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Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and Hippo pathway effectors by Notch1

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Abstract

The Notch pathway plays a pivotal role in regulating cell fate decisions in many stem cell systems. However, the full repertoire of Notch target genes *in vivo* and the mechanisms through which this pathway activity is integrated with other signaling pathways are largely unknown. Here, we report a transgenic mouse in which the activation of the Notch pathway massively expands the neural stem cell (NSC) pool in a cell context-dependent manner. Using this *in vivo* system, we identify direct targets of RBPJ/N1ICD in cortical NSCs at a genome-wide level through combined ChIP-Seq and transcriptome analyses. Through a highly conservative analysis of these data sets, we identified 98 genes that are directly regulated by N1ICD/RPBJ *in vivo*. These include many transcription factors that are known to be critical for NSC self-renewal (*Sox2, Pax6, Tlx,* and *Id4)* and the transcriptional effectors of the Wnt, SHH and Hippo pathways, *TCF4, Gli2, Gli3, Yap1, and Tead2*. Since little is known about the function of the Hippo-Yap pathway in NSCs, we analyzed *Yap1* expression and function in NSCs. We show that *Yap1* expression is restricted to the stem cell compartment in the developing forebrain and that its expression is sufficient to rescue Notch pathway inhibition in NSC self-renewal assays. Together, the results of this study reveal a previously underappreciated complexity and breadth of *Notch1* targets *in vivo* and show direct interaction between Notch and Hippo-Yap pathways in NSCs.

Keywords

Neural stem cells; Notch pathway; Hippo-Yap pathway; transcriptional targets; ChIP-seq

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There is no conflict of interest.

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Introduction

In the developing nervous system, neural stem cells (NSCs) in the neuroepithelium give rise to neurons and glia in a spatio-temporally controlled manner. During early stages of brain development, the naïve neuroepithelium expands laterally through symmetric stem cell divisions. As the brain matures, the symmetric divisions are gradually replaced by asymmetric divisions that generate a stem cell and a lineage-restricted progenitor cell or a postmitotic neuron, resulting in thickening of the cortex radially [1]. Hence, regulation of the symmetric to asymmetric division transition is a pivotal step in NSC maturation. This transition is regulated by both cell-intrinsic and -extrinsic pathways, including a large number of signaling pathways originating within the brain and its surrounding tissues.

Classical studies in *Drosophila* development have shown that Notch signaling plays a critical role in specifying the appropriate numbers and types of cells arising from an equivalent pool of precursor cells [2]. Notch function is mediated through cell-cell communication among adjacent cells (juxtacrine signaling). When a ligand (DELTA and JAGGED family members) binds to a NOTCH receptor (NOTCH 1-4 in mammals), NOTCH is cleaved through highly regulated step-wise processes and the intracellular domain of NOTCH (NICD) is released from the membrane, and then enters the nucleus and forms a transcriptional complex with RBPJ. RBPJ is a DNA-binding protein that is engaged in a transcriptional repressor complex in the absence of NICD. The current model is that when NICD binds to RBPJ, it displaces the repressive co-factors bound to RBPJ and recruits a transcriptional activator complex, which initiates transcription of its downstream target genes [3].

In mice, loss-of-function mutations in Notch pathway genes result in reduced numbers of neural stem/progenitor cells, precocious neurogenesis, and induction of apoptosis [4–8]. In contrast, previous *in vivo* gain-of-function studies report mixed and inconsistent findings, depending on the methods used to manipulate the N1ICD levels and the numbers and types of cells affected by the manipulation [6, 9–11]. Nevertheless, Notch signaling is elevated in many human cancers and implicated in regulating self-renewal of stem cell-like cells in tumors [12–14].

While the Notch pathway has been implicated in diverse biological processes, from angiogenesis to dendrite morphogenesis to tumorigenesis to stem cell maintenance, mechanistic details of its varied functions in different cellular contexts and biological processes remain to be resolved. Because NICD functions as a transcriptional co-factor to RBPJ, arguably the most informative approach to gaining insights into the mechanisms of Notch function is to identify its direct downstream target genes in different contexts. Currently, *Hes* and *Hey* gene family members are the best-characterized and most widely accepted direct targets of the NICD/RBPJ complex [15]. More recent studies have suggested that *GFAP*, *BLBP*, *SOX2* and *p53* are also downstream targets of Notch in the brain [16–18]. To date, however, these studies have been performed either on a gene-by-gene basis or in manipulated cells in culture [19–23]. Hence, the full repertoire of genes that are regulated by direct binding of NICD/RBPJ *in vivo* is unknown.

Here we report a genome-wide analysis of direct RBPJ/N1ICD targets in NSCs *in vivo*, combining chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-seq) with transcriptome analyses. We use a mouse strain in which elevation of N1ICD level induces massive expansion of the NSC pool but not progenitor cells. Using very stringent criteria, we report 98 genes that are directly regulated by N1ICD/RPBJ in NSCs. These include many of the known transcription factors that control NSC proliferation and self-renewal, suggesting that *Notch1* is a “master regulator” of NSCs. Surprisingly, *Notch1* also

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directly activates expression of transcriptional effectors of the Wnt, SHH, and Hippo pathways. Since little is known about the function of Hippo-Yap pathway in NSC regulation, we examined YAP1 expression and function in NSCs. We show that ectopic *Yap1* expression is sufficient to compensate for Notch signaling inhibition in NSCs. In summary, this study reveals an unexpected complexity and breadth of the transcriptional network downstream of N1ICD/RBPJ *in vivo*. It also suggests that activities of multiple signaling pathways converge to regulate an overlapping set of transcription factors, effectively integrating multiple signaling inputs into a single, discrete cell fate decision.

Materials and Methods

Please refer to Supplementary Methods for details.

Mouse strains

Gt(ROSA)26Sor$^{tm1(Notch1)Dam}$/J(stock# 8159), FVB-Tg(GFAP-cre)25Mes/J (stock # 4600), 129P2(Cg)-Foxg1<tm1(cre)Skm>/J (stock# 6084), B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J (stock# 3474), B6;129P-Tg(Neurog1-cre/ESR1)1Good/J (stock# 8529), and *Tg(dlx6a-cre)1Mekk/J (Stock# 8199).* All mouse work was performed according to the protocols approved by The Jackson Laboratory ACUC. All phenotype analyses were performed comparing at least three pairs of littermate control and transgenic embryos.

Neural stem cell culture

Single cells from freshly dissociated cortex or basal ganglia were cultured at 1 cell/μl density in NSC medium (DMEM/F12 + B27+ bFGF(10ng/ml) + EGF (20ng/ml)), unless noted otherwise. Neurospheres are counted 6–7 days later.

Histology and immunofluorescence analysis

BrdU-birthdating was performed by injecting pregnant dames at E11.5 and harvesting embryos for analysis at E13.5. Standard immunofluorescence protocols were used with antibodies listed on the figures.

RT-PCR

Realtime RT-PCR was performed using standard protocols using BioRad iQ5. For PCR conditions and primer sequences, see Supplementary Methods section. For statistical analysis, t-tests were used.

ChIP-Seq

Control and *GFAP-Cre;N1ICD* cortices (three each from littermates) were pooled together before the immunoprecipitation step with an antibody against RBPJ (Santa Cruz # SC-28713). High-throughput sequencing was performed using Illumina Genome Analyzer II. Peaks were identified as regions of the genome where significantly more reads align from the ChIP compared to input DNA samples (Bernoulli p-value < 0.05 for at least 20 consecutive bp).

Microarrays

RNA samples were isolated from 5 wildtype and 5 *GFAP-Cre:N1ICD* E15.5 cortices and were hybridized to Affymetrix Gene ST 1.0 arrays. Differentially expressed genes were identified by an analysis of variance (ANOVA) model [24] and by fold change (q-value < 0.05, and fold change > 1.5).

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RNA-Seq

Pools of RNA from the wildtype and *GFAP-Cre:N1ICD* samples that were used for Affymetrix microarray analysis were sequenced using an Illumina Genome Analyzer II. Expression levels were quantified as RPKM values [25], and differentially expressed genes were identified by modeling read distributions between samples as a Bernoulli random variable (FDR adjusted p-value < 0.05).

