

## Groucho augments the repression of multiple Even skipped target genes in establishing parasegment boundaries

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### SUMMARY

**Groucho acts as a co-repressor for several *Drosophila* DNA binding transcriptional repressors. Several of these proteins have been found to contain both Groucho-dependent and -independent repression domains, but the extent to which this distinction has functional consequences for the regulation of different target genes is not known. The product of the pair-rule gene *even skipped* has previously been shown to contain a Groucho-independent repression activity. In the Even skipped protein, outside the Groucho-independent repression domain, we have identified a conserved C-terminal motif (LFKPY), similar to motifs that mediate Groucho interaction in Hairy, Runt and Hückebein. Even skipped interacts with Groucho in yeast and in vitro, and *groucho* and *even skipped* genetically interact in vivo. Even skipped with a mutated Groucho interaction motif, which abolished binding to Groucho, showed a significantly reduced ability to rescue the *even skipped* null phenotype when driven by the complete *even skipped* regulatory region. Replacing this motif with a heterologous Groucho**

**interaction motif restored the rescuing function of Even skipped in segmentation. Further functional assays demonstrated that the Even skipped C terminus acts as a Groucho-dependent repression domain in early *Drosophila* embryos. This novel repression domain was active on two target genes that are normally repressed by Even skipped at different concentrations, *paired* and *sloppy paired*. When the Groucho interaction motif is mutated, repression of each target gene is reduced to a similar extent, with some activity remaining. Thus, the ability of Even skipped to repress different target genes at different concentrations does not appear to involve differential recruitment or function of Groucho. The accumulation of multiple domains of similar function within a single protein may be a common evolutionary mechanism that fine-tunes the level of activity for different regulatory functions.**

Key words: Repressor, Co-repressor, Embryogenesis, Segmentation, *Drosophila*

### INTRODUCTION

The development of multicellular organisms requires precise temporal and spatial control of gene expression. In *Drosophila*, segmentation is a well-studied example, wherein the embryo is subdivided along the anterior-posterior axis into repeating patterns of gene expression, which leads to the formation of morphological segments (Akam, 1987; Ingham, 1988). This process is controlled by a temporal hierarchy of zygotically active genes, the gap, pair-rule and segment-polarity genes (Nüsslein-Volhard and Wieschaus, 1980), in which spatial domains of expression are sequentially refined down to the single cell level (Fujioka et al., 1995; Manoukian and Krause, 1992). A pair-rule gene at the center of this process is *even skipped* (*eve*), which encodes a homeobox protein (Macdonald et al., 1986). Although like other pair-rule genes, it is expressed in a striped pattern that repeats every two segments, the *eve* null mutant phenotype at late embryonic stages is devoid of repeating pattern elements. This is due to the crucial role of *eve* in the establishment of all of the parasegment boundaries, which serve as organizational centers for the remainder of

embryonic development (DiNardo et al., 1994). The even-numbered and odd-numbered parasegment boundaries are set up by distinct mechanisms (DiNardo et al., 1988), but the Even skipped (*Eve*) protein is required in both cases (Goto et al., 1989) for activation of the segment polarity gene *engrailed* (*en*) in the proper cell rows. *en* then marks and helps to maintain each parasegment boundary throughout later development (DiNardo and O'Farrell, 1987).

Although *eve* is required to activate *en*, *Eve* has been shown to have a potent repression activity in cultured *Drosophila* cells (Han et al., 1989; Jaynes and O'Farrell, 1988), and to repress transcription in vitro (Li and Manley, 1998; Manley et al., 1996). The apparent paradox can be explained by considering intermediary genes in the segmentation cascade. *Eve* represses three genes (Fujioka et al., 1995) that themselves repress *en* (Cadigan et al., 1994; Grossniklaus et al., 1992; Manoukian and Krause, 1993), as well as one gene that is required to activate *en* (DiNardo and O'Farrell, 1987). The combination of these activities allows the activation of *en* in a single-cell-wide stripe at the edge of each *Eve* stripe (Fujioka et al., 1995; Morrissey et al., 1991).

An alanine/proline-rich repression domain was mapped in Eve using transient transfection assays (Han and Manley, 1993; Jaynes and O'Farrell, 1991), and similar repression domains have been found in other proteins (Hanna-Rose and Hansen, 1996). For one of these repressors, Engrailed (En), the domain responsible for activity in transient assays was shown to provide only part of the repression activity in vivo (Tolkunova et al., 1998), while the major activity in vivo was attributable to a conserved peptide, eh1. The eh1 domain showed strong activity both in vivo (Jiménez et al., 1997; Smith and Jaynes, 1996) and on stably integrated target genes in cultured cells, but was virtually inactive in transient transfections of the same cells (Tolkunova et al., 1998), suggesting that these repression domains have significant mechanistic differences. Similar conclusions have been drawn from studies showing that some repressors can act in vivo over greater distances than others (reviewed by Gray et al., 1995).

The eh1 motif is found in several classes of homeodomain proteins (Smith and Jaynes, 1996), where it functions by binding the co-repressor Groucho (Gro) to mediate repression activity (Jiménez et al., 1997; Jiménez et al., 1999; Mailhos et al., 1998; Tolkunova et al., 1998). The co-repressor function of Gro was first identified through its interaction with Hairy (Paroush et al., 1994). Subsequently, a diverse array of proteins with different types of DNA-binding domains have been shown to interact with Gro (Chen and Courey, 2000; Fisher and Caudy, 1998; Parkhurst, 1998; Paroush et al., 1997), including dTCF/LEF-1 (Cavallo et al., 1998) and Dorsal (Dubnicoff et al., 1997). Many of them have Gro interaction domains (GIDs) that contain sequence similarity either to the eh1 motif, such as Goosecoid and Pax proteins (Choi et al., 1999; Eberhard et al., 2000; Jiménez et al., 1999; Ren et al., 1999), or to the Hairy family's GID (Fisher et al., 1996). The latter group includes Runt domain proteins (Aronson et al., 1997; Levanon et al., 1998) and *Drosophila* Hückebein (Goldstein et al., 1999). In contrast, the repression domain of Eve mapped in transient transfections was shown to act independently of Gro (Jiménez et al., 1997).

In this report, we show that Eve possesses a second, previously unrecognized repression domain, similar to those of Hairy, Runt and Hückebein, which interacts with Gro, mediates a Gro-dependent repression activity in early embryos, and is crucial for the function of Eve in segmentation. We have tested whether this Gro-dependent activity is required on target genes whose expression patterns show a sensitive dose-dependent response to Eve, and find that it is responsible for part, but not all, of their repression. Thus, Eve utilizes its two repression domains in concert to repress multiple target genes.

## MATERIALS AND METHODS

### Plasmids

For rescue experiments, a construct containing the endogenous *eve* locus from -6.4 to +8.4 kb (Fujioka et al., 1999) was used. To create point or deletion mutants of the Eve C-terminal LFKPY motif, the region from +1093 to +1360 bp (*StyI* to *SpeI*) was amplified by nested PCR, subcloned and sequenced. Correctly mutagenized fragments were then transferred into the parental transgene vector. For yeast two-hybrid and His-tag pull-down assays, Eve C-terminal fragments were cloned into the vectors pAS2-1 or pET15b, respectively.

A Gro full-length cDNA in the vector pET3a (a gift from G.

Jiménez) was used in His-tag pull-down assays, while a Gro full-length cDNA in pACT2 (a gift from M. Caudy and A. Fisher) was used for yeast two-hybrid assays.

### Construction of *hb-Hairy<sup>Eve</sup>*

Sequences encoding either Eve amino acids 238-376 (*NdeI* to normal C terminus), for *hb-Hairy<sup>Eve</sup>*, or 238-366, followed by a stop codon, for *hb-Hairy<sup>EveΔ</sup>*, were fused to *hairy* sequences encoding amino acids 1-268, then inserted into pCaSpeR4-*hbh* (Goldstein et al., 1999; Jiménez et al., 1997).

