INTRODUCTION

Cell rearrangement drives many morphogenetic processes. During vertebrate gastrulation, non-epithelial mesodermal cells intercalate and converge toward the midline in a process designated convergent extension, resulting in dramatic elongation of the embryonic axis (Warga and Kimmel, 1990; Keller et al., 1985). Rearrangement of non-epithelial cells also occurs during invertebrate development, including the formation of Drosophila ovarian terminal filaments and the migration of ovarian border cells (Godt and Laski, 1995; Montell, 1999).

Cells can also rearrange while remaining constrained within an epithelial sheet. This type of rearrangement is responsible for elongation of both the Drosophila germ band and the C. elegans dorsal epidermis (Irvine and Wieschaus, 1994; Heid et al., 2001). Similarly, epithelial cell rearrangement causes elongation of developing tubes, including the sea urchin archenteron, C. elegans intestine, and Drosophila posterior spiracles (Ettensohn, 1985; Leung et al., 1999; Brown and Castelli-Gair Hombría, 2000).

For a field of epithelial cells to change shape in a coherent and directed manner, cell rearrangement within the epithelium must be oriented. In the case of the elongating Drosophila germ band, this orientation depends on the patterning of the anteroposterior axis of the embryo (Irvine and Wieschaus, 1994). Later, however, during development of epithelial organs, cell rearrangement is oriented to newly established axes that are specific to the organ itself. Examples include the elongation of the insect Malpighian tubule relative to signals from the tip cell, epithelial migration during tracheal system development relative to localized FGF signals in surrounding mesoderm, and eversion of the leg imaginal disc in response to segmentally localized Notch signaling (Skaer, 1993; Bradley and Andrew, 2001; Rauskolb and Irvine, 1999). A central problem in epithelial morphogenesis, therefore, is to understand how the positional cues (i.e. spatial patterning) that orient cell rearrangement are established.

Elongation of the Drosophila embryonic hindgut epithelium occurs by a process of oriented cell rearrangement requiring the genes drummerstick (drm) and lines (lin). The elongating hindgut becomes subdivided into domains – small intestine, large intestine and rectum – each characterized by a specific pattern of gene expression dependent upon normal drm and lin function. We show that drm encodes an 81 amino acid (10 kDa) zinc finger protein that is a member of the Odd-skipped family. drm expression is localized to the developing midgut-hindgut junction and is required to establish the small intestine, while lin is broadly expressed throughout the gut primordium and represses small intestine fate. lin is epistatic to drm, suggesting a model in which localized expression of drm blocks lin activity, thereby allowing small intestine fate to be established. Further supporting this model, ectopic expression of Drm throughout the hindgut produces a lin phenotype. Biochemical and genetic data indicate that the first conserved zinc finger of Drm is essential for its function. We have thus defined a pathway in which a spatially localized zinc finger protein antagonizes a globally expressed protein, thereby leading to specification of a domain (the small intestine) necessary for oriented cell rearrangement.

Key words: Drosophila melanogaster, Hindgut, Cell rearrangement, Patterning, Zinc finger, Relief of repression

SUMMARY

Drumstick is a zinc finger protein that antagonizes Lines to control patterning and morphogenesis of the Drosophila hindgut

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members of the odd family, a ‘zinc-fingerless’ drm-specific probe was synthesized from a PCR-amplified fragment of the drm cDNA 3′UTR. The upstream and downstream primer sequences were 5′-CAAAACTCAGAACAACTA-3′ and 5′-GAGTTGTGTTGTGTGTT-3′, respectively. The specificity of this probe was confirmed by its inability to hybridize to embryos homozygous for the drm −/−deficiency (which deletes drm, but not soh, odd, or bowl). Other probes were made from cDNA templates of lin (Hatini et al., 2000), upd (Harrison et al., 1998) (also known as outstretched, os – FlyBase) and hh (Lee et al., 1992). For transverse sections, embryos were labeled with anti-Crb prior to mounting in Epon. Two μm serial sections were cut and stained with 0.01% Toluidine Blue, 0.025% Methylene Blue, 0.025% sodium tetraborate. Whole-mount embryos were analyzed with a Zeiss (Thornwood, NY) Axiopt microscope using differential interference contrast optics. Sections were analyzed by phase contrast microscopy. Images were acquired with a Sony DCK-5000 digital camera and embryos were staged based on morphology (Campos-Ortega and Hartenstein, 1997). Larval feeding assays were performed as described previously (Pankratz and Hoch, 1995).

Molecular manipulations

Standard techniques were used for molecularly characterizing the drm gene (Sambrook et al., 1989). The breakpoints of the drmP1 and drmM2 deletions were determined by plasmid rescue (3′ end) and inverse PCR (5′ end) from the residual P element. The drmP1 element insertion was localized by Southern blot and molecularly mapped by inverse PCR. Total RNA for northern blot was isolated from 0- to 17-hour embryos using TRIzol (Invitrogen), and poly(A) RNA isolated by PolyATtract (Promega). Probes were constructed by radiolabeling BamHI fragments of DS01379 P1 phage clone (Kimmerly et al., 1996) for Southern blot, or LD26791 EST clone (Research Genetics) for northern blot. The drm-specific primers used for 5′ and 3′ RACE were 5′-GATATGCTCGTGTGTTGTTGCT-3′ and 5′-CTTAAGCCCGGCAACAGTACACA-3′, respectively. Amplification was performed on RACE-adapted cDNA from 0 to 4-hour embryos using the Marathon cDNA Amplification Kit (Clontech).

