Requirement for JAK/STAT signaling throughout border cell migration in *Drosophila*

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Summary

The evolutionarily conserved JAK/STAT signaling pathway is essential for the proliferation, survival and differentiation of many cells including cancer cells. Recent studies have implicated this transcriptional pathway in the process of cell migration in humans, mice, *Drosophila* and *Dictyostelium*. In the *Drosophila* ovary, JAK/STAT signaling is necessary and sufficient for the specification and migration of a group of cells called the border cells; however, it is not clear to what extent the requirement for cell fate is distinct from that for cell migration. We found that STAT protein is enriched in the migrating border cells throughout their migration and is an indicator of cells with highest JAK/STAT activity. In addition, *stat^{ts}* mutants exhibited border cell migration defects after just 30 minutes at the non-permissive temperature, prior to any detectable change in the expression of cell fate markers. At later times, cell fate changes became evident, indicating that border cell fate is labile. JAK/STAT signaling was also required for organization of the border cell cluster. Finally, we show that both the accumulation of STAT protein and nuclear accumulation are positively regulated by JAK/STAT activity. The activity of the pathway is negatively regulated by overexpression of a SOCS protein and by blocking endocytosis. Together, our findings suggest that the requirement for STAT in border cells extends beyond the initial specification and delamination of cells from the epithelium.

Key words: Stat, Cell migration, Border cells, *Drosophila*

Introduction

Cell migration is essential for embryonic development and adult homeostasis, and can contribute to pathological conditions such as inflammation and tumor metastasis. A variety of signaling pathways are known to function in cell migration. One that has recently been implicated in the regulation of cell migration during gastrulation in the zebrafish, in wound healing in the mouse, and in *Drosophila* oogenesis is the Janus Kinase (JAK)/Signal Transduction and Activator of Transcription (STAT) signaling pathway (Hou, 2003).

The JAK/STAT pathway is activated when an extracellular signal, such as a cytokine, binds to a receptor that constitutively associates with a JAK (Levy, 2002). Ligand binding activates JAK, leading to its autophosphorylation, as well as to phosphorylation of tyrosine residues on the receptor, which serve as docking sites for STAT to bind via its SH2 domain. STAT is then phosphorylated by JAK, dimerizes, and translocates into the nucleus where it activates the transcription of target genes. This pathway can be regulated at various steps. For example, SOCS proteins are thought to inhibit STAT function by blocking its activation or by promoting JAK protein degradation (Alexander, 2002), whereas PIAS proteins inhibit STAT activity in the nucleus (Kotaja, 2002).

JAK/STAT signaling is well known to promote cell proliferation, survival and cell fate determination (Levy, 2002). In addition, recent studies have identified a requirement for STAT in cell migration in vivo. STAT3 knockout mice are embryonic lethal, dying during gastrulation (Takeda et al., 1997). Conditional knockout of STAT3 in keratinocytes inhibits wound healing in the mouse and keratinocyte migration in a monolayer-wounding assay (Sano et al., 1999). In zebrafish, STAT3 is essential for the migration of sheets of cells during gastrulation, independent of an effect upon cell fate (Yamashita et al., 2002). Recently, STAT has been shown to be essential for the migration of primordial germ cells in *Drosophila* (Li et al., 2003).

Border cell migration in the *Drosophila* ovary is an excellent model for the study of developmentally regulated cell motility. The *Drosophila* ovary is composed of egg chambers, each of which contains 16 germline cells and about 900 somatic cells, called follicle cells (Fig. 1A). Early in oogenesis a pair of special follicle cells forms at each end of the egg chamber, the so-called polar cells. As oogenesis proceeds the follicle cells differentiate into several sub-types, including stalk cells, which separate adjacent egg chambers, squamous follicle cells, which cover the nurse cells, outer follicle cells, which cover the oocyte, and the border cells. At the beginning of stage nine of oogenesis, the border cell cluster forms when the anterior polar cells recruit a group of four to eight cells from the adjacent follicular epithelium (Grammont and Irvine, 2002; Montell, 2003; Xi et al., 2003). The cells delaminate and migrate as a cluster surrounding the two central anterior polar cells. Over a

6-hour period, the border cell cluster migrates to the oocyte and eventually contributes to the formation of a structure called the micropyle, which is the site of sperm entry and is required for fertilization to occur.

JAK/STAT signaling is essential for border cell migration (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001; Xi et al., 2003). The best-characterized components of the JAK/STAT signaling pathway in *Drosophila* are a secreted ligand called UPD, its receptor, called Domeless (DOME), a JAK, referred to as HOP, and STAT92E (STAT). There also appear to be additional *upd*-like and *domeless*-like genes in the *Drosophila* genome (Hombria and Brown, 2002). UPD is expressed and required in the polar cells to recruit the surrounding cells to form the border cell cluster (Silver and Montell, 2001). Loss of either *hop* or *stat* in the border cells inhibits their recruitment into the cluster and their subsequent migration. Furthermore, activation of the JAK/STAT pathway is sufficient to induce additional follicle cells to become invasive (Silver and Montell, 2001). In the current study, we asked whether STAT is required continuously during border cell migration, after the fate of the cells has been determined. We present evidence that the STAT protein accumulates in response to activation of the JAK/STAT pathway and is highly enriched in the migrating border cells throughout the migration. Using a temperature-sensitive (ts) allele, we show that we can separate the requirement for *stat* in migration from the requirement in cell recruitment and specification. We also present evidence that activity of this pathway is regulated by positive feedback, by the presence of inhibitors and by

endocytosis. Together, our results demonstrate a continuous requirement for STAT signaling during border cell migration and indicate the importance of regulating the level of this activity.

## Materials and methods

Drosophila genetics

The *stat^ts^* stock was a gift of Charles Dearolf. All crosses with *stat^ts^* were performed at permissive temperature (18°C) and then shifted to non-permissive temperature (29°C) for the stated time periods. At least 50 egg chambers were examined for each genotype and each time point analyzed. Crosses with the *slbo*-GAL4;UAS-*Rac^N17^* stocks were performed at 25°C. Ovaries from *c306*-GAL4; UAS-*hop^TUM^*, and *slbo*-GAL4; UAS-*shibire^K44A^* (DN), crosses were dissected after flies were fattened overnight at 29°C, while ovaries from *slbo*-GAL4 and *c306*-GAL4;UAS-*SOCS* crosses were fattened overnight at 25°C. The UAS-*shibire^K44A^* (DN) line was obtained from the Bloomington Stock Center. The UAS-*SOCS* stocks were gifts of Bernard Mathey-Prevot. The UAS-*DOME* stocks were gifts of James Castilla-Hombria and Stephanie Noselli. The MA33 enhancer trap line was a gift of Trudi Schupbach. The UAS-*UPD* line was a gift of Norbert Perrimon and the UAS-*UPD^TM^* line was a gift of Doug Harrison and Judith Lengyl.

To generate *stat* mosaic mutant follicle cells, *stat92E^397^*, FRT82B flies were crossed to hs-FLP; ubiquitin-nuclear-GFP, FRT82B. Clones marked by loss of GFP were induced as described (Silver and Montell, 2001). Flies of the appropriate genotype were heat shocked for one hour three times a day, for 2-3 consecutive days, and were dissected seven days later.

