A Transcription Factor Network Coordinates Attraction, Repulsion, and Adhesion Combinatorially to Control Motor Axon Pathway Selection

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http://dx.doi.org/10.1016/j.neuron.2014.01.038

SUMMARY

Combinations of transcription factors (TFs) instruct precise wiring patterns in the developing nervous system; however, how these factors impinge on surface molecules that control guidance decisions is poorly understood. Using mRNA profiling, we identified the complement of membrane molecules regulated by the homeobox TF Even-skipped (Eve), the major determinant of dorsal motor neuron (dMN) identity in Drosophila. Combinatorial loss-and gain-of-function genetic analyses of Eve target genes indicate that the integrated actions of attractive, repulsive, and adhesive molecules direct dMN axon guidance. Furthermore, combined misexpression of Eve target genes is sufficient to partially restore CNS exit and can convert the guidance behavior of interneurons to that of dMNs. Finally, we show that a network of TFs, comprised of eve, zfh1, and grain, induces the expression of the Unc5 and Beaten-path guidance receptors and the Fasciclin 2 and Neuroglian adhesion molecules to guide individual dMN axons.

INTRODUCTION

How axons follow specific paths depends on receptors expressed on their membranes that elicit a coordinated response to extracellular signals. Numerous guidance receptors and cell adhesion molecules (CAMs) have been discovered in the last two decades and they are generally classified into four nonexclusive categories as mediators of long-range or contact-mediated attraction or repulsion (Kolodkin and Tessier-Lavigne, 2011). Accordingly, as axons navigate, they respond to several cues of different nature in a complex extracellular environment to choose their correct path (Tessier-Lavigne and Goodman, 1996). However, it remains unclear how the combined expression and function of different receptors and CAMs controls the pathfinding decisions of individual neurons (Pecot et al., 2013) or how it is transcriptionally regulated.

Combinatorial codes of transcription factors (TFs) are essential for axonal pathway selection (Dasen and Jessell, 2009; Thor and Thomas, 1997; Tsuchida et al., 1994). It is generally assumed that transcriptional codes regulate the expression of guidance receptors, although few functional links have been established (Bonanomi and Pfaff, 2010; Zarin et al., 2014). For example, Zic2, a determinant for the retinal ganglion cells whose axons do not cross the optic chiasm, regulates the expression of the EphB1 receptor (Herrera et al., 2003). In addition, molecules that act together to control specific aspects of neuronal function as the battery of genes responsible for the synthesis, packaging, and degradation of acetylcholine as well as choline reuptake in motoneurons are all regulated by a single TF, UNC-3 in C. elegans (Kratsios et al., 2012). Thus, available data support a model in which transcriptional regulators that act as determinants for specific subsets of neurons coordinate the expression of several molecular programs to impart wiring specificity and to confer specific functional properties.

The Drosophila neuromuscular system is particularly suited for an exploration of the functional relationships between TF determinants of motor neuron identity and the cell surface molecules that direct wiring specificity. It consists of a segmentally reiterated arrangement of 30 muscles innervated by 36 motoneurons that fasciculate together into three main branches, the transverse nerve (TN), intersegmental nerve (ISN), and the segmental nerve (SN) (Landgraf and Thor, 2006). The pioneer neurons for the ISN, the aCC and RP2 motoneurons, fasciculate, project away from the CNS, navigate through the muscle field, and innervate their dorsal muscle targets (Figure 1B). The homeodomain TF Even-skipped (Eve) largely determines the specific guidance characteristics of the aCC and RP2 dorsal motoneurons (dMNs) (Doe et al., 1988; Fujioka et al., 2003; Landgraf et al., 1999). When eve is absent in dMNs, they no longer project away from the CNS (Figure 1C) and the ISN stops short of its most dorsal target muscles (Fujioka et al., 2003; Landgraf et al., 1999). eve regulates the expression of the guidance receptors Unc-5 (Labrador et al., 2005); however, unc-5 has a relatively mild guidance phenotype when compared to eve mutants (Labrador et al., 2005). The fact that the eve mutant phenotype is so much stronger than phenotypes observed in guidance receptor and CAM mutants suggests that eve is likely to control several different
guidance pathways that work together to control the correct trajectory of dorsal motor axons.

We now provide evidence that eve orchestrates the expression of several axon guidance pathways in dMNs and propose that eve together with zfh1 and grn comprise a transcriptional code required for the combinatorial expression of these guidance molecules. Using mRNA profiling of fluorescence-activated cell-sorted (FACS) wild-type and eve mutant dMNs in microarrays, followed by in vivo single-cell resolution expression analyses, we show that eve regulates the Beat Ia and Unc-5 receptors and the adhesive CAMs Fas2 and Nrg. These molecules are all expressed in dMNs as their axons start to navigate toward the muscle field in wild-type dMNs, but their mRNA levels are significantly reduced or undetectable in eve mutant dMNs. The TFs Grn and Zfh1 regulate overlapping subsets of these guidance molecules in dMNs. Individual loss of function of these guidance genes generally result in mild guidance phenotypes; however, their combined elimination shows a progressively stronger phenotype in which the ISN stops short of its targets and aCC and RP2 fail to exit the CNS, as is observed in eve mutants. Combinatorial reintroduction of these molecules in eve mutants can lead to CNS exit. This effect is achieved through the combination of different activities: (1) a repulsive action of Unc-5 directing aCC and RP2 away from the midline Netrin source, (2) the attractive effect of the Beat-Ia receptor toward the CNS exit points, and (3) fasciculation of aCC and RP2 mediated by the Nrg and Fas2 CAMs. Additionally, we show that eve can induce the expression of Fas2, beat Ia, Nrg, and unc-5 in EW commissural interneurons and redirect their axons away from the midline toward the muscle field. This effect is replicated by the combined expression of the eve-regulated guidance genes. Finally, we show that zfh1 can also induce the expression of
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Cooperative Regulation of a Combinatorial Code

Fas2, beat la, and unc-5 and that the combined expression of eve and zf h-1 or grn induces a stronger expression of the guidance genes in EW neurons and promotes increased exit of their axons. Thus, the dorsal motoneuron determinant eve partially controls the response of individual motor axons by regulating the precise expression of receptors and CAMs and can cooperatively regulate their levels together with grn and zf h-1 to integrate their different responses (attraction, repulsion, and adhesion) into the correct guidance choice.

