Klumpfuss, a putative *Drosophila* zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage

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The ~300 distinct neurons comprising each hemineuromere of the *Drosophila* embryonic central nervous system are derived from a segmentally reiterated array of ~30 progenitor cells, neuroblasts (NBs). Each NB has a unique identity and undergoes repeated cell divisions to produce several smaller secondary precursor cells, ganglion mother cells (GMCs); each GMC divides once to produce two neurons and/or glia, thereby generating a specific lineage of neurons/glia. Understanding the generation of neuronal diversity requires not only elucidation of the molecules and mechanisms that specify NB identity but also those that act to differentiate between the cell types produced within one NB lineage. Here we show that the *Drosophila* Zn finger protein Klumpfuss (Klu), which shows sequence similarities to the mammalian Wilm's tumor suppressor (WT-1), acts to differentiate between the identities of the first two secondary precursor cells produced from one NB lineage. Klu is expressed in the NB4-2 lineage only after two rounds of NB cell division, in the second born GMC (GMC4-2b). In loss-of-function mutant embryos, the first born GMC (GMC4-2a) as well as its progeny neurons are duplicated; we show that this duplication of the GMC4-2a sublineage arises because GMC4-2b adopts the identity of GMC4-2a and divides to produce the GMC4-2a progeny. Moreover, when Klu is ectopically expressed in GMC4-2a, it fails to acquire its normal identity and fails to produce correctly specified progeny. *klu* therefore acts to specify the identity of GMC4-2b and to make it distinct from GMC4-2a. Our findings further suggest that the determination of GMC cell fate occurs in two steps; the initial GMC identity is the consequence of inheritance from the maternal NB, however, the subsequent stabilization of this identity requires functions like *klu* in the GMC.

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The *Drosophila* embryonic central nervous system (CNS) provides an excellent model for elucidating the developmental mechanisms that lead to the generation of neural cell diversity. The neurons and glia of the *Drosophila* embryonic CNS are arranged in a stereotypic pattern that is bilaterally symmetric and segmentally reiterated. The ~350 distinct neurons and glial cells present in each of the hemineuromeres (cell number estimated from Ito et al. 1955; Bossing et al. 1996b) are derived from ~30 progenitor cells called neuroblasts (NBs) and glioblasts (GBs) (Campos-Ortega, 1993; Doe and Technau 1993; Goodman and Doe 1993; Jimenez and Modolell 1993; Doe and Skeath 1996). Because of the availability of a large number of enhancer trap and antibody markers

(Doe 1992; Broadus et al. 1995), as well as their distinct morphology and position, many of the neurons and glial cells and essentially all of the progenitor NBs can be identified unambiguously.

NBs are formed in several temporal waves with each NB possessing a unique cellular identity that is defined by the time of its birth and its position within the neuroectoderm (Hartenstein and Campos-Ortega 1984; Doe 1992; Chu-LaGriffa and Doe 1993; Skeath et al. 1995). The unique identity of each NB is manifested in the specific combination of genes (NB identity genes) it expresses and by the unique lineage of neurons and/or glia it generates. Each NB undergoes several asymmetric cell divisions to generate, in a distinct temporal order, a specific set of unique intermediate precursor cells called ganglion mother cells (GMCs); each GMC divides to produce two postmitotic neuron/glia. These observations suggest (see Doe 1992) that NB identity is dynamic and

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evolves in a unidirectional manner. Each GMC has a unique identity that is defined initially by determinants inherited from the parental NB and ultimately by a specific combination of expressed genes (GMC identity genes).

It has been shown that individual neuroectodermal cells can be labeled to reveal complete embryonic NB lineages (Bossing and Technau 1994). The lineages of three NBs—NB 1-1 (Udolph et al. 1993; Prokop and Technau 1994), NB 2-2 (Bossing et al. 1996a), and NB 4-2 (Chu-LaGraff et al. 1995; Udolph et al. 1995) have been delineated previously. NB1-1 was found to generate the aCC/pCC neurons, several unidentified neurons and the A- and B-glia. These findings indicate that neurons and glia are not necessarily produced by distinct progenitors but that the decision to adopt a neural or glial fate can occur within one lineage. This type of progenitor cell can be described as neuro-glioblasts and additional progenitor cells, for example NB2-2, have similar properties. More recent work has resulted in the elucidation of the terminal lineages of 17 NBs (Bossing et al. 1996b).

To understand the developmental mechanisms that lead to the generation of cell diversity within the CNS, it is important to define not only the genes that act to specify the unique cellular identities of each of the NBs but also genes that act to differentiate between the cellular identities that are produced within one stem cell lineage. For this purpose, we have focused on NB4-2, genetically the best characterized NB lineage in the *Drosophila* embryonic CNS (see Fig. 1). The first-born GMC of NB4-2, designated GMC4-2a (nomenclature according to Doe 1992; see also legend to Fig. 1), divides to produce the RP2 motoneuron and a sibling cell. A number of useful molecular markers for GMC4-2a and the RP2 neuron have been described (Fig. 1).

Several genes have been described previously whose activities are required for the generation of a correctly specified RP2 neuron. The secreted gene product of *wingless* (*wg*) (Chu-LaGraff and Doe 1993) is required for the formation and specification of NB4-2. In addition, the homeobox genes *fushi tarazu* (*ftz*) and *even-skipped* (*eve*) (Doe et al. 1988a,b) and the POU domain genes *pdm-1* and *pdm-2* (Billin et al. 1991; Dick et al. 1991; Lloyd and Sakonju 1991) are expressed in GMC4-2a and are necessary for specifying its cellular identity and for the formation of the RP2 neuron (Bhat et al. 1995; Yeo et al. 1995). The homeobox gene *prospero* (*pros*) (Doe et al. 1991; Matsuzaki et al. 1991; Vaessin et al. 1991) is expressed on the basal cortex of many dividing NBs and inherited by their GMC progeny (Hirata et al. 1995; Knoblich et al. 1995; Spana and Doe 1995), including GMC4-2a. Total loss of *pros* function affects the identities of many GMCs and neurons including those of GMC4-2a and RP2. In addition, the putative transcription factor Hucklebein appears to regulate aspects of GMC and neuronal identity required for the correct differentiation of motoneurons arising from the NB4-2 lineage (Chu-LaGraff et al. 1995) as well as for glial development and axon pathfinding in the NB1-1 and NB 2-2 lineages (Bossing et al. 1996a).

