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# The *Drosophila* gene *Medea* demonstrates the requirement for different classes of Smads in *dpp* signaling

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## SUMMARY

**Signals from transforming growth factor-β (TGF-β) ligands are transmitted within the cell by members of the Smad family, which can be grouped into three classes based on sequence similarities. Our previous identification of both class I and II Smads functioning in a single pathway in *C. elegans*, raised the issue of whether the requirement for Smads derived from different classes is a general feature of TGF-β signaling. We report here the identification of a new *Drosophila* class II Smad, *Medea*, a close homolog of the human tumor-suppressor gene *DPC4*. Embryos from germline clones of both *Medea* and *Mad* (a class I Smad) are ventralized, as are embryos null for the TGF-β-like ligand *decapentaplegic* (*dpp*). Loss of *Medea* also blocks *dpp* signaling during later development, suggesting that *Medea*, like *Mad*, is universally required for *dpp* signaling. Furthermore, we show that the necessity for these two closely related, non-redundant Smads, is due to their different signaling properties – upon activation of the Dpp pathway, Mad is required to actively translocate Medea into the nucleus. These results provide a paradigm for, and distinguish between, the requirement for class I and II Smads in Dpp/BMP signaling.**

Key words: TGF-β, Smad, *Mad*, BMP, Pattern formation, *Drosophila*, *Medea*

## INTRODUCTION

Members of the transforming growth factor-β (TGF-β; Roberts et al., 1981) superfamily have important roles in the development of multicellular animals, including the growth regulation of tissues and various patterning events (reviewed in Massagué et al., 1992; Kingsley, 1994; Hogan, 1996). The first invertebrate member of the TGF-β superfamily to be identified was the *Drosophila* gene *deceapentaplegic* (*dpp*), of the Bone Morphogenetic Protein (BMP) family (Padgett et al., 1987; Wozney et al., 1988). *dpp* plays many key roles in *Drosophila* development, including determination of the embryonic dorsal-ventral axis (Irish and Gelbart, 1987), larval morphogenesis (Segal and Gelbart, 1985), and imaginal disk growth and patterning (Spencer et al., 1982; Segal and Gelbart, 1985).

In recent years, the cell surface receptors responsible for binding TGF-β-like ligands have been identified and intensively studied (reviewed in Kingsley, 1994; Massagué, 1996). These receptors fall into two related classes of serine/threonine kinases called type I and type II receptors. During signaling, the ligand first binds constitutively phosphorylated type II receptor, which then recruits the type I receptor and phosphorylates it, which in turn transduces the signal intracellularly (Wrana et al., 1994). The *dpp*/BMP ligands bind their type I and II receptors simultaneously, rather than sequentially. Receptors for Dpp, namely Saxophone (Sax) and Thick veins (Tkv; type I receptors), and Punt (type II receptor) have been identified and shown to be required for *dpp* activity (Affolter et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995).

The identification of *dpp* as a functionally conserved member of the BMP subfamily (Padgett et al., 1987, 1993) enabled the genetic study of TGF-β signaling in *Drosophila*. Genetic screens for modifiers of *dpp* phenotypes have resulted in the identification of the genes *Mothers against dpp* (*Mad*) and *Medea* (Raftery et al., 1995; Sekelsky et al., 1995). The cloning of *Mad* and the homologous *C. elegans* genes, *sma-2*, *sma-3* and *sma-4*, and the characterization of their roles in TGF-β-like pathways (Sekelsky et al., 1995; Savage et al., 1996), identified these genes, referred to as the Smad family (for *sma* and *Mad*; Derynck et al., 1996), as conserved transducers of TGF-β-like signals. In vertebrates, the functional requirement for these genes was first shown for human *DPC4* (also referred to as *Smad4*). Loss of heterozygosity of this locus is highly correlated with the development of pancreatic carcinoma (Hahn et al., 1996).

Smad family members are characterized by two highly conserved domains (N-terminal MH1 and C-terminal MH2),

separated by a linker region of variable sequence. Based on sequence similarities, the family can be divided into three highly related groups (reviewed in Padgett et al., 1998). The class I Smads are similar to *Drosophila* Mad, the class II Smads comprise *C. elegans* SMA-4 and vertebrate Smad4, while the class III Smads comprise *Drosophila* Dad (Daughters against dpp; Tsuneizumi et al., 1997), and vertebrate Smad6 and Smad7 (Hayashi et al., 1997; Inamura et al., 1997; Nakao et al., 1997).

Upon activation of the receptor complex by the ligand, the class I Smads become phosphorylated (Hoodless et al., 1996; Liu et al., 1996; Yingling et al., 1996) directly by the type I receptor (Macias-Silva et al., 1996; Zhang et al., 1996; Kretzschmar et al., 1997). They then translocate to the nucleus (Hoodless et al., 1996; Liu et al., 1996; Maduzia and Padgett, 1997; Newfeld et al., 1997), where they can bind DNA (Kim et al., 1997) and activate target gene expression in conjunction with specific transcription factors (Chen et al., 1996). Recently, the class III Smads were shown to antagonize signaling (Hayashi et al., 1997; Inamura et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997) by competing with the class I Smads for receptor binding (Hayashi et al., 1997; Inamura et al., 1997; Nakao et al., 1997).

We have previously demonstrated that mutations in any one of the three *C. elegans* genes, *sma-2* and *sma-3* (both class I), and *sma-4* (class II), result in small body size and male-tail abnormalities, indicating that they act non-redundantly in signaling (Savage et al., 1996). This conclusion has been supported for the vertebrate Smads in cell culture studies, where it was shown that, for example, Smad3 could synergize with Smad4 to effect the TGF-β signal (Zhang et al., 1996). It has also been suggested that Smad4 is a common effector of signaling, based upon its ability to act with either Smad1 to effect the BMP signal (Lagna et al., 1996), Smad2 to effect the activin signal (Lagna et al., 1996), or Smad3 for the TGF-β response (Zhang et al., 1996). While these systems indicate synergy between the Smad classes in specific signaling contexts, they do not, however, address the issue of whether this synergy represents a universal requirement for class I and II Smads in all developmental contexts.

In this study, we report the identification of a new *Drosophila* class II Smad. This Smad is encoded by the gene *Medea* (Raftery et al., 1995), and is more closely related to the human tumor-suppressor Smad4 (Hahn et al., 1996) and *C. elegans* SMA-4 (Savage et al., 1996) than to any other Smad. The examination of the role of this novel Smad in *Drosophila* development indicates that this gene is universally required for *dpp* signaling. We present evidence indicating that *Medea* and *Mad* (Sekelsky et al., 1995) function non-redundantly in *dpp* signaling. While these two Smads are phenotypically indistinguishable, in cell culture, they behave differently in response to stimulation of the pathway. Furthermore, we show that, upon signaling, Mad is required to actively translocate Medea to the nucleus.

