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Huckebein-mediated autoregulation of Glide/Gcm triggers glia specification

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Cell specification in the nervous system requires patterning genes dictating spatio-temporal coordinates as well as fate determinants. In the case of neurons, which are controlled by the family of proneural transcription factors, binding specificity and patterned expression trigger both differentiation and specification. In contrast, a single gene, *glide cell deficient/glial cell missing* (*glide/gcm*), is sufficient for all fly lateral glial differentiation. How can different types of cells develop in the presence of a single fate determinant, that is, how do differentiation and specification pathways integrate and produce distinct glial populations is not known. By following an identified lineage, we here show that glia specification is triggered by high *glide/gcm* expression levels, mediated by cell-specific protein-protein interactions. Huckebein (Hkb), a lineage-specific factor, provides a molecular link between *glide/gcm* and positional cues. Importantly, Hkb does not activate transcription; rather, it physically interacts with Glide/Gcm thereby triggering its autoregulation. These data emphasize the importance of fate determinant cell-specific quantitative regulation in the establishment of cell diversity.
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Introduction

Cell specification requires the activity of patterning genes and cell fate determinants. Proneural genes trigger both neuronal differentiation and specification, and lack of any of them leads to the absence of identified neuronal subpopulations (Skeath, 1999; Bertrand *et al*, 2002). Multiple features allow proneural transcription factors of the basic helix–loop–helix (bHLH) family to generate diversity. Their expression is controlled by patterning genes along dorso/ventral (D/V), antero/posterior (A/P) and temporal axes; moreover, each factor is able to bind specific E boxes (Powell *et al*, 2004) and cofactors (Ramain *et al*, 2000; Lee and Pfaff, 2003; zur Lage *et al*, 2004).

A functional nervous system also relies on different types of glial cells (Ito *et al*, 1995; Bossing *et al*, 1996b; Schmidt *et al*, 1997; Van De Bor and Giangrande, 2002; Rowitch, 2004). Patterning genes have been shown to define broad gliogenic territories (for reviews see Skeath and Thor, 2003; Rowitch, 2004; Soustelle and Giangrande, 2005); however, the molecular cues underlying glia specification at the cellular level within and among these territories are still poorly understood. Knowing the gene that induces glial differentiation and being able to follow identified lineages make it possible to tackle this issue in *Drosophila melanogaster*. *glide/gcm* gene (referred throughout the text as *gcm*, for the sake of simplicity) is expressed and required in all lateral glial cells of the fly central nervous system (CNS) (Hosoya *et al*, 1995; Jones *et al*, 1995; Vincent *et al*, 1996). The glia to neuron transformation observed in *gcm* embryos and the fact that *gcm* ectopic expression leads to the differentiation of additional glial cells at the expense of endogenous developmental programs identify *gcm* as a molecular switch during cell fate decision (Hosoya *et al*, 1995; Jones *et al*, 1995; Vincent *et al*, 1996; Akiyama-Oda *et al*, 1998; Bernardoni *et al*, 1998).

These findings raise the question of how are different types of glial cells specified. In particular, does glia specification depend on the single *gcm* fate determinant or on a parallel pathway? Also, how and where do cell differentiation and specification integrate in order to produce the precise array of glial cells that characterize the nervous system? By analyzing the neuroglioblast 1-1 abdominal (NGB1-1A) lineage of the fly CNS, we here show that integration occurs at the level of the Gcm protein, the amount of which is controlled by Huckebein (Hkb). We also show for the first time that Hkb, a repressor controlling terminal patterning in the fly embryo (Weigel *et al*, 1990; Bronner and Jackle, 1991; Bronner *et al*, 1994), does not work as a transcription factor. Rather, its direct interaction with Gcm triggers *gcm* autoregulation and thereby promotes differentiation of a specific type of subperineural glia (SPG). Thus, we demonstrate the importance of fate determinant quantitative regulation in cell specification. *hkb* represents one of the lineage-specific factors controlled by patterning genes in the nervous system (Skeath, 1999). By identifying its mode of action, we provide the molecular link between patterning and specification here.

Results

Hkb is necessary for gcm expression in the NGB1-1A lineage

Each segment of the fly embryonic ventral cord contains about 60 stereotypically organized lateral glial cells (Figures 1A and 2A), most of which arise from neuroglioblasts (NGBs), mixed precursors producing both neurons and glia

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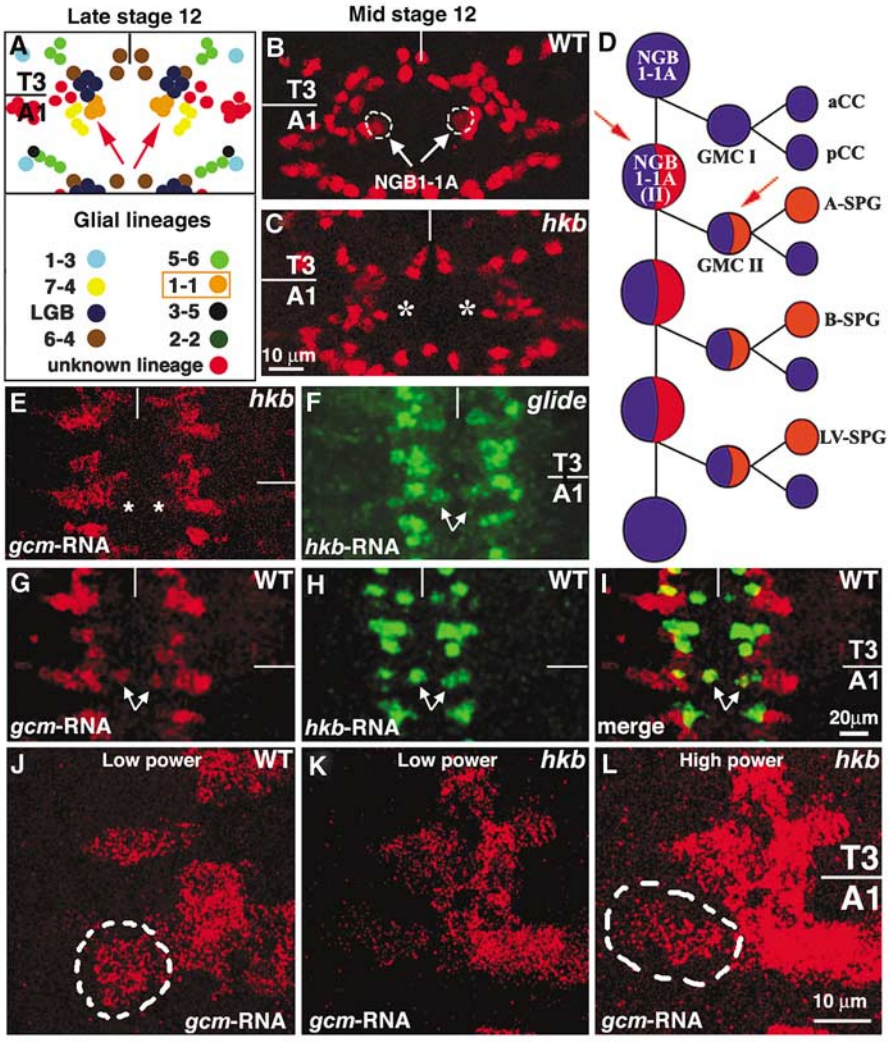


