Targeted localized degradation of Paired protein in *Drosophila* development

L. Raj*, P. Vivekanand*, T.K. Das*, E. Badam*, M. Fernandes*, R.L. Finley Jr†, R. Brent‡, L.F. Appel*, S.D. Hanes§ and M. Weir*

Background: Selective spatial regulation of gene expression lies at the core of pattern formation in the embryo. In the fruit fly *Drosophila*, localized transcriptional regulation accounts for much of the embryonic pattern.

Results: We identified a gene, *partner of paired* (*ppa*), whose properties suggest that localized receptors for protein degradation are integrated into regulatory networks of transcription factors to ensure robust spatial regulation of gene expression. We found that the Ppa protein interacts with the Pax transcription factor Paired (Prd) and contains an F-box, a motif found in receptors for ubiquitin-mediated protein degradation. In normal development, Prd functions only in cells in which *ppa* mRNA expression has been repressed by another segmentation protein, Even-skipped (Eve). When *ppa* was expressed ectopically in these cells, Prd protein, but not mRNA, levels diminished. When *ppa* function was removed from cells that express *prd* mRNA, Prd protein levels increased.

Conclusions: Ppa co-ordinates Prd degradation and is important for expression of Prd to be correctly localized. In the presence of Ppa, Prd protein is targeted for degradation at sites where its mis-expression would disrupt development. In the absence of Ppa, Prd is longer-lived and regulates downstream target genes.

Addresses: *Department of Biology, Wesleyan University, Middletown, Connecticut 06459, USA. †Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, Michigan 48201, USA. ‡The Molecular Sciences Institute, 2168 Shattuck Avenue, Berkeley, California 94704, USA. §Molecular Genetics Program, Wadsworth Center, New York State Department of Health and Department of Biomedical Sciences, State University of New York-Albany, New York 12208, USA.

Correspondence: Michael Weir
E-mail: mweir@wesleyan.edu

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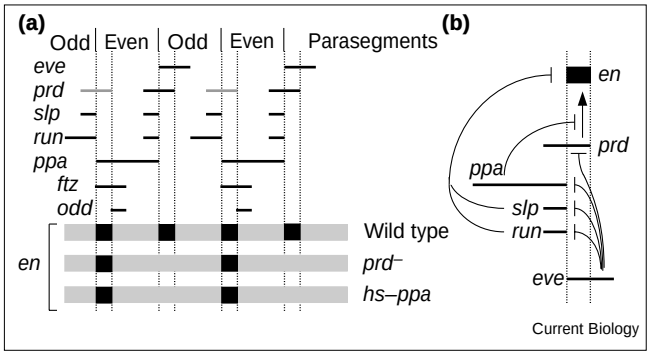
Background

In the early development of *Drosophila*, expression of the segmentation genes is gradually refined into smaller and smaller regions of the embryo, the gene products at each tier of spatial refinement working in various combinations to define progressively more refined patterns of gene expression. The transcription factor Paired (Prd) is a member of the pair-rule family of segmentation proteins and lies at an intermediate position in the hierarchy of gene products (coordinate → gap → pair-rule → segment polarity) that pattern the *Drosophila* embryo along the anteroposterior axis [1]. Working with other pair-rule proteins, it regulates the expression of several segment-polarity genes, including *engrailed* (*en*), which defines the posterior compartment of each segment (see Figure 1). Prd works with Even-skipped (Eve) to activate *en* expression in stripes that are 1–2 cells wide in the odd-numbered parasegments [2]. Prd functions as a transcriptional activator of *en*, whereas Eve represses three repressors of *en*, excluding them from the posterior-most cell row of Prd stripes, thereby allowing activation of *en* (Figure 1b). The genes encoding the three repressors are *sloppy-paired* (*slp*) [3,4], *runt* (*run*) [4–6] and *partner of paired* (*ppa*; this study). Activation of *en* stripes in the even-numbered parasegments is under separate control. In the even-numbered stripes, Fushi tarazu (Ftz) is responsible for activating *en* in cell rows that do not also express the Odd-skipped (Odd) repressor (Figure 1a) [2,6]. In this way, the combined activities of several pair-rule genes, each expressed in two-segmental patterns, together define the one-segmental pattern of *en*.

Figure 1



Spatial relationship between *en* stripes and its pair-rule regulators at the time of gastrulation. (a) The more intense primary *prd* stripes (full black stripes; compare with the stippled secondary stripes) overlap with the *en* stripes in the odd-numbered parasegments. The *en* stripes are absent in *prd*– embryos and weakened in embryos expressing a *ppa* transgene coupled to the *hsp70* promoter (*hs–ppa*). (b) The *en* stripes in the odd-numbered parasegments are activated by Prd. Eve activates these stripes indirectly by repressing *ppa*, *slp* and *run*.

Here, we report the identification of *ppa*, which encodes a receptor for degradation of Prd protein. We found that *ppa* expression is localized in stripes immediately adjacent to the Prd stripes, thereby downregulating any aberrant Prd expression in these cells. The *ppa* gene is repressed by *eve* in Prd-expressing cells, thus allowing Prd to activate its downstream genes.

