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Evolutionary Conserved Regulatory Mechanisms of the JAK/STAT Pathway

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ACADEMIC DISSERTATION
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Contents

List of original communications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-III.

I **Grönholm J.**, Ungureanu D., Vanhatupa S., Rämet M., Silvennoinen O. Sumoylation of *Drosophila* transcription factor STAT92E. *J Innate Immun*. 2:618-24, 2010.

II Kallio J.*, Myllymäki H.*, **Grönholm J.**, Armstrong M., Vanha-aho L.M., Mäkinen L., Silvennoinen O., Valanne S., Rämet M.
Eye transformer is a negative regulator of *Drosophila* JAK/STAT signaling. *FASEB J.* 24:4467-79, 2010.

III **Grönholm J.**, Kaustio M., Myllymäki H., Kallio J., Saarikettu J., Kronhamn J., Valanne S., Silvennoinen O., Rämet M.
Not4 enhances JAK/STAT pathway-dependent gene expression in *Drosophila* and in human cells. *FASEB J*. 26:1239-50, 2012.

The publication No II has also been used in the doctoral thesis of Jenni Kallio.
* Equal contribution.

Abbreviations

AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
BRWD3	Bromo-domain-containing protein disrupted in leukemia 3
CaMK	Calcium/Calmodulin-dependent protein kinase
CBM	Cytokine binding module
CBP	CREB-binding protein
CIS	Cytokine inducible SH2-containing protein
CNTF	Ciliary neurotrophic factor
CT-1	Cardiotrophin-1
DAP	Diaminopimelic acid
DCV	*Drosophila* C virus
DeSI-1	DeSumoylating Isopeptidase-1
Dif	Dorsal-related immunity factor
Dsp1	Dorsal switch protein 1
dsRNA	double-stranded ribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Elk-1	E twenty six-like-1
Epo	Erythropoietin
ERK	Extracellular-signal-regulated kinase
ET	Eye transformer
Eve	Even skipped
FBS	Fetal bovine serum
FERM	Four point one, erzin, radixin and moesin
FnIII	Fibronectin-type III
GAS	Gamma activated sequence
GBP-1	Guanylate binding protein-1
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	glycoprotein 130
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hop	Hopscotch
IFN	Interferon
IκB	Inhibitor of kappa-light-chain-enhancer of activated B cells
IKK	IκB kinase
IL	Interleukin
Imd	Immune deficiency
IP10	Interferon-γ-inducible protein 10 kDa
IRF-1	Interferon regulatory factor-1

ISGF3	IFN-stimulated gene factor 3
JAK	Janus kinase
JH	Jak homology
JNK	JUN N-terminal kinase
kDa	kiloDalton
LAP-1	Liver-enriched transcription-activating protein-1
LEF1	Lymphoid enhancer-binding factor 1
LIF	Leukemia inhibitory factor
L-PEI	Linear polyethyleneimine
LPS	Lipopolysaccharide
Luc	Luciferace
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MIG	Monokine induced by interferon-γ
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene (88)
NaF	Sodium fluoride
NEM	N-ethylmaleimide
Nf-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OSM	Oncostatin M
PBS	Phosphate buffered saline
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PIAS	Protein inhibitor of activated STAT
PML	Promyelocytic leukemia protein
PMSF	Phenylmethylsulfonyl fluoride
PO	Phenoloxidase
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1B
PTP-BL	Protein tyrosine phosphatase-Basophil like
RanBP2	Ran-binding protein 2
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RT	Room temperature
S2	Schneider 2
SAE	SUMO-activating enzyme
SAP	Scaffold attachment factor-A/B, acinus and PIAS
SCID	Severe combined immune deficiency
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SENP	Sentrin/SUMO-specific protease
SH2	Src homology-2
SIM	SUMO interacting motif
siRNA	Small interfering ribonucleic acid
SLIM	STAT-interacting LIM protein
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription

Su(var)	Suppressor of variegation
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
T-ALL	T cell acute lymphoblastic leukemia
Tap-1	Transporter associated with antigen presentation
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOPORS	Topoisomerase I-binding, arginine/serine-rich
Tot	Turandot
Tpo	Thrombopoietin
Tum-l	Tumorous-lethal
TYK2	Tyrosine kinase 2
UFDS	Ubc9 fusion-directed SUMOylation system
Upd	Unpaired
WT	Wild type

Abstract

Cytokines are secreted proteins or glycoproteins that are responsible for mediating cell to cell signals and orchestrating numerous biological events including the activation, proliferation and differentation of the cells of the immune system. Cytokine signals are mediated through specific transmembrane receptors on the surface of their target cells. The binding of a hematopoietic cytokine to its receptor triggers the activation of receptor-associated Janus kinases (JAKs). This is followed by the activation of their downstream targets, Signal transducers and activators of transcription (STATs) through tyrosine phosphorylation. Activated STATs dimerize and translocate to the nucleus, where they modulate the transcription of their target genes. The activity of this signaling cascade is under the control of regulatory proteins, such as Protein inhibitors of activated STAT (PIAS), Suppressors of cytokine signaling (SOCS) and various protein tyrosine phosphatases (PTPs). Moreover, JAKs and STATs are regulated by different post-translational modifications, like in the case of STAT1 and STAT5 through the covalent conjugation of a small ubiquitin-like modifier (SUMO).

The JAK/STAT pathway has been conserved throughout evolution. In the fruit fly, *Drosophila melanogaster,* the JAK/STAT cascade plays a crucial role in various developmental events as well as in the immune response, and it is activated by a set of secreted mediators called Unpaired (Upd). In this thesis *Drosophila melanogaster* was used as a model organism to study the regulation of the JAK/STAT pathway. The only known *Drosophila* STAT transcription factor, Stat92E, was shown to be modified with SUMO at a single lysine, K187. Mutating this SUMO conjugation site increased the Upd-induced transcriptional activity of Stat92E, suggesting that the sumoylation of Stat92E negatively regulates the *Drosophila* JAK/STAT pathway. This indicates that sumoylation is an evolutionary conserved regulatory mechanism of STAT-mediated signal transduction.

RNA interference (RNAi) has been found to function exceptionally well in *Drosophila* cells, and also *in vivo* in adult flies. A genome-wide RNAi screen was conducted in *Drosophila* S2 cells to reveal novel JAK/STAT pathway associated genes. As a result six potential JAK/STAT pathway regulating genes were found, of which two; *Eye transformer* (*ET*) and *Not4* were studied in more detail. *ET* encodes a gp130-related transmembrane protein that was found to inhibit Upd-induced Stat92E tyrosine phosphorylation, thus it functions as a negative regulator of the *Drosophila* JAK/STAT pathway. RNAi of *Not4* was found to repress the *Drosophila* JAK/STAT pathway target gene expression in *Drosophila* cells, indicating that Not4 is needed for the JAK/STAT pathway to function properly. Furthermore, overexpression of *Not4* resulted in enhanced Stat92E-mediated gene responses, confirming Not4 as a positive regulator of the *Drosophila* JAK/STAT pathway. We found that Not4 is able to interact with Stat92E, but does not affect Stat92E tyrosine phosphorylation. Finally, our experiments indicated that Not4 is needed for Stat92E to properly bind to its target DNA sequence at the promoter of the stress gene *TurandotM* (*TotM*). The Not4 mammalian homologue CNOT4 was also shown to participate in STAT1- and STAT6-mediated gene expression in human cells, indicating that Not4/CNOT4 is an evolutionary conserved regulator of JAK/STAT signaling.

Tiivistelmä

Sytokiinit ovat liukoisia välittäjäaineita, jotka säätelevät monia biologisia tapahtumia vaikuttamalla kohdesolujensa toimintaan. Sytokiinien vaikutukset välittyvät kohdesolujen pinnalla olevien reseptorimolekyylien kautta. Hematopoieettisen sytokiinin kiinnittyminen sille spesifiseen reseptoriin laukaisee reseptorialayksiköiden solunsisäisiin osiin liittyneiden JAK (Janus kinase) -proteiinien aktivaation. Aktivoidut JAK-proteiinit fosforyloivat reseptorimolekyylien solun sisäisissä osissa tietyt tyrosiini-aminohappotähteet, joihin sytoplasmassa vapaana olevat STAT (Signal transducer and activator of transcription) -transkriptiotekijät voivat kiinnittyä, tullen niin ikään fosforyloiduiksi JAK-proteiinien toimesta. Aktivoiduttuaan fosforylaation seurauksena STAT-proteiinit pariutuvat ja siirtyvät solun tumaan, missä ne sitoutuvat spesifisten kohdegeenien promoottorialueille aiheuttaen muutoksia kohdegeenien ilmentymisessä. JAK/STAT -tiedonsiirtoreitin aktiivisuus on tarkoin säädelty useiden säätelijämolekyylien kautta, joista tutkituimpia ovat PIAS (Protein inhibitor of activated STAT) ja SOCS (Suppressor of cytokine signaling) -proteiinit sekä proteiinityrosiinifosfataasit. Myös erityyppiset JAK- ja STAT-proteiinien translaation jälkeiset muokkaukset ovat tärkeässä osassa reitin aktiivisuuden säätelyssä. Tästä esimerkkinä voidaan mainita SUMO (Small ubiquitin-like modifier) -nimisen proteiinin kovalenttinen liittyminen STAT1- ja STAT5-proteiineihin, aiheuttaen niiden aktiivisuuden hiljentymisen.

JAK/STAT -tiedonsiirtoreitti on säilynyt muuttumattomana eläinlajien evoluutiossa. Banaanikärpäsessä (*Drosophila melanogaster*) JAK/STAT -reitin on osoitettu osallistuvan immuunipuolustuksen ja useiden eri elinten kehityksen säätelyyn. Tässä tutkimusprojektissa käytettiin banaanikärpästä malliorganismina tavoitteena löytää uusia JAK/STAT -signalointireitin säätelyyn osallistuvia proteiineja sekä selvittää, säädelläänkö banaanikärpäsen ainoaa STAT-transkriptiotekijää, Stat92E:tä, SUMO-konjugaation välityksellä. Tulostemme perusteella SUMO kiinnittyy Stat92E-proteiinissa

lysiini 187 aminohappotähteeseen. Kun SUMO:n kiinnittyminen Stat92E:hen estettiin mutatoimalla kyseinen aminohappotähde arginiiniksi, lisääntyi JAK/STAT kohdegeenien luenta, kun reitti aktivoitiin yli-ilmentämällä sen kautta vaikuttavaa välittäjäainetta, Upd:ta. Tämä osoittaa, että nisäkäshomologiensa STAT1:n ja STAT5:n tavoin Stat92E on negatiivisesti säädelty sumolaation välityksellä.

Geenien hiljentämisen RNA-häirintämenetelmällä (RNAi) on osoitettu toimivan erityisen hyvin banaanikärpäsen soluissa, kuten myös elävissä kärpäsissä. Tätä ominaisuutta hyödyntäen banaanikärpäsen S2-solulinjassa tehtiin koko genomin kattava RNA-häirintään perustuva seula, jonka tarkoituksena oli löytää uusia JAK/STAT -reitin aktiivisuuteen vaikuttavia geenejä. Tuloksena löydettiin kuusi potentiaalista geeniä, joista kaksi, *ET* ja *Not4* valittiin tarkempiin jatkotutkimuksiin. Osoitimme, että ET-proteiini toimii banaanikärpäsen JAK/STAT -reitin negatiivisena säätelijänä, joka inhiboi Upd-induktion aikaansaamaa Stat92E:n tyrosiinifosforylaatiota. *Not4* geenin ekspression hiljentäminen banaanikärpäsen soluissa vähensi huomattavasti JAK/STAT -reitin kohdegeenien ilmentymistä. *Not4* geenin yli-ilmentämisen banaanikärpäsessä huomattiin puolestaan lisäävän merkittävästi Stat92E:n kohdegeenien luentaa. Biokemialliset kokeemme osoittivat, että Not4 kykenee sitoutumaan Stat92E-proteiiniin mutta ei vaikuta sen tyrosiinifosforylaatiotasoon. Osoitimme lisäksi, että Not4 vaaditaan Stat92E-transkriptiotekijän DNA:han sitoutumiseen. Ihmissoluissa tekemiemme kokeiden perusteella päättelimme, että Not4-proteiinin ihmishomologi CNOT4 osallistuu STAT1- ja STAT6-välitteiseen kohdegeenien ilmentymiseen, osoittaen Not4/CNOT4:n olevan JAK/STAT -signalointireitin evolutiivisesti konservoitunut säätelijä.

1. Introduction

Myriad soluble mediators are responsible for the fate of cells. These growth factors and hormones regulate the maintenance, differentiation and proliferation of cells, as well as their death. The immune system is orchestrated by secreted glycoproteins called cytokines that signal through their specific cell membrane anchored receptors. The intracellular signals of the hematopoietic cytokines from the receptor into the nucleus are mediated via a defined Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway. This tyrosine phosphorylation driven cascade is activated when a hematopoietic cytokine binds to its cell surface receptor leading to receptor dimerization or oligomerization following the transphosphorylation of receptor-associated JAKs. Activated JAKs then phosphorylate specific tyrosine residues in the receptors, and in this manner produce docking sites for their cytoplasmic downstream targets, STATs. When bound to receptors via their SH2 domains, STATs become activated through JAK-mediated tyrosine phosphorylation. In response to activation STATs rapidly dimerize and translocate to the nucleus where they bind to their target gene promoters in order to activate gene expression. With multifold JAKs and STATs, mammalian cells provide the possibility for each hematopoietic cytokine to signal through a particular combination of JAKs and STATs, thus making the mammalian JAK/STAT pathway highly redundant.

The JAK/STAT pathway has been well conserved during evolution from small insects to humans. In the fruit fly, *Drosophila melanogaster* the JAK/STAT pathway contains only one transmembrane receptor, Domeless (Dome), one JAK (Hopscotch) and a single STAT (Stat92E), which mediate the signals of three secreted ligands termed Unpaired (Upd, Upd2, Upd3). Nonetheless, the *Drosophila* JAK/STAT pathway is highly similar to the mammalian cascade, offering a less redundant model for studying the function of the pathway. In addition to maintaining the function of hematopoietic cells, the JAK/STAT cascade is also responsible for the regulation of multiple steps in *Drosophila* development.

The JAK/STAT pathway needs to be under strict control at every level of the cascade. Factors that are responsible for the fine tuning and for shutting down the JAK/STAT pathway include protein tyrosine phosphatases, Protein inhibitor of activated STAT (PIAS) proteins and Suppressor of cytokine signaling (SOCS), all of which are conserved in *Drosophila melanogaster*. Like numerous other transcription factors, mammalian STAT1 and STAT5 are also negatively regulated by the covalent conjugation of a small ubiquitin-like modifier (SUMO).

Still, to date the molecular mechanisms of the regulation of the JAK/STAT pathway are largerly unknown, and it is very likely that all of the JAK/STAT pathway associated regulators have not yet been discovered. This study was aimed at exploring the molecular mechanisms of the JAK/STAT pathway regulation using *Drosophila melanogaster* as a model organism.

2. Review of the literature

2.1 Cytokines and the immune system

Humans and other animals are constantly threatened by infectious pathogens that can cause various diseases. During evolution organisms have developed efficient and sophisticated mechanisms to overcome these intruders. In humans, the immune system consists of an innate and an adaptive immunity. The innate immunity provides a fast defence against pathogens, whereas the adaptive immune system provides a more specific, but slower response (Kvell et al. 2007). The immune system is orchestrated by soluble mediators called cytokines. Cytokines are proteins or glycoproteins, which are released basically from any nucleated cell in response to infection or injury. Cytokines signal through cell surface receptors that are specific to each cytokine. When bound to a receptor, cytokines activate intracellular signaling cascades leading to altered gene expression in the target cell. This way, cytokines are able to drive the proliferation, differentiation or survival of their target cells (Oppenheim 2001). The first cytokines were found as early as is the 1950s, and were named interferons (IFNs) (Isaacs and Lindenmann 1957). After that, a growing number of cytokines with various functions have been described (Oppenheim 2001, O'Shea and Plenge 2012).

Cytokines are divided into various groups according to their structural and functional characteristics as well as the type of receptor they bind. Based on their structural features cytokine receptors can be divided into two families, class I and class II cytokine receptors (Table 1). Cytokines that signal through class I receptors are commonly called hematopoietic cytokines, because they mainly target blood cells. Hematopoietic cytokines include the majority of interleukins (IL), erythropoietin (Epo), thrombopoietin (Tpo), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), colony-stimulating factors for granulocytes (G-CSF) and granulocyte-macrophages (GM-CSF). Class I cytokine receptors also mediate the signals of some hormones such as growth hormone (GH) and prolactin (PRL). Class I cytokine receptors

are divided into four subfamilies based on the number of their subunits and the utilization of the common signaling chains. The first subfamily is composed of single chain receptors, which both bind the cytokine and pass on the signal after ligand induced homodimerization or oligomerization. Receptors in the other three subgroups contain a cytokine specific ligand binding subunit coupled to a common signal transducing receptor subunit. These three subfamilies are the common γ-chain, common β-chain and gp130 or gp130-related subunit class I cytokine receptor families (Silvennoinen et al. 1997).

Class II cytokine receptors use at least two different subunits, which are both capable of ligand binding and signal transduction (Silvennoinen et al. 1997). Class II cytokine receptors can be divided into four smaller subgroups: type I IFN receptors, which mediate the signals of 13 different IFN-αs, IFN-β and IFN-ω, IFN-κ and IFN-ε; type II IFN receptor is a IFN-γ receptor; the third subgroup contains the receptors for IL-10 and IL-10-related cytokines (IL-19, IL-20, IL-22, IL-24 and IL-26), and the fourth subgroup, which is the most recently discovered subclass of class II cytokine receptors, is a type III IFN receptor complex that mediates signals for interleukins 28A, 28B and 29 (also known as IFN-λ2, IFN-λ3 and IFN-λ1, respectively) (Kotenko and Pestka 2000, Fickenscher et al. 2002, Kotenko et al. 2003, Kotenko and Langer 2004, Kotenko 2011). The type III IFN receptor complex consists of two subunits: IFN-λR1 and IL-10R2 (IL-10Rβ), which is also shared with the IL-10 receptor and some other IL-10 family receptor complexes (Kotenko 2011). The signal transducing receptor subunits of the cytokine receptors are responsible for the activation of the intracellular signal transduction cascades that mediate the secondary message of the cytokine to the cell nucleus. The most common signaling cascade is the JAK/STAT pathway that mediates the secondary messages of the hematopoietic cytokines and most of the cytokines that use class II cytokine receptors, discussed in more detail in the next chapter (Silvennoinen et al. 1997, Kotenko and Pestka 2000).

Table 1. Cytokine receptor families (Silvennoinen et al. 1997, Kotenko and Langer 2004, Schindler and Plumlee 2008, Kotenko 2011)**.**

Cytokine receptor family	**Ligands**
Class I cytokine receptors	
Single chain receptors	Epo, Tpo, GH, PRL
Receptors that share common γ-chain	IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21
Receptors that share common β-chain	IL-3, IL-5, GM-CSF
gp130 family	IL-6, IL-11, LIF, CNTF, OSM, G-CSF*, CT-1, IL-12*, IL-23, IL-27, IL-31, IL-35
Class II cytokine receptors	
Type I IFN receptors	IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-ε
Type II IFN receptors	IFN-γ
Receptors for IL-10 family of cytokines	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26
Type III IFN receptors	IL-28A, IL-28B, IL-29

*Receptors for G-CSF and IL-12 share structural features with gp130, but does not require interaction with gp130 for signal transduction.

2.2 The JAK/STAT pathway

The signal from the receptors of the hematopoietic cytokines and class II cytokine receptors to the nucleus is mediated through a defined evolutionary conserved signal transduction cascade called the JAK/STAT pathway. Mammals have four members of the Janus kinase (JAK) family: JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase 2). JAKs activate their downstream targets, Signal transducers and activators of transcription (STATs), a family of transcription factors, which contains seven members in mammals: STATs 1–4, STAT5A, STAT5B and STAT6 (Leonard and O'Shea 1998). Depending on the cytokine receptor, JAKs and STATs form different combinations thus adding variability to the signaling cascade.

The activation of the JAK/STAT pathway is mediated through the tyrosine phosphorylation of signaling molecules inside the cell. Hematopoietic cytokine receptors lack tyrosine kinase activity, and are therefore dependent on JAKs that are associated

with the intracellular regions of the receptors. Signaling is initiated when a ligand binds to its receptor leading to receptor dimerization or oligomerization depending on the receptor type. A subsequent conformational change in the receptor subunits allows the associated JAKs to transphosphorylate one another on specific tyrosine residues. JAK-mediated tyrosine phosphorylation of the receptors creates a docking site for inactive cytoplasmic STAT molecules. When bound to receptors via their SH2 domains, STAT molecules too become activated through JAK mediated tyrosine phosphorylation. Following tyrosine phosphorylation STAT transcription factors dimerize via an SH2 domain phospho-tyrosine-mediated interaction creating either homo- or heterodimers that rapidly translocate to the nucleus in order to activate the expression of their target genes (Silvennoinen et al. 1997, Schindler 1999, Schindler 2002).

2.2.1 Biological roles of the JAK/STAT pathway

The JAK/STAT signaling cascade is important for the regulation and maintenance of hematopoietic cells, and disturbances in the pathway can lead to various pathological consequences such as tumorigenesis. STAT3 has been found to be constitutively active in over 50% of lung and breast tumors and in more than 50% of head and neck tumors (Dreesen and Brivanlou 2007). Like STAT3, the persistent activation of STAT5 has been linked to various solid tumors. This is due to increased secretion of cytokines and growth factors by tumor and stromal cells as well as dysfunction of JAK/STAT pathway regulators in tumor cells (Seavey and Dobrzanski 2012). Like in solid tumors, the JAK/STAT cascade is involved in a majority of hematopoietic tumors and disturbances (Seavey and Dobrzanski 2012). For example, JAK3 has been reported to be consitutively active in several cases of B-lineage lymphoid malignancies, including B-lineage acute lymphoblastic leukemia (Uckun et al. 2011). According to the most recent results, somatic mutations in the *Stat3* gene were found in 40% of patients with T-cell large granular lymphocytic leukemia. Furthermore, the patients with a mutated *Stat3* suffered more often of neutropenia and rheumatoid arthritis than patients with no *Stat3* mutations (Koskela et al. 2012). Several mutations in the JAK-homology 2 (JH2) domain of JAK2 have been linked to human hematological diseases, among them the V617F mutation,

which results in a constitutively active JAK2, and is found in up to 97% of cases of polycythaemia vera and in approximately 50% of patients with essential thrombocythaemia or idiopathic myelofibrosis (Baxter et al. 2005, James et al. 2005, Jones et al. 2005, Kralovics et al. 2005). The JAK/STAT pathway regulates signaling in the immune response and therefore dysregulation or altered activity of the pathway may also cause several immunological diseases, such as severe combined immune deficiency (SCID) where a mutation in the *Jak3* gene is seen in some patients (Macchi et al. 1995). As another example, STAT6 has been shown to have a central role in the pathogenesis of asthma due to an increased density of STAT6 expressing cells in the airways of asthma patients. This leads to the hyperexpression of IgE (Christodoulopoulos et al. 2001).

2.2.2 Domain structure of JAKs

JAK1-3 and TYK2 form the family of Janus kinanes, which range from 120 kDa to 140 kDa in size. JAK1, JAK2 and TYK2 are ubiquitously expressed, whereas JAK3 is normally expressed by hematopoietic cells and associates with the γ-chain of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Schindler et al. 2007). JAKs share a structure, which consists of seven JAK-homology (JH) domains (Fig. 1). JH domains are numbered from the carboxyl-terminus (C-terminus) to the amino-terminus (N-terminus). The JH1 domain in the C-terminus harbors the essential kinase activity. This domain is regulated by the adjacent JH2 domain, known as the pseudokinase domain. Recently, the pseudokinase domain in JAK2 was shown to harbor a kinase activity and to regulate JAK2 activity by phosphorylation of two negative regulation sites in JAK2: serine 523 and tyrosine 570 (Ungureanu et al. 2011). The N-terminal JH7-5 domains and half of the JH4 domain form the FERM domain that mediates receptor association (Schindler et al. 2007). The rest of the JH4 domain and the JH3 domain between the FERM and the pseudokinase domain constitute the SH2-like domain with no well-defined function (Schindler et al. 2007).

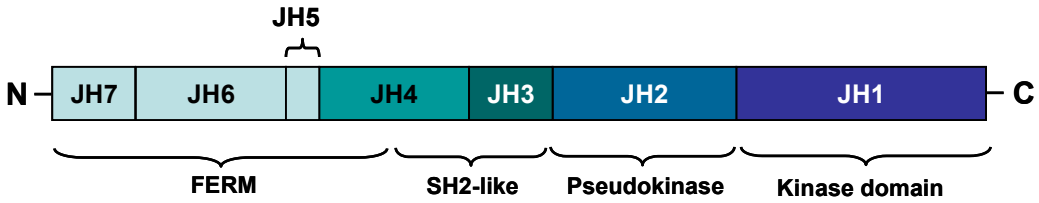

Figure 1. Domain organization of JAKs. JAKs are composed of seven JH domains which are numbered from the C-terminus to the N-terminus of the protein. The JH1 domain harbors a kinase activity, which is essential for JAK function. The regulatory JH2 domain is also known as the pseudokinase domain. The SH2-like domain is located in the middle of the protein. The N-terminal FERM domain mediates receptor interactions. Phosphorylation sites are not illustrated in the figure.

2.2.3 Domain structure of STATs

All of the seven mammalian STATs share a conserved domain structure illustrated in Figure 2. The first, approximately 125 amino acid residues compose the N-terminal domain that mediates the dimerization of inactive STATs (Baden et al. 1998, Meyer and Vinkemeier 2004, Mao et al. 2005). Deletion of the N-terminal domain was shown to result in a constitutively active STAT1, implying that the N-terminal domain is required for the dephosphorylation of tyrosine 701, the amino acid responsible for STAT1 activation (Shuai et al. 1996). The N-terminal domain also mediates interactions between STATs and other proteins, for example STAT1 is shown to interact with the CREB-binding domain of the transcriptional co-activator CBP/p300 via its N-terminal domain (Zhang et al. 1996). Furthermore, the N-terminal domain has been suggested to participate in the nuclear import and export of STATs as well as in cooperative DNA binding to tandem GAS-elements (Xu et al. 1996, Strehlow and Schindler 1998, Schindler et al. 2007).

Next to the N-terminal domain is a coiled-coil domain that consists of 4 α-helices and mediates interactions with other proteins and the nuclear export machinery (Chen et al. 1998, Becker et al. 1998, Begitt et al. 2000, Mowen and David 2000, Schindler et al. 2007). The DNA binding domain, located between amino acids ~320-480 consists of a β-

barrel immunoglobulin fold, and mediates the DNA binding of STATs (Schindler et al. 2007). A linker domain connects the DNA binding domain with dimerization domains, and is also thought to participate in the nuclear export of STATs through its nuclear export signal (Bhattacharya and Schindler 2003).

The SH2 domain is the most highly conserved domain found in STATs. It mediates interactions with the phosphorylated receptor, thus playing a critical role in the activation and dimerization of activated STATs (Schindler 2002). Upon activation by JAKs, a specific tyrosine residue within the C-terminal tail segment of the SH2 domain (amino acid ~700) is phosphorylated and this phospho-tyrosine interacts with the SH2 domain of another STAT allowing parallel dimer conformation to occur (Chen et al 1998, Schindler 2002). The C-terminus of the STATs is the least conserved region between different STATs and is responsible for transcriptional activation, and is thus called the transactivation domain (TAD). The TAD mediates many interactions with proteins involved in transcription, such as histone acetyltransferases CBP/p300, reported to function as coactivators for all STAT family members (Zhang et al. 1996, Bhattacharya et al. 1996, Pfitzner et al. 1998, Gingras et al. 1999, McDonald and Reich 1999, Paulson et al. 1999). In addition to tyrosine phosphorylation of the C-terminal tail segment of the SH2 domain, also serine phosphorylation of TAD participates in the regulation of STAT-mediated signaling. Serine 727 phosphorylation of STAT1 modulates interactions with co-activators such as MCM5 (a member of mini-chromosome maintenance protein family) and selectively enhances STAT1 target gene expression (Zhang et al. 1998, Varinou et al. 2003). Moreover, serine 727 phosphorylation is known to enhance STAT1 sumoylation (serine phosphorylation is discussed in more detail in the Chapter 2.3.4.1) (Vanhatupa et al. 2008).

Figure 2. Schematic presentation of the STAT domain architecture. The N-terminal domain and coiled-coil domain participate in molecular interactions. The SH2 domain mediates interactions with phosphorylated tyrosine residues. DNA BD stands for DNA binding domain and TAD for C-terminal transactivation domain. The tyrosine phosphorylation site located approximately at amino acid 700 in the C-terminal tail segment of the SH2 domain is illustrated with a red phosphate.

2.3 Regulation of the JAK/STAT pathway

The JAK/STAT pathway is activated through ligand binding to the cytokine receptor followed by the rapid tyrosine phosphorylation of JAKs, intracellular domains of the receptors and STATs, leading to the nuclear translocation of dimeric STATs. To avoid hyperactivity, and in order to switch off the pathway, several conserved mechanisms take place in the cells. These regulatory mechanisms include various post-translational modifications of JAKs and STATs, as well as the action of regulatory proteins such as protein tyrosine phosphatases, Suppressors of cytokine signaling (SOCS) and Protein inhibitors of activated STATs (PIAS). These regulatory mechanisms are discussed in more detail in the next chapters.

2.3.1 Protein tyrosine phosphatases

Dephosphorylation by protein tyrosine phosphatases (PTPs) is essential in order to inactivate JAKs and STATs. Several mammalian PTPs have been shown to dephosphorylate JAKs and STATs, including SHP1, SHP2, CD45, PTP1B, T cell PTP (TC-PTP), Receptor-type tyrosine-protein phosphatase T (PTPRT) and PTP-Basophil like (PTPBL) (Xu and Qu 2008).

SHP1 is an SH2 domain containing PTP, expressed in hematopoietic cells (Neel et al. 2003). SHP1 has been shown to downregulate erythropoietin (Epo) signaling by binding to the Epo receptor and by dephosphorylating Epo receptor-associated JAK2 (Klingmüller et al. 1995). SHP1 has also been suggested to dephosphorylate JAK1, JAK3 and STAT3. The silencing of SHP1 is associated with leukemia and lymphomas (David et al. 1995, Chim et al. 2004, Han et al. 2006, Xu and Qu 2008). SHP2 shares structural similarities with SHP1, but it is ubiquitously expressed (Xu and Qu 2008). Enhanced and prolonged interferon-γ-induced STAT1 Tyr701 and Ser727 phosphorylation was observed in *Shp2*$^{-/-}$ cells, and purified GST-SHP2 was shown to dephosphorylate STAT1 on Tyr701 and Ser727 (Wu et al. 2002). In addition to STAT1, SHP2 has also been suggested to interact and dephosphorylate STAT5 and to negatively regulate STAT3 mediated signaling (Yu et al. 2000, Chen et al. 2003, Xu and Qu 2008).

The regulatory affect of protein phosphatases on JAK/STAT signaling may not always be inhibitory, SHP2 has also been suggested to promote JAK2/STAT5 mediated prolactin signaling by dephosphorylating Tyr1007 in JAK2, a phosphotyrosine that mediates the interaction of JAK2 with its inhibitor SOCS1. When this residue was dephosphorylated by SHP2 *in vitro*, JAK2 was released from the inhibitory effects of SOCS1 (Ali et al. 2003). This indicates that SHP2 may first promote prolactin signaling at the level of JAK2, and then inhibit the activated pathway by dephosphorylating STAT5.

CD45 is a transmembrane protein, which is highly expressed in hematopoietic cells, and has an important role in the regulation of T and B cell receptor signaling. CD45 can dephosphorylate all of the JAK family members in murine cells, and it has been reported to dephoshorylate JAK1 and JAK3 in human cells as well (Irie-Sasaki et al. 2001, Yamada et al. 2002, Xu and Qu 2008). A recent study revealed a loss of function mutation in a gene encoding CD45 in patients with T cell acute lymphoblastic leukemia (T-ALL), resulting in increased activation of the JAK/STAT pathway. Furthermore, knockdown of CD45 significantly increased JAK1 and STAT5 phosphorylation levels in the human T-ALL cell line KE-37 (Porcu et al. 2012).

PTP1B is a phosphatase expressed in many tissues, and it has been shown to dephosphorylate the JAK2 and TYK2 kinases (Myers et al. 2001). TC-PTP shares structural similarities with PTP1B, but is mainly expressed in hematopoietic cells and contains a nuclear localization signal in its C-terminus. TC-PTP has been shown to dephosphorylate STAT1 and STAT5A and STAT5B in the nucleus (Aoki and Matsuda 2002, Ten Hoeve et al. 2002, Bourdeau et al. 2005). Moreover, JAK1, JAK3 and STAT3 are thought to be TC-PTP substrates (Simoncic et al. 2002, Yamamoto et al. 2002).

One of the STAT3 phosphatases is a Receptor-type tyrosine-protein phosphatase T (PTPRT). Overexpression of PTPRT in colorectal cancer cells significantly reduced the expression of STAT3 target genes (Zhang et al. 2007). Of other phosphatases PTP-Basophil like (PTP-BL) was found to interact with STAT4 in a yeast two-hybrid screen and was shown to dephosphorylate STAT4 and STAT6. Depletion of PTP-BL from $CD4^+$ T cells enhanced Th1 and Th2 differentation in response to increased and prolonged STAT4 and STAT6 activation (Nakahira et al. 2007).

A growing amount of evidence indicates that various protein tyrosine phosphatases play an essential role in the regulation of JAK/STAT signaling. Still, their specificity and the exact molecular mechanism of their function remain elusive.

2.3.2 Suppressors of cytokine signaling, SOCS

The suppressor of cytokine signaling (SOCS) protein family consists of eight members, SOCS1–7 and cytokine inducible SH2-containing protein (CIS). SOCS have been linked to the regulation of over 30 cytokines, including IFN-γ, IL-2, IL-4 and IL-6 (Croker et al. 2008). All eight SOCS contain a central SH2 domain, which is able to bind phosphorylated tyrosines, and a 40 amino acid SOCS box domain at the C-terminus of the protein. The SOCS domain is known to interact with elongins B and C and Cullin5 in order to catalyze the ubiquitination of target proteins such as JAK2 for SOCS1 (Zhang et al. 1999, Kamizono et al. 2001, Ungureanu et al. 2002, Babon et al. 2009). The amino-terminal region in SOCS proteins is variable in length and does not contain any known

functional domains, except a kinase inhibitory region (KIR) in SOCS1 and SOCS3, adjacent to the SH2 domain. This 12 amino acid long region directly inhibits the catalytical activity of JAKs. SOCS proteins may also compete with STATs for binding to tyrosine phosphorylated receptors (Croker et al. 2008). Recently SOCS3 was shown to downregulate JAK1, JAK2 and TYK2 but not JAK3. This selection is based on a three-amino acid motif, GQM, in the JAK insertion loop. The motif is present in JAK1, JAK2 and TYK2 but absent in JAK3. Mutations in the first and last amino acid of this motif abolished SOCS3 mediated inhibition of JAK2, while a mutation in the middle residue (Q1072 in JAK2) had only a minor effect on JAK2 activity. An NMR analysis in the same study suggested that SOCS3 interacts simultaneously with JAK2 and cytokine receptor subunit gp130. Furthermore, it was shown that SOCS3 does not compete in binding with either ATP or substrate, and therefore acts as a noncompetitive inhibitor of JAK2. Moreover, SOCS3 did not reduce ATP hydrolysis by JAK2, but instead inhibited phosphate transfer to a tyrosine residue of the substrate (Babon et al. 2012).

Several SOCS genes have a STAT binding site in their promoter region and the expression of SOCS is induced by various cytokines, such as IFN-γ, Epo and numerous IL-family members. Due to this, SOCS proteins are thought to form a negative feedback loop in the regulation of the JAK/STAT signaling pathway (Matsumoto et al. 1997, Greenhalgh and Hilton 2001, Alexander and Hilton 2004, Croker et al. 2008).

2.3.3 Protein inhibitors of activated STAT, PIAS

Four different protein inhibitors of activated STAT (PIAS) proteins have been described in mammals; PIAS1, PIAS3, PIASx (PIAS2) and PIASy (PIAS4), and all of these, except PIAS1, have two isoforms (Shuai 2006, Rytinki et al. 2009). The size of PIAS proteins varies from the 510 amino acids of PIASy to the 651 amino acids of PIAS1 and the proteins share a conserved domain architecture, illustrated in Figure 3. The N-terminal SAP (scaffold attachment factor-A/B, acinus and PIAS) domain is involved in DNA binding (Aravind and Koonin 2000, Rytinki et al. 2009). Incorporated within the SAP domain, PIAS proteins have an LxxLL motif that has been suggested to mediate

interactions with the nuclear receptors. It has also been shown that the LxxLL motif in PIASy is essential for the PIASy mediated downregulation of STAT1 activity, but it is not required for the STAT1-PIASy interaction (Heery et al. 1997, Liu et al. 2001). Next to the SAP domain lies the PINIT domain, which is thought to regulate the subcellular localization of at least PIAS3L, the long form of PIAS3, found in mouse embryonic stem cells (Duval et al. 2003, Rytinki et al. 2009). The RING-finger-like zinc-binding domain (RLD) in the middle of the protein is the most conserved region among the PIAS family members, and is needed for the SUMO (small ubiquitin-like modifier) E3 ligase function of PIAS proteins (Hochstrasser 2001, Rytinki et al. 2009). Adjacent to the RING domain lies a SUMO interacting motif (SIM), which possibly mediates noncovalent interactions with SUMO (Rytinki et al. 2009). The C-terminal serine/threonine-rich region (S/T) is the least conserved region in PIAS proteins, and is not present in PIASy (Sharrocks 2006, Rytinki et al. 2009).

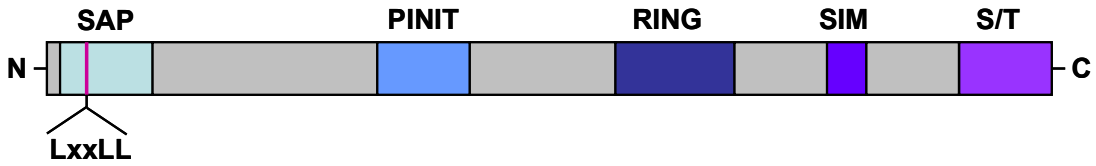

Figure 3. Schematic illustration of the PIAS protein structure. The N-terminal SAP domain mediates DNA binding and protein interactions. SAP domain contains an LxxLL motif. PINIT and RING domains are located in the central part of the protein. The SIM domain mediates interactions with SUMO. The C-terminal region is the least conserved among PIAS family members and the S/T region is absent from PIASy.

PIAS proteins were initially described as regulators of STAT3 and STAT1 signaling, and were correspondingly named PIAS3 and PIAS1. PIAS3 was found to interfere with STAT3 DNA binding without having an effect on STAT1, while PIAS1 inhibited STAT1 DNA binding and transcriptional activity (Chung et al. 1997, Liu et al. 1998). The interaction between PIAS and STAT requires cytokine stimulation, and it has been suggested that PIAS proteins interact only with dimeric STATs (Liao et al. 2000). While at first PIAS proteins were found to interrupt STAT1 and STAT3 DNA binding, nowadays PIAS proteins are known to affect STAT-mediated gene responses also

through other mechanisms. For example, PIASy and PIASx have been shown to inhibit STAT1 and STAT4 activity, respectively, without having an effect on DNA binding activity. PIASx was shown to interact with DNA bound STAT4 and a histone deasetylase inhibitor abolished PIASx mediated inhibition suggesting that PIASx may function as a co-repressor by recruiting HDACs to inhibit transcription on STAT4 responsive promoters (Liu et al. 2001, Arora et al. 2003).

Pias1 knockout mice are viable, but smaller in size when compared to their wild type littermates. Studies with *Pias1*$^{-/-}$ mice have revealed that PIAS1 selectively regulates interferon responsive gene expression. In their study, Liu *et al.* (2004) found that the expression of only 9% of IFN-γ target genes was altered in the absence of PIAS1. These genes included *Guanylate binding protein-1* (*Gbp-1*), *CXC chemokine ligands 9 and 10* (*Cxcl9* and *Cxcl10*), whereas the expression of genes such as *Interferon regulatory factor-1* (*Irf-1*), *Socs1* and *Inducible nitric oxide synthase 2* (*Nos2)* was not altered. In chromatin immunoprecipitation assays *Pias1* depletion resulted in enhanced STAT1 binding to the *Gbp-1* promoter, without having an effect on binding to the *Irf-1* gene promoter under IFN-γ stimulation. Although PIAS1 regulates only a subset of IFN-inducible genes, the protein seems to be important for the innate immune response. *Pias1*$^{-/-}$ cells showed increased antiviral activity towards mouse γ-herpes virus-68 (MHV-68) infection, based on reduced expression of viral proteins in infected knockout macrophages, when compared to infected wild type macrophages. *Pias1*$^{-/-}$ mice were also more resistant to *Listeria monocytogenes* bacterial infection than wild type mice (Liu et al. 2004).

In addition to STATs, PIAS proteins have also been shown to regulate a broad range of other transcription factors, including Nuclear factor-κB (Nf-κB) and lymphoid enhancer-binding factor 1 (LEF1) (Sharrocks 2006, Shuai 2006). PIAS proteins have also been shown to function as SUMO E3 ligases promoting SUMO conjugation to target proteins, such as STAT1 and STAT5 (Rogers et al. 2003, Ungureanu et al. 2003, Van Nguyen et al. 2012). This role of PIAS proteins is discussed in Chapter 2.4.

