g).

Together these data point very strongly to a specific role for HES5 in regulating FBW 7β transcription.

Hes5 Represses Fbw7β Transcription

To further validate FBW 7β as a direct transcriptional target of HES5, NICD1 was ectopically expressed in HCT116 colon cancer cells. NICD1 expression resulted in increased HES5 mRNA levels, but had no effect on HES1. Moreover, FBW 7β and to a lesser extent FBW 7α mRNA levels were strongly repressed (Figure 3a). shRNA-mediated knock-down of HES5 reversed the repression of FBW 7α and FBW 7β expression (Figure 3b). Similar results were obtained in NSCs (Figure S4a,b).

The direct repression of FBW 7β expression, and to a lesser extent, of FBW 7α, by HES5 implies that NICD1, HES5, and FBW 7β are connected through a feedback loop. This leads to the unexpected prediction that overexpression of FBW 7β should result in a cell-autonomous increase in NICD1 protein levels (Figure 3c), but that this increase should be impaired in cells deleted for the E3 ligase (that is, FBW 7) regulating NICD turnover. To test this hypothesis, we used a set of human colon cancer HCT116 cell lines that have homozygous isoform-specific FBW 7β-null mutations [2].

GFP-tagged-HES5 or GFP alone was overexpressed in HCT116 FBW 7-αt, FBW 7α-null, and FBW 7β-null cells followed by intracellular NICD staining and FACS analysis. FACS analysis on GFP+ gated cells revealed that NICD1 protein levels in HCT116 are not uniform, rather that there are two distinct subpopulations with different NICD1 levels. This resembles the bistability observed when lateral inhibition operates, and thus should be affected by the intracellular NICD1→Hes5→ FBW 7→ NICD positive feedback loop. In line with this, the majority of FBW 7-αt cells were in the low-NICD state, while a greater proportion of FBW 7β-null cells were in the high-NICD state (Figures S5a–c and S6a). Expression of HES5-GFP shifted these proportions, leading to a marked cell-autonomous increase in the percentage of cells in the high-NICD1 state in FBW 7-αt and FBW 7α-null cells, but this increase was drastically impaired in FBW 7β-null cells (Figure 3c and Figure S5). Conversely, silencing HES5 (sh–HES5-GFP) led to a cell-autonomous reduction in the percentage of cells in a high-NICD state in FBW 7-αt and FBW 7α-null cells, which was compromised in FBW 7β-null cells (Figure 3d and Figure S5). These data imply that FBW 7β is the predominant isoform involved in the NICD1/HES5/FBW 7β feedback loop.

To formally show that FBW 7β regulates NICD degradation, we performed cycloheximide chase experiments for NICD turnover in FBW 7-αt, FBW 7α-null, and FBW 7β-null cells. We found that NICD turnover was reduced in FBW 7β-null cells by comparison with FBW 7-αt and FBW 7α-null cells (Figure 3e, 3f). Accordingly, we observed less ubiquitination of NICD in FBW 7β-null cells (Figure S5c). Q-PCR analysis performed in the same set of FBW 7β-mutant cell lines confirmed that only loss of FBW 7β resulted in increased HES5 mRNA levels (Figure S6b). Together, these data demonstrate a crucial role of FBW 7β in regulation of NICD turnover.

Fbw7 Haploinsufficiency Requires Hes5 Function

To further investigate HES5 function in our proposed loop, we characterized the phenotype of Hes5−/− mice in the intestine and NSCs. Hes5−/− mice are viable, but mutant phenotypes in various organ systems such as the eye, inner ear, and nervous system have been described [26–28]. However, the function of HES5 in the intestine and in NSCs has not been analysed. The absence of HES5 led to a significant increase in intestinal goblet cell number by approximately 50% (Figure 4a,b). Q-PCR analysis revealed increased Fboa 7α mRNA levels among mice (Figure 4a,b). Q-PCR analysis revealed increased HES5 expression, and also the mRNA levels of the HES target gene Dll1 and the goblet cell marker Muc2 were augmented while F boa 7α transcript levels remained unchanged (Figure 4c). Loss of HES5 in the brain caused no obvious phenotypic abnormalities, consistent with previous observations [29]. However, NSCs cultured from Hes5−/− animals showed significant premature differentiation of Nestin-positive cells with a concomitant mild increase of Map2 positive neurons (Figure 4d,e). Deletion of Hes5 in NSCs also led to a significant increase in Fboa 7β and Dll1 expression (Figure 4f).

