C-terminal deletion of NOTCH1 intracellular domain (N1ICD) increases its stability but does not amplify and recapitulate N1ICD-dependent signalling

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Since the generation of a mouse strain conditionally expressing the active intracellular domain of Notch1 (N1ICD), many laboratories have exploited this model (RosaN1-ICD) to assess the impact of constitutive Notch1 signalling activation in normal and pathological processes. It should be underscored that Cre-recombination leads to the expression of a C-terminally truncated form of N1ICD (N1ICDdC) in the RosaN1-ICD mutant mice. Given that no studies were undertaken to delineate whether deletion of this region leaves intact N1ICD function, stable cell lines with single targeted integration of inducible N1ICD and N1ICDdC were generated. We found that C-terminal deletion of N1ICD stabilized the protein but did not promote the activity of Notch responsive promoters. Furthermore, despite higher expression levels, N1ICDdC failed to phenocopy N1ICD in the promotion of anchorage-independent growth. Our results thus suggest that the C-terminal region of N1ICD plays a role in shaping the Notch response. Therefore, it should be taken into consideration that N1ICD is truncated when interpreting phenotypes of RosaN1-ICD mutant mice.

The Notch pathway is a highly conserved signalling pathway with a relatively simple architecture. The trans-membrane Notch receptors (NOTCH 1–4) undergo a series of proteolytic cleavages upon ligand binding releasing the Notch intracellular domain (NICD). The NICD then translocates into the nucleus where, in association with CSL (CBF1, Su(H) and LAG-1) and MAML1 (Mastermind-like 1), forms a core transcriptional activation complex impacting on gene expression. The release of NICD thus constitutes a limiting step for activation of this signalling pathway devoid of amplification process. Although the precise mechanisms remain to be clarified, NICD turnover, consequent to its proteasomal degradation, dismantles the NICD/CSL/MAML1 ternary complex and put an end to Notch activity viz. Notch-dependent gene regulation. The PEST domain, located C-terminally of NICD, was shown to play a critical role in NICD turnover.

The Notch signalling pathway orchestrates many developmental processes as well as ensures tissue homeostasis in the adult. Notably, aberrant Notch signalling is frequently observed in different cancer types underscoring the need to maintain Notch signalling under tight regulation to preserve tissue homeostasis. To better define the impact of Notch activation in physiological or pathological contexts, mutant mouse with targeted insertion of mouse Notch1 intracellular domain (N1ICD) into the GT(ROSA)26Sor locus was generated (RosaN1-ICD). This mouse strain is now available through The Jackson Laboratory and is used in conjunction with Cre-recombinase expressing strain to generate cell type/tissue-specific expression of N1ICD. Up to now, over 125 publications reported diverse phenotypes taking advantage of this RosaN1-ICD mouse strain. It is of particular note that the sequence encoding the mouse N1ICD in the RosaN1-ICD model lacks the last C-terminal 238 amino acids. Although

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the original paper did not explicitly provide as to why the entire N1ICD coding sequence was not used, this RosaN1ICD strain is exploited to generate mouse models with cell type/tissue-specific constitutive activation of Notch1 signalling.

It is becoming clear that the relatively simple architecture of the Notch signalling pathway must hide complex regulatory mechanisms contributing to the coordinated nuclear outcomes of the N1ICD/CSL/MAML1 ternary complex. Previous studies have suggested that the N1ICD/CSL/MAML1 transcriptional platform is assembled in a precise stepwise manner and other interacting factors most likely joined this platform to ensure efficient transcription of target genes. Accumulating evidence also support roles for post-transcriptional modifications such as phosphorylation, acetylation, methylation and ubiquitination in the coordinated assembly and disassembly of the Notch1-dependent transcriptional platform. Notably, methylation of Notch1 within its C-terminal domain recently appeared critical in dosing the Notch response.

Given that, upon its release from the transmembrane receptor, N1ICD undergoes sequential post-translational modifications such as phosphorylation on amino acids that remain to be identified, and that the C-terminal domain of N1ICD potentially harbours sites targeted by phosphorylation, methylation and/or ubiquitination, this study was undertaken to determine whether a N1ICD deleted of its C-terminal domain (N1ICDdC) can substitute for N1ICD in functional studies. Herein, we provide evidence that despite higher expression levels, the transcriptional output of N1ICDdC is distinct from N1ICD. Moreover, N1ICD fails to phenocopy N1ICD in promoting anchorage-independent growth. Therefore, given these discrepancies in function between N1ICD and N1ICDdC, we should be careful when interpreting the functional impact of Notch1 activation on the basis of results obtained with models using a deleted version of N1ICD such as the RosaN1ICD mouse strain.