The 'FLP-out' GAL4 (AyGAL4) system was used to express UAS-*upd* and UAS-*upd(TM)*. Female flies were heat shocked at 37°C for 1 hour and incubated for 1-2 days at 25°C. Clones were detected by the expression of UAS-*lacZ* using an anti-β-galactosidase antibody.

Immunohistochemistry

Ovaries were dissected in Grace's medium containing 10% fetal calf serum. Immunohistochemical staining was performed as described (Silver and Montell, 2001). The following antibodies were used: affinity-purified rabbit anti-STAT at a dilution of 1:1000 (a generous gift from Stephen Hou); mouse anti-Fasciclin III at 1:20 (Developmental Studies Hybridoma Bank); rabbit
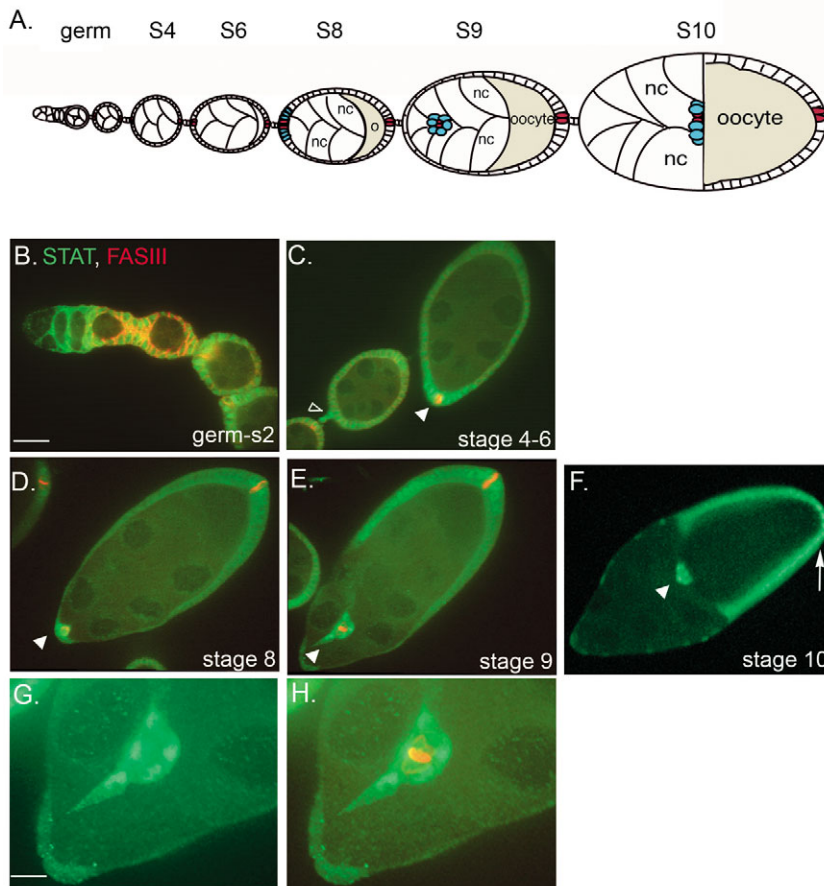
Fig. 1. STAT expression in wild-type egg chambers. (A) Schematic drawing of a *Drosophila* ovariole, with stages (S) indicated above. Pre-migratory and migratory border cells are indicated in blue and polar cells are indicated in red. (B-H) Confocal micrographs of egg chambers stained with anti-STAT (green) and anti-FasIII (red) antibody. (B) The germarium through stage 2 of egg chamber development; (C-F) egg chambers at stages (C) 4-6, (D) 8, (E) 9 and (F) 10. (G,H) High magnification images of the stage 9 border cell cluster shown in E, with and without FasIII staining to mark the interface between the two polar cells. Border cell clusters are indicated by the white arrowheads, stalk cells are indicated by the open arrowhead, and the outer follicle cells are indicated by the arrow. nc, nurse cells; o, oocyte. Scale bars: in B, 50 μm for B-F; in G, 10 μm for G,H.

anti-Domeless at 1:200 (S. Noselli); rat anti-DE-cadherin at 1:20 (DSHB); rabbit anti-GFP at 1:2000 (Promega); mouse anti-singed at 1:1 (DSHB); rabbit anti-β-galactosidase at 1:1000 (Promega); and mouse anti-armadillo at 1:100 (DSHB). Staining with DAPI and phalloidin was performed as described (Silver and Montell, 2001).

Results

### STAT is enriched in border cells throughout migration

To investigate whether JAK/STAT signaling was activated throughout border cell migration, we assessed the expression and subcellular distribution of STAT protein using a STAT antibody (Chen, 2002). The STAT antibody should recognize both unphosphorylated and phosphorylated STAT protein in the cell, which localize to the cytoplasm and nucleus, respectively.

We found STAT to be expressed throughout oogenesis in a highly specific and dynamic pattern. Early in oogenesis, STAT was expressed in the germarium, at the border between regions IIA and IIB (Fig. 1B). This region is thought to contain the somatic stem cells that give rise to all follicle cells, including the border cells (Margolis and Spradling, 1995). STAT protein was not detected in the germline. Although STAT appeared to be mostly cytoplasmic in the anterior and posterior polar cells, it was enriched in nuclei in the stalk cells at early stages (Fig. 1C), consistent with the known function of STAT in specifying stalk cell fate (Grammont and Irvine, 2002; McGregor et al., 2002; Xi, 2003).

At stage eight, when STAT localization was primarily cytoplasmic in most follicle cell types, it was highly enriched in the nuclei of about 10 anterior follicle cells, most of which become incorporated into the border cell cluster (Fig. 1D). STAT nuclear localization was maintained in the border cells

throughout their migration (Fig. 1E-H). Because activated STAT translocates from the cytoplasm into the nucleus, STAT localization in the nucleus is a strong indicator of the cells with active JAK/STAT signaling. STAT was also expressed, although primarily in the cytoplasm, in the squamous follicle cells covering the nurse cells as well as in those in contact with the oocyte (Fig. 1D). During stage nine, STAT was also enriched in the nuclei of posterior follicle cells (Fig. 1E,F, Fig. 2C), consistent with the previously described role for JAK/STAT in promoting posterior follicle cell fates (McGregor et al., 2002).