We next tested whether the NICD1/HES5/FBW 7β feedback loop might underlie the functional haploinsufficiency of FBW 7β. We generated compound mutant mice heterozygous for F boa 7α in a Hes5−/− background (F boa 7α+/Hes5−/−, F boa 7α+/Hes5−/− mice). Strikingly, goblet cell numbers were restored to wild-type levels in F boa 7α+/Hes5−/− mutant mice (Figure 5a,b), as were the numbers of Nestin-positive and Map2-positive cells in NSC

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Figure 3. Hes5 represses Fbw7β transcription and induces an increase in NICD in a cell autonomous manner. (a) Q-PCR analysis of Fbw7α, Fbw7β, Hes5, and Hes1 in HCT116 cells transfected with pcDNA3 or pcDNA3-NICD. (b) Q-PCR analysis of Fbw7α, Fbw7β, Hes5, and Hes1 in HCT116 cells transfected with pcDNA3-NICD in combination with either p-Super-sh-control or p-Super-sh-Hes5-1 and p-Super-sh-Hes5-2 (specific silencers for Hes5). (c) FACS analysis of intracellular NICD in HCT116-Wt, HCT116-Fbw7-α-null, or HCT116-Fbw7-β-null cells transfected with either empty-GFP or Hes5-GFP. (d) FACS analysis of intracellular NICD in HCT116-Wt, HCT116-Fbw7-α-null, or HCT116-Fbw7-β-null cells transfected with either sh-control-GFP or sh-Hes5-GFP. (e) Western blot analysis of NICD and actin in HCT116-Wt, HCT116-Fbw7-α-null, or HCT116-Fbw7-β-null cells treated with CHX for 0, 30, 60, 120, 240, and 360 minutes.
differentiation cultures (Figure 5d,e). Importantly, the repression of Fbw7 transcription in heterozygotes was rescued in Fbw7\textsubscript{D\textsubscript{G}/+}; Hes5\textsubscript{2/2}, and Fbw7\textsubscript{D\textsubscript{N}/+}; Hes5\textsubscript{2/2} mutant mice (Figure 5c,f). Thus, HES5 deficiency and FBW7 heterozygosity rescue each other, providing strong evidence that the two proteins are connected by a feedback loop.

Mathematical Modelling of the Effects of the Fbw7 Feedback Loop in the Delta-Notch Lateral Inhibition Circuit

Our experiments imply that, overlaid on the standard gene regulatory circuit of Delta-Notch-mediated lateral inhibition, there is an intracellular feedback loop involving Fbw7: NICD stimulates expression of Hes5; Hes5 represses Fbw7; and Fbw7 drives degradation of NICD. The net action of this NICD—\textrightarrow Hes5 — NICD feedback loop is positive: it tends to amplify the effect of any change in any of the three components. This can explain why Fbw7 is haploinsufficient, in the sense that loss of just one allele of the gene is enough to cause a marked shift in the ratio of secretory (low NICD) to absorptive (high NICD) cells in the gut, or of neurons to progenitors in the brain.

Intuitive arguments are, however, untrustworthy when applied to systems with feedback. We have therefore investigated a mathematical model of the Delta-Notch lateral inhibition circuitry incorporating the intracellular Fbw7 feedback loop, to see whether

Figure 4. Increased goblet cell number and increased NSC differentiation in Hes5 KO mice. (a) H&E and AB/PAS staining in the intestinal tissue of Hes5\textsuperscript{+/+} or Hes5\textsuperscript{--/--} mice. (b) Quantification of goblet (AB-PAS\textsuperscript{+}) cells in the intestines of Hes5\textsuperscript{+/+} or Hes5\textsuperscript{--/--} mice. (c) Q-PCR analysis of Fbw7\textsubscript{a}, Fbw7\textsubscript{b}, Dll1, Hes5, and Muc2 in intestinal cells isolated from Hes5\textsuperscript{+/+} or Hes5\textsuperscript{--/--} mice (relative fold induction after normalizing to actin \pm SEM, n=3 for each genotype). (d) Nestin, Map2, and DAPI staining on NSCs of Hes5\textsuperscript{+/+} or Hes5\textsuperscript{--/--} mice, 3 d after differentiation. (e) Quantification of Nestin\textsuperscript{+} and Map2\textsuperscript{+} cells of Hes5\textsuperscript{+/+} or Hes5\textsuperscript{--/--} NSCs, 3 d after differentiation (percentage positive cells \pm SEM, n=10 for each genotype). (f) Q-PCR analysis of Fbw7\textsubscript{a}, Fbw7\textsubscript{b}, Dll1, and Hes5 from NSCs isolated from Hes5\textsuperscript{+/+} or Hes5\textsuperscript{--/--} mice (relative fold induction after normalizing to actin \pm SEM, n=3 for each genotype).

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it can indeed give rise to the observed phenomena. In Figure 6, we compare the predicted multicellular patterns of differentiation under four conditions, corresponding to the genotypes $Fbw7^{+/+}$, $Hes5^{+/+}$; $Fbw7^{+/-}$; $Hes5^{-/-}$ (b), $Fbw7^{+/-}$; $Hes5^{-/-}$ (c), $Fbw7^{+/-}$; $Hes5^{-/-}$ (d), and $Fbw7^{+/-}$; $Hes5^{-/-}$ (e). In (d), where $Hes5$ is absent, the proportion of secretory cells is increased; in (e), where $Hes5$ is present but one of the two $Fbw7$ gene copies is defective, we see the opposite effect, reflecting haploinsufficiency of $Fbw7$; and in (c), where both types of mutation are present, their effects cancel out, restoring the normal ratio of secretory to absorptive cells. These results depend, of course, on the values assumed for the parameters in the model, for many of which we can only make rough guesses. The results of the modelling should therefore be viewed not so much as quantitative predictions, but rather as a demonstration that the experimental observations (Figures 1a, 4a, and 5a) are indeed consistent with a mechanism of the type proposed.

