The endocytic control of JAK/STAT signalling in *Drosophila*

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Summary

Domeless (Dome) is an IL-6-related cytokine receptor that activates a conserved JAK/STAT signalling pathway during *Drosophila* development. Despite good knowledge of the signal transduction pathway in several models, the role of receptor endocytosis in JAK/STAT activation remains poorly understood. Using both in vivo genetic analysis and cell culture assays, we show that ligand binding of Unpaired 1 (Upd1) induces clathrin-dependent endocytosis of receptor-ligand complexes and their subsequent trafficking through the endosomal compartment towards the lysosome. Surprisingly, blocking trafficking in distinct endosomal compartments using mutants affecting either *Clathrin heavy chain*, *rab5*, *Hrs* or *deep orange* led to an inhibition of the JAK/STAT pathway, whereas this pathway was unchanged when *rab11* was affected. This suggests that internalization and trafficking are both required for JAK/STAT activity. The requirement for clathrin-dependent endocytosis to activate JAK/STAT signalling suggests a model in which the signalling 'on' state relies not only on ligand binding to the receptor at the cell surface, but also on the recruitment of the complex into endocytic vesicles on their way to lysozomes. Selective activation of the pool of receptors marked for degradation thus provides a way to tightly control JAK/STAT activity.

Key words: Endocytosis, JAK/STAT, Border cells

Introduction

Many cytokines, including interferons, interleukins, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and growth factors, are major activators of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling pathway in mammals. JAK/STAT activity has been associated with several diseases, including cancer (leukaemia), myocardial hypertrophy and asthma, and knock-out studies indicate a central role for JAK/STAT signalling in haematopoiesis, immune function, cell growth, differentiation and development (Agaisse and Perrimon, 2004; Arbouzova and Zeidler, 2006; Hombria and Brown, 2002; Hou et al., 2002; Kisseleva et al., 2002; Luo and Dearolf, 2001; O'Shea et al., 2002; Rawlings et al., 2004). Cytokines bind to a large family of receptors that are active either as homodimers or heterodimers. In the absence of ligand, the cytoplasmic tail of the inactive receptors is constitutively associated with JAK tyrosine kinases. Ligand binding induces a conformational change activating JAK, which then phosphorylates cytokine receptors to create a docking site for SH2-domain-containing STATs. STATs become phosphorylated by JAK on a single tyrosine residue, leading to their nuclear localization and to the regulation of specific gene expression. Thus, STATs are both signal transducers and transcriptional activators linking receptor-dependent activation at the membrane to a specific transcriptional programme in the nucleus.

In *Drosophila*, a well-conserved JAK/STAT signalling pathway has been identified that plays various roles during development, including embryonic segmentation, eye development, immune response, stem cell development, and border cell differentiation and migration during oogenesis (Arbouzova and Zeidler, 2006; Baeg et al., 2005; Dostert et al., 2005; Hombria and Brown, 2002; Hou et al., 2002; Mukherjee et al., 2005; Mukherjee et al., 2006; Muller, 2000). In contrast to the situation in mammals, in which many different genes code for several ligands, receptors, JAKs and STATs, there is only one *Drosophila* JAK gene (*hopscotch*, *hop*) (Binari and Perrimon, 1994) and one STAT (*Stat92E* or *marelle*) (Hou et al., 1996; Yan et al., 1996). Three ligands, named Unpaired 1, Unpaired 2 and Unpaired 3 (Upd1, Upd2 and Upd3, respectively), can activate the JAK/STAT pathway in flies (Agaisse et al., 2003; Gilbert et al., 2005; Harrison et al., 1998; Hombria et al., 2005), and one receptor gene, *domeless* (*dome*), has been shown to transduce the Upd signal in several tissues (Brown et al., 2001; Chen et al., 2002; Ghiglione et al., 2002). Whether the Dome-like gene *CG14225*, the function of which remains uncharacterized, could serve as an alternate receptor in some tissues remains to be established. Dome is most similar to the IL-6 receptor family, containing a distant cytokine-binding module (CBM) found in leukaemia inhibitory factor receptor (LIFR) and ciliary neurotrophic factor receptor (CNTFR) (Brown et al., 2001). *Drosophila* thus represents a good model system in which to study JAK/STAT signalling in a developing organism.

Receptor endocytosis emerges as an important and versatile mechanism by which signalling can be modulated both quantitatively and qualitatively. After internalization, receptors can be recycled back to the membrane or can be targeted to the lysosome for degradation and pathway desensitization (Le Roy and Wrana, 2005a; Le Roy and Wrana, 2005b). Recent work has shown that endocytosis can also be used to sort different

ligand-receptor complexes and elicit specific responses, as is the case for the epidermal growth factor (EGF) receptor (Seto et al., 2002; Sorkin and Von Zastrow, 2002). The study of receptor-mediated endocytosis has challenged the simple view in which only plasma membrane-associated receptors would be active and intracellular ones, on their way for degradation, would be inactive. Studies on the EGF receptor and the transforming growth factor (TGF)-β pathways have shown that the active receptors are not restricted to the membrane, but that several discrete intracellular compartments are competent for signalling (signalosome) (Hoeller et al., 2005). Despite the important role of receptor endocytosis in several systems, the trafficking of cytokine receptors, as well as the role of endocytosis, has not been addressed in the context of JAK/STAT signalling in vivo.

Drosophila oogenesis is a good model system in which to study JAK/STAT signalling. Egg chambers develop in ovaries to produce mature eggs, following a series of well-described developmental stages. Egg chambers are made of 15 nurse cells and one oocyte, and are surrounded by follicle cells organized into a monolayer epithelium. Two pairs of follicle cells, the polar cells, are present at each pole and specifically express the Upd ligand (Fig. 1A). At stage 9, follicle cells abutting anterior, Upd-expressing polar cells are recruited to become outer border cells (oBCs), which, collectively with anterior polar cells, are called border cells (BCs) (Montell, 2003; Rorth, 2002). BCs then delaminate from the epithelium and migrate through the nurse cell compartment as a cluster. In the absence of JAK/STAT signalling, oBCs are not recruited and the BC cluster does not form, indicating that the ligand produced by polar cells is received by oBCs to control their differentiation (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001).