### Protein interaction assays

Yeast two-hybrid assays and in vitro pull-down assays with GST-En and -En(F→E) were performed as previously described (Tolkunova et al., 1998). Recombinant derivatives of Eve containing a (His)<sub>6</sub> tag were incubated with in vitro translated <sup>35</sup>S-labeled Gro at 4°C, and pulled down using TALON<sup>®</sup> Metal Affinity Resin (Clontech). After washing (50 mM NaPO<sub>4</sub> (pH 7.0), 150 mM NaCl, 5 mM imidazole), bound Gro was eluted (50 mM NaPO<sub>4</sub> (pH 7.0), 300 mM NaCl, 150 mM imidazole) and analyzed by autoradiography following 10% SDS-PAGE. The relative quantitation of signals (Fig. 1) was calculated using Imagemaster (Pharmacia Biotech AB).

### *Drosophila* strains and embryo analysis

The alleles of *eve* used were *Df(2R)eve* and *eve<sup>3</sup>* (*eve<sup>RL3</sup>*), both null alleles, and *eve<sup>1</sup>* (*eve<sup>LD19</sup>*), a temperature-sensitive hypomorph. Mutations of *groucho* were: *gro<sup>E48</sup>*, a point mutant that is a strong hypomorph; *Df(3R)E(spl)<sup>BX22</sup>* (*gro<sup>BX22</sup>*), a deficiency encompassing *m5*, *m7*, *m8* and *gro*; and *gro<sup>E73</sup>*, a putative antimorph (gifts from S. Artavanis-Tsakonas).

P-element-mediated transformation was performed as described previously (Fujioka et al., 2000; Rubin and Spradling, 1982). Transgenic lines of *hb-Hairy<sup>Eve</sup>* inserted on the X-chromosome were maintained in males using an attached-X chromosome (*C(1)M3*); insertions on the autosomes were kept as unbalanced stocks, selecting in each generation for transformant males and non-transformant females. Five lines (two X-linked) were generated that contained *hb-Hairy<sup>Eve</sup>* and three lines (one X-linked) that contained *hb-Hairy<sup>EveΔ</sup>*. Each of these lines was crossed to *yw* females to assess female-specific lethality (progeny were scored for their sex and eye color). Two X-linked *hb-Hairy<sup>Eve</sup>* lines were crossed to *gro<sup>BX22</sup>* or *gro<sup>E48</sup>* mosaic females, so that all female progeny carried the transgene; eggs were harvested in 1-3 hour collections, dechorionated in bleach and fixed in 4% formaldehyde, PBS and heptane for 15 minutes.

Embryos were fixed and stained for in situ hybridization as described previously (Tautz and Pfeifle, 1989). This was followed by antibody staining with anti-Eve antiserum (a generous gift from M. Frasch). Biotinylated secondary antibodies were detected using streptavidin-conjugated horseradish peroxidase (Chemicon International), as described (Mullen and DiNardo, 1995). For Sex lethal immunohistochemistry, embryos were preincubated for 1 hour at room temperature in PBS, 5% normal goat serum (Sigma) and 0.1% Triton (United Technologies Packard); incubated overnight at 4°C with a monoclonal antibody specific to the female form of Sex lethal (Bopp et al., 1991) in PBS, 5% normal goat serum and 0.1% Triton; washed extensively in PBS and 0.1% Tween (Sigma); preincubated for 1 hour in PBS, 0.1% BSA and 0.1% Tween; incubated 2-4 hours with goat α-rabbit-alkaline phosphatase secondary antibody (Jackson Immunoresearch Laboratories); washed extensively in PBS and 0.1% Tween; stained in NBT/BCIP (Boehringer Mannheim); mounted in methacrylate (JB-4, Polyscience); and examined under Nomarski optics. For cuticle preparation, embryos were collected for 4 hours, aged (for either 18 or 32 hours) and processed without devitelinization, as described previously (Fujioka et al., 1999).

Embryos that lack maternal *gro* activity were derived from mosaic females with either *gro<sup>BX22</sup>* or *gro<sup>E48</sup>* mutant germline clones, obtained using the FLP-DFS technique (Chou et al., 1993). Males

carrying an *FRT [82B] ovo<sup>DI</sup>* chromosome and a *yw hs-FLP X*-chromosome were crossed to females carrying an *FRT [82B] gro* chromosome. Progeny of this cross were heat-shocked (37°C for 1.5 hours for *gro<sup>E48</sup>*, and 2.5 hours for *gro<sup>BX22</sup>*) on each of days 3, 4 and 5 after egg deposition (AED), and allowed to develop at 25°C. Non-heat-shocked females were sterile, as expected, and all developing eggs laid by heat-shocked females displayed a severe neurogenic phenotype, as expected for *gro<sup>mat-</sup>* embryos (Paroush et al., 1994; Schrons et al., 1992).

## RESULTS

### An Even skipped C-terminal conserved region interacts with Groucho

The Even skipped protein (Eve) has previously been shown to contain a Gro-independent repression domain (Jiménez et al., 1997). In a search of known repressors for sequence motifs similar to the previously identified Gro interaction domains (GIDs) in Hairy, Runt and Hückebein, we discovered that the Eve C-terminus contains such a motif (Fig. 1A, LFKPY), separated from the previously defined repression domain by over 100 amino acids. The motif is most similar to that in Hückebein, which also contains a phenylalanine at the first position of the core four amino acid motif (Goldstein et al., 1999), originally identified in the Hairy protein as WRPW (Fisher et al., 1996). Comparison with Eve homologs in other species has shown that the region immediately surrounding the motif is conserved (Fig. 1A).

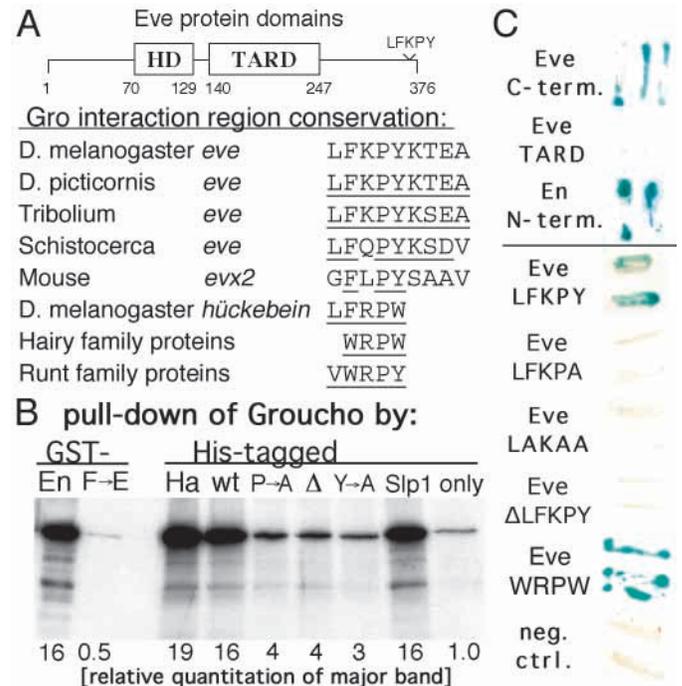
Both *in vitro* (Fig. 1B, lane 4) and in yeast two-hybrid assays (Fig. 1C) the Eve C-terminus binds Gro. To test whether the conserved LFKPY residues are important for the Eve-Gro interaction, we introduced point mutations into the GID, as well as deleting it altogether. Mutations in the GID abolished the interaction (Fig. 1B,C), as did similar mutations in the Hairy, Runt and Hückebein GIDs (Aronson et al., 1997; Fisher et al., 1996; Goldstein et al., 1999; Paroush et al., 1994). As we detect no residual interaction in the two-hybrid assay, we attribute the *in vitro* signals from the mutated proteins, which are close to background, to nonspecific interactions. Thus, Eve contains a conserved GID at its C terminus, separate from its Gro-independent transient assay repression domain (TARD).

