Temporal patterning of Drosophila medulla neuroblasts controls neural fates

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In the Drosophila optic lobes, the medulla processes visual information coming from inner photoreceptors R7 and R8 and from lamina neurons. It contains approximately 40,000 neurons belonging to more than 70 different types. Here we describe how precise temporal patterning of neural progenitors generates these different neural types. Five transcription factors—Homothorax, Eyeless, Sloppy paired, Dichaete and Tailless—are sequentially expressed in a temporal cascade in each of the medulla neuroblasts as they age. Loss of Eyeless, Sloppy paired or Dichaete blocks further progression of the temporal sequence. We provide evidence that this temporal sequence in neuroblasts, together with Notch–dependent binary fate choice, controls the diversification of the neuronal progeny. Although a temporal sequence of transcription factors had been identified in Drosophila embryonic neuroblasts, our work illustrates the generality of this strategy, with different sequences of transcription factors being used in different contexts.

Figure 1 | The developing medulla. a, Model of a larval brain showing that the neuropil (blue) gives rise to the lamina on the lateral (L) side and to the medulla on the medial (M) side. A wave of neurogenesis (light red) converts neuroepithelium (NE) cells (blue) into neuroblasts (NBs) (red). a, anterior; D, dorsal; P, posterior; V, ventral; VNC, ventral nerve cord. b, Surface view showing neuroepithelium (phalloidin, blue), medulla neuroblasts (Dpn, red), and lamina neurons (Elav, purple). c, Cross-sectional model showing neuroblasts (red), GMCs (green) and neurons (purple). d, Cross-sectional view showing the neuropil (DE-cadherin, blue), medulla neuroblasts (Dpn, red), medulla GMCs (Pros, green), medulla and lamina neurons (elav-ga42 > UAS-CD8::GFP, purple). In all panels, the small red arrow depicts the wave of neurogenesis.

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A temporal gene cascade in medulla neuroblasts

In the developing medulla, the wave of conversion of neuroepithelium into neuroblasts makes it possible to visualize neuroblasts at different temporal stages in one snapshot, with newly generated neuroblasts on the lateral edge and the oldest neuroblasts on the medial edge of the expanding crescent-shaped neuroblast region (Fig. 1a, b). We conducted an antibody screen for transcription factors expressed in the developing medulla and identified five transcription factors, Homothorax (Hth), Eyeless (Ey), Sloppy-paired 1 and 2 (Slp), Dichaete (D), and Tailess (Tll), that are expressed in consecutive stripes in neuroblasts of increasing ages, with Hth expressed in newly differentiated neuroblasts, and Tll in the oldest neuroblasts (Fig. 2a, b). This suggests that these transcription factors are sequentially expressed in medulla neuroblasts as they age. Neighbouring transcription factor stripes show partial overlap in neuroblasts with the exception of the D and Tll stripes, which abut each other. We and others had previously reported that Hth31 and Ey30 were expressed in medulla neuroblasts, but they had not been implicated in controlling neuroblast temporal identities. Hth and Tll also show expression in the neuroepithelium.

To address whether each neuroblast sequentially expresses the five transcription factors, we examined their expression in the neuroblast progeny (Fig. 1c, d). Hth, Ey, and Slp1 are expressed in three different layers of neurons that correlate with birth order, that is, Hth in the first-born neurons of each lineage; Ey or Slp1 in correspondingly more superficial layers, closer to the neuroblasts. This suggests that they are born sequentially in each lineage (Fig. 2c, d, j). D is expressed in two distinct populations of neurons. The more superficial population inherits D from D+ neuroblasts (Fig. 2e, above dashed line). D+ neurons in deeper layers (corresponding to the Hth and Ey layers) turn on D expression independently and will be discussed later (Fig. 2e, below dashed line). We generated single neuroblast clones and examined the expression of the transcription factors in the neuroblast and its progeny. Single neuroblast clones in which the neuroblast is at the Ey+ stage include Ey+ GMCs/neurons as well as Hth+ neurons (Fig. 2f). This indicates that Ey+ neuroblasts have transitioned through the Hth+ stage and generated Hth+ neurons. Clones in which the neuroblast is at the D+ stage contain Slp1+ GMCs and Ey+ neurons (Fig. 2g), suggesting that D+ neuroblasts have already transitioned through the Slp1+ and Ey+ stages. This supports the model that each medulla neuroblast sequentially expresses Hth, Ey, Slp1 and D as it ages, and sequentially produces neurons that inherit and maintain expression of the transcription factor.

slp1 and slp2 are two homologous genes arranged in tandem and function redundantly in embryonic and eye development22,23,35. Slp2 is expressed in the same set of medulla neuroblasts as Slp1 (Supplementary Fig. 1a). We will refer to Slp1 and Slp2 collectively as Slp.

