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Ancestry-independent fate specification and plasticity in the developmental timing of a typical *Drosophila* neuronal lineage

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In the *Drosophila* CNS, combinatorial, interdependent, sequential genetic programs in neuroectodermal (NE) cells, prior to the formation of neuroblasts (NBs), determine the initial identity of NBs. Temporal factors are then sequentially expressed to change the temporal identity. It is unclear at what levels this positional and temporal information integrates to determine progeny cell identity. One idea is that this is a top-down linear process: the identity of a NB determines the identity of its daughter, the ganglion mother cell (GMC), the asymmetric division of the GMC and the fate specification of daughter cells of the GMC. Our results with *midline* (*mid*), which encodes a T-box protein, in a typical lineage, NB4-2→GMC-1→RP2/sib, suggest that at least part of the process operates in GMCs. That is, a GMC or a neuronal identity need not be determined at the NB or NE level. This is demonstrated by showing that Mid is expressed in a row 5 GMC (M-GMC), but not in its parent NB or NE cell. In *mid* mutants, M-GMC changes into GMC-1 and generates an RP2 and a sib without affecting the expression of key genes at the NE/NB levels. Expression of Mid in the M-GMC in *mid* mutants rescues the fate change, indicating that Mid specifies neurons at the GMC level. Moreover, we found a significant plasticity in the temporal window in which a neuronal lineage can develop. Although the extra GMC-1 in *mid* mutants is born ~2 hours later than the bona fide GMC-1, it follows the same developmental pattern as the bona fide GMC-1. Thus, a GMC identity can be independent of parental identity and GMC formation and elaboration need not be strictly time-bound.

KEY WORDS: Neurogenesis, *Drosophila*, Midline, Transcription, Ancestry

INTRODUCTION

In the eukaryotic central nervous system (CNS), multi-potential neural progenitor cells undergo a series of asymmetric cell divisions to self-renew and to generate several rounds of neurons of distinct identities. Thus, a complex array of different types of neurons is formed from a few precursor cells. The CNS of the *Drosophila* embryo provides an experimentally advantageous model system in which to investigate the mechanisms that generate the metazoan nervous system. In the *Drosophila* embryo, the primary neuronal precursors, neuroblasts (NBs), divide by asymmetric mitosis to self-renew and to produce a chain of ganglion mother cells (GMCs). A GMC, although bipotential, does not self-renew; instead, it divides asymmetrically to generate two different post-mitotic neurons. Thus, from the ~30 NBs in a given hemisegment, ~320 distinct and highly specialized neurons and 30 glial cells are generated (reviewed by Gaziova and Bhat, 2006).

For several years, we have been focusing on a few typical NB lineages with the aim of understanding neuronal lineage elaborations in the ventral nerve cord of the *Drosophila* embryo. One such lineage is NB4-2→GMC-1→RP2/sib, a well-studied neuronal lineage (Chu-LaGraff and Doe, 1993; Bhat and Schedl, 1994; Bhat et al., 1995; Bhat, 1996; Bhat and Schedl, 1997; Duman-Scheel et al., 1997; Bhat, 1998; Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999; Bhat et al., 2000; Mehta and Bhat, 2001; Yedvobnick et al., 2004; Bhat and Apsel, 2004; Bhat, 1999; Gaziova and Bhat, 2006). NB4-2 is a row 4, column 2 cell, and is formed as one of ~30 NBs in a hemisegment; it is an S2 NB formed during the second wave of NB delamination. The identity of this NB is determined at the neuroectodermal (NE) level: the segmentation protein Patched (Ptc) represses the expression of Gooseberry (Gsb) in the precursor NE cells, allowing Wingless (Wg) signal reception and the specification of NB4-2 identity in this cell. This NB4-2 specification allows the cell to generate its first GMC, GMC-1 (also known as GMC4-2a); GMC-1 then divides asymmetrically into a motoneuron called RP2 and a sibling cell of as yet undetermined fate. Thus, a sequence of events governed by combinatorial and interdependent genetic programs set in motion prior to the formation of a NB appears to guide the identity of progeny neurons. This theory has been further extended by the observation that once a NB divides, its gene expression program changes, and this then dictates the identity of the next GMC, as well as the identity of the daughter cells generated from this GMC (Isshiki et al., 2001). Thus, an ancestry-dependent mechanism, i.e. gene expression programs in precursor cells, appears to strictly guide lineage development in the *Drosophila* CNS. However, it is unclear if the integration of the positional and temporal cues occurs in a linear fashion (i.e. one sets the other) or whether cells at the level of GMCs and neurons can respond to temporal cues independently of positional cues. A strict linear model seems unlikely because temporal factors are not present in parent cells. Could it be, then, that the positional cues are priming cells so that they can receive signals later in development?

In a screen for mutants that affect the RP2/sib lineage, we identified *extra*, a mutation characterized by an RP2-like extra neuron in each hemisegment at the periphery of the nerve cord (K.M.B. and P. Wai, unpublished). Our genetic analysis of *extra* indicated that it is allelic to the previously identified mutation *midline* (*mid*) (Nusslein-Volhard et al., 1984) or *lost in space* (*los*) (Kolodziej et al., 1995). *mid* mutants exhibit cuticle defects, suggesting that Mid protein is required for ectodermal patterning (Nusslein-Volhard et al., 1984). *mid* mutants also exhibit defects in

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the lateral chordotonal axons, with shorter and defasciculated dorsally routed axons in the peripheral nervous system (Kolodziej et al., 1995). Given these results, we sought to examine *mid* mutants in detail.

Cloning of the gene by us and others revealed that it encodes a T-box (Tbx) protein. The Tbx proteins, which are highly conserved among metazoans, are defined by the presence of a T-box domain, a 180-230 amino acid DNA-binding domain. One of the most striking attributes of Tbx proteins is their dosage sensitivity, i.e. developmental processes appear to be sensitive to the levels of some Tbx proteins. For example, upper limb malformation and congenital heart disease in Holt-Oram syndrome are caused by a haploinsufficiency of *TBX5* (Li et al., 1997; Basson et al., 1999). Haploinsufficiency of the Tbx genes mouse brachyury and human *TBX3* and *TBX1*, also produces dominant phenotypes: short tails/tailless, Ulnar-Mammary syndrome and DiGeorge syndrome, respectively (Bamshad et al., 1997; Merscher et al., 2001).

Whereas a previous study, based on limited analysis, suggested that the extra neuron present at the periphery of the nerve cord in *mid* mutants is not an RP2 neuron (Buescher et al., 2006), our analyses indicate that it is indeed an RP2 neuron. We found that loss of Mid activity causes a GMC from a row 5 NB (M-GMC) to adopt the GMC fate of the RP2/sib lineage (GMC-1) without affecting the expression pattern of key genes at the parent NB or NE level. Expression of Mid in the M-GMC in *mid* mutants is sufficient to suppress the fate change, indicating that Mid specifies neurons at the GMC level (or just as post-mitotic cells are produced). We also found that there is significant plasticity in the temporal window in which a neuronal lineage can develop. Although this extra GMC-1 in *mid* mutants is born ~2 hours later than the bona fide GMC-1, it follows the same developmental pattern as the bona fide GMC-1. Within the CNS, there appears, in general, to be a temporally progressive restriction on the ability of a NB to generate earlier-born neurons. Our results indicate that at the organismal level, an earlier lineage can be generated at a later point in development (although in a different NB lineage). In summary, our results show that a GMC or a neuronal identity can be independent of parental identity and their formation and elaboration need not be developmentally time-bound.

MATERIALS AND METHODS

Drosophila strains and genetics

mid mutant alleles used were mid^{1}, mid^{2} and los^{1}, and a deficiency that removes both *mid* and *H15* genes ($Df^{H15,mid}$ or mid^{df}; Bloomington stock number 7498). The original *extra* mutation was a hypomorphic allele isolated in a second chromosome screen (K.M.B. and P. Wai, unpublished), but has since been lost. The other lines used were: a deficiency that removes the two *pdm* genes (Df^{pdm}; Bloomington stock number 7416), wg^{cx4}, $insc^{22}$, $numb^{796}$, *scabrous-GAL4* (*sca-GAL4*), *ptc-GAL4*, *en-GAL4*, *wg-GAL4*, *ftz-GAL4* and *RKK-GAL4* (*eve-GAL4*). As wild type, we used the w^{1118} strain. The various mutant combinations were constructed using standard genetics.

Molecular characterization of *mid* alleles

Based on intron-exon prediction, four sets of primers were designed to amplify all four exons and surrounding parts of the introns of the *mid* gene. Genomic DNA from embryos homozygous for each of the *mid* alleles was amplified and PCR products from three independent reactions were sequenced.

Rescue experiments with *mid*

A full-length *mid* cDNA (RE27439; Berkeley *Drosophila* Genome Project) was subcloned into the pUAST *P*-element vector. Transgenic flies carrying one or two copies of a *UAS-mid* construct in the $Df^{H15,mid}$ mutant background were crossed with $Df^{H15,mid}$ or los^{1} carrying a specific transgenic *GAL4* driver. Several drivers were tested for their ability to rescue/suppress the *mid* phenotype: *sca-GAL4*, *ptc-GAL4*, *wg-GAL4*, *ftz-GAL4* and *eve-GAL4.* The rescue efficiency was analyzed at 22°C and 26°C.

