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The *Tbx20* Homologs *midline* and *H15* Specify Ventral Fate in the *Drosophila melanogaster* Leg

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The *Tbx20* homologs *midline* and *H15* specify ventral fate in the *Drosophila melanogaster* leg

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Regional fates in the developing limbs of *Drosophila melanogaster* are controlled by selector gene transcription factors. Ventral fate in the fly leg is specified by the expression of the ligand Wingless. We present evidence that *midline* and *H15*, members of the Tbx20 class of T-box transcription factors, are key mediators of the Wingless signal in the formation of the ventral region of the fly leg. *midline* and *H15* are restricted to identical ventral domains of expression through activation by Wingless and repression by the dorsal signal Decapentaplegic. *midline* and *H15* function redundantly and cell autonomously in the formation of ventral-specific structures. Conversely, *midline* is sufficient to induce ventral fate. Finally, the induction of ectopic ventral fate by *mid* is compromised when Wingless signaling is attenuated, suggesting that Wingless acts both upstream and in parallel with *midline*/*H15* to specify ventral fate. Based on these results, we propose that *midline* and *H15* may be considered as the selector genes for ventral leg fate.

KEY WORDS: T-box transcription factor, Limb development, Pattern formation, Selector gene

INTRODUCTION

Selector genes subdivide the developing limbs of *Drosophila melanogaster* into distinct regions (Curtiss et al., 2002; García-Bellido, 1975; Mann and Carroll, 2002). Selector gene expression is necessary and sufficient to assign a regional fate: groups of cells expressing a selector gene will assume one fate, whereas cells not expressing the selector gene will either assume a default fate or fail to survive altogether (Curtiss et al., 2002; Mann and Carroll, 2002). As an example, the *engrailed* (*en*) gene and its paralog *invected* (*inv*) encode homeodomain transcription factors expressed in the posterior halves of all imaginal discs, including the limb primordia (Brower, 1986). Loss of *en*/*inv* expression autonomously transforms posterior limb cells into an anterior fate, and ectopic expression transforms anterior cells into a posterior fate (García-Bellido and Santamaria, 1972; Lawrence and Struhl, 1982; Lawrence et al., 1979; Morata and Lawrence, 1975; Simmonds et al., 1995; Tabata et al., 1995; Zecca et al., 1995).

Whereas anterior versus posterior (A/P) fate is controlled by *en*/*inv* expression, the selection of proximal versus distal (P/D) fate and dorsal versus ventral (D/V) fate in the fly leg is controlled through distinct interactions downstream of the secreted signals Wingless (Wg) and Decapentaplegic (Dpp). Dpp is a BMP ligand that is expressed at high levels in a stripe of dorsal cells at the boundary between A and P cells. Wg, a Wnt ligand, is expressed in ventral cells near the A/P boundary (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). Wg and Dpp act cooperatively to specify distal fates. Cells in the center of the leg imaginal disc receive high levels of both Wg and Dpp and are specified as distal through the Wg- and Dpp-dependent induction of several genes, including *Distal-less* (*Dll*) (Kojima, 2004; Lecuit and Cohen, 1997), which acts as a selector gene for distal versus proximal fate (Gorfinkiel et al., 1997). The D/V decision is regulated by antagonistic signaling between Wg and Dpp. Dpp represses *wg*, limiting its expression in dorsal cells, and ventral Wg in turn reduces *dpp* expression in the ventral leg. Dpp expression specifies dorsal fate and represses ventral, whereas Wg specifies ventral fate and represses dorsal (Fig. 1A) (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffmann, 1996; Theisen et al., 1996).

One problem is how cells in the distal leg, which are exposed to high levels of both Wg and Dpp, are able to properly discriminate between the two signals in order to assume either dorsal or ventral fate. One solution would be the ventral- or dorsal-specific expression of a selector gene downstream of Wg and Dpp signaling. Candidates for such a selector are *H15* and its paralog *midline*. An enhancer trap in *H15* is activated downstream of Wg and is repressed by Dpp (Brook and Cohen, 1996; Estella and Mann, 2008; Wilder and Perrimon, 1995). *H15* and *mid* (also known as *neuromancer 1* and *neuromancer 2*, respectively) are members of the Tbx20 class of T-box transcription factors and have previously been shown to be required redundantly in several developmental processes (Buescher et al., 2004; Buescher et al., 2006; Miskolczi-McCallum et al., 2005; Qian et al., 2005; Reim et al., 2005). In this report, we show that the ventral-specific expression of *H15* and *midline* downstream of Wg and Dpp is both necessary and sufficient to specify ventral fate. Based on our results, we argue that *mid* and *H15* act as selector genes for ventral fate.

