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Notch post-translationally regulates β-catenin protein in stem and progenitor cells

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Cellular decisions of self-renewal or differentiation arise from integration and reciprocal titration of numerous regulatory networks. Notch and Wnt/β-catenin signalling often intersect in stem and progenitor cells and regulate each other transcriptionally. The biological outcome of signalling through each pathway often depends on the context and timing as cells progress through stages of differentiation. Here, we show that membrane-bound Notch physically associates with unphosphorylated (active) β-catenin in stem and colon cancer cells and negatively regulates post-translational accumulation of active β-catenin protein. Notch-dependent regulation of β-catenin protein did not require ligand-dependent membrane cleavage of Notch or the glycogen synthase kinase-3β-dependent activity of the β-catenin destruction complex. It did, however, require the endocytic adaptor protein Numb and lysosomal activity. This study reveals a previously unrecognized function of Notch in negatively titrating active β-catenin protein levels in stem and progenitor cells.

Canonical Wnt signals are mediated by the transcriptional effector β-catenin. In the absence of Wnt signalling, β-catenin is phosphorylated by a destruction complex of glycogen synthase kinase 3β (GSK-3β), adenomatous polyposis coli (APC) and axin[1]. Wnt signalling disrupts the destruction complex, enabling the unphosphorylated β-catenin protein at Ser 37 or Thr 41 to accumulate and function as a co-activator for the transcription factor TCF/LEF (T-cell factor/lymphoid enhancer factor; ref. 1).

The TCF/β-catenin complex targets many genes that promote the cell cycle and simultaneously regulates transcription of some members of the Notch signalling pathway, establishing reciprocal interactions between Wnt and Notch signals[2,3]. The canonical Notch signalling pathway is initiated when transmembrane ligands bind to the extracellular domain of transmembrane Notch receptors. This leads to cytoplasmic cleavage of the Notch intracellular domain (NICD) by γ-secretase and its rapid translocation to the nucleus. In the nucleus, Notch functions as a co-activator for the DNA-binding transcription factor RBP-J to activate Notch target genes, which often promote cellular differentiation[4].

We recently demonstrated that Notch1 antagonizes Wnt/β-catenin signalling by reducing levels of active β-catenin in cardiac progenitor cells (CPCs; ref. 5), which represent a multipotent transient amplifying cell population. However, the mechanism of Notch's negative regulation of β-catenin protein and the breadth of this event in other stem-cell types were unknown.

To determine if Notch negatively regulates β-catenin protein levels in stem cells, we used a *Notch1* short interfering RNA (siRNA) to decrease Notch1 levels in E14 embryonic stem cells (ESCs). We found that reduced Notch1 levels did not noticeably affect the levels of total or amino-terminal phosphorylated (Ser 37) β-catenin protein but resulted in an increase in the dephosphorylated, transcriptionally active form of β-catenin protein (Fig. 1a). The active form of β-catenin normally constitutes a small fraction of total β-catenin and was detected with antibodies that specifically recognize dephosphorylated β-catenin at Ser 37 and Thr 41 (ref. 6). In agreement with this finding, knockdown of Notch1 in ESCs showed significantly more TCF/β-catenin-dependent luciferase activity than controls (Fig. 1b; Supplementary Fig. S1). Interestingly, knocking down transcripts of all four Notch receptors (*Notch1, 2, 3, 4*) by applying *Notch1–4* siRNAs further increased β-catenin activity but the degree of increase was mild (Fig. 1b; Supplementary Fig. S1). This indicates that Notch1 is the predominant Notch receptor involved in regulating TCF/β-catenin activity in ESCs, consistent with the high level of Notch1 in ESCs (ref. 7). The increase in TCF/β-catenin-dependent luciferase activity was also observed in *Notch* siRNA-treated neural stem cells (NSCs; Fig. 1c) and in mouse CPCs lacking Notch1 *in vivo* and *in vitro*[5], indicating that Notch1 may function to negatively regulate active β-catenin levels in stem-cell populations.

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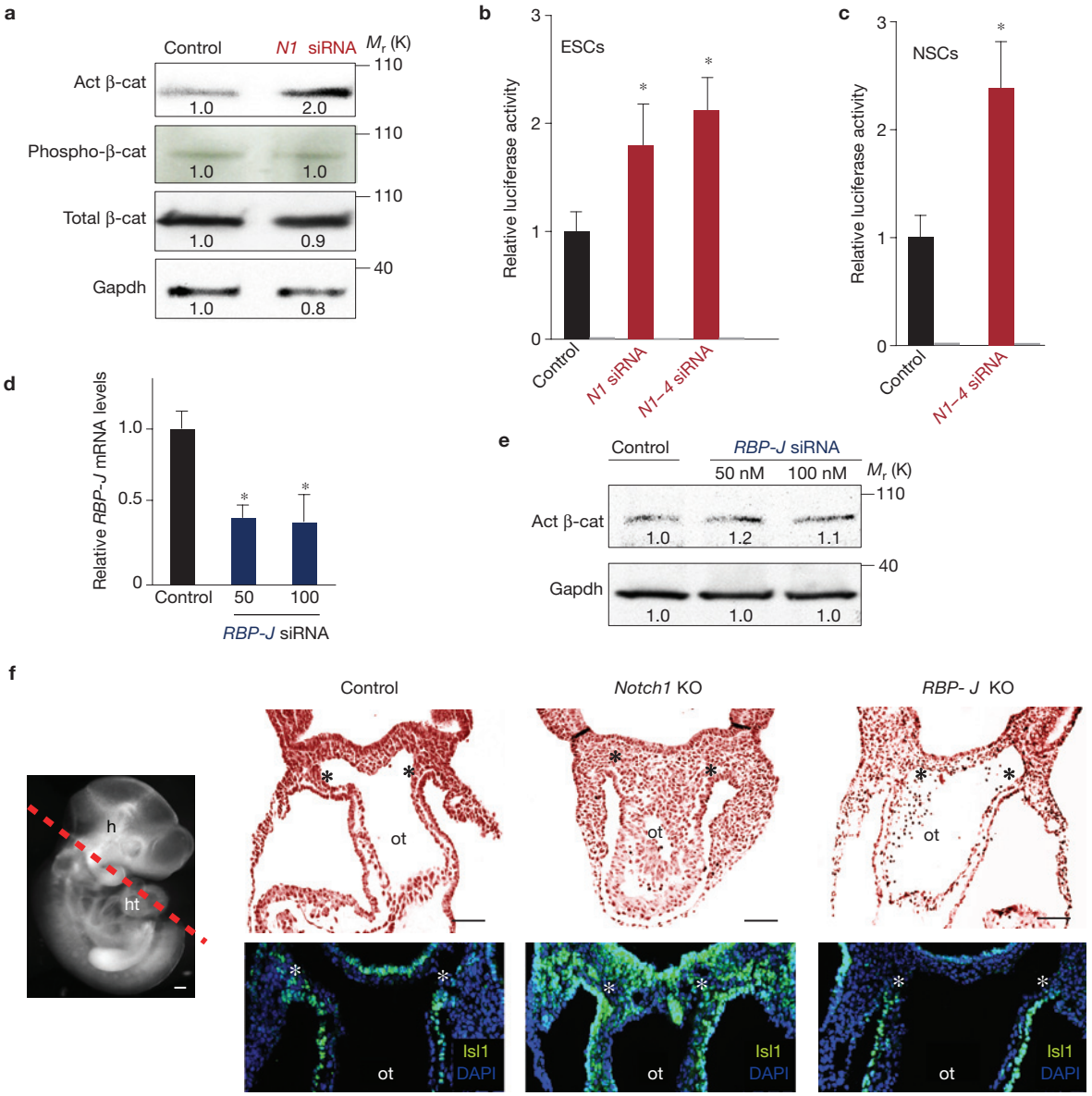

Figure 1 Notch negatively regulates active β-catenin in stem cells independently of RBP-J. (**a**) Western analysis of ESCs transfected with control or *Notch1* (*N1*) siRNA with active (Act), phospho- (Ser 37) or total β-catenin antibodies that detect N-terminal-dephosphorylated β-catenin. (**b**,**c**) Relative β-catenin/TCF-directed luciferase activity in ESCs (**b**) or NSCs (**c**) transfected with control siRNA or siRNA against *Notch1* or *Notch1–4* (*N1–4*). β-catenin/TCF activity was measured by co-transfecting cells with a luciferase reporter downstream of multiple TCF binding sites (Topflash). A mutant reporter (Fopflash) exhibited negligible activity in all luciferase assays done in this study. (**d**) Relative *RBP-J* expression levels by qPCR in ESCs after transfection with control or *RBP-J* siRNA, determined by qPCR. (**e**) Western analysis of ESCs transfected with control or *RBP-J* siRNA (50 or 100 nM) with Act β-cat antibodies. (**f**) Transverse sections of control, *Notch1* knockout (KO) (Isl^{Cre};$Notch1^{tm2Rko}$) or *RBP-J* KO (Isl^{Cre};$RBP\text{-}J^{flox/flox}$) embryos stained with haematoxylin and eosin (H&E) (top) or Isl1 antibody (green, bottom) at embryonic day 9.5, at the level of the outflow tract (ot). The asterisks indicate precardiac mesoderm containing CPCs. 4,6-diamidino-2-phenylindole (DAPI; blue) was used to counterstain the nuclei. The cutting plane is indicated by a dotted line (left). Scale bars, 100 μm. All luciferase values were normalized to *Renilla* activity (mean ± s.d.; $n = 4$; $^*P < 0.01$). *P* values were determined using a two-tailed Student *t*-test, type II (see Methods). Gapdh antibody was used as a loading control. Numbers on western blots correspond to relative quantification. h, head; ht, heart tube. Uncropped images of blots are shown in Supplementary Fig. S8.