Results

Generation of inducible U2OS Flp-In™ T-REx™ cell lines expressing N1ICD or N1ICDdC. To characterize the functional impact of deleting the C-terminal domain of N1ICD, stable cell lines expressing doxycycline inducible GFP-N1ICD or GFP-N1ICDdC were generated. We took advantage of the U2OS Flp-In™ T-REx™ cells in order to target GFP-N1ICD and GFP-N1ICDdC integration at a single transcriptionally active genomic locus and ensuring expression levels comparable to endogenous expression levels. The single targeted integration allowed minimizing for difference between GFP-N1ICD and GFP-N1ICDdC cell populations owing to variable integration sites. Of note, the encoded N1ICDdC is the corresponding human sequence of the mouse N1ICD inserted into the Rosa locus of the RosaN1ICD mouse strain. To confirm N1ICD and N1ICDdC expression in our stable U2OS Flp-In™ T-REx™ GFP-N1ICD and U2OS Flp-In™ T-REx™ GFP-N1ICDdC cell populations (hereafter named U2OS GFP-N1ICD and U2OS GFP-N1ICDdC, respectively), cells were induced with doxycycline. Solely the U2OS GFP-N1ICD and U2OS GFP-N1ICDdC cells, and not the parental U2OS Flp-In™ T-REx™ cells, expressed a GFP fusion protein at the expected molecular weight (~140 kDa for GFP-N1ICD and ~90 kDa for GFP-N1ICDdC) upon doxycycline exposure (Fig. 1a). In addition to recognizing the endogenous transmembrane NOTCH1 subunit, a NOTCH1 specific antibody detected the GFP-N1ICD protein but not GFP-N1ICDdC most likely owing to the loss of the C-terminal epitope on the GFP-N1ICDdC protein. Of note, the endogenous NOTCH1 expression levels were not modulated by the concomitant expression of GFP-N1ICD or GFP-N1ICDdC. Moreover, the N1ICD, interacting partners CSL and MAML1 were expressed at comparable levels in the U2OS cell populations although retarded migration on SDS-PAGE of MAML1 was regularly detected in induced U2OS GFP-N1ICD cells (Fig. 1a).

Increased expression levels of the Notch target HES1 were detected in the induced U2OS GFP-N1ICD and GFP-N1ICDdC cell populations (thereafter named U2OS GFP-N1ICD and U2OS GFP-N1ICDdC, respectively), cells were induced with doxycycline. Solely the U2OS GFP-N1ICD and U2OS GFP-N1ICDdC cells, and not the parental U2OS Flp-In™ T-REx™ cells, expressed a GFP fusion protein at the expected molecular weight (~140 kDa for GFP-N1ICD and ~90 kDa for GFP-N1ICDdC) upon doxycycline exposure (Fig. 1a). In addition to recognizing the endogenous transmembrane NOTCH1 subunit, a NOTCH1 specific antibody detected the GFP-N1ICD protein but not GFP-N1ICDdC most likely owing to the loss of the C-terminal epitope on the GFP-N1ICDdC protein. Of note, the endogenous NOTCH1 expression levels were not modulated by the concomitant expression of GFP-N1ICD or GFP-N1ICDdC. Moreover, the N1ICD, interacting partners CSL and MAML1 were expressed at comparable levels in the U2OS cell populations although retarded migration on SDS-PAGE of MAML1 was regularly detected in induced U2OS GFP-N1ICD cells (Fig. 1a).

Increased expression levels of the Notch target HES1 were detected in the induced U2OS GFP-N1ICD and GFP-N1ICDdC cell populations suggesting that N1ICD and N1ICDdC are able to promote Notch signalling.

We previously provided evidence that endogenous N1ICD is phosphorylated upon ligand-dependent and -independent NOTCH1 activation. To test whether the expressed N1ICD undergoes similar regulatory mechanisms in U2OS, phosphorylation levels of N1ICD and N1ICDdC were evaluated. Phosphorylation assays demonstrated that N1ICD was efficiently phosphorylated in U2OS whereas N1ICDdC was barely subjected to such a post-translational modification (Fig. 1b). Moreover, upon immunoprecipitation of proteins phosphorylated on serine or threonine residues using an anti-MPM2 antibody, only N1ICD was detected by immunoblotting (Fig. 1c). These results suggest that, similarly to endogenous N1ICD and N1ICDdC, N1ICD is post-translationally modified by phosphorylation whereas N1ICDdC lacks regulatory phospho-sites.

Albeit using U2OS Flp-In™ T-REx™ cells for single targeted integration, greater expression levels of N1ICDdC as compared to N1ICD were consistently observed after prolonged doxycycline exposure (see Fig. 1a) suggesting post-transcriptionally events regulating protein expression. To dissect the N1ICD and N1ICDdC expression profile upon doxycycline addition, a time-dependent response was performed. As shown in Fig. 2a, N1ICD expression reached a steady-state level upon 5–6 hours addition of doxycycline whereas N1ICDdC expression continuously increased over time. Given that the C-terminal domain of N1ICD was suggested to participate in protein turnover, cycloheximide and MG132 treatment were carried out to evaluate N1ICD and N1ICDdC stability and proteasomal degradation. The expression of N1ICD decreased upon cycloheximide addition (Fig. 2b) and increased upon MG132 treatment (Fig. 2c) suggesting that N1ICD is relatively unstable most likely owing to its proteasome-dependent degradation. In opposition, N1ICDdC was only slightly modulated by protein synthesis or proteasome inhibition (Fig. 2b and c). These results are thus in accordance with the reported requirement of the C-terminal domain for N1ICD proteasomal turnover. Altogether, we generated inducible U2OS cell populations expressing either GFP-N1ICD or GFP-N1ICDdC. The expressed N1ICD and N1ICDdC display characteristics similar to what was previously reported i.e. increased stability of N1ICDdC as compared to the rapid turnover of N1ICD.