## MATERIALS AND METHODS

### *Drosophila* methods

*Medea* alleles were identified via non-complementation of the $Med^1$ allele. *st e* males were mutagenized using 50 mM EMS, and crossed to balancer females in bottles. 10,000 *st e*/ TM3, *Sb* male offspring were crossed singly in individual vials to multiple $Med^1$/ TM3, *Sb Ser* females. Vials lacking *Sb*+ progeny were retested against a *Medea* deficiency, Df(3R)E40.

The single amino acid substitution in *saxophone* (Q263D) and *thick veins* (Q199D) were made using PCR, and then verified by sequencing. Transgenic lines of activated receptors and *Ubi-Medea* were generated in a *yw* background using standard protocols.

*w*, P{FRT}82B, P{*w*+}90E was used to generate recombinant FRT, *Medea* flies for clonal analysis. *w*; P{*w*+}30C P{FRT}40A was used to generate recombinant FRT, *Mad* lines. FRT recombinants were made as described (Xu and Rubin, 1993). *w*; P{FRT}82B P{*ovoD1*}3R1 P{*ovoD1*}3R2 was used to generate germline clones of *Medea*. *w*; P{FRT}40A P{*ovoD1*}2L1 P{*ovoD1*}2L2 was used to generate *Mad* germline clones. *y w* $hsFLP^{12}$ line was used to provide heat shock. All FRT and FLP stocks were kindly provided by the Bloomington stock center.

### PCR, DNA isolation and sequencing

PCR was performed using degenerate primers (described in Savage et al., 1996). Of over 50 clones sequenced, *Mad* and *Medea* were recovered in roughly equal proportions. Primers containing a *Sfi*I site were designed against the 172 bp *Medea* fragment isolated by degenerate PCR and inverse PCR performed on Nick Brown's *Drosophila* 4-8 hour library (Brown and Kafatos, 1988) to isolate a full-length cDNA. The product was restriction digested with *Sfi*I, self-ligated, and transformed into bacteria. The largest clone (3.7 kb) obtained was used as a probe to isolate other full-length cDNAs from the E library (Poole et al., 1985). After sequencing, the largest open reading frame was chosen as the deduced primary sequence. While none of the putative start sites have a good *Drosophila* consensus initiation sequence, Met27 (Fig. 1A) provides the best match with Smad4.

For the $Med^1$ allele, the mutation was identified by first constructing a genomic phage library from *e* $Med^1$ */ TM3, Sb* flies and from control flies carrying the same balancer. Several phage positive for *Medea* were isolated, and their ends sequenced to identify polymorphisms that could distinguish between the balancer and mutant chromosomes. One clone of each kind was then sequenced with primers designed to the *Medea* cDNA.

For the other alleles ($Med^{19}$, $Med^{21}$, $Med^{23}$ and $Med^{26}$), the entire genomic region of *Medea* was amplified by PCR. Sequence polymorphisms were used to distinguish mutant clones from balancer clones. $Med^{25}$, a strong hypomorph (data not shown), was not sequenced. DNA preparation, PCR, cloning and sequencing were done as described (Xie et al., 1994).

All sequence analyses were performed using the Genetics Computer Group (GCG) Program. Pileup was performed using full-length sequences, distances plotted using the Jules-Kimura algorithm and the dendogram made using the Growtrees function. Boxes were drawn using the MacBoxshade program.

### Somatic and germline clones

Clones were made using the FLP/FRT system (Xu and Rubin, 1993). Somatic clones were generated by crossing the recombinant FRT, *Medea* lines into the FRT, *w*+ line. The FLP enzyme was provided from a *yw* $hsFLP^{12}$ line. Clones in the eye were induced by subjecting first- or second-instar larvae to a heat shock of 37°C for 90-120 minutes. Clones were marked by the absence of the *white* gene.

A P{$ovo^{D1}$} insertion on the second or third chromosome (Chou et al., 1993) was used to make germline clones of *Mad* and *Medea*, respectively. Late larvae or early pupae were subjected to γ-irradiation from a $^{137}$Ce source for doses ranging from 1000 rads to 2000 rads. Female progeny (>300) of the genotype $Med^1/ovo^{D1}$ were crossed to $Med^{25}$/TM6B*Tb* males (both strong alleles) and $Mad^{12}/ovo^{D1}$ females were crossed to $Mad^{10}/Gla$ males. Vials were incubated at 25°C and frequently checked for the presence of dead eggs. Cuticle preparations were performed using standard protocols. Using γ-irradiation, 10-15%

of females were fertile ($Med^1/Med^1$), while the FLP/FRT system gave a 60-70% fertility rate ($Mad^{12}/Mad^{12}$).

### Scanning electron microscopy

Heads of flies with eye clones were detached without fixing and mounted for scanning electron microscopy; the heads remained intact for 30 to 45 minutes in vacuum without collapsing.

### *Drosophila* cell line transfections

Epitope-tagged versions of *Mad* and *Medea* were cloned into the plasmid vector pMK33. An HA tag was fused to the C terminus of the predicted open reading frame of Medea and a Flag tag was fused to the N terminus of Mad. Clones of *dpp*, *punt*, *tkv* and activated *tkv* (*tkv**) were also generated in pMK33.

All studies were carried out in *Drosophila* S2 cells. The cells were cultured in Schneider's Insect Medium (Sigma) with 12.5% Fetal Calf Serum (Gemini Bio-Products). Transfections were carried out as previously described (Maduzia and Padgett, 1997). 24 hours after transfection, expression of the various constructs from the metallothionein promoter was induced by the addition of $CuSO_4$ to a final concentration of 0.7 mM. Cells were harvested 10-12 hours later and assayed for immunofluorescence.

## RESULTS

### Identification of *Medea* as a new *Drosophila* Smad

The identification of multiple Smad members in both *C. elegans* and vertebrates (Savage et al., 1996) argued for the existence of multiple Smads in *Drosophila* as well. To identify additional Smads, we used degenerate primers for PCR on a *Drosophila* cDNA library and identified two species of clones. One was *Mad* (Sekelsky et al., 1995) and the other was a novel member of the Smad family. A full-length cDNA was isolated and found to encode a protein with a deduced primary sequence of 771 amino acids (Fig. 1A). While sharing homology with all other members of the family, this Smad contained motifs more related to *C. elegans* SMA-4 (Savage et al., 1996) and human Smad4 (Hahn et al., 1996) than to any of the other Smads (Fig. 1B). Smad4 and this new *Drosophila* Smad are over 80% identical in the two domains of approximately 350 residues that define members of the Smad family.

