

**Molecular Analysis of the *Drosophila* JAK/STAT  
Pathway Receptor Complex**

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## Abstract

The JAK/STAT signalling pathway plays a central role in numerous biological processes contributing to development and maintenance of homeostasis. *Drosophila melanogaster* offers a conserved JAK/STAT pathway with much lower redundancy. For this reason, the fruit fly was used as a model organism to investigate genetic interactions and functions of the JAK/STAT pathway in the context of the whole organism. However, very little is known regarding the molecular mechanisms governing the *Drosophila* JAK/STAT pathway.

Here, we present a molecular analysis of the sole receptor of the JAK/STAT pathway in *Drosophila*, Dome. We show that Dome shares characteristics with different sub-families of mammalian cytokine receptors. Specifically, the identified JAK binding site in Dome is reminiscent of that found in IFN $\gamma$  receptor, while constitutive endocytosis leading to lysosomal degradation shares similarities with the Leptin receptor. An increase in tyrosine phosphorylation and a shift in the ubiquitination pattern of the receptor in response to ligand binding are also described.

Furthermore, the structure-function analysis of *socs36E*, the only SOCS-like protein in the *Drosophila* genome that can potently suppress the JAK/STAT pathway, revealed two independent mechanisms of action. Firstly, SOCS36E affects stability of the receptor, most likely by forming ubiquitin ligase via the SOCS box domain, a mechanism well described for all mammalian SOCS proteins. Secondly, regulation of Dome phosphorylation by the N-terminal domain of SOCS36E contributes to suppression of the JAK/STAT pathway in a SOCS box independent manner.

Finally, two alleles of the *Drosophila* JAK that give rise to a phenotype reminiscent of human leukaemia, *hop*<sup>*Tuml*</sup> and *hop*<sup>*T42*</sup>, are shown to increase transcriptional activity of the pathway reporter without increasing phosphorylation of STAT. Both mutations cause constitutive activation of the kinase independently of the receptor. Moreover, autophosphorylation kinetics of both mutants are unaltered, compared to the wild-type Hop, suggesting non-canonical signalling to be the underlying cause of oncogenicity.

## **Declaration**

I declare that this thesis is the result of my own work except where the work of others is cited, either explicitly or via the list of references.

No part of this thesis has been submitted for a degree, diploma or other qualification at any other university.

Wojciech J. Stec

August 2013

## **Publications**

Oscar Marino Vidal, Wojciech Stec, Nina Bausek, Elizabeth Smythe and Martin P. Zeidler (2010) Negative regulation of *Drosophila* JAK-STAT signalling by endocytic trafficking. *Journal of Cell Science*, **123**: 3457-3466

Wojciech J. Stec and Martin P. Zeidler (2011) *Drosophila* SOCS Proteins. *Journal of Signal Transduction*, **2011**

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## Abbreviations

ALL	Acute Lymphoblastic Leukaemia
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CBM	Cytokine Binding Module
cDNA	complementary DNA
CIS	Cytokine Inducible SH2 protein
CME	Clathrin Mediated Endocytosis
CML	Chronic Myeloid Leukaemia
DNA	Deoxyribonucleic Acid
Dome	Domeless
dsRNA	double stranded RNA
DTT	Dithiothreitol
ECM	Extracellular Matrix
ECS	Eloning-Cullin-SOCS complex
EDTA	Ethylenediaminetetraacetic Acid
EGF(R)	Epidermal Growth Factor (Receptor)
Epo(R)	Erythropoietin (Receptor)
ER	Endoplasmic Reticulum
ESCRT	Endocytic Sorting Required for Transport
ET	Essential Thrombocythemia
FBS	Foetal Bovine Serum
FERM	four-point-one, ezrin, radixin, moesin
FL	Firefly Luciferase
FNIII	Fibronectin type II
G-CSF(R)	Granulocyte Colony Stimulating Factor (Receptor)
GFP	Green Fluorescent Protein
GH(R)	Growth Hormone (Receptor)
HCl	Hydrochloric Acid
Hop	Hopscotch
HRP	Horseradish Peroxidase
IFN	Interferon
IL	Interleukin
JAK	Janus Kinase
JH	JAK homology
LIF(R)	Leukaemia Inihbitory Factor (Receptor)
mRNA	messenger RNA
MVB	Multivesicular Body
NaCl	Sodium Chloride
NEM	N-Ethylmaleimide
N-ESS	N-extended SH2 Sequence
NS	Not Significant
Ob-R	Obesity Receptor or Leptin Receptor

OSM(R)	Oncostatin M (Receptor)
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline TritonX-100
PCR	Polymerase Chain Reaction
PDGF(R)	Platelet Derived Growth Factor (Receptor)
PIAS	Protein Inhibitors of Activated STATs
PMF	Primary Myelofibrosis
PRL(R)	Prolactin (Receptor)
PTP	Protein Tyrosine Phosphatase
pTyr/pY	phosphotyrosine
PV	Polycythaemia Vera
qRT-PCR	quantitative Real-Time PCR
RL	Renilla Luciferase
RLU	Relative Light Units
RNA	Ribonucleic Acid
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SH2	Src-Homology 2
SHP	SH2 domain-containing tyrosinePhosphatase
SOCS	Suppressor of Cytokine Signalling
STAT	Signal Transducers and Activators of Transcription
TAE	Tris Acetate EDTA
TE	Tris EDTA
TGFβ	Transforming Growth Factor Beta
TNF	Tumour Necrosis Factor
Tpo(R)	Thrombopoietin (Receptor)
UPD	Unpaired

# Chapter I Introduction

## I.1. Signalling pathways

Development and maintenance of homeostasis of multicellular organisms requires precise cell-to-cell communication, a process which orchestrates numerous cellular processes in the context of a tissue or a whole organism. Signalling pathways provide means of communication that allow for a temporal and spatial regulation at the level of a single cell. Numerous signal transduction pathways have been characterised, although the exact number depends on the categorization criteria. However, the high number of signalling pathways seems numerically insignificant when considered in the context of the complexity of development and the subsequent maintenance of multicellular organisms. This means that individual signalling pathways must contribute towards numerous processes by cross-modulation and complementation. Not surprisingly, mutations arising in the components of the signalling pathways during development can have a profound impact on the whole organism. While many examples have been described, one of the common diseases contracted due to mutations in the signalling pathways is cancer.

## I.2. The JAK/STAT signalling pathway

### I.2.1 Function

The JAK/STAT pathway was first identified as mediating response to Interferon following Influenza virus infection (LINDENMANN *et al.*, 1957). Numerous studies focusing on the JAK/STAT pathway showed its importance in inflammation and immune response. Indeed that JAK/STAT pathway mediates cytokine and growth factor signalling, which play central role in development and functioning of the haematopoietic and immune systems (Levy and Darnell, 2002). Other processes regulated by the JAK/STAT pathway involve reproduction, embryonic development, sexually dimorphic

growth, wound healing, mammary gland development and lactation as well as adipogenesis, evidence for which comes from numerous studies including murine knockout models (Table.I.1) (reviewed in, O'Shea *et al.*, 2002).

Table.I.1 Murine knockout models

Gene	Phenotype
<i>jak1</i>	Prenatally lethal, small
<i>jak2</i>	Embryonic lethal, no erythropoiesis
<i>jak3</i>	SCID
<i>tyk2</i>	Immunodeficient
<i>stat1 and stat2</i>	Immunodeficient
<i>stat3</i>	Early fetal death
<i>stat4</i>	Unresponsive to IL-12, immunodeficient
<i>stat5a</i>	Lactation defective, impaired PRL signalling
<i>stat5b</i>	Loss of dimorphic growth, defective GH signalling
<i>stat5a/stat5b</i>	Female infertility, immunodeficient
<i>stat6</i>	Immunodeficient

Adapted from Igaz *et al.*, 2001.

### ***1.2.2 General structure of the pathway***

The canonical JAK/STAT signalling pathway comprises a linear cascade responsible for the transduction of extracellular signals into transcriptional responses (Fig.I.1). Binding of a diffusible ligand to the extracellular portion of the trans-membrane receptor is considered to be the activating step. Receptors for the JAK/STAT pathway exist as preformed dimers that are stabilized and undergo conformational change following ligand binding. Consequently, receptor pre-associated Janus kinases (JAKs) come into close proximity with each other allowing for trans-phosphorylation and activation. JAKs phosphorylate the receptors on tyrosines, creating docking sites for Signal Transducers and Activators of Transcription (STATs). During transient interactions with the receptor, STATs become tyrosine phosphorylated, dissociate from the receptor complex and dimerize in the cytoplasm. Following nuclear translocation STAT dimers

bind to the palindromic consensus DNA motifs found in the promoters of pathway target genes and induce their transcription.

*Figure.I.1 Conceptual JAK/STAT pathway*

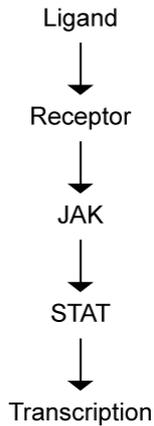


Figure.I.1 Conceptual JAK/STAT pathway. Schematic representation of linearity of simplified core JAK/STAT pathway.

### ***1.2.3 Ligands***

The common generalization that cytokines activate the JAK/STAT pathway is not entirely accurate as the superfamily of cytokines comprises hundreds small soluble molecules capable of stimulating the immune system. Not all of them act through the JAK/STAT pathway, with prime examples being TGF- $\beta$  and TNFs, the structures of which are mainly characterized by  $\beta$ -sheets. Only a subset of cytokines, described as helical cytokines, due to the dominance of  $\alpha$ -helices in their structure, have the ability to activate the JAK/STAT pathway (Grotzinger *et al.*, 1999). This group includes all interferons (IFN) and interleukins (IL), with exception of IL-1, IL-17 and IL-8 families, and haematopoietins. Additionally, the JAK/STAT pathway mediates signalling of a subset of growth factors and hormones that share structural similarities with helical cytokines, including prolactin, leptin, granulocyte colony-stimulating factor (G-CSF) and growth hormone (GH) (reviewed in Mohr *et al.*, 2012). A list containing selected ligands of the JAK/STAT pathway and associated receptors is presented in Table.I.2.

Table.I.2 Interaction partners

Ligand	Receptor	JAK	STAT	SOCS
IL-6	IL-6R, gp130	JAK1, JAK2, Tyk2	STAT1, STAT3	SOCS3 SOCS1
OSM	OSMR, gp130	JAK1, JAK2, Tyk2	STAT3, STAT1	SOCS3
LIF	LIFR, Gp130	JAK1, JAK2, Tyk2	STAT3, STAT1	SOCS3
G-CSF	G-CSFR	JAK2, JAK3	STAT3	SOCS3
Leptin	Ob-R	JAK1	STAT3	SOCS3, CIS, SOCS2
IFN $\alpha$	IFNAR1 IFNAR2	JAK1, Tyk2	STAT1, STAT2 STAT5a STAT5b	SOCS1
IFN $\gamma$	IFNGR1 IFNGR2	JAK1, JAK2	STAT1	SOCS1
Epo	EpoR	JAK2	STAT5a, STAT5b	SOCS3, SOCS2, CIS
Tpo	TpoR	JAK2, Tyk2	STAT5a, STAT5b	SOCS1, SOCS3
PRL	PRLR	JAK2	STAT5a, STAT5b	SOCS2, CIS
GH	GHR	JAK2	STAT3, STAT5a, STAT5b	SOCS2

Adapted from Linossi *et al.*, 2013; Baker *et al.*, 2007; Schindler and Plumee, 2008; Crocker *et al.*, 2008.

### ***1.2.4 Receptors of the JAK/STAT pathway***

Receptors activating the JAK/STAT pathway include not only selected cytokine receptors, but also receptors of growth factors and hormones reported to activate the JAK/STAT pathway described previously (listed in Table.I.2). However, the literature does not make such distinction, therefore the term ‘cytokine receptor’ will be used here to describe general population of over thirty JAK/STAT receptors described to date. As the majority of JAK/STAT pathway ligands share  $\alpha$ -helical structure, their receptors predominantly contain  $\beta$ -sheets at the sites of ligand interaction, often referred to as cytokine binding module (CBM) (Fig.I.2). The CBM subdivides the cytokine receptor family into the type I subfamily characterised by the presence of four conserved disulfide-linked cysteins in the N-terminal portion of CBM and at least a semi-conserved WSxWS motif in the C-terminal portion, while receptors of the type II subfamily lack the WSxWS motif entirely (x represents any amino acid) (Bazan, 1990a, 1990b; Thoreau *et al.*, 1991). The type I cytokine receptors represent the large majority of the receptor superfamily with the exception of the interferon receptors. In addition, the immunoglobulin-like domain present in several type I cytokine receptors has been reported to perform many functions, including stabilization of protein trafficking in the case of IL-6R and contributes towards ligand:receptor complex assembly as reported for PDGF:PDGFR interactions (Miyazawa *et al.*, 1998; Vollmer *et al.*, 1999).

*Figure.I.2 Structure of cytokine receptor*

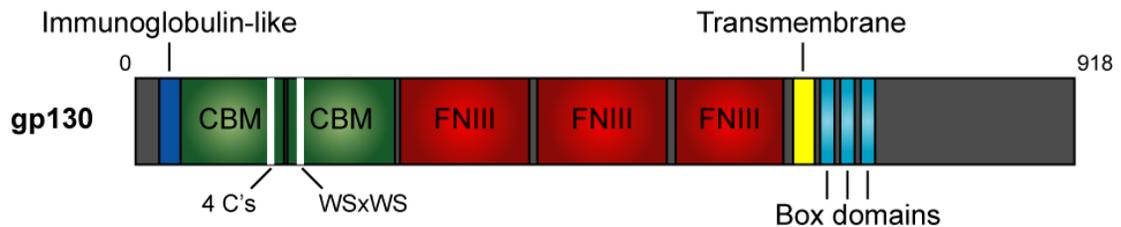


Figure.I.2 Structure of cytokine receptor. Structure of a prototype cytokine receptor gp130. CBM – cytokine binding motif, FNIII – fibronectin type III domain; 4C's – four conserved cysteins. Numbers at extremities represent protein size.

Adapted from Brown *et al.*, 2001.

More membrane proximal are fibronectin type-III domains (FNIII), characterised as mediators of interactions with heparins as well as other constituents of the cell surface environment. The exact number of FNIII domains varies between different receptors, ranging between two and four. Moreover, position-specific residues in the FNIII domains have been implicated in receptor dimerization as reported for the shared gp130 receptor (Timmermann *et al.*, 2002). Conversely, erythropoietin receptor (EpoR) has been shown to homodimerize in a transmembrane domain-dependent manner (Constantinescu *et al.*, 2001). Interestingly, juxtamembrane domains of cytokine receptors have been deemed necessary for receptor activation following ligand binding via phenomenon commonly referred to as conformational change (Kurth *et al.*, 2000; Constantinescu *et al.*, 2001; Greiser *et al.*, 2002; Couturier and Jockers, 2003; Seubert *et al.*, 2003; Staerk *et al.*, 2011).

The cytokine receptors exist as stable or transient preformed complexes on the cell surface. Composition of those complexes varies between cytokine receptors - EpoR and IFN $\gamma$ R form homodimers, while gp130 forms hetero-dimers/oligomers with OSMR, IL6R and LIFR (Liu *et al.*, 1994; Modrell *et al.*, 1994; Livnah *et al.*, 1999; Giese *et al.*, 2005; Krause *et al.*, 2006; Tenhumberg *et al.*, 2006, reviewed in Mohr *et al.*, 2012). The transient heterodimers/oligomers of gp130 as well as homodimers of TpoR are stabilized by ligand binding which also triggers conformational change thought to alter the relative orientation of their cytoplasmic domains (Staerk *et al.*, 2011; Dagil *et al.*, 2012).

The intracellular domain of cytokine receptors generally contains classical Box domains common amongst JAK/STAT pathway receptors, which have been shown to mediate receptor:JAK and receptor:STAT interactions (Fig.I.2) (Hackett *et al.*, 1997; Hirano *et al.*, 1997; Taga and Kishimoto, 1997; Grant and Begley, 1999). Located proximally to the plasma membrane, the Box 1 has been described to specifically mediate interactions with JAK kinases via PxP motif (x represents any amino acid) (Stahl *et al.*, 1994). However, only a semi-conserved PxP motif has been reported sufficient to mediate interaction between IFN $\gamma$ R1 and JAK1 (Kaplan *et al.*, 1996). Box 2 domain of several cytokine receptors has been characterized as essential for JAK binding (Witthuhn *et al.*, 1993; Lebrun *et al.*, 1995; Zhu *et al.*, 1998; Haan *et al.*, 2000).

### 1.2.5 Janus kinases

Despite the large number of cytokines and their receptors present, only four JAKs are encoded by human genome, *jak1-3* and *tyk2* (Wilks *et al.*, 1991; Firmbach-Kraft *et al.*, 1990; Takahashi and Shirasawa, 1994). JAKs are characterized as non-receptor protein tyrosine kinases that associate with cytokine receptors via Box1/2 in receptors as described previously and band 4.1, Ezrin, radixin and moiesin (FERM) domain in JAKs (Chishti *et al.*, 1998; Hilkens *et al.*, 2001; Huang *et al.*, 2001; Radtke *et al.*, 2002; He *et al.*, 2003). Differences in the structures of FERM domains provide the selectivity in interactions with the receptors (Table.I.2). Situated in the N-terminal portion of JAKs, FERM domains are composed of Jak homology (JH) domains 7 through 4 (Fig.I.3) (Wilks *et al.*, 1991). FERM domains have been shown to interact directly with and regulate the catalytic activity of the kinase (Funakoshi-Tago *et al.*, 2008).

Figure.I.3 Structure of JAK kinase

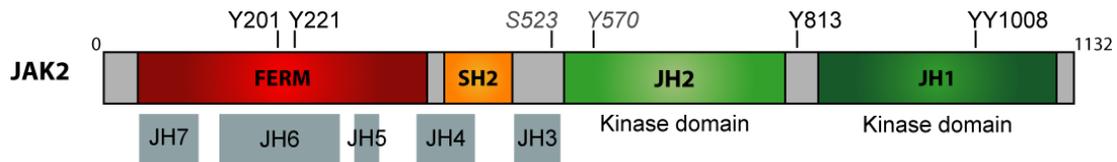


Figure.I.3 Structure of JAK kinase. Schematic representation of JAK2 kinase. JH – JAK homology domains, as described originally, shown below. JH1 and JH2 are kinase domains. Selected phosphorylated regulatory residues are shown above, negative regulators are italicised and in gray. The numbers at extremities represent size of the protein, 0 represents N-terminus.

Centrally located JH3-4 domains are reminiscent of Src homology 2-like (SH2-like) domain, however, they have not been shown to mediate interactions with phosphorylated tyrosine residues (Kampa and Burnside, 2000; Radtke *et al.*, 2005; Haan *et al.*, 2006). The C-terminal portion of JAKs contain the JH1 and JH2 domains, with JH1 situated at the proteins extreme (Fig.I.3). JH1 constitutes a classical tyrosine kinase domain that is required for substrate phosphorylation and contains a double tyrosine motif (YY), phosphorylation of which is crucial for activation of JAK1 and 2 (Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997). Conversely, the JH2 domain was considered to

be a pseudo-kinase domain, with kinase-like structure missing some key residues required for catalytic activity (Wilks *et al.*, 1991). More recently, it has been shown that the JH2 domain of JAK2 is actually a double-specificity functional kinase domain (Ungureanu *et al.*, 2011). The role of JH2 domain is to inhibit JH1 domain from functioning, with two models of action proposed: steric inhibition and autophosphorylation (Saharinen *et al.*, 2000; Saharinen and Silvennoinen, 2002; Saharinen *et al.*, 2003; Shuai and Liu, 2003). Autophosphorylation of JAK kinases can have different outcomes, depending on the residue modified - S523 and Y570 downregulate JAK2 kinase activity, while Y201, Y221, Y813 and other tyrosines up-regulate it (Fig.I.3) (Argetsinger *et al.*, 2004; Kurzer *et al.*, 2004; Matsuda *et al.*, 2004; Yan *et al.*, 2012). This intrinsic auto-regulation does not require an external kinase for activation. Instead, it is thought that ligand binding and conformational change of the receptors cause JAKs to come into close proximity allowing for trans-phosphorylation (Matsuda *et al.*, 2004).

Besides catalytic activity, JAK kinases play important role in regulation of receptor stability. Numerous reports indicate the receptors of the pathway to be stabilised by JAK binding (Radtke *et al.*, 2002; He *et al.*, 2005; Royer *et al.*, 2005; Haan *et al.*, 2006). Indeed, the receptor:JAK complex has been proposed to behave similarly to the receptor tyrosine kinases, and dissociation of JAK2 from EpoR following ligand-mediated stimulation has been described to destabilize the receptor (Funakoshi-Tago *et al.*, 2006; Haan *et al.*, 2006). Interestingly, association of JAK2 with EpoR has been suggested to occur in the endoplasmic reticulum, where the kinase is hypothesised to assist in correct folding of the receptor (Huang *et al.*, 2001). Regulation of receptor stability on the plasma membrane by JAKs often involves physical masking or phosphorylation of the internalization motifs present on the receptors (Ragimbeau *et al.*, 2003; Radtke *et al.*, 2006).

### ***1.2.6 Signal Transducers and Activators of Transcription***

The human genome encodes seven STATs, *stat1-4*, *stat5a* and *5b* and *stat6* (Shuai *et al.*, 1993; Improta *et al.*, 1994; Yamamoto *et al.*, 1994; Zhong *et al.*, 1994a, 1994b; Azam *et al.*, 1995; Quelle *et al.*, 1995). In canonical JAK/STAT signalling STAT

proteins bind to the tyrosine phosphorylated receptors via SH2 domains and become phosphorylated by JAKs (Fig.I.1). Upon phosphorylation, STATs dissociate from the receptor complex and dimerize in the cytoplasm via their SH2 domains (Shuai *et al.*, 1993). Dimerization allows for nuclear translocation to modulate transcription of pathway target genes (Koster and Hauser, 1999, reviewed in Igaz *et al.*, 2001; Kisseleva *et al.*, 2002). Despite sharing conserved SH2 domain, structural differences between STATs result in selectivity towards receptor:JAK complexes, with the phospho tyrosine sites on the receptor being the direct sites of interaction (Table.I.2) (Kotenko *et al.*, 1996; Kohlhuber *et al.*, 1997, reviewed in Lim and Cao, 2006). Tertiary structure of STATs is well conserved across species, indicating common ancestry (Fig.I.4) (reviewed in Jatiani *et al.*, 2010). The N-terminal domain of STATs is critical for function as it is implicated in nuclear import and export, receptor interaction, modulation of DNA binding and the formation of inactive dimers. Downstream of the N-terminal domain is the coiled-coil region involved in receptor and regulator interaction, followed by a highly conserved DNA-binding domain required for binding to conserved recognition sequences in promoters of target genes (Xu *et al.*, 1996; O'Shea *et al.*, 2002). The next domain is the linker domain required for structural integrity, followed by SH2 domain necessary for interaction with tyrosine phosphorylated substrates, formation of homo- and heterodimers, nuclear translocation and DNA binding. The C-terminally located transactivation domain is the most variable element of STAT proteins but it is crucial for transcriptional activation of target genes (reviewed in Schindler and Strehlow, 2000).

*Figure.I.4 Structure of STAT*



Figure.I.4 Structure of STAT. Schematic representation of STAT1 with indication of recognised domains.

Adapted from Jatiani *et al.*, 2001.

Besides canonical signalling, STATs have received a lot of attention due to their emerging roles in non-canonical signalling. Evidence for microtubule binding affecting

migration, regulation of oxidative phosphorylation, activation of Akt pathway and modulation of the epigenetic landscape have been reported (reviewed in Mohr *et al.*, 2012).

### **I.3. Regulation of the JAK/STAT pathway**

Roles of the JAK/STAT pathway in the regulation of numerous vital processes requires tight regulation of pathway activity. Multiple levels of regulation have evolved, including proteins acting in *trans*-, interactions with other signalling pathways as well as processes such as endocytosis, transcriptional editing, epigenetic regulation and polarization of the pathway components. A selection of these regulatory processes relevant to this thesis are described below.

#### ***I.3.1 Suppressors of Cytokine Signalling***

The family of Suppressors of Cytokine Signalling (SOCS) proteins has been described, as the name suggests, as negative regulators of the JAK/STAT pathway which act in a negative feedback loop. The family consists of eight members, SOCS1-7 and CIS (Endo *et al.*, 1997; Masuhara *et al.*, 1997; Minamoto *et al.*, 1997; Naka *et al.*, 1997; Hilton *et al.*, 1998; Starr and Hilton, 1998, 1999, reviewed in Croker *et al.*, 2008). All of the SOCS proteins share a centrally located SH2 domain that mediates interaction with tyrosine phosphorylated substrates and a C-terminally located SOCS box domain (Fig.I.5). The SH2 domain is immediately flanked by N- and C-extended SH2 domain regions, which help to orientate interaction with phosphorylated tyrosines (Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999; Babon *et al.*, 2006). SOCS proteins appear to differ in their affinities for substrates, which include JAKs, receptors and JAK:receptor complexes (Yasukawa *et al.*, 1999; Piganis *et al.*, 2011; Kershaw *et al.*, 2013). The SOCS box domain has been shown to mediate interaction with Elongins B/C and Cullin 5, which in turn recruits Rbx2 thereby forming an active E3 ubiquitin ligase (Kamura *et al.*, 1998; Zhang *et al.*, 1999; Kamura *et al.*, 2004). The Elongin-Cullin-SOCS (ECS) complex mediates transfer of ubiquitin moieties from E2 ubiquitin conjugating enzymes onto substrates, targeting them for degradation. SOCS proteins perform substrate recognition

role in the ECS complexes, however the affinity of each SOCS protein towards Elongins B/C and Cullin 5 differs (Kile *et al.*, 2002; Babon *et al.*, 2009).

Figure.I.5 SOCS family of proteins

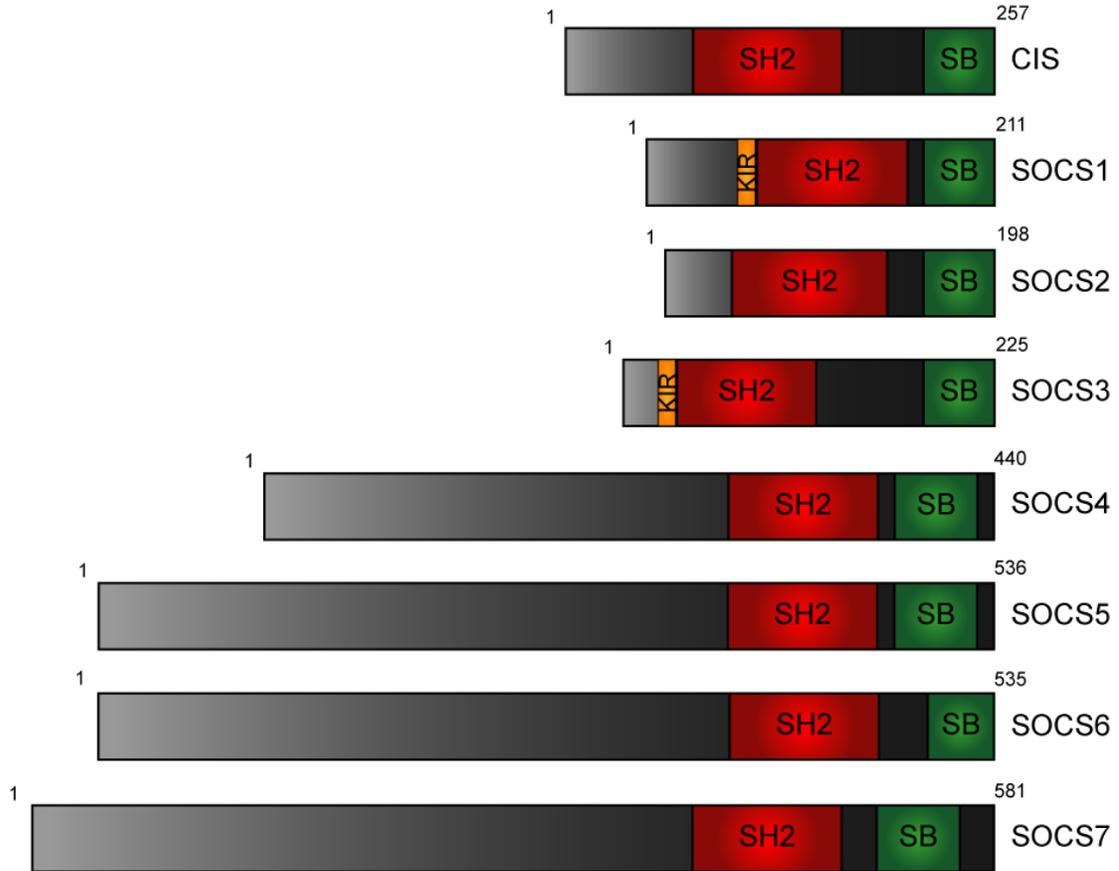


Figure.I.5 SOCS family of proteins. Schematic representation of SOCS family of proteins with indication of recognised domains, SB – SOCS box, KIR – kinase inhibitory region. Numbers at extremities indicate protein sizes.

Adapted from Yoshimura, 2001.

The N-terminal domain of SOCS proteins display low conservation among family members and no easily distinguishable structures are present within this domain. Exception to this are SOCS 1 and 3, which contain a kinase inhibitory region (KIR) located immediately upstream of the SH2 domain (Nicholson *et al.*, 1999; Sasaki *et al.*, 1999; Piganis *et al.*, 2011; Doti *et al.*, 2012). Recently, the crystallographic structure of SOCS3 bound to JAK2 has been resolved, showing that the KIR directly blocks substrate association with the catalytic groove of the kinase (Kershaw *et al.*, 2013). Function of the

long N-terminal domains of SOCS4-7 remains enigmatic, with only handful of reports providing insight into their roles. Structural analysis of the N-terminal domains of SOCS4-7 revealed them to be largely disordered (Feng *et al.*, 2011). A cryptic but more organised region in the N-terminal domains of SOCS4 and 5 has been determined, with indication of a role in protein interactions (Feng *et al.*, 2011). Indeed, the N-terminal of SOCS5 is sufficient for interaction with IL-4 in a phosphotyrosine independent fashion, and was reported to be required for interaction with EGFR (Seki *et al.*, 2002; Kario *et al.*, 2005; Nicholson *et al.*, 2005, reviewed in Croker *et al.*, 2008).

The three domains – SH2, SOCS box and N-terminal KIR – correspond to the mechanisms utilized by SOCS proteins to regulate the JAK/STAT pathway. SH2 domain mediates interactions with substrates, which might lead to competitive inhibition in case of phosphotyrosines used for binding of STATs (Endo *et al.*, 2003; Lavens *et al.*, 2006). As described previously, the SOCS box domain mediates the formation of a ubiquitin ligase resulting in ubiquitination and subsequent degradation of SOCS substrates. JAKs can undergo such modification, however evidence suggests that the ubiquitination of cytokine receptors is the most common of the three mechanisms (Nicholson *et al.*, 2000; Kapuria *et al.*, 2011; Linossi and Nicholson, 2012). Direct interference with JAK:STAT interaction via KIR has been suggested for SOCS1 and 3 only, however it constitutes a major mechanism for both of those proteins (Linossi *et al.*, 2013).

### ***1.3.2 Protein tyrosine phosphatases***

With tyrosine phosphorylation at the core of JAK/STAT pathway activity, protein tyrosine phosphatases represent an important regulatory element. Five protein tyrosine phosphatases belonging to three divergent families have been implicated in the regulation of the JAK/STAT pathway. SH2-containing phosphatases (SHP) 1 and 2 are cytoplasmic proteins that recognise their substrates via SH2 domains present in their structures (Yi *et al.*, 1993; Jiao *et al.*, 1997). Expression of SHP1 is restricted predominantly to the haematopoietic system and mice knockouts have been reported to have elevated phosphorylation levels of JAK1 and 2, IFN $\alpha$ R1, GHR and EpoR (Klingmuller, 1997; Migone *et al.*, 1998; Alicea-Velázquez *et al.*, 2013, reviewed in Valentino and Pierre, 2006). Expression of SHP2 is more ubiquitous, with its knockout resulting in lethality in

mouse models. SHP2 has been associated with inhibition of IL6 and gp130 containing receptors (Lehmann *et al.*, 2003). Interestingly, SHP2 becomes phosphorylated by JAKs resulting in dissociation from receptor complex, suggesting a transient interaction or a regulatory loop (Lu *et al.*, 2001, 2003).

Second pair of phosphatases involved in regulation of the JAK/STAT pathway are protein tyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TC-PTP) (Tonks *et al.*, 1988, reviewed in Stuible *et al.*, 2008). Both of those proteins selectively recognise the double tyrosine motif present in JAKs, however they display different specificity with PTP1B preferentially binding to JAK2 and Tyk2 and TC-PTP associating with JAK1 and JAK3 (Carbone *et al.*, 2012, reviewed in Levine and Wernig, 2006). A splice variant of TC-PTP has been shown to dephosphorylate nuclear STATs, contributing to their nuclear export and termination of signalling (ten Hoeve *et al.*, 2002).

The final phosphatase reported to act on the JAK/STAT pathway is CD45, a transmembrane tyrosine phosphatase expressed specifically in haematopoietic cells (Irie-Sasaki *et al.*, 2001). CD45 mice knockouts displayed elevated phosphorylation levels of JAK1 and 3, however direct association with JAKs has not been shown.

### ***1.3.3 Protein Inhibitors of Activated STATs***

Four proteins constitute the family of Protein Inhibitors of Activated STATs (PIAS), PIAS1 and 3, PIASx and PIASy (reviewed in Shuai and Liu, 2005). STAT proteins have been shown to undergo SUMOylation, which affects their transcriptional activity, however the enzymes responsible remain unknown (Begitt *et al.*, 2011). PIAS proteins are SUMO-E3-ligases, making them prime suspects for such activity (Schmidt and Muller, 2002). At the same time, PIAS1 and 3 have been shown to interact directly with STAT1 and 3, blocking their DNA binding activity (Chung *et al.*, 1997; Liu *et al.*, 1998). Conversely, PIASx and PIASy recruit co-repressors, such as histone deacetylases to inhibit transcriptional activity of STAT1 and 4 (Liu *et al.*, 2001).

### ***1.3.4 Src family of kinases***

A significant number of signalling pathways have been shown to affect JAK/STAT pathway in a process commonly called cross-talk. The Src family of non-receptor tyrosine kinases has been shown to positively regulate the JAK/STAT pathway via more than one way (for a review on Src kinases, see Thomas and Brugge, 1997). Firstly, Src kinases can activate STATs by direct phosphorylation, leading to a model of Src kinases being positive regulators of the JAK/STAT pathway (Silva and Shupnik, 2007). This model is supported by recent findings in *Drosophila*, which indicate that Src kinases polarize STAT subcellular localization via a non-catalytic activity, thereby sensitizing the pathway (Sotillos *et al.*, 2013). Finally, Src kinases were shown to directly phosphorylate SOCS proteins, leading to their inactivation, thereby leading to an increase in JAK/STAT pathway activity (Sommer *et al.*, 2005).

### ***1.3.5 Endocytosis***

The majority of cytokine receptors undergo clathrin-mediated endocytosis (CME) either in response to ligand binding or as a constitutive process (Fig.I.6) (Doherty and McMahon, 2009). In either case, endocytosis plays a three-fold role. Internalization of receptors from cell surface regulates cell's sensitivity to ligands, which most often represents desensitization. Secondly, the endocytic pathway has been shown to modulate the quality of the signalling output by enriching endosomal compartments with distinct signalling/accessory molecules, a concept described as signalling endosomes (Howe and Mobley, 2004). Modulation of the JAK/STAT pathway signalling output by endocytosis is however poorly understood (reviewed in Mohr *et al.*, 2012). Finally, endocytosis leads to termination of signalling via degradation of the receptor complex or its recycling to the plasma membrane following ligand decoupling and receptor dephosphorylation (Grant and Donaldson, 2009; Raiborg and Stenmark, 2009).

Endocytosis is a dynamic and fluid process, with several stages that can be distinguished. CME is initiated by a process of cargo selection followed by internalization into the intracellular endocytic vesicles, at which stage the affected receptor loses contact with extracellular environment but can still signal through its

Figure.I.6 Schematic endocytic pathway

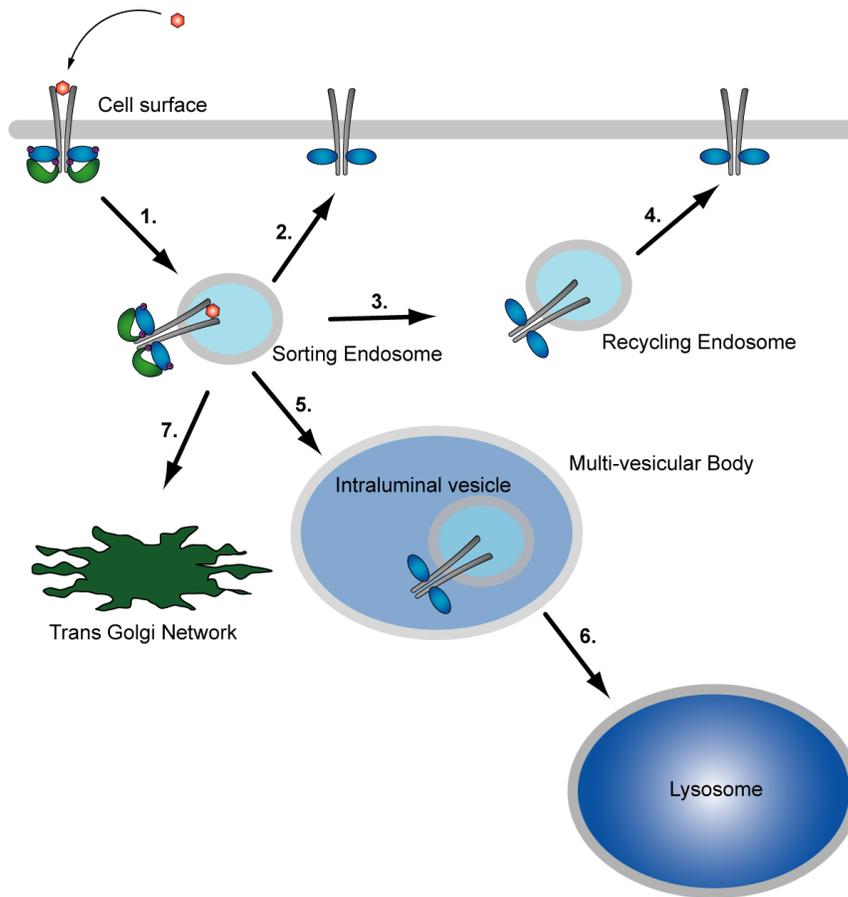


Figure.I.6 Schematic endocytic pathway. Schematic representation of clathrin-mediated endocytosis of cytokine receptors. **1.** Ligand-induced internalization of the receptor into clathrin coated vesicles concludes with fusion of the vesicles with the Sorting Endosome. At this point receptors intracellular domain remains in the cytoplasm allowing for activation of cytoplasmic STATs. Fate of receptor is determined in the Sorting Endosome and either of the four routs can take place. **2-4** Following uncoupling of the ligand and dephosphorylation, receptor can be recycled to the plasma membrane directly (**2.**) or through the Recycling Endosome (**3. and 4.**). **5.** Following maturation of the Sorting Endosome, receptor in vesicles is transported and incorporated into Multi-vesicular Bodies, while remaining in Intraluminal Vesicles. Contact with cytoplasm is lost and no signalling can occur. **6.** Fusion with lysosome destroys the receptor. **7.** Receptor is transported to the Trans Golgi Network from where it can undergo several processes. Proteosomal degradation and maturation of the Sorting Endosome into Late Endosome is not shown.

