PNAS
www.pnas.org

Supplementary Information for

β-Catenin Signaling Dynamics Regulate Cell Fate in Differentiating Neural Stem Cells

Alyssa B. Rosenbloom, Marcin Tarczyński, Nora Lam, Ravi S. Kane*, Lukasz J. Bugaj*, David V. Schaffer*

Corresponding Authors:
David V. Schaffer (schaffer@berkeley.edu)
Lukasz Bugaj (bugaj@seas.upenn.edu)
Ravi S. Kane (ravi.kane@chbe.gatech.edu)

This PDF file includes:

Figures S1 to S12
SI References

www.pnas.org/cgi/doi/10.1073/pnas.2008509117

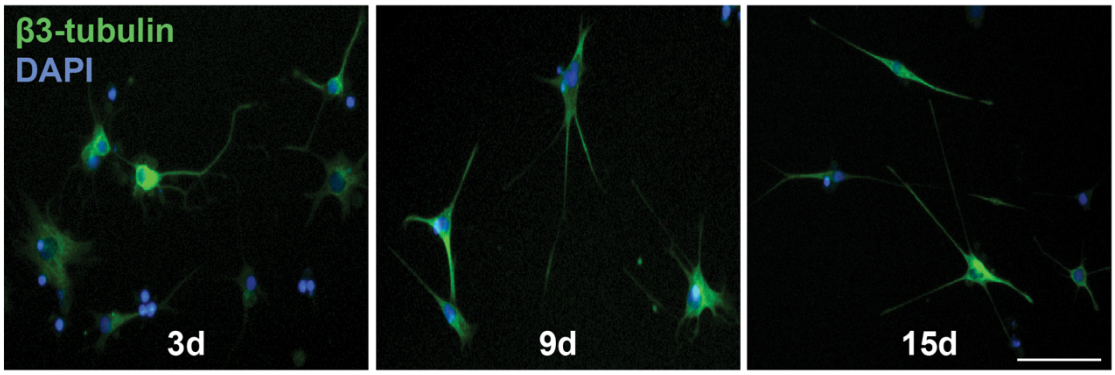


Fig. S1. Long-term maintenance of light-induced neurogenesis.

NSCs expressing optoWnt were stimulated with blue light for 3, 9, and 15 days. Neurogenesis was observed in all conditions. Long-term illumination produced neurons with elongated processes relative to those produce by short-term (3-day) illumination. Scale bar = 500 μm.

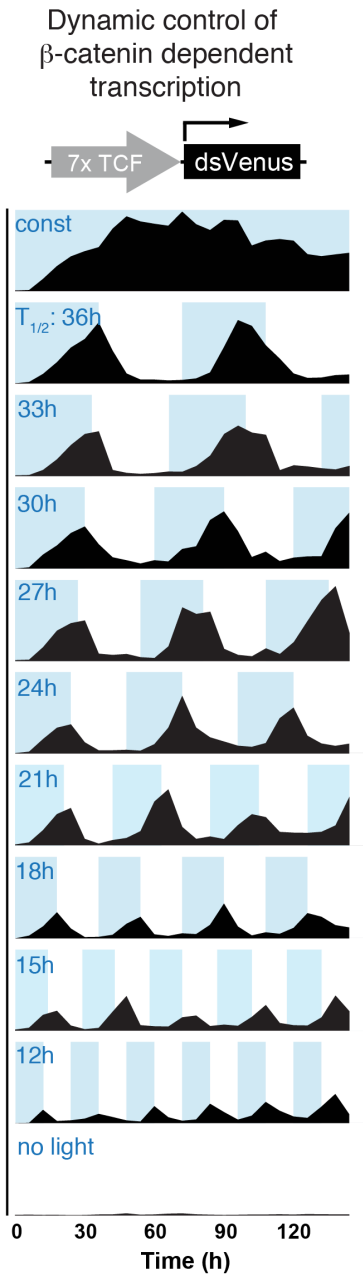


Fig. S2. OptoWnt drives dynamic β-catenin-dependent transcription in NSCs.

