# Specification of individual Slouch muscle progenitors in *Drosophila* requires sequential Wingless signaling

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## Summary

**The patterning of the *Drosophila* mesoderm requires Wingless (Wg), one of the founding members of a large family of secreted glycoproteins, the Wnt family. Little is known about how Wg provides patterning information to the mesoderm, which is neither an epithelium nor contains the site of Wg production. By studying specification of muscle founder cells as marked by the lineage-specific transcription factor Slouch, we asked how mesodermal cells interpret the steady flow of Wg. Through the manipulation of place, time and amount of Wg signaling, we have observed that Slouch founder cell cluster II is more sensitive to Wg levels than the other Slouch-positive founder cell clusters. To specify Slouch cluster I, Wg signaling is required to maintain high levels of the myogenic transcriptional regulator Twist. However, to specify cluster II, Wg not only maintains high Twist levels, but also provides a second contribution to activate Slouch expression. This dual requirement for Wg provides a paradigm for understanding how one signaling pathway can act over time to create a diverse array of patterning outcomes.**

Key words: Wingless, Twist, Slouch, Muscle, Mesoderm, Founder cell, *Drosophila*

## Introduction

Patterning a developing organism involves both long range and short range signaling. While much is known about how the Wnt family transduces its signal, we are only beginning to understand, at a molecular level, how this signaling cascade provides patterning information to a field of cells. A role for Wingless (Wg) in organizing cell fates in a number of *Drosophila* epithelial tissues has been described: the embryonic epidermis, where it specifies distinct cuticular fates; the eye imaginal disc, where it limits progression of the eye morphogenic furrow; the leg imaginal disc, where it patterns the anterior-ventral territory; and the wing imaginal disc where it specifies the dorsal-ventral boundary (Hatini and DiNardo, 2001; Lee and Frasch, 2000; Theisen et al., 1996; Treisman and Rubin, 1995; Wilder and Perrimon, 1995; Zecca et al., 1996). How Wg achieves these different outcomes on a mechanistic level appears to be context dependent. In some systems, Wg works combinatorially with other signaling pathways to subdivide a larger territory into smaller, distinct fates. In other systems, the absolute levels of Wg signal appear to be instructive, leading to differential activation of target genes. Hints as to how the former is accomplished have come from the study of patterning in the embryonic epidermis where Wg, Epidermal Growth Factor (EGF) and Hedgehog (Hh) signaling subdivide this tissue into different domains (Hatini and DiNardo, 2001). By contrast, for the latter, Wg in the wing imaginal disc acts as a morphogen, that is, it directly activates target genes *neuralized* and *distal-less* in a concentration-dependent manner (Zecca et al., 1996).

Less is known about how Wg provides patterning information to a group of cells in which the target field is neither a uniform epithelium nor contains the production site of the Wg signal. In vertebrates, for example, Wnts secreted by the neural tube have been shown to be important in specifying sclerotome and promoting proper muscle differentiation (Munsterberg et al., 1995; Tajbakhsh and Cossu, 1997). Likewise in *Drosophila*, Wg secreted by the ectoderm is essential for mesoderm development (Baylies and Michelson, 2001; Frasch, 1999). Analysis of *wg* null embryos reveals that the heart and particularly, the body wall muscles, either do not form or do not form normally (Baylies et al., 1995; Wu et al., 1995; Ranganayakulu et al., 1996).

The requirement for Wg signaling has been linked to several steps in *Drosophila* larval muscle formation. Body wall muscles arise from somatic mesoderm that is set aside in the posterior domain of each segment. The somatic mesoderm is marked by expression of high levels of Twist, a crucial tissue-specific transcriptional regulator for mesoderm and muscle development (Bate, 1993; Borkowski et al., 1995). High Twist levels direct these cells to adopt a body muscle fate. When Twist levels are reduced in these cells, body muscles fail to form (Baylies and Bate, 1996; Castanon et al., 2001). Within the region of high Twist expression, 19 pre-muscle clusters or equivalence groups expressing Lethal of scute (L'sc) (Carmena et al., 1995) subsequently emerge. A single muscle progenitor cell is singled out from each equivalence group through the combined actions of Notch and Ras signaling (reviewed by Frasch, 1999; Baylies and Michelson, 2001). This progenitor

cell divides asymmetrically to give two muscle founder cells, or a muscle founder cell and an adult muscle progenitor cell (Carmena, 1998b; Ruiz Gomez and Bate, 1997). Founder cells then fuse with surrounding fusion-competent cells, attach to appropriate sites on the epidermis and are properly innervated (Bate, 1990; Bate, 1993; Dohrmann et al., 1990). Wg acts on the mesoderm to maintain high Twist levels (Bate and Rushton, 1993), initiate L'sc expression (Carmena et al., 1998a) and regulate some founder cell identity gene expression (Baylies et al., 1995; Ranganayakulu et al., 1996; Wu et al., 1995). It has been shown, in one case only, that of the muscle founder identity gene *even-skipped* (*eve*), that the Wg transcriptional effector DTCF or Pangolin directly binds to the *eve* muscle enhancer (Halfon et al., 2000; Knirr and Frasch, 2001).

While the cells of the mesoderm undergo positional rearrangements and cell fate changes, the position and amount of ectodermal Wg remains constant. If Wg is required throughout mesodermal development, how do the cells of the mesoderm interpret the steady flow of the Wg signal correctly and, as a result, respond with activation of different target genes at different times in development? To address this question, we analyzed the requirement for Wg signaling in the specification of muscle founder cells that express the identity gene *slouch*. In *wg* mutant embryos, all Slouch-positive founder cell clusters are lost. We now report that Wg regulates each Slouch cluster differently. To specify Slouch-expressing cluster I in the mesodermal hemisegment, Wg signaling is required to maintain high Twist levels. However, to specify Slouch-expressing cluster II in that same hemisegment at a later time, Wg not only needs to maintain high Twist levels, but also needs to provide a second, Twist-independent contribution to activate Slouch expression. Thus, Wg controls the temporal and spatial activation of Slouch expression in the individual clusters through distinct signaling mechanisms. This dual requirement for Wg in specifying cluster II provides a novel insight to how one signaling pathway can be used repeatedly throughout development to impart patterning information within a target field.

## Materials and methods

### *Drosophila* strains

The following strains were used: $wg^{CX4}$ and $wg^{IG22}$, both null alleles; $dAPC2^{d40}$, a hypomorphic allele [a gift of M. Peifer and B. McCartney (McCartney et al., 1999)]; $wg^{NE1}$ and $wg^{PE6}$, two hypomorphic alleles [a gift of A. Bejsovec (Dierick and Bejsovec, 1998)]; $wg^{21.2}$, a third hypomorphic *wg* allele (a gift of A. Martinez-Arias); *w; twist (twi)GAL4* $wg^{cx4}$*/CyO ftz-lacZ, w; UAS-arm*$^{S10}$ $wg^{IG22}$*/CyO ftz-lacZ, w; UAStwi* $wg^{cx4}$*/CyO ftz-lacZ*, $hh^{21}$, a strong loss of function allele (Ingham and Hidalgo, 1993); and *twiGAL4;hh*$^{AC}$*/TM3Ubx-lacZ, UASwg;hh*$^{8}$*/TM6B* [a gift of R. Bodmer (Park et al., 1996)]. The GAL4 and UAS lines (Brand and Perrimon, 1993) were: *w;twiGAL4, w;twiGAL4;twiGAL4, w;twiGAL4;Dmef2GAL4*, for mesoderm-specific expression; *UAStwist(twi)* (Baylies and Bate, 1996); *UASwg*$^{E}$ (containing a wild-type *wg* cDNA, a gift of A. Martinez-Arias); *UASarm*$^{s10}$ (Pai et al., 1997); and *UASΔNtcf* (van de Wetering et al., 1997). All GAL4/UAS experiments were performed at 25°C unless otherwise indicated.

Temperature-shift experiments were carried out as follows: embryos carrying a hypomorphic allele of *wg* ($wg^{IL114}$*/CyO,ftz-lacZ*) were kept in laying pots at 18°C (permissive temperature), and were synchronized by changing apple juice plates every hour. After 15 hours (very late stage 11), 13 hours (late stage 11) or 12 hours (mid-stage 11) at 18°C, embryos were dechorionated and either immediately fixed, or shifted to 25°C (nonpermissive temperature) for another 2-3 hours to develop until very late stage 11. Embryos were then fixed according to standard protocols (Rushton et al., 1995). In a parallel experiment, embryos were raised at 25°C for 8 hours (very late stage 11), then fixed as usual. Slouch expression was then examined using the antibody staining protocol described below.

