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Mad acts downstream of Dpp receptors, revealing a differential requirement for *dpp* signaling in initiation and propagation of morphogenesis in the *Drosophila* eye

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SUMMARY

Decapentaplegic (Dpp), a member of the TGF-β family of cytokines, has been implicated in many patterning processes in *Drosophila*, including the initial steps of pattern formation in the developing eye. We show that the *Mothers against dpp* (*Mad*) gene is required for *dpp* signaling during eye development. Clonal analysis demonstrates a cell-autonomous function for *Mad* and genetic interactions indicate that *Mad* is an essential component of the signal transduction pathway downstream of the Dpp receptors in responding cells. Mad-mediated *dpp* signaling is absolutely required for the initiation of the morphogenetic furrow in the eye, but has only a minor role in its subsequent propagation across the eye disc. We also present evidence for the repression of *wingless* transcription by *dpp* signaling.

Key words: Decapentaplegic (Dpp), *Mad*, *Drosophila*, pattern formation, eye, *wingless*

INTRODUCTION

Intercellular communication and inductive interactions are important mechanisms controlling growth and patterning during development. Signaling molecules belonging to the transforming growth factor β (TGF-β) family of secreted proteins have been implicated in many processes during development and differentiation in all vertebrates and invertebrates examined (reviewed in Wall and Hogan, 1994). The *Drosophila decapentaplegic* (*dpp*) gene encodes a member of this family that is closely related to the vertebrate Bone Morphogenetic Proteins (BMP) 2 and 4, together with which it constitutes a subfamily of TGF-β-like proteins. Dpp and BMP-4 are functional orthologs (Padgett et al., 1993; Sampath et al., 1993), suggesting that the respective signaling pathways are conserved in higher eucaryotes. Thus, the study of *dpp* signaling in *Drosophila* development can provide general insights into TGF-β functions in vivo as well as information pertaining to reception and transduction of TGF-β signals.

Dpp protein is required for the control of proliferation, global patterning and induction of specific cell fates. For example, Dpp has the properties of a morphogen during early embryogenesis, inducing different cell fates along the dorsoventral axis in a concentration-dependent manner (Ferguson and Anderson, 1992; Wharton et al., 1993). Later in development Dpp is essential for the growth of imaginal disc tissue and Dpp-expressing cells serve as organizers during pattern formation in discs (Capdevila and Guerrero, 1994; Diaz-Benjumea et al., 1994; Zecca et al., 1995).

In the eye disc, *dpp* is expressed in the morphogenetic furrow (MF) (Masucci et al., 1990; Blackman et al., 1991), where uncommitted cells begin to assemble into ommatidial preclusters and differentiate as photoreceptor neurons. As the MF traverses the eye disc from posterior to anterior, it forms a moving boundary between uncommitted pluripotent cells anterior to it and cells that have started to differentiate posterior to it (reviewed in Heberlein and Moses, 1995). Expression of *dpp* in the MF depends on secretion of the signaling molecule Hedgehog (Hh) by differentiating photoreceptors (Heberlein et al., 1993; Ma et al., 1993). Hh diffuses anteriorly and antagonizes the repression of *dpp* by *patched* (*ptc*) and *PKA* (*pka-C1*, catalytic subunit of protein kinase A). Ectopic activation of the Hh pathway anterior to the MF is sufficient to induce ectopic *dpp* expression and subsequent ectopic photoreceptor differentiation (Heberlein et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). In other imaginal discs, *hh* expression in the posterior compartment induces *dpp* expression in nearby anterior cells, this is again mediated by antagonizing *ptc* and *PKA* activity (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995).

During eye development, Dpp is necessary for normal cell proliferation and has also been implicated in patterning and differentiation processes in the MF (Heberlein et al., 1993). Interestingly, *dpp* is already expressed at the posterior margin of the eye disc before formation of the MF and prior to expression of *hh*. Thus Dpp could play a role in both initiation and anterior propagation of the MF. Nevertheless, a specific requirement for Dpp in both processes has never been satisfactorily demonstrated, in part because of the diffusible nature of the Dpp protein. As a secreted growth factor, Dpp is likely

to signal over some distance and thus a non-autonomous rescue of *dpp* mutant tissue could be expected. Downstream components of the Dpp signaling pathway are required autonomously in the responding cells and can therefore be used to investigate the requirement for Dpp signaling in initiation of the MF and its subsequent propagation.

Receptors for several TGF-β family members have been identified (reviewed in Massagué, 1992). They consist of two types of transmembrane proteins referred to as type I and type II receptors. Type II receptors are constitutive serine/threonine kinases. The following mechanism has been proposed for the TGF-β and activin receptors: the type II receptor binds its ligand, the resulting complex recruits the type I receptor and *trans*-phosphorylates its intracellular domain. The phosphorylated type I receptor is thought to trigger an intracellular signaling cascade (reviewed in Massagué, 1992; Attisano et al., 1994; Wrana et al., 1994). Although the mode of activation of the receptors is reasonably well understood, it remains unclear how this signal is relayed to the nucleus. The recent identification of the Dpp receptor genes, *thick veins*, *saxophone* and *punt* (Letsou et al., 1995; Ruberte et al., 1995 and refs therein) should facilitate a genetic dissection of this signaling pathway.

Genetic screens in *Drosophila* have identified mutations that interact with *dpp*. Two of these genes, *Mothers against dpp* (*Mad*) and *Medea*, have been isolated as dominant maternal enhancers of specific *dpp* alleles (Raftery et al., 1995; Sekelsky et al., 1995). It has been proposed that these genes are involved in multiple *dpp*-dependent signaling events. However, no genetic or molecular data have been available to relate their function to a Dpp signaling cascade. In contrast, a transcription factor encoded by *schnurri* has been shown to act downstream of *dpp* signaling (Arora et al., 1995; Grieder et al., 1995), but it is not required in all Dpp-mediated responses and is thus unlikely to be a common component of the signaling pathway. In addition, the *shortsighted* (*shs*) gene interacts genetically with *dpp* signaling during eye development and *shs* is homologous to a mouse TGF-β responsive gene (Treisman et al., 1995). *Mad* homologues have been identified in *C. elegans* (Savage et al., 1996) and a human gene related to *Mad* has recently been isolated as a tumour suppressor gene mutated in pancreatic carcinomas (*DPC4*, Hahn et al., 1996).

We have used *Mad* loss-of-function mutants to investigate the possible involvement of *dpp* signaling in different stages of *Drosophila* eye development. Our observation of a cell-autonomous requirement for *Mad* function in growth and differentiation, as well as genetic interactions, indicate that *Mad* is an essential component of the signal transduction pathway downstream of the Dpp receptors. We provide evidence that the *dpp* response mediated by *Mad* plays a central role in the initiation of the morphogenetic furrow (MF) but is largely dispensable for its subsequent anterior propagation. In addition, we find that loss of *dpp* signaling can lead to ectopic expression of Wingless and pattern duplications in the eye imaginal disc.

