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A GATA/homeodomain transcriptional code regulates axon guidance through the Unc-5 receptor

Aref Arzan Zarin*, Amanda C. Daly*, Jörn Hülsmeier, Jamshid Asadzadeh and Juan-Pablo Labrador‡

SUMMARY

Transcription factor codes play an essential role in neuronal specification and axonal guidance in both vertebrate and invertebrate organisms. However, how transcription codes regulate axon pathfinding remains poorly understood. One such code defined by the homeodomain transcription factor Even-skipped (Eve) and by the GATA 2/3 homologue Grain (Grn) is specifically required for motor axon projection towards dorsal muscles in *Drosophila*. Using different mutant combinations, we present genetic evidence that both Grn and Eve are in the same pathway as Unc-5 in dorsal motoneurons (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.

KEY WORDS: Axon guidance, Transcription, Combinatorial code, GATA, Unc-5, *Drosophila*

INTRODUCTION

Transcriptional codes within a neuron direct the expression of an array of receptors and adhesion molecules that will eventually determine its guidance and connectivity properties. A considerable number of guidance systems that control axon pathfinding and target selection have been identified in the last two decades (Kolodkin and Tessier-Lavigne, 2010). Nevertheless, how different transcriptional codes regulate the expression of those guidance systems is only starting to emerge.

Some reports have already established some links between transcription factor codes regulating axon guidance and the effector molecules they regulate in either motoneurons (Kania and Jessell, 2003; Broihier et al., 2004; Labrador et al., 2005; Shirasaki et al., 2006; Luria et al., 2008) or commissural neurons (Crowner et al., 2002; Pak et al., 2004; García-Frigola et al., 2008; Lee et al., 2008; Wilson et al., 2008). However, how this regulation ultimately leads to specific guidance decisions is still an open question.

The *Drosophila* neuromuscular system is composed of 36 motoneurons innervating a stereotyped array of 30 somatic muscles. In this system, two main nerve branches can be identified, the intersegmental nerve (ISN) and the segmental nerve (SN). Two motoneurons, aCC and RP2, within the ISN specifically project to the most dorsal muscles and a transcriptional code determined by the homeodomain transcription factor Even-skipped (Eve) (Doe et al., 1988; Landgraf et al., 1999; Fujioka et al., 2003) and the GATA2/3 homologue Grain (Grn) determines the specific guidance characteristics of these *Drosophila* dorsal motoneurons (dMNs) (Garces and Thor, 2006). The *eve-grn* code is the only one identified that has been dissected to single motoneuron resolution and Garces and Thor have shown that both transcription factors can work in parallel or sequentially in a cell-specific manner (Garces and Thor, 2006) making dMNs an ideal system to study transcriptional code regulation of axon guidance. Nevertheless, the target guidance systems they control in each cell or whether they regulate independent or common guidance systems is unknown.

To gain a better understanding of how the expression of receptors on the cell surface is regulated through transcriptional codes, 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.

MATERIALS AND METHODS

Genetics

The following stocks were used in misexpression experiments: *UAS-grn#2;UAS-mEGFP^F*, *UAS-grn#2,UAS-eve;UAS-mEGFP^F*, *UAS-eve/TM3* (Brown and Castelli-Gair Hombría, 2000; Garces and Thor, 2006), *UAS-HA-unc5* (Keleman and Dickson, 2001) and *dMP2Gal4/CyO* (Miguel-Aliaga and Thor, 2004). *RN2Gal4; grn^{SPJ9}/TM3 and UAS-HA-*

Smurfit Institute of Genetics, Institute of Neuroscience, Trinity College Dublin, Dublin 2, Ireland.

*These authors contributed equally to this work
‡Author for correspondence (labradoj@tcd.ie)

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unc5;grn^{7L12}/TM3 stocks were used in rescue experiments. The following stocks were also used: *unc-5^{8}/CyO*, *XTE-18/CyO* (*unc-5* deficiency) (Labrador et al., 2005), *Df(2R)eve, RP2A/CyO; RN2-Gal4,UAS-tau_lacZ* (Fujioka et al., 2003), *RN2-Gal4, UAS-tau_lacZ; grn^{7L12}/TM3*, *dMP2Gal4,XTE18/CyO;UAS-tau-myc/TM2*, *Unc5^{8}/CyO;grn^{7L12}/TM3*, *dMP2Gal4/CyO;UAS-tau-myc/TM2*, *Unc5^{8},UAS-grn#2,UAS-eve*, *unc-5-Gal4* (*Bloomington 47230*), *Df(2R)eve, RP2A/CyO; UAS-NLS-Venus and Df(2R)eve, RP2A/CyO; unc-5-Gal4*.