Since *Mad* and *Medea* were the only novel loci that had been reported from screens for dominant modifiers of *dpp* phenotypes (Raftery et al., 1995; Sekelsky et al., 1995), and since *Mad* had previously been identified as a class I Smad (Sekelsky et al., 1995), we hypothesized that our transcript might represent *Medea*. To test this idea, we performed PCR against a series of Yeast Artificial Chromosomes (YACs) from the region to which *Medea* had been genetically mapped (Raftery et al., 1995) and localized our transcript to the YAC R16-72, which covers the region from 100C4-5 to 100D3-4 on the right arm of chromosome III.

To determine whether this Smad is encoded by *Medea*, we tested the ability of this transcript to rescue *Medea* lethality. Transgenic expression of our new Smad under the control of a *Ubiquitin* promoter (Brummel et al., 1994) rescued *Medea* lethality to significant levels (50-90% for various *trans*-allelic combinations, compared to 0% in the absence of the transgene), proving that the new Smad transcript corresponds to the genetic locus of *Medea*.

### Lesions in the *Medea* transcript

To further demonstrate that we had cloned *Medea*, we sequenced mutant *Medea* alleles. Novel alleles were obtained by performing a non-complementation screen (see methods) with the $Med^1$ allele (Shearn and Garen, 1974; Raftery et al., 1995). The alleles were confirmed by several criteria, including the failure to complement the *Medea* deficiency Df(3R)E40 (Raftery et al., 1995). All five of the mutations that we have identified map to the coding region of the new Smad transcript (Fig. 1A).

While two of the five mutations, Gln283Ter ($Med^1$) and Gln457Ter ($Med^{23}$), predict truncated versions of Medea, the other three mutations that we have identified, Gly609Ala ($Med^{21}$), Gly609Ser ($Med^{26}$) and Asp712Ala ($Med^{19}$), result in amino acid substitutions in MH2. Recently, the crystal structure of the C-terminal domain of Smad4 was solved and reported as a crystallographic trimer (Shi et al., 1997). We have mapped our mis-sense mutations on this structure (Fig. 1C). Gly609Ala ($Med^{21}$) and Gly609Ser ($Med^{26}$) map to an invariant residue in the three-loop/helix region (Shi et al., 1997), and may disrupt the core structure of the protein. Asp712Ala ($Med^{19}$) maps to an exposed, class II-specific residue, and possibly disrupts the trimer-interface. The identical amino acid (Asp493) in Smad4 is found mutated in pancreatic carcinoma (Hahn et al., 1996).

### Germline clones of *Medea* and *Mad* yield ventralized embryos

In order to assess the requirements of *Medea* for *dpp* signaling, we analyzed *Medea* mutant phenotypes during both embryonic and imaginal development.

*Medea* was identified as a maternal effect enhancer of *dpp* (Fig. 4A; Raftery et al., 1995), indicating that *Medea* mRNA is maternally deposited into the embryo . Hence wild-type *Medea* product is present even in genetically (or zygotically) null mutant embryos and, as a consequence, embryonic phenotypes may not be as severe as embryos that completely lack *Medea*. In fact, *Medea* homozygotes die as pupae (data not shown). To examine the phenotype resulting from the complete loss of both the maternal and zygotic components of *Medea*, we generated germline mosaics of this gene using a dominant female-sterile system (see methods; Chou et al., 1993). When eggs derived from germline clones of $Med^1$ are fertilized by sperm carrying the allele $Med^{25}$ (both strong alleles), the resultant embryos are devoid of any wild-type *Medea* product ($Med^-$ embryos). Cuticular preparations of these $Med^-$ embryos (Fig. 2C) revealed phenotypes identical to those of $dpp^-$ embryos (Fig. 2B; Irish and Gelbart, 1987). Ventrolateral structures, such as the denticle bands, had expanded into dorsal regions and severe head defects were observed. This phenotype was partially rescued when a wild-type copy of *Medea* was introduced from the father, into the maternally null embryo (data not shown). Such embryos were observed as having less severe ventralization phenotypes and reduced head defects (data not shown).

To examine the functional overlap between *Mad* and *Medea* during early embryonic development, we also generated germline clones of *Mad*. Embryos lacking both maternal and zygotic *Mad* (Fig. 2D) exhibit the same ventralized phenotype that $Med^-$ and $dpp^-$ embryos do. This phenotype can also be

