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Hedgehog is required for activation of *engrailed* during regeneration of fragmented *Drosophila* imaginal discs

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SUMMARY

Surgically fragmented *Drosophila* appendage primordia (imaginal discs) engage in wound healing and pattern regulation during short periods of in vivo culture. Prothoracic leg disc fragments possess exceptional regulative capacity, highlighted by the ability of anterior cells to convert to posterior identity and establish a novel posterior compartment. This anterior/posterior conversion violates developmental lineage restrictions essential for normal growth and patterning of the disc, and thus provides an ideal model for understanding how cells change fate during epimorphic pattern regulation. Here we present evidence that the secreted signal encoded by *hedgehog* directs anterior/posterior conversion by activating the posterior-specific transcription factor *engrailed* in regulating anterior cells. In the absence of *hedgehog* activity, prothoracic leg disc fragments fail to undergo anterior/posterior conversion, but can still regenerate missing anterior pattern elements. We suggest that *hedgehog*-independent regeneration within the anterior compartment (termed integration) is mediated by the positional cues encoded by *wingless* and *decapentaplegic*. Taken together, our results provide a novel mechanistic interpretation of imaginal disc pattern regulation and permit speculation that similar mechanisms could govern appendage regeneration in other organisms.

Key words: *Drosophila*, Imaginal disc, Pattern regulation, Regeneration, Duplication, Compartment, *hedgehog*, *engrailed*, *wingless*

INTRODUCTION

Epimorphic limb regeneration requires that cells change fate in order to replace lost or damaged structures (Morgan, 1901). In most cases, very little is known about how molecular cues stimulate regenerating limb cells to change their identity, although it can be assumed that signals employed in development are reused during regeneration. We are investigating cell fate respecification in cut fragments of *Drosophila* imaginal discs as a means of gaining insight into pattern formation during normal development and as a general model for understanding fate changes during appendage regeneration in other organisms.

Drosophila thoracic imaginal discs originate as distinct polyclones of anterior (A) and posterior (P) embryonic disc precursor cells (Crick and Lawrence, 1975). In larvae, these cells heritably maintain their A/P identity through three successive instars of exponential proliferation, resulting in mature discs comprising distinct A and P lineage compartments (Garcia-Bellido et al., 1973; Garcia-Bellido, 1975; reviewed in Cohen, 1993). Interactions across the A/P compartment boundary serve as the basis for pattern formation and axis specification during development (Fig. 1A; reviewed in Blair, 1995; Lawrence and Struhl, 1996).

In the leg disc, cells assume position-specific identities in response to spatial cues provided by localized transcription of secreted signals encoded by *wingless* (*wg*; Couso et al., 1993), *decapentaplegic* (*dpp*; Masucci et al., 1990) and *hedgehog* (*hh*; Lee et al., 1992; Basler and Struhl, 1994). The requirement for localized transcription of these genes can be demonstrated by misexpressing them outside their endogenous expression patterns, either in random cell clones (*flp*-out; Struhl and Basler, 1993) or targeted expression domains (*Gal4/UAS*; Brand and Perrimon, 1993). In general, misexpression results in overproliferation, duplication of the limb proximodistal axis, loss of selected pattern elements with concomitant multiplication of others and occasional changes in appendage identity (Struhl and Basler, 1993; Maves and Schubiger, 1995, 1998a; Johnston and Schubiger, 1996). Surprisingly, these pattern abnormalities closely resemble the effects of cutting discs into fragments and allowing them to heal and re-grow during short periods of in vivo culture.

Disc growth and patterning normally culminate at the end of the third larval instar. At this stage, leg disc cells achieve rigidly determined states (Schubiger, 1968) and assume significantly reduced rates of cell division (Graves and Schubiger, 1982) as they await metamorphic cues that trigger differentiation. Relative stasis is maintained even if discs are afforded the opportunity to undergo continued proliferation and patterning during short periods of in vivo culture (Kiehle and Schubiger, 1985). Under identical in vivo conditions,

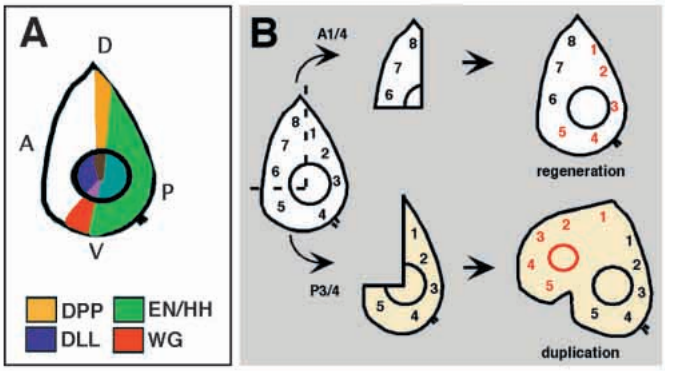

Fig. 1. Pattern formation and regulation in the L1 imaginal disc. (A) Schematic representation of pattern gene expression domains. Mature discs are comprised of lineage-restricted A and P compartments (Garcia-Bellido et al., 1973; Garcia-Bellido, 1975). *engrailed* (*en*) is expressed in all P cells (Kornberg et al., 1985) and cell-autonomously activates a signal encoded by *hedgehog* (*hh*; Lee et al., 1992; Tabata et al., 1992) which is secreted from P cells to activate *wingless* (*wg*) in anterioventral cells and *decapentaplegic* (*dpp*) in anteriodorsal cells along the compartment boundary (Basler and Struhl, 1994). WG and DPP, themselves secreted signals (Gelbart, 1989; van den Heuvel et al., 1989; González et al., 1991; Nellen et al., 1996; Zecca et al., 1996), act antagonistically to specify the leg dorsal-ventral axis (Penton and Hoffmann, 1996; Thiesen et al., 1996; Jiang and Struhl, 1996; Brook and Cohen, 1996), and synergistically to maintain the distal leg-determining transcription factor Distalless (DLL) in the center of the disc (Lecuit and Cohen, 1997). (B) Regeneration and duplication of L1 disc fragments. A whole disc is divided into A1/4 and P3/4 fragments. Each contains a subset of the positional values present in the original disc (1-8). For simplicity, we have assumed a uniform distribution of positional values. In the A1/4 fragment, missing positional values (1-5 in red) are regenerated. In the P3/4 fragment, existing positional values (1-5 in red) are duplicated. In both cases, the A/P lineage restriction is violated (Abbott et al., 1981). Note that the same set of values is generated during culture of both fragments (adapted from Schubiger, 1971).

however, cut and cultured third instar disc fragments engage in an aggressive program of proliferation and fate respecification called pattern regulation (Bryant, 1978). Despite considerable theoretical attention (French et al., 1976; Meinhardt, 1983; Kondo, 1992; Campbell and Tomlinson, 1995), the functional basis for this phenomenon is essentially unknown.

