Development 122, 1555-1565 (1996)
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DEV5046

The TGF-β signaling pathway is essential for *Drosophila* oogenesis

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SUMMARY

We examine roles of signaling by secreted ligands of the TGF-β family during *Drosophila* oogenesis. One family member, the DPP ligand encoded by the *decapentaplegic* (*dpp*) gene, is required for patterning of anterior eggshell structures. This requirement presumably reflects the expression pattern of *dpp* in an anterior subset of somatic follicle cells: the centripetally migrating and the nurse cell-associated follicle cells. Similar requirements are also revealed by mutations in the *saxophone* (*sax*)-encoded receptor, consistent with the idea that DPP signaling is, at least in part, mediated by the SAX receptor. A loss of germline *sax* function results in a block in oogenesis associated with egg chamber degeneration and a failure of the transfer of nurse cell contents to the oocyte, indicating that TGF-β signaling is required for these events. Some phenotypes of *sax* mutations during oogenesis suggest that SAX responds to at least one other TGF-β ligand as well in the posterior follicle cells.

Key words: TGF-β, signaling, oogenesis, decapentaplegic, *Drosophila*

INTRODUCTION

Cell-cell communication is crucial for cell migration and for specifying pattern during development. During *Drosophila melanogaster* oogenesis, extensive cell migrations occur as somatic and germline cells cooperate to generate a viable egg (reviewed by Spradling, 1993). The female ovary consists of 15-20 ovariole strands, each representing an independent and progressive chain of maturing egg chambers (King 1970; reviewed by Spradling, 1993). At the apical end of each ovariole is the germarium, where somatic and germline stem cells proliferate. Within the germarium, germline cells undergo four incomplete cell divisions forming a 16-cell cyst interconnected by cytoplasmic bridges, termed ring canals. Descendants of somatic stem cells migrate cortically and enter the germarium to enclose each cyst with a monolayer of follicle cells. The enclosed cyst, the egg chamber, exits the germarium. The somatic follicle cells continue dividing up to stage 6 (where they number ~1100) while the nurse cells enlarge and transfer small amounts of specific RNAs and proteins to the oocyte where yolk becomes visible by stage 8. By stage 9 nearly all the follicle cells migrate posteriorly over the oocyte. At this time, the anteriormost 6-10 follicle cells (termed border cells) delaminate and migrate posteriorly between the nurse cells toward the anterior pole of the oocyte.

By stage 10A, the oocyte spans the posterior half of the egg chamber while the anterior half is composed of the nurse cells. The chamber remains covered by a layer of follicle cells that have reorganized so that ~95% are a columnar epithelium over the oocyte and ~5% are thinly stretched over the nurse cells. The columnar centripetally migrating follicle cells (CMFC) at the oocyte-nurse cell boundary begin a migration inward between the oocyte and nurse cells and pause as they approach the border cells, which are centered immediately anterior of the oocyte. At stage 10B, cells from the dorsal anterior columnar epithelium overlying the oocyte are recruited to form respiratory filaments, the dorsal appendages. Later in stage 10B, the nurse cells begin rapidly transferring their cytoplasmic contents, causing an anterior retreat of the diminishing nurse cells and a concomitant enlargement of the oocyte. This transfer is completed in approximately 30 minutes. The CMFC and border cells are then thought to direct the production of anterior eggshell structures: the micropyle (the sperm entry point), the operculum (the hatch from which the embryo exits) and the ventral collar (a structure circumscribing the operculum). By stage 14, the nurse cell nuclei degrade and anterior eggshell structures are completed.

Considerable cell communication and migration is required for these events. The signals that orchestrate follicle cell migrations and the transfer of nurse cell contents to the oocyte have not been determined. Here we investigate roles of the Transforming Growth Factor-β (TGF-β) signaling in potentiating such activities (reviewed by Attisano et al., 1994; Kingsley, 1994; Miyazono et al., 1994). In *Drosophila melanogaster*, three members of the TGF-β superfamily are encoded by the genes *decapentaplegic* (*dpp*; Padgett et al., 1987), *60A* (Wharton et al., 1991; Doctor et al., 1992) and *screw* (*scw*; Arora et al., 1994). *dpp* has been shown to be capable of

signaling across germ layers. During early embryogenesis, dorsal ectoderm expression of *dpp* induces determination of dorsal versus ventral mesoderm (Staehling-Hampton et al., 1994; Frasch 1995). Later, mesoderm expression of *dpp* directs cell fate changes in the underlying endoderm (Panganiban et al., 1990; Immerglück et al., 1990).

TGF-β-related factors are thought to signal through type I and type II serine/threonine receptor kinases, distinguished by their sequence and ligand-binding characteristics (reviewed by Derynck 1994; Massagué and Polyak 1995). Recent characterization of DPP receptors has also shown a requirement for both type I and type II receptor subunits. Two DPP type I receptors are encoded by the *saxophone* (*sax*) and *thick veins* (*tkv*) genes (Brummel et al. 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994) and a DPP type II receptor is encoded by the *punt* gene (Letsou et al., 1995; Ruberte et al., 1995). All three receptors are thought to contribute to DPP signaling, though the requisite level of each receptor and the nature of the in vivo heteromeric receptor complex are not fully understood. Further, it is not known if these receptors participate in the signaling of the other known *Drosophila* ligands 60A and SCW.

Previous work has shown that *dpp* is not required in the germline during oogenesis (Irish and Gelbart, 1987). In this report, we show *dpp* function is required in the somatic component of *Drosophila* ovaries. We find that *dpp* expression is restricted to a subset of migrating anterior follicle cells. We demonstrate an essential germline requirement for *sax*, suggesting that a TGF-β signaling event may be transmitted from the soma to the germline during oogenesis. Our analysis of oogenesis in females mutant for DPP signaling elements demonstrate that this TGF-β pathway is necessary to maintain egg chamber integrity, generate anterior eggshell structures and transfer nurse cell contents to the oocyte. In addition, there is likely to be a contribution of an additional TGF-β signal for proper patterning within the posterior egg chamber.

MATERIALS AND METHODS

Drosophila strains

Canton-S or *y* w^{67c23} strains were used as wild type. The mutants used in this study are referenced in FlyBase (1996) unless otherwise noted. sax^{1rv5} is a loss-of-function *sax* allele recovered by Marnie Gelbart as an EMS-induced dominant suppressor of *Df(2L)Mad* sax^{1} / $Mad^{+}sax^{+}$ female sterility. Loss-of-function alleles sax^{4} and sax^{5} were EMS-induced on a *nub b pr* isogenic chromosome as recessive lethals exposed by *Df(2R)H23* (and all other available deletions of the 43E18-43F2 region) and are rescued by *sax* transgenes (V. T., unpublished results). Strains were cultured on standard cornmeal, yeast extract, dextrose medium at 25°C, unless stated otherwise.

Characterization of *dpp*^{10638}

dpp^{10638} is a P element insertion initially characterized by the Berkeley *Drosophila* Genome Project. Plasmid rescue was employed to recover the left half of the dpp^{10638} transposon and adjoining genomic sequences. Three clones were recovered extending from an *Xba*I site in the middle of the PZ transposon (Jacobs et al., 1989) to an *Xba*I or *Nhe*I site in the *dpp* gene at molecular map coordinate 83 (St. Johnston et al., 1990). Clone pPR11 was sequenced outward from the P element into the *dpp* genomic DNA. The transcription units of *dpp* and the *lacZ* gene in PZ are divergently oriented.

hs-tsax and *hs-dpp* plasmid and strain constructions

P{w^{+mC} $sax^{\Delta IC.1}$*=hs-tsax.T-G}1.1, (P{hs-tsax})*

The truncated SAX receptor was generated as in Graff et al. (1994). This modified cDNA was cloned into the pHsp70 vector (Pirotta, 1988) and transformed into *y* w^{67c23} embryos.

