The Rpd3 histone deacetylase is required for segmentation of the *Drosophila* embryo

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ABSTRACT Previous studies have implicated histone deacetylation and chromatin condensation as critical mechanisms of transcription repression in yeast and mammals. A specific histone deacetylase, Rpd3, interacts with a variety of sequence-specific transcriptional repressors, including Mad–Max heterodimers and members of the nuclear receptor superfamily. Here, we present evidence that a strong hypomorphic mutation in the *Drosophila* Rpd3 gene causes embryonic lethality and a specific pair-rule segmentation phenotype. The analysis of a number of segmentation genes suggests that the repressor function of Even-skipped (Eve) may be diminished, causing an indirect loss of Ftz-mediated activation of engrailed. The relatively mild defects observed in Rpd3 mutants suggest that the recently identified Groucho and dCtBP corepressor proteins do not function solely through the recruitment of histone deacetylases. We discuss the possibility that Eve mediates multiple mechanisms of repression, so that Rpd3 mutants disrupt the regulation of just a subset of Eve target genes.

Transcriptional repression is essential for the segmentation of the *Drosophila* embryo. Anteroposterior patterning depends on a broadly distributed Hunchback repressor gradient that establishes localized patterns of other gap repressors, including Giant, Krüppel, and Knirps (1, 2). The gap repressors subsequently define the borders of segmentation stripes of pair-rule gene expression (3–5).

There are eight zygotic pair-rule genes; each appears to encode a sequence-specific transcription factor. Cross-regulatory interactions help refine the patterns of pair-rule gene expression, and the encoded products are essential for the regulation of segment polarity genes (6–8). Two homeobox-containing pair-rule genes, *even-skipped* (*eve*) and *fushi-tarazu* (*ftz*), have been shown to play a particularly important role in subdividing each segment primordium into anterior and posterior compartments through the regulation of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) (9, 10). Eve activates *en* but represses *wg* expression in odd parasegments, whereas Ftz activates *en* but represses *wg* in even parasegments.

There is a loss of all 14 middle-body *en* stripes in mutant embryos that completely lack Eve function (6, 11, 12). Eve is thought to function as a dedicated transcriptional repressor that regulates *en* expression indirectly (7). The odd-numbered *en* stripes are activated by Paired and repressed by Sloppy-paired and Runt, whereas the even-numbered *en* stripes are activated by Ftz and repressed by Odd-skipped (Odd) (7, 8, 13). The differential repression of these target genes is thought to create small domains where the Paired and Ftz activators can initiate *en* expression. For example, higher concentrations of Eve are needed to repress *ftz* than *odd* (7). Consequently, a single cell within the *eve* stripe contains a relatively high concentration of the Ftz activator and low levels of Odd, thereby permitting the activation of the even-numbered *en* stripes.

Given the importance of Eve in segmentation, we have become interested in determining how it functions as a transcriptional repressor. Previous studies suggest that Eve interferes with the binding or function of TATA box-binding protein (TBP) at the core promoter (14–16). Recent studies have shown that a number of repressors in the early embryo interact with corepressor proteins, such as Groucho and dCtBP (17). It is conceivable that Eve also interacts with corepressor proteins. However, Eve activity does not appear to be impaired in mutant embryos lacking Groucho or dCtBP, although such mutants exhibit severe patterning defects because of the inactivation of other repressors such as Krüppel, Snail, and Hairy. Studies in both yeast and mammals have identified histone deacetylases as corepressors of a number of sequence-specific transcriptional repressors (18–20). The Rpd3 deacetylase is a major histone deacetylase in yeast (21), and the mammalian homolog of this protein appears to mediate repression by the thyroid hormone and retinoic acid receptors and the Mad–Max complex (20).

A *Drosophila* homolog of Rpd3 was identified in a genetic screen for mutations affecting position-effect variegation of white gene expression in the eye (22). However, this mutation affects Rpd3 expression only in eye imaginal discs. Searches of *Drosophila* genomic databases identified a P-element-induced mutation in the 5′ untranslated region of the Rpd3 deacetylase gene. This mutation causes a severe reduction in the expression of Rpd3. The analysis of embryos derived from Rpd3 germline clones revealed a pair-rule segmentation phenotype that can be attributed to a partial loss of Eve repressor function.