Immunohistochemistry

Embryo collection, fixation and immunostaining were performed according to standard procedures. The following antibodies were used: anti-Eve (gift of Manfred Frasch, 1:2000), anti-Zfh1 (gift of Eric Lai, 1:400), 22C10 [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, 1:1], anti-Wg (DSHB, 1:5), anti-En (DSHB, 1:1), anti-Gsb (gift of Bob Holmgren, 1:3), anti-Gsb-n (gift of Bob Holmgren, 1:1), anti-Slp (gift of Ken Cadigan, 1:400), anti-β-galactosidase (Cappel, 1:100) and anti-Mid (this study, 1:100). For color visualization, AP-conjugated and HRP-conjugated secondary antibodies were used. For double staining, secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 635 were used.

Generation and purification of anti-Mid antibodies and western blot analysis

A DNA fragment corresponding to 125 amino acids (aa 49-173) of the N-terminal portion of the Mid protein, which has the least homology to H15, was cloned into pMal-KK-1 to create a fusion with the maltose-binding protein (MBP). The MBP-Mid fusion protein was expressed in *E. coli* BL21-CodonPlus (Stratagene) and purified using amylose columns. About 0.6 ml of the MBP-Mid fusion protein (0.5 mg/ml) was used for rat immunization according to a standard protocol (Covance). To remove any antibodies specific to MBP from the polyclonal serum, we applied the serum to AminoLink Plus coupling gel (Pierce) immobilized with MBP2 protein and then affinity purified Mid-specific antibodies using AminoLink Plus coupling gel immobilized with MBP-Mid fusion protein. For western analysis, extracts from 30 *Drosophila* embryos per lane were used (mutants were identified by the absence of GFP balancer) using the anti-Mid antibody at 1:100. As a loading control, we used anti-Tubulin antibody (Abcam, 1:2000).

Transactivation assay

Two reporter vectors were prepared to analyze Mid and H15 activation potential in vitro. The luciferase gene in pGL3-Basic vector was fused with the *Hsp70Bc* minimal promoter. We inserted either the double-stranded oligonucleotide 5′-TTAATTTCACACCTAGGTGTGAAATT-3′, to create a T-site consensus reporter (see Kispert and Herrmann, 1993), or 367 bp of the *gsb-n* promoter (–1279 to –912) containing the TBE putative T-site upstream of the *hsp70* promoter. Full-length *mid* cDNA (RE27439) and *H15* cDNA (IP01538) were fused with the 6×His epitope sequence of the pAc5.1/V5-HisA vector. The predicted full-length cDNA of *org-1* was amplified by RT-PCR of RNA from *Drosophila* embryos and fused with the 6×His epitope sequence of pAc5.1. As a control, an expression vector containing the EGFP gene fused with the 6×His epitope sequence of pAc5.1 was used. *Renilla* luciferase (Rluc) under the control of the HSV thymidine kinase (HSV-TK) promoter in pRL-TK vector showed weak activation; an *Nhe*I-*Xba*I fragment containing the entire Rluc gene was fused with the *actin* gene promoter in the pAc5.1 vector via subcloning in pBluescript to create the normalization vector *Ac*-Rluc (see Porsch at al., 2005). *Drosophila* S2 cells were grown in Shields and Sang Medium (Sigma) with 10% FBS at 25°C. For transient transfection experiments, S2 cells were seeded in 24-well plates at a dilution of $1.2\text{-}1.5\times10^{6}$ and transfected 24 hours later using calcium phosphate precipitation. Each well containing 0.5 ml of S2 cells was treated with 25 μl of transfection mixture. For each transfection, 20 ng of *Ac*-Rluc and 40 ng of T-site reporter, or 10 ng of *Ac*-Rluc and 50 ng of *gsb-n* T-site reporter, were used per 500 ml of transfection mixture. The expression vectors were used at three different concentrations: 100 ng, 500 ng and 2 μg per 500 ml of transfection mixture. Total DNA concentration was adjusted with pBluescript SK+ to 10 μg per 500 ml. The transfected S2 cells of each well were lysed 48 hours later in 120 μl of Passive Lysis Buffer (Promega) and luciferase assays performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol and analyzed on a Veritas Microplate Luminometer. The transfections were set up in triplicate. The relative luciferase activity values shown are the mean of three independent experiments after normalization.

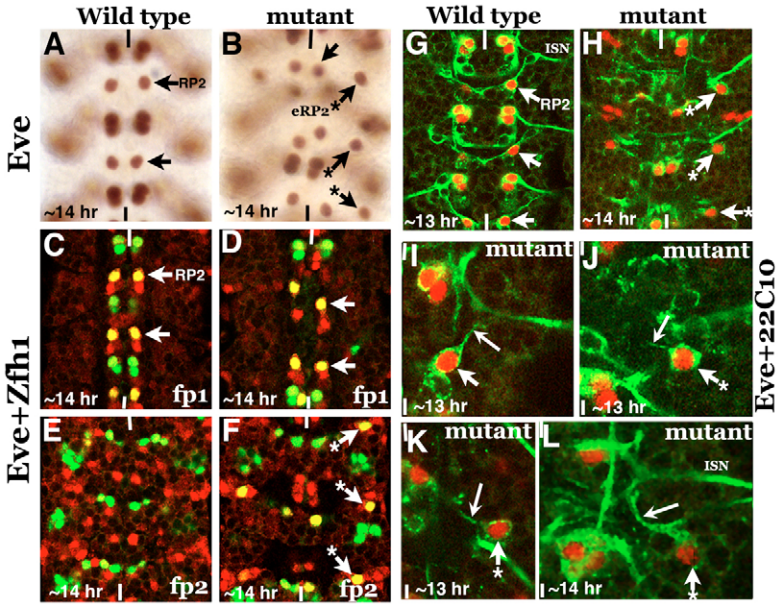

Fig. 1. The extra RP2 neuron in *Drosophila mid* mutants. Wild-type and *mid* mutant *Drosophila* embryos, 13 to 14 hours old, were stained with antibodies to Eve (**A**,**B**), Eve+Zfh1 (**C-F**) or Eve+22C10 (**G-L**). Anterior is up, the midline is marked by vertical lines. Arrow, RP2; arrow with asterisk, the extra RP2 (eRP2); small arrow, axon projection from an RP2 or eRP2. ISN, intersegmental nerve bundle; fp1, focal plane 1; fp2, focal plane 2.

RESULTS

An additional Even skipped-positive neuron is formed in *mid* mutants at an ectopic site within the nerve cord

We sought to examine in detail whether the NB4-2→GMC-1→RP2/sib lineage is affected in embryos mutant for *mid* using an antibody against Even skipped (Eve), a GMC-1→RP2/sib lineage marker. This lineage is generated by NB4-2, which is formed as an S2 NB at ~4.5 hours of development. It generates its first GMC (GMC-1) by a self-renewing asymmetric division at ~6-6.5 hours of development. This GMC-1 divides into an RP2 and a sib at ~7.45 hours of development. The RP2 begins to project its axon ipsilaterally towards the intersegmental nerve bundle (ISN) by ~10 hours of development and innervates muscle numbers 2, 9 and 11.

There are several well-established ways to distinguish GMC-1, an RP2 and a sib (Doe, 1992; Bhat and Schedl., 1994; Gaziova and Bhat, 2006). Both the nuclear division and cytokinesis of GMC-1 are asymmetric, and thus there is a size difference between GMC-1 (7.5 μm), an RP2 (~5 μm) and a sib (~3 μm). Similarly, the nucleus of GMC-1 is ~6.5 μm, whereas that of an RP2 is 4 μm and that of a sib 2.5 μm. There is also a difference in the level of marker gene expression between an RP2 and a sib, as well as a difference in the temporal dynamics of expression of these markers; the future RP2 cell expresses markers such as Eve more strongly than does a future sib. The cell that assumes a sib identity undergoes a size reduction and further downregulation of RP2-specific marker genes. By ~14 hours of development, expression of all RP2-specific markers is lost from the sib. Finally, there is a subset of markers that only a mature RP2 expresses but not the sib or GMC-1. These include Futsch (MAP1B) as detected by Mab 22C10, which stains the membrane and the axonal projection of only a subset of neurons in each hemisegment (Fujita et al., 1982), allowing us to visualize the axon morphology of these neurons (see Fig. 1G), and Cut, a transcription factor.

When ~14-hour-old embryos mutant for *mid* were examined with an antibody against Eve, a strongly Eve-positive cell was observed close to the periphery of the nerve cord (Fig. 1B). This cell is located between two clusters of EL neurons of the adjacent hemisegments; EL neurons are a group of 8-10 Eve-positive neurons (Fig. 1B). This phenotype was observed in all the *mid* mutants, the strongest phenotype observed being in a deficiency that removes *mid* and an adjacent gene that encodes another T-box protein, H15 (Table 1). Among the *mid* alleles, mid^{1} was genetically the strongest. We also observed variation in the penetrance of the defect in some of the alleles depending on whether the embryos were collected from balanced (*CyO* balancer) parents or parents in a wild-type background (Table 1), indicating a parental balancer-induced influence on the phenotype (see Bhat et al., 2007).