P{w^{+mC} $dpp^{hs.P\text{-}BP}$*=hs-dpp.B-P}, (8xP{hs-dpp})*

Genomic *dpp* sequences extending beyond the polyadenylation site were substituted for 3′ sequences of the *dpp* cDNA, E55 (Padgett et al., 1987). These sequences were cloned into the pHsp70 vector and injected into *y* w^{67c23} embryos (Pirotta, 1988). A strain homozygous for four distinct insertions (at 2B, 16F-17A, 61E and 97) was generated.

Histochemical staining and ovariole phenotypes

1- to 2-day-old females were conditioned for 3 days at 25°C on yeasted media and ovaries were then dissected in *Drosophila* Ringer's solution + 0.05% BSA (DR+BSA), unless stated differently in figure legends. Ovarioles were dissociated, fixed for 5 minutes in 4% formaldehyde and washed 3× with DR+BSA. To assay β-galactosidase activity, ovaries were incubated in X-gal-staining solution (Bellen et al., 1989) saturated with X-gal at 37°C for 12-16 hours. Ovaries were mounted in 50% glycerol and examined under Nomarski optics. Rhodamine-conjugated phalloidin staining was performed as in Cooley et al. (1992). Phalloidin-labelled ovaries were mounted in Vectashield and photographed with a Zeiss Axiophot.

Characterization of eggshell phenotypes

dpp^{e87} / dpp^{hr56} females were maintained at 29°C and egg collections spanned days 4 through 7. *8xP{hs-dpp}* females were maintained at 25°C and heat-shocked for 45 minutes at 37°C on both day 3 and day 4. Eggs were harvested 8 hours after the second heat-shock. *P{hs-sax} / + ;* sax^{-} */ Df(2R)sax-H9* females were maintained at 18°C and collections were performed from day 2 to 6. $tkv^{7}sax^{+}$ / $tkv^{+}sax^{1}$ females were maintained at 25°C and eggs were harvested on days 4 through 7. Eggs were harvested from yeasted grape juice agar plates, mounted in 3:1 Hoyer's : lactic acid and photographed under dark-field optics.

Eggshell measurements

Eggshells and an optical micrometer were photographed at the same magnification and the resulting images were used for measurements. Egg lengths were measured from the collar to the posterior aeropyle. Opercula were measured from the ventral collar to the anterior dorsal appendage attachment point. Dorsal appendage measurements span the distal tip to the posterior attachment point. For wild type at 25°C, the mean length of the egg, dorsal appendage and operculum were 0.58±0.03 mm, 0.32±0.01 mm and 0.11±0.01 mm, respectively (n=25).

Germline clonal analysis of *sax*

Females containing FRT constructs, *P{>*w^{hs}*>} sax*$^{+}$*sca bw*, *P{>*w^{hs}*>} sax*^{4} *sha*^{1} */ CyO* and *P{>*w^{hs}*>} sax*^{5} *sha*^{1} */ CyO* (kindly provided by Dr Matthew Singer) were crossed to *P{hs-FLP} / Y ; P{>*w^{hs}*>} P{ovo*^{D1}*=18}32X9 / CyO* males. These adults were transferred every 48 hours to new culture bottles. The progeny of this cross were heat-shocked at 24 hours after the onset of pupation, (±24 hours), as in Chou et al. (1993). Within single vials, 1- to 2-day-old individual females of the genotype *P{hs-FLP} / + ; P{>*w^{hs}*>}## / P{>*w^{hs}*>} P{ovo*^{D1}*=18}32X9* (where *##=*sax^{+} *sca bw*, sax^{4} sha^{1}, or sax^{5} sha^{1}) were continuously provided three males and egg production was monitored every other day as the culture vial was transferred.

RESULTS

The expression of *decapentaplegic* during oogenesis

A P-element enhancer trap insertion in *dpp* accurately

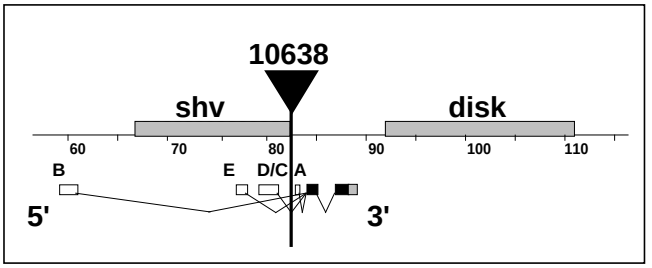

Fig. 1. Location of the P element in *dpp*^{10638}. Schematic representation of the insertion site (triangle) of *P{PZ}l(2)10638* in the *decapentaplegic* genomic region (St. Johnston et al., 1990). The coding exons and the four known 5′ untranslated exons are denoted below the genomic walk as black and white rectangles, respectively. The shaded rectangles above the genomic walk represent the *cis*-regulatory shv (shortvein) and disk (imaginal disk) domains.

reflects *dpp* expression during oogenesis. This insertion, originally isolated by Karpen and Spradling (1992) is a recessive embryonic lethal *dpp* allele and is now named *dpp*^{10638}. The insertion site is 218 bp 5′ of the *dpp* transcript A start site (Fig. 1). In addition to recapitulating the *dpp* embryonic and imaginal disk expression patterns (data not shown), *dpp*^{10638} also exhibits ovarian *lacZ* expression, a location for which *dpp* expression has not been previously described. RNA in situ hybridization to whole-mount ovaries were performed to ensure that the *lacZ* expression seen with *dpp*^{10638} represented the endogenous *dpp* ovary expression pattern. *dpp* RNA was detected in the same temporal and spatial pattern in the ovary visualized by *lacZ* expression of *dpp*^{10638} (Fig. 2A,H). In the following section, we will refer to this *lacZ* expression pattern in the ovary as the *dpp* expression pattern.

dpp expression is first detectable at the end of stage 8 in approximately 20 to 30 somatic follicle cells at the anterior tip of the egg chamber (Fig. 2B). The number of cells expressing *dpp* increases as they migrate cortically and posteriorly until expression is seen over the anterior third of the egg chamber at the end of stage 9 (Fig. 2B). The progression of the most posterior transverse plane of *dpp*-expressing cells is roughly the same plane as that of the border cells, which themselves do not express *dpp* (data not shown). By stage 10A, all follicle cells have completed their posterior movement. Here, the number of *dpp*-expressing cells has increased and includes ~50 thinly stretched nurse cell follicle cells (NFC) and approximately 20 columnar follicle cells overlying the nurse cell-oocyte boundary (Fig. 2C,D). The increase in the number of *dpp*-expressing cells during stages 8 through 10A may arise from maintenance of expression in the daughter cells of cells previously expressing *dpp* or through newly induced expression. At stage 10B, the cells at the nurse cell-oocyte boundary begin a centripetal migration inward between the oocyte and the nurse cells. Here, *dpp* is expressed in the leading edge (~20 cells) of the approximately 150 columnar centripetally migrating follicle cells (CMFC), which move adjacent to the border cells (Fig. 2D,E). From stage 11 onward, *dpp* expression continues in the NFC and CMFC populations as they migrate or are displaced anteriorly over the expanding oocyte (Fig. 2F). At stages 13-14, *dpp* expression is limited to the NFC and a two cell tall cylinder encircling the nearly complete micropyle (Fig. 2G).