N1ICDdC does not recapitulate N1ICD. Despite a much greater expression levels of N1ICDdC as compared to N1ICD upon prolonged doxycycline exposure, comparable HES1 expression levels were detected (Fig. 1a). In attempt to better discern the capacity of N1ICD and N1ICDdC to modulate Notch target(s), a kinetic of HES1 expression at shorter time point was performed given that N1ICD and N1ICDdC were expressed at comparable levels.
within the first 5 hours of doxycycline addition (Fig. 2a). No significant differences in HES1 expression levels were noted in U2OS GFP-N1ICD as compared to U2OS GFP-N1ICDdC cells upon doxycycline addition although U2OS GFP-N1ICD cells appeared to achieve HES1 steady-state levels faster (2 h) than U2OS GFP-N1ICDdC cells (4 h) (Fig. 3a). Luciferase assays also failed to unveil any significant difference in the ability of N1ICD and N1ICDdC to promote Hes1 promoter activity in U2OS (Fig. 3b). However, only N1ICD significantly stimulated the activity of the Notch pathway responsive reporter CSL-luciferase (Fig. 3c). The CSL-luciferase reporter gene contains multimerized CSL DNA-binding site upstream of the luciferase gene reflecting more global Notch-dependent transcription as opposed to Hes1-luciferase that monitors activity only on the Hes1 promoter. Our results thus suggest that N1ICDdC and N1ICD may not be equipotent in modulating gene expression since the higher expression levels of N1ICDdC was not converted into a higher transcriptional output or elevated expression of the Notch target HES1.

To verify this in another system, transient transfection of N1ICD and N1ICDdC were performed in the pancreatic cancer cell line MIA PaCa-2. As opposed to N1ICD, N1ICDdC was unable to significantly up-regulate the activity of the reporter genes Hes1-luciferase and CSL-luciferase in that cell model (Fig. 3d). Additionally, although expressed at much higher levels, N1ICDdC was not as competent than N1ICD to promote HES1 protein expression (Fig. 3e). Altogether, these results suggest that N1ICD and N1ICDdC may have distinct transcriptional potential.

Nuclear translocation of N1ICD and its association with its transcriptional partners CSL and MAML1 are mandatory to impact on gene expression. Both N1ICD and N1ICDdC were detected within the nuclear compartment (Fig. 4a) although a smaller proportion of N1ICDdC was found within the nucleus as compared to the 80% of N1ICD localized within the nucleus (Fig. 4b). Of note, most likely due to its higher expression levels, expression levels of N1ICDdC detected within the nucleus were at least as much as the N1ICD nuclear expression levels (not shown). So,
the amount of nuclear N1ICDdC could not account for the reduced transcriptional capacity observed in luciferase assays (Fig. 3b and c). The altered subcellular distribution only affected N1 ICDdC as CSL and MAML1 were distributed in a similar manner in the U2OS GFP-N1 ICD and GFP-N1 ICDdC cell populations i.e. mainly in the nuclear compartment. Co-immunoprecipitation studies indicated that N1 ICDdC was still able to interact with CSL and MAML1 (Fig. 4c and d). Therefore, N1 ICDdC retains its capacity to localize within the nucleus and interact with its partners CSL and MAML1.

