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Genetic Analysis of Punt, a Type II Dpp Receptor That Functions Throughout the *Drosophila melanogaster* Life Cycle

Karl Simin, Emily A. Bates, Michael A. Horner and Anthea Letsou

Department of Human Genetics, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah 84112

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ABSTRACT

TGF-β- (transforming growth factor-β-) mediated signal transduction affects growth and patterning in a variety of organisms. Here we report a genetic characterization of the Drosophila *punt* gene that encodes a type II serine/threonine kinase TGF-β/Dpp (Decapentaplegic) receptor. Although the *punt* gene was originally identified based on its requirement for embryonic dorsal closure, we have documented multiple periods of *punt* activity throughout the Drosophila life cycle. We demonstrate that potentially related embryonic *punt* phenotypes, defects in dorsoventral patterning and dorsal closure, correspond to distinct maternal and zygotic requirements for *punt.* In addition, we document postembryonic requirements for *punt* activity. The tight correspondence between both embryonic and postembryonic loss-of-function *punt* and *dpp* phenotypes implicates a role for Punt in mediating virtually all Dpp signaling events in Drosophila. Finally, our comparison of *punt* homoallelic and heteroallelic phenotypes provides direct evidence for interallelic complementation. Taken together, these results suggest that the Punt protein functions as a dimer or higher order multimer throughout the Drosophila life cycle.

CYTOKINES of the TGF-β superfamily evoke a wide range of eukaryotic developmental and physiological responses. These include modulation of cell growth and proliferation, regulation of immune and endocrine function, and control of axial patterning (for review, see Massagué 1996). Based on similarities in biological function and sequence, the members of the TGF-β superfamily have been divided into three subgroups: TGF-βs, activins, and Dpp/BMPs (bone morphogenetic proteins) (for review, see Kingsley 1994).

The diversity in responses elicited by the various members of the TGF-β superfamily derives, at least in part, from heterogeneity in TGF-β receptor complexes (Mathews and Vale 1991; Attisano *et al.* 1992). Biochemical studies *in vitro* (Wrana *et al.* 1994) indicate that a dimeric ligand from either the TGF-β or the activin subgroup forms a complex with two types of transmembrane serine/threonine kinase receptor, the type I and type II receptors. The type II receptor, a constitutively active kinase, is the primary determinant in TGF-β and activin subgroup binding. Once the type II receptor has bound ligand, it complexes with a type I receptor kinase and activates it by serine/threonine phosphorylation. By virtue of its interaction with Smad proteins, the type I receptor then transduces the TGF-β signal intracellularly (Sekelsky *et al.* 1995; Macias-Silva *et al.* 1996; Kim *et al.* 1997).

Corresponding author: Anthea Letsou, Department of Human Genetics, 15 N 2030 E RM 2100, University of Utah, Salt Lake City, UT 84112-5330 E-mail: anthea.letsou@genetics.utah.edu

Studies of the *Drosophila melanogaster* type II receptor encoded by *punt* (*put*) indicated that signaling by the third subgroup of TGF-β ligands, the Dpp/BMPs, differs only slightly from the paradigm described above: for cytokines in this subgroup, the type II receptor is not the primary determinant in ligand binding. Moreover, these studies revealed dual-ligand specificity by type II receptors to represent yet another mechanism by which TGF-β superfamily members could elicit diverse cellular responses. Although Punt was characterized originally as an activin receptor in assays of function and sequence homology (Childs *et al.* 1993), we observed that Punt binds Dpp/BMP-type ligands *in vivo* when a type I Dpp/BMP receptor is coexpressed (Letsou *et al.* 1995). This finding led to our hypothesis that Punt has dual-ligand specificity: Punt binds activin ligands directly and Dpp/BMP ligands in combination with a type I receptor. Consistent with our model for indirect ligand selection by type II Dpp/BMP receptors are two additional observations: (1) binding of BMP 4 by the mammalian type II receptor BRK-3 (BMP Receptor Kinase-3) is enhanced in cultured cells when a type I BMP receptor is coexpressed (Nohno *et al.* 1995), and (2) binding of BMPs 2 and 7 by the mammalian type II receptor BMPR-II (BMP Receptor Type II) in cultured cells is enhanced by coexpression of various type I BMP or activin receptors (Liu *et al.* 1995).

Three Drosophila gene products [Dpp, 60A, and Screw (Scw)] belong to the Dpp/BMP family of TGF-βs (Padgett *et al.* 1987; Wharton *et al.* 1991; Arora *et al.* 1994) and can potentially serve as Punt ligands *in vivo.*

The function of Dpp has been well characterized both molecularly and genetically. During oogenesis, the *dpp* gene product is required for patterning the anterior eggshell (Twombly *et al.* 1996). During embryonic dorsoventral axis formation, Dpp acts as a morphogen; increasing concentrations of the *dpp* gene product result in the formation of more dorsal structures (Ferguson and Anderson 1992; Wharton *et al.* 1993). Later in embryogenesis, the *dpp* gene product is required for dorsal closure and for normal constriction of the midgut (Immerglück *et al.* 1990; Panganiban *et al.* 1990). In addition, Dpp functions postembryonically as a long-range morphogen during imaginal disc development and patterning of the visual centers of the developing brain (Hoffmann 1991; Kaphingst and Kunes 1994; Lecuit *et al.* 1996; Nellen *et al.* 1996). Specifically, mutations in *dpp* result in wing, leg, eye, notal, antennal, genital, and anal defects (Spencer *et al.* 1982). Far less is known of *scw* and *60A* gene functions. Like mutations in *dpp*, *scw* mutations disrupt dorsoventral patterning (Arora *et al.* 1994). The third ligand, 60A, is less well characterized and has yet to be assigned a function.

A definitive role for Punt in mediating certain aspects of Dpp signaling has been documented. Initial insight into the *punt* gene's function was obtained in analyses of the original *punt* mutations, *punt*^{135} and *punt*^{P1}, which were identified in genetic screens for embryonic lethals affecting cuticular pattern. The single ethylmethane sulfate- (EMS-) induced allele, *punt*^{135}, is a recessive lethal that disrupts embryonic processes of dorsal closure and gut development (Jürgens *et al.* 1984; Letsou *et al.* 1995; Ruberte *et al.* 1995). The second *punt* allele, *punt*^{P1}, was identified in a *P*-element mutagenesis screen and similarly affects embryogenesis; its recessive lethal phenotype is indistinguishable from that of *punt*^{135} (Letsou *et al.* 1995; Ruberte *et al.* 1995). Postembryonic *punt* functions have been identified in direct assays for function. Both *punt*^{135} and *punt*^{P1} exhibit a maternal-effect ventralizing phenotype (Letsou *et al.* 1995; Ruberte *et al.* 1995). Whereas embryos derived from wild-type mothers secrete a cuticle that bears distinctive dorsal and ventral pattern elements, embryos derived from mothers homozygous for temperature-sensitive alleles of *punt* and embryos derived from mothers harboring *punt* germline clones are severely ventralized and secrete a cuticle that is circumscribed by ventral denticle belts. Finally, clonal analyses of *punt* function in imaginal discs demonstrated additional roles for *punt* in postembryonic patterning events that are mediated by *dpp* (Burke and Basler 1996; Penton and Hoffmann 1996).

In the current article we describe genetic studies that were designed to assess requirements for the *punt*-encoded type II receptor throughout the Drosophila life cycle. First, we generated new *punt* alleles. These alleles constitute an allelic series, exhibiting phenotypes of varying severities that range from full viability to embryonic lethality. The *punt* gene is pleiotropic, and our observation that all *punt* phenotypes were characterized originally as *dpp* phenotypes (Spencer *et al.* 1982) is particularly notable. The tight correspondence between *dpp* and *punt* mutant phenotypes prompts us to suggest that (1) Dpp repeatedly functions as the Punt ligand throughout the Drosophila life cycle, and (2) the *punt*-encoded type II receptor is sufficient to fulfill most type II receptor requirements for Dpp signaling. Second, we exploited the temperature sensitivity of *punt* alleles to investigate temporal requirements for Punt function. These analyses resolved a long-standing question: whether or not defects in dorsal closure are primary or secondary consequences of mutations in *punt.* Our studies indicated that rather than being secondary to earlier defects in embryonic dorsoventral patterning, defects in embryonic dorsal closure are a primary consequence of *punt* mutations. Finally, our genetic interaction studies revealed that the bioactive form of Punt is multimeric.