Pattern regulation initiates in vivo. Disc fragment cut edges heal together, establishing a region of localized cell proliferation and fate respecification termed a blastema (French et al., 1976). Fate changes in the blastema can be mapped by forcing cultured fragments to differentiate and then scoring for adult cuticle structures relative to uncultured control fragments. Only cells within or adjacent to the blastema participate in pattern regulation (Abbott et al., 1981), which results in either replacement of missing (regeneration) or reiteration of existing (duplication) disc pattern elements (Schubiger, 1971; Bryant, 1971). Generally, one fragment of a bisected disc regenerates while its complement duplicates. In addition, cells within a cultured fragment occasionally undergo changes in overall appendage identity termed transdetermination (Hadorn, 1965; reviewed in Hadorn, 1978; and Maves and Schubiger, 1998b).

Pattern regulation is described in leg, eye-antenna, wing, genital and labial disc fragments (reviewed in Bryant, 1978; Maves and Schubiger, 1998b). Among these, fragments of the prothoracic leg (L1) disc possess unique and remarkable regulative potential. In accordance with the maxim that one disc fragment regenerates while its complement duplicates, L1 anterior 1/4 (A1/4) fragments almost always regenerate, while complementing posterior 3/4 (P3/4) fragments almost always duplicate (Fig. 1B, Schubiger, 1971). In both cases, A cells convert directly to P identity (Abbott et al., 1981), in direct violation of lineage restrictions imposed during normal development (Garcia-Bellido et al., 1973, Garcia-Bellido, 1975). Curiously, mesothoracic leg (L2) discs lack this ability (M. C. G. and G. S., unpublished data), suggesting that the capacity for intercompartmental conversion derives from a unique property of the L1 disc.

Here we investigate the molecular basis for pattern regulation in cultured fragments of the L1 imaginal disc. Specifically, we ask why L1 A1/4 fragments regenerate while P3/4 fragments duplicate and how regulating A cells convert to P identity. We show that: (1) different regulative behaviors in the two fragments do not reflect a binary decision to regenerate or duplicate, but rather a uniform response to fragmentation; (2) HH signaling induces A/P conversion in both fragments by activating *engrailed* (*en*) in regulating anterior cells, and (3) a distinct population of squamous *en/hh*-expressing peripodial cells (specific to L1) fuse to the disc cut edge and act as a transient ectopic source of HH in cultured disc fragments. Based on these and other results, we suggest that fragmentation and wound-healing result in distinct A/P conversion and/or intracompartmental integration modes of pattern regulation. In addition, we discuss implications of our results for understanding cell fate changes during limb regeneration in other systems.

MATERIALS AND METHODS

In vivo culture

Leg discs were dissected at 96 hours after egg deposition (AED), fragmented in insect Ringer's solution (as described in Schubiger, 1971) and injected into the abdomens of freshly eclosed, etherized female hosts. Precise staging of disc donors was crucial because overaged pupally committed late third instar discs evert during culture, interfering with and/or obfuscating pattern regulation. The use of freshly eclosed hosts facilitated recovery of fragments and ensured a consistent, larva-like cell cycle period in regulating blastema cells (Kiehle and Schubiger, 1985). Host animals were maintained in the presence of males on standard food supplemented with yeast paste. Fragments were recovered from host abdomens in a drop of Ringer's solution on a siliconized slide and either fixed for immunocytochemistry or injected into late-wandering third instar larvae for cuticular analysis after the general method of Ephrussi and Beadle (1936) as described in Maves and Schubiger (1998a). Adult leg cuticle and differentiated implants were mounted in Faure's water-based medium and analyzed after the leg disc fate map of Schubiger (1971). In all experiments, discs were fragmented with tungsten needles and transferred in the tip of a P-20 Pipetteman lubricated by repetitive transfer of larval fat body.

Fly stocks and temperature shifts

We used *wg-lacZ* (Struhl and Basler, 1993) and P30 *hh-lacZ* (Lee et al., 1992) to identify gene expression domains and hh^{ts2} (Ma et al., 1993) to block *hh* activity during in vivo culture. For these experiments, hh^{ts2} larvae were raised to the late third instar at 15°C

(permissive) and shifted to 25°C (restrictive) 6 hours prior to fragmentation and injection into hosts. Host animals were raised at 25°C for 4 days for experimental groups or shifted back to 15°C for 10-14 days for controls. We used 25°C rather than higher temperatures (28°C, Basler and Struhl, 1994) to avoid potentially confounding effects of temperature shock on wound healing and regulative processes. At 29°C, hh^{ts2} A1/4 fragments were not recoverable from hosts, presumably due to lack of growth or degeneration (data not shown). For clone induction (described below) we used *hs-flp; Act5c>CD2>Gal4, UAS-GFP* (Pignoni and Zipursky, 1997; Neufeld et al., 1998). Ectopic *wg* was induced using the *flp*-out technique of Struhl and Basler (1993) after the method of Maves and Schubiger (1995).