NSCs were transduced with a transcriptional reporter of β-catenin activity comprising 7 TCF binding sites upstream of a destabilized Venus reporter. Reporter NSCs were then transduced to express optoWnt and were stimulated with blue light in square wave pulse trains of varying pulse lengths ($T_{1/2}$). Blue bars indicate illumination times. Black traces indicate total dsVenus fluorescence per image, as measured by live-cell fluorescence imaging. In all cases, β-catenin transcriptional response dynamically tracked the optogenetic stimulus. Response kinetics and amplitude appeared to be independent of stimulus history. Traces for $T_{1/2}$ = 12, 24, 30, and 36 hours are reproduced from Figure 2.

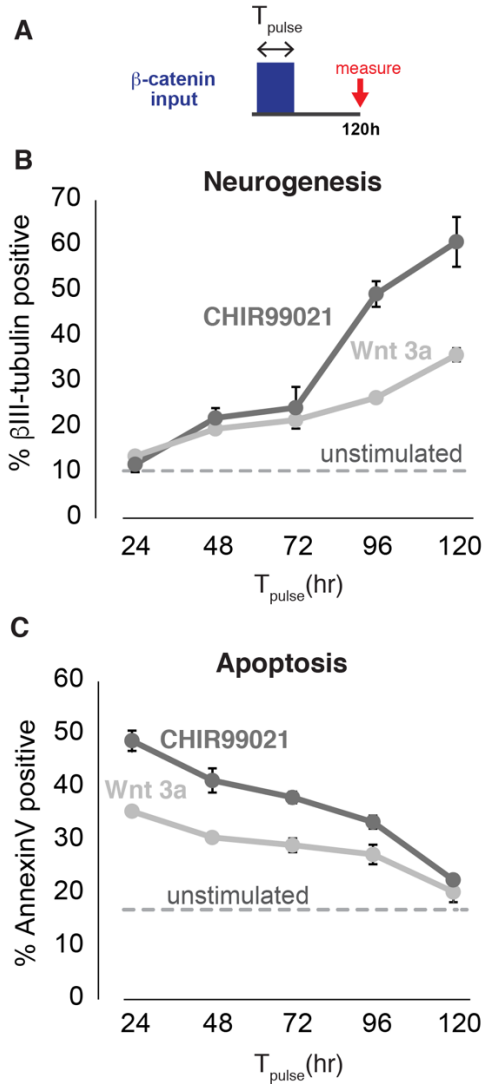


Fig. S3. β-catenin pulse-width specifies NSC differentiation or apoptosis independent of pathway agonist.

A) NSCs were treated with variable-length pulses of β-catenin pathway agonists CHIR99021 and Wnt3a (as in the optogenetic experiment depicted in Figures 2F and 2G). After 5 days, cells were probed for neurogenesis (B) and apoptosis (C) via fixed cell immunofluorescence and Annexin V staining, respectively. Neurogenesis increased with increasing duration of β-catenin stimulation. Apoptosis peaked at 1 day of stimulation and decreased upon increased signal duration. Dotted lines represent measurements from cells that received no pathway stimulation. Data points represent means +/- 1 s.e. from 3 biological replicates.

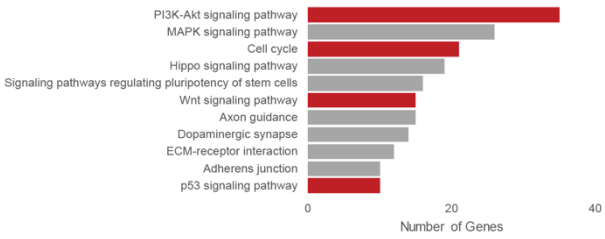
A

GO_Term	P-Value	Benjamini	FDR
protein phosphorylation	1.8E-05	1.1E-02	3.4E-02
positive regulation of gene expression	2.8E-06	2.9E-03	5.1E-03
brain development	1.6E-08	6.7E-05	2.9E-05
positive regulation of apoptotic process	6.1E-06	4.3E-03	1.1E-02
cell proliferation	2.6E-07	5.5E-04	4.8E-04
positive regulation of cell migration	2.0E-05	1.0E-02	3.7E-02
negative regulation of neuron apoptotic process	8.5E-07	1.2E-03	1.6E-03
peptidyl-serine phosphorylation	1.1E-04	4.3E-02	2.1E-01
myelination	5.4E-06	4.6E-03	1.0E-02
chromosome segregation	6.2E-05	2.9E-02	1.2E-01
protein targeting to plasma membrane	8.2E-05	3.4E-02	1.5E-01