MATERIALS AND METHODS

Fly Stocks and Generation of Germline Clones. Germline clones were produced by using the FLP-dominant female sterile (DFS) technique as described (23, 24). Males heterozygous for the DFS mutation *ovo*[D1] and containing a heat-inducible *hsp70*-FLP transgene were obtained by mating yw *hsp70*-FLP; CxD/TM3, Sb females with w; FRT[3L] *ovo*[D1]/TM3, Sb males. The resulting males, yw *hsp70*-FLP; FRT[3L] *ovo*[D1]/ TM3, Sb, were mated with w; FRT[3L] l(3)04556/TM3, Sb females (kindly provided by N. Perrimon). Larvae were heat-shocked for 3 hr at 37° for 3 consecutive days on days 3, 4, and 5 after egglaying to induce the expression of the FLP recombinase. Sb[+] females were mated with w; FRT[3L] l(3)04556/ TM3, Sb males, and embryos were collected and fixed for *in situ* hybridization. Embryos were also collected from an *eve*[R13] stock (Bloomington Stock Center no. BL-299).

Cuticle Preparation. Advanced-stage embryos were dechorionated in bleach, transferred to a microscopic slide, and

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Abbreviation: TBP, TATA box-binding protein.
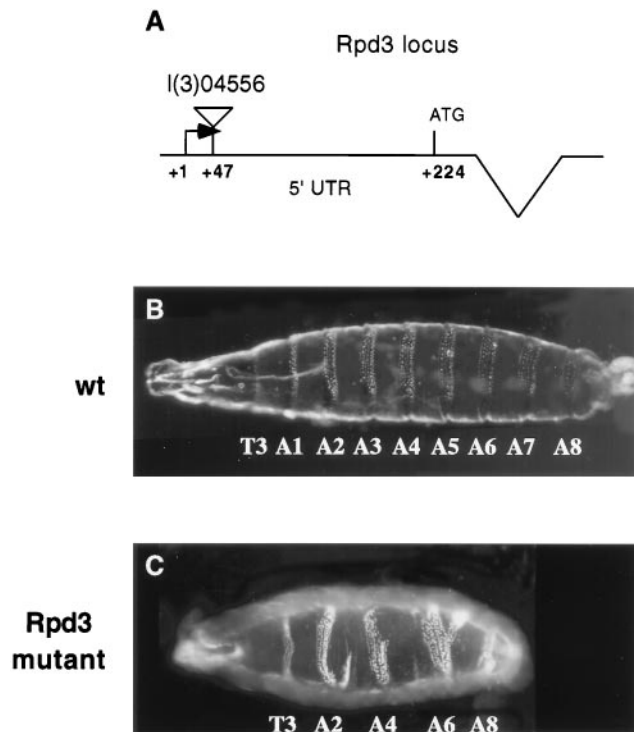*To whom reprint requests should be addressed. e-mail: mlevine@ uclink4.berkeley.edu.

FIG. 1. Summary of the Rpd3 mutation. The Berkeley Genome Project identified a P-element lethal mutation that maps within the Rpd3 gene. The location of the P-element within the 5′ UTR is summarized in *A*. This P-element mutation, l(3)04556, was previously examined in a large-scale screen of maternally expressed, zygotic lethals (24). Embryos derived from l(3)04556 germline clones exhibit a variable pair-rule phenotype (Rpd3 mutant, *C*). A wild-type embryo is shown for comparison (wt, *B*).

cleared with lactic acid at 65° for 1.5 hr. They were subsequently photographed using darkfield microscopy.