MATERIALS AND METHODS

Fly maintenance and stocks: Balancers, marker mutations, and dorsal-open class mutations, including *punt*^{135}, *punt*^{P1}, and *tkv*^{8} have been described (Jürgens *et al.* 1984; Nüsslein-Volhard *et al.* 1984; Lindsley and Zimm 1992; Letsou *et al.* 1995; Ruberte *et al.* 1995). Flies were maintained on cornmeal/molasses/agar medium at room temperature (22°) unless otherwise stated.

***P*-element excision:** The *punt* alleles *punt*^{51}, *punt*^{62}, *punt*^{88}, and *punt*^{136} were isolated in a screen for lethal excisions of the PZ transposon p[*lacZ ry*+] in *punt*^{P1} heterozygotes. The *rosy*+ (*ry*+) -marked *P* element was mobilized by an external transposase source (Robertson *et al.* 1988). Fly lines in which the *P*-element sequences had been excised were identified by their rosy eye color. The new *punt* alleles were identified in rosy lines failing to complement the dorsal closure defect of the *punt*^{P1} mutation. One *punt* allele (*punt*^{10}) was identified in the collection of excision lines that complemented the embryonic lethal *punt*^{P1} phenotype. Two additional adult-viable *punt* alleles, *punt*^{24} and *punt*^{97}, were isolated in a second PZ mobilization screen. In this screen of 75 fly lines, imprecise excisions of an unmarked (*ry*) *P* element in *punt*^{136} heterozygotes were identified by polymerase chain reaction (PCR) analyses using *punt* primers P1 (5′ GGGCCTGTTTTCAAGC GAT 3′) and P2 (5′ GGAATTCATTGTCTCACTACCAGCC 3′) to amplify genomic DNA fragments spanning the original transposon insertion site.

Gamma- (γ-) irradiation: γ-radiation was employed to generate deficiencies spanning the *punt* locus. *punt*^{P1} *ry*/*TM3, Sb Ser* males were exposed to 4000 rads of gamma radiation from a ^{137}*Cs* source. Irradiated males were mated to *CxD, ry*/*MKRS* virgin females, and rosy progeny were identified. Deficiencies mapping to the endogenous *ry* locus were distinguished from those mapping to *punt* by an analysis of bristle and wing markers.

Temperature shift manipulations: To determine the temperature-critical period for embryonic Punt function, adult flies were placed in laying blocks at either the permissive (18°) or the restrictive (25°) temperature and induced to lay eggs on grape-juice agar plates supplemented with fresh yeast paste. Since the total duration of Drosophila development is a function of temperature, all experimental time intervals were

standardized and expressed as 25° standard time intervals (× 1.75 for 18° and × 1 for 25°; Powsner 1935). Embryos were collected for 2-hr standard time intervals and then transferred to freshly yeasted vials. Embryonic development proceeded initially at the collection temperature and was followed by the indicated shift. After a 24-hr standard time interval, all animals completed development at 18°. To assay postembryonic Punt function, animals underwent embryogenesis at 18° and were shifted up to the restrictive temperature (25°) after a 24-hr standard time interval.

Phenotypic analyses: Cuticular phenotypes were examined using the Hoyer's mount technique (van der Meer 1977). Three classes of dorsal-open, embryonic lethal phenotype were distinguished. Embryos exhibiting single, large holes on their dorsal surface were scored as strongly defective in the process of dorsal closure. Embryos displaying defects that include a tail-up phenotype, increased curvature in abdominal segments, and noticeably reduced dorsal cuticle were scored as moderately defective. Embryos exhibiting more subtle or no visible defects were scored as weakly defective. Postembryonic *punt* phenotypes were scored in viable and pharate adults, as well as in prepupae. We distinguished prepupae from pupae by scoring for the occurrence of head eversion. Prepupae and pupae were dissected from pupal cases after boiling for 2 min in water. Due to the fragility of the structure, we were unable to examine wing phenotypes at this developmental stage. Both phase and scanning electron microscopy (SEM) were employed to image adult and pupal phenotypes. For SEM, samples were air-dried for 24 hr, attached to mounting stubs with conducting glue, and sputter-coated (Desk-1, Denton, Cherry Hill, NJ) with a $^{60}/_{40}$ gold/palladium alloy. Micrographs were examined using a S-450 scanning electron microscope (Hitachi, Mountain View, CA) at a working distance of 15 mm at an accelerating voltage of 15 kV.

Molecular analyses: Molecular lesions in *punt* alleles were identified by Southern hybridization, PCR, and DNA sequence analyses. Southern hybridization analyses were performed according to published procedures (Sambrook *et al.* 1989). Probes included 1) a 2 kb *Eco*RI genomic fragment that spans the transposon insertion site and 2) pBS5′P (the generous gift of D. McKearin, Southwestern Medical Center, Dallas) that corresponds to 540 bp in the 5′P sequence (Rubin and Spradling 1983). The size of insertions in $punt^{10}$, $punt^{24}$, and $punt^{97}$ was determined in PCR analyses using *punt* primers P1 and P2 to amplify genomic DNA fragments spanning the transposon insertion site. The precise lesions in $punt^{10}$, $punt^{24}$, and $punt^{97}$ were determined by DNA sequence analysis. Briefly, primers P1 and P2 were employed in a PCR reaction using genomic DNA from *punt* homozygotes as template. The double-stranded, amplified product was gel purified and sequenced on both strands with a DNA sequencer model 373 (Applied Biosystems, Foster City, CA). Sequence