MATERIALS AND METHODS

Fly stocks and generation of *Mad* mutants

The *Mad* allele l(2)k5807 was isolated in a screen of ~2300 lethal P element insertions on the second chromosome (Török et al., 1993) for dominant enhancers of a *roE-sca* phenotype (Ellis et al., 1994). Complementation against the *Mad* null allele Mad^{12} and a small deficiency Df(2L)*C28* covering the *Mad* locus (Sekelsky et al., 1995) identified l(2)k5807 (later referred to as Mad^{B1}) as a *Mad* allele. Reversion analysis and rescue experiments using a Ubiquitin promoter construct expressing the *Mad* cDNA c28 (Sekelsky et al., 1995) proved that the P element was responsible for the *Mad* phenotype. Imprecise excision mutagenesis was used to generate additional *Mad* alleles (see Table 1). The *PKA* allele *89/18* and dpp^{blk} have been described elsewhere (Masucci et al., 1990; Strutt et al., 1995).

Table 1. Classification of new *mad* alleles

Class	Allele	Size of eye discs	Larval survival rate (% of expected)
I	B1	absent	25
II	1-2	very small	35
	4-1		39
	6-2		39
	7-1		30
	17-3		42
	20-2		12
	22-3		30
III	8-2	intermediate	68
	18-3		47
IV	1-1	wild-type	93
	9-2		97

Larval survival rate was determined as the ratio of homozygous to heterozygous prepupae, standardized against a WT control. Class IV alleles produce rare adult escapers with wing and leg defects (not shown).

Molecular analysis

Standard molecular methods are described in Sambrook et al. (1989). The P element insertion in l(2)k5807 was mapped to 2L 23D4-6 by in situ hybridization to polytene chromosomes using a Biotin/Avidin-HRP detection system (Vector). The insertion point relative to the published map of the *Mad* locus (Sekelsky et al., 1995) was determined by Southern blotting and sequencing of rescue plasmids. Characterization of the newly induced alleles (see Table 1 and above) by Southern blot analysis indicated that all 12 lines retain P element sequences in the original position, suggesting that heterologous sequences in the first intron of *Mad* are required for the mutagenic effect.

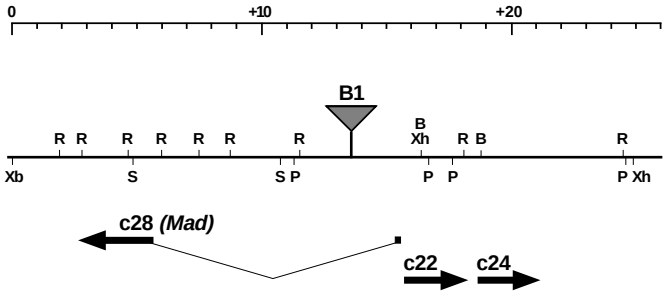

Fig. 1. The P element in Mad^{B1} is inserted in the first intron of the *Mad* gene. The insertion point of the B1 P element at position +13.5 of the genomic map of the Mad locus is indicated by a triangle. Note that the neighboring transcripts c22 and c24 (V. W. and M. M., unpublished; Sekelsky et al., 1995) are not affected by the P insertion. B, *Bam*HI; R, *Eco*RI; P, *Pst*I; S, *Sal*I; Xb, *Xba*I; Xh, *Xh*oI. Map positions (top) in kb.

Construction of *pGMR-Tkv^{Q253D}* and generation of transgenic flies

A point mutation in the *tkv* cDNA (Nellen et al., 1994), changing a glutamine (Q) residue at amino acid position 253 to aspartic acid (D) was generated in a two-step PCR reaction using mutagenic primers. The existence of a single point mutation was confirmed by sequencing. Subsequently the *tkv^{Q253D}* cDNA was inserted as an *Eco*RI/*Stu*I fragment into pGMR-1 (Hay et al., 1994) and transformed into flies (Spradling and Rubin, 1982). Several independent transformant lines showed a dominant eye roughening.

Histology

Mitotic clones were induced in first instar larvae using the FLP/FRT recombination system (Xu and Rubin, 1993). 3rd instar eye discs were prepared as described previously (Tomlinson and Ready, 1987; Strutt et al., 1995). An *arm-lacZ* construct inserted at 28A (Vincent et al., 1994) and a *dpp-lacZ* reporter construct on the 3rd chromosome (Blackman et al., 1991) were used for X-Gal staining. Discs were counterstained using a rat monoclonal antibody against the neuronal antigen Elav (kind gift of G. Rubin) or a rabbit polyclonal antiserum directed against the Wg protein and the respective secondary antibodies (Biorad). Expression of the *svp^{7842}* enhancer trap line (Mlodzik et al., 1990) was visualized using a monoclonal mouse anti-β-Galactosidase antibody (Promega). For scanning electron microscopy heads were dehydrated, critical point dried, coated with 20 nm gold-palladium mix and viewed on a prototype SEM. Histological sections of adult eyes were performed as previously described (Tomlinson and Ready, 1987).

RESULTS

Isolation and characterisation of new *Mad* alleles

To identify new genes involved in early eye development, we carried out a sensitized genetic screen for dominant enhancers of a mild eye roughening caused by overexpressing the Scabrous protein in the MF (Ellis et al., 1994; V. W. and M. M., unpublished). This screen has previously identified components of the Hh and Dpp pathways (*pka-C1*, Strutt et al., 1995; *shs*, V. W. and M. M., unpublished). From a collection of lethal P-element insertions (Török et al., 1993), an insertion mapping to 23D4-6 on the second chromosome, l(2)k5807, was identified. Larvae homozygous for l(2)k5807 survive to the late 3rd instar larval stage and lack imaginal discs. Additional defects can be found in a dramatic reduction of the larval fat body and optic lobes of the CNS, and in abnormal formation of the anterior midgut (data not shown). Complementation analysis revealed that l(2)k5807 was allelic to mutations in the previously identified *Mothers against dpp* (*Mad*) gene. *Mad* was isolated as a dominant enhancer of weak *dpp* alleles and displays very similar phenotypes to l(2)k5807 (Sekelsky et al., 1995).