Adapted from Platta and Stenmark, 2011.

cytoplasmic domain (reviewed in Traub, 2009). Internalization of cytokine receptors has been shown to require binding of a tetrameric AP-2 adaptor protein, which mediates interaction with Clathrin (Bonifacino and Traub, 2003). However, the process leading to AP-2 binding differs between cytokine receptors. As AP-2 requires non-phosphorylated tyrosine residues for binding, IFN $\alpha$ R has to be dephosphorylated on Y466 by PTP1B to allow for interaction (Carbone *et al.*, 2012). Intriguingly, the dileucine motif (an alternative AP-2 binding motif) of OSMR is masked by bound JAK1 which has to dissociate for internalization to occur (Radtke *et al.*, 2002). Similar process has been reported for internalization of IFN $\alpha$ R1 and Tyk2 (Ragimbeau *et al.*, 2003). Finally, ubiquitination has been reported to be strongly associated with regulation of cytokine receptor stability, including receptor internalization. Both, internalization and endocytic shuttling of G-CSFR and Prolactin receptor, among others, is regulated by site specific ubiquitination (Swaminathan *et al.*, 2008; Varghese *et al.*, 2008; Wölfler *et al.*, 2009). All of the aforementioned processes are not mutually exclusive and different combinations of these processes might be required for receptor internalization, as has been shown for EGFR (Goh *et al.*, 2010).

Following internalization of the receptor and arrival at the sorting endosome, receptors can be recycled directly or indirectly to the plasma membrane or Golgi or, alternatively they can be targeted for lysosomal or proteosomal degradation (Fig.I.6) (reviewed in Pfeffer, 2009). The means by which receptors are sorted are still not fully understood, however some evidence suggest motifs in the cytoplasmic domains of the receptors may be determining factors (Hitchcock *et al.*, 2008). In addition, a further signal controlling receptor fate determination is ubiquitination, as shown in case of PRLR and G-CSFR (Thrower *et al.*, 2000; Peng *et al.*, 2003; Pickart and Fushman, 2004; Lauwers *et al.*, 2009).

#### **I.4. JAK/STAT pathway in states of disease**

The JAK/STAT pathway plays a central role in the regulation of cellular proliferation, survival, apoptosis and differentiation, therefore it is not surprising that mutations affecting the function of the JAK/STAT pathway lead to a wide variety of

diseases, including erythrocytosis, acromegaly, inflammatory disorders and haematopoietic malignancies. Pathogenic mutations affecting the JAK/STAT pathway are most prominent in the haematopoietic and immune system, due to the central role of the pathway in the relevant tissues (reviewed in Vainchenker and Constantinescu, 2013). A brief overview of those diseases and their aetiology is given below.

#### ***1.4.1 TEL-JAK2 fusion***

The best described gene fusion involving JAK/STAT pathway components is translocation ETS leukemia-JAK2 (TEL-JAK2) fusion which leads to oligomerization of the catalytic domains of JAK2 resulting in constitutive activity (Lacronique *et al.*, 1997). Besides the aberrant activation of the JAK/STAT pathway, PI3K, RAS/ERK, p38 and NF- $\kappa$ B pathways are also activated (Nguyen *et al.*, 2001). The TEL-JAK2 mutations is associated with T-cell childhood acute lymphocytic leukaemia (ALL) and atypical chronic myeloid leukaemia (CML).

#### ***1.4.2 JAK2 mutations***

The JAK2 V617F mutation is found in over 90% of patients with polycythaemia vera (PV) and 50% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Baxter *et al.*, 2005; James *et al.*, 2005a; Kralovics *et al.*, 2005). These haematological malignancies represent the classical non-BCR-ABL myeloproliferative neoplasias, characterized by production of excess mature blood cells (Tefferi, 2006). The V617F mutation resides in the JH2 domain of the kinase, and is thought to function by suppressing the autoinhibitory effects of this domain, as described previously. This leads to hypersensitivity to cytokines or cytokine-independent pathway activation. The molecular mechanism of action has been extensively studied, however some controversy exists regarding potential non-canonical aspects of mutant protein activity (Dawson *et al.*, 2009; Girodon *et al.*, 2011, reviewed in James *et al.*, 2005b; Quintas-Cardama and Verstovsek, 2013).

A significant proportion of patients suffering from myeloproliferative diseases that are both BCR-ABL- and V617F-negative are diagnosed with *jak2* exon 12 somatic

mutations located in the JH2 domain (Pietra *et al.*, 2008). Resulting hyperactivation of the JAK2 leads to phenotypes identical to those caused by V617F mutation. Moreover, a deletion of a small region in the JH2 domain ( $\Delta$ IREED) as well as mutations of R683 in this region have been found in patients with B-cell ALL (Malinge *et al.*, 2007). Finally, a single point mutation in the FERM domain of JAK2, R340Q, has been associated with chronic myeloproliferative neoplasias (Aranaz *et al.*, 2010).

#### ***1.4.3 Mutations in the remaining JAKs***

Somatic mutations in FERM and JH1 domains (including a JAK2 V617F equivalent, V658F) of JAK1 were found in 10-20% of patients with T-cell ALL and less frequently in B-cell ALL (reviewed in Vainchenker and Constantinescu, 2013). Also patients with acute myeloid leukaemia were found to carry mutated JAK1 (Xiang *et al.*, 2008).

The Y100C mutation in the FERM domain of JAK3 causes a decreased interaction with IL-2 receptor in B-cells, resulting in autosomal severe combined immunodeficiency (SCID) (Cacalano *et al.*, 1999). The gain of function mutations L156P, E183G and R172Q residing in the same domain of JAK3 are associated with adult T-cell leukaemia/lymphoma (Elliott *et al.*, 2011).

#### ***1.4.4 Mutations in the remaining components of the JAK/STAT pathway***

Although mutations in the cytokine receptors have only rarely been associated with human diseases, mutations mimicking the activated state of TpoR are associated with ET and PMF (Pikman *et al.*, 2006). Furthermore, T617D mutation in G-CSFR has been associated with JAK2 hyperactivation, resulting in familial neutrophilia (Plo *et al.*, 2009).

Mutations arising in the exon 21 of *stat3* have been found in 40% of patients suffering from large granular lymphocytic leukaemia (Jiang *et al.*, 2009; Koskela *et al.*, 2012). Mutations were localised to the SH2 domain of STAT3 and resulted in increased phosphorylation and transcriptional activity of STAT3. Patients carrying these mutations are also more susceptible to neutropenia and rheumatoid arthritis. Interestingly, also

mutations in *stat5b* have been reported to produce large granular lymphocytic leukaemia in patients that were tested negative for *stat3* mutations (Rajala *et al.*, 2013).

## **I.5. Conservation of the JAK/STAT pathway in *Drosophila melanogaster***

### ***I.5.1 Overview of the pathway***

The *Drosophila* JAK/STAT pathway is conserved on all levels of the cascade, however it displays much lower redundancy (reviewed in Arbouzova and Zeidler, 2006). The only functional receptor of the pathway, Domeless (Dome), can be activated by any of the three ligands of the pathway: Upd, Upd2 or Upd3 (Fig.I.7) (Harrison *et al.*, 1998; Brown *et al.*, 2001; Agaisse *et al.*, 2003; Gilbert *et al.*, 2005; Hombría *et al.*, 2005). No significant differences in pathway transcriptional output were observed in response to stimulation with individual ligands, however, dissociation dynamics as well as *in vivo* temporo-spatial expression is likely to differentiate these ligands (Hombría *et al.*, 2005; Bina *et al.*, 2010; Wright *et al.*, 2011). In addition, the tertiary structure of Upd3 has recently been shown to be related to mammalian helical cytokines, while human helical cytokine leptin was shown to activate the *Drosophila* JAK/STAT pathway, implying strong conservation of signalling mechanisms between human and fly (Rajan and Perrimon, 2012; Oldefest *et al.*, 2013).

Genetic interaction studies as well as RNAi-mediated genome-wide screens confirmed existence of only a single JAK kinase, termed Hopscotch (Binari and Perrimon, 1994). Initially identified as a regulator of pair-rule and segment-polarity gene expression in the *Drosophila* embryo, Hop was soon after identified as a component of a conserved JAK/STAT pathway contributing to numerous biological processes. The homology to mammalian JAKs was first determined based on the presence of two kinase domains, JH1 and JH2. Loss of *hop in vivo* leads to under-proliferation of cells constituting larval imaginal discs, precursors of organs in the adult fly (Perrimon and Mahowald, 1986; Mukherjee *et al.*, 2005).

Figure.I.7 JAK/STAT pathway in *Drosophila*

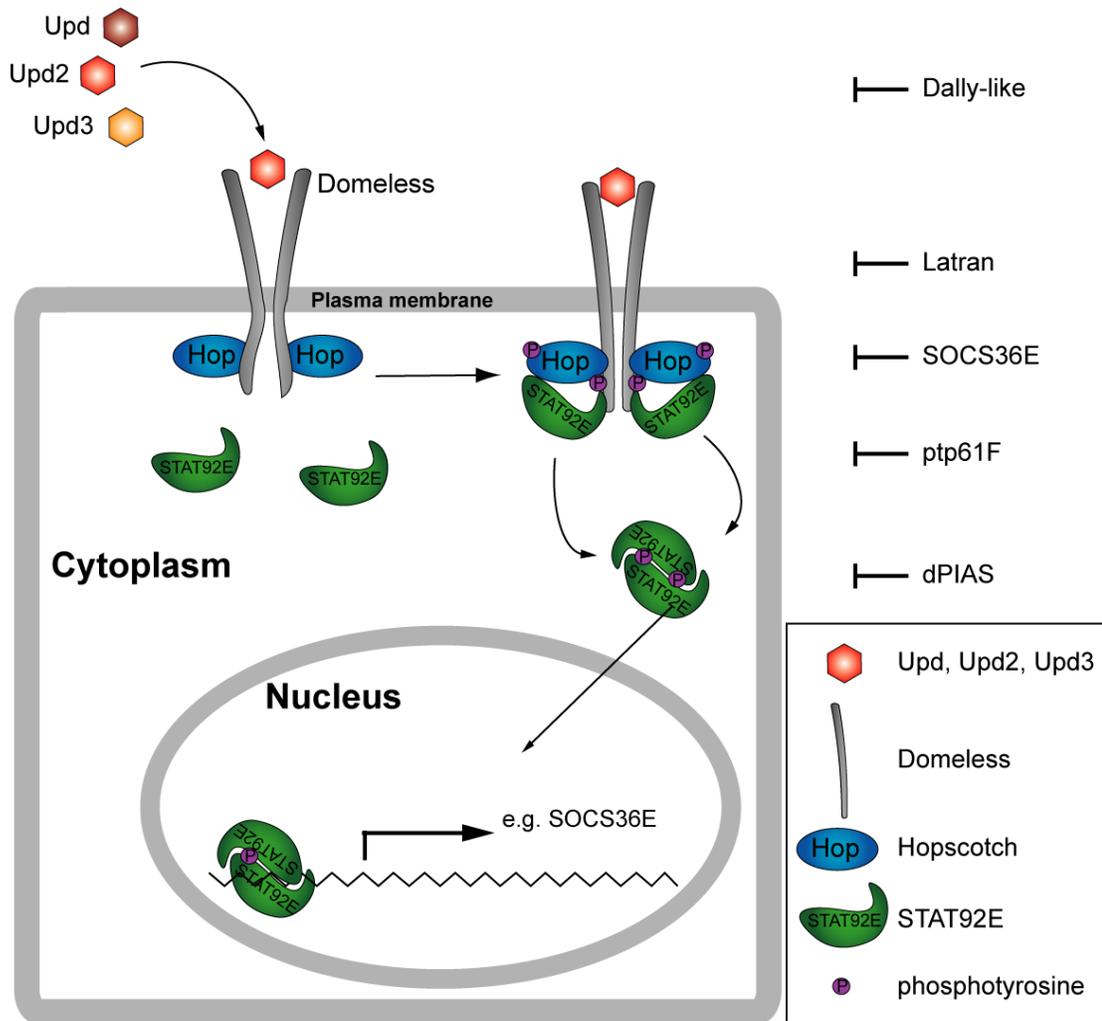


Figure.I.7 JAK/STAT pathway in *Drosophila*. Schematic representation of the JAK/STAT signalling pathway as described in *Drosophila*. Negative regulators of the pathway are indicated on the right. Their position relative to the pathway represents the level of the signalling cascade at which the regulator acts. Regulators located in between levels act on both levels below and above.

Adapted from Arbouzova and Zeidler, 2006.

The *Drosophila* genome encodes a single STAT, called *stat92E* (Hou *et al.*, 1996; Yan *et al.*, 1996b). STAT92E has been shown to be core pathway component, transducing signal in the process of canonical signalling, thereby contributing to growth of imaginal discs, as described previously. However, once the JAK/STAT pathway signalling is suppressed, STAT92E performs an anti-proliferative role (Mukherjee *et al.*,

2005). This ability to change proliferative functions has been attributed to the non-canonical signalling performed by STAT92E (reviewed in Arbouzova and Zeidler, 2006). This accounts for activation of STAT92E by receptor tyrosine kinases as well as by Src kinases (Li *et al.*, 2003; Read *et al.*, 2004). Moreover, emerging evidence suggests that STAT92E can modulate the epigenetic landscape, similarly to the mammalian STATs, by affecting heterochromatin stability (Shi *et al.*, 2006; Brown and Zeidler, 2008; Shi *et al.*, 2008).

### ***1.5.2 Regulation of the Drosophila JAK/STAT pathway***

The *Drosophila* JAK/STAT pathway has been shown to be regulated already at the level of ligand secretion and diffusion. Polarized mRNA expression of Upd, leading to polarized secretion pattern has been observed in the *Drosophila* ovary, while Dally and Dally-like glypicans have been shown to regulate extracellular distribution pattern of Unpaired in the developing eye (Fig.I.7) (van de Bor *et al.*, 2011; Zhang *et al.*, 2013). Reception of the ligand is further regulated by Dome homodimerization and its heterodimerization with Latran/Eye transformer, a negative regulator of the pathway expressed in response to physical insult, such as wasp infestation, in a tissue specific manner (Brown *et al.*, 2003; Kallio *et al.*, 2010; Makki *et al.*, 2010) Fisher *et al.*, *in prep*). Cells constituting ectodermal tissues in *Drosophila* were reported to have STAT92E localization to sub-apical regions of the cell, resulting in sensitization of the pathway (Sotillos *et al.*, 2008, 2013).

The major regulators of the mammalian JAK/STAT pathway are also conserved in *Drosophila*. The SOCS family of proteins is represented by three SOCS-like molecules, SOCS16D, SOCS36E and SOCS44A, however only SOCS36E acts in a negative feedback loop and potently suppresses the JAK/STAT pathway signalling (Callus and Mathey-Prevot, 2002; Karsten *et al.*, 2002; Rawlings *et al.*, 2004, reviewed in Stec and Zeidler, 2011). SOCS44A has been shown to mainly affect the EGFR signalling pathway while function of SOCS16D has not been determined to date.

So far, only Ptp61F, a homologue of human PTP1B, has been identified as a phosphatase regulating the fly pathway while the SHP2 homologue, Corkscrew, has no discernable effect on the *Drosophila* JAK/STAT pathway (Baeg *et al.*, 2005; Müller *et*

*al.*, 2005). Epistasis experiments determined Ptp61F to act on the level of STAT92E and Hop (Baeg *et al.*, 2005; Müller *et al.*, 2005), however *in vitro* experiments put it on the level of the receptor complex (Fig.I.7) (Fragiadaki and Fisher *et al.*, *in prep*). Moreover, a single PIAS protein, referred to as dPIAS or Su(var)2-10, has been identified and shown to interact with STAT92E and suppress ectopically activated pathway *in vivo* (Betz *et al.*, 2001; Hari *et al.*, 2001).

Finally, the process of endocytosis has been shown to regulate the *Drosophila* JAK/STAT signalling pathway (Müller *et al.*, 2005), however the only two reports on the matter suggest different regulatory outcomes (Devergne *et al.*, 2007; Vidal *et al.*, 2010). Never-the-less, the core endocytic machinery is well conserved between humans and flies and emerging evidence suggests a modulatory role in cell signalling for the *Drosophila* endocytic pathway (Huang *et al.*, 2010; Robinson and Moberg, 2011; Katja Vogt, personal communication).

### ***1.5.3 JAK/STAT pathway in Drosophila development and maintenance of homeostasis***

Classically, *Drosophila* has been used as a developmental model organism. Not surprisingly, studies on the *Drosophila* JAK/STAT pathway are predominantly conducted in a developmental context. The JAK/STAT pathway in *Drosophila* was originally identified as necessary for correct segmentation within the embryo as well as for the formation of embryonic structures such as the gonads, hindgut, trachea and posterior spiracles (Small *et al.*, 1996; Yan *et al.*, 1996a; Brown *et al.*, 2001; Johansen *et al.*, 2003; Brown *et al.*, 2006; Sotillos *et al.*, 2010). During larval stages, JAK/STAT pathway regulates cell proliferation in the developing eye and wing discs, thereby contributing to tissue size and patterning in the adult (Bach *et al.*, 2003; Mukherjee *et al.*, 2005). Interestingly, those processes have been shown to depend on both, canonical and non-canonical signalling by STAT92E (reviewed in Arbouzova and Zeidler, 2006; Brown and Zeidler, 2008).

The reproductive systems in male and female flies also involves the JAK/STAT pathway, which plays essential roles in maintenance of the stem cell populations as well as specification and correct border cell migration in the ovary (Kiger *et al.*, 2001; Tulina and Matunis, 2001; Decotto and Spradling, 2005; Silver *et al.*, 2005). Emerging evidence

suggests essential roles for the JAK/STAT pathway in maintenance of the intestinal stem cell niche and immune response in this tissue, suggesting strong functional parallels between human and fly (Buchon *et al.*, 2009a, 2009b; Jiang *et al.*, 2009). Despite lack of conservation of the adaptive immune system in *Drosophila* and drastic differences in “blood” composition, parallels can also be drawn regarding involvement of the JAK/STAT pathway in haematopoiesis (Rizki and Rizki, 1992; Sorrentino *et al.*, 2002; Meister and Lagueux, 2003; Crozatier *et al.*, 2007; Krzemien *et al.*, 2007). This comparison is particularly interesting considering hyperactivation of the *Drosophila* JAK/STAT pathway by pathogenic Hop mutants, Hop<sup>Tum1</sup> and Hop<sup>T42</sup>, leads to formation of melanotic masses phenotypically reminiscent of human haematopoietic malignancies (Harrison *et al.*, 1995; Luo *et al.*, 1997).

## **I.6. Synopsis**

The aim of this study is to investigate the molecular mechanisms governing the *Drosophila* JAK/STAT signalling pathway. Particular emphasis is put on the dissection of the structure-function relationship of core pathway components and pathway regulators. Questions regarding interaction sites between Dome and downstream pathway components as well as endocytosis of the receptor are addressed in Chapter III. Chapter IV focuses on the structure of SOCS36E and how it relates to regulation of the JAK/STAT pathway. Finally, the oncogenic Hop mutants, Hop<sup>Tum1</sup> and Hop<sup>Tum1</sup>, are dissected on the molecular level, with particular focus on the hyperactivation of the canonical JAK/STAT pathway, in Chapter V. Conclusions of this study in broader perspective as well as suggestions regarding future directions are provided in Chapter VI.

## **Chapter II Materials and methods**

### **II.1. Molecular techniques**

#### ***II.1.1 Polymerase Chain Reaction***

All Polymerase Chain Reactions (PCR) used in the generation of protein expression plasmids were conducted using Herculase II Fusion DNA Polymerase (Agilent Technologies) according to manufacturer instructions on a PTC-200 thermocycler (MJ Research). Primers used were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) and their sequences are listed in Table.II.1. Previously generated cDNA clones were used as templates for amplification: Dome (LD46805; DGRC), SOCS36E (SD04308; DGRC), Hop (Binari and Perrimon, 1994). All PCR products were sequenced for mutations and correct orientation following sub-cloning into destination vectors at Core Genomic Facility (Medical School, University of Sheffield, Sheffield, UK).

#### ***II.1.2 Cloning into Gateway vectors***

PCR amplified fragments were inserted into Gateway System Entry vector using pENTR Directional TOPO Cloning Kit (Invitrogen) and subsequently cloned into destination vectors pAWF or pAWH (*Drosophila* Gateway Vector Collection) using Gateway LR Clonase II Enzyme Mix (Invitrogen), according to manufacturer's instructions. Gateway destination vectors were obtained from *Drosophila* Genomics Resource Centre (Indiana, USA).

#### ***II.1.3 Cloning SOCS36E constructs into pRSETA plasmid***

Forward and reverse primers (Table II.1) containing BglII and KpnI restriction enzyme sites, respectively, were used to PCR amplify SOCS36E truncations using cDNA template (SD4308; DGRC). PCR product was poly-A tailed and sub-cloned into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen). SOCS36E SH2\* mutation was

introduced at this stage as described in section II.1.4. All vectors were sequenced and selected against unwanted mutations. Plasmids were cut with BglIII (New England BioLabs) or Asp718 (Roche) restriction enzymes according to manufacturer instructions. Reactions were resolved on 1% agarose electroporesis gel and required bands were extracted using QIAquick Gel Extraction kit (QIAGEN) according to manufacturer guidelines. Purified DNA fragment was ligated into pre-cut pRSET A plasmid (Invitrogen) using T4 DNA ligase (New England BioLabs).

**Table.II.1 Primers used for cloning**

Gene	Primer	Sequence
<b>Gateway cloning</b>		
Dome	Forward	caccATGGTGGCCCAGGAGCAGC
	Reverse	GAGGACGTGCCGATTGTGGGC
Hop	Forward	caccATGGCCCTGGCCAACGG
	Reverse	CTCGGCATCCGTCGGCTGATTCGGC
SOCS36E	Forward	caccATGGGTCATCACCTTAGCAAGTTCTCAGCA
	Reverse	TACATTGCCGTAGTACGGCATCG
SOCS36EΔN	Forward	caccATGCACTGCCTGGTTCCCGATCT
	Reverse	TACATTGCCGTAGTACGGCATCG
SOCS36EΔSB	Forward	caccATGGGTCATCACCTTAGCAAGTTCTCAGCA
	Reverse	GGAGAAGGTCTGCCTTCTGTGCAG
<b>pRSET A cloning</b>		
SOCS36E	Forward	GCAAGATCTATGGGTCATCACCTTAGCAAGTTCTCAGCA
	Reverse	ACATTGCCGTAGTACGGCATCGGGTACCTAC
SOCS36EΔN	Forward	TCTAGATCTATGCACTGCCTGGTTCCCGATCT
	Reverse	ACATTGCCGTAGTACGGCATCGGGTACCTAC
SOCS36EΔSB	Forward	GCAAGATCTATGGGTCATCACCTTAGCAAGTTCTCAGCA
	Reverse	GGAGAAGGTCTGCCTTCTGTGCAGGGTACCGGA

### ***II.1.4 Generation of single point mutations***

Single point mutations in Dome, Hop and SOCS36E were introduced using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and primers listed in Table.II.2. Gateway entry vector carrying gene of interest was used as DNA template for site directed mutagenesis reaction. Sequencing was undertaken to exclude unintended mutations as described previously (Section.II.1.1). Mutated DNA was subsequently cloned into pAWH or pAWF expression vectors, as described previously (Section.II.1.2).

**Table.II.2 Primers for site-directed mutagenesis**

<b>Gene</b>	<b>Mutation</b>	<b>Sequence</b>
Dome	P925I-F	GATATCGGTCTAGTGCTGATTCAGGGAATCATGGAGACC
	P925I-R	GGTCTCCATGATTCCCTGAATCAGCACTAGACCGATATC
	Y914F-F	CTACCTCGTGTACAAGAAATTCCGCAAGATGTCCGATATCG
	Y914F-R	CGATATCGGACATCTTGCGGAATTTCTTGTACACGAGGTAG
	Y1022F-F	GTTGGTCCGCCACCAGCTTCCTGGCCATGCGGCATGGC
	Y1022F-R	GCCATGCCGCATGGCCAGGAAGCTGGTGGGCGGACCAAC
	Y1070F-F	CAGAGTTGCACCAATGGTTTCATCAAGCCCACACAGATG
	Y1070F-R	CATCTGTGTGGGCTTGATGAAACCATTGGTGCAACTCTG
	Y1219F-F	CAAAATGGCTGACATCGGCTTTACCACCATGGAGCAGTTGC
	Y1219F-R	GCAACTGCTCCATGGTGGTAAAGCCGATGTCAGCCATTTTG
	L985R-F	GTGAGAGCTCGAAACGGCTGCTGCGCACAGC
	L985R-R	GCTGTGCGCAGCAGCCGTTTCGAGCTCTCAC
	Y966F-F	GACGACTCGCCGCCATTACGCCGCAGGATCTG
	Y966F-R	CAGATCCTGCGGCGTGAATGGCGGCGAGTCGTC
Hop	G341E-F	GGATTCCCTGGAGCCTGGACTCAAGGTGGCCAGGG
	G341E-R	CCCTGGCCACCTTGAGTCCAGGCTCCAGGGAATCC
	E695K-F	GCGGCATGCACTATTTGAAGGACAACAAGATT
	E695K-R	AATCTTGTGTCTTCAAATAGTGCATGCCGC
	K926Q-F	CCGAGCAGGTTGCCATCCAGATGCTGAACACCATG
	K926Q-R	CATGGTGTTCAGCATCTGGATGGCAACCTGCTCGG
SOCS36E	R499E-F	GGCACGTTCTGCTGGAGGACTCCGCCAGGAG
	R499E-R	CTCCTGGGCGGAGTCCTCCAGCAGGAACGTGCC

### ***II.1.5 Plasmid propagation and purification***

NEB-10 $\beta$  cells (New England BioLabs) were transformed according to manufacturer instructions, plated on LB-agar plates with appropriate antibiotic selection and grown overnight at 37 $^{\circ}$ C according to standard protocols. Individual colonies were picked and grown in 5ml of LB medium supplemented with appropriate antibiotic overnight at 37 $^{\circ}$ C. DNA was extracted using Plasmid Mini kit (QIAGEN) and restriction digests were performed to determine orientation and identity of the insert. If correct, large-scale bacterial culture was grown overnight and DNA was extracted using Plasmid Midi or Plasmid Maxi kit (QIAGEN) according to manufacturer guidelines.

### ***II.1.6 Extraction of genomic DNA from wild-type flies***

50 wild-type flies (w<sup>1118</sup>) were frozen at -80 $^{\circ}$ C overnight, homogenized in 400 $\mu$ l of DNA extraction buffer (0.1M NaCl, 0.2M Sucrose, 0.1M Tris HCl pH9, 50mM EDTA, 0.5% SDS) and incubated at 65 $^{\circ}$ C for 30 minutes. 120 $\mu$ l of 8M KOAc was added and further incubated for 30 minutes on ice. Reaction was centrifuged at 13,000 rpm for 5 minutes at 4 $^{\circ}$ C and 750 $\mu$ l of the supernatant were aliquoted into a fresh eppendorf. The volume was doubled with 100% ethanol and incubated for 5 minutes at room temperature. Reaction was centrifuged at 13k rpm for 5 minutes at 4 $^{\circ}$ C, supernatant was discarded and the pellet was washed with 70% ethanol and air-dried. Pellet was resuspended in 400 $\mu$ l of TE buffer. 2  $\mu$ g/ml RNase A was added and incubated at 37 $^{\circ}$ C for 30min to remove any RNA. 40 $\mu$ l of StratClean (Stratagene) was then used to remove remaining protein contaminants for 1 minute and centrifuged at 13,000 rpm for 5min at 4 $^{\circ}$ C. Supernatant was transferred into a fresh eppendorf and 1/10<sup>th</sup> volume of 5M NaCl and 2 volumes of 100% ethanol were added. Reaction was incubated overnight at -80 $^{\circ}$ C for 2 hours to allow for DNA precipitation and centrifuged at 13,000 rpm for 10 minutes at 4 $^{\circ}$ C. Pellet was washed with 70% ethanol and resuspended in 50 $\mu$ l of TE buffer.

### ***II.1.7 Generation of double stranded (ds)RNAs***

Wild-type fly genomic DNA was used as a template for PCR using primers listed in Table.II.3. Genomic DNA used for generation of LacZ dsRNA was obtained from flies

expressing LacZ and was a kind gift from Victoria M. Wright. PCR products were transcribed *in vitro* using MEGAScript T7 transcription kit (Ambion), according to manufacturer instructions. dsRNAs were checked for size by separation on standard 1% agarose gel. Efficiency of knockdowns was assessed by qPCR (Fig.II.1).

**Table.II.3 Primers to make dsRNAs**

Gene	Primer	Sequence
Cullin 5	Forward	TAATACGACTCACTATAGGGAGAGCAGCACCTCTACCTCCAA
	Reverse	TAATACGACTCACTATAGGGAGCTATCCATCAGGCGGAACAT
Elongin C	Forward	TAATACGACTCACTATAGGGAGCCCACGTCCTACAAAAGGTC
	Reverse	TAATACGACTCACTATAGGGAGTCTCGCAGGGACAATCTTCT
Elongin B	Forward	TAATACGACTCACTATAGGGAGCTGCCGTCTCAACTGCAATA
	Reverse	TAATACGACTCACTATAGGGAGGTTGCTCAGTTCGCACATA
Rab5	Forward	TAATACGACTCACTATAGGGGGCAACCACTCCACGCA
	Reverse	TAATACGACTCACTATAGGGTCCTGGCCAGCCGTGT
TSG101	Forward	TAATACGACTCACTATAGGGATCCCTCAAATCCCAGTTCC
	Reverse	TAATACGACTCACTATAGGGAAAGTGGCGCTGTGGTG
Dor	Forward	TAATACGACTCACTATAGGGGTGTTTAACTCAGGGTGAC
	Reverse	TAATACGACTCACTATAGGGGCGTGCTCGGCTATCA
Hop	Forward	TAATACGACTCACTATAGGGCCTTGCTTCGTTCTAATCGC
	Reverse	TAATACGACTCACTATAGGGATGCAGTGTTACATTGGGCA
Dome	Forward	TAATACGACTCACTATAGGGGCTCACTGGGCTGATAAAGC
	Reverse	TAATACGACTCACTATAGGGTACAGGTTCTGGGTTCTGGG
STAT92E	Forward	TAATACGACTCACTATAGGGAAGCTGCTTGCCCAAACTA
	Reverse	TAATACGACTCACTATAGGGGTCGACGATAAAGGCAGAGC
SOCS36E	Forward	TAATACGACTCACTATAGGGGAGCGTCACTCCAACCTCCTC
	Reverse	TAATACGACTCACTATAGGGCCAGCGATTACGAAAAGCTC
LacZ	Forward	TAATACGACTCACTATAGGGAGACAGTGGCGTCTGGCGGAAA
	Reverse	TAATACGACTCACTATAGGGAGATCCGAGCCAGTTTACCCGC
Ptp61F	Forward	TAATACGACTCACTATAGGGAGCGAAACCAAAAGCAAAGA
	Reverse	TAATACGACTCACTATAGGGGAAGGGCAAAAAGAGTGCTG

Figure.II.1 Knockdown efficiency

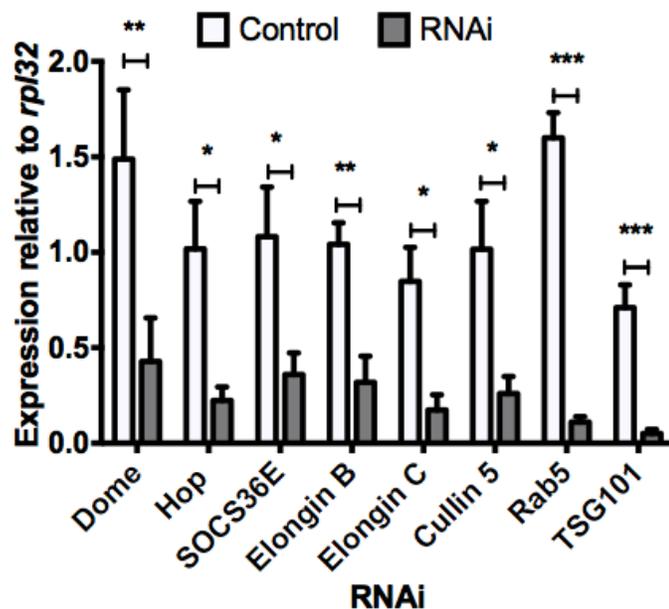


Figure.II.1 Knockdown efficiency. Results of quantitative RT-PCR on cells treated with control dsRNA or targeting gene of interest, as indicated.

### II.1.8 Isolation of mRNA from cells

mRNA that was used for qPCR analysis of dsRNA-mediated knockdown efficiency was extracted from cells using Trizol extraction (Chomczynski and Mackey, 1995). Briefly, cells grown in monolayer were washed once with ice cold PBS and lysed in the culture dish directly, using 1ml of TRIzol Reagent (Invitrogen) per well in 6-well plate and 0.5ml per well in 12-well plate. 0.2ml of chloroform per 1ml of Trizol was added, vortexed for 15s and incubated for 3min. Phase separation was achieved by centrifugation at 12,000g for 15min at 4°C. Aqueous phase was transferred into a fresh tube and mixed with isopropanol, volume of which was defined as half the starting volume of Trizol. Following 10min incubation at room temperature, samples were centrifuged at 12,000g for 10min at 4°C. Pellet was washed twice with 1ml of 75% ethanol, air dried and resuspended in DEPC treated water.

### ***II.1.9 Reverse transcription***

Concentration of mRNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies). 2µg of mRNA were used for reverse transcription reaction using High Capacity RNA-to-cDNA Kit (Applied Biosystems), according to manufacturer guidelines. For quantitative Real-Time PCR, 20µl cDNA reaction was topped up to 30µl volume with water.

### ***II.1.10 quantitative Real Time-PCR***

qRT-PCR was carried out on C1000 Touch Thermal Cycler (Bio-Rad) in 96-well plates, according to manufacturer instructions. The total volume of reaction per well was 10µl, consisting of 5µl of SYBR® Green JumpStart Taq ReadyMix for Quantitative PCR (Sigma), 1µl of diluted cDNA, 0.1µl per primer and 3.8µl water. Final primer concentration of 1µM, was used per reaction. Primer sequences used are provided in Table.II.4. Results were analysed using the  $\Delta\Delta C_T$  method (Livak and Schmittgen, 2001) and normalised using the housekeeping gene *rpl32* (Dostert *et al.*, 2005). Results of qRT-PCRs are available in the Fig.II.1.

## **II.2. Tissue culture techniques**

### ***II.2.1 Maintenance of cell cultures***

Kc<sub>167</sub> and S2R<sup>+</sup> cells were maintained in *Drosophila* Schneider's Medium (Gibco), supplemented with 10% Foetal Bovine Serum (FBS) (Sigma) and 5% Penicillin-Streptomycin (Gibco) at 25°C in a humidified incubator at atmospheric levels of CO<sub>2</sub>. Unfortunately, following change to the composition of the media in January 2012, by Gibco, Kc<sub>167</sub> cells no longer survived, and experiments using S2R<sup>+</sup> cells were also affected. All protocols were readjusted, and the latest ones are presented here. The Kc<sub>167</sub> cell line was reacquired and cultured in Shields's and Sang M3 Insect Medium (Caisson Labs) supplemented with 1g/L of yeast extract (Sigma), 2.5g/L peptone (Fisher Scientific), 10% FBS (Sigma) and 5% Penicillin-Streptomycin (Gibco). However, some

experiments with Kc<sub>167</sub> cells could never be replicated following media change and are not presented in this thesis.

Table.II.4 qRT-PCR primers

Gene	Primer	Sequence
Rpl32	Forward	GACGCTTCAAGGGACAGTATCTG
	Reverse	AAACGCGGTTCTGCATGAG
Dome	Forward	ACTTTCGGTACTCCATCAGC
	Reverse	TGGACTIONCACCTTGATGAG
Tsg101	Forward	GAGGAGACACAAATAACAAAGTACC
	Reverse	TGAGTGTCCATCAACCAAATAC
Rab5	Forward	CACAGCTTAGCTCCCATGTA
	Reverse	TGTTTGACAAATCTGCCTTG
Socs36E	Forward	AGTGCTTTACTGCTGCGACT
	Reverse	TCGTGAGTATTGCGAAGT
Hop	Forward	GCATTCACGCACAATATAGC
	Reverse	TTCATCTCCTACCTGGGTATCT
Elongin B	Forward	CTACGCGGATGTGGGTTA
	Reverse	GTGCAAGCGAAGTAGGAAAA
Elongin C	Forward	GTATCTCCCGGAAATGCAC
	Reverse	GCCATGTACGTGAAGCTGAT
Cullin5	Forward	CGTCGTCCCGTCTCATATAC
	Reverse	GCACACAAGAGGCCATTATC

### ***II.2.2 Transfections***

Transient transfections of cells were performed using Effectene transfection kit (Qiagen). For transfections, cells were plated at following concentrations: 1x10<sup>6</sup> per well in 24-well plate, 2x10<sup>6</sup> per well in 12-well plate, 5x10<sup>6</sup> per well in 6-well plate, 12.5x10<sup>6</sup> per T25 flask, 37.5x10<sup>6</sup> per T75 flask. All plastic-ware was acquired from Corning. Amount of

transfected DNA was always totalled at 2 $\mu$ g per well in 6-well plate and scaled accordingly. Amount of individual plasmids used in transfections was determined based on comparable amounts of protein expressed, assessed by western blotting. Cells were incubated at 25 $^{\circ}$ C for at least 2 days to allow proteins to express, before harvesting for western blotting. For RNAi experiments, transfected cells were incubated for 24h before treating with dsRNA (section II.2.3).