To make constructs for GAL4-UAS expression, the following oligonucleotides were used:

(a) 5′-CAGTGATATCCAGATCACATACAGGCCTTGAAA-3′,
(b) 5′-GTCAAGATTCCAGATGCCCCGAAAGTTGCGCGTATT-3′,
(c) 5′-GTAAGATTCCAGATGCCCCGAAAGTTGCGCGTATT-3′,
(d) 5′-CTGAGGATTAACAGGCGGGTTTCTGCTGTA-3′,
(e) 5′-CTAGCTCGAATATGGTATGTGCCATTAAG-3′,
(f) 5′-GACTTCCTGAGTTACTGCGACCTCCGACACATCT-3′,
(g) 5′-CTGAGATTCCAGATGCCCCGAAAGTTGCGCGTATT-3′,
(i) 5′-CGTGATATCCAGATCACATACAGGCCTTGAAA-3′.

The various drm domains (see Fig. 6) were amplified from either cn bw sp cDNA or the LD26791 EST clone using the following restriction site-modified oligo pairs (cut sites underlined above): Drm (a and c); C-Drm (b and e); N-Drm (c and f). Amplified products were digested and subcloned into either BglII/XhoI-digested (for Drm) or EcoRI/XhoI-digested pUAST (Brand and Perrimon, 1993). Point mutations were created by site-directed mutagenesis with the QuickChange XL Kit (Stratagene) using the following oligonucleotides and their complements (altered nucleotides underlined above): R46C (h) and C57G (i). HA-tagged derivatives of each construct were made by incorporating the HA epitope sequence, in frame, into the PCR primers. A UAS-DrmEST construct expressing the full-length drm cDNA was also generated by subcloning the 2.1 kb XhoI fragment from LD26791 into pUAST. The gain-of-function phenotypes of UAS-Drm and UAS-DrmEST were indistinguishable (data not shown). The drm genomic DNA rescue construct was generated by subcloning the 18.1 kb SgrAI (blunt-ended)/NotI fragment from DS01379 into NotI/Hpal-digested pCaSpeR-4 (Thummel and Pirrotta, 1992). The drm-GAL4 construct was generated by

of subdomains) have been identified (Lengyel and Iwaki, 2002). In particular, the genes drumstick (drm) and lines (lin) are required for both patterning in the prospective small intestine and for the cell rearrangement that drives elongation of the hindgut epithelium. While drm is required to commit cells to the small intestine fate, lin represses this fate (Iwaki et al., 2001).

By analysis of single and double mutants, we show here that drm and lin interact genetically, and that lin is epistatic to drm. The lin gene encodes a novel, globally expressed protein that is thought to be a transcriptional regulator (Hatini et al., 2000). We show here that drm encodes a small, zinc finger protein expressed in a dynamic, spatially localized pattern that is consistent with the drm mutant phenotype. Exptopic expression and biochemical interaction studies indicate that Drm antagonizes Lin activity, probably through direct binding. Thus, a relief-of-repression mechanism allows expression of genes that define the small intestine. The specification of this domain is essential for the oriented cell rearrangement that elongates the hindgut.

MATERIALS AND METHODS

Fly stocks

Previously described mutant alleles used were: drmP1 (Liu et al., 1999), linP1 (Hatini et al., 2000), Df(2L)tim62 (Myers et al., 1995), and Df(2L)led (Reuter and Szidonya, 1983). The strong UAS-lin 8 (used here) and other weaker UAS-lin stocks (Hatini et al., 2000), the byn-GAL4 hindgut-specific driver (Iwaki and Lengyel, 2002), and the I-3/fkh-GAL4 driver (Fuss and Hoch, 1998) have been previously described. The drmP1 linP1 double mutant was generated by recombination. UAS-lacZ is from the Bloomington Stock Center (Indiana University, Bloomington, IN).

Generation of drm alleles

Df(2L)ldrmp1 and Df(2L)drmm2 were generated by mobilization of the l(2)k10101 P element (Török et al., 1993). New point mutations in drm were generated by ethyl methanesulfonate mutagenesis (Grigliatti, 1986). Briefly, mutagenized cn bw sp males were crossed to GluSM6b females; 17,757 F1 progeny chromosomes were tested for failure to complement either Df(2L)drmP1 or Df(2L)drmmP2. To minimize the effects of second site mutations, newly isolated drm chromosomes were recombined with ast1 dpmp6 bo ed1 dpmp1 cl1 and screened for cross-over events between ed (24D3) and dp (25A), thus replacing ~80% of the mutagenized chromosome. To identify altered nucleotides, new drm chromosomes were balanced over CyO-GFP (Casso et al., 2000) for selection and sequencing of homozygous genomic DNA. The three exons were amplified individually by PCR (performed in triplicate), subcloned into pCR2.1 (Invitrogen), sequenced in both directions, and compared to the cn bw sp parent chromosome.

Phenotypic analysis

Antibody staining was performed according to standard protocols (Ashburner, 1989) using the following antibodies: anti-Crumb (Crb, 1:100; labels the apical surface of ectodermally derived tissues including the hindgut) (Tepass et al., 1990) and anti-En (1:5) were obtained from the Developmental Studies Hybridoma Bank at The University of Iowa, Department of Biological Sciences (Iowa City, IA); anti β-gal (1:1000, Promega). In situ hybridization to whole-mount embryos was performed as previously described (Tautz and Pfeifle, 1989) using digoxigenin-labeled RNA probes (Roche Molecular Biochemicals). To avoid cross-reactivity with other
amplifying 7.1 kb of the drm promoter (primers d and g, cut sites underlined above), digesting with KpnI and BglII, and subcloning upstream of GAL4 in KpnI/BamHI-digested pGuTB (Brand and Perrimon, 1993). The 10.3 kb KpnI/NorI drm-GAL4 fragment was then subcloned into pCaSpeR-4.

