HHS Public Access

Author manuscript

Nat Cell Biol. Author manuscript; available in PMC 2012 April 01.

Published in final edited form as:

Nat Cell Biol. ; 13(10): 1244–1251. doi:10.1038/ncb2313.

Notch Post-Translationally Regulates β-Catenin Protein in Stem and Progenitor Cells

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Abstract

Cellular decisions of self-renewal or differentiation arise from integration and reciprocal titration of numerous regulatory networks. Notch and Wnt/β-Catenin signaling often intersect in stem and progenitor cells and regulate one another transcriptionally. The biological outcome of signaling 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.

Keywords

Notch; β-Catenin; Numb; stem cells; progenitors; cancer; DAPT; NSAID

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

Competing interests: D.S. serves on the scientific advisory board of iPierian Inc and RegeneRx Pharmaceuticals.

Canonical Wnt signals are mediated by the transcriptional effector, β-Catenin. In the absence of Wnt signaling, β-Catenin is phosphorylated by a destruction complex of glycogen synthase kinase-3β (GSK3β), adenomatous polyposis coli (APC), and axin [1]. Wnt signaling disrupts the destruction complex, allowing the unphosphorylated β-Catenin protein at the serine at residue 37 (Ser37) or threonine at residue 41 (Thr41) to accumulate and function as a co-activator for the transcription factor TCF/LEF [1].

The TCF/β-Catenin complex targets many genes that promote the cell cycle and simultaneously regulates transcription of some members of the Notch signaling pathway, establishing reciprocal interactions between Wnt and Notch signals [2,3]. The canonical Notch signaling 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 signaling by reducing levels of active β-Catenin in cardiac progenitor cells (CPCs) [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* 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 N-terminal phosphorylated (Ser37) 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 Ser37 and Thr41 [6]. In agreement with this finding, knockdown (KD) of Notch1 in ESCs showed significantly more TCF/β-Catenin-dependent luciferase activity than controls (Fig. 1b, 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, s1). This indicates that Notch1 is the predominant Notch receptor for this event in ESCs, consistent with the high level of Notch1 in ESCs [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], suggesting that Notch1 may function to negatively regulate active β-Catenin levels in stem cell populations.

We next sought to determine if the regulation of β-Catenin protein occurs through the canonical Notch signaling pathway involving the transcription factor, RBP-J. We introduced an *RBP-J*-specific siRNA into ESCs to reduce RBP-J levels. Despite a ~70% KD of *RBP-J* mRNA (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} [8] (*Notch1* floxed allele) or *RBP-J*$^{flox/flox}$ mice [9] with mice

containing Cre recombinase in the *Isl1* locus (*Isl1*^{Cre}) [10]. Isl1 marks an undifferentiated pool of CPCs [11], whose expansion depends on Wnt/β-Catenin signaling [12,13]. Unlike embryos with a *Notch1* deletion, the resulting *RBP-J* mutant embryos showed no expansion of CPCs (Fig. 1f). These data suggested 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 signaling 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* KD ESCs, although *Axin2* and *Cyclin D1*, direct targets of TCF/β-Catenin [15,16], were significantly upregulated in *Notch1* KD cells (Fig. 2a). This raised the possibility that Notch affects β-Catenin protein at the post-translational level. Since the key step in activation of Wnt signaling 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 (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 (Figs. 2b, c). The decrease was also evident in ESCs deficient for RBP-J or Mastermind-like (MAML), an essential co-transcriptional regulator for Notch signaling [18] (Fig. 2c), providing additional evidence that Notch negatively regulates β-Catenin in a transcription-independent fashion. Furthermore, reduced levels of Notch1 increased β-Catenin activity even beyond that seen in BIO-treated ESCs (Fig. 2d). This suggested 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 *β-Catenin(ex3)*^{loxP}; *Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$*/J* mice. The *β-Catenin(ex3)*^{loxP} allele contains *loxP* sites surrounding exon 3 of *β-catenin* that encodes amino acids required for GSK3β-mediated degradation, generating stabilized β-Catenin upon *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, allowing tissue-specific overexpression of Notch1 upon *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 *β-Catenin(ex3)*^{loxP} mice, but were reduced close to baseline levels upon 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 performed 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 (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 (Fig. s4). This suggested 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 due 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 suggest 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 performing 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 domains also showed reduced repressive activity, implying these domains also contribute to repression (Fig. 2k).