Figure 1 *hkb* controls glial differentiation in the NGB1-1A lineage. Unless otherwise specified, panels in this and in following figures show ventral views of the embryonic ventral cord; T3 and A1 indicate, respectively, third thoracic and first abdominal segments; anterior is to the top and the vertical line indicates the midline. (**A**) Schematic drawing of Repo labeled cells in T3 and A1 segments of a wild-type (WT)_ embryo at late stage 12. Glial subsets are identified by the expression of lineage-specific markers indicated by different colors. Symbols as in Ragone *et al* (2003). (**B, C**) Mid stage 12 embryos, WT (B) or *hkb* (C), labeled with glial-specific antibody anti-Repo. Arrows and dashed lines indicate NGB1-1A-derived glia. Asterisks in (C) indicate the absence of Repo labeling at the position normally taken by NGB1-1A-derived glia. (**D**) NGB1-1A lineage as proposed by Udolph *et al* (2001). Glial and neuronal potentials/components are indicated by red and blue, respectively. The first division of NGB1-1A gives rise to a ganglion mother cell (GMC I) that produces neurons (aCC and pCC), whereas the second division (NGB1-1A (II)) gives rise to a GMC (GMC II) that produces one neuron (n) and one glial cell (A-SPG). B-SPG and LV-SPG arise from later divisions. Red arrows indicate the two cells in which *gcm* mRNA is detected at stage 11. (**E–L**) Late stage 11 embryos. (E) *hkb* embryo labeled with *gcm* riboprobe (asterisks indicate the position normally taken by *gcm* expressing cells in the NGB1-1A lineage). (F) *gcm* embryo labeled with *hkb* riboprobe. (G–I) WT embryo labeled with *gcm* (G) and *hkb* (H) riboprobes. (I) Merge of (G and H). Arrows in (F–I) indicate cells of the NGB1-1A lineage. (J–L) WT (J) or *hkb* (K, L) embryos labeled with *gcm* riboprobe. Note the very low levels of *gcm* expression in mutant (K) compared to WT (J, dashed line) NGB1-1A lineage. (L) Same embryo as in (K) analyzed at high photomultiplicator power to amplify signal. Scale bars: 10 μm in (B, C, J–L) and 20 μm in (E–I).

(Bossing *et al*, 1996b; Schmidt *et al*, 1997). In the absence of Gcm, glia are absent (Figure 2B) and transform into neurons (Hosoya *et al*, 1995; Jones *et al*, 1995; Akiyama *et al*, 1996; Vincent *et al*, 1996; Schreiber *et al*, 1997; Bernardoni *et al*, 1998; Miller *et al*, 1998; Akiyama-Oda *et al*, 1999). This phenotype is also observed in *hkb* embryos (Figure 1C), but restricted to lateral glia derived from NGB1-1A (Bossing *et al*, 1996a). These data suggest that Hkb and Gcm work in the same pathway in NGB1-1A, prompting us to use this lineage as a model to understand the bases of glia specification.

NGB1-1A lineage contains six to eight neurons and three glial cells of the SPG class (Broadus *et al*, 1995; Ito *et al*, 1995; Bossing *et al*, 1996b) (Figure 1A). NGB1-1A first produces aCC and pCC motoneurons and subsequently gives rise to several ganglion mother cells (GMCs), each producing a neuron and a glial cell that do not divide further (Udolph *et al*, 2001) (Figure 1D). *hkb* transcripts are first detected at the time gliogenesis starts (Chu-LaGraff *et al*, 1995) (Figures 1H and 3D–F) and colocalize with *gcm* RNA within the NGB1-1A lineage (Figures 1G–I and 3A–C, J–M). Expression of both mRNAs starts at stage 11 in a single, dividing, cell (Figures 3A–M). By late stage 11, two cells express *gcm*; one of them is somewhat larger, more apically located than the other, and corresponds to NGB1-1A (II) (Figure 3N). This cell expresses

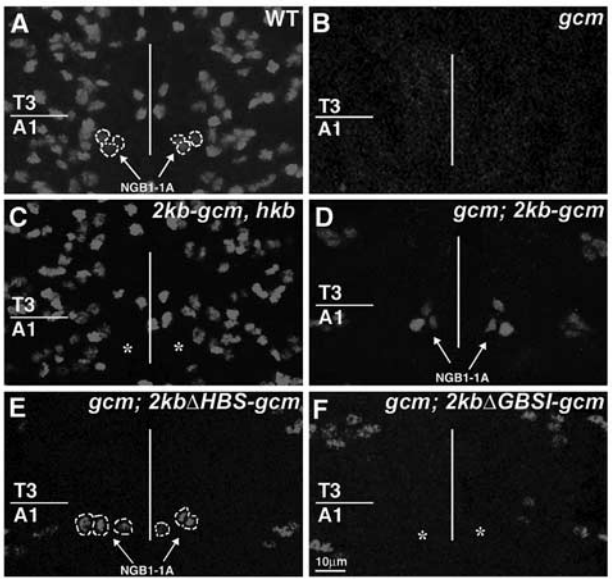


Figure 2 NGB1-1A-derived glial cells depend on *2kb-gcm* promoter. Late stage 12 embryos labeled with anti-Repo. (**A**) NGB1-1A glia are shown in a wild type (WT) by arrows and dashed lines. (**B**) No Repo positive nuclei are present in a *gcm* embryo (*gcm*). (**C**) NGB1-1A glial cells are absent in *hkb* embryo carrying the *2kb-gcm* transgene (see asterisks), but present in *gcm* embryo carrying the same (**D**) or *2kbΔHBS-gcm* (**E**) transgenes (see arrows) (100% of the animals, $n=15$). (**F**) NGB1-1A glia are absent in *gcm* embryos carrying the *2kbΔGBSI-gcm* transgene (40% of the animals, $n=50$), see asterisks. Note that several transgenes were analyzed for each construct. To take into account position effects, transgenes of comparable strength were used. Scale bar: 10 μm.

both *gcm* and *hkb*, whereas the basal, small, cell corresponds to ganglion mother cell II (GMC II) (see Figure 1D) and only expresses *gcm* (Figure 3N). Repo glial-specific marker (Campbell *et al*, 1994; Xiong *et al*, 1994; Halter *et al*, 1995) starts being detected in one cell of the lineage by mid stage 12 (Figure 1B), three cells per hemisegment being labeled at late stage 12 (Figures 1A and 2A).

As NGB1-1A glia are missing in *hkb* embryos (Figure 1C), we determined whether *hkb* controls *gcm*. Very low levels of *gcm* transcripts are indeed present in the NGB1-1A lineage of *hkb* embryos (Figures 1J–L), *gcm* expression remaining unaffected in the rest of the ventral cord. In contrast, NGB1-1A *hkb* expression is not affected by the absence of *gcm* (Figure 1F).

Previous analyses have allowed us to identify the first *2kb* upstream of *gcm* as sufficient to drive *gcm* expression in NGB1-1A lineage (Ragone *et al*, 2003). When introduced into a *gcm* background, a transgene carrying the *gcm* coding sequences together with the *2kb* upstream region (*2kb-gcm*) rescues differentiation of NGB1-1A derived glial cells (Figure 2D). In contrast, the same transgene does not rescue NGB1-1A glia when introduced into *hkb* embryos (Figure 2C). Thus, the *2kb* promoter that drives NGB1-1A glia development requires Hkb.

