DOI: 10.1534/genetics.109.105585

Functional Analysis of *saxophone*, the Drosophila Gene Encoding the BMP Type I Receptor Ortholog of Human ALK1/ACVRL1 and ACVR1/ALK2

Vern Twombly,* Erdem Bangi,[†,1] Viet Le,[†] Bettina Malnic,[‡] Matthew A. Singer[§] and Kristi A. Wharton[†,2]

**Department of Biological Sciences, University of Pittsburgh, Pennsylvania 15260, [‡]Departamento de Bioquímica, Instituto de Química University of São Paulo, 05508-900 Brazil, [§]Stemgent, San Diego, California 92121 and [†]Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Manuscript received May 29, 2009
Accepted for publication June 13, 2009

ABSTRACT

In metazoans, bone morphogenetic proteins (BMPs) direct a myriad of developmental and adult homeostatic events through their heterotetrameric type I and type II receptor complexes. We examined 3 existing and 12 newly generated mutations in the Drosophila type I receptor gene, *saxophone* (*sax*), the ortholog of the human *Activin Receptor-Like Kinase1* and *-2* (*ALK1/ACVRL1* and *ALK2/ACVR1*) genes. Our genetic analyses identified two distinct classes of *sax* alleles. The first class consists of homozygous viable gain-of-function (GOF) alleles that exhibit (1) synthetic lethality in combination with mutations in BMP pathway components, and (2) significant maternal effect lethality that can be rescued by an increased dosage of the BMP encoding gene, dpp^+. In contrast, the second class consists of alleles that are recessive lethal and do not exhibit lethality in combination with mutations in other BMP pathway components. The alleles in this second class are clearly loss-of-function (LOF) with both complete and partial loss-of-function mutations represented. We find that one allele in the second class of recessive lethals exhibits dominant-negative behavior, albeit distinct from the GOF activity of the first class of viable alleles. On the basis of the fact that the first class of viable alleles can be reverted to lethality and on our ability to independently generate recessive lethal *sax* mutations, our analysis demonstrates that *sax* is an essential gene. Consistent with this conclusion, we find that a normal *sax* transcript is produced by sax^J, a viable allele previously reported to be null, and that this allele can be reverted to lethality. Interestingly, we determine that two mutations in the first class of *sax* alleles show the same amino acid substitutions as mutations in the human receptors ALK1/ACVRl-1 and ACVR1/ALK2, responsible for cases of hereditary hemorrhagic telangiectasia type 2 (HHT2) and fibrodysplasia ossificans progressiva (FOP), respectively. Finally, the data presented here identify different functional requirements for the Sax receptor, support the proposal that Sax participates in a heteromeric receptor complex, and provide a mechanistic framework for future investigations into disease states that arise from defects in BMP/TGF-β signaling.

THE type I serine-threonine receptors of the transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) signaling pathway are critical for the transduction and specificity of signals initiated by the secreted ligands of this superfamily. A dimeric ligand elicits a vast range of biological responses by binding to the extracellular domain of a heterotetrameric receptor complex comprising two type I and two type II serine/threonine kinase receptors that then transduce a signal by phosphorylation of intracellular transcriptional regulators (reviewed by Yamashita *et al.* 1994; Weis-Garcia and Massague 1996; Kirsch *et al.* 2000a,b; Shi and Massague 2003; ten Dijke and Hill 2004). Both receptor types have a cysteine-rich, ligand-binding extracellular domain, a single transmembrane domain, an intracellaular kinase domain, and associated regulatory domains. Additionally, type I receptors contain a glycine-serine repeat (GS) domain, which is required for full kinase activation (Franzen *et al.* 1995). When complexed, the constitutive kinase activity of the type II receptor transphosphorylates the type I GS domain, activating the type I receptor, which binds and phosphorylates primarily receptor-mediated Smad proteins (R-Smad) (Carcamo *et al.* 1994; Wrana *et al.* 1994; Chen *et al.* 1998b; Macias-Silva *et al.* 1998; Chen and Massague 1999). Mutations in the human type I receptors ALK1/ACVRL1, ALK2/ACVR1, ALK3/BMPR1A, ALK4/ACVR1B, ALK5/TGFβR1, and ALK6/BMPR1B have been identified and in each case are associated

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.105585/DC1.

[1]*Present address:* Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029.

[2]*Corresponding author:* Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Box G-L370, Providence, RI 02912. E-mail: kristi_wharton@brown.edu

with a disease and/or developmental syndrome with very specific manifestations, *e.g.*, hereditary hemorrhagic telangiectasia type 2 (HHT2) (ALK1/ACVRL1), fibrodysplasia ossificans progressiva (FOP) (ALK2/ACVR1), juvenile polyposis syndrome (ALK3/BMPR1A), pancreatic adenocarcinoma (ALK4/ACVR1B), Loeys-Dietz syndrome (ALK5/TGFβR1), and brachydactyly type A2 (ALK6/BMPR1B) (Howe *et al.* 2001; Su *et al.* 2001; Zhou *et al.* 2001; Kim *et al.* 2003; Loeys *et al.* 2005; Lehmann *et al.* 2003, 2006; Abdalla and Letarte 2006; Bayrak-Toydemir *et al.* 2006; Shore *et al.* 2006; Wehner *et al.* 2006; Olivieri *et al.* 2007). In general, the specific effects of each mutation are not well understood in terms of signal transduction and functional consequences. In this report, we describe the identification of new mutations and the functional characterization of two distinct classes of *saxophone* (*sax*) alleles in the *Drosophila melanogaster* ortholog of human ALK1/ACVRL1 and ALK2/ACVR1 type I receptors.

In addition to *saxophone* (*sax*), two other Drosophila type I receptors are encoded by the *thick veins* (*tkv*) and *baboon* (*babo*) genes (Childs *et al.* 1993; Brummel *et al.* 1994, 1999; Nellen *et al.* 1994; Penton *et al.* 1994; Xie *et al.* 1994). Like the ligands, the Drosophila type I receptors have both overlapping and distinct domains of expression and functions that are essential to the development of a variety of tissues and organs (Schupbach and Wieschaus 1989; Affolter *et al.* 1994; Brummel *et al.* 1994, 1999; Nellen *et al.* 1994; Penton *et al.* 1994; Terracol and Lengyel 1994; Xie *et al.* 1994; Bangi and Wharton 2006b). Tkv and Sax are both essential mediators of BMP signaling, where the loss of *tkv* results in a complete loss of the phosphorylated form of the BMP-specific R-Smad protein, Mad (pMad), while loss of *sax* leads to reduced pMad levels (Singer *et al.* 1997; Dorfman and Shilo 2001; Bangi and Wharton 2006b).

The Drosophila orthologs of vertebrate BMP2/4 and BMP5/6/7/8 ligands are encoded by *decapentaplegic* (*dpp*) and *glass bottom boat* (*gbb*), respectively (Padgett *et al.* 1987; Wharton *et al.* 1991; Doctor *et al.* 1992). A third more divergent Drosophila BMP encoded by *screw* (*scw*), (Arora *et al.* 1994) appears to be specifically required for embryonic dorsal/ventral patterning (reviewed by O'connor *et al.* 2006). Tkv acts as a high affinity Dpp receptor on the basis of data from genetic and biochemical studies while Sax exhibits a higher affinity for Scw and Gbb (Haerry *et al.* 1998). In two well-characterized developmental processes, Tkv and Sax receptors have been shown to mediate signaling from the Drosophila BMPs, Dpp and Scw, in embryonic dorsal/ventral patterning, and Dpp and Gbb in the generation of a BMP activity gradient required for patterning the wing imaginal disc (Brummel *et al.* 1994; Nellen *et al.* 1994; Burke and Basler 1996; Zecca *et al.* 1996; Singer *et al.* 1997; Chen *et al.* 1998a; Khalsa *et al.* 1998; Neul and Ferguson 1998; Nguyen *et al.* 1998; Ray and Wharton 2001; O'connor *et al.* 2006; Bangi and Wharton 2006a,b).

Several other *sax* loss-of-function studies indicate that Sax also contributes to patterning the anterior eggshell, as well as to the maintenance of germline stem cell divisions (Schupbach and Wieschaus 1989; Xie *et al.* 1994; Twombly *et al.* 1996; Xie and Spradling 1998). The contribution of Sax to overall BMP signaling appears to be more complex than originally thought as indicated by its ability to play both a positive and a negative role in signaling (Bangi and Wharton 2006b). The ability of Sax to mediate signaling requires the presence of Tkv and thus, the molecular basis of Sax's dual role has been proposed to depend on the combination of type I receptors that make up the signaling complex, whereby Tkv–Sax complexes promote signaling while Sax–Sax complexes bind ligand but are unable to transduce a signal.

Differential signaling output dependent on type I receptor composition has also been suggested for ALK1 and ALK5 (Goumans *et al.* 2003; Finnson *et al.* 2008). In most contexts, human TGF-β signals through the ALK5 receptor; however, in endothelial cells and chondrocytes ALK1 is also involved in the transduction of TGF-β signals (Chen and Massague 1999; Oh *et al.* 2000; Goumans *et al.* 2003; Finnson *et al.* 2008). In response to TGFβ1, ALK5 is essential for ALK1 kinase activation and the phosphorylation of Smad1/5, while ALK5 alone acts in Smad3 phosphorylation. ALK1 acts to inhibit TGFβ1-induced Smad3 phosphorylation and thus, the presence of different levels of ALK1 and ALK5 in both endothelial cells and chondrocytes will likely have a dramatic influence on overall signaling output.

Prior to the work reported here, a limited number of *sax* mutations were available for the study of Drosophila BMP receptor function. The original *sax* alleles, sax^{1} and sax^{2}, were identified as maternal effect lethal (MEL) mutations, in which the maternal genotype is the overriding factor in determining the mutant phenotype of the progeny (Brummel *et al.* 1994; Nellen *et al.* 1994; Penton *et al.* 1994; Xie *et al.* 1994). To gain a better understanding of the range of *in vivo* functions of *sax*, we undertook genetic screens to isolate new mutant alleles. Here, we report the generation of such *sax* alleles, their characterization and their interactions with other BMP signaling pathway elements. We show that the preexisting alleles, sax^{1} and sax^{2}, as well as sax^{P}, are mutations that behave in a manner distinct from deficiencies that lack the *sax* coding region. In fact, the MEL of these alleles can be reverted to recessive lethal alleles and thus, we classify sax^{1}, sax^{2}, and sax^{P} alleles as gain-of-function (GOF) alleles indicating that the product produced by these alleles has a function beyond that of the wild-type protein. In contrast to the behavior of these GOF alleles, the new *sax* mutations are partial or complete loss-of-function (LOF) on the basis of their similarity to deficiencies in a number of assays.

Our studies provide insight into the functional consequences of different types of receptor mutations and will aid in understanding the molecular mechanisms by which Sax-like type I receptors contribute to signaling and the effects of specific lesions associated with human disease.

MATERIALS AND METHODS

Fly strains and culture conditions: All strains used in this study are referenced in FlyBase (2003) and cultured by standard methods. Canton-S and y^1 *Df(1)67c23* strains represent wild type. All dpp^{hr} strains are described previously in (Wharton *et al.* 1993, 1996). *Dp(dpp⁺)* refers to *Dp(2;2)DTD48*, dpp^{dho}, or *Dp(2;2)VT1*, dpp^+ (also designated *Dp(2;2)B16*), which are both tetrasomic for dpp^+, and Mad^{null}, refers to *Df(2L)JS17*. The sax^1 mutation is no longer homozygous viable, presumably due to a secondary, unassociated lethal mutation on the same chromosome. *Df(2R)P32* (43A3-43F6) and *Df(2R)H23* (43C1-43F2) both lack *sax* function (Brummel *et al.* 1994; Nellen *et al.* 1994; Xie *et al.* 1994) while *Df(2R)ST1* (42B3-43E18) retains *sax* function. The inducible sax^+ rescue construct, *P[hs-sax]*, contains a *sax*-RA cDNA with the potential to encode both Sax-PA and Sax-PB (Brummel *et al.* 1994).

Genetic and phenotypic studies: *Maternal sax^1 and sax^2 assays:* For the lethal phase: Females of a given *sax* genotype were separately crossed to males of indicated genotypes (Figure 1, Tables 2, and 3). Resulting embryos were harvested 8–12 hr after egg lay (AEL) and transferred to a grid on standard fly culture media. Embryonic lethality and viability was assessed at 12, 24, and 36 hr AEL and larval viability at 10–15 days AEL. Adult eclosion was assessed for up to 18 days AEL. Embryonic lethal phenotypes were examined as Hoyer's mounted embryonic/first instar larval cuticle preparations and as live embryos under halocarbon oil.

Maternal loss-of-function assays: Genotypes and crosses were performed by standard methods as described in Twombly *et al.* (1996). Briefly, germline clones were induced by heat shock of late third instar larvae and early pupae of the genotype *P{hs-FLP}/Y; P{>w⁺>} P{ovo^{D1}=18}32X9/P{>w⁺>} sax^5 sha^1*. Several hundred such females possibly containing sax^5 sha^1 homozygous germline clones were mated to appropriate tester males and progeny were processed as described above.

Zygotic loss-of-function studies: Heterozygous sax^A/+ females were mated to males heterozygous for a second allele (sax^B/*SM6a*) and the progeny were counted. All crosses were tested in two assays: (1) lethal phase and (2) zygotic lethal phenotype. Lethal phase was treated as above. For lethal phenotypes, sax^3, sax^4, sax^5, and sax^6 were mated to *Df(2R)H23* males. All strains utilized *CyO, P{ry[+t7.2]=en1}wg[en11]*, permitting the unambiguous identification of hemizigous larvae. Fifty larvae of each hemizygous genotype were examined for morphological abnormalities. Larval and pupal samples in 50% glycerol were documented with bright field and Nomarsky photomicroscopy.

Amnioserosa cell counts: Progeny from *sax* mutant females were crossed to Canton-S males and the resulting embryos were harvested from a 4- to 7-hr egg lay and aged 9 hrs before fixation. Anti-Krüppel antibody labeling was performed as described previously in Wharton *et al.* (1993). In triplicate, 20 embryos were scored for each genotype. For each 20, the four high and four low counts were removed, then the mean was determined. This was necessary to uncover statistically different means, in an assay that has considerable variation.

Genetic interactions studies: For maternal interactions with *dpp*, females of the relevant *sax** genotype were mated to heterozygous dpp^{hr} males. Cultures were neither overcrowded nor sparse. All progeny were scored through day 18. The percentage of survivorship of dpp^{hr} was calculated as $\{[(sax^*/dpp^{hr} + dpp^{hr}/CyO) \div 2] \div (sax^*/CyO)\} \times 100$. Control crosses, performed identically, reversed the sex of the genotypes. For dominant maternal *Mad–sax* interactions, the lethality of the progeny from Mad^* sax/Mad^+ sax^* females were determined as described for lethal phase studies. The zygotic phenotypes of *Df(2L)JS17* sax^1/dpp^{d12} adults were scored by examination of each leg of the adult for the presence of tarsal claws. For zygotic interactions with *dpp* in the wing, dpp^{hr56}/*SM6a* females were crossed to dpp^{d5}*/*SM6a* males, where * is sax^1, sax^4, sax^5 or *Df(2R)H23*. Wings of *dpp* mutant progeny were mounted in DPX mountant (EM Sciences) and scored for patterning defects in the longitudinal veins. Images were collected on a Nikon FXA with a SPOT-RT camera (Diagnostic Instruments).

sax complementation and rescue: Percentage of survivorship was calculated as $([(sax^A/sax^B) \div [(sax^A/SM6a + sax^B/SM6a) \div 2]) \times 100$. Crosses were performed in both directions. The viability of sax^3 hemizygotes utilized *sax* deficiencies and sax^1 revertants. All rescue experiments were performed as per Brummel *et al.* (1994). Percentage of rescue equals $\{sax^*/Df(2R)sax \div [(sax^*/SM6a + Df(2R)sax/SM6a) \div 2]\} \times 100$.