### *even skipped* interacts genetically with *groucho*

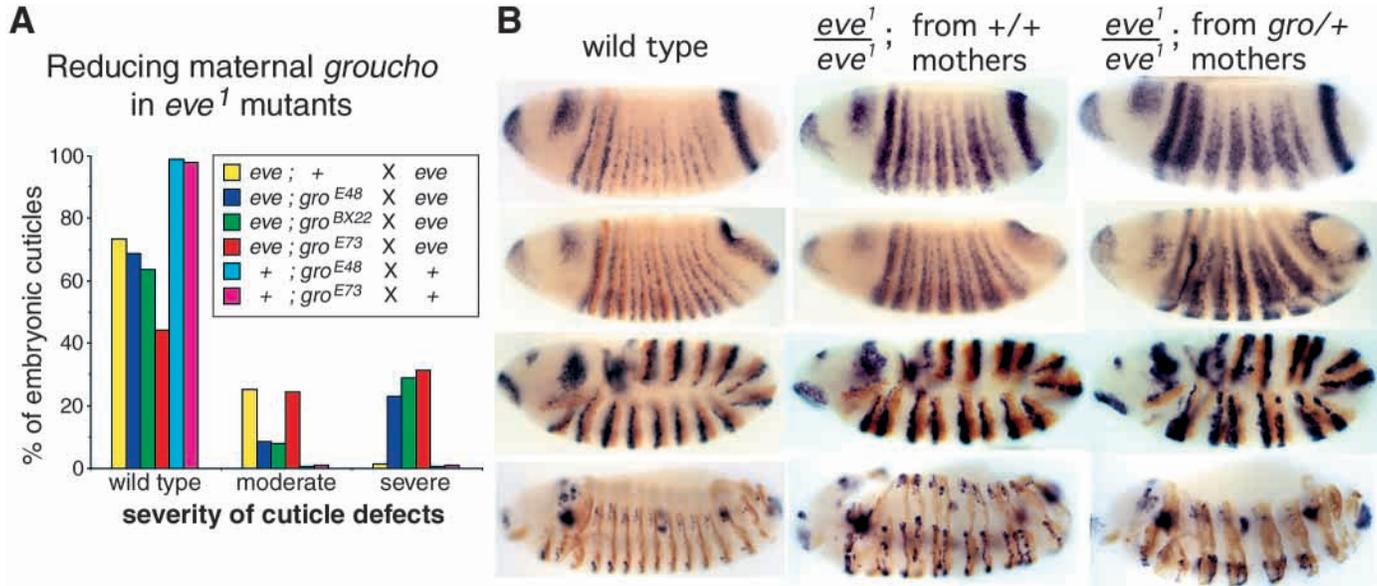
If the Eve-Gro interaction is functionally significant, we might see a dose-dependent genetic interaction in *Drosophila* embryos between the genes encoding these proteins. To test this, we used an *even skipped* (*eve*) hypomorphic allele (*eve<sup>1</sup>*, also known as *ID19*) in combination with several alleles of *groucho* (*gro*). This *eve* mutant encodes a protein with a single amino acid substitution in its homeodomain (Frasch et al., 1988), and therefore a normal C-terminal region (including the GID). We crossed females heterozygous both for *eve* and for *gro* to males heterozygous for *eve*. As *gro* has a strong maternal contribution, this reduces the amount of Gro supplied to each of the progeny. Compared with progeny from a cross of *eve* heterozygotes with normal maternal *gro* dosage, the severity of defects among the homozygous *eve* progeny was increased (Fig. 2A; data not shown). The degree of the effect increased with the strength of the *gro* allele used.

The *gro*-enhanced cuticle defects are pair-rule in nature,

affecting primarily the odd-numbered parasegments, and are similar to the defects seen in stronger alleles of *eve* (data not shown). Thus, they are consistent with Gro assisting Eve at the early stages of embryogenesis, during segmentation. This is also reflected in the patterns of expression of *en* and *wingless* (*wg*), two genes that are just downstream of *eve* in the segmentation cascade. Both patterns showed an increase in the severity of defects when *gro* function was reduced, over that



**Fig. 1.** A conserved Eve motif interacts with Gro. (A) Eve: the homeodomain (HD), transient assay-defined repression domain (TARD), which is Gro-independent, and the Gro interaction domain (GID, LFKPY) are shown, with corresponding amino acid numbers. The LFKPY region is conserved between Eve HD proteins, and is similar to GIDs in other protein families, as indicated. Amino acids that have high similarity to the *D. melanogaster* sequence are underlined. (B) Pull-down assay of full-length Groucho by His-tagged Eve and derivatives: positive control, GST-En (GST-tagged En; amino acids 1-348); negative control, F→E (GST-En with a single point mutation in the eh1 GID; F<sup>175</sup>→E; Tolkunova et al., 1998). *In vitro* transcribed and translated (labeled) Gro was mixed with equal amounts (400ng) of (His)<sub>6</sub>-tagged fusion protein containing either the Eve C-terminal region (amino acids 238-376) (wt) or the same region with these alterations in the LFKPY motif: HA, the Hairy family GID WRPW in place of FKPY; P→A, LFKAY; Δ, a STOP codon inserted just before LFKPY, removing the C-terminal 10 amino acids; Y→A, LFKPA; Slp1, the Sloppy paired 1 protein (which contains an eh1-like motif); or 'only', binding buffer alone. (C) Yeast two-hybrid assays using Eve peptides as bait (fused to the Gal4 DNA-binding domain) for full-length Gro (fused with the Gal4 activation domain). (Top) Bait was either the Eve C-terminal region as in B, or the Eve TARD, or the same En region as in B. (Bottom) Bait was either the wild type Eve C terminus (LFKPY), the same region with the indicated alterations or the mouse p53 protein as a negative control (neg. ctrl.). In each case, the Gro-coding region in pACT2 was co-transformed into yeast strain y190 with each of the bait plasmids (in pAS2-1). Each alteration in the Eve C terminus reduced the signal to background, except that the Hairy-like substitution resulted in an increased signal.



**Fig. 2.** Genetic interaction between *eve* and *gro*. (A) Effects of *gro* maternal gene dosage on the severity of pattern defects in *eve*<sup>1</sup> embryonic cuticles. The legend indicates the parental genotypes (female × male): females heterozygous for *eve*<sup>1</sup> (*eve*) or wild type (+) in *trans* to a wild-type 2nd chromosome, and simultaneously heterozygous for the indicated *gro* allele (or wild type, +) in *trans* to a wild-type 3rd chromosome were crossed with males heterozygous for either *eve*<sup>1</sup> or wild type, in *trans* to wild type. The graph shows the severity of cuticle defects in the progeny, grown at 18°C. 25% of the embryos are expected to be *eve* mutant, and when *gro* dose is wild type in the mother, they show moderately severe cuticle defects (fewer than four pairs of abdominal denticle bands fused; fewer than 2% had more severe defects, as the graph indicates). Note that as the effective maternal dosage of *gro* was reduced, by using stronger *gro* alleles (shown in order of increasing strength), the fraction of severely defective cuticles increased, and, concurrently, the total number of defective cuticles increased, while *gro* alone did not cause a significant fraction of cuticle defects. (B) A reduction in maternally supplied Gro reduces the activity of Eve in segmentation. All embryos in all figures are oriented anterior towards the left and dorsal upwards. Wild-type and *eve*<sup>1</sup>/*eve*<sup>1</sup> embryos were collected and stained for *wg* RNA (blue) and En protein (brown). *eve* mutant embryos were collected from *eve*/+ mothers either wild type for *gro* (2nd column) or *gro*<sup>E48</sup>/+ (3rd column). As gastrulation begins (top row), *wg* is ectopically expressed in the trunk, expanding within each domain of *eve* expression (Ingham et al., 1988). Note that the expansion is more severe when *gro* is reduced. During early germ band extension (2nd row), this *wg* pattern persists, as En begins to appear just posterior to each *wg* stripe (visualization of protein is slightly delayed relative to that of RNA; *en* RNA is induced concomitantly with *wg* in wild-type embryos). The posterior expansion of *wg* at the anterior edge of each *eve* domain shows how the odd-numbered parasegments (the primary *eve* domains) are reduced in width at later stages (full germ band extension, 3rd row, and retraction, bottom row). These defects are enhanced by reduction of maternal *gro*. The parasegment spacing defects apparently result from very early defects in cell fate specification (seen in the top two rows), which are a direct consequence of a reduction in early *eve* activity.