B

KEGG_Pathway	P-Valu	Benjami	FDR
p53 signaling pathway	4.5E-02	4.0E-01	4.5E+01
Adherens junction	5.6E-02	4.1E-01	5.3E+01
ECM-receptor interaction	3.6E-02	3.7E-01	3.9E+01
Dopaminergic synapse	9.4E-02	5.4E-01	7.3E+01
Axon guidance	4.9E-02	4.0E-01	4.9E+01
Wnt signaling pathway	9.3E-02	5.4E-01	7.2E+01
Signaling pathways regulating pluripotency of stem cells	5.0E-02	3.9E-01	4.9E+01
Hippo signaling pathway	1.5E-02	2.5E-01	1.7E+01
Cell cycle	3.0E-04	7.8E-02	3.9E-01
MAPK signaling pathway	4.3E-02	4.1E-01	4.4E+01
PI3K-Akt signaling pathway	1.2E-02	2.4E-01	1.5E+01

C



D

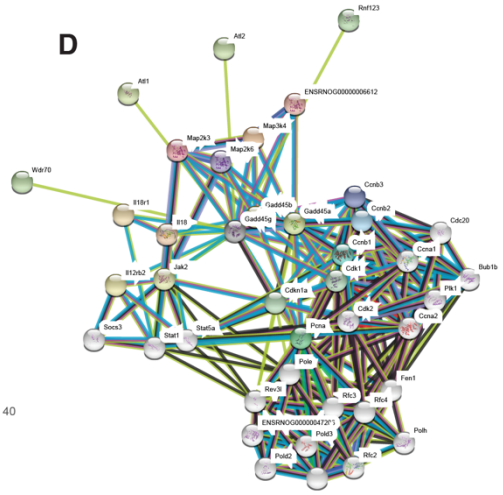


Fig. S4. GO and KEGG analysis of dynamically regulated genes.

GO (A) and KEGG (B) analysis of dynamically responsive genes identified by RNAseq analysis as detailed in the Methods. C) Quantification of differentially regulated genes from enriched KEGG pathways. D) STRING analysis of human Gadd45γ showing 2 shells of interaction. Interaction keys and algorithms are described at string-db.org[1].

