

# The pipsqueak-domain proteins Distal antenna and Distal antenna-related restrict Hunchback neuroblast expression and early-born neuronal identity

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## SUMMARY

A fundamental question in brain development is how precursor cells generate a diverse group of neural progeny in an ordered manner. *Drosophila* neuroblasts sequentially express the transcription factors Hunchback (Hb), Krüppel (Kr), Pdm1/Pdm2 (Pdm) and Castor (Cas). Hb is necessary and sufficient to specify early-born temporal identity and, thus, Hb downregulation is essential for specification of later-born progeny. Here, we show that *distal antenna* (*dan*) and *distal antenna-related* (*danr*), encoding pipsqueak motif DNA-binding domain protein family members, are detected in all neuroblasts during the Hb-to-Cas expression window. Dan and Danr are required for timely downregulation of Hb in neuroblasts and for limiting the number of early-born neurons. Dan and Danr function independently of Seven-up (*Svp*), an orphan nuclear receptor known to repress Hb expression in neuroblasts, because Dan, Danr and *Svp* do not regulate each other and *dan danr svp* triple mutants have increased early-born neurons compared with either *dan danr* or *svp* mutants. Interestingly, misexpression of Hb can induce Dan and *Svp* expression in neuroblasts, suggesting that Hb might activate a negative feedback loop to limit its own expression. We conclude that Dan/Danr and *Svp* act in parallel pathways to limit Hb expression and allow neuroblasts to transition from making early-born neurons to late-born neurons at the proper time.

**KEY WORDS:** *Drosophila*, Temporal identity, Seven-up, Dan, Danr, Hb

## INTRODUCTION

The generation of neuronal diversity during development is a tightly controlled process in which multiple regulatory factors provide neural stem cells with both spatial and temporal information. Much progress has been made in understanding spatial patterning, in which neural stem cells are topographically organized according to their unique dorsoventral and anteroposterior positions that specify cell fate (Doe and Technau, 1993; Skeath and Thor, 2003; Guillemot, 2005). However, much less is known regarding temporal patterning, a process in which neural stem cells change their transcriptional signatures over time to generate distinct progeny (Pearson and Doe, 2003).

In the developing *Drosophila* embryo, each of the 30 neuroblasts in a hemisegment can be uniquely identified by its stereotyped position, molecular markers and unique cell lineage (Doe, 1992; Broadus et al., 1995; Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999; Baumgardt et al., 2009; Karlsson et al., 2010). Neuroblasts undergo a series of asymmetric cell divisions that give rise to a self-renewed neuroblast and a smaller ganglion mother cell (GMC), which typically divides one more time to produce two postmitotic daughter cells (Goodman and Doe, 1993). Each neuroblast generates distinct sets of progeny, but always in a stereotyped birth order. This is accomplished by the sequential expression of the zinc-finger transcription factors Hunchback (Hb) and Krüppel (Kr), the redundant POU domain transcription factors

Pdm1 (Nubbin – FlyBase) and Pdm2 (henceforth both called Pdm), and the zinc finger transcription factor Castor (Cas) (Isshiki et al., 2001; Novotny et al., 2002). Because daughter cell fate is governed by the transcriptional profile of the neuroblast at the time of its birth, the timing and duration of expression of each temporal identity factor in neuroblasts is crucial for specification of the progeny cell type and its abundance. For example, use of the Gal4-UAS system to extend the length of Hb expression in neuroblasts results in extra neuronal cell types with early-born identities at the expense of those with later-born identities (Isshiki et al., 2001; Novotny et al., 2002). Thus, regulation of the timing and duration of Hb expression is an early and essential step in the generation of neuronal diversity.

Here, we show that the pipsqueak motif nuclear proteins Distal antenna (Dan) and Distal antenna-related (Danr) function in a parallel pathway to that of *Svp* to limit Hb neuroblast expression and the number of early-born progeny in multiple neuroblast lineages.

## MATERIALS AND METHODS

### Fly stocks

We used the following fly stocks to analyze mutant phenotypes at 23°C: *dan danr<sup>ex56</sup>/TM3 Ubx-lacZ*, *danr<sup>ex35</sup>/TM3 Ubx-lacZ*, *dan<sup>ems3</sup>/TM3 Ubx-lacZ* (Emerald et al., 2003), *hb<sup>P1</sup> hb<sup>FB</sup>/TM3 hb-lacZ* to remove Hb CNS expression (Hulskamp et al., 1994; Isshiki et al., 2001), and *svp<sup>e22</sup>/TM3 Ubx-lacZ* (Kanai et al., 2005). *svp<sup>e22</sup>/TM3 Ubx-lacZ* and *dan danr<sup>ex56</sup>/TM3 Ubx-lacZ* were recombined on chromosome 3 to generate triple mutants lacking *svp*, *dan* and *danr*. We used the following gal4 lines: *engrailed (en)-gal4* (Harrison et al., 1995; Isshiki et al., 2001; Pearson and Doe, 2003) for expression in the posterior compartment of each segment, *inscuteable (insc)-gal4 (1407-gal4*, Bloomington Stock Center) on chromosome 2 and *scabrous (sca)-gal4* (Cleary and Doe, 2006) for expression in all neuroblasts. For Hb misexpression studies, *en-gal4* was crossed to *UAS-hb* on chromosomes 2 and 3 (Wimmer et al., 2000). For

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Dan rescue experiments, the following stocks were generated and crossed to *UAS-dan: dan dan<sup>ex56</sup>/TM3, Ubx-lacZ: en-gal4; dan dan<sup>ex56</sup>/TM3, Ubx-lacZ, insc-gal4; dan dan<sup>ex56</sup>/TM3, Ubx-lacZ and sca-gal4; dan dan<sup>ex56</sup>/TM3, Ubx-lacZ. *yw* embryos were used for wild-type expression analysis.*

### Molecular markers and immunostaining

Antibody staining was performed according to standard methods (Rothwell and Sullivan, 2000). Primary antibodies, dilutions and sources were: rat anti-Dan 1:400 (kind gift from J. Curtiss, New Mexico State University, NM, USA); mouse anti-Eve 1:50 (3C10-c), mouse anti-Engrailed (4D9) 1:10, mouse anti-Islet 1:200 and mouse anti-Eagle 1:100 (8B11) (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA); rabbit anti-Cut 1:500 (kind gift from Y. N. Jan, UCSF, San Francisco, CA, USA); rabbit anti-Hb 1:200 (Tran and Doe, 2008); rat anti-Zfh2 1:200 (Tran et al., 2010); guinea pig anti-Runt 1:500 and guinea pig anti-HB9 1:500 (East Asian Distribution Center for Segmentation Antibodies, Mishima, Japan); rabbit anti-Castor 1:1000 (kind gift from W. Odenwald, Bethesda, MD, USA); anti- $\beta$ -galactosidase 1:500 (Promega, Madison, WI, USA); mouse anti-Svp 1:500 and rat anti-Svp 1:500 (kind gift from T. Isshiki, National Institute of Genetics, Japan) (Kanai et al., 2005); guinea pig anti-Miranda 1:400 (Lee et al., 2006). Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies were from Molecular Probes (Invitrogen, Eugene, OR, USA); Cy5- or Dylight 549-conjugated secondary antibodies and Cy3- or Dylight 405-conjugated Streptavidin were from Jackson ImmunoResearch (West Grove, PA, USA). Biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA, USA) and Jackson ImmunoResearch.

### Neuroblast lineage markers

Markers used to identify neurons from different neuroblast (NB) lineages were:

NB7-1: Eve (U1-U5; listed by birth-order), Hb (early-born U1, U2), Zfh2 (U2-U5), Runt (U4, U5), Castor (U5), Cut (U3-U5).