**RESULTS**

**Comparative mRNA Profiling of Wild-Type and eve Mutant Dorsal Motoneurons Identifies Multiple eve-Regulated Genes**

To determine transcriptional profiles of individual motoneurons, we developed a system to isolate aCC, RP2, and pCC neurons. We used the UAS/Gal4 system with the R N2Gal4 driver expressing mCD8GFP in wild-type or eve mosaic mutant embryos (Figure 1D), synchronized and aged to late stage 12, at onset of axon guidance. To overcome the early patterning effects due to eve loss of function, we used eve mosaic mutants, in which eve function is exclusively eliminated in aCC, pCC, and RP2 neurons (Fujioka et al., 2003). We purified GFP-positive dMNs by FACS (Figure 1E), extracted total RNA (Figure 1F), and labeled cRNA was hybridized to GeneChip Drosophila Genome 2.0 Arrays. A confirmation of microarray results by in situ hybridization and confocal imaging allowed us to achieve differential transcriptional profiles with single-cell resolution.

We identified 561 genes differentially expressed between wild-type and eve mutants displaying a false discovery rate (FDR) <0.05 and at least a 1.5-fold expression-level difference. Most of the identified genes showed downregulation in eve mutants. Gene Ontology (GO) of differentially expressed genes was analyzed and manually annotated. Based on biological processes, 227 genes were categorized into 55 clusters. Among the top 10% of clusters identified were “Neurological System Process” and “Neuron Projection Morphogenesis,” where GO terms “Axon Guidance” and “Axonogenesis” were included (Table S1 and Figures S1 and S2 available online). Microarray results were confirmed by in situ hybridization for genes in the top two enriched neuronal GO categories in wild-type and eve mosaic mutants, where the cell bodies of aCC and RP2 dMNs were also labeled by protein markers (RN2Gal4 driving UAS-tdTomyc) (Figure 2 and Figure S2). This allowed us to quantify the changes in their mRNA levels with single-cell resolution. All of the genes in these categories (13) except synj (Figure S2) showed significant reduction (p < 0.001) of mRNA signal in eve mutant dMNs. A comparison of the genes identified in our screen and a previous study in which genome-wide eve binding was examined through DamID (Pym et al., 2006) reveals that Eve can bind within 2 kb of at least 10% of the genes we identified (T. Southall, R. Baines, and A. Brand, personal communication).

**Eve Regulates Attraction, Repulsion, and Adhesion**

We focused on genes coding for membrane molecules annotated with axonal-related processes differentially expressed between wild-type and eve mutant aCC and RP2 dMNs and identified several not previously known to be regulated by eve: Beat la (Fambrough and Goodman, 1996), an attractive receptor (Siebert et al., 2009) for Sidestep (Sink et al., 2001) (Figures 2D–2F), the CAM Neuroglian (Nrg), a Drosophila homolog of the vertebrate L1CAM (Bieber et al., 1983) (Figures 2G–2I), and Fasciclin 2 (Fas2) (Grenningloh et al., 1991), the Drosophila homolog of NCAM (Figures 2A–2C). Together with Drosophila Unc-5, a repulsive receptor for Netrins (Harris et al., 1998; Mitchell et al., 1996) that has been shown to be regulated by eve (Labrador et al., 2005) (Figures 2J–2L), they all belong to the immunoglobulin superfamily and may act together to mediate guidance downstream of eve. We analyzed the mRNA expression of these genes by fluorescent in situ hybridization in wild-type and eve mutant aCC and RP2 dMNs and quantified the fluorescent signal (Figures 2C, 2F, 2I, and 2L). While all of these genes are expressed in dMNs in wild-type (eve+/+) embryos at stage 15 (Figures 2A, 2D, 2G, and 2J), their mRNA levels are significantly reduced in eve mutant dMNs (Figures 2B, 2C, 2E, 2F, 2H, 2I, 2K, and 2L). Protein expression for Fas2 and Nrg is also reduced (Figure S3). Our results indicate that eve regulates mRNA levels of Fas2, beat la, nrg, and unc-5 in aCC and RP2 and suggest that eve may program the guidance decisions of aCC and RP2 through the regulation of these cell surface molecules.

**zf h1 and grn Regulate the Expression of Overlapping Subsets of eve-Regulated Cell Membrane Molecules**

Previous studies have shown the involvement of two other transcriptional regulators of aCC and RP2 guidance, Zfh1, a zinc finger homeodomain family member, and Grain (Grn), a GATA family TF. While zf h1 is expressed in all motoneurons including dMNs in the ISN nerve (Layden et al., 2000), grn expression in motoneurons is restricted to dMNs (Garces and Thor, 2006). Both can be regulated by eve or work in parallel to direct axon guidance (Garces and Thor, 2006). Furthermore, we have previously shown that grn can collaborate with eve to regulate unc-5 (Zarin et al., 2012). Therefore, we investigated their requirement for Fas2, beat la, nrg, and unc-5 expression in dMNs (Figures 2M–2X) and found that, while Fas2, beat-la, and unc-5 are downregulated in aCC and RP2 neurons lacking zf h1 (Figures 2M–2U), nrg’s mRNA is unaffected (data not shown). In grn mutants, only unc-5 expression is reduced (Figures 2V–2X and data not shown). Together, these data indicate that zf h1 and grn are required to selectively control overlapping subsets of eve-regulated genes and that zf h1 is required for the regulation of a broader range of guidance molecules than grn.

