Targeted localized degradation of Paired protein in Drosophila development


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. Wh en 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.

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

\textbf{Results and discussion}

\textbf{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 × 10^9 primary transformants, we identified 22 classes of clones by restriction analysis; mRNA \textit{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 \textit{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 \textit{robo} gene). The protein interaction.

The \textit{ppa} protein sequence. (a) Schematic illustration and (b) sequence of the 531 amino-acid ORF of \textit{ppa}, which has 11 LRRs (numbered), an F-box, and a motif rich in Ala, His and Pro that is similar to repression domains found in transcriptional repressors [13–16]. \textit{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 \textit{ppa} sequence is AF187980. Other cDNAs from this screen will be described elsewhere. To test the specificity of the interaction of \textit{Ppa} with Prd, the \textit{ppa} cDNA was retransformed into yeast and tested using a mating assay [9] against a panel of different baits, including Prd. \textit{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, \textit{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 \textit{ppa} provided insights into its possible functions (Figure 2). The \textit{Ppa} open reading frame (ORF) contains 11 leucine-rich repeats (LRRs). These 29–29 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 \textit{Ppa}, encompassing three LRRs (Figure 2a,b), was sufficient for the interaction of \textit{Ppa} with Prd, the Prd stripes, thereby downregulating any aberrant Prd expression in these cells. The \textit{ppa} gene is repressed by \textit{eve} in Prd-expressing cells, thus allowing Prd to activate its downstream genes.

\textbf{Figure 2}

![Figure 2](current_biology_vol10_no22_1266_f2.png)

\section{Alignments of LRR sequences. Like its closest homologs, yeast (\textit{S.c}) \textit{Gr1}, \textit{C. elegans} (\textit{Ce}) ORF CO2FS5.7 and human (\textit{H.s}) \textit{Ppa} (genomic sequence, AC001582; sequence of cDNA 3 untranslated region, AF007128), Drosophila (\textit{D.m}) \textit{Ppa} is in a subfamily of LRR proteins with \textit{Cys} at position 10 [10]. Crystall structure analysis of porcine ribonuclease inhibitor indicates that the protein has alternating A- and B-type LRRs (RNase Inhib(B)) which each form an a-helix and \textit{β}-strand [10]. The LRR consensus sequence is based on collated sequences in [10] and consists of Ala, 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).

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 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 end 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.
Functional analysis of ppa. (a,b) Spatial regulation of ppa mRNA. The ppa mRNA was expressed uniformly in (a) homozygous eve127 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 eve127 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. (e) 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 4c), 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
consistent with previous observations that stripe 4 when compared with stripe 2 (Figure 4g, Table 1), correlating with the ectopic 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], wild-type and hs-ppa embryos. This was based on analysis of differences between the means of log10(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).

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 repressed 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

### Table 1

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

*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

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).
Table 2

<table>
<thead>
<tr>
<th>Embryo genotype</th>
<th>prd expression</th>
<th>Stripe 2*</th>
<th>Stripe 4</th>
<th>Stripe 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>mRNA</td>
<td>Relative pixel densities</td>
<td>Protein: mRNA ratio</td>
</tr>
<tr>
<td>Wild type†</td>
<td>100</td>
<td>100</td>
<td>82 ± 3.9</td>
<td>0.82</td>
</tr>
<tr>
<td>ppa‡</td>
<td>100</td>
<td>100</td>
<td>92 ± 3.2</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*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 5g,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 4). 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 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.
(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 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 (p[K101]) 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 pG4–5 [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 Haell I added directly to the PCR. Gel electrophoresis 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 a 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 BspE1 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 Sbf1 site of pCaSpeR-hsFRT. We constructed pCaSpeR-hsFRT by inserting into the Hpal 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 5B P[+2,3]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 coverslips 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% Clorex) 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].

Acknowledgements
We thank Laura Grabel and Steve Devoto for discussion of the manuscript; Fred Cohen for advice on statistics; Jim Muironey for technical advice; Joel D’Angelo for technical help; Tom Kidd and Guy Tear for sharing unpublished results; Miki Fukjio for eve transgenic lines; Claude Desplan for Prd antibody; Manfred Frasch for Eve antibody; BDGP for genomic sequence in the eve locus; and the Wadsworth Center Molecular Genetics Core Facility for RNA sequencing. This work was funded by grants from NIH and the American Cancer Society to W.M., and from the American Cancer Society to S.D.H.

References
11. Li FN, Johnston M: Grl1 of Saccharomyces cerevisiae is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. EMBO J 1997, 16:5629-5638.