### Immunocytochemistry

Immunocytochemistry in embryos (Rushton et al., 1995) was performed using antibodies to S59 (Slouch; 1:200) (Baylies et al., 1995), β-galactosidase (1:1000; mouse, Promega), Twist (1:5000; provided by S. Roth), and biotinylated secondary antibodies (Jackson Immunoresearch) used in combination with Vector Elite ABC kit (Vector Laboratories). Specimens were embedded in Araldite. Images were captured using an Axiocam with accompanying software (Zeiss). Different focal planes were combined into one picture using Adobe Photoshop software. Immunofluorescent staining was carried out using anti-S59 (1:100) or anti-Krüppel (1:500) (provided by J. Reinitz). Slouch was visualized using a secondary antibody conjugated to horseradish peroxidase (Vector Laboratories), followed by FITC tyramide (Vector Laboratories). Kr was visualized using a biotinylated secondary followed by Cy3 conjugated to streptavidin (Vector Laboratories). Immunofluorescent signals in co-localization studies were analyzed using a Zeiss LSM 510 confocal microscope.

## Results

### Wg is required for Slouch expression in founder cells

In *wg* mutant embryos, the heart and approximately half the body wall muscles are lost (Bate, 1993; Baylies et al., 1995; Ranganayakulu et al., 1996; Wu et al., 1995). One subset of these Wg-dependent body wall muscles can be visualized using an antibody to the NK-homeodomain protein Slouch (S59) (Dohrmann et al., 1990; Knirr et al., 1999). Slouch expression arises in a precise, stereotypic pattern during embryonic development (Fig. 1A,B). It is first expressed in a single progenitor cell during early stage 11 of embryonic development; this cell divides to give rise to two founder cells (Ia and Ib) which together form cluster I (cI). During late stage 11, two additional Slouch-positive progenitors appear at a different ventral location and divide sequentially to form four founder cells that make up cluster II (Carmena et al., 1995). Still later, at stage 12, a single progenitor arises dorsally and divides to give rise to cluster III (Fig. 1A,B). These muscle founder cells contain all the information needed to create a particular subset of muscles and contribute to the stereotypic set of larval muscles in each abdominal segment (Bate, 1990; Bate, 1993; Dohrmann et al., 1990). After stage 12, Slouch expression is maintained in a subset of these founder cells that give rise, in the final muscle pattern, to muscle VT1 (from cI), VA2 (from cII) and DT1 (from cluster III) (Bate, 1993; Ruiz-Gomez et al., 1997). Maintenance of Slouch expression in these founder cells is crucial to the development of these muscles; removal of *slouch* leads to complete (VT1) and partial muscle transformations (VA2; DT1) (Knirr et al., 1999). In this study, we focused on the role of Wg in patterning the Slouch muscle founder cells. For simplicity, we focused solely on two ventral Slouch clusters (I and II) (Fig. 1A), which develop independently and arise in a similar position along the dorso-

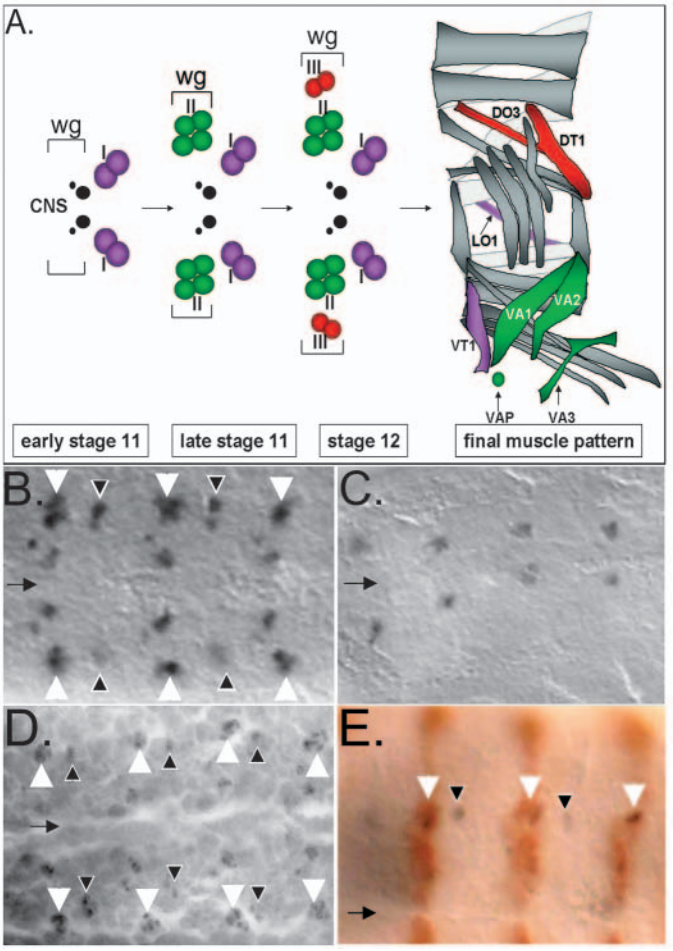



**Fig. 1.** Wingless is required for proper development of Slouch-expressing founder cells (FCs). (A) Schematic showing the temporal progression of Slouch-positive FC development from early (left) to late (right) stages of embryogenesis. Brackets indicate position of the Wg-expressing epidermal cells. Slouch is first expressed in a single progenitor cell during early stage 11 (5 hours AEL) of embryonic development; this cell divides to give rise to two FCs (purple), which together form cI located posterior to Slouch-expressing CNS cells (black). During late stage 11 (6 hours AEL), two additional Slouch-positive progenitors appear and divide sequentially to form four FCs that make up cII (green). At stage 12, a single progenitor arises dorsally and divides to give rise to cIII (red). Slouch expression is maintained in a subset of these founder cells that give rise, in the final muscle pattern, to muscle VT1 (25 from cI), VA2 (27 from cII) and DT1 (18 from III). In the figure, all muscles formed from these three clusters, as well as the ventral adult progenitor that comes from cII, are shown in color. Remaining gray muscles arise from non-Slouch-expressing FCs. (B-E) Ventral views of stage 11 embryos are shown; black arrows mark the midline, black arrowheads mark cI, white arrowheads mark cII. Abdominal segments 5-8 are shown; posterior is right. (B) High magnification of a wild-type embryo. cII is in line with the Slouch-expressing CNS cells, while cI is posterior to cII. (C) High magnification of a $wg^{CX4}$ embryo. All mesodermal Slouch expression is absent, indicating that Wg is necessary for the formation of these clusters. Slouch-expressing cells in the CNS remain. (D) High magnification of a $wg^{CX4}$, *twiGAL4>UASarm*$^{S10}$, $wg^{IG22}$ embryo shows normal patterning of all Slouch-expressing FCs. The ability of activated Armadillo to support normal patterning of both cI and cII suggests that the Wg pathway is sufficient to pattern these mesodermal clusters. (E) High magnification of a *CyO, wglacZ* embryo. Wg-expressing cells (brown, white arrowheads) and Slouch-expressing cells (black) are shown. The ectodermal Wg stripe specifically overlaid Slouch cII.

