Development 133, 429-437 doi:10.1242/dev.02229

Timing of identity: spatiotemporal regulation of *hunchback* in neuroblast lineages of *Drosophila* by Seven-up and Prospero

Ulrike Mettler, Georg Vogler and Joachim Urban*

Neural stem cells often generate different cell types in a fixed birth order as a result of temporal specification of the progenitors. In *Drosophila,* the first temporal identity of most neural stem cells (neuroblasts) in the embryonic ventral nerve cord is specified by the transient expression of the transcription factor Hunchback. When reaching the next temporal identity, this expression is switched off in the neuroblasts by *seven up* (*svp*) in a mitosis-dependent manner, but is maintained in their progeny (ganglion mother cells). We show that *svp* mRNA is already expressed in the neuroblasts before this division. After mitosis, Svp protein accumulates in both cells, but the downregulation of *hunchback* (*hb*) occurs only in the neuroblast. In the ganglion mother cell, *svp* is repressed by Prospero, a transcription factor asymmetrically localised to this cell during mitosis. Thus, the differential regulation of *hb* between the neuroblasts and the ganglion mother cells is achieved by a mechanism that integrates information created by the asymmetric distribution of a cell-fate determinant upon mitosis (Prospero) and a transcriptional repressor present in both cells (Seven-up). Strikingly, although the complete downregulation of *hb* is mitosis dependent, the lineage-specific timing of *svp* upregulation is not.

KEY WORDS: *Drosophila*, Temporal specification, Neuroblast, *Seven-up*, *Prospero*, *Hunchback*

INTRODUCTION

The central nervous system derives from neural stem cells generating progeny that often acquire specific neural fates in a fixed temporal sequence. Thus, the patterning of the neural tissue occurs not only in spatial but also temporal dimensions. One of the best-studied examples for temporal patterning is the development of the retina in vertebrates. It has been shown that the multipotent precursor cells produce lineages that consist of a number of different cell types that are produced in a certain temporal order (Cepko, 1999; Harris, 1997; Ohnuma et al., 1999). Similarily, the neural stem cells of the mammalian cortex always generate the cells of the different cortical layers in the same sequence (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell, 1992; McConnell and Kaznowski, 1991). In both examples, the combination of extrinsic and intrinsic factors is needed for the correct development of the stem cells along the time axis. However, the mechanisms that lead to the temporal specification of cell fates are still largely unknown.

Recently, the embryonic neuroblasts (NBs) of *Drosophila* have been established as a model system to tackle this issue (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). These NBs are multipotent precursor cells that divide in a stem cell mode generating a chain of ganglion mother cells (GMCs). These GMCs subsequently perform one additional division to produce two postmitotic cells, which can be neuronal or glial in nature. Each NB in the developing ventral nerve cord (VNC) can be identified individually (Broadus et al., 1995), and produces a specific and reproducible set of progeny, always in the same temporal sequence (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). In *Drosophila*, this change of temporal identity of NBs is thought to occur cell autonomously, and is linked to the sequential expression of the transcription factors Hunchback (Hb), Krüppel (Kr), Pdm1/Pdm2, Castor (Cas) and Grainyhead (Grh) (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). The genes encoding these transcription factors are consecutively expressed in a given NB in certain time windows. At the end of each time window, the expression is switched off in the NB but stays on in the GMC and its progeny. For two of these factors, Hb and Kr, it has been shown that they are indeed necessary and sufficient to specify the fate of the early born cells in certain NB lineages (Isshiki et al., 2001; Novotny et al., 2002). Moreover, *hb* has been shown to be able to keep certain NBs in a multipotent state, which becomes restricted after *hb* expression has vanished from the cell (Pearson and Doe, 2003). Thus, the temporal specification of NB progeny, as well as the developmental competence of the NB, depends strongly on the correct timing of the on and off switch of the temporal specification genes within the NB. Recently it has been shown that *svp* (*seven-up*) is required to switch off *hb* at the proper time (Kanai et al., 2005). Unlike for the other temporal specification genes, this off switch is dependent on the process of mitotic cell division (Isshiki et al., 2001; Großkortenhaus et al., 2005).

In this work, we concentrate on two questions: how is the timing of *svp* activity regulated and why is *hb* switched off only in the NB and not in the GMC after mitotic division? Our results show that the maintenance of *hb* expression in the GMC is dependent on the asymmetrically distributed cell-fate determinant Prospero (Pros). This transcription factor antagonises *svp* activity in the GMC, thereby inhibiting the downregulation of *hb* in this cell. Additionally, we provide evidence that the *svp* mRNA is not translated efficiently before mitosis; this probably leads to the observed link of *svp* activity to mitosis. Finally, we show that the lineage-specific timing of *svp* expression is independent of the number of NB divisions.

Institut für Genetik, Universität Mainz, 55099 Mainz, Saarstraße 21, Germany.

*Author for correspondence (e-mail: jurban@uni-mainz.de)

Accepted 29 November 2005

MATERIALS AND METHODS

Fly stocks and genetics

The following fly strains were used in this work: Oregon R (wild type, Bloomington Stock Center), *pros^{C7}/TM3-ftz-lacZ* (F. Matsuzaki, Kobe, Japan), *pros^{17}/TM6b* and *Tb* (A. Hildago, Birmingham, UK), *svp^{e22}/TM3-Ubx-lacZ* (M. Hoch, Bonn, Germany), and *Df (3L)H99/TM6b* and *abd A-lacZ* (H. Steller, New York, USA). The *svp^{e22}, pros^{C7}/TM3-ftz-lacZ* and *pros^{C7}, Df(3L)H99/TM3-ftz-lacZ* double mutant strains were generated by classical genetic recombinations. Ectopic expression of *svp* and *pros* was accomplished by use of the GAL4/UAS strategy (Brand and Perrimon, 1993). To misexpress *svpI*, we combined a second and third chromosomal insertion of UAS-*svpI* (a gift of M. Hoch), crossed this line to *en*-GAL4 (Bloomington Stock Center) and let the embryos develop at 29°C (for scoring the NB7-1 lineage). To misexpress *pros*, we crossed UAS-*pros* (H. Vässin, Columbus, OH) to *en*-GAL4 at 29°C for scoring the NB7-3 lineage.

Generation of the anti-Hb antibody

For protein expression, BL21 bacteria were transformed with the expression plasmid pAR-6His (Kosman et al., 1998). Protein purification was carried out by the use of Ni-NTA spin columns (Qiagen) under denaturing conditions, as described in the manual. After purification, the protein was dialysed with phosphate buffered saline (PBS, pH 7.4). Guinea pigs were immunised with purified protein (BioGenes, Berlin), and the obtained serum tested in embryos for specific Hb staining and then used preabsorbed without further purification.

Immunohistochemistry

Overnight egg collections at 25°C or 29°C were stained and dissected, as previously described (Nose et al., 1992; Patel, 1994). The following primary antibodies were used: rabbit anti-Hunchback (1:500, J. Reinitz), guinea pig anti-Hunchback (1:1000), mouse anti-Eagle (1:10, M. Freeman and C. Q. Doe, Eugene, OR), rabbit anti-Eagle (1:500) (Dittrich et al., 1997), mouse anti-Svp (Kanai et al., 2005) (signal amplification with TSA-Kit, Perkin Elmer), rat anti-Zfh-2 (1:200, M. Lundell, San Antonio, USA), rabbit anti-β-Gal (1:1000, Cappel), rabbit anti-Repo (1:500) (Halter et al., 1995), rabbit anti-Eve (1:5000, M. Frasch, New York, USA), mouse anti-Eve (1:2, Developmental Studies Hybridoma Bank). The following secondary antibodies were used: anti-rabbit-FITC, anti-guinea pig Cy3, anti-mouse Cy5, anti-rat Cy3 (1:250, all obtained from Dianova). Flat preparations of embryos were mounted in Vectashield mounting medium (Vector Laboratories). Embryos were analysed with a confocal laser scanning microscope (Leica TCS SP2). Scanning images were processed with Adobe Photoshop. All images show projections of multiple focal planes. Original scans will be provided upon request. In all images anterior is upwards.