seen with the *eve* mutation alone (Fig. 2B). This effect is seen when *wg* and *en* are first initiated, and the defects are consistent with the known functions of *eve* in repressing *wg* and in setting correct parasegmental spacing during the process of segmentation (Frasch et al., 1988; Ingham et al., 1988). The defects seen at early stages persist to late stages of embryogenesis (Fig. 2B), consistent with the increased severity of cuticle defects seen when *gro* function is reduced.

### The Gro interaction domain is essential for normal Even skipped function

If a direct Eve-Gro interaction is physiologically relevant, then specifically mutating the GID should strongly reduce Eve function. Previous work identified a 16 kb region from the *eve* locus (Sackerson et al., 1999) that could completely rescue *eve*-null mutants (Fujioka et al., 1999). We introduced separately into this transgene the small deletion and the two single amino acid changes that abolished the Gro interaction *in vitro*, and assayed the rescuing activity of the resulting constructs. Several lines were examined for each construct, and in each case, the ability to rescue viable adults was completely or almost completely abolished (Fig. 3A).

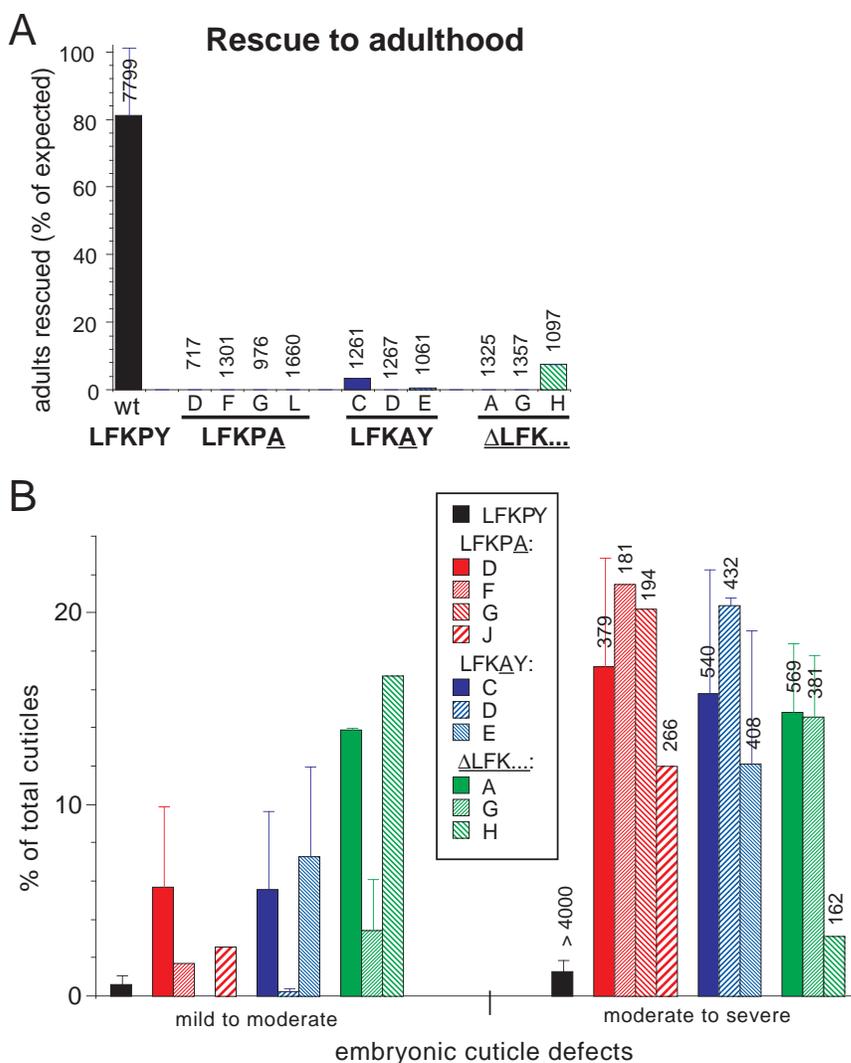
To determine whether the inability of GID-altered Eve to rescue *eve* mutants to adulthood was due to a deficiency in early Eve function, we examined embryonic cuticles from several lines (Fig. 3B). For the three GID mutants, the cuticle phenotype was similar to that of an *eve* hypomorph, with pair-rule defects in the pattern of abdominal denticle bands.

To rule out the possibility that the inability to rescue was due to aberrant protein accumulation, we stained embryos using polyclonal  $\alpha$ -Eve antiserum. In most of the transgenic lines, for each of the GID changes protein staining was at least as robust as with the wild-type rescue construct (data not shown), showing that none of the protein changes reduced protein stability to any noticeable extent. Overall, the analysis of GID mutants showed that without the ability to interact with Gro, Eve function during segmentation is seriously impaired.

### The GID mediates the genetic interaction between *groucho* and *even skipped*

If *eve* requires *gro* for its repressor function in segmentation, and if this requirement is mediated by the GID, then the rescuing ability of *eve* transgenes with mutations in the GID

**Fig. 3.** Alterations in the GID severely compromise the ability of Eve to rescue *eve* null mutations. (A) Rescue of *eve* null mutants to adulthood. Homozygous transgenic insertions expressing either the wild-type Eve-coding region (in the context of the entire *eve* locus) or a coding region with the indicated alterations in the LFKPY motif (altered amino acids are underlined) were crossed into two *eve*-null mutant backgrounds (*Df(eve)/eve<sup>3</sup>*, *eve<sup>3</sup>/eve<sup>3</sup>*, see Materials and Methods). Similar results were obtained for both; the graph shows the combined data. Rescued mutant flies were identified by their lack of the balancer marker (*Curly*). For wild type, the graph shows the average and range of seven independent insertion lines; for GID mutants, results are shown for individual insertion lines. The numbers above the bars are the total number of flies scored. Note that each of the alterations, which abolish the Gro interaction, cause Eve to rescue very poorly or not at all. (B) Rescue of the embryonic cuticle pattern. Embryos from the transgenic lines in A were collected within 23 hours AED, and cuticles were prepared. Abdominal denticle bands were counted and classified into the following categories: mild to moderate (fewer than three ventral abdominal denticle bands fused or deleted; most embryos in this category had two to three denticle bands deleted) and moderate to severe (three or more denticle bands fused or deleted; most had three to four denticle bands deleted). For wild type, the average and range of results from seven lines are shown, and in each case, the total number analyzed is shown above the bar. Note that the percentage of defective embryos is high for each of the mutants, but low for the wild-type lines.

