Development 125, 1407-1420 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5194

The *Drosophila Medea* gene is required downstream of *dpp* and encodes a functional homolog of human Smad4

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Accepted 30 January; published on WWW 18 March 1998

SUMMARY

The Transforming Growth Factor-β superfamily member *decapentaplegic* (*dpp*) acts as an extracellular morphogen to pattern the embryonic ectoderm of the *Drosophila* embryo. To identify components of the *dpp* signaling pathway, we screened for mutations that act as dominant maternal enhancers of a weak allele of the *dpp* target gene *zerknüllt*. In this screen, we recovered new alleles of the *Mothers against dpp* (*Mad*) and *Medea* genes. Phenotypic analysis of the new *Medea* mutations indicates that *Medea*, like *Mad*, is required for both embryonic and imaginal disc patterning. Genetic analysis suggests that *Medea* may have two independently mutable functions in patterning the embryonic ectoderm. Complete elimination of maternal and zygotic *Medea* activity in the early embryo results in a ventralized phenotype identical to that of null *dpp* mutants, indicating that *Medea* is required for all *dpp*-dependent signaling in embryonic dorsal-ventral patterning. Injection of mRNAs encoding DPP or a constitutively activated form of the DPP receptor, Thick veins, into embryos lacking all *Medea* activity failed to induce formation of any dorsal cell fates, demonstrating that *Medea* acts downstream of the *thick veins* receptor. We cloned *Medea* and found that it encodes a protein with striking sequence similarity to human SMAD4. Moreover, injection of human *SMAD4* mRNA into embryos lacking all *Medea* activity conferred phenotypic rescue of the dorsal-ventral pattern, demonstrating conservation of function between the two gene products.

Key words: *Drosophila*, *Medea*, *Smad4*, *dpp*, *Mad*, TGF-β signal transduction, Dorsal-ventral patterning

INTRODUCTION

A striking example of the conservation of molecular mechanisms throughout development has been the finding that closely related members of the Transforming Growth Factor-β (TGF-β) superfamily, DPP in *Drosophila melanogaster*, BMP-4 in *Xenopus laevis*, and BMP-2/4 in the Zebrafish *Danio rerio*, play essential roles in the specification of pattern along the embryonic dorsal-ventral axis (Dale et al., 1992; Ferguson and Anderson, 1992b; Hammerschmidt et al., 1996; Irish and Gelbart, 1987; Jones et al., 1992; Mullins et al., 1996). In *Drosophila*, *dpp* is transcribed at uniform levels in the dorsal 40% of the embryonic nuclei and is required within this expression domain for the production of all dorsal structures (Irish and Gelbart, 1987; St. Johnston and Gelbart, 1987). Genetic and embryological experiments have shown that *dpp* acts as an extracellular morphogen to promote dorsal cell fates in a dose-dependent fashion (Ferguson and Anderson, 1992a; Wharton et al., 1993). Similarly, in *Xenopus*, BMP-4 is expressed ventrally (Dale et al., 1992; Jones et al., 1992) and functions in a concentration-dependent fashion to pattern the ectodermal and mesodermal germ layers of the embryo (Dosch et al., 1997; Wilson et al., 1997). These observations raise a fundamental question regarding the conserved molecular mechanisms of DPP and BMP-4 action: how do the intracellular signal transduction pathways downstream of the DPP/BMP-4 ligands faithfully transduce small differences in DPP/BMP-4 activity to produce differential developmental outputs?

The intracellular signal transduction machinery that responds to the DPP/BMP-4 ligand is likewise highly conserved. In all species examined, TGF-β ligands signal through receptor complexes containing two types of transmembrane serine-threonine kinases, type I and type II. Biochemical experiments with mammalian TGF-β receptors have shown that upon ligand binding, the constitutively-active type II kinase phosphorylates and activates the type I kinase (Massagué, 1996; Wrana et al., 1994). In *Drosophila*, the products of the *thick veins* (*tkv*), *saxophone* (*sax*) and *punt* genes function as components of the *dpp* receptor complex (Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). The *tkv* and *sax* genes encode type I receptors, while the *punt* gene encodes a type II receptor.

Two additional genes involved in *dpp* signaling, *Mothers against dpp* (*Mad*) and *Medea*, were identified in a genetic screen for mutations that act as maternal-effect enhancers of a weak allele of *dpp*, dpp^{hr4} (Raftery et al., 1995). Molecular cloning of *Mad* (Sekelsky et al., 1995), isolation of the related *C. elegans* genes *sma-2*, *sma-3* and *sma-4* (Savage et al., 1996), and identification of vertebrate *Mad* homologs (Baker and Harland, 1996; Graff et al., 1996; Hoodless et al., 1996) have

shown that this family of genes, known as Smads, are extremely well conserved across metazoan phyla. Their defining structural features are two highly conserved '*Mad* homology' domains, MH1 (N-terminal) and MH2 (C-terminal), separated by a linker region of variable sequence and length (Hoodless et al., 1996). Subsequent work has shown that members of the Smad family of proteins are essential cytoplasmic components of the TGF-β signal transduction pathway (Heldin et al., 1997).

Functional and structural analysis indicates that Smad family members can be subdivided into three major classes. The first class of Smads transduce signals downstream of specific TGF-β family members. For example, Smad1 and Smad5 signal downstream of the bone morphogenetic protein (BMP) family of ligands and receptors, whereas Smad2 and Smad3 transduce signals from the TGF-β and activin receptors (Eppert et al., 1996; Graff et al., 1996; Hoodless et al., 1996; Lagna et al., 1996; Macias-Silva et al., 1996; Zhang et al., 1996). The second class of Smads is defined by human SMAD4 and its presumptive orthologs (Hahn et al., 1996; Lagna et al., 1996; Savage et al., 1996). Human *SMAD4* was isolated as a tumor suppressor gene (originally named DPC4 for Deleted in Pancreatic Carcinoma-4) that was mutated or absent in a majority of pancreatic carcinomas (Hahn et al., 1996; Schutte et al., 1996; Thiagalingam et al., 1996). Smad4 family members show significant structural differences from the receptor specific Smads in both the MH1 and MH2 regions. Furthermore, functional analysis indicates that they are involved in signaling downstream of all TGF-β superfamily members examined (Chen et al., 1997; Lagna et al., 1996; Zhang et al., 1997). A third class of Smads, including Smad6, Smad7 and *Drosophila* Dad, was recently identified as negative regulators of TGF-β signal transduction pathways (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997).

A paradigm for Smad function during TGF-β signal transduction has been developed from a variety of experimental systems (Heldin et al., 1997). Upon activation by a TGF-β superfamily ligand, a type I/type II receptor complex associates transiently with the appropriate receptor-specific Smad and phosphorylates carboxy-terminal serines that are necessary for Smad activation. The phosphorylated Smad then associates with Smad4 and translocates into the nucleus to regulate the expression of target genes. In *Xenopus*, Smad2 and Smad4 have been shown to associate together with the winged helix transcription factor, FAST-1, to form a tripartite complex that binds to an enhancer element upstream of an activin responsive gene (Chen et al., 1996, 1997). In *Drosophila*, the MH1 domain of MAD has been shown to bind specific DNA sequences necessary for the transcription of the target gene *vestigial* in the wing imaginal discs, while in tissue culture cells human SMAD4 binds sequences upstream of the TGF-β-responsive element 3TP-Lux (Kim et al., 1997; Yingling et al., 1997). Thus, the Smad family of proteins are components of a signal transduction pathway that extends directly from the receptor to the nucleus.

Here we report the isolation of additional alleles of *Medea* and present a detailed phenotypic characterization of its role in embryonic dorsal-ventral patterning. Molecular characterization of *Medea* indicates that it encodes the *Drosophila* homolog of Smad4. We obtained embryos completely lacking maternal and zygotic *Medea* activity, and we used the technique of mRNA injection to demonstrate functional conservation between human SMAD4 and MEDEA.

MATERIALS AND METHODS

Drosophila stocks

zen^{f16} p^{p}, *ry P720*, *ry* $P1575^{ttk}$, $P\{hsFLP\}^{1}$, $FRT^{3R\text{-}82B}$, and $FRT^{3R\text{-}82B}$ $P\{ovo^{D1}\}^{3R}$ fly stocks were obtained from the Bloomington *Drosophila* Stock Center (FlyBase, 1997). zen^{f16} is also known as zen^{1}. *P720* and $P1575^{ttk}$ refer to the P-element insertions $P\{ry^{+t7.2}=PZ\}l(3)00720$ and $P\{ry^{+t7.2}=PZ\}ttk^{02667}$ at cytological positions 100B5-7 and 100D1-2, respectively. $P\{hsFLP\}^{1}$, $FRT^{3R\text{-}82B}$, and $P\{ovo^{D1}\}^{3R}$ are as described (Chou and Perrimon, 1996). *Df(3R)E40* (Locke et al., 1988) was obtained from K. Tartof. Med^{4} (Raftery et al., 1995) and *Df(3R)KpnA* were obtained from L. Raftery. Mad^{12} and *Df (2L)C28* stocks were obtained from W. Gelbart (Sekelsky et al., 1995). Additional information about *Drosophila* stocks is available in FlyBase (1997).

Recovery of mutations in *Mad* and *Medea*

The zen^{f16} p^{p} chromosome was subjected to unselected recombination with wild-type (Oregon R) chromosomes to eliminate extraneous recessive lethal loci. Hatching of homozygous zen^{f16} p^{p} embryos was reduced from 98% (n=346) to 23% by the introduction of one copy of a weak *dpp* mutant allele, dpp^{hr56} (n=195). Thus, a very small decrease in *dpp* signaling greatly enhanced the penetrance of the lethal phenotype of zen^{f16} (Wakimoto et al., 1984). We reasoned that a similarly small decrease in the efficiency of *dpp* signaling, as might be expected from a 50% decrease in the activity of a component in the *dpp* signaling pathway, might also be sufficient to enhance the penetrance of the zen^{f16} mutant phenotype. Furthermore, we reasoned that, like the *dpp* receptors, other essential *dpp* signaling components were likely to be expressed maternally. Accordingly, we screened for maternal-effect mutations that dominantly enhanced the zen^{f16} phenotype.