In situ hybridisation

Fixed embryos were incubated for 10 minutes in 0.1% sodium borohydride in PBT to reduce autofluorescence. Whole-mount RNA in situ hybridisation was carried out as described previously (Jiang et al., 1991) using digoxigenin-(Dig) or FITC-labelled RNA probes made from *hb* (Margolis et al., 1995) and *svp1* (Mlodzik et al., 1990) cDNA. Probes were detected using anti-Dig-POD or anti-FITC-POD, depending on the labelling (1:1000, Roche), and the signal was amplified using the TSA amplification kit with Tyramide-Cy5 or Tyramide-Cy3 (Perkin Elmer). Additional antibody staining was performed after the final amplification step.

RESULTS

Maintenance of *hb* expression within the GMCs is mediated by Prospero

It has recently been shown that the up- and downregulation of Hb in the NBs is regulated on the transcriptional level (Großkortenhaus et al., 2005), and that it is switched off by the activity of Svp, a member of the orphan receptor family of zinc finger transcription factors (Kanai et al., 2005). However, *svp* mRNA expression has already started before the NB divides, after which *hb* expression is terminated. As a result both progeny, the NB and the GMC, inherit *svp* mRNA and produce Svp protein, although the GMC continues to express *hb* (Kanai et al., 2005). This suggests that one or more GMC-specific factors are able to suppress the Svp-mediated repression of *hb* within the GMC. Good candidates for such factors are the asymmetrically segregating cell-fate determinants Numb and Prospero (Pros). Both of these proteins form a basal crescent within the NB prior to division, and both are inherited only by the newly formed GMC (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). Therefore, we analysed the *hb* expression in loss-of-function alleles of these genes. Although we did not find an obvious difference in the number of Hb-positive (Hb$^+$) cells in the absence of Numb function (data not shown), there was a strong reduction in the number of these cells in *pros* mutant embryos (compare Fig. 1A and 1B).

pros codes for a homeodomain transcription factor that enters the nucleus of the GMC after mitosis, subsequently regulating GMC-specific gene expression (Chu-Lagraff et al., 1991; Doe et al., 1991; Spana and Doe, 1995; Vässin et al., 1991). To confirm that the observed reduction of Hb$^+$ cells is indeed due to a lack of *hb* maintenance within the GMCs and their progeny, we compared the timing of *hb* expression within different lineages between wild type and the *pros* loss-of-function alleles *pros^{C7}* and *pros^{17}*. We analysed the lineages of the thoracic NB2-4T and NB6-4T, as well as the abdominal NB7-3. As in wild type, NB7-3 in both *pros* alleles is initially Hb$^+$ and generates a Hb$^+$ GMC (GMCa) after its first division (compare Fig. 2A and 2G). At early stage 12, the NB is Hb-negative (Hb$^-$) and generates a second GMC (GMCb) that is Hb$^-$ too. At this stage, GMCa maintains *hb* expression in wild type, whereas this is reduced or already undetectable in *pros* mutants (compare Fig. 2B and 2H). In stage 14 *pros* mutant embryos, 100% of the NB7-3 derived cell clusters (n=110) do not show any Hb$^+$ cells, whereas there are two cells in wild type, the EW1 and GW neurons (compare Fig. 2C and 2I). To rule out that the lack of Hb$^+$ cells in NB7-3 is due to a loss of these cells by programmed cell death, we recombined *pros^{C7}* with the deficiency *H99* to prevent apoptosis (White et al., 1994). Again, only Hb$^-$ progeny of NB7-3 were found in later stages (Fig. 2O, n=70), confirming that the phenotype is indeed due to lack of *hb* maintenance. Consistent with its role as a repressor of *hb*, we see the opposite phenotype in *svp* mutants: here the NB stays Hb$^+$ after its first division and produces at least one additional Hb$^+$ GMC before becoming Hb$^-$ (Fig. 2D-F).

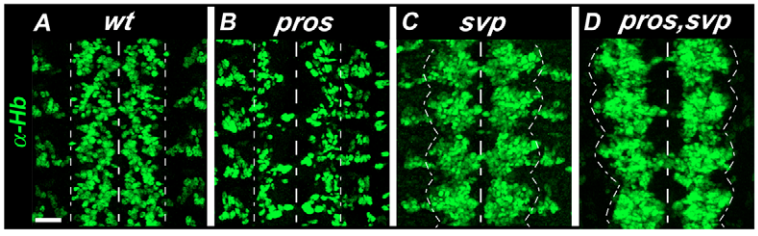

Fig. 1. *pros* and *svp* mutants show an opposite phenotype with respect to neural Hunchback expression. Dorsal views of wild-type (**A**), *pros^{C7}* (**B**), *svp^{e22}* mutant (**C**) and *pros^{C7}, svp^{e22}* double mutant (**D**) embryos at stage 14, stained for Hb protein (green). Thin dashed lines indicate lateral borders of the ventral nerve cord; thick dashed line indicates the midline. (B) In the absence of *pros* function there are fewer Hb-expressing cells within the ventral nerve cord than in wild type (A), whereas *svp^{e22}* mutant (C) and *pros^{C7}, svp^{e22}* double mutant embryos (D) show more Hb-expressing cells. Scale bar: 20 μm.