### ***II.2.3 RNAi-mediated knockdowns***

Cells were plated at the same concentrations as outlined above and allowed to settle for 2 hours. Media was substituted for serum-free medium (prepared as the media used for maintenance, without FBS) containing diluted dsRNAs. For volumes and concentrations see Table.II.5. After 1 hour incubation at 25 $^{\circ}$ C, volume was doubled with standard maintenance media supplemented with 10% FBS. Knockdown was allowed to occur for 4-5 days, depending on the experimental requirements.

Table.II.5 Concentration of dsRNAs per well

Plate	Volume per well ( $\mu$ l)	Amount of dsRNA ( $\mu$ g)
6-well plate	1500	12
12-well plate	600	6
24-well plate	300	3
96-well plate	75	1

### ***II.2.4 Preparation of Upd2-GFP conditioned media***

Kc<sub>167</sub> cells in 6-well plate were transfected with 2 $\mu$ g pAc-Upd2-GFP plasmid, as described previously. Cells were incubated until reaching confluence, resuspended and moved into a T75 flask. A single well was put into one flask and volume was topped up to 15ml with maintenance medium. After 5 days, medium was collected, filtered through 0.2 $\mu$ M filters (Thermo Scientific) divided into 1ml aliquots and frozen at -80 $^{\circ}$ C. The ability of the conditioned media to induce transcriptional response of the pathway in a

luminescence reporter assay (Section II.2.5), was used as a determining factor for amount of conditioned media used in future experiment with each batch. To generate mock-conditioned media cells were transfected with pAc5.1C/V5-HisC plasmid (Invitrogen). Protocol was adapted from (Vidal *et al.*, 2010).

### ***II.2.5 Luminescence assays***

Cells were co-transfected with required plasmid, 500ng of *pAc-6x2xDrafLuc*, 250ng of *pAc-Renilla-Luc* and topped up to 2µg with pAc5.1/V5-HisC vector (Invitrogen) per well in 6-well plate (Müller *et al.*, 2005). Following 24h incubation, wells were moved into 96-well plates, seeding 80k cells per well, topped up to 50µl with maintenance media and left for 2hour to settle. If required, dsRNAs were applied as described previously, however the maintenance media to double the volume was supplemented with mock or Upd2-GFP-conditioned media. If no dsRNA was used, volume was topped up to 100µl with mock or Upd2-GFP-conditioned media and incubated for 4-5 days. On the day of analysis, media was removed and replaced with 40µl of lysis buffer (BL buffer and 0.3% Triton-X100) per well and incubated for 10 min at room temperature. 60µl of firefly luciferase buffer (BL buffer, B2 buffer and 35.7mM D-Luciferin (Apollo Scientific)) was added to each well and the firefly luciferase luminescence recorded using a Mithras LB 940 plate reader (Berthold Technologies). 60µl of Renilla luciferase buffer (BL buffer, B2 buffer and 0.2mM Coelenterazine (Apollo scientific)) was added per well and the luminescence measured again using a 500nm short pass emission filter (ie. a filter that allows all wavelengths shorter than 500nm to pass through it while blocking light of longer wavelengths).

Schematic representation of reporters is shown in Fig.II.2.

BL buffer : 50mM Hepes pH 7.6, 0.5mM EDTA, 0.33mM Phenylacetic acid, 0.07mM

Oxalic acid

B2 buffer : 415mM DTT, 33mM ATP, 1mM AMP

Protocol was adapted from (Wright *et al.*, 2011).

*Figure.II.2 Transcriptional pathway activity reporters*

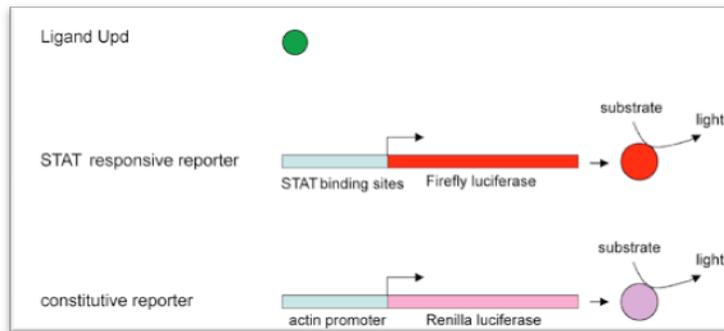


Figure.II.2 Transcriptional pathway activity reporters. Schematic representation of reporters used to assess transcriptional activity of the JAK/STAT pathway.

### ***II.2.6 Immunohistochemistry***

3 days after transfection as described above, cells were seeded at  $3 \times 10^5$  per 10mm glass coverslip in a well in 24-well plate. Cells were allowed to settle for 4 hours, washed three times with PBS (Sigma) and fixed with 4% formaldehyde in PBS for 20min. Fixed cells were washed 3x in PBS, incubated with PBST (PBS with 0.1% TritonX-100) for 20min and incubated with blocking media (PBST supplemented with 3% BSA) for 30min. Primary antibodies diluted in blocking media, concentrations are provided in Table.II.6, were applied onto cells and incubated at 4°C overnight. Primary antibodies were washed off with three washes in PBST (each 10min long) and incubated in secondary antibodies in blocking media for 2 hours at room temperature (for concentrations, see Table.II.6). Alexa Fluor conjugated phalloidin (Invitrogen) at 1:50 concentration was added to the secondary antibody mixture. Media was changed to PBST with Hoechst at 2µg/ml final concentration and incubated for 10min. Cells were washed with PBST three times for 10min each time and dry slide was mounted using Paramount (Thermo Fisher).

### ***II.2.7 Dextran uptake assay***

Cells treated with dsRNAs as described above, were seeded at  $3 \times 10^5$  per 10mm glass coverslip in a well in 24-well plate and allowed to settle for 4 hours. Cells were washed with PBS and incubated with media containing Alexa Fluor conjugated dextran

Figure.II.3 Dextran uptake analysis

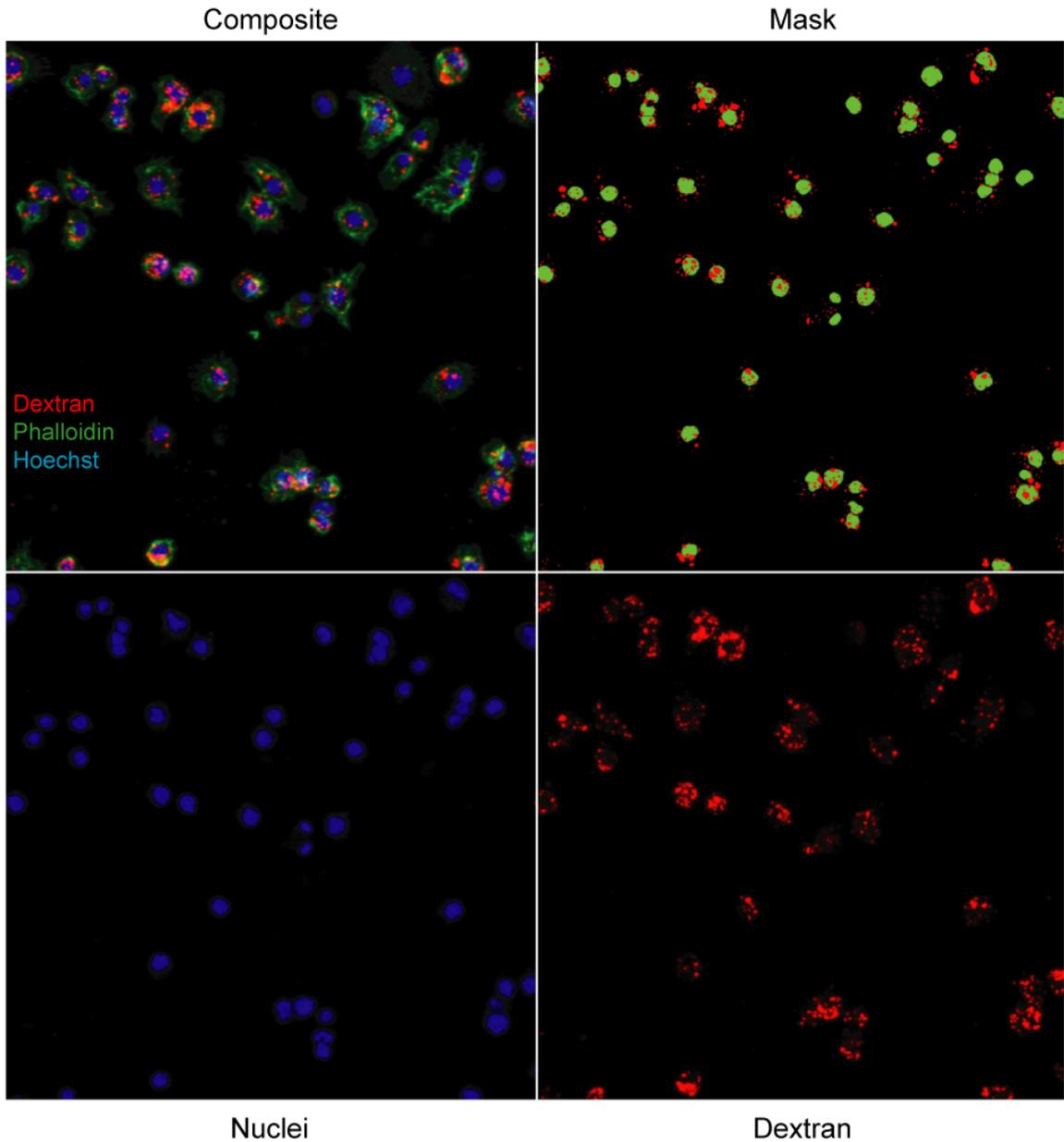


Figure.II.3 Dextran uptake analysis. The original image input into the MetaXpress software (top left), was separated into Red (bottom right) and Blue (bottom left) channels, corresponding to dextran and Hoechst, respectively. Those two channels were used to generate quantification mask (top right) used for analysis.

(MW=10,000) (Invitrogen) at 1 $\mu$ g/ $\mu$ l and mock or ligand-conditioned media. At indicated times, cells were washed with ice cold PBS, fixed and prepared as described above.

### ***II.2.8 Confocal microscopy and image analysis***

All slides were visualised using Zeiss LSM 510 confocal microscope. The standard image size is 1024 x 1024 pixels. Cells were visualised using a 63X oil immersion objective. Images were prepared using Image J (v1.43) or Adobe Photoshop CS v8.0. Z-stacks were obtained when imaging slides for dextran uptake assay, which were then analysed as maximal projections using MetaXpress 3.1 software (MDS). Analysis was automated using Transflour protocol, with exemplary images generated by the software shown in Fig.II.3. Following parameters were used to determine internalized dextran vesicles, from which intensity was determined: vesicle size between 0.5 and 2.5 $\mu$ m with intensity of at least 50 grey levels above proximal background. At least 40 cells per condition were analysed in a single replicate.

### ***II.2.9 Biotinylation***

Cells transfected with pAc-Dome-FLAG, as described above, were washed three times with serum-free maintenance media and three times with ice cold PBS. Ice cold PBS with 0.25mg/ml of EZ-link NHS-Biotin (Thermo Fisher) was left on cells for one hour at 4<sup>o</sup>C, washed away with three washes in PBS and three washes of maintenance media supplemented additionally with 0.2% BSA to sequester any unbound biotin. Cells were incubated with maintenance media for required periods of time in maintenance media at 25<sup>o</sup>C and lysed in standard lysis buffer: 50mM Tris-HCl pH7.4, 1mM EGTA, 1 mM EDTA and 0.5% Triton X-100, freshly supplemented with Complete Mini EDTA-Free tablet protease inhibitor cocktail tablet (Roche). Lysates were incubated for 30min on a rocking shaker at 4<sup>o</sup>C, centrifuged at 7,000g for 5min at 4<sup>o</sup>C and supernatant was incubated with Streptavidin Magnetic Beads (New England BioLabs) at 4<sup>o</sup>C overnight. Following incubation, beads were washed three times with lysis buffer, boiled in 3x Laemmli buffer and resolved by SDS-PAGE (Section II.3.2).

### ***II.2.10 Pharmacological agents***

To block *de novo* protein synthesis, cells transfected with pAc-Dome-FLAG were incubated with maintenance media supplemented with cyclohexamide at 10µg/ml (Sigma) for 30min at room temperature prior to experiment. In experiments utilising pharmacological inhibitors of degradation, cells were incubated with maintenance media supplemented with cyclohexamide and 10µM MG132 (Tocris Bioscience), 0.1µM Bafilomycin A1 (Sigma), 10µM N-ethylmaleimide (NEM) (Sigma) or appropriate carrier control (DMSO or ethanol) for 30min prior to the experiment and for the duration of the experiment.

## **II.3 Biochemical techniques**

### ***II.3.1 Immunoprecipitation***

Cells were prepared as described previously and treated as required with mock or ligand-conditioned media. They were subsequently lysed for 30min at 4°C on horizontal shaker. In experiments focusing on protein phosphorylation, Kinase immunoprecipitation lysis buffer (Kinase IPLB; 50mM Tris-HCl pH7.4, 1mM EGTA, 1mM EDTA, 5mM β-Glycerophosphate, 2.5mM Na-Pyrophosphate, 1mM Na-Orthovanadate, 0.5% Triton X-100 supplemented freshly with a Complete Mini EDTA-Free protease inhibitor cocktail tablet (Roche)) was used, otherwise standard lysis buffer was used (Section II.2.9). Lysates were cleared by centrifugation at 7,000g for 5min at 4°C and incubated with primary antibodies for 4h at 4°C with gentle agitation, followed by incubation with 1:5 Dynabeads (Novex, Invitrogen) overnight at 4°C with gentle agitation. Proteins were eluted into 3x Laemmli Buffer by boiling and stored on ice for analysis by SDS-PAGE and western blotting.

### ***II.3.2 SDS-PAGE and western blotting***

Appropriate volumes of samples were loaded on to Mini-PROTEAN TGX 4-15% gradient gels (Bio-Rad) and run for 1h 30min at 90V in Running Buffer (25mM Tris, 19mM Glycine, 0.04% SDS). Proteins were transferred onto Nitrocellulose

membranes (GE Healthcare) in Transfer Buffer (47mM Tris, 38mM Glycine, 0.037% SDS, 20% methanol) for 1h 15min at 70V. Membranes were incubated in blocking solution (5% Horse Serum (Sigma) in 0.5% TBS-Tween-20 (TBST)) for 30min at room temperature and incubated with primary antibodies diluted in blocking solution. Primary antibodies and concentrations are listed in Table.II.6. Following overnight incubation at 4°C, membranes were washed 3 times with TBST and incubated with secondary antibodies (listed in Table.II.6) in blocking solution for 2 hours at room temperature. Subsequently, membranes were washed 3 times with TBST and developed using ECL Western Blotting Detection Reagents (GE Healthcare) and Hyperfilm ECL (GE Healthcare) on Optimax 2012 X-Ray Film Processor (Protec).

TBS: 135mM NaCl, 25mM Tris, 2.5mM KCl; pH adjusted to 7.4 with HCl.

Table.II.6 Antibodies used

Antibody	Microscopy	Immunoprecipitation	Blotting
<b>Primary antibodies</b>			
Anti-FLAG M2 (Sigma)	1:500	1:200	1:2,500
Anti-HA (Roche)	1:500	1:200	1:2,500
Anti-pTyrosine (Calbiochem)			1:400
Anti- $\alpha$ -Tubulin (Sigma)			1:5,000
Anti-STAT92E (Santa Cruz)		1:50	1:1,000
Anti-GFP (Abcam)			1:250
Anti-Ubiquitin (Cell Signalling)			1:500
<b>Secondary antibodies</b>			
HRP anti-rabbit (Dako)			1:5,000
HRP anti-rat (Dako)			1:5,000
HRP anti-mouse (Dako)			1:5,000
HRP anti-goat (Dako)			1:5,000
AF647 anti-mouse (Invitrogen)	1:1,000		
AF647 anti-rat (Invitrogen)	1:1,000		

### ***II.3.3 Isolation of SOCS36E protein***

SOCS36E constructs in pRSET A plasmid were used for *in vitro* synthesis using PURExpress kit (New England BioLabs), according to manufacturer instructions. Constructs were labelled with <sup>35</sup>S-Methionine (PerkinElmer).

Alternatively, FLAG-tagged SOCS36E constructs were expressed in cells and pulled down with anti-FLAG antibody and bound to beads, as described above (Section II.3.1). Following overnight incubation, beads were washed 3x with lysis buffer and incubated with 3x FLAG peptides to elute protein of interest (Sigma), according to manufacturer guidelines. Eluted proteins were used for further experiments.

### ***II.3.4 in vitro kinase activity assay***

Cells transfected with HA-tagged Hop or Hop mutants were lysed and immunoprecipitated as described previously. Kinases bound to beads were washed three times with Kinase IPLB and three times with Kinase activity assay reaction buffer (KAAB): 20mM HEPES pH7.4, 10mM NaCl, 20mM MgCl<sub>2</sub>, 100mM NaF, 0.2mM NaOva, 2mM DTT and 10mM MnCl<sub>2</sub>, and resuspended in 25µl of 2xKAAB. 20µl of water or proteins obtained as described above, was added to the reaction and incubated at 30°C with agitation for 10min. Enzymatic reaction was initialised by addition of 5µl of 1mM ATP spiked with <sup>32</sup>P γ-ATP (PerkinElmer) to ca. 1x10<sup>6</sup>cpm per µl. Reactions were incubated at 30°C for 20min with occasional agitation and terminated by adding 200µl of 20mM EDTA. Three washes with Kinase IPLB preceded elution into 3xLaemmli buffer by boiling. Eluted Hop was resolved by SDS-PAGE and either transferred to nitrocellulose membrane (GE Healthcare) or dried and exposed to phospho-screen (Kodak) and quantified using Molecular Imager FX (Bio-Rad). Linearity between amount of bead bound kinase and <sup>32</sup>P radioactivity was checked (Fig.II.4). Analysis was performed using Quantity One software (Bio-Rad). Protein levels were determined by blotting the nitrocellulose membrane for HA or staining gel with Bio-Safe Coomassie G-250 Stain (Bio-Rad).

Figure.II.4 Linearity of  $^{32}\text{P}$  incorporation

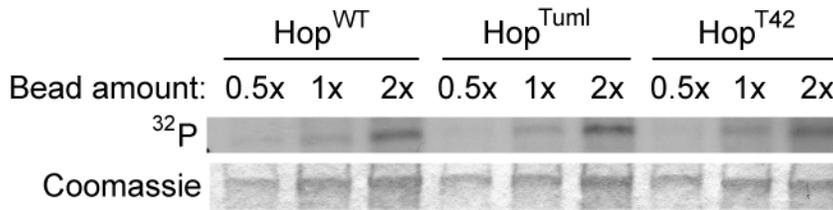


Figure.II.4 Linearity of  $^{32}\text{P}$  incorporation. *In vitro* kinase activity assay was conducted with batch of kinases bound to beads that were subsequently split into three samples with increasing volume of beads, as indicated. Samples were then resolved on SDS-PAGE gel and the amount of  $^{32}\text{P}$  incorporated assessed by exposure to luminescence film.

### II.3.5 Preparation of MultiDsk beads

MultiDsk-GST plasmid was a kind gift of Jesper Svejstrup (Cancer Research UK, London Research Institute). BL21 bacteria strain were transformed and grown in 5ml media overnight at  $37^{\circ}\text{C}$ . 600ml of media was inoculated with the overnight grown culture and grown at  $37^{\circ}\text{C}$  until optical density of 0.6 at  $\text{OD}_{600}$  was reached. At this point bacteria was induced with IPTG at 1mM final concentration and incubated at  $30^{\circ}\text{C}$  for 4 hours. Bacteria were centrifuged at 3,500g at  $4^{\circ}\text{C}$  for 20min and the pellet was resuspended in 20ml of STE buffer supplemented with  $100\mu\text{g/ml}$  lysozyme (Sigma) and incubated for 15min on ice. DTT was added to 5mM final concentration and vortexed briefly. N-lauryl sarcosine was added to 1.5% final concentration and vortexed for 5s. Suspension was sonicated 4x with probe sonicator (MSE UK) at amplitude 5 microns in periods of 15s, spaced 30s apart. This sample was centrifuged at 10,000g for 10min at  $4^{\circ}\text{C}$  and filtered through a cheesecloth. TritonX-100 was added to a final concentration of 3% and DTT concentration was re-adjusted to 5mM. Mixture was incubated for 5min at  $4^{\circ}\text{C}$  and 1ml of 50% slurry of BD BaculoGold Glutathione Agarose Beads was added (BD Biosciences) and incubated for 4h at  $4^{\circ}\text{C}$  on a roller. Beads were washed in 20ml of wash buffer 1, followed by batch wash in wash buffer 2. Beads were then moved into empty polystyrene purification columns (Thermo Fisher) and washed with 10ml of wash

buffer 1, followed by 10ml of wash buffer 2, allowing both to filter through by gravity-flow. Beads were then moved into eppendorf, equilibrated by 3x washes with PBS and stored at 4°C in PBS supplemented with 0.1% Sodium Azide. Expression of MultiDsk was checked at each stage (Fig.II.5).

Protocol adapted from (Wilson *et al.*, 2012).

STE Buffer: 10mM Tris pH8, 1mM EDTA, 100mM NaCl, supplemented with proteinase inhibitor tablets

Wash buffer 1: 1xPBS, 450mM NaCl, 10% glycerol, 0.1mM EDTA, 0.1% TritonX-100, 2mM DTT, supplemented with proteinase inhibitor tablets

Wash buffer 2: 50mM KPhos buffer pH7.4, 50mM NaCl, 10% glycerol, 1mM  $\beta$ -Mercaptoethanol, 0.2% TritonX-100, supplemented with proteinase inhibitor tablets

*Figure.II.5 Generation of MultiDsk bound to beads*

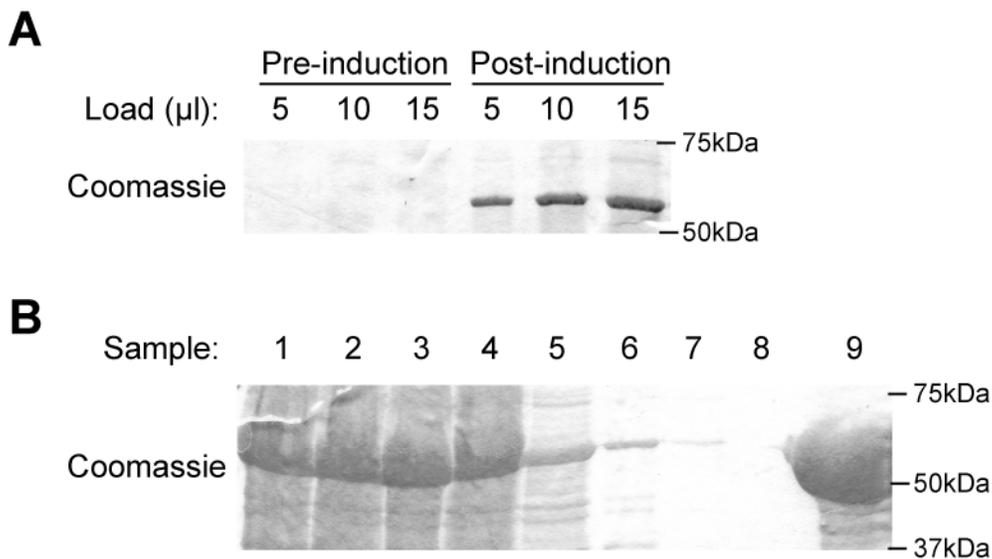


Figure.II.5 Generation of MultiDsk bound to beads. (A) Effectiveness of BL21 induction with IPTG. Molecular weight of MultiDsk-GST is 58kDa. (B) Samples were taken at each step in sequence of the protocol on MultiDsk bound beads generation: 1. Suspension in STE; 2. Sonication; 3. Centrifugation at 10,000g; 4. Supernatant from incubation with GST-beads; 5. Batch wash with wash buffer 1; 6. Batch wash with wash buffer 2; 7. Column wash with wash buffer 1; 8. Column wash with wash buffer 2; 9. Eluted from beads.

### ***II.3.6 Assessment of Dome ubiquitination***

Cells transfected with Dome-FLAG were treated with mock or ligand conditioned media for 30min at room temperature and lysed in standard lysis buffer freshly supplemented with 2mM iodoacetic acid (Sigma), 10 $\mu$ M MG132 (Sigma) and 10 $\mu$ M NEM (Sigma) and protease inhibitor tablets (Roche). Following 30min lysis at 4<sup>o</sup>C, samples were centrifuged at 7,000g for 5min at 4<sup>o</sup>C. Supernatants were used for immunoprecipitation as described previously. Proteins were analysed by western blotting for Ubiquitin, or incubated with MultiDsk beads generated as described previously and analysed by western blotting for FLAG.

### **II.4. Statistics and reproducibility**

All experiments were replicated with at least three biologically independent repeats. Images of western blots are representative. Quantification and statistical analysis was performed using Excel (Microsoft) or Prism 6 (GraphPad) software. Statistical significance was tested with paired Student's t-test or ANOVA followed by Bonferroni's multiple comparisons test, as appropriate. Legend to statistical significance: \*\*\* -  $p < 0.005$ ; \*\* -  $p < 0.01$ ; \* -  $p < 0.05$ .

## Chapter III Molecular analysis of Dome

### III.1. Introduction

#### *III.1.1 Shared homology between Dome and mammalian cytokine receptors*

*Domeless* (*Dome*), also called *master of marelle*, was the last core component of the *Drosophila* JAK/STAT pathway identified (Brown *et al.*, 2001; Chen *et al.*, 2002). It has been characterised as most similar to mammalian IL-6 cytokine receptor family, mainly gp130 and LIFR (Fig.III.1A and B) (Brown *et al.*, 2001; Chen *et al.*, 2002). Sequence analysis tools show that Dome contains three FNIII domains and a CBM (Fig.III.1). The CBM is only partially conserved in Dome, with incomplete WSxWS motif, although extensive evidence suggests that it can none-the-less bind Upd ligands (Silver *et al.*, 2005; Vidal *et al.*, 2010). Consistent with this, recent report has shown that human Leptin can signal via Dome, indicating the level of functional conservation of the ligand binding domain, even though the shared identity in amino acid sequence between Dome and Leptin receptor, Ob-Rb, is fairly low (Fig.III.1B) (Rajan and Perrimon, 2012).

Despite conservation of FNIII and CBM domains, an Immunoglobulin-like domain common to mammalian type I cytokine receptors is not present in Dome (Fig.III.1A). Besides Ig-like domains, Dome also does not contain any classical Box domains (Fig.III.1A) (Hirano *et al.*, 1997; Taga and Kishimoto, 1997; Grant and Begley, 1999; Brown *et al.*, 2001; Chen *et al.*, 2002). This structural difference is interesting as genetic interactions between Dome:Hop and Dome:STAT92E have been established *in vivo* as well as *in vitro* (Brown *et al.*, 2001; Chen *et al.*, 2002; Kallio *et al.*, 2010, reviewed in Arbouzova and Zeidler, 2006).

#### *III.1.2 JAK/STAT pathway regulation at the level of the receptor*

Regulation of the JAK/STAT signalling pathway occurs at numerous levels of the signalling cascade, as described previously (Section.I.5.2). This includes regulation of receptor's post-translational modifications, its stability and interaction partners, including

Figure.III.1 Homologies between Dome and mammalian cytokine receptors

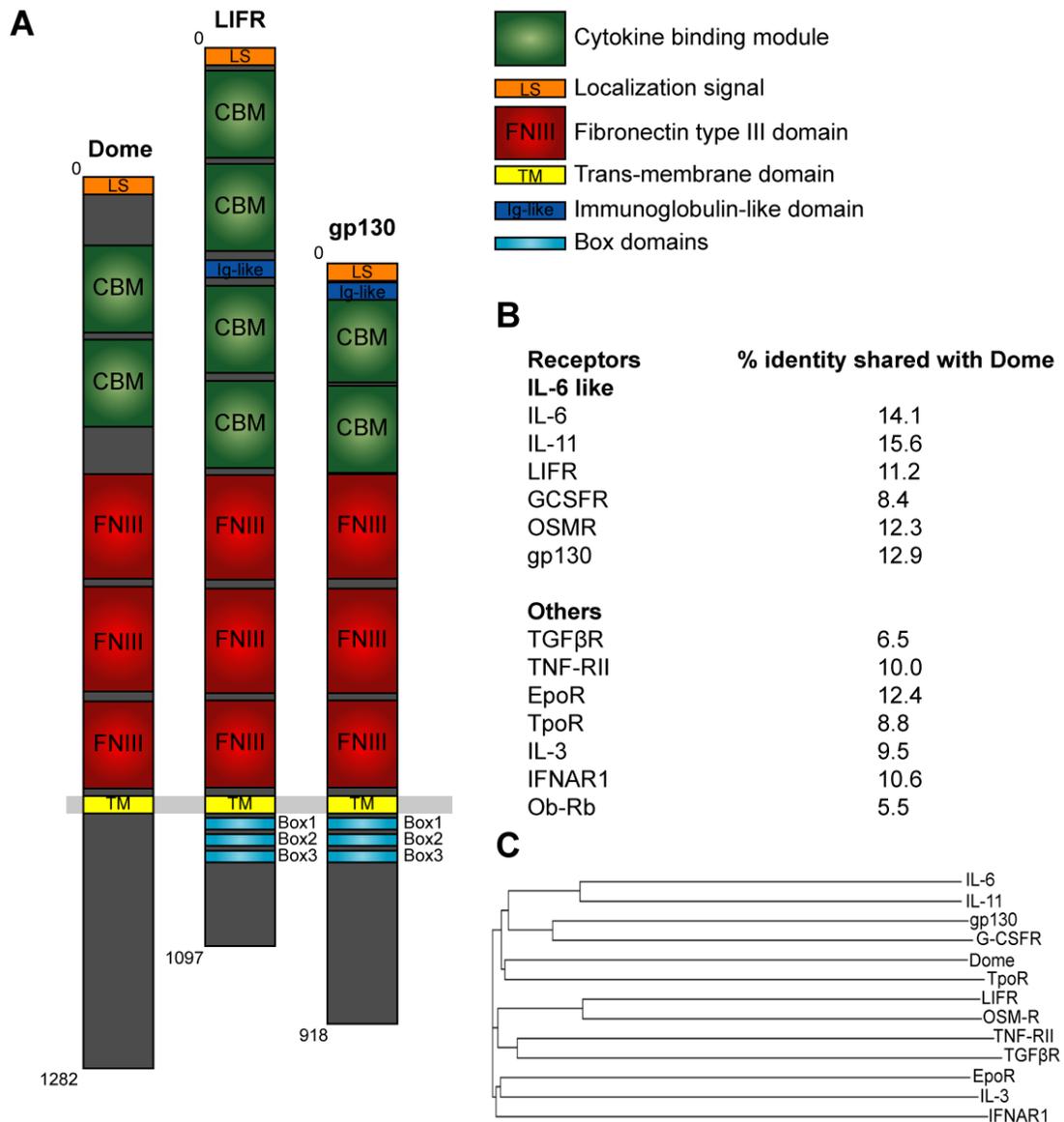


Figure.III.1 Homologies between Dome and mammalian cytokine receptors. (A) Schematic representation of Dome, LIFR and gp130 receptors. Domains are colour-coded, as described. Numbers indicate the amino acid length of proteins. Adapted from Brown *et al.*, 2001. (B) Table representing shared identity between Dome and indicated human receptors. Numerical values obtained using ClustalW2 sequence alignment analysis tool. (C) Phylogram representing common ancestry of Dome and human cytokine receptors. Phylogram generated by ClustalW2 sequence alignment analysis tool.

oligomerization. Numerous, but certainly not all, mammalian cytokine receptors have been investigated for various aspects of regulation, with abundant evidence suggesting a link between receptor activation by ligand binding, phosphorylation, ubiquitination and receptor endocytosis. In addition, structure-function studies of those receptors have also been undertaken. However, no common pattern that could be applied to all receptors has been proposed; a testimony to how divergent the mammalian family of cytokine receptors truly is, as represented by phylogram (Fig.III.1C).

The *Drosophila* JAK/STAT pathway contains only a single positively acting receptor and an additional negatively acting regulator that behaves like a constitutively inactive receptor termed Eye-transformer or Latran, that can heterodimerize or oligomerize with Dome (Brown *et al.*, 2001; Chen *et al.*, 2002; Kallio *et al.*, 2010) (Makki *et al.*, 2010). Under homeostatic conditions, Dome has been shown to homodimerize in a ligand-independent but tissue-specific manner (Brown *et al.*, 2003). The exact mechanisms governing dimer formation as well as its modulators remain to be determined, however, *in vivo* analysis indicates that homodimerization is required for JAK/STAT pathway activation, a phenomenon that has also been described for EpoR and the Leptin receptor (Ob-R) in mammalian systems (Couturier and Jockers, 2003).

Endocytosis has been established as a regulator of the JAK/STAT pathway activity in mammals as well as in *Drosophila* (Devergne *et al.*, 2007; Vidal *et al.*, 2010, reviewed in Platta and Stenmark, 2011). However, both reports on regulation of the JAK/STAT pathway by endocytosis in *Drosophila*, focused on the general outcomes of signalling following manipulation of endocytic machinery. As a tool, endocytosis of GFP-fused ligands has previously been visualised in the Zeidler lab and while interaction of ligand:Dome has been shown, the fate of the receptor has not been investigated. Considering the fact that receptors can potentially be recycled from numerous endocytic compartments, it is interesting to see what endocytic process the *Drosophila* Dome receptor undergoes and what governs this process (Grant and Donaldson, 2009; Raiborg and Stenmark, 2009; Platta and Stenmark, 2011).

## III.2. Results

### ***III.2.1 Over-expression of tagged wild-type Dome does not affect pathway activity***

Of the handful of antibodies against Dome generated by various labs (Ghiglione *et al.*, 2002; Stephen Brown, personal communication) all have been used for imaging, rather than biochemical techniques. Unfortunately, neither antibody was sufficiently specific for my requirements, therefore I used tagged Domeless constructs expressed from a constitutive Actin promoter in Kc<sub>167</sub> and S2R<sup>+</sup> cells. Investigation of the expression pattern of Dome-FLAG in S2R<sup>+</sup> cells by confocal microscopy shows an even distribution of Dome throughout the cell, with enrichment around the plasma membrane (Fig.III.2A). Expression of Dome-FLAG in much smaller Kc<sub>167</sub> cells appeared cytoplasmic with numerous puncta throughout the cell and potentially enriched in the epinuclear region (Fig.III.2B). The puncta observed in Kc<sub>167</sub> cells appeared bigger compared to the ones found in S2R<sup>+</sup>, however the exact subcellular localization was not investigated. This indicates that expression of the constructs used by us is similar to the one reported for endogenous protein (Ghiglione *et al.*, 2002).

Over-expression of proteins in cells can have detrimental effect on physiology of cells, often caused by a dominant negative effect. To exclude this possibility, I investigated the JAK/STAT pathway activity using a previously established STAT92E transcriptional activity reporter termed *6x2xDrafLuc*, based on a Firefly luciferase gene downstream of a minimal promoter and six multimerised regions from the *Drosophila* Raf promoter each of which contains two potential STAT92E binding sites (activity reporter) and Rennila (constitutively expressed viability reporter) luciferase (for detailed description see materials and methods) (Müller *et al.*, 2005). Expression of Dome-FLAG or Dome-HA did not have any effect on transcriptional pathway activity following ligand stimulation (Fig.III.2C). Considering that cytokine receptors become phosphorylated in response to ligand stimulation, I also investigated whether Dome constructs were tyrosine phosphorylated in response to cytokine stimulation (Fig.III.2D). Immunoprecipitation of Dome followed by anti-phosphotyrosine western blotting revealed that Dome is phosphorylated under steady state conditions, a modification that increases significantly following stimulation. Based on these results, it can be concluded that Kc<sub>167</sub> cells

Figure.III.2 Expression of Dome constructs does not affect pathway activity

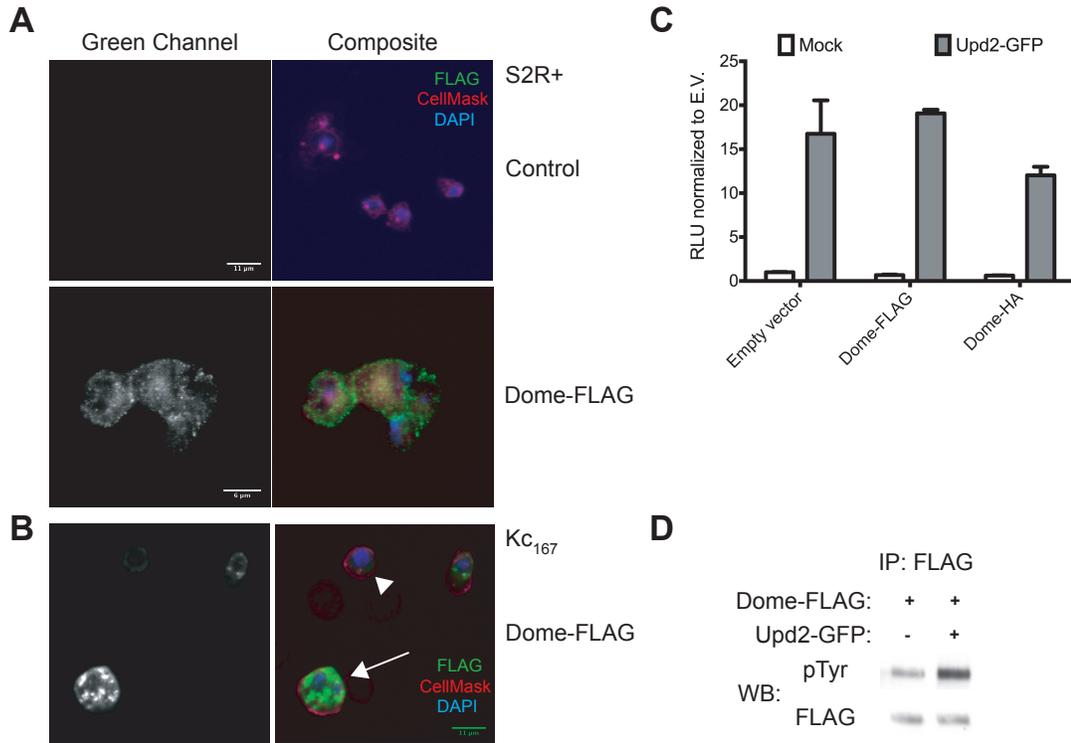


Figure.III.2 Expression of Dome constructs does not affect pathway activity. (A, B) Confocal images of S2R+ (A) or Kc<sub>167</sub> (B) cells transiently transfected with indicated plasmid and stained with appropriate antibodies. (A) Transfected Dome has even cytoplasmic distribution with enrichment around plasma membrane in large S2R+ cells. Blue channel was boosted to show weak DAPI stain. (B) Numerous intracellular puncta with perinuclear enrichment observed in Kc<sub>167</sub> cells. Arrows indicate transfected cells, arrowheads point to not transfected cells. (C) Kc<sub>167</sub> cells co-transfected with *6x2xDraf* luciferase reporters and plasmids carrying HA or FLAG tagged Dome under Actin promoter were incubated with mock or Upd2-GFP conditioned media for 4 days. At this point luminescence was measured as described in material and methods section (II.2.5). Results were normalized to Empty vector transfected cells treated with mock conditioned media. Statistical significance was checked with two-way ANOVA followed by Bonferroni's multiple comparisons test. No statistically significant difference was observed between Empty vector and Dome-FLAG or Dome-HA transfected cells in mock or Upd2-GFP treated group. Error bars represent SEM. (D) Kc<sub>167</sub> cells transfected with Dome-FLAG were treated with mock or Upd2-GFP conditioned media for 10min and lysed. Lysates were subjected to anti-FLAG immunoprecipitation and resolved on SDS-PAGE gels followed by blotting with anti-phosphotyrosine or anti-FLAG antibody, as described in materials and methods (II.3.2).

expressing tagged Dome is phosphorylated as expected and does not affect JAK/STAT pathway transcriptional activity.