Immunohistochemistry

Embryos were collected for 24 hours and processed as previously described (Labrador et al., 2005). ISN motor axonal projections at embryonic stage 16/17 in A2-A6 abdominal hemisegments were quantified in different genetic backgrounds. The following antibodies were used: anti-c-Myc 9E10 (1:50), anti-Fas2 1D4 (1:50) (Developmental Studies Hybridoma Bank), anti-HA (Covance; 1:500) and rabbit anti-β-gal (Cappel; 1:5000). Secondary antibodies were purchased conjugated to Alexafluor488 (Jackson ImmunoResearch Laboratories) and Cy3 (Molecular Probes) and used at 1:1000 and 1:500, respectively. Double-labelled images were false coloured for the benefit of colour-blind readers. Stacks of images were obtained with Zeiss Confocal LSM700 Microscope and a 40× oil immersion objective was used.

mRNA in situ hybridization

unc-5 in situ hybridization in aCC and RP2 dorsal motor neurons as well as dMP2 interneurons was performed with digoxigenin-labelled probes as previously described (Labrador et al., 2005). Hybridized probes were detected with anti-digoxigenin HRP, and Cy3-labelled tyramide was used as substrate. Double labelling of aCC, RP2 and dMP2 neurons was performed with anti-myc and Alexa 488 secondary antibody. For fluorescence quantification in aCC and RP2 motor neurons, heterozygous embryos and mutant siblings were isolated from the same embryo collection and identified on the basis of the presence (heterozygotes) or absence (homozygous mutants) of β-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 were 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. Using SPSS 16 software (SPSS), histograms were generated and statistical significance was determined. For analysis of genetic interaction between *unc-5* and *eve* as well as *unc-5* and *grn*, Kruskal–Wallis one-way analysis of variance was used. For all other comparisons, two-independent samples *t*-test was used.

Plasmids

unc-5 genomic fragments were amplified from genomic DNA and TA cloned into pCR8/GW/TOPO (Invitrogen) according to the manufacturer's protocol. Fragments were then cloned using LR Clonase II Plus enzyme (Invitrogen) into pGWA-NLS-Venus. NLS-Venus was amplified and inserted (*Xba*I/*Xho*I) into a pUAS-attB vector with an inserted Gateway A cassette (*Pst*I/*Bgl*II) to generate pGWA-NLS-Venus. All constructs were inserted by site-specific recombination into the attP2 site at 68A4 on 3L.

RESULTS

unc-5 and *grn* function in the same pathway to mediate motoneuron pathfinding

We have previously shown that the Unc-5 receptor is required in dMNs to reach its target muscles. In its absence, the ISN nerve will cross segment boundaries and even fasciculate with ISN from neighbouring segments as it presumably fails to sense the repulsive signal from the Netrins (Labrador et al., 2005), a phenotype similar to those observed for Netrin mutations (Mitchell et al., 1996). A homeobox transcription factor, Even-skipped (Eve) is largely responsible for the specification of aCC and RP2 dMNs (Doe et al., 1988; Landgraf et al., 1999; Fujioka et al., 2003) and regulates the expression of *unc-5* (Labrador et al., 2005). In order to understand how guidance is transcriptionally regulated in individual motoneurons, we screened for other transcriptional regulators that presented a genetic interaction with *unc-5*. First, we determined whether *unc-5* and *eve* were able to interact genetically in a transheterozygous combination. 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 (Fig. 1A,B; Table 1). 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 (Fig. 3D,E) (Garces and Thor, 2006). Furthermore, in *grn* mutants, motoneurons are properly specified because they are still able to induce Mad phosphorylation (Garces and Thor, 2006) and express several motoneuron markers (supplementary material Fig. S1). Thus, we generated transheterozygous combinations of *unc-5* and *grn*. Although *grn*/+