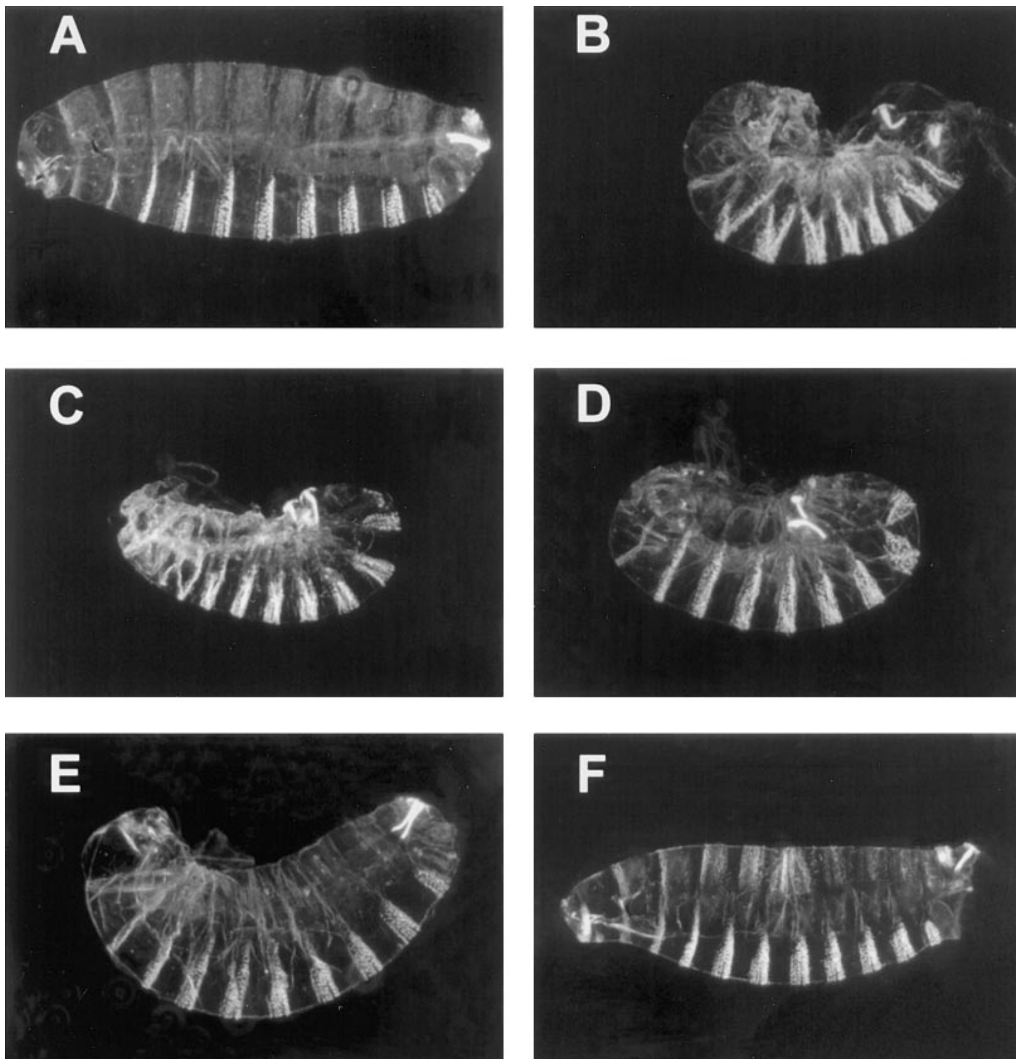


Figure 1.—Dark-field images of cuticles from wild-type and mutant embryos raised at 25°. The cuticle of a wild-type embryo (A) displays characteristic pattern elements. In contrast, cuticles from embryos homozygous for certain alleles of *tkv*, for example, *tkv8* (B), and *punt*, for example, $punt^{P1}$ (C) and $punt^{62}$ (D) can exhibit severe head defects and large holes in the dorsal hypoderm. *punt* phenotypes, however, are variably expressed. In contrast to the severe defects seen in (C) and (D), moderately affected cuticles from $punt^{62}/punt^{62}$ embryos exhibit the tail-up phenotype, as well as an increase in the curvature of abdominal segments and a reduction in the dorsal hypoderm (E). Weakly affected $punt^{62}/punt^{62}$ cuticles complete dorsal closure, exhibiting only subtle defects in dorsal cuticle (F). In all panels, dorsal is up, and anterior is to the left.

TABLE 1

***punt* alleles exhibit variable expressivity and temperature sensitivity**

Allele	Temperature	Phenotype			Number scored
		Strong	Intermediate	Weak	
51	18°	<1[a]	31	69	458
	29°	31	37	31	251
88	18°	0	69	31	131
	25°	39	31	31	242
62	18°	2	49	49	224
	25°	17	42	42	125
	29°	55	23	22	176
P1	25°	18	43	39	28
	29°	72	9	19	47

[a] Values presented are percentage of total cuticles

reactions were primed with oligos P1 and P2 and terminated with dye-labeled dideoxynucleotides.

RESULTS

***punt* mutations disrupt dorsal closure:** Wild-type embryos undergo dorsal closure between 8 and 12 hr after egg lay (AEL), corresponding to embryonic stages 12–15 (Campos-Ortega and Hartenstein 1985). During this process, epidermal cells change their shape [becoming rectangular in dorsal positions and remaining polygonal in more lateral positions (Young *et al.* 1993)], and lose their continuity with the extraembryonic membrane, the amnioserosa. Epidermis and amnioserosa overlap transiently, the latter is incorporated into the embryo, and closure is complete when the edges of the epidermal sheet meet and fuse at the dorsal midline. Since epidermal cells secrete cuticle, wild-type cuticle completely encases the animal and displays characteristic pattern elements (Figure 1A). In contrast, cuticles derived from well-characterized dorsal closure mutants, $punt^{P1}$ or $punt^{135}$ homozygotes (Jürgens *et al.* 1984; Letsou *et al.* 1995; Ruberte *et al.* 1995) are grossly abnormal as evidenced by a single, large hole in the dorsal cuticle (Figure 1C). Embryos lacking the type I Dpp receptor encoded by the *thick veins* (*tkv*) gene display the same dorsal-open phenotype (Figure 1B).

We generated several new embryonic-lethal *punt* alleles after mobilization of the *P* transposon in $punt^{P1}$. In this report, we show that the $punt^{51}/punt^{51}$, $punt^{62}/punt^{62}$, and $punt^{88}/punt^{88}$ homozygous embryonic lethal phenotypes resemble those of $punt^{135}/punt^{135}$ and $punt^{P1}/punt^{P1}$; each is fully penetrant but variably expressed at all temperatures tested (18°, 25°, and 29°) (Table 1). As an example, at 25°, 17% of $punt^{62}$ homozygotes exhibited the strong dorsal-open phenotype that has been described previously (Figure 1D). Forty-two percent of mutant embryos exhibited an intermediate dorsal-open phenotype, displaying a tail-up phenotype, an increased curvature in abdominal segments, and a noticeably reduced dorsal cuticle (Figure 1E). The remaining 42% of $punt^{62}$ homozygotes completed the process of dorsal closure and exhibited only minor defects in their dorsal cuticle (Figure 1F). Additional analyses of cuticles derived from all homoallelic combinations of *punt* alleles revealed the embryonic lethal *punt* phenotypes to be temperature-sensitive: in all cases the strong dorsal-open *punt* phenotype was more prevalent at higher temperatures (Table 1).

***punt* mutations disrupt wing patterning:** We similarly employed transposon mobilization to generate three new adult-viable *punt* alleles. We had shown previously

TABLE 2

Viable *punt* alleles are temperature-sensitive and exhibit defects in wing venation

Mating	Temperature	*punt/balancer*	*punt/punt*		*P* value
			Viability	Wing phenotype	
A. $punt^{P1}/MKRS \times punt^{10}/MKRS$	22°	80 (60)[a]	53 (40)[a]	15 (28)[a]	1[c]
B. $punt^{62}/MKRS \times punt^{10}/MKRS$	22°	224 (66)	117 (34)	39 (33)	0.20
C. $punt^{51}/MKRS \times punt^{10}/MKRS$	22°	267 (68)	128 (32)	9 (7)	$<10^{-6}$
D. $punt^{88}/MKRS \times punt^{10}/MKRS$	22°	224 (73)	81 (27)	1 (1)	$<10^{-6}$
E. $punt^{135}/MKRS \times punt^{10}/MKRS$	22°	267 (73)	100 (27)	1 (1)	$<10^{-6}$
F. $punt^{10}/MKRS \times punt^{10}/MKRS$	25°	24 (67)	12 (33)	0	1[d]
G. $punt^{10}/MKRS \times punt^{10}/MKRS$	18°	67 (87)	10 (13)	0	0.0015

[a] The numbers in parentheses indicate the percentage of total progeny. Because the *MKRS/MKRS* progeny are not viable, the expected frequency for the balanced class is 67% and for the unbalanced class is 33%.

[b] The numbers in parentheses indicate the percentage of total *punt/punt* homozygotes exhibiting a defect in wing venation.

[c] The tested parameter was wing phenotype. Observed and expected values (72% wt: 28% mutant) were compared using chi square analysis. *P* values < 0.05 were considered significant.

[d] The tested parameter was viability. Observed and expected values (67% *punt/balancer*: 33% *punt/punt*) were compared using chi square analysis. *P* values < 0.05 were considered significant.

that in contrast to homoallelic combinations, certain heteroallelic combinations of *punt* were fully viable at low temperatures. Because ∼ 10% of these viable *punt/punt* adults exhibited defects in wing venation (Letsou *et al.* 1995), we reasoned that weak hypomorphic *punt* alleles might be recovered in screens of viable excisions of the mutagenic transposon in $punt^{P1}$. Using wing venation as a criterion for Punt function, we screened our collection of viable excision homozygotes for weak alleles of *punt.* No visible defects were observed in these fly lines ($n > 500$ flies/line). One weak *punt* allele, $punt^{10}$, however, was identified in screens of viable alleles in *trans* to the embryonic lethal allele $punt^{P1}$; although fully viable at 25°, 28% of $punt^{10}/punt^{P1}$ adults exhibited a defect in wing venation (Table 2A; Figure 2, A and B). The defect, ectopic venation stemming from the posterior cross-vein into the second posterior cell, was recovered as either a unilateral or a bilateral condition. Furthermore, we found $punt^{10}$ to be a cold-sensitive allele. Whereas $punt^{10}$ homo-zygotes were fully viable at 25°, they exhibited a semilethal phenotype at 18° ($P = 0.00015$; Table 2, F and G). Our molecular characterization of the lesion in $punt^{10}$ (see below), as well as our failure to detect cold-sensitive lethality in fly lines in which the *P* element has excised precisely (data not shown), lead us to conclude that cold-sensitive lethality is a direct consequence of mutation at the *punt* locus.