Figure 1. The early born cells of the NB4-2 lineage. This diagram depicts the cells derived from the first two NB divisions and the antibody and enhancer trap markers used in this study for their identification. NB4-2a and GMC4-2a are the progeny of NB4-2 after its first asymmetric division. GMC4-2a divides to produce the RP2 motoneuron and its sibling cell. NB4-2b and GMC4-2b are the progeny of the NB4-2a cell division, the second NB division in this lineage. The identities of the progeny of GMC4-2b are not known. Anti-Eve labels cells in the GMC4-2a sublineage. Anti-Pros marks all GMCs including GMC4-2a and GMC4-2b. *klu*^{P1741} expresses β -Gal in NB4-2b and GMC4-2b. RP2 neuron is also labeled by both anti-Zfh1 and mAb 22C10.

In a screen designed to identify mutations that alter RP2 cell number, we isolated loss-of-function mutations of *klumpfuss*, (*klu*), which encodes a Wilms' tumor suppressor (WT-1)-like Zn finger protein (Klein and Campos-Ortega 1997). Here we demonstrate that Klu protein expression is nuclear and can be seen in many NB lineages; within the NB4-2 lineage, Klu protein expression commences only following two rounds of NB cell division in the GMC4-2b nucleus. In *klu* loss-of-function mutant embryos, the RP2 motoneuron appears to be duplicated; clonal analysis indicates that the duplicated neurons arise within the NB4-2 lineage. By following the fates of GMC4-2b and GMC4-2a in mutant animals, we show that loss of *klu* function causes a GMC4-2b to GMC4-2a cell fate transformation; thereby duplicating the GMC4-2a sublineage. Furthermore, we demonstrate that misexpression of Klu in GMC4-2a, which normally does not

express Klu, prevents it from adopting a correct GMC4-2a cell identity and prevents the formation of correctly specified RP2 neurons. Taken together, our work demonstrates that *klu* acts to differentiate between the identities of the first two GMCs generated within the NB4-2 lineage; its expression in the second-born GMC, GMC4-2b, is necessary to specify the identity of this cell, making it distinct from the first-born GMC, GMC4-2a.

Results

The *klu* mutant alleles

We isolated the enhancer trap line *P1741* from an anti- β -Gal screen of lethal enhancer trap lines provided by the Bloomington Stock Center. From ~500 enhancer trap lines screened, 37 lines with β -Gal expression in all or subsets of NBs were identified. *P1741* (cytological position 68A1-2) showed an RP2 duplication phenotype (see below). *P1741* (*klu*^{*P1741*}) was inserted in *klu* that had been cloned and for which a number of mutant alleles already existed (Klein and Campos-Ortega 1997). A summary description of the nature of the *klu* alleles used in this study and their molecular defects are given (Fig. 2). The various alleles show similar CNS defects (see below) at different expressivity. Our phenotypic analysis focused primarily on two null alleles of *klu*; *Df(3R)*klu*^{*XR19*}* is a one band (cytological) deficiency obtained as a *w*⁻ revertant induced by X-rays; *klu*^{*P212IR51C*}, generated by the imprecise excision of *klu*^{*P212*}, a P element inserted near the 3'-end of the *klu* transcription unit, contains a small, molecularly mapped, deletion that removes at least three exons from the 3'-end of *klu* (T. Klein and J.A. Campos-Ortega, in prep.). Both of these alleles are antigen-minus when stained with an antibody raised against a Klu fusion protein (see Materials and Methods).

Klu protein is expressed in the developing embryonic CNS

The temporal and spatial expression pattern of Klu has

been characterized with an anti-Klu antibody (Fig. 3) and is essentially identical to the anti- β -Gal pattern observed in both the *klu*^{*P1741*} and *klu*^{*P212*} enhancer trap insertions (data not shown). Consistent with its putative role as a transcription regulator, the antibody shows nuclear staining in embryos. The earliest Klu expression is nuclear and occurs before syncytial blastoderm (Fig. 3A). The staining disappears after cellularization. At stage 10, anti-Klu staining reappears in the nuclei of a subset of NB and in the procephalic region. The staining becomes more prominent in the NBs and brain at stage 11; some epithelial cells start to express Klu and are stained as patches on the lateral sides of the embryos (Fig. 3B). The staining in the ventral cord and brain continues at stage 12. At stage 16, the staining seen in the ventral cord and the brain remains but is weaker; the epithelial cells are stained as three- to four-cell-wide stripes; the posterior spiracles are also Klu⁺ (Fig. 3C,D).

The expression of Klu in neuroblasts is shown (Fig. 3E–J). The earliest NB staining (nuclear) is seen at stage 10 (SIII), when six NBs are initially stained by the antibody (Fig. 3E,H); they are row 5 NBs (NB5-2, NB5-3, and NB5-6); row 7 NBs (NB7-1 and NB7-4), and NB3-5. At stage 11 (SIV), with the exception of the five NBs (NB2-4, NB3-3, NB4-4, NB5-4, and NB6-4), all NBs show nuclear staining (Fig. 3F,I). At late stage 11 (SV), only two NBs (NB2-3 and NB6-4) do not express Klu (Fig. 3G,J). Klu is also expressed in some GMCs (see below).

RP2 neuron is duplicated in *klu* embryos

To detect possible cell fate changes and defects in the developing embryonic CNS of *klu* mutant embryos, an array of marker antibodies were used, including anti-Eve (Frasch et al. 1986), anti-Ftz (W. Gehring laboratory), anti-En (Patel et al. 1989b), anti-Zfh1 (Lai et al. 1991), mAbBP102 (C. Goodman laboratory), mAb 22C10 (Fujita et al. 1982), and mAb1D4 (Van Vactor et al. 1993). The only marker antibodies that gave obviously altered stain-

Figure 2. The molecular lesions associated with two *klu* alleles. Genomic DNA spanning 63 kb that contains the transcribed region of *klu* is shown. The regions that hybridize to a full-length *klu* cDNA are indicated on the map. The *klu* transcribed region contains at least five exons. The positions of the insertion sites of *klu*^{*P1741*} and *klu*^{*P212*} are indicated. *klu*^{*212IR51C*} removes all or part of the three 3'-most exons; its 5' breakpoint lies within a 3-kb region (double dash line); *Df(3R)*klu*^{*XR19*}* (data not shown) deletes one cytological band and removes the entire region shown here (Klein and Campos-Ortega 1997)