***In Situ* Hybridization and Immunohistochemistry.** *In situ* hybridization assays were performed with digoxigenin-labeled
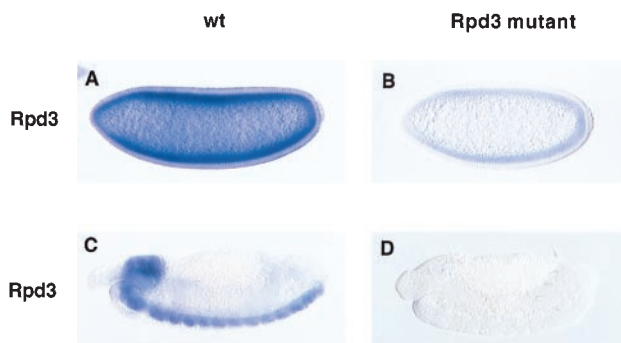

FIG. 2. The l(3)04556 mutation causes a substantial loss in Rpd3 expression. Embryos were hybridized with an Rpd3 digoxigenin-labeled antisense RNA probe and are oriented with dorsal up and anterior to the left. (*A* and *C*) Cellularizing (*A*) and retracted (*C*) wild-type embryos. Maternal Rpd3 products are ubiquitously distributed throughout early embryos (*A*). In contrast, zygotic products appear to exhibit a more localized distribution, with peak expression in the central nervous system, including both the brain and the ventral nerve cord (*C*). (*B* and *D*) Cellularizing (*B*) and retracted (*D*) l(3)04556 mutants. Embryos were collected from germline clones after mating with l(3)04556/+ males. Half the embryos lack both maternal and zygotic Rpd3 activity, and presumably, the embryo shown in *D* corresponds to such an example. There is at least a 5-fold reduction in the levels of maternal Rpd3 products (*B*) as compared with wild-type (*A*). There is a virtual loss in detectable products in advanced-stage l(3)04456/l(3)04556 homozygotes.

eve, *ftz*, *en*, *odd*, and Rpd3 antisense RNA probes as described (25, 26). Embryos were double stained for Ftz protein and *odd* RNA as described (7). Fixed embryos were first incubated with a 1:500 dilution of a Ftz polyclonal antibody (kindly provided by H. Krause) and subsequently stained with horseradish peroxidase by using the Vectastain Elite kit (Vector Laboratories). These embryos were then hybridized with an *odd* antisense RNA probe and histochemically stained after incubation with an alkaline-phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim).

RESULTS

Identification of a P-Element-Induced Mutation in Rpd3. Rpd3 maps within the 64C region of chromosome 3 (22). The Berkeley Drosophila Genome Project (27) identified a P-induced lethal mutation (l(3)04556) that maps 47 bp downstream of the Rpd3 putative transcription start site (Fig. 1 and http://www.fruitfly.org/p_disrupt/). As shown previously
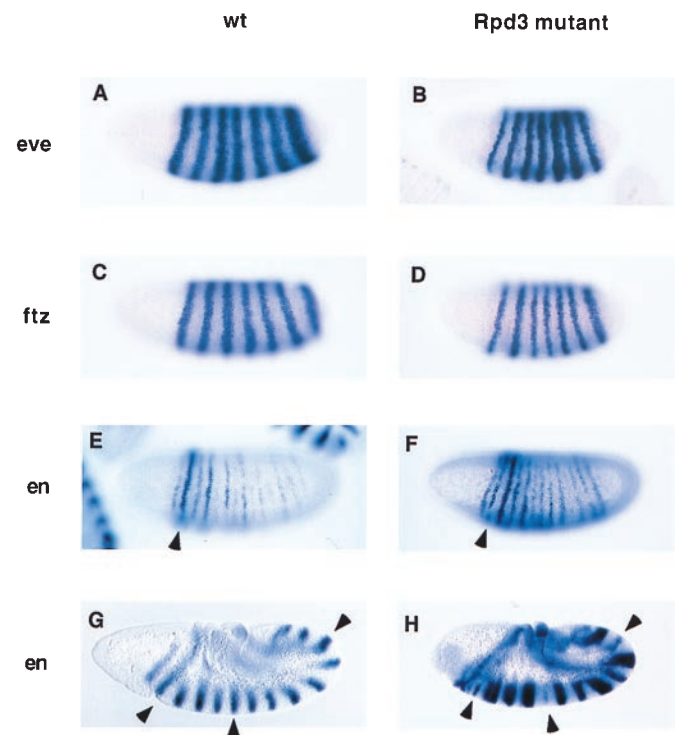