Two additional adult-viable alleles, $punt^{24}$ and $punt^{97}$, were recovered in an independent screen for excisions of an unmarked *P* element in $punt^{136}$ heterozygotes. Molecular characterization of the lesions associated with these alleles revealed an obvious relationship to that in $punt^{10}$ (see below); hence, only $punt^{10}$ was characterized genetically.

To place the *punt* alleles in an allelic series, we examined the phenotypes of $punt^{51}/punt^{10}$, $punt^{62}/punt^{10}$, $punt^{88}/punt^{10}$, $punt^{135}/punt^{10}$, and $punt^{P1}/punt^{10}$ heteroallelic combinations. Although defects in venation were observed in most classes of *punt* homozygotes, these defects were recovered at high frequencies only in $pun^{62}/punt^{10}$ and $punt^{P1}/punt^{10}$ homozygotes ($P < 0.00001$; Table 2, A–E). In particular, 32% of $punt^{62}/punt^{10}$ adults exhibited defects in wing venation identical to those already identified in $punt^{P1}/punt^{10}$ animals. Taken together, our phenotypic analyses of *punt* mutations allowed us to order three classes of *punt* alleles with respect to decreasing severity as follows: Class I [$punt^{P1} = punt^{62}$] > Class II [$punt^{51} = punt^{88} = punt^{135}$] > Class III [$punt^{10} = punt^{24} = punt^{97}$].

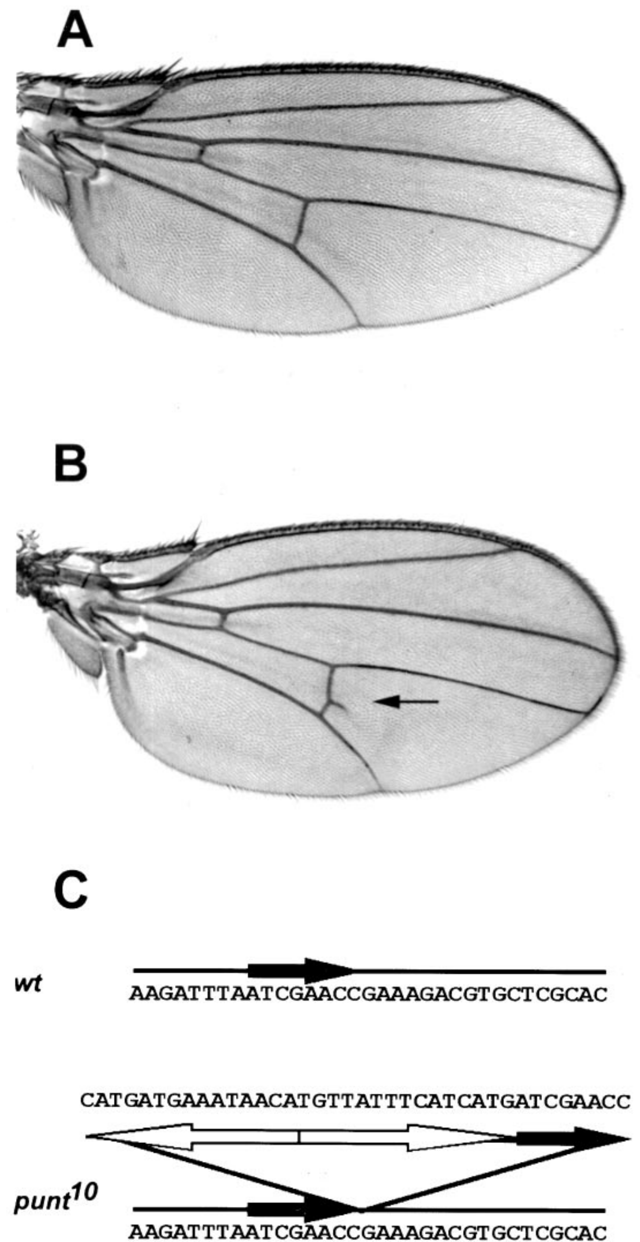


Figure 2.—Wing venation defects in *punt* mutants. Wild-type wing venation (A) is compared to that in $punt^{10}/punt^{62}$ mutants (B). Twenty-eight percent of $punt^{10}/punt^{62}$ animals exhibit an ectopic vein (arrow) stemming from the posterior cross-vein. In panel (C), the wild-type and $punt^{10}$ sequences are compared. Imprecise excision of a *P*-element transposon left a 38-bp insertion in the 5′ UTR of $punt^{10}$. Inserted sequences include: 1) 15-bp inverted repeats (open arrows) that are vestiges of the parental transposon's 31-bp inverted repeats, and 2) an 8-bp sequence (solid arrow) that accompanied the insertion of the parental transposon.

punt allele strength is correlated with insertion length: We had determined previously that the *P* transposon insertion site in $punt^{P1}$ mapped to the 5′ untranslated region (UTR) of the *punt* gene, 2 bp from its 5′ end (Letsou *et al.* 1995). To examine the molecular nature of the *punt* mutations that arose after transposon mobilization, we employed Southern hybridization and PCR analyses. These studies revealed a correlation between the molecular and genetic natures of the mutations (Table 3). All embryonic lethal *punt* alleles arising from mobilization retained *P*-element sequences in the 5′ UTR of the *punt* gene. Southern analyses revealed that more than 3 kb remained at the insertion

TABLE 3

Molecular and genetic analysis of *punt* alleles

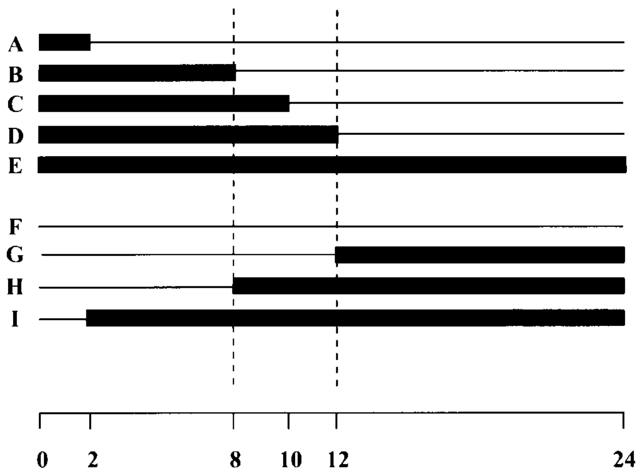
Class	Allele	Strength	Mutation
I	*punt*^{P1}	strong	14-kb insertion
	punt^{62}	strong	>3-kb insertion
II	*punt*^{51}	moderate	>3-kb insertion
	punt^{88}	moderate	~420-bp insertion
	punt^{135}	moderate	missense[a]
III	*punt*^{10}	weak	38-bp insertion
	punt^{24}	weak	38-bp insertion
	punt^{97}	weak	40-bp insertion

[a] Ruberte *et al.* (1995)

site in *punt*^{51} and *punt*^{62}. PCR analyses revealed the insertion in *punt*^{88} to be considerably smaller, ~420 bp in length. The smallest insertions mapped to the 5′ UTR of the three adult-viable *punt* alleles (*punt*^{10}, *punt*^{24}, and *punt*^{97}). To precisely define these lesions, we isolated a PCR fragment that contained the insertion and sequenced it. In *punt*^{10} and *punt*^{24}, two independently derived alleles, we identified identical 15 bp inverted repeats between the 8-bp target site duplication (Figure 2C). In *punt*^{97}, the inverted repeat unit was increased in length by a single base pair.