In this study, we show that the diffusible Upd ligand can induce receptor-mediated endocytosis both in Schneider cells and in egg chambers. Clathrin-dependent endocytosis is shown to be essential for JAK/STAT activity, controlling both the amount of membrane-bound receptor and signalling. Thus, binding of the ligand to membrane-bound receptors is not sufficient for JAK/STAT pathway activation. Both the interaction with clathrin and trafficking through the endosomal compartment are required for normal JAK/STAT activity. We propose a model whereby internalization of ligand-receptor complexes and trafficking through the endosomal compartment regulates signalling, thus ensuring that the active receptors are those marked for degradation.

Results

Ligand-dependent internalization of Domeless

JAK/STAT signalling is essential for follicle cell patterning (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001; Xi et al., 2003). We showed previously that Dome is activated by Upd and is essential for oBC recruitment at the anterior pole of egg chambers (Ghiglione et al., 2002). Interestingly, antibody staining showed that, close to the anterior and posterior polar cells, the source of Upd in egg chambers, Dome accumulates apically in a gradient of intracellular vesicles ranging over 5-6 cell diameters (Fig. 1A-C). The ectopic expression of Upd is sufficient to generate ectopic Dome-containing vesicles (Ghiglione et al., 2002), suggesting that Dome is internalized following ligand binding. To confirm this conclusion, clonal analysis was used to make mutant polar cells that do not express Upd (Fig. 1D,E). In such mutant egg chambers, Dome was no longer observed in intracellular vesicles, indicating that the Upd ligand is essential for Dome internalization.

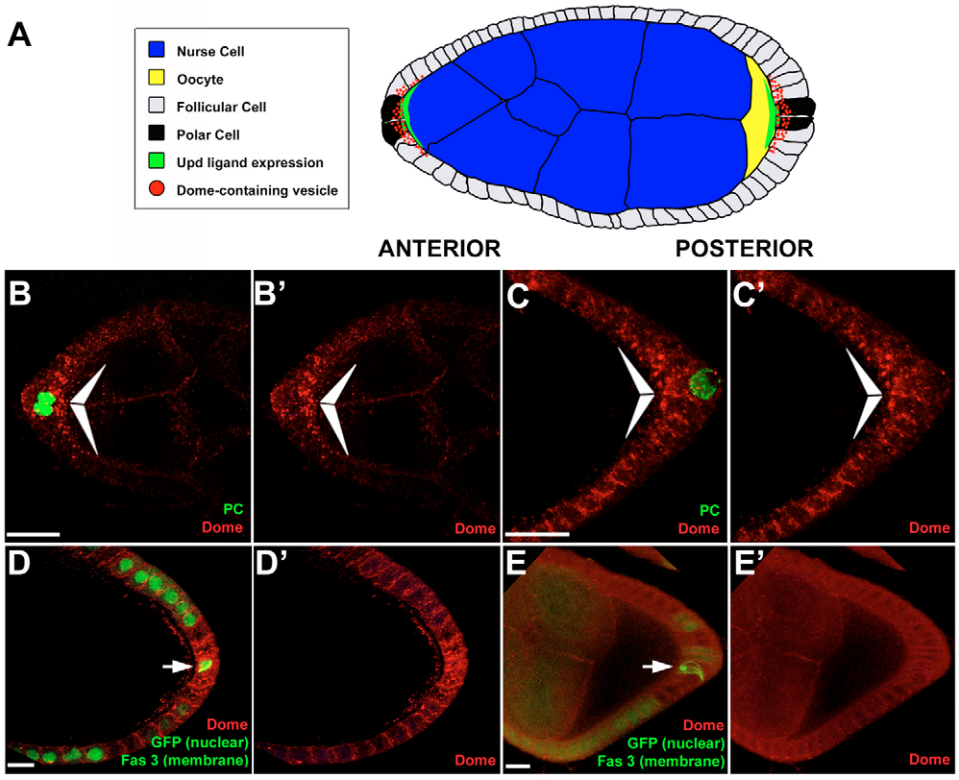

Fig. 1. Dome internalization is dependent on Upd ligand binding. (A) Schematic representation of a stage 8 egg chamber. The gradient of apically located vesicles is represented by red dots. Polar cells are in black. (B,C) Dome is expressed in a gradient (white wedges) of apically located vesicles at the anterior (B) and posterior (C) poles of the egg chamber. The abundance and distribution of Dome vesicles depend on the distance from the polar cells (marked with A101-β-galactosidase; green), the source of the Upd ligand. (D) When polar cells (arrow; marked with Fas III, membrane marker, green) are wild type for Upd (i.e. nuclear-GFP positive), Dome is present in intracellular vesicles. (E) By contrast, when polar cells (arrow) are mutant for Upd (i.e. nuclear-GFP negative), Dome is not detected in intracellular vesicles. In D and E, polar cells are marked using the FasIII antibody (membrane green staining). The clonal marker is GFP (nuclear green staining). Bars, 10 μm.