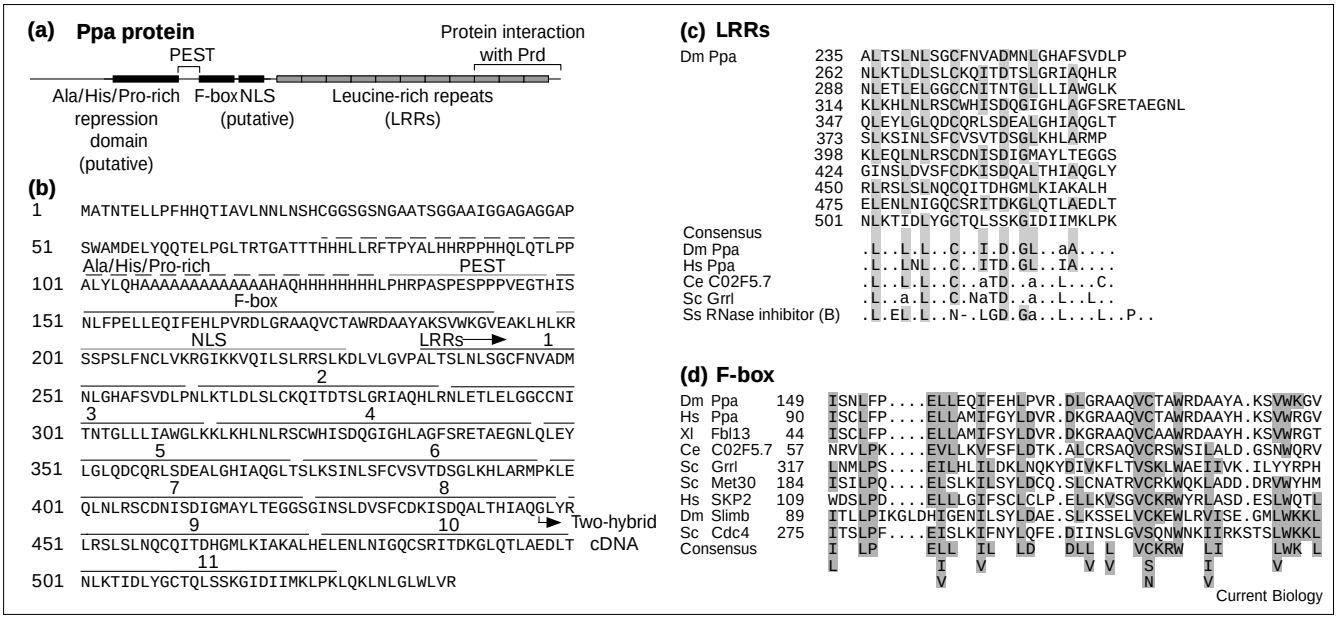
Results and discussion

Prd interacts with an F-box protein

To gain further insight into the combinatorial regulation by Prd and Eve, we performed a yeast two-hybrid screen of a cDNA library derived from 0–12 hour old embryos [7], using as bait a 140 amino-acid fragment of Prd that included its homeodomain. From a total of 2.4×10^6 primary transformants, we identified 22 classes of clones by restriction analysis; mRNA *in situ* hybridization analysis of representatives from each class indicated that one of the cDNAs from the screen is expressed in a pair-rule pattern of stripes (see below). We named this cDNA *ppa* (previously listed by us as *I-55* in FlyBase, http://flybase.bio.indiana.edu; and listed [8] as cDNA 5-1 in the vicinity of the *robo* gene). The other cDNAs from this screen will be described elsewhere. To test the specificity of the interaction of Ppa with Prd, the *ppa* cDNA was retransformed into yeast and tested using a mating assay [9] against a panel of different baits, including Prd. Ppa interacted with the original homeodomain-containing Prd fragment, or a fragment containing both the homeodomain and the Prd domain, but not with homeodomain-containing fragments of Ftz or Bicoid, or with several unrelated control baits (data not shown). In addition, Ppa did not interact with Prd containing a Ftz homeodomain substitution, suggesting that the Prd homeodomain sequences are required for the protein interaction.

Sequence analysis of *ppa* provided insights into its possible functions (Figure 2). The Ppa open reading frame (ORF) contains 11 leucine-rich repeats (LRRs). These 20–29 amino-acid motifs have Leu residues at characteristic positions as illustrated in Figure 2c, and have been implicated in protein–protein interactions [10]. Indeed, the carboxy-terminal 92 amino acids of Ppa, encompassing three LRRs (Figure 2a,b), was sufficient for the interaction

Figure 2



The Ppa protein sequence. (a) Schematic illustration and (b) sequence of the 531 amino-acid ORF of Ppa, which has 11 LRRs (numbered), an F-box, and a motif rich in Ala, His and Pro. LRRs generally mediate protein interactions [10] and the carboxy-terminal three LRRs of Ppa were sufficient for interaction with Prd in yeast two-hybrid assays. F-boxes are found in receptors for ubiquitin-mediated protein degradation [12]. The amino-terminal region rich in Ala, His and Pro is similar to repression domains found in transcriptional repressors [13–16]. Ppa also has a PEST sequence, suggesting that the protein is short lived, and a predicted nuclear localization sequence (NLS). The GenBank accession number for the *ppa* sequence is AF187980. (c) Alignments of LRR sequences. Like its closest homologs, yeast (Sc) Grr1, *C. elegans* (Ce) ORF CO2F5.7 and human (Hs) Ppa (genomic sequence, AC005182; sequence of cDNA 3′untranslated region, AF007128), *Drosophila* (Dm) Ppa is in a subfamily of LRR proteins with Cys at position 10 [10]. Crystal structure analysis of porcine ribonuclease inhibitor indicates that the protein has alternating A- and B-type LRRs (RNase Inhib(B)) which each form an α-helix and β-strand [10]. The LRR consensus sequence is based on collated sequences in [10]; 'a' indicates Ala, Val, Leu, Ile, Phe, Tyr or Met. (d) F-box sequence alignments. The F-box consensus is based on [12]. Conserved sequences are highlighted in gray in (c,d).

