The Drumstick/Lines/Bowl regulatory pathway links antagonistic Hedgehog and Wingless signaling inputs to epidermal cell differentiation

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Hedgehog and Wingless signaling in the *Drosophila* embryonic epidermis represents one paradigm for organizer function. In patterning this epidermis, Hedgehog and Wingless act asymmetrically, and consequently otherwise equivalent cells on either side of the organizer follow distinct developmental fates. To better understand the downstream mechanisms involved, we have investigated mutations that disrupt dorsal epidermal pattern. We have previously demonstrated that the gene *lines* contributes to this process. Here we show that the Lines protein interacts functionally with the zinc-finger proteins Drumstick (Drm) and Bowl. Competitive protein–protein interactions between Lines and Bowl and between Drm and Lines regulate the steady-state accumulation of Bowl, the downstream effector of this pathway. Lines binds directly to Bowl and decreases Bowl abundance. Conversely, Drm allows Bowl accumulation in *drm*-expressing cells by inhibiting Lines. This is accomplished both by outcompeting Bowl in binding to Lines and by redistributing Lines to the cytoplasm, thereby segregating Lines away from nuclearly localized Bowl. Hedgehog and Wingless affect these functional interactions by regulating *drm* expression. Hedgehog promotes Bowl protein accumulation by promoting *drm* expression, while Wingless inhibits Bowl accumulation by repressing *drm* expression anterior to the source of Hedgehog production. Thus, Drm, Lines, and Bowl are components of a molecular regulatory pathway that links antagonistic and asymmetric Hedgehog and Wingless signaling inputs to epidermal cell differentiation. Finally, we show that Drm and Lines also regulate Bowl accumulation and consequent patterning in the epithelia of the foregut, hindgut, and imaginal discs. Thus, in all these developmental contexts, including the embryonic epidermis, the novel molecular regulatory pathway defined here is deployed in order to elaborate pattern across a field of cells.

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Specialized groups of cells known as organizers establish the pattern of cell differentiation and morphogenesis across fields of progenitor cells. Although many organizer signals and their signal transducers have been identified, the pathways that link organizer signaling activity with subsequent cellular patterning and morphogenesis remain to be elucidated (Hatini and DiNardo 2001b). The embryonic epidermis in *Drosophila* has been contributing general insights into the mechanism of organizer function (Hatini and DiNardo 2001; Sanson 2001b). The pattern of cell differentiation across this epidermis is organized by two conserved signals, Hedgehog and Wingless, produced from adjacent sources that flank the boundary between parasegments (PS) (Baker 1987; Lee et al. 1992; Mohler and Vani 1992). Following the establishment of the sources of Hedgehog and Wingless production (DiNardo et al. 1988; Martinez Arias et al. 1988; Bejsovec and Martinez-Arias 1991; Heemskerk et al. 1991), each signal inhibits cellular responses elicited by the other signal. Wingless inhibits Hedgehog activity by repressing Hedgehog target gene expression anterior to the source of Hedgehog production. Hedgehog, however, inhibits Wingless activity posterior to the source of Wingless production by several distinct mechanisms (Sanson et al. 1999; Pfeiffer et al. 2000; Piepenburg et al. 2000; Dubois et al. 2001; Hatini and DiNardo 2001a). This results in polarized activity of Hedgehog and Wing-

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less from the organizer, with Hedgehog organizing the pattern of posterior cells and Wingless organizing the pattern of anterior cells. Ultimately, this initial polarity generates an asymmetric pattern of epidermal cell differentiation (O'Keefe et al. 1997; Szüts et al. 1997; Alexandre et al. 1999; Gritzan et al. 1999; Payre et al. 1999; Hatini et al. 2000a; Piepenburg et al. 2000; Hatini and DiNardo 2001a). The mechanisms by which epidermal cells respond to Hedgehog and Wingless signaling activities are not fully understood. In the ventral epidermis, Hedgehog and Wingless activity divide the PS into smaller territories, each a focus for a patterning signal (Alexandre et al. 1999; Gritzan et al. 1999; Wiellette and McGinnis 1999). As a consequence of this subdivision, Hedgehog and Wingless along with Serrate and Spitz organize the final pattern (Alexandre et al. 1999; Hatini and DiNardo 2001a). In dorsal epidermis, although Hedgehog and Wingless are again the primary organizing signals, Serrate and Spitz are not involved, and it is unclear what mechanisms produce the final pattern. As an approach to identify the genes involved, we selected mutants in which the normal asymmetric pattern of epidermal cell differentiation was replaced with a symmetric pattern. Using this approach, we have previously selected the gene *lines* for further analysis (Bokor and DiNardo 1996). We have subsequently shown that Lines exhibits asymmetric subcellular distribution across the PS, with enriched nuclear accumulation in cells signaled by Wingless and enriched cytoplasmic accumulation in cells signaled by Hedgehog. We have found that this arises because Wingless promotes nuclear accumulation of Lines, and suggested that Hedgehog antagonizes Wingless signaling by localizing Lines to the cytoplasm. We have also shown that Lines mediates the cellular responses dependent on Wingless signaling, antagonizes those responses dependent on Hedgehog signaling, and in this manner contributes to the pattern of epidermal cell differentiation. We have therefore proposed that Hedgehog and Wingless signaling regulate the asymmetric subcellular distribution and consequent action of Lines across the embryonic epidermis (Hatini et al. 2000). That study, however, neither pinpointed the molecular mechanism by which Hedgehog and Wingless regulate the subcellular distribution and function of Lines, nor the mechanism by which Lines promotes Wingless signaling inputs and antagonizes Hedgehog signaling inputs. Finally, based on genetic analysis, we have proposed that *lines* functions as a stage- and tissue-specific modulator of the Wingless signaling pathway by acting either in concert or in parallel to *armadillo* and *dtcf/pangolin*, the nuclear effectors of the Wingless signaling pathway. Here we provide a revised model for the mechanism of Lines function.

More recent analysis of mutants that affect gut morphogenesis has shown that *lines* mutants exhibit phenotypes related to those of *drm* and *bowl* mutants, raising the possibility that the three genes act along the same pathway (Iwaki et al. 2001). Formal genetic analysis has suggested that the three genes act in a linear genetic pathway to regulate the morphogenesis of the hindgut and foregut epithelia—*lines* inhibits *bowl* to maintain large intestine or foregut fate, respectively, except in localized areas where *drm* is expressed. In these areas, *drm* antagonizes *lines*, thereby allowing *bowl* to specify small intestine fate in the hindgut primordium and proventricular fate in the foregut primordium (Green et al. 2002; Johansen et al. 2003). These studies investigated neither the molecular mechanisms by which the Drm and Lines proteins regulate the activity of the Bowl protein, nor the possible involvement of this genetic pathway in other developmental processes. Here we demonstrate that the three proteins interact physically and functionally along a molecular regulatory pathway in order to regulate the spatial pattern of Bowl protein accumulation. Depending on context, this molecular regulatory pathway can elicit specific responses such as epidermal differentiation, gut morphogenesis, and formation of leg joints and distal leg structures. In all these contexts, this pathway is engaged by organizer signals or other positional cues in order to specify distinct cell fates across fields of progenitor cells, either directly or indirectly through the production of new signals. In the embryonic epidermis, this pathway is engaged by and implements the antagonistic activities of Hedgehog and Wingless signaling. Thus, our findings define the mechanism of action of a novel molecular regulatory pathway, and demonstrate general roles for this pathway in patterning a variety of epithelial tissues.