**ISN Guidance Requires the Combined Action of Attraction, Repulsion, and Adhesion**

In eve mutants, the ISN almost never reaches its appropriate dorsal target muscles (Fujioka et al., 2003; Landgraf et al., 1999), whereas beat la, nrg, and unc-5 single mutants only show partially penetrant defects in ISN projections (Fambrough and Goodman, 1996; Hall and Bieber, 1997; Labrador et al., 2005) (Figure 3). We reasoned that, since the expression of these genes is substantially decreased or absent in eve mutant motoneurons (Figure 2), their concerted function may be required for proper ISN guidance. To test this hypothesis, we analyzed...
The observed phenotypes are organized in order of severity, taking as a reference three branch points in the ISN from ventral to dorsal: first branch point (FB), second branch point (SB), and third branch point (TB), respectively (Figure 3A). Phenotypes present after TB are late phenotypes, between SB and TB intermediate phenotypes, and before FB the most severe early phenotypes (Figures 3N, 3O, and 3P, respectively). Double mutants display phenotypes not previously observed in single mutants (Table S2); for example, nrg; unc-5 double mutants (Figure 3G).
show aberrant crossings to the adjacent segment before the FB point (Figures 3C–3F). Additionally, the number of late defects is also substantially increased (double mutants 18% ± 0.02494%, nrg 4.3% ± 0.01381%, and unc-5 6.6% ± 0.01436%, respectively). Similarly, double mutants for nrg and beat la (Figure 3H) or unc-5 and beat la (Figures 3I and 3J) exhibit stalling before FB (9% ± 0.03149% and 21% ± 0.02846%, respectively) only occasionally observed in beat la mutants (3% ± 0.01086%) (Figures 3E and 3F). Consistently, triple mutants present more severe phenotypes than double mutants (Figures 3K and 3L); in particular, ISN crossing in the ventral muscle field (from 2% ± 0.020%, 8.2% ± 0.029%, or 10% ± 0.044% in nrg unc-5 double, nrg, beat la double, or unc-5, beat la double, respectively, to 15.3% ± 0.04382% in triple mutants; Table S2). In summary, single mutants generally present late phenotype, while double mutant phenotypes are more severe and obvious in more ventral positions. The earliest, most severe, phenotypes are almost exclusively seen in double and triple mutants. ISN stall, in particular, is very similar to the defects observed in eve mosaic mutant embryos (Figure 3M). eve is still present in aCC and RP2 in these triple mutants, suggesting that neither these genes nor a target-derived BMP signal is required for eve expression (Aberle et al., 2002; Garces and Thor, 2006; Marqués et al., 2002) (Figure S4). Thus, dorsal targeting of the muscle field by the ISN is progressively affected by sequentially removing receptors and CAMs, suggesting the requirement of their combined action.

With the exception of Unc-5, these guidance molecules are not exclusively expressed in aCC and RP2 motoneurons within the ISN and their combined guidance defects also reflect their interactions with other axons within the same nerve at later stages of pathfinding. Therefore, some of the observed ISN phenotypes in compound mutants of guidance molecules are probably dependent on the loss of guidance molecules in the ISNs in addition to the pioneers such as early crosses as they are not observed in eve mutants. Therefore, we wanted to investigate the effect of mutations in these molecules at earlier stages of axon guidance, before aCC and RP2 leave the CNS, where they act as pioneers (Jacobs and Goodman, 1989; Sánchez-Soriano and Prokop, 2005). Given that Netrins are expressed in the midline (Harris et al., 1996; Mitchell et al., 1996) and Sidestep labels the path followed by aCC and RP2 (Siebert et al., 2009), we reasoned that combined mutations in their receptors may present exit phenotypes in these neurons as well. We used the Rn2-Gal4 driver to specifically label aCC and RP2 motor axons and examined their ability to exit the CNS in single and different mutant combinations (Figures 3Q–3V; Table S2). No single mutant presented any aCC or RP2 exit phenotype (Figure 3V; Table S2), consistent with the weak ISN guidance phenotypes of single mutants (Figures 3C–3F; Table S2). However, when both guidance receptors were eliminated, exit defects were observed (10% hemisegments [Figures 3S and 3T; Table S2]). Interestingly, embryos where either one of the receptors and the CAM Nrg were eliminated did not present any defect (Figures 3R and 3V; Table S2) and the elimination of nrg (or Fas2; data not shown) in the double receptor mutant did not significantly increase the observed exit defects. Unfortunately, we were unable to generate quadruple mutant animals to determine the effect of removing both CAMs. These results suggest that directed guidance toward the muscle field and away from the midline is an important driving force for aCC and RP2 exit and the combined action of Unc-5 and Beat la contributes to this process. Nevertheless, the strong phenotypes observed in eve mutants and relatively mild ones in double receptor mutants indicate that eve regulates pioneer exit through additional genes not analyzed in the present study.

ev Interacts Genetically with nrg, beat la, and unc-5
In order to dissect the functional link between eve and its downstream targets in dMN guidance, we examined genetic interactions between them. We have previously shown a transheterozygous interaction between eve and unc-5, suggesting that both genes work together during ISN guidance (Zarin et al., 2012). We used this transheterozygous combination as a sensitized background to further reduce beat la levels to 50% and identified significantly increased ISN defects (number of axons exhibiting stalls from 3.0% ± 0.010% to 7.5% ± 0.022% in eve<sup>3DRP2</sup>+/+, unc-5<sup>3F</sup>+/+ transheterozygous and eve<sup>3DRP2</sup>+/+, unc-5<sup>5F</sup>+/+, beat la<sup>1I</sup>/+ triple transheterozygous, respectively [Figures 4D and 4F; Table S2] or from 8.1% ± 0.020% to 23.1% ± 0.036%, when eve<sup>3</sup> and beat-la<sup>C163</sup> null alleles were used [Figure 4G; Table S2]). We next compared ISN defects in nrg/Y hemizygote mutants with nrg/Y; eve+/+ animals. Removing one copy of eve in nrg mutant embryos resulted in more severe ISN guidance defects with significant increases in stalls (from 3% ± 0.014% to 21.2% ± 0.038% in nrg<sup>Y</sup>/Y or nrg<sup>Y</sup>/Y; eve<sup>3DRP2</sup>+/+, respectively; similar effects, although somewhat weaker, were found when a hypomorphic nrg<sup>2</sup> allele was used [Figures 4C and 4E; Table S2]). These results suggest that, in addition to nrg, eve regulates other genes that work in parallel to nrg as part of its guidance output.