In summary, *dpp* is expressed in the outer somatic layer of follicle cells in the anterior portion of the developing egg chamber. This expression almost completely surrounds the

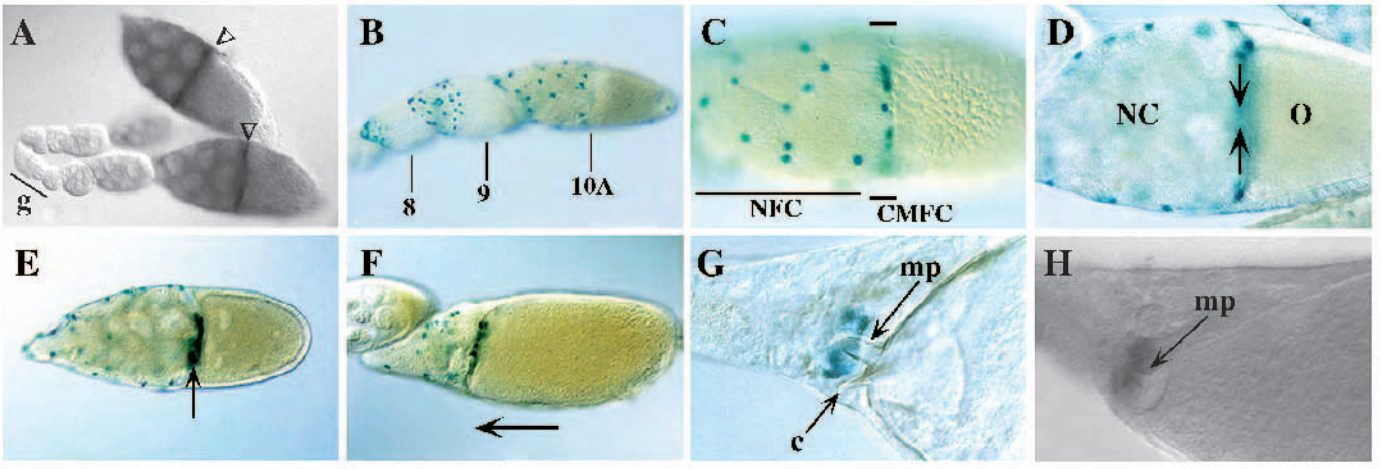

Fig. 2. *dpp* expression during oogenesis. Localization of *dpp* transcript accumulation by RNA in situ hybridization (A,H) and by *lacZ* expression of the *dpp*^{10638} enhancer trap (B-G). Anterior is to the left in this and subsequent figures, unless stated otherwise. Dorsal is up in E-H. (A) *dpp* RNA is absent from the germarium (g) and early stage egg chambers, but is expressed at later stages in the centripetally migrating follicle cells (CMFC, triangle). *dpp* RNA was also detected in the nurse cell follicle cells (not visible in this focal plane). (B) Superficial views of *dpp*^{10638} *lacZ* expression begins in late stage 8 chambers. The domain of *lacZ* expression extends posteriorly during stages 9 through 10A. (C) Superficial view of stage 10A expression in the nurse cell follicle cells (NFC) and a subset of the CMFC. (D) An optical cross-section of a stage 10B chamber shows *lacZ* expression in the leading edge of the CMFC, which have begun to migrate inward (in the direction of the arrows), between the nurse cells (NC) and the oocyte (O). (E,F) By the end of stage 10B, the *lacZ*-expressing CMFC (arrow) have completed their centripetal migration (E) and remain immediately anterior of the expanding oocyte (F). (G) From stage 12 to 14, expression remains on in the NFC (not visible in this focal plane) and the CMFC encircling the developing micropyle (mp). The collar (c), demarks the edge of the operculum. (H) Endogenous *dpp* transcripts are clearly visible in follicle cells around the micropyle at stage 14.

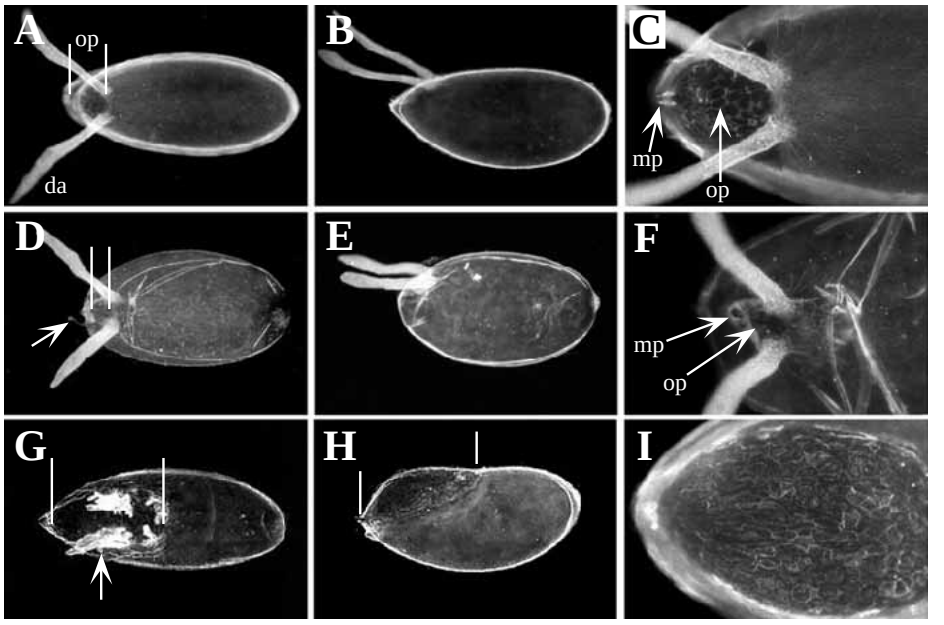

Fig. 3. Eggshell phenotypes associated with alterations in DPP signaling. The eggs in B, E and H are side views, with dorsal up. The other eggs are top views. (A-C) Morphologically wild-type eggs produced by control females. (A) The dorsal appendages (da) are broader at the distal end than they are proximally. The operculum (op) comprises the region between the vertical bars. (B) Lateral view; (C) high magnification of the anterior eggshell structures. Note the micropyle (mp) and the thinner appearance of the eggshell of the operculum. (D-F) Eggs resulting from defects in DPP signaling. These eggs are derived from dpp^{e87} / dpp^{hr56} females reared at 18°C and then cultured at restrictive temperature (29°C). (D) The egg length, operculum and dorsal appendages are all reduced. Small anterior protrusions of ectopic eggshell (arrow) were commonly observed. (E) Lateral view; (F) high magnification reveals a complete loss of the operculum and a malformed micropyle. (G-I) Mutant phenotypes from heat-shocked *8xP{hs-dpp}* females. (G,H) Eggs display enlarged opercula and deranged or absent dorsal appendages (arrow in G, missing in H). (I) The enlarged operculum retains some elements of the wild-type follicle cell imprint pattern (see Fig. 3C).

germline nurse cells and includes cell types implicated in the production of anterior eggshell structures.

Altered levels of *dpp* affect anterior eggshell structures

Because of the zygotic lethality associated with loss-of-function *dpp* alleles, we had to rely on conditional partial loss-of-function alleles to examine the contribution of *dpp* to oogenesis. At 25°C, dpp^{e87} / dpp^{hr56} animals die while, at 18°C, many survive to adulthood (Wharton et al., 1996). To assess maternal effects of *dpp* on oogenesis, such surviving dpp^{e87} / dpp^{hr56} females were mated to wild-type males and cultured at permissive (18°C) and restrictive (29°C) temperatures. Unlike the case for control females (wild type cultured at 29°C and dpp^{e87} / dpp^{hr56} at 18°C; Fig. 3A-C), a subset of eggs generated by mutant females reared at 29°C display anterior eggshell defects. Some abnormal eggshells exhibit extremely reduced opercula (Fig. 3F) and slightly reduced dorsal appendages while the other class displays a moderately shortened egg length (Table 1; Fig. 3D,E). In many cases, the operculum defects are accompanied by small protrusions of ectopic eggshell extending off the anterior pole and, more rarely, malformations of the micropyle (Fig. 3F). Thus, diminished levels of DPP are associated with a loss of anterior eggshell structures. Since *dpp* is completely dispensible in the germline (Irish and Gelbart, 1987), we infer that these partial loss-of-function phenotypes are due to reduced expression of DPP in the anterior follicle cells.

