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Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless

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SUMMARY

In the developing eye, *wingless* activity represses proneural gene expression (and thus interommatidial bristle formation) and positions the morphogenetic furrow by blocking its initiation in the dorsal and ventral regions of the presumptive eye. We provide evidence that *wingless* mediates both effects, at least in part, through repression of the basic helix-loop-helix protein Daughterless. *daughterless* is required for high proneural gene expression and furrow progression. Ectopic expression of *wingless* blocks Daughterless expression in the proneural clusters. This repression, and that of furrow progression, can be mimicked by an activated form of *armadillo* and blocked by a dominant negative form of *pangolin/TCF*. Placing *daughterless* under the control of a heterologous promoter blocks the ability of ectopic *wingless* to inhibit bristle formation and furrow progression. *hedgehog* and *decapentapleigic* could not rescue the *wingless* furrow progression block, indicating that *wingless* acts downstream of these genes. In contrast, Atonal and Scute, which are thought to heterodimerize with Daughterless to promote furrow progression and bristle formation, respectively, can block ectopic *wingless* action. These results are summarized in a model where *daughterless* is a major, but probably not the only, target of *wingless* action in the eye.

Key words: *wingless*, Signal transduction, *Drosophila*, *daughterless*, Morphogenetic furrow, bHLH proteins

INTRODUCTION

The *Drosophila* eye has proved to be an excellent tissue for the analysis of genetic circuits during development. It consists of repeated units called ommatidia, which contain photoreceptor neurons, cone cells (which secrete the lens), pigment cells and interommatidial bristles, which are mechanosenory organs (Wolff and Ready, 1993). The differentiation of all of these cell types is triggered by a coordinated wave of cell shape changes known as the morphogenetic furrow (MF). Starting at the beginning of the third larval instar stage, the columnar cells at the posterior edge of the presumptive eye, i.e., the eye imaginal disc, contract apically. This contraction is transient, but induces the adjacent cells to act similarly, thus causing the MF to sweep across the eye in an anterior direction. Behind the advancing MF, cells begin to differentiate, starting with the R8 photoreceptors. The other cell types are added in subsequent rounds of induction (Dickson and Hafen, 1993; Wolff and Ready, 1993).

The process of MF initiation and progression has been extensively studied at the molecular genetic level. The MF is initiated through a complex hierarchy initiated by the Pax6 homologs *twin of eyeless* (Czerny et al., 1999) and *eyeless* (Halder et al., 1998) and the secreted protein Hedgehog (Hh) (Dominguez and Hafen, 1997). These genes regulate the TGFβ homolog *decapentaplegic* (*dpp*) and the nuclear proteins Eyes absent, Sine oculis and Dachshund (Bonini et al., 1997; Curtiss and Mlodzik, 2000; Halder et al., 1998; Niimi et al., 1999). The MF then progresses through the eye via circular loops of gene expression in the furrow. These gene include *hh* (Dominguez and Hafen, 1997; Ma et al., 1993; Royet and Finkelstein, 1997), *dpp* (Burke and Basler, 1996; Chanut and Heberlein, 1997; Heberlein et al., 1993; Wiersdorff et al., 1996) and the proneural genes *atonal* (*ato*) (Jarman et al., 1995) and *daughterless* (*da*) (Brown et al., 1996). Behind the MF, Da is expressed ubiquitously at a low level while Ato resolves into evenly spaced cells. Ato is thought to heterodimerize with Da to promote specification of R8 photoreceptors (Jarman et al., 1995; Brown et al., 1996). Ras/Raf signaling is also required for MF progression (Hazelett et al., 1998; Greenwood and Struhl, 1999). Owing to the circular nature of the loop, the exact relationship between the MF progression genes is not exactly known, though the published data suggest an order of *hh* → *dpp*/Ras signaling → *ato/da* (Brown et al., 1996; Hazelett et al., 1998; Greenwood and Struhl, 1999).

The initiation of the MF at the posterior edge of the presumptive eye depends on the expression of *hh*, *dpp* and *wingless* (*wg*). *wg* encodes a secreted glycoprotein of the Wnt

family (Cadigan and Nusse, 1997). *hh* and *dpp* are expressed at the posterior edge (Chanut and Heberlein, 1997; Borod and Heberlein, 1998; Royet and Finkelstein, 1997) and Dpp signaling represses *wg* expression (Wiersdorff et al., 1996), restricting it to the dorsal and ventral edges of the eye disc. Removal of *wg* activity causes ectopic MF initiation from the dorsal and ventral edges (Treisman and Rubin, 1995; Ma and Moses, 1995) and activation of Wg signaling in the interior of the eye blocks MF progression (Heslip et al., 1997; Lee, 2001; Treisman and Rubin, 1995). Coexpression of activated *ras* with *wg* can suppress its ability to block the MF, while *dpp* can not, suggesting that *wg* acts downstream of *dpp* and upstream of *ras* (Hazelett et al., 1998).

In addition to its role in positioning the MF, we have previously shown that ectopic *wg* expression in the eye inhibits the formation of interommatidial bristles, part of the fly's peripheral nervous system (Cadigan and Nusse, 1996). These mechanosensory organs are derived from sensory organ precursors (SOPs) that arise from groups of cells expressing the proneural genes *achaete* (*ac*) and *scute* (*sc*) (Campuzano and Modolell, 1992). Wg acts by blocking proneural gene expression in the early pupal eye (Cadigan and Nusse, 1996).

In this report we further explore the mechanism by which Wg blocks the MF and bristle formation. The starting point was a screen for modifiers of a sensitized bristle phenotype caused by limited misexpression of *wg*. We identified three new alleles of *da* in this screen. We show that *da* is required for *ac* expression in the proneural clusters and confirm the previous report (Brown et al., 1996) that *da* is required for *hh* expression and MF progression. Ectopic *wg* represses Da expression and expression of endogenous *wg* is correlated with the normal lack of Da expression at the periphery of the eye. Coexpression of *da* with *wg* rescues the block in bristle formation and MF initiation. The simple model of *wg* acting solely through *da* to regulate bristles and the MF is complicated by the result that *sc* and *ato* can rescue the *wg* block of bristle formation and MF initiation, respectively. This suggests that *wg* regulates these processes at multiple levels.