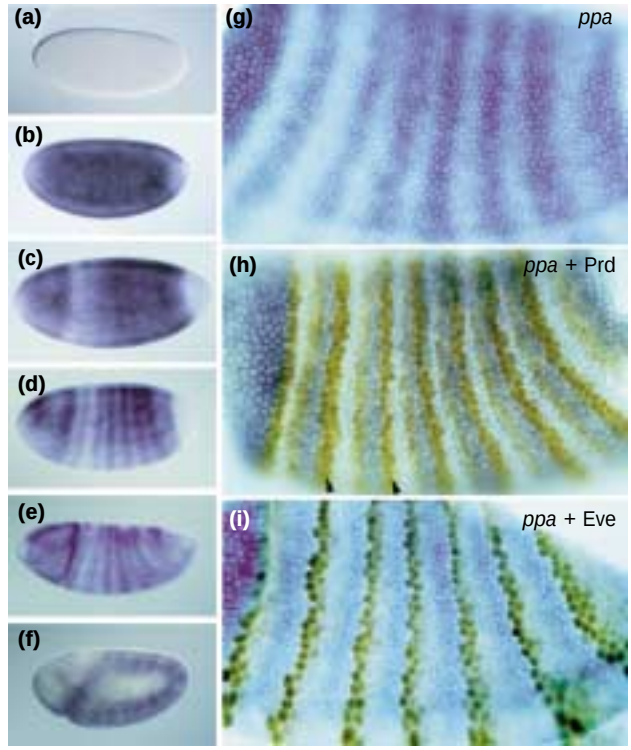
with Prd in our two-hybrid screen. Sequence alignments (Figure 2c) indicated that the LRRs in Ppa are similar to those found in yeast glucose repression regulator 1 (Grr1), *Caenorhabditis elegans* CO2F5.7, an ORF of unknown function, and a human hypothetical ORF that we have named Ppa because it is 63% identical (78% similar) to the carboxy-terminal 392 amino acids of the *Drosophila* protein.

Like GRR1 [11], CO2F5.7 and the human ORF, *Drosophila* Ppa also contains an F-box motif amino-terminal to the LRRs (Figure 2d). Previously characterized F-box proteins, including Grr1 and yeast Cdc4, have been shown to be receptors that target their substrates for ubiquitin-mediated protein degradation (reviewed in [12]). These proteins interact through their F-boxes with Skp1, which associates with Cdc53/Cullin, forming an SCF complex (Skp1/Cullin/F-box). The SCF complex functions as a ubiquitin ligase enzyme (E3), which facilitates transfer of ubiquitin from a ubiquitin conjugation enzyme (E2) to the substrate. The F-box proteins provide a vital link between this machinery and specific substrates to be degraded, the substrate interaction typically being mediated through WD40 or LRR protein-interaction motifs within the F-box protein. Thus, the F-box proteins provide for specificity of substrate choice. Unlike Grr1, CO2F5.7 or other described F-box proteins, Ppa also contains a region rich in Ala, His and Pro, which is similar to Ala-rich domains observed in previously identified transcriptional repressor proteins, including Kruppel, Knirps, Eve and En (Figure 2b) [13–16]. The presence of the F-box and Ala/His/Pro motifs suggests that Ppa might function as a receptor for protein degradation, or as a transcriptional co-repressor, or both.

The *ppa* gene is expressed in stripes

The *ppa* mRNA was not detected in unfertilized embryos (Figure 3a), suggesting that *ppa* is not expressed maternally. Uniform expression throughout the embryo was first detected at nuclear cycle 10 (data not shown), and gradually increased in intensity during cycles 11–14. Ppa expression diminished in the pole regions during cycle 13 (Figure 3b). During cycle 14 and early gastrulation, the expression of *ppa* transformed into a pair-rule striped pattern with the formation of interbands within which *ppa* expression was lost (Figure 3c–e). This was followed during germ-band elongation by splitting of the *ppa* stripes to generate a one-segment-repeated pattern of reiterated interbands (Figure 3f). The *ppa* stripes did not have sharp borders (Figure 3g). Expression of *ppa* was lost throughout the ventral region of the embryo, which contributes to the ventral furrow during gastrulation (data not shown), presumably as a result of dorsoventral regulators. The *ppa* mRNA was localized in the basal regions of cells, in contrast to the apical localization of most pair-rule gene mRNAs (data not shown).

