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Dissecting the functional link between transcription factor codes and axon guidance genes in Drosophila motor neurons

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A thesis submitted to The University of Dublin for the degree of

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Declaration

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Dedicated to my dear father, mother and wife.
Summary

As axons navigate through the extracellular environment they are exposed to different guidance cues that will steer them when sensed by receptors and cell adhesion molecules (CAMS) present on their membranes. The intricate neuronal network of even the simplest nervous systems together with the comparatively limited number of guidance molecules encoded in the genome suggests that axon guidance must be combinatorial in nature. However, there is little evidence that combinatorial expression and activities of guidance receptors and CAMS direct axon guidance in vivo or how the deployment of such a repertoire of surface molecules would be coordinated. We now take advantage of mRNA profiling of FACS sorted Drosophila dorsal motor neurons (dMNs) combined with confocal imaging to identify the complement of membrane molecules expressed within single motor neurons. Analysis of this collection in a mutant background for the homeobox Even-skipped (eve) transcription factor, the dMN determinant that directs the motor axon projections of the intersegmental nerve (ISN) into the dorsal muscle field, allows us to identify the array of receptors (i.e. Unc-5 and Beat-1a) and CAMS (i.e. Fas2 and Nrg) regulated by eve in individual dMNs. Analysis of their combined mutant phenotypes indicates that these molecules act combinatorially to determine ISN guidance. Rescue experiments where these receptors and CAMS are re-expressed in eve mutant dMNs reveal the combined requirement of their distinct functions to guide individual dMN axons. Finally ectopic expression of eve can reprogram EW interneurons to express the array of membrane molecules present in dMNs and ectopically direct their axons into the muscle field. Thus, the dorsal motoneuron determinant, eve, regulates the expression of a combinatorial array of receptors and CAMS that direct guidance away from the midline and into the muscle field in a bundle that will pioneer the ISN.

Combinatorial transcription factor codes play an essential role in neuronal specification and axonal guidance in both vertebrate and invertebrate organisms. Grain (Grn) is a GATA family transcription factor that, along with Eve, is specifically expressed in dMNs. To gain a better understanding of how Eve regulates the expression of its downstream guidance genes and to test weather the GATA factor, Grn, contributes to Eve in this regulation, we have studied the regulation of Unc-5
receptor in dMNs. We show that the combinatorial function of Even-skipped (Eve) and Grain (Grn) is required to generate sufficient levels of Unc-5 for proper pathfinding of dMNs- via a promoter element in Unc-5 intron 5. Using different mutant combinations, we present genetic evidence that both Grn and Eve are in the same pathway as Unc-5 (Netrin receptor) dMNs. In grn mutants, in which dMNs fail to reach their muscle targets, dMNs show significantly reduced levels of unc-5 mRNA expression and this phenotype can be partially rescued by the reintroduction of Unc-5. We also show that both Eve and Grn are required independently to induce expression of Unc-5 in dMNs. Reconstitution of the Eve-Grn transcriptional code of a dMN in dMP2 neurons, which do not project to lateral muscles in Drosophila, is able to reprogramme those cells accordingly; they robustly express Unc-5 and project towards the muscle field as dMNs. Each transcription factor can independently induce Unc-5 expression but Unc-5 expression is more robust when both factors are expressed together. Furthermore, dMP2 exit is dependent on the level of Unc-5 induced by Eve and Grn. Taken together, our data strongly suggests that the Eve-Grn transcriptional code controls axon guidance, in part, by regulating the level of unc-5 expression.
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Chapter 1: An introduction to transcriptional regulation of motor neuron identity and connectivity
Transcriptional regulation of axon guidance in motor neurons

During development, motor axons are instructed to navigate to their target muscles by simultaneously integrating multiple extracellular signals along the pathway; migrating axons continuously adapt their response by modulating the expression of guidance receptors and their intracellular signaling cascades (Tessier-Lavigne and Goodman, 1996). The subset of transcription factors expressed in different motor neurons is crucial for not only neural identity, but also for the innervations of axons into the appropriate muscles (Butler and Tear, 2007; Polleux et al., 2007; Shirasaki and Pfaff, 2002). Indeed transcriptional regulation in postmitotic motor neurons is thought to be a key mechanism regulating selectivity in axon guidance decisions, as it is one of the fundamental ways of controlling the repertoire of receptors expressed by each neuron. However, the intricate molecular mechanisms of this regulation are yet to be understood. To shed light on these mechanisms animals such as mice and fruit fly have been used as model organisms, and results have indicated a remarkable similarity in both the general motor neuron organization and type of axon guidance molecules between vertebrate and invertebrates.

Establishment of generic motor neuron identity in vertebrates

In vertebrates, motor neurons and several classes of interneurons are created in response to graded extrinsic signals of sonic hedgehog (Shh) and retinoic acid (RA) which act along the dorsoventral axis of the neural tube. Shh and RA signaling establishes the identity of five ventral progenitor cell domains (p0, p1, p2, pMN, and p3) via inducing the expression of distinct combinations of transcription factors in neural progenitors. The mechanisms by which these gradient signals work have been reviewed elsewhere (Dessaud et al., 2008; Jessell, 2000; Shirasaki and Pfaff, 2002). The bHLH proteins Olig2, Ngn1 and Ngn2 and the homeodomain factors Pax6, Nkx6.1, and Nkx6.2 are transcription factors expressed in the progenitor domain that gives rise to motor neurons (Ma et al., 2009; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; Vallstedt et al., 2001; Zhou and Anderson, 2002). Once postmitotic, motor neurons (MNs) express a set of homeodomain transcription factors (notably Hb9, Lhx3, Lhx4, Isl1, and Isl2), that
are responsible for the common features found in all spinal MNs and also the later aspects of MNs subtype specification (Arber et al., 1999; Pfaff et al., 1996; Sharma et al., 1998; Thaler et al., 1999; Thaler et al., 2004). As MNs innervate various peripheral targets throughout the body, their number, identity, and axon trajectory vary drastically along the rostro-caudal axis of the spinal cord. Based on anatomic positioning, MN cell bodies are categorized into five main columnar classes: phrenic motor column (PMC), lateral motor columns (LMCs), hypaxial motor column (HMC), preganglionic column (PGC) and median motor column (MMC). PMC consists of motor neurons innervating diaphragm muscles and their firing is prerequisite for mammalian respiration (Philippidou et al., 2012). Two LMCs are generated only at brachial and lumbar limb levels of the spinal cord and innervate limb muscles. PGC and HMC include motor neurons that reside only at thoracic levels, where the PGC innervates sympathetic ganglia and cells of the HMC projects to the intercostal and abdominal wall musculature (Gutman et al., 1993; Prasad and Hollyday, 1991). In contrast to the segmentally restricted columns described above, motor neurons innervating axial muscles are located in the median motor column (MMC) which is found in all segmental levels of the spinal cord (Gutman et al., 1993).

**Columnar fate determination of vertebrate motor neurons by Hox factors**

Several studies have shown that members of the vertebrate Hox gene family play crucial roles in establishing both columnar fate along the rostrocaudal axis and MN pool fate within the LMC; these genes encode homeodomain transcription factors and consist of 39 members located on four chromosomal clusters (Kmita and Duboule, 2003; Lemons and McGinnis, 2006; McGinnis and Krumlauf, 1992). Like Shh and RA gradient signals responsible for dorsoventral patterning of transcription factors within neural progenitor domains, multiple signaling molecules including FGFs, RA and members of the transforming growth factor (TGF) b superfamily (Gdf11) regulate the expression of Hox genes within the central nervous system (CNS)(Bel-Vialar et al., 2002; Liu, 2006; Liu et al., 2001). Similar to other transcription factors, Hox proteins have both transcriptional activator and repressor functions that are crucial for MNs diversification. These functional differences perform distinct but coherent roles in specification of motor
neuron subtypes. In contrast to the dorsoventrally homeodomain cross-repressive interactions which occur within neural progenitor cells (Briscoe and Ericson, 2001), the rostrocaudal Hox cross-repression is predominantly restricted to postmitotic neurons (Dasen et al., 2003).

PMC neurons are located in rostrocervical segments of spinal cord. They can be distinguished by selective expression of Hoxa5 and Hoxc5, and the exclusion of other Hox factors as well as their accessory factor FoxP1. Continuous function of Hox5 (Hoxa5 and Hoxc5) function is needed for different aspects of PMC neurons, including neuronal migration, clustering, axon projection toward the diaphragm and diaphragm innervation (Philippidou et al., 2012). The netrin receptor coding gene Unc5c is one of possible targets regulated by Hoxa5 and/or Hoxc5 factors, as this receptor is required for normal projection of phrenic motor neurons, and phrenic axons fail to reach the diaphragm in mice homozygous for null mutation in Unc5c (Burgess et al., 2006). Furthermore, genes encoding the cell adhesion molecule ALCAM, known to regulate the guidance and fasciculation of motor and retinal axons (Weiner et al., 2004) and the NgR ligand, NogoA, implicated in the visual cortex plasticity (McGee et al., 2005) are among the potential candidates that could be directly regulated by Hox5 (Philippidou et al., 2012).

Brachial (forelimb) LMC neurons are characterized by their selective expression of Hox6 proteins, thoracic PGC and HMC neurons by expression of Hox9 proteins, and lumbar (hindlimb) LMC neurons by expression of Hox10 proteins (Choe et al., 2006; Dasen et al., 2003; Jung et al., 2010; Liu et al., 2001). Cross-repressive interactions between Hox6, Hox9, and Hox10 proteins is required for refinement of Hox profiles along the rostrocaudal axis, whereas their activator functions determine LMC, HMC and PGC identities as well as their peripheral target connectivity (Dasen et al., 2003; Dasen et al., 2005; Shah et al., 2004). For example, in the absence of Hoxc9, thoracic motor neurons acquire an LMC identity which is accompanied by ectopic induction of Hoxc6 and several other Hox proteins at thoracic levels (Jung et al., 2010). In addition, Genome-wide ChIP-seq analyses have demonstrated the direct binding of Hoxc9 protein to multiple regions within the Hox loci subsequently prohibiting their expression (Figure 1.1) (Jung et al., 2010).
Two recent studies have demonstrated that the Forkhead transcription factor FoxP1 acts as an essential cofactor and conducts the Hox-dependent program of differentiation in LMC and PGC neurons (Dasen et al., 2008; Rousso et al., 2008). Interestingly, FoxP1 is exclusively expressed in neurons belonging to two Hox sensitive columns: LMC and PGC motor neurons (Dasen et al., 2003). In the absence of Foxp1, expression of generic motor neuron markers is not altered but LMC and PGC motor neurons gain HMC fate causing the spinal cord to form two continuous MMC and HMC columns (Figure 1.1) (Arber, 2008; Dasen et al., 2008).

Hox networks specify LMC MN pools via regulating downstream effectors

Within the LMC, each group of MNs innervating a dedicated target muscle in the limb are clustered into MN pools, segregating the vertebrate LMC into approximately 50 distinct groups of MNs each of which occupies a specific rostro-caudal subdomain. (Hollyday and Jacobson, 1990; Landmesser, 1978a). Several other Hox proteins have been shown to be involved in the specification of these MN pool identities. For example, within the chick brachial LMC, while rostral pools are specified by expression of Hox5 proteins, caudal pools are marked by Hox8 proteins (Dasen and Jessell, 2009; Dasen et al., 2005; Vermot et al., 2005). Hox5 proteins in mouse rostral LMC induce the downstream intermediate transcription factor Runx1, a runt related protein, in MNs that innervate the scapulohumeralis posterior (Sca) muscle. Furthermore within a single rostro-caudal level, the intrasegmental fate determination of multiple MN pools residing in parallel is achieved through cross-repressive interactions between several Hox proteins (Dasen et al., 2005). For example, the diversification of muscle innervating MN pools within the Hoxc8+ domain of brachial LMC requires the actions of Hox4, Hox6, and Hox7 paralogs, resulting in selective expression of ETS transcription factor, Pea3, in MN pool innervating pectoralis (Pec) muscle and POU domain transcription factor, Scip, in MN pool projecting to flexor carpiulnaris (FCU) muscle (Figure 1.1) (Dasen et al., 2005). In another study it has been shown that the homeodomain transcription factor Nkx6.1 is involved in Hox-dependent organization of axon trajectories in a defined set of MN pools (De Marco Garcia and Jessell, 2008). Nkx6.1 is transiently expressed in all MN progenitors, but by the time motor axons start projecting toward their target muscles its expression is
restricted to lumbar level MN pools innervating adductor, tibialis anterior (Ta) and gracilis posterior (Gp) muscles. Genetic experiments are in favor of the model that suggests a postmitotic role for Nkx6.1 in instructing thigh innervating MN pools to accurately project toward their distinct targets. The Hox transcriptional network appears to control MN pool specific expression of Nkx6.1 (De Marco Garcia and Jessell, 2008). Interestingly, in animals lacking Hox cofactor Foxp1, motor neurons lack the expression of molecular markers of LMC divisional and pool identities, including LMC transcription factors (e.g., Lhx1, Pea3, Nkx6.1) as well as axon guidance and synaptic specificity molecules (EphA4, Sema3E, Cad20) (Dasen et al., 2008). Together, these findings support the notion that Hox networks have a key role in the diversification of MNs at columnar and pool levels, and they are deterministic for the acquisition of subpopulation-specific gene expression profiles and formation of peripheral connectivity.

The Hox factors control motor neurons diversification. A) Hox factors are expressed at specific locations along the rostrocaudal axis, where they control the generation of different motor columns in their appropriate positions. B) Cross-repressive interactions between Hox proteins define their expression domains, hence columnar positioning of motor neurons. Hox6/10 induce higher levels of the cofactor Foxp1 in forelimb and hind limb LMC neurons, while Hox9 induces lower levels of Foxp1 in PGC neurons. Foxp1 is necessary for the formation of LMC and PGC columns as well as for motor pools diversification within the LMC. The Hox/FoxP1 complex is required for transcriptional regulation of multiple downstream genes including those coding for transcription factors, signaling molecules, and a repertoire of axon guidance molecules. For example the enzyme RALDH2 is induced by Hox/FoxP1 complex, which in turn triggers the expression of Lim1 in lateral LMC neurons via production of retinoic acid (RA). C) Hox5 proteins (Hoxa5 and Hoxc5) are responsible for production of motor pool in rostral LMC through induction of the transcription factor Runx1 in those neurons. On the other hand Hoxc8 plays a pivotal role in generation of Pea3 and Scip positive motor pools in caudal LMC neurons. Furthermore, the repressor activity of Hoxc8 restricts the expression of Hox5 proteins to rostral LMC. Figure adapted from three journal articles (Dalla et al., 2008; Dasen and Jessell, 2009; Philippidou et al., 2012).
**Lim proteins direct LMC and MMC axons to target muscles**

**LMC motor projections:** In vertebrates, as in *Drosophila*, the expression of distinct combinations of LIM-homeodomain proteins defines the axon-outgrowth pathways for different MNs subtypes. In other words, the combinatorial code of LIM proteins specifies MN diversity via enabling different classes of MNs to choose their appropriate projections routes (Sharma et al., 1998; Sharma et al., 2000; Tsuchida et al., 1994). LIM codes confer MNs with this ability apparently by controlling the genes involved in responsiveness to either attractive or repulsive signals emanating from both midway environmental cue and final target muscles. In the chick and rodent spinal cord, lateral motor column (LMC) can be further split into medial (LMCm) and lateral (LMCl) divisions projecting to the ventral and the dorsal part of the limb, respectively (Landmesser, 1978b; Tosney and Landmesser, 1985a; Tosney and Landmesser, 1985b). LMCm neurons express Lim proteins Isll and Isl2 whereas LMCl neurons express Isl2 and Liml (Kania et al., 2000). Columnar Hox proteins (Hox6/10) partition the LMC into medial and lateral divisions via activation of Isll in LMCm and Lim1 in LMCl (Dalla et al., 2008; Dasen and Jessell, 2009). Most of the LIM-proteins downstream targets remain unknown; however, genes encoding members of the Eph-receptors family and their membrane-bound ligands, the ephrins, are known to be regulated by Lim proteins (Kania and Jessell, 2003; Luria et al., 2008). Isll induces the expression of EphB1 in LMCm and Lim1 promotes EphA4 expression in LMCl. On the other hand, LMX1B induces ephrin-B2 and represses ephrin-A5 in dorsal limb, leading to expression of ephrin-B2 in dorsal limb and ephrin-A5 in ventral limb. As a result, EphA4+ LMCm is guided to dorsal limb due to repulsion from ephrin-A5, and EphB1+ LMCm is repelled from ephrin-B2 and projects to ventral limb (Figure 1.2) (Kania and Jessell, 2003; Luria et al., 2008).