The punt locus is haplo-insufficient: Because *punt* maps to a region of the Drosophila genome for which a deficiency has never been recovered, we screened genetically for deletions of the locus. First, we screened 200 *rosy* lines that were generated by mobilization of the ry^+-bearing *P* transposon in *punt*^{P1} and identified no deletions that extend into the *punt* locus. Second, we irradiated *punt*^{P1} *ry*/++ males and screened 12,641 *punt*^{P1*} *ry*/+ *ry* progeny for loss of the ry^+ eye color marker carried by the mutagenic transposon. Although we recovered deletions of the endogenous ry^+ gene that is carried by the balancer chromosome (4 *ry* mutations in 2921 irradiated ry^+ chromosomes), we recovered no *ry* deletions at the *punt* locus in viable adults ($P = 2.4 \times 10^{-5}$). Intriguingly, this mutagenesis protocol produced two *rosy* inviable adults; these animals failed to emerge from their pupal cases and exhibited an array of phenotypes, including medial notal clefts and eye and leg defects, which we have documented in viable *punt* homozygotes (see below). Although not proven through molecular analyses, we believe that these pharate adults display the haplo-insufficient *punt* phenotype.

Dorsal closure defects are a primary consequence of mutations at the punt locus: We showed previously that heteroallelic combinations of either *punt*^{51} or *punt*^{88} with *punt*^{135} result in a temperature-sensitive embryonic lethality. Whereas *punt*^{51}/*punt*^{135} and *punt*^{88}/*punt*^{135} animals abort development midway through embryogenesis at 25° due to defects in dorsal closure, adult-viable *punt*^{51}/*punt*^{135} and *punt*^{88}/*punt*^{135} homozygotes are recovered at 18° at the expected Mendelian frequency (Letsou *et al.* 1995). We exploited this temperature sensitivity of *punt* heteroalleles to determine the temperature-critical period for embryonic *punt* gene function. *punt*^{88}/*punt*^{135} embryos were generated in matings of *punt*^{88}/+ and *punt*^{135}/+ heterozygotes at 25°. Next, F_1 embryos were shifted from the restrictive temperature (25°) down to the permissive temperature (18°) at



	# *punt*/*punt*	# progeny scored	% expected
A	29	132	66
B	17	129	40
C	0	104	0
D	0	136	0
E	0	333	0
F	79	237	100
G	7	45	47
H	0	176	0
I	0	84	0

Figure 3.—Temperature-critical period determination for *punt.* In the first experiment (A–E), embryonic development was initiated at the restrictive temperature (25°, bar), and at successive embryonic time points, including 2, 8, 10, and 12 hr AEL, embryos were moved to the permissive temperature (18°, horizontal line). In the second experiment (F–I), embryonic development was initiated at the permissive temperature, and at successive embryonic time points, including 2, 8, and 12 hr AEL, embryos were moved to the restrictive temperature. In all trials, postembryonic development was completed at 18°. Because the rate of Drosophila development is reduced as temperature decreases, time intervals spent at 18° were multiplied by 1.75, the equivalency factor of Powsner (1935). Data are thus integrated into a single schematization with shift points indicated as 25° standard. Dashed vertical lines delimit the *punt* temperature-critical period. In adjacent columns, the number of viable *punt* homozygotes that survived to adulthood, the total number of adult progeny that were produced in each experiment, as well as the percentage of expected *punt* homozygotes, are indicated.

TABLE 4

Adult patterning defects in *punt* homozygotes

Structure	Phenotypes			Number scored	Percent penetrance
	Mutant		Wild type		
Notum	deleted	cleft			
	1	130	0	131	100
Leg	truncated				
	126		1	127	99
Eye	deleted	reduced			
	29	41	2	72	97
Antenna	duplicated	truncated			
	41	7	19	67	72

discrete times, either 2, 8, 10, 12, or 24 hr AEL. Viable *punt*^{88}/*punt*^{135} adults were recovered when downshifts were performed at 2 or 8 hr AEL, but no *punt* homozygotes were recovered in later shifts at 10, 12, or 24 hr AEL (Figure 3, A–E). These results indicate that zygotic Punt is not essential during the first 8 hr of embryogenesis. In a reciprocal set of upshift experiments, we produced viable *punt*^{88}/*punt*^{135} adults when shifts from 18° to 25° were performed at 12 or 24 hr AEL, but not in earlier shifts at 2 or 8 hr AEL (Figure 3, F–I). These results indicate that zygotic Punt is essential for development prior to 12 hr AEL. Taken together, results from the temperature-shift experiments demonstrate an absolute requirement for zygotic Punt between 8 and 12 hr of embryogenesis, coincident with the process of dorsal closure and well after dorsoventral cell fate has been established.

As a further test of our hypothesis that Punt functions in midembryogenesis to effect dorsal closure, we examined the mutant cuticular phenotype of *punt* homozygotes in both 8-hr upshift and 12-hr downshift experiments. Our observation that all mutant embryos exhibited an intermediate dorsal-open phenotype (data not shown) is consistent with our hypothesis that Punt plays a primary role in the process of dorsal closure.

Post-embryonic *punt* phenotypes mimic dpp phenotypes: Temperature-shift manipulations were also employed to investigate postembryonic requirements for Punt. As we had shown previously to be the case for two insertion alleles of *punt* (*punt*^{51} and *punt*^{88}), the heteroallelic combination of *punt*^{62} with *punt*^{135} results in a temperature-sensitive embryonic lethality due to defects in dorsal closure: *punt*^{62}/*punt*^{135} homozygotes are inviable at 25° and fully viable at 18° (data not shown). The progeny of *punt*^{62}/+ and *punt*^{135}/+ heterozygotes were allowed to complete embryogenesis at the permissive temperature, 18°. At 24-hr standard AEL, animals were shifted to the restrictive temperature, and thus larval and pupal development proceeded to completion at 25°. *punt*^{62}/*punt*^{135} homozygotes generated by this protocol exhibited a marked reduction in viability. In a cross expected to produce 156 *punt*^{62}/*punt*^{135} homozygotes, representing 25% of the total progeny, we recovered only eight homozygotes, corresponding to less than 2% of the total progeny. Further examination of all developmental stages revealed that *punt*-dependent lethality occurred postembryonically; 131 of the missing *punt* homozygotes were identified as dead prepupae (36) and pupae (95). Lethal prepupae exhibited defects in head eversion (data not shown).

To more precisely examine the cause of death in *punt* homozygotes, we dissected the 131 dead animals from their pupal cases. The presumed *punt*^{62}/*punt*^{135} genotype of dissected animals was confirmed by analysis of bristle markers. All *punt* homozygotes, including the eight fully viable adults, were grossly deformed (Table 4). All *punt* homozygotes exhibited notal defects; these were almost exclusively medial notal clefts (Table 4 and Figure 4). Ninety-nine percent of *punt* homozygotes exhibited leg defects, including truncations, bifurcations, and abnormal twists (Table 4 and Figure 5). Distal pattern elements were deleted in at least one limb in 126 of 127 animals examined. All six legs were

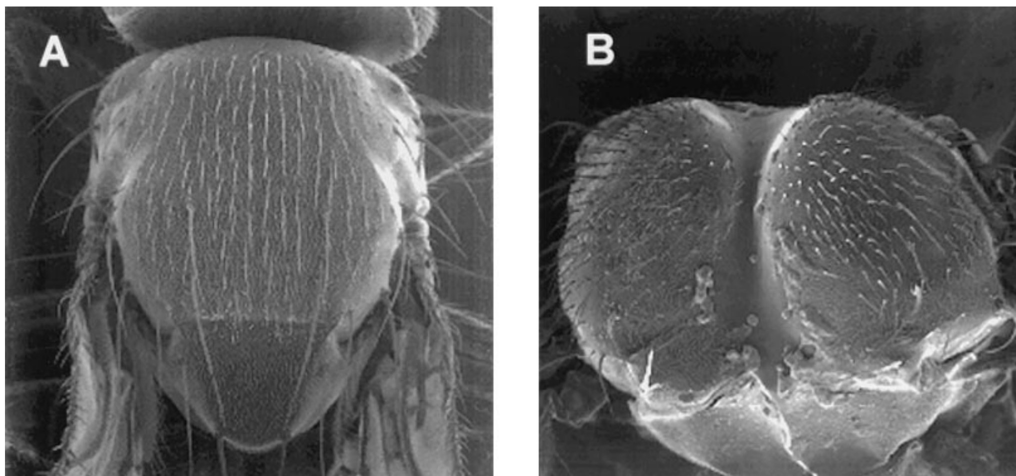


Figure 4.—Scanning electron microscopy of adult nota. A wild-type notum (A) is compared to that of a *punt*^{88}/*punt*^{135} mutant (B). The *punt* mutant exhibits a deep medial cleft; its head and wings were removed for imaging. In both A and B, dorsal views are presented, and anterior is up. Magnification is ×100.