The l(2)k5807 P insertion, subsequently referred to as *Mad^{B1}*, maps to position +13.5 kb on the genomic map of the *Mad* locus (Fig. 1 and Sekelsky et al., 1995). Alignment of the *Mad* cDNA with genomic sequences revealed the presence of a previously uncharacterized non-coding first exon 2 kb

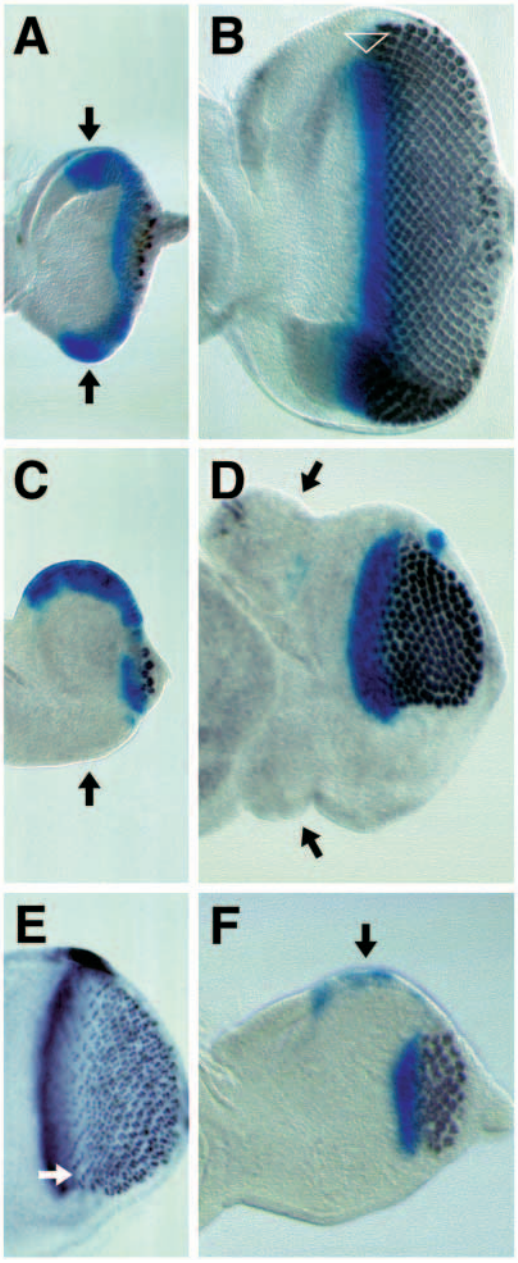

Fig. 2. Morphogenetic furrow initiation defects in eye discs of hypomorphic *Mad* alleles. All discs are shown with anterior to the left and dorsal up. (A,B) Wild-type expression pattern of a *dpp-lacZ* reporter gene (blue) and the neuronal marker elav (black). (A) Early 3rd instar disc shortly after initiation of the morphogenetic furrow (MF). Note that *dpp-lacZ* is expressed at the lateral margins of the disc (arrows) until the MF has passed. (B) At mid-third instar stage the MF (open arrowhead) forms a straight line across the disc. *dpp-lacZ* expression can no longer be detected at the margins. (C,D) Eye discs from animals trans-heterozygous for the hypomorphic alleles *Mad$^{9-2}$* and *Mad$^{1-2}$* display defects in *dpp* expression and MF initiation. (C) Early 3rd instar disc. *dpp-lacZ* expression is absent from the ventral disc margin (arrow). (D) Mid-third instar disc. MF and photoreceptor differentiation are limited to the dorsal region of the disc. Note that areas giving rise to anterior head cuticle show normal growth (arrows), whereas the rest of the disc appears reduced in size. (E) *Mad$^{9-2}$* / *Mad$^{1-2}$* disc stained with an anti-β-Gal antibody to visualize *dpp-lacZ* expression in the MF and expression of a *svp* enhancer trap line in photoreceptors R3/4, 1/6. The equator of ommatidial rotation (white arrow) is shifted to the ventral edge of the area of differentiation. (F) Eye disc homozygous for *dpp^{blk}*. Note that the area of photoreceptor differentiation and the extent of the MF is limited along the D/V axis. Traces of *dpp-lacZ* expression (arrow) are still visible at the dorsal but not at the ventral margin.

upstream of the P-element insertion point. A newly discovered transcription unit c22 (Fig. 1) in the immediate vicinity of the first *Mad* exon is not affected by the Mad^{B1} P insertion, as a construct expressing the *Mad* cDNA under the control of the Ubiquitin promoter (*UmMad*; Sekelsky et al., 1995) completely rescues the zygotic phenotype of Mad^{B1} (not shown). Additional *Mad* alleles were induced by imprecise excision of the Mad^{B1} P-element. The strength of these new *Mad* alleles was determined by a number of criteria, which placed them into three classes (II-IV in Table 1). In general they are hypomorphic in nature and weaker than our original P allele.

Hypomorphic *Mad* alleles cause defects in morphogenetic furrow initiation

To determine the role of *Mad* during eye patterning, we analyzed the eye phenotypes of several hypomorphic alleles that give rise to imaginal discs of reduced size (Table 1). In wild type, *dpp* is expressed prior to MF initiation at the posterior and lateral margins of early 3rd instar discs (Fig. 2A, and not shown). The MF is initiated in a small central region at the posterior edge by an unknown mechanism. Uniform propagation of differentiation from this point would lead to a curved MF. However the furrow seems to continue to initiate along the posterior margin faster than it is propagated in the center of the disc, thus ensuring that it forms a straight line across the eye disc during later stages (Fig. 2B; Heberlein and Moses, 1995). The furrow is generally more curved in discs of hypomorphic *Mad* alleles, possibly due to a delay in initiation at the lateral edges of the disc. In more extreme cases, furrow initiation is incomplete along the posterior margin, often failing in the ventral part of the disc (Fig. 2C,D). In wild-type discs, *dpp* is expressed at the lateral margin until the MF has passed (Fig. 2A). This expression is absent at this stage in the ventral part of *Mad* mutant discs (Fig. 2C), suggesting that *Mad* function is required for the expression of *dpp* at the margin. Once a partial initiation of the MF has occurred, furrow progression appears unaffected across the anterior eye field and the MF-associated *dpp-lacZ* expression is normal in this part of the disc (Fig. 2D). Photoreceptor differentiation seems unable to spread ventrally once the MF has lost contact with the margin (see Discussion). Consequently, the eyes that develop in such discs, although largely normal in the A/P axis, are reduced along the dorsal-ventral (D/V) axis.

In wild-type eyes, the equator demarcates a line of mirror-image symmetry between the dorsal and ventral halves of the eye. On either side of this line the developing ommatidia rotate in different directions (Tomlinson, 1988; Ready, 1989). In the eye discs of hypomorphic Mad alleles, the equator runs very close to the ventral edge of the differentiating eye field (Fig. 2E). Thus, although only few ommatidia show 'ventral' orientation, the position of the equator with respect to the dorsal eye field is relatively normal.

