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An eh1-Like Motif in Odd-skipped Mediates Recruitment of Groucho and Repression In Vivo

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***Drosophila* Groucho, like its vertebrate Transducin-like Enhancer-of-split homologues, is a corepressor that silences gene expression in numerous developmental settings. Groucho itself does not bind DNA but is recruited to target promoters by associating with a large number of DNA-binding negative transcriptional regulators. These repressors tether Groucho via short conserved polypeptide sequences, of which two have been defined. First, WRPW and related tetrapeptide motifs have been well characterized in several repressors. Second, a motif termed Engrailed homology 1 (eh1) has been found predominantly in homeodomain-containing transcription factors. Here we describe a yeast two-hybrid screen that uncovered physical interactions between Groucho and transcription factors, containing eh1 motifs, with different types of DNA-binding domains. We show that one of these, the zinc finger protein Odd-skipped, requires its eh1-like sequence for repressing specific target genes in segmentation. Comparison between diverse eh1 motifs reveals a bias for the phosphoacceptor amino acids serine and threonine at a fixed position, and a mutational analysis of Odd-skipped indicates that these residues are critical for efficient interactions with Groucho and for repression in vivo. Our data suggest that phosphorylation of these phosphomeric residues, if it occurs, will down-regulate Groucho binding and therefore repression, providing a mechanism for posttranslational control of Groucho-mediated repression.**

Negative transcriptional regulation is a strategy that has been commonly selected in evolution for setting up and maintaining gene expression patterns. A striking case in point is the process of segmentation in the early *Drosophila* embryo. This developmental system is regulated almost exclusively by transcription factors, many of which are repressors that silence the expression of their targets (35; reviewed in reference 57). Mutations in genes encoding these transcriptional repressors lead to the loss of repressor activity that normally restricts the expression domains of downstream genes, causing disruptions in the metameric subdivision of the fly embryo.

One key principle to emerge from studies on *Drosophila* segmentation and on developmental processes in other model organisms is the fact that DNA-binding repressors in general do not operate on their own. Instead, they complex with nuclear coregulators, called corepressors, tethering them to promoters whose expression is subsequently blocked (43). Groucho (Gro), one such ubiquitously expressed corepressor that is highly conserved throughout evolution from worms to humans, has been shown to interact with and to potentiate the repressor function of a vast number of transcription factors, including many of those acting in segmentation (8). It is not fully understood how Gro and its Transducin-like Enhancer-of-split (TLE) mammalian homologues elicit transcriptional repression, although given that these corepressors associate with histones and bind histone deacetylases, they are likely to participate in establishing regional repressive chromatin structures (9, 23, 49).

Gro/TLEs do not bind DNA directly but, like other corepressors, are recruited to target promoters via protein interactions with specific transcription factors (8). Analysis of Gro/TLE-dependent repressors has revealed that short conserved polypeptide motifs mediate binding to Gro/TLE. The first of these motifs, WRPW, was recognized in Hairy and the Enhancer of Split basic helix-loop-helix proteins (51). WRPW-related tetrapeptide motifs, later found also in other *Drosophila* transcription factors such as Huckebein, Runt, Even-skipped, and Brinker and in their vertebrate homologues, bind Gro/TLE in vitro and are necessary for Gro-dependent repression in vivo (3, 26, 27, 33, 38, 40). The second motif, originally identified in the homeodomain-containing proteins Engrailed (En) and Goosecoid (Gsc) and referred to as En homology 1 (eh1), similarly mediates physical and functional interactions with Gro/TLE (36, 37, 56, 60). Sequences related to the eh1-like motif have been subsequently found in vertebrate homeodomain proteins involved in neural tube differentiation, as well as in Pax5, which relies on this motif for transcriptional repression in B-cell maturation (21, 42, 44).

In this report, we describe a yeast two-hybrid selection assay showing that eh1-related peptide sequences are present in a wide range of presumptive transcription factors belonging to different DNA-binding domain superfamilies. Importantly, these motifs are good predictors of a protein's ability to physically bind Gro. Taking the zinc finger (ZnF) Odd-skipped (Odd)

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segmentation factor as an example, we show that this protein contains a functional eh1-like sequence that associates with Gro and mediates repression in the early embryo. We further provide evidence that the phosphoacceptor serine (Ser) and threonine (Thr) residues, prevalent at a conserved position within the majority of eh1 motifs, are vital for efficient binding to Gro and for repressor function. A mutation replacing the Thr amino acid in the eh1 domain of Odd with the phosphomimetic aspartic acid (Asp) blocks both activities, leading us to propose that if phosphorylation of these Ser/Thr residues does occur, it is likely to downregulate the recruitment of Gro/TLE and, hence, repressor activity of Gro/TLE-dependent transcriptional repressors.

MATERIALS AND METHODS

Fly culture. Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract-agar medium at 24°C.

Germ line transformations. P element-mediated transformations were performed as previously described (26). The *hb-hairy^{odd}* transgene causes female-specific lethality, so X chromosome insertions of the *hb-hairy^{odd}* and *hb-hairy$^{odd\Delta eh1}$* stocks are therefore maintained in males with an attached X chromosome [*C*(*1*)*M3*]; insertions on the autosomes are kept as unbalanced stocks, with transformant males and nontransformant females selected for at each generation.

Plasmids. Molecular manipulations were conducted according to standard protocols. Full-length or partial cDNA fragments were generated by PCR amplification and subcloned, first into pBluescript (Stratagene) and, once sequenced, into appropriate vectors and sites. *hb-hairy^{odd}* and *hb-hairy$^{odd\Delta eh1}$* were constructed as described by Jiménez et al. (36). For overexpressing *odd* in embryos, cDNA fragments were inserted into the pCaSpeR4-hs vector. Point mutations in Odd were introduced by site-directed mutagenesis (QuikChange; Stratagene). Additional details are available on request.

In situ hybridization and antibody staining. For heat shock-inducible expression in *Drosophila* embryos, either *yellow white* (*yw*) control or homozygous transgenic males were mated to *yw* females and embryos collected over a 40-min period and then aged for 2 h and 20 min prior to a 10-min incubation at 36.5°C. Following recovery for 25 min at 25°C, embryos were dechorionated in bleach and fixed in 4% formaldehyde–phosphate-buffered saline (PBS)–heptane for 20 min. Patterns of target gene expression were visualized by whole-mount in situ hybridization with digoxigenin-UTP-labeled antisense RNA probes and anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche). Immunodetection of Sxl has been described before (26). Embryos were mounted in methacrylate (JB-4; Polysciences) and examined under Nomarski optics.