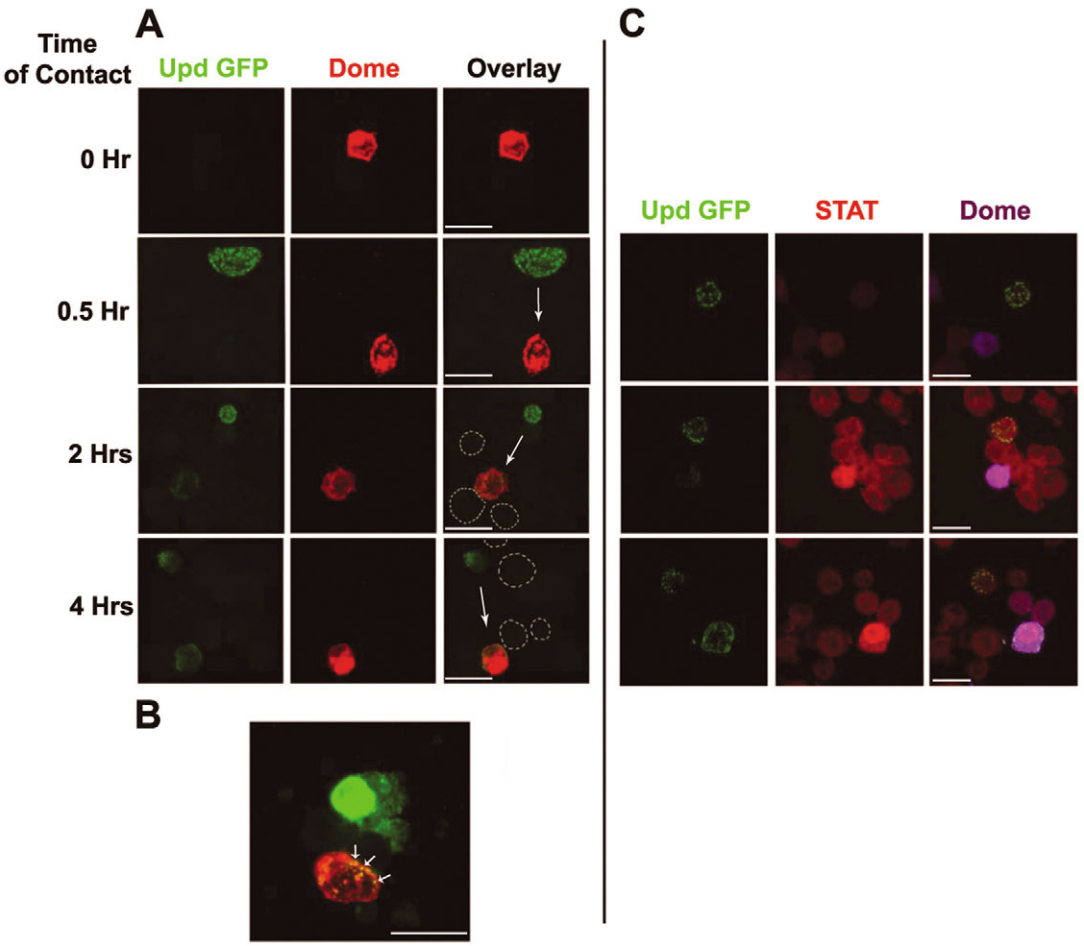

Fig. 2. Ligand diffusion and internalization leads to STAT nuclear localization in S2 cells. (A-C) Cells expressing either Upd-GFP or Dome were mixed and cultured together for 0.5-4 hours. The 0 hour time-point corresponds to cells transfected with Dome only. Upd-GFP diffuses in the medium and binds to Dome-expressing cells in a time-dependent manner. Cells that do not express Dome are outlined by broken lines. Upd-GFP is first found at the membrane of Dome-expressing cells, and (B) becomes internalized in Dome-containing vesicles (arrows) after 6 hours. (C) Binding of Upd-GFP leads to the nuclear accumulation of Stat in a Dome- and time-dependent manner. Dome-expressing cells in C are stained with a β-galactosidase marker (purple). Bars, 10 μm.

Upd is a diffusible ligand undergoing internalization in Dome-containing vesicles

One likely explanation for the observed gradient of Dome vesicles is that Upd diffuses away from its source to induce receptor internalization in neighbouring cells. Upd has been shown to be a secreted, glycosylated protein that is bound to the extracellular matrix (Harrison et al., 1998). In order to demonstrate that Upd can move in the extracellular space, we set up a co-culture assay using *Drosophila* Schneider S2 cells. Cells expressing either Upd-GFP or Dome were mixed and cultured together, and the GFP fluorescence analyzed at different time intervals. For standardization, only GFP/Dome cells that were at a distance of 2-4 cell diameters from each other were analyzed. After 0.5 hours co-culture, Upd-GFP was detected at the surface of Dome-expressing cells (Fig. 2A). The Upd-GFP signal found in Dome cells increased over time and was seen both over the whole surface of the cell (Fig. 2A) and intracellularly in vesicles containing Dome (Fig. 2B). These data demonstrate that Upd-GFP can diffuse in the medium over several cell diameters, and be internalized upon binding to the receptor. Internalization of Upd-GFP was strictly dependent on the presence of the receptor, because it was only detected in cells expressing Dome (Fig. 2). Indeed, even cells contacting Upd-GFP-expressing cells did not show any membrane-attached or intracellular Upd-GFP if they did not express Dome (Fig. 2A,C and data not shown).

One major output of JAK/STAT pathway activation is the nuclear translocation of the transcription factor STAT. Following exposure of Dome-expressing cells to Upd-GFP-expressing cells, endogenous STAT was found to be upregulated in the cytoplasm and in the nucleus after 2 hours (Fig. 2C). By 4 hours, a maximum of nuclear translocation was observed. Translocation of STAT was only observed in cells expressing Dome and was strictly dependent on the presence of Upd, indicating that nuclear translocation reflects a direct response to Dome activation.