The high levels of STAT protein in cells known to require JAK/STAT signaling suggested that the STAT expression level might increase in response to activation of the JAK/STAT pathway. To test this hypothesis, signaling through the pathway was increased by overexpressing either UPD or activated HOP^{TUM} using $c306$-GAL4, which is expressed at high levels in border cells, and in a larger number of anterior and posterior follicle cells (Fig. 2A), beginning earlier in development than another border cell driver, $slbo$-GAL4 (Fig. 2B). This treatment is known to induce extra border cells to form and migrate (Silver and Montell, 2001). Under such conditions, STAT protein was dramatically enriched in all of the migrating cells, and was localized strongly to the nuclei, as well as throughout the cytoplasm (Fig. 2C-H). Thus, the accumulation of STAT protein correlated with activity of the pathway. STAT protein levels were also proportionally reduced in $stat$ heterozygous and homozygous mutant clones, demonstrating the specificity of the antibody and its sensitivity to changes in protein concentration (Fig. 3A-C). These effects were specific because no change in the overall level of STAT was detected in $slbo$ mutants, or in egg chambers that overexpressed either constitutively activated raf or hh, all of which exhibit border cell migration defects (Fig. 3D, data not shown).
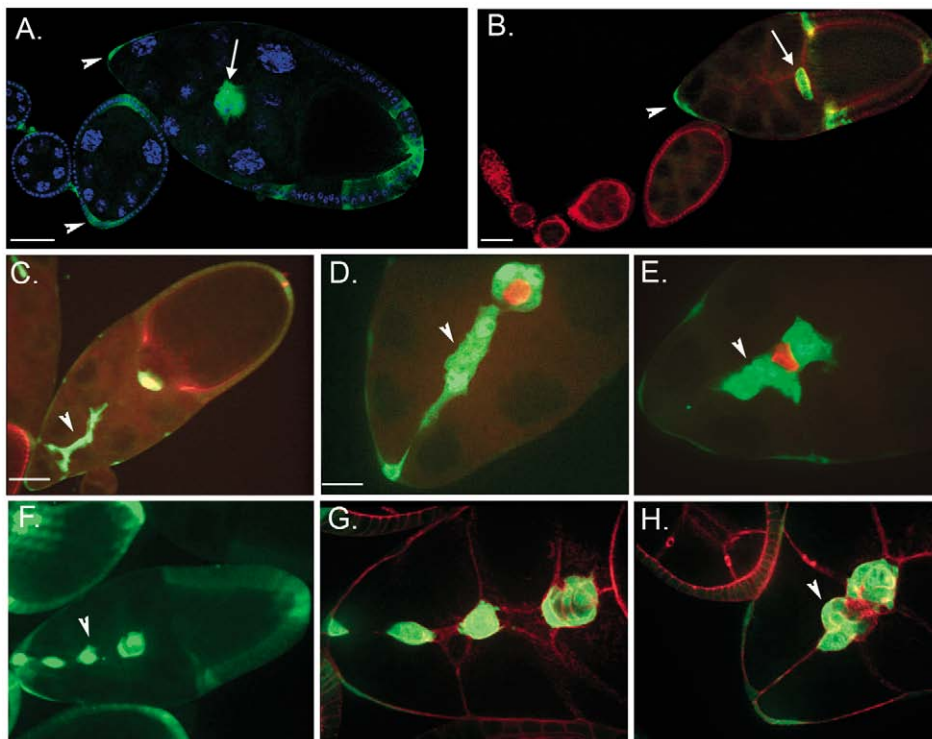
Fig. 2. Overexpression of HOP^{TUM} increases STAT levels in border cells. (A) An ovariole from a $c306$-GAL4; UAS-mCD8-GFP fly stained with DAPI (blue), showing the pattern of expression of the GAL4 driver (green). (B) An ovariole from a $slbo$-GAL4; UAS-mCD8-GFP fly stained with an antibody against Armadillo (red), showing the GAL4 pattern of expression (green). Arrow indicates the normal border cell cluster; arrowheads indicate the anterior cells that express the GAL4 driver but do not normally migrate. (C-H) Egg chambers from $c306$-GAL4; UAS-hop^{TUM} flies stained with antibodies against STAT (green). (C-E) Antibody staining for FASIII (red); (G,H) phalloidin staining of F-actin (red). Arrowheads indicate some of the ectopic border cells that formed. In all examples, anterior is to the left. Scale bars: 50 µm in A,B; in C, 50 µm for C-F; in D, 10 µm for D,E,G,H.

Fig. 3. STAT expression is reduced in *stat* mutants. (A-C) Outer follicle cells of *stat*[397] mosaic egg chambers. (A) Bright GFP (green) indicates homozygous wild-type cells, intermediate GFP staining indicates heterozygous cells, whereas lack of GFP depicts *stat* mutant cells. (B) STAT staining (red). (C) Co-localization of GFP and STAT (merged image of A and B). (D) STAT (green) and rhodamine phalloidin (red) staining in *slbo*[LY6]/*slbo*[E7b] mutant egg chambers. (E,F) STAT (green) and FASIII (red) staining of *stat*[1681]/*stat*[ts] egg chambers, after (E) 0 hours and (F) 2 hours at 29°C. (G,H) STAT (green) and rhodamine phalloidin (red) staining in *stat*[3391]/*stat*[ts] egg chambers kept at 29°C for (G) 0 hours and (H) 6 hours. Arrowheads indicate border cells with reduced expression of STAT. Scale bars: in A, 50 µm for A-C; in D, 10 µm for D-H.
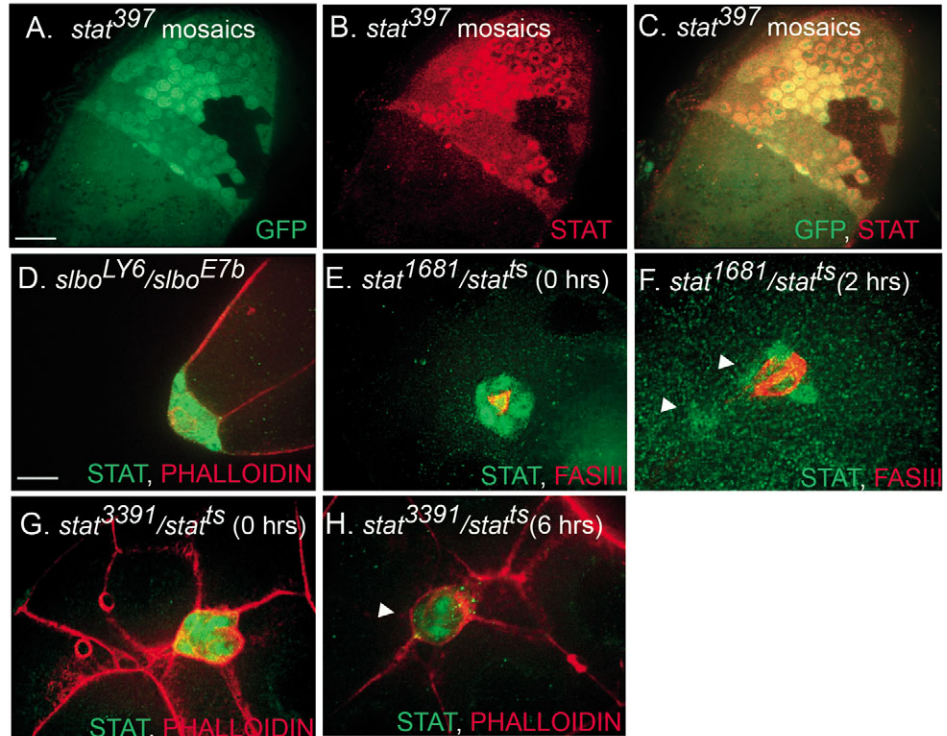
STAT activity is required throughout border cell migration

Because STAT was highly enriched in the nuclei of border cells throughout their migration, we postulated that STAT is required not only to specify border cells and initiate their migration, but also during their migration. To address this theory, we investigated whether there were *stat* mutants in which border cell migration defects occurred in the absence of effects on cell number or cell fate. In a hypomorphic allelic combination of *stat*[397]/*stat*[ep3391], STAT protein staining was barely detectable in border cells (data not shown). In such egg chambers, border cell migration was dramatically reduced (69% of egg chambers exhibited a border cell migration defect, *n*=81), yet the average number of cells in the cluster was similar to in wild type (5.6 versus 6, respectively). Consistent with this finding, egg chambers with mosaic clones of *stat*, analyzed 3-5 days after clone induction, exhibit border cell migration defects but do not show altered border cell number or expression of downstream targets such as SLBO (Beccari et al., 2002; Silver and Montell, 2001).