To determine whether the apparent distinct transcriptional potential of N1ICDdC was functionally relevant, the growth properties of U2OS GFP-N1 ICD and U2OS GFP-N1ICDdC cell populations were evaluated. The anchorage-dependent growth curve of the parental U2OS Flp-In TM T-REx TM cell line as well as the stable cell populations expressing either GFP-N1 ICD or GFP-N1ICDdC were similar (Fig. 5a). To evaluate whether Notch signalling mediated by N1 ICD or N1ICDdC could impact on a trait of transformed cells, anchorage-independent growth was assessed. Uninduced U2OS cell populations were able to form colonies in soft agarose but with limited capacity (Fig. 5b and c). Solely U2OS cells induced to express N1 ICD constantly and significantly formed more colonies in soft agarose as...
Figure 3. Elevated N1ICDdC expression levels as compared to N1ICD do not lead to higher expression of Notch responsive genes. (a) U2OS GFP-N1ICD and GFP-N1ICDdC cell populations were induced with doxycycline (Dox) for the indicated time period. HES1 and ACTIN expression levels were analysed using specific antibodies. Representative immunoblots are shown. Cropped blots are displayed and full-length blots are included in Supplementary Information. A graphical representation of the mean HES1 expression levels ± SEM of 4 independent experiments is shown where HES1 expression levels were normalized to ACTIN and HES1/ACTIN ratio in uninduced cells was set at 1. (b) Uninduced U2OS GFP-N1ICD and GFP-N1ICDdC cells were transfected with the Hes1-luciferase and Renilla-luciferase reporter constructs, and cells were left uninduced (without dox) or induced with doxycycline (with dox) for 24 h. The experiment was performed twice in triplicate. The data are expressed as the means ± SEM of Hes1-luciferase activity/Renilla-luciferase activity where the relative activity in uninduced cells (without dox) was set at 1. ***p < 0.001, **p < 0.01 as compared with uninduced cells. ns = not significant. (c) Uninduced U2OS GFP-N1ICD and GFP-N1ICDdC cells were transfected with the CSL-luciferase and Renilla-luciferase reporter constructs, and cells were left uninduced (without dox) or induced with doxycycline (with dox) for 24 h. The experiment was performed 4 times in triplicate. The data are expressed
as the mean ± SEM of CSL-luciferase activity/Renilla-luciferase activity where the relative activity in uninduced cells (without dox) was set at 1. ***p < 0.001 as compared with uninduced cells. ###p < 0.01. (d) MIA PaCa-2 cells were transfected with pDEST53 (empty vector), pDEST53-N1ICD (N1ICD) or pDEST53-N1ICDdC (N1ICDdC) together with the Hes1-luciferase or CSL-luciferase and Renilla-luciferase reporter constructs. Luciferase activities were measured the following day. The experiment was performed 3 times in quadruplicate. The data are expressed as the mean ± SEM of Hes1-luciferase or CSL-luciferase activity/Renilla-luciferase activity where the relative activity in empty vector transfected cells was set at 1. *p < 0.05. **p < 0.01. ***p < 0.001 as compared with empty vector transfected cells. ###p < 0.001. ns = not significant. (e) MIA PaCa-2 cells were transfected with pDEST53 (empty vector), pDEST53-N1ICD (GFP-N1ICD) or pDEST53-N1ICDdC (GFP-N1ICDdC). The following day, total cell lysates were analysed for N1ICD and N1ICDdC expression using an anti-GFP antibody. An anti-NOTCH1 antibody was also used for detection of endogenous NOTCH1 and GFP-N1ICD. The asterisk * denotes the expected molecular weight of GFP-N1ICDdC not detected by the anti-NOTCH1 antibody. Expression levels of MAML1, CSL, HES1 and ACTIN were analysed by immunoblotting using specific antibodies. Cropped blots are displayed and full-length blots are included in Supplementary Information.

compared to their uninduced counterpart (Fig. 5b and c). So, despite lower expression levels, N1ICD appears more potent than N1ICDdC in promoting anchorage-independent growth of U2OS cells.

Discussion

Many studies in the literature have exploited the RosaN1ICD mouse strain to direct Notch1 activation in specific cell types and evaluate its impact on developmental processes or carcinogenesis. Noteworthy, upon Cre-mediated recombination, RosaN1ICD mutant mice express a deleted version of the mouse N1ICD lacking its last C-terminal 238 amino acids. Given the increasing evidence that this region harbours potential regulatory sites, this study was undertaken to test whether Notch1 signalling triggered by a C-terminally truncated N1ICD (N1ICDdC) is indistinguishable from N1ICD-mediated signalling particularly in the context of a human N1ICD.

Essentially, our observations support previous data demonstrating an increased stability of N1ICDdC owing to its escape from proteasomal degradation. However, we uncovered that this higher expression levels of N1ICDdC is not converted into an elevated Notch-mediated transcriptional output on the Hes1 promoter or a CSL-responsive reporter gene. The impact on Hes1 promoter activity was mitigated since N1ICDdC, particularly in U2OS cells, was still able to upregulate the activity of the Hes1-promoter and increase HES1 protein expression levels. However, deletion of the C-terminal domain of N1ICD dramatically impaired its ability to induce the activity of the Notch-dependent construct CSL-luciferase. Of note, 4X CSL binding sites are in tandem in the CSL-luciferase reporter gene whereas 2 CSL binding sites are positioned head to head on the Hes1-luciferase reporter gene. Altogether, our results suggest that N1ICDdC might be competent in influencing a subset of Notch target genes, but is probably unable to faithfully recapitulate the repertoire of N1ICD targets.

We showed that N1ICDdC was still able to localize within the nucleus and associate with CSL and MAML1. This may not be surprising given that the domains involved in CSL and MAML1 interaction are still present on N1ICDdC. Still, we cannot totally exclude the possibility that the N1ICDdC/CSL/MAML1 ternary complex could be more efficiently assembled than the N1ICD/CSL/MAML1 complex, as the stoichiometric of the proteins of the ternary complex was regularly dissimilar in the N1ICD vs. N1ICDdC immunocomplexes. It is also possible that solely the conformation of the N1ICD/CSL/MAML1 ternary complex engages additional interactors potentially modulating its transcriptional activity. In support of this possibility, we frequently observed post-translational modifications on MAML1 associated with N1ICD (see Fig. 4c) suggesting that the N1ICDdC/CSL/MAML1 ternary complex, but not N1ICD/CSL/MAML1, may recruit additional regulators of the Notch core transcriptional complex. Further studies are clearly needed to identify the mechanisms orchestrating the assembly of the N1ICD/CSL/MAML1 complex as the regulatory events occurring after NOTCH1 cleavage/N1ICD release up to its integration into a transcriptional platform remain elusive.