2.3.4 Post-translational modifications in the regulation of the JAK/STAT pathway

2.3.4.1 Tyrosine and serine phosphorylation, methylation, acetylation, O-glycosylation and ISGylation

Of the known post-translational modifications to proteins, tyrosine phosphorylation plays the most important role in terms of the regulation of the activity of the JAK/STAT pathway. As discussed in Chapter 2.2, the activation of the JAK/STAT cascade is dependent on the phosphorylation of the certain tyrosine residues in receptor-associated JAKs, the cytoplasmic domains of the receptor subunits and in STATs. Ligand binding to the receptor induces the auto- or transphosphorylation of several tyrosine residues in JAKs. In the case of JAK2 more than 20 tyrosines are known to undergo phosphorylation (Argetsinger et al. 2010). Tyrosine phosphorylation is essential for the activation of JAK2, but certain tyrosine residues serve also as negative regulatory sites, like Tyr570, which is phosphorylated by a regulatory JH2 domain (Argetsinger et al. 2004, Ungureanu et al. 2011). JAKs are also regulated through serine phosphorylation. In its inactive state JAK2 is consitutively phosphorylated at Ser523 and the JH2 domain has also been shown to promote Ser523 phosphorylation (Ishida-Takahashi et al. 2006, Ungureanu et al. 2011).

In the canonical model of the JAK/STAT pathway, STATs are activated through the JAK-mediated phosphorylation of a single tyrosine residue around position 700. In addition to tyrosine phosphorylation, the activity of all of the STATs, with the exception of STAT2, is modulated through serine phosphorylation on at least one serine residue, which most often falls within the C-terminal TAD (Decker and Kovarik 2000, Schindler et al. 2007). In both STAT1 and STAT3, Ser727 phosphorylation is required, together with tyrosine phosphorylation, for full activation in response to IFNs and IL-6, respectively (Wen et al. 1995, Varinou et al. 2003). Moreover, Ser727 phosphorylation in STAT1 is required for proper association with the co-activators CBP and MCM5 (Zhang et al. 1998, Varinou et al. 2003). Ser727 phosphorylation has also been linked to negative regulation of STAT1 signaling by promoting the sumoylation of STAT1 (Vanhatupa et

al. 2008). Furthermore, STAT1 is regulated through an additional serine, Ser708, which is phosphorylated by IκB kinase ε (IKKε) (TenOever et al. 2007). Most recently, Ser708 phosphorylation was shown to inhibit STAT1 homodimerization, thus promoting heterodimerization with STAT2 and an association with IRF9 for the formation of the transcription factor complex IFN-stimulated gene factor 3 (ISGF3), which activates the expression of the IFN-I responsive genes needed for a proper antiviral response (Ng et al. 2011). In their recent study, Chen *et al.* (2011) showed that upon viral infections STAT6 is recruited to the endoplasmic reticulum by an adaptor protein called STING (also called MITA/ERIS). This is followed by Ser407 phosphorylation by TANK-binding kinase 1 (TBK1) and JAK-independent Tyr641 phosphorylation leading to the homodimerization and nuclear translocation of Tyr/Ser -phosphorylated STAT6 in order to activate the transcription of a specific set of target genes (Chen et al. 2011). This is an example of how STATs can be phosphorylated also through alternative pathways, in addition to the classical receptor/JAK-mediated activation mechanisms. Serine phosphorylation of STATs can be induced by the cytokines responsible for their tyrosine phosphorylation but also by an extracellular stimulus that does not lead to simultaneous tyrosine phosphorylation. These include inflammatory stimuli such as LPS or TNF-α as well as stress agents like ultraviolet radiation (Decker and Kovarik 2000). Many serine kinases are responsible for STAT phosphorylation including p38-mitogen-activated protein kinase (MAPK) in the case of STATs 1, 3 and 4; extracellular-signal-regulated kinase 2 (ERK2), c-Jun N-terminal kinase (JNK) and mammalian target of rapamycin (mTOR) have been reported to phosphorylate Ser727 in STAT3. STAT1 Ser727 is also phosphorylated by protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase (CaMK) II. These kinases have been mainly identified through the use of inhibitors, dominant-negative alleles and *in vitro* kinase assays (Decker and Kovarik 2000, Yokogami et al. 2000, Schindler et al. 2007). Serine phosphorylation of STATs offers a way for crosstalk between different intracellular signaling pathways, and maintains cellular homeostasis and the proper expression of STAT regulated genes.

In addition to phosphorylation, other post-translational modifications such as methylation, acetylation, O-glycosylsation and ISGylation have been reported to

participate in the regulation of STAT-mediated signal transduction, but the mechanisms and biological impacts of these modifications are not so well defined. As an example, STAT3 is found to undergo reversible dimethylation on lysine 140, and this methylation has been suggested to have a negative role on STAT3 transcriptional activity, at least at some of the STAT3-dependent promoters (Yang et al. 2010, Stark and Darnell 2012).

2.3.4.2 Ubiquitination

Ubiquitin is a highly conserved 76 amino-acid protein that can be covalently linked to lysine residues in other proteins through an enzymatic cascade that utilizes E1 (Ubiquitin activating enzyme), E2 (Ubiquitin conjugation enzyme) and E3 (Ubiquitin ligase) enzymes. Substrate proteins can be modified with a single ubiquitin molecule (monoubiquitination) or with polyubiquitin chains, through coupling of further ubiquitin proteins linked through integral lysine residues (polyubiquitination). All of the seven lysines (K6, K11, K27, K29, K33, K48, and K63) in Ubiquitin can be further conjugated by other Ubiquitins to form polyubiquitin chains (Chen and Sun 2009). In addition to non-linear polyubiquitin chains linked through the internal lysine residues, Ubiquitins may also polymerize in a linear manner, in which the carboxy terminus of one Ubiquitin is linked to the amino-terminal methionine of the adjacent Ubiquitin (Jiang and Chen 2011). Target proteins can also be mono- or polyubiquitinated simultaneously through multiple lysine residues in their amino acid sequence. The best charactericed consequence of protein polyubiquitination is proteosomal degradation of the substrate protein by 26S proteasomes, specifically in the case of proteins modified with K48 or K11 linked polyubiquitin chains (Clague and Urbé 2010). Polyubiquitin chains linked through the other internal lysines in Ubiquitin, linear polyubiquitin chains as well as monoubiquitination have also been reported to have nonproteolytic functions (Chen and Sun 2009). For example, K63-linked polyubiquitination has been suggested to play role in DNA repair and protein kinase activation, largely through proteasome-independent mechanisms (Liu and Chen 2010, Jiang and Chen 2011).

Ubiquitination also has a role in the regulation of the JAK/STAT pathway. As discussed in Chapter 2.3.2, SOCS proteins are able to mediate JAK degradation by the Ubiquitin-proteosome pathway (Ungureanu et al. 2002, Croker et al. 2008). In addition to this STAT proteins have also been reported to undergo ubiquitination through interactions with nuclear E3 Ubiquitin ligase STAT-interacting LIM protein (SLIM). SLIM was found to inhibit especially STAT1- and STAT4-mediated gene expression and to promote STAT1 and STAT4 ubiquitination and proteosomal degradation. Furthermore, SLIM enhanced STAT4 dephosphorylation independently of the proteosome pathway, thus providing an additional mechanism for STAT inhibition (Tanaka et al. 2005, Ungureanu and Silvennoinen 2005). Of the post-translational modifications related to ubiquitination, also sumoylation plays a role in the regulation of the activity of the JAK/STAT pathway, and will be discussed in the next chapter.

2.4 Sumoylation

Small ubiquitin-like modifier (SUMO) is a ubiquitin-related protein moiety, which can be covalently conjugated to a specific lysine residue in a target protein via an enzymatic cascade that resembles the ubiquitination pathway. Yeasts and invertebrates contain only one *Sumo* gene (known as *Smt3* in *Saccharomyces cerevisae* and in *Drosophila melanogaster*), whereas mammals have four: *Sumo-1 – Sumo-4*. SUMO-1 is ~11 kDa protein that shares 18% sequence identity with Ubiquitin. SUMO-2 and -3 have very similar structures, differing only by three N-terminal amino acids. Due to this similarity SUMO-2 and -3 are often referred as SUMO-2/3. SUMO-1 shares approximately 50% sequence identity with SUMO-2/3 (Johnson 2004, Wilkinson and Henley 2010). Similar to Ubiquitin, SUMO-2/3 has the ability to form chain structures through internal lysine residues (K11), whereas SUMO-1 has been reported to form chains only *in vitro* (Tatham et al. 2001, M. Yang et al. 2006). In contrast to the *Sumo-1 – Sumo-3* genes, the *Sumo-4* gene lacks introns and has been proposed to be a pseudogene (Wilkinson and Henley 2010). Expression of *Sumo-4* mRNA has been detected in certain tissues, but the SUMO-4 protein has not been found anywhere (Bohren et al. 2004, Guo et al. 2004).

Furthermore, the amino acid sequence of the SUMO-4 precursor suggests that the protein is unable to mature and covalently conjugate to targets (Proline 90, which is absent from other SUMOs, inhibits maturation) (Owerbach et al. 2005).

SUMO is conjugated to target proteins via an enzymatic cascade in a process called sumoylation (illustrated in Figure 4). SUMO proteins are produced as precursors with a C-terminal extension of 2-11 amino acids. SUMO becomes mature through C-terminal cleavage by SENP (Sentrin/SUMO-specific protease) proteases. This proteolytical processing exposes a di-glycine motif in the C-terminus of SUMO, which serves as the site for the covalent linkage between SUMO and the target protein. SUMO is then activated in an ATP-dependent manner by an activating enzyme complex, a heterodimer (E1) that contains SAE1 and SAE2 (SUMO-activating enzyme 1 and 2), also known as Aos1 and Uba2, repectively. In this step SUMO forms a thioester bond between the active site cysteine residue of SAE2 and the C-terminal glycine residue of SUMO. In the second step SUMO is passed on to the active site of the conjugating (E2) enzyme Ubc9 (Ubiquitin-conjugatin 9), again via a thioester bond. Finally SUMO is covalently conjugated to a specific lysine residue in the substrate protein by Ubc9 via C-terminal glycine residue of SUMO. Most often the lysine residue in the target protein is located within the sumoylation consensus motif ψKxD/E (where ψ stands for a large hydrophobic amino acid, x can be any amino acid). It has been proposed that the conjugating enzyme Ubc9 itself first binds to a consensus motif and that this interaction is required for SUMO transfer to the lysine residue (Sternsdorf et al. 1999, Sampson et al. 2001, Kim and Baek 2009, Wilkinson and Henley 2010). The presence of this consensus sequence is not absolutely essential for sumoylation, and not all proteins containing this motif are sumoylated. According to the literature in approximately 75% of the reported sumoylation sites the lysine is surrounded by this consensus motif (Xu et al. 2008).

The transfer of SUMO to the substrate is promoted by growing number of E3 ligases. These ligases are not known to form a covalent linkage with SUMO, but they are able to interact with the SUMO-Ubc9 complex and to bring it into contact with the substrate or additionally, to hold the SUMO-Ubc9 thioester bond in a position favorable for the

transfer of SUMO to the substrate. The first SUMO E3 ligases identified were the yeast Siz1 and Siz2 proteins that contain a RING domain homologous to the one in Ubiquitin E3 ligases. Deletion of these genes almost completely abolished sumoylation in yeast *Saccharomyces cerevisiae in vivo* (Johnson and Gupta 2001, Takahashi et al. 2001). Human PIAS proteins are homologues to Siz proteins and have also been shown to function as SUMO E3 ligases (Wilkinson and Henley 2010). In addition to the PIAS protein family, other RING domain containing proteins have been described to function as SUMO E3 ligases as well. One of these is TOPORS (Topoisomerase I-binding, arginine/serine-rich), a protein that is capable of transferring both SUMO and Ubiquitin to the substrate molecules (Weger et al. 2005).

Another type of mammalian SUMO E3 ligase is Ran-binding protein 2 (RanBP2), a large protein located in nuclear pore complexes. RanBP2 binds both Ubc9 and SUMO-1 and promotes sumoylation of Sp100, Histone deacetylase 4 (HDAC4) and Promyelocytic leukemia protein (PML). Interestingly, RanBP2 lacks the RING domain and does not bind directly to its substrate, meaning that it has an alternative E3 ligase function mechanism (Kirsh et al. 2002, Pichler et al. 2002, Tatham et al. 2004). Another example of an E3 ligase that does not contain a RING domain is the Polycomp2 (Pc2) protein, suggested to function as a scaffold by binding directly to its substrate protein CtBP (C-terminal-binding protein) and the SUMO-Ubc9 complex (Kagey et al. 2003).

Sumoylation is a reversible post-translational protein modification, and SUMO may be cleaved from the target protein by the same SENP proteases that in the maturation process cleave the C-terminus of SUMO precursors. The SUMO-specific proteases Ulp1 and Ulp2 were first identified in budding yeast (Li and Hochstrasser 1999, Li and Hochstrasser 2000). Six SENP variants have been described in mammals: SENP1–3 and SENP5–7. The numbering lacks SENP4, because originally it was identified as a gene with sequence similarities with Ulp1, but then SENP3 and 4 turned out to be the same protein (Kim and Baek 2009). The different SENPs vary in their subcellular localization, SUMO paralogue specificity and selectivity for their SUMO maturation versus deconjugation activities. SENPs can be divided into three groups. The first group is

shared with SENP1 and SENP2, known to function in precursor processing and deconjugation of both SUMO-1 as well SUMO-2/3 from numerous substrates. SENP1 localizes to the nucleous, and SENP2 is found in nuclear envelope associated compartments and nuclear pores (Gong et al. 2000, Nishida et al. 2001, Hang and Dasso 2002, Zhang et al. 2002, Wilkinson and Henley 2010). SENP3 and SENP5 have nucleolar localization and prefer to deconjugate monomeric SUMO-2/3 from substrates (Nishida et al. 2000, Di Bacco et al. 2006, Gong and Yeh 2006). The third group consists of SENP 6 and 7, which are found in the nucleoplasm. They are responsible for editing and deconjugating SUMO-2/3 polychains (Mukhopadhyay et al. 2006, Mukhopadhyay and Dasso 2007, Shen et al. 2009, Wilkinson and Henley 2010).

Recently, a completely novel SUMO cleaving enzyme called DeSumoylating Isopeptidase-1 (DeSI-1) was found. DeSI-1 was shown to desumoylate, but not to deubiquitinate, the transcriptional repressor BZEL (the BTB-ZF protein expressed in effector lymphocytes). Furthermore, DeSI-1 was able to desumoylate both SUMO-1 and SUMO-2/3 modified BZEL as well as to break SUMO-2/3 polychains, but did not participate in SUMO precursor processing. The substrate specificity and cellular localization of DeSI-1 differs from those of SENPs. DeSI-1 is also expressed in the cytoplasm instead of only in the nucleus or nuclear structures like are the SENPs (Shin et al. 2012).

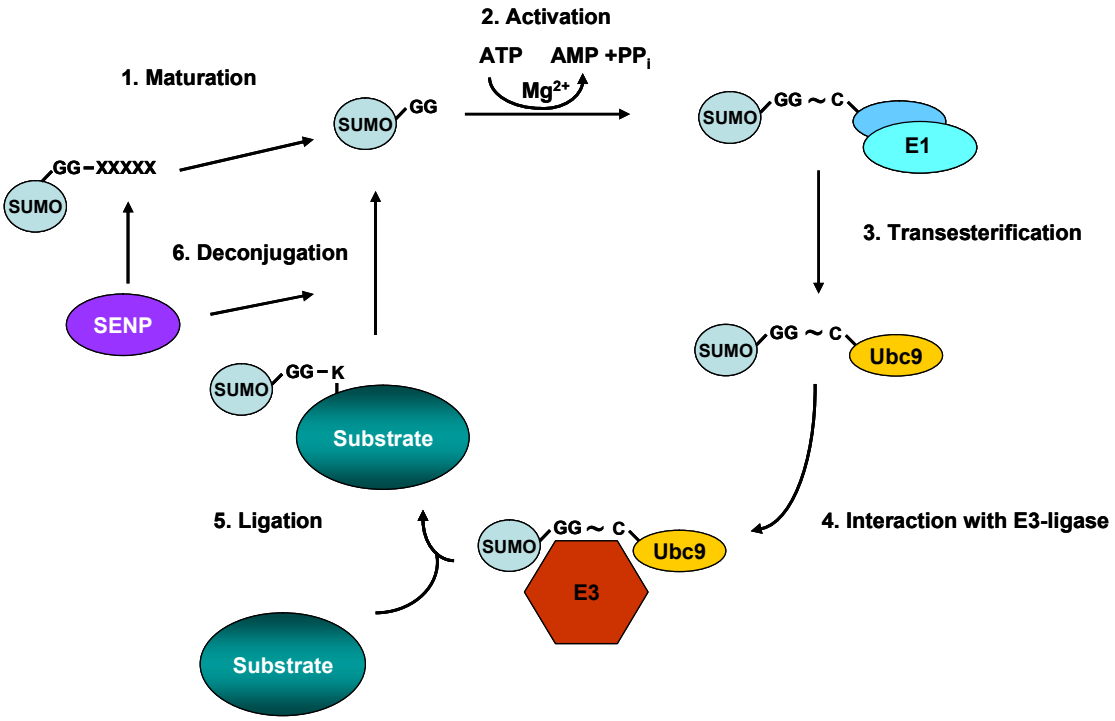

Figure 4. SUMO conjugation pathway. Pre-SUMO is maturated through C-terminal cleavage by SENPs (1.), followed by the formation of a thioester bond with a cysteine in the activating enzyme complex E1 (2.). The activated SUMO is then transferred to the conjugating E2 enzyme, Ubc9 (3.). Ligation to a spesific lysine of the substrate protein is promoted by an associated E3 ligase (4. and 5.). Sumoylation is a reversible post-translational modification and SUMO may be cleaved from the substrate by SENPs (6.).

2.4.1 Physiological consequences of protein sumoylation

To date, hundreds of proteins from diverse functional categories have been reported to undergo sumoylation. These proteins include chromatin modifying factors, DNA repair and genome stability proteins, stress-related proteins, proteins involved in translation and RNA processing, metabolic enzymes and transcription factors (Kim and Baek 2009, van der Veen and Ploegh 2012). Sumoylation leads to various molecular consequences on the target proteins. SUMO binding may block the substrate binding site of an enzyme, or disrupt protein-protein or protein-DNA interactions. Conversely, sumoylation may also recruit new protein interaction partners by creating a new binding face on the target protein. SUMO conjugation may also change substrate protein conformation leading to

altered target protein activity, or reveal binding sites that have been masked in the unconjucated substrate (Gill 2005, Wilkinson and Henley 2010). Sumoylation has also been reported to antagonize ubiquitination, an example of which is the Nf-κB regulator IκBα (inhibitory κBα) (Desterro et al. 1998). Furthermore, sumoylation has been suggested to regulate protein ubiquitination by modifying enzymes that are are part of the ubiquitination machinery (Wilkinson and Henley 2010).

One important target protein group of SUMO is transcription factors, and in most cases sumoylation has a repressive role in regulating their activity. This has been mainly studied by overexpressing proteins with mutated sumoylation sites in cultured cells. The mechanisms for how SUMO inhibits transcription are in most cases unknown. It has been suggested that SUMO conjugation may cause changes in protein interactions or DNA binding. SUMO attachment may block or compete with other modifications in the transcription factor. One mechanism for how sumoylation is postulated to inhibit transcription factor activity is through the recruitment of repressor complexes or histone deacetylases (HDACs), to the promoter site (Gill 2005). This has been reported to occur with the transcription factor E twenty six-like-1 (Elk-1). When sumoylated, Elk-1 associates with HDAC2, leading to decreased histone acetylation and downregulation of the transcription of the Elk-1 target genes (Yang and Sharrocks 2004). Another SUMO associated co-repressor is a protein called Daxx, which interacts with the sumoylated transcription factor Smad4, leading to repressed Smad4 transcriptional activity (Chang et al. 2005). Transcription factor sumoylation may also play a role in DNA methylation, like in case of the murine transcription factor Sp3. Stielow *et al.* (2010) found that lack of Sp3 sumoylation in mice expressing sumoylation deficient Sp3 E553D caused aberrant expression of certain spermatocyte-specific genes (*Dmc1* and *Dnahc8*) and certain neuronal genes (*Paqr6*, *Rims3* and *Robo3*) in somatic tissues and in non neuronal tissues, respectively. Furthermore, there was a loss of H3K9 and H4K20 tri-methylation, impaired recruitment of repressive chromatin modifying enzymes and a loss of DNA methylation on the promoters of these Sp3 target genes in mouse embryonic fibroblasts (MEFs) expressing Sp3 E553D mutant, indicating that sumoylation of Sp3 is essential for maintenance of tissue-spesific gene silencing.

2.4.2 Sumoylation of STATs

STAT1 was found to be sumoylated at Lys703 by two independent research groups in 2003 (Rogers et al. 2003, Ungureanu et al. 2003). Both studies suggested that overexpression of PIAS proteins increased the amount of modified STAT1, indicating that PIAS proteins may function as E3 ligases in the STAT1 sumoylation process. STAT1 has a SUMO consensus sequence in its transactivation domain from isoleucine 702 to glutamate 705 (^{702}IKTE^{705}). Mutating lysine 703 to arginine totally abolished the sumoylation of STAT1, indicating that Lys703 is the only SUMO site in STAT1. In addition to Lys703, also isoleucine 702 and glutamate 705 are indispensable for STAT1 sumoylation. Sumoylation deficient STAT1 showed increased transcriptional activity on certain STAT1 responsive promoters. These included the *Gbp-1* and *Tap-1* (*Transporter associated with antigen presentation-1*) genes, which have low affinity STAT1 responsive promoters, whereas *Irf-1* gene expression, from a high affinity promoter, was not affected by the STAT1 K703R mutation. Interestingly, this expression profile of the *Gbp-1* and *Irf-1* genes corresponded with findings from *Pias1*$^{-/-}$ mice, further suggesting that PIAS1 might have role in STAT1 regulation through sumoylation (Liu et al. 2004, Ungureanu et al. 2005). Sumoylation deficient STAT1 K703R also showed prolonged nuclear localization and DNA binding upon an IFN-γ stimulus (Ungureanu et al. 2005, Song et al. 2006). IFN-γ mediated activation of STAT1 enhanced the sumoylation of STAT1 (Ungureanu et al. 2003). Furthermore, serine 727 phosphorylation of STAT1, mediated by MAP-kinases was found to promote sumoylation, indicating that also other signaling cascades may participate in the SUMO-mediated regulation of STAT1 (Vanhatupa et al. 2008).

The sumoylation target Lys703 is in a close proximity of the STAT1 activating Tyr701 phosphorylation site. The first studies of STAT1 sumoylation suggested that the phosphorylation status of Tyr701 would not be significantly altered in response to sumoylation. These studies were based on a comparison of the wild type STAT1 phosphorylation status to the phosphorylation rate of a sumoylation deficient STAT1 mutant (Ungureanu et al. 2005, Song et al. 2006). Later it was shown that sumoylated

STAT1 is much less Tyr701 phosphorylated compared to the unmodified STAT1 both *in vivo* and *in vitro*, using the Ubc9 fusion-directed SUMOylation system (UFDS). In this system a STAT1-Ubc9 fusion protein is sumoylated up to 40% on Lys703 with endogenous SUMO, yielding considerably more STAT1 sumoylation than by only overexpressing SUMO in cells (Jakobs et al. 2007). Furthermore, Tyr701 phosphorylation of STAT1 was found to hinder the sumoylation of Lys703, suggesting that sumoylation and phosphorylation at Tyr701 would be mutually exclusive modifications (Zimnik et al. 2009). More recently, tyrosine phosphorylated STAT3 transcription factors were suggested to form paracrystal structures within the nucleus and to serve as a reservoir for activated STAT3s. STAT1 was able to form similar structures only when sumoylation was inhibited by mutating the SUMO conjugation site (Droescher et al. 2011a). In the same study, it was proposed that sumoylation, by inhibiting Tyr701 phosphorylation, drove STAT1s into semiphosphorylated dimer structures, interacting through their N-terminal domains instead of a phospho-tyrosine-SH2 domain-mediated interaction, which is typical for an activated STAT1 dimer (Droescher et al. 2011a). Additional studies using cells from knock in mice expressing SUMO-free STAT1 further strengthened this hypothesis. Furthermore, SUMO-induced STAT1 paracrystal dissolution was suggested to lead to accelerated dephosphorylation of STAT1. STAT1 sumoylation was postulated to protect cells from hyperresponsiveness to IFN-γ (Begitt et al. 2011, Droescher et al. 2011b). Although SUMO modulation of STAT1 has been widely studied, results are controversial and many aspects of this regulatory mechanism of STAT1-mediated IFN signal transduction remain elusive.

More recently murine STAT5 too was reported to be a target for SUMO-2 conjugation. STAT5 sumoylation reduced both tyrosine phosphorylation and lysine acetylation and led to STAT5 inactivation. In *Senp1*$^{-/-}$ mice T and B cell development was impaired, and sumoylated STAT5 was found to accumulate in these cell lineages, but not in myeloid cells. These results indicate that SENP1 is a crucial regulator of B and T cell development acting as a SUMO protease that cleaves SUMO-2 from conjugated STAT5. After SUMO is removed, STAT5 can re-enter the activation-inactivation cycle. (Van Nguyen et al. 2012).

2.5 *Drosophila* as a model organism in biomedical research

Drosophila melanogaster, or the fruit fly, has been used as a model organism since the beginning of the 20th century. This small insect has several benefits: its life-cycle is relatively short, and it is easy and cheap to rear, *Drosophila melanogaster* is also seen as ethically more acceptable for scientific studies, than are more developed organisms such as mice, which are also widely used as model organisms in biomedical research. The complete *Drosophila* genome has been sequenced and it is known to contain ~15,000 genes divided into four pairs of chromosomes (Adams et al. 2000, St Johnston 2002). An analysis of the *Drosophila* genome sequence revealed that *Drosophila* genes are very similar to mammalian genes. Furthermore, it is estimated that up to 77% of the known human disease genes have a *Drosophila* homolog, this makes *Drosophila* an exellent model for genetic research (Rubin and Lewis 2000, Reiter et al. 2001, St Johnston 2002).

Drosophila melanogaster has been widely used for RNAi-based genome-wide screens. Transfection of *Drosophila* S2 cells with syntethic dsRNA corresponding to a sequence of a desired target gene induces very efficient nuclease mediated degradation of the mRNA products of the target gene, thereby resulting in gene silencing (Hammond et al. 2000). To date, several genome-wide dsRNA mediated RNAi screens have been performed in *Drosophila* cells to identify components participating in cellular processes, such as signaling pathways and phagocytosis (Rämet et al. 2002a, Baeg et al. 2005, Müller et al. 2005, Valanne et al. 2010). In addition to cell culture based methods, RNAi can be used effectively also *in vivo* in flies through the tissue-spesific expression of RNAi constructs with the UAS-GAL4 expression system (Brand and Perrimon 1993). The availability of a genome-wide collection of RNAi fly lines has further speeded-up and simplified genetic research in *Drosophila* (Dietzl et al. 2007).

2.6 Overview of the *Drosophila* immune system

The immune system of *Drosophila* differs from the human immune system in that it lacks an adaptive immunity. Still, *Drosophila* has very powerful innate defence mechanisms to combat infections. The *Drosophila* innate immune response can be divided into the cellular response orchestrated by spesific hemocytes, and the humoral response, which is mediated by secreted effector molecules. In addition to these, also the cuticles of the fly and its epithelial cells have their own defence mechanism towards infective agents. The first line of defence in *Drosophila,* like in every organism, is the physical barrier formed by epithelia on the surface of body parts that come into contact with the environment, such as the alinmentary tract and trachea (Lemaitre and Hoffmann 2007). In addition to being a physical barrier, epithelial cells are able to produce antimicrobial peptides (AMPs) and reactive oxygen species (ROS), when they come into contact with microbes. This so-called local immune response is essential for flies living in and feeding in decomposing materia full of bacteria and other microbes. AMP gene expression has been detected in cells of the epidermis, respiratory tract, reproductive tract and alinmentary tract (Tzou et al. 2000, Ferrandon et al. 1998). Some AMPs are constitutively expressed, while others are expressed only upon bacterial infection. This inducible local AMP expression has been found to be triggered only by a Gram-negative bacterial infection through the Immune deficiency (Imd) pathway (discussed in the Chapter 2.6.2) (Tzou et al. 2000). ROS production is independent of the Imd pathway and is induced rapidly in *Drosophila*, providing an additonal local response to the bacterial challenge (Lemaitre and Hoffmann 2007). The AMPs are discussed in more detail in the Chapter 2.6.2.

2.6.1 Cellular response

Drosophila lacks a closed circulatory system. Instead, hemolymph floats freely inside its body cavity. In addition to the local immune response *Drosophila* has a repertoire of hemocytes in its hemolymph to combat microbes and participate in wound healing. *Drosophila* hemocytes are divided into three subgroups according to their structural and functional characteristics: plasmatocytes, crystal cells and lamellocytes. These cells

resemble mammalian myeloid lineages. Due to the lack of an adaptive immune system, *Drosophila* has no equivalent of a lymphoid lineage (Meister and Lagueux 2003, Lemaitre and Hoffmann 2007). The most common hemocytes are plasmatocytes, which are responsible for the phagocytic removal of microbial pathogens and dead cell depris. In an uninfected animal up to 95% of the hemocytes in its hemolymph are plasmatocytes (Williams 2007). Approximately 5% of circulating cells represent crystal cells, which are responsible for the melanization of invading pathogens and for wound healing (Meister and Lagueux 2003, Williams 2007). The third cell type, lamellocytes, are not present in the hemolymph under normal conditions. Lamellocytes are large and flat cells, which participate in the encapsulation of pathogens, such as parasitoid wasp eggs, that are too large for phagocytosis. And like crystal cells, lamellocytes are also involved in melanization (Agaisse and Perrimon 2004, Williams 2007).

Phagocytosis and encapsulation are the most essential mechanisms of the cellular immune response of *Drosophila*. Phagocytosis is an evolutionary conserved defence mechanism against intruders such as bacteria and yeast. The initiation of phagocytosis requires receptor mediated recognition of the particle, which is to be ingested into the phagocytic cell into vesicles called phagosomes, which then fuse together with lysosomes to create phagolysosomes. Finally, the phagocytosed particles are enzymatically degraded at the optimal pH in the phagolysosome (Stuart and Ezekowitz 2005, Ulvila et al. 2011). Still, the exact mechanism for how these foreign objects are destroyed remains elusive. Several groups of the phagocytic receptors have been identified on the surface of plasmatocytes. Among these Croquemort, a member of the CD36 superfamily has been shown to participate in the phagocytosis of apoptotic cells (Franc et al. 1996). Originally, it was believed that Croquemort does not participate in bacterial uptake, but later it was reported that it binds and mediates the internalization of *Staphylococcus aureus* bacteria into plasmatocytes (Franc et al. 1999, Stuart et al. 2005). Another member of this family is a protein called Peste, which is important for the phagocytic uptake of mycobacteria (Philips et al. 2005). Other known receptors involved in bacterial recognition include a member of the scavenger receptor family (dSR-CI), and the EGF-like repeat containing proteins Nimrod C1 and Eater (Pearson et al. 1995, Rämet et al. 2001, Kocks et al. 2005,

Kurucz et al. 2007). Eater is expressed primarily on plasmatocytes and on their precursors, whicn are known as prohemocytes, and which have a crucial role in mediating the phagocytosis of both Gram-negative and Gram-positive bacteria (Kocks et al. 2005). Also other receptors involved in phagocytosis are known, including Down syndrome cell adhesion molecule (Dscam) and peptidoglycan recognition protein LC (PGRP-LC), which is involved in the phagocytosis of Gram-negative but not Gram-positive bacteria (Rämet et al. 2002a, Watson et al. 2005). The most recent addition to the growing list of *Drosophila* phagocytic receptors is Integrin βv. Flies that lack Integrin βv showed reduced phagocytosis of *Staphylococcus aureus* and had an increased sensitivity to fatal septic *Staphylococcus aureus* infections when compared to wild type flies. Integrin βv was found to cooperate with a receptor called Draper in bacterial recognition (Shiratsuchi et al. 2012). In addition to transmembrane receptors, also opsonization has been shown to play a role in the phagocytosis in *Drosophila*. Here, thioester-containing proteins (TEPs), six variants of which are encoded by the *Drosophila* genome (TEPs 1-6), are thought to have at least a modest role in the phagocytosis of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (Stroschein-Stevenson et al. 2005).

In addition to phagocytosis, *Drosophila* larvae can combat an invading organism by encapsulation (Williams 2007). This reaction takes place when the object is too big for plasmatocyte-mediated phagocytosis. This reaction has been found to occur when for example parasitoid wasps lay their eggs into *Drosophila* larvae. Initially wasp eggs are recognized and surrounded by plasmatocytes. This activates the differentiation of numerous lamellocytes in the lymph glands. Once they are released from the lymp glands, lamellocytes are attracted to the site of the parasite by plasmatocytes in order to form a multilayered capsule around the wasp egg, following blackening of the capsule due to melanization by lamellocytes and activated crystal cells. Eventually the parasite is killed by released ROS and intermediates of the melanization cascade. Encapsulation requires that all hemocytes types function synergistically. The JAK/STAT signaling cascade and the Toll pathway are thought to be activated during the encapsulation process (Wertheim et al. 2005, Lemaitre and Hoffmann 2007, Williams 2007).

Melanization occurs rapidly at the cuticle wound sites, and after the encapsulation of intruders. Crystal cells serve as storage cells for large amounts of prophenoloxidase (pro-PO) in their cytoplasmic granules (or crystals, where the name of the cell type comes from). In melanization activated crystal cells release their content into the hemolymph. After release, the pro-PO is converted to the active phenoloxidase (PO) by a cascade of serine proteases. Active PO then catalyzes melanin polymerization, which is important in wound healing (Rämet et al. 2002b, Meister and Lagueux 2003, Bidla et al. 2007). At the injury sites melanization is mediated solely by crystal cells, but in encapsulation also by lamellocytes, which are known to express one pro-PO called DoxA3 (Irving et al. 2005). In wound healing, as far as it is known, melanization is triggered through a JNK pathway-mediated signal (Rämet et al. 2002b).

2.6.2 Humoral response

The humoral response against invading pathogens is mainly mediated by AMPs secreted from the fat body, an organ with characteristics comparable to the mammalian liver (Lemaitre and Hoffmann 2007). The fat body is a large organ, which is surrounded by hemolymph and is able to detect floating microbes in the hemolymph (Lemaitre and Hoffmann 2007). Shia et. al (2009) found that ablation of hemocytes from *Drosophila* larvae caused a significant decrease in AMP expression in the fat body upon both septic injury and oral infection. Furthermore, they suggested that signaling between hemocytes and the fat body would be mediated by the Toll pathway ligand Spätzle. Still, it is not completely understood how considerable role the hemocytes play in the initiation of the humoral response. AMPs are not expressed under normal conditions, but during infection AMPs are rapidly expressed and secreted into the hemolymph. This process is mediated by two distinct signaling pathways, the Toll and the Imd pathway, which will be discussed in more detail further on. The family of AMPs is composed of four variants of Attacins, four Cecropins, two Diptericins, seven Drosomycins and single variants of Drosocin, Defencin and Metchnikowin, with partly overlapping microbe specifities (Lemaitre and Hoffmann 2007). The exact molecular mechanism for how AMPs inactivate or kill their target microbes is still elusive.

The *Drosophila* Toll pathway is activated in response to Gram-positive bacteria and fungi. It also has an important role in the development of the *Drosophila* embryo, but this chapter concentrates on the Toll pathway's role in the immune response (Lemaitre and Hoffmann 2007). *Drosophila* Toll is a transmembrane cytokine receptor that is not able to directry bind to the pathogens. Instead, bacterial recognition is mediated by circulating pattern recognition factors, including peptidoglycan recognition protein SA (PGRP-SA) (Michel et al. 2001). The recognition of the pathogen leads to proteolytic activation of the soluble Toll ligand Spätzle, which then binds to Toll and induces the activation of a intracellular signaling cascade that resembles mammalian Nf-κB signaling. Toll activates an oligomeric complex that contains MyD88, Tube and a kinase called Pelle, consequently leading to the phosphorylation and proteolytic degradation of the NF-κB inhibitory protein Cactus. Normally Cactus is bound to the transcription factors Dif and Dorsal and in this manner inhibits their function. The degradation of Cactus releases Dif and Dorsal allowing them to translocate into the nucleus, where they bind DNA and activate the expression of their target genes, such as the AMPs *Drosomycin* and *Defencin* (Meng et al. 1999, Valanne et al. 2011).

Another important signaling cascade in AMP production is the Imd pathway, responsible for the immune response against Gram-negative bacteria (Lemaitre et al. 1995). Peptidoglycan (PGN), produced by Gram-negative bacteria, is recognized by two peptidoglycan recocnition proteins (PGRPs), PGRP-LC and PGRP-LE. PGRP-LC functions as a transmembrane receptor in the Imd pathway (Choe et al. 2002, Gottar et al. 2002, Rämet et al. 2002a, Rämet et al. 2002b, Choe et al. 2005), whereas PGRP-LE has both, intra- and extracellular functions (Kaneko et al. 2006). Full-length PGRP-LE is known to act as an intracellular receptor for the diaminopimelic acid-containing (DAP-type) PGN of intracellular bacteria such as *Listeria monocytogenes*, whereas the shorter form of PGRP-LE, which contains only the PGRP domain, is secreted into the extracellular matrix and enhances PGRP-LC-mediated peptidoglycan recognition on the cell suface (Kaneko et al. 2006). The binding of the peptidoglycan of Gram-negative bacteria to PGRP-LC activates the intracellular Imd-protein through an interaction

between the death domain of Imd and the intracellular domain of PGRP-LC. This leads to the activation of a complex protein cascade that finally activates the transcription factor Relish through C-terminal cleavage. Active Relish translocates to the nucleus and induces the transcription of AMPs such as Diptericin (Kaneko and Silverman 2005, Lemaitre and Hoffmann 2007). For a long time it was believed that the *Drosophila* JAK/STAT pathway does not participate in AMP production, but in their recent study Goto *et al.* (2010) found that the expression of a novel AMP-like protein called Listericin, which is active against *Listeria monocytogenes*, is cooperatively regulated by PGRP-LE and the JAK/STAT pathway. This, and the role of the JAK/STAT pathway in the regulation of other essential functions of the *Drosophila* immune system, are discussed in the Chapter 2.7.1.

2.7 The *Drosophila* JAK/STAT pathway

The JAK/STAT pathway has been conserved in evolution, and all the main components of the pathway, discussed in Chapter 2.2, are present in *Drosophila melanogaster*. An overview of the *Drosophila* JAK/STAT pathway is shown in Figure 5. In *Drosophila* the JAK/STAT pathway is activated through a single transmembrane receptor, called Domeless (Dome or Mom) and the intracellular signal is transduced via a single Janus-kinase, Hopscotch (Hop) and a single STAT transcription factor Stat92E (also known as Marelle) (Hanratty and Dearolf 1993, Binari and Perrimon 1994, Brown et al. 2001, Chen et al. 2002, Hou et al. 1996, Yan et al. 1996a). The extracellular part of Dome contains five fibronectin type domains, two of which resemble the cytokine binding module (CBM) found in class I mammalian cytokine receptors. Due to sequence similarities Dome has been suggested to be ancestor of the IL-6 family of cytokine receptors, sharing 18% identity with LIFR and 26% identity with CNTFR (Brown et al. 2001, Chen et al. 2002). Dome is activated when the ligand Unpaired (Upd) binds to its extracellular domain (Chen et al. 2002). The *Drosophila* genome encodes three *Upd* genes; *Upd*, *Upd2* and *Upd3* (Harrison et al. 1998, Agaisse et al. 2003, Hombría et al. 2005). The first identified Upd was reported to be a secreted glycoprotein that associates with the extracellular matrix, thereby functioning locally to activate the JAK/STAT pathway

(Harrison et al. 1998). The exact molecular mechanism for how ligand binding activates Dome is still more or less unclear. In the canonical model of the JAK/STAT pathway ligand binding induces rapid receptor dimerization followed by the activation of downstream targets. It has been suggested that, in addition to monomeric reseptor subunits, *Drosophila* Dome also forms homodimer structures in the resting state, independently of ligand binding. Furthermore, the same study presented evidence that Upd was only capable of activating the JAK/STAT pathway in cells where Dome was in a pre-dimerized state. In this model, ligand binding is predicted to cause conformational changes in the receptor homodimer, allowing interaction between associated Hop-molecules (Brown et al. 2003).

The *Hopscotch* gene encodes a 1117 amino acid protein, which shares 27% identity with human JAK2 (Binari and Perrimon 1994). The single *Drosophila Stat92E* locus encodes two highly similar splicing variants. The larger protein product is 761 amino acids long, while the smaller one is seven amino acids shorter. Stat92E shares 37% sequence identity with human STAT5 and approximately 35% identity with human STAT6, its domain structure is similar to that of mammalian STATs. The region with the highest identity was mapped to the SH2 domain, which is also the most conserved domain in mammalian STATs. Stat92E is phosphorylated on the conserved tyrosine 711 (Tyr704 in the short splicing variant) in response to Hop-mediated activation (Hou et al. 1996, Yan et al. 1996a). The phosphorylated Stat92E is then believed to form a homodimer, which is followed by nuclear translocation. Phosphorylated Stat92E binds to a spesific consensus sequence TTCnnnGAA on the promoters of its target genes. Stat92E is able to bind and activate transcription through both 3n and 4n spaced binding sites, but according to EMSA experiments by Rivas *et al*. (2008), it seems to prefer 3n consensus sequences. Several target genes for Stat92E have been described, and they participate in various biological processes. These include the pair-rule gene *even skipped* (*eve*), a gene that is responsible for proper embryonic segmentation, *Socs36E,* a negative regulator of Stat92E signaling, complement-like protein *Tep-1* and the *Turandot* (*Tot*) stress genes (Yan et al. 1996a, Hou et al. 1996, Karsten et al. 2002, Agaisse et al. 2003, Lagueux et al. 2000).