Germline transformation
Transformation was performed using standard techniques (Ashburner, 1989). Briefly, constructs were coprecipitated at a 2:1 ratio with the pP[Delta2-3] transposase plasmid (Laski et al., 1986), resuspended overnight at 4°C in injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 7.8), and filtered through a 0.2 μm cellulose acetate Spin-X column (CoStar) before loading into needles pulled from 1 mm Kwik-Fil borosilicate glass capillaries (World Precision Instruments). DNA was injected using a Transjector microinjector (Eppendorf) into KpnI digestion sites of 1 g UAS-Myc-Lin, 2.5 μg UAS-HA-Drm (or Drm derivatives), and 3 μg ubiquitin-GAL4 in 600 μl volume. Transfected was removed after 12 hours, and cells were harvested 48 hours later. Individual plates of cells were lysed in 400 μl 2X injection buffer (5 mM Tris pH 7.5, 400 mM NaCl, 5 mM EDTA, 1% NP-40, 50 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1.4 μg/ml pepstatin) for 30 minutes and incubated with mouse anti-HA (1:40; monoclonal HA.11 clone 16B12, Covance). Antibody-antigen complexes were collected with Protein G Sepharose HA (1:40; monoclonal HA.11 clone 16B12, Covance). Antibody-rabbit anti-Myc primary antibody (1:1000; polyclonal c-Myc A-14; Santa Cruz Biotechnology) and HRP-conjugated anti-rabbit secondary antibody (1:3000; Vector Laboratory). Proteins were visualized by ECL Plus detection (Amersham Pharmacia Biotech).

RESULTS

lin is epistatic to drm
In both drm and lin embryos, the hindgut is wider and shorter than that of wild-type embryos (Fig. 1A-C) (Iwaki et al., 2001). Beyond this superficial similarity, however, drm and lin hindguts are quite distinct. The drm hindgut is smaller in diameter, and its epithelium consists of an undulating layer of columnar cells resembling those of the immature wild-type hindgut primordium (Fig. 1F). In contrast, the lin hindgut appears distended, consisting of a uniform layer of cuboidal cells similar in appearance to those of the wild-type small intestine (Fig. 1G). The strongly Crb-stained boundary cells, which form two parallel rows running the length of the large intestine, are duplicated in drm but are absent in lin hindguts (Fig. 1E-G) (Iwaki et al., 2001).

As revealed by gene expression studies, drm and lin hindguts are improperly patterned, with opposite effects on specification of the large and small intestine. Expression in the dorsal large intestine of engrailed (en) is retained in drm but absent from lin hindguts (Fig. 1I-K). Expression in the small intestine of unpaired (upd), encoding a ligand for the JAK-STAT pathway) is missing from drm but greatly expanded in lin hindguts (Fig. 1M-O). Similarly, expression in the small intestine of hedgehog (hh) is reduced in drm but greatly expanded in lin hindguts (Fig. 1Q-S). These data indicate that, in drm embryos, the large intestine is present and the small intestine is greatly reduced or absent; in lin embryos, in contrast, the small intestine is greatly expanded and the large intestine is missing.

Because their phenotypes are opposite and easily distinguishable, we used epistasis analysis to ask whether drm and lin interact genetically. By all criteria applied, the hindgut of the drm lin double mutant is remarkably like that of the lin single mutant. As in lin embryos, the drm lin hindgut is short and distended (Fig. 1D), consists of cuboidal epithelial cells, and lacks boundary cell rows (Fig. 1H). Similarly, engrailed expression is absent, and both upd and hh expression are expanded posteriorly (Fig. 1L,P,T). All of these observations taken together show that lin is epistatic to drm in the hindgut.

Since similarities exist in the genetic regulation of foregut and hindgut development (Pankratz and Hoch, 1995), we asked whether lin is also epistatic to drm in the foregut. In drm embryos, the proventriculus (a multi-layered valve-like structure at the foregut-midgut junction that forms by epithelial folding) does not form, and the foregut is long and narrow (Fig. 1V) (Liu et al., 1999). In both lin and drm lin embryos, the proventriculus also fails to form, but the foregut is short and wide (Fig. 1W,X). We conclude that lin is epistatic to drm in the foregut as well.

The gene expression and phenotypic data presented here and previously (Iwaki et al., 2001) demonstrate that lin represses and drm promotes the small intestine fate. Taken together with the epistasis of lin to drm, this indicates that drm specifies small intestine by antagonizing the repressive effect of lin. To understand the molecular basis for this relief of repression, we have molecularly characterized the drm gene and analyzed the interaction, both in vivo and in vitro, between Drm and Lin.

drm encodes a small zinc finger protein related to odd-skipped
The drm1 allele was originally identified as a spontaneously occurring lethal mutation mapping between 23F6 and 24A2 (Liu et al., 1999). drm was mapped more precisely by using P element mobilization to generate two overlapping deletions, drmP1 and drmP2 (Fig. 2A and Table 1). The left and right breakpoints of these deletions were defined molecularly (see Materials and Methods), thereby localizing drm to a 60 kb interval between tim and sob. Southern blot analysis of drm1 genomic DNA revealed an insertion within the 11.9 kb BamHI fragment, characterized by inverse PCR as an I element upstream of the predicted gene CG10016 (Rubin et al., 2000b). Three expressed sequence tag (EST) clones from this gene have been identified (Rubin et al., 2000a); the longest, LD26791, is 2.3 kb and contains three exons (Fig. 2B).