Clone induction

Random Green Fluorescent Protein (GFP)-labeled clones were induced with a 30 minute-long 37°C heat shock to *hs-flp; Act5c>CD2>Gal4, UAS-GFP* larvae at 72 hours AED. At 96 hours AED, experimental discs were fragmented and cultured in vivo for an additional 72 hours. After culture, we used confocal microscopy to analyze GFP^+ clone position relative to the nascent A/P boundary (as defined by EN) in explanted duplicated disc fragments. Girton and Russell (1980) showed that as few as 20 cells participate in pattern duplication. Accordingly, we induced a relatively large number of clones in the disc in order to observe clones in the duplicate. In uncut control discs, our protocol produced a large number of small (4- to 8-cell) clones at the time of fragmentation. In experimental material, many clones were observed in non-regulating parts of the disc fragment, while an average of just 1.3 clones was observed in the duplicated area. 1-hour heat shocks resulted in too many clones for meaningful analysis (data not shown).

Immunocytochemistry and imaging

Whole discs and cultured disc fragments were fixed, immunostained and imaged after the methods of Maves and Schubiger (1998a) except that 4% paraformaldehyde fixation was for 1.5 hours on ice and disc material was preblocked in Normal Goat Serum for 1 hour prior to staining. Primary antibodies were used at the following dilutions: mouse anti-EN 4D9 (gift of Tom Kornberg) 1:1000; rabbit anti-phosphohistone H3 mitosis marker (Upstate Biotech) 1:2000; rabbit (Cappel) or mouse (Boehringer Mannheim) anti-β-galactosidase 1:500; mouse anti-WG (gift of Roel Nusse) 1:5, and rabbit anti-DLL (gift of Grace Panganiban) 1:200. All rinses were carried out in concave glass culture dishes to reduce loss of material. Images were collected with a Bio-Rad MRC 600 confocal microscope system and assembled into figures using Adobe Photoshop 5.0 and Canvas 5. Leg discs and disc fragments were oriented using the stalk (anterior-dorsal) and nerve (posterior-ventral). The position of the nerve allowed us to discern between endogenous and duplicated gene expression domains in cultured P3/4 disc fragments.

RESULTS

EN expression during pattern regulation

The observation that A cells switch to P identity during pattern regulation in L1 disc fragments (Abbott et al., 1981) is a curious exception to the rule of compartmental lineage restriction during *Drosophila* appendage development. This prompted us to ask how A/P conversion is achieved on the molecular level. Since EN is normally expressed in all P cells (Kornberg et al., 1985; Fig. 2A), we tested whether A/P conversion correlated with activation of EN in cultured disc fragments. Third instar L1 discs were cut into A1/4 and P3/4 fragments and cultured in vivo. Approximately 50% of A1/4 (11/20) and P3/4 (17/34) fragments produced novel EN expression domains (Fig. 2B,C) within 96 hours in vivo. The size and shape of nascent posterior compartments varied from

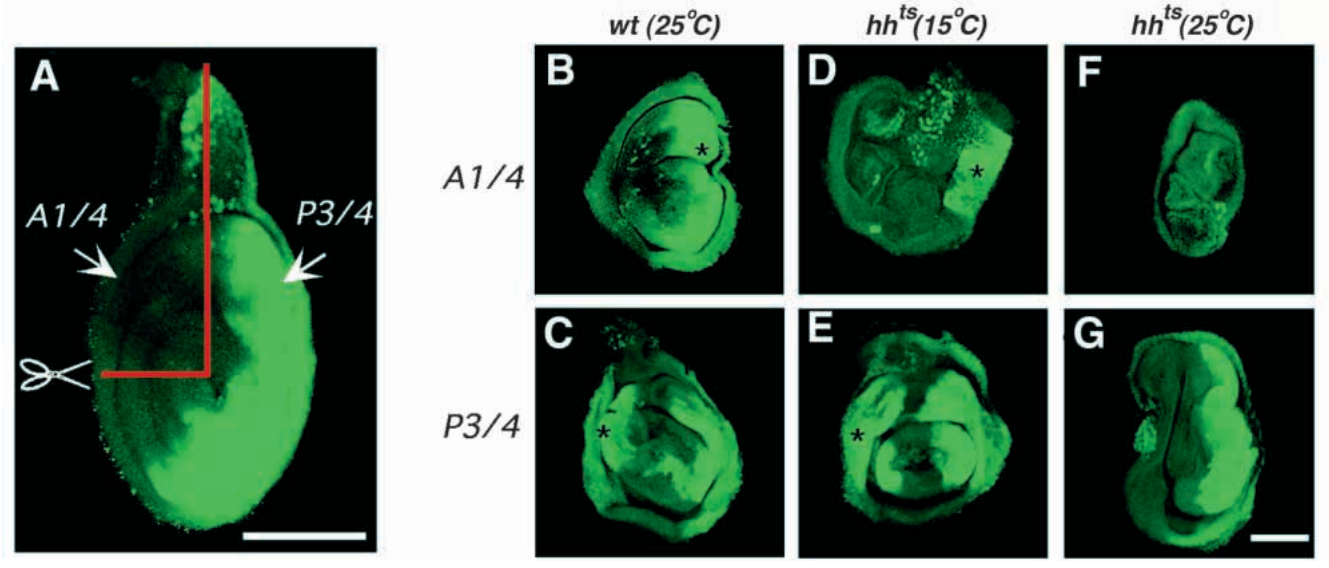

Fig. 2. HH signaling is required for activation of *en* during regeneration of A1/4 and P3/4 disc fragments. All discs and disc fragments in this and subsequent Figures are oriented dorsal, up; ventral, down; posterior, right; anterior, left. All fragments were immunostained for EN (green), an asterisk (*) indicates a nascent EN domain. (A) Whole third instar disc. Lines indicate the approximate position of surgical cuts used to generate fragments. (B,C) Wild-type (wt) control A1/4 and P3/4 fragments cultured for 96 hours at 25ºC. Note clearly regenerated EN domain in B, and mirror-symmetric duplication of EN domains in C. (D,E) A1/4 and P3/4 hh^{ts2} fragments cultured at a permissive temperature (15ºC) for 14 days (the culture period was corrected to compensate for lower temperature). Note disrupted morphology of the regenerated A1/4 fragment shown in D. Such defects were common and may reflect weak hypomorphic effects of the hh^{ts2} allele at low temperature. (F,G) hh^{ts2} A1/4 and P3/4 fragments cultured at the restrictive temperature for 96 hours did not form new EN domains. Bars, 50 μm.

small clusters of EN-expressing cells to large domains almost indistinguishable from the endogenous P compartment – suggesting that stochastic variations (subtle differences in cut sites, disc morphology and precise age of the disc donor) might influence the timing and extent of pattern regulation. In a significant number of cases, however, A1/4 disc fragments 'regenerated' a new P compartment, which restored the proportions of a whole disc (Fig. 2B), and P3/4 fragments 'duplicated' a new EN-expression domain in mirror-symmetric opposition to the endogenous P compartment (Fig. 2C).

