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Specification of *Drosophila* aCC motoneuron identity by a genetic cascade involving *even-skipped*, *grain* and *zfh1*

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During nervous system development, combinatorial codes of regulators act to specify different neuronal subclasses. However, within any given subclass, there exists a further refinement, apparent in *Drosophila* and *C. elegans* at single-cell resolution. The mechanisms that act to specify final and unique neuronal cell fates are still unclear. In the *Drosophila* embryo, one well-studied motoneuron subclass, the intersegmental motor nerve (ISN), consists of seven unique motoneurons. Specification of the ISN subclass is dependent upon both *even-skipped* (*eve*) and the *zfh1* zinc-finger homeobox gene. We find that ISN motoneurons also express the GATA transcription factor Grain, and *grn* mutants display motor axon pathfinding defects. Although these three regulators are expressed by all ISN motoneurons, these genes act in an *eve→grn→zfh1* genetic cascade unique to one of the ISN motoneurons, the aCC. Our results demonstrate that the specification of a unique neuron, within a given subclass, can be governed by a unique regulatory cascade of subclass determinants.

KEY WORDS: Axon pathfinding, Even-skipped, Grain, Neuronal fate specification, Combinatorial code, *Drosophila*

INTRODUCTION

During the past decade, motoneuron specification has been intensely studied and work from both invertebrates and vertebrates has shown that motoneuron subclass identity is determined by combinatorial transcription factor codes (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). However, how individual identities, within a related pool of motoneurons, are determined is still not understood. In the abdomen of the developing *Drosophila* embryo, reiterated sets of ~80 motoneurons are generated in each segment of the ventral nerve cord (VNC). These motoneurons project along distinct nerves to innervate peripheral target muscle fields and, based upon their peripheral axonal projections, they are typically grouped into six well-defined classes (Landgraf et al., 1997). The motor nerve innervating the dorsal-most muscle field, the intersegmental nerve (ISN), contains axons from seven well-defined motoneurons; the aCC, RP2 and the five U motoneurons, each with a well-defined and specific muscle target (Jacobs and Goodman, 1989; Johansen et al., 1989; Landgraf et al., 1997). The *even-skipped* (*eve*) regulatory gene is specifically expressed in ISN motoneurons and *eve* is both necessary and sufficient for ISN motor axon pathfinding (Landgraf et al., 1999). However, *eve* is expressed in all ISN motoneurons and is cell-autonomously crucial for their axonal exit out of the VNC (Fujioka et al., 2003). Recent studies reveal that the zinc-finger/homeodomain gene *zfh1* is also expressed by ISN motoneurons (Layden et al., 2006). However, *zfh1* is expressed by most if not all motoneurons, and important for many motor axons to exit the VNC. Together, these results suggest that regulators other than *eve* and *zfh1* are necessary to explain the specification of each individual ISN motoneuron identity.

To gain further insight into motoneuron specification, we have addressed the role of the *Drosophila* GATA transcription factor *grain* (*grn*). We find that *grn* is specifically expressed within the ISN motoneuron subclass and plays a crucial role for ISN axon projections. Genetic analysis reveals that the regulatory interplay between *eve*, *grn* and *zfh1* varies between the different ISN motoneurons. Within the postmitotic aCC motoneuron, these three regulators act in a unique *eve→grn→zfh1* genetic cascade that is crucial for the correct specification of aCC identity. Misexpression of *zfh1* (Layden et al., 2006) or co-misexpression of *eve* with *grn*, can trigger lateral axonal exit from the ventral nerve cord. *grn* and *zfh1* are, furthermore, sensitive to Notch signaling within this ISN motoneuron, whereas they are insensitive to Notch in other ISN motoneurons. These findings reveal the existence of a unique genetic program for the aCC motoneuron fate, consisting of factors expressed by all ISN motoneurons.

MATERIALS AND METHODS

Drosophila stocks

The grn^{lacZ} allele *l(3)05930* was identified in a survey of the BDGP *lacZ* collection (Spradling et al., 1999) for lines with restricted expression pattern in the embryonic VNC. grn^{GAL4} was generated by *P* element conversion of grn^{lacZ} as previously described (St Pierre et al., 2002). For *grn* mutant analysis, grn^{7l} and grn^{SPJ9} (Brown and Castelli-Gair Hombria, 2000) were placed over deficiency *Df(3R)dsx3*, and both allelic combinations showed the same pathfinding phenotype and no detectable Grn expression (not shown). For *grn* misexpression and rescue experiments, we used *UAS-grn#2* (Brown and Castelli-Gair Hombria, 2000). $UAS\text{-}mEGFP^{F}$ is a c-myc epitope-tagged membrane-targeted EGFP reporter line (Allan et al., 2003). Other lines used were: *islet-τ-myc-EGFP* (S.T., unpublished); *RN2-GAL4*, *CQ2-GAL4*, *Df(2R)eve,ΔRP2A/CyO,P[wg-lacZ];RN2-GAL4,UAS-τlacZ*, *Df(2R)eve/CyO,P[wg-lacZ];ΔRP2B* (Fujioka et al., 2003); *UAS-eve* and eve^{ID19} (Landgraf et al., 1999). $zfh1^{2}$, $zfh1^{65.34}$, $zfh1^{75.26}$ alleles were obtained from R. Lehmann and *UAS-zfh1* from the Bloomington stock center. $Hb9^{GAL4}$, $Hb9^{KK30}$, *UAS-vnd*, $mam^{l(2)04615}$, $spdo^{G104}$ were provided by J. B. Skeath and H. T. Broihier. $UAS\text{-}Notch^{ICD}$ was obtained from S. Artavanis-Tsakonas.

Quantification of pathfinding phenotypes

ISN motor axonal projections were scored at embryonic stage 16/17 in A2-A6 abdominal hemisegments using anti-Fasciclin 2, *RN2-GAL4/UAS-mEGFP*^{F} or *CQ2-GAL4/UAS-mEGFP*^{F}. Phalloidin-Texas Red (Molecular Probes) was used to visualize the musculature.

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Antibody production and staining of embryos

grn cDNA encoding amino acids 1-166 was cloned into pGEX-2T (Amersham) for protein expression and purification (J. Castelli-Gair Hombria, unpublished). Fusion protein was used to immunize rabbits and rats (Covance). Grn antibodies were used at 1:200 and their specificity was verified by the absence of staining in *grn* mutants. Immunolabeling was carried out as previously described (Thor et al., 1999). The following antibodies were used: α-c-Myc 9E10 (1:50), α-Fas2 1D4 (1:50), α-Even skipped 2B8 (1:5) and α-β-gal 40-1a (1:10) (all from Developmental Studies Hybridoma Bank). Rabbit α-β-gal (Cappel; 1:5,000), rabbit α-pMad (Tanimoto et al., 2000) (1:2,000), rabbit α-Zfh1 (Van Doren et al., 2003) (1:5,000), rabbit α-Hb9 (Broihier and Skeath, 2002) (1:500) and rabbit α-Vnd (Shao et al., 2002) (1:1,000). Double-labeled images were false colored for the benefit of color-blind readers. Prior to use, the polyclonal α-β-gal, -pMad, -HB9, -Vnd and -Grn antibodies were pre-absorbed against early-stage wild-type embryos.

RESULTS

grain is expressed in subsets of developing motoneurons and interneurons

To identify genes controlling motoneuron specification, we analyzed the expression patterns of a number of *lacZ* enhancer trap lines, surveying for lines with expression in the embryonic VNC (see Materials and methods). One line that showed restricted expression in subsets of cells in the VNC is an insertion in the *grain* (*grn*) gene. *grn* encodes a GATA transcription factor previously shown to control cell rearrangements in the developing leg imaginal disc and in the posterior spiracle (Brown and Castelli-Gair Hombria, 2000). Previous studies revealed that *grn* expression commences at the cellular blastoderm stage, and rapidly becomes localized to the dorsal part of the embryo, being most prominent in the procephalic region. From stage 11, expression is evident in the posterior spiracles, in the midgut and in a patch of cells in the lateral ectoderm (Brown and Castelli-Gair Hombria, 2000; Lin et al., 1995b). We generated Grn-specific antibodies and found that the expression of Grn closely matches the grn^{lacZ} and grn^{GAL4} reporter expression in these structures (not shown), as well as in the VNC (Fig. 1A1-3, 1E1-3; not shown).