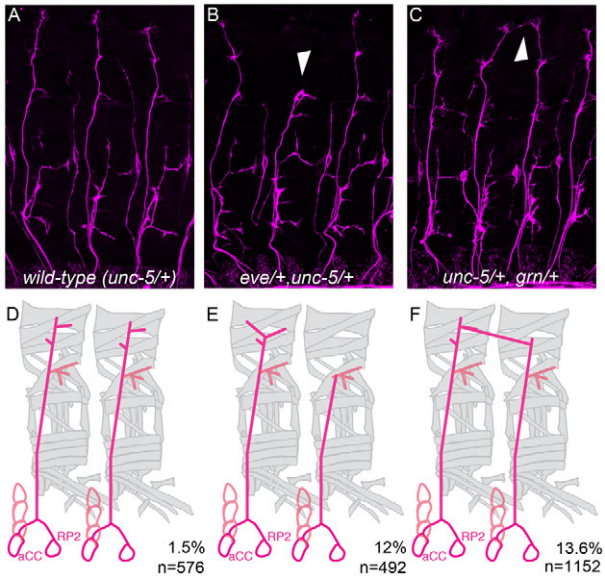

Fig. 1. *unc-5* interacts genetically with *eve* and *grn*. Motor axon projections of flat mounted late st-16 to st-17 embryos visualized with anti-Fas2. Anterior is left and dorsal is up in all panels. Partial genotypes are indicated below each panel. (**A**) Wild type (*Unc-5*/+). In wild type, the 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 1 for detailed description.

Table 1. Quantification of genetic interactions between *unc-5* and *eve* or *grn*

	Number of embryos	Penetrance*	Number of hemisegments	Hemisegments with ISN defects‡ (*n*)			
				ISN stall	ISN cross	ISN bifurcation	Total ISN defects
$eve^{\Delta RP2}$/+	20	20%	240	0.9% (2)	0% (0)	1.6% (4)	**2.5% (6)**
grn^{7l12}/+	29	14%	348	0.45% (1)	0.45% (1)	0.6% (3)	**1.5% (5)**
$unc\text{-}5^{8}$/+	48	12%	576	0.2% (1)	0.4% (2)	0.7% (4)	**1.3% (7)**
$eve^{\Delta RP2}$/+,+/$unc5^{8}$	41	65%	492	3.1% (15)	1.9% (10)	7% (34)	**12% (59)**
$unc\text{-}5^{8}$/+, grn^{7l12}/+	96	64%	1152	0.6% (7)	8% (92)	5% (57)	**13.6% (156)**

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

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. $P<0.001$; Fig. 1C; Table 1), an average of 24% defective ISNs per embryo presenting a phenotype (Fig. 1C, Table 1). This result strongly suggests that Unc-5 and Grn function in the same pathway to mediate guidance of dMNs.

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 dorsal-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 (Fig. 2B,D) when compared with their heterozygous siblings (Fig. 2A,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 (Fig. 2E). 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 (Fig. 2F; supplementary material Fig. S2). These results strongly suggest that *grn* is required for *unc-5* expression in both aCC and RP2 dMNs.

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 (Fig. 3A,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) (Fig. 3D,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 (Fig. 3F,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. ($P<0.006$) (Fig. 3G). 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%; Fig. 4B,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%; Fig. 4C,F). This result suggests that *eve* and *grn* can also work in parallel pathways to induce *unc-5* expression in dMNs.

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 (Fig. 5A). We identified a single region that could drive expression in neurons in the embryonic nervous system. This region drives expression in aCC, RP2 dMNs (Fig. 5B-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* (Fig. 5E-H), as the GFP signal visible in *eve*/+ embryos in aCC and RP2 (Fig. 5E,F) is absent in *eve/eve* mutants (Fig. 5G,H). Importantly, the GFP signal in motoneurons from the segmental nerve (SN in Fig. 5F,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

