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## Differentiation of the *Drosophila* serotonergic lineage depends on the regulation of Zfh-1 by Notch and Eagle

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### Abstract

Elucidating mechanisms that differentiate motor neurons from interneurons is fundamental to understanding CNS development. Here we demonstrate that within the *Drosophila* NB 7-3/serotonergic lineage, different levels of Zfh-1 are required to specify unique properties of both motor neurons and interneurons. We present evidence that Zfh-1 is induced by Notch signaling and suppressed by the transcription factor Eagle. The antagonistic regulation of *zfh-1* by Notch and Eagle results in Zfh-1 being expressed at low levels in the NB 7-3 interneurons and at higher levels in the NB 7-3 motor neurons. Furthermore, we present evidence that the induction of Zfh-1 by Notch occurs independently from canonical Notch signaling. We present a model where the differentiation of cell fates within the NB 7-3 lineage requires both canonical and non-canonical Notch signaling. Our observations on the regulation of Zfh-1 provide a new approach for examining the function of Zfh-1 in motor neurons and larval locomotion.

### Keywords

Zfh-1; Notch; Eagle; NB7-3; Serotonin

### Introduction

Zfh-1 is a zinc-finger/homeodomain protein (Fortini et al., 1991) conserved across the animal kingdom, that functions as a transcriptional repressor by binding to E boxes (Postigo et al., 1999). In *Drosophila*, it is expressed throughout the embryonic mesoderm, mesodermal-derived structures, and in the developing CNS including most, if not all, motor neurons (Lai et al., 1991; Layden et al., 2006). Zfh-1 has been shown to be required for gonad, muscle, and cardiac tissue differentiation as well as axonal guidance (Broihier et al., 1998; Garces and Thor, 2006; Lai et al., 1993; Layden et al., 2006; Moore et al., 1998; Postigo et al., 1999; Su et al., 1999). The *C. elegans* homolog of Zfh-1, ZAG-1, is expressed both in muscle and the nervous system and is required for differentiation and axonal guidance of both motor neurons and interneurons (Clark and Chiu, 2003; Wacker et al., 2003). Vertebrates have two Zfh-1 homologs that are widely expressed and have been shown to affect development in a variety

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of tissues such as thymus, skeletal, muscle, lens and specific neural crest cells (Bassez et al., 2004; Higashi et al., 1997; Maruhashi et al., 2005; Miyoshi et al., 2006; Muraoka et al., 2000; Postigo et al., 1999; Takagi et al., 1998; Van de Putte et al., 2003; Yoshimoto et al., 2005). The mouse homologs have been shown to bind Smads and can regulate BMP signaling (Postigo, 2003). In this manuscript we investigate the role of Zfh-1 in the specification and differentiation of the *Drosophila* serotonergic lineage.

The serotonergic neurons of the *Drosophila* ventral nerve cord (VNC) arise from neuroblast 7-3 (NB 7-3) (Lundell et al., 1996). Neuroblasts are stem cells that undergo an invariant number of asymmetric cell divisions to produce several ganglion mother cells (GMCs). GMCs divide once to form a pair of neuronal or glial progeny (Hartenstein et al., 1987). There are 30 neuroblasts in each hemisegment of the VNC (Doe, 1992) that give rise to approximately 350 neurons and 30 glial cells by the end of embryogenesis (Schmid et al., 1999). Fig. 1A schematically outlines the divisions of NB 7-3 (Bossing et al., 1996; Dittrich et al., 1997; Higashijima et al., 1996; Isshiki et al., 2001; Karcavich and Doe, 2005; Lundell and Hirsh, 1998; Lundell et al., 2003; Novotny et al., 2002; Schmid et al., 1999). Three GMCs are derived from NB 7-3. GMC-1 produces two cells; GW, a motor neuron, and EW1, the more medial serotonergic neuron. GMC-2 produces EW2, the more lateral serotonergic neuron, and a sister cell that undergoes apoptosis. GMC-3 produces EW3, a neuron that synthesizes the neuropeptide corazonin and a sister cell that undergoes apoptosis. It has also been suggested that GMC-3 may not always undergo division but differentiate directly into EW3 (Karcavich and Doe, 2005). The three EW interneurons project axons across the midline to the contralateral side of the VNC and bifurcate in both the anterior and posterior directions. The GW motor neuron projects an ipsilateral axon that exits the CNS via the intersegmental nerve and innervates muscles 15–17 (Bossing et al., 1996; Dittrich et al., 1997; Higashijima et al., 1996; Schmid et al., 1999). The GW motor neuron can be distinguished from the EW interneurons by its high level of Zfh-1 immunoreactivity (Isshiki et al., 2001).

All the cells of the NB 7-3 lineage express the transcription factor Eagle (Eg). Eg is a zinc finger protein homologous to the steroid receptor family (Higashijima et al., 1996; Rothe et al., 1989). It is expressed in only four neuronal lineages including NB 7-3 (Higashijima et al., 1996) and transiently in the embryonic gonad (Rothe et al., 1989). Mutant alleles of *eg* show the correct number of NB 7-3 progeny early in development, but there is a dramatic reduction in the number of detectable serotonin cells and the few remaining neurons have abnormal axon projections (Dittrich et al., 1997; Higashijima et al., 1996; Lundell and Hirsh, 1998). This suggests that Eg does not have a role in specification of cell identity in the NB 7-3 lineage but is important in the terminal differentiation of the progeny. The target genes of Eg regulation are unknown.

Previously, we have shown that Notch signaling is responsible for inducing apoptosis in the sister cells of EW2 and EW3 (Lundell et al., 2003). During division of the GMCs, Numb, a membrane-associated inhibitor of Notch signaling (Guo et al., 1996), is asymmetrically partitioned into the EW neurons. Numb inactivates the Notch receptors in these cells and prevents apoptosis (Karcavich and Doe, 2005; Lundell et al., 2003). Therefore, Numb inhibition of Notch signaling in NB 7-3 induces an interneuron cell fate, producing either serotonin or corazonin neurons, whereas activation of Notch signaling in NB 7-3 leads to apoptosis or the GW motor neuron cell fate.

Notch signaling is an evolutionarily conserved pathway that plays a critical role in both the specification and differentiation of cells. Various pathologies including cancer have been associated with aberrant regulation of Notch signaling (reviewed in Harper et al., 2003; Weinmaster and Kopan, 2006). Numerous studies in *Drosophila* have identified a canonical Notch signaling pathway where Delta and Serrate acting as ligands, lead to the proteolytic

release of the Notch intracellular domain (N<sup>ICD</sup>). The N<sup>ICD</sup> then translocates to the nucleus and associates with the transcription factor Suppressor of Hairless (Su(H)) and other proteins to form a complex that activates gene transcription (recent reviews Bray, 2006; Le Borgne et al., 2005; Louvi and Artavanis-Tsakonas, 2006; Roegiers and Jan, 2004; Schweisguth, 2004). More recently, Notch signaling that is independent of Su(H) has been described (Fuwa et al., 2006; Hayward et al., 2005; Hori et al., 2004; Langdon et al., 2006; reviewed in Martinez Arias et al., 2002; Wilkin and Baron, 2005). The molecular mechanism of Su(H)-independent Notch signaling is uncertain and it is possible there may be more than one mechanism. It is unclear how different intracellular Notch-signaling mechanisms would be integrated in specific developmental pathways. This paper supports a model where both Su(H)-dependent and Su(H)-independent Notch signaling are required for the specification and maturation of cells within the serotonergic cell lineage of the *Drosophila* CNS.

In this manuscript we report that *Zfh-1* is necessary for development of both the GW motor neuron and the EW interneurons in the NB7-3 cell lineage and that *zfh-1* expression is regulated antagonistically by Notch and Eg. We also observe that both *Zfh-1* and Notch signaling influence the direction of axonal projection in the NB 7-3 cell lineage. Finally we use our observations on the regulation of *Zfh-1* to specifically reduce *Zfh-1* expression in motor neurons and examine its role in larval locomotion.

## RESULTS

### Eagle immunoreactivity decreases more rapidly in the GW motor neuron than in the EW interneurons

Fig. 1A outlines the different stages of NB 7-3 development (Bossing et al., 1996; Dittrich et al., 1997; Higashijima et al., 1996; Isshiki et al., 2001; Karcavich and Doe, 2005; Lundell and Hirsh, 1998; Lundell et al., 2003; Novotny et al., 2002; Schmid et al., 1999). During late stage 11, NB 7-3 delaminates from the neural ectoderm and produces three GMCs. Numb protein is asymmetrically partitioned during division of each GMC, producing three EW progeny that receive Numb and three progeny cells that do not receive Numb, the GW cell and two apoptotic cells. The early NB 7-3 progeny can be detected with either an Eg antibody (Fig. 1B), an *eg-Gal4* transgene (*eg<sup>mz360</sup>*) (Figs. 1C,D) or an *eg-lacZ* transgene (*eg<sup>289</sup>*) (Fig. 3A). Hunchback (Hb) immunoreactivity is detected only in the GMC1 progeny, EW1 and GW (Figs. 1B–E). At stage 12, all six progeny cells can be detected with Eg immunoreactivity (Fig. 1B). The sister cells of EW2 and EW3 rapidly undergo apoptosis such that they are no longer detectable by stage 15 (Fig. 1C). At stage 15, *Zfh-1* immunoreactivity is detected only in the GW motor neuron (Fig. 1C). Eg expression begins to decrease after stage 15, and it does so more rapidly in the GW cell such that by stage 16 the GW is no longer detectable with Eg antibody or the *eg-Gal4/UAS-PON-GFP* transgenes (Fig. 1D). Stable lacZ produced by the *eg-lacZ* transgene (*eg<sup>289</sup>*) can be detected in later stages (Lundell and Hirsh, 1998). By the end of stage 15 the EW cells begin to synthesize terminal products, Dopa decarboxylase (Ddc) and serotonin in EW1 and EW2, and corazonin in EW3. These markers become clearly detectable by stage 17 (Figs. 1E, 2E).

