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Integration of JAK/STAT receptor-ligand trafficking, signalling and gene expression in *Drosophila melanogaster* cells

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MS TITLE: Novel regulatory mechanisms of JAK/STAT activation in Drosophila cells

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ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, all three reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper. I think that they can all be answered without the need for further experiments.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

The authors investigate the role of endocytosis in regulating cytokine induced JAK-STAT signalling in Drosophila. The Drosophila cytokine receptor Domeless has already been shown to require clathrin-mediated endocytosis (CME) (Devergne et al, 2007) and various molecular requirements have been identified. This has also previously been shown to regulate JAK-STAT localisation and pathway activation. In the current study the authors formally identify a di-leucine motif (classically associated with CME) that is essential for internalisation of Domeless – although this also relies on the presence of surrounding sequence elements. The requirement for clathrin and endocytic sorting mediators was investigated (Figure 2) and concurred with previous observations (Devergne et al, 2007). In this case additional reporters of STAT activation were examined, and variable dependencies were seen in the outputs - ie. interestingly not all targets of Dome require endocytosis. The authors show that STAT92E phosphorylation is required for transcription of Dome targets but this is not sensitive to endocytosis – therefore the intriguing observation of a differential dependence on endocytosis unfortunately remains unresolved. The work provides additional molecular underpinnings for JAK-STAT regulation in Drosophila and poses some new questions likely to stimulate further work.

Comments for the author

The data are straightforward and generally with appropriate controls. My main comments relate to:
1. For the dsRNA, is this a library or a specific sequence? More than one sequence example for each target should be used to increase confidence that the effects are specific. Blots needs to be shown to shown for all dsRNA examples that demonstrate that the intended targets have been knocked down (accompanied by loading control blots).
2. Data presentation. Some Figure legends are difficult to discern. The use of colours instead of formal labels in Figure 5 is not ideal.

Reviewer 2

Advance summary and potential significance to field

Moore et al investigated regulatory mechanisms of JAK/STAT signaling using cultured Drosophila S2 cells in which they can stimulate the pathway by adding the ligand Upd2. They first identified an evolutionarily conserved internalization LL motif in the cytoplasmic tail of the receptor Dome. They then demonstrated that internalization and endocytic trafficking of activated Dome to specific endosomes affect the JAK/STAT transcriptional output, using a reporter gene. They also showed that this regulation is independent of STAT Y704 phosphorylation of STAT. Finally, they used mass spec and mutagenesis to identify Threonine702 as being essential for Y704 phosphorylation of Drosophila STAT, as well as STAT nuclear translocation and transcription activity. The work is thorough and makes a significant and novel contribution to our understanding of the regulation of the JAK/STAT pathway, and should be publishable in Journal of Cell Science.

Comments for the author

I have only minor suggestions as the following.

The title can be more specific.

Figure 1a: Should explain how curve fitting was done or provide the equations used for fitting data points with curves.

Page 6, first paragraph, explain AP2, Hrs, etc when they first appear (only found later in the text).

Figure 1d: better just show relative levels of surface Dome-FLAG (relative to number of cells or some uniform marker instead of total transfected Dome-Flag)

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Figure 4: the difference between 30min and 0 min represented in b looks much more dramatic than the images shown or in other panels. In other words, it doesn't appear there is a 100-fold increase in nuclear localization from 0 to 30 min.
The apparent nuclear STAT without activation should be noted and referenced. Other groups have shown that STAT proteins shuttle between nucleus and cytoplasm regardless of its phosphorylation status, and there is unpohosphorylated nuclear STAT with noncanonical functions.

Page 9, The finding that Dome internalization affects STAT transcriptional activity but not its phosphorylation is intriguing and a little confusing. Perhaps some scenarios should be discussed in more detail in Discussion.

Page 10 , 2nd paragraph: "This analysis identified Threonine47…", should add "in addition to Y704" as being phosphorylated. Last sentence, "phosphorylation of Threonine702, but not Threonine47 or Serine227, is required for JAK/STAT signaling" should include Y704.

Figure 5d: If the authors have Y704E data, it should be included or mentioned.

Page 11, 3rd paragraph: As mentioned above, the statement "STAT92ET702V no longer translocates into the nucleus following Upd2-GFP stimulation" should be rephrased.