In the VNC, *grn* expression commences at early stage 12. The position and morphology of grn^{lacZ}- and grn^{GAL4}-expressing cells suggested a postmitotic and neuronal identity. Using grn^{GAL4}/*UAS-τlacZ*, we observed that *grn* is expressed in a diverse set of interneurons and motoneurons that extend axons along the major axon tracts (Fig. 1B,F). Double labeling with the glial-specific marker Repo showed that, with the exception of one dorsal glial cell per hemisegment (Fig. 1I), Grn (and grn^{lacZ} or grn^{GAL4}) expression is restricted to neurons. To resolve the identity of *grn*-expressing neurons further, we assayed for overlap with regulators known to be expressed in restricted sets of neurons, such as *isl*, *lim3*, *Hb9*, *zfh1*, *apterous* and *even-skipped* (*eve*) (Fig. 1B-D,F-H; not shown). Of these genes, only *eve* and *zfh1* showed apparent overlap with *grn*, specifically in the intersegmental nerve (ISN) motoneurons: aCC, RP2 and the five Us (U1-5 or CQ) (Fig. 1D,H). The ISN motoneurons are born during early embryogenesis with aCC and RP2 born at stage 9, and the U motoneurons born sequentially during stage 9-11 (Broadus et al., 1995; Doe et al., 1988a; Weigmann and Lehner, 1995). Expression of *grn* and Grn in ISN motoneurons commences at stage 11-12, subsequent to Eve expression, and expression of *grn* and Grn is maintained in ISN motoneurons into larval stages (not shown). Thus, *grn* is expressed in subsets of interneurons, and in a distinct subclass of motoneurons that innervate the dorsal-most muscles in the *Drosophila* embryo (Fig. 1J).

grain is required for ISN motor axon pathfinding

To determine if *grn* plays a role in ISN motoneuron specification, we analyzed motor axon projections in *grn* mutants. In *Drosophila* embryos, motor axonal projections are stereotyped and can be revealed using an antibody directed against the surface molecule Fasciclin 2 (Fas2) (Vactor et al., 1993). The aCC and U1 motor axons are known to innervate the dorsalmost muscles 1 and 9, respectively, while the RP2 and U2 motor axons innervate the dorsal muscles 2 and 10 respectively (Fig. 1J) (Jacobs and Goodman, 1989; Johansen et al., 1989; Landgraf et al., 1997). Fas2 reveals the high reproducibility of these projections in the wild-type embryo (Vactor et al., 1993) (Fig. 2A; 100% innervation, *n*=96; throughout the text, *n* refers to the numbers of hemisegments counted). In *grn* mutants, we find that the ISN motor axons are stalled at muscles 2/10, leading to a near complete loss of innervation of the dorsal-most muscles 1/9 (12% innervation; *n*=136) (Fig. 2B). To better resolve the *grn* pathfinding phenotype we used both an aCC/RP2-specific and a U-specific GAL4 driver line (*RN2-GAL4* and *CQ2-GAL4*, respectively) (Fujioka et al., 2003; Landgraf et al., 2003) and expressed a membrane targeted EGFP reporter (*UAS-mEGFP*^{F}) (Allan et al., 2003). In the wild type, *RN2-GAL4/UAS-mEGFP*^{F} clearly visualizes the peripheral projections of aCC and RP2 onto muscles 1 and 2 (arrow and arrowhead, respectively, in Fig. 2D), as well as their terminal processes (Fig. 2G). In *grn* mutants, muscle 2 is innervated with near wild-type frequency, but, by contrast, muscle 1 is innervated in only 15% of hemisegments (*n*=146) (Fig. 2E,K). Using *CQ2-GAL4/UAS-EGFP*^{F} in *grn* mutants, we observed a similar phenotype – apparently normal innervation of muscles 2/10 but only 18% muscles 1/9 innervation (*n*=88) (Fig. 2I,J,K). In addition, using Fas2, *RN2-GAL4* or *CQ2-GAL4* as markers, we noticed aberrant projections onto muscle 8 (Fig. 2B,E,H,J). We quantified this phenotype using *RN2-GAL4* or *CQ2-GAL4*, and found that whereas control embryos (*RN2-GAL4/UAS-EGFP*^{F} or *CQ2-GAL4/UAS-EGFP*^{F}) displayed no innervation of muscle 8 (0%; *n*=87 and *n*=72, respectively), *grn* mutants displayed frequent innervation of muscle 8. This phenotype was observed more often with *RN2-GAL4* than with *CQ2-GAL4* as marker (35%; *n*=140 versus 21%: *n*=146). In affected hemisegments, we observed a grossly normal pattern of axonal projections to the dorsal muscles 2/10 (Fig. 2B,E,J). This indicates that aCC and/or RP2, and at least one of the U motoneurons project aberrantly to muscle 8. These results show that *grn* is crucial for proper motor axon pathfinding of ISN motoneurons.

grain acts cell-autonomously in ISN motoneurons

Although *grn* is expressed in ISN motoneurons, it is also expressed in a patch of ectodermal cells in the lateral body wall that underlie the SNa muscle field, muscles 21-24 (Brown and Castelli-Gair Hombria, 2000) (not shown). In *grn* mutants, we observe a partially penetrant muscle patterning phenotype, evident as an imprecise insertion of muscles 21-24 into the body wall (Fig. 2A-F,I,J). Although the ISN motoneurons do not normally innervate this muscle field, it still raised the concern that the motor axon pathfinding defect observed in *grn* mutants may not result from a cell-autonomous role for *grn* in ISN motoneurons. To address this issue, we used the *RN2-GAL4* and *CQ2-GAL4* drivers to provide *grn* activity in aCC/RP2 and U motoneurons, respectively. We find that *RN2-GAL4* efficiently rescues *grn* mutant axon pathfinding (100% muscle 1/9 innervation; *n*=88) (Fig. 2C,K). By contrast, the *CQ2-GAL4* driver only partially rescued the *grn* phenotype; 54% of muscles 1/9 (*n*=132) (Fig. 2K). Together, these results show that *grn* acts cell-autonomously in ISN motoneurons to ensure proper axon pathfinding to the dorsal-most muscles (Fig. 2L,M).