### ***III.2.2 Mutation of Proline<sub>925</sub> in Dome weakens the interaction with Hop and acts as a dominant negative mutation***

JAK/STAT pathway is a phosphorylation dependent signalling pathway, where virtually all components of the pathway, with the exception of the ligand, are phosphorylated by the JAK kinase. Dome has been reported to physically associate with Hop, however the site of interaction was not determined (Kallio *et al.*, 2010). Identification of the interaction site might be of added importance in our studies, considering reports on stabilization of the receptor following JAK binding (described in Section.I.2.5).

Lack of Box domains in Dome sequence indicated that Dome might share interaction motifs common to class II cytokine receptors, such as interferon receptors. Literature searches identified the <sup>266</sup>LPKS sequence in IFN $\gamma$ R1 to be required for interaction with JAK1 (Kaplan *et al.*, 1996). The proline residue in the sequence has been reported to be essential for interaction with JAK. I identified a similar motif in Dome - <sup>924</sup>LPQG, with conserved leucine and proline and very similar structurally remaining two residues. Moreover, this motif is located in the same position as the one in IFN $\gamma$ R1, precisely 13 amino acids downstream of the trans-membrane domain. I hypothesised that mutation of the proline residue might disrupt Dome interaction with Hop, therefore I generated a construct in which proline was substituted for isoleucine, generating Dome P925I. As a control, pull down of wild-type Dome results in a clear co-immunoprecipitation of Hop (Fig.III.3A). Introduction of P925I mutation caused a significant decrease in Dome:Hop interaction. The remaining weak interaction observed might be attributed to Dome P925I dimerizing with endogenous wild-type Dome, therefore indirectly associating with Hop. While the change in wild-type Dome phosphotyrosine levels in response to ligand stimulation was fairly modest (especially by comparison to Fig.III.2D), likely caused by higher level expression of exogenous Hop. However, no change in pTyr levels of Dome P925I was observed following stimulation with ligand-conditioned media (Fig.III.3A). The decrease in association with Hop was

Figure.III.3 Proline<sub>925</sub> is required for Dome interaction with Hop

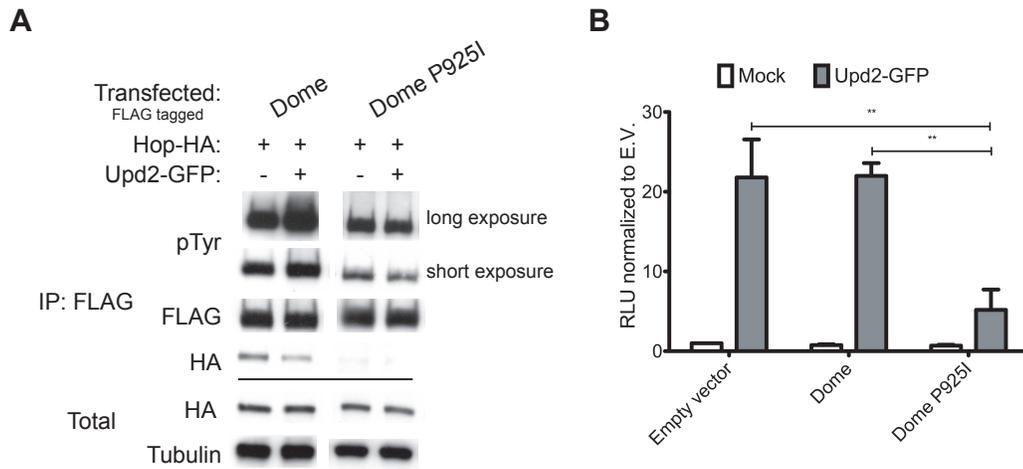


Figure.III.3 Proline<sub>925</sub> is required for Dome interaction with Hop. (A) Kc<sub>167</sub> cells co-transfected with FLAG tagged Dome or Dome P925I constructs and Hop-HA were stimulated with mock or Upd2-GFP conditioned media as indicated for 10min and lysed with Kinase lysis buffer. Lysates were subjected to anti-FLAG immunoprecipitation and analysed by SDS-PAGE and western blotting using anti-phosphotyrosine, anti-FLAG or anti-HA antibodies, as described in materials and methods section (II.3.2). Top two panels represent different film exposure times. (B) Transcriptional activity of Kc<sub>167</sub> cells transfected with indicated FLAG-tagged constructs and incubated with mock or Upd2-GFP conditioned media for 4 days was analysed using luminescence assay as described in materials and methods section (II.2.5). Results were normalized against Empty vector transfected cells treated with mock conditioned media. Statistical significance was measured by two-way ANOVA followed by Bonferroni's multiple comparison test. Significance between indicated samples is shown with \*\*, p<0.01. No significant difference was observed between the mock treated cells. Error bars represent SEM.

correlated by a marked decrease in Dome P925I tyrosine phosphorylation at basal levels. Moreover, expression of Dome P925I mutant resulted in a significant decrease in stimulated pathway activity induced by ligand conditioned media (Fig.III.3B). Considering the likely dimerization of Dome (Brown *et al.*, 2003), this decrease in pathway activity suggests a dominant negative activity of the Dome P925I mutation. These results imply that P<sub>925</sub> residue is critical for Dome interaction with Hop and indicates that this interaction is required for ligand-mediated pathway activation.

### ***III.2.3 Knockout of tyrosine<sub>966</sub> in Dome does not have any effect on pathway activity***

Tyrosine phosphorylation of a receptor by its associated JAK kinase allows for STAT binding and therefore is required for pathway activation. Out of 10 tyrosine residues present in the cytoplasmic tail of Dome, only one complies with the mammalian STAT3 consensus binding motif of YxxQ (where x represents any amino acid) – this is the tyrosine at position 966 (Y<sup>966</sup>TPQ) (Fig.III.4A) (Stahl *et al.*, 1995; Gerhartz *et al.*, 1996). Another putative STAT92E binding site is Y1022, whose context is similar to the mammalian STAT5 consensus motif YLx[VIL] (May *et al.*, 1996). I attempted to generate single point mutations substituting aforementioned tyrosines for phenylalanines and therefore impair association with STAT92E. Unfortunately, protein containing the Y1022F mutation did not express or was not stable and this construct was not pursued any further. Expression of Dome Y966F mutant in cells did not affect JAK/STAT pathway transcriptional activity as indicated by the *6x2xDrafLuc* reporter (Fig.III.4B). I further investigated pY levels of this mutant as well as its ability to interact with STAT92E (Fig.III.4C). No change in phosphorylation of the receptor was observed upon introduction of single-point mutation, which is not surprising considering the number of tyrosine residues present in the cytoplasmic tail of Dome. In addition, interaction with STAT92E was not affected by ligand stimulation or introduction of the Y966F point mutation. Taken together, these results suggest that tyrosine residue at position 966 is not essential for JAK/STAT pathway activity.

Figure.III.4 Mutation of tyrosine<sub>966</sub> residue does not affect pathway activity

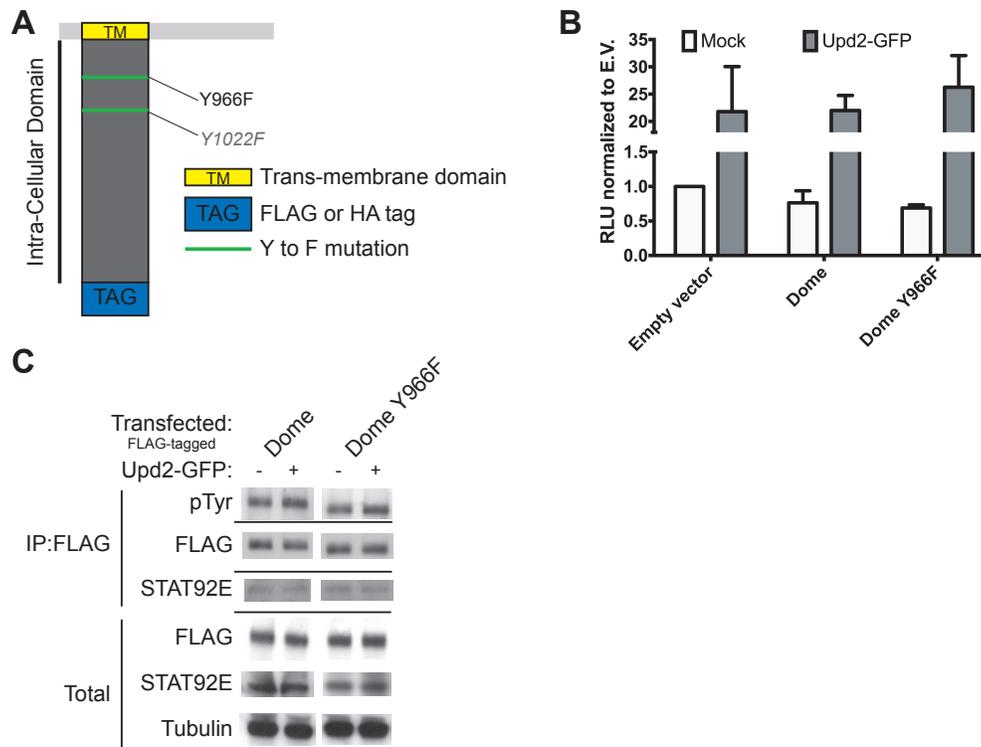


Figure.III.4 Mutation of tyrosine<sub>966</sub> residue does not affect pathway activity. (A) Schematic representation of the cytoplasmic domain of Dome. Tyrosine at position 966 was mutated to phenylalanine. Construct that did not express is shown in grey and italicised. (B) Transcriptional activity of Kc<sub>167</sub> cells transfected with FLAG-tagged indicated constructs was measured with the luminescence assay as described in materials and methods section (II.2.5) after 4 days of incubation with mock or Upd2-GFP conditioned media. Results were normalised to Empty vector transfected cells treated with mock conditioned media. No statistically significant difference between samples within mock or Upd2-GFP treated group was observed upon analysis with two-way ANOVA followed by Bonferroni's multiple comparisons test. Error bars represent SEM. (C) S2R<sup>+</sup> cells transfected with Dome-FLAG or Dome P925I-FLAG constructs were treated with mock or Upd2-GFP conditioned media as indicated for 10min prior to lysis with Kinase IPLB buffer. Lysates were subjected to co-immunoprecipitation of endogenous STAT92E following pulldown with anti-FLAG antibody as described in materials and methods section (II.3.1). SDS-PAGE with subsequent western blot analysis was undertaken using anti-pTyr, anti-FLAG or anti-STAT92E antibodies.

Figure.III.5 Dome undergoes constitutive degradation

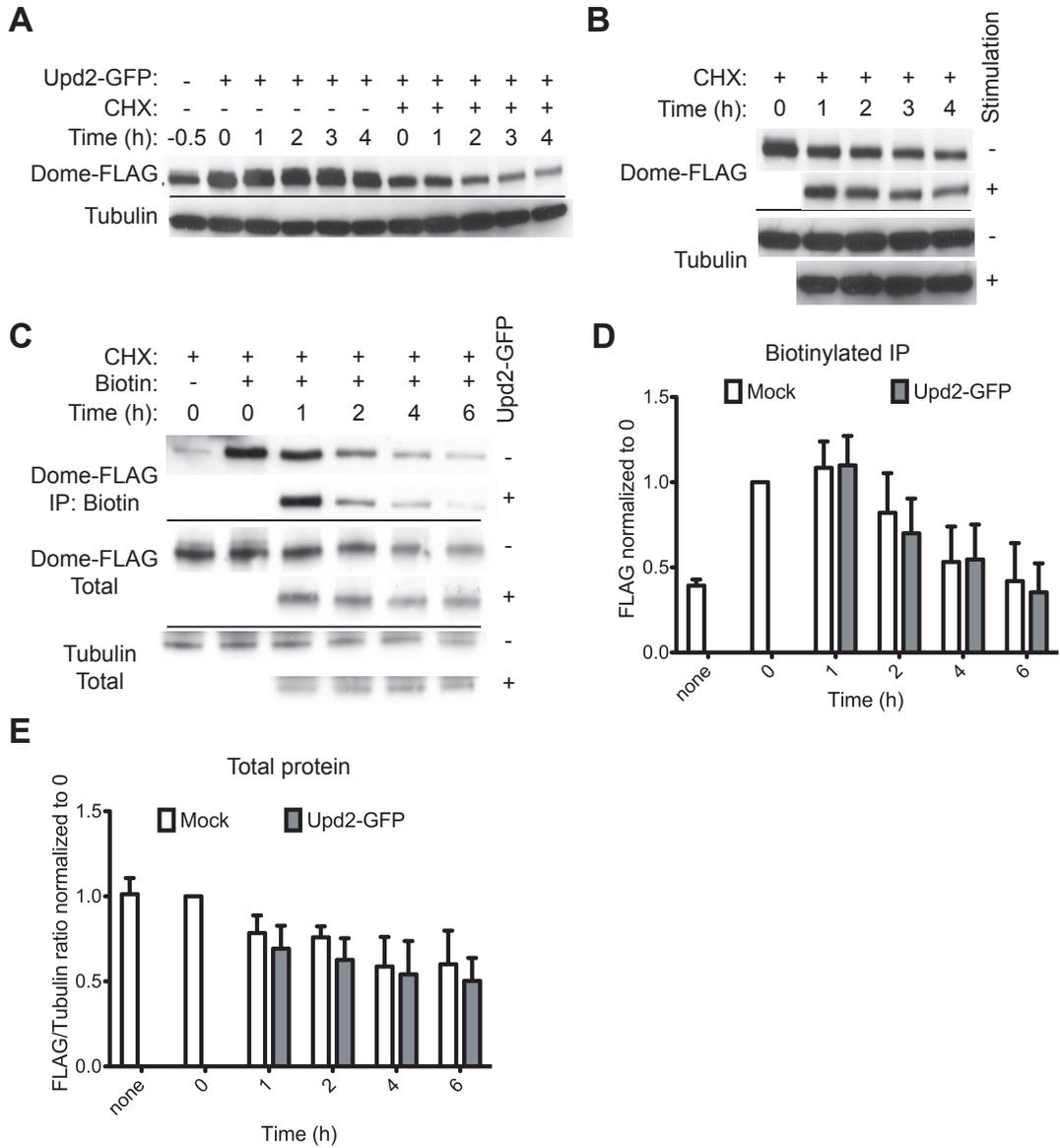


Figure.III.5 Dome undergoes constitutive degradation. (A)  $KC_{167}$  cells batch-transfected with Dome-FLAG were treated with cycloheximide at  $10\mu\text{g/ml}$  or its carrier as indicated for 30min prior to incubation with Upd2-GFP conditioned media containing CHX at the same concentration. Cells were lysed at indicated time points and analysed by SDS-PAGE followed by western blotting. (B) Dome-FLAG batch-transfected  $KC_{167}$  cells were incubated with CHX at  $10\mu\text{g/ml}$  for 30min prior to as well as during incubation with mock (top panel) or Upd2-GFP conditioned media (upper middle panel) for indicated periods of time, lysed and analysed with SDS-PAGE and western blotting. (C) Dome-FLAG batch transfected  $KC_{167}$  cells were treated with NHS-biotin to label cell-surface proteins for 1h at  $4^{\circ}\text{C}$ . Following wash, cells were incubated with mock or Upd2-GFP conditioned media for indicated periods of time at which point they were lysed. Lysates were subjected to Streptavidin immunoprecipitation (top two panels) followed by SDS-PAGE and western blot analysis. Total protein levels were assessed alongside (middle two panels). Experiment was performed in the presence of CHX as described in A. (D and E) Quantification of the biotinylated fraction (D) and total protein fraction (E) in C. Results were normalised against mock treated biotinylated sample at timepoint 0. "none" corresponds to not biotinylated control cells. Two-way ANOVA analysis followed by Bonferroni's multiple comparisons test did not reveal any statistically significant differences between mock or Upd2-GFP stimulated cells in the same timepoint. Error bars represent SEM.

### ***III.2.4 Dome undergoes rapid turnover irrespective of ligand binding***

An integral component of regulation of cytokine signalling is endocytosis of the receptor, a process that concludes with receptor degradation or recycling back to the plasma membrane following ligand uncoupling (Raiborg and Stenmark, 2009; Platta and Stenmark, 2011). Due to the necessity of expressing receptor constructs from the exogenous *actin* promoter, I could not study stability of the receptor without blocking *de novo* protein translation using cyclohexamide (Fig.III.5A). Stimulation of cells with ligand conditioned media resulted in a build-up of Dome protein over time in control cells. Conversely, in the presence of cyclohexamide I observed a decrease in levels of the receptor over time, indicating that Dome is degraded in the presence of ligand (Fig.III.5A). Closer investigation of the degradation dynamics of the receptor revealed that stimulation with Upd2-GFP resulted in only a mild increase in degradation rate compared to the steady state unstimulated conditions (Fig.III.5B). However, the total protein fraction investigated might include proteins that are undergoing trafficking to the plasma membrane or proteins still undergoing folding in the chaperones. In order to obtain better understanding of Dome degradation dynamics, I therefore biotinylated cell surface proteins prior to stimulation with mock or ligand-conditioned media (Fig.III.5C). Quantification of the biotinylated-receptor levels (ie. that proportion of the receptor pool present at the plasma membrane at the beginning of the experiment) indicated that plasma-membrane-localised Dome is degraded at a constant rate, with potentially weak trend towards enhanced degradation following pathway stimulation (Fig.III.5D). This trend was also reflected by quantification of the total receptor levels, which indicates that differences between mock and ligand-conditioned media treated cells at all time-points are not statistically significant (Fig.III.5E). The degradation time frame is in line with previous report by Devergne and colleagues (Devergne *et al.*, 2007). Taken together these results indicate that Domeless is degraded at a constant rate that is only modestly enhanced by ligand stimulation.

Endocytosis of the receptor begins with an internalization event, in which the receptor is removed from contact with extracellular environment. Hypothesising that degradation rate should correlate with internalization rate, I used dextran uptake assay, as a measurement tool for the receptor-mediated fluid phase endocytosis, to investigate

internalization rate of endogenous Domeless population. Cells were treated with mock or ligand conditioned media containing fluorescently labelled dextran for indicated periods of time and then washed to remove any extracellular dextran (as described in materials and methods). Uptake of dextran appeared not to be homogenous across the cell population (Fig.III.6A), therefore I utilized large sample pools and automated analysis software to quantify differences between samples. This quantification of dextran intensity indicated initially faster uptake by cells treated with mock conditioned media, with visible difference at 10min of treatment (Fig.III.6B). Within 30min the difference in dextran intensity between control and ligand stimulated cells disappeared, indicating potential stabilization of the receptor at the plasma membrane following ligand binding. In order to confirm that the dextran internalization observed is mediated by liquid phase endocytosis of the receptor, I performed RNAi-mediated knockdown of Dome and measured dextran uptake (Fig.III.6C). At 15min of treatment, cells depleted of Dome accumulated much less dextran compared to the control cells, indicating that Dome is responsible for significant portion of constant plasma membrane turnover in Kc<sub>167</sub> cells.

### ***III.2.5 Internalization motifs in Dome remain unknown***

Endocytosis begins with internalization of the receptor that might be triggered by different events depending on the context (as described previously in Section.I.3.5). Previous reports indicated clathrin mediated endocytosis to act as a regulator of the *Drosophila* JAK/STAT pathway (Devergne *et al.*, 2007; Vidal *et al.*, 2010). Clathrin mediated endocytosis of cytokine receptors depends heavily on adaptor molecule AP-2 to mediate Clathrin's interaction with the cargo molecule. The  $\alpha$  subunit of AP-2, called  $\alpha$ -adaptin, was identified as a negative regulator of the JAK/STAT pathway in a directed RNAi mediated screen (Vidal *et al.*, 2010). Considering the high conservation of the molecule across species, I hypothesised that the AP2 binding motif in the receptor will also be conserved. Two distinct consensus AP2 binding motifs are commonly accepted:

Figure.III.6 Fluid phase endocytosis is affected by Dome knockdown but not by ligand presence

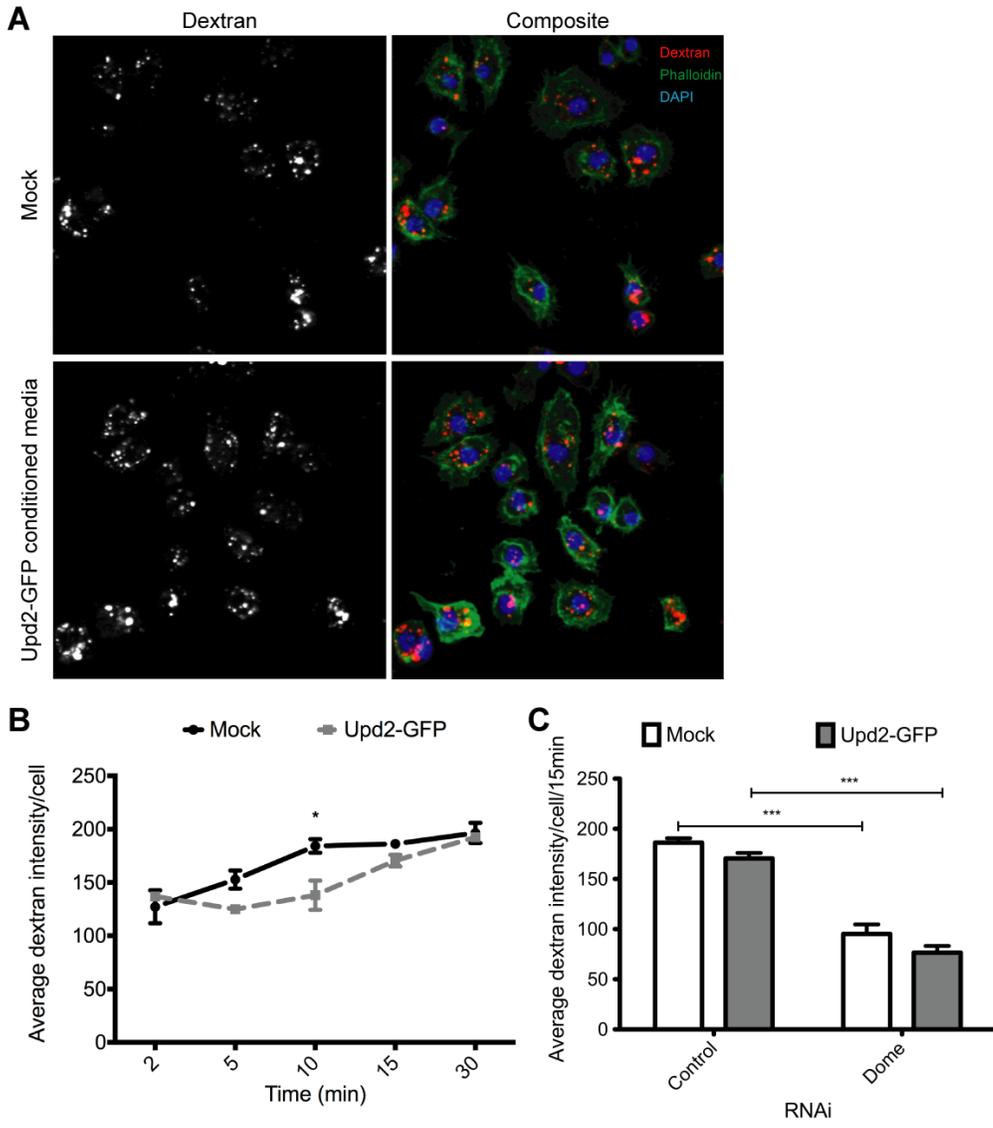


Figure.III.6 Fluid phase endocytosis is affected by Dome but not by ligand presence. (A-C) Kc<sub>167</sub> cells treated with Control or otherwise indicated dsRNA for 4 days were incubated with mock or ligand-conditioned media supplemented with fluorescently labelled dextran at 1mg/ml, fixed and imaged using Confocal microscope, as described in materials and methods section (II.2.7). (A) Representative images of Kc<sub>167</sub> cells treated with Control dsRNA at 15min of treatment with mock or Upd2-GFP conditioned media. (B) Quantification of the intensity of dextran accumulated in cells at indicated periods of incubation with mock (black) or Upd2-GFP (grey) conditioned media. At least 40 cells per condition per timepoint per replicate were analysed using maximal projection of Z-stacks obtained, as described in materials and methods section (II.2.8). Student's T-test was used for statistical analysis with \*\*\*, p<0.05; \*\*, P<0.01; \*, p<0.005. Error bars represent SEM. (C) Quantification of average dextran intensity per cell at 15min of incubation with mock or Upd2-GFP conditioned media in cells treated with Control or Dome dsRNA, as described in B.

Figure.III.7 Individual internalization motifs do not play a role in the regulation of Dome endocytosis or JAK/STAT pathway activity

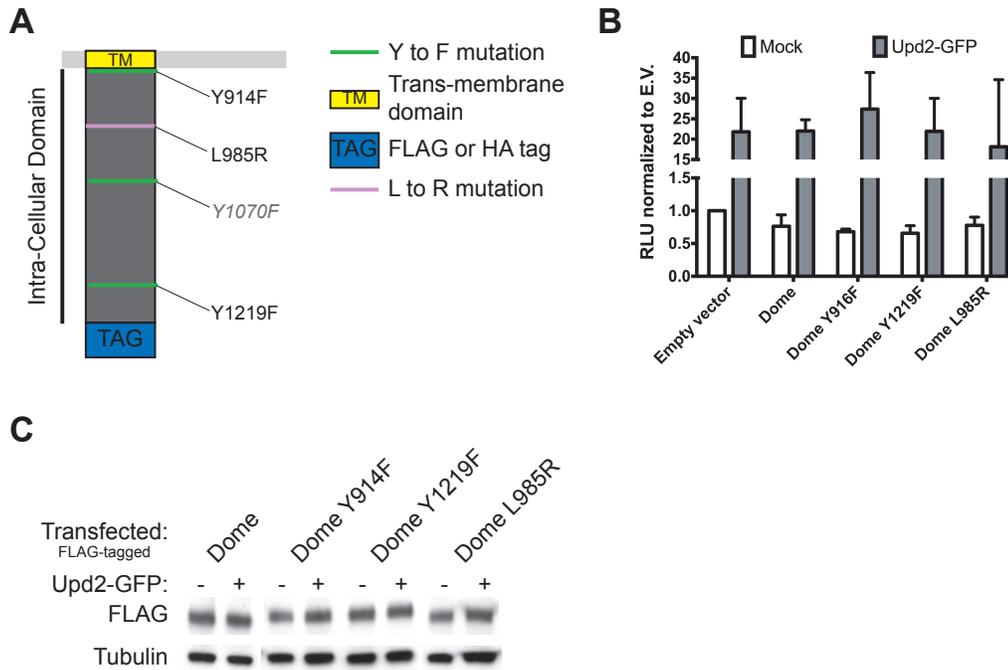


Figure.III.7 Internalization motifs in Dome individually do not play a role in regulation of JAK/STAT pathway activity. (A) Schematic representation of cytoplasmic domain of Dome with indicated mutated residues. Construct that was not cloned is shown in grey and italicised. (B) Transcriptional activity of the pathway in Kc<sub>167</sub> cells expressing indicated FLAG-tagged constructs was measured after 4 days of incubation with mock or Upd2-GFP conditioned media using luminescence assay, as described in materials and methods section (II.2.5). Results were normalised to Empty vector transfected cells treated with mock conditioned media. Two-way ANOVA followed by Bonferroni's multiple comparisons test analysis did not indicate any statistically significant differences among samples treated with mock or Upd2-GFP conditioned media. Error bars represent SEM. (C) Kc<sub>167</sub> cells transfected with FLAG-tagged indicated constructs were treated with mock or Upd2-GFP conditioned media for 15min prior to lysis. To determine stability of transfected constructs, lysates were analysed using SDS-PAGE and western blotting, as described in materials and methods section (II.3.2)

Yxx $\phi$  motif ( $\phi$  represents bulky hydrophobic amino acid) or acidic dileucine motif [E/D]xxxL[L/I] (Ohno *et al.*, 1995; Ehrlich *et al.*, 2001; Bonifacino and Traub, 2003). Analysis of Dome intra-cellular domain revealed presence of four tyrosine-based motifs: <sup>914</sup>YRKM, <sup>1022</sup>YLAM, <sup>1070</sup>YIKP and <sup>1219</sup>YTTM; and a single dileucine motif: <sup>981</sup>ESSKLL (Fig.III.7A). I therefore attempted to generate single point mutations that would impair AP2 binding by mutating tyrosine to phenylalanine residues in the putative binding motifs. Unfortunately, despite numerous attempts I was unable to generate a molecule carrying the Y1070F mutation, which failed due to unknown reasons while the Y1022F mutation failed to express, as mentioned previously. However, I was able to generate a single point mutation of leucine to arginine at position 985 in the dileucine motif (Fig.III.7A). Considering that knockdown of proteins involved in clathrin-mediated endocytosis resulted in an increase in *6x2xDrafLuc* pathway reporter activity (Müller *et al.*, 2005; Vidal *et al.*, 2010), I hypothesised that mutation of sites involved in interaction with AP2 would have a similar effect on this reporter. However, none of the mutations generated affected reporter activity (Fig.III.7B). Moreover, I did not observe the expected increase in stability of the receptor (Fig.III.7C). Although care should be taken when interpreting negative results, my data suggests that individual putative AP-2 interaction sites do not affect the receptor or signalling output of the pathway, potentially due to the redundancy between them, as reported for the EGF receptor (Goh *et al.*, 2010). Unfortunately time constraints and focus on other aspects of my project did not allow me to generate combinatorial mutations and test this hypothesis for Dome.

### ***III.2.6 Proteins involved in regulation of Dome phosphorylation have no effect on Dome stability***

Activation of cytokine receptors by ligand binding is followed by phosphorylation events that often lead to receptor endocytosis. Proteins regulating phosphorylation can directly or indirectly regulate receptor stability – association with JAKs has been shown to stabilize the receptors (Haan *et al.*, 2006; Radtke *et al.*, 2006), while SHP2 has been shown to dephosphorylate Y466 of IFN $\alpha$ R therefore allowing AP2 binding (Carbone *et al.*, 2012). I showed previously that Dome P925I mutant's interaction with Hop is

Figure.III.8 Hop does not affect stability of the receptor

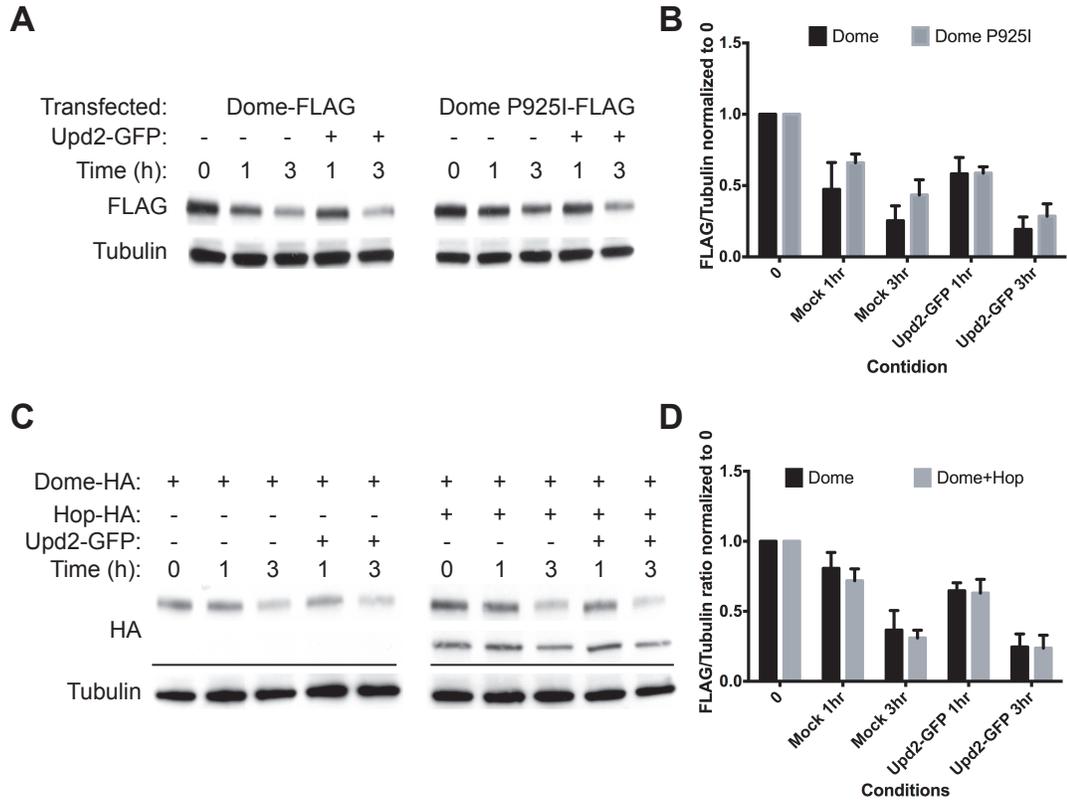


Figure.III.8 Hop does not affect stability of the receptor. (A) S2R+ cells transfected with Dome-FLAG or Dome P925I-FLAG constructs were treated with mock or Upd2-GFP conditioned media for indicated periods of time and lysed. Lysates were analysed by SDS-PAGE and western blotting. Experiment was performed in the presence of CHX. (B) Quantification of blots as shown in C, n=3. Results were normalised to value obtained from cells transfected with the same construct at timepoint 0. Student's T-test revealed no statistical differences between Dome and Dome P925I transfected samples at matching timepoints. Error bars represent SEM. (C) S2R+ Cells transfected with Dome-HA alone or co-transfected with Hop-HA and stimulated with mock or ligand-conditioned media for indicated periods of time and lysed. Lysates were analysed with SDS-PAGE and western blotting. Experiment was performed in the presence of CHX. (D) Quantification of blots as shown in C, n=3. Results were normalised to value obtained from cells transfected with the same construct combination at timepoint 0. Student's T-test revealed no statistical differences between Dome and Dome with Hop co-transfected samples at matching timepoints. Error bars represent SEM.

impaired, therefore it is a suitable model to study whether Hop regulates Dome stability. To investigate this aspect, cells expressing wild-type Dome or Dome P925I were stimulated with mock or ligand-conditioned media and stability of the transfected constructs was assessed (Fig.III.8A). Quantification of the blot revealed no statistically significant differences between the two constructs, although Dome P925I was trending towards increased stability compared to wild-type Dome under steady state conditions, but not following stimulation with ligand (Fig.III.8C and D). In the converse experiment, I expressed Dome on its own or together with exogenous Hop. Again, I observed no difference in receptor stability (Fig.III.8E and F). Taken together, these results indicate that Dome stability is not regulated by Hop.

### ***III.2.7 Genes involved in endocytosis affect Dome stability and JAK/STAT pathway activity***

Previous reports on the role of endocytosis in regulating JAK/STAT pathway in *Drosophila* suggested that endocytic processing of ligands was delayed following the silencing of genes involved in the process (Vidal *et al.*, 2010). To confirm that the receptor follows the same endocytic path as ligands do, I knocked down proteins that play key roles in endocytosis - Rab5 is essential for early endosome formation, Deep orange is involved in multivesicular body biogenesis and TSG101 is a component of the ESCRT-I complex required for sorting of ubiquitinated cargo in the endocytic pathway (Raiborg and Stenmark, 2009; Platta and Stenmark, 2011). RNAi-mediated knockdown of those proteins resulted in stabilisation of the receptor (Fig.III.9A). Moreover, stimulated but not basal JAK/STAT pathway activity was increased in cells treated with dsRNAs against endocytic genes, as reported previously (Fig.III.9B) (Müller *et al.*, 2005; Vidal *et al.*, 2010). Interestingly, knockdown of SOCS36E resulted in stabilisation of the receptor and increased pathway activity following treatment with mock or ligand-conditioned media. This result will be expanded on in the following chapter. Taken together, this data suggests the same route of endocytosis for the receptor and the ligand.

Figure.III.9 Genes involved in endocytosis affect Dome stability and pathway activity

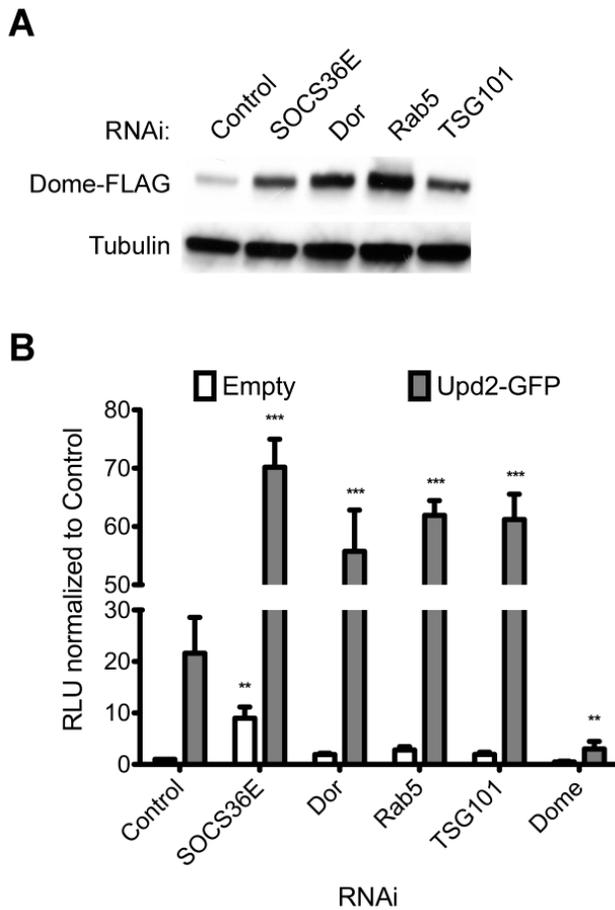


Figure.III.9 Genes involved in endocytosis affect Dome stability and pathway activity. (A)  $Kc_{167}$  cells batch transfected with Dome-FLAG were split and treated with indicated dsRNAs for 4 days prior to lysis. Levels of Dome were assessed by SDS-PAGE and western blotting. (B) Transcriptional activity of the pathway in  $Kc_{167}$  cells treated with indicated dsRNAs and stimulated with mock or Upd2-GFP conditioned media was assessed using luminescence assay, as described in materials and methods section (II.2.5). Results were normalised against Control cells treated with mock conditioned media. Statistical significance was determined using two-way ANOVA analysis followed by Bonferroni's multiple comparisons test. Stars indicate statistically significant difference between dsRNA treatments and Control cells within mock or Upd2-GFP stimulated groups, with \*\*\*,  $p < 0.005$ ; \*\*,  $p < 0.01$ . Error bars represent SEM.