Figure 3. The expression pattern of Klu protein in the embryo and in identified neuroblasts during early CNS development. (A) Lateral view of a pre-blastoderm embryo showing Klu protein expression in syncytial nuclei (arrow) before cellularization. (B) Lateral view of a stage-11 embryo showing Klu protein expression in the nuclei of the procephalic region (arrow), patches of epithelial cells (small arrowhead) and cells of the developing CNS (arrowhead). (C) Dorsal view of a stage-16 embryo. Nuclei of cells in the brain lobes (arrow) and posterior spiracles (arrowhead) are stained by anti-Klu. (D) Ventral view of same embryo seen in C. Nuclear staining in cells of the ventral cord (arrow) and stripes of epithelial cells (arrowhead). (E–G) Klu expression pattern in identified neuroblasts during three stages of NB segregation. Ventral views of one hemisegment each of a stage-10 (following SIII segregation, E), a stage-11 (following SIV segregation, F) and a late stage-11 (following SV segregation, G) embryo are shown, focused at the level of the NBs. The Klu expression patterns shown in E–G are illustrated diagrammatically in H–J, respectively; Klu-expressing NBs are in black. For A–D, anterior is left; dorsal is up in A and B. For E–J, anterior is up; dashed line indicates the midline and arrowhead marks the parasegmental groove.

ing patterns in *klu* mutant embryos were anti-Eve, mAb 22C10 and anti-Zfh1, markers that are expressed in the RP2 neuron (see Fig. 1). The anti-Eve antibody stains ~20 neurons in each embryonic hemineuromere (Patel et al. 1989a) and is a particularly useful marker for the early part of the NB4-2 lineage (Fig. 1). During neurogenesis, NB4-2 divides to produce its first ganglion mother cell (GMC4-2a) at stage 10. The GMC4-2a becomes Eve⁺ and undergoes terminal division to produce two Eve⁺ cells at stage 11. One of the progeny cells retains Eve expression (until the end of embryogenesis) and differentiates into the mature RP2 motoneuron with its characteristic position in the CNS scaffold, anterior lateral axon projection, and expression of marker genes by stage 13. RP2 sibling cell loses Eve expression completely at stage 13 and its final fate is unknown.

When stage-15 *klu* embryos were stained with anti-Eve, we observed two Eve⁺ cells per hemisegment (Fig. 4B), compared with only one Eve⁺ cell per wild-type hemisegment (Fig. 4A), at the characteristic RP2 position in the embryonic CNS. The RP2 duplication phenotype is position dependent; the frequency of duplication in T2, T3, and A1 segments is much higher than that in the other abdominal segments (A2–A8). The anteriormost neuromeres are difficult to score at late stages because of curvature of the CNS. The percentage of hemisegments containing two Eve⁺ cells at RP2 position varied among

the different mutant alleles. For the *klu*^{p1741} homozygotes, 49% (118/240) of the T2–A1 hemisegments showed duplicated RP2 neurons but no duplications (0/560) were seen in the A2–A8 hemisegments. In *klu*^{p212IR51C} homozygotes, 99% (238/240) of the T2–A1 hemisegments exhibited duplicated RP2 neurons; the RP2 duplication also occurred in A2–A8 (26%; 145/560), but at a lower frequency. Homozygotes for *Df(3R)kluXR19*, which removes the entire *klu* transcription unit, showed similar expressivity [T2–A1: 99% (238/240); A2–A8: 22% (123/560)] to *klu*^{p212IR51C}, indicating that *klu*^{p212IR51C} is an amorphic allele. The majority of our analyses are based on *klu*^{p212IR51C} unless otherwise specified.

Both of the Eve⁺ cells at the RP2 position in *klu* embryos express additional markers consistent with a RP2 identity. Zfh1 is a Zn finger homeodomain protein, expressed in a variety of tissues in the embryos, including CNS neurons and muscles (Lai et al. 1991). Zfh1 protein is expressed in the RP2 and aCC motoneurons starting stage 13. mAb 22C10 (Fujita et al. 1982) labels the membrane of a subset of CNS neurons including the RP2 motoneuron. Like their wild-type counterpart (Fig. 4A,C,E), the duplicated RP2 neurons are stained by mAb 22C10 (Fig. 4D) and anti-Zfh1 (Fig. 4F); their axon projections appear to be in an anterior ipsilateral direction (Fig. 4D), identical to those of wild-type RP2 neurons (Fig. 4C). Therefore, we conclude that both Eve⁺ cells at

Figure 4. In the *klu* mutant embryonic CNS, two RP2-like neurons are produced from the NB4-2 lineage. (A,B) Dorsal views of dissected wild-type (A) and *klu* mutant (B) stage-15 embryos stained with anti-Eve antibody; whereas only a single Eve⁺ RP2 neuron (arrow) is seen per hemisegment in wild type, two eve⁺ cells (arrows in B) per hemisegment are present at the characteristic RP2 position in the mutant embryonic CNS. (C,D) Dorsal views of dissected wild-type (C) and mutant (D) stage-15 embryos double-labeled with anti-Eve (black, nuclear staining) and mAb 22C10 (brown, cell surface and axon staining). The wild-type RP2 neuron (arrow, C) is labeled by both antibodies and exhibits an ipsilateral axon projection (arrowhead). Note that in the mutant embryo, both of the Eve⁺ cells at the RP2 position (arrows, D) are stained by anti-Eve and mAb 22C10 and the ipsilateral axon projection (arrowhead) appears to be identical to that seen in wild type. (E,F), Ventral views of superimposed confocal images of wild-type (E) and mutant (F) stage-15 embryos stained with anti-Eve (red) and anti-Zfh1 (green). In wild-type hemisegments (E), the RP2 neuron (arrow) is double positive (yellow) for Eve and Zfh1; the anterior corner cell (aCC, arrowhead) is also double positive (yellow) whereas the posterior corner cell (pCC, small arrowhead) is positive only for Eve (red). In mutant hemisegments (F), both of the Eve⁺ cells at the RP2 position (arrows), like the wild-type RP2 neuron, also express Zfh1 (yellow). Note that whereas the wild-type pCC (small arrowhead in E) expresses Eve but not Zfh1 (red), the mutant pCC (lower arrowhead in F) often expresses both Eve and Zfh1 (yellow). (G,H) Dorsal views of Tau- β -Gal expressing clones (see text and Materials and Methods) from dissected wild-type (G) and mutant (H) embryos double stained with anti- β -Gal (membrane and axon staining, brown) and anti-Eve (nuclear staining, black), focused at the level of the RP2 neuron. A single Tau- β -Gal and Eve double positive RP2 (G, arrow) is seen from a wild-type NB4-2 clone. A clone generated from a mutant embryo includes both RP2 neurons (arrows in H), indicating that the duplicated RP2 neurons are derived from the same NB4-2 lineage. Anterior is up.

the RP2 position show characteristics of mature RP2 neurons.