Yeast two-hybrid interaction assays. Yeast two-hybrid interaction assays were performed as previously detailed (51). A *Drosophila* embryonic cDNA library was screened with a bait comprising the C-terminal portion of Gro ($Gro_{251-719}$, Fig. 1A). Clones encoding 29 distinct Gro interactors were selected from over three million independent yeast transformants. Notably, some of these encode well-established Gro-dependent repressors such as Runt and several of the Hairy-related *E*(*spl*) basic helix-loop-helix proteins (3, 40, 51), validating our yeast selection protocol. Novel clones will be presented elsewhere.

GST pulldown assays. Glutathione *S*-transferase (GST) fusion proteins were expressed from the pGEX2T vector (Pharmacia) in a protease-deficient *Escherichia coli* strain (BL21/DE3) containing a plasmid for the overexpression of thioredoxin to enhance GST fusion protein solubility (63), either for 2 h at 30°C or overnight at 18°C, and purified on glutathione-agarose beads (Sigma) as previously described (26). [^{35}S]methionine-labeled Gro, synthesized with the Quick TNT-coupled rabbit reticulocyte lysate system (Promega), was added to each preincubation mixture, and the binding reactions were carried out for 3 h at 4°C. Beads were washed four times in 1 ml PBS–290 mM NaCl and boiled in sample buffer, and aliquots were examined by electrophoresis. Pulldown assays in Fig. 1 were performed with equal amounts of fusion proteins ($\sim$2 μg) bound to 50 μl of beads and preincubated for 1 h at 4°C in 1 ml of binding buffer (PBS, 0.34 M NaCl, 1 mM EDTA, 0.1% Igepal CA-630 [Sigma], 10% bovine serum albumin). For pulldown assays (see Fig. 4 and 5), preincubation and washing were carried out with PBS–5% glycerol–1 mM EDTA–1 mM dithiothreitol–0.1% Igepal CA-630. Coomassie staining confirmed the integrity and quantity of all GST fusion proteins, and autoradiography was used to detect the amount of retained Gro. Quantitative analysis of protein binding was performed on a Fujix BAS1000 phosphorimager with Tina 2.0 software.

RESULTS

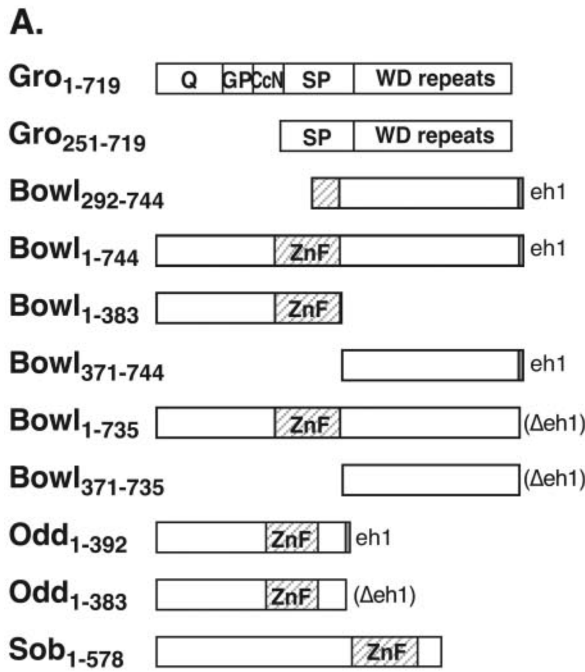
The ZnF proteins Bowel and Odd-skipped physically bind Gro via eh1-like domains. Gro regulates a large number of embryonic and postembryonic developmental processes by mediating the repressor activities of a myriad of negative transcriptional regulators (reviewed in reference 8). In a yeast two-hybrid screen aimed at identifying novel Gro partner proteins (see Materials and Methods), three clones encoding potential Gro-interacting proteins deriving from genes encoding known transcription factors that are required for normal embryonic patterning were isolated, i.e., *brother of odd with entrails limited* (*bowl*), *sloppy paired* (*slp*), and *dorsocross* (*doc*) (29, 32, 53, 62).

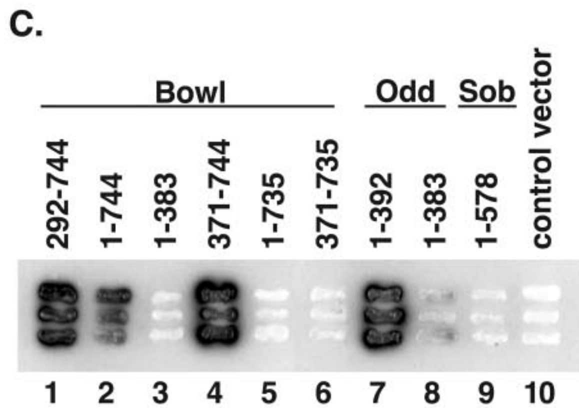
The Bowl protein is a member of a small family of transcription factors, all containing highly conserved C_2H_2 ZnF DNA-binding domains (Fig. 1A) (28, 32, 62). *bowl* was originally cloned based on its similarity to *odd-skipped* (*odd*), a pair rule segmentation gene (13, 32, 62); yeast two-hybrid and GST pulldown assays show that, like Bowl, the Odd protein also binds Gro (Odd_{1-392}; Fig. 1C and D).

We set out to delineate the Gro interaction interfaces in Bowl and Odd. The two proteins share high sequence similarity across their ZnF domains (87.5% identity) (32); however, these conserved regions do not mediate the interactions with Gro (Fig. 1). Accordingly, Bowl's N terminus, which includes the ZnF domain, does not associate with Gro ($Bowl_{1-383}$; Fig. 1A and C), whereas its C terminus, lacking the ZnF domain, effectively does so ($Bowl_{371-744}$; Fig. 1A and C). A closer inspection revealed several conserved amino acids at the extreme C termini of both Odd and Bowl that resemble the eh1 sequence in En (Fig. 1B), which was previously shown to bind Gro and to mediate repression in vivo (36, 60). Elimination of these eh1-like domains in Odd and Bowl leads to a marked reduction in their ability to bind Gro ($Bowl_{1-735}$, $Bowl_{371-735}$, and Odd_{1-383}; Fig. 1A, C, and D). Conversely, the eh1-like domain in Odd is sufficient to bind Gro in vitro, as a chimera comprising only the last 15 amino acids of Odd fused to GST ($Odd_{378-392}$) pulls down Gro, albeit less readily than full-length GST-Odd (Fig. 1D). Furthermore, a third Bowl and Odd family member of unknown function encoded by the gene *sister of odd and bowl* (*sob*) (32), which contains a highly related ZnF domain but lacks an eh1-like motif, fails to interact with Gro in yeast or in vitro (Fig. 1A, C, and D). Taken together, these results indicate that the eh1-like motifs of Bowl and Odd are both necessary and sufficient for binding to Gro.

An interaction between Odd and Gro is consistent with genetic studies showing that Odd acts as a repressor during segmentation (24, 45, 54). For this reason, and because Bowl is poorly characterized, we decided to focus on Gro's relationship with Odd.