Dome is internalized in clathrin-coated vesicles

To further characterize Dome internalization, specific markers of the endosome were expressed in BCs, including the human transferrin receptor (hTfR) (Strigini and Cohen, 2000), 2XFYVE-GFP (Wucherpfennig et al., 2003), *Drosophila* Rab5-GFP (Wucherpfennig et al., 2003) and *Drosophila* Rab7-GFP (Entchev et al., 2000). hTfR undergoes clathrin-mediated internalization and recycling in several cell types (Strigini and Cohen, 2000). When expressed in BCs, using *slbo-GAL4*, endogenous Dome was found to co-localize with hTfR in intracellular vesicles (Fig. 3A). Furthermore, the early endosomal markers 2XFYVE-GFP (Fig. 3B), and Rab5-GFP (Fig. 3C) or the late endosomal marker Rab7-GFP (Fig. 3D), were also found to co-localize with Dome in intracellular vesicles. Co-localization was observed before, during and after migration (data not shown; see below), suggesting that Dome is internalized during all the main steps of BC migration,

during which an active JAK/STAT pathway has been shown to be required (Silver et al., 2005). Together, these results suggest that Dome is internalized and is localized in the endosomal compartment.

Endocytosis controls the localization of membrane-bound Dome receptor

We tested directly the role of clathrin-mediated endocytosis by analyzing the localization of Dome in clones of cells that were mutant for the gene encoding Clathrin heavy chain (*Chc*) (Bazinet et al., 1993). In *Chc* clones, Dome was found to accumulate at the membrane (Fig. 4A), confirming that clathrin-mediated internalization controls Dome levels at the cell surface. Because we observed accumulation in all parts of the egg chamber at the same level, we conclude that all follicle cells can undergo constitutive Chc-mediated endocytosis. This activity is not likely to correspond to Rab11-dependent recycling, because Rab11 mutations did not affect the localization of Dome (data not shown). An increase in membrane Dome was also observed in cells mutant for Rab5 (Fig. 4B) (Wucherpfennig et al., 2003), indicating that Rab5 co-localizes with Dome (Fig. 3C) and regulates it.

Our double-labelling experiments indicate that Dome is found in early and late endosomes (Fig. 3), suggesting that, upon internalization, the Dome receptors are targeted to the lysosome for degradation. The *Drosophila deep orange* (*dor*) gene is homologous to the class C yeast vacuolar sorting protein (VPS) 18, and is involved in late endosomal functions by transporting lysosomal enzymes from the Golgi to the lysosome (Sevrioukov et al., 1999; Sriram et al., 2003). In cells that were mutant for *dor*, the lysosome was no longer functional, leading to an accumulation of multivesicular bodies (MVBs). In *dor* mutant follicular cells, Dome strongly accumulated in large intracellular vesicles that probably corresponded to enlarged MVBs (Fig. 4C,D). Interestingly, a clear gradient of large bodies was found around the poles, covering 5-6 cell diameters, as was the case for the endogenous wild-type Dome-containing vesicles (Fig. 1). Large vesicles were not observed in *dor* clones that were distant from the poles (Fig. 4C,D), indicating that these structures are formed in regions of high ligand concentration and probably correspond to internalization of ligand-bound receptors targeted for lysosomal degradation.

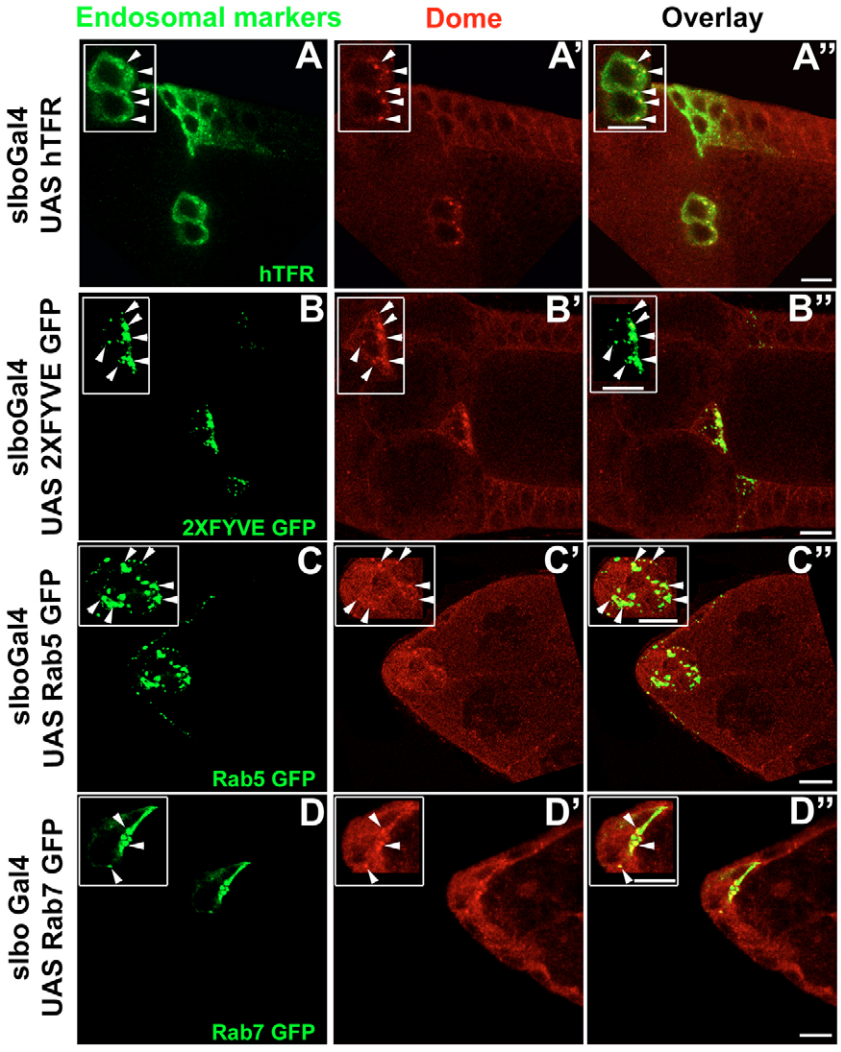

Fig. 3. Nature of Dome-containing vesicles. (A) Co-localization of Dome with the human transferrin receptor (hTfR), which was expressed in border cells using the slbo-Gal4 driver. (B-D) Dome co-localizes with specific markers (green) of the early (B, 2XFYVE-GFP; C, Rab5-GFP) and late (D, Rab7-GFP) endosomal compartments in the border cells. Arrowheads in insets show double-labelled intracellular vesicles. Bars, 10 μm.