The duplicated RP2 neurons are derived from the same NB lineage

As a first step in understanding the origin of the duplicated RP2 neurons, we examined their lineage relationship. Clonal analysis in *klu* embryos demonstrates that both RP2 neurons are derived from the same lineage. For these experiments, the *actin-tau-lacZ* gene in *klu* mutant embryos containing both the *actin-tau-lacZ* “flip cassette” and *HS-flippase* constructs can be permanently activated by controlled heat-shock treatment (see Materials and Methods). If the activation of *actin-tau-lacZ* occurs in an NB before its first division, all progeny cells derived from that NB will express *tau-lacZ* and form a β -Gal⁺ clone in the ventral cord. Clones containing RP2 neurons can be easily identified by staining the embryos with anti-Eve and anti- β -Gal. We found three such clones with duplicated RP2 neurons in stage 15–16 *klu* embryos and, in all cases, both RP2 neurons were labeled by anti- β -Gal antibody together with other β -Gal⁺ progeny cells (Fig. 4H). In two of these cases, the clones appeared to be about the same size as wild-type NB4-2 clones. These results (in combination with other

results shown below) clearly indicate that both RP2 cells in *klu* embryos are derived from the NB4-2 lineage.

klu expression within the NB4-2 lineage commences following two rounds of NB cell division, in GMC4-2b and NB4-2b

To elucidate the cell fate transformation that must have occurred within the NB4-2 lineage to generate the duplicated RP2 neurons, we first assessed the expression of Klu within this lineage. Pros is expressed essentially in all nuclei of GMCs in the developing CNS and can be used as a general GMC marker. At late stage 10 (SIII), anti-Pros antibody staining shows that there is a Pros⁺ cell dorsal to NB4-2 (Fig. 5C), indicating that the first round of NB4-2 cell division is complete and GMC4-2a is formed at this stage; the neuroblast seen at the NB4-2 position at this time is NB4-2a; anti-Klu and anti-Pros double-labelings indicate that NB4-2a and GMC4-2a are both Klu⁺ (Fig. 5A–F). Double labeling of stage-11 (SIV) embryos with anti-eve and anti-Klu antibodies confirms that GMC4-2a, which has by this stage commenced Eve-expression, is not labeled by anti-Klu (Fig. 5E); interestingly, there is a small Klu⁺ cell just dorsal to the Klu⁺ NB4-2b and close to GMC4-2a (Fig. 5F). Further experiments show that this Klu⁺ cell is also labeled by the

Figure 5. Klu protein expression within the NB4-2 lineage. Confocal images of an anti-Klu and anti-Pros double-labeled stage-10 embryo (A–C), an anti-Klu and anti-Eve double-labeled stage-11 embryo (D–F), and an anti-Klu and anti-Pros double-labeled stage-11 embryo (G–I). (A) Klu expression in NBs (green). Note that NB4-2a (circled by dots) does not express Klu at this stage. (B) Pros expression (red) in the same embryo shown in A but focused at the GMC level; GMC4-2a (arrowhead) is formed. (C) Superimposed image of A and B; GMC4-2a is located just dorsal to NB4-2a (circled by dots). (D) Klu expression (blue) at stage 11 (SIV segregation), focusing at the NB level. NB4-2b (circled by dots) is Klu⁺. (E) Superimposed image of anti-Klu (green) and anti-Eve (red) double-staining of the same embryo shown in D but focused at the GMC level; GMC4-2a is Eve⁺ (large arrowhead, red) and GMC4-2b is Klu⁺ (small arrowhead, green). (F) Overlay of D and E, showing that at SIV segregation, when only two GMCs are dorsal to NB4-2b (circled by dots), one of them is GMC4-2a (red, Eve⁺) and the other is GMC4-2b (light blue, Klu⁺); an enlargement of the relevant region is shown in the inset. (G) Klu expression (blue) at SIV, focusing at the NB level; NB4-2b (circled by dots) is Klu⁺. (H) Superimposed images of the same embryo shown in G double-labeled for Klu (green) and Pros (red), focused at the GMC level. (I) Overlay of G and H, showing that at SIV only two GMCs are dorsal to NB4-2b: GMC4-2a (Pros⁺Klu[−], red, large arrowhead) and GMC4-2b (Pros⁺Klu⁺, yellow, small arrowhead); an enlargement of the relevant region is shown in the inset. Anterior is up. The inverted double arrowheads mark the positions of the parasegmental grooves and the midline.

anti-Pros antibody (Fig. 5H), revealing its identity as a GMC. Because there are only two Pros⁺ GMCs dorsal and in close proximity to NB4-2b (at SIV), and one of them is the Eve⁺ GMC4-2a, we conclude that this second Klu⁺ and Eve[−] cell is GMC4-2b. Therefore, these data indicate that the earliest expression of Klu within the NB4-2 lineage occurs in GMC4-2b and NB4-2b, after the NB has undergone two rounds of cell division.

Transformation of GMC4-2b to GMC4-2a in *klu* embryos

Both the *klu* mutant phenotype and the Klu expression pattern are consistent with the possibility that the extra RP2 neuron found in the mutant results from a GMC4-2b to GMC4-2a cell fate transformation; to ascertain

whether the mutant GMC4b does in fact behave like GMC4-2a, that is, express markers consistent with a GMC4-2a identity and divides like GMC4-2a to produce another RP2 and another sibling cell, we require a marker to follow GMC4-2b in *klu* mutants. The absence of an antigen-positive mutant allele of *klu* precludes the use of anti-Klu as a marker for the mutant GMC4-2b. Nevertheless, we reasoned that it should be possible to use the *klu*^{P1741} enhancer trap allele to follow the fate of the mutant GMC4-2b. The *klu*^{P1741} β-Gal expression pattern is identical to that of anti-Klu staining (data not shown) and β-Gal expression can be detected in GMC4-2b and not in GMC4-2a (see Fig. 5). Moreover, *klu*^{P1741} homozygotes exhibit the RP2 duplication phenotype at a reasonably high expressivity (49% in T2–A1). We reasoned that because the initial identity of GMC4-2b

should be determined by the identity of its mother, NB4-2a, the mutant GMC4-2b in *klu* mutants should be born with an GMC4-2b identity because NB4-2a does not express Klu and its identity should be unaffected in the mutant; GMC4-2b should therefore initially express β -Gal even in a mutant background (e.g., *klu*^{P1741}/*klu*^{P1741}); the β -Gal that is produced should perdure and continue to mark the mutant GMC4-2b (and its progeny) even if it were to adopt a GMC4-2a identity later attributable to limiting *klu* function. Therefore, we should be able to follow the fate of the mutant GMC4-2b in the *klu*^{P1741} homozygote by virtue of the β -Gal expression and perdurance.