Surprisingly, the LD26791 EST contains only one small open reading frame (ORF) with two zinc finger motifs (Fig. 2D). To address the possibility that larger proteins might arise from this gene by alternative splicing, we characterized CG10016 transcripts by several approaches. A northern blot of embryonic RNA with probes from LD26791 identifies a single 2.5 kb transcript, similar in length to the EST clones (Fig. 2C). RT-PCR with intron-spanning primers failed to identify any splice variants (data not shown). An alternative first exon was identified in a small fraction (~10%) of 5’ RACE products, and several alternative poly(A) addition sites were identified by 3’ RACE; none of these variants affects the ORF length.

Sequence of all EST clones, RT-PCR products, 5’ and 3’ RACE
**Table 1. Mutations in the drm gene**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation</th>
<th>Location</th>
<th>Gut defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>drm¹</td>
<td>I element insertion*</td>
<td>35 bp upstream of transcription start</td>
<td>PV, HG</td>
</tr>
<tr>
<td>drm²</td>
<td>R46H (CGC→CAC)¹</td>
<td>1° zinc finger</td>
<td>PV</td>
</tr>
<tr>
<td>drm³</td>
<td>roo element insertion²</td>
<td>At exon 2 splice donor site</td>
<td>PV, HG</td>
</tr>
<tr>
<td>drm⁴</td>
<td>E45K (GAG→AAG)³</td>
<td>1° zinc finger</td>
<td>PV</td>
</tr>
<tr>
<td>drm⁵</td>
<td>E25K (GAG→AAG)³</td>
<td>1° zinc finger</td>
<td>PV</td>
</tr>
<tr>
<td>drm⁶</td>
<td>R46C (CGC→TCC)⁴</td>
<td>1° zinc finger</td>
<td>PV, HG</td>
</tr>
<tr>
<td>Df[2L]drm²¹</td>
<td>97 kb deletion from l(2)k10101 through drm¹</td>
<td>Deletion of drm</td>
<td>PV, HG</td>
</tr>
<tr>
<td>Df[2L]drm²²</td>
<td>165 kb deletion from l(2)k10101 through l(2)k06860⁰</td>
<td>Deletion of drm, sob and odd</td>
<td>PV, HG</td>
</tr>
</tbody>
</table>

Generation of drm mutations and phenotypic characterization are discussed in text. PV, proventriculus; HG, hindgut.

*See Fig. 2B for insertion location.
¹Altered nucleotide is underlined, see Figs 2D and 3C for residue location.
²See Fig. 3C for insertion location.
³See Fig. 2A for deletion breakpoints.

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**Fig. 1.** lin is epistatic to drm. Whole-mount embryos were stained with anti-Crb (A-H,U-X) or anti-En (I-L), hybridized in situ with probes for upd (M-P) or hh (Q-T), or fixed and sectioned transversely (E-H). The wild-type hindgut (A) consists of three morphologically distinct domains: small intestine (SI), large intestine (LI), and rectum (RE). Boundary cell (BC) rings, labeled strongly with anti-Crb, separate the SI and LI anteriorly and the LI and RE posteriorly. Two boundary cell rows also run the length of the LI dividing it dorsoventrally, as seen in transverse section (two arrows in E). In drm embryos (B), the SI is missing, the cells of the LI are columnar, and the BC rows are duplicated (four arrows in F). In both lin (C,G) and drm lin (D,H) embryos, the LI is missing, the cells are cuboidal, and the BC rows are absent. In wild-type embryos, En is expressed in the dorsal portion of the LI (I, black arrowhead). In drm embryos (J), En is expressed throughout dorsal hindgut (black arrowhead), but is not expressed in the rectum. In lin (K) and drm lin (L) embryos, En-expressing cells are not present (white arrowhead). In wild-type embryos (M, arrowhead), upd is expressed within the SI. In drm embryos (N), upd is not expressed (white arrowhead). In lin (O) and drm lin (P) embryos, expression of upd is expanded throughout most of the hindgut (wide arrowhead), but not the RE. In wild-type embryos, hh is expressed in both the SI and RE (Q, arrowheads). In drm embryos (R), hh is expressed in the RE, the ventral portion of the LI, and at the junction between the hindgut and midgut (arrowheads). In lin (S) and drm lin (T) embryos, expression of hh is expanded throughout most of the hindgut (wide arrowhead), but not the RE. In Crb-stained wild-type embryos (U), the proventriculus (PV) forms a multi-layered valve-like structure between the esophagus (ES) and anterior midgut (arrows in U-X indicate junction with anterior midgut). In drm embryos (V), the PV does not fold properly. In both lin (W) and drm lin (X) embryos, the entire foregut appears shorter and wider, and the PV does not form (arrows).
products, and genomic DNA (Adams et al., 2000) is consistent with the conclusion that CG10016 is an 8.5 kb transcription unit. Three exons are spliced to form a 2.5 kb mRNA encoding an 81 amino acid, 10,120 Da predicted protein (Wilkins et al., 1998).