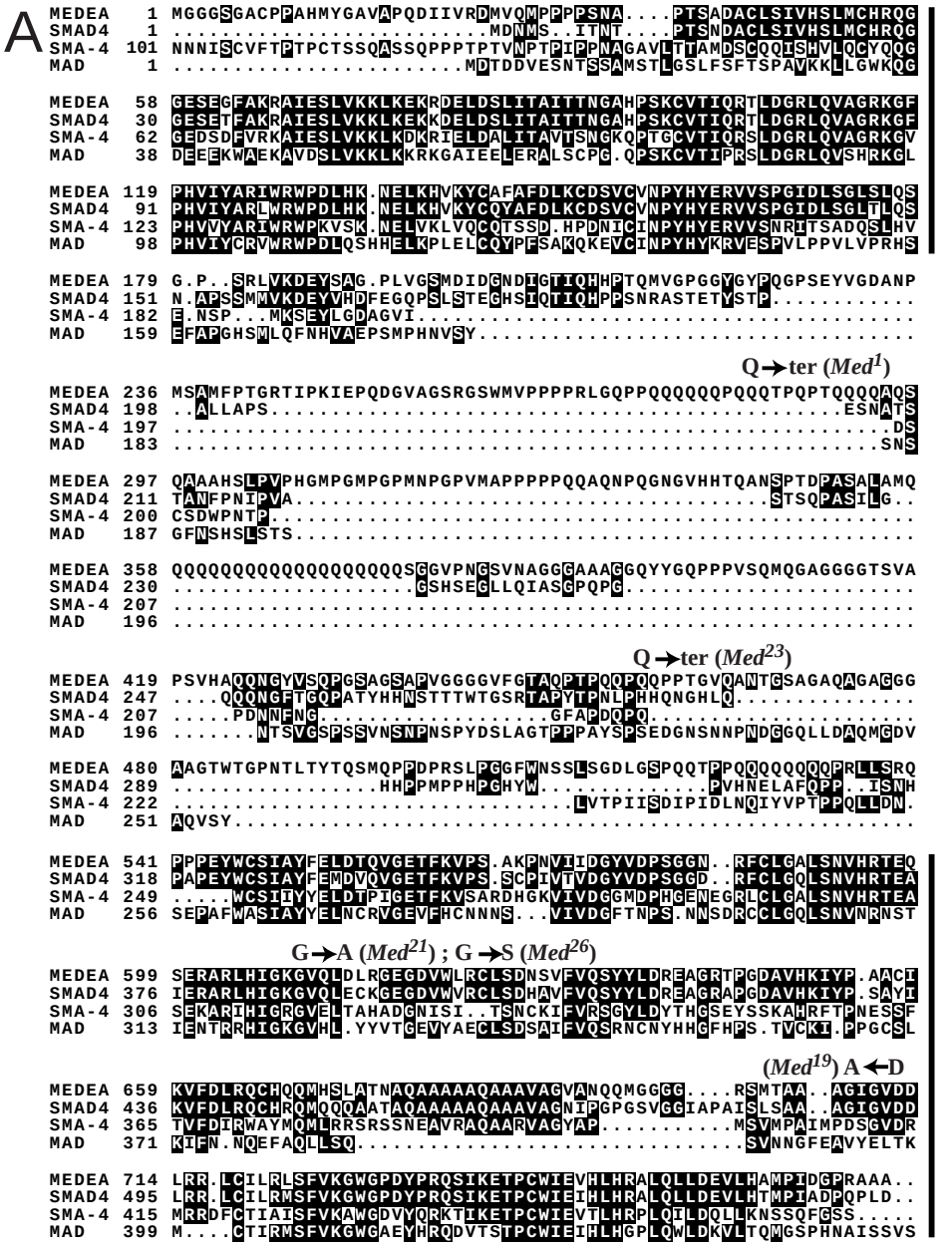
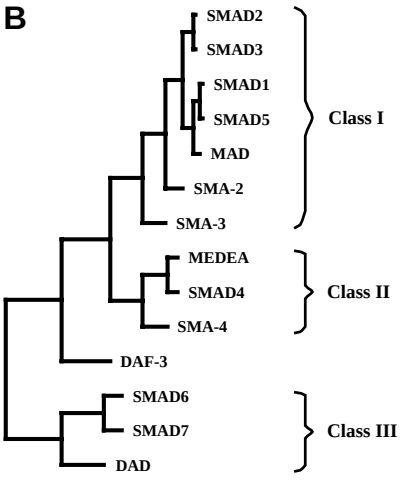
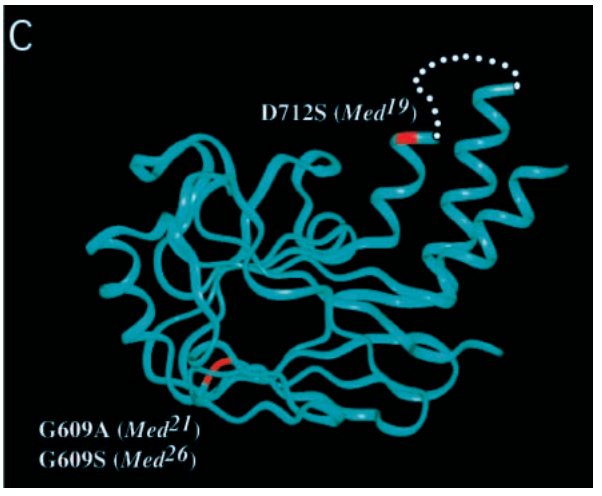



**Fig. 1**. *Medea* encodes for the *Drosophila* homolog of *Smad4* (*DPC4*). (A) Protein sequence alignment of the deduced primary sequence of Medea (Accession number AF041439), vertebrate Smad4, *C. elegans* SMA-4 (all class II Smads), and *Drosophila* Mad, a class I Smad. Dots indicate gaps introduced to maximize alignments. Highlights indicate identical residues in any two sequences. The black bars on the right mark MH1 (upper bar) and MH2 (lower bar). Mutations in five *Medea* alleles are shown above the corresponding residues. (B) Dendrogram showing the relationship between members of the Smad family. The three Smad classes have unique sequence motifs as well as functions. *C. elegans* DAF-3 is equally related to the class I and class II Smads, yet functions negatively, like the class III Smads (Patterson et al., 1997). (C) Mapping of Medea mutations on the crystal structure of Smad4 MH2 (91% identity). Gly 609 is mutant in two alleles.



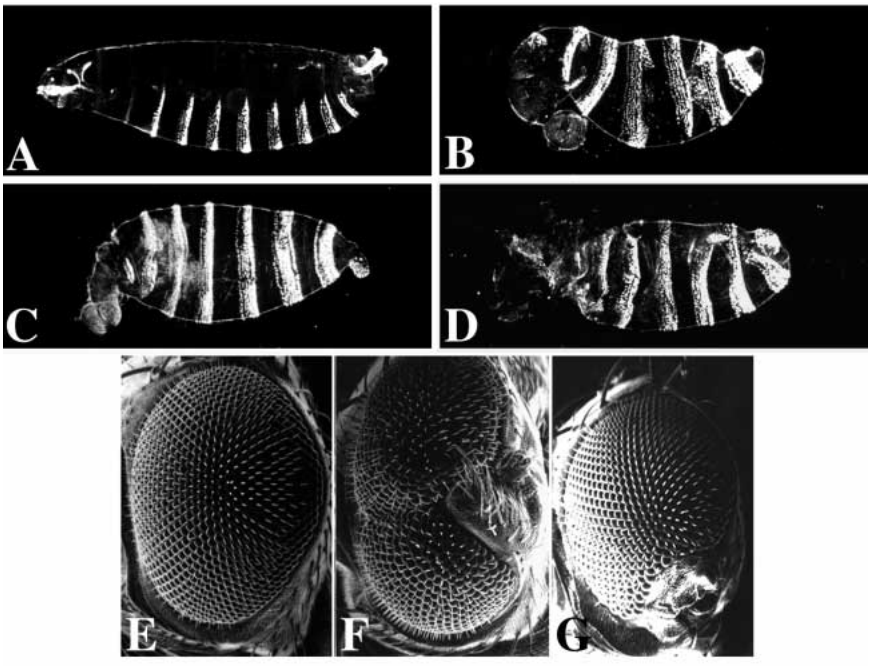



**Fig. 2**. Clonal analysis of *Medea* reveals *dpp*-like mutant phenotypes. (A-D) Cuticle preparations of embryos, with anterior to the left. (A) Wild-type embryo. (B) An embryo of the genotype $dpp^{H61}/dpp^{H48}$ showing loss of dorsal tissue and the expansion of the lateral denticle bands into dorsal regions. Head-involution defects result in the extruded globular structures at the anterior of the embryo. Embryos derived from germline clones are maternally and zygotically null for (C) *Medea* and (D) *Mad*, and show the identical ventralization as seen in B. The embryo in C was derived from a $Med^{1}$/*ovoD* mother and a $Med^{25}$ sperm (both strong alleles), while the embryo in D was from a $Mad^{12}$/*ovoD* mother and a $Mad^{10}$ sperm (both strong alleles). (E) Scanning electron micrograph (SEM) of a wild-type *Drosophila* eye. (F,G) Two examples of clones of null *Medea* alleles in the posterior of the eye. The mutant $Med^{-}$ cells have undergone a fate transformation from eye tissue to head cuticular structures. While both clones are of the $Med^{1}$ allele, similar phenotypes were obtained with the $Med^{26}$ allele.

partially rescued by the introduction of a paternal wild-type sperm (data not shown). Earlier attempts to generate such germline clones for *Mad* had proved unsuccessful, and had led to models that *Mad* may have roles in oogenesis that do not require *Medea*. Our data however, argue against that notion, and support the model that both *Mad* and *Medea* are required for all aspects of *dpp* signaling. Therefore, our results show that both *Medea* and *Mad* are required for *dpp* signaling in the dorsoventral patterning of the embryo.

