Developmental Cell *11*, 775–789, December, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2006.09.015

Prospero Acts as a Binary Switch between Self-Renewal and Differentiation in *Drosophila* Neural Stem Cells

Semil P. Choksi,[1,2,4] Tony D. Southall,[1,4] Torsten Bossing,[1] Karin Edoff,[1] Elzo de Wit,[3] Bettina E. Fischer,[2] Bas van Steensel,[3] Gos Micklem,[2] and Andrea H. Brand[1,*]

[1] The Gurdon Institute and Department of Physiology, Development and Neuroscience
University of Cambridge
Tennis Court Road
Cambridge CB2 1QN
United Kingdom

[2] Department of Genetics
University of Cambridge
Downing Street
Cambridge CB2 3EH
United Kingdom

[3] Netherlands Cancer Institute
1066 CX Amsterdam
The Netherlands

Summary

Stem cells have the remarkable ability to give rise to both self-renewing and differentiating daughter cells. *Drosophila* neural stem cells segregate cell-fate determinants from the self-renewing cell to the differentiating daughter at each division. Here, we show that one such determinant, the homeodomain transcription factor Prospero, regulates the choice between stem cell self-renewal and differentiation. We have identified the in vivo targets of Prospero throughout the entire genome. We show that Prospero represses genes required for self-renewal, such as stem cell fate genes and cell-cycle genes. Surprisingly, Prospero is also required to activate genes for terminal differentiation. We further show that in the absence of Prospero, differentiating daughters revert to a stem cell-like fate: they express markers of self-renewal, exhibit increased proliferation, and fail to differentiate. These results define a blueprint for the transition from stem cell self-renewal to terminal differentiation.

Introduction

Stem cells have the capacity to renew themselves at each division while producing a continuous supply of differentiating daughters for the generation, and subsequent repair, of tissues (reviewed in Weissman et al., 2001). Discovering how stem cells maintain their multipotent state and how their progeny differentiate into distinct cellular fates is of fundamental importance not only to understanding development but also to exploiting the therapeutic potential of stem cells. A better understanding of the molecular mechanisms that underlie the behavior of stem cells and their progeny may also identify novel targets for cancer treatment. Recent results suggest that tumors arise from cancer stem cells, in which the normal control of self-renewing divisions is overridden (reviewed in Bjerkvig et al., 2005). Stem cells must strike a balance so as to produce a sufficient number of self-renewing daughters to progress through development and repair damage, but not to produce so many self-renewing daughters that cancerous growth occurs (reviewed in Reya et al., 2001).

The *Drosophila* nervous system has proved to be a fertile ground for studying asymmetric stem cell division. *Drosophila* neural stem cells, or neuroblasts, divide in a regenerative fashion, producing one large daughter, which self-renews, and a second, smaller daughter called a ganglion mother cell (GMC). The GMC divides only once, to give rise to two terminally differentiating neurons or glial cells. At each division, cell fate determinants, such as the PTB domain protein Numb and the homeodomain transcription factor, Prospero, are segregated from the neuroblast to the GMC (reviewed in Jan and Jan, 1998).

Much work has been done to understand the mechanisms by which neuroblast polarity is established and maintained and by which the spindle is oriented along the apico-basal axis (reviewed in Wodarz and Huttner, 2003). However, little is known about the function of the proteins that are asymmetrically localized. *prospero* mRNA localization is dispensable for the appropriate differentiation of GMCs (Broadus et al., 1998), while Numb appears to function only in subsequent cell-fate decisions, not in the decision between self-renewal and differentiation (Buescher et al., 1998). Transcriptional modulation mediated by Prospero protein is thought to be one of the key factors that distinguishes neural stem cells from their daughters. For example, Prospero is proposed to arrest cell division in GMCs by blocking the expression of cell-cycle regulators such as *cyclin A*, *cyclin E*, *E2F*, and *string* (Li and Vaessin, 2000). Recent studies identified a novel asymmetrically segregated determinant in neuroblasts, the translational regulator Brain Tumor or Brat (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006b). *brat* mutants generate excess neuroblasts that form tumors in the larval brain (Arama et al., 2000). *brat* mutants fail to partition Prospero to the GMC (Bello et al., 2006; Lee et al., 2006b), suggesting that the loss of Prospero is the root cause of tumor formation. Bello and colleagues further demonstrate that targeted expression of Prospero in *brat* mutant clones is sufficient to rescue the lack of cell-cycle exit and differentiation and prevent the formation of tumors. This strongly suggests that Prospero's transcriptional control of genes is the primary mechanism controlling neuroblast proliferation.

Prospero is conserved in vertebrates, where the Prox (for *Pr*ospero-related homeob*ox*) family of atypical homeodomain transcription factors appears to play a role in initiating the differentiation of progenitors in various tissues. However, mechanistic insights into the role of Prox in differentiation have been elusive. In the rat forebrain, Prox1 expression is upregulated coincident

*Correspondence: ahb@mole.bio.cam.ac.uk
[4] These authors contributed equally to this work.

with the transition of Nestin-positive stem cells to early MAP2-expressing neurons (Torii et al., 1999). In addition, homozygous *prox1* mutant mice show increased proliferation of retinal progenitor cells, while Prox1 overexpression in retinal explants results in premature cell-cycle exit (Dyer et al., 2003). Prox1 is also expressed in the developing lens, where it is activated at a time when proliferative, epithelial lens precursors transition to lens fiber cells (Wigle et al., 1999). Prox1 has also been shown to be necessary and sufficient for the differentiation of endothelial precursors into lymphatic endothelial cells (reviewed in Hong and Detmar, 2003; Wigle et al., 2002; Wigle and Oliver, 1999).

The nuclear localization of Prospero is one of the first molecular differences between a self-renewing neuroblast and a differentiating cell (Knoblich et al., 1995; Spana and Doe, 1995) and may be the key to deciphering the genetic networks governing the regulation of stem cell division versus differentiation. Here, through a combination of in vivo binding-site mapping and in silico motif searching, we identify Prospero binding sites throughout the genome. We show by genome-wide expression profiling and in situ hybridization that not only does Prospero directly repress neuroblast genes and cell-cycle genes, but it also, surprisingly, regulates genes required in terminally differentiated neurons. We conclude that Prospero controls, at the level of transcription, the transition from self-renewal to differentiation. If this is correct, then GMCs lacking Prospero should be transformed into neural stem cells in the embryo. Here, we show, at the single cell level, that a GMC lacking Prospero is transformed into a neuroblast: it continues to divide, expresses neuroblast markers, and does not differentiate. Prospero therefore acts as a binary switch between self-renewal and differentiation in neural stem cells.

Results

Identifying Prospero Binding Sites in the *Drosophila* Genome

To identify sites within the *Drosophila* genome to which Prospero binds, we made use of an in vivo binding-site profiling technique, DamID (van Steensel et al., 2001; van Steensel and Henikoff, 2000). DamID is an established method of determining the binding sites of DNA- or chromatin-associated proteins (Bianchi-Frias et al., 2004; de Wit et al., 2005; Greil et al., 2003; Orian et al., 2003; Song et al., 2004; Sun et al., 2003; Tolhuis et al., 2006; van Steensel et al., 2001; van Steensel and Henikoff, 2000). Target sites identified by DamID have been shown to match targets identified by chromatin immunoprecipitation (ChIP) (Song et al., 2004; Sun et al., 2003; Tolhuis et al., 2006) or mapping to polytene chromosomes (Bianchi-Frias et al., 2004). DamID enables binding sites to be tagged in vivo and later identified on DNA microarrays. In brief, the DNA or chromatin-binding protein of interest is fused to an *Escherichia coli* adenine methyltransferase (Dam), and the fusion protein is expressed in vivo (Figure 1A, i). The DNA-binding protein targets the fusion protein to its native binding sites, and the Dam methylates local adenine residues in the sequence GATC (Figure 1A, ii). The sequences near the protein-DNA interaction site are thereby marked with a unique methylation tag, over approximately 2–5 kilobase pairs (kb) from the binding site (van Steensel and Henikoff, 2000). The tagged sequences can be isolated after digestion with a methylation-sensitive restriction enzyme, such as DpnI (Figure 1A, ii).

Dam was fused to the N terminus of Prospero, and transgenic flies were generated. The fusion protein is expressed from the uninduced minimal Hsp70 promoter of the UAS vector, pUAST (Brand and Perrimon, 1993), as high levels of expression of Dam can result in extensive nonspecific methylation and cell death (Bianchi-Frias et al., 2004; van Steensel and Henikoff, 2000) (T.S. and A.H.B., unpublished data). As a control for nonspecific Dam activity, animals expressing Dam alone were generated. To assess the sites to which Prospero binds during neurogenesis, genomic DNA was extracted from stage 10–11 embryos, approximately 4–7 hr after egg laying (AEL), expressing either the Dam-Prospero fusion protein or the Dam protein alone. The DNA was digested with DpnI and amplified by PCR (see Experimental Procedures). DNA from Dam-Prospero embryos was labeled with Cy3, and control DNA with Cy5. The samples were then cohybridized to genomic microarrays. We designed microarrays that tile the entire euchromatic *Drosophila melanogaster* genome (see Experimental Procedures). A 60 base oligonucleotide was printed for approximately every 300 bp of genomic DNA, resulting in roughly 375,000 probes on a single array (Nimblegen Systems).

Log-transformed ratios from four biological replicates (two standard dye configurations plus two swapped dye configurations) were normalized and averaged (see Experimental Procedures). Regions of the genome with a greater than 1.4-fold log ratio (corresponding to approximately a 2.6-fold enrichment) of Dam-Prospero to the control over a minimum of four adjacent genomic probes were identified as in vivo Prospero binding sites (see Experimental Procedures). Using these parameters, we identified a total of 1,602 in vivo Prospero binding sites in the *Drosophila* genome (data not shown). Our work demonstrates that it is possible to map in vivo binding sites across the whole genome of a multicellular organism.

Prospero is known to regulate the differentiation of photoreceptors in the adult eye, and recently Cook et al. characterized sites to which Prospero can bind upstream of two Rhodopsin genes, *Rh5* and *Rh6*. A variant of the Prospero consensus sequence (Figure 1A) is found four times upstream of *Rh5* and four times upstream of *Rh6* (Table S4; see the Supplemental Data available with this article online). Prospero was shown to bind this sequence in vitro, by band shift assay, and also by a 1-hybrid interaction assay in yeast (Cook et al., 2003). In addition, deletion analysis demonstrated that the consensus sequence is required for the Pros-DNA interaction both in vivo and in vitro (Cook et al., 2003). We find that 67% of in vivo binding sites contain at least one Prospero binding motif (Figure 1A, iv) (see Experimental Procedures). Combining our in vivo binding-site data with searches for the Prospero consensus sequence reveals 1,066 distinct sites within the *Drosophila* genome to which Prospero binds during embryogenesis (Table S1).

