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Cloning and Characterization of *peter pan*, a Novel *Drosophila* Gene Required for Larval Growth

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We identified a new *Drosophila* gene, *peter pan* (*ppan*), in a screen for larval growth–defective mutants. *ppan* mutant larvae do not grow and show minimal DNA replication but can survive until well after their heterozygotic siblings have pupariated. We cloned the *ppan* gene by P-element plasmid rescue. *ppan* belongs to a highly conserved gene family that includes *Saccharomyces cerevisiae* SSF1 and SSF2, as well as *Schizosaccharomyces pombe*, *Arabidopsis*, *Caenorhabditis elegans*, mouse, and human homologues. Deletion of both SSF1 and SSF2 in yeast is lethal, and depletion of the gene products causes cell division arrest. Mosaic analysis of *ppan* mutant clones in *Drosophila* imaginal disks and ovaries demonstrates that *ppan* is cell autonomous and required for normal mitotic growth but is not absolutely required for general biosynthesis or DNA replication. Overexpression of the wild-type gene causes cell death and disrupts the normal development of adult structures. The *ppan* gene family appears to have an essential and evolutionarily conserved role in cell growth.

INTRODUCTION

The developmental program of cell proliferation in *Drosophila* has been well characterized (Foe *et al.*, 1993; Edgar and Lehner, 1996). By the end of embryogenesis, cell proliferation has ceased, and most cells in the embryo are arrested in the G_1 phase of the cell cycle (Hartenstein and Campos-Ortega, 1985). These cells remain quiescent through the first 8–12 h of larval development, until the first G_1–S transitions begin in response to feeding (Britton and Edgar, 1998). Two different kinds of cell cycles are present in larval tissues; endoreplication and mitotic cycles. Endoreplication occurs in most larval tissues and is responsible for larval growth, whereas adult progenitor cells in the nervous system and the primordial tissues called imaginal discs undergo mitotic replication. The G_1 arrest in early larvae may be like the G_0 seen in serum-deprived tissue culture cells and can be maintained by allowing the larvae to hatch on media containing an energy source (sucrose) but lacking amino acids. Under these conditions the animal supports virtually no DNA replication or growth (Britton and Edgar, 1998). Exit from the G_1 arrest of the first larval instar is an event in *Drosophila* development that provides a unique opportunity to study the relationships among nutrition, growth, and the cell cycle.

We took advantage of this opportunity by performing a genetic screen for larval growth–defective (LGD) mutants. We hypothesized that LGD mutants would be good candidates to have defects in growth or initiation of DNA replication. This hypothesis was based on the observation that first instar larvae blocked in G_1 with DNA replication inhibitors remain small because they cannot undergo the DNA endoreplication required for most of *Drosophila* larval growth (Edgar, unpublished observation). Here we describe the characterization and cloning of the mutant *peter pan* (*ppan*), identified by its phenotype of first instar growth arrest and failure to undergo larval DNA replication. Our studies of the *ppan* mutant allow us to confirm some of the screen's original assumptions and to define a conserved novel gene family required for normal growth and DNA replication in *Drosophila* larvae.

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MATERIALS AND METHODS

Fly Stocks

l(3)6B6 was obtained from Y.N. Jan, and l(3)02231 was obtained from the Bloomington Stock Center. UAS-*ppan*, CASPR-ppan.xba I,

CASPR-ppan.eco RI, and CASPR-ppan.pst I lines were generated by P-element–mediated transformation. UAS-p35 was a gift from B. Hay, and en-GAL4 was a gift from A. Brand. M(3)95A^{Plac92} (Andersson *et al.*, 1994) and MS 1096-GAL4 (Capdevila and Guerrero, 1994) have been previously described.

UAS and Casper Transgenes

The CASPR-ppan.xba I, CASPR-ppan.eco RI, and CASPR-ppan.pst I lines were generated by ligation of *Xba*I, *Eco*RI, and *Pst*I genomic fragments into the *Xba*I, *Eco*RI, and *Pst*I sites of the vector CASPR. The UAS-*ppan* construct was generated by excision of the ppan cDNA from pNB using *Sal*I and *Xba*I. This fragment was subcloned into the vector pSL1180 (Pharmacia, Piscataway, NJ) and re-excised with *Eco*RI, and the resulting fragment was ligated into the *Eco*RI site in the polylinker of pUAST.

Yeast Strains

The yeast strain H50–16C (*Mat***a** *leu2-3112, trp1-1, ura3-1, ade2-1, his3-11,15, can1-100, ssf1-1::HIS3, ssf2::TRP1 [pGAL-SSF1.14, LEU2]*) was transformed to ura$^-$ with a yeast expression vector containing the *ppan* cDNA.

Histology

Larvae were fed 5-bromodeoxyuridine (BrdU) at a concentration of 100 μg/ml, dissected, and fixed in 6% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 1 h at room temperature. Immunohistochemistry was performed using standard techniques (Patel, 1994). Incorporated BrdU was detected using anti-BrdU (1:100) 1° antibody (Becton Dickinson, San Jose, CA) and HRP-coupled goat anti-mouse (1:350) 2° antibody (Jackson ImmunoResearch, West Grove, PA). Discs were also fixed in 6% paraformaldehyde and washed in PBS and 0.1% Tween 20 (Sigma, St. Louis, MO). Clonal markers were visualized using anti-myc (1:50) 1° antibody (Oncogene Science, Uniondale, NY) and preabsorbed goat anti-mouse FITC-conjugated (1:500) antibody (Jackson ImmunoResearch). Cell nuclei and cell outlines were visualized by incubation of fixed discs in DAPI (0.05 mg/ml in PBS) and rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing, discs were mounted in Fluoroguard antifade reagent (Bio-Rad, Hercules, CA). Ovaries were dissected from mature females and fixed in 6% paraformaldehyde in PBS. Tissues were washed three times with PBS and 0.1% Tween 20 and permeabilized by 1-h room temperature incubation in PBS and 1% Triton X-100 (Sigma). Permeabilized ovaries were then incubated with DAPI and rhodamine-conjugated phalloidin as described above, washed, and mounted.

Clonal Analysis in Discs and Ovaries

Clonal analysis was performed as described by Xu and Rubin (1993). *ppan* clones in imaginal discs were induced by heat shock induction of *flp* recombinase in *hsFLP; FRT* π*myc ppan*^{6B6}*/FRT* larvae at 48, 72, and 96 h after egg deposition (AED). Clones were scored at 120 h AED in wandering third instar larvae. For the *Minute* experiments, clones were induced in *hsFLP; FRT* π*myc ppan*^{6B6}*/FRT M(3)95A*^{Plac92} larvae and scored at 168 h AED. Mutant clones in discs were identified by antibody staining for π*myc. ppan* mutant clones were generated in the germ line using an *flp*/FRT *ovoD* system as described by Chou *et al.* (1993). Ovaries were dissected, permeabilized, and stained with DAPI and rhodamine-conjugated phalloidin as described above.