### Clonal analysis reveals functions for *Medea* in imaginal disk development

To determine roles for *Medea* during larval development, we have analyzed clones mutant for *Medea* in the eye. Dpp has an important role in the initiation and progression of the morphogenetic furrow (Chanut and Heberlein, 1997). The furrow is a dorsoventral indentation that traverses the eye disk from posterior to anterior and causes, in its wake, a series of cell cycle changes and cell fate determinations that are responsible for the proper development of the ommatidia that constitute the adult eye. Recently, clones of *Mad* mutant cells in the eye have been published (Wiersdorff et al., 1996). *Mad* clones in the posterior of the eye result in the loss of eye structures, which are instead replaced by head cuticular structures (data not shown; Wiersdorff et al., 1996). Wiersdorff et al. (1996) demonstrated that these clones showed the ectopic expression of *wingless* (*wg*), a gene that is normally repressed by *dpp* signaling and is required at the lateral margins of the eye disk to regulate the proper timing of furrow initiation and progression (Treisman and Rubin, 1995). Hence clones of *Mad* mutant cells were unable to transduce the Dpp signal and were unable to initiate the morphogenetic furrow. We generated clones of the strong *Medea* alleles, $Med^{1}$ and $Med^{26}$, and found that these clones gave very similar phenotypes (Fig. 2F,G) to *Mad* clones, such as loss of eye tissue. Such clones were observed only at the margins of the eye, most commonly the posterior margin, where the furrow initiates. This indicates that *Medea* has overlapping functions with *Mad* in *dpp* signaling during furrow initiation.

Clonal analysis with *Medea* has also revealed abnormalities in other tissues, in keeping with its involvement in *dpp* signaling. For example, we have observed partial duplications of the leg (data not shown), a phenotype reported for clones of the *dpp* receptor, *punt*, in the dorsal regions of the leg (Penton and Hoffmann, 1996). These analyses strongly suggest a closely related function for *Medea* and *dpp* during imaginal development.

### *Medea* is able to suppress the phenotype of an activated *dpp* receptor, *saxophone*

In order to directly address the role of *Medea* in the Dpp pathway, we tested the ability of *Medea* mutants to suppress ectopic signaling from Dpp receptors. A constitutively activated Dpp Type I receptor, Saxophone (Sax*), was generated by the substitution of a single amino acid (Q263D) near the GS box of the intracellular domain (Wieser et al., 1995). The activated receptor was expressed in a spatially controlled manner using the GAL4–UAS system (Brand and Perrimon, 1993). Under *engrailed*GAL4 (*en*GAL4) control, UAS-*Sax** produced a phenotype in the wing, characterized by posterior defects, such as overgrowth and ectopic venation (Fig. 3B). Removal of a single copy of a gene that is required for Sax signaling, for example *Mad*, suppressed this phenotype (Fig. 3C). This same suppression was observed when a single copy of *Medea* was removed from *en*Gal4,UAS-*Sax** transgenic flies (Fig. 3D).

Since two type I receptors have been identified for Dpp, we tested the ability of *Medea* to suppress signaling from the other activated receptor, Thick veins (Tkv*). Interestingly, *Medea* did not show the same ability to suppress a Tkv* phenotype, typified by ectopic vein material and severe blistering. In fact, a subset of our *Medea* alleles showed very low levels of

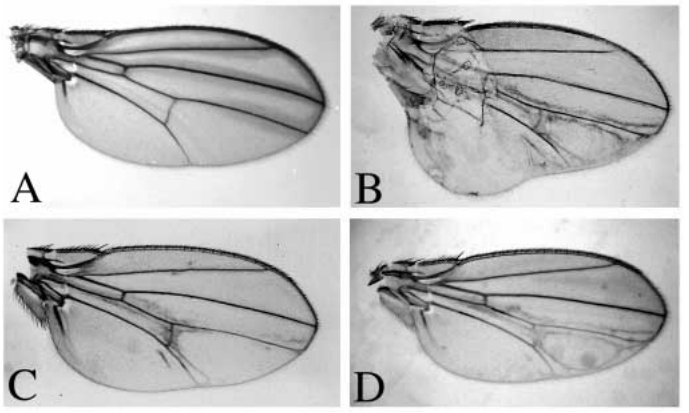



**Fig. 3**. *Medea* mutants suppress the phenotype of the activated *dpp* type I receptor, *saxophone**. (A) Wild-type *Drosophila* wing. (B) Wing from a UAS-Sax*/ *engrailed*GAL4, showing defects of the posterior compartment such as overgrowth and ectopic venation. The removal of one copy of (C) *Mad* (*Mad*$^{10}$) or (D) *Medea* (*Med*$^{1}$) results in the suppression of the phenotype. Other alleles of *Mad* (*Mad*$^{12}$) and *Medea* (*Med*$^{22}$, *Med*$^{23}$, *Med*$^{26}$) were also tested and observed to yield the similar levels of suppression.

suppression, and to a much lesser extent than *Mad* (data not shown). While *Mad*$^{10}$ and *Mad*$^{12}$ were able to revert the *tkv** wings to wild-type, *Medea* alleles (such as *Med*$^{1}$, *Med*$^{23}$, *Med*$^{26}$ and *Med*$^{27}$) only caused a slight reduction in the blistering. One explanation for the differential ability of *Medea* mutants to suppress the Sax* and Tkv* phenotypes is that the two activated receptors achieve different levels of signaling. In addition, *Medea* may not be a limiting component in Tkv* signaling. Therefore, the removal of one copy of *Medea* may be insufficient to affect the high levels of Tkv* signaling, but may be enough to influence the weaker Sax* signal. These data show that Medea and Mad function in signaling from Dpp receptors.