Combinatorial Expression of Cell Surface Molecules Reveals Their Individual Function and Restores CNS Exit
In eve mutants, aCC and RP2 fail to exit the CNS and thus provide an ideal genetic background to determine the individual function of the identified guidance molecules in aCC and RP2 guidance. To test this idea, we expressed unc-5, beat la, nrg, and Fas2 individually or in combination in eve mutant dMNs.

Expressing either of these cell surface molecules alone in eve mutants leads to exit of a single motoneuron of the pair per hemisegment (only when unc-5 is re-expressed 11% ± 0.014% of hemisegments show dual exit [Figures 5B–5E and 5N; Table S3]). Axonal exit ranged from no further exit beyond what is seen in eve mutants with nrg (10% ± 0.036% of hemisegments with single motoneuron exit) to some increase of dorsal projections with Fas2, beat la, or unc-5 (25% ± 0.024%, 36% ± 0.052%, and 67% ± 0.030% of hemisegments, respectively, with single motoneuron exit).

Expressing two of these surface molecules leads to a more robust exit where many hemisegments show exit of both motoneurons (Figures 5F–5J and 5N). The only exception is when both CAMs are re-expressed (26% ± 0.036% single motoneuron exit [Figure 5N; Table S3]). We observe two different types of dual exit: unfasciculated, both axons from the same hemisegment chose a different nerve root, or fasciculated,
endogenously contribute. Together, these findings suggest that the concerted expression and coordinated function of guidance receptors and CAMs can induce the proper exit from the CNS and fasciculation of motor axons.

Figure 3. The Coordinated Function of Attractive, Repulsive, and Adhesive Molecules Is Required for ISN Axon Guidance

(A) Schematic depictions of wild-type and different mutant phenotypes in late stage 16 embryos illustrating ISN motor axons (green) and body wall muscles (magenta). Black arrows point to the first (FB), second (SB), and third (TB) branch points of the ISN. (B–M) Flat-mounted late stage 16 embryos stained with anti-Fas2 and anti-myosin antibody to visualize the motor axons (green) and muscles (magenta), respectively. Anterior is shown on the top in all panels. Partial genotypes are indicated above each panel. In wild-type (B), ISN innervates dorsal muscles and respects segment boundaries. In nrg/Y; eve/+ embryos (C) or unc-5/+; beat Ia/+; eve/+ embryos (D), some ISNs stall before reaching their target muscles (white arrowhead) and some others cross the segment boundary (white star). (E–G) Quantification of total ISN defects in embryos heterozygous for eve, hemizygous for nrg, or heterozygous for eve and hemizygous for nrg together (E). Quantification of total ISN defects in embryos heterozygous for eve, beat Ia, or unc-5 and their different transheterozygous combinations (F). Quantification of total ISN defects in embryos heterozygous for eve, beat Ia,Y, or unc-5 and their different transheterozygous combinations (G). Data are represented as mean ± SEM. **p < 0.005.
Figure 5. Combined Expression of Cell Surface Molecules Partially Restores CNS Exit

Axonal projections of aCC and RP2 visualized with an RN2-Gal4 driver expressing UAS-tau-LacZ (magenta) in flat-mounted stage 16 embryos. To better localize and trace the motor nerve exit of these motoneurons, embryos were also stained with anti-Fas2 antibody (green). Anterior is shown on top in all panels and partial genotypes are indicated on the left of each panel.

(A) aCC and RP2 axons in over 90% of hemisegments in eve mosaic embryos fail to exit the CNS and some of these axons cross the midline; a phenotype that is absent in wild-type aCC and RP2 neurons (M).

(B–E) Individual re-expression in eve mutant aCC and RP2 motoneurons of UAS-nrg (B), UAS-Fas2 (C), UAS-beat la (D), or UAS-unc-5 (E).

(F–J) Simultaneous re-expression of two membrane molecules: UAS-beat la and UAS-nrg (F), UAS-beat la and UAS-Fas2 (G), UAS-beat la and UAS-unc-5 (H), UAS-nrg and UAS-unc-5 (I), or UAS-Fas2 and UAS-unc-5 (J).

(K and L) Combinatorial re-expression of UAS-unc-5, UAS-beat la, and UAS-nrg (K) or UAS-unc-5, UAS-beat la, and UAS-Fas2 (L) in eve mutant aCC and RP2 restore exit and fasciculation in 66% and 69% of hemisegments, respectively.

(N) Quantification of total exit for aCC and RP2 in different genetic backgrounds divided between dual exit (either fasciculated or not) of both motoneurons and single exit of either one of them per hemisegment. Single re-expression of any individual gene leads to almost exclusively single exit. Single or dual re-expression of both CAMs (Nrg or Fas2) leads only to single exit in 26% of the hemisegments.

(O) Quantification of dual exit in different backgrounds in which two or more genes are reintroduced in eve mutant aCC and RP2. Dual exit is divided between fasciculated or nonfasciculated exit. The ratio of fasciculated to unfasciculated exit increases when a CAM is re-expressed with one or both guidance receptors (unc-5 or beat la). From 25%/21% fasciculated/unfasciculated when unc-5 and beat la are reintroduced to 66%/13% or 69%/11% fasciculated/unfasciculated when both receptors are reintroduced with nrg or Fas2, respectively. Data are represented as mean ± SEM.
Ectopic Expression of eve in Interneurons Induces the Expression of Cell Surface Molecules of dMNs

Our previous data show that eve is required for the coordinated expression of an array of cell surface molecules in aCC and RP2 motoneurons (Figure 2). To determine whether eve is sufficient to induce their expression in other neurons, we identified a group of eagle interneurons that do not express eve or any of those four surface molecules, the EW neurons, which project axons across the posterior commissure (Higashijima et al., 1996) (Figures 6A–6D). We used the eagleGAL4 driver (Dittrich et al., 1997) to
misexpress eve in EW neurons and analyzed the induction of Fas2, unc-5, beat la, and nrg by in situ hybridization. While none of these genes’ mRNA was expressed in wild-type EW neurons (Figures 6A–6D), ectopic expression of eve resulted in their transcriptional induction (Fas2 in 96%, unc-5 in 66%, beat la in 81%, and nrg in 69% of the scored hemisegments [Figures 6E–6H and 6R–6U]). Additionally, Fas2 and Nrg protein expression is absent in wild-type EWs (Figures S5A, S5B, S5E, and S5F) but eve misexpression led to their ectopic induction (Figures S5C, S5D, S5G, and S5H). These results show that eve is sufficient to transcriptionally reprogram EW interneurons to express the same array of molecules it regulates in dorsal motoneurons.