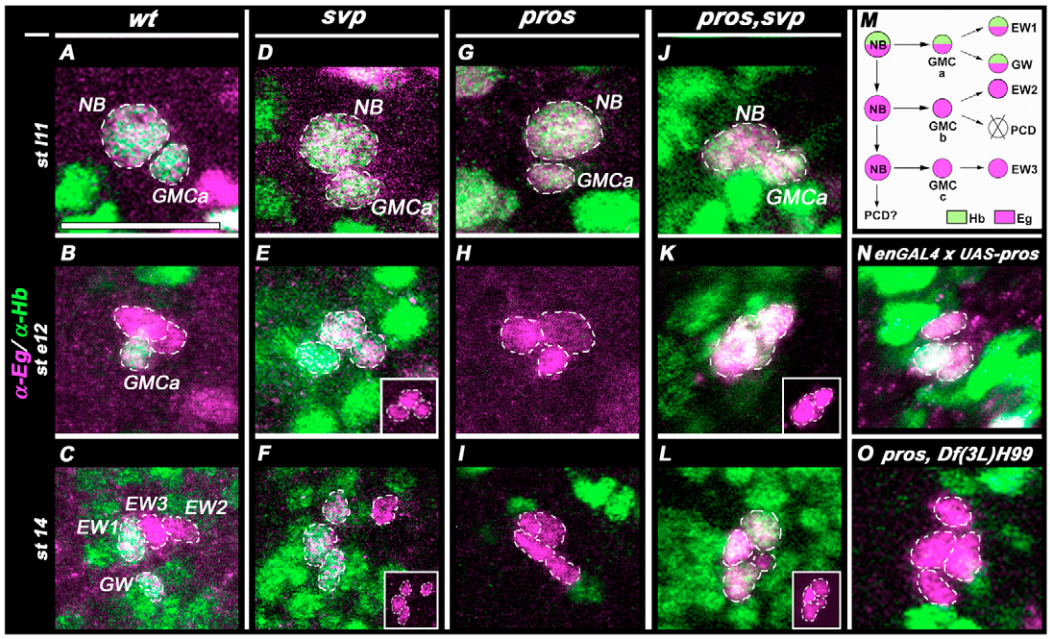

Fig. 2. Regulation of *hb* within the NB7-3 lineage. Embryos double labelled for the NB7-3 lineage marker Eg (magenta) and Hb (green). Midline is towards the left. Insets show Eg expression separately. (**A-C**) Hb expression during normal NB7-3 lineage development. At late stage 11 the NB has divided once to generate the Hb$^+$ GMC7-3a (A). After *hb* downregulation the NB has divided again, and Hb is only detectable in GMC7-3a (B). At stage 14 the progeny of GMC7-3a (GW and EW1) express Hb, whereas the progeny of GMC7-3b (EW2) and GMC7-3c (EW3) are Hb$^-$ (C). (**D-F**) Hb expression in *svp*^{e22}. Even after the generation of the second GMC, the NB has not yet downregulated Hb (E). At stage 14, three instead of two Hb$^+$ neurons can be detected in many cases (F). Note that not all NB7-3-derived cells express Hb. (**G-I**) Hb expression in *pros*^{C7}. In *pros*^{C7}, the NB generates a Hb$^+$ GMCa as normal (G) but the expression is not maintained (H,I). (**J-L**) Hb expression in *svp*^{e22}, *pros*^{C7}. After the first division Hb has not been downregulated in the NB (K), which results in a *svp*-like phenotype at stage 14 (L). (**M**) Scheme of normal NB7-3 lineage development, with markers used in our analysis. (**N**) Continuous expression of Pros within the NB inhibits the downregulation of Hb. All cells of the NB7-3 cluster are Hb$^+$. (**O**) *pros*^{C7}*/Df(3L)H99* double mutant. There are no NB7-3-derived Hb$^+$ cells present in the absence of apoptosis. Scale bar: 20 μm.

A similar result was obtained for the thoracic NB6-4T lineage. This NB is special because its first division produces a glial precursor instead of a GMC that gives rise to three glial cells (Akiyama-Oda et al., 2000; Freeman and Doe, 2001; Isshiki et al., 2001; Ragone et al., 2001). Again, the glial precursor and its progeny normally maintain *hb* expression, whereas it is switched off in the parental NB (Fig. 3A). As is expected in *pros* mutants, the *hb* expression in the glial cells is not maintained (compare Fig. 3A and 3E), whereas there is a considerable delay in switching off *hb* in *svp* mutants (compare Fig. 3A and 3C). This lack of *svp* function leads to one additional Hb$^+$ glial cell in 56% of the hemineuromeres (n=34; compare Fig. 3G and 3H). Concomitantly, we observed a reduction of the number of NB6-4T-derived neurons in almost all hemineuromeres in *svp* mutants (compare Fig. 3B and 3D).

Because NB7-3 and NB6-4T terminate *hb* expression after their first division, we next asked whether *pros* is also necessary for *hb* maintenance in NBs that produce two Hb$^+$ GMCs. NB7-1 generates such a lineage and it has already been shown that it also produces additional Hb$^+$ progeny in *svp* mutant embryos (Kanai et al., 2005). Unfortunately we could not analyse this lineage in *pros* mutants because the expression of *even skipped* (*eve*), which is needed as a marker for the detection of the first NB7-1 progeny, is itself *pros* dependent (Doe et al., 1991; McDonald et al., 2003). Therefore, we analysed NB2-4T, which we also found to be a neuroblast generating two Hb$^+$ GMCs leading to four Hb$^+$ neurons (Fig. 4A-C). In this lineage too, *hb* expression stays switched on longer in *svp*-mutant embryos, and as a result there are about five to eight Hb$^+$ cells in 86% of the analysed thoracic hemineuromeres (n=35; Fig. 4D-F). In *pros* mutants, the *hb* expression within the NB2-4T lineage seems initially to be normal (Fig. 4G,H), but at stage 14, in about 53%, there are only two Hb$^+$ neurons detectable (n=40; Fig. 4I). Thus, in all lineages analysed Pros seems to counteract the *hb*-downregulating activity of Svp.

To test whether Pros is not only necessary but also sufficient for *hb* maintenance, we made use of the GAL4/UAS-system to express *pros* ectopically within the NBs (Brand and Perrimon, 1993). We used *engrailed*-GAL4 (*en*-GAL4) to drive *pros* expression within NB7-3 and its progeny. Ectopic Pros caused a precocious stop in cell divisions within this lineage in all hemineuromeres analysed. This was expected, as Pros has been shown to activate *dacapo*, which subsequently inhibits further mitotic divisions (Li and Vässin, 2000; Liu et al., 2002). Nevertheless, in some hemineuromeres we could identify three NB7-3-derived cells. In most of these cases, all three cells were Hb$^+$ (Fig. 2N). This shows that Pros activity is indeed sufficient for maintaining *hb* expression, because one of these cells must be the NB that has divided at least once.

Prospero antagonises Seven up activity

The opposite phenotypes of *svp* and *pros* mutants suggest that *hb* maintenance in the GMC is due to Pros activity, which inhibits the repressive function of *svp*. If this is the case, a concomitant loss of Pros and Svp function should show a *svp*-like phenotype. To test this, we generated a svp^{e22}, *pros*^{C7} double mutant and stained the embryonic CNS for *hb* expression. We indeed found generally more

Hb$^+$ cells, which is similar to the phenotype in *svp* single mutant embryos (compare Fig. 1C and D). This was also confirmed on the lineage level: in the NB7-3 derived cluster of stage 14 *svp*-mutant embryos, there were three or four Hb$^+$ cells in 75% of the hemineuromeres (*n*=103). This is similar to the double mutants, which showed this in 67% of the hemineuromeres (*n*=91; Fig. 2L), thus supporting our hypothesis that Pros antagonises Svp activity in the GMC. But on which level does this occur? One possibility is that *svp* transcription, which is initiated before mitosis, is suppressed by Pros in the GMC after division. Alternatively, Pros could suppress the activity of the Svp protein. To distinguish between these two possibilities, we analysed the dynamics of *svp* mRNA expression in the NB7-3 lineage in wild-type and *pros* mutant embryos. In both genotypes, *svp* expression in the NB starts before its first division (Fig. 5A,E) and *svp* mRNA is still present in the NB after mitosis (Fig. 5B,C,F). However, when we examined *svp* mRNA expression in GMCa before the NB divides again, we found a difference between the wild-type and *pros* mutant embryos. In wild type, 70% of these GMCs (*n*=26) were negative for *svp* mRNA (Fig. 5B). In contrast to that, all GMCs examined in *pros* mutants expressed *svp* mRNA, although on a lower level than the NBs did (*n*=16; Fig. 5F). After the birth of GMCb, there is detectable *svp* mRNA in only eight out of 21 cases in GMCa in wild type, whereas in *pros* mutants 13 out of 20 are still positive for this transcript (Fig. 5D,G). This suggests that Pros might participate in the GMC-specific transcriptional downregulation of *svp*. However, overexpression of *pros* could not eliminate *svp* expression within the NBs (data not shown).

