

Localized JAK/STAT signaling is required for oriented cell rearrangement in a tubular epithelium

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SUMMARY

Rearrangement of cells constrained within an epithelium is a key process that contributes to tubular morphogenesis. We show that activation in a gradient of the highly conserved JAK/STAT pathway is essential for orienting the cell rearrangement that drives elongation of a genetically tractable model. Using loss-of-function and gain-of-function experiments, we show that the components of the pathway from ligand to the activated transcriptional regulator STAT are required for cell rearrangement in the *Drosophila* embryonic hindgut. The difference in effect between localized expression of ligand (Unpaired) and dominant active JAK (Hopscotch) demonstrates that the ligand plays a cell non-autonomous role in hindgut cell

rearrangement. Taken together with the appearance of STAT92E in a gradient in the hindgut epithelium, these results support a model in which an anteroposterior gradient of ligand results in a gradient of activated STAT. These results provide the first example in which JAK/STAT signaling plays a required role in orienting cell rearrangement that elongates an epithelium.

Supplementary figures available on-line

Key words: JAK/STAT pathway, Cell rearrangement, Tubulogenesis, Morphogenesis, *Drosophila melanogaster*

INTRODUCTION

The generation of complex shapes, both of the whole embryo and its constituent tissues and organs, depends on the coordination of multiple cellular processes; these include cell shape change, proliferation, cell migration and epithelial-mesenchymal transitions. An essential contributor to the form-generating processes of gastrulation and epithelial morphogenesis is oriented cell rearrangement. During gastrulation in frogs and fish, cells intercalate in the mediolateral axis, and converge towards the midline; this process has been termed convergent extension and results in dramatic elongation of the embryonic anteroposterior axis (reviewed by Keller et al., 2000; Wallingford et al., 2002). Although these cell rearrangements during gastrulation occur primarily among mesenchymal cells, cell rearrangement can also occur among cells that are bound together in an epithelium. Elongation of a number of epithelial sheets, such as the *Drosophila* germband and the *C. elegans* dorsal epidermis, has been shown to occur by mediolateral intercalation relative to the midline (Irvine and Wieschaus, 1994; Heid et al., 2001). Elongation of epithelial tubes such as the sea urchin archenteron, *C. elegans* intestine, insect renal tubule and *Drosophila* posterior spiracles and hindgut is also driven by cell rearrangement; in these cases, cell intercalation is oriented circumferentially (perpendicular to the tube proximodistal axis) rather than towards the midline (Fig. 1B)

(Ettensohn, 1985; Leung et al., 1999; Skaer, 1993; Brown and Castelli-Gair Hombria, 2000; Iwaki et al., 2001) (reviewed by Wallingford et al., 2002).

An unsolved problem of great interest is the source of the polarizing information that causes rearranging cells to intercalate in one axis, and not in another. Although convergent extension in both frogs and fish has been shown to require the planar cell polarity pathway initiated by non-canonical Wnt signaling, the nature of the positional cues that orient the rearranging cells has not been defined (reviewed by Wallingford et al., 2002). Similarly, known genes required for oriented cell rearrangement in the *C. elegans* dorsal epidermis and *Drosophila* posterior spiracles encode putative transcription factors that are thought to confer morphogenetic capacity, rather than regulate expression of cue-providing molecules (Heid et al., 2001; Brown and Castelli-Gair Hombria, 2000). As localized, secreted signals have been demonstrated to guide various types of cell migration and epithelial outgrowth in both *Drosophila* and mammals (Duchek et al., 2001) (reviewed by Metzger and Krasnow, 1999; Hogan and Kolodziej, 2002; Moore, 2001; Rollins, 1997), an appealing hypothesis is that rearranging cells orient with respect to spatially localized cell signaling molecules.

The conserved JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) signaling pathway is widely used; it has been shown to play a required role in a variety of processes including hematopoiesis, sex determination,

lymphocyte migration, and border cell migration (reviewed by Ward et al., 2000; Luo and Dearolf, 2001; Sefton et al., 2000; Moore, 2001; Vila-Coro et al., 1999; Silver and Montell, 2001; Beccari et al., 2002). A suggestion that JAK/STAT signaling might be involved in epithelial cell rearrangement comes from the observation that the ligand for the *Drosophila* JAK/STAT pathway, Unpaired (Upd; Os – FlyBase) is expressed in a highly localized position at the anterior of the embryonic hindgut epithelium (Iwaki et al., 2001). Mutants that block the elongation of the hindgut, which occurs largely by cell rearrangement, also alter the localized pattern of *upd* expression (Iwaki et al., 2001).

Exploiting the genetic simplicity of *Drosophila*, we present evidence that the JAK/STAT pathway orients cell rearrangement in the hindgut, a simple epithelial tubule. In contrast to the situation in mammals where there are four different JAKs and seven different STATs (Imada and Leonard, 2000), the *Drosophila* genome encodes only one known ligand (*upd*), one receptor (the cytokine-like *domeless*, *dome*, also known as *master of marelle*), one JAK (*hopscotch*, *hop*) and one STAT (*Stat92E*, also known as *marelle*) (reviewed by Castelli-Gair Hombria and Brown, 2002). For examining cell movement during tubulogenesis, the hindgut is a particularly useful model as it elongates by cell rearrangement without either cell proliferation or apoptosis (Iwaki et al., 2001) (reviewed by Lengyel and Iwaki, 2002). Analysis of loss-of-function mutants shows that the key components of the *Drosophila* JAK/STAT pathway are required to achieve a fully elongated hindgut; gain-of-function (overexpression) studies show that uniform high level activation of the pathway is not sufficient, while localized production of ligand is necessary to promote oriented cell rearrangement. Our results support a model in which an anteroposterior gradient of ligand activates STAT activity in a similar gradient, leading to orientation of cell rearrangement. This is the first example of a required role for JAK/STAT signaling in orienting cell rearrangement that drives elongation of an epithelium.

MATERIALS AND METHODS

Drosophila stocks

Mutant alleles used (the strongest available) were: *upd^{os1A}* (a deletion) (Ferrus et al., 1990); *hop^{C111}* (Perrimon and Mahowald, 1986); *Stat92E⁰⁶³⁴⁶* (Hou et al., 1996); *drm³*, *drm⁶* and *drm^{P1}* (a deletion) (Green et al., 2002); *byn^{apro}* (Murakami et al., 1995); *bowl¹* (Wang and Coulter, 1996); *lin²* (Hatini et al., 2000); *wg^{7L74}* (Tearle and Nusslein-Volhard, 1987); and *twi¹* (Nusslein-Volhard et al., 1984). For temperature shift experiments, the temperature-sensitive allele *wg^{LL114}* (Nusslein-Volhard et al., 1984) was used. Three GAL4 lines that drive expression uniformly throughout the hindgut were used: *bynGAL4* drives strong expression starting at stage 7, while *fkhGAL4* and *455.2GAL4* drive progressively weaker expression starting at stages 8 and 9, respectively (San Martin and Bate, 2001; Fuss and Hoch, 2002; Iwaki and Lengyel, 2002) (D. D. I., unpublished). *drmGAL4* drives expression only in the small intestine domain of the hindgut (Green et al., 2002). UAS lines used were *UAS-upd* (Zeidler et al., 1999), *UAS-hop^{TML}* (Harrison et al., 1995), *UAS-Stat92E* (from Steven Hou, unpublished) and *UAS-dome^{ΔCYT3.2}* (deletion of the Dome cytoplasmic domain) (Brown et al., 2001).