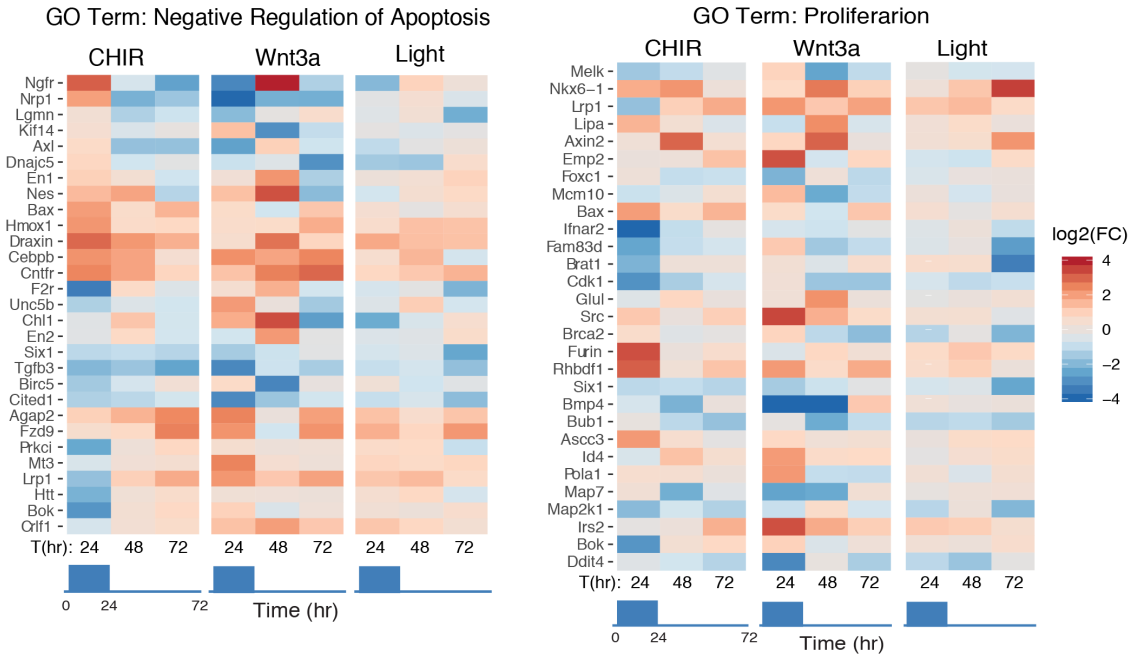
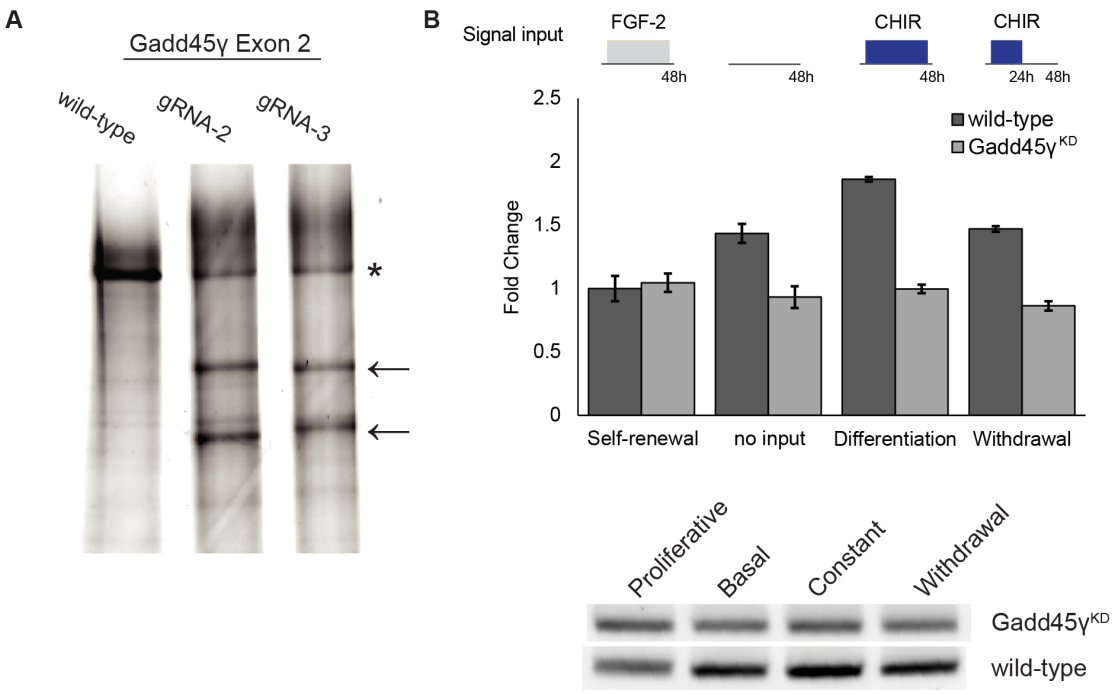


Fig. S5. Gene expression trends of dynamically regulated genes within select Gene Ontology categories.

Heatmaps depict gene expression time courses of differentially-regulated genes from the experiment depicted in Figure 3. Genes were clustered by expression trends obtained from CHIR treatment, as described in the Methods section.



C

```
5′- 3′
Exon 2                                   PAM        gRNA-2
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCAGACAGCCCTGGCGCTGCGCCGACAGCAGAAGTTCGTGCAATGCTTT
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCA-------------------GACAGCAGAAGTTCGTGCAATGCTTT
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCAGACAGCG------CTGCGCCGACAGCAGAAGTTCNTGCAATGCTTT
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCAGA----------CTGCGCCGACAGCAGAAGTTCGTGCAATGCTTT
GGACTTTGGCGGACTCGTAGACGCCAGCGGANGTNCTGC----------GCNNACAGCAGAANNNCGNGNNNNGCTTT
GGACTTTGGCGGACTCGTAGACGCCA--------------------GCGCCGACAGCAGAAGTTCGTGCAATGCTTT
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCAGACAGCCCNG-------------GACAGCGTTCGNGCAATGCTTN
GGACTTTGGCGGACTCGTAGACGCCAGCGG--------------------CNGANNGCAGAAGTTCGTGCNNNGCTTN
------------------------------------------------------------TTCGTGCAATGCTTT
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCAGAC-----------------------------------------
GGACTTTGGCGGACTCGTAGACGCCAGCGGTCAGACAGCCCT--------GCCGACAGCAGAAGTTCGTGCAATGCTTN
GGACTTT-----------------------------------------------------------------------
```

Fig. S6. Successful CRISPR/Cas9 mediated disruption of Gadd45γ in rat NSCs.