Intuitively, it seems that the $Fbw7$ loop superimposed on the standard lateral-inhibition circuitry should tend to amplify the differences between neighbouring cells and perhaps speed up the creation of a salt-and-pepper pattern. Moreover, as we have argued, it could explain why loss of a single $Fbw7$ gene copy has an unexpectedly large effect on the ratio of differentiated cell types in this final pattern.

**Discussion**

Notch signalling is a key pathway that controls differentiation decisions in a vast number of cell types. SCF($Fbw7$) is an important negative regulator of NICD function [30,31], and many, though not all, of the phenotypes observed in $Fbw7$ mutant animals can be attributed to deregulation of Notch activity [4,5,21,32–34]. In this study we show that $FBW7$ is the isoform responsible for NICD degradation and also reveal that the functional relationship between $FBW7$ and Notch is not unidirectional, but that $FBW7$ and NICD are connected through a double-negative, i.e. positive, feedback loop.

We propose that the NICD/HESS5/$FBW7$ feedback loop functions to refine the classical lateral inhibition mechanism (Figure 6a). Notch signalling represses transcription of Notch ligands, which leads to unequal levels of Notch signalling in neighbouring cells. We propose here that increasing levels of Notch activity results in reduced expression of $Fbw7$ mutant animals can be attributed to deregulation of Notch activity [4,5,21,32–34]. In this study we show that $FBW7$ is the isoform responsible for NICD degradation and also reveal that the functional relationship between $FBW7$ and Notch is not unidirectional, but that $FBW7$ and NICD are connected through a double-negative, i.e. positive, feedback loop.

We propose that the NICD/HESS5/$FBW7$ feedback loop functions to refine the classical lateral inhibition mechanism (Figure 6a). Notch signalling represses transcription of Notch ligands, which leads to unequal levels of Notch signalling in neighbouring cells. We propose here that increasing levels of Notch activity results in reduced expression of $Fbw7$, which in turn will lead to a further increase in NICD1 protein levels. Similarly, attenuation of Notch signalling will decrease NICD1 levels, as $Fbw7$ will be more highly expressed. Whereas NICD/Notch ligand regulation operates non-cell-autonomously, the NICD/HESS5/$FBW7$ loop results in a cell-autonomous amplification of inequalities in Notch activity. This mechanism will help the cell to stably attain a Notch-high or Notch-low state, thereby solidifying cell fate decisions.

NICD1 stands out among all the SCF($Fbw7$) substrates as it is the only substrate that is noticeably increased in $Fbw7^{+/-}$ heterozygous cells. Mechanistically, this is explained by the positive feedback causing repression of the wild-type $Fbw7$ allele in $Fbw7^{+/-}$ heterozygous cells. Thus, instead of being reduced to just 50% of normal, $Fbw7$ mRNA levels are reduced even further.
Figure 6. Mathematical modelling of the effects of the Fbw7 feedback loop in the Delta-Notch lateral inhibition circuit. (a) Diagram of the postulated gene regulatory network for a pair of adjacent cells. (b–e) Patterns of differentiation predicted by the mathematical model for an extended two-dimensional cell array; low-NICD cells are coloured green, high-NICD cells red. Four different genotypes are compared, showing the effect of halving the Fbw7 gene dosage under conditions where Hes5 is $(Hes5^{+/+})$ or is not $(Hes5^{-/-})$ functional. The low-NICD cells correspond to secretory cells in the gut or to neurons in the brain. The percentage of low-NICD cells is shown below the picture of a typical pattern for each genotype. With the chosen model parameters, the predicted percentages of low-NICD cells for the four genotypes are in approximately the ratios observed in the gut (see Figures 4b and 5b). (In comparing the model with the real tissue, note that in the gut, the future absorptive cells continue dividing after the secretory cells have stopped [9], scaling up the observed numbers of absorptive cells relative to secretory cells in all genotypes.) See Materials and Methods and Data S1 for details of the modelling.

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Absolute quantification of Fbw7 mRNA abundance in the intestine and NSCs has shown that the reduction in total Fbw7 mRNA in heterozygous animals cannot be accounted for solely by the reduction in levels of the Fbw7b isoform. We believe that the small but consistent reduction of the more abundant Fbw7α mRNA (reflecting its moderate regulation by Hes5; see Figure 3a,b) contributes to the overall regulation of total Fbw7 mRNA levels in heterozygous cells.