Ectopic Overexpression

Ectopic overexpression of *ppan* in wing imaginal discs was achieved using the GAL4 expression system (Brand and Perrimon, 1993). The *engrailed* GAL4 driver was used to overexpress *ppan* in the posterior compartment of the discs, and the MS1096 GAL4 driver (Capdevila and Guerrero, 1994) was used to preferentially overexpress *ppan* on the dorsal side of the disc.

P-Element Reversion

Reversion of the P-element presumptively conferring the *ppan* phenotype was accomplished by crossing *w; P[w*$^+$ *l(3)6B6]/TM3 Sb* flies with *w;* Δ*2-3 Sb Ly/TM6 Ubx e* flies carrying the Δ2-3 stable transposase source (Robertson *et al.*, 1988). Dysgenic males of the genotype *w/Y; P[w*$^+$ *l(3)6B6]/*Δ*2-3 Sb Ly* were then mated to *w; P[w*$^+$ *l(3)6B6]/TM3 Sb* females. *w; Sb*$^+$ *Ly*$^+$ revertants were scored for viability to the adult stage, and several hundred were recovered. Twenty-one lethal excision mutants were also isolated from *w; P[revertant]/TM3 Sb* progeny of the above cross.

Molecular Biology

Plasmid rescue was performed on the *P[w*$^+$ *l(3)6B6]* allele and a fragment corresponding to that rescued from *P[w*$^+$ *l(3)2231]* was subcloned. This fragment was already mapped to a P1 genomic clone. Using the rescued fragment from the *P[w*$^+$ *l(3)6B6]* as a probe, we isolated *Eco*RI and *Xba*I genomic clones (see Figure 3A). The *Eco*RI fragment is completely included in the *Xba*I fragment. Subfragments of these were in turn used to probe a plasmid-based cDNA library (Brown and Kafatos, 1988). Although four cDNAs were found to span the *Xba*I fragment, in both alleles, the P-element appears to be inserted into the 5′ end of the coding sequence of just one of those cDNAs (see Figure 3B).

Three different genomic fragments from around the *ppan* locus (Figure 3A) were transformed into flies to confirm that disruption of the *ppan* cDNA results in the observed larval growth arrest phenotype. These fragments are 1) the 7-kb *Xba*I fragment, which includes *ppan*, another complete cDNA, and fragments of two other cDNAs; 2) the 4-kb *Eco*RI fragment, which includes *ppan* and parts of two other cDNAs; and 3) a 2-kb *Pst*I fragment, which includes only *ppan* and a small piece of the 3′ end of an adjacent cDNA.

RESULTS

ppan Is a Larval Growth Arrest Mutant

The initial *ppan* allele, *P[w*$^+$ *l(3)6B6]*, was identified in a visual screen of 352 P-element lethal mutants (courtesy of Y.N. Jan), based on its striking growth arrest phenotype. *ppan* is a recessive P-element mutant with biphasic lethality. Forty-five percent of the homozygotes die in late embryogenesis with no obvious morphological defects and a normal cuticle (our unpublished results). Fifty-five percent of homozygous larvae hatch and live for 4–8 d but remain very small (Figure 1A). These mutant larvae move and feed normally, and no morphological defects were noted. Mutant larvae were weighed at time points after hatching, and we found that their growth closely resembled that of sucrose-fed wild-type larvae, which remain arrested in the first instar without protein accumulation (Figure 1B). Twenty percent of the *ppan* larvae that hatch survive for at least 8 d, 3 d after their heterozygous siblings pupariate (Figure 1C). Thus, the *ppan* mutant is specifically deficient in growth, but this deficiency is not immediately lethal.

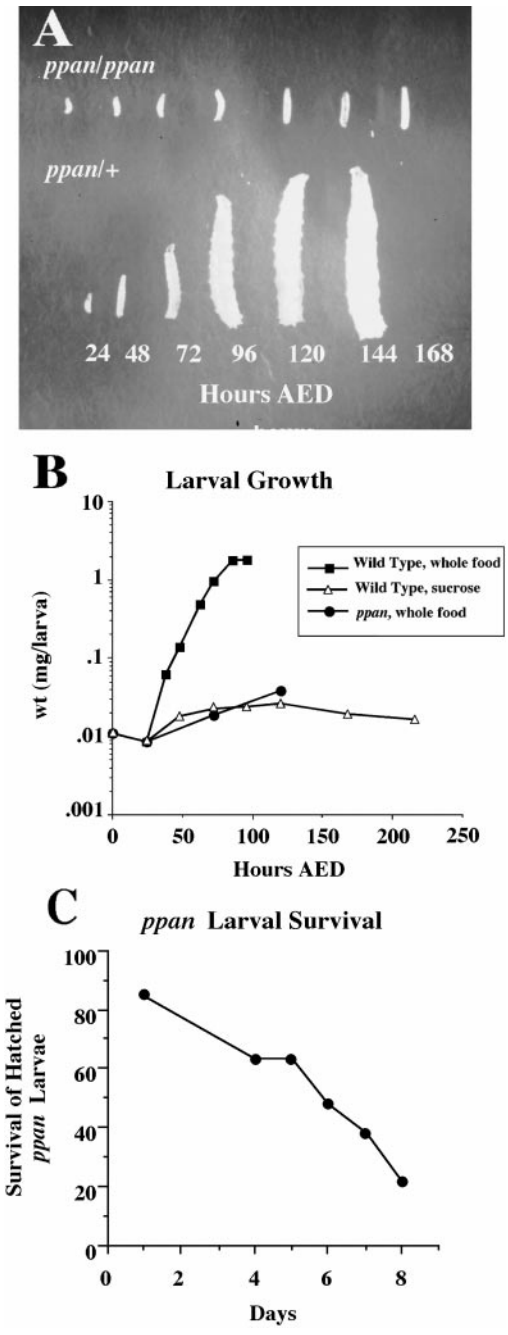

Figure 1. Larval growth arrest phenotype and survival of *ppan* mutant larvae. (A) Heterozygous and homozygous *ppan* larvae at 24, 48, 72, 96, 120, 144, and 168 h AED. (B) Larval growth curve for normally fed wild-type (black squares), sucrose-fed wild-type (open triangles), and normally fed homozygous *ppan* (black circles) larvae. Groups of ≥20 larvae were weighed at various times after hatching, and an average weight per larva was determined. (C) Survival of hatched *ppan* larvae Mutant *ppan* larvae were counted for several days after hatching.