The overexpression of N1ICD results in excessive cytoplasmic accumulation as well as nuclear localization (Fig. s5). We therefore investigated whether ligand-dependent cleavage of Notch to free the NICD, which is essential for canonical Notch signaling, was necessary for the Notch regulation of active β-Catenin protein, or if membrane-bound Notch was sufficient.

Notch1 intracellular cleavage occurs between amino acids G1743 and V1744 in a highly conserved manner; mutations of V1744 (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 comparable to the repression mediated by the well-known Wnt inhibitor, Dkk1 (Fig. 3c). The tethered Notch-mediated repression occurred independent of RBP-J (Fig. s6). In addition, endogenous active β-Catenin immunoprecipitated with the tethered form of Notch (Figs. 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 (V1744) in early embryoid bodies (before induction of mesodermal cells) derived from ESCs containing GFP in the endogenous mesoderm-specific *Brachyury* (*Bry*) gene [26]. Fluorescence-activated cell sorting revealed that the number of Bry^+ cells was significantly reduced upon 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 signaling 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 [28], had a significant reduction of active β-Catenin activity and protein levels in a dose-dependent fashion (Figs. 3g, h). This trend was also observed in hESCs, NSCs and bone marrow mesenchymal stem cells (Figs. 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 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 utilized *Notch1*$^{lbd/lbd}$ ESCs where endogenous Notch1 lacks the 11 and 12th 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 signaling 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), suggesting 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 upon overexpression of N1ICD (Fig. 4b).

These data suggested 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 (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 additional 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 TCF/β-Catenin-dependent transcriptional activity, β-Catenin protein, and a decrease in 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 unlikely 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 γ-secretase inhibitory (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 upon Ibuprofen treatment of cancer cells was not observed after knockdown of *Notch1-4* (Fig. 5i). This suggests 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 signaling 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 signaling, where inactivation of the destruction complex resulted in higher levels of active β-Catenin. Thus, in the presence of Wnt signaling, Notch might serve to titrate active β-Catenin levels to temper the proliferative state of expanding cells and affect cellular decisions.

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

Generally, GSIs, such as DAPT, mimic canonical Notch loss-of-function mutations. However, our findings suggest 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.

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 with standard maintenance media (GMEM with 10% FBS with 1xESGRO (Chemicon), Glutamax, sodium pyruvate, MEM Non-Essential amino acid). Human ESCs were cultured on matrigel-coated dishes in mTesR (STEMCELL Technologies). SW480, HT-29, NE4C were cultured as suggested by ATCC.

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 kindly provided by Drs. R. Kopan (Washington University, St. Louis, MO) and M. Nakafuku (Cincinnati Children's Hospital, Cincinnati, OH), respectively. Active β-Catenin construct (S33Y) was obtained from Addgene. We used 75–100ng 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 performed 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 quantitative PCR data were normalized and standardized with SDS2.2 software. The constructs to measure Notch/RBP-J (JH23A) were kindly provided by Dr. 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 hours (with/without BIO, 2 μM). Cells were scraped off the 100-mm dish and lysed in 1 ml of lysis buffer (1 mM PMSF, 1 mM EDTA, 10 mM Tris-HCl, 0.1% Triton X100, 1x 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 hour. The resulting outputs were washed with lysis buffer and subjected to western blot analysis. For western blotting/immunochemistry, samples were analyzed using primary antibodies against active-β-Catenin (anti-ABC, 1:500, Millipore), phospho-β-Catenin (Ser33/37/Thr41, 1:1000), Axin1 (C7B12, 1:1000), GSK3β (27C10, 1:1000), FLAG (2368, 3768, 1:50), MYC (71D10, 1:200), APC (2504, 1:1000, Cell Signaling), Lamp1 (ab24170, 1:200), Numb (ab14140, 1:1000, Abcam), Isl1 (1:100, DSHB), Myc (9E10, 1:500, Stratagene), β-Catenin (sc-1496, 1:200), β-TrCP (C-18, 1:200), Gapdh (1:2000, 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 Cat#565784), ibuprofen (99% pure, Sigma), Batimastata (TOCRIS Cat#2961), Ilomastat (SIGMA Cat # M5939), Bafilomycin A1(TOCRIS Cat#1334), or BIO (CALBIOCHEM Cat#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 supplimented with N2, and B27 suppliments. The Becton Dickinson FACS Calibur flow cytometer was used for quantifying Bry-GFP^{+}cells.