Along with forward signaling, ephrin dependent reverse signaling is important for LMC axon guidance. In contrast to EphA and EphB mediated forward signaling which leads to repulsive response in LMC axons, ephrin-A and ephrin-B reverse signaling which exists in, respectively, lateral and medial LMC neurons result in motor axon attraction (Kao and Kania, 2011). For example EphA4 and EphA7 are expressed by dorsal limb mesenchyme where they act as "ligands" for ephrin-As...
present in LMCl axons, leading to attraction and growth of LMCl to dorsal limb muscles via reverse signaling (Bonanomi et al., 2012; Dudanova et al., 2012; Kao and Kania, 2011; Marquardt et al., 2005). Bonanomi et al. have recently demonstrated that the receptor tyrosine kinase Ret is required for ephrin-A reverse signaling (Figure 1.2) (Bonanomi et al., 2012).

The cis-interaction between co-expressed Ephs and ephrins in LMC motor axons where ephrins attenuate forward signaling of co-expressed Ephs has recently been reported as another guidance mechanism. Kao et al. have demonstrated the cis-attenuation of EphA function by ephrin-As and cis-attenuation of EphB function by ephrin-Bs (Kao and Kania, 2011). The cis-silencing of Ephs forward signaling is probably achieved through limiting their availability to bind exogenous ephrin ligands in trans, hence increasing the accuracy of motor axon trajectory. This study also proposes that ephrin levels might be responsible for switching between cis-attenuation and trans-signaling states by altering the subcellular localization of ephrins and Ephs (Kao and Kania, 2011; Kao et al., 2012).

Limb-derived growth factors such as glial cell line-derived neurotrophic factor (GDNF) are also pivotal in motor axon navigation. In addition to participating in ephrin-A reverse signaling, Ret mediates GPI-anchored GFRα1 signaling in response to GDNF. GDNF binds to a receptor complex composed of GPI-anchored GFRα1 receptor (Gfrα1) and Ret (Airaksinen and Saarma, 2002). GDNF is expressed at the dorsoventral trajectory point within the hindlimb, whereas Ret and GFRα1 are expressed by limb-innervating motor neurons (Kramer et al., 2006). Previous studies have reported that motor axons expressing Ret and GFRα1 are attracted to gradients of GDNF (Dudanova et al., 2010), and that Gdnf, Gfrα1, and Ret mutant embryos are defective in peroneal nerve projection (Gould et al., 2008; Kramer et al., 2006). Bonanomi et al. further suggest a synergistic interaction between GDNF and EphA signals where GDNF might potentiate ephrin-A reverse signaling indirectly, via GFRα1-mediated co-compartmentalization of Ret and ephrin-A into membrane rafts in axon growth cone (Figure 1.2) (Bonanomi et al., 2012).
In addition to Eph/ephrin and GDNF/Ret pathways, genetic experiments in mouse and chick have evidenced the contribution of repulsive Sema3:Neuropilin signalings to LMC axon navigation. According to these studies, some medial LMC axons expressing Npn-2 receptor are repelled from Sema3F ligands in the dorsal limb, thereby leading to their ventral diversion (Huber et al., 2005). In addition, interactions between Sema3A and its receptor Npn-1 expressed in entire brachial LMC neurons control the timing of motor axon limb innervation as well as the extent of fasciculation of both lateral and medial LMCs (Huber et al., 2005; Moret et al., 2007). Sema3:Neuropilin guidance pathways may act in parallel or perhaps synergistically via direct interactions with ephrin:Eph signaling components to increase the robustness of dorsoventral navigation at the base of the limb.

**MMC motor projections:** Lim proteins also play a pivotal role in axon guidance of median motor column (MMC) MNs which innervate axial muscles (Sharma et al., 2000). The LIM factor Lhx3 selectively triggers expression of FGF receptor 1 (FGFR1) in MMC neurons, thereby making their motor axons attractive to fibroblast growth factors (FGFs). Acting as a secreted long-range chemoattractant, FGF is expressed in dermomyotome, a temporary structure that is later converted to MMC target cells, the axial muscles. Interestingly, forced expression of Lhx3 in LMC MNs reprograms their identity to MMC cells and induces FGFR1 expression (Figure 1.2) (Shirasaki et al., 2006). This interconversion of LMC into MMC motor neurons is associated with increased projections to the FGF+ dermomyotome (Sharma et al., 2000; Shirasaki et al., 2006). MMC motor neurons also express EphA3 and EphA4 receptors; however, their regulation by Lhx3 has not been shown yet. EphA3+ and EphA4+ MMC motor axons are repelled by ephrin-As expressed by sensory neurons of dorsal root ganglion (DRG), highlighting the crucial role of heterotypic trans-axonal signaling and interaction between sensory and motor axons in proper assembly of sensory-motor circuits. Likewise, ephrin-As are expressed in ventral mesenchyme, preventing MMC axons from innervating limb muscles (Gallarda et al., 2008). In double knockouts of EphA3 and EphA4 MMC axons misproject into DRG, a phenotype which is also observed in FgrR1 homozygous mutants (Figure 1.2) (Gallarda et al., 2008; Shirasaki et al., 2006).
These findings on LMC and MMC guidance reinforce the idea that transcription factors control axonal projections by regulating the expression of cell surface molecules (e.g. guidance receptors and their ligands). However, how combinations of several transcription factors fine-tune the expression of functionally different axon guidance receptors or ligands is an intriguing question that has to date not been fully elucidated. Furthermore, future work will be required to better understand how functionally different guidance receptors are coordinated with one another, and how their different responses are combined to make the pathfinding decisions in motor axons which are exposed to different guidance cues.
Figure 1.2. Specification of LMC and MMC motor axon projections to target muscles. A) Schematic illustration of a cross section through developing spinal cord and limb bud, representing the expression of different guidance receptors and ligands by subsets of motor neurons, intermediate environment and target muscles. See the text for detailed description of their functions. B) Top panels illustrating the distribution of transcription factors as well guidance molecules in motor neurons and muscles, and Bottom panels showing the transcriptional link between TFs and genes coding for guidance molecules. Question marks (?) indicate possible but not evidenced relationship.
Transcriptional Specification of sensorimotor circuits

In order to establish a functional mature nervous system, neurons should be assembled into precisely interconnected neuronal circuits during development. Assembly of multiple sensory and motor neurons into functional locomotor circuits in vertebrates is one of the best examples highlighting the connectional specificity. The sensory neuron cell bodies are located in DRG; they also have central and peripheral branches that extend toward the spinal cord and periphery (muscle) respectively. Peripheral branches of different subclasses of proprioceptive sensory neurons fasciculate with motor axons projecting to different peripheral muscles, and their central branches preferentially synapse with the dendrites of the same motor neurons in the spinal cord, thereby forming unique sensory-motor circuits during development. These precisely specified circuits between motor and sensory neurons are crucial for accurate control of movements (Arber, 2012).

In addition to controlling the peripheral axon pathfinding of different subclasses of motor neurons, transcriptional programmes also regulate the formation of functional sensory-motor circuits. Two members of E-twenty-six (ETS) transcription factors, Pea3 and Er81, are expressed in subsets of MN pools and sensory neurons (proprioceptive afferents) respectively, and regulate sensory-motor connectivity (Arber et al., 2000; Livet et al., 2002). The onset in the expression of these ETS transcription factors coincides with the stage when sensory or motor axons begin invading their peripheral targets, which is due to exposure to peripheral neurotrophic factors, GDNF and NTF (Ladle et al., 2007). GDNF triggers Pea3 expression in cutaneous maximus (CM) and latissimus dorsi (LD) MNs (Haase et al., 2002), whereas Neurotrophin 3 (NT3) is required for Er81 expression in proprioceptive afferents (Patel et al., 2003). Interestingly, Hox dependent programs are necessary for conferring the motor neurons sensitivity to the retrograde inductive effect of GDNF (Dasen and Jessell, 2009; Vermot et al., 2005). In Er81 mutants, the axons of proprioceptive sensory neurons stall prematurely in more dorsal regions of the spinal cord and fail to arborize near the motor neuron dendrites (Arber et al., 2000). Pea3 mutant mice are defective in MN cell body positioning, MN dendrite elaboration, and sensory-motor connectivity. In the absence of Pea3 function, Cad-8 and Sema3E are repressed in CM and LD.
motor neurons and Cad-7 is ectopically induced in those neurons (Livet et al., 2002). The mispositioning of motor pools observed in Pea3 mutants is partly due to the modified expression of type II cadherins (i.e. cad-7 and Cad-8) as a similar phenotype is also observed in chick embryos with deregulated type II cadherins (Price et al., 2002). Furthermore, it has been shown that expression of Sema3E by selected MN pools, and its high-affinity receptor plexin D1 (Plxnd1) by proprioceptive sensory neurons, is a critical recognition system for organization of sensory–motor circuits in mice (Pecho-Vrieseling et al., 2009).

Together, these results indicate that induction of new a set of transcriptional programs by target-derived trophic factors is deterministic for accomplishment of functional neural circuits, whereby they regulate final cell-body position, axon arborization within targets, and dendritic arborization within spinal cord. Identifying the downstream effectors regulated by Pea3 and Er81 such as guidance receptors, ligands, and cell adhesion molecules will shed further light on molecular mechanisms of cell-body localization as well as axonal and dendritic elaborations.

**Specification of MN identity and axonal projections in fruit fly**

The segmented central nervous system (Ventral Nerve Cord; VNC) of *Drosophila* is composed of two identical left and right sides known as hemisegments (half segment). In the central abdominal segments, each of hemisegments contains 36 motor neurons (MNs) innervating to 30 different body wall muscles of the same hemisegment. Based on the route that they choose, the somatic MNs are categorized into the intersegmental nerve (ISN), the segmental nerve (SN) and a minor route, the transverse nerve (TN). Most ISN, ISNb and ISNd motor neurons make synapse with muscles in the next posterior segment (thus the name "intersegmental"), whereas motor neurons of the segmental nerves (SNa and SNC) project to muscles of the same segment (Landgraf et al., 1997; Kohsaka et al., 2012). Depending on the dorsoventral positioning of their target muscles, ISN and SN motor neurons can be further subdivided into ISN⁰ (MNs projecting to dorsal muscles), ISN¹ and SNa (MNs projecting to lateral muscles), ISNb, ISNd and SNC (MNs projecting to ventral muscles). On the other hand and according to internal vs external localization, muscles can be grouped into internal muscles, spanning the width of a segment, which are innervated by ISN and external muscles, with
transverse orientation, which are innervated by SN and TN motor neurons (Landgraf et al., 2003; Butler and Tear, 2007). This division into ISN-internal and SN/TN external motoneuron-muscle units in the *Drosophila* larva is somewhat comparable to the musculature of vertebrate limbs where dorsal vs ventral muscles groups are innervated by motor neurons projecting through divergent routes (Figure 1.3) (Kania and Jessell, 2003; Ryan et al., 1998).

In contrast to vertebrates where MN progenitors are restricted to a particular sector, Drosophila MNs are generated from a number of different neuroblasts, which are distributed along both the mediolateral and dorsoventral axes of the central nervous system (CNS) (Briscoe and Ericson, 2001; Jessell, 2000; Schmid et al., 1999). As in vertebrates, however, *Drosophila* MNs that project axons through common trajectory pathways, share similar sets of transcription factors (e.g. homeodomain (HD) proteins), (Figure 1.3) and these unique combinatorial codes are probably responsible for expression of different set of cell surface receptors in each distinct MN subclass (Kania and Jessell, 2003; Landgraf and Thor, 2006). Zfh1 is a motor generic zinc finger homeobox protein expressed postmitotically by all MNs as well as some interneurons, and is believed to play a general role in specification of MNs. In embryos lacking this transcription factor motor axon guidance in both ISN and SN is severely impaired. On the other hand ectopic Zfh1 expression in interneurons with axons normally residing in the central nervous system leads to lateral projection of their axons out of the CNS (Layden et al., 2006). This redirection could be the result of a change in transcriptional profile of those neurons; hence changing the repertoire of guidance molecules in their axon growth cones, which in turn causes a different response to environmental cues.

Apart from the generic factor Zfh1, different sets of transcription factors are expressed in ventral MNs as compared to dorsal ones (Figure 1.3). Concerted action of *dHB9* and *Nkx6* is required for specification of ventral MNs (Broihier and Skeath, 2002; Broihier et al., 2004; Odden et al., 2002), partly through activating lim3/islet gene expression within those neurons (Broihier et al., 2004). Dorsally projecting motor neuron identity is defined by yet another homeobox gene called *even-skipped (eve)* (Doe et al., 1988; Fujioka et al., 2003; Landgraf et al., 1999). Interestingly, there is cross-repressive interaction between dorsal and ventral fate
determinants, so that parallel collaboration of Nkx6 and dHB9 restricts eve expression to dorsal MNs. In dHB9 and Nkx6 double mutant background eve is ectopically expressed in six extra neurons per hemi segment. On the other hand, dHB9 is derepressed in eve mutant dorsal MNs. Pan-neural mis-expression of eve represses dHB9 and Nkx6 expression, which in turn inhibits defasciculation of ventral MNs, and all MNs project their axons toward dorsal regions. In addition, Lim3 and Islet expression in ventral MNs is repressed by pan-neuronal Eve expression, demonstrating that Eve can repress Lim3 and Islet. On the other hand, neural misexpression of dHB9 results in nearly complete loss of Eve expression in all dorsal MNs, and prevents their axons from innervating their appropriate target muscles. Also, misexpression of Nkx6 in all postmitotic neurons dramatically reduces Eve expression in the vast majority of neurons, with the exception of two of dorsal MNs and an interneuron (Broihier and Skeath, 2002; Broihier et al., 2004; Fujioka et al., 2003; Garces and Thor, 2006; Landgraf et al., 1999; Odden et al., 2002). Drosophila Nkx6, dHb9 and Eve proteins contain domains that in vertebrates interact with the Groucho co-repressor, suggesting that they function as transcriptional repressors (Broihier and Skeath, 2002; Fujioka et al., 2003; Muhr et al., 2001; Uhler et al., 2002). Indeed, Eve represses dHB9 through its Groucho-interaction domain (Broihier and Skeath, 2002). Also, it has been suggested that Nkx6 and dHB9 repress Eve transcription via direct binding to its enhancer and recruiting the Groucho-dependent repressor complex (Broihier and Skeath, 2002; Broihier et al., 2004). Nkx6 and Hb9 have similar roles in MN specification in Drosophila and in vertebrates; however, the genetic network in which they work is different between these two groups of animals. In vertebrates Nkx6 is upstream of Hb9, while in Drosophila Nkx6 and dHb9 exhibit a side by side requirement for MN fate determination (Arber et al., 1999; Broihier et al., 2004; Sander, 2000; Vallstedt et al., 2001). Furthermore, Nkx6 is necessary to promote axon growth and guidance in Nkx6-positive neurons and axongenesis in ISNbs is severely compromised in Nkx6 mutant embryos. In support of this, Nkx6 is necessary for the expression of the neural adhesion molecule Fasciclin III in these neurons. It is still unknown whether Nkx6 regulates Fasciclin III directly or indirectly through its downstream factors (e.g. Lim3 and Isl1). However, a recent study has suggested that Nkx6 can work as both transcriptional activator and inhibitor (Syu et al., 2009), raising the
possibility that positive regulation of Fasciclin III could be directly performed by Nkx6. Generally these findings indicate that Nkx6 plays two different pivotal roles: cell fate specification and axon guidance, thereby linking the neuronal subtype identity to neuronal morphology and connectivity (Figure 1.3) (Broihier et al., 2004).

Two Lim-homeodomain proteins, Islet and Lim-3, are involved in specification as well as axon guidance of two groups of ventral MNs, ISNd and ISNb, each of which innervate different subset of target muscles. While islet is expressed in both ISNd and ISNb neurons, expression of Lim-3 is limited to ISNb MNs (Thor et al., 1999). lim-3 mutant embryos show ticker ISNd branches along with abnormal ISNb innervations. In the absence of lim-3, ISNb MNs are converted to ISNd type neurons, hence leading to uprooted innervations of ISNd-specific muscles by ISNb MNs (Thor et al., 1999). On the other hand, misexpression of lim-3 in ISNd MNs reroute their axons to ISNb-specific muscles, highlighting that combinatorial function of endogenous Islet with ectopic Lim-3 in ISNd results in an identity switch to ISNb MNs (Thor et al., 1999). Deciphering the target genes regulated by these transcription factors is prerequisite to better understand how these combinatorial codes specify neural identity and axonal projections.