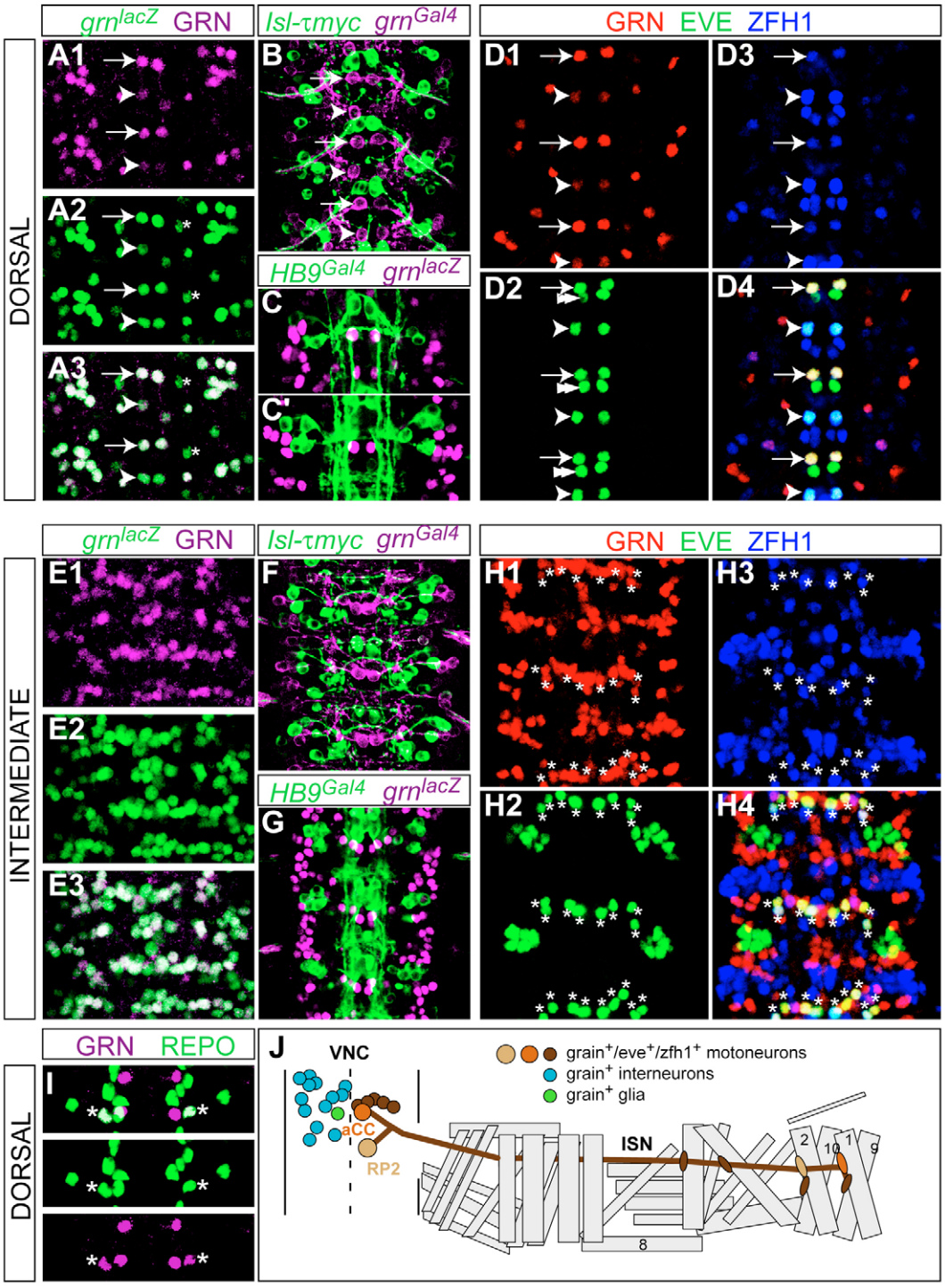

Fig. 1. Grain is expressed in ISN motoneurons and in subsets of interneurons. Stage 14 (**A-H**) and stage 12 (**I**) embryos stained for Grn (A,E,I), Repo (I), β-gal (A,C,E,G) and Myc (B,C,D,F,G,H). Dorsal (A-D,I), mid-dorsal (C′) and intermediate (E-H) focal planes of the VNC. Anterior is upwards in all panels. Grn expression within one (C,C′,I), two (A,E) or three (B,C,F-H) segments. *grn*^{lacZ} is expressed in all Grn-positive neurons (A,E). We noticed consistently weaker expression of Grn and *grn*^{lacZ} (or *grn*^{GAL4}) in the RP2 motoneuron (arrowhead) compared with other *grn*-expressing neurons (A,D). Mutually exclusive expression patterns of *grn*^{GAL4}*/UAS-τlacZ* with *islet-τmycEGFP* (B,F), and with *Hb9-GAL4* (C,C′,G) in subsets of motoneurons and interneurons. Overlap of Grn with Eve and Zfh1 in aCC, RP2 (D) and the U motoneurons (asterisks, H). The pCC interneuron, which is located posterior to aCC does not express *grn*^{lacZ} (A) or Grn (D). In stage 12 embryos, we find overlap of Grn and Repo (I) in one glia cell (*). This glia cell rapidly becomes Grn negative at later stages (compare with A1) but maintains β-gal expression when probed with *grn*^{lacZ} (A2) probably owing to the stability of the β-gal protein. (**J**) Schematic showing *grn*-expressing cells in the VNC and the *grn*-expressing ISN motor axon projections in the periphery. The five U motoneurons are depicted in dark brown.

An *eve→grn→zfh1* regulatory cascade in the aCC motoneuron

eve is expressed in a small subset of transiently identified GMC (ganglion mother cell) and derived aCC, RP2 and U motoneurons. Studies show that *eve* is both necessary and, at least in part, sufficient for dorsal motor axon projections (Landgraf et al., 1999). Given that *eve* and *grn* show similar mutant phenotypes in dorsally projecting motoneurons, we wanted to address whether these two genes regulate each other or act at the same genetic level. As *eve*-null mutants display severe segmental defects, a temperature-sensitive (ts) allele (*eve*^{ID19}), was previously used to study the role of eve in motoneuron specification (Landgraf et al., 1999). However, recent studies have shown that the *eve* ts allele does not completely remove *eve* function in ISN motoneurons. Using a sophisticated strategy, Fujioka et al. have succeeded in restoring *eve* function in all *eve*-expressing cells, except in the aCC and RP2 neurons, in an otherwise *eve*-null background (Fujioka et al., 2003). Using this ‘composite’ *eve* allele, *eve*$^{\Delta RP2}$ (denoted *eve* mosaic herein), we reproduced the recently described aCC/RP2 *eve*-null phenotype; a failure of these two motoneurons to project out of the VNC (Fig. 3A,B,F,G). This is coupled both with ectopic expression of the *Hb9* homeobox gene and loss of Grn expression within these cells. In aCC, these effects

are highly penetrant and observed at several stages, whereas in RP2 the effects are partly penetrant at stage 12 and almost absent at stage 15 (Fig. 3C-E,H-J). However, in *grn* mutants, we did not observe any evidence of Eve downregulation in aCC, RP2 or U motoneurons (Fig. 5A,B,D,E; not shown). We also addressed whether *grn* is important for repressing *Hb9* in these motoneurons, but found no evidence for ectopic expression of Hb9 in aCC (or in RP2) in *grn* mutants (Fig. 5G,H).

Zfh1, a Zn-finger-homeodomain protein, has been reported to be expressed in aCC and RP2, as well as in many other motoneurons (Lai et al., 1991). Recent analysis of *zfh1* reveals that is indeed expressed in all identifiable motoneurons, and genetic analysis reveals that it is necessary for proper motor axon pathfinding (Layden et al., 2006). In stage 15 embryos, we find that Zfh1 expression is dependent both upon *eve* and *grn*, but only in aCC and not in RP2 (Fig. 4A-E, Fig. 5D,E). As expected, when *grn* function is rescued (*RN2-GAL4/UAS-grn;grn*), Zfh1 expression is restored in aCC (Fig. 5I). In line with the notion that *eve* and *grn* act upstream of *zfh1*, Eve or Grn expression is unaffected in *zfh1* mutants (Fig. 5C,F).

Drosophila motoneurons depend upon a target-derived BMP signal for proper maturation (Aberle et al., 2002; Marques et al., 2002). Consistent with the failure of aCC and RP2 axons to exit the VNC in *eve* mosaic mutants, we observe a complete loss of pMad staining in both aCC and RP2 (0% pMad in aCC and RP2; n=32) (Fig. 4F,G), indicating that these neurons are unable to receive the peripheral BMP retrograde signal. By contrast, in *grn* and *zfh1* mutants, where aCC and RP2 still project into the periphery, we detect wild-type staining for pMad (100% pMad in aCC and RP2; n=46 and n=48, respectively) (Fig. 5A-C). These observations indicate that in *grn* and *zfh1*, ISN motoneurons maintain a 'generic' motoneuronal identity and further indicate that embryonic activation of the BMP pathway does not rely on the establishment of functional contacts between motoneurons and their proper muscle targets.

Within the aCC motoneuron, we are thus able to place these three genes in an *eve*→*grn*→*zfh1* regulatory cascade, with the added complexity that *eve* also acts to suppress *Hb9*. By contrast, there is only partial crossregulation between *eve*, *grn*, *zfh1* and *Hb9* in the RP2 motoneuron.