Odd-skipped contains a potent repression domain at the C terminus. Odd's ability to complex with Gro in vitro indicates that its repressor activity depends on Gro. We therefore set out to establish whether Odd contains a functional repression domain and, if so, whether the eh1 Gro-binding sequence is an integral part of it. To this end, we made use of an embryonic in





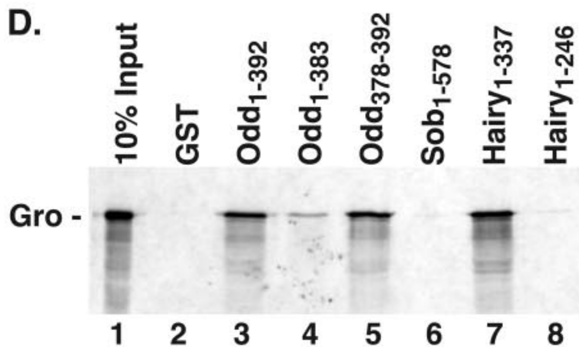


FIG. 1. The eh1 domains of Bowel and Odd-skipped mediate physical interactions with Gro in yeast and in vitro. (A) Schematic representation of full-length Gro, Bowl, Odd, and Sob and of their derivatives used in protein interaction assays. $Gro_{251–719}$ and $Bowl_{292–744}$ are the Gro derivative used as bait and the partial Bowl clone selected in the yeast two-hybrid screen, respectively. (B) Sequence alignment of the C termini of Odd and Bowl with the eh1 motifs of En and Gsc. (C and D) eh1 domains mediate the interactions with Gro in yeast and in vitro. (C) Activation domain-tagged proteins were tested for the ability to interact with LexA-$Gro_{251–719}$ in the yeast two-hybrid assay (51). $Bowl_{1-744}$, Odd_{1-392}, and Sob_{1-578} are full-length proteins (lanes 2, 7, and 9, respectively). $Bowl_{292-744}$ is the C-terminal region of Bowl selected in the yeast screen (lane 1). $Bowl_{1-383}$ (lane 3) includes the ZnF domain, whereas $Bowl_{371-744}$ (lane 4) does not. The C-terminal nine amino acids, and hence the respective eh1 domains, have been truncated in $Bowl_{1-735}$ (lane 5), $Bowl_{371-735}$ (lane 6), and Odd_{1-383} (OddΔeh1; lane 8). The pJG4-5 backbone expression vector was used as a negative control (lane 10). (D) GST pulldown assay. GST fusion proteins immobilized on glutathione agarose beads were incubated with [^{35}S]methionine-radiolabeled Gro and then washed of nonspecifically bound protein. Gro is specifically retained by GST-Odd (lane 3), GST-$Odd_{378-392}$ (Odd eh1; lane 5), and GST-Hairy (lane 7) but not by GST alone (lane 2), GST-OddΔeh1 (lane 4), GST-Sob (lane 6), or GST-$Hairy_{1-246}$ lacking its Gro-interacting WRPW motif (lane 8). Lane 1, 10% input of labeled Gro. Note that GST-$Odd_{378-392}$ (lane 5) is in vast molar excess of GST-Odd (lane 3) (not shown); thus, their respective affinities for Gro only appear to be equivalent.

vivo assay that determines the ability of any given polypeptide to act as a repression domain (36). Briefly, this assay is based on the silencing of the sex-determining gene *Sex-lethal* (*Sxl*) by the Hairy repressor. *Sxl* is normally expressed uniformly early only in females, but not in males. When *hairy* is misexpressed prematurely, under the regulation of the early *hunchback* (*hb*) promoter in the anterior half of the embryo, it brings about repression of *Sxl* in females (50). As Hairy-mediated transcriptional silencing depends on the binding of its C-terminal WRPW motif to Gro, truncation of the Hairy C terminus abolishes *Sxl* repression in this assay (36). However, when truncated Hairy is fused to a repression domain taken from another negative regulator, *Sxl* is repressed (21, 26, 33, 36–38).

We generated transgenic flies in which we replaced Hairy's C-terminal repression domain with the region in Odd harboring the eh1-like motif (residues 336 to 392, just C terminal to the ZnF region; $Hairy^{Odd}$ in Fig. 2A). As shown in Fig. 2B, $Hairy^{Odd}$ represses *Sxl* efficiently in the anterior region of transgenic female embryos, suggesting that Odd's C terminus comprises a potent repression domain. Furthermore, expression of this transgene also leads to full female-specific lethality, due to the role of *Sxl* in dosage compensation (not shown; 36, 50). Importantly, removal of just the eh1 domain ($Hairy^{Odd\Delta eh1}$; Fig. 2A) suppresses both repression of *Sxl* (Fig. 2C) and the associated female-specific lethality (not shown). These results reveal an inherent repressor activity in Odd that is dependent on the eh1-like Gro-binding domain.

Odd-skipped requires the eh1 domain for repression of specific segmentation genes. Embryos devoid of *gro* display a pleiotropic phenotype, owing to the widespread requirement for *gro* in early development (51). For this reason, we could not reliably establish whether the deregulation of Odd target genes seen in these mutant embryos (data not shown) is directly due to the failure of Odd to repress in the absence of Gro or