Previous reports have generated Fbw7β-specific knockout mice, which are viable, but comprehensive analyses of Notch-mediated phenotypes in brain or gut were not performed [33]. The same holds true for Hes5−/− mice, which had not been reported to have abnormalities in intestinal or NSC differentiation. In our analysis we have clearly shown that decreased levels of Fbw7b or loss of Hes5 have a profound effect on patterns of differentiation in the intestine and in NSCs.

There are various reports regarding the localisation of Fbw7β and its contribution to substrate turnover. Some accounts report that Fbw7β localises to the cytosol [1,36], whereas others have found it in the ER and Golgi [35]. Also in the cells we studied Fbw7β localised predominantly in the cytoplasm but some nuclear localisation could also be observed, especially in response to proteasome inhibitor treatment (Figure S7a–c). Conversely Fbw7β is able to interact with both endogenous NICD and overexpressed NICD (Figure S8a,b) and the ubiquitylation of overexpressed NICD is severely impaired in HCT116–Fbw7β-null cells (Figure S8c). The cytoplasmic presence of Fbw7β might even explain why the observed haploinsufficiency of Fbw7α/a animals is restricted to NICD1. Many Fbw7 substrates are predominantly nuclear, whereas NICD shuttles from the cytoplasm into the nucleus and is thus present in both subcellular compartments. On a similar note, Ye et al. have shown that Fbw7β is the predominant isoform responsible for CYCLIN-E turnover, which is primarily nuclear, but shuttles between cytoplasm and nucleus, like NICD [37].

A recent study, using homozygous isoform-specific Fbw7-null mutations in human colon cancer HCT116 cells, has shown that Fbw7α is the major isoform contributing to c-MYC and SREBP degradation [2]. We have used those cells to show that Fbw7β is the isoform regulating NICD degradation. While our data suggest that Fbw7β is the major isoform regulating NICD degradation, Fbw7α possibly also contributes to the proposed feedback loop. Further, we can confirm previous studies showing that c-MYC is primarily degraded by Fbw7β (Figure S8c) and [2]. The isoform specificity and absolute abundance of the Fbw7 isoforms, together with their heterogeneous tissue distribution, could also possibly explain the varying penetrance of Fbw7 deletion in different organs.

Fbw7β is frequently mutated in a large variety of human tumours [24]. In particular, loss-of-function mutations in Fbw7β are very commonly found in human CRC [38]. Interestingly, about 70% of Fbw7β mutations are mono-allelic, and only about 30% of the colorectal tumours with Fbw7β mutations show loss-of-heterozygosity (LOH) [39,40]. Fbw7β mRNA levels are significantly lower in human CRC tumour tissues than in normal intestinal tissue, and low Fbw7β expression correlates with poor prognosis [41]. In a mouse model for human CRC, it was clearly shown that Hes5 expression was upregulated in tumours carrying Fbw7β heterozygous mutations when compared to tumours wild-type for Fbw7β [3]. Thus Fbw7β heterozygosity results in increased Hes5 expression both in human colorectal tumours and in the APCmin;Fbw7−/− mouse model, suggesting that the NICD/HES5/Fbw7β positive feedback loop is the molecular mechanism that underlies Fbw7 haploinsufficiency in tumour suppression.

Thus the feedback loop created through repression of Fbw7β by NICD plays a crucial part in Notch-regulated cell fate decisions, not only in normal tissues but also in the evolution of a large class of cancers.

Materials and Methods

Mouse Lines

Fbw7+/−, Villin-cre, Nestin-cre, APCmin/+ and Hes5−/− mice have been described before [19,20,42–44].

Cell Culture and Transfection

HCT116-wt, HCT116–Fbw7-β-null, and Fbw7-β-null cells have been described previously [2]. Cells were cultured in DMEM and 10% FBS. Cells were plated at subconfluence and transfected with Lipofectamine 2000 (Invitrogen).

NSCs were isolated as spheres from E13.5 fore- and midbrain of Fbw7+/−, Fbw7+/−/−, Fbw7−/−, Hes5−/− and Hes5−/− mouse embryos. Cells were initially cultured as spheres under self-renewal conditions, as previously described [4]. Adherent NSC cultures were derived as previously described [45] with minor modifications. Briefly, primary spheres were plated in Neurobasal Medium (Invitrogen) supplemented with 1% Penicillin/Streptomycin (10,000 U/ml; Invitrogen), 1% L-glutamine (200 mM; Invitrogen), 2% B27 supplement (Invitrogen), 1% N-2 supplement (Invitrogen), 20 ng/ml EGF (PeproTech), 20 ng/ml FGF-basic (PeproTech), and 1 µg/ml laminin (Sigma). All experiments were performed using adherent NSCs.

For differentiation, growth factors were withdrawn from the medium and 10% NeuroCult Differentiation Supplement (Stem-Cell Technologies) was added. Under differentiation conditions, cells were plated on poly-L-ornithine (0.01% solution; Sigma; diluted 1:10 in 150 mM disodium tetraborate; Sigma) coated cover slips.

For transfection, NSCs were plated at subconfluence and transfected with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen).