MATERIALS AND METHODS

Drosophila strains

The P[*sev-wg^{ts}*] transgene was constructed by inserting the *Cla*I/*Xho*I fragment of pMH13 (van den Heuvel et al., 1993) into the pSEWa vector (Fortini et al., 1993). This places a *wg* cDNA, encoding a temperature sensitive Wg protein, under the control of the *sevenless* (*sev*) promoter. The P[*sev-wg*]^{2A} and P[*sev-wg*]^{2Aw} (where the white minigene is inactivated) transgenic stocks are as previously described (Cadigan and Nusse, 1996). Reporter genes used were *dpp-lacZ BS3.0* (Blackman et al., 1991) and *wg^{P}* (Kassis et al., 1992). The Gal4 drivers were P[*dpp-Gal4*] (Staehling-Hampton et al., 1994) and P[*GMR-Gal4*] (Freeman, 1996). UAS lines were P[*UAS-wg*] (Simmonds et al., 2001), P[*UAS-lacZ*] (Brand and Perrimon, 1993) P[*UAS-TCFΔN*] (van de Wetering et al., 1997), P[*UAS-arm^{SC10}*] (Pai et al., 1997), P[*UAS-da*] and P[*UAS-sc*] (Hinz et al., 1994)), P[*UAS-ato*] (Chien et al., 1996), P[*UAS-hh*] (Tabata and Kornberg, 1994) and P[*UAS-dpp*] (Pignoni and Zipursky, 1997). The *da* alleles *da^{2}* and *da^{3}* are amorphic (Cronmiller and Cummings, 1993) and immunonegative for an anti-Da polyclonal antibody (Cronmiller and Cummings, 1993). The P[*da^{+}*] rescue construct contains approximately 7.5 kb comprising the *da* locus (Brand and Campus-Ortega, 1990). *In(1)sc$^{10\text{-}1}$* removes or disrupts *ac* and *sc* expression, respectively (Villares and Cabrera, 1987). For mosiacs, *da* alleles were recombined onto a P[*FRT, hs-neo*]^{40A} chromosome as described previously (Xu and Rubin, 1993). The double mutant chromosomes *emc^{1}, h^{IL}* P[*FRT, hs-neo*]^{80B} and *emc^{1}, h^{C1}* P[*FRT, hs-neo*]^{80B} (Brown et al., 1995) were also used. Clonal markers were P[*arm-lacZ*]^{2L} (Pan and Rubin, 1995)and a P[*arm-lacZ*] on 3L (D. Lessing and R. N., unpublished). Mitotic clones were induced with P[hs-flp]^{1} (Golic and Lindquist, 1989) or P[*eye-flp*]^{T12} (Newsome et al., 2000).

Isolation of new *da* alleles

The *da* alleles were identified in a screen for modifiers of the P[*sev-wg^{ts}*] partial loss of interommatidial bristle (when reared at 17.6°C) phenotype. A P[*sev-wg^{ts}*] transgene was mobilized onto a *CyO* chromosome. w; *Sp*/*CyO* P[*sev-wg^{ts}*] flies were then mated with males previously fed the mutagen ethyl methane sulfonate (20 mM). The F_1 progeny were scored for unusually low (less than 50/eye) or high (more than 300/eye) bristle number. Putative positives were backcrossed to w; *Sp*/*CyO* P[*sev-wg^{ts}*] to confirm that a modifier was present and to fix the mutation in the germline. Subsequent crosses to balancer stocks mapped the modifier to one of the four chromosomes. A more detailed description of the screen will be described elsewhere. Three enhancers belonged to a single lethal complementation group and were subsequently found to be allelic to *da*. Two alleles (*da^{5B5}* and *da^{11B6}*) appear to be null (see Results).

Histochemical staining

Larval and pupal eyes were dissected and immunostained as described (Blochlinger et al., 1993). Double stains with horseradish peroxidase and alkaline phosphatase-conjugated secondary antibodies were performed as described previously (Cadigan et al., 1994). Clones of *da* in pupal eyes were observed in *yw* P[*hs-flp*]^{1}/+; da P[*FRT, hs-neo*]^{40A}/P[*arm-lacZ*]^{2L} P[*FRT, hs-neo*]^{40A} females and were identified by the lack of β-gal staining. *emc*, *h* clones were identified the same way in P[*eye-flp*]^{T12}/P[*sev-wg*]^{2Aw}; *em^{1}, h* P[*FRT, hs-neo*]^{80B}/P[*arm-lacZ*]3L animals.

The primary antibodies used were, an affinity purified rabbit anti-Da (1:50) (Cronmiller and Cummings, 1993) and affinity purified rabbit anti-Wg (1:50) (Cadigan et al., 1998); a mouse monoclonal against Ac (1:5) (Skeath and Carroll, 1991) a rat monoclonal against Elav (1:100) (O'Neill et al., 1994); rabbit polyclonal antibodies against β-gal (1:500; Cappel), Hairy (H; 1:50) (Brown et al., 1995) and Extramacrochaetae (Emc; 1:1000) (Brown et al., 1995). For fluorescence microscopy either donkey FITC anti-mouse (1:100) or donkey Cy3 anti-rabbit (1:300) from Jackson Immunochemicals were used. Confocal images were collected with a Bio-Rad MRC 1000 or Zeiss LSM500 confocal laser setup. Images were imported into Adobe Photoshop for presentation.

Histology

Clonal analysis of *da* in adult eyes were performed using the flies described above. Clones were identified by the lack of pigment (from the mini-white in the P[*arm-lacZ*] transgenes). Flies were anesthetized and then decapitated. The clones were photographed with a Leica M10 stereomicroscope using Kodak EP-135 film.

Flies were prepared for scanning electron microscopy (SEM) as described (Cadigan and Nusse, 1996), except that hexamethyldisilazane was used instead of Freon 113. The samples were viewed with a scanning electron microscope and photographed using Polapan 400 film (Kodak).

RESULTS

da interacts with *wg* in the eye

We have reported previously that P[*sev-wg*] flies lack interommatidial bristles, due to Wg repression of proneural

gene expression (Cadigan and Nusse, 1996). To utilize this phenotype as a starting point to identify genes that interact with *wg*, we created P[*sev-wg^{ts}*] flies that express a temperature-sensitive form of Wg (van den Heuvel et al., 1993). At 25°C, these animals have the normal (600/eye) number of bristles (Fig. 1A). At 16°C, where the Wg^{ts} protein is almost fully active (Couso et al., 1994), less than 50 bristles remain (data not shown). At 17.6°C, approximately 150-200 bristles form (Fig. 1B). This temperature was chosen to generate a sensitized background with which to screen for dominant modifiers.

In this report, we focus on three enhancers of the P[*sev-wg^{ts}*] bristle phenotype. All three reduce the number of bristles to between 10-50/eye (Fig. 1C). These modifiers form one lethal complementation group, which was meiotically mapped to an area between 30-32 on the cytological map. Complementation with deficiencies narrowed the region to 31B-32A, a location that includes the *da* gene (Caudy et al., 1988; Cronmiller et al., 1988). Four lines of evidence demonstrate that these enhancers are alleles of *da*. First, they fail to complement lethal alleles of *da* and are rescued by a P[*da^{+}*] rescue construct (data not shown). Second, null alleles of *da* dominantly enhance the P[*sev-wg^{ts}*] phenotype (Fig. 1D). Third, clones of two modifiers were negative for Da antibody staining (data not shown). Finally, identical effects on proneural gene expression, bristle formation and MF progression were observed in clones of the modifiers and known alleles of *da* (see below).