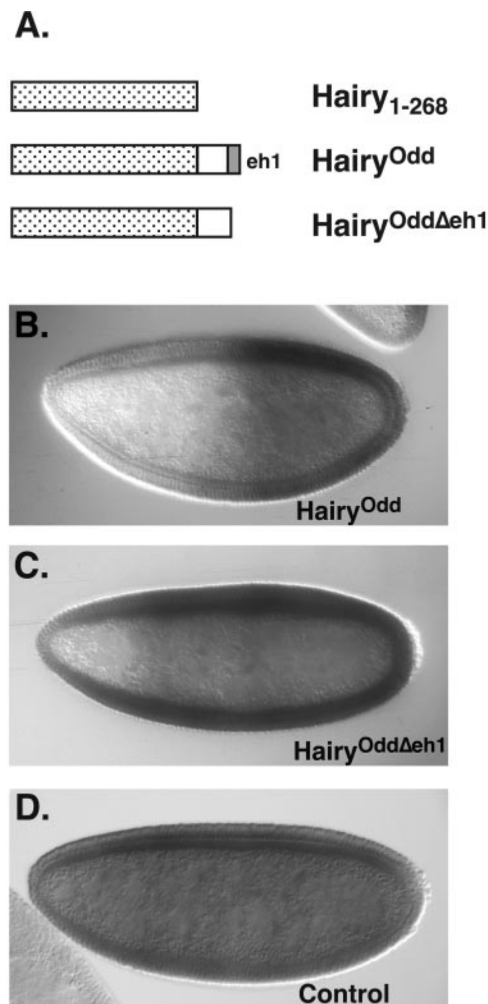


FIG. 2. Odd-skipped harbors a potent, transferable repression domain. (A) Schematic representation of the transgenes expressed in the embryonic Sxl repression assay (36). Stippling represents $\text{Hairy}_{1\text{–}268}$ lacking its C-terminal 71 amino acids and hence its WRPW Gro-binding domain; white indicates the C terminus of Odd; gray indicates Odd's eh1 domain. (B to D) Antibody staining for the female-specific form of Sxl. Shown are female embryos ectopically expressing the $\text{Hairy}^{\text{Odd}}$ (B) or $\text{Hairy}^{\text{Odd}\Delta\text{eh1}}$ (C) transgene in their anterior halves. (D) Wild-type control stained for Sxl. Note that the misexpression of $\text{Hairy}^{\text{Odd}}$ results in Sxl repression, whereas the removal of the eh1 domain leads to relief of repression. The residual repression observed in the anterior tip in panel C is also seen when $\text{Hairy}_{1\text{–}268}$ is expressed alone (not shown).

whether this is due to indirect effects. Thus, to determine whether Odd needs to associate with Gro in order to repress its endogenous target genes, we generated transgenic flies in which the *hsp70* promoter drives the ubiquitous expression of either native Odd (*hs-odd*) or an Odd derivative lacking its eh1 motif (*hs-odd*Δ*eh1*). Transgenic embryos were subjected to heat shock early in their development, which induced the ubiquitous accumulation of *odd* transcripts (data not shown). The ensuing cuticular phenotypes, as well as the resulting expression patterns of potential Odd downstream targets, were then assessed.

We observed a range of cuticular defects in experimental embryos, similar to those reported by others (54). In a typical experiment, about 80% of *hs-odd* embryos show classical pair rule or more severe compound segmental phenotypes (Fig. 3B), whereas the remaining 20% are largely unaffected. In contrast, >80% of heat-shocked *hs-odd*Δ*eh1* embryos appear completely normal, with the rest displaying subtle segmental defects (Fig. 3C). Thus, nearly all of the defects caused by the misexpression of *odd* are mediated by the eh1 motif.

We extended the above findings by staining gastrulating heat-shocked embryos for expression of putative Odd targets. During embryonic development, Odd represses transcription of several genes, including *engrailed* (*en*), *wingless* (*wg*), *gooseberry* (*gsb*), *even-skipped* (*eve*), and *paired* (*prd*) (5, 24, 45, 54). We find that *en*, *wg*, and *gsb* are rapidly and efficiently silenced by ubiquitously expressed Odd (Fig. 3E, H, and K), but not by ectopic $\text{Odd}^{\Delta\text{eh1}}$ (Fig. 3F, I, and L; compare with D, G, and J). Similarly, silencing of the anterior-most stripe of *eve* by Odd in early stage 5 *hs-odd* embryos is also eh1 dependent (Fig. 3M to O, arrows). Thus, repression of at least four Odd target genes is fully dependent on its eh1 Gro recruitment domain.

In contrast, we find that repression of *prd* by Odd appears partly independent of the eh1 motif. In wild-type cellularizing embryos, *prd* is expressed in 14 alternating primary and secondary stripes (Fig. 3P) (30). Whereas ectopic Odd weakens the intensity of primary *prd* stripes and completely blocks transcription of the seven secondary *prd* stripes, $\text{Odd}^{\Delta\text{eh1}}$ has no effect on the primary stripes but reduces the level of *prd* secondary stripe expression (Fig. 3Q and R, dots). Thus, $\text{Odd}^{\Delta\text{eh1}}$ retains some repressor activity, likely accounting for the minor cuticular segmentation defects observed in a small number of heat-shocked *hs-odd*Δ*eh1* embryos.

Clearly, the most plausible interpretation of the above results is that repression of Odd's segmentation targets depends almost entirely on its eh1 Gro-binding domain and, by inference, on the recruitment of the Gro corepressor.

Gro interacts with diverse types of transcription factors containing eh1-like motifs. The eh1 Gro recruitment domain was originally defined as a heptapeptide motif, which is conserved in members of the En family of homeodomain proteins and their vertebrate homologues (36, 56, 60). More recently, eh1-dependent binding to Gro has also been demonstrated in vitro for various other *Drosophila* and mammalian proteins, nearly all of which contain homeodomains (e.g., references 21 and 44). Given that Bowl and Odd, two non-homeodomain ZnF transcription factors, contain this motif and interact with Gro (Fig. 1), we explored the possibility that eh1 motifs are prevalent among additional non-homeodomain transcription factor families. Indeed, our unbiased yeast screen for Gro-interacting proteins selected two additional transcriptional regulators that contain eh1-like motifs, namely, Sloppy-paired (Slp; Forkhead related) and Dorsocross (Doc; T box) (Fig. 4A). Alignment of the eh1-like sequences of Bowl, Odd, Slp, and Doc with those of En and Gsc revealed three conserved amino acids: phenylalanine-x-isoleucine-x-x-isoleucine (Fig. 4A; Phe-x-Ile-x-x-Ile, where x is any amino acid). Subsequent database searches for presumptive *Drosophila* transcription factors containing this minimal peptide sequence identified a wide range of potential negative regulators belonging to different superfamilies as classified by their distinct DNA-binding domain types. Remarkably, eh1-related motifs have been preserved in many human homologues of these fly proteins (e.g., Fig. 4B), indicating that the ability to bind Gro/TLE has been