If *dpp* plays a role in the fating or activity of anterior follicle cells, then ubiquitous follicle cell expression of *dpp* might be expected to increase the extent of eggshell structures produced by the anterior follicle cell populations. Indeed, eggs produced by females ubiquitously expressing *dpp* (through an Hsp70-*dpp* transgene) display expanded anterior eggshell structures (Fig. 3G-I). *8x P{hs-dpp}* females received a 37°C heat-shock and were then maintained at 29°C. Nearly all resulting eggs have abnormal dorsal appendages and 58% display abnormalities that we interpret as vastly enlarged opercula (Table 1). The opercula are minimally 150% larger than those of wild-type eggs and can span 50% of the egg length (Fig. 3G,H). Nearly a third of these eggs completely lack dorsal appendages while one-sixth display multiple spurs of dorsal appendage material or branching ‘antler-like’ dorsal appendages along the circumference of the operculum (Fig. 3G). Eggs from control females (wild type at 29°C and *8xP{hs-dpp}* maintained at 18°C) show none of these abnormalities. Thus, ubiquitous expression of *dpp* has the opposite effect on eggshell development - expansion of anterior eggshell domains - to that generated by partial loss of *dpp* expression.

Receptor mutations also produce defective anterior eggshell structures

We have examined the effects of several mutations in the SAX receptor on oogenesis. Reduction of *sax* activity in ovaries was accomplished by rescuing zygotic lethal, loss-of-function *sax* mutations with an Hsp70-sax^{+} transgene and maintaining the resulting rescued females at non-heat shock temperatures (18°C). These *sax* mutant females produce abnormal eggs that fall into two classes: a frequently encountered class of eggs with abnormal dorsal appendages and infrequently encountered short eggs (Table 1). The short eggs are 70-80% of wild-type length and also display short abnormal dorsal appendages (Fig. 4D-F). Similar rescued females, but maintained at 25°C and 29°C, do not display such abnormalities, presumably because more sax^{+} product is produced by the *hs-sax* transgene at higher temperatures.

We also observed a nearly identical eggshell phenotype produced by females doubly heterozygous for *sax* and *tkv*

Table 1. Eggshell phenotypes associated with disruptions in the TGF-β signaling pathway

	Dorsal appendages (DA)					Operculum (OP)		Egg shape		
Genotype	*n*	Wild-type	Abnormal[a]	Fused	Absent	Reduced[b]	Enlarged[c]	Wild-type	Short[d]	Ventralized
Canton S @ 29°C	408	89%	10%	0	0	0	0	99%	1%	0
dpp^{e87}/dpp^{hr56} @ 29°C	374	2%	98%	0	0	93%	0	41%	59%	0
Canton S @ 29°C with heat shock	387	86%	4%	0	0	0	0	97%	3%	0
8 x hs-dpp @ 29°C with heat shock	307	0	71%[e]	0	29%	0	58%	100%	0	0
Canton S @ 18°C[f]	1655	92%	7%	0.3%	0	na	na	97%	0.5%	2.1%
hs-sax/+ ; sax^{1rv1}/Df(2R)sax-H9 @ 18°C[f]	858	69%	29%	2%	0	na	na	94%	4.3%	1.4%
hs-sax/+ ; sax^{5}/Df(2R)sax-H9 @ 18°C[f]	1247	81%	15%	4%	0	na	na	96%	1.5%	2.5%
hs-sax/+ ; sax^{5}/sax^{1rv1} @ 18°C[f]	927	55%	33%	12%	0	na	na	89%	10.7	0.2%
tkv^{7} +/+ sax^{1rv5} @ 25°C	189	61%	39%	0	0	na	na	99%	0.5%	0
tkv^{7} +/+ sax^{1} @ 25°C	437	7%	5%	48%	0	na	na	69%	31%	0

na = not applicable. This is because the CMFC may not migrate to a position that would engender the phenotypes seen from *dpp* mutants.
[a]DA had one or more of the following phenotypes; short, thin, broad, or the two DA of a single egg were substantially different lengths.
[b]OP were 0-50% of wild-type length.
[c]OP were greater than 150% of wild-type length.
[d]Egg lengths were approximately 70-85% of wild-type length.
[e]DA reductions were different than in the above three genotypes and were associated with enlarged operculums. In this case 42% of the total had moderately broadened DA, 14% displayed DA 3-4 times the wildtype width, and 21% exhibited spurs of DA material along the edge of the operculum or they 'antler-like' DA branches.
[f]Virgin females were crossed at 18º for 3 days at which point egg collections began. Eggs were collected daily and scored for 8 days.

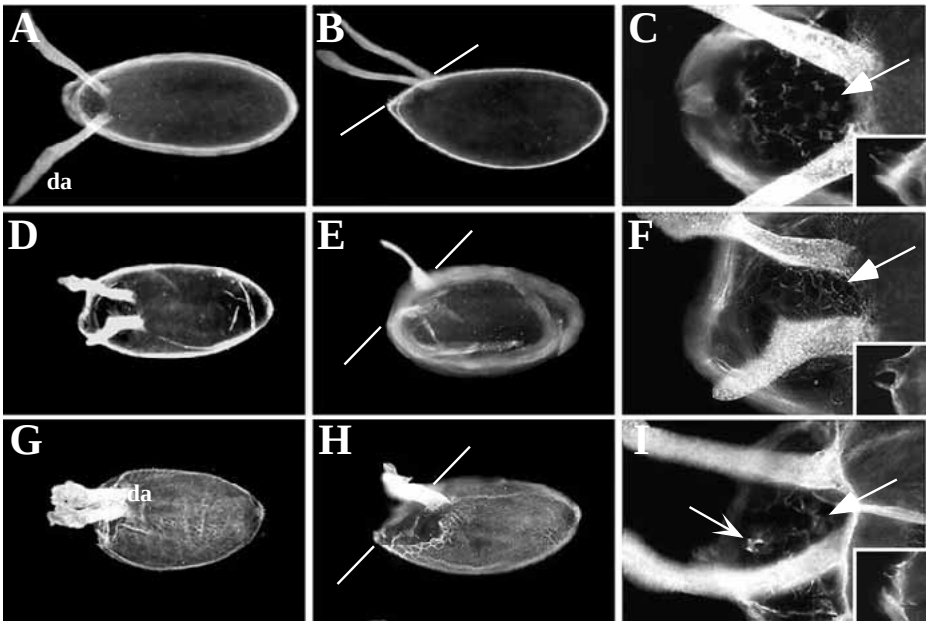

Fig. 4. Eggshell phenotypes associated with reductions in receptor activity. The eggs in B, E and H are side views, with dorsal up. The other panels are top views. (A-C) Eggs from control females. (A) Morphologically normal egg length; dorsal appendages (da); (B) side view; (C) high magnification of the operculum. Note the follicle cell patterning of the operculum (arrow). The inset is a lateral view of the micropyle (mp). (D-F) Eggs produced with reduced maternal SAX receptor levels from *P{hs-sax} / + ; sax^{1rv1} / Df(2L)sax-H9* females at 18°C. (D) Short egg with short abnormal dorsal appendages. (E) The opercula of these eggs are shifted toward the anterior pole of the egg. (F) Note the follicle cell imprints in the posterior operculum are reduced (arrow) and that the micropyle is abnormal (inset). (G-I) Eggs produced with reduced maternal SAX and TKV receptor levels, from *tkv^{7}sax^{+} / tkv^{+}sax^{1}* mutant females cultured at 25°C. (G) A short eggshell with fused dorsal appendages. (H). The operculum is shifted toward the anterior pole. (I) The operculum has irregular follicle cell imprints. The micropyle (small arrow) and collar are reduced in size relative to wild type.

mutations. Females that are *sax^{1} / sax^{+}* or *tkv^{7} / tkv^{+}* or *tkv^{7}sax^{+} / tkv^{+}sax^{1rv5}* generate morphologically normal eggs. However, 42% of the eggs produced by *tkv^{7}sax^{+} / tkv^{+}sax^{1}* females have reduced dorsal appendages and 31% are short with fused appendages (Table 1; Fig. 4G-I). The opercula are shifted toward the anterior pole (Fig. 4H) and appear to lack

normal follicle cell imprint patterns (Fig. 4I). In summary, mutations in what are thought to be DPP receptors produce short eggs with reduced anterior structures. While the same anterior structures are affected in eggs derived from either *dpp* or receptor mutant females, the latter produce more severe defects.