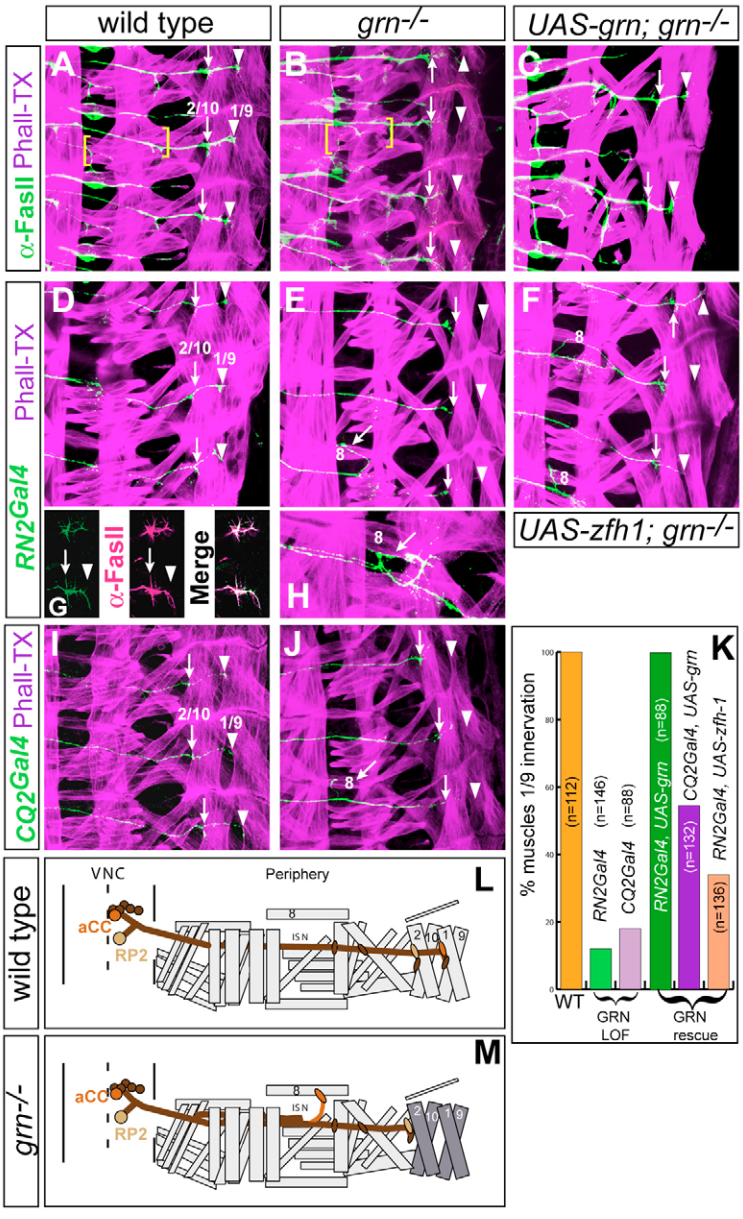

Fig. 2. *grain* is required for ISN motor axon projections. Stage 16 embryos stained with α-Fas2 (green in A-C,G), *RN2-GAL4* driving *UAS-mEGFP^F* (green in D-H), *CQ2-GAL4* driving *UAS-mEGFP^F* (green in I,J) and Phalloidin-TX (magenta in A-F,H-J). Arrows and arrowheads indicate axons terminals contacting dorsal (2 and 10) and dorsal-most (1 and 9) muscles, respectively. (**A**) In wild type, the ISN nerve innervates muscles 2/10 and 1/9. (**B**) In *grn* mutants, ISN fails to innervate muscles 1/9, but axonal projections are seen contacting muscles 2/10. Bracket denotes a partially penetrant muscle patterning phenotype, evident as an imprecise insertion of muscles 21-24 into the body wall. (**C**) In *grn* rescue (*RN2-GAL4/UAS-grn; grn$^{-/-}$*) ISN innervates muscles 2/10 and 1/9 as in wild type. (**D**) In control, *RN2-GAL4/UAS-EGFP^F* reveals muscle 1 innervation by aCC and muscle 2 innervation by RP2. (**E**) In a *grn* mutant background, *RN2-GAL4/UAS-EGFP^F* reveals that although muscle 1/9 is not innervated by aCC, axon terminals from aCC and/or RP2 contact muscles 2/10. (**F**) *zfh1* can partially rescue *grn* mutants (*RN2-GAL4/UAS-zfh1; grn$^{-/-}$*) and the lack of muscle 1 innervation (arrowheads) is less severe than in *grn* mutant. (**G**) Overlap between *RN2-GAL4/UAS-EGFP^F* (green) and α-Fas2 (magenta) revealing axons terminals for aCC and RP2. This reporter allows for a precise analysis of aCC and RP2 terminals in the periphery. (**H**) In *grn* mutants, 36% of hemisegments (n=69) show ectopic innervation of muscle 8 together with defasciculation of aCC and RP2 motor axons (see also oblique arrow in E). (**I**) In control, *CQ2-GAL4/UAS-EGFP^F* reveals muscle 9 innervation by U1 and muscle 10 innervation by other U motoneurons. (**J**) In a *grn* mutants, *CQ2-GAL4/UAS-EGFP^F* reveals that muscle 9 is not innervated (by U1), while U axon terminals contact muscles field 2/10. (**K**) Quantification of muscles 1/9 innervation in different genetic backgrounds. (**L**,**M**) Schematic showing the *grn* mutant phenotypes (M) compared to wild type (L).

eve and *grain* play additional roles outside of the *eve→grn→zfh1* regulatory cascade

Do *eve* and *grn* act solely in the *eve→grn→zfh1* regulatory cascade to specify aCC motoneuron identity, or do these regulators play additional roles during aCC specification? To address this question, we attempted to rescue the motoneuron pathfinding phenotype of *eve* mutants with *UAS-grn,* and, similarly, to rescue *grn* mutants with *UAS-zfh1* (using in both cases *RN2-GAL4*). First, we find that *grn* does not rescue the *eve* phenotype in aCC; a failure of aCC to project its axon out of the VNC and activate Zfh1 expression (Fig. 6A-E). Second, we find that *UAS-zfh1* can only partially rescue the *grn* motoneuron phenotype; muscle 1/9 innervation is increased to 34% (*n*=136) compared with the more severe (12%) *grn* mutant phenotype (Fig. 2F,K).

The dMP2 peptidergic neurons project posteriorly in the VNC (Hidalgo and Brand, 1997) and exit the VNC to innervate the hindgut (Miguel-Aliaga and Thor, 2004). dMP2 neurons do not express Eve, Grn or Zfh1 (Fig. 6F; not shown). Recent studies show that misexpression of *zfh1* in dMP2 neurons can potently trigger lateral axonal exit from the VNC (45% lateral exit) (Layden et al., 2006). To test whether misexpression of *eve* and/or *grn* can similarly alter axonal projections of dMP2 neurons, we misexpressed them alone and in combination. We find that although *eve* can trigger lateral VNC exit at low frequency (5.5%; *n*=36; Fig. 6H), *grn* has no such effect (0%; *n*=28). By contrast, co-misexpression of *eve* and *grn* leads to a high frequency of lateral exit (40.5%; *n*=84; Fig. 6G,H). To our surprise, the combinatorial misexpression of *eve* and *grn* alters axon pathfinding without any obvious sign of ectopic Zfh1 expression (Fig. 6G). Thus, misexpression of either *zfh1* alone or of *eve/grn* together, can act equally well in triggering dMP2 lateral axonal exit. These rescue and misexpression results indicate that although *eve* and *grn* act in an *eve→grn→zfh1* regulatory cascade within aCC, both genes play additional roles to ensure proper aCC identity.

The *eve→grn→zfh1* regulatory cascade and integration of the Notch pathway

In the aCC neuron, *grn* and *zfh1* are positively regulated by *eve*. aCC and its sibling, the pCC interneuron, is a well-studied sibling pair. The pCC neuron also expresses Eve, as well as the Nkx-family member *vnd* (ventral nervous system defective) (McDonald et al., 1998). Using *eve* mosaic mutants, we find that Vnd expression in pCC is completely dependent upon *eve* (Fig. 4H,I). Thus, *eve* acts in both sibling cells to regulate different downstream genes in each neuron; *grn* and *zfh1* in aCC, and *vnd* in pCC. Studies have shown that the aCC versus pCC cell fate decision is dependent upon Notch signaling, with pCC being dependent upon Notch activation (Skeath and Doe, 1998). Although Eve expression in aCC and pCC

