Genes implicated in stem cell identity and temporal programme are directly targeted by Notch in neuroblast tumours

Evanthia Zacharioudaki1,2,3, Benjamin E. Housden1,*, George Garinis2,3, Robert Stojnic1,4, Christos Delidakis2,3,† and Sarah J. Bray1,‡

ABSTRACT
Notch signalling is involved in a multitude of developmental decisions and its aberrant activation is linked to many diseases, including cancers. One example is the neural stem cell tumours that arise from constitutive Notch activity in Drosophila neuroblasts. To investigate how hyperactivation of Notch in larval neuroblasts leads to tumours, we combined results from profiling the upregulated mRNAs and mapping the regions bound by the core Notch pathway transcription factor Su(H). This identified 246 putative direct Notch targets. These genes were highly enriched for transcription factors and overlapped significantly with a previously identified regulatory programme dependent on the proneural transcription factor Asense. Included were genes associated with the neuroblast maintenance and self-renewal programme that we validated as Notch regulated in vivo. Another group were the so-called temporal transcription factors, which have been implicated in neuroblast maturation. Normally expressed in specific time windows, several temporal transcription factors were ectopically expressed in the stem cell tumours, suggesting that Notch had reprogrammed their normal temporal regulation. Indeed, the Notch-induced hyperplasia was reduced by mutations affecting two of the temporal factors, which, conversely, were sufficient to induce mild hyperplasia on their own. Altogether, the results suggest that Notch induces neuroblast tumours by directly promoting the expression of genes that contribute to stem cell identity and by reprogramming the expression of factors that could regulate maturity.

KEY WORDS: Drosophila, Neuroblast, Notch, Gene regulation, Stem cell

INTRODUCTION
The Notch pathway is a cell communication mechanism that is involved in many developmental decisions and in stem cell homeostasis of adult tissues. Furthermore, abnormal Notch activity is linked to various diseases, including several forms of cancer. Indeed, in some cancers Notch is thought to have a role in the initiation and maintenance of cancer stem cells (CSCs) (Capaccione and Pine, 2013; Ntziahristos et al., 2014). One context in which high Notch activity causes stem cell hyperplasia, with similarities to CSCs, is in Drosophila neural stem cells, the so-called neuroblasts (NBs). Notch is normally active in NBs but is rapidly inactivated in their progeny. Sustained activity of the pathway in the NB lineages results in brain tumours, where the overproliferation of NBs at the expense of neurons gives rise to large NB masses in the brain that compromise the survival of the animals to adulthood (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010). It is therefore important to understand how sustained Notch activity alters the balance between self-renewal and differentiation to result in tissue tumorigenesis.

In normal circumstances, the larval NBs undergo repeated rounds of asymmetric division to generate neurons appropriate for the adult CNS. At each division the larger cell maintains NB properties and regrows to sustain many rounds of division (Knoblich, 2008; Sousa-Nunes and Somers, 2013). The majority are Type I NBs, identified by expression of the transcription factors (TFs) Deadpan (Dpn) and Asense (Ase), whose small daughter cell, the ganglion mother cell (GMC), divides terminally to produce two neurons and/or glia. A small number of NBs, the so-called Type II NBs (eight per brain lobe), express Dpn but not Ase and follow a more complex pattern of division. When these divide asymmetrically, their smaller daughter is an immature intermediate neural progenitor (INP), which reaches maturation within a few hours and then itself divides asymmetrically a few times. In this case, the daughter is a GMC similar to that of Type I NBs. The existence of INPs enables Type II NBs to generate a large number of progeny in a short period of time (Bayraktar and Doe, 2013; Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izerigina et al., 2009; Kang and Reichert, 2014; Knoblich, 2008). At the end of larval life, both Type I and Type II NBs exit the cell cycle and cease proliferation, under the influence of temporal factors (Chai et al., 2013; Maurange et al., 2008), the steroid hormone ecdysone (Homem et al., 2014) and other cues (Chai et al., 2013).

Notch pathway activity is detected in NBs and contributes to their maintenance. During mitosis, one of the key determinants that is segregated asymmetrically into the GMC daughter is Numb, a potent inhibitor of Notch signalling (Babaoglan et al., 2009; Connor-giles et al., 2003; Guo et al., 1996; Le Borgne et al., 2005; Rhyu et al., 1994; Spana and Doe, 1996; Wang et al., 2006). Perturbations in Numb function lead to uncontrolled proliferation of NBs and the formation of brain tumours. This is largely caused by the ectopic Notch activity that ensues, a condition that is mimicked by expression of a constitutively active Notch fragment (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010). Upon interaction with its ligands [Delta (Dll) or Serrate (Ser)], the Notch receptor undergoes two proteolytic cleavages to release the Notch intracellular domain (Niced), which translocates into the nucleus where it interacts with the CSL (also known as RBPJ) DNA-binding protein Suppressors of Hairless (Su(H)) in Drosophila and activates the transcription of target genes (Bray, 2006; Kopan and...
Ilagan, 2009). Expression of Niced or of a transmembrane fragment mimicking the first ligand-activated cleavage (NAeCd), results in similar brain tumours to those caused by loss of Numb.