NB1-1: Eve, Cut and Hb (early-born aCC, pCC).

NB3-1: Isl and Hb9 (RP1, RP4, RP3, RP5; listed by birth-order), Hb (early-born RP1, RP4).

NB7-3: Eagle (EW1/GW siblings, EW2, EW3; listed by birth-order), Hb (early-born EW1/GW), Hb9 (EW1-3).

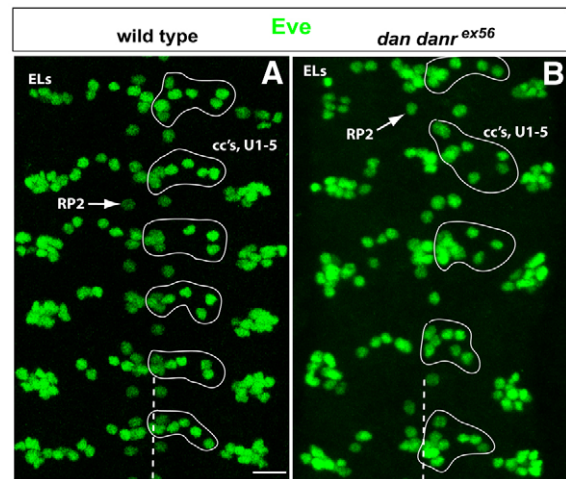
### In situ hybridization

*dan* and *danr* cDNA were obtained from the Drosophila Gene Collection. To generate RNA probes to detect *CG34110* specifically, we used the Superscript One-Step RT-PCR Kit (Invitrogen). We used gene-specific primers to obtain *CG34110* cDNA (forward, TCGACAGGATTCATTTGCGAGAC and reverse, TTGAGCAGACTCACAAACCGAGAC) with the T7 promoter sequence on the reverse primer). Digoxigenin-labeled RNA probe was transcribed using the Roche DIG Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA). In situ hybridization was performed as described previously (Grosskortenhaus et al., 2005).

## RESULTS

### *distal antenna* and *distal antenna-related* double mutants generate extra early-born Eve<sup>+</sup> neurons

To identify genes that regulate early-born versus late-born temporal identity in *Drosophila* embryonic neuroblast lineages, we conducted a forward genetic screen of ~100 second and third chromosomal deficiency lines for mutants that had altered numbers of Even-skipped (Eve)<sup>+</sup> early-born neurons. Eve labels a small number of identifiable early-born neurons from multiple neuroblast lineages: anterior corner cell (aCC) and posterior corner cell (pCC) from NB1-1, RP2 from NB4-2, and the first five motor neurons (U1-U5) from NB7-1 (Fig. 1A). Here, we describe the phenotype of deficiency *Df(3R)Exel6201*, in which nearly half of all hemisegments showed extra early-born aCC/pCC, RP2 or U1/U2 neurons (46.6%; 99 hemisegments counted from nine embryos).

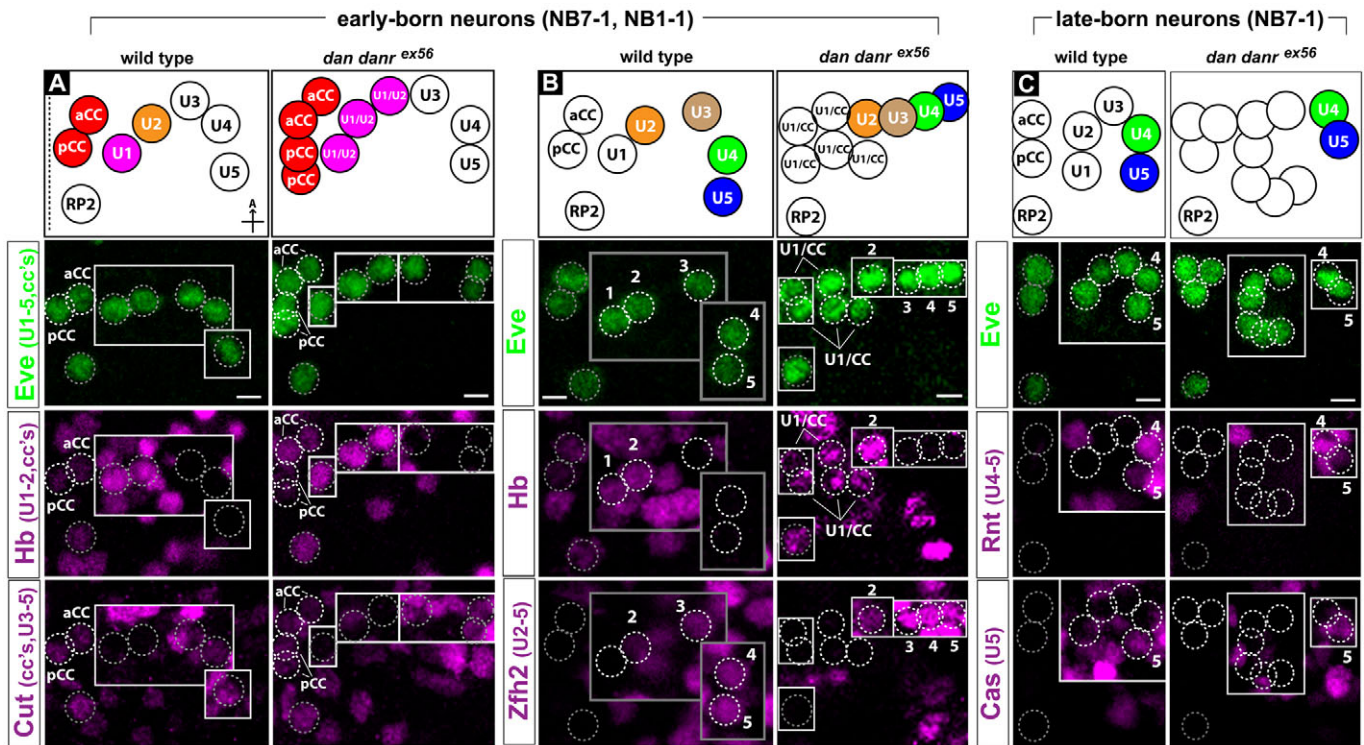


**Fig. 1. A deficiency removing *dan danr* and *CG34110* has ectopic Eve<sup>+</sup> neurons. (A,B)** Stage 16 embryonic CNS stained for Even-skipped (Eve) and shown as a maximum intensity projection. Eve marks anterior and posterior corner cells (aCC/pCC) from the NB1-1 lineage (cc's), U1-U5 neurons from the NB7-1 lineage (U1-U5 circled in the right hemisegments), the RP2 neuron from NB4-2 and the Eve lateral (EL) neurons from NB3-3. Anterior is up, segments T3-A5 are shown. The ventral midline is indicated by the vertical dashed line. Note the seven aCC/pCC/U1-U5 neurons on each side of the midline in the wild-type embryo (A). The U1 neuron is often obscured by the aCC/pCC neurons. The *dan danr<sup>ex56</sup>* (*dan danr CG34110*) mutant embryo (B) demonstrates an increase in aCC/pCC/U1-U5 neurons. Embryo morphology is fairly normal in the mutant embryo although there is some variability in the position and/or number of RP2 neurons. Scale bar: 10  $\mu$ m.