Statistical Analyses

The two-tailed Student's *t*-test, type II, was used for data analyses. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. R. Kopan (Washington University, St. Louis, MO), M. Nakafuku (Cincinnati Children's Hospital, Cincinnati, OH), T. Honjo (Kyoto University, Kyoto, Japan) and Dr. P. Stanley for providing tethered Notch constructs, Notch deletion constructs, *RBP-J*^{flox} mice and *Notch1*$^{lbd/lbd}$ ES cells (Albert Einstein College of Medicine), respectively. The authors thank G. Howard and S. Ordway for editorial assistance, Srivastava and Kwon lab members for helpful discussions, B. Taylor for assistance with manuscript and figure preparation, and B. Bruneau for critical reading of the manuscript. We also thank J. Fish and C. Miller in the Gladstone Histology core. C.K. was supported by grants from AHA Beginning Grant-in-Aid and NHLBI/NIH (1K99HL092234, 4R00HL09223); D.S. was supported by grants from NHLBI/NIH and the California Institute for Regenerative

Medicine and the Younger Family Foundation. This work was supported by NIH/NCRR grant (C06 RR018928) to the Gladstone Institute.

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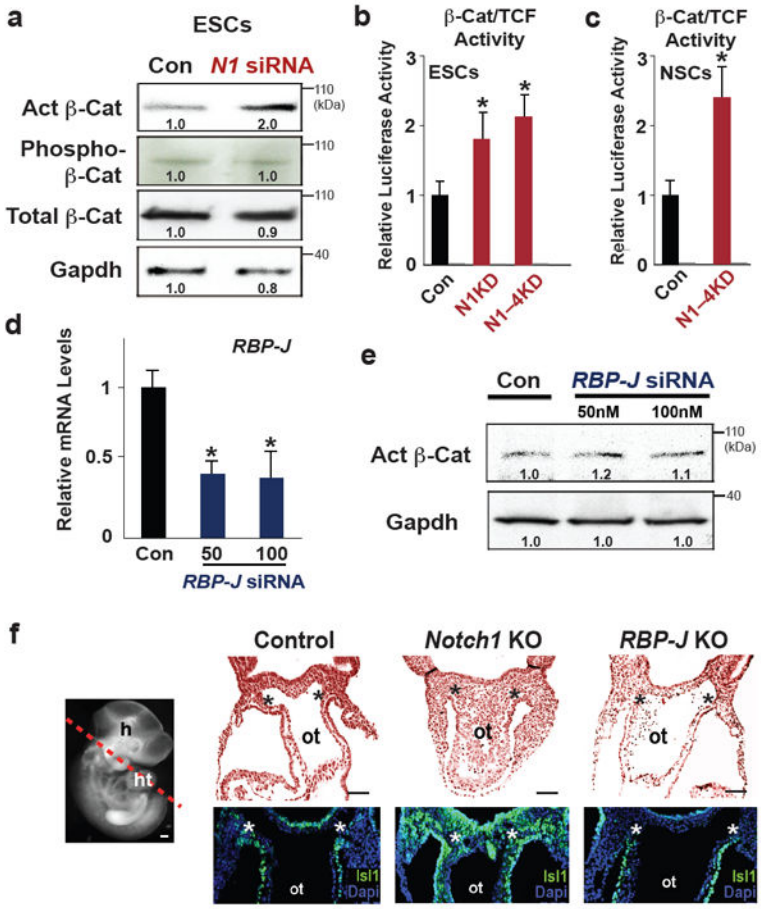

Figure 1.