### *Zfh-1* is required for the differentiation of both the GW motor neuron and EW interneurons

To investigate the function of *Zfh-1* in the NB 7-3 lineage we examined the protein-null allele, *zfh-1<sup>2</sup>* (Lai et al., 1993), in stage 17 embryos (Fig. 2. and Table 1). In the wild-type VNC, each hemisegment has two Ddc immunoreactive cells and one corazonin immunoreactive cell (Fig. 2A). In the *zfh-1<sup>2</sup>* mutant over 84% of the hemisegments have an extra cell positive for Ddc immunoreactivity (Figs. 2C,D,F, H and Table 1). This additional Ddc cell is also immunoreactive for Hb similar to the EW1 cell (Fig. 2D and Table 1). These results suggest that a loss of *Zfh-1* in the GW motor neuron converts it to an EW1 interneuron cell fate and

that Zfh-1 is necessary for motor neuron identity in the NB 7-3 lineage. It has previously been suggested that Zfh-1 is not required for specification of cell identity in the CNS (Lai et al., 1993; Layden et al., 2006), clearly this is not the case for the GW motor neuron and there may be other undetected examples.

Despite the fact that Zfh-1 is not detectable in the EW interneurons with the Zfh-1 antibody, we were surprised to find that the *zfh-1<sup>2</sup>* allele also has an effect on terminal differentiation of the EW cells. In the *zfh-1<sup>2</sup>* mutant 88% of the detectable Ddc-positive cells, including the ectopic cell, fail to produce serotonin unlike the wild-type EW interneurons (Figs. 2E,F and Table 1). In addition, the number of cells detectable with corazonin antibody decreases 30% (Table 1). The axons of the Ddc-positive neurons, including the ectopic Ddc cell, appear to cross the midline; however, these axon bundles show defasciculation and increased varicosities relative to the wild-type axons (Figs. 2G,H). It has previously been observed that serotonin autoregulates the varicosity density of serotonin neurons (Budnik et al., 1989; Sykes and Condron, 2005). The reduction in serotonin levels induced by the *zfh-1<sup>2</sup>* mutation may be responsible for stimulating an increase in varicosity density. In summary, Zfh-1 is required for proper development of both the GW motor neuron and EW interneurons in the NB 7-3 lineage.

### Eagle suppresses *zfh-1* expression

As demonstrated in Fig. 1, Eg immunoreactivity declines more rapidly in the GW neuron. This suggests that *eg* expression might need to be suppressed in order to maintain high levels of Zfh-1. To test this possibility, we examined Zfh-1 immunoreactivity in the hypomorphic allele *eg<sup>289</sup>*. In the absence of Eg, all four cells in the NB7-3 lineage become Zfh-1 positive, including the EW interneurons (Fig. 3B). In a wild-type stage 15 VNC, the ratio of the number of Zfh-1 immunoreactive cells to the number of Eg immunoreactive cells (Zfh-1/Eg) is 23%, whereas in the *eg<sup>289</sup>* genotype at stage 15 the ratio of Zfh-1/Eg immunoreactivity is 93% (Table 2).

When a *UAS-eg* allele is over-expressed in the lineage using the *eg-Gal4* driver, 95% of the GW neurons are no longer detectable with Zfh-1 immunoreactivity (Fig. 3C, Table 2). Occasionally this results in two EW1-type neurons that are both Ddc and Hb positive (data not shown), which is identical to the *zfh-1<sup>2</sup>* mutant phenotype (Fig. 2D). The suppression effect that Eg has on the expression of *zfh-1* can be dramatically demonstrated if *eg* is mis-expressed throughout the CNS using the pan-neural driver *elav-Gal4*. Zfh-1 immunoreactivity is diminished in nearly all cells except for a few near the midline (Figs. 3D,E).

Together, these results demonstrate that Eg is both necessary and sufficient to suppress Zfh-1 expression in the NB 7-3 lineage. Presumably the preferential loss of Eg in the GW cell in a wild-type embryo (Fig. 1C) allows for the continued expression of *zfh-1*, whereas the high expression of Eg in the EW neurons reduces *zfh-1* expression to very low levels.

### Notch induces Zfh-1 expression in the NB 7-3 lineage

Since the GW neuron does not inherit Numb and has active Notch signaling, we asked whether Notch signaling could be responsible for inducing expression of Zfh-1 in the GW motor neuron. First, we investigated whether Zfh-1 immunoreactivity is detectable in the sister cells of EW2 and EW3 prior to apoptosis, since these cells also do not receive Numb. Fig. 4A shows a stage 12 embryo where all six Eg positive cells of the lineage are detected, three of the cells are immunoreactive for Zfh-1. By stage 15 the sisters of EW2 and EW3 have undergone apoptosis and only the GW shows Zfh-1 immunoreactivity (Fig. 4B). Thus, all the cells in the NB 7-3 lineage that have active Notch signaling are positive for Zfh-1 immunoreactivity.

Previously we reported that expression of a constitutively active Notch allele, *UAS-Notch<sup>ACT</sup>* (Doherty et al., 1996), within the NB 7-3 lineage, induces apoptosis of EW2 and

EW3 and produces a second cell immunoreactive for Zfh-1 (Lundell et al., 2003). This result suggests that in the presence of ectopic Notch the EW1 cell converts to a GW cell fate. Here we show that if we express both *UAS-Notch<sup>ACT</sup>* and *UAS-p35* (an apoptosis inhibitor) with an *eg-Gal4* driver, all detectable cells of the lineage are immunoreactive for Zfh-1 (Fig. 4C). The ratio of Zfh-1/Eg immunoreactivity is 95% (Table 2). Even the EW neurons that normally lack Zfh-1 are positive for Zfh-1 immunoreactivity when Notch is ectopically expressed. The *eg-Gal4/UAS-p35* control shows that the rescued apoptotic cells are Zfh-1 positive, but that the EW neurons remain Zfh-1 negative (Fig. 4D). These results demonstrate that in the NB 7-3 lineage Notch signaling is sufficient to induce *zfh-1* expression and shows a positive correlation between cells that have active Notch receptors and Zfh-1 expression.

### Canonical Notch signaling in the NB 7-3 lineage is necessary for apoptosis but not for Zfh-1 expression

To demonstrate that Notch is required for *zfh-1* expression we tested three conditions where canonical Notch signaling is inhibited: a *Dl/Ser* double mutant null allele (Fig. 4E), the ectopic expression of *numb* (Fig. 4F) and a *Su(H)* null allele (Fig. 4G). We found that for all three genotypes, 74–90% of the hemisegments at stage 15 show rescue of at least one of the apoptotic sisters of EW2 and EW3. Zfh-1 immunoreactivity is still detected in the GW neuron and is also detected in the rescued apoptotic cells. The ratio of Zfh-1/Eg immunoreactivity is approximately 40% (Table 2). Although there is variability in the number of cells rescued from apoptosis when Notch signaling is inhibited, these rescued cells are always positive for Zfh-1 immunoreactivity. A *Su(H)<sup>1</sup>:eg<sup>289</sup>* double mutant not only shows rescue of the apoptotic cells, but also shows that the expanded Zfh-1 immunoreactivity detected in the EW neurons due to *eg<sup>289</sup>*, also occurs in the absence of Su(H) (Fig. 4H, Table 2). Thus, while Notch-induced apoptosis in the NB 7-3 lineage is clearly dependent on the Notch/Delta/Numb/Su(H) canonical signaling pathway, disruptions in canonical Notch signaling do not effect the expression of *zfh-1*.

### Mis-expression of Zfh-1 and Notch prevent axonal midline crossing of the EW neurons

We next asked whether Notch signaling or Zfh-1 expression is important for the distinction between the direction of GW and EW axonal projection. The EW interneurons send axons across the midline to the contralateral side, whereas the GW motor neuron extends an axon on the ipsilateral side (Bossing et al., 1996; Dittrich et al., 1997; Higashijima et al., 1996; Schmid et al., 1999). To visualize the axons, we used *eg-Gal4* to drive a membrane bound GFP (*UAS-Pon-GFP* or *UAS-mCD8-GFP*). Fig. 5A shows the wild-type axonal projection at stage 15 of both NB 7-3 and another Eg immunoreactive lineage, NB 3-3. The three EW axons cross the midline through the posterior commissure (closed arrow), the single GW axon remains ipsilateral (arrowheads) and the axons of the NB 3-3 lineage cross the midline through the anterior commissure (open arrow). We focused our study on the midline crossing of the EW neurons since the single axon of the GW neuron is more difficult to detect.