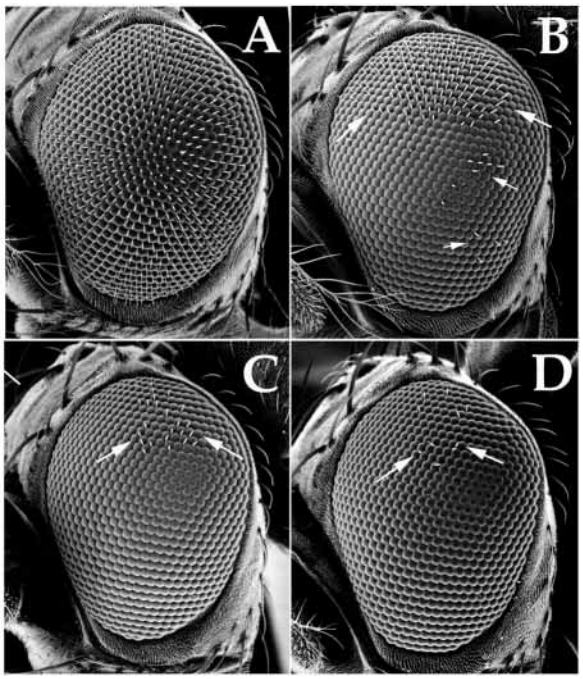

Fig. 1. *da* dominantly enhances the P[*sev-wg^{ts}*] bristle phenotype. Scanning electron micrographs of adult fly heads. P[*sev-wg^{ts}*]/+ flies reared at 25°C (A) or 17.6°C (B). P[*sev-wg^{ts}*]/*da^{11B6}* (C) and P[*sev-wg^{ts}*]/*da^{3}* (D) flies reared at 17.6°C. P[*sev-wg^{ts}*]/+ flies reared at the lower temperature had about 150 bristles/eye (compared to approximately 600 observed at 25°C) grouped in the dorsal third of the eye (large arrows) with a few bristles in the ventral half (small arrows in B). When heterozygous for strong *da* alleles, bristle number was reduced to 10-25 bristles/eye, and no bristles were seen in the ventral half of the eye.

da is required for normal proneural gene expression in the eye

Mechanosensory bristles are four-cell external sensory organs that are derived from single sensory organ precursors (SOPs). In the wing and eye, SOP specification requires the proneural genes *ac* and *sc*, which encode bHLH transcription factors (Campuzano and Modolell, 1992). These genes are first expressed in groups of cells known as proneural clusters. As one cell reaches the threshold of Ac/Sc expression necessary to trigger SOP formation, it represses proneural gene expression in the other cells in the cluster. This occurs through a process referred to as lateral inhibition, involving Notch signaling (Artavanis-Tsakonas et al., 1999).

The Ac and Sc proteins are thought to promote SOP formation by acting with Da, another bHLH protein (Cabrera and Alonso, 1991; Van Doren et al., 1992). *da* alleles dominantly suppress the ectopic bristle phenotypes caused by the misexpression of *sc* (Lehman et al., 1990) and *lethal of scute* (*lsc*), a gene that mimics *ac*/*sc* (Hinz et al., 1994). Da can bind to Ac or Sc, and the heterodimers can bind to specific DNA sequences known as E boxes (Murre et al., 1989). In cultured cells, Da and Ac or Sc synergistically activate reporter genes with promoters containing E boxes, including the proximal promoter of the *ac* gene (Van Doren et al., 1992). Unlike Ac and Sc, all cells examined express some Da (Cronmiller and Cummings, 1993), though there is significant modulation of levels in some embryonic tissues (Giebel et al., 1997) and the eye (Brown et al., 1996). Because most of the spatial information is manifested in Ac and Sc, Da is thought of as a proneural gene co-factor.

To further examine the relationship between Da and Ac/Sc and bristle formation, we examined clones of *da* in the eye and wing. While large clones in the eye resulted in a total lack of eye development (data not shown) because of a block in MF progression (Brown et al., 1996), small clones differentiated eye tissue that completely lacked interommatidial bristles (Fig. 2A). Ac expression was reduced at 3 hours after pupae formation (h APF) in *da* clones (Fig. 2B). This reduction of Ac protein and *sc* mRNA expression was also seen in the presumptive wing margin (Fig. 2C,D). Thus, *da* is required for normal proneural gene expression, most likely at the level of autoactivation, preventing the high expression levels needed for SOP specification.

Wg blocks bristle formation by repressing Da

At 3h APF, every cell in the basal portion of the eye (where Ac is expressed) expressed Da (Fig. 2B). However, groups of two to three cells had a much higher level of expression. Double staining with Ac indicated that these are the proneural clusters (data not shown). In P[*sev-wg*] eyes, where Ac expression is greatly reduced (Cadigan and Nusse, 1996), Da expression was also significantly lower (Fig. 2F). In P[*GMR-Gal4*], P[*UAS-wg*] (*GMR/wg*) eyes, ectopic *wg* was expressed at a much higher level than P[*sev-wg*] (data not shown). *GMR/wg* eyes had almost no detectable Da (Fig. 2G, Fig. 4F) or Ac (Fig. 4E) expression.

Does the ability of ectopic *wg* to repress Da and Ac expression reflect a normal role for *wg* in bristle inhibition? Normal adult eyes lack interommatidial bristles at the periphery of the eye (Cagan and Ready, 1989). We have found