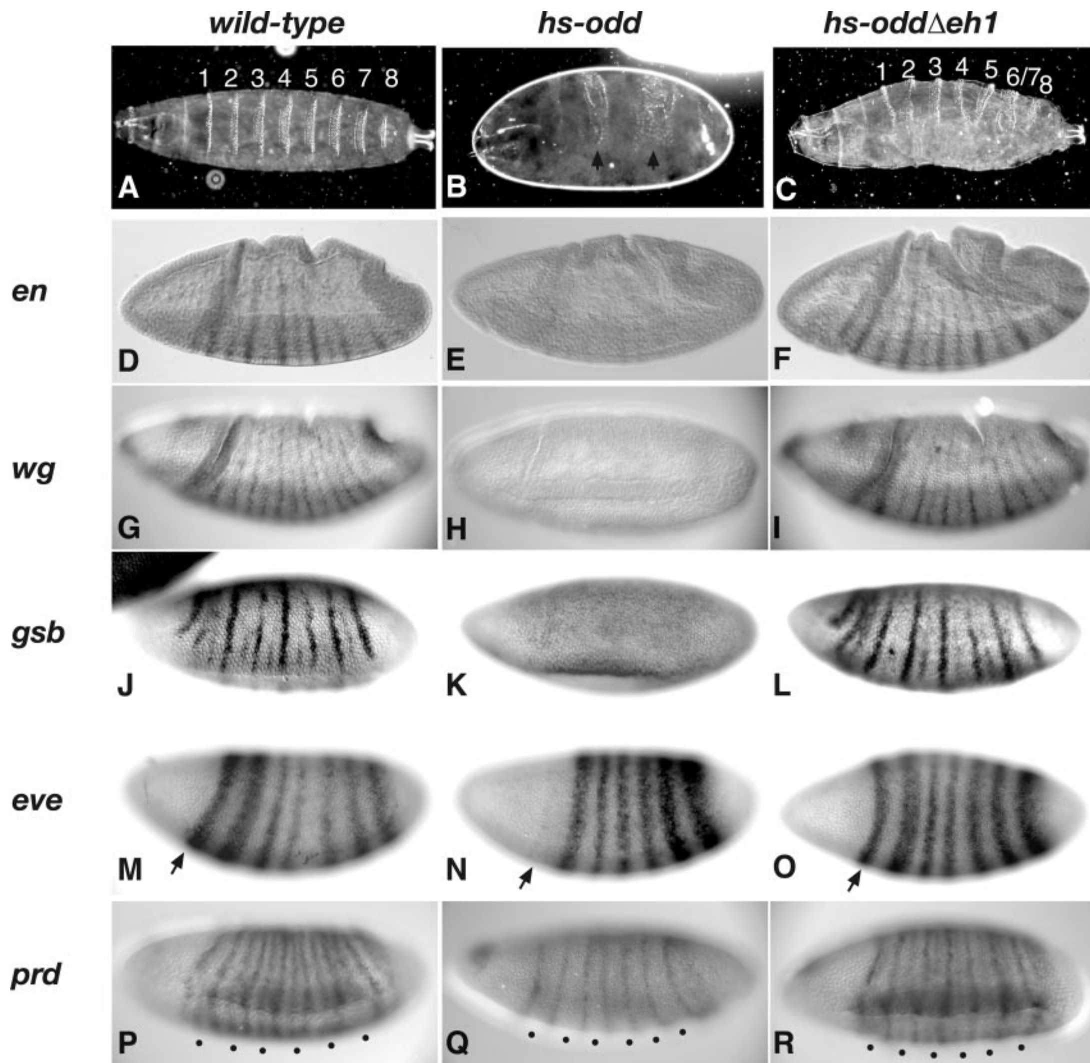


FIG. 3. Odd-skipped requires its eh1 domain for repressing its endogenous targets. Shown are cuticle preparations (A to C) and gene expression patterns (D to R) in stage 5 to 7 embryos. Panels: D to F, *en*; G to I, *wg*; J to L, *gsb*; M to O, *eve*; P to R, *prd*. Left column, wild-type embryos; middle column, transgenic *hs-odd* embryos; right column, transgenic *hs-odd*Δ*eh1* embryos. A high percentage of heat-shocked *hs-odd* embryos have segmental phenotypes, showing deleted or fused denticle belts (B, arrowheads; 80%; $n = 82$), whereas *hs-odd*Δ*eh1* embryos subjected to the same heat shock regimen are mostly wild type (not shown; 85%; $n = 100$), with the remainder generally showing partial deletions or fusions of denticle belts A2 and/or A6 (C). (E, H, and K) Misexpressed *odd* efficiently blocks *en*, *wg*, and *gsb* expression in stage 6 embryos. (F, I, and L) Most heat-shocked *hs-odd*Δ*eh1* embryos show normal gene expression patterns that are indistinguishable from those observed in heat-shocked *yw* controls treated identically in parallel (D, G, and J). *eve* stripe 1 is repressed in stage 5 embryos only by ectopic *odd* (N, arrow) but not by *odd*Δ*eh1* (O, arrow). *prd* secondary stripes are repressed by both ectopic *odd* and *odd*Δ*eh1* (Q and R, dots).

evolutionarily conserved in human transcriptional regulators and that this sequence may have been widely adopted throughout the proteome as a Gro recruitment domain.

We tested several representatives, corresponding to different transcription factor families, for the ability to bind Gro in biochemical assays. Where possible, we obtained full-length expressed sequence tags encoding these proteins; otherwise, we PCR amplified from genomic DNA single exons containing the eh1-like sequence. Each polypeptide was assessed for the ability to pull down radiolabeled Gro in vitro. As shown in Fig. 4C, GST-tagged Slp (full length; lane 1) and Doc (amino acids 254 to 391; lane 2) readily retain Gro, as do Eyes absent (Eya, full length; a novel nuclear protein; lane 3) and the homeodomain proteins Ventral nervous system defective (Vnd, 1 to 465), Bagpipe (Bap, 1 to 129), BarH1 (full length), and Empty spiracles (Ems, 1 to 360) (lanes 4 to 7), as well as the orphan nuclear hormone receptor DHR96 (full length; lane 8). To confirm that these interactions rely on intact eh1-related sequences, we mutated the eh1 motif of one of these—BarH1—by substituting glutamic acid for Phe at position 1, finding that its binding to Gro is reduced by >60% (Fig. 4D).

Based on our data, as well as on previous studies on En and Gsc (36, 37, 60), we conclude that the eh1 peptide sequence, found in various proteins that belong to a wide range of distinct transcription factor families, is a good predictor of Gro-binding capability in vitro. Moreover, our in vivo analysis of Odd's eh1 motif indicates that the above eh1 sequences impart Gro-mediated repression to a multitude of transcription factors.