Plasmids and Reagents

Cycloheximide was used at a final concentration of 100 µg/ml (Sigma). The Notch expression vector (Notch-IC-D/A05) was a gift from Anna Bigas [46]. p-Super-sh-control, p-Super-sh-Hes5-1, and p-Super-sh-Hes5-2 were generated by cloning short hairpin containing oligos into pSuper vector following the manufacturer’s instructions (Oligogene). Silencing oligo sequences were sh-Hes5-1 (cagctgccagacgacct; sh-Hes5-2 (ggaagccgctgtggagga). pCMV6-Hes5-gfp was purchased from Origene. pDest-FLAG was purchased from Invitrogen. pDest-Hes5-FLAG was generated by Gateway cloning of PCR-amplified Hes5 into pDest-FLAG. The oligonucleotide sequences used to amplify the DNA fragments for luciferase constructs are: pGL3–Fbw7a–efmut: N box motif was modified via Geneart; pGL3–Fbw7a–cd mut: N box motif was modified via Geneart; pGL3–Fbw7b–ef mut: 5’ ATTGTCCCTGAAGGTAGTTGTG 3’; pGL3–Fbw7b–cd rev: 5’ GTCCTACAGTCCTTTCCGTTATTATTTGC 3’; pGL3–Fbw7α–cd fwd: 5’ TTTTGAACGCGGATAGTCCTGGCT 3’; pGL3–Fbw7β–cd rev: 5’ TTTTGAACGCGGATAGTCCTGGCT 3’; pGL3–Fbw7β–cd mut: N box motif was modified via Geneart; pGL3–Fbw7α–cd mut: N box motif was modified via Geneart.

Reporter Gene Assay

HCT116 cells were transfected with the indicated plasmids with Lipofectamine 2000 (Invitrogen). Transient transfections of the experimental samples and controls of Firefly and Renilla luciferase reporters were performed and measured using the Dual-Luciferase Reporter Assay System (Promega), 36 h posttransfection. Data are...
expressed as fold induction after being normalised using tk-renilla luciferase (mean ± SD; n = 3).

**ChIP**

ChIP analysis was performed as described previously [47]. Cells were transfected with Lipofectamine 2000 [Invitrogen] with empty-flag or Hes5-flag prior to collection. Immunoprecipitations were carried out with anti-Flag antibody directly conjugated to agarose beads. The oligonucleotide sequences used to amplify the DNA fragments by q-PCR are: Fbw7α (ab-fw): 5'-TGAAATATCATGAAAGATGCTGGTACG-3'; Fbw7α (ab-rev): 5'-TCTAAGCAGTGTGCTTTCACTG-3'; Fbw7β (cd-fw): 5'-TGAGCGGTCCATTAGCAGAATG-3'; Fbw7β (cd-rev): 5'-TTGCATCTGGACCCATTTCCTAGTCACTG-3'; Fbw7β (ef-fw): 5'-TGGCCGAAGAGGGTTTGGAT-3'; Fbw7β (ef-rev): 5'-GCCAGGCACACCCAATATCC-3'; Ngn3-fw: 5'-CCCTGTCAGGAGATGCTG-3'; Ngn3-rev: 5'-CTGTCCAGGGCACCCTCAGA-3'; Gapdh-fw: 5'-TGACGAGTCCGGTGCTCACTA-3'; Gapdh-rev: 5'-AACAGATCGCCGCCTGACGTG-3'; Actin-fw: 5'-GGATGCAGAAGAGGATGACTG-3'; Actin-rev: 5'-GAGCTCACGAGGAGTACTGG-3'; Cyclin1-fw: 5'-CGGCCGACCCCTTCAG-3'; Cyclin1-rev: 5'-CGGCCCCAGACCCCTTAGA-3'.

Quantitative real-time PCR was accomplished with SYBR Green incorporation (Platinum Quantitative PCR SuperMix-UDG w/ROX, Invitrogen) using an ABI7900HT (Applied Bioscience), and the data were analyzed using the SDS 2.3 software.

**Quantitative RT-PCR**

For qRT-PCR analysis, total mRNA was isolated from ileum fraction obtained as described before [5]. Total RNA was used from adherent NSC cultures. Results, normalized to β-actin, were presented as fold induction over control mice. The list of primers that were used for qPCR analysis of mouse tissues were: F-c-Jun: 5'-TGAAATATCATGAAAGATGCTGGTACG-3'; R-c-Jun: 5'-TGAAATATCATGAAAGATGCTGGTACG-3'; F-Fbw7 (exon5): 5'-GCCAGGCACACCCAATATCC-3'; R-Fbw7 (exon5): 5'-GCCAGGCACACCCAATATCC-3'; F-Hes5: 5'-CATGAAACGATGCTTGAAGAGACGCTT-3'; R-Hes5: 5'-GTGGCAAGGCCGGCTGACGTG-3'; F-β-actin: 5'-GCCGGCAGACCCCTCAGA-3'; R-β-actin: 5'-GCCGGCAGACCCCTCAGA-3'.