Our data revealed that only the forced expression of N1ICD significantly promotes the anchorage-independent growth capacity of U2OS. Therefore, the transformed phenotype cannot be extrapolated from N1ICD vs. N1ICDdC expression levels. A previous study reported similar observation where varied levels of N1ICD expression was not correlating with the extent of transformation of immortalized RKE cells. One lesson from our study is thus that the Notch-induced phenotype and transcriptional output on limited but common Notch-dependent reporter genes cannot be deduced from N1ICD or mutant N1ICD expression levels.

Our results also imply that the Notch response is likely to be affected by the level of Notch activation. It has been shown that high expression levels of N1ICD reduces cervical cancer cell proliferation by interfering with expression of the human papilloma viral oncogene E6 and E7 whereas moderate expression levels of N1ICD cooperates with E6/E7 to transformed keratinocytes. The extent of Notch1 activation was also showed to shape the phenotype of mammary epithelial cells as well as being determinant in influencing hematopoiesis and T-ALL initiation. So, as for many signalling pathways, it is becoming clear that the amplitude and the duration of the Notch-dependent transcriptional output will impact on the cellular outcomes. From our observations, it is tempting to speculate that the C-terminal domain of N1ICD harbours regulatory sites that affect its function. In the same line of idea, other studies proposed a role for the C-terminal domain, not only in the stabilization of the protein, but also on the transcriptional potential of N1ICD. Notably, Gerhardt et al. demonstrated that deletion of amino acids 2193 to 2396 in mouse N1ICD (amino acids 2203 to 2421 in human N1ICD) led to higher expression levels of N1ICD but the latter had a reduced capacity to bind Notch responsive elements. The generation of knock-in mice expressing this truncated form of N1ICD allowed them to conclude that this region is not critical for all Notch1 functions but may play a role in enhancing or facilitating the expression of a subset of Notch1 target genes.
genes. More recently, methylation of mouse N1ICD on 5 conserved arginine residues was shown to shape the Notch response21. Three out of these 5 arginine residues are deleted in our N1ICDdC construct. It is noteworthy that methylation-defective N1ICD is more stable, still associates with CSL but displays reduced transcriptional activity and is biologically less active, observations that closely remind our results. Interestingly, the authors developed a mathematical model in which they predicted that N1ICD would produce a robust but short transcriptional response whereas methylation-defective N1ICD would lead to a dampened but more prolonged transcriptional output. This model fits well with our findings in U2OS demonstrating that higher expression levels of N1ICDdC as compared to N1ICD leads to comparable expression levels of the Notch target HES1.

It is worth mentioning that Notch signalling was reported to elicit CSL-dependent and -independent cellular responses34–36. Notably, ChIP-seq and bioinformatics recently showed that DNA binding sites of N1ICD and CSL are not entirely overlapping suggesting CSL-independent gene regulation by N1ICD37. Of interest, in Drosophila, there is indication that CSL-independent Notch-mediated signals require the C-terminal region of Notch34, 38. These observations thus support both CSL-dependent and -independent mechanisms involved in shaping the Notch response. Given that we mainly monitor CSL-dependent activity in our models, we cannot exclude that the cellular response to N1ICD and N1ICDdC expression, particularly on anchorage-independent growth, is consequent of changes in the proportion of CSL-dependent and -independent Notch signalling. Therefore, it could be interesting to perform ChIP-seq experiments along with microarrays in order to relate N1ICD vs. N1ICDdC vs. CSL DNA binding sites with gene regulation. These experiments could be informative in revealing differentially regulated CSL-dependent and -independent gene networks by N1ICD and N1ICDdC.

Overall, we have generated cell models with single targeted integration of human N1ICD or N1ICDdC in order to test the requirement of the C-terminal domain in withstanding N1ICD function. Despite leading to increased
stability of N1ICD, deletion of the C-terminal domain did not increase the ability of N1ICD to modulate some Notch responsive promoters. Furthermore, deletion of this region limited the capacity of N1ICD to promote anchorage-independent growth. In light of these results, it is worth asking whether the RosaN1-ICD mouse strain, expressing a truncated version of mouse N1ICD similar to our human N1ICDdC, faithfully recapitulate the full spectrum of N1ICD function. Of note, with regards to the role of Notch1 signalling during development, truncated forms of N1ICD have never been detected in normal embryonic or adult tissues. C-terminally deleted forms of NOTCH1 have been identified, particularly in T-ALL, but not yet in solid tumours. In the future, it would be interesting to examine in greater details whether full length or truncated forms of NOTCH1, by modulating distinctly the amplitude and the duration of CSL-dependent and –independent NOTCH1 signalling, permit the optimal gene expression profile required to transform cells or promote tumour progression in a concerted action with cell context.

Methods

Plasmid expression constructs. The human N1ICD and N1ICDdC were amplified by PCR from pcDNA3-NOTCH1 expressing vector (kindly provided by Stephen C. Blacklow, Boston). The N1ICD sequence encodes for amino acids 1754–2555 whereas N1ICDdC encodes for amino acids 1754–2301 of human NOTCH1. The oligonucleotides included the BP recombination sites attB. To generate the entry clones pDONR 221-N1ICD and pDONR 221-N1ICDdC, BP Clonase® (Life Technologies) was used for BP recombination reaction between the attB-containing PCR products and the attP-containing donor vector pDONR 221. To generate N-terminally GFP-tagged N1ICD and N1ICDdC expressing plasmids, LR Clonase™ (Life Technologies) was used for LR recombination reaction between attL-containing pDONR 221-N1ICD and pDONR 221-N1ICDdC and attR-containing destination vector pDEST53 or pgLAP1.