Genetic screens for *sax* alleles: *sax^1 reversion screen*: G_0 irradiated (4000 rads, Cs^{137}) cn^1 sax^1 bw^1 *sp/SM6a* males were mated to nco^{Sco} cn^1 sax^2 bw^1/*SM6a* females. Cross A: 1362 [5 G_1 sax^{1*}/nco^{Sco} sax^2 females] were mated to wild-type males to test for a reduction in the expected 100% MEL. Cross B: 1920 [5 G_1 sax^{1*}/*SM6a* females] were crossed to dpp^{hr4} *sp/SM6a* males to test for the loss of maternal enhancement of *dpp* mutations [scored for presence of *Cy*+ (dpp^{hr4}/sax^{1*}) progeny]. In the absence of a sax^1 revertant, few or no progeny resulted from cross A, while a candidate sax^{1*} reversion was indicated by the presence of ≥15 progeny. The presence of a sax^{1*} reversion in cross B led to *Cy*+ (dpp^{hr4}/sax^{1*}) progeny.

Hobo element mutagenesis screen: The homozygous viable, fertile enhancer trap *H{Lw2}SW283* (Smith *et al.* 1993) was mobilized using the *Hobo* transposase source, *CyO-P{HBL1}2* (Calvi and Gelbart 1994). *H{Lw2}SW283* complements all *sax* mutations. Twenty-four thousand G_1 progeny were scored for changes in w^+ eye color, resulting in 214 independent mobilizations that were tested for zygotic lethality and MEL in *trans* to *Df(2R)P32* and sax^{1rv1}, respectively. Two independently derived zygotic lethal 2nd chromosome mutations were recovered, which retained the original *Hobo* and a new insertion with an adjacent deletion (data not shown). These lines were further mobilized and no reversion was observed.

Mad-sax^1 reversion screen: A double mutant, *Df(2L)JS17* cn^1 sax^1, chromosome (referred to as *Df(Mad)sax^1*) was generated and balanced over a CyO containing a dpp^+ rescue transgene (Wharton *et al.* 1993), which zygotically rescues the embryonic lethality of this strain. Male *Df(2L)JS17* cn^1 sax^1/*CyO23* flies were EMS treated (Lewis and Bacher 1968) and crossed to wild-type females. Resulting G_1 *Df(2L)JS17* cn^1 sax^1/+ + females were tested for fertility. In the absence of a reversion event, few adult progeny were observed, while a revertant resulted in a modest number of progeny (≥15).

F_2 lethal screen: Isogenized nub^1 b^1 pr^1 males were EMS treated and crossed to a balancer strain. Individual F_1 males were crossed to tester sax^{1rv1} and *Df(2R)sax-H9* females. A test *sax* rescue cross was performed at 25°, which exhibits 10–15% rescue (Brummel *et al.* 1994). Lethal mutations that could be rescued were assayed for viability over *Df(2R)H23* and *Df(2R)ST1*.

sax^P excision screen: The *P*-element insertion was mobilized using *CyO,P[HBL1]2*. Female *y Df(1)67c23; In(2LR)Gla/CyO,*

P[HBL1]2 were mated to *Df(1)67c23; sax^{p}/SM6a* males and the F_1 Cy, Gla^+ male progeny were recovered. These *y Df(1)67c23; sax^{p}/CyO,P[HBL1]2* males were mated to *Df(1)67c23; In(2LR)Gla/SM6a* females in individual culture vials and *Cy, Gla^+, w^-* male progeny were recovered and stocked by crosses to *Df(1)67c23; In(2LR)Gla/SM6a* females. Of 50 F_1 males crossed, 42 w^- excision lines (*sax^{PE}*) were recovered and tested for lethality in *trans* to the *sax^4*, *sax^5*, and *Df(2R)H23* chromosomes.

Molecular analysis and DNA sequencing: Genomic DNA was isolated from larvae hemizygous (*sax*/Df(2R)H23*) for *sax* mutations, *sax^3*, *sax^4*, *sax^5*, and *sax^6*, according to standard procedures (Wharton *et al.* 1996). Regions of the *sax* gene were amplified by PCR and sequenced using the dsDNA cycle sequencing system (GIBCO BRL). PCR-induced sequence changes were eliminated by sequencing two or more independent PCR amplifications. The entire coding sequence of all the alleles was determined. The primer pairs used were (5′ to 3′) TAGGCTCGGACAAATAAC and CATTAGCTATG GACAGGC3 or TGATGACGCACTACTATC and GTCTTGTA CTTGGATTAG.

For RT–PCR, RNA was isolated from *yw^{1118}* and *yw; FRT^{G13} sax^{p}* homozygous third instar larvae ($n = 10$) using QIAGEN RNeasy and treated with Promega RQ1 DNase before cDNA synthesis. cDNA was synthesized using MLV RTase (Sigma) and oligo $(dT)_{12\text{-}18}$ primer (Invitrogen). RT–PCR analysis was conducted using the following primers: (sax 9396 fwd) GCTGTGCCGGTGATTA CTG and (sax 11119 rev) GTCTTGTACTTGGATTAG; (P{lacW} 9661 fwd) GGATCTTC TTGAGATCC and (P{lacW}10648 fwd) GGATGTCTCTTGCC GACGGG; (8103) CGTTTCTGCTGTACAATAATGCCAG and (10228) GCCCATTAGCTATGGACAGGC.

The *sax^5* mutation was generated in a *sax*-RA cDNA clone (gift from Mike O'Connor) by primer extension using *Pfu*Ultra DNA Polymerase (Stratagene) with the following primer GGCGAAAGCATCGACGTGAAGATAT.

Cell-based BMP signaling assay: A cell-based BMP signaling assay using S2 cells has been described previously (Muller *et al.* 2003; Bangi and Wharton 2006b) and depends on the endogenous expression of BMP signaling components. In this assay, a reporter construct expressing *lacZ* is controlled by a *Su(H)* transcriptional activation response element as well as a *brk* transcriptional silencer element (*Su(H)/brk-lacZ*). Transcription is activated by cotransfection of the reporter construct with plasmids encoding Su(H) and an activated form of Notch (N*). Activation of BMP signaling leads to repression of *lacZ* expression due to the presence of the *brk* silencer element, and thus, a reduction in β-galactosidase activity. BMP signaling levels are inversely correlated with the level of β-galactosidase activity. Plasmids containing the coding sequences of *tkv* (pAcpA-tkv1-FLAG), *sax* (pAcpA-sax-FLAG), or *sax^5* (pAW-sax^5) were cotransfected with Su(H), N*, Su(H)/brk-lacZ, and luciferase plasmids, all under the control of the actin 5C promoter using Effectene Transfection (QIAGEN). β-Galactosidase values were measured using the dual luciferase assay system (Dual-Light, Applied Biosystems) and normalized to luciferase for each sample.

RESULTS

Maternal contribution of *sax^1* or *sax^2* affect BMP signaling: The *saxophone* (*sax*) gene was originally identified by genetic screens for mutations resulting in recessive MEL (Schupbach and Wieschaus 1989). When crossed to wild-type males, females homozygous for a *sax* mutation, *sax^1* or *sax^2* (*sax^1/sax^1*, *sax^2/sax^2*, or *sax^1/sax^2*), produce phenotypically normal eggs that die as embryos (Table 1, compare crosses F, H and G, O). Cuticle preparations indicate that these lethal embryos exhibit a weakly ventralized phenotype, reminiscent of weak embryonic lethal *dpp* alleles (Schupbach and Wieschaus 1989; Wharton *et al.* 1993; Brummel *et al.* 1994). The loss of BMP-dependent dorsal patterning is also supported by the observed reduction in the number of Kr-positive amnioserosa cells (the dorsal most embryonic fate) in embryos laid by *sax^1/sax^2* mutant mothers (Table 1).

MEL of *sax^1* and *sax^2* rescued by *dpp$^+$*: We find that this MEL induced by *sax^1* or *sax^2* can be significantly rescued if the fathers possess two extra copies of the wild-type *dpp* gene (Table 1, crosses I and P), such that all progeny carry three copies of *dpp$^+$*. This rescue of MEL suggests that an increase in zygotic *dpp$^+$* copy number leads to an increase in ligand level and a subsequent increase in BMP signaling that compensates for a loss of maternal *sax* function.

Interestingly, we discovered that when homozygous, *sax^1* and *sax^2* mothers induce a higher percentage of embryonic lethality than when either allele is in *trans* to a *sax* deficiency (*i.e.*, *sax^1/Df(2R)P32* or *sax^1/Df(2R)H23*) (Table 1, crosses L, N, S, U). This finding raised the possibility that *sax^1* and *sax^2* are GOF alleles and not standard null, LOF alleles. Both *sax^1* and *sax^2* are missense mutations that alter conserved amino acid residues within the kinase domain of the Sax receptor (Brummel *et al.* 1994), possibly producing a defective Sax receptor protein that is in some way detrimental to normal BMP signaling in the early embryo. The MEL induced by hemizygous *sax^1* or *sax^2* is also rescued by *dpp* duplications (Table 1, crosses M and T), again indicating that the lethality induced by aberrant *sax* function in the mother can be compensated for by increased BMP signaling. The higher embryonic lethality produced by homozygous *sax^1 or sax^2* mutant mothers could reflect the fact that BMP signaling in the early embryo is particularly sensitive to the absolute level of a defective Sax protein.

Mutant *sax* maternal contribution reduces *dpp* function: We further investigated the possibility that *sax^1* and *sax^2* negatively impact BMP signaling by examining the ability of these alleles to enhance *dpp* haplolethality (Brummel *et al.* 1994; Nellen *et al.* 1994; Penton *et al.* 1994; Xie *et al.* 1994). *dpp^{hr4}/+* males crossed to +/+ females result in viable heterozygous *dpp^{hr4}/+* progeny (Figure 1A). However, when *dpp^{hr4}/+* males are crossed to *sax^1/+* or *sax^2/+* females, *all* the resulting *dpp^{hr4}/+* progeny die. Importantly, deficiencies known to lack the *sax* coding sequences show little maternal enhancement of *dpp^{hr4}/+* lethality, as is also true of deficiencies that do not uncover *sax* function (*Df(2R)ST1*) (Figure 1) (Brummel *et al.* 1994; Nellen *et al.* 1994; Nicholls and Gelbart 1998). To test if this effect was specific to *dpp^{hr4}*, we examined a series of *dpp^{hr}*

TABLE 1

Maternal effect lethality of *sax*^{1} and *sax*^{2} mutations

Cross	Female genotype	Male genotype	% lethality					Kr^{+} AS cells
			Embryonic	Larval	Pupal	Total	*n*	No.[b]
A	*Df(2R)ST1/+*	+/+	11.3	5.1	4.4	20.9	273	
B	*Df(2R)H23/+*	+/+	1.5	4.1	0.7	6.4	266	
C	*sax^{1}/+*	+/+	0	0.7	1.7	2.4	293	
D	*sax^{2}/+*	+/+	1.6	3.5	0.8	5.9	254	
E	*sax^{1}/+*	*sax^{2}/+*	1	0.6	1.3	2.9	521	
F	+/+	*sax^{1}/sax^{2}*	1	2.7	0.3	4.1	293	
G	+/+	*sax^{2}/sax^{2}*	0.7	1.7	0.7	3.2	286	
H	***sax^{1}/sax^{2}*** [a]	+/+	**100**	0	0	100	971	28 ± 5
I	*sax^{1}/sax^{2}*	*Dp(dpp^{+})/Dp(dpp^{+})*	37.7	45.8	6	89.5	284	
J	*sax^{1}/Df(2R)ST1*	+/+	4.4	2.9	1.4	8.8	274	
K	*sax^{1}/Df(2R)ST1*	*Dp(dpp^{+})/Dp(dpp^{+})*	10.6	7.6	1	19.2	198	
L	***sax^{1}/Df(2R)H23***	+/+	**85.2**	12.4	0.3	97.9	290	
M	*sax^{1}/Df(2R)H23*	*Dp(dpp^{+})/Dp(dpp^{+})*	8.2	4.9	4.1	17.2	245	
N	*sax^{1}/Df(2R)P32*	+/+	91.7	7.2	0.7	99.7	279	42 ± 4
O	***sax^{2}/sax^{2}***	+/+	**81.7**	14.3	0.8	96.8	252	
P	*sax^{2}/sax^{2}*	*Dp(dpp^{+})/Dp(dpp^{+})*	9.7	8.9	3.1	21.8	257	
Q	*sax^{2}/Df(2R)ST1*	+/+	2.2	11.9	4.1	18.4	267	
R	*sax^{2}/Df(2R)ST1*	*Dp(dpp^{+})/Dp(dpp^{+})*	11	10.5	2.9	24.4	209	
S	***sax^{2}/Df(2R)H23***	+/+	**41.7**	37.1	6.7	85.5	283	
T	*sax^{2}/Df(2R)H23*	*Dp(dpp^{+})/Dp(dpp^{+})*	5	5	0	10	238	
U	*sax^{2}/Df(2R)P32*	+/+	64.8	27.7	3.7	96.1	267	69 ± 5

Boldface genotypes and lethality values highlight the distinct behavior of *sax*/sax** or *sax*/sax* vs. sax*/Df(2R)H23* or *sax*/Df(2R)H23* females. Underlined values indicate the failure of *Df(2R)ST1* to uncover the *sax* maternal effect lethality.

[a] *sax^{1}/sax^{1}* homozygotes are not shown due to the presence of a second site, unrelated, lethal on the chromosome, acquired since the isolatio of *sax^{1}*.

[b] Compared to 164 ± 23 Kr^{+} aminoserosa (AS) cells present in wild-type embryos (Raftery *et al.* 1995).

alleles, *dpp^{e87}*, *dpp^{hr56}*, *dpp^{h90}*, *dpp^{hr4}*, and *dpp^{hr27}*, which represents decreasing Dpp activity (respectively) and lesions in three different essential regions of the gene (Wharton *et al.* 1993, 1996). We found that both *sax^{1}/+* and *sax^{2}/+* females are strong maternal enhancers of all *dpp^{hr}* mutations tested. We also found that this maternal enhancement of *dpp* haplolethality by *sax^{1} or sax^{2}* is rescued by a *dpp* duplication, suggesting that an increase in *dpp* levels can overcome the lethality caused by defective maternal *sax* function (Figure 1A).

Dominant Mad-sax genetic interaction dominant interactions have been observed between *sax^{1}* and *sax^{2}* and mutations in *Mothers against dpp* (*Mad*) and *Medea* (*Med*), two genes encoding the R-Smad and co-Smad intracellular mediators of BMP signaling, respectively (reviewed by Raftery and Sutherland 2003) (Figure 1B). Strong MEL is observed when double transheterozygous females *Df(Mad) sax^{+}/Mad^{+} sax^{1}*, *Mad^{12} sax^{+}/Mad^{+} sax^{1}* or *Mad^{12} sax^{+}/Mad^{+} sax^{2}* are crossed to wild-type males (Figure 2A). In addition to this dominant interaction between *Mad-sax* in the mother, we also observed a zygotic interaction, at later stages of development, in the adult appendages (Figure 2B). The presence or absence of the most distal element of the adult leg, the tarsal claw, is easily quantified and represents a sensitive assay for *dpp* function, whose highest activity is required in the distal most elements of adult appendages (Spencer *et al.* 1982). In *dpp^{d12}Mad^{+} sax^{+}/dpp^{+} Df(Mad) sax^{+}* transheterozygotes, 78% of the individuals have a full complement of legs (6) with tarsal claws and the remaining having at least three legs with tarsal claws. By introducing the *sax^{1}* mutation into this genotype, 53% of the *dpp^{d12}Mad^{+} sax^{+}/dpp^{+}Df(Mad) sax^{1}* adults lack all tarsal claws and none (0%) have tarsal claws on all six legs. A similar effect is seen with defects in wing venation (data not shown). Taken together, our data from the MEL studies, the maternal enhancement of *dpp* mutant genotypes, and the dominant interaction between *Mad* and *sax^{1} and sax^{2}* alleles, indicate that *sax^{1}* and *sax^{2}* produce more severe phenotypes than *sax* deficiencies, consistent with being GOF alleles. Furthermore, the GOF activity of *sax^{1}* and *sax^{2}* appears to impact the output of Dpp/BMP signaling at multiple stages of development.