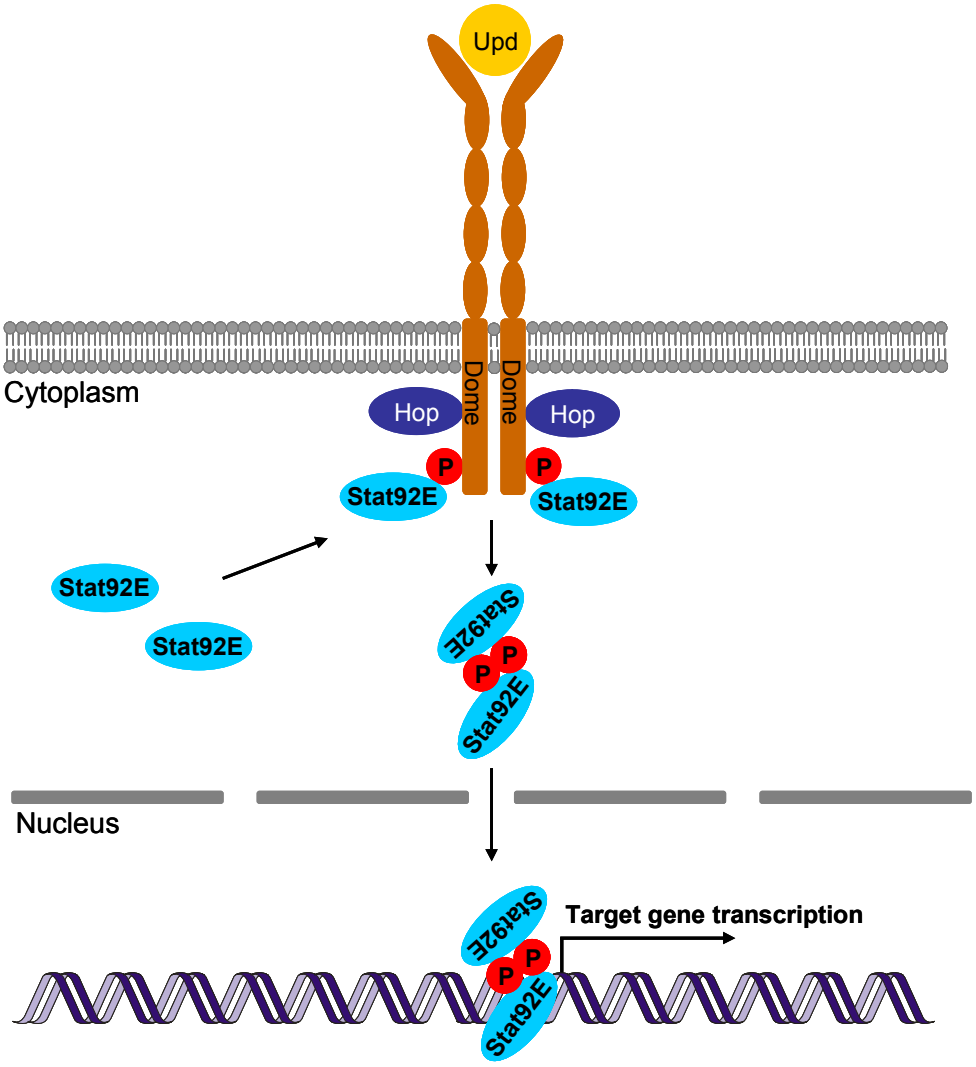

Figure 5. Schematic presentation of the *Drosophila* JAK/STAT pathway. Binding of Upd to its receptor Dome induces Hop-mediated Stat92E tyrosine phosphorylation and homodimerization. Stat92E homodimers translocate to the nucleus and bind to responsive DNA elements in the promoters of target genes and initiate gene expression.

2.7.1 Roles of the JAK/STAT pathway in *Drosophila* development and the immune response

Drosophila melanogaster has been used extensively as a model organism in developmental research. These studies have revealed the essential role of the the JAK/STAT pathway in many developmental processes. When first described, the *Hop* and *Stat92E* genes were found to participate in embryonic segmentation through *eve* gene

activation (Binari and Perrimon 1994, Hou et al. 1996, Yan et al. 1996a). The JAK/STAT pathway was also found to be required for stem cell maintenance in male gonads, as the inactivating mutations in *Hop* and *Stat92E* caused male sterility. (Kiger et al. 2001, Tulina and Matunis 2001). Furthermore, the JAK/STAT pathway also has an essential role in oogenesis in female flies (McGregor et al. 2002). In addition to fly fertility, the JAK/STAT pathway is indispensable for the organogenesis of, for example, the trachea and the eye, and it takes part in sculpting wing veins (Yan et al. 1996b, Luo et al. 1999, Brown et al. 2001).

Like in mammals, the JAK/STAT pathway participates in the regulation of the immune response in *Drosophila*. As was discussed in Chapter 2.6, the *Drosophila* immune response is divided into the humoral and cellular responses. As part of the humoral response and in cooperation with PGRP-LE, the JAK/STAT pathway regulates the expression of an AMP called Listericin upon *Listeria monocytogenes* infection. *L. monocytocenes* is a Gram-positive intracellular pathogen, which exeptionally expresses a DAP-type PNG on its surface, and is thus recogniced by cytoplasmic PGRP-LE (Bierne and Cossart 2007, Yano et al. 2008). *Listericin* was found to suppress the growth of DAP-type PNG containing bacteria, such as *L. monocytogenes,* as well as Gram-negative bacteria, but it had no effect on the growth of lysine-type PGN-containing Gram-positive bacteria. The promoter of the *Listerincin* gene contains a putative Stat92E binding site, but it remains to be verified if *Listerincin* is a direct Stat92E target gene (Goto et al. 2010). While the expression of AMPs is mainly regulated through the Toll and Imd pathways, the JAK/STAT pathway plays a crucial role in regulating the expression of other humoral factors in *Drosophila.* Upon septic injury Upd3 triggers Stat92E activation in the fat body, which leads to expression of the *TotA* stress gene (Ekengren et al. 2001, Agaisse et al. 2003). *TotA* belongs to a family of *Turandot* stress genes, which are expressed under various stress conditions such as bacterial infection, heat shock and ultraviolet radiation (Ekengren and Hultmark 2001, Ekengren et al. 2001). Also the expression of other *Tot* family members (*TotC* and *TotM*) is controlled by the JAK/STAT pathway (Agaisse and Perrimon 2004). The exact mechanism for how the protein products of these genes function is still elusive (Ekengren and Hultmark 2001).

Another secreted Stat92E target gene product, *Tep-1,* was mentioned earlier. There is evidence that TEP proteins may function as opsonins, which recognize microbes and promote their phagocytosis (Stroschein-Stevenson et al. 2005). A recent study revealed that TEPs are expressed in potential immune cells and tissues, such as in hemocytes, the fat body and in some epithelia. Although the immune challenge normally induces TEP expression in these tissues, *Tep-1*, *Tep-2*, *Tep-3*, *Tep-4* deficient flies were not more susceptible to bacterial infections compared to wild type flies, indicating that TEP proteins are not essential for *Drosophila* immunity, but may still have a role in the defence against a restricted subset of pathogens (Bou Aoun et al. 2011).

Gain-of-mutations in *Hop* (Hop^{Tum-l} and T42) result in strong overproliferation of lamellocytes that eventually contribute to melanotic tumors (Hanratty and Dearolf 1993, Luo et al. 1995, Luo et al. 1997, Agaisse and Perrimon 2004). Furthermore, it has been found that larvae carrying a loss-of-function mutation in Hop are unable to conduct encapsulation upon wasp paratization (Agaisse and Perrimon 2004). Together, these results indicate that the JAK/STAT pathway regulates lamellocyte differentation, thus contributing to the cellular immune response. The JAK/STAT pathway also contributes to the renewal of intestinal stem cells and to their differentation into enterocytes after damage and stress-induced Upd production in the *Drosophila* midgut (Jiang et al. 2009). In addition, the JAK/STAT pathway was shown to be important for the survival of flies after an intestinal *Serratia marcescens* bacterial infection (Cronin et al. 2009). Stat92E has been proposed to participate in the downregulation of Relish target genes in the *Drosophila* immune response by forming a repressome complex with the transcription factor AP-1 and Dsp1 (Dorsal switch protein 1). This complex was shown to replace Relish at the *Attacin-A* gene promoter. Furthermore, this repressome complex was also found to recruit histone deasetylases to the Relish-dependent promoter sites to inhibit transcription. In this manner Stat92E participates in fine-tunig the strenght of the innate immune response in *Drosophila melanogaster* (Kim et al. 2007).

The JAK/STAT pathway is also required for antiviral defence in *Drosophila*. Dostert *et al.* (2005) found, using high-density DNA microarrays, that a *Drosophila* C virus (DCV)

infection triggers a unique gene expression profile compared to genes activated in bacterial and fungal infections. Moreover, some of these DCV-induced genes including *vir-1* (*virus-induced RNA 1*), *CG12780* and *CG9080*, were found to contain Stat92E binding sites in their 5' upstream sequences, and a DCV-infection increased Stat92E binding to its optimal DNA-binding sequence in EMSA experiments, suggesting that Stat92E is activated upon viral infection. The expression of *vir-1* and *CG12780* was reduced or abolished in DCV-infected *Hop* loss-of-function flies ($Hop^{M38/msvl}$), but these genes were not overexpressed in Hop^{Tum-l} flies, indicating that *Hop* is required, but not sufficient, for an antiviral response against DCV in *Drosophila* (Dostert et al. 2005). The upregulation of the same genes in response to an infection with three different DCV strains was observed by Hedges and Johnson (2008) as well. Interrestingly, this upregulation was not detected when flies were infected with inactivated DCV and only a modest increase in *CG12780* and *CG9080* expression was observed when flies were injected with purified DCV-dsRNA, indicating that viral dsRNA by itself is not sufficient to activate the JAK/STAT pathway (Hedges and Johnson 2008).

2.7.2 Regulation of the *Drosophila* JAK/STAT pathway

The *Drosophila* JAK/STAT pathway shares similarities with the corresponding mammalian cascade but has less redundancy. The regulatory mechanisms that affect the *Drosophila* JAK/STAT pathway have been studied, but mostly their molecular details remain enigmatic. Like in mammals, PIAS has been reported to influence STAT-mediated signal transduction in *Drosophila* (Betz et al. 2001). dPias (also called Zimp) is the only PIAS protein in *Drosophila melanogaster,* and is encoded by the *Su(var)2-10* (*Suppressor of variegation 2-10*) locus (Hari et al. 2001). *Drosophila* Pias was shown to interact with tyrosine phosphorylated Stat92E. Furthermore, genetic studies revealed that depletion of *dPias* enhanced melanotic tumor formation in flies overexpressing a constitutively active Hop^{Tum-l} mutant. As expected, the overexpression of dPias was found to reduce tumorigenesis caused by JAK/STAT pathway hyperactivation. In addition, a balanced Stat92E/dPias ratio was shown to be crucial for normal eye development. As a conclusion, these results indicate that dPias is a negative regulator of

Stat92E (Betz et al. 2001). In addition to dPias, the *Drosophila* genome encodes three SOCS proteins, Socs36E, Socs16D and Socs44A (Callus and Mathey-Prevot 2002, Karsten et al. 2002, Rawlings et al. 2004). The *Socs36E* gene was identified first, and its promoter region was found to contain 19 Stat92E binding sites. Moreover, *Socs36E* mRNA expression was upregulated by ectopic activation of the JAK/STAT pathway, and was significantly reduced in flies lacking either Upd or Hop, indicating that *Socs36E* is a JAK/STAT pathway target gene (Karsten et al. 2002). Socs16D has not yet been connected to the JAK/STAT pathway, whereas both Socs36E and Socs44A have been reported to function as negative regulators of the *Drosophila* JAK/STAT pathway. Like mammalian SOCS (described in detail in Chapter 2.3.2), Socs36E seems to form a classical negative feedback loop in the regulation of the activity of the JAK/STAT pathway (Rawlings et al. 2004, Stec and Zeidler 2011). In addition to full length Stat92E the *Drosophila* genome encodes an N-terminally truncated Stat92E (ΔN-Stat92E), which is able to dimerize with full length Stat92E and to bind DNA. Overexpression of *ΔN-Stat92E* suppressed Stat92E activity, indicating that ΔN-Stat92E acts as a negative regulator of Stat92E (Henriksen et al. 2002).

As discussed earlier, *Drosophila* offers an excellent tool for genome-wide RNAi screens. In order to find novel genes that participate in the JAK/STAT signaling cascade, two such screens have been conducted using dsRNAs targeting the majority of the *Drosophila* transcriptome (Baeg et al. 2005, Müller et al. 2005). Both screens revealed a number of potential regulators for *Drosophila* JAK/STAT signaling, but interrestingly the number of common hits was very small. Müller *et al.* (2005) concentrated their further studies on two novel JAK/STAT pathway regulators: a bromo-domain-containing protein disrupted in leukemia 3 (also known as bromo-domain and WD repeat-containing protein 3, BRWD3) and tyrosine phosphatase Ptp61F. BRWD3 was found to function as a positive regulator of the JAK/STAT pathway, but the mechanism for this remain unclear. Ptp61F was also found in the screen by Baeg *et al.* (2005). As expected, RNAi mediated knockdown of *Ptp61F* resulted in an increase in JAK/STAT pathway driven reporter gene expression in both screens, indicating that Ptp61F is a negative regulator of the pathway. Furthermore, *Ptp61F* RNAi caused hyperphosphorylation of both Hop and

Stat92E, thus raising the possibility that Ptp61F may function as a phosphatase, which dephosphorylates Hop or both of these signaling molecules (Baeg et al. 2005). In the same study Baeg *et al.* also found that dsRNA-mediated knockdown of *Drosophila* homologs of *RanBP3* (*CG11763*) and *RanBP10* (*CG10225*) caused increase in phospho-Stat92E nuclear accumulation upon Upd stimulation, indicating that these factors may control nucleocytoplasmic transport of Stat92E.

3. Aims of the study

Drosophila melanogaster and vertebrates share the main components of the conserved JAK/STAT signaling pathway. The regulatory mechanisms of the JAK/STAT pathway have been widely studied, but little is known about the molecular details of the behaviour of the known JAK/STAT pathway regulators, such as SUMO. Moreover, as it has become evident that the regulation of the intracellular signaling cascades is generally complex, it is most likely that critical factors involved in JAK/STAT-mediated signal transduction remain to be discovered. The JAK/STAT pathway plays a crucial role in many biological processes including blood formation and the regulation of immune responses. Disturbances in JAK/STAT pathway-mediated signal transduction can lead to various diseases, as discussed in the Chapter 2.2.1. So, it is essential to understand the molecular basis of the JAK/STAT pathway regulation in order to develop treatments and better diagnostic tools for these pathological conditions. *Drosophila melanogaster* is an excellent model organism for studying signaling pathways with powerful genomic approaches such as genome-wide RNAi screening. The aims of this study were to reveal if sumoylation has an evolutionary conserved role in the regulation of STAT-mediated signal transduction, and to characterize novel components of the JAK/STAT pathway, using *Drosophila melanogaster* as a model organism.

The specific aims of the study were:

1. To study if the *Drosophila* transcription factor Stat92E is a target for SUMO modification and to reveal the functional effect of sumoylation on Stat92E-mediated gene expression.
2. To analyze how *ET/CG14225* participates in the regulation of the *Drosophila* JAK/STAT pathway activation.
3. To investigate the role of *Not4/CG31716* and its mammalian homolog *CNOT4* in STAT-mediated gene expression.

4. Materials and methods

4.1. Plasmid constructs and cloning

The *Drosophila Stat92E* full length cDNA (clone RE13194) was purchased from the *Drosophila* Genomics resource Center (Indiana University, Bloomington, IN, USA) and was cloned between the Not1 and Kpn1 restriction sites in the pcDNA3.1(-) expression vector after PCR amplification with the following the primers:
5'-CGGGCCGCGGCCGCGCCGCCATGAGCTTGTGGAAGCGC-3' to the 5'-end containing Not1 restriction site and 5'-CGCGGTACCCTACTACTTGTCATCGTCGTC GTTGTAGTCAAAGTTCTCAAAGTTTGTAATC-3' to the 3'-end containing Kpn1 restriction site and Flag epitope-tag.

In order to clone a Flag-tagged *Stat92E* into the *Drosophila* expression vector pMT-V5-HisA (Invitrogen, Carlsbad, CA, USA), *Stat92E* (clone RE13194) was amplified by PCR using primers as follows:
5'-CGGGCCGCGGCCGCGCCGCCATGAGCTTGTGGAAGCGC-3' to the 5'-end containing a Not1 restriction site and 5'-CGGGTTTAAACCTACTACTTGTCATCGT CGTCCTTGTAGTCAAAGTTCTCAAAGTTTGTAATC-3' with a Pme1 restriction site and the Flag epitope-tag.

The *pMT-Stat92E K187R-Flag* mutant was created using the QuikChange® Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions with the following primers: Forward primer: 5'-GGTATGGT CACACCCAGGGTGGAGCTGTACGAG-3' and Reverse primer: 5'-CTCGTACA GCTCCACCCTGGGTGTGACCATACC-3'.

The *pcDNA3-SUMO-1-Flag* and the *pcDNA3-SUMO-1-Flag-Flag* expression vectors were kindly provided by Dr. H Yasuda. *pSG5-SUMO-1-His* was a kind gift from Dr. A.

Dejean. *SENP1-Flag*, the catalytically inactive *SENP-1 C603S-Flag*, *SUMO-1 GA-His* and *SUMO-3-HA* were provided kindly by Prof. J. Palvimo (Xu et al. 2006).

The *pcDNA3-p53* expression vector and the *PG-13-luciferase* reporter containing 13 synthetic p53 binding sites were kind gifts from Dr. M. Laiho (Lee et al. 1997, Subler et al. 1994).

The *GAS-luciferase* reporter construct contains a GAS sequence TTTCCCCGAAA from the *Irf-1* gene promoter region cloned upstream of the thymidine kinase (TK) driving the firefly *luciferase* gene (Pine et al. 1994). The *Igε-luc* (*pfNεN4-luc*) construct contains four copies of IL-4 responsive elements inserted upstream of the c-*fos* minimal promoter in front of the *Photinus pyralis luciferase* gene (Pesu et al. 2000).

The *TotM-luc* reporter and the *Actin 5C-β-galactosidase* reporter plasmids were provided by Dr. J.-L. Imler. $Hop^{Tum\text{-}l}$, which was provided by J.-L. Imler as well, contains a substitution of glycine 341 to glutamic acid creating a constitutively active form of *Hop* (Luo et al. 1995). The *Smt3-HA* plasmid was obtained from Dr. A.J. Courey (Bhaskar et al. 2000) and the *pMT-Upd* expression vector was a generous gift from Dr. M. Boutros. The *10xStat92E-luc* construct contains five tandem repeats of the Stat92E binding sites from the *Socs36E* enhancer region inserted upstream of a minimal heat shock promoter-driven firefly *luciferase* gene (Baeg et al. 2005). This construct was kindly provided by Dr. E. Bach.

The *Drosophila CG31716/Not4* cDNA (clone RE04975) was purchased from *Drosophila* Genomics resource Center (Indiana University, Bloomington, IN, USA). The cDNA was amplified by PCR using the following oligonucleotides as primers: 5'-CACA CAGCGGCCGCATGAACGGCCTGAGCAGC-3' containing a Not1 restriction site and 5-'CACACATCTAGAAACGAATTGACGGCTTTTTAAAAACG-3' containing an Xba1 restriction site. The PCR product was inserted into the pMT-V5-HisA expression vector (Invitrogen, Carlsbad, CA, USA). The pCOHygro plasmid containing Hygromycin resistance gene was purchased from Invitrogen (Carlsbad, CA, USA).

4.2 Antibodies

Table 2. Antibodies used in the study.

Name	Description	Manufacturer	Used in
Anti-Actin	Clone C4, mouse monoclonal	Millipore, Billerica, MA, USA	III
Anti-Flag	clone M2, mouse monoclonal	Sigma-Aldrich, St. Louis, MO, USA	I, III
Anti-HA (anti-Influenza virus hemagglutinin)	clone 16B12, mouse monoclonal	Covance, Princeton, NY, USA	I, III
Anti-phospho-tyrosine	PY99, mouse monoclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	II, III
Anti-Stat92E-N-terminus	dN-17, goat polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	I, II, III
Anti-V5	mouse monoclonal	Invitrogen, Carlsbad, CA, USA	III
Anti-STAT1 (N-terminus)	Clone 1, mouse monoclonal IgG1	BD Biosciences, Franklin Lakes, NJ, USA	Results, Fig. 8
Anti-phospho-STAT1 (Y701)	rabbit polyclonal	Cell signaling,	Results, Fig. 8

4.3 Cell culture

Cos-7 (SV-40-transformed green monkey kidney cells) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% (vol/vol) FBS (Gibco), 2 mM L-glutamine (Sigma-Aldrich St. Louis, MO, USA), 100 U/ml penicillin and 50 μg/ml streptomycin (Lonza, Basel, Switzerland) at +37ºC. Human HeLa (cervix adenocarsinoma cell line) cells were cultured in DMEM supplemented with 10% (vol/vol) FBS (Gibco), 2 mM L-glutamine (Sigma-Aldrich St. Louis, MO, USA), 100U/ml penicillin and 50 μg/ml streptomycin (Lonza, Basel, Switzerland) and

1% (vol/vol) non-essential amino acids (Lonza, Basel, Switzerland) at +37 ºC. Schneider 2 *Drosophila* cells (S2, derived from macrophage-like embryonic cell lineage) were cultured in Schneider's medium (Sigma-Aldrich, St. Louis, Mo, USA) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 50 µg/ml streptomycin at +25ºC.

4.4 Generation of stable overexpression S2 cell lines

S2 cell lines for stable overexpression of the *pMT-Not4-V5* or the *pMT-V5-HisA* control plasmid were generated by transfecting S2 cells with 9.5 µg of the *pMT-Not4-V5* or *pMT-V5-HisA* plasmids, together with 0.5 µg of the pCOHygro plasmid (Invitrogen, Carlsbad, CA, USA) using FuGENE® 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. After 72 hours 300 µg/ml Hygromycin-B (Calbiochem, San Diego, CA, USA) was added to the Schneider's growth medium supplemented with FBS and antibiotics as described earlier. Cells were cultured at +25ºC.

4.5 Transfections

A total amount of $5x10^6$ Cos-7 cells were transfected using electroporation with a Bio-Rad Gene Pulser® (Bio-Rad Laboratories, Hercules, CA, USA) at 260 V and 960 µF. For each transfection 3 µg of plasmid DNA was mixed with herring sperm DNA for a total amount of 40 µg of DNA in each sample. The DNA was mixed with Cos-7 cells in 250 µl of DMEM in GenePulser® electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA, USA) prior to electroporation. After electroporation, cells were incubated in the cuvettes for 10 minutes at RT and then plated onto 10 cm diameter cell culture dishes. Transfected cells were cultured at +37 ºC for 48 hours prior to cell lysis.

HeLa cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmids were mixed with Lipofectamine 2000 in Opti-MEM® (Invitrogen, Carlsbad, CA, USA) and added to the

plated cells. After 4 hours the medium was changed to the DMEM HeLa culture medium described above.

S2 cells were transfected using either the L-PEI transfection reagent (Boussif et al. 1995, Huh et al. 2007) or the FuGENE® 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Approximately $1.5x10^7$ S2 cells and a total amount of 10 μg of plasmid DNA were used in L-PEI transfections.

4.6 Immunoprecipitation, Co-immunoprecipitation and Western blotting

A total amount of $5x10^6$ Cos-7 cells were transfected using electroporation with a total amount of 3.0 μg of indicated plasmid DNAs. To prepare Cos-7 whole cell lysates for the detection of Stat92E sumoylation, cells were harvested by scraping them from culture dishes and lysed in 1% Triton-X lysis buffer (50 mM Tris-HCl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF) supplemented with vanadate, aprotinin PMSF and 5 mM NEM.

In order to study Stat92E sumoylation in *Drosophila* cells, S2 cells were transfected with 4 μg of *pMT-Stat92E-Flag* or *pMT-Stat92E K187R-Flag* together with 6 μg of *Smt3-HA* plasmids using L-PEI as described in the articles of Boussif *et al.* (1995) and Huh *et al.* (2007). Two hours after transfection the medium was changed and supplemented with 500 μM $CuSO_4$ to induce the expression of *Stat92E* WT and the K187R mutant from the pMT expression vector. Cells were incubated for 72 hours and harvested by pipeting from culture dishes into 15 ml Falcon tubes following centrifucation. The cell pellet was then washed once with ice cold PBS and suspended in 1% Triton X lysis buffer supplemented with aprotinin, vanadate, PMSF and 5mM NEM as described earlier. Protein concentrations were measured using the Bio-Rad DC Protein Assay kit (Bio-Rad laboratories, Hercules, Ca, USA).

4.6.1 Immunoprecipitation

Equal amounts of protein lysates were subjected to immunoprecipitation assay using either anti-Flag (Sigma-Aldrich) or anti-Stat92E (Santa Cruz) antibodies. Protein-antibody complexes were then collected with Protein G Seprahose beads (GE Healthcare, Little Chalfont, UK). Proteins were separated by SDS-PAGE and immunoblotted with anti-Stat92E-N-terminal (Santa Cruz), anti-phospho-tyrosine (Santa Cruz) or anti-HA (Covance) antibodies.

4.6.2 Co-immunoprecipitation

For co-immunoprecipitation experiments *pMT-Not4-V5* overexpressing and *pMT-V5-HisA* overexpressing control S2 cells were treated with 500 μM $CuSO_4$ in order to induce transcription from the pMT-expression vector. After 48 hour of incubation at +25ºC cells were lysed using a buffer containing 20 mM HEPES pH 8.0, 100 mM NaCl, 1% TritonX-100, 10% Glycerol, 1 mM EDTA, 50 mM NaF supplemented with vanadate, aprotinin and PMSF. Equal amounts of protein lysates were incubated with either an anti-Stat92E-N-terminal antibody (Santa Cruz) or an anti-HA antibody (Covance) as a control for 2 hours rotating at +4ºC, followed by a one hour incubation with Protein G Seprahose beads (GE Healthcare, Little Chalfont, UK). The beads were then washed four times with TritonX-100 lysis buffer supplemented with inhibitors and the co-immunoprecipitated proteins were released from the beads by adding 2xSDS loading buffer. Co-immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting using an anti-V5 antibody (Invitrogen).

4.6.3 Western blotting

Proteins were separated by SDS-PAGE and transferred onto Protran® nitrocellulose filters (Whatman, Dassel, Germany). Membranes were blocked with 5% non-fat dried milk in TBS 0.1% Tween 20 or with 5% BSA in TBS 0.1% Tween 20. Immunoblotting was performed by incubating membranes with specific primary antibodies followed by

incubation with biotinylated secondary antibodies (Dako A/S, Glostrup, Denmark) and incubation with a strepavidin-biotin horseradish peroxidase complex (GE-healthcare, Little Chalfont, UK). Immunodetection was performed with an enhanced chemiluminescence (ECL) system (GE Healthcare, Little Chalfont, UK).

4.7 dsRNA synthesis and RNA interference

For RNA interference (RNAi) in S2 cells, targeted dsRNAs were generated. At first double-stranded DNA was amplified by two-step PCR using gene specific primers and *Drosophila* cDNA as a template. The primers for the second PCR contained T7 promoter sequence (GAATTAATACGACTCACTATAGGGAGA) at their 5'-end. The product from the second PCR was then used as a template for dsRNA synthesis using the T7 MegaScript® RNA polymerase kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

The following primers were used for dsRNA synthesis:
Stat92E 3'-UTR: 5'-CGTAATATGGTCCTCGTGTC-3' and 5'-AACAACTTC AGCTCACAGCC-3' for the first PCR and 5'-GAATTAATACGACTCACTATAGGG AGAGGCAATCGCATGTGGC-3' and 5'-GAATTAATACGACTCACTATAG GGAGAGGAATGTGTCCGCACG-3' for the second PCR.

dPias: 5'-GCCATTTCCGTCTCTTCAGG-3' and 5'-CTTTGACGTTTACGTTCGGCG-3' for the first PCR and 5'-GAATTAATACGACTCACTATAGGGAGATCTCAT TCGCTGGACG-3' and 5'-GAATTAATACGACTCACTATAGGGAGACTCC ACCAGGCAAAAG-3' for the second PCR.

A dsRNA containing the sequence for green fluorescent protein (GFP) was used as a negative control. Other dsRNA constructs used are described in original communications II and III.

For siRNA experiments in HeLa cells gene specific siRNAs were purchased from Ambion (Austin, TX, USA).

4.8 Luciferase reporter gene assay

A total amount of $0.1x10^6$ HeLa cells were seeded on 24-well culture dishes 24 hours prior to transfection with 0.2 μg of the *luciferace* reporter and 0.1 μg of the *CMV-β-galactosidase* reporter plasmids simultaneously with 20 pmol of specific siRNA using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 hours cells were serum starved for an additional 24 hours and either treated or left untreated with 100 ng/ml of human IFN-γ or 10 ng/ml of human IL-4. Cells were lysed in Promega's Reporter Lysis Buffer (RLB) (Promega, Madison, WI, USA) following luciferase activity measurement using the Luciferace Assay Substrate (Promega, Madison, WI, USA) and Luminoscan Ascent (ThermoElectron Corporation, Finland). Luciferase values were normalized against β-galactosidase values of the lysates.

For luciferase assay experiments in S2 cells, $1.0x10^6$ cells were plated on 24-well culture plates and transfected with the indicated plasmid DNAs and dsRNAs using FuGENE® 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were lysed 48 – 72 hours after the transfection following luciferase and β-galactosidase activity measurements as described above.

4.9 RNAi screen to identify novel JAK/STAT pathway regulators

For the genome-wide RNAi screen a total of 16,025 dsRNAs were produced. Of these, 13,625 dsRNAs were synthesized using commercial *Drosophila* genome RNAi library (MCR Geneservice) derived PCR products with dual T7 promoter sequences as templates. An additional 2,400 dsRNAs were synthetized from a S2 cell-derived cDNA library (Rämet et al. 2002a, Ulvila et al. 2006). The T7 MegaScript® RNA polymerase kit

(Ambion, Austin, TX, USA) was used for dsRNA synthesis as described in the Chapter 4.7. The dsRNA concentrations were measured using the picoGreen® dsRNA Quantitation kit. The dsRNA synthesis for the genome-wide RNAi screen is also described in detail in the doctoral thesis of Jenni Kallio. The activity of the Stat92E-responsive *TotM-luc* reporter was used to monitor the activity of the JAK/STAT pathway and the *Actin 5C-β-galactosidase* reporter was used to monitor cell viability. S2 cells were plated on 24-well culture dishes and transfected with 0.1 µg of *TotM-luc* and 0.1 µg of *Actin 5C-β-galactosidase* reporters together with 0.5 µg of dsRNA using FuGENE® 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Stat92E dependent *TotM-luc* expression was activated by co-transfecting the cells with 0.1 µg of $Hop^{Tum\text{-}l}$ and RNAi against *Stat92E* and *GFP* were used as a positive and negative control, respectively. Cells were lysed 72 hours after the transfection and luciferase and β-galactosidase activities were measured as described in the Chapter 4.8. Genes targeted by RNAi treatments that repeatedly decreased *TotM-luc* activity by more than 50% without significantly affecting cell viability (Actin 5C-β-galactosidase activity) were considered as novel positive JAK/STAT pathway regulators, whereas genes targeted with dsRNA treatments that repeatedly increased *TotM-luc* activity by more than 50% without significantly affecting cell viability were considered potential negative JAK/STAT pathway regulators. The corresponding templates from the original library were TA cloned and sequenced. Gene-specific primers for targeted dsRNA synthesis against these novel regulators were designed based on sequencing data. Targeted dsRNAs were synthetized as descriped in the Chapter 4.7. Transfections and luciferase assay measurements were repeated with these targeted dsRNAs similarly as described above, in order to confirm that the RNAi phenotypes in the original screen were not caused by a contaminant dsRNA or any off-target effect. The endogenous *TotM* and *Actin 5C* expression levels from $Hop^{Tum\text{-}l}$ and dsRNA transfected cells were measured using qRT-PCR, in order to confirm that our screening results were not caused by reporter assay related artifacts (described in the thesis book of Jenni Kallio).

4.10 Electrophoretic mobility shift assay (EMSA)

S2 cells were transfected with 2.0 µg of *Hop*$^{Tum\text{-}l}$ to induce the JAK/STAT pathway or with an empty vector as a control together with 5.0 µg of different dsRNAs using FuGENE® 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cell were incubated for 48 hours at +25ºC and lysed with lysis buffer 10 mM HEPES-KOH pH 8.0, 10 mM KCl, 0.1% NP-40, 1 mM EDTA supplemented with 10 mM NaF, 1 mM DTT, vanadate, aprotinin and PMSF. After centrifugation the supernatant was collected as cytoplasmic extract. The pellet was resuspended into a buffer containing 20 mM HEPES-KOH pH 8.0, 400 mM NaCl, 10% glycerol, 1 mM DTT, 10 mM NaF supplemented with vanadate, aprotinin and PMSF and rotated at +4ºC for 30 minutes following centrifugation in order to extract the nuclear lysate. Double-stranded DNA (dsDNA) probes containing the Stat92E binding site sequence (TTCTTAGAAT) from the *TotM* gene promoter region were generated by annealing oligonucleotide 5'-CAAAAAAACAGTTCTTAGAATGCAATCAATAC-3' with its complement. Annealed dsDNA probes were then end-labelled with [γ-^{32}P]-labeled ATP using T4-polynucletide kinase. For the binding assay cytoplasmic and nuclear lysates were mixed in a 3:1 ratio together with the [γ-^{32}P]-labeled dsDNA oligonucleotides in the presence or without a Stat92E-N-terminal antibody (Santa Cruz) in binding buffer (20 mM HEPES-KOH pH 8.0, 50 mM NaCl, 10% glycerol, 0.01% Triton X-100, 1 mM DTT) for 30 minutes at room temperature. The complexes were resolved by 6% PAGE in 0.5 x TAE, before drying and autoradiography.

5. Results

5.1 Sumoylation of Stat92E (Article I)

5.1.1 Lysine 187 is a putative sumoylation site in Stat92E

Numerous transcription factors are modified with a small ubiquitin-like modifier (SUMO) (Gill 2005). Human STAT1 was the first STAT transcription factors shown to be SUMO conjugated. This sumoylation on Lys703 in the C-terminal transactivation domain of STAT1 was shown to inhibit the expression of certain STAT1 driven genes (Rogers et al. 2003, Ungureanu et al. 2003, Ungureanu et al. 2005). The main components of the *Drosophila* JAK/STAT pathway are comparable to those of the human pathway. Also regulators of the human JAK/STAT pathway, such as PIAS and SOCS are found in *Drosophila melanogaster* (Betz et al. 2001, Hari et al. 2001, Callus and Mathey-Prevot 2002). In order to find out if *Drosophila* Stat92E is sumoylated like its mammalian counterpart STAT1, the sequence of Stat92E was analyzed for putative sumoylation sites. Sumoylation most often occurs on lysine residues within the consensus motif ψKxD/E (where ψ stands for a large hydrophobic amino acid, and x can be any amino acids) (Sternsdorf et al. 1999). A sequence analysis of Stat92E revealed one complete SUMO consensus site in the coiled-coil domain region of Stat92E between amino acids 186 and 189 (PKVE), where Lys187 would be the SUMO acceptor. The sumoylation site Lys703 in human STAT1 is in close proximity to tyrosine 701, a phosphorylation site required for STAT1 activation. A corresponding lysine residue is conserved also in Stat92E (Lys713), but the sequence around it does not create a perfect sumoylation consensus motif (I, Fig. 2A). The discovery that Stat92E contains a putative SUMO-conjugation site prompted us to do further biochemical studies to investigate Stat92E sumoylation.

5.1.2 Stat92E is sumoylated in mammalian cells

Sumoylation is a highly dynamic modification and usually only a few target proteins are sumoylated at a given time in the cell. This poses a challenge for detecting protein sumoylation. The mammalian Cos-7 cell line is widely used in sumoylation studies because of the well-defined SUMO-conjugation machinery found in these cells. To this end, we chose to study Stat92E sumoylation first by overexpressing Flag-tagged *Stat92E* together with either Flag-tagged, tandem-Flag-tagged *SUMO*-1 or HA-tagged *SUMO-3* in Cos-7 cells. Stat92E was immunoprecipitated from total cell lysates using an anti-Flag antibody and immunoblotted with a Stat92E specific antibody. In the lanes with co-transfected *SUMO-1-Flag*, *SUMO-1-Flag-Flag* or *SUMO-3-HA*, slowly migrading bands at around 116 kDa were detected with the Stat92E antibody indicating that Stat92E is SUMO-1/3-modied in Cos-7 cells (I, Fig. 1A). Furthermore, overexpression of the double Flag-tagged *SUMO-1* created a band shift corresponding to the size of the Flag-tag, further indicating that the bands represent Stat92E-SUMO complexes, instead of other possible post-translational modifications to Stat92E caused by the forced expression of *SUMO-1/3*.

To further verify that the antibody was detectecting, Stat92E-SUMO-1/3 bands, Cos-7 cells were co-transfected with *Stat92E-Flag* and *SUMO-1-GA-His*, a mutant which cannot be SUMO-conjugated. As expected, the 116 kDa band was not present in the SUMO-1-GA-His lane when probed with the anti-Stat92E antibody. Sumoylation is a reversible post-translational modification and SUMO is cleaved from target proteins by specific SUMO proteases (SENPs) (Mukhopadhyay and Dasso 2007). Thus, we wanted to study if the overexpression of *SENP1* abolishes Stat92E sumoylation. To this end, Cos-7 cells were co-transfected with *Stat92E-Flag*, *SUMO-1-His* and *SENP1* WT or a catalytically inactive *SENP1 C603S* mutant. The *SENP1* co-transfection totally abolished the upper, slower migrating band detected by anti-Stat92E, whereas co-transfection of a catalytically dead *SENP1 C603S* mutant slightly increased the amount of Stat92E-SUMO-1 (I, Fig. 1B). These findings suggest that Stat92E can be sumoylated in Cos-7 cells and SENP1 can cleave SUMO-1 from Stat92E.

5.1.3 Stat92E is sumoylated in *Drosophila* S2 cells and lysine 187 is the acceptor site for SUMO conjugation

As described above, Stat92E has a SUMO consensus site in its coiled-coil domain between amino acids 186-189 (PKVE). To determine if this is the SUMO binding site, we mutated lysine 187 to arginine. *Stat92E K187R-Flag* and *Stat92E WT-Flag* were then transfected into *Drosophila* S2 cells with or without an HA-tagged *Drosophila* SUMO variant, *Smt3*. The expression of endogenous *Stat92E* was inhibited by adding a dsRNA targeting *Stat92E-3'-UTR* to the cells. After cell lysis, Stat92E was immunoprecipitated from the lysates with an anti-Flag antibody. As expected, *Smt3* overexpression created a band of ~ 116 kDa, which was detected with both the Stat92E specific antibody and an HA-antibody detecting Smt3, indicating that Stat92E is also modificated by Smt3 in S2 cells. Moreover, the K187R mutation totally abolished this band, indicating that lysine 187 is the only sumoylation site in Stat92E (I, Fig. 2B).

5.1.4 Sumoylation negatively regulates Stat92E driven gene expression

In order to determine if SUMO attachment affects the transcriptional activity of Stat92E, luciferase reporter assay experiments were carried out in S2 cells. Two independent *luc*-reporters, *TotM-luc* and *10xStat92E-luc* were used to detect JAK/STAT pathway activity. In the absence of a Stat92E activating stimulus, overexpression of WT *Stat92E* and the sumoylation deficient *Stat92E K187R* mutant equally increased the activities of both *luc*-reporters (approximately 3-fold). When the JAK/STAT pathway was activated with co-transfection of the pathway ligand *Upd*, Stat92E K187R displayed significantly increased transcriptional activity on both of the Stat92E driven reporters. These data suggest that sumoylation has an inhibitory effect on Upd-induced Stat92E-mediated gene transcription (I, Fig. 3A and B).

PIAS proteins have been shown to promote SUMO conjugation to human STAT1 and dPias has also been suggested to negatively regulate Stat92E-mediated signaling (Betz et al. 2001, Rogers et al. 2003, Ungureanu et al. 2003). This prompted us to investigate if

dPias could affect the sumoylation of Stat92E. As shown in original communication I, Fig. 3C, *dPias* RNAi increased Stat92E driven *TotM-luc* reporter activity, supporting the previously published data that dPias negatively regulates the *Drosophila* JAK/STAT pathway (Betz et al. 2001). However, we were not able to detect any alterations in the sumoylation status of Stat92E with *dPias* RNAi in our immunoblot studies (data not shown), and therefore the possible role of dPias in Stat92E sumoylation remains unclear.

5.2 *CG14225/ET* and *CG31716/Not4* as regulators of the *Drosophila* JAK/STAT pathway (Articles II and III)

5.2.1 *CG14225* and *CG31716* are novel *Drosophila* JAK/STAT pathway regulators found in a genome-wide RNAi screen in *Drosophila* S2 cells

In order to identify novel JAK/STAT pathway regulators, a genome-wide RNAi screen was performed in *Drosophila* S2 cells. This screen is described in more detail in the doctoral thesis of Jenni Kallio and in original communication II. The pathway was activated by overexpressing the constitutively active *Hopscotch* mutant $Hop^{Tum\text{-}l}$, and expression from the Stat92E target gene *TurandotM (TotM)-luc* reporter was used as an indicator of the activity of the JAK/STAT pathway. *Act5C-β-galactosidase* reporter was used to monitor cell viability and transfection efficiency. In this set-up, 16,025 dsRNA treatments were used to find genes which affect *Drosophila* JAK/STAT signaling. The knockdown of seven genes repeatedly caused an over 50% decrease in *TotM-luc* activity, indicating that these genes may act as positive regulators of the *Drosophila* JAK/STAT pathway (II, Fig. 1C). The genes included the previously identified JAK/STAT pathway members *Stat92E* and *Hop*, as well as four novel JAK/STAT pathway regulator candidate genes: *CG14225, CG31716*, *Med27* and *Taf1*. The seventh gene was a gene called *enok*, which was also found in two previously published screens by Baeg *et al*. (2005) and Müller *et al*. (2005). In addition to the candidates for positive regulators, the screen also revealed one interesting putative negative regulator, *kayak*.

To investigate, if these effects are specific for the *TotM*-promoter, or if they have a more general affect on JAK/STAT signaling, dsRNA-mediated knockdown of the candidate genes was studied with another Stat92E responsive *luciferase* promoter, *10xStat92E-luc*, containing five doublet repeats of Stat92E binding sites from the *Socs36E* gene enhancer region upstream of the *luciferase* gene (Baeg et al. 2005). Like in the original screen, RNAi targeting *CG14225* and *CG31716* significantly reduced *10xStat92E-luc* activity (Fig. 6 and III, Fig. 1B). Similarly to the original screen, *kayak* RNAi lead to a notable enhancement in *luciferase* activity also on the *10xStat92E*-promoter (Fig. 6). Interestingly, dsRNAs targeting *Med27*, *Taf1* or *enok* had no effect on Stat92E-mediated gene expression in this setting, indicating that these factors may have more selective roles in JAK/STAT pathway regulation.