The position of the I element in \textit{drm} \textit{1}, and the endpoints of the deletions in \textit{drm} \textit{P1} and \textit{drm} \textit{P2}, strongly suggest that CG10016 is the \textit{drm} gene. To obtain further evidence supporting this relationship, we performed rescue and mutagenesis experiments. We generated germline transformants of an 18.1 kb fragment of genomic DNA (Fig. 2A) that includes CG10016 plus 6.9 kb of upstream and 2.7 kb of downstream DNA (but no other predicted genes). Although this construct does not rescue \textit{drm} lethality, it does rescue the \textit{drm} hindgut and foregut phenotypes (data not shown). We also generated and molecularly characterized five new EMS-induced alleles (see Materials and Methods). Four of these are point mutations (in three different residues) within the first zinc finger, and one is a \textit{roo} element insertion in the second exon splice donor site (Fig. 2D, Table 1); no mutations were found outside the ORF. In all of these mutants, the proventriculus is both morphologically and functionally defective (assessed by

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**Fig. 2.** Molecular characterization of \textit{drm}. (A) Physical map between \textit{male-specific lethal-2} (\textit{msl-2}) and \textit{foraging} (\textit{for}) (cytological regions 23F3 to 24A3). Genomic DNA in P1 phage clones DS01379 and DS01340 is shown above the map, with the 18.1 kb fragment used for genomic rescue experiments shaded in gray. Characterized genes are shown with arrows representing their directions of transcription, as annotated in GadFly (Rubin et al., 2000b). The \textit{l(2)k10101} P element, mobilized to generate deficiency alleles, is represented by an inverted triangle. Below, deficiencies are represented by open bars with breakpoint uncertainties shown in gray. \textit{Df(2L)tim} \textit{02} and \textit{Df(2L)ed} \textit{1} were used for rough mapping of the \textit{drm} \textit{1} allele. The breakpoints of \textit{Df(2L)drm} \textit{P1} and \textit{Df(2L)drm} \textit{P2} were mapped molecularly. B, BamHI. (B) Expanded view of the 11.9 kb BamHI fragment that includes the \textit{drm} gene. The I element in the \textit{drm} \textit{1} chromosome is inserted 35 bp upstream of the transcription start site. The \textit{drm} transcription unit is 8.5 kb with three exons spliced together to form a 2.5 kb mRNA. An alternate first exon ('1') was found in 10% of 5' RACE products, and several alternate transcriptional stop sites (dotted lines in exon 3) were present in 3' RACE products. The \textit{drm} ORF (black) spans the second splice junction. (C) Northern blot of 50 \textmu g total embryonic RNA (0-17 hours) and 1 \textmu g poly(A) RNA shows the approximately 2.5 kb \textit{drm} mRNA. (D) Schematic representation of the Drm protein folded to form one C\textsubscript{2}H\textsubscript{2} and one C\textsubscript{2}HC zinc finger. Residues conserved in the canonical C\textsubscript{2}H\textsubscript{2} zinc finger are shaded gray and residues altered in the \textit{drm} point mutants are black (see Table 1).
lellar feeding assays; data not shown), while the hindgut is defective only in flies carrying the stronger alleles. This deleterious effect has been found in other C2H2 zinc finger proteins (compare mouse and human Osr proteins). Identical and similar residues are shaded black and gray, respectively (BOXSHADE, v. 3.2). Dashes indicate gaps in the alignment, and dots indicate amino acid residues (not shown) outside the zinc finger domains. Conserved residues in the canonical C2H2 zinc finger are shown below. Asterisks above the Drm sequence denote single residues altered in drm mutants (see Table 1 and Fig. 2D), and the diamond denotes the second exon splice donor site where the drm1 roo element is inserted.

Expression of drm during embryogenesis
Consistent with the gut defects observed in drm embryos, drm is expressed dynamically in cells that will give rise to the foregut and hindgut (Fig. 4). In the posterior gut primordium, drm mRNA is first detected at stage 5 in a ventral crescent at 10% embryonic length (EL) (Fig. 4A). Cells in this region are fated to give rise to the epithelia of the posterior midgut, Malpighian tubules and hindgut (Campos-Ortega and Hartenstein, 1997). At stage 6, the posterior crescent expands dorsally to encircle the amnioproctodeal plate (Fig. 4B). By stage 7, drm is expressed in a ring within the proctodeal invagination (Fig. 4C). During germ band extension (stages 8
and 9), drm expression is seen in a region overlapping the junction of the posterior midgut and hindgut primordia (Fig. 4D,E). At stage 10, drm is expressed transiently in the evaginating buds of the Malpighian tubules (Fig. 4F). Between stages 11 and 13, drm mRNA is detected in the posterior midgut, the ureters of the Malpighian tubules, and the most anterior cells of the small intestine (Fig. 4G,H); expression in these domains persists throughout the remainder of embryogenesis.

Drm is also expressed in the anterior gut primordium starting at stage 5 (Fig. 4A). Expression increases in this domain until stage 10 when it is internalized as the invaginating stomodeum (Fig. 4F). Dm expression is then refined to a narrow ring of cells at the junction of the foregut and anterior midgut (Fig. 4H). Like odd and sob (Hart et al., 1996), drm is also expressed in a seven-stripe segmental pattern at stage 5 (Fig. 4A); this pattern evolves into fourteen stripes that mark the anterior margin of each segment (Fig. 4H).