Cell culture and treatments. The human pancreatic cancer cells MIA PaCa-2 (American Type Culture Collection) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamax...
in humidified 5% CO₂ atmosphere at 37 °C. The U2OS Flp-InT-M T-RExTM cell line was cultured in DMEM medium supplemented with 10% FBS, 2 mM glutamax and 100 μg/mL Zeocin and 5 μg/mL Blasticidin-HCl. Stable U2OS GFP-N1ICD and GFP-N1ICDCD cell populations were obtained by transfecting the U2OS Flp-InT-M T-RExTM cell line with pgLAP1-N1ICD or pgLAP1-N1ICDCD plasmid along with the Flp-recombinase expressing plasmid POG44 followed by 10-days selection with 100 μg/mL Hygromycin B and 5 μg/mL Blasticidin-HCl. Of note, for each U2OS GFP-N1ICD and GFP-N1ICDCD, two independent stable cell populations were generated and displayed similar characteristics. Results obtained with all cell lines are presented and included within graph representation and statistical analyses.

Induction of GFP-N1ICD and GFP-N1ICDCD protein expression was carried out by addition of 1 μg/mL doxycycline for 24 h or the indicated time period. When indicated, cells were incubated with the protein synthesis inhibitor cycloheximide (25 μg/mL) or the proteasome inhibitor MG132 (10 μM) for the indicated time period.

**Extracts, Immunoblotting and antibodies.** Cells were washed with ice-cold PBS before being lysed in Triton buffer (1% Triton X-100, 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.2 mM orthovanadate, 40 mM β-glycerophosphate, 50 mM NaF, 10% glycerol, 1 mM PMSF, 0.5 μg/mL aprotinin, 0.5 μg/mL leupeptin and 0.7 μg/mL pepstatin). Total cell lysates were cleared of cellular debris by centrifugation (10 000 rpm, 10 min, 4 °C). Subcellular fractionation was performed as previously described. Protein concentrations were measured using the bicinchoninic acid (BCA) reagent procedure from Pierce with bovine serum albumin as standard. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were detected immunologically after electrotransfer onto nitrocellulose or polyvinylidene difluoride membranes as previously described. The anti-GFP, anti-LAMB1 and anti-NOTCH1 antibodies were from Santa Cruz Biotechnology and anti-ACTIN from EMD Millipore. The anti-MAML1, anti-CSL, anti-cleaved NOTCH1, anti-HES1 and anti-GAPDH were from Cell Signaling Technology. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were from Jackson Immunoresearch Laboratories.

**Immunoprecipitation and phosphatase assays.** Immunoprecipitation and phosphatase assays were performed essentially as previously described. Briefly, 1–2 μg of cleared lysates were incubated with GFP-Trap agarose beads (Chromotek) or anti-MPM2 antibody (EMD Millipore) for 2 h at 4 °C under agitation. SureBeads Protein G magnetic beads (Bio-Rad) were subsequently added to MPM2 immunocomplexes for 1 h at 4 °C. Cell lysates were then washed thrice with lysis buffer before boiling for 5 min in 4X Laemmli sample buffer (1X = 62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol, 1 mM PMSF, 0.005% bromophenol blue and 5% β-mercaptoethanol). For phosphatase assays, immunocomplexes were washed twice with 1X NEBuffer pack for Protein MetalloPhosphatase (New England Biolabs Inc.) supplemented with 1 mM MnCl₂ and 1 mM PMSF, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin and 0.5 μg/mL aprotinin. The beads were then equally split in two Eppendorf tubes. 400 units of lambda protein phosphatase (New England Biolabs Inc.) were added to one tube and both tubes were incubated for 30 min at 30 °C. The reaction was stopped by adding 4X Laemmli sample buffer.

**Transient transfections and luciferase assays.** Experiments were performed essentially as described. Briefly, for transient transfection, MIA PaCa-2 cells were transfected with the indicated plasmids, lysed 24–48 h post-transfection and prepared for immunoblotting. For luciferase assays, cells were transfected with firefly-luciferase reporter construct (CSL-luciferase, gift from Nicholas Gaisano Addgene plasmid #26897; Hes1-luciferase, kind gift from Ruth S. Slack, Ottawa), Renilla-luciferase reporter plasmid (pRL-thymidine kinase luciferase) and an expressing vector when indicated (pDEST53, pDEST53-N1ICD or pDEST53-N1ICDCD). Cells were harvested 24–48 h post-transfection in passive lysis buffer (Promega) and luciferase activities were determined using the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instruction. The data are expressed as firefly-luciferase activity normalized to Renilla-luciferase activity.