We next sought to determine if the regulation of β-catenin protein occurs through the canonical Notch signalling pathway involving the transcription factor RBP-J. We introduced an *RBP-J*-specific siRNA into ESCs to reduce RBP-J levels. Despite about 70% knockdown of *RBP-J* messenger RNA (Fig. 1d), active β-catenin levels were unchanged (Fig. 1e). To determine if RBP-J mediates the Notch regulation of β-catenin *in vivo*, we deleted *Notch1* or *RBP-J* in CPCs by inter-crossing $Notch1^{tm2Rko}$ (ref. 8) (*Notch1* floxed allele) or $RBP\text{-}J^{flox/flox}$ mice[9] with mice containing Cre recombinase in the *Isl1* locus ($Isl1^{Cre}$; ref. 10). Isl1 marks an undifferentiated pool of CPCs (ref. 11), whose expansion depends on Wnt/β-catenin signalling[12,13]. Unlike embryos with a *Notch1* deletion, the resulting *RBP-J*-mutant

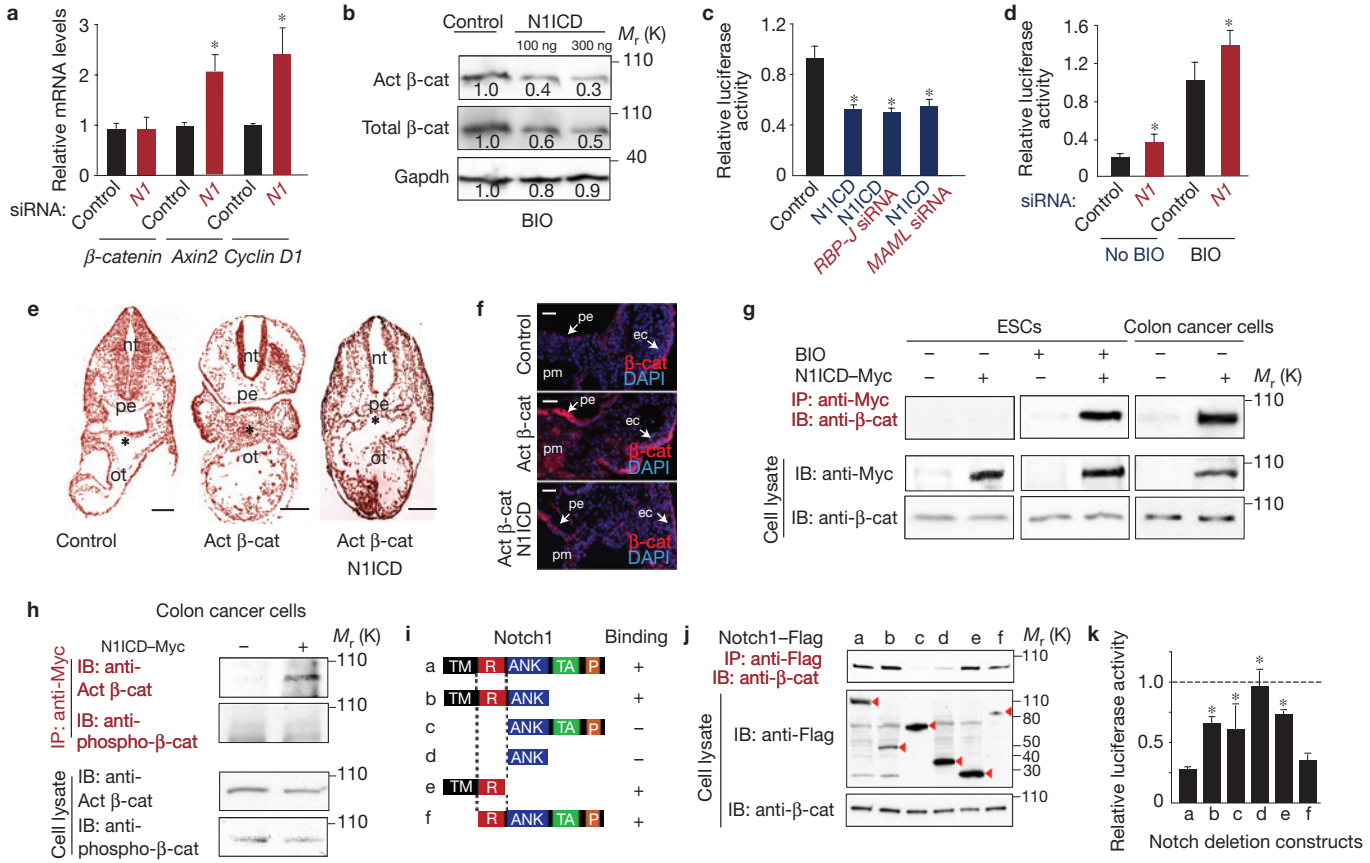

Figure 2 Notch1 negatively regulates active β-catenin in ESCs and physically interacts with β-catenin. (**a**) Relative expression of *β-catenin* and *Cyclin D1* mRNA in ESCs transfected with control or *Notch1* siRNA (100 nM), determined by qPCR. (**b**) Western analysis of ESCs transfected with control plasmids or plasmids encoding N1ICD (100 or 300 ng) and cultured with BIO. Gapdh antibody was used as a loading control. (**c**) Relative β-catenin/TCF luciferase activity of BIO-treated ESCs transfected with control or N1ICD ± *MAML* or *RBP-J* siRNA. (**d**) Relative β-catenin/TCF luciferase activity of ESCs transfected with control or *Notch1* siRNA and cultured with or without BIO. (**e**,**f**) Transverse sections of control, Isl^{Cre};β-$catenin(ex3)^{loxP}$ (Act β-cat) or Isl^{Cre};β-$catenin(ex3)^{loxP}$;$Gt(ROSA)26Sor^{tm1(Notch1)Dam}/J$ (Act β-cat; N1ICD overexpression) embryos at embryonic day 9.5, stained with H&E (**e**) or β-catenin antibody (red, **f**). The asterisks indicate precardiac mesoderm containing CPCs (**e**). Scale bars, 100 μm (**e**) or 25 μm (**f**). DAPI (blue) was used to counterstain the nuclei (**f**). nt, neural tube; ot, outflow tract; pe, pharyngeal endoderm; ec, pharyngeal ectoderm; pm, precardiac mesoderm. (**g**,**h**) ESCs treated with or without BIO (**g**) or SW480 (human colon cancer) cells (**g**,**h**) were transfected with expression constructs for Myc (−) or N1ICD–Myc (+), immunoprecipitated (IP) with anti-Myc antibody and immunoblotted (IB) with β-catenin antibody recognizing its carboxy terminus (**g**), dephosphorylated (active) form or the phosphorylated N-terminus (**h**). Notch expression was detected with anti-Myc antibody (**g**). (**i**) Schematic representation of Notch1 deletion constructs and their interactions with β-catenin. TM, transmembrane domain; R, RAM domain; ANK, Ankyrin repeats; TA, transactivation domain; P, PEST domain. (**j**) Co-IP of BIO-treated ESCs with the Notch1 deletion constructs shown in **i** using the antibodies indicated. Arrowheads indicate Notch1 expression. (**k**) Relative β-catenin/TCF-dependent luciferase activity when compared with control (dashed line) of BIO-treated ESCs transfected with the Notch constructs shown in **i**. BIO was used at 2 μM. All qPCR or luciferase values were normalized to Gapdh or *Renilla* activity, respectively. (mean ± s.d.; $n = 4$; $^*P < 0.01$.) *P* values were determined using a two-tailed Student *t*-test, type II (see Methods). Numbers on western blots correspond to relative quantification. Uncropped images of blots are shown in Supplementary Fig. S8.

embryos showed no expansion of CPCs (Fig. 1f). These data indicated that Notch-mediated regulation of active β-catenin protein in ESCs and CPCs did not involve RBP-J-dependent transcriptional regulation.

RBP-J-independent Notch signalling has been described in vertebrates and invertebrates[14] and is thought to involve Notch-mediated transcription through other DNA-binding proteins. However, quantitative PCR (qPCR) revealed that levels of *β-catenin* transcripts were not altered in *Notch1*-knockdown ESCs, although *Axin2* and *Cyclin D1*, direct targets of TCF/β-catenin (refs 15,16), were significantly upregulated in *Notch1*-knockdown cells (Fig. 2a). This raised the possibility that Notch affects β-catenin protein at the post-translational level. As the key step in activation of Wnt signalling is regulation of the amount and localization of β-catenin by GSK3β-dependent phosphorylation of its N terminus in an APC-based destruction complex, we investigated whether the effects of Notch were mediated by this complex. We first confirmed that a pharmacological GSK3β inhibitor, 6-bromoindirubin-3'-oxime (BIO), specifically inhibits GSK3β activity and inactivates the destruction complex[17], resulting in the accumulation of active β-catenin (Supplementary Fig. S2). Overexpression of the Notch1 intracellular domain (N1ICD) in ESCs reduced active and total β-catenin protein levels, but not mRNA, and decreased its activity in the presence of BIO (Fig. 2b,c). The decrease was also evident in ESCs deficient for RBP-J or Mastermind-like (MAML), an essential co-transcriptional regulator

for Notch signalling[18] (Fig. 2c), providing further evidence that Notch negatively regulates β-catenin in a transcription-independent fashion. Furthermore, reduced levels of Notch1 resulted in increased β-catenin activity even beyond that seen in BIO-treated ESCs (Fig. 2d). This indicated that Notch-mediated negative regulation of β-catenin protein *in vitro* is independent of GSK3β activity.