The domains in which drm is expressed during embryogenesis include a region predicted to become small intestine (Fig. 4C-G). By stage 13, however, drm mRNA is not seen in the small intestine (Fig. 4H). To determine if drm is expressed early in cells fated to become small intestine, we generated a drm-GAL4 driver and used it to drive UAS-b-gal, this provides important, the presence of b-gal in the small intestine of stage 16 embryos (Fig. 4K) demonstrates that some of the drm-expressing cells in the early embryo do indeed give rise to the small intestine.

drm and lin interact in vivo
If, as suggested above, spatially localized expression of drm in the anterior hindgut allows specification of the small intestine by antagonizing lin, then expression of drm throughout the hindgut should inhibit endogenous lin, thereby producing a lin-like hindgut phenotype. We tested this by driving UAS-drm using the hindgut-specific byn-GAL4 driver. The result of this gain-of-function expression of drm is a hindgut phenotype that resembles lin loss-of-function mutants: the hindgut is short and distended, consisting of a uniform layer of cuboidal cells similar to those of the wild-type small intestine, and the boundary cell rows are absent (Fig. 5A,C). en is not expressed in the hindgut of these embryos, but apd and hh expression is greatly expanded posteriorly (Fig. 5E,G,I). Like lin mutations, the byn-GAL4:UAS-drm combination is lethal. Overall, it appears that in both lin mutants and embryos expressing drm throughout the hindgut the small intestine is expanded at the expense of the large intestine. We conclude that drm functions in the hindgut by antagonizing lin.

If drm acts primarily by inhibiting lin activity in the hindgut,
undulating layer of columnar cells with a duplication of the boundary cell rows (Fig. 5B,D). As seen in drm embryos, en is expressed throughout the dorsal hindgut, upd expression is absent, and hh expression in the small intestine is reduced (Fig. 5F,H,J). Like drm mutations, the byn-GAL4:UAS-lin combination is lethal. Since weaker hindgut drivers (e.g. 14-3fh-GAL4) and weaker UAS-lin transformants do not produce a phenotype as severe as with the strong byn-GAL4 driver or the strongest UAS-lin stocks (R. Green, unpublished observations), we conclude that the repressive effect of drm can be titrated only by relatively high levels of lin.

Drm interacts physically with Lin

The epistasis and in vivo overexpression studies indicate that drm inhibits lin activity, either directly or indirectly. As we observe no reduction of lin expression in the drm expression domains of wild-type embryos (Hatini et al., 2000; Iwaki et al., 2001), or any changes in drm or lin expression in lin or drm mutant embryos, respectively (data not shown), we conclude that drm does not affect lin at the transcriptional level. We therefore used a biochemical approach to ask whether Drm might interact physically with Lin. When expressed together in cultured cells, full-length Drm and full-length Lin interact with each other, as demonstrated by coimmunoprecipitation assays (Fig. 6B, lane 1), indicating that the two proteins are in a complex. Since Drm and Lin interact with each other in a yeast two-hybrid assay (data not shown), the Drm-Lin interaction is likely mediated by direct binding.

To map the protein interaction domain, we generated deletion and point mutation constructs of Drm (Fig. 6A) and tested their ability to coimmunoprecipitate with Lin (Fig. 6B). The N-terminal portion of Drm (N-Drm) is unable to bind Lin, while the C-terminal portion of Drm, containing the two zinc finger motifs (C-Drm), retains full Lin-binding activity. Because these results map the protein-protein interaction domain to the zinc fingers, we tested the requirement of each individual zinc finger for Lin-binding activity. A mutation in the C2H2 first finger (R46C, identical to the mutation in the drmnull allele) abolishes Lin-binding activity, while disruption of the C2HC second finger (C57G, a substitution in one of the conserved zinc-binding cysteine residues) reduces Lin-binding activity, but does not abolish it completely. To confirm these results, we made similar point mutations in the truncated C-Drm construct and tested their ability to coimmunoprecipitate with Lin (Fig. 6B). The N-terminal portion of Drm (N-Drm) is unable to bind Lin, while the C-terminal portion of Drm, containing the two zinc finger motifs (C-Drm), retains full Lin-binding activity. Because these results map the protein-protein interaction domain to the zinc fingers, we tested the requirement of each individual zinc finger for Lin-binding activity. A mutation in the C2H2 first finger (R46C, identical to the mutation in the drmnull allele) abolishes Lin-binding activity, while disruption of the C2HC second finger (C57G, a substitution in one of the conserved zinc-binding cysteine residues) reduces Lin-binding activity, but does not abolish it completely. To confirm these results, we made similar point mutations in the truncated C-Drm construct and tested their effect on Lin-binding activity. Again, a mutation in the C2H2 first finger in C-Drm(R46C) abolishes Lin-binding activity while disruption of the C2HC second finger in C-Drm(C57G) reduces Lin-binding activity (Fig. 6B). We conclude that the C2H2 zinc finger is essential for binding to Lin, while the C2HC finger contributes to binding, perhaps by stabilizing the interaction.

Structure-function analysis of Drm-Lin antagonism in vivo

We next asked which portions of the Drm protein are required to block Lin function in vivo by expressing, throughout the hindgut, the same Drm deletion and point mutation constructs used in the coimmunoprecipitation studies (Fig. 6A). We assayed the activity of these mutated proteins by their ability to induce a lin-like hindgut

Fig. 5. Drm and Lin antagonize each other in vivo. Whole-mount embryos were stained with an anti-Crb (A-D) or anti-En (E,F), hybridized in situ with probe for upd (G,H) or hh (I,J), or fixed and sectioned transversely (G,H). Ectopic expression of Drm throughout the hindgut with the byn-GAL4 driver produces a lin-like phenotype as observed by morphology of the hindgut (A; compare Fig. 1C), by hindgut epithelial cell shape and absence of boundary cell rows (C; compare Fig. 1G), by the lack of expression of En in the large intestine (E, white arrowhead; compare Fig. 1K), and by the posterior expansion of upd and hh from the small intestine (G and I, wide arrowheads; compare Fig. 1O and 1S). Ectopic expression of Lin throughout the hindgut using the byn-GAL4 driver produces a drm-like phenotype as observed by the morphology of the hindgut (B; compare Fig. 1B), hindgut epithelial cell shape and duplication of boundary cell rows (D, four arrows, compare with Fig. 1F), the expression of En in the dorsal large intestine (F, black arrowhead; compare Fig. 1J), the absence of upd expression in the small intestine (H, white arrowhead; compare Fig. 1N), and the expression pattern of hh (J, black arrowheads; compare Fig. 1R).