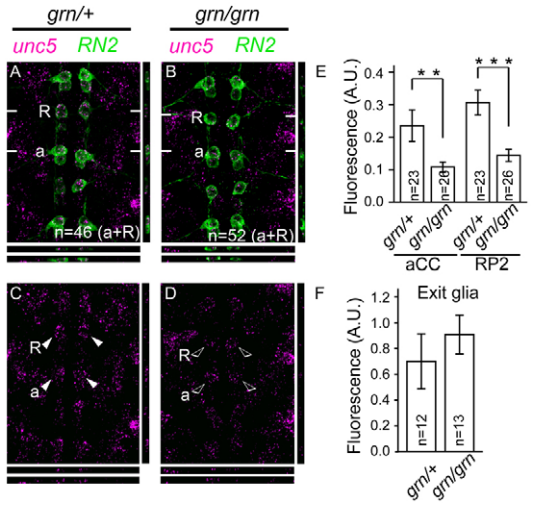

Fig. 2. Endogenous *grn* regulates *unc-5*. (**A-D**) *unc-5* mRNA expression was examined in aCC and RP2 motoneurons of Stage 15 *grn* mutant embryos (B,D) and their heterozygous siblings (A,C). Anterior is up and dorsal is right in all panels. The top panels (A,B) show *unc-5* in situ signal in magenta 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.

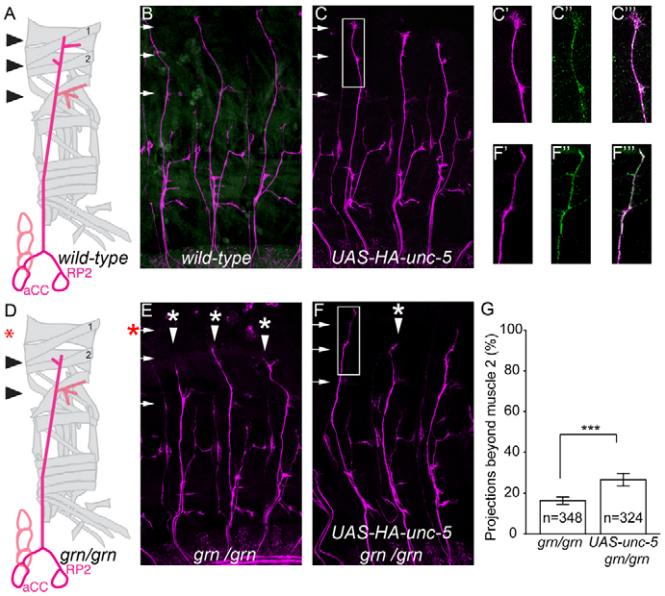

Fig. 3. *unc-5* partially rescues *grn* mutants. (**A-C**) Motor axon projections of flat mounted late st-16 to st-17 embryos visualized with anti-Fas2. Anterior is left and dorsal is up in all panels. Partial genotypes are indicated below each panel. Wild-type embryos (A,B) or animals expressing *HA-unc-5* 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.

difference in *eve* mutants as its elimination leads to an almost complete downregulation of endogenous *unc-5* (Fig. 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* (Fig. 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.

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 (Fig. 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 (Fig. 6A, Fig. 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 (Fig. 6B,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 (Fig. 7), suggesting that *eve* or *grn* can induce *unc-5* expression independently of each other. When both transcription factors were

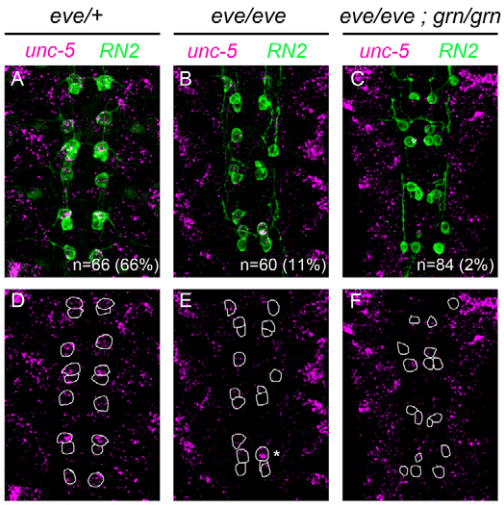

Fig. 4. *unc-5* levels are further reduced in *eve* and *grn* double mutants. (**A-F**) *unc-5* mRNA expression was examined in aCC and RP2 motoneurons of Stage 15 *eve* (E,F) or double *eve, grn* (C,F) mutant embryos and in *eve* heterozygous embryos (A,D). Anterior is up, dorsal is right and in situ 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.

