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

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Neutral 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). Similarly, 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.
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-fz-lacZ (F. Matsuzaki, Kobe, Japan), pros^{TM6b} and Tb (A. Hildago, Birmingham, UK), svp^{22}/TM3-Usb-lacZ (M. Hoch, Bonn, Germany), and Df (3L)H99/TM6b and abd A-lacZ (H. Steller, New York, USA). The svp^{22}, pros^{C7}/TM3-fz-lacZ and pros^{C7}, Df (3L)H99/TM3-fz-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 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-Pros (1:500) (M. Frasch, New York, USA), mouse 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 bh (Margolis et al., 1995) and svp (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; Spany 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; Spany 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^{C17}. 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).
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 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 (Canai 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.

**Propero 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 GMCs 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 GMCs 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).

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. 5L). 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 (Ishikii et al., 2001; Grobortenhaus 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+ 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. 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+ 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+ cells.
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. 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 division (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) stg⁴. 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 stg⁴ 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) stg⁴. 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, 1997; Spana and Doe, 1995). 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.](image)
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 (Sadursan 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, generation of their first GMCs (e.g. NB2-4T and NB7-1), suggesting that 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., 1995). New neuroblast markers and the origin of the acCtPCC neurons in the Drosophila central nervous system. Mech. Dev. 53, 393-402.