Acute deletion of RBPJ in NSCs

NSCs from E15.5 $RBPJ^{fl/fl}$ embryos were isolated and cultured in NSC medium. Small neurosphere cells at passage 0 or 1 were transduced with adenovirus expressing EGFP (control) or Cre-IRES-EGFP. RNA was harvested 48 hours after the addition of virus.

Results

Notch1 promotes symmetric stem cell divisions to expand the NSC pool *in vivo*

It has been shown that endogenous Notch activity level is high in NSCs but low in neural progenitor cells [26]. To test whether high-level Notch pathway activity is sufficient to confer and/or maintain stemness in the developing nervous system, we conditionally expressed an activated form of the NOTCH1 receptor (N1ICD) in maturing neuroepithelium at three different stages. To do so, we used the *Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$*/J* (herein called N1ICD) transgenic mouse, which contains a single-copy insertion of the *N1ICD* transgene in the *ROSA26* locus [27].

First, to determine the functional consequence of sustaining high levels of Notch activity in NSCs, we utilized *Foxg1-Cre* [28] mice to drive N1ICD expression in early NSCs. *Foxg1-Cre* is activated in the murine cortex at E9, when the neuroepithelium is entirely composed of NSCs that expand rapidly through symmetric stem cell divisions. Elevated and sustained Notch signaling in *Foxg1-cre;N1ICD* brains resulted in a dramatic expansion of the neuroepithelium (Fig. 1A), similar to what has been observed by activating Wnt signaling in the forebrain [29]. Expression analyses of markers for NSCs (SOX2, PAX6), progenitors (TBR2), and neurons (TBR1, TuJ1, NeuN) indicated expansion of the NSC pool and reduction of neural progenitors and neurons at all ages examined (Supplementary Fig. 1 and not shown). Interestingly, the proportion of BrdU+ (S-phase) cells was reduced in the *Foxg1-cre;N1ICD* transgenic cortex, compared to the control cortex (Supplementary Fig. 1), suggesting that elevated Notch signaling does not necessarily enhance proliferation. These data suggest that maintaining high-level Notch1 activity in NSCs is sufficient to drive symmetric stem cell divisions and that switch to asymmetric divisions requires downregulation of Notch activity.

Notch1 expands the NSC pool in a dose-dependent manner *in vivo*

Previously Yang et al., reported that transgenic expression of N1ICD in the developing nervous system results in increased apoptosis and premature gliogenesis [9]; however, we did not observe any increased apoptosis or gliogenesis in *Foxg1-cre; N1ICD* brains (not shown). To test whether these discrepancies between the two studies are due to differences in expression levels or the cell types in which N1ICD is expressed, we activated the *N1ICD* transgene using *GFAP-Cre* mice [30]. In these mice, Cre recombinase is activated in the cortical NSCs and progenitors in the VZ from E12.5 onward. H&E analyses of *GFAP-Cre;N1ICD* double transgenic embryos with one copy of the N1ICD transgene showed an obvious expansion of the neuroepithelium compared to control littermates from E13.5 onward (Fig. 1B). We did not observe any precocious gliogenesis or apoptosis in cells over-expressing N1ICD (not shown). To test whether this is due to different levels of N1ICD expression, we generated embryos in which two copies of the transgene are expressed.

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GFAP-Cre;N1ICD embryos with two copies of the transgene consistently showed significantly greater lateral expansion of the neuroepithelium than those with a single copy (Fig. 1B, $0.0004<p<0.05$). At the same time, *GFAP-Cre;N1ICD* transgenic cortices were significantly thinner than those of control littermates (Fig. 1B), suggesting a decreased generation of progenitors and neurons. Western blot analysis of NSC markers confirmed increased levels of SOX2, PAX6, and BLBP proteins in transgenic cortices (Fig. 1C). Immunofluorescence analyses with markers showed a significantly increased number of NSCs (SOX2, PAX6) and decreased numbers of progenitor cells (TBR2) and neurons (TBR1, MAP2, β-III-tubulin, NeuN) in transgenic cortices at E15.5 (Fig. 1D,E and not shown). Hence, elevation of Notch activity in NSCs and progenitors did not expand both proliferating cell types; rather, it specifically increased the NSC number while reducing the progenitor number. Again, we did not observe increased apoptosis or premature astrocytogenesis in *GFAP-Cre;N1ICD* brains with high doses of the transgene (not shown).

To functionally test whether *GFAP-Cre;N1ICD* cortices contain increased numbers of NSCs, freshly dissociated cells from E15.5 control and transgenic cortices were plated at a clonal density (1 cell/μl). We plated them in normal NSC medium containing both bFGF (basic Fibroblast Growth Factor) and EGF (Epidermal Growth Factor) or in medium containing either bFGF or EGF alone. As shown in Figure 1F, significantly higher numbers of neurospheres formed in the transgenic culture grown in normal NSC medium, and even more dramatic differences were observed in cultures grown in bFGF alone. Interestingly, while the number of sphere-forming cells was consistently higher, the size of each neurosphere was smaller in the *GFAP-Cre;N1ICD* cultures (not shown). This is consistent with our *in vivo* observation that high-level Notch activity prevents formation of highly proliferative neural progenitor cells (Fig. 1, Supplementary Fig. 1). Increased self-renewal of bFGF-responsive NSCs in the transgenic cultures is consistent with increased expression of FGFR3 (Fibroblast Growth Factor Receptor 3) in the transgenic cortices at E15.5 (Fig. 1G). No significant differences were observed between the control and transgenic neurospheres cultured in EGF alone (Fig. 2E). Together, these observations suggest that high-levels of Notch activity cooperate with the FGF but not EGF pathway to expand the NSC pool.

Notch1 activation is insufficient to confer NSC phenotype to neural progenitors

To formally test whether the increased number of NSCs in *GFAP-Cre;N1ICD* brains resulted from selective expansion of the NSC pool and not from conversion of neural progenitors to NSCs, we activated N1ICD expression only in committed neural progenitors and differentiating neurons in the VZ and SVZ of developing brain. As shown in Fig. 2A, *dlx6a-cre*[31] is active in proliferating progenitors (PCNA+, MASH1+) and maturing neurons in the basal ganglia SVZ from E11.5 onwards. In *dlx6a-Cre;N1ICD* embryos, there was no significant change in proliferation as determined by BrdU and phosphorylated histone3 (PH3) staining (Fig. 2B). BrdU birthdating and a neuronal marker (TuJ1) analyses indicate that neurogenesis is also grossly normal (Fig. 2B). Isolation of NSCs from E13.5 basal ganglia showed that the number of NSCs also had not changed (Fig. 2C), despite high levels of the transgene expression and elevation of *Hes1* and *Hes5* expression (Fig. 2D). We made similar observations using *Ngn1-CreER* mice to activate N1ICD expression in cortical progenitors (Fig. 2E). These observations are in stark contrast to the same transgene expression only in NSCs in *Foxg1-Cre;N1ICD* embryos (Fig. 1). The fact that ectopic *N1ICD* expression is not sufficient to confer the stem-cell phenotype to committed progenitors or enhance progenitor cell proliferation indicates that the lateral expansion of the neuroepithelium in *GFAP-Cre;N1ICD* brain is due to selective expansion of NSCs. Together, our observations suggest that Notch function is highly cell context-specific *in vivo* and is insufficient to confer stemness to non-stem cells.