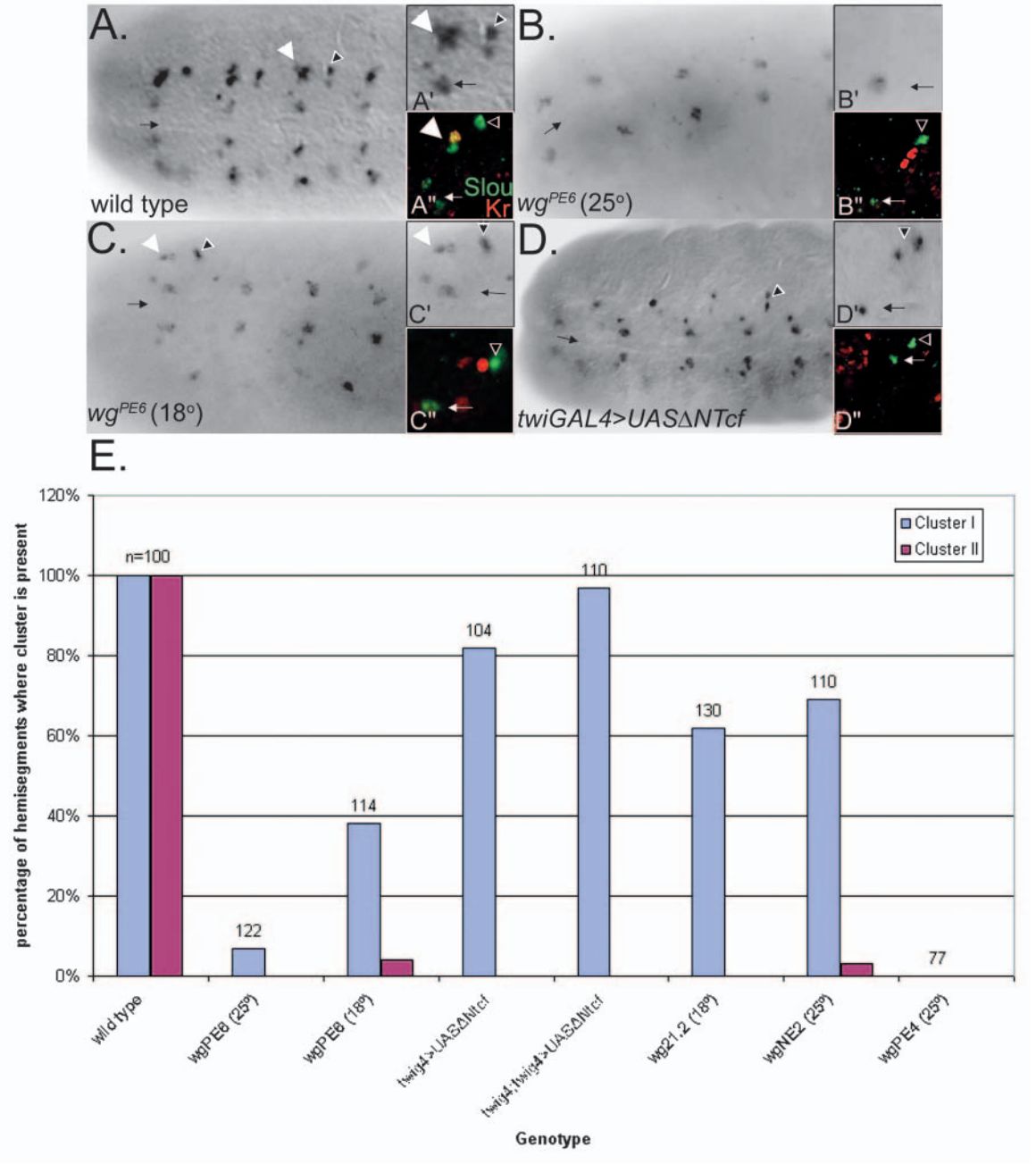
ventral axis but have different anterior-posterior positions within each abdominal hemisegment.

In *wg* mutant embryos, mesodermal Slouch expression never appeared during specification of these founder cells, indicating a requirement for Wg in this process [Fig. 1C; compare with Baylies et al. (Baylies et al., 1995)]. Consequently, all Slouch-dependent muscles are missing in *wg* mutant embryos (data not shown) (Baylies et al., 1995). Previous work suggested that the effect of Wg on the mesoderm was direct (Baylies et al., 1995). To first rule out the possibility that the mesodermal defects seen in *wg* mutant embryos were due to a requirement of Wg for the induction of a secondary signaling pathway which, in turn, patterns Slouch clusters I and II, we pan-mesodermally expressed an activated form of the Wg transcriptional activator Armadillo (Fig. 1D). Activated Armadillo was sufficient to pattern these clusters in a *wg* null embryo, indicating that Wg must be the primary pathway acting on the mesoderm to pattern these clusters. Hence, Wg signaling is both necessary and sufficient for specification of all Slouch clusters.

Expression of *lacZ* driven by *wg* promoter elements in wild-type embryos revealed that, during the differentiation of the Slouch founder cells, Wg-producing cells in the ectoderm directly overlie cII Slouch-expressing cells but not those of cI (Fig. 1E). Wg expression in the embryo changes in two important ways over the course of mesoderm development. Wg protein is initially detected symmetrically on either side of the Wg-expressing cells. Then, the protein expression becomes restricted to the anterior side after stage 9 when the mesoderm begins the allocation of cells to different fates. Also, during stage 11, the continuous ectodermal stripe of Wg expression breaks into two regions, one dorsal and one ventral, leaving a small gap laterally (Gonzalez et al., 1991). The Slouch-positive founders cells in cI and cII arise ventrally and are therefore exposed to a continuous supply of Wg. However, because of its position relative to that of the constant source of Wg from overlying ectodermal cells, cII is likely to receive higher levels of Wg. Therefore, we hypothesized that cII required a different amount of Wg to be patterned, and that this difference is instructive in specifying the identity of cII versus cI and hence the muscles that arise from them.

## Partial loss of function in the Wingless pathway leads to loss of Cluster II but not Cluster I

To test whether cII required an increased level of Wg signaling, we analyzed embryos in which Wg signaling was reduced. Two assays were used to determine cI and cII identity in these mutant backgrounds: morphology (that is, position relative to Wg-insensitive Slouch-positive central nervous system cells) and co-expression of a second founder cell identity gene, *Krüppel* (*Kr*). In wild-type embryos, cII always aligned with the Slouch-positive central nervous system cells, while cI was located in the mesoderm just posterior to these cells (Fig. 2A,A′). Kr had been shown to co-localize with Slouch in cII but not cI (Fig. 2A″) (Ruiz-Gomez et al., 1997). We manipulated Wg levels using different alleles of genes in




the Wg pathway and reagents that altered Wg signal transduction, and tested these embryos for alterations in Slouch cI and cII.

Although we analyzed a number of different *wg* alleles (Table 1), two particular alleles, $wg^{21.2}$ and $wg^{PE6}$, highlighted the differential response of the Slouch clusters to levels of Wg signaling. Embryos carrying these temperature-sensitive alleles showed ectodermal phenotypes associated with decreased Wg signaling when raised at the nonpermissive temperature. These phenotypes included a reduction in Wg target gene expression in the epidermis at stage 11 (i.e. *engrailed*), and *wg* cuticular phenotypes (i.e. reduction in naked cuticle, lack of denticle diversity) (Dierick and Bejsovec, 1998; Owen, 1994). At the permissive temperature, embryos carrying these alleles have ectodermal target gene expression restored ventrally (overlying where the mesodermal Slouch clusters arise) and nearly wild-type cuticles (Dierick and Bejsovec, 1998; Owen, 1994).

When embryos carrying the $wg^{PE6}$ allele were raised at the nonpermissive temperature, we detected a reduction in the Slouch-expressing clusters. However, cII was reproducibly affected more strongly than cI (Fig. 2B,E; Table 1). When $wg^{PE6}$ embryos were raised at the permissive temperature, the mesoderm showed some rescue (Fig. 2C,E; Table 1). We noted that reappearance of cII was always coupled with cI rescue within the same hemisegment. Since cI and cII are not related by lineage, these data indicated that, within a hemisegment, when Wg levels were high enough to properly pattern cII, they were high enough to pattern cI. This, coupled with the observation that cI could form in the absence of cII, supported the model that cII requires higher levels of Wg than cI.

