Mechanisms of JAK/STAT pathway negative regulation by the short coreceptor Eye Transformer/Latran

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ABSTRACT Transmembrane receptors interact with extracellular ligands to transduce intracellular signaling cascades, modulate target gene expression, and regulate processes such as proliferation, apoptosis, differentiation, and homeostasis. As a consequence, aberrant signaling events often underlie human disease. Whereas the vertebrate JAK/STAT signaling cascade is transduced via multiple receptor combinations, the Drosophila pathway has only one full-length signaling receptor, Domeless (Dome), and a single negatively acting receptor, Eye Transformer/Latran (Et/Lat). Here we investigate the molecular mechanisms underlying Et/Lat activity. We demonstrate that Et/Lat negatively regulates the JAK/STAT pathway activity and can bind to Dome, thus reducing Dome:Dome homodimerization by creating signaling-incompetent Dome:Et/Lat heterodimers. Surprisingly, we find that Et/Lat is able to bind to both JAK and STAT92E but, despite the presence of putative cytokine-binding motifs, does not detectably interact with pathway ligands. We find that Et/Lat is trafficked through the endocytic machinery for lysosomal degradation but at a much slower rate than Dome, a difference that may enhance its ability to sequester Dome into signaling-incompetent complexes. Our data offer new insights into the molecular mechanism and regulation of Et/Lat in Drosophila that may inform our understanding of how short receptors function in other organisms.

INTRODUCTION The development of multicellular organisms requires individual cells to coordinate their behavior. Communication events are frequently initiated by secreted ligands, which bind to transmembrane receptors, triggering intracellular signaling cascades. In many cases, these signaling pathways alter target gene expression and modulate cellular behavior. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway represents one such communication system. The pathway has been evolutionarily conserved and is essential for many developmental processes, including cellular proliferation, innate immune responses, and stem cell maintenance (Arbouzova and Zeidler, 2006; Bausek, 2013; Morin-Poulard et al., 2013; Amoyel et al., 2014). Furthermore, dysregulation of intercellular signaling is frequently associated with human diseases, including immune disorders and cancers (Teng et al., 2014; Thomas et al., 2015; Villarino et al., 2015). Given the importance of appropriate pathway activation, multiple regulatory mechanisms act at many levels to tightly control both the strength and duration of activity (Arbouzova and Zeidler, 2006; Stec et al., 2013).

In vertebrates, the JAK/STAT pathway transduces signaling of multiple transmembrane receptors, which can bind extracellular cytokines, including interleukins (ILs), growth factors, and interferons. Of these, the IL-6 family of vertebrate cytokine receptors, most similar to Drosophila Dome, can be broadly split into two categories: long β-receptors, such as GP130, LIFRβ, and OSMRβ, and shorter α-receptors, such as IL-6Rα, IL-11Rα, and IL-13Rα2 (Rahaman et al., 2002; reviewed in Taga and Kishimoto, 1997; Heinrich et al., 2003).
Binari and Perrimon, 1994), and one STAT (STAT92E; Hou et al., 2010). In the fly, only three ligands—Upd, Upd2, and Upd3 (Harrison et al., 1998; Hombria et al., 2005; Wright et al., 2011)—one signaling receptor (Dome; Brown et al., 2001), one JAK (Hopscotch [Hop]; Binari and Perrimon, 1994), and one STAT (STAT92E; Hou et al., 1996; Yan et al., 1996)—make up the complete core signaling cascade. Regulatory mechanisms mediated by factors such as SOCS36E (Sect et al., 2013) and the tyrosine phosphatase PTP61F (Müller et al., 2005) have also been conserved.