To determine if Notch could suppress β-catenin activity *in vivo*, independent of GSK3β activity, we expressed a form of β-catenin that cannot be degraded by the destruction complex, with or without *Notch1*, in the domain of precardiac mesoderm. This was done by crossing $Isl1^{Cre}$ mice with $\beta\text{-}catenin(ex3)^{loxP}$;$Gt(ROSA)26Sor^{tm1(Notch1)Dam}/J$ mice. The $\beta\text{-}catenin(ex3)^{loxP}$ allele contains *loxP* sites surrounding exon 3 of *β-catenin*, which encodes amino acids required for GSK3β-mediated degradation, generating stabilized β-catenin on *Cre* expression[19], and the $Gt(ROSA)26Sor^{tm1(Notch1)Dam}/J$ allele contains a *loxP-stop-loxP* sequence before *Notch1* in the *Rosa* locus, enabling tissue-specific overexpression of Notch1 on *Cre* expression[20]. Co-expression of stabilized β-catenin and Notch1 completely rescued the abnormal expansion of precardiac mesoderm induced by activated β-catenin alone (Fig. 2e). Immunochemical analyses revealed that β-catenin protein levels were increased in the domains of $Isl1^{Cre}$ expression (pharyngeal mesoderm, ectoderm and endoderm) in $\beta\text{-}catenin(ex3)^{loxP}$ mice, but were reduced close to baseline levels on Notch1 expression in the same domains (Fig. 2f). These findings provide evidence that Notch can negatively titrate β-catenin protein levels *in vivo*, independent of GSK3β activity, and thereby regulate expansion of progenitor cells.

Given that Notch expression did not require GSK3β activity to regulate β-catenin protein, we examined if Notch modulates active β-catenin protein levels through a direct physical interaction. We expressed N1ICD in ESCs by transfecting cells with a Myc-tagged N1ICD construct and carried out co-immunoprecipitation (Co-IP) assays with anti-Myc antibodies with or without BIO. Comparable introduction of control Myc and Myc–N1ICD constructs into ESCs was confirmed by qPCR (Supplementary Fig. S3). We did not detect an interaction of endogenous β-catenin with Notch1 in the absence of BIO (Fig. 2g). However, when treated with BIO, which greatly increases active β-catenin levels by inactivating the destruction complex, Notch1 co-precipitated with endogenous β-catenin (Fig. 2g), but not with APC, Axin, Gsk3β or TrCP (Supplementary Fig. S4). This indicated that Notch might selectively interact with active β-catenin, whose levels are normally very low in ESCs. To investigate this possibility further, we used a human colon cancer cell line, SW480, which contains high levels of active β-catenin owing to an APC mutation that causes colon cancer[21]. When expressed in SW480 cells, Notch strongly associated with endogenous β-catenin even without BIO treatment (Fig. 2g). Further analysis of the precipitated β-catenin confirmed enrichment of active β-catenin, but not of N-terminal phosphorylated β-catenin (Fig. 2h). These data indicate that Notch physically associates with the active form of β-catenin, although we cannot exclude the possibility that interaction with the phosphorylated form is below the level of detection.

Next, we mapped the domains of Notch responsible for β-catenin association by carrying out Co-IP experiments with a series of truncated Notch mutants that lacked the extracellular domain[22] (Fig. 2i). We found that Notch mutants lacking the RAM domain had limited association with β-catenin (Fig. 2j). To determine if the RAM domain, also required for RBP-J interaction[23], was necessary for Notch regulation of β-catenin transcriptional activity, we expressed control and mutant Notch constructs in BIO-treated ESCs with the β-catenin luciferase reporter. In agreement with the Co-IP result, deleting the RAM domain significantly compromised Notch's ability to repress β-catenin activity (Fig. 2k). Interestingly, Notch without the transactivation or PEST (proline–glutamic acid–serine–threonine) domains also showed reduced repressive activity, implying that these domains also contribute to repression (Fig. 2k).

The overexpression of N1ICD results in excessive cytoplasmic accumulation as well as nuclear localization (Supplementary Fig. S5). We therefore investigated whether ligand-dependent cleavage of Notch to free the NICD, which is essential for canonical Notch signalling, was necessary for the Notch regulation of active β-catenin protein, or whether membrane-bound Notch was sufficient.

Notch1 intracellular cleavage occurs between amino acids Gly 1743 and Val 1744 in a highly conserved manner; mutations of Val 1744 (V1744K or V1744L) block intracellular cleavage, leaving Notch tethered to the membrane[24] (Fig. 3a). We confirmed that the tethered form of Notch (V1744L) remained uncleaved in ESCs and exhibited negligible levels of Notch/RBP-J-dependent luciferase activity (Fig. 3a,b). Surprisingly, expression of the tethered forms of Notch in ESCs decreased β-catenin transcriptional activity to a comparable extent to the repression mediated by the well-known Wnt inhibitor Dkk1 (Fig. 3c). The tethered Notch-mediated repression occurred independent of RBP-J (Supplementary Fig. S6). In addition, endogenous active β-catenin immunoprecipitated with the tethered form of Notch (Fig. 3d) in the presence of BIO. In agreement with the Co-IP result, active and total β-catenin protein levels were considerably lower in cells with the tethered form of Notch (Fig. 3e).

Membrane-bound Notch has no transcriptional activity and would conventionally be considered biologically inert. To determine if negative titration of active β-catenin protein by membrane-bound Notch has biological consequences, we assayed the effects of tethered Notch on Wnt-dependent differentiation of ESCs to mesoderm[25]. We expressed tethered Notch (V1744L) in early embryoid bodies (before induction of mesodermal cells) derived from ESCs containing green fluorescent protein (GFP) in the endogenous mesoderm-specific *Brachyury* (*Bry*) gene[26]. Fluorescence-activated cell sorting revealed that the number of Bry^+ cells was significantly reduced on expression of tethered Notch. This occurred with or without BIO (Fig. 3f). This result indicates that membrane-bound Notch can negatively titrate a cellular response mediated by Wnt/β-catenin signalling in stem cells.

To determine if endogenous membrane-bound Notch negatively regulates active β-catenin protein, we blocked Notch endoproteolysis, which is mediated by the presenilin–γ-secretase complex[27]. We found that mouse ESCs treated with the γ-secretase inhibitor (GSI) DAPT (ref. 28), had a significant reduction of active β-catenin activity and protein levels in a dose-dependent fashion (Fig. 3g,h). This trend was also observed in hESCs, NSCs and bone marrow mesenchymal stem cells (MSCs) (Fig. 3g,h) and, importantly, occurred in the absence of any overexpression. The number of Bry^+ cells was also decreased in embryoid bodies when treated with DAPT (Fig. 3i). Similarly, blocking α-secretase activity, required for ligand-mediated

