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Molecular Regulation of Janus Kinases (JAKs)

Focus on the Pseudokinase Domain

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ACADEMIC DISSERTATION
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PunaMusta Oy – Yliopistopaino
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To my Family

TIIVISTELMÄ

JAK-STAT-(vapaasti suomennettuna Janus-kinaasi - signaalinvälittäjä ja transkriptioaktivaattori) reitti välittää yli 50 sytokiinin signaaleja, jotka säätelevät solun selviytymistä, jakaantumista, migraatiota, geenieksprессiota, sekä muita elintärkeitä prosesseja kuten immuunivastetta. Siksi myös virheellisesti toimiva JAK-STAT signalointi aikaansaa vakavia seurauksia. Aktivoivat JAK-mutaatiot aiheuttavat hematologisia syöpiä sekä myeloproliferatiivisia tauteja, kun taas vajaatoimintainen JAK-signalointi voi johtaa muun muassa vakavaan immuunivajaukseen sekä autoimmuunisairauksiin.

Tämä tutkimus keskittyi JAKeissa (JAK1-3 ja tyrosiinikinaasi 2, TYK2) olevaan pseudokinaasiosaan (JH2) joka ei ole kinaasiaktiivinen, kuten sitä muistuttava kinaasiosa (JH1). Biokemialliset tutkimuksemme osoittavat, että JAK pseudokinaasiosat eroavat muun muassa nukleotidin (ATP:n) sitoutumisominaisuuksien osalta. Solupohjaisten kokeiden avulla näytimme, että mutatoimalla kohdennetusti tiettyjä JH2 alueita, pystymme vaikuttamaan JAK-aktiivisuuteen. Vertailimme myös näiden mutanttien vaikutusta signalointiin, riippuen siitä mihin JAK-perheen jäseneen mutaatio kohdentuu. Havaitsimme, että yksittäisen JAKin ja sen pseudokinaasiosan rooli on keskeinen toiminnallisessa signaloinnissa, mutta se voi vaihdella riippuen reseptorikompleksista, jossa JAK kulloinkin toimii.

Koska hyvälaatuista täyspitkää JAK rakennetta ei ole saatavilla, D.E. Shaw research (N.Y.) toteutti laskennallisen mallin JAK2-erytropoientin -reseptori kompleksista, jonka me yhteistyössä vahvistimme rakenneperustaisen mutaatioanalyysin avulla. Mallimme kattaa sekä aktiivisen dimeerin, että inaktiivisen monomeerisen JAK2 rakenteen. Inaktiivisessa konformaatiossa JAKin sisäisen JH2-JH1 interaktio sulkee rakenteen ja estää aktiivisten osien transfosforylaation. Aktiivisessa, aukinaisessa rakenteessa JH2-JH2 interaktio kahden JAK2 proteiinin välillä vahvistaa aktiivisen

reseptorikompleksin muodostumista. Myös viimeaikaset tutkimukset tukevat pseudokinaasiosan osallistumista dimerisaatioon, ja sitä kautta aktivaatioon. Mallimme antaa myös teoreettista taustaa huomiollemme, jonka mukaan ATP:n sitoutuminen pseudokinaasiosaan mahdollistaa patogeenisten mutaatioiden aktivoitumisen: ATP-sitoutumiskohta on rakenteellisesti tärkeä osa pseudokinaasia, ja vaikuttaa suoraan sen rakenteeseen, sekä dynaamiseen vaihteluun aktiivisen ja inaktiivisen konformaation välillä.

Edellä kuvatut tulokset lisäävät ymmärrystä niistä ominaisuuksista, jotka määrittävät moninaisten JAK-signalointireittien spesifisyyden. Lisäksi työmme valottaa mekanismeja, joilla patogeeniset JAK-mutaatiot aiheuttavat pysyvän signaloinnin aktivoitumisen, sekä tukee uusien, entistä vaikuttavampien JAK-inhibiittoreiden kehitystä.

ABSTRACT

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway mediates the transduction of over 50 cytokines that regulate cell survival, proliferation, migration, gene expression and other vital processes such as immune response. On the other hand, defects in the JAK-STAT signalling have severe impacts. Activating JAK (JAK1-3 and tyrosine kinase 2, TYK2) mutations cause haematological cancers and myeloproliferative disorders while impaired JAK-signalling leads to severe combined immunodeficiency (SCID) and autoimmune diseases.

The results presented in this thesis focus on the JAK pseudokinase domain (JH2) that is inactive but has a crucial role in regulating the JAK activity. Our biochemical and cell-based studies show that all JAK JH2s bind ATP, but that the binding properties vary among the JAK-family. Clinical and structure-based mutation studies show that modulation of JH2 can be used to effectively alter the activity. In addition, by introducing homologous mutations into all JAK members, we observed that an individual JAK can have varying functions depending of the signaling systems it is attached to. The results highlight that each JAK within the signaling complex, and specifically JH2, is important for the signaling.

As no well-defined structures exist of the full-length JAKs, a molecular dynamic (MD) simulation model of the full-length JAK2 with erythropoietin receptor was constructed in collaboration with D.E. Shaw research (N.Y.). The model depicts JAK2 in different states of activation and is supported by structure-based mutation analysis. In the inactive, monomeric conformation the interaction between JH2 and the kinase domain (JH1) in part closes the JAK2 structure and hinders the transphosphorylation of the active kinase domains. The active conformation is a more open, dimeric structure formed partly via JH2-JH2 interactions between the opposing JAK2 proteins. The model also gives theoretical background to our observation that ATP-binding to JH2 is crucial for the pathogenic JAK activation. It implies that the ATP-binding site is structurally important region within the pseudokinase that directly affects to the dynamic shifting between the active and inactive JH2 conformations.

The results summarized above allow us to better comprehend the characteristics that dictate the specificity among JAK-signaling pathways. Moreover, they bring insight into the mechanism of pathologic JAK activation and support the development of novel, more potent JAK inhibitors.

CONTENTS

ABBREVIATIONS

ALL: acute lymphoblastic leukemia

ATP: Adenosine triphosphate

CD: Crohn´s disease

CIS: Cytokine-inducible SH2 containing protein

CLCF: Cardiotrophin-like cytokine factor

CNTF: Ciliary neurotrophic growth factor

CT-1: Cardiotrophin

DSF: Differential scanning fluorimetry

EGFR: Epidermal growth factor receptor

EPO(R): Erythropoientin (receptor)

FAK: Focal adhesion kinase

FGF: Fibroblast growth factor

GAS: Interferon-g activated site

GH: Growth hormone

G-SCSF: Granulocyte-colony stimulating factor

HER: Human epidermal growth factor receptor

IFN(R): Interferon (receptor)

IL: Interleukin

IRF: IFN regulatory factor

ISRE: IFN-stimulated response element

JAK: Janus kinase

JH1-7: Janus homology 1-7

KSR: Kinase suppressor of Ras 1

LIF: Leukemia Inhibitory Factor

MANT: 2´/3´-O-(N-methyl-anthraniloyl)

MAPK: Mitogen-activated protein kinase

MPN: Myeloproliferative neoplasms

NP: Neuropoietin

OSM: Oncostatin M

PDGF: Platelet-derived growth factor

PEAK1: Pseudopodium-enriched atypical kinase 1

PIAS: Protein inhibitors of STATs

PTK7: Tyrosine-protein kinase-like 7

PV: Polycythaemia vera

ROR: Receptor tyrosine kinase-like orphan receptor

SH2- Src-homology 2

SHP: SH2-containing protein tyrosine phosphatases

SOCS: Suppressor of cytokine signaling

Src: Sarcoma family kinases

STRADα: STE20-related adaptor-α

SUMO: Small ubiquitin-related modifiers

TSA: Thermal stability assay

UC: Ulcerative colitis

TRIB: Tribbles family of pseudokinases (TRIB1-3)

TSLP: Thymic stromal lymphopoietin

ZAP70: TCR-zeta associated protein kinase 70

LIST OF ORIGINAL COMMUNICATIONS

I. Raivola J, Hammarén H, Virtanen AT, Bulleeraz V, Ward AC, Silvennoinen O. (2018) Hyperactivation of Oncogenic JAK3 Mutants Depend on ATP Binding to the Pseudokinase Domain**.** Frontiers in Oncology, 8, 560.

II. Raivola J, Haikarainen T, Silvennoinen O. (2019) Characterization of JAK1 Pseudokinase Domain in Cytokine Signalling. Cancers, *12*(1), 78

III. Pelin A., Hammarén H.M., Raivola J., Abraham B.G., Sharon D., Hubbard S.R., Silvennoinen O., Shan Y., Shaw D.E. "Structural models of full-length JAK2 kinase" Manuscript submitted.

1 INTRODUCTION

Cell communication, interaction with neighbouring cells and responding to cues from the environment, is crucial for organism to survive. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) cascade is one of the major pathways that transduce extracellular signals to biological responses. The pathway is critical for the development and function of myeloid and lymphoid cells and has especially important role in maintaining the homeostasis of the immune system.

Cytokines, hormones and growth factors are extracellular messenger molecules that are secreted in response to changes in the cells´ microenvironment, but can also work in a systemic level. These messengers activate the JAK-STAT pathway and have a crucial role in the induction homeostasis of the immune system, together with other important functions. Cytokines bind to a specific receptor on the cell surface that are expressed depending of the cell type and tissue. This enables a single cell to integrate signals simultaneously from multiple cytokine receptors. The four members of the JAK-family (JAK1-3 and Tyrosine kinase 2, TYK2) bind to the intracellular part of the receptor chains and can form various JAK-receptor complexes. Once stimulated by cytokines, JAKs activate their downstream effectors (STATs) that initiate the transcription of a specific set of genes in the nucleus.

The members of the JAK-family share a general structure with four domain modules. These enable the receptor binding (the FERM-SH2 domains) and the cytokine responsive activation (the JH2-JH1 module) of JAKs. A characteristic feature of JAKs is the pseudokinase domain (JH2) that is homologous to the active kinase domain (JH1). Despite the resemblance, JH2 has no- or very limited kinase activity, but the domain is crucial for both regulating the JAK function and in the cytokine-induced activation.

Deregulated JAK signalling can have severe effects, such as cancers, leukaemia and autoimmune diseases. Hence, JAK inhibitors are gaining traction both for the treatment of diseases driven by JAK mutations (ruxolitinib for myelofibrosis) and

for inflammatory disorders where the JAK-STAT pathway is an integral part of the pathogenesis (e.g. tofacitinib and baricitinib for the treatment of rheumatoid arthritis). However, the efficacy of the current JAK inhibitors is not optimal and can cause milder and more severe side effects. With normal dosage, approximately half of the patient do not respond to the treatment.

Despite the long-lasting studies of the JAK-STAT pathway, many aspects are still elusive. What are the molecular mechanisms of the JAK activation, how JAKs can accurately transduce specific signals driven by dozens of different cytokines, what kind of various (pathogenic) mechanism may lead to the alteration of the activity and how the constitutive activation of JAKs could be efficiently and safely reduced are examples of the critical questions. In general, the JAK-STAT pathways is constitutively activated either by overt cytokine production and activating JAK mutations, other upstream oncogenes or more rarely in STATs.

We focused our studies on the mechanistic aspects of the Janus kinase function. The aim was to bring forth novel details about how the allosteric sites within JAKs contribute to the regulation of the signaling, focusing on the important function of JH2 as a regulatory "switch" between the active and basal states. Together with cell-based and biochemical studies, we used molecular dynamics simulations to model the conformational and mechanistic changes that by activation occur in the signaling complex. In addition, we compared the IFNγ, IFNα and IL-2 signaling pathways, addressing both the wild type and pathological JAK-signalling. Our aim was to find if, and how the receptor complex can define the function of JAKs. Moreover, we wanted to bring insight to the cytokine-dependent activation of the JAK-STAT signaling.

Here I present a compact literature review of the current state of the research surrounding the JAK-STAT signalling. After the summary of the results described in the original works, I will discuss their implications in finding novel ways to modulate cytokine signalling via JAK inhibitors.