### *Mad* and *Medea* show partial reciprocal rescue of maternal effect lethality with *dpp*

Since *Mad* and *Medea* are separately mutable, it is expected that they function non-redundantly and cannot substitute for each other. Consistent with this model, ubiquitous expression of *Mad* (*Ubi-Mad*) cannot rescue *Medea* lethality (data not shown). To further examine the relationship between these two Smads, we have used a sensitized assay system. This assay utilizes the dominant maternal effect lethality of *Mad* and *Medea* with *dpp* (Fig. 4A). The extent of this lethality depends on the strength of the *Mad* or *Medea* allele and the *dpp* allele it is crossed to. Crossing a strong, hypomorphic *dpp* allele, *dpp*$^{hr27}$, to the strongest available alleles of *Mad*, (*Mad*$^{12}$) or *Medea* (*Med*$^{1}$), results in 100% lethality of both *dpp* classes among the progeny. As expected, *Ubi-Mad* can rescue the maternal effect lethality of *Mad*$^{12}$, and *Ubi-Medea* that of *Med*$^{1}$ (Fig. 4B).

We then examined the effects of introducing *Ubi-Medea* or *Ubi-Mad* from *Mad*/+ or *Medea*/+ females, respectively. Interestingly, a *Ubi-Medea* transgene can reduce the maternal effect lethality of *Mad*$^{12}$/+ females with *dpp* from 100% to 12%, while *Ubi-Mad* reduces that of *Med*$^{1}$/+ females to 68% (Fig. 4B). To assay whether this rescue was simply due to increased levels of *dpp* pathway components, we used a *Ubi-tkv* line in the same assay system. While this line is able to rescue a *tkv* mutant (Brummel et al., 1994), it cannot rescue the maternal effect lethality associated with *Mad* or *Medea* (Fig. 4B). The lower extent of *Ubi-Mad* rescue of *Medea* maternal effect lethality, may be due to the fact that *Med*$^{1}$ may be an antimorphic allele. Alternatively, this may be indicative of an important aspect of Smad function (see Discussion).

While it is clear that *Mad* and *Medea* cannot substitute for

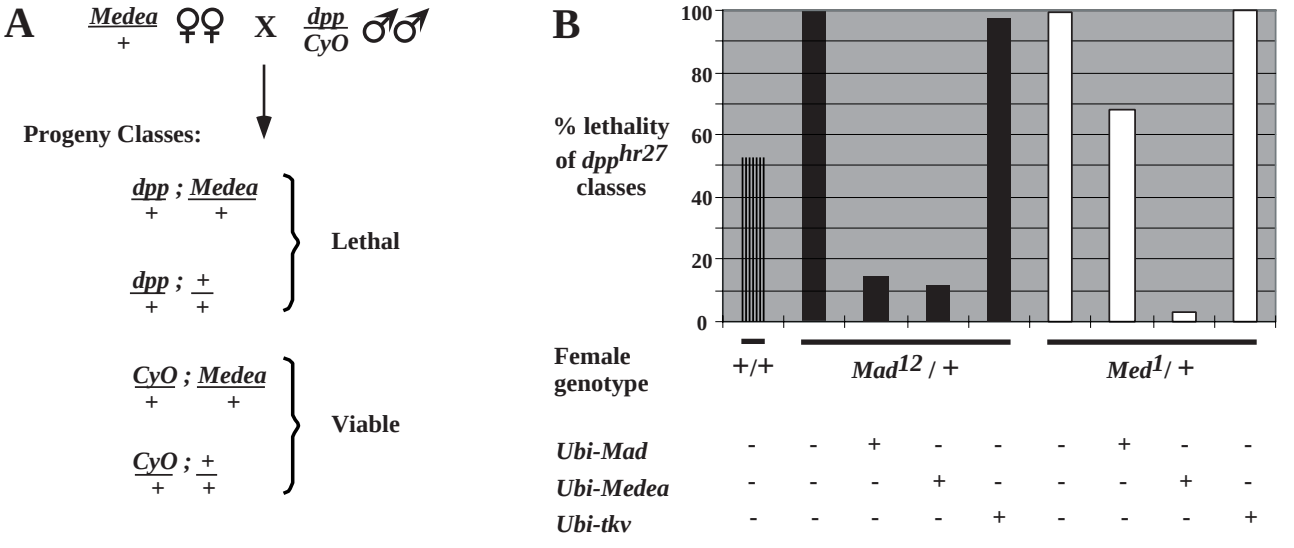



**Fig. 4**. *Mad* and *Medea* show reciprocal maternal effect rescue with *dpp*. (A) A simplified outline of the maternal effect interaction between *Medea* females and *dpp* males. When strong alleles of *Medea* and *dpp* are used, both classes of *dpp* progeny die. *Mad* interacts with *dpp* in a similar fashion. (B) The ubiquitous expression of *Medea*, *Mad* or *tkv* result in varying degrees of rescue of this maternal interaction. *Ubi-Medea* rescues the maternal effect of both *Med*$^{1}$ and *Mad*$^{12}$ with *dpp*. *Ubi-Mad* rescues the maternal effect of *Mad*$^{12}$ completely, and of *Medea* only partially, while *Ubi-tkv* does not rescue the maternal effect lethality of *Mad* or *Medea*.