Reduced receptor signaling causes egg chamber defects

To determine the developmental defects that may underlie these eggshell abnormalities, we analyzed developing egg chambers from *sax* mutant females (*P{hs-sax} / + ; sax^{1rv1} / Df(2L)sax-H9* maintained at 18°C). Control females contain ovarioles with well-represented stages; 81% contain stages 2-9 followed by a stage 10-13 chamber (*n*=80 ovarioles; Fig. 5A,D). Degraded egg chambers are rarely observed. In comparison, 58% of *sax* mutant ovarioles display no chambers older than stage 9 (*n*= 62 ovarioles; Fig. 5B,C). These ovarioles contained degenerating chambers immediately posterior to abnormal stage 9 chambers (Fig 5B). The few older chambers observed between stages 9 and 10 are elongated and the yolk is inappropriately localized (Fig. 5C). These degenerating chambers might be a direct consequence of reduced receptor signaling or may indirectly result from blockage of the oviduct by abnormal eggs, as has been seen in other circumstances (D. St. Johnston, personal communication).

We utilized a heat-shock inducible dominant negative *sax* receptor transgene, *P{hs-tsax}*, to attempt to temporally block endogenous SAX function at a variety of stages upon induction. This transgene lacks the intracellular kinase domain of the receptor and is structurally similar to other dominant negative TGF-β receptor constructs (Graff et al., 1994). *P{hs-tsax} / + ; $dpp^{10638}sax^{-}$ / $dpp^{+}sax^{+}$* ovaries dissected 9 hours after heat-shock treatment display degenerating late stage 9 chambers similar to those seen in Fig. 5B as well as stage 8-9 chambers with narrowed posterior poles (data not shown). In addition, we observed chambers in which large nurse cells remain anterior of abnormally short but mature eggs (Fig. 5E). In these chambers, the eggshell, micropyle and dorsal appendages were formed in the absence of complete transfer of nurse cell contents into the oocyte, resulting in short eggs (Fig. 5E,F, compare with Fig. 5D). We also observed stage 10B-11 chambers with uniformly thick columnar follicular epithelium over the oocyte (data not shown). In wild-type stage 10B chambers, the epithelium is only this thick in the dorsal anterior region of the columnar follicle cells. The mutant chamber phenotypes observed are consistent with the production of short eggs, anterior defects and degenerative events which block further progression of oogenesis.

Receptor mutations disrupt F-actin localization in egg chambers

In wild type, from stage 3 to early stage 10, F-actin filaments are subcortically localized in all cells of the egg chamber (Fig. 6A,B; reviewed by Cooley and Theurkauf, 1994). During and after stage 10, large arrays of cytoplasmic F-actin filaments form in the nurse cells near the ring canals and at the nurse cell-oocyte boundary (Fig. 6C) Mutations in several loci encoding F-actin-binding proteins prevent assembly of cytoplasmic actin arrays. This results in a failure of the nurse cells

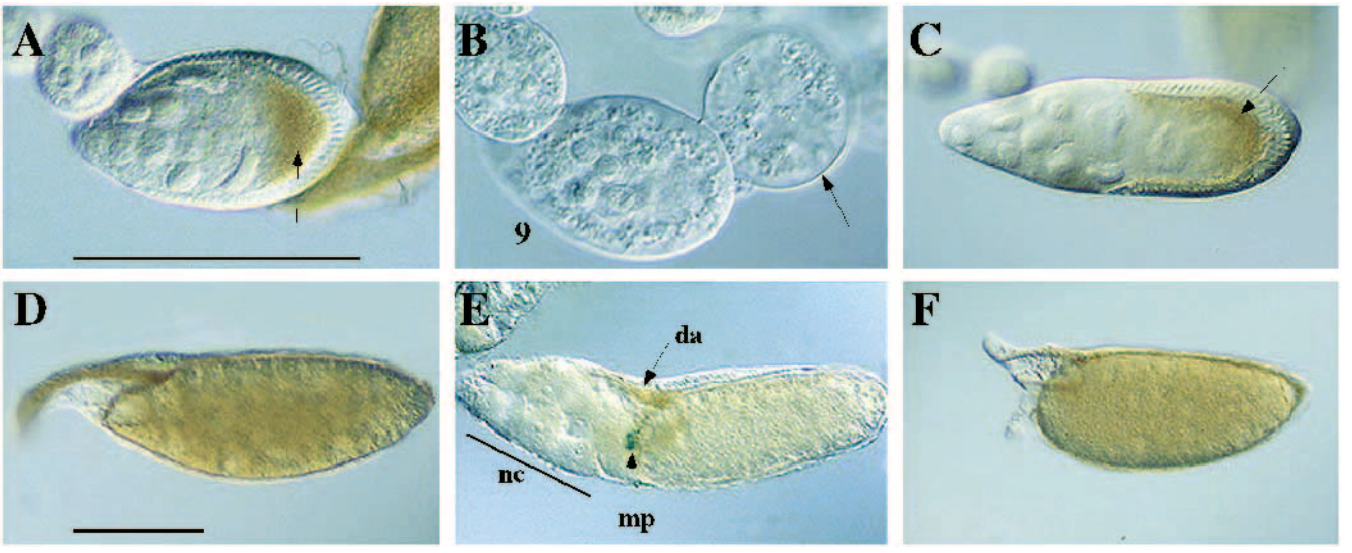

Fig. 5. Egg chamber morphology in receptor mutant ovaries. (A-C) At more than twice the magnification of D-F, as indicated by the horizontal bars in A and D. (D-F) Dorsal is up. (A,D) Control egg chambers. (A) Stage 9 chamber. The oocyte at the posterior end, contains yolk (arrow) and is covered by thickened follicular epithelium. (B,C) *sax* mutant egg chambers from *P{hs-sax} / + ; sax^{1rv1} / Df(2L)sax-H9* females cultured at 18°C. (B) This appears to be an abnormal stage 9 chamber with no yolk accumulation followed by a degraded chamber (arrow). (C) A late stage 9 chamber with elongated morphology and asymmetrically localized yolk (arrow). (D) A mature wild-type stage 14 egg chamber from a control female. (E,F) Egg chambers produced with temporally reduced SAX signaling from *P{hs t-sax} / + ; $dpp^{10638}sax^{1rv1}$ / sax^{+}* heat-shocked mothers. (E) Stage 14. The nurse cells (nc) anterior to a mature oocyte have not completed transfer of their cytoplasmic contents. Dorsal appendages, (da). There is *dpp^{10638} lacZ* expression around the micropyle (mp). (F) A short stage 14 egg chamber presumably resulting from a lack of cytoplasmic transfer from the nurse cells to the oocyte, as indicated in E. Ovaries were dissected from females cultured as follows: (A-C) wild-type and mutant females at 18° for six days; (D-F) wild-type and mutant females at 25°C except for 40 minute heat shocks at 37°C on day 3 and day 4.