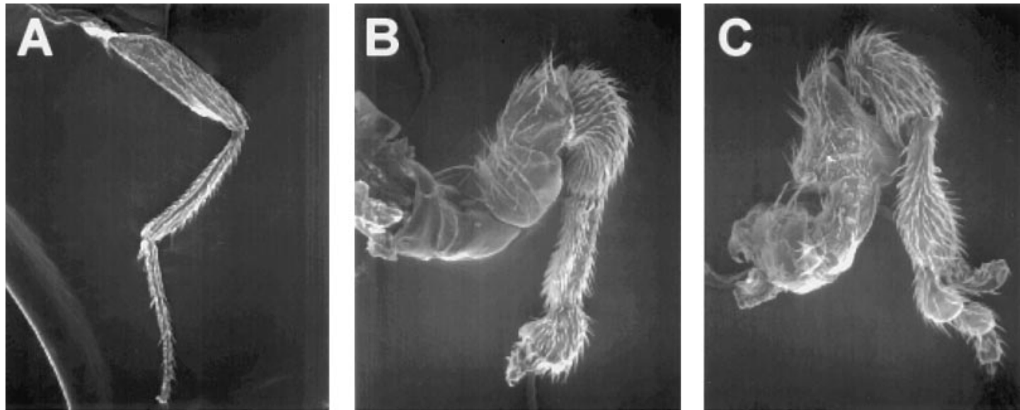


Figure 5.—Scanning electron microscopy of adult hindlimbs. A wild-type hind limb (A) is compared to those of two *punt*^{88}/*punt*^{135} mutants (B and C). Although *punt* limb phenotypes were highly penetrant (>99%), they were variably expressed. Most mutants exhibited shortened and bent femurs, as well as deleted and deformed tarsi (B). In addition, a significant fraction (59%) exhibited pattern duplications, including duplicated metatarsi (C) and duplicated sex combs (data not shown). Wild-type magnification is ×63; mutant magnification is ×150.

rarely affected to the same degree, and defects could be unilateral or bilateral with respect to each pair of legs. Although the posterior leg pair was more frequently affected than were the anterior pairs, duplicated sex combs were readily discernible on several of the mutant's forelegs (data not shown). Sex comb duplication is a hallmark of ventralization of patterning in imaginal discs (Theisen *et al.* 1996). A very large fraction (97%) of *punt* homozygotes exhibited gross eye and antennal defects (Table 4 and Figure 6). The eyes of *punt* homozygotes were highly disorganized and showed striking reductions in ommatidial number. Antennal defects, like leg defects, included deletions and duplications of distal pattern elements. Both eye and antennal defects could be unilateral or bilateral. None of these defects were identified in the 469 wild-type siblings that were produced by the initial mating of *punt* heterozygotes. With one exception, the bifurcation of legs in *punt* mutant animals, all of these defects were identified in *dpp* mutant animals (Spencer *et al.* 1982). With respect to the single, apparently unique *punt* phenotype, it is notable that a role for Dpp signaling in this type of limb patterning has been suggested by Theisen *et al.* (1996). These investigators observed a high rate of bifurcations in the ventral leg after disruption of the type I Dpp receptor that is encoded by *tkv.*

DISCUSSION

Although Punt was originally identified as a type II receptor for activin-type and not Dpp/BMP-type ligands, we and others have suggested that Dpp indeed functions as the physiologically relevant Punt ligand *in vivo.* This hypothesis is based primarily upon four lines of evidence. First, zygotic loss-of-function mutations in the *punt* gene result in a dorsal-open embryonic phenotype, indistinguishable from that seen in loss-of-function alleles of *tkv,* the type I Dpp receptor. Second, maternal loss-of-function mutations in the *punt* gene result in a ventralized embryonic phenotype, indistinguishable from that seen in loss-of-function *dpp* alleles. Third, under certain conditions, Punt can bind Dpp *in vitro.* And fourth, as yet no activin-type ligand has been identified in Drosophila (Letsou *et al.* 1995; Ruberte *et al.* 1995). In the current article, we describe additional genetic tests of this hypothesis. Our studies indicate that Punt regulates multiple developmental decisions in response to the Dpp signal.

Punt function is required for dorsal closure between 8 and 12 hr AEL: The possibility that defects in dorsal closure result secondarily from defects in dorsoventral patterning has often been invoked to explain how mutations in *punt* and other Dpp signaling molecules, in-

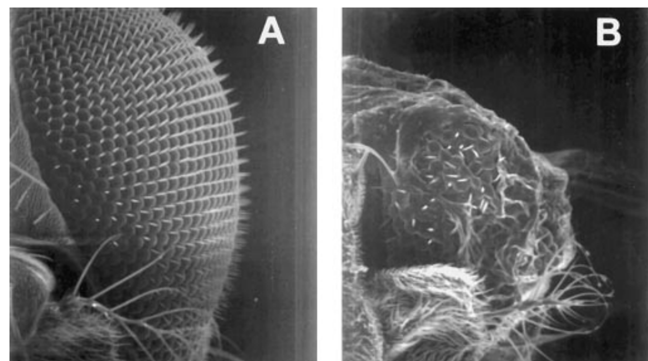


Figure 6.—Scanning electron microscopy of adult eyes. A wild-type eye (A) displays a regular pattern of ommatidia and interommatidial bristles. In contrast, *punt*^{88}/*punt*^{135} mutants exhibited a reduced number of ommatidia and bristles, and consequently were highly disorganized overall (B). Magnification is ×300.

cluding *tkv* and *schnurri* (*shn*), first affect dorsal patterning, and subsequently, dorsal closure (Nellen *et al.* 1994; Penton *et al.* 1994; Terracol and Lengyel 1994; Arora *et al.* 1995). We considered the alternative hypothesis that defects in dorsoventral patterning and dorsal closure are each primary consequences of the mutation and result from independent requirements for the *punt* gene during two different stages of embryogenesis. Using temperature-sensitive *punt* alleles, we demonstrated that the embryonic requirements for *punt* correspond to distinct time intervals; thus, the temporal requirement for *punt* during dorsal closure is separable from its earlier requirement for dorsoventral pattern formation. Our demonstration of a dual requirement for Punt during embryogenesis indicates that the dorsal-open *punt* phenotype is a primary effect of the zygotic mutation and is consistent with the documented roles for Dpp and Tkv in dorsal closure. In addition to the recent demonstration by several groups that *dpp* is a transcriptionally regulated target of Drosophila Jun-amino-terminal kinase (DJNK) signaling in leading-edge dorsal epithelial cells (Riesgo-Escovar and Hafen 1997; Hou *et al.* 1997; Glise and Noselli 1997), Riesgo-Escovar and Hafen (1997) have shown that ectodermal expression of *dpp* or *tkv* transgenes partially rescues the dorsal-open phenotype in a DJNK signaling-deficient embryo.

Targets of Punt-mediated signaling during dorsal closure have yet to be identified. Several likely candidates, however, include cytoskeletal components of the dorsal epithelium. Some of these proteins play structural roles in transforming epithelial cell shape and consequently effect dorsal closure. Specifically, mutations in the nonmuscle myosin heavy chain (Young *et al.* 1993), the Drosophila homologue of band 4.1 (Fehon *et al.* 1994), and the *canoe* gene product (Miyamoto *et al.* 1995) all produce the same gross defects in dorsal closure that we identify in *punt* mutants.

Punt functions postembryonically as a Dpp receptor: We additionally exploited temperature-sensitive *punt* alleles to demonstrate a clear requirement for *punt* postembryonically. Since the phenotypes of *punt* animals that we generated by manipulation of temperature mimicked the array of *dpp* syndromes documented by Gelbart and colleagues over a decade ago (Spencer *et al.* 1982), we conclude that Dpp functions as a Punt ligand repeatedly throughout the Drosophila life cycle. Not only are both Punt and Dpp required for embryonic patterning events, including dorsoventral axis formation and dorsal closure and gut development, but both gene products are similarly required for imaginal patterning events in the leg, wing, thoracic, and eye/antennal discs (see Figures 4–6).