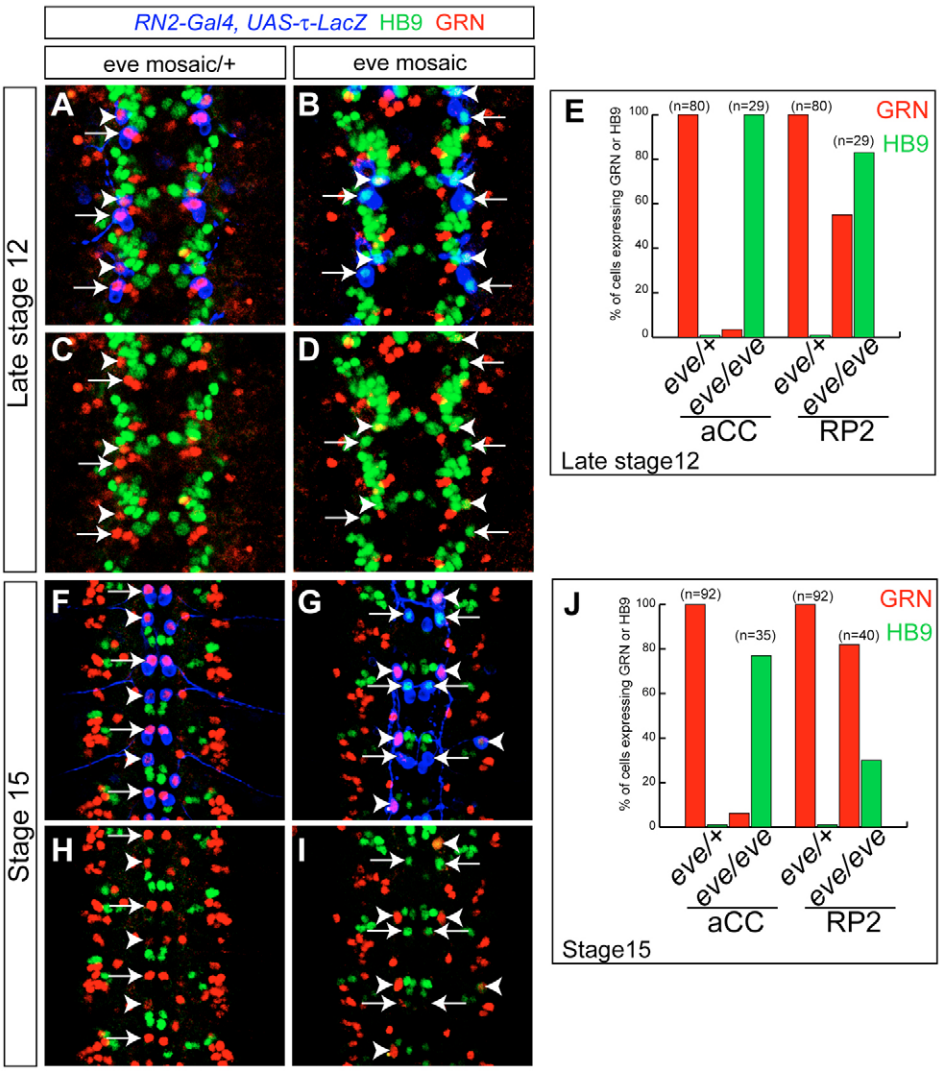

Fig. 3. *eve* is necessary for *grain* expression and for *Hb9* repression in both aCC and RP2 motoneurons. Stage 12 (A-D) or stage 15 (F-I) $eve^{\Delta RP2A/+}$ heterozygote (*eve* mosaic/+) (A,C,F,H) and $eve^{\Delta RP2A}$ homozygote mutant (*eve* mosaic) (B,D,G,I) embryos. Arrows and arrowheads indicate aCC and RP2, respectively (visualized using *RN2-GAL4/UAS-τlacZ*). (**A**,**C**,**F**,**H**) *eve* mosaic/+ *RN2-GAL4/UAS-τlacZ* showing that Grn is expressed in aCC and RP2 at stage 12 and stage 15, while Hb9 is not. (**B**,**D**) In stage 12 *eve* mosaic mutant, Hb9 is derepressed in aCC and RP2 while Grn expression is not detectable in aCC but maintained in RP2. (**G**,**I**) In stage 15 *eve* mosaic mutants, Hb9 remains derepressed in aCC and partly in RP2, while Grn expression is not detectable in aCC but maintained in RP2. At this stage, Grn expression in RP2 appears even stronger in *eve* mosaic compared with wild type. (**E**,**J**) Quantification of these phenotypes.

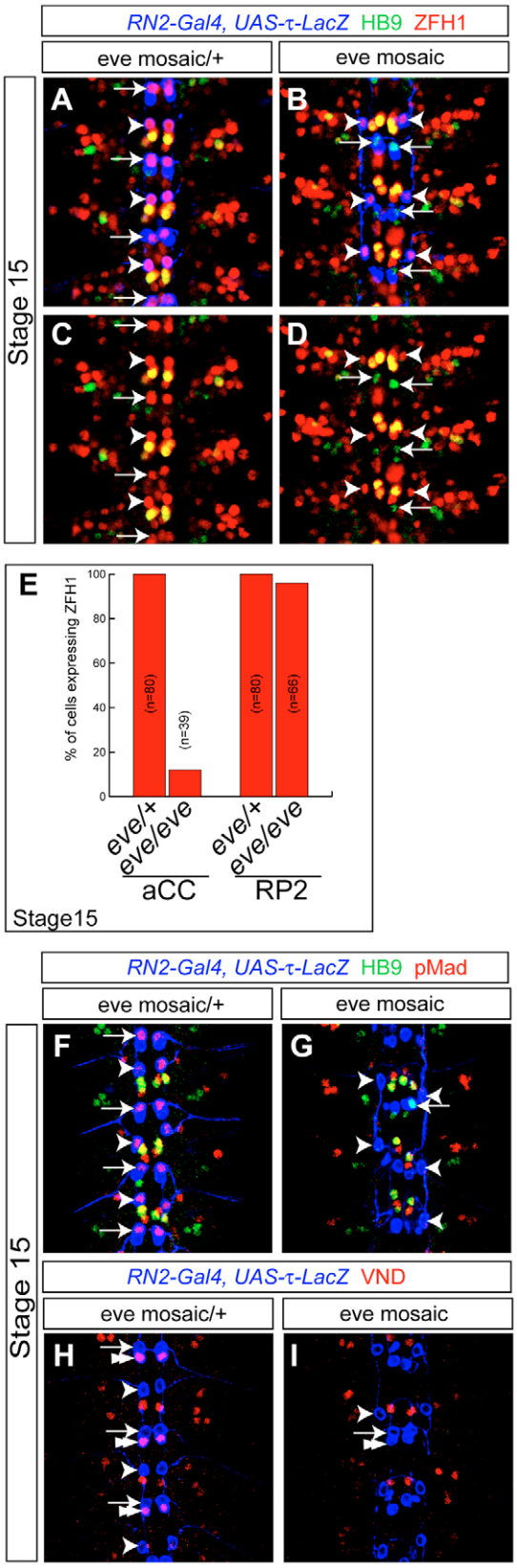

Fig. 4. Zfh1, pMad and Vnd expression is affected in *eve* mutants. (A-D,F-I) Stage 15 *eve* mosaic/+ (A,C,F,H) and *eve* mosaic (B,D,G,I). Arrows and arrowheads indicate aCC and RP2, respectively (visualized using *RN2-GAL4/UAS-τlacZ*). (**A**,**C**) Zfh1 expression is robust in control aCC and RP2 motoneurons. (**B**,**D**) In *eve* mosaic mutants, Zfh1 is lost from aCC, but unaffected in RP2 motoneurons. (**E**) Quantification of these phenotypes. (**F**) In control, pMad staining is evident in both aCC and RP2, but lost from these neurons in *eve* mosaic mutants (**G**). (**H**) In control, Vnd is specifically expressed by the pCC interneuron (double arrowhead) but expression is lost in *eve* mosaic mutants (**I**).

does not respond to alterations in the Notch pathway, expression of both Zfh1 and Vnd in these siblings has been shown to be sensitive to Notch signaling (Lear et al., 1999). To address whether *grn* also responds to Notch activity in the aCC/pCC cell pair, we analyzed grn^{lacZ}, grn^{GAL4} and Grn expression in two mutants affecting the Notch pathway, *sanpodo* ($spdo^{G104}$) and *mastermind* ($mam^{l(2)04615}$). *spdo* facilitates N signaling specifically during asymmetric cell divisions, and mutants permit normal N signaling during early neurogenesis (O'Connor-Giles and Skeath, 2003). Likewise, *mam* is needed for nuclear events downstream of N signaling, but has a maternal contribution (Skeath and Doe, 1998). This allows, in both cases, for the examination of N function at later stages of neuronal development. In *spdo* and *mam* mutants, we find activation of both Grn (and grn^{GAL4}) expression in pCC (Fig. 7E,F,H). As previously reported, we find that Vnd expression is lost in pCC (Fig. 7A,B,B′). Conversely, ectopic Notch activation in aCC, using the *RN2-GAL4* driver to express the intracellular (activated) $UAS\text{-}Notch^{ICD}$ transgene (Doherty et al., 1996), produces the reverse phenotype: de-repression of Vnd in aCC (but not in RP2) and repression of *grn* in aCC and RP2 (Fig. 7C,G,I). Thus, in the *eve→grn→zfh1* regulatory cascade, only *grn* and *zfh1* respond to Notch signaling.

We next asked whether *grn* was sufficient to activate aCC-specific or to suppress pCC-specific genes, respectively? Although *grn* is necessary for Zfh1 expression in aCC, we find that misexpression of *grn* in pCC neither suppresses Vnd nor activates Zfh1 (Fig. 8A-C; not shown). This is in agreement with the fact that we never observed Vnd expression in aCC in *grn* mutants (data not shown). Likewise, using *RN2-GAL4/UAS-vnd*, we asked whether *vnd* was sufficient to suppress aCC-specific markers but find that *vnd* cannot suppress Grn expression in aCC (Fig. 8D-F).