Layden et. al. (2006) previously concluded that when Zfh-1 is ectopically expressed in the EW neurons using *UAS-zfh-1* and the *eg-Gal4* driver, many of the EW neurons abnormally extend axons laterally out of the CNS. Using the same genetic background we find a similar result that 25% of the hemisegments (n=130) show EW neurons with aberrant projections and varicosities (Fig. 5B arrow and arrowheads). Some of these axons project laterally as previously reported (arrow), but we additionally see that some axons project to the midline but are inhibited from crossing (arrowheads). Apparently ectopic Zfh-1 either represses some factor required for midline crossing or triggers a repulsive cue that prohibits midline crossing.

We obtain a more dramatic result on the projection of EW axons when *Notch<sup>ACT</sup>* and *p35* are coexpressed using the *eg-Gal4* driver. In this genetic background midline crossing of EW axons

is completely abolished (n=202). Instead, the NB 7-3 cluster makes a single thick ipsilateral projection (Fig. 5C). A control showing ectopic expression of *p35* alone shows rescue of the apoptotic cells but does not alter midline crossing of the EW neurons (Fig. 5D). In a *numb<sup>1</sup>* mutant, Notch repression is lost in the EW neurons and only the EW1 cell survives Notch-induced apoptosis (Lundell et al., 2003). In 91% of the *numb<sup>1</sup>* hemisegments (n = 69) the EW1 axons fail to cross the midline. Often the axons seem to wander aimlessly (Fig. 5E arrowhead). These results suggest that Notch signaling must be repressed by Numb for midline crossing of the EW neurons and that active Notch signaling in the GW neurons may direct ipsilateral projection of the axon. In summary, both Notch and Zfh-1 can influence axon projections in the NB 7-3 lineage.

### Ectopic expression of Eagle in motor neurons decreases OK6 gene expression and larval locomotion

Although Zfh-1 has been shown to be expressed in most motor neurons its role in locomotor activity has not been tested directly because all *zfh-1* mutant alleles, including *zfh-1<sup>2</sup>*, are embryonic lethal. Having established that ectopic expression of Eg can suppress Zfh-1 expression (Fig. 3), we asked whether we could use Eg suppression to reduce Zfh-1 levels specifically in motor neurons, thus avoiding mesodermal defects and maintaining viability. To test this we mis-expressed Eg with the *OK6-Gal4* enhancer trap that has been shown to express in most identifiable motor neurons (Aberle et al., 2002; Sanyal et al., 2003). When the *OK6-Gal4* is used to drive expression of *UAS-GAP-GFP* a large number of cell bodies and axons are detected in a wild-type stage 17 embryonic VNC (Fig. 6A). In the *zfh-1<sup>2</sup>* mutant this same GFP fluorescence is dramatically reduced (Fig. 6B), suggesting that the loss of Zfh-1 substantially alters development of these cells so that they are no longer capable of expressing the motor neuron marker OK6. This could be due either to an intrinsic alteration of cell fate or due to alterations in axonal projections that prevent the cells from responding to extrinsic signals responsible for OK6 induction. When *UAS-eg* is mis-expressed in this same set of cells with the *OK6-Gal4* driver the level of fluorescence is intermediate between wild-type and the *zfh-1<sup>2</sup>* mutant (Fig. 6C). This effect of ectopic Eg is presumably due to its ability to suppress *zfh-1* (Fig. 3).

The similarity of *zfh-1<sup>2</sup>* and *UASeg/OK6-gal4* phenotype is also observed with FasII immunoreactivity, which detects three interneuronal fascicles in the longitudinal connectives and lateral projecting motor neuron axons (Fig. 6D). In both the *zfh-1<sup>2</sup>* mutant (Fig. 6E) and the *UAS-eg/OK6-Gal4* line (Fig. 6F) the lateral projecting motor neuron axon bundles are disorganized and not always detectable. The interneuronal longitudinal axonal bundles are also disorganized and show defasciculation. Some axons leave their appropriate tract and cross to a neighboring tract (Fig. 6E,F arrowheads). The defasciculation of the longitudinal bundles is similar to that what we previously observed for the EW neurons (Fig. 2H). Thus the loss of Zfh-1 and ectopic expression of Eg with *OK6-Gal4* have similar detrimental effects on motor neuron development and fasciculation throughout the VNC.

Whereas the *zfh-1<sup>2</sup>* mutation is an embryonic lethal the ectopic expression of *eg* with *OK6-Gal4* produces viable larvae (77% survival). Compared to wild-type the *UAS-eg/OK6-Gal4* larval VNC shows a significant reduction in both Zfh-1 immunoreactivity (Figs. 7A,B) and OK6-Gal4/UAS-GAP-GFP fluorescence (Figs. 7C,D). When these same larvae are tested in a standard locomotor crawling assay, the activity of the *UAS-eg/OK6-Gal4* larvae is reduced by 50% compared to the control larvae (Fig. 7E). The ectopic expression of *eg* appears to immobilize the posterior end of the larvae. Whereas wild-type larvae will roam over the entire surface of the Petri plate, the locomotor activity in the ectopic Eg larvae is restricted to about 25% of the test plate area. This loss in locomotor activity is consistent with a disruption of the motor system, although the exact nature of the motor neuron defect induced by ectopic *eg*

expression will require further investigation. Using Eg to suppress expression of Zfh-1 in specific subsets of cells will allow further examination of the role of Zfh-1 in motor neuron development without the contributing mesodermal defects that exist in *zfh-1* mutant embryos.

## Discussion

### Eagle/Notch regulation of *zfh-1* expression in NB 7-3 lineage

Our data shows that in the NB 7-3 lineage, *zfh-1* is induced by Notch and repressed by Eg. Zfh-1 is expressed in all cells where Notch signaling is active (GW and apoptotic cells, Fig. 4A) and ectopic expression of Notch can induce ectopic Zfh-1 in the EW neurons (Fig. 4C). In addition we have previously shown that a loss of Notch signaling with a *sanpodo* mutant can lead to a conversion of the GW neuron to an EW1 neuron (Lundell et al., 2003) similar to the Zfh-1 mutation presented in Fig. 2. However, we have also shown that three different mutations (*Dl<sup>RevF10</sup>Ser<sup>RX82</sup>*, *UAS-numb* and *Su(H)<sup>1</sup>*) in the canonical Notch signaling pathway do not reduce Zfh-1 expression (Figs. 4E–G), suggesting Zfh-1 expression is independent of canonical Notch signaling. We propose that an alternative non-canonical Notch pathway can induce *zfh-1* expression. This would explain how Notch can be sufficient to induce *zfh-1* expression (Fig. 4C), but work independently of Delta, Numb and Su(H) (Figs. 4E–H). Furthermore, since canonical Notch signaling is repressed in the EW neurons by Numb, a non-canonical pathway would explain how *zfh-1* expression could be induced in the EW neurons, which we have shown becomes detectable in the *eg<sup>289</sup>* mutant (Fig. 3B) and is necessary for EW maturation (Fig. 2). An alternative explanation of our results would be that all three mutations we have used maintain some residual level of canonical Notch signaling which is sufficient to induce *zfh-1*, but insufficient to induce apoptosis. Although a formal possibility we think this unlikely to be the case, since it's doubtful that all three of our independent analyses would have suffered from leaky Notch signaling. In addition, if the distinguish between apoptosis and *zfh-1* expression were solely dependent on levels of canonical Notch signaling one would expect variability in these mutants where some of the cells would have low enough Notch levels to both rescue cells from apoptosis and inhibit *zfh-1* expression, but all rescued cells are consistently Zfh-1 positive. Therefore our results favor an interpretation where alternative mechanisms of Notch signaling induce *zfh-1* expression and apoptosis.

We have shown that Eg efficiently suppress *zfh-1* expression in NB 7-3 lineage (Figs. 3B,C) and other Zfh-1 expressing neurons when ectopically expressed (Fig. 3E). In a wild-type fly, the preferential loss of Eg in the GW by stage 16 (Fig. 1D) insures that detectable Zfh-1 is limited just to the GW motor neuron. Although the regulation of *eg* expression is unknown, we have previously shown that Eg can negatively regulate its own expression in the GW neuron (Lundell and Hirsh, 1998). It is also possible that *eg* expression might be reciprocally suppressed by Zfh-1. Since detectable Eg expression is limited to only four neuroblast lineages in the wild-type CNS, it may be necessary to globally restricted Eg expression throughout the CNS to allow for *zfh-1* expression that is found in many lineages. In summary, the levels of *zfh-1* expression in the NB 7-3 lineage are carefully regulated by both Notch and Eg.

The role of Notch in the NB 7-3 lineage is similar to asymmetric divisions of mesodermal progenitor cells in the heart where the cells that have active Notch signaling, also have Zfh-1 expression, and the Zfh-1 expression appears to be independent of the Numb/Notch pathway (Su et al., 1999). On the other hand, these results are fundamentally different from the aCC/pCC and RP2/RP2sib asymmetric neuronal lineages where Zfh-1 is expressed in the cell where Notch signaling is inactivated by Numb. In these lineages mutations in the canonical Notch signaling pathway lead to a de-repression and expansion of Zfh-1 expression (Skeath and Doe, 1998). Our results present a possible explanation for these lineage differences in that the variation could be due to multiple Notch mechanisms that induce *zfh-1* expression and/or

various cell specific regulators of *zfh-1* expression, similar to the function of Eg presented in this manuscript.