MATERIALS AND METHODS

Drosophila stocks

Flies were grown under standard conditions at 25°C. To generate the *H15 mid* double mutant chromosome necessary for mosaic analysis, we screened 4500 EMS-treated $H15^{X4}$ b^{1} cn^{1} chromosomes (Buescher et al., 2004) and found a mutant, mid^{1a5}, that failed to complement mid^{1} embryonic lethality. Sequence analysis indicated that mid^{1a5} is a nonsense mutation at codon 144, truncating the protein just prior to the T-box domain, and is probably null because it is in a similar location to two other *mid* null mutations, mid^{1} and mid^{GA174} (see Fig. S3E,F in the supplementary material), and because the $H15^{X4}$ mid^{1a5} double mutant has a lethal phenotype similar to embryos with *mid* and *H15* deleted (data not shown).

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Other stocks were obtained from Bloomington Indiana and Kyoto Stock Centers or have been described previously (Brook and Cohen, 1996; Buescher et al., 2004; Lecuit et al., 1996).

Genotypes shown in figures: Fig. 1C,D, Oregon-R; Fig.1E-H, *y w hsFLP*; *H15^{X4} mid^{1a5} FRT40A/y^{+} ry^{+}25F FRT40A*; Fig. 2A, *y w hsFLP*; *H15^{X4} mid^{1a5} FRT40A/w^{+} GFP FRT40A*; *BS3.0(dpp-lacZ) ry^{506}*/+; Fig. 2B, *y w omb-lacZ hsFLP*; *H15^{X4} mid^{1a5} FRT40A/w^{+} GFP FRT40A*; Fig, 2C,E,F, *y w hsFLP*; *H15^{X4} mid^{1a5} FRT40A/w^{+} GFP FRT40A*; Fig. 2D, *H15-lacZ b cn*; Fig. 3A,C,D, *omb*-GAL4; UAS-*mid2.12*/+; Fig. 3B, *y w omb-lacZ hsFLP*; Fig. 3E-H, *y w hsFLP*; *Ay*GAL4 UAS-*GFP*/+; UAS-*mid2.12*/+; Fig. 4B,C, *y w hsFLP*; UAS-*Lef1*/*Ay*GAL4 UAS-*GFP*; and Fig. 4D, *y w hsFLP*; UAS-*Lef1*/*Ay*GAL4 UAS-*GFP*; UAS-*mid2.12*/+.

Genetics mosaics and ectopic expression of *midline*

H15^{X4} mid^{1a5} loss-of-function clones, null for both *H15* and *midline*, were induced with the Flp/FRT technique (Xu and Rubin, 1993) at 24-48 or 48-72 hours after egg laying (ael) with similar outcomes. *H15^{X4} mid^{1a5}* loss-of-function clones were generated by crossing *y w*; *H15^{X4} mid^{1a5} P{neoFRT}40A*/*CyO* to *y w hsFLP*; *P{w^{+} GFP}33 P{neoFRT}40A* to detect GFP clones in imaginal discs or to *y w hsFLP*; *P{y^{+}}25F P{neoFRT}40A* to detect *y* clones in adult cuticle. Ectopic *mid* expression was driven with *omb*-GAL4 (Lecuit et al., 1996), *rn*-GAL4 (St Pierre et al., 2002) and *NP2113*-GAL4 (Hayashi et al., 2002). Clones expressing UAS-*arm^{s10}* (Pai et al., 1997), *UAS-tkv** (Lecuit et al., 1996), *UAS-Lef1* (Riese et al., 1997) or UAS-*mid* and/or *Lef1* were induced using the *y w*; *P{w^{+} Ay*GAL4*}25 P{w^{+}*UAS-*GFP}T2* driver.