The extra cell in *mid* mutants behaves as an RP2 neuron

Next we examined whether this cell has any of the characteristics of an RP2 neuron. We stained embryos mutant for *mid* with an antibody against Zfh1, a zinc-finger protein. Zfh1 is expressed in an

Table 1. The penetrance of the extra RP2 lineage phenotype in various *mid* alleles

Parental genotype	Percentage of hemisegments affected in mutant embryos (*n*)
los^{1}/+	25 (418)
los^{1}/*bal*	41 (184)
mid^{2}/+	30 (450)
mid^{2}/*bal*	29 (224)
mid^{1}/+	38 (864)
mid^{1}/*bal*	35 (504)
Df(H15, mid)/+	89 (900)
Df(H15, mid)/*bal*	96 (204)
los^{1}/mid^{2}/+	24 (272)
los^{1}/mid^{2}/*bal*	25 (256)
los^{1}/mid^{1}/+	30 (442)
los^{1}/mid^{1}/*bal*	39 (796)
los^{1}/*Df(H15, mid)*/+	57 (586)
los^{1}/*Df(H15, mid)*/*bal*	65 (186)
mid^{2}/mid^{1}/+	33 (428)
mid^{2}/mid^{1}/*bal*	34 (410)
mid^{2}/*Df(H15, mid)*/+	50 (442)
mid^{2}/*Df(H15, mid)*/*bal*	56 (292)
mid^{1}/*Df(H15, mid)*/+	63 (728)
mid^{1}/*Df(H15, mid)*/*bal*	66 (396)

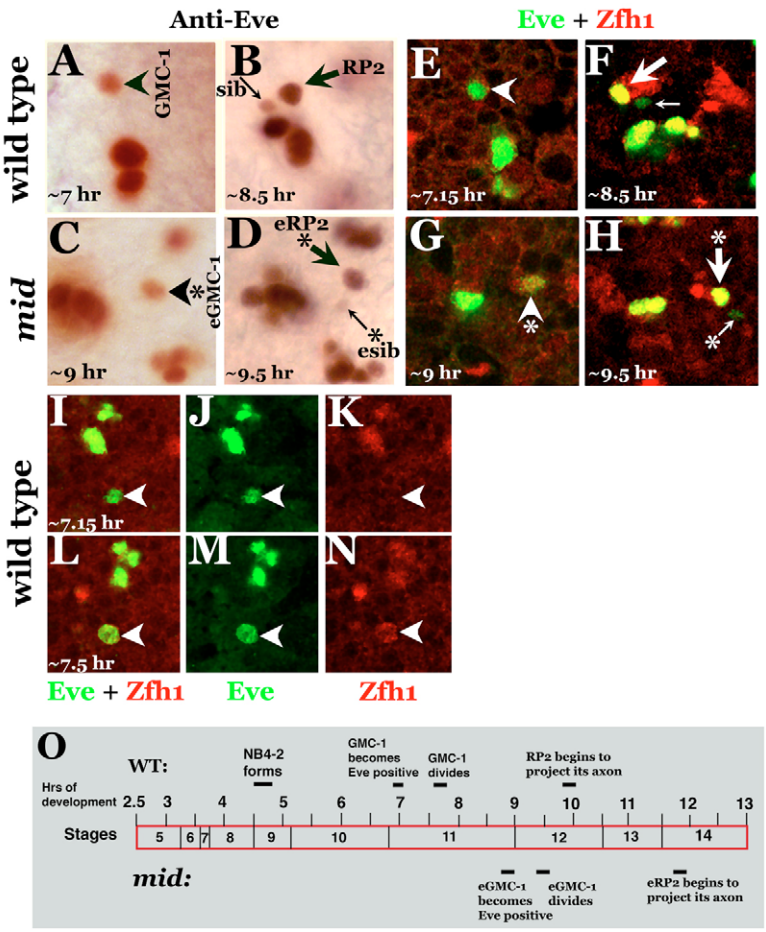

Fig. 2. The elaboration of the extra RP2 lineage in *mid* mutants. (**A-N**) Wild-type and *mid* mutant *Drosophila* embryos of the indicated ages were stained with antibodies to Eve (A-D,J,M), Zfh1 (K,N), or Eve+Zfh1 (E-H,I,L). Only one hemisegment is shown in each panel; anterior is up. Arrowhead, GMC-1; arrowhead with asterisk, the extra GMC-1; arrow, RP2; arrow with asterisk, eRP2; thin arrow, sib; thin arrow with asterisk, esib. (**O**) The developmental timing of the bona fide RP2/sib lineage in wild-type *Drosophila* (WT) and the development of the eRP2 lineage in *mid* mutants.

RP2 (Fig. 1C,D) but not in a sib or an early GMC-1. The extra Eve-positive neuron was also Zfh1-positive (Fig. 1F). We then examined this neuron in the mutant embryo using Mab 22C10. One of the 22C10-positive neurons is RP2, with its ipsilateral axon projection (Fig. 1G). As shown in Fig. 1H,J-L, the extra neuron is indeed 22C10-positive; it first projects its axon anterior and towards the midline (Fig. 1J,K) and then outwards, often fasciculating with the ISN (Fig. 1L), along with the projection from an RP2.

Using Eve staining, we next sought to visualize the GMC and the sibling cell for this extra neuron. In the case of the bona fide RP2 lineage, we can detect a GMC-1 by Eve staining as early as 7 hours of development and an RP2 and a sib by 8 hours of development. With the extra neuronal lineage, we were able to observe a GMC-1-like cell (eGMC-1) by ~9 hours of development (Fig. 2C) and an RP2 (eRP2) and a smaller sib-like cell (esib) by ~9.5 hours (Fig. 2D). We note that an Eve-positive esib was observed in ~50% of the hemisegments in this extra lineage in *mid*^{kdf} embryos. It appears that in the remaining ~50% of the hemisegments, Eve expression only became apparent following eGMC division; because the *eve* gene is not transcribed in a sib, that esib cells are not detected in such hemisegments is not surprising.

We further examined the lineage development using Zfh1 staining. We found that the eGMC in a ~9-hour-old embryo is weakly positive for Zfh1 (Fig. 2G), and that the Zfh1 expression becomes stronger in the eRP2 (Fig. 2H) but not in its smaller esib cell (Fig. 2H). The above result led us to carefully examine Zfh1 expression in the bona fide GMC-1→RP2/sib cells. We found that the bona fide GMC-1 has no Zfh1 expression as an early GMC-1 (Fig. 2I-K), but a late GMC-1 does have low levels of Zfh1 (Fig. 2L-N). Most of the Zfh1 appeared to segregate into an RP2; however, rarely, we also observed low levels of Zfh1 in a sib that are likely to be inherited from a GMC-1 (data not shown). These results refine our knowledge of the temporal expression of Zfh1 in this lineage and, more importantly, suggest that the expression of Zfh1 in the eGMC is not unusual or is perhaps even a distinct property of that cell. Thus, the Zfh1 results indicate that the extra lineage in *mid* mutants behaves as an RP2/sib lineage (see below) and that it is formed 2-2.5 hours after the development of the bona fide RP2 lineage (Fig. 2O).

inscuteable, *numb*, *pdm1/2* and *wingless* RP2 phenotypes are epistatic to the *mid* RP2 phenotype

It has been shown that the asymmetric division of a GMC is guided by the asymmetric localization of proteins such as Numb (Uemura et al., 1989). In the RP2 and sib lineages, we and others have shown that the asymmetric division of GMC-1 involves several proteins, including Notch, Delta, Inscuteable (Insc) and Numb (Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999) (reviewed by Gaziova and Bhat, 2006). Asymmetric localization of Insc to the apical end of the cell causes a basal localization of Numb, resulting in one of the two cells inheriting Numb. This Numb prevents the reception of Notch signaling in this cell by inhibiting the proteolytic cleavage of the intracellular domain of Notch. As a result, this cell becomes an RP2. The cell that does not inherit Numb is able to respond to Notch signaling and becomes a sib.

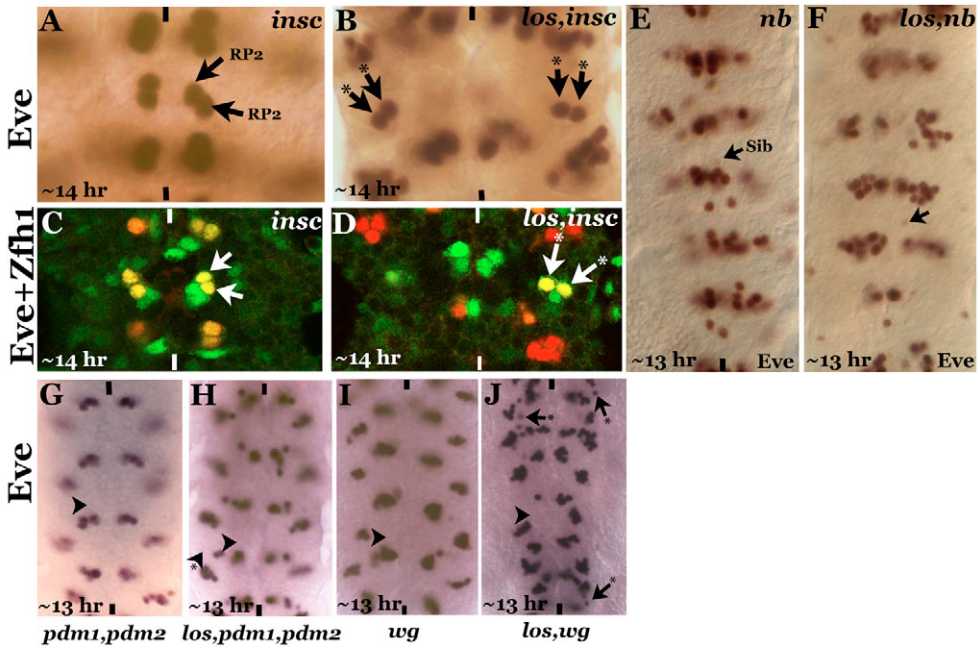

Fig. 3. Double-mutant analysis between *mid* and *insc*, *numb*, *pdm* and *wg*. *Drosophila* embryos of the indicated genotypes and ages were stained with antibodies to Eve (**A**,**B**,**E-J**) or Eve+Zfh1 (**C**,**D**). Anterior is up; vertical lines indicate the midline. Arrow, RP2; arrow with asterisk, eRP2; smaller arrow, sib; arrowhead indicates missing RP2 lineage; arrowhead with asterisk indicates missing eRP2 lineage.