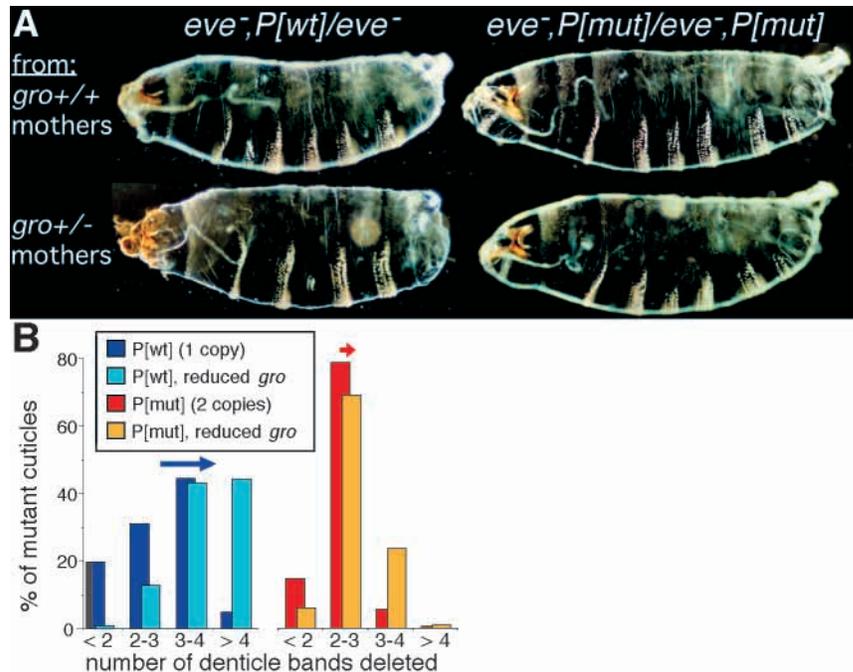


should not be further compromised by the genetic removal of *gro*. To test this, we crossed such altered transgenic copies of *eve* into an *eve*-null mutant background, and tested for a genetic interaction with *gro*, as before (Fig. 2). We compared rescue with two copies of the mutated transgene to one copy of the wild type transgene because these gave similar degrees of rescue, allowing us to directly compare their phenotypes. As Fig. 4 shows, a reduction of maternal *gro* caused an increase in severity of denticle band deletions when *eve* function was provided by the wild-type *eve* transgene. On average, more than one extra denticle band was deleted when *gro* was reduced. In contrast, when *eve* function was provided by transgenes encoding GID-mutated proteins, there was a considerably smaller effect of reducing *gro*. On average, less than an additional one-third of a denticle band was deleted per embryo in this case (see Fig. 4 legend for details). The latter genetic interaction may be due to an indirect effect of *gro* through other gene products such as Engrailed, which is known to interact physically and functionally with Gro (Jiménez et al., 1997; Smith and Jaynes, 1996; Tolkunova et al., 1998). Thus, the *eve-gro* genetic interaction can be attributed to a direct physical interaction between the proteins.

### The Even skipped C terminus is a Groucho-dependent, transferable repression domain

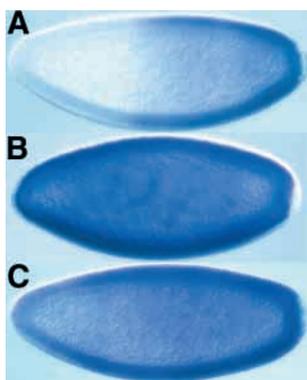
As Eve binds DNA (through its homeodomain) and represses transcription, we explored the possibility that the Eve C terminus constitutes a repression domain that acts to directly recruit the co-repressor Gro. We first determined that the Eve C terminus is a transferable repression domain, using an in vivo repression assay (Jiménez et al., 1997). Previous studies have shown that expressing the pair-rule gene *hairy* prematurely, under the *hunchback* (*hb*) promoter, brings about repression of *Sex lethal* (*Sxl*) expression in the anterior of syncytial blastoderm stage embryos (Parkhurst et al., 1990). Presumably, the Hairy protein mimics the activity of Deadpan, a known *Sxl* repressor (Younger-Shepherd et al., 1992) that is structurally related to Hairy (Bier et al., 1992). Because *Sxl* is required for proper dosage compensation in females, this causes female-specific lethality (Parkhurst et al., 1990). In this assay, Hairy also represses *Sxl* when its own repression domain is substituted for by heterologous repression domains (Jiménez et al., 1997). We therefore generated transgenic lines carrying a *hb-Hairy<sup>Eve</sup>* construct, in which the repression domain of Hairy was replaced with the Eve C-terminal 139 amino acids, including the GID (see Materials and Methods). As shown in

**Fig. 4.** The *eve-gro* genetic interaction is dependent on the GID. (A) Enhancement of *eve* partial rescue phenotypes by *gro*. On the left, cuticles of embryos with *eve* function provided by one wild-type transgenic copy (2nd chromosome genotype *eve*<sup>3</sup>, *P[Eve-wt]/eve*<sup>3</sup>) from mothers either wild type for *gro* (top) or heterozygous for *gro* (bottom, 3rd chromosome maternal genotype *gro*<sup>E48/+</sup>). On the right, *eve* function is provided by two transgenic copies of Eve without the GID (2nd chromosome genotype *eve*<sup>3</sup>, *P[Eve-ΔLFKPY]*). Note that the reduction in Gro more strongly enhances the wild-type rescued phenotype. (B) For each population of embryos described in A, cuticle phenotypes were categorized according to the number of abdominal denticle bands deleted. The graph shows how the distribution of phenotypes changed in response to the reduction in maternal Gro. The length of the arrow above the graph indicates, for each population, how much the average number of denticle bands deleted was changed by the reduction of Gro. Note that the response to *gro* dosage was strongly reduced by removal of the GID.



**Fig. 5A,** *Sxl* is efficiently repressed at the anterior of transgenic female embryos expressing this fusion protein. Importantly, this repression leads to a partial female-specific lethality (only 50-70% of the expected number of females survived to adulthood; data not shown). Thus, the Eve C-terminal region is a functional repression domain.

To test the Gro-dependence of the Eve C terminus, we

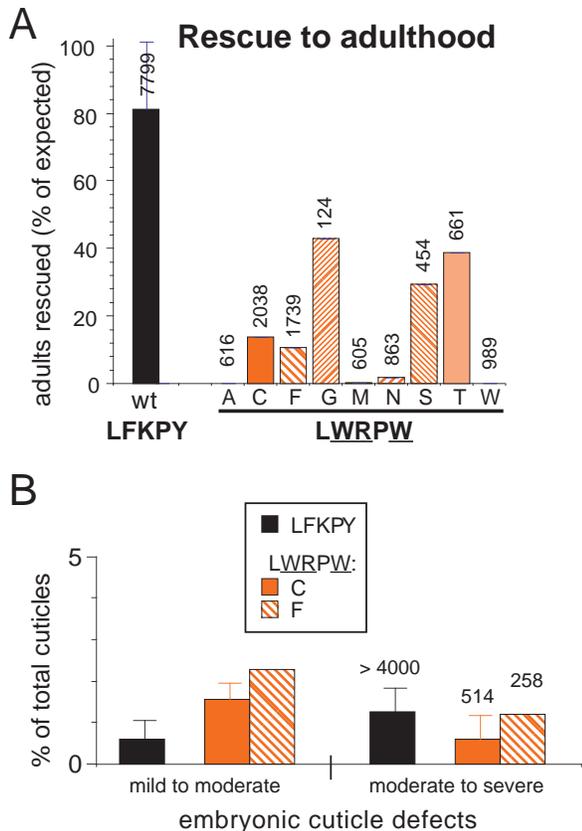


**Fig. 5.** The Eve GID acts as a *gro*-dependent repression domain in vivo. (A) Embryo carrying a *hb-Hairy*<sup>Eve</sup> transgene, stained for *Sxl* RNA. Note the repression of *Sxl* in the anterior (to the left), where *hb-Hairy*<sup>Eve</sup> is expressed in an anterior gradient. (B) Embryo derived from a *gro*<sup>E48</sup> mutant germ-line clone, which removes the maternal contribution of Gro (see Materials and Methods), carrying the same *hb-Hairy*<sup>Eve</sup> transgene as in A, stained for *Sxl* RNA. Note that in the absence of Gro, *Hairy*<sup>Eve</sup> is unable to repress *Sxl*. (C) Embryo carrying a *hb-Hairy*<sup>EveΔ</sup> transgene, stained for *Sxl* RNA. Note that when the LFKPY motif is removed from Eve, repression of *Sxl* is almost completely eliminated. The residual effect in these embryos is similar to the degree of *Sxl* repression seen with a *hb-Hairy* construct that lacks its repression domain (data not shown).