Even-skipped (Eve), a homeodomain protein, and Grn, a GATA family protein, are two transcription factors specifically expressed in dorsally projecting MNs (ISN°)(Fujioka et al., 2003; Garces and Thor, 2006; Landgraf et al., 1999). Unc-5, a repulsive receptor for Netrin, is also expressed in dorsal MNs and is required for proper guidance of their motor axons(Keleman and Dickson, 2001; Labrador et al., 2005). It has been found that Eve is required for Unc-5 transcription in two dorsal MNs called aCC and RP2 (Labrador et al., 2005). In the absence of Eve, nearly all of aCC and RP2 axons fail to exit the CNS toward the muscle field, and the expression of Unc-5 is almost completely suppressed in these neurons (Labrador et al., 2005). In grn mutant embryos, around 85% of aCC and RP2 motor axons are unable to innervate their target muscles, instead they stall midway and some of them ectopically project to wrong target muscles (Figure 1.3) (Garces and Thor, 2006).
Figure 1.3. Neuromuscular connectivity and motor neuron specific transcriptional codes of *Drosophila* embryo. A) Muscles (numbered rectangles according to Bate (1990)) and the innervating motor axons are shown in concurring colors (Bate, 1990). The color-matched boxes in the left show accordance between the external or internal positioning of muscles (top) and the projecting motor route (bottom). Majority of motor neurons exit from the CNS through two major nerves, the segmental nerve (SN) and intersegmental nerve (ISN), from which they defasciculate to innervate distinct groups of muscles. Subgroups of motor neurons express different set of transcription factor codes which play a crucial role in targeting them to their appropriate muscle fields. B) Schematic representation of dorsal (upper half) and ventral (lower half) projecting motor neurons in *Drosophila*. Dorsal motor neurons or ventral motor neurons (left) are shown projecting to either dorsal or ventral muscles. Some transcription factors required for their specification (center) and receptors expressed by those motoneurons (right) are also represented. Dashed lines and "?" represent unknown interactions or components. See the text for detailed description.
Evolution of motor neurons from invertebrates to vertebrates: Chordate Bottleneck Model
In vertebrates, all somatic motor neurons express orthologs of *Nkx6, hb9, islet* and *Lim3*; however, while in *Drosophila* their expression are restricted to ventral motor neurons (Sander, 2000). On the other hand vertebrate *evx-1* and *evx-2* genes, orthologs of *Drosophila* *even-skipped*, are not found in any developing motor neurons, and instead are restricted to subsets of commissural interneurons (Moran-Rivard et al., 2001). These differences have led to a model called "chordate bottleneck" based on which *Drosophila* *Nkx6+, Hb9+, Islet+* and *Lim3+* motor neurons are homologous to vertebrate somatic motor neurons and the *Drosophila* *Eve+* dorsal motor neurons have no vertebrate motor neuron counterpart. This model suggests that *Drosophila* dorsal motor neurons may have been “lost” during the invertebrate to chordate transition (Landgraf and Thor, 2006; Thor and Thomas, 2002). *grn* is another transcription factor present in *Drosophila* dorsal motor neurons, nevertheless its vertebrate orthologs *Gata2/3* are not expressed in spinal motoneurons, further supporting the chordate bottleneck model (Garces and Thor, 2006; Karis et al., 2001; Pata et al., 1999).

Transcriptional regulation of guidance cues in *Drosophila* muscles
The accurate axon guidance of motor neurons is achieved by the selective responsiveness to environmental cues. In parallel to controlled expression of guidance molecules on axons, regulation of such cues in target and non-target muscles as well as environments in which axons travel also plays a pivotal role in motor axon pathfinding in both vertebrates and invertebrates. Target muscle selection is achieved through attraction to the target cells and repulsion from non-target cells. (Inaki et al., 2007; Kania and Jessell, 2003; Kania et al., 2000; Luria et al., 2008; Shishido, 1998; Taniguchi et al., 2000). An invertebrate example of such regulation is the transcriptional repression of a repulsive cue in a group of *Drosophila* muscles (Inaki et al., 2010). *Tey* is a putative DNA binding transcription factor, the expression of which is confined to a single muscle, M12, among the 30 muscles in the body wall. *Tey* is involved in negative regulation of transcription and inhibits the expression of its target genes (i.e. *toll*) (Inaki et al., 2010). *Toll* is a transmembrane receptor with 22 leucin rich repeats (LRRs) expressed on muscles but not motor neurons, which serves as a repulsive cue in
the development of *Drosophila* neuromuscular junctions (Rose et al., 1997). Toll is differentially expressed in M13 and some other surrounding muscles but not in the neighboring M12, and by acting as a repellant it locally prevents the innervation of M12 specific motor neuron axons onto M13. The inhibitory function of Toll is negatively suppressed in M12 by the transcription factor Tey, which works as a transcriptional repressor. In *tey* mutant animals, Toll is ectopically induced in M12 and synapse formation on M12 is impaired (Inaki et al., 2010). On the other hand, ectopic Tey in M13 prevents Toll expression in the muscle, and M13 is innervated by ectopic motor axons. This is an excellent example of how a target cell can be specified via repression of an inhibitory signal in one among a group of target cells expressing the signal.

The identities of *Drosophila* body wall muscles are determined by several transcription factors that are differentially expressed in subsets of muscle and/or their progenitor cells (reviewed in Tixier et al., 2010). Furthermore, various target recognition molecules, including homophilic cell adhesion molecules (CAMs), secreted factors and heterophilic ligands or receptors are expressed in specific muscles (reviewed in A. Nose, 2012). It is likely that the expression of genes coding for these target recognition molecules are controlled by muscle specific transcription factors; thereby regulating neuromuscular target specificity.

**Aims:**

How the axon of an individual neuron makes different decisions during pathfinding is dependent on the expression of a variety of surface molecules that interact in a complex manner with the surrounding environment in order to reach their targets. To date, numerous receptors have been identified, however their precise expression on individual motoneurons as well as how they are regulated is not known.

In this study, which aimed to shed light on how transcriptional codes regulate the expression of cell surface molecules, we used two *Drosophila* dorsal MNs, aCC and RP2 as a model. There were several advantages for using these neurons as a model: i) aCC and RP2 could be individually labeled and isolated. ii) Using a specific GAL4 driver, RN2 GAL4, it was possible to over-express or mis-express different genes exclusively in aCC and RP2, and examine their effects. iii) Studying the phenotype of ISN\textsuperscript{DM} was quite
simple. iv) It was previously established that the homeodomain transcription factor, Even-skipped or eve, as well as the GATA factor, Grn, played an essential role on the specification of motor neurons that project to dorsal muscles in Drosophila. The main aims of this project were as follows:

A) To decipher the mechanisms through which Eve acted in dorsal MN specification and identify novel genes regulated by it, studies were initiated in which mRNA profiles of wild type aCC and RP2 MNs were compared with eve mosaic neurons using microarray and ISH methods (Chapter 2).

B) To dissect the functional and transcriptional links between Eve and its downstream genes identified in Aim 1, several experiments including multiple loss of function and gain of function approaches were designed and conducted (Chapter 3).

C) The Netrin receptor, Unc-5, was used as a model to understand how Eve and Grn combinatorial transcriptional code regulates the expression of this axon guidance receptor in aCC and RP2 MNs (Chapter 4).
Chapter 2: Comparative mRNA profiling of wild type and eve mosaic dorsal motoneurons identifies multiple eve-regulated genes.
2.1 Introduction
How the axon of an individual motoneuron makes different decisions during pathfinding is dependent on receptors expressed on its membrane, and a coordinated response to several extracellular signals will be responsible for their proper guidance to its given muscle target. Thus, the precise spatial and temporal expression of these membrane molecules will directly determine motoraxon behavior. To understand how guidance is programmed in individual neurons we have focused on the intersegmental nerve (ISN) of the Drosophila embryo and specifically on the aCC and RP2 motoneurons. Both motoneurons fasciculate, project away from the CNS and after navigating through the muscle field they finally innervate their targets, the most dorsal muscles. Their role is essential since they pioneer the ISN and influence the guidance decisions of the follower motoneurons. The homeodomain transcription factor Even-skipped (Eve) largely determines the specific guidance characteristics of aCC and RP2 dorsal motoneurons (dMNs) in Drosophila. When eve is absent in dMNs they no longer project away from the CNS (Figure 2.1) and the ISN stops short of its most dorsal target muscles. Given the profound effect of eve on dMN guidance an analysis of the transcriptional profiles in wild-type and eve mutant dMNs would allow us to understand with single cell resolution how guidance is programmed on them (Landgraf et al., 1999).

2.2 FACS based isolation of RNA from dorsal motoneurons followed by microarray hybridization:
We used the UAS/Gal4 system to specifically label only 3 cells per hemisegment, the aCC, RP2 and the pCC interneurons with the R2Gal4 driver expressing mCD8GFP in both wild-type or eve mutant Drosophila embryos (Figure 2.1). Since the developmental stage we were interested on analyzing was the earliest stages of ISN axon guidance we synchronized and aged the embryos accordingly. After single cell dissociation of embryos by trypsinization, we purified GFP positive dMNs by fluorescent-activated cell sorting (FACS) and extracted total RNA. Finally, labeled cRNA, was hybridized to GeneChip Drosophila Genome 2.0 Arrays (Figure 2.1). To overcome early patterning and non-cell autonomous effects of eve loss of
function we used eve mosaic mutants where *eve* is only absent in the aCC, pCC and RP2 neurons (Fujioka et al., 2003).

**Figure 2.1. Sorting of wild type and eve mosaic aCC and RP2 motor neurons followed by total RNA extraction, and microarray hybridization.** A) Cartoon representing the relative position of aCC and RP2 motoneurons in a Drosophila embryo nerve cord with laterally projecting motoneurons; the area shadowed in yellow is represented in cartoons B and C. B-C) Schematic cartoons illustrating magnified view of panel A inset in *wild-type* (B) and *eve* mosaic embryos where *eve* is only absent in aCC, RP2 and pCC neurons (C). In *wild-type* embryos aCC and RP2 axons exit laterally and innervate their most dorsal target muscles, whereas in *eve* mosaic animals majority of them fail to exit the CNS and project toward muscles field. D) Stage 13 embryos carrying RN2 driving GFP either in a *wild-type* background (upper panel) or an *eve* mosaic background (lower panel). E) Embryos were dissociated and GFP positive aCC and RP2 neurons were FACS sorted based on their fluorescence. F) Total RNA was extracted from FACS sorted cells and analyzed for quality by electrophoresis based on the presence of the double band of ribosomal RNA (rRNA).
2.3 Microarray data normalization and analysis:
Three biological replicates and three technical replicates were performed for each group. Files were subjected to Bioconductor’s affyPLM to detect if any blemishes were present on the GeneChips. GeneChips without major blemishes were used for further quality control assays, including examination of Normalized Unscaled Standard Error (NUSE) and Relative Log Expression (RLE) boxplots (Brettschneider et al., 2007). GeneChip CEL files were then loaded to oneChannelGUI Package (Sanges et al., 2007) and were processed with the GC-RMA algorithm. Data were converted to log2 scale and filtered to eliminate genes with lower intensity or with unchanged expression level between WT and the eve mutant. Principal component analysis (PCA) and Hierarchical clustering (HCL) confirmed that all arrays have good quality to be used for further bioinformatic analysis; principal component analysis (PCA) of arrays resulted in clear segregation of wild type arrays from eve mutant arrays (Figure 2.2 A) and hierarchical clustering (HCL) of arrays produced two major clusters: one containing arrays hybridized with wild type samples and the other containing array hybridized eve mutant samples (Figure 2.2 B). MA and Volcano plots of differentially expressed probe sets indicate that removal of eve causes a substantial change in expression profile of dorsal motor neurons, mainly leading to downregulation of genes normally expressed in those neurons (Figure 2.2 C and D).
**A** Samples PCA plot

**B** Samples HCL

**C** MA plot
- absolute fc=1.5, p-value=0.05

**D** Volcano plot
- absolute fc=1.5, p-value=0.05
Figure 2.2. (A-B) Quality control (QC) plots (PCA and HCL) of arrays used in our study. A) Principal component analysis (PCA) of arrays shows a clear separation of wild-type (WT) samples from mutant samples (mu). The first principal component (PC1 on X axes) mutant arrays are positioned on the right half and wild-type arrays on the left half of the plot. Samples labeled with R are technical replicates. B) Hierarchical clustering (HCL) of arrays indicates homogenous clusters of wild-type arrays versus eve mutant arrays highlighting the difference in general expression profile between wild-type and mutant groups. Arrays with names ending in R are the technical replicates of arrays with same name but lacking R. (C-D) MA and Volcano plots for differentially expressed probe sets, red dots, detected by limma method. C) An MA-plot for the expression level data which plots the differences of the log intensities between wild-type and eve mutant samples (M, y-axis) versus the mean log-intensities (A, x-axis) across the two groups. A -5 value represents a 5.0 fold downregulation in eve mutants neurons. D) Volcano plot of differentially expressed probes in which the x-axis denotes fold change (a negative value represent downregulation in eve mutant neurons versus wild-type neurons) and y-axis denotes minus the log of p-value; hence a y-axis value of 2.0 and 4.0 represents a p-value of 0.001 and 0.00001 respectively. The volcano plot shows the presence of genes (represented by red dots) that have high gene expression profile changes and that are also highly statistically significant as evidenced by the p-values and the large fold change values. Both MA and volcano plots highlight that elimination of eve from aCC and RP2 motor neurons has resulted in strong reduction in transcription of genes that are normally expressed in wild-type neurons.

Using the Limma package we carried out statistical analysis to determine differentially expressed genes between WT and mutant conditions. The p-value of the moderated t-test was adjusted for multiple hypotheses testing, controlling the false discovery rate (FDR) using the Benjamini- Hochberg procedure. We identified 561 genes that were differentially expressed between wild-type and eve mutant dMNs displaying a false discovery rate (FDR) <0.05 and at least a 1.5 fold difference. Most of the identified genes showed down-regulation in eve mutants compared with controls. The differentially expressed genes were analyzed by DAVID tool with respect to their Gene Ontology category (Huang et al., 2009) as well as manually annotated for function searching three databases: GOToolBox (Martin et al., 2004), Flybase (McQuilton et al., 2012) and FlyTF (Pfreundt et al., 2010). Based on their involvement in specific biological processes, 227 genes were categorized into 55 clusters.

Interestingly, among the top 10% clusters are two neuronal related GO terms: Neuron Projection Morphogenesis and Neurological System Process, with downregulation of genes that are assigned for these terms (Table 2.1). Genes linked to these GO terms were examined by DAVID 2D heat map, a tool that is useful to better interpret the biological relevance of inter-related genes (Figure 2.3).
2.3). According to heat map data, genes belonging to Neuron Projection Morphogenesis could also be subgrouped to other GO terms such as axogenesis and axon guidance. Genes assigned to Neurological System Process could be subdivided to different GO terms such transmission of nerve impulse, synaptic transmission, neurotransmitter transport, vesicle mediated transport and exocytosis (Figure 2.3).

Consistent with previous findings, we also identified downregulation of the zinc-finger/homeodomain gene zfhl in eve mutant dMNs (Layden et al., 2006). Furthermore, unc-5 (Labrador et al., 2005) or grn (Garces and Thor, 2006), known to be down regulated in eve mutant dMNs showed a clear trend towards down regulation, that our statistical analysis, however, did not consider significant. This demonstrates that the selected FDR thresholds employed for data analysis were stringent enough to minimize the false positive results, thereby highlighting genes with real differential expression.
Figure 2.3. Gene Term 2D heat map. This map that was made by DAVID tool shows the relationship among highly related genes and their annotation. Green square means that there is reported evidence for corresponding gene-term association, and black square means that for corresponding gene-term association no evidence has been reported yet. Neuron Projection Morphogenesis (A) and Neurological System Process (B) were among top enriched GO terms. Using 2D heat map, the genes belonging to these terms were further analyzed to understand the common biology and pinpoint the key differences among related genes. Of particular importance to our study axonogenesis and transmission of nerve impulse are among the over represented GO terms. We chose three genes linked to these annotations, *Fas2*, *nrg* and *beat la* to perform additional functional and transcriptional experiments.