FIG. 3. *eve*, *ftz*, and *en* expression in Rpd3 mutants. Embryos were hybridized with the indicated digoxigenin-labeled antisense RNA probes and are oriented with anterior to the left and dorsal up. Rpd3 mutant embryos were derived from l(3)04556 germline clones. (*A* and *B*) Wild-type (wt) and mutant (Rpd3 mutant) embryos undergoing cellularization after hybridization with an *eve* probe. The mutant embryo is reduced in size, but the seven-stripe *eve* pattern appears essentially normal. (*C* and *D*) Wild-type and mutant embryos undergoing cellularization after hybridization with a *ftz* probe. The mutant embryo exhibits an essentially normal seven-stripe pattern. (*E* and *F*) Wild-type and mutant embryos at the onset of gastrulation after hybridization with an *en* probe. In wild-type embryos, the even-numbered *en* stripes are stronger than the odd-numbered stripes. The arrowhead indicates *en* stripe 2. Note that it is stained more intensely than the adjacent stripe 3. In mutant embryos, there is a relative loss in the even-numbered *en* stripes so that *en* stripe 2 (arrowhead) is significantly weaker than stripe 3. (*G* and *H*) Wild-type and mutant embryos undergoing the rapid phase of germband elongation. The normal *en* pattern includes 14 stripes that extend along the length of the germband (*G*). The mutant embryos show a variable loss or reduction in the even-numbered *en* stripes. For example, the arrowheads in *H* indicate *en* stripes 2, 6, and 12. These stripes are reduced in the mutant, but are expressed at the same levels as the other stripes in wild-type embryos (see arrowheads in *G*).

(24), embryos derived from l(3)04556 homozygous germline clones exhibit pair-rule patterning defects that are similar to those observed in *ftz*⁻ embryos (Fig. 1). In the present study, we have attempted to determine the basis for this disruption in segmentation.

Because Rpd3 is expressed maternally, it was necessary to produce female flies homozygous for the l(3)04556 mutation. This was accomplished by making mosaic flies containing clones of homozygous germ cells by using the FLP-dominant female sterile technique (23). We first examined Rpd3 expression in mutant embryos derived from l(3)04556 germline clones (Fig. 2). Embryos were hybridized with a digoxigenin-labeled Rpd3 antisense RNA probe. There is at least a 5-fold reduction in the levels of Rpd3 transcripts in mutant vs. wild-type embryos (Fig. 2 *A* and *B*). There is no detectable expression of Rpd3 in advanced-stage mutant embryos (Fig. 2 *C* and *D*).

Most Repressors Are Active in Rpd3 Mutant Embryos. The primary pair-rule genes *eve* (Fig. 3 *A* and *B*), *hairy,* and *runt* (not shown) exhibit essentially normal patterns of expression in mutant embryos, although sharpening of the *eve* stripes might be delayed in gastrulating embryos (not shown). This result indicates that all of the gap gene repressors are active in the Rpd3 mutant. The secondary pair-rule gene *ftz* also exhibits a normal pattern of expression in mutant embryos (Fig. 3 *C* and *D*), although there may be a delay in the refinement of the stripes as seen for *eve* (not shown). These

results suggest that the Hairy and Runt repressors function normally in Rpd3 mutants.

We next examined the expression of the segment polarity gene *en* (Fig. 3 *E*–*H*). In normal embryos, the even-numbered *en* stripes are initially stronger than the odd-numbered stripes. However, these stripes are weaker than the odd-numbered stripes in Rpd3 mutants (Fig. 3*F*). This effect becomes more dramatic in older embryos (compare Fig. 3 *G* with *H*) where some of the even-numbered *en* stripes are very weak or completely missing. There is a variable loss of the even-numbered *en* stripes in early embryos and a corresponding variation in the cuticular defects observed in older embryos (data not shown).