**Combined Misexpression of eve with grn or zfh1 Induces Robust Expression of Cell Surface Molecules from dMNs in Interneurons**

Since zfh1 and grn are partially required for the transcriptional regulation of a subset of eve downstream genes in aCC and RP2 neurons (Figures 2M–2X), we tested whether they were also sufficient to induce them in EWs. Similar to eve, neither grn nor zfh1 are expressed in these interneurons (Figures S6A, S6B, S6E, and S6F). Individual misexpression of zfh1 in EWs induces Fas2, beat-la, and unc-5 expression in 89%, 64%, and 48% of scored hemisegments, respectively (Figures 6l–6K and 6R–6U); however, grn alone is not sufficient to induce expression of any of these genes (Figures 6R–6U). Given their partial requirement for the induction of some of these genes (Figures 2M–2X), we hypothesized that coexpression of eve with either grn or zfh1 may elicit a more robust transcriptional response. Indeed, combined misexpression of eve with zfh1 robustly induced Fas2 in 100% of the segments in EWs, with a much stronger signal than eve alone (fluorescence intensity 10.9 ± 1.7 a.u. and 19.3 ± 1.1 a.u. for eve alone and eve, zfh1 dual misexpression, respectively; *p < 0.005*), as well as unc-5 and beat-la (Figures 6L–6N and 6R–6T) but not nrg (Figure 6U). In addition, misexpression of eve and grn together lead to a significant increase of EWs expressing Fas2 (fluorescence intensity 18.4 ± 1.4 a.u.; *p < 0.005*) and unc-5 (100% and 85% hemisegments, respectively [Figures 6O, 6P, 6R, and 6S]) but not a significant increase in beat la or nrg expression beyond eve induction alone (Figures 6T and 6U). Importantly, eve does not induce zfh1 or grn in EWs (Figure S6), indicating that they work in parallel to eve in EWs to promote transcription of these receptors and CAMs.

These data suggest that zfh1 is sufficient to promote transcription of some of the dMN guidance molecules in EWs but grn is not. However, both zfh1 and grn can work together with eve to transcriptionally reprogram EWs and regulate guidance receptors and CAMs normally expressed in dMNs. Additionally, the combinatorial expression of eve with zfh1 or grn induces a more robust transcriptional reprogramming of EWs than each of them alone (Figure 6Q).

**The unc-5 Neuronal Enhancer Is Combinatorially Regulated In Vivo by eve, zfh1, and grn**

To understand how these regulators work together in vivo to regulate gene expression, we took advantage of the previously characterized eve-dependent unc-5 neuronal enhancer that drives expression in aCC and RP2 dMNs (Zarin et al., 2012). We used this enhancer to generate a GFP reporter that is expressed in aCC and RP2 under the control of eve (Figure S7) and tested whether eve is sufficient to regulate the reporter in EW neurons independently of zfh1 and grn. The unc-5 reporter is not expressed in EWs (Figures 7A and 7B). In contrast, eve misexpression in EWs leads to GFP reporter activation (17% of hemisegments [Figures 7C, 7D, and 7K]). These results indicate that eve is able to regulate transcription through the unc-5 neuronal enhancer independently of zfh1 and grn. In order to determine whether zfh1 or grn were also sufficient to regulate this enhancer, we misexpressed them in EWs. Only zfh1 was able to induce the GFP reporter expression through this enhancer element (14% of hemisegments [Figures 7E, 7F, and 7K]). As eve can work together with grn and zfh1 to induce...
unc-5 expression in EWs (Figures 6M and 6P), we reasoned that this regulatory region might be able to integrate the effect of combinations of these TFs. Hence, we misexpressed eve and grn (Figures 7G and 7H) or eve and zfh1 combinations in EWs in the presence of the reporter (Figures 7I and 7J). Combined expression of either zfh1 or grn with eve led to a substantial induction of the reporter in EWs (53% and 68% of the hemisegments when eve is combined with grn or zfh1, respectively [Figure 7K]), indicating that eve and grn or zfh1 can act synergistically to induce unc-5 through the same regulatory region. As grn and zfh1 are not induced by eve in EW neurons, this cooperative activation requires the individual presence of each of these TFs. Together, these data indicate that eve and zfh-1 are sufficient to regulate transcription in vivo through the unc-5 neuronal enhancer element. Additionally, grn and zfh1 can collaborate with eve to induce a more robust transcriptional response through the same regulatory region that regulates unc-5 expression in aCC and RP2 dMNs.

**Misexpression of eve or Combined Misexpression of Its Downstream Cell Surface Molecules Can Reprogram the Guidance Behavior of Interneurons**