ppan Is Required for Endoreplication and Normal Mitotic Proliferation in the Whole Animal

To determine whether the growth defect was accompanied by defects in larval DNA replication, analysis of the incorporation of the nucleotide analogue BrdU was performed on heterozygous and homozygous *ppan* larvae at 24-h intervals after hatching (Figure 2). At 0–24 h after hatching, the homozygous mutants showed greatly reduced BrdU labeling compared with heterozygous siblings. Between 24 and 48 h after hatching, there was no more DNA replication in the homozygotes except in the mushroom body neuroblasts in the brain, which continue proliferation even in nutrient-deprived larvae (Britton and Edgar, 1998). Thus, in the mutant *ppan*, there is little or no DNA replication in either endoreplicating tissues such as the gut or in mitotically proliferating tissues such as the brain and larval histoblasts. The presence of DNA replication in the mushroom body neuroblasts indicates that ppan is not essential for DNA replication per se.

Isolation of the ppan Gene

Having established *ppan* to be an interesting mutant with defects in larval growth and DNA replication, we set about to identify the gene defective in the mutant. The P-element lethal allele *P[w$^+$ l(3)6B6]* was the first identified, and a second allele, *P[w$^+$ l(3)2231]*, was found in the *Drosophila* Genome Project database. Both *P[w$^+$ l(3)6B6]* and *P[w$^+$ l(3)2231]* were mapped by the *Drosophila* Genome Project to the right arm of the third chromosome (93B10-11) using the *P[w$^+$ l(3)2231]* allele. The *P[w$^+$ l(3)6B6]* allele is the subject of most of the work described in this paper, but the phenotype of the *P[w$^+$ l(3)2231]* allele is indistinguishable from that of *P[w$^+$ l(3)6B6]*. To be sure that the P-element insertion is responsible for the LGD phenotype and the lethality, the *P[w$^+$ l(3)6B6]* was mobilized. In doing so, we reverted the phenotype and the lethality, confirming that there is no mutation elsewhere on the chromosome that caused the observed growth defects.

We used the plasmid rescue technique to isolate the gene defective in the mutant *ppan* (Cooley *et al*., 1988). Plasmid rescue was performed on the *P[w$^+$ l(3)6B6]* allele, and a rescued fragment was used to isolate genomic DNA fragments (Figure 3A), which were in turn used to probe a plasmid-based cDNA library. Four cDNAs were found to span the largest genomic fragment (an *Xba*I fragment), but in both alleles, the P-element is inserted into the 5′ end of the coding sequence of just one of those cDNAs (Figure 3B). As these P-elements are inserted in the putative coding region of the *ppan* gene, it is likely that these mutants are null for function.

To confirm that disruption of the *ppan* cDNA is in fact responsible for the larval growth arrest pheno-

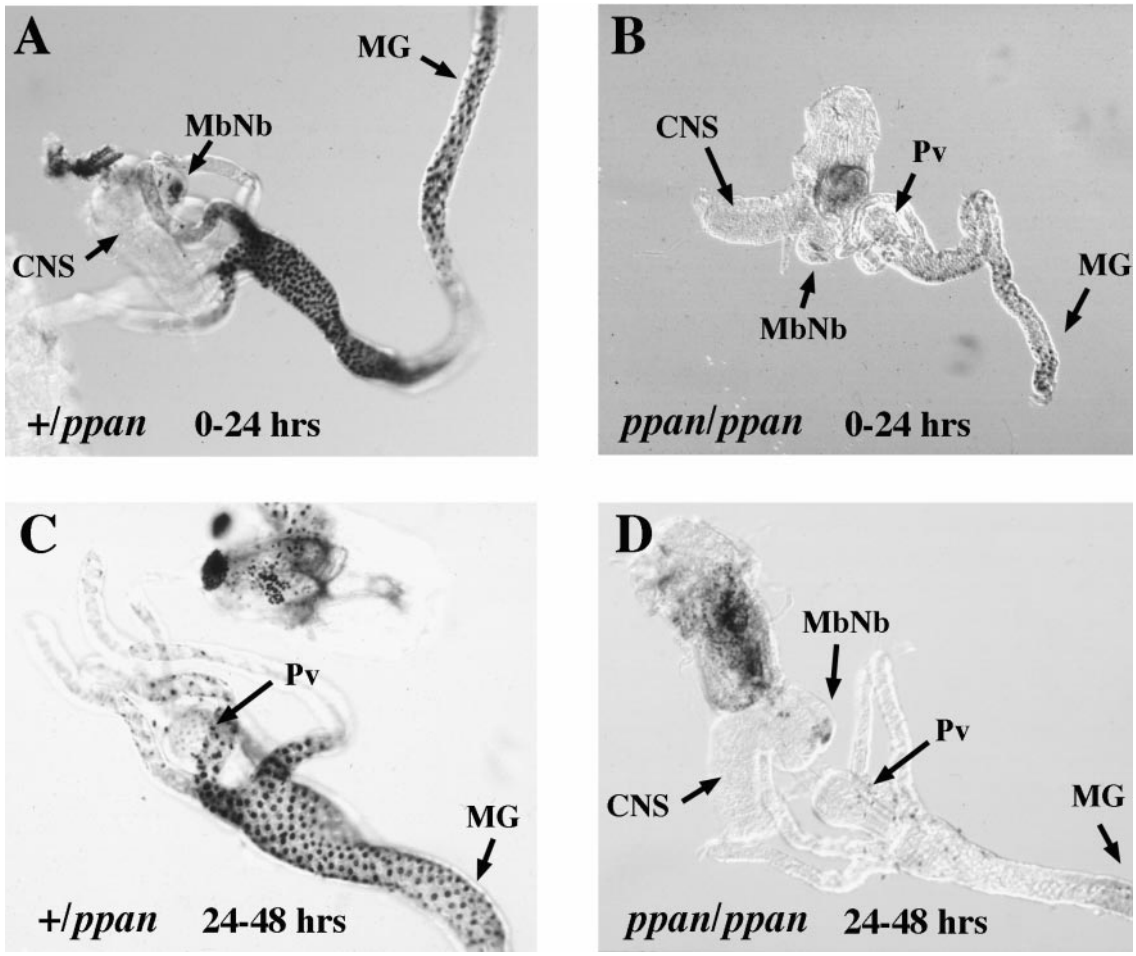

Figure 2. *ppan* mutants show decreased DNA replication. *ppan* heterozygotes (A and C) and homozygotes (B and D) were fed the nucleotide analogue BrdU (100 μg/ml) during the periods 0–24 h (A and B) and 24–48 h (C and D). Internal organs were dissected and histochemically stained to detect incorporated BrdU (black). MG, midgut; Pv, proventriculus; MbNb, mushroom body neuroblasts.

