SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Drosophila stocks:

Notch loss of function clones:

w; FRT82B Dl^{rev10} e Ser^{RX106}/ TM6B;

w; FRTG13 mam^{8}/ CyO;

Double mutant mosaic analysis of *grh* and *wor*:

FRTG13 grh^{B37}; UAS-whiteRi (BL35573)

FRTG13 grh^{B37}; UAS-worRi (V6248)

FRTG13; UAS-worRi (V6248)

FRTG13; UAS-whiteRi (BL35573)

Mosaic analysis coupled with hyperactivation of Notch

UASNΔecd; FRT82B armlacZ

UASNΔecd; FRT82B svp^{e22}

UASNΔecd; FRT82B hth^{C1}

UASNΔecd: FRT82B hth^{B2}

UASNΔecd; FRT82B $antp^{25}$

UASNΔecd; FRT82B cas^{24}

UASNΔecd; FRT82B $P^{[gro+]}$ Df(3R)E(spl)$^{b32.2}$

FRTG13; UASNicd

FRTG13 dpn^{7};UASNicd

FRTG13 grh^{B37}; UASNicd

FRT42D $lola^{5D2}$; UASNicd

Reporters:

Release 5 coordinates of the cloned NRE fragments were:

grh: chr2R: 13.702.359 –13,703,723;

cas-prox: chr3R: 1,545,445-1,553,980

cas-med: chr3R: 1.553.134 – 1.553.980

cas-dist: chr3R: 1.554.461 - 1.555.027

svp: chr3R: 8.116.871 – 8.118.854

Primary Antibodies

Primary antibodies were goat-anti-Hth (Santa Cruz, Sc26187) guinea pig-anti-Dpn (1:5.000; courtesy of J.Skeath, Washington University School of Medicine, St.Louis, U.S); mouse anti-Antp (1:100, DHSB); mouse anti-Cut (1:100, DHSB); mouse-anti-CycE [1:100, (Richardson et al., 1995)]; mouse-anti-GFP (1:100, Molecular Probes); mouse anti-Mira [1:100, (Ohshiro et al., 2000)]; mouse anti-Pros MR1A (1:50, DHSB); mouse anti-Svp [1:100, (Kanai et al., 2005)]; mouse anti-Wor [1:100, (Cai et al., 2001)]; rabbit anti-Ase [1:100, (Brand et al., 1993)]; rabbit anti-Ase (1:5000, courtesy of Y.N.Jan); rabbit anti-β-gal (1:10000, Cappel); rabbit anti-Castor [1.3000(Kambadur et al., 1998)]; rabbit-anti-GFP (1:100000, Minotech); rabbit anti-Grh (1:2000,gift of Christos Samakovlis); rabbit anti-Hth [1:1000, (Noro et al., 2006)]; rabbit anti-Lola [1:100, (Giniger et al., 1994)]; rabbit anti-Numb [1:20, (Schober et al., 1999)]; rat anti-Dpn [1:1, (Boone and Doe, 2008)], rat-anti-Elav 7E8A10 (1:50, DSHB); rat anti-Grh [1:1000, (Baumgardt et al., 2009)].

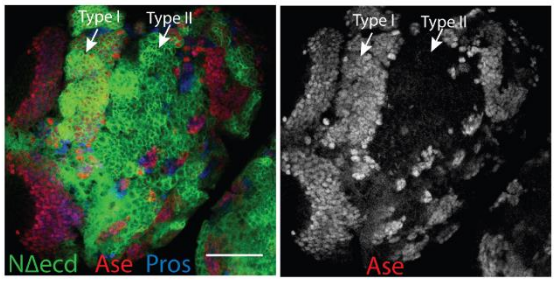
Expression arrays

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) from Drosophila larval CNSs of control and NΔecd overexpressing animals. 300 CNSs were used per biological replica and 4 replicas made for each genotype. Synthesis of double-stranded cDNA and biotin-labelled cRNA was performed according to manufacturer's instructions (Affymetrix). Fragmented cRNA preparations were hybridized to *Drosophila* genome oligonucleotide arrays (GeneChip Drosophila Genome 2.0 Array (3' IVT Expression_Affymetrix)] using an Affymetrix hybridization Oven 640, washed, and then scanned on a GeneChip Scanner 3000. Initial data extraction and normalization within each array were performed with GCOS software (Affymetrix). The quantile normalised log2 intensities per probe set were processed with Limma (limma_3.26.2) as per default settings for single channel analysis of two groups. In the first step a linear model "lmFit" was fitted to the data based on the two samples types (Notch and Control). Then using the "eBayes" implementation, the moderated t-statistics was computed. The p-values were then adjusted for multiple testing using Benjamini & Hochberg ("fdr") with "topTable".

Testing for gene enrichment (Table S3)

All genes on the microarray were binned according to their logFC expression (without p-value cutoff) and the proportion within each bin calculated (All genes: bin/total). Using the set of genes that have a Su(H) peak within 20kb of gene boundary, the number within each bin was ascertained and the proportion of the gene set in each bin calculated (Su(H) genes: bin/total). For a random subset of genes, the proportion falling into each of the bins would be the same as for the total (enrichment =1). Enrichment values >1 indicate that there is a higher proportion of Su(H) genes than expected amongst the genes within the indicated range of logFC. To examine the statistical significance of the enrichments, 10,000 random ChIP datasets (of same size) were generated with a similar distribution of TSS distance to those in the Su(H) dataset and the proportion within each bin calculated for each dataset. These were used as a null distribution to calculate empirical P-values for individual bins (by counting how many random ChIP datasets have higher or equal enrichment in each bin).