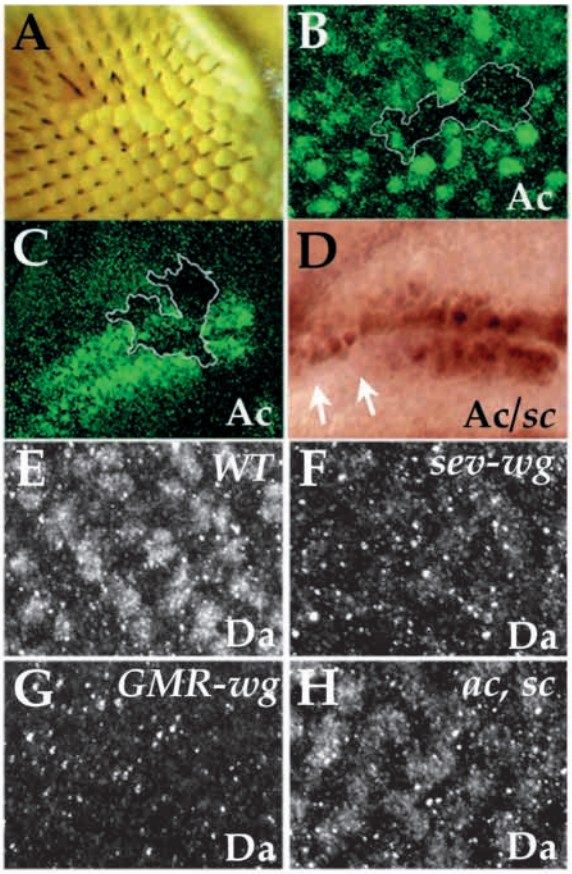

Fig. 2. Wg represses Da expression, which is required for proneural gene expression and bristle formation. (A) Micrograph of an adult fly head containing a small clone of da^{5B5} cells (marked by absence of pigment). Bristles fail to form inside the clone. (B,C) Confocal images of pupal eye (B) and larval wing imaginal disc (C) containing da^{5B5} clones and immunostained for Ac. Clonal boundaries were marked by the absence of β-gal staining (not shown; indicated by white lines). Ac expression is markedly reduced inside the *da* clones in both tissues. (D) Wing imaginal disc containing a da^{5B5} clone stained for Ac protein (orange) and *sc* mRNA (blue). The approximate location of the *da* clone is indicated by the reduction of Ac expression (white arrows). This area also contains reduced *sc* transcript. (E-H) Pupal eyes stained for Da protein. (E) Wild type; (F) P[*sev-wg*]; (G) P[*GMR-Gal4*], P[*UAS-wg*]; (H) sc^{10-1} (deficiency removing the *ac* and *sc* loci). While Da expression is significantly repressed by ectopic Wg (F,G), it is only mildly affected by removal of *ac* and *sc* (H). All pupal eyes were at 3h APF.

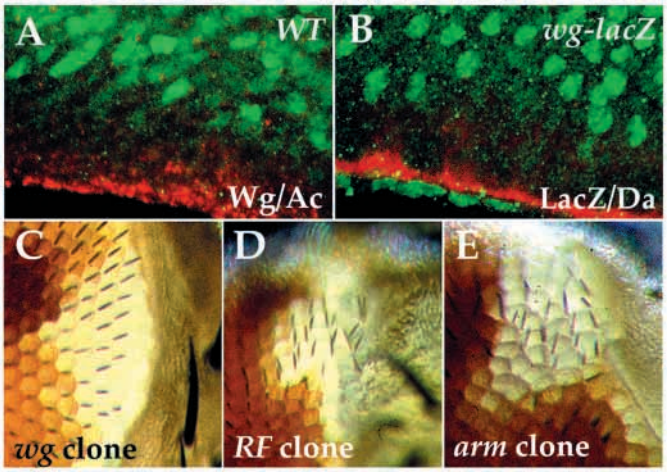

Fig. 3. Endogenous Wnt signaling represses Da and Ac expression and bristle formation at the periphery of the eye. (A) Wild-type pupal eye (3h APF) stained for Wg (red) and Ac (green). (B) P[*wg-lacZ*] pupal eye stained for β-gal (red) and Da (green). In both cases, Ac and Da expression is absent from the edge of eye, where Wg is expressed. The Da signal observed at the edge of the eye in B is from peripodal membrane staining. (C-E) Micrographs of adult fly heads containing clones of wg^{CX4} (C), *Df(2L)RF* (D) or arm^{25B} (E). Inside the *wg* clone, bristles are still inhibited at the edge of the eye. In the *Df(2L)RF* or *arm* clones shown, bristles are found up to the edge of the eye. At the top of the *arm* clone, ectopic bristles are present but are out of focus. See Results for further description of the clonal analysis.

an inverse correlation between Wg expression and that of Ac (Fig. 3A) and Da (Fig. 3B) at the edge of early pupal eyes. However, clones removing *wg* activity are identical to wild type, with no extra bristles at the eye's periphery (Fig. 3C). Clones of *arm* on the other hand, almost always (95%; *n*=20) cause bristles to form right up to the edge of the adult eye (Fig. 3E). Since loss of arm activity is normally associated with a block in Wg signaling (Cadigan and Nusse, 1997), this result suggest that endogenous Wg signaling may repress bristle formation at the periphery.

The lack of ectopic bristles in *wg* clones could be explained by the fact that *wg* clones often act non cell-autonomously (Morata and Lawrence, 1977) due to Wg diffusing in from surrounding wg^{+} tissue. However, temperature shifts (from 12 hours before pupation to 12 h APF) with wg^{ts} animals resulted in only occasional ectopic bristles (data not shown). Clones of *Df(2L)RF* exhibited ectopic bristles (Fig. 3D) one third (7/22) of the time. This deficiency has reported breakpoints of 27F2-4-28A3 (Tiong et al., 1989). While it may delete up to 30 annotated genes (Adams et al., 2000) besides *wg* (27F3), these include three other *Wnt* genes; *Dwnt4* (27E7-27F1) (Graba et al., 1995) *Dwnt6* (27F3-5) and *Dwnt10* (27F5-6). The removal of *Dwnt4* was confirmed by in situ hybridization of *Df(2L)RF* homozygous embryos (data not shown). Thus it is possible that one or more of these Wnts acts through *arm* to repress bristle formation at the edge of the eye.

Expression of *wg* at high levels behind the furrow (via the *GMR* promoter) results in a dramatically reduced eye completely lacking bristles (Fig. 4A). The reduced eye size is not due to a lack of MF progression (data not shown). *GMR/wg* eyes have a large degree of apoptosis during pupal development that is partially responsible for the reduction in eye size (A. Rogulja and K. M. C., unpublished). Coexpression of *wg* with a dominant negative form of *TCF* (*pangolin*-FlyBase), the transcription factor that mediates many Wg transcriptional effects (Brunner et al., 1997; van de Wetering et al., 1997) suppressed the size reduction of the *GMR/wg* eye and bristle inhibition (Fig. 4G). Ac and Da levels were also greatly elevated compared to *GMR/wg/lacZ* controls (Fig. 4H,I). These results, plus the requirement for *arm* (Fig. 3E) shown here and previously for P[*sev-wg*] (Cadigan and Nusse, 1996) indicate a canonical Wnt pathway mediating these effects.