EN is activated in anterior cells

Previously, clonal analysis has been used to show that antennal cells convert directly to wing identity (Gehring, 1967) and that A leg cells can convert directly to P identity during pattern regulation (Abbott et al., 1981; Girton and Russell, 1981). In these studies, conclusions about clone behavior were inferred from analysis of genetically marked cuticle structures. To directly observe the cellular origin of nascent EN domains in regulating disc fragments, we induced random GFP-labeled clones by heat-shocking *hs-flp; Act5c>CD2>GAL4, UAS-GFP* larvae 1 day prior to fragmentation and injection into host animals. Fig. 3A shows an uncut control disc, note the relatively uniform distribution of small GFP$^+$ clones in both compartments. P3/4 disc fragments containing GFP$^+$ clones were cultured in vivo for an additional 72 hours. Duplicated fragments were then scored for the presence of marked clones, that contained both A (EN$^-$) and P (EN$^+$) cells (Fig. 3B,C).

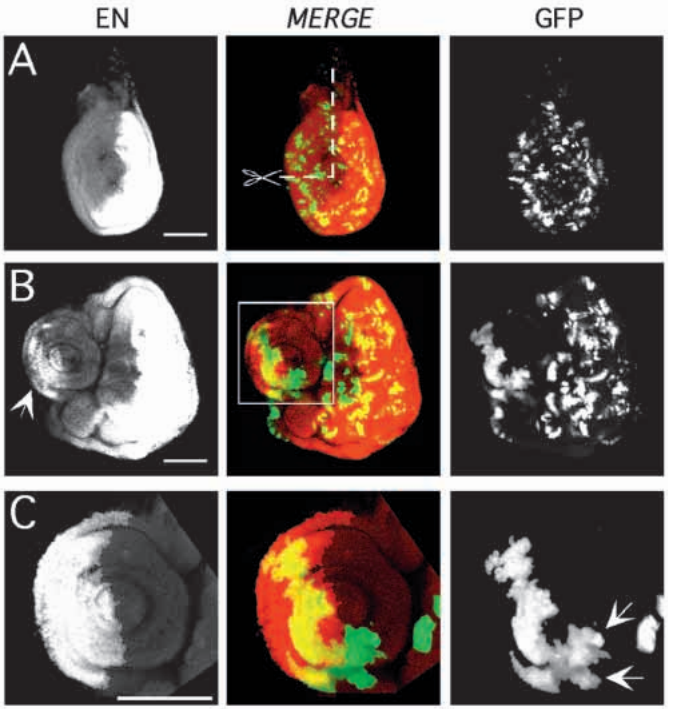

Fig. 3. GFP-marked cell clones cross nascent compartmental boundaries in regulating L1 P3/4 disc fragments. (A) 96-hour-old whole third instar disc showing small clones of GFP$^+$ cells (green) and a single EN-expressing posterior compartment (red). (B) Single confocal plane view of a duplicated P3/4 disc fragment, showing GFP$^+$ cells (green) and two distinct EN$^+$ posterior compartments (red). The nascent posterior compartment is indicated by an arrow. Note the difference in clone density between the duplicate (roughly within the box) and the rest of the disc. (C) High-magnification confocal Z-series of the box indicated in B. A single, large GFP$^+$ clone crosses the nascent compartmental boundary. Arrows indicate two potential points of clone origin in the A compartment. The whole disc in A contains about 20,000 cells (Kiehle and Schubiger, 1985), based on a rough area measurement, we estimate that the 96-hour-old clone in B contains 3,000 cells. This suggests an estimated doubling time of 8 hours, generally consistent with a leg disc cell doubling time of 7 hours during normal development (Graves and Schubiger, 1982). Bars, 50 μm.

17/34 clone-bearing P3/4 fragments produced a duplicated EN domain; 12 of these had a well-defined novel compartment boundary suitable for detailed analysis. Of these 12 duplicates, two contained no GFP$^+$ clones, six had one clone, two had two clones and two had three clones in the duplicated area. Four duplicates contained a contiguous GFP$^+$ clone comprising both A (EN$^-$) and P (EN$^+$) cells (Fig. 3C). Because our protocol generated an average of 1.3 clones/duplicated area (16 clones/12 duplicates), we infer that these four cases represent single large clones that violate the A/P lineage restriction rather than two or more smaller clones fused exactly at the nascent A/P boundary. These observations confirm the results of Abbott et al. (1981), demonstrating that A cells directly convert to P identity during pattern regulation in L1 disc fragments.

The size and distribution of GFP$^+$ clones in duplicated discs provided additional insights into the dynamics of proliferation in the blastema. Nascent P compartments were never entirely composed of GFP$^+$ cells (0/12), indicating that A/P conversion is a polyclonal event in duplicating disc fragments. We also observed that single GFP$^+$ clones often occupied up to 10-20% of the whole duplicated area, suggesting that as few as 5-10 founder cells participated in duplicative growth. This agrees with a previous clonal analysis, which suggests that similar cell numbers generate disc duplications during cell-lethality-mediated pattern regulation (Girton and Russell, 1980).