Western Blot Analysis

Immunoblots were carried out as previously described [4,5]. Antibodies to c-JUN (BD biosciences), p-c-JUNser73 (Cell Signaling), p-c-MYC (Cell Signaling), c-MYC (Santa Cruz), CYCLIN-E (Santa Cruz), and β-ACTIN (Sigma) were used.

**Histological Analysis**

Mice were euthanized by cervical dislocation and the small intestines prepared for histology as described before [48]. Sections were cut at 4 μm for Haematoxylin & Eosin staining and PAS/AB staining. To quantify goblet cells, AB/PAS+ cells were quantified from at least 100 villi from comparable intestinal regions from at least 5 mice from each genotype and the data represented as the mean ± SEM.

**Intracellular Staining and FACS Analysis**

HCT116 cells transfected with the indicated plasmids were fixed for 10 min in 1% PFA, permeabilized in PBS+0.5% Triton for 10 min at RT, and blocked in PBS+2% FCS for 30 min. After blocking, cells were incubated with anti-NICD antibody (1:500 dilution in PBS+2% FCS) for 30 min. Cells were washed in PBS+2% FCS and incubated with donkey-anti-rabbit-Alexa-647 secondary antibody (1:1000 in PBS+2% FCS) for 30 min in the dark at RT. Cells were analysed in an LSRII cytometer. Overlay Histograms (Hes-GFP or sh-Hes5-GFP versus their controls) were represented as NICD-Alexa-647 versus cell numbers on GFP+ gated cells. The number of GFP+ cells quantified for each individual sample, the single histograms, and the percentage of cells in high-NICD and low-NICD state are indicated in Figure S5.

**Immunofluorescence**

Cells from differentiation cultures were fixed for 20 min in 4% paraformaldehyde and permeabilized in ice-cold Methanol for 20 min. For immunocytochemistry, antibodies against NESTIN (BD [monoclonal]) and MAP2 (Sigma [monoclonal]) were used. DNA was counterstained with 4′,6-Diamidino-2-phenylindole (DAPI; Sigma).

**Mathematical Modelling**

To describe the Delta-Notch-Fbw7-Hes gene regulatory circuit, we adapted a standard simple Delta-Notch lateral-inhibition model, adding the Fbw7 feedback loop as in Figure 6a. We represented the dynamics by a set of differential equations, which we solved numerically using Mathematica to determine the final steady state of a two-dimensional array of cells. The model assumes that there are two relevant Hes genes, HesX and Hes5, where HesX stands for one (or more) of the many other members of the Hes/Hey family that are expressed in gut and CNS. HesX (by itself) represses Delta, while HesX and Hes5 act in parallel to repress Fbw7. Loss of functional Hes5 thus leads roughly to a doubling of Fbw7 expression and can be compensated by a halving of the Fbw7 gene dosage. With our chosen model parameters, the Fbw7 positive feedback loop gives rise to bistability, allowing a cell exposed to a given level of Delta signalling from its neighbours (above a certain low Delta threshold) to exist in either a low- or a high-NICD state (as suggested by the data; see Figures 5c,d and S5a). This biases the outcome of Delta-Notch-mediated lateral inhibition. In the version of the model used to compute Figure 6, we postulate molecular lifetimes such that the dynamics of the Fbw7 loop are fast compared with the dynamics of the Delta-Notch loop. Each cell then moves rapidly to a low- or high-NICD state, with a relative probability dependent on the starting conditions and genotype, creating an initial random multicellular pattern that is subsequently adjusted by lateral inhibition. The adjustments follow a simple rule: thanks to bistability, low-NICD cells can persist regardless of the states of their neighbours, but any high-NICD cell that is entirely surrounded by other high-NICD cells is eventually converted to a low-NICD state. This is because high NICD entails a near-zero level of Delta production, and the high-NICD state becomes unstable when levels of Delta signalling from neighbours fall very low.
The model assumes that cells all start in an approximately similar state but with some small random variation from cell to cell, reflecting genetic noise, whose consequences are amplified through the Fbw7 and Delta-Notch feedback loops to give a final pepper-and-salt pattern. Results of the computation are shown for a 10×10 hexagonal array of cells, with cyclic boundary conditions.

Mathematical details of the model and values of the parameters are given in Data S1. The Mathematica program is available on request from julian.lewis@cancer.org.uk.

### Statistical Analysis

Statistical evaluation was performed by Student’s unpaired t test. Data are presented as mean ± SEM. *p<0.05 was considered statistically significant. **p<0.01 was considered highly statistically significant. ***p<0.001 was considered very highly statistically significant.