examined the ability of the transgene to repress *Sxl* in embryos lacking maternal *gro* (see Materials and Methods). *gro*<sup>BX22</sup> or *gro*<sup>E48</sup> germline mosaic females were crossed to males carrying an X-linked *hb-Hairy*<sup>Eve</sup>, thus ensuring that all female embryos inherit the transgene. Indeed, *Sxl* is expressed at full-level throughout all progeny examined (Fig. 5B). Notably, the Gro-dependent repression activity of Eve is mediated by the GID: when males carrying a chimeric *hb-Hairy*<sup>EveΔ</sup> transgene, in which the LFKPY motif is deleted, were crossed to wild-type females, full-level *Sxl* expression is seen almost throughout all female embryos (Fig. 5C), and no female-specific lethality ensues. Thus, the Eve C terminus requires an intact GID for its repressor activity, and this activity is completely dependent on Gro.

### Conversion of the Groucho-recruitment motif LFKPY to LWRPW

The Eve GID sequence is different from the Hairy GID, yet both can bind Gro in vitro, in yeast and in vivo. We therefore asked whether the two GIDs are functionally equivalent, or whether the differences in sequence reflect differing activities. First we tested whether WRPW could substitute for the Eve GID in vitro and in yeast. As shown in Fig. 1B, lane 3 (Ha) and Fig. 1C (Eve WRPW), the Hairy GID interacts more strongly with Gro than does the Eve GID. Second, we assayed for function in vivo, and found that an Eve<sup>WRPW</sup> transgene can rescue *eve* null mutants to adulthood (Fig. 6A). The degree of rescue, although certainly greater than the GID mutants that abolish the Gro interaction (compare with Fig. 3A), is not as high as with wild-type Eve. Importantly, the inability of Eve<sup>WRPW</sup> to rescue fully does not appear to be due to aberrant Eve<sup>WRPW</sup> function in segmentation, as embryos rescued by Eve<sup>WRPW</sup> showed very few cuticle defects (comparable to those rescued by wild type Eve, Fig. 6B). Thus, Eve<sup>WRPW</sup> is able to rescue embryonic development, but, perhaps because of the stronger binding to

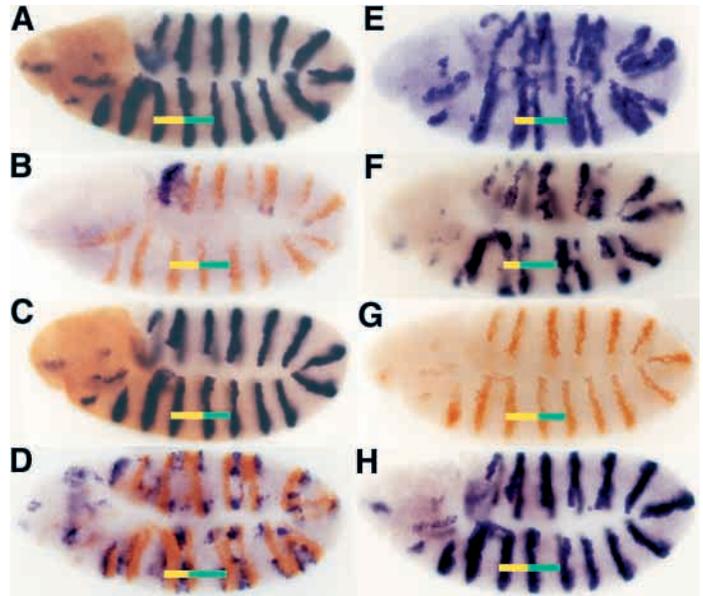


**Fig. 6.** The Hairy GID can substitute for the Eve GID in early Eve function. (A) Rescue of *eve* null mutants to adulthood. Homozygous transgenic insertions expressing either the wild-type Eve-coding region (in the context of the entire *eve* locus) or with the Hairy-like LWRPW in place of the LFKPY motif were crossed into two *eve* null mutant backgrounds (*Df(eve)/eve<sup>3</sup>*, *eve<sup>3</sup>/eve<sup>3</sup>*, see Materials and Methods). Similar results were obtained for each; the graph shows the combined data. Rescued mutant flies were identified by their lack of the balancer marker (*Curly*). For wild type, the graph shows the average and range of seven independent insertion lines (as in Fig. 3); for the Hairy substitution, results are shown for individual insertion lines. The numbers above the bars are the total number of flies scored. Note that while some WRPW-containing lines rescue to adulthood, they do not do so as efficiently or as consistently as does wild-type Eve. (B) Rescue of the embryonic cuticle pattern. Embryos from the indicated transgenic lines were collected within 23 hours AED, and cuticles were prepared. Abdominal denticle bands were counted and categorized as in Fig. 3. For wild type, the average and range of results from seven lines are shown, and in each case, the total number analyzed is shown above the bar. Note that the percentage of defective embryos is low for both the wild-type lines and the lines that carry the WRPW substitution (the scale here is much smaller than in Fig. 3B), indicating that embryonic Eve function is effectively provided by the Hairy GID.

Gro, is able only to rescue adult development partially (see Discussion).

### Groucho directly assists Even skipped in establishing correct parasegment spacing

When comparing the activities of altered proteins in their normal context in vivo, it is desirable to have a reliable dose-sensitive assay for function. *eve* is weakly haplo-insufficient,



**Fig. 7.** Effects of gene dose and the GID on the function of Eve in establishing parasegment spacing. In each panel, the yellow bar spans parasegment 3 and the green bar spans parasegment 4. (A) Embryo with one endogenous copy and two transgenic copies of *eve* (*eve<sup>3</sup>/CyO,hb-Z; P[Eve-wt]*) stained for *en* RNA (blue) and  $\beta$ -galactosidase protein (brown, balancer marker). The *en* pattern is indistinguishable from wild type: two transgenic copies provide the equivalent of one endogenous copy. (B) Embryo with one endogenous copy only (*eve<sup>3</sup>/CyO,hbZ*) stained for En protein (brown). Note that the odd-numbered parasegments are slightly narrower than the even-numbered ones. (C) Embryo with two endogenous and two transgenic copies of *eve* (*CyO,hb-Z; P[Eve-wt]*) stained as in A; the posterior extent of  $\beta$ -galactosidase staining indicates that it carries two copies of the balancer. Note that the odd-numbered parasegments are slightly wider than the even-numbered ones. (D) Embryo with one transgenic copy of *eve* only (*Df(eve); P[Eve-wt]/+*) stained for *wg* RNA (purple) and En protein (brown). Severe spacing defects result from *eve* activity equivalent to about 1/2 of one endogenous copy. (E) *eve* hypomorphic embryo (*eve<sup>1</sup>/eve<sup>1</sup>*, grown at 25°C), stained for *en* RNA. Note the similarity to D. (F) Embryo deficient for endogenous *eve*, rescued with two copies of a GID-mutated transgene (*Df(eve); P[Eve-LFKPA]*), stained for *en* RNA. Note the similarity to D,E, and the lack of rescue relative to G,H. (G) Embryo deficient for endogenous *eve*, rescued with two copies of a transgene with a Hairy-like GID (*Df(eve); P[Eve-WRPW]*), stained for En protein (brown). Parasegment spacing is almost normal, with some expanded odd-numbered parasegments, indicating more than wild-type activity. (H) Embryo deficient for endogenous *eve*, rescued with two copies of a wild-type transgene (*Df(eve); Eve-P[wt]*), stained for *en* RNA. Odd-numbered parasegments are slightly reduced, indicating activity about equal to one endogenous *eve* gene (as in B).

showing a reduced viability when only one copy is present (Nüsslein-Volhard et al., 1984). Although most single copy embryos have no apparent defects at the end of embryogenesis, most of them do have abnormally narrow odd-numbered parasegments at early stages of development (Fig. 7B). Conversely, introducing extra copies of *eve* using the rescuing transgene results in abnormally wide odd-numbered parasegments (Fig. 7C). The hypomorphic (pair-rule) *eve* phenotype arises from odd-numbered parasegments that are

severely reduced in width (Fig. 7E), and are unstable, so that at later stages they are eliminated by processes that repair patterning defects. Thus, one copy of the endogenous gene is near a threshold of sufficiency for generating stable parasegments, and the spacing of parasegments is a dose-dependent assay for early *eve* function.