We next used a temperature-sensitive *stat* allele (Baksa, 2002) to ablate STAT function after the border cells had been specified and migration initiated. This allele has a closely linked background lethal mutation making it impossible to examine homozygous *stat*[ts] egg chambers. Therefore, we used *stat*[ts] heterozygous with *stat*[1681], *stat*[397] or *stat*[3391]. STAT staining was absent in mosaic clones of *stat*[1681] and *stat*[397], supporting the genetic data suggesting that these are null alleles (Fig. 3A-C). When *stat*[1681]/*stat*[ts] and *stat*[3391]/*stat*[ts] flies were kept at the non-permissive temperature for short periods of time, STAT expression was dramatically reduced (Fig. 3E-H). We previously showed that about 10% of *stat*[397]/+ and *stat*[1681]/+ egg chambers exhibit border cell migration defects

(Silver and Montell, 2001), similar to those observed in *stat*[1681]/*stat*[ts] at the permissive temperature of 18°C (Fig. 4A). After just 30 minutes at the non-permissive temperature (29°C), about 30% of the *stat*[1681]/*stat*[ts] egg chambers exhibited incomplete border cell migration, with no decrease in average border cell number (Fig. 4B, Table 1), and after two hours about 50% exhibited migration defects (Fig. 4D). After 4 or 6 hours at the non-permissive temperature, border cell migration was significantly inhibited in 90% of egg chambers, again with no measurable reduction in border cell number (Fig. 4E,F). The *stat*[3391]/*stat*[ts] egg chambers also showed an overall reduction in migration with no effect upon border cell number, after 5 hours at 29°C (data not shown). The discernible effect on migration observed after just 30 minutes at non-permissive temperature indicated that the cells stopped migrating immediately after the temperature shift. Therefore *stat* is required throughout border cell migration, independent of its requirement in initial cell fate specification, recruitment to the cluster and separation from the epithelium.

Previous studies have shown that reducing JAK/STAT signaling can cause squamous follicle cells to form at the

Table 1. Average number of border cells in *stat* mutants

Time after temperature shift	*stat*[1681]/*stat*[ts]	*stat*[3391]/*stat*[ts]	*stat*[ts]/TM3
0	6	6	6
30 minutes	5.8	6	6
1 hour	5.8	6	6
2 hours	5.8	6	6
4 hours	5.5	6	6
6 hours	4.7	5.9	6

For each time point and genotype, at least 50 egg chambers were examined.
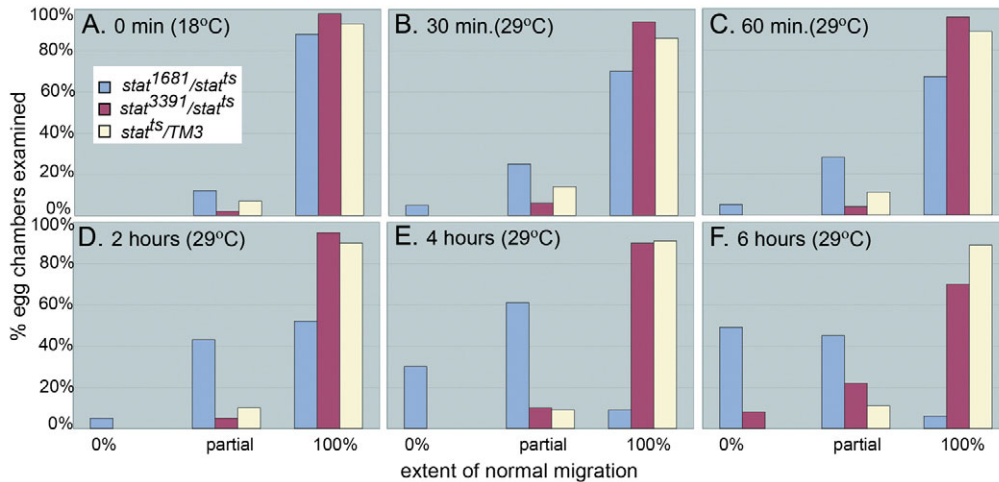
Fig. 4. *stat* is required throughout border cell migration. (A-F) Graphs depicting the percentage of egg chambers of *stat* mutants of the genotypes indicated that exhibit border cell migration defects. Flies were shifted to 29°C for (A) 0 hrs, (B) 30 minutes, (C) 1 hour, (D) 2 hours, (E) 4 hours and (F) 6 hours. For each genotype, the percentage of egg chambers is shown in which the border cells migrated 0%, partially, or 100% with respect to the extent of migration observed in wild-type egg chambers. For each time point at least 50 egg chambers were examined.

expense of border cells (Xi, 2003). Therefore, we tested whether the acute migration defects that were observed following brief incubations at the non-permissive temperature were due to a rapid change in fate from border cells to squamous cells. The MA33 enhancer trap marker is normally expressed at high levels in squamous follicle cells but is undetectable in most border cells. After one hour at non-permissive temperature, when border cell migration defects were evident, MA33 expression in $stat^{397}/stat^{ts}$ egg chambers was similar to that in wild-type egg chambers (Fig. 5A-D). However, MA33 expression became evident at low levels in the border cells after 2.5 hours at non-permissive temperature, indicating that JAK/STAT signaling also appears to function in maintaining border cell identity during migration (data not shown). The $stat^{3391}/stat^{ts}$ egg chambers that exhibited some migration defects after 6 hours at the non-permissive temperature did not express MA33 in the border cells (data not shown). Expression of two border cell markers, Armadillo (Fig. 5E-H) and Myosin VI (data not shown), was maintained in border cells of this genotype after two hours at non-permissive temperature. Together, these results demonstrate that *stat* function is required throughout border cell migration, both to repress squamous cell fate and to promote migration.