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Identification of *Notch1* targets *in vivo* at the genome level

To begin to understand the molecular mechanisms through which *Notch1* maintains "stemness" in NSCs, we sought to identify direct downstream targets of the N1ICD/RBPJ transcriptional complex by taking advantage of the *in vivo* system in which N1ICD expression expands the NSC pool (Fig. 3A). First, E15.5 cortices from littermate control and *GFAP-Cre;N1ICD* embryos were micro-dissected and their transcriptomes were analyzed by RNA-seq (Supplementary Table 1) and Affymetrix Gene Chip (Supplementary Table 2) methods. As anticipated, RNA-seq revealed a greater number of differentially expressed genes due to greater signal resolution (Fig. 3B). The overlap between the two methods of transcriptome analysis was 280 genes (Fig. 3B, Supplementary Table 3). Both methods indicated that known canonical Notch target genes, *Hes1*, *Hes5, Hey1, and Hey2*, were higher expressed in *GFAP-Cre;N1ICD* than control cortices, validating our analysis. In addition, *GFAP-Cre;N1ICD* cortices expressed higher levels of known NSC regulators, including *Pax6*, *Sox2, Nr2e1 (Tlx),* and *Id4,* and lower levels of neurogenesis regulators, *Neurod1* and *Neurod4* (Supplementary Table 3). Also, the list of 280 genes included key components of the Notch, Wnt, SHH, TGFβ, FGF, and Hippo pathways, including *Dll3, Fgf14, FGFR3, Fzd2, Fzd8, Fzd9, Gli2, Gli3, Notch1, SFRP2, SFRP3, Smo, TCF4 (Tcf7l2), Tead2, TAZ(Wwtr1), and Yap1 (*Supplementary Table 3).

To identify which of these genes are directly regulated by the NICD/RBPJ complex, E13.5 cortices from wildtype embryos were processed with a standard ChIP protocol to pull down chromosomal regions that are bound by the N1ICD/RBPJ transcriptional complex. Since RBPJ protein directly contacts DNA, we used an antibody against RBPJ that was successfully used in another ChIP study [32]. A total of 5719 genes (9056 peaks) were identified to be within 15kb of significant peaks (Fig. 3C, $p<0.05$; see Methods for details). In *GFAP-Cre;N1ICD* cortices, 8640 genes (19, 261 peaks) were associated with peaks above the cutoff threshold, and of these, 4838 genes (6693 peaks) overlapped with the wildtype cortex (Figure 3C, Supplementary Tables 4, 5). Interestingly, over 85% of the RBPJ binding sites were located within 15kb of a coding sequence, and they are scattered approximately evenly in up/downstream, intronic, and exonic sequences (Fig. 3D), similar to other genome level ChIP studies of transcription factors [33, 34]. The general pattern of binding sites was nearly identical between wildtype and *GFAP-Cre;N1ICD* transgenic cortices (Fig. 3E), indicating high reproducibility of these binding patterns. The known *Notch1* target genes *Hes1, Hes5, Hey1, Hey2,* and *Nrarp* were all bound by N1ICD/RBPJ (Fig. 3E, Supplementary Fig. 2), validating our approach. In addition, we observed significant binding to many known NSC-regulating genes, including *Pax6, Sox2, Nr2e1(Tlx), Msi, Bmi, Trp53, Ctnnb1(β-catenin),* and *Id4,* as well as progenitor genes, *Tbr2* and *Neurogenin1* (Fig. 3E, Supplementary Fig. 2, Supplementary Tables 4,5). We also observed direct binding to many components of the Wnt, SHH, and FGF pathways (Supplementary Fig. 2, Supplementary Tables 4,5). We confirmed specificity and reproducibility of the ChIP pull down by randomly selecting 17 peaks associated with NSC maturation and testing them in an independent ChIP preparation. All 17 regions were validated by quantitative realtime PCR analysis (Fig. 3F).

To identify direct targets of N1ICD/RBPJ with the highest level of confidence, we focused on the genes that show direct binding and differential expression by N1ICD expression. By intersecting the list of genes that showed differential expression levels in both transcriptome analyses and significant DNA binding by RBPJ in wildtype cortices, we identified 98 genes that are most likely direct, endogenous targets of the N1ICD/RBPJ complex (Fig. 4A). Of these 98 genes, 77 were up-regulated and 21 were down-regulated in *GFAP-Cre;N1ICD* transgenic cortices compared to control littermates (Table 1). GO term analysis of these genes indicated that they include regulators of stem cell maintenance, axon guidance, synaptic transmission, cytoskeleton, and transcription (Supplementary Table 6 and 7),

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consistent with known functions of the Notch pathway in the nervous system [4]. Gene set analysis of these 98 genes revealed that angiogenesis and the Notch pathway are among the most significantly enriched pathways (Fig. 4B). This is consistent with a large number of studies that indicate a critical role of the Notch pathway genes in angiogenesis [35, 36]. Interestingly, the Wnt pathway is also significantly enriched (Fig. 4B). N1ICD/RBPJ complex directly regulates transcription of many Wnt pathway components (Fig. 4C, Table 1, Supplementary Fig. 2). In addition, components of the SHH and FGF signaling pathways were identified as direct targets of the N1ICD/RBPJ complex and validated by realtime RT-PCR analysis (Fig. 4C, Table 1). Remarkably, these targets include critical transcriptional effectors of the pathways, including *Tcf7l2(Tcf4*), *Gli2* and *Gli3.*

Interestingly, a previously reported consensus sequence for RBPJ from *in vitro* studies is not enriched in the peaks we identified, although they are present in nearby sites (denoted by circles in Fig. 3,5, Supplementary Fig. 2). Analysis of *de novo* enriched sequence motifs within the significant peaks identified a GC-rich motif (Supplementary Fig. 3) within 311 of the 347 WT peaks near up-regulated genes, and in 42 of 61 peaks near down-regulated genes. To test whether there are distinct co-factor binding sites near up- and down-regulated genes, we analyzed sequences within 15kb of the transcription start sites of the up- and down- regulated genes. We found significant enrichment near the down-regulated genes for HIF1, E2F, and GAL4 (permutation p-value < 0.001) binding sites; and near the up-regulated genes we found enrichment for NMYC ($p<0.001$) and others (not shown).

To validate that the canonical Notch pathway modulates the endogenous expression of newly identified target genes, we measured the expression level changes of selected target genes after acutely inactivating the Notch pathway in NSCs. To do this, we isolated NSCs from E15.5 cortices of $RBPJ^{f/f}$, a conditional deletion allele of *RBPJ* embryos [37], and used Adeno-Cre-EGFP or Adeno-EGFP (control) virus transduction to acutely delete RBPJ expression. As shown in Fig. 4D, previously identified Notch targets, *Hes1* and *Hes5*, as well as newly identified Notch targets, *Sox2, Pax6,* and *Id4*, showed significantly reduced expression levels in *Cre*-transduced NSCs, confirming that these are endogenous targets of the canonical Notch pathway in NSCs.

To complement the loss-of-function analysis, we transiently transfected an N1ICD expression vector in murine NSC cell line C17-2 [38]. RNA was harvested 24 hours after transfection (within 12 hrs of N1ICD expression onset), and analyzed by realtime RT-PCR. We observed significant up-regulation of *Hes1*, *Sox2,* and *Gli2* in N1ICD-transfected cells compared to control GFP-transfected cells (Fig. 4E, $p<0.04$), indicating that N1ICD is sufficient to induce expression of these genes. These transient loss- and gain-of-function analyses provide corroborating evidence that the genes we have identified are directly regulated by N1ICD/RBPJ at the transcriptional level in NSCs and that the increased transcript level we observed in *GFAP-Cre;N1ICD* cortices is not simply due to an increased number of NSCs in transgenic embryos.

Yap1 is a significant downstream target of N1ICD in NSCs

An unanticipated finding from our genome-level analysis is the large number of NOTCH1 targets that are integral components of other signaling pathways (Table 1). In particular, we were intrigued by the interaction between the Notch and Hippo pathways, since little is known about the function of the Hippo pathway in NSCs. Hippo pathway is a cascading kinase pathway and the activation of the Hippo pathway results in phosphorylation and cytoplasmic retention of the YAP1 protein[39]. When the pathway is inactive, YAP1 translocates to the nucleus and form a transcriptional activation complex with TEAD proteins and YAP1/TEAD complex activates downstream target genes that promote proliferation and survival [40, 41]. In chick neural tube, *Yap1* expression induces neural

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progenitor proliferation [42], suggesting that Hippo-Yap1 pathway is involved in neural cell proliferation and survival in vertebrates. Since YAP1 is a rate limiting transcription factor in the Hippo pathway [43–45], we analyzed its expression and function in NSCs in the developing brain.