Genetic screens for new *sax* alleles: Given that *sax^{1}* and *sax^{2}* exhibit GOF activity, we reasoned that it should be possible to revert this behavior such that any potential revertants would behave similarly to deficiencies of *sax*. We carried out five different genetic screens for (1) revertants of *sax^{1}* or *sax^{2}* or for (2) the isolation of new *sax* alleles (Table 2; supporting information, Figure S1; materials and methods). Here,

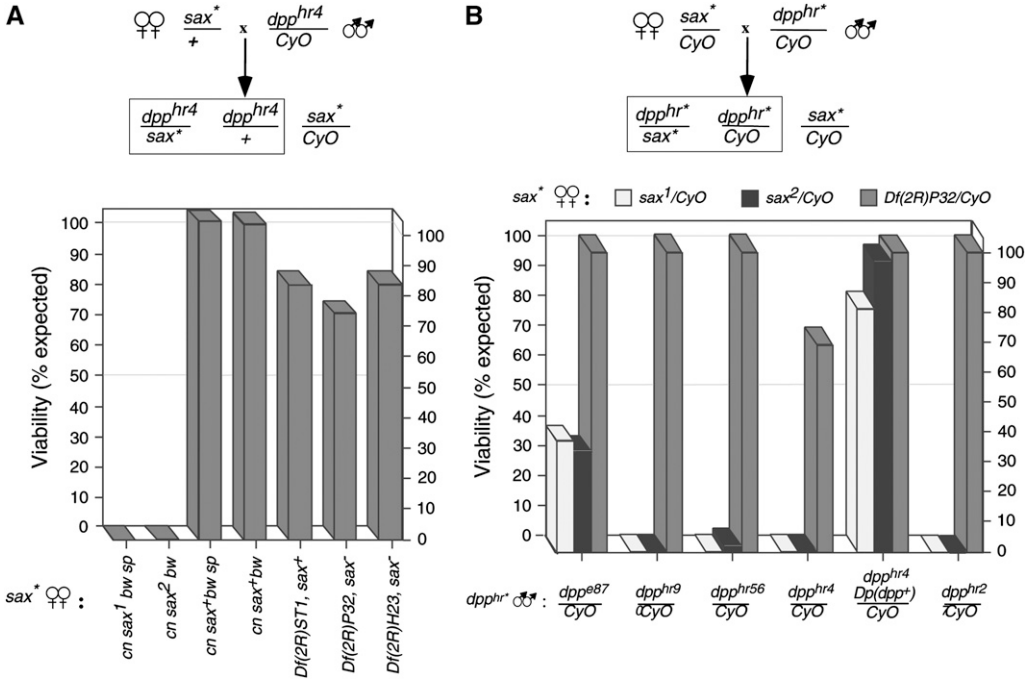


FIGURE 1.—*sax*[1] and *sax*[2] maternal enhancement of *dpp* mutant progeny. Females heterozygous for the maternal effect mutations, *sax*[1] or *sax*[2], dominantly interact with *dpp*[hr] alleles. Percentage of survivorship of *dpp*[hr]/+ adults (boxed genotypes) is shown on the *y*-axis. (A) Females heterozygous for *sax*[1] or *sax*[2], the parental chromosomes (*cn bw sp* and *cn bw*) or deficiencies that retain (*Df(2R)ST1*) or lack (*Df(2R)P32*, *Df(2R)H23*) *sax* function were tested for maternal enhancement of *dpp*[hr4]/+ lethality. The number of progeny examined was ≥577. (B) Survivorship of different *dpp*[hr] alleles when crossed with females heterozygous for a given *sax* mutation is shown as the percentage of expected *dpp*[hr] heterozygous progeny. The female genotypes are indicated by different bar shading. *dpp* alleles are listed along the *x*-axis. The number of progeny examined from each cross was ≥200.

we briefly describe our rationale for each screen and the results. For the screens aimed at identifying *sax* revertants, we used the observation that *sax* mutant mothers exhibit 100% MEL (no progeny survive from a cross between *sax*[1]/*sax*[2] females and +/+ males), while a cross between *Df(sax)*/*sax*[2] females and +/+ males results in ~30–50% survivorship of the embryonic progeny. Similarly, heterozygous *sax*[1]/+ and *sax*[2]/+ females give rise to no viable *dpp*[hr4]/+ adults, while *Df(sax)*/+ females give rise to 85% viable *dpp*[hr4]/+ adults.

sax[1] γ*-ray reversion:* In an F_2 *sax*[1] γ-ray reversion screen, we simultaneously screened for revertants of *sax*[1]/*sax*[2] MEL (cross A) and revertants of the *sax*[1]/+ enhancement of *dpp*[hr]/+ (cross B) (Figure S1, A). A total of 16,410 mutagenized haploid genomes were screened; 6810 by cross A and 9600 by cross B. One revertant from each cross was recovered and designated *sax*[1rv2] and *sax*[1rv1]. Both revertants are lethal in *trans* to *sax* deficiencies and genetically behave like a *sax* deficiency. For instance, *sax*[1rv1]/*sax*[2] females exhibit MEL comparable to *Df(2R)H23*/*sax*[2] females. The identification of *sax*[1rv1] and

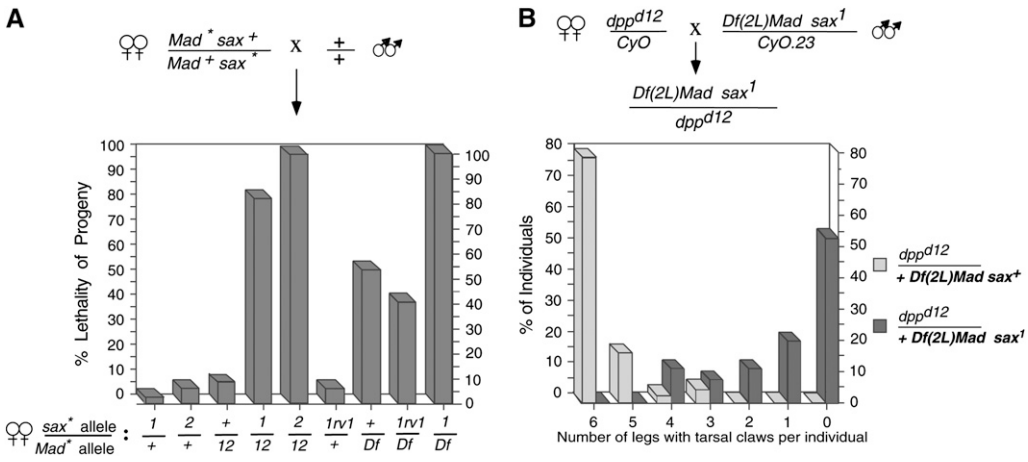


FIGURE 2.—Dominant *Mad–sax* interactions. (A) *Mad–sax* MEL. The percentage of lethality of progeny from females heterozygous for various *Mad* and *sax* alleles crossed to wild-type males is depicted by bars. Transheterozygous (*Mad* +/+ *sax*) females show significant maternal effect lethality. Female genotypes are listed on the *x*-axis. $n \geq 300$ embryos scored for control crosses and $n \geq 475$ embryos for experimental crosses. (B) *Mad–sax* zygotic enhancement of the *dpp*[d12] disk mutation. The number of legs per individual with tarsal claws was quantified in progeny from *dpp*[d12]/+ females crossed to males bearing a *Mad*[−] *sax*[−] double mutant chromosome (*Df(2L)JS17 sax*[1]/*CyO23*) (dark bars) or only *Mad*[−] (light bars). A lowering of both *Mad* and *sax* dosage results in an enhancement of tarsal claw loss associated with a reduction in *dpp* function.

TABLE 2

Summary of new *sax* alleles

Allele	Mutagen	Screen selection	Cytology
Extant			
sax^{1}	EMS	Recessive female sterile (MEL)[a]	Normal
sax^{2}	EMS	Recessive female sterile (MEL)[a]	Normal
sax^{P}	*P* element	2nd chromosome lethal[b]	ND
New			
sax^{1rv1}	γ-ray	Reversion of sax^{1} DME	Normal
sax^{1rv2}	γ-ray	Reversion of sax^{1} MEL	Dp(2;1)43C-F; 46B3-14;20
sax^{1rv5}	EMS	Reversion of *Mad* sax^{1} MEL	Normal
Df(2R)sax-H9	Hobo	*Df(2R)P32* lethal	Df(2R)43F1-2
Df(2R)sax-H30	Hobo	*Df(2R)P32* lethal	Df(2R)43F1-2
sax^{3}	EMS	*Df(2R)H23* lethal	Normal
sax^{4}	EMS	*Df(2R)H23* lethal	Normal
sax^{5}	EMS	*Df(2R)H23* lethal	Normal
sax^{6}	EMS	*Df(2R)H23* lethal	Normal
sax^{PE5}	*P* excision	*Df(2R)sax-H9* lethal	ND
sax^{PE7}	*P* excision	*Df(2R)sax-H9* lethal	ND
sax^{PE10}	*P* excision	*Df(2R)sax-H9* lethal	ND

MEL, maternal effect lethality; DME, dominant maternal enhancement (of *dpp* mutant phenotypes).
[a] Isolated by Schupbach and Weischaus (1989).
[b] Isolated by Török *et al.* (1993) and this allele, and its homozygous viable phenotypes are described in detail by Nellen *et al.* (1994).

sax^{1rv2} demonstrate that sax^{1} is indeed a GOF allele and this function can be eliminated.

Hobo Mobilization Screen: Bolstered by the recovery of sax^{1} revertants that were lethal, we chose to perform an insertional mutagenesis using a $white^{+}$ marked, homozygous viable, Hobo element insertion adjacent to *sax* (43E10-43E15) (*H[J21.31,*w^{+}*J]*) as the mutagen (Figure S1, B) (Smith *et al.* 1993). We expected that the element would either transpose locally or cause a local genomic aberration that may affect the *sax* coding region. Two strains, *Df(2R)sax-H9* and *Df(2R)sax-H30*, that exhibited lethality over tester chromosomes were identified and each was shown to result in a deletion of the 43F1-2 polytene interval (data not shown).

Maternal Df(Mad)/sax^{1} *reversion screen:* Success in the previous screens led to a third assay based upon the dominant interactions observed between sax^{1} and sax^{2} and *Mad* (Figure 2). While strong MEL is observed when double transheterozygous females, *i.e., Df(Mad)* sax^{+}/ Mad^{+} sax^{1}, are crossed to wild-type males (Figure 2A), we found that females bearing one of the new *sax* revertants, *Df(Mad)* sax^{+}/Mad^{+} sax^{1rv1}, exhibit only 40% MEL instead of >90% by *Df(Mad)* sax^{+}/Mad^{+} sax^{1} females. Thus, we reasoned that a screen designed to recover mutations that revert the *sax* GOF interaction with *Mad* mutants could also result in LOF *sax* alleles (Figure S1, C). Of ~8000 mutagenized genomes screened, one revertant was recovered, sax^{1rv5} and is lethal in *trans* to *sax* deficiencies.

F_2 lethal screen: The three screens described above recovered five *sax* alleles that were lethal. These screens were unbiased in their identification of lethal alleles because they were designed to either revert the maternal effects of the original sax^{1} mutation or to identify a Hobo mobilization. Nonetheless, we asked whether lethal *sax* alleles could be recovered independently of a reversion assay and performed a standard F_2 lethal screen for mutations in the 43E18-F2 region. Lethal mutations were then tested for rescue by a *sax* transgene (*P{hs-sax}*) when in *trans* to a *sax* deficiency *Df(2R)sax-H9/SM6a*). By this method, 5610 mutagenized genomes were screened and four new lethal alleles were recovered, sax^{3}, sax^{4}, sax^{5}, and sax^{6}.

sax^{P} *excision screen:* Finally, a *P*-element excision screen was performed using the sax^{P} allele previously reported to be a *placW* insertion in *sax* (Nellen *et al.* 1994). Forty-two independent $white^{-}$ excisions were selected (sax^{PE}) and 12 were shown to be lethal in *trans* to three different LOF *sax* alleles (sax^{PE}/sax^{4}, sax^{PE}/sax^{5}, and sax^{PE}/*Df(2R)H23*). Three lines sax^{PE5}, sax^{PE7}, and sax^{PE10} were retained for further examination (Table 2).

Characterization of new *sax* alleles: From five genetic screens, a total of 12 new *sax* alleles were generated (Table 2). All new alleles were first characterized by complementation analysis and all failed to complement *Df(2R)H23* (Figure 3). Although lethality was not used as a criteria to select for mutations in two of the screens, lethal *sax* alleles were still identified and proved to be lethal over other alleles isolated from subsequent screens aimed at specifically selecting lethal alleles, each done in different genetic backgrounds. On the basis of viability studies and the failure to complement a deficiency, two general classes of *sax* alleles emerged.

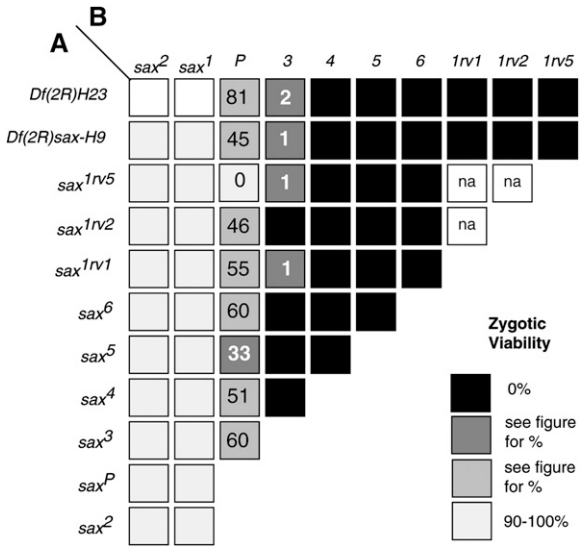


FIGURE 3.—Interallelic complementation between *sax* alleles. Females (A) and males (B) heterozygous for the listed *sax* alleles were crossed and the lethality of sax*^{A}/sax*^{B} progeny was quantified. Shading of boxes indicates the percentage of progeny exhibiting zygotic viability of each allelic combination (key on right). The percentage of viability was calculated as the number of Cy^{+}, divided by the number in the Cy parental class that survived least well ($n \geq 300$ adults scored for each cross).

One class consisting of sax^{1}, sax^{2}, and sax^{P} shows little or no lethality when in *trans* to *Df(2R)H23*. sax^{1} and sax^{2} are not only 100% viable in *trans* to *sax* deficiencies but also display 100% viability over all new alleles (termed *sax** here) (Figure 3). Similarly, sax^{P}/*Df(sax)* and sax^{P}/*sax** exhibit robust viability. The second class of alleles, consisting of sax^{3}, sax^{4}, sax^{5}, sax^{6}, sax^{1rv1}, sax^{1rv2}, sax^{1rv5}, all sax^{PE} alleles, *Df(2R)sax-H9*, and *Df(2R)sax-H30* show complete lethality in *trans* to *sax* deficiencies (*sax**/*Df(sax)*), as well as to one another [with the exception of sax^{3}, which shows a very small percentage (1–2%) of escapers] (Figure 3). To test whether the lethality of the new alleles is in fact due to a loss of *sax* function, we assayed for the ability of an inducible sax^{+} transgene (*P[hs-sax]*) to rescue the lethality associated with these alleles. Eight of 12 new *sax* alleles were rescued in *trans* to *Df(2R)sax-H9* and in *trans* to *Df(2R)H23* (Table S1). sax^{1rv2}, sax^{PE7}, *Df(2R)sax-H9*, and *Df(2R)sax-H30* failed to be rescued. The failure of these alleles to be rescued reflects the fact that the lesion associated with each of these mutations most likely disrupts more than just the *sax* locus. This is clearly evident from the cytology of sax^{1rv2}, *Df(2R)sax-H9*, and *Df(2R)sax-H30* (Table 2). Furthermore, Southern blot analysis of sax^{PE7} indicates that genomic sequences beyond the *sax* locus have been deleted (data not shown). The rescue of the remaining eight alleles by the sax^{+} transgene indicates that the lethality associated with these mutations must result from a reduction in *sax* function.