Reviewer 3

Advance summary and potential significance to field

This study investigates the link between a membrane receptor, Domeless, and key downstream tyrosine kinases (JAK kinase, Hop; STAT kinase, STAT92E) that mediates signal transduction and cellular responses. This is a well-done piece of work and worthy of publication in JCS. My specific points are detailed below.

Comments for the author

This is a well-executed piece of work from the authors but needs careful attention to the points raised below.

Major points:
1) The authors use endocytosis as a single term to describe membrane protein internalisation, but the current picture is that multiple pathways mediate delivery to endosomes in eukaryotes (REFS). This is perhaps less clear in Drosophila compared to mammalian systems, but the authors need to better consider clathrin-dependent endocytosis (CDE) vs. clathrin-independent endocytosis (CIE), especially in the introduction and discussion. In the case of CIE, lipid raft-dependent and -independent mechanisms can be postulated to regulate both signalling and endocytosis. This is especially relevant here, but only mentioned at the beginning of the Results section, and in passing. This needs to be carefully considered in both the introduction and discussion and how the findings stand in this context.

2) Page 6, para 1. It is unclear how the data in Fig. 1 and Fig. S1 show requirement for CDE at low conc. of ligand (Upd2) and both CDE and CIE at high Upd2 conc. The data needs to be presented in a different and more coherent way.
This is an important point.

3) Page 6, para 2, Fig. 1C. In examining the data for the effects of di-leucine mutations, there is higher levels of cell surface Domeless (Fig. 1C), but this is statistically non-significant by the error bars presented. However, effects are more clear-cut when endocytosis is measured (Fig. 1F). If the authors want to suggest that the mutations have an effect on endocytosis and cell surface levels of Domeless, they have to vary clearly (and specifically) mention the % changes in cell surface levels and in endocytosis in each situation, and but also say where this was non-significant by the statistical tests used here.

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4) Pages 7-8, Fig. 2. Looking at the activation of reporter expression and SOCS mRNA levels as a function of Domeless activation, it is clear that AP2 and Hrs have effects but not anything else. This might suggest that different pools of Domeless have different signalling functions here. As AP2 regulates early endocytic events and delivery to early endosomes, whereas Hrs is part of the ESCRT-0 complex already on early endosomes, this may argue for multiple compartmentalised signalling events.

5) Pages 12-15. Discussion. I find the discussion a bit confusing as the lines of thinking are unclear in fitting into a pathway leading from the cell surface, endosomes to nuclear gene expression, although this is a complex topic. The issues are as follows: multiple endocytic pathways occurring dependent on low and high ligand concentrations, all ending up in endosomes and likely different pools of inactive and activated receptors and receptor-ligand complexes positioned along the pathway. Could this be helped by having a little schematic added as a small panel (Fig. 6x) to show this? One important previous study, quoted here is by di Fiore and colleagues (Sigismund et al., 2005) where they show that depending on EGF concentration, different endocytic routes come into play and this is important for downstream EGF-EGFR signalling AND trafficking. I think the authors need to rework the discussion substantially.

Minor points:
1) Use single amino acid designations when discussing specific residues in specific proteins and enzymes e.g. T702, Y704 and mutations/phosphorylation events e.g. T702A, pY704. This is done in figures but not consistently done in the text of the manuscript.

2) I am not sure the title of the study accurately reflects what the study is about. Needs more thought e.g. 'Integration of receptor-ligand trafficking,
signalling and gene expression in Drosophila melanogaster'

First revision

Author response to reviewers' comments

We thank all three reviewers for their very constructive comments on our manuscript. We have addressed each of their individual comments below and feel that the manuscript is now much improved because of their input.

Reviewer 1 Comments for the Author:
The data are straightforward and generally with appropriate controls. My main comments relate to:
1. For the dsRNA, is this a library or a specific sequence? More than one sequence example for each target should be used to increase confidence that the effects are specific. Blots needs to be shown to shown for all dsRNA examples that demonstrate that the intended targets have been knocked down (accompanied by loading control blots).