then overexpression of lin might overcome the repressive effect of endogenous drm, thereby producing a drm-like phenotype. Consistent with this notion, overexpression of lin produces a hindgut phenotype that resembles drm loss-of-function mutants: the hindgut is short and wide, consisting of an
phenotype when ectopically expressed with byn-GAL4 (similar to the gain-of-function phenotype induced by ectopic expression of full-length Drm). Expression of N-Drm, which lacks the zinc fingers, results in a morphologically wild-type hindgut (Fig. 6C), while expression of C-Drm produces a lin-like phenotype similar to the gain-of-function phenotype produced by ectopic expression of full-length Drm (compare Fig. 5A). Expression of R46C results in a wild-type hindgut while C57G produces a lin-like phenotype. +++ or +, strong or weak interaction, respectively; −, no interaction.

**DISCUSSION**

**Drm relief of Lin repression is required for both patterning and cell rearrangement in the hindgut**

We showed previously that drm is required for specification of the small intestine, while lin represses this fate (Iwaki et al., 2001). Here we demonstrate that lin is epistatic to drm, indicating that lin acts downstream of drm in a genetic pathway. In our molecular characterization, we determine that, in contrast to the uniform expression of lin (Hatini et al., 2000), drm is expressed in a highly localized pattern at the anterior of the hindgut primordium. We further show that uniform expression of drm throughout the hindgut results in a lin-like phenotype. When co-expressed in cultured cells, Drm and Lin are found in a complex. Taking into account these genetic, molecular and biochemical data, we propose a model (Fig. 7) in which Lin (or a complex containing Lin) functions as a transcriptional regulator that represses the genetic program promoting small intestine fate. In the anterior of the hindgut, Drm blocks the activity of Lin by direct physical association, thereby allowing specification of small intestine fate. The Drm-Lin interaction in the hindgut thus defines a pathway in which the spatially localized expression of a small regulatory protein inhibits the repressive effect of a uniformly expressed transcriptional regulator.
Putative transcriptional regulators have been shown to be required for a number of processes of epithelial cell rearrangement, die-1 encodes a zinc finger protein required for intercalation of dorsal epidermal cells in C. elegans (Heid et al., 2001), grain a GATAc factor required for stigmatophore elongation (Brown and Castelli-Gair Hombriá, 2000), and ribbon (rib) a BTB domain protein required for tracheal branching morphogenesis (Bradley and Andrew, 2001). All of these genes are expressed throughout and are required within the cells undergoing cell rearrangement. In contrast, drm is expressed only at one end of the prospective hindgut, but is required for cell rearrangement throughout much of the epithelium.

As the drm, bowl and lin phenotypes suggest that juxtaposition of properly specified small and large intestine is required for hindgut cell rearrangement (Iwaki et al., 2001), we speculate that expression of signaling molecules specifically in the small intestine orients the rearrangement of cells within the large intestine. Hedgehog (Hh), Wingless (Wg) and Serrate (Ser) are expressed in the termini of the hindgut, but they appear to play either a minor or no role in controlling morphogenesis (Iwaki et al., 2001; Iwaki and Lengyel, 2002). One signaling molecule that is required for hindgut cell rearrangement is Upd, the Drosophila ligand activating JAK/STAT signaling, which is expressed specifically in the small intestine (K. A. J. and J. A. L., unpublished). Further characterization of the upd pathway and its downstream effectors may provide insight into how patterning of the small intestine affects oriented cell rearrangement.

**Drm is a protein-binding member of the Odd-skipped zinc finger family**

Both epistasis and ectopic expression experiments indicate that, in the hindgut, Drm acts primarily through Lin. The association between Drm and Lin, demonstrated both in cultured cells and in yeast, suggests that, rather than binding to DNA, Drm functions by binding to protein. Drm contains two zinc fingers that differ structurally, and perhaps functionally, from those of its family members. The first, canonical C2H2 finger lacks the conserved TG(E/Q)(K/R)/P linker present in other Odd family zinc fingers, which is required in other canonical C2H2 fingers for stabilization of high-affinity DNA-protein binding (Laity et al., 2000). Furthermore, as shown here, this C2H2 finger is both necessary and sufficient for Lin-binding in vitro and Lin-inhibiting activity in vivo. A protein-binding function for the Drm C2H2 finger is consistent with the demonstrated role of other C2H2 fingers, including those of the Ikaros family, in homo- and heterodimerization (Sun et al., 1996; Morgan et al., 1997; Kelley et al., 1998). Although we cannot rule out a DNA-binding function for Drm, our data are consistent with a model in which the primary role of the C2H2 first finger is in binding to Lin.

C2HC fingers have also been shown to act as protein binding motifs: both mammalian FOG and Drosophila Ush bind to zinc fingers in their partner proteins via specific C2HC fingers (Tsang et al., 1997; Fox et al., 1999; Haenlin et al., 1997). When co-expressed in cultured cells and assessed by coimmunoprecipitation, the Drm C2HC second finger does indeed contribute to the binding of Drm to Lin (although it is not absolutely necessary). In overexpression studies in vivo, however, the Drm C2HC finger was not necessary for Lin-inhibiting activity. Consistent with this in vivo observation, no mutations in the Drm C2HC finger were identified in our screen of 18,000 mutagenized chromosomes. Thus, while a protein-binding function is suggested by the in vitro data, we have not detected a required in vivo function for the Drm C2HC finger.