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 (Ser37), 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 neural stem cells (NSCs) (**c**) transfected with control *siRNA* or *siRNA* against *Notch1* or *Notch1–4*. 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}; $Notch1^{tm2Rko}(ex3)^{loxP}$) or *RBP-J* KO ($Isl^{Cr}$; $RBP\text{-}J^{flox/flox}$) embryos stained with H&E (top) or Isl1 antibody (green, bottom) at embryonic day 9.5, at level of outflow tract (ot). Asterisks indicate precardiac mesoderm containing cardiac progenitor cells. 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 two-tailed Student's *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; Con, control; N1KD, *Notch1 siRNA*; N1–4KD, *Notch 1–4 siRNA*.

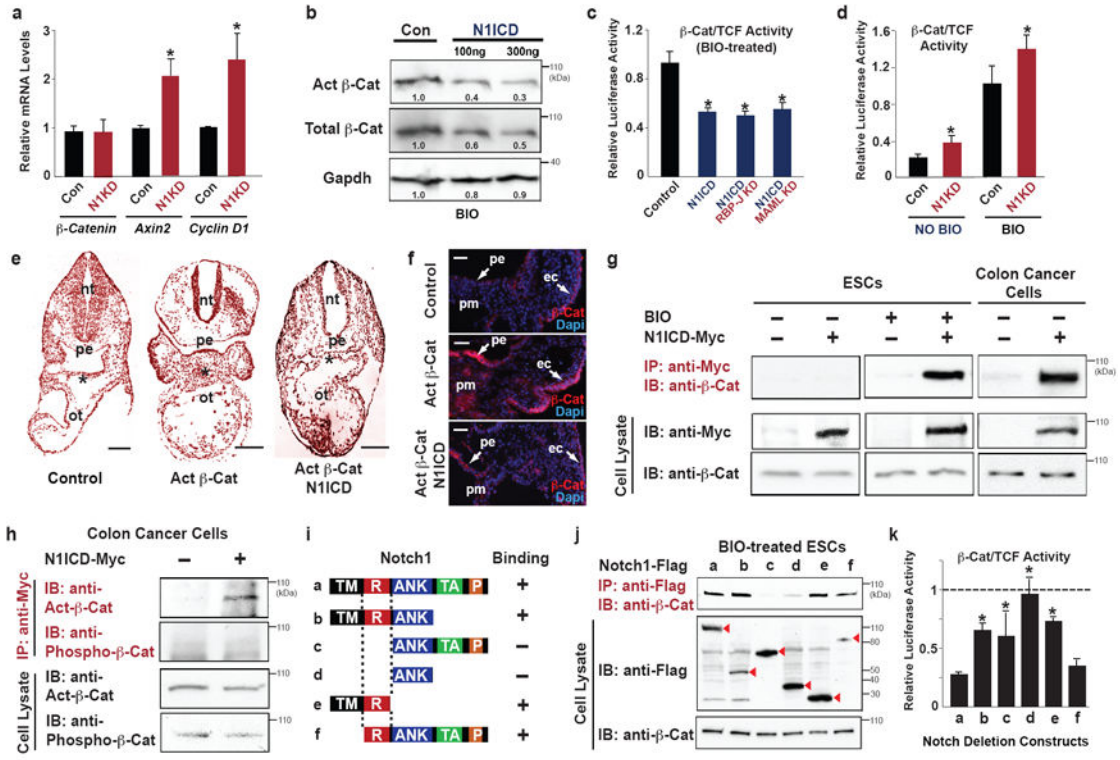

Figure 2.

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 or Notch1 intracellular domain (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};*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**). Asterisks indicate precardiac mesoderm containing cardiac progenitor cells (**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 C-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. **j**, Co-IP of BIO-treated ESCs with Notch1 deletion constructs shown in (**i**) using antibodies indicated. Arrowheads indicate Notch1 expression. **k**, Relative β-Catenin/TCF activity of BIO-treated ESCs transfected with Notch

constructs shown in (**i**). TM (transmembrane domain), R (RAM domain), ANK (Ankyrin repeats), TA (transactivation domain), P (PEST domain). 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 two-tailed Student's *t*-test, type II (see Methods). Numbers on Western blots correspond to relative quantification. Con, control; N1KD, *Notch1* siRNA.