### The role of Zfh-1 in motor neuron development

Zfh-1 has previously been shown to be expressed in most if not all motor neurons and has been implicated in the differentiation of motor neurons (Garces and Thor, 2006; Lai et al., 1991; Layden et al., 2006). Here we show that the GW motor neuron of the NB 7-3 lineage converts to an EW1 interneuron fate in the absence of Zfh-1 (Fig. 2D). Although a *zfh-1* mutation has been shown to disrupt cell fates in mesodermal tissues (Broihier et al., 1998; Lai et al., 1993; Su et al., 1999), this is the first report of a neuronal conversion in cell fate.

When we examined the motor system in the *zfh-1<sup>2</sup>* null mutant more globally, we found that the motor neuron marker OK6 is completely disrupted and that both longitudinal and lateral FasII axon bundles show disorganization and defasciculation (Fig. 6). Layden et al. recently reported that a loss of function in *zfh-1* prevents some motor axons from exiting the *Drosophila* (Layden et al., 2006). A mutation in *zag-1*, the *C. elegans zfh-1* homolog, also shows errors in axon guidance, fasciculation and branching of both motor neurons and interneurons (Clark and Chiu, 2003; Wacker et al., 2003). Finally we have shown that ectopic Eg can suppress both *zfh-1* and OK-6 expression throughout the CNS (Figs. 3E,7B,7D) and produce larvae with obvious locomotor defects (Fig. 7E). All of these results support the hypothesis that Zfh-1 has a critical role in motor neuron differentiation.

### The role of Zfh-1 in interneuron development

We were surprised to find that the *zfh-1<sup>2</sup>* mutant allele also affects maturation of the EW interneurons. The Zfh-1 antibody does not detect protein in the EW interneurons (Fig 1. C), except when Eg suppression is removed (Fig. 3B). These results suggest that, in a wild-type fly, Zfh-1 is expressed in the EW neurons at low undetectable levels.

The *zfh-1<sup>2</sup>* allele disrupts expression of the terminal products, serotonin and corazonin and also causes defasciculation and ectopic branching of the EW axons (Fig. 2). In *C. elegans*, a mutation in *zag-1* disrupts the development of the HSN serotonergic neurons (Clark and Chiu, 2003). The HSN neurons show aberrant axon projections, branch extensively and downregulate tryptophan hydroxylase the rate limiting enzyme in serotonin biosynthesis. Ectopic expression of *zfh-1* leads to aberrant projections of the EW axons (Fig. 5B and Layden et al., 2006). We have previously reported a similar mutant phenotype for *eg<sup>289</sup>* (Lundell and Hirsh, 1998), which we have shown leads to ectopic *zfh-1* expression (Fig. 3B). Therefore although Zfh-1 is required for fasciculation and maturation of the EW interneurons, the level of Zfh-1 in these cells must be carefully maintained by Eg suppression to prevent detrimental effects that can be induced by excessive Zfh-1.

### The role of Zfh-1 and Notch in axonal guidance of NB 7-3 progeny

Because the GW motor neuron and the EW interneurons have very different axon projections we asked whether Zfh-1 expression or Notch signaling might be responsible for this variation. We found dramatic ipsilateral rerouting of EW axons when *Notch* is overexpressed (Fig. 5C). Notch appears to have a fundamental role in differentiating between ipsilateral and contralateral projections. Presumably the unrestricted canonical Notch signaling in the GW neuron would direct an ipsilateral projection, whereas Numb blocks this function in the EW neurons allowing contralateral projections. Notch signaling has been reported to direct axonal guidance in other lineages of *Drosophila* (Crownier et al., 2003; Giniger, 1998; Giniger et al., 1993; Skeath and Doe, 1998)



We observe that ectopic Zfh-1 can misdirect EW axons away from the midline (Fig. 5B), as previously reported (Layden et al., 2006). However, we also found that axons that project toward the midline can be abruptly repelled when expressing ectopic Zfh-1 (Fig. 5B). This suggests that ectopic Zfh-1 might make the cells more sensitive to a midline repulsive cue, such as Slit or Netrin. This could occur by either inducing the expression of a member of the Roundabout (Robo) family, the Slit receptor (reviewed in Couch and Condron, 2002; Dickson, 2002) or UNC5 a repulsive Netrin receptor (Keleman and Dickson, 2001). Alternatively Zfh-1 might downregulate Commissureless (Comm), an intracellular protein that induces Robo degradation. Robo, Comm and Slit have been shown to be involved in the midline crossing of the EW neurons (Couch et al., 2004). We have previously observed that *eg<sup>289</sup>* can also prevent midline crossing of the EW interneurons (Lundell and Hirsh, 1998).

Therefore, ectopic expression of both Notch and Zfh-1 can prevent midline crossing in the NB 7-3 lineage. This may not be surprising since we have shown that Notch can induce *zfh-1* expression. The observation that ectopic Notch signaling produces a more severe phenotype than ectopic Zfh-1 suggests that the role of Notch in guidance of NB 7-3 axons is probably more complex than simply inducing *zfh-1* expression. The specific epistatic relationship of Notch and Zfh-1 with respect to the growth of the GW axon remains to be determined.

### The role of canonical and non-canonical Notch signaling in NB 7-3 lineage

Our results suggest a model where both canonical and non-canonical Notch signaling have a role in development of the NB 7-3 lineage (Fig. 8). We propose that canonical/Su(H)-dependent Notch signaling is required for inducing apoptosis and ipsilateral axonal guidance, whereas non-canonical/Su(H)-independent Notch signaling is responsible for inducing *zfh-1* expression.

In EW interneurons, Su(H)-dependent Notch signaling is blocked by the asymmetric distribution of Numb; this prevents both apoptosis (Karcavich and Doe, 2005; Lundell et al., 2003) and ipsilateral axonal projection (Fig. 5E). Zfh-1 is induced in the EW interneurons by Su(H)-independent Notch signaling but Eg counteracts this pathway keeping Zfh-1 at very low (undetectable) levels (Fig. 3B). The low level of Zfh-1 maintained in the EW interneurons is critical for axon fasciculation and terminal differentiation (Figs. 2F,H).

The GW motor neuron not only has Su(H)-dependent Notch signaling, but the preferential loss of Eg expression in GW (Fig. 1D) might also activate the Su(H)-independent Notch signaling pathway. Both of these pathways could contribute to the high levels of Zfh-1 unique to the GW neuron. The high concentration of Zfh-1 specifies the GW motor neuron cell fate (Fig. 2C) and contributes to determining the ipsilateral projection of its axon (Fig. 5B and Layden et al., 2006).

Apoptosis within the NB 7-3 lineage is clearly driven by canonical Notch signaling since a *numb* mutant induces cell death (Karcavich and Doe, 2005; Lundell et al., 2003) and *DT<sup>-</sup>*, *Su(H)<sup>-</sup>* or *UAS-numb* alleles are all capable of rescuing the cells from cell death (Fig. 4E-H). Despite the fact that the apoptotic cells express Zfh-1 (Fig. 4A), apoptosis is not dependent on Zfh-1 since these cells still die in *zfh-1<sup>2</sup>* mutant flies (Fig. 2C).

Thus, our model suggests that canonical and non-canonical Notch signaling have specific functions within the NB 7-3 lineage. It remains to be determined how Su(H)-independent Notch signaling activates Zfh-1 expression, how Eg can block this process and how the intracellular components of two different Notch signaling mechanisms are linked or resolved in the GW motor neuron. Furthermore, Wingless (*wg*) has been proposed as a ligand for Notch that can antagonize Su(H)-independent Notch signaling (reviewed in Martinez Arias et al., 2002). A *wg* mutation result in a loss of detectable NB 7-3 progeny (Deshpande et al., 2001).

Examination of the relationship of Notch and Wnt signaling in the NB 7-3 lineage should provide further insight into the crosstalk between these pathways.

## EXPERIMENTAL METHODS

### Drosophila Stocks

The following fly lines were used in this work: *eg-lacZ* (*eg*<sup>289</sup>), *eg-Gal4* (*eg*<sup>mz360</sup>), *UAS-eg* (from G. Technau); *UAS-Pon-GFP*, *numb*<sup>1</sup> (from C. Doe); *UAS-N<sup>ACT</sup>* (Notch with a deleted extracellular domain but intact transmembrane and intracellular domains), *UAS-numb* (from Y. N. Jan); *OK6-Gal4* (from B. Zhang); *zfh-1*<sup>2</sup> (a protein null allele from Z.C. Lai); *Dl<sup>RevF10</sup>Ser<sup>RX82</sup>* (a lethal null allele, where both mutations are deletions), *Su(H)*<sup>1</sup> (a lethal null allele, where the molecular lesion is unknown), *Elav-Gal4*, *UAS-GFP*, *UAS-zfh-1*, *UAS-GAP-GFP*, *UAS-p35*, *UAS-mCD8-GFP* and Canton S (from the Bloomington stock center).