HH activates *en* in regulating disc fragments

Endogenous *en* expression in the L1 disc is activated during embryogenesis by the transcription factor Fushi tarazu (FTZ; Ingham et al., 1988). However, *ftz-lacZ* expression was not detected in cultured disc fragments (data not shown), suggesting an alternate mechanism for activation of *en* during pattern regulation. Ectopic *hh* activity induces *en* in A cells of the abdominal tergites (Struhl et al., 1997), wing (de Celis and Ruiz Gomez, 1995; Guillen et al., 1995) and leg imaginal discs (Laura Johnston, personal communication), indicating that *hh* might be required to activate *en* in cultured disc fragments. To test this hypothesis, L1 discs from *hh*^{ts2} larvae raised at permissive temperature (15ºC) were fragmented and cultured under restrictive (25ºC) conditions (Fig. 2). In the absence of HH, cultured A1/4 fragments were small and extremely difficult to recover from hosts. Of those recovered, only 13% (3/23) regenerated new EN domains, compared with 37% (7/19) at the permissive temperature. The effect of *hh* loss was more pronounced in P3/4 fragments; none (0/27) duplicated at restrictive temperature while 39% (15/38) duplicated under permissive conditions. Loss of *hh* clearly blocked *en*-activation in both fragments (Fig. 2D-G). At non-permissive temperature, *hh*^{ts2} disc fragments also failed to produce regenerated/duplicated posterior leg structures upon forced differentiation in host larvae (Fig. 4). Taken together, these experiments

Regeneration/duplication of leg structures during culture of P3/4 fragments

	n	genotype	days *in vivo*	% duplicated structures Sc⁻8	Sc3	BH⁻	2GSt	% regenerated structures St8	Sc⁺5
L1	77	wt	0	0	0	0	0	0	0
	24	wt	3	54	71	83	50	25	17
	13	hh^{ts}	3	0	7	0	0	93	64
L2	13	wt	0	0	0	0	0	23	0
	14	wt	7	26	14	21	0	93	78

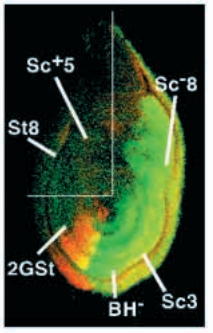

Fig. 4. Regeneration/duplication of leg structures in different P3/4 disc fragments cultured at 25ºC (restrictive for hh^{ts2}). Image at right (L1 disc immunostained for EN (green) and *wg-lacZ* (red)) indicates the approximate fate-map positions of trochanter ($Sc^{-}8$, Sc3, 2Gst and $Sc^{+}5$) and coxa (BH^{-} and St8) structures referred to in the table, after Schubiger (1971): $Sc^{-}8$, posterior group of 8 sensilla campanifomia in naked cuticle; Sc3, posterior group of 3 sensilla campaniformia; BH^{-}, single posterior Bristle on naked cuticle; 2GSt, 2 dense anterior groups (Gruppen) of Sensilla trichodea; St8, anterior group of 8 Sensilla trichodea; $Sc^{+}5$, anterior group of 5 Sensilla campaniformia in a region of dense hairs. '% duplicated structures' and '% regenerated structures' refer to the percentage of fragments containing new copies of each structure after the specified number of days in vivo. '23% regeneration' in L2 fragments cultured in vivo for 0 days may be attributed to pupal delay of the larval host in one case. This delay might allow for pattern regulation prior to metamorphosis. Note that all duplicated structures are present in the original P3/4 fate map.

demonstrate that *hh* is necessary to activate *en* and establish a new P compartment in cultured L1 disc fragments. Because loss of *hh* did not affect endogenous EN expression in cultured P3/4 fragments (Fig. 2G), we can rule out the possibility that HH is simply required to maintain EN during culture.

Without HH, P3/4 fragments switch from a duplicative to a regenerative program

P3/4 fragments normally duplicate existing pattern elements, but only rarely regenerate missing structures. In the absence of *hh*, however, P3/4 fragments gained the ability to regenerate all missing anterior leg structures (Fig. 4). This observation suggests a paradox: if *hh* is required for normal development, how do P3/4 fragments regenerate in its absence? During normal leg disc development, HH secreted from P cells acts primarily through induction of *wg* and *dpp* in A cells along the compartment boundary (Basler and Struhl, 1994). Secreted WG and DPP subsequently pattern both compartments through a variety of mechanisms (reviewed in Fig. 1), including activation of *Distalless* (*Dll*) at the center of the disc (Lecuit and Cohen, 1997). Once established, late-third instar *wg* and *dpp* expression domains may be sufficient to direct anterior pattern regulation even if HH levels are severely reduced. In intact discs, ectopic WG interacts with DPP to cause overgrowth and anterior pattern duplications (Johnston and Schubiger,1996), suggesting that *wg*/*dpp* interactions might be sufficient to direct anterior compartment regeneration without direct input from *hh*. This assertion is supported by the fact that *hh* is not required for maintenance of *dpp* in third instar wing discs (Capdevila et al., 1994), and predicts that WG and DLL domains are maintained in the absence of HH in cultured disc fragments.

To test this hypothesis, P3/4 fragments cultured in the absence of *hh* were immunostained with antibodies directed against WG and DLL (Fig. 5). Fig. 5C-F shows that hh^{ts2} P3/4 fragments cultured at restrictive temperature maintained WG and DLL at reduced levels (relative to wild-type) in their endogenous domains. These fragments did not produce duplicated WG or DLL domains. At permissive temperature WG and DLL levels were also significantly reduced (possibly due to weak hypomorphic effects of hh^{ts2}), but both domains were duplicated or expanded in 9/11 fragments analyzed (Fig. 5E,F). These data show that wild-type levels of HH are required for activation, but not maintenance, of WG and DLL domains in cultured disc fragments. We conclude that P3/4 fragments regenerate missing anterior pattern elements in an