A



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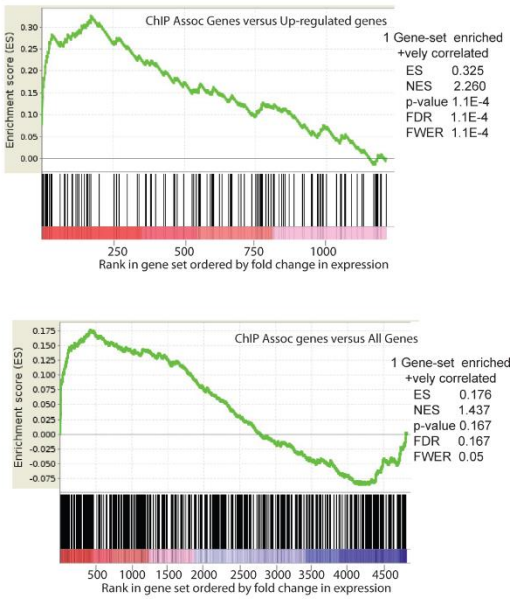

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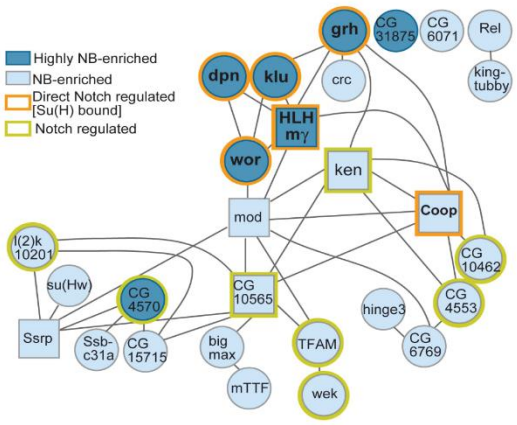


D

Rank	Target	PWM	Motif ID	Raw score	P–value
1	ttk		Dmelanogaster–FlyFactorSurvey–ttk.PA_SANGER_5_FBgn0003870	4.32	2.05e–71
2	Su(H)		Su(H)_Bray	4.25	4.21e–43
3	lola		Dmelanogaster–FlyFactorSurvey–lola.PY_SANGER_2.5_FBgn0005630	1.41	3.46e–37
4	Kr		Dmelanogaster–FlyFactorSurvey–Kr_SANGER_5_FBgn0001325	1.22	1.52e–27
5	pad		Dmelanogaster–FlyFactorSurvey–pad_SANGER_5_FBgn0038418	2.43	7.11e–26
6	Kr		Dmelanogaster–FlyFactorSurvey–Kr_FlyReg_FBgn0001325	1.3	3.13e–22
7	lola		Dmelanogaster–FlyFactorSurvey–lola.PD_SANGER_5_FBgn0005630	3.46	3.04e–21
8	Trl		Dmelanogaster–JASPAR_CORE–Trl–MA0205.1	4.31	4.11e–19
9	vis		Dmelanogaster–FlyFactorSurvey–Vis_Cell_FBgn0033748	0.997	1.68e–17
10	Kr		Dmelanogaster–JASPAR_CORE–Kr–MA0452.1	2.45	2.48e–17
11	ttk		Dmelanogaster–FlyFactorSurvey–ttk.PF_SANGER_5_FBgn0003870	1.64	3.03e–17
12	z		Dmelanogaster–JASPAR_CORE–z–MA0255.1	1.29	9.91e–17
13	vvl		Dmelanogaster–FlyFactorSurvey–vvl_SOLEXA_5_FBgn0086680	1.27	1.5e–14
14	E(spl)mbeta–HLH		melanogaster–FlyFactorSurvey–HLHmbeta_SANGER_10_FBgn0002733	3.11	2.81e–14
15	Met		Dmelanogaster–FlyFactorSurvey–Met_SANGER_5_FBgn0002723	1.53	4.24e–14
16.5	Met		Dmelanogaster–FlyFactorSurvey–Met_Clk_SANGER_5_FBgn0002723	2.26	1.24e–13
16.5	Clk		Dmelanogaster–FlyFactorSurvey–Met_Clk_SANGER_5_FBgn0023076	2.26	1.24e–13
18	vis		Dmelanogaster–JASPAR_CORE–vis–MA0252.1	1.03	1.28e–13
19	pad		Dmelanogaster–FlyFactorSurvey–pad_SOLEXA_5_FBgn0038418	4.35	1.5e–13
20	CG16778		Dmelanogaster–FlyFactorSurvey–CG16778_SANGER_5_FBgn0003715	2.44	2.11e–13
21	hth		Dmelanogaster–FlyFactorSurvey–Hth_SOLEXA_FBgn0001235	1.05	2.43e–13
22	E(spl)m5–HLH		Dmelanogaster–FlyFactorSurvey–HLHm5_FlyReg_FBgn0002631	1.81	3.94e–13
23	Aef1		Dmelanogaster–FlyFactorSurvey–Aef1_FlyReg_FBgn0005694	2.6	8.65e–13
24	btd		Dmelanogaster–JASPAR_CORE–btd–MA0443.1	6.1	1.53e–12
25	btd		Dmelanogaster–FlyFactorSurvey–btd_NAR_FBgn0000233	6.1	1.93e–12
26	achi		Dmelanogaster–JASPAR_CORE–achi–MA0207.1	1.09	4.1e–12
27.5	Clk		Dmelanogaster–FlyFactorSurvey–tai_Clk_SANGER_5_FBgn0023076	3.96	7.16e–12
27.5	tai		Dmelanogaster–FlyFactorSurvey–tai_Clk_SANGER_5_FBgn0041092	3.96	7.16e–12
29	hth		Dmelanogaster–FlyFactorSurvey–Hth_Cell_FBgn0001235	0.931	8.73e–12
30	hth		Dmelanogaster–FlyFactorSurvey–hth_SOLEXA_2_FBgn0001235	1.03	1.13e–11

Fig. S1: Features of hyperplastic NB lineages; Enrichment of gene sets and over-representation of motifs in the Notch regulated genes

(A) Hyperplastic dorsal brain-lobes from NΔecd expression (using *grhNB-Gal4; tubGal80ts)*. Ase+ve staining distinguishes the TypeI NB-like hyperplasia from Type II NB-like hyperplasia (eg. Arrows) (B) Transcriptional network of 28 TFs found to be differentially expressed in NBs (Berger et al., 2012) with hubs represented by squares and the extent of differential NB expression indicated by shading [strong, dark blue; moderate, pale blue; (Berger et al., 2012)]. A subset of the TFs were identified as direct Notch targets in our experiments (orange outline) and many others were up-regulated following NΔecd expression (green outlines). (C) Top scoring enriched TF binding motifs in Su(H) bound regions from ChIP (Robert Stojnic and Diego Diez, 2014).