We did not observe any gross morphological deficits in the CNS of the *Df(3R)Exel6201* mutant embryos. Phenotypes of other deficiencies will be described elsewhere. *Df(3R)Exel6201* is a small chromosomal deletion affecting only three genes: *distal antenna* (*dan*), *distal antenna-related* (*danr*) and the uncharacterized gene *CG34110*. To control for any phenotypes in the deficiency caused by background mutations on this chromosome, we obtained a second small deficiency (*dan danr<sup>ex56</sup>*) removing the same three genes (*dan*, *danr* and *CG34110*) that was independently generated on a different genetic background (Emerald et al., 2003). We confirmed that the ectopic Eve early-born neuron phenotype was also present in *dan danr<sup>ex56</sup>* mutants and in the *dan danr<sup>ex56</sup>/Df(3R)Exel6201* transheterozygous mutants (Fig. 1B).

To determine which of these three missing genes caused the observed CNS phenotype, we assayed their gene expression patterns and performed rescue experiments. Both *dan* and *danr* were highly expressed in neuroblasts and neurons of the developing CNS, whereas *CG34110* was expressed in a subset of sensory neurons at late stages but was expressed at low or undetectable levels in the CNS (see Fig. S1 in the supplementary material). Based on expression patterns, we focused on the *dan* and *danr* genes. Dan and Danr are two closely related nuclear proteins containing a pipsqueak motif DNA-binding domain; this family of proteins is conserved from fungi to vertebrates (Lehmann et al., 1998). Dan and Danr were previously reported to have overlapping expression patterns and redundant functions in antennal development (Emerald et al., 2003) and have been shown to regulate cell specification in the retina (Suzanne et al., 2003; Curtiss et al., 2007). We used the Gal4-UAS system to misexpress





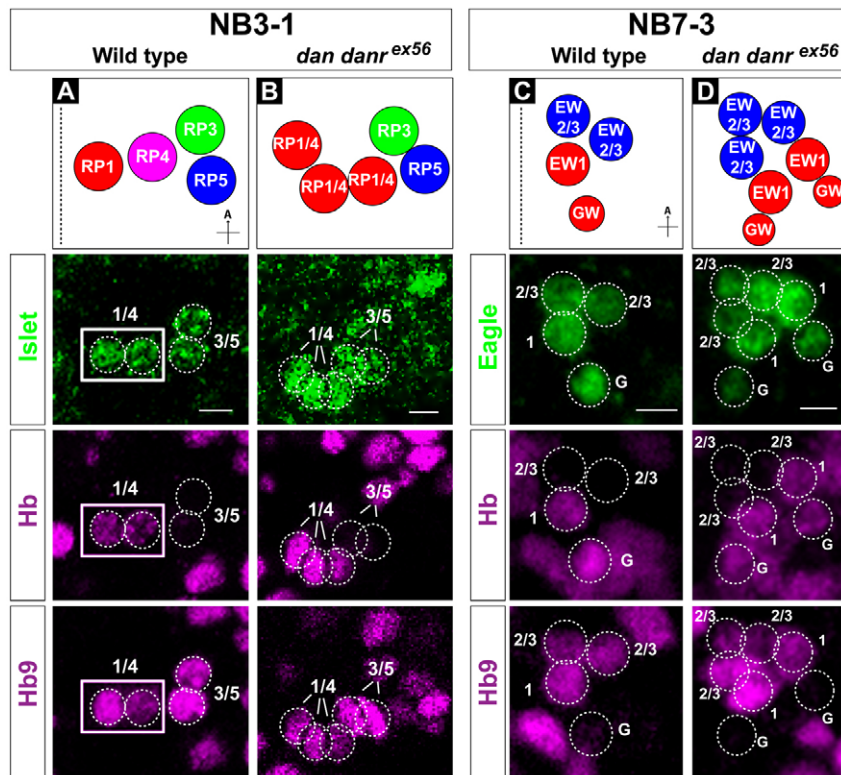
**Fig. 2. *dan danr* mutants have ectopic early-born neurons in NB7-1 and NB1-1 lineages.** (A) Left: wild-type pattern showing the first-born aCC/pCC siblings from NB1-1, and the first-born to fifth-born U1-U5 neurons from the NB7-1 lineages (each U neuron has an Eve<sup>-</sup> sibling, not shown). Right: in *dan danr<sup>ex56</sup>* double mutants, there is often an extra aCC, an extra pCC and extra early-born U neurons (U1 or U2) based on Eve, Hb and Cut expression (aCC/pCC neurons are Eve<sup>+</sup>Hb<sup>+</sup>Cut<sup>+</sup> whereas U1/U2 from NB7-1 are Eve<sup>+</sup>Hb<sup>+</sup>Cut<sup>-</sup>). Note the extra pair of Eve<sup>+</sup> Hb<sup>+</sup> Cut<sup>+</sup> aCC/pCC neurons and the extra Eve<sup>+</sup> Hb<sup>+</sup> Cut<sup>-</sup> U1/2 neuron in the *dan danr* mutant. Later-born U3-U5 are also Eve<sup>+</sup>Hb<sup>+</sup>Cut<sup>+</sup>, but their more lateral positions in the ventral nerve cord distinguishes them from aCC/pCC in the midline. (B) Zfh2 and Eve label U2-U5 neurons from the NB7-1 lineage, and U2 is Eve<sup>+</sup>Hb<sup>+</sup>Zfh2<sup>+</sup>. *dan danr* mutants show a wild-type pattern of Zfh2 expression, suggesting that the extra Eve<sup>+</sup>Hb<sup>+</sup>Cut<sup>-</sup> neurons found in panel A represent extra U1 neurons. Note the extra Eve<sup>+</sup>Zfh2<sup>-</sup> neuron (this could be a CC or U1 neuron) in the *dan danr* mutant. (C) U4 and U5 neurons from the NB7-1 lineage are Eve<sup>+</sup> Run<sup>+</sup> (U4) and Eve<sup>+</sup> Run<sup>+</sup> Cas<sup>+</sup> (U5). *dan danr* mutants generally have wild-type numbers of these later-born neurons. Colored cells in schematics at the top indicate which progeny can be identified by the stains below them. All panels show one representative hemisegment with anterior up, midline left (see schematic of wild type in A). Eve (shown in green surrounded by dashed circle in all panels) and the markers directly below it represent markers that were stained together. Wild-type and mutant images are matched for stage and focal plane; white boxes show insets of different z-axis focal planes at their approximate xy coordinates (insets containing U1 are shifted to the right because U1 is normally directly below aCC/pCC). Scale bars: 3  $\mu$ m. aCC, anterior corner cell; pCC, posterior corner cell.

*dan* in all neuroblasts and found that it could partially rescue the *dan danr<sup>ex56</sup>* phenotype (see Fig. S2 in the supplementary material), so we conclude that loss of *dan* and/or *danr* causes the observed CNS phenotype (described in more detail below).

Dan and Danr act redundantly in antennal development (Emerald et al., 2003) and had similar expression in the embryonic CNS (see Fig. S1 in the supplementary material), raising the possibility that they act redundantly in the CNS. We found that the hypomorphic *dan<sup>ems3</sup>* mutant (Emerald et al., 2003; Curtiss et al., 2007) has no Eve CNS phenotype, but the presumptive null *danr<sup>ex35</sup>* mutant (Emerald et al., 2003) has an Eve CNS phenotype in the NB1-1 lineage similar to that of the *dan danr* double mutant and a weak phenotype in the NB7-1 lineage. These observations suggest that endogenous levels of Dan are not sufficient for normal CNS development (see Fig. S2A in the supplementary material). Importantly, overexpression of Dan alone rescued nearly all aspects of the *dan danr* double mutant phenotype (see Fig. S2B in the supplementary material), suggesting that elevated levels of Dan alone can compensate for Danr loss. We propose that Dan and Danr have redundant or similar functions in the CNS, but both proteins are required for normal development.