Results

drm, lines, *and* bowl *operate in a relief-of-repression pathway to control dorsal epidermal patterning*

The *Drosophila* embryonic epidermis is composed of a series of PS. *lines* is required in the epithelium of the dorsal epidermis to specify one of the four (1°–4°) cell fates present across each PS, such that in *lines* mutants the 4° fate is missing and all the cells adopt only the 1°–3° fates (Fig. 1, cf. A and B; Heemskerk and DiNardo 1994; Bokor and DiNardo 1996; Hatini et al. 2000). If *lines* operates in the context of the *drm/lines/bowl* regulatory pathway to control epidermal patterning, *drm* and *bowl* should have phenotypes opposite to *lines*, as they do in the gut. To test this hypothesis, we examined the cuticle phenotype of *drm* and *bowl* mutants either alone or in combination with *lines*. Indeed, we found that the *drm* and *bowl* mutant phenotypes were opposite to *lines*. In both mutants, the 1°–3° fates were replaced with 4° (Fig. 1C,D). In addition, gain-of-function phenotypes for *lines* and *drm* paralleled those observed in the gut—while *lines* gain-of-function phenocopied a *drm* mutant, *drm* gain-of-function phenocopied a *lines* mutant (Fig. 1E,F). Therefore, similarly to *lines*, *drm* and *bowl* control cell fate decisions across the dorsal embryonic epidermis. In all three mutants, cells make abnormal fate decisions early during development, which are reflected later during development in specific abnormalities in the cuticle pattern. Finally, the epistatic relationships between *lines* and *bowl* and between *drm* and *lines* were the same as those observed in the gut: *lines bowl* double

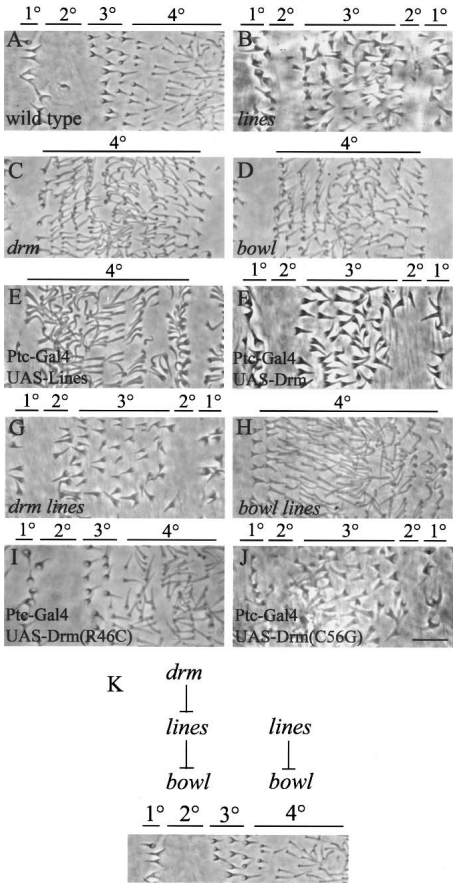

Figure 1. The Drm/Lines/Bowl regulatory pathway is deployed for patterning the embryonic epidermis. One PS of dorsal cuticle, 24 h after egg laying (AEL) (anterior to *right*). (*A*) Wild type. (*B*) *lines*: 4° fate was replaced with 1°–3° (Hatini et al. 2000). In *drm* (*C*) and *bowl* (*D*): 1°–3° fates were replaced with 4°. (*E*) Ectopic *lines*: 1°–3° were replaced with 4° fate. (*F*) Ectopic *drm*: 4° fate was replaced with 1°–3° fates. Ectopic *bowl*: no gain-of-function phenotype in embryonic epidermis (data not shown). (*G*) *drm lines*. (*H*) *bowl lines*. (*I*) Ectopic Drm(R46C): subtle or no phenotype. Lines associated with much reduced affinity with this variant in co-IP assays (Green et al. 2002). (*J*) Ectopic Drm(C56G). (*K*) The genetic results lead to a model whereby *drm*, *lines*, and *bowl* operate in a relief-of-repression pathway to distinguish anterior from posterior fates (see text). Similar genetic interactions between *drm* and *lines* and between *lines* and *bowl* distinguish small and large intestine in the developing hindgut, and proventriculus and foregut proper in the developing foregut (Green et al. 2002; Johansen et al. 2003). Bar, 10 μm.

mutants looked like *bowl* single mutants (Fig. 1H), while *drm lines* mutants looked like *lines* (Fig. 1G). These results imply that the three genes act in a linear relief-of-repression pathway to pattern the dorsal embryonic epidermis—*lines* inhibits *bowl* across the PS allowing specification of the 4° cell fate, while *drm* inhibits *lines* in a subset of cells, allowing *bowl* to specify the 1°–3° cell fates (Fig. 1K). Consistent with this model, expression of *lines* (Hatini et al. 2000) and *bowl* (Wang and Coulter 1996) mRNA is ubiquitous, whereas expression of *drm* mRNA is localized (Figs. 3A, 4A, below).