First, we validated that activation of Notch results in elevated *Yap1* and *Tead2* RNA levels in the *GFAP-Cre;N1ICD* cortices by realtime RT-PCR analysis (Fig. 5A, $p < 0.01$). In addition, RBPJ binds to regulatory regions of *Yap1* and *Tead2* in both wildtype and *GFAP-Cre;N1ICD* cortices (Fig. 5B), indicating that they are endogenous targets of N1ICD. To test whether endogenous *Yap1* and *Tead2* expression depends on the canonical Notch pathway, we acutely deleted RBPJ expression from NSCs in culture and measured the consequent *Yap1* and *Tead2* expression levels (Fig. 5C). Similar to other Notch target genes (Fig. 4D), *Yap1* expression level was significantly reduced in *RBPJ−/−* NSCs ($p<0.0007$), while the reduction in *Tead2* level did not quite reach statistical significance ($p<0.0881$). To test whether acute N1ICD expression is sufficient to induce *Yap1* and *Tead2* expression, we transiently transfected C17-2 cells with N1ICD and measured *Yap1* and *Tead2* levels within 12 hours after the transgene expression (<24hr after transfection). As shown in Fig. 5D, N1ICD is sufficient to induce *Yap1* expression in these cells although it appears other factors are needed to activate *Tead2* in these cells. Together, these results suggest that *Yap1* is a direct target of N1ICD.

To test whether YAP1 protein is expressed and is functional in mammalian NSCs, we analyzed YAP1 expression pattern by immunofluorescence analysis on wildtype mouse brains at E13.5 and E15.5. As shown in Fig. 5E, YAP1 expression is high in the stem cell compartment (VZ) of wildtype cortex and basal ganglia and significantly lower in the progenitor and differentiation zones (SVZ and mantle). Double labeling with a marker for neural progenitors (TBR2) suggests that most progenitor cells in the SVZ have very low to undetectable levels of nuclear YAP1, in contrast to many cells in the VZ that have both nuclear and cytoplasmic YAP1 (Fig. 5F). Consistent with its expression in NSCs, YAP1 immuno-reactivity is observed in radial glial fibers (Fig. 5F). To better visualize nuclear localization of YAP1 and to test whether YAP1 localization is regulated by cellular density, we plated wildtype NSCs onto coverslips at low and high densities. YAP1 is nuclear, albeit at different levels, in all SOX2+ cells at low density (Fig 5G) but becomes weaker in some SOX2+ cells at high density (Fig 5H). Furthermore, western blot analysis of nuclear and cytoplasmic fractionations of E15.5 wildtype cortex shows that YAP1 is present in both the nucleus and cytoplasm (Fig. 5I). Together, these results indicate that YAP1 is expressed in the right cell type to mediate Notch function in NSC proliferation and survival.

To functionally test whether *Yap1* is a significant downstream target of *Notch1*, we ectopically expressed *Yap1* in wildtype NSCs *in vitro.* Treatment of wildtype NSCs with DAPT, a Notch pathway inhibitor, significantly reduced neurosphere formation (Fig 5J). In contrast, *Yap1* expressing cells were relatively resistant to DAPT treatment, indicating that ectopic expression of *Yap1* can rescue Notch pathway inhibition (Fig 5J). Realtime RT-PCR analysis of control and *Yap1*-expressing cells show that expression of human *Yap1* transgene elevated endogenous mouse *Yap1* but not TAZ expression, suggesting an auto-feedback loop (Fig 5K). Interestingly, *Yap1* also elevated *Hes5,* but not *Hes1* or *N1ICD*, expression, suggesting another point of potential cross-talk between the Notch and Hippo-Yap pathways. Together, these results validate that *Yap1* is an important downstream effector of the Notch pathway in NSC self-renewal and suggest mutual cross-regulation between the Notch and Hippo pathways.

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Discussion

The Notch pathway has been shown to play a critical role during normal development and homeostasis of multiple stem cell compartments [4, 46, 47]. Aberrant activation of the Notch pathway is implicated in multiple types of human cancer, including brain tumors [48–50]. Hence, it is imperative to understand molecular mechanisms of Notch function in order to safely manipulate normal stem cells or treat cancer.

Interestingly, the age-dependent effect of N1ICD elevation is more dramatic than the dose-dependent effect (Fig. 1,2). Specifically, single copy expression of N1ICD in *Foxg1-Cre*+ cells leads to a much more dramatic phenotype than two-copy expression in *GFAP-Cre*+ cells in older neuroepithelium. These observations suggest that young NSCs may be more competent or responsive than older NSCs to *Notch1* activity. This idea is further supported by several of our observations. First, N1ICD transgenic NSCs were highly responsive to bFGF but not to EGF (Fig. 2E). Early neuroepithelial stem cells depend on bFGF for proliferation, while the more mature progenitor cells in the SVZ depend on EGF [51]. Second, N1ICD directly activates expression of *FGFR3* (Fig 2F), presumably enhancing responsiveness of N1ICD expressing cells to FGF signaling, consistent with a previous report that FGF and Notch signaling cooperate to promote neural precursor proliferation [11]. Third, N1ICD expression enhances symmetric stem cell divisions (not shown), the dominant mode of cell division by early NSCs during the neuroepithelium expansion phase. It has been reported that stem cell numbers diminish with age due to both cell-autonomous and non-autonomous effects, and that NSCs require different molecular mechanisms for self-renewal at different ages [52–54]. *GFAP-cre;N1ICD* adult mice have increased numbers of NSCs in the SVZ compared to littermate controls of the same age (not shown). However, we do not yet know whether this is due to maintenance of higher numbers of NSCs throughout life or whether N1ICD directly promotes adult NSC self-renewal in mature *GFAP-Cre;N1ICD* brains.

We determined that Notch activation is not sufficient to confer the stem cell phenotype to non-stem cells. This observation complements other studies that suggested that Notch signaling is not necessary for the generation of NSCs but is required to maintain NSCs [55]. *Notch1* directly activates expression of *Sox2, Pax6* and many other NSC regulators but does not bind to DNA sequences near other stem cell regulators such as *Nanog* and *Oct4/Pou5f1*, suggesting that only a subset of the stem cell network genes is directly regulated by N1ICD/RBPJ. Together, these observations indicate that Notch is a permissive rather than instructive factor for the NSC fate. In the future, it will be important to identify distinct molecular events required for the acquisition vs. maintenance of stemness and the types of epigenetic changes that accompany progenitor specification and loss of competence to respond to N1ICD.

Our findings suggest that Notch activity must be repressed in maturing wildtype neural progenitor cells, and multiple molecular mechanisms are in place to ensure reduced Notch activity in neural progenitors. For example, earlier studies have shown that NUMB and NUMB-LIKE antagonizes Notch and these proteins are asymmetrically localized to differentiating daughter cells [56–58]. More recently, it has been shown that degradation of NICD by FBW7, an E3-ligase that ubiquitinylates NICD, plays a critical role in NSC maturation *in vivo* [59]. Identification of Notch targets in this study provides a potential explanation for this necessity: in addition to repressing neurogenic bHLH proteins by activating *Hes* gene expression, N1ICD/RBPJ actively represses the transcription of many genes associated with neural progenitor specification and differentiation (Table 1). According to the dogma in the field, N1ICD functions as a transcriptional co-activator; however, our results suggest that more than 20% (21/98) of the direct targets *in vivo* are

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repressed by Notch activation. Hence, *Notch1* simultaneously promotes NSC fate and actively inhibits progenitor/neuron specification at the molecular level.

It should be noted that the genes we identified are endogenous targets of NICD/RBPJ in physiological *in vivo* conditions since the DNA binding occurs in wildtype cortices (Fig. 4E) and endogenous levels of all activated genes tested are decreased when RBPJ is inactivated (Fig. 5D, 6C). In addition to our own data, there are corroborating evidence in the literature that these are true targets of Notch: 1) the short list of 98 genes includes most well-established canonical targets of Notch previously reported (such as *Hes1, Hes5, Hey1*); 2) NOTCH1 target genes identified in ES cells [23] overlap with the genes we identified (including *Pax6, Sox9, Id4*); 3) previous genetic analyses of many of the newly identified target genes, including *Pax6, Sox2, Tlx,* and *Id4,* showed overlapping phenotypes with Notch pathway loss- and gain-of-function in the NSC compartment [60–64]. Interestingly, we did not observe significant enrichment of previously identified "consensus RBPJ" motif, although they were often present near the peaks we identified (denoted with green dots in ChIP peak figures). There are multiple possible variables that might explain this result. First, our work indicates that cellular context has a strong influence in modulating Notch/RBPJ function. Previously identified "consensus sequences" were optimized *in vitro* or in cultured cells, as opposed to *in vivo* tissues, where cell:cell contact and microenvironmental cues may be drastically different. Second, RBPJ binding site preferences may be inherently different between NSCs and other cell types used in earlier studies. In addition, epigenetic status of NSCs and other cell types are most likely very different, altering the accessibility of RBPJ binding sites. We estimate that our analysis is very conservative and that Notch1 targets include many more genes than the 98 genes we prioritized. In the future, it will be informative to test the functions of those genes whose roles in NSC regulation are unknown, particularly many of the transcription factors, cell adhesion molecules, and kinases.