Homozygous zen^{f16} p^{p} males were starved for 8 hours, fed 25 mM ethylmethane sulfonate (EMS) in 1% sucrose for 24 hours, and mated for 6 days to zen^{f16} p^{p} females. Approximately 5000 mutagenized F_1 zen^{f16} p^{p} females, each bearing a mutagenized haploid genome, were collected and mated individually to zen^{f16} p^{p} males in egg laying blocks. The viability of embryos from approximately 4000 productive matings was assessed by visual inspection with a dissecting microscope. F_1 females that produced 75-100% inviable embryos with cuticular phenotypes characteristic of partial ventralization were considered as candidate dominant maternal enhancer mutants. Secondary matings of F_1 female mutant candidates to zen^{f16} p^{p}/*TM3 Sb e* males allowed the recovery of the enhancer mutations from some of the viable zen^{f16} p^{p}/*TM3 Sb e* progeny (Fig. 1).

Eight enhancer mutations on chromosome 3 were recovered in *trans* to the balancer chromosome *TM3 Sb e* through matings of individual candidate females to zen^{f16} p^{p} *e/TM3 Sb e* males and assessment of the progeny of each female for dominant enhancement of the zen^{f16} mutation. The *Sb* and *e* markers were used to follow the segregation of the zen^{f16} p^{p} chromosome bearing the enhancer mutation. One enhancer mutation was recovered in *trans* to the chromosome 2 balancer *CyO* from iterated matings of individual females to *Sp/CyO*; zen^{f16} p^{p} males.

The identities of the mutant genes were assessed both by multi-factorial recombinant mapping with respect to visible marker mutations and by complementation analysis. The enhancer mutation on chromosome 2 was localized to the chromosomal interval between the *aristaless* and *dumpy* loci. It was identified as an allele of *Mad* by non-complementation with the phenotypically null mutant Mad^{12} and *Df(2L)C28*; it has been named Mad^{Ez}. The eight mutations on chromosome 3, numbered *11* through *18*, were shown by

complementation analysis to be allelic to one another. A deficiency of *Medea*, *Df(3R)E40*, failed to complement mutants *11*, *15* and *16*, and two mutants, 13 (L Raftery, personal communication) and *15*, failed to complement Med^4 (Raftery et al., 1995), identifying the eight enhancer mutations as alleles of *Medea*; they have been named Med^{11} through Med^{18}. The deficiency chromosome *Df(3R)KpnA* also failed to complement Med^{11} and Med^{16}, although Southern blot analysis has indicated that it contains *Medea* DNA sequences (not shown).

Analysis of *Mad* and *Medea* mutant phenotypes

Embryonic cuticle preparations were performed as described by Wieschaus and Nüsslein-Volhard (1986). β-galactosidase expression in the amnioserosa from the *P{Kr-lacZ}* reporter construct was assayed as described by Ferguson and Anderson (1992a).

Wings dissected from adult flies were mounted in Gary's magic mountant (Ashburner, 1989). Adult flies were fixed for scanning electron microscopy first in 70% ethanol and then in PBS with 2.5% glutaraldehyde. Fixed flies were returned to 70% ethanol, dissected as necessary, and dehydrated first in ethanol and then in hexamethyldisilazane. Samples were mounted onto stubs with either tape or a graphite bed, coated with gold in a sputter coater (Technics), and viewed with a JEOL JSM-840A scanning electron microscope at a working distance of 39 mm and an accelerating voltage of 15 kV.

Germline clones

A *ru h* $FRT^{3R\text{-}82B}$ *sr e* $Medea^{13}$ chromosome was generated and placed in *trans* to $FRT^{3R\text{-}82B}$ $P\{ovo^{D1}\}^{3R}$ in virgin females that also carried $P\{hsFLP\}^1$ on the X chromosome. Expression of the *FLP* recombinase was induced by heating pupae or adults to 37°C for 2 to 3 hours. Females were mated after heat shock to $Medea^{14}$, *P{Kr-lacZ} /TM3 P{Ubx-lacZ}* males to produce embryos for phenotypic analysis. Expression in the amnioserosa of β-galactosidase from the *P{Kr-lacZ}* construct was assayed 20 hours after egg laying at 18°C. Embryos that received a wild-type copy of *Medea* from the *TM3* balancer were identified by *Ubx-lacZ* expression.

For mRNA injection experiments, 500-1000 females generated as above were mated with Med^{14} *P{Kr-lacZ}/TM3 P{Ubx-lacZ}* males at 18°C in an egg-laying cup on apple juice plates. Eggs were collected for injections every 2-3 days on fresh apple juice plates smeared with yeast paste. Flies were kept in the dark during collections and kept in constant light on fresh apple juice plates without yeast between collections. Each 2 hour collection produced approximately 25-50 embryos for injection; a second successive plate was sometimes also productive. In practice, a mating cup became productive after a week of pre-mating and remained so for only approximately 10 days.

Molecular cloning of *Medea*

The *Medea* gene was localized between the two P[ry^+] insertions *P720* at 100B5-7 and *P1575* at 100D1-2 by three-factor meiotic recombination mapping. Females were generated with *st ry P720* and *st ry P1575* chromosomes in *trans* to *st ry ca* Med^{15} and *st ry* Med^+ recombinant chromosomes with breakpoints between the *Medea* gene and each P-element insertion were collected in *trans* to *st ry* Med^{11} or *st ry* Med^{13}. The genetic distance between *Medea* and *P720* was 0.3 cM (21 *st ry* Med^+ recombinants among 7021 *st* and *st ry* progeny) and that between *Medea* and *P1575* was 0.06 cM (7 *st ry* Med^+ recombinants among 11890 *st* and *st ry* progeny). These recombinant chromosomes, as well as an additional seven *P1575* recombinant chromosomes, were used in RFLP analysis.

P1 phage clones with inserts from the 100D region (Berkeley *Drosophila* Genome Project) were obtained from T. Kaufman (clones DS00235, DS03999, and DS07056 are referred to here as 235, 3999, and 7056, respectively). Overlap among P1 genomic inserts was determined by restriction enzyme analysis and by Southern blotting with ^{32}P-labeled terminal subclones. Gaps between P1 inserts were partially filled with cosmids (including clone 91C5) obtained from I. Siden Kiamos (Foundation for Research and Technology - Hellas) or recovered from *Drosophila* genomic DNA libraries constructed in a cosmid vector (S. King library, provided by C.-I. Wu; clones K17 and K31) or in λEMBL3 (Tamkun et al., 1992), provided by A. Mahowald; clone λ7. The genomic inserts of these clones defined two contiguous regions (Fig. 5A). Subcloned restriction fragments from the genomic DNA clones were used as probes for RFLP analysis to align the physical and genetic maps and to localize *Medea*. Other probes included a *tramtrack* (*ttk*) cDNA (Xiong and Montell, 1993) and a genomic fragment from *abnormal wing discs* (*awd*) (Dearolf et al., 1988).

RFLPs (restriction fragment length polymorphisms) were identified by Southern blot hybridization of these probes to DNA prepared from Med^{15} and each of the two P-element insertion stocks, after digestion with a panel of eleven restriction enzymes having 4-base recognition sites (*Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II or *Msp*I, *Nla*III, *Rsa*I, *Sau*3AI or *Dpn*II, *Scr*FI, and *Taq*$^{\alpha}$I). Probes that revealed a RFLP between the P-element insertion chromosome and the Med^{15} chromosome were used to probe Southern blots with restriction digested DNA from the 14 (P1575) or 21 (P720) recombinant chromosomes. The Med^{15} lesion was shown by this analysis to lie in a 40 kb region defined by two such probes: a *Taq*$^{\alpha}$I RFLP identified by the probe NN.1 and an *Nla*III RFLP identified by the probe GN.1.

cDNAs from three distinct transcription units within the delimited region were isolated by screening embryonic and imaginal disc cDNA libraries (Brown and Kafatos, 1988) with ^{32}P-labeled cosmid K17 or subcloned fragments from P1 3999. The *Medea* cDNA clone O513 was obtained from a 0- to 4-hour embryonic cDNA library; four additional *Medea* cDNA clones (H12, H25, H36, and H44) were recovered from a 4- to 8-hour embryonic cDNA library. The two other transcription units were represented by the cDNA clone H31, isolated from the 4- to 8-hour embryonic library, and the imaginal disc cDNA clone I7114.

Sequence analysis of *Medea* gene

The sequence of the *Medea* cDNA clone O513 was determined in both directions from subcloned restriction fragments spanning the length of the cDNA and has been placed into GenBank (accession number AF039232). The cDNA sequence ends with fourteen consecutive adenosines, twelve of which represent the adenosines in the primer that was used to synthesize the cDNA (Brown and Kafatos, 1988).

Genomic *Medea* DNA sequence was determined from DNA isolated from the *Medea* homozygous mutants (Med^{15} and Med^{17}) and from *Medea/TM3* heterozygotes. After digestion with *Bss*HII and *Nco*I, the 3 to 5 kb DNA fraction was excised from a 1% agarose gel, extracted with GlasPac/GS (National Scientific Supply Co.), and cloned into *Mlu*I- and *Nco*I-digested pGEM-5Zf(–) (Promega). *Medea* clones were identified by hybridization to a ^{32}P-labeled 550 bp *Bst*XI fragment from the *Medea* cDNA, and wild-type clones derived from the *TM3* balancer chromosome were excluded by way of a *Hin*fI polymorphism. The *Medea* mutant DNA clones were sequenced in both directions with primers spaced 400 to 500 bp apart. Wild-type sequence of the *Medea* genomic region was determined as a consensus from the mutant sequences and has been placed into GenBank (accession number AF039233). This sequence begins at position 315 of the cDNA and ends 537 bp downstream of the cDNA 3′ terminus. Nine exons were identified by comparison of the cDNA and genomic sequences. The sizes and placement of the eight introns are: intron 1 after cDNA base 682, 236 bp; intron 2 after base 951, 86 bp; intron 3 after base 1306, 201 bp; intron 4 after base 1528, 156 bp; intron 5 after base 2143, 60 bp; intron 6 after base 2426, 61 bp; intron 7 after base 2595, 56 bp; and intron 8 after base 2722, 59 bp.