Results of double labeling *klu*^{P1741} embryos with anti-

β -Gal and anti-Eve are shown in Figure 6. In wild-type control embryos, [*klu*^{P1741}/*TM3[ubx-lacZ]*], GMC4-2a becomes Eve⁺ at stage 11 (SIV) (Fig. 6A) and later divides to produce two Eve⁺ postmitotic neurons (Fig. 6B). One of the postmitotic neurons continues to express Eve and differentiates into the RP2 neuron and the other (the sibling cell) extinguishes Eve expression (Fig. 6C,D). In control embryos, no more than two Eve⁺ cells are seen in the RP2-sib cluster and neither are labeled by anti- β -Gal.

In *klu*^{P1741} homozygous embryos, GMC4-2a becomes Eve⁺ at stage 11 (Fig. 6F), same as in wild-type (Fig. 6A); later, a second Eve⁺ cell that is also labeled by the anti- β -Gal antibody appears in the close proximity to GMC4-2a dorsal to NB4-2b (Fig. 6G). Anti- β -Gal labeling of one

Figure 6. GMC4-2b adopts GMC4-2a identity in the enhancer trap line *klu*^{P1741}. The embryos were double labeled with anti- β -Gal (red) and anti-Eve (green). Co-expression of Eve and β -Gal is yellow. In wild-type [*klu*^{P1741}/*TM3[ubx-lacZ]*] embryos, GMC4-2a (arrowhead, A) becomes Eve⁺ at stage 11; later it divides to produce two Eve⁺ postmitotic neurons (arrowheads, B). At stage 12 (C), one of the Eve⁺ cells continues to express Eve and differentiates into the RP2 neuron (arrow), whereas the sibling cell (small arrow) loses Eve expression gradually. By stage 15 only the mature RP2 remains Eve⁺ (arrow, D). A diagram summarizing the process of RP2 formation seen from A–D is shown (E). In *klu*^{P1741} homozygotes, GMC4-2a (arrowhead, green) becomes Eve⁺ at stage 11 (F) as in wild-type (A). Shortly after that, the transformed GMC4-2b (arrowhead, yellow) can be seen that is Eve⁺ and β -Gal⁺ (G). Dotted circle in G indicates the position of NB4-2. Later three Eve⁺ cells are seen in the cluster (H): two β -Gal[−] postmitotic neurons generated by GMC4-2a (green) as in wild type and the transformed β -Gal⁺ GMC4-2b (yellow). At late stage 11, there are four Eve⁺ cells in the cluster (I): two β -Gal⁺ postmitotic neurons (yellow) derived presumably from the transformed GMC4-2b and the β -Gal[−] RP2 and sibling cell (green) derived from GMC4-2a. At stage 12, two RP2 (arrow) and two sibling cells (small arrow) are obvious (J). Note that one set of RP2 and sibling cell, presumably derived from the transformed GMC4-2b, are β -Gal[−] (yellow). At stage 15, two mature Eve⁺ RP2s are seen (K); note that β -Gal is no longer present at this late stage. The probable process of formation of two RP2 neurons in *klu* embryo as seen from F–K is diagrammatically summarized (L). Anterior is up. Dashed line marks the midline. (Arrowhead) Eve⁺ GMCs and postmitotic neurons. (Arrow) RP2 neuron. (Small arrow) Sibling cell.

of the two Eve⁺ cells indicates that these two cells are not the postmitotic neurons derived from the GMC4-2a as seen in Figure 6B. This Eve⁺ and β -Gal⁺ cell is the same cell that we identified earlier, using anti-Eve and anti-Klu double labelings, as GMC4-2b (see Fig. 5). In *klu* embryos, the mutant GMC4-2b ("GMC4-2b") adopts GMC4-2a identity and becomes Eve⁺; GMC4-2a divides first and gives rise to two Eve⁺ postmitotic neurons (Fig. 6H); at late stage 11, "GMC4-2b" also divides and produces two postmitotic neurons that are both Eve and β -Gal⁺ and at this time Eve expression starts to fade in the sibling cell produced from the GMC4-2a division (Fig. 6I); the progeny of the "GMC4-2b" cell division behave in an identical way to those of the GMC4-2a cell division with respect to Eve expression and by stage 12, two RP2s and two sibling cells become obvious (Fig. 6J); the RP2 and sibling cells derived from the "GMC4-2b" are also β -Gal⁺. Ultimately, Eve expression becomes totally extinguished from both sibling cells, β -Gal becomes undetectable in the "GMC4-2b" sublineage and two indistinguishable Eve⁺ RP2 neurons are seen at stage 15 (Fig. 6K).

These results, schematically represented in Figure 6L, indicate that in *klu* embryos two RP2 neurons are produced by two different GMCs—GMC4-2a and transformed "GMC4-2b". The "GMC4-2b" sublineage in *klu* embryos, which is labeled by anti- β -Gal, is an exact, although delayed, replica of the GMC4-2a sublineage. We conclude from these results that *klu* functions to specify GMC4-2b identity and in the absence or under limiting *klu* function, GMC4-2b takes on a GMC4-2a identity.

Ectopic expression of Klu reduces the number of Eve⁺ RP2 neurons

Two observations—(1) Klu is expressed in GMC4-2b but not GMC4-2a and (2) in *klu* mutants, GMC4-2b takes on a GMC4-2a identity—argue that *klu* is not only required to specify GMC4-2b identity but also acts to differentiate between the cellular identities of GMC4-2a and GMC4-2b. Therefore, we might expect that if Klu were to be ectopically expressed in GMC4-2a, where it is not normally expressed, GMC4-2a might undergo the opposite cell fate transformation leading to its loss, thereby eliminating RP2 and its sibling from the NB4-2 lineage. We set out to test these predictions.

First we looked to see if the ectopic expression of Klu causes the loss of Eve-expressing RP2 neurons. In embryos homozygous for a *HSP70-klu* transgene (see Materials and Methods), ectopic Klu protein induced by a 10-min heat shock at 37°C became obvious within 30 min and reached a maximum level within an hour, at 25°C; the Klu protein remained in the heat-shocked embryos for another hour and was gradually degraded afterward (data not shown). The extent to which Eve⁺ RP2 neurons are lost depends on the developmental stage at which the heat-shock is administered (Fig. 8, see below). Embryos heat-shocked at early stage 10 (4.5 hr after egg laying) showed the maximum inhibition of Eve⁺ RP2 formation (Figs. 7B and 8), ~83% (99/120) of the hemiseg-