Generation of germline clones

Germline clones of *hop* and *Stat92E* were made using the FLP-DFS

technique (Chou et al., 1993). For *hop*, larvae of the genotype *hop^{C111} FRT101/ovo^{D1} FRT101*; *hsFLP38* were heat shocked at 37°C for 2 hours on each of days 4 and 5 after egg deposition. The eclosed females of this genotype were then mated to *FM7, act-lacZ* males to obtain progeny, half of which lacked both maternal and zygotic contributions of *hop* (referred to as *hop^{m-z}*). For *Stat92E*, larvae of the genotype *hsFLP122;FRT82B ovo^{D1}/FRT82B Stat92E^{P1681}* were similarly heat shocked and mated to *Stat92E⁰⁶³⁴⁶/TM3 ftz-lacZ* males to obtain *Stat92E^{m-z}* progeny.

Histology

Whole-mount in situ hybridization was carried out as described (Pignoni and Zipursky, 1997; Tautz and Pfeifle, 1989). Digoxigenin-labeled RNA probes (Roche Molecular Biochemicals) were made from cDNA templates of *upd* (Harrison et al., 1998), *dome* (Brown et al., 2001), *hop* (Binari and Perrimon, 1994), *Stat92E* (Hou et al., 1996; Yan et al., 1996), *drm* (Green et al., 2002), *Ser* (Thomas et al., 1991), *hh* (Lee et al., 1992) and *dri* (Gregory et al., 1996). Antibody staining of embryos was performed using standard techniques (Ashburner, 1989). Antibodies (and dilutions) used were α-Crb (1:100), α-Wg (1:100) and α-En (1:5), all available from the Developmental Studies Hybridoma Bank at the University of Iowa Department of Biological Sciences; α-Stat92E (Chen et al., 2002) (1:1000); α-phosphorylated histone H3 (Upstate Biotechnology, 1:500); α-Con (Meadows et al., 1994) (1:30); and α-β-Galactosidase (Promega, 1:1000, Cappel, 1:500). For transverse sections, embryos were fixed in either 2% glutaraldehyde plus 1% osmium tetroxide, or in 4% formaldehyde, embedded in Epon and sectioned at 2 μm. Sections were stained with 0.025% Methylene Blue and 0.01% Toluidine Blue in 0.025% sodium tetraborate buffer. Light microscopy was carried out using a Zeiss Axiophot microscope, and images were captured with a Sony DKC-5000 digital camera. Confocal microscopy was carried out using a Carl Zeiss LSM 310 with a 40× objective and 1.5× digital zoom; images were acquired and processed using Zeiss LSM software. Hindgut lengths were measured as previously described (Iwaki et al., 2001) using images acquired with a Hamamatsu camera and Axiovision software. Total hindgut cell number in embryos of different mutant genotypes was determined by counting nuclei (identified by anti-β-Galactosidase staining) that were expressing *lacZ* due to the presence of either *byn^{apro}* or *bynGAL4, UAS-lacZ*.nls, following previously published procedures (Iwaki et al., 2001). The number of cells in the hindgut circumference was determined by counting cells in eight to ten serial transverse sections through the large intestine of three to four different embryos. Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

RESULTS

Candidate cues for hindgut elongation

The *Drosophila* hindgut epithelium elongates and narrows during a 10-hour period of embryogenesis (Fig. 1A), driven largely by cell rearrangement (Fig. 1B) (reviewed by Lengyel and Iwaki, 2002). This cell rearrangement must be oriented by some type of polarity, which probably arises from locally expressed signaling molecules. The developing hindgut is patterned into three morphologically distinct regions along its anteroposterior axis: the small intestine at the most anterior (to which the renal tubules connect), the large intestine and the rectum at the most posterior. Genes encoding at least seven cell signaling molecules are expressed differentially in these three regions (Fig. 1C). The putative transcriptional regulator encoding genes *drumstick* (*drm*), *bowel* (*bowl*) and *lines* (*lin*) control patterned expression of these signaling molecules (in

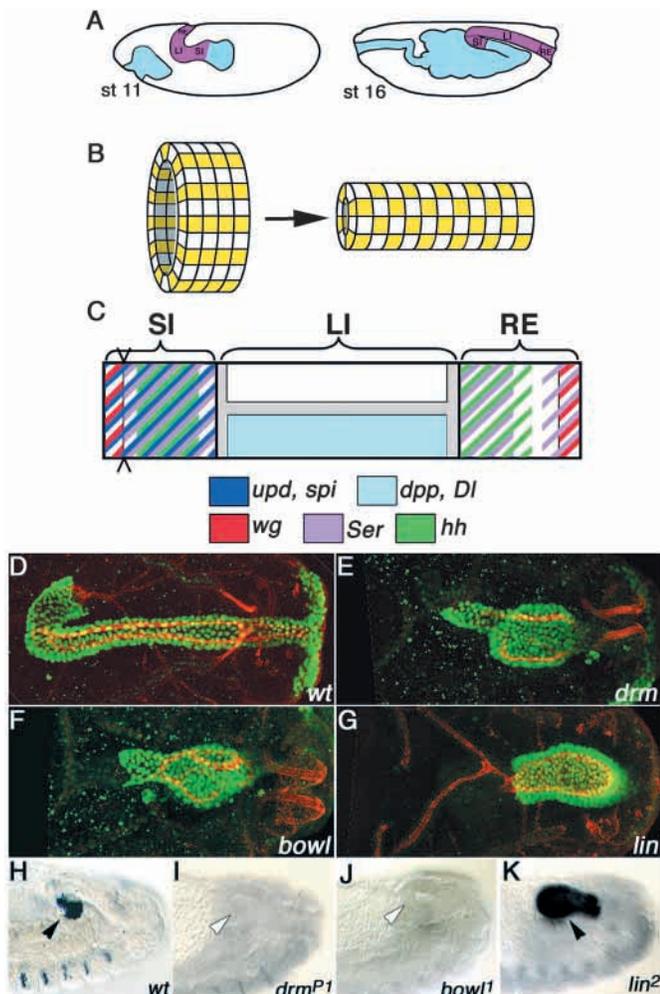


Fig. 1. Signaling molecules expressed in the hindgut. The hindgut epithelium (pink) elongates during stages 11 to 16, changing from a short, wide tube to a long, narrow tube (A); the cell rearrangement that is a major contributor to this process is shown in B. The three domains of the hindgut (SI, small intestine; LI, large intestine; RE, rectum), and the genes encoding signaling molecules expressed therein, are indicated in (C); anterior is towards the left, dorsal is upwards and the gray region indicates boundary cells. The points of attachment of the renal tubules are indicated near the anterior of the SI by 'V'. Hindgut morphology, outlined by apical staining with anti-Crb (red) and nuclear staining with anti- β -Gal (green), is shown in stage 16 wild-type (D), *drm* (E), *bowl* (F) and (G) *lin* embryos, all carrying the *byn^{ap^{ro}}* enhancer trap that drives expression of *lacZ* in the nuclei of the hindgut. (H-K) *upd* expression, detected by in situ hybridization in stage 13 embryos, is seen only in the anterior hindgut (small intestine) of wild-type (H, black arrowhead), is missing from *drm* (I) and *bowl* (J) embryos (white arrowheads), and is expanded throughout most of the hindgut in *lin* embryos (K, black arrowhead).