Tll is expressed in the oldest medulla neuroblasts. The oldest Tll+ neuroblasts show nuclear localization of Prospero (Pros) (Fig. 2h), suggesting that they undergo Pros-dependent cell-cycle exit at the end of their life, as in larval nerve cord and central brain neuroblasts34. Tll+ neuroblasts and their progeny express glial cells missing (gcm) (Supplementary Fig. 1b), and the progeny gradually turn off Tll and turn on Repo (red) while migrating (along the dashed arrow) to become medulla neuroepithelial glia (arrowhead). In contrast, the Tll- neuroblasts (small arrows) lose Tll (cyan), and turn on Repo (red) while migrating (along the dashed arrow) to become medulla neuroepithelial glia (arrowhead). Schematic model. For simplicity, the overlap between transcription factors is not shown; only one neuroblast/GMC is shown for each stage. D expression in the deeper neuron population is not shown. Empty cells indicate that a subset of neurons born during the Ey, Slp or D windows do not maintain the neuroblast transcription factor. Model showing that each neuroblast sequentially expresses five transcription factors.

Figure 2 | A temporal sequence of transcription factors in medulla neuroblasts. a, b, Surface views showing that neuroblasts sequentially express Hth (red), Ey (blue), Slp1 (green) and D (red) (a), and D (red) and Tll (cyan) (b). c–f, Cross-sectional views showing the expression of the five transcription factors in neuroblasts and their progeny. c, Hth (red), Ey (blue) and Dpn (green). d, Ey (blue) and Slp1 (green). e, D (red). The dashed line separates the two populations of D+ neurons (see text). n, neurons. f, In a neuroblast clone (β-Gal; green in left, dashed circles in right), the neuroblast is Ey+ (blue, small arrow), whereas its progeny are Ey− or Hth- (red, open arrows). g, In a neuroblast clone (β-Gal; white in inset), the neuroblast is D+ (red, small arrow). It has generated Slp- (green) GMCs (arrowhead), and Ey- (blue) neurons (open arrows). h, The oldest neuroblasts (small arrows) express Tll (cyan in top), Dpn (red) and nuclear Pros (blue in bottom). i, Tll- neuroblast progeny (small arrows) lose Tll (cyan), and turn on Repo (red) while migrating (along the dashed arrow) to become medulla neuroepithelial glia (arrowhead). j, Schematic model. For simplicity, the overlap between transcription factors is not shown; only one neuroblast/GMC is shown for each stage. D expression in the deeper neuron population is not shown. Empty cells indicate that a subset of neurons born during the Ey, Slp or D windows do not maintain the neuroblast transcription factor. k, Model showing that each neuroblast sequentially expresses five transcription factors.
between tll misexpressing neuroblasts (Supplementary Fig. 2e, g–i and data not shown). Only transcription factor or repress the previous transcription factor in blasts or in large neuroblast clones is not sufficient to activate the next. However, misexpression of Hth, Ey, Slp1 or Slp2, or D in all neuroblasts is sufficient to repress D expression (Fig. 3d, e and Supplementary Fig. 2f), suggesting that neuroblasts maintain the expression of Ey and do not progress to eye expression is not expanded into oldest neuroblasts, suggesting that Slp is required to repress Ey and activate D or Tll (Fig. 3d, e and Supplementary Fig. 2f), indicating that D is required to repress ey and activate D. In clones of a deficiency mutation, slpS37A, that deletes both slp1 and slp2 (ref. 33), neuroblasts normally transit from Hth to Ey, but older neuroblasts maintain the expression of Ey and do not progress to express D or Tll (Fig. 3d, e and Supplementary Fig. 2f), suggesting that Slp is required to repress ey and activate D. Similarly, in D mutant clones, neuroblasts are also blocked at the Slp stage, and do not turn on Tll (Fig. 3f, g), indicating that D is required to repress slp and activate tll. Finally, in tll mutant clones, D expression is not expanded into oldest neuroblasts, suggesting that tll is not required for neuroblasts to turn off D (Supplementary Fig. 2j). Thus, in the medulla neuroblast temporal sequence, ey, slp and D are required for turning on the next transcription factor. slp and D are also required for turning off the preceding transcription factor (Fig. 3h). We also examined gain-of-function phenotypes of each gene. However, misexpression of Hth, Ey, Slp1 or Slp2, or D in all neuroblasts or in large neuroblast clones is not sufficient to activate the next transcription factor or repress the previous transcription factor in neuroblasts (Supplementary Fig. 2e, g–i and data not shown). Only misexpressing tll in all neuroblasts is sufficient to repress D expression (Supplementary Fig. 2k).