Two copies of the wild-type *eve* transgene, inserted at various chromosomal locations (Fujioka et al., 1999), do not completely rescue the parasegment spacing defects of *eve* null mutants. Rather, a homozygous transgene phenocopies heterozygous *eve* (Fig. 7H versus 7B), while a single (heterozygous) *eve* transgene produces a hypomorphic phenotype (Fig. 7D). This provides a means with which to compare the activities of altered Eve proteins expressed in their normal pattern, in either one or two copies.

When we examined the early parasegment spacing in *eve* mutant embryos rescued by a homozygous transgene with a point mutation in the GID, we found that odd-numbered parasegments were reduced, closely resembling an *eve* hypomorph (Fig. 7F versus 7E). This was consistently observed with each of the GID mutants that abolished the Gro interaction (data not shown). In contrast, in transgenic flies in which the wild-type Eve motif was replaced with the Hairy GID, odd-numbered parasegments were normal, or sometimes

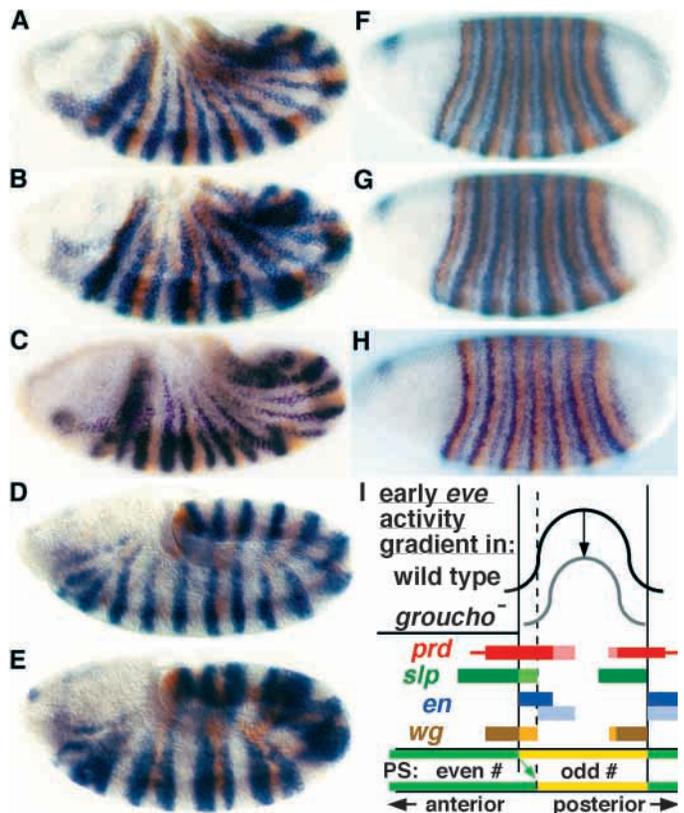
even slightly increased relative to even-numbered parasegments (Fig. 7G). Thus, the parasegment spacing parallels the relative strength of the in vitro interaction with Gro. The similarity in phenotype produced by one copy of the wild-type transgene and two copies of the GID-mutant transgene suggests that the activity of Eve is reduced about twofold by mutation of the GID. The more complete rescue by the Hairy GID-containing protein suggests that it has a stronger activity than does wild-type Eve, consistent with the in vitro interaction data of Fig. 1 and the embryonic pattern rescue (Fig. 6B). However, the apparently more complete rescue of embryonic pattern was not fully reflected in the ability to rescue to adulthood (Fig. 6A, see Discussion).

### Groucho acts with Even skipped on two target genes with distinct repression thresholds

What is the significance of the two distinct Eve repression activities, only one of which is dependent on Gro? Gro could be required to repress a subset of Eve targets, whereas repression of other target genes might be Gro independent. Alternatively, the two repression activities might function cooperatively, in which case both activities might be required for repression of each target gene. Extensive molecular and genetic studies have identified several target genes that are likely to be directly repressed by Eve. The best characterized of these genes are *sloppy paired* (*slp*), *paired* (*prd*) and *odd skipped* (*odd*). The posterior boundaries of expression of *slp* and *prd* correspond to the anterior and posterior borders, respectively, of the odd-numbered *en* stripes. As these *en* stripes shift posteriorly, both when the dose of *gro* is reduced and when the GID is mutated, the boundaries of *slp* and *prd*

**Fig. 8.** Eve and Gro collaborate to repress two target genes.

(A-E) Embryos were hybridized with probe for *slp* RNA (blue), followed by staining with  $\alpha$ -Eve polyclonal antiserum (brown). (F-H) Embryos were similarly probed for *prd* RNA and Eve protein. (A,D,F) Wild type. (B,E,G) *eve* deficient, rescued by two copies of a GID-mutated transgene (genotype *Df(eve)*, *P[Eve-LFKPA]*). Note the expansion of *slp* and *prd* expression anterior to each Eve domain (anterior is to the left in all cases). (C,H) *eve* deficient, rescued by two copies of a GID-altered transgene that preserves the Gro interaction (genotype *Df(eve)*, *P[Eve-WRPW]*). Note the rescue of near-normal *slp* and *prd* expression. (I) Model of how Gro assists Eve in establishing parasegment boundaries. Reduction of Gro concentration in the syncytial blastoderm reduces the repression activity of Eve, causing changes similar to a reduction in *eve* genetic dose or *eve* function (Fig. 7). Concentration-dependent effects at the anterior edge of each Eve stripe include expanded *prd* and *slp* expression (lighter colored bars). Prd activates both *en* and *wg*, while Slp represses *en*. Thus, Slp (and other *en* repressors, such as Runt, which is expressed similarly to Slp at this stage) can effectively subdivide the *prd* domain into *wg* and *en* domains. *wg* is repressed by both Eve and En. It expands posteriorly when *eve* activity is reduced (Fig. 2B), then maintains its border with the posteriorly shifted *en* stripe while contracting to one cell row at later stages (Figs 2B, 7D). The border between *wg* and *en* becomes the parasegment boundary, and the overall width of the parasegment is determined by the location of this border. The shifted parasegment boundary is indicated by a broken line. The net effect of reducing Gro or mutating the GID is to reduce the width of the odd-numbered parasegments, and to sometimes expand the odd-numbered *en* stripes, as *prd* sometimes expands more than does *slp*. This is also similar to what is seen in *eve* hypomorphs, i.e. it is not specific to a reduction in Gro. There may also be effects at the posterior border of the Eve stripe, but these appear to be relatively minor; for example, *ftz* expression does not appreciably expand in the absence of *eve* (Fujioka et al., 1995) and *odd* still clears from the anterior-most cell row of the *ftz* stripe (an *eve*-dependent effect) when Gro is reduced, allowing activation of the even-numbered *en* stripes. This still occurs both when Gro is reduced and in *eve* hypomorphs, so this function of *eve* is less concentration dependent than is repression of *prd* and *slp*.



may be coordinately shifted. We examined *slp* and *prd* expression in embryos rescued with a GID-mutated transgene. Both *slp* (Fig. 8B,E) and *prd* expression (Fig. 8G) were expanded in the *eve* domains, relative to wild-type embryos (Fig. 8A,D,F). The degree of expansion of each gene correlates with the shift of *en* stripes seen in Fig. 7F. Furthermore, both the width of individual *en* stripes and their spacing are very similar to those in *eve* hypomorphs, such as that shown in Fig. 7E. Thus, the removal of the GID has an effect that is similar to that of a general reduction of *eve* activity on both targets, *slp* and *prd*. This expansion of *slp* and *prd* expression was reversed, in each case, when the Eve GID motif was replaced by that of Hairy (Fig. 8C,H). These results suggest that Gro is required by Eve to a similar degree for its repression activity on each of these genes.