Endocytosis, but not recycling, regulates JAK/STAT signalling

The levels of STAT are steady in wild-type follicle cells. In vivo, JAK/STAT signalling, as well as nuclear STAT, follow a gradient distribution peaking at anterior and posterior poles, the regions in which the ligand is the most concentrated (Xi et al., 2003) (Fig. 5A). These results suggest that STAT localization and level in follicle cells depend on the concentration of the ligand and strength of signalling, as was observed in cell cultures (Fig. 2). Indeed, we observed a strong reduction or absence of nuclear and cytoplasmic STAT protein in *dome* mutant cells (Fig. 5B).

To test the role of internalization and trafficking on JAK/STAT signalling, we first made clones of cells that were mutant for *Chc*. Surprisingly, blocking endocytosis of Dome using *Chc* mutations led to a dramatic decrease of STAT expression and nuclear localization, indicating that JAK/STAT signalling is strongly downregulated in these conditions (Fig. 5C), despite an increase in the concentration of the receptor at the membrane (Fig. 4A). These data suggest that clathrin recruitment and the formation of budding vesicles are required for JAK/STAT signalling, and that binding of Upd to the membrane-bound Dome receptor is not sufficient to

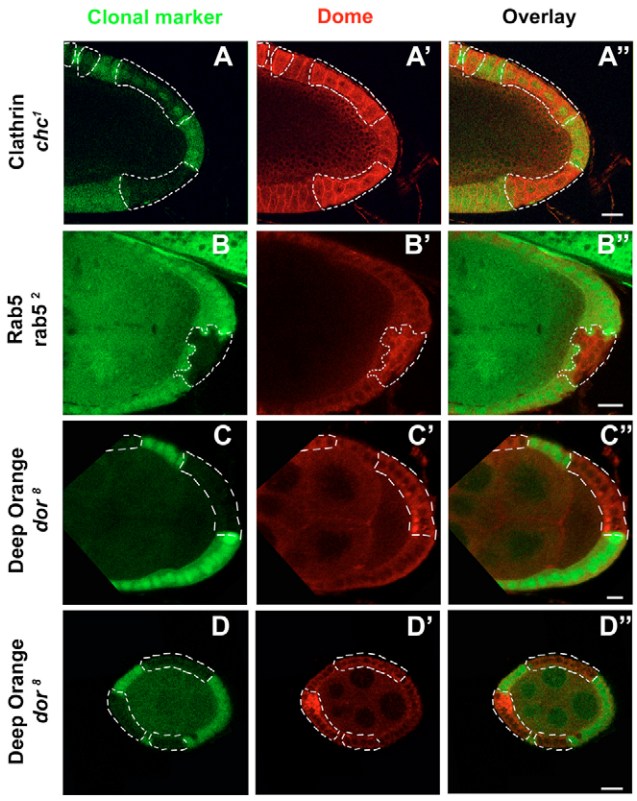

Fig. 4. Localization of Dome in mutants with affected intracellular trafficking. (A-D) Immunostaining using anti-Dome antibodies (red) showing Dome localization in *Clathrin heavy chain* (*Chc*, A), *rab5* (B) and *dor* (C,D) mutant cells. (A) Mosaic egg chamber containing *Chc* mutant clones (mutant cells are shown enclosed by a broken line and do not express the GFP clonal marker, green). In *Chc* mutant clones, Dome is concentrated at the membrane, indicating that Dome is internalized in clathrin-coated vesicles. (B) Mutations in *rab5* induce Dome accumulation in follicle cells. Notice the formation of a multi-layered epithelium, as previously observed in follicle cells of *rab5* mutant clones (Lu and Bilder, 2005). (C,D) Loss of *dor* function leads to a strong accumulation of Dome in large intracellular structures corresponding to enlarged multivesicular bodies (MVBs). This phenotype is only seen in *dor* mutant cells close to the ligand source at the posterior (C) and anterior (D) poles of the egg chamber. Bars, 10 μm.

activate the pathway. To confirm that the *Chc* mutation leads to a decrease of STAT transcriptional activity, we analyzed the expression of the *pointed* (*pnt*) target gene, using a *pnt-lacZ* reporter line (Xi et al., 2003). We found that both STAT and *pnt-lacZ* expression were strongly reduced in some clones (Fig. 6A,B), indicative of a reduction of STAT activity. Not all clones showed a visible reduction in expression of *pnt-lacZ*, however, probably reflecting the perdurance of the β-galactosidase marker and/or of STAT activity.

The formation of clathrin-coated vesicles is followed by fusion with the early endosome, which depends on the small GTPase Rab5. Rab5 has been shown to be involved in basement membrane dynamics and migration of BCs (Medioni and Noselli, 2005), and in epithelial polarity (Lu and Bilder, 2005). Follicle cells mutant for Rab5 showed a strong reduction of cytoplasmic and nuclear STAT accompanied by perinuclear localization (Fig. 5D), all indicative of a decrease in signalling. These results suggest that fusion to the early endosome and a functional early endosomal structure are also important for signalling. STAT is less affected in *rab5* than in *Chc* mutant conditions, suggesting that signalling could begin after *Chc* function, but would need Rab5 for full activation. Alternatively, it could also suggest redundancy at the level of Rab5.