### **distal antenna and distal antenna-related double mutants generate extra early-born neurons in the NB7-1 and NB1-1 lineages**

To determine the identity of the ectopic Eve neurons in the *dan danr* mutant, we used molecular markers that distinguish early-born progeny of the NB7-1 and NB1-1 lineages. In the NB7-1 lineage, the first five divisions produce GMCs that generate the Eve<sup>+</sup> U1-U5 motor neuron and their Eve<sup>-</sup> siblings. The following markers can distinguish all five U neurons: Hb (U1 and U2), Zfh2 (U2-U5), Cut (U3-U5), Rnt (U4 and U5) and Cas (U5 only) (Isshiki et al., 2001) (Fig. 2A). In the NB1-1 lineage the first GMC generates the Eve<sup>+</sup> aCC/pCC neurons that are also Hb<sup>+</sup>. The aCC/pCC and U1/U2 neurons are adjacent, but can be distinguished because only aCC/pCC are Cut<sup>+</sup>. Using these markers, we found that both NB1-1 and NB7-1 generated extra early-born neurons in *dan danr* mutants. In the NB1-1 lineage, *dan danr* mutants showed ectopic aCC/pCC neurons in 34.3 $\pm$ 6.2% of hemisegments (ranging from one to eight extra corner cells), with the average number of ectopic aCC/pCC neurons being 2.6 $\pm$ 0.2 ( $n=110$  hemisegments from 11 embryos; Fig. 2). In the NB7-1 lineage, *dan danr* mutants showed ectopic U1 neurons in



**Fig. 3. *dan danr* mutants have ectopic early born neural progeny in NB3-1 and NB7-3 lineages.** (A,B) Neurons in the NB3-1 lineage are shown for one hemisegment in a stage 16 embryo. In wild type (A), NB3-1 makes the RP1,3,4,5 motor neurons marked by Islet (green) and Hb9 (magenta). The first-born RP1/RP4 neurons are Hb<sup>+</sup> (magenta). In *dan danr<sup>ex56</sup>* double mutants (B), there are ectopic Hb<sup>+</sup> early-born RP1/RP4 neurons (showing one extra early-born RP neuron). (C,D) Neurons in the NB7-3 lineage are shown for one hemisegment in a stage 16 embryo. In wild type (C), Eagle (green) marks all four neurons in the lineage, and the first-born GW/EW1 neurons are Hb<sup>+</sup> (magenta). *dan danr<sup>ex56</sup>* double mutants (D) show the rare ectopic early-born GW/EW1 neuron phenotype, and the more common ectopic late-born EW2/EW3 neuron phenotype. A partial or complete loss of neuron phenotype is also observed (not shown). In all panels, the ventral midline is the left border of the panel, anterior is up, white boxes show insets of different z-axis focal planes at their approximate xy coordinates (insets containing RP1/4 are shifted to the left because they normally lie directly above RP3). Scale bars: 3 μm.

24.3±5.3% of hemisegments (ranging from one to three extra early-born U neurons;  $n=98$  hemisegments from ten embryos; Fig. 2). Later-born U4-U5 neurons were affected, but to a lesser degree than early-born progeny (only 4.8±2.4% of  $n=42$  hemisegments from three embryos had ectopic U4/5 neurons). We conclude that Dan/Danr act to limit the number of early-born neurons generated in both the NB1-1 and NB7-1 lineages.

### distal antenna and distal antenna-related double mutants generate extra early-born neurons in the NB3-1 and NB7-3 lineages

NB3-1 sequentially gives rise to four GMCs that subsequently generate four RP motor neurons (and siblings) in the order RP1/sib→RP4/sib→RP3/sib→RP5/sib (Tran and Doe, 2008). All four RP motor neurons are Islet<sup>+</sup> Hb9<sup>+</sup> (Tup and Exex, respectively – FlyBase), but only the early-born RP1 and RP4 neurons are Hb<sup>+</sup> (schematic, top panel of Fig. 3A). *dan danr* mutants showed ectopic RP1/RP4 neurons in 34.5% of hemisegments (ranging from one to three extra early-born RP cells;  $n=58$  hemisegments from 12 embryos; Fig. 3B). We conclude that Dan and Danr function to limit the number of early-born neurons in the NB3-1 lineage, similar to their role in the NB1-1 and NB7-1 lineages. Additionally, similar to the NB7-1 lineage, we found at low frequency extra Hb<sup>-</sup> later-born neurons (12.1% of the hemisegments,  $n=58$ ), suggesting that although Dan and Danr might be primarily required for limiting the number of early-born progeny, they might also have a broader role in regulating temporal identity throughout the Hb→Cas expression window (see Discussion).

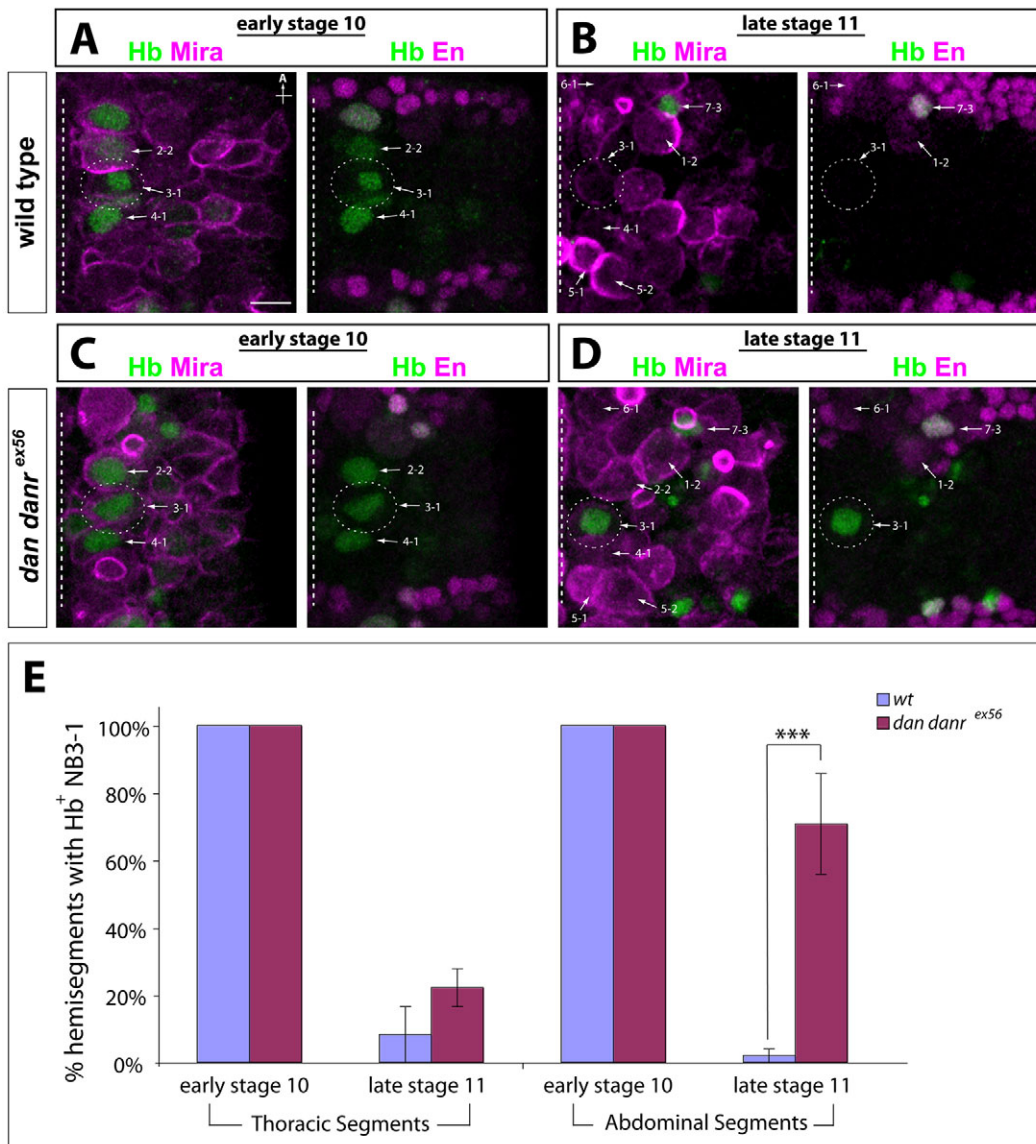
NB7-3 generates a short lineage: the first GMC makes the EW1 interneuron and its GW motor neuron sibling, followed by GMCs that generate EW2/sib and then EW3 (Isshiki et al., 2001). All four neurons in this lineage are Eagle<sup>+</sup>, but only the first-born EW1/GW neurons are Hb<sup>+</sup> (schematic, top panel of Fig. 3C). In the NB7-3 lineage, *dan danr* mutants showed ectopic EW1/GW neurons;