type, flies were transformed with three different genomic fragments from the *ppan* locus (Figure 3A). A single copy of any of these constructs was able to rescue homozygous *ppan* mutants. The smallest fragment (a 2-kb *Pst*I fragment) includes only *ppan* and a small piece of the 3′ end of an adjacent cDNA. Surprisingly, this fragment contains only 175 bp of DNA 5′ to the putative initiating methionine that are not a part of the 3′ region of the adjacent cDNA. Thus it appears that a transcriptional regulatory region sufficient for function is very small and compact. The presence of such a small regulatory region suggests that *ppan* transcription is not subject to complex regulation.

ppan Expression

Only 55% of homozygous *ppan* larvae hatch. The homozygotes that die as embryos appear normal. This indicates that the maternally derived *ppan* gene product is sufficient to support most or all of embryonic development. Examination of *ppan* expression by Northern blot indicated that ppan mRNA is loaded into the oocyte and that mRNA levels decrease by ~50% during embryogenesis. RNA in situ hybridization performed on embryos showed that ppan mRNA is ubiquitously expressed and that RNA levels are highest in the early embryo. This ubiquitous distribution is consistent with the small regulatory region we have observed.

ppan Is Similar to the SSF1 and SSF2 Genes in Saccharomyces cerevisiae

Sequencing of the *ppan* cDNA identified it as a *Drosophila* homologue of the yeast genes *SSF1* and *SSF2* (Figure 4). *ppan* shares 28% amino acid identity with the yeast genes and 35% amino acid identity with the

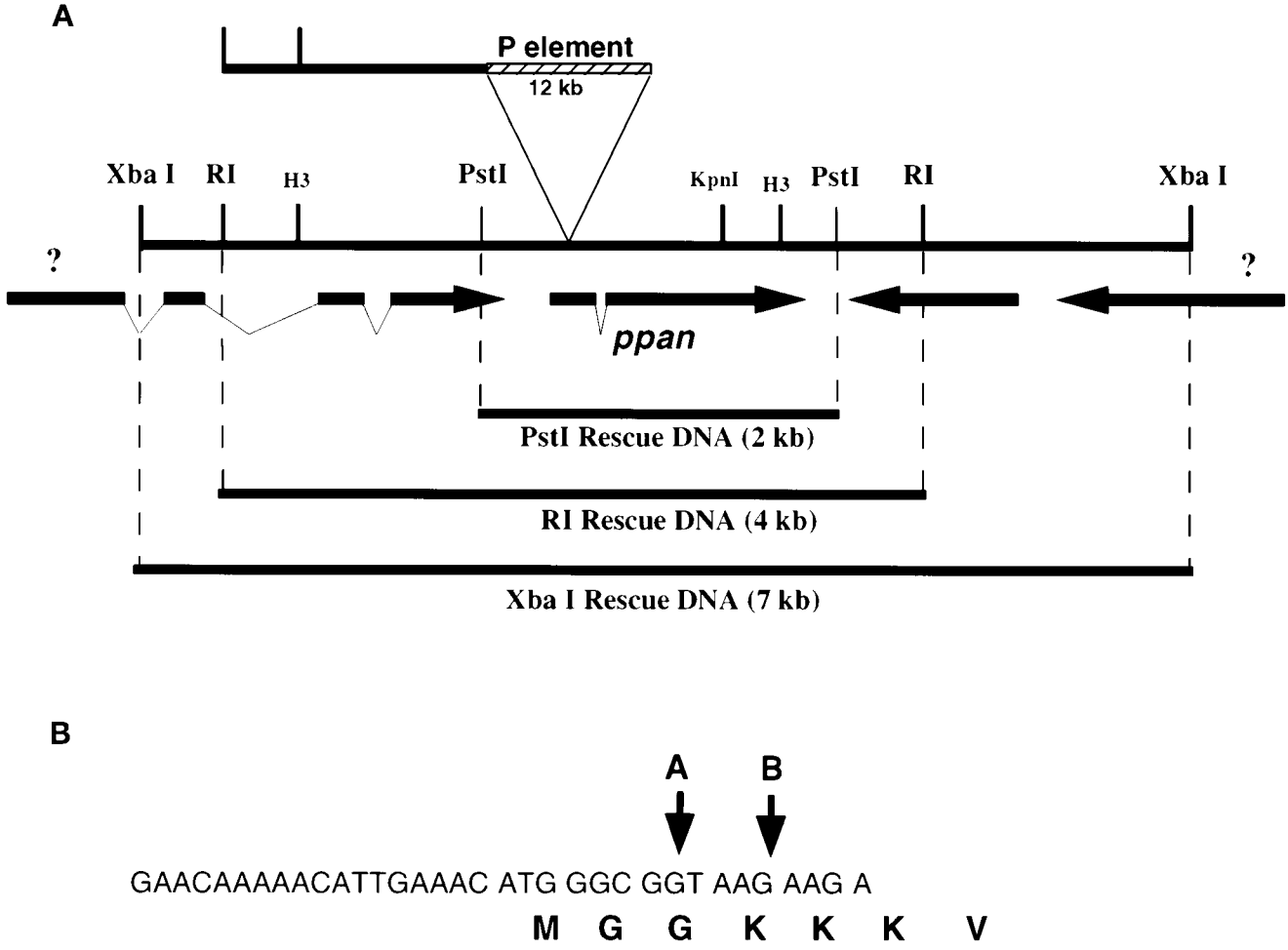

Figure 3. *ppan* genomic region and P-element location. (A) Map of genomic region showing the location of the P-element insertion (top), restriction sites, cDNAs (arrows), and DNA fragments used for rescue of the *ppan* mutant (below). (B) Nucleotide map of P-element insertion sites as determined by sequencing of genomic DNA recovered by plasmid rescue. P-element insertions sites just inside the *ppan* ORF for *P[w$^+$ l(3) 6B6]* (A) and *P[w$^+$ l(3) 02231]* (B) are indicated by the arrows.