Since Wg signaling represses both Da and Ac expression,

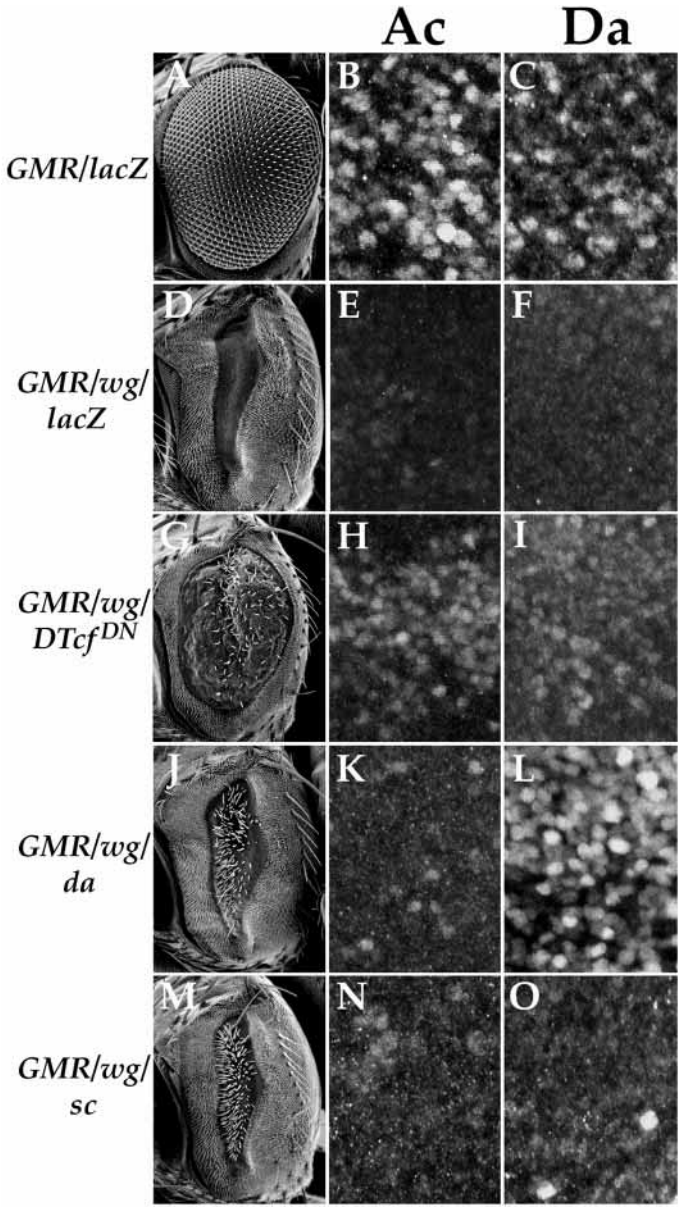

Fig. 4. Coexpression of a dominant negative version of *DTcf* (*DTcf*^{DN}), *da* or *sc* suppresses Wg-induced bristle inhibition. Scanning electron micrographs of adult fly heads (A,D,G,J,M) and pupal eyes (6 h APF) immunostained for Ac (B,E,H,K,N) or Da (C,F,I,L,O). (A-C) P[*GMR-Gal4*], P[*UAS-lacZ*]; (D-F) P[*GMR-Gal4*], P[*UAS-wg*], P[*UAS-lacZ*]; (G-I) P[*GMR-Gal4*], P[*UAS-wg*], P[*UAS-TCF*^{DN}]; (J-L) P[*GMR-Gal4*], P[*UAS-wg*], P[*UAS-da*]; (M-O) P[*GMR-Gal4*], P[*UAS-wg*], P[*UAS-sc*]. Ectopic expression of *wg* via the *GMR* promoter causes a severe reduction in eye size and completely blocks bristle formation (D). *GMR/wg* causes a huge decrease in Ac (E) and Da (F) expression. Co-expression of *wg* with *TCF*^{DN} partially suppresses the small eye and loss of bristles (G), as well as the Ac (H) and Da (I) inhibition. Co-expression of *wg* with *da* or *sc* does not significantly alter the small eye phenotype, but fully suppresses the bristle loss (J,M). *GMR/wg/da* causes a modest increase in Ac levels (K; compared with E). *GMR/wg/sc* also causes a modest increase in Ac (N) and Da (O) levels. All cultures were maintained at 25°C.

and Ac expression depends on *da* activity, it is possible that Wg represses Ac through inhibition of Da. One piece of evidence in support of this is that Da levels are only modestly affected in animals lacking *ac* and *sc* (Fig. 2H). Because lack of *ac/sc* does not cause lack of Da, the simplest model is that Ac is repressed by Wg signaling due to Da inhibiton.

To further test this hypothesis, we attempted to rescue the Wg-induced bristleless phenotype by coexpression with either Da or Sc. If the simple model were correct, placing *da* under a heterologous (i.e., *GMR*) promoter would restore bristles to *GMR/wg* eyes, but expressing *sc* in the same way would not. If *GMR/wg* repressed both *da* and *sc* directly (by direct, we mean without influence of the other gene), then neither *da* or *sc* heterologous expression would restore bristles.

Surprisingly, the results do not follow either of the above models. Both *da* (Fig. 4J) and *sc* (Fig. 4M) coexpression rescue the bristleless phenotype of *GMR/wg* eyes. Not every bHLH protein can rescue the bristles; *GMR/wg/ato* eyes are still completely bristleless (data not shown). The *GMR/wg/da* eyes have a significant but modest increase in Ac levels (compare Fig. 4K with 4E) while *GMR/wg/sc* eyes show a similar degree of increase of Ac (Fig. 4N) and Da (Fig. 4O) expression. These results suggest a more complicated situation, though caution is needed when interpreting overexpression studies (see Discussion).

da is required for *wg* to block MF initiation

In addition to its role in SOP specification, *da* is also known to be required for the initiation and progression of the MF (Brown et al., 1996) (and data not shown). Da is expressed at higher levels in the MF than elsewhere in the eye imaginal disc (Brown et al., 1996). It is thought to form heterodimers with the bHLH protein Atonal (Jarman et al., 1995) to specify R8 differentiation, which then promotes MF progression (White and Jarman, 2000).

wg is known to be required for the proper orientiation of the MF. Removal of *wg* causes ectopic furrow initiation from the dorsal and ventral borders of the eye disc (Ma and Moses, 1995; Treisman and Rubin, 1995). In addition, ectopically expressed *wg* can block MF initiation and progression (Treisman and Rubin, 1995). Having established that Wg blocks bristle formation through (at least in large part) Da repression prompted us to examine a similar connection in MF initiation.

Misexpression of *wg* using a Dpp-Gal4 driver, which is active at the posterior edge of the eye disc (Staehling-Hampton et al., 1994), causes a complete block in MF initiation (Fig. 5B) (Treisman and Rubin, 1995). Co-expression with the dominant negative *TCF* construct significantly rescued the block (Fig. 5C). Expression of an activated form of *arm* also blocks MF initiation (Fig. 5K), though not quite to the same extent as *wg*. As with bristle inhibition, *wg* appears to block MF initiation through a canonical Wnt pathway.