Rpd3 Mutants Alter Eve Repressor Function. The preceding results suggest that the Rpd3 mutation might impair the Ftz or Ftz-F1 activators because these are required for the expression of the even-numbered *en* stripes (28–30). Alternatively, the loss of Rpd3 might lead to a change in the expression pattern of a repressor, which in turn inhibits Ftz activity. To distinguish between these possibilities, we examined the expression of *odd*, a known repressor of *en* (8, 13, 31, 32) (Fig. 4 *A* and *B*). *odd* is initially expressed in a pair-rule pattern of seven stripes, but during gastrulation seven additional secondary stripes are formed to generate a 14-stripe expression pattern (33). In normal embryos, these stripes are evenly spaced (Fig. 4*A*), whereas in Rpd3 mutants they are not (Fig. 4*B*). In the mutant embryos there is a partial pair-wise
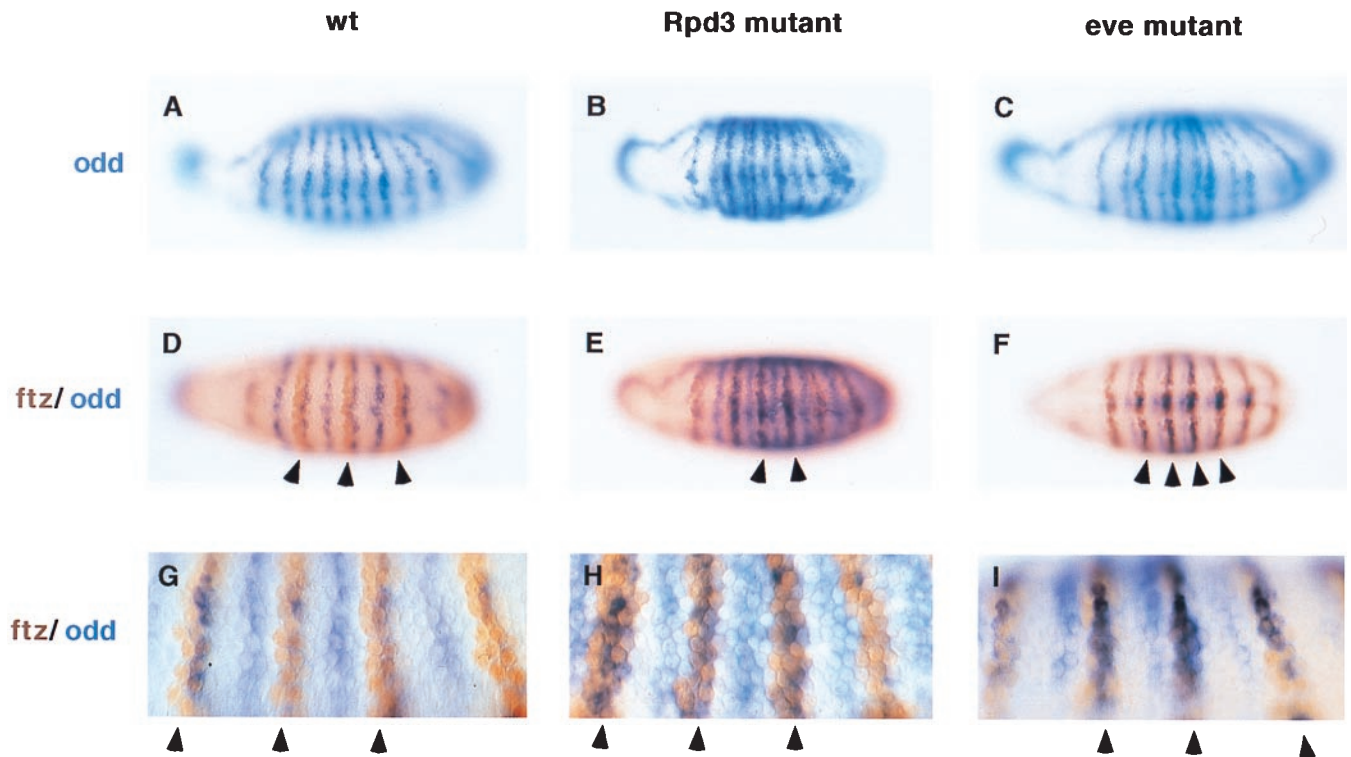