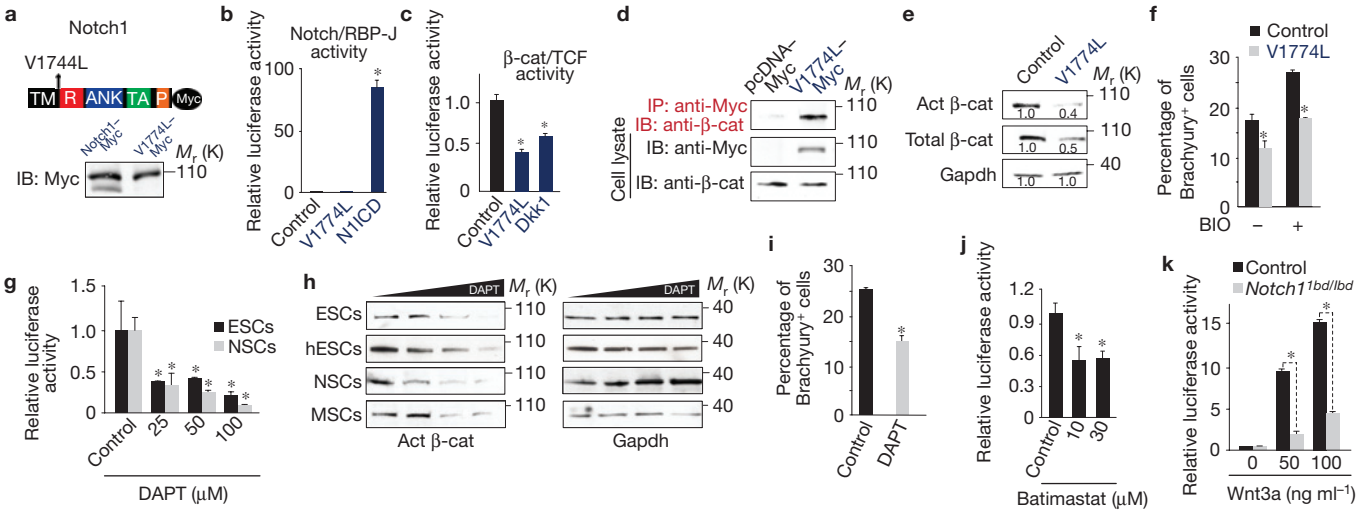

Figure 3 Membrane-bound Notch1 negatively regulates active β-catenin levels through Numb and Numb-like in stem cells. (**a**) Schematic representation of a cleavage-site-mutated tethered form of Notch1 (V1774L, top) and western analysis of ESCs transfected with plasmids encoding Myc-tagged wild-type Notch1 or tethered Notch1 (V1774L) and blotted with anti-Myc antibody (bottom), showing the lack of a cleaved protein band. (**b**) Relative RBP-J-responsive luciferase activity of mESCs transfected with control plasmids or plasmids encoding tethered Notch1 or N1ICD. (**c**) Relative β-catenin/TCF luciferase activity of ESCs transfected with the control or tethered Notch1 (V1774L) construct shown in **a** or treated with Dkk1 (50 ng ml^{-1}). (**d**) BIO-treated ESCs transfected with control or tethered Notch1–Myc constructs immunoprecipitated (IP) with anti-Myc antibody and immunoblotted (IB) with β-catenin antibody. Notch1 expression was detected with anti-Myc antibody. (**e**) Western analysis of active or total β-catenin in BIO-treated ESCs transfected with control plasmids or plasmids encoding tethered Notch1. (**f**) Percentage of *Brachyury*–GFP^{+} cells after 3 days of differentiation of mouse ESCs with tethered Notch (V1774L) or control in the presence or absence of BIO (0.5 μM; mean ± s.d.; $n = 4$; $^{*}P < 0.01$). (**g**) Relative β-catenin/TCF luciferase activity of ESCs or NSCs treated with increasing doses of DAPT for 72–96 h. (**h**) Western analysis of active β-catenin in mouse or human ESCs, NSCs or bone marrow MSCs treated with increasing doses of DAPT (0, 25, 50 or 100 μM) for 72–96 h. (**i**) Percentage of *Brachyury*–GFP^{+} cells after 3.5 days of differentiation of mouse ESCs with control or 75 μm DAPT (mean ± s.d.; $n = 4$; $^{*}P < 0.01$). (**j**) Relative β-catenin/TCF luciferase activity of ESCs treated with control or batimastat, an α-secretase inhibitor. (**k**) Relative β-catenin/TCF luciferase activity of wild-type ESCs (control) or ESCs with ligand-binding-site-deleted Notch1 (*Notch1*$^{lbd/lbd}$) treated with Wnt3a. All luciferase values were normalized to *Renilla* activity (mean ± s.d.; $n = 4$; $^{*}P < 0.01$). *P* values were determined using a two-tailed Student *t*-test, type II (see Methods). Gapdh antibody was used as a loading control. Numbers on western blots correspond to relative quantification. BIO was used at 2 μM. Uncropped images of blots are shown in Supplementary Fig. S8.

cleavage of the Notch extracellular domain, resulted in a significant reduction of active β-catenin activity (Fig. 3j). To further test the ligand-independent function of Notch, we used *Notch1*$^{lbd/lbd}$ ESCs where endogenous Notch1 lacks the 11 and 12th epidermal growth factor (EGF) repeats required for ligand binding[29]. When stimulated with Wnt3a, we found that *Notch1*$^{lbd/lbd}$ ESCs exhibited significantly lower β-catenin-dependent luciferase activity than controls (Fig. 3k).

Membrane-bound Notch is regulated by endosomal sorting pathways, leading to either recycling or lysosomal degradation[30]. In *Drosophila*, the conserved endocytic adaptor protein Numb, which is present as two orthologues, Numb and Numb-like (Numbl) in mammals, negatively regulates Notch[31,32]. As one mechanism, Numb inhibits Notch signalling by trafficking membrane-bound Notch into the lysosome for degradation[33]. We found that endogenous Numb was also co-immunoprecipitated with the tethered form of Notch (Fig. 4a), indicating that Numb may be involved in Notch-mediated degradation of active β-catenin.

To determine if Numb activity was required for degradation of active β-catenin complexed with membrane-bound Notch, we knocked down *Numb* and *Numbl* with siRNAs in ESCs in the presence of the tethered form of Notch (V1744L). Tethered Notch was unable to repress β-catenin transcriptional activity in *Numb*- and *Numbl*-deficient ESCs (Fig. 4b). In agreement with this finding, knockdown of Numb and Numbl abrogated the ability of tethered Notch to lower active β-catenin protein levels (Fig. 4c). Similarly, Numb and Numbl deficiency relieved repression of β-catenin activity observed on overexpression of N1ICD (Fig. 4b).

These data indicated that Numb and Numbl were involved in lysosomal trafficking of the Notch-β-catenin complex for degradation. In agreement with this, inhibition of lysosomal activity with bafilomycin A1, a potent and specific inhibitor of vacuolar proton ATPases[34], abrogated the DAPT-induced decrease in active β-catenin protein in ESCs (Fig. 4d). Furthermore, immunocytochemistry revealed that tethered Notch1 (or NICD) and active β-catenin co-localized with the lysosomal protein Lamp1 (Supplementary Fig. S7). These findings indicate that the Notch-β-catenin complex is present in the lysosome and that lysosomal activity is important for the Notch-mediated decrease in active β-catenin.

Extrapolating our results from stem/progenitor cells, we hypothesized that membrane-bound Notch could affect β-catenin levels in *APC*-mutated human cancer cells containing elevated active β-catenin protein. We knocked down *Notch 1–4* in SW480 human colorectal cancer cells and found a prominent increase in active β-catenin protein levels (Fig. 5a). This result provided further evidence for regulation of β-catenin by Notch, independent of the destruction complex. Conversely, treatment of two human colorectal cancer cell lines, SW480 and HT-29, with DAPT, which chemically prevents NICD cleavage, resulted in a paradoxical dose-dependent decrease in

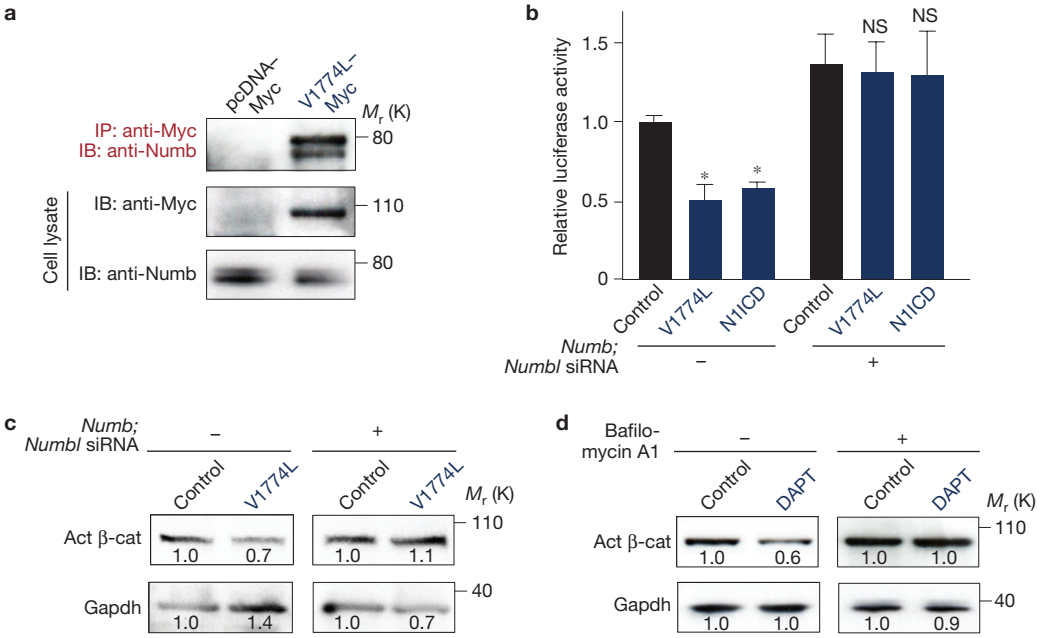

Figure 4 Notch-mediated degradation of β-catenin requires Numb and lysosomal activity. (**a**) Human colon cancer cells (SW480) transfected with pcDNA–Myc or tethered Notch (V1774L)–Myc constructs, immunoprecipitated (IP) with anti-Myc antibody and immunoblotted (IB) with anti-Numb antibody. Expression of tethered Notch was detected with anti-Myc antibody; expression of pcDNA–Myc was confirmed by PCR. (**b**) Relative β-catenin/TCF luciferase activity of ESCs transfected with control plasmids or plasmids encoding N1ICD or tethered Notch (V1774L) in the presence or absence of *Numb/Numbl* siRNA and cultured in BIO for 72 h. (**c**) Western analysis of active β-catenin in ESCs transfected with control plasmids or plasmids encoding tethered Notch (V1774L) in the presence or absence of *Numb/Numbl* siRNA. (**d**) Western analysis of active β-catenin in ESCs with control or DAPT treatment in the presence or absence of bafilomycin A1, which inhibits lysosomal activity. All luciferase values were normalized to *Renilla* activity (mean ± s.d.; $n = 4$; $^*P < 0.01$; NS, not significant). *P* values were determined using a two-tailed Student *t*-test, type II (see Methods). Gapdh antibody was used as a loading control. Numbers on western blots correspond to relative quantification. Uncropped images of blots are shown in Supplementary Fig. S8.