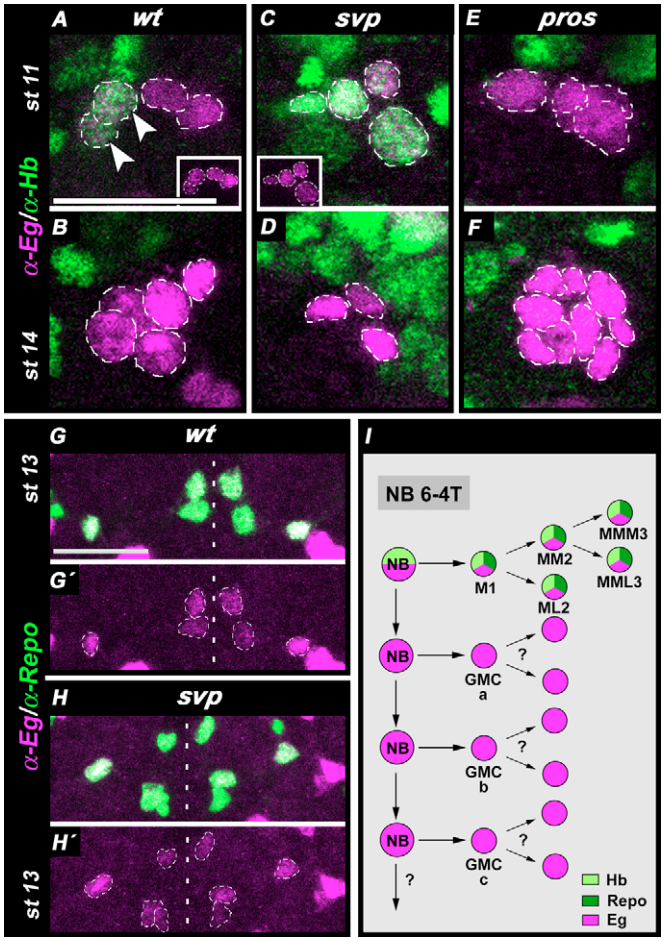

Fig. 3. Regulation of *hb* within the NB6-4T lineage. Embryos double labelled with the lineage marker Eg (magenta), and Hb (green) or the glial marker Repo (green), as indicated. Midline is towards left (A-F) or marked by a dashed line (G,G′,H, H′). Insets show Eg expression separately. (**A**,**B**,**G**,**G′**) Wild type. At stage 11 there are four cells generated by this lineage: two Hb$^+$ glial cells (arrowheads), one Hb$^-$ GMC and the Hb$^-$ neuroblast (A). At stage 14 the lateral neuronal cluster consists mostly of six cells (B). At stage 13, one of the two glial cells in A has divided again and the three resulting cells have migrated towards the ventral midline: two (MMM3 and MML3) near the midline and one (ML2) left behind (outlined in G′). (**C**,**D**,**H**,**H′**) *svp*^{e22}. At stage 11, all four cells (inset in C) are often Hb$^+$, indicating a prolonged expression of Hb (C). Concomitantly, the size of the lateral cluster is reduced (D). In many hemineuromeres there is one additional NB6-4T-derived glial cell at the ventral midline (H,H′). (**E**,**F**) *pros*^{C7}. At stage 11, all four cells are Hb$^-$ (E), showing that Hb expression could not be maintained. The lateral neuronal cluster is enlarged (F), probably due to the deregulation of *dacapo*. (I) Scheme of NB6-4T lineage development, with markers used in our analysis. Scale bar: 20 μm.

Interestingly, the observed difference in *svp* mRNA expression between wild-type and *pros* mutant NB7-3 lineages was not seen in the Svp protein distribution; we found no or only a weak level of Svp protein in NB7-3 before division in both genotypes (Fig. 5H,K). Likewise, after division both cells are always Svp$^+$ (Fig. 5I,L). After the second neuroblast division, GMCa remained positive for Svp protein in nearly all cases in wild type (*n*=30), as well as in *pros* mutant embryos (*n*=25; Fig. 5J,M). At this stage, Hb protein normally has completely vanished from the NB but is maintained in GMCa despite the presence of Svp protein. Taken together, this suggests that Pros acts on both a transcriptional and a post-transcriptional level to downregulate Svp activity in the GMC.

svp activity but not transcription is mitosis dependent

It has been shown that *hb* downregulation in the NB is mitosis dependent, because Hb is maintained in *string* (*stg*) mutant NBs, where mitosis is blocked at the G2/M transition (Isshiki et al., 2001; Großkortenhaus et al., 2005). However, in NB7-3, *svp* mRNA begins to be expressed prior to the division that leads to *hb* downregulation (Kanai et al., 2005) (this work). This timing of *svp* expression seems to be a general feature, as we also see this in other lineages. NB6-4T, which generates only one *hb*-positive progeny, switches on *svp* expression before its first division (Fig. 6A,A′), whereas NB2-4T and NB7-1, which both generate two Hb$^+$ GMCs, start *svp* expression before the Hb$^+$ GMCb is born (Fig. 6B,B′,F,F′). This suggests that either there is no *svp* mRNA expression in *stg* mutant NBs, or that the *svp*-mediated *hb*-repressing activity is post-transcriptionally upregulated after division.

To distinguish between these two possibilities, we analysed *svp* mRNA expression in *stg* mutant embryos in Eg-positive NBs at different developmental time points. We found a normal onset of *svp* expression within NB2-4T and NB7-3 (Fig. 6C,C′), showing that lack of *hb*-downregulation in *stg* mutants in these NBs is not due to a lack of *svp* transcription. To test whether the regulation could be on the level of protein translation, we analysed *stg* mutant embryos for the presence of Svp protein in the NBs. Indeed, we found only a low or undetectable amount of this protein in these cells up to early stage 12, suggesting that the translation of the *svp* mRNA is very low (Fig. 6D,D′). The reason for this might be the unusual localisation of the *svp* mRNA: when comparing the distribution of *hb* and *svp* mRNAs, we realised that almost all of the visible *svp* mRNA is localised in the nucleus, whereas the *hb* mRNA is enriched in the cytoplasm (compare Fig. 7C′ and 7C′′). This nuclear localisation of