Hkb does not directly activate gcm transcription

Hkb is known to bind specific sequences (Kuhnlein *et al*, 1997) and was first identified as a putative transcription factor based on the presence of a glutamine-rich domain (Bronner *et al*, 1994). More recently, it was shown that Hkb interacts with the Groucho corepressor and negatively regulates the expression of several target genes, such as *snail* and *brachyenteron* (Goldstein *et al*, 1999). Interestingly, we have found that Hkb-predicted open reading frame (ORF) does not contain the glutamine-rich region (putative activation domain), as further confirmed by the genome annotation (release 4.1, 2005) of the Genome Sequence Project. The published sequence contains a two-nucleotide deletion that changes the ORF in the N-terminal (Nt) region, whereas a third deletion of one nucleotide upstream of the Zn-finger motifs restores the correct ORF (Figure 4A). Thus, Hkb protein does contain three Zn-finger motifs in the C-terminal (Ct) region as well as the Groucho binding motif (FRPW) (Goldstein *et al*, 1999) (Figure 4B), but does not contain the putative activation domain.

Both genetic data and expression profile analysis prompted us to determine whether Hkb induces NGB1-1A glial differentiation by regulating *gcm* expression directly. Four Hkb putative binding sites (HBS) were identified in the *2kb-gcm* promoter (Figure 4D), the region that requires Hkb. These sites bind purified recombinant GST–Hkb fusion protein (Figure 5A). Binding specificity was confirmed by using mutagenized primers, as a two-nucleotide mutation abolishes interaction with Hkb (Figure 5B). Furthermore, we challenged the Hkb–DNA complex with increasing amounts of specific and nonspecific DNA competitors. Only specific cold competitor displaces the GST–Hkb protein from labeled oligonucleotides (Figure 5C). When tested in CAT assays, however, none of the HBSs induces gene expression upon cotransfection with an Hkb expression vector (Figure 5D).

Finally, we mutagenized the four HBSs present in the *2kb-gcm* transgene (*2kbΔHBS-gcm*) and determined the ability of this mutant transgene to rescue glial cells of NGB1-1A lineage in *gcm* embryos. In line with the cotransfection data, the *2kbΔHBS-gcm* transgene rescues NGB1-1A glia (Figure 2E), indicating that Hkb does not work as a transcription factor.

Hkb–Gcm interaction controls gcm positive autoregulation

It has been shown that Gcm positively and directly auto regulates *in vitro*, and that *in vivo*, this positive feedback loop requires cell-specific cofactors (Miller *et al*, 1998; Ragone *et al*, 2003). We speculated that Hkb represents one such factor and regulates *gcm* expression indirectly by binding the Gcm protein. Indeed, the *2kb-gcm* promoter that is sufficient to drive rescue of NGB1-1A glia contains a Gcm binding site (GBS) (Miller *et al*, 1998; Ragone *et al*, 2003) (Figure 4D). We performed GST pull-down assays by using an Hkb–GST fusion expressed in bacteria and found that *in vitro*-translated Gcm binds GST–Hkb, but not GST alone (negative control) (Figure 4C). Binding specificity was further confirmed by using *in vitro*-translated Luciferase instead of Gcm (Figure 4C). Deletion analyses indicate that binding is mediated by Gcm Nt and HKB C-terminal (Ct-Hkb) regions (Figures 4C and E). Gcm–Hkb interactions were also confirmed in two-hybrid assay (Supplementary Figure 1).

We then determined the functional relevance of Hkb-Gcm interaction in DNA binding and CAT assays. The binding profile of Gcm Nt region (amino acids 1–261) to its site is modified by adding Hkb, which, on its own, does not bind GBSI (the site present in the *2kb-gcm* promoter) (Figure 6A).

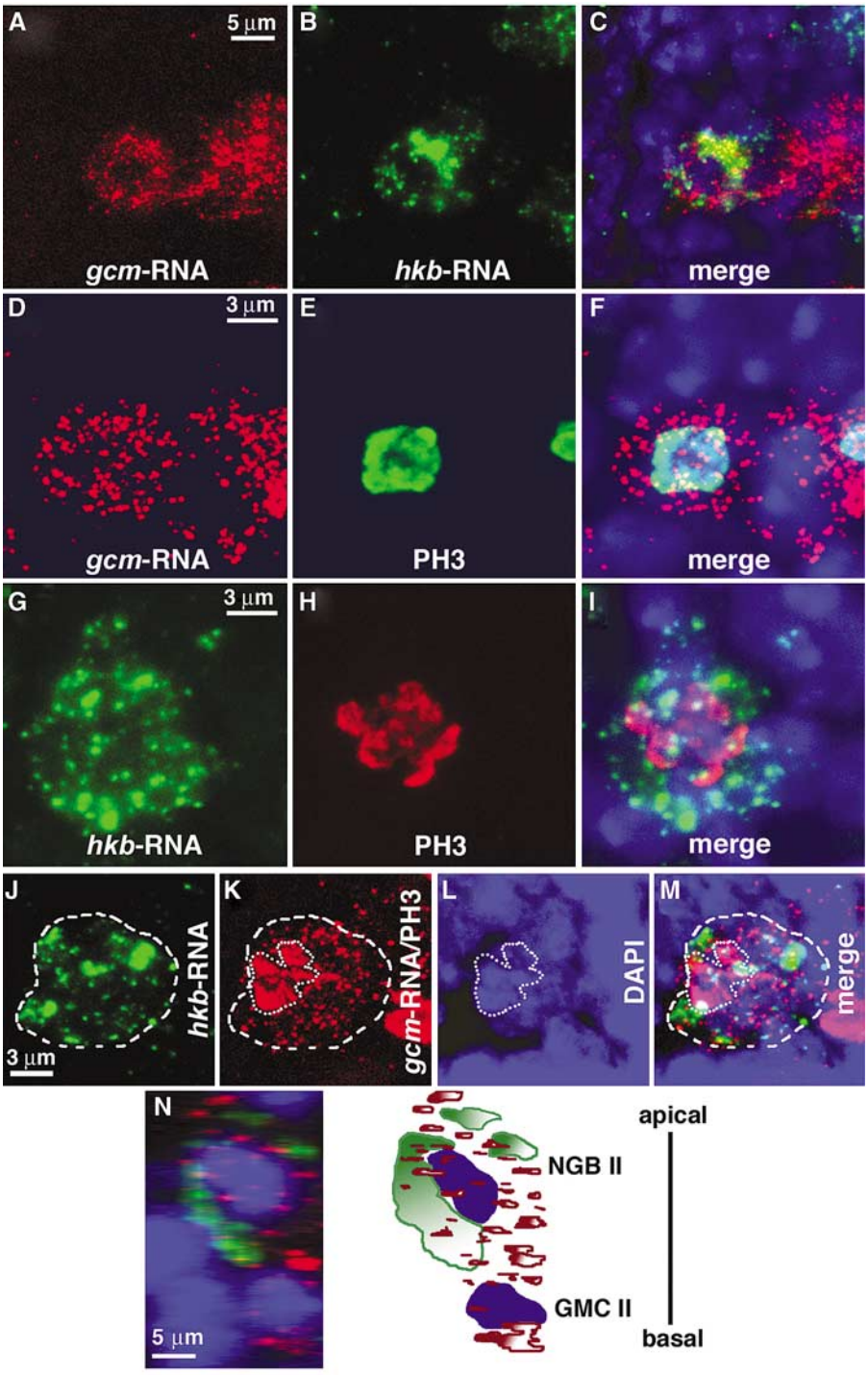


Figure 3 *hkb* and *gcm* are coexpressed in NGB1-1A (II). NG1-1A lineage multiple labelling in wild-type (WT) embryos. (**A–C**) Triple labeling: (A) *gcm* riboprobe, (B) *hkb* riboprobe, (C) is a merge showing DAPI (blue) nuclear labeling. (**D–F**) Triple labeling: (D) *gcm* riboprobe, (E) anti-PH3 mitotic marker, (F) is a merge showing DAPI. (**G–I**) Triple labeling: (G) *hkb* riboprobe, (H) anti-PH3, (I) is a merge showing DAPI. (**J–M**) quadruple labeling: (J) *hkb* riboprobe (dashed line), (K) anti-PH3/*gcm* riboprobe, (L) DAPI labeling (dotted line), (M) is a merge. In (K), the same secondary antibody was used to reveal *gcm* RNA (dashed line) and PH3 (dotted line) because primary antibodies used were raised in the same species, but subcellular localization of *gcm* RNA and PH3 enables to distinguish between the two stainings. (**N**) 90° rotation to show labeling along the *Z*-axis. Triple labeling at two cell stage: *hkb* riboprobe (green), *gcm* riboprobe (red) and DAPI (blue). *hkb*-RNA is localized only in NGB1-1A II, whereas *gcm*-RNA is localized both in NGB and in GMC II, as schematically represented in the right panel. Scale bars: 5 μm in (A–C and N) and 3 μm in (D–M).