The $(A)_{14}$ at the 3′ terminus of the *Medea* cDNA is encoded within the genomic DNA and does not represent 3′ polyadenylation. A single AATAAA poly(A) specification consensus sequence identified within the genomic sequence, 142 bp downstream of the cDNA terminus, may encode the true poly(A) signal sequence. However, preliminary sequencing has indicated that the *Medea* cDNA clones H36 and H44

terminate in polyadenosines after a single G insertion at position 3180 and after position 3188, respectively, perhaps reflecting the partial use of the cryptic poly(A) site AATATA at position 3158.

The lesions in six *Medea* mutants were identified by double stranded sequencing of the complete coding region of each mutant chromosome. The Med^{11} mutation is a 405 bp deletion, starting in intron 4 and continuing into exon 5 through cDNA position 1888, into which the 5 base duplication GTCTT has been inserted; the presence of this deletion has been confirmed by Southern blot analysis. Med^{12}, Med^{14} and Med^{16} have the single base substitutions G2481A, G2145A and C1804, respectively. Med^{15} has the missense mutations C2732T and C2837A. Med^{17} has the missense mutation C2839A and the silent substitution G2874A that ablates a *Pst*I restriction site (confirmed by Southern blot analysis). Sequencing was performed on an ABI Prism 377 or 377XL DNA sequencer at the University of Chicago Cancer Research Center DNA Sequencing Facility. Sequence was analyzed with Sequencher (Gene Codes Corp.) and Lasergene (DNASTAR) software.

mRNA preparation and injection into embryos

The full-length *Medea* cDNA of plasmid O513, consisting of a *Hin*dIII-*Not*I fragment and a *Not*I fragment, was cloned into the pGEM-11Zf(−) vector (Promega) at its *Hin*dIII and *Not*I sites to generate p11Zf-*Medea*; p11Zf-*Medea* was digested with *Xba*I at a unique vector site 3′ of the cDNA insert and transcribed with the SP6 Message Machine kit (Ambion). The cDNAs H31 and I7114 in the pNB40 vector were linearized after the 3′ UTR with *Not*I and transcribed with the SP6 Message Machine kit. *dpp* mRNA was synthesized as described by Holley et al. (1996). pSP35T-*tkv-a* was constructed from *tkv-a* cDNA (Holley et al., 1996) that was PCR-amplified from pBluescript KS-*tkv-a* and cloned into pSP35T via *Nco*I and *Xba*I sites in the 5′ and 3′ PCR primers, respectively (J. Neul, personal communication); pSP35T-*tkv-a* was linearized with *Eco*RI and transcribed using the SP6 Message Machine kit. pSP35T-*Mad* was constructed from *Mad* cDNA (Sekelsky et al., 1995) that was PCR-amplified from pBluescript-*Mad* and cloned into pSP35T via *Nco*I and *Xba*I restriction sites in the PCR primers; pSP35T-*Mad* was linearized with *Sac*I and transcribed with the SP6 Message Machine kit. Human *SMAD4* cDNA (Hahn et al., 1996) in the pGEM-3Z vector (Promega) was digested with *Bam*HI and transcribed with the SP6 Message Machine kit.

Capped mRNAs were injected into the dorsal side of pre-blastoderm stage embryos, and the resulting phenotypes were analyzed as previously described (Ferguson and Anderson, 1992a; Holley et al., 1996).

DNA and RNA analysis

Southern blotting, northern blotting, restriction digestion, and subcloning techniques were conducted according to standard procedures (Sambrook et al., 1989).

Photomicroscopy

Photomicroscopy was performed with an Axiophot microscope (Zeiss) with DIC Nomarski, transmitted light, dark field, or phase contrast optics. Photographic images were digitized using a Polaroid Sprint Scan 35 slide scanner or UMAX Powerlook flatbed scanner. Some images were obtained with a Progres 3012 digital camera (Kontron Elektroniks). Figures were assembled using Adobe PhotoShop 3.0 and printed using a Kodak XL dye-sublimation printer.

RESULTS

Recovery and genetic characterization of mutant alleles of *Mad* and *Medea*

To identify genes that participate in *dpp* signal transduction,

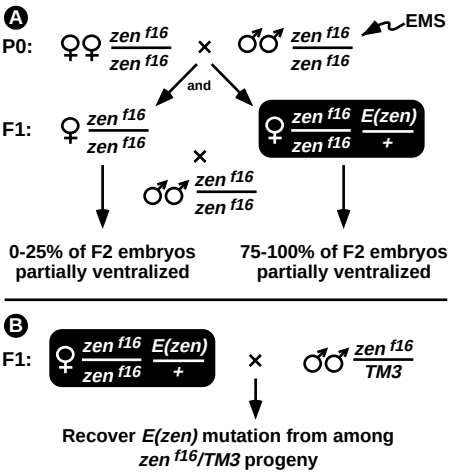


Fig. 1. A genetic screen for dominant maternal enhancers of a homozygous viable allele of *zen*, zen^{f16}.

we conducted a genetic screen for dominant maternal enhancers of a weak allele of *zerknüllt* (*zen*), a downstream target of *dpp* (Ferguson and Anderson, 1992a; Rushlow and Levine, 1990). The *zen* gene encodes a homeodomain protein expressed at cellular blastoderm stage in the dorsal 10% of nuclei, which are fated to become amnioserosa (Rushlow et al., 1987). Mutant embryos lacking *zen* activity do not differentiate amnioserosa, and they die with a partially ventralized cuticular phenotype (Arora and Nusslein-Volhard, 1992; Rushlow and Levine, 1990). The allele we chose for this screen, zen^{f16}, confers a subliminal phenotype: 98% of zen^{f16} mutant embryos hatch (Wakimoto et al., 1984) (Fig. 1 and Materials and Methods). We recovered nine mutations that act as dominant maternal-effect enhancers of zen^{f16}. In females carrying any of these mutations, 75-100% of zen^{f16} progeny die as embryos with a partially ventralized cuticular phenotype. Eight of the newly induced mutations were mapped to the third chromosome and shown to be alleles of *Medea*. These have been named Med^{11} through Med^{18}. The remaining mutation mapped to the second chromosome and proved to be an allele of *Mad*; it has been named Mad^{Ez}.

Phenotypic analysis indicates that Mad^{Ez} is a hypomorphic allele of the gene. Homozygous Mad^{Ez} flies are viable and fertile at 25°C. However, flies carrying Mad^{Ez} in *trans* to the phenotypically null allele Mad^{12} display imaginal disc defects (data not shown) similar to those observed in other partial loss-of-function allelic combinations of *Mad* mutations (Sekelsky et al., 1995). When rare egg-laying competent females of genotype Mad^{Ez}/Mad^{12} are mated with wild-type males, all their progeny die as embryos. One-half (55%, n=533) of the embryos, of presumptive genotype $Mad^{12}/+$, have a weakly ventralized phenotype (compare Fig. 2A and Fig. 2E). The remaining embryos, of presumptive genotype $Mad^{Ez}/+$, have a variably expressive dorsal-open phenotype (Fig. 2F), similar to the zygotic phenotypes caused by mutations in the *dpp* receptor genes *punt* and *thick veins* (Affolter et al., 1994; Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1994) or in the gene encoding the zinc-finger transcription factor *schnürri* (Arora et al., 1995; Grieder et al., 1995).

Even though all eight *Medea* alleles recovered in our screen

enhance zen^{f16} to a similar degree, they result in a variety of phenotypes. Six strong alleles (Med^{11}, Med^{12}, Med^{13}, Med^{14}, Med^{16} and Med^{18}) are lethal in *trans* to each other and to a deficiency of the locus, *Df(3R)E40* (data not shown). Trans-heterozygous larvae arrest with a phenotype identical to that described by Raftery et al. (1995). In contrast, flies homozygous for either of the remaining two mutations, Med^{15} or Med^{17}, are viable without obvious phenotypic abnormalities.

Phenotypic analysis of embryos from homozygous Med^{15} females reveals the direct role of *Medea* in patterning the embryonic dorsal-ventral axis. When Med^{15} females are mated with wild-type males, all of their progeny die with a partially ventralized phenotype (compare Fig. 2A with Fig. 2C) that is nearly identical to that of embryos lacking *zen* activity. Consistent with their cuticular phenotypes, we found that these embryos do not differentiate amnioserosa as assayed by β-galactosidase staining of a *Kr-lacZ* transgene (compare Fig. 2B with Fig. 2D).

The function of *Medea* in patterning the imaginal discs was revealed by analysis of transheterozygotes between Med^{15} and any of the lethal *Medea* alleles. While some trans-heterozygotes die at the pharate adult stage, many escapers eclose with a range of phenotypes similar to those exhibited by *dpp* mutants defective in imaginal disc patterning (Spencer et al., 1982). The observed defects include split nota (Fig. 3A,B), intercalary and terminal gaps in L4 wing veins, absent or gapped posterior crossveins (Fig. 3C,D), and foreshortened legs that lack tarsal claws and often lack several tarsal segments (Fig. 3E,F).