One significant target of Notch activity in NBs is Enhancer of split my [E(spl)m{y}-HLH], a HES family gene that is dependent on Notch for expression (Almeida and Bray, 2005). However, mutations removing the entire E(spl) complex [E(spl)-C] of Notch-responsive genes have only minor effects on NB maintenance, suggesting that additional targets exist. Indeed, E(spl)m{y}-HLH appears to function semi-redundantly with dppn, another HES family gene, which has both Notch-dependent and Notch-independent modes of regulation in NBs (San-Juan and Baonza, 2011; Zacharioudaki et al., 2012; Zhu et al., 2012). In addition, expression of the zinc-finger protein Klumpfuss (Klu) may also be Notch regulated in this context (Berger et al., 2012; Xiao et al., 2012). Overexpression of E(spl)m{y}-HLH, dppn or klu can cause NB hyperplasia (Berger et al., 2012; San-Juan and Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012); however, their effects are generally weaker or more spatially limited than that of Niced or NAeCd. It therefore appears that these Notch targets do not account for the full scope of Notch functions in normal NBs, nor in the hyperactive Notch-induced NB tumours.

To characterise the repertoire of genes activated by Notch in overproliferating NB tumours we compared the transcriptional profiles from the CNS of Notch-induced NB hyperplasia with wild type (WT) and integrated these data with maps of the regions bound by Su(H) in the Notch hyperplasia. The Notch targets identified in this way were highly enriched in genes encoding TFs associated with NB maintenance and the self-renewal programme, as well as TFs that are implicated in the temporal programming of the stem cells. Validating these targets and their functions in vivo suggests that stemness and temporal TFs might cooperate to sustain Notch-induced hyperplasias. Furthermore, the redundancy between the identified targets potentially gives a robustness to the signalling output that could explain why the previously known targets are insufficient to account for the Notch activation phenotype.

RESULTS
Identification of Notch target genes involved in NB hyperplasia
Constitutively active Notch (NAeCd) results in NB overproliferation at the expense of neurons (Bowman et al., 2008; Wang et al., 2006). To identify genes acting downstream of Notch to produce NB hyperplasia, we first characterised the population of RNAs showing elevated expression, since the Niced complex results in transcriptional activation (Fig. 1A). NAeCd was expressed for 24 h in larval NBs (via grhNB-Gal4 Gal80ts) to produce CNS dominated by Dpn-expressing NBs (Fig. 1A), which included many ase Type II NB-like cells as well as ase Type I NB-like cells (Fig. S1A). A comparison of their RNA expression profile with that of control CNS of a similar stage identified 1576 upregulated transcripts in NAeCd hyperplastic CNS (FDR≤0.1) (Table S1). We note that, as this reflects changes in the entire CNS transcriptome, the effects of NAeCd will be attenuated by the non-NB cells and there will be indirect effects from the altered ratios of cell types in the hyperplastic brains.

To distinguish which genes may be directly regulated by Notch activity, genomic regions occupied by Su(H) in hyperplastic brains were identified by ChIP. The 595 bound regions were significantly enriched for sequences matching the consensus Su(H) binding motif YGTGRGAA (P=4.11×10^{-43}), strengthening the likelihood that they represent bona fide binding sites. Other enriched motifs included those for Transtrack (Tik), which is expressed in glial cells (Badenhorst, 2001) and may thus repress the enhancers in those lineages, for bHLH repressor proteins (CRGCGT) and for the homeodomain protein Homothorax (Hth) (TGACA), which is discussed further below (Fig. 1D, Fig. S1C).

Our criteria for direct Notch-regulated genes are that they should be both upregulated in the Notch-induced hyperplasia and associated with Su(H)-occupied regions. This implies that Su(H)-bound genes should be enriched among those that are upregulated by NAeCd, which we confirmed by two approaches. First, gene set enrichment analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) using a pre-ranked gene list from the expression analysis (3717 genes, FDR≤0.1) indicated a significant enrichment of Su(H)-bound genes among those with upregulated mRNAs (normalised enrichment score=1.68, P=0.037; Fig. 1B). Second, a comparison of the Su(H)-bound genes with gene lists generated from 10,000 randomly generated ChIP peak sets demonstrated that the former were preferentially enriched among the more highly upregulated genes (log2 fold change (FC)>0.5; see Materials and Methods and Table S3).