### Immunohistochemistry

Embryonic and larval CNS were dissected, fixed in 4% paraformaldehyde and subsequently incubated with primary and secondary antisera as previously described (Lundell and Hirsh, 1994). Primary antibodies were: rabbit anti-Eg (1:2000, G. Technau), guinea pig anti-Hb (1:600, East Asian Distribution Center for Segmentation Antibodies), rabbit anti-Ddc (1:50, M. Lundell), rat anti-Ddc (1:200, M. Lundell), rabbit anti-Crz (1:200, C. Doe), rabbit anti-Zfh-1 (1:400, R. Lehman), guinea pig anti-Zfh-1 (1:450, Z. C. Lai), rat anti-5-HT (1:100, Accurate Chemical), mouse anti-FasII monoclonal 1D4 (1:5, Developmental Studies Hybridoma Bank) and rat anti-lacZ (1:5000, C. Doe). All Texas Red, FITC and Cy5 secondary antibodies were from Jackson Laboratories and used at 1/200. Images were obtained using a BioRad 1024 laser-scanning microscope and edited with Adobe Photoshop 7.0.

### Larval Locomotor Assay

Larvae were subjected to a 12-hour light/dark cycle. The assay was done two hours before the light-to-dark transition time. Third-instar larvae were randomly sampled and placed on a Petri dish (8.5 × 1.4 cm) that was coated with 10 ml of nonnutritive 0.8% agar. After two minutes to allow for acclimatization, larval locomotion was tested for three minutes. To measure the trail distance, the Petri dish was placed on top of a light box that had a marked grid (5×5mm). The number of grid squares the larva crossed in three minutes was counted. At least 20 larvae of each genotype were tested.

### Statistical Analysis

Statistical analysis was performed using SAS software (SAS Institute, Inc.). The larval locomotor assay was analyzed using analysis of variance (ANOVA) followed by Tukey's Studentized Range Test to compare larval activity means in four genotypes: Canton S, *OK6-Gal4*, *UAS-eg* and *OK6-Gal4/UAS-eg*.

### Acknowledgments

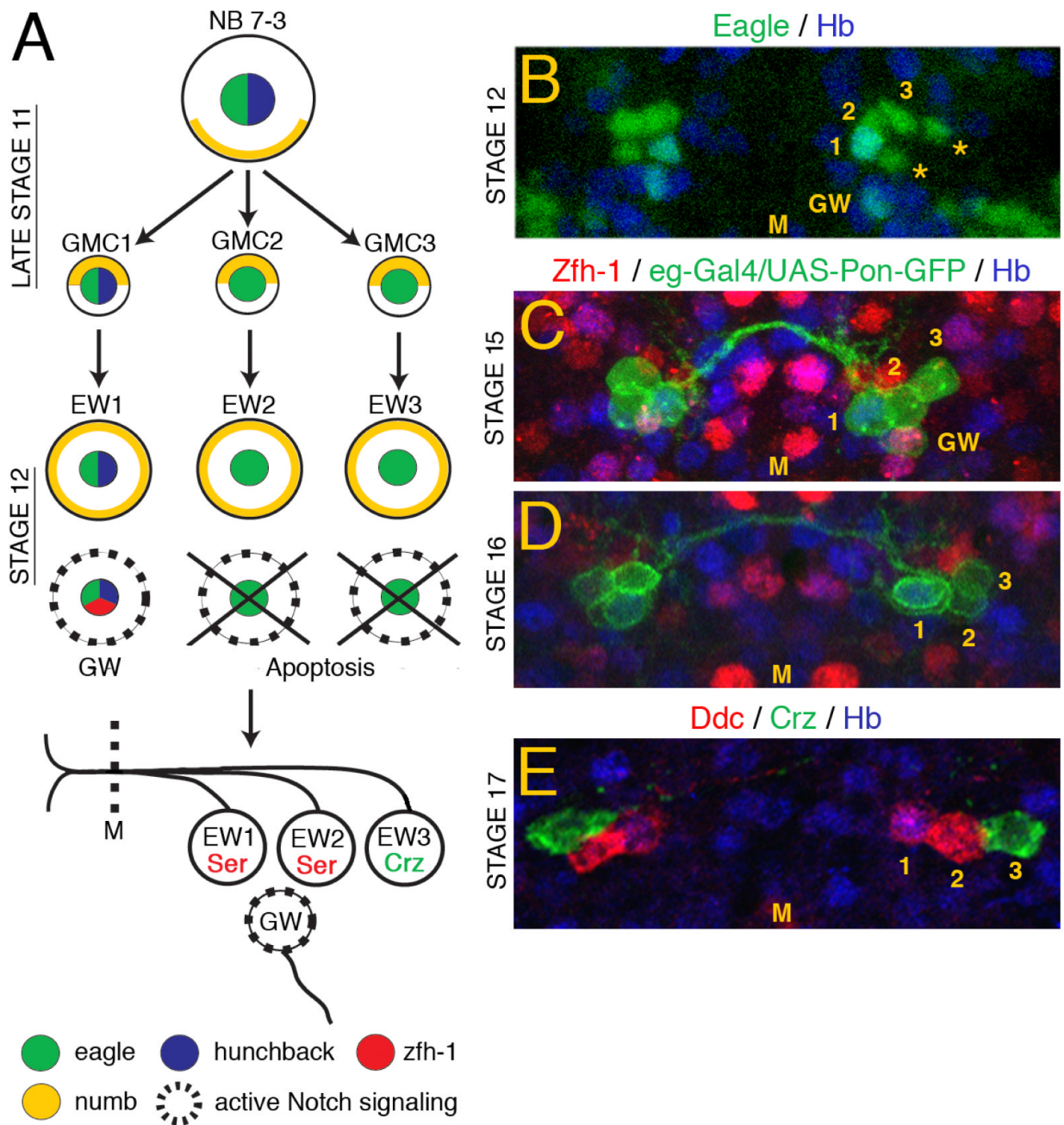
This work was supported by a NIH/MBRS/SCORE grant GM 08194 to M.J.L. We thank C. Doe, Y. N. Jan, Z.C. Lai, G. Technau, B. Zhang and the Bloomington stock center for provision of fly stocks. We thank C. Doe, R. Lehman, Z.C. Lai, G. Technau, the Iowa Hybridoma Bank and East Asian Distribution Center for provision of antibodies. We thank K.W. Lee for assistance with the behavioral assay. We thank E. Wheeler and P. Mueller for their comments on the manuscript.