**Fig. 2.** Slouch-expressing cells in cluster II require more Wingless than Slouch cluster I. (A-D) In this and all subsequent figures ventral views of late stage 11 abdominal segments 5-8 are shown. Insets depict one representative hemisegment at a higher magnification. Black arrowheads mark cI; white arrowheads mark cII. Black arrows denote the midline in panels; black arrows in A′-D′ and white arrows in A″-D″ indicate Slouch-expressing CNS cells. (A) Wild-type embryo shows stereotypic repeating pattern of Slouch-positive clusters. (A′) Immunocytochemistry of a single hemisegment reveals that cII (white arrowhead) aligns with the CNS, while cI (black arrowhead) localizes just posterior to cII. (A″) Confocal micrograph of a wild-type hemisegment; in cII (white arrowhead) Kr (red/Cy3) and Slouch (green/FITC) co-localize (yellow), while in cI (black arrowhead) Slouch (green/FITC) alone is expressed. Slouch is also expressed in the CNS (white arrow). Kr is also expressed in the CNS. Arrowheads and colors are maintained for all figures. (B) $wg^{PE6}$ embryo at the nonpermissive temperature (25°C) lost Slouch expression. (B′) Immunocytochemistry reveals an example in which both cII and cI are missing. (B″) Confocal micrograph of a single hemisegment shows an example in which Slouch staining is absent from the position where cII is normally found, but is present for cI. Slouch expression does not overlap with Kr. The identity of these Kr-positive cells is unknown. (C) $wg^{PE6}$ at the permissive temperature (18°C) showed expression of cI and cII in some hemisegments. (C′) Immunocytochemistry reveals an example where both cII (white arrowhead) and cI (black arrowhead) are present. (C″) Confocal micrograph shows another example in which Slouch staining is absent from the normal cII position, and Slouch does not co-localize with Kr in cI (black arrowhead). The identity of these Kr-positive cells is unknown. (D) *twiGAL4; twiGAL4>UASΔNTcf* embryos in which dominant-negative Tcf is expressed pan-mesodermally; this construct specifically repressed expression of cII and did not affect cI. (D′) Immunocytochemistry shows that cII is missing but cI (black arrowhead) is present. (D″) Confocal micrograph of a single hemisegment shows that Slouch staining is absent from its normal position of cII, and Slouch expression does not overlap with Kr in cI (black arrowhead). (E) Quantification of Slouch cluster loss in Wg pathway partial loss-of-function embryos. Graphs show percentage of hemisegments in which cI (blue) or cII (red) are present. *n*, number of hemisegments counted.

Consistent with the morphological studies described above, confocal microscopy studies predominantly showed a failure of Kr to co-localize with Slouch, indicating that the vast majority of the Slouch clusters that did appear in the embryos carrying the different *wg* alleles were cI and not cII (Fig. 2B″, C″; Table 1; data not shown). Thus, from the analysis of embryos carrying different *wg* alleles, we concluded that Slouch cII requires more Wg than Slouch cI.

This differential sensitivity of cII was confirmed by overexpressing a dominant-negative form of the Wg transcriptional effector dTCF/Pangolin (ΔNTcf) (van de Wetering et al., 1997). Pan-mesodermal expression of this construct, which is missing the Armadillo binding domain, gave a weak *wg* phenotype: Slouch cI was always present whereas cII was always affected (Fig. 2D,E; Table 1). Increasing the expression of this construct by using two copies of the pan-mesodermal GAL4 driver again led to a complete loss of cII, and, in addition, cI was missing in 3% of hemisegments (Fig. 2E; Table 1). It could be argued that the ability of UASΔNTcf to repress Slouch cII but not cI completely was due to the delay of the GAL4-UAS system, or that sufficient levels of the dominant-negative Tcf protein had not accumulated in time to block formation of Slouch cI. However, we believe that this is not the case, as dominant-negative Tcf does affect other mesodermal targets such as L'sc and Eve at late stage 10 (A. Carmena, unpublished). Taken together, in situations in which Wg signaling is reduced but not completely eliminated, cII is preferentially lost.

## Gain of function in the Wg pathway increases Slouch cluster II

Given that cII showed a greater response to reduced Wg signaling, we next investigated whether cII was also more responsive to increased levels of Wg signaling. The GAL4/UAS system was used to ectopically express Wg throughout the mesoderm using the *twist (twi)GAL4* driver (Brand and Perrimon, 1993; Baylies and Bate, 1996). When we expressed Wg throughout the mesoderm, cII was significantly expanded in all hemisegments, while cI always retained its normal size (Fig. 3A,B,G; Table 1). While cII size enlarged reproducibly in response to increased Wg, the number of cells per cluster varied from hemisegment to hemisegment (5-15 cells, mode=12 cells versus 4 cells in wild-type). No correlation could be drawn between Wg levels and the number of cells in cII. In addition, we found no evidence that early exposure to higher Wg amounts leads to earlier Slouch activation in cII, as the onset of Slouch cII expression was the same as that found in wild-type embryos. To reinforce that this effect was mediated by the Wg signal transduction pathway autonomously in the mesoderm, an activated form of Armadillo, $UASarm^{s10}$, was expressed throughout the mesoderm. Once again, cII was significantly expanded in all hemisegments (100%), while cI remained at wild-type size (Fig. 3C,G; Table 1). These data indicated that cII was indeed more responsive to increased Wg signaling and that the effects on cII were mediated by the classical Wg pathway, at least through the level of Armadillo.

Similar results were obtained when we asked whether loss of function in a negative regulator of the Wg pathway, *dAPC2*, specifically enlarged cII (McCartney et al., 1999). Embryos derived from homozygous mothers and fathers carrying the hypomorphic allele $dAPC2^{d40}$ displayed an increase in cII size in every segment of every embryo examined. We also detected a change in cI size in 4% of hemisegments analyzed (Fig. 3D,G; Table 1). Again, no change in the timing of Slouch expression was found. Thus, in situations where Wg signaling was increased and uniformly expressed, cII was preferentially affected.

The specific effect on Slouch cII with loss and gain of Wg signaling was suggestive of a differential requirement of the two clusters for Wg signaling. We could not, however, rule out the formal possibility that cI had not yet shown a response to increased Wg simply because we had not supplied enough Wg to the mesoderm under our experimental conditions. To rule out this possibility, the level of Wg supplied to the mesoderm was increased in two ways. First, the dose of the pan-mesodermal GAL4 driver was increased from one to two copies to drive Wg expression (Fig. 3E,G; Table 1). Wg overexpression in this manner specifically expanded Slouch cII while cI remained at wildtype size. This suggested that a factor other than Wg levels limits cI size. Interestingly, the increased Wg levels obtained using two copies of the GAL4 driver did not expand cII further than that observed using one copy, suggesting an upper limit of Wg responsiveness.

Overexpression of an activated Armadillo construct under the same conditions had identical results (Fig. 3G; Table 1 and data not shown).

Second, we tested whether the combination of increased levels and increased length of time that the mesoderm was exposed to Wg might now affect cI as well as cII. We overexpressed Wg using flies that carried two different GAL4 drivers (*twiGAL4; Dmef2GAL4*), which led to maintained, high levels of mesodermal expression throughout embryogenesis. Ectopic Wg expressed in this manner led to a specific increase in cII size, with no notable increase in the size of cI (Fig. 3F,G; Table 1). Likewise, no additional increase in cII size was noted beyond what was seen under previous conditions. Overexpression of activated Armadillo similarly caused an increase in cII without changing cI, further supporting the assertion that neither the amount nor the length of time exposed to Wg signaling can alter Slouch cI (Fig. 3G, Table 1, data not shown). In addition, despite increased amounts and time of Wg signaling, no change in the onset of Slouch expression was detected. Moreover, these data suggested that neither parameter could further affect the size of Slouch cII. Thus, only a limited number of cells can respond to the Wg signal and

**Table 1. Quantification of Slouch cluster loss and gain**

| Genotype | *n* | Cluster I present: Number of hemisegments | Cluster I present: Percentage of hemisegments | Cluster II present: Number of hemisegments | Cluster II present: Percentage of hemisegments |
|---|---|---|---|---|---|
| Loss of Wg function | | | | | |
| Wild type | 100 | 100 | 100 | 100 | 100 |
| $wg^{PE6}$ (25°) | 122 | 9 | 7 | 0 | 0 |
| $wg^{PE6}$ (18°) | 114 | 43** | 38 | 5 | 4 |
| *twiGAL4>UASΔNTcf* | 104 | 85 | 82 | 0 | 0 |
| *twiGAL4;twiGAL4> UASΔNTcf* | 103 | 100 | 97 | 0 | 0 |
| $wg^{21.2}$ (18°) | 130 | 81 | 62 | 0 | 0 |
| $wg^{NE2}$ (25°) | 77 | 53 | 69 | 2 | 3 |
| $wg^{PE4}$ (25°) | 71 | 0 | 0 | 0 | 0 |
| $wg^{NE1}$ (25°) | 110 | 0 | 0 | 0 | 0 |

