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A genetic screen for modifiers of *Drosophila decapentaplegic* signaling identifies mutations in *punt, Mothers against dpp* and the BMP-7 homologue, *60A*

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SUMMARY

***decapentaplegic (dpp)* is a Transforming Growth Factor beta (TGF-β)-related growth factor that controls multiple developmental processes in *Drosophila*. To identify components involved in *dpp* signaling, we carried out a genetic screen for dominant enhancer mutations of a hypomorphic allele of *thick veins (tkv)*, a type I receptor for *dpp*. We recovered new alleles of *tkv, punt, Mothers against dpp (Mad)* and *Medea (Med)*, all of which are known to mediate *dpp* signaling. We also recovered mutations in the *60A* gene which encodes another TGF-β-related factor in *Drosophila*. DNA sequence analysis established that all three *60A* alleles were nonsense mutations in the prodomain of the *60A* polypeptide. These mutations in *60A* caused defects in midgut morphogenesis and fat body differentiation. We present evidence that when *dpp* signaling is compromised, lowering the level of *60A* impairs several *dpp*-dependent developmental processes examined, including the patterning of the visceral mesoderm, the embryonic ectoderm and the imaginal discs. These results provide the first in vivo evidence for the involvement of *60A* in the *dpp* pathway. We propose that *60A* activity is required to maintain optimal signaling capacity of the *dpp* pathway, possibly by forming biologically active heterodimers with Dpp proteins.**

Key words: *dpp, punt, Mad, 60A*, Modifier screen, TGF-β, *Drosophila*

INTRODUCTION

The Transforming Growth Factor-beta (TGF-β) superfamily is a family of conserved polypeptide growth factors that regulate diverse biological activities (reviewed by Kingsley, 1994a; Massagué, 1996). In particular, within the TGF-β superfamily, the Bone Morphogenetic Proteins (BMPs) are critical regulators of cell proliferation, cell death, cell fate specification and organogenesis (reviewed by Kingsley, 1994b; Hogan, 1996). The versatile signaling capacity of TGF-β-related factors partially stems from extensive post-translational modifications that occur during the maturation process. These factors are synthesized as large precursor molecules, which undergo homomeric or heteromeric dimerization and subsequent proteolytic cleavage to yield the bioactive carboxy terminal portion (Roberts and Sporn, 1990). Upon secretion, their association with extracellular binding proteins is important for regulating their access to cell surface receptors (reviewed by Miyazono et al., 1993). The interaction with a heteromeric transmembrane receptor system composed of two distinct serine/threonine kinases (type I and II receptors) adds one more level of complexity to TGF-β-related signaling (Massagué and Weis-Garcia, 1996). In order to understand the complex processes involved in TGF-β signaling, we focus on *decapentaplegic* (*dpp*), a *Drosophila* gene that is functionally interchangeable with mammalian BMP-2 and BMP-4 (Padgett et al., 1993; Sampath et al., 1993).

dpp has a dynamic expression pattern and multiple functions throughout *Drosophila* development. At the blastoderm stage, *dpp* transcripts are restricted dorsally (St. Johnston and Gelbart, 1987) where it functions as a morphogen to specify distinct dorsal structures (Ferguson and Anderson, 1992a,b; Wharton et al., 1993). Later during embryogenesis, *dpp* is expressed in the ectoderm (Jackson and Hoffmann, 1994), where it induces dorsal mesoderm differentiation (Staehling-Hampton et al., 1994). In the visceral mesoderm, *dpp* is expressed in discrete domains (Panganiban et al., 1990b) to regulate the expression of several homeotic genes in different tissue layers (Bienz, 1994). *dpp* is also expressed in specific positions in the larval imaginal discs (Masucci et al., 1990) to control the proliferation and patterning of adult appendages (reviewed by Brook et al., 1996).

In addition to *dpp*, *60A* and *scw* are two other BMP-related genes in *Drosophila* (Arora et al., 1994; Doctor et al., 1992; Wharton et al., 1991). Scw proteins have been proposed to enhance *dpp* activity during early embryogenesis by forming heterodimers with Dpp (Arora et al., 1994). The role of *60A* in

development was unclear due to the lack of knowledge of the phenotypic consequences of disrupting *60A* function.

In *Drosophila*, two type I receptors encoded by *saxophone* (*sax*) and *thick veins* (*tkv*) and one type II receptor encoded by *punt* have been shown to be functional *dpp* receptors (Penton et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Ruberte et al., 1995; Letsou et al., 1995; Xie et al., 1994). *Mothers against dpp* (*Mad*), *Medea* (*Med*) and *schnurri* (*shn*) were identified through genetic interactions with *dpp* (Raftery et al., 1995; Sekelsky et al., 1995; Staehling-Hampton et al., 1995; Grieder et al., 1995; Arora et al., 1995). *shn* encodes a protein related to human zinc finger transcription factor PRDII/MBPI/HIV-EP1 (Staehling-Hampton et al., 1995; Arora et al., 1995; Grieder et al., 1995). *Mad*-related proteins (Smads) have been isolated from a wide range of distantly related organisms. Genetic and biochemical evidence indicated that Smads are key signal transducers, linking events between receptor activation and changes in target gene expression (Derynck and Zhang, 1996; Massagué, 1996; Wrana and Pawson, 1997).

The dosage-sensitive nature of *dpp* signaling prompted us to use modifier genetics to identify additional components in the *dpp* pathway. This approach exploits synergistic interactions between components in the same biological pathway. We sensitized the *dpp* pathway using a hypomorphic *dpp* receptor, tkv^6, which had a mild visible phenotype. We reasoned that, in this genetic background where *dpp* signaling is below optimal level, a two-fold reduction in the activities of other signaling components in the pathway would produce a modified phenotype. So we screened for mutations that dominantly modified tkv^6 phenotype. Such an approach has successfully identified components in several *Drosophila* signal transduction pathways, including the *sevenless* receptor tyrosine kinase pathway (Simon et al., 1991), the *Abelson* cytoplasmic tyrosine kinase pathway (Gertler et al., 1990) and the *dpp* pathway (Raftery et al., 1995).

In our screen, we recovered new alleles of *tkv, punt, Mad* and *Med*; all are known to mediate *dpp* signaling. Two other complementation groups were identified that potentially represent new components in the pathway. Most significantly, mutations in *60A*, the *Drosophila* homologue of BMP-7, were recovered as enhancers for the sensitized *dpp* pathway. We describe the loss-of-function phenotypes of *60A* and present the first in vivo evidence that *60A* acts synergistically with *dpp* in several developmental processes.