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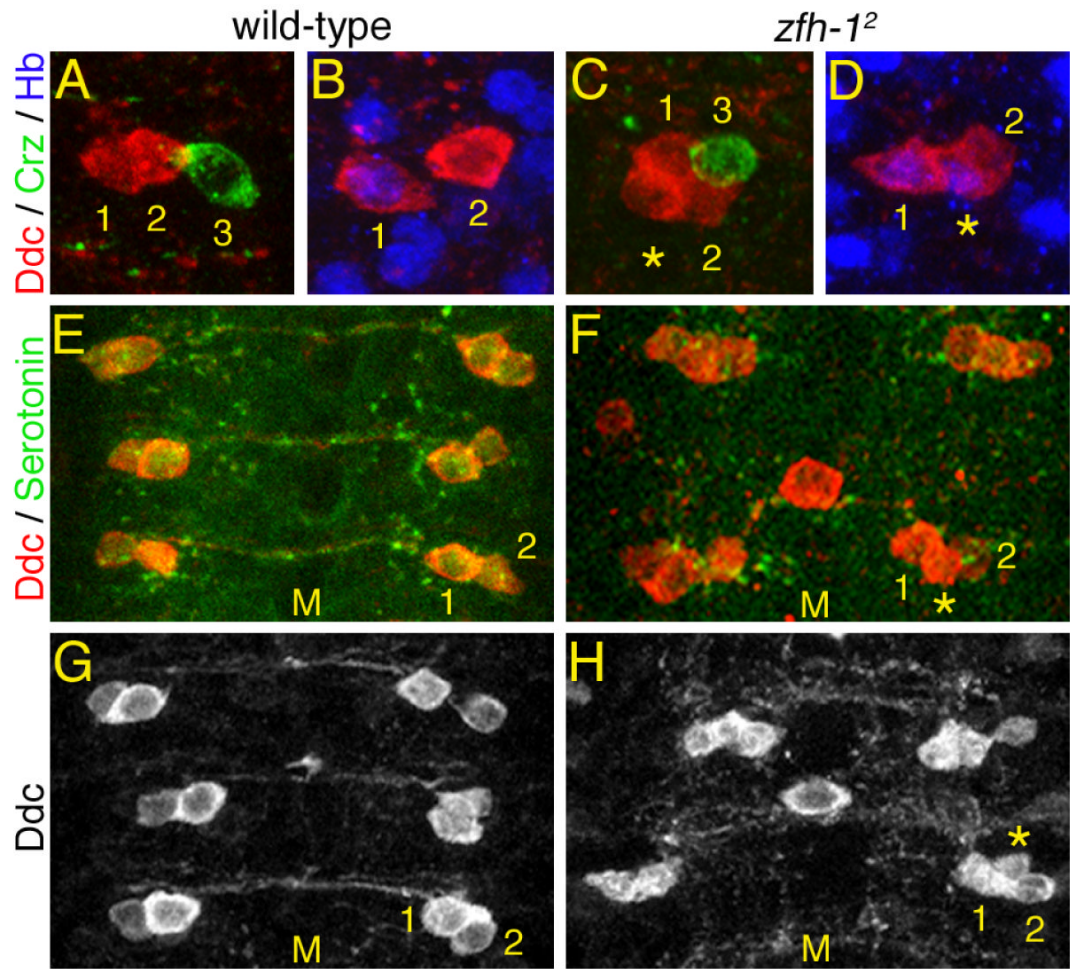
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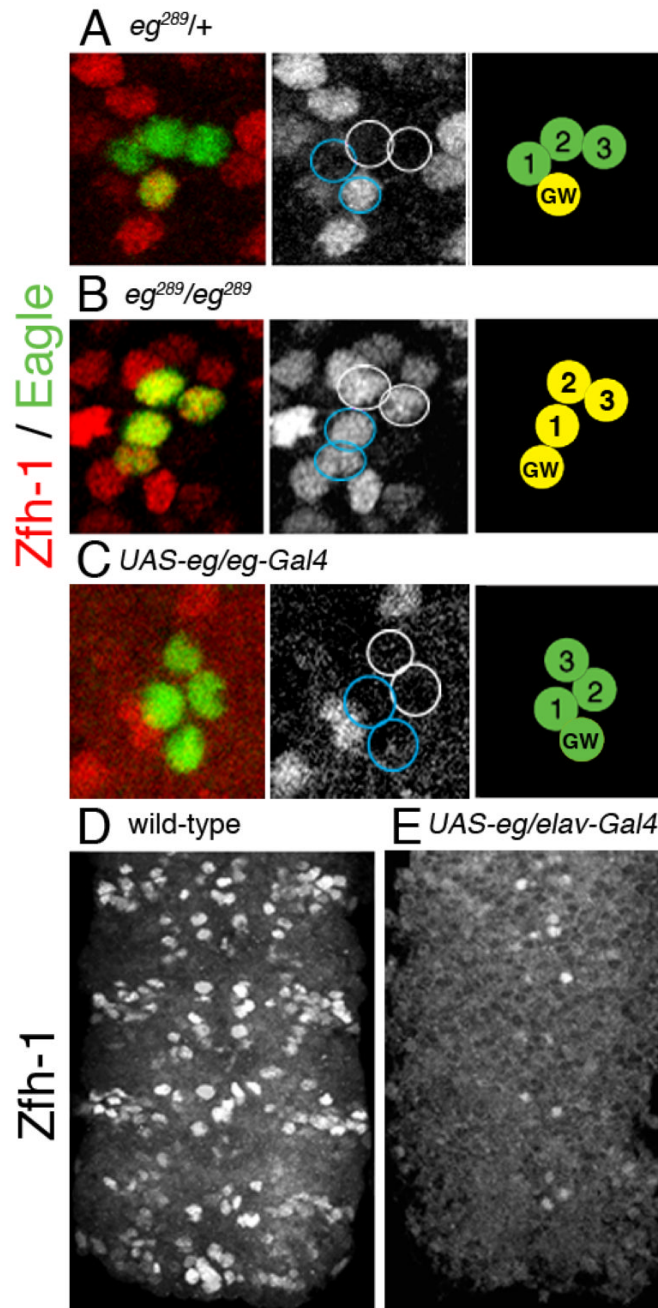
**Fig. 1. Developmental stages and molecular markers of the NB 7-3 lineage**

(A) Schematic of NB 7-3 development, see text for details. (B) A stage 12 wild-type VNC abdominal segment showing 5–6 Eg positive progeny of the NB 7-3 lineage, only the GMC1 progeny are immunoreactive for Hb. The asterisks indicate sister cells of EW2 and EW3 that will undergo apoptosis. (C) A stage 15 VNC showing that only the GW motor neuron is immunoreactive for Zfh-1. (D) A stage 16 VNC showing that the GW neuron no longer expresses detectable levels of Eg. (E) A stage 17 VNC showing expression of the terminal markers Ddc and Corazonin. The yellow numbers 1, 2 and 3 indicate the three EW neurons. M indicates the midline.



**Fig. 2. *zfh-1<sup>2</sup>* mutant phenotypes of the NB 7-3 lineage**

All images are VNC abdominal segments from stage 17 embryos. The numbers 1, 2 and 3 indicate the EW neurons, the asterisks indicate ectopic Ddc neurons and M indicates the midline. (A,C) Embryos homozygous for *zfh-1<sup>2</sup>* show one additional Ddc positive cell per hemisegment. (B,D) The ectopic Ddc cell in the *zfh-1<sup>2</sup>* mutant is Hb positive, suggesting the GW has converted to an EW1 cell fate. (E,F) Embryos homozygous for *zfh-1<sup>2</sup>* show a severe reduction in serotonin immunoreactivity. (G,H) Embryos homozygous for *zfh-1<sup>2</sup>* show defasciculation of the EW axons. The CNS of *zfh-1<sup>2</sup>* mutant embryos is extended such that length of three abdominal segments in a wild-type VNC is equal to two abdominal segments in a *zfh-1<sup>2</sup>* VNC. Panels F and H also show a midline dopaminergic neuron that is immunoreactive for Ddc.

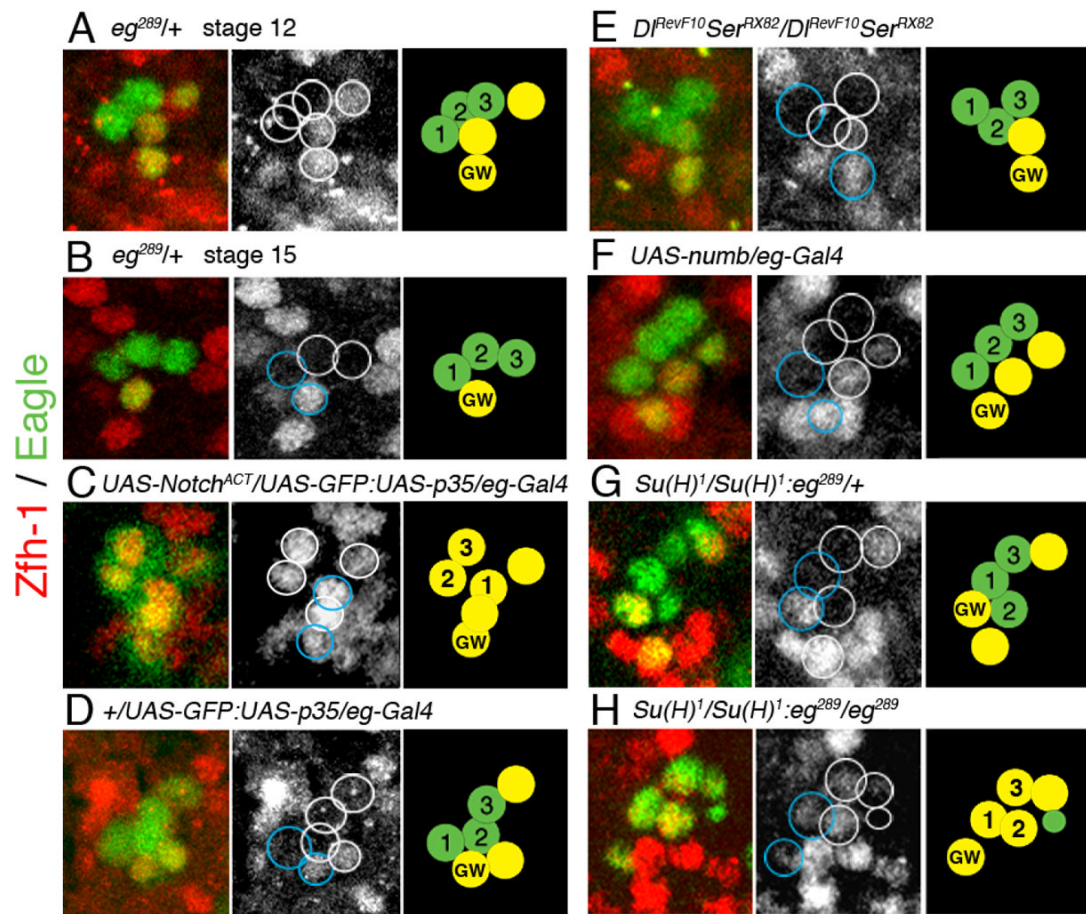


### Fig. 3. Eagle suppresses *zfh-1* expression

(A–C) Abdominal hemisegments from stage 15 VNC. All VNC were immunostained for Zfh-1 (red). Eg expression (green) was detected with either anti-lacZ antibody for the *eg-lacZ* (*eg<sup>289</sup>*) transgene (A,B) or anti-Eg antibody (C). Black and white panels show the Zfh-1 immunoreactivity separately for the same hemisegment. Blue circles indicate the GMC1 progeny that were determined by Hb immunoreactivity (actual immunoreactivity not shown). In the schematic the numbers 1, 2, and 3 indicate the three EW neurons, green cells are Eg positive and yellow cells are both Eg and Zfh-1 positive. The midline is to the left. (A) A wild-type (*eg<sup>289</sup>/+*) VNC showing that only the GW neuron is Zfh-1 positive. (B) Embryos homozygous for *eg<sup>289</sup>* show Zfh-1 immunoreactivity in all four NB 7-3 progeny. (C) Ectopic