however, the phenotype was quite rare, observed in only 3.6±1.6% of hemisegments ( $n=136$  hemisegments from eight embryos; Fig. 3D). As per NB7-1 and NB3-1 lineages, we also infrequently observed hemisegments with extra late-born EW2/EW3 neurons (in two of the 136 hemisegments quantified, e.g. Fig. 3D) and, more frequently, examples in which there were fewer or a total loss of cells in this lineage (21.3% of hemisegments;  $n=136$  from eight embryos). This latter phenotype might arise in part from the failure of NB7-3 to form (data not shown). We conclude that Dan and Danr limit the early-born neurons in all four lineages examined (1-1, 7-1, 3-1 and 7-3). However, the fact that there was lineage-specific variability in how many extra cells were generated, how many hemisegments per embryo had a phenotype, as well as in the specific type of phenotype (i.e. how frequently extra late-born neurons were observed) suggests that additional lineage-specific regulatory components interact with Dan and/or Danr (see Discussion).

### *dan danr* mutant neuroblasts have prolonged Hb expression

One mechanism by which *dan danr* mutants could generate an extra early-born GMC in multiple lineages is by a slight extension of Hb neuroblast expression. We focused our attention on NB3-1, which is easy to identify owing to its relatively late delamination and persistent superficial position. In wild-type embryos, the Hb<sup>+</sup> NB3-1 formed at early stage 10 and downregulated Hb by late stage 11 (Fig. 4B,E). In wild type, Hb downregulation in NB3-1 was tightly correlated with the formation of NB7-3, so we used this as a temporal reference point. In *dan danr* mutants, neuroblasts delaminated at their normal developmental stages, including NB3-1 at early stage 10 (Fig. 4C), and NB7-3 at late stage 11 (Fig. 4D) but NB3-1 maintained Hb expression past the time of NB7-3 formation. This was most apparent in the abdominal segments, in which 70.8% of the 3-1 neuroblasts still maintained Hb expression ( $n=24$  from





**Fig. 4. *dan danr* mutants show prolonged Hb expression in NB3-1.** (A-D) Wild-type (A,B) or *dan danr* mutant (C,D) embryos stained for Hb and the pan-neuroblast marker Miranda (Mira; magenta) or the row 6/7 neuroblast marker Engrailed (En; magenta). All panels show one hemisegment bordered by the engrailed domain from two adjacent hemisegments. Anterior is up and midline is to the left (dashed line). Scale bar: 10  $\mu$ m. In wild-type at early stage 10 (A), NB3-1 has formed and is Hb<sup>+</sup>. By late stage 11 (B), NB3-1 has downregulated Hb concurrent with formation of NB7-3. In *dan danr* mutant embryos at early stage 10 (C), NB3-1 has formed and is Hb<sup>+</sup>. By late stage 11 (D), NB3-1 shows prolonged Hb expression past the time that NB7-3 forms. (E) Quantification of Hb expression in NB3-1 in wild-type and *dan danr* embryos. \*\*\**P*<0.001, calculated using the two-sample *t*-test. Error bars indicate s.e.m.

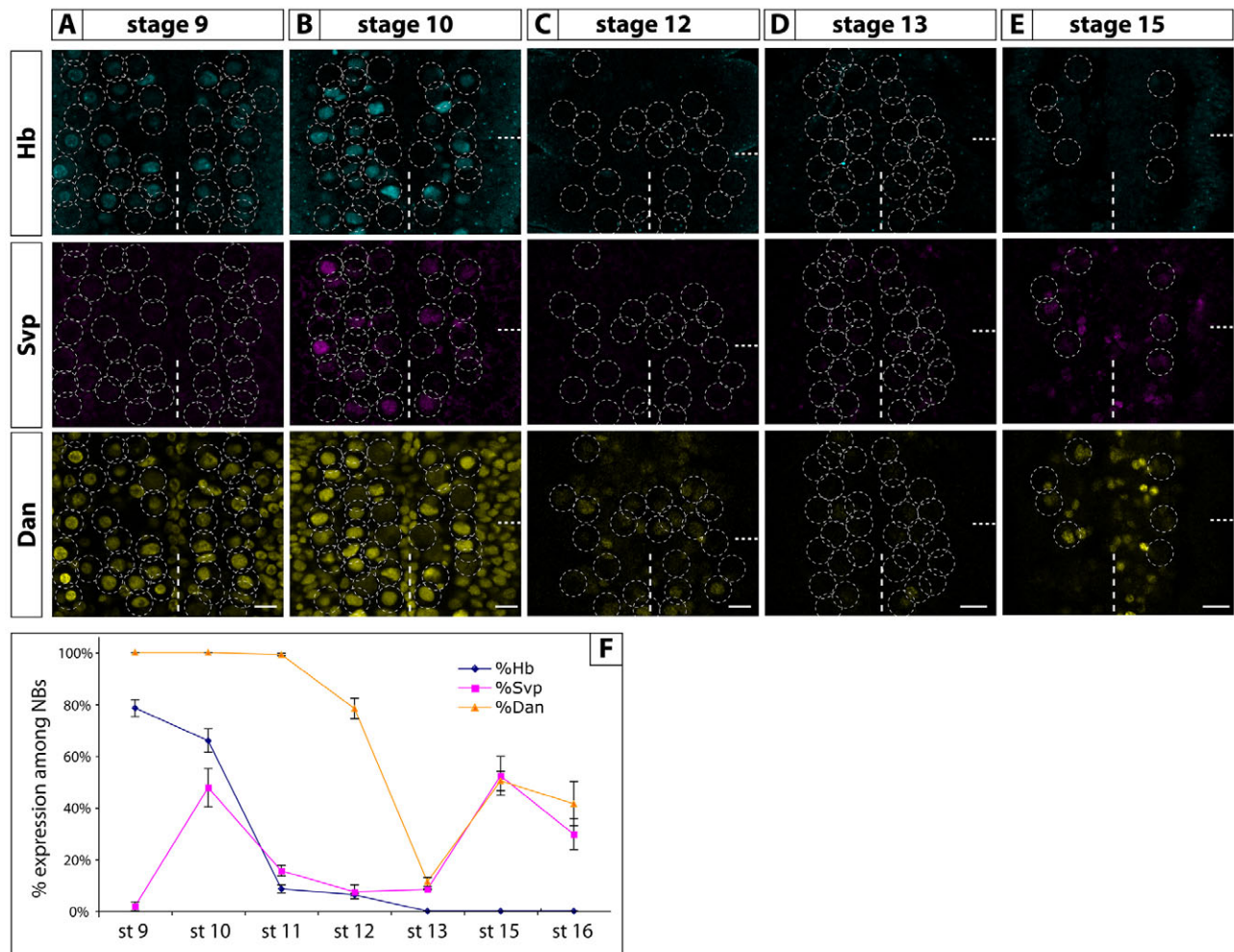
three embryos) compared with only 2.0% in wild type ( $n=42$  hemisegments from five embryos) (Fig. 4D,E). NB3-1 in thoracic segments were also similarly affected, though to a lesser degree (Fig. 4E). These data indicate that Hb expression in NB3-1 is prolonged in *dan danr* mutant embryos, consistent with the presence of ectopic RP1/4 early-born neurons in this lineage, and suggest that the same occurs in other neuroblasts that produce extra early-born neurons in the *dan danr* mutant embryos. However, misexpression of Dan (using *en-gal4 UAS-dan*) did not result in premature loss of *hb* expression or lack of early-born neurons in the NB7-1 lineage (data not shown), suggesting that the timely downregulation of Hb in neuroblasts does not occur through direct repression of *hb* by Dan.