2 REVIEW OF THE LITERATURE

2.1 Eukaryotic Protein kinases and cytokine signalling

2.1.1 The eukaryotic protein kinase family

In 1992, the Nobel Prize in Physiology or Medicine was awarded to Edmond H. Fischer and Edwin G. Krebs on their seminal discovery of how the reversible phosphorylation of proteins regulates cell function. Since the initial studies dating back to 1955, protein phosphorylation has been under extensive research. It has been established that phosphorylation regulates crucial cellular functions such as metabolism, transcription, apoptosis and cell movement, and that more than two-thirds of human proteins are phosphorylated, making it the most abundant post translational modification occurring in cells (Ardito et al. 2017; Khoury, Baliban, and Floudas 2011). A large family of enzymes called the protein kinases are specialized in catalysing phosphorylation, i.e. transferring the nucleotide γ-phosphate to a protein substrate. In addition, 20 protein kinase-like families that cover metazoans, prokaryotes and plants have been described (Kannan et al. 2007; Oruganty et al. 2016).

This thesis focuses on the eukaryotic protein kinase family that encompasses more than 500 members and is encoded by a whopping 2% of the entire human genome. Eukaryotic protein kinases can be classified either as serine, threonine or tyrosine kinases based on the substrate residue (amino acid) they phosphorylate. Most kinases act on both serine and threonine (serine/threonine kinases) while some act on tyrosine (tyrosine kinases, TKs). Dual-specificity kinases act on all three residues (Ardito et al. 2017). Tyrosine kinases can be further divided into receptor- and non-receptor tyrosine kinases (Hubbard and Till 2000). The epidermal growth factor receptor (EGFR) is a prototypical receptor tyrosine kinase, but also other growth factor and insulin receptor families, among other, belong to this category. Receptor tyrosine kinases consist of the extracellular portion where the ligand binds, a

transmembrane helix, and a cytoplasmic side of the receptor where the active kinase site resides. Non- receptor tyrosine kinases are intracellular kinases that attach to the cytoplasmic part of the receptor that does not have an intrinsic kinase domain. They are important regulators of the immune system and include the Src, JAK, Abl and SYK (Zap70) family kinases.

2.1.2 Structure of a canonical kinase

Protein kinases consist of conserved regions that have evolved to enable the kinase activity. The protein kinase A (PKA) is a prototypical kinase and among the first discovered. In this thesis, PKA is used as a model for the prototypical kinase fold and the numberings of amino acid residues are based on PKA, if not stated otherwise. Kinases consist of an N-lobe and larger C-lobe between which the ATP (or other nucleotide) binds. The nucleotide typically requires two complementary cations (e.g. magnesium or other divalent cation) that orient it correctly for the catalysis (Endicott, Noble, and Johnson 2012; Taylor and Kornev 2011). The dynamic opening and closing of the active site cleft allows the transfer of the nucleotide phosphate, but also global changes in the protein conformation play a crucial role in the activation (McClendon et al. 2014).

Several highly conserved residues are critical for the kinase activity (Roskoski 2015; Taylor et al. 2012), and a fraction of eukaryotic protein kinases that lack some of these residues are classified as atypical protein kinases (Hanks, Quinn, and Hunter 1988) (Figure 1). Autophosphorylation of the activation loop is essential for the activity of most protein kinases. Depending on the kinase, the phosphorylated residue can be either tyrosine (Tyr), serine (Ser) or threonine (Thr). Phosphorylation of the activation loop stabilizes the kinase into an active conformation and helps in the phosphotrasferase reaction, possibly by increasing the binding of the substrate and the nucleotide (Cheng, Zhang, and McCammon 2006). The catalytic loop, on the other hand, contains residues needed for the kinase reaction including the catalytic aspartate (D166) from the highly conserved His-Arg-Asp (HR**D**)-motif and the conserved Asp184 from the Asp-Phe-Gly (**D**FG) motif (Endicott et al. 2012; Kannan et al. 2007; Taylor and Kornev 2011). The DFG motif locates at the beginning of the catalytic loop and the D184 interacts with the catalytic magnesiumm

and directs the ATP γ-phosphate to the protein substrate (see Figure 1). Reorientation of the DFG motif positions the Phe185 (D**F**G) to complete the active, so-called "DFG-in" conformation. In the inactive form, the region is in the "DFG-out" state where the Phe185 blocks the ATP-binding (Steichen et al. 2012). The DFG-out state reflects also to the position of the αC-helix (shown in Figure 7). The αC-helix is an essential feature that is conserved among protein kinases. When in an active conformation, the N-terminus of the αC-helix interacts with the activation loop phosphate and the C-terminus of the helix is a part of the hinge at the base of the active site cleft (see Figure 1).

Essential and highly conserved lysine (Lys72) in the beta three (β3) strand positions the γ-phosphate for the transfer to the substrate, likely by stabilizing the α- and β-phosphate of ATP (Endicott et al. 2012; Taylor and Kornev 2011). In addition, the positively charged Lys72 couples with a Glu91 in the αC-helix and orients it to an active conformation. The glycine-rich loop is another important site for nucleotide binding, and especially Gly52 is required for the correct positioning of the ATP γ-phosphate. The loop (also known as P-loop) is located between the N-lobe β1 and β2 loops (Figure 1).

In addition to the above-mentioned regions and motifs, two hydrophobic spines connect the N- and C-lobes. These are critical for the dynamic conformational change that enables the proper function of kinases (Hu et al. 2015). The regulatory R-spine folds when the kinase becomes active and connects the HRD- and DFG-motifs, the αC-helix and the Leu106 residue from the β4-strand. The catalytic (C-) spine consists of two conserved hydrophobic residues at the N-lobe and six from the C-lobe. The fold of the C-spine is completed after the ATP binds and the adenine ring within the nucleotide closes the two lobes. Briefly, the assembly of the R- spine defines activation whereas the assembly of the C-spine sets the kinase for catalysis (Hu et al. 2015; Taylor and Kornev 2011).

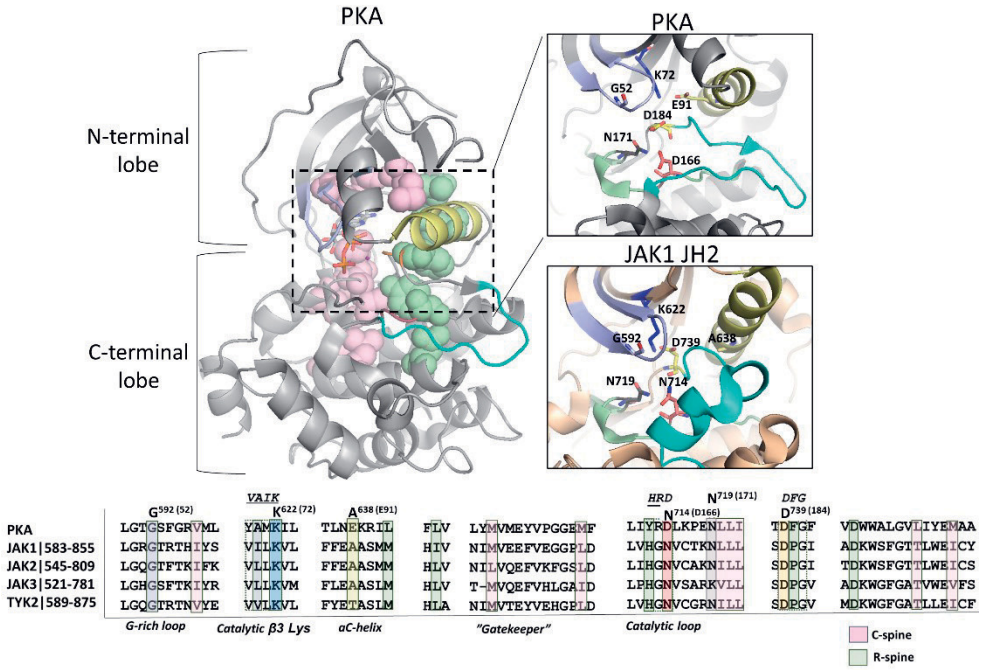


Figure 1. Kinase fold of a prototypical kinase, PKA. The αC-helix is depicted as yellow and the C- and R-spine as pink and green, respectively. The activation loop is coloured as **turquoise**. Right panel: Comparison of the ATP-binding sites between PKA (PDB: 4WB5) and JAK1 pseudokinase domain (JH2) (PDB: 4L00). For clarity, the C- and R-spines are not shown in the ATP-site close-ups. The catalytic loop is only shown in the close-up figures in **green**. Below: An alignment of the PKA versus human JAK JH2 sequences. Typical functionally important residues are coloured as in the structures above, and the residues are named according to JAK1 JH2, with the PKA numbering (or residues, if vary) in the brackets.

2.1.3 Pseudokinases

Two decades ago, Manning and colleagues catalogued 518 putative protein kinase genes within the human genome (Manning et al. 2002). Out of these, approximately 10% are classified as pseudokinases. Pseudokinases lack at least one of the conserved catalytic residues (namely the β3 lysine (K72), HDR Asp (D166) or DFG Asp (D166), described in the previous chapter) and thus do not necessarily have biologically relevant kinase activity (Byrne, Foulkes, and Eyers 2017; Manning et al. 2002). However, pseudokinases have a significant role in regulating and driving cell signalling.

Due to the less conserved primary structure, pseudokinases have more functional variation than prototypical eukaryotic protein kinases (Table 1 depicts some of these features regarding the pseudokinases discussed in this chapter and thesis). For example, JAK and MLKL (mixed lineage kinase domain like pseudokinase) have a pseudokinase domain that allosterically modulates the activity of the adjacent kinase domain. On the other hand, other pseudokinases such as KSR (kinase suppressor of RAS), STRADα (STE20-related kinase adaptor protein α) and HER3 (human epidermal growth factor receptor 3, or ErbB3) function as scaffolds to another kinases and hence promote the assembly of signalling complexes (of note, catalytically active kinases can also act as scaffolds independently of their catalytic activity). A single pseudokinase may also have multiple ways to convey its function (reviewed by Kung & Jura, 2016, 2019). The Tribbles family of pseudokinases (TRIB1-3) promote an E3 ligase-dependent ubiquitination of their protein substrates and have an additional role as a scaffold for the MAPK (mitogen-activated protein kinase) and AKT signaling. The Wnt-binding PTK7 (protein tyrosine kinase 7) and ROR1/2 (receptor tyrosine kinase like orphan receptor) are another example of multifunctional pseudokinases. They allosterically modulate the activity of other kinases but also recruit/scaffold intracellular signaling molecules.

Some pseudokinases have measurable catalytic activity in vitro (KSR2, JAK2 JH2 and HER3) (Brennan et al. 2011; Shi et al. 2010; Ungureanu et al. 2011). However, the consensus opinion is that this activity is too low to be physiologically relevant (Hammaren, Virtanen, and Silvennoinen 2015). Instead, the ATP binding is likely important for stabilization of the protein and regulation of the allosteric/scaffolding functions of pseudokinases. However, most pseudokinases do not bind nucleotides (e.g. ROR1 and PTK7) and due to the variation in the nucleotide binding mechanisms, pseudokinases can be classified based on these differences (Murphy et al., 2013; Hammarén et al., 2016; Kung & Jura, 2019).

Some pseudokinases bind ATP but without or only one cation present (JAK2 JH2) (Murphy et al. 2013). PEAK1 (inactive tyrosine-protein kinase) is an example of a pseudokinase that has a binding site that occludes ATP but binds cations. The biological relevance of the Mn^{2+} binding is not known, and cations where not depicted in the crystal structure of PEAK1 pseudokinase domain, but the structure allows cation (but not ATP) binding (Ha and Boggon 2018; Murphy et al. 2013). The

mammalian endoribonuclease RNase L is the only known pseudokinase displaying a canonical nucleotide-binding mode with two cations required for the binding (Han et al. 2014). It is, however, considered as a pseudokinase due to its scaffolding function and the inability to auto-/phosphorylate.

Knowledge of the pseudokinases has grown fast throughout the decades and expanded our understanding of what is "inactive" or "pseudo" when kinases are considered. Importantly, majority of pseudokinases have been associated to diseases including a wide range of cancers and autoimmune diseases (reviewed in Kung and Jura 2019; Reiterer, Eyers, and Farhan 2014). Knowledge of the varying activation mechanisms, specifically the distinct features of the ATP-binding pockets compared with prototypical kinases, has brought interesting possibilities and extensively increased the interest to target the pseudokinome. Future studies will continue to reveal novel biochemical and biological properties of the currently known pseudokinases, and new kinase-like proteins is also likely to be found, especially regarding plants, fungi and bacteria where pseudokinases can cover up to 50 % of the total kinome (Kwon et al. 2019; Lopez et al. 2019).