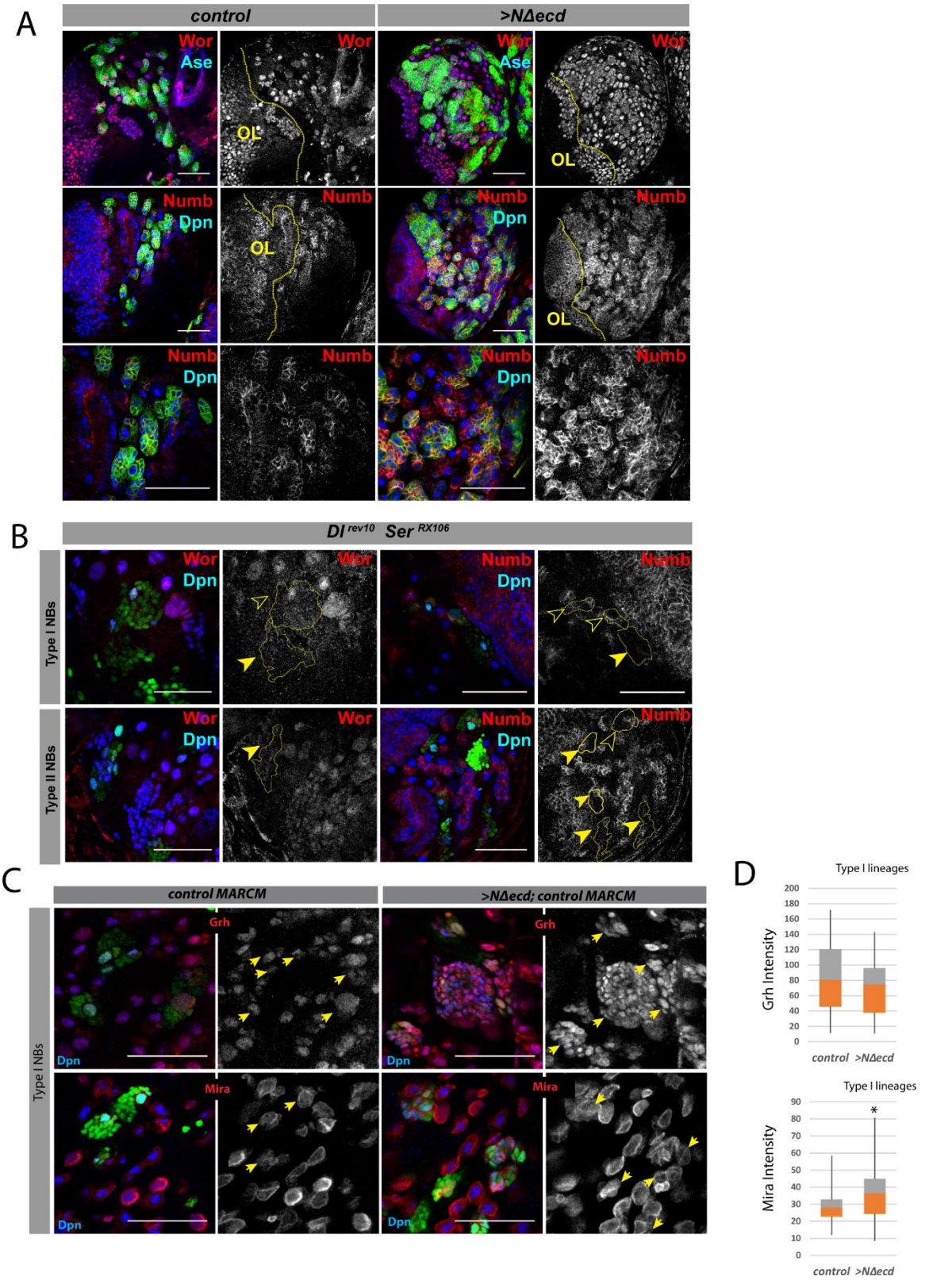


Fig. S2: Responses of NB stem cell network genes to Notch signaling

(A) Wor and Numb are upregulated in the presence of ectopic Notch activity. Expression of Wor (upper panels; red, white) and Numb (lower two rows of panels; red, white) in wild-type (control) and NΔecd expressing NBs, marked by CD8-GFP (Green). Dpn, blue, marks NBs. Yellow dashed lines distinguish central brain from optic lobe (OL).

(B) Wor and Numb expression undergoes subtle changes upon disruption of Notch pathway in Type I lineages (upper) and Type II lineages (lower). Wor (left panels; red, white) or Numb (left panels; red, white) expression was analyzed in GFP marked clones mutant for Dl^{rev10} Ser^{RX106} (green), Dpn (blue) marks NBs. Yellow outlines mark mutant Type I and II lineages, filled yellow arrowheads indicate clones with reduced or no expression whereas open arrowheads indicate clones retaining expression.

(C) Response of Grh and Mira to ectopic Notch activity. Expression of Grh (upper panels; red, white) and Mira (lower panels; red, white) in wild-type (control) and NΔecd expressing MARCM clones (Green) of Type I NB lineages. Dpn (blue) marks NBs. Yellow arrows indicate NBs of the MARCM clones.

(D) Intensity of the expression of Grh or Mira in the NBs from control vs >NΔecd expressing MARCM clones of Type I lineages in the VNC. Box represents the interquartile range (IQR), orange/grey interface indicates median and whiskers indicate ± 1.5xIQR. Asterisk indicates statistically significant difference of expression in >NΔecd from control NBs; *, $p<0.05$; Student's t-Test.