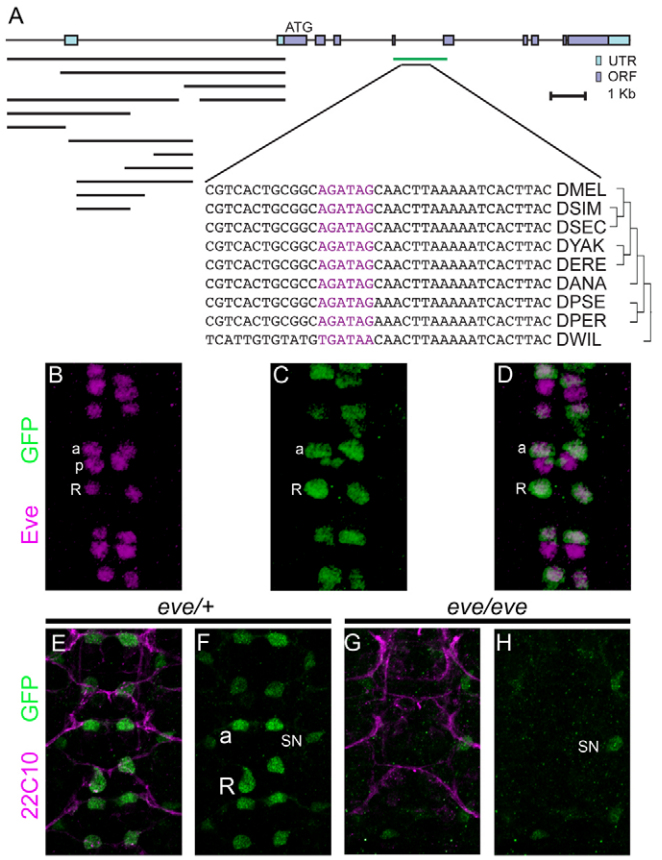

Fig. 5. An enhancer element for dMNs is regulated by Eve. (**A**) Mapping the location of the *unc-5* neuronal transcriptional enhancer. A schematic of the *unc-5* locus is shown at the top and the DNA fragments used in enhancer-reporter transgene expression analysis are depicted below. All constructs 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.

expressed in dMP2 neurons there was a further increase in the level and number of neurons expressing *unc-5* (86% of neurons; Fig. 6C), 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*.

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 (Garces and Thor, 2006) and follow the ISN nerve similarly to dMN projections (Fig. 8C) but each factor alone does not have this effect (Fig. 8B) (Garces and Thor, 2006). Likewise, *unc-5* misexpression in dMP2 neurons can also lead to aberrant axonal exit (Fig. 8D), similarly to apterous interneurons (Keleman and Dickson, 2001; Allan et al., 2003). Because *eve* and *grn* are upstream of *unc-5* in dMNs and *unc-5* is required as part of their guidance output (Figs 1, 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* (Fig. 8E,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 (Fig. 8E,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* (Fig. 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.

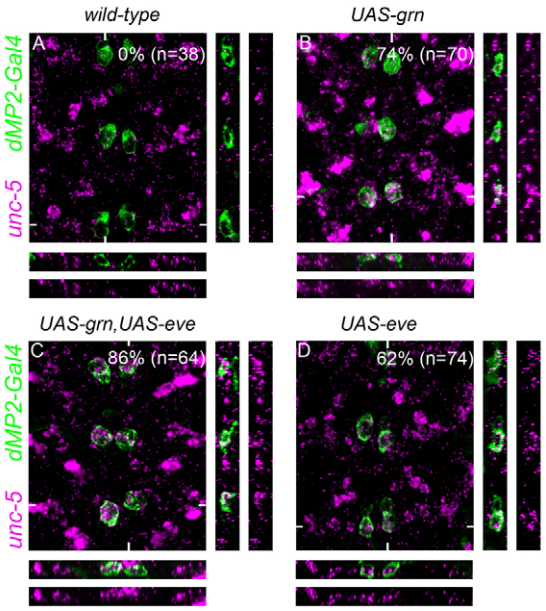

Fig. 6. Individual or combinatorial misexpression of *eve* and *grn* in dMP2 cells induce *unc-5* expression. (**A-D**) *unc-5* mRNA expression was examined in dMP2 neurons of stage-15 embryos. *unc-5* signal is in magenta, and myc antibody to localize *UAS-tau-myc* under the control of *dMP2Gal4* is 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.