wt   **Rpd3 mutant**   **eve mutant**

FIG. 4. Altered patterns of *ftz* and *odd* expression in Rpd3 mutants. Gastrulating embryos were stained with an *odd* RNA probe (*A*–*C*) or with a mixture of an *odd* RNA probe and anti-Ftz antibodies (*D*–*I*). (*A*) Wild-type embryo (wt) that was hybridized with the *odd* RNA probe. *odd* is initially expressed in a seven-stripe pattern during cellularization (not shown), but is expressed in 14 stripes during gastrulation. Note that the stripes are evenly spaced along the germ band. (*B*) Same as *A* except that the mutant embryo was derived from l(3)04556 germline clones (Rpd3 mutant). There is a pairwise alignment of adjacent *odd* stripes, including stripes 2 + 3, 4 + 5, 6 + 7, etc. (*C*) Same as *A* and *B* except that the embryo is homozygous for a null mutation in *eve* (*eve*^R13). As seen in the Rpd3 mutant, there is a pairwise alignment of adjacent stripes, including 2 + 3, 4 + 5, 6 + 7, etc. (*D*) Wild-type embryo stained to show both *odd* RNAs (blue) and Ftz protein (brown). Note that each Ftz stripe is shifted just anterior of each odd-numbered *odd* stripe. Consequently, *odd* and Ftz are coexpressed in posterior regions of each Ftz stripe, whereas Ftz alone is expressed in anterior regions. The latter sites are thought to correspond to the locations of the even-numbered *en* stripes (arrowheads). This overlap in the two patterns is more easily seen at high magnification (*G*, arrowheads). (*E*) Same as *D*, except that the embryo was obtained from an l(3)04556 germline clone. The Ftz and *odd* patterns largely coincide, so that the anterior portion of each Ftz stripe also expresses *odd* (arrowheads, see high-magnification view in *H*). (*F*) Same as *D* and *E*, except that the embryo is homozygous for the *eve* null mutation. There is a similar failure of the Ftz and *odd* patterns to resolve (arrowheads, see high-magnification view in *I*).

alignment of adjacent *odd* stripes. A similar change is observed in *eve*⁻ embryos (Fig. 4C).

Previous studies suggest that both *ftz* and *odd* stripes are under the control of the Eve repressor (7). Differential repression of *ftz* and *odd* resolves the two patterns, so that each *ftz* stripe is normally shifted anterior of each odd-numbered *odd* stripe (Fig. 4D). In Rpd3 mutants, the *ftz* and *odd* patterns fail to resolve (Fig. 4 E and H), so that odd-numbered *odd* stripes mostly coincide with the *ftz* stripes. We suggest that this failure in *ftz*–*odd* resolution is responsible for the pair-rule phenotype observed in Rpd3 mutant embryos (see *Discussion*). A prediction of this proposal is that *eve* mutants should exhibit similar alterations in the *ftz* and *odd* expression patterns. Double staining assays reveal that *eve*⁻/*eve*⁻ embryos exhibit a similar failure to resolve the *ftz* and *odd* expression patterns (Fig. 4 F and I).

DISCUSSION

We have analyzed mutant embryos derived from germline clones of a P-element mutation in the Rpd3 gene. Although the mutation does not eliminate Rpd3⁺ gene activity, there is at least a 5-fold reduction in maternal Rpd3 products. This reduction causes a pair-rule phenotype that is similar to the one observed in *ftz*⁻ mutants (34), whereby the odd-numbered abdominal segments are lost. This phenotype demonstrates a surprisingly specific role of the Rpd3 histone deacetylase, even though maternal Rpd3 products are ubiquitously distributed throughout the early embryo. It would appear that the majority of the embryonic repressors are active in mutant embryos, including the gap repressors Hunchback, Giant, Krüppel, and Knirps. This situation contrasts with the patterning defects caused by mutations in two other corepressors present in the early embryo, Groucho and dCtBP (35–38). Mutations in either gene cause compound patterning defects because of the loss of multiple repressors. For example, removal of maternal dCtBP products causes a reduction or loss in the repression activities of Krüppel and Knirps (38) and a corresponding disruption in both thoracic and abdominal segments. An implication of these observations is that neither Groucho nor dCtBP functions solely by the recruitment of Rpd3.

Given the importance of Rpd3 as a corepressor of both yeast and mammalian transcriptional repressors, we anticipated that the Rpd3 mutants would exhibit more severe patterning defects. Instead, it would appear that this histone deacetylase does not represent a major pathway of repression in the early embryo. Of course, it is conceivable that the complete loss of Rpd3 products would cause more severe patterning defects. Unfortunately, it might not be possible to produce germline clones for a null mutation in the Rpd3 gene because the present hypomorphic allele produces very few eggs and mutations in genes that encode associated proteins such as Sin3 (not shown) and Mi-2 (39) fail to produce viable germline clones. An alternative explanation for the relatively mild Rpd3 patterning defects is that there is redundancy among different deacetylases. Indeed, two additional histone deacetylases are maternally expressed and ubiquitously distributed throughout the early embryo (M.M., unpublished results).