In summary, we have shown that Notch signaling acts downstream of, or in parallel to, *eve* to restrict *grn* and *zfh1* to aCC, and *vnd* to pCC. However, these determinants are not involved in cross-repressive interactions within these post-mitotic sibling cells (Fig. 9). We furthermore find that although both aCC and RP2 express *eve*, *grn* and *zfh1*, their regulatory interactions differ between aCC and RP2.

DISCUSSION

Specification of unique motoneuron identities

During motoneuron generation, combinatorial codes of regulators act to specify important aspects of subclass identity (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). However, within any given subclass, there exists a further refinement, apparent in *Drosophila* and *C. elegans* at single-cell resolution. Our findings suggest that unique motoneuron identities may be defined by the unique interplay between subclass determinants (i.e. *eve*/*grn*/*zfh1* in the ISN subclass). Our findings, combined with previous studies of the aCC/pCC and RP2/RP2sib pairs (Doe et al., 1988b), reveal a remarkable difference in the genetics of aCC and RP2 specification. A summary of the specification of these cells is presented in Fig. 9 and highlights how a unique genetic cascade allows for the specification of the aCC

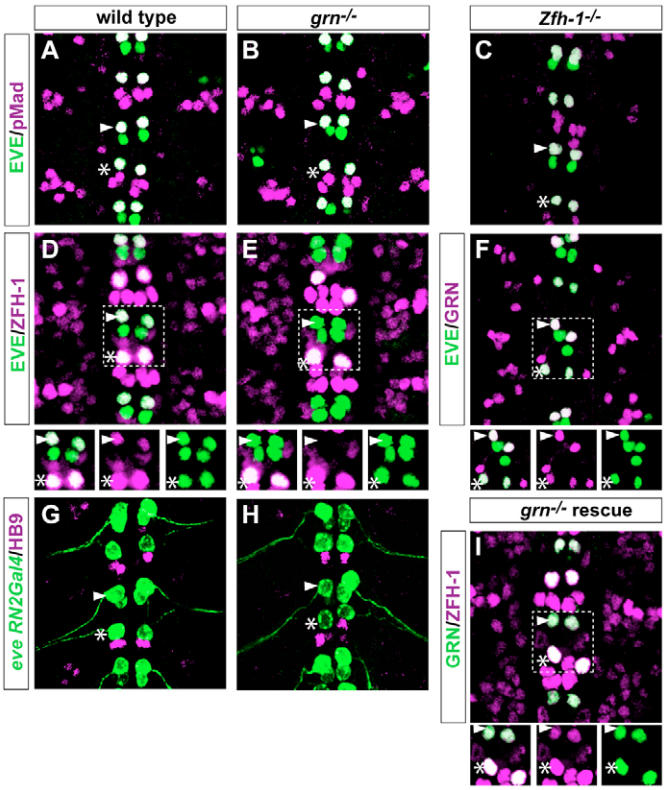

Fig. 5. In *grain* mutants, loss of Zfh1 expression is restricted to the aCC motoneuron. Stage 15 wild-type (A,D,G), *grn* mutant (B,E,H), *zfh1* mutant (C,F) and *grn* rescue (I) (using *RN2-GAL4/UAS-grn; grn–/–*) embryos stained for Eve and pMad (A-C), Eve and Zfh1 (D-F) or Grn and Zfh1 (I). (G,H) *RN2-GAL4/UAS-mEGFP^F* embryo stained with α–Hb9. (**A-C**) pMad staining in *grn* and *zfh1* mutants appears unaffected within aCC and RP2. (**D-F**) In *grn* mutants, Zfh1 expression is not detectable in the aCC motoneuron, but RP2 maintains Zfh1 expression. Grn expression is not affected in aCC or RP2 in *zfh1* mutants. (**G**,**H**) Hb9 expression is unaffected in *grn* mutants. (**I**) In *grn* rescue experiments, Zfh1 expression is restored in aCC showing the cell autonomous effect of *grn* on Zfh1 expression in this motoneuron. Arrowheads and asterisks indicate aCC and RP2, respectively.

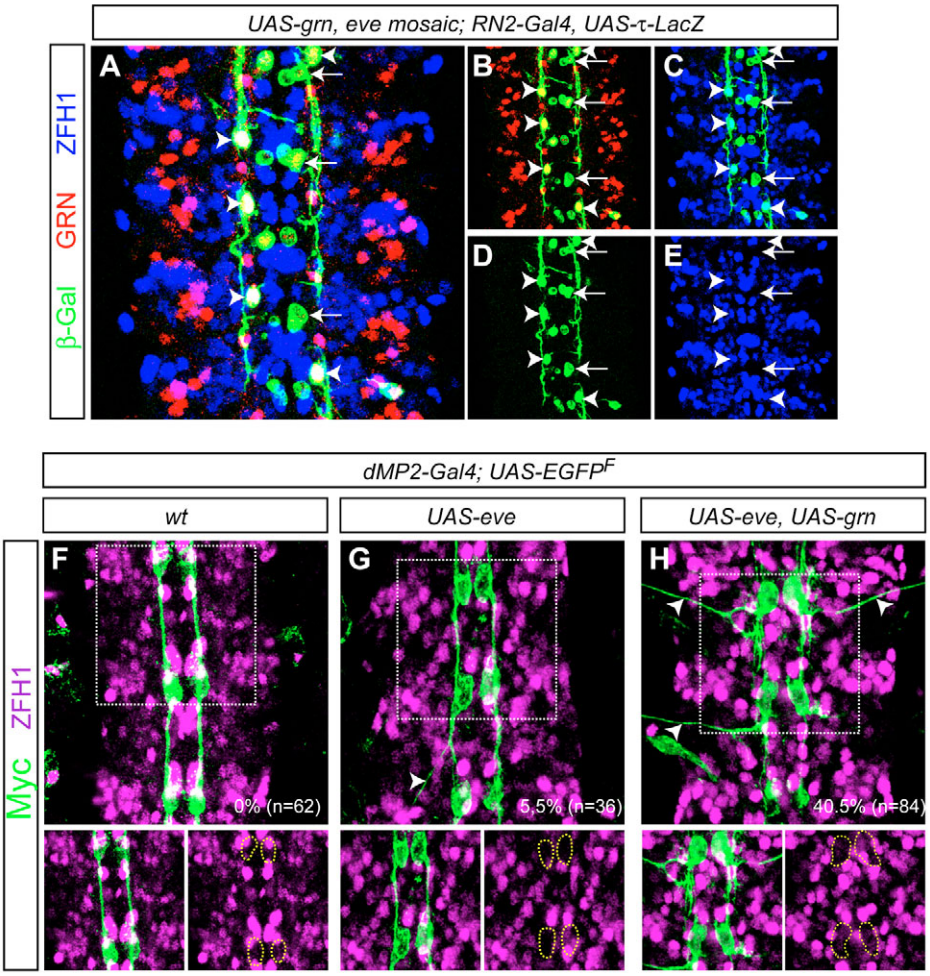

Fig. 6. *eve* and *grain* play additional roles outside of the *eve→grn→zfh1* cascade. (**A-E**) Stage 15 embryo stained for Grn (A,B) β-Gal (A-D) and Zfh1 (A,C,E). B-E are identical to A but with different combinations of color channels to facilitate the observation of Grn and Zfh1 expression in aCC (arrows) and RP2 (arrowheads). *grn* is unable to rescue *eve* mosaic mutants (*UAS-grn, eve* mosaic*; RN2-GAL4, UAS-τlacZ*), evident as a failure of aCC and RP2 to project axons out of the VNC, and of aCC to express Zfh1. (**F-H**) Stage 15 embryo stained for Myc and Zfh1, expressing only *UAS-EGFP^F* (F), *UAS-eve* (G) or co-misexpressing both *eve* and *grn* (H). (F) In the control, dMP2 axons project posteriorly in the longitudinal connective and never exit the VNC laterally (*n*=62). (G) Ectopic *eve* triggers lateral VNC exit, but only in 5% of hemisegments. (H) Ectopic *eve* and *grn* (*UAS-eve, UAS-grn, dMP2-GAL4; UAS-EGFP^F*) triggers lateral VNC exit in 40% of hemisegment (*n*=84). There is no evidence of Zfh1 expression in dMP2 neurons (yellow circles), in the control (F) or in the misexpression backgrounds (G). Arrowheads indicate dMP2 axons exiting the VNC.