Drm outcompetes the binding of Lines to the N-terminal zinc finger of Bowl to liberate Bowl from negative regulation

We next investigated whether direct molecular interactions underlie these genetically defined inhibitory interactions. Drm and Bowl are members of the conserved Odd-skipped family of zinc-finger proteins. The *bowl* gene encodes a protein containing five C_2H_2 fingers (Fig. 2A, lane 1; Wang and Coulter 1996). *drm* encodes an 81-amino-acid peptide containing a single C_2H_2 finger most similar to the first zinc finger of Bowl (Green et al. 2002). *lines* encodes a pioneer protein, conserved in mammals, with no motifs that would suggest a biochemical function (Hatini et al. 2000). Previously, we have shown that Lines binds to the N-terminal C_2H_2 finger of Drm (Green et al. 2002). This finger shares a high degree of homology with the N-terminal finger of Bowl, suggesting that Lines inhibits Bowl by binding to this finger. Using protein–protein interaction assays, combined with deletion and point mutation analyses (Fig. 2A), we next investigated this hypothesis. Yeast two-hybrid and coimmunoprecipitation (IP) assays suggested direct interactions between Bowl and Lines (Fig. 2A, lane 1; data not shown). The zinc-finger domain (ZFD) was sufficient for the interaction with Lines (Fig. 2A, lane 4). Within this domain, a mutation in the first finger (R258C) (Fig. 2A, lanes 2,5) abolished interaction with Lines, while a mutation in the second finger (C268G) (Fig. 2A, lanes 3,6) had little or no effect. Because the N-terminal zinc fingers of Bowl and Drm were each essential for binding to Lines, one likely mechanism for Drm to antagonize Lines is to disrupt, by competition, the Lines–Bowl interaction. We tested this hypothesis by cotransfecting Lines and Bowl into Schneider line 2 cells (S2), with increasing amounts of Drm. We found that in the absence of Drm, Lines coimmunoprecipitated with Bowl (Fig. 2B, lane 1). However, cotransfection with increasing amounts of Drm decreased the amount of Lines associated with Bowl, and did so in a dose-dependent manner, supporting our hypothesis (Fig. 2B, lanes 2,3).

Lines decreases the steady state accumulation of Bowl while Drm increases it by inhibiting Lines

In principle, the physical interactions between Lines and Bowl and between Drm and Lines could influence either the activity or the abundance of Bowl, the key downstream effector of this pathway. To determine whether these interactions affect Bowl abundance in vivo, we investigated the distribution of Bowl protein in wild-type embryos. While Bowl mRNA is expressed uniformly (Wang and Coulter 1996), Bowl protein accumulated in the nuclei of only two cell rows in each PS (Fig. 2C,G, red), the posteriormost Engrailed cells and a row of cells just posterior to this (Fig. 2G, arrow and arrowhead, respectively). These two cell rows flank the segment border. In addition, the formal genetics suggested particular roles for *lines* and *drm* is this regulation. In agreement, in *drm* mutants, the normal discrete accumulation of

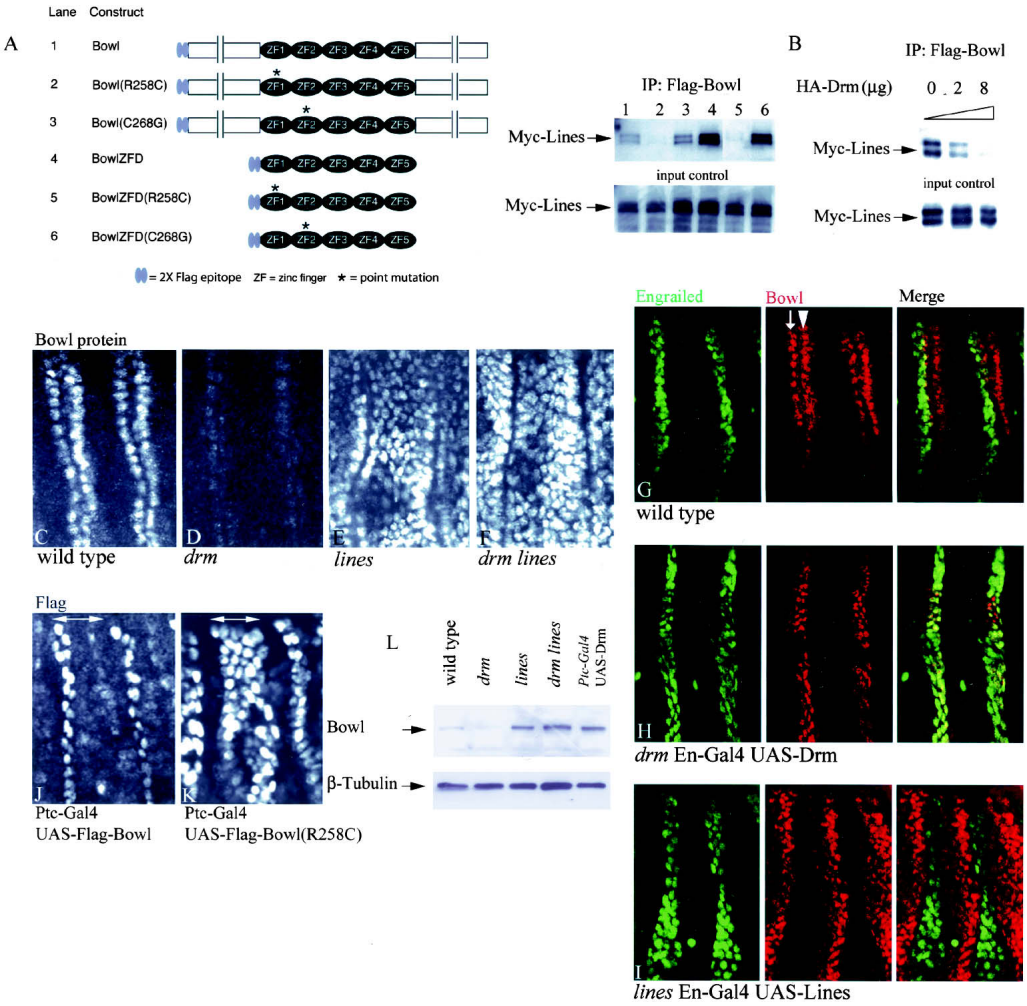

Figure 2. Lines decreases the steady-state accumulation of Bowl by binding to its N-terminal zinc finger, whereas Drm increases the steady-state accumulation of Bowl by outcompeting the Lines–Bowl interaction. (*A*) Coimmunoprecipitation of tagged Myc-Lines and Flag-Bowl variants transfected together into S2 cells. Schematic representation of deletion and point mutant variants of Flag-Bowl used in each lane. Lines co-IPed with full-length Bowl protein, its zinc-finger domain—BowlZFD—and a point mutant in the second zinc finger—Bowl(C268G). Lines associated with much reduced affinity with a point mutant in the first finger—Bowl(R258C). A similar point mutation in Drm that disrupts the first finger—Drm(R46C)—reduced interaction with Lines and abolished gain-of-function phenotypes in vivo (Green et al. 2002). (*B*) Constant amounts of Myc-Lines and Flag-Bowl transfected into S2 cells, together with increasing amounts of Drm: Lines–Bowl interaction was decreased in a dose-dependent manner. (*C–F*) Bowl immunostains; the boundaries of two PSs are shown. (*C*) Wild type: Bowl accumulated in cells flanking the segment boundary. (*D*) *drm*: Bowl was barely detected. However, Bowl accumulated broadly in *lines* (*E*) and *drm lines* (*F*). (*G–I*) Engrailed cells (green), Bowl (red); the *right* panel is Merge. (*G*) Wild type: Endogenous Bowl accumulated in cells flanking the segment border, the posteriormost En-expressing cell row (arrow, anterior to the segment border; yellow in *H*), and in one cell row just posterior to this (arrowhead, posterior to the segment border). (*H*) *drm En-GAL4* UAS-Drm: Bowl accumulated in Drm-expressing cells. Residual Bowl expression was detected in cells anterior to the En domain due to earlier Engrailed expression in these cells. (*I*) *lines En-GAL4* UAS-Lines: Bowl was down-regulated in Lines-expressing cells. (*J*) *Ptc-GAL4* UAS-Flag-Bowl. (*K*) *Ptc-GAL4* UAS-Flag-Bowl(R258C): the *Ptc-Gal4* expression domain is marked using a double arrow in *J* and *K*. (*L*) Western blot: 7–11-h AEL embryonic extracts for Bowl from indicated genotypes and β-tubulin as a loading control. Bars: *C–K*, 10 μm.