Originally identified on the basis of cytokine-binding motifs similar to those present in Dome (Hombria and Brown, 2002), the short receptor Eye Transformer (Et/Latran [Lat]) is located adjacent to the long Dome receptor in the genome and is likely to be the product of an evolutionarily recent duplication event. With a primary structure similar to that of vertebrate α-receptors (Hombria and Brown, 2002), Et/Lat was also identified in two studies that named the protein Eye Transformer (Kallio et al., 2010) and Latran (Makki et al., 2010), respectively, subsequently referred to in this study as Et/Lat. One study focused on the in vivo mutant phenotype of Et/Lat, which demonstrated a requirement in innate immune responses. On parasitic wasp infestation, Et/Lat is up-regulated, whereas Dome expression is down-regulated. This increase in the Et/Lat/Dome ratio reduces pathway activity and thus allows hemocyte precursors within the lymph gland to differentiate into lamellocytes, which in turn attempt to smother the wasp egg (Makki et al., 1996; May et al., 1996). Once bound to the receptor complex, the STATs are tyrosine phosphorylated before translocating into the nucleus, where they bind DNA and mediate gene expression.

The Drosophila JAK/STAT cascade represents a highly conserved yet lower-complexity system in which to study JAK/STAT signaling. In this system, the three STATs—STAT92E (Müller et al., 2005), one JAK (Hopscotch [Hop]; Binari and Perrimon, 1994), and one STAT (STAT92E; Hombria et al., 2005)—one signaling receptor (Dome; Brown et al., 2001), one JAK (Hopscotch [Hop]; Binari and Perrimon, 1994), and one STAT (STAT92E; Hou et al., 1996; Yan et al., 1996)—make up the complete core signaling cascade. Regulatory mechanisms mediated by factors such as SOCS36E (Sect et al., 2013) and the tyrosine phosphatase PTP61F (Müller et al., 2005) have also been conserved.

RESULTS AND DISCUSSION

Et/Lat disrupts formation of Dome:Dome dimers

Although it was previously shown that Et/Lat negatively regulates JAK/STAT signaling and binds to the Dome receptor complex, the molecular mechanism underlying this activity is not understood. We first set out to confirm that Et/Lat is indeed a negative regulator of JAK/STAT signaling, using an established STAT92E-responsive luciferase reporter in Drosophila Kc167 cells (Müller et al., 2005). Although increasing concentrations of cotransfected pathway ligand resulted in higher STAT92E activity, coexpression of additional Dome had no effect on signaling. However, expression of Et/Lat-HA in these cells dramatically reduced signaling output in a dose-dependent manner (Figure 1A). Furthermore, expression of additional Dome was sufficient to at least partially relieve the Et/Lat-induced suppression (Figure 1A), findings that are similar to those of Makki et al. (2010). Consistent with a previous report (Kallio et al., 2010), we found that Et/Lat physically associates with Dome, as shown by coimmunoprecipitation of tagged receptors expressed in cells (Figure 1B). Furthermore, we also found that Et/Lat can itself form homodimers (Figure 1B), although the relative affinity of these associations and their potential functional relevance is not clear.

To better understand the nature of receptor interactions, we tested the effect of Et/Lat on Dome:Dome homodimers. Using coimmunoprecipitation as an assay, we found that increasing levels of Et/Lat can reduce the Dome:Dome interaction (Figure 1C). To confirm the immunoprecipitation results, we also developed a cell-based molecular complementation assay, termed β-luciferase, previously used to report Dome dimers (Brown et al., 2003). In this assay, the dome-coding region is fused to two LacZ mutants termed Δα and Δω. These mutants each produce truncated, enzymatically inactive β-galactosidase (β-gal) proteins (Supplemental Figure S1). However, when brought into close enough proximity (e.g., via interaction of the proteins to which they are fused), they are able to complement one another and thus reconstitute a functional β-gal enzyme (Rossi et al., 1997). We first expressed Dome-Δα and Dome-Δω in Kc167 cells before retransferring them with increasing concentrations of Et/Lat-hemagglutinin (HA). Visualization of β-gal activity showed that higher levels of Et/Lat were able to reduce β-gal activity and complementation (Figure 1D). This suggests that either Dome:Dome dimer formation is reduced or dimer breakdown is enhanced by Et/Lat, with the approaches used here not able to differentiate between these mechanisms. To quantify this more accurately, we went on to use a substrate that is cleaved by β-gal to release luciferin. This luciferin acts as a substrate in a firefly luciferase reaction, giving a quantifiable measurement of β-gal activity (Hannah et al., 2003). Despite higher levels of background associated with this assay, we found that the signal from Dome-Δα:Dome-Δω dimers was significantly reduced at the highest doses of both Et/Lat-HA and Dome-HA, with the latter as a positive control (Figure 1E).