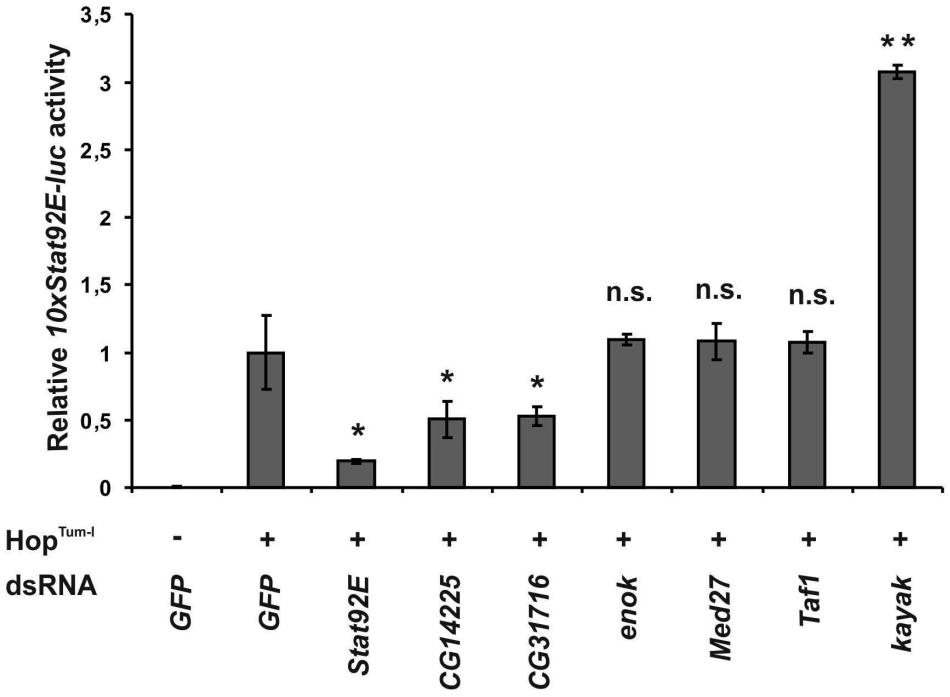

Figure 6. The effect of RNAi against the potential novel JAK/STAT pathway regulators on Hop^{Tum-l}-induced *10xStat92E-luc* activity. *CG14225* and *CG31716* RNAi impairs JAK/STAT pathway mediated *10xStat92-luc* activity, while kayak RNAi results in the opposite effect. *Enok, Med27 and Taf1* have no effect on JAK/STAT pathway activity in this setting. *Stat92E* RNAi was used as a positive control. The error bars indicate SD, calculated from three independent transfections. *p≤0.05, **p≤0.001, n.s.= non significant.

As shown in Fig. 6, *kayak* RNAi caused a notable enhancement in Stat92E responsive *10xStat92E-luc* activity. This prompted us to study this gene in more detail. *Kayak* encodes a protein homologous to mammalian FosB. Thus, we wanted to investigate if *FosB* RNAi also affects the activity of the human JAK/STAT pathway. Human STAT1 is a well-studied transcription factor essential for IFN-γ signaling. In order to study if *FosB* RNAi has an effect on STAT1 responsive *GAS-luc* activity, human HeLa cells were transfected with either Scrambled (Scr) siRNA as a negative control, a siRNA targeting *Stat1* or a siRNA targeting *FosB*. Cells were stimulated by incubating them in the presence of human IFN-γ for 6 hours prior to lysis. In contrast to results obtained from *Drosophila* S2 cells with *kayak* RNAi, *FosB* knockdown in mammalian cells seemed to have a negative effect on STAT1-mediated reporter gene expression (Fig. 7). Of note, the possible effect of FosB on STAT1 activating tyrosine 701 phosphorylation was studied in HeLa cells as well. As a result, we did not detect any alterations in the STAT1 tyrosine 701 phosphorylation status with *FosB* RNAi, when compared to control scrambled siRNA transfected cells at different IFN-γ induction time points (data not shown). Because of these negative results we decided not to study kayak/FosB further.

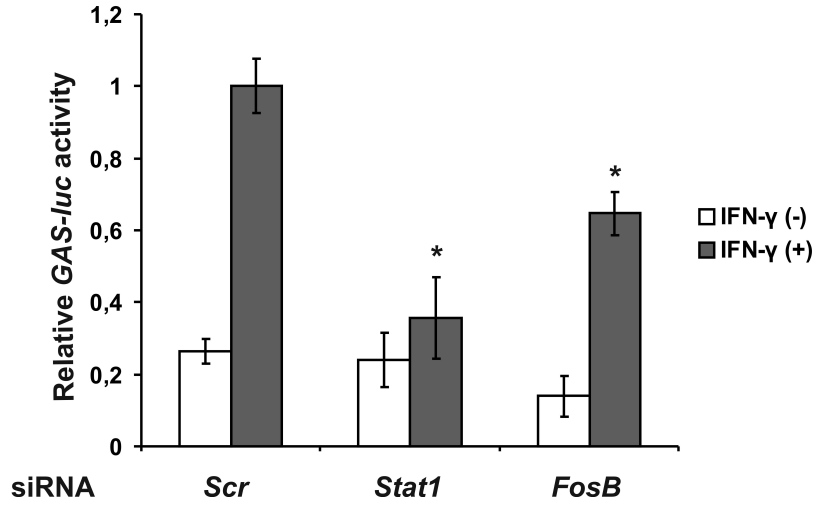

Figure 7. Effect of *FosB* siRNA on STAT1 mediated *GAS-luciferase* reporter activity. Values are mean ± SD from three independent transfections, *p≤0.05.

The candidate genes which had a positive effect on JAK/STAT signaling in the screen were then studied under more physiological conditions in *Drosophila* S2 cells using overexpression of the Dome receptor ligand *Upd* to activate the JAK/STAT pathway. Strikingly, in this experimental setting dsRNA mediated RNAi against *CG14225* had a significant positive influence on two independent Stat92E responsive *luc*-reporters, *TotM-luc* and *10xStat92E-luc* (described in the thesis of Jenni Kallio and in II, Fig. 2A and B). This unexpected result prompted us to concentrate on this gene as a novel regulator of the *Drosophila* JAK/STAT pathway. As in the original screen, dsRNA treatment targeting *CG31716* had a significant negative effect on both reporters, also when induced with the overexpression of *Upd*, whereas the effect of knocking down the remaining three genes of interrest differed depending on the promoter (Described in thesis book of Jenni Kallio and II, Fig. 2A and B). Because of these context dependent effects of *enok*, *Med27* and *Taf1* on Stat92E responsive promoters, we chose *CG31716* for further studies. The goal of my thesis was to study, in detail, the molecular mechanisms behind *CG14225*- and *CG31716*-mediated regulation of JAK/STAT signaling.

5.2.2 *CG14225* encodes a gp130 related transmembrane protein called Eye transformer (ET) (Article II)

A sequence analysis revealed that *CG14225* is a 3.3 kb gene containing three different coding sequences, and interestingly, it is located next to *Dome* on the *Drosophila* X chromosome. The protein product of the *CG14225* gene is 713 amino acids long and contains a putative transmembrane domain. The CG14225 protein shares structural similarities with Dome mainly in its extracellular domains. Like Dome, the extracellular part of CG14225 contains a putative sequence for a cytokine binding module (CBM). CBM in vertebrate interleukin receptors consists of two FnIII domains, the N-terminal domain is usually identifiable through four conserved cystein residues and the C-terminal domain contains a conserved WSXWS motif (Bazan 1990). The conserved cystein residues can also be found in the CG14225 sequence, but the WSXWS motif is incomplite (NTLWS). In contrast to Dome, CG14225 lacks three additional extracellular

FnIII domains near the transmembrane domain (II, Fig. 3A). According to our BLAST results the closest human homologue for CG14225 is gp130 with 12.7% sequence identity. The intracellular part of CG14225 was not found to share homology with any known protein.

Our *in vivo* results showed that crossing flies carrying *CG14225* RNAi under an eye-specific driver (*ey-GAL4*) with an *Upd* overexpressing fly strain causes severe eye overgrowth in the offspring. These experiments are discussed in more detail in the doctoral thesis of Henna Myllymäki (in preparation) and in II, Fig. 7C and D. Thus, the protein encoded by *CG14225* was named Eye transformer (ET).

5.2.3 ET negatively regulates Upd-induced Stat92E phosphorylation (Article II)

Stat92E activation requires Hop-mediated phosphorylation of a single C-terminal tyrosine residue, which under physiological conditions is a response to Upd-mediated Dome activation (Dearolf 1993, Binari and Perrimon 1994, Hanratty and Hou et al. 1996, Yan et al. 1996a, Brown et al. 2001, Chen et al. 2002). According to our results, ET was found to negatively regulate Upd-induced Stat92E-mediated gene responses. ET was shown to interact with Dome and Hop, and furthermore, ET was found to function at the level or upstream of the JAK/STAT pathway receptor Dome (discussed in detail in original communication II, Fig. 4). Together with the sequence data, this suggests that ET is a Dome-Hop complex associated transmembrane regulatory protein.

Next, we investigated if the negative effect of ET on Stat92E target gene expression is a consequence of an altered Stat92E phosphorylation status. To this end, S2 cells were transfected with *ET* dsRNA or control dsRNAs together or without *Upd* to activate the JAK/STAT pathway. After cell lysis Stat92E phosphorylation was studied by immunoblotting with a Stat92E specific antibody. Strikingly, an additional slower migrating Stat92E band was detected in the lanes where *Upd* was transfected together with *ET* dsRNA (II, Fig. 5B, upper panel). To verify that the observed band represents a phosphorylated form of Stat92E, endogenous Stat92E was immunoprecipitated from

lysates with an anti-Stat92E antibody and immunodetection was performed with an anti-phospho-tyrosine specific antibody. As shown in original communication II, Fig. 5B (two middle panels), the phospho-tyrosine specific antibody detects the corresponding band, indicating that the band represents tyrosine phosphorylated Stat92E. Furthermore, the band could be detected only weakly in the lanes with the *GFP* control dsRNA and was totally abolished with RNAi against *Stat92E* or *Hop*. These results indicate that depletion of ET causes hyperphosphorylation of Stat92E upon Upd induction. In addition to this, the simultaneous transfection of a dsRNA targeting *Dome* or *Hop* together with an *ET* targeted dsRNA almost totally abolished Stat92E hyperphosphorylation caused by the *ET* RNAi, indicating that ET-mediated Stat92E regulation is dependent on *Dome* and *Hop* (II, Fig. 5B, lower panel). Taken together, ET negatively regulates JAK/STAT-mediated signal transduction at the level or upstream of Dome by inhibiting Upd-induced Stat92E tyrosine phosphorylation.

5.2.4 *CG31716* overexpression enhances JAK/STAT pathway mediated gene responses in S2 cells (Article III)

As described earlier, *CG31716/Not4* RNAi reduced the activities of the *TotM-luc* and the *10xStat92E-luc* reporter genes when the JAK/STAT pathway was activated by both $Hop^{Tum\text{-}l}$ and *Upd* overexpression. Furthermore, *CG31716/Not4* RNAi was shown to inhibit endogenous *TotM* expression, verifying that these results were not caused by artifacts in the reporter gene assay (described in detail in III, Fig. 1E and F). In addition to RNAi studies, we investigated the effect of *CG31716/Not4* overexpression on Stat92E-mediated gene expression. For this, S2 cells were transfected with *CG31716/Not4* in the pMT-V5 expression vector together with the *TotM-luc* or *10xStat92E-luc* reporter construct and with or without $Hop^{Tum\text{-}l}$ to activate the JAK/STAT pathway. As shown in original communication III, Fig. 2A and B, *CG31716/Not4* overexpression causes a significant increase in Stat92E-dependent reporter gene activity, both in uninduced cells and upon $Hop^{Tum\text{-}l}$-mediated Stat92E activation. A repeat experiment where the pathway was activated with *Upd* yielded similar results. In summary, forced *CG31716/Not4* expression enhances Stat92E-responsive reporter gene expression in S2 cells, indicating

that *CG31716/Not4* positively regulates the *Drosophila* JAK/STAT pathway. A similar effect on *TotM* and *TotA* gene expression was detected in *CG31716/Not4* overexpressing fly strains *in vivo.* Both genes were up-regulated in *CG31716/Not4* overexpressing flies compared to control flies under normal conditions. Septic injury caused by an *Enterobacter cloacae* infection resulted in a significant increase in *TotM* expression in *CG31716/Not4* overexpressing flies compared to control flies. The effect on *TotA* expression was less clear in this experimental setting (discussed in more detail in III, Fig. 3A and B).

5.2.5 *Drosophila CG31716* gene encodes Not4, a protein related to mammalian CNOT4 (Article III)

CG31716/Not4 was found to positively regulate *Drosophila* JAK/STAT signaling. *CG31716/Not4* gene encodes a 1051 amino acid protein that shares structural similarities with the yeast and human CNOT4 proteins (Albert et al. 2000, Albert et al. 2002). The domain structures of Not4 and human CNOT4 are illustrated in original communication III, Fig. 4A. The most N-terminal domain of human CNOT4 is the RING domain, known to harbor a Ubiquitin E3 ligase activity. Adjacent to this is the RRM domain that mediates interactions with ssDNA and ssRNA. The zinc-finger domain mediates possible DNA and protein interactions (Albert et al. 2002). The N-terminus of *Drosophila* Not4 was found to resemble the N-terminus of human CNOT4, while the C-terminus of *Drosophila* Not4 is less conserved and does not contain any identifiable domains.

5.2.6 Human CNOT4 regulates STAT1- and STAT6-mediated signal transduction (Article III)

The mammalian JAK/STAT pathway is highly similar to the corresponding *Drosophila* cascade. In order to study whether Not4 is a conserved regulator of the JAK/STAT cascade, we investigated if siRNA-mediated knockdown of the *Not4* human homologue, *CNOT4*, affects STAT1-responsive reporter gene activity in human HeLa cells. As shown in III, Fig. 3C, *CNOT4* RNAi causes a significant reduction in *GAS-luc* activity upon

IFN-γ stimulation. We also observed a similar effect on STAT6-responsive *Igε-luc* activity after IL-4 induction (III, Fig. 3D). These results suggest that CNOT4 participates in human JAK/STAT signaling. Of note, a *CNOT4* siRNA treatment had no effect on *PG-13-luc* activity (III, Fig. 3E), or on CMV-promoter driven *β-galactosidase* values used for monitoring transfection efficiency in the experiments, indicating that CNOT4 does not affect transcription in HeLa cells in a global manner.

5.2.7 *Drosophila* Not4 is a Stat92E interacting protein (Article III)

Because of the strong evidence that Not4 affects *Drosophila* JAK/STAT pathway we established a stable *Not4-pMT-V5* overexpression cell line for further studies. In order to study if Not4 interacts with Stat92E, *Not4-V5* overexpressing S2 cells and *pMT-V5-HisA* control S2 cells were lysed, and endogenous Stat92E was immunoprecipitated from the whole cell lysates with an anti-Stat92E antibody. After SDS-PAGE, co-immunoprecipated Not4-V5 was visualized with an anti-V5 antibody. As shown in original communication III, Fig. 5, Not4 does indeed co-immunoprecipitate with Stat92E. Next, we investigated if the JAK/STAT pathway activation affects the Stat92E-Not4 interaction. We transfected *Not4-V5* overexpressing S2 cells with increasing amounts of the JAK/STAT pathway ligand *Upd*, and performed a co-immunoprecipitation assay with the method described earlier. As a result we observed a consistent enhancement in the Stat92E-Not4 interaction with different amounts of transfected *Upd* (III, Fig. 5B).

5.2.8 Not4/CNOT4 does not affect STAT tyrosine phosphorylation (Article III)

In study II, ET was found to regulate the tyrosine phosphorylation of Stat92E. To investigate if Not4 is required for Stat92E activation, we studied how *Not4* RNAi affects the tyrosine phosphorylation status of Stat92E in S2 cells. As a result we did not observe a difference in Upd-induced Stat92E tyrosine phosphorylation between *Not4* dsRNA treated and control *GFP* dsRNA treated cells either with an anti-Stat92E antibody or a phospho-tyrosine-specific antibody (III, Fig. 6A). To validate this result with more

detectable amounts of phosphorylated Stat92E, *Not4-V5* overexpressig cells and control *pMT-V5-HisA* cells were transiently transfected with a Flag-tagged *Stat92E* together or without $Hop^{Tum\text{-}l}$ to induce Stat92E phosphorylation. Cells were lysed and equal amounts of Stat92E were immunoprecipitated from the lysates with an anti-Flag antibody and immunoblotted with an anti-Stat92E specific antibody. $Hop^{Tum\text{-}l}$ overexpression caused a considerable Stat92E band shift that was the same in *Not4-V5* and *pMT-V5-HisA* cell lysates. Furthermore, *Not4-V5* overexpression on its own did not produce a detectable band shift on the Stat92E immunoblot (III, Fig. 6B). In summary, these results suggest that Not4 does not affect Stat92E tyrosine phosphorylation. Thus it affects Stat92E-mediated gene responses through a different mechanism.

CNOT4 was found to affect STAT-mediated gene expression in human cells. To this end, we also wanted to investigate if *CNOT4* RNAi would mitigate STAT1 phosphorylation in HeLa cells. In order to do this, Hela cells were transfected with either *Scrambled* (*Scr*) or *MyD88* siRNA as a negative control or with siRNA targeting *CNOT4*. *Stat1* siRNA was used as a positive control. Phosphorylation of endogenous STAT1 was induced by incubating cells in the presence of hIFN-γ for six hours prior to lysis. As shown in Fig. 8, *CNOT4* RNAi had no significant effect on STAT1 tyrosine 701 phosphorylation when compared to *Scr* or *MyD88* siRNA transfected control cells, indicating that CNOT4 is not indispensable for STAT1 phosphorylation in human cells.

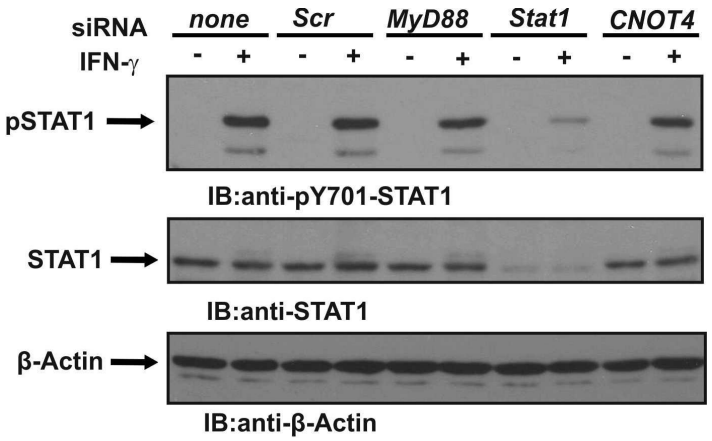

Figure 8. *CNOT4* RNAi does not alter IFN-γ-induced tyrosine 701 phosphorylation of human STAT1.

5.2.9 Not4 is required for efficient Stat92E DNA binding (Article III)

Activated Stat92E translocates to the nucleus and binds to the consensus DNA sequence TTCnnnGAA at the promoters of its target genes (Rivas et al. 2008, Yan et al. 1996a). As discussed above, we did not observe any alterations in Stat92E phosphorylation in either *Not4* dsRNA treated cells or in *Not4-V5* overexpressing cells, suggesting that Not4 functions downstream of Stat92E phosphorylation. Thus, we studied if Not4 affects Stat92E DNA binding in S2 cells using an EMSA assay with probes containing a Stat92E binding site from the *TotM* promoter. A $Hop^{Tum\text{-}l}$ transfection was used to induce Stat92E phosphorylation and *GFP* and *Stat92E* dsRNA transfections were used as controls. Interestingly, *Not4* RNAi almost totally abolished Stat92E binding to the DNA probe upon $Hop^{Tum\text{-}l}$ induction, indicating that Not4 is needed for Stat92E DNA binding (III, Fig. 6C). As a conclusion, Not4 positively regulates the *Drosophila* JAK/STAT pathway by enhancing Stat92E DNA binding.

6. Discussion

6.1 *Drosophila melanogaster* as a model for studying the JAK/STAT signaling cascade

Drosophila melanogaster has made an important contribution to our understanding of the immune system as we know it. A famous example of this is the discovery of the Toll-like receptors (TLRs), which are essential for the recognition of invading pathogens. Originally, *Drosophila* Toll was found to mediate immune signaling and to be essential for defending against fungal infections (Rosetto et al. 1995, Lemaitre et al. 1996). Soon after this, TLRs were reported to participate in mammalian immune signaling via Nf-κB as well (Medzhitov et al. 1997). As for recognizing the importance of *Drosophila* in biomedical research, the *Drosophila* researcher Jules Hoffmann was awarded the Nobel Prize in physiology or medicine in 2011 together with immunologists Bruce Beutler and Ralph Steinmann. The studies that lead to Hoffmann's Nobel Prize concentrated on the very foregoing Toll receptor. *Drosophila* in turn is an exceptional model organism for genetic studies due to its small size, short reproduction cycle and relatively short life (Rämet 2012). Fruit flies are also cheap and easy to rear. Moreover, using *Drosophila* in biomedical research is ethically less problematic than is the use higher organisms such as rodents.

Like the Toll pathway, the JAK/STAT signaling pathway has been highly conserved in evolution. Human JAK/STAT pathways consist of seven STATs and four JAKs, thus making the cascades reduntant and complicated to sudy (Leonard and O'Shea 1998). The corresponding cascade in *Drosophila melanogaster* is much simpler consisting of only one STAT transcription factor, Stat92E, one Janus kinase, Hopscotch (Hop), and a single defined transmembrane recepror, Dome (Binari and Perrimon 1994, Hou et al. 1996, Yan et al. 1996a, Brown et al. 2001, Chen et al. 2002). These molecules seem to share conserved domain architectures with their mammalian counterparts, and the signal

transduction route follows a canonical tyrosine phosphorylation dependent pattern originally described in the mammalian system (Binari and Perrimon 1994, Hou et al. 1996, Yan et al. 1996a, Leonard and O'Shea 1998). In addition to the common core components, also the best defined JAK/STAT pathway regulators are found in *Drosophila*: PIAS, SOCS and protein tyrosine phosphatase, the last of which was found in two independent genome-wide RNAi screens (Betz et al. 2001, Callus and Mathey-Prevot 2002, Karsten et al. 2002, Baeg et al. 2005, Müller et al. 2005). These findings support the idea that the *Drosophila* JAK/STAT pathway is similar to the mammalian cascade, thus the novel JAK/STAT pathway regulators found in *Drosophila* are likely to be involved in the mammalian systems as well. Nevertheless, the results obtained from *Drosophila* studies cannot, of course, be directly extrapolated to hold true for all species, but need to be repeated in other organisms as well.

Two of the original communications (II and III) in this thesis are based on a genome-wide RNAi screen conducted in *Drosophila* S2 cells, which was carried out to identify genes that modulate the activity of the JAK/STAT pathway. The main goal was to identify genes that are also involved in human JAK/STAT signaling, in order to better comprehend the biology of this disease related cascade. Of the novel *Drosophila* JAK/STAT pathway regulators described in this thesis, the *Not4* mammalian homologue *CNOT4* was also shown to be important for human STAT1- and STAT6-mediated gene responses, functioning downstream of STAT phosphorylation. Although, the results from *Drosophila* cells indicated that Not4 is reguired for Stat92E DNA binding, the mechanism in mammalian cells may be different and warrants further studies. The screen described in original communication II revealed that *kayak* RNAi had a positive effect on *TotM* gene expression and a similar result was obtained from a *10xStat92E-luc* reporter assay, indicating that *kayak* is a potential negative regulator of Stat92E-mediated gene expression. To find out if the effect was conserved in humans, a similar experiment with an RNAi against the closest mammalian homolog, *FosB*, was conducted in human HeLa cells, but this yielded an opposite outcome (Fig. 7).

6.2 Sumoylation in the regulation of transcription factor activity

Sumoylation is a post-translational modification with diverse consequences on the fate of target proteins. To date numerous transcription factors have been reported to be regulated through sumoylation, among these the human STAT1 and murine STAT5 transcription factors (Rogers et al. 2003, Ungureanu et al. 2003, Van Nguyen et al. 2012). In both cases sumoylation was found to repress the activity of these transcription factors. Despite intensive studies, in most cases the molecular mechanisms for how SUMO conjugation alters the activity of a transcription factor are yet to be described. In many cases SUMO conjugation is associated with the recruitment of repressor proteins such as HDACs or protein repressor complexes to the sumoylated substrates, like has been reported for p300, Elk-1 and liver-enriched transcription-activating protein-1 (LAP-1) (Girdwood et al. 2003, Yang and Sharrocks 2004, Wang et al. 2008).

In both mammals and in *Drosophila* the activity of the transcription factor Sp3 is strongly inhibited by the SUMO modification (Sapetschnig et al. 2002, Stielow et al. 2008). An RNAi-based genome-wide screen in *Drosophila* Kc_{167} cells revealed that *Drosophila* Sp3 is downregulated by a repressor complex, which is attracted by the Sp3 conjugated SUMO. This complex was found to be composed of MEP-1, Mi-2 and Sfmbt, which were found to interact with each other and SUMO (Stielow et al. 2008). This study shows that like in mammals, sumoylation dependent chromatin-associated transcriptional repression complexes are also present in insects.

In addition to mediating or interrupting transcription factor-protein interactions, SUMO conjugation may also affect other post-translational modifications such as phosphorylation or acetylation, like is the case for murine STAT5 (Van Nguyen et al. 2012). Sumoylation has also been reported to alter the subcellular localization of some transcription factors (Gill 2005).

6.3 SUMO as a regulator of STAT-mediated gene expression

Like its mammalian counterparts STAT1 and STAT5, *Drosophila* Stat92E too was found to be a target for SUMO modification. While the SUMO acceptor lysine 701 in STAT1 is located in the transactivation domain, just two amino acids from the indispensable phosphorylation site, tyrosine 701, the sumoylation consensus sequence in Stat92E falls within the predicted coiled-coil domain region in the N-terminus of the protein. Although SUMO seems to inhibit both of these transcription factors, the molecular mechanism may be completely different. STAT1 sumoylation has been under intensive research from the day it was discovered. Recent studies suggest that sumoylation and tyrosine 701 phosphorylation are mutually exclusive modifications, regulating STAT1 partitioning between active and inactive states (Jakobs et al. 2007, Zimnik et al. 2009, Begitt et al. 2011, Droescher et al. 2011a). Analysis of the crystal structure of DNA bound STAT1 and STAT3 homodimers revealed that the coiled-coil domain consists of four α-helices pointing out from the STAT core structure (Becker et al. 1998, Chen et al. 1998). The coiled-coil domain of mammalian STATs is known to mediate protein interactions and participates in the nuclear export of STATs (Horvath et al. 1996, Begitt et al. 2000, Mowen and David 2000). The function of the Stat92E coiled-coil domain or the Stat92E crystal structure has not been studied, but it is possible that the coiled-coil domain of Stat92E shares similar features with the mammalian STATs and is well exposed to protein-protein interactions. Thus it is likely that conjugated SUMO (called Smt3 in *Drosophila*) may interfere with the molecular interactions of Stat92E and proteins, such as transcriptional co-activators, in the nucleus. Another possibility is that SUMO recruits transcriptional repression complexes to the Stat92E-bound promoter site, like has been reported for the sumoylated *Drosophila* transcription factor Sp3 (Stielow et al. 2008).

The IFN-γ-induced nuclear localization of the sumoylation deficient human STAT1 has been reported to be prolonged compared to wild type STAT1 (Ungureanu et al. 2005). We studied if the sumoylation abolishing K187R mutation in Stat92E affected the localization of Stat92E upon Upd or pervanadate induction, but there was no difference in the cytoplasmic versus nuclear localization of WT Stat92E and Stat92E K187R in these

experiments (data not shown). However, studying the time-dependent localization of a transcription factor is technically challenging due to lack of cytokines whose amount and timing can be controlled when used to activate the *Drosophila* JAK/STAT pathway. Sumoylation has been suggested to increase the solubility of STAT by suppressing its paracrystal assembly in the nuclei of cytokine stimulated cells (Droescher et al. 2011a). Whether the tyrosine phosphorylated Stat92Es also have a tendency to polymerize into nuclear paracrystals has not been studied. The possible role of paracrystallization in Stat92E-mediated signaling and the role of sumoylation in this process are interresting questions for further studies.

The effect of sumoylation on Stat92E DNA binding was not studied. The coiled-coil domain of STATs is not thought to participate directly in promoter binding. The crystal structures of DNA bound STAT1 and STAT3 homodimers support this notion by proposing that coiled-coil domains point out from the complex without being in contact with the DNA (Becker et al. 1998, Chen et al. 1998). Thus it is unlikely that SUMO attachment to the coiled-coil domain would directly interfere with STATs ability to bind DNA. Still, this does not rule out the possibility that attaching SUMO to the coiled-coil domain changes the molecular structure of Stat92E in a way that leads to altered DNA binding of the dimerized Stat92E. Although it is not likely that SUMO conjugation to the N-terminal coiled-coil domain of Stat92E would affect the phosphorylation of Tyr711 or block the phospho-tyrosine-SH2 domain mediated dimerization of activated Stat92Es, these possibilities should also be addressed in future studies.

Targeting *dPias* with RNAi enhanced *TotM-luc* expression, supporting previously published data that dPias negatively regulates Stat92E (Betz et al. 2001). RNAi against *Stat92E 3'-UTR*, which was used as a positive control in the experiment, caused a significant decrease in $Hop^{Tum\text{-}l}$-induced *TotM-luc* expression. Interestingly, transfection of *Stat92E 3'-UTR* dsRNA into the S2 cells increased the basal level of *TotM-luc* expression (I, Fig. 3C). It has been suggested that *Turandot* stress genes are not entirely under the control of the JAK/STAT pathway, but are cooperatively regulated by the JAK/STAT and Imd pathways together with the MAPK kinase kinase (MAPKKK)

Mekk1 (Brun et al. 2006). Most likely, the expression of *TotM-luc* is induced through these additional pathways in response to the *Stat92E 3'-UTR* dsRNA transfection. Although the Pias proteins have been shown to induce the sumoylation of human STAT1 (Rogers et al. 2003, Ungureanu et al. 2003), we did not observe a difference in the sumoylation of Stat92E in *dPias* dsRNA and *GFP* control dsRNA treated S2 cells. Although this data indicates that *dPias* is not indispensable for SUMO conjugation to Stat92E, further *dPias* overexpression studies would clarify if dPias can promote Stat92E sumoylation.

In original communication I, Stat92E was shown to be sumoylated on Lys187 and a sumoylation deficient K187R mutant showed increased transcriptional activity compared to wild type Stat92E on two independent Stat92E responsive promoters. This difference was only detected when the JAK/STAT pathway was activated with *Upd* overexpression. In summary, these data suggest that activated Stat92E is inhibited by sumoylation, but the molecular mechanism remains enigmatic.

6.4 Genome-wide screens to identify genes that regulate the activity of the JAK/STAT pathway

We characterized two novel components of the *Drosophila* JAK/STAT pathway, ET and Not4. ET was shown to inhibit Upd-induced Stat92E tyrosine phosphorylation causing repressed target gene expression and Not4 was suggested to enhance Stat92E DNA binding to the *TotM* promoter. Furthermore, the mammalian homologue of Not4, CNOT4 was shown to be involved in STAT1- and STAT6-mediated reporter gene expression in human cells. Both genes were found in an RNAi-based genome-wide screen carried out in *Drosophila* S2 cells. Two similar RNAi based screens for novel JAK/STAT pathway associated factors have been published earlier (Baeg et al. 2005, Müller et al. 2005). Although the basic approach in all of the screens was similar, including the use of a *luciferase*-reporter assay to monitor pathway activity, there was a huge divergence in the findings. Only *enok* was found in all of the three screens. The differences may be caused

by several variables. The cell lines used in the screens were different: we used S2 cells, while Baeg *et al.* used a derivative of S2 cells, a cell line called S2-NP and Müller *et al*. used Kc_{167} cells in their screen. Also the assay design varied between the screens. The *luciferase* reporter was different in all of the screens: while we used the *TotM-luc* reporter, Baeg *et al*. had a *10xStat92E-luc* construct, and Müller *et al.* monitored the activity of the *6x2xDraf-luc*, a reporter construct that contains six repeats of a 165 bp fragment with two Stat92E binding sites from the promoter of the Stat92E target gene *raf*. One significant difference between the screens was the mechanism of pathway activation. S2-NP cells used by Baeg *et al*. express sufficient levels of endogenous Upd2 to activate the JAK/STAT pathway, while Müller *et al.* stimulated the pathway by overexpressing Upd, and we activated the pathway through the forced expression of a constitutively active $Hop^{Tum\text{-}l}$. There was also variation in induction and RNAi times between the conducted screens: the S2-NP cells in the screen of Baeg *et al.* were lysed 96 hours posttransfection, Müller *et al.* used incubation time of 5 days (120 hours), while in our screen reporter activities were measured 72 hours after transfections. In addition, different normalization approaches may add divergence to the outcome (Baeg et al. 2005, Müller et al. 2005, Müller et al. 2008).

CG14225/ET RNAi was first found to decrease *TotM-luc* activity upon $Hop^{Tum\text{-}l}$ induction, but interestingly, the opposite effect was observed in a more physiological context when the pathway was activated with the Dome ligand Upd. Our further biochemical studies revealed that ET downregulates Upd-induced Stat92E phosphorylation, thus functioning as a negative regulator of the *Drosophila* JAK/STAT pathway. Soon after we had published our article, another research group demonstrated that *ET* (called *Latran* in their study) controls the JAK/STAT pathway-mediated cellular immune response in the larval lymph gland (Makki et al. 2010). They too showed that ET/Latran is a class I cytokine receptor-related protein, which is able to form heteromers with Dome and can inhibit downstream Stat92E activation. Their study confirms the importance of ET in the regulation of *Drosophila* immune signaling.

The molecular mechanism for how ET regulates Stat92E phosphorylation still remains to be explained, but a few posibilities can be envisioned. ET is a transmembrane protein with a putative cytokine binding module in its extracellular domain. Therefore, one possibility would be that ET functions as a decoy receptor on the cell surface, inhibiting Upd binding to its normal receptor Dome. Evidence that short receptor variants may act as dominant negative receptors has also been reported for the mammalian system, like in the case of porcine prolactin signaling (Trott et al. 2011). According to our data, ET does not affect Dome homodimerization or interfere with interaction between Hop and Dome, instead ET was found to co-immunoprecipitate with both Hop and Dome. Thus it is possible that ET forms complexes with Hop and Dome at the plasmamembrane. Through its interactions with Dome, ET may directly disrupt Upd binding to Dome, or cause conformational changes in Dome upon Upd binding leading to disturbances in Hop activation.

STAT activation is mediated through tyrosine phosphorylation following dimerization and translocation to the nucleus where STAT dimers bind DNA in order to activate the transcription of STAT responsive genes (Schindler 1999). STATs are not able to initiate transcription on their own. Instead, interactions with other co-activators and regulatory proteins are needed. This cooperation then leads to the recruitment and activation of the RNA polymerase and transcriptional apparatus. STAT coregulators have been mainly studied in mammalian systems, but similar factors are also likely to be present, at least to some extent, in *Drosophila* cells.

Not4 was shown to be important for JAK/STAT pathway mediated signaling. According to our co-immunoprecipitation experiments, Not4 interacts with Stat92E and *Upd* overexpression enhances this interaction. Interestingly, Upd-induction also increased the total amount of Not4 protein expressed by a methallothionein promoter. Of note, Stat92E RNAi decreased Not4 protein levels in *pMT-Not4-V5* overexpressing cells (data not shown). Therefore it is possible that Upd-induction stabilizes the Not4 protein Stat92E-dependently, rather than enhances the expression of Not4. Furthermore, we found that Not4 was essential for optimal Stat92E DNA binding, but it did not influence the

phosphorylation status of Stat92E. The exact molecular mechanism for how Not4 affects Stat92E DNA binding or expression of Stat92E responsive genes still remains elusive, but it is possible that Not4 is a component of the Stat92E enhanceosome complex.

Drosophila Not4, like its human homologue CNOT4, associates with a multifunctional Ccr4-Not complex, but is not a stable component of the complex (Lau et al. 2009, Temme et al. 2010). The Ccr4-Not complex, which has been mainly studied in yeast, participates in many cellular events including DNA repair, histone methylation, transcription elongation and initiation as well as mRNA degredation through poly-A tail deadenylation (Bianchin et al. 2005, Mulder et al. 2005, Laribee et al. 2007, Mulder et al. 2007). Furthermore, the N-terminal RING domain of CNOT4 has been reported to act as a Ubiquitin E3 ligase, and is essential for the regulation of histone methylation through polyubiquitintion of the demethylase Jhd2 (Laribee et al. 2007, Mersman et al. 2009). Our results indicated that the RING domain of *Drosophila* Not4 is not needed for JAK/STAT signaling, whereas the C-terminal part of Not4 was found indispensable (III, Fig. 4). The C-terminal part of Not4 does not contain any known functional domains, thus further studies are needed in order to reveal its mechanistic role in the regulation of Stat92E-mediated signaling. Whether Not4/CNOT4 participates in the regulation of the JAK/STAT pathway regulation on its own or as a part of the Ccr4-Not complex, also remains to be investigated.

This study revealed three novel mechanisms for regulating the *Drosophila* JAK/STAT signaling cascade: sumoylation of Stat92E, ET, which negatively regulates Stat92E phosphorylation and a positive regulator Not4, which is needed for proper Stat92E DNA binding. The mammalian homologue of Not4, CNOT4, was also found to participate in JAK/STAT signaling in human cells. This demonstrates that mechanisms found in *Drosophila* can be conserved in mammalian cells. The main findings of this thesis are recapitulated in Figure 9, wherein they are referred to their corresponding original communications with the Roman numerals.

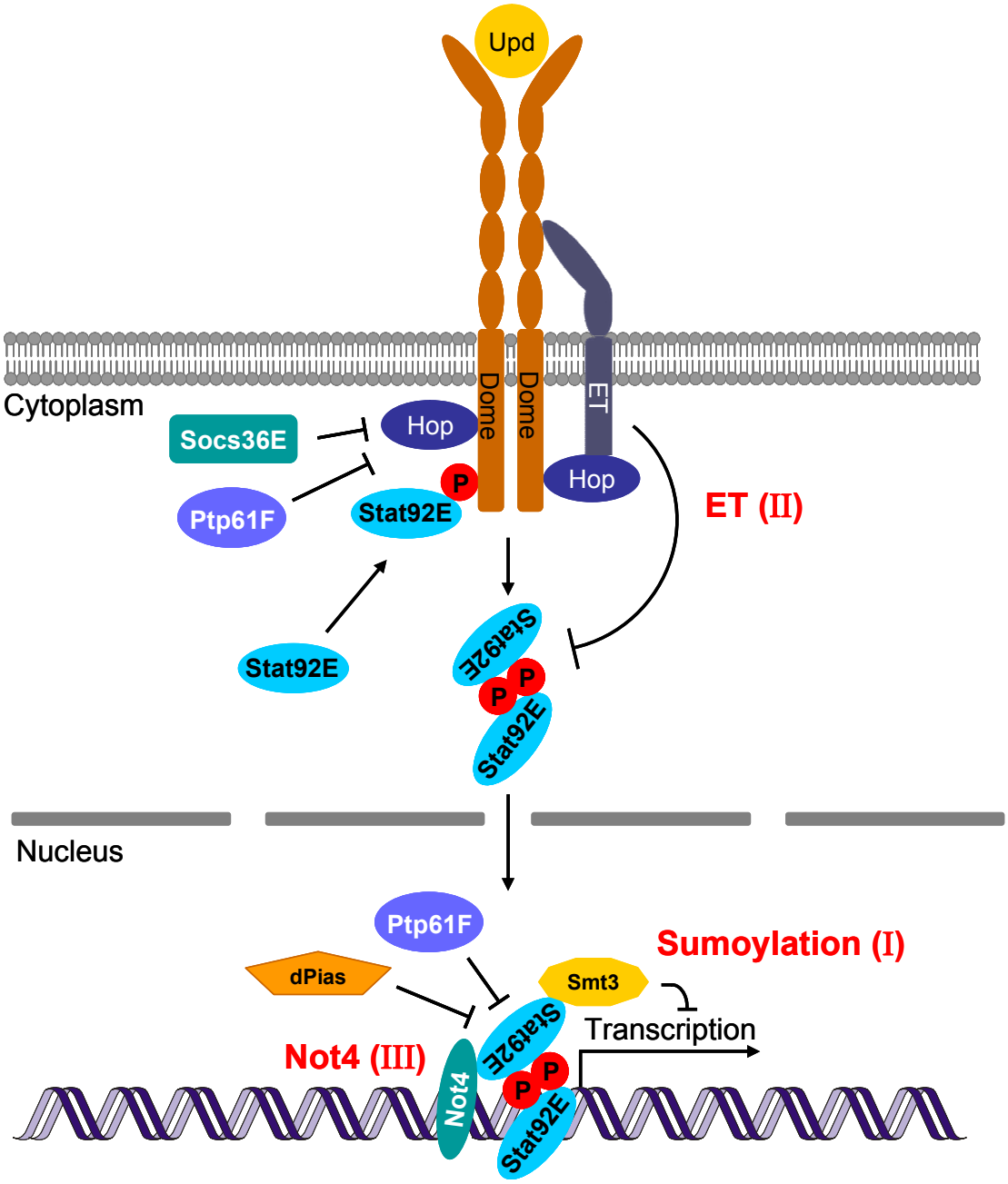

Figure 9. The main findings of this thesis (I-III) and the regulators of *Drosophila* JAK/STAT signaling. Sumoylation of Stat92E was found to inhibit JAK/STAT pathway target gene transcription (I). ET negatively regulates Upd-induced Stat92E tyrosine phosphorylation (II). Not4 interacts with Stat92E and is required for proper Stat92E DNA binding (III).