We next wanted to test whether expression of eve in EW neurons would also alter their guidance behavior. Indeed the axons of EW neurons that express eve no longer cross the CNS midline remaining ipsilateral and some project toward the muscle field (24% ± 0.029%) (Figures 8H and 8N). We reasoned that if the surface molecules that eve regulates in them mediate the guidance switch it promotes in EW interneurons, we might be able to reprogram their guidance by ectopically expressing these molecules. To test this idea, we used eagleGAL4 to misexpress each of these four genes alone or in different combinations and traced their axons (Figures 8B–8F). Individual genes did not lead to EW projections into the muscle field except for a small number when unc-5 was misexpressed (3% ± 0.012% hemisegments [Figures 8C and 8G]). Expression of unc-5, however, prevented EW axons from crossing the midline, similar to eve misexpression (Figure 8H). Expression of double, triple, and quadruple combinations of membrane molecules had an increasing effect on CNS exit (Figures 8D–8G). Interestingly, a combination of both CAMs (nrg and Fas2) had no effect on exit but each one individually or in combination had an enhancing effect in the presence of unc-5 or both guidance receptors (from 5% ± 0.019% exit when beat la and unc-5 are combined to 20% ± 0.041% when nrg is also present and up to 33% ± 0.049% if the four are misexpressed [Figures 8D and 8G]). Moreover, EW axon rerouting by eve is significantly suppressed (to 14% ± 0.013% and midline crossing is also partially restored when eve is misexpressed in a beat la and unc-5 double mutant background (Figures 8I and 8N). To determine the effect of these molecules on guidance in the muscle field, we also misexpressed them in ventral MNs (vMNs). eve misexpression in vMNs leads to dorsalization of the ISNb branch, where it overshoots ventral muscles and projects into the dorsal muscle field (Landgraf et al., 1999). Since eve regulates all of these guidance molecules, we would predict that their combined misexpression would lead to a stronger rerouting of vMNs. Indeed, combined misexpression leads to an increasingly stronger dorsal projection when both receptors or both receptors and CAMs are expressed (Figure S8).

Our data demonstrate that the combinatorial expression of unc-5, beat la, nrg, and Fas2 can redirect EW axons to join the motor axon roots and exit toward the muscle field as motoneurons do. eve can not only reprogram EW interneurons to express these genes but can also alter their guidance decisions to resemble dMNs. Furthermore, this reprogramming of axonal projections is dependent on the molecules it normally regulates in aCC and RP2.

**Combined Misexpression of eve with zfh1 or grn Leads to a Stronger Reprogramming of the Guidance Behavior of Interneurons**

Considering the robust expression of guidance molecules by a combinatorial expression of eve and zfh1 or grn (Figures 6 and 7), our prediction was that their combined misexpression would also lead to a more significant axonal exit from the midline. Consistent with its ability to induce expression of different eve downstream targets (Figures 6I–6K), ectopic expression of zfh1 induced EW exit in 24% ± 0.065% of the hemisegments. Within a given segment, some EW axons still crossed the midline and others projected laterally (Figures 8K and 8N), as previously reported (Layden et al., 2006). In line with its inability to induce expression in EWs, grn alone did not change their projection patterns (Figures 8J and 8N). However, combined ectopic expression of eve with either grn or zfh1 led to a strong lateral exit of EW axons in 72% ± 0.058% and 80% ± 0.050% of hemisegments, respectively (Figures 7L, 7M, and 7N).

Consistent with their combined effect on the expression of receptors and CAMs, coexpression of eve with grn or zfh1 redirects EW axons more robustly than when eve is misexpressed alone. These results highlight the combinatorial requirement of membrane guidance molecules as well as the combined action of eve with zfh1 and grn for efficient reprogramming of EW’s axonal projections.

**DISCUSSION**

Microarray analysis and single-cell resolution expression data have allowed us to establish how eve, a motoneuron subclass determinant, regulates axonal trajectories partially through the coordinated regulation of a series of membrane receptors and CAMs. We also show that this repertoire of molecules functions in individual dorsal pioneer motoneurons to orchestrate adhesive, repulsive, and attractive forces that can work together to steer their axons in the correct path and pioneer the ISN nerve branch. Furthermore, we also show that two other transcriptional regulators expressed in aCC and RP2 dMNs, grn and zfh1, can work in parallel and in cooperation with eve to regulate the expression of subsets of these molecules.

**Regulation of a Full Program of Axon Guidance in Dorsal Motoneurons**

The connection between TFs and the guidance genes that they regulate has been generally elusive. Transcriptional regulators with tissue or cell-specific expression patterns may control local expression of multiple pathfinding genes, frequently broadly
Figure 8. Misexpression of eve or the Combined Misexpression of Its Downstream Cell Surface Molecules Can Reprogram the Guidance Behavior of EW Interneurons

An eagle-Gal4 driver is used to express tau-LacZ and visualize the axonal projections of EW interneurons in flat mounted stage 16 embryos (magenta). To better localize and trace the EW axons, we also stained embryos with anti-Fas2 antibody (green). Partial genotypes are indicated to the left of each panel and a cartoon representing EW projections to the right.

(A) Axons of wild-type EW neurons fasciculate and project across the posterior commissure.

(B) No significant difference is observed in axonal projection of EW neurons misexpressing UAS-beat Ia.

(C) EW neurons misexpressing UAS-unc-5 do not cross the midline but fail to project laterally.

(D) Combinatorial misexpression of UAS-unc-5 with UAS-nrg results in lateral exit of 8% of EW axons.

(E and F) Triple misexpression of UAS-unc-5, UAS-beat Ia, and UAS-nrg (E) or quadruple misexpression of UAS-unc-5, UAS-beat Ia, UAS-nrg, and UAS-Fas2 (F) in EW neurons leads to lateral redirection of axons in 20% or 33% of hemisegments, respectively.

(G) Quantification of lateral projection of EW axons in different genetic backgrounds. F, Fas2; n, nrg; b, beat Ia; u, unc-5.

(H–N) The combined misexpression of eve with grn or zfh1 leads to a stronger lateral exit of EW axons than eve alone. (H) In almost all of EW neurons, misexpressing UAS-eve axons fail to cross the midline and in 25% of hemisegments project laterally and join the motor roots. (I) CNS exit from EW neurons misexpressing UAS-eve is partially suppressed in the absence of unc-5 and beat Ia (15%), and midline crossing is partially restored (white asterisks). (J) Individual misexpression of grn has no effect on EW guidance behavior. (K) Misexpression of UAS-zfh1 leads to EW exit (24% of hemisegments) and thinning of the commissural projections. (L and M) Combinatorial misexpression of eve with grn (L) and eve with zfh1 (M) induces strong exit of EW axons in 72% and 80% of hemisegments, respectively. (N) Quantification of lateral projection of EW axons in different genetic backgrounds. Data are represented as mean ± SEM. ***p < 0.005.
expressed and whose individual elimination leads in general to mild phenotypes. In our study, we provide several lines of evidence to demonstrate that eve partially controls guidance of dorsal motoneuron axons through the regulation of at least two guidance receptors (Unc-5 and Beat-Ia) and two CAMs (Nrg and Fas2), highlighting the requirement of multiple guidance systems downstream of eve.