In summary, cross-regulation among transcription factors is required for at least some of the transitions. We did not observe cross-regulation between hth and ey. Because ey is already expressed at low levels in the neuroepithelium and in Hth neuroblasts, an as yet unidentified factor might gradually upregulate ey and repress hth to achieve the first transition. As tll is sufficient but not required to repress D expression, additional factors must act redundantly with Tll to repress D.

**Notch–dependent binary fate choice**

The temporal sequence of neuroblasts described above could specify at least four neuron types plus glia (in fact more than ten neuron types plus glia considering that neuroblasts divide several times at each stage with overlaps between neighbouring temporal transcription factors; see Discussion). As this is not sufficient to generate the 70 medulla neuron types, we asked whether another process increases diversity in the progeny neurons born from a neuroblast at a specific temporal stage. Apterous (Ap) is known to mark about half of the 70 medulla neuron types31. In the larval medulla, Ap is expressed in a salt-and-pepper manner in subsets of neurons born from all temporal stages (Fig. 4a, b). Cross-sectional view: in ey mutant clones (GFP, white in left, dashed line in middle and right), neuroblasts continue to express Ap (blue) and do not turn on Tll (cyan). g. Summary model. h. Model summarizing cross-regulations between the five transcription factors. Asterisk denotes sufficient but not required.
The intermingling of $Ap^+$ and $Ap^−$ neurons raised the possibility that asymmetric division of GMCs gives rise to one $Ap^+$ and one $Ap^−$ neuron. We generated two-cell clones to visualize the two daughters of a GMC. In every case ($n = 11$), one neuron is $Ap^+$ and the other is $Ap^−$ (Fig. 4c and Supplementary Fig. 3b), suggesting that asymmetric division of GMCs diversifies medulla neuron fates by controlling $Ap$ expression.

Asymmetric division of GMCs in *Drosophila* involves Notch (N)-dependent binary fate choice\(^{37-39}\). In the developing medulla, the N pathway is involved in the transition from neuroepithelium to neuroblast, and loss of Su(H), the transcriptional effector of N signalling, leads to faster progression of neurogenesis and neuroblast formation\(^{34}\). However, $Su(H)$ mutant neuroblasts still follow the same transcription factor sequence and generate GMCs and neuronal progeny (Supplementary Fig. 3e, f and Fig. 4f, open arrow), allowing us to analyse the effect of loss of N function on GMC progeny diversification. Notably, neurons completely lose $Ap$ expression in $Su(H)$ mutant clones. All mutant neurons born during the $Hth^+$ stage still express $Hth$, but not $Ap$, suggesting that the $N^{ON}$ daughters of $Hth^+$ GMCs are the neurons expressing both $Ap$ and $Hth$ (Supplementary Fig. 3h). In contrast to wild-type clones (Fig. 4d), all $Su(H)$ mutant neurons born during the $Ey^+$ neuroblast stage express $Ey$ and none express $Ap$ (Fig. 4e). Similarly, all mutant neurons born during the $Slp^+$ neuroblast stage express $Slp$ but lose $Ap$ (Supplementary Fig. 3g and data not shown). These data suggest that, for $Ey^+$ or $Slp^+$ GMCs, the $N^{OFF}$ daughter maintains the neuroblast transcription factor expression, whereas the $N^{ON}$ daughter loses this expression but expresses $Ap$. In the wild-type progeny born during the $D^+$ neuroblast stage, $Ap^+$ neurons co-express $D$. Both $D$ and $Ap$ are lost in $Su(H)$ mutant clones in the $D^+$ neuroblast progeny (Fig. 4f, arrow), confirming that $D$ is transmitted to the $Ap^+$ $N^{ON}$ daughter of $D^+$ GMCs. By contrast, the $D^+$ $Ap^+$ neurons in the deeper layers (corresponding to the $N^{OFF}$ progeny born during the $Ey^+$ and $Hth^+$ neuroblast stages, see above) are expanded in $Su(H)$ mutant clones at the expense of $Ap^+$ neurons (Fig. 4f, asterisk). Therefore, the deeper layer of $D$ expression is turned on independently in the $N^{OFF}$ daughters of $Hth^+$ and $Ey^+$ GMCs.