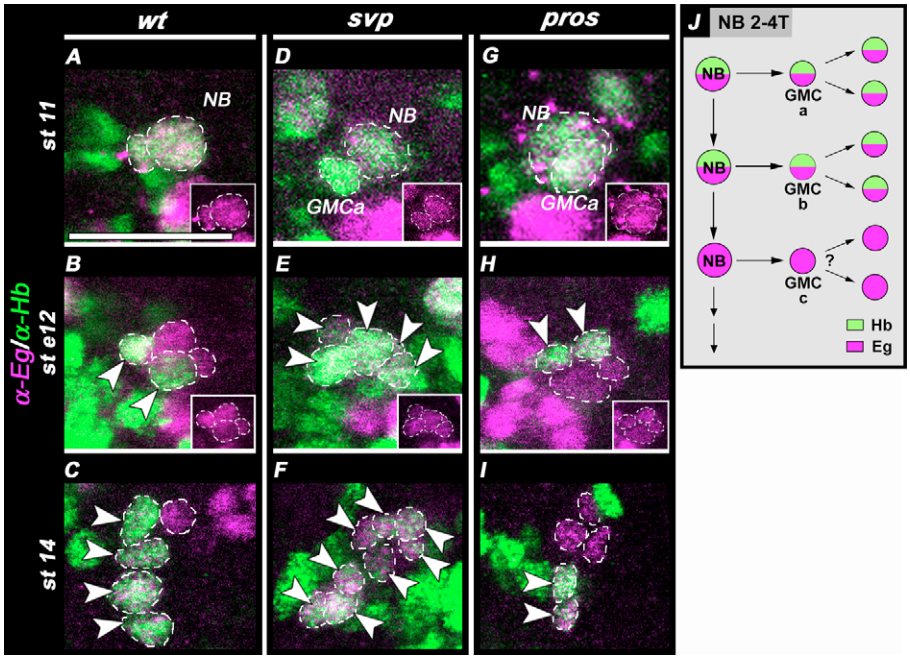

Fig. 4. Regulation of *hb* within the thoracic NB2-4T lineage. Embryos are double labelled for the NB2-4T lineage marker Eg (magenta) and Hb (green). NB2-4T-derived cells are outlined; midline is towards the left. Insets show Eg expression separately. (**A-C**) Wild type. At early stage 11 NB2-4T has divided once to give rise to the Hb$^+$ GMC2-4Ta (A). At early stage 12 the NB has generated three GMCs, two of them are the first-born Hb$^+$ GMC2-4Ta and GMC2-4Tb (B, arrowheads). At stage 14, four neurons are Hb$^+$, which are the progeny of the two first born GMCs (C, arrowheads). (**D-F**) *svp*^{e22}. Hb expression is not downregulated after the second mitosis (E), resulting in up to eight Hb$^+$ neurons (F, arrowheads). (**G-I**) *pros*^{C7}. Hb is initially expressed in the first and second born GMCs (H, arrowheads), but the expression is often not maintained in one GMC (most likely GMC2-4Tb), resulting in only two Hb$^+$ neurons at stage 14 (I, arrowheads). (**J**) Scheme of early NB2-4T lineage development with markers used in our analysis. Scale bar: 20 μm.

the *svp* mRNA is also evident in the in situ hybridisation for *svp* mRNA combined with the antibody staining for Hb protein in *stg* mutant embryos (Fig. 7A-A′′). We assume that this localisation might prevent efficient translation of the Svp protein, which takes place in the cytoplasm. However, some of the *svp* mRNA molecules seem to escape from the nucleus, as we could detect a low level of Svp protein in NBs from around stage 12 onwards (Fig. 6D,D′, Fig. 7B-B′′). This seems to lead to a reduction of *hb* expression because the amount of *hb* mRNA and protein in the *svp*-expressing NBs is lower than in the other cells (Fig. 7).

The observation that, in *stg* mutants, not only NB6-4T and NB7-3 but also NB2-4T expressed *svp* mRNA was unexpected because, in this NB, *svp* mRNA is normally only detectable after the birth of the first GMC (compare Fig. 6A,A′ and 6B,B′). The generation of this cell is obviously not necessary for *svp* upregulation because otherwise NB2-4T would remain *svp* mRNA negative in *stg* mutant embryos. The same was observed for the En$^+$ NB7-1; although normally becoming *svp* positive after the birth of its first GMC at the beginning of stage 10 (compare Fig. 6E,E′ and 6F,F′), *svp* expression started at exactly the same time in *stg* mutants, despite of lack of cell division (compare Fig. 6F,F′ and 6G,G′). Thus, in contrast to *hb* downregulation, the timing of *svp* upregulation is mitosis independent in the analysed lineages.

DISCUSSION

Pros maintains *hb* expression by repressing *svp* function within GMCs

Common to all genes of the temporal specification cascade is the fact that after division they are downregulated within the NB but remain expressed in the newly generated GMCs and their progeny. For *hb*,

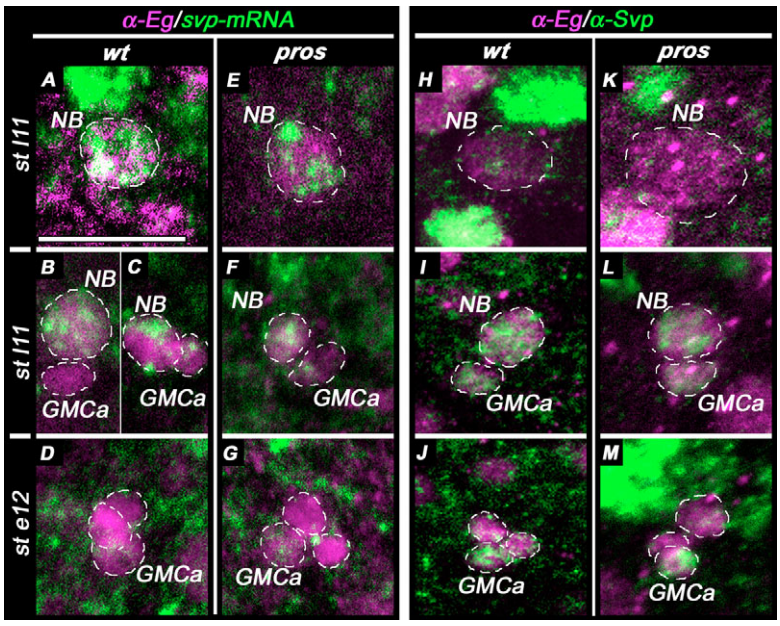

Fig. 5. *svp* mRNA and protein expression in early NB7-3 lineage development. Embryos are double labelled for the NB7-3 lineage marker Eg (magenta), and *svp* mRNA (green) or Svp protein (green), as indicated. NB7-3-derived cells are outlined; midline is towards left. (**A-D**) *svp* mRNA expression in wild type. NB7-3 initiates *svp* expression before its first division (A). After this division, *svp* mRNA can be detected only in the NB (B) or in both cells (C). After the second NB division there is no detectable mRNA in most cases (D). (**E-G**) *svp* mRNA expression in *pros*^{C7}. As in wild type, NB7-3 expresses *svp* mRNA before the first division takes place (E). After this division, both cells are usually positive for *svp* mRNA, although the amount of transcripts in GMCa seems to be generally lower than in the NB (F). After the second NB division there is still detectable *svp* mRNA in GMCa in most cases (G). (**H-J**) Svp protein expression in wild type. There is only a little or no protein in NB7-3 prior to the first mitosis (H). After division, both cells are Svp$^+$ (I). After the birth of GMCb, all cells are Svp$^+$ but the highest amount of Svp protein is found in GMCa (J). (**K-M**) Svp protein expression in *pros*^{C7} is similar to wild type. Scale bar: 20 μm.