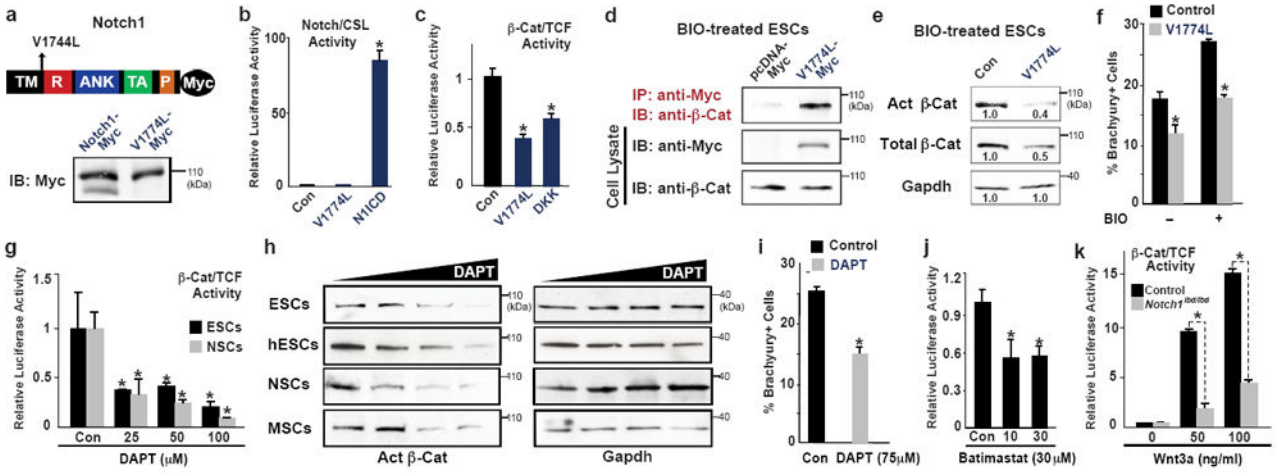

Figure 3.

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 Myc-tagged wild type Notch1 or tethered Notch1 (V1774L) and blotted with anti-myc antibody (bottom), showing lack of cleaved protein band. **b**, Relative RBP-J responsive luciferase activity of mESCs transfected with control, tethered Notch1 or N1ICD. **c**, Relative β-Catenin/TCF luciferase activity of ESCs transfected with control or tethered Notch1 (V1774L) construct shown in (**a**) or treated with Dkk1 (50 ng/ml). **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 or tethered Notch1. **f**, % 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, a γ-secretase inhibitor, for 72–96 h. **h**, Western analysis of active β-Catenin in mouse or human ESCs, NSCs, or bone marrow mesenchymal stem cells (MSCs) treated with increasing doses (0, 25, 50 or 100 μM) of DAPT for 72–96 h. **i**, % of *Brachyury*-GFP^{+} cells after 3.5 days of differentiation of mouse ESCs with control or 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 two-tailed Student's *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. Con, control.