MATERIALS AND METHODS

Drosophila stocks

Drosophila stocks were cultured on standard cornmeal yeast extract sugar medium at 25°C. *Canton S.* was used as the wild-type stock. sax^5 was described in Twombly et al. (1996). Mad^P is described in Sekelsky et al. (1995). Med^4 was described in Raftery et al. (1995). All other mutants and chromosomes are referenced in Flybase (http://cbbridges.harvard.edu).

Isolation and analysis of enhancer mutations of tkv^6

The initial attempts to recover modifiers of tkv^6 in an F_1 screen was not successful because of greatly reduced viability and fertility of the flies with enhanced phenotypes. Thus an F_2 screen was carried out.

pr cn was recombined onto the tkv^6 chromosome and the stock was made isogenic for the second and the third chromosomes. tkv^6 *pr cn/CyO* males were mutagenized with 3.4 mM ethylnitrosourea (ENU) and mated to females with a translocation between *CyO* and *TM6,B* to force the co-segregation of the second and third chromosome. Individual male progeny were mated to tkv^6*/CyO; TM2/TM6,B* females. Enhancement of the phenotypes of the imaginal-disc-derived structures were screened in the progeny homozygous for tkv^6. The enhancer mutations were recovered from the siblings of the enhanced progeny and crossed to tkv^6*/CyO; TM2/TM6,B* females for retesting and to establish balanced stocks.

The number of complementation groups was determined from inter se crosses among enhancers. For enhancers on the second chromosome, a *tkv* transgene on the third chromosome (Y. C. and F. M. H., unpublished data) was used to compensate for the tkv^6 mutation present on the second chromosome. Allelism with known mutations was established by genetic non-complementation and by meiotic and deletion mapping. The *Sp Bl* L^{rm} *Bc* Pu^2 Pin^B chromosome was used for meiotically mapping enhancers on the second chromosome.

Sequencing of mutant alleles

Total RNAs were isolated from heterozygous *Mad* and *punt* females and heterozygous *60A* males using the Tri Reagent (Molecular Research Center, Inc.). Gene-specific cDNAs were reverse transcribed and amplified using the Superscript Preamplification System (GIBCO BRL). The PCR products were subcloned into TA cloning vectors (Invitrogen) and sequenced on an automated sequencer (ABI 373). Sequencing multiple alleles of the same gene allowed identification of the polymorphisms specific to the mutagenized chromosomes. For $60A^{D4}$ and $60A^{D8}$, the genomic region of *60A* was sequenced in a similar fashion because the mutant cDNAs were under-represented.

Preparation of larval gut, cuticle and adult appendages

Larval gut was dissected and mounted according to Masucci and Hoffmann (1993). Cuticles were prepared as described previously (Struhl, 1989). Wings and legs were mounted in Gary's magic mounting media (Ashburner, 1989).

Antibody staining and identification of mutant embryos

Anti-Scr, Anti-Ubx, Anti-Lab antibodies were gifts of Dr Matthew Scott, Stanford University. Anti-Wg antibody was a gift of Dr Roel Nusse, Stanford University. Anti-Dpp antibody was described in Panganiban et al. (1990a). Antibody stainings were done as previously described (Panganiban et al., 1990b; Staehling-Hampton and Hoffmann, 1994; Reuter et al., 1990). All stocks used for antibody staining were balanced over a *CyO* chromosome with an *elav-lacZ* enhancer trap to allow unambiguous identification of mutant embryos.

RESULTS

shn and *punt* enhance tkv^6 phenotypes

tkv^6 is a mutation in a splice acceptor site that results in aberrant in-frame splicing, deleting two extracellular amino acids of the receptor. When expressed in COS1 cells, the mutant receptor fails to bind BMP-2 homodimers (Penton et al., 1994). However, tkv^6 behaves genetically as a hypomorph. In contrast to the embryonic lethal *tkv* null alleles, tkv^6 is homozygous viable and the only visible phenotype is the thickened wing veins (Fig. 1B). All other imaginal-disc-derived structures of tkv^6 homozygotes appear normal (Figs 1C, 3A,C). Interestingly, tkv^6*/Df(2L)tkv2* flies are phenotypically identical to tkv^6 homozygotes (data not shown). To test if tkv^6 is a suitable genetic background for a modifier screen, we examined the effects of lowering the activity of other known *dpp* pathway components. We found that

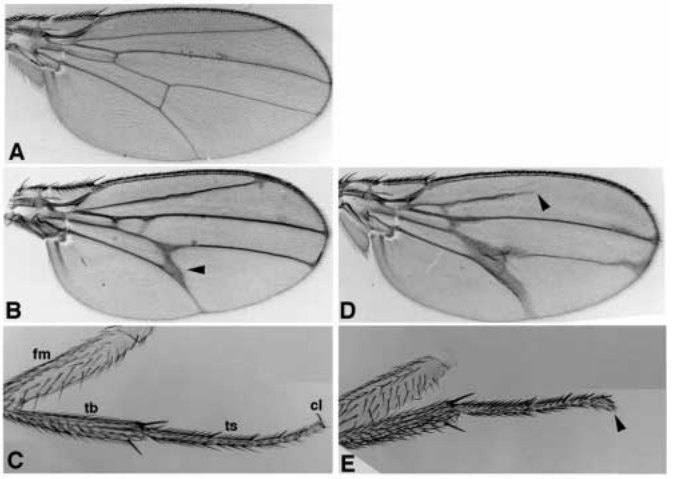

Fig. 1. *shn* enhanced tkv^6 homozygous phenotypes. (A) Wild-type wing. (B) tkv^6 wing. Thickening of the cross veins and the terminals of the longitudinal veins were evident (arrowhead). (D) $tkv^6 shn^{IB}/tkv^6$ wing. Besides the thickened wing veins, the longitudinal vein 2 was truncated (arrowhead). (C) Phenotypically normal tkv^6 mesothoracic leg. (E) $tkv^6 shn^{IB}/tkv^6$ mesothoracic leg. The distal tarsal segments and the claws were missing (arrowhead). fm, femur; tb, tibia; ts, tarsal segments; cl, claws.