Antibodies and reporter constructs

Discs were stained as in Pattatucci and Kaufman (Pattatucci and Kaufman, 1991). *H15*, *omb* and *dpp* expression was monitored with *H15-lacZ*, *omb-lacZ* and *BS3.0(dpp-lacZ)* reporters. Primary antibodies were mouse anti-β-Gal (1:1000, Promega), rabbit anti-β-Gal (1:1000, Jackson ImmunoResearch Laboratories, Cedarlane), rabbit anti-Nmr1 and rabbit anti-Nmr2 [1:2000 and 1:100, which recognize H15 and Mid, respectively; Jim Skeath, Washington University School of Medicine, St Louis, MO, USA (Leal et al., 2009)], mouse anti-Wg (1:50, DHSB), and mouse anti-Scr (1:100, DHSB). Secondary antibodies were as in Ciechanska et al. (Ciechanska et al., 2007).

RESULTS AND DISCUSSION

mid and *H15* mediate a subset of Wg functions in the ventral leg

Wg signaling specifies ventral fate in the fly leg. The Wg-dependent domain is best delineated in the second leg tarsus, where eight rows of bristles are organized around the circumference and run the length of all five tarsal segments (Held et al., 1994) (Fig. 1B). Wg is secreted from a stripe of cells between the primordia of the two ventral-most rows of bristles (1 and 8) (Joshi et al., 2006), which are distinct from more dorsal rows because they are peg-shaped instead of rapier-shaped (Held et al., 1994) (Fig. 1B,C). The Wg morphogen diffuses to pattern a wedge of the imaginal disc that is broader than and centered on the *wg* expression domain. In *wg* hypomorphic mutants, rows 1 and 8 are replaced with a mirror image duplication of dorsal rows 3 through to 6, resulting in a leg with double dorsal symmetry (Held et al., 1994). Similar transformations are observed in clones of cells blocked for Wg signaling, where the row 1/8 bristles are transformed to rapier-shape (Heslip et al., 1997). Other prominent Wg-dependent ventral structures include the apical bristle (AB) of the distal ventral tibia in the second leg (Fig. 1C) and the ventral transverse rows (TRs) and sex combs (SCs) of the first leg (Fig. 1D).

The *Tbx20* homologs *mid* and *H15* are essential for the proper development of the Wg-dependent structures in the leg. In the imaginal discs, *mid* and *H15* are expressed in identical ventral domains that are broader than and centered on the Wg domain (see