Thus, in *insc* mutant embryos, the GMC-1 of the RP2/sib lineage symmetrically divides into two RP2s (Fig. 3A,C), whereas in *numb* mutants it symmetrically divides into two sibs (Fig. 3E). To obtain additional lines of evidence that the extra lineage is indeed an additional RP2/sib lineage, we generated double-mutant embryos between *insc* and *mid* as well as between *numb* and *mid*. If the extra lineage is an eRP2 lineage, our expectation is that the eGMC should yield two eRP2s in *insc*, *mid* double mutants and two esibs in *numb*, *mid* double mutants. Indeed, we observed two eRP2 neurons in *insc*, *mid* double mutants (Fig. 3B,D). In *numb*, *mid* double mutants we observed a *numb* phenotype with two esib cells (Fig. 3F). Note that *numb* is maternally deposited, and that in this *numb* mutant allele the penetrance of the defect is restricted to ~50% of the hemisegments. We observed full penetrance of the *numb* phenotype in the eRP2/sib lineage. This is likely to be a consequence of the late formation of this extra lineage: by the time this eGMC-1 is dividing, the maternally inherited Numb must have been exhausted, resulting in complete penetrance of the *numb* phenotype in the extra lineage.

We examined the identity of the extra lineage further. The two POU transcription factors, Pdm1 (also known as Nubbin) and Pdm2 (also known as Miti-mere), are expressed in the GMC-1 of the RP2/sib lineage and are known to be involved in the specification of GMC-1 identity (Bhat and Schedl, 1994; Bhat et al., 1995; Yeo et al., 1995). Thus, a mutant for both genes causes a fully penetrant loss of the Eve-positive GMC-1→RP2/sib lineage (Fig. 3G). We constructed triple mutants between *pdm1*, *pdm2* and *mid* and examined these embryos with Eve staining. As shown in Fig. 3H, the eRP2 neurons are completely missing from the triple mutant, indicating that, just as with the bona fide RP2 lineage, this extra lineage is dependent on *pdm1*/*2* for the specification of its identity.

It has been shown that Wg signaling is necessary for the specification of the RP2/sib lineage (Chu-LaGraff and Doe, 1993; Bhat, 1996; Bhat, 1998). In *wg* mutants, the Eve-positive RP2 lineage is missing owing to an NB4-2 formation and specification defect (Fig. 3I). We constructed double mutants between *wg* and *mid* and examined these embryos with Eve staining for the extra RP2 lineage. These double-mutant embryos were missing the eRP2 lineage in 75-96% of the hemisegments (Fig. 3J; n=12 embryos). It has been reported that in *wg* mutants, the formation and/or specification of row 4 and 6 NBs are affected, but row 5 NBs are normal (Chu-LaGraff and Doe, 1993). Accordingly, our *mid*, *wg* double-mutant result suggests that the eRP2 lineage originates from either a row 4 or a row 6 NB; a failure in the formation or specification of the parent NB will cause the *wg* phenotype to be epistatic to the *mid* phenotype. Alternatively, as loss-of-function of *wg* also affects row 5 NBs, the eRP2 lineage might originate from one of the NBs in row 5 (see below).

Expression of Mid in the CNS: defining the M-lineage and its transformation into an extra RP2 lineage

A fine mapping of *mid* mutations using deletions led us to a gene that encodes a Tbx transcription factor of 580 amino acids that is located in close proximity to another T-box gene, *H15*. We sequenced the gene in three different *mid* alleles and found mutations in all of these alleles in this gene (Fig. 4A). The mid^1 allele had a C→T change that produced a stop codon at amino acid position 128 (this was the strongest allele), mid^2 had a C→T change at 361, whereas in los^1 a 22 bp deletion caused a deletion of seven amino acids at position 321 and a frame shift leading to a stop codon at amino acid position 350 (thus, the protein in this allele had 28 amino acids that were different from wild type, in addition to the truncation). In the meantime, a report was published showing that this gene corresponds to *mid* (Buescher et al., 2006), although they reported a different mutation in the gene for the mid^2 allele.

Because we repeated the sequencing several times, we are confident that the mutational change in mid^2 is as reported in this study. We further confirmed the identity of the gene by performing

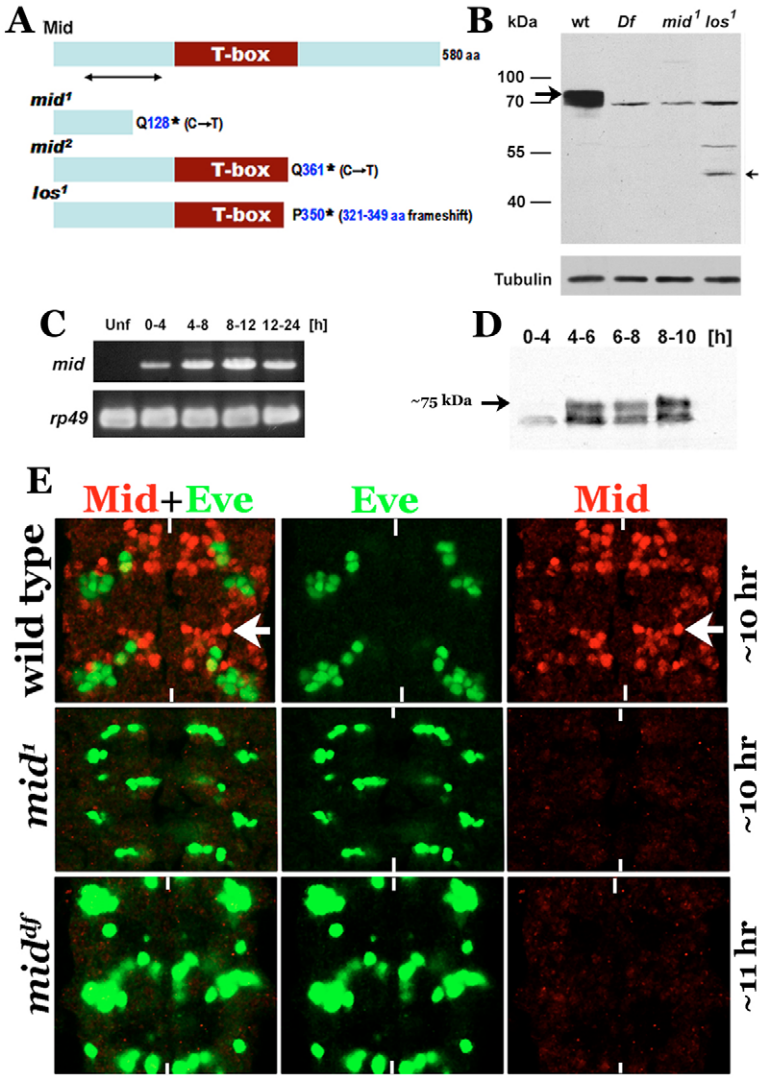

Fig. 4. Characterization of various *mid* alleles and the Mid antibody. (**A**) Mid protein in wild-type *Drosophila* and in three different *mid* alleles. The double-headed arrow indicates the region of Mid used to raise the antibody. (**B**) Western analysis of the Mid protein in the wild type and in various *mid* alleles and a *mid*, *H15* deficiency. The large arrow indicates the Mid-specific band in the wild type; the small arrow indicates the truncated Mid protein in los^1. (**C**) There is no maternal deposition of *mid* transcript to embryos. RT-PCR for *mid* transcript from unfertilized eggs (Unf) and embryos at 0-4, 4-8, 8-12 and 12-24 hours of development. *rp49* was used as control. (**D**) There is no maternal deposition of Mid protein to embryos. Western blotting of embryos at 0-4, 4-6, 6-8 and 8-10 hours of development. The Mid band (arrow) is separated from a non-specific band in this blot. (**E**) Mid antibody is specific to Mid protein. Embryos are stained with Mid and Eve antibodies. Anterior is up; vertical lines mark the midline. In the wild type, Mid is expressed in a large number of neurons. The neuron that appears to change into an extra RP2 is indicated by the arrow. In mid^1 embryos, no detectable Mid was present. This was also the case in *mid* and *H15* deficiency (mid^{df}) embryos.

western analysis (Fig. 4B) using a polyclonal antibody generated against Mid. In the wild type, we observed a band of ~75 kDa, which is larger than that expected for a protein of 580 amino acids. This band was absent in embryos that were homozygous for a deficiency that removes both *mid* and *H15*. In mid^1 we did not observe any Mid protein, whereas in los^1 and mid^2 we observed a truncated protein of expected size from the sequence data. There was no maternal deposition of *mid* RNA (Fig. 4C) or Mid protein (Fig. 4D) to developing embryos.