It is somewhat surprising that Notch activates expression of so many transcription factors (28 out of 98 genes), including key regulators of NSC proliferation and differentiation, suggesting that Notch1 is a "master regulator" of NSCs. It is even more surprising that Notch activates transcription factors that function as effectors of many other signaling pathways involved in stem cell regulation. Stem cells *in vivo* are under the influence of multiple signaling pathways and at each cell division, the input from all these pathways must be integrated into a single, discrete, cell fate decision. Emerging studies show that there is cross-talk among different signaling pathways via protein:protein interactions among the canonical pathway proteins or via cross-regulation of classical target genes or ligand expression [65]. For example, it was recently reported that β-catenin, a transcriptional effector of the Wnt pathway, binds to N1ICD to enhance *Hes1* expression [66]. Others have reported that the SHH pathway activates *Hes1*, independent of Notch activation [67, 68]. Similarly, the TGF/BMP pathway has been shown to antagonize or synergize with Notch1 activation [69]. Our findings demonstrate yet another level of cross-regulation among the pathways: N1ICD regulates RNA levels of transcriptional effectors in the Wnt, SHH, and Hippo signaling pathways (Fig. 4D,E, Fig. 5A,B,C, Table 1). We propose a working model in which activation of the Notch pathway positively feeds into the activities of the other signaling pathways (Wnt, SHH and Hippo) by raising the RNA levels of their transcriptional effectors, priming them for rapid response upon their pathway activation (Fig. 6).

Another unanticipated discovery of our study is the direct crosstalk between the Hippo and Notch pathways in NSCs. The Hippo pathway responds to cellular density *in vivo* and *in vitro* by sensing cell:cell contact[39]. Hence, it is logical that the Notch and the Hippo pathways would be intimately interconnected; however, there is little evidence for direct interaction at the molecular level, especially in mammalian cells. In *Drosophila,* genetic studies have suggested that the Hippo pathway modulates Notch target gene expression in

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posterior follicle cells [70], and that the Hippo pathway is involved in regulating the Notch receptor level [71]. Recently, Reddy et al., have shown that expression of activated *Yki* expands the neuroepithelial cells in Drosophila optic disc by inhibiting differentiation, and suggested that this is mediated, at least in part, through regulation of *Delta1* expression. Interestingly, they also reported that the neuroblasts are refractory to the Fat-Hippo pathway [72], similar to our observation that neural progenitors are refractory to Notch activation (Fig. 2). We show that *Notch1* directly activates *Yap1* mRNA levels in murine NSCs and that *Yap1* activates *Hes5* expression, although it remains to be tested whether *Hes5* is a direct target of *Yap1*. By combining published data by others and our observations, we propose that the coordinated activities of the Hippo and Notch pathways play a determining role in regulating the stem cell number and ultimately organ size during embryogenesis.

Conclusion

In summary, we show that Notch is prominently positioned in a transcriptional regulatory network that controls NSCs by directly regulating the expression of many stem cell factors and key components of other signaling pathways (Table 1). Clearly, N1ICD/RBPJ does not solely regulate the expression of these genes but most likely interacts with other factors that control transcription of these genes in a cooperative or competitive manner. Together with emerging studies from other laboratories, data we present here suggest a new model in which signaling pathways that are traditionally considered to act in parallel interact much more intimately than previously appreciated to control NSC fates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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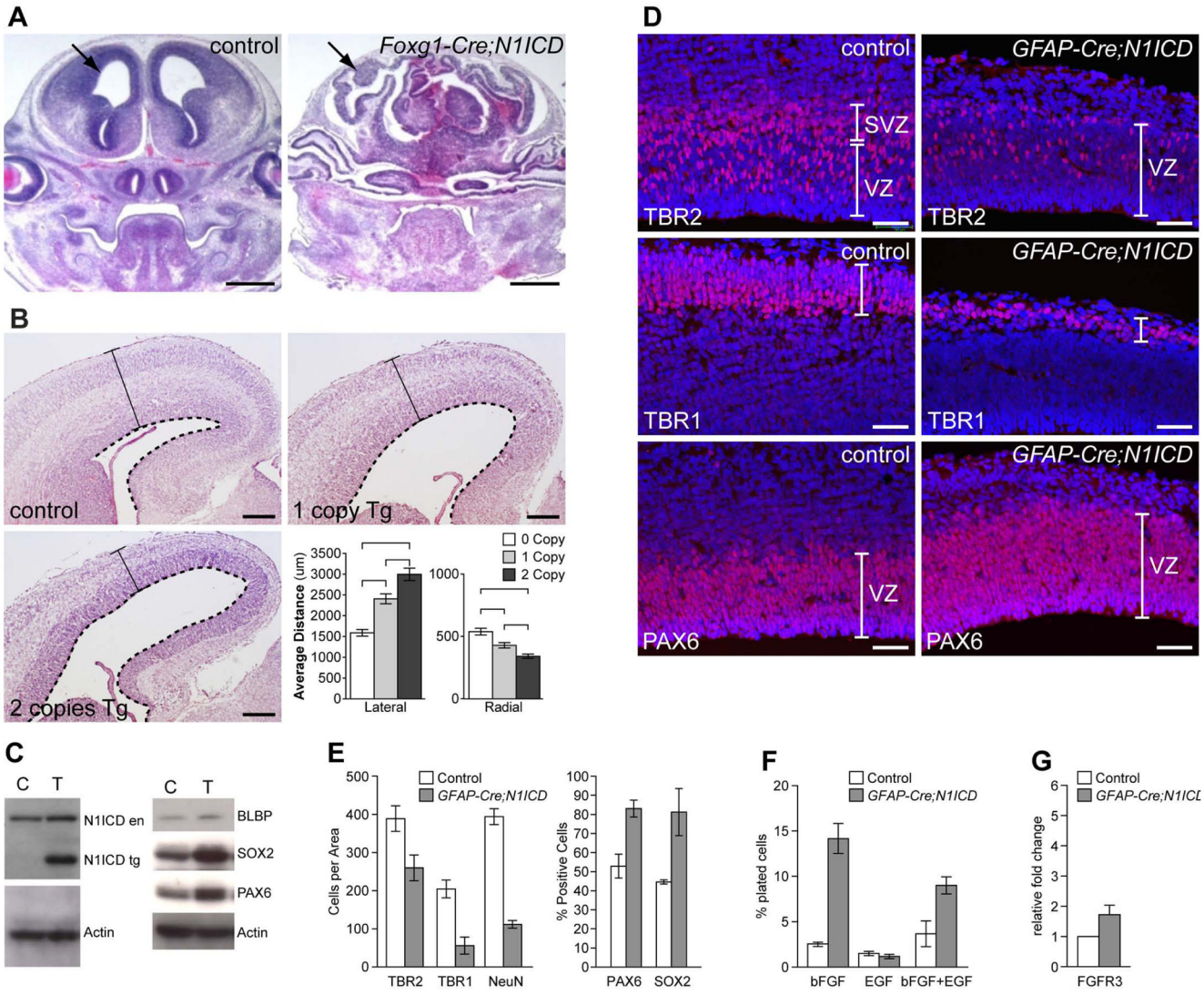