An anti-Hkb, but not a nonspecific antibody, eliminates the band shift observed in the presence of Gcm and Hkb (Figure 6A), further demonstrating that Gcm–Hkb complex binds to GBS. Moreover, cotransfection assays using a reporter vector-containing GBSI show that Hkb does not trigger any detectable CAT activity (Figure 6B) and that combined Gcm and Hkb expression leads to two-fold increase of CAT activity, compared to that found with Gcm alone (Figure 6B).

This effect is abolished upon GBSI mutagenesis (Figure 6B). Similar synergistic effects were obtained when a different GBS (site 1, present upstream of the *2kb* promoter fragment; Miller *et al*, 1998; Ragone *et al*, 2003) was used in cotransfection assays as seen in Figure 6C. Nt-Hkb does not have any activation effect, whereas Ct-Hkb has almost the same effect as that observed with Hkb full-length protein, when cotransfected with Gcm (Figure 6B), in agreement with the pull-down data. Thus, the Zn-finger-containing region of Hkb triggers *gcm* autoregulation, even though this is not mediated by DNA binding, as Hkb does not bind GBS (Figure 6A). In addition, *2kbΔGBSI-gcm* transgene that carries a mutated GBSI does not rescue NGB1-1A glia in a *gcm* background (Figure 2F). Finally, combined Gcm and Hkb expression does not trigger any detectable CAT activity when the reporter vector contains HBS (HBS1 and HBS2), reinforcing the idea that Gcm and Hkb do not act via Hkb-mediated transcription (Figure 5D). Our *in vitro* and *in vivo* data demonstrate the importance of *gcm* autoregulation in NGB1-1A glia specification and the pivotal role of Hkb in the process.

As hkb^2 embryos do express *gcm* albeit at very low levels (Figures 1K and L), we asked whether this allele is a hypomorph by sequencing its cDNA. hkb^2 carries a G→A mutation in the third nucleotide of the start codon. Moreover, no protein can be detected by Western blot on extracts from hkb^2 embryos (Supplementary Figure 2). These data altogether indicate that hkb^2 represents a null allele and that *hkb* is necessary to amplify *gcm* expression, but not to induce the first boost of transcription.

Thus, in the NGB1-1A lineage, *hkb* does not work as a transcription factor. Rather, it triggers *gcm* autoregulation *via* protein–protein interaction.

Hkb amplifies gcm expression in vivo

The above data suggest that Hkb controls NGB1-1A glia specification by mediating positive *gcm* autoregulation. If Hkb is sufficient to trigger this process, its overexpression should lead to excess of Hkb-dependent glia and to Gcm overexpression. We overexpressed Hkb throughout the CNS and analyzed flies carrying the UAS-*hkb* construct (Myat and Andrew, 2002), the *sca-GAL4* driver and the enhancer-trap line *P101* (Klambt and Goodman, 1991). This enhancer trap line specifically expresses β-galactosidase (β-gal) in SPG, including NGB1-1A-derived SPG (A- and B-SPG) (Figures 7A–A″). Hkb overexpression does induce the formation of additional Repo positive cells (Figure 7B′), most of which also express the *P101* SPG marker (Figures 7B and B″). The presence of additional SPG is accompanied by ectopic *gcm* expression (compare Figure 8A with 8B). Moreover, *gcm* mRNA persists until stage 14 (Figure 8D), whereas in wild-type (WT) NGB1-1A, it fades away by the end of stage 12 (Figure 8C).

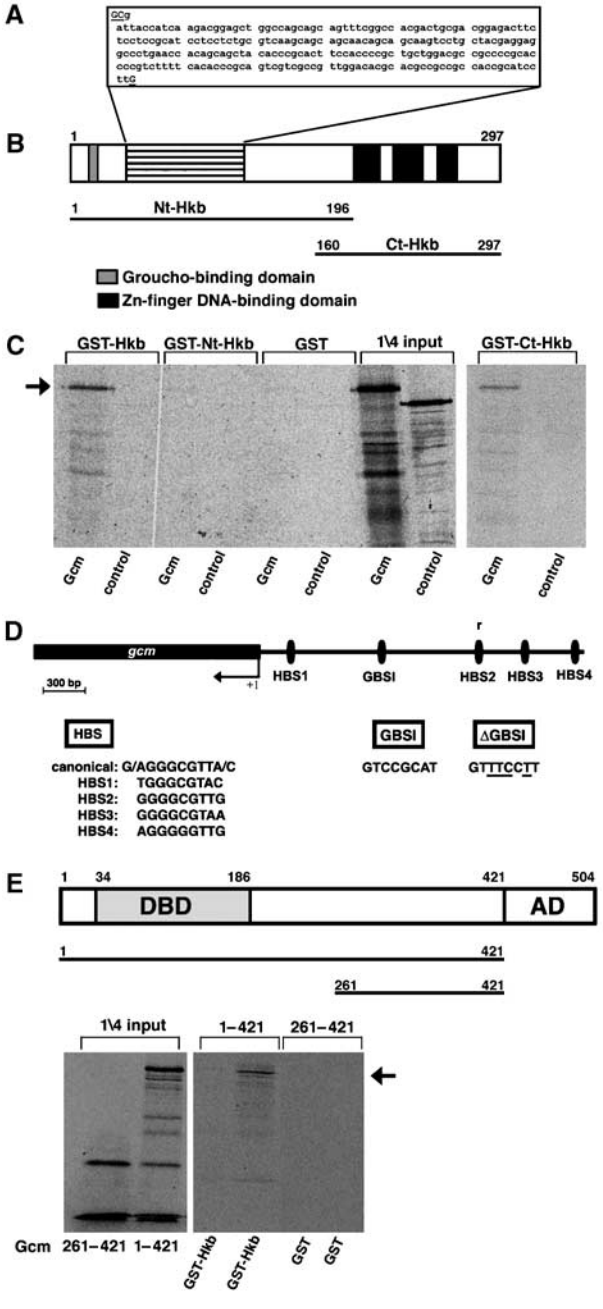
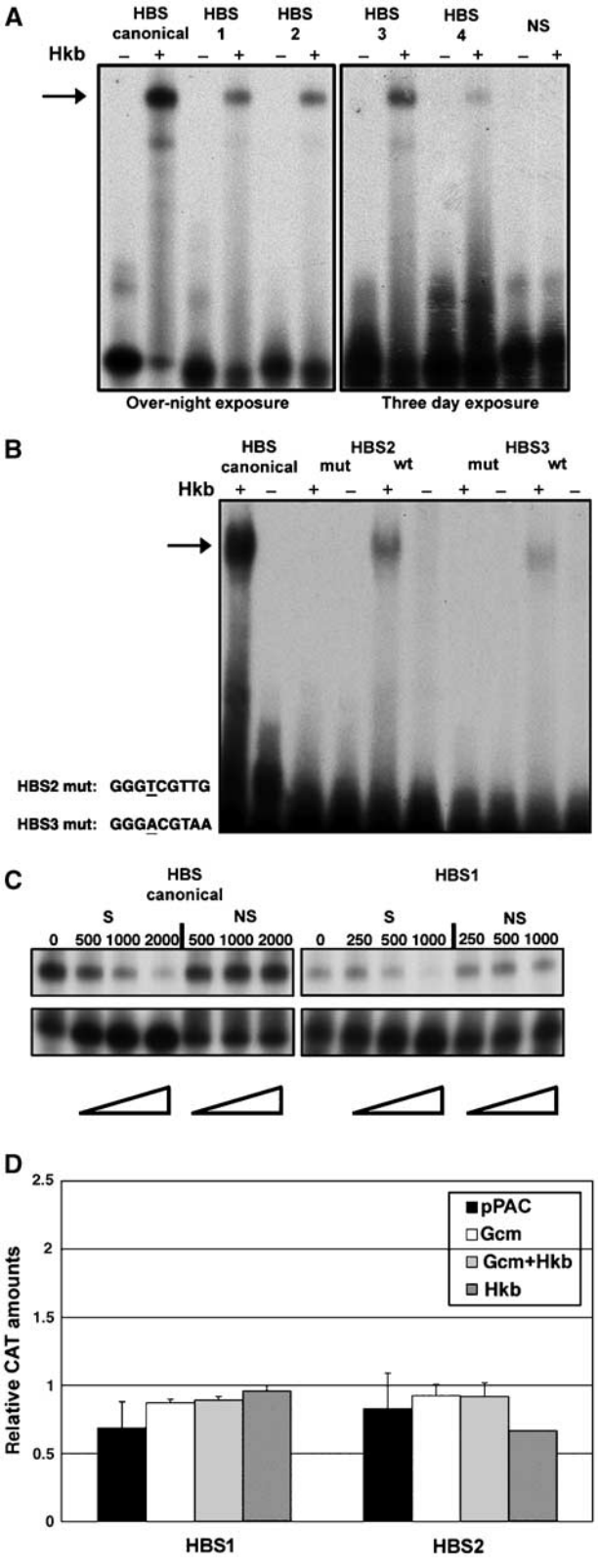