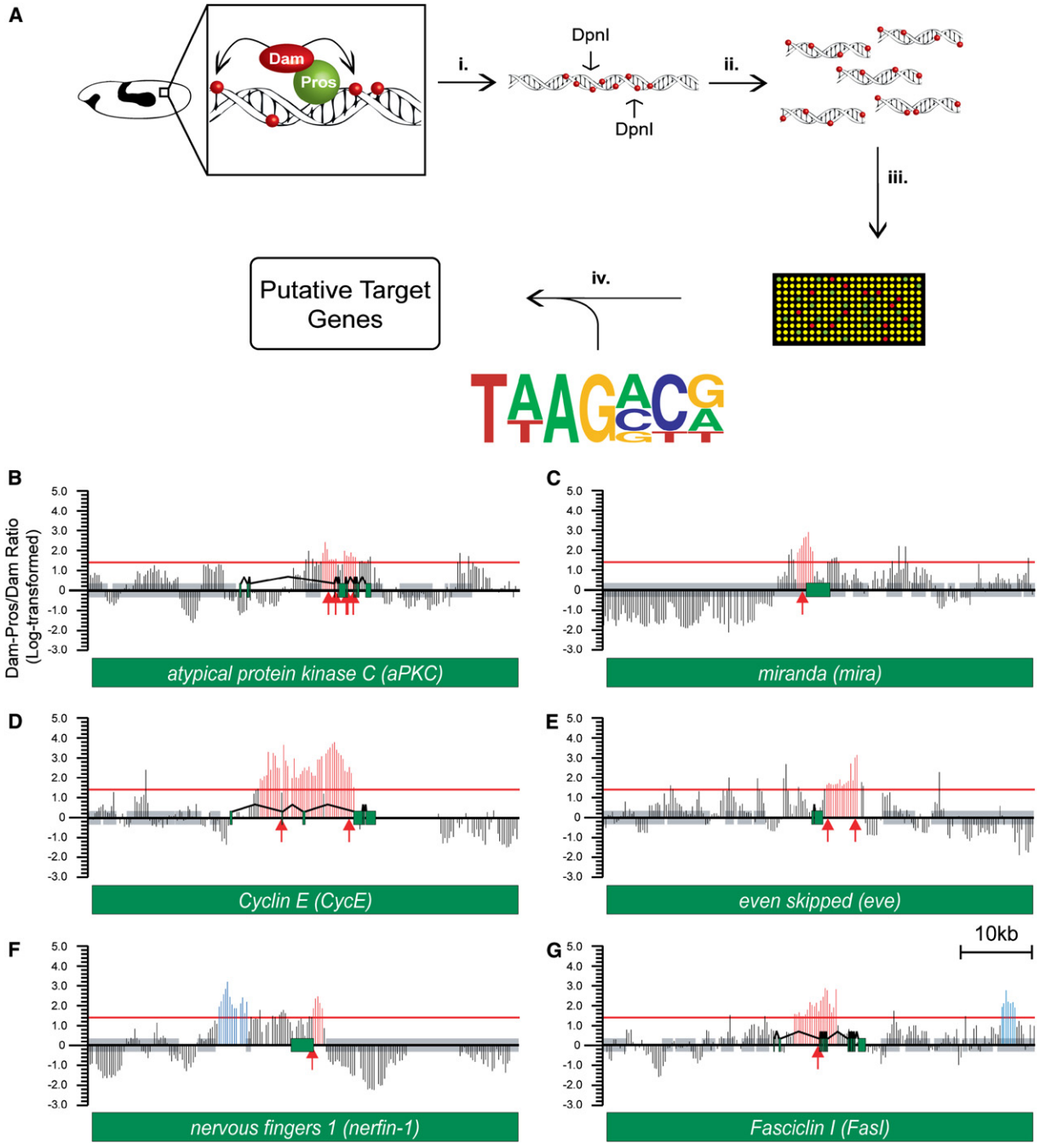


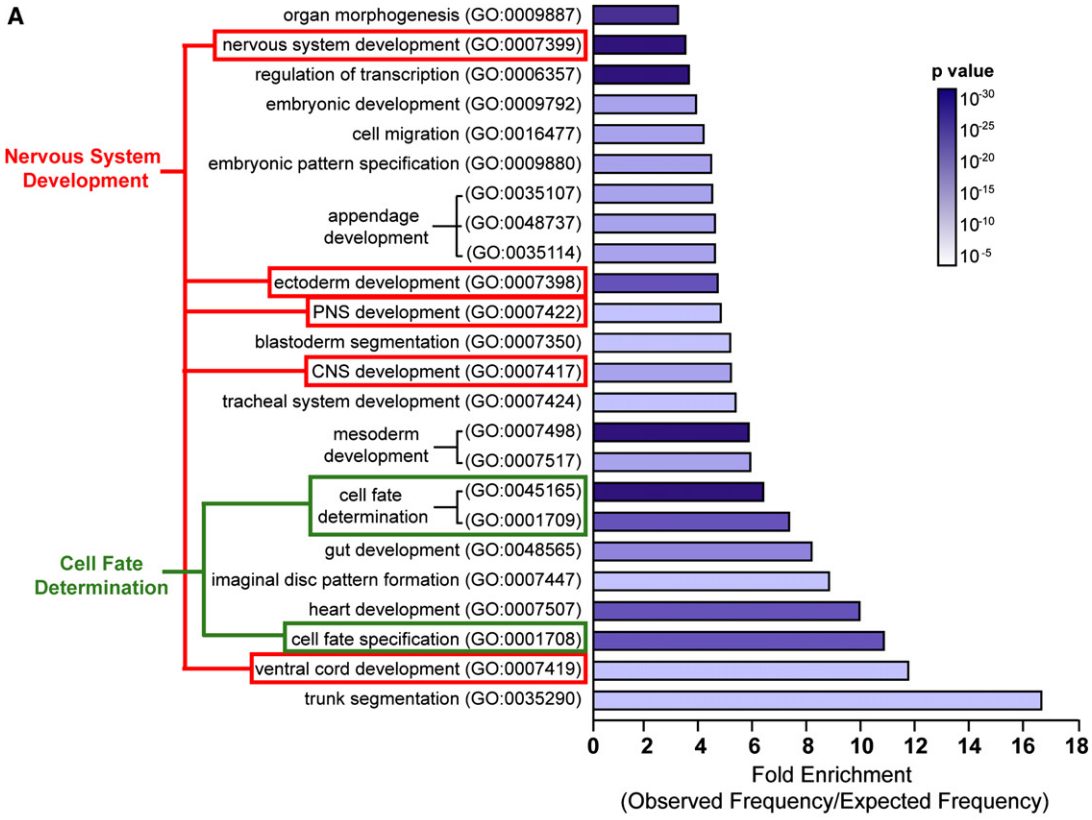
Figure 1. Genome-Wide Mapping of Prospero Binding Sites

(A) A schematic diagram of the DamID technique.

(B–G) The binding sites associated with *aPKC* (B), *mira* (C), *CycE* (D), *eve* (E), *nerfin-1* (F), and *FasI* (G) are shown in red; predicted Prospero binding motifs are indicated with red arrows. The genome is a black horizontal line; exons of the gene of interest are green; other transcription units in the region are gray. Vertical bars indicate the position of oligonucleotides on the genomic microarray. Bar heights are proportional to the average of normalized log-transformed ratio of intensities from four replicate DamID in vivo binding-site-mapping experiments. Genomic regions with a Dam-Prospero/Dam ratio over 1.4 (red horizontal line) for at least four consecutive probes were identified as Prospero binding sites (red and blue vertical bars). In all diagrams, the transcription start of the gene of interest is to the left. The total length of genomic DNA displayed for each gene is 60 kb.

Prospero Binds near both Neuroblast Genes and Glial Genes

A total of 730 genes have one or more of the 1,066 Prospero binding sites located within 1 kb of their transcription unit (Table S1). We performed statistical analyses to determine GO annotation (Ashburner et al., 2000) enrichment on the members of our list that had some associated annotation (519) by using a web-based set of tools, GOToolbox (Martin et al., 2004) (see Experimental Procedures). Using Biological Process (GO: 0008150) as the broadest classification, we generated a list of over-represented classes of genes.



B **Neuroblast Fate Genes**

achaete (ac)
scute (sc)
lethal of scute (l(1)sc)

asense (ase)
deadpan (dpn)

snail (sna)
escargot (esg)

miranda (mira)
inscuteable (insc)
bazooka (baz)
atypical protein kinase C (aPKC)

hunchback (hb)
Kruppel (Kr)
pdm (nub + pdm2)
grainyhead (grh)

Cell Cycle Genes

Cyclin E (CycE)
string (stg)
E2f

Glial Genes

glial cells missing (gcm)
bangles and beads (bnb)
gilgamesh (gish)

GMC Genes

even skipped (eve)
fushi tarazu (ftz)

Figure 2. Prospero Binds Genes Involved in Neurogenesis and Cell-Fate Determination

(A) GO annotation classes that are overrepresented in the list of 519 annotated Prospero target genes (relative to the annotated genes in the genome). Bars show fold enrichment compared to that expected at random. The color of the bar represents the Bonferroni-corrected p value, indicating the significance of the overrepresentation. A minimum of a 3-fold enrichment with a significance level of $p < 2 \times 10^{-10}$ yields a total of 24 overrepresented classes of genes. Unexpected classes of overrepresented genes, including mesoderm development and trunk segmentation, may identify previously uncharacterized roles for Prospero, although many of the genes involved in nervous system development and cell-fate determination are also involved in these processes.

(B) Manual annotation of the list of putative Prospero targets yields numerous genes involved in neuroblast fate determination, cell-cycle regulation, GMC cell fate, and glial development.

The three most significant classes of genes enriched in our list of putative Prospero targets are Cell Fate Commitment (GO: 0045165, $p < 2 \times 10^{-31}$), Nervous System Development (GO: 0007399, $p < 2 \times 10^{-27}$), and Regulation of Transcription (GO: 0006357, $p < 2 \times 10^{-27}$) (Figure 2A). This unbiased analysis validates the list of putative Prospero targets as the most significantly overrepresented genes broadly concur with what is known about Prospero function.