The dsRNA used were specific sequences, apart from the *C. elegans* control dsRNA which was a mixture of 3 sequences. They were obtained from the Sheffield RNAi Screening Facility whose dsRNA database is based on the HD2.0 generated by Next-RNAi (Horn et al. 2010). We have clarified the text in the methods section on dsRNA to include the following text.
'The dsRNA collection we use is from Michael Boutros' lab and is the redesigned, non-off target effect library, HD2.0 generated using the software next-RNAi (developed by Thomas Horn). Low complexity regions and sequence motifs that induce off-target effects have been excluded. dsRNA probe sizes vary from 81 to 800bp covering ~14000 protein encoding genes and ~1000 non-coding genes (~98.8% coverage). The dsRNA design covers every isoform of each gene and have been optimised for specificity and avoidance of low complexity region'

To our knowledge there are no antibodies available that reliably cross react with Dome, AP2 or TSG101 from *Drosophila*. In the course of our studies an antibody to Hrs became available

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which we used to show the knockdown was effective (see blot which we enclose for the reviewer only). However, this was not used for the experiments presented in the manuscript. Instead of using antibodies, we routinely used pPCR to determine efficiencies of knockdown, using rpl32 as a reference control. We have now included these data in the supplemental material (Fig S1B).

[A figure provided for the reviewer has been removed]

2. Data presentation. Some Figure legends are difficult to discern. The use of colours instead of formal labels in Figure 5 is not ideal.
We have modified Figure 5 as suggested by the reviewer to indicate the relevant mutations. We have also converted all of the figures, except for Figure 1, to black and white. We believe that the colour in Figure 1C-E, indicating different mutations within Dome, makes the figure clearer and easier to follow because the quantitation can be directly related to the sample blots.

Reviewer 2 Comments for the Author:
I have only minor suggestions as the following.

1. The title can be more specific.
As requested by this reviewer and reviewer 3, we have changed the title to: Integration of JAK/STAT/receptor-ligand trafficking, signalling and gene expression in *Drosophila melanogaster*

2. Figure 1a: Should explain how curve fitting was done or provide the equations used for fitting data points with curves.
The data were fitted in Prism and we have amended the text in the legend to Figure 1a to reflect this (amended text indicated in red).

3. Page 6, first paragraph, explain AP2, Hrs, etc when they first appear (only found later in the text).
As suggested by the reviewer we have amended the Introduction to include a description of the roles of AP2 and Hrs. The following text has been included (indicated in red in the manuscript).
Activated receptors can be internalised into cells by multiple endocytic pathways of which clathrin mediated endocytosis (CME) is the best characterised. Receptor complexes internalised by CME are clustered into clathrin coated pits. The assembled clathrin lattice is linked to the cytoplasmic domains of transmembrane receptors via adaptor proteins, including the AP2 adaptor complex (Mettlen et al., 2018; Owen et al., 2004). In addition to CME, several clathrin independent (CIE) pathways exist which are important for the uptake of particular cargoes (Mayor et al., 2014). Following internalisation, activated receptors are delivered to the early endosome where they may be recycled or targeted to late endosomes and lysosomes for degradation. The Endosomal Sorting Complexes Required for Transport (ESCRT) protein complexes are key for sorting receptors into late endosomes and lysosomes. Hrs is a component of ESCRT-0, acting as an adaptor to select ubiquitinated cargo for targetting to lysosomes. TSG101 is a component of ESCRT I complexes which recruit other ESCRT complexes, which are key in allowing the inward invaginations of the late endosome to form intraluminal vesicles (Henne et al., 2013). Results from *in vivo* and *in vitro* experiments indicate that endocytosis can regulate receptor signalling quantitatively through removal of activated receptors from the cell surface and targeting them to lysosomes for degradation. Endocytosis can also qualitatively regulate signalling by establishing ‘signalosomes’, which are membrane microdomains within endosomal compartments that allow the recruitment of specific scaffolds, adaptors, kinases and phosphatases, thus resulting in different downstream signalling outputs (Carroll and Dunlop, 2017; Lawrence et al., 2019; Moore et al., 2018; Sigismund and Scita, 2018; Villasenor et al., 2016). The route of entry of activated receptors (CME versus CIE) can also influence signaling output as demonstrated for Notch signaling in *Drosophila* (Shimizu et al., 2014) and TGF-beta signaling in mammalian cells (Di Guglielmo et al., 2003). CME is a major entry portal which has been shown to regulate JAK/STAT signalling following activation of several different cytokine receptors in mammalian cells (Cendrowski et al., 2016; Chmiest et al., 2016; German et al., 2011; Kermorgant and Parker, 2008; Marchetti

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et al., 2006).