Figure 1. Notch activation expands the NSC pool in a dose-dependent manner

(A) H&E staining of *Foxg1-cre;N1ICD* embryos at E13.5 shows greatly expanded neuroepithelium containing NSCs compared to control littermates. Arrows point to cortex. (B) Coronal sections of littermate control and *GFAP-Cre; N1ICD* embryos at E15.5 with 1 copy or 2 copies of the transgene, stained with H&E. Quantification of the lateral width and radial thickness of littermate cortices shows statistically significant changes in the transgenic cortex (p-values <0.004). Dashed lines: apical surface of the cortical epithelium, solid bars: radial thickness of cortex. (C) Western blot analyses with antibodies against NOTCH1, BLBP, SOX2 and PAX6 show increased expression in *GFAP-Cre;N1ICD* cortices at E15.5. N1ICD en: endogenous N1ICD, N1CID tg: transgenic N1ICD. (D) Marker analysis for progenitors (TBR2), neurons (TBR1) and NSCs (PAX6) at E15.5. (E) Quantification of NSCs (SOX2+, PAX6+) or progenitors (TBR2+) and neurons (TBR1+, NeuN+) in equivalent areas of control and transgenic cortices (n=3). (F) Neurosphere assays show increased number of NSCs isolated from E15.5 cortices of *GFAP-Cre;N1ICD* compared to control littermates, cultured with bFGF, EGF, or bFGF+EGF. (G) Relative expression levels of FGFR3 in control and *GFAP-Cre; N1ICD* cortices at E15.5, measured by realtime RT-

PCR. VZ: ventricular zone, SVZ: subventricular zone. Error bar: standard deviation of the mean. Also see Supplementary Figure 1.

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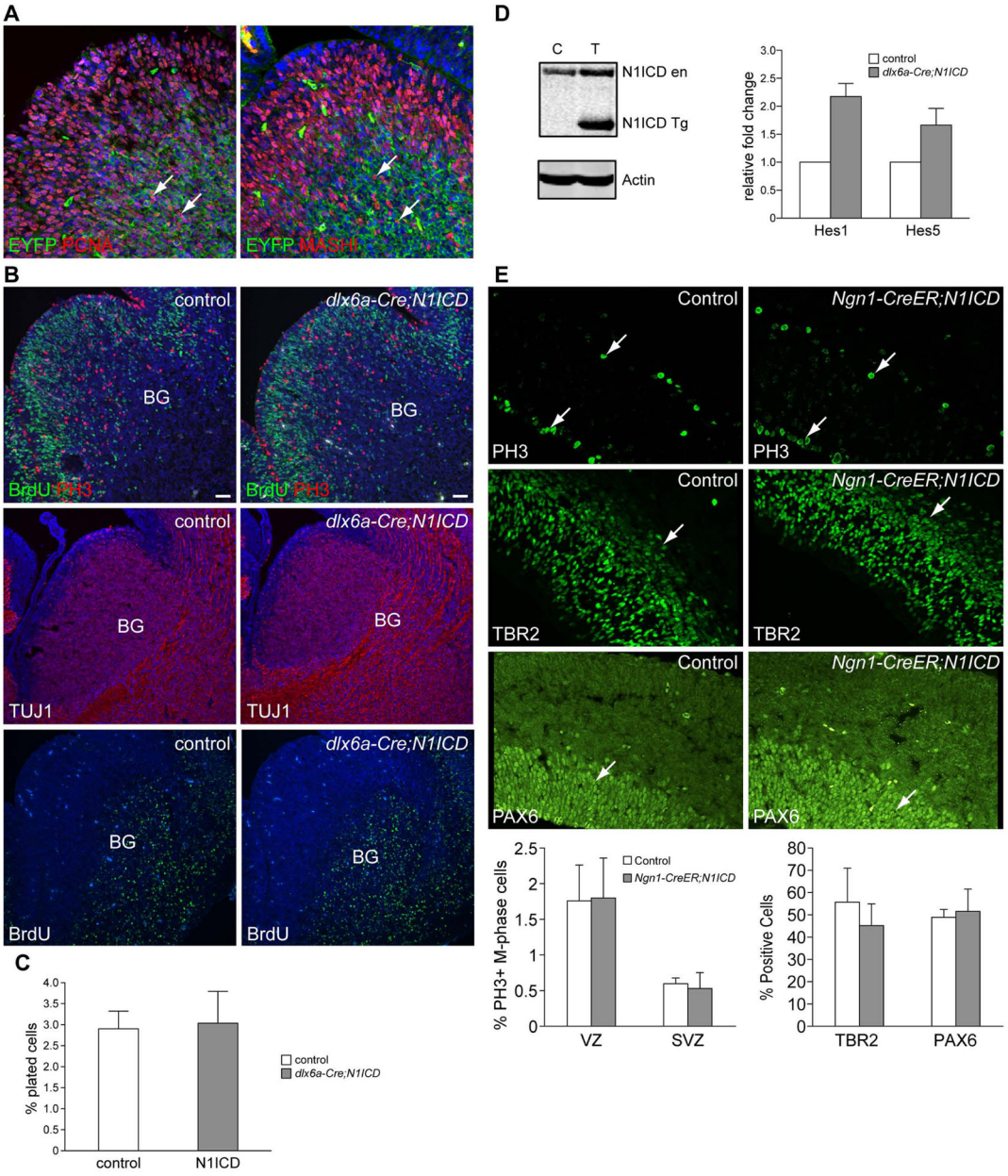

Figure 2. N1ICD expression in neural progenitors is not sufficient to confer stem cell phenotype

(A) *dlx6a-cre;ROSA-EYFP* reporter analysis shows that CRE is active in proliferating progenitors (PCNA+) in the SVZ. (B) Proliferation (BrdU/PH3) and neurogenesis (TuJ1 and BrdU birthdating: inject E11.5-analysis at E13.5) are unaltered in *dlx6a-cre;N1ICD.* (C) Self-renewal assay of neurospheres isolated from basal ganglia of control and *dlx6a-cre;N1ICD* embryos confirm no significant change in NSC numbers. (n=3) (D) N1ICD expression and target gene (*Hes1* and *Hes5*) activation in the basal ganglia of *dlx6a-cre;N1ICD.* (E) No significant changes in proliferation (PH3+ cells), progenitors (TBR2+), or stem cells (PAX6+) are observed in *Ngn1-creER;N1ICD* cortices treated with Tamoxifen at E11.5 and harvested at E13.5. PH3: phosphorylated Histone3, BG: basal ganglia, arrows point to positive cells in VZ and SVZ.