Consistent with the reduction in Dome:Dome dimers observed, high doses of Et/Lat-HA were also sufficient to reduce significantly the expression of the STAT92E transcriptional target socs36E (Bina et al., 2010; Figure 1F), whereas high Dome-HA levels were not. This indicates that whereas Et/Lat and Dome can both interact with a signaling-competent receptor complex, only Et/Lat has the ability to block downstream signaling.

Et/Lat can bind to intracellular JAK/STAT signaling components

To determine the mechanism underlying how Et/Lat negatively regulates JAK/STAT signaling, we set out to investigate whether Et/Lat could physically associate with other components of the JAK/STAT pathway. Examination of the protein sequence alignments between intracellular Dome and Et/Lat revealed the presence of a conserved potential Hop-binding site located at the same position relative to the transmembrane domain within both molecules (HBS in Figure 2A). This LP motif is similar to the 266LKS sequence in interferon-γ receptor 1 previously shown to be required for binding to JAK1 (where P is the critical residue required for binding; Kaplan et al., 1996). To test whether this site does indeed confer Hop binding, we mutated the proline at amino acid 925 (P925I). As expected, Dome

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P925I showed very little binding to Hop even when coexpressed at high levels (Figure 2B). Furthermore, whereas tyrosine phosphorylation of wild-type Dome was increased after stimulation with Upd2-conditioned medium, the basal phosphorylation level of Dome P925I was lower and remained unchanged upon stimulation (Figure 2B). We went on to test the signaling activity of Dome P925I using a STAT92E-responsive reporter assay. We found that increasing the level of Dome did not affect the reporter activity compared with empty vector–transfected cells (Figure 2C). However, expression of Dome P925I substantially reduced pathway activity, suggesting that Dome P925I is acting as a dominant-negative receptor, possibly via the formation of heterodimers with endogenous Dome (Figure 2C). Taken together, these data suggest that the LP motif within Dome is a Hop-binding site and also suggest that two Hop molecules are required to form a signaling receptor complex. Given that the sequence and the relative position of this site are conserved between Dome and Et/Lat (Figure 2A), we suggest that this site could also act as a binding site for Hop in Et/Lat. To test this