4. Figure 1d: better just show relative levels of surface Dome-FLAG (relative to number of cells or some uniform marker instead of total transfected Dome-Flag)

As the reviewer has suggested, we have included a Figure (FigS1C) that shows the relative levels of expression of wild-type and mutant Dome compared to ¤-actin as a marker for total cell protein. All of the mutants appear to transfect slightly more effectively than the wild-type construct. For greater clarity, we have moved Figure 1B to the supplementals (Figure S1D) since it shows that the levels of cell surface Dome as a proportion of the total exogenously expressed Dome are similar and yet there are significant differences in their endocytic uptake (Figure 1D and E). We have also amended the text (line 183 onwards) to reflect this. This showed that while expression of the mutants was somewhat more efficient than transfection of wild-type Dome (Figure S1C), plasma membrane expression all of the constructs was comparable (Figure S1D). *See also response to Reviewer 3.*

5. Figure 4: the difference between 30min and 0 min represented in b looks much more dramatic than the images shown or in other panels. In other words, it doesn't appear there is a 100-fold increase in nuclear localization from 0 to 30 min.
We agree with the reviewer that the way we presented the data was sub-optimal. This is because we initially normalised the data by using the 0 minute time point as a background measure of STAT92E in the nucleus. We now present a revised Figure 4B where we have not subtracted the 0 time point value but present the ratio of nuclear-to-cytoplasmic GFP, as a percentage of that at 30 minutes (when maximal nuclear accumulation occurs). This shows that between 0 and 30 minutes, the nuclear to cytoplasmic ratio of STAT92E-GFP increases by 36%.

6. The apparent nuclear STAT without activation should be noted and referenced. Other groups have shown that STAT proteins shuttle between nucleus and cytoplasm regardless of its phosphorylation status, and there is unpohosphorylated nuclear STAT with noncanonical functions.

We agree with the reviewer's point and have included the following in the description of the data shown in Figure 4 (line 293 onwards):
In the absence of ligand there appears to be low levels of STAT92E-GFP in the nucleus. This is consistent with reports that STATs shuttle between the nucleus and cytoplasm in a phosphorylation-independent manner and that unphosphorylated nuclear STATs can perform non-canonical functions (Brown and Zeidler, 2008). The levels of nuclear STAT92E- GFP we observe in the absence of Upd2 is also in keeping with reports of GFP-tagged proteins entering the nucleus independently of a nuclear localisation signal (Seibel et al., 2007).

7. Page 9, The finding that Dome internalization affects STAT transcriptional activity but not its phosphorylation is intriguing and a little confusing. Perhaps some scenarios should be discussed in more detail in Discussion.

The classical view of JAK/STAT signalling is that Tyr704 phosphorylation of STAT92E is necessary and sufficient for target activation. Our results indicate that this is not always the case and that while Tyr704 is necessary, it is not always sufficient for gene transcription. Our results show that endocytic uptake and, presumably delivery to a particular endosomal compartment, is required to allow STAT92E to become transcriptionally competent for at least a subset of target genes. We have substantially revised the Discussion and to clarify the possible scenarios that might allow STAT92E to become transcriptionally active (acquisition of additional posttranslational modifications and/or a chaperone protein).
The specific text that we have included in the Discussion is included below:

When the endocytic pathway is disrupted, phosphorylated STAT92E can still translocate into the nucleus but it is no longer fully signalling competent. This implies that Dome needs to reach a particular endosomal subcompartment or microdomain in order to allow STAT92E to become transcriptionally competent. Of particular interest is the post-Hrs and pre-TSG101 compartment required for *socs36E* expression (Figure 6D). Hrs is a component of ESCRT-0