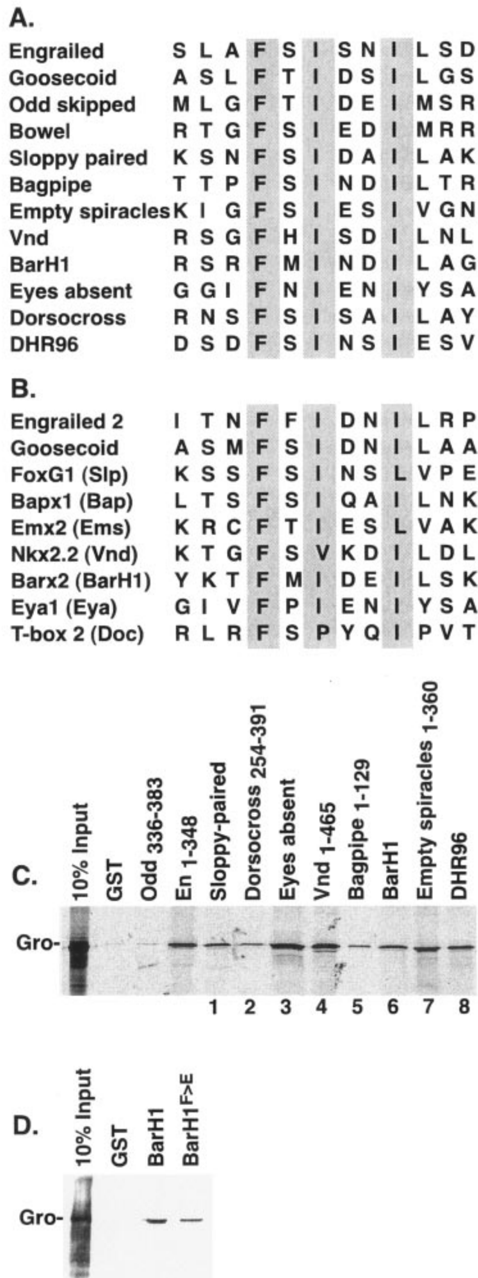


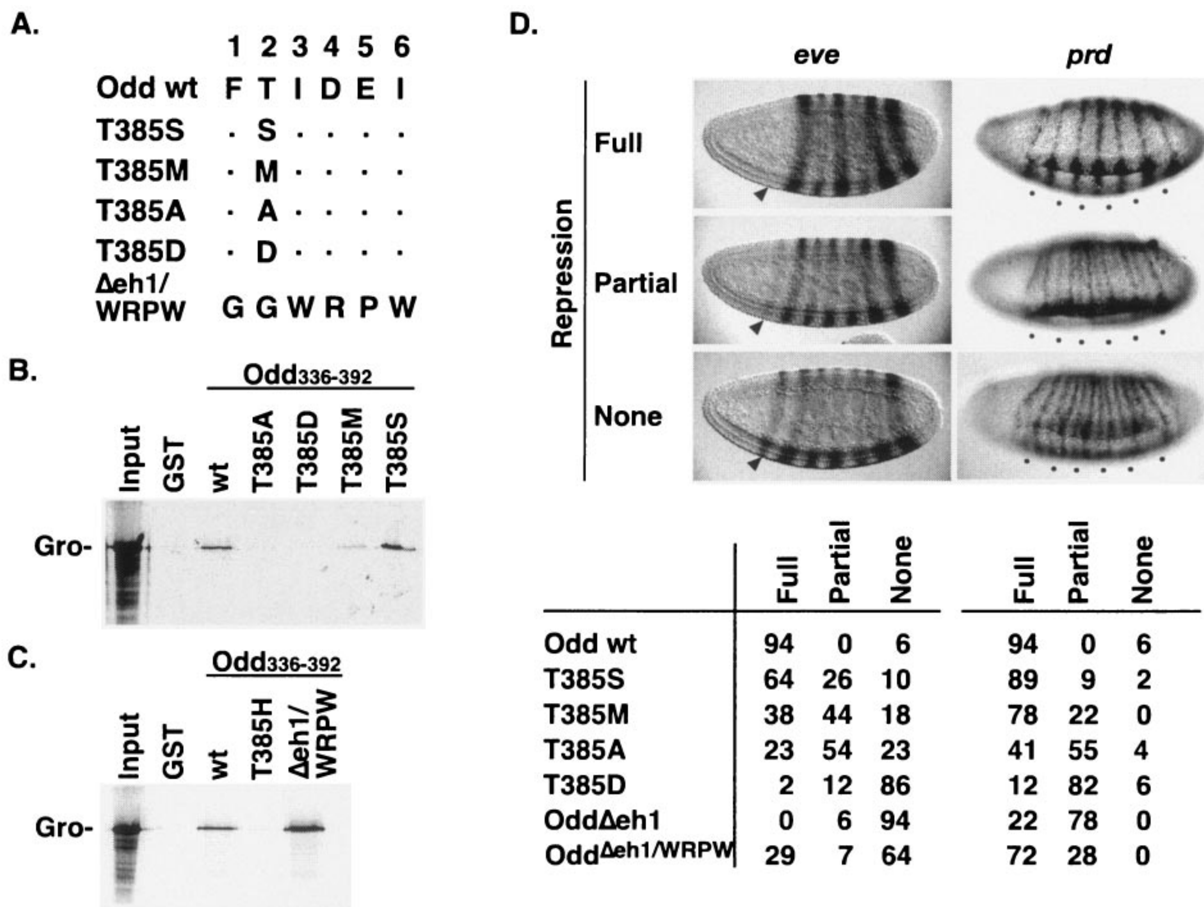
FIG. 4. Protein interactions between Gro and various transcriptional regulators containing eh1-like motifs. (A) Alignment of eh1-like sequences found in *Drosophila* transcription factors that bind Gro in vitro (C). In many cases, the eh1 motif has been conserved in related human proteins (B). (C) Several transcription factors containing eh1-like motifs, selected in an unbiased manner in yeast two-hybrid screens or in database searches in silica, were expressed in bacteria as GST fusion proteins and assayed for the ability to bind radiolabeled Gro. The GST-En_{1-348} chimera, comprising En's N terminus including its eh1 domain, served as a positive control. GST-$Odd_{336-383}$, lacking its eh1 domain, and GST alone are negative controls. (D) Pulldown assay showing that a mutation in BarH1's eh1 motif (Phe to Glu) significantly decreases its ability to bind Gro.

The amino acid threonine is an essential element of Odd-skipped's eh1 domain. Comparison of more than 80 different eh1 regions revealed several recurring features, such as the prevalence of negatively charged amino acids at positions 4 and 5 between the two Ile residues, as well as a striking bias (>60%) toward Ser/Thr residues in position 2 adjacent to the invariant Phe residue (Fig. 4A and B) (44). Given that phosphorylation of transcription factors has been well documented as a mechanism for regulating transcriptional outcomes (e.g., see reference 61), we questioned the importance of these potential phosphoacceptor amino acids for binding to Gro by replacing the Thr residue in Odd's eh1 motif (T385) with other amino acids (Fig. 5A). We find that the C-terminal 57-amino-acid portion of Odd binds Gro in a GST pulldown assay, whereas changes to alanine (T385A), methionine (T385M), or histidine (T385H) markedly reduce this interaction (Fig. 5B and C). In fact, the only construct tested that retains full binding to Gro is one in which Thr was changed to Ser (T385S) (Fig. 5B). Thus, the Odd-Gro interactions appear highly sensitive to amino acid modifications at residue T385.

To test if the above alterations affect Odd's repressor activity, we next generated flies carrying Odd derivatives harboring modifications in T385. These were expressed throughout embryos under heat shock control and tested for the ability to repress two Odd targets, namely, *eve* stripe 1 and the secondary stripes of *prd*, both of which are fully repressed in close to 100% of embryos expressing native Odd (Fig. 3N and Q and 5D). For simplicity of quantification, target gene repression was classified as "full," "partial," or "none," with results depicted as the percentage of embryos displaying the corresponding expression pattern (representative embryos shown in Fig. 5D). We find that expression of the Odd T385S transgene leads to strong repression of both targets, consistent with this variant's ability to bind Gro in vitro (Fig. 5B and D). In contrast, the T385A and T385M alterations brought about a significant loss of repressor activity, particularly of *eve* and less dramatically of *prd* (Fig. 5D). Thus, consistent with the biochemical protein interaction assay, a Ser/Thr residue appears to be a crucial element of the eh1 Gro recruitment domain for Odd, and presumably for other repressors as well.