Figure 4 Hkb predicted ORF and interaction with Gcm. (**A**) Nucleotide sequence in the region indicated by horizontal lines in (**B**). Underlined nucleotides are absent in the published sequence (Bronner *et al*, 1994). (B) Organization of the predicted Hkb ORF. Boxes indicate the Zn-finger motifs (black) and the Groucho binding domain (gray) (Goldstein *et al*, 1999). Lines in (B) below the ORF indicate the Hkb truncated forms used to map interaction domains in GST pull-down assays. (**C**) Autoradiography of a pull-down assay using full length Gcm (amino acids 1–504) and GST–Hkb derivatives immobilized on glutathione-agarose beads. Binding of full-length Hkb protein (GST–Hkb) and Hkb C-terminal part (GST–Ct-Hkb) is indicated by an arrow. Luciferase (Luc) *in vitro* translated protein is used as a control. 1/4 of the input is shown in the right part of the panel. (**D**) Four Hkb binding sites (HBS1–4) are present in the *2kb-gcm* promoter, one of which is in the opposite orientation (r). Canonical HBS represents the site identified by Kuhnlein *et al* (1997). GBSI indicates the Gcm binding site present in the *2kb-gcm* promoter (Ragone *et al*, 2003). +1 indicates the transcription start site. (**E**) Gcm ORF: DBD indicates DNA binding domain, and AD, activation domain (see for a review Van De Bor and Giangrande, 2002). Lines below ORF indicate the *in vitro* translated products used to map interaction domains in GST pull-down assay shown in the bottom panel. Arrow indicates binding of GST–Hkb with translated Gcm 1-421 product. Note that GST pull-down assays entail a DNAse treatment, which eliminates possible DNA contamination.

To confirm the role of Hkb as a positive cofactor in *gcm* autoregulation, we analyzed *rA87/sca-GAL4*; UAS-*hkb* embryos. As in the *rA87* enhancer trap line the *lacZ* gene is under the control of *gcm* promoter, β-gal expression can be used to trace autoregulation *in vivo* (Figures 7F–F″) (Miller *et al*, 1998). The additional Repo positive cells present in animals overexpressing *hkb* also express β-gal (Figures 7G–G″), meaning that Hkb positively acts on the *gcm* promoter and regulates its expression. In summary, the *in vivo* data show that *hkb* amplifies *gcm* levels by positive autoregulation and that this triggers NGB1-1A glia specification. The importance of *gcm* autoregulation is further confirmed by the observation that *gcm* overexpression in a WT background induces many more Repo positive cells than in a *gcm* mutant background (Figure 9).

The effects of *hkb* overexpression on *gcm* autoregulation and SPG specification prompted us to determine the relative contribution of *gcm* in this process. Indeed, co-overexpression of *gcm* and *hkb* induces many more Repo and SPG positive cells (Figures 7C–C″) compared to those observed upon *hkb* overexpression (Figures 7B–B″). Finally, overexpression of *gcm* alone is sufficient to induce SPG (Figures 7D–D″), even though less efficiently than in combination with *hkb*. Further confirming the importance of Gcm threshold levels, the presence or absence of endogenous *hkb* does not seem to modify the phenotype induced by Gcm overexpression (Figures 7D–D″ and E–E″, respectively), which overrides the need for cell-specific autoregulation. In order to quantify the effect of *gcm* overexpression, the number of SPG was determined. Values obtained by counting 24 hemisegments per embryo ($n = 3$) confirm that *hkb*, *gcm* coover-expression leads to many more SPG (average: 820 nuclei/embryo) than *gcm* expression (average: 576 nuclei/embryo) and that *hkb* absence does not affect the phenotype induced by *gcm* overexpression (average: 549 nuclei/embryo). It is worth noting that, while differences can be observed with respect to SPG labeling, the three genotypes display a similar number of Repo positive cells, further confirming that *hkb* acts on glia specification.

Thus, *hkb* is necessary to sustain *gcm* expression in NGB1-1A lineage, which in turn induces SPG specification.



Discussion

We here show that Hkb controls glia specification by binding Gcm glia promoting factor and inducing high levels of *gcm* expression. Thus, cell-specific autoregulation of a fate deter-

Figure 5 Hkb binds to its target sequences but does not act as a transcription factor. (**A**) Gel-shift assay showing DNA binding of a purified GST-–Hkb fusion protein (Hkb). Labeled 27-mers corresponding to each of the four HBSs, to the canonical HBS (Kuhnlein *et al*, 1997) or to nonspecific DNA (NS: GCATGGACC AACATTGACACCGCTTTG) were used in the assay. Binding is indicated by arrows. (**B**) Hkb binding is abolished when HBS2 or HBS3 carrying point mutations are used (HBS2mut and HBS3mut, respectively, mutant nucleotides underlined). Binding to the canonical site is shown as a positive control. Arrow indicates the position of bound 27mers. (**C**) Competition gel-shift assay on canonical HBS and HBS1. S and NS indicate specific and nonspecific cold competitors, respectively (X indicates folds of excess, 0 indicates the absence of competitor). (**D**) pBLCAT5 reporter constructs containing either HBS1 or HBS2 (HBS1 and HBS2) were cotransfected with one (pPAC, pPAC-Gcm or pPAC-Hkb) or two (pPAC-Gcm and pPAC-Hkb) expression vectors. CAT assay data were normalized by using control reporter vector pBLCAT5. Each bar represents the average of at least three measurements, and error bars indicate standard error.

minant coordinates patterning and differentiation and is crucial for the establishment of cell diversity.