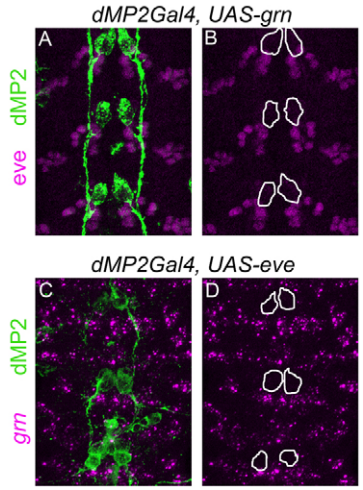

Fig. 7. *eve* and *grn* do not cross-regulate each other in dMP2 neurons. Anti-myc antibody was used to visualize dMP2 neurons expressing *UAS-tau-myc* through *dMP2Gal4* in green. (**A**) Eve protein expression was examined in St-15 embryo misexpressing *UAS-grn* in dMP2 cells. Anti-eve antibody in 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.

DISCUSSION

grn controls motor axon guidance through the Unc-5 receptor

Different transcriptional codes regulate axon guidance but the guidance systems they regulate are still unknown. The GATA factor, Grn, is a major determinant of guidance within dorsal MNs. Garces and Thor have previously determined a strong guidance phenotype in *grn* mutants, in which ISN axons fail to reach their targets in >85% of the segments (Garces and Thor, 2006). However, none of the downstream molecules required for guidance downstream of *grn* has been identified to date (Landgraf and Thor, 2006). Several lines of evidence indicate that the Unc-5 receptor mediates guidance downstream of the GATA transcription factor Grn. First, both genes interact genetically in trans and this type of genetic interaction is often seen between two genes the gene products of which directly interact, such as Slit and Robo (Kidd et al., 1999). Second, there is also a partial requirement of *grn* for *unc-5* expression in dMNs as *unc-5* mRNA levels are reduced in aCC and RP2 in *grn* mutants. Further support for the role of *unc-5* downstream of *grn* comes from the partial rescue of the *grn* phenotype obtained by exogenously providing *unc-5* specifically in aCC and RP2.

eve mediates dMN guidance through *unc-5*

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). Similarly, *eve* regulates guidance of dMNs through *unc-5* (Labrador et al., 2005). In at least some of these situations the regulation is unlikely to be direct because the regulators are thought to mediate their function through a repressive activity (Muhr et al., 2001; Fujioka et al., 2003). Indeed, *eve* repressive activity has been shown to be 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) (Fujioka et al., 2003; Broihier et al., 2004), which would confer those motoneurons with a ventral fate. Our analysis of the *unc-5* neuronal enhancer regulated by *eve* failed to identify any conserved Eve consensus homeodomain binding site, suggesting that *eve* regulation of *unc-5* is not mediated through a direct binding to this element.

eve and *grn* can regulate *unc-5* through independent parallel pathways

Combinatorial codes of transcription factors play an instructive role in the generation of subclass diversity within the vertebrate spinal cord (Dasen and Jessell, 2009; Bonanomi and Pfaff, 2010). In invertebrates, in which it is possible to analyse 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 *zfh1*)

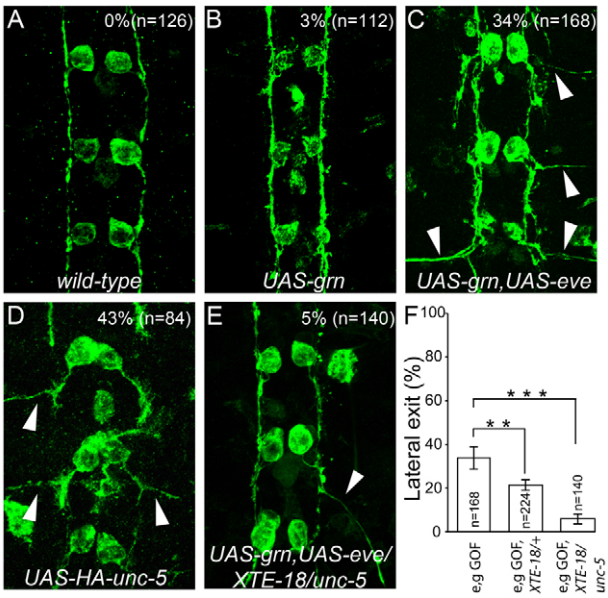

Fig. 8. Lateral axon exit of dMP2 neurons promoted by *eve* and *grn* is partially dependent on *unc-5*. (**A-E**) Axonal projections of dMP2 neurons on flat mounted st-15 embryos visualized with anti-GFP. Partial genotypes are indicated below each panel. Axons in dMP2 neurons expressing EGFP through *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*) (Labrador et al., 2005). $^{**}P<0.02$, $^{***}P<0.0001$.