### ***III.2.8 Endocytosis of Dome terminates in the lysosome***

Previous reports showed internalised Upd2-GFP localises to lysosomes (Vidal *et al.*, 2010). This data coupled with the role of genes central to multivesicular body biogenesis in the regulation of Dome stability suggests that the lysosome is likely to be the site of receptor degradation. To assess this possibility, I utilised a pharmacological approach. Cells expressing Dome-FLAG were stimulated with ligand conditioned media in presence of cyclohexamide and either Bafilomycin A1, MG132 or DMSO (Fig.III.10A). Addition of the lysosomal inhibitor Bafilomycin A1 very potently inhibited Dome degradation in comparison to DMSO, a carrier control. Treatment with MG132, widely used as an inhibitor of proteosomal degradation, caused a slight increase in protein stability, that was not statistically significant upon quantification (Fig.III.10B). These results provide a clear indication that Dome is degraded in the lysosome following ligand binding.

Similar Dome degradation dynamics between the steady state and ligand stimulated conditions indicate similar degradation route under both conditions. To confirm this, I compared degradation of Dome with and without ligand stimulation in the presence of Bafilomycin (Fig.III.10B). Again, I observed efficient inhibition of degradation that was irrespective of ligand presence. Moreover, Bafilomycin efficiency remained unchanged even at half the recommended concentration (50nM). This suggests that Dome undergoes lysosomal degradation irrespective of ligand binding.

### ***III.2.9 Dome undergoes dynamic ubiquitination***

While testing pharmacological agents during my investigation I observed that addition of N-Ethylmaleimide (NEM), a known deubiquitinase inhibitor, to ligand conditioned media resulted in increased stability of a prominent high molecular weight band (arrowhead), not visible in stimulated control cells, but present in cells prior to stimulation (Fig.III.11A). Unfortunately, NEM interferes with tubulin assembly (Phelps and Walker, 2000), therefore no loading control is available and no conclusion can be made regarding overall Dome stability. Increased stability of the top band in the presence of deubiquitinase inhibitor implies that Dome is not only ubiquitinated, but also

Figure.III.10 Dome undergoes degradation in the lysosome

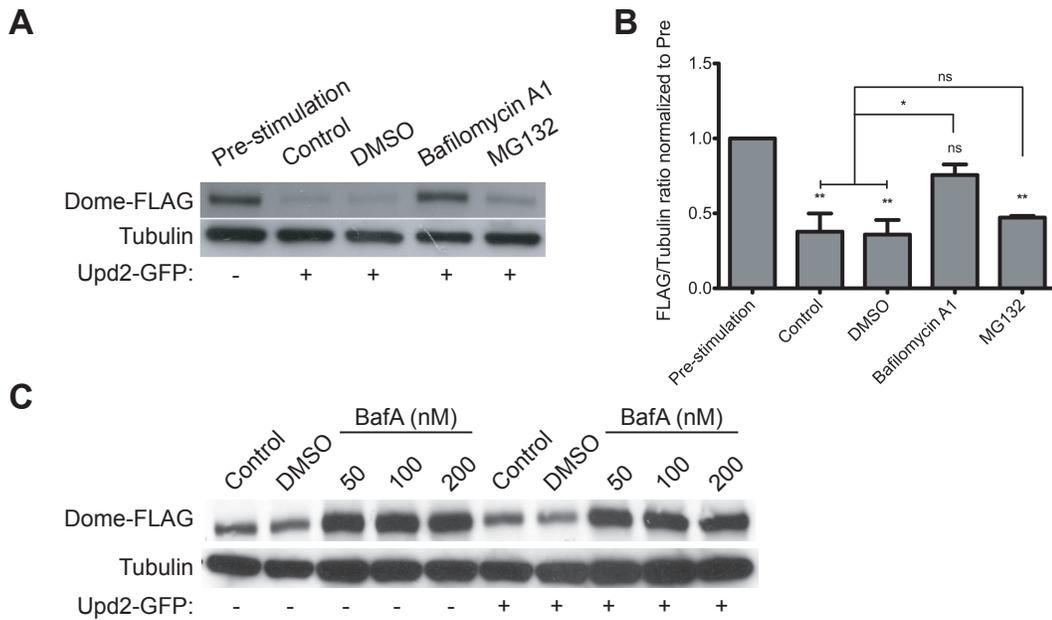


Figure.III.10 Dome undergoes degradation in the lysosome. (A) Kc<sub>167</sub> cells batch-transfected with Dome-FLAG were incubated with 0.2 $\mu$ M Bafilomycin A1, 10 $\mu$ M MG132 or DMSO and stimulated with Upd2-GFP conditioned media for 2 hours. Control sample was not treated with any chemical agent, “pre-stimulation” sample was lysed at the start of stimulation with Upd2-GFP conditioned media. Lysates were analysed by SDS-PAGE and western blotting. Experiment was performed in the presence of CHX. (B) Quantification of data in A, n=3. Quantified values were normalised against Pre-stimulated sample. Stars above columns reflect statistical significance towards Pre-stimulated sample, stars above bars reflect statistical significance between indicated samples. Statistical significance was determined using ANOVA analysis followed by Bonferroni’s multiple comparisons test, with \*\*, p<0.01; \*, p<0.05; ns, not significant. Error bars represent SEM. (C) Kc<sub>167</sub> cells batch-transfected with Dome-FLAG were treated with increasing concentrations of Bafilomycin A1, as indicated, and stimulated with mock or ligand-conditioned media for two hours prior to lysis. Lysates were analysed by SDS-PAGE and western blotting. Control refers to cells not treated with pharmacological agent or carrier. Experiment was performed in the presence of CHX.

deubiquitinated. To directly assess ubiquitination of Dome, I immunoprecipitated Dome followed by blot with anti-ubiquitin antibody (Fig.III.11B). Anti-ubiquitin blot revealed double band pattern, suggesting that Dome must exist as two differentially ubiquitinated forms. I have not however determined whether the bottom band represents mono-ubiquitinated fraction or which ubiquitin chains are prevalent, although the presence of nine lysine residues in the cytoplasmic domain of Dome implies numerous possibilities regarding ubiquitination pattern and ubiquitin chain species. However, the lack of difference in the band size or pattern between ligand stimulated and non-stimulated samples prompted me to investigate further. I therefore employed a more sensitive method, based on pull-down of all ubiquitinated intracellular proteins using an Ubiquitin-specific high affinity resin termed MultiDsk (Fig.III.11C) (Wilson *et al.*, 2012). Surprisingly, a stable poly-ubiquitinated Dome population, represented by top band, was observed in both stimulated and non-stimulated samples. However, the lower weight band was present only in the stimulated fraction. This implies that ligand mediated pathway stimulation causes a distinct change in pattern of Dome ubiquitination, while poly-ubiquitination is a common feature of Dome, that is independent of ligand treatment.

### **III.3. Discussion**

This study provides molecular characterization of the only functional receptor of the *Drosophila* JAK/STAT signalling pathway. I showed that despite the lack of Box motifs characteristic for type I cytokine receptors, Dome associates with Hop via P952. This interaction is crucial for pathway activity as mutation of this residue results in a loss-of-function phenotype that is dominant in the presence of endogenous Dome. Mutation of Hop binding site did not have any effect on receptor stability or its degradation rate, indicating that Dome stability is not regulated by phosphorylation. Furthermore, I showed that Dome undergoes constitutive lysosomal degradation that is not affected by ligand presence. Unfortunately, I was not able to pinpoint the AP-2 or STAT92E binding sites in Dome.

Figure.III.11 Dome ubiquitination status changes following stimulation

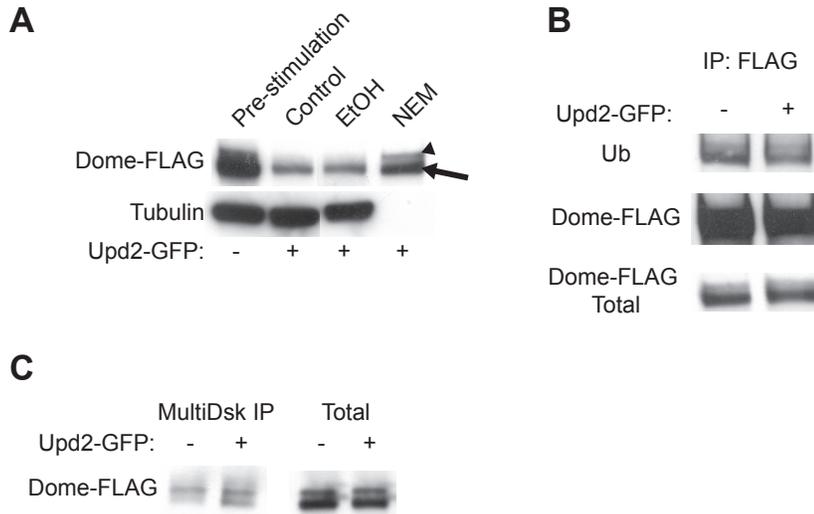


Figure.III.11 Dome ubiquitination status changes following stimulation. (A)  $Kc_{167}$  cells batch transfected with Dome-FLAG were treated with N-Ethylmaleimide (NEM) at  $10\mu M$  or EtOH and stimulated with Upd2-GFP conditioned media for two hours and lysed. Lysates were analysed by SDS-PAGE and western blotting. Control refers to non-treated cells, pre-stimulation refers to cells lysed at the start of incubation with conditioned media. Experiment was performed in the presence of CHX. (B)  $Kc_{167}$  cells transfected with Dome-FLAG were treated with mock or Upd2-GFP conditioned media for 30min prior to lysis in modified lysis buffer, as described in materials and methods section (II.3.6). Lysates were subjected to immunoprecipitation with anti-FLAG and analysed by SDS-PAGE and western blotting. (C)  $Kc_{167}$  cells were prepared and treated as in B. Lysates were subjected to immunoprecipitation using MultiDsk - ubiquitin high affinity resin followed by SDS-PAGE and western blotting with anti-FLAG antibody.

### ***III.3.1 Interactions with Hop***

Dome has been described as most homologous to mammalian gp130 family of cytokine receptors, which share a common Box motifs mediating interaction with JAKs (Hackett *et al.*, 1997; Taga and Kishimoto, 1997; Grant and Begley, 1999). I have shown that Dome:Hop interaction occurs via a motif that has been characterized for type II cytokine receptors. However, position of the Hop interaction site remains in close proximity to the membrane, a similarity which might be of particular importance for activation of the kinase in response to ligand binding. The juxtamembrane region of numerous mammalian receptors that form homodimers has been shown to be essential for activity as it mediates conformational change resulting in a shift in orientation of cytoplasmic domain, bringing JAKs in close proximity (Kurth *et al.*, 2000; Constantinescu *et al.*, 2001; Greiser *et al.*, 2002; Seubert *et al.*, 2003; Staerk *et al.*, 2011). Proximity of JAKs is required for their *trans*-phosphorylation and activation (Matsuda *et al.*, 2004). This concept is particularly relevant considering the dominant negative nature of P925I mutation in Dome, which suppresses stimulated pathway activity when expressed in the presence of endogenous Dome (Fig.III.3B). In a situation where wild-type Dome:Dome P925I dimers form, conformational changes likely bring the cytoplasmic domains of Dome constructs in close proximity, however only one Hop is present, therefore no *trans*-phosphorylation can occur.

The SH2 domain present in the STAT family of proteins provides specificity towards cytokine receptors (Lim and Cao, 2006). STAT92E was characterised as a homologue of mammalian STAT5, however, the SH2 domains of mammalian STATs are relatively well conserved among family members (Pawson and Schlessingert, 1993; Hou *et al.*, 1996). I have identified a single tyrosine residue in the cytoplasmic tail of Dome that was reminiscent of STAT5 consensus motif. Unfortunately, mutation of this residue produced a construct that did not express. Conversely, mutation of a tyrosine in the conserved STAT3 consensus sequence did not have any effects on interaction with STAT92E. This implies that STAT92E does not share the same consensus motif with STAT3 or there is more than one tyrosine residue able to mediate such interaction. The physical association of Dome with STAT92E under steady state conditions and the lack of observed increase in this interaction following ligand-mediated pathway stimulation

suggests that biochemical techniques are inadequate for such studies, possibly because of the transient nature of the interaction.

### ***III.3.2 Constitutive degradation of Domeless***

Modulation of a cell's competence to receive signals as well as termination of signalling from numerous cytokine receptors is frequently achieved by receptor endocytosis, which also plays a role in the modulation of signalling output quality (reviewed in Raiborg and Stenmark, 2009; Platta and Stenmark, 2011). For this reason it is important to understand how receptor endocytosis is initiated and regulated. Numerous interlinked processes, such as post-translational modifications, structural changes or association partners, can regulate cytokine receptor stability and serve as internalization triggers. In contrast to a large majority of cytokine receptors that become endocytosed in response to ligand binding, Dome undergoes constitutive endocytosis, even in the absence of ligand (Fig.III.5). However, trends indicate that stimulation with ligand-conditioned media does increase degradation kinetics. Even though quantification of this process seems to be statistically insignificant, implications for cell physiology and signalling output might be very relevant. The observed delay in internalization in cells treated with ligand-conditioned media (Fig.III.6B) might indicate a lag on the plasma membrane which, in the context of signalling endosomes, might have implications on the quality of the signal. Therefore, a more detailed analysis, including sub-cellular localization and kinetics of degradation, will be required for detailed characterization of the process.

Some mammalian cytokine receptors are stabilized by JAK kinase binding. However, Dome P925I mutant as well as expression of exogenous Hop did not affect degradation rate of the receptor. Moreover, degradation of Dome appeared to be higher compared to that of Hop, when co-expressing both proteins together (Fig. III.8C). This observation is contrary to the suggestion that receptor:JAK complexes behave like RTKs, as this result suggests that Hop is likely to dissociate from the receptor prior to degradation in *Drosophila* cells (Haan *et al.*, 2006).

Unfortunately, my attempts at identifying the motifs responsible for the initiation of endocytosis, which most likely involve AP-2 binding sites, were unsuccessful. First of

all, I was unable to generate mutations of all putative sites, with Dome Y1022F and Dome Y1070F constructs not expressing. Therefore, it is possible that either of those residues is required for AP-2 interaction. Another possibility is that AP-2 interaction sites are redundant, as demonstrated for EGFR and OSMR (Radtke *et al.*, 2006; Goh *et al.*, 2010). It would be interesting to generate constructs with all putative AP-2 interaction sites mutated and investigate its impact on pathway activity.

Finally, ubiquitination of the receptor might modulate receptor stability. My data indicates that Dome is constitutively poly-ubiquitinated, a finding which correlates with constitutive degradation of the receptor (Fig.III.11B and C). As ubiquitination of the cytokine receptors has been shown to regulate not only stability but also determine the degradation route and kinetics, it is very likely that the same process takes place for Dome (Haglund *et al.*, 2003; Belouzard and Rouillé, 2006; Shabek *et al.*, 2009). Interestingly, I observed a change in Dome ubiquitination pattern in response to pathway stimulation, with a lower molecular weight band appearing in response to ligand treatment. Whether this modification is causative for the delay in receptor-mediated fluid phase endocytosis (Fig. III.6) and the trend of increased degradation kinetics following pathway stimulation (Fig.III.5), remains to be determined. Further evidence for how dynamic the relationship between ubiquitination and degradation is, comes from stabilization of the higher molecular weight band (corresponding to the polyubiquitinated Dome) following treatment with deubiquitinase inhibitors. The process of dynamic ubiquitination, where deubiquitinases play important roles was previously described for mammalian cytokine receptors, further support the similarity between Dome and mammalian cytokine receptors (reviewed in Clague and Urbé, 2010).

## Chapter IV *Drosophila* SOCS36E regulates the JAK/STAT pathway via two independent mechanisms

### IV.1. Introduction

JAK/STAT signalling pathway plays numerous essential roles in development and maintenance of homeostasis (Arbouzova and Zeidler, 2006; Vainchenker and Constantinescu, 2013). Not surprisingly, numerous regulators of the pathway activity exist. One of the best characterised families of negative regulators is SOCS family of proteins. Similarly to the core pathway components, SOCS proteins have been conserved *in Drosophila at lower redundancy with the Drosophila genome encoding three SOCS-like proteins* (Callus and Mathey-Prevot, 2002; Karsten *et al.*, 2002; Rawlings *et al.*, 2004), however only SOCS36E can potently suppress the JAK/STAT pathway (reviewed in Stec and Zeidler, 2011).

#### IV.1.1 *Drosophila* JAK/STAT pathway activity induces *socs36E* expression

Previous studies have characterised *socs36E* mRNA expression profile to be similar to the one observed for Upd expression and in line with the JAK/STAT pathway activity *in vivo* (Karsten *et al.*, 2002; Stec and Zeidler, 2011). Indeed, *socs36E* promoter region contains 19 putative STAT92E binding sites (Karsten *et al.*, 2002) and cell culture experiments confirmed *socs36E* to be a strong transcriptional target for STAT92E (Bina *et al.*, 2010). Flies mutant for JAK/STAT pathway ligands or Hop kinase display much lower *socs36E* mRNA expression (Karsten *et al.*, 2002; Rawlings *et al.*, 2004), while ectopic-activation of the pathway with Hop<sup>TumI</sup> kinase or ectopic ligand expression causes an increase in *socs36E* transcription (Karsten *et al.*, 2002). This indicates that SOCS36E acts in a classical negative feedback loop, similar to the mammalian SOCS proteins (Starr *et al.*, 1997).

#### ***IV.1.2 SOCS36E regulates the JAK/STAT pathway activity***

Role of SOCS36E as the negative regulator of the JAK/STAT pathway has been established both *in vivo* and *in vitro* (reviewed in Stec and Zeidler, 2011). Indeed, numerous cell culture assays, including genome wide RNAi screens, used SOCS36E as a control (Baeg *et al.*, 2005; Müller *et al.*, 2005; Vidal *et al.*, 2010; Wright *et al.*, 2011). However, the function of SOCS36E *in vivo* remains cryptic, as null mutants are homozygous viable and fertile (Bellen *et al.*, 2004; Almudi *et al.*, 2009). At the same time, over-expression of Hop leads to lethality that can be rescued by SOCS36E expression (Callus and Mathey-Prevot, 2002). Detailed analysis of JAK/STAT pathway reveals that ectopic expression of SOCS36E has been reported to cause wing venation and wing posture defects characteristic for *upd*, *hop* or *stat92E* mutants (H.J.Muller, 1930; Yan *et al.*, 1996a; Callus and Mathey-Prevot, 2002). Moreover, SOCS36E has been shown to regulate stem cell maintenance and niche integrity in the testis as well as specification and migration of border cells in the ovary, processes that require JAK/STAT pathway activity (Silver *et al.*, 2005; Singh *et al.*, 2010; Monahan and Starz-Gaiano, 2013). Also the size and number of melanotic tumours in flies heterozygous for the constitutively active Hop<sup>Tum1</sup> allele can be reduced by ectopic expression of SOCS36E, while the converse situation, mediated by silencing of *socs36E mRNA*, is also true (Luo *et al.*, 1997; Heinrich *et al.*, 1998; Rane and Reddy, 2000; De Celis and Diaz-Benjumea, 2003; Bina *et al.*, 2010).

#### ***IV.1.3 SOCS36E plays a role in regulation of the EGFR pathway***

The JAK/STAT and EGFR signalling pathways have been found to cross-talk in numerous model systems (Shilo, 2003; Rawlings *et al.*, 2004). Not surprisingly, the regulatory mechanisms are shared by the two pathways, with mammalian SOCS4 and 5 proteins involved in regulation of the stability of EGF receptor (Kario *et al.*, 2005; Nicholson *et al.*, 2005; Bullock *et al.*, 2007). Studies on photoreceptor cell specification in the developing *Drosophila* eye and wing venation implicate SOCS36E to be involved in regulation of the EGFR pathway in a JAK/STAT independent manner (Callus and Mathey-Prevot, 2002; Almudi *et al.*, 2009). Moreover, interaction between Sevenless and SOCS36E has been found to be direct and dependent on the SH2 domain

Figure.IV.1 Homology of SOCS36E

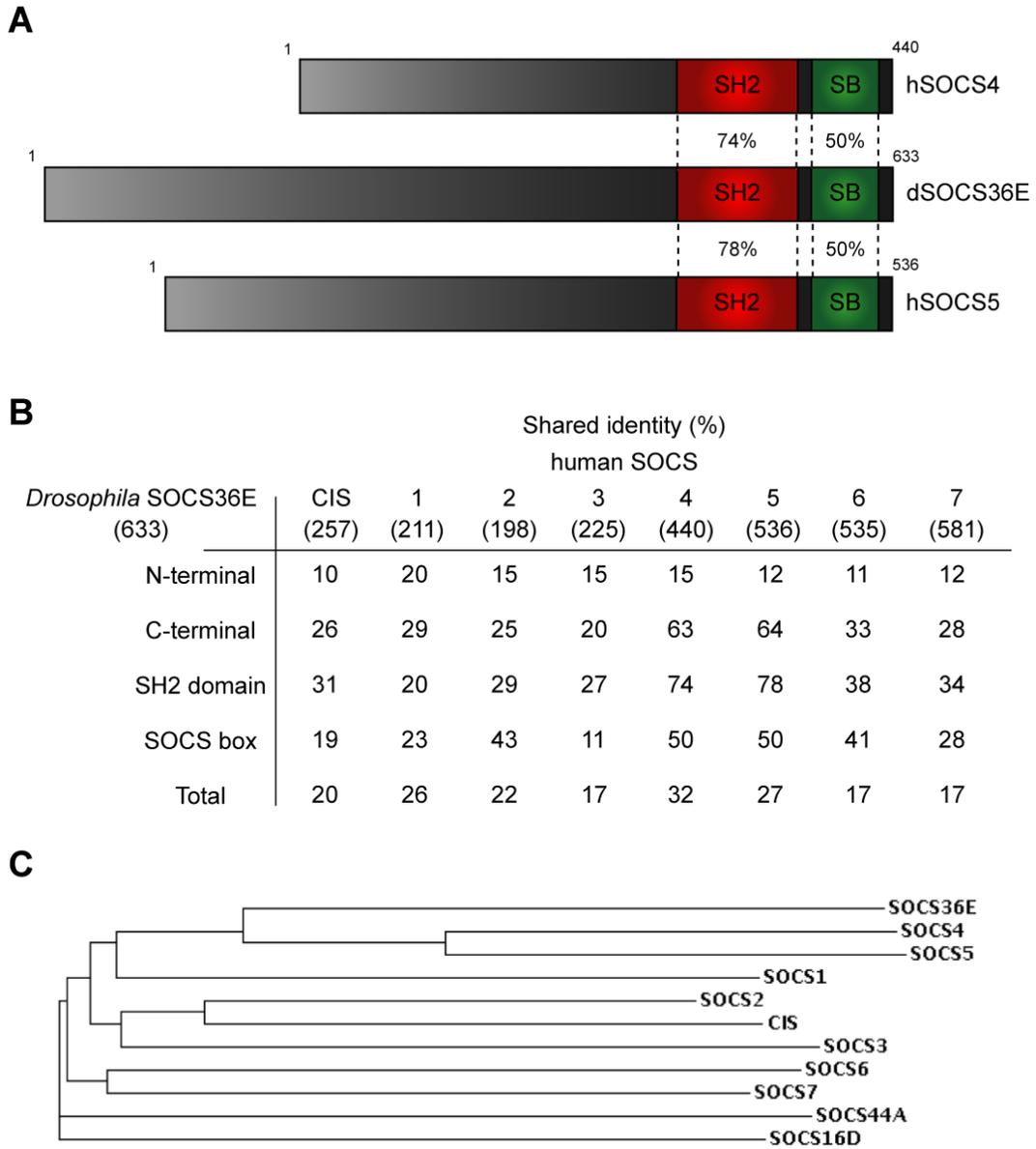


Figure.IV.1 Homology of SOCS36E. (A) Schematic representation of human SOCS4 and 5 and *Drosophila* SOCS36E and homology shared between the proteins. Numbers between dashed lines indicate shared identity in the region indicated. SB indicates SOCS box domain. (B) Shared identity in the indicated regions between human SOCS molecules and dSOCS36E. Numbers in brackets represent amino acid sequence length. Domains determined by NCBI Conserved Domains Search tool. (C) Phylogram representing common ancestry of human and *Drosophila* SOCS proteins. Phylogram generated by ClustalW2 sequence alignment analysis tool.

(Almudi *et al.*, 2010). While regulation of the EGFR pathway is of tremendous importance for physiology, this aspect of SOCS36E activity has not been investigated in this study.

#### ***IV.1.4 Structure of SOCS36E resembles mammalian SOCS4 and 5***

Sequence alignment tools clearly indicate SOCS36E to be most homologous to mammalian SOCS4 and 5 (Fig.IV.1A and B) (Callus and Mathey-Prevot, 2002). Most of the homology between proteins is located in the SH2 and SOCS box domains, while the N-terminal is not conserved. (Fig.IV.1B). Regions of homology are not surprising, considering the same regions are the most conserved within the members of the mammalian SOCS family (Larsen and Röpke, 2002). The N-terminal domain of SOCS36E is larger than that of mammalian SOCS proteins, even the long N-terminal domains of SOCS4-7. Computational analysis has not revealed presence of kinase inhibitory region in SOCS36E, characteristic for SOCS1 and 3.

Phylogenetic analysis suggests that mammalian short N-terminal SOCS proteins emerged after the divergence of mammalian and insect SOCS proteins, suggesting a common ancestry for SOCS36E and SOCS4 and 5 (Fig.IV.1C). Interestingly, SOCS-like molecules with long N-terminals have been described in other invertebrate model organisms, including *Manduca sexta* and *Tribolium* (Elliott and Zeidler, 2008; Bäumer *et al.*, 2011).

#### ***IV.1.5 SOCS36E might play a role in endocytic regulation of the JAK/STAT pathway***

Despite being used in numerous cell culture based assays, the mechanism of function of SOCS36E remains unknown. During investigation of the roles of endocytic machinery in regulation of the JAK/STAT pathway activity, a previous PhD student in the Zeidler lab, Oscar Vidal, observed a delay in clearing of the internalised Upd2-GFP from cells deprived of SOCS36E, a phenotype similar to that observed upon knockdown of genes involved in endocytosis (Fig.IV.2) (Vidal, 2010). Considering that Upd and Upd2 bind to the only *Drosophila* cytokine receptor Domeless, it is therefore possible that SOCS36E might be involved in endocytic processing of the ligand-receptor complex

Figure.IV.2 SOCS36E is involved in degradation of internalized Upd2-GFP

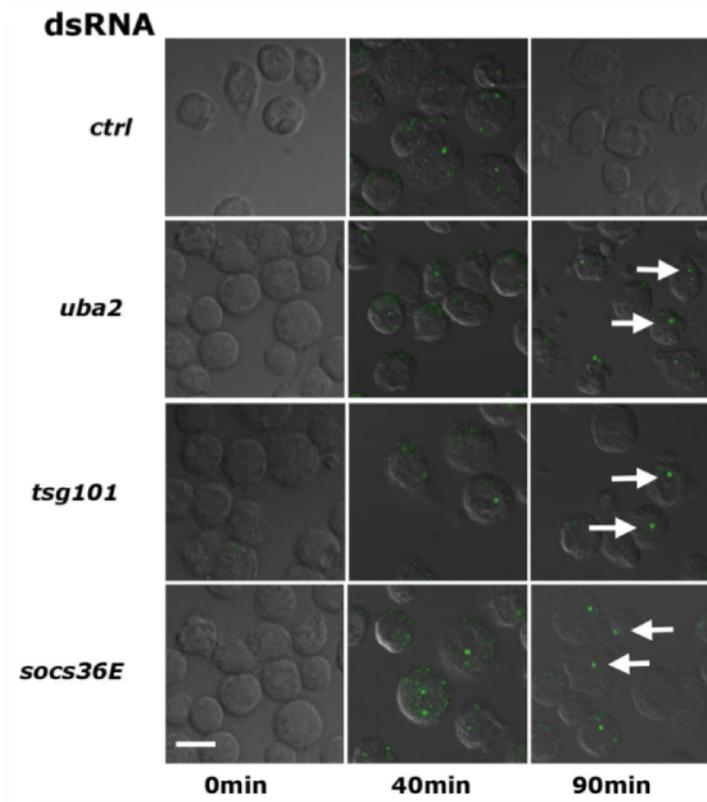


Figure.IV.2 SOCS36E is involved in degradation of internalized Upd2-GFP.  $KC_{167}$  cells treated with indicated dsRNAs for 4 days were pulsed with Upd2-GFP conditioned media at 4°C and shifted to room temperature. At indicated time points cells were washed with acidic buffer to remove any ligand remaining on the plasma membrane. At this point, cells were fixed and imaged on Confocal microscope. Image adapted from Vidal, 2010.

Figure.IV.3 SOCS36E co-localizes with Dome in the follicle cells in *Drosophila* ovary

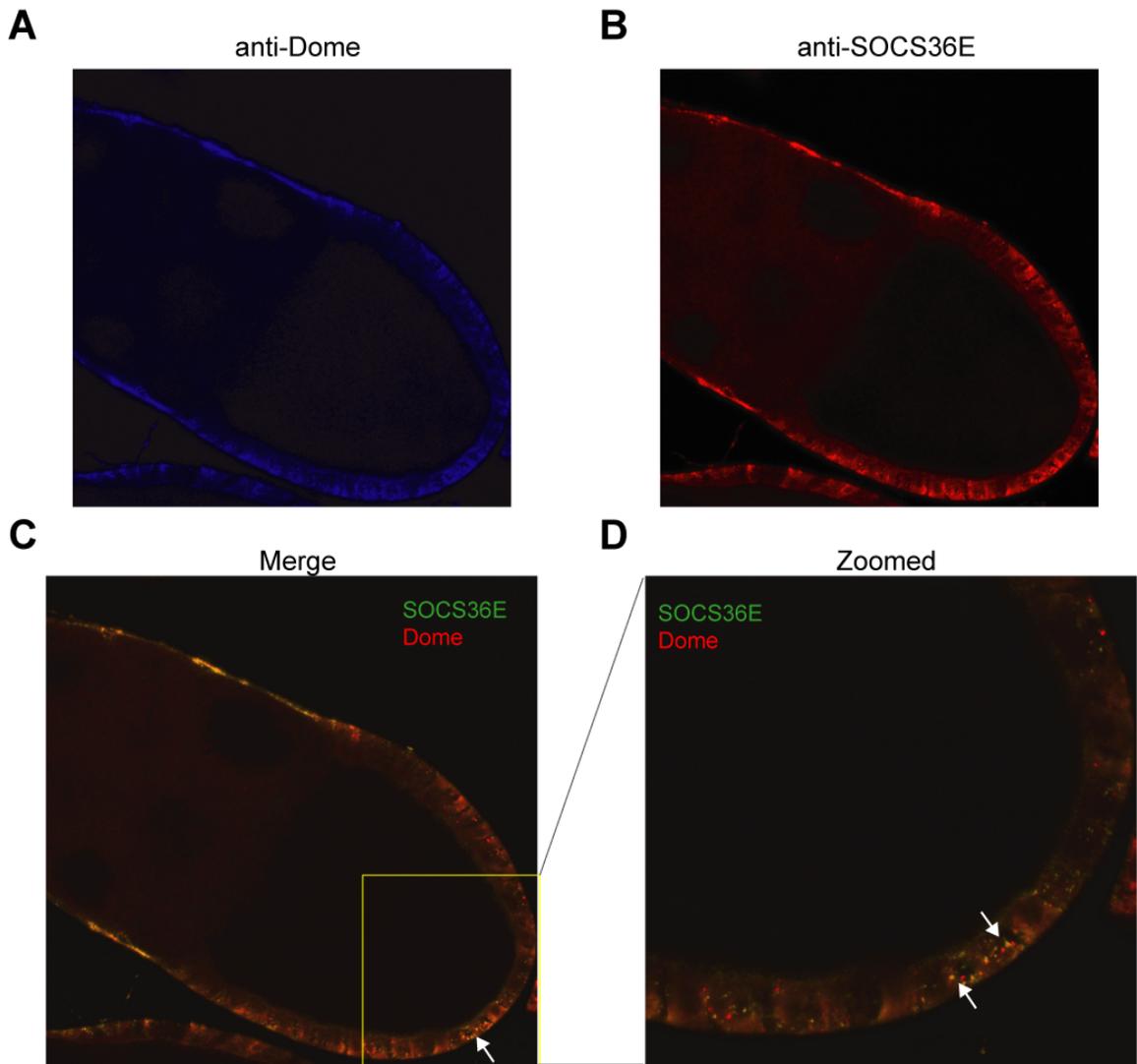


Figure.IV.3 SOCS36E co-localizes with Dome in the follicle cells in *Drosophila* ovary. (A-D) Ovaries from the wildtype fly were dissected and stained with antibodies against Dome and SOCS36E. (A) Dome has cytoplasmic distribution with occasional puncta. (B) SOCS36E is dispersed in the cytoplasm and accumulates in the puncta. (C) Merge of A and B, Dome shown in red, SOCS36E shown in green. Yellow box marks the region zoomed on in D. White arrows point to overlapping puncta. Images obtained by Nina Bausek, unpublished data.

(Devergne *et al.*, 2007; Vidal *et al.*, 2010). This hypothesis is supported by co-localization of SOCS36E with Dome in follicle cells in the ovary (Nina Bausek, unpublished data). Interestingly, co-localization of Dome with SOCS36E is observed in the intracellular puncta, which might represent endocytic vesicles or endosomes, internalised structures which have been shown previously to be the destination organelle for internalized ligands (Devergne *et al.*, 2007; Vidal *et al.*, 2010). Based on the current understanding of the function of mammalian SOCS molecules and the data obtained by Nina Bausek and Oscar Vidal, I hypothesised that SOCS36E might regulate the JAK/STAT pathway activity by affecting stability of the receptor.

## **IV.2. Results**

### ***IV.2.1 Elongins B/C and Cullin 5 are negative regulators of the activated JAK/STAT pathway***

Given the similarity to mammalian SOCS proteins, I hypothesised that SOCS36E might be involved in formation of E3 ubiquitin ligase complexes, as described previously (Section I.3.1). ElonginB, ElonginC and Cullin 5 are well conserved between human and fly, sharing 55%, 90% and 65% identity, respectively. I therefore assessed transcriptional activity of the pathway in cells deprived of the putative ubiquitin ligase complex components, Elongins B/C and Cullin 5. RNAi-mediated knockdown of Elongins B/C, Cullin 5 and SOCS36E caused an increase in pathway activity following stimulation with Upd2-GFP conditioned media (Fig.IV.4A), suggesting that components of the ECS complex act as negative regulators of the activated pathway. The increase caused by knockdown of Elongins B/C and Cullin 5 was quantitatively similar to that observed upon ablation of proteins involved in endocytosis - Rab5, TSG101 and Dor. Knockdown of SOCS36E increased pathway activity to a higher degree than knockdown of Elongins B/C and Cullin 5 following stimulation (Fig.IV.4A). Investigation of the pathway activity under steady state conditions in the same RNAi backgrounds indicated that Elongins B/C and Cullin 5 do not regulate the basal activity of the pathway, similarly to genes involved in endocytosis (Fig.IV.4B). Only knockdown of SOCS36E caused an increase in basal pathway activity. Taken together these results indicate that Elongins B/C and Cullin 5

Figure.IV.4 Components of the ECS complex negatively regulate the activated JAK/STAT pathway

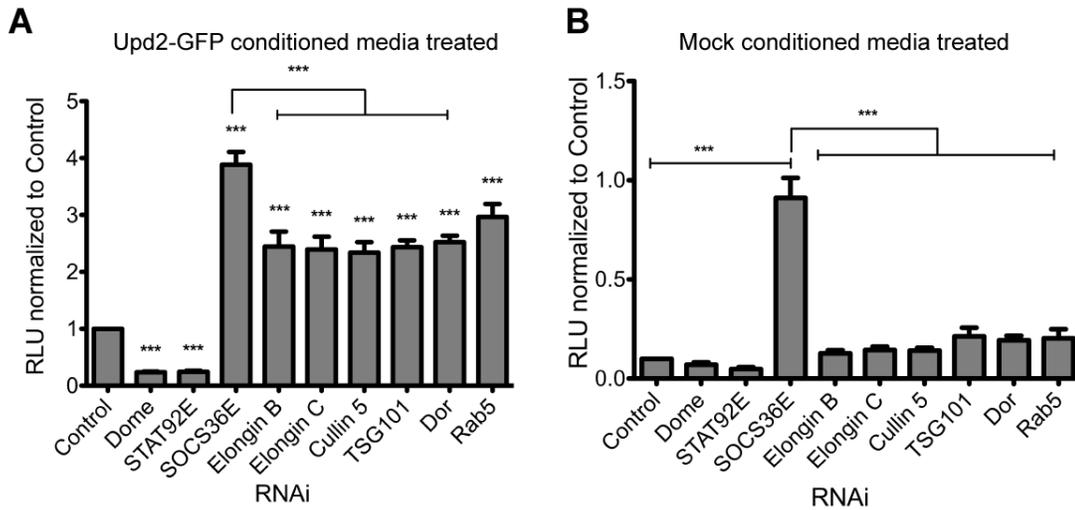


Figure.IV.4 Components of the ECS complex negatively regulate the activated JAK/STAT pathway. Transcriptional pathway activity was measured by *6x2xDrafLuc* reporter in Kc<sub>167</sub> cells treated with indicated dsRNAs and incubated with Upd2-GFP (A) or mock conditioned media (B), as described in materials and methods section (II.2.5). Dome and STAT92E knockdowns act as controls. Results were normalised to Control cells in both conditions. To represent lower pathway activity in mock treated cells compared to Upd2-GFP treated cells, normalisation was performed to 0.1. Stars above columns represent statistical significance between the column and Control sample. Stars above bars indicate statistical significance between indicated samples. Statistical significance was analysed by ANOVA followed by Boferroni's multiple comparisons test, with \*\*\*, p<0.005; \*\*, p<0.01; \*, p<0.05. Error bars represent SEM.

regulate the activity of the pathway only following stimulation, while SOCS36E is involved in regulation of both basal and activated pathway signalling.