Benchling's CRISPR/Cas9 genome editing design suite was used to design multiple gRNAs against exon 2 of the rat Gadd45γ genomic sequence. A) Two gRNAs demonstrated successful disruption (Surveyor Nuclease Assay). B) Quantitative PCR demonstrates that Gadd45γ knockdown prevents Gadd45γ transcript accumulation under differentiation conditions relative to wild-type cells. C) Sequencing of edited cells confirmed a wide range of disruptive deletions. Sequences were obtained by PCR amplification of the edited genomic DNA, insertion of the PCR amplicon into a bacterial vector, bacterial transformation, and Sanger sequencing of extracted plasmid from individual bacterial clones.

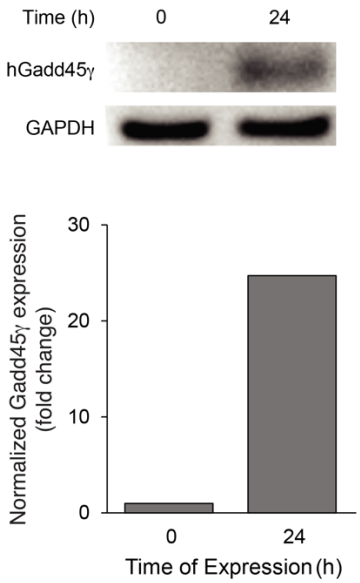


Fig. S7. Validation of Gadd45γ overexpression.

Rat NSCs were transduced with retrovirus encoding human Gadd45γ. hGadd45γ expression was assessed via western blot of NSC lysate prior to and 24 hours post viral transduction.

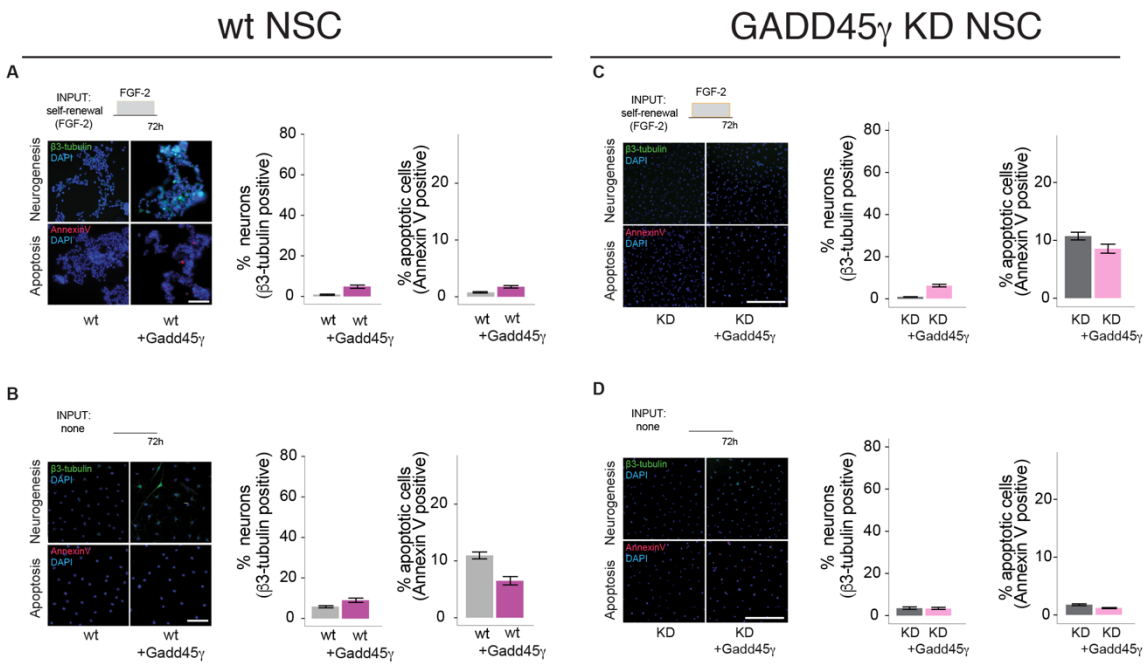


Fig. S8. NSCs exhibit low basal levels of neurogenesis and apoptosis in the absence of input or under self-renewal conditions.