Finally, in wild type, we observe a considerable amount of apoptotic cells dispersed among neurons, suggesting that one daughter of certain GMCs undergoes apoptosis in some of the lineages (Supplementary Fig. 3i). Together these data suggest that Notch-dependent asymmetric division of GMCs further diversifies neuronal identities generated by the temporal sequence of transcription factors (Fig. 4g).

### Temporal transcription factors control neuronal fates

How does the neuroblast transcription factor temporal sequence, together with the Notch-dependent binary fate choice, control neuronal identities in the medulla? We used transcription factor markers specifically expressed in subsets of medulla neurons, but not in neuroblasts, including Brain-specific homeobox (Bsh) and Drifter (Dfr)\(^{31}\), as well as other transcription factors identified in our antibody screen, for example, Lim3 and Toy. Bsh is required and sufficient for the Mi1 cell fate\(^{40}\), and Dfr is required for the morphogenesis of nine types of medulla neurons, including Mi10, Tm3, TmY3, Tm27 and Tm27Y (ref. 31). We first investigated at which neuroblast temporal stage these neurons were born by examining co-expression with the inherited neuroblast transcription factors. We then examined whether the neuroblast transcription factors regulate expression of these markers and neuron fates. The results for each neuroblast stage are described below.

#### Hth$^+$ neuroblast stage

Bsh is expressed in a subset of $Hth^+$ neurons\(^{31}\) that also express $Ap$ (Fig. 5a), suggesting that Bsh is in the $N^{ON}$ daughter of $Hth^+$ GMCs. Indeed, Bsh expression is lost in both $Su(H)$ and $hth$ mutant clones (Fig. 5b, c). Thus, both Notch activity and Hth are required for specifying the Mi1 fate, consistent with the previous report that Hth is required for the Mi1 fate\(^{41}\). Ectopic expression of Hth in older neuroblasts is also sufficient to generate ectopic Bsh$^+$ neurons, although the phenotype becomes less pronounced in later parts of the lineage (Fig. 5d). These data suggest that Hth is necessary and sufficient to specify early born neurons, but the competence to do so in response to sustained expression of Hth decreases over time. This is similar to...
Figure 5 | Hth and Ey are required for neuronal diversity. All images are cross-sectional views of larval medulla. a, In wild type, Bsh (blue) is in neurons expressing both ap-LacZ (ap-Z; green), an enhancer trap that perfectly mimics Ap expression, and Hth (red). b, Bsh (blue), but not Hth (red), is lost in Su(H) mutant clones (GFP, green). c, Bsh (blue) is lost in hth

mutant clones (GFP, green). d, Bsh (blue) is ectopically expressed when UAS-GFP::Hth is driven by tub-gal4 in a MARCM clone (GFP, red). e, In wild type, Dfr (red) is expressed in two-three rows of Ap (green) neurons. There are also Dfr

Ey neurons in neuroblasts and the NOFF progeny of the last-born Ey subsets of neurons born from Ey neurons. The expanded Dfr

ey–null mutant clones marked by lack of GFP (green in left, dashed line in right). h, Dfr (red) neurons are expanded in slp mutant clones (GFP, green). In this region there are very few Dfr

dac–neurons. The expanded Dfr

ey–null mutant clones do not express Dac.

Ey+ neuroblast stage

Lim3 is expressed in all Ap

neurons, where ectopic Hb is only able to specify early born neurons during a specific time window. Ap

expression in the Ey progeny layer, followed by loss of Toy in the Slp and D progeny layer (Supplementary Fig. 4e).