Following fusion, endocytic vesicles are sorted to undergo either recycling or further trafficking through the endosomal compartment. Recycling of receptors back to the membrane is a common mechanism to maintain receptor levels and thus signal receptivity of cells, and the Rab11 GTPase has been shown to be a main regulator for the recycling of clathrin-coated vesicles (Ullrich et al., 1996; Green et al., 1997). We found that Rab11 mutant cells (Satoh et al., 2005) show normal Stat levels and localization (Fig. 5E), indicating that Rab11 and recycling do not significantly control JAK/STAT signalling. Altogether, these data suggest that the main pathway of Dome trafficking and activation goes from the membrane to the lysosome through the early and late endosomal compartments.

Blocking late endosomal function and blocking sorting to MVBs affects JAK/STAT signalling

We showed that blocking lysosomal function using *dor* mutations led to a strong accumulation of Dome in enlarged MVBs. If Dome is active in this abnormal compartment, then an increase or at least a normal level of signalling should ensue. Like in the *rab5* mutant conditions, we observed that *dor* mutant cells had little or no nuclear STAT (Fig. 5F) and a reduction in *pnt-lacZ* expression (Fig. 6C), indicative of a decrease in JAK/STAT activity. These results suggest that Dome is mostly inactive in the MVBs. It is also possible that blocking trafficking at the MVB level induces regulatory feedback loops reducing upstream endocytosis, and hence signalling.

Reaching the MVB requires proteins to be post-translationally modified through mono-ubiquitylation (Hicke and Dunn, 2003). The ubiquitin tag is recognized by proteins harbouring ubiquitin-recognition motifs; Hrs, which is implicated in the formation of the MVB (Lloyd et al., 2002), is an example of such a protein. As expected, disrupting Hrs activity led to an inhibition of JAK/STAT signalling, indicating that Hrs is a positive regulator of signal transduction (Fig. 5G).

Discussion

Using genetic analysis in a developing tissue, we show that several regulators of the endocytic pathway are required for normal JAK/STAT signalling in vivo. The membrane-bound Dome receptors undergo ligand-dependent internalization in clathrin-coated vesicles (Figs 1, 2), which are then targeted to the sorting endosome via Rab5 (Figs 3, 4). The function of Hrs is required for JAK/STAT activation and to direct most of the active receptors to the MVBs, targeting them to the lysosome for degradation (Fig. 5).

One important question is to know whether the trafficking of ligand-bound receptors has any effect on signalling. We addressed this question by looking at Stat nuclear localization, which represents a robust readout to assess JAK/STAT activity (Figs 2, 5) and *pnt-lacZ* expression (Fig. 6).