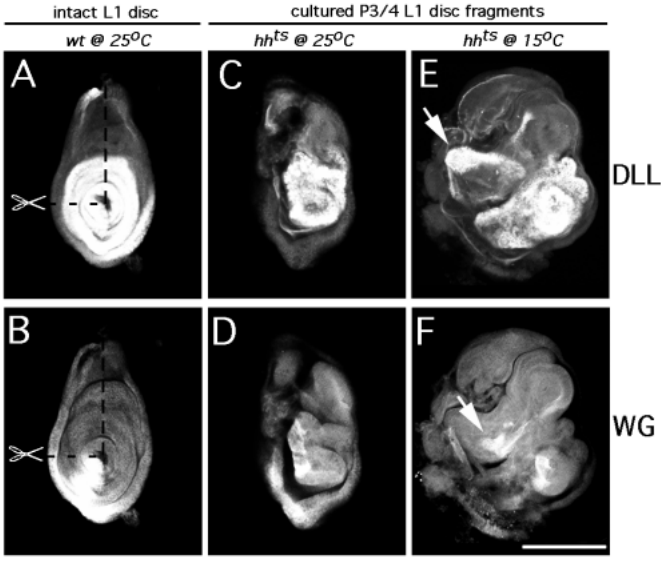

Fig 5. HH is not required to maintain a low level of WG or DLL during culture of P3/4 disc fragments. Intact control L1 discs stained with antibodies to DLL (A) and WG (B). (C,D) hh^{ts2} P3/4 fragments cultured at 25ºC for 96 hours. In the absence of *hh* function, both WG and DLL are maintained at slightly lower levels than in controls, and their expression domains are marginally reduced but not duplicated. Reduced protein levels are approximately equivalent to those seen in hh^{ts2} discs cultured at the permissive temperature, suggesting that the hh^{ts2} allele may have weak hypomorphic effects throughout development. However similar the protein levels, both WG and DLL domains only duplicated in the presence of HH (arrows, E,F). (E,F) Cultured at 15ºC for 10 days. Bar, 100 μm.

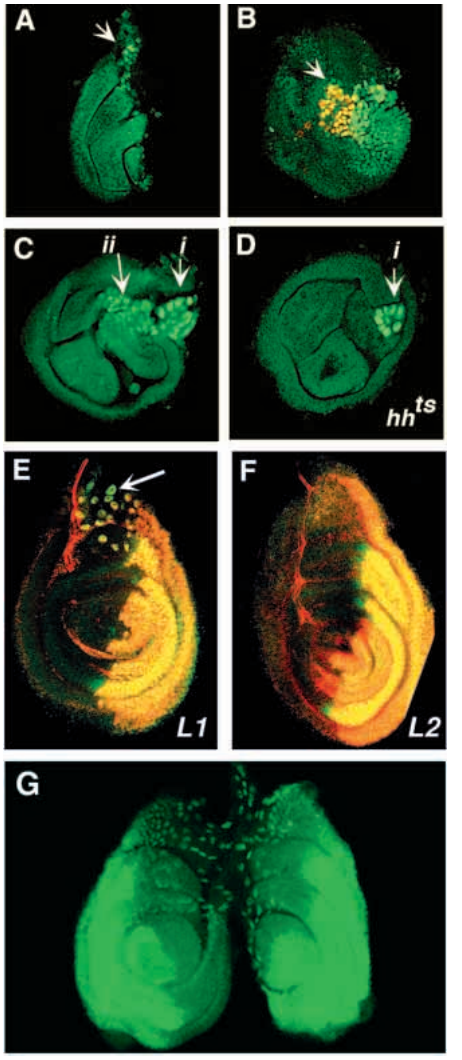

Fig. 6. HH signaling from peripodial cells stimulates EN expression in the regenerating disc epithelium. (A) Uncultured A1/4 fragment immunostained to show EN-expressing dorsal peripodial cells prior to culture (arrow). (B) A1/4 fragment stained to show *hh-lacZ* (red) and EN (green) expression after 48 hours in vivo. *hh-lacZ* is only expressed in the large peripodial nuclei visible at center (arrow), while A cells induced to express EN are visible as small green nuclei in the disc epithelium. At this stage, *hh-lacZ* is not expressed in the new posterior domain, indicating that early phases of growth within the nascent P compartment are likely *hh*-independent. (C) A1/4 fragment immunostained for EN (green) after 48 hours in vivo. (i) Large EN-expressing peripodial cells fuse to the wounded disc epithelium and secrete HH, which in turn (ii) induces EN in regulating A cells. (D) A1/4 fragment cultured for 48 hours in the absence of HH, (i) large EN-expressing peripodial cells fuse to the regenerating epithelium, but do not induce EN in nearby cells. (E and F) Whole third-instar discs immunostained for EN (green) and *hh-lacZ* (red). Note the presence of large EN/*hh*-expressing peripodial cells in L1 (E, arrow), but not L2 (F). EN expression in the L3 peripodial membrane is similar to L2. There is no clear peripodial A/P boundary as defined by expression of EN. (G) Fused left/right L1 discs stained for EN (green). Note the dorsolateral distribution of EN-expressing peripodial cells over the anterior compartment of both discs.

hh-independent fashion and suggest that this occurs by a process of integration between established *wg/dpp* domains juxtaposed through wound healing.

To confirm that *hh* is not directly required for leg pattern homeostasis after the third instar, hh^{ts2} larvae were shifted to the restrictive temperature late in development (90 hours AED, the same time used in hh^{ts2} fragmentation experiments). These larvae pupariated but categorically failed to eclose. Analysis of pharate legs revealed largely normal cuticle patterns, with the exception of loss of the anterior claw at the distal tip of L1, L2 and L3 (data not shown). Although claw specification has previously been attributed to *dpp* function (Held et al., 1994), our observations are consistent with a reported late and direct requirement for *hh* in specification of intervein distances and sensory organ precursors along the A/P boundary in wing discs (Mullor et al., 1997), and thus a late role for HH in direct specification of some structures at the leg disc compartmental boundary.