Figure 3

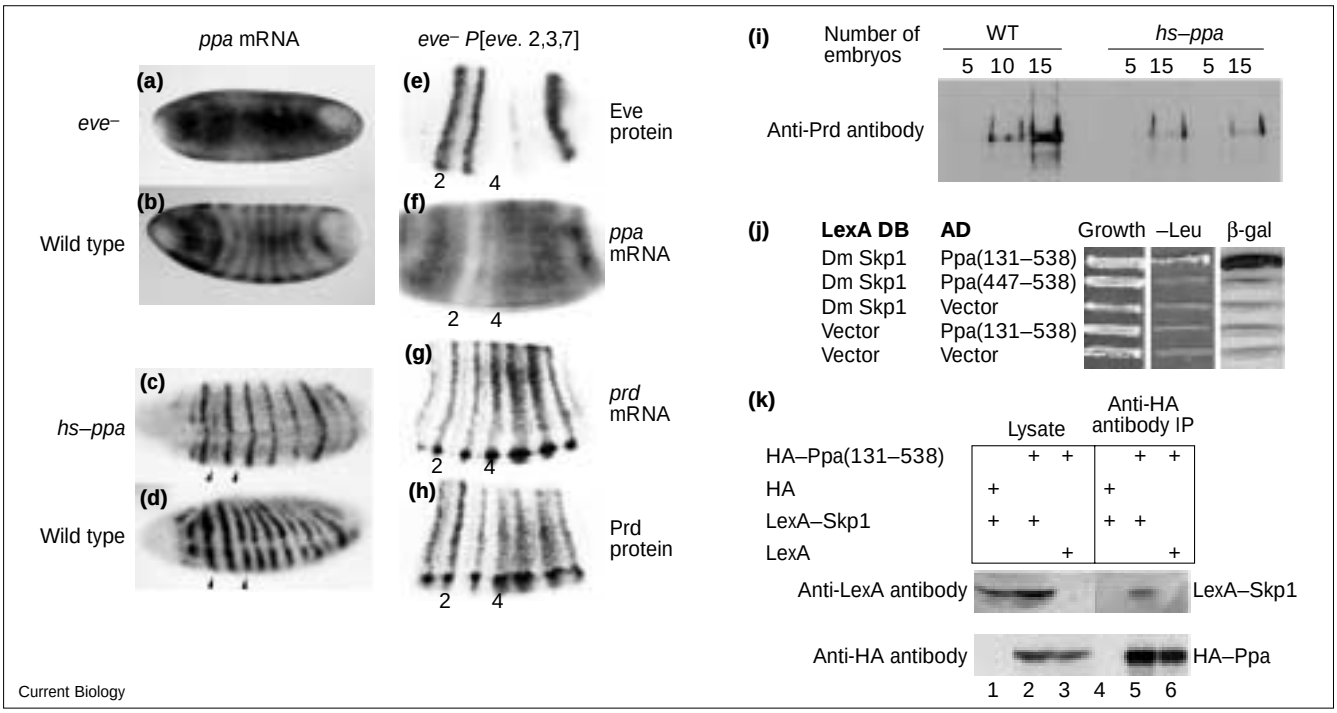


Expression of *ppa* mRNA in wild-type embryos. **(a–f)** The *ppa* mRNA was not detected in (a) unfertilized eggs, but expression progressed from (b) almost uniform to (c–f) striped expression. The embryonic stages are (b) cycle 13; (c) early cycle 14; (d) mid-cycle 14; (e) gastrula; and (f) germ-band elongation. **(g–i)** Fillets of recently gastrulated embryos showing (g) *ppa* mRNA alone, or double-stained with (h) Prd protein or (i) Eve protein. (h) The *ppa* mRNA has cleared from the posterior cells of the primary Prd stripes (arrowheads) at the time when Prd activates *en* transcription in these cells. (i) Eve protein expression coincides with *ppa* mRNA interbands during cycle 14. Note that, by gastrulation, Eve expression diminishes in the posterior regions of *ppa* interbands.

To assess the possible functional relationships with Prd and Eve, we double-stained embryo fillets for *ppa* mRNA and Prd or Eve protein (Figure 3h,i). During the early stages of cycle 14, when *ppa* expression was being restricted to stripes, there were significant levels of *ppa* expression overlapping the stripes of Prd protein (data not shown). As cycle 14 proceeded, the posterior regions of the forming *ppa* stripes overlapped transiently the anterior regions of the primary Prd stripes but, by early gastrulation, the Prd and *ppa* stripes were almost distinct (Figure 3h). This transient but limited overlap in the expression of *ppa* and Prd is consistent with the model that Ppa negatively regulates Prd protein function (see below).

Comparison of *ppa* mRNA with Eve protein showed almost reciprocal expression of the two genes (*ppa* interbands coincided with Eve stripes; Figure 3i), raising the possibility that