It is significant that no unique phenotypes were attributed to mutations in *punt*, and this observation suggests that Punt is a dedicated Dpp receptor. However, we cannot exclude the possibility that Punt mediates signaling by additional ligands because the *punt* alleles included in this study are not null. In contrast, a slightly wider range of structural abnormalities is evident in some *dpp* mutant animals. For example, Dpp is required for patterning of adult terminalia (Spencer *et al.* 1982) and for patterning of the anterior egg shell during oogenesis (Twombly *et al.* 1996). With respect to a potential requirement for Punt in genital morphogenesis, no combination of *punt* alleles produced the gross defects in terminal structures that are characteristic of certain mutations in *dpp* (data not shown). Again, this negative result might be misleading because we have been unable to study the *punt* null phenotype. We are unable to comment upon an oogenic requirement for Punt, because our genetic studies did not permit us to examine this very early developmental stage. Despite these caveats, the overall striking phenotypic similarities in *punt* and *dpp* mutants, including embryonic defects in dorsoventral patterning, dorsal closure, and gut development, and disc defects in leg, thoracic, eye, and antennal development, clearly implicate a role for Punt in most, and perhaps all, Dpp-mediated signaling events in Drosophila.

The ability of molecules belonging to the BMP/Dpp subgroup of the TGF-β cytokine superfamily to form bioactive, disulfide-linked heterodimers in cultured cells indicates that these complexes are likely to have natural biological functions (Aono *et al.* 1995; Hazama *et al.* 1995; Israel *et al.* 1996). In addition to Dpp, two other gene products, Scw and 60A, belong to the Drosophila Dpp/BMP family of TGF-β signaling molecules (Padgett *et al.* 1987; Wharton *et al.* 1991; Arora *et al.* 1994), and hence the ability to form heterodimeric ligands might contribute to the diversity of biological responses elicited by Dpp/BMP signaling cascades in flies. The data presented in the current article, that *punt* functions as a Dpp receptor throughout the Drosophila life cycle, are certainly consistent with this view. The correspondence between *dpp* and *punt* loss-of-function phenotypes could reflect Punt function as a receptor for either homodimeric Dpp ligands or heterodimeric Dpp ligands. Scw, like Dpp and Punt, is required for differentiation of dorsal structures (Arora *et al.* 1994), and perhaps a Dpp/Scw heterodimer is a signal for dorsal cell fate differentiation *in vivo.* As we showed previously, combined maternal and zygotic loss of *punt* function results in a more severely ventralized phenotype than that observed in *scw* null mutants, and thus we think it unlikely that Punt acts solely as a Scw receptor during embryogenesis (Letsou *et al.* 1995). Since roles for the *60A* gene product have been documented in oogenesis and in gut and wing development (K. A. Wharton, personal communication), it is tempting to speculate that like Dpp and Scw, Dpp and 60A can heterodimerize to form a bioactive cytokine.

***punt* mutations define an allelic series:** Based upon molecular and genetic analyses, mutant *punt* alleles were

grouped into three distinct classes, Classes I through III, with Class I alleles being defined as strongest and Class III alleles being defined as weakest. Class I and II alleles are genetically very similar; they are heat-sensitive and exhibit a fully penetrant embryonic lethality at all temperatures tested (see Table 1). Class III alleles, in contrast, are cold-sensitive and fully viable at 25°.

The original transposon insertion allele, *punt*^{P1}, as well as three alleles that were generated by *P*-element mobilization, *punt*^{51}, *punt*^{62}, and *punt*^{88}, fall into two classes, I and II. Molecular analyses revealed all four to be regulatory mutants, harboring large insertions (>420 bp) between the second and third nucleotides in the 5′ untranslated region of *punt.* The temperature-sensitive nature of all four alleles indicates that Class I alleles, albeit capable of evoking a stronger phenotype than Class II alleles, retain partial function and are hypomorphic. It is likely that the size of the insertions in these alleles dictates the strength of the mutation because 1) the largest insertion (14 kb) maps to a Class I allele, *punt*^{P1} and 2) the smallest insertion (∼420 bp) maps to a Class II allele, *punt*^{88}. Consistent with this hypothesis is our identification of even smaller insertions in adult-viable *punt* alleles (see Table 2).

The original EMS-induced allele *punt*^{135} was identified as a recessive lethal that disrupts the embryonic process of dorsal closure (Jürgens *et al.* 1984) and was reported to represent the null phenotype (Ruberte *et al.* 1995). Molecular studies (Ruberte *et al.* 1995), as well as genetic studies reported here and elsewhere (Letsou *et al.* 1995; Ruberte *et al.* 1995), have led, however, to our designation of *punt*^{135} as a temperature-sensitive Class II allele. In *punt*^{135}, a missense mutation (Ala-376→Thr) maps to the conserved kinase domain VIII that is required for substrate recognition (Ruberte *et al.* 1995). The conclusion that the Thr substitution at position 376 does not completely abolish the kinase activity encoded by *punt*^{135} at any of the temperatures tested is based on the following observations. First, functional serine/threonine kinases harbor a conserved Ala, Ser, or Pro at this position (Hanks *et al.* 1988), and Ser→Thr substitutions can be considered to be conservative. Second, the *punt*^{135} phenotype is not the strongest in our collection (see Table 3). Finally, our observation that *punt*^{135} phenotypes are variably expressed, albeit not itself a criterion for a hypomorphic allele, is consistent with our conclusion that the kinase encoded by *punt*^{135} retains some catalytic activity (see Figure 1).

Class III alleles, *punt*^{10}, *punt*^{24}, and *punt*^{97}, harbor very small insertions, either 38 or 40 bp in length. In all three alleles, transposon-derived sequences remaining at the insertion site are oriented as inverted repeats and are capable of forming perfectly base-paired stem-loops, either 15 or 16 bp in length. The molecular definition of these mutations suggests that mRNA secondary structure reduces the efficiency of translation of the very weak Class III hypomorphic alleles (for review, see Brown and Schreiber 1996). Consistent with this hypothesis is our observation that conditions that stabilize stem-loop structures, such as lower temperatures, enhance the mutant phenotype of *punt*^{10}.

It is notable that all *punt* alleles described to date exhibit a temperature-sensitive phenotype. It is tempting to speculate that the pathway is itself temperature sensitive, but since the number of mutant alleles studied is relatively small, we favor the hypothesis that these are allele-specific temperature sensitivities and that these alleles retain partial function at the permissive temperatures.

Consistent with our hypothesis that the *punt* alleles described here are hypomorphic, but not null, are genetic studies indicating that the *punt* null phenotype is haplo-insufficient. First, *punt* maps to one of the few regions in the Drosophila genome for which a deficiency has never been recovered, and regions such as this are likely to harbor haplo-lethal loci (Lindsley *et al.* 1972). Even more meaningful were our specific efforts to generate null *punt* alleles in screens for deletions of the *punt* locus; in this regard, our failure to recover a deletion of *punt* was statistically significant ($P = 2.4 \times 10^{-5}$). Our conclusion that *punt* is a haplo-insufficient gene indicates that it is a dosage-sensitive component of the Dpp signaling pathway. While this finding does not rule out the possibility that Dpp is another dosage-sensitive component of the pathway, it is nonetheless intriguing. In standard paradigms for signaling, ligand is the single dosage-sensitive component of the pathway.

Punt functions as a multimer: In analyzing the genetic interactions of specific *punt* alleles, we observed striking differences in the phenotypes of animals harboring homoallelic and heteroallelic *punt* combinations. Homoallelic combinations of Class I and II *punt* alleles (*punt*^{P1}, *punt*^{51}, *punt*^{62}, *punt*^{88}, and *punt*^{135}) produced a fully penetrant embryonic lethality; viable adults were never recovered at any temperature (see Table 1). In contrast, heteroallelic combinations of *punt*^{135} with alleles arising from mobilization of the *P* element in *punt*^{P1} (*punt*^{51}, *punt*^{62}, and *punt*^{88}) were viable at 18°, and phenotypically normal adults were recovered at the expected Mendelian frequency (see Figure 3). Our observation that heteroallelic *punt* combinations produced a weaker phenotype than homoallelic combinations led us to speculate that there was a direct interaction between *punt* gene products in heteroallelic animals.