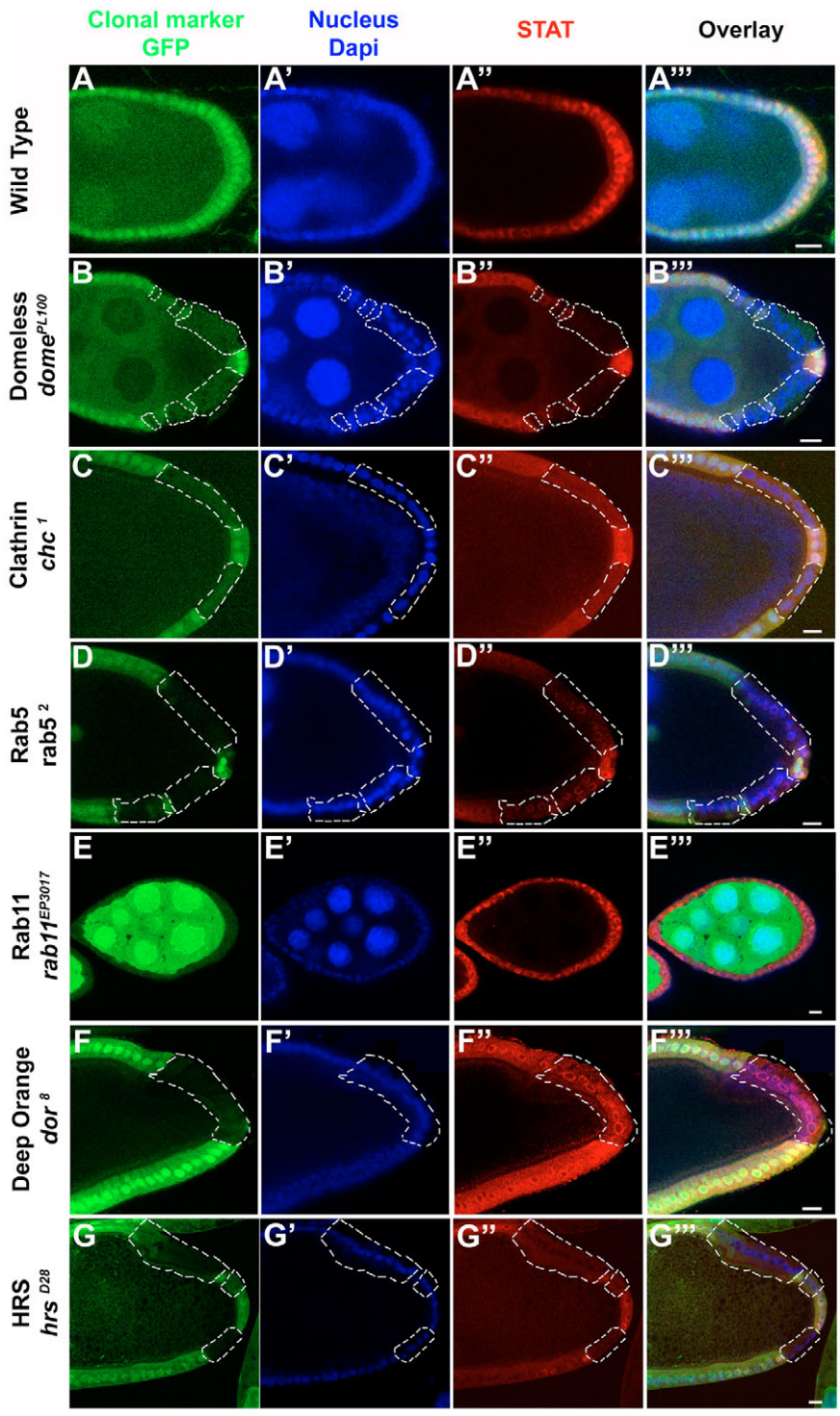

Fig. 5. Endocytosis controls STAT nuclear localization and pathway activity. (A-G) STAT intracellular localization was analyzed using anti-STAT antibodies (red) to measure JAK/STAT pathway activation in wild-type egg chambers (A) and in *dome* (B), *Chc* (C), *rab5* (D), *rab11* (E), *dor* (F) and *Hrs* (G) mutant backgrounds. In cells mutant for *Chc* (C), *rab5* (D) or *Hrs* (G), Stat disappears from the cytoplasm and nucleus, as in *dome* mutant cells (B), indicating that JAK/STAT signalling is strongly affected in these conditions. Mutations in *dor* (F) also reduce JAK/STAT signalling but to a lesser extent, as evidenced by weak residual STAT staining in the periphery of the nucleus (F). By contrast, cells mutant for *rab11* (E) show normal Stat staining, suggesting that it is not required for JAK/STAT signalling. Broken lines mark the mutant cells. Bars, 10 μm.

The effect of Hrs is opposite on the JAK/STAT pathway compared with its effect on other pathways. Indeed, in the egg chamber, Hrs plays a positive role on JAK/STAT activity (this study), whereas it has been shown to downregulate the EGFR, Notch and TGF-β pathways in the same tissue (Jekely and Rorth, 2003). Interestingly, HRS has been shown to interact with STAM in the same mono-ubiquitylated recognition complex (Lohi and Lehto, 2001). STAM is a known JAK/STAT activator (Pandey et al., 2000), suggesting that HRS could control STAT signalling through its interaction with STAM. So, Hrs could play two crucial roles: first, allowing the sequestration and the sorting of the receptor to the lysosome and, second, activating the ligand-receptor complex in collaboration with STAM.

Our data challenge the simple view whereby binding of the ligand to the receptor at the membrane would be sufficient to activate the pathway. Indeed, we found that equally essential is the need of clathrin for the activation of JAK/STAT signalling. Thus, activation can occur only when the ligand-receptor complex is assembled into clathrin-coated vesicles. In this view, activation would proceed in two steps, requiring both binding of the ligand and interaction with clathrin. The role of clathrin could be to concentrate/cluster receptors and/or bring them together with other signal transducers in the endosomal compartment. This finding is in agreement with a recent work showing that, in mammals, clathrin is required to transduce JAK/STAT signals through the IFNα-receptor, but not the IFNγ-receptor, suggesting a conserved function for clathrin in JAK/STAT signalling (Marchetti et al., 2006). Interestingly, like in mammals, JAK/STAT signalling in *Drosophila* might be controlled in a cell-type-specific manner by Chc-dependent endocytosis. Indeed, in eyes, Vps25 and TSG101 mutations lead to Upd upregulation and JAK/STAT activation in a Notch-dependent manner (Moberg et al., 2005; Vaccari and Bilder, 2005).

What is the significance of clathrin function and, more generally, of the requirement for internalization, in JAK/STAT signalling? It has been shown for several signalling pathways that internalization brings together membrane receptors and intracellular pathway components in the endosomal compartment, which thus serves as a platform for signalling. The fact that Dome internalization and activation are coupled to

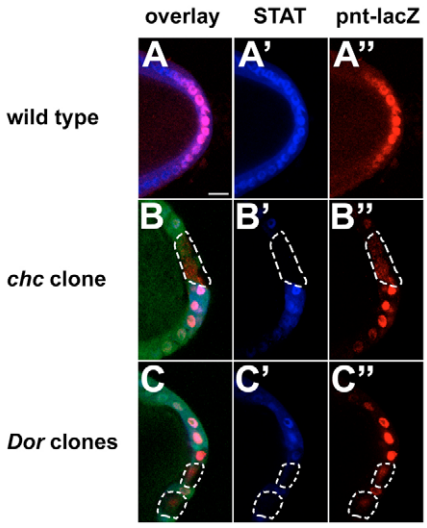

Fig. 6. Expression of the STAT target gene *pointed* (*pnt*) is affected in *Chc* and *dor* mutant cells. Expression of STAT (blue) and the activity of the JAK/STAT pathway, monitored using a *pointed-lacZ* reporter gene (red). Staining of wild-type (A-A″), *Chc*^{1} (B-B″) and *dor*^{8} (C-C″) egg chambers. The clonal marker is GFP (green in B-C″). Broken lines mark the mutant cells. Bar, 10 μm.

degradation has important consequences. Making signalling complexes only active in the endosomal compartment is a powerful mechanism to control the number of active complexes in the cell. Their targeting to the lysosome allows the control of their lifetime as active receptors, providing a temporal – hence quantitative – control on signalling.