#### ***IV.2.2 Elongins B/C and Cullin 5 affect Dome stability***

Having established that components of the ECS complex negatively regulate the pathway, I next investigated whether they affect stability of the receptor. RNAi-mediated knockdown of ECS components resulted in increased stability of the receptor, however the increase was not as potent as in the case of Dor knockdown (Fig.IV.5A). This indicates that ECS complex is involved in regulation of Dome stability under steady state conditions, however it is not necessary for the process.

Similar conclusion was made upon investigation of Dome degradation in SOCS36E or Elongin B RNAi background upon ligand-mediated pathway stimulation (Fig.IV.5B). Knockdown of SOCS36E or Elongin B caused mild stabilization of the receptor at 5 hours post treatment with mock or ligand conditioned media, compared to control cells. Again, lack of complete inhibition of degradation implies that ECS complex is not the only mechanism implicated in Dome degradation, but rather ECS is accessory to the process.

#### ***IV.2.3 SOCS36E negatively regulates the JAK/STAT pathway not only via ECS complex formation***

Pathway activity following individual knockdown of SOCS36E and Elongins B/C and Cullin 5 suggested that SOCS36E is more potent negative regulator than other components of the ECS complex (Fig.IV.4). This result was in line with the original report characterising SOCS36E *in vivo* that indicated negative regulatory activity of the protein even after SOCS box truncation (Callus and Mathey-Prevot, 2002). Hypothesising that SOCS36E can utilise mechanism separate from ECS, I ablated different combinations of ECS components in cells and investigated pathway activity. Cells treated with three dsRNAs targetting Elongins B/C and Cullin 5 did not display additive effect of knockdown under ligand-stimulated conditions (Fig.IV.6A). Considering that formation of the ECS complex requires presence of all components, this

Figure.IV.5 ECS complex components regulate stability of Domeless

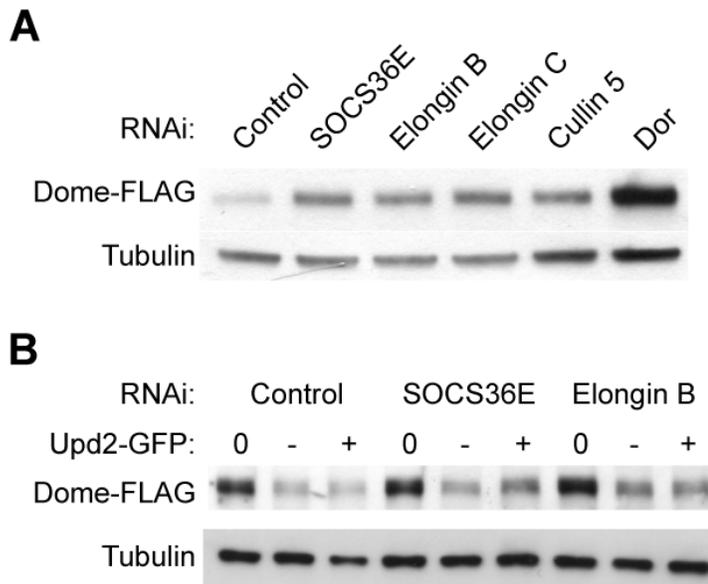


Figure.IV.5 ECS complex components regulate stability of Domeless. (A) Dome-FLAG batch-transfected Kc<sub>167</sub> cells were treated with indicated dsRNAs for 4 days. At this point cells were lysed and lysates analysed with SDS-PAGE and western blotting. (B) Dome-FLAG batch-transfected S2R+ cells were treated with indicated dsRNAs for 4 days and treated with mock or Upd2-GFP conditioned media. After 5 hours of treatment, cells were lysed and lysates were analysed with SDS-PAGE and western blotting. Sample indicated as "0" was lysate at the start of stimulation.

Figure.IV.6 SOCS36E regulates the JAK/STAT pathway via mechanism additional to and independent of ubiquitin ligase formation

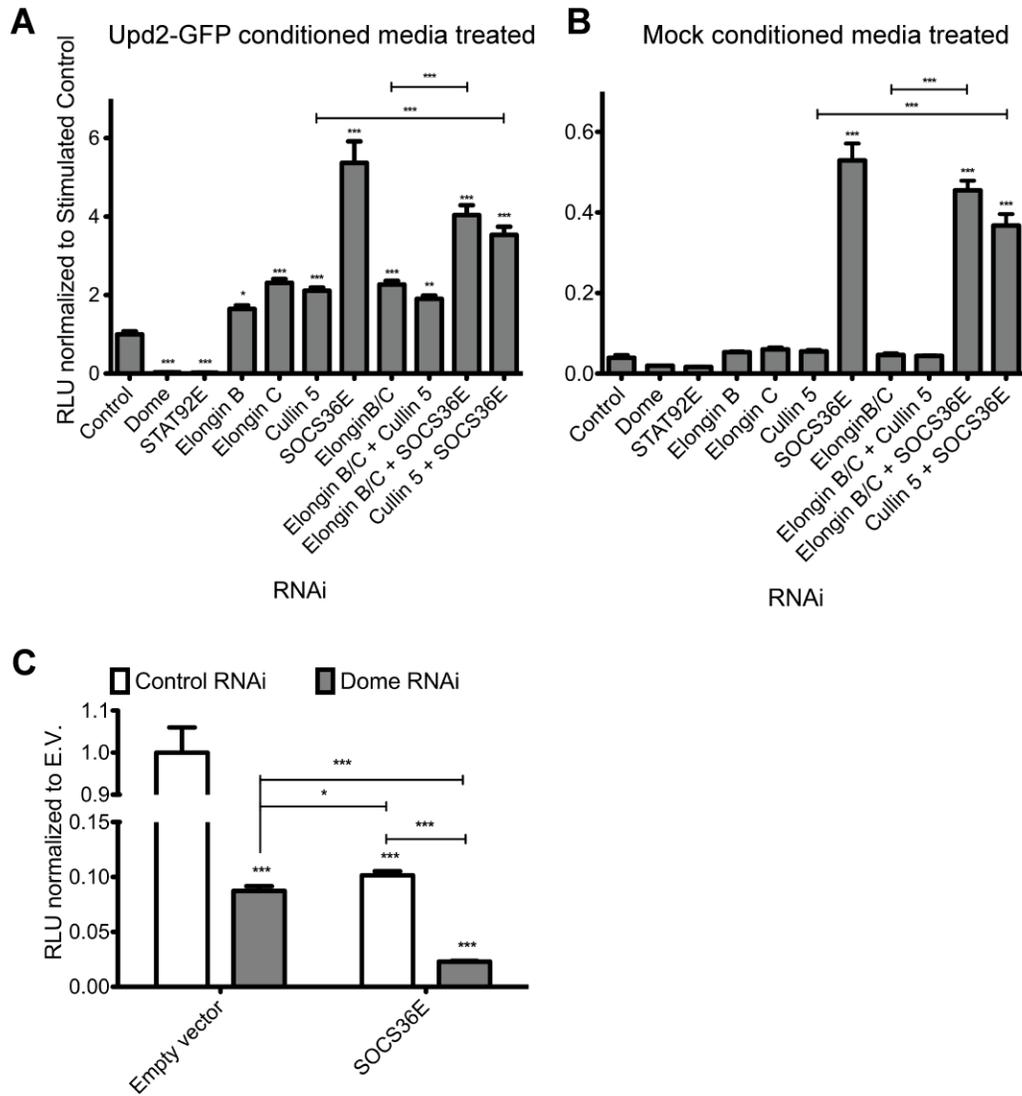


Figure.IV.6 SOCS36E regulates the JAK/STAT pathway via mechanism additional to and independent of ubiquitin ligase formation. (A, B) Transcriptional activity in Kc<sub>167</sub> cells treated for 4 days with indicated dsRNAs was measured with *6x2xDraf* luminescence assay, as described in materials and methods section (II.2.5). Individual genes were targeted by the same quantity of dsRNA with the total amount of dsRNA kept constant by the addition of control dsRNA. Along with the dsRNA treatment cells were incubated with Upd2-GFP (A) or mock conditioned media (B). (C) Transcriptional pathway activity in Kc<sub>167</sub> cells transfected with Empty vector or SOCS36E and treated with Control or Dome dsRNA for 4 days prior to measurement with *6x2xDraf* luminescence assay. During dsRNA treatment, cells were stimulated with Upd2-GFP. Stars above columns indicate statistical significance relative to Control cells within the experimental conditions (A and B) or Empty vector transfected cells treated with Control dsRNA (C). Results were normalised to the same samples. In B normalisation was performed to 0.1 to symbolise lower pathway activity relative to A. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test, with \*\*\*,  $p < 0.005$ ; \*\*,  $p < 0.01$ ; \*,  $P < 0.05$ .

result is not surprising (Babon *et al.*, 2009). However, knockdown of SOCS36E on its own or in combination with Elongins B/C or Cullin 5 resulted in a more dramatic increase in pathway activity compared to knockdowns of ECS components alone (Fig.IV.6A). This indicates that SOCS36E can suppress the JAK/STAT pathway via mechanism additional to ubiquitin ligase formation.

I previously observed that knockdown of Elongins B/C and Cullin 5 had no effect on basal pathway activity under steady state conditions (Fig.IV.4B). In addition, combinatorial knockdowns did not produce additive effect on the basal pathway activity either (Fig.IV.6B). In contrast, knockdown of SOCS36E by itself, or simultaneously with the remaining components of ECS complex resulted in increased accumulation of the luciferase reporter under steady state conditions. This indicates that SOCS36E suppresses the basal JAK/STAT pathway activity even in the absence of other ECS components via mechanism that must therefore be independent of ECS complex formation.

Over-expression of SOCS36E causes suppression of the JAK/STAT pathway activity *in vivo* (Callus and Mathey-Prevot, 2002; Rawlings *et al.*, 2004; Bina *et al.*, 2010; Singh *et al.*, 2010). I observed similar effect in tissue culture cells (Fig.IV.6C). Suppression of the pathway caused by SOCS36E over-expression was quantitatively similar to the one observed following RNAi-mediated knockdown of Dome. However, those two conditions were additive as simultaneous over-expression of SOCS36E and ablation of Dome resulted in further suppression of the pathway (Fig.IV.6C). This suggests that SOCS36E can suppress the pathway not only by regulating receptor stability.

#### ***IV.2.4 Truncation of the N- or C-terminal of SOCS36E does not change the sub-cellular localization***

To investigate the additional mechanism utilised by SOCS36E, I undertook a structure-function analysis of the molecule. I generated N- and C-terminal truncations, referred to as  $\Delta N$  and  $\Delta SB$  respectively (Fig.IV.7A). Based on reports indicating positional requirements for arginine residue in the SH2 domain for interaction with pY, I

generated a single point mutation in the SH2 domain, substituting arginine residue for glutamine acid (R499E) – construct referred to as SH2\* (Fig.IV.7A) (Marengere and Pawson, 1992; Kamura *et al.*, 1998). This mutation should prevent binding to tyrosine phosphorylated substrates and previous report indicated this mutation to render protein unable to suppress the JAK/STAT pathway when over-expressed *in vivo* (Callus and Mathey-Prevot, 2002). As a first step in assessing generated constructs, I adjusted the amount of DNA transfected so as to express equivalent protein levels as assessed by western blotting (Fig.IV.7B). Sub-cellular localization was investigated by immunofluorescence with all constructs localizing predominantly to the cytoplasm with epinuclear enrichment in case of the full-length,  $\Delta$ N and  $\Delta$ SB constructs (Fig.IV.7D-F). SOCS36E SH2\* construct appeared predominantly cytoplasmic (Fig.IV.7G). Both truncation and SH2 domain mutation constructs displayed sub-cellular localization similar to the full-length protein, which was reminiscent of reports describing the endogenous or over-expressed SOCS36E both *in vivo* and *ex vivo* (Almudi *et al.*, 2009, 2010; Herranz *et al.*, 2012).

#### ***IV.2.5 N- and C-termini can suppress the JAK/STAT pathway separately***

I next investigated the generated constructs on the functional level, using the *6x2xDrafLuc* reporter of pathway transcriptional activity. Expression of the full-length protein as well as the C-terminal truncation construct resulted in a decrease in basal pathway activity under steady state conditions (Fig.IV.8A). In contrast, ablation of the N-terminal did not decrease STAT92E transcriptional activity, indicating that the SOCS box domain does not modulate the basal activity of the pathway. Interestingly, expression of the point mutation in the SH2 domain (SH2\*) thought to prevent binding to pY substrates caused an increase in pathway activity, potentially due to sequestration of unidentified co-factors that would otherwise interact with endogenous SOCS36E, however this possibility was not investigated any further (Fig.IV.8A). Taken together, these data suggests a role for the N-terminal in suppression of spontaneous firing of the JAK/STAT pathway under steady state conditions.

Figure.IV.7 SOCS36E constructs

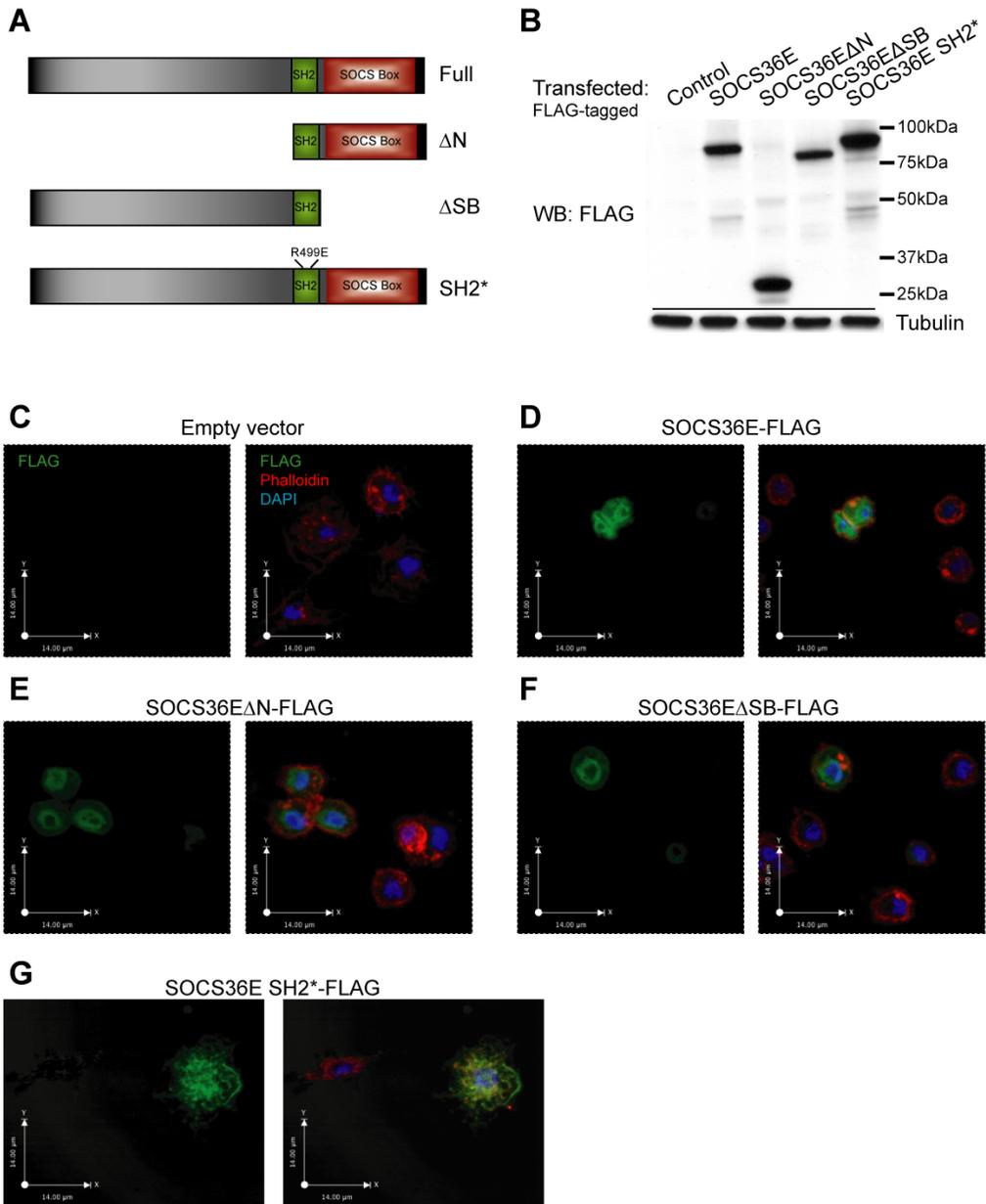


Figure.IV.7 SOCS36E constructs. (A) Schematic representation of generated SOCS36E constructs. (B) Western blot analysis of constructs shown in A expressed in Kc<sub>167</sub> cells. (C-G) Images from Confocal microscopy showing sub-cellular localization of SOCS36E constructs. Colour legend for panels C-G shown in C.

Confirmation of functionality of the N-terminal came from investigation of pathway activity following stimulation with Upd2-GFP conditioned media. Both truncated versions of SOCS36E were able to suppress pathway activity compared to the control cells, however that suppression was not as efficient as that elicited by the full-length protein (Fig.IV.8B). The SH2\* mutant did not affect pathway activity, implying that binding to phosphorylated tyrosine residue is required for both activities of SOCS36E. These results indicate that both N- and C-termini along with a functional SH2 domain are required for regulation of the pathway activity following stimulation.

As truncation of the N-terminal left only the SH2 domain and SOCS box intact, I investigated whether the  $\Delta$ N mutant can affect pathway activity in an ECS independent manner. Therefore, I expressed the SOCS36E constructs in cells treated with dsRNA targeting Elongins B/C and Cullin 5 and incubated cells with pathway ligand conditioned media. Knockdown of Elongins B/C and Cullin 5 increased the pathway activity as shown previously (Fig.IV.6). This increase was suppressed by the full-length SOCS36E and SOCS36E $\Delta$ SB constructs. By contrast, the  $\Delta$ N mutant was unable to decrease the pathway activity under these conditions (Fig.IV.8C). Considering that the N-terminal truncated construct was able to suppress the pathway in the presence of Elongins B/C and Cullin 5, this confirms that SOCS box of SOCS36E is indeed required for formation of the putative ECS ubiquitin ligase. Moreover, these results strongly suggest that the N-terminal of SOCS36E functions as a negative regulator of the pathway via mechanism independent of ECS complex formation.

#### ***IV.2.6 SOCS box and SH2 domains are required for regulation of Dome levels***

As an independent approach to examine the separate functions of the N- and C-terminal of SOCS36E, I next assessed the effect of expressing the SOCS36E constructs on Dome levels. I co-transfected constant levels of Dome with increasing dosage of SOCS36E constructs and subsequently examined the steady state level of Dome protein. The full-length SOCS36E construct negatively affected stability of the receptor even at very low expression levels (Fig.IV.9). In contrast, high levels of protein were required to obtain the similar effect with the SOCS36E $\Delta$ N construct. SOCS36E $\Delta$ SB and SH2\* constructs had no effect on Dome stability, even at high protein levels. This indicates that

Figure.IV.8 N- and C-termini of SOCS36E are required for suppression of the JAK/STAT pathway

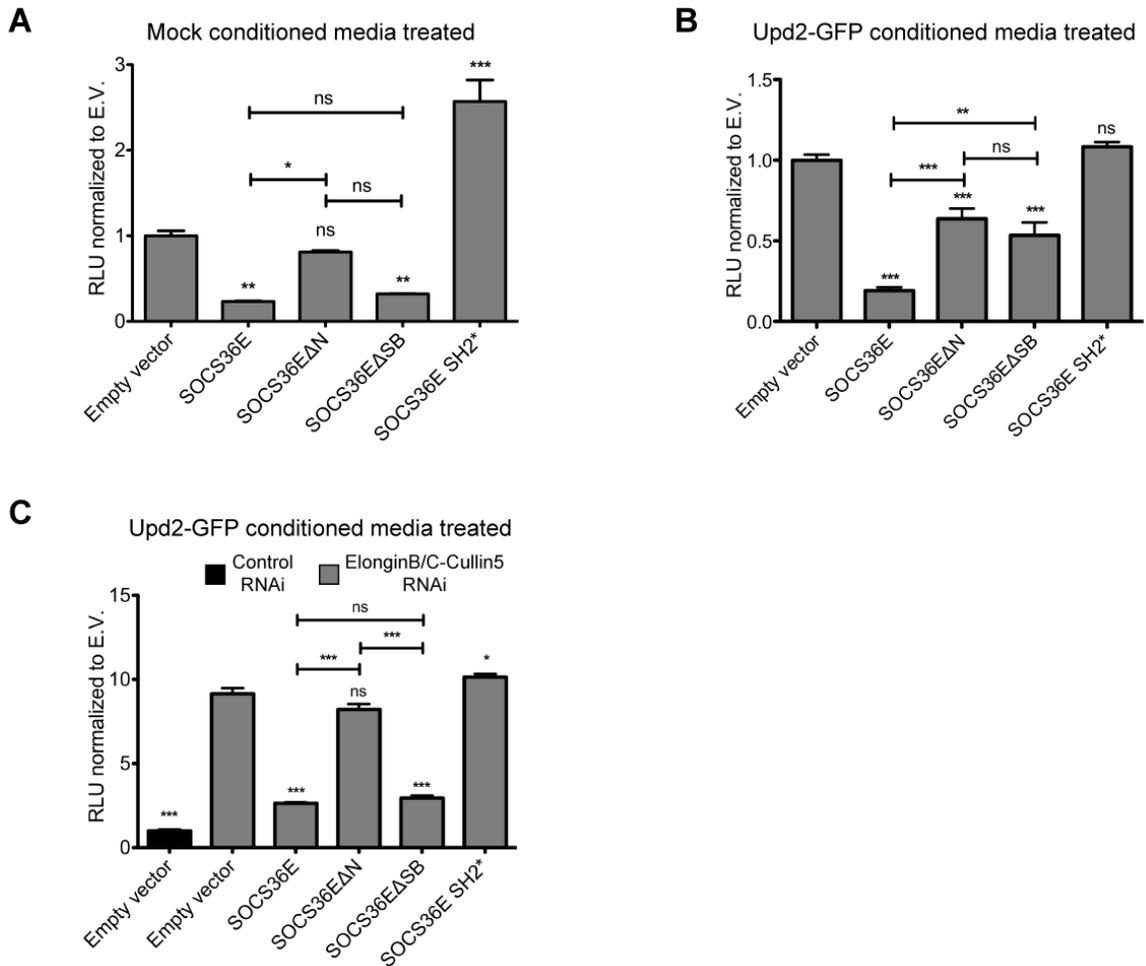


Figure.IV.8 N- and C-termini of SOCS36E are required for suppression of the JAK/STAT pathway. (A-C) Transcriptional JAK/STAT pathway activity in cells expressing SOCS36E constructs was measured by *6x2xDrafLuc* reporter. Cells were treated with mock (A) or Upd2-GFP conditioned media (B and C) for 4 days prior to quantification. (C) In addition to transfection, cells were treated with Control or a combination of ElonginB/C and Cullin 5 dsRNAs. Total amount of dsRNA was kept constant, as described in materials and methods section. All results were normalised to Empty vector transfected cells (treated with Control dsRNA in C). Stars above columns reflect statistical significance between the Empty vector and indicated sample (in C, ElonginB/C and Cullin5 treated Empty vector cells were used to analyse statistical significance against). Statistical significance was analysed with ANOVA with Bonferroni's multiple comparisons test with \*\*\*,  $p < 0.005$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .

*Figure.IV.9 SOCS box and a functional SH2 domain are required for regulation of Dome stability*

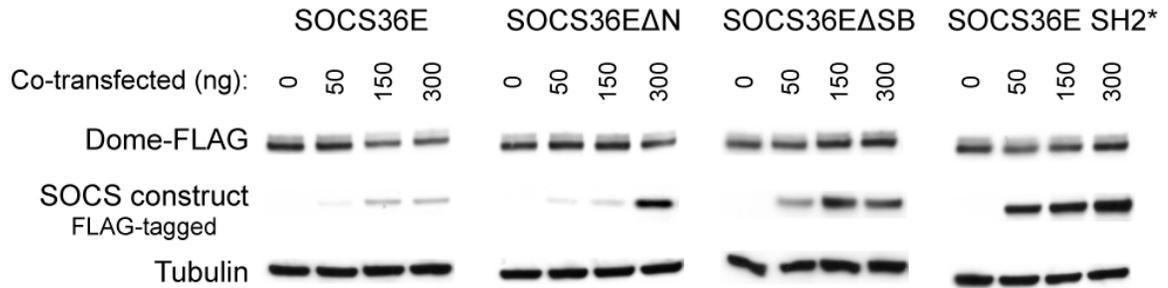


Figure.IV.9 SOCS box and a functional SH2 domain are required for regulation of Dome stability. S2R+ cells co-transfected with constant amount of Dome and increasing concentrations of SOCS36E constructs, as indicated.

regulation of Dome stability requires the SOCS box domain of SOCS36E together with an intact SH2 domain.

#### ***IV.2.7 Interaction of Dome with SOCS36E requires intact SH2 domain and is stabilised by the N-terminal domain of SOCS36E***

Mammalian SOCS1 has been shown to interact preferentially with JAKs, while the remainder of mammalian SOCS proteins have been reported to have higher affinity for the associated receptors or the JAK:receptor complexes (Piganis *et al.*, 2011; Kershaw *et al.*, 2013, reviewed in Croker *et al.*, 2008; Yoshimura, 2009). To determine binding partners of SOCS36E, I next co-expressed Dome or Hop together with SOCS36E and pulled down Dome or Hop. Interaction of SOCS36E with Dome was much stronger than that with Hop (Fig.IV.10A). Furthermore, knockdown of Hop did not affect the Dome:SOCS36E interaction, implying that this interaction is direct. By contrast, SOCS36E interaction with Hop was much less robust and was further weakened by Dome knockdown. This indicates that SOCS36E:Hop interaction is at least partially dependent on Dome, a phenomenon also observed for mammalian SOCS proteins (Fig.IV.10A) (Starr and Hilton, 1998; Yoshimura, 2009). Interestingly, interaction of SOCS36E with the receptor or JAK remained unaffected by stimulation of the pathway. While I have previously shown that Dome is constitutively tyrosine phosphorylated,

Figure.IV.10 The N-terminal and SH2 domains are required for SOCS36E interaction with Dome

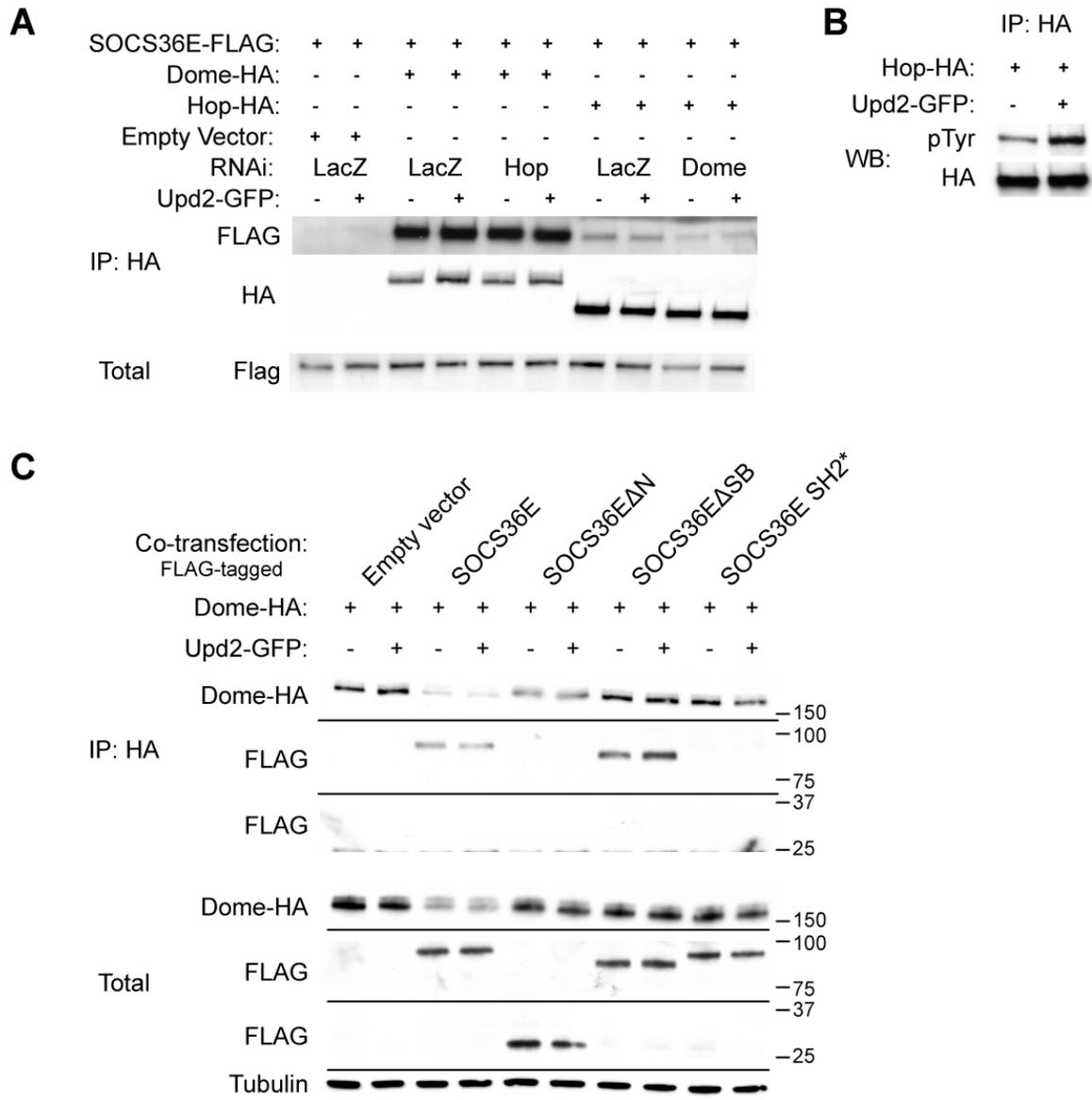


Figure.IV.10 The N-terminal and SH2 domains are required for SOCS36E interaction with Dome. (A) Kc<sub>167</sub> cells co-transfected with SOCS36E-FLAG and Dome-HA, Hop-HA or Empty vector were treated with Control (LacZ), Hop or Dome dsRNAs, as indicated, for 4 days. Prior to lysis, cells were stimulated for 15min with mock or Upd2-GFP conditioned media. Lysates were subjected to anti-HA immunoprecipitation and subsequent analysis with SDS-PAGE and western blotting. (B) Kc<sub>167</sub> cells transfected with Hop-HA were treated for 15min with mock or Upd2-GFP conditioned media and lysed with lysis buffer supplemented with phosphatase inhibitors. Lysates were subjected to anti-HA immunoprecipitation followed by SDS-PAGE and western blotting. (C) S2R+ cells were co-transfected with Dome-HA and FLAG-tagged SOCS36E constructs, as indicated. Prior to lysis, cells were incubated with mock or ligand conditioned media for 15min. Lysates were subjected to anti-HA immunoprecipitation followed by SDS-PAGE and western blot analysis.

therefore enabling interaction with SOCS36E via its SH2 domain (Fig.III.3), I also investigated Hop phosphorylation levels. I observed that tyrosine phosphorylation of Hop under steady state conditions was increased upon pathway stimulation, similar to that observed for Dome (Fig.IV.10B, Fig.III.3, respectively). These results indicate that SOCS36E preferentially interacts with the constitutively phosphorylated receptor of the pathway.

In order to identify the regions necessary for the interaction of SOCS36E with Dome I made use of the SOCS36E truncations (Fig.IV.10C). I found that full-length SOCS36E as well as the SOCS36E $\Delta$ SB constructs co-immunoprecipitate with Dome in a ligand independent manner. As expected, SOCS36E SH2\* did not precipitate with Dome, confirming that Dome:SOCS36E interaction is mediated by the SH2 domain. No detectable interaction with SOCS36E $\Delta$ N implies involvement of the N-terminal region in interaction (Fig.IV.10C). This would also explain why high levels of  $\Delta$ N construct expression were required to destabilise the receptor (Fig.IV.9). Taken together, these results demonstrate that SOCS36E:Dome interaction requires both an intact SH2 domain and the N-terminal region.

#### ***IV.2.8 Full-length SOCS36E can suppress phosphorylation of Dome in response to ligand-mediated pathway stimulation***

Involvement of the N-terminal region of SOCS36E for interaction with Dome does not however explain why the SOCS36E $\Delta$ SB construct can suppress pathway activity (Fig.IV.6). I hypothesised that the N-terminal of SOCS36E may regulate kinase activity. As cytokine receptors are substrates of their associated JAK kinases, I analysed the pTyr levels of Dome and Hop in SOCS36E RNAi background. Stimulation with pathway ligand resulted in increased pTyr levels of both Dome and Hop in control and SOCS36E RNAi background (Fig.IV.11A). However, knockdown of SOCS36E caused elevated pTyr levels of Dome under steady state as well as stimulated conditions. Hop phosphorylation remained unaffected by SOCS36E ablation. This indicates that SOCS36E might be involved in the regulation of Dome phosphorylation.

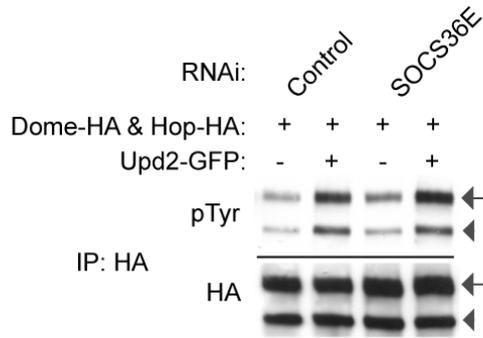
In the converse experiment, I co-expressed Hop and Dome with SOCS36E constructs and quantified blots of Dome and Hop pTyr levels from three independent experiments to identify any trends. Co-expression of full-length SOCS36E prevented Dome phosphorylation in response to pathway stimulation (Fig.IV.11B and quantified in C). In contrast, SOCS36EAN and SH2\* constructs had no effect on Dome phosphorylation, while SOCS36EΔSB mildly increased basal levels of Dome phosphorylation. These results indicate that both N- and C-termini as well as the SH2 domain of SOCS36E are required to suppress Dome phosphorylation upon ligand binding.

#### ***IV.2.9 SOCS36E constructs do not affect Hop phosphorylation***

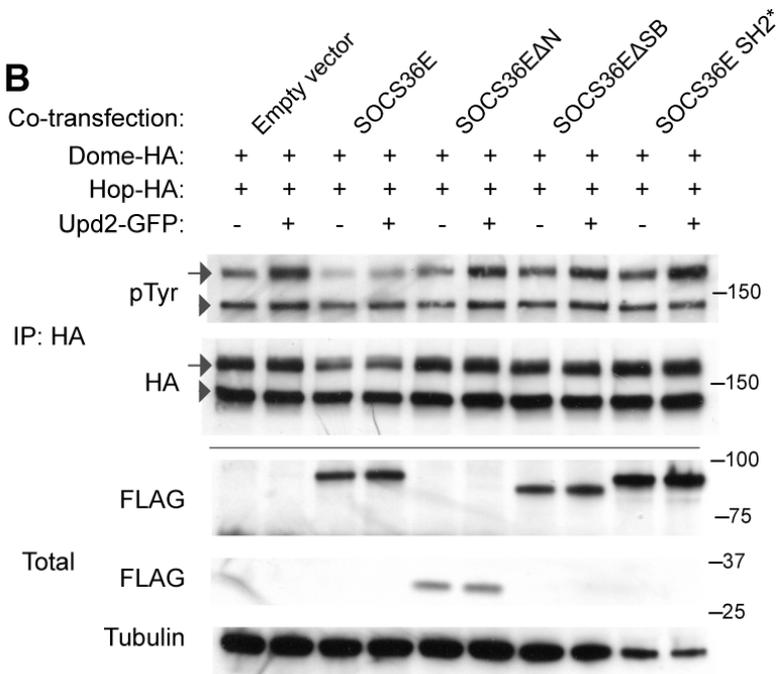
Based on previous results, I hypothesised that the N-terminal of SOCS36E might be involved in either masking of tyrosine residues on Dome or regulating the catalytic activity of Hop. Quantification of Hop tyrosine phosphorylation when co-expressed with SOCS36E constructs indicated that both truncations and the SOCS36E SH2\* constructs lead to a mild elevation of Hop phosphorylation under steady-state conditions (Fig.IV.11B and quantified in D). The full-length SOCS36E had no effect on Hop phosphorylation and neither of the SOCS36E constructs affected Hop phosphorylation in response to ligand-mediated pathway stimulation. To clarify whether SOCS36E constructs can affect Hop catalytic activity, I made use of the fact that JAKs undergo auto-phosphorylation (Saharinen *et al.*, 2000; Matsuda *et al.*, 2004; Funakoshi-Tago *et al.*, 2006). Based on this phenomenon, I established and conducted *in vitro* kinase activity assays, using incorporation of radiolabelled phosphate from <sup>32</sup>P γ-ATP as the readout of Hop auto-activity. SOCS36E constructs were synthesised *de novo* to avoid contamination with potential co-factors or extracted from cells to ensure correct folding (Fig.IV.12A and B, respectively). Irrespective of method used, no change in Hop auto-phosphorylation was observed (Fig.IV.12C). While inherently a negative result, this at least suggests that SOCS36E does not affect Hop catalytic activity.