### Dan is expressed during and after the Hb neuroblast expression window

To gain better insight into how Dan and Danr might be regulating Hb expression in neuroblasts, we analyzed the timing of Dan neuroblast expression relative to that of Hb and Svp, a previously identified negative regulator of Hb expression (Fig. 5; see Fig. S3 in the supplementary material). We found that Dan was broadly and robustly expressed in all newly delaminated Hb<sup>+</sup> neuroblasts, and

its expression extended well beyond the Hb temporal window, fading at early stage 12 (Fig. 5B,C) at the beginning of the Cas window (see Fig. S4 in the supplementary material). In situ hybridization using probes specific to *dan* or *danr* revealed that the two genes have similar expression patterns: robust expression by neuroepithelia at late stage 8 and young neuroblasts at stages 9-10, and expression by a subset of neurons at late embryonic stages (see Fig. S1 in the supplementary material, in situ images for neurons not shown). We cannot rule out the possibility that there might be subtle differences in the levels of *dan* versus *danr* expression in specific NB lineages, yet our in situ data indicate that *dan* and *danr* have remarkably similar expression patterns in the CNS. In contrast to the temporally broad expression of both *dan* and *danr*, we confirmed previous observations (Kanai et al., 2005) that Svp is only transiently expressed in neuroblasts just as Hb is being downregulated (Fig. 5). Such distinct temporal expression of Dan and Svp in neuroblasts suggests they have very different functions in limiting the Hb expression window (see Discussion).

Interestingly, Dan and Svp both displayed a second wave of neuroblast expression that peaked at stage 15 (Fig. 5E,F); the function of this later expression is unknown. Dan expression was



**Fig. 5. Temporal profile of Hb, Svp and Dan neuroblast expression.** (A-E) Wild-type embryos of the indicated stages quadruple labeled for Hb (cyan), Svp (magenta), Dan (yellow) and the pan-neuroblast marker Miranda (dashed circles; staining not shown, but see Fig. S3 in the supplementary material). Segments T3 and A1 are shown separated by horizontal dashed line; ventral midline indicated by vertical dashed line. Hb is expressed in newly formed neuroblasts at stages 9-10 (A,B) and becomes undetectable by stage 12 (C). Svp is detected transiently at stage 10 (B) as Hb levels are declining. Dan is expressed in newly formed neuroblasts until stage 12 (A-C) and is undetectable by stage 13 (D). Note the late burst of Svp and Dan neuroblast expression at stage 15 (E). (F) Quantification of Hb, Svp and Dan expression in neuroblasts at stages 9-16. Percentages indicated are the average of three to four embryos. Error bars indicate s.e.m. 100-200 neuroblasts were scored for expression of Hb, Dan and Svp in each embryo for each developmental stage. Scale bars: 10  $\mu$ m.

also observed in post-mitotic neurons (see Fig. S5 in the supplementary material), predominantly the early-born neurons in the deeper layers of the CNS (Isshiki et al., 2001). Most early-born Hb<sup>+</sup> neurons were also Dan<sup>+</sup>, although there were a few Hb<sup>+</sup> neurons that did not express Dan (see Fig. S5 in the supplementary material). By contrast, Dan expression did not overlap with the superficially localized later-born Cas<sup>+</sup> neurons. Because Dan expression overlaps with that of Hb in neuroblasts and post-mitotic neurons, we conclude that Dan and Danr are likely to act indirectly, rather than by direct repression, to limit Hb expression in neuroblasts.

### Dan and Svp function independently to downregulate Hb in neuroblasts

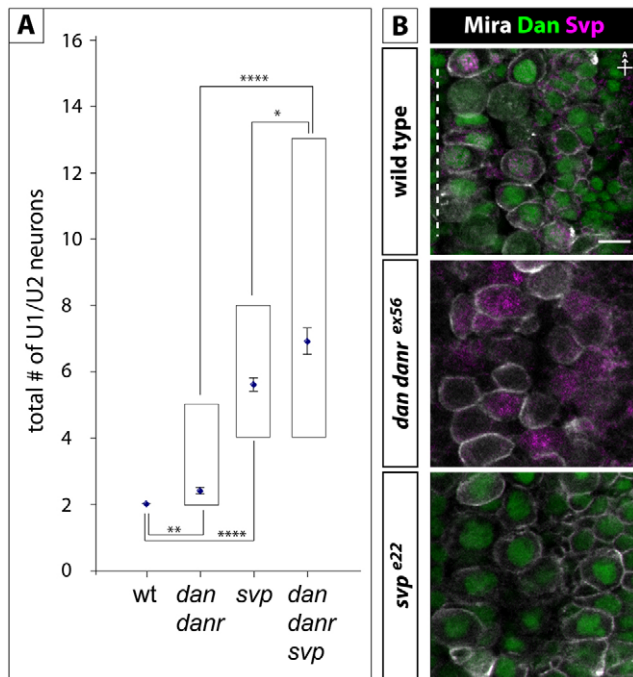
The difference in Dan and Svp neuroblast expression patterns suggests that they have very different functions in specifying temporal identity. Here, we test whether Dan and Svp act in a linear pathway or parallel pathways to limit the number of early-born neurons. We generated a *dan danr svp* triple mutant and observed

an additive phenotype that showed significantly more early-born neurons in the NB7-1 lineage than that observed in either *dan danr* or *svp* mutants alone. In the wild-type NB7-1 lineage, there were 2.0 Hb<sup>+</sup>, early-born neurons. In the *dan danr* mutant there was an average of 2.4 early-born neurons, in the *svp* mutant there was an average of 5.6 early-born neurons, whereas in *dan danr svp* triple mutants there were 6.9 of these neurons (Fig. 6A). In addition, Dan expression was not lost in *svp<sup>e22</sup>* mutants, and Svp expression was not lost in *dan danr* mutants (Fig. 6B). We conclude that Dan/Danr and Svp function in independent pathways that are both required for the timely downregulation of Hb in neuroblasts.