**Cell growth and Soft Agarose Assay.** For anchorage-dependent growth, 300 000 cells from U2OS Flp-InT-M T-RExTM, uninduced (without doxycycline added) U2OS GFP-N1ICD or U2OS GFP-N1ICDCD population were seeded in duplicate in 35 mm dishes in medium containing doxycycline. At each time point, cells were trypsinized and cell number evaluated by cell counting using a hemacytometer. Anchorage-independent growth was tested as previously described. Briefly, 6-well dishes were precoated with 1.5 mL/well mixture (1:1) of DMEM 2X without phenol red and agarose type VII 1.4% (Sigma-Aldrich). Cells were then seeded on top of the precoated wells by adding 2 mL of DMEM-agarose mixture (1:1) containing 100 000 cells, after which the plates were allowed to solidify. Fresh DMEM 1X without phenol red supplemented with 10% FBS together with or without doxycycline (1 μg/mL) was added to the surface of the agarose and changed daily. After 3–4 weeks, colonies were stained by adding 500 μg/mL aminocyanine (Calbiochem) at 0.5 mg/mL in PBS to the surface of the agarose and incubated for 5 h at 37 °C in 5% CO₂. Images were acquired and colonies were counted using the CellProfiler 2.2.0 software.

**Statistical Analysis.** Densitometric analyses were performed using the ImageJ software version 1.48v. Data were analyzed by Prism 7 version 7.0a (GraphPad Software, Inc.). Except for luciferase assays that were analyzed by unpaired two-tailed t-test, comparison of multiple groups was done by two-way ANOVA. Results are expressed as mean and error bars represent SEM. Differences were considered statistically significant when p < 0.05.

**References**
1. Braune, E. B. & Lendahl, U. Notch—a goldilocks signaling pathway in disease and cancer therapy. Discov Med 21, 189–196 (2016).
2. Bray, S. J. Notch signalling in context. Nat Rev Mol Cell Biol 17, 722–735, doi:10.1038/nrm.2016.94 (2016).
5. Ranganathan, P., Weaver, K. L. & Capobianco, A. J. Notch signalling in solid tumours: a little bit of everything but not all the time.

6. Van Vlierberghe, P. & Ferrando, A. The molecular basis of T cell acute lymphoblastic leukemia.

36. Zeng, C., Xing, R., Liu, J. & Xing, F. Role of CSL-dependent and independent Notch signaling pathways in cell apoptosis.

37. Liu, H., Zhou, P., Lan, H., Chen, J. & Zhang, Y. X. Comparative analysis of Notch1 and Notch2 binding sites in the genome of BxPC3 pancreatic cancer cells.

31. Mazzone, M.

29. Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. & Bishop, J. M. Neoplastic transformation by truncated NOTCH1/TAN1 and NOTCH2.

7. Murtaugh, L. C., Stanger, B. Z., Kwan, K. M. & Melton, D. A. Notch signaling controls multiple steps of pancreatic differentiation.

11. Vasquez-Del Carpio, R., et al. Assembly of a Notch transcriptional activation complex requires multimerization.

12. Yatim, A.

13. Chiang, M. Y. et al. Identification of a conserved negative regulatory sequence that influences the leukemogenic activity of NOTCH1.

15. Ishitani, T.

14. Foltz, D. R. & Nye, J. S. Hyperphosphorylation and association with RBP of the intracellular domain of Notch1. Biochem Biophys Res Commun 286, 484–492, doi:10.1016/j.bbrc.2001.5421 (2001).

16. Romchini, C. & Capobianco, A. J. Notch(c)-ER chimeras display hormone-dependent transformation, nuclear accumulation, phosphorylation and CBFI activation. Oncogene 19, 3914–3924, doi:10.1038/sj.onc.1203719 (2000).

17. Santos, N. et al. Phosphorylation of Notch1 by Pim kinases promotes oncogenic signaling in breast and prostate cancer cells. Oncotarget 7, 43220–43238, doi:10.18632/oncotarget.9215 (2016).

18. Tremblay, I., Pare, E., Arsenault, D., Douziech, M. & Boucher, M. J. The MEK/ERK pathway promotes NOTCH signaling in pancreatic cancer cells. PLoS One 8, e58502, doi:10.1371/journal.pone.0058502 (2013).

19. Guarani, V. et al. Acetylation-dependent regulation of endothelial Notch signaling by the SIRT1 deacetylase. Nature 473, 234–238, doi:10.1038/nature09917 (2011).

20. Popko-Scibor, A. E., Lindberg, M. J., Hansson, M. L., Holmlund, T. & Wallberg, A. E. Ubiquitination of Notch1 is regulated by the mammalian Sel-10 homolog. J Biol Chem 286, 26261–26270, doi:10.1074/jbc.M600036200 (2011).

21. Hein, K. et al. Site-specific methylation of Notch1 controls the amplitude and duration of the Notch1 response. Sci Signal 8, ra30, doi:10.1126/scisignal.2005892 (2015).

22. Oberg, C. et al. The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. J Biol Chem 276, 35847–35853, doi:10.1074/jbc.M103992200 (2001).

23. Krejci, A. & Bray, S. Notch activation stimulates transient and selective binding of Su(H)/CSL to target enhancers. Genes Dev 21, 1322–1327, doi:10.1101/gad.240607 (2007).