Neurogenesis and apoptosis were measured in wt NSCs, in NSCs with Gadd45γ knockdown (KD), and in wt or KD cells that overexpress Gadd45γ. A) Wild-type NSCs showed low levels of neurogenesis and apoptosis under self-renewal conditions, both in the presence and absence of exogenous Gadd45γ. B) Both cell lines showed enhanced apoptosis in the absence of FGF2 input. C) NSCs with Gadd45γ KD, and KD NSCs that overexpress exogenous Gadd45γ (KD + Gadd45γ), showed similarly low levels of neurogenesis compared to wt NSCs but showed enhanced levels of apoptosis under self-renewal conditions. D) Conversely, KD and KD + Gadd45γ NSCs showed minimal levels of both neurogenesis and apoptosis in the absence of FGF2 input. Data represent means +/- 1 s.e. of biological triplicates. Scale bars = 500 μm.

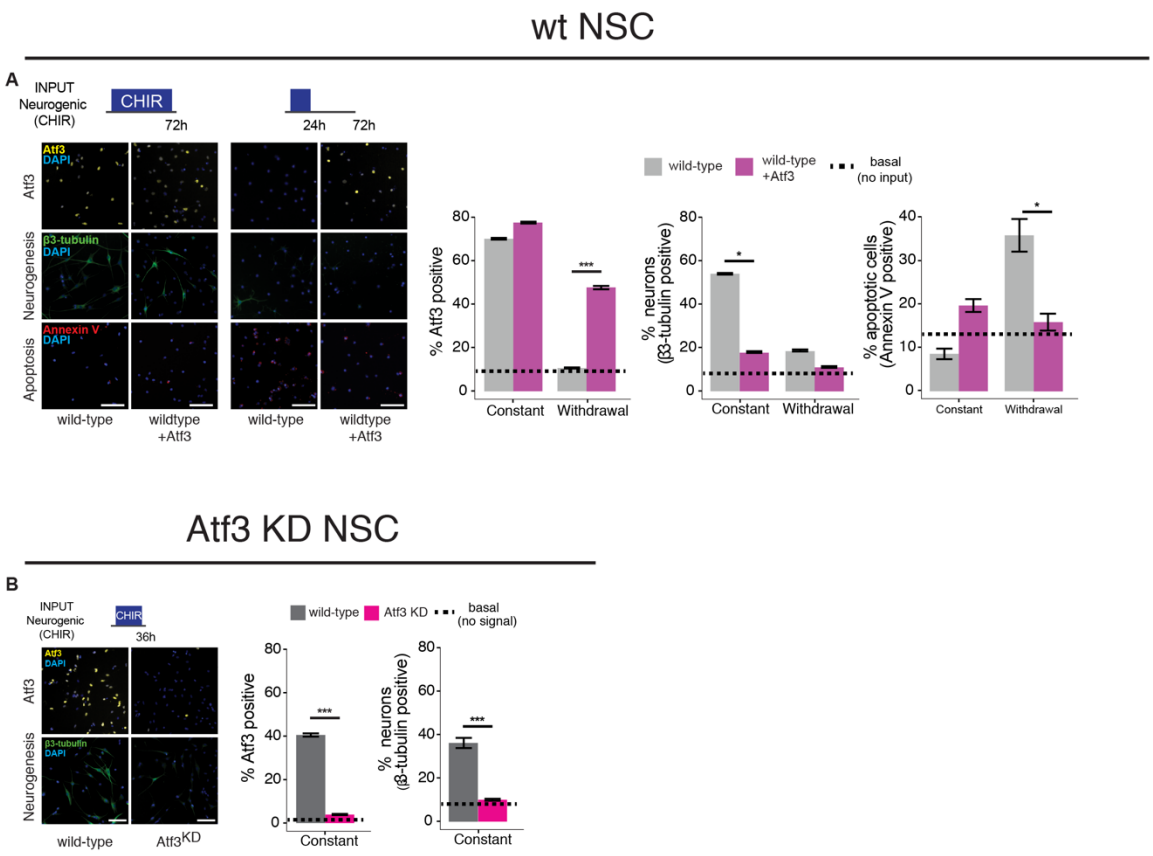


Fig. S9. Atf3 upregulation and downregulation both inhibit NSC neurogenesis.