Utilizing GO annotation, we find that nearly 41% of all annotated neuroblast fate genes (11 of 27; Neuroblast Fate Determination, GO: 0007400) are located near

Prospero binding sites and that approximately 9% of known cell-cycle genes (13 of 144; Mitotic Cell Cycle Genes, GO: 0000278) are near Prospero binding sites. These include the neuroblast genes *achaete* (*ac*), *scute* (*sc*), *asense* (*ase*) (Figure 2B), *aPKC*, and *mira* (Figures 2B, 1B, and 1C) and the cell-cycle regulators *stg* (Figure 2B) and *CycE* (Figures 2B and 1D). In addition, we find that the *Drosophila* homolog of the mammalian *B lymphoma Mo-MLV insertion region 1* (*Bmi-1*) gene, *Posterior sex combs* (Brunk et al., 1991; van Lohuizen et al., 1991), is located near a Prospero binding site (Table S1). Bmi-1 is a transcription factor that has been shown to regulate the self-renewal of vertebrate hematopoetic stem cells (reviewed in Raaphorst, 2003). We conclude that Prospero is likely to regulate neuroblast identity and self-renewal genes as well as cell-cycle genes directly, repressing their expression in the GMC (see below).

Prospero enters the nucleus of GMCs, and its expression is maintained in glial cells but not in neurons (Spana and Doe, 1995). We therefore searched the list of targets for genes annotated as glial development genes (Gliogenesis, GO: 0042063). Prospero binds near 45% of genes involved in gliogenesis (9 of 20). Among the glial genes, we find that the master regulator of glial development, *glial cells missing* (*gcm*) (Hosoya et al., 1995; Jones et al., 1995), and *gilgamesh* (*gish*), a gene involved in glial cell migration (Hummel et al., 2002), are both near Prospero binding sites (Figure 2B) and are likely directly activated by Prospero in glia.

In summary, Prospero binds near, and is likely to regulate directly, genes required for the self-renewing neural stem cell fate such as cell-cycle genes. We also find that Prospero binds near most of the temporal cascade genes: *hb*, *Kruppel* (*Kr*), *nubbin* (*nub/pdm1*), and *grainyhead* (*grh*) (Table S1 and Figure 2B) and to genes required for glial cell fate. Our in vivo binding-site mapping experiments are supportive of a role for Prospero in regulating the fate of *Drosophila* neural precursors by directly controlling their mitotic potential and capacity to self-renew.

Prospero Represses Neuroblast Genes

The *Drosophila* ventral nerve cord develops in layers, in a manner analogous to the mammalian cortex (Isshiki et al., 2001). The deepest (most dorsal) layer of the VNC comprises the mature neurons, while the superficial layer (most ventral) is made up of the mitotically active, self-renewing neuroblasts (Figure 3). Neuroblast cell-fate genes and cell-cycle genes are normally expressed only in the most ventral cells, while Prospero is found in the nucleus of the more dorsally lying GMCs. If in GMCs, Prospero normally acts to repress neuroblast cell-fate genes and cell-cycle genes, then in a *prospero* mutant, expression of those genes should expand dorsally. Conversely, ectopically expressed Prospero should repress gene expression in the neuroblast layer.

The neuroblast genes *mira* (Figures 3A and 3B), *ase* (Figure 4A), and *insc* (Figure 4C) and the cell cycle genes *CycE* (Figure 4E) and *stg* (data not shown) show little or no expression in differentiated cells of wild-type stage 14 nerve cords. We assayed expression of these neuroblast-specific genes in the differentiated cells layer of *prospero* embryos and found that they are derepressed throughout the nerve cord of mutant embryos (Figures 3 and 4). *mira* (Figures 3C and 3D), *ase* (Figure 4B), *insc* (Figure 4D), *CycE* (Figure 4F), and *stg* (data not shown) are all ectopically expressed deep into the normally differentiated cell layer of the VNC. To check whether Prospero is sufficient to repress these genes, we expressed Prospero with the *sca-GAL4* driver (Klaes et al., 1994), forcing Prospero into the nucleus of neuroblasts (A.J. Schuldt and A.H.B., unpublished data). Prospero expression is sufficient to repress *mira* (Figures 3E–3H), *ase* (Figures 4G and 4H), *insc* (Figures 4I and 4J), *CycE* (Figures 4K and 4L), and *stg* (data not shown) in the undifferentiated cell layer of the VNC. These data, combined with the Prospero binding-site data, demonstrate that Prospero is both necessary and sufficient to directly repress neuroblast genes and cell-cycle genes in differentiated cells. This direct repression of gene expression is one mechanism by which Prospero initiates the differentiation of neural stem cells.

Prospero Is Required for Activation of Differentiation Genes

Having shown that Prospero directly represses genes required for neural stem cell fate, we asked whether Prospero also directly activates GMC-specific genes. Alternatively, Prospero might regulate a second tier of transcription factors, which are themselves responsible for the GMC fate. Of the few previously characterized GMC genes (Doe, 1992), we find that Prospero binds to *eve* (Figure 1E) and *fushi-tarazu* (*ftz*) (Table S1). In our list of targets we expected to find several more GMC genes, but not genes involved in neuronal differentiation, as Prospero is not expressed in neurons (Doe et al., 1991). Much to our surprise, however, we discovered that 18.8% of neuronal differentiation genes are located near Prospero binding sites (26 of 138) (Figure 2B and Table S2) (see Discussion).

To determine Prospero's role in regulating these neuronal differentiation genes, we carried out in situ hybridization on *prospero* mutant embryos. We find that Prospero is necessary for the expression of a subset of differentiation genes, such as the adhesion molecules FasciclinI (FasI) (Figures 1G, 4Q, and 4R) and FasciclinII (FasII) (Figures 4S and 4T), which have roles in axon guidance and/or fasciculation (Elkins et al., 1990; Lin et al., 1994). Netrin-B, a secreted protein that guides axon outgrowth (Mitchell et al., 1996), and Encore, a negative regulator of mitosis (Hawkins et al., 1996), also both require Prospero for proper expression (Figures 4U, 4V, 4W, and 4X). Therefore, in addition to directly repressing genes required for neural stem cell self-renewal, Prospero binds and activates genes that direct differentiation. Our data suggest that Prospero is a binary switch between the neural stem cell fate and the terminally differentiated neuronal fate.

Prospero Represses Neural Stem Cell Genes and Is Required to Activate Neuronal-Differentiation Genes

To test to what extent Prospero regulates the genes to which it binds, we carried out genome-wide expression profiling on wild-type and *prospero* mutant embryos. While the DamID approach identifies Prospero targets in all tissues of the embryo, here we assayed specifically for genes regulated by Prospero in the developing

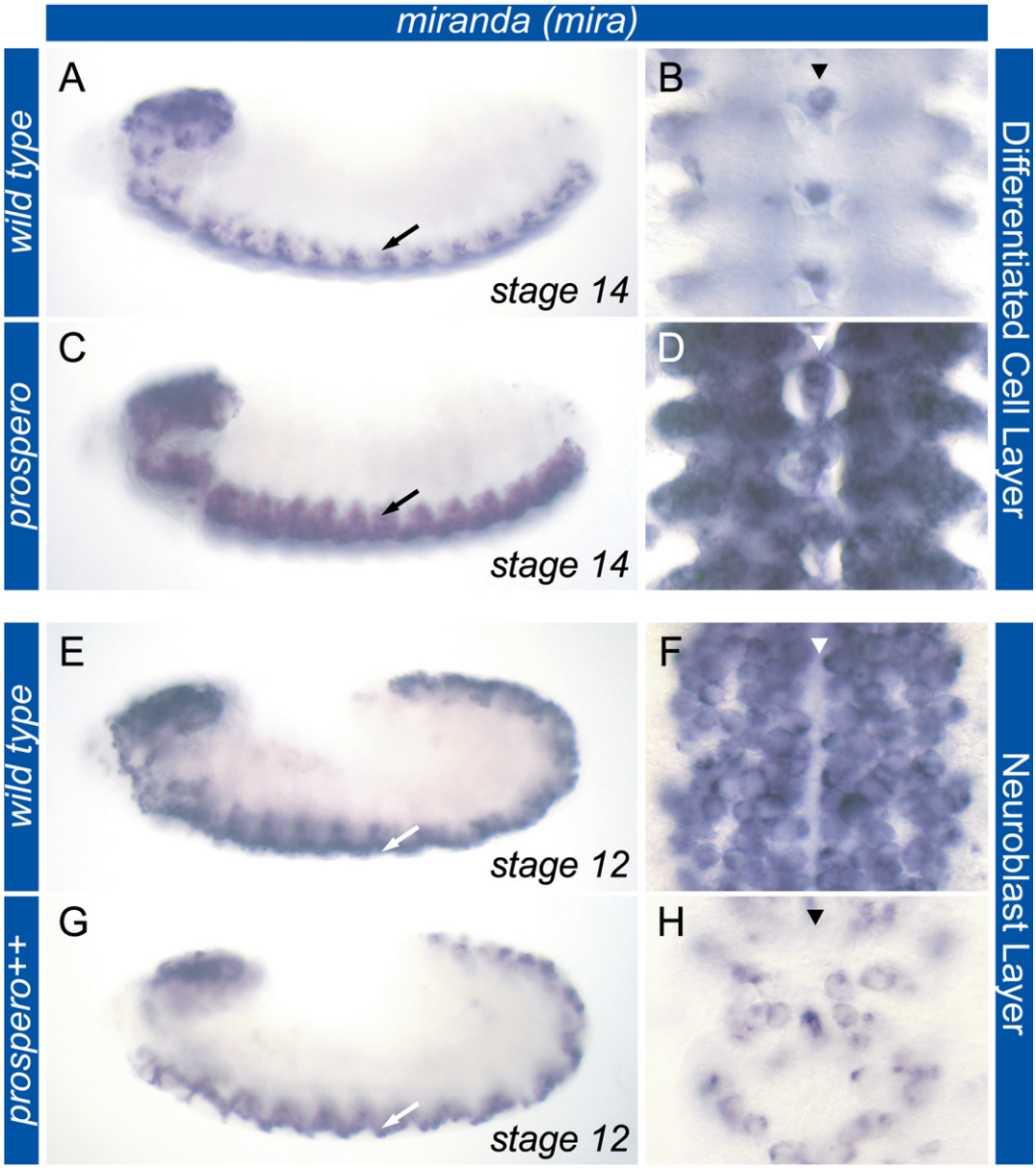


Figure 3. Prospero Represses the Neuroblast Gene, *miranda*, in Differentiated Cells