Molecular lesions associated with new sax alleles: The molecular lesions associated with sax^{3}, sax^{4}, sax^{5}, and sax^{6} were identified as single nucleotide changes within the *sax* coding region (Table 3), indicating that these new alleles specifically affect *sax* function. The sax^{3} and sax^{4} mutations both result in a premature stop codon in the extracellular domain of the Sax protein, while sax^{5} and sax^{6} are missense mutations altering highly conserved residues in different regions of the cytoplasmic kinase domain. The sax^{5} A289D mutation affects a region of the molecule critical for ATP binding. Specific molecular lesions associated with sax^{1rv1} and sax^{1rv5} were not identified and while it was determined that sax^{PE5} and sax^{PE10} exhibit abnormal restriction patterns 3′ to the sax^{P} insertion site (data not shown), the exact boundaries of the lesions were not determined.

Lethal sax alleles fail to maternally enhance dpp phenotypes: sax^{P}/+ shows a strong maternal enhancement of dpp^{hr4}/+ lethality as discussed above for sax^{1} and sax^{2} (Figures 1 and 4). sax^{P}/+ females also enhance dpp^{hr56}, dpp^{hr90}, and dpp^{e87} heteozygotes resulting in 26, 41, and 55% viability, respectively. In contrast, the majority

TABLE 3

Molecular lesions associated with *sax* alleles

Allele	Nucleotide change	Codon change	sax^{n}/*Df(sax)* phenotype
Newly described alleles			
sax^{3}	CAG→TAG	Gln 121 stop	Lethal*
sax^{4}	CAA→TAA	Gln 114 stop	Lethal
sax^{5}	GCC→GAC	Ala 289 Asp	Lethal
sax^{6}	CGC→CAG	Arg 541 His	Lethal
sax^{P} [b]	*P*[lacW] insertion	After aa 34 of Sax-PA	Viable
Previously described alleles			
sax^{1a}	ACC→ATC	Thr 434 Ile	Viable
sax^{2a}	GGA→GAA	Gly 412 Glu	Viable

sax^{n}, new *sax* allele; *sax^{3}/*Df(sax)* exhibits weak (0.6–2%) viability.

[a] Isolated by SCHUPBACH and WEISCHAUS (1989).

[b] Isolated by TÖRÖK *et al.* 1993; previously described in NELLEN *et al.* 1994 as a *P*-element insertion after the first 36 amino acid residues of the SAX protein.

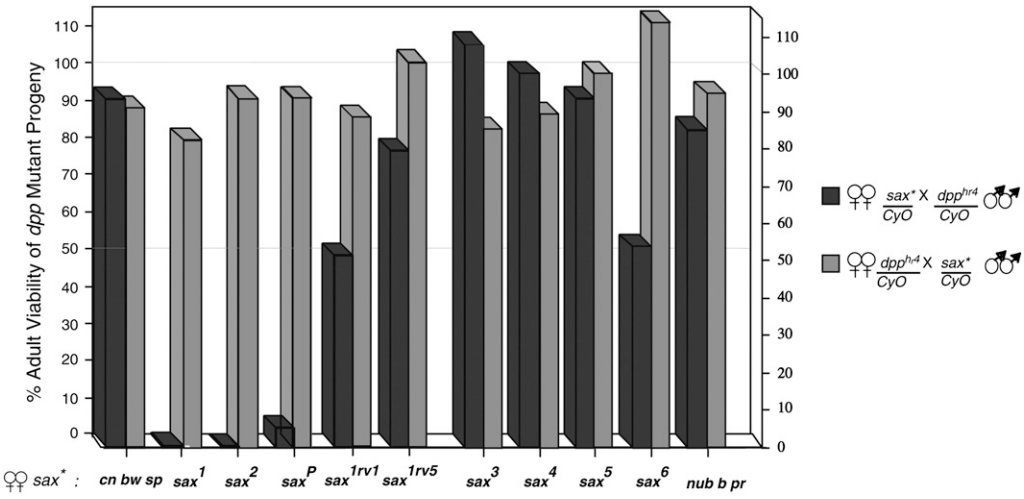


FIGURE 4.—Maternal enhancement of *dpp* mutant progeny by all *sax* alleles. Test crosses between all *sax* alleles and dpp^{hr4} were performed in both directions, with respect to the genotypes of the females and males. Crosses in which the females were mutant for *sax* are represented by the solid bars, and the male *sax* mutant crosses are represented by shaded bars. The genotypes of tested chromosomes are listed below. cn^1 bw^1 sp^1 is the parental chromosome for sax^1, sax^{1rv1}, and sax^{1rv5}. nub^1 b^1 pr^1 is the parental chromosome for sax^3, sax^4, sax^5, and sax^6. For female *sax* mutant crosses, the number of progeny scored per cross was ≥550 (except sax^P, $n = 417$). For male *sax* mutants crosses, the number of progeny scored per cross was ≥345 (except sax^2, $n = 155$; sax^{PE4}, $n = 269$).

of alleles in the second class of *sax* mutations (the lethal alleles) show no specific maternal enhancement of dpp^{hr}/+ lethality. Of this class, only sax^{1rv1} and sax^6 show a moderate maternal enhancement. Given the importance of *dpp* in BMP signaling in the embryo, the mutations giving rise to sax^1, sax^2, and sax^P must clearly impact *sax* function in a manner that more significantly affects this role for *dpp* in the embryo than the lethal *sax* alleles do, such that dpp^{hr}/+ animals are no longer viable.

As another measure of the effect of the sax^1, sax^2, and sax^P alleles on embryonic *sax* function, we examined the possibility that these alleles genetically interact with *scw* mutations. The haplolethality of *dpp* is associated with its role in embryonic dorsal–ventral patterning and *scw* is intimately involved in establishing the BMP activity gradient critical for dorsal–ventral patterning (ARORA *et al.* 1994; NEUL and FERGUSON 1998; SHIMMI *et al.* 2005). We found that sax^1, sax^2, and sax^P alleles exhibit a dominant maternal enhancement of scw^{E1} and scw^{E2} mutations (Table S2) and as with their failure to enhance *dpp* lethality, the lethal *sax* alleles do not show this maternal enhancement (data not shown). Interestingly, scw^{E1} and scw^{E2} are GOF alleles thought to alter *dpp* activity (RAFTERY *et al.* 1995) and while sax^1, sax^2, and sax^P heterozygous mothers generate synthetic lethality when crossed to these alleles, they show no enhancement when crossed to LOF *scw* alleles (Table S2; crosses A, B, G, H, M). Given the critical role of Dpp:Scw heterodimers in the generation of dorsal/ventral patterning in the embryo, it is likely that the GOF nature of scw^{E1} and scw^{E2} reflects their ability to dominantly influence the function of Dpp when heterodimerized with Scw mutant protein, such that a more significant reduction in the effectiveness of BMP signaling is observed.

We also examined the ability of *sax* mutations to modify a later *dpp* function by separately scoring the various aspects of the dpp^{d5}/dpp^{hr56} resulting wing phenotype in different *sax* mutant backgrounds in addition to any effects on adult viability (Table 4). A deficiency of *sax* leads to a very slight suppression of the dpp^{d5}/dpp^{hr56} phenotype as expected from our previous work indicating that Sax receptors can inhibit BMP signaling

TABLE 4

Zygotic Enhancement of *dpp* Phenotypes

	% wings showing phenotype							Viability	
Genotype	*n*	Intact	L2 gap	L2 intact	L4 gap	L4–L5 reduced	L4–L5 fused	*n*	% Exp
dpp^{d5} +/dpp^{hr56} +	168	59	41	100	0	63	38	517	50
dpp^{d5} *Df(2R)H23*/dpp^{hr56} +	135	**65**	**35**	**100**	**0**	**94**	**6**	ND	ND
dpp^{d5} sax^4/dpp^{hr56} +	136	62	38	100	0	88	12	521	55
dpp^{d5} sax^5/dpp^{hr56} +	105	47	53	99	1	68	32	227	23

All crosses have a genotype (dpp^{d5}/dpp^{hr56}) with reduced *dpp* signaling which results in a wing tissue that is "sensitized phenotypically" and responds readily to further perturbation of BMP signaling. Boldface percentages are those of the control class, dpp^{d5} *Df(2R)H23*/dpp^{hr56} sax^+. Importantly, note that the sax^4 allele closely mimics the results of the deficiency of *sax* (underlined).

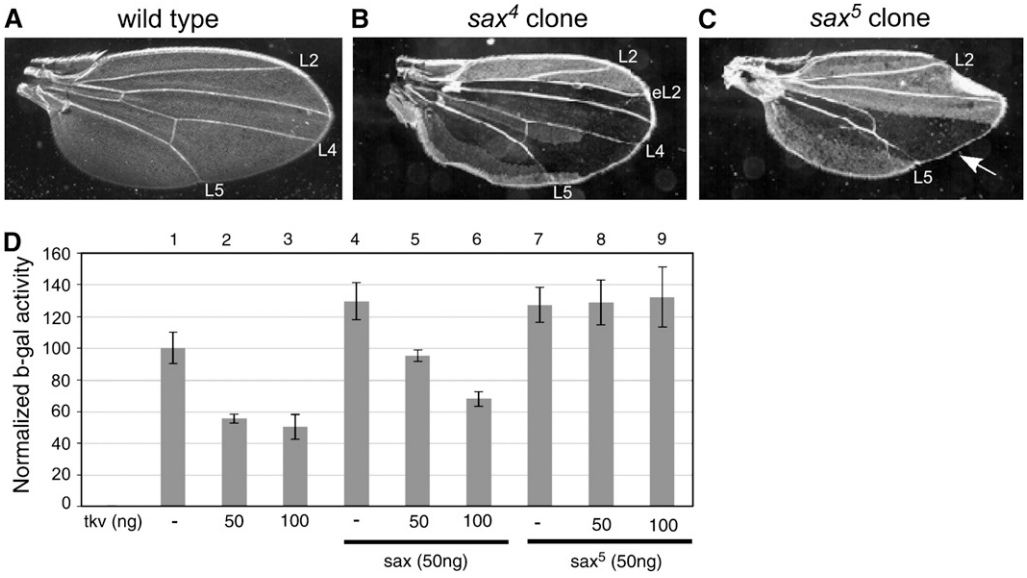


FIGURE 5.—sax^5 produces more severe phenotypes than sax^4. (A) Dark field image of a wild-type wing. Longitudinal veins 2 (L2), 4 (L4), and 5(L5) are indicated. (B and C) Wings resulting from *sax* mutant clones as described in BANGI and WHARTON (2006b). Clones marked with *shv* appear dark in images. (B) A *sax^4* clone encompassing the entire posterior compartment shows no patterning defects. Consistent with previous studies (SINGER *et al.* 1997; BANGI and WHARTON 2006b), a small *sax^4* clone in the anterior compartment leads to an ectopic L2 (eL2) vein. (C) A *sax^5* clone in the posterior compartment results in the loss of L4 (arrow) and a narrowing of the L4/L5 intervein, a phenotype never seen in an equivalent *sax^4* clone. The more severe phenotype of *sax^5* suggests that the presence of a defective Sax receptor is more detrimental to BMP signaling during wing patterning than the complete loss of the Sax receptor. (D) A cell-based BMP signaling assay indicates that the *sax^5* mutation is able to negatively affect BMP signaling mediated by Tkv. S2 cells were cotransfected with the *Su(H)/brk-lacZ* reporter construct, *Su(H)*, and *N** constructs to stimulate transcription (sample 1), and *tkv*, and/or *sax* and *sax^5* constructs under the control of the actin 5C promoter (samples 2–9). Values depicted are the fold activation of β-galactosidase over the basal activity of the reporter construct alone. All values represent the average of samples measured in triplicate and normalized for transfection efficiency.

in the Drosophila wing (BANGI and WHARTON 2006b). Consistent with the expectation that the *sax^4* mutation leads to a complete loss of Sax protein, *sax^4* behaves identically to the *sax* deficiency and causes a slight suppression of the *dpp^{d5}/dpp^{hr56}* wing phenotype (Table 4). Interestingly, *sax^5* does not show this same suppression and in fact shows an enhancement of lethality associated with *dpp^{d5}/dpp^{hr56}* not seen when *sax* dosage is reduced by a deficiency.

sax^5 exhibits dominant negative behavior: The difference in the ability of *sax^5* to enhance *dpp* lethality compared to *sax^4* or a *sax* deficiency (*Df(2R)H23*), prompted us to investigate in more detail the possibility that *sax^5* may exhibit a mild dominant-negative effect. We had previously examined the role of *sax* in wing patterning (BANGI and WHARTON 2006b) and sought to compare the phenotype associated with a *sax^4 vs.* a *sax^5* clone in the adult wing. While large posterior clones of *sax^4* show no wing patterning abnormalities, large clones of *sax^5* show a significant loss of longitudinal vein 4 (L4) and a narrowing of the L4/L5 intervein, a phenotype associated with a loss of *dpp* function (Figure 5). This result is consistent with the enhancement of *dpp^{d5}/dpp^{hr56}* lethality by *sax^5* (Table 4) and supports the conclusion that *sax^5* is able to negatively impact *dpp* function.

We next made use of a cell-based BMP signaling assay to assess the ability of the *sax^5* mutation to affect BMP signaling. As described previously, *lacZ* expression in this assay is repressed by BMP signaling in a quantitative manner and thus, β-galactosidase activity is inversely correlated with the level of BMP signaling (BANGI and WHARTON 2006b; MULLER *et al.* 2003). As observed previously, Tkv exhibits some degree of signaling when wild-type *tkv* constructs are transfected into S2 cells alone (samples 2 and 3, Figure 5D), while Sax does not (sample 4, Figure 5D). We have found that S2 cells express *gbb* (T. Akiyama, unpublished results) and transfection with a wild-type *sax* construct appears to block endogenous Gbb signaling, likely as a result of ligand being bound by nonsignaling Sax–Sax complexes (BANGI and WHARTON 2006b). When cotransfected with *tkv*, a wild-type *sax* construct results in the antagonism of signaling in a dose-dependent manner (samples 5 and 6, Figure 5D). In agreement with our genetic analysis of *sax^5* mutants, cotransfection of *tkv* with a *sax^5* construct leads to a complete inhibition of Tkv-mediated BMP signaling (samples 8 and 9, Figure 5D), indicating that the Sax^5 protein can completely disrupt successful signaling.

MEL and zygotic lethality induced by lethal sax alleles is not rescued by increased dpp dosage: We have shown that increasing the dosage of *dpp$^+$* can rescue the MEL associated with the GOF *sax^1* and *sax^2* alleles. All new *sax* alleles exhibit significant MEL (Table S3), therefore, we tested for the ability of increased *dpp$^+$* dosage to rescue *sax^3*, *sax^4*, *sax^5*, *sax^6*, and *sax^P*, in *trans* to *sax^{1rv1}*. As Figure S2 shows, increasing the dosage of *dpp$^+$* to three copies in *sax*/sax^{1rv1}* animals fails to rescue all but *sax^P* and *sax^3*.

No rescue of the other new alleles was observed even in the presence of four copies of dpp^+ (data not shown). The rescue of sax^P and sax^3 may indicate that they retain some residual wild-type *sax* function.