## JAK/STAT signaling promotes organization of the border cell cluster

Extra and ectopic border cells can be induced in at least two different ways. When ectopic polar cells form, for example in *eyes absent* or *costal 2* (*costa* – FlyBase) mutant clones, or following mis-expression of activated Notch, they recruit surrounding cells into a cluster and these are capable of migration (Bai and Montell, 2002; Grammont and Irvine, 2002; Liu and Montell, 1999). Alternatively, ectopic expression of UPD, HOP or HOP^{Tum} is sufficient to induce large numbers of ectopic migrating border cells (Silver and Montell, 2001). These cells migrate in a variety of sizes of clusters, which lack polar cells, and can even migrate as individual cells (Fig. 2). These findings indicate that UPD might be the only factor produced by polar cells that functions to recruit border cells and sustain their motility. To test this hypothesis, we induced ectopic expression of UPD in single anterior follicle cells, or in pairs of cells, to see whether UPD expression alone was as effective as polar cells in recruiting
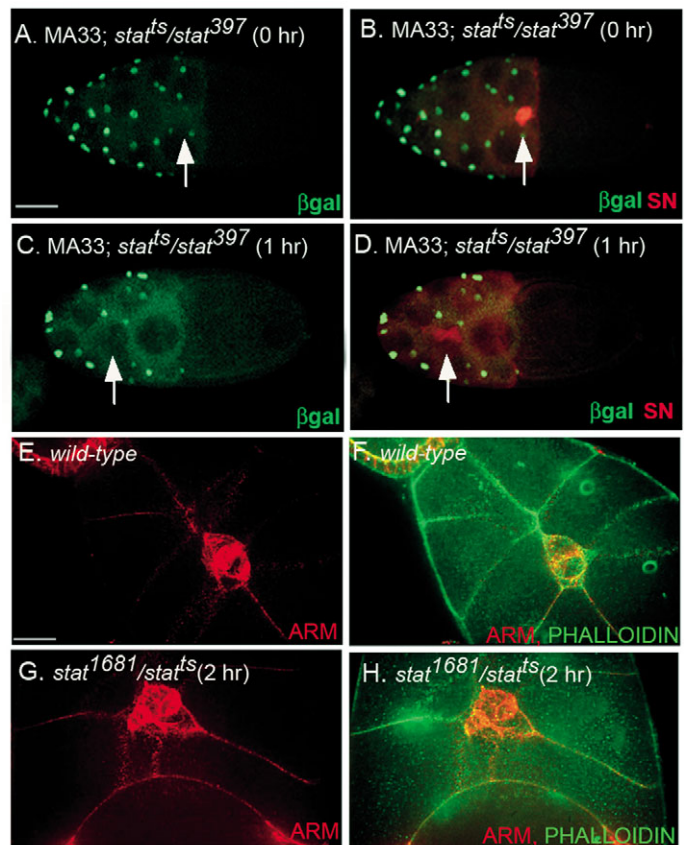
Fig. 5. *stat* migration defects are evident prior to cell identity changes. (A-D) Confocal immunofluorescence images of two representative $stat^{ts}/stat^{397}$; MA33 egg chambers kept at 18°C (A,B) or at non-permissive temperature for 1 hour (C,D). Singed staining (red) indicates the migrating border cells; the enhancer trap MA33 (green) is a marker for squamous follicle cell fate. Arrow indicates the border cell cluster. (E-H) Armadillo staining (red) indicates the border cells, and phalloidin (green) indicates all cells in wild-type (E,F) and $stat^{ts}/stat^{1681}$ (G,H) egg chambers; anterior at the top. Scale bars: in A, 50 μm for A-D; in E, 10 μm for E-H.

border cells. For comparison, we also used the same method to express a form of UPD that contains a transmembrane domain (UPD^{TM}). Expression of the wild-type form of UPD
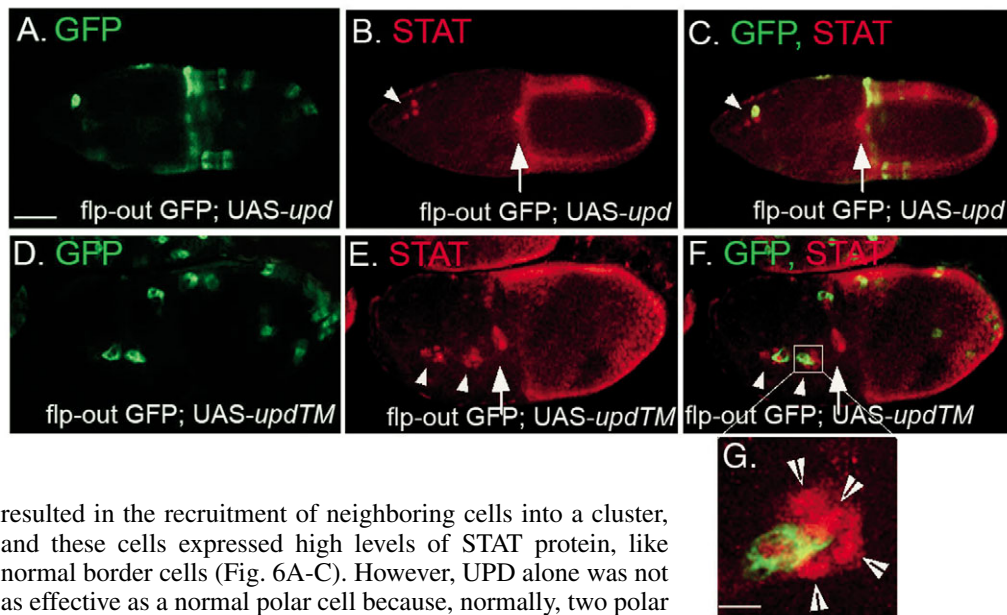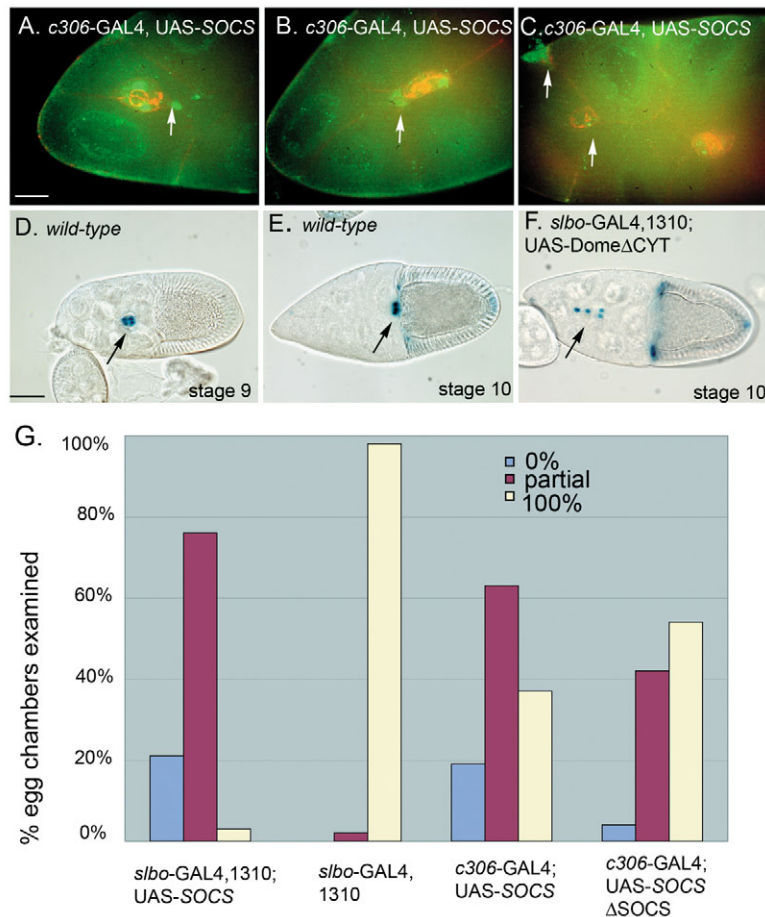
Fig. 6. Ectopic expression of UPD and UPD[TM] induces extra migratory border cells. (A-G) Confocal immunofluorescence images of egg chambers stained with anti-GFP to detect those cells expressing UAS-UPD (green) and those expressing STAT (red). (A-C) HS-FLP-GFP; UAS-*upd* egg chamber and (D-F) HS-FLP-GFP; UAS-*upd*[TM] egg chamber. (G) An example of the ectopic border cells that surround the cell expressing UAS-*upd*[TM]. Arrows indicate normal border cell clusters; arrowheads indicate ectopic border cell clusters. Scale bars: in A, 50 µM for A-F; in G, 10 µM.