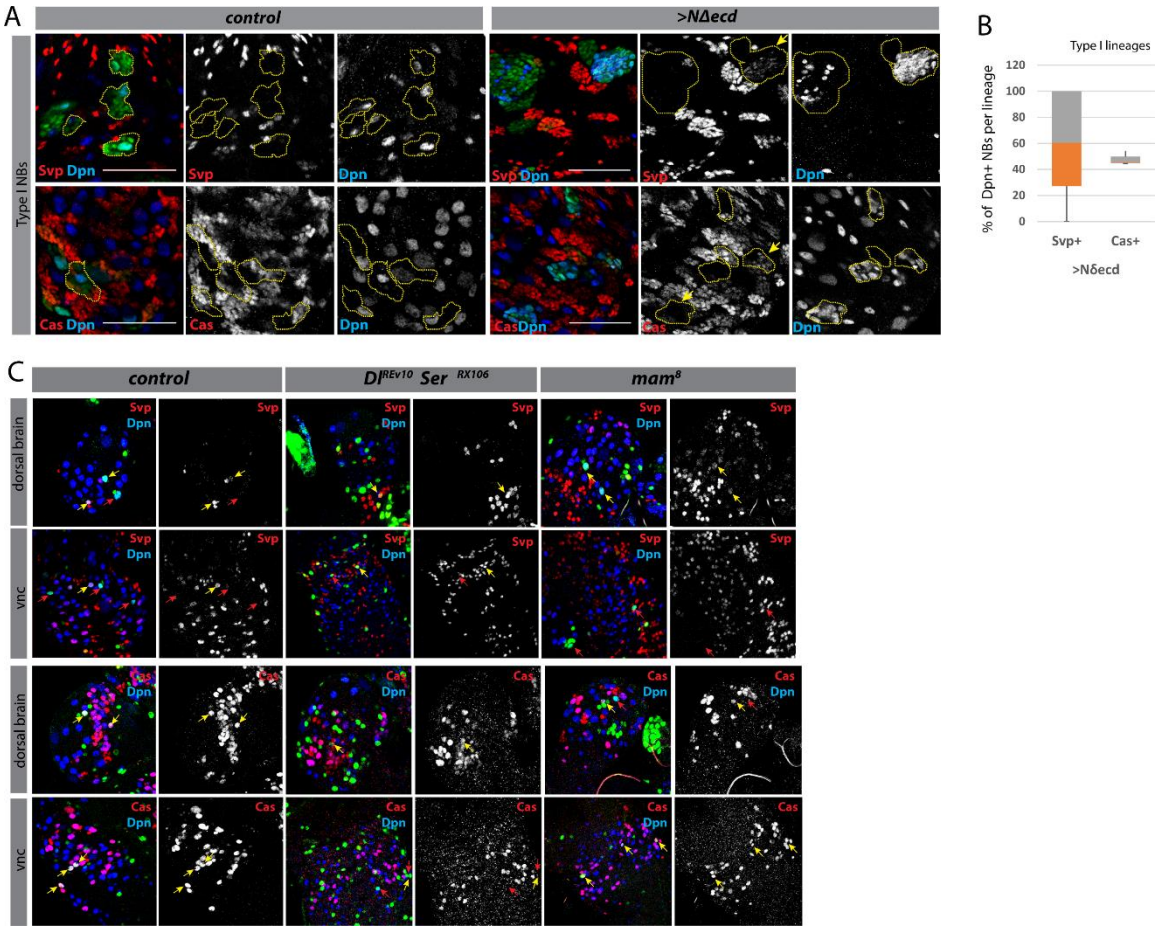


Fig.S3: Svp and Cas are ectopically expressed in NB-like cells upon ectopic Notch activity but are unaffected by reductions in Notch activity at early larval stages

(A) Svp and Cas are ectopically expressed in NB-like cells upon ectopic Notch activity. Expression of Svp (upper panels; red, white 2nd and 5th column) and Cas (lower panels; red, white 2nd and 5th column) in wild-type (control; green; left) and NΔecd expressing MARCM clones (green; right) of Type I NB lineages. Dpn (blue or white 3rd or 6th column) marks NBs. Yellow outlines mark MARCM clone boundaries in white channels. **(B)** Proportion of Dpn+ cells that are also expressing Svp or Cas in >NΔecd expressing MARCM clones of Type I VNC lineages. Control Type I lineages contain 1 Dpn^{+} NB that is always negative for Svp or Cas expression. Box represents the interquartile range (IQR), orange/grey interface indicates median and whiskers indicate ± 1.5xIQR. (C) Disruption of Notch activity (Dl^{rev10} Ser^{RX106} or mam^{8}) does not perturb Svp or Cas expression in Type I lineages in early larval life (30-50h ALH). GFP marked clones (green) with genotypes indicated on top stained for Svp or Cas (red or white) and Dpn (blue) to indicate NBs. Two different anatomical regions are displayed [dorsal central brain, VNC]. Yellow arrows mark early NBs of mosaic clones with expression of Svp or Cas whereas red arrows indicate early NBs with no expression of the genes. This lack of expression cannot be attributed to the disruption of Notch signaling, since many neighbouring wt NBs also lack Svp or Cas expression.