Bowl protein accumulation was decreased dramatically in these two cell rows (Fig. 2D). Conversely, in *lines* mutants, Bowl protein accumulated ubiquitously across the PS (Fig. 2E), even when *drm* function was also removed (Fig. 2F). These effects on Bowl accumulation were cell-autonomous, as the localized expression of Drm (Fig. 2H, green; *En-Gal4/UAS-Drm*) in *drm* mutants resulted in the increased accumulation of Bowl (Fig. 2H, red) only in cells that expressed Drm (Fig. 2H, Merge), while localized expression of Lines (Fig. 2I, green; *En-Gal4/UAS-Lines*) in *lines* mutants resulted in the decreased accumulation of Bowl (Fig. 2I, red) only in cells that expressed Lines (Fig. 2I, Merge). Finally, to confirm that the Lines–Bowl protein–protein interaction is necessary for the regulation of Bowl accumulation in vivo, we compared the distribution of wild-type Bowl to

that of Bowl(R258C), which is compromised for binding to Lines. We expressed these proteins across the embryonic epidermis using *Ptc-Gal4*, a driver expressed across most but not all cells of the PS (Fig. 2J,K; the expression domain within one PS is marked by a double arrow). We found that epitope-tagged wild-type Bowl accumulated to the greatest degree in cells that normally express *drm* (Fig. 2J). This is roughly a single-cell-wide stripe since the domains of *Ptc-gal4* and *drm* overlap in only the posterior *drm*-expressing cells. In contrast, an epitope-tagged form of Bowl(R258C), compromised for binding to Lines, accumulated in all cells in which it was expressed (Fig. 2K, double-headed arrow). We thus conclude that changes in the nuclear abundance of Bowl across the embryonic epidermis are dependent on regulated physical interaction between Lines and Bowl.

Changes in the intensity of the Bowl immunofluorescent signals could reflect either changes in the steady-state level or subcellular distribution of the Bowl protein. We distinguished between these possibilities by immunoblotting embryonic extracts from different genotypes. Lower levels of Bowl were detected in *drm* mutants compared to wild type, and approximately fivefold higher levels of Bowl were detected in *lines* mutants, *drm lines* double mutants, or in embryos overexpressing *drm* (Fig. 2L). Thus, these data confirmed that *drm* and *lines* control the steady-state level of Bowl protein. We conclude that the Lines protein regulates Bowl protein accumulation post-translationally by physically binding to Bowl, consistent with Lines activity leading either directly or indirectly to the degradation of Bowl protein. Drm may inhibit the degradation of Bowl by antagonizing *lines* in the narrow domain of cells that express *drm* (see below; Fig. 3).

Drm localizes Lines to the cytoplasm, thereby segregating Lines from nuclear Bowl

We next investigated whether Drm antagonizes other aspects of Lines function. We reported previously that, across a PS, the Lines protein exhibits distinct subcellular localization that correlates with its genetic requirement (Hatini et al. 2000). An epitope-tagged version of Lines, when expressed either broadly using *Arm-GAL4* (Fig. 3B) or more discretely using *Ptc-Gal4* (Fig. 3G), accumulated in the nuclei of cells where *lines* is required genetically, but was either less focused to nuclei (Fig. 3B) or quite cytoplasmically enriched (Fig. 3G) within a narrow domain where *lines* is not required genetically. The cytoplasmic enrichment of Lines occurs in a region that flanks the segment border (marked by arrowhead), which is where *drm* is transcribed (Fig. 3A) and Bowl protein