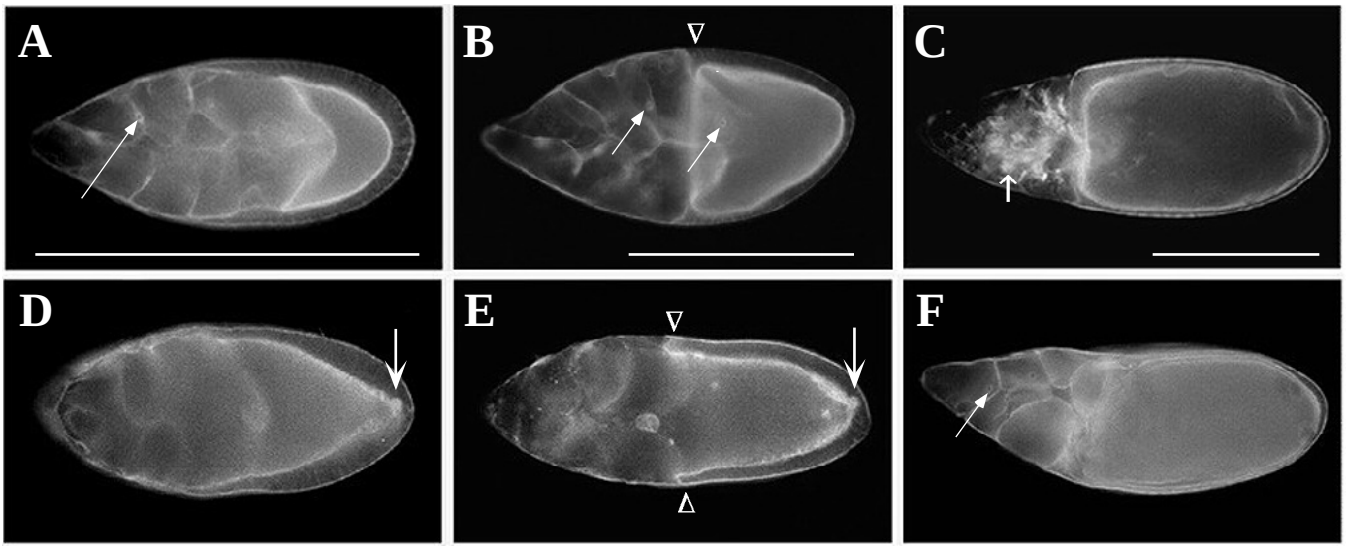

Fig. 6. Effect of reduced *dpp* receptor levels on F-actin formation in egg chambers. F-actin was visualized with rhodamine-phalloidin. All panels are optical cross sections of egg chambers and dorsal is up in B, C and F. Magnification bar in A also refers to D, the bar in B refers to E and the bar in C refers to F. (A-C) Morphologically wild-type egg chambers from *P{hs-tsax}* females without heat-shock. (A) Stage 9: F-actin is localized at the ring canals (closed white arrow; also in B and F) and subcortically in the nurse cells, oocyte, and follicle cells. (B) Stage 10A: F-actin remains subcortical. The dorsal anterior follicular epithelium has thickened (triangle) and the future centripetally migrating follicle cells have extended slightly between the nurse cells and oocyte. (C) Stage 11: subcortical F-actin remains while new cytoplasmic bundles have formed in the nurse cells near the ring canals and at the nurse cell-oocyte boundary. (D-F) Abnormal egg chambers from heat-shocked *P{hs-tsax}* females. (D) Stage 9: F-actin deposits in the posterior region appear irregular and the posterior pole follicle cells are unusually thin (open vertical arrow). (E) Stage 10A: the anterior edge of the follicular epithelium overlying the oocyte (triangles) has failed to thicken prior to centripetal migration, (compare with B). The nurse cells have deviated from wild-type morphology. (F) Stage 11: new cytoplasmic F-actin arrays have failed to form in the nurse cells and at the nurse cell-oocyte boundary (compare with C).

to complete the transfer of their contents to the oocyte, producing short eggs similar to those observed in this study (Fig. 3B, Cooley et al., 1992; Fig. 4D,G in this study).

We examined the effects of disrupted TGF-β signaling upon F-actin distribution in egg chambers from females carrying the inducible dominant negative *sax* receptor, *P{hs-tsax}*. Mutant stage 9 and 10A chambers display normal subcortical F-actin localization but exhibit morphologically abnormal nurse cells (Fig. 6D,E, compare with Fig. 6A,B) and irregular actin deposits at the posterior pole. Some stage 10A mutant chambers display atypical follicular epithelium over the oocyte, of which the most anterior cells (triangles, Fig. 6E) appear to fail to undergo centripetal migration (compare with wild type, Fig. 6B). Several late stage chambers from *P{hs-tsax}* heat-shocked females entirely lack nurse cell cytoplasmic F-actin arrays (Fig. 6F). We also observed chambers in which the oocyte cytoplasm included ring canals and nurse cell nuclei (data not shown). These results suggest that disrupted TGF-β signaling directly or indirectly causes abnormalities in F-actin organization in the nurse cell-oocyte complex.

SAX receptor function is required in the germline

sax is expressed in both the germline and the somatic follicle cells, and the receptor mutant phenotypes that we observe may be due to defects emanating from either tissue. To begin to understand the contribution to the *sax* mutant phenotypes, we examined individuals lacking *sax* activity only in the germline. Germline clones homozygous for loss-of-function *sax* mutations were produced by the FLP-FRT system (Chou and Perrimon, 1992; Chou et al., 1993) through induced mitotic recombination in developing females *trans*-heterozygous for a *sax* mutation and a germline-autonomous dominant female sterile mutation, ovo^{D1} (Fig. 7A). Germline clones were generated with a control sax^{+} chromosome and with two different loss-of-function *sax* alleles.

Females bearing *sax* mutant clones produced 10-25% as many eggs and over a much briefer time period than sax^{+} control clones (Fig. 7B). *sax* mutant clones only produced eggs in the first 6 to 9 days of the experiment, while sax^{+} clones continued to produce eggs for up to 40 days (Fig. 7B). Most of the eggs from the *sax* mutant clones were morphologically normal, with a minority (~20%) displaying aberrant dorsal appendages and short lengths. The residual eggs produced by *sax* mutant clones may result from perdurance of the sax^{+} gene product. Clones were induced at a time when *sax* is highly transcribed and when early pupal egg chambers (stages 3 to 5) have formed (King, 1970). The failure of continued egg production confirms that removal of SAX signaling from the germline of ovaries ultimately blocks oogenesis.