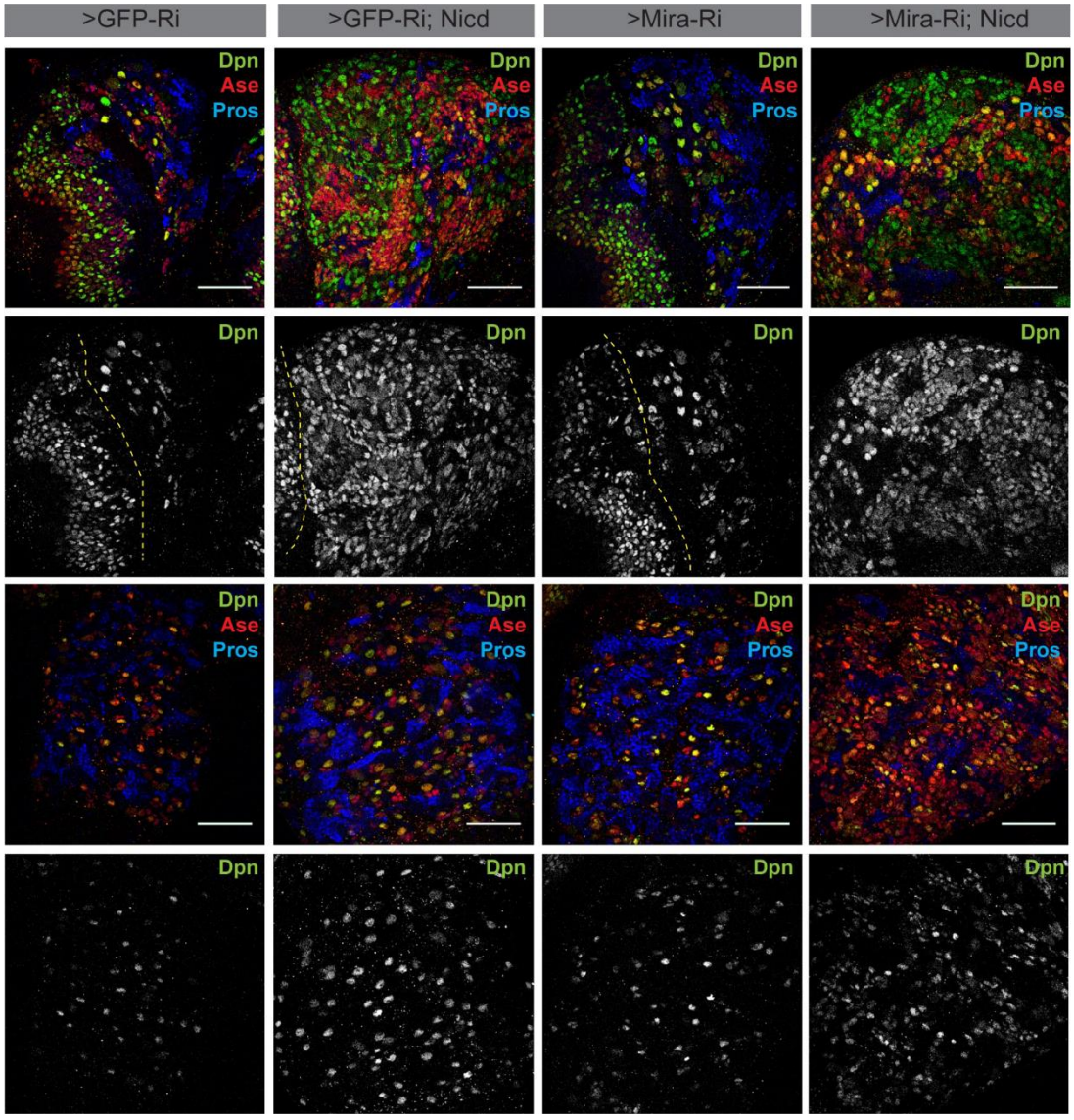


Fig.S4: Reduction in *mira* expands the Notch induced hyperplasias

Depletion of *mira* enhances the Notch induced hyperplasia not only in the dorsal brain but also in the VNC. Distribution of NBs marked by Dpn (green or white) is shown in two anatomical regions, dorsal brain (upper panels) and VNC (bottom panels) from animals where all NB lineages express GFP-RNAi (control), GFP-RNAi coupled with ectopic Notch activity, *mira*-RNAi with ectopic Notch activity or *mira*-RNAi alone. Ase (red) marks intermediate progenitors (GMCs or INPs) and Pros (blue) marks neurons. Yellow dashed lines separate central brain from the optic lobe (OL).