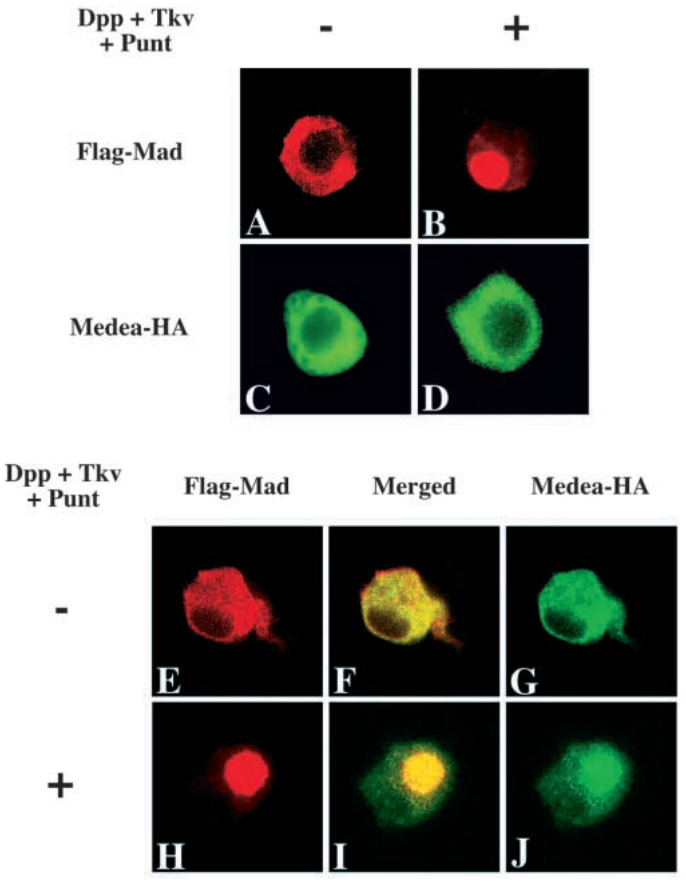



**Fig. 5**. Medea requires Mad for nuclear translocation. Mad protein is visualized with anti-Flag antibody (in red), while Medea is visualized with anti-HA antibody (in green). Yellow indicates regions of overlap between Mad and Medea localization. (B,D,H-J) Cells that were stimulated by the co-expression of Dpp, Tkv, and Punt. (A) Mad and (B) Medea are both cytoplasmic in the absence of stimulus. Upon receptor activation, and when expressed alone, (C) Mad becomes translocated to the nucleus, while (D) Medea remains cytoplasmic. (E-G) When expressed together, and in the absence of stimulation, Mad and Medea are both cytoplasmic. (H-J) Upon signaling from Dpp, and in the presence of Mad, Medea becomes localized to the nucleus.

each other, our genetic data argue that a reduction in one class of Smads can be at least partially compensated by augmenting the dosage of the other Smad class. This compensation may be a Smad-specific feature, as elevated levels of *tkv* do not yield the same results. The simplest explanation for these genetic observations is that increased levels of one class of Smads may enhance the ability of the other class to signal.

### Mad actively translocates Medea into the nucleus

*Mad* and *Medea* are closely related, yet separately mutable, genes required for Dpp signaling. To gain insight into the functional relationship between these Smads, we examined the subcellular localization of Mad and Medea proteins in *Drosophila* Schneider 2 (S2) cells, in the presence or absence of stimulation of the Dpp pathway. To activate the pathway, we co-transfected constructs of *dpp* and its receptors, *punt* and *thick veins*. This strategy provided a more powerful stimulus than transfection of activated *dpp* type I receptors.

In the absence of signaling, Flag-Mad showed predominantly cytoplasmic staining (Fig. 5A). This is consistent with what has been reported for Mad (Maduzia and Padgett, 1997; Newfeld et al., 1997) and, further, for the vertebrate class I Smads, Smad1 (Hoodless et al., 1996; Liu et al., 1996) and Smad2 (Macias-Silva et al., 1996). The same cytoplasmic staining was also observed for Medea-HA in the absence of stimulus (Fig. 5C). Only a small number of cells (6% for Mad, and 1.5% for Medea) showed predominantly nuclear staining. However, when co-expressed with the ligand and receptors, they revealed an important difference. The localization of Medea, in the presence of Punt, Tkv and Dpp, remained cytoplasmic in the majority of cells (Fig. 5D). However, Mad, in the presence of stimulus, was localized to the nucleus in about 95% of transfected cells (Fig. 5B). A similar, but lower, response was observed when activated Tkv* was used to stimulate the pathway. In this case, Mad was seen localizing to the nucleus in about 40% of cells. Hence, when expressed alone, and in the presence of stimulus, Mad was able to translocate to the nucleus, while Medea was not.

We then assayed the response when Mad and Medea were expressed together, with or without stimulus. When co-expressed, and in the absence of stimulus, both Smads were seen to be predominantly cytoplasmic (Fig. 5E-G), consistent with their responses when expressed alone. However, in the presence of stimulus, we observed that both Mad and Medea were now localized to the nucleus in about 40% of cells (Fig. 5F). In some cells, Mad was nuclear, and Medea was both cytoplasmic and nuclear, while in other cells, both localized primarily to the nucleus.

Hence, the co-expression of Mad is required for Medea to change its subcellular localization in response to stimulus. We have also observed that full-length Mad and Medea associate directly in two-hybrid assays (C. Evangelista and R. W. P., unpublished data), and co-immunoprecipitation (IP) experiments (data not shown). Furthermore, vertebrate Smads have also been shown to physically associate in two-hybrid (Wu et al., 1996) and IP experiments (Lagna et al., 1996; Kretzschmar et al., 1997). Taken together, these data suggest a model whereby activated Mad interacts directly with Medea to actively translocate it to the nucleus.

## DISCUSSION

### *Medea* is required for *dpp* signaling, at several stages of development

Our results show that *Medea* is essential for *dpp* signaling at multiple stages of development. *Medea* is required for the *dpp*-mediated dorsoventral patterning of the embryo. Hence, elimination of both zygotic and maternal *Medea* gives rise to embryos that produce only ventrolateral cell fates. This phenotype is identical to that produced by the complete loss of *Mad*, *dpp*, or its receptors. Later examples of *dpp*-mediated patterning include the morphogenetic furrow initiation and movement during eye development in the larval and pupal stages. Clonal analysis at this stage proves that *Medea* is essential for these processes. In addition, mutations in *Medea* can suppress the phenotype of an activated Dpp receptor, indicating that *Medea* functions downstream of the Dpp receptor complex. Therefore, in all the tissues that we have

examined, *Medea* and *dpp* have identical functions, indicating that *Medea* is required for all *dpp* signaling.

Furthermore, *Medea* and *Mad* function together in these multiple developmental contexts. We show that *Mad* mutants suppress the phenotype of an activated Dpp receptor, similar to *Medea* mutants. Our germline clonal analysis indicates that maternal and zygotic loss of *Mad* results in ventralized embryos that resemble those from germline clones of *Medea*. Our imaginal clonal analyses for *Medea* and *Mad* have yielded similar mutant phenotypes in the eye. In addition, Medea and Mad interact in two-hybrid assays and immunoprecipitation experiments. All these results indicate that both Medea and Mad function together in transducing the Dpp signal during embryonic and larval morphogenesis.

### Analysis of *Medea* mutations

We find that the mutations in *Medea* result in the premature truncation of, or cause the substitution of specific residues in, MH2, as do the majority of mutations in other members of the Smad family (described in Shi et al., 1997). MH2 is required for the formation of hetero-oligomeric complexes between the two classes of Smads (Lagna et al., 1996). Hence, two of the mutations, Gln283Ter and Gln457Ter, lead to a mutant form of Medea that probably lacks the ability to form hetero-oligomers with Mad. The other mutations, Asp712Ala, Gly609Ala and Gly609Ser, predict the disruption of the core structure of the protein (Shi et al., 1997).