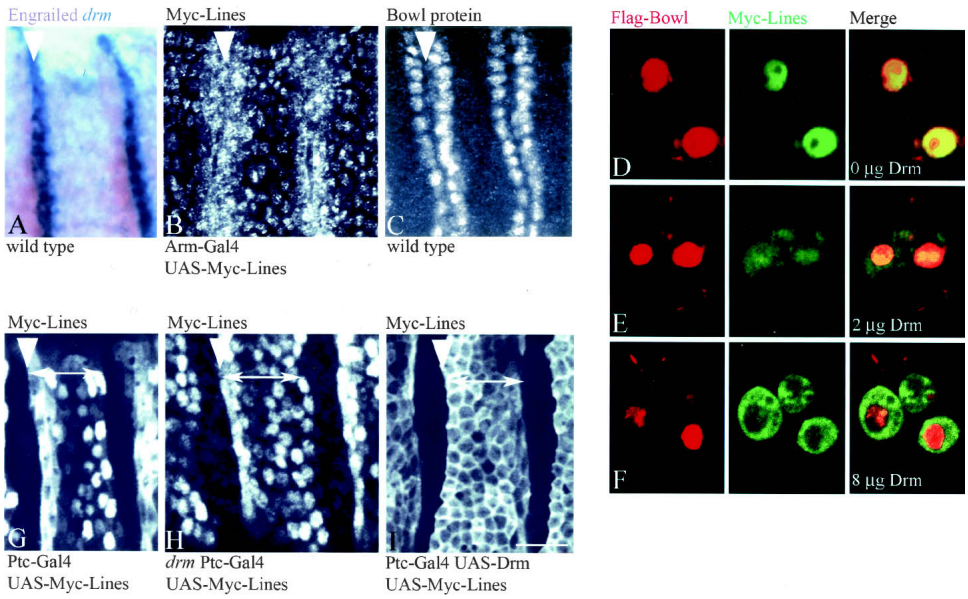

Figure 3. Drm redistributes Lines to the cytoplasm, thereby segregating Lines away from nuclear Bowl. (*A*) Wild type, En protein (brown), *drm* RNA (purple): *drm* was expressed in two cell rows flanking the segment border (marked by arrowhead). (*B*) *Arm-GAL4* UAS-Myc-Lines: nuclear accumulation of Myc-Lines, except in cells flanking the segment border, where the localization is diffuse and more cytoplasmic. (*C*) Bowl accumulated in cells flanking the segment border (where *drm* was expressed and Lines was enriched in the cytoplasm). (*D–F*) S2 cells transfected with constant amounts of UAS-Flag-Bowl (red) and UAS-Myc-Lines (green) and increasing amounts of UAS-Drm (0, 2, and 8 µg of UAS-Drm in *D–F*, respectively). The *right* panel is Merge. We suspect that Lines was unable to efficiently down-regulate Bowl in *D* due to the high level of Bowl gene expression in transfected cells. (*G*) *Ptc-GAL4* UAS-Myc-Lines: The strength and timing for this driver differ from that for *Arm-GAL4*; it is expressed in the domain marked by double-headed arrows, and it is not expressed in Engrailed/Hedgehog cells that appear unstained. Pronounced cytoplasmic accumulation of Lines posterior to the segment border (Hatini et al. 2000). (*H*) *drm Ptc-GAL4* UAS-Myc-Lines: fairly uniform nuclear accumulation of Lines. Residual cytoplasmic accumulation of Lines posterior the segment border may result from either a residual activity of the drm^3 allele, or from a weak activity of *odd* and/or *sob*, which are related to *drm* in both sequence and expression pattern. (*I*) *Ptc-GAL4* UAS-Myc-Lines UAS-Drm: uniform cytoplasmic accumulation of Lines. Bar: *A–C*,*G–I*, 10 µm; *D–F*, 3 µm.

accumulates (Fig. 3C). Since the subcellular distribution of Lines was independent of *bowl* function (data not shown), we tested whether it was controlled by *drm*. The reduced nuclear accumulation of Lines in cells flanking the segment border suggested that Drm disrupts the Lines–Bowl interaction by segregating Lines away from nuclearly localized Bowl. This was investigated by co-transfecting cells with constant amounts of Lines and Bowl together with increasing amounts of Drm. Consistent with our hypothesis, Lines and Bowl localized to the nucleus in the absence of transfected *drm* (Fig. 3D). However, Lines redistributed to the cytoplasm with increasing amounts of cotransfected *drm* (Fig. 3E,F). To determine whether this interaction occurred in vivo as well, we examined the subcellular distribution of Lines in *drm* mutants or when *drm* was ectopically expressed. In wild type, the epitope-tagged form of Lines was cytoplasmic posteriorly adjacent to the segment border, and nuclear in remaining cells that express *Ptc-Gal4* (Fig. 3G; the double-headed arrow marks the *Ptc-GAL4* expression domain; Engrailed/Hedgehog cells do not express *Ptc-Gal4* and therefore remain unstained). In *drm* mutants, the epitope-tagged form of Lines was nuclear in all cells in which it was expressed by *Ptc-Gal4* (Fig. 3H), while in embryos coexpressing *lines* and *drm*, Lines was cytoplasmic in all cells expressing the two proteins (Fig. 3I). To confirm that the interaction between Drm and Lines was functionally significant, we investigated the biological activities of a mutant derivative of Drm, Drm(R46C), that failed to bind to Lines in co-IP assays and failed to elicit gain-of-function phenotypes in the gut in ectopic expression assays (Green et al. 2002). Ectopic expression of Drm(R46C) failed to transform the cuticle pattern (Fig. 1I, see figure legend), failed to redistribute Lines to the cytoplasm, and failed to increase the steady-state accumulation of Bowl (data not shown). Thus, each of the newly discovered in vivo activities of the Drm protein defined here required the interaction between Drm and Lines. We conclude that, in those cells requiring Bowl activity for patterning, Drm is expressed and inhibits Lines through a dominant interfering mechanism. The Drm peptide disrupts the Lines–Bowl interaction, alters the subcellular distribution of Lines, and thereby allows the nuclear accumulation and consequent action of Bowl. Drm localizes Lines to the cytoplasm either by stimulating nuclear export or by inhibiting nuclear import of Lines. Although our findings do not distinguish between these two possible mechanisms, we suspect that Drm disrupts the Lines–Bowl interaction in nuclei, and subsequently stimulates nuclear export of Lines, and in this manner eliminates residual activity of Lines in the nucleus.

Hedgehog promotes Bowl protein accumulation by promoting drm *expression, while Wingless antagonizes Hedgehog function and Bowl accumulation by repressing* drm *expression*

We next investigated the operation of the Drm/Lines/Bowl regulatory pathway in the context of the epidermal organizer. Across the dorsal embryonic epidermis, Hedgehog and Wingless are the key pattern-organizing signals (Heemskerk and DiNardo 1994; Hatini et al. 2000). Hedgehog specifies cell fate in half the PS (the 1°–3° cell fates), while Wingless specifies the remaining cell fate (the 4° cell fate) in the complementary half. To investigate whether Hedgehog and Wingless engage the Drm/Lines/Bowl regulatory pathway, we examined *drm* gene expression and Bowl protein accumulation under conditions of loss or excess of Hedgehog or Wingless signaling. We found that expression of *drm* was decreased in *hedgehog* mutants (Fig. 4B), and expanded posteriorly in embryos expressing the secreted form of Hedgehog in Engrailed/Hedgehog-expressing cells (Fig. 4D). Two points are noteworthy here. First, while Hedgehog can