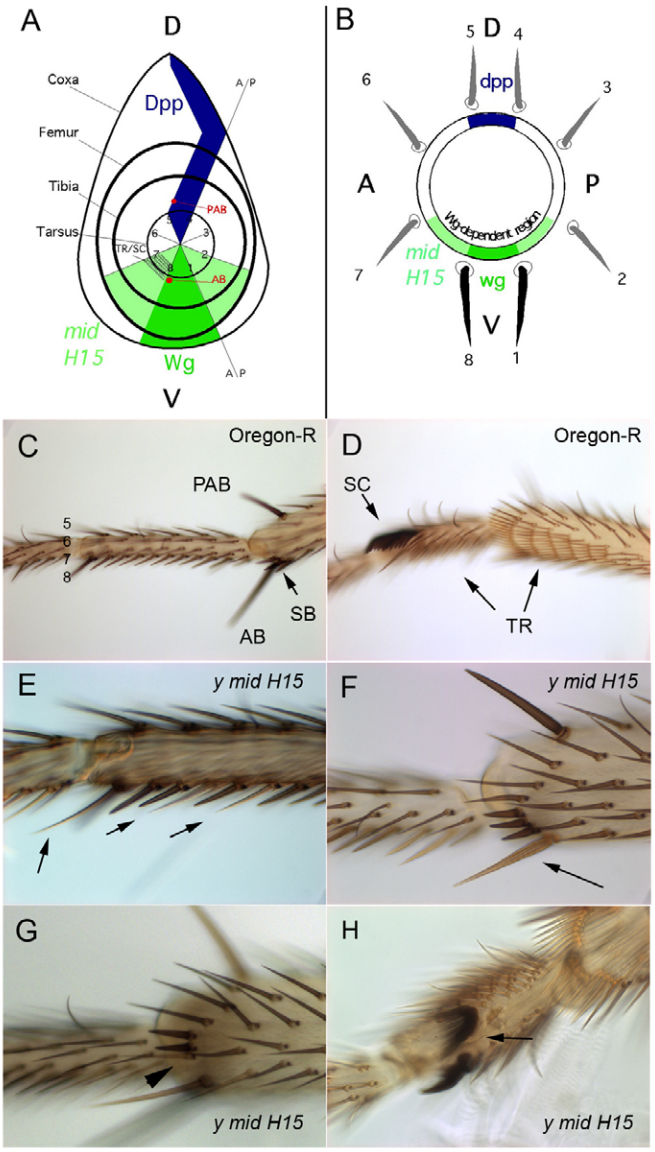

Fig. 1. *mid* and *H15* are required for Wg-dependent ventral leg structures. (**A**) Diagram of a leg imaginal disc showing the expression domains of Wg, Dpp, *mid* and *H15*. The A/P boundary, dorsal (D) and ventral (V) ends, and the distal (center) and proximal (outer) segments of the leg are indicated. The longitudinal bristle rows of the adult leg (1-8) are projected onto the tarsal region of the disc. Areas fated to give ventral apical bristle (AB), dorsal pre-apical bristle (PAB), and transverse rows (TR) and sex combs (SC) are shown. (**B**) Cross-section of adult tarsus with the positions of the eight longitudinal bristle rows (1-8) and the expression of Dpp, Wg, *mid* and *H15* (as in A). The expression domain of *mid* and *H15* (light green) corresponds to the Wg-dependent domain (dark green) deleted in *wg* mutants (Held et al., 1994). (**C**) Wild-type second leg showing the peg bristles of row 8 on the ventral basitarsus, and the pre-apical bristle (PAB), apical bristle (AB) and spur bristles (SB, arrow) on distal tibia. In this and all cuticle images, distal is to the left and dorsal is up. (**D**) Wild-type male first leg showing transverse rows (TR) on distal tibia and on the basitarsus and the sex comb (SC). (**E**) *mid H15 yellow* loss-of-function (LOF) clones in the peg row are transformed to the dorsal rapier shape (arrows). (**F**) A *mid H15 yellow* LOF clone in the ventral distal tibia results in the loss of the AB and the formation of a PAB-like phenotype (arrow). (**G**) A *mid H15 yellow* LOF clone in the distal ventral tibia results in a spur to rapier-shaped bristle transformation (arrowhead). (**H**) A *mid H15 yellow* LOF clone in the basitarsus is associated with a gap in the sex comb (arrow).

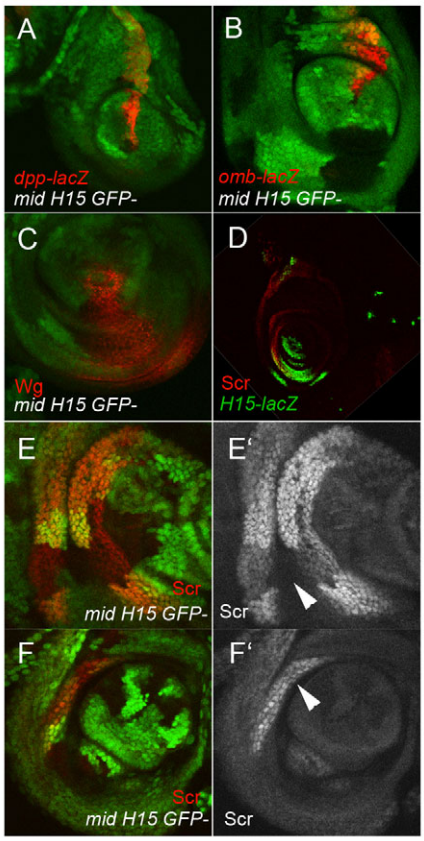

Fig. 2. *mid* and *H15* are required for ventral Scr expression but are not required to regulate dorsal gene expression. All stained disc images are oriented dorsal up and anterior to the left. (**A-C**) *mid H15* loss-of-function clones (GFP–) do not change the expression of dorsal markers *dpp-lacZ* (A) and *omb-lacZ* (B) or the ventral marker Wg (C) (red). (**D**) Scr expression (red) in the first leg counterstained with antibodies to β-galactosidase to visualize *H15-lacZ* expression (green). (**E**,**E′**) Decreased Scr expression (arrowhead in E′) is seen in a *mid H15* ventral LOF clone (GFP–). (**F**,**F′**) A dorsal *mid H15* LOF clone with normal Scr expression (arrowhead in F′).