particular *upd*), and are concomitantly required for cell rearrangement and elongation of the hindgut (Fig. 1C-K). The signal-encoding genes *Ser*, *Dl*, *hh*, *dpp* and *spi*, however, are not required for hindgut cell rearrangement (Iwaki and Lengyel, 2002; Fuss and Hoch, 2002; Hoch and Pankratz, 1996; Takashima and Murakami, 2001) (data not shown).

wg is expressed throughout the hindgut primordium starting

at stage 5; by late stage 10 it is expressed at the most anterior and most posterior of the hindgut (Fig. 1C). To assess the required role of *wg* expression at these different stages, we carried out temperature shift experiments with a temperature-sensitive *wg* mutant (see supplementary figure S1 at <http://dev.biologists.org/supplemental/>). While early lack of *wg* function results in a dramatically smaller hindgut, the elimination of *wg* function during stage 10 (prior to the period of major elongation of the hindgut) or later allows essentially normal hindgut elongation. Thus, localized expression of *Ser*, *hh*, *spi*, *dpp*, *Dl* and *wg* does not appear to play a required role in hindgut cell rearrangement.

Localized expression of *upd*, *dome* and *Stat92E* in the developing hindgut

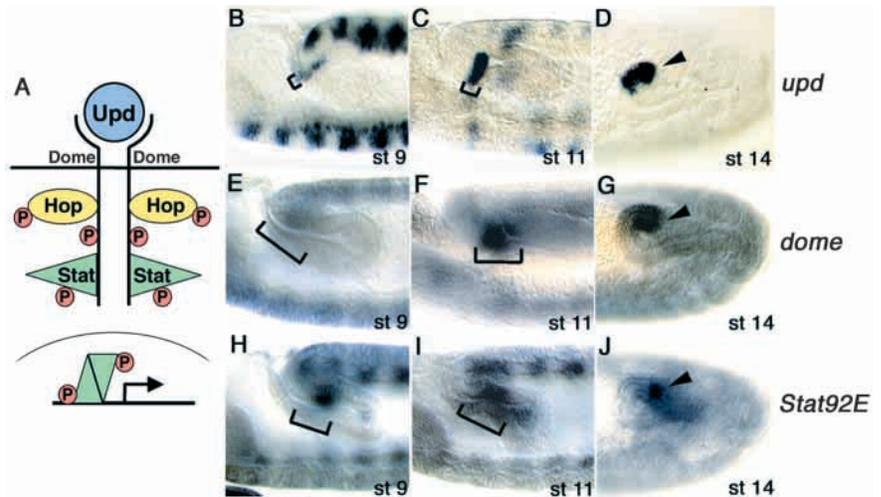
upd, encoding the ligand for the *Drosophila* JAK/STAT pathway, is only expressed in the small intestine (Fig. 1C,H) and is regulated by genes controlling hindgut cell rearrangement. In *drm* and *bowl* mutants, expression of *upd* is missing from the small intestine (Fig. 1I,J), while in *lin* mutants, *upd* expression is expanded throughout much of the hindgut (Fig. 1K). These results raise the possibility that localized Upd might provide an orienting cue for rearranging hindgut cells.

If it plays a role in hindgut cell rearrangement, *upd* must be expressed before and during the period of major hindgut elongation, i.e. between stages 11 and 16 (Fig. 1A); genes encoding the other known components of the *Drosophila* JAK/STAT signaling pathway, summarized in Fig. 2A, should also be expressed at the same stages, both within and adjacent to *upd*-expressing cells. We used in situ hybridization to characterize the expression of *upd*, *dome*, *hop* and *Stat92E* during stages just prior to and during hindgut elongation; characterization of *Stat92E* protein expression is presented in a subsequent section.

Expression of *upd* in the hindgut is first detected at stage 9 in a narrow ring of cells that will become the small intestine (Fig. 2B). Expression in the prospective small intestine is maintained during stages 10 and 11 (Fig. 2C), where it can be seen just posterior to the everting renal tubules (note that in the hindgut at these germband-extended stages, 'posterior' is toward the head). During stages 12-14, when the hindgut undergoes a major part of its elongation, *upd* expression is seen throughout the now distinct small intestine (Fig. 2D). Expression of *upd* is maintained throughout the small intestine during the remainder of embryogenesis.

In addition to *upd*, we examined expression in the hindgut of genes encoding the JAK/STAT receptor (*dome*), JAK (*hop*) and STAT (*Stat92E*). *hop* is expressed uniformly throughout the embryo, including the hindgut as it elongates (data not shown) (Binari and Perrimon, 1994). Expression of both *dome* and *Stat92E* is detected weakly at the anterior of the hindgut beginning at stage 9, becomes significantly stronger by stage 11, and is maintained through stage 14 (Fig. 2E-J). For both the receptor- and STAT-encoding genes, expression domains in the hindgut epithelium overlap with and extend beyond the narrow domain of *upd* expression (Fig. 2, brackets). Most significantly, expression of *dome* and *Stat92E* extends to a more posterior position in the hindgut epithelium than does expression of *upd* (Fig. 2, compare C,F,I, brackets). Thus, the mRNA expression of the ligand, receptor and STAT

Fig. 2. *upd* and JAK/STAT pathway components are expressed at the anterior of the elongating hindgut. The components of the *Drosophila* JAK/STAT signaling pathway (ligand Upd, receptor Dome, JAK Hopscotch and STAT) are shown in A and discussed in text. Beginning at stage 9, *upd* expression is detected in a narrow ring of cells at the anterior of the developing hindgut (at junction with posterior midgut) (B), while expression of *dome* and *Stat92E* in this domain is weaker and more diffuse (E,H). By stage 11, expression of *upd* in the prospective small intestine is strong (C), and increased expression of *dome* and *Stat92E* is observed in domains overlapping with, but more extensive than that of *upd* (F,I, respectively). These expression patterns for *upd*, *dome* and *Stat92E* are maintained throughout embryogenesis (D,GJ, respectively; stage 14). Expression in the anterior hindgut, detected by in situ hybridization, is indicated by brackets (B,C,E,F,H,I) and arrowheads (D,G,J).



components in the hindgut prior to and during its elongation is consistent with a role for JAK/STAT signaling in hindgut cell rearrangement.

Required role of *upd* and JAK/STAT pathway components in hindgut elongation

To assess the requirement for *upd* and JAK/STAT signaling in hindgut elongation, we examined hindgut morphology, length and circumference in whole mounts and transverse sections of embryos deficient for *upd*, *dome*, *hop* or *Stat92E*. In embryos lacking zygotic *upd* function, the hindgut reaches only about half its normal length and is somewhat wider (Fig. 3B). Hindguts are also incompletely elongated and wider in embryos lacking both maternal and zygotic activity of either *hop* or *Stat92E* (Fig. 3C,D). Embryos lacking zygotic *dome* function have hindguts only slightly shorter than wild type (data not shown), presumably because of the maternal contribution of *dome* (Brown et al., 2001). However, when a dominant negative form of Dome (*UAS-dome^{ΔCYT3,2}*) is expressed uniformly using *bynGAL4*, hindguts are significantly shorter and wider (Fig. 3I). Length measurements reveal that *upd*-, *hop*- and *Stat92E*-deficient, as well as *Dome^{DN}*-expressing hindguts, while not as short and wide as those of *drm*, *bowl* and *lin* embryos (compare Fig. 1B-D), are nevertheless 40-50% shorter than those of wild-type (summarized in Fig. 3Q). Consistent with their wider appearance in whole-mount embryos, *upd*, *dome*, *hop* and *Stat92E*-deficient hindguts have a greater number of cells in their circumference (19-27) than do wild-type hindguts (12) (Fig. 3F-H,M,Q). Overall, the shorter and wider appearance of the hindgut is roughly similar among embryos lacking the different components of the JAK/STAT pathway.