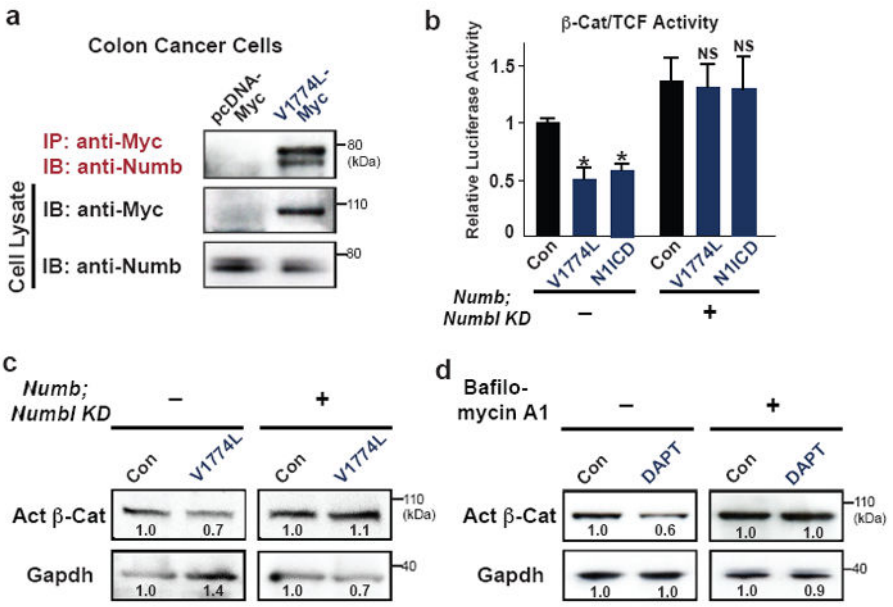

Figure 4.

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, 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 or tethered Notch (V1774L) in the presence or absence of *Numb/Numbl* siRNA. **d**, Western analysis of active β-Catenin in ESCs treated with control or DAPT 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 two-tailed Student's *t*-test, type II (see Methods). Gapdh antibody was used as a loading control. Numbers on Western blots correspond to relative quantification. Con, control.

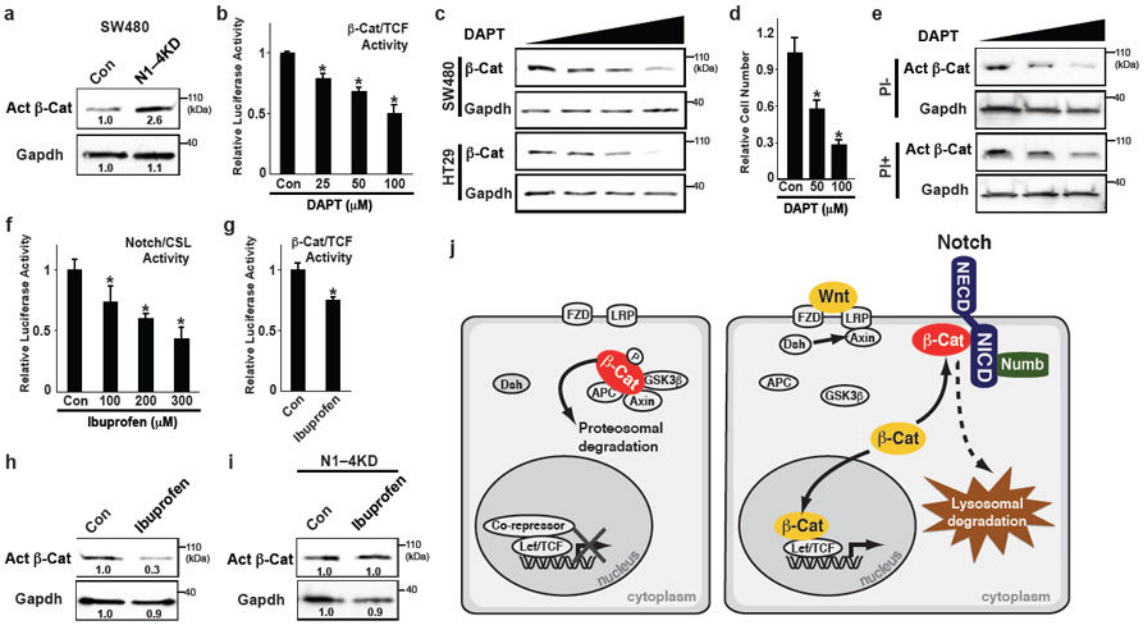

Figure 5.

γ-Secretase Inhibitors (GSIs) Suppress Expansion of Human Colon Cancer Cells by Blocking Notch Cleavage. **a**, Western analysis of active β-Catenin in SW480 human colon cancer cells transfected with control or siRNA against *Notch1–4* (N1-4KD, 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 panel since 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 for 72 h. **i**, Western analysis of active β-Catenin in SW480 cells treated with control or Ibuprofen and 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 two-tailed Student's *t*-test, type II (see Methods). Numbers on Western blots correspond to relative quantification. Con, control; N1–4KD, *Notch 1–4* siRNA. **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 TCF/LEF. 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.