Med^{17} confers a less severe phenotype than does Med^{15}. 98% (n=550) of the embryos from homozygous Med^{17} females hatch. In addition, transheterozygotes between Med^{17} and any of the lethal *Medea* alleles are viable and do not display any disc patterning defects. However, when these trans-heterozygous females are mated with wild-type males, between 28 and 90% of their progeny die with a weakly ventralized phenotype (Table 1).

We performed a series of genetic crosses to compare the effect of each lethal mutation with that of a known deficiency of *Medea*. Like the alleles identified in the screen of Raftery et al. (1995), all of our lethal *Medea* alleles acted as strong dominant enhancers of the moderate dpp^{hr4} mutation (Table 1). However, the percentage enhancement of a weaker *dpp* allele, dpp^{hr56}, varied among alleles. Approximately 50% of the dpp^{hr56}/+ progeny from females heterozygous for the *Medea* deficiency *Df(3R)E40* or for the alleles Med^{13}, Med^{14} or Med^{16} survived to adulthood. However, proportionally fewer dpp^{hr56}/+ progeny survived from females heterozygous for the other three mutations, Med^{11}, Med^{12} and Med^{18}. A similar phenotypic series was observed by measuring the percentage of ventralized embryos arising from females carrying each lethal *Medea* allele in trans to the weak allele, Med^{17}. This antimorphic or dominant negative character of the Med^{11}, Med^{12} and Med^{18} mutations suggests that they may produce polypeptides that interfere with the signaling activity of the wild-type *Medea* gene.

The Med^{15} and Med^{17} alleles confer homozygous viability; therefore they must retain some *Medea* activity. However, Med^{15} and Med^{17} can enhance other *dpp*-pathway mutants as strongly as does a deficiency of the *Medea* locus. Specifically, Med^{15} and Med^{17} enhance zen^{f16} and dpp^{hr4}/+ as strongly as do null *Medea* alleles, and Med^{15} enhances dpp^{hr56} as strongly as do null *Medea* alleles (Table 1). These results are consistent with the hypothesis that the Med^{15} and Med^{17} compromise one of two mutations

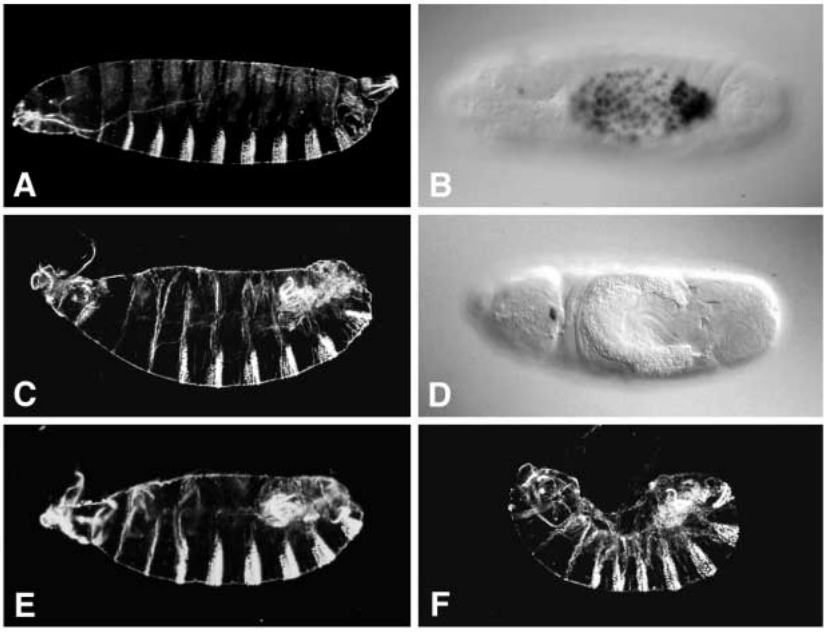


Fig. 2. Maternal effect phenotypes of *Mad* and *Medea* mutants. (A) A dark-field photomicrograph of a lateral view of a cuticle of a wild-type embryo, dorsal up, anterior left. The ventral-most ectodermal cells form the neurogenic ectoderm, characterized by the segmentally repeated pattern of ventral denticle bands. Dorsolateral cells differentiate dorsal hairs, only faintly visible in the cuticle preparation. The filzkörper, respiratory structures of the tail, are derived from cells in a dorsolateral position in the blastoderm. The dorsal-most cells form the amnioserosa, which does not contribute to the embryonic cuticle but can be visualized in a dorsal view of a wild-type stage 15 embryo (B) after staining for β-galactosidase activity from a *P{Kr-lacZ}* construct expressed in the amnioserosa. Amnioserosa cells are large and squamous and some cells can be individually visualized after staining for β-galactosidase activity. (C,D) One of the mutants recovered in the *zen* enhancer screen, Med^{15}, is homozygous viable but displays a maternal-effect ventralization of embryonic dorsal-ventral pattern. Females homozygous for the Med^{15} mutation lay eggs that are partially ventralized. In the lateral view of the cuticle shown in C, filzkörper are internalized due to lack of amnioserosa, evident in D from the absence of β-galactosidase activity after staining a stage 15 embryo carrying the *P{Kr-lacZ}* construct. The residual anterior staining reflects expression of the *P{Kr-lacZ}* construct in the Bolwig's organs (Schmucker et al., 1992), which are paired in the wild-type but fused at the dorsal midline in the mutant embryo. (E,F) Cuticles secreted by embryos laid by females of genotype Mad^{Ez}/Mad^{12} that have been mated with wild-type males are of two phenotypes: one half of the embryos are weakly ventralized (E) and are of presumptive genotype Mad^{12}/+, while the other half of the embryos develop a dorsal open phenotype (F) and are of presumptive genotype Mad^{Ez}/+.

Table 1. Dominant phenotypic interactions of *Medea* mutant alleles with mutations in the *dpp* pathway

Medea allele	Percentage survival to adulthood of flies from females heterozygous for the *Medea* mutation*			Percentage hatching of embryos from females of the *Medea* mutation in *trans* to Med^{17}†
	dpp^{hr4}/+	dpp^{hr56}/+; *Med*/+	dpp^{hr56}/+; *TM3*/+	
Df(3R)E40	0	56	94	50
11	0	12	24	45
12	0	3	101	40
13	0	88	104	72
14	0	72	75	53
15	0	60	42	93
16	0	63	72	52
17	1	94	95	98
18	0	16	26	11

*Adult viability of progeny from *Medea/TM3* females mated to dpp^{hr4}/*CyO* or dpp^{hr56}/*CyO* males. Heterozygous *dpp* mutant progeny were identified as straight-winged adults. Their survival was normalized to the survival of progeny bearing the *CyO* chromosome, recognized by the dominant *Cy* phenotype on *CyO*. Survival of dpp^{hr4}/+ progeny includes both *Med*/+ and *TM3*/+ progeny. Survival of dpp^{56}/+ progeny is presented separately for *Med*/+ and *TM3*/+ progeny; the latter are distinguished by the dominant *Sb* phenotype on *TM3*.

†Embryonic viability of progeny from a mating of transheterozygous females of Med^{17} and the indicated *Medea* mutant allele with wild-type males.

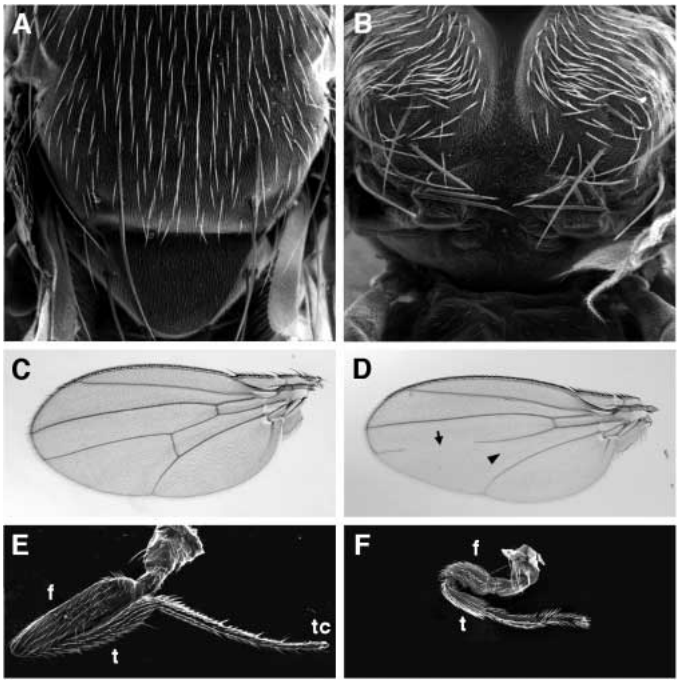


Fig. 3. Adult flies transheterozygous for Med^{15} and the lethal *Med* mutations display a range of abnormalities in the adult cuticle, indicating that defects occur during imaginal disc development. These phenotypes are similar to those observed in various dpp^{disk} mutants (Spencer et al., 1982). (A) During metamorphosis, the proximal portions of the wild-type wing discs fuse on the dorsal side to form the notum. (B) The notum of a Med^{15}/Med^{11} transheterozygous individual that has a large medial cleft, caused by the failure of the dorsal portions of the imaginal epithelia to fuse. (C) Wild-type wings have five major longitudinal veins. (D) A wing of a Med^{15}/Med^{13} transheterozygote that did not differentiate the posterior crossvein (arrowhead) and contained a large discontinuity in the L4 vein (arrow). (E) A wild-type leg indicating the femur (f), tibia (t), and tarsal claws present on the fifth tarsal segment (tc). (F) A leg from a Med^{15}/Med^{11} transheterozygote at the same scale as the wild type in E, showing shortening of the femur and tibia and loss of the fourth and fifth tarsal segments.

independently mutable functions of *Medea*, while leaving a second function intact. To test this hypothesis, we determined whether a further reduction in maternal and zygotic *Medea* activity would worsen the dorsal-ventral patterning defects caused by Med^{15}. Specifically, we compared the phenotypes of embryos from Med^{15} females mated with wild-type males to the phenotypes of embryos from Med^{15}/Med^{16} females crossed to Med^{13}/TM3 males (Med^{16} and Med^{13} are null alleles of *Medea*). All of the embryos from this latter cross are thus derived from females that contain a two-fold reduction in the dose of Med^{15}, and one fourth of the progeny embryos, of genotype Med^{13}/Med^{16}, completely lack zygotic *Medea* activity. We found that this further reduction in *Medea* activity resulted in only a slight increase in the phenotypic severity of the mutant embryos: while all embryos from a Med^{15} mother mated to wild-type males have phenotypes similar to those caused by a weak allele of *dpp*, dpp^{hr56}, some embryos from the latter cross had phenotypes similar to those caused by a slightly stronger *dpp* allele, dpp^{hr4} (Wharton et al., 1993; data not shown). Both of these phenotypes are much weaker than the phenotype caused by complete loss of *Medea* activity (see below) and support the hypothesis that Med^{15} severely compromises, but does not totally eliminate, one of two independently mutable activities of the Medea protein.