| Genotype | *n* | Cluster I expanded: Number of hemisegments | Cluster I expanded: Percentage of hemisegments | Cluster II expanded: Number of hemisegments | Cluster II expanded: Percentage of hemisegments |
|---|---|---|---|---|---|
| Gain of Wg function | | | | | |
| Wild type | 100 | 0 | 0 | 0 | 0 |
| *twiGAL4>UASwg*$^{E}$ | 103 | 0 | 0 | 103 | 100 |
| *twiGAL4>UASarm*$^{s10}$ | 106 | 1* | 0.90 | 106 | 100 |
| *dAPC2*$^{\Delta S}$ | 110 | 8* | 7 | 110 | 100 |
| *dAPC2*$^{d40}$ | 35 | 1* | 4 | 35 | 100 |
| *twiGAL4;twiGAL4>UASwg* | 100 | 0 | 0 | 100 | 100 |
| *twiGAL4;twiGAL4>UASarm** | 108 | 1 | 0.90 | 108 | 100 |
| *twiGAL4;Dmef2GAL4>UASwg* | 135 | 0 | 0 | 135 | 100 |
| *twiGAL4;Dmef2GAL4>UASarm** | 112 | 0 | 0 | 112 | 100 |

| Genotype | *n* | Cluster I present: Number of hemisegments | Cluster I present: Percentage of hemisegments | Cluster II present: Number of hemisegments | Cluster II present: Percentage of hemisegments |
|---|---|---|---|---|---|
| Temporal shifts of Wg expression | | | | | |
| Wild type | 100 | 100 | 100 | 100 | 100 |
| $hh^{21}$ | 100 | 74** | 74 | 5 | 5 |
| *twiGAL4>UASwg;hh*$^{AC}$*/hh*$^{13C}$ | 103 | 103 | 100 | 102 | 99 |
| $wg^{IL114}$ 25° | 100 | 0 | 0 | 0 | 0 |
| $wg^{IL114}$ 18° | 100 | 100 | 100 | 100 | 100 |
| $wg^{IL114}$ 18° 13 hours, 25° 2 hours | 154 | 154 | 100 | 101 | 66 |
| $wg^{IL114}$ 18° 12 hours, 25° 3 hours | 56 | 56 | 100 | 0 | 0 |

| Genotype | *n* | Cluster I present: Number of hemisegments | Cluster I present: Percentage of hemisegments | Cluster II present: Number of hemisegments | Cluster II present: Percentage of hemisegments |
|---|---|---|---|---|---|
| Rescue of *wg* mutants with Twist | | | | | |
| $wg^{CX4}$ | 100 | 0 | 0 | 0 | 0 |
| $wg^{CX4}$*,twiGAL4>UAStwi* | 58 | 22 | 38 | 0 | 0 |
| $wg^{CX4}$*,twiGAL4>UAStwi* (29°C) | 84 | 27 | 32 | 0 | 0 |
| $wg^{CX4}$*,twiGAL4,Dmef2GAL4>UAStwi* | 70 | 31 | 44 | 0 | 0 |
| *twiGAL4;twiGAL4>UAStwi,UAStwi* | 112 | 4 | 4 | 112 | 100 |

*, cluster I expanded from two to four cells (see text).
**, cluster I also rescued in hemisegments where cluster II was rescued (see text).

increases in amount or time of exposure cannot further expand this domain. Considering these data together, we favor the conclusion that, although both ventral clusters require Wg for their specification, cII displays an increased sensitivity to Wg levels.

### Wingless sets up a region competent to express Slouch and is required later to specify the fate of cluster II

We have shown that, although both Slouch cI and cII require Wg, cII is more sensitive to Wg signaling than cI. We noted that cII arises directly under the ectoderm cells that produce Wg. We also observed that cI appears in the mesoderm at early stage 11 whereas cII appears at late stage 11, and that these two clusters are not related by lineage. Moreover, providing ectopic, high levels of Wg does not cause an earlier activation of Slouch in the clusters, suggesting that the accumulation of Wg over time cannot explain Slouch expression in cI and cII. Taking all this information together, two models can explain our observations: (1) Slouch cII cells simply need a greater amount of Wg signaling compared with Slouch cI cells; or (2) since the clusters arise at different times, Slouch cII requires two sequential Wg signaling events, whereas Slouch cI requires a single dose of Wg signaling. To test whether the contribution of Wg to Slouch cluster fate was temporally separable, we examined Slouch expression in two genetic backgrounds: (1) embryos carrying null alleles of *hedgehog* ($hh^{21}$, $hh^{AC}$ and $hh^{8}$) that do not maintain Wg expression past early stage 11 (DiNardo et al., 1994); and (2) embryos carrying the *wg* temperature-sensitive allele, $wg^{IL114}$, which have been shifted to non-permissive temperatures at different points in Slouch cluster development (Fig. 4).

In *hh* mutant embryos, the pattern of Wg expression is initially set up properly. However, this pattern is not maintained: Wg expression decreases and finally fades by late stage 11 (DiNardo et al., 1994), when cII would normally appear. In $hh^{21}$ mutant embryos, which are null for Hh signaling, cI was seen in the majority of hemisegments. cII, however, was detected only in 5% of hemisegments, all of which also contained cI (Fig. 4A,G; Table 1). We ruled out the possibility of a specific requirement