Figure.IV.11 Full-length SOCS36E regulates Dome phosphorylation

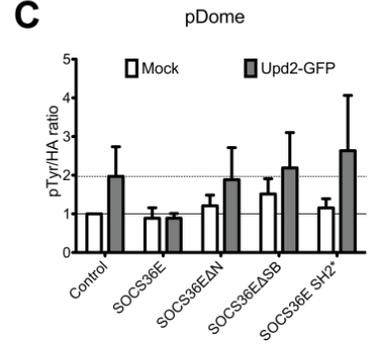
**A**



**B**



**C**



**D**

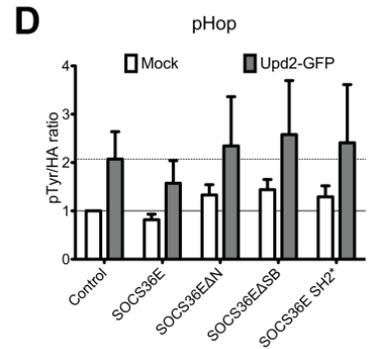


Figure.IV.11 Full-length SOCS36E regulates Dome phosphorylation. (A)  $Kc_{167}$  cells co-transfected with Dome-HA and Hop-HA were treated with Control or SOCS36E dsRNAs for 4 days. Prior to lysis with lysis buffer supplemented with phosphatase inhibitors, cells were incubated for 10min with Upd2-GFP or mock conditioned media. Lysates were subjected to anti-HA immunoprecipitation and subsequent SDS-PAGE and western blot analysis. Arrow indicates Dome, arrowhead indicates Hop. (B)  $Kc_{167}$  cells co-transfected with Dome-HA, Hop-HA and FLAG-tagged indicated SOCS36E construct were treated and analysed as described in A. (C, D) Quantifications of Dome (C) or Hop (D) phosphorylation levels, determined by ratio of band observed in pTyr blot and HA blot, under experimental settings as shown in B, n=3. Error bars indicate SEM. No statistically significant difference between mock or Upd2-GFP stimulated cells transfected with the same constructs was observed following analysis with two-way ANOVA and Bonferroni's multiple comparisons test.

Figure.IV.12 SOCS36E does not affect catalytic activity of Hop

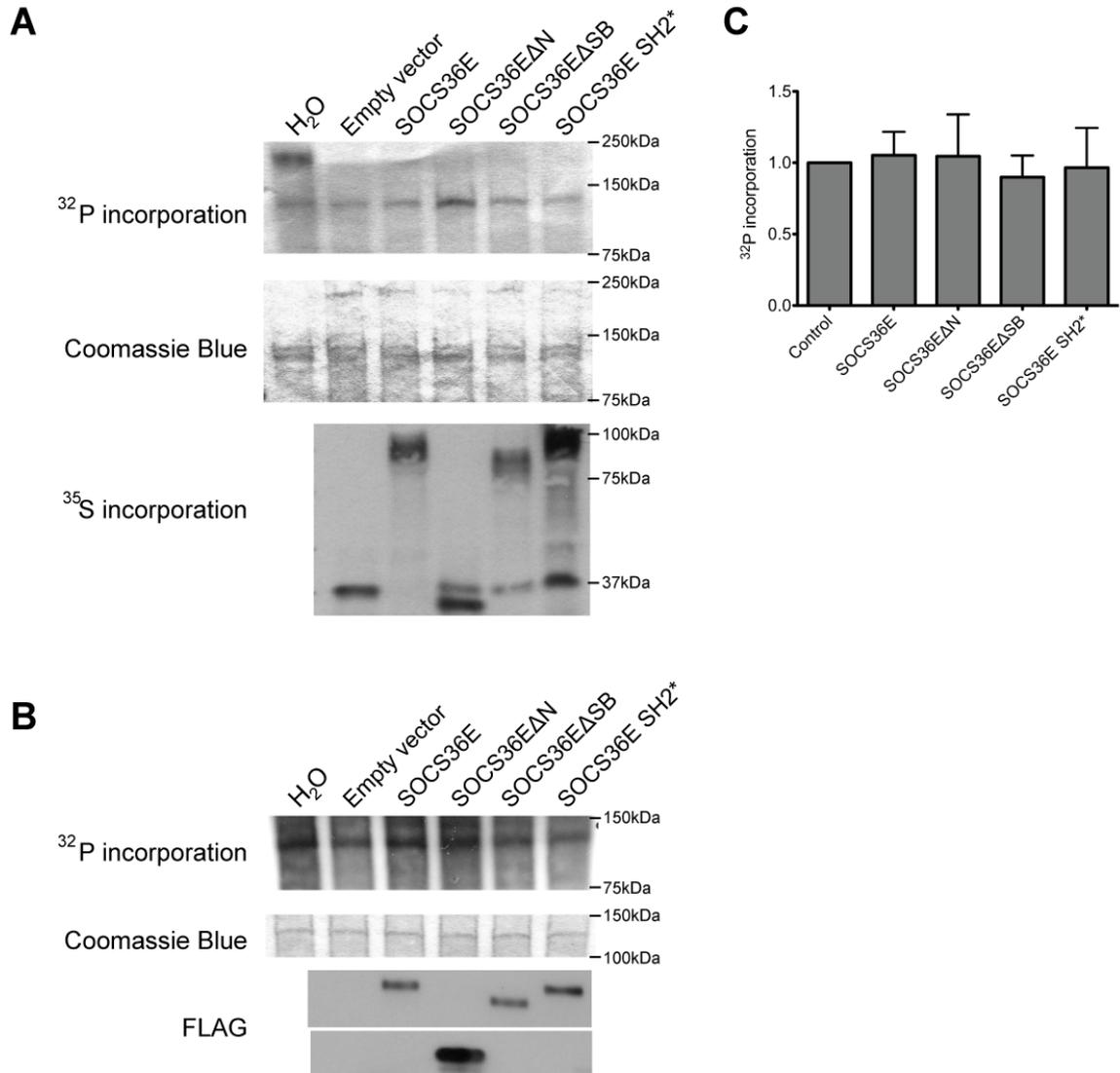


Figure.IV.12 SOCS36E does not affect catalytic activity of Hop. (A, B) S2R+ cells were transfected with Hop-HA and lysed. Hop-HA was immunoprecipitated and left on beads that were used in *in vitro* kinase activity assay. Assays were performed using  $^{32}\text{P}$   $\gamma$ -ATP and assessed by incorporation of radioactive phosphate onto Hop-HA, as described in materials and methods section (II.3.4). (A) Kinase activity assay reaction was supplement with SOCS36E constructs obtained by *de novo* synthesis in E.Coli extracts, as described in materials and methods section (II.3.3). Synthesised SOCS36E constructs were labelled with  $^{35}\text{S}$ -Methionine. (B) SOCS36E constructs used to supplement the kinase activity assay reaction were obtained by immunoprecipitation of FLAG-tagged SOCS36E constructs from transfected S2R+ cells and subsequent elution using 3xFLAG peptide. See materials and methods for details. (C) Amount of incorporated  $^{32}\text{P}$  into Hop-HA in kinase activity assays performed as shown in A and B was quantified using phosphorimager. Quantifications were normalised to Control reaction within experiment. Results were pooled irrespective of method used to obtain SOCS36E constructs with total n=6. No statistically significant difference was observed between samples when analysed with two-way ANOVA followed by Bonferroni's multiple comparisons test. Error bars represent SEM.

#### ***IV.2.10 SOCS36E suppresses STAT92E phosphorylation***

The ability of full-length SOCS36E to suppress Dome phosphorylation in response to ligand stimulation without affecting Hop auto-phosphorylation implies steric inhibition of Hop by SOCS36E. As transduction of the signal by the JAK/STAT pathway involves phosphorylation of STAT92E by Hop, we investigated whether SOCS36E can affect STAT92E phosphorylation. Unfortunately, STAT92E pTyr levels in Kc<sub>167</sub> cells proved difficult to detect, as no signal was observed upon stimulation with pathway ligand in cells treated with control dsRNA (Fig.IV.13A). However, ablation of SOCS36E or Ptp61F produced a strong pTyr-STAT92E band that was detectable even in cells not stimulated with pathway ligand. Cells deprived of SOCS36E showed an increase in STAT92E phosphorylation upon pathway stimulation, while knockdown of Ptp61F caused constitutive phosphorylation of STAT92E. This indicates that SOCS36E and Ptp61F negatively regulate phosphorylation of pathway components.

In the converse experiment, I attempted to utilise SOCS36E constructs to investigate effects of individual domains of SOCS36E on STAT92E phosphorylation. I used S2R+ cells in this experiment as STAT92E pTyr levels appeared much higher in this cell-line, leaving head-room for potential suppression from SOCS36E constructs. I observed phosphorylation of STAT92E following stimulation with conditioned media, however the effect of SOCS36E was variable between experiments (Fig.IV.13B and C). The reason for the variability of this assay remains perplexing and no conclusions regarding effect of SOCS36E constructs on STAT92E phosphorylation could be drawn.

### **IV.3. Discussion**

This study provides molecular characterization of the negative regulator of *Drosophila* JAK/STAT signalling pathway, SOCS36E. I showed that SOCS36E is involved in regulation of the receptor stability, most likely by formation of ubiquitin ligase complexes with Elongins B/C and Cullin 5. Indeed, all components of the ECS complex regulate stability of the receptor Domeless, and therefore are negative regulators of the ligand-activated JAK/STAT pathway. An intact SOCS36E SH2 domain is required

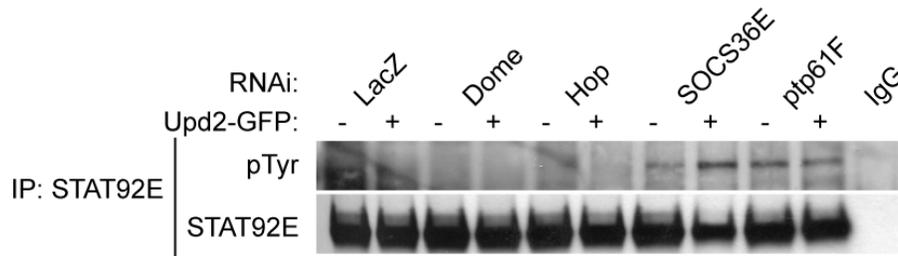
for this process, together with the SOCS box domain. Moreover, SOCS36E is able to negatively regulate ligand-induced as well as basal activity of the JAK/STAT pathway in a manner independent of SOCS box and Elongins B/C and Cullin 5. This suppression of pathway signalling is mediated by the unconserved and undefined N-terminal region of SOCS36E. Both, N- and C-termini of SOCS36E are required for suppression of Dome phosphorylation in response to ligand-mediated pathway stimulation, however the catalytic activity of Hop remains unaffected. The exact molecular mechanism by which the N-terminal of SOCS36E operates remains unresolved. Taken together, SOCS36E negatively regulates the *Drosophila* JAK/STAT pathway via two independent mechanisms.

#### ***IV.3.1 SOCS36E with Elongins B/C and Cullin 5 affect Dome stability***

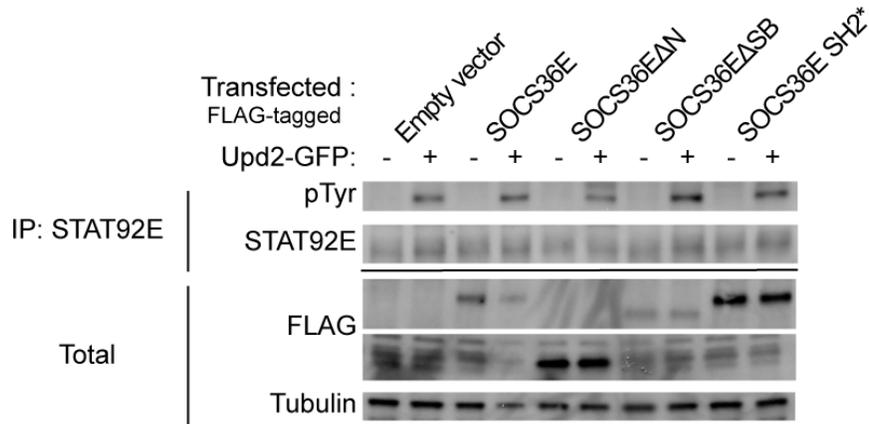
Mammalian SOCS proteins have been shown to affect internalization and endocytosis of cytokine receptors by more than one mechanism. SOCS1 has been shown to regulate INF- $\alpha$ R1 receptor stability by sequestration of associated JAK resulting in destabilization of the receptor (Piganis *et al.*, 2011). Granulocyte-Colony Stimulating Factor Receptor is targeted for degradation via ubiquitination of key residue by Elongin-Cullin-SOCS3 complex (Hörtner *et al.*, 2002; Zhuang *et al.*, 2005; Wölfler *et al.*, 2009). Our data indicates that SOCS36E co-localizes with Dome *in vivo* (Fig.IV.3) and co-precipitates *in vitro* (Fig.IV.10A), strongly suggesting a direct interaction. As changes in SOCS36E expression due to RNAi-mediated knockdown (Fig.IV.5) or protein over-expression (Fig.IV.9) resulted in opposing change in receptor levels, it is plausible that SOCS36E targets Domeless for degradation. Numerous reports have indicated that SOCS molecules can form E3 ubiquitin ligase complexes in vertebrates, by associating with Elongins B/C and Cullin 5 (Babon *et al.*, 2009; Linossi and Nicholson, 2012). Those complexes mediate ubiquitination of their substrates, often receptors. As I have observed Dome to be ubiquitinated (Fig.III.12), it is very likely that SOCS36E can form ubiquitin ligases similarly to its mammalian orthologues and mediate receptor ubiquitination. Indeed, I observe that knockdown of Elongins B/C and Cullin 5 resulted in stabilization of the receptor (Fig.IV.5).

Figure.IV.13 SOCS36E suppresses phosphorylation of STAT92E

**A**



**B**



**C**

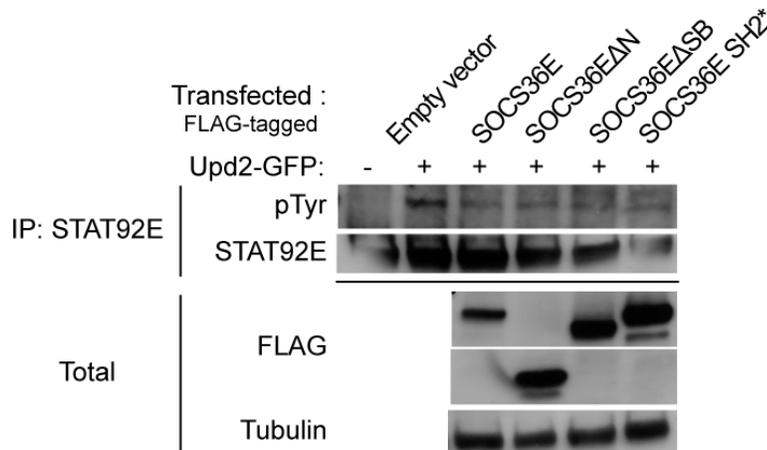


Figure.IV.13 SOCS36E suppresses phosphorylation of STAT92E. (A-C) Cells were lysed with lysis buffer supplemented with phosphatase inhibitors and lysates were subjected to anti-STAT92E immunoprecipitation followed by SDS-PAGE and western blotting. (A) Kc<sub>167</sub> cells were treated with indicated dsRNAs for 4 days, treated with mock or Upd2-GFP conditioned media for 10min and lysed. (B) Cells were transfected with FLAG-tagged SOCS36E constructs, stimulated with mock or Upd2-GFP conditioned media for 10min and lysed. (C) Cells treated as described in B, stimulated only with Upd2-GFP conditioned media.

Interestingly, I observed constitutive degradation of Dome (Fig.III.5) that correlated with its poly-ubiquitination (Fig.IV.12). While knockdown of Elongins B/C and Cullin 5 did not increase basal activity of the pathway (Fig.IV.4B), it increased the stability of the receptor under steady state conditions (Fig.IV.5). The lack of correlation between pathway activity and receptor stability can be explained by the fact that only ligand-bound receptor can signal. However, the observed increase in receptor levels following knockdown of ECS complex components was not as strong as in the case of Dor knockdown (Fig.IV.5A), nor knockdown of SOCS36E or Elongin B caused complete inhibition of degradation (Fig.IV.5B) as was the case following inhibition of lysosomal degradation using Bafilomycin (Fig.III.10). It is possible that ECS complex is involved in only partial ubiquitination of the receptor, therefore performing an accessory role in the process. In the light of previous reports on the negative regulatory role of endocytosis in regulation of the *Drosophila* JAK/STAT pathway (Devergne *et al.*, 2007; Vidal *et al.*, 2010), this accessory role of ECS complex becomes essential for fine-tuning pathway output. This is particularly important especially following stimulation of the pathway with a ligand, which might be evidenced by the second, lower band in MultiDsk immunoprecipitation (Fig.III.12). This hypothesis would assume existence of another ubiquitin ligase responsible for poly-ubiquitination as well as redundancy. Alternatively, it is also possible that ubiquitination might not be required for Dome degradation, but rather for modulation of the process.

#### ***IV.3.2 SOCS36E regulates JAK/STAT pathway activity via mechanism independent of ubiquitin ligase formation***

Only SOCS1 and 3 have been reported to regulate the JAK/STAT pathway via multiple mechanisms, while the remainder of the proteins in the SOCS family depend predominantly on formation of the ECS ubiquitin ligase as their mechanism of function (reviewed in Croker *et al.*, 2008; Yoshimura, 2009; Linossi *et al.*, 2013). My data implies that SOCS36E can suppress the activated JAK/STAT pathway via mechanism independent of Elongins B/C and Cullin 5 (Fig.IV.6A and Fig.IV.8B and C). This is further supported by the fact that truncation of the SOCS box domain, which in

mammalian SOCS proteins is required for association with Elongins B/C and Cullin 5 (Kamura *et al.*, 1998; Zhang *et al.*, 1999), does not render the construct inactive (Fig.IV.8B). Similar results have been reported *in vivo*, where SOCS box-truncated version of SOCS36E was still able to mildly suppress pathway signalling (Callus and Mathey-Prevot, 2002). This implies that the SOCS box domain of SOCS36E is required for effective suppression of the activated pathway signalling, however it is not the only mechanism utilised by SOCS36E.

Another line of evidence for secondary mechanism of function of SOCS36E comes from previous reports indicating ability of SOCS36E to suppress Hop<sup>TumI</sup> signalling *in vivo*, resulting in decreased tumour index (Bina *et al.*, 2010). This is particularly important considering Hop<sup>TumI</sup> independence from Dome in the same assay (Nina Bausek and Samira Bina, personal communication), an indication supported by the result in Fig.IV.6C indicating additive effect of SOCS36E overexpression and Dome knockdown.

The existence of a SOCS-box-independent mechanism is also supported by observations that SOCS36E but not Elongins B/C or Cullin 5 inhibits the basal activity of the pathway (Fig.IV.6B and Fig.IV.8A). Consistent with this, truncation of the SOCS box domain does not affect suppression of the basal activity of the pathway (Fig.IV.8A). This implies that this secondary mechanism is required for suppression of the basal activity of the pathway. Recognition of the importance of SOCS36E in regulation of pathway signalling in two different states of activation, basal and stimulated, is an important one, as SOCS36E was thus far believed to act only as part of a negative feedback loop (ie. suppressing activity only after stimulation). In this context, SOCS36E resembles SOCS4 and 5 that are believed to be constitutively present in cells, while SOCS1-3 and CIS are induced upon pathway activation (reviewed in Linossi *et al.*, 2013). However, SOCS36E is a strong transcriptional target of the pathway, suggesting a possibility that protein levels dictate the balance between two mechanisms of function.

### ***IV.3.3 N-terminal of SOCS36E has a role in suppression of the JAK/STAT pathway signalling***

Lack of suppression of basal pathway activity (Fig.IV.8A) by construct missing the N-terminal domain, implies that this domain is important for SOCS36E function. Also in the absence of Elongins B/C and Cullin 5, SOCS36EAN can not suppress the ligand-stimulated pathway (Fig.IV.8C), indicating again that the N-terminal domain is required for protein function. Despite numerous studies on mammalian SOCS molecules, only a handful of reports have indicated a role for the N-terminal in SOCS4 and 5. A report from Seki and colleagues (Seki *et al.*, 2002), indicated N-terminal of SOCS5 to be sufficient for interaction with IL-4 receptor in a phosphotyrosine independent fashion. Moreover, SOCS5 has been reported to associate with EGFR in N-terminal dependent manner (Kario *et al.*, 2005; Nicholson *et al.*, 2005). Although the reported interactions between SOCS36E and EGFR signalling pathway was not investigated in this study, it is important to note that truncation of the N-terminal domain of SOCS36E also decreased interaction with Sevenless, *Drosophila* EGFR receptor (Almudi *et al.*, 2010). I also observed that SOCS36EAN construct did not co-immunoprecipitate with Dome (Fig.IV.10C), which might explain why high expression levels of this construct was required to destabilize Dome (Fig.IV.9). This implies that the N-terminal of SOCS36E plays a similar role to the N-terminal of SOCS3, which has been characterized to contain an N-extended SH2 domain (N-ESS) involved in orientation of interaction with phosphorylated tyrosine residue (Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999; Babon *et al.*, 2006). Interestingly, a recent report from Feng and colleagues (Feng *et al.*, 2011) indicated presence of a conserved motif in the N-termini of SOCS4 and 5 that has potential role in protein interaction. As SOCS36E is a homologue of mammalian SOCS4 and 5, my results provide further evidence for the conserved functional role of the long N-termini in SOCS molecules.

I also observed that, similarly to mammalian SOCS molecules, SOCS36E requires an intact SH2 domain to interact with its substrates. This conclusion is supported by evidence from co-immunoprecipitation with Dome (Fig.IV.10C) as well as functional assays (Fig.IV.8, 9 and 11). This is in line with reports of SOCS36E interaction with Sevenless being dependant on an intact SH2\* domain and SOCS36E substrate tyrosine

phosphorylation (Almudi *et al.*, 2010). As phosphorylated tyrosine residues are required for interaction with SH2 domain and given that SOCS36E co-immunoprecipitated with Dome and Hop irrespective of ligand-mediated stimulation of the pathway, it is plausible that Dome and Hop are constitutively phosphorylated on tyrosines that are not essential for signal transduction, or STAT92E binding in this case. However, I could not exclude the possibility that SOCS36E binds to the same phosphorylated tyrosine residues as STAT92E does, which would classify as competitive binding.

#### ***IV.3.4 Regulation of phosphorylation of the JAK/STAT pathway components***

Only SOCS1 and 3 have been reported to interact directly with JAK kinases and obscure their catalytic activity by steric hindrance (Kershaw *et al.*, 2013). While structurally SOCS36E is distinct to SOCS1 or 3, it is involved in regulation of Dome phosphorylation in response to pathway stimulation (Fig.IV.11) as well as inhibition of STAT92E phosphorylation (Fig.IV.13A). I have considered two possible mechanisms by which SOCS36E can regulate phosphorylation of pathway components. Firstly, it could affect catalytic activity of Hop. This possibility is less likely to be true, based on the lack of change in Hop phosphorylation levels in cells (Fig.IV.11) and *in vitro* kinase activity assays assessing Hop's ability to auto-phosphorylate (Fig.IV.12). It should be noted however, that the stoichiometry of the *in vitro* reactions was unknown. Therefore, it is possible that insufficient amounts of SOCS36E constructs were present in the reaction mixture to effectively inhibit activity of Hop. Furthermore, Dome was not present in the reaction mixtures in the kinase assay. Considering my observation that SOCS36E preferentially binds to Dome, it is possible that Dome is required for SOCS36E to position itself in a way that allows for inhibition of Hop's catalytic activity, similarly to SOCS3 (Kershaw *et al.*, 2013). The second possibility regarding SOCS36E mechanism of function is that SOCS36E "masks" Tyrosine residues on substrates from the Hop kinase, thereby preventing phosphorylation. Investigation of this possibility would require crystallographic studies, which might be very interesting but also very challenging technically. The fact that SOCS36E suppresses phosphorylation of Dome and STAT92E might imply that the first possibility is more likely, however it cannot be

excluded that phosphorylation of STAT92E is a consequence of increased phosphorylation of Dome (Fig.IV.11).

## **Chapter V Analysis of the catalytic activity of Hopscotch and its gain-of-function mutants**

### **V.1. Introduction**

#### ***V.1.1 Mutations in JAK kinases often lead to pathologies***

The JAK/STAT signalling pathway plays a central role in numerous developmental processes as well as in the maintenance of homeostasis. Phosphorylation of pathway components, mediated by JAK kinases is essential for pathway activity. Not surprisingly, mutations affecting catalytic activity of JAKs are often pathological in nature. Interestingly, such mutations do not have to arise in the JH1 kinase domain, as other domains provide regulatory function, as described previously (Section I.2.5). Investigation of the pathological significance of those mutations at molecular level is challenging in mammals due to redundancies between receptors, JAKs, STATs and negative regulators. Moreover, pathogenicity of mutations in JAKs depends not only on the residue affected, but also on the tissue or cell population affected. This is particularly important in case of JAK3 mutations, as expression of JAK3 is strongly associated with the immune system (Ghoreschi *et al.*, 2009).

*Drosophila* does not possess adaptive immune system, therefore it is hard to justify using this organism to model effects of JAK mutations on the immune system, such as Y100C in JAK3. However, fruit fly is a suitable model organism to study tumour development. Two gain-of-function Hop alleles, Hop<sup>Tum1</sup> and Hop<sup>T42</sup>, have been reported to produce black melanotic masses, phenotypes reminiscent of myeloproliferative neoplasias (Harrison *et al.*, 1995; Luo *et al.*, 1997).

#### ***V.1.2 Hop as a model to study pathogenic JAK mutations***

Similarly to mammalian JAKs, Hop contains FERM domain located towards the N-terminal and JH1 and JH2 domains towards the carboxy terminal (Fig.V.1A). This

Figure.V.1 Homology between Hop and mammalian JAK kinases

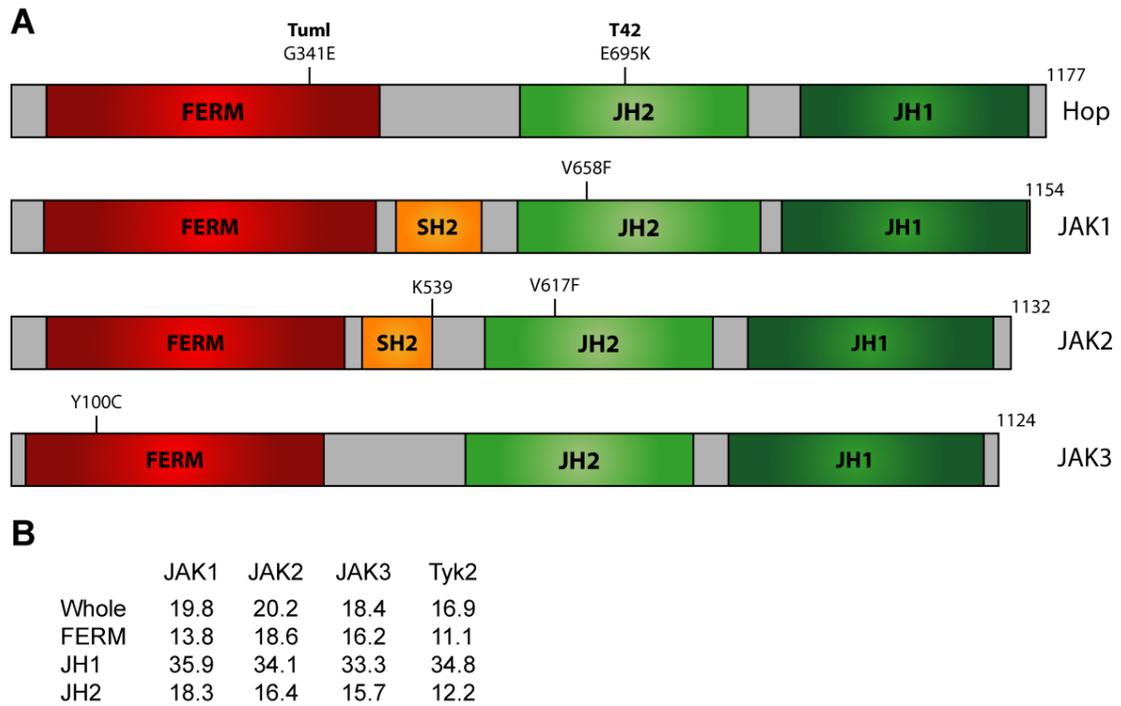


Figure.V.1 Homology between Hop and mammalian JAK kinases. (A) Schematic representation of the *Drosophila* Hop and mammalian JAK1-3 proteins. Individual domains are colour coded and labelled. Numbers at the right indicate amino acid length of proteins. Selected single point mutations that are associated with diseases are indicated for each protein. Names given to oncogenic Hop alleles are in bold font over the mutation indicators. (B) Sequence shared identity with Hop across indicated portions of proteins, generated with ClustalW2 sequence alignment analysis tool. Domain location and sizes were determined using ExPASy Prosite analysis tool.

structural similarity is not reflected in the amino acid sequence, with only 20% shared identity between Hop and JAK2, its closest human homologue (Fig.V.1B). Mammalian JAKs share much stronger amino acid conservation between them than with Hop.

Interestingly, also pathogenic mutations seem to be localized to the similar sites in Hop. Hop<sup>Tum1</sup> mutation is a single amino acid substitution, G341E, located in the FERM domain similarly to Y100C mutation in JAK3 or R340Q in JAK2 (Cacalano *et al.*, 1999). Hop<sup>T42</sup> is more reminiscent of a well studied JAK2 V617F pathogenic mutant, which is strongly associated with myoproliferative neoplasias. The Hop<sup>T42</sup> mutation is a single point mutation substituting glutamic acid at position 695 to lysine (E695K). Interestingly, despite affecting different domains of the kinase, both Tum1 and T42 alleles cause the same phenotype of blood cell over proliferation leading to the formation of melanised tumours (Harrison *et al.*, 1995; Luo *et al.*, 1997). This phenotype was reproduced by tissue specific expression of mutated Hop proteins in the lymph gland, *Drosophila* equivalent of mammalian haematopoietic organ. Also strong over-expression of wild-type Hop resulted in similar phenotype, leading to characterisation of both mutants as gain-of-function mutations causing increased pathway activity. However, hyperphosphorylation of the kinase and STAT92E has been reported for both T42 and Tum1 mutants, but not upon over-expression of wild-type Hop (Harrison *et al.*, 1995). Similar observations regarding hyperphosphorylation of pathway components were reported for mammalian JAK gain-of-function mutants. Studies on how two mutations in distant domains of Hop can result in a similar phenotype offers a unique opportunity to gain insight into structure-function relationship of Hop and molecular aetiology of its mutants.

## V.2. Results

### V.2.1 Hop<sup>T42</sup> and Hop<sup>Tum1</sup> activate JAK/STAT pathway more potently than wild-type Hop

Both, Hop<sup>Tum1</sup> and Hop<sup>T42</sup> have been previously shown to elevate transcriptional pathway activity *in vivo* and *in vitro*, and Hop<sup>Tum1</sup> has been used as a dominant genetic background in which to conduct genetic screens and screen validation

Figure.V.2 Increased activity of GOF Hop mutants is not correlated by their autophosphorylation or STAT92E phosphorylation

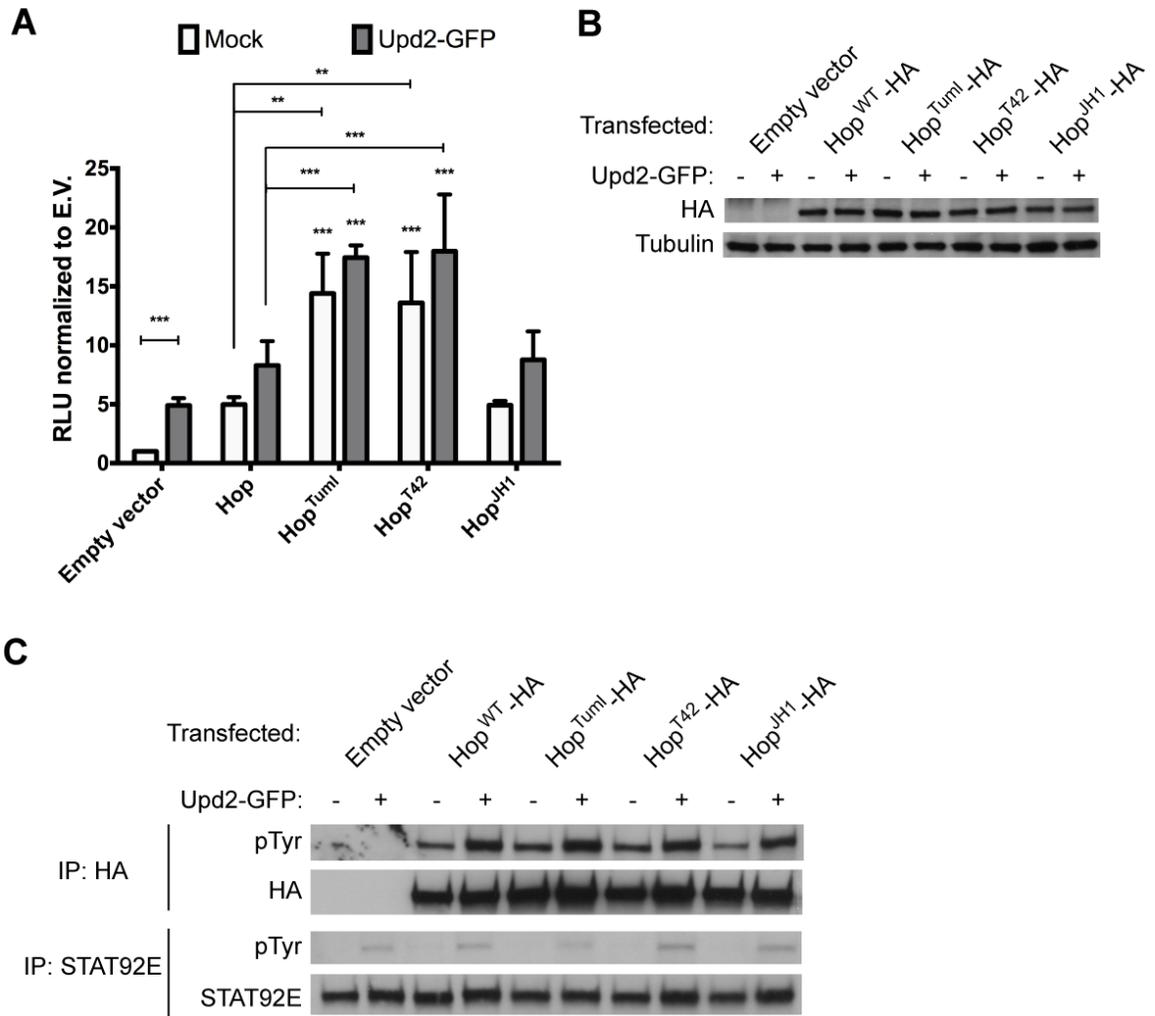


Figure.V.2 Increased activity of GOF Hop mutants is not correlated by their autophosphorylation or STAT92E phosphorylation. (A) Transcriptional activity of the pathway in Kc<sub>167</sub> cells transfected with indicated HA-tagged constructs and treated with mock or Upd2-GFP conditioned media for 4 days was measured by luminescence assay. Results were normalized to mock treated Empty vector transfected cells. Stars above columns indicate statistical significance compared to Empty vector cells within mock or Upd2-GFP treated group, determined by two-way ANOVA followed by Bonferroni's multiple comparisons test. Statistical significance between mock and Upd2-GFP treated cells transfected with one ligand was determined by Student's t-test. \*\*\*, p<0.005; \*\*, p<0.01; \*, p<0.05. Error bars represent SEM. (B) Kc<sub>167</sub> cells transfected with indicated Hop constructs were treated with mock or Upd2-GFP conditioned media for 10min and lysed. Lysates were used for SDS-PAGE followed by western blot analysis. (C) Kc<sub>167</sub> cells transfected with indicated HA-tagged Hop constructs were treated with mock or Upd2-GFP conditioned media for 10min prior to lysis with lysis buffer supplemented with phosphatase inhibitors. Lysates were subjected to HA (top two panels) or STAT92E (bottom two panels) immunoprecipitation followed by SDS-PAGE and western blot analysis.

(Harrison *et al.*, 1995; Luo *et al.*, 1997; Müller *et al.*, 2005; Kallio *et al.*, 2010). As a first step to study Hop in a cell based system, and to validate that HA-tagged Hop mutant constructs work in my assays, I assessed transcriptional pathway activity in cells transfected with these constructs. Additionally, I attempted to generate a kinase dead version of Hop by mutating a conserved lysine residue, K926 in the JH1 domain to glutamine. This missense change was selected based on sequence homology with JAK2 and was predicted to be essential for ATP binding by sequence analysis software (ExpASy Prosite). This mutant was abbreviated as Hop<sup>JH1</sup> and used alongside the GOF mutants in these experiments, intended as negative control. Expression of exogenous wild-type Hop resulted in increased basal and stimulated activity of the pathway, however the increase was not as dramatic as in the case of GOF mutants (Fig.V.2A). No statistical significance was observed between mock and ligand stimulated conditions in cells transfected with Hop constructs, however data indicated a trend in this direction. The Hop<sup>Tum1</sup> and Hop<sup>T42</sup> mutations were equally potent in increasing pathway activity. Increases compared to endogenous were over three-fold following stimulation with pathway ligand and over fifteen-fold under steady state conditions. Surprisingly, the Hop<sup>JH1</sup> mutant also caused an increase in pathway activity to a similar degree as the wild-type Hop, suggesting that the lysine mutated was most likely not essential for catalytic activity. To exclude the possibility of transcriptional differences arising from differences in kinase dosage, protein levels of transfected constructs were assessed. (Fig.V.2B).