resulted in the recruitment of neighboring cells into a cluster, and these cells expressed high levels of STAT protein, like normal border cells (Fig. 6A-C). However, UPD alone was not as effective as a normal polar cell because, normally, two polar cells recruit four to eight cells to surround them, whereas UPD alone resulted in the recruitment of an average of only 1.1 border cell per UPD-expressing cell (n=9). Cells expressing the UAS-UPD[TM] were actually more effective at recruiting border cells than cells expressing wild-type UPD. A single cell expressing the membrane-tethered form of UPD recruited an average of 3.25 cells to surround it (n=11; Fig. 6D-G), similar to normal or ectopic polar cells (Fig. 1) (Bai and Montell, 2002; Liu and Montell, 1999). In the case of the membrane-tethered form of UPD, ectopic migratory cells were only observed adjacent to the UPD-expressing cells. These findings suggested that JAK/STAT signaling contributes to the organization of the migrating border cell cluster. We investigated this further by analyzing the organization of migrating border cells following the reduction of JAK/STAT function.

Suppressors of cytokine signaling (SOCS) are thought to inhibit the JAK/STAT pathway, either by blocking JAK or STAT function via a SOCS SH2 domain, or by causing the destruction of these proteins by ubiquitination (Alexander, 2002). There are three SOCS genes in *Drosophila* but, to date, no loss-of-function mutants have been reported (Callus and Mathey-Prevot, 2002; Karsten, 2002). Using both

slbo-GAL4,1310 and *c306*-GAL4 drivers (Fig. 2A,B), we found that overexpression of wild-type SOCS36E inhibited border cell migration and recruitment, and overexpression of SOCS36E lacking the SOCS domain weakly inhibited

Fig. 7. Overexpression of SOCS and Domeless causes defective border cell migration and organization of the cluster. (A-C) Confocal micrographs of egg chambers from *c306*-GAL4; UAS-*SOCS* flies kept at 29°C, stained with STAT (green) and Armadillo (red). The border cell clusters appear to be less cohesive than normal and can exhibit reduced STAT staining (arrows). (D-F) Nomarski images of (D,E) wild-type and (F) *slbo*-GAL4,PZ1310;UAS-DomeΔCYT egg chambers. Egg chambers were stained for β-galactosidase activity (blue). PZ1310 is an enhancer trap insertion into the *slbo* locus. Arrow indicates the location of the border cell cluster. (G) Graph depicting the extent of border cell migration in the indicated genotypes. For each genotype at least 50 egg chambers were examined. Scale bars: in A, 50 µm for A-C; in D, 50 µm for D-F.

migration (Fig. 7A-C,G). By contrast, overexpression of a SOCS36E protein that lacked the SH2 domain, or of *slbo*-GAL4,1310 or *c306*-GAL4 alone failed to disrupt border cell migration (Fig. 7G, data not shown). Compared with an average border cell number of six for wild-type egg chambers, *slbo*-GAL4,1310;UAS-SOCS and *c306*-GAL4;UAS-SOCS egg chambers had an average number of border cells of 3.3 and 4.9, respectively. This is consistent with previous findings that reduction of JAK/STAT activity inhibits border cell recruitment (Ghiglione et al., 2002; Silver and Montell, 2001). Those border cells that did form and migrate, frequently did so as single cells rather than as a cluster. In addition, the border cells had reduced levels of STAT (Fig. 7A-C).

Consistent with a requirement in cluster organization, when JAK/STAT signaling was reduced by overexpression of a dominant-negative form of *dome* lacking the cytoplasmic domain, border cells migrated slower than wild-type cells, and frequently as single cells (Fig. 7D-F). This is consistent with the finding that in *dome* mosaic egg chambers, border cells often fail to migrate in a cluster (Ghiglione et al., 2002). Taken together, these results support the idea that signaling through the JAK/STAT pathway is responsible for the organization of the border cell cluster.

JAK/STAT signaling is regulated by endocytosis

Border cells appear to be sensitive to levels of JAK/STAT signaling, as both gain-of-function and loss-of-function mutants of the JAK/STAT pathway cause migration defects. Receptor-mediated endocytosis is known to decrease signaling through the EGF receptor, but can increase signaling of RTK, and increase DPP signaling (Jekely and Rorth, 2003; Zhu and Scott, 2004). We tested the effects of inhibiting endocytosis on signaling through the JAK/STAT pathway and on border cell migration. We overexpressed, specifically in border cells, a dominant-negative form of *shibire*, which is the fly homolog of Dynamin, a GTPase required for endocytosis (Ramaswami et al., 1993). This treatment induced a border cell migration defect in 95% ($n>50$) of egg chambers (Fig. 8A,B).

We then investigated whether the expression of JAK/STAT components was affected in these mutants. In wild-type egg chambers, the receptor Domeless is expressed at a low level in all follicle cells, including border cells (Ghiglione et al., 2002) (Fig. 8C). By contrast, in egg chambers in which dominant-negative *shibire* was expressed specifically in border cells and posterior follicle cells, there was an increase in the level of Domeless protein at the cell surface (Fig. 8E). This was also apparent when using a UAS-domeless-GFP fusion protein (Fig. 8D,F). Whereas in wild-type follicle cells Dome-GFP was concentrated in intracellular puncta, in follicle cells expressing dominant-negative dynamin, Dome-GFP was concentrated at the cell surface. This effect was specific because the distribution of E-Cadherin, another cell surface receptor, appeared to be normal in cells expressing dominant-negative dynamin (Fig. 8G,H). This result indicates that the level of Domeless protein at the cell surface is normally dynamically regulated by endocytosis.

Our finding that STAT protein levels were elevated and more concentrated in nuclei in cells in which JAK/STAT signaling is active, such as the border cells (Figs 1, 2), indicated that the STAT protein level and subcellular distribution can be used to detect the level of pathway activity. Therefore, we stained egg
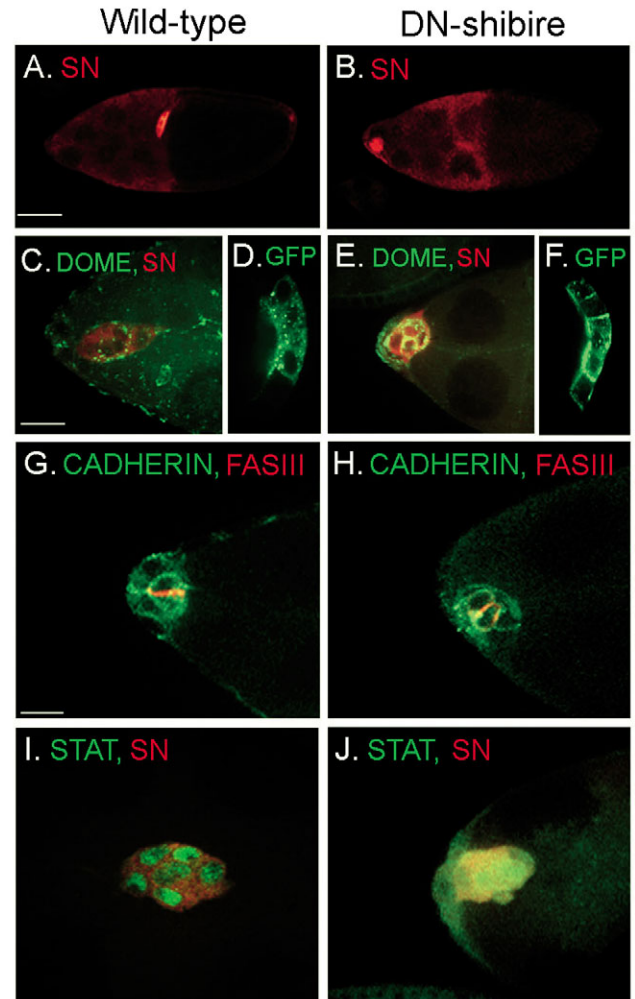
Fig. 8. Overexpression of DN-Shibire inhibits border cell migration and alters Dome and STAT protein distribution. (A-J) Confocal immunofluorescence images showing egg chambers of wild type (A,C,G,I) and *slbo*-GAL4;DN-shibire (B,E,H,J) stained with the following antibodies: (A-C,E) Singed (red); (C,E) Domeless (green); (G,H) Cadherin (green) and FasIII (red); or (I,J) STAT (green) and Singed (red). (D,F) Egg chambers expressing *slbo*-GAL4; UAS-dome-GFP (D) or *slbo*-GAL4; DN-shibire; UAS-dome-GFP (F) with GFP marking the border cells (green). Scale bars: in A, 50 μm for A,B; in C, 10 μm for C-F; in G, 10 ∝m for G-J.