**FIGURE 1:** Et/Lat can disrupt formation of Dome homodimers. (A) STAT92E transcriptional reporter activity assay. Pathway activity increases after the cotransfection of Upd ligand. Cotransfection of increasing quantities of Et/Lat blocks signaling capability in a dose-dependent manner. Expression of additional Dome does not significantly alter signaling (column 6) in the absence of Et/Lat but is sufficient to significantly increase signaling inhibited by Et/Lat coexpression (compare column 3 to column 7 and column 5 to column 9). Numbers indicate relative levels of Dome- and Et/Lat-expressing plasmids transfected. (B) Coimmunoprecipitation assays using FLAG-tagged Dome (D) or Et/Lat (L) to pull down HA-tagged Dome or Et/Lat indicate that both Dome and Et/Lat can form both homodimers and heterodimers. Constructs transfected are indicated. α-Tubulin was used as a loading control. (C) Coimmunoprecipitation assays using Dome-FLAG to pull down Dome-HA from cells. Quantification of this blot is shown as percentage of control levels. Increasing the level of Et/Lat cotransfected into cells reduces the amount of Dome-HA that can be coimmunoprecipitated. EV, empty vector. (D) Cells transfected with the Dome-Δα and Dome-Δω dimerization assay and stained with X-gal (blue) to visualize bimolecular complementation. Owing to transient transfection, not every cell expresses both constructs. Increasing amounts of Et/Lat retransfected into previously transfected cells progressively block Dome homodimerization. (E) Dome homodimerization as described in D but quantified using β-Glo reagent to measure β-galactosidase activity levels. (F) Quantitative PCR of the JAK/STAT pathway target gene socs36E. mRNA levels are reduced after increased expression of Et/Lat but not after increased Dome. The analysis used unpaired t tests with Welch’s correction. *p < 0.05, **p < 0.01, and ***p < 0.001. ns, not significant.
**FIGURE 2:** Et/Lat can bind to intracellular JAK/STAT components. (A) Visualization of Dome and Et/Lat protein domain structure. Clustal sequence alignment for intracellular portion of Dome and Et/Lat. Transmembrane domain (TM; black bar), putative binding sites for Hop (HBS), and STAT92E (SBS) are highlighted. Tyrosine residues in Et/Lat are shown with bold letters. SP, signal peptide; CBM, cytokine-binding motif; FNIII, fibronectin 3 domains; TM, transmembrane domain; HBS, Hop-binding site; SBS, STAT binding site; aa, amino acids). Percentage identity between the indicated domains. (B) Dome-FLAG can coimmunoprecipitate Hop-HA, but this is greatly reduced when the putative Hop-binding site is mutated (Dome P925I). Tyrosine phosphorylation (pTyr) of Dome is also reduced in the Dome-P925I. (C) STAT92E transcriptional reporter activity assay. JAK/STAT pathway signaling is greatly reduced in cells expressing Dome P925I even in the presence of Upd2 ligand, \( **p < 0.01 \). (D) Immunoprecipitation assays show that both Dome-FLAG and Et/Lat-FLAG can coprecipitate Hop-HA. (E) Immunoprecipitation assays after transfection of the indicated Dome-FLAG and Et/Lat-FLAG proteins show that both proteins can coprecipitate endogenous STAT92E. dsRNA targeting the labeled mRNAs was added as indicated; ctrl, a nontargeting dsRNA. Knockdown of endogenous Dome (final lane) did not disrupt the ability of Et/Lat to bind to STAT92E. Upd2-conditioned medium was added for 15 min before protein harvesting. EV, empty vector. (F) Immunoprecipitation of endogenous STAT92E from cells expressing FLAG-tagged Dome or Et/Lat shows that both receptors are coprecipitated. Treatment with dsRNAs targeting the endogenous receptors suggests that interactions detected are independent of potential heterodimer formation.
possibility, we also tested for physical interactions between Hop and Et/Lat. As predicted, immunoprecipitation of both Dome and Et/Lat is able to coprecipitate Hop (Figure 2D).

Previous studies of mammalian receptors identified STAT-binding sites within the intracellular domain of multiple cytokine receptors. STAT5 was shown to bind to a YXXL motif in EPO-R (Klingmüller et al., 1996), and STAT3 can associate with GP130 via a YXXQ motif (Stahl et al., 1995). Consistent with these vertebrate receptors, Dome also contains a YXXQ motif, which may represent a STAT92E-binding site (SBS in Figure 2A; Chen et al., 2002). Neither STAT consensus binding motif is present within the Et/Lat intracellular domain; however, five tyrosine residues (highlighted in Figure 2A), including two YXXN sites (solid gray boxes in Figure 2A), are present. Somewhat surprisingly, we find that Et/Lat is able to bind to endogenously expressed STAT92E, an interaction that we detect in reciprocal precipitation experiments of both receptor and STAT92E (Figure 2, E and F). Furthermore, we find that the interaction is not destabilized upon Dome RNAi treatment and is not modulated by addition of ligand (Figure 2, E and F), suggesting that STAT92E is binding directly to Et/Lat in this system. Given the similarity of the glutamine residue within the Dome YXXQ STAT-binding motif and the amide side chain of asparagine, it is tempting to suggest that the two YXXN motifs present in the cytosolic tail of Et/Lat may represent the STAT92E binding sites in this molecule.