7. Conclusions

Our immune system constantly protects us from microbial challenge. In order to function properly it relies on a sophisticated signaling system, in which soluble signaling molecules, cytokines, play a crucial role. The JAK/STAT pathway transduces signals of numerous cytokines and growth factors essential for various cellular functions. To maintain cellular homeostasis, the activity of the JAK/STAT signaling pathway needs to be under strict control. During the past decade, a growing number of studies have confirmed that the JAK/STAT signaling cascade in *Drosophila* is regulated through mechanisms similar to the mammalian system. This thesis was aimed at exploring novel regulatory mechanism of the JAK/STAT pathway using *Drosophila melanogaster* as a model organism.

Like its mammalian counterparts STAT1 and STAT5, *Drosophila* Stat92E was found to be a target for SUMO conjugation (original communication I). Further studies revealed that sumoylation inhibits Upd-induced Stat92E target gene expression. Protein post-translational modifications, such as phosphorylation, ubiquitination and other ubiquitin-like modifications, like sumoylation, play a major role in the regulation of the intracellular signal transduction cascades of the immune system. These modifications can have diverse effects on their targets. The same modification to a target protein may have completely different consequences for target protein behaviour, depending on the position of the acceptor amino acid in the target protein. Like in case of JAKs, tyrosine phosphorylation is known to be essential for the regulation of kinase activity. This regulation may be either positive or negative, depending on the phosphorylation site within the protein. Sumoylation and ubiquitination are closely related post-translational modifications, and both are associated with the downregulation of the JAK/STAT pathway (Ungureanu et al. 2005, Ungureanu and Silvennoinen 2005). In some cases the effects of these modifications may be completely opposite. Ubiquitination plays an indispensable role in the activation of the Nf-κB cascades. In the canonical model for the Nf-κB pathway, the activation of Nf-κB transcription factors requires the ubiquitin-

proteasome system-mediated degradation of inhibitor of kappaB protein (IκB). Ubiquitination of IκB is preceded by IκB kinase (IKK) complex-mediated serine phosphorylation of the IκB, and the activation of this protein complex is dependent on the K68-linked polyubiquitination of its regulatory subunit NEMO (Liu and Chen 2010). Both SUMO and Ubiquitin are covalently linked to specific lysine residues within their target proteins. This enables SUMO and Ubiquitin to, in some cases, compete for the same binding sites, thus having antagonistic effects on the target protein. The interplay of post-translational modifications has a major role in the regulation of human STAT1 activation, as the sumoylation of Lys703 and the phosphorylation of Tyr701 have been suggested to be mutually exclusive modifications. In addition, Ser727 phosphorylation has a role in both the sumoylation and transcriptional activation of STAT1. Although the molecular mechanism for how sumoylation inhibits Stat92E-mediated signaling remains unknown, the *Drosophila* system offers an excellent model for future studies on the physiology of sumoylation in the regulation of the JAK/STAT pathway.

ET and Not4 were characterized as novel JAK/STAT pathway regulators from a genome-wide RNAi screen in *Drosophila* S2 cells. We found that ET is a transmembrane protein that negatively regulates Upd-induced Stat92E tyrosine phosphorylation. This finding opens up a completely new chapter in the regulation of JAK/STAT signaling. The experiments described in original communication II were conducted only in *Drosophila*, raising the question if a similar regulatory mechanism is also present in mammalian signaling systems. In addition to the negative regulators studied in this thesis, we studied Not4, which was found to function as a positive regulator of JAK/STAT-mediated gene expression in *Drosophila* as well as in mammalian cell line (original communication III). The RING domain of Not4 was found dispensable for Stat92E target gene expression. This indicates that the function of Not4 in JAK/STAT signaling is not, at least directly, based on Ubiquitin transfer. As Not4 was found to interact with Stat92E, it is possible that it mediates interactions between Stat92E and other proteins such as transcriptional coregulators or RNA polymerase II, which has been reported to interact with the Ccr4-Not complex in yeast. Although our experimental data suggest that Not4 regulates

Stat92E DNA binding, the exact molecular mechanism for how Not4 is utilized for STAT-mediated signaling requires further studies.

This study has added two complitely novel components, ET and Not4, to the *Drosophila* JAK/STAT pathway, revealing the complexity of this signaling cascade. The discovery that SUMO has an evolutionary conserved role in the regulation of the JAK/STAT pathway verifies the importance of this post-translational modification in cytokine signaling. The findings of this thesis further demonstrate that *Drosophila* can be utilized as a model organism to study complex signaling cascades. The present study provides new aspects for further studies for solving the puzzles of cytokine signaling in immunology.

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Sumoylation of *Drosophila* Transcription Factor STAT92E

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Key Words
Signal transducer and activator of transcription · STAT · Small ubiquitin-like modifier · Sumo · Sumoylation · Post-translational modification

Abstract
STAT92E is an essential transcription factor in *Drosophila melanogaster* for the development of several organs and the immune system. The JAK/STAT pathway employs different evolutionary conserved regulatory mechanisms to control biological processes. Numerous transcription factors in both mammals and invertebrates have been shown to be either activated or inhibited by a covalent modification with a small ubiquitin-like modifier (Sumo). Here, we show that *Drosophila* STAT92E is modified by Sumo at a single lysine residue 187 in S2 cells. Mutation of Lys187 increases the transcriptional activity of STAT92E, thus suggesting that sumoylation of STAT92E has a repressive role in the regulation of the JAK/STAT pathway in *Drosophila melanogaster*.

Introduction

The JAK/STAT pathway is highly conserved in the evolution from invertebrates to humans. The JAK/STAT signaling cascade is essential for several biological processes, including the control of hematopoiesis and immune responses, as well as for cellular homeostasis and embryonic development [1, 2]. The human JAK/STAT pathway consists of 7 STATs and 4 JAKs [3]. In *Drosophila melanogaster*, the pathway employs only a single Janus kinase Hopscotch (Hop) and the transcription factor STAT92E as well as the receptor Domeless (Dome) [2], thus making *Drosophila* a useful non-redundant model to study this pathway.

The activation mechanism of the JAK/STAT pathway is shared by invertebrates and humans. In *Drosophila*, 3 secreted ligands (Unpaired 1–3, Upd1–3) [4–6] induce the homodimerization of the Dome receptors allowing the Hop kinases to phosphorylate tyrosine residues in the receptors, thus creating docking sites for the SH2 domain of STAT92E. Following the interaction with Dome, STAT92E becomes phosphorylated by Hop, leading to the homodimerization and nuclear translocation of STAT92E. In the nucleus, STAT92E binds to its target DNA sequences and acts as activator of transcription for

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several target genes such as *Tep 2* and *Turandot* stress genes [1, 7].

Cellular homeostasis requires tight regulation of the JAK/STAT signaling cascade, and deregulation or disturbances of the pathway are associated with or cause several human diseases such as myeloproliferative diseases, cancer, immune deficiencies, autoimmunity and allergy [8–10]. In *Drosophila,* deregulation of the JAK/STAT pathway causes overproliferation of plasmatocytes, tumor formation and developmental abnormalities [2, 11]. The basic regulatory mechanisms of the JAK/STAT pathway appear to be well conserved between *Drosophila* and mammals. Previously known negative regulators of the *Drosophila* JAK/STAT pathway include a family of SOCS-like *Drosophila* genes, dPIAS, also known as Zimp or Su(var)2–10, Ptp61F (protein-tyrosine phosphatase 61F) and an N-terminally truncated form of STAT92E (ΔNSTAT92E) [12–15]. Transcriptional activity of STAT92E is inhibited by direct interaction with dPIAS, and the correct dPIAS/STAT92E ratio is crucial for normal hematopoiesis and organogenesis in *Drosophila* [13]. PIAS1 was initially shown to interfere with the promoter-binding activity of tyrosine-phosphorylated STAT1 in mammals [16]. PIAS1 functions also as an E3 ligase for small ubiquitin-like modifier (Sumo), and STAT1 was identified as a substrate for PIAS1-induced sumoylation. Sumoylation of STAT1 has an inhibitory effect on STAT1-mediated transcriptional activation [17, 18].

Several transcription factors in mammals as well as in *Drosophila* are regulated by sumoylation. Sumoylation is a reversible post-translational modification in which a Sumo moiety is covalently conjugated to the lysine residues on target proteins through a process analogous to ubiquitination. The sumoylation pathway is conserved in all eukaryotic organisms and consists of E1-activating enzymes, E2-conjugating enzymes and E3 ligases as well as of the family of the Sumo cleaving proteases [19, 20]. The Sumo conjugation pathway is less redundant in invertebrates; for example, in vertebrates, 4 Sumo variants (Sumo 1–4) are present, while *Drosophila* expresses only 1 Sumo ortholog known as Smt3 [19]. Sumoylation can affect target proteins by multiple mechanisms, altering their subcellular localization, molecular interactions, stability or the enzymatic activity of the proteins [20]. At the transcriptional regulation level, sumoylation is mostly associated with the repression of gene expression. The mechanism of Sumo-mediated transcriptional inhibition is often associated with the recruitment of various chromatin-modifying or -associated proteins or protein complexes [21]. For example, the transcriptional activity of *Drosophila* Sp3 is inhibited by sumoylation-mediated recruitment of a repression complex formed by MEP-1, Mi-2 and Sfmbt [22]. This study aims to investigate whether sumoylation is involved in the regulation of the JAK/STAT pathway in *Drosophila*.

Methods

Antibodies and Reagents

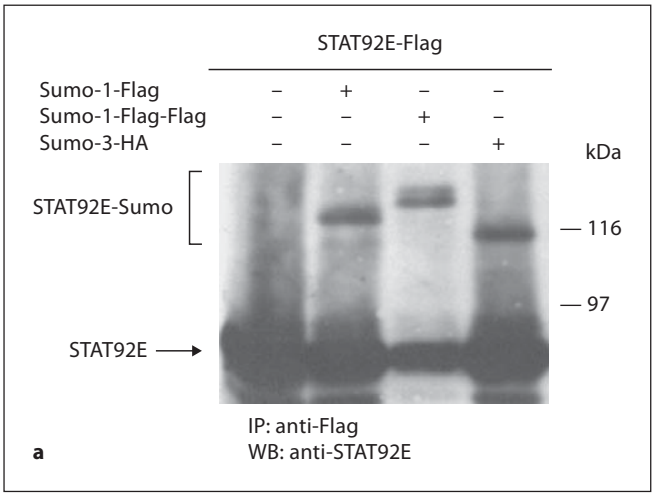
The following antibodies were used: anti-Flag M2 (Sigma-Aldrich); anti-influenza hemaglutinin (HA) epitope antibody (Covance); anti-*Drosophila* STAT92E-N-terminus (dN-17; Santa Cruz); biotinylated anti-mouse (DakoCytomation, Glostrup, Denmark) and biotinylated anti-goat (DakoCytomation). Also, streptavidin-biotin horseradish peroxidase conjugate (GE-Healthcare), protein G sepharose (GE-Healthcare) and N-ethylmaleimide (Sigma-Aldrich) were used.

Plasmid Constructs

STAT92E (clone RE14194) was obtained from the Drosophila Genomics Resource Center (Indiana University, Bloomington, Ind., USA) and amplified by PCR using 5′-primer containing Not1 site and 3′-primer containing Flag epitope tag and Pme1 restriction site. *STAT92E* was cloned into a pMT/V5-HisA expression vector (Invitrogen). PCR-amplified C-terminally Flag-tagged *STAT92E* was also cloned into Not1 and Kpn1 sites of the pcDNA3.1(–) expression vector. The pMT-STAT92E K187R-Flag was created by PCR mutagenesis with the following primers: 5′-GGTATGGTCACACCCAGGGTGGAHCTGTACGAG-3′ and 5′-CTCGTACAGCTCCACCCTGGGTGTGACCATACC-3′. Sumo-1-Flag and Sumo-1-Flag-Flag were a kind gift from Dr. H. Yasuda. Senp1-Flag, Senp1 C603S, Sumo-1 GA-His and Sumo-1-His as well as Sumo-3-HA were provided by Prof. J. Palvimo, *Turandot M-luciferase (TotM-luc), Actin 5C-β-galactosidase* reporters and Hop-Tum-l were provided by Prof. J.-L. Imler. Smt3-HA plasmid was a kind gift from Dr. A.J. Courey. *10xSTAT-luciferase* reporter plasmid was a kind gift from Dr. E. Bach, and pMT-Upd plasmid was kindly provided by Dr. Michael Boutros.

Cell Culture, Transfection and Immunodetection

Cos-7 cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin and 50 mg/ml streptomycin. S2 cells were cultured at 25°C in Schneider medium (Sigma-Aldrich) supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin and 50 mg/ml streptomycin. Cos-7 cells (5×10^6) were transfected by electroporation with a total amount of 3.0 μg plasmid DNA using Bio-Rad gene-pulser at 260 V and 960 μF. Cells were lysed in 1% Triton X lysis buffer containing 5 mM N-ethylmaleimide [17]. One milligram of protein from total cell lysates was subjected to immunoprecipitation, SDS-PAGE electrophoresis and Western blotting as previously described [17]. Approximately 1.5×10^7 S2 cells were transfected with a total amount of 10 μg DNA using polyethyleneimine [23]. Two hours after transfection, the medium was changed to a fresh medium containing 10 μg of *STAT92E 3′-UTR* dsRNA to inhibit the expression of endogenous STAT92E, and



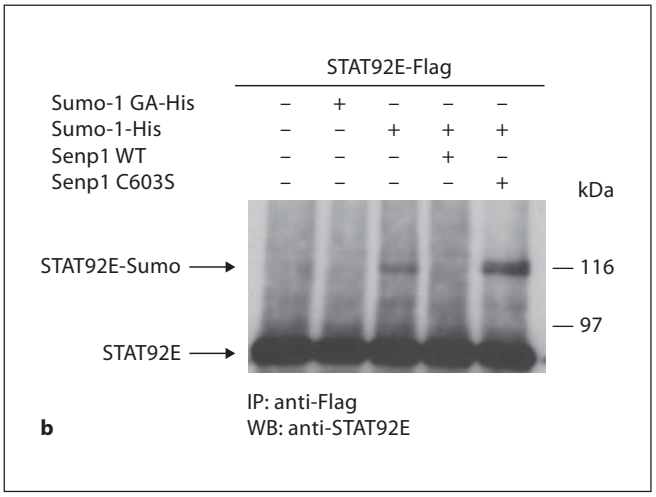


Fig. 1. STAT92E is sumoylated in Cos-7 cells. **a** Sumoylation of STAT92E in Cos-7 cells by human Sumo-1 and human Sumo-3. Cos-7 cells were transiently transfected with STAT92E-Flag with or without different Sumo constructs as indicated. Cells were lysed 48 h after transfection. Equal amounts of lysates were immunoprecipitated by using anti-Flag antibody, followed by SDS-PAGE and immunoblotting with anti-STAT92E antibody. **b** Senp1 desumoylates STAT92E. Cos-7 cells were transfected with STAT92E-Flag together with Sumo-1-His or Sumo-1 GA-His as indicated and with Senp1 or with catalytically inactive Senp1 C603S. Cell lysis, immunoprecipitations and immunoblotting were carried out as described above. IP = Immunoprecipitation; WB = Western blot.

500 μM $CuSO_4$ to induce the expression of the pMT vector. The cells were lysed after 72 h and immunoprecipitations were carried out as described [17].

RNA Interference

For RNA interference (RNAi) assay with endogenous STAT92E, double-stranded DNA was synthesized from the cDNA of the 3′-UTR of *STAT92E* using 2-step PCR with nested primers, the second primers containing a T7 promoter sequence at the 5′ end. *STAT92E 3′-UTR* dsRNA was synthesized from the product of the second PCR with the T7 MegaScript RNA polymerase (Ambion), according to the manufacturer's instructions. dPIAS and GFP dsRNAs were created as above, but the PCR primers were designed in the middle of the protein coding sequence of the genes. dsRNAs were added to S2 culture medium 72 h prior to cell lysis.

Luciferase Assay

S2 cells (1.0×10^6) were transfected with 0.1 μg *TotM-luc* reporter plasmid or with 0.001 μg *10xSTAT-luciferase* reporter plasmid simultaneously with 0.1 μg *Actin 5C-β-galactosidase,* with or without 0.1 μg pMT-Upd and with either 0.2 μg empty pMT vector, pMT-STAT92E or pMT-STAT92E K187R using Fugene 6 transfection reagent, according to the manufacturer's instructions (Roche). $CuSO_4$ (500 μM) was added to the medium 24 h after transfection to induce the expression of the pMT vector. Cells were lysed after 72 h with reporter lysis buffer (Promega), according to the manufacturer's instructions. The luciferase values were normalized against β-galactosidase values.

Results

STAT92E Is Modified by Sumo in Mammalian Cells

In order to investigate if *Drosophila* STAT92E is a target for sumoylation, the initial experiments were performed in mammalian Cos-7 cells with well-defined mammalian components of the sumoylation pathway. Cells were transfected with Flag-tagged STAT92E alone or together with Flag-tagged human Sumo-1, or tandem Flag-tagged human Sumo-1, or HA-tagged human Sumo-3. Cell lysates were immunoprecipitated with anti-Flag antibody, and anti-*Drosophila* STAT92E-N-terminal antibody was used in immunoblotting. Anti-STAT92E antibody detected protein bands corresponding to the molecular mass of STAT92E (fig. 1a). In addition, slower migrating bands were detected when STAT92E was expressed together with Sumo-1 or Sumo-3, indicating that these bands are STAT92E-Sumo-1/3 conjugation complexes. The band shift of the slower migrating band with tandem Flag-tagged Sumo-1 compared with single Flag-tagged Sumo-1 corresponds to the size of a Flag-tag, showing that the upper bands are STAT92E-Sumo-1 bands. The STAT92E-Sumo-1/3 complex is a branched chain molecule, and thus, its migration in the SDS-PAGE gel is abnormal, creating bands of approximately 116 kDa.

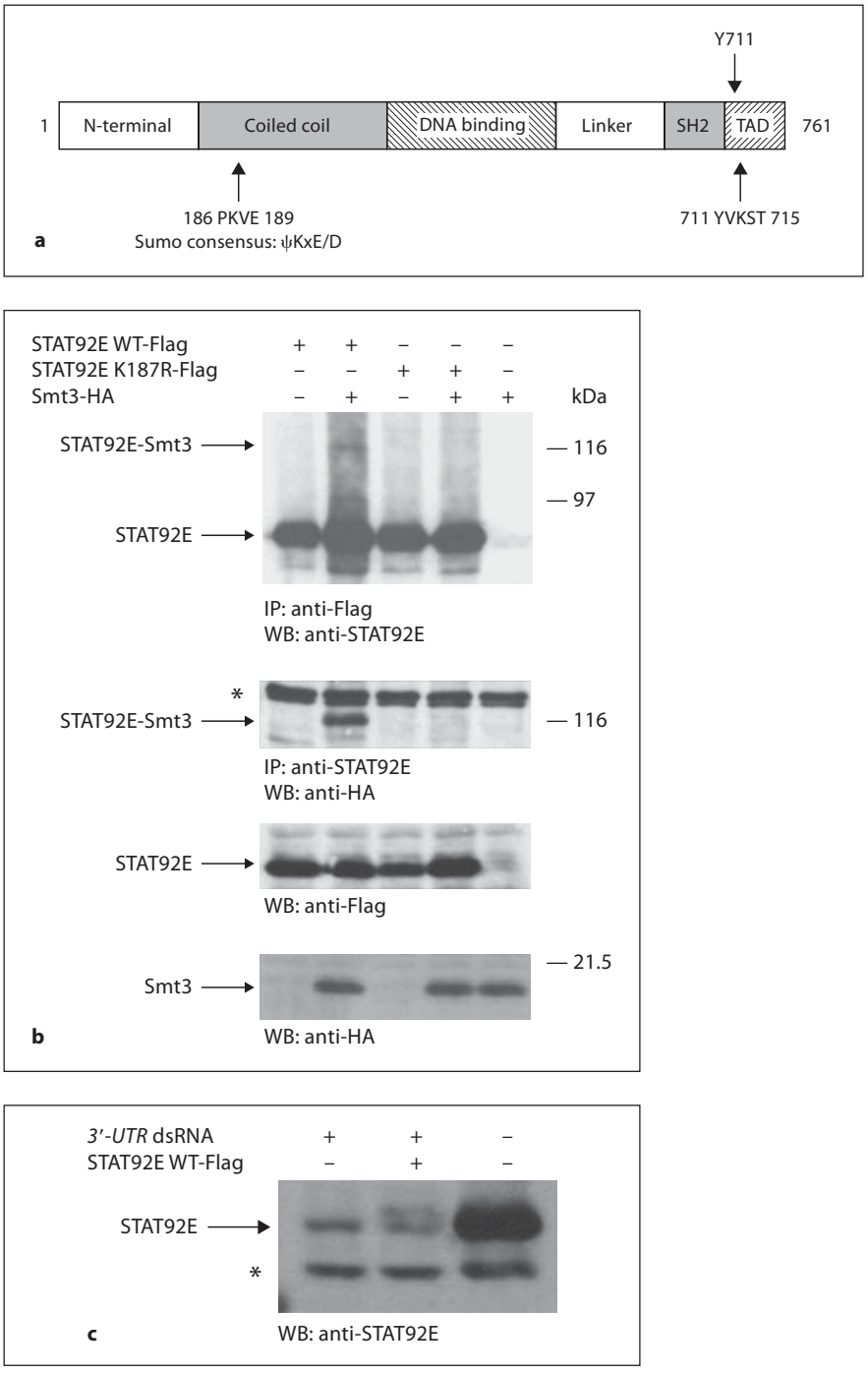


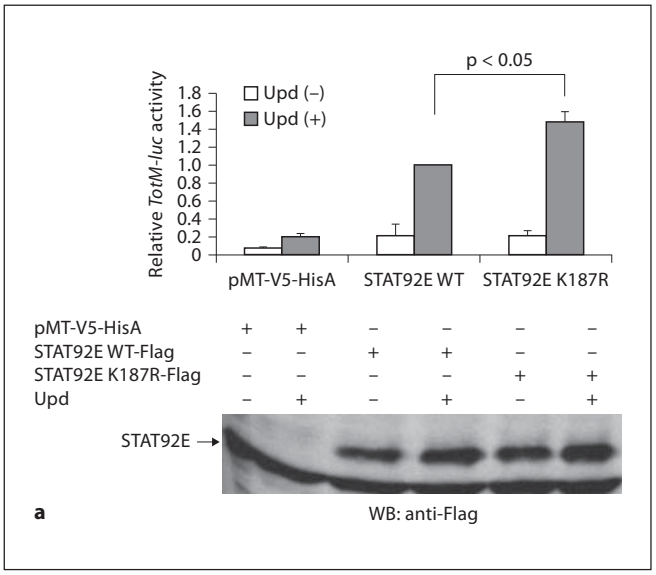
Fig. 2. STAT92E is sumoylated at lys187. **a** Schematic domain structure of STAT92E based on sequence comparison with mammalian STATs. Amino acid sequence of the Sumo consensus site in the coiled coil domain is indicated. The Sumo consensus sequence is not complete in the C-terminal site, although it resembles the human STAT1 sumoylation site (Tyr711 is the critical phosphorylation site in the activated STAT92E). **b** The K187R mutation abrogates sumoylation of STAT92E. S2 were transiently transfected with STAT92E WT-Flag or STAT92E K187R-Flag with or without Smt3-HA as indicated. Two hours after transfection, 10 μg of *STAT92E 3′-UTR* dsRNA was added to the medium to inhibit the expression of the endogenous STAT92E. Equal amounts of lysates were immunoprecipitated by using anti-Flag or anti-STAT92E-N-terminal antibodies following SDS-PAGE and immunoblotting with anti-STAT92E-N-terminal and anti-HA antibodies. **c** Effect of RNA interference on endogenous STAT92E levels in S2 cells by double-stranded RNA targeted on *STAT92E 3′-UTR*. dsRNA was added to cells 2 h after DNA transfection. Unspecific bands are marked with an asterisk. IP = Immunoprecipitation; WB = Western blot.

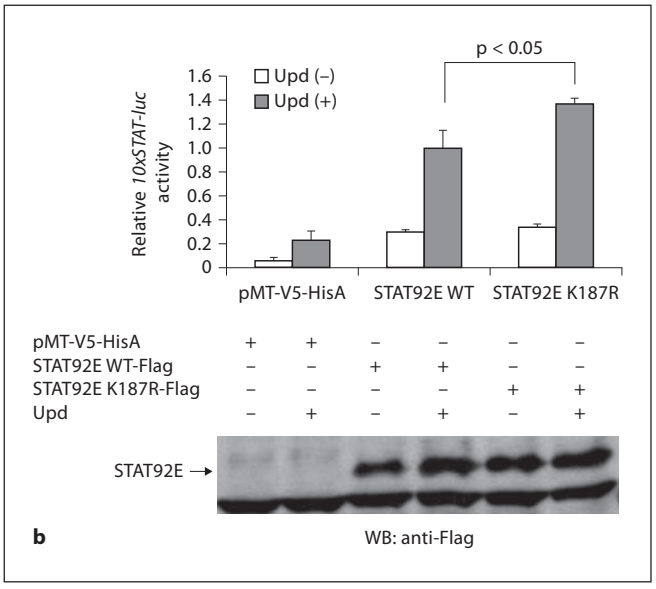
To obtain further proof that the slower migrating STAT92E proteins are sumoylated, we investigated if the Sumo-specific protease Senp1 could remove Sumo molecules from STAT92E and subsequently eliminate the slower migrating bands. Cos-7 cells were transfected with STAT92E and His-tagged Sumo-1 constructs with or without Senp1 expression constructs. A catalytically inactive mutant Senp1-C603S and a conjugation-deficient mutant of Sumo-1 (Sumo-1 GA-His) were used as controls [24]. As shown in figure 1b, expression of Senp1

abolished the protein band corresponding to Sumo-1 conjugation of STAT92E, while the expression of Senp1-C603S slightly increased the amount of sumoylated STAT92E. The Sumo-1 GA-His mutant was not attached to STAT92E, thus confirming the specificity of the conjugation reaction. Sumo-1-His and Sumo-1 GA-His expression levels were detected (data not shown). These results suggest that *Drosophila* STAT92E can be sumoylated in cells.

STAT92E Is Sumoylated in Drosophila S2 Cells on Lysine 187

Next, we wanted to study if STAT92E is sumoylated also in a more physiological environment and *Drosophila* S2 cells were used. Sumo is conjugated to a lysine residue in target proteins most often within a consensus sequence site, ΨKxE/D, where Ψ is a large hydrophobic amino acid [20]. The protein sequence analysis of STAT92E displays a single putative sumoylation site between amino acids 186–189 (PKVE) (fig. 2a). To investigate if this is a Sumo-binding site in STAT92E, Lys187 was mutated to arginine. Flag-tagged STAT92E WT and Flag-tagged STAT92E K187R were transfected into *Drosophila* S2 cells with or without HA-tagged Smt3. The expression of the endogenous STAT92E protein was inhibited using RNAi with *STAT92E 3′-UTR* dsRNA (fig. 2c). S2 cell lysates were im-





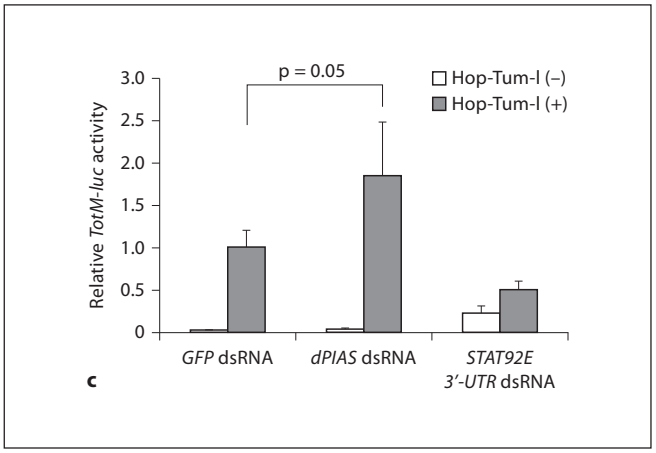


Fig. 3. K187R mutation increases STAT92E transcriptional activity. **a** S2 cells were transiently transfected with the pMT-V5-HisA vector, STAT92E WT-Flag or STAT92E K187R-Flag together with *TotM-luc* reporter and *Actin 5C-β-galactosidase* with or without Upd to activate the JAK/STAT pathway. Twenty-four hours after transfection, 500 μM $CuSO_4$ was added to the medium to induce expression of STAT92E and Upd from the pMT vectors. Seventy-two hours after transfection, the cells were lysed with reporter lysis buffer and luciferase activities were measured and normalized against β-galactosidase activities. The relative *TotM-luc* activity of Upd-induced STAT92E WT was set as one. Equal amounts of lysates were subjected to SDS-PAGE following immunoblotting with anti-Flag antibody in order to detect the expression of transfected STAT92E WT and STAT92E K187R. **b** K187R mutation increases STAT92E transcriptional activity on *10xSTAT-luc* reporter. Transfections and lysis were carried out as in **a**. The relative *10xSTAT-luc* activity of Upd-induced STAT92E WT was set as one. Immunoblotts for cell lysates were carried out as described above. **c** dPIAS RNAi increases *TotM-luc* activity. S2 cells were transiently transfected with *TotM-luc* reporter and *Actin 5C-β-galactosidase* together with indicated dsRNAs and with or without constitutively active Hopscotch (Hop-Tum-l) to activate the JAK/STAT pathway. Cells were lysed 72 h after transfection. Error bars indicate SEM, calculated from 3 independent transfections. WB = Western blot.

munoprecipitated with anti-Flag and anti-STAT92E antibodies followed by Western blotting with anti-STAT92E and anti-HA antibodies. As shown in figure 2b, the K187R mutation abrogated the modification by Smt3, indicating that Lys187 is the only sumoylation site in STAT92E.

K187R Mutation Increases STAT92E Transcriptional Activity

Sumoylation is known to regulate the activity of several transcription factors, including human STAT1. Next, we wanted to study if sumoylation affects the transcriptional activity of STAT92E. The transcriptional activities of STAT92E WT and the sumoylation-deficient STAT92E K187R were analyzed in S2 cells using a reporter gene driven by the STAT92E-inducible stress gene *Turandot M* promoter. Expression of the Dome ligand Upd resulted in a 3-fold induction of transcription with endogenous JAK/STAT proteins. In the absence of Upd, the expression of both STAT92E WT and K187R induced transcription at similar levels, but upon Upd stimulation, the sumoylation-deficient STAT92E mutant K187R showed a significantly higher level of transcriptional activity (fig. 3a). Equal amounts of lysates were subjected to SDS-PAGE following immunoblotting with anti-Flag antibody to show that STAT92E WT and STAT92E K187R were expressed equally (fig. 3a, lower panel). To verify this observation, we used a reporter gene driven by a different STAT92E responsive element from the *SOCS36E* enhancer region. Figure 3b shows that the STAT92E K187R mutant had an increased transcriptional activity compared with STAT92E WT after Upd stimulation with the *10xSTAT92E-luc* reporter gene as well. Taken together, these results suggest that sumoylation has an inhibitory effect on STAT92E-induced gene expression.

Discussion

Sumoylation is a common posttranslational modification for transcription factors, which in most cases has an inhibitory effect on transcription. In this study, we demonstrate that STAT92E is subject to Sumo modification and that sumoylation appears to play a negative regulatory role in the *Drosophila* JAK/STAT pathway.

Sumoylation has been challenging to study due to the highly dynamic and reversible nature of the modification resulting in low cellular levels of Sumo-modified proteins. Also, the Sumo-modified fraction of STAT92E was notably smaller than the unmodified STAT92E, and overexpression of STAT92E and Sumo was needed to detect the Sumo-modified STAT92E. The results with Senp1 coexpression are in line with the specificity of Sumo conjugation. Sumo is deconjugated very rapidly by Sumo-specific proteases, and expression of the inactive Senp1-C603S resulted in increased levels of STAT92E sumoylation, possibly due to competitive inhibition of endogenous Senps.

STAT92E was found to become sumoylated at Lys187. Human STAT1 is sumoylated at Lys703, which is localized in the C-terminus close to the phosphorylated activating Tyr701 residue [17]. *Drosophila* STAT92E has a lysine in a similar position (Tyr711-Lys713), but mutation of this lysine did not alter the amount of sumoylation when compared with STAT92E WT (data not shown). Thus, Lys187 appears to be the only sumoylation site in STAT92E. This lysine is not conserved in mammalian STATs, but there is a putative Sumo consensus site found at least in the coiled coil domain of human STAT2 (Lys234). Sumoylation of human STAT2 has not been reported.

Mutation of Lys187 resulted in increased transcriptional activity of STAT92E, suggesting that sumoylation has an inhibitory effect on STAT92E. Removal of endogenous Sumo E3 ligase dPIAS by dsRNA increased the STAT92E activity on *TotM* promoter approximately to the same level as K187R mutation in STAT92E (fig. 3c), suggesting that sumoylation of STAT92E is involved in dPIAS-mediated inhibition of STAT92E.

The mechanisms of how sumoylation is affecting STAT92E are presently unknown, but several possible mechanisms can be envisioned. The sumoylation site Lys187 is localized in the coiled coil domain, which in the mammalian system is involved in nuclear transport of STATs. The coiled coil domain of STATs is composed of 4 α-helixes that are pointing out from the DNA-bound STAT dimer, forming a hydrophilic surface able to interact with other molecules [9]. Thus, sumoylation of Lys187 may interrupt the interaction between STAT92E and its transcriptional coregulators or the proteins involved in its nuclear translocation. Alternatively, sumoylation may lead to the recruitment of histone deacetylases to the promoter or allow the interaction with a transcription repression complex similarly to *Drosophila* Sp3 [22]. The effect of sumoylation on DNA-binding properties of STAT92E was not analyzed, but the coiled coil domain is not contacting DNA, suggesting that direct effects upon the promoter-binding activity are less likely.

In the mammalian system, sumoylation has been implicated in the regulation of interferon-induced gene responses. STAT1 and other coregulatory proteins have been shown to become sumoylated [25], but the physiological role of this modification remains to be revealed. The sumoylation pathway is well conserved during evolution and the lower redundancy of the *Drosophila* system provides a useful system for functional and mechanistic analysis of the pathway. Our findings that STAT92E is subject to Sumo modification imply that the *Drosophila* system can now be utilized to investigate the physiological function of sumoylation in JAK/STAT signaling.

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Eye transformer is a negative regulator of *Drosophila* JAK/STAT signaling

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ABSTRACT **JAK/STAT signaling pathway is evolutionarily conserved and tightly regulated. We carried out a reporter-based genome-wide RNAi *in vitro* screen to identify genes that regulate *Drosophila* JAK/STAT pathway and found 5 novel regulators. Of these, CG14225 is a negative regulator structurally related to the *Drosophila* JAK/STAT pathway receptor Domeless, especially in the extracellular domain, and to the mammalian IL-6 receptor and the signal transducer gp130. CG14225 coimmunoprecipitates with Domeless and its associated kinase hopscotch in S2 cells. *CG14225* RNAi caused hyperphosphorylation of the transcription factor Stat92E in S2 cells on stimulation with the *Drosophila* JAK/STAT pathway ligand unpaired. *CG14225* RNAi *in vivo* hyperactivated JAK/STAT target genes on septic injury and enhanced unpaired-induced eye overgrowth, and was thus named the *eye transformer (ET)*. In the gastrointestinal infection model, where JAK/STAT signaling is important for stem cell renewal, *CG14225/ET* RNAi was protective *in vivo*. In conclusion, we have identified ET as a novel negative regulator of the *Drosophila* JAK/STAT pathway both *in vitro* and *in vivo*, and it functions in regulating Stat92E phosphorylation.—Kallio, J., Myllymäki, H., Grönholm, J., Armstrong, M., Vanha-aho, L.-M., Mäkinen, L., Silvennoinen, O., Valanne, S., Rämet, M. *Eye transformer* is a negative regulator of *Drosophila* JAK/STAT signaling. *FASEB J.* 24, 4467–4479 (2010). www.fasebj.org**

Key Words: RNAi screen · cytokine signaling · stress response · Domeless · unpaired

The evolutionarily conserved janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway controls responses to hematopoietic cytokines that orchestrate inflammatory and immune responses in mammals (1). In humans, selective utilization of 4 different Janus kinases (JAKs) and 7 STAT transcription factors leads to specific changes in the activity of a set of target genes providing complexity for JAK/STAT-mediated responses. Disturbances in JAK/STAT signaling may cause serious human diseases, including cancer, polycytemia vera, severe immune deficiencies such as SCID, autoimmunity, allergies, and neurological defects (1–5).

Drosophila has been widely used as a model for JAK/STAT signaling. The core signaling pathway is conserved in evolution from flies to humans, but *Drosophila* has only 1 JAK, hopscotch (6, 7), and a single STAT transcription factor, Stat92E (8, 9), making the pathway simpler and less redundant. Like in mammals, the *Drosophila* JAK/STAT pathway is also involved in multiple processes, which include embryonic segmentation, larval hematopoiesis and development of various organs, regulation of stem cell maintenance, and cellular proliferation. In addition, *Drosophila* JAK/STAT signaling is required to control immune and stress responses (7, 10; reviewed in refs. 11–14). After septic injury, the activation of JAK/STAT pathway leads to the expression of a number of genes, including *Turandot* (*Tot*) stress genes in the fatbody (15–17).

JAK/STAT pathway activation is mediated by secreted cytokine-like molecules unpaired (upd), upd2, and upd3 (18–20), which bind to the transmembrane receptor Domeless (Dome) (21, 22). Dome shares homology with members of the interleukin 6 (IL-6) receptor family and, like the mammalian cytokine receptors, forms dimers (22, 23). In the canonical model of JAK/STAT signaling cascade, ligand binding induces a conformational change in the receptor leading to activation of associated JAKs by auto- and/or trans-phosphorylation. Activated JAKs phosphorylate tyrosine residues in the receptors, thereby creating docking sites for STATs, which subsequently become phosphorylated by JAKs too. Activated STATs dimerize and translocate in the nucleus, where they bind their target sites in DNA and act as transcriptional activators (8, 24). In *Drosophila,* the *Socs36E* (suppressor of cytokine signaling 36E) gene is known to be a target gene of the JAK/STAT pathway, forming a negative feedback loop by inhibiting hop activity (25, 26). *Drosophila* protein inhibitor of activated stat (dPIAS) is shown to

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be a negative regulator of the *Drosophila* JAK/STAT pathway too, but the mechanism remains elusive (27) compared to humans, where, for example, hPias1 is shown to promote sumoylation of hStat1 (28).

JAK/STAT signaling has been studied in the *Drosophila* system because of its importance in human diseases. S2 cell-based large-scale RNAi screening provides a powerful tool to identify theoretically all genes required for given cellular function (29, 30). Two *Drosophila in vitro* RNAi screens have been carried out to find genes involved in JAK/STAT signaling (31, 32), and several new modifiers of this pathway have been identified. Curiously, however, there were differences between the sets of identified genes. To study regulation of the *Drosophila* JAK/STAT signaling and to elucidate the events involved in the signaling, we carried out a genome-wide RNAi based *in vitro* screen in *Drosophila* S2 cells. As the intracellular part of the JAK/STAT pathway downstream of the JAK kinase is particularly well conserved, we chose to activate the signaling using the constitutively active form of the *Drosophila* JAK kinase *hopscotch* ($hop^{Tum\text{-}l}$) (33, 34). Activity of the pathway was monitored using a Stat92E responsive *TotM-luciferase* (*TotM-luc*) reporter-based assay. We screened 16,025 dsRNAs for their effect on *TotM* reporter activity. In addition to the known JAK/STAT signaling pathway components, we identified 5 genes that regulated *TotM* response in S2 cells. Of these, the *CG14225* gene we call *eye transformer (ET)* was identified as a negative regulator of JAK/STAT signaling both *in vitro* and *in vivo*.

MATERIALS AND METHODS

dsRNA synthesis

The dsRNAs used in the RNAi screen were produced from a commercial *Drosophila* genome RNAi library consisting of a set of 13,625 PCR products with dual T7 promoter sequences [Medical Research Council (MRC) Geneservice Ltd., Cambridge, UK]. An additional 2400 dsRNAs were transcribed from the S2 cell-derived cDNA library (35). Targeted dsRNAs were synthesized from S2 cDNA essentially as described in ref. 36. pMT/BiP/V5-His/GFP plasmid (Invitrogen, Carlsbad, CA, USA) was used as a template for the production of the negative control *GFP* dsRNA. Primers used for targeted dsRNAs were *GFP* 5′-T7+GCTCGGGAGATCTCC-3′ and 5′-T7+CTAGACTCGAGCGGC-3′; *Stat92E* 5′-T7+CCGATTAGCCAACGC-3′ and 5′-T7+GGACCCCAGTGATCT-3′; *hop* 5′-T7+GGAGCAGCAGATAGC-3′ and 5′-T7+GGCGGTAGAGGAACT-3′; *Dome* 5′-T7+TAACGGCAAGAGCGC-3′ and 5′-T7+AGGTTCTGGCCAGGT-3′; *ET dsRNA*[1] 5′-T7+TGCGAAGGCAGGGCACAATAGAATC-3′ and 5′-T7+CAAGTCTGGTTGGGCGTTTGTATCA-3′; *ET dsRNA*[2] 5′-T7+CGGAGAATGCGTTGC-3′ and 5′-T7+AGTTGGGCAGCTTGG-3′; *ET dsRNA*[3] 5′-T7+GACATCCGGGATCGACG-3′ and 5′-T7+CGTGGGCTCCTCTTCCG-3′. Additional information related to primers is presented in Supplemental Table S1.

Cell culture, transfections, dsRNA treatments, and overexpression constructs

Drosophila S2 cells were cultured in Schneider medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 25°C. Transfections and dsRNA treatments were performed essentially as described previously (37). We transfected 1.0×10^6 S2 cells with 0.2 μg of a constitutively active form of the Janus kinase $hop^{Tum\text{-}l}$ together with *TotM-luc* reporter plasmid for activating the JAK/STAT pathway and for quantifying *TotM* expression, respectively. Cells were also transfected with 0.2 μg *Act5C-β-gal* reporter plasmid for monitoring cell viability and transfection effiency. We used 0.5 μg of control and experimental dsRNAs for RNAi. Reporter activities were measured 72 h after transfection. Transfections with other reporters were carried out similarly. *CG14225* was cloned from S2 cell cDNA to *Eco*RI and *Not*I sites of *Drosophila* expression vector pMT-HisA. Protein production was induced by addition of $CuSO_4$ to a final concentration of 500 μM 24 h prior to cell lysis.