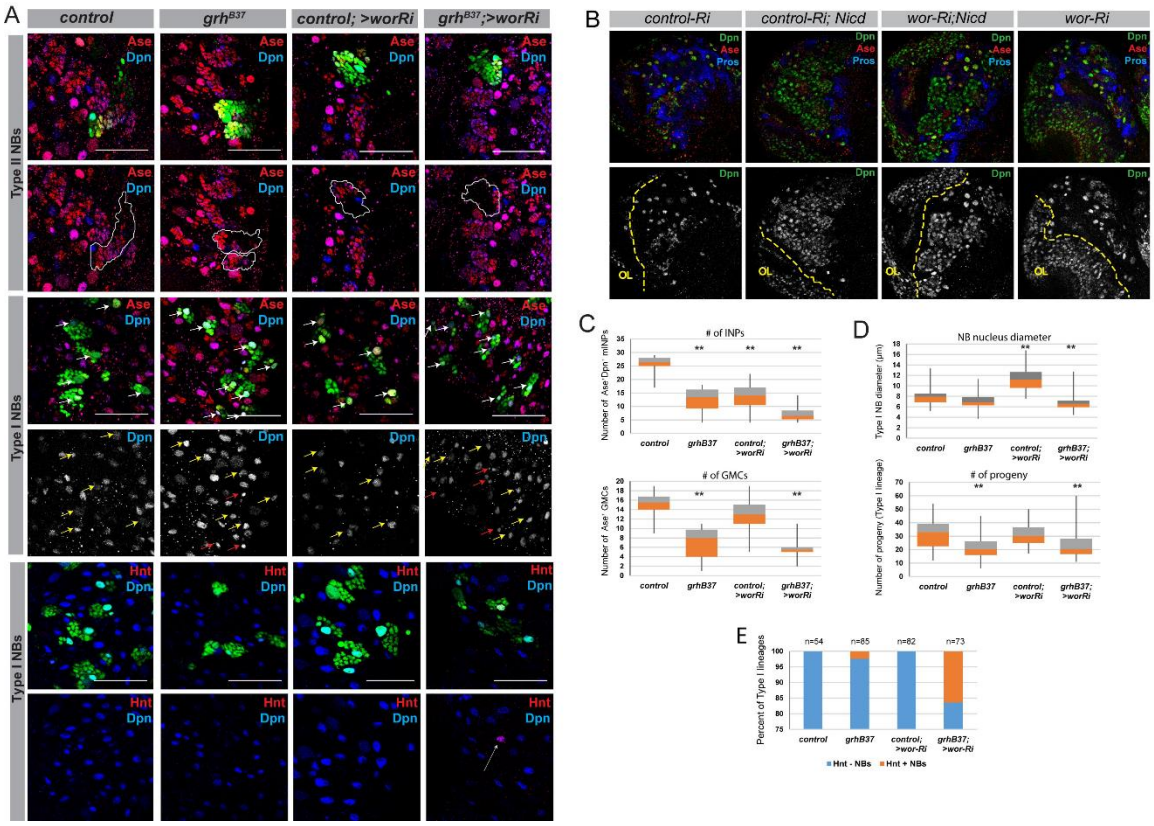
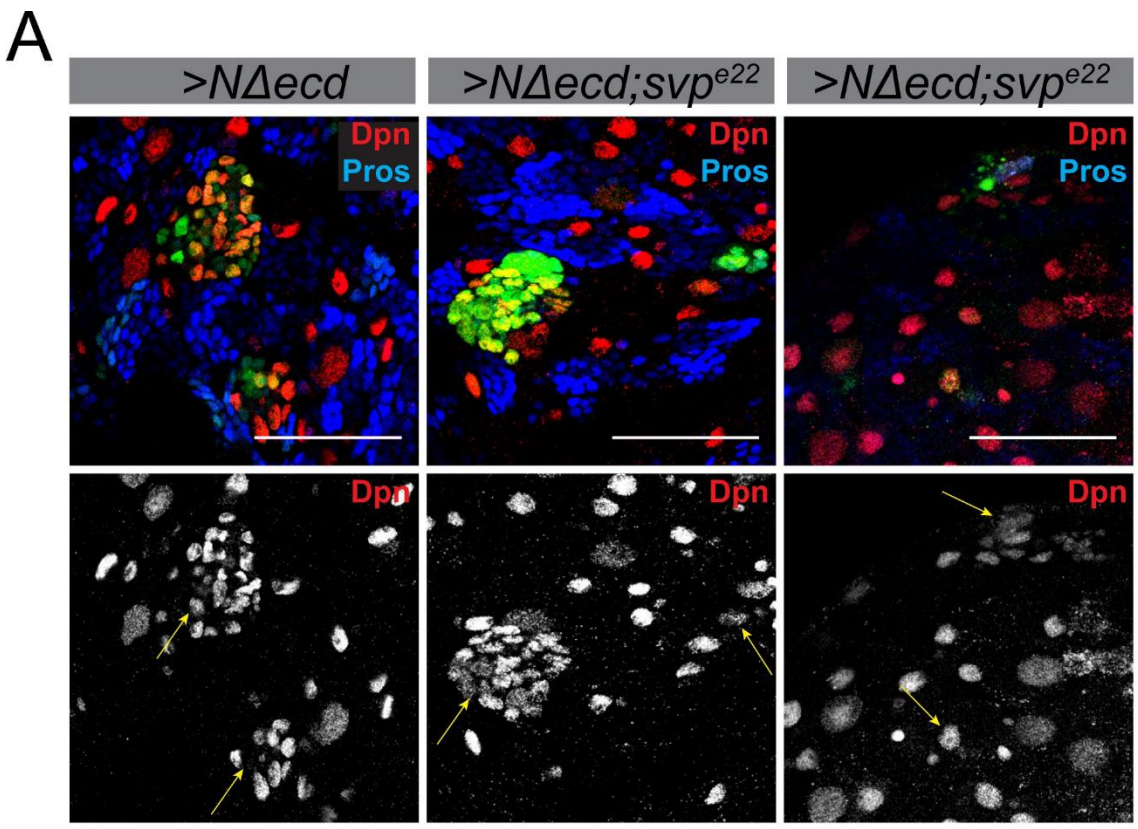
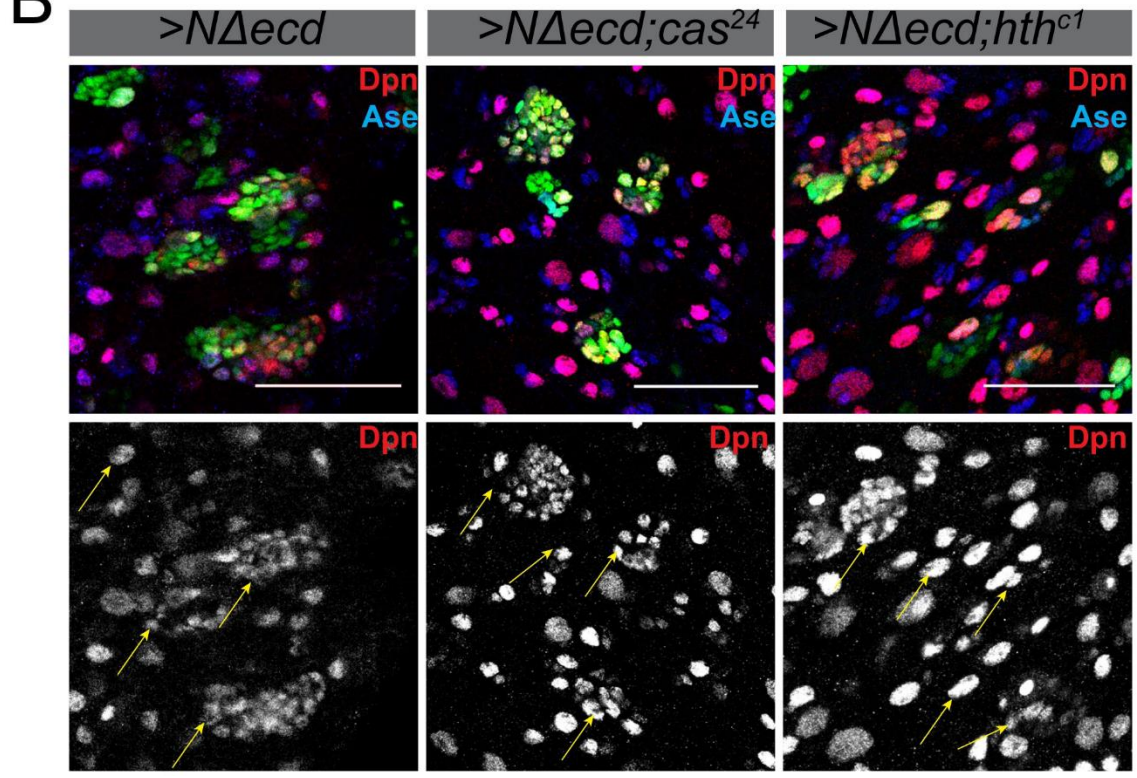


Fig. S5: Effects from perturbation of NB stem cell genes in pathological Notch induced hyperplasia and in normal conditions.