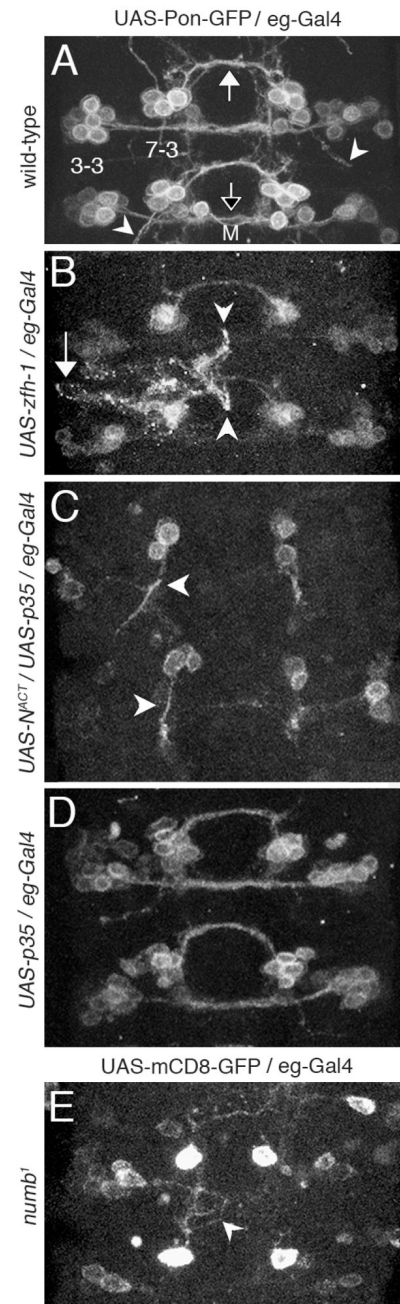


expression of *eg* using the *eg-Gal4* driver results in a loss of Zfh-1 immunoreactivity in the GW neuron. (D,E) Stage 15 VNC showing that ectopic expression of *eg* using the *elav-Gal4* driver results in a loss of Zfh-1 immunoreactivity throughout the VNC.



#### Fig. 4. Notch regulation of *zfh-1* expression

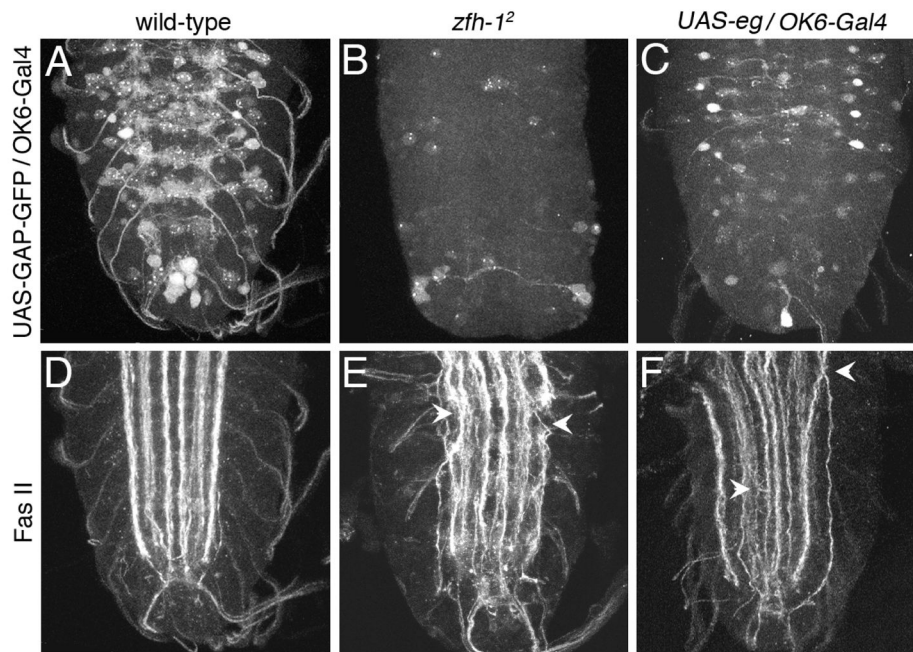
All images are abdominal hemisegments from stage 15 VNC, except for panel A which is stage 12. All VNC were immunostained for Zfh-1 (red). Eg expression (green) was detected with either anti-lacZ antibody for the *eg-lacZ* (*eg<sup>289</sup>*) transgene (A,B,G,H), GFP fluorescence for the *eg-gal4/UAS-GFP* transgenes (C,D), or anti-Eg antibody (E,F). The black and white panels and the schematic are as described in the legend for Fig. 3. Panel B is repeated from Fig. 3. (A) A wild-type (*eg<sup>289/+</sup>*) stage 12 VNC showing all six NB 7-3 progeny. The GW neuron and the cells undergoing apoptosis are Zfh-1 positive. (B) A wild-type (*eg<sup>289/+</sup>*) stage 15 VNC showing that after apoptosis only the GW neuron is Zfh-1 positive. (C) Ectopic *Notch* expression induces Zfh-1 immunoreactivity in all NB 7-3 progeny. (D) Ectopic *p35* rescues cells from apoptosis but does not alter Zfh-1 immunoreactivity. (E–G) Three genetic conditions that reduce canonical Notch signaling all show rescue of apoptotic cells but Zfh-1 immunoreactivity is identical to wild-type. (H) Embryos homozygous for *Su(H)<sup>1</sup>* and *eg<sup>289</sup>* show both the rescue of apoptotic cells and expansion of Zfh-1 immunoreactivity into the EW neurons. The number of cells that can be rescued from apoptosis is variable: (C,D,F,G) show the rescue of both cells, (E) shows the rescue of only one cell and (H) shows the rescue of one cell and the other undergoing apoptosis.



**Fig. 5. Zfh-1 and Notch regulate axonal projections of NB 7-3 progeny**

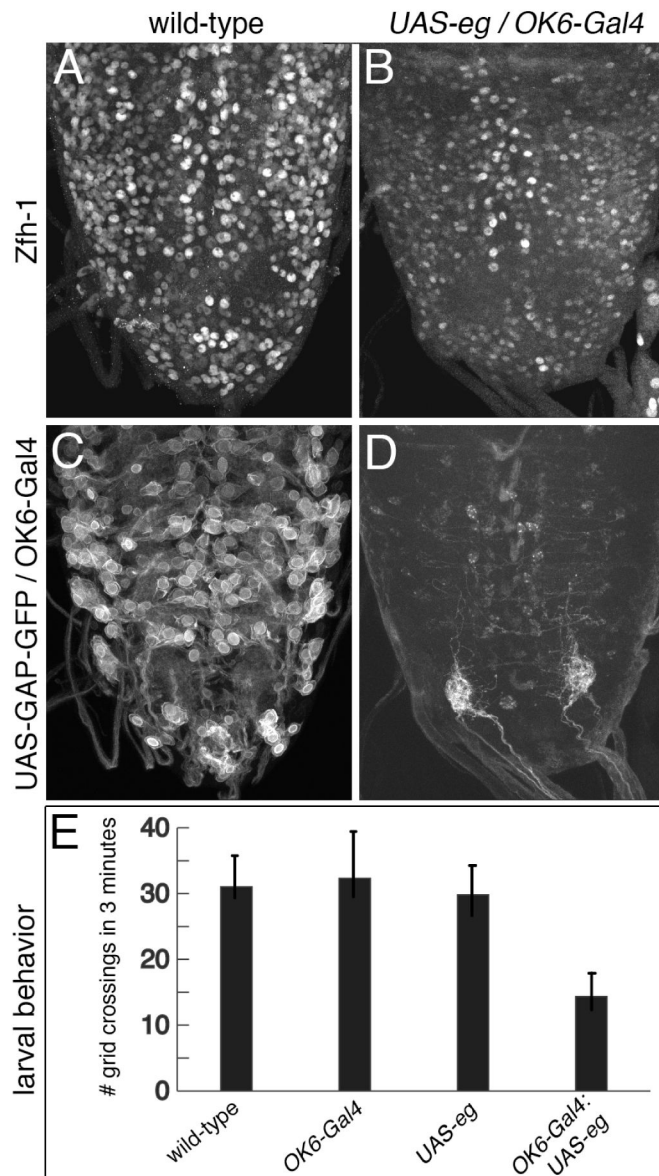
All images are VNC abdominal segments from stage 15 embryos. To visualize axons *eg-Gal4* was used to drive expression of a membrane bound GFP marker (either *UAS-Pon-GFP* or *UAS-mCD8-GFP*). M indicates the midline. (A) A wild-type VNC showing the EW axonal bundle of the NB 7-3 lineage crossing the midline through the posterior commissure (closed arrow), the NB 3-3 axonal bundle crossing the midline through the anterior commissure (open arrow) and the GW ipsilateral axons (arrowheads). (B) The ectopic expression of *zfh-1* shows aberrant EW axon projections (arrow and arrowheads) but all hemisegments maintain some EW midline crossing. (C) The ectopic expression of *Notch<sup>ACT</sup>* blocks all midline crossing of the EW neurons and produces a thick ipsilateral projection (arrowheads). (D) The ectopic

expression of *p35* rescues cells from apoptosis but has no effect on EW midline crossing. (E)  
The *numb<sup>l</sup>* mutation blocks midline crossing of EW1.