Figure 4



Functional analysis of *ppa*. (a,b) Spatial regulation of *ppa* mRNA. The *ppa* mRNA was expressed uniformly in (a) homozygous *eve*$^{1.27}$ mutant embryos, unlike (b) their heterozygous siblings (which are wild type in phenotype), suggesting that repression by *eve* stripes is required for *ppa* interband formation. In *eve*$^{1.27}$ mutants, *ppa* was not derepressed at the embryo pole regions, suggesting that it is under separate control at these sites. (c,d) Ectopic *ppa* represses *en* activation. (c) Heat-treated *hs–ppa* and (d) wild-type embryos were stained for *en* mRNA. Ectopic *ppa* caused partial or complete deletion of *prd*-dependent, odd-numbered *en* stripes (compare the regions indicated by the arrowheads). (e–h) Localized ectopic expression of *ppa* reduces Prd protein levels. (e) The *P*[*eve*.2,3,7] transgene [4] drives strong expression of Eve stripes 2, 3 and 7, and weak or undetectable expression of stripe 4 and other stripes. (f) In *eve*⁻ *P*[*eve*.2,3,7] embryos, well-formed *ppa* interbands were seen only at the sites of strong Eve stripes, including stripe 2 but not stripe 4, confirming that *eve* represses *ppa* expression. (g) The corresponding, overlapping *prd* mRNA stripes showed stronger expression at stripe 4 compared with stripe 2, because Eve represses and refines stripe 2 but not stripe 4. (h) In contrast, the Prd protein stripe 4 was weaker than stripe 2, correlating with the local ectopic expression of *ppa* at stripe 4 (see text and Table 1) and suggesting that Ppa mediates Prd degradation. (i) Western analysis of hand-selected *hs–ppa* and wild-type embryos probed with anti-Prd antibody. Embryos were heat-treated during cycle 14 and fixed shortly after gastrulation. Prd protein signals were reduced in *hs–ppa* embryos compared with the wild type. Ponceau-S staining of total protein (not shown) suggested similar loading and transfer for wild-type and *hs–ppa* lanes. Similar results were observed in two independent experiments. (j) Ppa interacts with *Drosophila* Skp1 in a yeast mating assay. LexA DNA-binding domain (DB) fused to *Drosophila* (Dm) Skp1 (LexA–Skp1), co-expressed with Ppa (amino acids 131–538) fused to an activation domain (AD; B42), activated LexA–operator–LEU2 to permit growth on leucine drop-out plates (–Leu), and activated LexA–operator–lacZ to provide β-galactosidase (β-gal) activity on X-gal plates. Expression of either LexA–Skp1 or Ppa (amino acids 131–538) alone did not activate either LexA–operator target, nor did LexA–Skp1 co-expressed with Ppa (amino acids 447–538), which has no F-box. All tested combinations grew on complete media plates ('growth'). The *Drosophila* Skp1 full ORF (163 amino acids) was encoded by LD07173 (BDGP) starting at nucleotide 307. *Drosophila* Skp1 is also referred to as SkpA in FlyBase (http://flybase.bio.indiana.edu). (k) LexA-tagged Skp1 co-immunoprecipitated with HA-tagged Ppa (amino acids 131–538; lane 5). Co-immunoprecipitation was not observed with the HA (lane 4) or LexA (lane 6) tags alone. Immunoprecipitation (IP) was carried out with anti-HA antibody, and detection was performed with anti-HA or anti-LexA antibodies.

Eve might repress *ppa* expression, thereby giving rise to the *ppa* interbands. This interpretation was supported by examination of *eve* mutant embryos, which had uniform (Figure 4a) instead of striped *ppa* expression (Figure 4b) during cycle 14 and germ-band elongation. Moreover, adding back a transgene (*P*[*eve*.2,3,7], referred to as E+L-eve in [4]) that expresses *eve* stripes 2, 3 and 7 in an otherwise *eve* mutant background (Figure 4e), resulted in *ppa* interbands corresponding to these three Eve stripes (Figure 4f).

Ectopic *ppa* reduces Prd protein

The spatial expression and sequence of *ppa* suggest that Ppa might negatively regulate Prd, either by transcriptional co-repression or degradation of the Prd protein. To test these possibilities, we expressed *ppa* ectopically in the Prd-expressing cells to determine whether activation of *en* transcription or levels of Prd protein were affected. A transgene with the full ORF of *ppa* driven by an *hsp70* promoter (*hs–ppa*) was introduced into embryos. Heat treatment of

Table 1

Localized ectopic *ppa* reduces Prd protein:mRNA ratios.

Embryo genotype	*prd* expression	Stripe 2*	Stripe 4		Number of embryos
			Relative pixel densities*†	Protein:mRNA ratio‡	
eve⁻ *P*[*eve*.2,3,7]	Protein	100	75 ± 2.4	0.50	17
	mRNA	100	151 ± 4.4		17
Wild type§	Protein	100	88 ± 2.1	0.82	38
	mRNA	100	107 ± 3.2		29
hs–ppa§	Protein	100	98 ± 1.3	0.95	21
	mRNA	100	105 ± 3.8		13

*Protein or mRNA expression at stripes 2 and 4 are presented as ratios (% ± SEM) of pixel densities relative to stripe 2 values in the same embryo. The relative pixel intensities should be interpreted with caution because of possible non-linearities in the signal detection. †The arithmetic differences between the means for protein and mRNA were calculated with 95% confidence limits on the basis of standard errors of these differences between means. For *eve*⁻ *P*[*eve*.2,3,7] embryos, the difference between means was 76% ± 10% (± 95% confidence limits), which was significantly larger than the difference between means for *hs–ppa* embryos (7% ± 7%). Both of these differences between means were distinct from the value for wild-type embryos (19% ± 7%). ‡The protein:mRNA ratios were significantly different among *eve*⁻ *P*[*eve*.2,3,7], wild-type and *hs–ppa* embryos. This was based on analysis of differences between the means of $\log_{10}$(stripe 4/stripe 2) ratios. §The *hs–ppa* embryos were heat-treated at 37°C with two 5 min pulses separated by 30 min, and fixed 20 min after the end of the second heat treatment. Heat treatment of a portion (37%) of the wild-type embryos had no effect on stripe 4/stripe 2 ratios (by the Student's *t*-test).

hs–ppa embryos during cycle 14 had pronounced effects. The odd-numbered, Prd-dependent, *en* stripes were weakened or completely absent, suggesting that Prd activation of these stripes was repressed (Figure 4c, summarized in Figure 1a). This was not observed in heat-treated wild-type embryos processed in parallel (Figure 4d).