TCF/β-catenin-dependent transcriptional activity, β-catenin protein and cell expansion (Fig. 5b–d). Proteasome inhibitors that block the destruction-complex-mediated degradation of β-catenin resulted in increased active β-catenin levels but failed to prevent the Notch-mediated decrease in β-catenin protein (Fig. 5e). This indicates that Notch regulation of β-catenin protein is probably not proteasome mediated and supports the earlier evidence showing Numb dependence and potential involvement of the lysosome.

Chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) in humans has frequently been reported to lower the risk of developing primary and recurrent colorectal cancer[35,36]. A subset of NSAIDs also has significant GSI activity[37], and we correspondingly found that ibuprofen induced a dose-dependent decrease of canonical Notch transcriptional activity, determined by Notch/RBP-J-dependent luciferase activity in SW480 cells (Fig. 5f). Ibuprofen treatment also lowered levels of active β-catenin transcriptional activity and protein (Fig. 5g,h). Importantly, the reduction of β-catenin protein levels on ibuprofen treatment of cancer cells was not observed after knockdown of *Notch1–4* (Fig. 5i). This indicates that NSAIDs act, at least in part, through Notch to decrease active β-catenin protein levels, and this regulation may contribute to the overall protective effects of NSAIDs on colorectal cancers. This result is consistent with the observation that GSI treatment in APC-mutant mice reduces proliferating adenomas in the intestine[38,39].

In the present study, we show that Notch negatively regulates protein levels of active β-catenin in a post-translational manner and thereby serves to titrate Wnt/β-catenin signalling in stem and progenitor cells (Fig. 5j). In our experiments, the interaction between these two critical regulatory proteins did not require ligand-dependent cleavage of Notch, and membrane-bound Notch could form a complex with the active form of β-catenin. This was observed in cells with active Wnt signalling, where inactivation of the destruction complex resulted in higher levels of active β-catenin. Thus, in the presence of Wnt signalling, Notch might serve to titrate active β-catenin levels to temper the proliferative state of expanding cells and affect cellular decisions.

Although NICD can interact with β-catenin in the cytosol and co-localizes with the lysosomal marker on overexpression, this is unlikely to be its normal function given its low cytoplasmic levels under physiological conditions. Instead, it may be the membrane-bound Notch that serves to titrate the active form of β-catenin in cells responding to Wnt signalling. The evolutionary conservation of this process is striking, as Notch also interacts with Armadillo in endocytic vesicles in *Drosophila* and negatively regulates Wnt signalling[40].

Generally, GSIs, such as DAPT, mimic canonical Notch loss-of-function mutations. However, our findings indicate that GSI treatment paradoxically decreases Wnt/β-catenin signals through membrane-bound Notch, which effectively reduces active β-catenin levels and activity. Biochemical approaches to purify the Notch-β-catenin complex may reveal the more precise mechanism by which Notch affects active β-catenin accumulation/degradation and provide more specific approaches to disrupt or promote the Notch–β-catenin interaction. □

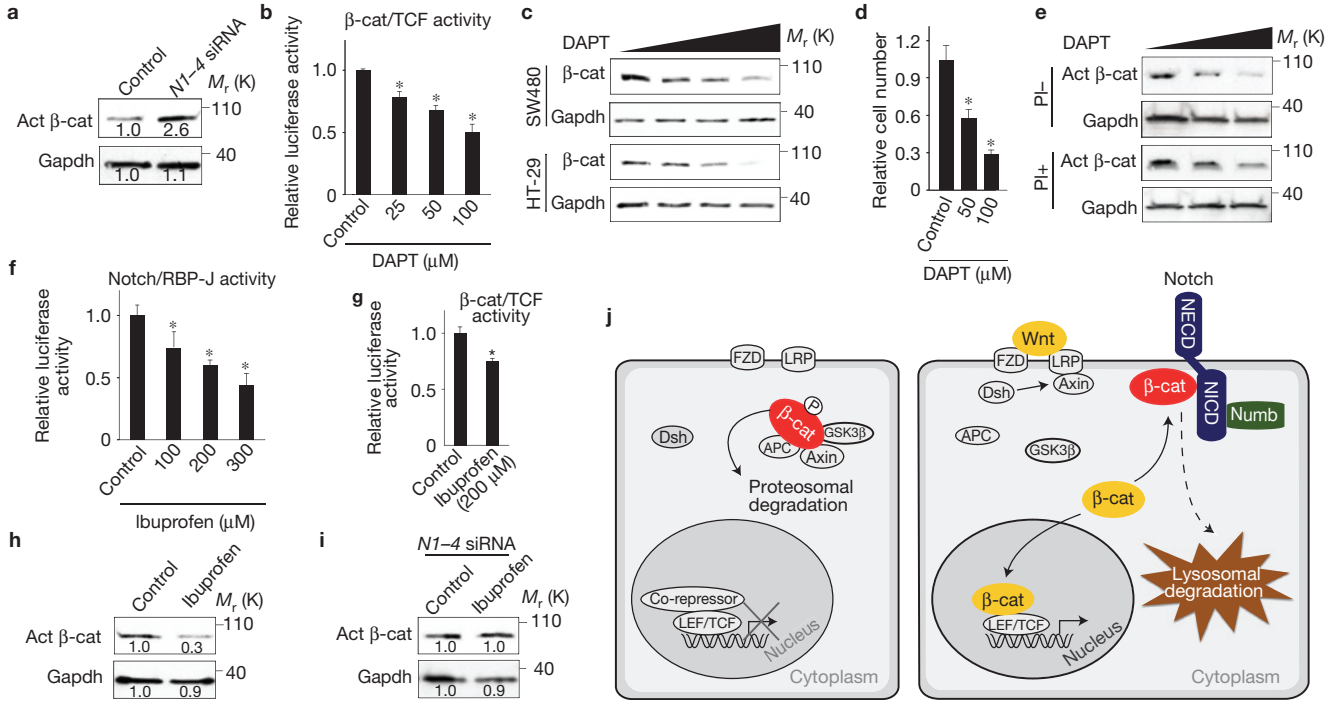

Figure 5 GSIs negatively regulate Wnt signalling and cell expansion in colon cancer cells by blocking Notch cleavage. (**a**) Western analysis of active β-catenin in SW480 human colon cancer cells transfected with control siRNA or siRNA against *Notch1–4* (100 nM each). (**b**) Relative β-catenin/TCF luciferase activity of SW480 cells treated with increasing doses of DAPT for 96 h. (**c**) Western analysis of β-catenin levels in SW480 and a second colon cancer cell line, HT-29, treated with increasing doses (0, 25, 50 or 100 μM) of DAPT for 96 h. (**d**) Relative number of SW480 cells treated with DAPT (50 or 100 μM) for 72 h (mean ± s.d.; $n = 4$; $^*P < 0.01$). (**e**) Western analysis of active β-catenin levels in SW480 cells with increasing DAPT in the presence or absence of proteasome inhibitor (PI) MG-132 (5 nM) for 72 h. Fewer PI-treated cells were loaded in the right-hand lane because they exhibit higher levels of β-catenin. (**f**) Notch/RBP-J luciferase reporter activity (multimerized RBP-J binding sites) of SW480 cells treated with increasing doses of ibuprofen. (**g**) Relative β-catenin/TCF luciferase activity of SW480 cells treated with ibuprofen for 72 h. (**h**) Western analysis of active β-catenin in SW480 cells treated with ibuprofen (200 μM) for 72 h. (**i**) Western analysis of active β-catenin in SW480 cells with control or ibuprofen (200 μM) treatment transfected with *Notch1–4* (100 nM each) siRNA. Gapdh antibody was used as a loading control. All luciferase values were normalized to *Renilla* activity (mean ± s.d.; $n = 4$; $^*P < 0.01$). *P* values were determined using a two-tailed Student *t*-test, type II (see Methods). Numbers on western blots correspond to relative quantification. (**j**) Model for post-translational regulation of β-catenin protein by Notch. In the absence of Wnt, the destruction complex of Axin, APC and GSK3β phosphorylates β-catenin, leading to its proteasomal degradation (left). When the destruction complex is inactivated by Wnts, dephosphorylated (active) β-catenin functions as a transcriptional activator with LEF/TCF. We show that active β-catenin protein levels can be negatively regulated by interaction with Notch in a Numb-dependent manner, involving the lysosome. Notch-mediated degradation of β-catenin is independent of the APC-dependent destruction complex. FZD, frizzled; LRP, low-density lipoprotein receptor-related protein; Dsh, dishevelled; NECD, Notch extracellular domain; NICD, Notch intracellular domain. Uncropped images of blots are shown in Supplementary Fig. S8.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

C.K. designed, carried out and supervised *in vivo* and *in vitro* work and wrote the manuscript. P.C. designed and carried out *in vivo* and *in vitro* work and wrote the manuscript. I.N.K. carried out Notch Co-IP and western analyses. P.A. cultured embryonic stem cells and carried out luciferase assays. L.S. carried out immunocytochemistry and confocal microscopy. V.N. isolated mesenchymal stem cells and carried out western analyses. D.S. designed and supervised this work and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

D.S. is a scientific co-founder of iPierian Inc. and is a member of the Scientific Advisory Board of iPierian Inc. and RegeneRx Pharmaceuticals.