Similarly, in several well-established systems, individual TFs play a critical role in axon guidance. For example, during dorsal motor axon growth in the limb, the Lim1/Lhx1, a limb family TF, is exclusively expressed in the lateral set of neurons within the lateral motor column (LMC(l)) that projects to the dorsal side of the limb mesenchyme. Its absence renders LMC(l) motoneurons unable to distinguish between dorsal and ventral limb (Kania et al., 2000). Lhx1 regulates EphA4 in LMC(l) and misexpression of EphA4 can redirect ventral axons dorsally (Kania and Jessell, 2003). Nevertheless, other guidance cues have been identified that together with EphA4 control dorsal steering of LMC(l) motor axons such as Ret and ephrinAs (Bonanomi et al., 2012; Duda-nova et al., 2010, 2012; Kramer et al., 2006). However, whether Lhx1 also controls these other pathways is unknown.

How Does eve Regulate Dorsal Motoneuron Guidance Receptors?

Eve has a dual role in motoneuron fate. First, it blocks the expression of the ventral motoneuron-specific TFs HB9, Nkx6, and Lim3 (Broihier et al., 2004; Broihier and Skeath, 2002; Fujioka et al., 2003; Odden et al., 2002), which in turn regulate ventral-specific adhesion molecules such as Fas3 (Broihier et al., 2004). Second, eve can regulate the expression of two other TFs, Grn and Zfh1 (Garces and Thor, 2006), responsible for the expression of guidance genes in dMNs such as unc-5 (Zarin et al., 2012). In grn (Garces and Thor, 2006) or zfh1 (Layden et al., 2006) mutants, the ISN fails to reach its dorsal muscle targets, although their phenotype is less severe than that of eve alone, suggesting that each one might be responsible for only part of eve’s guidance output (Garces and Thor, 2006; Layden et al., 2006). Our results suggest that this is the case as mRNA levels of only subsets of eve-regulated guidance receptors and CAMs are controlled by grn or zfh1 in dMNs (Figure 2).

While zfh1 and grn are the only identified effectors regulating guidance downstream of eve, they also work in parallel in a cell-dependent manner (Garces and Thor, 2006; Zarin et al., 2012). We show that eve is able to induce expression of Fas2, beatia, unc-5, and nrg in a grn- and zfh1-independent manner, indicating that eve can also work at the same hierarchical level as grn and zfh1. Similarly, in vMNs, isl (Thor and Thomas, 1997) and Lim3 (Thor et al., 1999) work in parallel to dfr to specify vMN projections to the correct ventral muscles in part through the differential regulation of beat ic (Certel and Thor, 2004).

We provide evidence in this work that the eve/zfh1/grn dMN combinatorial code of transcriptional regulators may also be required to cooperatively regulate levels of receptors in a cell-specific manner. Our data indicate that individual members of the eve, grn, zfh1 code are differentially required or sufficient to promote expression of dMN guidance molecules. Nevertheless, their combined action leads to a more robust transcriptional response that in the case of unc-5 is mediated through a single regulatory element (Figures 6 and 7). Importantly, this enhanced transcriptional effect is also translated into a more robust guidance response (Figure 8). Expression levels of dMN guidance molecules are likely to be very tightly regulated as indicated by the gene dosage-sensitive genetic interactions they present (Zarin et al., 2012; Figure 4). Therefore, our results strongly suggest that the combined action of these transcriptional regulators is required to attain the required levels of expression of dMN guidance molecules.

Toward a Code of Receptors for Motoneuron Guidance

While eve mutants show severe ISN guidance phenotypes in which all the ISNs fail to reach their dorsal muscle targets, individual mutations in the different receptors regulated by eve result in much milder phenotypes. These phenotypes are almost exclusively restricted to the most dorsal muscle area and have low expressivity. In contrast, we show that their combination increases the expressivity of the phenotype to 65% of the hemisegments and ISN axons stall at earlier points in their path, as they do in eve mutants (Fujikura et al., 2003; Landgraf et al., 1999) (Figure 3). These results indicate that these molecules act together to determine guidance of the ISN and their concerted action gives robustness to the guidance system. The coordinated action of these guidance receptors and adhesion molecules is further supported by their ability to promote CNS exit of aCC and RP2 into the muscle field and rescuing the eve phenotype. In this situation, we can analyze the individual contribution of each one of the molecules regulated by eve by reintroducing them individually or in combination in aCC and RP2. Each individual gene has little or no effect, but the combined action of all of them is able induce substantial CNS exit; in this experiment, the guidance receptors Beat-Ia and Unc-5 together can promote significant CNS exit of both motoneurons. Interestingly, they mainly induce exit where aCC and RP2 are defasciculated, joining either the ISN or the SN branch. These experiments also demonstrate the requirement for an adhesive system to allow the fasciculation of RP2 and aCC. Neither the directed guidance imparted by Unc-5 and Beat-Ia, nor the adhesive function provided by Nrg and Fas2 are alone sufficient to mediate eve’s guidance activity. Only when both guidance receptors and CAMs are expressed simultaneously in aCC and RP2 motoneurons can they project out of the CNS together fasciculated in a bundle. Nevertheless, the relatively mild CNS exit phenotypes of compound mutants suggest that eve also regulates other genes in this process. Similarly, in the Drosophila eye, L3 lamina neurons require in a cell-autonomous manner the combined adhesive function of CadN and the repulsive action of Sema-1a for targeting the outer medulla (Pecot et al., 2013). One might predict that other CAMs with restricted motoneuron expression, such as Connectin (Nose et al., 1992) and Fas3 (Patel et al., 1987), may provide selective fasciculation and specificity for the SNA and ISNb motor branches, respectively.