(A) *mira* transcripts are restricted to the neuroblast layer at stage 14 and are nearly absent from differentiated cells (arrow).
(B) Ventral view of differentiated cell layer.
(C) In *prospero* mutant embryos, *mira* is expressed throughout the VNC, including differentiated cells (arrow).
(D) Ventral view of differentiated cell layer.
(E) *mira* transcript is found in most neuroblasts at stage 12 (arrow).
(F) Ventral view.
(G) Ectopic Prospero in neuroblasts reduces *mira* transcription in the stem cell layer (arrow; *sca-GAL4/+;UAS-YFP-Pros/+*).
(H) Ventral view. (A, C, E, and G) Lateral views, anterior to the left; (B, D, F, and H) ventral views of nerve cords, anterior at top. Arrowheads mark the ventral midline.

central nervous system. We isolated small groups of neural stem cells and their progeny (on the order of 100 cells) from the ventral nerve cords of living late stage 12 embryos with a glass capillary. The cells were expelled into lysis buffer, and cDNA libraries generated by reverse transcription and PCR amplification (based upon methods described by Iscove et al., 2002, and Osawa et al., 2005). cDNA libraries prepared from neural cells from six wild-type and six *prospero* null mutant embryos were hybridized to full genome oligonucleotide microarrays, together with a common reference sample. Wild-type and *prospero* mutant cells were compared indirectly through the common reference.

In the group of Prospero target genes that contain a Prospero consensus sequence within 1 kb of the transcription unit, 91 show reproducible differences in gene expression in *prospero* mutants (Figure 5 and Table S3). Seventy-nine percent of these genes (72) exhibit at least

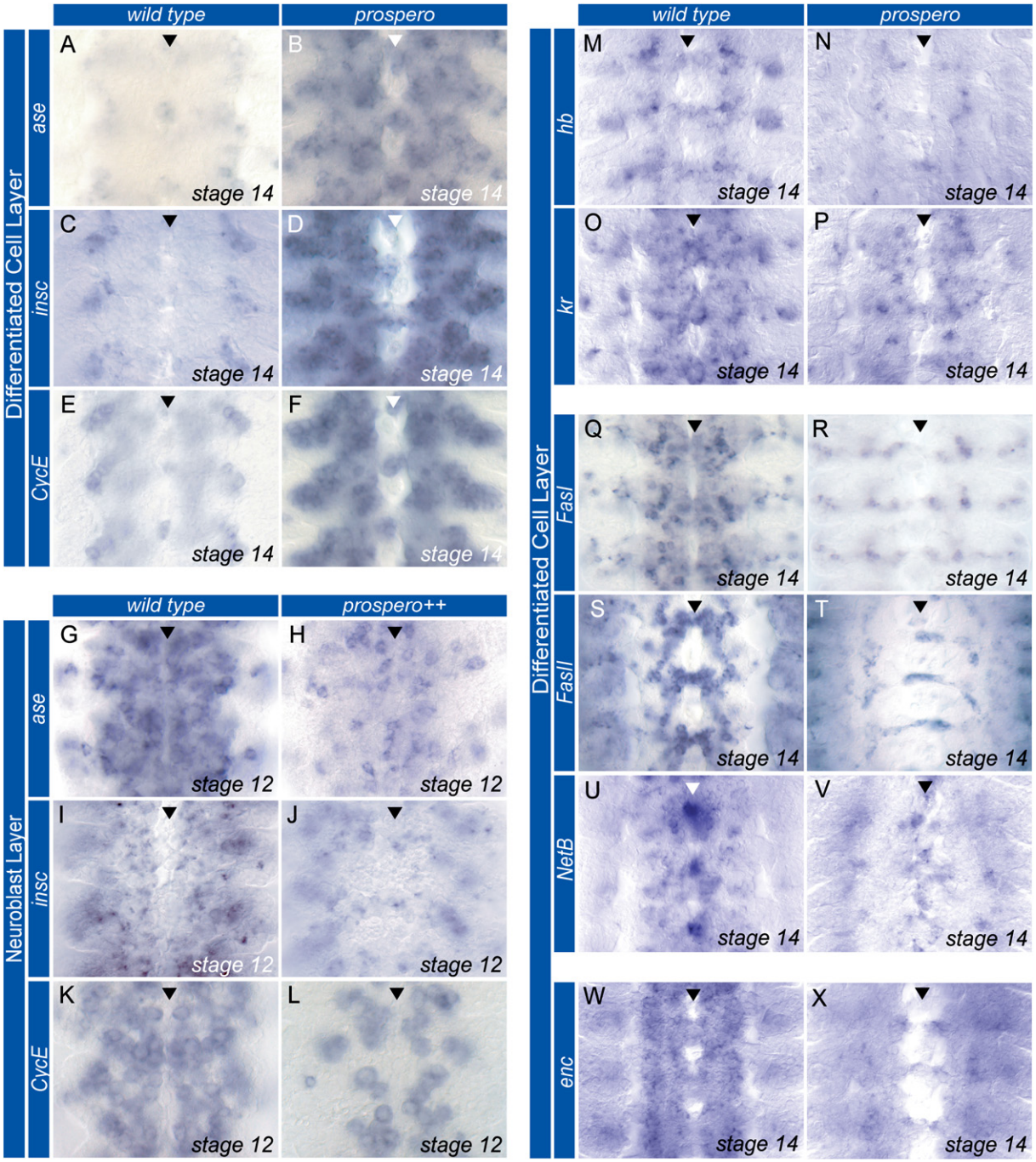


Figure 4. Prospero Is Required to Repress Neuroblast Gene Expression, to Maintain Temporal Cascade Genes, and to Activate Neuronal Genes

ase, *insc*, and *CycE* transcripts are nearly absent from the differentiated cells of wild-type VNCs (A, C, and E) but are derepressed throughout the VNC of stage 14 *prospero* embryos (B, D, and F). *ase*, *insc*, and *CycE* are expressed throughout the neuroblast layer at stage 12 (G, I, and K). Overexpression of Prospero in neuroblasts (prospero++: *sca-GAL4/+;UAS-YFP-Pros/+* embryos) is sufficient to reduce transcription of all three neuroblast genes in the stem cell layer (H, J, and L). Expression of the temporal cascade genes *hb* and *kr* is reduced in *prospero* mutant embryos (N and P) compared to wild-type (M and O). *FasI* and *FasII* are expressed in many neurons at stage 14 (Q and S) and expression is almost completely lost in *prospero* mutants (R and T). *NetB* is expressed in a subset of neurons and on the midline (U); expression is severely reduced in *prospero* mutants (V). *enc* also shows reduced expression in *prospero* mutants (W and X).Ventral views of nerve cords, anterior up. Arrowheads mark the ventral midline.

a 2-fold change in levels of expression. Many of the known genes involved in neuroblast fate determination and cell-cycle regulation (e.g., *asense*, *deadpan*, *miranda*, *inscuteable*, *CyclinE*, and *string*) show increased levels in a *prospero* mutant background, consistent with their being repressed by Prospero. Genes to which Prospero binds, but which do not contain an obvious consensus sequence, are also regulated by Prospero: *CyclinA* and *Bazooka* show elevated mRNA levels in the absence of Prospero, as does *Staufen*, which

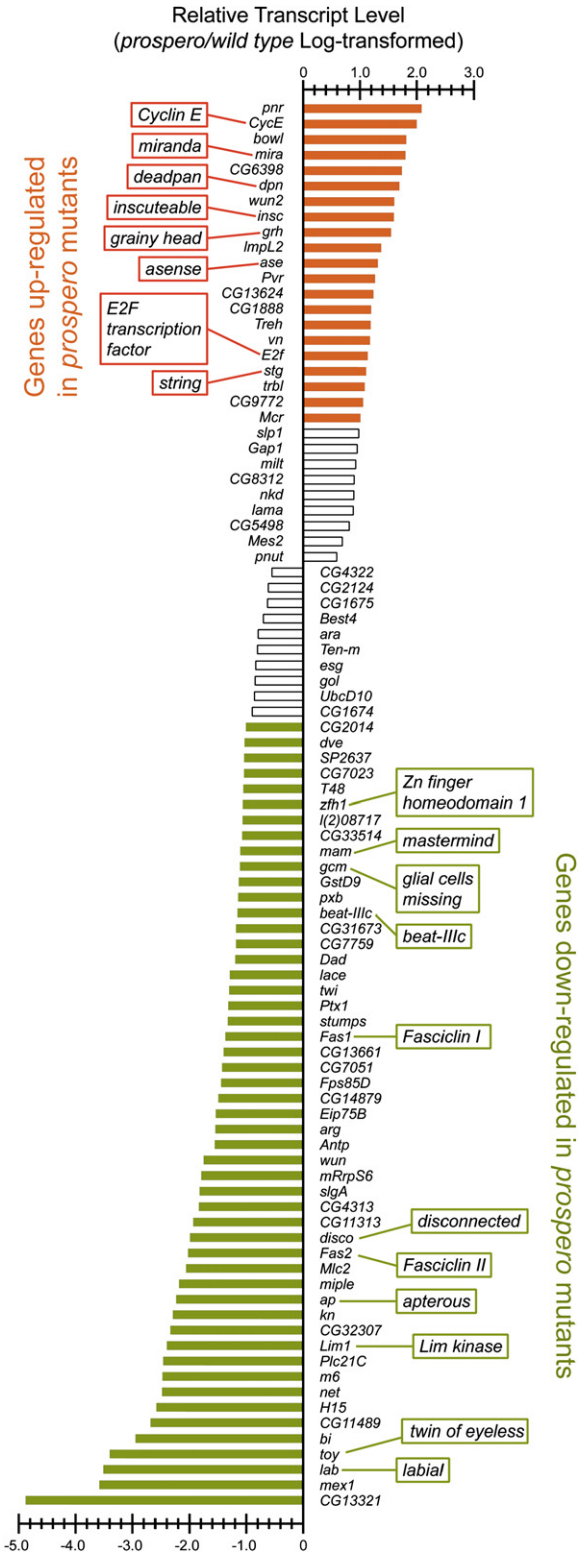


Figure 5. Prospero Represses Neural Stem Cell Genes and Is Required for Activation of Neuronal Differentiation Genes

Gene expression profiling of cells from the VNC identifies 91 genes whose expression is significantly altered in *prospero* mutants and which are located within 1 kb of a Prospero binding site. Genes upregulated in *prospero* mutant embryos are shown in red, and downregulated genes are shown in green. Genes outlined in red were manually annotated as having a known role in neuroblast fate or cell-cycle regulation; genes in green were annotated as neuronal differentiation genes (involved in either neuronal or glial differentiation).

encodes a dsRNA binding protein that binds to both Miranda and to *prospero* mRNA (Table S3).