We next examined what effect phosphorylation might have on Odd's repressor function by introducing a negative charge at this site through the exchange of Odd's Thr residue for Asp (T385D). As Fig. 5B shows, the C-terminal portion of Odd, containing the T385D alteration, does not associate with Gro in a GST pulldown assay. Furthermore, this pseudophosphorylated form of Odd is a much weaker repressor of *eve* stripe 1 and secondary *prd* stripes than T385M or T385A, being as ineffective as $Odd^{\Delta eh1}$ (Fig. 5D). This suggests that if the Thr residue in the eh1 domain of Odd is subjected to phosphorylation, this modification would probably block binding to Gro and transcriptional repression.

Gro recruitment domains are not simply interchangeable. We next tested if the eh1- and WRPW-like Gro recruitment domains are interchangeable in vivo, by exchanging the eh1 sequence in Odd for a WRPW motif ($Odd^{\Delta eh1/WRPW}$; Fig. 5A). The WRPW motif has been shown to confer repressor potential on a number of transcription factors (e.g., Gal4 and Notch; 22, 58), and in line with this, the $Odd^{\Delta eh1/WRPW}$ variant binds Gro vigorously in vitro (Fig. 5C). Unexpectedly, however, when misexpressed throughout the embryo, this transgene represses Odd target genes rather weakly (Fig. 5D). This result suggests that, at least with regard to Odd, the eh1 and WRPW motifs are not simply interchangeable. We surmise that Odd's



	eve Full	eve Partial	eve None	prd Full	prd Partial	prd None
Odd wt	94	0	6	94	0	6
T385S	64	26	10	89	9	2
T385M	38	44	18	78	22	0
T385A	23	54	23	41	55	4
T385D	2	12	86	12	82	6
OddΔeh1	0	6	94	22	78	0
Odd$^{\Delta eh1/WRPW}$	29	7	64	72	28	0

FIG. 5. The threonine residue within Odd-skipped's eh1 domain is essential for binding to Gro and for repression in vivo. (A) Shown is the core sequence of Odd's eh1 motif and the point mutations specifically introduced at the T385 position. (B and C) Pulldown assays of labeled Gro by GST-Odd$_{336–392}$ chimeras carrying mutations in their eh1 domains: T385A, T385D, T385M, T385S (B), T385H, and Δeh1/WRPW (C). Note that of the five T385 exchanges, only T385S does not disrupt the ability of the Odd eh1 domain to interact with Gro. wt, Odd's native eh1 domain. (D) Repressor potential of distinct Odd derivatives in the embryo. Native and mutant *odd* variants were misexpressed in early embryos under inducible *hs* promoter regulation as in Fig. 3. Also tested, in a similar manner, was the repressor capability of Odd$^{\Delta eh1/WRPW}$. Embryos were stained by in situ hybridization for the expression of Odd target genes. Particularly, transgenes were scored for the ability to bring about repression of *eve* stripe 1 (arrowheads) and the secondary *prd* stripes (dots), either in full (Full), only partially (Partial), or not at all (None). Representative embryos are shown in the upper panel, and the percentage of embryos displaying each respective expression pattern is presented in the tables below the corresponding column. Two or more independent transgenic lines were tested for each construct, and 40 to 100 embryos were scored for each target gene. wt, wild type.

eh1-like sequence necessitates a particular structural and/or conformational context, or the cooperation of other distinct domains within Odd, for presenting Gro in a way that will mediate efficient transcriptional repression in vivo.

DISCUSSION

Recruitment of Gro by repressors containing eh1-like motifs. Transcription factors bind their coregulators via short conserved polypeptide domains, and Gro-dependent repressors are no exception. Of the two Gro recruitment motifs identified to date, eh1 was recognized predominantly in homeodomain proteins. Notably, however, due to the limited sequence similarity between various eh1 motifs (e.g., Fig. 4A and B), their presence in transcriptional regulators belonging to other protein families had been largely overlooked. In this report, we have shown that eh1 motifs are prevalent among members of various transcription factor families and have further demonstrated that one of these, specifically, the ZnF protein Odd, requires its eh1 Gro-binding domain for blocking gene expression in vivo. While our work was in progress, three additional eh1-containing proteins, shown in this paper to complex with Gro in vitro, were found to behave as repressors in vivo, i.e., Slp (1, 2), BarH1 (41), and Vnd (16, 39). These reports independently support our conclusions and, along with the results presented here, suggest that a significant number of uncharacterized transcriptional regulators, containing eh1-related motifs, function as Gro-dependent repressors.

The alignment of various eh1 motifs reveals substantial degeneracy in this short polypeptide sequence. The amino acids Phe-x-Ile-x-x-Ile constitute a minimal consensus sequence for a subset of eh1 motifs (Fig. 4A); however, some mammalian homeodomain proteins have the amino acid Ile at position 3 or 6 replaced with valine or leucine or have histidine replacing

Phe at position 1 (Fig. 4B) (44). Furthermore, the Gro-binding octapeptide motifs of Pax2, -5, and -8 contain a Tyr residue in place of Phe (21). Future studies will help ascertain the full attributes of the eh1-like Gro-binding motif.

Interestingly, our database searches have also revealed eh1-like motifs within a number of cytoplasmic and membranal proteins. However, given that Gro is strictly a nuclear factor, we doubt that these proteins are involved in transcriptional regulation. We suggest that eh1-like sequences in nonnuclear proteins may serve as docking sites for cytoplasmic WD repeat proteins in a manner analogous to that described here for Gro.

Another region(s) in Odd, besides the eh1-like domain, must also be contributing to its effective binding to Gro and, consequently, to repression. Thus, for example, Gro's affinity for the full-length Odd protein is greater than its affinity for Odd's eh1 domain in isolation while, conversely, an Odd variant lacking its eh1 sequence still binds weakly to Gro (Fig. 1C and D). In line with this, a weak Gro-binding activity was mapped to Odd's N-terminal 181 amino acids, in which there are no identifiable WRPW- or eh1-like sequences; joint deletion of both this region and the eh1 domain completely abolishes Odd's binding to Gro (unpublished data).