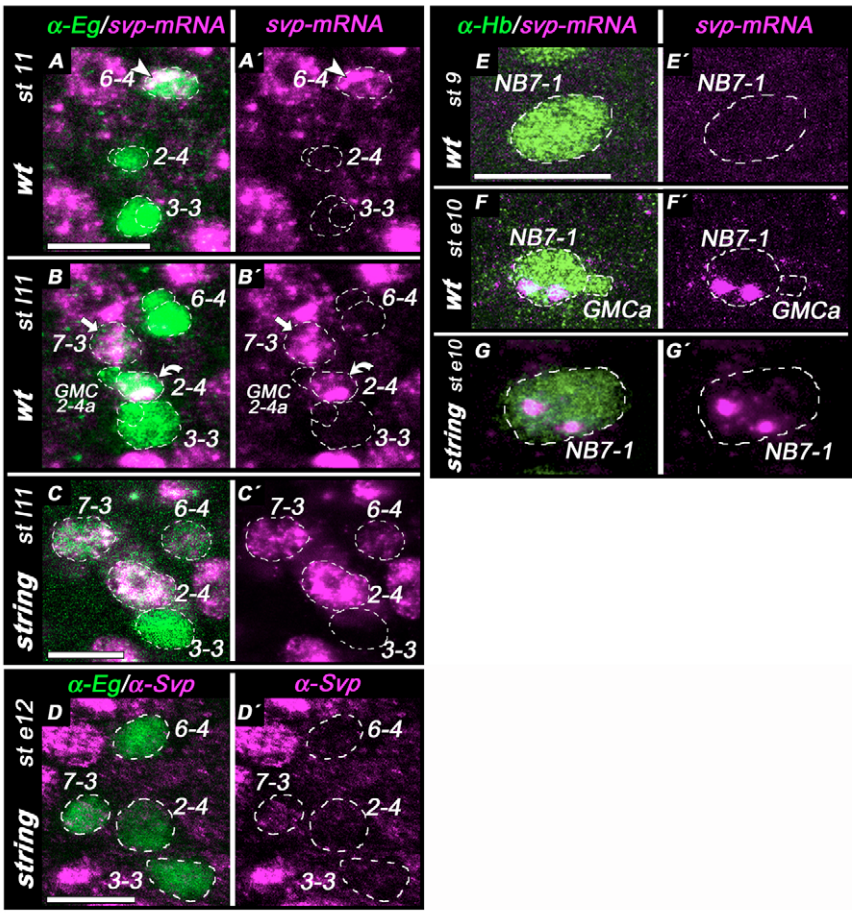

Fig. 6. Temporal regulation of *svp* mRNA expression. In situ hybridisation for *svp* mRNA (magenta, A-C′,E-G′) combined with anti-Eg (green, A-C) and anti-Hb (green, E-G), and double staining with anti-Eg (green, D) and anti-Svp (magenta, D,D′). NBs and their GMCs are outlined. (**A**,**B**) Wild type. NB6-4T expresses *svp* before it undergoes the first mitosis (arrowhead in A,A′). After mitosis, *svp* expression stops immediately (6-4T cells in B,B′). Likewise, NB7-3 expresses *svp* before its first divison (B,B′, arrow). NB2-4T generates one GMC (small 2-4T cell in A,A′) before it expresses *svp* (curved arrow in B,B′). There is no detectable *svp* mRNA within GMC2-4Ta (B,B′). NB3-3 and its progeny do not express *svp* mRNA at these early stages. (**C**) *stg*^{4}. At late stage 11, NB6-4T, 2-4T and 7-3 still express *svp* mRNA in cell cycle-arrested embryos, indicating that *svp* expression without cell division is not sufficient to switch off *hb* expression. (**D**) Even at early stage 12 there is hardly any Svp protein detectable in NB2-4T, 6-4T and 7-3 in *stg*^{4} mutant NBs. (**E**,**F**) Wild type. NB7-1 (identified by position and En expression, not shown) is initially negative for *svp* mRNA (E,E′). *svp* expression starts after the generation of its first GMC (F,F′). (**G**) *stg*^{4}. Like NB2-4T, NB7-1 also starts to express *svp* mRNA at normal time, even without the generation of its first GMC. Scale bar: 20 μm.

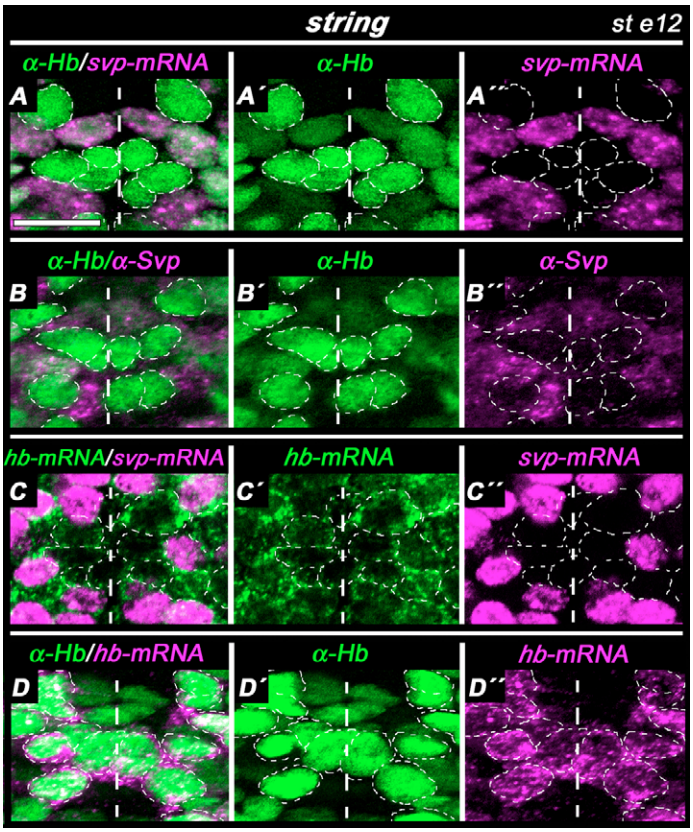

Fig. 7. Nuclear *svp* mRNA localisation and attenuation of *hb* expression in *stg* mutant embryos. Dashed line indicates the midline. Cells expressing high levels of Hb protein are outlined. (**A**) Double labelling for Hb protein (green) and *svp* mRNA (magenta). Cells that express high levels of Hb, do not express *svp* mRNA. In contrast to that, *svp*-expressing cells show low levels of Hb protein. Note the complete colocalisation of the nuclear Hb protein with *svp* mRNA. (**B**) Double labelling for Hb (green) and Svp protein (magenta). There is no Svp protein detectable in cells that show high expression of Hb protein. Those cells that are weakly Hb^{+} show a concomitant low level of Svp protein. (**C**) Double labelling for *hb* mRNA (green) and *svp* mRNA (magenta). Although hb mRNA is enriched in the cytoplasm of the NBs, the svp mRNA is found mainly in the nucleus. (**D**) Double labelling for Hb protein (green) and *hb* mRNA (magenta). Low Hb protein levels correlate with low hb mRNA levels, suggesting a transcriptional downregulation of *hb* by Svp. Scale bar: 20 μm.

it has recently been shown that this downregulation is dependent on Svp, whose mRNA is already expressed within the neuroblast before the generation of the Hb^+ GMC and is symmetrically distributed to both cells after NB division (Kanai et al., 2005) (this work). Why then does *svp* downregulate *hb* only within the NB and not in the GMC? Our results show that this is due to the activity of Pros, a homeodomain transcription factor that is asymmetrically distributed only to the GMC (Hirata et al., 1995; Knoblich et al., 1995; Li et al., 1997; Spana and Doe, 1995). Earlier work by other groups has suggested that Pros is involved in the regulation of GMC-specific gene activity (Doe et al., 1991; Vässin et al., 1991; Li and Vässin, 2000) In principle this is also true for the function of *pros* in the context of *hb* regulation, because it inhibits the NB-specific *svp*-mediated downregulation of *hb*. How is this antagonistic activity of Svp and Pros achieved at the molecular level? On the one hand, our data suggest that Pros downregulates *svp* transcription, because the *svp* mRNA in the first GMC of NB7-3 was present longer in *pros* mutants than in wild type. On the other hand, it seems likely that Pros also inhibits Svp activity, because the Hb^+ GMC often possesses Svp protein even after the parental NB is Hb^-. An attractive model for this would be that Pros neutralises Svp repressor function by binding to the same regulatory region of *hb*. In fact, we found an evolutionarily conserved enrichment of putative Svp-binding sites in the vicinity of a potential Pros-binding site (Cook et al., 2003; Hassan et al., 1997; Zelhof et al., 1995), within a regulatory region that is necessary for neural *hb* regulation (J. Margolis, PhD thesis, University of California at San Diego, 1992). We are currently testing whether these sequences are indeed functional in the proposed context.

svp activity is regulated at a post-transcriptional level

Because blocking the transition between the G2 and M phase prevents *hb* from being downregulated (Isshiki et al., 2001; Großkortenhaus et al., 2005) (this work), the repressing activity of *svp* must somehow depend on mitosis. This regulation cannot be at the level of the transcriptional activation of *svp* because its mRNA is already present before the NBs enter the decisive M-phase. Moreover, in *stg* mutant embryos, where the G2/M transition is blocked, we find co-expression of *svp* mRNA and Hb for several hours, although at later stages the average amount of Hb molecules seems to be generally lower than in cells without *svp* expression. At the protein level the situation is somewhat different: in wild-type embryos we do hardly see any Svp protein before the NB divides, suggesting that the *svp* mRNA cannot be efficiently translated before mitosis occurred. This might be due to a low translation rate, because in *stg* mutants we found only a slowly increasing Svp protein level in the NBs despite a permanently strong *svp* mRNA expression. One reason for this might be the nuclear localisation of the *svp* mRNA, which we found in NBs of *stg* mutant embryos as well as in wild type. This localisation might be able to largely prevent *svp* mRNA from becoming translated before the cell divides. Clearly, further work is needed to test this interesting hypothesis.