Expression of genes required for neuronal differentiation is decreased in the *prospero* mutant cells, consistent with Prospero being required for their transcription (Figure 5). These include *zfh1* and *Lim1*, which specify neuronal subtypes (Garces and Thor, 2006; Lilly et al., 1999), and *FasI* and *FasII*, which regulate axon fasciculation and path finding (Elkins et al., 1990; Lin et al., 1994).

Prospero Is a Tumor Suppressor

The stem cell-like division of neuroblasts generates two daughters: a self-renewing neuroblast and a differentiating GMC. Prospero represses stem cell self-renewal genes and activates differentiation genes in the newly born GMC. In the absence of *prospero*, therefore, neuroblasts should give rise to two self-renewing neuroblast-like cells.

We followed the division pattern of individual neuroblasts in *prospero* mutant embryos by labeling with the lipophilic dye, DiI. Individual cells were labeled at stage 6, and the embryos allowed to develop until stage 17. S1 or S2 neuroblasts were examined, as determined by their time of delamination. Wild-type neuroblasts generate between 2 and 32 cells, producing an average of 16.2 cells (n = 26) (Figures 6A and 6C). Most of the clones exhibit extensive axonal outgrowth, as seen in the neuroblast 3-2 clone (Figure 6A, arrows). In contrast, *prospero* mutant neuroblasts generate between 8 and 51 cells, producing an average of 31.8 cells (n = 26) (Figures 6B and 6C) ($p < 0.0001$, Student's t test). Moreover, *prospero* mutant neural clones exhibit few if any projections (Figure 6B, arrow), and the cells are smaller in size. Thus, *prospero* mutant neuroblasts produce much larger clones of cells with no axonal projections, suggesting that neural cells in *prospero* mutants undergo extra divisions and fail to differentiate.

GMCs Are Transformed into Neural Stem Cells in *prospero* Mutants

Recently it was shown, in the larval brain, that clones of cells lacking Prospero or Brat undergo extensive cell division to generate undifferentiated tumors (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006b). Given that Prospero is nuclear in the GMC but not in neuroblasts, the expanded neuroblast clones in *prospero* mutant embryos might arise from the overproliferation of GMCs: the GMCs lacking Prospero may divide like neuroblasts in a self-renewing manner (Figure 6D, I). It is also possible, however, that neuroblasts divide more frequently in *prospero* mutant embryos, giving rise to supernumerary GMCs that each divide only once (Figure 6D, II) (see also discussion in Lee et al., 2006b). To distinguish between these two possibilities, we followed the division pattern of individual GMCs in *prospero* mutant embryos.

S1 or S2 neuroblasts were labeled with DiI as before. After the first cell division of each neuroblast, the

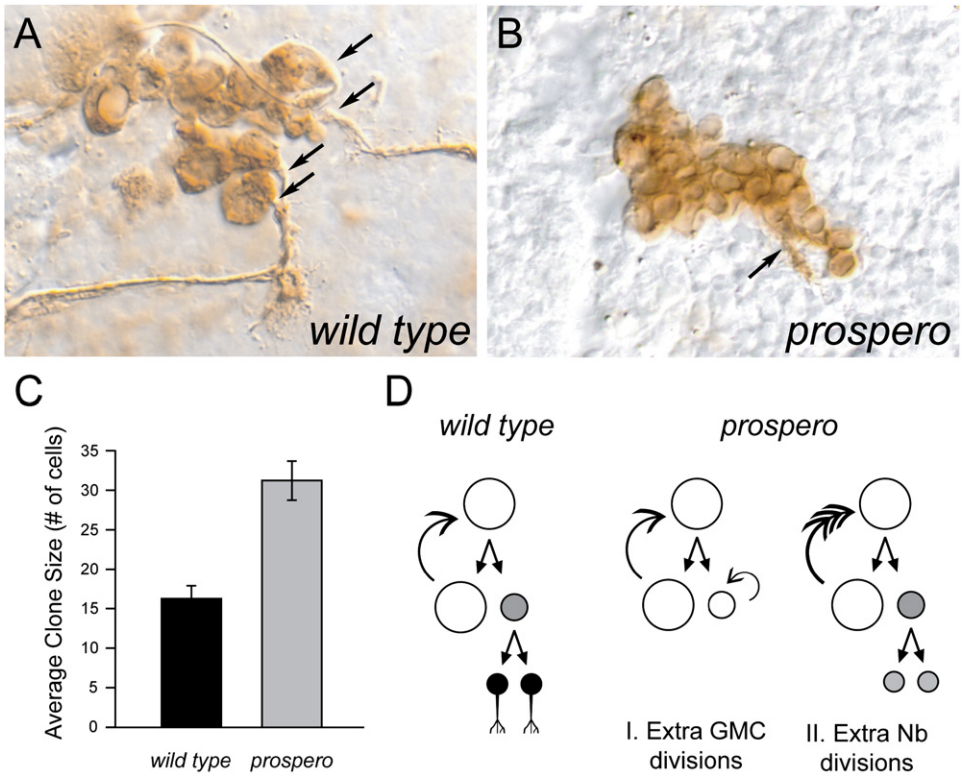


Figure 6. Neuroblasts Overproliferate and Fail to Differentiate in *prospero* Mutants

(A) Wild-type clones from a single S1 or S2 neuroblast labeled with DiI extend axonal projections (arrows) that often exit the CNS.

(B and C) *prospero* mutant clones give on average twice as many cells without axonal projections ($p < 0.001$, Student's t test). Instead, they occasionally exhibit short, blunt outgrowths that never exit the CNS (B, arrow).

(D) Wild-type neuroblasts (white) divide in a self-renewing manner to produce a neuroblast and a GMC (gray). The GMC divides only once to produce two neurons or glial cells (black). In *prospero* mutant embryos, GMCs either divide in a self-renewing manner (I), or mutant neuroblasts divide more frequently, generating GMCs that divide normally (II). Ventral views of embryos, anterior up.

neuroblast was mechanically ablated, leaving its first-born GMC. All further labeled progeny derive, therefore, from the GMC (Figures 7A and 7B). Embryos were allowed to develop until stage 17, at which time the number of cells generated by a single GMC was determined.

Wild-type GMCs consistently give rise to just two daughter cells (5/5) (Figures 7C and 7E), which extend axons such as those shown in Figure 7C (arrowheads). In contrast, 5/6 *prospero* mutant GMCs give rise to more than two daughter cells (Figures 7D and 7E). In two cases, a single *prospero* mutant GMC divided to generate seven cells. These GMC offspring exhibited few if any signs of differentiation, rarely producing projections.

To determine whether mutant GMCs are transformed to a stem cell-like state, stage 14 embryos were stained for the three neuroblast markers: Miranda (Mira), Worniu (Wor), and Deadpan (Dpn). In wild-type embryos at stage 14, the most dorsal layer of cells in the VNC consists mostly of differentiated neurons. As a result, few or none of the cells in this layer express markers of self-renewal. Mira- (Figure 7F), Wor- (Figure 7H), and Dpn- (Figure 7J) expressing cells are only found on the midline (arrowheads) or in lateral neuroblasts of the differentiated cell layer of wild-type nerve cords. In contrast, a majority of cells in the differentiated cell layer of stage 14 *prospero* mutant embryos express all three markers: Mira is found cortically localized in most cells of the dorsal layer of *prospero* nerve cords (Figure 7G); Wor is nuclear in most cells of mutant VNCs (Figure 7I); Dpn is ectopically expressed throughout the nerve cord of *prospero* mutants (Figure 7K).

We assayed expression of neuroblast markers in the ventral-most layer of the nerve cord (the neuroblast layer), to exclude the possibility that a general disorganization of cells within the VNC contributes to the increased number of Mira-, Wor-, and Dpn-positive cells in the dorsal layer. The number of neuroblasts in a *prospero* mutant embryo is normal in stage 14 embryos, as assayed by Wor (Figure S1), Dpn, and Mira expression (data not shown). Thus, the increased expression of neuroblast markers in *prospero* mutants is the result of an increase in the total number of cells expressing these markers in the differentiated cell layer. We conclude that *prospero* mutant neuroblasts divide to give two stem cell-like daughters. GMCs, which would normally terminate cell division and differentiate, are transformed into self-renewing neural stem cells that generate undifferentiated clones or tumors.

Discussion

Here, we show that the homeodomain transcription factor, Prospero, regulates the switch from stem cell self-renewal to differentiation in the *Drosophila* nervous