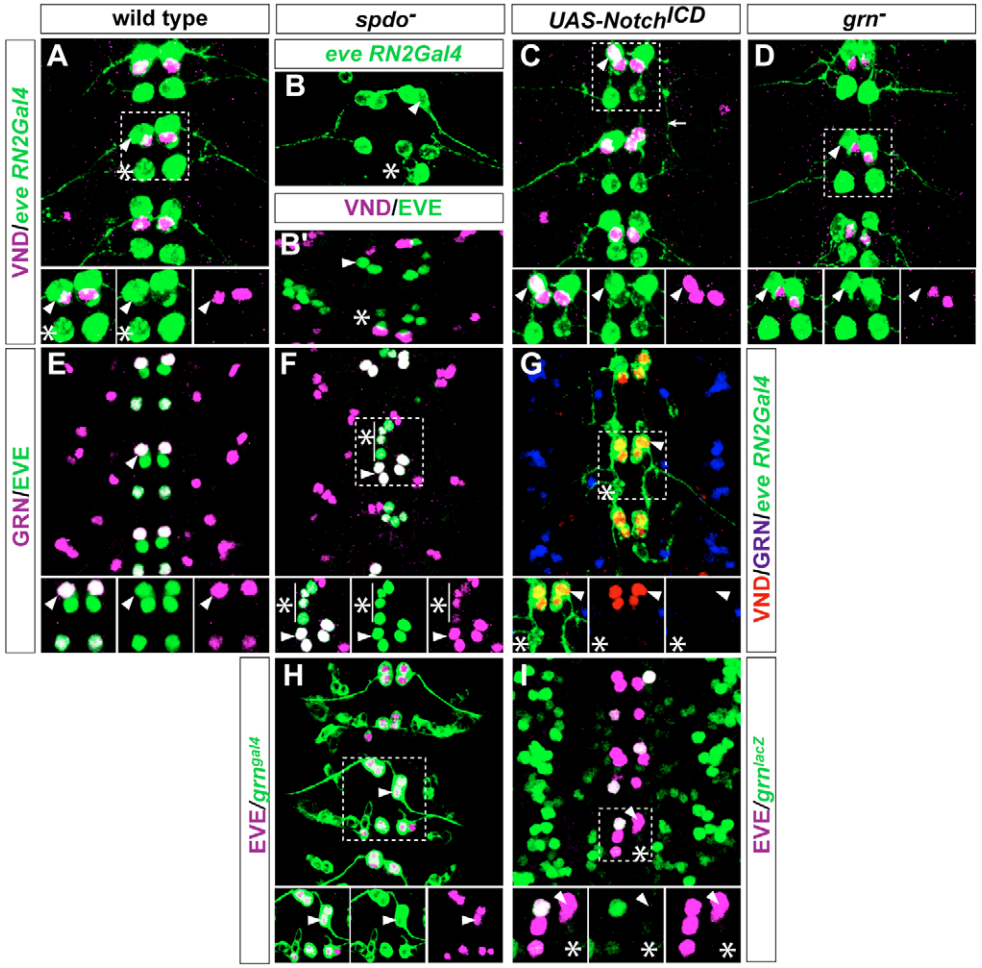

Fig. 7. *grain* expression is under the control of Notch signaling. (**A**) In wild type, Vnd is expressed in the pCC interneuron, but this expression is lost in *spdo*^{G104} mutant (**B**,B′). (**C**,G) Vnd is derepressed in the aCC motoneuron when Notch signaling is activated using *RN2-GAL4/UAS-Notch*^{ICD} (intracellular domain of a constitutive activated form of Notch). The arrow indicates aberrant axonal projection (probably from aCC and/or RP2). (**D**) In *grn* mutants, derepression of Vnd is not observed in aCC (or in RP2) suggesting that *grn* does not repress Vnd in this sibling neuron. (**E**) In wild type, Grn is not expressed in pCC. (**F**,**H**) In *spdo*^{G104} mutants, Grn (and *grn*^{GAL4}) is derepressed in the pCC neuron. Grn is also derepressed in the RP2sib; 4 Eve-positive neurons (observed in B) are indicated by a vertical bar and an asterisk. (**G**,**I**) Activation of Notch (*RN2-GAL4/UAS-Notch*^{ICD}) led to a loss of Grn (and *grn*^{lacZ}) expression in aCC and RP2. Arrowheads and asterisks indicate aCC and RP2, respectively.

motoneuron. But why do these three genes act in a unique fashion in aCC, and why is *grn* and *zfh1* sensitive to Notch specifically in this ISN motoneuron? One explanation may be that the differential input from upstream regulators, such as Ftz, Pdm1, Hkb and Pros (McDonald et al., 2003), acts to modify the genetic interactions between *eve*, *grn* and *zfh1*. Another possibility is that the relative level of each factor plays an important role in dictating different cellular fates. Studies of the related *Isl1* and *Isl2* LIM-homeobox genes suggest that their involvement in motoneuron subclass specification is not primarily the result of the unique activity of each gene, but rather by the combined 'generic', tightly temporally controlled, *Isl1* and *Isl2* levels (Thaler et al., 2004). Similarly, the different expression levels of the transcription factor Cut have been shown to play instructive roles during the specification of neuronal cell identities within the PNS (Grueber et al., 2003). We have as well noticed different levels of expression of Grn and Zfh1; while Grn is strongly expressed in aCC and weakly in RP2, Zfh1 expression shows an opposite distribution. It is tempting to speculate that these levels may be instructive for ISN motoneuron specification.

Cross-repressive interactions and Notch signaling specify neural fates

In the VNC, we observe mutually exclusive expression between Grn and Hb9 (and Islet) in different subsets of interneurons and motoneurons. Cross-inhibitory interactions between *eve* and *Hb9* has been shown to contribute to their mutually exclusive expression patterns, and functional studies demonstrate that *eve* and *Hb9* regulate axonal trajectories of dorsally and ventrally projecting axons, respectively (Broihier and Skeath, 2002; Doe et al., 1988b; Fujioka et al., 2003; Landgraf et al., 1999). These observations are reminiscent of the cross-repressive interactions between classes of regulators that act to determine, refine and maintain distinct progenitor domains along the dorsoventral axis of the vertebrate neural tube (Briscoe et al., 2000). We have shown that *eve* is important for proper *grn* and *zfh1* expression in aCC, but not in RP2. These results are consistent with previously reported observations that the requirement for *eve* in axonal guidance is somewhat more stringent in aCC than in RP2, leading the authors to propose that there may be different target genes for Eve in these two motoneurons (Fujioka et al., 2003).

Zfh1 expression was previously shown to depend upon Notch signaling activity in the aCC/pCC sibling pair as mutations in *spdo* or *mam*, members of the Notch signaling pathway, lead to de-repression of Zfh1 in pCC (Skeath and Doe, 1998). Using the same allelic combinations, we also observed de-repression of *grn* in pCC. Whether or not *grn* is directly suppressed by the Notch pathway remains to be seen, but it is interesting to note that in vertebrates, *gata2/3* have been identified as targets of Notch during the differentiation of specific hematopoietic lineages (Amsen et al., 2004; Kumano et al., 2001).

aCC, RP2 and U motoneurons – several pioneers for ISN?