**Et/Lat is unable to bind Upd ligands**

Because Et/Lat is able to bind the key intracellular signaling pathway components Hop and STAT92E, it is possible that an inability to bind to extracellular ligands may explain its negative pathway activity. Despite clear homology between the cytokine-binding motifs of both Dome and Et/Lat (Makki et al., 2010), we investigated whether this molecule was in fact able to bind to any of the Upd family of ligands (Upd, Upd2, Upd3; Harrison et al., 1998; Hombria et al., 2005; Wright et al., 2011). First, we cotransfected cells with green fluorescent protein (GFP)-tagged versions of the Upd, Upd2, and Upd3 cytokines previously shown to be active (Wright et al., 2011), along with HA-tagged Dome or Et/Lat. We find that Et/Lat is unable to bind to any of the pathway ligands at detectable levels under conditions in which interactions with Dome are clearly detected (Figure 3A). We next sought to confirm that the interactions between Dome and pathway ligands were not arising from their coexpression and therefore repeated this experiment using Upd2-conditioned medium. We found that Dome coprecipitated with GFP-tagged ligand present in the conditioned medium, suggesting a stable interaction at the plasma membrane. However, under the same conditions, no interaction was detected between Upd2-GFP and Et/Lat. In addition, RNAi targeting the untranslated region (UTR) of endogenous dome mRNA had no effect on this result, indicating that endogenous Dome is not affecting receptor/ligand interactions in this assay and suggesting that Dome:Et/Lat heterodimers are also incapable of binding ligand. This failure to interact with Upd2-GFP suggests that the shorter receptor is either unable to bind ligand or that any interaction that does occur is weaker than the Dome:ligand interaction and is disrupted under the experimental conditions used (Figure 3B).

Although independently identified as a negative regulator of JAK/STAT signaling in a number of assays, the mechanism underlying how Et/Lat expression reduces pathway activity has been unclear. It was previously shown that Dome homodimerization is required to form a functional signaling complex, whereas monomeric Dome is not active (Brown et al., 2003). By inference, a single molecule of Dome is either not capable of binding to ligand or unable to transduce signaling in response to binding. Here we demonstrate that Dome can effectively form heterodimers with Et/Lat and that the cytosolic domains of both receptor molecules are capable of binding to Hop and STAT92E. Furthermore, we demonstrate that whereas Dome homodimers are able to bind to each of the three pathway ligands, no such binding could be detected between Et/Lat and Upd, Upd2, or Upd3 (Figure 3).

Our data suggest two potential mechanisms via which Et/Lat negatively regulates downstream pathway activity. First, Et/Lat could act as a negative regulator by forming heterodimers with Dome. This generates receptor complexes containing only one molecule capable of binding to extracellular ligands, an arrangement likely to be incapable of transducing pathway activation to intracellular components. The second potential mechanism is that Et/Lat homodimers may simply bind to positively acting components of the JAK/STAT pathway such as Hop and STAT92E, sequestering these into signaling incompetent complexes. It is most likely that both mechanisms contribute toward negative regulation of the pathway at some level. However, the ability of additional Dome to partly relieve Et/Lat-induced pathway suppression (Figure 1A) argues against a “squelching” mechanism and instead suggests that Et/Lat is likely to negatively regulate JAK/STAT signaling via formation of inactive Dome:Et/Lat heterodimers.
Et/Lat regulates JAK/STAT signaling

Et/Lat has increased protein stability compared to Dome

We and others previously demonstrated that Dome is rapidly internalized and targeted for lysosomal degradation even in the absence of ligand stimulation, a trafficking process that results in attenuation of signaling (Vidal et al., 2010; Stec et al., 2013; Ren et al., 2015). We therefore investigated whether the turnover dynamics of Et/Lat was similar to that of Dome, using a time-course experiment that involved ligand stimulation in the presence of cyclohexamide (CHX) to block new protein translation. Consistent with previous findings (Stec et al., 2013), we observed a rapid decrease in Dome protein levels (Figure 4, A and A’). By contrast, Et/Lat appeared to be much more stable, showing only a modest decrease in protein levels even after 10 h of stimulation (Figure 4, A and A’). To determine whether this difference was the result of alternative degradation mechanisms, we examined the effect of the proteosomal inhibitor MG132 and the lysosomal inhibitor bafilomycin A1 on the stability of both receptors. As is also the case for Dome, Et/Lat degradation occurs within the lysosome, as shown by the increased stability of the receptor after treatment with bafilomycin A1 (Figure 4, B and B’; Stec et al., 2013).