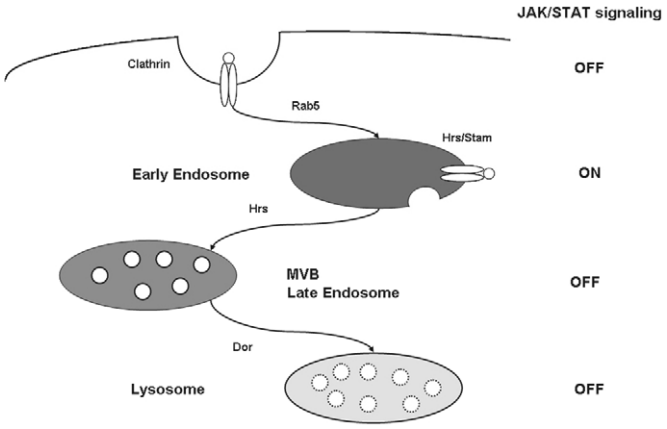

Fig. 7. Model of endocytic control of Dome trafficking and JAK/STAT signalling. Dome intracellular trafficking in egg chambers. Following ligand binding, Dome is directed to the early endosome and is then sorted to the lysosome, via the multivesicular body (MVB) and the late endosomal compartment. The off/on/off model (right): to become active, the JAK/STAT signalling pathway requires the formation of the ligand-receptor complex at the cell membrane and the formation of Chc-containing budding vesicles. These two conditions allow JAK/STAT signalling to be activated only when the receptor is on its way to degradation. Finally, the degradation of Dome in the MVB/lysosome eliminates active receptors, thus dynamically controlling JAK/STAT pathway activity.

Activation of JAK/STAT follows an off/on/off model in which two conditions are required for correct JAK/STAT activation (Fig. 7): (i) formation of a ligand-receptor complex (as proposed in the classical model), followed by (ii) the internalization of the complex via Chc-containing budding vesicles. The sole formation of the ligand-receptor interaction would lead to an inactive complex (off). However, interaction with Chc and subsequent internalization activate the complex (on), thus ensuring that only the complexes targeted for degradation are activated. Arrival of the complex in the MVB/lysosome turns it into the off state (Fig. 7).

Altogether, our results thus show a crucial role for endocytosis in the activation and in the regulation of JAK/STAT signalling in a developing tissue, with strong parallels to mammalian IFNα signal transduction (Marchetti et al., 2006).

Materials and Methods

Drosophila genetics

A description of genetic markers and chromosomes can be found at FlyBase (http://flybase.bio.indiana.edu). The loss-of-function mutant alleles used in this study are *Chc*^{1} (lethal), *rab5*^{2} (null allele), *dor*^{8} (null allele), *Hrs*^{D28} (non-sense mutation leading to a truncated Hrs protein, abolishing its function), *upd*^{4} (lethal) and *dome*^{PL100} (lethal). The following *Drosophila* stocks have been used: *neur*^{A101} (CG11988); *dome*^{PL100} FRT19A/FM7i (CG14226); *upd*^{4} FRT19A/FM7i (CG5993); *Chc*^{1} FRT19A/FM7i (CG9012); *rab5*^{2} FRT40A/Cyo (CG3664, a gift from M. Gonzalez-Gaitan, University of Geneva, Switzerland); *dor*^{8} FRT19A/FM6 (CG3093, a gift from H. Kramer, UT Southwestern, Dallas, TX); *Hrs*^{D28} FRT40A/Cyo (CG2903, a gift from H. Bellen, Baylor College, Houston, TX); *rab11*^{EP3017} FRT82B/TM2 (CG5771, a gift from D. F. Ready, Purdue University, West Lafayette, IN); *slbo1*, *slbo*-Gal4/Cyo; UAS-*rab5GFP*, UAS-*rab7GFP*; UAS-*2XfyveGFP* (gifts from M. Gonzalez-Gaitan), UAS hTfR (a gift from S. Cohen, EMBL Heidelberg, Germany); UBGFP FRT19A; *e22c*-Gal4, UAS-flp/Cyo; *T155*-Gal4, UAS-flp; UBGFP FRT40A; *e22c*-Gal4 UAS-flp/Cyo; UBGFP FRT 82B. Homozygous follicle cell clones for *upd*, *Chc*, *dome*, *rab5*, *dor*, *rab11* and *Hrs* were induced using the UAS-FLP method (Duffy et al., 1998). Mosaic egg chambers were analyzed in *dome*^{PL100} or *upd*^{4} or *Chc*^{1} *or dor*^{8}, FRT19A/UB-GFP, FRT19A; *e22c*-GAL4, UAS-flp/+ females, in *T155*-Gal4, UAS-flp/+, *rab5*^{2} or *hrs*^{D28}, FRT40A/FRT40A UB-GFP, females, and in *e22c*-GAL4, UAS-flp/+; *rab11*, FRT82B/UB-GFP, FRT82B females.

Antibodies, immunostaining and imaging

Immunostaining of egg chambers was performed as described previously (Ghiglione et al., 2002). The following primary antibodies have been used: rabbit anti-Dome (1:200), rabbit anti-Stat92E (1:500, a gift from S. Hou, NCI-Frederick, Frederick, MD), mouse anti-Fas3 [1:100; 7G10, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-β-galactosidase (1:1000, Promega), mouse anti-hTfR (1:200, Pharmingen anti-human CD71). Secondary antibodies were anti-mouse Alexa-Fluor-488 (1:400) and anti-rabbit Alexa-Fluor-546 (1:400) from Molecular Probes. DAPI has been used at 10 μg/ml.

Confocal images were taken using a Leica TCS-SP1 or a Zeiss LSM 510 META confocal microscope.

Cell culture and transfection

S2 cells were transiently transfected with pUASt-*updGFP* and pAc5-Gal4 (UpdGFP-expressing cells) or with pUASt-*dome*, pAc5-Gal4 and pAc5-*lacZ* (Dome expressing cells), using Cellfectin transfection reagent (Invitrogen) according to the manufacturer's instructions. At 3 days after transfection, Upd-GFP-expressing cells and Dome-expressing cells were co-cultured for 0.5, 2, 4 and 6 hours in LabTech1 chamber slides (Nunc). Cells were then fixed and stained with rabbit anti-Dome, mouse anti-β-galactosidase or rabbit anti-Stat92E using standard protocols.

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