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complex that packages ubiquitinated signalling cargo into inward invaginations of the endosomal membrane to form ILVs and ultimately multivesicular bodies. TSG101 is required for later stages of ILV formation (Vietri et al., 2019). As such both these components are found within the same limiting membrane. It has been proposed that membrane microdomains of defined composition, containing signalling molecules, must be able to form within endosomal membranes to generate local signalling competent (signalosome) domains (Shimizu et al., 2014; Teis et al., 2002). Within these specialised signalosomes, STAT92E is likely either to undergo additional posttranslational modifications or to acquire a chaperone protein that facilitates its ability as a transcription factor for a subset of target genes. Support for an Hrs signalosome comes from studies that demonstrate that the Hrs interacting protein STAM is required for downstream signalling following IL2-R activation (Takeshita et al., 1997; Tognon et al., 2014). In mammals, STAMs are phosphorylated in response to a range of cytokines and growth factors (Pandey et al., 2000). The Hrs/STAM complex remains an interesting link between signalling and endocytosis, as it has been shown to have both positive and negative roles in the regulation of RTK signalling in Drosophila, which are dependent on the specific tissue and developmental stage (Chanut-Delalande et al., 2010).

8. Page 10 , 2nd paragraph: “This analysis identified Threonine47…”, should add “in addition to Y704” as being phosphorylated. Last sentence, “phosphorylation of Threonine702, but not Threonine47 or Serine227, is required for JAK/STAT signaling” should include Y704.

We have modified the text to make it clearer as suggested by the reviewer (indicated in red). This indicates that phosphorylation of Thr702 as well as Tyr704, but not Thr47 or Ser227, is required for JAK/STAT signalling.

Figure 5d: If the authors have Y704E data, it should be included or mentioned.
We did not generate a Y704E mutation in STAT92E. This was because a conversion to glutamic acid is unlikely to mimic a phosphotyrosine residue because, while gaining a negative charge, the essential hydrophobic nature of the tyrosine residue would be lost. Furthermore, we felt it was unclear what additional information would be gained by testing this mutation.

Page 11, 3rd paragraph: As mentioned above, the statement “STAT92ET702V no longer translocates into the nucleus following Upd2-GFP stimulation” should be rephrased.
The wording of this sentence has been rephrased as indicated in red: We found that Upd2- GFP does not stimulate STAT92E^{T702V} translocation into the nucleus

Reviewer 3 Comments for the Author:
This is a well-executed piece of work from the authors but needs careful attention to the points raised below.

Major points:
1) The authors use endocytosis as a single term to describe membrane protein internalisation, but the current picture is that multiple pathways mediate delivery to endosomes in eukaryotes (REFS). This is perhaps less clear in Drosophila compared to mammalian systems, but the authors need to better consider clathrin-dependent endocytosis (CDE) vs. clathrin-independent endocytosis (CIE), especially in the introduction and discussion. In the case of CIE, lipid raft-dependent and -independent mechanisms can be postulated to regulate both signalling and endocytosis. This is especially relevant here, but only mentioned at the beginning of the Results section, and in passing. This needs to be carefully considered in both the introduction and discussion and how the findings stand in this context.

We acknowledge the important points made by this reviewer as well as reviewer 2 and have substantially amended the text in both the Introduction and Discussion to describe in more detail the roles of CIE and CME in receptor uptake and signalling. As indicated in response to Reviewer 2, we have included more description of CME and CIE in the Introduction. In response to the suggestion of Reviewer 3 we have substantially modified the Discussion as follows: Our results support a role for CIE, in addition to CME, in uptake of activated Dome in S2R+ cells. While dsRNA-mediated knockdown of CHC and AP2 inhibits internalisation of Upd2-GFP/Dome, the extent of inhibition depends on the concentration of the Upd2-GFP ligand. At low concentrations (3 nM) of Upd2-GFP, there is an absolute requirement for CHC and AP2,