Figure 7. Ectopically expressed Klu protein inhibits formation of Eve⁺ GMC4-2a and mature RP2 neuron. (A–C) Dorsal views of dissected stage-15 embryos stained with anti-Eve to visualize mature RP2 neurons. (A) A control non-heat-shocked embryo homozygous for a *HS70-klu* insertion on the third chromosome showing the wild-type pattern of one Eve⁺ RP2 neuron (black arrow) in every hemisegment. (B) A heat-shocked embryo (see Materials and Methods) homozygous for the same third chromosomal *HS70-klu* insertion; note that for most hemisegments, an Eve⁺ RP2 neuron is absent (white arrows indicate the positions of the absent RP2s); see text and Fig. 8 for quantitation. (C) An embryo containing both the *ftz-gal4* and the *uas-klu* transgenes; note the absence of mature RP2 neurons in all hemisegments (see text). (D–F) Ventral views of stage-11 embryos stained with anti-Eve to visualize GMC4-2a. (D) A control, non-heat-shocked embryo carrying the *HS70-klu* transgene showing the wild-type pattern of one Eve⁺ GMC4-2a (black arrowhead) per hemisegment. (E) A heat-shocked embryo carrying the *HS70-klu* transgene; note the absence of Eve⁺ GMC4-2as in most of the hemisegments (white arrowhead) of Eve⁺ GMC4-2as in most of the hemisegments (see text). (F) An embryo containing both the *ftz-gal4* and the *uas-klu* transgenes; note the absence of Eve⁺ GMC4-2as in most hemisegments (see text). (G–I) Superimposed confocal images of anti-Eve (green) and anti-Klu (red) double-labeled stage-11 embryos. Arrowhead indicates GMC4-2a. (G) Wild-type embryo. (H) Embryo containing both *ftz-gal4* and *uas-klu*. (I) Enlarged image of GMC4-2a from H; note that this GMC4-2a expresses both Eve and Klu (yellow), albeit at low levels. Anterior is up.

ments fail to exhibit Eve⁺ mature RP2 neurons (scored at stage 14 or later); whereas the non-heat-shocked controls (Fig. 7A) and heat-shocked wild-type embryos (data not shown) showed the wild-type anti-Eve staining pattern. The frequency of RP2 loss dropped drastically from

Figure 8. The temporal dependence of Klu ectopic expression in causing the loss of mature RP2 neurons. A 10-min heat shock was given at various stages of development to embryos homozygous for a third chromosome insertion containing a *HSP70-klu* transgene; the embryos were aged until stage 15–16 and scored for the presence or absence of mature Eve⁺ RP2 neurons (see Materials and Methods). Heat shock treatment at stage 10 results in the maximal loss of mature RP2 in late embryos. Number of hemisegments scored is given in parenthesis.

~80% to only 10% when the heat shock is administered 2 hr later at mid-stage 11 (Fig. 8). There is a temporal window within which ectopic Klu expression can prohibit formation of the mature RP2 neuron. This window coincides with the time frame of the birth of GMC4-2a in the developing embryonic CNS, suggesting that the ectopically expressed Klu might act at the GMC (GMC4-2a) level to prevent mature RP2 formation.

To directly examine this possibility, a similar heat shock experiment was carried out to look at the effects of ectopic Klu on the formation of Eve⁺ GMC4-2a (scored at stage 11). Embryos heat-shocked at 4.5 hr after egg-laying (early stage 10) lose Eve-expressing GMC4-2a (see Fig. 7E) from 77% (230/300) of their hemisegments. This is consistent with the frequency of RP2 loss in heat-shocked embryos and lends further credence to the idea that ectopically expressed Klu can act to prevent GMC4-2a from being specified correctly, thereby preventing Eve-expressing RP2 neurons from being made. Both considerations of the timing of ectopic expression and the fact that the identity of GMC4-2a is affected argue against the defects being mediated at the level of the post-mitotic neurons. However, because of the crude nature of the heat shock paradigm, other possibilities cannot be rigorously ruled out; for example, it is conceivable that as the result of ectopically expressing Klu, NB4-2 might adopt a NB4-2b fate, thereby failing to produce the first two GMCs in the lineage, GMC4-2a and GMC4-2b, as well as their progeny.

Ectopically expressed Klu in GMC4-2a is sufficient to prevent the formation of Eve⁺ GMC4-2a and mature RP2 neuron

To demonstrate more rigorously that ectopically ex-

pressed Klu does act at the GMC4-2a level to prevent the formation of Eve-expressing RP2 neurons, experiments of targeted ectopic expression of *klu* in GMC4-2a were carried out with the Gal4 system (Brand and Perrimon 1993). We used a *ftz-gal4* driver that is expressed in ~20 GMCs, including GMC4-2a, but not in NBs. Animals homozygous for either the *ftz-gal4* or *uas-klu* transgene alone did not show any abnormalities in the anti-Eve staining pattern (data not shown). In embryos carrying both the *ftz-gal4* and *uas-klu* transgenes, 82% (214/260) of the hemisegments failed to exhibit an Eve⁺ RP2 neuron (Fig. 7C). Ectopic *klu* expression driven by the *ftz* promoter can be detected by the anti-Klu antibody in GMC4-2a in stage 11 embryos (Fig. 7H,I), albeit at a low level. In embryos carrying both *ftz-gal4* and *uas-klu* transgenes, Eve expression in GMC4-2a was either lost completely or was weak (Fig. 7F,H,I). Seventy-nine percent (283/360) of hemisegments failed to produce a Eve⁺ GMC4-2a at stage 11. This experiment demonstrates that ectopic expression of Klu in GMC4-2a is sufficient to prohibit the formation of Eve⁺ GMC4-2a.

The possibility that ectopically expressed Klu protein in NB4-2 might cause it to adopt an NB4-2b identity (thereby eliminating GMC4-2a and GMC4-2b and their respective progeny from the lineage) can be eliminated because the *ftz-gal4* driver does not express in any NB. Based on these ectopic expression experiments, we conclude that ectopic expression of Klu in GMC4-2a is sufficient to alter the identity of GMC4-2a, leading ultimately to a decrease in the number of mature RP2 neurons. It is possible that ectopically expressed Klu in GMC4-2a forces it to adopt a GMC4-2b identity; however, because of the lack of information on the nature of the progeny of GMC4-2b, we cannot directly test this prediction.

Discussion

The role of klu within the context of genes affecting the NB4-2 lineage

A number of processes and mechanisms need to be studied in an effort to understand how cellular diversity is generated in the CNS—the establishment of the initial identity of the NBs; the asymmetric NB cell division; the modulation of NB identity with each cell cycle; and related to this issue, the establishment of distinct GMCs within each NB lineage; the GMC cell division and the signalling events that must occur at the level of the post-mitotic neurons to establish asymmetric cell fate. To identify the molecules and elucidate the mechanisms involved in these processes and how they work with respect to a single NB lineage, the NB4-2 lineage, considerable attention has been focused on mutations that affect RP2 cell number.