Attenuation of *hb* expression by Svp – a possible mechanism to discriminate the fates of two consecutively born Hb^+ GMCs

The fact that we find Svp protein in NBs in *stg* mutants that reduces but does not switch off *hb* expression might offer an explanation as to how the different fates of two Hb^+ GMCs might be determined. A well-studied case is NB7-1, where the first GMC gives rise to the Zfh2-negative U1 neuron, whereas the second generates a Zfh2-positive U2 neuron. Earlier work by Pearson and Doe (Pearson and Doe, 2003) provided evidence that U2 is specified by a reduction of *hb* activity within the NB or GMC. Thus, it is possible that a low level of Svp protein present in the NB before the second GMC is born might be responsible for this. Indeed, when *svp* is expressed in NB7-1 prematurely before the birth of the first GMC, there is no U1 neuron and the chain of U neurons often starts with only one Hb^+ neuron, which has a U2 identity (Kanai et al., 2005) (U.M. and J.U., unpublished). According to our hypothesis, this would be due to a reduced Hb activity caused by the premature *svp* expression. Likewise, in the absence of *svp* function the NB first produces many additional Hb^+ U1 neurons before it eventually generates the other U neurons starting with U2 (Kanai et al., 2005) (U.M. and J.U., unpublished). In this case, *hb* expression level might

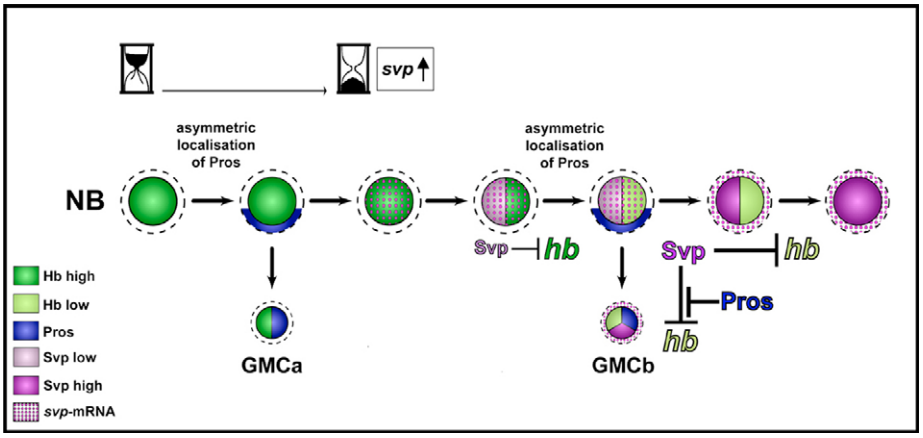

Fig. 8. Model of *hb* regulation in NB lineage development. Expression of *svp* mRNA starts in the NB in a lineage-specific time window, which is dependent on an unknown timing mechanism. In the case of two Hb-dependent GMCs with different fates, *svp* expression starts after the birth of the first GMC (GMCa). Before the next mitosis, most of the *svp* mRNA is kept in the nucleus, allowing only a limited translation of Svp protein. The resulting Svp activity leads to a reduction of *hb* expression, which is necessary to specify the fate of GMCb. Subsequently, as a result of the next mitotic division, the translation of *svp* mRNA is enhanced and *hb* expression is switched off in the NB. Within GMCb, Svp is inhibited by Pros, which has segregated into the GMC during mitosis, thereby maintaining *hb* expression in this cell.

initially remain high resulting in the production of several U1 neurons before it drops down leading to the specification of a U2 neuron.

The lineage-specific timing of *svp* expression is cell cycle independent

It has been shown that the lineage-specific timing of the switching on of *svp* expression defines the end of the Hb^+ time window, and thereby the number of the progeny generated during this phase (Kanai et al., 2005) (U.M. and J.U., unpublished). How is this timing regulated? In one group of NBs, the expression of *svp* already starts before its first division (e.g. NB7-3 and NB6-4T). This could be directly dependent on the activity of proneural genes. Indeed, the early expression of *svp* within the developing Malphigian tubules has been shown to be regulated by these genes (Sudarsan et al., 2002). In this context, it is interesting to note that, in *Drosophila*, *svp* expression in certain NB lineages has already begun in their proneural clusters within the neuroectoderm (Broadus and Doe, 1995). A second group of NBs show *svp* upregulation after the generation of their first GMCs (e.g. NB2-4T and NB7-1), suggesting that the mitotic division is the trigger for this event. To our surprise, this is not the case: in *stg* mutant embryos, NB7-1 upregulates *svp* at the same time as in wild type, although no division has occurred. The same was found for NB2-4T. Thus, lineage-specific timing of *svp* expression is independent of the number of cell divisions. However, currently we cannot rule out that earlier stages of the cell cycle, like the S-Phase, could be the trigger instead. Interestingly, the sequential transitions of the temporal specification genes acting after *hb* expression have recently been shown to occur independently of the cell cycle (Großkortenhaus et al., 2005). According to our results, this might be also true for the timing of *svp* expression.

Conclusion

To our knowledge, the regulatory interactions between *hb*, *svp* and *pros* are the first example where mitosis-dependent gene activity acts together with an asymmetric cell fate determinant to regulate differential gene expression in space and time (Fig. 8). We currently do not know whether such a regulation also exists in other organisms. Interestingly, Svp shows a high homology with COUP-TF orphan receptors from vertebrates, which are also necessary for CNS development (Pereira et al., 2000). Prox1, the vertebrate homologue of Pros is not asymmetrically distributed during division but is expressed and needed during neurogenesis (Tomarev et al., 1998; Yamamoto et al., 2001). During retinal development, Prox1 is involved in the specification of the fate of the early born horizontal neurons (Dyer et al., 2003). Future investigations will show whether during vertebrate CNS development these homologous factors play a role comparable to Svp and Pros in *Drosophila*.

We thank Alicia Hidalgo, Jim Posakony, Marc Freeman, Chris Doe, Manfred Frasch, Martha Lundell, John Reinitz, Michael Hoch, Fumio Matsuzaki, Harald Vässin, Marek Mlodzik, Hermann Steller, Yasushi Hiromi and the Bloomington Stock Center for providing us the various antibodies, fly stocks and cDNA used in this study. We are very grateful to Yasushi Hiromi for communicating unpublished results. We also thank Ulrike Kalkowski and Simone Renner for excellent technical assistance, and Lisa Meadows, Christian Berger, Tor Erik Rusten, Ana Rogulja-Ortmann and Gerd Technau for critical reading of the manuscript. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to J.U. (Ur 42/3-5).