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whereas at higher concentrations (20 nM), uptake of Upd2-GFP/Dome in cells treated with dsRNA targeting CHC and AP2 is inhibited by approximately 50% compared to cells treated with dsRNA targeting Dome. This is consistent with studies in *Drosophila* where uptake of Notch and Delta through different endocytic pathways (CME and CIE) leads to delivery to different endosomal compartments and differential signalling and the balance of flux between these pathways allows cells to respond to different environmental conditions (Shimizu et al., 2014). Similarly, in mammalian cells, activated receptor tyrosine kinases such as TGF-beta receptors and EGFR can be taken up by CME and CIE, with CME being favoured at lower ligand concentrations (Di Guglielmo et al., 2003; Sigismund et al., 2005). As with Notch signalling, the route of entry of the receptors can determine signalling outcome and receptor fate (Sigismund et al., 2013; Vander Ark et al., 2018). The concept of endocytosis modulating Dome target gene expression in different cells and tissues is supported by previous *in vitro* and *in vivo* studies (Devergne et al., 2007l; Silver et al., 2005; Vidal et al., 2010). Our experiments, which have focussed on CME of activated Dome, indicate that endocytosis also regulates a subset of Dome signalling in S2R+ cells. Mutation of the internalisation motif not only prevents Dome uptake but also prevents Dome activation of *10XSTAT-Luciferase*, consistent with a role for endocytosis in activation of target genes. It is noteworthy that we observe constitutive internalisation and recycling of Dome in the absence of ligand, as has been observed, in mammalian cells, for other cytokine receptors (Thiel et al., 1998). Regulation of constitutive recycling provides cells with a mechanism to control cell surface levels of receptor, which in turn will impact on the magnitude of signalling (Moore et al., 2018).

2) Page 6, para 1. It is unclear how the data in Fig. 1 and Fig. S1 show requirement for CDE at low conc. of ligand (Upd2) and both CDE and CIE at high Upd2 conc. The data needs to be presented in a different and more coherent way. This is an important point.

We have modified the text (line 149 onwards) to clarify the results and the differential effect of CHC knockdown at low and high ligand concentrations:

It has been shown that Dome is internalised into *Drosophila* Kc167 cells by CME (Vidal et al., 2010). To investigate if this is the case in S2R+ cells, we measured internalisation of Upd2-GFP, as a proxy for receptor internalisation, using an anti-GFP ELISA assay (Wright et al., 2011). We first treated cells with dsRNA targeting Dome and found that there is a significant reduction in the rate (-38%) and extent (-50%) of uptake of Upd2-GFP at both high (20 nM, Figure 1A) and low (3 nM, Fig. S1A) concentrations of Upd2-GFP. Under these conditions levels of Dome mRNA are reduced by ~90% (Figure S1B). The residual uptake of Upd2- GFP in the absence of Dome is likely due to non-specific fluid phase uptake of ligand. When cells were incubated with 20 nM Upd2-GFP, knockdown of CHC and AP2 reduced the uptake of Upd2-GFP by approximately 60% compared to knockdown of Dome alone (Figure 1A). Since levels of CHC and AP2 mRNA were reduced by ~80% following dsRNA knockdown, this suggests that the Upd-2-GFP complex can be internalised by CIE as well as CME, as has been shown for several receptors in mammalian cells (Sigismund et al., 2005; Vander Ark et al., 2018) and for Notch and Delta in *Drosophila* (Shimizu et al., 2014). By contrast, when S2R+ cells were incubated with low concentrations of Upd2-GFP (3 nM), knockdown of CHC reduced the uptake of Upd2-GFP to the level observed following Dome knockdown (Figure S1A). Together this suggests that at low concentrations of Upd2-GFP, Dome is primarily internalised by CME, but that increasing concentrations of ligand results in Dome also being internalised via CIE.

We hope that this now addresses the important point made by the reviewer.

3) Page 6, para 2, Fig. 1C. In examining the data for the effects of di-leucine mutations, there is higher levels of cell surface Domeless (Fig. 1C), but this is statistically non-significant by the error bars presented. However, effects are more clear-cut when endocytosis is measured (Fig. 1F). If the authors want to suggest that the mutations have an effect on endocytosis and cell surface levels of Domeless, they have to vary clearly (and specifically) mention the % changes in cell surface levels and in endocytosis in each situation, and but also say where this was non-significant by the statistical tests used here.

The reviewer has raised an important point which we realise was confusing. Figure 1D showed

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the levels of surface expression of wild-type and mutant Dome in the absence of Upd2-GFP. We included it to show that the proportion of surface Dome was similar in the transfected cells. Together with the comments made by Reviewer 2, point 4, above, we realise that this is unclear and as described in response to Reviewer 2, we now include in FigS1C the data showing the level of expression of Dome wild-type and mutants normalised to the levels of ¤-actin measured in the cell lysates. We have applied a students t-test to the data in both Figure S1C and S1D and find that there is no statistical difference between wild- type and mutant expression or cell surface expression. This is now indicated in the legend in red.