The shorter hindgut length is not due to a deficiency of cells, nor is the excess number of cells in the circumference due to overproliferation, as the total number of hindgut epithelial cells is 96, 89 and 85% of wild-type for *upd*, *hop* and *Stat92E* mutant embryos, respectively (Fig. 3Q). Further, staining of *upd* embryos with anti-phosphorylated histone H3, a marker of mitosis, did not detect more cell division than observed in wild

type (data not shown). The number of cells in the wild-type hindgut at stage 11 is ~50; by stage 16, this number has been reduced dramatically (to ~12) by cell rearrangement (Iwaki et al., 2001). Because by stage 16 the number of cells in the circumference of *upd*, *hop* and *Stat92E* embryos is reduced only partially (to roughly 20-30), while total cell number is essentially the same as seen in wild-type (Fig. 3Q), it must be concluded that, in the JAK/STAT loss-of-function mutants tested, there is a defect in hindgut cell rearrangement.

Requirement for localized JAK/STAT signaling

If localized JAK/STAT signaling provides an orienting cue for cell rearrangement, then ectopic JAK/STAT signaling throughout the hindgut would be expected to disrupt this process. To test this, we used *bynGAL4* to drive various UAS constructs uniformly in the hindgut epithelium. Uniform expression of *upd* results in hindguts that elongate to only about 65% of the wild-type length and have about 50% more than the normal number of cells in their circumference (Fig. 3J,N,Q). In the testis, eye and hemocytes, ectopic expression of *upd* or activated JAK causes increased cell proliferation (Kiger et al., 2001; Tulina and Matunis, 2001; Chen et al., 2002; Luo et al., 1997). However, total cell number in hindguts ectopically expressing *upd* is only 76% of normal (Fig. 3Q); consistent with this, staining with anti-phosphorylated histone H3 did not detect excess cell proliferation (data not shown). As uniform expression of *upd* in the hindgut does not result in an increase (but rather a reduction) in hindgut cell number, the excess number of circumferential cells seen in *upd* overexpressing hindguts must arise from a defect in cell rearrangement.

To ask whether localized activation of other components of the JAK/STAT pathway is required for hindgut elongation, we expressed uniformly an activated form of the *Drosophila* JAK (*UAS-hop^{TML}*). Similar to what was seen for uniform expression of *upd*, total cell number did not differ significantly from wild type, but the resulting hindguts were shorter and had more circumferential cells (Fig. 3K,O,Q). When both *UAS-hop^{TML}* and *UAS-Stat92E* are driven by *bynGAL4* (Fig. 3L,P),

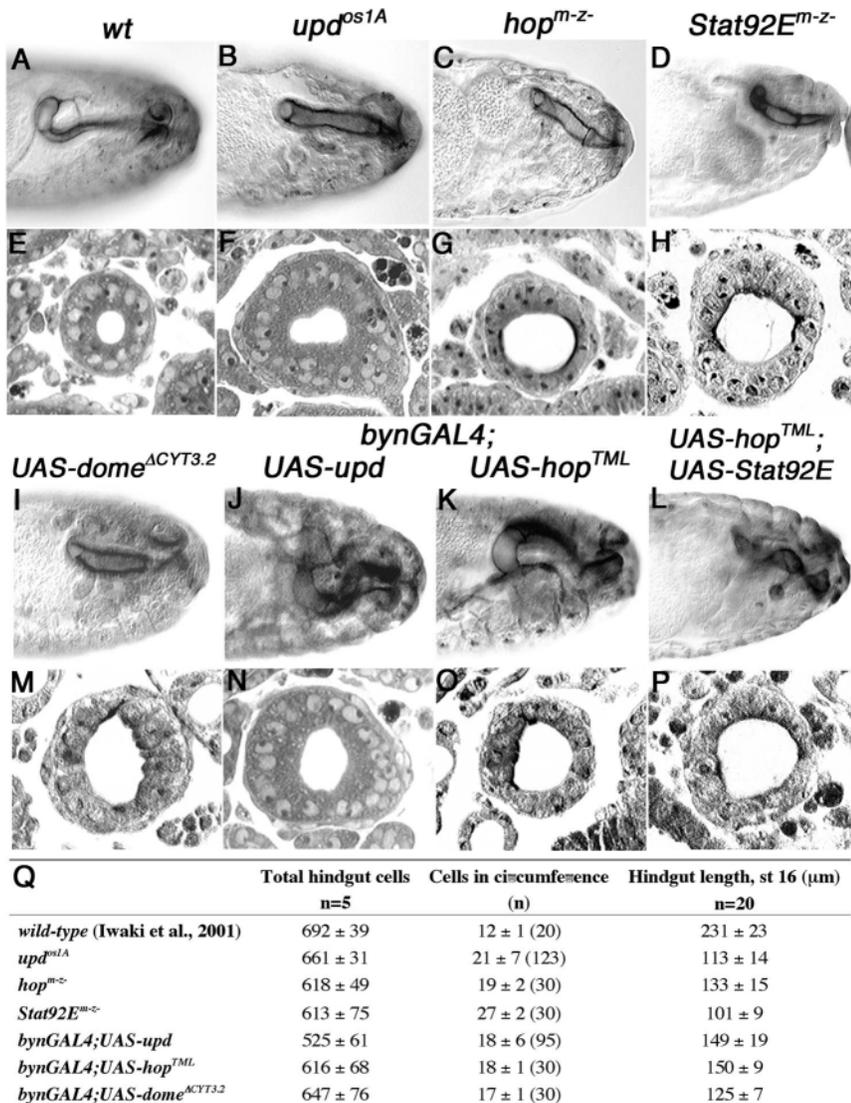


Fig. 3. Spatially localized JAK/STAT signaling is required for hindgut elongation and cell rearrangement. Compared with wild type (A), the hindgut is shorter and wider in *upd^{os1A}* (B), *hop^{m-z}* (C) and *Stat92E^{m-z}* (D) embryos. As seen in transverse section, compared with wild-type (E), there are more cells in the hindgut circumference of *upd^{os1A}* (F), *hop^{m-z}* (G) and *Stat92E^{m-z}* (H) embryos. The hindgut is also shorter and wider when *bynGAL4* is used to drive uniform hindgut expression of UAS constructs that activate JAK/STAT signaling, namely *UAS-upd* (J) and *UAS-hop^{TML}* (K); the defect is more severe when both *UAS-hop^{TML}* and *UAS-Stat92E* are driven together (L). Inhibition of JAK/STAT signaling by *bynGAL4* driven expression of the dominant negative *UAS-dome^{ΔCYT3.2}* also results in a shorter, wider hindgut (I); transverse sections of embryos of these genotypes similarly reveal a greater than normal number of cells in the hindgut circumference (M-P). All embryos shown are at stage 16; wholemounts are stained with anti-Crb and sections are of the large intestine. Averages of total number of hindgut cells, cells in hindgut circumference, and hindgut lengths observed in the loss-of-function mutants and in *bynGAL4* embryos expressing various JAK/STAT components are summarized in(Q).

the hindgut elongation defect is more severe than *UAS-hop^{TML}* alone.