We would predict that the sax^3 mutation should generate a truncated Sax protein (121 amino acids of the extracellular domain) that is likely to be unstable and, therefore, should lack all *sax* function. However as shown in Figure 3, sax^3 unexpectedly exhibits a very low percentage of viability (0.6–2%) in *trans* to *Df(2R)H23*, sax^{1rv1}, sax^{1rv5}, and *Df(2R)sax-H9*. Hemizygous adult escapers display wing venation defects (50% penetrance) similar to sax^P/Df while females display 100% MEL with severely reduced egg production, averaging 5.7 eggs/female ($n = 54$ females). In all other assays, sax^3 is indistinguishable from sax^4, a mutation expected to truncate the Sax protein after amino acid 114. We tested the possibility that a suppressor mutation was induced at a second site on the sax^3 chromosome by generating meiotic recombinants proximal and distal to the mutation and finding that a few $sax^3/Df(2R)sax\text{-}H9$ escapers are still generated. While this result does not completely eliminate the possibility of a closely linked second site suppressor, an alternative hypothesis is that the sax^3 mutation allows altered splicing or translational read through (Cartegni *et al.* 2002). Regardless of the molecular explanation, our genetic analysis thus far suggests that sax^3 retains some very low level of *sax* function, and significantly less than sax^P. The fact that dpp^+ duplications can rescue lethality associated with sax^3/sax^{1rv1} mutants indicates that the defect to BMP signaling in sax^3/sax^{1rv1} animals can be compensated for by an increase in ligand levels and signaling, presumably, through another receptor, such as Tkv.

sax^P retains significant function: sax^P was previously reported to be a null allele, on the basis of the identification of a *P*-element insertion in the *sax* locus immediately following codon 36 of the *sax* PA open reading frame (Nellen *et al.* 1994). However, given that the sax^P allele exhibits traits in common with both sax^1 and sax^2 (missense mutations that likely produce aberrant proteins), we considered the possibility that the *P*-element insertion responsible for sax^P may not completely disrupt normal *sax* transcription and may allow for the production of a wild-type protein product or one that is abnormal in some way. We performed RT–PCR on RNA from homozygous sax^P animals and detected the clear presence of an mRNA derived from sequences downstream of the proposed *placW* insertion site (Figure 6B). The *sax* open reading frame (PA) (Brummel *et al.* 1994; Nellen *et al.* 1994) may be generated by one of two possible transcripts from the *sax* locus, the *saxRA* transcript (see www.flybase.org). A second transcript, *saxRB*, can generate a second Sax protein (PB) that initiates at the methionine at codon 36 of the PA open reading frame. We considered the possibility that a transcript from the sax^P locus could encode an open reading frame similar to PB. We first determined the orientation of the *placW* element responsible for sax^P by genomic Southerns (data not shown) and then to verify the site of the *P*-element (*placW*) insertion, we produced cDNA from sax^P homozygotes, generated PCR products, and sequenced the junction between the P3′ end and the *sax* locus. In contrast to the previous report (Nellen *et al.* 1994), we found that the *placW* element is inserted just 5′ to the GAC codon encoding aa 35 of the SaxPA open reading frame and thus, upstream from the ATG encoding methionine aa 36 (underlined in Figure 6A). Thus, the SaxPB open reading frame remains intact in sax^P mRNA. Two additional ATG codons are upstream within the P3′ end sequences and could encode methionine codons in frame with SaxPB, providing other possible alternative translational start sites (Figure 6C).

Our identification of lethal, null *sax* alleles and the genetic analyses indicating that sax^P retains some function, led us to conclude that the SaxPB protein (or a SaxPB protein with 2–12 additional amino acids) must have activity and importantly, must contribute in part to BMP signaling during development. sax^P homozygotes are viable and display only weak mutant phenotypes, indicating that most requirements for *sax* function throughout development can be met by the SaxPB protein. While similar to sax^1 and sax^2 in the maternal enhancement of $dpp^{hr}/+$ lethality and in its ability to be rescued by dpp^+ duplications, the reduced eye and mutant wing phenotype of sax^P/sax^* adults (a loss of the ACV and ectopic vein material near distal L2) set this allele apart and suggest that sax^P retains less wild-type *sax* function than sax^1 and sax^2.

***sax* zygotic lethal phase and phenotypes:** We examined the lethal phase and mutant phenotypes of the new *sax* alleles (Figure S3, Figure S4). The zygotic lethal phases were determined in crosses of $sax^4/+$ to $sax^3/+$, $sax^4/+$, $sax^5/+$, $sax^6/+$, $sax^{1rv1}/+$, and $Df(2R)H23/+$ (collectively *sax**) where lethality of sax^4/sax^* is as expected (~25% when the lethality of control classes is taken into account). The zygotic lethal phase is primarily larval and to a lesser extent pupal. More than 70% of $sax^*/Df(2R)H23$ third instar larvae are transparent (Figure S4), a phenotype not seen in $sax^1/Df(2R)H23$ and $sax^2/Df(2R)H23$ animals. This transparency reflects in part a qualitative alteration in the morphology of the mutant fat body (S. Ballard, K. Wharton, unpublished data). Despite minimal embryonic lethality, the lethal *sax* alleles exhibit abnormal embryonic midgut morphology, mainly consisting of a failure in the second midgut constriction, a phenotype not shown by sax^1 mutant embryos (C. Savery, K. Wharton, unpublished results). Only a small percentage (≤5%) of hemizygous mutant larvae ($sax^3/Df(2R)H23$, $sax^4/Df(2R)H23$, $sax^5/Df(2R)H23$, and $sax^6/Df(2R)H23$) exhibit other defects, such as developmental delay, lethargy, a reduction in the size of imaginal

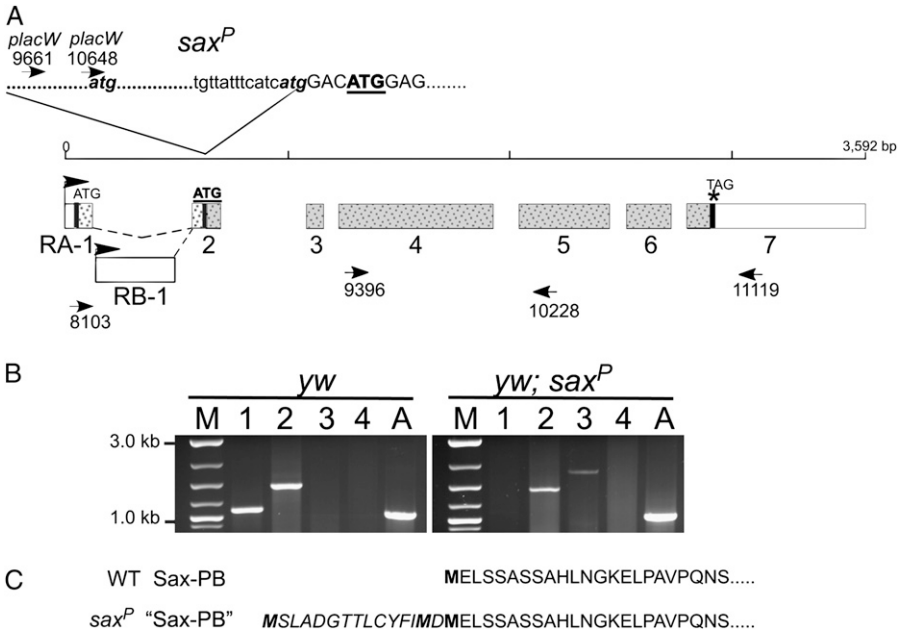


FIGURE 6.—*P*-insertion site of *sax*^{P} does not disrupt transcription. (A) Genomic structure of *sax* locus shown with exon (numbered) distribution for the two splice forms of *sax* mRNAs (RA and RB). The locations of two ATG initiation codons and the TAG termination codon (vertical thick solid lines) indicate the two overlapping open reading frames (speckles) giving rise to the putative protein products PA and PB (shaded speckles). Positions of PCR primers are indicated by arrowheads. The site and junctional sequence of the *placW* insertion giving rise to *sax*^{P} is shown at the top. The endogenous second ATG within the *sax* transcription unit is bold and underlined. (B) RT–PCR products from different primer pairs generated from RNA isolated from control (*yw*) (left) and *sax*^{P} homozygous mutant flies (right). Lane 1, primers 8103 + 10228; lane 2, primers 9396 + 11119; lane 3, primers placW10648 + 11119; lane 4, primers placW9661 + 10228. Note the insertion of *placW* disrupts the wild-type transcription unit initiating at RA-1 (lane 1) but allows transcription to initiate within the *placW* element between primers placW9661 and placW10648 (presence of PCR product in lane 3 of *sax*^{P} animals and not in wild type, or in lane 4 of *yw* or *sax*^{P}). Transcription in both genotypes extends through the expected translational termination site (PCR product present in lane 2 of both genotypes). M, marker lane. A, actin control. (C) The predicted amino acid sequence of SaxPB produced by open reading frame initiating at second endogenous **ATG** (bold and underlined in A) is shown in normal type with potential additional amino acids (italics) if translation initiated at an **atg** within the 3′ *P*-element of *placW* (shown in A).

discs, brain, and midgut structures, as well as tracheal truncations (Figure S4). The pupal lethal phase is variable occurring anywhere from imaginal disc eversion to pharate adults (data not shown).

DISCUSSION

Here we describe a mutational analysis of the *saxophone* gene encoding the Drosophila ortholog of the human ACVR1/ALK2 and ALK1/ACVRL1 type I receptor. We show that the two defining mutations in the gene, *sax*^{1} and *sax*^{2}, are GOF alleles on the basis of our ability to revert them to alleles that (1) are lethal, (2) do not display maternal effect lethality, and (3) do not enhance *dpp* mutant phenotypes. The majority of the new lethal alleles represent either the partial or complete loss-of-function state of *sax* given that in functional assays they primarily behave like deficiencies of the *sax* locus. Furthermore, we show that *sax*^{P}, previously reported to be a *sax* null allele (NELLEN *et al.* 1994), in fact retains significant *sax* function, which can be eliminated by *P*-element excision. Our studies demonstrate that *sax* is an essential gene that is required for multiple developmental processes and the genetic characteristics of different alleles appear to reflect the site of the mutation and the fact that the Sax receptor acts in a multimeric receptor complex in BMP signaling.

Genetic nature of new lethal sax alleles: Of the 10 lethal alleles, *sax*^{3}, *sax*^{4}, *sax*^{5}, and *sax*^{6} arise from point mutations and *Df(2R)saxH9*, *Df(2R)saxH10*, and *sax*^{PE} alleles appear to be deletions of the *sax* locus on the basis of polytene chromosome *in situ* hybridizations and Southern blot analysis (data not shown). The molecular lesions associated with revertants *sax*^{1rv1}, *sax*^{1rv2}, *sax*^{1rv5} have yet to be identified but were not found within the *sax* transcription unit. Without exception, these alleles are lethal as hemizygotes and distinct from the original *sax*^{1} and *sax*^{2} GOF alleles. Unlike the GOF alleles, the lethal alleles are not rescued by increased dosage of *dpp*$^{+}$ or *gbb*$^{+}$ (data not shown) consistent with their designation as LOF alleles; increasing ligand level cannot facilitate signaling through an absent or inactive receptor. The genetic screens used to identify these new alleles each selected mutations according to different criteria: (a) reversion of the maternal effect lethality of *sax*^{1} and *sax*^{2}, (b) reversion of *dpp* enhancement, or (c) by lethality over regional deficiencies. The frequency at which these new alleles were generated (one per 1400) is comparable with that of other LOF alleles, unlike the rate at which *sax*^{1} and *sax*^{2} were recovered (one per 4000; SCHUPBACH and WIESCHAUS 1989).

Lethal sax phenotypes: The lethal phase of *sax* LOF alleles is primarily larval with some early pupal lethality (Figure S3). In general the mutant phenotypes observed are consistent with a role for *sax* in BMP signaling throughout development, including dorsal/ventral patterning, midgut, and tracheal development, imaginal disc patterning, and neuromuscular junction formation

and function (reviewed in Immergluck *et al.* 1990; Panganiban *et al.* 1990; Affolter *et al.* 1994; Vincent *et al.* 1997; Marques *et al.* 2002; McCabe *et al.* 2003; O'connor *et al.* 2006; Rawson *et al.* 2003). The low frequency, variable expressivity, and general pleiotropy of zygotic *sax* mutant phenotypes could reflect the substantial maternal contribution of *sax*, and/or the dual role of Sax in BMP signal transmission (Bangi and Wharton 2006b). Maternal *sax* mRNA and/or protein is preloaded into the oocyte and is likely to persist through embryogenesis, such that the maternal contribution of wild-type *sax* can compensate for early functions despite the absence of zygotic *sax* in complete LOF mutants. Sax requires a second type I receptor Tkv to transduce a BMP signal but alone it influences the availability of ligand. Thus, slight shifts in the balance between receptor and ligand pools can likely result in very different phenotypes (Bangi and Wharton 2006b).

With regard to the maternal contribution, we have previously generated germline clones to eliminate the contribution of wild-type maternal *sax* mRNA to the egg and found that *sax* function is required during oogenesis and removal of all *sax* function from ovaries causes the cessation of egg production within days of inducing clones (Twombly *et al.* 1996; Xie and Spradling 1998). Of the few *sax* null embryos produced by germline clones, a dramatic shift in the lethal phase from larval to embryonic was observed, as would be expected from a reduction in *sax* maternal mRNA due to its clear role in early dorsal/ventral patterning. However, the small number of recovered embryos did not allow a thorough analysis.

Molecular lesions and functional consequences of receptor mutations: The molecular lesions of sax^{3}, sax^{4}, sax^{5}, sax^{6} and a previously described *P*-element insertion allele, sax^{P}, were determined. Both sax^{3} and sax^{4} are nonsense mutations in the extracellular domain of the Sax receptor and sax^{5} and sax^{6} are missense mutations within the intracellular kinase domain (Table 3, Figure 7). The precise location of the *placW* element inserted in *sax* was determined for the sax^{P} allele (Figure 6A) and RT–PCR analysis indicates that transcription of this mutant gene must initiate within the 3′ *P*-element end between the positions of primers *placW*9661 and *placW*10648 and extend into the *sax* locus. The resulting sax^{P} mRNA contains an open reading frame either identical to SaxPB or with a short amino extension (Figure 6, B and C). These results indicate that sax^{P} is not an RNA null. The generation by imprecise *P*-element excisions of sax^{P} revertants (sax^{PE*}) that are lethal over *sax* deficiencies and LOF alleles (Table 2) support our conclusion that sax^{P} is not a functional null. Instead, sax^{P} retains partial function, sufficient for viability (Figure 3), but interestingly the resulting protein product produced by sax^{P} in some way interferes with *dpp* function in the early embryo (Figure 4). It is possible that this difference in function reflects a difference in the role of Sax-PA and Sax-PB proteins in either the early functions of *sax* or in its interaction with Dpp. The weaker affect of sax^{P} germaria on stem cell loss than that observed in *dpp* mutant germaria (Xie and Spradling 1998) likely reflects, not a minor role for Sax in the maintenance of the stem cell niche, but rather a mild hypomorphic nature of the sax^{P} allele for this function and a less dramatic difference between the contributions of Sax-PA and Sax-PB to germ cell niche maintenance.

The two alleles, sax^{5} and sax^{6}, arising from missense mutations within the Sax kinase domain, are clearly LOF alleles on the basis of their lethality and inability to maternally enhance *dpp* lethality (Figure 4). Both mutations are likely to destabilize the kinase domain and the dominant-negative behavior exhibited by sax^{5} is consistent with this proposal (Figure 5). While the Sax5 protein is null for signaling activity as indicated by multiple genetic assays and a cell-based signaling assay, the dominant-negative behavior of the sax^{5} mutation appears to stem from the ability of a defective Sax5 receptor to interfere with the function of Tkv, presumably by forming inactive Tkv–Sax5 receptor complexes and effectively reducing the number of functional receptor complexes for BMP signaling. While wild-type Sax possesses a dual function, whereby it can both inhibit and facilitate signaling depending on the relative levels of Sax to Tkv, Sax5 is clearly unable to facilitate signaling as assayed by both phenotypic and cell-based signaling criteria.

Consequences of analogous mutations in human sax orthologs: Mutations in the two human orthologs of *sax*, *ALK1/ACVRL1* and *ACVR1/ALK2*, result in hereditary hemorrhagic telangiectasia (HHT2, Osler-Weber-Rendu disease) and fibrodysplasia ossificans progressiva (FOP), respectively (see www.hhtmutation.org; Shore *et al.* 2006). Interestingly, HHT2, a vascular disorder, and FOP, a devastating heterotopic bone disorder, are both dominant, immediately indicating that the combination of mutant receptors with wild-type receptors is disruptive to normal signaling.