Caenorhabditis elegans homologue. *SSF1* and *SSF2* were identified in a screen for multicopy suppressors of a yeast mating pathway defect. The mating pathway defect was a temperature-sensitive mutation in the G_β subunit of the heterotrimeric G-protein that couples the mating factor receptor to its downstream effects on cell cycle and cell morphology. Overexpression of *SSF1* increased the mating efficiency of the mutant strain at the restrictive temperature. A second gene, *SSF2*, was found to share 94% amino acid identity and functional interchangeability with *SSF1*. Deletion of both yeast genes is lethal, and depletion of the gene products causes cell division arrest (Yu and Hirsch, 1995). SSF1 and SSF2 are believed to be localized in the nucleus (Kim and Hirsch, 1998). Analysis of *ppan* sequence using the sequence analysis program PSORT (Nakai and Kanehisa, 1992; Horton and Nakai, 1997) suggests that ppan has a 0.987 probability of being nuclearly localized. Murine, human, *Arabidopsis*, *C. elegans*, and *Schizosaccharomyces pombe* homologues have also been identified (Figure 4). Thus, these genes are likely to have an evolutionarily conserved role in growth and cell cycle progression.

Expression of the Drosophila Homologue in SSF1, SSF2 Mutant Yeast Can Suppress the Yeast Growth Defect

Given that the *ppan* gene is conserved across species, we examined whether the molecular function is also conserved by testing whether expression of *ppan* could rescue loss of *SSF1* and *SSF2* in *S. cerevisiae*. We used a strain of yeast in which *SSF1* and *SSF2* were deleted and *SSF1* had been added back under the control of galactose promoter (Yu and Hirsch, 1995). Cells were depleted of the *SSF1* gene products by switching the

Figure 4. Comparison of amino acid sequences of *ppan* with putative homologues from other species. Homologues were first aligned with the program Clustal-W and then hand aligned. The names and sources of the sequences is as follows: DM, *D. melanogaster* ppan, sequencing of both strands of a cDNA; CE, *C. Elegans* cDNA, GenBank accession number AF043700; SP, *S. Pombe* cDNA, GenBank number Z98531; SSF1, *S. cerevisiae* cDNA, GenBank number P38789; SSF2, *S. cerevisiae* cDNA, GenBank number Q12153; MM, *Mus musculus*, compilation of expressed sequence tag fragments AA451276, AA475332, AA068339, AA237717, AA517621, AA270523, AA756790, AA028364, AA575760, AA239726, AA561626, and AA265569; HS, *Homo sapiens*, compilation of expressed sequence tag fragments N34073, N40373, AI147481, AI1084732, AA321112, AA300789, and AA258103; AT, *A. thaliana.*

yeast from media containing galactose to media containing dextrose. In the dextrose-containing media, the cells arrested after 12 h. This strain was transformed with either a *ura*-marked plasmid containing the *ppan* cDNA under the transcriptional control of the GAPDH promoter or the same plasmid without the

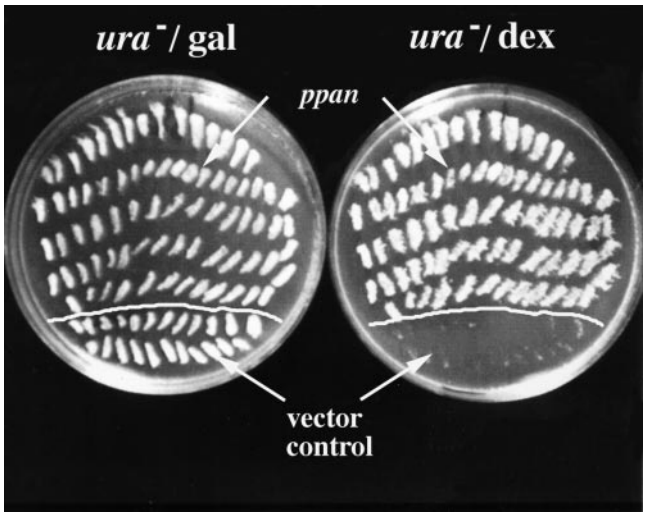

Figure 5. The *ppan* gene complements *ssf1* and *ssf2* function. The yeast strain H50[hyphem]16C is deficient for *ssf1* and *ssf2* but has the ssf1 gene added back on a plasmid under the control of a galactose promoter. Thus these cells can only grow in media containing galactose. These cells were transformed to *ura*$^-$ galactose plates with either the empty pAB23BXN plasmid or the same plasmid with *ppan* subcloned into it under the transcriptional control of the strong constitutive GAPDH promoter. The streaked colonies from both the control and *ppan* transformations were replated onto *ura*$^-$ dextrose plates. The *ppan* expressing colonies (pAB23BXN-ppan) were able to grow, whereas the control colonies (pAB23BXN) were not.

cDNA as a control. *ura*$^+$ colonies from both transformations were replated onto *ura*$^-$ dextrose plates (Figure 5). Only the colonies transformed with the *ppan* cDNA were able to grow; the control plasmid transformed colonies were not. Thus, the *ppan* gene complements *SSF1* and *SSF2* function.

ppan Mutant Cells Have a Growth Defect

Although the lack of growth and DNA endoreplication in *ppan* mutant larvae demonstrates that *ppan* is required for these processes in the whole animal, it does not address whether the *ppan* gene product is required cell autonomously. BrdU-labeling experiments showed no DNA replication in the mitotically replicating brain of *ppan* mutant larvae, except in the mushroom body neuroblasts, which continue to divide just as in amino acid–deprived larvae. However, in wild-type larvae growth of the endoreplicating larval tissues is required to start replication in the mitotic tissues such as the brain (Britton and Edgar, 1998). Therefore, the lack of BrdU labeling in homozygous *ppan* larval brains may be due to the absence of growth and endoreplication in other tissues.

To characterize the proliferative capability of *ppan* mutant cells, clonal analysis was performed in imaginal discs using the FLP/FRT mitotic recombination system (Xu and Rubin, 1993). This technique allowed us to generate clones of homozygous *ppan* mutant cells in a *ppan* heterozygous background. We induced mutant clones at various developmental stages and then scored the marked clones in imaginal discs from wandering stage larvae (120 h AED) and in adult eyes (Table 1). We also inspected adult flies carrying clones of mutant cells for appendage and cuticle abnormalities. The fate of *ppan* mutant cells depended on when the clones were induced (Table 1). When clones were induced early in disc development (24–48 h AED) we were unable to detect homozygous *ppan* mutant clones, even though their adjacent twin spots (homozygous wild-type sister clones) were present. When clones were induced late in disc development (110–114 h AED), we saw small *ppan* clones with larger twin spots. Close inspection of the anterior wing margin revealed occasional defects in some chemo- and mechanosensory bristles. These defects included deletion and disruption of these bristles. Thus, homozygous *ppan* clones induced early are eliminated by "cell competition" (Simpson, 1979),