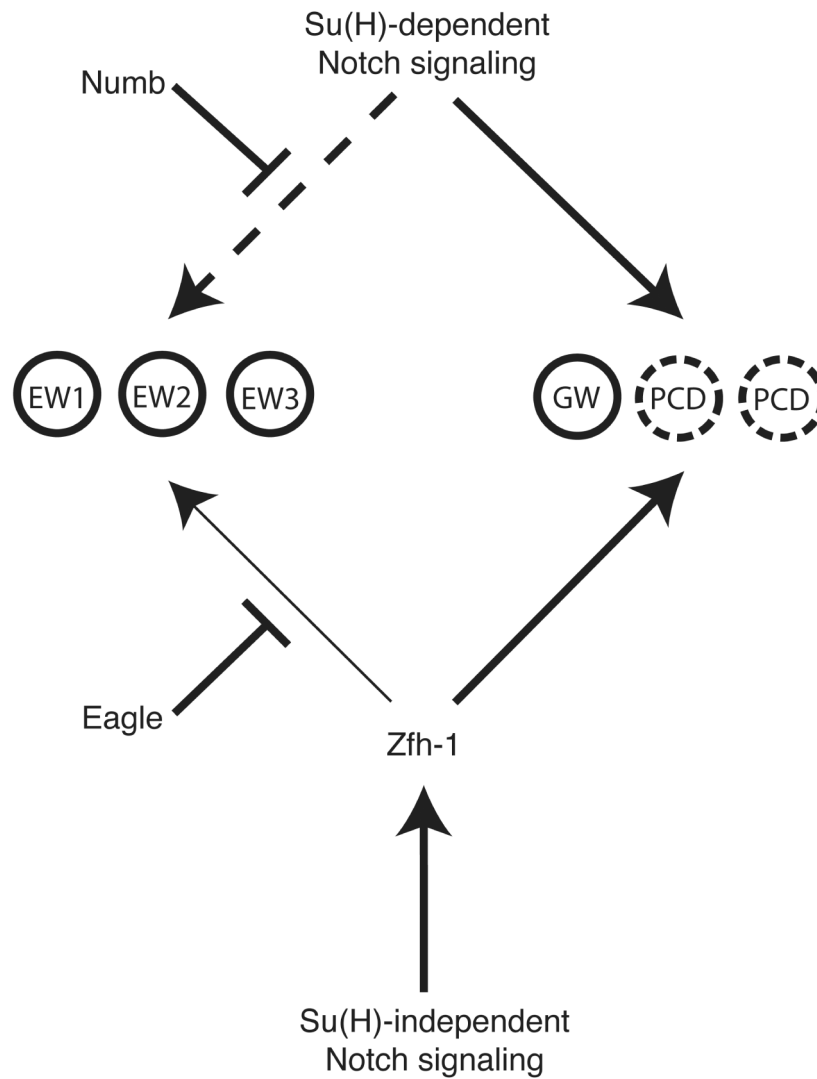
**Fig. 6. Zfh-1 is required for motor neuron development and axon fasciculation**

All images show a VNC from stage 17 embryos. (A–C) Motor neuron cell bodies and axons are detected using *OK6-Gal4* to drive expression of *UAS-GAP-GFP*. Both the *zfh-1<sup>2</sup>* mutation and ectopic expression of *eg* reduces the number of cells detected with *OK6-GFP*. (D–F) Lateral projecting motor neurons and longitudinal axon bundles are detected with *FasII* immunoreactivity. Both the *zfh-1<sup>2</sup>* mutation and the ectopic expression of *eg* result in disorganized lateral motor neurons and defasciculation of the longitudinal bundles. Arrowheads indicate aberrant crossing between adjacent longitudinal bundles.



**Fig. 7. Reduced levels of Zfh-1 alter larval locomotor behavior**

(A,C) A third instar larval VNC showing wild-type Zfh-1 immunoreactivity and *OK6-Gal4/UAS-GAP-GFP* fluorescence. (B,D) A third instar larval VNC showing that ectopic expression of *eg* reduces both Zfh-1 immunoreactivity and OK6-GFP. (E) Ectopic expression of *eg* in motor neurons that express *OK6* reduces third instar larval locomotor activity by 50% relative to three control lines with a significant difference in the mean activity of  $P < 0.0001$ . At least 20 larvae were tested for each line.



**Fig. 8. A model for the regulation of NB 7-3 development by Notch, Eagle and Zfh-1**

Su(H)-dependent Notch signaling is active in the GW motor neuron and apoptotic sister cells (dashed circles) but is inhibited by Numb in the EW interneurons (dashed arrow). Zfh-1 is expressed at high levels in the GW motor neuron and apoptotic cells but is limited in expression in the EW interneurons by Eg (thin arrow). The results presented in this manuscript suggest that Zfh-1 may be induced by Su(H)-independent Notch signaling. Thus both Su(H)-dependent and Su(H)-independent Notch signaling have specific functions within the lineage.

**Table 1**  
**Analysis of molecular marker expression in the NB 7-3 lineage of wild-type and *zfh-1*<sup>2</sup> mutant embryos at stage 17**

For each genotype, hemisegments in abdominal segments 1–6 were counted for the number of NB 7-3 cells that expressed the specific antigen. The results are presented as a percentage of total number of hemisegments counted (n) and as an average number of cells per hemisegment

Antigen	Genotype	Number of immunoreactive cells/hemisegment						
		0	1	2	3	4	n	avg. ± s.d.
Ddc	wild-type	0.6%	1.9%	<b>91.9%</b>	5.6%	---	161	2.02±0.32
	<i>zfh-1</i> <sup>2</sup> / <i>zfh-1</i> <sup>2</sup>	1.4%	---	14.4%	<b>82.0%</b>	2.2%	139	2.80±0.50
hunchback	wild-type	2.0%	<b>91.2%</b>	6.9%	---	---	102	1.05±0.29
	<i>zfh-1</i> <sup>2</sup> / <i>zfh-1</i> <sup>2</sup>	4.0%	15.8%	<b>80.2%</b>	---	---	101	1.76±0.51
serotonin	wild-type	---	3.4%	<b>93.9%</b>	2.7%	---	148	1.99±0.25
	<i>zfh-1</i> <sup>2</sup> / <i>zfh-1</i> <sup>2</sup>	<b>88.8%</b>	10.5%	0.8%	---	---	134	0.12±0.35
corazonin	wild-type	2.4%	<b>97.6%</b>	---	---	---	166	0.98±0.15
	<i>zfh-1</i> <sup>2</sup> / <i>zfh-1</i> <sup>2</sup>	28.9%	<b>68.5%</b>	2.7%	---	---	149	0.56±0.55



**Table 2****Analysis of Eg and Zfh-1 expression in the NB 7-3 lineage at stage 15**

For each genotype, hemisegments in abdominal segments 1–6 were counted for the number of NB 7-3 cells that expressed either the Eg or Zfh-1 antigen. The results are presented as an average number of cells per hemisegment. The two averages for each genotype are also presented as a ratio of Zfh-1 immunoreactive cells to Eg immunoreactive cells

Genotype	Antigen	n	average number of cells $\pm$ s.d.	Zfh-1/Eg
<i>eg</i> <sup>289</sup> /+ (wild-type)	Eg-lacZ Zfh-1	113	3.81+0.48 0.89+0.43	23.4%
<i>eg</i> <sup>289</sup> / <i>eg</i> <sup>289</sup>	Eg-lacZ Zfh-1	68	4.03+0.60 3.74+0.77	92.8%
<i>eg-gal4/UAS-eg</i>	Eg Zfh-1	86	4.10+0.51 0.21+0.41	5.1%
<i>eg-Gal4/UAS-Notch/UAS-p35</i>	Eg-GFP Zfh-1	69	4.20+0.83 4.00+0.87	95.2%
<i>eg-Gal4/UAS-p35</i>	Eg-GFP Zfh-1	87	4.43+0.82 1.97+0.75	44.5%
<i>Dl</i> <sup>RevF10</sup> <i>Ser</i> <sup>RX82</sup> / <i>Dl</i> <sup>RevF10</sup> <i>Ser</i> <sup>RX82</sup>	Eg Zfh-1	74	5.12+0.60 2.00+0.60	39.1%
<i>eg-gal4/UAS-Numb</i>	Eg Zfh-1	85	5.00+0.73 1.93+0.75	38.6%
<i>Su(H)</i> <sup>l</sup> / <i>Su(H)</i> <sup>l</sup>	Eg-lacZ Zfh-1	59	5.29+0.64 2.20+0.69	41.7%
<i>Su(H)</i> <sup>l</sup> / <i>Su(H)</i> <sup>l</sup> : <i>eg</i> <sup>289</sup> / <i>eg</i> <sup>289</sup>	Eg-lacZ Zfh-1	26	5.42+0.70 5.04+0.72	92.9%