References

Akiyama-Oda, Y., Hotta, Y., Tsukita, S. and Oda, H. (2000). Mechanism of glia-neuron cell-fate switch in the Drosophila thoracic neuroblast 6-4 lineage. *Development* **127**, 3513-3522.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Broadus, J. and Doe, C. Q. (1995). Evolution of neuroblast identity: seven-up and prospero expression reveal homologous and divergent neuroblast fates in Drosophila and Schistocerca. *Development* **121**, 3989-3996.

Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G. and Doe, C. Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system. *Mech. Dev.* **53**, 393-402.

Brody, T. and Odenwald, W. F. (2000). Programmed transformations in neuroblast gene expression during Drosophila CNS lineage development. *Dev. Biol.* **226**, 34-44.

Cepko, C. L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. *Curr. Opin. Neurobiol.* **9**, 37-46.

Chu-Lagraff, Q., Wright, D. M., McNeil, L. K. and Doe, C. Q. (1991). The prospero gene encodes a divergent homeodomain protein that controls neuronal identity in Drosophila. *Development* **2**, 79-85.

Cook, T., Pichaud, F., Sonneville, R., Papatsenko, D. and Desplan, C. (2003). Distinction between color photoreceptor cell fates is controlled by Prospero in Drosophila. *Dev. Cell* **4**, 853-864.

Desai, A. R. and McConnell, S. K. (2000). Progressive restriction in fate potential by neural progenitors during cerebral cortical development. *Development* **127**, 2863-2872.

Dittrich, R., Bossing, T., Gould, A. P., Technau, G. M. and Urban, J. (1997). The differentiation of the serotonergic neurons in the Drosophila ventral nerve cord depends on the combined function of the zinc finger proteins Eagle and Huckebein. *Development* **124**, 2515-2525.

Doe, C. Q., Chu-LaGraff, Q., Wright, D. M. and Scott, M. P. (1991). The prospero gene specifies cell fates in the Drosophila central nervous system. *Cell* **65**, 451-464.

Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat. Genet.* **34**, 53-58.

Frantz, G. D. and McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* **17**, 55-61.

Freeman, M. R. and Doe, C. Q. (2001). Asymmetric Prospero localization is required to generate mixed neuronal/glial lineages in the Drosophila CNS. *Development* **128**, 4103-4112.

Großkortenhaus, R., Pearson, B. J., Marusich, A. and Doe, C. Q. (2005). Regulation of temporal identity transitions in Drosophila neuroblasts. *Dev. Cell* **8**, 193-202.

Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A. and Technau, G. M. (1995). The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of Drosophila melanogaster. *Development* **121**, 317-332.

Harris, W. A. (1997). Cellular diversification in the vertebrate retina. *Curr. Opin. Genet. Dev.* **7**, 651-658.

Hassan, B., Li, L., Bremer, K. A., Chang, W., Pinsonneault, J. and Vaessin, H. (1997). Prospero is a panneural transcription factor that modulates homeodomain protein activity. *Proc. Natl. Acad. Sci. USA* **94**, 10991-10996.

Hirata, J., Nakagoshi, H., Nabeshima, Y. and Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. *Nature* **377**, 627-630.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-521.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. *Genes Dev.* **5**, 1881-1891.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S. J. and Odenwald, W. F. (1998). Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS. *Genes Dev.* **12**, 246-260.

Kanai, M. I., Okabe, M. and Hiromi, Y. (2005). *seven-up* controls switching of transcription factors that specify temporal identities of Drosophila neuroblasts. *Dev. Cell* **8**, 203-213.

Knoblich, J. A., Jan, L. Y. and Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature* **377**, 624-627.

Kosman, D., Small, S. and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. *Dev. Genes Evol.* **208**, 290-294.

Li, L. and Vaessin, H. (2000). Pan-neural Prospero terminates cell proliferation during Drosophila neurogenesis. *Genes Dev.* **14**, 147-151.

Li, P., Yang, X., Wasser, M., Cai, Y. and Chia, W. (1997). Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during Drosophila neuroblast cell divisions. *Cell* **90**, 437-447.

Liu, T. H., Li, L. and Vaessin, H. (2002). Transcription of the Drosophila CKI gene

dacapo is regulated by a modular array of cis-regulatory sequences. *Mech. Dev.* **112**, 25-36.
Margolis, J. S., Borowsky, M. L., Steingrimsson, E., Shim, C. W., Lengyel, J. A. and Posakony, J. W. (1995). Posterior stripe expression of hunchback is driven from two promoters by a common enhancer element. *Development* **121**, 3067-3077.
McConnell, S. K. (1992). The control of neuronal identity in the developing cerebral cortex. *Curr. Opin. Neurobiol.* **2**, 23-27.
McConnell, S. K. and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-285.
McDonald, J. A., Fujioka, M., Odden, J. P., Jaynes, J. B. and Doe, C. Q. (2003). Specification of motoneuron fate in Drosophila: integration of positive and negative transcription factor inputs by a minimal eve enhancer. *J. Neurobiol.* **57**, 193-203.
Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S. and Rubin, G. M. (1990). The Drosophila seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* **60**, 211-224.
Nose, A., Mahajan, V. B. and Goodman, C. S. (1992). Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in Drosophila. *Cell* **70**, 553-567.
Novotny, T., Eiselt, R. and Urban, J. (2002). Hunchback is required for the specification of the early sublineage of neuroblast 7-3 in the Drosophila central nervous system. *Development* **129**, 1027-1036.
Ohnuma, S., Philpott, A., Wang, K., Holt, C. E. and Harris, W. A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. *Cell* **99**, 499-510.
Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount Drosophila embryos and larvae using antibody probes. *Methods Cell Biol.* **44**, 445-487.
Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in Drosophila. *Nature* **425**, 624-628.
Pereira, F. A., Tsai, M. J. and Tsai, S. Y. (2000). COUP-TF orphan nuclear receptors in development and differentiation. *Cell Mol. Life Sci.* **57**, 1388-1398.
Ragone, G., Bernardoni, R. and Giangrande, A. (2001). A novel mode of asymmetric division identifies the fly neuroglioblast 6-4T. *Dev. Biol.* **235**, 74-85.
Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.
Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J. and Technau, G. M. (1997). The embryonic central nervous system lineages of Drosophila melanogaster. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Dev. Biol.* **189**, 186-204.
Spana, E. P. and Doe, C. Q. (1995). The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. *Development* **121**, 3187-3195.
Sudarsan, V., Pasalodos-Sanchez, S., Wan, S., Gampel, A. and Skaer, H. (2002). A genetic hierarchy establishes mitogenic signalling and mitotic competence in the renal tubules of Drosophila. *Development* **129**, 935-944.
Tomarev, S. I., Zinovieva, R. D., Chang, B. and Hawes, N. L. (1998). Characterization of the mouse Prox1 gene. *Biochem. Biophys. Res. Commun.* **248**, 684-689.
Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. and Jan, Y. N. (1991). prospero is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in Drosophila. *Cell* **67**, 941-953.
White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-683.
Yamamoto, S., Nagao, M., Sugimori, M., Kosako, H., Nakatomi, H., Yamamoto, N., Takebayashi, H., Nabeshima, Y., Kitamura, T., Weinmaster, G. et al. (2001). Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. *J. Neurosci.* **21**, 9814-9823.
Zelhof, A. C., Yao, T. P., Evans, R. M. and McKeown, M. (1995). Identification and characterization of a Drosophila nuclear receptor with the ability to inhibit the ecdysone response. *Proc. Natl. Acad. Sci. USA* **92**, 10477-10481.