Mutations in the *ALK1* gene have been found with an incidence of 1/10,000 in either the extracellular and intracellular domains of the protein (www.hhtmutation.org) and in both cases mutations cause vascular dysplasias, such as mucocutaneous telansiectases and arterovenous malformations, that can result in life-threatening complications (Lesca *et al.* 2007). The penetrance of these mutations is quite variable even within a family and with an increase in age, leading to considerable controversy as to whether mutations exhibit GOF or LOF behaviors (Massagué *et al.* 2000; Lesca *et al.* 2004). *ALK1* is expressed in the distal capillaries (Mahmoud *et al.* 2009) and HHT2 mutations are thought to affect endothelial cell metabolism, angiogenesis, and vascular remodeling with no clear

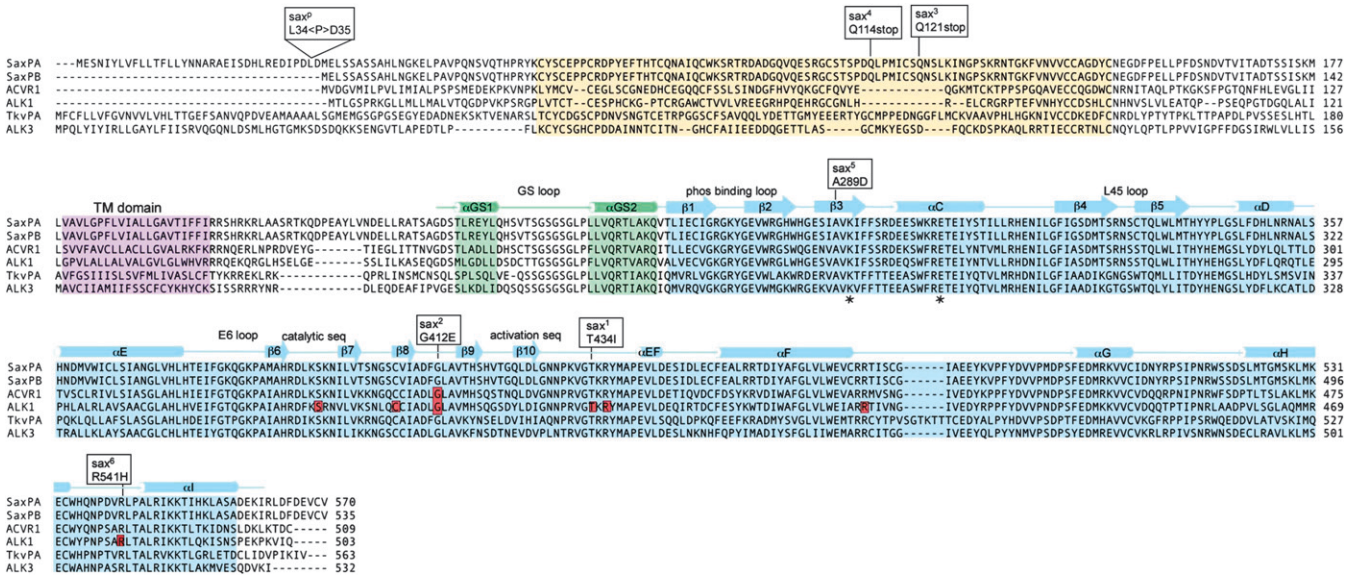


FIGURE 7.—Sequence comparison of Sax (PA and PB isoforms), ACVR1 (ALK2), ALK1 (ACVRL1), Tkv (PA isoform), and ALK3. The extracellular ligand binding domain (yellow), the transmembrane domain (purple), the intracellular GS activation (green), and serine/threonine (blue) kinase domains are shaded. The structural elements of the cytoplasmic GS and kinase domains (based on the TβR1 structure) (HUSE *et al.* 1999) are indicated above the sequence alignment. The positions of mutations associated with the *sax* alleles discussed are indicated above the sequence alignment. The positions of specific HHT2 and FOP mutations in ALK1 and ACVR1, respectively, are highlighted in red within the sequence. The asterisks mark the invariant Lys and Glu residues critical for stabilization of the catalytic segment with the N and C lobes of the kinase.

explanation for the variability in expressivity of mutant phenotypes (LENATO *et al.* 2007).

The most frequent mutation in *ACVR1* that gives rise to FOP has been found to result in an R206H alteration near the GS loop, the site of phosphorylation by the type II receptor (SHORE *et al.* 2006). Seven other less common FOP variants have been shown to alter highly conserved residues within the kinase domain (KAPLAN *et al.* 2009). Protein structural homology modeling has suggested that the amino acid substitutions associated with FOP mutations result in an ACVR1 protein conformational change that impacts the activity of the receptor molecule (GROPPE *et al.* 2007; KAPLAN *et al.* 2009). Classic FOP is characterized by great toe malformations and progressive heterotopic ossifications in the medial tibia (osteochondromas), the cervical spine, and femoral neck, often with thumb malformations. Patients with atypical FOP show progressive heterotopic ossifications with abnormalities in other organs, such as the eye and in hair growth (KAPLAN *et al.* 2009). The variability in the expressivity of the clinical features of FOP and its progressive nature is of great interest but at present is not well understood.

Interestingly, we found that the conserved residues altered by *sax²*, *sax¹*, and *sax⁶* mutations have also been identified as residues altered by various HHT2 and FOP mutations (Figure 7). The Arg near the C-terminal end of the receptor substituted in *sax⁶* is altered in HHT2 mutations (R479L, R479E, R479X) (LESCA *et al.* 2004; BAYRAK-TOYDEMIR *et al.* 2006). Given its location in the structural scaffold of the kinase domain, these mutations likely destabilize the kinase. The conserved core of all protein kinases consists of an N-terminal lobe and a large C-terminal lobe separated by a cleft where ATP is bound (HUSE and KURIYAN 2002). Conformational changes in the αC-helix and its interaction with the activation segment are associated with activation of kinase activity. Furthermore, alterations to residues within the activation segment and/or catalytic segment within the cleft have been shown to modulate kinase activiy (HUSE *et al.* 1999). Both *sax¹* and *sax²* mutations affect conserved residues within the activation segment on either side of the β9 and β10 β-sheets (Figure 7). The identical amino acid substitution of Thr to Ile found in *sax¹* (T434I) is found in several HHT2 patients (ALK1 T372I) (WEHNER *et al.* 2006; LENATO *et al.* 2007). Threonine 434 is one of the 12 nearly invariant amino acids found in all kinase domains. The substitution of a nearby Arg in ALK1 R374W has been shown to result in normal expression levels of a mutant protein (FERNANDEZ *et al.* 2005), suggesting that defects in this region do not necessarily lead to protein destabilization and degradation. In fact, it appears that all individual missense mutations within this region of the ALK1 protein (S333I; C344Y; R374W; R411Q) are expressed and mutant proteins present on the cell surface (GU *et al.* 2006; OLIVIERI *et al.* 2007). S333I and C344Y display dominant-negative activity when overexpressed in zebrafish embryos while R411Q exhibits lower activity (GU *et al.* 2006).

In *sax²* mutants, Gly 412 of Sax is substituted (G412E) and mutations of this same residue are found in both

HHT2 (ALK1-G350S, ALK1-G350R) (Abdalla *et al.* 2005; Letteboer *et al.* 2005; Schulte *et al.* 2005) and FOP patients (ACVR1-G356D) (Kaplan *et al.* 2009). Protein modeling has suggested that the ACVR1-G356D substitution could interfere with ion pairing between conserved Lys235 and Glu248 residues that is normally observed in kinases that are in the active state (Kornev *et al.* 2006; Kaplan *et al.* 2009).

The functional data we have presented here clearly show that the *sax[1]* and *sax[2]* mutations are not null and that the resulting mutant receptors must retain sufficient function for viability. It is of particular interest that these mutations could affect either substrate recognition and/or the orientation of the αC helix. The orientation of the αC helix is critical for maximal kinase activity depending on stabilization of its interaction with the phosphates of bound ATP and the activation and catalytic segments. In either case, the unique behaviors displayed by *sax[1]* and *sax[2]* that the lethal LOF *sax* mutations do not possess, such as maternal effect lethality and the maternal enhancement of *dpp* phenotypes, strongly suggest that Sax^1 and Sax^2 proteins are not simply dominant negative as would be expected of a fully kinase-defective receptor (such as Sax^5). Rather, these proteins appear to be capable of participating in productive signaling complexes that allow for normal zygotic development and viability. However, when the only source of maternal Sax protein is the Sax^1 and/or Sax^2 mutant form, the resulting signaling output is abnormal such that the developmental process most sensitive to disruptions in BMP signaling, early embryonic dorsal/ventral specification, is affected. The fact that homozygous or transheterozygous *sax[1]* and *sax[2]* females show higher MEL than hemizgous females (Table 1) suggests that the dosage or level of mutant Sax receptor protein is critical in generating the observed phenotypes. Assuming that protein production can be correlated with gene dosage, more maternally loaded aberrant Sax receptor must be more detrimental than less mutant receptor. Given that type I receptors must participate in a heteromeric receptor complex to transduce a signal, it is likely that the ratio of receptor complexes containing only Tkv (Tkv:Tkv) *vs.* Tkv:Sax^1 will change when the dosage of the *sax* mutant chromosome is reduced. It appears that some developmental processes are more sensitive to a particular Tkv:Sax to Tkv:Tkv ratio than others for optimal signaling. It is not yet clear how Sax^1 or Sax^2 may affect signaling complex formation but the fact that the mutations that give rise to these aberrant proteins can be reverted is consistent with their assignment as gain of function. It is conceivable that their affect on BMP signaling can be one of partial loss of function, as well as with some antimorphic properties, depending on the presence of other proteins important for complex formation.

In conclusion, the isolation and characterization of new *sax* alleles has clarified the null state of *sax* and highlighted the consequences of mutations in different domains of this BMP type I receptor. Given the homology between Sax and the human ALK1/ACVRL1 and ACVR1/ALK2 type I receptors, these studies show promise in making use of genetic analyses and unbiased screens to identify mutations with a range of activities. Such functional characterizations of mutations known to cause the human disorders HHT2 and FOP should continue to provide invaluable data on their mechanistic consequences.

We thank D. Gubb for deficiency stocks, C. Rushlow for Kr antibody, Mike O'Connor for pAcpA-tkv and pAcpA-sax, and T. Akiyama for discussions. We thank M. Gelbart and G. Krateberg for superb technical assistance, and Diane Duplissa, Lorainne Lucas, and Alex Makovetsky for excellent facilities support. This work was supported in part by National Institutes of Health grants to K.A.W (GM068118) and William M. Gelbart (5 R37 GM28669), a National Research Service Award Trainee Grant to V.T. and to V.L. (T32 GM 007601), a National Research Service Award Postdoctoral Fellowship to M.A.S., a Fundação de Amparo à Pesquisa do Estado de São Paulo award to B.M., and a research grant from The Center for Research in Fibrodysplasia Ossificans Progressiva from The University of Pennsylvania to K.A.W. This work was performed in the laboratories of Bill Gelbart and K.A.W.

LITERATURE CITED

Abdalla, S. A., U. Cymerman, D. Rushlow, N. Chen, G. P. Stoeber *et al.*, 2005 Novel mutations and polymorphisms in genes causing hereditary hemorrhagic telangiectasia. Hum. Mutat. **25:** 320–321.

Abdalla, S. A., and M. Letarte, 2006 Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J. Med. Genet. **43:** 97–110.

Affolter, M., D. Nellen, U. Nussbaumer and K. Basler, 1994 Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel functions of TGFβ homologs during *Drosophila* embryogenesis. Development **120:** 3105–3117.

Arora, K., M. Levine and M. O'Connor, 1994 The *screw* gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. Genes Dev. **8:** 2588–2601.

Bangi, E., and K. A. Wharton, 2006a Dpp and Gbb exhibit different effective ranges in the establsihment of the BMP activity gradient critical for Drosophila wing patterning. Dev. Biol. **295:** 178–193.

Bangi, E., and K. A. Wharton, 2006b Dual function of the Drosophila Alk1/Alk2 ortholog, Saxophone shapes the BMP activity gradient in the wing imaginal disc. Development **133:** 3295–3303.

Bayrak-Toydemir, P., J. McDonald, B. Markewitz, S. Lewin, F. Miller *et al.*, 2006 Genotype-phenotype correlation in hereditary hemorrhagic telangiectasia: mutations and manifestations. Am. J. Med. Genet. A **140:** 463–470.

Brummel, T. J., V. Twombly, G. Marqués, J. L. Wrana, S. J. Newfeld *et al.*, 1994 Characterization and relationship of *dpp* receptors encoded by the *saxophone* and *thick veins* genes in *Drosophila*. Cell **78:** 251–261.

Brummel, T., S. Abdollah, T. E. Haerry, M. J. Shimell, J. Merriam *et al.*, 1999 The Drosophila activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development. Genes Dev. **13:** 98–111.

Burke, R., and K. Basler, 1996 Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. Development **122:** 2261–2269.

Calvi, B. R., and W. M. Gelbart, 1994 The basis for germline specificity of the hobo transposable element in Drosophila melanogaster. EMBO J. **13:** 1636–1644.

Carcamo, J., F. Weis, F. Ventura, R. Wieser, J. Wrana *et al.*, 1994 Type I receptors specify growth-inhibitory and transcrip-

tional responses to transforming growth factor β and activin. Mol. Cell. Biol. **14:** 3810–3821.

Cartegni, L., S. L. Chew and A. R. Krainer, 2002 Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet. **3:** 285–298.

Chen, Y., M. J. Riese, M. A. Killinger and F. M. Hoffmann, 1998a A genetic screen for modifiers of Drosophila decapentaplegic signaling identifies mutations in punt, Mothers against dpp and the BMP-7 homologue, 60A. Development **125:** 1759–1768.

Chen, Y. G., A. Hata, R. S. Lo, D. Wotton, Y. Shi *et al.*, 1998b Determinants of specificity in TGF-beta signal transduction. Genes Dev. **12:** 2144–2152.

Chen, Y. G., and J. Massague, 1999 Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. J. Biol. Chem. **274:** 3672–3677.

Childs, S. R., J. L. Wrana, K. Arora, L. Attisano, M. B. O'Connor *et al.*, 1993 Identification of a *Drosophila* activin receptor. Proc. Natl. Acad. Sci. USA **90:** 9475–9479.

Doctor, J. S., D. Jackson, K. E. Rashka, M. Visalli and F. M. Hoffmann, 1992 Sequence, biochemical characterization, and developmental expression of a new member of the TGF-β superfamily in *Drosophila melanogaster*. Dev. Biol. **151:** 491–505.

Dorfman, R., and B. Z. Shilo, 2001 Biphasic activation of the BMP pathway patterns the Drosophila embryonic dorsal region. Development **128:** 965–972.

Fernandez, L. A., F. Sanz-Rodriguez, R. Zarrabeitia, A. Perez-Molino, R. P. Hebbel *et al.*, 2005 Blood outgrowth endothelial cells from hereditary haemorrhagic telangiectasia patients reveal abnormalities compatible with vascular lesions. Cardiovasc. Res. **68:** 235–248.

Finnson, K. W., W. L. Parker, P. ten Dijke, M. Thorikay and A. Philip, 2008 ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. J. Bone Miner. Res. **23:** 896–906.

FlyBase, 2003 The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res. **31:** 172–175.

Franzen, P., C. H. Heldin and K. Miyazono, 1995 The GS domain of the transforming growth factor-beta type I receptor is important in signal transduction. Biochem. Biophys. Res. Commun. **207:** 682–689.

Goumans, M. J., G. Valdimarsdottir, S. Itoh, F. Lebrin, J. Larsson *et al.*, 2003 Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. Mol. Cell **12:** 817–828.

Groppe, J. C., E. M. Shore and F. S. Kaplan, 2007 Functional modeling of the ACVR1 (R206H) mutation in FOP. Clin. Orthop. Relat. Res. **462:** 87–92.

Gu, Y., P. Jin, L. Zhang, X. Zhao, X. Gao *et al.*, 2006 Functional analysis of mutations in the kinase domain of the TGF-beta receptor ALK1 reveals different mechanisms for induction of hereditary hemorrhagic telangiectasia. Blood **107:** 1951–1954.