Table 1: Pseudokinases show more variation in the HRD and DFG motifs, as well as the catalytic β3 lysine. The pseudokinases addressed in this thesis, classified based on nucleotide-binding properties. In addition, the conservation of the three critical residues within the kinase fold is indicated. The specific residue in the motif is highlighted with red, and Y or **N** indicate whether the residue is conserved or not, respectively. The table is modified from (Hammaren, Virtanen, et al. 2015).

ATP-binding mode	Pseudokinase	Conserved motifs (that are altered)		
		HRD	DFG	β3 Lys
Does not bind nucleotides/cations	ROR1	Y	Y	Y
	TRIB1	Y	**N**	Y
	PTK7	Y	**N**	Y
Binds nucleotides in the absence of cations	JAK3 JH2	**N**	**N**	Y
	STRADα	**N**	**N**	**N**
	TRIB2-3	Y	**N**	Y
	MLKL	**N**	**N**	Y
Binds cations but not nucleotides	PEAK1	Y	Y	Y
Pseudokinases that bind to nucleotides and cation(s)	HER3	**N**	Y	Y
	JAK1,2,TYK2 JH2	**N**	Y	Y
	KSR	Y	Y	**N**
	Rnase L	Y	Y	Y

2.2 JAK-STAT signalling

In 1957, Alick Isaacs and Jean Lindenmann established the existence of an innate host defense system that, once triggered by virus infection, causes the infected cells to produce a substance that alters the properties of the cells and protects the ones not yet infected. Furthermore, they observed that all this occurs within hours, without waiting for antibody production. The protein responsible was named "interferon" (Isaacs, Lindenmann, and Andrewes 1957). Today, interferon (IFN)-mediated antiviral responses are not only known to be central to host defense, but also play an important role in the Janus kinase-Signal transducer and activator of transcription (JAK-STAT) signaling. Janus kinases (JAK1, JAK2, JAK3 and TYK2 (Tyrosine kinase 2)) are critical players in intercellular signaling, governing growth and energy homeostasis, hematopoiesis and immunity. JAKs were discovered at the beginning of 1990´s (Firmbach-Kraft et al. 1990; Silvennoinen et al. 1993; Wilks et al. 1991; Witthuhn et al. 1994) together with the cloning and describing of the seven STATs (STAT1-6 including STAT5a and STAT5b) (Darnell, Kerr, and Stark 1994; Shuai et al. 1992; Wakao, Gouilleux, and Groner 1995; Zhong, Wen, and Darnell 1994). Simultaneously, unique sets of JAKs and STATs were found to mediate various signaling pathways including interleukin (IL)-2, interferon (IFN)γ and IFNα systems (reviewed by Larner & Finbloom, 1995 and Gaffen, 2001). Despite the long-lasting and extensive studies, the specific mechanisms determining the course of the JAK-STAT pathway are still partly undetermined, and new questions have arisen. The most pressing among these, is how the JAK-STAT signaling could be harnessed therapeutically to treat the related diseases.

2.2.1 Cytokines and their receptors

JAKs are versatile in transmitting signals that initiate from multiple extracellular signals, cytokines. Cytokines are small extracellular peptides that bind and activate transmembrane receptors. The term "cytokine" encompasses interferons, interleukins, chemokines, mesenchymal growth factors, the tumor necrosis factor family and adipokines (Dinarello 2007). Cytokines are especially important to

immunity, functioning as primary lymphocyte growth factors as well as pro- and anti-inflammatory triggers. In addition, cytokines contribute to a broad range of other biological functions from the formation of erythrocytes and osteoclasts to having a role in neuronal development and function.

The interaction between JAK and its receptor is crucial for the signal transduction, and studying the receptor structure and function is an important part of understanding the mechanism of JAK activation (Fujii, 2008; Haan et al., 2001). This thesis focuses on the molecular mechanism of the JAK activation, but the following chapter provides a compact introduction of the characteristics of the receptors that interact with JAKs. In addition, Table 2 presents a list of the receptors and JAKs, STATs they activate, and typical biological functions related to each pathway.

Receptor complex composes of individual receptor chains that together form homo-, hetero- or multimeric complexes (Figure 2). The chains may have specific roles within the receptor, e.g. some have high affinity towards the cytokine while others only weakly bind the extracellular signaling molecule. For example, the common gamma chain (γc) of the interleukin-2 receptor (IL2R) complex has low IL-2 affinity but once the it binds to the other chains (IL2Rα and IL2Rβ), the γc completes and stabilizes the high-affinity IL2R-complex (Waickman, Park, and Park 2016).

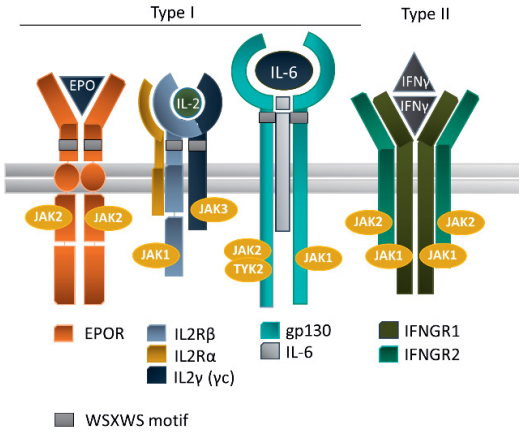


Figure 2: A schematic presentation of a selected Type I and Type II receptors used by different JAK combinations. The receptor chains are specified below (EPOR: erythropoietin receptor, IL2R interleukin-2 receptor, IFNGR: interferon-γ receptor).

Both the type I and type II receptors contain a conserved juxtamembrane box1 and box2 sequences that are required for JAK binding (Ferrao and Lupardus 2017; O'Shea, Holland, and Staudt 2013; Schwartz et al. 2016). Type I receptors include the erythropoietin (EPO) and the growth hormone (GH) receptors and several of the interleukin- (IL) receptors. They share an extracellular WSXWS motif that is suggested to be important for the correct folding of the receptor, binding of the substrate, and for the substrate-induced conformational changes (Dagil et al. 2012; Olsen and Kragelund 2014). Type II receptors have a similar overall structure but they do not contain the WSXWS motif. This group consists of receptors for type I and type II interferons (IFNαβ and IFNγ, respectively).

The question whether the receptors exist as monomers that oligomerize in a cytokine-induced manner, or if they are pre-formed dimers undergoing conformational changes that activate JAKs, has been under extensive research. Although studies supporting the latter are not singularity in the field (Brown et al. 2005; Constantinescu et al. 2001; Gent et al. 2002; Tenhumberg et al. 2006; Waters and Brooks 2015), the current data favors of the model where cytokine receptors are monomeric until stimulation (Hammaren et al. 2018; Wilmes et al. 2020). It is also plausible that an equilibrium between the two states shifts according to the stimulation status. According to the cytokine-induced dimer model, cytokine-binding drives the dimerization of the receptors and brings the JAK JH1 domains together, enabling their trans activation. Recent studies suggest that also the interaction between the JH2-JH2 and FERM-FERM domains of the opposing JAKs contribute to the dimerization (see Chapters 2.2.2 and 2.2.3). However, these studies concentrated on the JAK2-bound homomeric receptors (EPOR, TPOR, PRLR, GHR) (Wilmes et al. 2020) and further research is required to better understand the molecular details of the JAK-receptor activation (discussed further in Chapter 6.2).

Table 2: JAK-related cytokines, receptors and the STATs they activate. The table is modified from (Hammaren, Virtanen, Raivola, et al. 2019). In addition, the main biological functions are indicated.

		Cytokine	Receptor chain(s)		JAKs	STATs	Main biological function
TYPE II CYTOKINE RECEPTORS	IFN family	IFN-α (type I)	IFNAR1	IFNAR2	**JAK1**, TYK2	**STAT1/2**/3 /4 (STAT5/6)	Suppresses allergic inflammatory processes, promotes antiviral activity (Gonzales-van Horn and Farrar 2015).
		IFN-γ (type II)	IFNGR1	IFNGR2	**JAK1, JAK2**	**STAT1**	Important for antiviral immunity and cell apoptosis, regulates the activation of macrophages and differentiation of Th1 cells (Lin and Young 2014; Schroder et al. 2004).
		IL-10	IL-10Rα	IL-10Rβ	**JAK1, TYK2**	**STAT3**/1	Regulates and represses the expression of proinflammatory cytokines during the recovery phase of infections and reduces the tissue damage caused by these cytokines. (Ouyang et al. 2011)
		IL-19	IL-20Rα	IL-20Rβ	JAK1, JAK2	**STAT3**/1	Promotes B-cell proliferation and differentiation. Promotes allergic inflammation (Noelle and Nowak 2010).
		IL-20, IL-24 / mda7	IL-20Rα or IL-22R	IL-20Rβ	JAK1, JAK2	**STAT3**/1	Promotes wound healing and is upregulated in viral infections (Mitamura et al. 2020)
		IL-22 / IL-TIF†	IL-22R	IL-10Rβ	JAK1, TYK2	**STAT3**/1, (STAT5)	Contributes to the tissue repair and host defense (Lu et al. 2016)
		IL-26 / AK155	IL-20Rα	IL-10Rβ	JAK1, TYK2	**STAT3**/1	Induces the secretion of proinflammatory cytokines (IL-1β, TNFα, IL-6) and has antimicrobial activity (Larochette et al. 2019)
		IL-28a, IL-28b, IL-29	IL-28R / IFNLR1	IL-10Rβ	**JAK1**, TYK2	**STAT1/2**/3 /5	IFN-like antiviral activity (Ouyang et al. 2011).

		Cytokine	Receptor chain(s)		JAKs	STATs	Main biological function
TYPE I CYTOKINE RECEPTORS	gp130 family	IL-6	IL-6Rα	gp130	JAK1, JAK2, TYK2	STAT3/1	Propagates chronic inflammation, T-and B-cell differentiation and antibody production. Regulates the level proinflammatory cytokines and chemokines (Tanaka, Narazaki, and Kishimoto 2014).
		IL-11	IL-11Rα	gp130	**JAK1,** JAK2, TYK2	**STAT3/**1	Inhibits the production of proinflammatory cytokines (TNFα, IL-6, IL-1β) (Johnson, Wood, and Serio 2004).
		LIF	LIFRβ	gp130	**JAK1,** JAK2, TYK2	**STAT3/**1	Highly pleiotropic. Role in the progression of the inflammatory state and cartilage destruction in rheumatoid arthritis. Response to stress. Increase neural, heart and skeletal muscle development and proliferation (Nicola and Babon 2015).
		CNTF	CNTFRα LIFRβ	gp130	JAK1, (JAK2, TYK2)	**STAT3,** (STAT1)	Induce the differentiation of neuronal cells, rescue them from naturally occurring death, and trigger neuronal regeneration.(Sariola et al. 1994)
		CLCF1, NP	CNTFRα LIFRβ	gp130	JAK1, (JAK2)	**STAT3/**1	Affect kidney and lung pathology, osteoarthritis and haematopoiesis.NP inhibits adipose differentiation (Sims 2015).
		CT-1	CNTFRα LIFRβ	gp130	JAK1, (JAK2, TYK2)	**STAT3**	Stimulate the survival of both cardiac and neuronal cells (Latchman 1999).
		OSM	OSMRβ or LIFRβ	gp130	JAK1, (JAK2, TYK2)	**STAT3/**1	Pleiotropic functions e.g. regulation of the inflammatory response. Supports growth inhibition of various solid tumors (Hintzen et al. 2008).
		IL-27 (p28+EBI3)	IL-27Rα	gp130	JAK1, JAK2, TYK2	**STAT1/3/**4 (STAT5)	Expansion and function of T-cells, induction of IFN-γ production (Fabbi, Carbotti, and Ferrini 2017).
		IL-35 (p35+EBI3)	IL-12Rβ2	gp130	JAK1, JAK2	STAT1/4	Anti-inflammatory. Produced by e.g. Treg-, B-cells, macrophages and various tumor cells (Kong et al. 2016).
		IL-12 (p35+p40)	IL-12Rβ2	IL-12Rβ1	**TYK2,** JAK2	**STAT4**	Required for activation and differentiation of T cells and induction of IFN-production (Vacaflores et al. 2017).
		IL-23 (p19+p40)	IL-23R	IL-12Rβ1	**TYK2,** JAK2	**STAT3/4/**1	Proinflammatory. Involved in formation of osteoclast. Related to psoriasis, chronic inflammatory bowel disease and rheumatoid arthritis (Duvallet et al. 2011).
		G-CSF	GCSFR / CSF3R		JAK1, (JAK2)	**STAT3**	Growth and differentiation factor for granulocyte and macrophage populations. Extend the life span of neutrophils by preventing apoptosis (Jiang and Schwarz 2010).
		IL-31	IL-31Rα / GLMR	OSMRβ	JAK1, (JAK2)	STAT3/5/1	Associated with inflammation in the skin, lung and gut. Chemokine-inducing activity. Regulates hematopoietic progenitor cell homeostasis (R&DSystems n.d.).