The specificity of the antibody was further confirmed by immunohistochemistry (Fig. 4E). In the wild type, a large number of neurons express Mid, but no detectable staining was observed in embryos homozygous for the deficiency that eliminates *mid* and *H15* or in mid^1. This also indicates that the Mid antibody does not cross-react with H15.

We next examined the expression of Mid in NBs in developing embryos (Fig. 5). We found that the expression of Mid in NBs is restricted to midline NBs (MNBs), three NBs in row 7 (NB7-1, 7-2 and 7-4) and two NBs in row 1/2 (NB 2-5 and 3-2) at ~4.5 hours of development (Fig. 5A,B). At ~6.5 hours of development, NB3-2, NB7-1 and NB7-2 became negative for Mid, but NB1-2, NB6-4 and NB6-2 in row 6 started to express Mid (Fig. 5D,E). In a 10-hour-old embryo, we observed a Mid-positive cell in the same location as the eRP2 (Fig. 5G,H) and named this cell an M-neuron (M for Mid). This cell is located in the row of cells expressing Wg (Fig. 5I) and thus the cell appears to originate in row 5. No NBs in this location expressed Mid, indicating that the parent NB of this M-GMC does not express Mid.

Since the Mid antibody detects Mid protein in los^1 embryos (Fig. 4B), we examined whether the M-neuron is the same as that which gets transformed into the eRP2 by double staining los^1 mutant embryos with Mid and Eve antibodies. We found that this is indeed the case (Fig. 5J-L). These results argue that Mid functions in this M-lineage to prevent it from developing into a second RP2/sib lineage.

We next sought to examine the development of the M-lineage in detail. First, we stained wild-type embryos with Mid and Engrailed (En) antibodies. The results indicate that the M-GMC is located adjacent to the anterior row of En-expressing cells and appears at ~7.45 hours of development (Fig. 6B); no such cell was observed in or near that location in a ~7-hour-old embryo (Fig. 6A). Thus, the GMC must have formed between 7 and 7.45 hours of development. This cell divides into a larger M-neuron and a smaller M-sib cell at ~9-9.5 hours of development (Fig. 6C-F). Interestingly, the smaller M-sib cell appears to become Mid-

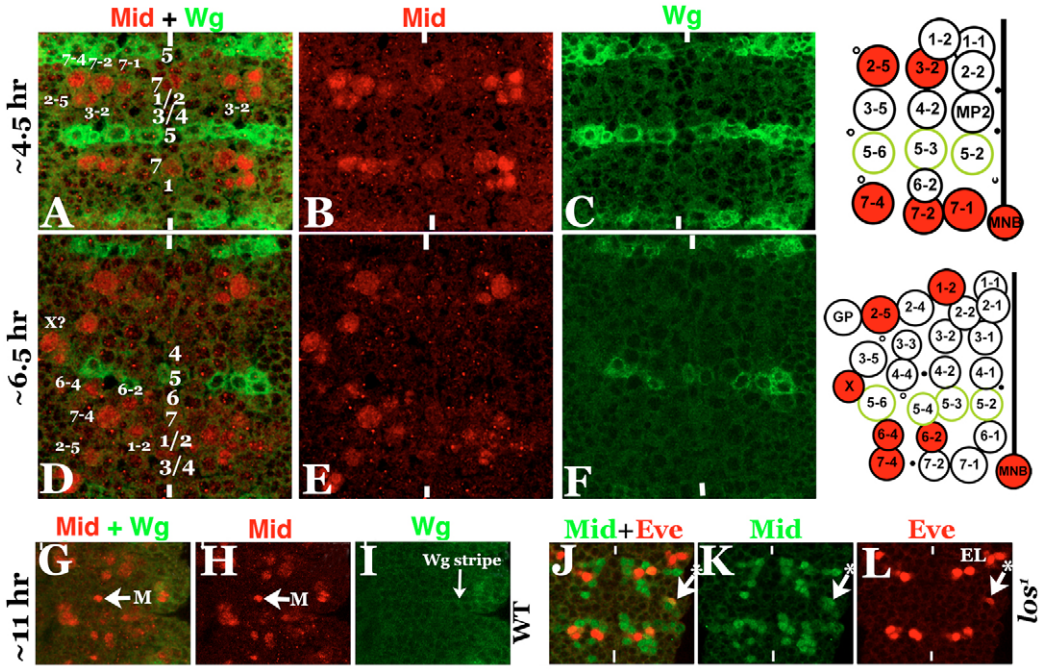

Fig. 5. Mid expression in NBs, GMCs and neurons. Wild-type (**A-I**) and *los*^{1} mutant (**J-L**) *Drosophila* embryos of the indicated ages stained with Mid (red) and Wg (green) antibodies. Anterior is up; vertical lines mark the midline. M, M-neuron; X?, this Mid-positive cell appears to be the X-cell; arrow with asterisk, the extra RP2. Rows of NBs are indicated by numbers at the midline. The schematics illustrate NB maps corresponding to A-C and D-F.

negative soon after its birth as it is no longer visible by Mid staining (Fig. 6G,H). The same conclusions were also reached from the results obtained with Mid and Eve staining (Fig. 6I-L). Note that in the wild-type embryo, the M-neuron is smaller than an RP2 (Fig. 6L). In embryos older than 15 hours, the level of Mid in the M-neuron seemed to fade (Fig. 6M). A similar pattern was also observed in at least one other (and possibly more) Mid-positive lineage in which the GMC divides to generate two asymmetric cells: the smaller cell inherited a reduced amount of Mid (Fig. 6N).

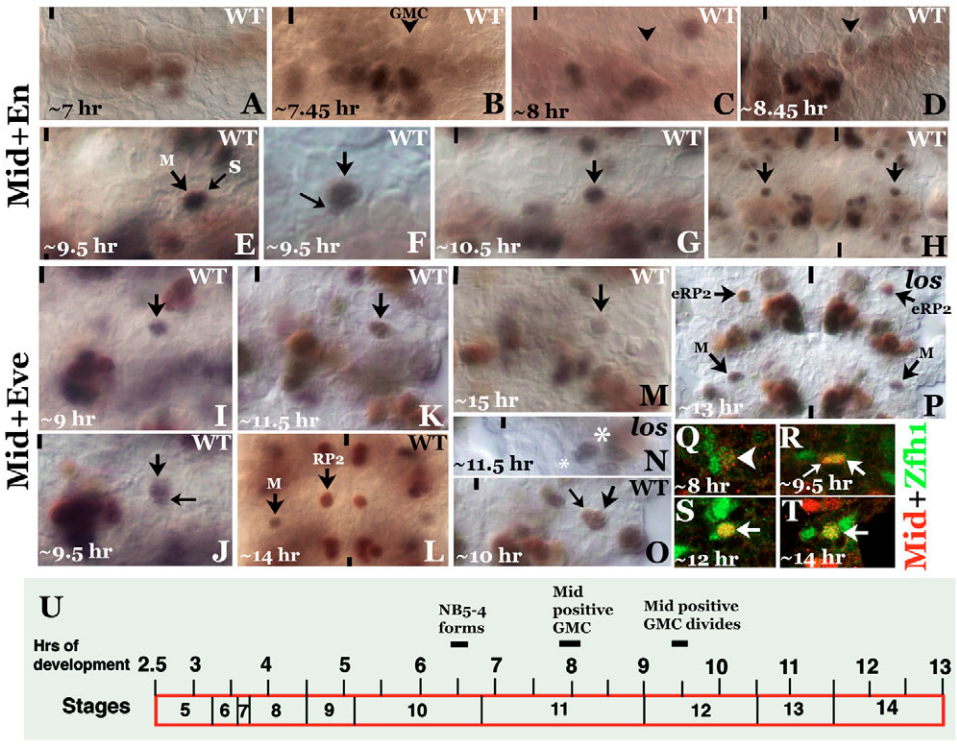

Fig. 6. Development of the M-lineage. (**A-T**) Wild-type (WT) and *los* mutant *Drosophila* embryos of the indicated ages were stained with antibodies to Mid and En (A-H), Mid and Eve (I-P), or Mid+Zfh1 (Q-T). Anterior is up; midline is marked by vertical line(s). Arrowhead, M-GMC; large arrow and M, M-neuron; small arrow and s, M-sib; white asterisks in N indicate another Mid-positive neuron and its smaller sibling cell. (**U**) The developmental timing of the M-lineage.