By intersecting the 1576 upregulated genes with those located within 20 kb of Su(H) peaks, we identified a set of 246 candidates for direct Notch targets in the CNS stem cells, of which 185 had log2 FC>0.5 (Fig. 1C, Table S2). These included E(spl)m{y}-HLH and dppn (Fig. 1F), two genes previously associated with Notch regulation in larval NBs. Furthermore, the gene set was significantly enriched in NB-expressed genes (P=5.47×10^{-5}), based on two different experiments in which NB transcription profiles were analysed (Fig. 1C) (Berger et al., 2012; Southall et al., 2013). Conversely, the gene set was not enriched for genes expressed in sensory organ proneural clusters (Reeves and Posakony, 2005), where Notch has an opposing function and is off in the neural precursor.

Functional characteristics of the 246 putative direct Notch targets were assessed using gene ontology (GO) and protein domain annotations (Fig. 1E). Besides general development-related categories (‘post-embryonic organ morphogenesis’, ‘cell fate determination’), enriched categories included ‘regulation of transcription’ and ‘DNA binding’, along with three neurogenesis-related categories (‘neuron differentiation’, ‘sensory organ development’, ‘compound eye development’; Fig. 1E). Several targets in the transcription category have been implicated in NB maintenance [E(spl)m{y}-HLH, dppn, klu, wor, grh, klu (Almeida and Bray, 2005; Berger et al., 2012; Cenci and Gould, 2005; San-Juan and Baonza, 2011; Song and Lu, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012; Zhu et al., 2012)] or in their temporal regulation [cas, syp, hth (Li et al., 2013; Maurange et al., 2008)].

Strikingly, Notch-regulated genes in NB hyperplasia were generally different from those in an epithelial hyperplasia caused by excessive Notch activity in wing imaginal discs (Djiane et al., 2013). First, the overall functional characteristics differed: in NB hyperplasia the target genes were associated with cell fate commitment and transcription (Fig. 1E), whereas in epithelial hyperplasia they were enriched for signalling pathways and proliferation control (Djiane et al., 2013). Second, only nine genes appeared to be directly Notch induced in both conditions (Fig. 1C; P=0.028). Even when genes from a similar Su(H)-induced wing disc hyperplasia were added, the overlap only increased to 18 genes, although this is a significant enrichment demonstrating that the responses are related (P=1.091×10^{-46}). Besides E(spl)m{y}-HLH, the overlap included dppn (also HES related) and Myc. The latter is widely regulated by Notch in many contexts both in flies and mammals (Djiane et al., 2013; Klinakis...
et al., 2006; Krejci et al., 2009; Weng et al., 2006). Finally, even for those genes that were regulated in both tissues, the Su(H) binding profiles differed (e.g. Fig. 1F). For example, in CNS, Su(H) was detected at a region 5′ of the dpn gene body, which overlapped with a previously identified NB enhancer (San-Juán and Baonza, 2011), whereas in wing-discs it was predominantly bound at an intronic enhancer (Babaoglan et al., 2013). These results demonstrate that Notch responses can differ extensively, even in circumstances when the eventual outcomes for the tissue are similar (i.e. tissue overgrowth).

**Notch regulates genes linked to stem cell identity**

Two previous studies have investigated gene networks involved in regulating NBs. One evaluated the enrichment of RNAs in NBs compared with neurons in the larval CNS and then proposed a regulatory network of TFs (Berger et al., 2012). This TF network is significantly represented amongst our CNS direct Notch targets \((P=7.278\times10^{-17}; \text{Fig. S1B})\). Indeed, our results confirm Notch regulation of a core network of these genes. The second used the binding profile of the proneural protein Ase to identify genes involved in NB programming in the embryo (Southall and Brand, 2009). Again, the Notch target genes were highly enriched in these Ase-bound genes: 69/246 were identified targets of Ase \((P=4.75\times10^{-16}; \text{Fig. 2A})\). This subset of Notch targets was also enriched in genes bound by two other TFs involved in regulating NB lineages, namely Dpn and Pros \((P=7.794\times10^{-26}; \text{Fig. 2A})\) (Southall and Brand, 2009). By contrast, the 177 genes that were not Ase targets showed less overlap with Dpn/Pros regulation \((P=0.005;\)
Fig. 2A). Nevertheless, both Ase+ and Ase− subsets were similarly enriched in NB-expressed genes, suggesting that the latter are also important in NBs.

Altogether, these comparisons reveal that a significant proportion of the Notch targets have been implicated in regulatory networks coordinating the NB stem cell programme (e.g. Fig. 2B). We
therefore sought to verify their response to Notch signalling by testing whether expression from a subset (grh, wor, mira, numb) was modified when NBs were subjected to a pulse of N\textsubscript{Aecd} for 24 h. All four genes were expressed in overproliferating NB-like cells generated under these conditions (grh, mira, Fig. 2C; wor, numb, Fig. S2A). Indeed, Mira levels were significantly higher in the N\textsubscript{Aecd} lineages (Fig. S2C,D), although we note that, in general, the major effect is one of more cells expressing these genes rather than a large increase in expression levels per cell. Nevertheless, there is widespread expression of the stem cell genes in response to ectopic Notch activity in vivo, in agreement with the expression array results.