Table 2.1. Description of top 10% GO enriched clusters
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<td>regulation of neurotransmitter levels</td>
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2.4 Eve coordinately regulates attractive, repulsive and adhesive cell membrane molecules

While numerous receptors responsible for motoneuron guidance have been identified to date, their precise expression on individual motoneurons, how their coordinated expression is regulated and how their signals are integrated is not well understood. Our results provide us with a profile of genes expressed with single motoneuron resolution that is unique to understand how guidance decisions of individual motoneurons are integrated and transcriptionally regulated. Therefore, we decided to further analyze genes coding for membrane molecules annotated with axonal related processes showing differential expression in wild-type and eve mutant aCC and RP2 dMNs. Among the GO category “Neuron Projection Morphogenesis” we identified several cell surface molecules not previously known to be regulated by eve: Beat Ia (Fambrough and Goodman, 1996), an attractive receptor (Siebert et al., 2009) for Sidestep (Sink et al., 2001), the cell adhesion molecule (CAM) Neuroglian (Nrg), the Drosophila homolog of the vertebrate LI CAM (Hall and Bieber, 1997) and the CAM Fasciclin 2 (Fas2) (Grenningloh et al., 1991), the Drosophila homolog of N-CAM (neural cell adhesion molecule). Together with the Unc-5 Drosophila homolog, a repulsive receptor for Netrins (Harris et al., 1996; Mitchell et al., 1996) and also regulated by eve (Labrador et al., 2005) they all belong to the immunoglobulin superfamily (IgSF) and may mediate guidance together downstream eve. We examined the mRNA expression of these genes by fluorescent in situ hybridization. In this assay we used fluorescent RNA probes for each single gene in wild-type and eve/eve embryos where the cell bodies of aCC and RP2 dMNs were also labeled by protein markers (RN2Gal4 driving UAS-taumyc). While all of them are expressed in both dMNs in wild-type (eve/+ ) embryos by stage 13, their mRNA levels are significantly reduced in eve/eve dMNs from age-matched siblings (Figure 2.4). Our results indicate that eve regulates mRNA levels of Fas2, beat Ia, nrg and unc-5 in aCC and RP2 dMNs. Furthermore, they strongly suggest that eve programs the guidance decisions of both motoneurons at least partially through these cell surface molecules.
Figure 2.4. **eve** regulates genes coding for Beat1a and Unc-5 receptors and the cell adhesion molecules Nrg and Fas2. The expression of Fas2, beat 1a, nrg, and unc-5 mRNA was examined in aCC and RP2 motor neurons of stage-15 **eve** mosaic embryos and their heterozygous siblings using in situ hybridization and confocal imaging. An **RN2-Gal4** driver is used to express taumyc and identify the aCC and RP2 motoneurons (green) and in situ signals are in magenta. The two columns on the left contain panels for wild-type neurons and the two columns on the right show panels of **eve** mosaic neurons. XZ and YZ sections are displayed below and to the right of each panel. In wild-type embryos, clear expression of Fas2 mRNA (A-B), beat 1a mRNA (F-G), nrg mRNA (K-L) and unc-5 mRNA (P-Q) in both aCC and RP2 is observed. In **eve** mutant motor neurons, expression of these genes is substantially decreased Fas2 (C-D), beat 1a (H-I), nrg (M-N) and unc-5 (R-S). Graphs show the quantification mRNA expression in **eve** heterozygous and **eve** mutant aCC and RP2 neurons. X-axis indicates genotypes and number of cells used in quantification, and fluorescence intensity as arbitrary units [A.U.] is indicated on the Y-axis. Expression of all the genes shows statistically significant downregulation in **eve** mutant aCC and RP2 neurons. *** P<0.005.
2.5 Further Verification of Microarray data by ISH:
To further validate our microarray results we analyzed by in situ hybridization the expression of genes belonging to top enriched neuronal GO categories in wild-type and eve/eve aCC and RP2 dMNs (Figure 2.5). This validation allowed us to determine their expression and quantify their changes in mRNA levels with single cell resolution specifically in dMNs. All but 1 showed significant reduction (p<0.001) of mRNA signal in eve mutant dMNs (Figure 2.5).
Figure 2.5. Verification of microarray results using in situ hybridization. Genes downregulated in eve mosaic dMNs that belong to the top enriched GO terms in involved neuron projection morphogenesis or transmission of nerve impulse that were further validated. Validation was performed by in situ hybridization and single motoneuron analysis with confocal imaging. The expression of bsk (A, A', A"), gogo (B, B', B"), Ank2 (C, C', C"), wit (D, D', D"), Synaptogyrin (E, E', E"), cont (F, F', F"), cpx (G, G', G"), synj (H, H', H"), CanB (I, I', I"), nrv3 (J, J', J"), CG3408 (K, K', K"), and kek5 (L, L', L") mRNA was examined in aCC and RP2 eve mutant or heterozygous siblings at stage 15. An RN2-Gal4 driver is used to express taumyc and identify the aCC and RP2 motoneurons (green) and in situ signals are in magenta. The two columns on the left contain panels for wild-type neurons and the two columns on the right show panels of eve mutant neurons. XZ and XY sections are displayed below and at the right of each panel. Graphs show the quantification mRNA expression in eve heterozygous and eve mutant aCC and RP2 neurons for bsk (A"), gogo (B"), Ank2 (C"), wit (D"), Synaptogyrin (E"), cont (F"), cpx (G"), synj (H"), CanB (I"), nrv3 (J"), CG3408 (K"), and kek5 (L"). Genotypes and number of cells used in quantification are indicated on the X-axis, and fluorescence intensity as arbitrary units [A.U.] is indicated on the Y-axis. Expression of all the genes but synj shows statistically significant downregulation in eve mutant aCC and RP2 neurons. *** $P<0.005$, ** $P<0.05$. 
Chapter 3: Eve transcriptionally regulates a combinatorial code of cell surface guidance molecules composed of multiple receptors and CAMs.
**Introduction**

In the previous chapter, using cell-specific microarray and in situ hybridization (ISH) methods, we demonstrated that several axon guidance genes, including those coding for repulsive and attractive guidance cue receptors (*unc-5* and *beat1a* respectively) as well as cell adhesion molecules (*nrg* and *fas2*) are substantially downregulated in *eve* mutant dMNs. In this section, we present the data through which we have dissected the functional and transcriptional links between Eve and these four downstream genes.

**Combinatorial requirement of attraction, repulsion and adhesion in ISN guidance.**

Our previous results have identified several genes coding for membrane molecules that are downregulated in *eve* mutant dMNs. In *eve* mutants the ISN nerve almost never reach their appropriate dorsal target muscles (Fujioka et al., 2003; Landgraf et al., 1999), whereas *beat la, nrg* and *unc-5* single mutant embryos show only partially penetrant defects in ISN projections (Fambrough and Goodman, 1996; Hall and Bieber, 1997; Labrador et al., 2005; Siebert et al., 2009)(Figure 3.1). We reasoned that, since the expression of these genes is substantially decreased or absent in *eve* mutant motoneurons (Figure 2.4), the expression of all these genes is coordinated and their concerted function would be required for proper ISN guidance; hence, compound mutants of these genes should result in more severe pathfinding defects. To test this hypothesis we analyzed the motoraxon guidance phenotypes in double and triple mutants and compared them with the single mutants. We have organized the observed phenotypes by order of severity taking as a reference 3 branching points in the ISN from ventral to dorsal FB, SB and TB respectively (Figure 3.1 A), where phenotypes present before FB are early phenotypes, between SB and TB intermediate phenotypes and after TB late phenotypes (Figure 3.1 N, 0, P). Double mutants present phenotypes not previously observed in single mutants, for example *nrg/Y, unc-5/unc-5* double mutants start to present crossing before FB while this phenotype is not present in the single mutants. 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* or *unc-5* and *beat la* present stalls before FB (9% ±0.03149 and 21% ±0.02846, respectively)
while this severe phenotype is observed only occasionally in beat 1a mutants (3% ± 0.01086). Consistently, triple mutants present more severe phenotypes than double mutants. In particular, ISN crossing in the ventral muscle field is significantly increased in triple mutants when compared to double mutants (from 2% ± 0.020, 8.2% ± 0.029 or 10% ± 0.044 in nrg, unc-5 double, nrg, beat 1a double or unc-5, beat 1a double respectively to 15.3% ± 0.04382 in triple mutants). In summary, single mutants generally present late phenotypes while double mutant phenotypes are more severe and are also present in more ventral positions. The earliest most severe phenotypes are almost exclusively present in double and triple mutants and there is gradual increase in the number and severity of the phenotypes from double mutants to triple mutants.

The phenotypes observed when different combinations of mutants are analyzed reveal the requirement of all these molecules at various stages during dorsal motor axon guidance and at earlier stages than previously reported for any of them. Our results strongly suggest that the robustness of motoraxon guidance is provided by the combinatorial and coordinated attractive, repulsive and adhesive function provided by these surface molecules. Furthermore, co-regulation of all these genes by eve indicates that their concerted action is also regulated in place and time transcriptionally.

Table 3.1. Quantification of ISN phenotypes for eve, nrg, unc-5, and beat-1a and their compound mutants.

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<th>Penetrance</th>
<th>Number of hemicorpora</th>
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<th>intermediate defects</th>
<th>early defects</th>
<th>Hemisegments with ISN defects (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ISN bifurcation</td>
<td>ISN dorsal cross</td>
<td>ISN stall (SB)</td>
<td>ISN ventral cross</td>
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<td>nrg/Y</td>
<td>23</td>
<td>62%</td>
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<tr>
<td>unc-5/unc-5*</td>
<td>21</td>
<td>90%</td>
<td>210</td>
<td>6.6% (14)</td>
<td>10% (21)</td>
<td>0.5% (1)</td>
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<tr>
<td>beat 1a/beat 1a*</td>
<td>21</td>
<td>96%</td>
<td>210</td>
<td>1.9% (4)</td>
<td>5.2% (11)</td>
<td>13.3% (28)</td>
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<td>eve/beat 1a</td>
<td>10</td>
<td>100%</td>
<td>100</td>
<td>0.0% (0)</td>
<td>0.0% (0)</td>
<td>63.0% (63)</td>
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<td>nrg/Y; unc-5/unc-5*</td>
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<td>100%</td>
<td>100</td>
<td>18.0% (16)</td>
<td>9.0% (9)</td>
<td>4.0% (4)</td>
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<tr>
<td>nrg/Y; beat 1a/beat 1a*</td>
<td>11</td>
<td>100%</td>
<td>110</td>
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<td>0.0% (0)</td>
<td>11.8% (13)</td>
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<td>100%</td>
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<td>10.0% (17)</td>
<td>9.4% (16)</td>
<td>11.7% (20)</td>
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Penetrance: embryos presenting a phenotype. Dorsal cross and ventral cross, segmental cross of ISN after SB or before SB respectively. ISN stall (SB) stalls after SB. ISN stall (FB) stalls at FB or before.
Figure 3.1. The coordinated function of attractive, repulsive and adhesive molecules is required for ISN axon guidance. A) Schematic depictions of wild-type as well as different mutant phenotypes in late stage-16 embryos illustrating ISN motor axons in green and body wall muscles in magenta. Black arrows point to the fist (FB), second (SB) and third (TB) branching point of the ISN. The severity of phenotypes increases from left to right cartoons and different symbols for each phenotype observed are to the right of their description. 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 left and dorsal is up in all panels. Partial genotypes are indicated above each panel. B) In wild type ISN innervates dorsal muscles and respect segment boundaries. C) nrg/Y mutant embryo in which one ISN stall before reaching the dorsal muscles (arrowhead). D) Embryos lacking unc-5 gene show ISN dorsal crossing (white arrow). E-F) beat la mutant embryos showing ISN stall (tip loss; arrowheads in E and F), ISN ventral crossing (star in F) and ISN early stall (diamond in E). G) nrg/Y; unc-5/unc-5 double mutants showing ISN bifurcations (asterisk) and ISN dorsal crossing (arrow). H) nrg/Y; beat la/beat la double mutant embryo in which two ISNs stall very early (diamonds) and one ISN stall before the reaching the most dorsal muscles (arrowhead). I-J) beat la/beat la, unc-5/unc-5 double mutant animals having early ISN ventral crossing (star in I) and early ISN stalls (diamonds in J). K-L) nrg/Y; beat la/beat la, unc-5/unc-5
compound mutants with ISN bifurcation (asterisk in K), ISN ventral crossing (star in K) and ISN early stalls (diamond in L). M) ISNs in eve mosaic embryos lacking eve in aCC and RP2 motor neurons show both early (diamonds) and late (arrowheads) stalls. N-P) Quantification of ISN defects in different genetic backgrounds. Based on severity, the phenotypes were divided into three categories: N) ISN bifurcation as late phenotype (mild in severity), O) ISN tip loss and ISN dorsal cross as intermediate phenotypes (moderate in severity), and P) ISN early stall and ISN ventral cross as early phenotypes (most severe). Single mutants for guidance receptors and cell adhesion molecules largely show late and intermediate phenotypes. eve mosaic embryos as well as double and triple compound mutants mainly show early and intermediate phenotype, highlighting the increase in severity of guidance defects in compound mutants compared to single ones. For a detailed description and quantification of phenotypes see Table 3.1.

ev<sub>e</sub> interacts genetically with nrg, beat lα and unc-5

In order to dissect the functional link between eve and its downstream target genes in dMN guidance we examined possible genetic interactions among them during ISN guidance. First, we determined whether unc-5 and eve were able to interact genetically in a transheterozygous combination (eve/<sup>+</sup>, unc-5/<sup>+</sup>). This is a genetic test in which the levels of two genes are halved and if the combination shows a phenotype not present on each single heterozygous, it strongly suggests that both genes are in the same pathway. Reducing unc-5 and eve levels to 50% did indeed result in a transheterozygous interaction (Figure 3.2 and Table 3.2). In addition, this transheterozygous combination provided a sensitized background to test for more components of the same pathway in ISN axon guidance. This type of approach has been very successfully to identify novel components of the slit/robo pathway such as kuzbanian (Coleman et al., 2010). We therefore used the eve/<sup>+</sup>, unc-5/<sup>+</sup> genetic background to determine if beat lα works together with them during dMN guidance. Reducing eve, unc-5 and beat lα levels to 50% did indeed result in a triple transheterozygous interaction where ISN stalls and segment boundary crosses are very significantly increased (crosses from 2.0%±0.005 to 3.8%±0.015 and stalls from 3.0% ±0.010 to 7.5%±0.022 in eve/<sup>+</sup>, unc-5/<sup>+</sup> and eve/<sup>+</sup>, unc-5/<sup>+</sup>, beat lα/<sup>+</sup> respectively). Neither eve/<sup>+</sup>, unc-5/<sup>+</sup> or beat lα/<sup>+</sup> animals showed any significant ISN stalls or crosses (Figure 3.2 and Table 3.2). We next compared ISN defects in nrg/Y hemizygote mutant embryos with nrg/Y;eve/<sup>+</sup> animals. We found that removing one copy of eve in nrg mutant embryos resulted in more severe ISN guidance defects with very significant increases in stalls (from
3%±0.014 to 21.2%±0.038 in nrg/Y or nrg/Y; eve/+ respectively). These dose sensitive genetic interactions reveal that the genes identified in our mRNA profiling screen also work together downstream of eve to control dorsal motor axon guidance (Figure 3.2 and Table 3.2).

**Figure 3.2. eve interacts genetically with nrg, beat la and unc-5.** A) Schematic depiction of single hemisegment in wild type late stage-16 embryo illustrating ISN motor axons (green) and body wall muscles (Magenta). Black arrows point to the fist (FB), second (SB) and third (TB) branching point of the ISN B-D) 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 left and dorsal is up in all panels. Partial genotypes are indicated above each panel. In wild type (B), ISN innervates dorsal muscles and respect segment boundaries. In nrg/Y; eve/+ embryos (C) and in unc-5/+; beat la/+; eve/+ embryos (D) some ISNs stall before reaching their target muscles (white arrowhead) and some others cross the segment boundary (white star). 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 la or unc-5, transheterozygous for eve and unc-5 or triple transheterozygous for eve, unc-5 and beat la. Number of hemisegments (N) analyzed is indicated to the right of each genotype. For a detailed description and quantification of phenotypes see Table 2.
unc-5 and beat la partially phenocopy eve CNS exit phenotype

It has been previously established that aCC and RP2 dMN fail to exit the CNS in eve mutants (Fujioka et al., 2003). Therefore, we wanted to investigate if the guidance molecules that work downstream of eve present similar phenotypes. We used the RN2-Gal4 driver to specifically label aCC and RP2 motor axons and examined their ability to exit the CNS in single, different mutant combinations. While CNS exit was normal in all single mutants, we found a CNS exit failure in combinations where both unc-5 and beat la were eliminated (5% of hemisegments) (Figure 3.3). Therefore, the eve motor axon exit defects are partially phenocopied in compound mutants of both guidance receptors and strongly suggest that the combinatorial action of these guidance receptors mediate the guidance program established by eve in the aCC and RP2 dMNs.
Figure 3.3. Unc-5 and Beat-1a are required for lateral exit of aCC and RP2 motoneurons. An RN2-Gal4 driver is used to express taumyc and visualize the axonal projections of aCC and RP2 motor neurons in flat mounted stage-16 embryos. Anterior is up in all panels and partial genotypes are indicated above each panel. A) Motor axons of wild-type aCC and RP2 neurons fasciculate with each other and exit the CNS. B) The majority of these motor axons fail to exit the CNS in eve mutant aCC and RP2 motoneurons. C) Lateral projection of aCC and RP2 motor axons in nrg/Y;beat la/beat la double mutant embryos is normal. D-E) aCC and RP2 motor axons fail to exit the CNS in 5% of hemisegments in beat la/beat la,unc-5/unc-5 (D) or nrg/Y;beat la/beat la, unc-5/unc-5 (E) compound mutants indicating the joint requirement of both Unc-5 and Beat la receptors for dorsal motoneuron exit.