The ectopic expression studies presented here demonstrate that, while components of JAK/STAT signaling are required, activation of the pathway at uniformly high levels throughout the hindgut is not compatible with normal cell rearrangement. Experiments to be presented in a subsequent section further support the idea that spatially restricted JAK/STAT signaling is necessary for hindgut cell rearrangement.

Disruption of JAK/STAT signaling does not affect hindgut patterning

As proper cell rearrangement is correlated with correct hindgut patterning (Iwaki et al., 2001), it could be argued that, rather than affecting the orientation of rearranging cells directly, *upd* and JAK/STAT signaling control cell rearrangement by affecting patterning. To test this idea, we assessed gene expression characteristic of the different hindgut regions (Fig. 4A) in *upd* mutants and in embryos ectopically expressing *upd* throughout the hindgut. Examination of all markers tested (except *wg*, see below) supports the conclusion that all three

domains – small intestine, large intestine, and rectum – as well as boundary cell rows and rings are present and correctly patterned in *upd* mutant hindguts (Fig. 4B; *upd^{os1A}* row). Similarly, all three hindgut domains and boundary cells are present when *upd* is uniformly expressed in the hindgut (Fig. 4B; *bynGAL4; UAS-upd* row). The only domain missing from hindguts lacking *upd* is a small, *wg*-expressing region at the extreme anterior of the small intestine that is established during stages 10/11 (Fig. 4A,B). As our

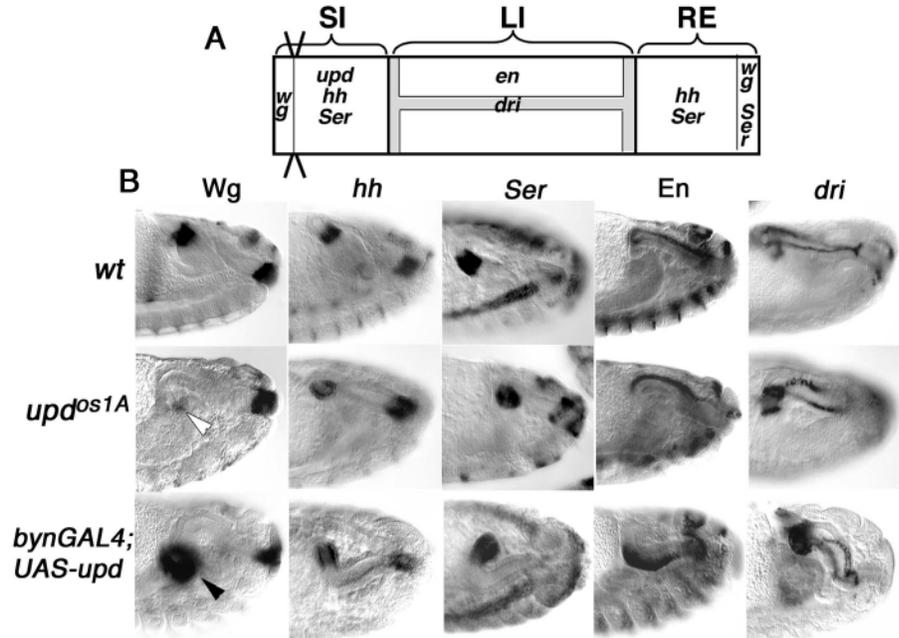
temperature-shift experiments showed that activity of *wg* is not required for hindgut elongation after stage 10, this defect in *wg* expression cannot be the basis for the effect of *upd* loss-of-function on hindgut elongation.

In summary, all domains of the hindgut, with the exception of a small number of cells at its anteriormost tip, are correctly patterned in hindguts either lacking or uniformly expressing *upd*. We therefore conclude that, rather than affecting patterning primarily and morphogenesis secondarily, *upd* and JAK/STAT signaling directly affect the cell rearrangement that elongates the hindgut.

Spatially restricted *upd* is required for hindgut cell rearrangement

If localized expression of *upd* at the anterior of the hindgut epithelium is required for hindgut cell rearrangement, then expression of *upd* in this domain should rescue the elongation defect in embryos lacking *upd*. We therefore used *drmGAL4* to drive expression of *UAS-upd* only at the anterior of the hindgut in embryos lacking *upd* function. The resulting hindguts appeared morphologically normal, with an average length

Fig. 4. *upd* has only minimal effect on hindgut patterning. Spatially localized patterns of gene expression in distinct domains of the stage 14 hindgut and posterior midgut are summarized in A; points of renal tubule attachment are indicated by 'V'. Gene expression patterns in A are shown in wholemounts of wild-type (wt), *upd* loss-of-function (*upd^{os1A}*) and *upd* gain-of-function (*byn-GAL4; UAS-upd*) embryos (B). In the absence of *upd* activity (*upd^{os1A}*), expression of Wg at the most anterior of the small intestine is reduced (white arrowhead), while expression patterns of *hh* and *Ser* in small intestine, En in large intestine, *dri* in boundary cells and *hh*, *Ser* and Wg in rectum are not affected. When *upd* is expressed uniformly throughout the hindgut (*byn-GAL4; UAS-upd*), expression of Wg in the anterior small intestine is slightly upregulated (black arrowhead), while all other spatially localized gene expression patterns examined appear normal. Expression of *hh*, *Ser* and *dri* was detected by in situ hybridization, that of Wg and En by antibody staining.



~93% that of wild type (Fig. 5C,D,I). Thus, anteriorly localized (in the small intestine) expression of *upd* in an *upd* mutant background is sufficient for elongation.

Is restriction of *upd* expression only to the hindgut anterior necessary for the cell rearrangement that drives elongation? To answer this, we used the uniformly expressed *bynGAL4* to drive *upd* in an *upd* mutant background. Rather than rescuing (like the *drmGAL4* driver), this resulted in severely defective, short and wide hindguts (Fig. 5A,B). It could be argued that the early and high level of expression driven by *bynGAL4* causes a level of activation of JAK/STAT pathway that inhibits cell rearrangement. We therefore performed the same experiment with *fkfGAL4* and *455.2GAL4*, which (respectively) drive expression uniformly in the hindgut at progressively lower levels and later times (Iwaki and Lengyel, 2002; Fuss et al., 2000; San Martin and Bate, 2001) (D. D. I., unpublished). We observed neither rescue of the *upd* hindgut phenotype nor a phenotype more defective than that of *upd* alone (in contrast to the result with *bynGAL4*; data not shown). Thus, anteriorly localized *upd* expression appears to be both necessary and sufficient for cell rearrangement in the large intestine.

An important issue is whether *upd* plays a permissive role in the hindgut (giving cells the ability to rearrange), or an instructive role (orienting cells as they rearrange). As none of three different levels of uniform *upd* expression rescued the *upd* loss-of-function phenotype, it seems unlikely that the function of JAK/STAT signaling in the hindgut is simply to promote the capacity of cells to rearrange. Rather, the data support the notion that the required role of localized expression of *upd* is to provide an instructive cue that orients cell rearrangement.