### Hb can induce Dan and Svp expression in neuroblasts

Dan and Hb are coexpressed in the neuroepithelia just prior to neuroblast delamination, and Svp expression is observed immediately following Hb in neuroblasts, raising the possibility that Hb might activate Dan, Danr and/or Svp. We observed no change in





**Fig. 6. Dan/Danr and Seven-up act independently to limit production of early-born neurons.** (A) *dan danr svp* triple mutants form more early-born U1/U2 neurons than either *dan danr* or *svp* mutants alone. All alleles used are null (see Materials and methods). U1/U2 neurons were identified as  $Eve^+ Hb^+ Cut^-$ . The blue diamond indicates the average, error bars indicate s.e.m. and the bar indicates the range. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\*\* $P \leq 0.0001$ . All  $P$  values were calculated using the two-sample T-test. (B) Dan and Svp do not regulate each other's expression. Neuroblast expression of Dan (green) is normal in *svp* mutants, as is Svp expression (magenta) in *dan danr* mutants. All panels show representative region in the neuroblast layer at stage 10. Anterior is up and midline is to the left (dashed line). Scale bar: 10  $\mu$ m.

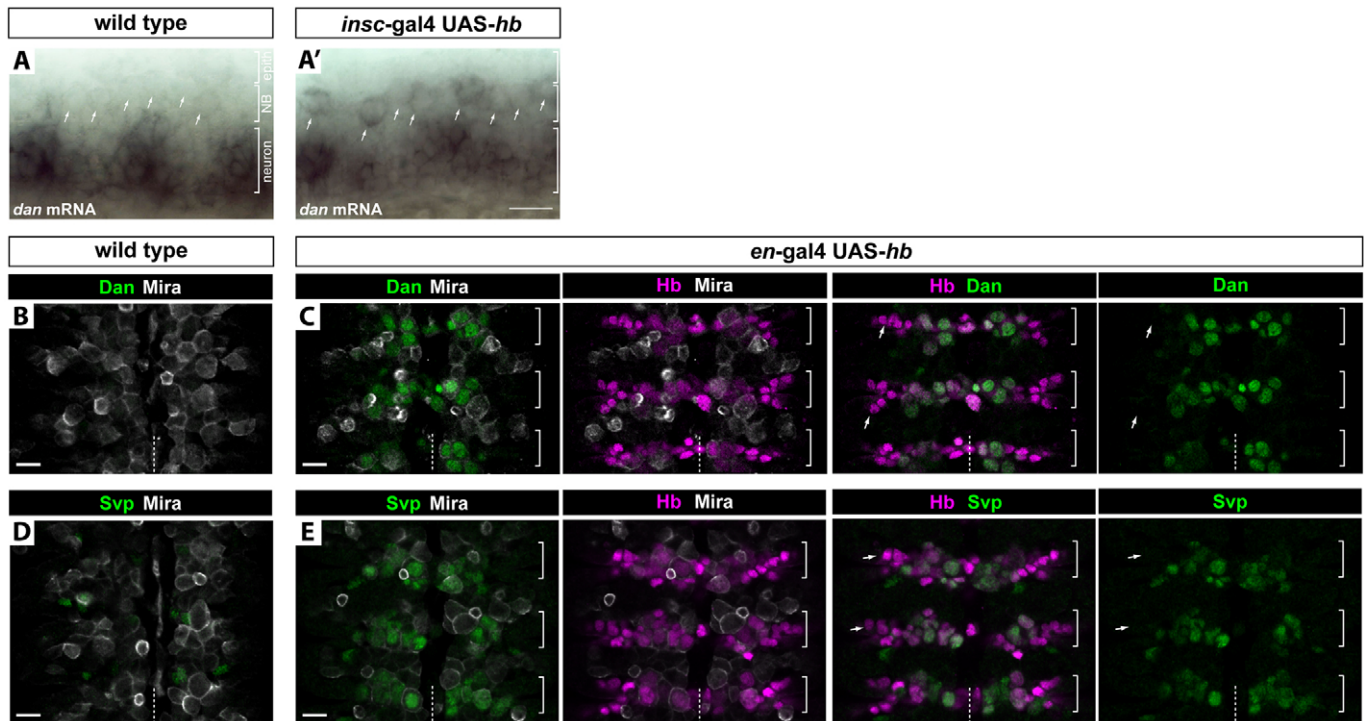
Dan expression in *hb* mutants in both young neuroblasts (see Fig. S6B in the supplementary material) and in postmitotic neurons (see Fig. S6E in the supplementary material). However, at the stage when Dan is normally no longer expressed by neuroblasts (stage 13; see Fig. S7, top left panel and Fig. S7B in the supplementary material) *dan* transcription could be strongly upregulated following Hb overexpression in all neuroblasts (Fig. 7A') or a subset of neuroblasts (Fig. 7C). Dan expression was also activated by Hb misexpression in neuroectoderm, although this was only detectable before stage 12 (data not shown). By contrast, Hb did not activate Dan expression in the dorsal non-neural ectoderm or in any epithelial cells past stage 12 (Fig. 7C, arrows), suggesting Hb activation of Dan is dependent on cell type and developmental stage. Similarly, Hb could induce Svp expression in neuroblasts (compare Fig. S7 in the supplementary material, top right panel and Fig. 7D with 7E), but not in neuroectoderm or epidermis (Fig. 7E, arrows). Thus, Hb can induce neuroblast expression of both Dan and Svp, suggesting that Hb itself participates in a feedback loop to help determine the precise timing with which the neuroblast generates later-born fates.

## DISCUSSION

Here, we show that Dan and Danr are required to limit Hb expression in neuroblasts and restrict the number of early-born neurons generated in multiple neuroblast lineages. The orphan

nuclear hormone receptor protein Svp also functions to limit Hb expression in neuroblasts, and our data strongly suggest that Dan/Danr and Svp function in parallel pathways that are each independently required. First, the temporal expression patterns of Dan and Danr versus Svp do not suggest their coordinated activity: Dan and Danr are expressed from the time of neuroblast formation (stage 9), beyond Hb downregulation (stage 10), until the time of strong Castor expression (stage 12). By contrast, Svp protein is very transiently detected in neuroblasts only at the onset of Hb downregulation. Second, Dan/Danr and Svp are not in a linear transcriptional hierarchy: neither mutant affects expression of the other gene. Third, *dan danr* and *svp* mutants have distinct phenotypes: for example, compared with the *dan danr* mutant, the *svp* mutant has many more early-born neurons in the NB7-1 lineage, whereas it does not have any extra early-born neurons in the NB1-1 lineage. Fourth, the *dan danr svp* null triple mutant has the summed phenotypes of the *dan danr* double mutant and the *svp* single mutant. Fifth, misexpression of Svp, but not Dan, can repress *hb* transcription in neuroblasts (Kanai et al., 2005) (data not shown). The fact that neither one appears to have an effect on cell fate when misexpressed in postmitotic neurons suggest that both Svp and Dan function at the level of the mitotic precursors. Taken together, it appears that Dan/Danr and Svp are each required to downregulate *hb* expression in neuroblasts, but do so using separate mechanisms. The data are consistent with Svp directly repressing neuroblast *hb* transcription (although this has not been shown) whereas Dan and Danr act more indirectly.