chambers in which endocytosis was inactivated in border cells to see whether endocytosis normally increases, decreases, or has no effect on JAK/STAT signal transduction. The effect was dramatic. STAT protein levels were higher overall in border cells expressing dominant-negative dynamin than they were in wild-type border cells, suggesting that endocytosis may normally target some STAT protein for degradation (Fig. 8I,J). However, nuclear enrichment was not as obvious as in wild type (Fig. 8I,J). This could be because endocytosis is required to allow activated STAT to translocate to the nucleus efficiently. Alternatively the level of STAT protein in the nucleus could be similar to in wild type but, because of the higher level of cytoplasmic protein, enrichment in the nucleus is not detected.

Overexpression of JAK/STAT components suppresses the border cell migration defects caused by dominant-negative Rac

The border cell migration defects that were observed in *stat^ts* mutants after very short times following temperature shift suggested that STAT might have a direct function in cell migration, independent of transcription. One signaling pathway that STAT may communicate with is the Rac GTPase pathway. Rac is a 21 kDa GTPase of the Rho family, and is known to regulate actin dynamics in migrating cells. Overexpression of a dominant-negative Rac GTPase in border cells inhibits their migration (Geisbrecht and Montell, 2004; Murphy and Montell, 1996). We found that overexpression of UPD, JAK or STAT suppressed the border cell migration defect caused by dominant-negative Rac, whereas overexpression of AWD, an inhibitor of STAT signaling did not (Zinyk, 1993) (see Fig. S1 in the supplementary material). The suppression was not due to titration of the GAL4 by the additional UAS sequences because UAS-GFP does not alter the *slboGal4;UAS-RacN17* phenotype (Geisbrecht and Montell, 2004). Moreover in a screen of 2300 EP lines tested for suppression of RacN17 migration defects, the vast majority had no effect (Geisbrecht et al., 2004). Therefore the suppression of the RacN17 migration defects by UAS-STAT and other STAT pathway components was significant.

Discussion

Requirement for continuous STAT activity throughout border cell migration

Previous work demonstrated that UPD secreted from polar cells activates JAK and STAT in neighboring follicle cells, which form the border cell cluster and initiate migration as a result (Silver and Montell, 2001). Loss of function of any known component of the JAK/STAT pathway including *upd*, *dome*, *hop* or *stat*, causes defective border cell fate specification, recruitment and migration (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001; Xi et al., 2003); however, it was not clear from any of these studies whether JAK/STAT signaling was required specifically at the initiation of migration to specify border cell fate, or whether there was a requirement for continuous signaling throughout the 6-hour migration, either to maintain cell fate or to sustain motility. The evidence presented in this study argues that STAT activity is essential not only to specify border cell fate and initiate migration, but also during the course of their migration.

The ligand for the JAK/STAT pathway, UPD, is expressed in the polar cells throughout border cell migration (Silver and Montell, 2001), and we show here that STAT protein is highly enriched in the nuclei of border cells throughout their migration. STAT protein was not detected in the central polar cells, suggesting that some mechanism must exist to attenuate signaling in these cells. This could result from the expression of an inhibitor of the pathway, or from an absence of expression of one or more activating components of the pathway.

Using a temperature-sensitive allele of *stat*, we show that border cell migration defects become apparent in as little as 30 minutes following the shift to the non-permissive temperature. These defects become increasingly severe at longer time points. This result is striking because in order to observe a defect after only 30 minutes, the cells must stop migrating almost immediately following the temperature shift. One possibility is that STAT could be required for the expression of mRNAs and proteins that are very short-lived and are essential for migration. Alternatively, there might be a direct role for STAT in cell motility in addition to its well-characterized function as a transcriptional activator. In support of this possibility, STAT1 and activated STAT3 are found in focal adhesions in mammalian fibroblasts and ovarian carcinoma cells (Silver and Montell, 2001; Xie et al., 2001). In mammalian cells, STAT3 has also been shown to physically interact with the active form of the Rac GTPase, which regulates the actin cytoskeleton (Bar-Sagi and Hall, 2000; Simon, 2000). In border cells, we showed that overexpression of UPD, HOP or STAT can suppress border cell migration defects caused by dominant-negative Rac. Although only genetic interactions have been observed to date, signaling through the JAK/STAT pathway could provide a means of rapid activation of Rac in border cells during their migration. It is also possible that STAT suppresses Rac migration defects indirectly, through a transcriptional mechanism, as STAT also regulates the expression of two actin-binding proteins, Singed and Profilin (D.L.S. and D.J.M., unpublished), and overexpression of Profilin alone is sufficient to suppress Rac^N17 migration defects (Geisbrecht and Montell, 2004). However, such an effect would presumably require a longer time course.

Up to two hours following the shift to non-permissive temperature in *stat^ts* mutants, border cells showed no detectable alteration in gene expression. However, in as little as 2.5 hours, expression of the nurse cell-associated follicle cell marker MA33 was detected in border cells. This indicates that the border cell fate is extremely labile. Similarly, germline stem cell fate in the *Drosophila* testis depends on JAK/STAT signaling and is labile. When *stat^ts* flies are shifted to the non-permissive temperature, the germline stem cells apparently differentiate (Brawley and Matunis, 2004). The organization of cells at the tip of the testis is similar to the organization of border cell clusters in that there is a central group of cells that express UPD. Germ cells that touch the UPD-expressing cells are exposed to high levels of JAK/STAT activity and remain as stem cells. Germ cells that become separated from the UPD-expressing cells, and thus lose JAK/STAT signaling, differentiate. It is unclear how common it will turn out to be for cell fate maintenance to depend upon continuous input from neighboring cell types. However, it is easy to see how such a mechanism could be useful to ensure that the proper ratios of particular cell types are maintained within a tissue.