We next examined whether the greater stability of Et/Lat might be a consequence of its recycling back to the plasma membrane after endocytic uptake. We therefore knocked down Rab4 and Rab11, two molecules key to the recycling of previously endocytosed proteins (Jones et al., 2006), and measured the degradation rate of both receptors over time (Figure 4C). Even after quantification and normalization for loading differences, we found no change in the stability of either receptor compared with controls (Figure 4, C and C’), suggesting that endocytic recycling does not play a significant role in the differing stability of the Dome and Et/Lat receptors.

Finally, we returned to our β-laulocomplementation assay to examine the steady-state circumstances under which Dome and Et/Lat homodimers and Dome:Et/Lat heterodimers accumulate in cells. We found that knockdown of shibire and Rab5, which encode components required for the formation of the early endosome, resulted in increased levels of each receptor dimer combination (Figure 4D). By contrast, knockdown of rab7 or hrs, two molecules involved in the formation of the late endosome and multivesicular bodies, respectively, or rab4 and rab11, required for recycling, have no effect on the steady-state levels of receptor complexes (Figure 4D). In the absence of antibodies, we are unable to confirm complete loss of these proteins; however, if taken at face value, this result suggests that blocking the formation of early endosomes is sufficient to cause an increase in the levels of receptor dimers at the plasma membrane. By contrast, blocking downstream aspects of endocytic trafficking has no effect.
on the levels of receptor dimers, suggesting that receptors are no longer dimerized in endosomal compartments.

We suggest that Et/Lat inhibits JAK/STAT signaling via the formation of signaling-competent Dome:Et/Lat heterodimers. If Et/Lat were to strongly down-regulate pathway signaling via competitive binding with Dome, we would require higher levels of Et/Lat in relation to Dome. Evidence from previous in vivo studies, as well as from our analysis of Et/Lat and Dome stability, suggests that this is indeed the case. In vivo, Et/Lat acts within the lymph gland to down-regulate pathway signaling after infection by the parasitic wasp Leptopilina boulardi. Strikingly, infection not only triggers the up-regulation of Et/Lat expression, but also simultaneously down-regulates dome mRNA levels, a combination that is likely to change the relative abundance of receptors present in vivo (Makki et al., 2010). In addition to changes in relative gene expression levels, we demonstrated that Et/Lat has a significantly longer protein half-life than Dome. As a result, expression of long-lived Et/Lat protein could produce strong and lasting effects with relatively small changes in transcription.

In vertebrates, multiple ligands, including IL-6 and IL-11, first bind to their respective α-receptors, forming ligand:receptor complexes, which then associate with long β-receptors to form functional signaling complexes (Heinrich et al., 2003). In this scenario, expression of α-receptors sensitizes cells to ligand-induced signaling and thus positively regulates downstream signaling. By contrast, a handful of short receptors, including IL-13Rα2, soluble forms of oncostatin M receptor (OSMR), and short forms of the prolactin receptor, act negatively (Perrot-Applanat et al., 1997; Diveu et al., 2006; Chandriani et al., 2014). In the case of IL-13Rα2 and soluble OSMR, short receptors do not interact with JAKs or STATs, but instead act as decoys that compete for ligand binding (Diveu et al., 2006; Chandriani et al., 2014). By contrast, the short, 291-amino acid form of the prolactin receptor is identical to the full-length form and can bind to JAK1 but is missing cytoplasmic domains required for STAT binding (Lebrun et al., 1995; Perrot-Applanat et al., 1997). This short receptor cannot activate downstream signaling and acts in a dominant-negative manner to reduce prolactin-mediated JAK/STAT pathway activation (Perrot-Applanat et al., 1997; Devi et al., 2009). Similarly to Dome and Et/Lat, prolactin receptors also predominate in the absence of ligand to form both long:long homodimers and long:short heterodimers (Qazi et al., 2006; Tan and Walker, 2010). Whereas homodimers signal through JAK2 and STAT5 after ligand stimulation, heterodimers are able to bind ligand but do not activate downstream JAK/STAT signaling (reviewed in Devi and Halperin, 2014).