		Cytokine	Receptor chain(s)			JAKs	STATs	Main biological function
TYPE I CYTOKINE RECEPTORS	γ_c family	IL-2	IL-2Rα	IL-2Rβ	γ_c	JAK1, JAK3, (JAK2)	STAT5/1 (STAT3)	Promotes T- and NK cell proliferation. Can increase the number of T_{reg} cells and prevent the uncontrolled expansion of immune responses. Regulates B cell activities (Lin and Leonard 2018).
		IL-4	IL-4Rα		γ_c	**JAK1, JAK3**	**STAT6**	Th2- and B-cell differentiation. Promotes B-cell and macrophage activities (Lin and Leonard 2018).
		IL-7	IL-7Rα		γ_c	**JAK1, JAK3**	**STAT5** (STAT3)	Drives lymphoid (especially T-cell) development, homeostasis and function (Mackall, Fry, and Gress 2011).
		IL-15	IL-15Rα	IL-2Rβ	γ_c	**JAK1, JAK3**	**STAT5** (STAT3)	Enhances the growth and functions of activated T, B, and NK cells, acts as a chemoattractant for NK-cells (Choi et al. 2004).
		IL-21	IL-21R		γ_c	JAK1, JAK3	**STAT3**/5 (STAT1)	Regulates the proliferation and function of mature T and B cells. Promotes expansion and cytotoxicity of NK cells (Parrish-Novak et al. 2000; Spolski and Leonard 2014).
		IL-9	IL-9Rα		γ_c	JAK1, JAK3	**STAT5**/3	Th9 cell differentiation, mast-cell proliferation, antitumor activities (Lin and Leonard 2018).
		TSLP	IL-7Rα		TSLPR / CRLF2	JAK1, JAK2	STAT1/3/4/5/6	Activation of DCs (Liu et al. 2007).
		IL-13	IL-4Rα		IL-13R	JAK1, JAK2, TYK2	**STAT6,** (STAT3)	Activates B cell functions and inhibits inflammatory cytokine production (Wynn 2003).
	IL-3 / β_c	IL-3	IL-3Rα		β_c (gp140)	JAK2, (JAK1)	**STAT5**/3	Multipotent hematopoietic growth factor. Induces proliferation and maturation of pluripotent hematopoietic stem cells. Supports vessel formation and tumour angiogenesis (Mangi and Newland 1999).
		IL-5	IL-5Rα		β_c (gp140)	JAK2	**STAT5**/1/3	Required for eosinophil and B-cell (in mice) differentiation and survival (Hitoshi et al. 1991).
		GM-CSF	GM-CSF-Rα		β_c (gp140)	JAK2	**STAT5**	Induces activation of monocytes/macrophage. At the site of inflammation, recruits myeloid cells and enhances their survival (Ushach and Zlotnik 2016).
	Single chain	EPO	EPOR			**JAK2**	**STAT5**	Stimulates erythropoiesis. Impairs the formation of pro-inflammatory factors such as TNF-α, IL-6, IL12/IL-23 (Nairz et al. 2012).
		GH	GHR			**JAK2**	**STAT5,** (STAT3)	Regulates growth, metabolism and aging processes (Vijayakumar, Yakar, and Leroith 2011).
		PRL	PRLR			**JAK2**	**STAT5**	Essential to the maturation of the mammary glands during pregnancy (Naylor et al. 2003).
		TPO	TPOR / MPL			**JAK2**	**STAT5**	Differentiation of megakaryocytes and platelets. Homeostasis of hematopoietic stem cells (Smith and Murphy 2014).

2.2.2 The JAK-STAT signalling pathway

JAKs are non-receptor tyrosine kinases (NRTKs) that drive the signaling of over 50 cytokines, growth-factors and hormones (Hammaren, Virtanen, Raivola, et al. 2019). JAKs are cytoplasmic proteins that constitutively bind to their cognate receptors and become active as the ligand binds to the extracellular part of the receptor (Witthuhn et al. 1993). The binding of the substrate induces conformational changes and dimerization (or oligomerization) of the receptors. This brings the JAKs into proximity and enables the transphosphorylation of the JAK kinase domains (Figure 3). Typically, the four JAKs form heterodimeric pairs, but JAK2 homodimerizes in the erythropoietin receptor (EPOR), thrombopoietin receptor (TPOR), growth hormone receptor (GHR) and prolactin receptor (PRLR) systems (Waters and Brooks 2015). Subsequently, the activated JAKs phosphorylate specific tyrosines in the cytoplasmic domain of the receptors, which creates a docking site for STATs and other signaling molecules. Finally, JAKs phosphorylate and STATs that, once activated, translocate to the nucleus and initiate the transcription of specific genes.

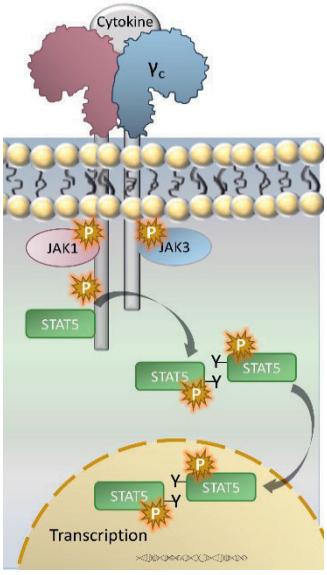


Figure 3: A schematic presentation of the JAK-STAT pathway, exemplified as the JAK1/JAK3-driven γc-signaling. In the pathway, cytokine-bound receptors dimerize and allow the transphophorylation of JAK1 and JAK3. STAT5 binds to the receptor where JAK can phosphorylate it. Activated STATs dimerize, translocate to the nucleus and initiate the transcription of specific genes.

2.2.3 Structure and function of the JAK domains

Structural studies provide valuable information that is used to deduce the function of a protein, find regulatory sites within kinases and to design small molecular weight inhibitors against them. However, proteins and especially kinases are highly dynamic, which is a feature not captured in the crystallized structures. Thus, both structural and functional studies are essential.

JAKs are relatively large proteins, constituting of approximately 1150 amino acids and weighing around 130 kDa. They consist of four functionally distinct parts, domains, that each has characteristic features (Figure 4). Although no high-resolution structure of the full-length JAK has been obtained, multiple structures of the individual JAK domains are available in the protein data banks. Only the structures of the JAK3 JH2 and the FERM-SH2 module remain unsolved. Still, reliable JAK3 models can be constructed by homology modeling the protein against known JAK structures. The following chapter briefly describes the features of the JAK domains.

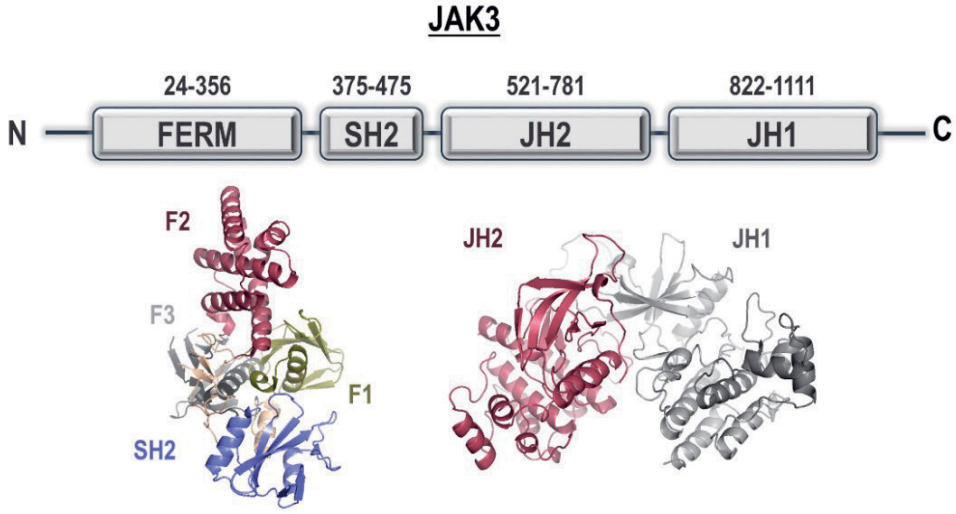


Figure 4: A schematic presentation of the JAK domains and the amino acid numbers for JAK3 (according to UniProt). Below, the solved structures of the FERM-SH2 module (JAK2 4Z32) and the JH2-JH1 module (TYK2 4OLI). The FERM F1-3 subdomains are also designated.

2.2.3.1 FERM-SH2

JAKs consists of seven Janus-homologue (JH) domains that together form four distinctive domains. The N-terminal FERM (band 4.1 protein, ezrin, radixin and moesin) domain (JH7-JH5 and parts of JH4) comprise the main receptor interaction site together with the adjacent Src homology 2 domain (SH2, containing the JH4-3 domains) (Ferrao and Lupardus 2017). The FERM-SH2 module binds to the proline-rich box1 and the more variable box2 domains of the receptor, which is crucial for the dimerization and activation of JAKs.

The FERM structure resembles the canonical "clover leaf" architecture of the ezrin, radixin and moesin and focal adhesion kinase (FAK) proteins, which are formed of F1, F2 and F3 subdomains. (Ferrao and Lupardus 2017; Frame et al. 2010; Haan et al. 2006; Tsukita, Yonemura, and Tsukita 1997). In JAKs, the F1 and F3 interact with the SH2 domain while the F2 likely contacts the cell membrane through a large hydrophobic interface (Wallweber et al. 2014; Wilmes et al. 2020).

Unlike SH2 domains in general, JAK SH2 does not bind phosphotyrosine residues (pY), but interact with the receptor box2 and together with FERM conveys the JAK-receptor interaction (Radtke et al. 2005). According to the available structures, the box2-JAK SH2 interaction is conserved across the JAK–receptor complexes but the features that determine the specific JAK-receptor interactions are not fully understood (Ferrao and Lupardus 2017). However, the conserved SH2-receptor interaction likely enables the binding of individual JAKs to several receptors.

The FERM F2 subdomain interacts with the receptor box1, but the conformation of the F2 deviates among JAKs and likely contribute to the specificity of the JAK receptor binding. Structural data suggests that the orientation of the JAK2 F2 α3-helix differs considerably from the one in JAK1 and TYK2, which creates incompatibility with certain box1 sequences. The sequence conservation between the receptors is generally low (sequence identity between the same receptor family is ~20 % (Broughton et al. 2012)) that further promotes specific interactions between JAKs. Moreover, considerable differences where observed in the FERM-SH2 module between the JAK1/IFNLR and JAK2 EPOR/LEPR structures, and also the

JAK1/IFNLR and TYK2/IFNAR1 interactions deviate significantly (Ferrao and Lupardus 2017).

In addition to its receptor binding function, FERM-domains can alter the conformation of the entire JAK-kinase and hence participate in the activation. Zhou and colleagues showed that JAK3 JH1 coimmunoprecipitates together with FERM. In the same study, the activity of isolated JH1 was increased when recombinantly expressed FERM was introduced into the solution (Zhou et al. 2001). Finally, the expression of the kinase-dead JAK1 K907E was shown to decrease the JAK1 binding to the IFNγ-receptor 1 (IFNGR1) (Haan et al. 2008). Together, these data support the direct interaction between JH1 and FERM.