A parallel set of sax^{5} germline clones was generated and ovaries of clone-bearing females were examined as their egg production waned on day 5. The $ovo^{+}sax^{5}$ homozygous egg chambers could be identified because they lacked the dominant ovo^{D1} phenotype. These $ovo^{+}sax^{5}$ clones displayed degenerating mid-stage chambers (similar to those in Fig. 5B,C). In addition, these clones also contained occasional ‘cup’-like eggs, in which the anterior end of the egg remains open (Fig. 7C). Mutations in several genes produce such ‘cup’ eggs

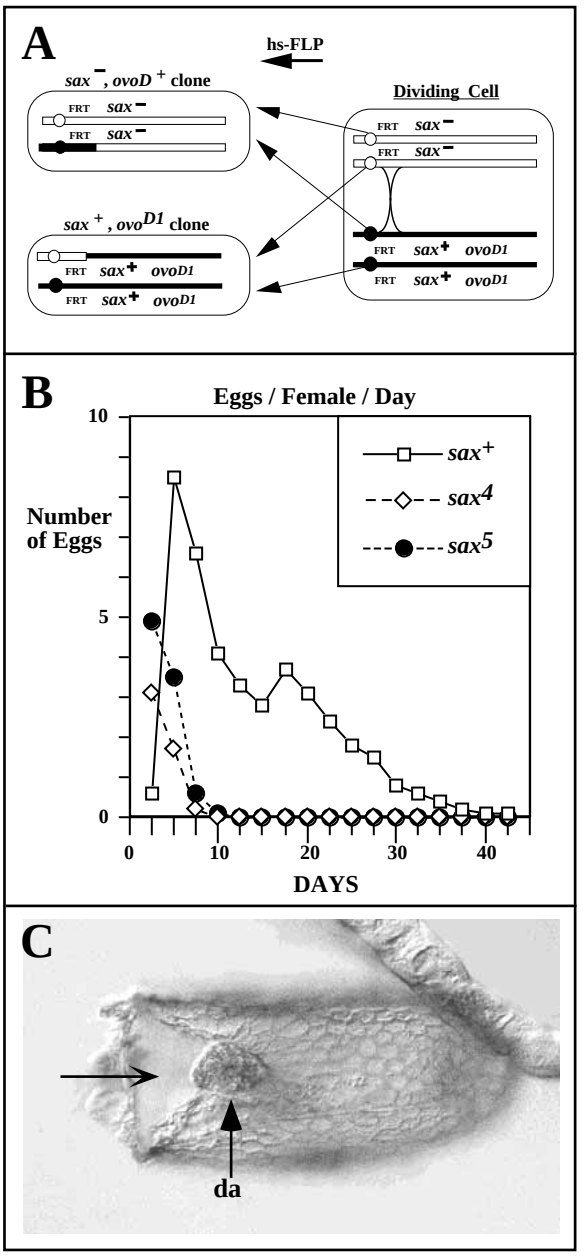

Fig. 7. *saxophone* germline mosaics produced by mitotic recombination. Clones of germline cells homozygous for a loss-of-function lethal *sax* mutation were generated utilizing the FLP-FRT system in conjunction with the germline-specific dominant female sterile mutation, ovo^{D1} (Chou and Perrimon 1992). (A) The heat shock promoted expression of FLP recombinase induces recombination at FRT sites in a dividing germline cell. Appropriate chromosomal segregation yields a daughter cell simultaneously sax^{-} and ovo^{+}. Such cells can contribute to the germline. (B) Adult females bearing *sax* mutant germline clones were mated and monitored for egg production. The average daily egg production per female is plotted for a sax^{+} control and sax^{4} and sax^{5} germline clones. Greater than 50 females were examined for each genotype. (C) A dorsal view of a 'cup' shaped egg produced by a female bearing sax^{5} germline clone. A deposit of dorsal appendage material (da) lies at the edge of the open anterior end (horizontal arrow).

because of a failure of CMFC migration (Schüpbach and Wieschaus, 1991).

grk and *sax* function control *dpp* expression in the posterior follicular epithelium

The product of the *gurken* (*grk*) gene, which encodes a TGF-α-like secreted protein (Neuman-Silberberg and Schüpbach, 1993), induces the posterior follicle cell fate of the egg chamber (Gonzalez-Reyes et al, 1995; Roth et al., 1995). In wild type, the GRK signal emanating from the germinal vesicle (the immature oocyte nucleus) induces the posterior follicle cells to adopt their posterior fate. In the absence of this signal, these cells retain their default anterior fate and thus can produce a second micropyle at the posterior pole. We examined the possibility that this anterior fate decision proceeded via mis-activation of *dpp* at the posterior pole. dpp^{10638} *lacZ* expression in the posterior follicle cells was assayed in chambers derived from *grk* mutant females. Ectopic expression of *dpp* occurs at the posterior pole of 77% of the *grk* mutant egg chambers examined from stage 9 to 14 (n=97; Fig. 8A,B) compared with 0.3% of control *grk* / + chambers (n=370; data not shown). *dpp* expression is indeed a more reliable marker of the *grk* mutant posterior to anterior fate change than the presence of an ectopic micropyle (data not shown). Suprisingly, *sax* mutant egg chambers exhibit similar posterior dpp^{10638} expression, albeit in far fewer cells but never form a second micropyle (Fig. 8C,D). Posterior *dpp* expression occurs in 13% of these stage 12 to 14 egg chambers (n=89). This suggests that SAX receptor function is required in the posterior follicle cells for the acquisition of at least some posterior fate.

DISCUSSION

Our results clearly demonstrate that one or more TGF-β signaling pathways are necessary for proper oogenesis in *Drosophila*. DPP signaling, most likely originating in the anterior somatic follicle cells, is essential during oogenesis for the determination of anterior eggshell structures. The SAX receptor is required for the formation of the anterior eggshell, egg chamber integrity and transfer of nurse cell contents to the oocyte. These conclusions are based on several observations. The *dpp* expression domain includes the most anterior centripetally migrating follicle cells, which are thought to be responsible for forming anterior eggshell structures (Spradling, 1993). Increases or decreases in DPP levels correlate with the presence of anterior eggshell defects. Females bearing mutations in the *sax* gene, or doubly heterozygous for *sax* and *tkv* mutations, produce short eggs with anterior eggshell defects. Ovaries bearing *sax* receptor mutations display defects in egg chamber integrity, nurse cell cytoplasmic transfer and cytoplasmic F-actin filament formation. Severe reductions of germline *sax* function results in a failure to complete oogenesis. Finally, *sax* mutations show a partial transformation of posterior toward anterior follicle cell fate. Some, but not all, of the mutant phenotypes just described can be rationalized as due to defects in a single signalling pathway.

TGF-β ligand/receptor interactions during oogenesis

Two issues complicate the interpretation of the genetic analysis

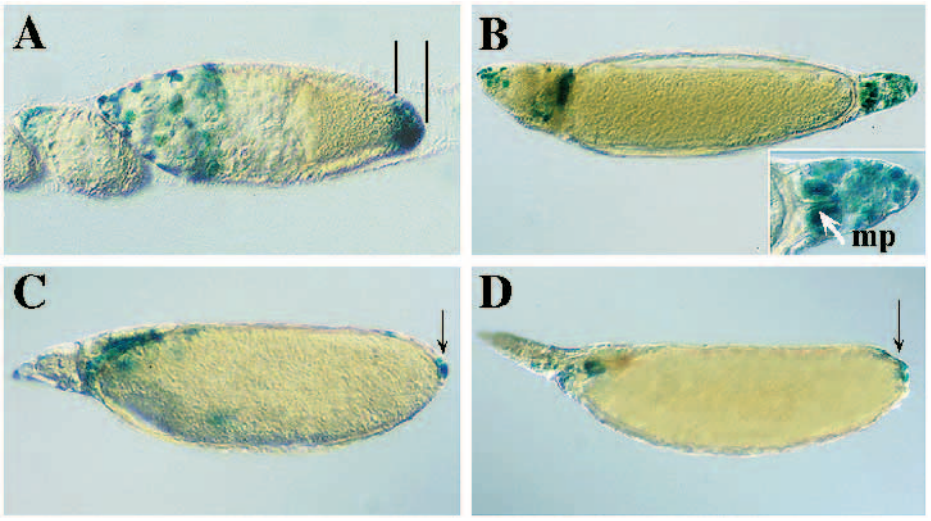

Fig. 8. *grk* and *sax* mutants ectopically express *dpp* in posterior follicle cells. (A,B) dpp^{10638} grk^{3} / $dpp^{+}grk^{3}$ egg chambers. (A) A stage 9 chamber exhibits ectopic posterior *lacZ* follicle cell expression (marked by vertical bars). (B) A late stage chamber with an ectopic posterior micropyle (mp, inset) encircled by *dpp* expression. (C,D) *P{hs-sax} / + ;* $dpp^{10638}sax^{1rv1}$ / $dpp^{+}Df(2L)sax\text{-}H9$ egg chambers. (C) A late stage chamber with several posterior follicle cells ectopically expressing *dpp* (arrow). (D) A stage 14 egg chamber with ectopic posterior expression of *dpp* (arrow).