Phosphorylation of STATs is commonly used as an indication of pathway activity in mammalian system. Previous reports indicated STAT92E to be hyperphosphorylated in cells transfected with GOF Hop mutants (Luo *et al.*, 1997). I therefore investigated tyrosine phosphorylation of Hop mutants and endogenous STAT92E by immunoprecipitation and subsequent western blotting with pY specific antibody. While phosphorylation of Hop and its mutants increased in response to stimulation with pathway ligand (Fig.V.2C), no clear difference between the various Hop constructs was observed. In addition, STAT92E was phosphorylated only in response to pathway stimulation, even upon expression of GOF Hop mutants. Interestingly, STAT92E

Figure.V.3 Hop and its mutants localize to the cytoplasm

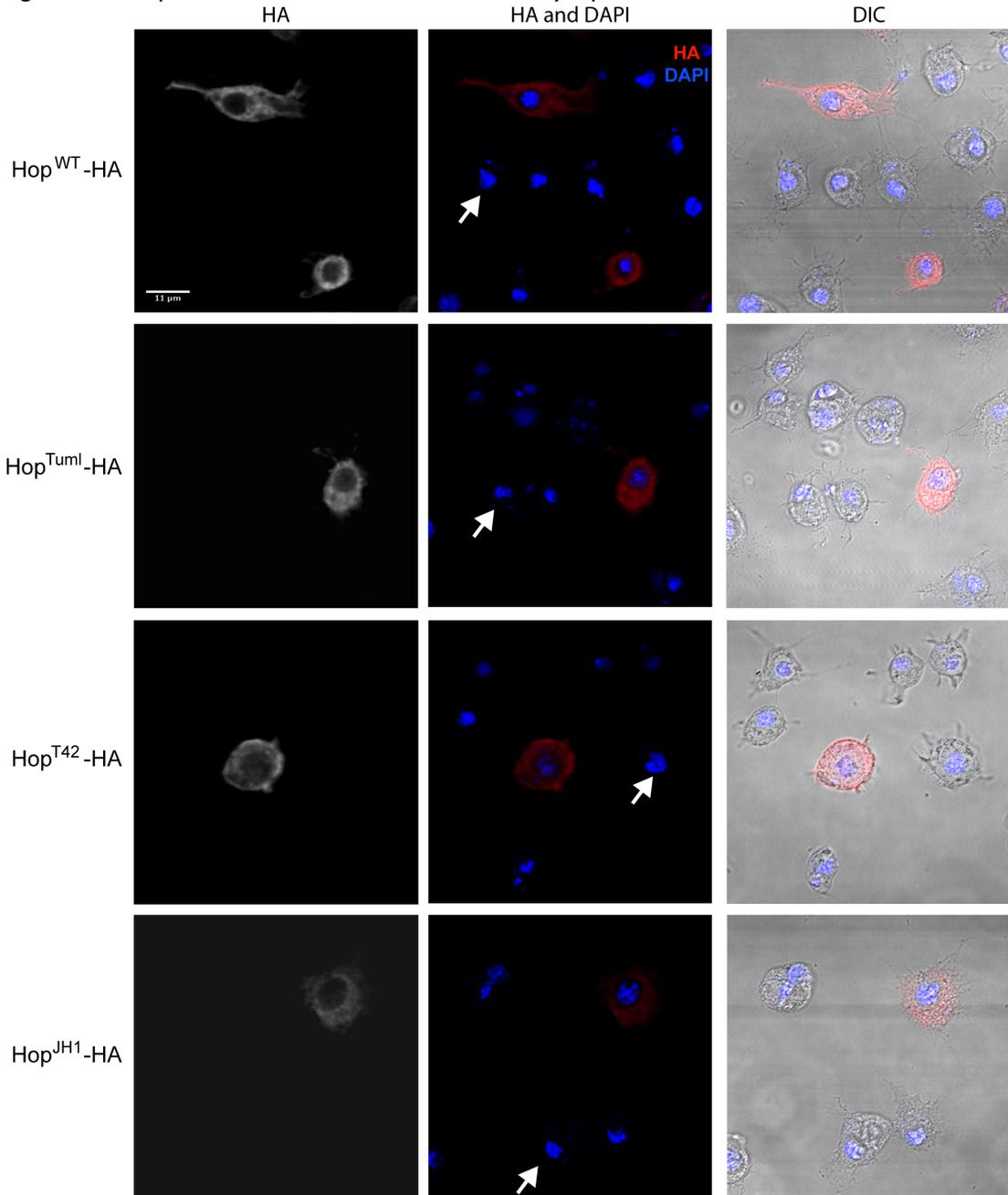


Figure.V.3 Hop and its mutants localize to the cytoplasm. Confocal microscopy images of cells transfected with indicated HA-tagged Hop constructs. White arrows point to not transfected cells, acting as controls. Left panel: HA staining in greyscale, middle panel: composition of DAPI and Ha staining, right: DIC (in grey) with DAPI and HA staining.

phosphorylation levels in cells transfected with Hop<sup>Tum1</sup> construct appeared to be slightly lower. These results indicate that GOF Hop mutants hyperactivate the JAK/STAT pathway without hyperphosphorylating STAT92E or themselves.

The lack of increased phosphorylation levels of STAT92E in cells transfected with Hop GOF mutants might be indicative of a non-canonical signalling exerted by those mutants, including potential cross-talk with other signalling pathways or modulation of epigenetic landscape, among others (Fig.V.2C). This is particularly relevant in case of Hop<sup>Tum1</sup> mutant, which exhibited even lower levels of STAT92E phosphorylation. Activation of another signalling pathway is only one of the means by which GOF kinase mutants have been reported to exert oncogenic effects (Busch *et al.*, 2009). However, alternative signalling pathways were not analysed in this study. Another possibility is nuclear translocation and alteration of epigenetic landscape, as reported in case of JAK2 V617F mutant (Dawson *et al.*, 2009). To account for this possibility, I inspected subcellular localization of Hop constructs. All of the constructs were evenly distributed across the cytoplasm (Fig.V.3). Enrichment at the plasma membrane reported for mammalian JAK proteins was not observed with Hop constructs, potentially due to expression of exogenous protein (Haan *et al.*, 2006). No clear nuclear localization was observed (Fig.V.3), however this method might not be sensitive enough to detect small amounts of proteins and cell fractionation might be required. It should be kept in mind that nuclear translocation of JAK2 V617F has been both proposed and questioned (Dawson *et al.*, 2009; Girodon *et al.*, 2011).

### ***V.2.2 Hop GOF mutants bind to Dome, however they do not depend on it***

Signalling by JAK2 V617F has been shown to depend on cytokine receptor presence, but not on ligand binding (Lu *et al.*, 2008). To investigate whether Hop GOF mutants require Dome for activity, I measured pathway transcriptional activity in cells transfected with Hop mutant constructs and treated with control or Dome RNAi (Fig.V.4A and B). Under steady state conditions knockdown of Dome caused decrease in basal pathway activity in control cells, indicating that Dome is required for this low level basal activity (Fig.V.4A). Alternatively, basal activity might represent activation of the pathway by small amounts of pathway ligand that are secreted by Kc<sub>167</sub> cells (Wright *et al.*, 2011). No statistically

significant difference in transcriptional activity was observed upon Dome knockdown in cells transfected with Hop constructs, with exception of Hop<sup>JH1</sup> transfected cells, suggesting that this mutant may have a higher dependence on the receptor than wild-type Hop. By contrast, measurement of the ligand-stimulated pathway activity revealed that knockdown of Dome decreased firefly luciferase (Fig.V.4B). This can be potentially attributed to the endogenous pathway being down-regulated, as is the case in cells transfected with the Empty vector. However, knockdown of Dome in cells expressing GOF Hop mutant constructs resulted in *6x2xDrafLuc* reporter accumulation to the same levels as in the Empty vector cells treated with control dsRNA. This implies that Dome is required but not necessary for GOF Hop mutants to activate the pathway.

In order to confirm the efficiency of Dome knockdown at the protein level, I assessed the phosphorylation level of wild-type Hop in RNAi background. I observed that cells deprived of Dome had decreased phosphorylation levels of Hop under steady state conditions and very mild increase was observed following stimulation with pathway ligand, most likely due to incomplete Dome knockdown (Fig.V.4C). Positive and negative control knockdowns produced interesting pattern of Hop phosphorylation, as knockdown of Ptp61F resulted in only a mild increase in Hop phosphorylation in response to pathway stimulation when compared to control cells. This increase was expected to be higher considering previous reports regarding the role of Ptp61F as a negative regulator of the *Drosophila* JAK/STAT pathway (Baeg *et al.*, 2005; Müller *et al.*, 2005). Knockdown of STAT92E, which was expected not to produce any change in Hop pTyr levels, resulted in elevated Hop phosphorylation independent of ligand (Fig.V.4C). While not investigated any further, it is possible that this is an indirect effect due to changes in the transcription of negatively acting pathway regulators such as SOCS36E (Callus and Mathey-Prevot, 2002, reviewed in Arbouzova and Zeidler, 2006).

Finally, I investigated the interaction of Hop GOF mutants with Dome by pulling down Dome. Dome interaction with Hop<sup>WT</sup> and Hop<sup>Tum1</sup> appeared to be stronger compared to Hop<sup>T42</sup> (Fig.V.4D). Dome P925I mutant, previously described to have impaired interaction with Hop (Fig.III.4), was used as a negative control. None of the Hop constructs used interacted with Dome P925I at a detectable level, indicating that

Figure.V.4 Hop GOF mutants display lower dependency on receptor presence

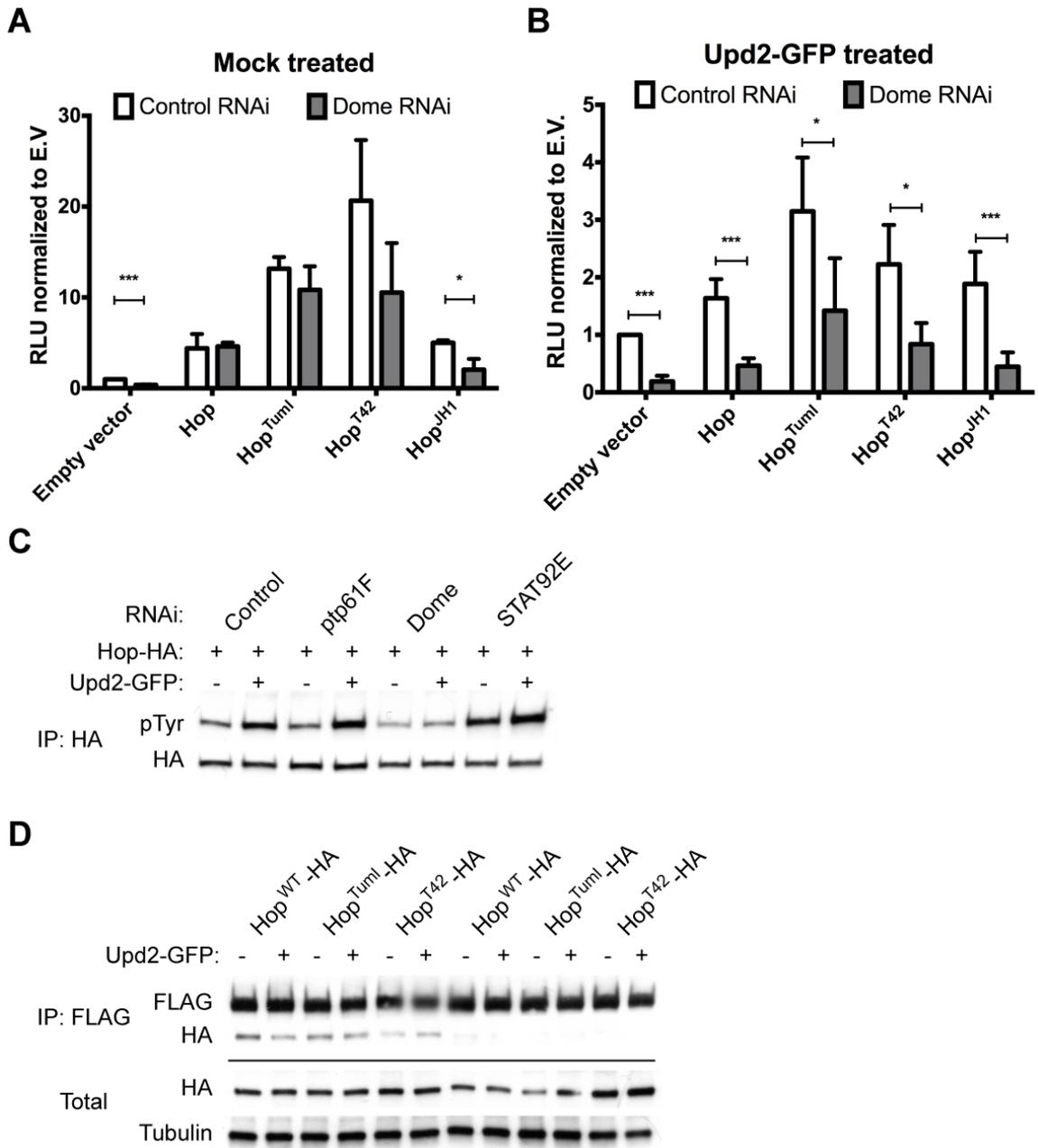


Figure.V.4 Hop GOF mutants display lower dependency on receptor presence. (A, B) Transcriptional pathway activity in Kc<sub>167</sub> cells transfected with indicated HA-tagged Hop constructs and treated with Control of Dome dsRNAs, as indicated, for 4 days was measured by *6x2xDraf* luminescence assay. Alongside the dsRNA treatment, cells were treated with mock (A) or Upd2-GFP conditioned media (B). Statistically significant difference is indicated by stars above the bars, as determined by Student's t-test with \*\*\*, p<0.005; \*\*, p<0.01; \*, p<0.05. Results were normalized to Empty vector transfected cells treated with control dsRNA. Error bars represent SEM. (C) S2R+ cells batch-transfected with Hop-HA were treated with indicated dsRNAs for 4 days and stimulated with mock or Upd2-GFP conditioned media for 10min prior to lysis with lysis buffer supplemented with phosphatase inhibitors. Lysates were used for HA immunoprecipitation and analysed with SDS-PAGE followed by western blot. (D) Cells co-transfected with Dome-FLAG or Dome P925I-FLAG and indicated HA-tagged Hop constructs were treated with mock or Upd2-GFP conditioned media for 10min and lysed. Lysates were subjected to immunoprecipitation with anti-FLAG antibody and analysed with SDS-PAGE and western blot.

each Hop mutant uses the same interaction site. These results indicate that GOF mutations in Hop do not stabilize interaction with Dome, while T42 mutation might mildly destabilize this interaction.

### ***V.2.3 Hop GOF mutants do not have increased autophosphorylation kinetics***

One of the potential mechanisms by which GOF JAKs hyperactivate the pathway is increased efficiency of the catalytic activity, in which more STAT proteins are phosphorylated prior to the termination of signalling. To investigate whether this is the case with Hop GOF mutants, I developed and conducted *in vitro* kinase activity assays. Due to lack of available substrates, I used Hop autophosphorylation as an indicator of catalytic activity (Saharinen *et al.*, 2000). The most striking observation was much lower incorporation of  $^{32}\text{P}$  by Hop<sup>JH1</sup> than the remainder of Hop constructs used at 10 and 30min of reaction (Fig.V.5A). I conducted a time course activity assay over one hour period and best fit curves were determined to characterise reaction kinetics (Fig.V.5B-D). As expected, Hop<sup>JH1</sup> mutant kinetics proved to be slower compared to the rest of the constructs. The plateau, representing saturation of phosphorylation sites in the population of kinases, was not achieved in the duration of the assay as all curves were still climbing (Fig.V.5C). For this reason it is impossible to determine whether any of the Hop mutants might plateau at a higher level and therefore reveal the possible existence of additional phosphorylation sites. The initial kinetics of Hop<sup>WT</sup>, Hop<sup>Tum1</sup> and Hop<sup>T42</sup> were very similar to each other. This implies that Hop GOF mutants' catalytic activities are not altered compared to the wild-type Hop, while mutation in the JH1 domain resulted in decreased autophosphorylation dynamics.

## **V.3. Discussion**

In this study I have investigated the differences between wild-type Hop and its GOF mutants, Hop<sup>Tum1</sup> and Hop<sup>T42</sup>, using *ex vivo* and *in vitro* approaches. I introduced a single point mutation in the JH1 domain that affected the kinetics of the kinase, but did not render it dormant as expected. With exception of lowered kinetics indicated by *in vitro* kinase activity assay, the Hop<sup>JH1</sup> mutant behaved very similarly to the wild-type

Figure.V.5 Autophosphorylation kinetics of Hop GOF mutants are similar

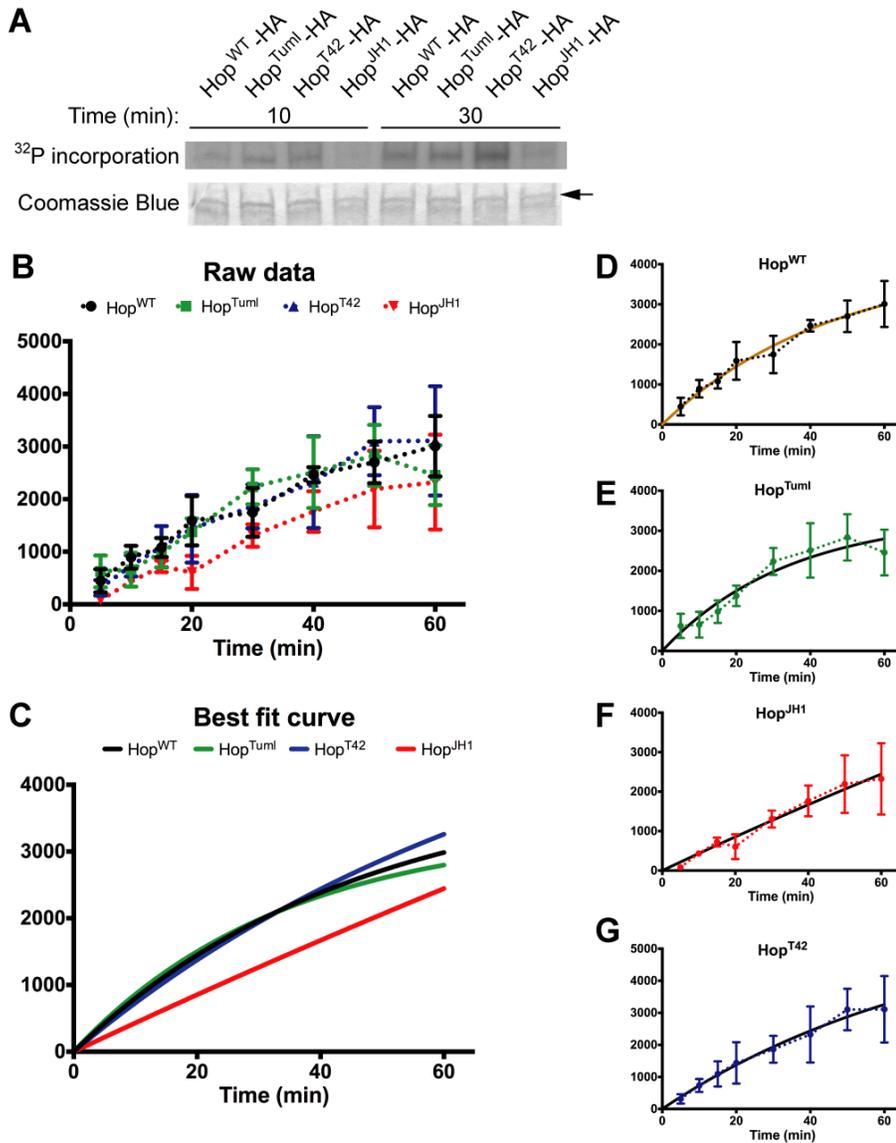


Figure.V.5 Autophosphorylation kinetics of Hop GOF mutants are similar. (A-G) Indicated HA-tagged Hop constructs were transfected into S2R+ cells and extracted by immunoprecipitation, left on beads and subjected to *in vitro* kinase activity assay, as described in materials and methods for indicated periods of time. (A) Representative image of the kinase activity assay, arrow points towards radioactive Hop construct band (top band). Bottom band is likely to be unspecific. (B-G) Quantification of autophosphorylation kinetics of indicated constructs, with raw quantification (B) and best fit curve, according to equation:  $Y=TOP*[1-\exp(K*X)]$  (C). Constructs are colour coded for convenience. (D-G) Raw data and best fit curve of individual constructs. Error bars indicate SEM.

Hop. Transcriptional hyperactivation of the pathway observed upon expression of two GOF Hop mutants was not correlated by phosphorylation of JAKs themselves or STAT92E, the only STAT protein in *Drosophila*. Also the autophosphorylation kinetics were not affected by both mutations. Despite both mutants interacting with Dome similarly to wild-type Hop, they did not require the receptor to induce pathway activity. Taken together, these results indicate GOF mutations in Hop act to hyperactivate the pathway via a mechanism that appears to be independent of Dome but which does not change the catalytic kinetics of the kinase or increase STAT92E tyrosine phosphorylation.

### ***V.3.1 Increased transcriptional pathway activity is not correlated by phosphorylation of pathway components***

Gain-of-function mutations in kinases often result in hyperactivation of the pathway, a description used to characterise either constitutively active pathway or pathway that is overly sensitive to ligand stimulation, leading to numerous malignancies (reviewed in Vainchenker and Constantinescu, 2013). Using transcriptional reporter of pathway activity I confirmed that activity of STAT92E is elevated in cells transfected with Hop GOF mutants (Fig.V.2A). This effect was particularly prominent under steady state conditions, with firefly luciferase reporter activity 15-fold higher than empty vector transfected cells. It is important to note that expression of wild-type protein produced an increase in pathway activity, especially noticeable under steady state conditions. While this effect appeared to be statistically insignificant, the trend is relatively clear. This implies that at high levels exogenous Hop<sup>WT</sup> can hyperactivate the pathway, a finding consistent with previous reports (Luo *et al.*, 1997). One of the mechanisms by which GOF Hop constructs can increase transcription of artificial reporter of pathway activity is by phosphorylating STAT92E in a manner different than wild-type kinase would, by hyper- or differential phosphorylation of additional residues. I did not observe the tyrosine hyperphosphorylation of STAT92E by GOF Hop constructs that was reported previously (Fig.V.2.B) (Luo *et al.*, 1997). Discrepancies in results might be caused by use of different cell lines, expression levels of constructs, technical differences or any combination of those reasons. The same reasons might apply to discrepancies in observed

phosphorylation levels of Hop and its mutants. Previous reports indicated Hop<sup>Tum1</sup> and Hop<sup>T42</sup> to be hyperphosphorylated compared to Hop<sup>WT</sup>, which is not observed in my hands (Harrison *et al.*, 1995; Luo *et al.*, 1997).

In either case, investigation of tyrosine phosphorylation of STAT92E might be insufficient and misleading, as serine phosphorylation of mammalian STATs has been reported to play a role in transcriptional regulation (Schuringa *et al.*, 2000; Friedbichler *et al.*, 2010, 2011; Steen *et al.*, 2013, reviewed in Decker and Kovarik, 2000; Schindler *et al.*, 2007). While JAKs are considered to phosphorylate their substrates only on tyrosine residues, the JH2 domain of JAK2 has been shown to be a dual specificity kinase domain (Ungureanu *et al.*, 2011), suggesting that serine/threonine residues within STAT92E might be phosphorylated by Hop. Alternatively, such phosphorylation might be an indirect effect elicited by cross-activation of PI3K/Akt/mTor pathway by hyperactive JAKs (Levine and Wernig, 2006; Busch *et al.*, 2009).

### ***V.3.2 Interplay between Hop GOF mutants and pathway receptor***

Investigation of Hop requirements for its receptor revealed that under steady state conditions transcriptional pathway activity is not affected by Dome knockdown in a background that expresses exogenous Hop (Fig.V.4A). On the other hand, pathway activity following ligand-mediated pathway stimulation was decreased by Dome RNAi in control cells and in cells transfected with wild type Hop constructs (Fig.V.4B). This decline in activity cannot be attributed only to the inability of the endogenous pathway to transduce signal, as no difference between steady state and stimulated conditions was observed in the absence of dsRNAs in cells expressing GOF Hop mutants (Fig.V.2A). However, GOF Hop mutants still elicited elevated levels of pathway activity in the absence of Dome. This implies that Dome facilitates Hop<sup>Tum1</sup> and Hop<sup>T42</sup> signalling but is not necessary for the process. This result is consistent with *in vivo* tumour formation assay which find that melanotic spot size and number are independent of Dome knockdown (Nina Bausek, unpublished data). Direct interaction of Hop with Dome was comparable between different Hop constructs, reinforcing the idea that GOF mutations do not affect interaction with the receptor. Another possibility worth considering is that in the absence of Dome another receptor can potentially interact with Hop. Latran has been

shown to function as an inactive receptor for the JAK/STAT pathway in *Drosophila* that can bind Hop (Kallio *et al.*, 2010). Even though Latran has not been shown to interact with STAT92E, it is possible that Latran can substitute for Dome as Hop or Hop mutants' binding partner.

### ***V.3.3 JH1 domain and kinetics of autophosphorylation***

When assayed *in vitro*, JAK2 has a relatively poor catalytic activity compared to other members of the mammalian JAK family (Saharinen and Silvennoinen, 2002). Given that JAK2 represents the closest Hop homologue, it may not be surprising that Hop also displays a relatively low kinetics of catalytic activity. Never the less, the comparison of kinetics of auto-phosphorylation of wild-type Hop with its GOF mutants did not reveal any significant differences between the constructs (Fig.V.5). However, the Hop<sup>JH1</sup> mutant autophosphorylated itself at a slower rate compared to the other three constructs. This is particularly interesting, considering that the construct did not demonstrate decreased activity in the functional assays compared to wild-type Hop construct (Fig.V.2). The only exception was a decrease in basal pathway activity upon Dome knock down, that was not observed with other constructs. This suggests that Hop<sup>JH1</sup> construct might be more dependant on interaction with the receptor, a factor that was not present in the *in vitro* kinase activity assay. It should be noted that the JH2 domain of Hop<sup>JH1</sup> construct remained intact. A possibility that JH2 domain play another role besides autophosphorylation, should be also considered.

The fact that mutations in the FERM or JH2 domains of Hop did not increase activity of Hop is baffling, as similar oncogenic mutations in mammalian JAKs have been reported to increase activity of JH1 domain (Yeh *et al.*, 2000; Zhao *et al.*, 2010; Ungureanu *et al.*, 2011). Considering homology between mammalian JAKs and Hop, it is probable that G341E and E695K mutations introduced structural alterations in the protein that did not have any steric but rather allosteric effects. This might relate to structural mimicry of activated state, enhanced trans-phosphorylation kinetics or the facilitation of interactions with substrates. At the same time, it should be considered that autophosphorylation of kinases, especially in the absence of the receptor, might not be the most appropriate measurement of kinase activity. While autophosphorylation has been

widely reported as a regulatory step, phosphorylation of STATs has direct physiological relevance (Saharinen and Silvennoinen, 2002; Mazurkiewicz-Munoz *et al.*, 2006; Yan *et al.*, 2012). Studies on JAK2 indicated that V617F mutation does not increase kinetics of reaction but rather increases affinity for the substrates (Zhao *et al.*, 2010). While these results provide an insight into the molecular mechanisms, more detailed studies will be required to dissect the differences and similarities between Hop GOF mutants *in vivo*.

## Chapter VI Conclusions and proposed directions

### VI.1. Introduction

The JAK/STAT signalling pathway plays essential roles in numerous biological processes that are vital for correct development and the maintenance of homeostasis. Misregulation of the pathway can have serious consequences for the organism, resulting in developmental defects as well as diseases, including solid and haematological malignancies (O'Sullivan *et al.*, 2007). In *Drosophila* the JAK/STAT pathway is fully conserved with lower redundancy between pathway components and regulators (Arbouzova and Zeidler, 2006). Hyperactivation of the pathway results in blood cell proliferation defects phenotypically reminiscent of human leukaemia (Crozatier and Meister, 2007). This makes *Drosophila* a suitable model organism to investigate molecular aspects of the JAK/STAT pathway activity.

### VI.2. Domeless

#### *VI.2.1 Evolutionary conservation of the JAK/STAT pathway*

Domeless is the only functional JAK/STAT pathway receptor in *Drosophila*. It has been described as similar to mammalian type I cytokine receptors based on its structural conservation (Brown *et al.*, 2001; Chen *et al.*, 2002). The *Drosophila* JAK/STAT pathway is strongly implicated in the immune response to wasp parasitisation, a process dependent on Dome and regulated on the level of the receptor (Crozatier and Meister, 2007; Makki *et al.*, 2010). Formation of lamellocytes represents a link between immunity and haematopoiesis in *Drosophila*, that has parallels to the mammalian system (O'Shea *et al.*, 2002). In this context, functional similarities between Dome and mammalian type I cytokine receptors are evident.

Missing structures such as identifiable Box domains in the cytoplasmic domain and Immunoglobulin-like domain in the extracellular portion of the receptor as well as

only a semi-conserved WSxWS motif in the CBM set Domeless apart from type I cytokine receptor family. However, I have shown that Dome interacts with Hop via a motif found in type II cytokine receptors, represented mainly by interferon receptors that are essential for the immune system and the functional evidence presented here and in previous reports suggests that these missing domains are not in fact essential for cytokine receptor function.

In addition, Clathrin-mediated endocytosis of mammalian cytokine receptors is predominantly initiated by ligand binding, leading to receptor degradation. Here I have shown that Domeless undergoes constitutive degradation irrespective of stimulation with exogenous ligand or activation of the downstream pathway components, although a similar observation has been made for the Leptin receptor in mammalian cells (Belouzard *et al.*, 2004). Interestingly, the human Leptin polypeptide was shown to be sufficient to activate *Drosophila* JAK/STAT pathway via interaction with Dome, indicating a functional conservation of the CBM (Rajan and Perrimon, 2012). In the same report, strong parallels between *Drosophila* Upd2 and human Leptin were suggested at a functional level, indicating both molecules are involved in regulation of organismal energy balance.

From an evolutionary perspective, it is striking that in mammalian system JAKs and STATs mediate signalling from numerous cell-surface receptors, regulating many biological processes, as outlined above. Conversely, multiple similar processes are regulated in *Drosophila* by a single receptor. This suggests that Dome represents an ancestor protein that likely gave rise to type I and II cytokine receptors, adipokine receptor (Ob-R), erythropoietin-like receptors and growth hormone receptor.

### ***VI.2.2 Dome as a model receptor for regulation of signalling output by endocytosis***

Negative regulatory functions of endocytosis on the JAK/STAT pathway has been described previously, however, the conclusions were made in regard to quantitative signalling, rather than qualitative differences (Howe and Mobley, 2004; Vidal *et al.*, 2010; Platta and Stenmark, 2011). Given the presence of a single receptor that undergoes constitutive degradation, the *Drosophila* JAK/STAT pathway represents an attractive

model to study the regulation of the JAK/STAT pathway by endocytosis. Such a suggestion is supported by numerous factors. Firstly, the lack of redundancy among cytokine receptors and receptor complex composition simplifies the pathway. Secondly, the receptor undergoes constitutive degradation, even in the absence of pathway stimulation. However, my data indicates that subtle differences in internalization and degradation kinetics exist when comparing steady state and ligand-stimulated conditions. Moreover, the ubiquitination pattern of the receptor changes in response to ligand stimulation, suggesting a potential molecular basis for the aforementioned differences in kinetics, while Hop binding seems not to play a role in Dome endocytosis – a characteristic which would allow for studies on the receptor itself, rather than investigation of the whole receptor complex. Despite the failure to identify internalization motifs present in Dome, my data indicates that the process of receptor internalization in *Drosophila* is reminiscent of the mammalian system. It would be interesting to identify the AP-2 interaction motifs in Dome. Finally, commonly accepted advantages of using *Drosophila* as a model organism for *in vivo* studies are applicable.

### **VI.3. Hopscotch**

#### ***VI.3.1 Activity of GOF Hop mutants***

Malignancies arising due to mutations in the JAK/STAT pathway are mostly associated with the kinase itself, with JAK2 V617F being the best characterised mutation. Localised within the JH2 domain, the V617F substitution affects the inhibitory role of the JH2 domain on JH1 domain (Vainchenker and Constantinescu, 2013) and causes an increase in the affinity of JAK2 for STATs (Zhao *et al.*, 2010). Interestingly, the report from Zhou and colleagues also indicated an important role for the JAK2 FERM domain in the regulation of kinase activity. Only few mutations in FERM domains have been reported to be oncogenic, however the molecular mechanisms involved remain unknown (reviewed in Vainchenker and Constantinescu, 2013).

Homologous to JAK2, *Drosophila* Hop shares the same domains as the mammalian JAKs, with the exception of dysfunctional SH2-like domain present in JAK1 and 2. Two independent gain-of-function dominant mutations, *hop*<sup>Tuml</sup> and *hop*<sup>T42</sup>,

localised to the FERM and JH2 domains respectively, result in a phenotype that has been described as being reminiscent of human leukaemia (Harrison *et al.*, 1995; Luo *et al.*, 1997). Due to the structural differences in the JH2 domain of Hop and mammalian JAKs, *hop*<sup>T42</sup> hyperactivates Hop via mechanism that is likely to be different on the structural basis than that observed in JAK2 V617F when considering the reports on  $\pi$ -stacking phenomenon caused by V617F mutation (Dusa *et al.*, 2010; Gnanasambandan *et al.*, 2010). Despite the structural differences, the functional outcome might be the same, as my data indicates that neither the *hop*<sup>T42</sup> nor *hop*<sup>Tuml</sup> mutation causes an increase in the catalytic activity of the Hop JH1 domain (measured by autophosphorylation) or an increase in STAT92E phosphorylation. While this data contradicts previous reports, I did observe an increase in transcriptional activity of the pathway. This indicates that melanotic tumours observed in flies carrying these mutations arise due to non-STAT mediated activity, including non-canonical signalling. Such mechanisms have already been reported for JAK2 V617F mutation, which is implicated in alteration of the epigenetic landscape as well as activation of other signalling pathways (Dawson *et al.*, 2009; Vainchenker and Constantinescu, 2013).

### ***VI.3.2 Proposed direction of research on Hop GOF mutations***

One of the major issues encountered during my investigation of Hop<sup>Tuml</sup> and Hop<sup>T42</sup> using cell culture approaches was caused by transfection of the exogenous kinase. Introduction of additional Hop kinase caused an increase in pathway activity even in the case of the wild-type Hop. While this increase was not as dramatic as in the case of GOF mutants, detailed analysis of the functions of Hop derived from such an approach may therefore be skewed. Therefore, flies carrying the mutations should be used to investigate molecular mechanisms of Hop hyperactivation. An alternative to *in vivo* approach is establishment of primary cell lines using cells extracted from Hop<sup>Tuml</sup> and Hop<sup>T42</sup> flies, which would allow analysis to be undertaken on proteins expressed at normal endogenous levels – although this might be technically challenging. Both approaches, cell culture and *in vivo*, have some advantages and disadvantages, however they might prove complementary to each other. As fly lines are readily available, I will briefly outline proposed experiments below.

The data presented in this thesis did not identify any changes in STAT92E tyrosine phosphorylation levels following expression of Hop GOF mutants. This should be validated using biochemical techniques *in vivo*. While technically challenging, immunoprecipitation of endogenous STAT92E from tissue expressing Hop or its GOF mutants at endogenous levels could prove a powerful tool. Cells residing in the *Drosophila* haemolymph would be a prime tissue for such investigation, however other tissues/organs might be suitable for the purpose. This approach is especially relevant considering the tissue specific drivers that would allow for exogenous expression or RNAi-mediated ablation of pathway regulators. The same biochemical techniques could be employed to study other pathways that might be affected by hyperactive kinases, such as Akt and Erk pathways reported to be activated by JAK2 V617F, as well as proteins implicated in epigenetic regulation. For example, Histone 3 has been reported to be phosphorylated by nuclear localised JAK2 V617F. Complementary to protein based assays, qPCR of pathway target genes can also be used to determine the activity of the pathways investigated. As another example, numerous cancers have been associated with silencing of SOCS proteins, it would be interesting to investigate whether expression of the pathway target SOCS36E is changed in flies carrying Hop GOF mutations.

A particularly appealing method to study differences between Hop<sup>WT</sup>, Hop<sup>T42</sup> and Hop<sup>Tuml</sup> at the protein level, is high resolution mass spectrometry (Mann *et al.*, 2013). Alternative and cheaper approach is a 2D differential in-gel electrophoresis, which, when undertaken at sufficient resolution, can identify differences in protein expression level as well as protein modifications. Such high throughput methods can provide a complete overview of processes affected by oncogenic mutations arising in Hop, which can then be followed-up by hypothesis-driven studies.

#### **VI.4. SOCS36E**

The SOCS family of proteins have received a lot of attention due to their roles as tumour suppressors (Elliott *et al.*, 2008). In particular, SOCS1 and 3 have been a focus of numerous studies because of the unique kinase inhibitory region in their N-terminals (Linossi *et al.*, 2013). By contrast, relatively little is known about the long N-terminal

domain group of SOCS proteins, namely SOCS4-7. Only a few reports have been published which indicate the importance of the N-terminal domain of these molecules for protein interactions (Crocker *et al.*, 2008). Only recently, computational modelling determined the presence of an unstructured motif in the N-terminals of SOCS4 and 5 that might mediate protein-proteins interaction (Feng *et al.*, 2011). My results indicate that the N-terminal domain of *Drosophila* SOCS36, a homologue of SOCS4 and 5, is also required for the interaction with Dome. Moreover, I have shown that the N-terminal domain of SOCS36E also plays a role in the suppression of pathway activity under steady state conditions as well as following pathway stimulation. This function is mediated by inhibition of Domeless phosphorylation, possibly via the obstruction of kinase function. However, autophosphorylation of the kinase was unaffected, therefore it seems unlikely that the N-terminal domain of SOCS36E is functioning in a fashion similar to the KIR present within SOCS1 and 3. This finding opens new avenues of research into the mechanisms of function for long N-terminal domain SOCS proteins.

I have also investigated the ability of SOCS36E to regulate Dome stability in a SOCS box-dependent manner. This is in line with reports indicating that all SOCS proteins are able to form ubiquitin ligases (Babon *et al.*, 2009). However, SOCS proteins are believed to function in a negative feedback loop, being transcriptional targets of the JAK/STAT pathway. Based on my data, a contrary model is suggested, where SOCS36E is constantly present in the cell, suppressing the basal activity of the pathway via its N-terminal and regulating stability of the receptor. I have shown that Dome is ubiquitinated, however it remains to be seen whether SOCS36E is responsible for this modification and whether ubiquitination triggers Dome endocytosis. An alternative model should be considered, where SOCS36E destabilizes Dome indirectly, by affecting another protein.

Finally, the fact that flies homozygous for null mutations in the SOCS36E locus are viable and display only minor defects related to the JAK/STAT pathway is interesting (Bellen *et al.*, 2004; Almudi *et al.*, 2009). Firstly, it suggests that degradation of Dome can occur in the absence of SOCS36E. Secondly, it indicates that regulators of other processes can compensate for the loss of SOCS36E. To provide an example, Ptp61F can potentially suppress phosphorylation of Dome under steady state conditions in the

absence of SOCS36E. It would be interesting to explore what compensatory mechanisms are activated in SOCS36E-null flies.

## **VI.5. Summary**

In this thesis I describe work characterising molecular mechanisms that regulate the *Drosophila* JAK/STAT pathway. I showed that Domeless interacts with Hop via a motif found in interferon receptors and that it undergoes constitutive degradation independent of ligand or kinase. However, its stability is regulated by the SOCS-box domain of SOCS36E, which is also implicated in regulation of Dome phosphorylation via the N-terminal domain. This function is novel for the long N-terminal SOCS molecules, which have not been investigated in detail in this context. Moreover, I investigated the mechanisms by which Hop gain-of-function mutants promote oncogenesis. My results indicate that the core JAK/STAT pathway components are not affected by these mutants, contradicting previous reports, however the pathway is hyperactivated. I suggest that an alternative approach based on *in vivo* studies may be worth pursuing. Taken together, this work provides good basis for further, more in-depth studies.

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