2.2.3.2 JH1-JH2

Janus kinases were named based on the two-faced Roman god Janus, referring to the highly homologous domains that together constitute the C-terminus of JAK. The far C-terminal domain (JH1) is a traditional kinase entailing the previously described motifs required for the phosphotransferase activity (DFG, HRD and the catalytic β3 lysine). In addition, JAK JH1 contains a conserved pair of tyrosines within the activation loop, the phosphorylation of which contributes to the activation of kinases. The pseudokinase domain (JH2) resides between SH2 and JH1 and is homologous to JH1. However, the domain lacks some critical residues required for the phosphotransferase reaction. These include the third Gly of the canonical GXGXXG motif, the conserved Phe residue (in the DFG motif) and the catalytic, nearly invariant Asp residue in the catalytic loop (HRD)(Hammaren, Virtanen, et al. 2015; Wilks et al. 1991)(Hammaren, Virtanen, et al. 2015; Leonard and O'Shea 1998) (see Figure 1 in Chapter 2.1.2). However, JH2 exerts an important negative regulatory function on JH1 and in this way maintains low STAT activity when substrate is not bound to the receptor (Lupardus et al. 2014; Saharinen and Silvennoinen 2002; Saharinen, Takaluoma, and Silvennoinen 2000; Yeh et al. 2000). The structure of the TYK2 JH2-JH1 revealed that JH2 binds JH1 adjacent to the active site that limits the conformational mobility of JH1, and hence the transphosphorylation and kinase activity (Lupardus et al. 2014).

Studying the activating and inactivating JAK mutations can give insight of their activation mechanisms both in health and in disease. A majority of the patient-derived JAK mutations localize in JH2, specifically at the JH2-JH1 interface (Silvennoinen and Hubbard 2015). This, together with the studies showing that disruption of JH2 and the JH1-JH2 interaction activates JAK-STAT signaling strongly support the regulatory function of JH2 (discussed further in Chapter 6.1) (Luo et al. 1997; Saharinen et al. 2000; Shan et al. 2014).

Allosteric regulation of the active JH1 by JH2 is not a unique feature of JAKs but similar kinase-(pseudo)kinase regulation has been observed e.g. in MLKL where the pseudokinase domain promotes the disengagement of an N-terminal four-helix bundle domain and hence the formation of an active tetramer. The RAF family kinases also form homo- and heterodimers among the kinases (ARAF, BRAF, CRAF) and their pseudokinase paralogs KSR1 and KSR2 that scaffold and allosterically regulate BRAF, and the entire Ras-Raf-MEK-ERK pathway (reviewed by Terrell and Morrison 2019). In addition to negatively regulating JH1 activity, JH2 aids in the cytokine-induced activation of the kinase (Saharinen and Silvennoinen 2002).

2.2.3.3 ATP binding and activity of JAK JH2

Although the DFG and HRD motifs are not conserved in the JAK pseudokinase domains, some residues such as the Asp184 in the DFG-motif and the Gly52 in the glycine-rich loop are present in JH2 (Hammaren, Virtanen, et al. 2015). These residues are important for the ATP binding and, indeed, all JAK JH2s bind ATP, despite lacking kinase activity (JAK2 JH2 is an exception, described below). The structures of JAK1, JAK2 and TYK2 JH2 show that the domain bind ATP in a non-canonical manner, with only one Mg^{2+} present that is coordinated by conserved Asn171. Currently, no crystal structure of JAK3 JH2 has been obtained.

The JAK pseudokinase domains, and pseudokinases in general, are considered as kinase inactive, which makes JAK2 JH2 an interesting exception, as JAK2 JH2 has been shown to entail kinase activity in vitro (Ungureanu et al. 2011). However, the detected activity is approximately 10 % of the JH1 activity. Furthermore, JAK2 JH2 becomes autophosphorylated on S523 and Y570 residues that negatively regulate the

kinase activity of both JH2 and JH1. These residues are not conserved in other JAKs (Mazurkiewicz-Munoz et al. 2006; Ungureanu et al. 2011).

2.2.4 STATs

Downstream of JAKs, STAT transcription factors initiate the transcription of specific target genes. Of note, the JAK-STAT pathway is called the "classical" STAT-signaling pathway but also e.g. PDGF (platelet-derived growth factor) and FGF (fibroblast growth factor) receptors can activate STATs (Paukku et al., 2000; reviewed by Lim & Cao, 2006).

STATs bind to the intracellular parts of the cytokine receptor via their conserved SH2 domain. This enables the docking to the receptor via the (JAK) phosphorylated tyrosine residues. Subsequently, STAT become phosphorylated by JAKs, which allows them to form homo- or heterodimerize, or higher order tetramers through their phosphorylated tyrosines and the SH2 domains (Chen et al. 1998). At least STAT1/STAT2, STAT1/STAT3 and STAT1/STAT4 heterodimers have been reported (reviewed by Levy & Darnell, 2002). STATs shuttle between the cytoplasm and nucleus, and only phosphorylated STATs are retained in the nucleus where they can recognize and bind to tens of thousands of DNA sites within the genome and regulate the transcription of thousands of protein-coding genes, microRNAs and long non-coding RNAs (reviewed by Mitchell & John, 2005). Each STAT has affinity towards specific DNA patterns, which can overlap between the family members. For example, most STATs bind to the gamma-activated sites (GAS) proximal to the transcriptional starting sites (promoters), but they can also bind to enhancers or other cis regulatory elements. Finally, STATs are released upon dephosphorylation by nuclear phosphatases (Marg et al. 2004; Meyer et al. 2003).

Many aspects promote the specific STAT-driven activation of transcription. These include the tendency for specific receptor-JAK-STAT complexes that promote the formation of varying STAT complexes. Moreover, the specificity of activated STATs towards DNA sequences, cooperation with distinct transcription factors (e.g. IFN regulatory factors), cell-type specific gene expression and the state of the chromatin

of the target gene contribute to the finely-tuned output of the JAK-STAT cascade (Ehret et al. 2001).

2.2.5 Regulation of the JAK-STAT signalling

Disassembling of the activated JAK-STAT cascade is as important as the timely and specific activation (reviewed by O'Shea et al., 2002). Regulation of the signaling consists of the reversible phosphorylation of JAKs and STATs, as well as other brakes that function to maintain control and prevent pathological proliferation and carcinogenesis (JAK-driven diseases are discussed in Chapter 2.3). The following chapter describes molecules that regulate the pathway by directly binding to JAKs (phosphatases and SOCS) or indirectly control the signal activation (protein inhibitor of activated STATs: PIAS and CIS).

2.2.5.1 Protein phosphatases

Protein tyrosine phosphatases (PTPs) dephosphorylate kinases and in this way downregulate the JAK signaling. SH2-containing protein tyrosine phosphatases (SHP)2 and SHP1 are two closely related phosphatases that are essential in hematopoiesis (both SHP1 and SHP2) and lymphopoiesis (SHP2) (reviewed by Babon et al., 2014; D. Xu & Qu, 2008). They consists of two tandem SH2 domains that recognize the phosphorylated tyrosines, mediate the interaction between the substrate and regulate the function of the classic protein phosphatase domain. An unstructured tail in the C-terminus includes two important tyrosine residues (Y542 and Y580) that regulate the activity. SHP1 can directly associate with all JAKs in addition to a number of other cytokine and growth factor receptors (e.g. EPOR). SHP2 regulates the JAK1/STAT1 signaling and the activation of STAT3 and STAT5 (reviewed in Xu and Qu 2008). Somewhat controversially, few studies suggest that SHPs may activate JAK-signaling but the mechanism is not fully understood. A possible model is that SHP2 competes with SOCS1 for binding to JAK2 (discussed in the next chapter) (Ali et al. 2003; Ungureanu et al. 2002). The binding of SOCS1 marks JAK2 to the ubiquitin-dependent degradation pathway and competition with

the SHP2 might decrease the JAK2 degradation and hence increase STAT5 activation.

In addition to SHPs, other PTPs dephosphorylate JAKs and STATs to maintain homeostatic signaling, and may contribute the formation of several malignancies (Ruela-de-Sousa et al. 2010). CD45 and PTP1B phosphatases are ubiquitously expressed while the T-cell PTP (TC-PTP) is found solely in hematopoietic cells (reviewed by D. Xu & Qu, 2008). TC-PTP and PTP1B have homologous catalytic domains and both dephosphorylate JAK1, STAT3 and STAT5. PTP1B also dephosphorylates JAK2 and TYK2 (Myers et al. 2001) while TC-PTP targets JAK1, JAK3 and STAT, and controls the IL-2 induced STAT5 activation (Simoncic et al. 2002).

2.2.5.2 SOCS

The suppressor of cytokine signaling (SOCS) family proteins are part of the negative feedback loop of the JAK-STAT signaling. The expression of SOCS is induced by the JAK-STAT function and they use several different mechanisms to regulate JAK-signaling (reviewed by Babon et al., 2012). Like SHPs, SOCS have a SH2 domain that is surrounded by an N-terminal domain and a C-terminal SOCS box domain. The latter promotes the ubiquitination and subsequent degradation of the protein substrates by recruiting the modules of E3 ubiquitin ligase. Using their SH2 domains SOCS binds to phosphotyrosine motifs within the cytoplasmic domain of (JAK-bound) cytokine receptors. Out of the eight SOCS encoded by the human genome, only SOCS1 and SOCS3 can directly bind JAK and inhibit JAK catalytic activity. Using the kinase inhibitory region, SOCS3 occludes the substrate-binding groove on JAK2 and blocks the substrate association. Interestingly, SOCS3 inhibits JAK1, JAK2 and TYK2 but cannot bind to the JAK3 due to the lack of the "GQM" motif that is present in all other JAK JH1s (Lucet et al. 2006).

The cytokine inducible SH2 containing protein (CIS) is a member of the SOCS family. CIS inhibits cytokine signal transduction by competing with STAT5 or other signaling molecules for docking sites on the receptor (Krebs and Hilton 2001).

2.2.5.3 Other regulatory proteins

The SH2B family of adapter proteins encompasses SH2B1, SH2B2 (APS) and SH2B3 (LNK). SH2Bs have a SH2 domain and a pleckstrin homology (PH) domain that directs the protein to the membrane. All SH2B proteins target the same site, pY813 of JAK2. The tyrosine is not conserved in JAK1 or TYK2 but JAK3 has a homologous tyrosine (Y785) that provides docking site for SH2B (Kurzer et al. 2004). While SH2B1 and APS have been demonstrated to activate JAK2, the seemingly similar LNK negatively regulates the JAK2 activity (Babon et al. 2014).

The protein inhibitors of STATs (PIAS) regulate the JAK-signaling at the STAT level. PIAS induce SUMOlyation of STATs (SUMO: small ubiquitin-related modifiers) and suppress transcriptional activity by reducing the STAT phosphorylation and translocation (Niu et al. 2018; Rabellino, Andreani, and Scaglioni 2017).

2.3 Disease-driving mutations in the JAK-STAT pathway

Wild type JAK singling plays important part in immunity and cell-growth and alterations in the pathway are associated to autoimmune diseases and cancers. Inherited mutations in JAK3 and TYK2 cause human immune deficiency syndromes while somatic JAK1, JAK2 and JAK3 mutants drive cells to cytokine independent signalling, leading e.g. to myeloproliferative neoplasms (MPNs) and leukaemia/lymphoma (reviewed by Casanova et al., 2012; Hammaren et al., 2019). Consequently, JAK-inhibition is used as a treatment strategy for situations where the body´s own immune defense is overtly active, i.e. autoimmune diseases such as rheumatoid arthritis (RA) and the host-versus graft diseases (Schroeder et al. 2018; Virtanen et al. 2019). Apart from JAKs, other components of the signaling cascade can be affected and cause severe symptoms. In his chapter, I focus on the JAK mutations but a short overview of pathogenic mutations in the JAK-binding receptors and STATs is also included.

Functional JAK signaling requires the conformational integrity of the NRTK receptors. For example, mutations that target the thrombopoietin receptor (TPOR) region that maintains the inactive conformation can cause myeloproliferative

neoplasms (Defour et al. 2013). Similarly, the premature termination of the EPOR cytoplasmic region disrupts the autoinhibitory region, causes hypersensitivity to EPO, and is associated with dominant familial erythrocytosis (Arcasoy et al. 2002). Truncations in the granulocyte colony-stimulating factor receptor (G-CSFR) promotes acute lymphocytic leukemia (ALL). These mutations remove the binding site for the negative regulators, SOCS, making the receptor resistant to ubiquitination (Irandoust et al. 2007). Lastly, mutations in the IL2GR gene causes X-linked severe combined immunodeficiency (discussed below) and also IL-12 and IFNGR1/2 mutations are associated to immune deficiencies (Wu & Holland, 2015).