Medea is required for signaling downstream of the *thick veins* receptor

The ventralized phenotype of embryos from Med^{15} homozygous females indicated that *Medea* is involved in embryonic dorsal-ventral patterning. To determine the phenotype caused by the complete loss of maternal and zygotic *Medea* activity, we used the FLP-FRT system coupled with ovo^{D1} (Chou and Perrimon, 1996) to induce mitotic recombination within the female germline and generate germ cells homozygous for the Med^{13} mutation. When mated to Med^{14}/*TM3* males, these germline clone (GLC) females laid eggs of two phenotypic classes. 48% (n=219) of the embryos had a partially ventralized phenotype (Fig. 4C) and were of presumptive zygotic genotype Med^{13}/*TM3*. These cuticular phenotypes were characterized by the presence of dorsal hairs, internalization of filzkörper, and elimination of dorsolaterally derived structures of the head. To determine whether any

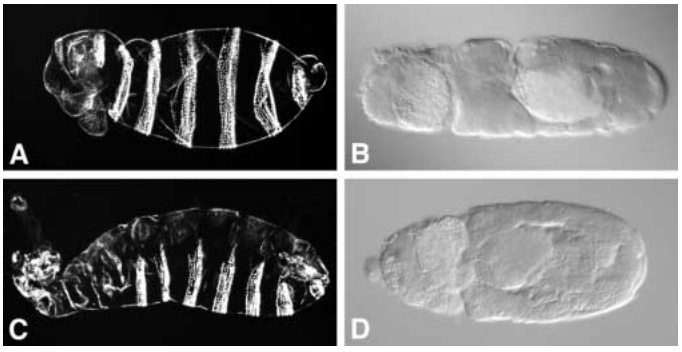


Fig. 4. Phenotypes caused by elimination of maternal *Medea* activity. Phenotypes of progeny of females of genotype *P{hsFLP}*^{1}/+; *FRT*$^{3R\text{-}82B}$ *Med*^{13}/ *FRT*$^{3R\text{-}82B}$ *P{ovo*^{D1}*}*^{3R} that were heat shocked as larvae and mated to males of genotype *Med*^{14}/*TM3, Sb* (A,C), *Med*^{14} *P{Kr-lacZ}/TM3 P{Ubx-lacZ}* (B), or *P{Kr-lacZ}* (D). *ovo*^{D1} prevents germ cell maturation; therefore all eggs from these females were derived from clones of germ cells that had undergone mitotic recombination to become homozygous for the *Med*^{13} mutation. (A) A cuticle produced by an embryo of zygotic genotype *Med*^{13}/*Med*^{14}. The cuticle lacks all dorsal structures and is phenotypically identical to those produced by embryos lacking all *dpp* activity. (B) A stage 15 embryo of zygotic genotype *Med*^{13}/*Med*^{14} *P{Kr-lacZ}* that did not differentiate any amnioserosa as assayed by β-galactosidase staining. (C) A cuticle produced by an embryo of zygotic genotype *Med*^{13}/*TM3* that is weakly ventralized. The cuticle contains some dorsal hairs, and has internalized filzkörper. (D) A stage 15 embryo of zygotic genotype *Med*^{13}/+ *P{Kr-lacZ}* that did not differentiate any amnioserosa as assayed by β-galactosidase staining.

embryos of this genotype formed amnioserosa, GLC females were mated with males carrying a *P{Kr-lacZ}* marker. 97% (*n*=152) of such embryos failed to differentiate any amnioserosa as assayed by β-galactosidase activity from *P{Kr-lacZ}*, (Fig. 4D), indicating a partial ventralization of the embryonic pattern. Thus, in the absence of maternal *Medea* activity, zygotic *Medea* function can only partially rescue the embryonic pattern.

The second class of embryos from the above cross, of presumptive zygotic genotype *Med*^{13}/*Med*^{14}, had cuticular phenotypes indistinguishable from those of *dpp* null mutant embryos (Fig. 4A). These embryos did not differentiate any dorsal structures, but instead differentiated ventral denticle bands around the entire embryonic circumference. In addition, no embryos of this genotype differentiated amnioserosa (Fig. 4B). These results indicate that *Medea*, like *dpp*, is required for the specification of all dorsal structures in the *Drosophila* embryo. In the remainder of the text, we refer to embryos from GLC females that lack both maternal and zygotic *Medea* activity as '*Medea* GLC-null' embryos.

To formally determine the order of action of the *Medea* gene relative to that of *dpp* and its receptor *tkv*, we injected mRNAs encoding DPP or a constitutively active form of the *tkv* receptor (*tkv-a*) into syncytial blastoderm embryos of various *Medea* mutant genotypes. Injection of either *dpp* or *tkv-a* mRNA into *dpp* null embryos induces amnioserosa, indicating that amnioserosa formation can result directly from the activity of the injected mRNA and not from any subsequent induction of *dpp* transcription (Holley et al., 1996). We first injected 4 μg/μl *dpp* or *tkv-a* mRNAs into embryos from *Med*^{15} mutant females. Injection of either mRNA resulted in the restoration of amnioserosa (*dpp*, 89%, *n*=166; *tkv-a*, 92%, *n*=52) in these mutant embryos (compare Fig. 5A,B with Fig. 2B). Furthermore, the majority of embryos differentiated a greater amount of amnioserosa than is present in the wild type. Thus, an increase in *dpp* signaling caused by the injection of either mRNA is sufficient to bypass a partial loss of *Medea* activity.

We then injected the same concentration of *dpp* or *tkv-a* mRNAs into embryos completely lacking maternal and zygotic *Medea* activity. Phenotypic analysis of the injected *Medea* GLC-null embryos demonstrated that *Medea* function is required for the dorsalizing activity of both injected mRNAs. Specifically, 0 of 54 *Medea* null embryos injected with 4 μg/μl *dpp* mRNA (Fig. 5C) and 0 of 31 injected with 4 μg/μl *tkv-a*

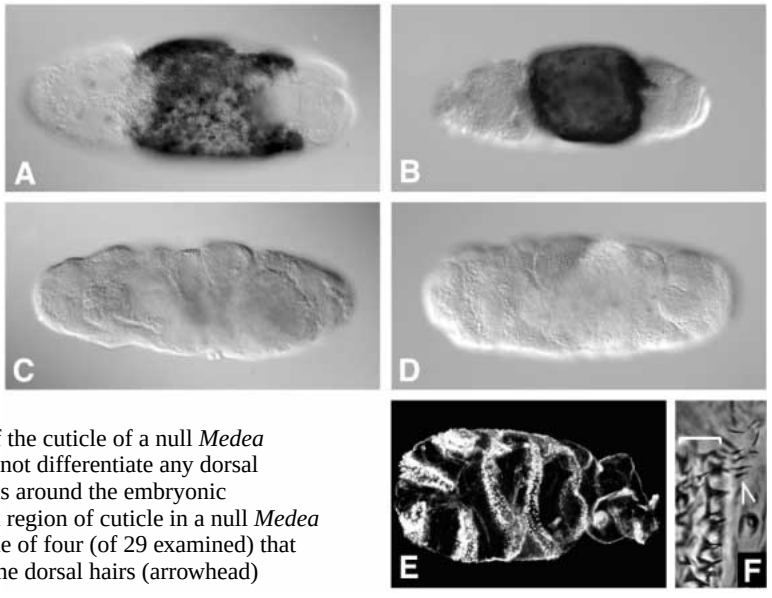
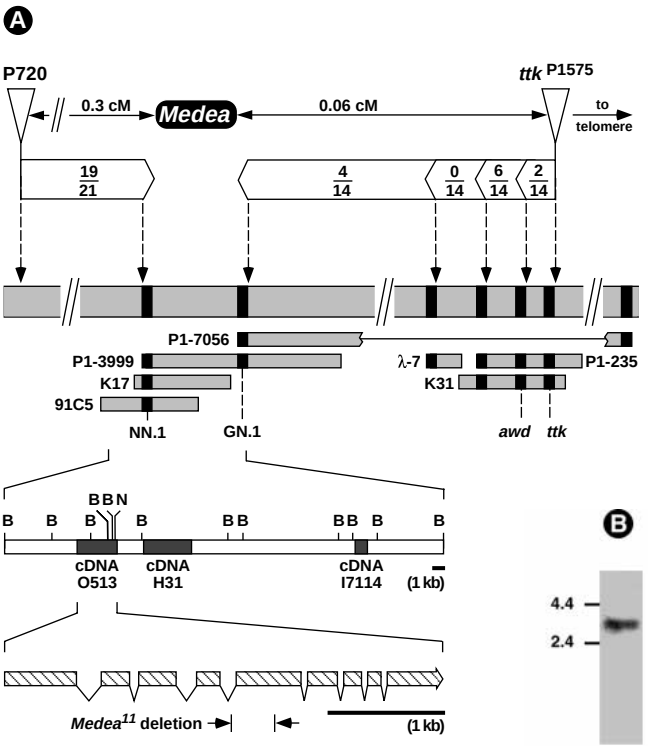