Because the *Drosophila* embryo develops very quickly, the segmentation gene products are expected to be short lived. This is indeed the case for those products examined [17] and is also likely to be true for the Prd protein, perhaps even in the absence of *ppa* function (see below). Indeed, it was difficult to assess whether Prd protein levels were reduced in *hs–ppa* embryos because of the fairly broad range of immuno-staining signals observed between different embryos, a problem inherent to the detection technique. To overcome this problem, we expressed *ppa* ectopically over only part of the embryo, so that the effects of ectopic *ppa* could be assessed relative to regions of the same embryo where *ppa* expression was normal. As shown earlier, *eve* mutant embryos with a transgene *P*[*eve*.2,3,7] that expresses only *eve* stripes 2, 3 and 7 had well-formed *ppa* interbands at these locations (Figure 4f). Thus, it was possible to compare Prd protein expression at stripe 2, which overlaps the *ppa* interband at *eve* stripe 2, with Prd expression at stripe 4, where *ppa* is expressed ectopically. Examination of *prd* mRNA signals in *eve*⁻ *P*[*eve*.2,3,7] embryos revealed strong expression of stripe 4 when compared with stripe 2 (Figure 4g, Table 1), consistent with previous observations that *eve* represses *prd* transcription, thereby contributing to refinement of *prd* stripes [4,18]. In contrast, Prd protein signal at stripe 4 was significantly lower than at stripe 2 (Figure 4h, Table 1), correlating with the ectopic *ppa* expression at stripe 4, and suggesting that Ppa regulates Prd protein levels. Even though there was 50% more mRNA signal at stripe 4 than stripe 2 after *ppa* upregulation, there was 25% less protein. Note that it is formally possible that the reduced Prd protein levels result from changes in genes other than *ppa* that are regulated by *eve*. Nevertheless, our analyses of *hs–ppa* and *ppa* mutant embryos suggest that regulation by *ppa* is responsible.

The equivalent analysis of wild-type embryos showed similar mRNA signals at stripes 4 and 2, whereas the Prd protein signal at stripe 4 was somewhat reduced compared with stripe 2 (Table 1), correlating with the residual *ppa* expression normally still present at gastrulation at the *ppa* interband corresponding to Prd stripe 4 (see, for example, Figure 3g). This decrease in Prd protein was less pronounced than in *eve*⁻ *P*[*eve*.2,3,7] embryos, presumably because the difference in *ppa* expression at stripes 2 and 4 was smaller. To confirm the interpretation that Ppa regulates Prd protein levels, we would expect the stripe 4 to stripe 2 ratios for mRNA and protein to be similar in heat-treated *hs–ppa* embryos, in which *ppa* was expressed ectopically at both stripes 2 and 4. This was indeed observed (Table 1), supporting our conclusion that *ppa* regulates Prd protein levels (see Table 1 for statistical analysis of the differences between the means of ratios). Because Ppa has an F-box, this regulation is most likely through targeted protein degradation rather than translational repression. Consistent with these data, western analysis of embryo extracts indicated that Prd protein levels were reduced by approximately 50% in *hs–ppa* embryos compared with the wild type (Figure 4i).

Increased Prd protein in *ppa* mutants

To confirm the role of Ppa in Prd degradation, we generated a small chromosomal deletion that removes the Ppa

Table 2

***ppa* mutants have increased Prd protein:mRNA ratios.**

Embryo genotype	*prd* expression	Stripe 2*	Stripe 4		Stripe 6		Number of embryos
			Relative pixel*† densities	Protein: mRNA ratio‡	Relative pixel densities*†	Protein: mRNA ratio‡	
Wild type§	Protein	100	82 ± 3.9	0.82	101 ± 4.5	0.83	13
	mRNA	100	100 ± 2.3		121 ± 4.5		17
ppa–§	Protein	100	92 ± 3.2	0.95	88 ± 4.1	0.98	12
	mRNA	100	97 ± 2.6		89 ± 5.5		15

*Prd protein or mRNA expression at stripes 2, 4 and 6 are presented as ratios (% ± SEM) of pixel densities relative to stripe 2 values in the same embryo. †The arithmetic differences between means for protein and mRNA were significantly higher (95% confidence limits) in wild-type (stripe 4, 18% ± 9; stripe 6, 20% ± 13) compared with *ppa*– (stripe 4, 5% ± 8; stripe 6, 1% ± 16) embryos. ‡The protein:mRNA ratios were significantly different for *ppa*– and wild-type embryos. This was based on an analysis of differences between the means of log10(stripe 4 or 6/stripe 2) ratios. §The stripe 4/stripe 2 mRNA ratios were similar in *ppa*– and wild-type embryos. For unknown reasons, the stripe 6/stripe 2 ratios were lower in *ppa*– mutants, perhaps reflecting a delay in posterior activation of *prd* mRNA.

ORF, starting 345 bp upstream and ending 304 bp downstream of the ORF. The deletion was derived from a *P*-element insertion in the *ppa* 3′ untranslated region (EP(2)0698) using transposase-mediated male recombination (P.V., L.F.A. and M.W., unpublished work). Consistent with our observation that high levels of *ppa* are normally only observed in regions where Prd function is not required, the homozygous *ppa* mutants survive to adulthood but with reduced viability and abnormal nuclear cycling (our unpublished data). To analyze the mutants, we took advantage of the normal anterior–posterior progression of *ppa* stripe development in wild-type embryos: at the gastrulation stage, the more anterior Prd stripes (for example, stripe 2) had little overlap with *ppa* expression, whereas the more posterior stripes (for example, stripes 4 and 6) still had significant overlap (Figure 3g,h). When *prd* mRNA and protein signals at stripes 4 and 6 were measured relative to stripe 2 in the same embryos (Table 2), the protein signals at stripes 4 and 6 were found to be significantly lower than the corresponding mRNA levels in wild-type embryos. In *ppa* mutant embryos, however, the mRNA and protein signals were similar, indicating that *ppa* normally reduces Prd protein expression.