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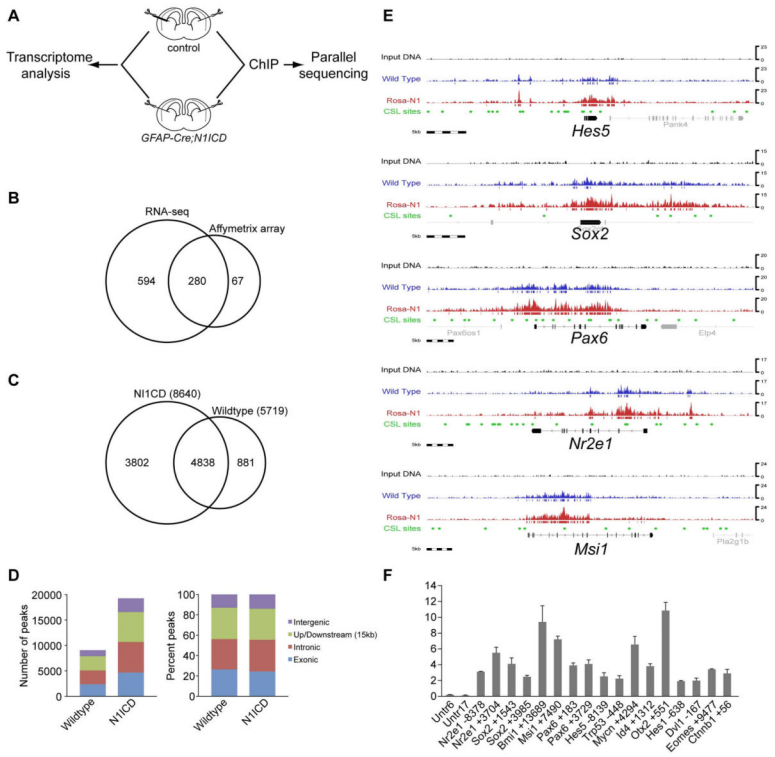
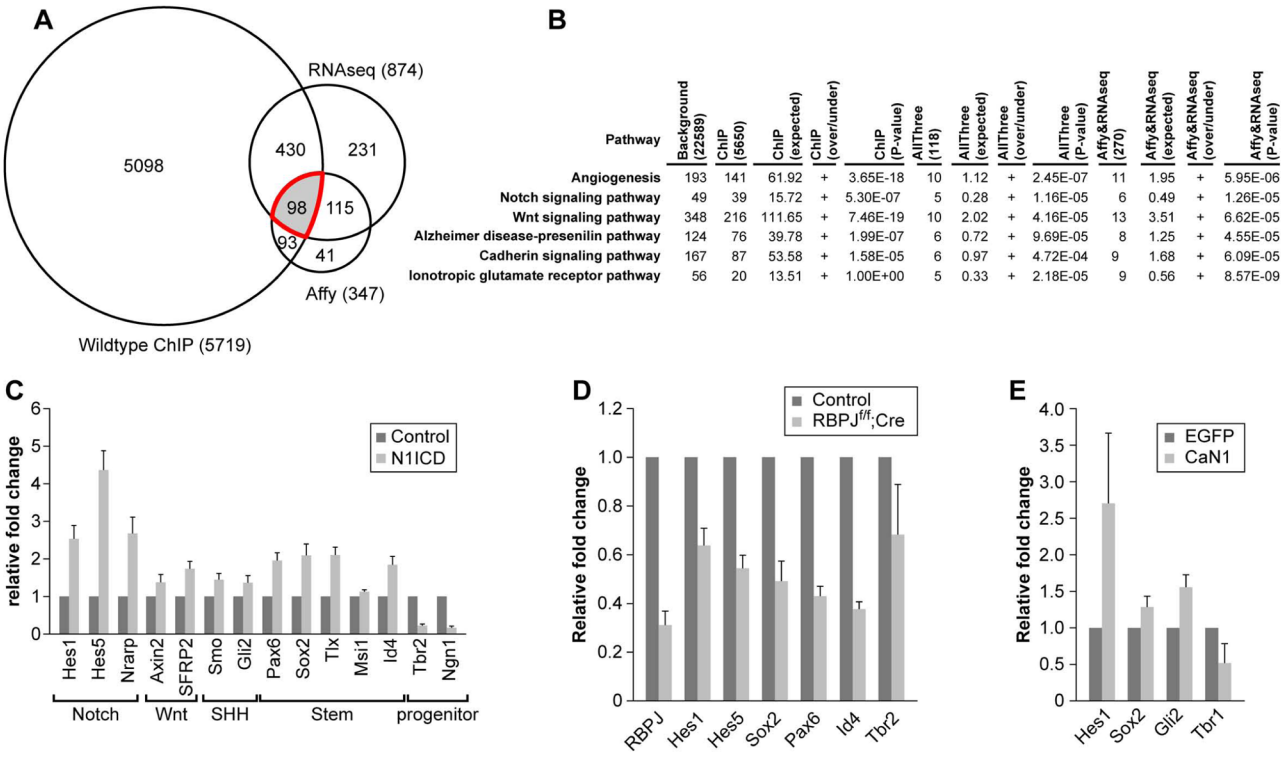


Figure 3. ChIP-seq and transcriptome analyses to identify N1ICD/RBPJ targets *in vivo*

(A) A schema of experimental design: littermate wildtype and *GFAP-Cre;N1ICD* cortices were isolated and analyzed by RNA-seq and Affymetrix St 1.0 microarrays and ChIP-seq. (B) Numbers of differentially expressed genes measured by RNA-seq and Affymetrix microarray methods. (C) Numbers of genes within 15kb of significant peaks in wildtype and *GFAP-Cre;N1ICD* cortices. When the window is expanded to 50kb, a total of 8343 genes are associated with significant peaks in wildtype cortices. (D) Distribution of genomic regions bound by RBPJ. (E) Representative histogram presentations of aligned ChIP-seq reads in wildtype and transgenic cortices. Significant peaks are marked with bars below each histogram. Consensus RBPJ binding sites from earlier studies are marked with dots. (F) Independent ChIP validation of selected target sequences by realtime PCR. *Untr6* and *Untr17* are negative control regions. Also see Supplementary Figure 2.



Pathway	Background (22589)	ChIP (5650)	ChIP (expected)	ChIP (over/under)	ChIP (P-value)	AllThree (118)	AllThree (expected)	AllThree (over/under)	AllThree (P-value)	Affy&RNAseq (270)	Affy&RNAseq (expected)	Affy&RNAseq (over/under)	Affy&RNAseq (P-value)
Angiogenesis	193	141	61.92	+	3.65E-18	10	1.12	+	2.45E-07	11	1.95	+	5.95E-06
Notch signaling pathway	49	39	15.72	+	5.30E-07	5	0.28	+	1.16E-05	6	0.49	+	1.26E-05
Wnt signaling pathway	348	216	111.65	+	7.46E-19	10	2.02	+	4.16E-05	13	3.51	+	6.62E-05
Alzheimer disease-presenilin pathway	124	76	39.78	+	1.99E-07	6	0.72	+	9.69E-05	8	1.25	+	4.55E-05
Cadherin signaling pathway	167	87	53.58	+	1.58E-05	6	0.97	+	4.72E-04	9	1.68	+	6.09E-05
Ionotropic glutamate receptor pathway	56	20	13.51	+	1.00E+00	5	0.33	+	2.18E-05	9	0.56	+	8.57E-09

Figure 4. Direct targets of N1ICD/RBPJ include transcription factors of the WNT, SHH, and Hippo pathway

(A) Venn diagram summarizing the overlap among ChIP-seq and transcriptome analyses to identify 98 genes with highest confidence of being direct targets of N1ICD/RBPJ complex. (B) Gene set analysis [73] of most enriched pathways represented by the 98 target genes. (C) RT-PCR validation of selected genes in wildtype and *N1ICD;GFAP-Cre* cortices at E15.5 (student t-test, 0.0001<p< 0.04). (D) Endogenous NOTCH1 target gene expression in control vs. *RBPJ−/−* NSCs, measured by realtime RT-PCR. (n=7, p< 0.05 for all except *Tbr2*, p<0.17) (E) Realtime RT-PCR analysis of C17-2 cells transiently transfected with N1ICD (CaN1) or EGFP control expression vector (p<0.04, n=3). Also see Supplementary Figure 3.

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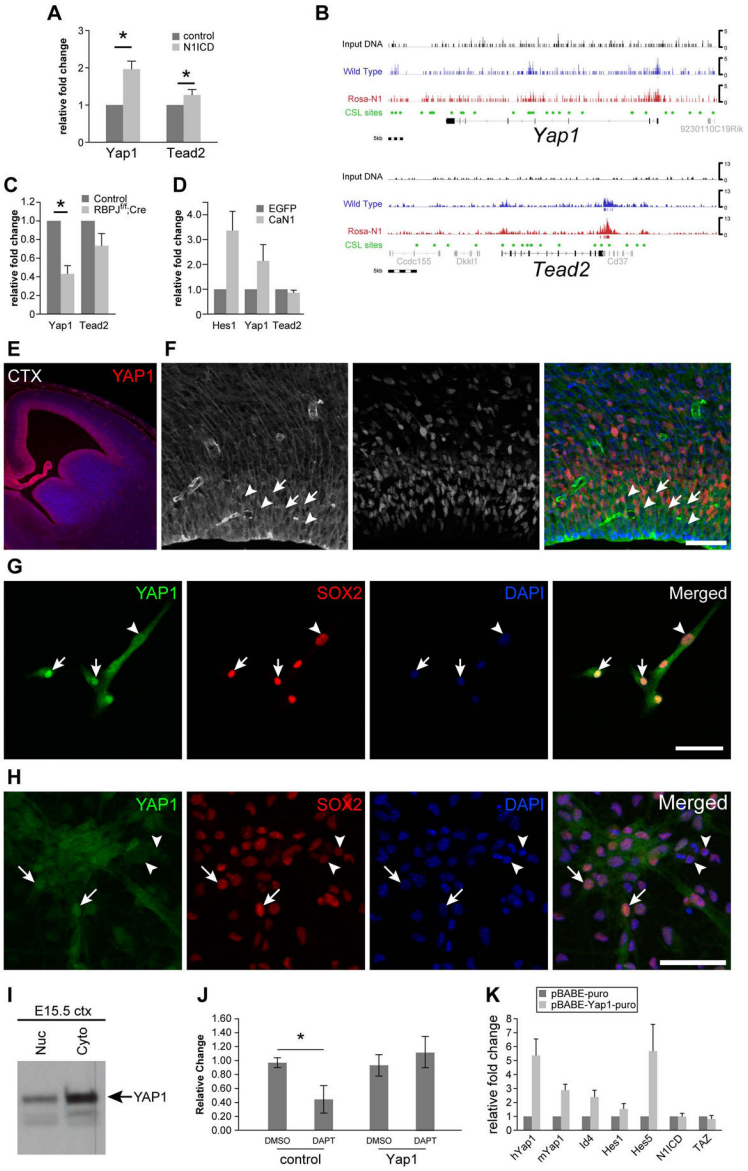