Table 2. Suppression of formation of the extra RP2 lineage

Genotype	Percentage of hemisegments affected in mutant embryos (*n*)
$Df^{H15,\ mid}$/UAS-mid, $Df^{H15,\ mid}$; wg-GAL4	49 (724)
UAS-mid, $Df^{H15,\ mid}$	89 (298)
los^{1}/UAS-mid, $Df^{H15,\ mid}$; wg-GAL4	13 (148)
los^{1}/UAS-mid, $Df^{H15,\ mid}$	58 (150)
en-GAL4, $Df^{H15,\ mid}$/UAS-mid, $Df^{H15,\ mid}$	85 (388)
en-GAL4, $Df^{H15,\ mid}$/$Df^{H15,\ mid}$	93 (468)
en-GAL4, los^{1}/UAS-mid, $Df^{H15,\ mid}$	62 (124)
en-GAL4, los^{1}/ $Df^{H15,\ mid}$	68 (246)
ptc-GAL4, $Df^{H15,\ mid}$/2xUAS-mid, $Df^{H15,\ mid}$	59 (210)
ptc-GAL4, $Df^{H15,\ mid}$/UAS-mid, $Df^{H15,\ mid}$	90 (134)
ptc-GAL4, $Df^{H15,\ mid}$/$Df^{H15,\ mid}$	92 (168)
sca-GAL4, $Df^{H15,\ mid}$/UAS-mid, $Df^{H15,\ mid}$	49 (80)
sca-GAL4, $Df^{H15,\ mid}$/$Df^{H15,\ mid}$	93 (152)
sca-GAL4, los^{1}/UAS-mid, $Df^{H15,\ mid}$	15 (176)
sca-GAL4, los^{1}/ $Df^{H15,\ mid}$	71 (174)
$Df^{H15,\ mid}$/UAS-mid, $Df^{H15,\ mid}$; ftz-GAL4	50 (596)
UAS-mid, $Df^{H15,\ mid}$	89 (341)
los^{1}/UAS-mid, $Df^{H15,\ mid}$; ftz-GAL4	14 (402)
los^{1}/UAS-mid, $Df^{H15,\ mid}$	60 (140)
$Df^{H15,\ mid}$/UAS-mid, $Df^{H15,\ mid}$; eve-GAL4	51 (160)
UAS-mid, $Df^{H15,\ mid}$	90 (285)
los^{1}/UAS-mid, $Df^{H15,\ mid}$; eve-GAL4	34 (212)
los^{1}/UAS-mid, $Df^{H15,\ mid}$	63 (218)

We also stained wild-type and los^{1} mutant embryos with Mid and Eve antibodies. As shown in Fig. 6O,P, we observed a larger eRP2 and a smaller esib that had both Mid and Eve. As los^{1} is a hypomorph, we observed not only hemisegments in which the M-neuron had changed into an eRP2 neuron, but also hemisegments where the M-neuron had not changed its identity (Fig. 6P). Thus, in hemisegments in which the M-neuron had changed into an eRP2, the cell was larger (akin to an RP2) and expressed both Eve and Mid, whereas in hemisegments where the M-neuron had not changed its identity, the cell was smaller and often elongated, and only expressed Mid. Additionally, we found that the M-lineage expresses Zfh1 in the M-GMC (Fig. 6Q), the newly formed M-neuron and its sib (Fig. 6R), and Zfh1 continued to be expressed in the M-neuron but not in its sib (Fig. 6S). It should be noted that when this lineage changes into an eRP2 lineage in *mid* mutants, the esib is negative for Zfh1, whereas the M-sib in the wild type is transiently positive for Zfh1.

The extra RP2 lineage in *mid* mutants originates from a row 5 NB

In order to determine from which row of NBs this GMC is generated, we performed rescue (this is also a suppression of formation of the eRP2 lineage) experiments by spatially expressing *mid* in the *mid* mutant using a *UAS-mid* transgene. We generated *UAS-mid* transgenic lines and sought to determine whether the mutant eRP2 lineage phenotype could be rescued by expressing the gene in different rows of NBs. We crossed the *UAS-mid* to the following GAL4 lines: *wg-GAL4* (in row 5), *en-GAL4* (rows 6 and 7 and one NB in row 1), *ptc-GAL4* (rows 2-5) and *sca-GAL4* (all NBs). As shown in Table 2, we found that expression of the *mid* transgene in row 5 significantly suppresses the formation of the eRP2 lineage (the M-GMC and its neuronal pairs now express Mid; data not shown). This was also the case with *ptc-GAL4*, although the suppression with *ptc-GAL4* was only observed at 26.5°C. The formation of the eRP2 lineage was also suppressed upon expression of *mid* using *sca-GAL4*. However, with *en-GAL4*, no suppression was observed (Table 2). These results argue that the extra lineage in *mid* mutants arises from a row 5 NB. Since staining of *mid* mutant embryos for Huckebein (Hkb) expression, a NB4-2 marker, did not reveal any additional Hkb-positive NBs in row 5 (or in rows 4 and 6; data not shown), it was possible that NB5-4 or NB5-5 is changed into NB4-2 in *mid* mutants; both these NBs express Hkb and therefore have the potential to change into NB4-2. Moreover, NB5-4 is formed as a S4 NB at ~6.5 hours of development and NB5-5 is formed as a S5 NB at ~7 hours of development, and the timing of their formation is consistent with a first GMC from either of these NBs changing to eGMC-1, of which NB5-4 seems the more likely by the timing (Fig. 6T; see also below).

Wingless is necessary for the proper formation and specification of row 5 cells

The suppression of the eRP2/sib lineage phenotype in *mid* mutants by the expression of *UAS-mid* using *wg-GAL4* is not consistent with our result that *los;wg* double mutants lack the eRP2 lineage in 75-96% of the hemisegments because, as previously determined, row 5 NBs are unaffected in *wg* mutants (Chu-LaGraff and Doe, 1993). The only way to reconcile the *los*, *wg* double-mutant results and the suppression data is to conclude that row 5 NBs are indeed affected in *wg* mutants. Therefore, we re-examined the formation of row 5 NBs in *wg*-null mutants by staining the mutant embryos with an antibody against Gsb (also referred to as Gsb-distal). In the wild type, Gsb is expressed in NBs from rows 5 and 6 and in NB7-1 in row 7 (Fig. 7A). In embryos mutant for *wg*, we found that row 5 NBs were indeed affected. Thus, ~70% of the hemisegments were missing one or more NBs, such as NB5-4 (which was affected the most), NB5-5 and NB5-2 (Fig. 7B). These results suggest that Wg function is necessary for the formation of some of the row 5 NBs, and it is therefore probable that the eRP2 lineage originates from a row 5 NB. Since we did observe NB5-4 (and NB5-5) in *wg* mutant embryos in ~30% of the hemisegments, the presence of the eRP2 lineage in *mid*, *wg* double mutants in some of the hemisegments is consistent with a row 5 NB, most likely NB5-4, giving rise to a GMC that transforms into eGMC-1 in the absence of Mid activity.

Fate transformation in *mid* mutants occurs at the GMC level and not at the NB level

The presence of Gsb in the precursor NE cells that give rise to NB4-2 will block Wg from specifying NB4-2 identity to a NB (Bhat, 1996) (reviewed by Bhat, 1999). The finding that the GMC that transforms into GMC-1 of the RP2/sib lineage (M-GMC) is Mid-positive, whereas none of the row 5 NBs is Mid-positive, suggests that the fate transformation is likely to be at the GMC level (Mid being a transcription factor). If this were true, row 5 NBs and the precursor NE cells should all have Gsb expression intact. As shown in Fig. 7C, all these NBs expressed Gsb. The precursor NE cells of the row 5 NBs also expressed Gsb (data not shown). These NBs also had normal Wg (Fig. 7E), Sloppy paired (Slp) and Hkb expression (data not shown).

There is a second *gsb* gene, *gsb-neuro* (*gsb-n*) (also known as *gsb-proximal*). This gene is expressed in GMCs and neurons that originate from the same parent NBs that express Gsb (Gutjahr et al., 1993). In *mid* mutants, among the cluster of Gsb-n-positive neurons, only the M-neuron was lacking Gsb-n expression (Fig. 7F-H; Eve-positive, Gsb-n-negative cell in the right hemisphere at the top), and double staining for Eve indicated that this neuron is indeed the eRP2. These results also confirm that eRP2 is generated from a row 5 NB.

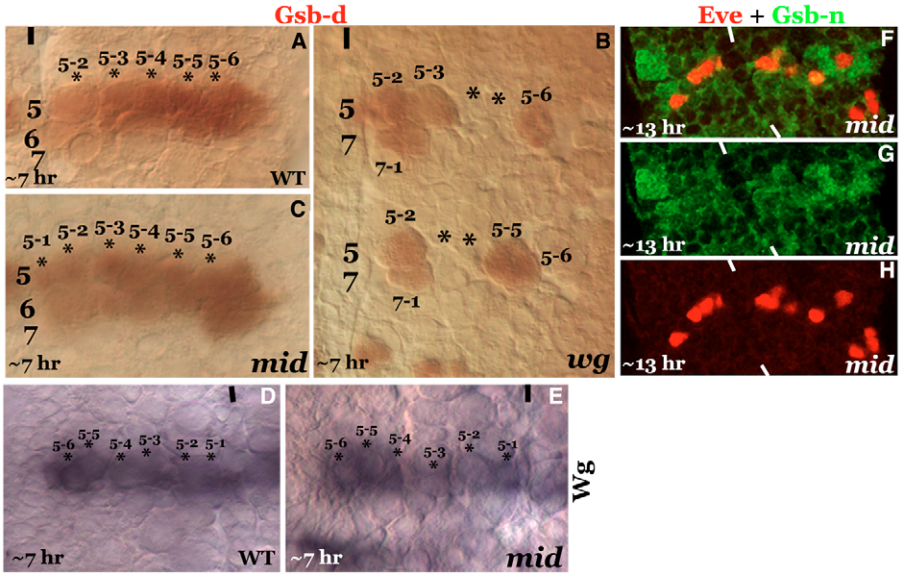

Fig. 7. Loss of Wingless activity affects the formation of row 5 NBs and expression of Gsb and Wg is unaffected in *mid* mutants but the expression of Gsb-n is lost from the eRP2. Wild-type (WT) and *mid* or *wg* mutant *Drosophila* embryos stained with antibodies to Gsb (Gsb-d) (**A-C**), Wg (**D**,**E**) or Gsb-n (**F-H**). Anterior is up; midline is marked by vertical line(s). Numbers in the midline indicate NB rows; NBs are indicated by row number followed by NB number (e.g. 5-6 = NB5-6 = NB6 on row 5).