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1. Logan, C. Y. & Nusse, R. The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20,** 781–810 (2004).
2. Radtke, F. & Clevers, H. Self-renewal and cancer of the gut: two sides of a coin. *Science* **307,** 1904–1909 (2005).
3. Hayward, P., Kalmar, T. & Arias, A. M. Wnt/Notch signalling and information processing during development. *Development* **135,** 411–424 (2008).
4. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. *Science* **284,** 770–776 (1999).

5. Kwon, C. *et al.* A regulatory pathway involving Notch1/β-catenin/Isl1 determines cardiac progenitor cell fate. *Nat. Cell Biol.* **11,** 951–957 (2009).
6. van Noort, M., Weerkamp, F., Clevers, H. C. & Staal, F. J. Wnt signaling and phosphorylation status of β-catenin: importance of the correct antibody tools. *Blood* **110,** 2778–2779 (2007).
7. Nemir, M., Croquelois, A., Pedrazzini, T. & Radtke, F. Induction of cardiogenesis in embryonic stem cells via downregulation of Notch1 signaling. *Circ. Res.* **98,** 1471–1478 (2006).
8. Yang, X. *et al.* Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev. Biol.* **269,** 81–94 (2004).
9. Tanigaki, K. *et al.* Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat. Immunol.* **3,** 443–450 (2002).
10. Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1,** 4 (2001).
11. Cai, C. L. *et al.* Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev. Cell* **5,** 877–889 (2003).
12. Kwon, C. *et al.* Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proc. Natl Acad. Sci. USA* **104,** 10894–10899 (2007).
13. Qyang, Y. *et al.* The renewal and differentiation of Isl1+ cardiovascular progenitors are controlled by a Wnt/β-catenin pathway. *Cell Stem Cell* **1,** 165–179 (2007).
14. Martinez Arias, A., Zecchini, V. & Brennan, K. CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? *Curr. Opin. Genet. Dev.* **12,** 524–533 (2002).
15. Yan, D. *et al.* Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/β-catenin signaling is activated in human colon tumors. *Proc. Natl Acad. Sci. USA* **98,** 14973–14978 (2001).
16. Tetsu, O. & McCormick, F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398,** 422–426 (1999).
17. Meijer, L. *et al.* GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* **10,** 1255–1266 (2003).
18. McElhinny, A. S., Li, J. L. & Wu, L. Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. *Oncogene* **27,** 5138–5147 (2008).
19. Harada, N. *et al.* Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. *EMBO J.* **18,** 5931–5942 (1999).
20. Murtaugh, L. C., Stanger, B. Z., Kwan, K. M. & Melton, D. A. Notch signaling controls multiple steps of pancreatic differentiation. *Proc. Natl Acad. Sci. USA* **100,** 14920–14925 (2003).
21. Korinek, V. *et al.* Constitutive transcriptional activation by a β-catenin–Tcf complex in APC-/- colon carcinoma. *Science* **275,** 1784–1787 (1997).
22. Yamamoto, N. *et al.* Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. *J. Biol. Chem.* **276,** 45031–45040 (2001).
23. Tamura, K. *et al.* Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J κ/Su(H). *Curr. Biol.* **5,** 1416–1423 (1995).
24. Schroeter, E. H., Kisslinger, J. A. & Kopan, R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393,** 382–386 (1998).
25. Lindsley, R. C., Gill, J. G., Kyba, M., Murphy, T. L. & Murphy, K. M. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* **133,** 3787–3796 (2006).
26. Fehling, H. J. *et al.* Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* **130,** 4217–4227 (2003).
27. De Strooper, B. *et al.* A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398,** 518–522 (1999).
28. Sastre, M. *et al.* Presenilin-dependent γ-secretase processing of β-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep.* **2,** 835–841 (2001).
29. Ge, C. & Stanley, P. Effects of varying Notch1 signal strength on embryogenesis and vasculogenesis in compound mutant heterozygotes. *BMC Dev. Biol.* **10,** 36 (2010).
30. Kanwar, R. & Fortini, M. E. Notch signaling: a different sort makes the cut. *Curr. Biol.* **14,** R1043–R1045 (2004).
31. Guo, M., Jan, L. Y. & Jan, Y. N. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17,** 27–41 (1996).
32. Zhong, W., Jiang, M. M., Weinmaster, G., Jan, L. Y. & Jan, Y. N. Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. *Development* **124,** 1887–1897 (1997).
33. McGill, M. A., Dho, S. E., Weinmaster, G. & McGlade, C. J. Numb regulates post-endocytic trafficking and degradation of Notch1. *J. Biol. Chem.* **284,** 26427–26438 (2009).
34. Tapper, H. & Sundler, R. Bafilomycin A1 inhibits lysosomal, phagosomal, and plasma membrane PH(+)-ATPase and induces lysosomal enzyme secretion in macrophages. *J. Cell Physiol.* **163,** 137–144 (1995).
35. Chan, A. T. *et al.* Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. *JAMA* **294,** 914–923 (2005).
36. Rostom, A. *et al.* Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force. *Ann. Intern. Med.* **146,** 376–389 (2007).
37. Eriksen, J. L. *et al.* NSAIDs and enantiomers of flurbiprofen target γ-secretase and lower Aβ 42 *in vivo*. *J. Clin. Invest.* **112,** 440–449 (2003).
38. Koch, U. & Radtke, F. Notch and cancer: a double-edged sword. *Cell Mol. Life Sci.* **64,** 2746–2762 (2007).
39. van Es, J. H. *et al.* Notch/γ-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435,** 959–963 (2005).
40. Sanders, P. G. *et al.* Ligand-independent traffic of Notch buffers activated Armadillo in *Drosophila*. *PLoS Biol.* **7,** e1000169 (2009).

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METHODS

Mouse genetics and cell culture. The *Isl1*^{Cre};*RBP-J*$^{flox/flox}$ or *Isl1*^{Cre};*Notch1*$^{flox/flox}$ embryos were obtained by crossing *Isl*^{Cre};*RBP-J*$^{flox/+}$ mice with *RBP-J*$^{flox/flox}$ or *Notch1*^{tm2Rko} mice, respectively[8,10]. *Isl1*^{Cre};*β-catenin*(*ex3*)$^{loxP/+}$ or *Isl1*^{Cre};*β-catenin*(*ex3*)$^{loxP/+}$; *Gt*(*ROSA*)*26Sor*$^{tm1(Notch1)Dam}$/*J* embryos were obtained by crossing *Isl*^{Cre} mice with *β-catenin*(*ex3*)$^{loxP/+}$, *Gt*(*ROSA*)*26Sor*$^{tm1(Notch1)Dam}$/*J* mice[20]. Mouse ESCs (E14) were cultured on gelatin-coated tissue culture dishes in standard maintenance medium (Glasgow minimum essential medium with 10% fetal bovine serum and 1×ESGRO (Chemicon), Glutamax, sodium pyruvate, MEM non-essential amino acids). Human ESCs were cultured on Matrigel-coated dishes in mTesR (STEMCELL Technologies). SW480, HT-29 and NSCs (NE4C) were cultured as indicated by the American Type Culture Collection.

Constructs, siRNA, transfection, gene expression and luciferase assays. For *Notch*, *RBP-J*, *MAML* or *Numb*/*Numbl* knockdown experiments, Notch1–4, RBPSUH, MAML or Numb/Numbl ON-TARGETplus SMARTpool (Dharmacon L-041110, L-044202, L-047867, L-046498, L-059179, L-007772 or L-046935/L-046983) or Block-iT Alexa Fluor Red (46-5, 318, Invitrogen) was used at concentrations of 50, 100, or 150 nM for cell transfection. Tethered and truncated forms of *Notch1* constructs were provided by R. Kopan (Washington University) and M. Nakafuku (Cincinnati Children's Hospital), respectively. Active β-catenin construct (S33Y) was obtained from Addgene. We used 75–100 ng of the constructs to transfect 3×10^5 cells. Cells were transfected with Lipofectamine LTX (Invitrogen) or Lipofectamine 2000 (Invitrogen) in single-cell suspensions. For gene expression analysis, qPCR was carried out with the ABI Prism system (7900HT, Applied Biosystems) with the following primers: *β-catenin* (Mm01350394_m1), *Cyclin D1* (Mm00432359_m1), *Axin2* (00443610_m1) or *Gapdh* (Mm99999915_g1). All samples were run at least in triplicate. Real-time qPCR data were normalized and standardized with SDS2.2 software. The constructs to measure Notch/RBP-J (JH23A) were provided by N. Gaiano (Johns Hopkins University). For luciferase assays, *Renilla* was used as an internal normalization control.