A combinatorial expression of cell surface molecules partially rescues the eve CNS exit phenotype.

Our previous results show that genes coding for repulsive and attractive guidance receptors (unc-5 and beat la respectively) as well as the CAMs (nrg and Fas2) are substantially downregulated or almost absent in eve mutant dMNs (Figure 2.4). Furthermore, motor axon CNS exit defects in unc-5, beat la double mutant embryos partially phenocopy those observed in eve mutant dMNs (Figure 3.3). Based on these data, we hypothesized that individual or combinatorial reintroduction of these four genes in eve mutant dMNs may partially rescue the lateral projection of their axons towards the muscle field. To test this hypothesis, we used the UAS/GAL4 system to re-express specifically in dMNs unc-5, beat la, nrg, and Fas2 individually or combinations of these genes.

Reintroduction of a single cell membrane molecule in eve mutant dMNs promoted different levels axonal exit ranging from no exit beyond what was already observed in eve mutants when nrg was re-expressed (10%±0.036 of hemisegments presenting exit of a single motoneuron) to some increase of lateral projections in
Fas2, beat la and unc-5 (25%±0.024, 36%±0.052 and 67%±0.030 of hemisegments presenting exit of a single motor axon, respectively). Nevertheless, the expression of a single gene leads only to the exit of a single motor axon of the pair per hemisegment rescued in most of the cases (only when unc-5 is re-expressed 11%±0.014 of hemisegments show dual exit) (Figure 3.4). A dual combination of surface molecules leads to a more robust rescue where both motoneurons present exit on each hemisegment (Figure 3.4). The only exception is when both CAMs are re-expressed since there is no further increase beyond that observed when Fas2 is reintroduced alone (26 ± 0.036 %, Figure 3.4). In the other combinations we observe two different types of dual exit: unfasciculated, where the axons of the motoneurons from the same hemisegment chose a different nerve root (Figure 3.4), or fasciculated when both axons join and exit through the ISN root (Figure 3.4). There is a higher ratio of fasciculated versus unfasciculated exit when combinations of a receptor and a CAM are re-expressed than when both receptors are expressed. Finally, reintroduction of triple combinations of surface molecules achieve the highest number of rescued hemisegments (85% ± 0.019 or 90% ± 0.029 when unc-5, beat and nrg or unc-5, beat and Fas2 are reintroduced respectively) and most of the rescued hemisegments show dual fasciculated exit (66% ±0.014 fasciculated versus 12% ±0.016 unfasciculated in unc-5, beat and nrg or 70%±0.055 fasciculated versus 11%± 0.034 unfasciculated in unc-5, beat and Fas2).

Together these findings suggest that the concerted expression and coordinated function of several cell surface molecules with their different roles is required downstream eve for the proper exit of motor axons from the CNS and correct nerve root choice.
Figure 3.4. A combinatorial expression of cell surface molecules partially rescues eve CNS exit phenotype. 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 up in all panels and partial genotypes are indicated on the left part 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 fully rescued exit and fasciculation in 66% and 69% of hemisegments respectively. O) Quantification of total exit for aCC and RP2 in different genetic backgrounds and 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 cell adhesion molecules (Nrg or Fas2) leads only to single exit in 26% if the hemisegments. P) Quantification of dual exit in different backgrounds where two or more genes are reintroduced in eve mutant aCC and RP2. Dual exit is divided between fasciculated or non fasciculated exit. The ratio of fasciculated to unfasciculated exit increases when a cell adhesion molecule 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 of Fas2 respectively. For a detailed quantification of phenotypes see Table 3.3.
Table 3.3. Rescue of eve/eve CNS exit phenotype

<table>
<thead>
<tr>
<th>Partial genotype*</th>
<th>Number of hemisegments</th>
<th>Hemisegments with exit</th>
<th>single exit</th>
<th>dual exit unfasciculated</th>
<th>dual exit fasciculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>120</td>
<td>9.17%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>UAS-nrg</td>
<td>167</td>
<td>9.72%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>UAS-Fas2</td>
<td>118</td>
<td>25%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>UAS-beat la</td>
<td>168</td>
<td>36.11%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>UAS-unc-5</td>
<td>132</td>
<td>55.72%</td>
<td>2.63%</td>
<td>8.28%</td>
<td></td>
</tr>
<tr>
<td>UAS-n,F</td>
<td>120</td>
<td>26.39%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>UAS-b,n</td>
<td>134</td>
<td>19.56%</td>
<td>0%</td>
<td>23.61%</td>
<td></td>
</tr>
<tr>
<td>UAS-b,F</td>
<td>159</td>
<td>24.17%</td>
<td>0.83%</td>
<td>25.33%</td>
<td></td>
</tr>
<tr>
<td>UAS-u,b</td>
<td>144</td>
<td>19.94%</td>
<td>20.78%</td>
<td>24.85%</td>
<td></td>
</tr>
<tr>
<td>UAS-u,n</td>
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<td>22.62%</td>
<td>13.07%</td>
<td>38.69%</td>
<td></td>
</tr>
<tr>
<td>UAS-u,F</td>
<td>72</td>
<td>22.10%</td>
<td>11.81%</td>
<td>44.17%</td>
<td></td>
</tr>
<tr>
<td>UAS-u,b,n</td>
<td>120</td>
<td>5.98%</td>
<td>12.58%</td>
<td>65.86%</td>
<td></td>
</tr>
<tr>
<td>UAS-u,b,F</td>
<td>72</td>
<td>8.33%</td>
<td>10.83%</td>
<td>69.17%</td>
<td></td>
</tr>
</tbody>
</table>

* All embryos are eve/eve;RN2Gal4+ the UAS constructs indicated. n: nrg, F: Fas2, b: beat la, u: unc-5.

Ectopic expression of eve is sufficient to induce the specific repertoire of cell surface molecules of dorsal motoneurons in interneurons.

Our previous data show that eve is required for the coordinated expression of an array of cell surface molecules on aCC and RP2 motoneurons (Figure 2.4). To determine if eve is sufficient to recapitulate the expression of these genes in other neurons we searched for a population of neurons that would not normally express the transcription factor or the cell surface molecules it regulates in dMNs. Expressing eve ectopically in those cells would allow us to examine its ability to induce its putative downstream genes. We identified a defined subpopulation of eagle expressing commissural interneurons, the EW neurons, which project axons across the posterior commissure of the adjacent anterior segment (Higashijima et al., 1996) that fulfill our requirements (Figure 3.5). Therefore, we used the eagleGAL4 driver (Dittrich et al., 1997) to misexpress eve in EW neurons and analysed the induction of Fas2, nrg, beat la and unc-5 by in situ hybridization. While none of these genes mRNA was expressed in wild type EW neurons, ectopic expression of eve resulted in the transcriptional induction of all of them (Fas2 in
96%; nrg in 69%; beat la in 81% and unc-5 in 66% of the scored hemisegments) (Figure 3.5). These results show that eve is sufficient to transcriptionally reprogram EW interneurons to express the same array of molecules it regulates in dorsal motoneurons.
Figure 3.5. Misexpression of *eve* in EW interneurons is sufficient to induce the repertoire of cell surface molecules of aCC and RP2 on them.

The expression mRNA of *Fas2, nrg, beat la* and *unc-5* was examined in EW neurons of stage-16 embryos using in situ hybridization and confocal imaging (in magenta). To identify EW cells an *eagleGal4* driver was used to express *UAS-tau-myc* (green). The two columns on the left (A, B, E, F, I, J, M, N) contain panels of wild-type EW neurons and the two columns on the right (C, D, G, H, K, L, O, P) show panels of EW neurons mis-expressing *UAS-eve* under the control of *eagleGal4*. XZ and YZ sections are displayed below and at the right of each panel respectively. There is no evidence for expression of *fasl* (A-B), *beat la* (E-F) *nrg* (I-J) or *unc-5* (M-N) mRNA in wild-type EW neurons. However, ectopic expression of *UAS-eve* in EW induces the expression of all of them in these cells; *fasl* (C-D), *beat la* (G-H), *nrg* (K-L) and *unc-5* (O-P). The percentage of cells expressing mRNA for each gene is shown on each panel.
Misexpression of *eve* or a combinatorial misexpression of its downstream cell surface molecules can reprogram the guidance behaviour of interneurons.

*eve* is required for guidance of dMNs and the projection of the ISN nerve to dorsal muscles (Doe et al., 1988; Fujioka et al., 2003; Landgraf et al., 1999). Additionally, our microarray analysis has identified a set of membrane molecules that work downstream of *eve* in dMNs mediating its guidance output (Figure 3.1-3.4). In fact, *eve* is able to induce the expression of these genes in other neurons that do not normally express them (Figure 3.5). While these results suggest that *eve* is able to transcriptionally reprogram those cells we wondered if expression of *eve* in EW neurons would be able to also reprogram their guidance. Therefore, we misexpressed *eve* in eagle-Gal4 positive neurons and could observe that EW axons no longer cross the CNS midline remaining ipsilateral and some of them project towards the muscle field as motoneurons would (24%±0.029) (Figure 3.6 B). We argued that if the guidance switch promoted by *eve* in EW interneurons is mediated by the surface molecules it regulates in them, we might be able to reprogram their guidance by changing these molecules rather than *eve*. To test this idea, we used *eagleGAL4* to misexpress each of these four genes alone or different combinations in eagle neurons and traced their axons (Figure 3.6). The expression of individual genes did not lead to EW projections into the muscle field except for a small number (3%±0.012 hemisegments) when *unc*-5 was misexpressed (Figure 3.6 D). Expression of *unc*-5, however, prevented EW axons from crossing the midline, similarly to when *eve* is misexpressed (Figure 3.6 D), probably due the repulsive activity of Netrin (Keleman and Dickson, 2001). Dual, triple and quadruple combinations of membrane molecules however had an increasing effect on CNS exit as the number of components of the system rose (Figure 3.6 E, F, G and H). Interestingly, a combination of both CAMs (*nrg* and *Fas2*) had no effect on exit but either of them, or both in combination had an enhancing effect when in the presence of *unc*-5 or both guidance receptors (Figure 3.6 G and H). For example exit increases from 5%±0.019 when both *beat la* and *unc*-5 are combined to 20%±0.041 when *nrg* is also present and up to 33%±0.049 if both CAMs are provided in the same background (Figure 3.6 H).
Our data demonstrate that eve can not only reprogram EW interneurons to express the array of cell surface molecules it normally regulates in aCC and RP2 but also their guidance decisions to resemble dMNs. Furthermore, the combinatorial expression of these guidance molecules can also redirect EW axons to join the motoraxon roots and exit towards the muscle as motoneurons do.

Figure 3.6. Misexpression of eve or a combinatorial misexpression of its downstream cell surface molecules can reprogram the guidance behaviour 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, embryos were also stained with anti-Fas2 antibody (green). Partial genotypes are indicated to the left of each panel and a cartoon representing EW projections to the left. A) Axons of wild-type EW neurons fasciculate and project across the posterior commissure of the adjacent anterior segment. B) In almost all of EW neurons misexpressing UAS-eve axons fail to cross the midline and in 24% of hemisegment project laterally and join the motor roots. C) No significant difference is observed in axonal projection of EW neurons misexpressing UAS-beat la. D) EW neurons misexpressing UAS-unc-5 do not cross the midline as when UAS-eve is misexpressed but fail to project laterally. E) Combinatorial misexpression of UAS-unc-5 with UAS-nrg results in lateral exit of 8% of EW axons. F-G) Triple misexpression of UAS-unc-5, UAS-beat la and UAS-nrg (F) or quadruple misexpression of UAS-unc-5, UAS-beat la, UAS-nrg and UAS-Fas2 (G) in EW neurons leads to lateral redirection of axons in 20% or 33% of hemisegments respectively. H) Quantification of lateral projection of EW axons in different genetic backgrounds. Single misexpression of either receptor or adhesion molecule does not lead to EW exit. Dual misexpression leads to exit in 5% or 8% of the hemisegments in unc-5, beat la misexpression or unc-5 with either adhesion molecule respectively. Dual misexpression of both adhesion molecules does not lead to exit. Triple or quadruple misexpression leads to increasing percentage of EW axons exiting through the nerve roots. F: Fas2, n: nrg, b: beat la, u: unc-5.
Chapter 4: A combinatorial code of GATA/Homeodomain transcription factors regulates Eve-dependent guidance receptor.
Introduction
According to the data explained in chapters 2 and 3, the homeodomain transcription factor Even-skipped (Eve) regulates the expression of four well-known axon guidance genes; *unc-5, fas2, nrg* and *beat-la* in dorsal motor neurons (dMNs). However, the exact mechanism by which Eve regulates these genes is not well defined. Combinatorial transcription factor codes play an essential role in neuronal specification and axonal guidance in both vertebrate and invertebrate organisms. Therefore, it is likely that other transcription factors may function in combination with Eve to tightly regulate the expression of its downstream effectors. One such code defined by Eve and the GATA 2/3 homologue Grain (Grn) is specifically required for motor axon projection towards dorsal muscles in *Drosophila*. To gain a better understanding of how Eve regulates the expression of its downstream guidance genes and to test whether the GATA factor, Grn, contributes to Eve in this regulation, we have studied Unc-5 receptor regulation in dorsal motoneurons.

We show that *grn* regulates Unc-5 receptor expression in dMNs. Through genetic analysis we show that both *grn* and *unc-5* function in the same pathway with *grn* working upstream of *unc-5* to promote guidance of dMNs. We also show that both *eve* and *grn* are required independently to induce expression of *unc-5* in dMNs and identify an enhancer element in the *unc-5* locus responsible for expression in dMNs that responds to *eve*. Furthermore, *grn* and *eve* can promote *unc-5* expression when misexpressed in dMP2 neurons. However, only the combined misexpression of the *eve-grn* dMN transcriptional code in dMP2 neurons can reprogramme their axonal pathfinding and promote axonal exit towards the muscle field (Garces and Thor, 2006). We now demonstrate that axonal exit of these 'reprogrammed' dMP2 neurons is directly dependent on the levels of *unc-5* that both factors are able to induce together.
unc-5 and grn function in the same pathway to mediate motoneuron pathfinding

We have previously shown a transheterozygous genetic interaction between eve and unc-5 (Figure 3.2 and Table 3.2). This genetic test relies on the limited availability of the products of the tested genes as the level of both genes is halved. Since the combination shows a phenotype not present on each single heterozygous it strongly suggests that both genes are in the same pathway. This dose-sensitive interaction indicated that an unc-5 heterozygous background could be used to identify other unc-5 regulators. In particular, this system could be used to screen for different transcriptional regulators present in dMNs that control unc-5 during axon guidance.

One potential candidate is the GATA 2/3 homologue, Grn, because it is required for proper guidance of the aCC and RP2 dMNs to their muscles (Figure 4.3 D,E). Furthermore, in grn mutants, motoneurons are properly specified because they are still able to induce Mad phosphorylation (Garces and Thor, 2006). Thus, we generated transheterozygous combinations of unc-5 and grn. Although grn/+ or unc-5/+ seldom showed any guidance defect, transheterozygous unc-5/++; grn/+ embryos presented significant guidance defects in their ISN motoneurons (13%±2 s.e.m. P<0.001; Figure 4.1C; Table 4.1) an average of 24% defective ISNs per embryo presenting a phenotype (Figure 4.1C; Table 4.1). This result strongly suggests that Unc-5 and Grn function in the same pathway to mediate guidance of dMNs.
Figure 4.1. *unc-5* interacts genetically with *eve* and *grn*. Motor axon projections of flat mounted late st-16 to st-17 embryos visualized with anti-Fas2. Anterior is left and dorsal is up in all panels. Partial genotypes are indicated below each panel. (A) Wild type (*Unc-5/+*). In wild type, the ISN nerve innervates dorsally to muscles 2/10 (by RP2 and U2 motor neurons) and muscles 1/9 (by aCC and U1 motor neurons), and respects the segment boundary. (B) In *unc-5/+;eve/+* transheterozygous embryo, some ISNs stall (arrowhead). (C) *unc-5/+;grn/+* transheterozygous embryo, some ISNs cross the segment boundaries fasciculating with ISNs in neighbouring segments (arrowhead). (D-F) Cartoons representing phenotypes on the ISNs of two adjacent segments and the cell body position of aCC, RP2 (magenta) and U motoneurons (magenta, lighter) in wild-type (D), *unc-5/+;eve/+* transheterozygous (E) and *unc-5/+;grn/+* transheterozygous (F) embryos. Quantification of ISN defects and number of hemisegments scored (n) is also shown in the cartoons. See Table 4.1 for detailed description.
**Table 4.1. Quantification of genetic interactions between *unc-5* and *eve* or *grn***

<table>
<thead>
<tr>
<th></th>
<th>Number of hemisegments</th>
<th>Hemisegments with ISN defects* (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ISN stall</td>
</tr>
<tr>
<td>eve*</td>
<td>20</td>
<td>20%</td>
</tr>
<tr>
<td>grn*</td>
<td>29</td>
<td>14%</td>
</tr>
<tr>
<td><em>unc-5</em></td>
<td>48</td>
<td>12%</td>
</tr>
<tr>
<td>eve* + <em>unc-5</em></td>
<td>41</td>
<td>65%</td>
</tr>
<tr>
<td><em>unc-5</em> / <em>grn</em></td>
<td>96</td>
<td>64%</td>
</tr>
</tbody>
</table>

*Embryos presenting a phenotype.
*ISN defects scored are hemisegments presenting crossing, bifurcation or stall.