In conclusion, our characterization of Drm adds to the growing number of C3H2 zinc fingers that act as protein-protein interaction motifs (Mackay and Crossley, 1998). The Drm protein is unique in that it is extremely small (10 kDa), is expressed in a highly spatially localized pattern, and appears to function entirely (at least in the hindgut) by associating with, and thereby antagonizing, the activity of a globally expressed protein, Lin.

**Globally-expressed Lin requires spatially localized cofactors for specificity**

lin is expressed relatively uniformly throughout the embryo, yet has tissue- and stage-specific effects, and both activating and repressing activities. A number of these functions have been shown to depend on spatially localized cofactors or signals. In the posterior spiracles and eighth abdominal belt, lin functions together with Abdominal B (which is expressed specifically in the posterior) to activate empty spiracles, cut and spalt (Castelli-Gair, 1998). In the dorsal epidermis, as a result of localized wg signaling, lin activates wg and represses veinlet expression (Hatini et al., 2000); lin is required to promote quaternary (4°) and represses tertiary (3°) cell fates (Bokor and DiNardo, 1996; Hatini et al., 2000). In the hindgut, lin represses expression of upd and hh (characteristic of small intestine fate); establishment of the small intestine requires the locally expressed cofactor Drm, which relieves repression by Lin.

Antagonism of repressors is an important mechanism by which transcriptional activity is spatially regulated (Courey and Jia, 2001). In Drosophila, localized cell signaling by receptor tyrosine kinases (e.g. Torso and Sevenless) or the canonical wg pathway is required to relieve global repression (in a number of cases by Groucho), thereby allowing proper patterning and/or differentiation of portions of the embryonic termini, segments, midgut and eye (Paroush et al., 1997; Jimenez et al., 2000; Waltzer and Bienz, 1998; Cavallo et al., 1998; Rebay and Rubin, 1995). While these relief-of-repression pathways are
dependent on localized cell signaling, the Drm-Lin interaction is, to our knowledge, the first described example in which relief of repression is initiated by spatially restricted production of a small transcriptional regulator.

Many questions remain about the Drm-Lin interaction and its mechanism of action. First, it is not clear how Lin acts as a transcriptional regulator. In the hindgut, and perhaps in other tissues, Lin might act as part of a repressosome complex (Courey and Jia, 2001), binding DNA directly or associating with chromatin on the basis of an interaction with other DNA-binding proteins. Binding of Drm to a Lin-containing complex might then inhibit the repressive activity of Lin. Second, activation of gene expression requires not only the absence (or inactivation) of repressors, but also the presence of transcriptional activators (Struhl, 1999). Thus, there must be as-yet-unknown transcriptional activators that promote expression of small intestine-specific targets once Drm has lifted the repression by Lin.

**Drm and Lin in other developmental contexts**

We have shown that *drm* is expressed dynamically not only in the hindgut, but also in the foregut and in the developing epidermis. This raises the question of whether Drm-mediated Lin inhibition might occur in tissues other than the hindgut. Both the spatially restricted expression of *drm* and the epistasis of *lin* to *drm* in the foregut epithelium (Fig. 1U-X) are consistent with the idea that Drm also blocks Lin activity in the proventricular primordium, thus allowing proper folding morphogenesis. In the dorsal epidermis, stripes of *drm* expression roughly correspond to defects in patterning seen in *drm* mutants (V. H. and S. DiNardo, unpublished observations); these defects are opposite to those seen in *lin* embryos (Hatini et al., 2000), suggesting that Drm-mediated relief of Lin repression may occur in dorsal epidermis patterning. We have also observed *lin* expression in leg imaginal discs and a required role for *lin* in leg development (V. Hatini and S. DiNardo, unpublished observations); taken together with the potential role of *drm* in the developing leg (Cohen et al., 1991), these results suggest that Drm and Lin might also control aspects of leg development. We conclude that, by antagonizing Lin, Drm controls multiple events of epithelial patterning and thereby morphogenesis.

Another gene in the *odd* family, *bowl*, has a hindgut phenotype very similar to that of *drm* (Wang and Coulter, 1996; Iwaki et al., 2001), suggesting that *bowl* might also function in the same pathway as *drm* and *lin*. However, in contrast to the spatially localized expression of *drm*, but similar to the spatial and temporal expression of *lin*, *bowl* expression appears uniform throughout the hindgut primordium until stage 12 (Wang and Coulter, 1996). Thus, despite its similarity in both mutant phenotype and sequence to *drm*, *bowl* function in the hindgut is likely different from that of *drm*. In mouse and humans, the genes *Osr1* and *Osr2* encode Osr-related proteins with three and five zinc fingers, respectively, that are most similar to those of *Sob* and *Bowl* (Fig. 3C) (So and Danielian, 1999; Lan et al., 2001). Mouse *Osr1* and *Osr2* are expressed during embryogenesis in dynamic, spatially localized patterns correlated with sites of active morphogenesis (So and Danielian, 1999; Lan et al., 2001). Given that Drm (and perhaps Bowl) is involved in a pathway with Lin, it may be significant that a mammalian protein, predicted from an EST clone and sharing homology with Lin over a 200 amino acid region, was recently identified (Venter et al., 2001). It will be interesting to determine if any parallels exist between the expression and function of these mammalian homologs and the *Drosophila* Lin and Odd family of proteins.

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