Importance of quantitative regulation during development

Autonomous and nonautonomous cues trigger specific cell fates in the nervous system and thereby guarantee its precise architecture. Morphogens elicit different fates depending on their concentration, a clear example being provided by Sonic hedgehog, the gradient of which specifies D/V positions within the neural tube (Poh *et al*, 2002). Autonomous cues, on the other hand, are known to trigger cell fates based on their cell-specific expression, emphasizing the importance of qualitative differences in the establishment of cell diversity (Bardin *et al*, 2004). Recent studies, however, call for a role of quantitative regulation even in the case of autonomous cues. Generation of neural precursors relies on progressive accumulation of proneural proteins, first in a group of cells called the proneural cluster and later on in one cell of the cluster, based on feedback loop interactions that control proneural protein levels in different cells (Skeath and Carroll, 1994; Baker, 2000; Ramain *et al*, 2000; zur Lage *et al*, 2004). Fate determinant levels are also important for glial differentiation: (i) the number of supernumerary glia depends on the amount of ectopic Gcm (Bernardoni *et al*, 1998); (ii) *gcm* RNA is unequally distributed in the dividing NGB, the presumptive neuroblast inheriting less RNA than the presumptive glioblast (Akiyama-Oda *et al*, 1999; Bernardoni *et al*, 1999; Ragone *et al*, 2001); (iii) *gcm* contains a PEST motif, characteristic of proteins at high turn over (Rogers *et al*, 1986), and an mRNA instability element (IE) (Shaw and Kamen, 1986) in the 3′ untranslated region, both of which are conserved throughout evolution (Hosoya *et al*, 1995; Kammerer *et al*, 1999; Kanemura *et al*, 1999; Tuerk *et al*, 2000; Wegner and Riethmacher, 2001; Hashemolhosseini *et al*, 2004). The present data show that *gcm* levels control not only the number but also the type of induced glia, demonstrating for the first time that autonomous cue quantitative regulation controls cell specification within a neural lineage.

Control of fate determinant levels has been shown to be mediated by cooperative pathways. Pannier prepatterning transcription factor interacts with proneural proteins and activates transcription through its DNA target sequences in order to modulate proneural gene expression (Ramain *et al*, 2000). Long-range-mediated interactions facilitate such cooperativity, as Chip (Morcillo *et al*, 1997) interaction with both Pannier and Achaete enhances Achaete autoregulation (Ramain *et al*, 2000). Also, autoregulation of Atonal (Ato) proneural protein, which is necessary for recruiting chordotonal sensory organ precursors (Jarman *et al*, 1993), depends

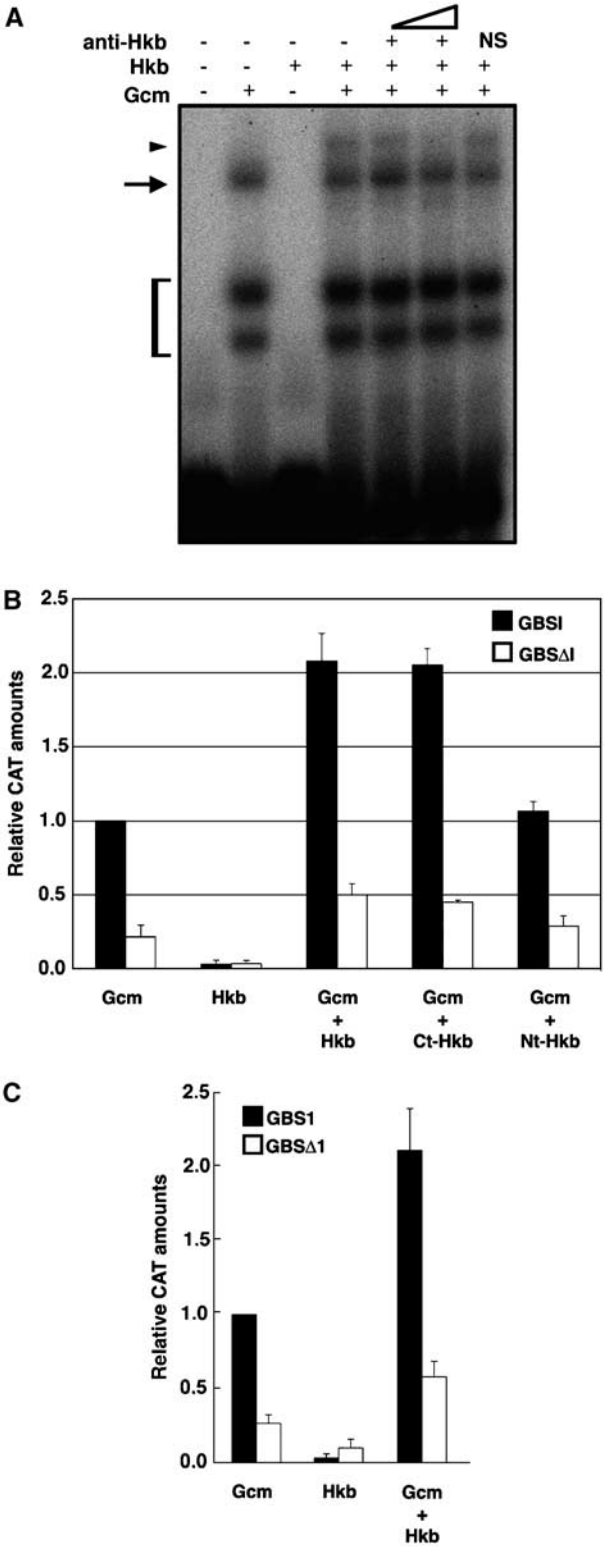


Figure 6 Gcm–Hkb synergistic activity. (**A**) Gel-shift assay showing DNA binding on a 30-mer containing GBSI. Arrow indicates binding of GST–Gcm N-terminal region (amino acids 1–261) to its target. Arrowhead indicates the additional band induced by incubation with both GST–Gcm N-terminal and GST–Hkb. Note that GST–Hkb does not, on its own, bind GBSI. Band shift induced by GST–Hkb is progressively removed by adding increasing amounts of anti-Hkb, but not by adding anti-Flag, used as nonspecific antibody (NS). Bracket indicates degraded GST–Gcm N-terminal products. (**B**) Cotransfection of reporter constructs containing either wild type (WT) or mutated GBSI (GBSI and ΔGBSI, see Figure 4) with pPAC-Gcm or pPAC-Hkb alone, or with pPAC-Gcm in combination with one of the Hkb-containing plasmids (pPAC-Hkb, pPAC-Ct-Hkb or pPAC-Nt-Hkb). (**C**) Cotransfection assays as in (B) but using reporter constructs containing either WT or mutated GBS1, a GBS that is three times more active than GBSI (Ragone *et al*, 2003; GBSC in Miller *et al*, 1998). CAT values obtained upon cotransfection with pPAC-Gcm and WT reporter were arbitrarily given a value of 1 and used for normalization. Each bar represents the average of at least three measurements, and error bars indicate standard error.