Co-immunoprecipitation, western and immunochemistry analyses. Cells were transfected with indicated constructs and cultured for 24 h (with/without BIO, 2 μM). Cells were scraped off the 100 mm dish and lysed in 1 ml of lysis buffer (1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, 10 mM Tris-HCl, 0.1% Triton X100, 1× Complete Protease Inhibitor Cocktail (Roche) in PBS). The lysates were spun down, and 1 μg of anti-c-Myc antibody (Sigma, M4439) or anti-Flag antibody (Sigma, F1804) was added to 500 μl of the supernatant. A 50–50 mixture of protein A Sepharose (Amersham) and protein G Sepharose (Amersham) was added to the lysate–antibody mixture for immunoprecipitation for 1 h. The resulting outputs were washed with lysis buffer and subjected to western blot analysis. For western blotting/immunochemistry, samples were analysed using primary antibodies against active β-catenin (anti-ABC, 1:500, Millipore), phospho-β-catenin (Ser 33/37/Thr 41, 1:1,000), Axin1 (C7B12, 1:1,000), GSK3β (27C10, 1:1,000), FLAG (2368, 3768, 1:50), Myc (71D10, 1:200), APC (2504, 1:1,000, Cell Signalling), Lamp1 (ab24170, 1:200), Numb (ab14140, 1:1,000, Abcam), Isl1 (1:100, DSHB), Myc (9E10, 1:500, Stratagene), β-catenin (sc-1496, 1:200), β-TrCP (C-18, 1:200) and Gapdh (1:2,000, Santa Cruz Biotechnology). Alexa 488 and 568 (1:200, Invitrogen) were used for secondary antibodies. Densitometry was carried out in Photoshop.

DAPT, ibuprofen, batimastat, ilomastat, bafilomycin A and BIO treatment. Cells were treated with DAPT (Calbiochem catalogue no 565784), ibuprofen (99% pure, Sigma), batimastat (TOCRIS catalogue no 2961), ilomastat (Sigma catalogue no M5939), bafilomycin A1 (TOCRIS catalogue no 1334), or BIO (Calbiochem catalogue no 361550) at the indicated concentrations.

ESC differentiation and flow cytometry. ESCs were trypsinized into single cells, and differentiated as standard embryoid bodies in 75% IMDM, 25% F12 supplemented with N2 and B27 supplements. The Becton Dickinson FACS Calibur flow cytometer was used for quantifying Bry–GFP$^+$ cells.

Statistical analyses. The two-tailed Student *t*-test, type II, was used for data analyses. $P < 0.05$ was considered significant.

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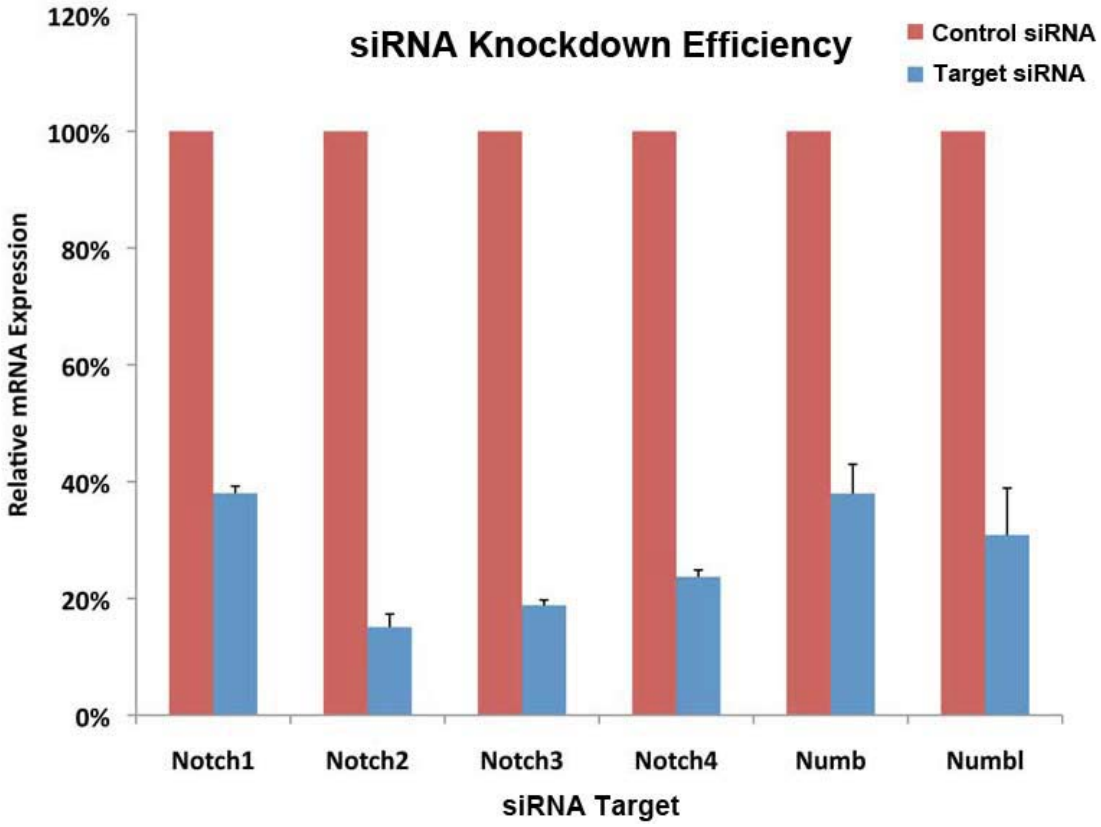
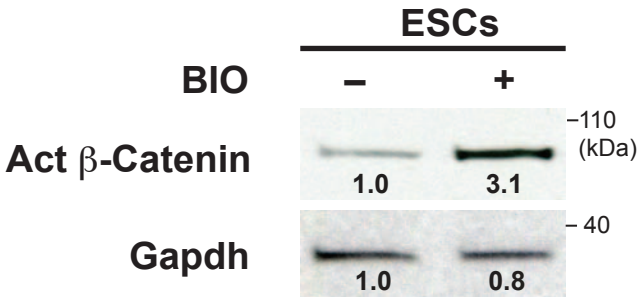
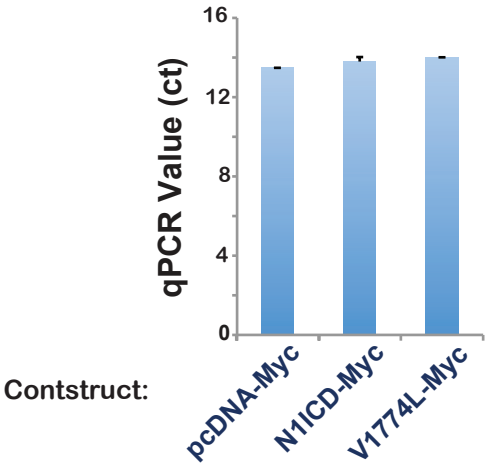


Figure S1 Knockdown efficiency of *Notch 1–4* and *Numb/Numbl* siRNA by qPCR (mean ± s. d.; n = 4; $*P < 0.01$). P values were determined using two-tailed Student's *t*-test, type II (see Methods).



Supp. Figure 2 - Kwon

Figure S2. Western analysis of active (Act) β-Catenin in embryonic stem cells (ESCs) treated with or without BIO (1μM). GAPDH antibody was used as a loading control.



Supp. Figure 3 - Kwon

Figure S3 qPCR quantification of pcDNA-Myc, N1ICD-Myc or tethered Notch1-Myc (V1774L-Myc) constructs introduced in ESCs for immunoprecipitation. PCR primers to ampicillin were used to quantify the constructs (mean ± s. d.; n = 3).

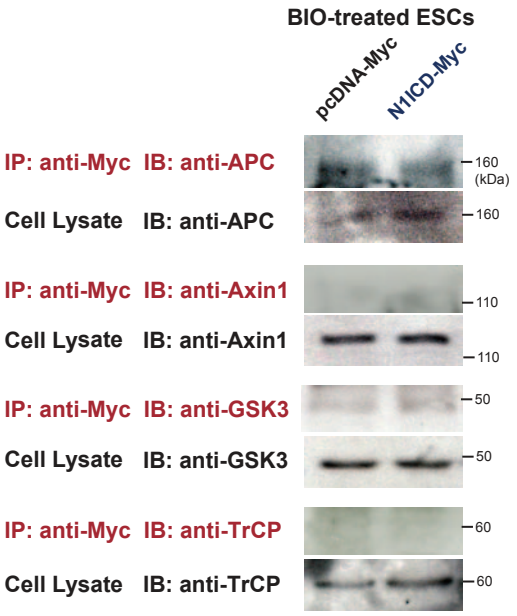


Figure S4 Immunoprecipitation assay. ESCs treated with BIO were transfected with expression constructs for pcDNA-Myc or NICD-myc, immunoprecipitated (IP) with anti-Myc antibody and immunoblotted (IB) with indicated antibodies.