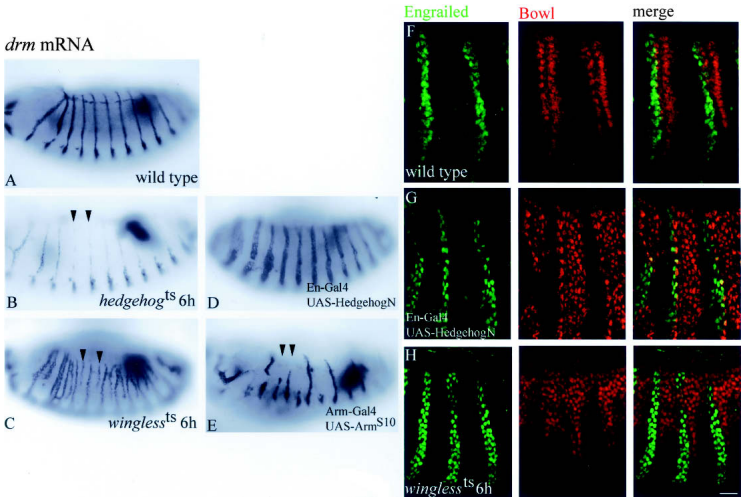

Figure 4. Hedgehog and Wingless define boundaries of *drm* expression and Bowl accumulation in the embryonic epidermis. (*A–E*) *drm* RNA in situ; embryos 10 h AEL, anterior to left, dorsal up. (*F–H*) Bowl (red) and En (green) immunostains; *right* panel is Merge. (*A*) Wild type. (*B*) *hedgehog*^{ts}, shifted to nonpermissive temperature at 6 h AEL: *drm* RNA expression was significantly reduced (arrowheads mark two segment border regions). (*C*) *wingless*^{ts}, shifted to the nonpermissive temperature at 6 h AEL: A second stripe of *drm* expression was induced anterior to the PS border, two of which are marked by arrowheads. (*D*) *En-Gal4* UAS-HedgehogN: expanded expression of *drm*. (*E*) *Ptc-Gal4* UAS-Armadillo^{S10} (Arm^{S10}): *drm* expression was repressed and stripes appear missing or interrupted (arrowheads). (*F–H*) Bowl protein (red), Engrailed protein (green), *right* panels Merge. (*F*) Wild type. (*G*) En-Gal4 UAS-HedgehogN: Phenotype is associated with expanded accumulation of Bowl protein (red) posterior to En-expressing cells (green). (*H*) *wingless*^{ts}: Phenotype is accompanied by an increased accumulation of Bowl (red). Bar: *A–D*, 20 µm; *E,F*, 10 µm.

directly control *drm* expression posterior to the Hedgehog domain, control within the Hedgehog domain is likely indirect since these cells cannot themselves respond to Hedgehog signaling (Eaton and Kornberg 1990; Tabata et al. 1992; Zecca et al. 1995). Second, the fact that excess Hedgehog did not induce *drm* expression in anterior cells suggested that Wingless signaling represses *drm* expression in this region. Consistent with this prospect, we found that *drm* expression was ectopically activated in *wingless* mutants and repressed upon ectopic activation of the Wingless pathway (Fig. 4C,E). Finally, we also found that changes in *drm* expression due to manipulations of Hedgehog and Wingless signaling largely led to the expected changes in Bowl protein accumulation. For instance, broadened *drm* expression caused by excess Hedgehog led to a broadened Bowl domain (Fig. 4G), while the ectopic stripe of *drm* expression in *wingless* mutants also led to increased Bowl accumulation (Fig. 4H), although Bowl accumulated rather more broadly than the narrow *drm* stripe would suggest. These changes in Bowl accumulation correlated nicely with the patterning changes observed with inactivation or activation of Hedgehog or Wingless signaling (Heemskerk and DiNardo 1994; Bokor and DiNardo 1996; Hatini et al. 2000). We conclude that the asymmetric response of *drm* to Hedgehog underlies the pattern of epidermal cell differentiation as *drm* promotes the accumulation of Bowl in *drm*-expressing cells and consequent cellular responses elicited by Bowl. Note that Bowl accumulates in two rows of cells but apparently is required for patterning across a broader region. This observation implies that Bowl controls expression of a new signal that further elaborates epidermal pattern.

drm *and* lines *regulate Bowl protein accumulation and consequent patterning in a variety of epithelial tissues*

We next investigated whether *drm* and *lines* regulate Bowl abundance in other epithelia and whether the restricted accumulation of Bowl in these epithelia controls distinct developmental fates, as it does across the embryonic epidermis. First, we investigated the regulation of Bowl accumulation in the gut. Genetically, *bowl* is required both in the foregut, where it distinguishes proventriculus from anterior gut, and in hindgut, where it distinguishes small from large intestine (Iwaki et al. 2001; Johansen et al. 2003). Indeed, we found that Bowl protein accumulated in two narrow domains in the gut: the primordia for the proventriculus (Fig. 5A) and for the small intestine (Fig. 5E). In addition, these domains coincide with the sites of *drm* expression, and in *drm* mutants, Bowl protein was barely detectable across these domains (Fig. 5B,F). Conversely, in *lines*, as well as *drm lines* double mutants, Bowl accumulated ubiquitously across the foregut and hindgut primordia (Fig. 5C,G and D,H, respectively). Thus, in the gut just as in the embryonic epidermis, the restricted accumulation of Bowl appears to control distinct developmental fates.

We next extended the analysis to the leg imaginal disc epithelia, where *bowl* has been shown to regulate distal leg identities and leg-joint morphogenesis. We found that the Bowl protein is detected at a set of five rings within the leg imaginal discs (Fig. 5M,O; de Celis Ibeas and Bray 2003), and *drm* mRNA is detected at a set of five similar rings (Fig. 5N,P), supporting the idea that the Drm/Lines/Bowl regulatory pathway also operates in this tissue (Hao et al. 2003). To determine whether *lines* controls Bowl accumulation in the leg also, we examined Bowl accumulation in clones of cells mutant for *lines*. We found a cell-autonomous increase in Bowl protein accumulation in these clones (Fig. 5Q,R). This ectopic Bowl accumulation disrupted the normal pattern of gene expression in the leg, as it led to cell-autonomous reduction of *bric-a-brac* expression, a target gene repressed by Bowl (Fig. 5Q,S, distal demarcated clone; de Celis Ibeas and Bray 2003). These regulatory interactions likely extend to several other imaginal disc epithelia, as we observed a strong correlation in the areas where Bowl was detected at high levels and the domains of *drm* expression in the wing (Fig. 5I,J) and eye–antennal disc (Fig. 5K,L).

Discussion

Our analysis in the epithelia of the embryonic epidermis, the foregut, the hindgut, and the imaginal discs provides compelling evidence that Drm, Lines, and Bowl are the core components of a novel regulatory pathway. Depending on context, this pathway can be engaged by a variety of positional cues. Once engaged, the pathway regulates the nuclear accumulation of Bowl and consequently patterning and morphogenesis in that tissue.

The Drm/Lines/Bowl pathway: mechanism of action

Drm and Bowl are structurally related proteins, and this forms the basis for the post-translational regulation of Bowl by Drm and Lines. Lines binds directly to Bowl and decreases the abundance of the Bowl protein, while, in cells that express *drm*, Drm binds directly to Lines and reverses this effect. Drm uses its N-terminal zinc finger in order to outcompete the binding of Lines to the N-terminal zinc finger of Bowl.