**grn is required for *unc-5* expression in aCC and RP2**

This genetic interaction led us to propose that *grn* and *unc-5* work together to mediate guidance of dMNs. These results also suggested that *grn* acts upstream of *unc-5* and regulates its expression. To test this hypothesis, we analysed *unc-5* mRNA levels in *grn/+* and *grn/grn* mutant embryos. We determined *unc-5* expression by in situ hybridization with fluorescent RNA probes and labelled dMN (aCC and RP2) cell bodies with protein markers (*RN2Gal4* driving *UASTauMycGFP*) (Fujioka et al., 2003). This double-labelling technique allows us to determine levels of *unc-5* mRNA and quantify its changes with single-cell resolution (Labrador et al., 2005). *unc-5* is normally expressed in aCC and RP2 motoneurons in *Drosophila* embryos beginning at stage 12. Double labelling of dorsally projecting motoneurons and *unc-5* mRNA showed that by stage 14 *unc-5* levels are substantially reduced in aCC and RP2 in *grn/grn* homozygous mutant embryos (Figure 4.2 B, D) when compared with their heterozygous siblings (Figure 4.2 A, C). *unc-5* mRNA was reduced in *grn* mutant aCC neurons from 23.6±4.8 to 10.7±1.5 (*P<0.02*) and in *grn* mutant RP2 from 30.6±3.8 to 14.3±1.9 (*P<0.005*) (arbitrary fluorescence units ± s.e.m.) when compared with *grn* heterozygous (Figure 4.2 E). Importantly, *unc-5* expression in *grn* homozygous mutants remained unchanged in cells in which *grn* is not normally expressed and is therefore not required for *unc-5* expression, such as exit glia (Figure 4.2 F). These results strongly suggest that *grn* is required for *unc-5* expression in both aCC and RP2 dMNs.
Figure 4.2. Endogenous grn regulates unc-5. (A-D) unc-5 mRNA expression was examined in aCC and RP2 motoneurons of Stage 15 grn mutant embryos (B,D) and their heterozygous siblings (A,C). Anterior is up and dorsal is right in all panels. The top panels (A,B) show unc-5 in situ signal in magenta and myc antibody in green to label tau-myc expressed in aCC and RP2 by the RN2-Gal4 driver. The bottom panels (C,D) show RNA signals in magenta. Anterior is up in all panels. White marks indicate the positions of the xz and yz sections displayed below and to the right of the main xy panels, respectively. Two separate xz sections are shown, one for aCC (a) and one for RP2 (R). (A) In grn heterozygous embryo, clear expression of unc-5 mRNA in both aCC and RP2 is observed, and the cells are easily identified even in the absence of the anti-myc labelling (C). (B) grn mutants show less than half the level of fluorescence in both aCC and RP2 neurons. The reduced unc-5 signal is obvious in the bottom panel in the absence of anti-myc labelling (D). (E,F) Quantification of unc-5 expression in aCC and RP2 neurons (E) or glial cells (F) of st-15 grn heterozygous and grn mutant embryos. Genotypes for each cell analysed are indicated on the x axis, and fluorescence intensity is indicated on the y axis. unc-5 mRNA expression in glia cells is not affected in grn mutants, and is within the same range in both grn/+ and grn mutant embryos (69±21 s.e.m. and 90±15 s.e.m. fluorescence units, respectively) (F); however, it is drastically reduced in aCC from 23.6±4.8 to 10.7±1.5 (P<0.02) and RP2 from 30.6±3.8 to 14.3±1.9 (P<0.005) compared with the heterozygous siblings (arbitrary fluorescence units ±s.e.m.) (E) *P<0.05, **P<0.005. Neuronal bodies for aCC (a) and RP2 (R) are indicated.
**unc-5 partially rescues pathfinding defects in grn mutant embryos**

unc-5 and grn are both required for proper guidance of dMNs although the grn mutant phenotype is more severe with >85% of the ISN nerves affected (Garces and Thor, 2006). The aCC and RP2 dMNs normally innervate muscles 1 and 2, respectively (Figure 4.3 A,B). However, in grn mutants the ISN nerve stops short of its most dorsal target and fails to innervate muscle 1 (Garces and Thor, 2006) (Figure 4.3 D,E). We reasoned that if Unc-5 functions downstream of Grn in dMNs and contributes to its pathfinding output, the grn guidance phenotype might be partially rescued by increasing unc-5 levels in aCC and RP2. Indeed, expression of HA-unc-5 in aCC and RP2 neurons in grn mutant embryos resulted in a partial rescue of the grn phenotype (Figure 4.3 F,G). We observed 10% more ISN branches projecting beyond muscle 2 and a reduction in defective segments from 83.6%±1.8 s.e.m. to 73%±3.1 s.e.m. (P<0.006) (Figure 4.3 G). These results further support the model that unc-5 is a downstream target of Grn, and is required for motor-guidance.

**unc-5 expression is dependent on eve and grn**

Both, eve and grn have been previously shown to be required for the proper specification of aCC and RN2. They can work in a linear pathway, as eve is required in aCC for grn expression, but they also work in independent pathways in both dMNs (Garces and Thor, 2006). If grn and eve are also working independently to induce unc-5 expression, we should be able to show this by analysing its expression in an eve/eve; grn/grn double mutant. We have previously shown that unc-5 levels are substantially reduced in eve mutant dMNs (Labrador et al., 2005); however, some cells still express unc-5 (11%; Figure 4.4 B,E asterisk). Therefore, we quantified the number of cells that still express unc-5 in the double-mutant embryos for eve and grn and were able to determine that the number of cells expressing unc-5 is further reduced (to 2%; Figure 4.4 C,F). This result suggests that eve and grn can also work in parallel pathways to induce unc-5 expression in dMNs.
Figure 4.3. *unc-5* partially rescues *grn* mutants. (A-C) Motor axon projections of flat mounted late st-16 to st-17 embryos visualized with anti-Fas2. Anterior is left and dorsal is up in all panels. Partial genotypes are indicated below each panel. Wild-type embryos (A,B) or animals expressing HA-*unc-5* in aCC and RP2 motoneurons through *RN2Gal4* (C) present a normal ISN branch. A magnification of the region in the rectangle is shown in C' (anti-Fas2), C'' (anti-HA) and C''' (merged). The most dorsal muscle 1 (top arrowhead in A, top arrows in B,C) is innervated by the aCC motor neuron. Muscle 2 is innervated by RP2 motor neuron (2nd arrowhead in A, 2nd arrows in B,C) and a 3rd branch from the ISN innervates muscle 3 (bottom arrowhead in A, bottom arrows in B,C). (D-F) In *grn* mutants (D,E), 85% of ISNs show defective muscle 1/9 innervations (arrowheads with asterisks in E), but axonal projections to muscles 2/10 are normal. Expression of HA-*unc-5* in *grn* mutants partially restores projections beyond muscle 2 (F, 2nd arrow), and decreases the percentage of failed ISN projections from 85% to 72%. A magnification of the region in the rectangle is shown in F' (anti-Fas2), F'' (anti-HA) and F''' (merged). Red asterisks indicate defective projections beyond muscle 2. (G) Quantification of ISN branches projecting beyond muscle 2 in different genetic backgrounds. *RN2Gal4; grn/grn*; wild-type hemisegments=16.4%±1.8 s.e.m., n=250 and *RN2Gal4, UAS-HA-unc-5; grn/grn* wild-type hemisegments=26.6%±2.9 s.e.m., n=240. ***P<0.006.
Figure 4.4 *unc-5* levels are further reduced in *eve* and *grn* double mutants. (A-F) *unc-5* mRNA expression was examined in aCC and RP2 motoneurons of Stage 15 *eve* (E,F) or double *eve, grn* (C,F) mutant embryos and in *eve* heterozygous embryos (A,D). Anterior is up, dorsal is right and in situ signal shows *unc-5* mRNA in magenta in all panels. The top panels (A-C) are also labelled with a myc antibody in green to reveal *tau-myc* expressed in aCC and RP2 by the RN2-Gal4 driver. The bottom panels (D-F) show RNA signals in magenta. In *eve* heterozygous embryos, clear expression of *unc-5* mRNA in both aCC and RP2 is observed, and the cells are easily identified even in the absence of the anti-myc labelling (A,D). *eve* mutants show very low levels of fluorescence in most aCC and RP2 neurons although some of them (asterisk, 11%) still express significant amount of *unc-5* mRNA (B,E). In double *eve, grn* mutants there is reduction of the number of cells in which *unc-5* is still present (2%) (C,F). Number of cells scored and percentage of cells expressing *unc-5* is also indicated on each panel. Neuronal cell bodies are outlined in D-F.
An *unc-5* embryonic neuronal enhancer drives expression in an *eve*-dependent manner

In order to identify the regulatory regions responsible for *unc-5* expression in dMNs, we scanned the *unc-5* locus from the stop of the preceding gene (*Hr51*) to the 5th intron within the *unc-5* gene using different genomic fragments fused to the GFP or *Gal4* genes (Figure 4.5 A). We identified a single region that could drive expression in neurons in the embryonic nervous system. This region drives expression in aCC, RP2 dMNs (Figure 4.5 B-D) and the segmental nerve a, (SNa) motoneurons (data not shown) in the same pattern as endogenous expression of *unc-5* (Keleman and Dickson, 2001; Labrador et al., 2005).

We reasoned that if this region is responsible for *unc-5* expression in dMNs, it should be regulated in the same way as the endogenous gene in those cells. To determine whether this enhancer is regulated by *eve* or *grn*, we stained for GFP signal driven by the enhancer in an *eve*- or a *grn*-null background. We showed that the enhancer is under the control of *eve* (Figure 4.5 E-H), as the GFP signal visible in *eve/+* embryos in aCC and RP2 (Figure 4.5 E,F) is absent in *eve*/eve mutants (Figure 4.5 G,H). Importantly, the GFP signal in motoneurons from the segmental nerve (SN in Figure 4.5 F,H), where *eve* is not expressed nor required, is still present.

The neuronal enhancer drives expression of *Gal4*, which in turn drives expression of GFP. Given the stability of the *Gal4* protein and its amplification effect on the UAS-driven reporter, we reasoned that we should be able to detect a difference only if the change is very considerable. We were, therefore, able to detect a difference in *eve* mutants as its elimination leads to an almost complete downregulation of endogenous *unc-5* (Figure 4.4) (Labrador et al., 2005). Not surprisingly, we failed to detect a significant effect in *grn* mutants as *unc-5* downregulation in *grn* mutants is less severe than in *eve*/eve (Figure 4.2).

Alternatively, it is possible that another enhancer not identified in this study might be responsible for *grn* regulation of *unc-5* in dMNs. We can therefore conclude that we have identified a neuronal enhancer element responsible for the *eve*-dependent expression of *unc-5* in dMNs.
Figure 4.5. An enhancer element for dMNs is regulated by Eve. (A) Mapping the location of the unc-5 neuronal transcriptional enhancer. A schematic of the unc-5 locus is shown at the top and the DNA fragments used in enhancer-reporter transgene expression analysis are depicted below. All constructs were site-directed to attP2 on 3L. A 1.6 kb fragment that spans the 5th intron was identified as a neuronal-specific enhancer element being the only one driving expression in aCC and RP2 (highlighted in green). There is a conserved GATA binding site present within the neuronal element in Drosophila species with a divergence time of >30 million years (from D. melanogaster, DMEL to D. willistoni, DWIL) that might be bound by Grn directly. The aligned sequence as well as the approximate position is highlighted under the neuronal element. (B-D) Unc-5 neuronal enhancer is expressed in Eve-positive aCC and RP2 neurons. Flat mounted st-17 embryos expressing UAS-NLS-GFP under the control of Unc-5-Gal4 were visualized with anti-Eve (B) and anti-GFP (C). Colocalization of Eve and the neuronal enhancer is observed in aCC and RP2 neurons (D). (E-H) unc-5 neuronal enhancer expression was examined in aCC and RP2 neurons of stage-13 eve mutant embryos (G,H) and their heterozygous siblings (E,F). The cell bodies and axonal projections of aCC and RP2 neurons are visualized with anti-22C10 and the unc-5 neuronal
enhancer expression was visualized with anti-GFP. Enhancer expression is absent in aCC and RP2 cells in eve mutant embryos. Anterior is up in all panels. a, aCC; p, pCC; R, RP2; SN, segmental nerve.

**Ectopic expression of grn or eve in dMP2 neurons is sufficient for unc-5 expression**

During Drosophila embryonic nervous system development, grn is downstream of eve in aCC, although it works independently of eve in RP2 (Garces and Thor, 2006). Thus, there is a different requirement for eve and grn in each individual dMN. Our results show that grn is required in both neurons for unc-5 expression (Figure 4.2), suggesting that grn might work sequentially or in a parallel pathway to eve to induce unc-5 transcription in a context-dependent manner. Additionally, our results also demonstrate that eve and grn also work independently to promote unc-5 expression. To test this hypothesis, we investigated whether grn or eve are sufficient to promote unc-5 expression in other neurons that would normally not express either of them (Figure 4.6 A, Figure 4.7). We misexpressed grn or eve in dMP2 neurons, which are hindgut-innervating peptidergic motor neurons (Miguel-Aliaga and Thor, 2004), with the dMP2Gal4 driver and examined unc-5 expression by in situ hybridization. Upon expression of either grn or eve in dMP2 neurons, we observed unc-5 mRNA expression in 74% and 62% of the neurons, respectively (Figure 4.6 B,D). These results show that grn is sufficient to induce unc-5 expression in vivo in a similar way as eve. Interestingly, we failed to detect grn in dMP2 neurons when eve was misexpressed or eve when grn was misexpressed (Figure 4.7), suggesting that eve or grn can induce unc-5 expression independently of each other. When both transcription factors were expressed in dMP2 neurons there was a further increase in the level and number of neurons expressing unc-5 (86% of neurons; Figure 4.6 C), further supporting the hypothesis that in dMP2 neurons both transcription factors can work in parallel to induce unc-5 transcription and the coordinated action of both leads to a more robust induction of unc-5.
Figure 4.6. Individual or combinatorial misexpression of eve and grn in dMP2 cells induce unc-5 expression. (A-D) unc-5 mRNA expression was examined in dMP2 neurons of stage-15 embryos. unc-5 signal is in magenta, and myc antibody to localize UAS-tau-myc under the control of dMP2Gal4 is in green. Two identical xz and yz sections are displayed below or at the right of each panel, top and left sections are the merged image of both channels and bottom or right section is the in situ signal alone. There is no evidence of unc-5 expression in wild-type dMP2 neurons (A). However, ectopic expression of UAS-grn (B), UAS-eve, UAS-grn (C), or UAS-eve (D) in dMP2 neurons, activates expression of the unc-5 gene in these cells. The percentage and number of cells expressing unc-5 mRNA is shown in each panel.
Figure 4.7. *eve* and *grn* do not cross-regulate each other in dMP2 neurons. Anti-myc antibody was used to visualize dMP2 neurons expressing UAS-tau-myc through dMP2Gal4 in green. (A) Eve protein expression was examined in St-15 embryo misexpressing UAS-grn in dMP2 cells. Anti-eve antibody in magenta and anti-myc in green. (B) The same image as in A without myc staining. Ectopic *grn* expression in dMP2 cells does not induce eve expression (encircled in white). (C) *grn* mRNA expression was also analysed in St-15 embryo misexpressing UAS-eve in dMP2 cells. *grn* in situ signal is in magenta and anti-myc antibody is in green. (D) The same image as in C but without myc staining. As in wild-type dMP2 neurons, there is no evidence of *grn* expression in dMP2 cells misexpressing *eve* (encircled in white), suggesting that in these cells *eve* does not regulate *grn* expression.