Peripodial membrane cells act as a source of HH during pattern regulation

Peripodial cells contribute to a squamous epithelium that covers the columnar epithelium of the disc proper and participates in disc eversion and metamorphosis (Milner et al., 1984; reviewed in Fristrom and Fristrom, 1993). In wing disc fragments, peripodial cells form a transient heterotypic contact with regulating columnar cells at the site of wound healing (Reinhardt et al., 1977). We find substantial evidence that a specific population of dorsolateral peripodial membrane cells act as the source of HH in cultured L1 disc fragments (Fig. 6). In both A1/4 and P3/4 disc fragments, peripodial cells express *hh-lacZ* and EN prior to and during in vivo culture (Fig. 6A,B), and appear to fuse with anterior cells in the regenerating disc epithelium during wound healing (Fig. 6B,C). In A1/4 disc fragments, *hh-lacZ* is not expressed in nascent P cells until after a new EN-domain is clearly visible (Fig. 6B). This rules out the possibility that loss of A/P conversion in hh^{ts2} mutants (Fig. 2) resulted from *hh*-dependent growth effects within the nascent P compartment and makes peripodial cells the sole potential source of HH in regenerating A1/4 fragments (Fig. 6A,B,E). These observations indicate that HH from peripodial cells activates *en* during a transient fusion between peripodial and columnar cells at the wound site. As confirmation, EN-expressing peripodial cells still fuse to the wounded epithelium in the absence of HH, but do not induce EN in surrounding columnar cells (Fig. 6D).

L2 discs lack EN/HH-expressing peripodial cells and cannot regenerate posterior leg structures

Ectopic HH induces duplicated *en* domains in intact L1 and L2 discs (Laura Johnston, personal communication), and since pattern formation in L1 and L2 discs is nearly indistinguishable, one might expect L1 and L2 disc fragments to regulate identically. However, L2 A1/4 fragments do not regenerate (data not shown), and L2 P3/4 fragments rarely duplicate but rather regenerate missing anterior structures during culture (Fig. 4). These regulative behaviors are nearly identical to those of hh^{ts2} L1 fragments cultured at restrictive temperatures, suggesting that reduction of *hh* converts L1 fragments to an L2 mode of pattern regulation. Do L2 discs lack the *hh*/EN-expressing peripodial cells proposed to activate

en in L1 disc fragments? Fig. 6E and F show that L2 discs have many fewer *hh*/EN-expressing peripodial cells (about 0-5 cells in L2 versus 20-30 in L1), explaining their diminished capacity for A/P conversion (Fig. 4). Because L1 and L2 discs are essentially identical in all other regards, these results are consistent with a unique role for *hh*-expressing peripodial cells in induction of A/P conversion in cultured L1 disc fragments.

DISCUSSION

A new interpretation of pattern regulation in L1 disc fragments

Analysis of leg disc fragments cultured at reduced levels of HH has allowed us to distinguish two distinct modes of pattern regulation. During integration, missing pattern is regenerated in a spatially appropriate manner within the A compartment. This process does not require wild-type HH signaling, and may proceed by interaction between established *wg* and *dpp* domains brought into contact through wound healing. During conversion, A cells activate EN and assume P identity. This process is strictly *hh*-dependent and occurs only in L1 disc fragments. Cultured fragments of L2 discs do not undergo A/P conversion. This observation led us to consider why L1 disc fragments possess such unique regulative potential.

In cultured L1 disc fragments, *hh*-expressing peripodial cells fuse with columnar cells at the wound site and provide a transient ectopic source of HH, which stimulates A/P conversion. Following this idea, L2 disc fragments lack *hh*-expressing peripodial cells (Fig. 6E,F) and hence fail to undergo A/P conversion during culture. The presence of the *hh*-expressing peripodial cells may imply an L1-specific developmental function. For example, left and right L1 primordia are distinguished from L2 and L3 in that they fuse together sometime during larval development. *hh*/EN-expressing peripodial cells localize to the precise dorsolateral position of L1 disc fusion (Fig. 6G), and are not detected in L2 or L3 discs. We infer that peripodial HH signaling could mediate right/left fusion in developing L1 discs and propose that other metamorphic disc fusion events could similarly depend on (this or other) intercellular signaling mechanisms.

A role for EN in anterior cells during development?

In wing and leg discs, EN expression is restricted to P cells for most of development, but becomes detectable in A cells along the A/P boundary late in the third larval instar (Blair, 1992; Maschat et al., 1998; Fig. 7A). Consistent with its role in A/P conversion, HH signaling is also thought to mediate *en* activation in these anterior boundary cells (de Celis and Ruiz-Gomez, 1995; Guillen et al., 1995). The developmental function of EN in A cells is not known, although recent evidence suggests a potential role in inhibition of *dpp* along the compartmental boundary (Mullor et al., 1997; Maschat et al., 1998). Levels of *dpp* are critical for normal growth and patterning; loss of function leads to truncated and defective appendages (Gelbart, 1989; Held et al., 1994) while ectopic expression elicits overgrowth and pattern respecification (Nellen et al., 1996). Since EN is known to inhibit *dpp* (Raftery et al., 1991), and *dpp* is not coexpressed with *en* in boundary cells, late-activated EN could inhibit *dpp* (Mullor et al., 1997) as a mechanism to restrict *dpp*-dependent growth and/or patterning at the end of larval life.

Why doesn't *hh*-mediated *en*-activation result in A/P conversion and pattern regulation at the compartmental boundary? Maschat et al. (1998) show that A boundary cells, which express EN, activate *polyhomeotic* (*ph*), which in turn represses *hh*. The authors argue that the *en/ph* circuit prevents *hh*-dependent induction of *patched* (*ptc*), *Cubitis interruptus* (*Ci*) and *dpp* in adjacent posterior cells – proscribing 'invasion' of the posterior compartment by anterior pattern markers. Similarly, results from de Celis and Ruiz-Gomez (1995) show that *hh* can activate *en* in A wing cells and that *groucho* inhibits expression of *hh* in A cells along the compartment boundary. We propose that during pattern regulation, A/P conversion occurs in cells distant from the A/P boundary which initially lack (or somehow downregulate) expression of these or other potential inhibitors of *hh* (such as *Ci*; Domínguez et al., 1996).