Fig. 5. The phenotypic response of embryos of various *Medea* genotypes to injection of *dpp* or activated *thick veins* (*tkv-a*) mRNAs. Induction of amnioserosa was assayed by β-galactosidase expression in stage 15 embryos carrying a *P{Kr-lacZ}* construct (A-D) or by dark-field (E) or phase contrast (F) photomicroscopy of embryonic cuticles. Injection of 4 μg/μl *dpp* mRNA (A) or 4 μg/μl *tkv-a* mRNA (B) induced amnioserosa in embryos laid by *Med*^{15} mutant females mated with *P{Kr-lacZ}* males. In the majority of embryos, more amnioserosa was induced than is present in wild-type embryos. (C-F) Null *Medea* embryos of genotype *Med*^{13}/*Med*^{14} *P{Kr-lacZ}* laid by germline clone females that were mated with *P{Kr-lacZ} Med*^{14}/*TM3 P{Ubx-lacZ}* males. The embryo in C was injected with 4 μg/μl *dpp* mRNA, while the embryo in D was injected with 4 μg/μl *tkv-a* mRNA. Neither embryo differentiated amnioserosa. (E) A dark-field photomicrograph of the cuticle of a null *Medea* embryo after injection of 4 μg/μl *tkv-a* mRNA. The embryo did not differentiate any dorsal structures, as evidenced by the presence of ventral denticle bands around the embryonic circumference. (F) A phase contrast photomicrograph of a small region of cuticle in a null *Medea* embryo injected with 4 μg/μl *tkv-a* mRNA. This embryo was one of four (of 29 examined) that differentiated any dorsal structures, in this case a grouping of nine dorsal hairs (arrowhead) adjacent to a row of ventral denticles (bracket).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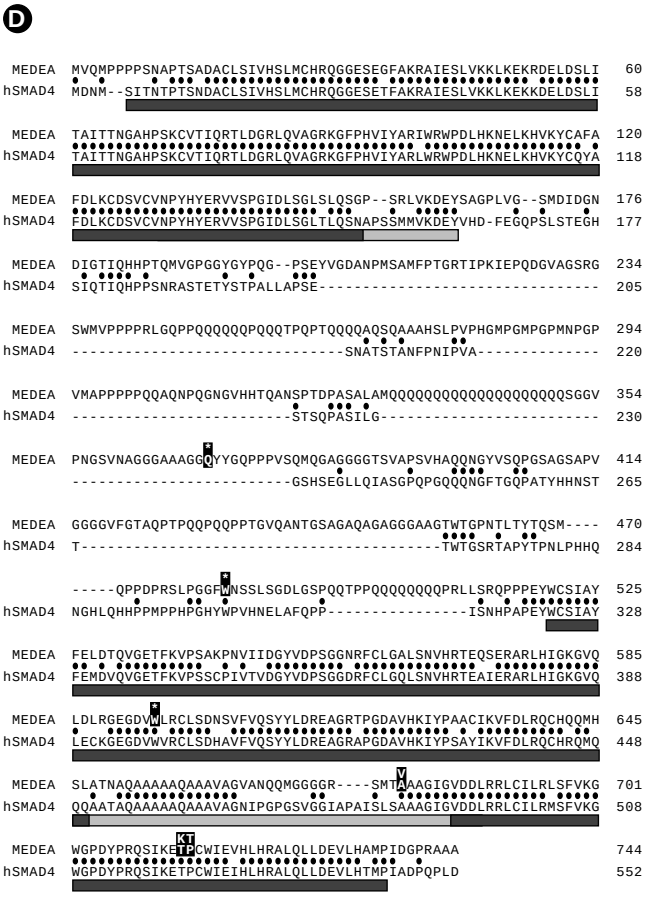
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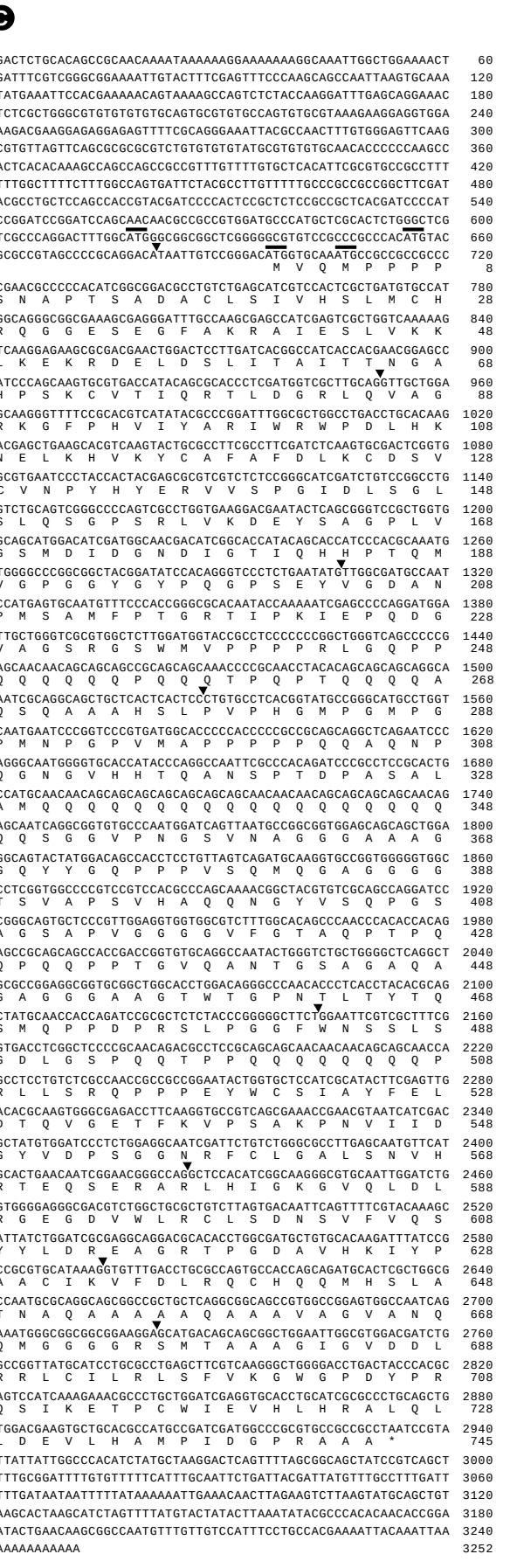
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ATTTGCGGATTTTGTGTTTTTCATTTGCAATTCTGATTACGATTATGTTTGCCTTTGATT 3060
ATTTGATAATAATTTTTATAAAAAATTGAAACAACTTAGAAGTCTTAAGTATGCAGCTGT 3120
AAAGCACTAAGCATCTAGTTTTATGTACTATACTTAAATATACGCCCACACAACACCGGA 3180
GATACTGAACAAGCGGCCAATGTTTGTTGTCCATTTCCTGCCACGAAAATTACAAATTAA 3240
AAAAAAAAAAAA 3252

Fig. 6. Molecular analysis of the *Medea* locus. (A) By three factor mapping, *Medea* was localized 0.3 cM distal to the P-element insertion P720 in 100B and 0.06 cM proximal to the *tramtrack* (*ttk*) locus at 100D1-2. To correlate the physical and genetic maps in the region, P1, cosmid, and λ clones that contained genomic inserts in 100D (narrow gray bars) were used as a source of probes (solid black inserts) for RFLP analysis between the Med^{15} chromosome and each of the P-element bearing chromosomes. The relative position of each clone on the physical chromosome (large gray box) is to scale; note however, that the chromosomal map contains three gaps of unknown length, indicated by double slashes. One of the P1 clones, P1-7056, contains non-contiguous DNA. The gap in the P1 is represented by a thin line. The locations of the genes *abnormal wing discs* (*awd*) and *ttk* are depicted on the physical map. Upon identification of a RFLP, the battery of recombinant chromosomes (21 chromosomes with breakpoints between P720 and *Medea*, and 14 chromosomes with breakpoints between *Medea* and P1575) was probed to map each recombination breakpoint relative to the RFLP. The numbers within the open, arrow-headed boxes indicate the fraction of the recombinants with breakpoints within the given physical interval. This analysis delimited an approximately 40 kb region, defined by the probes NN.1 and GN.1, that contained *Medea*. Three transcription units (black boxes) were localized within this 40 kb region (B = *Bam*HI, N = *Not*I). Med^{11} is a deletion that extends from the fourth intron into the fifth exon of the *Medea* gene, defined by the cDNA O513. (B) A northern blot containing poly(A)-enriched RNA from 0 to 2 hour embryos was probed with a 1.1 kb *Eco*RI fragment from the 3′ end of the *Medea* cDNA. The single *Medea* band was estimated to migrate as a 3.1 to 3.3 kb transcript, based upon the relative positions of the RNA markers. (C) The complete sequence of the *Medea* cDNA clone O513 is shown above that of its predicted protein product. The cDNA contains one large ORF with four potential translation start sites (solid bars); the third potential start site was chosen based on similarity of the MEDEA protein to human SMAD4. The location of each of the eight introns in *Medea* is indicated by solid triangles. (D) Alignment of the predicted MEDEA protein sequence with that of human SMAD4. Identical residues are indicated by black dots. The darkly shaded boxes beneath the protein sequences delineate the MH1 and MH2 domains that are conserved among all Smad family members as defined by Hoodless et al. (1996). The two lightly shaded boxes indicate regions of conservation that are specific to MEDEA and Smad4. Point mutations identified in the various *Medea* mutants are indicated above the protein sequence. $Medea^{16}$, $Medea^{14}$ and $Medea^{12}$ are nonsense codons at amino acid positions 370, 483, and 595 respectively. The $Medea^{15}$ chromosome has a pair of missense mutations, A679V and T714K; it is likely that the mutant phenotype is caused by the latter change. $Medea^{17}$ is a missense mutation, P715T. (GenBank accession numbers for the *Medea* cDNA clone 0513 and the wild type genomic region are AF039232 and AF039233, respectively).

mRNA (Fig. 5D) differentiated any amnioserosa tissue. Furthermore, the lack of any phenotypic effect of the *tkv-a* mRNA was not limited to the absence of induced amnioserosa; cuticle preparations from 25 of 29 *Medea* null embryos injected with 4 μg/μl *tkv-a* mRNA did not differentiate any dorsal structures (Fig. 5E). These embryos resembled uninjected embryos, indicating the complete lack of response to the constitutively active receptor. The remaining four embryos differentiated only a single small patch of cuticle containing a few (2-12) dorsal hairs (Fig. 5F). We postulate that this low level of response was due either to residual gene activity from the Med^{13} or Med^{14} alleles or to perdurance of wild-type *Medea* mRNA or protein from before the mitotic recombination event in the GLC females. We therefore conclude that *Medea* is absolutely required for signal transduction downstream of *dpp* and its receptor, *thick veins*, in the specification of embryonic dorsal-ventral pattern.