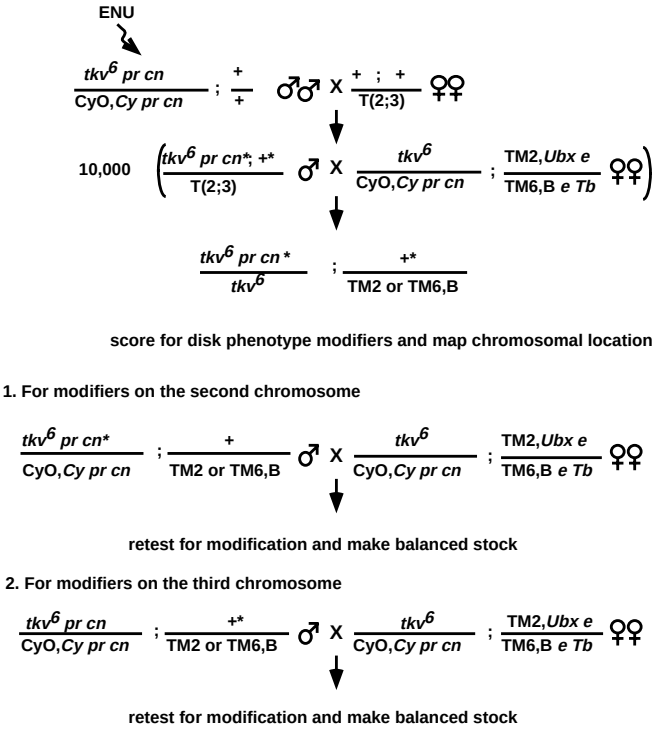

Fig. 2. Scheme for the F_2 enhancer screen of tkv^6. See Materials and Methods for detailed description of the procedure. ENU, Ethylnitrosourea; T(2;3), a translocation between *CyO* and *TM6,B*. Asterisks indicate the mutagenized chromosomes.

heterozygous mutations in *shn* or *punt* enhanced the tkv^6 homozygous phenotype. In the tkv^6 background, shn^{IB} was a dominant enhancer of the venation pattern in the wing (Fig. 1D) and the proximal/distal patterning of the leg (Fig. 1E). In the wing, longitudinal vein 2 failed to reach the wing margin (Fig. 1D). In the leg, distal elements such as claws and distal tarsal segments were deleted (Fig. 1E). Such phenotypes were reminiscent of hypomorphic *dpp* phenotypes (Spencer et al., 1982). $punt^{135}$ also enhanced the tkv^6 phenotypes (data not shown). Based on these observations, we reasoned that the *dpp* signaling output through the mutant receptor tkv^6 was near the threshold for proper patterning of the imaginal discs. It was therefore an appropriate genetic background for identifying new components essential for mediating *dpp* signaling.

Enhancers of *tkv* are phenotypically similar to *dpp* mutants

The modifier screen was conducted as outlined in Fig. 2. The F_2 progeny were screened for enhanced phenotypes in the imaginal-disc-derived structures. Over 10,000 mutagenized genomes were screened and fourteen dominant enhancers defining seven loci were recovered (Table 1). The enhancers were recessive lethal in a wild-type background. tkv^6 homozygotes that are heterozygous for the enhancer mutations had defects in imaginal disc development (Fig. 3). During pupal development, the dorsal proximal region of the two wing imaginal discs fuse to form the adult notum. In tkv^6 flies, the notum appeared normal with a smooth contour and orderly oriented sensory bristles (Fig. 3A). This pattern was disrupted by heterozygous *D1* mutation, resulting in a medial cleft in the notum, with abnormally parted bristles on both sides of the cleft (Fig. 3B). tkv^6 flies had normally patterned legs (Fig. 3C). However, heterozygous *D4* mutation caused deletions of distal and dorsal structures (Fig. 3D,E) and occasional duplication of ventrolateral structures such as sex combs on male prothoracic legs (Fig. 3E). These phenotypes were indistinguishable from

Table 1. Summary of enhancer mutations

Complementation group	Enhancement	Chromosomal location	Number of alleles	Allelic to
D1	notum	second	1	–
D2	notum	second	1	–
D3	legs and notum	second	5 (*D3*, *D14*, *D15*, *D16*, *D24*)	*Mad*
D17	legs and notum	second	1	*tkv*
D4	legs and notum	second	3 (*D4*, *D8*, *D20*)	*60A*
D5	legs and notum	third	1	*Med*
D13	legs and notum	third	2 (*D13*, *D18*)	*punt*

The assignment of the enhancer mutations to different complementation groups is based on genetic mapping and inter se complementation tests among enhancer mutations as described in Materials and Methods.

Table 2. Heterozygous interactions between enhancer mutations and *dpp* pathway mutations

	tkv^{6}	*Df(2L)tkv2*	*dpp*^{s5}	*shn*^{IB}	*sax*^{5}	*Mad*^{P}	*Med*^{4}	*punt*^{135}
tkv^{6}	–#	–#	–	–	–	–	–	–
tkv^{6}*D1*	67	71	64	60	–	–	31	40
tkv^{6}*D2*	43	53	34	–	31	–	-	41
tkv^{6}*D3*	94*	100*	96	100*	–	lethal	23	68
tkv^{6}*D15*	96*	91*	100	100*	–	lethal	29	78
tkv^{6}*D24*	100*	100*	100	100*	–	lethal	41	81
tkv^{6}*D14*	98	lethal	68	–	44	lethal	–	35
tkv^{6}*D16*	31	50	–	–	41	48	–	–
tkv^{6}*D17*	65	lethal	–	15	68	–	–	–
tkv^{6}*D4*	78	83	37	43	–	–	–	25
tkv^{6}*D8*	82	87	31	39	–	–	–	22
tkv^{6}*D20*	68	73	25	50	–	–	–	31
D5	47	52	ND	ND	ND	ND	lethal	–
D13	61	63	ND	ND	ND	ND	–	lethal
D18	62	67	ND	ND	ND	ND	–	lethal

Numbers represent the percentage of heterozygous progeny with disk phenotypes such as gaps in wing veins, cleft in the notum and/or distal truncation of the legs.

lethal, failure to recover the indicated progeny class.

*Severe reduction of the indicated progeny class (less than 10%).

Minus, no interactions observed.

ND, not done.

#Thickened wing veins only, due to non-complementation with *tkv* alleles.

those of *dpp*^{disk} alleles (Spencer et al., 1982), suggesting that these enhancers act in the *dpp* signal transduction pathway.

Additional evidence that the enhancers mediate *dpp* signaling came from their dosage-sensitive interactions with known mutations in the *dpp* pathway, including *dpp*^{s5}, *tkv*^{6}, *Df(2L)tkv2*, *sax*^{5}, *punt*^{135}, *Mad*^{P}, *Med*^{4} and *shn*^{IB} (Table 2). In many cases, the enhancers failed to fully complement these mutations and showed imaginal disc development defects ranging from gaps in wing veins to the notum and leg phenotypes described in Fig. 3. *tkv*^{6} alone showed no detectable heterozygous interactions with the *dpp* pathway mutations examined except for the thickened venation phenotype when in *trans* to *tkv*^{6} or *Df(2R)tkv2*, indicating that phenotypes observed were due to the presence of the enhancer mutations.