24. O'Gorman, S., Fox, D. T. & Wahl, G. M. Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science 286, 1351–1355 (1991).

25. Nam, Y., Sliz, P., Pear, W. S., Aster, J. C. & Blacklow, S. C. Cooperative assembly of higher-order Notch complexes functions as a switch to induce transcription. Proc Natl Acad Sci USA 104, 2103–2108, doi:10.1073/pnas.0610921104 (2007).

26. Wilton, J. J. & Kovall, R. A. Crystal structure of the CSL-Notch-Mastermind ternary complex. Cell 125, 985–996, doi:10.1016/j.cell.2006.01.035 (2006).

27. Nam, Y., Sliz, P., Song, L., Aster, J. C. & Blacklow, S. C. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Cell 124, 973–983, doi:10.1016/j.cell.2005.12.037 (2006).

28. Friedman, D. R., Wilson, J. J. & Kovall, R. A. RAM-induced allelestoy facilitates assembly of a notch pathway active transcription complex. J Biol Chem 283, 14781–14791, doi:10.1074/jbc.M709501200 (2008).

29. Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. & Bishop, J. M. Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. Mol Cell Biol 17, 6265–6273 (1997).

30. Lathion, S., Schaper, J., Beard, P. & Raj, K. Notch1 can contribute to viral-induced transformation of primary human keratinocytes. Cancer Res 63, 8687–8694 (2003).

31. Mazzone, M. et al. Dose-dependent induction of distinct phenotypic responses to Notch pathway activation in mammmary epithelial cells. Proc Natl Acad Sci USA 107, 5012–5017, doi:10.1073/pnas.0808961107 (2010).

32. Chiang, M. Y. et al. Leukemia-associated NOTCH1 alleles are weak tumor initiators but accelerate K-ras-initiated leukemia. J Clin Invest 118, 3181–3194, doi:10.1172/JCI35090 (2008).

33. Gerhardt, D. M. et al. The Notch1 transcriptional activation domain is required for development and reveals a novel role for Notch1 signaling in fetal hematopoietic stem cells. Genes Dev 28, 576–593, doi:10.1101/gad.227496.113 (2014).

34. Martinez Arias, A., Zecchinì, V. & Brennan, K. CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? Curr Opin Genet Dev 12, 524–533 (2002).

35. Sanalkumar, R., Dhanesh, S. B. & James, J. Non-canonical activation of Notch signaling/target genes in vertebrates. Cell Mol Life Sci 67, 2957–2968, doi:10.1007/s00018-010-0351-x (2010).

36. Zeng, C., Xing, R., Liu, J. & Xing, F. Role of CSL-dependent and independent Notch signaling pathways in cell apoptosis. Apoptosis 21, 1–12, doi:10.1007/s10495-011-0885-x (2016).

37. Liu, H., Zhou, P., Lan, H., Chen, J. & Zhang, X. Y. Comparative analysis of Notch1 and Notch2 binding sites in the genome of BxPC3 pancreatic cancer cells. J Cancer 8, 65–73, doi:10.7150/jca.16739 (2017).

38. Ramain, P. et al. Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. Curr Biol 11, 1729–1738 (2001).

39. Pear, W. S. & Aster, J. C. T cell acute lymphoblastic leukemia/lymphoma: a human cancer commonly associated with abberant NOTCH1 signaling. Curr Opin Hematol 11, 426–433 (2004).

40. Weng, A. P. et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306, 269–271, doi:10.1126/science.1102160 (2004).

41. Torres, J. Z., Miller, J. J. & Jackson, P. K. High-throughput generation of tagged stable cell lines for proteomic analysis. Proteomics 9, 2888–2891, doi:10.1002/pmic.200800873 (2009).

42. Marchand, B., Arsenault, D., Raymond-Fleury, A., Boisvert, F. M. & Boucher, M. J. Glycogen synthase kinase-3 (GSK3) inhibition induces prosurvival autophagic signals in human pancreatic cancer cells. J Biol Chem 290, 5592–5605, doi:10.1074/jbc.M11616714 (2015).
43. Marchand, B., Tremblay, I., Cagnol, S. & Boucher, M. J. Inhibition of glycogen synthase kinase-3 activity triggers an apoptotic response in pancreatic cancer cells through JNK-dependent mechanisms. *Carcinogenesis* 33, 529–537, doi:10.1093/carcin/bgr309 (2012).

44. Yu, X. et al. Notch signaling activation in human embryonic stem cells is required for embryonic, but not trophoblastic, lineage commitment. *Cell Stem Cell* 2, 461–471, doi:10.1016/j.stem.2008.03.001 (2008).

45. Vanderluit, J. L. et al. The Retinoblastoma family member p107 regulates the rate of progenitor commitment to a neuronal fate. *J Cell Biol* 178, 129–139, doi:10.1083/jcb.200703176 (2007).

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**Author Contributions**

J. Blain and J.B. performed experiments. J. Blain, M.T., F.M.B. and M.J.B. conceived the experiments. J. Blain and M.J.B. analysed the results. M.J.B. wrote the manuscript.

**Additional Information**

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