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 decapentaplegic 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 mechanosensory 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 (and thus interommatidial bristle formation and furrow progression. 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] transgene was constructed by inserting the Clal/Xhol 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] and P[sev-wg]2 were used (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 (Kassis et al., 1992). The Gal4 drivers were P[dp-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-TCF-AN] (van de Wetering et al., 1997), P[UAS-armSc10] (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 Zipsrky, 1997). The da alleles da and da are amorphic (Crommiller and Cummings, 1993) and immunonegative for an anti-Da polyclonal antibody (Crommiller and Cummings, 1993). The P[da*] rescue construct contains approximately 7.5 kb comprising the da locus (Brand and Campus-Ortega, 1990). In(1)Sc10 removes or disrupts ac and sc expression, respectively (Villares and Cabrera, 1987). For mosiacs, da alleles were recombined onto a P[FRT, hsn-10A chromosome as described previously (Xu and Rubin, 1993). The double mutant chromosomes emc, hsc/P[FRT, hsn-10B and emc, hsc/P[FRT, hsn-10B (Brown et al., 1995) were also used. Clonal markers were P[arm-lacZ] (Pan and Rubin, 1995) and P[arm-lacZ] on 3L (D. Lessing and R. N., unpublished). Mitotic clones were induced with P[hs-flp] (Golic and Lindquist, 1989) or P[eye-flp] (Newsonse et al., 2000).

Isolation of new da alleles

The da alleles were identified in a screen for modifiers of the P[sev-wg] partial loss of interommatidial bristle (when reared at 17.6°C) phenotype. A P[sev-wg] transgene was mobilized onto a CyO chromosome. w; Sp/CyO P[sev-wg] flies were then mated with males previously fed the mutagen ethyl methane sulfonate (20 mM). The F1 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] 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 (da585 and da11B6) 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]/+; da P[FRT, hsn-10B/P[arm-lacZ] (P[FRT, hsn-10B females and were identified by the lack of β-gal staining. emc, h clones were identified the same way in P[eye-flp]1/P[sev-wg]1w, eml, h P[FRT, hsn-10B/P[arm-lacZ]3L animals.

The primary antibodies used were, an affinity purified rabbit anti-Da (1:50) (Crommiller 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 Immunonomicals were used. Confocal images were collected with a Bio-Rad MRC 1000 or Zeiss LSM510 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 as described (Cadigan et al., 1994). Larval and pupal eyes were dissected and immunostained as described above. Clones of da were observed in yw P[hs-flp]/+; da P[FRT, hsn-10B/P[arm-lacZ] (P[FRT, hsn-10B females and were identified by the lack of β-gal staining. emc, h clones were identified the same way in P[eye-flp]1/P[sev-wg]1w, eml, h P[FRT, hsn-10B/P[arm-lacZ]3L animals.

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

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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-wgΔ]
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 WgΔ 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-wgΔ]
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-wgΔ] 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).

**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 acslc (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...
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 suggests 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 Wgts 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). 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,
This page contains a detailed description of the effects of Wingless (Wg) signaling on Daughterless (Da) activity in the eye. The text discusses the expression patterns of Ac and Da, and the results of experiments involving the expression of Wg and Daughterless (Da) in the eye. The text also describes the effects of misexpression of Wg and Daughterless (Da) on the development of eye structures.

The text mentions that Wg represses Da through inhibition of Da. One piece of evidence in support of this is that Da levels are only modestly decreased when Wg represses Ac through inhibition of Da. This suggests that the simple model, where Da is repressed by Wg signaling due to Da inhibition, would not be accurate. If the simple model were correct, placing Ac 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 orientation 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
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 UAS-wg and 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. In some cases, note that the dpp-lacZ 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*).

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 activity and Wg inhibits Da transcription by directly binding to the ac promoter (Van Doren et al., 1994; Ohnaka 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...
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 \(GMR/wg/\alpha\text{AC}Z\) 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 \(oto\) 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 possible 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 \(Emc/Hi\) 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 contrast 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. \(hh\rightarrow dpp/Ras\) signaling \(\rightarrow atolda\rightarrow hh\) and \(hh\rightarrow dpp/atolda\rightarrow Ras\) signaling \(\rightarrow hh\)) 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/TCF 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 \(da\) 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.
REFERENCES