Dominant enhancers of *tkv*^{6}: new alleles of *tkv, Mad, Med, punt* and *60A*

Meiotic mapping and complementation tests established seven complementation groups for the enhancers. As expected based on the initial evaluation of the *tkv*^{6} genetic background, new alleles of *tkv*, *Mad*, *Med* and *punt* were recovered (Table 1). We sequenced the coding regions of the new *Mad* and *punt* alleles to establish their molecular identity. Of the five *Mad* alleles, three had point mutations in the coding region (Fig. 4A). Mis-sense mutations were found in both new *punt* alleles (Fig. 4B). The *tkv*^{D17} and *Med*^{D5} allelism was based on genetic non-complementation. In addition, it was found that a *tkv* transgene rescued the lethality of *tkv*^{D17} homozygotes, supporting the view that *D17* is a *tkv* allele (data not shown).

Genetic and molecular characterizations of the *D4* complementation group revealed that it corresponded to the *60A* gene. *60A* encodes a BMP-7 homologue isolated based on sequence homology (Doctor et al., 1992; Wharton et al., 1991). Its function remained unknown due to the lack of mutations in *60A*. Three alleles of *60A* were confirmed by sequencing the mutant alleles. *60A*^{D8} and *60A*^{D20} are nonsense mutations in the prodomain due to single nucleotide substitutions. *60A*^{D4} has one nucleotide deletion, causing a frame-shift premature stop also in the prodomain (Fig. 4C).

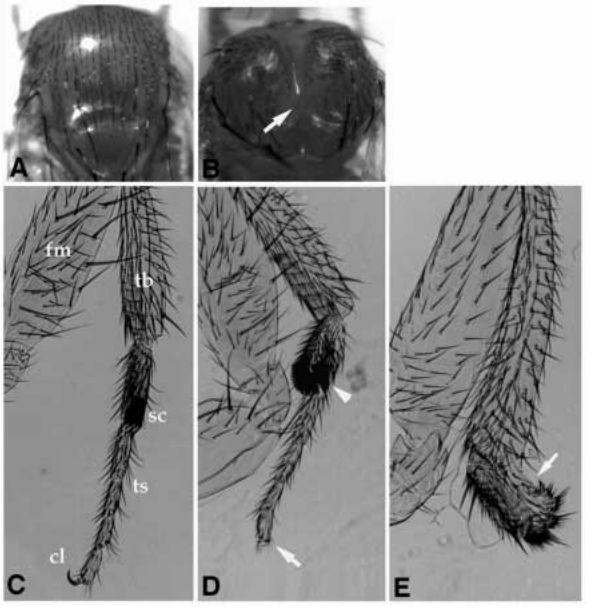

Fig. 3. Heterozygous enhancer mutations enhanced *tkv*^{6} phenotypes. (A) Phenotypically normal *tkv*^{6} notum. (B) *tkv*^{6}*D1/tkv*^{6} notum. The sensory bristles were parted to both sides and there was a profound medial cleft (arrow). The scutellum was often reduced in size (compare with A). (C) Phenotypically normal *tkv*^{6} male prothoracic leg. (D) *tkv*^{6}*60A*^{D4}*/tkv*^{6} male prothoracic leg. The most distal tarsal segments and claws were truncated (arrow) and the ventral lateral sex combs were duplicated (arrow head). (E) *tkv*^{6}*60A*^{D4}*/tkv6* metathoracic leg. Note the severe truncation of distal structures and the curved appearance of the leg caused by the loss of dorsal tissues (arrow). fm, femur; tb, tibia; sc, sex combs; ts, tarsal segments; cl, claws.

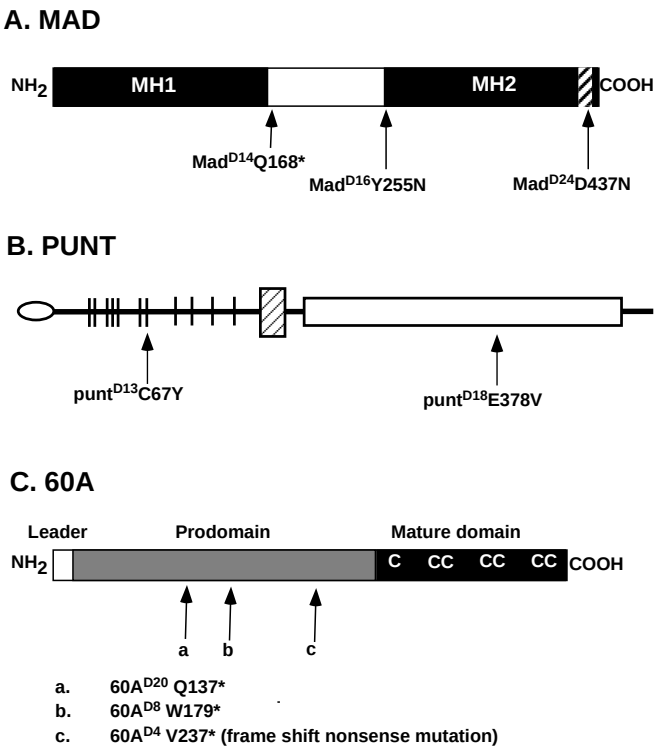

Fig. 4. Molecular lesions in new alleles of *Mad*, *punt* and *60A*. The position and nature of the mutations are indicated below the schematic diagram of the protein. Asterisk, nonsense mutation. (A) Mutations in new *Mad* alleles. The hatched box represents the mutation hot spot. (B) Mutations in new *punt* alleles. The structural features shown are the putative signal peptide (oval box), the extracellular cysteine residues (vertical bars), the transmembrane domain (hatched box) and the kinase domain (open box). (C) Mutations in *60A* alleles. Letter Cs within the mature domain represent conserved cysteine residues.

Loss-of-function phenotypes of *60A*

Animals lacking *60A* function died at late larval/early pupal stages. One of the striking phenotypes of *60A* mutant larvae is a transparent appearance due to the lack of fat body (Fig. 5B). In roughly 50% of the *60A* larvae, the gastric caecae were reduced in length (Fig. 5D), consistent with the expression of *60A* in the gastric caecae (Doctor et al., 1992). These phenotypes are similar to those of the *Mad* mutant larvae (Sekelsky et al., 1995).