***upd* signaling mediates *drm* function in hindgut**

Although *upd* cannot entirely mediate the effect of *drm* on hindgut cell rearrangement (as *drm* hindguts are shorter and wider than those of *upd*; Fig. 5E,F), we asked whether, and to

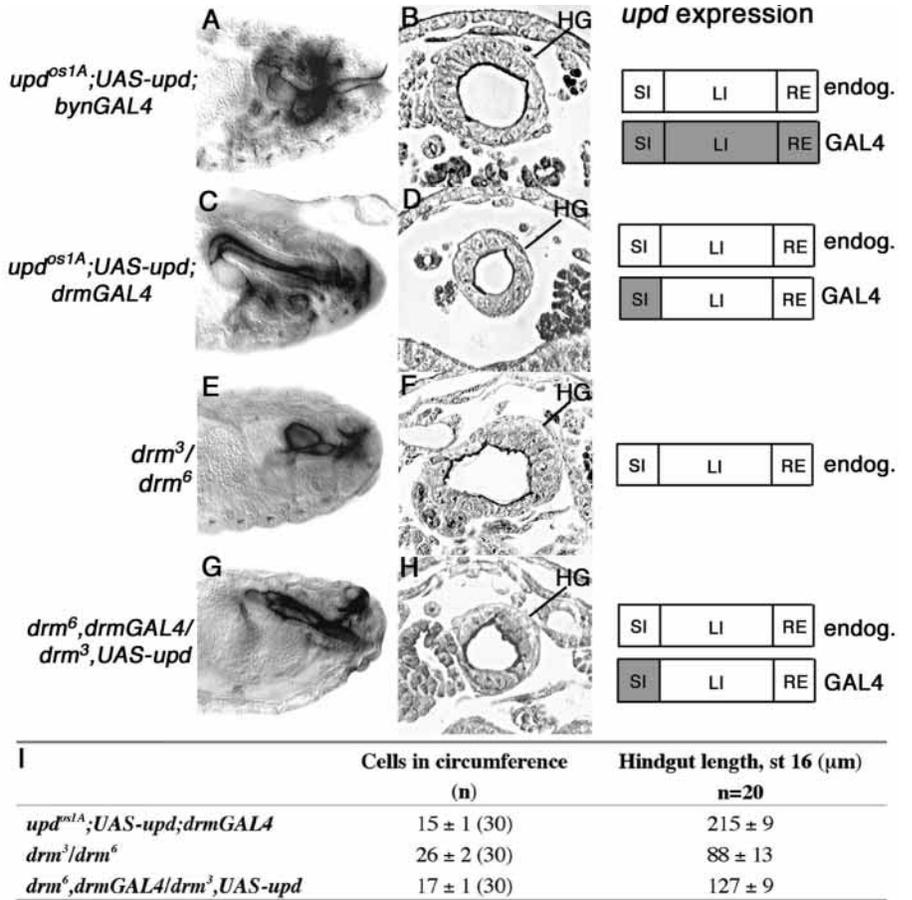
what extent, expression of *UAS-upd* under control of *drmGAL4* could rescue the *drm* loss-of-function phenotype. Strikingly, expression of *upd* at the anterior of the *drm* mutant hindgut is sufficient to bring about significant rescue of the *drm* hindgut phenotype, as assessed in both wholemounts and transverse sections (Fig. 5G,H). Compared with *drm* hindguts, the partially rescued hindguts are 45% longer, and have 35% fewer cells in their circumference (Fig. 5I). The rescue of the *drm* hindgut phenotype by anteriorly expressed *upd* thus demonstrates that *upd* is a key mediator of *drm* function in the hindgut.

Upd signal is received by cells of the large intestine

Given the small number of cells in its circumference by stage 16 (Fig. 3E) (Iwaki et al., 2001), the large intestine must undergo significant cell rearrangement as it elongates. Yet *upd*, which we have shown controls cell rearrangement throughout much of the hindgut, is expressed only in the small intestine, and not in the large intestine. Because action of Upd over a distance has been described in the eye disc (Zeidler et al., 1999), it seemed possible that Upd produced in the small intestine might control cell rearrangement by activating JAK/STAT signaling in the large intestine (Fig. 1C,H). During stages 11 and 12, as cell rearrangement is elongating the hindgut, the prospective large intestine is 50 to 60 μ m in length (Fig. 6A,B); this is the distance over which Upd would have to diffuse to affect the entire large intestine.

In addition to activating phosphorylation of Stat92E (Luo and Dearolf, 2001), expression of *upd* has also been shown to upregulate Stat92E protein levels during embryogenesis (Chen et al., 2002). We therefore investigated expression of *Stat92E* mRNA and protein as reporters for receipt of the Upd signal. Antibody staining shows that, in the stage 11 hindgut, Stat92E protein is greatly reduced in the absence of *upd* function, and dramatically upregulated when *upd* is uniformly expressed (Fig. 6C,D). This regulation appears to occur at the transcriptional level, as *Stat92E* mRNA is similarly reduced in the absence of

Fig. 5. Anteriorly localized *upd* expression is required for hindgut elongation. When *upd* is expressed throughout the hindgut (using *bynGAL4*) in an *upd^{os1A}* mutant background, the hindgut is shorter and more deformed (A) and has as many or more cells in transverse section (B) than that seen in *upd^{os1A}* embryos (compare with Fig. 3B,F). When *upd* is expressed only in the small intestine (using *drmGAL4*) in an *upd^{os1A}* mutant background, the length of the hindgut and number of cells in transverse section is almost indistinguishable from that seen in wild-type (C,D; compare with Fig. 3A,E). The *drm³/drm⁶* hindgut is short and wide (E) and has an excessive number of cells in its circumference (F). When *upd* is expressed only in the small intestine (using *drmGAL4*) in a *drm³/drm⁶* mutant background, the hindgut is more elongated (G), and has fewer cells in its circumference (H) than seen in *drm³/drm⁶*. This phenotype is similar to the rescue of the *drm³/drm⁶* hindgut that is seen when *drmGAL4* is used to drive *UAS-drm* (R. B. Green, PhD thesis, UCLA, 2002). Hindgut apical surfaces are outlined by anti-Crb staining; embryos are stage 16; *GAL4*-driven expression patterns are shown on the right of each pair of panels. Length measurements and circumference counts are summarized in I.



upd function, and upregulated when *upd* is uniformly expressed in the hindgut (Fig. 6E,F). We conclude that staining with anti-Stat92E identifies cells receiving Upd signal.

During stages 11 to 12, Stat92E protein is detected in a domain that extends more posteriorly than the *upd* expression domain at the hindgut anterior (compare Fig. 2C and Fig. 6G,I); this can be seen most clearly when both *upd* mRNA and Stat92E protein are labeled (Fig. 6K). Sagittal sections reveal that Stat92E is present in the nuclei of the hindgut epithelium at stages 11 and 12, (Fig. 6H,J); therefore, during these stages, at least some of the large intestine epithelial cells are receiving Upd signal. Significantly, Stat92E appears to be present in the hindgut as a gradient, with the most anterior cells (at the point of renal tubule evagination) most strongly labeled; during stages 11 to 12, this gradient extends to at least 20% to 45% of the length of the large intestine primordium (Fig. 6G-J). Because of limits in dynamic range of the anti-Stat92E assay, we cannot presently determine whether, at some point, this gradient extends over the entire large intestine.