Since domains 1 and 2 of the Smad family are highly related, it is interesting to note that most Smad mutations map to MH2. While MH1 of MAD has the ability to bind DNA (Kim et al., 1997), the loss of MH1 has been implicated in constitutive nuclear localization and signaling (Baker and Harland, 1996; Liu et al., 1996). In addition, MH1 may serve in an autoinhibitory capacity to prevent hetero-oligomerization prior to entry into the nucleus, and MH1 overexpression has a dominant negative effect on signaling (Hata et al., 1997). Interestingly, an analogous effect is seen in the antimorphic $Med^{1}$ allele (data not shown, Raftery et al., 1995), where a nonsense mutation predicts a protein lacking the linker and MH2.

### Class II Smads are essential for effecting TGF-β-like signals

We show here that *Medea* is important for multiple facets of *dpp* function. Together with the requirement for *sma-4* in *C. elegans* body size determination and male-tail morphogenesis (Savage et al., 1996), our data strongly indicate that class II Smads are an essential component of TGF-β-like signaling.

Biochemical and cell culture studies have suggested that Smad4 may synergize with specific class I Smads to effect signaling from different TGF-β superfamily ligands. These studies raise the possibility that Medea, the Smad4 homolog in *Drosophila*, may also function with specific class I Smads to transduce signals from different ligands. While two *Drosophila* TGF-β homologs, other than *dpp*, have been identified, their roles in patterning seem to either overlap with those of *dpp*, as in the case of *screw* (*scw*; Arora et al., 1994), or are unknown, as in the case of *60A* (Wharton et al., 1991; Doctor et al., 1992). *Medea* shows no genetic interaction with null alleles of *scw* (data not shown), while *60A* mutants have not been reported. It is possible that, like *scw*, *60A* also functions in concert with *dpp*. While our data prove the role of *Medea* in *dpp* signaling, they do not rule out the possibility that Medea may function to transduce the signal from other ligands as well. However, no *Medea* phenotypes were observed that differed from *Mad* or *dpp*. It is possible that other *Medea* phenotypes were masked by the severity of its *dpp*-related phenotype.

Kim et al. (1997) have recently shown that Mad is able to directly bind the promoters of Dpp target genes, such as *vestigial* and *labial*. Additionally, within these promoters, distinct Mad binding sites can be distinguished (Kim et al., 1997). It is possible that further in vitro and in vivo analyses will result in the identification of Medea-specific sites, and result in an understanding of how the two Smads act together to modulate the differential transcription of target genes. It is also likely that the association of the two Smads results in a more stable complex with specific transcription factors, such as FAST-1 (Chen et al., 1996, 1997).

### Class I and class II Smads respond differently to receptor activation

Our results demonstrate that Mad and Medea have biochemically distinct responses to Dpp signaling. Both Flag-Mad and Medea-HA are cytoplasmic when transfected alone into *Drosophila* S2 cells. Under conditions that result in Dpp receptor activation, Mad is able to translocate to the nucleus, while Medea remains cytoplasmic. In the presence of activated Mad, however, Medea translocates to the nucleus. These observations suggest that Mad, but not Medea, is a direct target of the signal, and that the signal from the activated receptor complex to Medea is mediated by Mad. Thus it is likely that Medea, unlike Mad, does not interact with the type I receptor. The distinct responses of these two closely related proteins to stimulation in cell culture, provide a biochemical explanation for the genetic requirement for *Mad* and *Medea* in *dpp* signaling.

The basis of this difference in response to receptor activation may lie in the major sites of phosphorylation of the Smads. The class I Smads have been shown to be phosphorylated in response to stimulus at C-terminal serines – the SSXS motif, an event that is important for signaling (Macias-Silva et al., 1996; Kretzschmar et al., 1997). This motif is absent in Medea and the other class II Smads, as well as in the class III Smads.

From these observations, it is possible to draw a model whereby the activation of Mad occurs before the activation of Medea during Dpp signal transduction. The levels of Mad that become activated (Mad*) determine the potential of the next, equally important step, which is its hetero-oligomerization with Medea. Thus, the higher levels of signaling achieved by the Dpp/Punt/Tkv activation system in cell culture, yield higher levels of Mad*, and cause high levels of nuclear Medea, while the lower Tkv* stimulus yields low levels of Mad*, and hence undetectable levels of nuclear Medea. As the formation of the Mad*-Medea complex is important for signaling, from this model it is also conceivable that a quantitative increase in the levels of Medea protein can compensate for a reduction of Mad, by increasing the likelihood of the hetero-oligomerization of Mad* with Medea, thereby explaining the ability of *Ubi-Medea* to rescue the maternal effect lethality of $Mad^{12}$/+ flies with $dpp^{hr27}$.

In summary, our data refine the current model for Dpp signaling as follows (see Fig. 6 for a general model): after Dpp

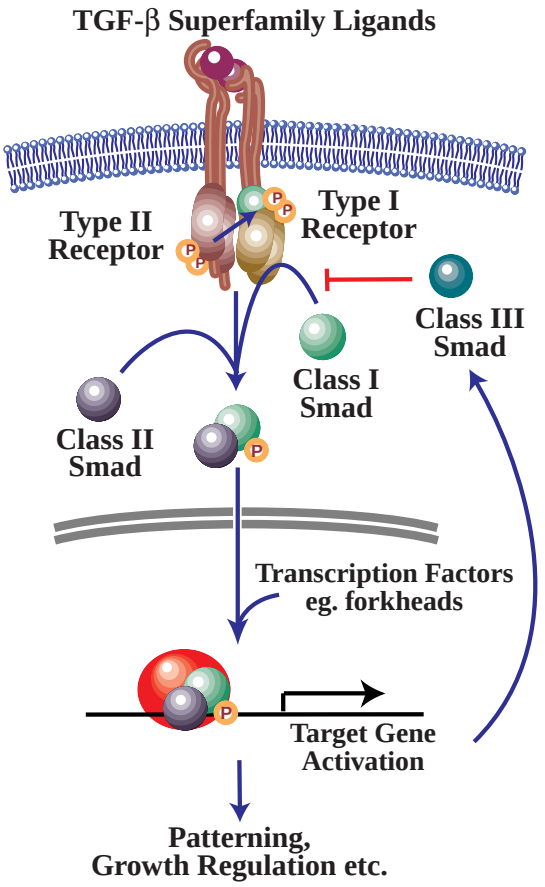



**Fig. 6**. A general model for TGF-β signaling.

complexes with its receptors Tkv or Sax (both type I), and Punt (type II), Punt phosphorylates Tkv or Sax. This results in the phosphorylation of Mad on C-terminal serines, which is now able to hetero-oligomerize with Medea, and translocate to the nucleus. Once in the nucleus, the hetero-oligomer can, in conjunction with specific transcription factors, effect the expression of the appropriate target genes. Given the conservation of TGF-β signaling components across species, the results described here may apply to other organisms as well.

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