During embryogenesis, *60A* is expressed throughout the visceral mesoderm of the developing midgut (Doctor et al., 1992) suggesting a function for *60A* in gut development. Indeed, embryos lacking *60A* failed to form the first constriction (Fig. 6E,F). The homeotic gene *Antennapedia (Antp)* is expressed in the visceral mesoderm around the first constriction and is required for its formation (Reuter and Scott, 1990; Tremml and Bienz, 1989). We examined the *Antp* expression in *60A* mutant embryos. Consistent with the lack of the first constriction, *Antp* expression was greatly reduced (Fig. 6B). Thus, *60A* is required for the formation of the first constriction of the midgut, likely through positively regulating the expression of *Antp* in the visceral mesoderm.

60A maintains an optimal level of *dpp* signaling in the visceral mesoderm

The identification of mutations in *60A* as dominant enhancers of tkv^6, thus *dpp* signaling, in the imaginal discs raised the possibility that *60A* is required for optimal signaling by the *dpp* pathway. To determine if there was a general requirement for *60A* in *dpp* signaling, we examined the effects of *60A* mutations on *dpp* signaling in the visceral mesoderm where both *dpp* and *60A* are expressed.

dpp is expressed in two discrete domains in the visceral mesoderm (Panganiban et al., 1990b). The anterior domain of *dpp* coincides with the gastric caecae primordia, which are immediately anterior to the expression domain of *Sex combs reduced (Scr)* in parasegment (ps) 4. The failure to initiate *dpp* expression in ps3 in dpp^{shv} mutants results in anterior expansion of *Scr* expression and arrested outgrowth of the gastric caecae (Panganiban et al., 1990b; Hursh et al., 1993), indicating a role for *dpp* in repressing *Scr* in ps3. tkv^6 homozygotes are homozygous viable, so it is not surprising that all the midgut gene expression patterns examined were essentially normal (Fig. 7A-D). *Scr* expression in tkv^6 and *60A* mutants was normal (Fig. 7A,E). However, in tkv^6 and *60A* double mutants, the *Scr* expression extended anteriorly into ps3 (Fig. 7I) as it did in dpp^{shv} mutants, suggesting that *60A* activity is required in ps3 for optimal *dpp* signaling.

To test whether *60A* also acts synergistically with *dpp* elsewhere in the midgut, we examined the gene expression of *dpp* and *Ultrabithorax* (*Ubx*) in ps7 and *wingless* (*wg*) in the

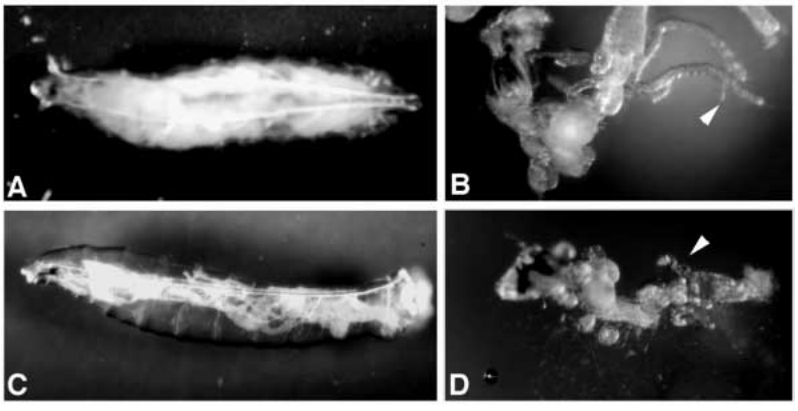

Fig. 5. Larval phenotypes of *60A*. (A) Wild-type third instar larva. (C) $60A^{D4}/60A^{D8}$ third instar larva had greatly reduced fat body and appeared transparent. (B) Wild-type third instar larval gut. Note the long and extended gastric caecae (arrowhead). (D) $60A^{D4}/60A^{D8}$ third instar larval gut. The gastric caecae were short in about half of the *60A* larvae (n=47) (arrowhead). Compared with the wild-type larva of the same stage, *60A* mutants grew more slowly and were somewhat reduced in size.

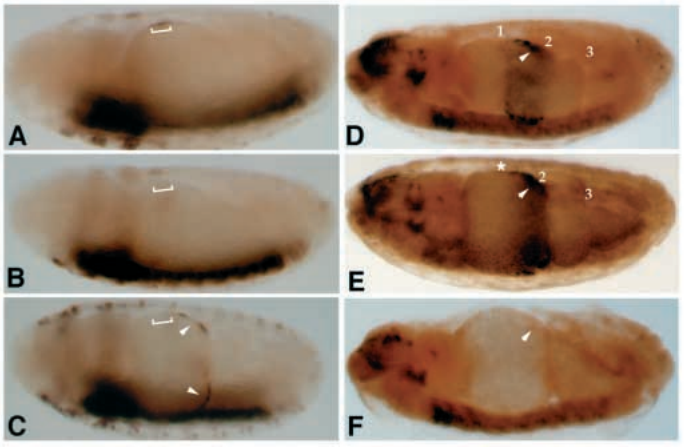

Fig. 6. *60A* mutants lacked the first constriction of the embryonic midgut. Lateral views of embryos stained with anti-Antp antibody (A-C) and anti-Lab antibody (D-F); stage 14 (A, B), stage 15 (C), stage 16 (D-F). Anterior is to the left and dorsal is up for this and subsequent figures. (A) Phenotypically normal visceral mesoderm expression of *Antp* in ps6 in tkv^{6} homozygous embryos (bracket). (B) $60A^{D4}/60A^{D8}$ embryos lacked ps6 *Antp* expression (bracket). (C) $tkv^{6}60A^{D4}/tkv^{6}60A^{D8}$ embryos lacked *Antp* expression in ps6 (bracket) but acquired an ectopic *Antp* domain in ps7 (arrowheads) presumably due to lacking *Ubx* expression in ps7 (see Fig. 7L). (D) tkv^{6} homozygotes had normal *lab* expression (arrowhead) in the endoderm and formed three constrictions indicated by numbers. (E) $60A^{D4}/60A^{D8}$ embryos failed to form the first constriction (asterisk) but the second and third constrictions still formed. *lab* expression was not altered (arrowhead). (F) $tkv^{6}60A^{D4}/tkv^{6}60A^{D8}$ embryos lacked *lab* expression (arrowhead) in the endoderm and only formed one constriction.

adjacent ps8. It has been established that ps7 expression of *dpp* is activated by the homeotic gene *Ubx* (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990; Capovilla et al., 1994; Sun et al., 1995) and maintained by an autostimulatory circuit involving *dpp*, *Ubx* and *wg* (Hursh et al., 1993; Thüringer et al., 1993a,b; Staehling-Hampton and Hoffmann, 1994). The proper expression of all three genes is interdependent and critical for maintaining a stable cellular differentiation commitment (Bienz, 1994). The expression of *dpp* and *wg* in the visceral mesoderm is required for the induction of the homeotic gene *labial* (*lab*) in the underlying endoderm (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990). The absence of *dpp* function in ps7 disrupts the autoregulatory loop and reduces the expression of *Ubx*, *wg*, *dpp* and *lab* in ps7, leading to the absence of the second constriction in *dpp* mutant embryos (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990).