Haerry, T. E., O. Khalsa, M. B. O'Connor and K. A. Wharton, 1998 Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila. Development **125:** 3977–3987.

Howe, J. R., J. L. Bair, M. G. Sayed, M. E. Anderson, F. A. Mitros *et al.*, 2001 Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat. Genet. **28:** 184–187.

Huse, M., Y. G. Chen, J. Massague and J. Kuriyan, 1999 Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. Cell **96:** 425–436.

Huse, M., and J. Kuriyan, 2002 The conformational plasticity of protein kinases. Cell **109:** 275–282.

Immergluck, K., P. A. Lawrence and M. Bienz, 1990 Induction across germ layers in *Drosophila* mediated by a genetic cascade. Cell **62:** 261–268.

Kaplan, F. S., M. Xu, P. Seemann, J. M. Connor, D. L. Glaser *et al.*, 2009 Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. Hum. Mutat. **30:** 379–390.

Khalsa, O., J.-w. Yoon, S. Schumann-Torres and K. Wharton, 1998 TGF-b/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the *Drosophila* wing. Development **125:** 2723–2734.

Kim, I. J., J. H. Park, H. C. Kang, K. H. Kim, J. H. Kim *et al.*, 2003 Identification of a novel BMPR1A germline mutation in a Korean juvenile polyposis patient without SMAD4 mutation. Clin. Genet. **63:** 126–130.

Kirsch, T., J. Nickel and W. Sebald, 2000a Isolation of recombinant BMP receptor IA ectodomain and its 2:1 complex with BMP-2. FEBS Lett. **468:** 215–219.

Kirsch, T., W. Sebald and M. K. Dreyer, 2000b Crystal structure of the BMP2-BRIA ectodomain complex. Nat. Struc. Biol. **7:** 492–496.

Kornev, A. P., N. M. Haste, S. S. Taylor and L. F. Eyck, 2006 Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proc. Natl. Acad. Sci. USA **103:** 17783–17788.

Lehmann, K., P. Seemann, S. Stricker, M. Sammar, B. Meyer *et al.*, 2003 Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. Proc. Natl. Acad. Sci. USA **100:** 12277–12282.

Lehmann, K., P. Seemann, J. Boergermann, G. Morin, S. Reif *et al.*, 2006 A novel R486Q mutation in BMPR1B resulting in either a brachydactyly type C/symphalangism-like phenotype or brachydactyly type A2. Eur. J. Hum. Genet. **14:** 1248–1254.

Lenato, G. M., P. Suppressa, P. Giordano, G. Guanti, E. Guastamacchia *et al.*, 2007 Hereditary haemorrhagic telangiectasia: a rare disease as a model for the study of human atherosclerosis. Curr. Pharm. Des. **13:** 3656–3664.

Lesca, G., H. Plauchu, F. Coulet, S. Lefebvre, G. Plessis *et al.*, 2004 Molecular screening of ALK1/ACVRL1 and ENG genes in hereditary hemorrhagic telangiectasia in France. Hum. Mutat. **23:** 289–299.

Lesca, G., C. Olivieri, N. Burnichon, F. Pagella, M. F. Carette *et al.*, 2007 Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. Genet. Med. **9:** 14–22.

Letteboer, T. G., R. A. Zewald, E. J. Kamping, G. de Haas, J. J. Mager *et al.*, 2005 Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum. Genet. **116:** 8–16.

Lewis, E. B., and F. Bacher, 1968 Method of feeding ethyl methane sulfonate (EMS) to *Drosophila* males. Dros. Inf. Serv. **43:** 193.

Loeys, B. L., J. Chen, E. R. Neptune, D. P. Judge, M. Podowski *et al.*, 2005 A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. Nat. Genet. **37:** 275–281.

Macias-Silva, M., P. A. Hoodless, S. J. Tang, M. Buchwald and J. L. Wrana, 1998 Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J. Biol. Chem. **273:** 25628–25636.

Mahmoud, M., G. M. Borthwick, A. A. Hislop and H. M. Arthur, 2009 Endoglin and activin receptor-like-kinase 1 are co-expressed in the distal vessels of the lung: implications for two familial vascular dysplasias, HHT and PAH. Lab. Invest. **89:** 15–25.

Marques, G., H. Bao, T. E. Haerry, M. J. Shimell, P. Duchek *et al.*, 2002 The Drosophila BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. Neuron **33:** 529–543.

Massagué, J., S. Blain and R. Lo, 2000 TGFbeta signaling in growth control, cancer, and heritable disorders. Cell **103:** 295–309.

McCabe, B. D., G. Marques, A. P. Haghighi, R. D. Fetter, M. L. Crotty *et al.*, 2003 The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the Drosophila neuromuscular junction. Neuron **39:** 241–254.

Muller, B., B. Hartmann, G. Pyrowolakis, M. Affolter and K. Basler, 2003 Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. Cell **113:** 221–233.

Nellen, D., M. Affolter and K. Basler, 1994 Receptor serine/threonine kinase implicated in the control of *Drosophila* body pattern by *decapentaplegic*. Cell **78:** 225–237.

Neul, J., and E. L. Ferguson, 1998 Spatially restricted activation of the SAX receptor by SCW modulates DPP/TKV signaling in *Drosophila* dorsal-ventral patterning. Cell **95:** 483–494.

Nguyen, M., S. Park, G. Marques and K. Arora, 1998 Interpretation of a BMP activity gradient in *Drosophila* embryos

depends on synergistic signaling by two type I receptors, SAX and TKV. Cell **95:** 495–506.

Nicholls, R. E., and W. M. Gelbart, 1998 Identification of Chromosomal Regions Involved in *decapentaplegic* Function in Drosophila. Genetics **149:** 203–215.

O'Connor, M. B., D. Umulis, H. G. Othmer and S. S. Blair, 2006 Shaping BMP morphogen gradients in the Drosophila embryo and pupal wing. Development **133:** 183–193.

Oh, S. P., T. Seki, K. A. Goss, T. Imamura, Y. Yi *et al.*, 2000 Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc. Natl. Acad. Sci. USA **97:** 2626–2631.

Olivieri, C., F. Pagella, L. Semino, L. Lanzarini, C. Valacca *et al.*, 2007 Analysis of ENG and ACVRL1 genes in 137 HHT Italian families identifies 76 different mutations (24 novel). Comparison with other European studies. J. Hum. Genet. **52:** 820–829.

Padgett, R., R. St. Johnston and W. Gelbart, 1987 A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature **325:** 81–84.

Panganiban, G. E. F., R. Reuter, M. P. Scott and F. M. Hoffmann, 1990 A *Drosophila* growth factor homolog, *decapentaplegic* regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development **110:** 1041–1050.

Penton, A., Y. Chen, K. Staehling-Hampton, J. L. Wrana, L. Attisano *et al.*, 1994 Identification of two bone morphogenetic protein type I receptors in *Drosophila* and evidence that Brk25D is a *decapentaplegic* receptor. Cell **78:** 239–250.

Raftery, L., V. Twombly, K. Wharton and W. Gelbart, 1995 Genetic screens to identify elements of the *decapentaplegic* signaling pathway in *Drosophila*. Genetics **139:** 241–254.

Raftery, L. A., and D. J. Sutherland, 2003 Gradients and thresholds: BMP response gradients unveiled in Drosophila embryos. Trends Genet. **19:** 701–708.

Rawson, J. M., M. Lee, E. L. Kennedy and S. B. Selleck, 2003 Drosophila neuromuscular synapse assembly and function require the TGF-beta type I receptor saxophone and the transcription factor Mad. J. Neurobiol. **55:** 134–150.

Ray, R., and K. Wharton, 2001 Context-dependent relationships between the BMPs *gbb* and *dpp* during development of the Drosophila wing imaginal disc. Development **128:** 3913–3925.

Schulte, C., U. Geisthoff, A. Lux, S. Kupka, H. P. Zenner *et al.*, 2005 High frequency of ENG and ALK1/ACVRL1 mutations in German HHT patients. Hum. Mutat. **25:** 595.

Schupbach, T., and E. Wieschaus, 1989 Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. Genetics **121:** 101–117.

Shi, Y., and J. Massague, 2003 Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell **113:** 685–700.

Shimmi, O., D. Umulis, H. Othmer and M. B. O'Connor, 2005 Facilitated transport of a Dpp/Scw heterodimer by Sog/Tsg leads to robust patterning of the Drosophila blastoderm embryo. Cell **120:** 873–886.

Shore, E. M., M. Xu, G. J. Feldman, D. A. Fenstermacher, M. A. Brown *et al.*, 2006 A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat. Genet. **38:** 525–527.

Singer, M. A., A. Penton, V. Twombly, F. M. Hoffmann and W. M. Gelbart, 1997 Signaling through both type I DPP receptors is required for anterior-posterior patterning of the entire *Drosophila* wing. Development **124:** 79–89.

Smith, D., J. Wohlgemuth, B. R. Calvi, I. Franklin and W. M. Gelbart, 1993 *hobo* enhancer trapping mutagenesis in Drosophila reveals an insertion specificity different from *P* elements. Genetics **135:** 1063–1076.

Spencer, F. A., F. M. Hoffmann and W. M. Gelbart, 1982 *decapentaplegic*: a gene complex affecting morphogenesis in *Drosophila melanogaster*. Cell **28:** 451–461.

Su, G. H., R. Bansal, K. M. Murphy, E. Montgomery, C. J. Yeo *et al.*, 2001 ACVR1B (ALK4, activin receptor type 1B) gene mutations in pancreatic carcinoma. Proc. Natl. Acad. Sci. USA **98:** 3254–3257.

ten Dijke, P., and C. S. Hill, 2004 New insights into TGF-beta-Smad signalling. Trends Biochem. Sci. **29:** 265–273.

Terracol, R., and J. A. Lengyel, 1994 The *thick veins* gene of *Drosophila* is required for dorsoventral polarity of the embryo. Genetics **138:** 165–178.

Török, T., G. Tick, M. Alvarado and I. Kiss, 1993 *P-lacW* insertional mutagenesis on the second chromosome of *Drosophila melenogaster*: isolation of lethals with different overgrowth phenotypes. Genetics **135:** 71–80.

Twombly, V., R. K. Blackman, H. Jin, J. M. Graff, R. Padgett *et al.*, 1996 The TGF-β signaling pathway is essential for *Drosophila* oogenesis. Development **122:** 1555–1565.

Vincent, S., E. Ruberte, N. C. Grieder, C. K. Chen, T. Haerry *et al.*, 1997 Dpp controls tracheal cell migration along the dorsoventral body axis of the Drosophila embryo. Development **124:** 2741–2750.

Wehner, L. E., B. J. Folz, L. Argyriou, S. Twelkemeyer, U. Teske *et al.*, 2006 Mutation analysis in hereditary haemorrhagic telangiectasia in Germany reveals 11 novel ENG and 12 novel ACVRL1/ALK1 mutations. Clin. Genet. **69:** 239–245.

Weis-Garcia, F., and J. Massague, 1996 Complementation between kinase-defective and activation-defective TGF-beta receptors reveals a novel form of receptor cooperativity essential for signaling. EMBO J. **15:** 276–289.

Wharton, K., R. Ray, S. Findley, H. Duncan and W. Gelbart, 1996 Molecular lesions associated with alleles of decapentaplegic identify residues necessary for TGF-β/BMP cell signaling in *Drosophila melanogaster*. Genetics **142:** 493–505.

Wharton, K. A., R. P. Ray and W. M. Gelbart, 1993 An activity gradient of *decapentaplegic* is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. Development **117:** 807–822.

Wharton, K. A., G. H. Thomsen and W. M. Gelbart, 1991 *Drosophila 60A* gene, another transforming growth factor β family member, is closely related to human bone morphogenetic proteins. Proc. Natl. Acad. Sci. USA **88:** 9214–9218.

Wrana, J. L., L. Attisano, R. Wieser, F. Ventura and J. Massagué, 1994 Mechanism of activation of the TGF-β receptor. Nature **370:** 341–347.

Xie, T., A. L. Finelli and R. W. Padgett, 1994 The *Drosophila saxophone* gene: a serine-threonine kinase receptor of the TGF-β superfamily. Science **263:** 1756–1759.

Xie, T., and A. C. Spradling, 1998 *decapentaplegic* is essential for the maintenance and division of germline stem cells in *Drosophila* ovary. Cell **94:** 251–260.

Yamashita, H., P. ten Dijke, P. Franzen, K. Miyazono and C. H. Heldin, 1994 Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β. J. Biol. Chem. **269:** 20172–20178.

Zecca, M., K. Basler and G. Struhl, 1996 Direct and long-range action of a wingless morphogen gradient. Cell **87:** 833–844.

Zhou, X. P., K. Woodford-Richens, R. Lehtonen, K. Kurose, M. Aldred *et al.*, 2001 Germline mutations in BMPR1A/ALK3 cause a subset of cases of juvenile polyposis syndrome and of Cowden and Bannayan-Riley-Ruvalcaba syndromes. Am. J. Hum. Genet. **69:** 704–711.

Communicating editor: T. Schupbach

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.105585/DC1

Functional Analysis of *saxophone*, the Drosophila Gene Encoding the BMP Type I Receptor Ortholog of Human ALK1/ACVRL1 and ACVR1/ALK2

Vern Twombly, Erdem Bangi, Viet Le,

Bettina Malnic, Matthew A. Singer and Kristi A Wharton

DOI: 10.1534/genetics.109.105585

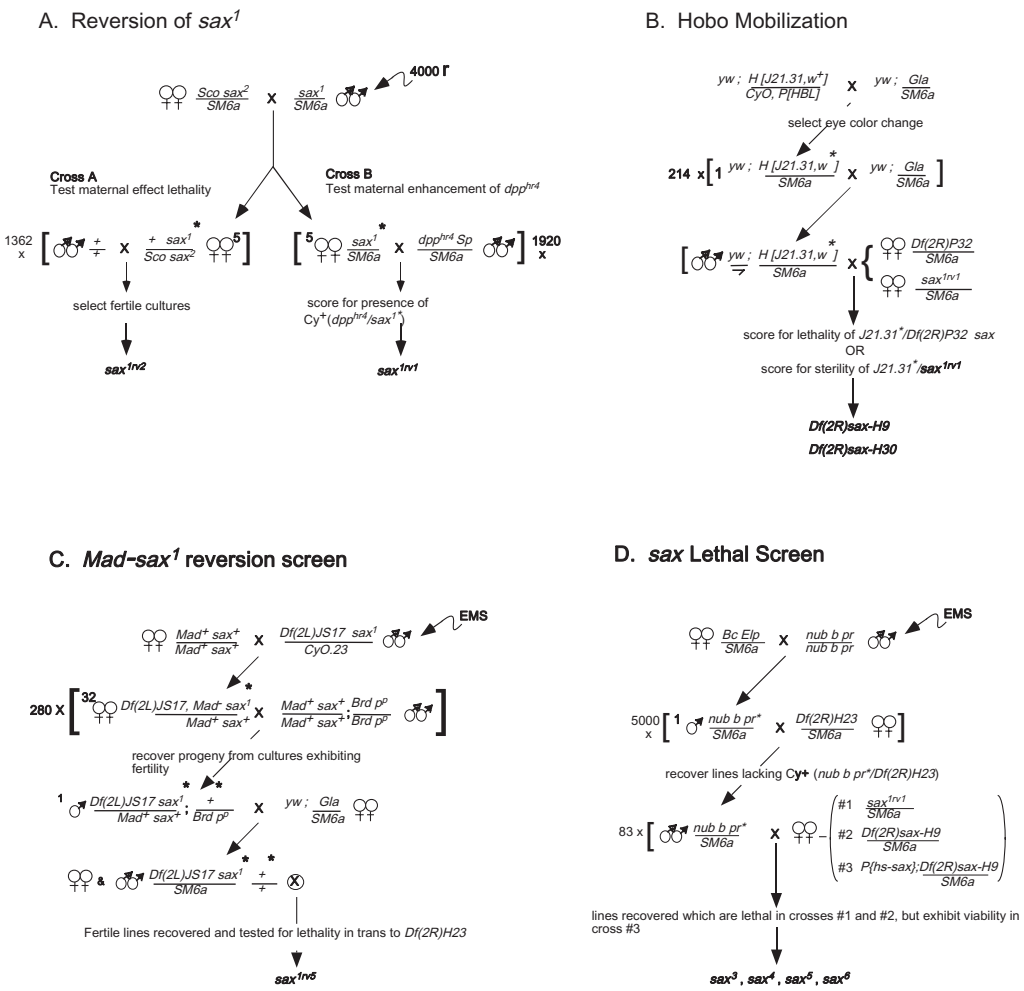


FIGURE S1.—Genetic screens for *sax* mutations. See Materials and Methods.