As Marc Tessier-Lavigne and Corey Goodman proposed in their landmark review:

Thus, an individual axon might be “pushed” from behind by a chemorepellent, “pulled” from afar by a chemotactrant, and “hemmed in” by attractive and repulsive...
local cues. Push, pull, and hem: these forces appear to act together to ensure accurate guidance. (Tessier-Lavigne and Goodman, 1998)

aCC and RP2 axons will be “pushed” from behind by Netrin, “pulled” by Sidestep, and “hemmed” by Neuroglian and Fasci-88lin2 to establish the intersegmental nerve branch. Since this “pulled” by Sidestep, and “hemmed” by Neuroglian and Fasci-88lin2 to establish the intersegmental nerve branch. Since this

**EXPERIMENTAL PROCEDURES**

**Genetics**
Fly stocks used were the following: UAS-HA-Unc5 (Kelemen and Dickson, 2001), Unc-5g;CyO (Labrador et al., 2005), UAS-beat la, beat la^{2176}/CyO, and beat la^{276}/CyO (Siebert et al., 2009), UAS-Fas2 (transmembrane, PEST iso-89form) (Lin et al., 1994), UAS-ngr^193 (Islam et al., 2003), and nrg^2/FM7 (Hall and Bieber, 1997). eve mosaic mutants used were the following: Df(2R)Rive, RP2A/Cyo; RN2-Gal4, UAS-tau-LacZ (Hal and Bieber, 1997). eve mosaic mutants used were the following: Df(2R)Rive, RP2A/Cyo; RN2-Gal4, UAS-tau-LacZ (Fujikawa et al., 2003), UAS-evet/TM3, RN2-Gal4, UAS-tau-myc-GFP, and UAS-tau-myc-GFP (Garces and Thor, 2009), ega-Gal4 (Dittrich et al., 1997), RN2Gal4, UASmCD8GFP, and Df(2R)Rive, RP2A, RN2Gal4, UASmCD8GFP/S6M-TM6. Lethal mutations/insertions were kept over FM7, CyO, TM2, TM3, and TM6 balance chromosomes with blue balancers. Detailed genotypes are described in the Supplemental Experimental Procedures.

**Cell-Specific mRNA Profiling**
Wild-type and eve mosaic aCC and RP2 neurons were isolated from RN2Gal4, UAS-mCD8GFP and Df(2R)Rive, RP2A, RN2Gal4, UASmCD8GFP/S6M-TM6 fly lines, respectively (see the Supplemental Experimental Procedures). Wild-type and eve mosaic embryos were aged for 8 hr at 25°C, trypsinized, and dissociated. GFP-positive neurons were purified using FACS and immediately transferred to TRIzol (Invitrogen). Total RNA was processed with the GeneChip Fly 81 system (Affymetrix) and hybridized to GeneChip Fly 81 Drosophila Genome Array (Affymetrix) and microarray hybridized. Three biological and three technical replicates were performed for each group. Microarray output GeneChip CEL files were analyzed with the oneChannelGUI (Bioconductor) and normalized using GC-RMA algorithm. To determine differential expres-
sionally expressed genes between two conditions, we performed two-tailed paired t test using the Limma package from the Bioconductor project (http://www.bioconductor.org/). The p value of the moderated t test was adjusted for multiple hypotheses testing, controlling for FDR with the Benjamini-Hoch-
berg procedure. Genes with FDR less than 0.05 (5%) and fold change greater than 1.5 were chosen for further examination. Other analyses were performed as described in detail in the Supplemental Experimental Procedures.

**Immunofluorescence**
Primary antibodies used were the following: anti-c-Myc 9E10 (1:50), anti-Fas2 1D4 (1:50) (Developmental Studies Hybridoma Bank), anti-HA (Cowance; 1:500), anti-muscle myosin (1:50), and rabbit anti-l-j-gal (Cappel; 1:5,000). Alexa Fluor 488 (Molecular Probes), HRP, and Cy3 (Jackson ImmunoResearch Laboratories) conjugated anti-mouse or anti-rabbit secondary antibodies were used at 1:1,000, 1:500, and 1:500, respectively. Cy3-labeled tyramide (PerkinElmer) was used as HRP substrate. ISN projections at embryonic stage 16/17 in A2–A6 abdominal hemisegments were stained with anti-Fas2 and examined in different genetic backgrounds. Stacks of images were obtained with a Zeiss Confocal LSM700 Microscope using a 40x oil immersion objective.

**RNP In Situ Hybridization**
In situ hybridization to analyze the mRNA expression of different genes in aCC and RP2 dorsal motor neurons as well as eagle interneurons was performed as previously described (Labrador et al., 2005). Full-length cDNAs were PCR amplified and probes were transcribed using digoxigenin-labeled ribonucleo-
tides. Hybridized probes were bound with anti-digoxigenin POD-conjugated Fab fragments and detected using Cy3-labeled tyramide. Anti-l-j-gal and anti-Myc antibodies were used for double labeling. Mutant embryos and het-
erozygous siblings were dissected, mounted on the same slide, and distin-
guished with l-j-galactosidase balancer chromosomes. Stacks of images were obtained as described above. Laser power and detector settings were optimized for detection of unsaturated fluorescent signal and kept constant for all the different genotypes. Fluorescence was quantified with ImageJ.

**Statistics**
Data are presented as mean values ± SEM. SPSS 16 software (SPSS) was used to generate histograms and determine statistical significance. For anal-
ysis of genetic interactions, Kruskal-Wallis one-way analysis of variance was used. For all other comparisons, two-independent samples t test was used. Significance levels are represented in figures with **p < 0.05 and ***p < 0.005.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.01.038.

**ACKNOWLEDGMENTS**
We would like to thank James Castelli-Gair Hombria, Barry Dickson, Herman Aberle, Luis Garcia Alonso, Miki Fujikawa, Alain Garces, and Brian McCabe for fly stocks and diverse reagents. We would also like to thank Richard Barnes, Tony Southall, and Andrea Brand for sharing their unpublished data with us. This work was supported by a Principal Investigator Grant 07/1N.1/B913 and the Research Frontiers Program 08/ RFP/NSC1617 from Science Foundation Ireland (to J.-P.L.), an IRCSET Embark Initiative Postgraduate Scholarship (to A.A.Z.), and NIH Grant NS054739 (to G.J.B.).

Accepted: January 7, 2014 Published: February 20, 2014

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