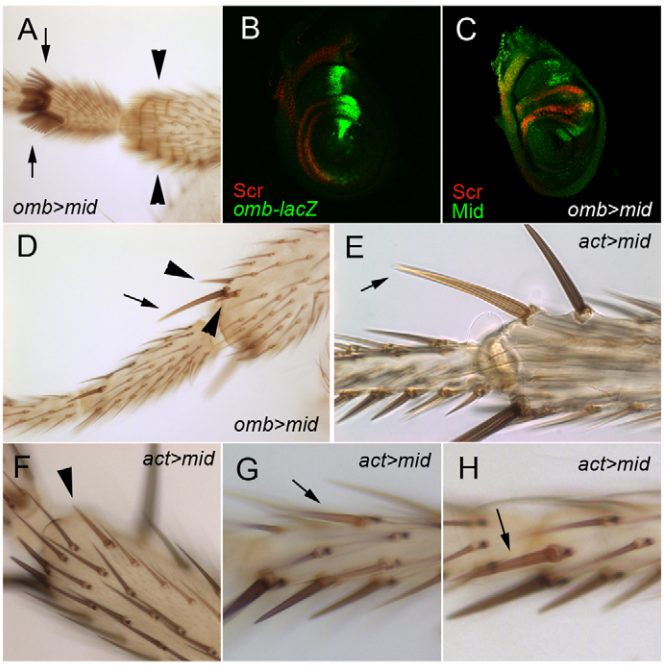

Fig. 3. Ectopic *mid* expression can induce ventral fate. (**A**) Expression of *mid* in the dorsal *omb* domain results in ectopic sets of sex combs (arrows) and transverse rows (arrowheads). (**B**) A high level of Scr (red) is excluded from the *omb-lacZ* expression domain (green). (**C**) Mid expression (green) in an *omb*-GAL4; UAS-*mid* leg results in Scr expression (red) in the *omb* domain. Note that the endogenous ventral Mid staining is often difficult to detect with this antibody. (**D**) A second leg showing the induction of an apical-like bristle (arrow) and spurs (arrowheads) resulting from the expression of *mid* in the dorsal *omb* domain. (**E**,**F**) Induction of an AB-like bristle (E, arrow) and an ectopic spur (F, arrowhead) by dorsal *mid*-expressing clones marked with *yellow*. (**G**,**H**) Clones expressing *mid* and marked with *yellow* can produce ventral-type bristles (peg-shaped) in dorsolateral (G, arrow) and ventrolateral (H, arrow) rows.

Fig. S1A,B,E in the supplementary material). In the tarsus, the *mid H15* domain is similar to the Wg-dependent domain, encompassing row 1 and 8 bristles and extending to, but not including, rows 2 and 7, as determined by co-staining with an antibody to Achaete, a bristle row marker (Fig. 1A,B; see Fig. S1C,D in the supplementary material). Both *mid* and *H15* are activated in ventral cells by Wg and restricted from dorsal cells by the dorsal morphogen Dpp (see Fig. S2 in the supplementary material), but neither *H15* nor *mid* alone is essential for leg development (see Fig. S3 in the supplementary material). However, loss of both *mid* and *H15* in marked clones caused the autonomous transformation of the Wg-dependent peg-shaped row 1/8 bristles into lateral or dorsal rapier-like bristles (Fig. 1E). In one sample, 54 out of 56 clones transformed bristles in row 1 or 8. Similar cell-autonomous transformations were observed in the second leg tibia, in which the ventral AB was lost in *mid H15* clones that span the distal tibia of the second leg. In 24 out of 26 such clones, a large bristle similar to the dorsally located pre-apical bristle (PAB) developed in place of the AB (Fig. 1F). The AB is associated with a cluster of peg-shaped bristles called spur bristles (SBs), which, like the row 1/8 bristles, were autonomously transformed to dorsal-like rapier-shaped bristles in *mid H15* clones (Fig. 1G). The SCs and TRs of the first leg were also deleted in *mid H15* clones (Fig. 1H). Other ventral structures were either lost or disorganized within *mid H15* clones (see Fig. S4 in the supplementary material). Clones located outside the *mid H15* expression domain were normal and the few ventral clones with no phenotype were small and located in structures that have no obvious D/V differences (see Fig. S4 in the supplementary material).