Mutations in STATs can drive both solid and hematopoietic cancers (reviewed in Schwartz et al., 2016; Shahmarvand et al., 2018). STAT3 and STAT5 are the most significant in cancer development and most of the mutations they entail target the SH2 domain (Andersson et al. 2016). STAT1 is the most frequently mutated in melanoma. The mechanism by which the STAT mutations function has not been studied extensively, but studies with designed and patient-derived STAT mutants have suggested that mutations can promote STAT oligomerization and cause constitutive tyrosine phosphorylation and DNA binding activity (Mertens et al. 2015; Onishi et al. 1998). Moreover, mutated STATs may promote signaling effectors that are not part of the JAK-STAT pathway (Verhoeven et al. 2020).

2.3.1 JAK loss of function (LOF) mutations

Albeit discovered only after other members of the family, JAK3 was the first to be associated with disease. JAK3 is mainly expresses in lymphoid cells where it transmits signals together with its sole receptor, the common gamma chain (γ_c, or IL2RG). The γ_c, interleukins (see Table 2) are important for immune development and regulation (Leonard and O'Shea 1998; Yamaoka et al. 2004). Highlighting its biological significance, damaged JAK3 signaling causes severe combined immunodeficiency (SCID): a disease resulting in complete lack of T- and natural killer (NK)-cells and reduced B-cell function. X-linked SCID arises from mutations in the IL2RG gene in the X chromosome and thus primarily affects male infants (Noguchi et al. 1993) while the autosomal recessive type of SCID is directly caused by mutations in JAK3. The latter accounts for 7-14% of heritable SCIDs.

Loss-of-function (LOF) mutations have been detected in all JAK3 domains, but like the JAK gain-of-function (GOF) mutations discussed in the next chapter, the majority of them cluster in JH2 (Figure 5, Table 3) (Hammaren, Virtanen, Raivola, et al. 2019; Notarangelo et al. 2001). JAK3 LOF mutations include early stop codons and frame shifts that lead to a truncated or improperly folded protein or otherwise abrogate JAK3 expression (e.g. FERM G36fsX146, SH2 R445X). Other LOF mutations disrupt the kinase activity (e.g. del 58A, D169E in FERM and G589S, C759R in JH2) while some (FERM Y100C) prevent the receptor binding (Cacalano et al. 1999; Candotti et al. 1997; Chen et al. 2000). The mechanism of function has only been proposed for some mutations. For example, C759R in the C-lobe and del 586-592 in the N-lobe of JH2 cause constitutive JAK3 autophosphorylation but decrease STAT activation, suggesting that these mutations alter the conformation of JH2 to allow the autophosphorylation, possibly by breaking the autoinhibitory state of JAK3 (Chen et al. 2000). Concurrently, the mutants likely hinder the dimerization and transphophorylation of JAK3 and JAK1, hence reducing the STAT activity and γ_c, signaling (Table 2).

Similar to JAK3, TYK2 deficient mice are viable. However, their host defense is impaired due to non-functional IL-12 and IL-18 signaling (Shimoda et al. 2002) and non-responsiveness to IFNα and IFNγ (Karaghiosoff et al. 2000). Currently, few patients with TYK2 deficiency have been found. The patients had multiple opportunistic virus and bacterial infections in various organs caused by the weakening of the IL-12 driven NK cell responses (Minegishi et al. 2006), and exhibited severely impaired cellular responses to IL-23 and IFNα signaling that creates high susceptibility to tuberculosis (Boisson-Dupuis et al. 2018).

Deficiency of JAK1 or JAK2 cause perinatal death in mice (Neubauer et al. 1998; Rodig et al. 1998), and only recently patient-derived LOF mutants were found in JAK1 and JAK2. However, the mutants were expressed solely in cancer cells, where JAK1/JAK2 deficient melanoma cells exhibited resistance against anti-tumor T-cell and IFNγ activity (Sucker et al. 2017). Moreover, undetectable JAK1 protein levels are linked to IFNγ insensitivity in human prostate adenocarcinoma cells (Dunn et al. 2005). Together, JAK1 (and JAK2) LOF mutations could be related to the immune evasion of cancers (Albacker et al. 2017; Shin et al. 2017).

Table 3: Mutation distribution between the JAK3 domains. Data is from (Hammaren, Virtanen, Raivola, et al. 2019) with some additions. The number of JAK3 GOFs and the SCID (LOF) mutations are shown separately.

Domain	FERM	SH2	JH2	JH1
Residues	1-375	375-476	477-820	821-1111
Total	**26**	**8**	**31**	**20**
SCID	16	5	18	10
Other (GOFs)	10	3	13	10

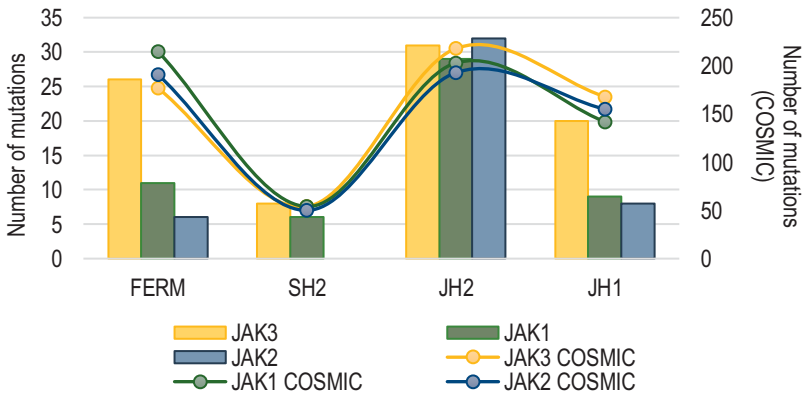


Figure 5: Mutation distribution between the JAK1, JAK2 and JAK3 domains. Data is compiled from the COSMIC database (Tate et al. 2018) and from the literary research by Hammarén et al. (Hammaren, Virtanen, Raivola, et al. 2019). JH2 of JAK1, JAK2 and JAK3 has higher number of mutations than the active JH1 domain. The number of FERM mutations is also high, especially in JAK3, which is likely due to the prevalent SCID mutations that disturb the receptor binding. Moreover, the JAK1 and JAK2 FERM mutations have lower incidence than the JH2 mutations, and many of the FERM mutations have not been characterized, leaving their biological function elusive.

2.3.2 JAK gain of function mutations (GOFs)

JAK gain-of-function (GOF) mutations drive cytokine-independent, constitutive activation of the JAK-STAT signaling. They are more common than LOF mutations that mainly target JAK3 (see previous chapter). Somatic GOF mutations particularly in JAK2 are found in patients with various types of leukemia and myeloproliferative neoplasms (Flex et al. 2008; Hammaren, Virtanen, Raivola, et al. 2019; Jeong et al. 2008; Ma et al. 2009). Screenings of several cancers revealed a low incidence of somatic JAK mutations in solid tumors. However, mostly JAK1 mutations were found in hepatocellular carcinoma xenografts and gynecologic tumors (reviewed in Hammaren, Virtanen, Raivola, et al., 2019) while few other where depicted in colorectal (JAK1, TYK2), gastric (JAK2, TYK2) and ovarian cancer samples (JAK3) (Bardelli et al. 2003; Greenman et al. 2007). None of the same mutations were detected in a following study, underlining the low frequency of the JAK mutants in solid cancers (Jeong et al. 2008).

JAK2 activation in human cancer was first noted by the finding of the gene fusion between TEL and JAK2 (translocation between chromosomes 9 and 12-JAK2). TEL is a transcription factor that regulates growth in hematological tissue and fusion with JAK2 causes constitutive JAK-signaling (Lacronique et al. 1997). Although point mutations, deletions and frameshifts are the main cause of non-functional JAK signaling, TEL-JAK2 and other JAK-fusion proteins have been related to the formation of lymphoid and myeloid leukemia (Chen et al., 2012; Vainchenker & Constantinescu, 2013; Wöss et al., 2019). Recently, the first activating TYK2 mutations were found in two primary leukemia patients (Waanders et al. 2017). Interestingly, the P760L and G761V mutations reside in the conserved JH2 DPG (DFG) motif and activate TYK2, STAT1, STAT3 and STAT5, plausibly by altering the conformation of the autoregulatory domain.

JAK2 V617F is the most frequent and widely studied JAK mutation. This JH2 mutation accounts for approximately 95% of the patients with polycythemia vera (PV) and 50% of the patients with essential thrombocytosis and primary myelofibrosis. Homologous mutant in JAK1 (V658F) causes acute lymphoblastic leukemia (ALL) and the homologous TYK2 V678F is constitutively active in cells although not found in patients (Staerk et al. 2005). Mechanistically, the mutation

stabilizes the αC-helix (αC) in the N-lobe of JH2 and induces cytokine independent dimerization of the receptors, likely by promoting the dimerization between the pseudokinase domains (JH2-JH2 dimerization discussed in Chapter 6.2.1) (Bandaranayake et al. 2012; Hammaren, Virtanen, Raivola, et al. 2019; Leroy et al. 2016, 2019). In addition, the mutation interacts with other residues within JH2 and forms the so-called FFV-triad (Gnanasambandan, Magis, and Sayeski 2010). The triad links the (JAK1) F575 in the SH2-PK linker, F636 in the αC-helix, and the V658 residues. Specifically, the Val to Phe mutation enables the formation of a π-π stacking interaction between the residues that causes stabilization of the αC (see Figure 13 in Chapter 5.3.4).

The majority of the JAK GOF mutations reside in JH2 but the activation mechanisms may differ depending on the intra- and interdomain interactions they alter (reviewed in Hammaren, Virtanen, Raivola, et al. 2019). In general, the JAK GOF mutants are highly dependent of the FERM-receptor interaction (Degryse et al. 2014; Losdyck et al. 2015). The most distinct mutation hotspot is at the JH1-JH2 interface, including the exon 12 region and the αC-helix (Haan, Behrmann, and Haan 2010; Shan et al. 2014). Another mutation rich region lies in the hinge between the JH2 N- and C- lobes and the flexible loop that reaches towards JH, likely interacting with JH1 and maintaining the inactive conformation (Figure 6). For example, pathogenic JAK1 mutants R724E/H/S and homologous mutations in JAK2 (R683G/S) and JAK3 (R657Q) reside in this regulative interface. Together with the neighboring residues, JAK1 R724 was modelled to form a salt bridge between JH1 and JH2 (JAK1 R724-E897 and K736-D899) (Canté-Barrett, Uitdehaag, and Meijerink 2016), and the interaction has been proven with charge-reversal mutants in JAK2 in cell-based assays (with JAK2 Y570R-K883E and R683E-D873N constructs) (Shan et al. 2014). In addition, the exon 12 region is frequently mutated in JAK2 and other JAKs (Sanz Sanz et al. 2014). It comprises an extended, loose N-terminal segment that is unique structure of the JAK pseudokinase domains. Exon 12 includes the SH2-JH2 linker, the αC helix and the β4 and β1 strands and has several contact points to JH1 (Figure 6). This makes it plausible that the exon 12 mutations loose the autoinhibitory JH1-JH2 interaction (Lupardus et al. 2014; Shan et al. 2014). Mutations in this region include the JAK2 M535I that causes acute megakaryoblastic leukemia and its homolog, the most frequently mutated residue in JAK3 (M511I) and driver of T-ALL (Hammaren, Virtanen, Raivola, et al. 2019).