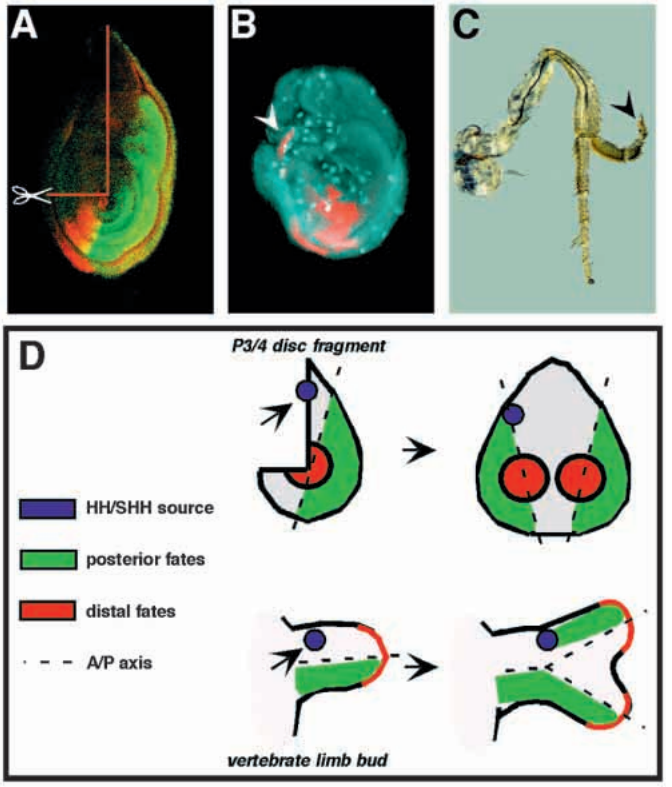

Fig. 7. Fusion of *hh*-expressing peripodial cells to regenerating leg disc epithelia mimics the effects of forcing gene expression in developing discs. (A) Whole leg disc stained for *wg-lacZ* (red) and EN (green); red lines indicate the approximate cut sites. Note position of the *wg* domain within the prospective P3/4 fragment, and slight overlap between EN and *wg-lacZ* in anterior leg boundary cells (yellow). (B) P3/4 fragment stained for *wg-lacZ* (red) and phosphohistone H3, a mitosis marker (blue) after 24 hours in vivo. Localized cell proliferation (bright blue nuclei) marks the position where wound healing occurs. An ectopic *wg* domain is activated at the wound site (arrowhead), resulting in leg structure duplications analogous to those caused by ectopic *wg* expression in developing discs. (C) Leg duplication induced by ectopic expression of *wg* during the second larval instar. (D) Cartoon demonstrating parallels between *hh*-dependent regeneration of P3/4 leg disc fragments and pattern regulation in chick limb buds stimulated with *Sonic Hedgehog*, adapted from Riddle et al. (1993).

In this scenario, EN activation in the blastema could lead to production and secretion of HH, subsequent *wg* and *dpp* activation in neighboring A cells, respecification of the proximodistal axis via *Dll*, and thus a partial recapitulation of leg disc development.

Parallels with ectopic gene expression

Consistent with the view that pattern regulation is driven by spatiotemporally inappropriate gene interactions, ectopic gene expression can clearly mimic the effects of disc fragmentation. For example, ectopic *hh* induces A/P conversion and pattern duplication in developing abdominal tergites (Struhl et al., 1997) and wing and leg imaginal discs (Guillen et al., 1995; de Celis and Ruiz-Gomez, 1995; Laura Johnston, personal communication). Previously, *hh*-mediated anterior leg pattern duplications have been attributed to positionally inappropriate activation of *wg* and *dpp* (Basler and Struhl, 1994; Lepage et al., 1995). Using a *wg-lacZ* transgene, we found that *wg* transcription is also activated at the precise location where *hh*-expressing peripodial cells fuse to the wound site in cultured L1 disc fragments (Fig. 7A,B). This 'ectopic' activation of *wg* at the wound site extends the analogy between *hh*-misexpression and fragmentation experiments, and explains why cultured P3/4 fragments differentiate duplicated anterior-ventral cuticular patterns akin to those elicited by clones of constitutive *wg* activity in developing discs (Struhl and Basler, 1993; see Fig. 7C).

Activation of *wg* at the wound site also provides a compelling explanation for leg-to-wing transdeterminative phenomena observed in cultured L1 disc fragments (Schubiger, 1971). In unfragmented leg discs, ectopic *wg* interacts with *dpp* to stimulate localized cell proliferation (Johnston and Schubiger, 1996) and leg-to-wing transdetermination via activation of the wing-specific transcription factor Vestigal (Maves and Schubiger, 1995, 1998; Johnston and Schubiger, 1996). A similar pathway could be executed during culture of L1 disc fragments, since *wg* is activated in close proximity to *dpp*-expressing cells. It is interesting to note that L2 disc fragments, which lack a peripodial source of HH, fail to transdetermine during culture (G.S., unpublished observations), suggesting that HH is needed to activate *wg* at the wound and *wg* is required for transdetermination to wing.

Parallels with vertebrate limb regeneration?

Imaginal disc pattern regulation is an experimental phenomenon, and cannot be directly compared to the highly specialized and adaptively-significant process of amphibian limb regeneration. However, the striking similarities in limb pattern regulation between flies and vertebrates (French et al., 1976) may suggest a common mechanistic basis. Saunders and Gasseling (1968), studying chick limb buds, demonstrated that grafting posterior cells into anterior limb sites induces mirror-symmetric distal limb duplications. It has since been shown that vertebrate *Sonic hedgehog* (*Shh*) is the molecular agent of this phenomenon (Riddle et al., 1993). The effects of *Shh* misexpression are inescapably analogous to those caused by HH in cultured P3/4 disc fragments, particularly respecification of A cells to P identity and duplication of distal limb primordia (see Fig. 7D). Previous authors have cited the parallel effects of HH misexpression in flies and vertebrates as evidence that *hh*-family members play an evolutionarily conserved role in appendage patterning (Fietz et al., 1994). Our results are consistent with this view and permit speculation that in vertebrates, as in flies, the ability to regenerate particular limb structures may be predicated upon the availability of SHH (or the ability to express or respond to SHH) at the site of limb transection. The recently reported expression of *Shh* in regenerating newt limb blastemas supports this notion (Imokawa et al., 1997).

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