To test whether stem cell network genes are sensitive to reduced Notch function, we generated marked clones of cells mutant for \textit{Dl}, \textit{Ser} [which compromises Notch activity in NBs (Zacharioudaki et al., 2012)] or \textit{for mastermind (mam)} (to prevent the transcriptional activation of targets). Such manipulations led to significantly altered expression of \textit{mira} (100% of lineages, \textit{n}=8; Fig. 2D), grh (85.72% of lineages, \textit{n}=7; Fig. 2D), wor (60% of lineages, \textit{n}=5) and numb (100% of lineages, \textit{n}=7) in Type II lineages (Fig. S2B, Table S4). The effects in Type I lineages were more subtle, as \textit{mira} was the only gene that was significantly reduced in expression (16.2% of lineages, \textit{n}=99; Fig. 2D, Table S4), although the levels of Numb and Wor were also reduced in some clones. Altogether, these data are consistent with these genes being under Notch regulation in some NB lineages. The fact that their expression is not abolished upon Notch loss of function in Type I NBs might be the consequence of additional, compensatory regulatory inputs, as has been shown for \textit{dpn} (San-Juán and Baonza, 2011; Zacharioudaki et al., 2012), or of Type I NBs being more resilient to reductions in Notch activity (Bowman et al., 2008; Wang et al., 2006).

\textbf{Notch regulates the expression of TFs implicated in temporal programming}

In addition to genes implicated in NB identity, which are expressed consistently and specifically in NBs, Notch targets also included genes with more dynamic NB expression (Fig. 2E). Several of these are involved in temporal programming of NBs and their progeny (Maurange et al., 2008). In particular, \textit{svp} and \textit{cas} are expressed in NBs at mid-larval stages and regulate a change in the size and identity of the neurons produced, as well as determining the time that NBs will undergo cell cycle exit or Reaper/Hid/Grim-dependent apoptosis (Maurange et al., 2008). \textit{hth} has a similar role in programming optic lobe NBs (Li et al., 2013) and exhibits dynamic expression in central brain NBs (data not shown), although its function there is not known. The fact that such genes have increased expression in Notch-induced NB tumours suggests a disruption to the temporal programme such that early expressed genes are transcribed at later times.

To substantiate the expression array results we compared the expression of \textit{svp}, \textit{cas} and \textit{hth} in WT CNS with that in which N\textsubscript{Aecd} was induced for 24 h. No expression of Cas or Svp was detected in NBs in late WT CNS (Fig. 2F, Fig. S3A); their expression was entirely complementary to that of Dpn, suggesting that they were predominantly neuronal. Expression of Hth was more complex, with variable levels in NBs as well as in GMCs and in neuronal progeny (Fig. 2F). In the presence of N\textsubscript{Aecd}, all three proteins were detected in the ectopic NB-like cells. Many Dpn\textsuperscript{+} cells expressed Svp and Cas and they exhibited greatly enhanced Hth levels (Fig. 2F, Fig. S3A,B). Thus, unexpectedly, sustained Notch activity results in ectopic expression of temporal genes in late stage NBs.