Molecular identification of the *Medea* gene

The *Medea* gene has been mapped distal to the eye color locus *claret* at meiotic map position 104 on the third chromosome, and it is deleted by *Df(3R)E40* which removes polytene bands 100C5 through 100F1-5 (Raftery et al., 1995). To undertake a molecular analysis of *Medea*, we first localized the gene more precisely by mapping it relative to two P-element insertions, P720 located in 100B and the P1575 insertion in the *tramtrack* (*ttk*) gene at 100D3 (Fig. 6A). Genetic recombination experiments indicated that Med^{15} was located 0.3 cM distal to the P720 insertion and 0.06 cM proximal to *ttk*. We obtained P1, cosmid, and λ clones with inserts of *Drosophila* genomic DNA from this region, and we used subclones from them as probes to identify restriction fragment length polymorphisms (RFLPs) between the Med^{15} chromosome and each of the two P-element chromosomes. We correlated our physical and genetic maps of this region by mapping each RFLP with respect to Med^{15} and the two P-element insertions, identifying a 40 kb genomic region expected to contain the *Medea* gene (Fig. 6A).

By screening cDNA libraries with DNA probes from the cosmid and P1 clones, we identified three transcripts from the 40 kb region (Fig. 6A). To determine whether any of these transcripts were derived from the *Medea* gene, mRNA from each cDNA was injected into embryos from Med^{15} females. mRNA from the O513 cDNA, but not from the cDNAs H31 and I7114, rescued the loss of amnioserosa (data not shown). Moreover, injection of 3 μg/μl mRNA from the O513 cDNA was sufficient to restore normal amnioserosa pattern (79%, n= 94) to *Medea* null embryos (compare Fig. 7A to Fig. 4B), demonstrating that the O513 cDNA encodes the *Medea* gene.

Sequence analysis of the *Medea* cDNA revealed an insert of 3252 bp, and northern blot analysis of mRNA from 0- to 2-hour embryos identified a *Medea* transcript of 3.1-3.3 kb (Fig. 6B), indicating that this cDNA is nearly full length. Comparison of the genomic DNA sequence with that of the cDNA indicated that the *Medea* transcript is contained within 4.2 kb of genomic DNA and is composed of nine exons. The cDNA sequence (Fig. 6C) contains a large open reading frame (ORF). The 5′ end of the ORF contains four in frame methionine codons. The sequences upstream of each methionine codon share approximately equal similarity with the consensus *Drosophila* translational initiation site (Cavener and Ray, 1991); however, we have chosen to represent the protein as initiating at the third methionine codon, based on sequence similarity with its human homolog (see below). Assuming that this AUG is used as the start codon, translation of the ORF would produce a 745 amino acid protein of predicted molecular mass 79×10^3.

The amino acid sequence of the deduced MEDEA protein was compared with sequences in the translated GenBank database. A high overall similarity was found between MEDEA and members of the Smad family of proteins. The highest degree of similarity was found between MEDEA and the murine and human Smad4 proteins (Fig. 6D). Smad family members are characterized by the presence of two domains of

high amino acid similarity, MH1 and MH2. The MEDEA protein is 97% identical to human SMAD4 over the entire MH1 domain, and is 82% identical to SMAD4 within MH2. The MH2 domains of MEDEA and SMAD4 differ from those of the other Smads in that they each contain an insert of 30 amino acids, which is 69% identical between the two proteins, in place of eight amino acids conserved among other Smads. The high degree of sequence similarity between MEDEA and SMAD4, coupled with the conservation of the SMAD4-specific domain within MEDEA, strongly suggests that MEDEA and hSMAD4 may be related by direct evolutionary descent, and thus they may be true orthologs. MEDEA and SMAD4 are largely unrelated in the linker region between the MH1 and MH2 domains, which includes two contiguous runs of glutamine residues in MEDEA that are not contained in the SMAD4 sequence.

We have localized DNA lesions in four of our six strong *Medea* alleles. One mutation, Med^{11}, is a 405 base pair deletion internal to the *Medea* gene, starting in intron 4 and extending into exon 5 (Fig. 6A). The remaining three mutations are single base pair changes that result in stop codons. Med^{16} and Med^{14} truncate the protein within the linker domain separating MH1 and MH2, at amino acid positions 370 and 483, respectively. Med^{12} truncates the protein at amino acid 595 within the MH2 domain. We note that Med^{12}, but not Med^{16} or Med^{14}, displays antimorphic characteristics in some genetic crosses (Table 1). This phenotypic difference could be due to differential stability of the three truncated polypeptides. Alternatively, it is possible that the presence of MH2 sequences are necessary for the non-functional protein-protein interactions that confer the antimorphic phenotype.

The homozygous viable *Medea* alleles, Med^{15} and Med^{17}, contain base pair changes that cause missense mutations within MH2. The Med^{15} chromosome has two such changes: a conservative amino acid change A679V and a non-conservative change T714K. The Med^{17} chromosome has the non-conservative change P715T. The adjacent amino acids affected by the Med^{15} and Med^{17} mutations, 714 and 715, map to the loop 3 (L3) region of the Smad4 MH2 domain crystal structure (Shi et al., 1997). The L3 region protrudes from the planar disk of the Smad4 trimer and has been hypothesized to mediate heteromeric interactions between Smad4 and other Smad family members.

Functional conservation between *Medea* and *hSMAD4*

We tested the ability of human *SMAD4*, as well as *Drosophila Mad*, to substitute for *Medea* function and restore *dpp* signaling in various *Medea* mutant embryos. Injection of 3 μg/μl of *SMAD4* mRNA was sufficient to restore amnioserosa in 39% (n=77) of embryos from Med^{15} females and 68% (n=53) of *Medea* null embryos from GLC females (Fig. 7C). While injection of 2 μg/μl *Mad* mRNA was sufficient to restore amnioserosa in 63% of embryos from Med^{15} females (n=191), injection of the same concentration of *Mad* mRNA did not restore amnioserosa in any *Medea* null embryos from GLC females (n=76) (Fig. 7B). Because *hSMAD4*, but not *Mad*, was able to restore normal dorsal-ventral pattern to embryos completely deficient for *Medea* activity, we conclude that hSMAD4 function can substitute for MEDEA in this patterning process.

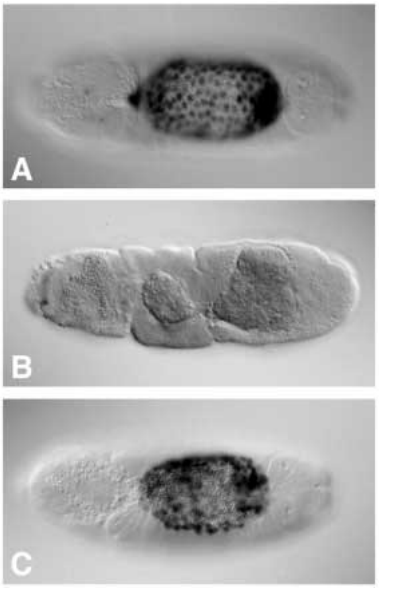

Fig. 7. Human *SMAD4* can rescue the dorsal-ventral patterning defects in *Medea*. null embryos produced by germ line clone females. All panels depict dorsal views of stage 15 embryos of genotype Med^{13}/Med^{14} *P{Kr-lacZ}* laid by germline clone females that have been mated with Med^{14} *P{Kr-lacZ}/TM3,P{Ubx-lacZ}* males. Each embryo was stained for β-galactosidase activity. (A) Restoration of amnioserosa by injection of 3 μg/μl *Medea* mRNA. (B) Lack of amnioserosa after injection of 2 μg/μl *Mad* mRNA. The same concentration of *Mad* mRNA restored amnioserosa after injection into embryos laid by Med^{15} females (not shown). (C) Restoration of amnioserosa by injection of 3 μg/μl *SMAD4* mRNA.