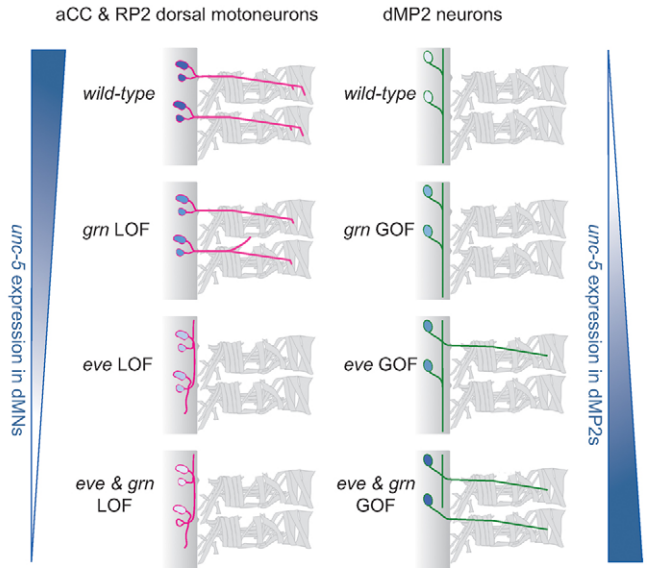

Fig. 9. Model for *unc-5* regulation. *unc-5* is regulated by both *eve* and *grn* in aCC and RP2 dMNs. Eliminating *grn*, *eve* or both transcription factors progressively reduces *unc-5* levels (left). In dMP2 neurons (right), neither *eve*, *grn* nor *unc-5* is expressed and axons project posteriorly 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.

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 *unc-5* expression, it is not the only factor required because *unc-5* mRNA is not completely absent from those cells and it also requires the presence of *eve*. In fact, *grn* or *eve* can independently induce *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 *eve* and *grn* might bring *unc-5* above the threshold required for exit. *unc-5* levels are definitely important for dMP2 exit because removing 50% of the gene dosage significantly suppresses the exit phenotype and this suppression is almost complete in an *unc-5* null background. Transheterozygous interactions identified between *unc-5* and *eve*, or *unc-5* and *grn* also suggest that their levels are tightly controlled. As both *eve* and *grn* can regulate *unc-5* it is likely that their combined activity in aCC and RP2 is essential to express the required levels of *unc-5* in both neurons. A model of *unc-5* regulation by *grn* and *eve* in each individual neuron is shown in Fig. 9.

The combinatorial nature of substrate recognition during motoneuron guidance and targeting is well established (Winberg et al., 1998; Kurusu et al., 2008). Mutants for guidance receptors or ligands often show partially penetrant phenotypes (Vactor et al., 1993) and this includes the *unc-5* phenotype in dMNs (Labrador et al., 2005). By contrast, phenotypes observed for transcription factor mutants that affect dMN guidance are far more severe. For example, Lim1 regulates EphA4 in vertebrate motoneurons but the EphA4 mutant phenotype is less severe than Lim1 mutant phenotype (Kania and Jessell, 2003). Similarly, 100% of ISNs in *eve* mutants (Landgraf et al., 1999; Fujioka et al., 2003) or 85% in *grn* mutants (Garces and Thor, 2006) are affected, suggesting that they regulate several guidance systems. It will be interesting to investigate the full array of guidance systems regulated by the *eve-grn* code. One potential candidate is *FasII* (*Fas2* – FlyBase) (Landgraf et al., 1999) as it is an essential molecule for the pioneer function of aCC and RP2 (Sánchez-Soriano and Prokop, 2005).

In summary, we have identified the guidance receptor Unc-5 as a novel target of the GATA transcription factor Grn. *unc-5* is key common target that mediates guidance downstream of both *eve* and *grn*. Furthermore, both transcription factors can promote transcription of *unc-5* independently, suggesting that their combined action is essential to attain the proper expression levels of the Unc-5 receptor in dMNs. Future investigations in cell type-specific expression profiling and chromatin immunoprecipitation sequencing (ChIP-seq) analysis will hopefully unravel other guidance systems regulated by the *eve-grn* code in dMNs. This knowledge will bring us closer to understanding the cell-specific transcriptional regulation of guidance.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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