We further tested the possibility raised above that the fate transformation occurs at the GMC level by suppression experiments. The *UAS-mid* transgene was induced with *ftz-GAL4* and *eve-GAL4*. These two GAL4 drivers are expressed in GMC-1 of the RP2 lineage. As shown in Table 2, the *UAS-mid* transgene expressed using *ftz-GAL4* and *eve-GAL4* suppressed the transformation defect, indicating that the fate transformation in *mid* mutants is at the GMC level.

Mid behaves as a weak transcriptional activator of the *gsb-n* promoter

The above result that the eRP2 in *mid* mutants lacks Gsb-n expression suggests that Mid might promote *gsb-n* transcription in this cell. This is consistent with the fact that Mid is a transcription factor. We examined the promoter of both *gsb* genes for the presence of a binding site for Tbx proteins. Although there were none in the *gsb* promoter, we found a degenerate palindromic T-box-binding element (TBE) in the *gsb-n* promoter (Fig. 8A). To determine whether this element functions as an activator of *gsb-n* transcription, we performed luciferase reporter assays using both the consensus Tbx site and the *gsb-n* promoter (Fig. 8B-D). We also used the following Tbx proteins in this assay: Mid, H15 and Optomotor-blind-related-1 (Org-1). Mid, H15 and Org-1 proteins belong to the Tbx1 subfamily; Mid and H15 show the highest homology to human TBX20, whereas Org-1 is homologous to Tbx1. Org-1 is strongly expressed during embryogenesis, where it is required for the patterning of the visceral mesoderm and imaginal discs, amongst others (Porsch et al., 1998; Lee et al., 2003). As shown in Fig. 8C, in control experiments with the consensus TBE, Org-1 activated the reporter gene strongly, whereas Mid and H15 activated transcription ~2- to 3-fold as compared with the control protein GFP. With the *gsb-n* promoter (Fig. 8D), H15 showed the highest activation: by

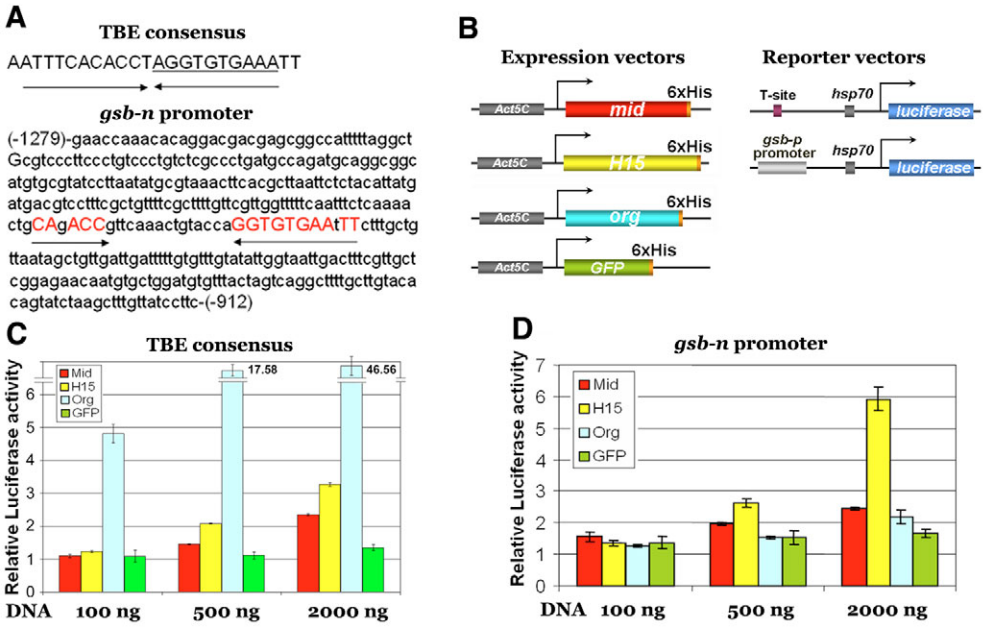

Fig. 8. Transcriptional activation by Mid, H15 and Org from the consensus T-box-binding element (TBE) and the *gsb-n* promoter. (**A**) Consensus TBE and the degenerate TBE (highlighted in red) present in the *Drosophila gsb-n* promoter. (**B**) Expression and reporter constructs used for the transcriptional activation assays. (**C**) Activation of the luciferase gene linked to the consensus TBE. (**D**) Activation of the luciferase gene linked to the *gsb-n* promoter.

~6-fold, at the highest DNA concentration tested. This was followed by Mid, which was ~1.5-fold that of the control, and then Org-1 (see Discussion).

DISCUSSION

That two cells converge to the same fate from different lineages has been well documented in *C. elegans*: except in the gut and germline, identical cells in all other tissues originate from multiple lineages. Body wall muscle cells that are almost identical morphologically and physiologically come from four different founder cells (Moerman and Fire, 1997). Similarly, identical neurons can be specified by different lineages. For instance, for bilateral neurons among the six sensory neurons involved in mechanosensation, although derived from the same founder cell, their lineages diverge four cell divisions prior to the terminal division (WormBase; www.wormbase.org). However, *Drosophila* is not driven by lineages, except for NBs, but they produce distinct progeny lineages specific to a given NB. Therefore, the above conclusion as drawn from studies in *C. elegans* was not an obvious, or expected, one in *Drosophila* and this makes our findings with *mid* significant. The fate of a cell is not specified simply by a single transcription factor, but instead by a complex combination of cell-autonomous and cell-non-autonomous genetic circuitry. Our results indicate that Mid plays a central role in this process in M-GMC, preventing it from becoming GMC-1 of the RP2/sib lineage. Absence of Mid activity initiates a cascade of events in M-GMC that ultimately transforms M-GMC into GMC-1.

A NB undergoes multiple self-renewing asymmetric divisions, each time producing a GMC of specific identity, which then generates two neurons of distinct identities. The identity of the first GMC from a NB is dependent upon the gene expression program in the NE cells from which the parent NB is delaminated, and this identity is thought to be invariant (Bhat, 1996; Bhat and Schedl, 1997; Duman-Scheel et al., 1997) (see also Chu-LaGraff and Doe, 1993) (reviewed by Bhat, 1999; Isshiki et al., 2001). Following division to generate a GMC, the gene expression program in the NB changes so that it produces a second GMC of different and distinct identity from the first GMC (Isshiki et al., 2001). Based on these and several other similar studies, it is currently believed that the identity of a GMC and its neuronal pairs is already determined in the NE and NB levels, i.e. it is ancestry-dependent. However, our results with *mid* show that this ancestry-dependent fate specification is not as stringent as once thought, and that the identity of a GMC can be altered without altering the gene expression program in the NB or NE level. Thus, a specific set of neurons (in this case RP2/sib) can be derived or specified from a GMC other than the bona fide GMC by altering the activity of a single gene, in this case *mid*. One should keep in mind that the ultimate specification of the identity of a GMC (in this case M-GMC/eGMC-1) certainly depends on a complex interplay of many gene products. Our study, however, identifies Mid as a key player in preventing M-GMC from becoming GMC-1 of the RP2 lineage. One should also point out that some GMCs, although being generated by different NBs, may have similar potentials and that there might be only one gene responsible for their differences; we believe that we have identified *mid* as one such gene.

Our results also show that duplication of the RP2/sib lineage, an extensively studied neuronal lineage, can occur by a mechanism or route that is different from those previously described. There are several ways the RP2 lineage can be duplicated. The most common way is through a second NB changing its identity into NB4-2, the parent of the RP2/sib lineage (Bhat and Schedl, 1997; Duman-Scheel et al., 1997). RP2 lineage duplication can also occur when a GMC-1 divides symmetrically to produce two GMC-1s (Yang et al., 1993; Bhat and Schedl, 1994; Bhat et al., 1995), each producing an RP2. A GMC-1 can also divide asymmetrically to self-renew and generate an RP2, and the self-renewed GMC-1 divides again to generate another (or more) RP2 or sib (Bhat and Schedl, 1994; Bhat and Apsel, 2004). A GMC-1 can also divide symmetrically to generate two RP2s (Mehta and Bhat, 2001; Bhat and Apsel, 2004). All these scenarios are different from the one we have described in *mid* mutants, in which an unrelated GMC (M-GMC) in a relatively distant location changes its identity to GMC-1 and generates a second set of RP2/sib cells at this distant site. This occurs without changing the expression of any of the genes known to be crucial for fate determination in the precursor NB or NE cells. This has not been observed before and as such adds to the novelty of our results.