The strategy to test an involvement of *da* in *wg*-mediated MF inhibition was similar to that employed in Fig. 4. Coexpression of *da* with *wg* with Dpp-Gal4 always restored some MF progression, and Fig. 5D-F shows representative samples of the three classes observed (see legend). *da* also caused a dramatic increase in MF progression in *Dpp/arm*^{act} eyes (Fig. 5L). Similar to what was found for *sc* with bristle

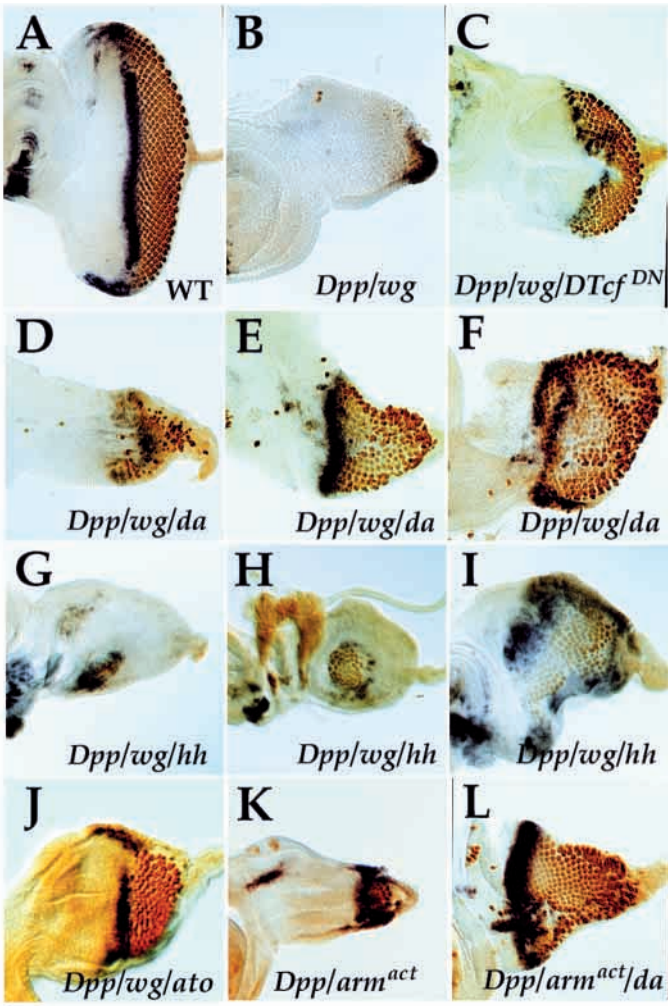

Fig. 5. Expression of *da* and *ato*, but not *hh* and *dpp*, can rescue the block in MF progression caused by ectopic activation of Wg signaling. Wandering third instar larval eye discs stained for β-gal (all samples have a P[*dpp-lacZ*] transgene) and Elav. (A) WT; (B) P[*dpp-Gal4*], P[*UAS-wg*]; (C) P[*dpp-Gal4*], P[*UAS-wg*], P[*UAS-TCF^{DN}*]; (D-F) P[*dpp-Gal4*], P[*UAS-wg*], P[*UAS-da*]; (G-I) P[*dpp-Gal4*], P[*UAS-wg*], P[*UAS-hh*]; (J) P[*dpp-Gal4*], P[*UAS-wg*], P[*UAS-ato*]; (K) P[*dpp-Gal4*], P[*UAS-arm^{act}*]; (L) P[*dpp-Gal4*], P[*UAS-arm^{act}*], P[*UAS-da*]. (A) At this stage, the MF is normally more than halfway across the eye primordia. (B) Ectopic expression of *wg* at the posterior edge of the eye completely blocks MF progression and differentiation of Elav-positive cells. (C) Coexpression of *TCF^{DN}* with *wg* significantly rescues MF progression. (D-F) Coexpression of *da* with *wg* always resulted in some MF progression, with approximately 40% of the eyes similar to D, 40% similar to E and the remainder rescued to the extent shown in F. Coexpression of *dpp* with *wg* resulted in no rescue (data not shown). (G-I) Coexpression of *hh* with *wg* did not rescue MF progression from the posterior edge, but did result in usually one (occasionally more) MF being initiated in the interior of the eye primordia and progressing concentrically. These ectopic MFs had progressed to different extents at the time of fixation, with some recent (G), some several rows of photoreceptors (H) and some progressed to the periphery of the eye (I; note that *dpp-lacZ* is at the periphery, indicating the leading edge of the MF). Coexpression of *ato* with *wg* resulted in considerable rescue of MF progression, though less than seen with *da* coexpression (the eye in J is one of the best rescues). Expression of an activated form of *arm* (*arm^{act}*) blocked MF progression, though not as well as *wg* (note some Elav-positive cells in K). Coexpression of *da* with *arm^{act}* resulted in significant rescue of MF progression (L). All crosses were maintained at 29°C before fixation.

formation, expression of *ato* with *wg* resulted in a significant rescue of MF progression (Fig. 5J). The degree of rescue was less than observed with *da* (Fig. 5J shows one of the best rescues). Expression of *sc* with *wg* also gave a modest rescue of the MF, with 7 of the 12 *Dpp/wg/sc* eyes examined showing some MF progression, similar to the eye shown in Fig. 5L (data not shown). This result is surprising, since *sc* has no known physiological role in regulating the MF and highlights the potential pitfalls of overexpression studies.

The genes *dpp* and *hh* have also been implicated in MF initiation and progression (Heberlein and Moses, 1995). Coexpression of *dpp* with *wg* did not result in any rescue (all eyes look identical to those in Fig. 5B; data not shown). Dpp-Gal4 driving P[*UAS-wg*] and P[*UAS-hh*] never resulted in MF rescue from the posterior edge (Fig. 5G,H,I). However, the majority of the eyes had at least one ectopic furrow that initiated from the anterior portion of the eye. These furrows apparently had different initiation times, since some were quite small (Fig. 5G), some had progressed to several rows of concentric photoreceptors (Fig. 5H) and some had progressed to fill most of the eye disc (Fig. 5I; note that the *dpp-lacZ*, marking the MF is at the posterior edge in this eye). At least in regard to the initiation of the endogenous furrow at the posterior end of the eye, the results suggest that Wg acts at the level of *da/ato*, rather than *hh* or *dpp*.

The role of *emc* and *h* in mediating the effects of *wg* in the eye

The two Wg readouts we have examined are the inhibition of bristle formation and MF initiation/progression. Since both processes depend on *da* activity and Wg inhibits Da expression, it is tempting to postulate that *da* is a direct target of the Wg signaling pathway. However, there are two other genes that are also known to regulate bristle formation and the MF, *hairy* (*h*) and *extramacrochaete* (*emc*).

Both *h* and *emc* encode HLH transcription factors that have distinct biochemical activities. The C terminus of H contains a sequence that mediates transcriptional repression (Fisher and Caudy, 1998). Emc lacks the DNA-binding basic portion of the bHLH motif, but can form heterodimers with other bHLH proteins (Cabrera et al., 1994; Van Doren et al., 1991). These heterodimers lack DNA binding. Thus Emc acts as a dominant negative bHLH factor. In this way, Emc is thought to repress bristle formation by acting as a negative regulator of Ac/Sc autoactivation (Cabrera et al., 1994; Van Doren et al., 1992). The H protein inhibits *ac* transcription by directly binding to the *ac* promoter (Van Doren et al., 1994; Ohsako et al., 1994).

In the eye imaginal disc, H is expressed in a stripe just anterior of the MF, while Emc is ubiquitously expressed, although it is found at much higher levels anterior of the H stripe (Brown et al., 1995) (Fig. 6A,C). If both genes are removed, the rate of MF progression is accelerated, as is neuronal differentiation (Brown et al., 1995). While the direct targets are not known, *ato* and *da* are likely candidates.