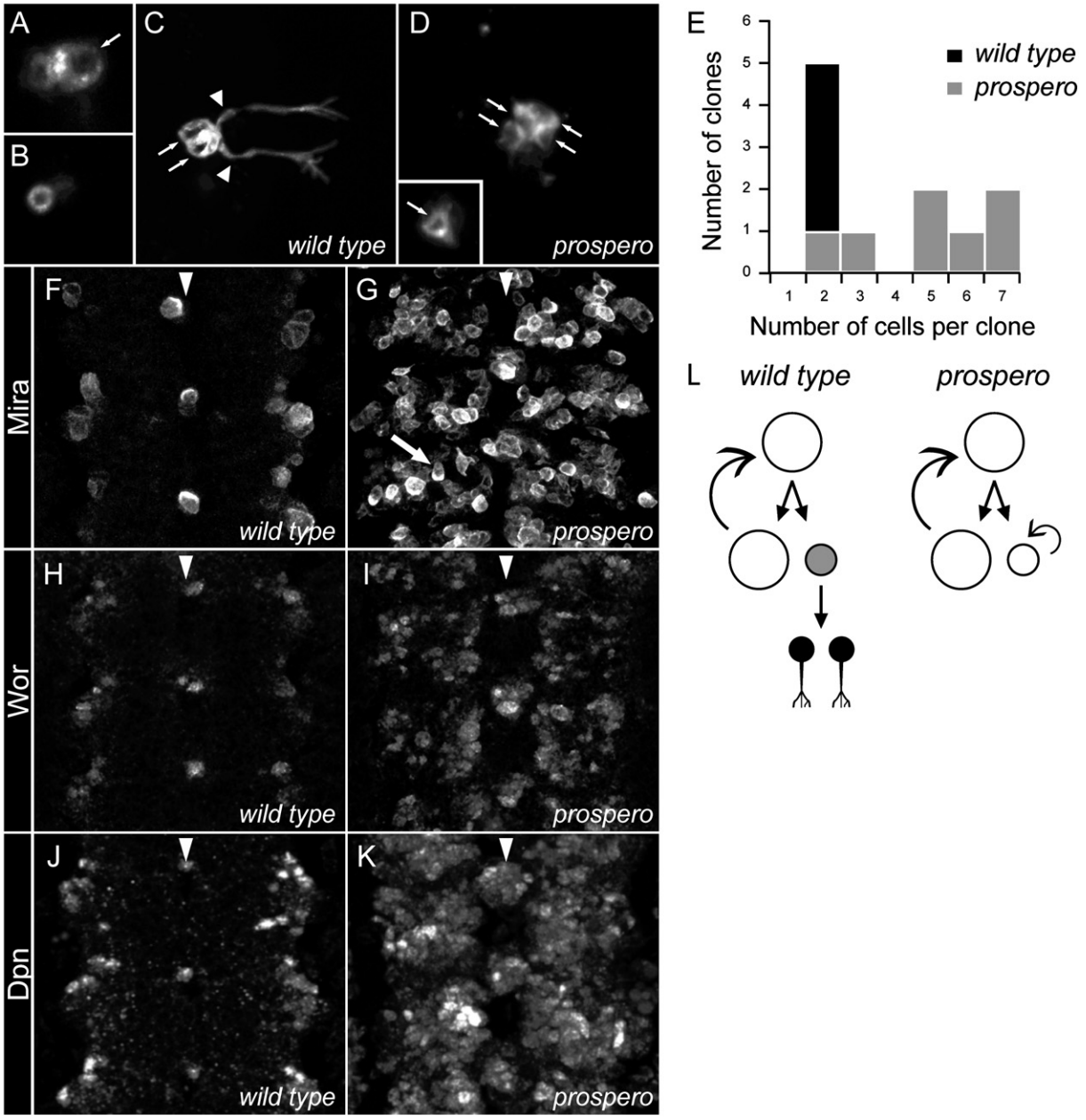


Figure 7. *prospero* Mutant GMCs Are Transformed into Self-Renewing Neural Stem Cells

(A–E) The division pattern of a single GMC lacking Prospero was determined after ablation of the neuroblast from which it was born. The neuroblast (A, arrow) was mechanically ablated after producing a single daughter GMC, leaving the GMC (B) intact. At stage 17, cell number was determined by counting nuclei (arrows; C, wild-type; D, *prospero* mutant). (C and E) Wild-type GMCs produce two cells with axonal projections (arrowheads); (D and E) *prospero* mutant GMCs produce between two and seven cells with few or no projections. Inset in (D) shows a single cell lying ventral to the main cluster.

(F, H, and J) Miranda, Worniu, and Deadpan protein are found in almost no differentiated cells of wild-type embryos, only in the ventral midline (arrowheads) and a few lateral neuroblasts.

(G, I, and K) In stage 14 *prospero* mutant nerve cords, all three neuroblast markers are expressed in a majority of cells in the differentiated layer of the nerve cord (arrow). In each image, three segments are shown (A2–A6); ventral view, anterior at top. Images are projections of single confocal sections spanning 4–6 μm.

(L) Neuroblasts and GMCs self-renew in *prospero* mutant embryos.

system. Prospero directly represses stem cell genes, including neuroblast fate genes and genes that promote proliferation. Prospero also regulates genes involved in neuroblast asymmetric cell division and in the temporal control of neuronal identity. Remarkably, we also find that Prospero directly activates the expression of neuronal genes, suggesting that Prospero promotes differentiation not merely by blocking cell division but by actively promoting the transcription of genes required for terminal differentiation. Loss of Prospero transforms GMCs to neuroblasts: mutant GMCs divide repeatedly and express neuroblast markers such as

Miranda, Worniu, and Deadpan. Mutant GMCs fail to initiate differentiation and instead resemble their self-renewing neuroblast siblings.

Prospero Function during Neurogenesis

Earlier studies on Prospero raise two questions: how does Prospero regulate the differentiation of neural progenitors, and what is the fate of GMCs that lack Prospero? Embryos mutant for *prospero* overexpress cell-cycle genes (*CycA*, *CycE*, *Rbf*, and *stg*). Prospero might repress these cell-cycle regulators directly, resulting in an exit from the cell cycle and subsequent differentiation. Alternatively, Prospero might activate differentiation genes directly, resulting in a downregulation of cell-cycle genes and exit from the cell cycle. Finally, Prospero might regulate these genes through a cascade of effector genes. Here, we show that Prospero is necessary and sufficient to repress *CycE*, *stg*, and *E2f* (data not shown), all of which exhibit DamID peaks and contain a Prospero consensus sequence. Prospero also regulates *CycA*, to which it binds but which has no obvious consensus, but not *Rbf*, which has neither a DamID peak nor a consensus sequence; Prospero also activates the gene *encore*, which represses germ-line mitoses (Hawkins et al., 1996) and is likely to play a role in repressing mitosis in neurons as well.

Prospero directly represses the transcription of many neuroblast genes (Table S1) and binds near most of the temporal cascade genes: *hb*, *Kruppel* (*Kr*), *nubbin* (*nub*/*pdm1*), and *grainyhead* (*grh*), which regulate the timing of cell-fate specification in neuroblast progeny (Brody and Odenwald, 2000; Isshiki et al., 2001). Prospero maintains *hb* expression in the GMC, and it has been suggested that this is through regulation of another gene, *seven-up* (*svp*) (Mettler et al., 2006). We show here that Prospero not only regulates *svp* expression directly but also maintains *hb* expression directly. In addition, Prospero maintains *Kr* expression and is likely to act in a similar fashion on other genes of the temporal cascade. Intriguingly, Prospero regulates several of the genes that direct asymmetric neuroblast division (*baz*, *mira*, *insc*, *aPKC*). *aPKC* has recently been shown to be involved in maintaining the self-renewing state of neuroblasts (Lee et al., 2006a).

Prospero initiates the expression of genes necessary for differentiation. This is particularly surprising as *prospero* is only transcribed in neuroblasts, not in GMCs or neurons (Broadus et al., 1998). Prospero mRNA and protein are then segregated to the GMC (Broadus et al., 1998; Li et al., 1997; Schuldt et al., 1998; Shen et al., 1998). Prospero binds near *eve* and *ftz*, which were previously shown to be downstream of Prospero (Doe et al., 1991; Vaessin et al., 1991), as well as to genes required for terminal neuronal differentiation, including the neural-cell-adhesion molecules FasI and FasII. Prospero protein is present in GMCs and not neurons (Matsuzaki et al., 1992; Vaessin et al., 1991), suggesting that Prospero initiates activation of neuronal genes in the GMC. The GMC may be a transition state between the neural stem cell and the differentiated neuron, providing a window during which Prospero functions to repress stem cell-specific genes and activate genes required for differentiation. There may be few, or no, genes exclusively expressed in GMCs.

Prospero acts in a context-dependent manner, functioning alternately to repress or activate transcription. This implies that there are cofactors and/or chromatin remodeling factors that modulate Prospero's activity. In support of this, although Prospero is necessary and sufficient to repress neuroblast genes, forcing Prospero into the nuclei of neuroblasts is not sufficient to activate all of the differentiation genes to which it binds (data not shown).

Regulation of Cell Cycle and Differentiation in the Absence of Prospero

Neuroblasts decrease in size with each division throughout embryogenesis. By the end of embryogenesis, they are similar in size to neurons (Hartenstein et al., 1987). A subset of these embryonic neuroblasts becomes quiescent and is reactivated during larval life: they enlarge and resume stem cell divisions to generate the adult nervous system (Truman and Bate, 1988). Neuroblasts in *prospero* mutant embryos divide to produce two self-renewing daughters but still divide asymmetrically with respect to size (Doe et al., 1991), producing a large apical neuroblast and a smaller basal neuroblast-like cell. The daughter may be too small to undergo more than three additional rounds of division during embryogenesis. *prospero* mutant cells eventually stop dividing, and a small number occasionally differentiate. This suggests that there is an inherent size limitation on cell division. The segregation of Brat (Betschinger et al., 2006), or an additional cell fate determinant, to the daughter cell may also limit the potential of the *prospero* mutant cells to keep dividing.

Prospero/Prox1: Universal Regulator of Progenitor Differentiation?

The Prox family of atypical homeodomain transcription factors has been implicated in initiating the differentiation of progenitor cells in contexts as varied as the vertebrate retina, forebrain, and lymphatic system (Dyer et al., 2003; Torii et al., 1999; Wigle and Oliver, 1999). Prospero/Prox generally regulates the transition from a multipotent, mitotically active precursor to a differentiated, postmitotic cell. In most contexts, Prox1 acts in a similar fashion to *Drosophila* Prospero: to stop division and initiate differentiation.

We propose that Prospero/Prox is a master regulator of the differentiation of progenitor cells. Many of the vertebrate homologs of the *Drosophila* Prospero targets identified here may also be targets of Prox1 in other developmental contexts. Prospero directly regulates several genes required for cell-cycle progression (Figure 2B), and it is possible that Prox1 will regulate a similar set of cell-cycle genes during, for example, vertebrate retinal development. In addition, we have identified numerous Prospero target genes whose orthologs may be involved in the Prox-dependent differentiation of retina, lens, and forebrain precursors.

Implications for Stem Cell Biology and Cancer Biology

One of the goals of therapeutic cloning is to expand a pool of stem cells and then induce their differentiation to a particular lineage for therapeutic purposes. In this work, we show that the expansion of neural stem cells

in *Drosophila* can be achieved by the removal of a single transcription factor. It may, therefore, be possible to induce the differentiation of this expanded pool by reintroducing Prospero. This would be one of the first steps toward directing stem cells to differentiate into specific cell types in vivo.