Table 1. Mosaic analysis: *ppan* mutant cells have a growth defect

HS (h AED)	Genotype	*ppan* clone survival (L3 discs)	Morphology/clone survival (adults)
96	*FRT ppan/FRT*	+++	Normal/many small clones
72	*FRT ppan/FRT*	+	Normal/many small clones
48	*FRT ppan/FRT*	−	Normal/no surviving clones
36	*FRT ppan/FRT*	−	Normal/no surviving clones
144	*FRT ppan/FRT Minute*	+++	Rough eyes, wing and cuticle defects/ND
120	*FRT ppan/FRT Minute*	+++	Rough eyes, wing and cuticle defects/ND
96	*FRT ppan/FRT Minute*	++ (>30 cells)	Pupal lethal/ND
72	*FRT ppan/FRT Minute*	++ (>50 cells)	Pupal lethal/ND

Data summary for studies of *ppan* clones allowed to grow for various times after heat shock (HS) induction of *flp* recombinase (see MATERIALS AND METHODS). For analysis in L3 discs, larvae were fixed at 120 h AED (wild-type background) and at 168 h AED (*Minute* background). Mutant clones in discs were identified using the *πmyc* marker and in adult eyes by pigmentation derived from the w^+ marker in $P[w^+\ ppan^{6B6}]$. +++, mutant clones equivalent in size to wild-type sister clones ("twin spots"); ++ and +, mutant clones smaller than twin spots; −, no detectable mutant clones; ND, clone survival was not determined.

whereas late induced clones can survive but grow slowly and often differentiate defectively in the adult. These results indicate that *ppan*-deficient cells have a cell-autonomous growth disadvantage and a compromised ability to produce differentiated structures.

To further study the growth and proliferation properties of *ppan* mutant imaginal cells, we induced clones in the genetic background of heterozygous *Minute l(3)95A* larvae. *Minute* mutants share the phenotype of dominant growth delay and recessive cell lethality. *M l(3)95A* is mutant in ribosomal protein rpS3 (Andersson *et al.*, 1994). Although homozygous *ppan* clones induced during early disc development (24–48 h AED) in a wild-type background were outcompeted and eliminated, clones induced at the equivalent stage in a slow-growing *Minute* background were easily detected and reached significant size (>50 cells). Nevertheless, wild-type clones induced at the same time were much larger and sometimes filled a complete compartment (Garcia-Bellido *et al.*, 1973; Simpson, 1979). This clearly demonstrates that *ppan* mutant cells grow much more slowly than wild-type cells. Without the competition of the wild-type cells, homozygous *ppan* cells can continue to divide and are not eliminated but grow more slowly than wild-type cells. We also found that early induction of *ppan* clones in the *Minute* background resulted in pupal lethality. Thus, the presence of large clones of *ppan* cells severely disrupts pupal development.

Three major conclusions may be drawn from these experiments. First, *ppan* is not absolutely required for mitotic proliferation. Second, although growth and mitotic proliferation do proceed in *ppan* mutant cells, these processes are slow, and the cells have a growth disadvantage. Finally, clones of *ppan* mutant cells that do survive through larval life result in either disruptions of adult structures such as mechano- and chemosensory bristles or in pupal lethality. Thus, although still able to grow and proliferate at a reduced rate, *ppan* mutant cells are deficient in a specific cellular function essential for differentiation.

ppan Is Required for Oogenesis

We thought we might gain further insight about the molecular function of *ppan* by examining the phenotypes of *ppan* mutant cells in the female germ line. *ppan* mutant clones were generated in the germ line using an FLP/FRT *ovoD* system (Chou *et al.*, 1993). *ppan* mutant clones in the ovary were never (0%) able to produce viable eggs. However, upon examination of the ovaries in which the mutant *ppan* clones were generated, we found that the *ppan* mutant oocytes progressed further than either the homozygous or heterozygous *ovoD* oocytes, sometimes even allowing the deposition of malformed, deflated eggs (Figure 6, A–C). Interestingly, *ppan* mutant oocytes often had incorrect numbers of nurse cells. Wild-type oocytes typically have 15 nurse cells (Figure 6, A and B), whereas *ppan* mutant oocytes exhibit a variety of nurse cell numbers ranging from 0 to >30 (Figure 6, E and F). This phenotype is indicative of a defect early in oogenesis when cysts pinch off from the germarium. However, judging from the size of the mutant cysts and the nuclei within, germ line proliferation, DNA endoreplication, and overall growth were not completely blocked.

ppan Overexpression Studies

Another approach to understanding the molecular function of *ppan* is to examine the effects of *ppan* overexpression. Using the GAL4 expression system (Brand and Perrimon, 1993), *ppan* was overexpressed in wing imaginal discs. Overexpression of *ppan* in the *engrailed* pattern in the posterior compartment of the wing imaginal disc caused the posterior part of the wing to take on a crinkled appearance and disrupted normal wing vein formation. The crinkled appearance was accompanied by increased opacity (Figure 7B). The disruption of wing vein formation consisted of ectopic wing vein formation, thickening of wing veins, and loss of wing veins.

We also overexpressed *ppan* using the MS1096 GAL4 driver (Capdevila and Guerrero, 1994), which expresses *GAL4* strongly on the dorsal side of the wing imaginal disc and weakly on the ventral side. Flies having one copy of this *GAL4* driver and one copy of UAS-*ppan* construct had wings that were slightly curved upward (our unpublished results). Closer examination of the trichomes on these wings showed that there were fewer trichomes on the dorsal side of the wing than on the ventral side. In some spots on the dorsal side there were multiple fused trichomes (Figure 7E). Although it is possible that each trichome comes from individual smaller cells, the even spacing suggests the multiple fused trichomes originate from single cells. Normally each trichome derives from an individual cell, and thus we infer that overexpressing ppan with the MS1096 GAL4 driver reduced the number of cells on the dorsal side of the wing. This may account for the curved wing phenotype. Increasing the number of copies of either the MS1096 GAL4 driver or the UAS-*ppan* transgene ultimately resulted in the near complete elimination of the wing (Figure 7C).