By contrast, reductions in Notch function had relatively little impact on \textit{cas}, \textit{hth} or \textit{svp} expression (Fig. 2G). In Type I lineages mutant for \textit{Dl \textsubscript{Ser}}, Cas and Svp were still present, both at L3 in neurons (Fig. 2G, Table S4) and at earlier stages when these proteins are normally expressed in NBs (L2, 30-50 h after larval hatching; Fig. S3C). In \textit{Dl \textsubscript{Ser}} mutant Type II lineages Hth and Svp were absent, suggesting that their expression might be dependent on Notch in these NBs. Thus, similar to the NB-specific genes, temporal programming genes are responsive to Notch overactivation but are largely resistant to Notch downregulation, except in Type II NBs, suggesting that compensatory mechanisms might be involved in regulating their NB expression.
Fig. 3. Su(H)-bound regions identify NB enhancers. (A) Genomic region spanning grh, svp and cas with graphs depicting Su(H)-bound regions (enrichment relative to input, AvgM, scale log2 0-4) in N\textsubscript{Aecd} brains (red) and in N\textsubscript{Aecd} wing discs (green). Grey bars indicate regions tested for enhancer activity, and the purple bar indicates a previously identified grh NB enhancer. Details as in Fig. 1F. (B) Su(H)-bound regions from grh, wor and mira respond to Nicd in transient transfection assays. Fold change in expression from WT enhancers was significantly different from those with Su(H) binding motifs mutated. *P \leq 0.05, **P \leq 0.001, \textit{t}-test. Average of three biological replicates; error bars indicate s.e.m. (C) Su(H)-bound region from grh, cas and svp direct NB-specific expression and respond to ectopic Notch activity. Expression from the indicated enhancers (red, white) in WT and N\textsubscript{Aecd}-expressing brain regions. Pros (blue) marks GMCs and neurons, and Mira (blue) marks NBs. (D) Intensity of expression from grh[NRE] in control and N\textsubscript{Aecd}-expressing dorsal brain NBs (20 NBs per brain lobe, five brains) was significantly different (*P <0.05, \textit{t}-test). Box represents the interquartile range (IQR), orange/grey interface indicates median and whiskers indicate ±1.5×IQR. (E) grh[NRE] is sensitive to reduced Notch signalling. grh[NRE]-lacZ (red, white) in WT (green, left) or \textit{Dlrev10 SerRx106} (green, right). Yellow arrows indicate NBs from GFP-marked WT or mutant Type I and Type II lineages. (F) Enhancers from cas show differing sensitivity to reduced Notch signalling. Expression from the indicated enhancers (red, white) in WT (green, left) or \textit{Dlrev10 SerRx106} (green, right) clones. Yellow arrows indicate NBs from clonally marked WT or mutant Type I and Type II lineages. (G) Percentages of Type I NBs with high (blue), medium (orange) or low/no (grey) levels of expression of the indicated reporters in control versus \textit{Dlrev10 SerRx106} lineages. Scale bars: 50 μm.
Role of stem cell identity genes in Notch-induced NB hyperplasia

Possible roles for Notch-regulated NB-expressed genes might be to inhibit pro-differentiation factors and/or to maintain the self-renewal characteristics of NBs. One candidate for the former is mira, which encodes a protein required to retain key factors at the cytoplasmic cortex of NBs. In Type I NBs these include Pros, a transcriptional regulator of proliferation/differentiation, which can drive proliferating larval NBs into quiescence (Lai and Doe, 2014). By upregulating the expression of mira, Notch activity could favour the sequestration of Pros and so promote self-renewal. To test whether Mira is important for Notch-induced hyperplasia, we analysed the consequences of Mira depletion (by RNAi) in the background of Niced overexpression (via inscGal4;UAS-Niced, a combination that induces weaker hyperplasia than the Naced used in earlier experiments; Fig. 4A). Under these conditions, Pros was detectable in the nuclei of NBs, consistent with perturbation of Mira function (Fig. 4A). However, there was no reduction in hyperplasia; instead, the extent of hyperplasia was exacerbated (Fig. 4A,B, Fig. S4). Thus, the hyperplasia cannot be explained by Mira-mediated sequestration of factors such as Pros.

We then tested whether hyperplasia is reduced when the NB-specific TFs are depleted, since previous studies showed that depletion of E(spl)m8-HLH from Naced-overexpressing clones drastically reduces overproliferation (Zacharioudaki et al., 2012) (see Fig. 5A). However, little rescue of the overproliferation occurred in Type I lineages when grh or dpm were mutant (Fig. 4C-E, Table S5), nor when wor was depleted (Fig. S5B). The number and extent of overproliferating Niced-expressing lineages that were mutant for dpm (28.2% of lineages, median of 2 Dpn+ cells, n=234) or grh (16.6% of lineages, median of 3 Dpn+ cells, n=229) was similar to Niced alone (13% of lineages, median of 3 Dpn+ cells, n=355).

We also tested the effects of eliminating the widely expressed TF Lola, since several of its isoforms were upregulated by Naced. A strong hypomorphic mutation affecting all lola isoforms (lola92D) had little impact on Type I lineage overproliferation (8.4% of lineages overproliferating, median of 6 Dpn+ cells, n=143). By contrast, the phenotypes in Type II lineages were partially suppressed by mutations affecting these genes (Fig. 4C-E, Table S5). For example, loss of grh decreased the Niced-induced overproliferation phenotype from 158 Dpn+ cells per Type II lineage (n=21) to 114 Dpn+ cells per lineage (n=15), and lola92D significantly rescued the Niced phenotype (33 Dpn+ cells, n=8; WT lineages have 27 Dpn+ cells). A dpm allele also decreased overproliferation of Type II NBs, as observed previously (Zacharioudaki et al., 2012), yielding a median of 84 Dpn+ cells (n=7), but as there was considerable variability the effect was not statistically significant in these experiments.