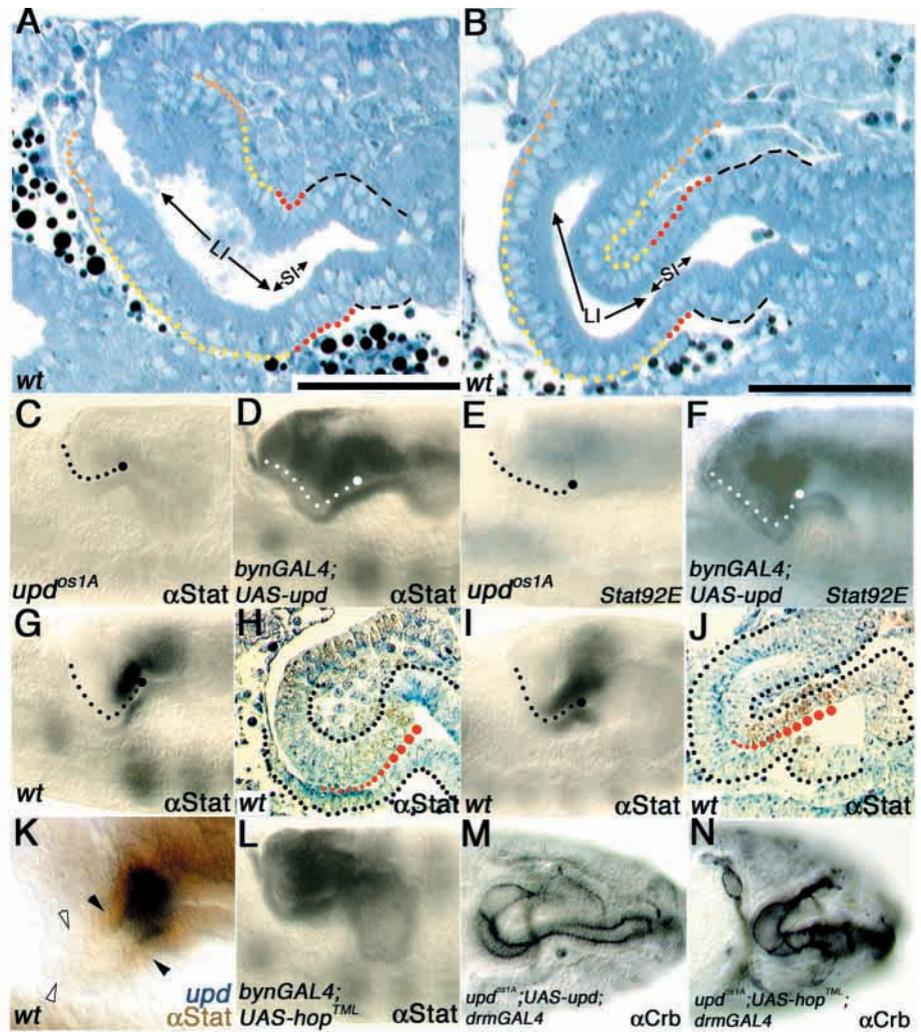
Stat92E is present not only in the nuclei of the hindgut epithelium, but also in the nuclei of the hindgut visceral mesoderm (Fig. 6J; see supplementary figure S2 at <http://dev.biologists.org/supplemental/>). However, as determined by staining with anti-Connectin, the hindgut visceral mesoderm is normal in *upd* mutant embryos (see supplementary figure S2); furthermore, the hindguts of *twi* mutants, which lack all mesoderm, are able to undergo a significant amount of elongation (see supplementary figure S2)

(San Martin and Bate, 2001). Thus, even though cells of the visceral mesoderm receive the Upd signal, they do not appear to play a significant role in the rearrangement of cells within the hindgut epithelium.

The expression of Stat92E in the hindgut epithelium, while suggestive, does not reveal whether receipt of Upd signal by prospective large intestine cells is required for their rearrangement. Because in an *upd* mutant embryo expression of *upd* at the anterior of the hindgut rescues elongation (Fig. 5C), we asked whether expression of dominant active Hop (*hop^{TML}*) might also rescue. As a control, we expressed *hop^{TML}* uniformly in the hindgut; similar to what we observed for *upd*, this results in a dramatic upregulation of Stat92E (Fig. 6, compare D with L). A key finding is that, in contrast to the essentially complete rescue of the *upd* hindgut phenotype when *upd* is expressed anteriorly (Fig. 6M), anterior expression of *hop^{TML}* in an *upd* background does not rescue hindgut elongation (Fig. 6N). These results demonstrate that activation of the JAK/STAT pathway only in cells of the prospective small intestine (i.e. the domain that expresses *upd*) is not sufficient for normal hindgut cell rearrangement. Thus, *upd* is required in a non-cell autonomous fashion for hindgut cell rearrangement.

The observed upregulation of Stat92E in cells of the hindgut posterior to the small intestine is consistent with the cell non-autonomous function of *upd* in the hindgut. The fact that cell rearrangement is severely abnormal when uniform levels of Stat92E and Hop^{TML} are driven together (Fig. 3L,P) further supports the idea that Stat92E must be distributed non-

Fig. 6. *upd* activates the JAK/STAT pathway in cells posterior to the small intestine. Sagittal sections through the hindgut at stages 11 (A) and 12 (B) show outlines of the evertting renal tubules (black dashes), small intestine primordium (red dots), large intestine primordium (yellow dots) and rectum primordium (orange dots). Estimates of the lengths of the small intestine (SI) and the large intestine (LI) are shown based on analysis of the sets of serial sections to which A and B belong. Scale bar: 50 μ m. Levels of Stat92E protein and mRNA are barely detectable in *upd^{os1A}* embryos (C and E, respectively), but dramatically upregulated in *bynGAL4;UAS-upd* embryos (D and F, respectively); Stat92E protein is also upregulated in *bynGAL4,UAS-hop^{TML}* embryos (L). Stat92E protein is detected along a significant region of the length of the anterior hindgut, as observed in both whole-mount (G,I) and sagittally sectioned embryos (H,J) at stages 11 (G,H) and 12 (I,J). The hindgut is outlined by black dots (H,J), and red dots indicate the observed anteroposterior gradient of Stat92E protein (H,J). Consistent with this, double staining for both *upd* mRNA (in situ hybridization) and Stat92E protein (antibody staining) shows expression of Stat92E in an anterior-to-posterior gradient (brown, posterior extent marked with open arrowheads) that extends posterior to the domain of *upd* expression (blue, posterior extent marked with black arrowheads) (K). Expression of *UAS-upd* with *drmGAL4* in an *upd* mutant background rescues the *upd* hindgut elongation defect (M), while expression of *UAS-hop^{TML}* with *drmGAL4* in an *upd* mutant background fails to rescue (N); α Crb is used to outline hindgut morphology. In C-G,I, the lumen of the hindgut is indicated with dots; the anterior limit of the small intestine is indicated with a larger dot.



uniformly, i.e. as a gradient, in order for cells to rearrange. The observed gradient of Stat92E, reaching to at least 20% of the length of the large intestine during stage 11, and to at least 45% during stage 12 (Fig. 6H,J), suggests that there is a corresponding gradient of the activating ligand Upd in the hindgut. Both the distance (roughly 20 to 40 μ m, see Fig. 6A,B) and time (stages 9 through 12, roughly 4-6 hours) over which this postulated Upd gradient is established are consistent with parameters described for formation of gradients of Upd, Dpp and Wg in imaginal disc tissues (Zeidler et al., 1999; Entchev et al., 2000; Teleman and Cohen, 2000; Strigini and Cohen, 2000), as described below.