4) Pages 7-8, Fig. 2. Looking at the activation of reporter expression and SOCS mRNA levels as a function of Domeless activation, it is clear that AP2 and Hrs have effects but not anything else. This might suggest that different pools of Domeless have different signalling functions here. As AP2 regulates early endocytic events and delivery to early endosomes, whereas Hrs is part of the ESCRT-0 complex already on early endosomes, this may argue for multiple compartmentalised signalling events.

We completely agree with the reviewer that we are observing compartmentalised signalling events. We apologise if this was unclear in the original manuscript and have now included the term 'compartmentalised signalling' as suggested by the reviewer as the running title and also in the opening paragraph of the Discussion (indicated in red, line 374). There is also a more detailed description of our model for endocytic regulation of JAK/STAT signalling in the Discussion. See also response to point 5.

5) Pages 12-15. Discussion. I find the discussion a bit confusing as the lines of thinking are unclear in fitting into a pathway leading from the cell surface, endosomes to nuclear gene expression, although this is a complex topic. The issues are as follows: multiple endocytic pathways occurring dependent on low and high ligand concentrations, all ending up in endosomes, and likely different pools of inactive and activated receptors and receptor- ligand complexes positioned along the pathway. Could this be helped by having a little schematic added as a small panel (Fig. 6x) to show this? One important previous study, quoted here is by di Fiore and colleagues (Sigismund et al., 2005) where they show that depending on EGF concentration, different endocytic routes come into play and this is important for downstream EGF-EGFR signalling

AND trafficking. I think the authors need to rework the discussion

Following on from the reviewer's suggestion, we have included a model in Figure 6D. We have also substantially rewritten the Discussion to more clearly describe how we envisage Dome signalling to be regulated at different stages of the endocytic pathway. We hope this is now clearer.

Minor points:
1) Use single amino acid designations when discussing specific residues in specific proteins and enzymes e.g. T702, Y704 and mutations/phosphorylation events e.g. T702A, pY704. This is done in figures but not consistently done in the text of the manuscript.

We have ensured that within the text, all of the amino acids are abbreviated to their three letter code, e.g. Tyr, as is usual. In the figures we have annotated them by their single letter abbreviation for greatest clarity.

2) I am not sure the title of the study accurately reflects what the study is about. Needs more thought e.g. 'Integration of receptor-ligand trafficking, signalling and gene expression in Drosophila melanogaster'

We have revised the title of the manuscript in line with the advice of reviewers 2 and 3 and used the suggestion of Reviewer 3. It is now called: Integration of JAK/STAT receptor- ligand trafficking, signalling and gene expression in *Drosophila melanogaster* cells

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Second decision letter

MS ID#: JOCES/2020/246199

MS TITLE: Integration of JAK/STAT receptor-ligand trafficking, signalling and gene expression in Drosophila melanogaster cells

AUTHORS: Rachel Moore, Katja L Vogt, Adelina E Acosta Martin, Patrick Shire, Martin Zeidler, and Elizabeth Mary Smythe
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

Moore et al investigated regulatory mechanisms of JAK/STAT signaling using cultured Drosophila S2 cells in which they can stimulate the pathway by adding the ligand Upd2. They first identified an evolutionarily conserved internalization LL motif in the cytoplasmic tail of the receptor Dome. They then demonstrated that internalization and endocytic trafficking of activated Dome to specific endosomes affect the JAK/STAT transcriptional output, using a reporter gene. They also showed that this regulation is independent of STAT Y704 phosphorylation of STAT. Finally, they used mass spec and mutagenesis to identify Threonine702 as being essential for Y704 phosphorylation of Drosophila STAT, as well as STAT nuclear translocation and transcription activity. The work is thorough and makes a significant and novel contribution to our understanding of the regulation of the JAK/STAT pathway. The revision addressed all my previous concerns. The revised manuscript is suitable for publication in Journal of Cell Science.

Comments for the author

I'm satisfied with the revision. I don't have any further comments.

Reviewer 3

Advance summary and potential significance to field

I am satisfied with the revised version of the manuscript.

Comments for the author

None.

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