We tested whether Ey in neuroblasts regulates Dfr expression in neurons. As expected, Dfr-expressing neurons are lost in ey-null mutant clones (Fig. 5g), suggesting that they require Ey activity in neuroblasts, even though Ey is not maintained in Ap

neurons. Furthermore, in slp mutant clones in which neuroblasts remain blocked in the Ey

state, the Ap

Dfr

neuron population is expanded into later-born neurons (Fig. 5h), suggesting that the transition from Ey

slp

in neuroblasts is required for shutting off the production of Ap

Dfr

neurons. In addition, Ap

Dfr

neurons are lost in Su(H) mutant clones (Supplementary Fig. 4b). Thus, Ey expression in neuroblasts and the Notch pathway together control the generation of Ap

Dfr

neurons.

Slp+ and D+ neuroblast stages

In addition to its expression with Ey in the N

progeny of the last-born Ey

GMCs, Toy is also expressed in Ap

(N

neurons in more superficial layers generated by Slp

and D

neuroblasts (Supplementary Fig. 4c, d and Fig. 6a, i). Consistently, in Su(H) mutant clones, we see an expansion of Toy

Ey

neurons in the Ey progeny layer, followed by loss of Toy in the Slp and D progeny layer (Supplementary Fig. 4e).

We tested whether Slp is required for the neuroblasts to switch from generating Toy

Ap

neurons, progeny of Ey

neuroblasts, to generating Toy

Ap

neurons. Indeed, in slp mutant clones, the Toy

Ap

neurons largely disappear, whereas Toy

Ap

neurons expand (Fig. 6b).

We examined Ap and Toy expression in specific adult neurons. Ortl-gal4 primarily labels Tm20 and Tm5 (C.-H. Lee, personal communication) plus a few Tm10 neurons, and these neurons express both Ap and Toy (Fig. 6c–f). To examine whether Slp is required for the specification of these neuron types, we generated

embryonic CNS neuroblasts, where ectopic Hb is only able to specify early born neurons during a specific time window.

We tested whether Ey in neuroblasts regulates Dfr expression in neurons. As expected, Dfr-expressing neurons are lost in ey-null mutant clones (Fig. 5g), suggesting that they require Ey activity in neuroblasts, even though Ey is not maintained in Ap

neurons. Furthermore, in slp mutant clones in which neuroblasts remain blocked in the Ey

state, the Ap

Dfr

neuron population is expanded into later-born neurons (Fig. 5h), suggesting that the transition from Ey

slp

in neuroblasts is required for shutting off the production of Ap

Dfr

neurons. In addition, Ap

Dfr

neurons are lost in Su(H) mutant clones (Supplementary Fig. 4b). Thus, Ey expression in neuroblasts and the Notch pathway together control the generation of Ap

Dfr

neurons.

Slp+ and D+ neuroblast stages

In addition to its expression with Ey in the N

progeny of the last-born Ey

GMCs, Toy is also expressed in Ap

(N

neurons in more
wild-type or slp mutant clones using the mosaic analysis with a repres- sible cell marker (MARCM) technique by heat-shocking for 1 h at early larval stage and analysed the number of OrtC1-gal4-marked
neurons in the adult medulla. In wild-type clones, OrtC1-gal4 marks
~100 neurons (95.1 ± 19.3 (mean ± s.d.), n = 8) per medulla (Fig. 6g
and Supplementary Fig. 4f, h). By contrast, very few neurons
(9.7 ± 11.2, n = 17) are marked by OrtC1-gal4 in slp mutant clones
(Fig. 6h and Supplementary Fig. 4g, i). Slp is unlikely to directly
regulate the Ort promoter because Slp expression is not maintained
in Ap+ Toy+ neurons. Furthermore, the expression level of OrtC1-
gal4 in lamina L3 neurons (C.-H. Lee, personal communication) is not
affected by slp mutation (Fig. 6h). These data suggest that loss of Slp
expression in neuroblasts strongly affects the generation of Tm20 and
Tm5 neurons.

In summary, our data show that the sequential expression of tran-
scription factors in medulla neuroblasts controls the birth-order-dependent
expression of different neuronal transcription factor markers, and thus
the sequential generation of different neuron types (Fig. 6i).

Discussion

Although a temporal transcription factor sequence that patterns
Drosophila nerve cord neuroblasts was reported more than a decade
to4, it was not clear whether the same or a similar transcription
factor sequence patterns neural progenitors in other contexts3. Our
identification of a novel temporal transcription factor sequence pat-
terning the Drosophila medulla suggests that temporal patterning of
neural progenitors is a common theme for generating neuronal
diversity, and that different transcription factor sequences might be
recruited in different contexts.