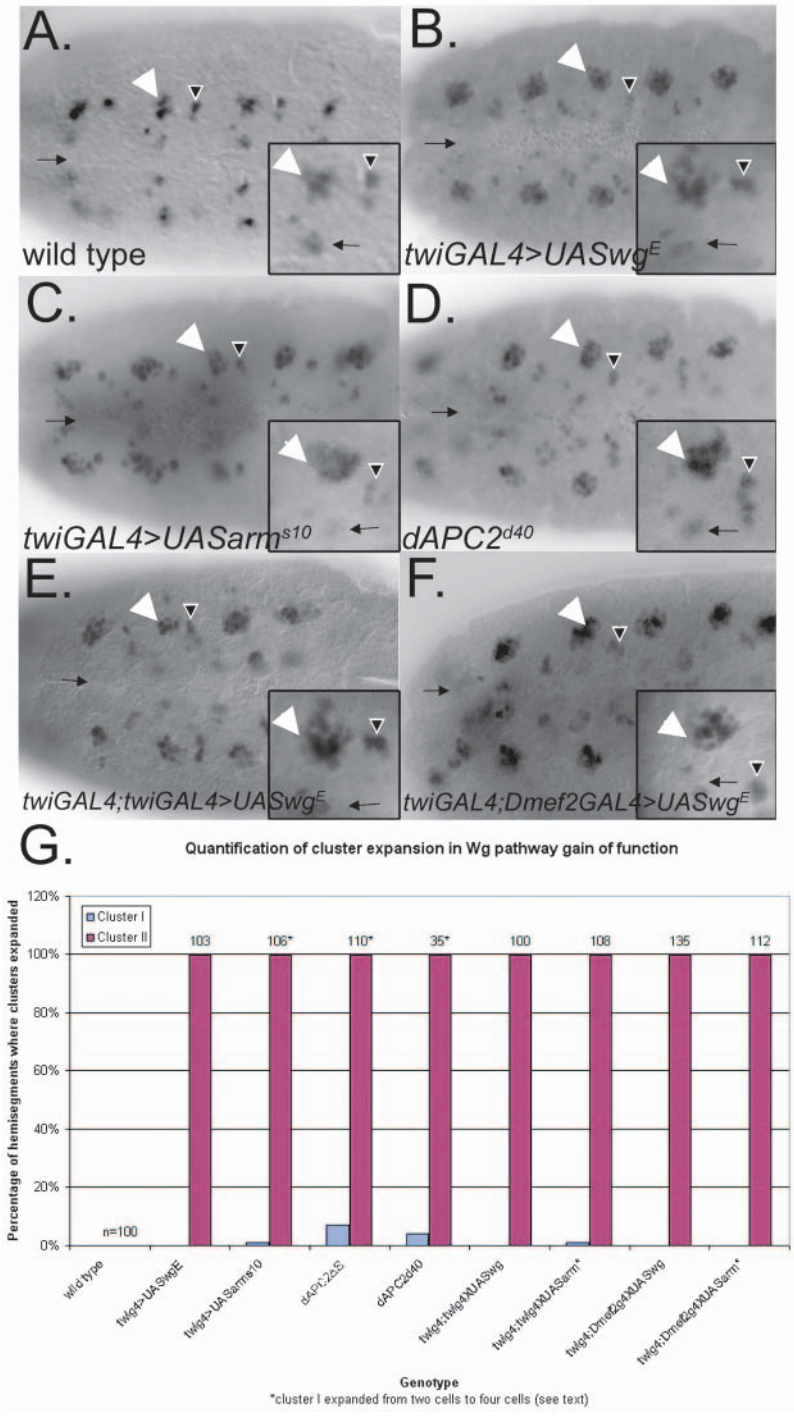



**Fig. 3.** Gain of function in the Wingless pathway specifically increases the size of Slouch cluster II. Ventral views of late stage 11 embryos were stained with Slouch antibody; ventral views are shown and anterior is left. cI (black arrowhead) and cII (white arrowhead) are indicated in both panel and insets. Black arrows denote the midline in panels and Slouch-expressing CNS cells in the insets. Insets show one hemisegment for each condition. The effect of different levels of Wg signaling were assayed in embryos of the following genotypes: (A) wild-type, (B) *twiGAL4>UASwg*$^{E}$, (C) *twiGAL4>UASarm*$^{s10}$, (D) *dAPC2*$^{d40}$, (E) *twiGAL4;twiGAL4>UASwg*$^{E}$ and (F) *twiGAL4;Dmef2GAL4>UASwg*$^{E}$. As shown in B-F, increased Wg signaling led to an increase in cII size (white arrowhead) to 5-15 cells, mode=12. cI size (black arrowhead) was unaffected. Four cells constitute cII in wild-type; two cells make up cI. Higher Slouch expression was detected in the visceral mesoderm in F. We detect displacement of cI towards the midline in these experiments. This effect may be due to the GAL4 drivers used to manipulate Wg signaling. (G) Quantification of cluster expansion in Wg pathway gain-of-function experiments. Graphs show percentage of hemisegments that show cluster expansion, cII (red) and cI (blue). **, conditions where cI was expanded from two cells; *n*, number of hemisegments counted. The mode number of cells increased in cII under gain-of-function conditions was 12 for each condition.

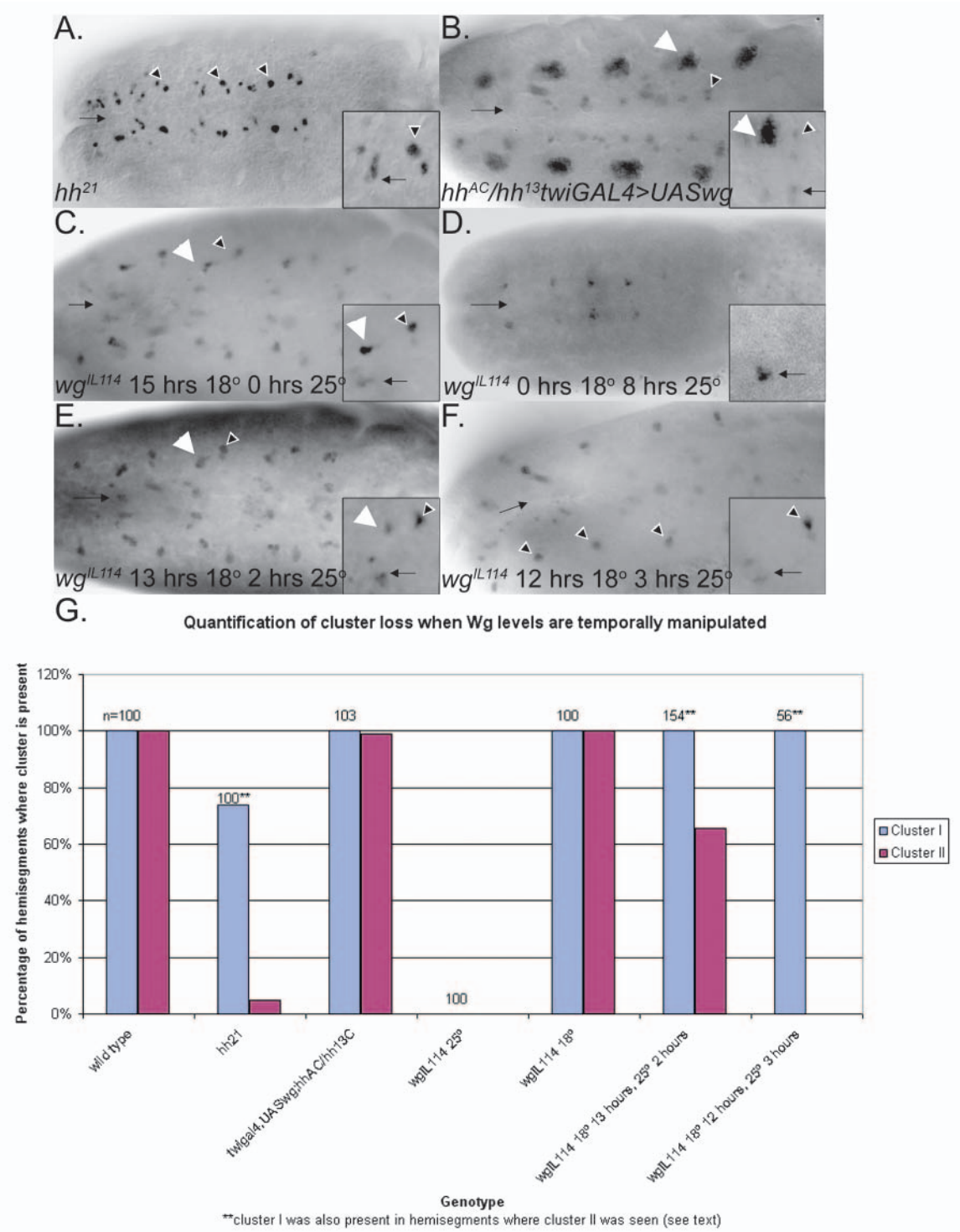



**Fig. 4.** Wingless acts sequentially to specify Slouch clusters I and II. (A-F) Ventral views of late Stage 11 embryos stained with Slouch; anterior is left. cI (black arrowhead) and cII (white arrowhead) are indicated in both panel and insets. Black arrows denote the midline in panels and Slouch-expressing CNS cells in insets. (A) Loss of *hh* function causes a loss of cII in most hemisegments but does not effect cI expression as strongly. (B) *hh* embryos in which *wg* is now expressed in the mesoderm reveals that Hh does not have a specific input to cII. cII and cI are specified normally. (C) Embryos carrying the hypomorphic allele $wg^{IL114}$ raised at 18°C for 15 hours show Slouch expression in both cI and cII. (D) Embryos carrying the hypomorphic allele $wg^{IL114}$ raised at 25°C for 8 hours show complete loss of mesodermal Slouch expression, although Slouch continues to be expressed in the CNS as in $wg^{CX4}$ embryos. (E) Embryos carrying the hypomorphic allele $wg^{IL114}$ raised at 18°C for 13 hours and shifted to 25°C for 2 hours show partial loss of Slouch expression in cII. (F) Embryos carrying the hypomorphic allele $wg^{IL114}$ raised at 18°C for 12 hours and 25°C for 3 hours show complete loss of Slouch cII and no loss of cI. (G) Quantification of Slouch cluster loss when Wg levels are manipulated by *hh* alleles and temperature shifts. Graphs show percentage of hemisegments in which cI (blue) or cII (red) are present. **, conditions in which cI and cII appeared in the same hemisegment; *n*, number of hemisegments counted.

for Hh itself in patterning the Slouch clusters by supplying Wg to the mesoderm of *hh* mutant embryos. In this genetic background, all Slouch clusters were rescued in all hemisegments (Fig. 4B,G). Thus, these data would suggest that cII has a temporally separable requirement for Wg.

We also performed temperature-shift experiments with embryos carrying the temperature-sensitive allele of *wg*, $wg^{IL1l4}$ (DiNardo et al., 1988). Embryos homozygous for this allele and raised at the permissive temperature throughout development showed a wildtype phenotype both in the ectoderm and mesoderm (Bejsovec and Martinez Arias, 1991) (Fig. 4C,G; Table 1). By contrast, $wg^{IL1l4}$ embryos raised at the non-permissive temperature showed a *wg* null phenotype both for epidermis (Bejsovec and Martinez Arias, 1991) and for mesoderm. Specifically, neither Slouch cI nor cII are specified (Fig. 4D,G; Table 1). We next removed *wg* function selectively during development by shifting from the permissive to the non-permissive temperature at mid-stage 11 (12 hours AEL) and at late stage 11 (13 hours AEL). Embryos shifted at the later timepoint showed expression of Slouch cI in all hemisegments and an occasional loss of Slouch cII (present in 66% of mutant hemisegments; Fig. 4E,G; Table 1). By contrast, embryos shifted at mid-stage 11 displayed a significant increase in the number of hemisegments in which cII was lost (present in 0% of mutant hemisegments); cI was present in all hemisegments analyzed (Fig. 4F,G; Table 1). Altogether our data suggested that specification of Slouch cII requires a temporally separable input of Wg signaling.