**A transcription code for axonal exit towards the muscle field is dependent on *unc-5***

Changing the transcription factor code in dMP2 neurons by expressing *grn* and *eve* together forces their axons to leave towards the muscle field and follow the ISN nerve similarly to dMN projections (Figure 4.8 C) but each factor alone does not have this effect (Figure 4.8 B) (Garces and Thor, 2006). Likewise, *unc-5* misexpression in dMP2 neurons can also lead to aberrant axonal exit (Figure 4.8 D), similarly to apterous interneurons (Allan et al., 2003; Keleman and Dickson, 2001). Because *eve* and *grn* are upstream of *unc-5* in dMNs and *unc-5* is required as
part of their guidance output (Figure 4.1, 4.2), the guidance behaviour of these transcriptionally 'reprogrammed' neurons might also be dependent on the Unc-5 receptor repulsive output. To test this hypothesis, we misexpressed both eve and grn in dMP2 neurons and reduced unc-5 levels or completely eliminated unc-5 (Figure 4.8 E,F). Aberrant axon exit was significantly reduced (P<0.02) from 34%±5.1 to 21%±2.4 s.e.m in an unc-5 heterozygous background and further reduced to a 6%±2.1 s.e.m exit (P<0.0001) in an unc-5 null background (Figure 4.8 E,F), indicating that unc-5 is required for projection of dMP2 neurons into the muscle field. These data, together with the ability of each individual transcription factor to induce unc-5 (Figure 4.6), suggests that unc-5 is a common determinant of guidance regulated by eve and grn. Furthermore, the dose-dependent effect of unc-5 in the suppression of the exit phenotype suggests that levels of the receptor are crucial to promote exit and only the combined effect of both transcription factors results in high enough levels of Unc-5.
Figure 4.8. Lateral axon exit of dMP2 neurons promoted by *eve* and *grn* is partially dependent on *unc-5*. (A-E) Axonal projections of dMP2 neurons on flat mounted st-15 embryos visualized with anti-GFP. Partial genotypes are indicated below each panel. Axons in dMP2 neurons expressing EGFP through *dMP2Gal4* fasciculate and project longitudinally through the ventral nerve cord (A). In dMP2 neurons misexpressing *grn*, axons do not significantly project into the lateral muscle field (B). Combinatorial misexpression of *uas-grn* with *uas-eve*, results in lateral exit of 34% of dMP2 axons (C). Ectopic expression of *HA-unc-5* in dMP2 neurons redirects 43% of dMP2 axons laterally (D). In an *unc-5* mutant embryo percentage of dMP2 lateral axonal exit triggered by *UAS-grn*, *UAS-eve* significantly decreases from 34%±5.1 s.e.m. to 21%±2.4 s.e.m. in an *unc-5/+* background and to 6%±2.1 s.e.m. exit in an *unc-5* null background (E). (F) Quantification of lateral projection of dMP2 axons in *UAS-grn*, *UAS-eve* overexpressing flies in a wild-type background (e.g. GOF), *unc-5/+* (e.g. GOF, XTE-18/+ ) or *unc-5/unc-5* (e.g. GOF, XTE-18/+ ) genetic backgrounds (XTE-18 is a deficiency that removes *unc-5*). **P<0.02, ***P<0.0001.
Figure 4.9. Model for *unc-5* regulation. *unc-5* is regulated by both *eve* and *grn* in aCC and RP2 dMNs. Eliminating *grn*, *eve* or both transcription factors progressively reduces *unc-5* levels (left). In dMP2 neurons (right), neither *eve*, *grn* nor *unc-5* is expressed and axons project posteriorly within the CNS. 'Reprogrammed' dMP2 neurons through *grn*, *eve* or both factors increasingly express *unc-5* and more axons project towards the muscle field as *unc-5* levels increase. GOF, gain of function; LOF, loss of function.
Chapter 5: Discussion
The assembly of neuronal circuits in both vertebrates and invertebrates depends on the accurate guidance of axons, which in turn is achieved by the selective responsiveness of axon growth cones to environmental cues. The expression of cell surface and extracellular molecules required for axon guidance (i.e. guidance receptors, ligands and cell adhesion proteins) is under tight regulation in motor neurons, environments in which motor axons travel, and in target muscles. Several transcription factors (TFs) that play pivotal roles in motor axon pathfinding are thought to do so via transcriptional regulation of such proteins. However, except for a few examples, the links between these transcription factors and their downstream effectors have not been well understood. Here we have shed light on downstream genes controlled by a Homeodomain protein, Eve, which is a major fate determinant transcription factor required for neuronal specification, as well as development of neuronal projections in dorsal motor neurons.

**Cell Specific RNA Profiling in Different Organisms**

Forward genetics screens, including chemical, radiation, and transposon-based genetic screens have been powerful tools to search for novel players in different biological processes. However, they are unlikely to be useful for identifying the functions of all genes since it is not possible to mutate all parts of the genome. Also, due to the presence of partially or fully redundant or compensatory genes and pathways, the phenotypes associated with the mutation of a single gene are often masked. Furthermore, elimination of a single component of a multi-component complex might weaken but not eradicate its function (St Johnston, 2002). Therefore, genome-wide mRNA expression analysis combined with reverse genetics experiments will improve our understanding of the roles played by related and possibly redundantly functioning genes.

Microarray analysis has proven to be a very powerful system for obtaining large amounts of gene expression information during several processes such as the *Drosophila* time course (Arbeitman et al., 2002), sex-differentially expressed genes (Arbeitman et al., 2004), circadian rhythms (Claridge-Chang et al., 2001; Mcdonald and Rosbash, 2001) and analysis of cell migration (Wang et al., 2006). However, when a small number of cells from an organism have to be analyzed it is not possible to use microarrays unless they are isolated since contamination with cells or tissues that are not the subject of study will dilute the specific signal to the point...
where it will not be detectable. Recently a number of different approaches have been attempted to circumvent this problem. Cell specific expression of tag-labeled cell translation components followed by purification of the bound mRNAs has been used in mice and *Drosophila* (Doyle et al., 2008; Heiman et al., 2008; Yang et al., 2005). TU-tagging is another method for cell type specific RNA isolation in which 4-thiouracil (4TU) is incorporated into transcribing mRNAs in cells specifically expressing the enzyme uracil phosphoribosyltransferase (UPRT). RNAs labeled with 4-thiouracil (4TU) can then be purified and analyzed using microarray, RNAseq or real time PCR techniques. This method has been used successfully in mice and flies (Gay et al., 2013; Miller et al., 2009).

In the present study we have used an approach, in which we have labeled *Drosophila* dorsal MNs with GFP, isolated wild type and *eve* mutant dorsal MNs by FACS, extracted mRNA from samples, and used affymetrix microarray chips to analyze our RNA samples. Notably, FAC sorting of specific cell types labeled by conditional GFP expression has previously been applied in mice (Arlotta et al., 2005; Lobo et al., 2006; Marsh et al., 2008; Tighe and Held, 2010; Tomomura et al., 2001), zebrafish (Cerda et al., 2009; Covassin et al., 2006; Stuckenholz et al., 2009) and flies (Borghese et al., 2006), highlighting it as a promising approach for transcriptomic studies in different organisms.

**Microarray mRNA profiling of WT and eve mosaic dorsal motor neurons using microarray technique**

This project set out to decipher what the cell surface molecular profiles of individual axons during their navigation and how these profiles can be transcriptionally regulated. We have specifically focused on two genetically amenable dorsal motor neurons, aCC and RP2, and have isolated and analyzed their mRNA from stage 13 WT and *eve* mosaic embryos using affymetrix microarrays. The most prominent phenotype observed in *eve* mosaic aCC and RP2 dMNs is their failure to project axons towards the muscle field. Normally these lateral projections start at embryonic stage 13. Therefore we sorted MNs of stage 13 animals to identify Eve-dependent axon guidance genes that are transcribed during this “critical” period. Consequently, this may have limited our ability to
detect differential expression of other Eve dependent genes, as they may be regulated by Eve at later stages of development. For example, processes such as dendritic guidance, dendritic branch patterning and territory formation occur at later stages of embryogenesis as well as during larval development. Genes involved in these processes are expected to be expressed shortly before they occur. Indeed, our microarray results show that several genes involved in axon guidance are downregulated in eve mosaic dMNs; these data was further validated by ISH. In addition to well-annotated axon guidance genes, we have identified differential expression of multiple genes with unknown functions in eve mosaic dMNs. Future reverse genetics studies will be required to understand the biological function of these genes, as they may have redundant roles in guidance and may be required for fine-tuning of axon targeting at multiple choice points.

**Eve regulates functionally different guidance molecules**

Among transcriptional codes that regulate motor axon pathfinding, specific Lim-HD codes are required for the proper guidance of vertebrate motoneurons, in part, through the regulation of the EphA (Kania and Jessell, 2003), EphB (Luria et al., 2008) or FGF receptors (Shirasaki et al., 2006). In *Drosophila*, Nkx6 (HGTX – FlyBase) is important for vMN specification and has been proposed to promote guidance through the expression of *fasciclinIII* (*Fasciclin 3 – FlyBase*) (Broihier et al., 2004). In this study we show that the homedomain protein, Eve, transcriptionally regulates several downstream genes, including those coding for attractive and repulsive receptors (*beat-1a* and *unc-5*) as well as two cell adhesion proteins (*nrg* and *fas2*). First, using cell-specific microarray profiling and a single cell resolution *in situ* hybridization technique combined with confocal imaging we confirm that the expression of *beat-1a*, *unc-5*, *nrg* and *fas2* genes are drastically decreased in eve mosaic aCC and RP2 MNs. These data indicate that Eve-mediated transcriptional programs are necessary for expression of these genes at proper levels required for correct axon guidance. Moreover, to decipher the functional link of Eve with *beat-1a*, *unc-5* and *nrg* genes, we examined motor axon guidance phenotypes in different genetic backgrounds where we reduced the expression levels of Eve and these downstream proteins. We observed remarkable pathfinding defects in these backgrounds, highlighting the occurrence of genetic interactions between Eve and Eve-dependent guidance molecules. Then, to
examine the sufficiency of Eve for expression of these genes, we used the robust GAL4-UAS system for transcriptional manipulation of a group of serotonergic interneurons, named EWs. We misexpressed Eve in EW interneurons and observed the ectopic induction of beat-1a, unc-5, nrg and fas2 genes in those neurons, suggesting that Eve is sufficient for transcriptional induction of these genes. There are several possible mechanisms by which Eve can regulate the expression of these downstream genes. It has been previously shown that the repressive activity of Eve is responsible for its guidance function in aCC and RP2 (Fujioka et al., 2003), suggesting that its function might be to repress the expression of other transcription factors, such as HB9 (Exex - FlyBase) (Broihier and Skeath, 2002; Fujioka et al., 2003; Odden et al., 2002), which would otherwise confer those motoneurons with a ventral fate. However, it has not been formally ruled out that Eve may also have an activator function and positively regulate its target genes. It is likely that the recruitment of different cofactors specifies which function prevails at a given promoter. In Drosophila, Nkx6 and Engrailed are among bifunctional transcription factors that have been shown to have both negative and positive targets, highlighting the possibility that Eve could also act as both transcriptional activator and repressor (Alexandre and Vincent, 2003; Syu et al., 2009).

A combinatorial code of Grn/Eve transcription factors is required for transcriptional regulation of common target genes

In vertebrates, combinatorial codes of transcription factors play an instructive role in the generation of subclass diversity within the spinal cord (Bonanomi and Pfaff, 2010; Dasen and Jessell, 2009). In Drosophila, in which it is possible to analyze individual motoneurons within a subclass, a further level of complexity is revealed. Within the subclass of motoneurons that project to dorsal muscles, aCC and RP2, subclass determinants (eve, grn and zfhl) can work in a sequential order in aCC specification or independently, in parallel pathways, within RP2 (Garces and Thor, 2006). Whereas Zfh1 is a general factor required in all motoneurons (Layden et al., 2006), eve and grn are specific to dMNs. It is plausible that each one of them might be important for specific aspects of specification within the same subclass of neurons (Garces and Thor, 2006) but together might be responsible for the regulation of common targets such as unc-5. Although grn is required in both aCC
and RP2 for \textit{unc-5} expression, it is not the only factor required because \textit{unc-5} mRNA is not completely absent from those cells and it also requires the presence of \textit{eve}. In fact, \textit{grn} or \textit{eve} can independently induce \textit{unc-5} expression in dMP2 neurons but only both factors expressed in combination are able to induce axon exit towards the muscle field. This combinatorial expression of \textit{eve} and \textit{grn} might bring \textit{unc-5} above the threshold required for exit. \textit{unc-5} levels are definitely important for dMP2 exit; removing 50% of the gene dosage significantly suppresses the exit phenotype and this suppression is almost complete in an \textit{unc-5} null background. Transheterozygous interactions identified between \textit{unc-5} and \textit{eve}, or \textit{unc-5} and \textit{grn} also suggest that their levels are tightly controlled. As both \textit{eve} and \textit{grn} can regulate \textit{unc-5} it is likely that their combined activity in aCC and RP2 is essential to express the required levels of \textit{unc-5} in both neurons. A model of \textit{unc-5} regulation by \textit{grn} and \textit{eve} in each individual neuron is shown in \textbf{Figure 4.9}. Through comparative bioinformatic analysis we have identified a conserved GATA binding site in the \textit{unc-5} neural enhancer element, suggesting that Grn may directly bind to the \textit{unc-5} promoter in dMNs and trigger its expression. However, our Chip-IP and DNA pull down assays failed to show the direct binding of Grn to this element (data not shown). It is plausible that Grn may also play a role in transcriptional regulation of \textit{fas2}, \textit{nrg} and \textit{beat-1a} genes.

In addition to Grn, transcription factors such as Zfh1 or some other un-known TFs may also cooperate with Eve and tightly regulate the expression of common downstream targets, including of \textit{fas2}, \textit{nrg}, \textit{unc-5} and \textit{beat-1a} genes. Interestingly, it has recently been shown that Zfhx1b, a vertebrate homolog of \textit{Drosophila} Zfh1, is required for transcriptional regulation of \textit{Unc5b} in migrating cortical interneurons, suggesting the presence of a similar transcriptional program in \textit{Drosophila} (van den Berghe et al., 2013).

\textbf{Coordinated function of attractive, repulsive and adhesive guidance molecule during axonal projection}

During the last two decades several attractive and repulsive receptors, as well as cell adhesion molecules responsible for MN axon guidance have been identified. However, whether these molecules function independently or have a combinatorial effect on motor axon guidance is still an open question. Our
present work is a unique study in which we examine the individual rules as well as the dual, triple and quadruple combinatorial effects of four axon guidance genes, with different biological functions, in two different neuronal populations: ISN motor neurons and serotonergic interneurons (EWs). First, we analyzed the axon guidance of ISN motor neurons in different genetic backgrounds where we individually or concomitantly eliminated \textit{nrg}, \textit{unc-5} and \textit{beat-la} genes. While single null mutants showed late ISN guidance defects (i.e. ISN bifurcations, dorsal crossing and late stall) in a subset of hemisegments, we observed more severe pathfinding errors (i.e. ISN ventral crossing and stall) with higher frequency in double mutant embryos. Accordingly, triple compound mutants lacking \textit{nrg}, \textit{unc-5} and \textit{beat-1a} genes showed higher rates of severe early guidance defects in the majority of hemisegments. These findings highlight the idea that the combinatorial and concerted action of attractive and repulsive receptors and adhesive molecules is a prerequisite for robust motor axon guidance. Given that we used anti-Fas2 staining for visualizing motor axons, we could not analyze motor axon defects in a genetic background where Fas2 was missing. Therefore, we excluded the \textit{fas2} gene from this part of study.

Furthermore, using a cell-specific GAL4 line, RN2 Gal4, we traced aCC and RP2 motor axons in the aforementioned genetic backgrounds. We found that the aCC and RP2 axons fail to exit the CNS in a small subset of hemisegments (5%) in compound mutants lacking Beat-1a and Unc-5 receptors, a phenotype which is similar to what we observe in the majority of hemisegments in \textit{eve} mosaic dMNs. The lower frequency of exit failure in these compound mutants compared to \textit{eve} mosaic embryos could be explained by compensatory effects of unidentified redundant genes which are still expressed in embryos lacking \textit{beat-1a} and \textit{unc-5} but are missing in \textit{eve} mosaic dMNS due to their dependency on the Eve TF. As mentioned before, we have detected differential expression of a high number of un-annotated genes, any of which could have a redundant role to already well-known axon guidance genes. Such redundant systems seem to be necessary for well-developed and complex biological processes such as axon guidance.