We found that animals mutant for either tkv^{6} or *60A* had normal expression of *dpp*, *wg* and *Ubx* (Fig. 7B-D,F-H). However, in tkv^{6} *60A* double mutants, *dpp* expression in ps3 and ps7 was greatly reduced (Fig. 7J). The initiation of *dpp* expression at earlier stages was not affected in the double mutants (not shown), suggesting that the reduction of *dpp* expression resulted from failure to maintain its expression at later stages. Similarly, in *Mad* mutants, the initiation of *dpp* expression in ps3 and ps7 is not affected but the maintenance of *dpp* expression does not occur (Newfeld et al., 1997). This is because *dpp* expression is activated directly by *Ubx* and only its maintenance requires positive feedback involving *dpp* signaling. In the double mutants, *Ubx* expression in ps7 and *wg* expression in ps8 were greatly reduced (Fig. 7K,L), suggesting the disruption of the positive regulatory loop. The

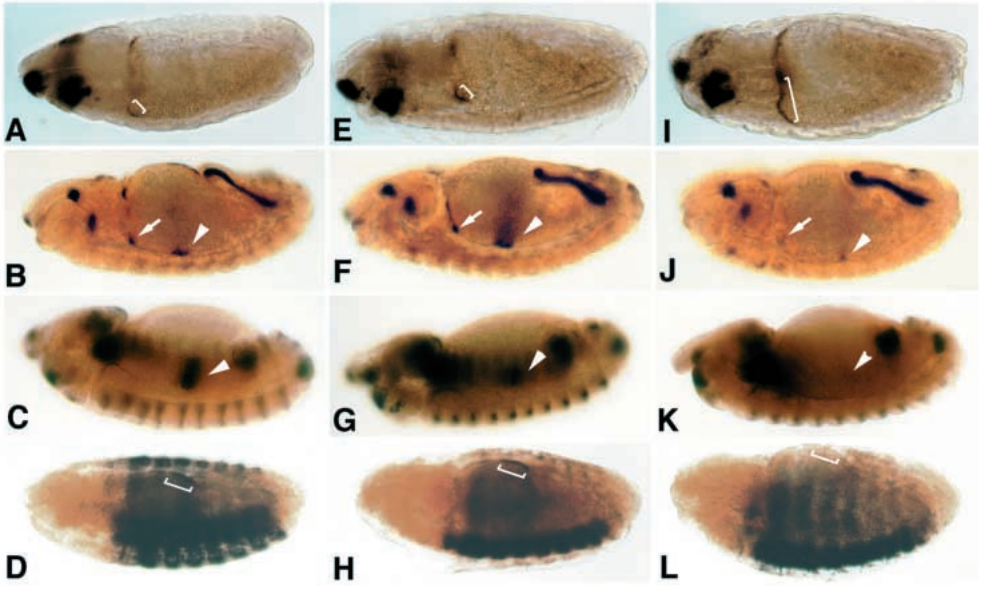

Fig. 7. *60A* enhanced tkv^{6} midgut phenotypes. Lateral views of embryos stained with anti-Scr antibody (A, E, I); anti-Dpp antibody (B, F, J); anti-Wg antibody (C, G, K) and anti-Ubx antibody (D, H, L). A-D, tkv^{6} homozygotes; E-H, $60A^{D4}/60A^{D8}$ embryos; I-L, $tkv^{6}60A^{D4}/tkv^{6}60A^{D8}$ embryos. C, G and K were at stage 14; all other embryos were at stage 15. *Scr* expression in ps4 was normal in tkv^{6} (A, bracket) or *60A* mutants (E, bracket). In tkv^{6}*60A* embryos, *Scr* extended anteriorly into ps3 (I, bracket). The visceral mesoderm expression of *dpp* in the single mutants were normal (B, F, arrows, gastric caecae; arrowheads, ps7). Double mutants had greatly reduced expression of *dpp* in gastric caecae (J, arrow) and in ps7 (J, arrowhead). *dpp* expression in the other domains were unaffected. *wg* was expressed normally in the single mutants (C, G, arrowhead), however, ps8 expression is undetectable in the double mutants (K, arrowhead). *Ubx* expression in ps7 was normal in tkv^{6} (D, bracket) or *60A* mutants (H, bracket), but was greatly reduced in the double mutants (L, bracket).

reduction of *dpp* in ps3 in the double mutants may explain the observed derepression of *Scr*.

Ubx is required for repressing *Antp* in ps6. In *Ubx* mutants, the *Antp* domain is extended posteriorly into ps7 (Tremml and Bienz, 1989), indicating a homeotic transformation of ps7 into ps6. A similar phenotype was observed for *tkv* null embryos (Affolter et al., 1994). Consistent with the argument that lacking *60A* compromises *dpp* signaling, there was also a posterior expansion of *Antp* in tkv^6*60A* double mutants (Fig. 6C). Interestingly, due to the *60A* mutation, the endogenous *Antp* expression was absent, such that there was only ectopic *Antp* in ps7, where *Ubx* would normally be.

We also examined the expression of *lab* in the endoderm. Consistent with the gene expression changes in the visceral mesoderm, *lab* expression was not affected by tkv^6 or *60A* mutations. However, it was greatly reduced in tkv^6*60A* double mutants (Fig. 6F). The gut of the double mutants only formed two chambers instead of the normal four chambers (Fig. 6G, compare to 6D). This phenotype likely resulted from the failure to form the first constriction due to lacking *60A* function and the failure to form the second constriction due to lacking *dpp* signaling. It is unclear why the position of the only constriction observed in the double mutants is somewhat more anterior than a normal third constriction.

The gene expression changes in the midgut of the tkv^6*60A* double mutants are consistent with *60A* playing a role in augmenting *dpp* signaling.

60A enhances the ectodermal phenotypes of tkv^6 homozygotes

Previous studies have established *dpp*'s role as a morphogen in patterning the embryonic ectoderm (Ferguson and Anderson, 1992a,b; Wharton et al., 1993). *dpp* signaling is also required for dorsal closure of the embryonic ectoderm (Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). We therefore examined if the level of *60A* affected the phenotype of the embryonic ectoderm.