that we describe. One is that each of the conditional genotypes used to modulate ligand or receptor levels might lead to different residual levels of protein product and, hence, different levels of signal transduction in target cells. The other is that *Drosophila* has at least three TGF-β ligands, encoded by the *dpp*, *60A* and *scw* genes (Padgett et al., 1987; Wharton et al., 1991; Doctor et al., 1992; Arora et al., 1994), and at least four TGF-β receptor subunits, encoded by the *sax*, *tkv*, *Atr-I* and *punt* genes (Childs et al., 1993; Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Wrana et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). Within the TGF-β superfamily, ligands can either be homo- or heterodimeric, and active receptor complexes are heteromeric. All four receptors are expressed in the germline, while *sax* and *punt* are also expressed ubiquitously in the soma of ovaries (Childs et al., 1993; Brummel et al., 1994; Wrana et al., 1994). Evidence exists that *tkv* function is required for oogenesis (Terracol and Lengyel, 1994) and our preliminary studies suggest that the same is true of the *punt* receptor (unpublished observations). Thus, many combinations of signals and receptor complexes might contribute to the oogenesis events defective in our several mutant genotypes. These two issues are not completely separable from one another. For example, some of our mutant receptor genotypes might act not only by reducing the levels of the mutant receptor subunit but, in addition, by 'poisoning' other receptor subunits as well. Indeed, our *sax* phenotypes are more severe than those previously reported for a putative *sax* null allele, sax^{P} (Nellen et al., 1994). Either the *sax* mutations that we have used in this report are more severe than the true *sax* null state represented by sax^{P}, perhaps because they poison the activity of another receptor subunit or, alternatively, the sax^{P} allele is actually a leaky mutation with some residual SAX receptor activity. We cannot critically rule out either possibility. However, the observations that sax^{4} is a nonsense mutation that should produce a very short extracellular protein fragment (B. Malnic and W. M. G., unpublished data) and that sax^{4} and sax^{5} are indistiguishable from deficiencies of *sax* in limited genetic assays lead us to favor the latter alternative.

While these caveats make it difficult to delineate the exact contribution of DPP signaling per se, we can still draw several reasonable conclusions. It is quite possible that the differences in the severity and frequency of anterior chorion defects in the *dpp* and receptor mutant genotypes are largely quantitative. Only one conditional *dpp* genotype exists and its conditional phenotype is due to the temperature sensitivity of dpp^{hr56} (Wharton et al., 1996). From its zygotic phenotypes, dpp^{hr56} clearly has residual activity under restrictive conditions. We know less about the residual activities in our receptor mutant genotypes, but it is quite possible that some of them, under continual maintenance at restrictive temperatures, lead to severely reduced *sax* activity in developing egg chambers. We can extend this argument to the defective egg chambers observed in certain receptor mutant genotypes. It is possible that the residual levels of *dpp* activity in our *dpp* mutant genotype are sufficient for egg chambers to mature, whereas in many of the receptor genotypes, *sax* activity is too low for normal maturation. Consistent with this possibility, in preliminary experiments in which unmarked dpp^{-} null follicle cell clones were generated, we have observed degenerating egg chambers and the absence of mature eggs (unpublished observations). This suggests that a severe reduction in *dpp* levels can produce phenotypes quite similar to the more severe *sax* phenotypes. Thus, we can consider many of the progressively more severe oogenesis defects as manifestations of a hypomorphic series of mutations reducing the activity of a DPP signaling pathway. A modest reduction in the activity of the DPP pathway causes defective anterior eggshell structures. Further reduction results in a failure of complete cytoplasmic transfer from the nurse cells to the oocyte, producing short eggs. Severe reduction, or possibly complete inactivation of the pathway, causes a failure to complete oogenesis due to mid-stage egg chamber degradation. In support of this model, the location of *dpp* expression in the CMFC is consistent with a role in movement of the CMFC to close off the anterior end of the eggshell, which is defective in some *sax* mutant chambers.

The mild phenotype of our one available *dpp* conditional genotype has not allowed us to compare the effects of signaling and receptor defects in the regulation of transfer of the nurse cell contents to the oocyte. Pronounced defects in transfer occur in some of our receptor mutant genotypes and these correlate with defects in the cytoplasmic F-actin arrays

required for this event (Gutzeit, 1986). We can only note that the expression pattern of *dpp*, essentially enwrapping the nurse cell complex in the anterior half of the mid-stage egg chamber, is consistent with a role for *dpp* in initiating the receptor activation necessary for normal cytoplasmic transfer from nurse cells to oocyte. The observations that BMP2, a functional DPP homolog, binds the SAX receptor in COS cell assays (Brummel et al., 1994) and that *dpp* and *sax* mutants share several developmental defects (Brummel et al., 1994; Nellen et al., 1994; Xie et al., 1994), are also consistent with this notion.

Some phenotypes that we observed suggest that other TGF-β signaling pathways play roles during oogenesis. One relates to the phenotype elicited by ubiquitous expression of *dpp* by heat shock induction of the *P{hs-dpp}* transgenes. Profound changes in anterior follicle cell structures and in the expression of at least one downstream marker of dorsal anterior follicle cells (*shortsighted*: L. Dobens et al., unpublished data) might well reflect the ectopic activation of receptors that are ordinarily activated by another TGF-β ligand. The second observation is even more difficult to rationalize in terms of the site of expression of *dpp* during oogenesis. Certain receptor mutant genotypes lead to mis-expression of the dpp^{10638} enhancer trap at the posterior pole of the embryo, as well as a morphologically mutant follicular epithelium in the posterior oocyte. These changes are suggestive of a partial transformation of posterior follicle cell fate toward anterior. It is not apparent how the anteriorly expressed *dpp* follicle cell signal could mediate these phenotypes. It is much more likely that another TGF-β ligand activates the SAX receptor in this posterior domain. Indeed, the 60A gene is expressed in these cells (K. Wharton, personal communication) and hence is a candidate to encode this ligand. Only the further analysis of somatic follicle cell and germline clones lacking various elements of the Drosophila TGF-β pathway will resolve the roles played by these signaling molecules.

We thank Fotis Kafatos, Len Dobens, Joe Duffy, Tien Hsu and Doug Harrison for local expertise and discussions. Excellent technical support was provided by Diane Duplissa and Genya Kraytsberg. We would like to express our gratitude for the extraordinary efforts and years of friendship with our 'Drosophila curator' Lorraine Lukas. For discussions, information and materials, we thank Celeste Berge, Laurel Raftery, Lynn Cooley, Ruth Lehmann, Daniel St. Johnston, Mike O'Connor, Norbert Perrimon, Matt Singer, Allan Spradling and Kristi Wharton. This work was supported by NSF grants (DCB 90-18618 and MCB 93-17701) to R. K. B., NIH grants to W. M. G. and a Council for Tobacco Research grant to R. W. P.; J. M. G. is a HHMI Physician Research Fellow; V. T. was a National Research Service Award genetics trainee.

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(Accepted 5 February 1996)

Note added in proof

During the proof stage it was brought to our attention that mutations in the nonmuscle myosin gene, *sqh*, cause phenotypes which are strikingly similar to DPP signaling mutant phenotypes. See Wheatley et al., *Development* **121**, 1937-1946.