To further study the effects of *ppan* overexpression, we examined cell death in imaginal discs overexpressing *ppan*. Acridine orange staining of apoptotic cells showed increased numbers of apoptotic cells in regions of the imaginal disc overexpressing *ppan* using *en*-GAL4 (Figure 7*G*). Thus, overexpression of *ppan* causes cell death, which in turn may be responsible for the observed phenotypes in the wings. In fact, even the most severe *ppan* overexpression wing phenotype

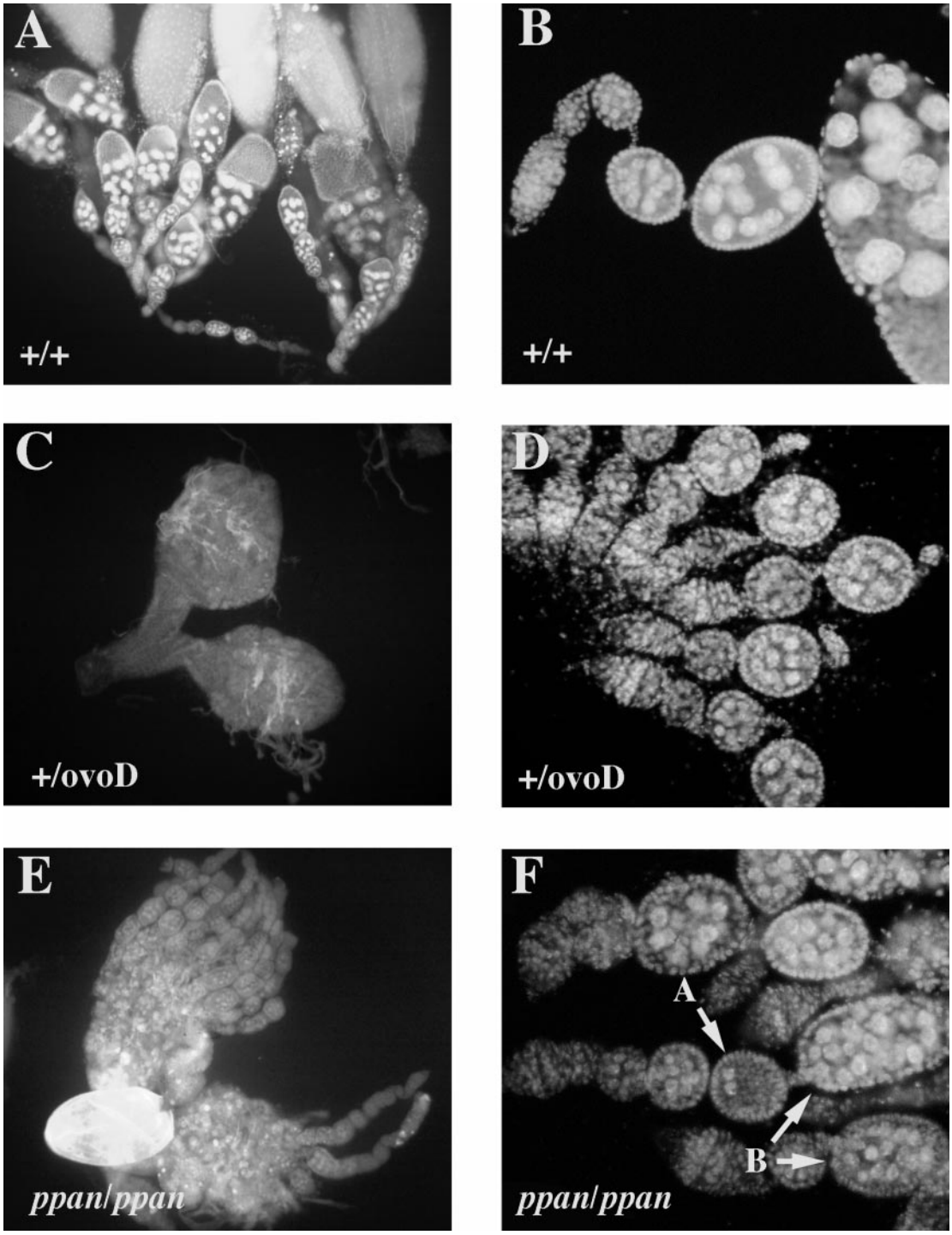

Figure 6. Germ line mosaic analysis. *ppan* is required to produce a viable oocyte. DAPI staining of the nuclei is shown in wild-type ovaries (A; magnification, 10×), wild-type ovarioles (B; 20×), *ovoD*/+ ovaries (C; 10×), *ovoD*/+ ovarioles (D; 20×), *ppan*/*ppan* ovaries (E; 10×), and *ppan*/*ppan* ovarioles (F; 20×). Arrows in F indicate cysts that deviate from the normal number of nurse cells (15).

can be rescued by coexpression of the baculovirus P35 protein (Figure 7*D*), which blocks cell death by inhibition of Caspases (Hay *et al.*, 1994). Clearly we can conclude that imaginal disc cells are sensitive to levels of *ppan* and that increased expression of *ppan* decreases cell viability.