Quantitative RT-PCR

Quantitative RT-PCR for *TotM, TotA,* and *CG14225* and *Act5C* levels was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) and the ABI7000 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Primers used for qRT-PCR were *Act5C* 5′-CGAAGAAGTTGCTGCTCTGG-3′ and AGAACGATACCGGTGGTACG; *TotM* 5′-ACCGGAACATCGACAGCC-3′ and 5′-CCAGAATCCGCCTTGTGC-3′; *TotA* 5′-CCCAGTTTGACCCCTGAG-3′ and 5′-GCCCTTCACACCTGGAGA-3′; *ET* 5′-CGGAGAAAGGAGCACCCA-3′ and 5′-GGGACTGCATCTCGCAGT-3′.

Sequence analysis

Sequences were analyzed with the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/). ClustalW alignments (http://www.ebi.ac.uk/Tools/clustalw2/index.html) were carried out in order to identify similar regions between ET, Dome, and gp130.

Coimmunoprecipitation

S2 cells were transfected with constructs of cDNAs cloned in the pMT/V5/HisA vector (Invitrogen). Protein production was induced with $CuSO_4$. The following tagged full-length constructs were used: hopscotch-V5, Dome-myc, ET-myc, ET-V5, and Dome-V5. Constructs were cotransfected in the combinations shown and immunoprecipitated with Protein G Sepharose beads (GE Healthcare, Little Chalfont, UK) or Protein G Dynabeads (Invitrogen), separated, transferred to nitrocellulose membrane, and detected essentially as described previously (38).

Immunodetection

We transfected 5.0×10^6 S2 cells with a total amount of 3.0 μg of dsRNA and 1.0 μg of pMT-upd plasmid or 1.0 μg of empty pMT-V5-HisA using Fugene6 reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Then 48 h after transfection, cells were treated with 500 μM $CuSO_4$ for 24 h. Cells were lysed in Triton-X lysis buffer. The protein amounts were determined by a Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% milk in TBS-0.1% Tween 20, incubated with anti-Stat92E-N-terminal antibody (dN-17;

Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with anti-phospho-Tyrosine antibody (PY99; Santa Cruz Biotechnology) and with a biotinylated anti-goat or anti-mouse secondary antibody (DakoCytomation, Copenhagen, Denmark). Immunodetection was performed with the enhanced chemiluminescence method (GE Healthcare). TBS buffer containing 1% β-mercapthoethanol and 0.2% SDS was used for stripping. Phospho-Stat92E bands were quantified by ImageQuant TL image analysis software (GE Healthcare) and analyzed after background subtraction.

Fly stocks and maintenance

Drosophila stocks were kept on a standard mashed potato diet at RT or at 25°C. The RNAi transgenic fly stocks were obtained from the Vienna *Drosophila* RNAi Center [VDRC; Vienna, Austria; VDRC transformants 19756 (*ET IR*^{1}), 100881 (*ET IR*^{2}), and 43866 (*Stat92E IR*)]. *UAS-RNAi* flies were crossed over a fatbody-specific *C564-GAL4* or ubiquitous *GeneSwitch-GAL4* driver flies or to w^{1118} flies for controls. In flies crossed over the *GeneSwitch-GAL4* driver, the *GAL4* construct was induced with Mifepristone (200 μM). Week-old offspring were used for experiments.

To study the eye phenotype, flies carrying *GMR-upd*Δ*3′* were first crossed over *ET* IR^{1}/*CyO* flies. The F1 flies with 1 copy of *GMR-upd*Δ*3′* and 1 copy of *ET* IR^{1} were then crossed over eye-specific driver *ey-GAL4* to induce *ET* RNAi. The offspring from the first cross without the *ET* RNAi construct were used as controls.

Fly infections

For *Enterobacter cloacae* infection, week-old flies were pricked with a thin tungsten needle dipped in a concentrated culture of bacteria. *Serratia marcescens* feeding infection experiment was performed as described previously (39, 40). Survival of the flies was recorded daily.

Data analysis

Statistical analyses of reporter assays and qRT-PCR results were carried out using 1-way ANOVA. Statistical analysis of fly survival experiments was carried out using the log-rank (Mantel-Cox) test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Luciferase-based reporter assay to monitor JAK/STAT signaling pathway activity in *Drosophila* S2 cells

Under stressful conditions, including septic injury, several *Tot* genes are expressed in the *Drosophila* fatbody, the functional equivalent of mammalian liver (15, 16). It has been shown that the activation of *TotA* is JAK/STAT pathway dependent but is also partly regulated by the Imd pathway and requires MAPKKK Mekk1 (17, 41). As shown in **Fig. 1*A***, *TotM-luc* reporter activity is induced by a constitutively active form of Janus kinase *hopscotch* (*hop*$^{Tum\text{-}l}$) in *Drosophila* S2 cells. This induction is STAT dependent, as RNAi targeting the transcription factor *Stat92E* blocks the *TotM* expression. On the other hand, RNAi targeting the Imd pathway transcription factor *Relish* has no effect on *hop*$^{Tum\text{-}l}$-induced *TotM* expression, demonstrating the specificity of our assay to JAK/STAT signaling (data not shown). These results indicate that *hop*$^{Tum\text{-}l}$-induced *TotM-luc* reporter activity can be used to study the regulation of the JAK/STAT pathway in *Drosophila* S2 cells.

Genome-wide RNAi analysis of the *Drosophila* JAK/STAT pathway in *Drosophila* S2 cells

To identify all regulators of the JAK/STAT pathway downstream of hop, we carried out a genome-wide RNAi screen and monitored the effects of 16,025 dsRNA treatments on *hop*$^{Tum\text{-}l}$-induced *TotM-luc* reporter activity in S2 cells. The dsRNAs were produced by *in vitro* transcription from a commercial *Drosophila* genome-wide library (MRC Geneservice; 13,625 PCR products), and an additional 2400 dsRNAs were transcribed from S2 cell-derived cDNA library (35). S2 cells were transfected with a *hop*$^{Tum\text{-}l}$ expression vector and *TotM-luc*-reporter together with experimental or control dsRNAs. *Act5C-*β*-gal* reporter was used to control cell viability. The luciferase and β-galactosidase activities were measured 72 h after transfection. dsRNA targeting *Stat92E* and dsRNA targeting a gene encoding *Green Fluorescence Protein* (*GFP*), which is not expressed in S2 cells, were used as positive and negative controls in each experiment, respectively. The luciferase and β-galactosidase values for *GFP* dsRNA-treated cells were used as reference values for experimental dsRNAs. As shown in Fig. 1*B*, most dsRNA treatments had little or no effect on *TotM-luc* or *Act5C-*β*-gal* activity. Notably, there were 7 dsRNA treatments that repeatedly decreased *TotM-luc* activity by more than 50% without significantly affecting *Act5C-*β*-gal* activity. These targeted 2 known JAK/STAT pathway components (*Stat92E* and *hop*), one gene previously shown to be involved in JAK/STAT pathway regulation (*enok*) (31, 32), and 4 novel regulators (*Taf1, CG31716, CG14225,* and *Med27*). Corresponding templates from the original library were TA-cloned and sequenced. Based on the sequencing results, we designed gene-specific primers and synthesized targeted independent dsRNAs against these novel regulators to confirm that the RNAi phenotype had been due to presumed dsRNA and not due to contaminating dsRNAs or any off-target effect. As shown in Fig. 1*C*, all 5 targeted dsRNA treatments decreased *TotM-luc* reporter activity comparably to the library dsRNAs.

To ensure that the obtained results were not caused by an artifact related to the reporter assay, we studied the endogenous *TotM* and *Act5C* expression levels of *hop*$^{Tum\text{-}l}$-transfected and dsRNA-treated S2 cells using qRT-PCR (Fig. 1*D*). RNAi targeting any of the identified genes resulted in at least a 50% reduction in relative *TotM* expression level, indicating that these genes are required for normal hop$^{Tum\text{-}l}$ -induced *TotM* response in S2 cells. Based on these results, *enok, Taf1,*

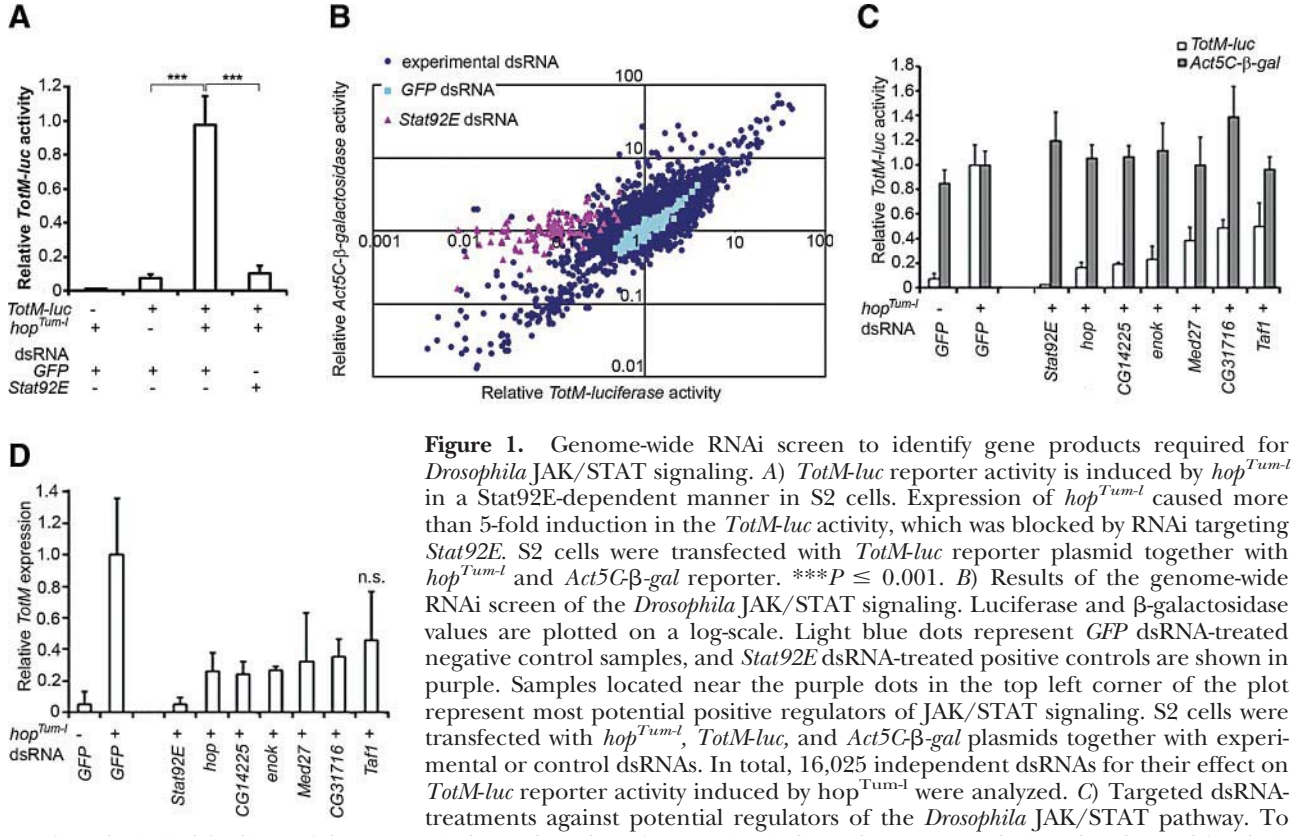

Figure 1. Genome-wide RNAi screen to identify gene products required for *Drosophila* JAK/STAT signaling. *A*) *TotM-luc* reporter activity is induced by hop^{Tum-l} in a Stat92E-dependent manner in S2 cells. Expression of hop^{Tum-l} caused more than 5-fold induction in the *TotM-luc* activity, which was blocked by RNAi targeting *Stat92E*. S2 cells were transfected with *TotM-luc* reporter plasmid together with hop^{Tum-l} and *Act5C-β-gal* reporter. $^{***}P \leq 0.001$. *B*) Results of the genome-wide RNAi screen of the *Drosophila* JAK/STAT signaling. Luciferase and β-galactosidase values are plotted on a log-scale. Light blue dots represent *GFP* dsRNA-treated negative control samples, and *Stat92E* dsRNA-treated positive controls are shown in purple. Samples located near the purple dots in the top left corner of the plot represent most potential positive regulators of JAK/STAT signaling. S2 cells were transfected with hop^{Tum-l}, *TotM-luc,* and *Act5C-β-gal* plasmids together with experimental or control dsRNAs. In total, 16,025 independent dsRNAs for their effect on *TotM-luc* reporter activity induced by hop^{Tum-l} were analyzed. *C*) Targeted dsRNA-treatments against potential regulators of the *Drosophila* JAK/STAT pathway. To confirm the initial findings of the screen, independent dsRNAs targeting indicated genes were designed and tested for their effect on hop^{Tum-l}-induced *TotM*-reporter activity as in panel *A*. All targeted dsRNAs decrease hop^{Tum-l}-induced *TotM-luc* reporter activity by more than 50% in S2 cells as compared to *GFP* dsRNA-treated cells. *D*) Endogenous *TotM* expression is reduced by dsRNA treatments targeting any of the 5 novel regulators of the *Drosophila* JAK/STAT pathway. dsRNAs targeting indicated genes were transfected into S2 cells together with hop^{Tum-l}. Endogenous *TotM* expression levels were measured by qRT-PCR and results normalized to *Act5C* expression values. All data are shown as means ± SD, $n \geq 4$. Values of $P \leq 0.05$ unless indicated otherwise.

CG31716, CG14225, and *Med27* are potential regulators of *Drosophila* JAK/STAT signaling, and these 5 genes were subjected to further studies.

CG31716 and CG14225 are general modifiers of JAK/STAT signaling, whereas enok, Med27, and Taf1 are more context sensitive

To assess the role of identified genes in JAK/STAT signaling in more physiological context, we activated the JAK/STAT signaling in S2 cells by overexpressing the ligand *upd,* and we used *TotM-luc* reporter to measure the pathway activity. As shown in **Fig. 2*A***, dsRNA treatments targeting *enok, Taf1,* and *CG31716* reduced upd-induced *TotM* expression in a similar manner compared to hop^{Tum-l}-induced *TotM* response, whereas RNAi against *Med27* showed no effect. Intriguingly, RNAi targeting *CG14225* caused strong hyperactivation of the *TotM* reporter activity in this setting.

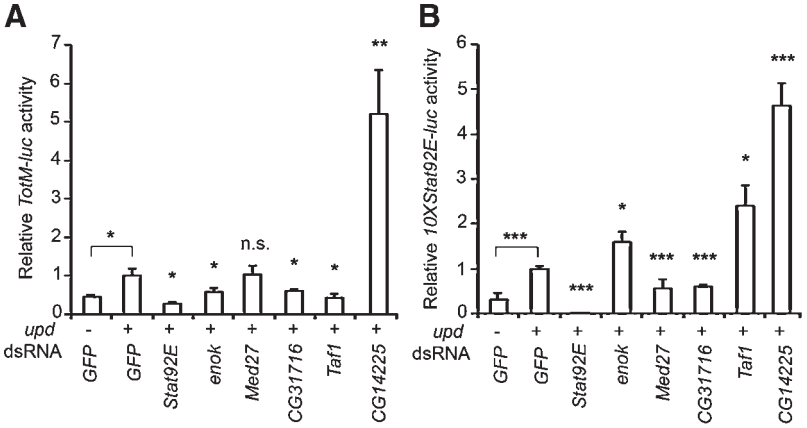

Figure 2. CG31716 and CG14225 are general modifiers of JAK/STAT signaling, whereas enok, Med27, and Taf1 are more context sensitive. *A*) RNAi targeting *enok, CG31716,* and *Taf1* decreases upd-induced *TotM-luc* activity in S2 cells, whereas *CG14225* dsRNA strongly enhances *TotM-luc* response. *B*) RNAi targeting *Med27* and *CG31716* decreases upd-induced *10xStat92E-luc activity* in S2 cells, whereas *CG14225* and *Taf1* RNAi enhances the response. S2 cells were transfected with *TotM-luc* (*A*) or *10xStat92E-luc* (*B*) and *Act5C-β-gal* reporter constructs together with indicated dsRNAs and *upd* to induce the pathway. Data are shown as means ± SD, $n \geq 4$, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Next, we tested whether these novel regulators are specific for regulating *TotM* expression, or whether they affect JAK/STAT target genes in a more general manner. To this end, we induced JAK/STAT signaling by expressing *upd,* and we used *10xStat92E-luc* reporter consisting of a sequence containing a double Stat92E binding site from a *SOCS36E* enhancer region multiplied 10 times, to measure the JAK/STAT pathway activity (31). As shown in Fig. 2*B, upd* expression caused 3.1-fold induction in *10xStat92E-luc* reporter activity. This induction is strongly inhibited by dsRNA treatments targeting *CG31716* and *Med27,* suggesting that these genes are required for Stat92E-dependent activation of target genes in *Drosophila* S2 cells. Interestingly, RNAi targeting *enok* or *Taf1* increased upd-induced *10xStat92E* reporter activity, indicating that these gene products have a more specific effect on regulating *TotM* expression. Of note, as enok also had been identified earlier as a negative regulator of *Drosophila* JAK/STAT signaling (31, 32), our results with *10xStat92E-luc* reporter are in line with the earlier reports. *CG14225* RNAi caused clear hyperactivation of *10xStat92E-luc* reporter also in this setting. Furthermore, *CG14225* dsRNA treatment caused a strong increase in another Stat92E-responsive reporter, *3 × 2xDraf-luciferase* activity (32) in response to *upd* expression in S2 cells (more than 10-fold induction compared to GFP dsRNA-treated controls, data not shown). These results related to the role of CG14425 in upd-induced JAK/STAT signaling are in striking contrast compared to our results with $hop^{Tum\text{-}l}$ induction and prompted us to study this gene in more detail.

CG14225/ET is a *Dome*-related gene that negatively regulates the JAK/STAT signaling in *Drosophila* S2 cells

CG14225/ET is a 3.3 kb gene comprising 3 separate coding sequences (**Fig. 3*A***). It is located next to the

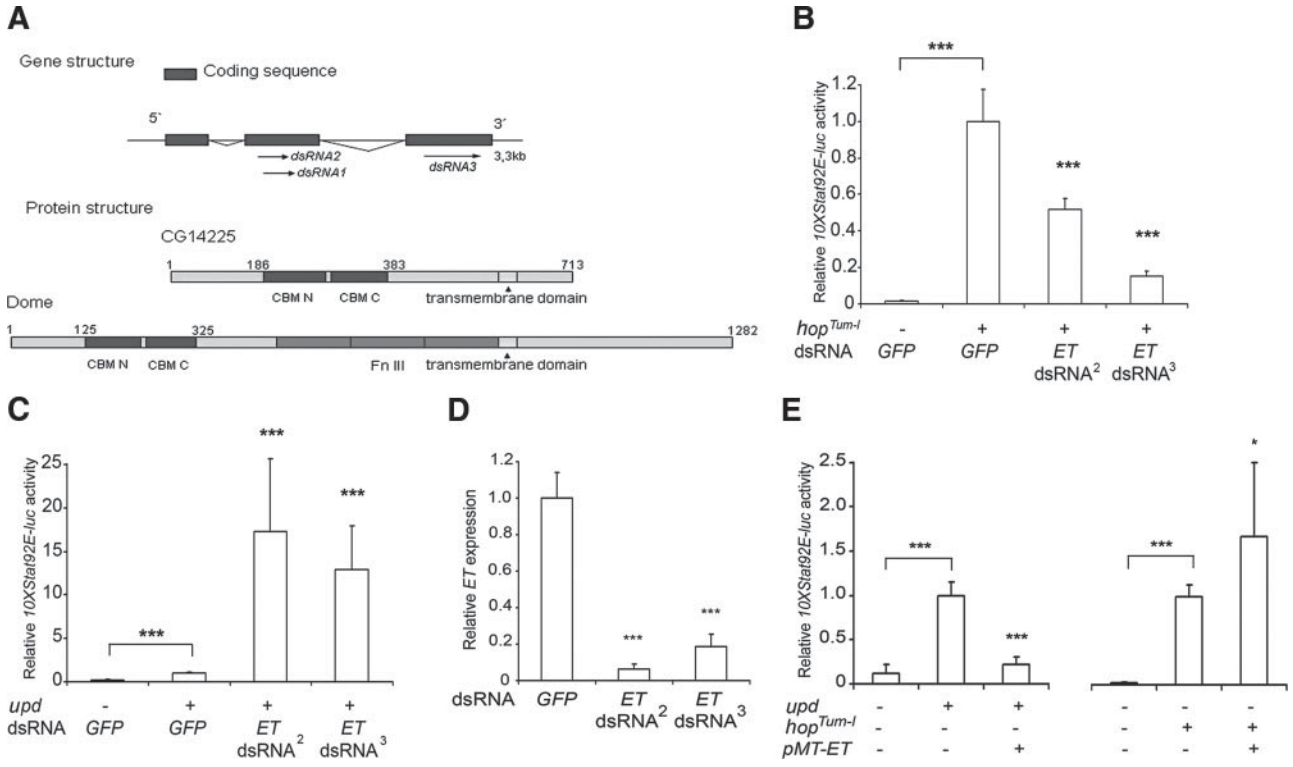

Figure 3. ET is a negative regulator of the *Drosophila* JAK/STAT pathway and is structurally related to Dome. *A*) Schematic representation of gene and protein structures of *CG14225/ET*. The *ET* gene consists of 3 exons and has no UTR regions. Sequence regions where *ET* dsRNAs ($dsRNA^{1-3}$) were designed are shown. Domain structure of ET protein is illustrated and compared to domain structure of Dome in the bottom panel. N-terminal fibronectin-type III domain of the cytokine-binding module (CBM N) and C-terminal fibronectin-type III domain of the cytokine-binding module (CBM C) are highly conserved in both proteins. The fibronectin-type III (FnIII) domain triplet near the transmembrane domain in Dome is absent in ET. *B, C*) RNAi targeting different regions of *ET* has a similar effect on both $hop^{Tum\text{-}l}$- and upd-induced *10xStat92E-luc* activity. S2 cells were transfected with *10xStat92E-luc* reporter plasmid together with $hop^{Tum\text{-}l}$ or upd constructs for induction of the pathway. Two dsRNAs targeting different parts of the *ET* gene decrease $hop^{Tum\text{-}l}$-induced *10xStat92E-luc* activity (*B*) and increase upd-induced *10xStat92E-luc* activity (*C*), compared to *GFP* dsRNA-treated controls in S2 cells. *D*) RNAi targeting *ET* strongly decreases the amount of *ET* transcripts in S2 cells compared to *GFP* dsRNA-treated controls. Endogenous *ET* mRNA levels after dsRNA treatments were measured using qRT-PCR. *E*) Overexpression of *ET* blocks upd-induced *10xStat92E-luc* activity in S2 cells, but enhances $hop^{Tum\text{-}l}$-induced *10xStat92E-luc* response. S2 cells were transfected with an *ET* overexpression construct or an empty vector together with $hop^{Tum\text{-}l}$ or upd to induce the pathway. All data are shown as means ± SD, $n \geq 4$. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Drosophila JAK/STAT pathway receptor *Dome* in the genome. Furthermore, the *ET* gene encodes a 713 amino acid type-I transmembrane protein that shares structural similarities with Dome (11). Like Dome, *ET* has a cytokine binding module (CBM) in the N terminus but lacks 3 FnIII domains near the transmembrane domain. The CBM domain of vertebrate interleukin receptors is composed of 2 FnIII domains, containing 4 conserved cysteine residues in the N-terminal domain and a conserved WSXWS motif in the C-terminal domain (42). CBM in Dome and CG14225/ET share these features, but the WSXWS motif is incomplete in both (NTLWS/GSPWS). Intriguingly, although Dome shares similarities with the mammalian interleukin-6 (IL-6) receptor family members, ET's closest human homologue is the signal transducing protein gp130 with 12.7% identity, mostly found in the extracellular region. In mammalian IL-6 signaling, gp130 associates with IL-6 receptors that have bound their ligand and dimerizes, therefore allowing transduction of the signal to activate target molecules, including JAKs and STATs (43). Thus, ET shares structural similarities with Dome and mammalian gp130, which plays an important role in regulation of JAK/STAT signaling. The structure of the *ET* gene product explains in part why we observed opposing results with *ET* RNAi depending on what level the JAK/STAT pathway was activated at, because with the transmembrane domain, *ET* is likely to function at the level of Dome, thus epistatically between upd and hop.

To verify that the observed *ET* RNAi phenotype was not due to off-target effects, we generated another dsRNA targeting the third exon of *ET* (the 3 different dsRNAs targeting *ET* are shown schematically in Fig. 3*A*). As shown in Fig. 3*B,* both targeted dsRNAs ($dsRNA^2$ and $dsRNA^3$) caused strong inhibition of $hop^{Tum\text{-}l}$-induced *10xStat92E-luc* reporter activity. Furthermore, both targeted dsRNAs caused hyperactivation of the upd-induced *10xStat92E-luc* reporter activity (Fig. 3*C*), indicating that independent dsRNAs targeting *ET* cause similar phenotypes. Furthermore, hyperactivation of *10xStat92E-luc* reporter was also observed when upd-conditioned medium (32) was added on *ET* dsRNA-treated cells (data not shown). To confirm efficiency of RNAi on the *ET* mRNA level, we treated S2 cells with both targeted dsRNAs against *ET* and analyzed mRNA levels using qRT-PCR (Fig. 3*D*). Both dsRNAs resulted in strongly decreased *ET* mRNA levels compared to GFP dsRNA-treated control cells, indicating that these treatments effectively suppress *ET* expression in S2 cells. Taken together, these results indicate that *ET* RNAi causes hyperactivation of upd-induced JAK/STAT response in S2 cells.

To test whether overexpression of *ET* has an effect on JAK/STAT signaling in S2 cells, we cloned *ET* to a *Drosophila* expression vector. As shown in Fig. 3*E,* overexpression of *ET* caused a marked decrease in upd-induced *10xStat92E-luc* reporter activity in S2 cells and consistently with the RNAi phenotype results, 1.6-fold increase in $hop^{Tum\text{-}l}$-induced *TotM-luc* activity. Of note, *ET* mRNA contains no UTR regions, and thus it was not possible to carry out a rescue experiment where endogenous *ET* would be knocked down by dsRNA targeting the UTR regions and the resulting phenotype would then be rescued by overexpression of *ET* construct.

To analyze whether *ET* RNAi affects signaling cascades in S2 cells in a more general manner, we investigated the Toll and the Imd pathway signaling in *ET* dsRNA-treated S2 cells. *ET* RNAi did not significantly affect the heat-killed *E. coli*-induced *Attacin* (Imd pathway target gene) reporter activity, and had only a minor effect on $Toll^{10b}$-induced *Drosomycin* (Toll pathway target gene) reporter activity in S2 cells (data not shown). These results indicate that *ET* is not a general regulator of signaling pathways in S2 cells, but its function is more specific to the JAK/STAT pathway.

ET is an intrinsic component of the Dome receptor complex, and it functions as a regulator of Stat92E phosphorylation

Curiously, RNAi targeting *ET* caused different phenotypes in *TotM-luc* and *10xStat92E-luc* reporter assays depending on whether $hop^{Tum\text{-}l}$ or upd was used to trigger the JAK/STAT pathway signaling. To investigate the function of ET, we tested whether *ET* RNAi phenotype can be suppressed by dual RNAi treatments targeting known regulators of *Drosophila* JAK/STAT signaling in S2 cells. S2 cells were treated with *ET* dsRNA and with dsRNA targeting known components of the JAK/STAT pathway, *hop, Stat92E,* or *Dome*. The JAK/STAT pathway activity was measured using *TotM-luc* reporter. *ET* RNAi caused a subtle activation of the *TotM-luc* reporter activity (**Fig. 4*A***), suggesting that ET acts as a constitutive negative regulator of the JAK/STAT pathway in S2 cells. RNAi targeting any of the known *Drosophila* JAK/STAT pathway components (*Dome, hop,* or *Stat92E*) prevented this activation, which suggests that ET function is dependent on these components and that ET acts upstream or at their level in S2 cells.

To gain a more mechanistic insight about ET-mediated inhibition of JAK/STAT signaling, we coimmunoprecipitated overexpressed V5-tagged components of the *Drosophila* JAK/STAT signalosome with myc-tagged ET in S2 cells (Fig. 4*B*). At first, we confirmed the method by hop and Dome coimmunoprecipitation, and as expected, hop-V5 coimmunoprecipitated with Dome-myc (Fig. 4*B*). Notably, both hop-V5 and Dome-V5 coimmunoprecipitated with ET-myc, suggesting that overexpressed ET interacts directly with key regulators of the *Drosophila* JAK/STAT signalosome in S2 cells. Of note, we were unable to coimmunoprecipitate upd or Stat92E with ET (data not shown).

To gain further insight to the molecular function of ET, we analyzed whether ET suppresses JAK/STAT signaling by affecting the dimerization of Dome. As shown in **Fig. 5*A***, Dome-Dome interaction, as analyzed by immunoprecipitating V5-tagged Dome with myc-

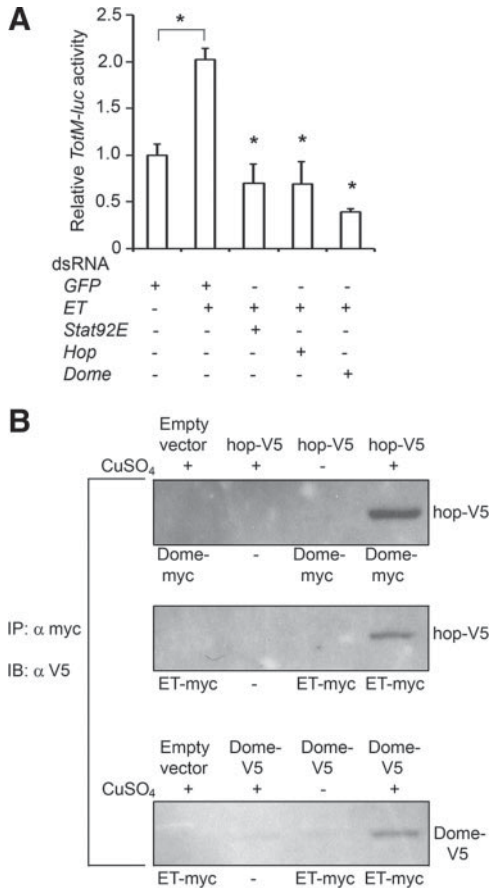

Figure 4. ET functions at the level or upstream of Dome and coimmunoprecipitates with hop and Dome. *A*) ET functions at the level or upstream of Dome. *TotM*-luc reporter was activated by RNAi targeting *ET*. RNAi targeting known components (*hop, Stat92E, Dome*) of the *Drosophila* JAK/STAT pathway abolishes *ET* RNAi -induced *TotM* activity, indicating that these components are located functionally downstream of ET. Data are shown as means ± SD, $n \geq 4$. $^*P \leq 0.05$. *B*) ET coimmunoprecipitates with both hop and Dome. S2 cells were transfected with either ET-myc or Dome-myc and hop-V5 or Dome-V5. Immunoprecipitation (IP) was done with anti-myc (α myc) antibody and immunoblotting (IB) with anti-V5 (α V5) antibody. $CuSO_4$ was used to induce the expression of the constructs.

tagged Dome, was not affected by overexpression of *ET* (or *ET* RNAi). Similarly, overexpression of *ET* (or *ET* RNAi) did not alter the interaction between hop-V5 and Dome-myc (Fig. 5*A*). These results indicate that ET is not likely to function by preventing dimerization of Dome, or by disrupting Dome-hop interaction in S2 cells.

Next, we studied whether ET affects the kinase activity of the *Drosophila* JAK/STAT signalosome by investigating Stat92E phosphorylation upon upd induction in S2 cells (Fig. 5*B*). S2 cells were transfected with indicated dsRNAs and *upd* to activate signaling leading to Stat92E phosphorylation. Then 72 h after transfection, S2 cell protein lysates were separated by SDS-PAGE and analyzed by Western blotting with anti-Stat92E N-terminal antibody. RNAi targeting *ET* caused Stat92E band shift upon activation with upd compared to *GFP* dsRNA-treated controls, suggesting hyperphosphorylation of Stat92E. To ensure that this upper band represents a phosphorylated form of the protein, Stat92E was immunoprecipitated from S2 cell lysates with anti-Stat92E N-terminal antibody and detected with phospho-tyrosine-specific antibody (Fig. 5*B*). Hyperphosphorylation of Stat92E was abolished when *Dome* or *hop* dsRNA was cotransfected with *ET* dsRNA (Fig. 5*B*), indicating that ET function is dependent on these factors. *Stat92E* dsRNA strongly decreases the signal demonstrating the specificity of the antibody.

Taking these data together, we see that ET is a negative regulator of the JAK/STAT pathway in *Drosophila* S2 cells. ET functions as a regulator of Stat92E phosphorylation and is located functionally at the level or upstream of the receptor Dome. Furthermore, ET appears as an intrinsic component of the Dome receptor complex as it coimmunoprecipitates with both hop and Dome when overexpressed in S2 cells, but it does not affect Dome dimerization or Dome-hop interaction. The mechanistic function of ET is shown schematically in Fig. 5*C*.

ET negatively regulates *Tot* gene expression *in vivo*

ET functions as a negative regulator of JAK/STAT signaling *in vitro*. JAK/STAT pathway is required for *Tot* gene expression under stressful conditions in *Drosophila in vivo* (17, 41). To investigate the role of *ET* in JAK/STAT signaling *in vivo,* we crossed fly lines carrying *UAS-RNAi* constructs targeting *ET* (*ET IR*^{1} and *ET IR*^{2}) (44) over *C564-GAL4* flies, which drive GAL4 expression in the adult fatbody. Thereafter the relative expression levels of both *TotM* and *TotA* in response to septic injury with *E. cloacae* were measured in experimental and control progeny flies by qRT-PCR (**Fig. 6*A, B***). As expected, *C564-GAL4*-driven expression of *UAS-Stat92E* RNAi strongly impaired both *TotM* and *TotA* response to *E. cloacae* compared to controls crossed over w^{1118} (Fig. 6*A, B*). On the contrary, *C564-GAL4*-driven *UAS-ET* RNAi markedly enhanced both *TotM* and *TotA* expression. These results are in agreement with the results obtained using S2 cells and indicate that *ET* negatively regulates *Tot* gene expression in adult *Drosophila*. Of note, infection with *E. cloacae* did not affect the level of *ET* expression, which suggests that *ET* expression is not regulated by the JAK/STAT (or the Imd) pathway (data not shown).

Genetic background may affect gene expression levels under experimental conditions. To avoid bias caused by genetic background, we analyzed the *in vivo ET* RNAi phenotype using a drug-inducible ubiquitous driver *GeneSwitch-GAL4,* which activates the expression of the RNAi construct when Mifepristone is added to the food vials. This enables monitoring the offspring from each cross with and without expression of the RNAi construct, therefore providing a genetically relevant control. Figure 6*C, D* shows that in Mifepristone-induced *ET* RNAi flies, both *TotM* and *TotA* expression in response to septic injury with *E. cloacae* are hyperac-

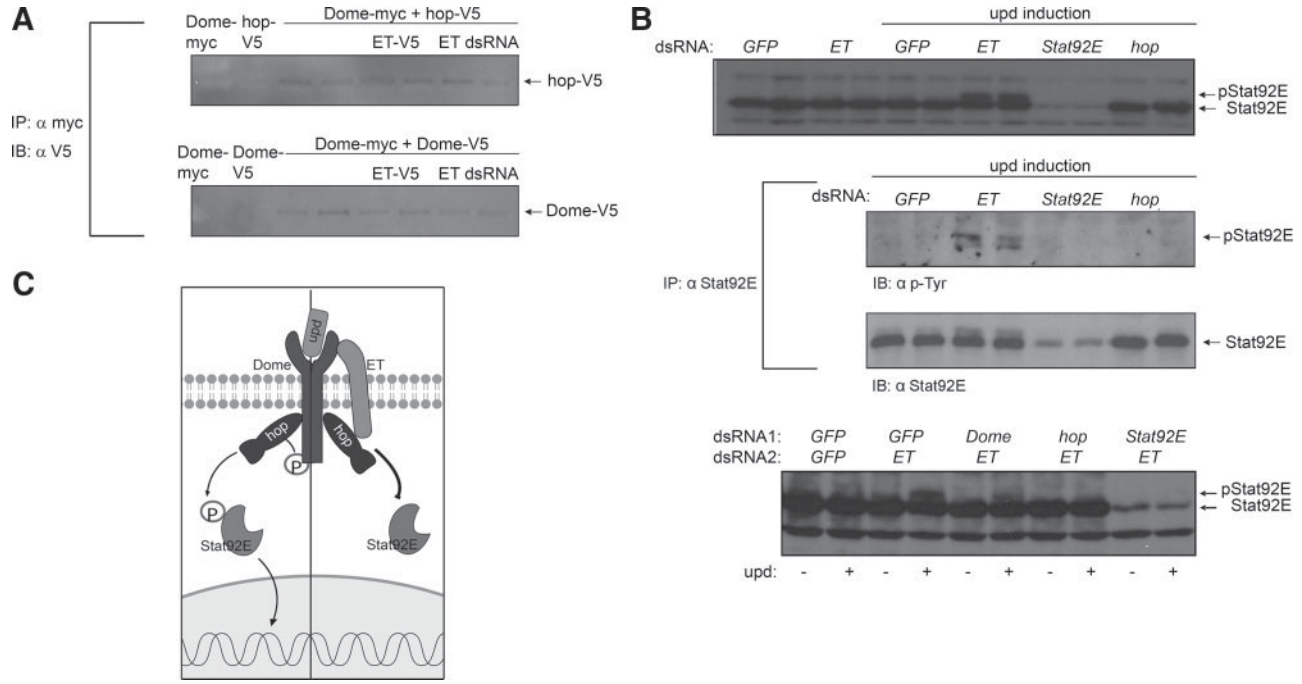

Figure 5. *ET* RNAi causes hyperphosphorylation of Stat92E in response to *upd* expression in S2 cells. *A*) ET does not affect the interaction between hop and Dome (top panel) or homodimerization of Dome (bottom panel). The effect of *ET* overexpression or RNAi was studied by transfecting S2 cells with Dome-myc and hop-V5 or Dome-myc and ET-V5 or treated with *ET* dsRNA. Immunoprecipitation (IP) was done with antimyc (α-myc) antibody and immunoblotting (IB) with anti-V5 (α V5) antibody. *B*) *ET* RNAi causes Stat92E hyperphosphorylation in response to activation of JAK/STAT signaling with upd (3 top panels). The intensity of the phosphorylation bands of Stat92E is significantly increased in *ET* dsRNA-treated samples compared to *GFP* dsRNA-treated controls. Hyperphosphorylation of Stat92E is abolished when *Dome* or *hop* dsRNA is cotransfected with *ET* dsRNA (bottom panel), indicating that function of ET is dependent on these factors. S2 cells were transfected with dsRNAs as indicated with or without *upd* to activate the JAK/STAT pathway. Cells were lyzed 72 h after transfection, and proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-Stat92E N-terminal antibody. IP was done with anti-Stat92E N-terminal antibody followed by SDS-PAGE and IB with antiphospho-Tyrosine antibody. After stripping, the same membrane was reprobed with anti-Stat92E N-terminal antibody showing equal amounts of protein in the immunoprecipitates (middle panels). *C*) Schematic representation of the canonical *Drosophila* JAK/STAT signaling (left) and the inhibitory function of ET (right).

tivated compared to control flies with a drug-free diet. As expected, *Stat92E* RNAi strongly inhibits both *TotM* and *TotA* induction under these conditions, demonstrating that the *GeneSwitch-GAL4* driver was operative.

Tot gene expression is controlled jointly by the JAK/STAT and by the Imd pathway on septic injury in *Drosophila* (17, 41). By crossing *UAS-ET* RNAi flies over *C564-GAL4* flies and measuring *Attacin B* expression on *E. cloacae* septic injury in the offspring, we confirmed that the effect of *ET* RNAi on *Tot* gene expression is not mediated by the Imd pathway (data not shown).

ET RNAi is protective in a gastrointestinal infection model with *Serratia marcescens*

Recently, the *Drosophila* JAK/STAT signaling has been shown to be important for survival of the flies after intestinal bacterial infection (45, 46). In response to infection or damage, enterocytes in the *Drosophila* midgut produce upd, upd2, and upd3, which activate JAK/STAT signaling in intestinal stem cells, leading to cell division and regeneration of the gut epithelium (45). *Serratia marcescens* is an entomopathogenic bacterium that can infect *Drosophila* through the digestive tract. It was shown that despite the local immune response induced, *S. marcescens* infection causes disruption of the gut morphology, which contributes to death of the flies that follows in a few days (40). Thus, we used the *S. marcescens* intestinal infection assay to study the role of ET in microbial resistance *in vivo* (39, 40, 46). *ET* RNAi lines (*ET IR*^{1} and *ET IR*^{2}) were crossed over the *GeneSwitch-GAL4* driver line, and w^{1118} and *Stat92E* over *GeneSwitch-GAL4* were used as controls. As shown in **Fig. 7**, *ET* RNAi flies survived better in food contaminated with *S. marcescens* than flies in which RNAi was not induced by Mifepristone. In contrast, flies with induced *Stat92E* RNAi were more susceptible than flies with no RNAi induction (Fig. 7*A, B*). These data support previous reports suggesting that JAK/STAT signaling is involved in survival of the flies from *S. marcescens* infection *in vivo,* and that *ET* RNAi enhances their resistance to *S. marcescens.* The enhanced resistance against gastrointestinal infection may be due to improved stem cell renewal caused by hyperactivated JAK/STAT pathway (45), although it is plausible that the protective effect of *ET* RNAi is caused by mechanisms independent of JAK/STAT signaling.