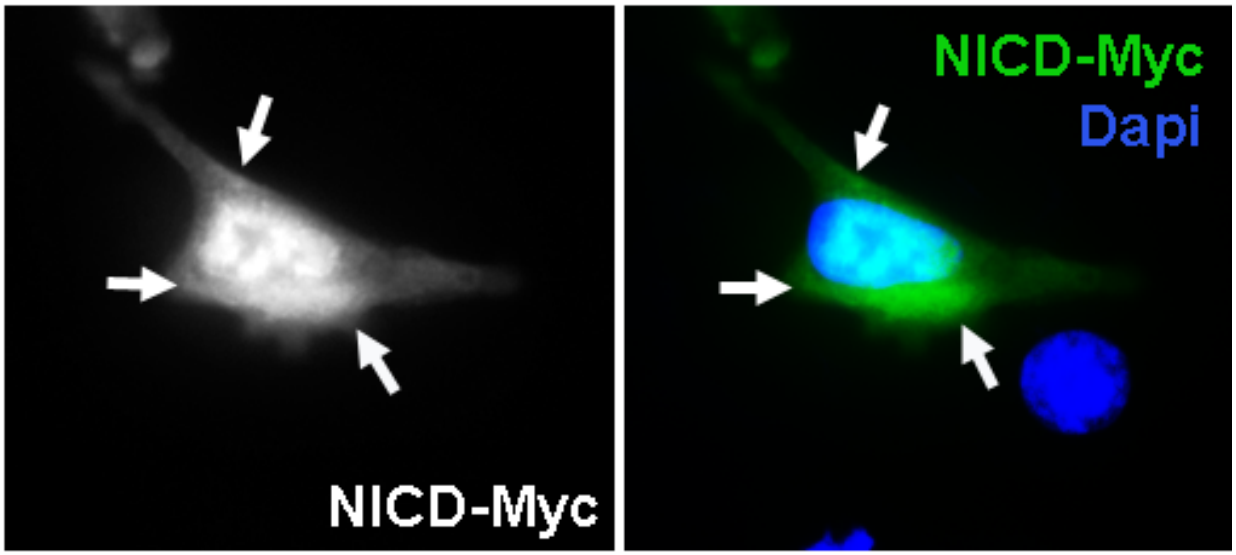


Figure S5 Confocal microscopy analysis of SW480 cells transfected with NICD-Myc construct. Cells were immunostained with Myc primary antibody and Alexa 488 secondary antibody. Arrows indicate cytoplasmic localization of NICD. Dapi (blue) was used to counterstain the nuclei.

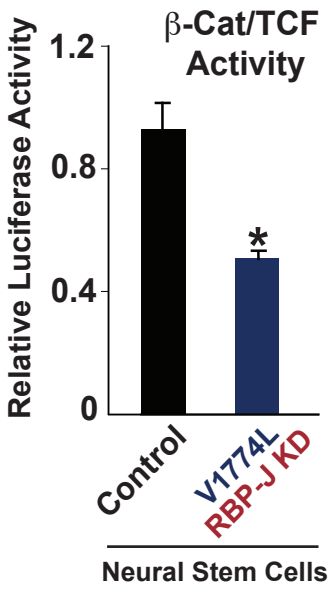


Figure S6 Relative β-Catenin/TCF luciferase activity of NSCs transfected with tethered Notch1 (V1774L) and *RBP-J* siRNA (mean ± s. d.; n = 4; **P* < 0.01). P values were determined using two-tailed Student's *t*-test, type II (see Methods).

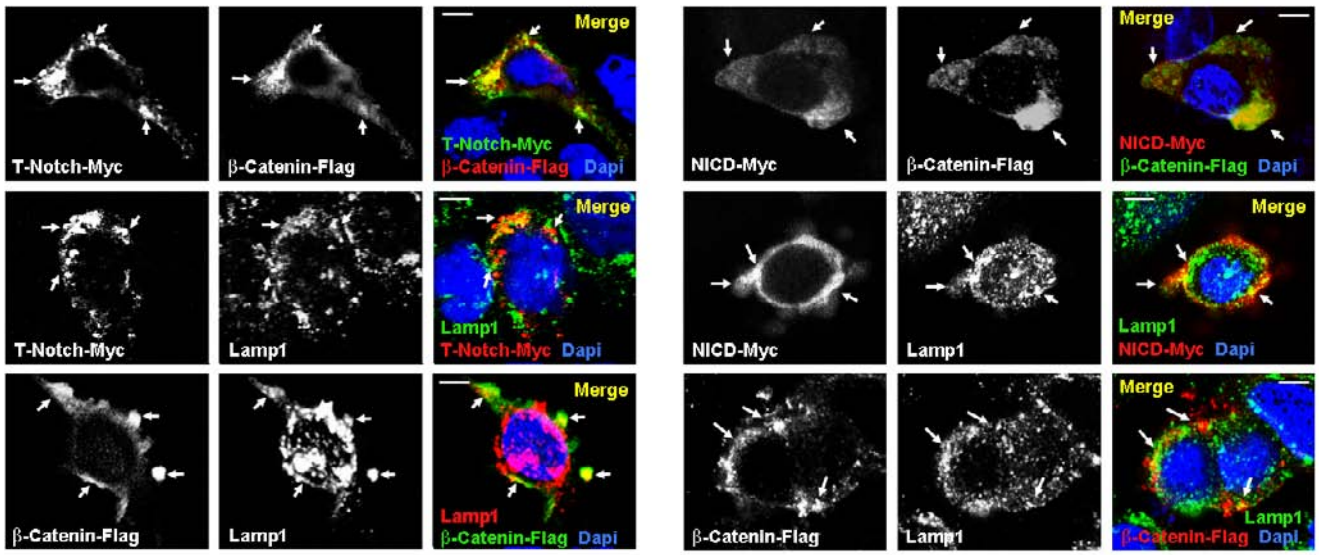


Figure S7 Confocal microscopy analysis of SW480 cells transfected with tethered Notch1-Myc (T-Notch-Myc, left) or NICD (right) and active β-Catenin-Flag (S33Y) constructs. Cells were immunostained with primary antibodies (Myc, Flag, Lamp1) detecting indicated proteins and fluorescent secondary antibodies (Alexa 488, 568). Arrows indicate co-localization. Dapi (blue) was used to counterstain the nuclei. Scale bars, 5μm.

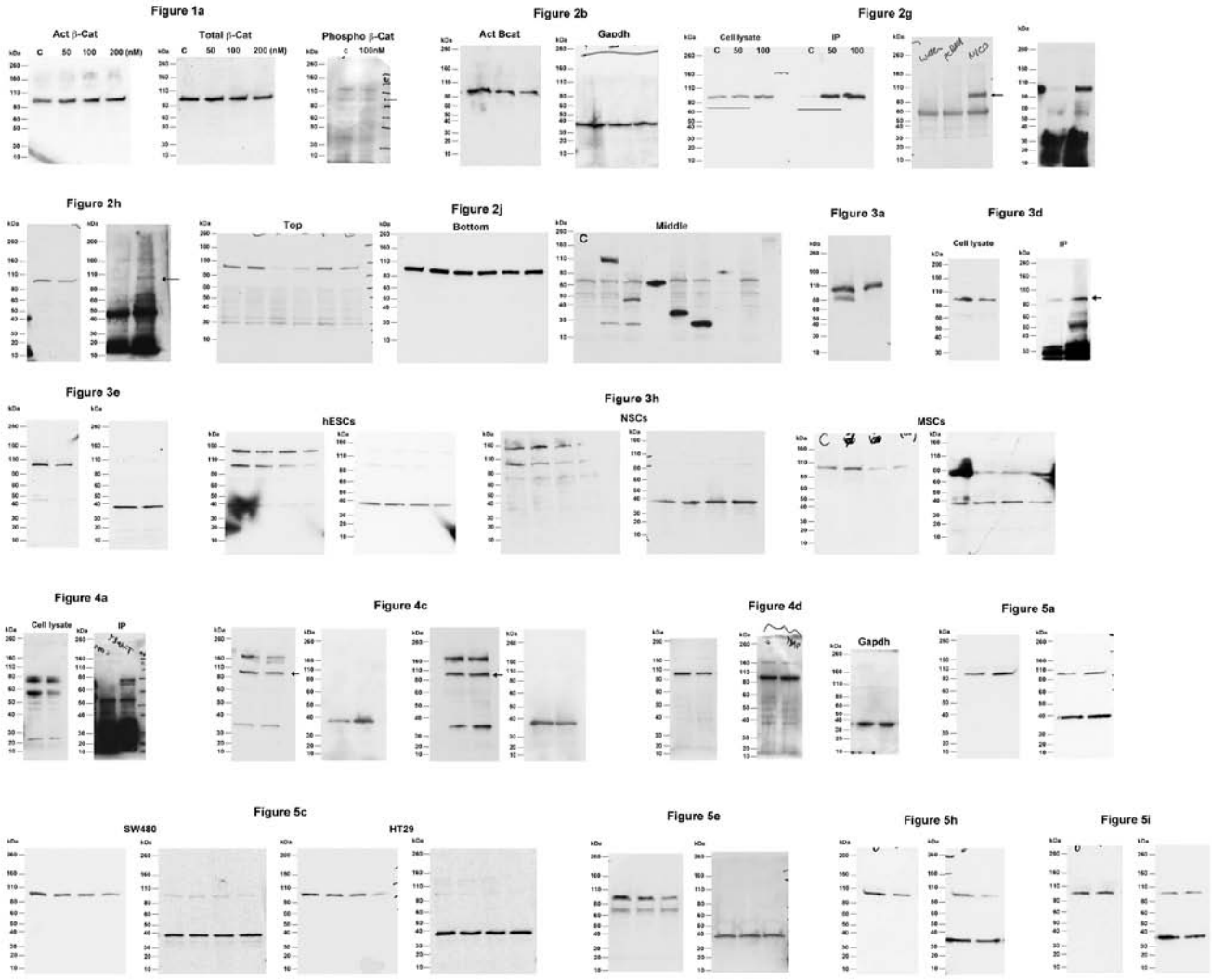


Figure S8 Full western blot scans