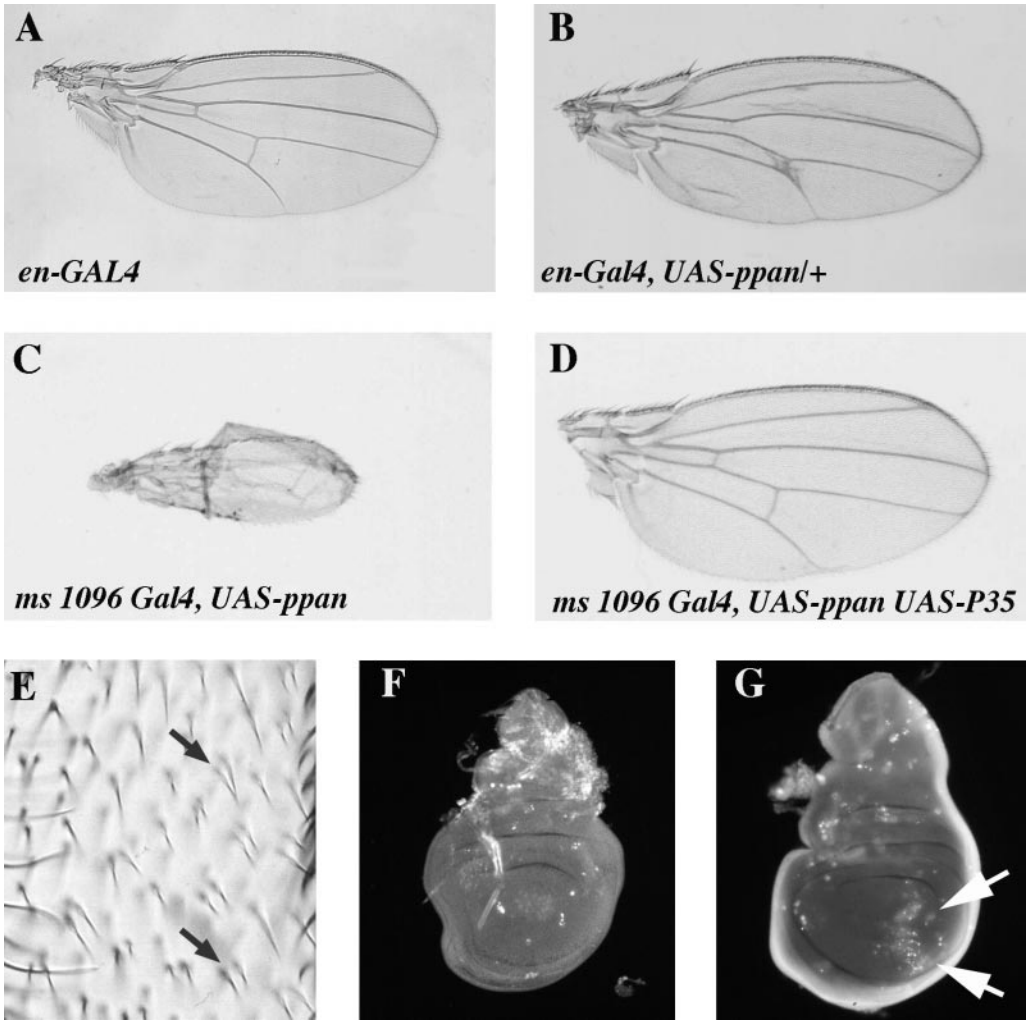

Figure 7. Ectopic overexpression of *ppan* causes cell death and disrupts wing development. Results of *ppan* overexpression in the posterior compartment of the imaginal wing disc using the *engrailed* GAL4 driver: (A) *en*-GAL4; (B) *en*-GAL4, UAS-*ppan*. Results of *ppan* overexpression in the imaginal wing disc using the ms 1096-GAL4 driver, which expresses GAL4 preferentially in the dorsal portion of the disc: (C) ms 1096 GAL4, UAS-*ppan*; (D) ms 1096 GAL4, UAS-*ppan,* UAS-P35. (E) Closeup of irregular and duplicated wing trichomes in ms 1096-GAL4, UAS-*ppan*/+ flies. Visualization of cell death in imaginal discs using acridine orange: (F) wild-type; (G) *en*-GAL4, UAS-*ppan.* Arrows indicate abnormal cell death in the posterior wing compartment.

DISCUSSION

Here we describe the characterization and cloning of the LGD mutant *ppan*. Close examination of the phenotype of *ppan* mutant larvae and clones of *ppan* mutant cells revealed that *ppan* is an essential gene required for a variety of developmental and cellular functions. Recent work in yeast has provided some clues to the molecular function of *ppan*. From studies of the *ppan* homologues *SSF1* and *SSF2*, Kim and Hirsch (1998) propose that *SSF1* and *SSF2* are nuclear proteins that affect mating efficiency by altering mating projection formation. Mating projection formation in yeast was prevented by depletion of *SSF* gene products and increased by overexpression of *SSF1*. Both mating projection formation and bud formation during vegetative growth are forms of polarized cell growth that involve significant actin cytoskeletal reorganization (Leberer *et al.*, 1997). A role for SSF1 and SSF2 in a specialized form of cell growth rather than general growth is supported by the observation that yeast cells depleted of *SSF* gene products continue to increase in size but are unable to form mating projections in response to mating factor (Kim and Hirsch, 1998). Another laboratory found SSF1 to be a weak suppressor of a temperature-sensitive *tor2* mutant (Schmidt and Hall, personal communication). TOR2, a target of FKBP–rapamycin complexes and a putative phosphatidylinositol-3 kinase, functions both in nutri-

tion-stimulated activation of protein synthesis and also in cell cycle–dependent polarized distribution of the actin cytoskeleton (Helliwell *et al.*, 1998).

These studies in yeast prompted us to investigate whether *ppan*, like SSF1 and SSF2, might also function in polarized cell growth. The biology of *Drosophila* and the genetic techniques available allowed us to study the requirements for *ppan* in a variety of organismal and cellular processes. In the ovary, we found that *ppan* mutant clones exhibited early defects in cytoarchitecture and nurse cell segregation. One step in this process that may require polarized growth, or at least actin cytoskeleton rearrangements, is the pinching off of cysts from the germarium. Despite defects in this process, *ppan* mutant cysts grew in size, and the nurse cells appeared to undergo multiple rounds of endoreplication before finally degenerating. Thus the *ppan* phenotype in the fly ovary bears some similarity to that found in the *SSF* depletion experiments in yeast, in which the mutant yeast grew in size but failed to form mating projections. The cytoarchetectural defects in *ppan* mutant ovaries might be attributed to defects in polarized growth or defective regulation of the actin cytoskeleton.

In other situations, however, loss of *ppan* function did not result in phenotypes readily explained by defects in polarized cell growth. For instance, actin staining of *ppan* mutant cells in imaginal discs did not reveal the expected defects in cell morphology or polarization. The fate of *ppan* mutant cells in imaginal discs depended on when the mutant clones were induced and the genetic background in which the analysis was performed. Early induced mutant clones in a wild-type background were lost, whereas those induced late, or in a *Minute* background, grew significantly. Our interpretation of these results is that *ppan* is not absolutely required for mitotic proliferation but that its absence confers on the mutant cells a general growth delay that results in their elimination. Cell clones deficient in *ppan* behaved quite differently from those deficient in genes required for cell cycle progression, such as *string, CyclinE,* and *Cdc2*, which cease proliferation but exhibit continued cell growth (Neufeld *et al.*, 1998), or from those deficient in protein synthesis, such as *rpS3* (*l(3) M95A)*, which neither proliferate nor grow (Andersson *et al.*, 1994). In addition to these growth defects, we found that the survival of *ppan* clones into the pupa resulted in the disruption of adult structures or in lethality. Thus it seems that *ppan* is required for some aspects of cell differentiation as well as for cellular growth.

The requirement for *ppan* in the germ line seemed to follow a similar pattern. *ppan* mutant ovaries produce no viable eggs, and thus the gene is required for normal oogenesis. However, a careful analysis of the mutant phenotype revealed that growth and DNA replication were not completely arrested. These observations seem paradoxical in light of the phenotype of homozygous *ppan* larvae, which exhibit a total inability to grow and never initiate global DNA replication. To reconcile these cellular and organismal phenotypes, we suggest that in *ppan* mutant larvae defects in a specialized cellular function, perhaps in a specific larval tissue, may feed back on and block growth and DNA replication throughout the animal. In mutant imaginal disc cells and oocytes these defects do not appear to be so closely monitored. In summary, at the organismal level *ppan* is required for initiation of larval growth and DNA replication, progression of oogenesis, and maturation of some imaginal tissues into adult structures. At the cellular level *ppan* is not absolutely required for growth, mitosis, or DNA endoreplication, but its absence does confer a growth delay, and it is also required for some aspects of normal cell differentiation. Identification of *ppan* as a homologue of the yeast genes SSF1 and SSF2 provides a useful framework in which to study the molecular, cellular, and organismal role of this gene.

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