DISCUSSION

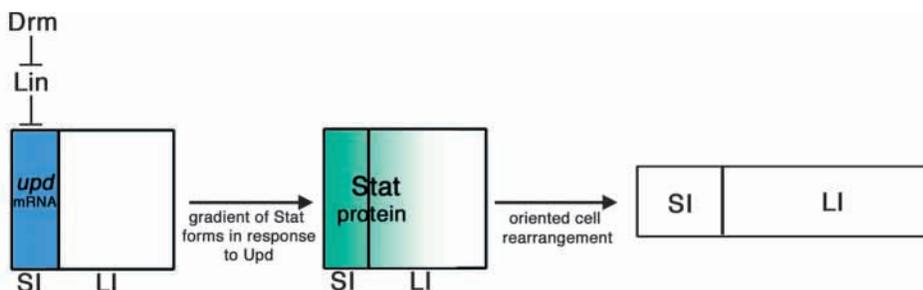
We have shown that elongation of the *Drosophila* hindgut by cell rearrangement requires the Upd ligand and the JAK/STAT pathway components Dome (receptor), Hop (JAK) and Stat92E. As elongation does not occur when expression of ligand or activation of the pathway is uniform, but only when

the source of ligand is localized to the hindgut anterior, the requirement for localized JAK/STAT signaling in hindgut elongation can be characterized as instructive, rather than permissive. As patterning is normal in hindguts both lacking and uniformly expressing *upd*, the required role of JAK/STAT signaling in hindgut morphogenesis is likely via direct effects on cell movement.

Upd is required non-autonomously for hindgut cell rearrangement

The rescue of the *upd* phenotype by anteriorly localized expression in the hindgut of *upd*, but not of activated JAK (Hopscotch), demonstrates that there is a requirement for *upd* function that is not cell autonomous. In other words, *upd* is required in cells (those of the large intestine that undergo the greatest rearrangement) different from cells that produce it (those of the small intestine). A number of examples have been described in which localized expression of a signaling molecule (including Upd) is required non-autonomously for cell rearrangement, morphogenesis or motility. In the *Drosophila* eye imaginal disc, expression of Upd at the midline

Fig. 7. Model for hindgut elongation by Upd/JAK/STAT signaling. Expression of *upd* in the small intestine (SI) under the control of Drm and Lin results in the formation of a gradient of Upd protein in cells posterior to the small intestine. In response to signaling by Upd, a gradient of Stat92E is established; this leads to the orientation of cell rearrangement in the hindgut.



is required to establish a dorsoventral polarity that orients ommatidial rotation (Zeidler et al., 1999). In both *Drosophila* tracheae and the vertebrate lung, branching morphogenesis of the epithelium depends on localized expression of FGF in adjacent mesenchyme (reviewed by Metzger and Krasnow, 1999; Hogan and Kolodziej, 2002).

Localized activation of JAK/STAT signaling has been shown to play a role in cell motility in a number of contexts. In *Drosophila*, localized expression of Upd in the anterior polar cells of the egg chamber acts to coordinate the migration of the adjacent border cells (Silver and Montell, 2001; Beccari et al., 2002). In mammals, cytokines expressed in target tissues act to attract both migrating lymphocytes and tumor (reviewed by Moore, 2001; Muller et al., 2001; Murphy, 2001; Vila-Coro et al., 1999). Our finding that localized (only in the small intestine) expression of *upd* is both necessary and sufficient for rearrangement of cells in the large intestine indicates that Upd must have an organizational, action-at-a-distance function in controlling cell rearrangement during tubular morphogenesis.

A predicted gradient of Upd in the hindgut

The rescue experiments discussed above establish that there is a cell non-autonomous requirement for *upd* in hindgut elongation. Consistent with this, there is evidence that Upd is present and required in an anteroposterior gradient in the hindgut. Prior to and during hindgut elongation, both *Stat92E* mRNA and Stat92E protein are detected not only in the small intestine epithelium (and the visceral mesoderm surrounding the small intestine), but also in the epithelium posterior to the small intestine; this expression of Stat92E appears to be in a gradient. In the *Drosophila* eye imaginal disc, a gradient of Upd is required to orient the rotation of ommatidial cell clusters (Zeidler et al., 1999); in addition, there is evidence for a gradient of Upd and Stat92E in patterning of the follicular epithelium of the *Drosophila* egg chamber (R. Xi, J. R. McGregor and D. A. Harrison, unpublished). As expression of Stat92E depends on *upd* (this work) (Chen et al., 2002), it is likely that Upd protein is present in the hindgut epithelium as an anteroposterior gradient, with its highest level in the *upd*-expressing cells of the small intestine, and lowest level in posterior, *upd* non-expressing cells of the large intestine. Expression of *SOCS36E* (*suppressor of cytokine signaling at 36E*), which is regulated by *upd*, overlaps with and extends significantly beyond the domain of *upd* expression (Karsten et al., 2002), further supporting the idea that there is a gradient of Upd in the hindgut. A model that summarizes the observed localized expression of *upd* mRNA, a gradient of Stat92E protein, and cell rearrangement leading to elongation is shown in Fig. 7.

In the *Drosophila* eye imaginal disc, anti-Upd staining and the behavior of clones of mutant cells that have lost components of the JAK/STAT pathway indicate that Upd is present in a gradient that extends at least 50 μm beyond its midline mRNA expression domain (Zeidler et al., 1999). In the *Drosophila* hindgut, we have shown that Stat92E is a reliable reporter for the presence of Upd. Two to four hours after *upd* is first expressed at the anterior of the hindgut (stage 9), Stat92E can be detected at least 30-40 μm from the site of *upd* expression (stages 11 and 12). These time and distance parameters are similar to those observed during generation of the Upd gradient in the eye, and the Dpp and Wg gradients in wing imaginal discs, which form over distances of roughly 40-80 μm in 1-8 hours (Zeidler et al., 1999; Entchev et al., 2000; Teleman and Cohen, 2000; Strigini and Cohen, 2000). Thus, it is reasonable to imagine that a gradient of Upd is established in the developing hindgut in a short enough time frame to affect cell rearrangement.

The essential consequence of JAK/STAT signaling is activation of the STAT protein, which leads to altered transcriptional programs (Darnell, 1997; Horvath, 2000). STAT has been shown in a number of contexts to be required for cell motility (Sano et al., 1999; Yamashita et al., 2002; Silver and Montell, 2001; Beccari et al., 2002), and therefore probably regulates expression of genes controlling cytoskeletal assembly and cell adhesion. In these contexts, however, activation of STAT does not appear to be required to orient cell movement, but rather to facilitate or promote it. As Stat92E is required for hindgut elongation, and its protein product appears to be present in a gradient along the anteroposterior axis, this raises the intriguing question of how a gradient of a transcription factor might orient cell rearrangement.

Concluding remarks

Our results demonstrate a new role for Upd and the JAK/STAT pathway, namely the control of cell rearrangement that elongates a tubular epithelium. Given the widespread occurrence of JAK/STAT signaling, further analysis of the mechanism by which JAK/STAT signaling controls hindgut cell rearrangement in *Drosophila* is likely to provide insights into the control of cell motility during many processes, including organogenesis, wound healing and cancer metastasis.

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