There are both similarities and differences between the two neuro-
blast temporal sequences. In the Hb-Kr-Pdm-Cas-Grh sequence, ectopi-
cally expressing one gene is sufficient to activate the next gene, and
repress the previous gene, but these cross-regulations are not necessary
for the transitions, with the exception of Castor7,11,12,13. In the Hb-Ey-
Slp-D-Tll sequence, removal of Ey, Slp or D does disrupt cross-regulations
necessary for temporal transitions (except the Hb-Ey transition). However,
in most cases these cross-regulations are not sufficient to ensure tem-
poral transitions, suggesting that additional timing mechanisms or
factors are required.

For simplicity, we represented the medulla neuroblasts as transiting
through five transcription factor stages, whereas in fact the number of
stages is clearly larger than five (Fig. 6i). First, neuroblasts divide more
than once while expressing a given temporal transcription factor, and
each GMC can have different sub-temporal identities. Furthermore,
there is considerable overlap between subsequent temporal neuro-
blast transcription factors: neuroblasts expressing two transcription
factors are likely to generate different neuron types from neuroblasts
expressing either one alone.

Although we are still investigating the complete lineage of medulla
neuroblasts, we show here how a novel temporal sequence of tran-
scription factors is required to generate sequentially the diverse neu-
rons that compose the medulla. The requirement for transcription
factor sequences in the medulla and in embryonic neuroblasts sug-
gests that this is a general mechanism for the generation of neuronal
diversity. Interestingly, the mammalian orthologue of Slp1, FOXG1,
acts in cortical progenitors to suppress early born cortical cell fates42.
Thus, transcription-factor-dependent temporal patterning of neural
progenitors might be a common theme in both vertebrate and inver-
tebrate systems.

METHODS SUMMARY

We screened ~200 antibodies against transcription factors from various sources
including: the polyclonal antibody collection against Drosophila segmentation
proteins43; various gifts from the Drosophila community; Developmental Studies
Hybridoma Bank; and a collection of antibodies generated by the modENCODE
project that were provided by N. Nègre and K. White. Wild-type or mutant
MARCM clones were generated by 37 °C heat-shocks at early larval stages. Wandering
third instar larvae or adults were analysed. For the generation of ey
mutant clones, we used a BAC containing the ey genomic region inserted on
chromosome 3L, recombined with FRT80B and Ubi-GFP, and crossed into an
ey11185;1 mutant background. This line was crossed with hs-Flp; FRT80B, ey11185;1
In(4)C3 and the progeny was heat-shocked for 1 h at 37 °C 3 days before dissec-
tion of wandering third instar larvae. Single neuroblast clones were generated
using AC225-gal4, which is expressed in the neuroepithelium-to-neuroblast
transition zone, driving UAS-FLP combined with act-FRT-STOP-FRT-nlumCZ
and tub-gal80Δ to provide temporal control. Two-cell clones were generated using
two methods: twin-spot MARCM10,44 or pros-gal4 (expressed in GMCs) driving
UAS-FLP with ubi-FRT-STOP-FRT-nuGFP and tub-gal80Δ.

Full Methods and any associated references are available in the online version of
the paper.

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Author Contributions C.D. planned the project and analysed the data together with X.L. and T.E., and T.E. performed the antibody screen; S.V. examined Slp2 expression; A.C. contributed to screening the modENCODE antibodies. K. White, N. Negre, D. Vasiliauskas and R. Johnston contributed to screening the modENCODE antibodies. Special thanks to C.-H. Lee for sharing unpublished information and the Ortl1-gal4 line. We thank R. Mann for suggestions and reagents; and Desplan laboratory members for discussion and support, especially R. Johnston, D. Vasiliauskas and N. Neriec for critically reading the manuscript. This work was supported by National Institutes of Health (NIH) grant R01 EY017916 to C.D.; The Robert Leet and Clara Guthrie Patterson Trust Postdoctoral Fellowship to X.L.; The Canadian Institutes of Health Research (CIHR) to T.E.; fellowships from EMBO (ALTF 680-2009) and HFSP (LT000077/2010-L) to C.D.; NIH grant GM058575 and a Career Development fellowship from the Leukemia and Lymphoma Society to R.V.