Wild-type and ATF KD NSCs were stably transduced with retrovirus encoding exogenous Atf3 (+ Atf3). A) Atf3 expression, neurogenesis, and apoptosis were assessed in cells after 72 hours of "constant" or "withdrawal" CHIR treatment (3 μM). Atf3 overexpression simultaneously inhibits neurogenesis under neurogenic "constant" treatment, but also inhibits apoptosis under "withdrawal" conditions. B) Atf3 knockdown (KD) efficiency and neurogenesis were assessed in NSCs treated with constant 3 μM CHIR for 36 hr. Similar to Atf3 overexpression, Atf3 KD inhibited neurogenesis. Scale bars = 500 μm.***p <0.001, *p<0.05, ANOVA

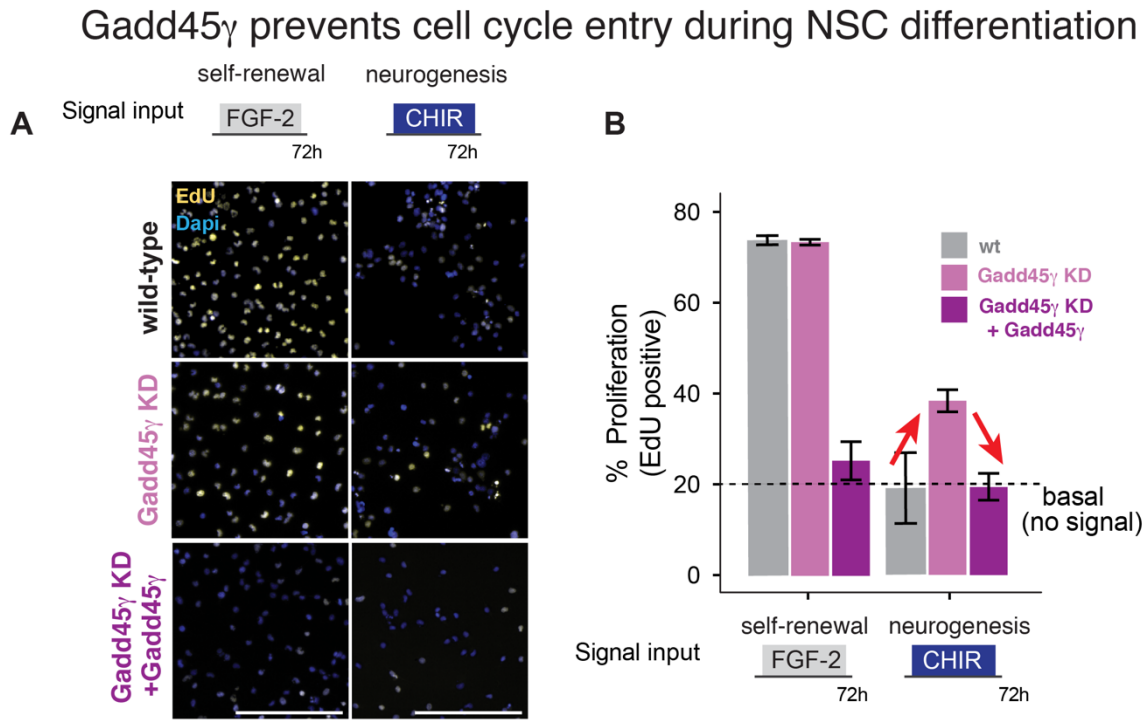


Fig. S10. Gadd45γ prevents cell cycle entry during NSC differentiation.

A) The role of Gadd45γ in NSC proliferation was assessed, as in Figure 5A,B,C. NSCs, KD NSCs, and KD + Gadd45γ NSCs were treated with self-renewal or neurogenic conditions, and Edu incorporation was assessed after 72 hours. Scale bars = 500 μm. B) Quantitation of images represented in A. Wild-type and KD NSCs showed strong proliferation under self-renewal conditions, but constitutive Gadd45γ overexpression dramatically blocked cell cycle entry, supporting the role of Gadd45γ as a cell cycle checkpoint protein. Neurogenic media reduced proliferation in wt NSCs, but loss of Gadd45γ increased proliferation, again supporting the role of Gadd45γ as a negative regulator of cell cycle progression. Data represent means +/- 1 s.e. of biological triplicates.