Given what is known about the roles of *h* and *emc* in MF movement and bristle formation, it is possible that *wg* could

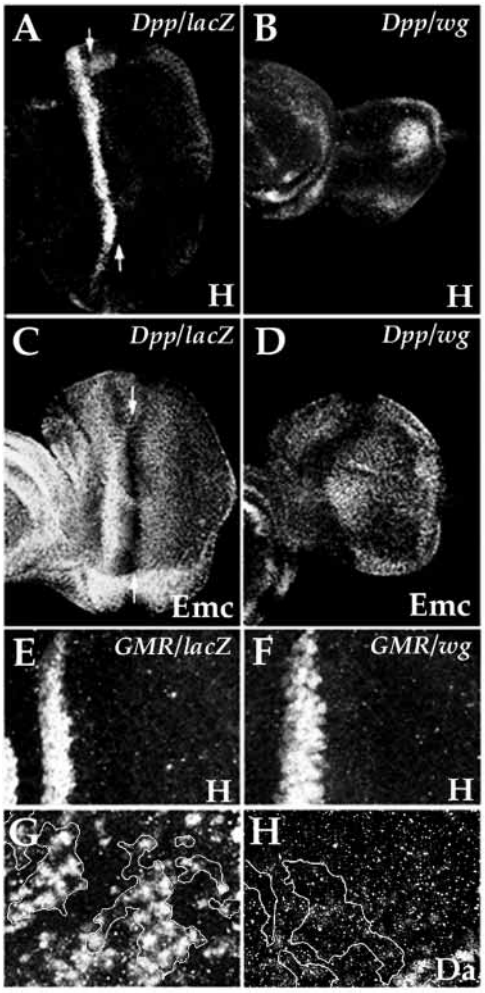

Fig. 6. Wg does not appear to induce Emc or H expression in the eye, but *emc* and/or *h* is required for Wg-dependent repression of Da expression and bristle formation. (A-D) Wandering third instar eyes; (E-G) pupal eyes (6 h APF) and adult P[*sev-wg*] eye with emc^{1}, h^{IL} clones (H). (A,C) P[*dpp-Gal4*], P[*UAS-lacZ*]; (B,D) P[*dpp-Gal4*], P[*UAS-wg*]; (E) P[*GMR-Gal4*], P[*UAS-lacZ*]; (F) P[*GMR-Gal4*], P[*UAS-wg*]; (G,H) clones of emc^{1}, h^{IL} in a P[*sev-wg*] (G) or P[*GMR-Gal4*], P[*UAS-wg*] (H) background. (A,B,E,F) H expression; (C,D) Emc expression; (G,H) Da expression. Clonal boundaries were marked with β-gal (not shown). H is normally expressed in a stripe several cell diameters ahead of the MF (marked with arrows). Emc is expressed in a stripe between the H stripe and MF. In *Dpp/wg* eyes, Emc is at the posterior edge of the eye (D) and H just anterior. This is consistent with the lack of MF progression in this background. Behind the MF, ectopic expression of wg (via the GMR promoter) does not induce H expression (F) or Emc (data not shown). Removal of *emc* and *h* blocks P[*sev-wg*] from inhibiting Da expression (G) but not P[*GMR-Gal4*], P[*UAS-wg*]-dependent Da repression (H). Clonal boundaries are indicated by white lines.

act by activating either gene's expression. To test this, we examined Emc and H protein levels in eyes misexpressing *wg*. In Dpp-Gal4, P[*UAS-wg*] eyes, no elevation of H or Emc was seen at the posterior edge of the eye (Fig. 6B,D). Likewise, levels of H and Emc were unchanged in GMR/wg or P[*sev-wg*] eyes compared to controls (Fig. 6F and data not shown). Hence, within the limits of detection, we found no evidence for Wg signaling activating H or Emc expression.

Despite the lack of activation of Emc or H expression by Wg signaling, these genes are required for the P[*sev-wg*] bristleless phenotype, as demonstrated by clonal analysis in adult eyes (data not shown). Moreover, Wg mediated repression of Da levels does not occur in clones lacking *emc* and *h* (Fig. 6G). However, when the dosage of *wg* is increased, as in a GMR/wg background, removal of *emc* and *h* does not block the ability of Wg to repress Da expression (Fig. 6H).

DISCUSSION

The role of Wnt signaling in eye development

Wg signaling is essential for the proper positioning of the MF during third larval instar (Treisman and Rubin, 1995; Ma and Moses, 1995). At this time, *wg* is expressed in the dorsal/posterior portion of the eye disc, and to a lesser extent in the ventral/posterior region (Ma and Moses, 1995; Royet and Finkelstein, 1997; Treisman and Rubin, 1995). After pupation, *wg* expression forms a ring around the entire eye (Fig. 3A,B and data not shown). This portion of the eye has low levels of Ac and Da (Fig. 3A,B) and bristles do not form there (Cagan and Ready, 1989). While ectopic expression of *wg* inhibits Da, Ac and bristle formation (Figs 2, 4) (Cadigan and Nusse, 1996) we could not demonstrate a requirement for endogenous *wg* activity in bristle inhibition at the periphery of normal eyes (Fig. 3C). However clones of *arm* at the edge of the eye have ectopic bristles (Fig. 3E), indicating that Wnt signaling is involved.

Though removal of *wg* does not block bristle inhibition (Fig. 3C), clones of a deficiency removing *wg* and three other *Wnts* (*DWnt4*, *DWnt6* and *DWnt10*) cause bristles to form at the edge ~30% of the time (Fig. 3D). This incomplete penetrance could be caused by diffusion of Wnts into the clone from surrounding tissue, or perhaps the presence of another Wnt (DWnt2, DWnt3 and DWnt8 lie outside the deficiency). Misexpression of Dwnt2, DWnt3 or DWnt4 does not block bristle formation (K. M. C. and R. N., unpublished). Testing the three remaining ones may help to resolve this mystery.

The genetic circuitry of Wg action

Our data supports a model for inhibition of bristle formation and the MF by Wg signaling through Arm/TCF. Clonal analysis (Cadigan and Nusse, 1996) (Fig. 3E) and overexpression of an activated form of *arm* (Fig. 5K) implicate Arm in both processes. A dominant negative version of the DNA-binding protein TCF can block Wg action in both contexts as well (Fig. 4G-I; Fig. 5C). Arm and TCF are thought to form a complex in the nucleus to regulate Wg target gene transcription (Cadigan and Nusse, 1997; Clevers and van de Wetering, 1997) and that appears to be the case in the eye as well.