To date, several genes have been identified that act at various levels to affect the genesis of this cell. At the NB level, Wg protein secreted by cells that are destined to form row 5 NBs is required for the formation/specification of NB4-2 as well as other row 4 and row 6 NBs

(Chu-LaGriffa and Doe 1993). In the absence or under conditions of limiting *wg* function, correctly specified GMC4-2a and RP2 are not made. Two other segment polarity genes, *patched* (*ptc*) and *gooseberry* (*gsb*), are also involved in controlling the *wg*-mediated specification of NB4-2 (Patel et al. 1989a; Zhang et al. 1994; Skeath et al. 1995; Bhat 1996). Although segment polarity genes like *wg*, *gsb*, and *ptc* have been shown to act before NB delamination (for review, see Doe and Skeath 1996) to specify NB identity along the anterior-posterior axis, few genes have been described that act to differentiate between the identities of NBs along the dorsal/ventral axis. A gene, *lottchen*, appears to act to differentiate between the identities of NB4-3 and NB4-2; in *lottchen* loss-of-function mutants, NB4-3 apparently takes on some of the characteristics of NB4-2 (Buescher and Chia 1997).

The understanding of cell fate specification within NB lineages has been hampered by the lack of lineage information and markers. The adaptation of the Dil labeling technique to *Drosophila* has facilitated the determination of terminal NB lineages; however information pertaining to the order in which these cells are generated and markers that facilitate the identification of these cells are largely lacking. Therefore, the analysis of cell fate specification within NB lineages has largely been limited to the first GMC and its progeny (see Introduction). Although it has been shown that gene expression changes in the course of an NB lineage (e.g., Cui and Doe 1992; Broadus et al. 1995), and it has been postulated that there must be genes that act to differentiate between cells born at different times within one stem cell lineage (Doe 1992), this point has not been demonstrated. In this study, we extend the analysis of the NB4-2 lineage to its second-born GMC. Our data show that a single genetic function encoded by *klu* is sufficient to differentiate between the second-born GMC4-2b from the first-born GMC4-2a. *Klu* is expressed in GMC4-2b but is absent in the earlier born cells in the lineage (NB4-2, NB4-2a, and GMC4-2a). Our results indicate that, consistent with its expression pattern, *Klu* acts at the GMC and not at the NB level. In the absence of *Klu*, GMC4-2b adopts a GMC4-2a identity, for example, expresses *Eve* and divides to produce progeny with characteristics normally associated with the progeny of GMC4-2a. Furthermore, the mis-expression of *Klu* in GMC4-2a prevents this cell from acquiring its correct cellular identity and prevents the formation of a mature RP2 neuron.

When does Klu act to establish GMC4-2b identity?

When is the GMC4-2b identity normally established? How and when does the mutant GMC4-2b adopt a GMC4-2a identity? The fact that the enhancer trap allele *klu*^{P1741} expresses β -Gal in phenotypically mutant GMC4-2b is interesting to consider; the fact that this cell initiates *klu* (β -Gal) expression suggests that the mutant GMC4-2b is born with an initially normal GMC4-2b identity (presumably because the cell identity of its mother NB4-2a, which does not express *Klu*, is not al-

tered in the mutant); however, this identity is clearly not maintained in the mutant because this cell proceeds to acquire characteristics of GMC4-2a. So at the time of its birth, the mutant GMC4-2b clearly has a distinct identity from GMC4-2a and initiates *klu* (β -Gal) expression. However, in the absence of the *Klu* function, this distinction from GMC4-2a cannot be maintained and this cell ultimately adopts a GMC4-2a identity.

These observations suggest that the initial identity of any GMC is specified by its mother NB. In this context, our observations lend support to the view that gene products present in the once divided NB, NB4-2a, are inherited by its daughter GMC, GMC4-2b; these gene products must act to initiate a program necessary to establish GMC4-2b cell identity. A key player that is normally activated in GMC4-2b to implement this program is *Klu*. Therefore, at least two types of genes are necessary to establish the identity of a GMC; first, genes that are expressed in the NB and presumably inherited by the GMC daughter cell which act to initiate a program necessary to establish GMC identity; second, genes encoding molecules like *Klu* that are expressed, presumably in response to the inherited gene products, and act exclusively in the GMC to establish its cellular identity.

Another point of interest is that, if this program for establishing GMC4-2b identity cannot be implemented attributable to the absence of or limiting *klu* function, GMC4-2b will adopt, as far as we can tell, all of the characteristics of GMC4-2a. GMC4-2b is clearly different from GMC4-2a early during its life because GMC4-2b will initiate *klu* expression, whereas GMC4-2a does not. Nevertheless, GMC4-2b appears to retain a default program that will confer a GMC4-2a cell identity if the program (involving *klu*) necessary to establish a GMC4-2b cell identity cannot be implemented, for example, in *klu* mutants. Therefore the absence or presence of a single gene product (*Klu*) determines whether the second-born GMC in the NB4-2 lineage adopts a GMC4-2a or a GMC4-2b cell identity.

Homeotic control

The loss-of-function phenotype of *klu* appears to be highly position dependent. In *klu* protein null alleles, the RP2 duplication phenotype occurs with essentially complete expressivity (>99% of the hemisegments exhibit RP2 duplications) in the more anterior segments, for example, T2–A1, whereas the expressivity in the more posterior segments (A2–A8) is much lower (see Results). The position dependence of the loss-of-function phenotype correlates well with the position-dependent expression of *Klu* in the CNS, where the levels of *Klu* protein expression appear to be much higher in the thoracic segments than in the abdominal segments (see Results). These observations suggest that the level of *klu* expression may be modulated by the homeotic genes.

There is precedence for homeotic regulation of the levels of gene expression in the CNS; previous findings further indicate that cell lineage regulation of insect CNS stem cells can be modified by homeotic genes (Taghert

and Goodman 1984; Prokop and Technau 1994). The *pdm-1* and *pdm-2* genes that are required for the specification of GMC4-2a identity (Yeo et al. 1995) are expressed in the developing CNS with higher levels in the thoracic segments and lower levels in the abdominal segments. The low level of expression in the abdominal segments appears to require genes of the bithorax complex (Lloyd and Sakonju 1991). A functional correlation to the positional dependence of the *pdm-1* and *pdm-2* expression levels has also been reported; when *pdm-2* is ectopically expressed, duplications of the GMC4-2a sublineage occur apparently because both of the postmitotic progeny of GMC4-2a adopt the identity of their mother; this duplication appears to occur preferentially in the thoracic segments (Yang et al. 1993). The relatively low phenotypic penetrance seen specifically in the abdominal segments of *klu* null alleles suggests that in this region of the CNS where *Klu* expression is kept at a low level (presumably through the actions of homeotic genes), there are other players whose functions overlap those of *klu*.

Materials and methods

Standard molecular biology techniques

All routine DNA work was performed as described by Sambrook et al. (1989).