Drm and Bowl most likely originated from a common ancestral gene by a process of gene duplication and divergence, as these genes map near one another and have a common aspect to their splicing pattern (Wang and Coulter 1996; Green et al. 2002; Johansen et al. 2003). Duplicated gene pairs typically perform redundant functions in a given tissue, or distinct but essential functions in different tissues as a consequence of diversification of *cis*-regulatory elements. However, in this particular case, *drm* and *bowl* do not appear to share *cis*-regulatory elements. Furthermore, the activities of Drm and Bowl are not redundant. Rather, Bowl is a nuclear protein (in cells in which it accumulates) and likely regulates target gene expression. In contrast, we provide strong evidence that Drm uses a dominant interference mechanism to liberate Bowl from negative regulation. We therefore propose that *drm* and *bowl* provide an example of duplicated genes that evolved to carry out distinct molecular

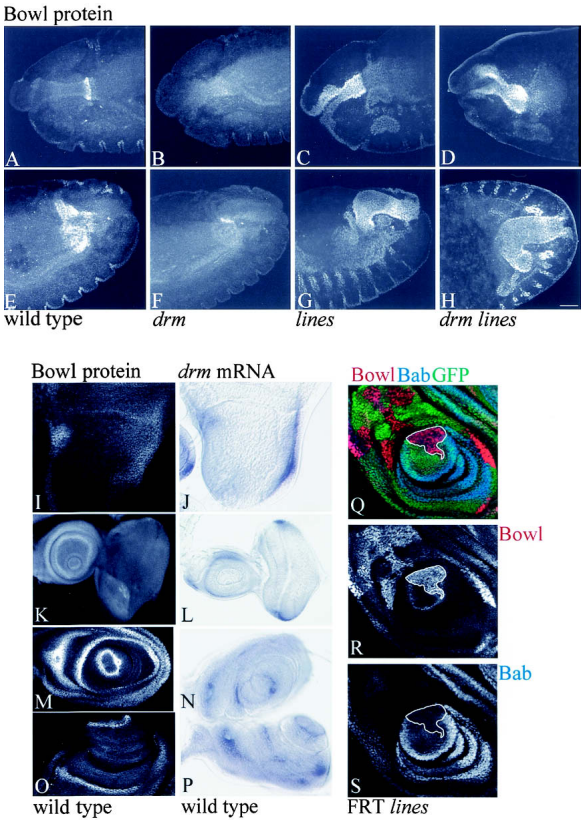

Figure 5. *drm* and *lines* control Bowl protein accumulation in the epithelia of the embryonic gut and the imaginal discs. Bowl protein in embryonic foregut (*A–D*) and hindgut (*E–H*). (*A*) Wild type: restricted accumulation of Bowl in proventriculus. (*E*) Wild type: restricted accumulation of Bowl in small intestine. (*B,F*) *drm*: Bowl accumulation was barely detected. (*C,G*) *lines*: Bowl accumulated broadly across the entire foregut and hindgut primordia, respectively. (*D,H*) *drm lines* mutants look like *lines* mutants. (*I–S*) Larval imaginal discs. Bowl protein accumulation (*I,K,M,O*) and *drm* RNA expression (*J,L,N,P*) overlapped in the epithelia of the wing (*I,J*), eye–antennal (*K,L*), and leg (*M,P*) imaginal discs. (*Q–S*) Leg disc, *lines* mutant clones (absence of green, *Q*): Bowl (red in *Q*, white in *R*) accumulated cell-autonomously in *lines* mutant clones. *bric-a-brac* (*bab*) expression (blue in *Q*, white in *S*) was lost cell-autonomously only within the distal demarcated mutant clone (white outline marks one clone). Bar: *A–H*, 20 µm; *I–S*, 10 µm.

roles within the same regulatory pathway. It is interesting to note that the role for Drm, in this duplication–divergence scenario is likely to protect Bowl from degradation by Lines. While regulated protein degradation is at the heart of several developmentally important signal transduction pathways, including those of Hedgehogs and Wnts (Maniatis 1999), the example revealed here—the likely inhibition of protein degradation by the patterned expression of a small peptide inhibitor (Drm)—appears to be novel.

While we have found that this regulatory pathway operates in several tissues, it is likely that in a few instances other factors can substitute for *drm*. For instance, while *bowl* is necessary for the specification of distal leg identities and leg-joint morphogenesis (de Celis Ibeas and Bray 2003; Hao et al. 2003), *drm* mutants do not exhibit an effect on these processes (Hao et al. 2003). It was therefore hypothesized that *drm* acts redundantly with either or both of two related genes mapping nearby, *odd* and *sob*, as these genes exhibit expression patterns similar to that of *drm* (Hao et al. 2003). This hypothesis implies that three related inhibitors—*drm*, *sob*, and *odd*—could regulate the Lines–Bowl interaction, depending on context. While gain-of-function experiments support this hypothesis, loss-of-function experiments and biochemical evidence are still lacking. Although other family members might substitute for Drm occasionally, it appears so far that Bowl is a dedicated target for regulation by Lines. A particularly interesting example of this is in the ventral embryonic epidermis. The normally trapezoid-shaped denticle belts are narrower, and more "line"-like in *lines* mutant embryos (Nusslein-Volhard et al. 1984). During normal development, there is no role for *drm* in denticle patterning, and, consistent with the lack of a defect in *drm* mutants, neither *drm* nor *odd* or *sob* is expressed ventrally at the appropriate stage. *bowl*, too, plays no role normally in ventral patterning, even though this gene is globally expressed in this tissue. What is striking is that the *lines* mutant phenotype is due to ectopic accumulation of Bowl protein, as the *lines* phenotype is completely suppressed if *bowl* function is also removed (V. Hatini and S. DiNardo, unpubl.). Since Bowl is the likely dedicated target for *lines* activity, we would predict that *lines* phenotypes in other tissues would also be due to ectopic Bowl protein accumulation and function.

The Drm/Lines/Bowl pathway: tissue-specific engagement

The most important biological implication of our findings is that the Drm/Lines/Bowl pathway can be engaged by a variety of positional cues, depending on context, to elaborate pattern across a field of cells. While Hedgehog and Wingless engage this regulatory pathway in the embryonic epidermis, these signals are not involved in the developing gut epithelia, and the relevant positional cues remain unknown. In the leg imaginal disc, two reports suggest that the Notch signaling pathway regulates *drm* expression (Hao et al. 2003) and Bowl accumulation (de Celis Ibeas and Bray 2003). The Notch pathway may engage *lines* and *bowl* in order to control the identity of distal leg identities and the morphogenesis of leg joints. The regulation of *bric-a-brac* expression by *lines* (Fig. 5Q–S) nicely substantiates this idea, as *bric-a-brac* itself specifies distal leg identities (de Celis Ibeas and Bray 2003). Taken together with the results presented here, we propose that the *drm* gene can integrate distinct signaling inputs depending on the specific tissue invloved.