Within the ISN subclass, the aCC motoneuron pioneers the ISN to innervate the dorsal-most muscle, muscle 1 (Jacobs and Goodman, 1989; Sanchez-Soriano and Prokop, 2005; Thomas et al., 1984). A

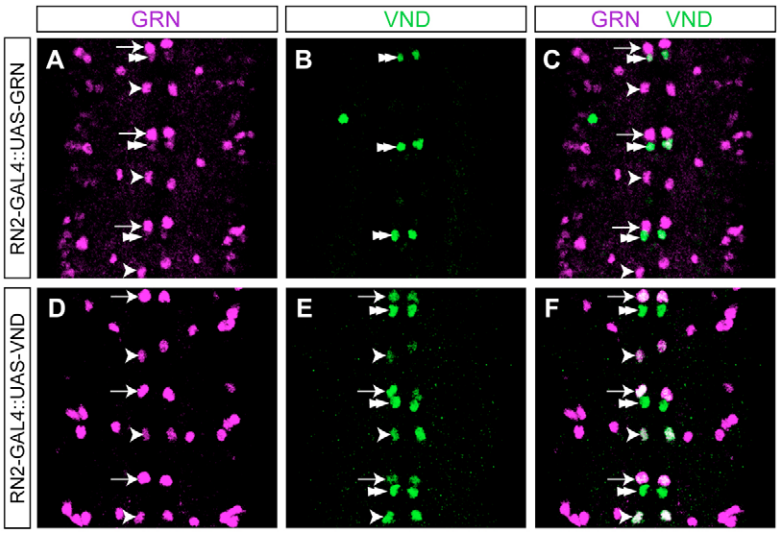

Fig. 8. *grain* and *vnd* do not act in a cross-repressive manner in aCC, pCC and RP2. (A-F) Stage 15 embryos stained for Grn and Vnd. (**A-C**) Ectopic *grn* expression in pCC (double arrowhead; *RN2-GAL4/UAS-grn*) does not suppress Vnd expression in this cell. (**D-F**) Ectopic Vnd expression in aCC (arrow) and RP2 (arrowhead; *RN2-GAL4/UAS-vnd*) does not suppress Grn expression in these cells.

number of genetic and cell-ablation studies have convincingly shown that aCC plays an instructive pioneer role and guides the follower U motoneurons along the ISN nerve (Fujioka et al., 2003; Lin et al., 1995a; Sanchez-Soriano and Prokop, 2005). Our results lend support for the proposed instructive role of aCC in ISN formation. However, our studies indicate that aCC may not be essential for ISN formation. First, using *RN2-GAL4* to visualize aCC and RP2, we frequently find (35% of hemisegments) aberrant innervation of muscle 8 in *grn* mutants. However, we simultaneously observe an axonal projection at the vicinity of the dorsal muscles 2/10. In *grn* mutants, *zfh1* expression is specifically lost in aCC but maintained in RP2. Given the role for *zfh1* in motor axon pathfinding, we propose that aberrant innervation of muscle 8 in *grn* mutants, is caused by aCC and not by RP2, and that RP2 pathfinds normally to the muscles 2/10. If so, RP2 may function as a pioneer motoneuron for muscle 2 and project there without the aCC axon. Second, although the rescue of *grn* mutants using *RN2-GAL4* is complete, we do find that using *CQ2-GAL4* to specifically rescue U motoneurons does lead to a partial rescue (54% muscles 1/9 innervated compared with 15% in *grn* mutants). Thus, even in the absence of aCC pioneer function, the Us (presumably U1) can still project to the dorsal-most muscles. This is in line with previous studies showing that in eve aCC/RP2 mosaic mutants and in aCC/RP2 cell ablation experiments, there is still partial innervation of muscle 1/9 (Fujioka et al., 2003; Lin et al., 1995a; Sanchez-Soriano and Prokop, 2005).

The *eve→grn→zfh1* genetic cascade contra other roles for *eve* and *grain*

We find that *grn* is part of an *eve→grn→zfh1* transcriptional cascade crucial for specification of aCC motoneuron identity. However, the failure of *grn* to rescue *eve*, and of *zfh1* to completely rescue *grn*, combined with the misexpression results, indicate additional roles for both *eve* and *grn*. These roles could be either in the regulation of other aCC determinants and/or in the regulation of genes directly involved in aCC axon pathfinding. Although we are unaware of obvious candidates for additional aCC determinants, recent studies point to a candidate axon pathfinding gene. The *Drosophila unc-5* gene encodes a netrin receptor and is expressed in subsets of neurons in the VNC (Keleman and Dickson, 2001). Misexpression of *unc-5* is sufficient to trigger ectopic VNC exit in subsets of interneurons (Allan et al., 2003; Keleman and Dickson, 2001). Recent studies now show that *unc-5* is specifically expressed in *eve* motoneurons, and that *eve* is necessary, but only partly sufficient for *unc-5*

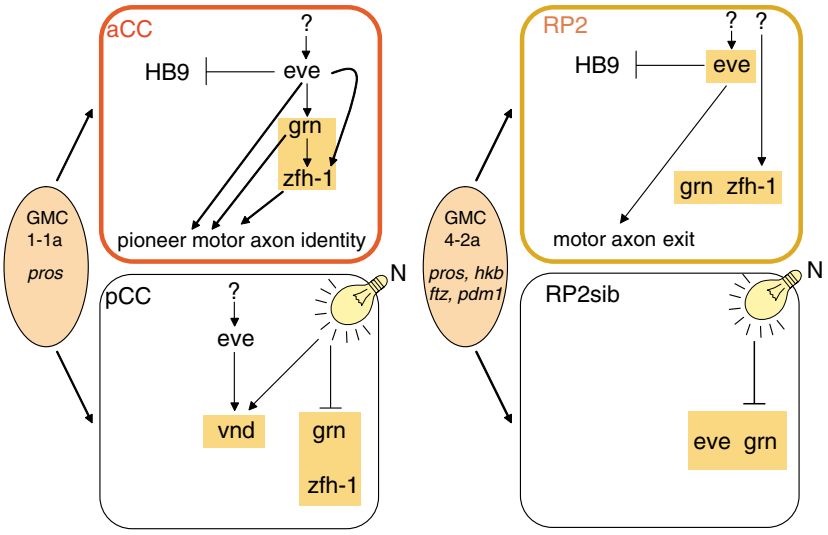

Fig. 9. An *eve→grn→zfh1* genetic cascade specifies aCC motor axon identity. Within the VNC, GMC1-1a is one of the first GMCs to divide and produces two postmitotic neurons: the aCC pioneer motoneuron and its sibling the pCC interneuron. In aCC and pCC, *eve* expression is independent of the activity of Notch signaling, whereas *grn* and *zfh1* are suppressed by Notch signaling, and *vnd* is activated by Notch. The GMC4-2a divides later and produces the RP2 motoneuron and the RP2sib. In contrast to aCC/pCC, in the RP2 neuron, *eve*, *grn* and *zfh1* do not regulate each other and in addition expression of all three genes is dependant upon Notch signaling. Although *pros* function is essential for proper GMC1-1a fate, GMC4-2a specification is under control of concerted activities of *pros*, *hkb*, *ftz* and *pdm1*. The orange boxes indicate genes regulated and/or sensitive to Notch signaling.

expression (Labrador et al., 2005). In line with these findings, we find that whereas single misexpression of *eve* or *grn* in dMP2 neurons has very minor effects, co-misexpression of *eve* and *grn* can efficiently trigger dMP2 lateral axonal exit. This combinatorial effect of *eve*/*grn* occurs without apparent activation of *zfh1*. However, misexpression of *zfh1* can also trigger dMP2 lateral exit (Layden et al., 2006). Thus, these genes appear to be able to act in an independent manner to trigger VNC exit, but in a highly context-dependent manner. A speculative explanation for not only the mutant and rescue results, but also these misexpression results, would be that all three regulators are needed for robust and context-independent activation of axon pathfinding genes such as, for example, *unc-5*.

Evolutionary conservation of GATA gene function

grn encodes a GATA Zn-finger transcription factor and is the ortholog of the closely related vertebrate *gata2* and *gata3* genes. In vertebrates, *gata2/3* are expressed in overlapping domains in the nervous system, but relatively little is known about their function. Expression data and evidence from gene targeting suggest an involvement in neurogenesis, neuronal migration and axon projection (Karis et al., 2001; Nardelli et al., 1999; Pandolfi et al., 1995; Pata et al., 1999). A role in specifying neuronal subtypes within the context of neural tube patterning is emerging (Karunaratne et al., 2002; Zhou et al., 2000) and recently a role for *gata2/3* during 5-HT neuron development has been reported (Craven et al., 2004; Tsarovina et al., 2004; van Doorninck et al., 1999). The role of *gata3* in the development of the inner ear has been of particular interest, and in humans, mutations in this gene have been linked to HDR syndrome, which is characterized by hypoparathyroidism, deafness and renal defects (Muroya et al., 2001; Van Esch et al., 2000). In the mouse, *gata3* is expressed in auditory but not vestibular ganglion neurons during development (Lawoko-Kerali et al., 2002; Rivolta and Holley, 1998). The mouse *gata3* mutant shows auditory ganglion neuron loss and efferent nerve misrouting, revealing that *gata3* regulates molecules associated with neural differentiation and guidance (Karis et al., 2001). These vertebrate studies, combined with our results, suggest that *gata2/3* genes, similar to other transcription factors specifying neuronal identities, such as *islet1/2*, *evx1/2* or *Hb9*, and their respective orthologs in *Drosophila*, have maintained similar functions throughout evolution (Broihier and Skeath, 2002; Fujioka et al., 2003; Thor and Thomas, 2002).

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