Interestingly, the nurse cell-associated follicle cell fate has also been proposed to require STAT activity, albeit a lower level of STAT than border cells (Xi, 2003). Yet, the nurse cell-associated follicle cell fate, as assessed by MA33 expression, did not change after temperature shift at any time point that we examined. Therefore, unlike border cells, this cell fate does not require the continuous activation of the JAK/STAT pathway to be maintained, thus not all STAT-dependent cell fates require sustained signaling. Taken together, these results indicate that continuous signaling through the JAK/STAT pathway is required to sustain border cell motility, as well as to suppress an alternative cell fate.

Regulation of STAT activity

Border cell migration is sensitive to either downregulation or hyperactivation of STAT activity, as either loss-of-function or

gain-of-function can cause defective migration. STAT activity appears to be regulated by a variety of mechanisms, including the regulation of protein abundance and nuclear translocation. It is well established that activation of JAK leads to nuclear translocation of STAT in mammalian cells (Levy, 2002), and we also found this to be true in border cells. Our studies also suggest that activation of the pathway leads to an increase in the overall level of STAT protein. In wild-type ovaries, we found that STAT protein was enriched in cells that neighbor the UPD-expressing cells, including stalk cells, border cells and posterior follicle cells. Furthermore, expression of an activated form of HOP in a large number of anterior follicle cells led to a dramatic increase in the level of accumulation of STAT protein in those cells. This observed increase in STAT protein in response to excess JAK activity is consistent with previous studies that showed that STAT protein levels are dramatically reduced, when compared with wild type, in *upd*, *hop* and *dome* (also called *mom*) mutant embryos (Chen et al., 2002), and are upregulated upon overexpression of *UPD* and HOP^{TUM} (Johansen et al., 2003). Thus, both the subcellular localization and the overall level of STAT protein respond to the level of activity of the pathway.

We also provide evidence that the activity-dependent nuclear translocation and activity-dependent STAT protein accumulation occur via distinct mechanisms. This conclusion is supported by the finding that, when receptor-mediated endocytosis was inhibited, STAT protein still accumulated in border cells, to even higher levels than in wild type. However, nuclear enrichment of STAT was not observed when endocytosis was inhibited, consistent with recent pharmacological studies in mammalian cells (Bild et al., 2002). One model that is consistent with these findings is that phosphorylation of STAT by JAK is sufficient to stabilize the STAT protein even without endocytosis. However, in the absence of endocytosis the phosphoprotein cannot be delivered to the nucleus efficiently. The receptor Domeless is probably normally actively recycled in a dynamin-dependent manner, as the protein was readily detected in puncta within wild-type cells but little in the way of cell surface protein was observed (this study) (Ghiglione et al., 2002). By contrast, in cells expressing dominant-negative dynamin, cell surface Domeless staining was far more prevalent than intracellular staining. As STAT did not accumulate specifically at the surface with Domeless, it is likely that dissociation of STAT from the receptor does not require endocytosis. EGF receptor signaling is downregulated by receptor-mediated endocytosis, whereas the results presented here indicate that endocytosis contributes both positively and negatively to modulate STAT activity.

In addition to regulation by pathway activation and endocytosis, STAT activity is controlled by proteins such as SOCS. We found that overexpression of wild-type SOCS36E inhibited border cell recruitment and migration. Interestingly, JAK/STAT signaling is both necessary and sufficient for the expression of SOCS36E in *Drosophila* embryos (Karsten et al., 2002). Together with a recent study that shows expression of SOCS36E mRNA in follicle cells flanking the polar cells (Rawlings et al., 2004), this suggests that SOCS36E may normally function to achieve the precise level of STAT activity that is required for border cell migration. Consistent with this, levels of STAT in the border cells were reduced in egg chambers overexpressing SOCS36E. However, analysis of a loss-of-function SOCS mutant, which is not yet available, will be the definitive test of that hypothesis.

Role of UPD and JAK/STAT in organizing the border cell cluster

Normally border cells migrate as a cohesive cluster with the non-migratory, UPD-expressing cells in the center and the migratory cells surrounding them. These two cell types are dependent upon each other, as the central cells cannot migrate and are carried by the surrounding cells, and the migratory cells cannot move in the absence of the UPD signal from the central cells. Thus, the organization of the border cell cluster is crucial for normal migration. In addition to its function in border cell specification and motility, several lines of evidence demonstrated the role of UPD/JAK/STAT in organizing the border cell cluster. Ectopic expression of UPD in single anterior follicle cells, for example, was sufficient to recruit adjacent cells to form a cluster capable of migration. In addition, we and others have shown that a variety of treatments that reduced STAT activity (*dome* mosaic clones, overexpression of dominant-negative Dome, and overexpression of SOCS) lead to disruptions of cluster formation (Ghiglione et al., 2002). Disruption of the cluster is likely to affect migration through the egg chamber. For example, PAR6, an epithelial protein required for polarity and the migration of border cells, is disrupted in border cells in which dominant-negative Dome is overexpressed (Pinheiro and Montell, 2004), lending support to the idea that JAK/STAT signaling helps to regulate the organization of cells within the cluster. Once the cluster is disrupted, the migratory cells become separated from the polar cells, presumably reducing STAT activity further and aggravating the migration defect. Thus, STAT activity promotes cluster organization, which feeds back to promote efficient UPD/DOME/JAK/STAT signaling.

Although ectopic expression of the normal, secreted form of UPD in a single anterior cell was sufficient to recruit an adjacent cell to form a small cluster, a single cell expressing the membrane-tethered form of UPD could recruit two to three cells. One explanation for this difference could be that the membrane-tethered protein becomes more concentrated locally, as presumably it cannot diffuse away. A polar cell pair can recruit six cells, suggesting that polar cells are able to concentrate UPD and limit its diffusion. UPD has been reported to bind to the extracellular matrix and to stay associated with the membranes of cultured *Drosophila* cells (Harrison et al., 1998). Polar cells may express higher levels of one or more extracellular matrix proteins than other anterior follicle cells, allowing them to retain UPD so that sufficient numbers of migratory cells are recruited.

Taken together, the results presented here demonstrate several inter-related properties of JAK/STAT signaling in the control of border cell migration and function. Both anatomical and biochemical mechanisms feed back upon each other to regulate the level of STAT activity precisely throughout the six hours of border cell migration. Positive-feedback mechanisms include maintaining close contact between UPD-expressing cells and the migratory cells, as well as stabilization and nuclear enrichment of STAT protein in response to signaling. One negative regulatory mechanism is the expression of SOCS36E.

The findings described here may also have relevance for understanding the requirement of STAT signaling in the progression of cancer. Constitutively activated STAT3 is associated with the aggressive clinical behavior of a number of cancers, including ovarian and renal cancers (Horiguchi et al.,

2002; Huang et al., 2000). Blocking STAT3 in pancreatic cancer cells inhibits tumor growth and metastases in mice, whereas expression of activated STAT3 promotes metastasis (Wei et al., 2003). Inhibiting STAT3 expression or activation in ovarian carcinoma cells impedes their motility in vitro (Silver et al., 2004). Thus, cancer cells too appear to require sustained activation of this pathway to survive, proliferate and migrate. The finding that JAK/STAT signaling appears to be tightly regulated by its own activity, by that of SOCS inhibitors and by endocytic processes suggests that these may provide points of clinical intervention in the treatment of STAT-dependent cancers.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/15/3483/DC1

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