We compared the cuticle phenotypes of single and double mutants. Since tkv^6 homozygotes were viable and *60A* mutants had no obvious defects until late in development, the cuticular patterns of these mutants were essentially normal (Fig. 8A,B). However, tkv^6*60A* homozygote embryos died and exhibited head defects and an excessive ventral curvature (Fig. 8C). Although the double mutant cuticles bore some resemblance to hypomorphic *dpp* mutants, they did not exhibit an obvious expansion of the ventral denticle belts (Wharton et al., 1993). The double mutant phenotype suggested, however, that, when *dpp* signaling was compromised in the embryonic ectoderm, removing *60A* activity further attenuated *dpp* signaling. We considered that the relatively mild phenotype of the double mutant embryo might reflect partial rescue by maternally contributed wild-type Tkv receptors. Indeed, a quarter of the embryos produced by mothers homozygous for tkv^6 and heterozygous for *60A* exhibited a dorsal open phenotype similar (Fig. 8D) to that of zygotic *tkv* null embryos (Penton et al., 1994). Therefore, in the absence of maternally provided wild-type Tkv, tkv^6*60A* double mutant embryos exhibit a phenotype indicative of defective *dpp* signaling during the process of dorsal closure.

DISCUSSION

The haploinsufficiency of the *dpp* locus reflects the sensitivity of developmental processes to a reduction in *dpp* signaling. We have carried out a genetic screen to search for modifiers of tkv^6, a hypomorphic type I *dpp* receptor. *tkv* has been implicated in all aspects of *dpp* signaling both in vitro and in vivo (Penton et al., 1994; Nellen et al., 1994; Brummel et al., 1994; Affolter et al., 1994; Burke and Basler, 1996; Singer et al., 1997). Therefore, the modifiers of tkv^6 are most likely to be integral components of the *dpp* signal transduction pathway.

Identification of new alleles of *tkv, Mad, Med, punt, 60A* and two new loci as dominant enhancers of tkv^6

New alleles of several genes known to mediate *dpp* signaling were identified, including *tkv*, *punt*, *Mad* and *Med*. Recovery of these mutations as dominant enhancers of tkv^6 validated the specificity of the screen.

We isolated five alleles of *Mad*, a key signal transducer in *dpp* signaling (Newfeld et al., 1996; Kim et al., 1997). Three of them have point mutations in the coding region (Fig. 4A). The molecular lesions correlate with their phenotypic properties. Mad^{D14} has a nonsense mutation predicted to produce a truncated protein with only the conserved MH1 domain. It behaves genetically as a null. Mad^{D16} has a tyrosine-to-asparagine change in the divergent linker region and behaves as a hypomorph with residual activity (Table 2). This suggests that the amino acid change only partially affects the protein function. The MH2 domains of Smads are highly conserved.

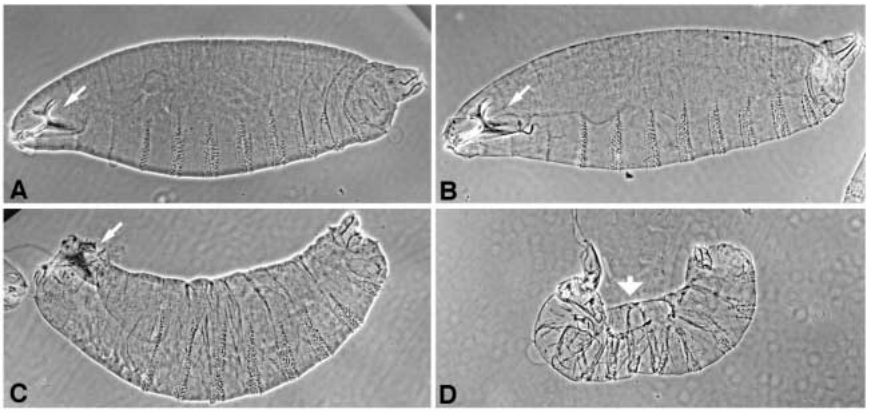

Fig. 8. *60A* enhanced the tkv^6 cuticle phenotypes. Phenotypically normal tkv^6 (A) and $60A^{D4}/60A^{D8}$ (B) cuticle. Note the fully internalized head skeleton (arrow). (C) $tkv^6 60A^{D4}/tkv^6 60A^{D8}$ cuticle. The partially deleted head skeletons remained external (arrow). (D) An embryo produced by $tkv^6 60A^{D4}/tkv^6$ females mated to $tkv^6 60A^{D8}/tkv^6$ males. Roughly a quarter of the embryos lacked dorsal hypoderm (arrow).

The three-dimensional structure of the MH2 domain of Smad4 indicates that Smads form homotrimers whose intact conformation is essential for the assembly of a hexamer with a different Smad homotrimer in response to receptor activation (Hata et al., 1997; Shi et al., 1997). Many of the Smad mutations associated with tumors or affecting development map to the MH2 domain. Based on the crystal structure of the carboxy domain of Smad4 (Shi et al., 1997), the invariant aspartic acid mutated to asparagine in *Mad*^{D24} maps to the trimer interface region critical for trimerization. The corresponding residue in Smad2 is mutated in colon cancers (Eppert et al., 1996). Interestingly, the *Mad*^{D24} mutation resulted in dominant female sterility (Y. C. and F. M. H., unpublished data) which is not observed with *Mad* null alleles, suggesting that it has a dominant negative effect, perhaps by forming inactive oligomers with the wild-type proteins in the heterozygotes.

Analysis of the two new *punt* alleles also provides evidence for the in vivo importance of conserved structural motifs in this type II *dpp* receptor (Fig. 4B). *punt*^{D13} has a cysteine-to-tyrosine change in the extracellular cysteine cluster characteristic of all receptors for TGF-β superfamily members (Massagué et al., 1994). *punt*^{D18} changes the highly conserved glutamic acid to a valine in the catalytic core of the kinase domain, where another punt mutation, *punt*^{135}, is mapped (Ruberte et al., 1995). Like *punt*^{135}, both new *punt* alleles display some temperature sensitivity (Y. C. and F. M. H., unpublished data), suggesting that they are not protein nulls. No null mutations in the *punt* locus have been reported, suggesting that like *dpp*, *punt* may be haploinsufficient.

We did not isolate any new alleles of *shn*, which enhanced *tkv*^{6} in the initial test. The enhancement by *shn*^{IB} may be allele specific, such that a particular form of mutant Shn protein is needed to produce an enhancement. Consistent with this, *shn*^{p}, which makes no detectable protein (Staehling-Hampton et al., 1995), failed to enhance the *tkv*^{6} phenotype (data not shown). No *dpp* alleles were recovered either, possibly due to the haploinsufficiency associated with the locus and the fact that most hypomorphic *dpp* mutations affect regulatory regions, which are less likely to be affected by chemical mutagens such as ENU.