Overexpression of Mad suppresses the phenotype of an eye-specific *dpp* allele

Homozygous flies carrying the eye-specific allele dpp^{blink} (dpp^{blk}) have eyes that are reduced along the D/V axis but relatively unaffected along the A/P axis (Fig. 3A; Masucci et al., 1990). dpp^{blk} eye discs (Fig. 2F) have a similar appearance to those of hypomorphic *Mad* alleles: the furrow initiates in a restricted area at the posterior edge and *dpp* expression is not maintained along the posterior margin. The ventral part of the eye is generally more affected than the dorsal part in both genotypes (Fig. 3A) and the equator is shifted to the ventral edge of the differentiating eye field (Masucci et al., 1990).

The similarity of the dpp^{blk} and *Mad* phenotypes prompted us to test if *Mad* and dpp^{blk} interact genetically. Homozygous dpp^{blk} flies were combined with a transgene that overexpresses Mad under control of the Ubiquitin promoter (*UmMad*; Sekelsky et al., 1995). Although *UmMad* has no apparent phenotype in wild-type eyes, it partially rescues the dpp^{blk} phenotype (compare Fig. 3A and B). Taken together with the fact that *Mad* was isolated as a dominant enhancer of specific *dpp* alleles in the embryo (Raftery et al., 1995; Sekelsky et al., 1995), these data further support the idea that *Mad* and *dpp* function in a common pathway.

The eye phenotype of an activated Dpp receptor is suppressed by *Mad* mutants

Clonal analysis indicates that *Mad* is required cell-autonomously (see below), suggesting that it might encode a downstream component mediating Dpp signaling. To determine the position of *Mad* with respect to the known components of the *dpp* signaling pathway, we analyzed genetic interactions between *Mad* and the Dpp receptors. Experiments with the TGF-β receptors have defined a point mutation that constitutively activates the type I receptor and renders it independent of activation via transphosphorylation by the type II receptor and therefore independent of ligand (Wieser et al., 1995). An activated form of the type I Dpp receptor Thick veins (Tkv^{Q253D}) was generated and expressed in all cells in and behind the MF under the control of an enhancer consisting of multiple binding sites for the transcription factor Glass (pGMR-1; Hay et al., 1994), subsequently called *GMR-*tkv^{Q253D}. Flies carrying this transgene have rough eyes. Analysis of sections of these eyes revealed that many ommatidia contain extra photoreceptors (Fig. 3C,E). This effect is largely restricted to the posterior region of the eye (see Discussion).

To test for an interaction between *Mad* and *tkv*, the *GMR-*tkv^{Q253D} flies were crossed to *Mad* mutants. The phenotype of *GMR-*tkv^{Q253D} was strongly suppressed by the removal of one functional copy of *Mad* (Fig. 3D,F). This was apparent both from the normal composition of nearly all ommatidia in sections and from the external morphology of the eye. These data indicate that, under these circumstances, Mad is a limiting component that functions downstream of the Dpp receptors in the signaling pathway.

Initiation of the furrow is affected in *Mad* mutant clones

Analysis of the functions of *dpp* signaling during eye development has been complicated by the fact that Dpp is diffusible. Downstream components of the pathway are required autonomously in the responding cells and should therefore provide a means to test the role of Dpp signaling in MF progression. Depending on the position of the mutant clones, we can distinguish between requirements for Dpp in initiation and in subsequent propagation of the MF. First, we examined eye clones of Mad^{B1} and a previously identified null allele, Mad^{12} (Sekelsky et al., 1995). Very few clones were recovered. They

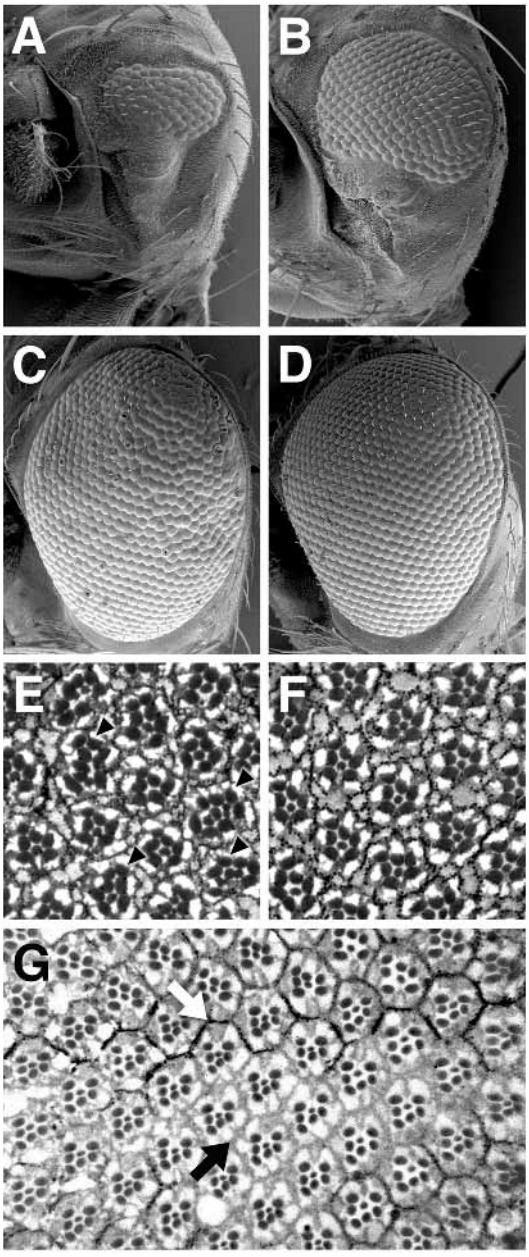

Fig. 3. Genetic interactions of *Mad* and *dpp* pathway mutants. (A,B) The reduced eye phenotype of dpp^{blk} is partially rescued by additional Mad expression under the control of the Ubiquitin promoter (*UmMad*). (A) Scanning EM of dpp^{blk} / dpp^{blk}. (B) Scanning EM of dpp^{blk}, *UmMad 2* / dpp^{blk}. (C-F) The *Mad* mutation dominantly suppresses the eye roughening caused by overexpression of an activated form of the Dpp receptor Tkv. (C) Scanning EM of *GMR-tkv*^{Q253D}/+. Note that roughening is more severe in the posterior part of the eye. (D) Scanning EM of Mad^{B1}/+; *GMR-tkv*^{Q253D}/+. The roughening is suppressed, but slight abnormalities in the posterior of the eye are still evident. (E) Eye section of *GMR-tkv*^{Q253D}/+. Note extra photoreceptor cells in the majority of ommatidia (arrowheads). (F) Eye section of Mad^{B1}/+; *GMR-tkv*^{Q253D}/+. Most ommatidia have normal appearance. (G) Section of a Mad^{B1} mutant eye clone. The clone forms a narrow stripe of cells marked by high levels of pigment (white arrow). Note that the WT 'twin spot' area next to it (black arrow) contains many more cells than the clone. No disruption of the WT arrangement of photoreceptors is observed.

were always very small and located next to large wild-type 'twin spot' clonal regions, suggesting that *Mad* function is autonomously required in imaginal disc cells for proliferation and/or survival (Fig. 3G). However, strong *Mad* alleles (class II in Table 1) give rise to virtually no discs when homozygous, but retain enough Mad activity to allow clones of reasonable size to develop.