The *da* gene is an attractive candidate for a direct target of Wg/Arm/TCF action. *da* is required for normal Ac expression (Fig. 2B-D) and bristle formation (Fig. 2A) and MF initiation/progression (Brown et al., 1996). Thus, loss of *da* has a very similar phenotype to misexpression of *wg*. Da levels are repressed by Wg (Fig. 2F,G; Fig. 4F) and placing *da* under a heterologous promoter can block Wg's ability to repress bristles (Fig. 4J) and the MF (Fig. 4D-F). These data are consistent with a relatively simple model of Wg affecting both bristle formation and the MF through repression of *da*.

Consistent with the simple model, *ato* is not required for Da expression in the MF (Brown et al., 1996) and elevated Da expression in the proneural clusters is only mildly affected by the absence of *ac* and *sc* (Fig. 2H). Thus, Wg cannot repress Da expression by inhibition of Ato, Ac or Sc expression.

Despite its attractiveness, the simple model outlined above is complicated by other experiments we performed. In the proneural clusters, Da and Ac or Sc are thought to heterodimerize to specify the SOP cell fate (Cabrera and Alonso, 1991; Van Doren et al., 1992). Given the data described above, coexpression of *sc* with *wg* should not block the ability of Wg to inhibit bristle formation, since Wg should still inhibit Da expression. However, misexpression of *sc* clearly does rescue the bristle inhibition (Fig. 4M). Da levels are higher in *GMR/wg/sc* eyes than *GMR/wg/lacZ* controls (compare Fig. 4O with 4E). This could be due to Sc activating *da* transcription or a post-translational stabilization of Da protein. More troubling to the simple model is Ac expression in *GMR/wg/da* eyes (Fig. 4K). If *GMR/wg* represses Ac expression by blocking synthesis of Da, then coexpressing *da* with *wg* should rescue Ac expression. However, only a slight elevation in Ac protein is observed, and this could be explained by post-translational stabilization due to *da* overexpression. This data suggests that Wg signaling may repress Ac (and by extension Sc) through a *da*-independent mechanism.

The relationship between Wg and Ato in MF progression mirrors that of Ac/Sc. The simple model of Wg signaling acting solely through Da is marred by the observation that coexpression of *ato* or *sc* with *wg* can significantly suppress the ability of Wg to block the MF (Fig. 5J and data not shown). It is important to note that the ability of *ato* and *sc* to suppress Wg's actions in the MF and bristle formation may be an artifact of overexpression. Perhaps residual Da can still heterodimerize with its overexpressed partners. Another possibility is that Sc or Ato homodimers can function when overexpressed. It has been shown that ectopic *sc* expression can rescue photoreceptor formation in *ato* mutants (Sun et al., 2000). However, it is also possible that Wg signaling represses *ato* expression in the eye independently of *da*.

Like Wg signaling, the genes *emc* and *h* are known to repress both the MF (Brown et al., 1995) and bristle formation (Cabrera et al., 1994; Ohsako et al., 1994; Van Doren et al., 1994). It is possibile that Wg signaling acts through transcriptional activation of either *emc* or *h*, but we found no evidence for this (Fig. 6A-F). However, *emc*, *h* activity is required for the P[*sev-wg*] transgene to repress Da expression (Fig. 6G), although, when the expression level of Wg is higher (i.e. GMR/*wg* eyes), the removal of *emc* and *h* activity did not prevent Wg from inhibiting Da levels (Fig. 6H). Therefore, we favor a model where Wg and Emc/H repress Da expression in parallel pathways.

As previously shown (Hazelett et al., 1998), coexpression of *dpp* with *wg* cannot suppress the Wg-induced MF block (Fig. 5). Coexpression of *hh* also did not rescue the endogenous furrow, though ectopic furrows did initiate in the anterior portion of the eye. These furrows were not observed when *hh* was expressed alone via the Dpp-Gal4 driver (data not shown). While we do not have a complete explanation for these data, it seems likely to be related to the results obtained with *pka* clones, which activate Hh signaling and induce ectopic furrows in the anterior eye (Dominguez and Hafen, 1997). These ectopic furrows initiate in the absence of Dpp signaling, which is in constrast to the endogenous furrow, where Hh signaling requires Dpp signaling (Hazelett et al., 1998).

Hazelett et al. (Hazelett et al., 1998) reported that activation of Ras signaling could suppress Wg's ability to block photoreceptor differentiation. Ras/Raf signaling has been implicated in the activation of Ato expression in the MF (Greenwood and Struhl, 1999). It is possible that Wg signaling blocks the MF solely through repression of Ras signaling, although we have found no evidence for Ras signaling playing a role in the inhibition of bristle formation by Wg (data not shown). It has also been reported that Ras signaling can block Wg signaling at the level of Wg degradation (Dubois et al., 2001) or *arm* (Freeman and Bienz, 2001), which could complicate the conclusions of Hazelett et al. (Hazelett et al., 1998). It is also possible that *ras* is downstream of *da*; this would explain our results and those of Hazelett et al. (Hazelett et al., 1998). In fact, misexpression studies with *ato* indicate that it (probably as a heterodimer with Da) can activate Ras signaling (White and Jarman, 2000).

With the caveat that much of the data described above is based on overexpression, a model where there are multiple positive feedback loops (e.g. *hh* → *dpp*/Ras signaling → *ato*/*da* → *hh* and *hh* → *dpp*/*ato*/*da* → Ras signaling → *hh*) may explain our results and those previously reported. Ultimately, the identification of direct targets by promoter analysis will be required to confirm these models.

Does Arm/TCF directly repress Da transcription?

It is thought that in the absence of Wg signaling, TCF bound to Groucho acts as a transcriptional repressor of Wg target genes (Cavallo et al., 1998; Yang et al., 2000). Wg signaling causes translocation of Arm to the nucleus, where it is thought to convert TCF to a transcriptional activator.

The data cited above suggest that Wg signaling regulates *da* expression through Arm/DTCF activation of a *da* repressor. Our results in Fig. 6 argue against *emc* or *h* fulfilling the role of a Wg-induced Da repressor. Another possibility is that Arm/TCF activates Delta/Notch signaling, which has been shown to inhibit Da expression in S2 cells and embryos (Wesley and Saez, 2000). Loss of Notch and Delta activity does suppress the ability of P[*sev-wg*] to inhibit bristle formation (Cadigan and Nusse, 1996). Further studies are needed to determine if the Wg and Notch signaling pathways are linked or act in parallel.

The alternative to indirect regulation of Da by Arm/TCF is direct repression of *da* expression. This possibility is strengthened by the finding that TCF sites in an enhancer of the *stripe* gene are required for Wg signaling to repress *stripe* expression (Piepenburg et al., 2000). We have found several sites in the *da* 5′ promoter and intron that are predicted to be TCF binding sites (A. Wardani and K. M. C., unpublished data). Mutating these sites in a Da-reporter gene chimera will be necessary to determine whether *da* is a direct target of Wg signaling.

In conclusion, the regulatory circuits by which Wg signaling regulates MF progression and bristle formation feature positive feedback loops and cross talk at multiple levels. Still, our data support a model where *da* is a major target of Wg action in the eye.

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