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Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.D. (cd38@nyu.edu).
METHODS
Antibodies and immunostaining. We screened ~200 antibodies against transcription factors from various sources including: the polyclonal antibody collection against *Drosophila* segmentation proteins, gifts from the fly community; Developmental Studies Hybridoma Bank (DSHB); and a collection of antibodies generated by the modENCODE project that were provided by N. Negre and K. White. The positive ones among them, and other antibodies used in this work, include rabbit anti Slp1 (1:200) and guinea pig anti Tll (1:200) (segmentation antibodies); rabbit anti-D (1:100) (modENCODE); rabbit anti-Hth (1:500) (from R. Mann), rat anti-Slp1 (1:200) and rat anti-Slp2 (1:200) (from K. Cadigan), guinea-pig anti-D (1:50) (from J. R. Namba), rabbit anti-DPN (1:500) (from Y.-N. Jan), guinea-pig anti-Dpn (1:1,000), guinea-pig anti-Lim3 (1:250) (from J. Skeath), rat anti-Ap (1:200) (from J. Thomas), guinea-pig anti-Br-h and rat anti-Dfr (from M. Sato), guinea-pig anti-Toy (1:500) (from U. Walldorf); mouse anti-Ey (1:10) (from P. Callaerts and DSHB), mouse anti-Pro (1:10), mouse anti-Repli (1:50), 24B10 (1:20), rat anti DE-cadherin (1:20), rat anti-DN-cadherin (1:50) and mouse anti-Dac (1:20) (all from DSHB); sheep anti-GFP (1:1,000, AbD Serotec), and chick anti-b-gal (1:200, Gallus Immunotech). Secondary antibodies are from Jackson or Invitrogen.

Immunostaining was done as described with a few modifications. Larval brains or adult brains were dissected in 1X PBS, and fixed in 4% formaldehyde for 30 min (larval) or 45 min (adult) on ice. Brains were incubated in primary antibody solution overnight at 4 °C, washed three times and incubated in secondary antibody solution overnight at 4 °C, washed three times and mounted in SlowFade. Images are acquired using a Leica SP5 confocal. Figures are assembled using Photoshop and Illustrator.

**Genetics and fly strains.** Canton S is used as wild-type controls. To generate M mutant MARCM clones, flies of y, w, hsFLP, UAS-CD8-GFP; ; tub-gal4, FRT2B, tub-gal80/TM6B were crossed with FRT82B, hth+/TM6B or FRT82B, hth1069+/TM6B flies (gifts from R. Mann). To generate elav mutant MARCM clones, flies of FRT19A, tub-gal80, hsFLP; UAS-LacZ/Cyo; tub-gal4/TM6B were crossed with FRT19A, exd1/FM7C flies (gift from R. Mann). The null mutant of ey; w; ey228/+ and In(4)eyC/eyC was obtained from Bloomington. To generate MARCM clone MARCM10 (see Supplementary Fig. 3). The flies of y, w, hsFLP, UAS-CD8-GFP; FRT40A, tub-gal80, tub-gal4/TM6B were crossed with FRT40A, slp5567/SM6-TM6B flies (gift from A. Tomlinson). To generate D mutant clones, flies of y, w, hsFLP, UAS-CD8-GFP; FRT40A, tub-gal80, tub-gal4/TM6B were crossed with FRT40A, slp5567/SM6-TM6B flies (gift from A. Tomlinson). To generate D mutant clones, flies of y, w, hsFLP, UAS-CD8-GFP; FRT40A, tub-gal80, tub-gal4/TM6B were crossed with FRT40A, ey228/+ and In(4)eyC/eyC flies (gift from F. Schweisguth). For these mutant clones, the progeny were heat-shocked at 37 °C at early larval stage, and dissected at wandering third instar stage or white pupa stage.

For targeted ey RNAi, Vxs-Gal4 was used to drive two UAS-ey-RNAi transgenes (UAS-ey-RNAI-P02501 from Bloomington, and UAS-ey-RNAI-kk107100 from VDRC stock centre) together with UAS-Dcr-2.