Mutant clones of such strong hypomorphic alleles were examined in eye imaginal discs for furrow initiation and propagation defects and in adult eyes for phenotypes during subsequent development and differentiation. Clones that are located within the eye field proper do not cause discernible defects in furrow propagation nor any obvious phenotype in the adult eye (Fig. 4A and not shown). In rare cases, mostly in clones of the null alleles, single photoreceptor cells can be missing in individual ommatidia (not shown). The analysis of clones of *dpp* null alleles is consistent with this result: even large *dpp* clones encompassing up to a quarter of the eye do not show abnormalities in furrow progression or photoreceptor recruitment (V. W., unpublished).

In contrast, clones of the same *Mad* alleles that include the posterior margin of the eye disc completely abolish initiation of the furrow within the mutant tissue (Fig. 4). Interestingly, the progressing furrow in the neighboring wild-type tissue is unable to spread laterally into the clone. Clones located ventral to the posterior center of the eye field allow initiation of an independent furrow in the wild-type tissue on the ventral side of the clone (see Discussion). Depending on the size and position of a clone, the two furrows can fuse (Fig. 4A-C) or remain separated as two independent eye fields (Fig. 4D-F). In both cases, the mutant clones give rise to head cuticle in adult flies and are often associated with tissue overgrowth. Initiation of secondary furrows was not observed when a clone was positioned dorsally to the posterior center. Large *Mad* clones, which cover the region of the disc where the normal furrow would initiate, cause a complete failure of initiation at the posterior margin. Nevertheless, an ectopic furrow can initiate at the ventral margin in such cases (Fig. 4G). Both, ectopic and endogenous furrows always form their own equators, regardless of their position within the developing eye (Fig. 4H,I).

In summary, the clonal phenotypes of strong *Mad* alleles together with the disc phenotypes of weaker hypomorphs suggest that Dpp signaling via Mad is required for the initiation of the morphogenetic furrow and plays only a minor role in its subsequent propagation.

Internal furrow initiation mediated by *PKA* mutant clones is repressed by *Mad*

It has been shown that initiation of an ectopic furrow and photoreceptor differentiation can be induced anterior to the MF by ectopic expression of Hh or by clones mutant for *PKA* or *ptc* (Heberlein et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1995; Strutt et al., 1995; Wehrli and Tomlinson, 1995). In such ectopic furrows, *dpp* expression is detected first, followed by photoreceptor differentiation, similar to the sequence of events during initiation of the endogenous MF. Subsequent radial propagation of the ectopic MF is mediated by the same Hh-dependent mechanism as for the endogenous furrow. *PKA* or *ptc* mutant clones located at the posterior margin have no apparent phenotype. To analyze