Mutations in the NB-specific genes might not alter Notch-induced hyperplasia if they have overlapping functions in stem cell renewal, as previously shown for dpm and E(spl)-C (Zacharioudaki et al., 2012). We therefore examined whether removal of ‘stemness’ regulators, alone or in pairs, results in any defects in a normal background. We generated NB lineages that were double mutant for different combinations of TFs [grh E(spl)-C, wor E(spl)-C, grh dpm, wor dpm and grh wor] and measured changes in the number of progeny for both Type I and Type II NBs. Of those tested, only grh wor double-mutant lineages showed significant differences in the number of INPs and GMCs compared with either single mutant alone (Fig. S5). In addition, the grh wor Type I NBs had smaller nuclei (Fig. S5). Thus, these two NB-specific TFs have overlapping roles in maintaining NB size and proliferative rate. This suggests a robustness in the transcriptional network regulating stem cell characteristics, which might explain why removal of a single factor is insufficient to fully suppress Notch-induced hyperplasia.

Role of temporal genes in Notch-induced hyperplasia

To decipher whether persistent expression of the temporal programming factors svp, cas and hth is important for NB hyperplasia, we used mutations to compromise their function while at the same time expressing Naced. Results showed that the hyperplasia in Type II lineages was highly susceptible to depletion of these TFs. Thus, the Naced-induced hyperplasia in Type II lineages (median of 135 Dpn+ cells/lineage, n=13) was rescued by removing either of the temporal genes svp or hth as well as by removing E(spl)-C (Fig. 5A-C, Table S5). The effects of removing svp were most dramatic: none of the lineages showed residual overproliferation and most lineages even had fewer Dpn+ cells (median 13 Dpn+ cells, n=8; Fig. 5C) than WT (median 28 Dpn+ cells). One way to reconcile this highly penetrant phenotype with the observation that only a subset of NB-like cells exhibit Svp expression is that the immunofluorescence only gives a snapshot of the expression at any given moment; many more NB-like cells might switch svp on at some stage to prompt the overproliferation. Alternatively, as we were only able to recover a relatively small number of clones of this genotype, it is possible that the phenotypic effects are overestimated. Eliminating hth in lineages expressing Naced also ameliorated the hyperplasia (Fig. 5B,C), with the median number of Dpn+ cells reduced by both hthb2 (64.5 Dpn+ cells, n=8) and hthC1 (58 Dpn+ cells, n=19). Finally, mutations affecting cas also resulted in decreased numbers of Dpn+ cells per Type II lineage (89 Dpn+ cells, n=25; Fig. 5C) although, because of the high variability, the effects were not statistically significant.

The effects on Type I lineages were less pronounced (Fig. 5B,C, Fig. S6). In these lineages, which were scored as hyperplastic when they contained two or more Dpn+ cells, the overexpression of Naced caused hyperplasia in 61.6% of lineages (n=86), with a median of 13 Dpn+ NB-like cells (which were usually intermediate in size between a normal NB and a GMC). Of the genes tested, only mutations in svp significantly rescued the Type I hyperplasia, although to a smaller extent than removing E(spl)-C (Zacharioudaki et al., 2012) (Fig. 5B,C). Specifically, only 13.3% of svp lineages (n=654) remained overgrown, with a median of 2 Dpn+ cells, although there was considerable variability. In addition, in a few lineages (1.7%) the NBs appeared to have undergone apoptosis. By contrast, mutations in cas or hth failed to significantly alter Type I NB hyperplasia; 47% cas (n=228), 40.8% hthC1 (n=94) and 58.5% hthb2 (n=174) lineages exhibited hyperplasia, with medians of 12, 14 and 11 Dpn+ cells, respectively.

Expression of wor and of temporal genes is sufficient to induce mild hyperplasia

From the loss-of-function experiments it appears that several Notch-regulated genes contribute to the hyperplasia. To investigate whether any of these targets is sufficient to induce excess NBs,
we assessed the consequences of their overexpression in larval lineages by scoring the numbers of Dpn⁺ cells (Fig. 6). We note that in some cases grh, E(spl)mγ-HLH, wor these manipulations would augment existing expression levels, whereas for svp they would result in ectopic expression.

Two of the three NB-specific genes tested resulted in significant hyperplasia. Notably, expression of wor was sufficient to promote an increase in NB-like cells both in the dorso-posterior brain, where Type II lineages reside, and in the ventral nerve cord (VNC), where Type I lineages occur (Fig. 6A,B). As previously shown, overexpression of E(spl)mγ-HLH also caused hyperplasia, primarily in the dorso-posterior brain, where it generated regions of contiguous Dpn⁺ Ase⁻ cells. Their size was intermediate between an INP and an NB (Fig. 6C) and they most likely arose from Type II lineages. By contrast, grh overexpression failed to cause any increase in NB numbers, even in these more susceptible Type II lineages (data not shown).