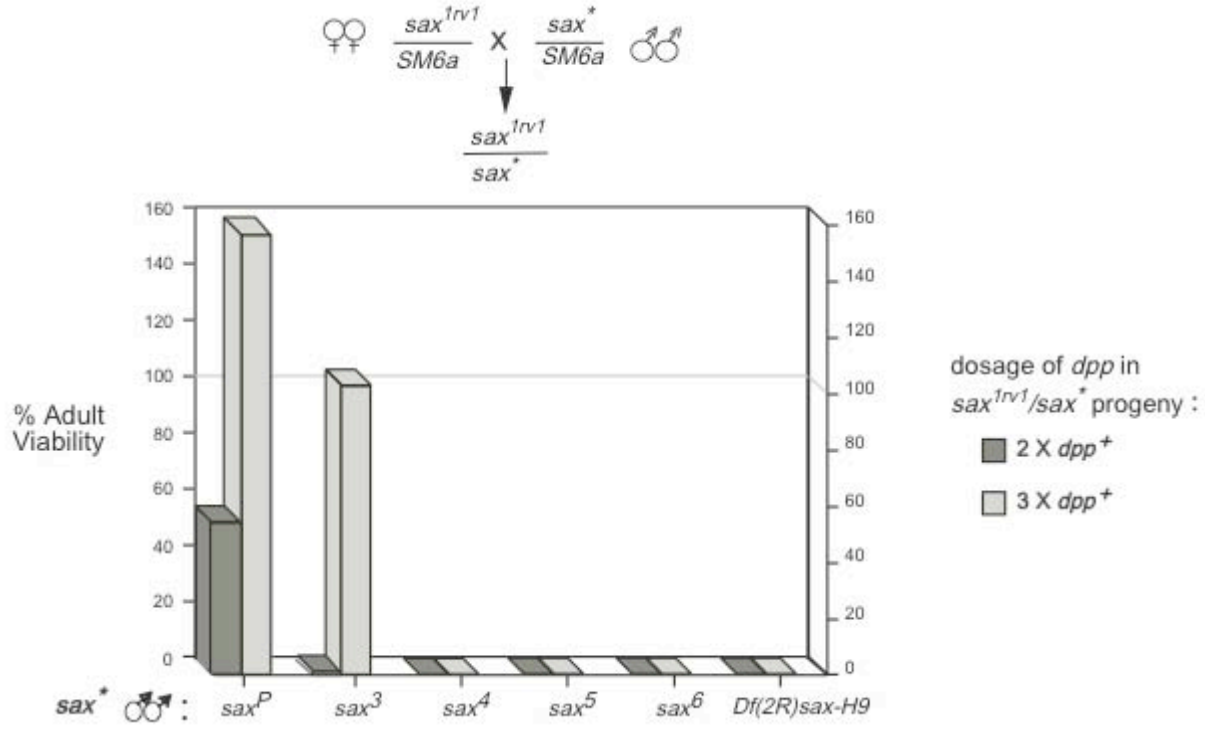


FIGURE S2.—Increased *dpp* dosage fails to rescue the zygotic lethality of most *sax* alleles. The viability of sax^{1rv1} in trans to six *sax* alleles was tested in the presence of increased dpp^+ dosage. While sax^P and sax^3 displayed complete rescue in the presence of three copies of dpp^+, the remaining alleles exhibited no rescue. $n \geq 200$ progeny scored per cross.

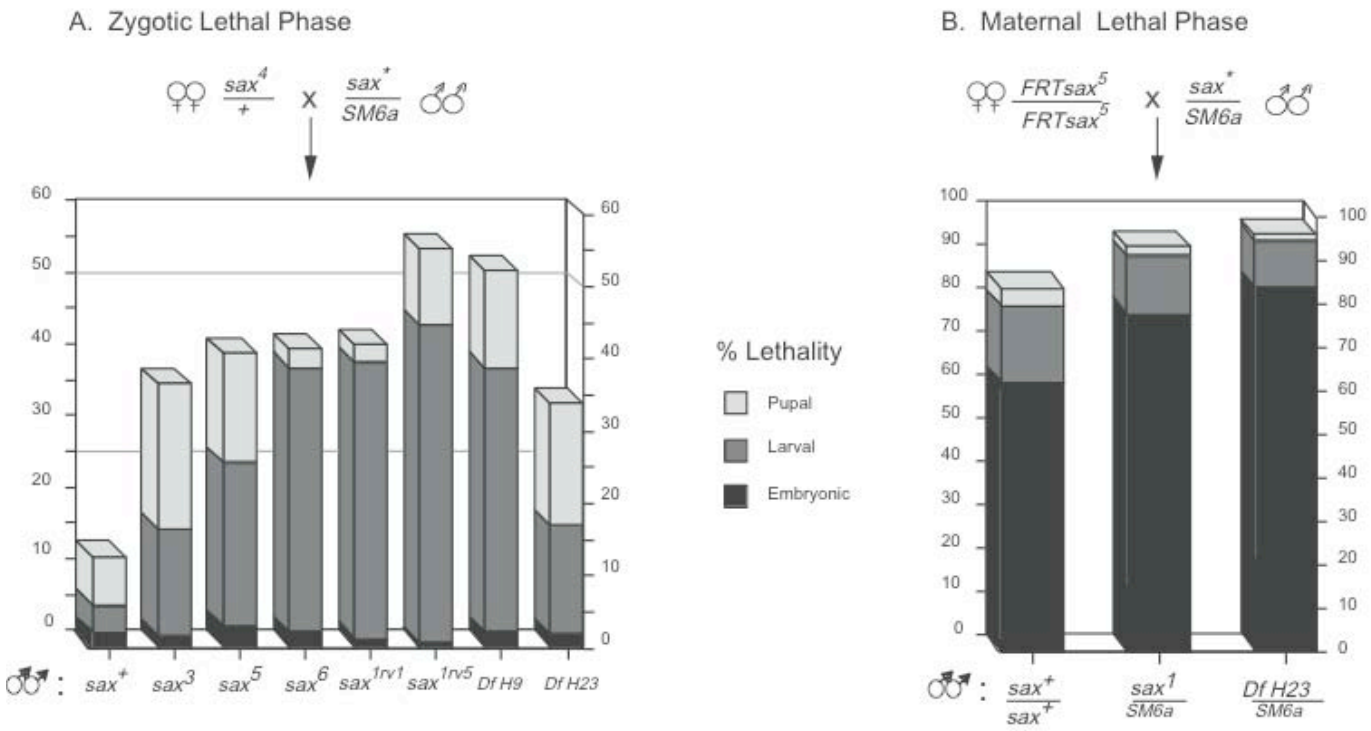
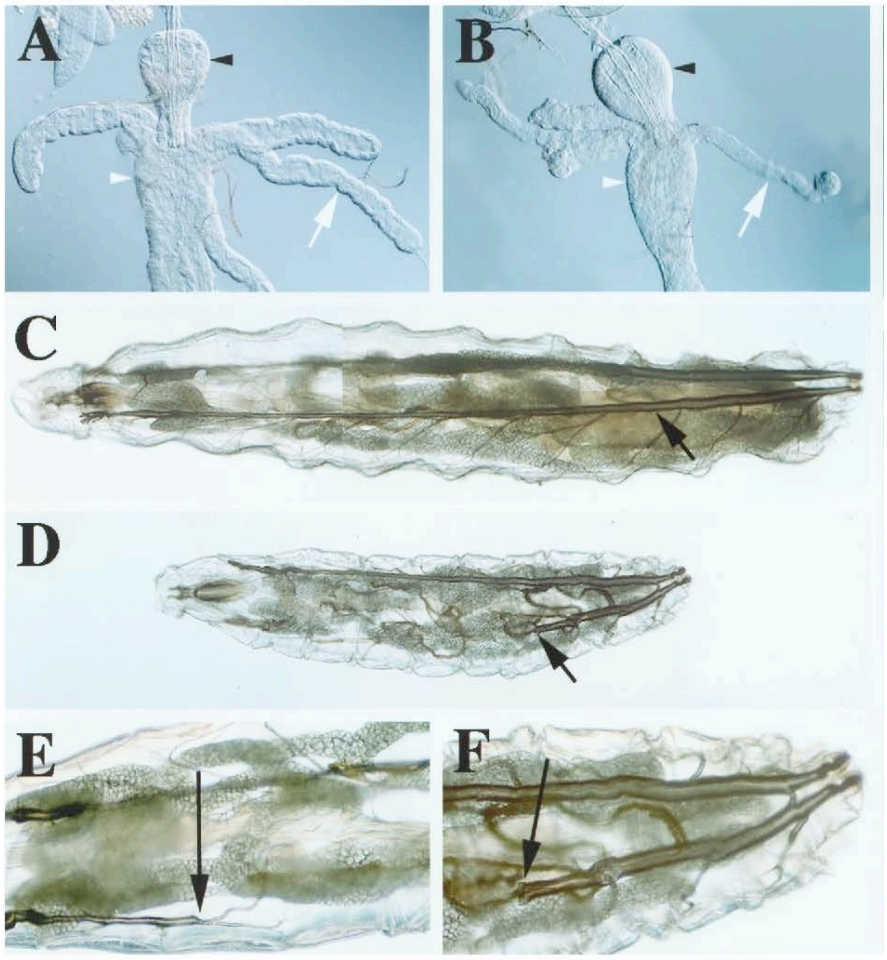


FIGURE S3.—*sax* lethal phase. A. The zygotic lethal phase associated with *sax* LOF alleles. Female $sax^4/+$ heterozygotes were crosses to several *sax* alleles and the resulting progeny were monitored through embryonic, larval and pupal development. The control cross of $sax^4/+$ females mated with wild type males exhibited 12% lethality, which may be due to the sax^4 mutation or the result of the genetic background upon which sax^4 was induced. $n \geq 467$ fertilized embryos examined for each cross. B. The lethal phase of embryos derived from sax^5 homozygous germline clones. Germline clones were induced in $FRT\ sax^5/FRT\ ovo^{D1}$ females, which were crossed to sax^+, sax^1 and *Df(2R)H23* males and the resulting progeny were monitored through embryonic, larval and pupal development. The number of fertilized embryos examined for each cross was n = 991, 792 and 672, respectively.



Genotype	% Transparent	n
sax^{1}/Df(2R)H23	0	11
sax^{2}/Df(2R)H23	0	14
sax^{3}/Df(2R)H23	30	23
sax^{4}/Df(2R)H23	70	20
sax^{5}/Df(2R)H23	76	25
sax^{PE5}/Df(2R)H23	75	16
sax^{PE10}/Df(2R)H23	67	18
sax^{1rv5}/Df(2R)H23	0	11

FIGURE S4.—*sax* lethal phenotypes. Anterior is left in all panels, except A and B. A-F. Zygotic phenotypes of hemizygous *sax^{5}/Df(2R)H23* larva. For A and B the apical portion of the gut is up. A. The proventriculus (black arrowhead), midgut (white arrowhead) and gastric caecae (white arrow) of a *sax^{5}/CyO, P[Lac-Z]* larva. B. The equivalent structures of a hemizygous *sax^{5}/Df(2R)H23* larva. Note the narrow diameter of the gastric caecae though the proventriculus and midgut appear wild type. C. A composite photograph of a 3rd instar hemizygous *sax^{5}/Df(2R)H23* larva from an oblique dorsal view. Note the two wild type main dorsal tracheal trunks (black arrow) running along the anterior-posterior axis. D. A dorsal view of an equivalently aged (+/- 12 hrs) hemizygous *sax^{5}/Df(2R)H23* larva. The size of the larva is reduced and one main dorsal tracheal trunk is disrupted (black arrow). E. A magnified view of a terminated dorsal trunk at the anterior end of a *sax^{5}/Df(2R)H23* larva (black arrow). F. A magnified view from panel D of the terminated dorsal trunk. G. The transparency of *sax*/Df(2R)H23* third instar larvae was assessed. n = number of total larvae of given genotype examined.

TABLE S1

Rescue of *sax* loss-of-function lethality by a *sax*$^{+}$-transgene

Female *y w P{hs-sax}51-35B;Df(2R)sax/SM6a* **X** *y w P{hs-sax}51-35B; sax***/SM6a* Male

heat shock

↓

Number of progeny of designated genotypes and phenotypes

*sax** allele [a]	*sax*/Df(2R)sax* (% rescue)	*sax*/SM6a* & *Df(2R)sax/SM6a*
sax^{1rv1}	346 (**49**) [b]	1407
sax^{1rv2}	0 (**0**)	367
sax^{1rv5}	47 (**62**)	151
sax^{3}	64 (**57**)	223
sax^{4}	68 (**54**)	253
sax^{5}	50 (**41**)	243
sax^{6}	3 (**4**)	131
sax^{PE5}	51 (**61**)	166
sax^{PE7}	0 (**0**)	109
sax^{PE10}	53 (**90**)	117

Df(2R)sax = *Df(2R)sax-H9* for the first seven crosses and *Df(2R)H23* for all *sax*^{PE} crosses.

[a] = *sax** males were hemizygous for the X-linked *sax* rescue transgene (*P{hs-sax}51-35B*).

[b] = This rescue has been presented previously, (BRUMMEL et al., 1994).

TABLE S2

***sax* maternal enhancement of *screw* mutations**

Cross	Female Genotype	Male Genotype	% Lethality	n
A)	***sax^{1}/CyO***	***scw^{E1}/CyO***	**100**	351
B)	***sax^{1}/CyO***	***scw^{E2}/CyO***	**100**	323
C)	*sax^{1}/CyO*	*scw^{E1R1}/CyO*	22	261
D)	***sax^{1}/CyO***	***Df(2R)OD16/CyO***	**11**	246
E)	*scw^{E1}/CyO*	*sax^{1}/CyO*	33	360
F)	*scw^{E2}/CyO*	*sax^{1}/CyO*	16	249
G)	***sax^{2}/CyO***	***scw^{E1}/CyO***	**100**	214
H)	*sax^{2}/CyO*	*scw^{E2}/CyO*	94	250
I)	***sax^{2}/CyO***	***scw^{E1R1}/CyO***	**20**	122
J)	*sax^{2}/CyO*	*Df(2R)OD16/CyO*	50	160
K)	*scw^{E1}/CyO*	*sax^{2}/CyO*	0	192
L)	*scw^{E2}/CyO*	*sax^{2}/CyO*	0	185
M)	***sax^{P}/CyO***	***scw^{E1}/CyO***	**99**	395
N)	***sax^{P}/CyO***	***scw^{E1R1}/CyO***	**0**	371

Bolded genotypes and percent lethality demonstrate that the synthetic lethality between *sax* and *scw* are limited to the gain-of-function alleles of both genes.

TABLE S3

Maternal effect lethality of new *sax* mutations

Cross	Female Genotype	Male Genotype	% Lethality	n
A)	*sax²/sax¹*	+/+	93.6	531
B)	*sax²/sax³*	+/+	95.1	405
C)	*sax²/sax⁴*	+/+	87.4	412
D)	*sax²/ FRT42 sax⁴*	+/+	94.3	474
E)	*sax²/sax⁵*	+/+	92.0	389
F)	*sax²/ FRT42 sax⁵*	+/+	93.6	454
G)	*sax²/sax¹ᵐ⁵*	+/+	94.9	372
H)	*sax²/saxᴾ*	+/+	98.4	385
I)	*sax²/saxᴾᴱ⁵*	+/+	97.9	326
J)	*sax²/saxᴾᴱ⁷*	+/+	81.6	267
K)	*sax²/saxᴾᴱ¹⁰*	+/+	96.1	304