Overexpression of *Mad* and *Medea* is not sufficient to perturb embryonic pattern

A variety of experiments in *Xenopus* embryos have shown that overexpression of various Smads after mRNA injection has phenotypic consequences for dorsal-ventral patterning as assayed by morphology or gene expression (Baker and Harland, 1996; Graff et al., 1996; Suzuki et al., 1997; Thomsen, 1996). To determine whether overexpression of *Mad* and *Medea* have similar phenotypic consequences in *Drosophila*, we co-injected equimolar amounts of *Mad* (3 μg/μl) and *Medea* (6 μg/μl) mRNAs into wild-type embryos and observed the effects on embryonic patterning. Ventral injection of these mRNAs had no phenotypic consequences on amnioserosa production (n=68). Furthermore, none of the cuticles from injected embryos displayed any alteration in the dorsal-ventral extents of either the dorsal epidermis or neurogenic ectoderm (n=106). Rather, many cuticles displayed a variable alteration in the anterior-posterior specification of the ventral denticles at the presumptive site of injection. Specifically, 14% of embryos differentiated normal cuticles, 42% of embryos differentiated cuticles with a truncation or fusion of one or two denticle bands, 32% of embryos differentiated cuticles in which three or more denticle bands were truncated or fused, and 12% of embryos differentiated cuticles with large ventral holes. Co-injection of these mRNAs on the dorsal side of the embryos had no phenotypic consequences, either at the level of amnioserosa production or

cuticular patterning (data not shown). The lack of dorsal-ventral pattern defects in these injected embryos is in marked contrast with the strong dorsalization of the embryonic pattern observed after injection of *dpp* mRNA (Ferguson and Anderson, 1992a). Moreover, in embryos that lack endogenous *dpp* signaling ('lateralized' embryos from females of genotype $snk^{rm4}Tl^{9Q}/snk^{229}$; Ferguson and Anderson, 1992a), co-injection of the same concentrations of *Mad* and *Medea* mRNAs was not sufficient to promote amnioserosa formation (n=155), as assayed by *Kr-lacZ* expression. Thus, over-expression of the *Mad* and *Medea* genes in the *Drosophila* embryo does not appear to be sufficient either to confer a ligand-independent response leading to amnioserosa formation or to perturb the embryonic dorsal-ventral pattern.

DISCUSSION

The conservation of the mechanisms of TGF-β signal transduction across metazoan phyla has facilitated rapid progress in the analysis of this signaling system since the identification of mutations in the *Mad* and *Medea* genes of *Drosophila* (Raftery et al., 1995; Sekelsky et al., 1995). We report here a genetic and molecular characterization of the *Medea* gene. We show that *Medea*, like the *dpp* receptor genes *tkv* and *punt*, is absolutely required for the *dpp*-dependent specification of dorsolateral and dorsal cell fates in the embryonic ectoderm (Irish and Gelbart, 1987; Letsou et al., 1995; Nellen et al., 1994; Ruberte et al., 1995; Terracol and Lengyel, 1994). Furthermore, we show formally that *Medea*, like *Mad* (Newfeld et al., 1997), is required downstream of *dpp* and the *tkv* receptor for the specification of dorsal cell fates. However, other results suggest that *Medea* may not be absolutely required to transduce all *dpp*-dependent signals during *Drosophila* development (Wisotzkey et al., 1998). We have cloned the *Medea* gene and shown that it shares significant sequence similarity with human *SMAD4*. The striking conservation of these signaling pathways is further illustrated by our finding that *SMAD4* mRNA can substitute for *Medea* activity in embryonic dorsal-ventral patterning.

Medea and the Smad family

The conservation of sequence and function between *Medea* and human *SMAD4* indicates that their protein products share essential structural features that mediate interactions with other signal transduction components. In particular, we expect that MEDEA and the hSMAD4 protein share the ability to interact productively with the *Drosophila* MAD protein and possibly other cofactors to control the activation and/or repression of downstream target genes in this patterning process. The sequence similarity between MEDEA and Smad4 is primarily restricted to the MH1 and MH2 domains. The MH1 domains of *Drosophila* MAD and human SMAD4 have DNA binding activity in vitro, and the MH2 domains of human SMAD1 and SMAD4 can activate transcription at a heterologous promoter (Kim et al., 1997; Liu et al., 1996; Yingling et al., 1997). We therefore anticipate that the MH1 and MH2 domains of MEDEA will be the essential domains in the transmission of *dpp* signals from receptor to nucleus, although the regulation of target gene expression by the *dpp* signaling system remains poorly understood. No function has yet been ascribed to the *SMAD4* linker domain. The observations that MEDEA and *SMAD4* diverge considerably within this region yet share all functions indicate that the linker may not carry out essential functions.

Separately mutable functions of *Medea*

Through our analysis of the two homozygous viable *Medea* alleles, Med^{15} and Med^{17}, we have suggested that the MEDEA protein contains at least two independently mutable activities in the transduction of *dpp* signals. In particular, we propose that Med^{17} and especially Med^{15} are compromised in the dosage-sensitive specification of amnioserosa, but that both mutant proteins retain a separable function required for the specification of dorsolateral cell fates in the embryo.

What could the two separately mutable activities of *Medea* represent? One possibility is that each activity represents a differential capacity to transduce a signal downstream of each of the two type I DPP receptors, TKV and SAX. Embryos that lack both maternal and zygotic *tkv* activity differentiate no dorsal structures, similar to the complete loss of *Medea* (Nellen et al., 1994; Terracol and Lengyel, 1994). In contrast, although the phenotypes of embryos completely lacking *sax* activity have not been reported because of a requirement for *sax* during oogenesis (Twombly et al., 1996), existing mutations in *sax* result only in the loss of amnioserosa, similar to the phenotype caused by the Med^{15} mutation (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). These parallels suggest that Med^{15} and Med^{17} mutants may be defective in the response to signals downstream of the SAX receptor, while still transducing signals from the TKV receptor.

In light of this proposal, we note that both Med^{15} and Med^{17} have amino acid substitutions in loop 3, an element of the Smad4 crystal structure that is implicated in productive heteromeric interactions with activated receptor-specific Smad proteins (Shi et al., 1997). The mutant MEDEA proteins might therefore have a diminished capacity to form particular heteromeric complexes with MAD in response to signaling by one receptor but not another. Alternatively, the mutant MEDEA proteins could have selective disruptions in interactions with other components of the signaling system, such as factors that may collaborate with MAD and MEDEA to regulate expression of specific target genes. Full evaluation of this proposal awaits biochemical characterization of signaling downstream of the TKV and SAX receptors in vivo.

Regulation of Smad function

Observations from a series of mRNA injection experiments suggest that MAD and MEDEA activities are tightly restricted by the spatial distribution and level of *dpp* signaling. Although injected *Mad* or *Medea* mRNA can restore essentially normal dorsal-ventral pattern to embryos that lack some or all endogenous *Medea* activity, neither induces further dorsalization of the embryo. Furthermore, while injection of *dpp* or activated *tkv* mRNA induces ectopic amnioserosa in a concentration-dependent fashion (Holley et al., 1996), overexpression of *Mad* and *Medea* in wild-type embryos has no effect on dorsal-ventral pattern. Overexpressed MAD and MEDEA proteins therefore require additional positive input for activity, such as MAD phosphorylation by the DPP receptors or the activation of other proteins required for *dpp* signaling.

We note that the *Drosophila* embryo differs in this regard from *Xenopus* and cell culture systems in which *Smad* overexpression elicits responses (Baker and Harland, 1996; Chen et al., 1996; Graff et al., 1996; Liu et al., 1997; Suzuki et al., 1997; Thomsen, 1996); responsiveness in these systems to ectopic Smad expression could reflect amplification of a low level of ambient signaling rather than de novo generation of a response.

Alternatively, the different responses of these systems to Smad overexpression may reflect inherent differences in the distributions of negative regulators such as *Drosophila* DAD and vertebrate Smad6 and Smad7, which are structurally related yet functionally opposed to the signaling Smads (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997). Such intracellular negative regulation of *dpp* signaling in the *Drosophila* embryo is also suggested by the observations that although genetic experiments demonstrate *Mad* activity is present in limiting quantity (Raftery et al., 1995), endogenous levels of *dpp* signaling in the embryo do not lead to detectable nuclear localization of MAD (Newfeld et al., 1996, 1997). Taken together, these observations suggest that a pool of cytoplasmic MAD may be needed to titrate a stoichiometric cytoplasmic inhibitor. Because *Medea*, like *Mad*, is easily rendered dosage sensitive, it may be subject to a similar negative regulation in the cytoplasm.

Conclusions

With this report and that of Raftery et al. (1995), two independent genetic screens have been conducted to identify maternally supplied, dosage-sensitive components of the *dpp* signaling pathway. Together these screens led to the recovery of five alleles of *Mad* and eleven alleles of *Medea* from among 6000 mutagenized haploid genomes. Additional work will be required to identify *dpp* signaling components that are less dosage sensitive than *Mad* and *Medea* and to identify negative regulators of *dpp* signaling. The recovery in this work of *Medea* alleles with a range of mutant phenotypes affords new opportunities for such efforts. Furthermore, our finding that human *SMAD4* can replace the function of the *Medea* gene will allow the dissection of *SMAD4* functions within a developmental context.

We thank William Gelbart, Stuart Newfeld, Eugene Xu, Chung-I Wu, Anthony Mahowald, Christopher Schonbaum, Laurel Raftery, Kenneth Tartof, Jeffrey Neul, Allen Shearn, Craig Montell, Joan Massagué, Janice Fischer, I. Siden Kiamos, Thomas Kaufman, and Roger Karess for fly stocks, cloned DNAs or genomic libraries. We thank Ed Williamson for assistance with scanning electron microscopy; Nipam Patel for advice and use of equipment for photomicroscopy; Terrence Banks, Christian Denes, Adriane Stewart, Deborah Dorsett for fly work, and Joseph June and Jim Miller for assistance in the preparation of genomic DNA. Genomic sequencing was performed at the University of Chicago Cancer Research Center DNA Sequencing Facility. We are grateful to Laurel Raftery and Richard Padgett for communication of results prior to publication. We thank Judith Austin, Jeffrey Neul, Eva Rosen and Harinder Singh for helpful comments on the manuscript. This work was supported by grants from the Mallinckrodt Foundation and from the American Cancer Society (DB-79090). J. B. H. and S. D. P. were supported by the NCI Training Program (5T32-CA09594), S. D. P. was supported by a N. I. H. postdoctoral fellowship (HD07959), and K. K. was supported by N. I. H. training grant GM07183. E. L. F. is a Pew Scholar in the Biomedical Sciences.

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