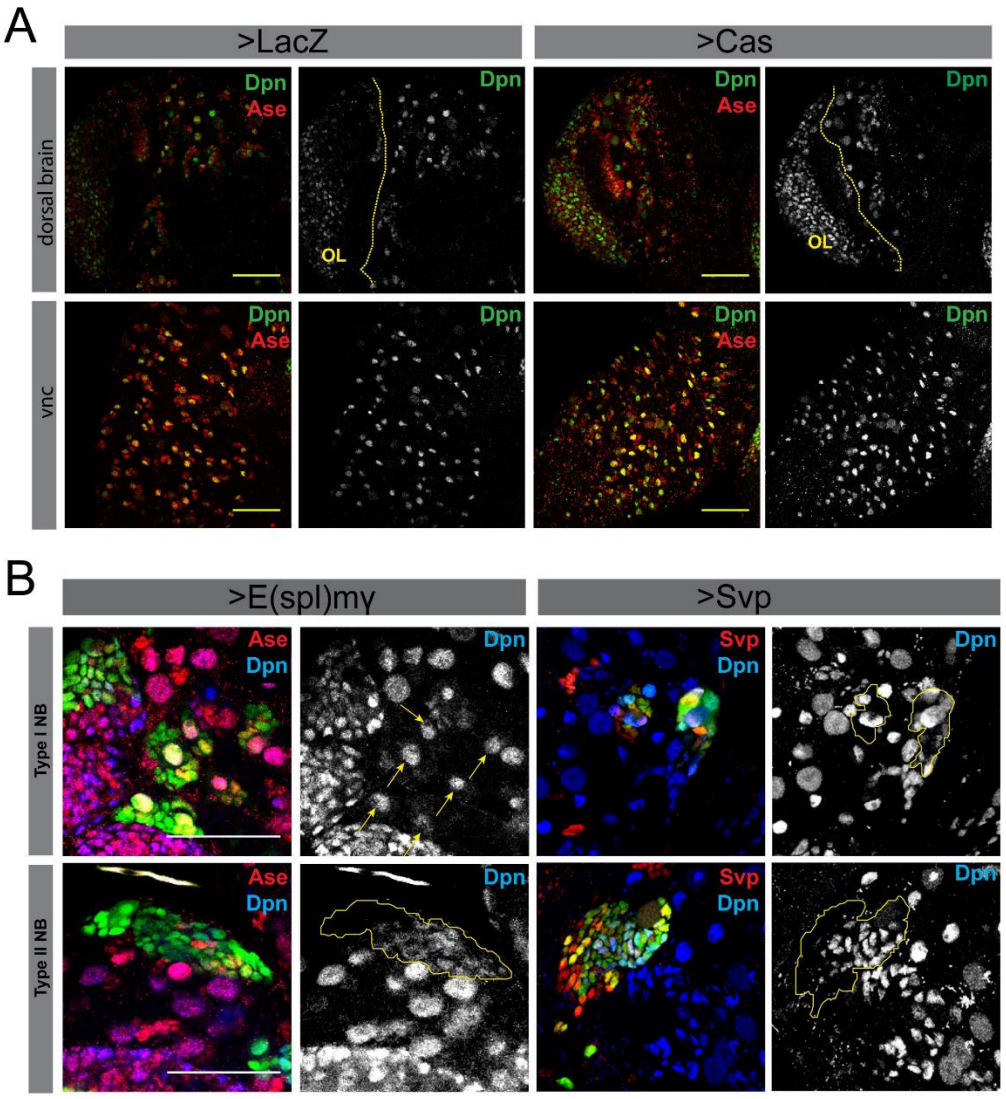
(A) Overlapping *grh* and *wor* functions in stem cell regulation. GFP marked larval CNS clones (green) of the genotypes indicated stained for Ase (red) and Dpn (blue). Top two rows: Type II lineages, fewer mature Dpn^{+}/Ase^{+} INPs and Ase^{+} GMCs are present in *grh*^{B37} *wor* compared to control and to *grh*^{B37} or *wor* alone, white lines outline the marked lineages. Middle two rows: Type I lineages, with Dpn only channels in white (bottom row). Yellow arrows indicate NBs with big nuclei whereas red arrows indicating NBs with smaller nuclei. White arrows point to NBs of GFP marked clones. Note the increase in the number of lineages with small nuclei (red arrows) in *grh*^{B37} *wor* mutant clones. Lower two rows: Type I lineages where Pebble/Hintsight [(Hnt) in red; a gene correlated with differentiation fates in other cell contexts (Terriente-Felix et al., 2013) but normally not expressed in larval NBs] is ectopically activated in a higher proportion of double *grh*^{B37} *wor* mutants compared to single *grh*^{B37} mutants and compared to

none in control and or *wor* mutants. White arrows indicate NBs with Hnt expression. NBs are marked with Dpn (blue). **(B)** Depletion of *wor* is unable to alleviate the excess number of NBs caused by ectopic Notch activity. Distribution of Dpn (green or white) positive NBs in the central brain where NB lineages express control-RNAi, control-RNAi coupled with ectopic Notch activity, *wor-RNAi* with ectopic Notch activity or *wor-RNAi*. Pros (blue) marks neurons and Ase (red) marks GMCs or intermediate progenitors. Yellow dashed lines divide optic lobe regions from central brain **(C)** Box plots scoring different phenotypes of the Type II NB lineages for the overlapping *grh* and *wor* functions: INP (median: WT =26.5, *grh* =13.5, *wor* =14, *grh wor* =6.5) and GMC numbers (median: WT =15.5, *grh* =8, *wor* =13, *grh wor* =5.5). Asterisks indicate significant difference from controls, $p<0.05$ (Analysis of variance (ANOVA), P-values were calculated using the "Tukey's HSD test**) (D)** Box plots scoring different phenotypes in Type I lineages: NB diameter (median WT=7.95µm, *grh*=6.87 µm, *wor* =11.22 µm, *grh wor*=6.59 µm) number of progeny (median WT=33, *grh*=20.5, *wor* =30, *grh wor*=20.5). The box represents the interquartile range (IQR), orange/grey interface indicates the median and whiskers indicate ± 1.5xIQR. Asterisks indicate significant difference from controls, $p<0.05$ (Analysis of variance (ANOVA), P-values were calculated using the "Tukey's HSD test**) (E)** Diagram depicting the percentage of Hnt positive NBs in each condition.