### Wg-dependent Twist expression is sufficient for Slouch cI but not cII

In *wg* mutant embryos, Twist expression is initiated correctly, but high levels of Twist are reduced by mid to late stage 11, the period during which the Slouch clusters appear (Bate and Rushton 1993). We hypothesized that to form cI, Wg needed only to activate Twist, as Wg was required first to sustain high Twist levels, and all founder cells, including the Slouch-positive ventral clusters, are derived from the high Twist domain (Baylies and Bate, 1996; Carmena et al., 1995). However, to specify the identity of cII, we proposed that Wg must provide an additional, Twist-independent signaling event. This does not rule out the possibility that other signals and transcriptional regulators such as *l'sc* (Carmena et al., 1995) and *ladybird* (Knirr et al., 1999) contribute to cI identity, but simply, that Wg was not required after the initial input to Twist expression for cI. Additional support for this idea comes from our studies of the different *wg* alleles; embryos in which cI was present but cII was missing had wild-type Twist levels (Fig. 2B,C; data not shown).

To test this hypothesis, we expressed Twist throughout the mesoderm in *wg* null mutant embryos (Fig. 5A,B). When we examined these embryos, we saw rescue of some Slouch expression (Fig. 5C,D,E; Table 1), in contrast to the complete loss of mesodermal Slouch expression seen in $wg^{CX4}$ embryos. To determine the identity of the rescued Slouch cells, both morphology and the co-localization of Kr and Slouch were assayed. When Twist was expressed in the mesoderm of *wg* mutant embryos, the ventral Slouch expression did not co-localize with Kr and did not align with the Slouch-positive nervous system cells, indicating that the cells rescued in these mutant embryos were not cII (Fig. 5D′,D″; Table 1). We had no additional independent marker for cI. Thus, we were unable to determine unequivocally that the identity of the rescued Slouch-positive cells represented cI; however, the ventral position of these cells and the lack of Kr staining would suggest a cI identity. Increasing Twist levels by using multiple copies of the GAL4 driver or different GAL4 drivers, led to a mild increase in cI appearance but not in cII (Fig. 5E; Table 1). These data indicate that Wg uses two distinct mechanisms to pattern these two clusters and direct the expression of Slouch: in the case of Slouch cI, Wg needs only to maintain high Twist levels, whereas the fate of Slouch cII additionally requires a temporally distinct input from Wg that is Twist-independent (Fig. 6).

## Discussion

### Wg operates a cascade of transcriptional regulators to pattern the *Drosophila* mesoderm

Through the manipulation of the amount and time of exposure to Wg signaling in the *Drosophila* mesoderm, we have shown that Slouch founder cell cII requires more Wg signaling than its neighbor, cI. Because cII arises in the mesoderm beneath the source of Wg signal, we initially thought that the sensitivity we detected would be due to Wg acting as a classic morphogen. Specifically, during stage 11, Wg would directly elicit concentration-dependent responses, leading to Slouch cI specification at low levels and cII at higher levels. Instead, our data suggest an alternative mechanism underlying this sensitivity. For Slouch cI, Wg signaling through Twist is sufficient for fate specification. However, for Slouch cII, a second, Twist-independent Wg signal is also necessary (Fig. 6).

It has previously been shown that *wg* mutants fail to maintain high levels of Twist (Bate, 1993). Overexpression of Twist led to expanded somatic mesodermal fates at the expense of other mesodermal fates, such as heart and gut muscle. Conversely, decreasing Twist levels led to a reduction in somatic mesodermal fate, while heart and gut muscle remained largely unaffected (Baylies and Bate, 1996). Our findings underscore the importance of high Twist levels for the proper implementation of somatic muscle fate. Because loss of high Twist levels leads to loss of muscle founder cells, including all Slouch-positive clusters of founder cells, it has always appeared that each Slouch cluster required the same amount of Wg signal (relayed through Twist) to assume its particular fate. In this study, we uncoupled the requirement for Wg in maintaining high Twist levels from the later role of Wg in specifying cII fate. The fact that Twist specifically rescues Slouch cI in a *wg* mutant background suggests that Slouch cII requires an additional, Twist-independent contribution from Wg for proper patterning. Consistent with these results, we found *wg* hypomorphs that provided sufficient signaling to maintain high Twist levels during early mesoderm development and therefore pattern cI, but that did not pattern cII. Temperature-shift experiments using *wg* temperature-sensitive alleles have shown that Slouch cII specification and *engrailed* expression in the ectoderm required Wg expression at later stages of embryonic development (Dierick and Bejsovec, 1998; DiNardo et al., 1994; Owen, 1994) (this study). Thus, the absence of Slouch cII in the different *wg* alleles, in *hh* mutant embryos and in a Twist rescued *wg* mutant embryo, all suggest that proper patterning requires not only an

earlier Wg-dependent regulation of Twist, but also an additional Wg contribution to specify its identity.

Our manipulations of Wg signaling also revealed two additional aspects of Wg signaling to the mesoderm. First, we found that the mesoderm, in general, has a different threshold for Wg signaling when compared with the ectoderm.