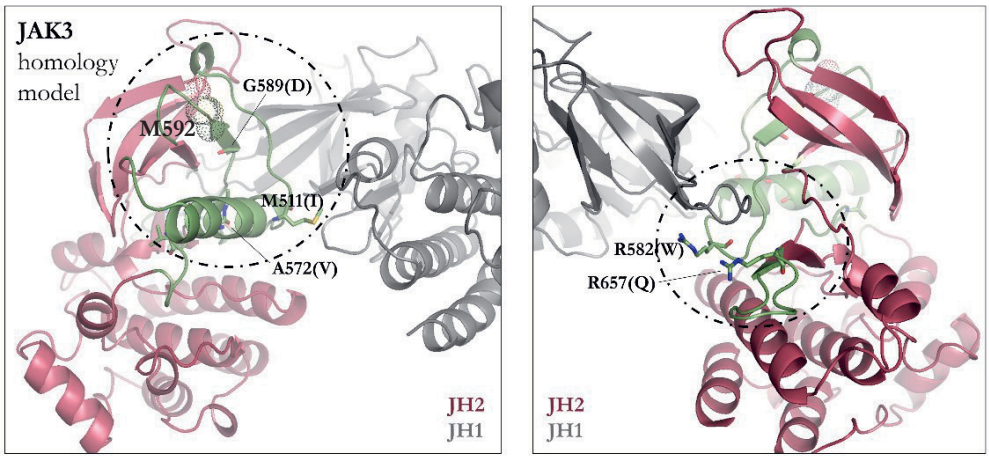


Figure 6: Hotspots of the JAK JH2 mutants shown in the JAK3 JH2(-JH1) homology model. The N-lobe of JAK JH2 has two regions where numerous pathogenic mutations cluster: Left, the region consisting of the αC-helix, SH2-JH2 linker and the β4-sheet (and linkers), and (right) the niche between N- and C-lobes consisting of a flexible loop that likely protrudes towards JH1. Example JAK3 GOF mutations are presented as sticks. The JAK2 V617F homolog JAK3 M592(F) is depicted as dots. The JAK3 M592F is not found in patients (see Chapter 5.3.4), but the residue is conserved in other JAKs and an effective driver of myeloproliferative neoplasms and leukemia. The R657(Q) and R582(W) residues that are mutated throughout JAKs are also depicted. The cartoon was constructed by superimposing a homology modelled JAK3 JH2 and the solved JAK3 JH1 structure (PDB: 1YVJ) above the TYK2 JH1-JH2 structure (PDB code 4OLI).

2.4 Inhibitors for JAK-signalling

Due to their role in the cancer pathogenesis and immune surveillance, kinases are highly prominent targets against a broad spectrum of disorders. At present, kinase inhibitors account for a quarter of all drug discovery efforts and FDA has approved 48 kinase inhibitors targeting e.g. EGFR, ERBB2, VEGFR (vascular endothelial growth factor receptor), B-Raf, PDGFR, Abl, Src, mTOR and the JAK-family (Roskoski 2019). However, the full potential of modulating the kinome, consisting of ~500 kinases, for immuno-oncology applications is yet to be discovered.

Kinase inhibitors can be classified based on their mechanism of action (Bhullar et al. 2018; Dar and Shokat 2011; Roskoski 2016). Type I inhibitors bind the hydrophobic

ATP-binding pocket of kinases in their active, DFG-in, conformation. Type II inhibitors occupy the pocket adjacent to the ATP-binding site of an inactive (DFG-out) kinases (Figure 7). The increased flexibility of the DFG-out conformation allows more conformational variation among kinases, making the binding of the type II inhibitors less conserved than the type I inhibitors. Type III aka allosteric inhibitors bind outside the active ATP-binding pocket (note that JAK JH2 is inactive and thus considered as allosteric site). Type IV aka substrate directed inhibitors are like type II but have reversible interaction with their target. Type V, or covalent inhibitors bind irreversibly (covalently) to their protein kinase target.

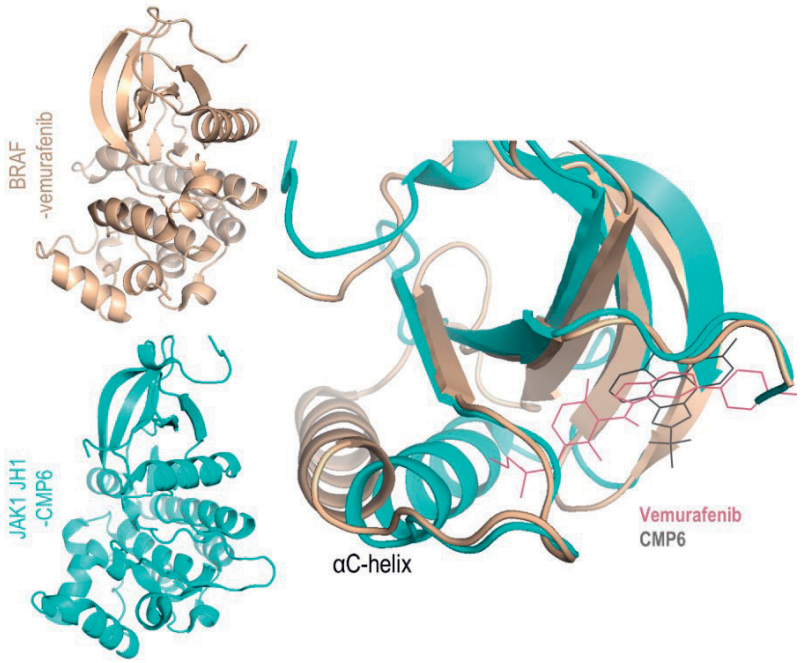


Figure 7: Comparison of the type I and type II inhibitors. Upper left panel: BRAF monomer with the type II inhibitor vemurafenib (in beige, PDB: 4RZV). Below: A classical type I inhibitor CMP6 bound to JAK1 JH1 (in cyan, PDB code: 3EYH). JAK1 JH1 is in active, DFG-in conformation while vemurafenib blocks BRAF to its inactive, DFG-out form (Bollag et al. 2012). Right: Close-up of the N-lobes, where the reorientation of the αC-helix is apparent between the DFG-in and DFG-out conformations. Structures of both inhibitors are shown in the close-up as sticks.

As discussed in Chapter 2.1.3, pseudokinases are important players in many signaling systems and majority of them contribute to human pathologies. Hence, targeting the pseudokinome has gained significant interest. Antibodies and small molecules have been developed against several pseudokinases, both with a targetable ATP-binding pocket (HER3, MLKL, JAK JH2) and ones that do not bind ATP. In the former case, modulators that target other allosteric sites in addition to the ATP-binding cleft can and have been developed, for example against ROR1 (Kung and Jura 2019; Sheetz et al. 2020).

In parallel with the kinase-targeted inhibitors, other approaches can be used to modulate cytokine signaling. Biological therapies are used in clinics for the treatment of autoimmune diseases and cancers (Clark, Flanagan, and Telliez 2014). They target the extracellular components of the signaling, i.e. the cytokines (TNFα and IL-12), cytokine receptors (IL1R, IL6R) (reviewed by Kopf et al., 2010), or other cell surface receptors that are expressed in lymphocytes or are able to prime them (CD20, CD80, and CD86) (Montgomery et al. 2002; Tokunaga et al. 2007). Unfortunately, the drugs must be administered intravenously and in constant intervals, which is inconvenient for the patient and raises the risk of infections at the injection site. Moreover, biological therapies are not without side effects. For example, antibodies directed against IL6Rα target both the membrane-bound and soluble forms of the receptor. This may lead to trans signaling with the accessory soluble gp130 receptor and unwanted effects on cell types that do not express the membrane-bound IL6Rα (Kopf et al. 2010). Kinase inhibitors can be taken orally, which minimizes the drug discontinuation rate over parenterally delivered drugs. Finally, the production of small kinase inhibitors is generally more cost-efficient due to the more compact structures.

In addition to biological inhibitors and kinase binders, inhibitors targeted directly to STATs are being developed but have not reached the clinics (Thomas et al. 2015). The lack of suitable binding sites hampers the development of the small-molecule inhibitors, namely because the regions that in theory could be modulated to inhibit the function of STATs are planar and hence difficult to target. Nevertheless, nucleic acid-based approaches such as RNA interference with small interfering RNAs (siRNAs) have been shown to inhibit STAT3 and STAT5 activation in cell models, suggesting that STATs can be potential therapeutic targets (Verhoeven et al. 2020).

Approved in late 2011 against myelofibrosis, ruxolitinib was the first FDA-approved JAK inhibitor. Tofacitinib (approved in late 2012) was the first JAK inhibitor accepted against autoinflammatory and autoimmune diseases (Clark et al. 2014; Virtanen et al. 2019). Currently five JAK inhibitors (JAKinibs): tofacitinib, baricitinib (against rheumatoid arthritis), ruxolitinib (myelofibrosis), upadacitinib (rheumatoid arthritis) and fedratinib (myelofibrosis) are in clinical use in the US and Europe. Each of the above-mentioned drugs are type I class kinase inhibitors. Tofacitinib was initially accepted against rheumatoid arthritis but since FDA has accepted its use against ulcerative colitis (UC) and Crohn's disease (CD). These inflammatory diseases are chronic but clinical remission can be achieved with treatment. Initially, tofacitinib was marketed as JAK3 specific but has since demonstrated to be a potent JAK1 inhibitor in addition to JAK3 (McInnes et al. 2019). The other inhibitors that are currently in clinical use mainly target JAK1 and/or JAK2.

At the present, almost a dozen JAKinibs are in stage II/III clinical trials (see Table 4 for examples), among them compounds that are not type I inhibitors (Virtanen et al. 2019). The JAK3 specific PF-06651600 is a powerful example of a covalent type IV inhibitor (Pei et al. 2018; Vazquez et al. 2018). It binds to a unique cysteine residue in JAK3 JH1 (Cys909 in the human sequence) and exhibits a significant specificity against JAK3: it inhibits the JAK3 kinase activity with 33.1 nM IC50. The homologous Cys residue is conserved only between 10 other protein kinases, including EGFR and TXK. However, the effect of PF-06651600 is considerably weaker than seen with JAK3 (11% and 7% inhibition for EGFR and TXK, respectively, 99% inhibition for JAK3). The compound is in clinical trials for alopecia areata, rheumatoid arthritis, Crohn's disease and ulcerative colitis (reviewed by T Virtanen et al., 2019). The novel type II inhibitor, CHZ868 is another example of inhibitors that do not bind to the conserved ATP-binding site. It targets the kinase domain of JAK2, locks it in an inactive conformation and has been successfully used to inhibit JAK2 signaling in MPN cells and in murine MPN models (Meyer et al. 2014; Wu et al. 2015).

Although the inhibition and modulation of the JAK-STAT signaling have significant potential to treat autoimmune diseases and cancers, a fair number of patients do not respond to the current therapies and side effects occur. Off-target effects of the first-generation JAKinibs include cytopenias, infections and hyperlipidemia and patients

in tofacitinib treatment have slightly increased risk of malignancies and lymphoma (similar symptoms are encountered with biological drugs) (Rubbert-Roth et al. 2016; Virtanen et al. 2019). Recently, small compounds that target bind JH2 have been developed and shown promising inhibitory potency and selectivity (Wrobleski et al. 2019). Bristol Myers Squibb and colleagues developed one of the most advanced JH2 specific inhibitors, BMS-986165 that targets TYK2 and has biochemical and biological properties that contribute to high potency (Wrobleski et al. 2019). It is currently in phase II clinical trials for the treatment of active psoriatic arthritis, ulcerative colitis (UC) and systemic lupus erythematosus (SLE). These next generation JAKinibs aim for improved selectivity and potency. To conclude, more knowledge is required for developing high potency JAK-inhibitors, and the recent advancements and challenges in this field are further discussed in Chapters 6.3 and 6.4.

Table 4: FDA approved inhibitors mentioned in this thesis. The biding-type/mechanism of action, targeted diseases and the stage of the drug development are indicated.

Inhibitor	Target (secondary binders)	Type	Disease	Status
Ruxolitinib	JAK2/JAK1 (JAK3)	I	MF, PV	FDA approved
Tofacitinib	Pan JAK	I	RA, PsA, UC	FDA approved
Baricitinib	JAK2 (JAK1)	I	RA	FDA approved
Upadacitinib	JAK1 (JAK2>JAK3)	I	RA	FDA approved
Fedratinib	JAK2 (JAK1>JAK3)	I	MF	FDA approved
Filgotinib	JAK2 (FLT3, BRD4)	I	RA,CD, AS, PsA	Phase III and II
PF-06651600	JAK3 (EGFR, TXK)	V	AA, RA, CD, UC	Phase II
BMS-986165	TYK2 JH2	III	PA, UC, SLE	Phase II
CHZ868	JAK2 JH2 (KIT, PDGFR, VEGFR)	II	MPN, B-ALL	Pre-clinical studies
Vemurafenib	BRAF	II	Late stage melanoma	FDA approved
Adalimumab	TNFα	antibody	RA, PsA, CD, UC	FDA approved
Methotrexate	variety of targets/mechanisms	RA, chemotherapy agent	PS, RA, several malignant neoplastic diseases	FDA approved

Abbreviations for the diseases: AA: alopecia areata, AS: ankylosing spondylitis, B-ALL: B cell acute lymphoid leukemia, CD: Crohn's disease, MF: myelofibrosis, MPN: myeloproliferative neoplasm, PS: psoriasis, PsA: psoriatic arthritis, PV: polycythemia vera, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, UC: ulcerative colitis.