Do Dan, Danr and Svp have lineage-specific functions? Despite the widespread expression of Dan and Danr in early neuroblasts (Fig. 5; see Fig. S1 in the supplementary material) and the widespread transient expression of Svp in most neuroblasts (Broadus et al., 1995; Kanai et al., 2005) (Fig. 5), it is likely that each has lineage-specific functions. For example, in the NB1-1 lineage, ectopic early-born neurons are generated in *dan danr* mutants, but not in *svp* mutants. Further comparing Dan versus Danr in this lineage, it appears that Danr is more important than Dan, because the *danr<sup>ex35</sup>* single mutant phenocopies the *dan danr<sup>ex56</sup>* double mutant in the number of ectopic aCC/pCC neurons generated and the number of hemisegments affected per embryo. In contrast to the NB1-1 lineage, Dan and Danr each appear to be required for limiting the number of early-born neurons in the NB7-1 lineage, as *danr<sup>ex35</sup>* single mutants had a weaker phenotype than the *dan danr* double mutant. Additionally, there are more early-born neurons in the NB7-1 lineage in *svp* mutants than in *dan danr* mutants, highlighting their lineage-specific differences. These differences might be due to different levels or functions of each protein in distinct neuroblasts. For example, there is variability in *dan* and *danr* mRNA levels between neuroblasts (see Fig. S1 in the supplementary material), suggesting that distinct neuroblasts might have different levels of Dan and/or Danr protein (although Dan protein levels appear constant between newly formed neuroblasts), or that they express Dan and/or Danr protein for different durations. Alternatively, or in addition, the lineage-specific variation might be due to unique cofactors present in different neuroblasts. This seems likely, as Hb misexpression in all neuroblasts has varying effects within different lineages. For example, NB1-1 generates only one to three ectopic early-born neurons in response to Hb misexpression, whereas NB7-1 generates ~20 ectopic early-born neurons (Isshiki et al., 2001; Pearson and Doe, 2003). Consistent with the notion that co-factors can alter the functional output of transcriptional regulators, recent evidence shows that the co-regulator CtBP forms complexes with distinct eye specification



**Fig. 7. Hb can activate Dan and Seven-up expression in neuroblasts.** (A) Wild-type stage 13 embryo lateral view showing that *dan* mRNA is not detected in neuroblasts (NB; arrows), but is present in neurons (white brackets). (A') *insc-gal4 UAS-hb* stage 13 embryo lateral view where Hb is overexpressed in all neuroblasts; note that *dan* mRNA is upregulated in neuroblasts (arrows). Epith, epithelia. (B, C) Overexpression of Hb upregulates Dan protein in neuroblasts. (B) Wild-type stage 13 embryo, ventral view of neuroblast layer, labeled with Dan (green) and the neuroblast marker Miranda (Mira; white). Note that Dan protein is mostly undetectable in neuroblasts at this stage. (C) *en-gal4 UAS-hb* stage 13 embryo, ventral view of neuroblast layer, labeled for Dan (green), Hb (magenta) and the neuroblast marker Miranda (white). Misexpression of Hb in the row 6/7 neuroblasts (brackets) results in Dan protein in these neuroblasts. Note that at this stage, Hb misexpression activates Dan in neuroblasts, but not in ectoderm (arrows). (D, E) Overexpression of Hb upregulates Svp protein in neuroblasts. (D) Wild-type stage 13 embryo, ventral view of neuroblast layer, labeled with Svp (green) and the neuroblast marker Miranda (white). Svp is detectable in only a few neuroblasts at this stage. (E) *en-gal4 UAS-hb* stage 13 embryo, ventral view of neuroblast layer, triple labeled for Svp (green), Hb (magenta) and the neuroblast marker Miranda (white). Misexpression of Hb in the row 6/7 neuroblasts (brackets) results in Svp protein in neuroblasts, but not in ectoderm (arrows). All panels show segments T2, T3 and A1 (top to bottom); ventral midline is indicated by the dashed line. Scale bars: 10  $\mu$ m.

factors, including Dan and Danr, to regulate proliferation versus differentiation during eye development in *Drosophila* (Hoang et al., 2010).

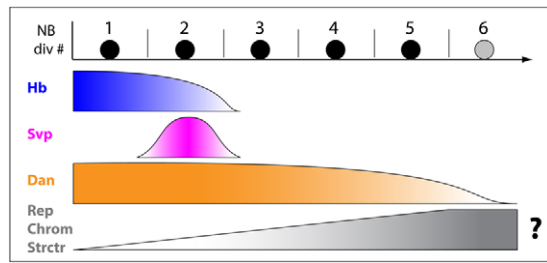
Do Dan and Danr function redundantly? We are unable to rigorously test this hypothesis owing to the lack of a *dan* null single mutant, but the available evidence suggests that they do have redundant functions. First, they have nearly identical expression patterns. But most crucially, overexpression of Dan in the *dan danr* double mutant can nearly completely rescue the CNS phenotype (see Fig. S2 in the supplementary material), suggesting that high enough levels of Dan can compensate for loss of Danr. However, a *danr* null single mutant shows a strong phenotype in the NB1-1 lineage and a partial phenotype in the NB7-1 lineage, suggesting that endogenous levels of Dan are insufficient for normal CNS development. The most parsimonious explanation is that each protein has equivalent function, but that both genes are required to generate sufficient levels of Dan/Danr protein.

We have observed that Hb overexpression can activate expression of both Dan and Svp. What is the significance of this activation? Previous work has shown that Hb can function both as a transcriptional activator and a repressor. Although its repressive functions are required for the neuroblast to specify early-born fates and maintain neuroblast competence (Tran et al., 2010), its

activator functions remain elusive. One possibility is that Hb-mediated activation of Svp, and the subsequent Svp-mediated downregulation of Hb, create a negative feedback loop to ensure timely progression of the neuroblast to later temporal fates. This is not unlike what has been observed for Cas, which activates feed-forward and feed-back transcriptional cascades to regulate temporal identity in the NB5-6 lineage (Baumgardt et al., 2009). By contrast, Hb activation of Dan expression might be part of the mechanism by which Hb maintains neuroblast competence, because Dan is unlikely to repress *hb* expression directly (see below).

What might be the mechanism by which Dan and Danr function to restrict the duration of Hb expression in neuroblasts? Some clues might come from the fact that Dan and Danr are found in a subgroup of pipsqueak-domain containing nuclear proteins that have been proposed to regulate higher order chromatin structure by targeting distal DNA elements (Siegmond and Lehmann, 2002). Pipsqueak, the founding member of the family, has been shown to recruit Polycomb group complexes to specific regions of the genome to mediate gene silencing (Huang et al., 2002). Perhaps Dan and Danr modify chromatin structure through recruitment of chromatin remodeling complexes, which indirectly affects *hb* transcription by changing the accessibility of the *hb* locus to other transcriptional regulators (Fig. 8). Such a function in modulating chromatin





**Fig. 8. Model for expression and function of Dan and Svp in NB7-1.** Schematic of NB7-1 after each cell cycle to produce the U1-U5 neurons. Hb (blue) is expressed at high levels in the newly formed NB7-1, but is downregulated after two divisions. Svp (magenta) is transiently expressed and might directly repress *hb* expression in neuroblasts. Dan (orange) is expressed at high levels past the Hb window and might establish a chromatin structure that reduces Hb expression and/or function, here represented by the increasing repressive chromatin structure (gray).

architecture might not be restricted to regulating just *hb* expression, but can extend to other temporal identity factors as well. Indeed, in NB7-1, the initial Hb→Cas ‘competence window’, during which the U1-U5 motor neurons are generated (Pearson and Doe, 2003; Cleary et al., 2006), matches nearly exactly the window of Dan and Danr expression. This raises the possibility that Dan and Danr might have a more global role in NB temporal progression by stabilizing ‘transition states’ between successive temporal identity factors (e.g. Hb→Kr, Kr→Pdm or Pdm→Cas). Such a function might explain the low frequency misregulation of later-born neuron numbers in several lineages (7-1, 3-1, 7-3), in addition to the extra early-born neurons phenotypes. Future experiments that address the role of Dan and Danr in later temporal identity transitions will provide a better understanding of the mechanisms that control the progression of temporal identity in neuroblasts.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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