Repression of another Eve target gene (*odd*) is required for the establishment of the even-numbered (*ftz*-dependent) *en* stripes (Manoukian and Krause, 1992). Intriguingly, these are established more or less normally in embryos rescued by the GID-mutated transgene (Fig. 7F), and examination of *odd* expression in those embryos showed it to be normal in the even-numbered parasegments (data not shown). However, repression of *odd* and the establishment of even-numbered *en* stripes are also normal when *eve* function is reduced in other ways (e.g. in the hypomorph; Fig. 7E; data not shown), suggesting that a lower threshold of Eve activity is required for this *eve* function than for proper repression of *slp* and *prd*. Therefore, this assay did not allow us to fully assess the contribution of Gro to *odd* repression by Eve (see Discussion).

## DISCUSSION

Here, we describe the discovery of a Gro-dependent repression domain in Eve, a homeodomain protein previously known to contain a Gro-independent repression activity. An emerging theme is that many transcriptional regulators have multiple domains that use distinct mechanisms to achieve similar end results. However, little is known about the functional relationships between such domains in the regulation of target genes during development. Eve is a central component of the segmentation cascade in *Drosophila*, which establishes repeating patterns of gene expression along the anterior-posterior axis of the embryo, and several specific target genes regulated by Eve are known. This, in conjunction with the ability to functionally substitute altered Eve proteins for endogenous Eve, using a rescuing transgene (Fujioka et al., 1999), allowed us to analyze how the distinct repression activities of Eve function in the regulation of its target genes.

### A requirement for Groucho in developmental functions of Even skipped

We have shown that Eve contains a conserved motif at its C-terminus that resembles a subset of previously identified Gro interaction domains in Hairy, Runt and Hückebein. The C-terminal region of Eve interacts with Gro in vitro and in yeast, and a conserved pentapeptide is essential for the interaction (Fig. 1). The previously known repression domain of Eve, identified in cell culture assays (Han and Manley, 1993) and shown to be Gro independent (Jiménez et al., 1997), is not included in this C-terminal region. We established that Gro is

involved in Eve function in two ways: (1) by showing a genetic interaction between mutations in the two genes during embryogenesis (Fig. 2); and (2) by removing the GID in the context of a transgene capable of rescuing *eve* null mutants to viability (Figs 3, 7). By both criteria, the Gro interaction is crucial to the required level of Eve activity in embryos. Without it, embryonic development is seriously perturbed, and there is almost no rescue of *eve*-null mutants to adulthood (Fig. 3). We further showed that the genetic interaction is mediated by physical interaction between the two proteins (Fig. 4). We established that the GID mediates repression in early embryos, and that this repression is Gro dependent (Fig. 5). These studies set the stage for an investigation of how Gro and Eve function together on specific target genes during development.

### Groucho augments the repression of multiple Even skipped target genes

Previous studies have shown that the parasegment spacing defects of *eve* hypomorphs are the result of a reduced activity on at least two target genes, *slp* and *prd* (summarized by Fujioka et al., 1995; see also Raj et al., 2000). Eve represses each of these target genes, but within each early Eve stripe, which is about five to six nuclei wide, *prd* is expressed in a region of intermediate Eve concentration where *slp* is repressed. Thus, these two target genes are differentially sensitive to Eve's repression activity. We examined how these target genes responded when the GID was mutated, and found that both expand their expression domains into regions of higher Eve concentration (Fig. 8). However, in *eve*-null mutants, both expand throughout the entire *eve* domain of expression (Fujioka et al., 1995; Gutjahr et al., 1993), so clearly both are still repressed to some extent without the GID. As expected from the fact that Eve contains a strong Gro-independent activity in early embryos (Jiménez et al., 1997), removal of the Gro-independent domain also has a strong effect on repression of these target genes (M. F., G. Yusibova, and J. B. J., unpublished). Thus, both of the repression domains of Eve contribute significantly to its activity, and we find no evidence of differential Gro activity on these target genes.

Another target gene of Eve that is repressed at this same stage of development is *odd*. Repression of *odd* in the cells that give rise to the even-numbered *en* stripes is not apparently affected by mutating the GID. Thus, *odd* is formally a Gro-independent target of repression by Eve, in contrast to *prd* and *slp*. However, repression of *odd* is also insensitive to reducing the activity of *eve* genetically. Therefore, as *odd* is not a dose-sensitive target gene, either the Gro-dependent or -independent activity of Eve may be sufficient for complete repression of *odd*. Overall, in the early embryonic (segmentation) function of Eve, Gro may be a constitutive partner that contributes a substantial fraction of the repression activity on each target gene.

### Functional distinctions among repression domains

Substituting the GID from Hairy for that of Eve provides a similar function in several assays, although it appears that there is a subtle difference in their activities. The Hairy GID is fully functional in segmentation, where its activity appears to be somewhat stronger than that of the Eve GID, consistent with the relative strengths of the in vitro interactions with Gro (Fig. 1). In embryos, both segmentation defects (Figs 6, 7) and

the ability to hatch (data not shown) are rescued by the Hairy GID, and the expression of downstream target genes is restored to normal or near normal (Fig. 8). However, the Hairy GID, in the context of Eve, may be improperly regulated or hyperactive at a later stage of development. With the Hairy GID, the ability of Eve to rescue *eve* mutant flies to adulthood is impaired (Fig. 6). Thus, in addition to the Hairy GID having a stronger activity than that of Eve, these two GIDs may respond differently to regulatory inputs, particularly at post-embryonic stages of development, where Eve is known to function in the regulation of neuroblast proliferation (Park et al., 1998; Park et al., 2001).

It is intriguing that although the GID of Eve provides a substantial amount of activity in vivo, it was not identified as a repression domain in transient assays in *Drosophila* S2 cells, even though Gro is abundant in these cells, and the GIDs from Hairy (Fisher et al., 1996) and Runt (Aronson et al., 1997) were shown to have activity in such assays. This situation is similar to that with the two En repression domains, where the activity of the GID (eh1) was not apparent in transient transfections, but was apparent in the same cells when stably integrated target genes were assayed (Tolkunova et al., 1998), consistent with its substantial contribution to En function in embryos (Smith and Jaynes, 1996). Thus, GIDs may not function as effectively in transient transfection assays as they do in vivo, for reasons that are still unclear.

Despite the identification of many repressors and several corepressors, the ultimate targets of their action remain largely unknown. Consistent with the involvement of chromatin structure, histone deacetylases have been shown to interact with Gro family members, and to account for part of their repression function (Chen et al., 1999; Mannervik and Levine, 1999; Sun and Taneja, 2000).

In conclusion, Gro-mediated repression is an important aspect of the function of Eve, collaborating with the Gro-independent repression activity of Eve on multiple target genes. Their combined action during embryonic segmentation is required to provide a precise level of repression of each of the dose-dependent target genes for Eve.

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### Note added in proof

A recent study identified an 'LFKPY' motif in the Brinker protein that interacts with Gro (Zhang et al., 2001).

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