Ppa interacts with *Drosophila* Skp1

Supporting the conclusion that Ppa mediates Prd degradation, we found that Ppa interacts with *Drosophila* Skp1, the component of the protein degradation machinery that is predicted to link Ppa to the ubiquitin-mediated degradation pathway. We identified expressed-sequence-tag (EST) cDNAs for *Drosophila* Skp1 from the Berkeley *Drosophila* Genome Project (BDGP; clot 1632; [19]) and showed by yeast two-hybrid assays that the Skp1 protein interacts with Ppa (amino acids 131–538, which lacks the Ala/His/Pro-rich region; Figure 4j). As expected, the smaller fragment of Ppa (amino acids 447–538) originally identified in our two-hybrid screen did not interact with Skp1, presumably because it has no F-box. The interaction between Ppa and Skp1 was confirmed by co-immunoprecipitation analysis of yeast cell extracts. Immunoprecipitation of a hemagglutinin (HA) epitope-tagged Ppa fragment (amino acids 131–538) using anti-HA antibody also brought down LexA-tagged Skp1, which was detected with anti-LexA antibody (Figure 4k, lane 5). Co-immunoprecipitation was not observed with the HA or LexA tags alone (Figure 4k, lanes 4,6). The Ppa–Skp1 interaction was also observed by immunoprecipitation with anti-LexA antibody, and the interaction of Ppa with Prd was also verified in these experiments (data not shown).

Localized F-box receptors co-ordinate localized protein expression

Our analysis of *ppa* function indicates that, when it is expressed ectopically in Prd-expressing cells, the levels of Prd protein diminish about twofold. A similar change in substrate stability (2–4-fold) is observed when *GRR1*, the yeast gene most similar to *ppa*, is mutated [20,21]. It is also possible that, in addition to reducing Prd protein levels, Ppa might function as a transcriptional co-repressor, interacting with Prd to reduce its activation of *en*. Together, these two repression functions would ensure robust negative regulation of Prd in the Ppa-expressing cells.

Ppa is the first example of an F-box receptor localized in stripes. Our loss- and gain-of-function analyses show that, while the presence of *ppa* expression in stripes is important for embryo development, it is the absence of *ppa* expression in the interbands that is crucial. Homozygous *ppa*– mutants survived to adulthood, but showed 50–70% lethality, consistent with the fact that Ppa works in conjunction with the transcriptional repressors *slp* and *run* to localize Prd function (Figure 1). The partial lethality may be due to altered Prd expression or abnormal nuclear cycling (P.V., L.F.A. and M.W., unpublished data). In contrast, in embryos with a functional *ppa* gene, it is absolutely essential that its expression be spatially regulated

(by *eve*). Even basal expression of one of our *hs–ppa* transgenes (two copies without heat treatment) caused complete lethality, whereas the same transformant was not lethal before removal of an FRT cassette that blocked transcription. This predicts that *cis*-regulatory mutations in *ppa* causing loss of spatial regulation will have profound detrimental effects on embryogenesis, and this could also apply to the vertebrate homologs of *ppa*, which have such striking sequence similarity.

With the recent cloning of F-box proteins and the realization that they provide specific links between substrates and the protein degradation machinery, it was predicted that F-box proteins would play important roles in development [12]. Because F-box-regulated degradation normally depends on phosphorylation of substrates [12,22,23], localized action of signal transduction systems can, in principle, lead to localized protein degradation. This is likely to be the case for the signal-dependent localized degradation of *Drosophila* Cactus, a homolog of vertebrate IκB, whose degradation is a prerequisite for nuclear import of the Dorsal transcription factor (a homolog of NFκB) in the ventral portion of the embryo [24–26]. Degradation of Cactus is mediated by the F-box protein Slimb (a homolog of β-TrCP), which is also implicated in Wingless and Hedgehog pathways [26,27]. In contrast to these signal transduction systems, the localized protein degradation in the Ppa system depends on spatially regulated expression of the Ppa F-box protein itself. By having its transcription regulated by a segmentation protein (Eve), and by targeting other segmentation proteins for degradation (Prd), the Ppa F-box protein forms an integrated link in the segmentation protein regulatory cascade that serves to strengthen the spatial refinement required for pattern formation. We predict that integration into transcriptional cascades may be a property of an important subfamily of F-box proteins, which, as suggested above, may also have recruited transcriptional repression functions to optimize their negative regulation of targeted transcription factors.

Conclusions

Ppa is the first example of a localized F-box receptor for protein degradation that works alongside transcription factors to ensure localized gene expression in the *Drosophila* segmentation cascade. Our analyses suggest that Ppa targets the Prd transcription factor for degradation in cell rows in which Prd function is inappropriate, and that it is crucial that *ppa* expression is removed, through repression by *eve*, from cell rows in which Prd function is required for normal embryonic development.