Whereas expression of Cas had no effect on NB lineages (Fig. 6B, Fig. S7A), overexpression of Svp did elicit supernumerary Dpn⁺ cells in Type II lineages (Fig. 6C,D, Fig. S7B). These were Ase⁺ and generally smaller than NBs (Fig. 6C). Excess Dpn⁺ cells were also produced following expression of a chimeric Hth, in which Hth is fused to the Engrailed (En) repressor domain to create a constitutive Hth repressor. These excess Dpn⁺ cells were Ase⁺ and...
were similar to, or smaller than, mature INPs. In addition, some hyperplasia was present in Type I NB lineages (Fig. 6C,D). As Hth has been reported to have both activator and repressor functions (Inbal et al., 2001; Wernet and Desplan, 2014), we cannot predict whether En-Hth would act as a positive or a dominant-negative factor in the conditions tested. Regardless of the mechanisms involved, altered activity of Svp and Hth is nevertheless sufficient to induce a modest level of hyperplasia, especially in Type II lineages.

DISCUSSION

Notch activity is sustained in post-embryonic NBs throughout their lifetime and, when activated inappropriately, is sufficient to confer NB-like properties on the progeny. Our analysis of the genes regulated by Notch under these circumstances reveals that it is likely to achieve its functions through multi-pronged regulation of the stem cell programme. Of the 246 putative direct Notch target genes that we identified in hyperplastic CNS, the majority (>55%) are enriched in the NB transcriptome and 28% are assigned to the NB network regulated by Ase. Many of these encode TFs that have been implicated in stem cell regulation, such as grh, klu, wor, dpn and E(spl)mγ-HLH, and others encode proteins involved in regulating asymmetric stem cell divisions, including mira and numb. An unexpected constituent of the Notch-upregulated genes were those implicated in the temporal programming of NBs, represented by syp, cas and hth. Such genes are thought to confer stage-specific NB characteristics and determine the ultimate timing of their cell cycle exit. For example, in larval NBs, syp and cas are expressed early in L2 and, if absent, a switch in neuronal identities fails to occur and the NBs fail to cease proliferating appropriately (Maurange et al., 2008). Similarly, hth has been implicated in the temporal cascade of optic lobe larval NBs (Li et al., 2013). By promoting the re-expression or extended expression of factors that are normally present transiently, Notch activity is likely to change the NB developmental clock. Sustained expression of such factors may perturb the ability of NBs to exit the cell cycle correctly. This would imply that persistent expression of such factors has similarly disruptive consequences to their total removal (Maurange et al., 2008). Similarly, hth has been implicated in the temporal cascade of optic lobe larval NBs (Li et al., 2013). By promoting the re-expression or extended expression of factors that are normally present transiently, Notch activity is likely to change the NB developmental clock. Sustained expression of such factors may perturb the ability of NBs to exit the cell cycle correctly. This would imply that persistent expression of such factors has similarly disruptive consequences to their total removal (Maurange et al., 2008). As Notch-regulated enhancers were enriched in target motifs for both E(spl)mγ-HLH and Hth, feed-forward regulatory loops might also be important in the regulation of NB maintenance and progression by Notch, although the mechanisms remain to be deciphered.
With the exception of mira, all of the Notch targets tested were found to contribute to Notch-induced hyperplasia. Type II NB hyperplasia was more sensitive to the attenuation of target genes, with all mutations reducing the overgrowth to a greater or lesser extent, whereas Type I NB hyperplasia was only ameliorated by E(spl)-C and svp. Furthermore, a subset, notably wor, svp, hth, dpn, E(spl)my-HLH and klu, were themselves sufficient to drive mild hyperplasia, especially in Type II lineages (see also Berger et al., 2012; San-Juan and Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012). Thus, both stemness and temporal TFs cooperate to sustain Notch-induced hyperplasia. Furthermore, although individual factors each make some unique contribution to NB maintenance, several [E(spl) my-HLH and Dpn, Grh and Wor] appear to have overlapping functions, which confers robustness on the regulatory network.
The regions bound by Su(H) identified NB enhancers, in agreement with the nearby genes being targets of Notch in NBs. However, an unexpected feature was that the NB expression of these genes did not appear to be strictly dependent on Notch activity. This might in part be explained by the regulated genes having multiple NB enhancers; for example, at least two further NB-specific enhancers have been identified for grh, besides grh[NRE] (Brody et al., 2012; Prokop et al., 1998). However, this cannot fully account for the lack of Notch dependency. Even when we focused on individual enhancers, loss of Notch signalling did not always eliminate their expression, although in most cases it did reduce it, as observed previously for $E(spl) m 8\cdot H L H$ (Zacharioudaki et al., 2012). The only target gene tested that is fully dependent on Notch for expression in NBs, being undetectable upon Notch loss of function, is $E(spl)m y\cdot H L H$ (Almeida and Bray, 2005; Zacharioudaki et al., 2012). It thus appears that in NBs many of the Notch targets respond to additional transcriptional cues that can partially compensate for the absence of Notch. It will be interesting to determine whether this is also true for the majority of Notch target enhancers in other tissues.