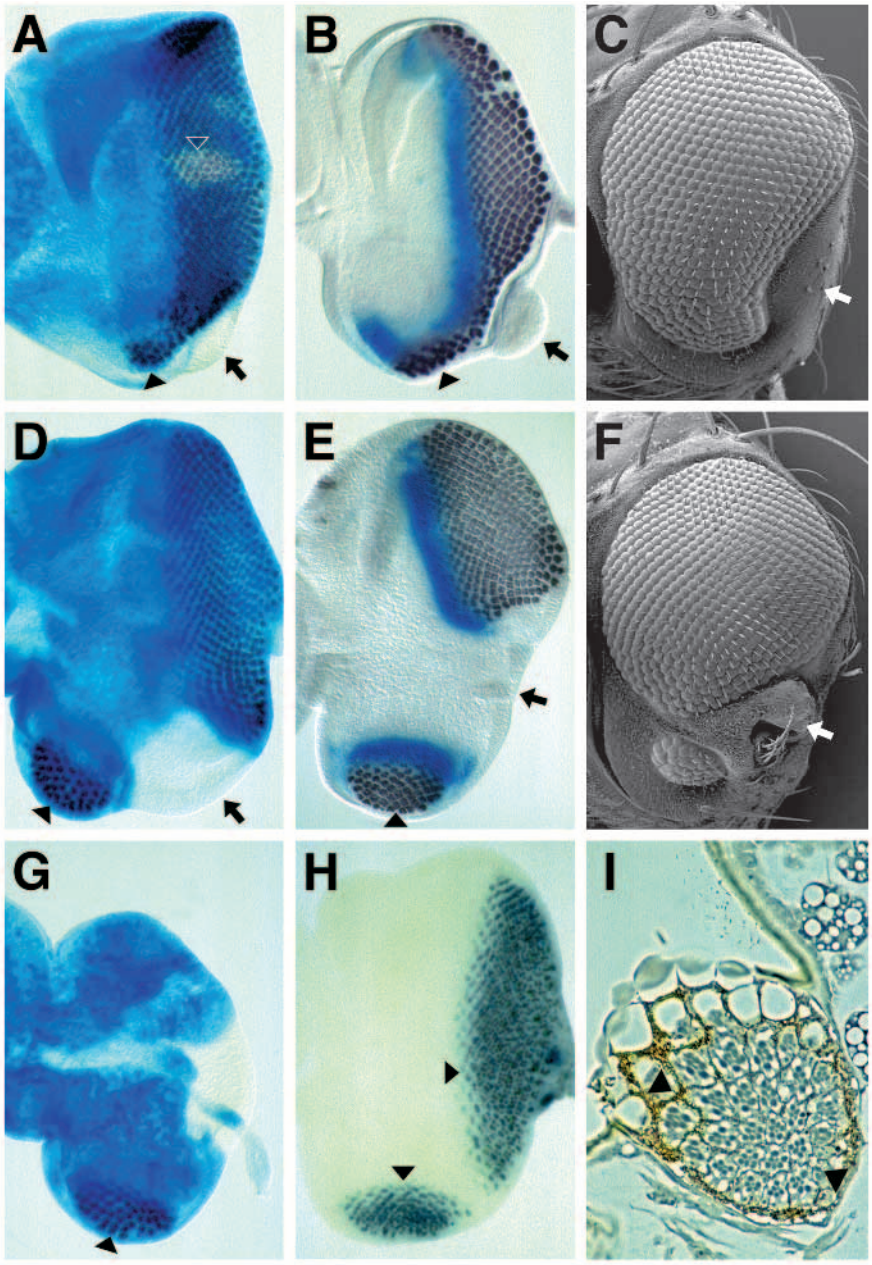

Fig. 4. *Mad* clones at the posterior eye margin cause failure of MF initiation and pattern duplications. Clones homozygous for Mad^{1-2} in 3rd instar eye discs are marked by the absence of blue staining of clonal marker *arm-lacZ* (A,D,G). Blue staining in B, E shows expression of the *dpp-lacZ* reporter gene in the MF. Photoreceptor differentiation is marked by Elav expression in black (A,B,D,E,G) or by anti-β-Gal staining of a *svp* enhancer trap (H). (A-F) Clones in the anterior region of the disc show no apparent defects in MF progression or ommatidial assembly (open arrowhead in A), clones at the posterior margin (arrows) do not participate in MF initiation and differentiation does not spread laterally into the mutant tissue (white in A,D). Instead, ectopic furrows initiate from the eye margin ventral to the clones (arrowheads in A,B,D,E). Depending on the size of the clone the two areas of differentiation fuse (A-C) or form separate eye fields (D-F). In the adult (C,F) the mutant tissue gives rise to head cuticle replacing part of the eye (white arrows). (G) Extreme case of a large clone (white) covering the posterior center of the eye disc. Note that although the MF cannot initiate from its normal position, an ectopic furrow is initiated at the ventral margin (arrowhead). (H) Separate areas of neuronal differentiation form their own equator of ommatidial rotation. Note that ommatidia turn in different directions on the left and right of the respective equators (arrowheads). (I) Tangential section through a 'secondary' adult eye induced by a neighboring *Mad* clone (compare F). Even small eye fields contain an equator (arrowheads) as visualized by the mirror image symmetry of ommatidia on either side of it.

the effects of *Mad* on internally initiating furrows, we induced clones doubly mutant for *PKA* and *Mad*.

At the posterior margin *Mad, PKA* double mutant clones show a very similar phenotype to *Mad* single mutant clones. They prevent furrow initiation in the mutant tissue and allow secondary furrows to initiate from the ventral margin. The only difference observed is that *Mad, PKA* clones are more frequently associated with overgrowth than single mutant *Mad* clones (Fig. 5A).

Mad, PKA double mutant clones located in more anterior positions show phenotypes characteristic of neither *PKA* nor *Mad* single mutant clones (Fig. 5B). Such clones are always associated with overgrowth (as are *PKA* single mutant clones), but unlike *PKA* clones they do not cause ectopic furrow initiation or photoreceptor differentiation. Ectopic *dpp* expression is initiated in these double mutant clones, but is not maintained and fades prematurely (Fig. 5C,D). In contrast to *Mad* single mutant clones, which appear wild-type, the stripe of *dpp* expression associated with the endogenous MF never enters the *Mad, PKA* mutant tissue and neuronal differentiation is excluded from these clones (Fig. 5D). As a consequence, *Mad, PKA* double mutant clones give rise to head cuticle in adult flies. Taken together with the phenotypes of the *Mad* clones at the posterior margin, this indicates that *Mad*-mediated *dpp* signaling is absolutely required for the initiation of a morphogenetic furrow anywhere in the eye.

Wingless is ectopically expressed in *Mad* mutant tissue

During imaginal disc development, *dpp* signaling often acts in concert with *wingless* (*wg*), a prototype of the Wnt family of secreted growth factors. For example, in leg discs, *dpp* is

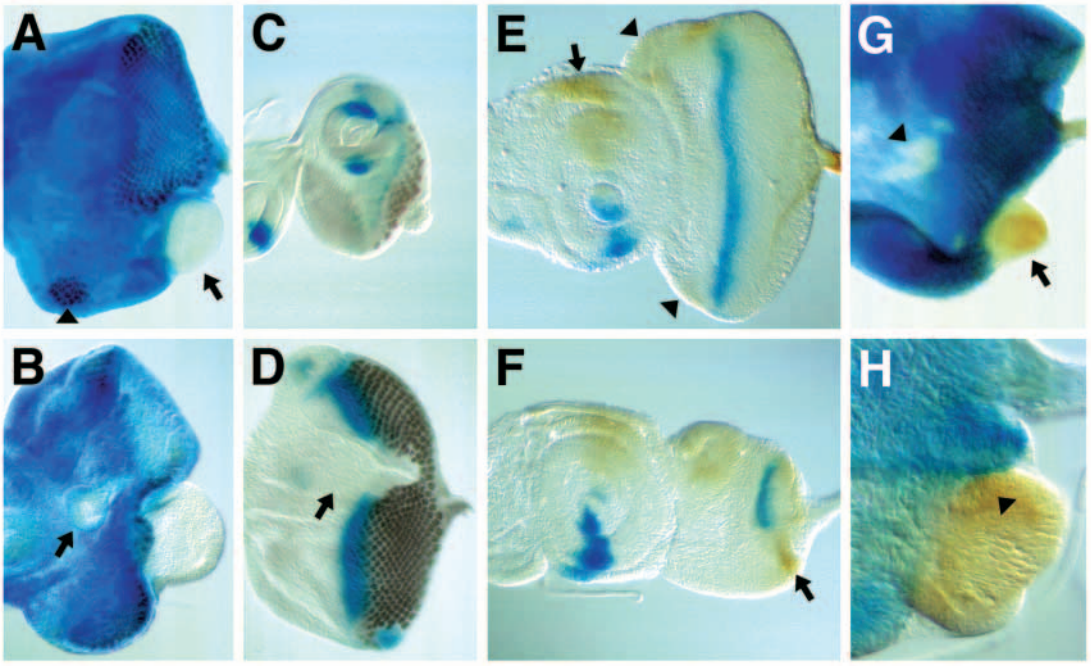

Fig. 5. Wingless expression is induced by lack of Dpp signaling. (A-D) Clones mutant for both *Mad* and *PKA* are excluded from neuronal differentiation. Mad^{1-2}, $pka^{89/18}$ clones are marked by absence of *arm-lacZ* staining (white in A,B). Anti-Elav antibody staining visualizes photoreceptor differentiation in black (A,B) or brown (C,D); *dpp-lacZ* reporter gene expression is shown in blue in panels C and D. (A) Posterior *Mad, PKA* clones (arrow) induce ectopic MF initiation (arrowhead) at the ventral margin, but generally display more overgrowth (compare to Fig. 4A,D). (B) Disc distorted by the overgrowth caused by multiple *Mad, PKA* clones. Note bulging of clone tissue in the center of the disc (arrow). (C) Early 3rd instar eye disc stained for *dpp-lacZ* expression. Local overgrowth in the anterior region indicates the presence of a *Mad, PKA* clone (compare B). Note ectopic *dpp-lacZ* expression in the overgrown area. (D) *Mad, PKA* clone in mid-third instar disc. Position of the clone is inferred from associated overgrowth (arrow) and the absence of neuronal differentiation, in analogy to clones marked by absence of *arm-lacZ* staining (panels A, B, and not shown). Note that neither the MF nor photoreceptor differentiation enter the clone from posterior. (E, F) Antibody staining of Wingless protein (yellow) in wild-type and dpp^{blk} eye discs; *dpp-lacZ* expression is shown in blue. (E) Wild-type eye/antennal imaginal disc complex showing Wg expression in the antennal disc (arrow) and in two separate areas of the anterior eye margin (arrowheads). Note more intense staining in the dorsal region. Depending on fixation conditions staining can also be observed in the photoreceptor cell axons leaving the disc posteriorly. (F) dpp^{blk} imaginal disc complex. MF progression as visualized by *dpp-lacZ* is restricted to the dorsal region of the eye disc. Note high levels of ectopic Wg expression that mark the ventral area fated to become head cuticle (arrow). (G) Mad^{1-2} clones marked by absence of clonal marker *arm-lacZ* (blue). Wg expression (yellow) can be seen in the overgrown clone at the posterior eye margin (arrow), but not in a clone of similar size in the center of the disc (arrowhead). (H) Detail of a posterior Mad^{1-2}, $PKA^{89/18}$ clone showing high levels of Wg protein and associated overgrowth. Note staining of apical vesicles inside cells of the clone (arrowhead).