Materials and methods

Two-hybrid screen

The protocol for our two-hybrid screen was essentially as described in [28]. Several *prd* cDNA segments were amplified by PCR and subcloned in pEG202. The LexA–Prd baits were tested for intrinsic activation of LexA–operator–lacZ and LexA–operator–LEU2. The baits were also tested for their ability to repress (most probably through interference) activation of a Gal4–UAS–lacZ reporter (pJK101) with LexA operator sequences between the GAL4-binding site and *lacZ*, thereby indicating expression of stable, nuclear-localized LexA–Prd baits. The LexA–Prd construct, which encodes a fragment of Prd from 61 amino acids amino-terminal of the homeodomain to 18 amino acids carboxy-terminal (and was the most effective in the repression assay and did not itself activate), was used to screen a *Drosophila* 0–12 h embryo cDNA library in pJG4–5 [7]. Initial *leu*$^+$ positives (1224 clones) were picked and replica-plated to glucose- or galactose-containing plates to distinguish clones requiring activation of the Gal-driven cDNA. The cDNA inserts of the galactose-dependent positives (320 clones) were amplified by PCR and digested with *Hae*III added directly to the PCR. Gel electophoresis revealed 22 classes of restriction patterns. Representatives from each class were sequenced and cDNA-encoded proteins examined by western analysis using anti-HA antibodies. The cDNA clones were tested for specificity of their interaction with Prd using a yeast mating assay [9]. Briefly, the cDNAs were retransformed into α cells, which were plated with an array of baits expressed in a cells. Growth of diploid cells was tested on leucine drop-out or X-gal plates. Similar mating assays were also used in our later characterization of the Ppa–Skp1 interaction.

Molecular characterization of ppa

Northern analysis was done with a single-strand DNA probe for a 1.1 kb *ppa* cDNA subcloned in M13mp18. A 4.2 kb mRNA was detected in total RNA from 0–3 h, 3–6 h and 6–12 h embryos. To obtain longer cDNAs, the 1.1 kb cDNA was used to screen by colony hybridization a 0–4 h cDNA library [29]. The resulting 2.4 kb cDNA was sequenced on both strands. Comparison of the 2.4 kb cDNA sequence with the BDGP revealed that BDGP had recently sequenced through the region (DS05609; BDGP, personal communication). The genomic sequence 5′ of the 2.4 kb cDNA was used to design primers for RT–PCR. The sequence immediately upstream of the 2.4 kb cDNA is GC rich (79%), and this necessitated the use of 'GC melt' (Clontech) to PCR through the region. An RT–PCR-derived cDNA was sequenced, revealing a 538 amino-acid ORF. Comparison with genomic sequence (DS05609) indicated that there were no introns in the *ppa* ORF, nor in the 1.1 kb 3′ untranslated region (UTR). To generate a cDNA with the complete ORF, 5′ cDNA sequence derived from RT–PCR was ligated to cDNA of the 2.4 kb clone using the *BspE*I restriction site at +412 bp relative to the translation start. A fragment of this composite cDNA, including 9 bp of the *ppa* 5′UTR, the full ORF, and the 1.1 kb 3′UTR was subcloned into the *Stu*I site of pCaSpeR-hsFRT. We constructed pCaSpeR-hsFRT by inserting into the *Hpa*I site of CaSpeR-hs, a 0.4 kb FRT cassette with *hsp70* polyadenylation sequences [30]. The resulting pCaSpeR-hsFRT-ppa was introduced into *yw; ry Sb P[ry*$^+$ *Δ2-3]99B/TM6* embryos by germ-line transformation. The FRT cassette was included in the transgene to ensure no transcriptional readthrough of the *ppa* sequence during establishment and maintenance of the transgenic line, as it was possible that basal expression of *ppa* might be deleterious to the embryo, a problem we have experienced previously with *prd* constructs. After establishment of transgenic lines, the FRT cassette was removed using FLPase derived from a *tubulin–FLPase* transgene, which expresses FLPase in the male germ line during spermatogenesis [31].

Embryo expression and ectopic expression analysis

In situ hybridization to mRNA was performed as described [32], and mRNA/protein double labeling was performed according to [5]. After color reactions, double-labeled embryos were filleted using glass needles and mounted under coverlips in glycerol. Filleting of embryos permitted clear examination of gene expression in a single ectodermal layer. For ectopic expression analysis, 30 min embryo collections were dechorionated (in 50% Clorox) 15 min before heat treatment. Wild-type and *hs–ppa* embryos were heat-treated at 37°C for two 5 min pulses at 140 and 175 min after the end of egg collections. Embryos were fixed at 10, 20, 30 or 40 min after the second heat pulse, and stored in methanol before *in situ* hybridization or antibody staining.

Equivalent results were obtained with two independent *hs–ppa* transgenic lines. For mRNA and protein signal comparisons at *prd* stripes 2, 4 and 6, recently gastrulated embryos were photographed, films were scanned with constant exposure, and digitized images were imported into NIH Image for pixel-density comparisons. Pixel densities were measured using two sample rectangles per embryo, each 20–30 μm by 60–100 μm. Within a dynamic range that did not saturate signals, similar results were obtained in stripe signal comparisons using different exposures of the same embryo. Western analysis of *hs–ppa* embryos was performed as described [33].

Co-immunoprecipitation analysis

Co-immunoprecipitation of HA-tagged Ppa with LexA-tagged Prd or Skp1 was performed essentially as described [11]. Cells were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM PMSF, 0.6 μM leupeptin, 1 μg/ml pepstatin A, 2 mM benzamidine, and the lysate was incubated overnight with anti-HA or anti-LexA antibody, followed by a 4 h incubation with protein G–Sepharose beads (Sigma), five washes with lysis buffer (LexA pull-down), or lysis buffer supplemented with 1% NP-40 and 0.1% SDS (HA pulldown), and a final wash in 10 mM Tris-HCl pH 7.5, 0.1% NP-40 and protease inhibitors. Immunoprecipitated proteins were run on SDS–polyacrylamide gels and transferred to Immobilin-P membrane (0.45 μm pore, Millipore). The membrane was probed with anti-HA (BAbCO) or anti-LexA (Santa Cruz Biotechnologies) antibodies, which were detected by ECL (Amersham).

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