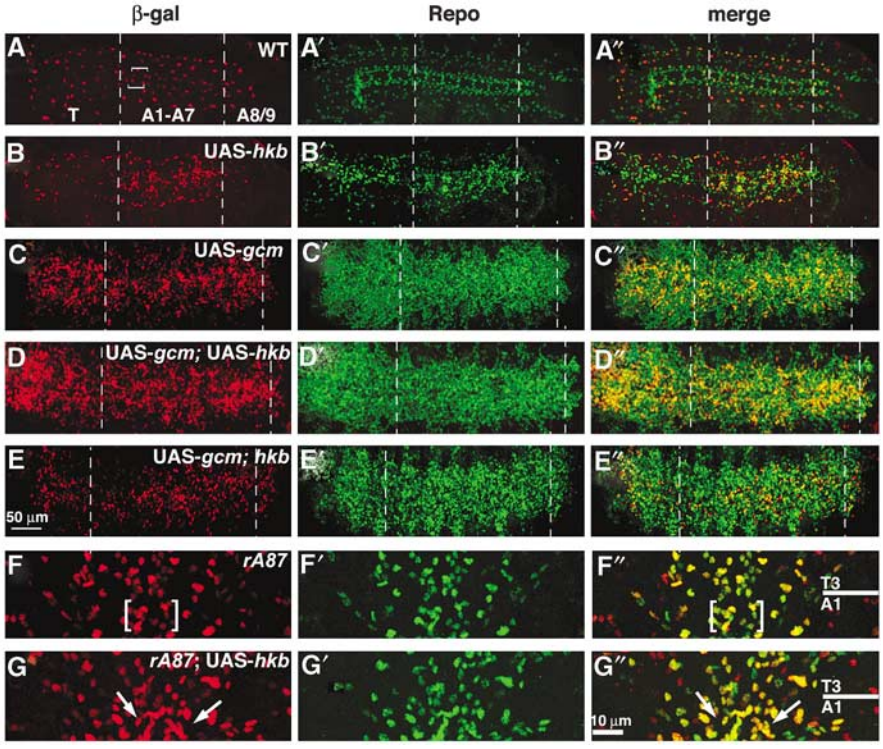


Figure 7 Role of Hkb and Gcm in NGB1-1A specification. (**A–E**″) Ventral views of stage 16 embryonic ventral cord carrying the *P101* SPG marker; T3 and A1 indicate, respectively, third thoracic and first abdominal segments; anterior to the top and vertical line indicates the midline. β-gal (SPG), Repo double labeling. Left panels show β-gal, mid panels, Repo and right panels, merges. In all overexpression experiments, *sca-GAL4* was used as a driver. (A–A″) labeling in wild-type (WT) embryo. Square brackets indicate A- and B-SPG (note that LV-SPG cannot be seen in this focal plane), arrowhead in (A) indicates lateral SPG, a cell that flanks A- and B-SPG cells, but is not derived from NGB1-1A lineage. (B–B″) labeling upon *hkb* overexpression induces additional SPG (B) and Repo (B′) labeling, close to the position at which A- and B-SPG are normally present. (C–C″) labeling upon combined *gcm* and *hkb* overexpression. Note the presence of additional SPG labeling in thoracic and abdominal segments (thick arrow). (D–D″) labeling upon *gcm* expression. (E–E″) labeling upon *gcm* expression in *hkb* embryo. (**F–G**″) Ventral views of stage 16 ventral cord of *rA87*/+ embryos. β-gal (*rA87*), Repo double labeling as above. (F–F″) labeling in a WT *rA87*/+ embryo. (G–G″) labeling in a *sca-GAL4/rA87*; UAS-*hkb*/+ embryo. Square brackets in (F and F″) indicate β-gal/Repo positive cells at the position of A- and B-SPG. Colocalization of additional Repo and β-gal labeling is indicated by arrows. Scale bar: 10 μm.

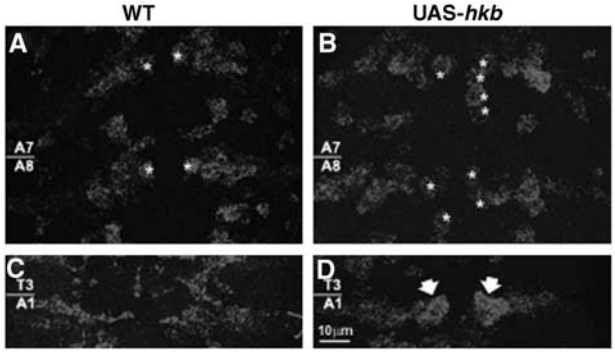


Figure 8 Hkb induces *gcm* expression *in vivo*. (**A**) *gcm* mRNA is expressed in one cell per hemisegment at stage 11 in a wild-type (WT) embryo (asterisks). (**B**) Several *gcm* expressing cells, indicated by white asterisks, are detectable in embryos overexpressing *hkb* (UAS-Hkb). *sca-GAL4* driver was used for overexpression. Abdominal segments 7 and 8 (A7 and A8) are shown in (A) and (B). *gcm* expression profile at stage 14 in WT (**C**) and UAS-*hkb* (**D**) embryos. Thick arrows indicate persistent *gcm* expression in *hkb* overexpressing embryos. Scale bar: 10 μm.

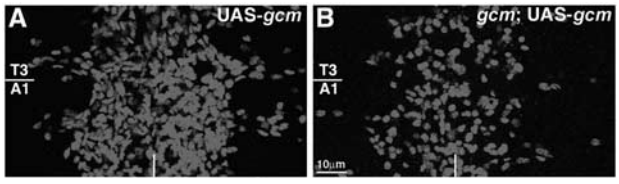


Figure 9 Glial differentiation requires *gcm* autoregulation. Ventral views of stage 16 embryos, Repo labeling upon *gcm* overexpression (*sca-GAL4*) in wild type (WT) (**A**) or *gcm* background (**B**). Symbols as in Figure 1. Scale bar: 10 μm.

on the cooperative activity of Ato and Pointed P1 transcription factors, which bind E box and ETS motifs, respectively (zur Lage *et al*, 2004). Strikingly, *gcm* autoregulation in NGB1-1A does not depend on cooperative activation of two transcriptional pathways. Rather, the presence of the GBS is sufficient *in vitro* and *in vivo* for cell-specific *gcm* autoregulation, thus pointing to a novel molecular strategy controlling fate determinant levels.

The role of Hkb is to sustain *gcm*-dependent transcription by acting on Gcm binding and transactivation potential. This is also supported by three observations: (i) Hkb works on different GBSs; (ii) mutagenized GBSI is active, although at low levels, in the presence of Hkb and Gcm, but not in the presence of Gcm alone; (iii) *gcm* overexpression does produce ectopic SPG in an *hkb* context, as this overcomes the need for autoregulation. In the establishment of terminal patterning in the fly embryo, *hkb* works as a repressor *via*

interaction of its Nt region with Groucho protein (Goldstein *et al*, 1999). This region, however, is neither relevant for Hkb binding to Gcm nor for activating *gcm* autoregulation. Thus, *hkb* works either as a repressor or as a coactivator by interacting with different proteins.

Cell differentiation and specification in the nervous system

Glia specificity (e.g., longitudinal versus SPG) could rely on genes that are activated by a pathway independent of Gcm and impose specification on postmitotic cells otherwise displaying a default 'pan-glia' fate. Specification of postmitotic cells does also take place during the development of neurons, where it refines/maintains early decisions taken in precursor cells and allows for further diversification (Allan and Thor, 2003, 2005). While establishing the possible need for postmitotic specification awaits the identification of novel glial markers, present data demonstrate the importance of cues working in glial precursors.