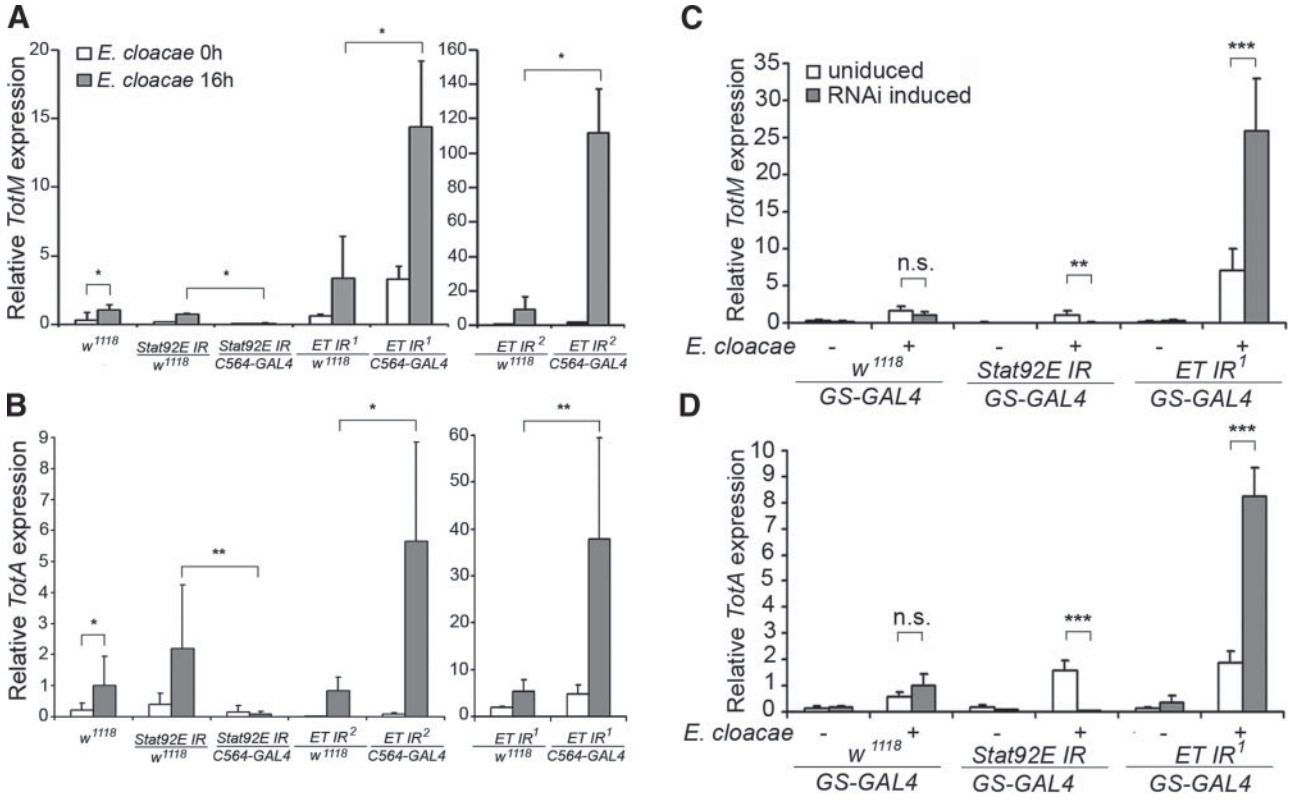

Figure 6. *ET in vivo* RNAi increases JAK/STAT pathway response. *A, B*) *ET* RNAi strongly increases *TotM* (*A*) and *TotA* (*B*) expression in response to septic injury in *Drosophila in vivo,* whereas *Stat92E* RNAi abolishes *Tot* gene expression. Flies carrying the *UAS*-RNAi constructs *ET IR* [1], *ET IR*[2], or *Stat92E IR* (positive control) were crossed over either *C564-GAL4* driver or w^{1118} flies (negative control). The offspring were infected with *E. cloacae* to induce JAK/STAT signaling. *TotM* and *TotA* expression levels were measured from extracted total RNAs by qRT-PCR. *C, D*) *ET* RNAi also dramatically increases *TotM* (*C*) and *TotA* (*D*) expression with an inducible ubiquitous *GeneSwicth-GAL4* driver as compared to flies with the same genetic background with no RNAi induction. In the positive control (*Stat92E* RNAi flies), induction of RNAi abolished *Tot* gene expression, whereas in the negative control (w^{1118}) *Tot* gene expression was not affected by induction. In flies crossed over *GeneSwitch-GAL4* driver, RNAi was induced by adding the drug Mifepristone to food vials, and flies with a drug-free diet (uninduced) were used as controls. All data are shown as means ± SD, $n \geq 4$. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

ET RNAi causes eye overgrowth in adult flies

To gain more direct evidence that ET negatively regulates JAK/STAT signaling *in vivo,* we investigated whether *ET* RNAi affects upd-induced eye overgrowth. JAK/STAT signaling has been shown to be important in regulation of *Drosophila* eye imaginal disc development (47, 48). This is demonstrated by the fact that hyperactivation of the pathway by ectopic overexpression of the ligand *upd* in the developing eye leads to distinct overgrowth (48; Fig. 7*C*). Furthermore, the overgrowth phenotype has been shown to be modulated by overexpression or removal of several JAK/STAT pathway components and regulators (*e.g.,* refs. 32, 48–50). To this end, we tested whether *ET* RNAi affects eye development. Expressing the *ET* RNAi construct under an eye-specific driver (*ey-GAL4*) alone did not affect eye development (Supplemental Fig. S1). However, when these flies were further crossed to *GMR-upd*Δ*3′* flies that overexpress *upd* in the eye, the resulting offspring had more severe eye overgrowth than flies from the same cross without *ET* RNAi (Fig. 7*C* and Supplemental Fig. S1). To evaluate the eye phenotypes objectively, we created a scoring system to quantify the observations. Pictures of each fly's eyes were independently evaluated by 5 experienced researchers from our group as a blind test, and the eye phenotypes were given scores 0–5, 0 representing wild type and 5 the most severe phenotype. The results in Fig. 7*D* show that the eye phenotype of flies carrying both *GMR-upd*Δ*3′* and *ET* RNAi construct together with the *ey-GAL4* driver is more severe than that of flies with only *GMR-upd*Δ*3′*. These results suggest that ET regulates JAK/STAT pathway-mediated eye overgrowth in *Drosophila.*

DISCUSSION

JAK/STAT signaling is involved in a variety of processes in both *Drosophila* and mammals. To identify gene products involved in regulation of *Drosophila* JAK/STAT signaling, we carried out a genome-wide reporter assay-based RNAi screen in S2 cells. A constantly active form of hop ($hop^{Tum\text{-}l}$) was chosen for pathway activation in order to focus on the more evolutionarily

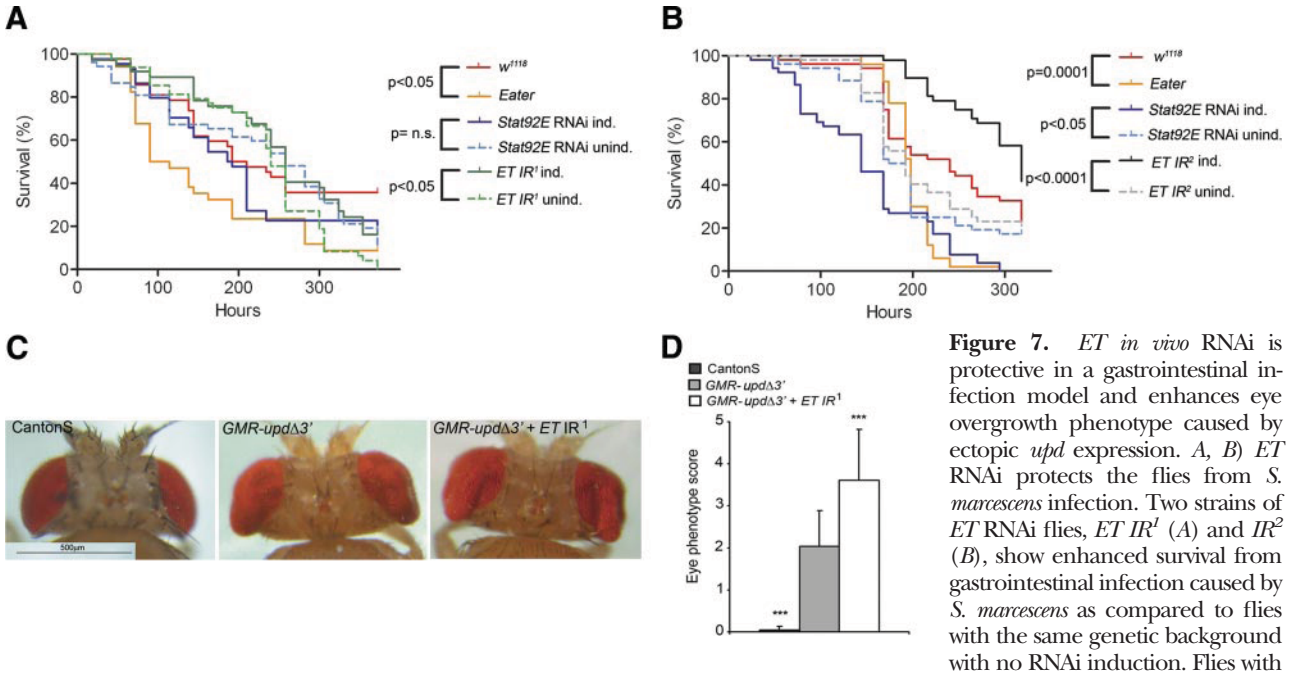

Figure 7. *ET in vivo* RNAi is protective in a gastrointestinal infection model and enhances eye overgrowth phenotype caused by ectopic *upd* expression. *A, B*) *ET* RNAi protects the flies from *S. marcescens* infection. Two strains of *ET* RNAi flies, *ET IR*^{1} (*A*) and *IR*^{2} (*B*), show enhanced survival from gastrointestinal infection caused by *S. marcescens* as compared to flies with the same genetic background with no RNAi induction. Flies with induced *Stat92E* RNAi show decreased survival, as well as *eater* mutant flies, which were used as controls. *ET* RNAi flies were crossed over the drug-inducible driver *GeneSwitch-GAL4*. Mifepristone was used to induce the expression of the RNAi construct. w^{1118} and *Stat92E* RNAi flies crossed over *GeneSwitch-GAL4* flies were used as an additional control. *C*) *ET* RNAi enhances upd-induced eye overgrowth. Eye overgrowth phenotype caused by overexpression of *upd* in the developing eye (*GMR-updΔ3'*) is more severe in flies that also express an *ET* RNAi construct with an eye-specific driver (*ey-GAL4*). *D*) The eye phenotype in flies with ectopic expression of both *upd* and *ET* RNAi in their eyes was significantly more severe than in flies with *upd* alone. Grades: 0 = wild-type, 5 = most severe eye phenotype. $n = 8$ for CantonS flies and $n \geq 16$ for *GMR-updΔ3'* and *GMR-updΔ3'* + *ET* IR^{1} flies. Data are shown as means ± SD. $^{***}P < 0.001$ *vs. GMR-updΔ3'*.

conserved intracellular part of the signaling cascade. To identify the most important regulators, we subjected the genes, whose RNAi repeatedly caused >50% decrease on the luciferase activity, to further studies. The original findings of the screen were confirmed *in vitro* by several means using different dsRNAs, different reporters, and different ways to induce the JAK/STAT signaling. In this way, we identified 5 novel putative regulators of *Drosophila* JAK/STAT signaling. This is a reasonable number of gene products that can be directly involved in JAK/STAT pathway regulation and probably excludes factors that affect JAK/STAT pathway activity indirectly *via* crosstalk with other signaling pathways. In addition, our screen found 2 previously known intracellular components of the pathway, *hop* and *Stat92E,* confirming the validity of our screen. Notably, we identified the gp130/IL-6R related transmembrane protein ET as a novel negative regulator of *Drosophila* JAK/STAT pathway.

ET is a negative regulator of *Drosophila* JAK/STAT signaling

Our RNAi screen was originally set out to identify novel positive regulators of *Drosophila* JAK/STAT pathway. *ET* RNAi caused strong decrease in hop^{Tum-l}-induced *TotM-luc* activity in our primary screen. Curiously, when the JAK/STAT signaling was induced using upd, the ligand of the pathway, the phenotype of *ET* RNAi was the exact opposite. In this setting, *ET* RNAi increased the activity of all reporters tested (*TotM-luc, 10xStat92E-luc,* and *3× 2xDraf-luc*) by at least 5-fold, indicating that the RNAi phenotype was not reporter sensitive, and therefore not likely to be highly context-dependent or due to an artifact. Furthermore, overexpression of *ET* in S2 cells also produced opposite luciferase assay phenotypes with different activating molecules. Even though these results are consistent with the RNAi phenotypes observed, the reason for different phenotypes of *ET* knockdown or overexpression observed depending on the activating molecule remains speculative. However, several pieces of evidence suggest partial explanations for this phenomenon.

In the *Drosophila* genome, the sequence coding for *ET* is located next to that of *Drosophila* JAK/STAT pathway receptor *Dome*. ET polypeptide carries a putative transmembrane domain and shares sequence similarity to Dome, especially in the extracellular domain (11); therefore, ET is likely a result of gene duplication.

Both the proposed protein structure of ET and our experimental data from double-RNAi and coimmunoprecipitation assays suggest that ET functions on the cell membrane, epistatically at the level upd and hop. hop^{Tum-l} causes strong activation of *Drosophila* JAK/STAT signaling, but this induction appears to take a somehow pathological form. Interestingly, Dome was not among the known components identified in our screen for positive regulators. We therefore tested the

RNAi phenotype for *Dome* with both upd and $hop^{Tum\text{-}l}$-induction in S2 cells. As expected, when upd was used, *Dome* RNAi abolished reporter activity. In line with our ET results, when $hop^{Tum\text{-}l}$ was used to activate JAK/STAT signaling, the phenotype for *Dome* RNAi was the opposite, the reporter activity being doubled (data not shown). These results implicate that the mutant hop is likely to behave abnormally in *Drosophila* cells acting independently of Dome, perhaps by using ET's short cytoplasmic tail as a docking site: This results in constitutive activation of JAK/STAT signaling and may explain the $hop^{Tum\text{-}l}$-mutant phenotype. Using ectopic expression of upd as an inducer of JAK/STAT pathway provides more physiological information about ET's function, and thus we conclude that ET is a negative regulator of the *Drosophila* JAK/STAT signaling.

ET's exact molecular function in regulation of *Drosophila* JAK/STAT signaling remains to be studied, but since it has putative cytokine binding motifs in the ectodomain, it could function as a decoy receptor that captures upd ligands from Dome. ET could also inhibit Dome activation by forming a nonsignaling heterodimer with Dome, or by inhibiting Dome homodimer-hop signalosome in some other manner. Since ET coimmunoprecipitated with Dome but did not affect Dome homodimerization, or interaction of hop with Dome, the latter statement appears more likely.

Regulation of JAK/STAT signaling in *Drosophila* and mammals: common mechanisms

The core JAK/STAT signaling pathway is evolutionarily conserved. Because of its role in diverse cellular processes, the JAK/STAT pathway needs to be strictly controlled at different levels of the cascade. Several regulatory mechanisms appear to be conserved from flies to humans, as many of the positive and negative regulators of *Drosophila* JAK/STAT signaling have been identified based on their homology with the mammalian counterparts. The *Drosophila* model has also provided important information about regulation of the JAK/STAT pathway. For example, the first evidence for the critical role of the JH2 pseudokinase domain in regulation of JAK activity and hematopoietic homeostasis was obtained in *Drosophila* (51). Of the known conserved negative regulators of the JAK/STAT pathway, *SOCS36E* is strongly induced by Stat92E, forming a negative feedback loop. dPIAS, on the other hand, interacts directly with Stat92E and affects its nuclear functions. According to our results, ET negatively regulates pathway at the level or upstream to Dome and is not induced *via* a negative feedback loop.

Both Dome and ET show homology with mammalian IL-6 receptor family members and the signal transducer gp130. In mammals, gp130 is able to form functional signaling complexes with several cytokine receptors, such as interleukin-6, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, cardiotrophin-1, the granulocyte colony-stimulating factor, IL-11, and IL-27 (43, 52–56). In these ligand-induced receptor complexes gp130 mediates the activation signal to multiple cytoplasmic signaling molecules *via* activation of JAKs that are constitutively associated with gp130 cytoplasmic domain. In *Drosophila,* the signaling cascade is simpler, and Dome is the only characterized JAK/STAT pathway receptor (22, 23). Dome homodimerization is needed for a proper upd-induced signal transduction and Stat92E activation (23), but it is still unclear if Dome participates in larger multisubunit receptor complexes resembling those seen in mammals.

The role of ET in *Drosophila in vivo*

Notably, we also assessed ET's role in *Drosophila* JAK/STAT signaling *in vivo*. Two *ET* RNAi strains crossed over a driver expressed in the fatbody and lymph glands (*C564-GAL4*) or a ubiquitous drug-inducible driver (*GeneSwitch-GAL4*) were tested by qRT-PCR for their effect on JAK/STAT pathway target gene expression in response to septic injury with *E. cloacae*. Both *ET* RNAi lines showed a significant increase in JAK/STAT pathway-dependent stress response compared to controls, indicating hyperactivation of JAK/STAT signaling. More direct *in vivo* model to study JAK/STAT activity in *Drosophila* is overexpression of *upd* in the developing eye, which leads to eye overgrowth due to hyperactivated JAK/STAT signaling (48). The eye overgrowth phenotype is shown to be modified by overexpression and removal of several JAK/STAT pathway components and regulators (32, 48–50). Notably, overexpression of *upd* together with *ET* RNAi construct under an eye-specific driver *ey-GAL4* lead to a significantly more severe eye overgrowth than overexpression of *upd* alone. In addition, *ET* RNAi appears to have a protective role in an *S. marcescens* gastrointestinal infection model. Enhanced JAK/STAT signaling activity may be advantageous in *S. marcescens*-infected flies for renewal of the injured gut wall (45). Accordingly, *Stat92E* RNAi flies were more susceptible to infection in this assay. It is possible, however, that the protective effect of *ET* RNAi is due to another mechanism unrelated to JAK/STAT signaling.

In summary, we identified 5 putative novel regulators of *Drosophila* JAK/STAT signaling in this study. Of these, ET is a negative regulator of JAK/STAT pathway signaling both *in vitro* and *in vivo*. ET is involved in Stat92E phosphorylation and coimmunoprecipitates with Dome and hop. The exact molecular mechanisms of how ET regulates Stat92E phosphorylation remains to be studied. FJ

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Not4 enhances JAK/STAT pathway-dependent gene expression in *Drosophila* and in human cells

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ABSTRACT **The JAK/STAT pathway is essential for organogenesis, innate immunity, and stress responses in *Drosophila melanogaster*. The JAK/STAT pathway and its associated regulators have been highly conserved in evolution from flies to humans. We have used a genome-wide RNAi screen in *Drosophila* S2 cells to identify regulators of the JAK/STAT pathway, and here we report the characterization of Not4 as a positive regulator of the JAK/STAT pathway. Overexpression of *Not4* enhanced Stat92E-mediated gene responses *in vitro* and *in vivo* in *Drosophila*. Specifically, Not4 increased Stat92E-mediated reporter gene activation in S2 cells; and in flies, *Not4* overexpression resulted in an 8-fold increase in *Turandot M* (*TotM*) and in a 4-fold increase in *Turandot A* (*TotA*) stress gene activation when compared to wild-type flies. *Drosophila* Not4 is structurally related to human CNOT4, which was found to regulate interferon-γ- and interleukin-4-induced STAT-mediated gene responses in human HeLa cells. Not4 was found to coimmunoprecipitate with Stat92E but not to affect tyrosine phosphorylation of Stat92E in *Drosophila* cells. However, Not4 is required for binding of Stat92E to its DNA recognition sequence in the *TotM* gene promoter. In summary, Not4/CNOT4 is a novel positive regulator of the JAK/STAT pathway in *Drosophila* and in humans.—Grönholm, J., Kaustio, M., Myllymäki, H., Kallio, J., Saarikettu, J., Kronhamn, J., Valanne, S., Silvennoinen, O., Rämet, M. Not4 enhances JAK/STAT pathway-dependent gene expression in *Drosophila* and in human cells. *FASEB J.* 26, 1239–1250 (2012). www.fasebj.org**

Key Words: Ccr4-Not complex · cytokine signaling · Stat92E · stress response

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has been well conserved during evolution from flies to humans. The human JAK/STAT cascade transduces signals from numerous hematopoietic cytokines and growth factors, leading to changes in target gene expression (1). Consequently, deregulation of the JAK/STAT pathway has been associated with several diseases, including cancer; myeloproliferative neoplasms; severe immune deficiencies, such as severe combined immune deficiency; allergies; and autoimmunity (2–5).

The human JAK/STAT pathway consists of 4 JAKs and 7 different STATs (6), whereas in the fruit fly *Drosophila melanogaster*, the JAK/STAT pathway consists of only one cytokine receptor-like transmembrane protein, domeless (Dome); one Janus kinase, termed hopscotch (Hop); and a single STAT transcription factor, Stat92E (7–11). In *Drosophila*, the JAK/STAT pathway orchestrates a diverse set of developmental events and cellular processes, including embryonic segmentation, larval hematopoiesis, sex determination, organogenesis, and the regulation of stem cell maintenance (12–14). As in mammals, the JAK/STAT pathway is also required for the control of immune and stress responses, including viral defense in *Drosophila* and other insects (15–16). An immune challenge induces the expression of several JAK/STAT pathway target genes, including the complement-like protein *thioester-containing protein 2* (*Tep2*) and *Turandot* (*Tot*) stress genes (17, 18).

Abbreviations: CMV-β-gal, cytomegalovirus-β-galactosidase; Dome, domeless; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; EMSA, electrophoretic mobility shift assay; ET, eye transformer; GAS-luc, γ-activated sequence-luciferase; GFP, green fluorescent protein; Hop, hopscotch; Hop^{Tum-l}, hopscotch tumorous-lethal; IFN-γ, interferon-γ; Ig, immunoglobulin; IL-4, interleukin-4; JAK, Janus kinase; mRNA, messenger RNA; PIAS, protein inhibitor of activated STAT; RING, really interesting new gene; RRM, RNA recognition motif; S2 cell, Schneider 2 *Drosophila* cell; SOCS, suppressor of cytokine signaling; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; STAT, signal transducer and activator of transcription; Tep2, thioester-containing protein 2; Tot, Turandot; Upd, unpaired.

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In the canonical model for JAK/STAT signaling, pathway activation occurs when a secreted ligand binds to a transmembrane receptor, leading to the dimerization or multimerization of the receptor molecules. The receptor-associated JAK molecules are then brought into a position that allows them to transphosphorylate each other and to phosphorylate tyrosine residues in the cytoplasmic portion of the receptor molecules that serve as docking sites for the Src homology 2 (SH2) domains of cytoplasmic STAT molecules. The receptor-bound STATs are activated by JAK-mediated tyrosine phosphorylation, which induces the dimerization and nuclear translocation of STATs. In the nucleus, STAT dimers bind to specific enhancer elements in the DNA and act as regulators of transcription (6). In *Drosophila,* 3 variants of Dome receptor ligands have been characterized, named unpaired 1–3 (Upd1–3; refs. 19–21). Upd binding to Dome leads to a rapid conformational change in dimerized Dome molecules and to the activation of the *Drosophila* JAK/STAT pathway (22).

The JAK/STAT pathway is tightly regulated in both the mammalian and *Drosophila* systems, which share many conserved regulatory factors. Known regulators include a family of suppressor of cytokine signaling (SOCS) molecules, consisting of 8 proteins in humans. Human SOCSs are usually transcriptionally regulated by the JAK/STAT pathway, and thus form a negative feedback loop (23, 24). In *Drosophila*, Socs36E has been reported to participate in JAK/STAT pathway regulation in a similar manner (25). Protein inhibitor of activated STAT (PIAS) proteins are known negative regulators of the JAK/STAT pathway, and *Drosophila* PIAS is shown to negatively regulate Stat92E, but the mechanism for this is still elusive (26). In humans, PIAS1 is known to affect STAT1 DNA binding and to act as an E3 SUMO ligase in the sumoylation of STAT1 (27). Like its mammalian counterpart, Stat92E was recently shown to be negatively regulated by a SUMO modification (28). Other known negative regulators of the *Drosophila* JAK/STAT pathway are the protein-tyrosine phosphatase 61F (Ptp61F) and an N-terminally truncated Stat92E (ΔN-Stat92E), which functions as a dominant-negative form of Stat92E (29, 30).

Recently, a new negative regulator of the *Drosophila* JAK/STAT pathway, called eye transformer (ET), was found in a genome-wide RNAi screen (31). Here, we characterize another novel regulator of the JAK/STAT pathway, CG31716, which encodes the *Drosophila* Not4 protein. *Drosophila* Not4 shares homology with the human CNOT4 protein. CNOT4 is a member of the evolutionarily conserved multifunctional Ccr4-Not complex, composed of 9 core subunits, originally found in yeast (32). The Ccr4-Not complex takes part in multiple cellular processes, including DNA repair, histone methylation, regulation of messenger RNA (mRNA) degradation, transcription initiation, and transcription elongation in yeast (32, 33). CNOT4 is also known to harbor a ubiquitin E3 ligase activity in its N-terminal really interesting new gene (RING) domain (34). In this study, we show that Not4 enhances JAK/STAT-mediated gene responses in *Drosophila* both *in vitro* and *in vivo*. Furthermore, we provide evidence that CNOT4 also participates in the regulation of JAK/STAT signaling in human cells.

MATERIALS AND METHODS

Plasmid constructs and double-stranded RNA (dsRNA) synthesis

The *Drosophila* CG31716/Not4 cDNA (clone RE04975) was purchased from the *Drosophila* Genomics Resource Center (Indiana University, Bloomington, IN, USA), was amplified by PCR using 5′-CACACAGCGGCCGCATGAACGGCCTGAGCAGC-3′ containing a *Not*I restriction site and 5-′CACACATCTAGAAACGAATTGACGGCTTTTTAAAAACG-3′ containing an *Xba*I restriction site, and was cloned into the pMT-V5-HisA expression vector (Invitrogen, Carlsbad, CA, USA). pMT-Stat92E-Flag (28), Hop tumorous-lethal (Hop^{Tum-l}), pMT-Upd, *TotM-luc*, *10XStat92E-luc*, and *Act5C-β-gal* plasmids (31), as well as *γ-activated sequence-luciferase* (*GAS-luc*), *cytomegalovirus-β-galactosidase* (*CMV-β-gal*), and *immunoglobulin* (*Ig*)*ε-luc* plasmids, have been described previously (27, 35). The Not4 deletion constructs ΔRING, ΔRINGΔRNA recognition motif (RRM), and ΔC terminus were cloned from an existing pMT-Not4 expression vector into the *Spe*I and *Xho*I sites (ΔRING, ΔRINGΔRRM) or the *Xba*I and *Eco*RI sites (ΔC terminus) of the *Drosophila* expression vector pMT-V5-HisA. The primers used for cloning the 3 deletion constructs were ΔRING, 5′-CACACAACTAGTATGCCAGCTGACTTCAAGCCGCT-3′ and 5′-CACACACTCGAGAACGAATTGACGGCTTTTTAAAAACG-3′; ΔRINGΔRRM, the same forward primer as for ΔRING and 5′-CACACAACTAGTATGACCAGCTTGGGGACAACC-3′; ΔC terminus, 5′-CACACAGAATTCATGAACGGCCTGAGCAGC-3′ and 5′-CACACATCTAGACAATTCATGTAAGTACATGCAGTCG-3′. *Not4–5′ UTR* dsRNA was synthesized from Schneider 2 *Drosophila* (S2) cell cDNA using primers 5′-T7+ATAGTTTGAGCTGTGG-3′ and 5′-T7+TTGATTAGTGATGAAG-3′; otherwise, the RNAi primers and dsRNA synthesis were as described elsewhere (31).

Cell culture, creation of stably overexpressing S2 cell lines

Drosophila S2 cells were cultured in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), as described previously (31). Stable pMT-V5-HisA control and pMT-Not4-V5-overexpressing cell lines were created by transfecting 5.0×10^6 S2 cells with 9.5 μg of the pMT-V5-HisA or pMT-Not4-V5 plasmids together with 0.5 μg of the pCoHygro plasmid. After 72 h, the medium was changed to Schneider's medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml hygromycin-B (Calbiochem, San Diego, CA, USA), and plates were cultured at 25°C. All S2 cell transfections were carried out with the FuGENE6 transfection reagent (Roche, Basel, Switzerland), according to the manufacturer's instructions. HeLa cells were cultured at 37°C in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 50 mg/ml streptomycin.

Luciferase assay

Transfections, dsRNA treatments, and *TotM-luc/10xStat92E-luc* reporter activity measurements were carried out as described previously (31). For luciferase assay experiments with overexpressed Not4, 0.2 μg of Not4-V5, Not4 deletion constructs, or a control plasmid was cotransfected into S2 cells;

otherwise, the transfection and measurements were carried out similarly to a protocol described earlier. HeLa cells (0.1×10^6) were transfected with 0.2 μg of *GAS-luc* or *Igε-luc* reporter plasmid with 0.1 μg of the *CMV-β-gal* reporter plasmid for monitoring cell viability and transfection efficiency simultaneously with 20 pmol of the indicated control or experimental siRNA. After 24 h, cells were serum starved for 24 h and stimulated with 100 ng/ml of human interferon-γ for 6 h or human IL-4 for 24 h prior to lysis. Luciferase and β-gal values were measured as described previously (31). Luciferase assays for the Toll and the Imd pathways were carried out as described previously (36).

Fly stocks and infections

All *Drosophila* stocks were reared at 25°C on a standard mashed potato diet [4% w/v mashed potato powder, 1.1% w/v dried yeast, 6% w/v dark syrup, 1% w/v agar and 0.85% w/v 10% methyl 4-hydroxybenzoate (Sigma-Aldrich) in ethanol].

The *UAS-Not4* transgenic stocks were obtained from Best Gene, Inc. (Chino Hills, CA, USA). To generate these, *Not4* was amplified by PCR and cloned into a pUAST vector (37) using the following PCR-primers: 5′-CACACAGCGGCCGCATGAACGGCCTGAGCAGC-3′, containing a *Not*I restriction site, and 5′-CACACATCTAGAAACGAATTGACGGCTTTTTAAAAACG-3′, containing an *Xba*I restriction site; for *Not4-myc*, 5′-GGCCGGTCTAGATTATTACAAGTCCTCTTCAGAAATGAGCTTTTGCTCAACGAATTGACGGCTTTTTAAAAACG-3′, containing a myc-tag sequence together with an *Xba*I restriction site, was used as a 3′-primer. Seven independent transformant lines with a *UAS-Not4* construct (lines *UAS-Not4*^{1} to *UAS-Not4*^{7}) incorporated into their genome and 6 lines carrying a *UAS-NOT4*-myc construct (lines *UAS-Not4-myc*^{1} to *UAS-Not4-myc*^{6}) were used to study the effect of *Not4* overexpression *in vivo*. Using the UAS-GAL4 binary system (37), we crossed *UAS-Not4* overexpression lines with *C564-GAL4* driver lines, to achieve overexpression of Not4 in the fat body of the progeny flies, as described previously (31). Activation of the JAK/STAT pathway by infecting 3- to 6-d-old flies with *Enterobacter cloacae* has also been described previously (31).

Total RNA extraction from flies

For RNA extractions, 3 female and 3 male flies per sample were anesthetized with CO_2 and snap-frozen on dry ice. Total RNA was isolated by homogenizing the flies in 300 μl of TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) by grinding with a micropestle grinder and then continuing with the RNA extraction according to the reagent manufacturer's instructions; 1;40 dilutions of the fly total-RNA samples were used for quantitative RT-PCR (qRT-PCR).

qRT-PCR

qRT-PCR for *TotM, TotA, Not4,* and *Act5C* levels was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) and the ABI7000 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers used for *Not4* quantification were 5′-ATGGCAAAGAGGCGGCTG-3′ and 5′-GCTGCTTCTCGTTTCGGG-3′. Other primers (*Act5C, TotM, TotA*) were as described previously (31).

Coimmunoprecipitation and immunodetection

For coimmunoprecipitation experiments, Not4-V5 expression from the stable pMT-Not4-V5-overexpressing S2 cells was induced by adding 500 μM $CuSO_4$ into the Schneider's growth medium. After 48 h, cells were lysed with coimmunoprecipitation lysis buffer (20 mM HEPES, pH 8.0; 100 mM NaCl; 1% TritonX-100; 10% glycerol; 1 mM EDTA; and 50 mM NaF) supplemented with vanadate, PMSF, and aprotinin. Protein concentrations were determined by a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equal amounts of protein were immunoprecipitated with an anti-Stat92E-N-terminal antibody (dN-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at +4°C following incubation with Protein G Sepharose beads (GE Healthcare, Little Chalfont, UK). Coimmunoprecipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany), and immunodetected with an anti-V5 antibody (Invitrogen, Carlsbad, CA, USA), as described previously (31).

Electrophoretic mobility shift assay (EMSA)

For EMSA, double-stranded DNA (dsDNA) probes were generated by annealing 5′-CAAAAAAACAGTTCTTAGAATGCAATCAATAC-3′ and 5′-GTATTGATTGCATTCTAAGAACTGTTTTTTTG-3′ oligos containing the Stat92E binding site (TTCTTAGAAT) from the *TotM* gene promoter region and end-labeled by T4-polynucleotide kinase using [γ-^{32}P]-labeled adenosine triphosphate. S2 cells (5×10^6) were transfected with 2.0 μg of $Hop^{Tum\text{-}l}$ or control plasmid together with 5.0 μg of indicated dsRNAs. After 48 h, cells were lysed with buffer containing 10 mM HEPES-KOH (pH 8.0), 10 mM KCl, 0.1% Nonidet P-40, 1 mM EDTA supplemented with 10 mM NaF, 1 mM DTT, vanadate, aprotinin, and PMSF. After 1 min of centrifugation at 10,000 *g*, the supernatant was collected as a cytoplasmic extract. The pellet was then resuspended into a buffer containing 20 mM HEPES-KOH (pH 8.0), 400 mM NaCl, 10% glycerol, 1 mM DTT, and 10 mM NaF, supplemented with vanadate, aprotinin, and PMSF, and rotated at +4°C for 30 min following centrifugation for 3 min at 14,000 *g* in order to obtain a nuclear lysate. Cytoplasmic and nuclear lysates were subjected to a binding reaction in a 3:1 ratio together with the radiolabeled dsDNA oligo with or without 200 ng of an anti-Stat92E-N-terminal antibody (dN-17; Santa Cruz Biotechnology) in binding buffer (20 mM HEPES-KOH, pH 8.0; 50 mM NaCl; 10% glycerol; 0.01% Triton X-100; and 1 mM DTT) for 30 min at room temperature. DNA-probe-bound proteins were then separated in a 6% PAGE in 0.5× TAE buffer, followed by autoradiography.

A dsDNA probe containing mutated Stat92E binding site was generated by annealing 5′-CAAAAAAACAGGGGAATCCCAGCAATCAATAC-3′ and 5′-GTATTGATTGCTGGGATTCCCCTGTTTTTTTG-3′ oligos. For competition assay, an unlabeled wild-type probe and unlabeled mutated probe were added to the binding reactions together with the labeled wild-type probe. Otherwise, the assay and analysis were carried out as described above.

Data analysis

Statistical analyses of reporter assays and qRT-PCR results were carried out using 1-tailed Student's *t* test with Welch's correction or 1-way ANOVA. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

CG31716/Not4 positively regulates Stat92E-inducible genes in S2 cells

The *Drosophila* gene *CG31716,* encoding the Not4 protein, was originally identified as a potential regulator of

the JAK/STAT pathway in a genome-wide RNAi screen in *Drosophila* S2 cells (31). To confirm the initial finding, we first performed controlled *Not4*-knockdown experiments. dsRNA-mediated RNAi targeting the *Not4* resulted in a >50% decrease in *TotM-luciferase* values on Hop^{Tum-l} induction when compared to the effect of a dsRNA targeting the *green fluorescent protein* (*GFP*), which was used as a control (**Fig. 1*A***). This finding prompted us to further investigate the role of *Not4* in the regulation of JAK/STAT-mediated signal transduction. First, a *10xStat92E-luc* reporter consisting of a sequence containing 10 doublets of Stat92E binding sites from the *SOCS36E* promoter region was used to measure the JAK/STAT pathway activity in response to Hop^{Tum-l} transfection. As shown in Fig. 1*B*, Hop^{Tum-l} expression induced robust *10xStat92E-luc* activation, which was strongly inhibited by *Stat92E* RNAi. Notably, the dsRNA targeting *Not4* caused a nearly 50% reduction in the *10xStat92E-luc* activity compared to the *GFP* RNAi control. To study the effect of *Not4* RNAi on the JAK/STAT pathway in more physiological conditions, the pathway was activated by cotransfecting Upd, the ligand of the transmembrane receptor Dome, together with different dsRNAs and reporter plasmids. In this setting, the activity of both of the Stat92E-dependent luciferase reporters, *TotM-luc* and *10xStat92E-luc*, was significantly reduced by a dsRNA treatment targeting *Not4* (Fig. 1*C*, D). dsRNAs against the previously published JAK/STAT pathway regulator *eye transformer* (*ET*; ref. 31) and against *Stat92E* were used as positive controls and dsRNAs against *GFP* as a negative control.

To investigate whether *Not4* RNAi affects gene expression in a more general manner, we induced Imd pathway activity by adding heat-killed *Escherichia coli* to S2 cells and measured the Imd pathway target-gene *Attacin* promoter-driven luciferase activity. As shown in Supplemental Fig. S1, *Not4* RNAi did not affect Imd pathway signaling in this setting. Of note, we observed a minor decrease in Toll pathway activity with both *Stat92E* and *Not4* dsRNA treatments. In summary, *Not4* RNAi does not have a general effect on gene expression in S2 cells. The meaning of the results related to Toll pathway signaling warrants further studies.

To verify that our results were not caused by artifacts in the reporter analysis, we studied the effect of *Not4* RNAi on the expression level of the endogenous Stat92E target gene *TotM* in S2 cells using qRT-PCR. S2 cells were transfected with dsRNA targeting *Not4* or with control dsRNAs together with Hop^{Tum-l} to activate Stat92E. *TotM* expression levels were normalized against endogenous *Act5C* expression levels. As shown in Fig. 1*E*, *Not4* RNAi significantly reduces endogenous

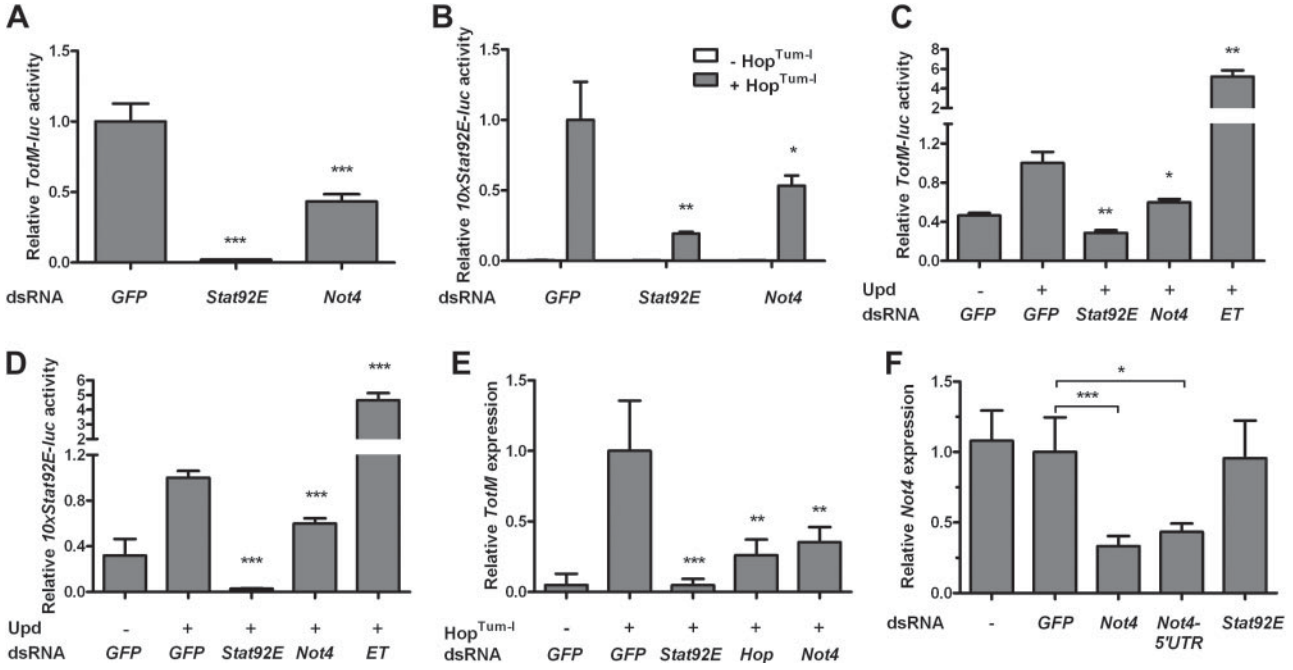

Figure 1. Depletion of *Drosophila* Not4 inhibits Stat92E-mediated gene expression in S2 cells. *A*) *TotM-luciferase* reporter analysis of dsRNA-treated S2 cells with constitutively active Hop^{Tum-l} induction. Cultured S2 cells were cotransfected with the *TotM-luciferase* reporter plasmid together with the *Act5C*-β-*galactosidase* reporter, the Hop^{Tum-l} expression vector, and the indicated dsRNAs. *Not4* dsRNA results in a statistically significant decrease in TotM-luciferase activity. *Stat92E* RNAi is shown as a positive control. *B*) Luciferase reporter analysis using the *10xStat92E-luc* reporter with or without Hop^{Tum-l} overexpression with indicated dRNAs. *C*) *TotM-luc* expression in response to coexpression of the ligand Upd. *D*) *10xStat92E-luc* expression in response to coexpression of Upd. *E*) Endogenous *TotM* gene expression in the presence of *Not4* dsRNA. S2 cells were transfected with the Hop^{Tum-l} expression vector to activate the JAK/STAT pathway and dsRNAs as indicated. qRT-PCR was used to monitor TotM-levels. *F*) Expression of endogenous *Not4* in the presence of two separate dsRNAs targeting different sequences in *Not4*. *Not4* marks the dsRNA used in the original screen and validation (31), whereas *Not4* 5′-UTR dsRNA is used in this study. S2 cells were transfected with the indicated dsRNAs, and Not4 mRNA levels were measured by qRT-PCR. Error bars indicate SD from 3–6 independent transfections. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

TotM gene expression in S2 cells after Hop^{Tum-l} induction, indicating that Not4 is required for Stat92E-mediated gene expression. As shown in Fig. 1*F*, endogenous *Not4* expression is efficiently inhibited by transfected *Not4* dsRNA in S2 cells.