Antibody preparation

A fusion protein was produced using the QIAexpress system from QIAGEN. A 840-bp *SphI* (1995)/*XhoI* (2835) fragment from the *klu* cDNA that encodes 280 amino acids (residues 433–712) from the carboxy-terminal region of KLU including the four Zn finger motifs was cloned in-frame with the 6His tag. Bacterially produced fusion protein was purified using Ni-NTA resin that is selective for proteins with an affinity tag of six consecutive histidine residues and injected into rabbits. Purified fusion protein (300 µg) was used for the initial inoculation (complete Freund's adjuvant) and subsequent boosts (incomplete Freund's adjuvant). Anti-Klu serum was diluted 1:1000 and preadsorbed against fixed embryos before use. The authenticity of the wild-type staining patterns seen with the serum was confirmed by showing that embryos homozygous for *Df(3R)klu^{XR19}* or *klu^{p212IR51C}* do not stain.

Immunocytochemistry and photomicroscopy

Single antibody staining was performed essentially as described by Yang et al. (1991). Embryos were collected, dechorionated with 50% bleach, fixed with 4% paraformaldehyde for 20 min, devitellinized with a 1:1 mixture of heptane/methanol, washed with methanol, and rehydrated with PBT. Photomicrographs were taken with a Zeiss axiophot using DIC optics and either a 63× plan-Apochromat or a 20× Neofluor lens. For immunofluorescence, FITC- and Texas Red-conjugated anti-mouse or anti-rabbit antibodies (Jackson laboratory) were used. Lethal *klu* alleles were balanced over a “blue balancer” chromosome carrying a *ubx-lacZ* insertion and homozygous mutant embryos were identified by double staining with anti-β-Gal antibodies (Promega or Cappel) based on the absence of *lacZ* expression. Confocal microscopy was done on a Biorad MRC 600, using Adobe Photoshop 3.0 for image processing.

Generation of β-gal-expressing “flip-out” clones

To label individual NB lineages through the expression of *Tauβgal*, we used the FRT/FLP system (Golic and Lindquist 1989; Xu and Rubin 1993). Using a homozygous viable second chromosome insertion carrying *hsFLP* and a second chromosome insertion carrying an *actin-Tauβgal* cassette (A. Marti-Subirana and R. Holmgren, pers. comm.), the stocks *hsFLP/CyO*; *klu^{p212IR51C}/TM3*, and *Tauβgal* cassette/*CyO*; *klu^{p212IR51C}/TM3* were established; virgins from the first stock were crossed to males of the second stock. The *actin-Tauβgal* transgene is normally inactive because the ubiquitous *actin5c* promoter is separated from the *Tauβgal* by a sequence, in the form of a FRT cassette, which prevents its expression; however, when the FRT cassette is deleted through the expression of FLP, *Tauβgal* will be expressed from the *actin5c* promoter. To irreversibly activate the expression of *Tau-βgal*, embryos were collected from the above cross in 2-hr intervals and aged for 2 hr at 25°C. Heat shock was performed by placing embryos into a moist chamber that was submerged in a waterbath for 15–20 min at 34°C. Subsequently, the embryos were allowed to develop at 18°C until stage 15–16, fixed and stained with anti-β-Gal (Cappel) and anti-Eve. Non-heat-shocked control embryos handled in the same manner had no β-Gal⁺ clones.

hsp70-klu and uas-klu constructs and transformants

To generate the *hsp70-klu* construct, full-length *klu* cDNA plasmid was linearized with *HindIII* and rendered blunt-ended by end-filling with Klenow polymerase; an ~4-kb *NotI*/end-filled *HindII* fragment, containing the whole of the *klu* coding region was generated by digesting with *NotI*. This fragment was inserted into *HpaI*/*NotI* cut pCaSpeR-hs (from Carl Thummel, University of Utah, Salt Lake City) for germ-line transformation.

To produce a full-length *klu* cDNA under the control of *gal4-uas*, an *EcoRI* site in the 5' untranslated region (UTR) of *klu* was engineered by PCR. The 5' primer (gaattcaagcttgaattccaataacgatcgcgcggt) contained an *EcoRI* site and sequence from the *klu* 5'-UTR. The 3' internal *klu* primer (atcgctgcagatctggca) used in the PCR reaction includes the *BgIII* site at position 487. The 387-bp PCR product was cut with *EcoRI* and *BgIII* and added to a ligation mix that contained the *gal4-uas* germ-line transformation vector, pUAST, cut with *EcoRI* and the remaining 3351-bp *BgIII*/*EcoRI* fragment from the *klu* cDNA. The final construct containing the 3738-bp *klu* cDNA was selected by restriction mapping.

The procedure described by Spradling (1986) was used to obtain germ-line transformants.

klu misexpression experiments

Heat shock-induced ectopic expression experiments were carried out with a homozygous transformant line that carried an *HSP70-klu* insertion on the third chromosome. Overnight collection of embryos were dechorionated; stage 7 embryos (2 hr 50 min to 3 hr) were hand-picked, transferred to a moist chamber and aged for various times at 25°C before being treated to a 10-min heat shock at 37°C. The heat-shocked embryos were kept in the moist chamber at 18°C and allowed to develop until stage 15–16; the embryos were fixed, stained with anti-Eve and Eve⁺ RP2s were scored. A similar experiment was carried out to assess the formation of Eve⁺ GMC-2a. Hand-picked stage-7 embryos were incubated in a moist chamber at 25°C for 1.5 hr. After a 10-min heat shock at 37°C the embryos were aged for another 3.5 hr at 25°C. The embryos were fixed and stained

with anti-Eve and Eve⁺ GMC4-2as were scored. Control embryos comprising of heat-shocked wild-type embryos and non-heat-shocked *HSP70-klu* embryos were treated in a similar way.

For targeted misexpression of Klu, the *ftz-gal4/TM3, Sb Gal4* driver line (from the C.S. Goodman laboratory, homozygous lethal) was used to drive the expression of a *uas-klu* insertion on the second chromosome. A stock, *uas-klu/Cyo; ftz-gal4/TM3*, carrying both the *ftz-gal4* and *uas-klu-myc* insertions was generated; overnight collections of embryos from this stock were used for the study. Control embryos homozygous for *ftz-gal4* or homozygous for *uas-klu* alone did not show any defects in RP2 formation.

Staging embryos

In general, we use the morphological criteria set out by Campos-Ortega and Hartenstein (1985). The criteria we use for identifying NB4-2, NB4-2a, and GMC4-2a as well as the criteria we use for making fine distinctions of the age of embryos from stage 10–13 have been described previously (Yang et al. 1993).

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Klumpfuss, a putative *Drosophila* zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage.

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