The specific interaction of *punt* alleles can be explained if active Punt protein functions as either a dimer or a higher order multimer in the heteromeric complex that also contains its partner and substrate, the type I receptor encoded by the *tkv* gene. In accordance with all models of interallelic complementation, each *punt* allele must supply a function in receptor

complex activation and signaling that is disrupted in the other.

We suggest that Dpp-mediated signal transduction is diminished in *punt*^{135} homozygotes due to the defect in the kinase substrate recognition site that was identified by Ruberte *et al.* (1995). In addition, we suggest that signaling is defective in insertion alleles such as *punt*^{88} because a 5′ UTR sequence insertion results in downregulation of Punt and its consequent inefficient multimerization. In our model for interallelic complementation in *punt*^{88}/*punt*^{135} animals, wild-type quantities of signaling-defective Punt^{135} increase the pool of receptor monomers and consequently increase the probability of forming a functional receptor dimer (or higher order multimer) that contains at least one wild-type Punt^{88} isoform (Figure 7).

Consistent with our genetic demonstration that type II receptor serine/threonine kinases function as multimers *in vivo* are the results of several biochemical studies performed in somatic cells. Similarities between the TGF-β receptor serine/threonine kinases and the well-characterized EGF (epidermal growth factor) homodimeric receptor tyrosine kinases that are capable of autophosphorylation *in vivo* (Lammers *et al.* 1990; Canals 1992) were first observed in structure/function studies (Wrana *et al.* 1994). In biochemical experiments designed to determine whether the stoichiometry of components in the TGF-β signaling complex is analogous to that in EGF signaling complexes, mink lung epithelial cells and COS-1 and -7 cells were transfected with type I and type II TGF-β receptors. In all cell types, homomeric receptor dimers were recovered (Chen and Derynck 1994; Henis *et al.* 1994; Yamashita *et al.* 1994).

The biological relevance of the type II receptor interaction is revealed in the genetic studies described in this article. That the type I receptor similarly functions as an oligomer *in vivo* has also been only recently demonstrated (Weis-Garcia and Massagué 1996). Whereas cotransfection of kinase-defective TβR-I (TGF-β type I receptor) mutants and activation-defective TβR-I mutants restores TGF-β responsiveness to R-1B cells, neither construct restores TGF-β responsiveness to R-IB cells when transfected alone. Taken to-

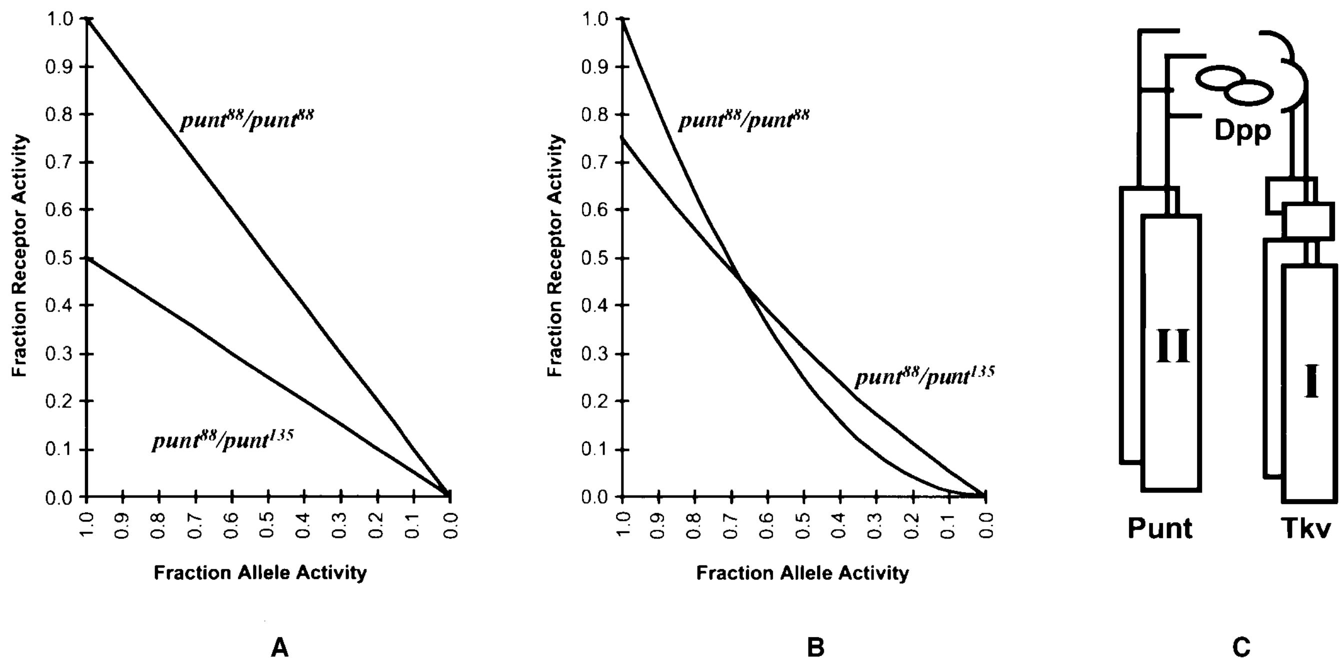


Figure 7.—Theoretical comparison of receptor activity in *punt*^{88}/*punt*^{88} and *punt*^{88}/*punt*^{135} mutants. For this theoretical comparison we have made the following simplifying assumptions: 1) Punt^{135} has no signaling activity, and 2) a dimer containing a single Punt^{88} subunit will have full signaling activity. In a monomer model for Punt function (A), the rate of receptor complex formation is linearly related to the relative concentration of Punt^{88}. In a *punt*^{88}/*punt*^{88} animal: Fraction(active Punt) $= K([\mathrm{Punt_{rel}}] + [\mathrm{Punt_{rel}}])2K = [\mathrm{Punt_{rel}}]$. In a *punt*^{88}/*punt*^{135} animal: Fraction(active Punt) $= K([\mathrm{Punt_{rel}}] + [0])/2K$. Because the *punt*^{88}/*punt*^{135} line always remains below the *punt*^{88}/*punt*^{88} line, this monomeric model for Punt function is inconsistent with our experimental observation of interallelic complementation. In a dimer model for Punt function (B), the fraction of active receptor complex formation is exponentially related to the relative concentration of wild-type *punt* gene product, $[\mathrm{Punt_{rel}}]$, that is produced. In a regulatory mutant (*e.g.*, *punt*^{88}/*punt*^{88}): Fraction(active Punt) $= K([\mathrm{Punt_{rel}}]^2 + 2[\mathrm{Punt_{rel}}][\mathrm{Punt_{rel}}] + [\mathrm{Punt_{rel}}]^2)/4K = [\mathrm{Punt_{rel}}]^2$. In a heteroallelic mutant that harbors a regulatory allele in combination with a kinase-defective allele (*e.g.*, *punt*^{88}/*punt*^{135}): Fraction(active Punt) $= K([\mathrm{Punt_{rel}}]^2 + 2[\mathrm{Punt_{rel}}][1] + [0]^2)/4K$. The dimeric model (or any multimeric model for Punt function) is consistent with our experimental observation of interallelic complementation: when regulatory mutant levels are low, the *punt*^{88}/*punt*^{135} model curve crosses over the *punt*^{88}/*punt*^{88} curve. In (C), a schematic representation of a signaling heteromer is presented.

gether, the interallelic complementation studies presented in the current article and elsewhere (Weis-Garcia and Massagué 1996) indicate that the structure of TGF-β receptor complexes, such as the Dpp receptor complex in Drosophila, is oligomeric and harbors at least two copies of the type I and II serine/threonine kinase receptors.

In summary, our characterization of three classes of temperature-sensitive *punt* alleles has led to a fuller understanding of Dpp receptor complex function *in vivo.* Our molecular and genetic studies revealed essential and repeated roles for the Dpp receptor encoded by the *punt* gene throughout the Drosophila life cycle. In addition, the shared loss-of-function phenotypes that we documented in *punt* and *dpp* mutants implicate Punt as an important mediator of virtually all Dpp signals. Finally, our observation that interallelic complementation depended on the presence of two types of *punt* mutation, one that specifically reduces kinase function and one that disrupts gene regulation, revealed the multimeric structure of Punt during signaling.

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