A third set of our results that we think are novel comes from the fact that a second GMC-1→RP2/sib lineage can be formed 2-2.5 hours after the formation of the bona fide GMC-1→RP2/sib lineage. This type of plasticity in the timing of formation of a lineage has, to our knowledge, never been shown before for this or any other lineage in the CNS. There is a certain degree of plasticity in the timing of formation and elaboration of a lineage in the CNS between hemisegments. For example, formation of NB4-2 and its division can be delayed by ~15 minutes between hemisegments. In the case of *gsb* or *en/invected* mutants, for example, NB5-3 (which is located close to NB4-2) transforms into NB4-2, thus duplicating the RP2 lineage. NB5-3 (whether transformed into NB4-2 as in these mutants, or not) is formed ~30 minutes prior to the formation of NB4-2. Thus, the sequential production of the duplication can be delayed by as much as 45 minutes in an embryo. A similar interval in the sequential production of the RP2 lineage is also observed in embryos mutant for *lottchen* (Buescher and Chia, 1997), in which a second NB (possibly NB3-2, located adjacent to NB4-2) changes into NB4-2. Our results with *mid* indicate that an additional GMC-1→RP2/sib lineage can be formed as much as ~2 hours later than normal for this lineage, and at a site relatively distant from the original location of this lineage. This indicates considerable plasticity in terms of the developmental timing of a neuronal lineage, and that the nerve cord is capable of generating an early forming neuronal lineage also at a later point in time. Moreover, in all previous cases in which a second RP2/sib lineage was formed, it was always formed close to the bona fide RP2/sib lineage. The duplication of the RP2/sib lineage in *mid* mutants is the first case in which the second lineage is formed at an ectopic site.

These results are also interesting from another angle. A NB loses it ability, later in development, to produce earlier neurons. In other words, there is a temporally guided progressive restriction on the ability of a NB to generate earlier-born neurons. Indeed, a previous study showed that NBs indeed gradually lose competence to generate earlier-born cells (Pearson and Doe, 2003). Although it is not clear whether this is true for all lineages, our results show that at an organismal level, an earlier lineage can be generated at a later point in development. Thus, whereas the same NB, later in its life, may lose its ability to generate an earlier-born neuron, an earlier-born neuron can still be generated in the CNS at a later point in development, albeit in a different NB or GMC lineage.

Our results show that Mid plays a unique role in preventing M-GMC from becoming GMC-1, ~2 hours after the formation of the bona fide RP2 lineage. It is possible that in the wild type, during evolution a combination of gene expression patterns converged at this ~2-hour time point with the potential to push the M-GMC into GMC-1, but because a nerve cord does not need two RP2s, evolution

found a way to prevent this from occurring via expression of Mid in this M-GMC. We suspect that a similar mechanism might exist in many more lineages than just the M-lineage.

The extra neuron in *mid* is an RP2 neuron

It has been suggested previously that the extra cell is not an RP2 neuron (Buescher et al., 2006). However, this conclusion was based on the observation that this cell does not have an axon projection similar to that of RP2. Since the location of this eRP2 is at the periphery of the nerve cord, one would not expect to observe an ipsilateral projection from this neuron. We found that the growth cone from this neuron projects anterior and towards the midline (Fig. 1), where a choice point for an RP2 projection might exist. This growth cone often fasciculates with the ISN along with the projection from the bona fide RP2. We have employed a number of experiments involving different markers, mutant combinations and a very detailed and thorough analysis of this extra lineage. Our analysis reveals that it is indeed an RP2: the GMC divides into a larger and a smaller cell akin to the division of the GMC-1 into an RP2 and a sib. One of the two cells, similar to a sib, loses the expression of Eve and does not express RP2-specific markers such as Zfh1. Furthermore, in a *mid*, *insc* double-mutant embryo, the esib adopts an RP2 fate, with both cells being the same size and expressing the same RP2 markers as the bona fide RP2 lineage in *insc* mutants. Similarly, in *mid*, *numb* double mutants, both cells become sibs. The two POU genes, *pdm1* and *pdm2*, are required for the specification of GMC-1 of the RP2/sib lineage. In *mid*, *pdm1*, *pdm2* triple mutants, the eGMC-1 fails to adopt a GMC-1 identity just as the bona fide GMC-1 also fails to adopt a GMC-1 identity.

However, there are temporal differences in the gene expression pattern between the bona fide RP2/sib lineage and the eRP2/sib lineage. For example, Eve expression begins later in the eRP2 lineage in at least 50% of the hemisegments, as late as subsequent to the eGMC-1 division. Thus, we often find hemisegments with no Eve-positive esib. Since loss-of-function for Eve has no drastic effect on the RP2/sib lineage, this late expression of Eve is likely to be non-consequential to the development of the lineage.

Plasticity in the developmental timing of the GMC-1→RP2/sib lineage

The bona fide GMC-1→RP2/sib lineage originates from NB4-2, an S2 NB formed at ~4.5 hours of development (at 22°C). The GMC-1 is formed at 6-6.5 hours of development, although it becomes Eve-positive at ~7 hours of development; it then divides at ~7.45 hours into an RP2 and a sib. The cells undergo a complex migration and then settle within the anterior commissure (Bhat, 2007). An RP2 begins to project its axon growth cone at ~10 hours of development. The eGMC-1 appears to be formed at ~8 hours, becoming Eve-positive at ~9 hours of development (Fig. 6). It then divides at ~9.5 hours and begins to project its axon at ~12 hours of development. This indicates that there is significant plasticity in terms of developmental timing as far as the ability of the embryo to generate an RP2 lineage is concerned. All the requisite genetic pathways must still be operational even after 2 hours of development of the bona fide RP2/sib lineage.

Transformation of a row 5 GMC into GMC-1 and not the transformation of a NB into NB4-2 is responsible for the extra RP2 lineage

Our results indicate that the M-GMC from a row 5 NB (most likely NB5-4) is transformed into GMC-1, as opposed to a NB being transformed into NB4-2. It has previously been shown that in order to specify a NB as NB4-2, that cell should be Gsb-negative. First, our 'suppression' results indicate that the eRP2 is generated by a row 5 NB and not a row 4 or 6 NB. However, none of the NBs in row 5 is Gsb-negative in *mid* embryos (Fig. 7); row 5 NBs also had normal expression of three other markers: Wg, Slp and Hkb. This indicates that the identity of these NBs is unlikely to be affected in *mid* mutants. Second, whereas none of the NBs in row 5 expresses Mid, a row 5 GMC that generates the neuron that transforms into an RP2 in the mutant expresses Mid (Fig. 6). It is possible that Mid is expressed in the parent NB of M-GMC but at an undetectable level. However, our conclusion was based on three sets of results: (1) by RNA in situ hybridization using a *mid* probe, we did not observe any *mid*-positive NBs at this location (data not shown); (2) we generated *mid*-promoter-*lacZ* transgenic lines and the expression of *lacZ* was basically the same as expression observed with the Mid antibody; and (3) we were able to rescue/suppress the *mid* phenotype (i.e. the formation of an extra RP2 lineage) by expressing Mid in M-GMC in *mid* mutants. Finally, the timing of NB versus GMC specification is also consistent with the conclusion that the transformation occurs at the NB level. We conclude that a row 5 GMC becomes GMC-1 of the RP2/sib lineage in the absence of wild-type Mid function.

One issue that we were not able to resolve conclusively is the identity of the parent NB for the M-lineage. Our current results indicate that it is NB5-4; the first GMC of this NB gives rise to the M-lineage. Alternatively, it might be NB5-5, in which case the NB has to generate the M-GMC within 1 hour, or it could be a later-born GMC of NB5-3, although based on the position of the M-GMC this latter possibility is unlikely. We have not been able to address the ultimate fate of the M-neuron or its sibling, as to whether they are motoneurons, interneurons or some other cell type (it is unlikely to be glial as they do not express Repo, a glial cell marker), or the function of these cells.

Our results indicate that row 5 NBs are affected in *wg* mutants (Fig. 6B), not just rows 4 and 6 as was previously thought (Chu-LaGraff and Doe, 1993). In the previous work, the authors used a temperature-sensitive (ts) mutant and an allele of *wg*, wg^{CX4}. Whereas the ts mutation is likely to be a hypomorph and retains some Wg activity, wg^{CX4} is considered a null. However, we have noticed that this allele carries a background mutation(s) that suppresses the *wg* loss-of-function effect; a partial recombination did eliminate the background suppressor mutation(s) and this 'cleaned up' wg^{CX4} mutation in trans to another allele of *wg*, wg^{IG22}, did have the missing row 5 NB defect. We believe that because of the effect of *wg* mutation on row 5 NBs, the *wg* phenotype is mostly epistatic to the *mid* phenotype in *wg*, *mid* double mutants in terms of the extra RP2 lineage defect.

Mid and H15 as transcriptional activators

The T-box-binding element (TBE) was first defined as a 20-bp degenerate palindromic sequence with the highest affinity for the Brachyury protein (Kispert and Herrman, 1993). However, analysis of the actual target genes reveals that the TBE is highly variable in sequence, number and distribution within their promoters. In our experiments, with the consensus TBE only the Org-1 protein showed strong activation of the reporter gene, whereas Mid or H15 showed only an ~2-fold increase in transcriptional activation over the GFP control. However, with the *gsb-n* promoter, which contains a degenerate palindromic TBE sequence, activation by Org-1 was only slightly greater than that by the control protein. By contrast, there was a significant level of activation (~4-fold that of the control) by H15 from the same promoter element (H15 shares 62% identity with Mid), and the level of activation by Mid was ~1.5-fold that of

the control, which is slightly more than the stimulation by Org-1. That Org-1 behaves differently to Mid and H15 is consistent with the fact that Mid and H15 belong to the Tbx20 subfamily, whereas Org-1 belongs to the Tbx1 subfamily. This result also shows that although these proteins are all in the Tbx family, they diverge significantly in their sequence preference with regard to the activation of transcription. The Tbx family of proteins is also known to repress transcription (Porsch et al., 2005). Whereas the Tbx domain binds to DNA, albeit with different specificities according to variations in DNA sequence in the binding site, the rest of the protein is likely to be responsible for either activation or repression.

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