One unexpected locus identified in our screen is *60A*, which encodes the BMP-7 homologue (Doctor et al., 1992; Wharton et al., 1991). The fact that in a screen of the entire genome, *60A* mutations were recovered multiple times as dominant enhancers of a mutant *dpp* receptor provides strong evidence for its involvement in *dpp* signaling. Nonsense mutations were found in all three alleles of *60A* in the prodomain of the precursor protein. Since these mutations are predicted to eliminate translation of the biologically active mature C-terminal domain, they most likely represent functional nulls of the *60A* gene.

The developmental functions of *60A* and its role in *dpp* signaling

Phenotypic analysis of *60A* single mutants and *tkv*^{6}*60A* double mutants revealed both *dpp*-independent and *dpp*-dependent functions for *60A*. *60A* is expressed broadly throughout development, with enrichment in the developing gut (Doctor et al., 1992), suggesting a role for *60A* in gut morphogenesis. *60A* mutants lack the first constriction of the embryonic midgut and *Antp* expression in ps6, indicating that *60A* is required for the formation of the first constriction, possibly through regulating *Antp* expression. This function is independent of *dpp* signaling, since mutations in *dpp* or its receptors only disrupt the formation of the second but not the first constriction (Panganiban et al., 1990b; Nellen et al., 1994; Ruberte et al., 1995). This also suggests that there is either redundancy or that a different receptor system is responsible for mediating *60A* signaling to pattern the first constriction. It would be interesting to see if AtrI (Childs et al., 1993), a type I receptor and STK-D (Ruberte et al., 1995), a type II receptor in *Drosophila*, both of unknown function, are mediators of *60A* signaling at the site of the first constriction.

The fact that *60A* mutations are dominant enhancers of a sensitized *dpp* pathway implicates *60A* in potentiating *dpp* signaling. This is most obvious in the visceral mesoderm of the midgut where *dpp* signaling is required to regulate homeotic gene expression and to maintain its own expression through a positive feedback mechanism. Although *dpp* signaling in the visceral mesoderm appears intact in *60A* mutants, a requirement for *60A* is revealed in *tkv*^{6}*60A* double mutants. When *dpp* signaling is attenuated through a mutant *tkv* receptor, eliminating *60A* function reduces the signaling to below threshold level. The derepression of *Scr* in the anterior midgut and the loss of expression of *dpp* target genes, *wg*, *Ubx* and *dpp*, in the visceral mesoderm and *lab* in the endoderm are consistent with inadequate *dpp* signaling. A similar requirement for *60A* is observed during dorsal closure of the embryonic ectoderm. The enhanced phenotypes of the adult appendages closely resemble those of the *dpp* hypomorphic mutants (Spencer et al., 1982), suggesting that *60A* activity is also required for imaginal disc patterning. It is interesting that the imaginal discs are more sensitive to the reduction of *60A* function, as a 50% reduction in *60A* function is sufficient to produce a phenotype in a *tkv*^{6} genetic background. This may reflect a differential threshold requirement for *dpp* signaling in different tissues. Taken together, our data argue for an involvement of *60A* in *dpp* signaling at different developmental stages and in various tissues.

In a signaling system with multiple interacting dimeric ligands, the interpretation of any single mutant phenotypes must consider the effect of losing both homomeric and possible heteromeric ligands. Therefore, the functions of the *dpp* pathway may be a composite input from Dpp homodimers, and Dpp/Scw and Dpp/60A heterodimers. Alternatively, 60A homodimers may function in an additive fashion with Dpp homodimers at sites of overlapping expression. However, the loss-of-function phenotypes of *dpp* are as severe as the loss-of-function phenotypes of its downstream components, such as *tkv* or *Mad* (Padgett et al., 1987; Penton et al., 1994; Nellen et al., 1994; Newfeld et al., 1996, 1997), suggesting that there is very little signaling, if any at all, from 60A homodimers in *dpp*-dependent events. Therefore, we believe it is unlikely that 60A homodimers play a significant role in *dpp*-dependent processes. Rather, we favor the interpretation that Dpp/60A heterodimers form at sites of overlapping expression and participate with Dpp homodimers in multiple signaling events.

The overlapping expression patterns of murine BMPs have led to the suggestion that they may act combinatorially during development (Lyons et al., 1995). Given the dimeric nature of TGF-β superfamily ligands, one mechanism to achieve such a

combinatorial effect is to form functional heterodimers. Heterodimers of *Xenopus* BMP-4 and BMP-7 have been generated in vitro (Hazama et al., 1995) and shown to be more potent in bone- (Aono et al., 1995) and mesoderm-inducing assays (Suzuki et al., 1997) than either homodimer. In *Drosophila*, the Scw protein is proposed to upregulate *dpp* activity by forming Dpp/Scw heterodimers in the dorsal/ventral patterning of the embryonic ectoderm (Arora et al., 1994). The broad distribution of 60A proteins provides an opportunity for forming Dpp/60A heterodimers. Unlike *scw* null mutations, no obvious disruption of *dpp* signaling is observed in *60A* null mutants, suggesting that Dpp/60A heterodimers are not as limiting as Dpp/Scw heterodimers, but partially redundant with Dpp homodimers. The first constriction phenotype of *60A* mutants is unique, suggesting that it may be a function of 60A homodimers.

The tkv^6 receptor failed to bind homomeric ligands when expressed in COS cells (Penton et al., 1994). Since *dpp* signaling is intact in tkv^6 mutants, it would be interesting to determine if the mutant receptor still binds Dpp/60A and/or Dpp/scw heterodimers. If this is the case, the severe midgut phenotypes of tkv^6*60A* double mutants could be due to additional loss of signaling from Dpp/60A heterodimers, which would be consistent with the proposal that *60A* potentiates *dpp* signaling by forming heterodimers with Dpp. The cuticular phenotypes of tkv^6*60A* double mutants without maternal wild-type Tkv receptor do not exhibit the altered dorsal/ventral polarity observed in *dpp* null embryos, possibly due to Dpp/Scw heterodimer signaling through the tkv^6 receptor during early embryogenesis.

In summary, we have isolated mutant alleles of genes involved in *dpp* signaling, including *60A*. Mutations in *60A* disrupt *dpp* signaling in multiple developmental processes when *dpp* signaling is compromised. We propose that *60A* participates in *dpp* signaling by forming heterodimers with Dpp protein. Our data support both *dpp*-dependent and *dpp*-independent functions for *60A*. It remains to be determined if these distinct functions reflect the qualitative differences between different forms of the ligands and if they are mediated by differentially activated receptors or distinct cytoplasmic signal transducers. The availability of *60A* mutations provides a genetic tool for dissecting the differential requirement of each component in this combinatorial signaling system.

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