### Supporting Information

**Data S1** Mathematical model.

(Figures S1-S8)

#### Figure S1
NICD target gene analysis in Fbw7AN/+ and Fbw7M/+ mice. (a) Quantification of NICD levels in intestine detected by Western blot in different experiments (pool of >3 mice each genotype per Western blot). Numbers represent fold induction over control after normalization to actin. (b) Q-PCR analysis of blots. Numbers represent fold induction over control after specific sequences for the four different sets of NICD target gene analysis in HCT116-wt, HCT116-Fbw7a-null and HCT116-Fbw7β-null cells. (a) Q-PCR analysis of intracellular NICD in HCT116-wt, HCT116-Fbw7a-null, or HCT116-Fbw7β-null cells. (b) Q-PCR analysis of Hey1, Hey2, Hes1, Hes5, Hes6, Jagged1, Jagged2, Dll1, Dll3, and Dll4 in wild-type or Fbw7AN/+ NSCs. (c) Q-PCR analysis of Hey1, Hey2, Hes1, Hes3, Hes6, Jagged1, Jagged2, Dll1, Dll3, and Dll4 in wild-type or Fbw7M/+ intestinal tissue (ud, undetectable). (d) Q-PCR analysis of Hes1 in wild-type, Fbw7M/+ intestinal tissue and Fbw7AN/+ NSCs using four different sets of Hes1 Q-PCR primers with specific sequences for the four different sets of Hey1 Q-PCR primers used.

(TIF)

#### Figure S2
Absolute abundance of Fbw7α and Fbw7β mRNA in NSCs, Guts, and HCT116. Data presented in the table contain the calculated amount of molecules per microriter of Fbw7α and Fbw7β mRNA calculated as an extrapolation of the Ct values (from each sample) to the equation of the regression curve obtained using serial dilutions of Fbw7α or Fbw7β plasmids.

(TIF)

#### Figure S3
Endogenous HES5 chromatin IP analysis. ChIP was performed using HCT116 cells. HES5 binding to the consensus sites in FBW7A, FBW7B, and MGN2 promoters was determined by Q-PCR. Data were represented as fold activation of percentage input versus IgG immunoprecipitated samples.

(TIF)

#### Figure S4
HES5 represses Fbsαβ transcription. (a) Q-PCR analysis of Fbsαβ, Fbw7β, Hey3, and Hes1 in NSCs transfected with pCDNA3 or pCDNA3-NICD. (b) Q-PCR analysis of Fbsαβ, Fbw7β, Hey3, and Hes1 in NSCs transfected with p-Super-sh-control or p-Super-sh-Hes5-1 and p-Super-sh-Hes5-2 (specific silencers for Hes5).

(TIF)

#### Figure S5
FACS analysis of sh-Hes3 and Hes5-GFP transfected HCT116-wt, HCT116-Fbw7α-null, and HCT116-Fbw7β-null cells. (a) Single histograms displaying NICD-Alexa547 versus number of cells in sh-control-GFP/sh-Hes5-GFP transfected cells. (b) Percentage of NICD-low/NICD-high in sh-control-GFP/sh-Hes5-GFP transfected cells. (c) Table containing the number of GFP+ counted cells in each sample, the percentage of NICD-low/NICD-high cells, and the percentage increase in NICD-high cells of sh-Hes5-GFP transfected cells compared to sh-control-GFP transfected cells. (d) Single histograms displaying NICD versus number of cells in empty-GFP/Hes5-GFP transfected cells. (e) Percentage of NICD-low/NICD-high in empty-GFP/Hes5-GFP transfected cells. (f) Table containing the number of GFP+ counted cells in each sample, the percentage of NICD-low/NICD-high cells, and the percentage increase in NICD-high cells of Hes5-GFP transfected cells compared to empty-GFP transfected cells.

(TIF)

#### Figure S6
NICD target gene analysis in HCT116-wt, HCT116-Fbw7α-null and HCT116-Fbw7β-null cells. (a) Q-PCR analysis of Hey1, Hey2, Hes1, Hes5, Hes6, Jagged1, Jagged2, Dll1, Dll3, and Dll4 in HCT116-wt, HCT116-Fbw7α-null and HCT116-Fbw7β-null cells. (b) Western blot analysis of c-MYC and TUBULIN in HCT116-wt, HCT116-Fbw7α-null, or HCT116-Fbw7β-null cells after treatment with cyclohexamide for the indicated time points.

(TIF)

#### Figure S7
Subcellular localisation of Fbw7β. (a) Immunofluorescence of Hela cells transfected in the presence or absence of proteasome inhibitor (MG132) with pEGFP-C2-Fbw7β. (b) Immunoblot of nuclear and cytoplasmic extracts of 293T cells transfected with pEGFP-C2-Fbw7β in the presence of proteasome inhibitor (MG132) for GFP, LAMINB, and TUBULIN. (c) Immunoblot of nuclear and cytoplasmic extracts of HCT116 cells transfected with different concentrations of pCMV-Fbw7β-flag for Flag, LAMINB, and TUBULIN.