There are several possible explanations for impaired Ftz function in Rpd3 mutants. It is conceivable that the Rpd3 mutation disrupts Ftz-mediated activation. However, we favor the idea that Rpd3 functions as a corepressor of Eve. The similarities in the Rpd3 and *ftz* mutant phenotypes may be caused by the coincident *odd* and *ftz* expression patterns observed in embryos derived from l(3)04556 germline clones (summarized in Fig. 5). The Odd repressor is thought to block Ftz-mediated activation of *en* (8, 13, 40). We have presented evidence that this expansion in Odd might result from an inability of Eve to repress *odd* expression in Rpd3 mutant embryos (Fig. 5). Consistent with this proposal, we find that *in*
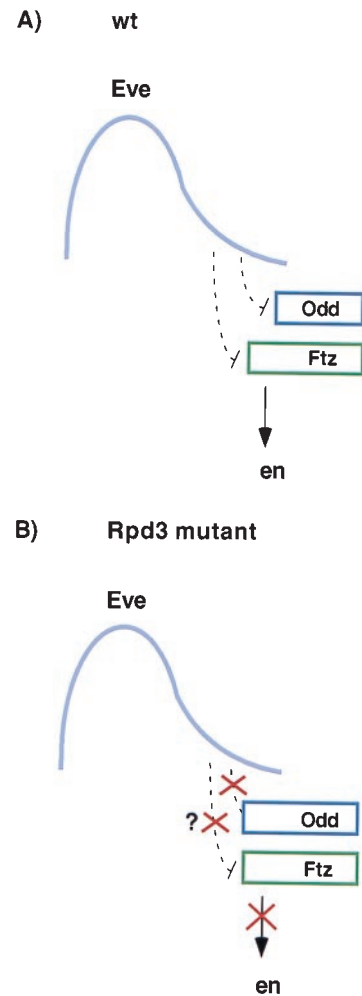

FIG. 5. Summary of segmentation defects in Rpd3 mutants. (*A*) The diagram represents a single Eve stripe in a wild-type gastrulating embryo. The stripe is asymmetric, with peak levels of Eve protein in anterior regions and progressively lower levels in posterior regions (6). Genetic studies suggest that this Eve gradient differentially represses the expression of *ftz* and *odd*, thereby generating overlapping but noncoincident *ftz* and *odd* expression patterns (7, 13). Ftz activates *en* in anterior regions lacking the Odd repressor (arrow). Odd restricts the expression of the even-numbered *en* stripes to a single cell. (*B*) In Rpd3 mutants, the *ftz* and *odd* patterns fail to resolve. Consequently, anterior cells in each Ftz stripe also express the Odd repressor, thereby attenuating the even-numbered *en* stripes. It is possible that Eve fails to repress *odd*, whereas *ftz* expression is unaffected. Alternatively, both the *odd* and *ftz* patterns might shift into more anterior regions. In either case, the two patterns coincide so that the Odd repressor blocks Ftz activity.

vitro translated Eve interacts with a glutathione *S*-transferase-Rpd3 fusion protein (data not shown). Because the Eve repressor is required for both the odd- and even-numbered *en* stripes, it would appear that the Rpd3 mutation does not cause a general loss of Eve function. For example, eve hypomorphs cause the loss of odd-numbered *en* stripes, whereas null mutations cause a loss of all *en* stripes (6, 12). It would therefore appear that Eve fails to repress certain promoters (e.g., *odd* and possibly *ftz*) in Rpd3 mutant embryos, but retains repressor function on other promoters (e.g., *paired* and *sloppy-paired*). This selectivity in the regulation of different target promoters is consistent with the notion that Eve mediates repression through multiple mechanisms, including the recruitment of corepressors and direct interactions with TBP (14–16). Multiple modes of repression may be mediated by other transcriptional repressors, such as Hairy, which appears to interact with different classes of corepressors (37, 41, 42).

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