The stem cell model of cancer attributes cancerous growth to the misregulation of stem cell self-renewal. Several genes have been shown to be involved in the regulation of self-renewal, including those encoding the transcription factor Bmi-1 and members of the Notch, Wnt, and Sonic Hedgehog signaling pathways (reviewed in Al-Hajj et al., 2003). Homeodomain genes are downregulated in numerous cancers (reviewed in Abate-Shen, 2002). Much of their function is likely to be the regulation of cell-cycle genes, but little is known about the direct molecular mechanisms by which homeodomain proteins regulate self-renewal in cancer (reviewed in Del Bene and Wittbrodt, 2005). *prospero* mutant neuroblasts have been shown to cause tumors when transplanted from the larval brain into the abdomen (Caussinus and Gonzalez, 2005). We show that Prospero-mediated transcriptional repression of stem cell genes, and activation of differentiation genes, prevents tumorigenic growth, suggesting that Prospero is in fact a tumor suppressor. Our single-cell analysis reveals that *prospero* mutants lose control of stem cell differentiation, leading to overproliferation in the embryo. The presence of Prospero in the daughter cells of *Drosophila* midgut stem cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) suggests that Prospero may be a versatile regulator of stem cell differentiation.

Experimental Procedures

Fly Lines

prospero^{17} is an RNA null mutation (Doe et al., 1991). The *prospero*^{17} mutation was maintained over a *TM3,Sb* green balancer chromosome (*Kruppel-GAL4*;*UAS-GFP*) to distinguish homozygous *prospero* mutant embryos from heterozygous and wild-type siblings. Wild-type refers to *OregonR* throughout the text, except where noted. *scabrous-GAL4* (Klaes et al., 1994) was used to drive UAS-YFP-Pros (A.J. Schuldt, C.B. Phelps, and A.H.B., unpublished data) throughout the ectoderm and nerve cord starting at late stage 10.

Plasmid Construction for DamID

To express Dam fusion proteins, we first generated a pUAST-NDam plasmid by cloning the Dam-Myc sequence from pNDamMyc (van Steensel et al., 2001) into the multiple cloning site of pUAST (Brand and Perrimon, 1993) after digestion with EcoRI and BglII. The pUASTNDam-Prospero construct was made by PCR amplification of the *prospero* cDNA from a *Drosophila* embryonic cDNA library and insertion into pUASTNDam with BglII and NotI sites. The *prospero* cDNA was amplified with the following primers: prospero-for (5′-GAGATCTGATGAGTAGCGCTGCCGCGGCTGCTGCG-3′) and prospero-rev (5′-GTACGCGGCCGCTTCCAGCTGCTCTAAAAAATT GGGCG-3′). Transgenic flies containing either pUAST-NDam (*UAS-Dam*) or pUAST-NDam-Prospero (*UAS-Dam-Prospero*) were generated as described previously (Brand and Perrimon, 1993), except that DNA was prepared with a Qiagen midiprep kit.

DNA Isolation for DamID

Stage 10–11 embryos (4–7 hr AEL) were collected from *UAS-Dam* (control) and *UAS-Dam-Prospero* flies. Genomic DNA was isolated from embryos with the Qiagen DNeasy kit. Fifty milligrams of embryos were homogenized in 180 μl of PBS, and then 4 μl of RNase A (100 mg/ml) was added and left to incubate for 2 min to remove RNA from the sample.

Digestion and PCR Amplification of Dam-Methylated DNA

DNA digestion and PCR amplification was done essentially as previously described (Greil et al., 2003). For selective PCR amplication of methylated DNA fragments, 2.5 μg of the isolated genomic DNA was digested for 16 hr at 37°C with ten units DpnI (NEB) in a total volume of 10 μl buffer 4 (NEB). After inactivation of DpnI at 80°C for 20 min, 1.25 μg of the DpnI digested genomic DNA was ligated to 40 pmol of a double-stranded unphosphorylated adaptor (top strand, 5′-CTAA TACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA-3′; bottom strand, 5′-TCCTCGGCCG-3′) for 2 hr at 16°C with five units T4-DNA Ligase (Roche) in a total volume of 20 μl ligation buffer (Roche). To prevent amplification of DNA fragments containing unmethylated GATCs, the adaptor-ligated DNA was cut with five units DpnII (NEB) for 1 hr at 37°C in a total volume of 80 μl DpnII buffer (NEB). Next, amplification was performed with 20 μl DpnII-cut DNA (313 ng), 1.6 μl PCR Advantage enzyme mix (Clontech), 16 nmole of each dATP, dCTP, dGTP, dTTP, and 100 pmole primer (5′-GGTCGCGGCCGAGGATC-3′) in 80 μl total volume of PCR Advantage reaction buffer under the following cycling conditions: activation of the polymerase and nick translation for 10 min at 68°C, followed by one cycle of 1 min at 94°C, 5 min at 65°C, and 15 min at 68°C; three cycles of 1 min at 94°C, 1 min at 65°C, and 10 min at 68°C; and 19 cycles of 1 min at 94°C, 1 min at 65°C, and 2 min at 68°C. The PCR products were purified with the QIAquick PCR purification kit (Qiagen).

DamID, Analysis, and Binding-Site Finding

To map Prospero binding sites on a genome-wide scale, a custom whole genome 375,000 feature tiling array, with 60-mer oligonucleotides spaced at approximately 300 bp intervals, was designed against Release 4.0 of the *Drosophila* genome (details available upon request). The control and experimental samples were labeled and hybridized to these custom arrays. Arrays were then scanned, and intensities extracted (Nimblegen Systems).

Four replicates of the Dam-Pros versus Dam only comparison were performed. Two of the experiments used a standard dye configuration, while the other two used a swapped dye configuration. Log2 ratios of each spot were normalized by a mean-centering normalization. The trimmed mean was used for normalization, utilizing only the middle 80% of the data. Dye-swap-corrected, normalized ratios were averaged across all four slides. Peaks were identified. A run-sums algorithm was used to identify regions of the genome bound by Pros, with parameters as follows: peak height threshold $\geq$ 1.4 log-fold change and length of peak $\geq$ 1200 bp. The score of a region was incremented by 1 for a spot with a greater than 1.4 log-fold increase of experimental over wild-type, while the score was decremented by 6 for a spot with a less than 1.4 log-fold increase. Regions with positive scores over a minimum of 1200 bp and spanning a region containing an in vitro-determined binding site (see Table S4) were considered true Pros binding sites (Perl scripts for analysis available upon request).

GO annotation overrepresentation analysis was performed with GOToolbox (Martin et al., 2004). Parameters for overrepresentation searches include: Biological Process, specificity $\geq$ 4, Hypergeometric testing, and Bonferroni correction of p values.

Isolation of Neural Stem Cells for Microarray Expression Profiling

Using a drawn out and beveled capillary, we extracted samples of ~100 cells in vivo from the ventral and intermedial columns of the ventral nerve cords of late stage 12 homozygous and heterozygous *prospero*^{17} embryos (*pros*^{17}*/TM3*, *Sb*, *Kruppel-GAL4*, *UAS-GFP*). The cells were expelled into 1.5 μl lysis buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, 0.5% NP40, 0.4 units/μl RNasin (Promega), 0.3 units/μl PrimeRNase Inhibitor [Eppendorf], 50 μM dNTP, 200 ng/ml anchor oligo dT primer TAT AGA ATT CGC GGC CGC TCG CGA $dT_{[24]}$) and a cDNA library generated by reverse transcription and PCR amplification (detailed protocol available on request; see also Iscove et al., 2002; Osawa et al., 2005, and Subkhankulova and Livesey, 2006) and were hybridized to microarrays (FL002; Flychip Cambridge Microarray Facility). One microgram of

the amplified DNA was labeled with the BioPrime DNA labeling kit (Invitrogen) in the presence of fluorescently labeled Cy3- or Cy5-dCTP (GE Healthcare) at 37°C for 3 hr, and the product purified with Sephadex G50 minicolumns. Slide hybridizations were performed for 16 hr at 51°C with a GeneTac hybridization station (Genomic Solutions). Posthybridization washes were performed according to the manufacturer's recommendation. For statistical analysis of the expression array data, see the Supplemental Experimental Procedures.

DiI Labeling and Ablations

S1 or S2 neuroblasts in *prospero* heterozygotes ("wild-type") or *prospero* homozygous mutant embryos were labeled with DiI. DiI labeling was performed as previously described (Bossing and Technau, 1994). For analysis of neuroblast clones, DiI was photoconverted with DAB. Cell ablations were carried out by labeling single cells with DiI at stage 6. S1 or S2 neuroblasts were ablated with a pulled and beveled capillary 40 min after delamination (stage 9–10), leaving only the first GMC daughter cell. Embryos were allowed to develop until stage 16 before imaging. DiI images were taken at 100× magnification, and projections were assembled manually.

Immunohistochemistry and In Situ Hybridization

See the Supplemental Experimental Procedures.

Microscopy and Imaging

All light-microscopic images were collected with a Zeiss Axioplan microscope equipped with a Progres $C10^+$ digital camera. Fluorescent images were captured on a Leica DMRE microscope fitted with a Leica TCS SP2 confocal scanhead or with a Nikon E800 microscope fitted with a BioRad MRC1024 confocal scanhead. Images were imported into Adobe Photoshop, and figures assembled in Adobe Illustrator. Two-dimensional projections of confocal stacks were created with Imaris. Eight to ten 0.5 μm sections were combined by using an average projection. Reconstructions of DIC images were performed by manually merging up to four different focal planes, to display neural clones that extend over several micrometers in the z axis.

Supplemental Data

Supplemental Data include two figures, four tables, two Excel workbooks (Tables S1 and S3), Supplemental Experimental Procedures, and references and are available online at http://www.developmentalcell.com/cgi/content/full/11/6/775/DC1/.

Acknowledgments

We thank F. Matsuzaki, X. Yang, C.Q. Doe, and the Developmental Studies Hybridoma Bank for antibodies; the Bloomington Stock Center for providing fly stocks; and J.M. Chell and G. Tanentzapf for critical reading of the manuscript. S.P.C. was funded by a Gates-Cambridge Scholarship. This work was funded by a Wellcome Trust Programme Grant and an MRC Project Grant to A.H.B.

Received: March 23, 2006
Revised: July 26, 2006
Accepted: September 19, 2006
Published: December 4, 2006

References

Abate-Shen, C. (2002). Deregulated homeobox gene expression in cancer: cause or consequence? Nat. Rev. Cancer *2*, 777–785.

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. USA *100*, 3983–3988.