Across the dorsal embryonic epidermis, the regulation of *drm* gene expression can explain how the Drm/Lines/Bowl pathway links the antagonistic inputs of Hedgehog and Wingless signaling to subsequent steps in epidermal differentiation. Indeed, changes in *drm* expression account nicely for the transformation of the epidermal pat-

tern observed in conditional *hedgehog* and *wingless* mutants. Loss of *drm* expression, as seen in *hedgehog* mutants, leads to the establishment of the 4° cell type in place of the 1°2°3° portion of the pattern, resulting in a 4°–4° pattern. In contrast, symmetric *drm* expression, as seen in *wingless* mutants, leads to the establishment of mirror-symmetric 3°2°1° fates in place of the 4°, resulting in a 1°2°3°–3°2°1° pattern. The asymmetric induction of *drm* expression is then used to modulate Lines and Bowl function. This is reflected by the asymmetry of Lines subcellular distribution and Bowl accumulation relative to the source of Hedgehog production. Although Bowl accumulates in only two cell rows in each PS, it has a remarkable influence on a broader field of cells that spans approximately six cell rows. Bowl may therefore organize the pattern indirectly by regulating expression of a new signal.

The Drm/Lines/Bowl pathway and the epidermal organizer

Pattern across each PS in the ventral embryonic epidermis is not organized by a single morphogen but by a combination of distinct signals, with each signal acting fairly locally. Early during development, the expression of Hedgehog and Wingless is established by reciprocal induction across the parasegment border. At a later stage, Hedgehog induces expression of *rhomboid* only on the segment border side within the anterior compartment. *rhomboid* controls the production of secreted Spitz, a TGFα homolog that activates the EGF-R pathway. In addition, Hedgehog and Wingless appear to act at a distance to restrict *Serrate* expression to the middle of the anterior compartment. Finally, cell differentiation is controlled by Hedgehog, Wingless, Spitz, and Serrate, each controlling a subset of cell fates (for review, see Hatini and DiNardo 2001b; Sanson 2001). For example, Hedgehog, Spitz, and Wingless each induce expression of the gene *stripe* by short-range inductive signaling, leading to tendon differentiation at three discrete positions across each abdominal PS (Hatini and DiNardo 2001a). While *rhomboid* and consequent EGF-R activation are crucial for ventral patterning, we were unsuccessful to detect a role for *rhomboid* in dorsal cuticle patterning. Our current findings suggest that the Drm/Lines/Bowl pathway organizes the pattern in response to Hedgehog signaling dorsally and thus substitutes for *rhomboid*. Although *drm* responds to Hedgehog asymmetrically, there is an important distinction between the regulation of *drm* expression and the regulation of other Hedgehog targets such *stripe* and *rhomboid*. While previously known Hedgehog targets are induced only in anterior compartment cells, the *drm* gene is induced in both anterior and posterior compartments, on either side of the segment border. The induction of *drm* expression in the posterior compartment is likely not due to Hedgehog directly, because Hedgehog-producing cells are refractory to Hedgehog signaling. There is likely a reciprocal induction between anterior and posterior compartment cells with Hedgehog inducing *drm* expression in the anterior compartment, and a new signal inducing *drm* in the posterior compartment. Understanding the logic underlying this regulation will require identifying the signal(s) downstream of Bowl that lead to broad patterning. Given that the Drm/Lines/Bowl regulatory pathway is conserved and operates reiteratively in development, such signals are likely to be used in patterning of other epithelial tissues.

Materials and methods

DrmHA constructs were previously described (Green et al. 2002). Bowl constructs were generated from cDNA clone RE32660. UAS-Bowl rescued the cuticle phenotype of $bowl^1$/$bowl^2$ mutant embryos to wild type. Variants of Bowl were generated by PCR amplification and fused in frame with two tandem Flag epitopes in pCS2 2X-Flag (provided by P. Klein, University of Pennsylvania, Philadelphia, PA) to generate N-terminally tagged derivatives (Fig. 2A). Three glycine residues separated the 2X-Flag epitope and the Bowl-coding region. The ZFD fragment spans the RPKKQF to HAVGEVN interval. Bowl(R258C) was generated by substituting an arginine in loop 3 of the first zinc finger with a cysteine. A similar mutation in the first finger of Drm eliminated the Drm–Lines interaction (Green et al. 2002). Bowl(C268G) was generated by substituting the first zinc-coordinating cysteine in the second zinc finger with a glycine. The primer sequences used are available upon request. Lines constructs were generated by PCR from a previously cloned cDNA (Hatini et al. 2000), and fused in frame with six tandem Myc epitopes in pCS26X-Myc (provided by P. Klein). Tagged cDNA inserts were cloned into pUAST.

Transgenic flies were generated by standard methods as previously described (Hatini et al. 2000). S2 cells were transfected using calcium phosphate in a 6-cm dish with 3 μg of *Ubiquitin-GAL4* and 2.5 μg of each construct. For dose-response assays, cells were transfected with 2 μg of UAS-Myc-Lines, 2 μg of UAS-Flag-Bowl, and 2.5 μg of Ubiquitin-Gal4 in the presence of increasing amounts of UAS-HA-Drm (0, 2, and 8 μg). For each dose, at least 50 double-labeled cells were scored, and Lines was redistributed in >80% of double-labeled cells (Fig. 3D–F). Each Western blot described in Figure 2A, B, and L was repeated at least three times, and a representative blot is shown for each experiment. To ensure sufficient protein production following transfection, cultured cells were fixed and immunostained 48 h following transfection. Cell suspensions were transferred to an eight-well lab-tek II chamber slide system (Nalge Nunc Int.) and incubated for 1 h to allow cells to adhere to the slide. Cells were then washed with PBS, fixed for 30 min, and processed for immunofluorescence with 1:1000 anti-Myc and anti-Flag antibodies. Immunoprecipitation assays were performed as previously described (Green et al. 2002) using anti-Flag antibodies (M2; Sigma) at 1:40 dilution, followed by immunoblotting with rabbit anti-Myc (A-14; Santa Cruz) at 1:1000 dilution. The amount of Myc-Lines in unprocessed lysates was used to normalize for variations in transfection efficiency.

Fixed embryos were analyzed using immunohistochemistry, immunofluorescence, and in situ hybridization, as described previously (Hatini et al. 2000; Hatini and DiNardo 2001a). *lines*, *drm*, and *lines drm* double-mutant alleles were maintained over the CyO Kr-Gal4 UAS-GFP balancer chromosome and staged seven to 11 AEL (after egg laying) mutant embryos lacking GFP fluorescence were selected using a fluorescent dissecting microscope. UAS-Drm was crossed to the *Ptc-Gal4* driver to uniformly express the transgenes in all progeny. Following dechorionation, embryos were homogenized in NET buffer (50 mM

Tris at pH 7.5, 5 mM EDTA, 1% NP-40, and protease inhibitors) and incubated for 30 min on ice. Lysates were then cleared by centrifugation and proteins were fractionated by SDS-PAGE. Blots were probed using anti-Bowl antibody (1:2000) (de Celis Ibeas and Bray 2003) and β-tubulin (Tu27, Covance, 1:2000).

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