The effects of *wg* mutants and clones of cells unable to detect the Wg signal differ from the effects of *mid H15* clones, because they also cause non-autonomous effects such as axis bifurcation or ectopic bristle rows (Heslip et al., 1997; Joshi et al., 2006; Theisen et al., 1994). The axis bifurcation caused by loss of Wg function is due to ectopic *dpp* expression. However, we found that neither *dpp-lacZ* (Fig. 2A) nor the dorsal marker *omb-lacZ* (Fig. 2B) were increased in *mid H15* clones located in ventral anterior cells. The ventral-to-dorsal transformation in *mid H15* clones is also not a result of a decrease in the expression of Wg, which was unchanged in ventral *mid H15* clones (Fig. 2C). The homeotic gene *Sex combs reduced* (*Scr*), which is required for the development of sex combs and TRs, is expressed at high levels in the anterior tibia and basitarsus segments (Fig. 2D) (Shroff et al., 2007). *mid H15* mutant

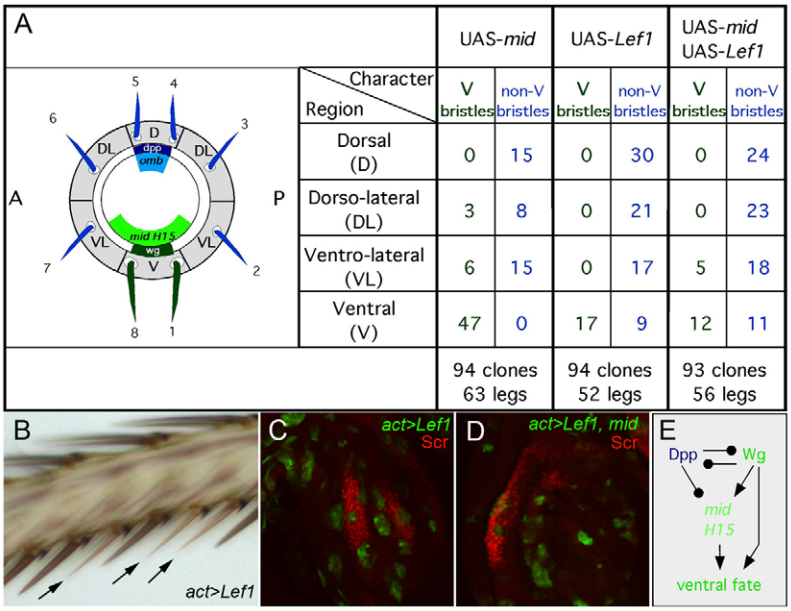

Fig. 4. Induction of ectopic ventral fate by *mid* is influenced by Wg signaling. (**A**) Summary of the effects of clones expressing *mid* and/or *Lef1* on the dorsal/lateral or ventral character of bristle rows 1 through to 8. Ventral rows 1/8 (green) are peg-shaped, whereas lateral and dorsal rows 2 through to 7 (blue) are rapier-shaped in the wild type. Clones expressing *mid* are over-represented in the ventral region and can transform ventrolateral and dorsolateral regions to ventral fate. Both of these effects are attenuated by the expression of *Lef1*. (**B**) *Lef1*-expressing clones can transform ventral peg to dorsal-type rapier bristles (arrows). (**C**,**D**) Scr expression (red) is turned off in clones expressing *Lef1* (C) and is not rescued when *mid* is coexpressed with *Lef1* (D). (**E**) Genetic pathway for ventral leg development.

clones in ventral (Fig. 2E,E′), but not lateral or dorsal (Fig. 2F,F′), positions downregulate Scr to background anterior levels. Taken together, these results indicate that *mid* and *H15* are required for the specification of ventral fate downstream of Wg and for some ventral gene expression. However, *mid* and *H15* are not required to repress dorsal gene expression.