Secondly, we show that simultaneous reinstatement of repulsive (Unc-5) and attractive (Beat-1a) receptors together with an adhesive molecule (Nrg or Fas2)
in eve mosaic dMNs leads to a more robust rescue of axonal exit from the CNS than when we restore these proteins individually or in double combinations. Our data suggest that repulsion from CNS via Unc-5 and attraction toward muscles via the Beat-1a receptor result in lateral projection of eve mosaic aCC and RP2 axons; and that the adhesive effect of Nrg or Fas2 molecules holds the rescued axons together, leading to fasciculation of these motor axons. We further examined the combinatorial function of these receptors and adhesion molecules by ectopically expressing them in a group of serotonergic interneurons, called EWs, the axons of which normally cross the midline and synapse with an unknown set of neurons on the contralateral side. We show that individual misexpression of Beat-1a, Fas2, and Nrg does not have a significant effect on the projection patterns of these axons. In fact, misexpression of Unc-5 only prevents them from crossing the midline which could be due to repulsion by Netrins expressed in the midline. Double or triple misexpression of Unc-5 with the other molecules leads to some degree of EW axonal redirection. However, concurrent misexpression of these four molecules significantly reprograms the projection patterns of EW axons; instead of crossing the midline they project laterally while fasciculating with motor axon's roots. These data are remarkably compatible with the rescue results, both reinforcing the idea that attractive, repulsive and adhesive guidance molecules work in a coordinated fashion during motor axon guidance. Here we only show the concerted function of four guidance molecules; however, it is conceivable that such coordination is likely to occur between other proteins involved in axon pathfinding. Future work will be required to see if there are any physical interactions between these receptors and adhesion molecules or overlap in their downstream effectors. Such physical interaction has recently been shown between GDNF and Ephrin signaling components during vertebrate motor axon guidance (Bonanomi et al., 2012).

Our findings on coordinated function of guidance molecules during lateral exit of ISN and EW axons are consistent with previous studies on molecular mechanisms of target muscle selection by motor neurons. Generally two models for axon guidance have been proposed. The relative balance model suggests that combinatorial expression of attractive and repulsive axon guidance molecules on
both axon growth cones and target muscle fibers is required for proper axon innervations (Winberg et al., 1998). On the other hand the lock and key model suggests that each individual target muscle is labeled by a specific set of molecules that is recognized by defined receptors present on the growth cone of an appropriate innervating axon (Hoang and Chiba, 1999). The relative balance model was first examined and supported by Winberg et al (1998) by studying the effects on ventral muscle innervation by motor axons of adding or subtracting Netrin A and B, Semaphorin II, and Fasciclin II alone or in dual combination (Winberg et al., 1998). In a more recent study in Drosophila, Kurusu et al (2008) have studied the role of Leucine-Rich Repeat proteins in the selection of proper larval target muscles by ventral motor axons; their data is consistent with the relative balance model (Kurusu et al., 2008).

**Future Perspectives**
Transcription factors play a crucial role in specification of motor neuron identity and connectivity in both vertebrates and invertebrates. The results presented provide a strong argument that the *Drosophila* homeodomain transcription factor, Eve, controls the dorsal axonal projection of the MNs, aCC and RP2, through regulating the expression of multiple cell surface guidance molecules each of which has a different function during axon pathfinding. However, the exact mechanism of this regulation is yet to be uncovered. Thus, it will be important to unravel whether Eve acts a transcriptional activator to directly induce axon guidance molecule coding genes, or acts as repressor to regulate these genes through indirect mechanisms. Analyzing the sufficiency of an Eve protein lacking different functional domains (e.g. the repressor domain or homeodomain) in inducing its downstream genes will be helpful to answer this question. Also, the investigation of Eve target genes using Chip-SEQ experiments will shed light on how it functions in dMNs. Further future work will be required to see if other transcription factors expressed in dMNs (i.e. Grn and Zfh1) cooperate with Eve to fine-tune the expression levels of common target genes. Comparative mRNA profiling of WT dMNs with those lacking Grn or Zfh1, as well as Chip-SEQ experiments to identify their targets will be informative in examining the contribution of these TFs in the specification of motor axon connectivity. Individual and combinatorial mis-expression of Eve, Grn and Zfh1 in
other neuronal populations (e.g. EW interneurons), followed by tracing their axonal projections and comparative analysis of their mRNA profiles may further shed light on how these TFs specify different aspects of dMNs.
Chapter 6: Materials and Methods
Genetics
The following stocks were used:

- UAS-eve/TM3 (Brown and Castelli-Gair Hombría, 2000), UAS-HA-Unc5 (Keleman and Dickson, 2001), Unc-5^+/CyO (Labrador et al., 2005), UAS-beat1a, beat^C163/CyO and beat^+/CyO (Siebert et al., 2009) (gifts from Hermann Aberle), UAS–Fasll (transmembrane, PEST+ isoform) (Lin and Goodman, 1994), UAS-nrg^180 (Hortsch et al., 1998) and nrg^+/FM7 (Hall and Bieber, 1997) (gifts from L. Garcia Alonso), Df(2R)eve, RP2A/CyO; RN2-Gal4, UAS-tau_LacZ and UAS-tau_LacZ (Fujioka et al., 2003), RN2-Gal4, UAS-tau-myC and UAS-tau-myC (Garces and Thor, 2006), eagle-Gal4 (Akiyama-Oda et al., 2000) RN2Gal4, UASmCD8GFP, Df(2R)eve, RP2A, RN2Gal4, UASmCD8GFP/S6-TM6, UAS-grn^+/2;UAS-mEGFP^+, UAS-grn^+/2;UAS-eve;UAS-mEGFP^+, UAS-eve/TM3, grn^+/TM3, grn^+/TM3 (Brown and Castelli-Gair Hombria, 2000; Garces and Thor, 2006) XTE-18/CyO (Unc-5 deficiency) (Labrador et al., 2005), Unc-5-Gal4 (Bloomington 47230) and dMP2Gal4/CyO (Miguel-Aliaga and Thor, 2004). Lethal mutations/insertions were kept over FM7, CyO, TM2, TM3, and TM6 balancer chromosomes. Fly stocks and recombinant chromosomes were generated using standard procedures. Homozygous mutant embryos were identified by lack of β-Gal (lacZ).

Cell specific mRNA profiling of WT and eve mosaic motor neurons.
To address what genes lie downstream eve specifically in the aCC and RP2 dMNs we analyzed wild-type and eve mutant neurons. Wild-type neurons were isolated from fly strains that carried two copies of RN2Gal4 and two copies of UASmCD8GFP. To obtain eve mutant cells we had to generate a fly strain we could use to isolate mutant cells. To this end we generated an eve mosaic mutant in aCC and RP2 on the 2nd chromosome that carried two copies of RN2Gal4 and two copies of UASmCD8GFP on the 3rd chromosome. To ensure that the eve mutant chromosome would segregate with the GFP marker both chromosomes were balanced over a compound balancer (SM6-TM6). In such a strain only eve homozygous or heterozygous cells are labeled. Since the developmental stage we were interested on analyzing was at the onset of axonogenesis we aged the embryos 8-9h and performed the cell dissociation followed by purification of GFP positive neurons by Fluorescent Activated Cell Sorting (FACS). Purified neurons were transferred into trizol and were sent to Affymetrix Microarray Facility to extract RNA, amplify cDNA,
produce labeled cRNA, and hybridize it to GeneChip Drosophila Genome 2.0 Array. Three biological replicates as well as three technical replicates were performed for each group. Microarray outputs were obtained as GeneChip CEL files, which were then loaded to oneChannelGUI Package (Bioconductor-oneChannelGUI) and were normalized using GC-RMA algorithm. After conversion to log2 scale, genes with lower intensity or with unchanged expression level between WT and mutant neurons were removed from data. To determine differentially expressed genes between two conditions, two-tailed paired t test was performed using the Limma package from the Bioconductor project. The p value of the moderated t test was adjusted for multiple hypotheses testing, controlling the false discovery rate (FDR) with the Benjamini-Hochberg procedure. Genes with FDR less than 0.05 (5%) and fold change larger than 1.5 were chosen for further examination. Other analyses were performed as described in detail in the Supplemental Data and Experimental Procedures.

Immunohistochemistry
24 hr old embryos were collected and fixed as previously described (Labrador et al., 2005). For embryo staining we used following primary antibodies: anti-c-Myc (DSHB-9E10; 1:50), anti-Fas2 1D4 (1:50) (Developmental Studies Hybridoma Bank), anti-HA (Covance; 1:500), anti-muscle myosin (home made; 1:50) and Rabbit anti-β-gal (Cappel; 1:5,000). Alexafluor488 (Molecular Probes), HRP and Cy3 (Jackson Immunoresearch Laboratories) conjugated anti-mouse or anti-rabbit secondary antibodies were used at 1:1000, 1:500 and 1:500 respectively. Cy3-labeled tyramide was used as HRP substrate. To study motor axon phenotypes, ISN projections at embryonic stage 16/17 in A2-A6 abdominal hemisegments were stained with anti-Fas2 and examined in different genetic backgrounds. For the benefit of color-blind readers, double-labeled images were false colored. Stacks of images were obtained with Zeiss Confocal LSM700 Microscope and a 40X oil immersion objective was used.

RNA 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 of different genes were PCR amplified, and in situ probes were transcribed from the PCR products using digoxigenin-labeled ribonucleotides. Hybridized probes were bound with antidigoxigenin POD-conjugated Fab fragments and detected using Cy3-labeled tyramide as POD substrate. Anti-b-gal and anti-myc antibodies were used, respectively, for double labeling of dorsal motor neurons and eagle interneurons in hybridized embryos.

In order to quantify fluorescent in situ signals in aCC and RP2 motor neurons, heterozygous embryos and mutant siblings were isolated from the same embryo collection and distinguished based on the presence (heterozygotes) or absence (homozygous mutants) of b-galactosidase in balancer chromosomes.

Embryos were dissected and mounted on the same slide. 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 in each experiment. Fluorescence within regions of interest was quantified with ImageJ.

**Statistical analysis**

Data are presented as mean values ± s.e.m. SPSS 16 software (SPSS Inc.) was used to generate histograms and examine the statistical significance. For analysis 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 ** for \( p<0.05 \) or *** if \( p<0.01 \).

**Genotype of Embryos Used for Different experiments:**

**Insitu Hybridization experiments in WT, grn mutant and eve mosaic aCC and RP2 neurons:**

\[
Df(2R)eve, RP2A/CyO; RN2-Gal4,UAS-tau_LacZ
\]

\[
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ
\]

\[
RN2-Gal4::taumycGFP; grn^{71.12/}\]

\[
RN2-Gal4::taumycGFP; grn^{71.12/\text{grn}^{SP^{79}}}
\]
Cell specific mRNA profiling of WT and eve mosaic motor neurons:
RN2Gal4, UASmCD8GFP
Df(2R)eve, RP2A, RN2Gal4, UASmCD8GFP, SM6-TM6

Genetics Interactions:
nrg^1/Y
Df(2R)eve, RP2A/+  
Unc-5^0/+  
beat^3/+  
Unc-5^0, beat^3/+  
nrg^1/Y; Df(2R)eve, RP2A/+  
Df(2R)eve, RP2A/+; Unc-5^0/+  
Df(2R)eve, RP2A/+; beat^3/+  
Df(2R)eve, RP2A/+; Unc-5^0, beat^3/+  
Unc-5^0/+; grn7112/+  

Individual and Compound mutants:
nrg^1/FM7; RN2-Gal4::taumycGFP/+  
Unc-5^0/Unc-5^0; RN2-Gal4::taumycGFP/+  
beat^3/beat^163; RN2-Gal4::taumycGFP/+  
nrg^1/Y; Unc-5^0/Unc-5^0; RN2-Gal4::taumycGFP/+  
nrg^1/Y; beat^3/beat^3; RN2-Gal4::taumycGFP/+  
Unc-5^0; beat^3/Unc-5^0, beat^3; RN2-Gal4::taumycGFP/+  
nrg^1/Y; Unc-5^0/Unc-5^0; RN2-Gal4::taumycGFP/+  
nrg^1/FM7; beat^3/Unc-5^0, beat^3; RN2-Gal4::taumycGFP/+  
nrg^1/FM7; Unc-5^0, beat^3/Unc-5^0, beat^3; RN2-Gal4::taumycGFP/+  
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4, UAS-tau_LacZ  
grn519/RN2-Gal4, UAS-tau_LacZ, grn7112  
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4, UAS-tau_LacZ, grn7112/RN2-Gal4, UAS-tau_LacZ, grn7112
Rescue of eve mosaic aCC and RP2 neurons:
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ
Df(2R)eve, RP2A,UAS-FasII /Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ
Df(2R)eve, RP2A, UAS-nrg180/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ
Df(2R)eve, RP2A, UAS-nrg180/Df(2R)eve, RP2A,UAS-FasII;RN2-Gal4,UAS-tau_LacZ
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-beat1a
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-HA-Unc5
Df(2R)eve, RP2A/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-HA-Unc5,UAS-beat1a
Df(2R)eve, RP2A, UAS-nrg180/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-HA-Unc5
Df(2R)eve, RP2A, UAS-FasII /Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-HA-Unc5
Df(2R)eve, RP2A, UAS-nrg180/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-beat1a
Df(2R)eve, RP2A, UAS-nrg180/Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-HA-Unc5,UAS-beat1a
Df(2R)eve, RP2A, UAS-FasII /Df(2R)eve, RP2A; RN2-Gal4,UAS-tau_LacZ/UAS-HA-Unc5,UAS-beat1a

Rescue of eve mosaic aCC and RP2 neurons:
RN2Gal4/+; grnSPP/grn71.12
RN2Gal4/UAS-HAunc5; grnSPP/grn71.12

Mis-expression of eve in eagle neurons:
UAS-taumycGFP/+; eagle-Gal4 /UAS-eve
UAS-taumycGFP/+; eagle-Gal4 /+

Mis-expression of eve, grn and unc-5 in dMP2 neurons:
dMP2Gal4/++;UAS-tau-myc/+ 
dMP2Gal4/UAS-HAunc5
dMP2Gal4/UAS-grn#2,UAS-eve;UAS-tau-myc/+ 
dMP2Gal4/UAS-grn#2;UAS-tau-myc/+
dMP2Gal4/UAS-eve;UAS-tau-myc/+  
dMP2Gal4/Unc5<sup>§</sup>,UAS-grn<sup>#2</sup>,UAS-eve;UAS-tau-myc/+  
XTE-18,dMP2Gal4/Unc5<sup>§</sup>,UAS-grn<sup>#2</sup>,UAS-eve;UAS-tau-myc/+  

Reprogramming of eagle neurons:  
UAS-tau<sub>La</sub>cZ/+; eagle-Gal4/+  

UAS-tau<sub>La</sub>cZ/+; eagle-Gal4/UAS-eve  
UAS-tau<sub>La</sub>cZ/UAS-FasII; eagle-Gal4/+  
UAS-tau<sub>La</sub>cZ/UAS-nrg<sup>180</sup>; eagle-Gal4/+  
UAS-tau<sub>La</sub>cZ/UAS-FasII, UAS-nrg<sup>180</sup>; eagle-Gal4/+  
UAS-tau<sub>La</sub>cZ/+; eagle-Gal4/UAS-HA-Unc5  
UAS-tau<sub>La</sub>cZ/+; eagle-Gal4/UAS-beat<sub>1a</sub>  
UAS-tau<sub>La</sub>cZ/+; eagle-Gal4/UAS-HA-Unc5, UAS-beat<sub>1a</sub>  
UAS-tau<sub>La</sub>cZ/UAS-FasII; eagle-Gal4/UAS-HA-Unc5, UAS-beat<sub>1a</sub>  
UAS-tau<sub>La</sub>cZ/UAS-FasII; eagle-Gal4/UAS-HA-Unc5  
UAS-tau<sub>La</sub>cZ/UAS-nrg<sup>180</sup>; eagle-Gal4/UAS-HA-Unc5, UAS-beat<sub>1a</sub>  
UAS-tau<sub>La</sub>cZ/UAS-nrg<sup>180</sup>; eagle-Gal4/UAS-beat<sub>1a</sub>  
UAS-tau<sub>La</sub>cZ/UAS-nrg<sup>180</sup>; eagle-Gal4/UAS-HA-Unc5  
UAS-tau<sub>La</sub>cZ/UAS-nrg<sup>180</sup>; eagle-Gal4/UAS-HA-Unc5, UAS-beat<sub>1a</sub>  

Expression Analysis of unc-5 enhancer element  
UAS-NLS-Venus/Unc-5-Gal4  
Df(2R)eve, RP2A/ Df(2R)eve, RP2A; UAS-NLS-Venus/Unc-5-Gal4  
UAS-tau<sub>La</sub>cZ/+; grnSPJ9/grn7L12,Unc-5-Gal4
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