Persistent Notch activity in several other tissues also causes extreme hyperplasia. Surprisingly, however, only 18 of the genes upregulated in the NB hyperplasia are also targeted in an epithelial hyperplasia caused by ectopic activity of Notch in wing discs. These include HES/ $E(spl)$ genes, which respond robustly to Notch signalling in most cellular contexts, and the growth regulator Myc, which is also a common target even in human pathologies, such as T-ALL (Klinakis et al., 2006; Weng et al., 2006). Thus, the mechanisms through which Notch induces hyperplasia differ in the two contexts. It is possible that this relates to the fact that the hyperplasia originates from a stem cell lineage in one case and an epithelium in the other. Indeed, comparisons between the genes regulated in the epithelial model and those regulated by Notch1 in a breast cancer cell line revealed a surprising extent of overlap (Djiane et al., 2013; Mazzone et al., 2010). It will therefore be important in future to make comparisons with different stem cell-related hyperplasias in mammals, to ask whether the Notch-regulated genes exhibit similar characteristics to those observed for NBs. Such comparisons will help to ascertain whether there are indeed common themes in the transcriptional outputs from Notch activation that reflect the different cellular contexts.

Expression arrays and genome-wide ChIP

For each expression array, RNA was isolated from the CNS of 300 flies using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using standard procedures before hybridising to Affymetrix GeneChip Drosophila Genome 2.0 Array 3 (see the supplementary Materials and Methods for details). Four replicate arrays were analysed for each genotype. Quantile normalised data were analysed using Limma (Smyth, 2004) to estimate the log$_2$ FC in Notch versus control samples and the Benjamini-Hochberg adjusted P-values (FDR). Data from expression arrays have been deposited in ArrayExpress (E-MTAB-3561).

For each ChIP, chromatin was prepared from the CNS of 50 flies and the Su(H) ChIP performed as described previously (Krejci et al., 2009). The products were amplified and hybridised to NimbleGen D. melanogaster 2.1 M Whole-Genome Tiling Arrays. Three biological replicates were performed and quantile normalisation was applied to replicates for each genotype. Bound regions (peaks) were identified using Tamapalps at T02P005 stringency [top 2%, $P \leq 0.005$ (Bieda et al., 2006)]. Genes in proximity to ChIP peaks were identified by the nearest genes upstream and downstream of each peak (using coordinates for the gene body) with a distance cutoff of 20 kb. ChIP data have been deposited in Gene Expression Omnibus (GEO series GSE68614).

Gene set enrichment analysis (Subramanian et al., 2005) was performed using the ‘pre-ranked’ option to analyse the gene list ranked by log$_2$ FC from the mRNA expression data (3717 genes, FDR $\leq 0.01$) for enrichment of Su(H)-bound genes. As recommended, the more conservative ‘classic’ scoring approach was used, which computes enrichment using only the gene’s ranking (with no increment for the absolute value of the ranking metric).

GO analysis was performed using the DAVID bioinformatics resource (Huang et al., 2009). Motif enrichment analysis utilised the Bioconductor package PWMEEnrich, which assesses the enrichment of each motif from a library of 650 experimentally derived Drosophila TF DNA motifs (Stojnic and Diez, 2014). Enrichments for NB-expressed genes, Ace targets, and NB stem cell networks were assessed using Fisher’s exact test, with the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) to correct for multiple sampling.

NRE reporters

Putative Notch-regulated enhancers (NREs) in grh, svp and cas (coordinates are provided in the supplementary Materials and Methods) were cloned in pBlueRabbit (pBR) (Housden et al., 2012). Flies carrying the pBR transgenes were generated by $\psi c 31$-mediated site-directed integration in the attP40 landing site.

Immunofluorescence

Fixation and immunohistochemistry of larval tissues were performed according to standard protocols. Details of primary antibodies are provided in the supplementary Materials and Methods. Mouse, rabbit, guinea pig or rat secondary antibodies were conjugated to Alexa 488, 555, 563, 643 or 647 (Molecular Probes) or to FITC, Cy3 or Cy5 (Jackson ImmunoResearch). Samples were imaged on a Leica SP2 or TCS SP8 confocal microscope (Confocal Facility, University of Crete or CAIC, University of Cambridge).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.Z. and B.E.H. performed experiments; E.Z., R.S., G.G., C.D. and S.J.B. analysed the data; E.Z., C.D. and S.J.B. designed experiments; E.Z., C.D. and S.J.B. wrote the manuscript; B.E.H., R.S. and G.G. edited the manuscript.