Ectopic *mid* expression induces ventral fate

Ectopic expression of *mid* is sufficient to induce ectopic Wg-dependent ventral structures. Since flies with *H15* deleted have normal ventral patterning (see Fig. S3 in the supplementary material), *mid* can mediate the function of both genes. Expression of *mid* in the dorsal *omb* (*bi* – FlyBase) domain resulted in ectopic SCs and TRs in the dorsal basitarsus and distal tibia of all male first legs (Fig. 3A). This was accompanied by the ectopic expression of *Scr* in the *omb* domain, which was appropriately restricted in the P/D axis to the basitarsus and tibia (Fig. 3C). In the second leg, ectopic expression of *mid* in the dorsal tibia under the control of the *omb*-GAL4 or in small clones of *mid*-expressing cells resulted in ectopic bristles similar to the AB and SBs (Fig. 3D,E,F). Small clones of *mid*-expressing cells either in or adjacent to rows 2/7 and 3/6 induced ventral row 1/8 bristles cell autonomously (Fig. 3G,H). We saw similar results using other GAL4 drivers expressed in the tarsus (see Fig. S5 in the supplementary material).

mid induces ectopic ventral fate in conjunction with Wg signaling

The regions of the leg where *mid* induces ectopic ventral structures are within the range of the ventral Wg signal, which reaches many dorsal and lateral cells to induce P/D genes such as *Dll* (Estella et al., 2008). This leaves open the possibility that Wg might act both upstream of and in parallel with *mid* to specify ventral fate. To test the requirement for Wg, we generated clones of cells that are compromised for Wg signaling. We expressed mouse *Lef1*, which acts as a dominant negative in Wg signaling in *Drosophila* (Riese et al., 1997), and compared its effects on ventral development with and without the expression of ectopic *mid*. We induced the clones in third instar larvae, at 84 to 108 hours, when the P/D axis is independent of Wg but Wg signaling is still necessary for specifying ventral fate (Campbell, 2002; Galindo et al., 2002). *mid*-expressing clones induced at earlier stages can cause more extensive repatterning, with the occasional repression of *dpp* and non-autonomous induction of *wg* (see Fig. S6 in the supplementary material). *Lef1* clones were distributed evenly in the dorsal, ventrolateral, dorsolateral and ventral regions of the tarsus (Fig. 4A). As expected, dorsal clones were normal and clones in the ventral-most rows (Fig. 4B) often showed transformation towards more dorsal fates (9/26). Clones expressing *mid* were recovered much more frequently in ventral regions, suggesting that dorsal *mid*-expressing clones either sort to more ventral positions or they are lost. Ventrolateral or dorsolateral *mid*-expressing clones are often transformed to ventral character. By contrast, clones expressing both *mid* and *Lef1* are recovered more often in lateral and dorsal cells, indicating that the sorting behavior of *mid*-expressing clones depends on the transduction of the Wg signal. Dorsolateral clones expressing both *mid* and *Lef1* do not transform towards ventral fate (0/23), whereas ventrolateral clones are still sometimes transformed to ventral fate. This is consistent with a requirement for Wg in ectopic ventral development, as the dorsolateral row 3/6 bristles are further from the source of Wg signal and would be expected to be more sensitive to the effects of *Lef1*. We observed a similar effect on Scr, where UAS-*Lef1* blocked Scr expression (Fig. 4C); this was not rescued by the simultaneous expression of UAS-*mid* (Fig. 4D). These results suggest that *mid* regulates ventral fate and *Scr* expression in conjunction with Wg (Fig. 4E).

Our results suggest that the ventral expression of *mid* and *H15* represents a major function downstream of Wg and Dpp in the D/V fate decision. The cell-autonomous requirement for *mid* and *H15* and the ability of ectopic *mid* expression to induce ventral fate and gene expression in dorsal cells mean that *mid* and *H15* meet the criteria to be defined as selector genes (Crick and Lawrence, 1975; García-Bellido, 1975). In the absence of *mid* and *H15*, ventral structures may assume a dorsal fate due to the low levels of Dpp signaling found in the ventral leg (Azpiazu and Morata, 2002). However, it is not likely that dorsal is the default fate in the leg, as lateral structures prevail when the expression of both *wg* and *dpp* is greatly reduced (Held et al., 1994). Ventral fate also requires Wg signaling, suggesting that *mid* and *H15* act to provide a molecular

context for the upstream Wg morphogen to direct ventral-specific patterns of gene expression, as has been observed for other selector genes (Curtiss et al., 2002; Mann and Carroll, 2002). The ventral-specific expression of *mid*, *H15* and *wg* is conserved throughout several arthropod orders, suggesting that it represents a fundamental mechanism in limb patterning (Janssen et al., 2008; Prpic et al., 2005).

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Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/16/2689/DC1

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