Integrating cell differentiation and specification via Gcm–Hkb interaction allows a single fate determinant to generate different types of glia. It will be interesting to determine whether Hkb also acts on *gcm* targets, as it is known that some of these targets are pan-glial (Repo: Campbell *et al*, 1994; Xiong *et al*, 1994; Halter *et al*, 1995), whereas others are lineage specific (Loco: Granderath *et al*, 2000). It will also be interesting to determine whether *gcm* autoregulation mediated by cell-specific factors is necessary in other glial lineages.

Our data show that NGB1-1A glia specification requires two equally important regulatory steps. The first one is *gcm* independent and results in low levels of *gcm* expression, whereas the second one is *gcm* and *hkb* dependent and induces high levels of *gcm*. Previous data show that *hkb* expression is induced by columnar genes, which control D/V regionalization in the nervous system (Chu *et al*, 1998; McDonald *et al*, 1998; Mellerick and Modica, 2002). Thus, cell-specific factors such as Hkb provide an intermediate step between patterning genes and fate determinants, thereby triggering the identity of neural precursors.

Supernumerary *P101* positive cells induced by Hkb overexpression are all located close to endogenous SPG. The central and abdominal position of supernumerary SPG suggests that cells within the NGB1-1A lineage are 'competent' to express the SPG fate. Furthermore, *hkb* gliogenic activity is also temporally restricted, since the first division is never affected and always produces aCC/pCC sibling neurons (Supplementary Figure 3). These data call for additional factors regulating *gcm* expression and SPG specification and, indeed, homeotic as well as temporal genes are known to control the NGB1A-A lineage (Udolph *et al*, 1993; Prokop and Technau, 1994; Isshiki *et al*, 2001). Thus, a grid of positional cues along spatial and temporal axes works through *gcm*, thereby triggering differentiation of the appropriate type of glia. Understanding the interplay of these cues will be the matter of further studies; however, our data already demonstrate that cofactor-mediated quantitative regulation plays a pivotal role in cell specification. Such fine-tuning and accurate orchestration of events highlights the complexity underlying nervous system differentiation. Integrating qualitative and quantitative regulation via cell-specific autoregulation likely applies to other developmental processes in which a single fate determinant triggers different phenotypes.

Materials and methods

Stocks

WT strain was *Sevelen*. gcm^{26} (null allele) and hkb^{2} (Weigel *et al*, 1990) were used as mutant strains. Homozygous mutant embryos were identified by using blue balancers (β-gal labeling as in Ashburner, 1989). *scabrous-GAL4* (*sca-GAL4*) was used to express UAS-*gcm* (Bernardoni *et al*, 1998), UAS-*hkb* (Myat and Andrew, 2002) and UAS-*Ubx* (Castelli-Gair *et al*, 1994). *rA87* line is described in Vincent *et al* (1996). *P101* enhancer trap line (Klambt and Goodman, 1991) was used as SPG marker. [*w; gcm*^{26}*/CyO twi-lacZ; P(2kb-gcm,w*$^{+}$*)*] and [*w; P(2kb-gcm,w*$^{+}$*); hkb*^{2}*/TM3, Ser, twi-lacZ*] lines carry the WT transgene in *gcm* or *hkb* background, respectively. [*w; gcm*^{26}*/CyO twi-lacZ; P(2kbΔHBS-gcm,w*$^{+}$*)*] and [*w; gcm*^{26}*/CyO twi-lacZ; P(2kbΔGBSI-gcm,w*$^{+}$*)*] carry the mutant transgenes in a *gcm* background.

In situ hybridization and immunolabeling

Embryo preparation, antibody incubation and *in situ* hybridization were performed as in Bernardoni *et al* (1997). Digoxigenin-labeled *gcm* and Fluorescein-labeled *hkb* riboprobes were obtained by using full-length cDNAs. Embryos were mounted in Vectashield medium (Vector). The following primary antibodies were used: rabbit anti-Repo (1:1000) (A Travers), mouse anti-Repo (1:100) (DHSB), rabbit anti-PH3 (1:10000) (Upstate Biotechnology), mouse anti-digoxigenin (1:100) (Boehringer), rabbit anti-fluorescein (1:1000) (Molecular Probes), mouse anti-β-gal (1:100) (DSHB), rabbit anti-β-gal (1:500) (Cappel). Secondary antibodies coupled to Cy3 and FITC (Jackson) were used at 1:400. Chromatin labeling was obtained by using DAPI at 100 ng/ml in PBS-0.3% Triton X-100. Preparations were analyzed by confocal microscopy (DMRE, Leica).

Cell transfection and CAT ELISA *assay*

Reporter vector pBLCAT5-GBSI and pBLCAT5-GBS1 as well as vectors pPAC5C (which we refer to as pPAC, C Thummel) and pPAC-Gcm are described in Miller *et al* (1998). pBLCAT5-ΔGBSI and pBLCAT5-ΔGBS1 were obtained by double-stranded mutagenesis (Clontech) on pBLCAT5-GBSI and pBLCAT5-GBS1, respectively, using the following primers:

ΔGBSI: 5′-GGATTCTAATGTTTCCCTTAAAGGATTC-3′;
ΔGBS1: 5′-GGATTCTGCAAGGGAAACATCTGGATTC-3′.

pPAC-Hkb, pPAC-Nt-Hkb and pPAC-Ct-Hkb were obtained by cloning the full-length, the first 471nt or the last 411nt of *hkb* cDNA in the pPAC vector, respectively. pPAC-LacZ was obtained by cloning the entire *lacZ* cDNA in pPAC vector. Transient transfection of *Drosophila* S2 line (Schneider, 1972) was performed using effectene (Qiagen), according to the manufacturer's instructions, using 1.15 μg of DNA containing the following: 100 ng of pPAC-LacZ, 50 ng of reporter DNA, 1 μg of expression vectors. Cells were harvested 48 h after transfection and normalized for β-gal activity. CAT levels were determined using the CAT ELISA kit (Boehringer).

In vitro GST pull-down assays

An *Eco*RI fragment containing the *hkb* full-length cDNA was cloned into the pGEX4T3 (Pharmacia) to produce a GST–Hkb fusion protein of 50 kDa. GST–Hkb was expressed in the protease-deficient *E. coli* strain BL21 and purified using GST-conjugated Sepharose 4B (Pharmacia), according to the manufacturer's instructions. The GST–Nt-Hkb and GST–Ct-Hkb were produced in the same manner by cloning the first 471nt or the last 411nt of *hkb* cDNA, corresponding to 157 amino acids of the amino terminal part or 137 amino acids of the carboxy terminal part of Hkb, respectively. Luciferase and Gcm (full length, Gcm 1–421 and Gcm 261–421) proteins were produced by *in vitro* transcription–translation in rabbit reticulocyte lysate in the presence of methionine, according to manufacturer's instructions (Promega, Madison, WI). Pull-down assays were performed using 1 μg of fusion protein, bound to 50 μl beads and preincubated with 1 ml of binding buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EGTA, 1% Triton X-100, 1 mM PMSF). [^{35}S]Gcm or Luciferase proteins were added to each preincubation mix and binding reactions were carried out overnight

at 4°C. Beads were washed several times with binding buffer, boiled for 5 min in sample buffer and aliquots were examined by electrophoresis. The amount of retained proteins was detected by autoradiography.

DNA-binding assay

An *Eco*RI fragment containing the first 261 amino acids of Gcm was generated by PCR and cloned into pGEX4T3 (Pharmacia) to produce GST–Gcm Nt fusion protein, which was used in gel-shift assays (Miller *et al*, 1998). Same 30-mer containing the GBSI as in Miller *et al* (1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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