induced dorsally and *wg* ventrally at the anterior border of the A/P boundary, where they synergistically activate downstream target genes. In the eye disc, *wg* expression (Fig. 5E) is associated with head cuticle development, and is required at the dorsal and ventral edges of the eye to repress premature furrow initiation from the lateral margins by antagonizing Dpp activity (Ma and Moses, 1995; Treisman and Rubin, 1995). Moreover, ectopic activation of the *wg* signaling pathway within the eye field blocks MF progression and causes a transformation of eye tissue to head cuticle (Treisman and Rubin, 1995). This effect is very similar to the phenotypes seen in posterior *Mad* clones and *PKA, Mad* double mutant clones. We therefore tested if Wg is involved in the generation of this phenotype.

Mad clones were found to express Wg protein in third instar imaginal discs. Similarly Wg is detected in *PKA, Mad* double mutant clones (Fig. 5G,H). Since *PKA* clones lead to *dpp* expression in the eye (Pan and Rubin, 1995; Strutt et al., 1995), ectopic production of Wg alone in the double mutant clones must be a consequence of the lack of *Mad* function. Interestingly, single mutant clones of strong *Mad* alleles lead to ectopic *wg* expression more frequently than weaker alleles, suggesting that weak alleles retain sufficient *Mad* activity to repress *wg* most of the time. Wg-expressing clones are always associated with tissue overgrowth. The requirement for functional *dpp* signaling in patterning *wg* expression is also evident in dpp^{blk} eye discs. Here, the ventral region of the disc that does not participate in eye tissue differentiation and forms head cuticle instead is marked by Wg (Fig. 5F).

In summary, these data demonstrate an antagonism between *dpp* and *wg* at the level of expression. Mad-mediated *dpp* signaling is not only required for the maintenance of *dpp* transcription, but also for repression of *wg* expression.

DISCUSSION

Mad acts downstream of Dpp receptors in the *dpp* signaling pathway

Mad, originally isolated as a maternal enhancer of specific *dpp* alleles, interacts with several *dpp*-associated phenotypes, suggesting that it could be a common component of Dpp signaling (Raftery et al., 1995; Sekelsky et al., 1995). We have identified *Mad* as a dominant enhancer of a morphogenetic furrow phenotype and show that Mad is a downstream component of the Dpp pathway. Firstly, overexpression of Mad partially rescues the eye phenotype of dpp^{blk}. Secondly, Mad appears to be a limiting factor downstream of Thick veins (Tkv), the Dpp type 1 receptor, as a strong loss-of-function *Mad* allele domi-

nantly suppresses an eye-specific activated Tkv phenotype. The analysis of *Mad* in different genetic backgrounds during Dpp-induced midgut morphogenesis in the embryo also places it downstream of *dpp* (Newfeld et al., 1996) and thus supports our results. Moreover, Mad appears to be a ubiquitously expressed cytoplasmic protein (Sekelsky et al., 1995; Newfeld et al., 1996), which is consistent with our genetic experiments placing it downstream of the receptors.

Several lines of evidence suggest that *Mad* is a conserved component of Dpp signaling and members of the TGF-β superfamily in general. Besides the similarity of *Mad* and *dpp* phenotypes, and their genetic interactions in all tissues analyzed (Raftery et al., 1995; Sekelsky et al., 1995, Newfeld et al., 1996, and this study), *Mad* homologues have been found in several invertebrates and vertebrates, such as *C. elegans*, *Xenopus* and humans (Sekelsky et al., 1995; Hahn et al., 1996; Savage et al., 1996; Newfeld et al., 1996). Furthermore, *Drosophila* Mad functions similarly to the *Xenopus* homologues when assayed in *Xenopus* for mesoderm induction (Newfeld et al., 1996). Taken together with our demonstration that Mad acts downstream of the receptors, this argues that Mad (and related proteins) constitute a conserved component of TGF-β signaling. The presence of multiple Mad homologues in *C. elegans* and *Xenopus* suggests that different TGF-β-like molecules in one animal might signal through different members of this protein family (Savage et al., 1996; Newfeld et al., 1996).

Mad and Dpp signaling are required for furrow initiation

The movement of the furrow during eye development can be divided into two stages: initiation at the posterior margin and progression. Expression of *dpp* and its dependence on Hh during progression have led to the proposal that *dpp* is involved in both processes (reviewed in Heberlein and Moses, 1995). Nevertheless, the analysis of *dpp* mutant clonal tissue does not conclusively support this idea. Mutant clones of strong $dpp^{hin\text{-}r}$ alleles very rarely showed posterior indentations of the eye, but no apparent phenotype was associated with anterior clones (Heberlein et al., 1993), which was consistent with a non-autonomous function for Dpp. The lack of defects was attributed to rescue of the mutant tissue by Dpp diffusing into the clone from neighboring wild-type tissue.

The analysis of a downstream component, *Mad*, that is required autonomously in responding cells allowed us to directly address the role of *dpp* signaling during different stages of furrow progression. Clones located within the eye field do not affect MF progression and have essentially a wild-type appearance, with only minor defects in few ommatidia. In contrast, *Mad* clones at the posterior edge of the eye field do not participate in furrow initiation and develop as head cuticle. Similarly, *Mad* is required for the initiation of ectopic furrows induced by loss of *PKA* function in the anterior region of the eye disc (Pan and Rubin, 1995; Strutt et al., 1995). In *Mad, PKA* double mutant clones, initial *dpp* expression fails to induce ectopic neuronal differentiation and an associated furrow. Instead anterior *Mad, PKA* clones give rise to adult head cuticle (see below). Both, *Mad* and *Mad, PKA* mutant clones indicate that Mad and Dpp are essential for initiation of the morphogenetic furrow. However, the lack of a phenotype of anterior *Mad* clones suggests that *dpp* signaling plays only a minor role during subsequent propagation of the morphogenetic furrow. Due to the use of hypomorphic alleles we cannot exclude a reduced requirement for Dpp signaling in the propagation.