To study the effect of *Not4* overexpression on Stat92E-mediated gene expression in S2 cells, we cloned the *CG31716/Not4* gene into the pMT-V5-HisA expression vector. As shown in **Fig. 2*A, B***, *Not4* overexpression enhanced both *TotM-luc* and *10xStat92E-luc* reporter activities in response to Hop^{Tum-l} induction in S2 cells. Ectopic *Not4* expression caused the activation of both of the reporters without Hop^{Tum-l} expression. Similar results were obtained in response to Upd ligand induction (Fig. 2*C, D*). In summary, *Not4* RNAi decreases Stat92E-dependent reporter gene activation and endogenous Stat92E target gene expression in stimulated S2 cells, while overexpression of *Not4* activates Stat92E-dependent reporter gene expression. These results indicate that Not4 positively regulates the *Drosophila* JAK/STAT pathway in S2 cells.

Overexpression of *Not4* enhances Stat92E-dependent gene expression *in vivo*

Next, we studied the effect of Not4 on JAK/STAT pathway target gene expression *in vivo* in *Drosophila*. For this, 8 independent *UAS-Not4* transformant lines (*UAS-Not4*^{1} to *UAS-Not4*^{7} and *UAS-Not4*-myc^{1}) were crossed with a *C564-GAL4*-driver strain, which activates transgene expression in the fat body of progeny flies. First, expression of the transgene in the *UAS-Not4/C564-GAL4* flies was verified by measuring the relative transcriptional levels of *Not4* in total RNA extracts with qRT-PCR. The level of *Actin* mRNA was used for normalization. In all flies overexpressing *Not4*, we observed a 4- to 15-fold increase in *Not4* expression compared to their respective w^{1118} controls. The average fold increase in *Not4* expression was roughly 9-fold (Supplemental Fig. S2*A, B*).

Overexpression of *Not4* resulted in the up-regulation of Stat92E target gene expression in S2 cells (Fig. 2). Septic injury causes the JAK/STAT pathway-dependent activation of *TotM* and *TotA* gene expression in adult flies, and we tested whether *Not4* overexpression affects *TotM* or *TotA* expression *in vivo* (17, 21). To investigate this, we assayed *UAS-Not4/C564-GAL4* adult flies overexpressing *Not4* for *TotM* and *TotA* expression by performing qRT-PCR and compared these expression levels to those in control w^{1118}, w^{1118}*/C564-GAL4,* and *UAS-Not4/*w^{1118} flies. Both unchallenged flies and flies infected with the gram-negative bacteria *Enterobacter cloacae* were analyzed. In accordance with our *in vitro* results, we found that both *TotM* and *TotA* expression were significantly up-regulated in *Not4*-overexpressing *UAS-Not4/C564-GAL4* flies compared to control flies (**Fig. 3*A, B*,** open bars). The results shown were averaged from all 8 *UAS-Not4* lines. A significant increase in *TotM* levels was detected in all transgene-expressing fly lines, and a significant increase in *TotA* levels was seen in most lines, with the exception of lines *UAS-Not4*^{3} and *UAS-Not4*^{5} (Supplemental Fig. S2*C, D*). On average, an 8-fold increase in the expression of *TotM* and an ~4-fold increase in the expression of *TotA* were observed in flies overexpressing *Not4* compared to the progeny of the control cross without the GAL4-driver (*UAS-Not4/*w^{1118}; Fig. 3*A, B*, open bars).

To test whether *Not4* overexpression affects *TotM* or *TotA* expression after septic injury, flies were infected with *E. cloacae*. RNA from these flies was extracted after an incubation period of 16 h, and the transcription levels of *TotA* and *TotM* were measured by qRT-PCR. Again, the results of all of the 8 *UAS-Not4* lines were averaged. We observed an increase in *TotA* and *TotM*

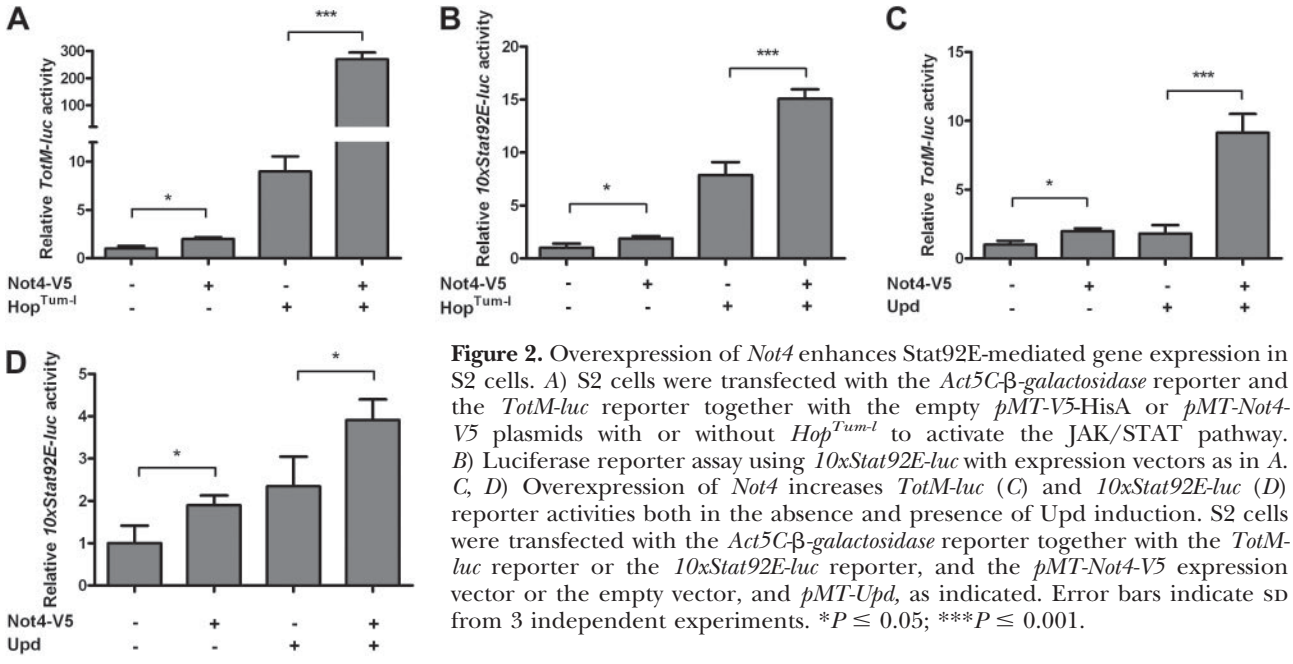

Figure 2. Overexpression of *Not4* enhances Stat92E-mediated gene expression in S2 cells. *A*) S2 cells were transfected with the *Act5C-β-galactosidase* reporter and the *TotM-luc* reporter together with the empty *pMT-V5*-HisA or *pMT-Not4-V5* plasmids with or without Hop^{Tum-l} to activate the JAK/STAT pathway. *B*) Luciferase reporter assay using *10xStat92E-luc* with expression vectors as in *A*. *C, D*) Overexpression of *Not4* increases *TotM-luc* (*C*) and *10xStat92E-luc* (*D*) reporter activities both in the absence and presence of Upd induction. S2 cells were transfected with the *Act5C-β-galactosidase* reporter together with the *TotM-luc* reporter or the *10xStat92E-luc* reporter, and the *pMT-Not4-V5* expression vector or the empty vector, and *pMT-Upd,* as indicated. Error bars indicate SD from 3 independent experiments. $*P \leq 0.05$; $***P \leq 0.001$.

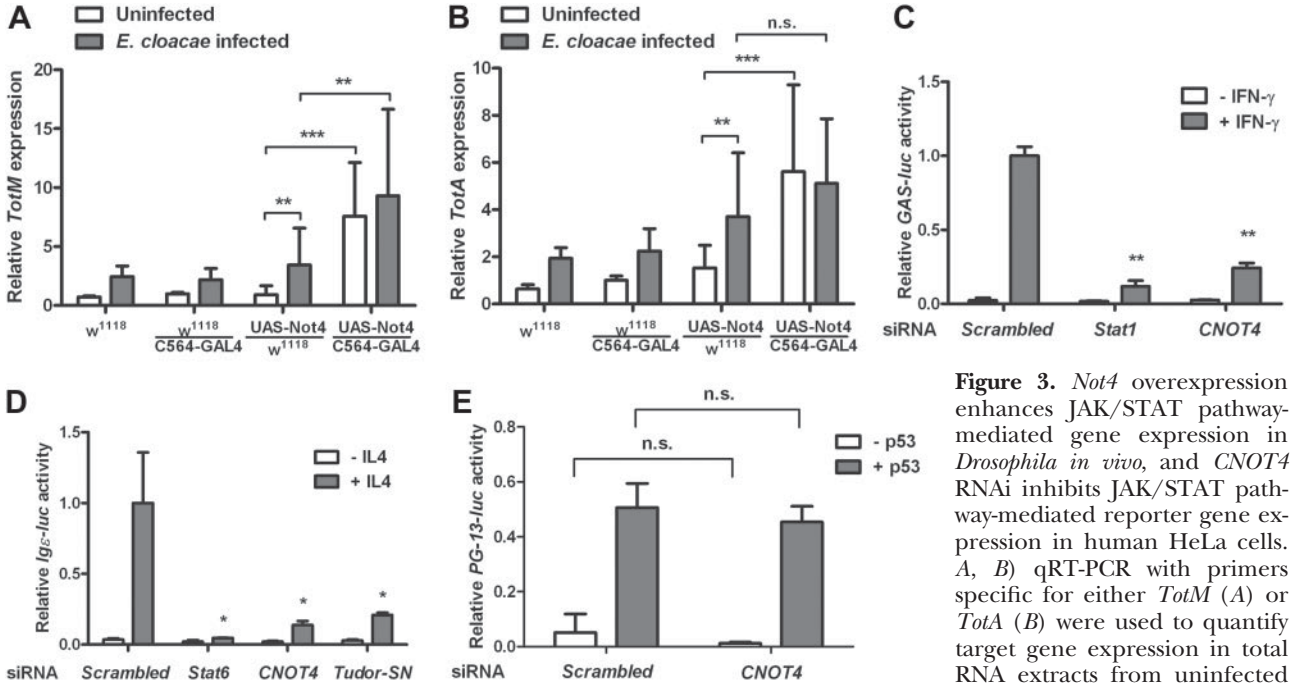

Figure 3. *Not4* overexpression enhances JAK/STAT pathway-mediated gene expression in *Drosophila in vivo*, and *CNOT4* RNAi inhibits JAK/STAT pathway-mediated reporter gene expression in human HeLa cells. *A*, *B*) qRT-PCR with primers specific for either *TotM* (*A*) or *TotA* (*B*) were used to quantify target gene expression in total RNA extracts from uninfected and infected flies overexpressing *Not4* in the fatbody. *C*) Effect of human CNOT4 depletion on IFN-γ-induced *GAS-luc* activity. HeLa cells were transfected with the *GAS-luc* reporter together with the *CMV*-β-*gal* reporter and the indicated siRNAs. After 48 h, STAT1-mediated signaling was activated by adding 100 ng/ml of human IFN-γ to cells for 6 h prior to lysis. *D*) Effect of human CNOT4 depletion on IL-4-induced *Igε-luc* activity. HeLa cells were transfected with the *Igε-luc* reporter plasmid together with *CMV*-β-*gal* and the indicated siRNAs. Activation of the STAT6-responsive *Igε-luc* promoter was induced by stimulating the cells for 24 h with human IL-4. *E*) *CNOT4* RNAi effect on *PG-13-luc* reporter activity. HeLa cells were transfected with the *PG-13-luc* reporter together with the *CMV*-β-*gal* reporter and a p53 expression vector to activate the *PG-13-luc* reporter expression. Error bars indicate SD from 3 independent transfections. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

expression levels in response to septic injury in most fly strains. Following septic injury, there was on average a 2- to 4-fold increase in the expression of both *TotM* and *TotA* in control flies (Fig. 3*A*, *B*, shaded bars). In infected flies, *Not4* overexpression significantly elevated *TotM* expression compared to the control cross (Fig. 3*A*), but no difference was detected in *TotA* expression on septic injury (Fig. 3*B*). Of note, there was no significant difference in *TotA* or *TotM* expression levels between uninfected and infected *Not4*-overexpressing flies. Similarly, no changes in *Not4* expression were observed in response to septic injury (Supplemental Fig. S2*A*). Taken together, these results indicate that *Not4* also up-regulates *Tot* gene expression in *D. melanogaster in vivo*.

Many regulatory factors of the JAK/STAT signaling pathway have been conserved in evolution, and *Drosophila Not4* shares homology with human CNOT4. This prompted us to study whether CNOT4 also takes part in the regulation of mammalian JAK/STAT signaling in human cells. Interferon-γ (IFN-γ) is a cytokine important for macrophage activation, and its transcriptional responses are mediated through STAT1 (27). To study whether CNOT4 takes part in STAT1-mediated signal transduction, we used siRNA transfection to knock down *CNOT4* in HeLa cells with cotransfection of STAT1 responsive *GAS-luc* and *CMV*-β-*gal* reporters. After 24 h, cells were serum starved overnight and stimulated with human IFN-γ for 6 h. After cell lysis, *GAS-luc* activity was measured and normalized against β-galactosidase activity. As shown in Fig. 3*C*, human *CNOT4* RNAi significantly decreases relative *GAS-luc* activity, indicating that knockdown of *CNOT4* impairs human STAT1-mediated signal transduction. Furthermore, a similar effect was observed with interleukin-4 (IL-4) induced *Igε-luc* activity, indicating that human STAT6 signaling is also modulated by CNOT4. To control for any general effects CNOT4 might have on gene expression in HeLa cells, we used a JAK/STAT pathway independent p53-responsive *PG-13-luc* construct, containing 13 synthetic p53 binding sites upstream of a luciferase gene. As shown in Fig. 3*E*, *CNOT4* siRNA does not alter *PG-13-luc* activity either in the presence of the *p53* expression vector or in the presence of a control plasmid. Of note, the β-*gal* expression was not affected by the *CNOT4* RNAi in our experiments. These data suggest that CNOT4 positively regulates JAK/STAT signaling in human cells.

Not4-mediated regulation of the *Drosophila* JAK/STAT pathway is independent of the RING and the RRM domains

Not4 is a 6.2-kb gene that produces a 1051-aa protein that contains a RING-finger domain, an RRM that is known to interact with single-stranded RNA (ssRNA) and single-stranded DNA (ssDNA), as well as proteins,

and a Zn-finger domain mediating possible protein-protein interactions (**Fig. 4*A***). *Drosophila* Not4 shares similar domain architecture with CNOT4 proteins from yeast to humans (38). In contrast to the N terminus, the C-terminal part of Not4 does not contain any known secondary structures and is less conserved. The yeast Not4 and the human CNOT4 have an E3 ubiquitin ligase activity, as the RING domain on CNOT4 can interact with ubiquitin-conjugating enzymes (UbcH5B, UbcH6, and UbcH9 in humans; refs. 34, 39).

The RING domain harbors the essential function of CNOT4, acting most likely as a mediator of ubiquitin E3-E2 ligase interactions (34). We wanted to examine whether the RING domain of *Not4* is also important for the JAK/STAT pathway in *Drosophila* cells. For this, we generated 3 deletion mutants of the *Not4* gene: deletion of the RING domain (ΔRING, aa 63–1051), deletion of the RING domain and the RRM (ΔRINGΔRRM, aa 186-1051), and one construct containing both the RING and RRM domains but missing the whole C-terminal part (ΔC terminus, aa 1–215). The proper expression of these constructs was determined by Western blot (Fig. 4*B*). To study the effect of these deletions on the *Drosophila* JAK/STAT pathway, the constructs were cotransfected into *Drosophila* S2 cells together with the *TotM-luc* reporter construct in addition to the Hop^{Tum-l} expression vector to induce the JAK/STAT pathway. Samples were treated with *Not4 5′ UTR* dsRNA to block expression of the endogenous *Not4* or *GFP* dsRNA for negative controls. An *Act5C*-β-galactosidase reporter

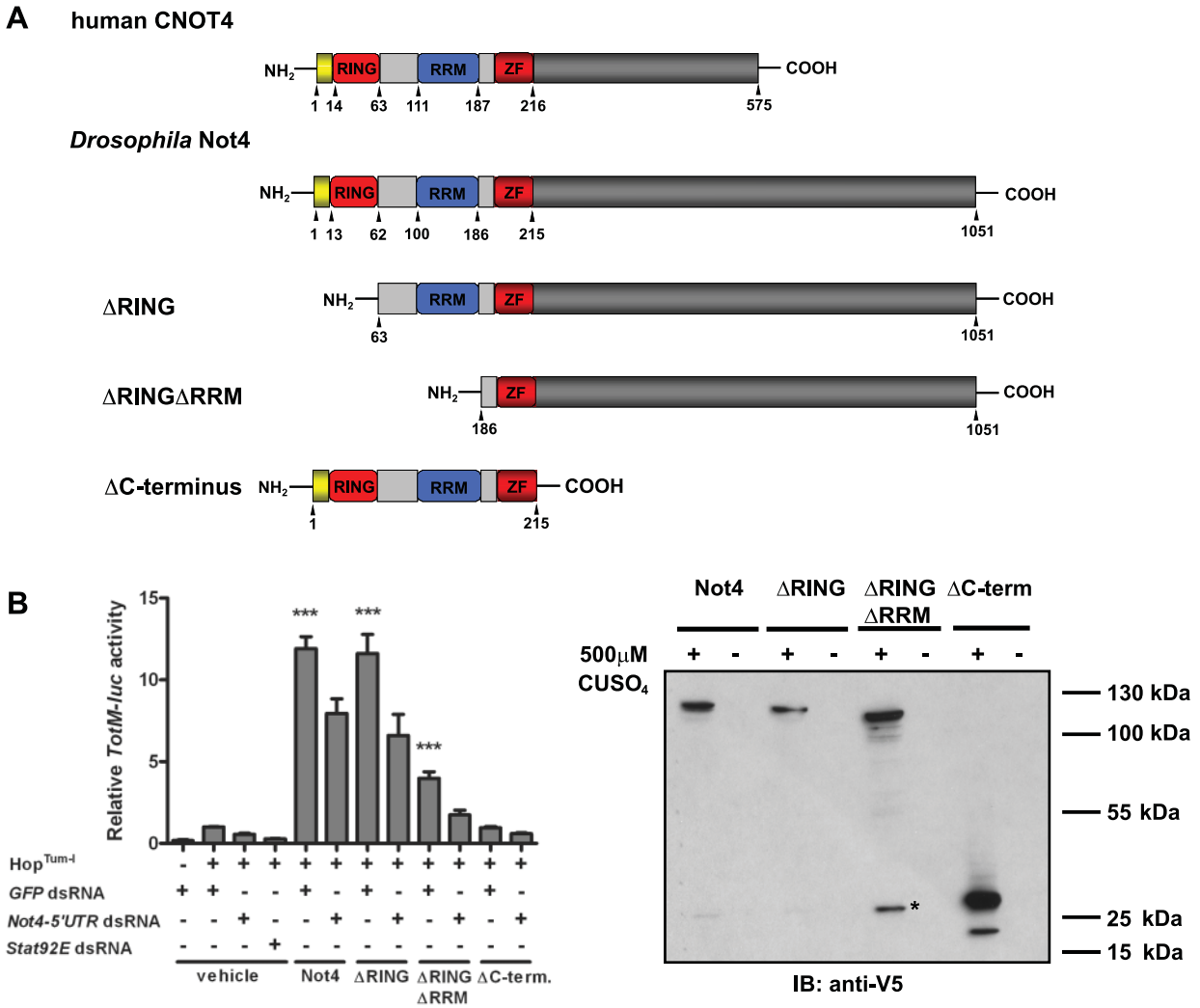

Figure 4. RING-finger and RRM domains in Not4 are not essential for JAK/STAT pathway regulation. *A*) Illustration of the domain organization of *human* CNOT4, full-length *Drosophila* Not4, and *Drosophila* Not4 deletion constructs. *Human* CNOT4 and *Drosophila* Not4 share 3 highly conserved domains: a RING-finger domain in the N terminus of the protein followed by an RRM domain and a zinc-finger domain (ZF). We generated 3 different deletion constructs of Not4, of which Δ*RING* lacks the RING domain, Δ*RING*Δ*RRM* lacks both the RING and the RRM domains, and Δ*C terminus* lacks the whole C-terminal part of the protein. The effect of these deletion constructs on reporter expression was then analyzed in S2 cells. *B*) *TotM-luc* reporter analysis of S2 cells cotransfected with the *TotM-luc* reporter plasmid, the *Act5C*-β-*gal* reporter, the Hop^{Tum-l}, the indicated dsRNAs, and *pMT-Not4* or the indicated *Not4* deletion constructs. Error bars indicate SE from 8 independent transfections. Expression of the wild-type Not4-V5 and indicated deletion constructs were verified using Western blot analysis; asterisk indicates a nonspecific band. ***$P \leq 0.001$.

was used for normalization. *Stat92E* dsRNA-treated cells were used as controls. Luciferase and β-galactosidase activities were measured at 72 h after transfection. As shown in Fig. 4*B*, transfection with pMT-Not4 significantly increased *TotM-luc* reporter activity. Transfection of the ΔRING deletion construct caused a similar increase in *TotM-luc* reporter activity, as did transfection with the intact gene (Fig. 4*B*). This suggests that this deletion construct still has the domains and functions required for the regulation of JAK/STAT target gene expression. Transfection with the deletion construct ΔRINGΔRRM also resulted in an increase in *TotM-luc* reporter activity compared to transfection with an empty vector, albeit to a lesser extent than the transfection with a full-length pMT-Not4 construct (Fig. 4*B*). Hence, Not4 lacking both the RING and the RRM domains still retains some functions needed for the positive regulation of JAK/STAT signaling. We also studied the effect of Not4 deletion constructs on *10xStat92E-luc* activity on Upd induction in S2 cells. Similarly to the *TotM-luc* reporter, overexpression of ΔRING and ΔRINGΔRRM deletion constructs caused a significant increase in *10xStat92E-luc* activity when compared to the transfection control, but the effect was slightly smaller than that obtained with overexpression of full-length *Not4* (data not shown). Conversely, transfection with the ΔC-terminal deletion construct seemed to have neither a positive nor a negative effect on *TotM/10xStat92E-luc* reporter activity. These data suggest that the Not4-mediated regulation of the *Drosophila* JAK/STAT pathway is independent of the RING and the RRM domains.

Drosophila Not4 interacts with Stat92E

It has been suggested that Not4 is localized in the cytoplasm of S2 cells (40). On the basis of our immunostaining results from stable pMT-Not4-V5 overexpression S2 cells, the majority of the Not4 protein is indeed localized in the cytoplasm in S2 cells, but a minor nuclear portion was also detected (data not shown). To determine whether Not4 interacts with Stat92E, S2 cells overexpressing a Not4-V5 or a pMT-V5-HisA vector control were lysed, and the endogenous Stat92E was immunoprecipitated using an anti-Stat92E-N-terminal antibody. Immunoprecipitated proteins were separated by SDS-PAGE following immunoblotting using an anti-V5 antibody to detect coimmunoprecipitated Not4. As shown in **Fig. 5*A***, Not4-V5 coimmunoprecipitates with endogenous Stat92E.

Next, we wanted to study whether JAK/STAT pathway activation affects the Stat92E-Not4 interaction. For this experiment, cells overexpressing pMT-Not4-V5 were transfected either with a vehicle or with increasing amounts of pMT-Upd in order to activate the JAK/STAT pathway. After 48 h, cells were lysed, and the coimmunoprecipitation experiment was carried out as described earlier. As shown in Fig. 5*B*, the amount of Stat92E interacting with Not4 increases on Upd stimulation. Also, the total amount of Not4-V5 was observed to be higher after stimulation of cells with Upd. Of note, *Stat92E* RNAi resulted in a decrease in Not4 protein levels in the stable Not4-V5 S2 cell line, indicating that Stat92E may have a role in regulating Not4 protein stability (data not shown).

Drosophila Not4 enhances Stat92E DNA binding without affecting Stat92E tyrosine phosphorylation

Activation of Stat92E by phosphorylation of the tyrosine 711 residue is considered to be essential for Stat92E target gene transcription. To further study the mechanism of how *Not4* affects *Drosophila* JAK/STAT signaling, we determined the Upd-induced tyrosine phosphorylation status of Stat92E in cells with *Not4* RNAi knockdown. S2 cells were cotransfected with Upd to induce endogenous Stat92E tyrosine 711 phosphorylation together with dsRNAs targeting *Not4* or *GFP* and *Stat92E* or *Hop* as negative and positive controls, respec-

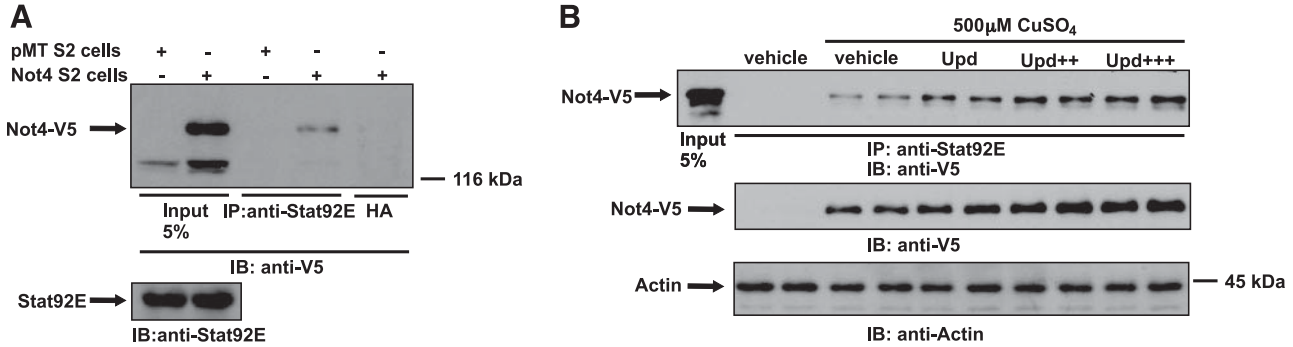

Figure 5. *Drosophila* Not4 interacts with Stat92E. *A*) Cultured pMT-V5-HisA-control S2 cells or pMT-Not4-V5 overexpression cells were treated with 500 μM $CuSO_4$ for 48 h to induce the expression of *Not4* from the pMT-Not4-V5 vector. Total cell lysates were used for immunopecipitation with an anti-Stat92E antibody, or as a control, with an HA-antibody, and coimmunoprecipitated Not4-V5 was detected with an anti-V5 antibody. Stat92E protein was equally expressed in both cell lines (bottom panel). *B*) Overexpression of *Upd* results in increased Stat92E-Not4 interaction. pMT-Not4-V5 overexpression S2 cells were transfected with either an empty vector (vehicle) or a Upd expression vector in order to activate the JAK/STAT pathway. Cells were treated with 500 μM $CuSO_4$ for 48 h following cell lysis. Coimmunoprecipitation was carried out with an anti-Stat92E-N-terminal antibody, followed by immunodetection with anti-V5. Middle panel shows Not4-V5 expression levels; bottom panel shows an anti-actin immunoblot to demonstrate that equal amounts of protein lysates were subjected to SDS-PAGE analysis.

tively. $CuSO_4$ (500 μM) was added to the medium to induce the expression of Upd from the pMT vector 48 h prior to cell lysis. Equal amounts of protein lysates were subjected to SDS-PAGE and immunoblotted with anti-Stat92E. As shown in **Fig. 6*A***, a weak, slower-migrating Stat92E band, corresponding to tyrosine phosphorylated Stat92E, was detected in Upd-induced samples (compare lane 1 to lanes 2 and 3). As expected, this band is absent in *Hop* or *Stat92E* RNAi samples. Interestingly, *Not4* RNAi did not affect Stat92E phosphorylation in this setting. To ensure that the Stat92E is phosphorylated, endogenous Stat92E was immunoprecipitated with an anti-Stat92E-N-terminal antibody and immunoblotted with an anti-phosphotyrosine antibody. The amount of phosphorylated Stat92E did not differ between cells with *GFP* and *Not4* knockdown (Fig. 6*A*, bottom panel). To further study the role of *Not4* in Stat92E phosphorylation, we overexpressed *Not4* in S2 cells. S2 cells stably overexpressing pMT-Not4-V5 or control S2 cells were cotransfected with Stat92E-Flag together with Hop^{Tum-l} to induce Stat92E phosphorylation or with an empty vector as a transfection control. Cells were treated with 500 μM $CuSO_4$ and lysed after 48 h. Equal amounts of protein were immunopreciptated with an anti-Flag antibody and separated by SDS-PAGE, followed by immunodetection with an anti-Stat92E-N-terminal antibody to detect immunoprecipitated Stat92E. As shown in Fig. 6*B*, there is a clear slower-migrating band in the Hop^{Tum-l}-induced samples (lanes 3 and 4), indicating Stat92E phosphorylation. Forced *Not4* expression did not affect Stat92E phosophorylation in this setting. Taken together, these results suggest that Not4 modulates Stat92E target gene expression downstream of the Stat92E phosphorylation event.

Tyrosine-phosphorylated Stat92E translocates to the nucleus, where it binds to the Stat92E consensus sequence in the promoter regions of target genes. We used EMSA to study whether *Not4* has a role in the DNA binding of Stat92E, using the sequence of the Stat92E

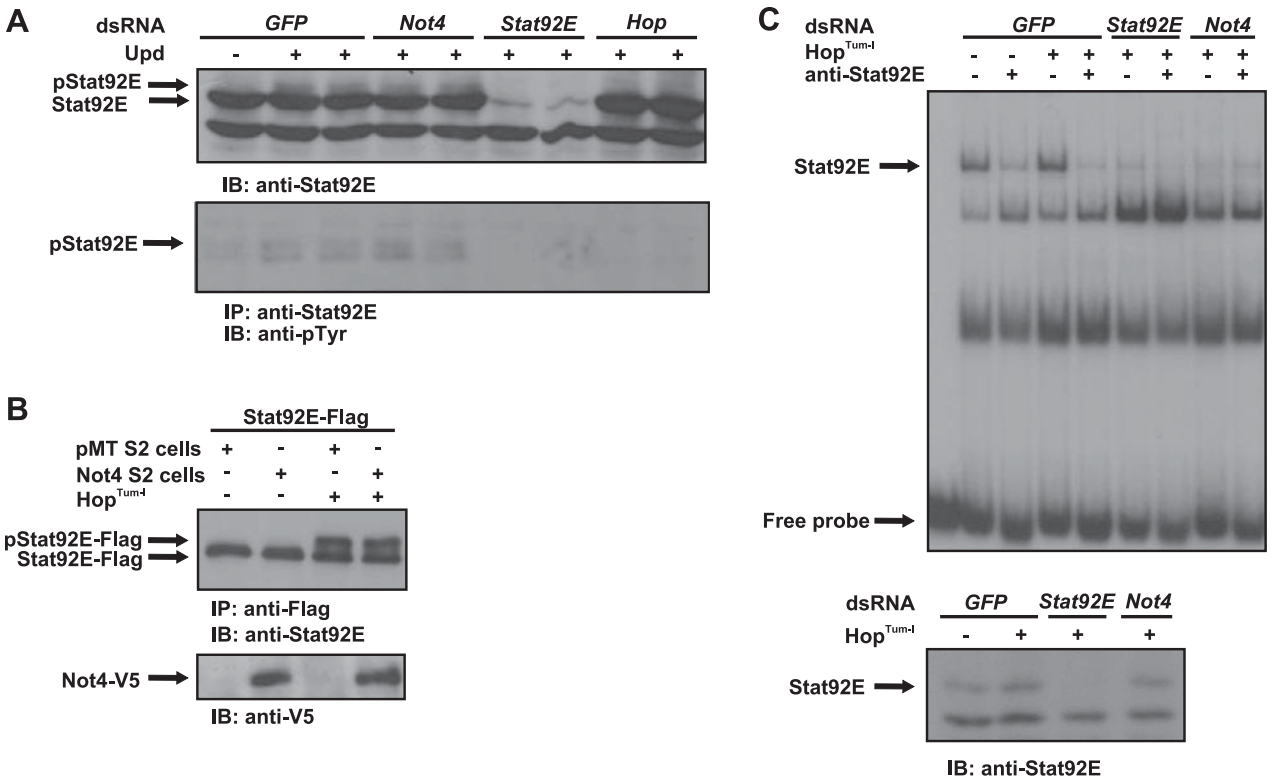

Figure 6. Not4 enhances Stat92E DNA binding without affecting Stat92E tyrosine phosphorylation. *A*) *Not4* RNAi does not affect tyrosine phosphorylation of Stat92E. S2 cells were cotransfected with different dsRNAs, and Upd to activate Stat92E phosphorylation, as indicated. Cells were treated with $CuSO_4$ to induce Upd expression 48 h prior to cell lysis. Total Stat92E was detected by immunoblotting with an anti-Stat92E-N-terminal antibody (top panel). Phosphorylated Stat92E was detected by immunoprecipitating Stat92E from total cell lysates using an anti-Stat92E-N-terminal antibody and immunoblotting with an anti-phospho-tyrosine (anti-pTyr) antibody (bottom panel). *B*) pMT-Not4-V5-overexpressing S2 cells or pMT-V5-overexpressing control S2 cells were transiently cotransfected with Stat92E-Flag in the presence or absence of the Hop^{Tum-l} expression vector to induce tyrosine phosphorylation of Stat92E. Equal amounts of total cell lysates were subjected to immunoprecipitation with an anti-Flag antibody and immunodetection with an anti-Stat92E-N-terminal antibody. *C*) *Not4* RNAi interrupts Stat92E binding to its DNA-recognition sequence in the *TotM*-promoter in an EMSA analysis. S2 cells were transfected with the indicated dsRNAs together with the Hop^{Tum-l} or the empty vector as a control. Equal amounts (15 μg) of cell lysates were incubated for 30 min at room temperature in the presence of the EMSA probe with or without an anti-Stat92E-N-terminal antibody, as indicated. Samples were separated in a 6% PAGE in 0.5× TAE. First lane represents the free probe only, without cell lysate. Equal amounts of protein were subjected to immunoblotting with an anti-Stat92E-N-terminal antibody to detect Stat92E levels from the lysates used in EMSA.

binding site in the *TotM*-gene promoter region as a probe. S2 cells were transfected with Hop^{Tum-l} to induce Stat92E phosphorylation and with dsRNA targeting *Not4*, or with *GFP* or *Stat92E* dsRNAs as controls. As shown in Fig. 6*C*, there is a clear band (indicated by an arrow), which likely represents the Stat92E-dsDNA-probe complex, as this band is hindered by both the anti-Stat92E antibody and by a dsRNA treatment targeting *Stat92E*. Furthermore, the intensity of this band is strongly reduced by competing with an unlabeled wild-type probe but not by competing with a probe containing a mutated Stat92E binding site (Supplemental Fig. S3). No supershift was detected with the anti-Stat92E antibody, likely because binding of the antibody to its epitope blocks the DNA binding ability of Stat92E. Notably, dsRNA treatment targeting *Not4* markedly reduced the intensity of this band compared to the *GFP* dsRNA control, suggesting that Not4 regulates the DNA binding ability of Stat92E. Taking these data together, we conclude that Not4 modulates Stat92E DNA binding without affecting Stat92E tyrosine phosphorylation.

DISCUSSION

The JAK/STAT pathway is regulated by a multitude of mechanisms that can affect receptor expression or shuttling, JAK kinase activity or receptor docking, phosphorylation of signaling proteins, chromatin modifications, DNA binding, or the activity of transcription factors. In the present study, we investigated the role of *Not4* in the regulation of JAK/STAT pathway-mediated gene responses. *Not4* RNAi was shown to cause an ~50% decrease in JAK/STAT signaling using 2 independent reporters, *TotM-luc* and *10xStat92E-luc*, on either Hop^{Tum-l} or Upd induction in S2 cells. Furthermore, endogenous Stat92E-dependent *TotM* expression was reduced in the absence of Not4 in S2 cells. Overexpression of *Not4* was associated with the hyperactivation of *TotM-luc* and *10xStat92E-luc* reporter activities on both Hop^{Tum-l} and Upd activation. The *Not4* overexpression-induced hyperactivation of the *10xStat92E-luc* promoter was not as drastic as the hyperactivation of the *TotM-luc* promoter on Hop^{Tum-l} induction, suggesting that Not4 may regulate Stat92E target genes differently depending on the promoter. In addition to activating reporter gene expression in *in vitro*, *Not4* overexpression caused strong hyperactivation of the JAK/STAT pathway responsive *TotM* and *TotA* stress genes *in vivo* in the fatbody of uninfected flies, as well as the hyperactivation of the *TotM* gene under septic injury in *E. cloacae*-infected flies.

The core components of the JAK/STAT signaling pathway are highly conserved in evolution, and most of the known regulatory mechanisms of JAK/STAT-mediated signal transduction are similar in *Drosophila* and humans. This appears to be the case also with Not4, whose human homologue CNOT4 was shown in our study to be essential for optimal STAT1, as well as STAT6-mediated transcriptional activation in human HeLa cells.

STAT-mediated target gene transcription requires a variety of coactivators

In the canonical model for the JAK/STAT pathway, STAT activation occurs through JAK-mediated tyrosine phosphorylation following dimerization and translocation to the nucleus, where STAT dimers bind to DNA enhancer elements in the promoters of target genes. When bound to DNA, interactions between STAT, other transcription factors, and many coregulatory proteins, including chromatin modifying coactivators are required to activate the basal transcription machinery and RNA polymerase II to finally initiate the transcription of the target gene. These include histone acetyltransferases CBP/p300, known to function as coactivators for all STAT family members in mammals (41–46), as well as Tudor-SN (p100), known to bridge human STAT6 to CBP (47). Of transcription factors, PU.1 is essential for the proper activation of the Stat6 response element in the Igε promoter and for Stat1-mediated transcriptional activation of the high-affinity Fcγ receptor (48, 49). Although the molecular mechanisms for how DNA-bound STAT dimers activate target gene transcription have been extensively studied in mammals, many aspects of this process remain enigmatic. This holds true even more in *Drosophila*, where Stat92E-associated transcriptional coregulators are completely unidentified, but certainly existent.

Our data suggest that *Not4* interacts with Stat92E in S2 cells, and knocking down *Not4* expression by RNAi inhibits the DNA binding of Stat92E. Therefore, it is possible that Stat92E binds DNA when it is in a complex with Not4. Not4 may also mediate interactions between Stat92E and other transcriptional coregulators or RNA polymerase II, which has been reported to interact with the Ccr4-Not complex in yeast, this interaction being essential for proper transcriptional elongation (33).

Ccr4-Not complex in gene regulation

The Ccr4-Not complex is associated with transcription and mRNA processing in many ways. In yeast, the Ccr4-Not complex was recently shown to promote histone H3 lysine 4 methylation (H3K4me3), a process that has been associated with gene activation (50, 51). Interestingly, the yeast Not4, and, furthermore, its RING domain, were shown to be crucial in the regulation of this process through the polyubiquitination of the demethylase Jhd2 (39). In our study, a Not4 deletion construct lacking the RING domain (ΔRING) was shown to affect Stat92E-dependent reporter gene expression to a similar extent as the full-length Not4. A similar result was obtained with a deletion construct lacking both the RING and the RRM domains (ΔRINGΔRRM) in the highly conserved N terminus of Not4, indicating that neither the RING nor the RRM domain is essential for the activation of Stat92E-medi-

ated gene expression in S2 cells. This suggests that Stat92E target gene activation does not require the Not4 ubiquitin ligase activity or the RRM domain-mediated ssRNA, ssDNA, or protein interactions. The C-terminal domain that harbors STAT activation properties does not possess identifiable protein domains, and the nature and mechanism of this regulatory function need further study.

In human cells, CNOT4 has been shown to associate with a 200-kDa protein complex, which is not stably integrated in the Ccr4-Not complex but has the ability to directly interact with the CNOT1 subunit of the Ccr4-Not core complex (52). Also, it has been suggested that Not4 is not a stable component of the Ccr4-Not complex in *Drosophila* (40). Furthermore, it was shown in the same study that knocking down Not4 has no detectable effect on the poly(A) tail length or the rate of deadenylation of mRNA in *Drosophila* cells (40). These data suggest that Not4 may also have functions independent of the Ccr4-Not complex.

DNA binding of Stat92E was inhibited when *Not4* was knocked down in S2 cells by RNAi, indicating that Not4 positively affects Stat92E DNA binding. Furthermore, the phosphorylation status of Stat92E was not altered by *Not4* RNAi or overexpression. This suggests that Not4 functions downstream of Stat92E phosphorylation in the JAK/STAT signaling cascade, perhaps in either nuclear transport or in the DNA binding of Stat92E in the nucleus. Not4 has been reported to be cytoplasmic, and our immunofluorescence studies confirm this, but also identified a smaller nuclear portion of the protein (data not shown). Of note, the distribution of Stat92E in cytoplasmic and nuclear lysates of $Hop^{Tum\text{-}l}$ transfected pMT-Not4-V5 overexpressing cells and $Hop^{Tum\text{-}l}$ transfected control S2 cells was the same (data not shown). Although a long stimulation time with $Hop^{Tum\text{-}l}$ transfection forces Stat92E transcription factors to shuttle between active and inactive states within the cell, this result indicates that Not4 more likely affects Stat92E DNA binding than its cellular localization.

In summary, we have identified Not4 as a novel positive regulator of the JAK/STAT pathway both *in vitro* and *in vivo* in *Drosophila* and in human cells. The well-conserved N-terminal RING and RRM domains of Not4 are not required for JAK/STAT pathway regulation in S2 cells. Not4 coimmunoprecipitates with Stat92E, and it participates in Stat92E DNA binding. The exact molecular mechanism for how Not4 affects Stat92E DNA binding and Stat92E target gene transcription remains to be studied both in *Drosophila* and in mammalian systems. FJ

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