Although they share some mechanistic similarities with the short form of the human prolactin receptor, we show that Et/Lat is unable to associate with extracellular ligands but is able to bind to both the JAK Hop and STAT92E, as well as dimerize with the long receptor Dome. In both examples, the inability to bind to one key component of the pathway results in a strong dominant-negative effect and the down-regulation of pathway signaling. As such, it is possible that insights into molecular mechanisms of short receptor antagonists in Drosophila may provide clues to similar mechanisms in vertebrate systems.

**MATERIALS AND METHODS**

**Cell culture**

Drosophila Kc167 cell lines were cultured, and conditioned medium was produced as previously described (Vidal et al., 2010). Cell transfactions were performed using Effectene (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Pathway activity was measured using the 6x2xDrAFLuc reporter assay, as previously described (Müller et al., 2005; Fisher et al., 2012), and is presented as the ratio of Firefly (reporter) to Renilla (constitutively expressed cell density control) luciferase. RNAi experiments, pharmacological treatments, and socs3Δε quantitative PCR were carried out as previously described (Stec et al., 2013). Templates for double-strand RNA (dsRNA) generation were provided by the Sheffield RNAi Screening Facility, using designs previously described (Horn et al., 2010; Fisher et al., 2012).

**β3- and β-Glo assays**

Kc167 cells were batch-transfected with pAc-Dome-LacZ-Δα, pAc-Dome-LacZ-Δω, and pAc-RLuc and incubated for 24 h. Cells were either split into 12-well plates for retransfection with Et/Lat-HA or control plasmid or changed into serum-free medium and split into 384-well white plates with 250 ng of dsRNA. In RNAi experiments, serum-containing medium was added after 1 h. After 5 d, cells were assayed for β-gal activity using β-Glo Assay System (Promega), followed by measurement of Renilla luciferase activity. For whole-cell assays, cells were fixed in 2% gluteraldehyde/phosphate-buffered saline before development using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), using standard techniques (Brown et al., 2003).

**Constructs**

The previously described plasmids were pAc-Dome-FLAG, pAc-Dome-HA, and pAc-Hop-HA (Stec et al., 2013), and pAc-Upd-GFP, pAc-Upd2-GFP, and pAc-Upd3-GFP (Wright et al., 2011). A full-length sequenced Et/Lat cDNA clone (MIP15047; a kind gift of the Susan Celniker lab [Berkeley, CA] and the Berkeley Drosophila Genome Project [BDGP]) was used as template for amplification. The PCR-amplified fragment was inserted into a Gateway System Entry vector using a pENTR Directional TOPO Cloning Kit (Invitrogen, Carlsbad, CA) and subsequently cloned into destination vector pAWH (Drosophila Gateway Vector Collection) using Gateway LR Clonase II Enzyme Mix (Invitrogen), according to manufacturer’s instructions. Gateway destination vectors were obtained from the Drosophila Genomics Resource Center (Bloomington, IN). Single point mutation in Dome was introduced with a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using the following primers: Dome-P925I-F, GATATCGGTC-CAGCACTAGACCGATATC. The PCR-amplified fragment was inserted into a Gateway System activation vector (Perrot-Applanat et al., 1997; Devi et al., 2009). Similarly to Dome and Et/Lat, prolactin receptors also predominate in the absence of ligand to form both long:long homodimers and long:short heterodimers (Qazi et al., 2006; Tan and Walker, 2010). Whereas homodimers signal through JAK2 and STAT5 after ligand stimulation, heterodimers are able to bind ligand but do not activate downstream JAK/STAT signaling (reviewed in Devi and Halperin, 2014).

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**Immunoprecipitation, Western blotting, and antibodies**

Immunoprecipitation and Western blotting were carried out as described previously (Stec et al., 2013). The 3% of the input used is shown in each experiment. Tubulin was used as a loading control on all westerns. Kilodalton markers are shown for each Western panel shown in each experiment. Tubulin was used as a loading control on all westerns. Kilodalton markers are shown for each Western panel.
Protein alignment
Alignment of amino acid sequences was carried out using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). Transmembrane and intracellular portions were aligned for Dome (amino acids 888–1112) and Et/Lat (amino acids 517–645).

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