In accordance with these results, the *GMR-*tkv^{Q253D} transgene, which expresses the activated Tkv protein in all cells in and posterior to the MF, has a much stronger phenotypic effect in the posterior part of the disc where the furrow initiates. Overactivation of the pathway elicited by the constitutively activated Tkv receptor leads to ommatidia with too many photoreceptors, consistent with a positive role of *dpp* signaling during photoreceptor induction.

The observation that *dpp* is not expressed in *Mad* eye margin clones and *Mad* hypomorphs raises the intriguing possibility that Dpp signaling is required for maintenance of *dpp* expression. In support of this, we observe that a mutation in *dpp* itself (dpp^{blk}) reduces expression of a *dpp-lacZ* reporter gene in the eye margin. Moreover, *dpp* expression is not maintained in *Mad, PKA* double mutant clones. We propose that Dpp and its signaling pathway (including Mad) are involved in a positive autoregulatory loop for *dpp* expression (Fig. 6). The lack of maintenance of *dpp* expression is accompanied by a change in fate of the mutant tissue, which loses its ability to respond to the differentiation signals associated with the endogenous furrow and is therefore excluded from photoreceptor differentiation.

The *dachshund* (*dac*) gene was previously proposed to be a specific factor required for furrow initiation (Mardon et al., 1994). The eye phenotype of *dac*, failure of MF initiation but only minor MF progression and differentiation defects, is very similar to the *Mad* phenotype. The only apparent difference between the *Mad* and *dac* clonal phenotypes in the eye relates to *dpp* expression, which at the posterior margin is maintained in *dac*, but not *Mad*, clones. By analogy to *Mad*, the *dac* phenotypes could be reinterpreted as related to *dpp* signaling. Thus, Dac might be a downstream effector of Dpp (and possibly Mad) during MF initiation, but not involved in the autoregulatory loop leading to persistent *dpp* expression (Fig. 6). Alternatively, *dac* could function in a parallel, *dpp*-independent pathway required for MF initiation.

Furrow initiation and patterning of the eye disc

The initiation of ventral ectopic furrows associated with posterior *Mad* clones indicates that the entire *dpp*-expressing eye margin is capable of initiating a furrow. However, the

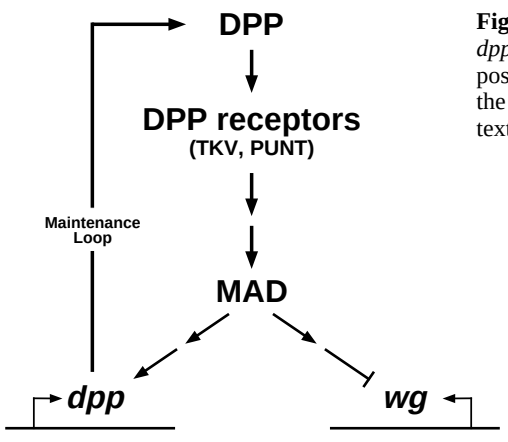

Fig. 6. Model for *dpp* signaling at the posterior margin of the eye disc. See text for details.

repressive effect that Wg has on furrow initiation at the dorsal edge of the eye disc (Ma and Moses, 1995; Treisman and Rubin, 1995) seems to limit ectopic furrows to the ventral domain of the disc. As is the case for the normal furrow, they always initiate with a small number of ommatidia and define their own equator around which ommatidia organize themselves (Chanut and Heberlein, 1995; Strutt and Mlodzik, 1995). Although there might be a signal that triggers regular initiation specifically at the posterior center of the eye disc in wild type, our data suggest that coordinated initiation can take place independently of such a signal. It is conceivable that local interactions between *dpp*-expressing cells in the eye margin are responsible for the selection of the initiation point. *Mad* mutant clones might interfere with this process and therefore create two isolated areas of the margin that each form their own center of initiation. Alternatively, *Mad* clones could affect an underlying global pattern in the eye disc established by a mechanism relying on diffusible signals (such as Wingless, see below); formation of ectopic furrows could therefore be an indirect consequence of this repatterning.

Dpp signaling represses *wingless* expression

Our analysis of *Mad* and *dpp* eye phenotypes reveals that *dpp* signaling is necessary to repress *wingless* expression in the eye disc. Ectopic Wg can be detected in *Mad* clones as well as *Mad*, *PKA* double mutant clones. This effect is more frequently observed with stronger *Mad* alleles, suggesting that partial activity of the signaling pathway is usually enough to repress *wg*. The Wg-expressing tissue forms head cuticle and is generally significantly overgrown in comparison to neighboring wild-type areas. Both these effects could be mediated by Wg.

Our observation that Wg protein is also ectopically expressed in dpp^{blk} discs is in contrast to the reported lack of ectopic expression of a *wg-lacZ* reporter (Treisman and Rubin, 1995). Interestingly, in dpp^{blk} discs, Wg is detected in an arc-like pattern, ventrally adjacent to the reduced MF and thus possibly induced by *hh* expression posterior to the furrow. Consistent with this, *wg* alleles are dominant suppressors of the dpp^{blk} phenotype (Treisman and Rubin, 1995). In eye discs mutant for a hypomorphic *dac* allele, initiation of the MF is slowed down in the lateral regions of the margin, giving rise to a curved furrow (Mardon et al., 1994). It is remarkable that this initiation defect is accompanied by ectopic *wg* expression at the eye margin (Treisman and Rubin, 1995). Taken together with the similarity of the phenotypes of *Mad* and *dac* mutant clones, this substantiates our hypothesis that both Mad and Dac are required in the *dpp*-signaling pathway, leading to repression of *wg* in the eye disc (Fig. 6).

Ectopic expression of Wg can also explain the failure of the MF to expand laterally into tissue with reduced levels of *dpp* signaling. Wg has been demonstrated to counteract both MF initiation and progression (Treisman and Rubin, 1995). It is therefore conceivable that localized ectopic Wg expression in posterior *Mad* clones and hypomorphic *Mad* or dpp^{blk} discs renders these areas non-responsive to MF progression signals.

In addition, we often observed bifurcations of antennae associated with *Mad* clones and *PKA*, *Mad* double mutant clones. Such clones express Wg and are overgrown when located in the dorsal region of the antennal disc (our unpublished results). Thus, the antagonistic effect of *dpp* signaling on *wg* expression is also observed in other discs and might be a general mechanism during *Drosophila* imaginal development.

In summary, we present evidence that Mad acts downstream of the Dpp receptors and is required in an autoregulatory loop that maintains *dpp* expression (Fig. 6). Failure to maintain *dpp* expression through *Mad* signaling leads to ectopic Wg expression, suggesting a reciprocal relationship between the expression and function of these two growth factor like molecules.

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