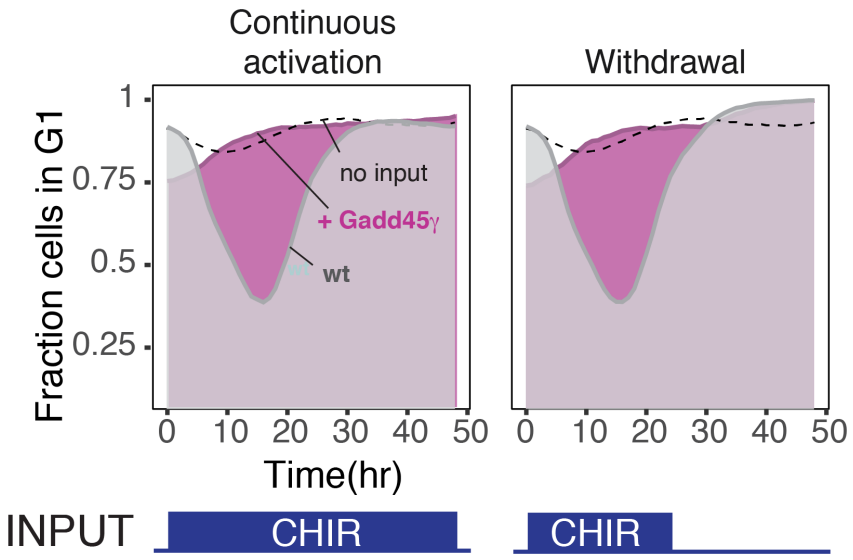


Fig. S11. Cell cycle dynamics are similar in the first 24 hr of continuous stimulation or withdrawal of CHIR treatment.

Fucci reporters were used to examine NSC cell-cycle dynamics, as in Figure 5E. In response to constant neurogenic stimulation (left), synchronized NSCs traverse through one cell cycle, as evidenced by the transient loss of cells on the G1 phase. Cells that constitutively overexpress Gadd45γ and cells that receive no input largely do not enter the cell cycle and remain in G1. Because the cell cycle transit lasts ~24 hours, the response to "sustained" and "withdrawal" conditions looks similar. The "continuous activation" condition is reproduced from Figure 5E, where it is labeled as the "differentiation" condition.

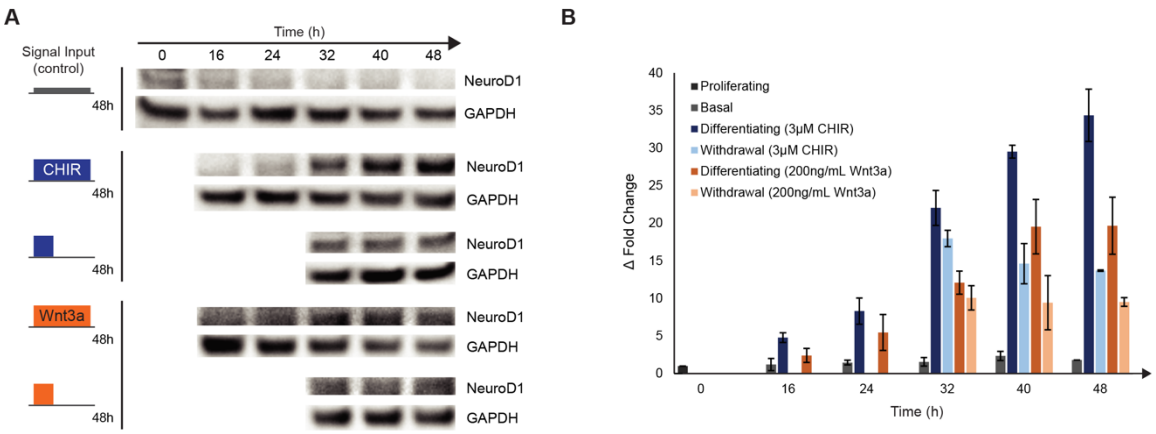


Fig. S12. Dynamic expression of NeuroD1 as a function of CHIR and Wnt stimulation pattern.

A) Western blot analysis of NeuroD1 expression over time in response to CHIR, Wnt3a, or control inputs, as indicated on the left. B) Quantification of Western blot analysis in (A). Values indicate fold change of normalized NeuroD1 levels relative to the zero timepoint sample. CHIR and control values were used to generate the plot in Figure 5F.

SI References

1. Szklarczyk, D. et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res* **43**, D447-452 (2015).