It is indeed common to find a combination of two independent Gro-binding domains within the same protein, where each interacts with a different portion of Gro/TLE. For instance, Pax5 and Bap interact with Gro/TLE through sequences besides their octapeptide/eh1 domains, and AML1 lacking its WRPY motif retains some Gro-binding activity (11, 21, 40). Importantly, the occurrence of multiple Gro-binding sites within repressors might explain why mutations in some eh1 domains lead to surprisingly mild phenotypes (12, 16, 39). It is conceivable that synergistic interactions between multiple weak binding domains within repressors and in Gro/TLE provide composite interfaces that allow for high-affinity protein interactions and hence for robust repressor capability in vivo.

Gro mediates repression by Odd-skipped in segmentation. Odd acts at the heart of the transcriptional hierarchy in *Drosophila* segmentation. Mutations in *odd* lead to a pair rule phenotype (14, 20, 24, 45). Reciprocally, ectopic expression of Odd throughout early embryos also results in a pair rule phenotype, likely due to repression of *eve* (54; this study). Odd has been reported to directly repress the primary pair rule gene *h* and the segment polarity genes *en* and *wg*, and it delimits the expression domains of the pair rule gene *ftz* in even-numbered parasegments (24, 54). Moreover, some of the phenotypes brought about by ectopic Odd even resemble those observed when gap genes are mutated, indicating a broad role for *odd* at all levels of the segmentation cascade (54). Now we provide molecular and genetic data strongly indicating that Gro mediates Odd's repressor activity in segmentation.

Altogether, Gro-mediated repression is essential for the function of at least seven segmentation gene products (*hkb*, *h*, *run*, *eve*, *en*, *slp*, and *odd*) (1, 3, 26, 36, 38, 51, 60; this study). Interestingly, Gro is required by both Eve (38) and Odd (this study), two pair rule gene products that participate in the correct spacing of the 14 *en* expression stripes and, hence, set up parasegment boundaries in the segmented trunk of the embryo. In fact, all of the repressors encoded by the segmentation genes depend on Gro for their activity, with the exception of the trunk gap gene products, several of which rely on the CtBP corepressor (46–48, 59). Since Gro and CtBP act via qualitatively distinct mechanisms (15, 52, 64), some feature of Gro must be making it an optimal corepressor for pair rule and segment polarity gene products, but not for those encoded by the central gap genes.

In addition to embryonic segmentation, Odd also regulates the subdivision of the adult leg. Loss-of-function clones for either *odd* or *bowl* in the leg disc result in limb joint fusion, whereas ectopic Odd or Bowl expression leads to novel segment-like groove formation (19, 31). Future experiments will show whether, in this setting, Odd and Bowl act as DNA-binding repressors whose activities depend on Gro. A putative target gene, *bric-a-brac* (*bab*), responds to these genetic manipulations as expected if Odd and Bowl were acting as direct *bab* repressors (19). Intriguingly, however, the misexpression of Sob or of a fourth related family member protein, Drumstick (Drm), also affects leg development similarly to ectopic Bowl or Odd, despite the fact that neither Sob nor Drm interacts with Gro (Fig. 1C and D; unpublished results) (28, 31). Thus, Odd/Bowl/Sob/Drm family members may be regulating different developmental processes by distinct molecular mechanisms, some of which may be Gro independent. For example, when overexpressed in the adult leg, Drm is thought to sequester an inhibitor of Bowl, Lines, thus elevating the levels of nuclear Bowl (34).

Gro/TLE-dependent repression and human health. In spite of its clear role as a global developmental corepressor, it is surprising that mutations that disable Gro/TLE have yet to be linked to human disease; one possibility is that the extensive overlap in expression domains of the four TLE genes leads to functional redundancy. Interestingly, human relatives of several *Drosophila* eh1-domain proteins have been associated with disease. For example, aberrations in Emx2, an Ems homologue, result in schizencephaly (6, 25); Emx2 negatively regulates FGF8 in the anterior-posterior division of the cortex, possibly in a Gro-dependent manner. Strikingly, a recent screening of patients with septo-optic dysplasia, a pituitary disorder, uncovered an individual with a homozygous mutation in the eh1 motif of the homeodomain protein Hesx1, whose murine homologue requires Gro (7, 18). Here, an Ile-to-Thr mutation at position 6 impedes binding of Hesx1 to Gro/TLE, supporting an in vivo role for Gro-mediated repression in pituitary development (7).

Regulating recruitment of Gro. In addition to switching off gene expression, Gro-mediated repression also maintains transcription in a temporarily silenced state, until gene activation is required (referred to as "default repression") (4). In this case, a physiological or developmental switch triggers the relief of Gro-dependent repression. One way of tilting the balance in favor of an activator mode is by down-regulating the affinity of transcriptional repressors for Gro (e.g., see reference 10). In fact, Odd itself has the ability to activate transcription (54), indicating that its binding to Gro might be regulated. Our results point to two mechanisms that might impinge on the interactions between Gro and repressors containing eh1 domains, which may promote the transition from transcriptional repression to activation.

First, we find that Sob displaces Gro from Odd competitively. *sob* and *odd* are coexpressed during segmentation (32; Ryan Green, personal communication), and the proteins they

encode can form homodimers or associate as Odd-Sob heterodimers in vitro (data not shown). While Odd bears all the hallmarks of being a Gro-dependent repressor, Sob does not contain an eh1 motif and does not interact with Gro in vitro (Fig. 1C and D). In pulldown assays, Sob outcompetes Gro for binding to Odd, but not to Hairy or other control proteins (data not shown). A model that fits these data well is that Odd homodimers recruit Gro and silence target gene expression; subsequent associations between Sob and Odd lead to the removal of Gro, preventing Odd from acting as a repressor. Likewise, we find that Buttonhead (Btd), an activator that has been reported to physically bind Ems and modulate its activity in the embryo, precludes Ems from binding to Gro in vitro (55; our unpublished results). Additionally, competition between Gro and Armadillo over binding to dTCF/Pangolin has recently been demonstrated (17). It thus appears that the recruitment of Gro by repressors is subject to competing associations with other interacting proteins in a way that alters transcriptional output. We postulate that similar principles apply to the interactions between other transcription factors and their respective coregulators.

Second, our results suggest that phosphorylation of the phosphomeric Ser/Thr amino acids, found at a fixed location in the majority of eh1 motifs, is likely to obstruct repression in vivo. The Thr residue of Odd is required for binding to Gro in vitro and for repression in vivo and therefore appears to be an indispensable element of Odd's repression domain. Changing it to a phosphomimetic Asp residue results in an Odd variant whose compromised repression activity is comparable to that of Odd$^{\Delta eh1}$ (Fig. 5D). Thus, spatiotemporally restricted phosphorylation of an eh1 domain might serve as a dynamic molecular switch alternating between transcriptional activation and repression. Future studies will show whether Odd or other eh1 domain-containing transcriptional regulators undergo phosphorylation in a developmentally regulated manner in a way that affects their repressor potential.

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