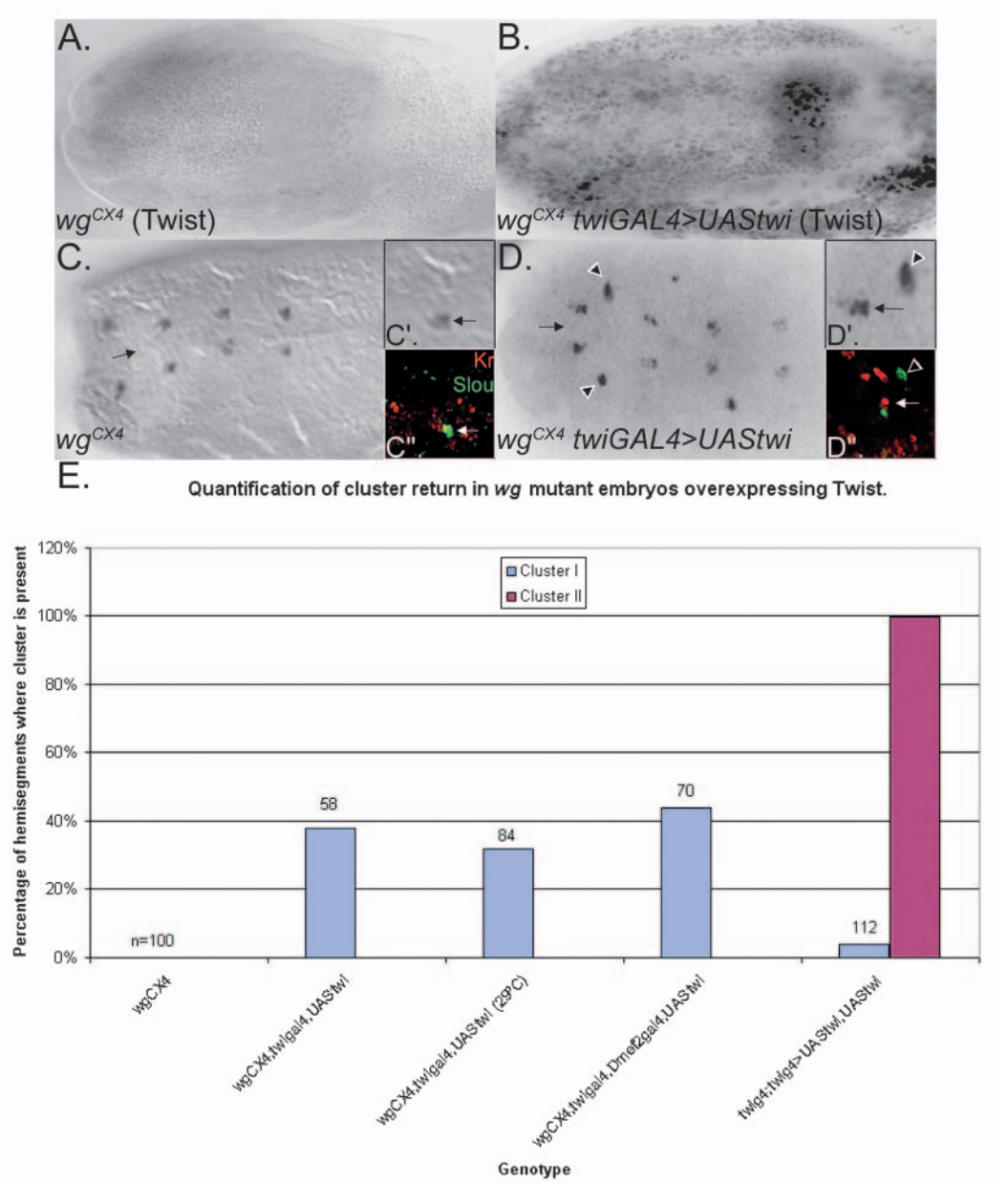



**Fig. 5.** Wingless sets up a region competent to express Slouch and is required later to specify the fate of cluster II. (A) Late stage 11 $wg^{CX4}$ mutant embryos fail to maintain Twist at high levels. (B) Ectopic Twist expression in $wg^{CX4}$ mutant embryos maintains Twist expression through stage 11. (C) Late stage 11 $wg^{CX4}$ mutant embryo showed loss of all mesodermal Slouch, although some expression remained in the CNS. cI (black arrowhead) and cII (white arrowhead) are indicated in both the panel and the inset. Black arrows denote the midline in the panel; black arrows in C′,D′ and white arrows in C″,D″ show Slouch-expressing CNS cells in the insets. (C) Immunocytochemical staining of a single hemisegment shows that both cII and cI are missing. (C″) Confocal micrographs of embryos stained with antibodies to Slouch (green/FITC) and Kr (red/Cy3). No co-localization of Kr and Slouch is detected in the mesoderm. White arrow indicates Slouch CNS expression. (D) $wg^{CX4}$,*twiGAL4>UAStwi* embryos showed rescue of mesodermal Slouch expression in positions corresponding to cI. (D′) Immunocytochemical staining shows that cII is absent but cI is present (black arrowhead). (D″) Confocal micrograph of a single hemisegment shows that Slouch staining is absent from the normal position of cII, and Slouch (green/FITC) does not co-localize with Kr (red/Cy3), supporting the identity of this cluster as cI (black arrowhead). We note that the amount of Twist maintained in these cells after specification is detrimental. While Twist is necessary for the specification of the Slouch clusters, maintained elevated expression can lead to repression of these clusters (V.T.C. and M.K.B., unpublished). (B) Quantification of Slouch cluster rescue in *wg* mutant embryos that overexpress *twist*. Graphs show percentage of hemisegments in which cI (blue) or cII (red) are present under conditions listed at the bottom of the graph. *n*, number of hemisegments counted.

Conditions that completely rescue the ventral ectoderm and epidermis ($wg^{PE6}$ at the permissive temperature) failed to completely rescue the mesoderm. Second, we find that different mesodermal targets respond differently to Wg signaling. For example, we find that expression of the ΔNTcf had mild effects on Twist but significant effects on Slouch cII. Although we predict that TCF binds *slouch* regulatory regions directly, we have found that Wg regulates Twist both directly through TCF and indirectly through the pair-rule gene *sloppy-paired* (V.T.C. and M.K.B., unpublished) (Lee and Frasch, 2000). Whether or not the difference in Wg regulation of *twist* and *slouch* is due to the structure of the regulatory regions, additional factors that integrate on these promoters in these contexts and the activity of the Arm/dTCF complex remains to be uncovered.

Our study also underscores the contribution that other factors make to position the Slouch clusters: ectopic Wg expression in the mesoderm does not produce uniform Slouch expression (Baylies et al., 1995; Brennan et al., 1999). This aspect of Wg signaling is reflected in other tissues such as the epidermis (Sampedro and Guerrero, 1991). Indeed, we were unable to further enlarge the size of Slouch cII beyond that seen when we initially increased Wg signaling (Fig. 3). This suggests a prepatterning mechanism, perhaps involving the activity of the pair-rule genes that have been shown to be responsible for segmentation of the mesoderm (Azpiazu et al., 1996; Riechmann, 1997), as well as the integration of other signal transduction pathways, such as EGF/FGF and Notch signaling (Brennan et al., 1999; Carmena et al., 2002; Carmena, 1998a). Our data suggest that Wg signaling then works on this prepattern to regulate the domain of Slouch expression.

The effect of Wg that we have described on muscle patterning is similar to that described for *even-skipped* muscle progenitor specification; that is, Wg signaling (in collaboration with such signals as Decapentaplegic) is first required to set up a region of 'competence' through activation of mesoderm-specific factors such as Twist and Tinman. Wg then later cooperates with these intrinsic factors to induce the expression of *even-skipped* in dorsal muscle progenitors (Halfon et al., 2000), much as we would suggest for Slouch cII. However, our observations suggest an important variation of Wg signaling in mesodermal patterning. In the case of Slouch patterning, Wg creates temporal as well as spatial diversity, while in patterning *eve* it only acts temporally. Wg signaling contributes to the expression of Slouch in its two discrete ventral patches by two distinctive mechanisms: through the regulation of an upstream transcription regulator (Twist), which is sufficient for one domain of expression; and through the cooperation of this factor with a second, temporally distinct Wg input for the second domain of expression. The expression of the same gene but at two different times and places, through two Wg-dependent means, gives insight into how an organism may generate diverse tissues in response to the same signal.

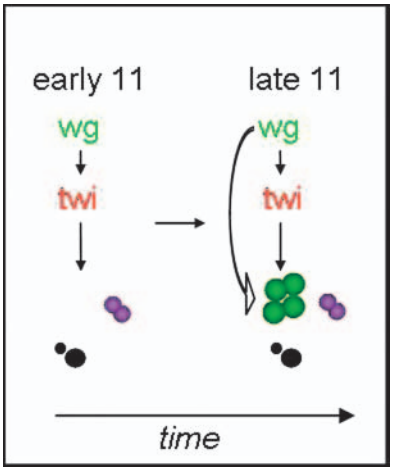



**Fig. 6.** A model for Wingless specification of Slouch clusters I and II. An illustration of the two-step process by which Wg specifies Slouch cI and cII in the developing mesoderm. Wg provides two contributions for Slouch cII: one through Twist and a second that is Twist independent. The Twist-independent Slouch activation is likely to involve transcriptional regulation by the Wg transcriptional effector Pangolin/Tcf. cI needs Wg only to generate a myogenic competency domain through Twist.

## A new molecular look for morphogens?

Work carried out in the wing imaginal disc suggested that Wg acts as a morphogen. In this tissue, Wg protein could be visualized in a graded distribution and it appeared to activate multiple target genes directly, in a concentration-dependent manner (Strigini and Cohen, 2000; Zecca et al., 1996). Based on these criteria, Wg was labeled as a classical morphogen. However, careful inspection of the molecular mechanisms underlying Wg activation of both short- and long-range targets in the wing have revealed that the pattern of Wg expression changes during wing imaginal disc development, and that Wg collaborates with other pathways to set up the expression of these genes. These studies have cast doubt on whether Wg is a true morphogen in this tissue (Martinez Arias, 2003).

Our work, investigating the molecular mechanisms that govern patterning of the embryonic mesoderm, similarly suggests that Wg does not act on Slouch clusters I and II as a classical morphogen. We discovered that Wg does not activate cI directly, but that, instead, it maintains high levels of Twist, which sets up a somatic mesodermal competency domain that is sufficient to create cI. Additional Wg is then required later to pattern cII. It can be argued that Wg acts as a morphogen to regulate Twist expression (at low levels), and then to control Slouch expression (at high levels) within cells of cII. However, the precise regulation and dependence of Slouch clusters I and II on Wg within both the dorsoventral and anteroposterior axes suggest that there must be additional patterning information available to properly place these two cell types. As more putative morphogens are held up to the lens of molecular biology, it will be interesting to see whether there are unexpected, new twists in the molecular underpinnings of morphogens.

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