Arama, E., Dickman, D., Kimchie, Z., Shearn, A., and Lev, Z. (2000). Mutations in the beta-propeller domain of the *Drosophila* brain tumor (brat) protein induce neoplasm in the larval brain. Oncogene *19*, 3706–3716.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. *25*, 25–29.

Bello, B., Reichert, H., and Hirth, F. (2006). The brain tumor gene negatively regulates neural progenitor cell proliferation in the larval central brain of *Drosophila*. Development *133*, 2639–2648.

Betschinger, J., Mechtler, K., and Knoblich, J.A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. Cell *124*, 1241–1253.

Bianchi-Frias, D., Orian, A., Delrow, J.J., Vazquez, J., Rosales-Nieves, A.E., and Parkhurst, S.M. (2004). Hairy transcriptional repression targets and cofactor recruitment in *Drosophila*. PLoS Biol. *2*, E178.

Bjerkvig, R., Tysnes, B.B., Aboody, K.S., Najbauer, J., and Terzis, A.J. (2005). Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat. Rev. Cancer *5*, 899–904.

Bossing, T., and Technau, G.M. (1994). The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labelling. Development *120*, 1895–1906.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–515.

Broadus, J., Fuerstenberg, S., and Doe, C.Q. (1998). Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. Nature *391*, 792–795.

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

Brunk, B.P., Martin, E.C., and Adler, P.N. (1991). *Drosophila* genes Posterior Sex Combs and Suppressor two of zeste encode proteins with homology to the murine bmi-1 oncogene. Nature *353*, 351–353.

Buescher, M., Yeo, S.L., Udolph, G., Zavortink, M., Yang, X., Tear, G., and Chia, W. (1998). Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. Genes Dev. *12*, 1858–1870.

Caussinus, E., and Gonzalez, C. (2005). Induction of tumor growth by altered stem-cell asymmetric division in *Drosophila melanogaster*. Nat. Genet. *37*, 1125–1129.

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.

de Wit, E., Greil, F., and van Steensel, B. (2005). Genome-wide HP1 binding in *Drosophila*: developmental plasticity and genomic targeting signals. Genome Res. *15*, 1265–1273.

Del Bene, F., and Wittbrodt, J. (2005). Cell cycle control by homeobox genes in development and disease. Semin. Cell Dev. Biol. *16*, 449–460.

Doe, C.Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. Development *116*, 855–863.

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.

Elkins, T., Hortsch, M., Bieber, A.J., Snow, P.M., and Goodman, C.S. (1990). *Drosophila* fasciclin I is a novel homophilic adhesion molecule that along with fasciclin III can mediate cell sorting. J. Cell Biol. *110*, 1825–1832.

Garces, A., and Thor, S. (2006). Specification of *Drosophila* aCC motoneuron identity by a genetic cascade involving even-skipped, grain and zfh1. Development *133*, 1445–1455.

Greil, F., van der Kraan, I., Delrow, J., Smothers, J.F., de Wit, E., Bussemaker, H.J., van Driel, R., Henikoff, S., and van Steensel, B. (2003). Distinct HP1 and Su(var)3-9 complexes bind to sets of developmentally coexpressed genes depending on chromosomal location. Genes Dev. *17*, 2825–2838.

Hartenstein, V., Rudloff, E., and Campos Ortega, J. (1987). The pattern of proliferation of the neuroblasts in the wild-type embryo of *Drosophila melanogaster*. Rouxs Arch. Dev. Biol. *196*, 473–485.

Hawkins, N.C., Thorpe, J., and Schupbach, T. (1996). Encore, a gene required for the regulation of germ line mitosis and oocyte differentiation during *Drosophila* oogenesis. Development *122*, 281–290.

Hong, Y.K., and Detmar, M. (2003). Prox1, master regulator of the lymphatic vasculature phenotype. Cell Tissue Res. *314*, 85–92.

Hosoya, T., Takizawa, K., Nitta, K., and Hotta, Y. (1995). glial cells missing: a binary switch between neuronal and glial determination in *Drosophila*. Cell *82*, 1025–1036.

Hummel, T., Attix, S., Gunning, D., and Zipursky, S.L. (2002). Temporal control of glial cell migration in the *Drosophila* eye requires gilgamesh, hedgehog, and eye specification genes. Neuron *33*, 193–203.

Iscove, N.N., Barbara, M., Gu, M., Gibson, M., Modi, C., and Winegarden, N. (2002). Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. Nat. Biotechnol. *20*, 940–943.

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.

Jan, Y.N., and Jan, L.Y. (1998). Asymmetric cell division. Nature *392*, 775–778.

Jones, B.W., Fetter, R.D., Tear, G., and Goodman, C.S. (1995). glial cells missing: a genetic switch that controls glial versus neuronal fate. Cell *82*, 1013–1023.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H., and Klambt, C. (1994). The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS. Cell *78*, 149–160.

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

Lee, C.Y., Robinson, K.J., and Doe, C.Q. (2006a). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature *439*, 594–598.

Lee, C.Y., Wilkinson, B.D., Siegrist, S.E., Wharton, R.P., and Doe, C.Q. (2006b). Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Dev. Cell *10*, 441–449.

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.

Lilly, B., O'Keefe, D.D., Thomas, J.B., and Botas, J. (1999). The LIM homeodomain protein dLim1 defines a subclass of neurons within the embryonic ventral nerve cord of *Drosophila*. Mech. Dev. *88*, 195–205.

Lin, D.M., Fetter, R.D., Kopczynski, C., Grenningloh, G., and Goodman, C.S. (1994). Genetic analysis of Fasciclin II in *Drosophila*: defasciculation, refasciculation, and altered fasciculation. Neuron *13*, 1055–1069.

Martin, D., Brun, C., Remy, E., Mouren, P., Thieffry, D., and Jacq, B. (2004). GOToolBox: functional analysis of gene datasets based on Gene Ontology. Genome Biol. *5*, R101.

Matsuzaki, F., Koizumi, K., Hama, C., Yoshioka, T., and Nabeshima, Y. (1992). Cloning of the *Drosophila* prospero gene and its expression in ganglion mother cells. Biochem. Biophys. Res. Commun. *182*, 1326–1332.

Mettler, U., Vogler, G., and Urban, J. (2006). Timing of identity: spatiotemporal regulation of hunchback in neuroblast lineages of *Drosophila* by Seven-up and Prospero. Development *133*, 429–437.

Micchelli, C.A., and Perrimon, N. (2006). Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. Nature *439*, 475–479.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S., and Dickson, B.J. (1996). Genetic analysis of Netrin genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. Neuron *17*, 203–215.

Ohlstein, B., and Spradling, A. (2006). The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. Nature *439*, 470–474.

Orian, A., van Steensel, B., Delrow, J., Bussemaker, H.J., Li, L., Sawado, T., Williams, E., Loo, L.W., Cowley, S.M., Yost, C., et al. (2003). Genomic binding by the *Drosophila* Myc, Max, Mad/Mnt transcription factor network. Genes Dev. *17*, 1101–1114.

Osawa, M., Egawa, G., Mak, S.S., Moriyama, M., Freter, R., Yonetani, S., Beermann, F., and Nishikawa, S. (2005). Molecular characterization of melanocyte stem cells in their niche. Development *132*, 5589–5599.

Raaphorst, F.M. (2003). Self-renewal of hematopoietic and leukemic stem cells: a central role for the Polycomb-group gene Bmi-1. Trends Immunol. *24*, 522–524.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105–111.

Schuldt, A.J., Adams, J.H., Davidson, C.M., Micklem, D.R., Haseloff, J., St Johnston, D., and Brand, A.H. (1998). Miranda mediates asymmetric protein and RNA localization in the developing nervous system. Genes Dev. *12*, 1847–1857.

Shen, C.P., Knoblich, J.A., Chan, Y.M., Jiang, M.M., Jan, L.Y., and Jan, Y.N. (1998). Miranda as a multidomain adapter linking apically localized Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in *Drosophila*. Genes Dev. *12*, 1837–1846.

Song, S., Cooperman, J., Letting, D.L., Blobel, G.A., and Choi, J.K. (2004). Identification of cyclin D3 as a direct target of E2A using DamID. Mol. Cell. Biol. *24*, 8790–8802.

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.

Subkhankulova, T., and Livesey, F.J. (2006). Comparative evaluation of linear and exponential amplification techniques for expression profiling at the single-cell level. Genome Biol. *7*, R18.

Sun, L.V., Chen, L., Greil, F., Negre, N., Li, T.R., Cavalli, G., Zhao, H., Van Steensel, B., and White, K.P. (2003). Protein-DNA interaction mapping using genomic tiling path microarrays in *Drosophila*. Proc. Natl. Acad. Sci. USA *100*, 9428–9433.

Tolhuis, B., Muijrers, I., de Wit, E., Teunissen, H., Talhout, W., van Steensel, B., and van Lohuizen, M. (2006). Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in *Drosophila melanogaster*. Nat. Genet. *38*, 694–699.

Torii, M., Matsuzaki, F., Osumi, N., Kaibuchi, K., Nakamura, S., Casarosa, S., Guillemot, F., and Nakafuku, M. (1999). Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development *126*, 443–456.

Truman, J.W., and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. Dev. Biol. *125*, 145–157.

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.

van Lohuizen, M., Frasch, M., Wientjens, E., and Berns, A. (1991). Sequence similarity between the mammalian bmi-1 proto-oncogene and the *Drosophila* regulatory genes Psc and Su(z)2. Nature *353*, 353–355.

van Steensel, B., and Henikoff, S. (2000). Identification of in vivo DNA targets of chromatin proteins using tethered dam methyltransferase. Nat. Biotechnol. *18*, 424–428.

van Steensel, B., Delrow, J., and Henikoff, S. (2001). Chromatin profiling using targeted DNA adenine methyltransferase. Nat. Genet. *27*, 304–308.

Weissman, I.L., Anderson, D.J., and Gage, F. (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu. Rev. Cell Dev. Biol. *17*, 387–403.

Wigle, J.T., and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. Cell *98*, 769–778.

Wigle, J.T., Chowdhury, K., Gruss, P., and Oliver, G. (1999). Prox1 function is crucial for mouse lens-fibre elongation. Nat. Genet. *21*, 318–322.

Wigle, J.T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M.D., Jackson, D.G., and Oliver, G. (2002). An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. *21*, 1505–1513.

Wodarz, A., and Huttner, W.B. (2003). Asymmetric cell division during neurogenesis in *Drosophila* and vertebrates. Mech. Dev. *120*, 1297–1309.