We used I407a-gal4 (an insertion into the inscutable locus), combined with tub-Gal80 to drive UAS-GFP::Hth, UAS-Ey (from Bloomington), UAS-Slp1 (from A. Tomlinson and G. Struhl), UAS-D (from J. Namba) or UAS-Tll (from M. Kurusu) in all neuroblasts, and the progeny were shifted from 18 °C to 29 °C 4 days before dissection of the wandering third instar larvae. For gain of function of Slp2, UAS-Slp2 (from M. Leptin) was crossed with y, w, hsFLP; UAS-LacZ; act-eY > gal4, and the progeny were heat-shocked for 8 min at 37 °C 3 days before dissection of the wandering third instar larvae. For gain-of-function of Slp1, flies of y, w, UAS-Slp1; FRT82B (from A. Tomlinson and G. Struhl) was crossed with y, w, hsFLP, UAS-CD8-GFP; ; tub-gal4, FRT82B, tub-gal80 /TM6B flies, and the progeny were heat-shocked for 1 h at 37 °C 3 days before dissection of the wandering third instar larvae.

To generate Ortl1-Gal4 wild-type or slp mutant MARCM clones, virgin females of y, w, hsFLP, UAS-CD8::GFP; FRT40A, tub-gal80/Cyo; Ortl1-Gal4, UAS-CD8::GFP/Tm2 (gift from C.-H. Lee) were crossed with FRT40A/Cyo or FRT40A, slp1378/Cyo males. The progeny were heat-shocked at 37 °C at early larval stage for 1 h, and the adult male progeny with the correct genotype were dissected and stained. To generate Ortl1-Gal4 flip-out clones, y, w; Ortl1-Gal4/ Cyo; Ortl1-Gal4/TM4 (gift from C.-H. Lee) were crossed with UAS-FRT-STOP- FRT-CD8-GFP, and the progeny were heat-shocked at late pupal stage, and dissected in the adult stage.

Other strains used included ap5564-gal4, ap5564-lacZ (ref. 47), yw; act-FRT-STOP-FRT-lacZ; UAS-FLP, and "UAS-Red-Stinger. UAS-FLP, ubi-FRT-STOP- FRT-NuGFP (G-TRACE)".

**Generation of ey mutant clones by BAC rescue.** A BAC that contains the ey genomic region (CH321-01A12, BacPac Resources) was inserted by PhiC31 transgenesis on chromosome 3L in attP site PIsacy+−attP-3B/YK00031. The resulting transgenic flies were tested for rescue of the ey null allele ey271. Subsequently, this ey BAC insertion was recombinated with FRT80B (P{neoFRT80B} and Ubi-GFP (P{Ubi-GFP/SM67TnmlS3L}. This chromosome arm was used to generate the strain yw, hsFlp; FRT80B; eyBAC, Ubi-GAL4/UFM6B; ey271 that served as a wild-type copy of ey on the third chromosome. To generate mitotic clones this strain was crossed to flies with genotype hsFlp; FRT80B; ey271+ In(3)eyC, ey3233 and the progeny were heat shocked for 1 h at 37 °C 3 days before dissection of the wandering third instar larvae. Clones in larvae that lacked both GFP fluorescence and staining with an anti-Ey antibody were further analysed.

**Generation of single-neuroblast clones.** Larvae of the genotype AC225-gal4 (which is expressed in the neuroepithelium-to-neuroblast transition), tub-gal80/TM6B, UAS-FLP and act-FRT-STOP-FRT-nuLacZ were grown at 18 °C, and shifted to 29 °C for 15 min to inactivate tub-gal80 only in scattered newly generated neuroblasts, and after another 3–6 days at 18 °C, the wandering third instar larvae were dissected and stained.

**Generation of two-cell clones.** Two methods were used. One is twin-spot MARCM (see Supplementary Fig. 3). The flies of elav-gal4; FRT40A, UAS-CD8::GFP, UAS-rCD2-RFP, UAS-mRNA/Cyo,y+ were crossed with hsFLP; FRT40A, UAS-rCD2-RFP, UAS-mRNA/Cyo,y+ (gifts from T. Lee), and the progeny larvae were heat-shocked at 37 °C for 8 min, and dissected 2 days later as wandering third instar larvae. The other method that was used for Fig. 4c was to treat the larvae with the genotype of preis-gal4 (that is expressed in GMCs), tub-gal80, UAS-FLP and ubi-FRT-STOP-FRT-nuGFP at 29 °C for 1 h to inactivate tub-gal80 only in scattered GMCs, and to perform the staining 2 days later on wandering third instar larvae. Only scattered GMCs flip out the STOP cassette, and transmit the GFP to the two daughters.

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