FigS6: Removal of *svp*, but not *cas* or *hth*, can alleviate Type I lineage overproliferation. Overproliferation induced by *NΔecd* is slightly suppressed in Type I lineages lacking *svp* **(A)** but remains unaffected in Type I lineages mutant for *cas* or *hth* **(B)**. MARCM clones (green) overexpressing *NΔecd* in WT, in *svp[e22]*, in *cas[24]* and in *hth[C2]* homozygous mutant backgrounds. Dpn (red, white) marks the neuroblasts, Ase (blue) marks NBs and GMCs, whereas Pros (Blue) marks neurons. Yellow arrows indicate some NBs of MARCM clones.



FigS7: Effects from ectopic activity of Notch target genes in the induction of NB overproliferation (A) Overexpression of Cas is unable to generate any hyperplasia in the larval CNS. LacZ (left) or Cas (right) is driven by insc-Gal4; tubG80ts in the NBs. Dpn (green, white) marks NBs and Ase (red) NBs and GMCs. Yellow dashed lines separate central brain from optic lobe (OL). **(B)** Overexpression of E(spl)mγ-HLH and Svp is sufficient to induce overproliferation in the dorsal brain of the larval CNS. Mosaic MARCM clones (green) of Type I (upper) or Type II (bottom) lineages where UAS transgenes are ectopically expressed. Ase (red) marks NBs and GMCs, Dpn (blue or white) marks NBs and Svp indicates cells with ectopic Svp activity (red). Yellow arrows point to NBs in MARCM clones and yellow outlines mark both Type I and Type II clones.

SUPPLEMENTARY TABLES

Table S1: Expression array results (xlsx file).

Click here to Download Table S1

Table S2: Putative Direct Notch targets in NB Hyperplasia (xls file)

Click here to Download Table S2

Table S3: Relationship of Su(H) bound genes with Expression data

	Log2 Fold change											
	> 3	3 to 2	2 to 1.5	1.5 to 1	1 to 0.5	0.5 to 0	0 to -0.5	-0.5 to -1	-1 to -1.5	-1.5 to -2	-2 to -3	< -3
#All genes/bin	29	93	182	521	1647	4118	4823	1915	510	134	47	15
#Su(H) genes/bin	7	20	34	101	261	617	720	245	79	21	8	2
All genes: bin/total	0.0021	0.0066	0.0130	0.0371	0.1174	0.2934	0.3437	0.1365	0.0363	0.0095	0.0033	0.0011
Su(H) genes: bin/total	0.0033	0.0095	0.0161	0.0478	0.1234	0.2917	0.3404	0.1158	0.0374	0.0099	0.0038	0.0009
Enrichment	**1.60**	**1.43**	**1.24**	**1.29**	**1.05**	0.99	0.99	0.85	1.03	1.04	1.13	0.88
p-value	**0.0158**	**0.0095**	**0.0133**	**0.0000**	**0.0562**	0.8870	0.7216	1.0000	0.1019	0.2671	0.2498	0.7612

Table S4: Altered expression of putative Notch targets in *Dl Ser* mutant lineages

	Type I lineages*				Type II lineages**			
Gene	***control***		***Dl^{Rev10} Ser^{RX106}***		***control***		***Dl^{Rev10} Ser^{RX106}***	
	N	**% high**	**N**	**% high**	**N**	**% high**	**N**	**% high**
grh	118	**100.0**	131	**100.0**	5	**100.0**	7	**14.3**
mira	102	**100.0**	99	**83.8**	4	**100.0**	8	**0.0**
wor	61	**100.0**	80	**98.8**	2	**100.0**	5	**40.0**
numb	103	**100.0**	21	**80.9**	6	**100.0**	7	**14.3**
svp	73	**12.3**	117	**15.4**	10	**30.0**	2	**0.0**
cas	114	**100.0**	63	**100.0**	4	**100.0**	3	**100.0**
hth	114	**59.6**	124	**54.8**	10	**80.0**	6	**0.0**
lola	96	**100.0**	56	**100.0**	NT	**NT**	NT	**NT**

*Type I lineages: lineages were not scored as “high” if levels in NBs or GMCs or neurons were reduced compared to neighbouring WT NBs with high levels of expression

**Type II lineages: lineages were not scored as “high” if the expression levels were reduced in NBs/INPS or if there was a significant fractions of INPs that were Dpn+ but negative for the gene of interest.

Table S5: Summary of Genetic Experiments

Genotype	Type I NB lineages		Type II NB lineages	
	Extent of hyperplasia	Proportion of hyperplastic NBs	Extent of hyperplasia	Proportion of hyperplastic NBs
control	None	-	None	None
>Nicd	**Mild**	**+**	**High**	**+++**
grh[B37]; >Nicd	Mild	+	High	++
dpn[7]; >Nicd	Mild	+	High	++
lola[5D2]; >Nicd	Mild	+	Mild	+++
mira-Ri; >Nicd	High	++	High	+++
wor-Ri; >Nicd	Mild	+	High	+++
>NΔecd	**Mild**	**++**	**High**	**+++**
>NΔecd; cas[24]	Mild	++	High	+++
>NΔecd; hth[B2]	Mild	++	High	+
>NΔecd; hth[C1]	Mild	++	High	+
>NΔecd; svp[e22]	Mild	+	None	None
>NΔecd; E(spl)[b32.2]	None	-	None	None

High: >80 Dpn+ cells (for both Type I and Type II lineages) Mild: 2-80 Dpn+ cells (Type I lineages); 28-80 Dpn+cells (Type II lineages)

+++: > 70% of lineages exhibit the indicated phenotype

++: 35%-70% of lineages exhibit the indicated phenotype

+: < 35% of lineages exhibit the indicated phenotype

Red Font indicates phenotypes were significantly different from controls ($p<0.01$; Wilcoxian rank-sum test)

SUPPLEMENTARY REFERENCES

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