(TIF)

#### Figure S8
Fbw7β binds and ubiquitylates NICD. (a) HCT116-wt cells were transfected with Flag-tagged FBW7-alpha or FBW7-beta. Cell extracts were immunoprecipitated with anti-Flag and immunoblotted with anti-NICD. (b) HCT-Fbw7α cells were transfected with Flag-tagged FBW7-alpha ± Myc-tagged NICD α FBW7-beta ± Myc-tagged NICD. Cell extracts were immunoprecipitated with anti-Flag and immunoblotted with anti-MYC. (c) HCT116-wt, HCT116-Fbw7α-null, or HCT116-Fbw7β-null cells were transfected with Myc-tagged NICD and His-Ubiquitin. Ubiquitylated NICD was pulled down by Ni²⁺-NTA agarose beads and immunoblotted with anti MYC antibody.

(TIF)

### Materials and Methods S1
Details of cell culture and transfections, IP assays, ubiquitylation assay, plasmids and reagents, and qRT-PCR used in supplementary figures.

(DOCX)

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### Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: RS SMB AB.
References

1. Welcker M, Oriol A, Grım JE, Eiermann RN, Churman BE (2004) A nuclear isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. Gen. Biol. 14: 1852–1857.

2. Grim JE, Gustafson MP, Hirata RK, Hagar AC, Swanger J, et al. (2008) Isoform- and cell cycle-dependent substrate degradation by the Fbw7 ubiquitin ligase. J Cell Biol 181: 913–920.

3. van Drogen F, Sangfelt O, Malyskova A, Matskova I, Yeh E, et al. (2006) Ubiquitination of cyclin E requires the sequential functional of SCF complexes containing distinct bCdc4 isofoms. Mol Cell 23: 37–48.

4. Hoek JD, Jandke A, Blake SM, Nye E, Spencer-Dene B, et al. (2010) Fbw7 controls neural stem cell differentiation and progenitor apoptosis via Notch and c-Jun. Nat Neurosci 13: 1365–1372.

5. Sancho R, Jandke A, Davis H, Diefenbacher ME, Tomlinson I, et al. (2010) Fbw7 regulates c-Myc and cell size. Development 137: 799–806.

6. Babari-Jadidi R, Li N, Saadatlinia A, Spencer-Dene B, Jandke A, et al. (2011) FBXN7 influences murine intestinal homeostasis and cancer, targeting Notch, Jun, and DEK for degradation. J Exp Med 208: 293–312.

7. Scoville DH, Sato T, He XC, Li L (2008) Current view: intestinal stem cells and signaling. Gastroenterology 134: 849–864.

8. Sancho E, Batlle E, Clevers H (2004) Signaling pathways in intestinal development and cancer. Annu Rev Cell Dev Biol 20: 137–164.

9. van Drogen F, Sangfelt O, Malyukova A, Matskova I, Yeh E, et al. (2006) Ubiquitination of cyclin E requires the sequential functional of SCF complexes containing distinct bCdc4 isofoms. Mol Cell 23: 37–48.

10. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, et al. (2005) Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435: 929–933.

11. Fre S, Huyge M, Mortuza P, Robine S, Louvard D, et al. (2005) Notch signals control the fate of immature progenitor cells in the intestine. Nature 435: 946–950.

12. Gotz M, Barde YA (2005) Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. Neuron 46: 369–372.

13. Corbin JG, Gaiasson N, Julioan SL, Pichol S, Stancic E, et al. (2008) Regulation of neural progenitor cell development in the nervous system. J Neurochem 106: 2372–2386.

14. Yoon KJ, Koo BK, Im SK, Jeong HW, Ghihn J, et al. (2008) Mind bomb 1-expressing intermediate progenitor genes generate Notch signaling to maintain radial glial cells. Neuron 58: 519–531.

15. Gaiasson N, Nye JS, Fishell G (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron 26: 395–404.

16. Borggreve T, Oswald F (2009) The Notch signaling pathway: transcriptional regulation at Notch target genes. Cell Mol Life Sci 66: 1631–1646.

17. Ferroni M (2008) Notch signaling: the core pathway and its posttranslational regulation. Dev Cell 16: 633–647.

18. Lewis J (1998) Notch signaling and the control of cell fate choices in vertebrates. Semin Cell Biol 9: 503–509.

19. el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, et al. (2011) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39: 186–193.

20. Jandke A, Da Costa C, Sancho R, Nye E, Spencer-Dene B, et al. (2011) The F-box protein Fbw7 is required for cerebellar development. Dev Biol 358: 201–212.

21. Tsunematsu R, Nakayama K, Oike Y, Nishiyama Y, Ishida N, et al. (2004) Mouse Fbw7/Sel-10/Cdc4 is required for notch degradation during vascular development. J Biol Chem 279: 9417–9423.

22. Natori AS, Riera-Sans L, Da Costa C, Behrens A (2004) The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. Science 303: 1374–1378.

23. Kepp DM, Schaefer LK, Ye X, Keyomarsi K, Chao C, et al. (2001) Phosphorylation-dependent ubiquitination of cyclin E by the SCF(Fbw7) ubiquitin ligase. Science 294: 173–177.

24. Welcker M, Churman BE (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer 8: 83–96.

25. Jensen J, Pedersen EJ, Galante P, Hald J, Heller RS, et al. (2000) Control of endodermal endocrine development by Hex-1. Nat Genet 24: 36–44.