Figure 5. Yap1 is a significant downstream target of N1ICD in NSCs

(A) RT-PCR validation of increased *Yap1* and *Tead2* expression levels in *GFAP-Cre;N1ICD* cortices. (B) A histogram representation of RBPJ binding in *Yap1* and *Tead2* genomic regions in wildtype and *GFAP-Cre;N1ICD cortices in vivo.* (C) *Yap1* and *Tead2* RNA levels in control (Adeno-EGFP) and RBPJ-deleted (Adeno-Cre-EGFP) neurospheres (n=7, Yap1 $p<0.0007$, Tead2 $p<0.08$). (D) Realtime RT-PCR analysis of control (EGFP) and N1ICD (CaN1) transfected C17-2 cells at <24hr after transfection (n=3). (E) Immunofluorescence analyses of YAP1 in wildtype forebrain at E13.5 at low magnification. (F) Double immunofluorescence analysis with antibodies against YAP1 (green) and TBR2 (red) show radial glial fiber and nuclear YAP1 expression in some of the VZ cells. Most TBR2+ cells only contain cytoplasmic YAP1. Arrows point to nuclear and cytoplasmic YAP1+ cells; arrowheads point to only cytoplasmic YAP1+ cells. (G,H) Nuclear YAP1 expression in wildtype NSCs cultured in low (G) and high (H) densities (YAP1: green, SOX2: red). Arrows point to strong nuclear YAP1 and arrowheads point to weaker nuclear

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YAP1 expressing cells. (I) YAP1 protein is present in the nucleus and cytoplasm in E15.5 wildtype cortex. (J) DAPT (10μM) treatment of control (pBABE-Puro) and *Yap1* over-expressing (pBABE-Yap1-puro) NSCs show that the reduced NSC self-renewal by Notch inhibition can be rescued by *Yap1* over-expression (p=0.05). (K) Realtime RT-PCR analysis of control (pBABE-puro) and Yap1-expressing cells (pBABE-Yap1-puro). All gene expression levels shown are normalized to the control sample levels. Scale bar= 50μm.

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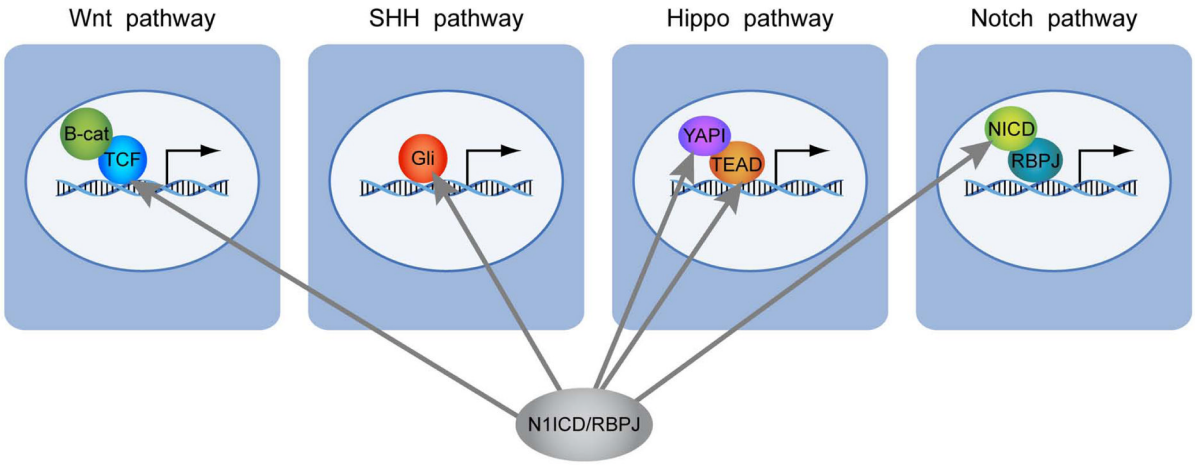

Figure 6.
A working model based on our observation that N1ICD/RBPJ directly binds and activates expression of transcriptional effectors of the Wnt, SHH, Hippo, and Notch pathways, priming these pathways for rapid and robust responses when the canonical pathways are activated.

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Table 1

Direct targets of N1ICD/RBPJ in NSCs in vivo

Up-regulated	**n**	**genes**
transcription	24	*Btg2, Gli2, Gli3, Hes1, Hes5, Hey1, Id4, Jun, Nfatc4, Notch1, Nr2e1 (Tlx), Pax6, Prdm16, Sall1, Sox2, Sox21,Sox9, Tcf3 (E2A/E47), Tcf7l2 (Tcf4), Tcfap2c, Tead2, Yap1, Zfhx4, Zhx2*
Notch pathway	5	*Hes1, Hes5, Hey1,Notch1, Nrarp*
Wnt pathway	8	*Fzd2, Fzd8, Fzd9, Nrarp, Ppap2b, Sfrp1, Slc9a3r1 (NHERF1), Tcf7l2 (Tcf4)*
SHH pathway	4	*Cdon, Gli2, Gli3, Smo*
Hippo pathway	2	*Tead2, Yap1*
other signaling	15	*AI464131, Angptl2, Cntfr, Dusp16, E130112L23Rik, Epb4.1l5, Fgfr3, Grik2, Igfbp5, Jun, Ltbp3,Prdm16, Rcn3, Rlbp1, Spata13*
calcium binding/signaling	6	*Casq1,Grik2, Ltbp3, Rcn3, Rhbdl3, Ttyh1*
kinase/phosphatase	5	*Alk, Axl, Camk2d, D8Ertd82e, Ppap2b*
cell adhesion	8	*Cdon, Celsr1, Epb4.1l5, Jub, Lamb2, Megf10, Tns3, Ttyh1*
extracellular matrix/membran	7	*Bcan, Igdcc4, Igfbp5, Kcnj10, Lamb2, Mfap2, Ptgfrn*
apoptosis	3	*Aldoc, Bcl2l11 (Bim), Ddit4*
metabolism	5	*Dbi, Aldoc, Ddit4, Prdm16, Slc27a1*
stress response	3	*Hhipl1, HSPB6, Nfatc4*
other	7	*1190002N15Rik,2010011I20Rik, Cbs, Chst3, Myo10, Pgpep1, Pgpep1,* ***Ston2***
down-regulated	n	genes
transcription	4	*Bcl11b, Fezf2, Nr4a3, Sox5*
Notch pathway	1	*Dll3*
other signaling	6	*Abr, Adamts3, Agap2, Cck, Cnih2, Dab1*
cell adhesion	1	*Dab1*
extracellular matrix/membran	3	*Adamts3, Islr2, Tnr*
calcium binding/signaling	1	*Necab3*
apoptosis	2	*Bcl11b, Cck*
stress response	1	*Necab3*
neural differentiation	5	*Accn1, Islr2, Slc17a7, Slit1, Sox5*
other	4	*Opcml, Sh3gl2, St3gal1, Unc13a*