3 AIMS OF THE STUDY

Throughout the decades of research, we have taken major leaps in depichering the biology and function of the JAK-STAT pathway. For example, it has been established that the catalytically inactive pseudokinase domain is a crucial regulator of the JAK activation, and that modulating the domain has great potential regarding the development of more potent JAK inhibitors. However, important details about the mechanisms underlying the physiological and pathogenic JAK-activation are not completely understood. These include the interdomain interactions in the basal and active states, as well as the cooperation between JAKs and their cognate receptors. In addition, the factors that dictate the specificity between the JAK-signalling cascades are not well defined. Further biochemical, structural and biological data from JAK1-3 and TYK2 are required to systematically compare their properties and to define the common and unique mechanisms that induce the flexible yet specific action of the numerous cytokine-driven pathways.

Specific aims of the current study:

1. Unravel the mechanism of JAK function, focusing on JAK1 and JAK3 JH2.

– Provide previsouly lacking biochemical information of the JAK3 JH2 (recombinant) protein.
– Elucidate the effects of clinical and structure-based mutations on regions that are important for the activation/regulation in both wild type and pathogenic backgrounds.
– Comprise a molecular dynamic simulation model to describe the inactive and active states of the JAK2-EPOR system, and to bring forth molecular understanding about the V617F activation and how it could be inhibited.

2. Characterize the known/hypothesized allosteric sites in JAK1/3 JH2.

– Compare the biochemical and biological effects between blocking the ATP binding to JH2 and modulating the outer face of the JH2 αC-helix. Decipher the inhibitory potential of these mutants against wild type and pathogenic JAK activation with and without cytokine stimulation.

3. Decipher the roles of JAK1 and its JH2 in different receptor systems.

– Elucidate the effects of individual JAKs between signalling systems and compare the roles of their pseudokinase domains on STAT activation.

4 MATERIALS AND METHODS

4.1 Plasmid constructs, cloning, and site-directed mutagenesis

4.1.1 Mammalian expression constructs (I–IV)

The following constructs for mammalian expression were done in pCI-neo expression vector (Promega, Madison, WI, USA) using SalI-NotI restriction sites: full-length human JAK1, JAK2, JAK3 and TYK2. Human STAT5A was in pXM vector (Wakao, Gouilleux & Groner 1994). JAKs and STAT5A were C-terminally tagged with the human influenza hemagglutinin tag (HA; amino acid sequence YPYDVPDYA).

4.1.2 Expression constructs for recombinant protein production in insect cells

Bac-to-Bac system was used for the recombinant expression of human proteins (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Constructs were cloned in pFastBac1 vector (Invitrogen) using SalI-NotI restriction sites (Table 5). The bacmids were produced in and isolated from DH10Bac cells (Invitrogen) according to manufacturer's instructions. JAK3 JH2 recombinant protein constructs were C-terminally tagged with tobacco etch virus (TEV) protease cleavage site followed by a hexahistidine tag (His_6). JAK1 JH2 constructs were tagged N-terminally with glutathione-S-transferase (GST) fusion protein and with TEV protein. The proteins were expressed in insect cells and purified with Ni-NTA beads or GSH-coupled resin (JAK3 and JAK1, respectively), after which they were subjected to size-exclusion filtration.

Table 5: Recombinant JH2 constructs used in this study. The amino acid number of the start and the end of the construct are shown with the C- or N-terminal tags.

JAK domain	Boundaries and tags	Mutants
JAK3 JH2	His6 511-790	-
		R657Q
		I535F
		K556A
JAK1 JH2	561–852 GST	-
		I597F
		K622A

4.1.3 Homology modelling of JAK3 JH2

The homology model of the pseudokinase domain of JAK3 was generated with the SWISS-MODEL server using the TYK2 structure (Protein Data Bank identification 4OLI, resolution 2.80 Å) as the modeling template. Sequence similarity bwetween full-length JAK3 and TYK2 is 39 % and between the pseudokinase domains 48 %. Graphical presentations were generated using the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA).

4.1.4 Site-directed mutagenesis

The JAK mutations and domain deletions were introduced using QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA) following manufacturer's instructions (see Table 6 for all point mutations used in the study). The constructs were analyzed and confirmed by Sanger sequencing.

Table 6: A short description of the mutations used in the studies.

	Mutation	Description	Article(s) where used
JAK1	ΔJH2 (583–855)	JAK1 with pseudokinase domain deletion	I, II
	ΔJH1 (875-1153)	JAK1 with kinase domain deletion	I, II
	L633K	At the solvent exposed face of the JH2 αC-helix, can inhibit STAT activation.	II
	I597F	Homologous to JAK2 I559F, but does not inhibit constitutive activity.	I, II
	V658F	Analogous to JAK2 V617F.	I, II
	K622A	Removes conserved β3 lysine in JH2. Designed to inhibit ATP binding. Homologous to JAK3 K566A and JAK2 K581A.	II
JAK2	E592R	Homologous to the JAK1 L633K.	II
	V617F	Hyperactivating MPN mutation	II,III
	I559F	Designed to sterically disrupt ATP binding to JH2. Verified to inhibit ATP binding and constitutive activity.	II
	P58A/E61A	Confirmation of the active dimer-model (FERM-JH2 interaction).	III
	R340A/R360A	Confirmation of the active dimer-model (FERM-JH2 interaction)	III
	E549K	Confirmation of the active dimer-model	III
	I324D	Confirmation of the active monomer-model (FERM-JH2 interaction). Decreases V617F activation.	III
	D348K	Confirmation of the active monomer-model (FERM-JH2 interaction). Decreases V617F activation.	III
	K497E	Confirmation of the active monomer-model (SH2-JH2 interaction). Decreases V617F activation.	III
	E666K	Confirmation of the active monomer-model. Decreases V617F activation.	III
	W298A	Confirmation of the inactive monomer-model. Further increases V617F activation.	III
	R300E	Confirmation of the inactive monomer-model. Further increases V617F activation.	III
JAK3	K566A	Homologous to K855A, disrupts ATP binding to JH2	I
	I535F	Homologous to JAK2 I559F, disrupts ATP binding to JH2	I,II
	L570F	Mutation designed to create the WT state as in JAK1, JAK2 and TYK2 The residue stacks with the mutated JAK2 V617F (or homolog) and enables the hyperactivation via the FFV-triad formation	II
	M592F	Homologous to JAK2 V617F	II
	E567R	At the solvent exposed face of the JH2 αC-helix, can inhibit STAT activation.	II
	R657Q	Found in T-ALL patients, constitutively active	I,II
	M511I	Found in T-ALL patients, constitutively active	I
	L857Q	und in T-ALL patients, constitutively active	I
	ΔJH2 (521-787)	JAK3 with pseudokinase domain deletion	I
	ΔJH1 (822-1124)	JAK3 with kinase domain deletion	I
	C759R	Found in SCID patients. Inhibits JAK3 kinase activity and reduces STAT5 activity.	I
	K855A	Kinase dead mutation: removes the catalytic lysine.	I

	V603F	Homologous to JAK2 I559F.	II
TYK2	L653R	Analogous to the JAK1 L633K.	II
	V678F	Homologous to JAK2 V617F, constitutively active in cells.	II

4.2 Mammalian cell culture, transfection, and cytokine stimulation

JAK2-deficient (γ2A), TYK2-ficient (11.1) and JAK1 and JAK3-deficient (U4Cγβ) human fibrosarcoma cells (Haan et al, 2011) were cultured using standard methods in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-Glutamine (Lonza), and 0.5% PenicillinStreptomycin (Lonza). Cells were cultured at 37 °C, 5% CO2 in a humidified incubator and split upon reaching ~80% confluence using Trypsin-EDTA (Lonza) according to manufacturer's instructions.

For transfection, cells were seeded onto 24- or 96-well tissue culture plates and transfected the following day using FuGENE HD (Promega) according to manufacturers' instructions. After 48 h, cells were washed with cold PBS and lysed using cold cell lysis buffer (50 mM Tris-Cl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF). The lysis buffer was supplemented with 2 mM vanadate (Sigma-Aldrich) for the inhibition of tyrosine phosphatases and protease inhibitors (8.3 μg/ml aprotinin (Sigma-Aldrich), 4.2 μg/ml pepstatin (Roche), and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich)). Lysates were centrifuged and used directly for SDS-PAGE and immunoblotting, or stored at -20 °C.

For cytokine stimulation experiments, cells were transfected for ~32 h and subsequently starved for 12 h (see the times for Luciferase-experiments in the following chapter) in serum-free DMEM (supplemented with L-Glutamine and antibiotics) before stimulation. The cells were stimulated either with IL-2, IFNγ or IFNα (all human and from Peprotech, Rocky Hill, NJ USA).

4.3 Luciferase reporter assay to decipher JAK downstream signaling

The Luciferase activity was measured with the Dual-Glo Luciferase assay kit (Promega) according to the manufactures´ instructions on 96-well plates. Briefly, γ2A, 11.1 or U4Cγβ cells were transiently transfected with the JAK-constructs, the STAT-responsive luciferase plasmid and the transfection control plasmid (renilla; pRL-TK). SPI-Luc was used for the detection of STAT5 activity (Sliva et al. 1994) and IRF-GAS/ISRE STAT1 reporter construct were used for IFNγ and IFNα detection, respectively. After 42 h transfection and 5 h starvation/stimulation the luminescence and absorbance were detected with an EnVision multiplate reader (Perkin Elmer).

In some experiments, the STAT transcriptional activity was detected with a parallel method using the Luciferase Assay Substrate, Promega (Article I). The protocol differed from the Dual-Glo assay in that β-galactosidase was used as a control for the transfection efficiency. After stimulation, cells were washed twice with ice- cold PBS and lysed using Reporter Lysis Buffer (Promega). The lysates were split for luciferase detection and for β-galactosidase normalization (with ortho-Nitrophenyl-β-galactoside (ONPG), Sigma-Aldrich) and measured as previously described.

4.4 SDS-PAGE and immunoblotting

Cell lysates or the recombinant protein preparations were run on a lab-made 7-10% SDS-PAGE gels or ready-made gels (4-15 % Mini-PROTEAN TGX Precast Protein Gel, Bio-Rad). Standard methods were used to transfer the lysates onto Protran 0.45 μm nitrocellulose membranes (GE Healthcare, Chicago, IL, USA) that were subsequently blocked with 4% BSA (Sigma-Aldrich) in 0.05% TBS-Tween. Blots were double-stained with rabbit and mouse primary antibodies and a mix of labelled secondary antibodies (Table 7). Signals were detected using the Odyssey CLx (LI-COR, Lincoln, NE, USA) and analyzed with Image Studio software (LI-COR).

Table 7: Antibodies used in the studies.

Name	Antibody	manufacturer	Cat. No	Used in Articles
HA	HA Tag	Aviva Systems Biology	OAEA00009	I-III
pSTAT1	Phospho-STAT1 (Y701)	Cell Signaling	7649	I-III
STAT1	Anti-STAT1	BD Bioscience	610116	I-III
pSTAT5		Cell Signaling	4322	I-III
αMouse	Goat anti-mouse IgG, DyLight 800	Thermo Scientific	35521	I-III
αRabbit	Goat anti-rabbit IgG, DyLight 680	Thermo Scientific	35568	I-III

4.5 Protein expression and purification

Proteins were expressed in Spodoptera frugiperda Sf9, or High Five (BTI-Tn-5B1-4) cells and affinity-purified as described in Articles I and Article II. Affinity-purified protein was used directly for differential scanning fluorimetry (DSF), if sufficient purity was obtained (evaluated by SDS-PAGE), or further purified with gel filtration for other biochemical assays. Gel filtration was done with Superdex200 10/300 GL column (GE Healthcare) on an ÄKTA (GE Healthcare) or Shimadzu (Shimadzu Corp., Kyoto, Japan) HPLC systems. The data from the gel filtration was used to estimate the functionality/folding of the protein before further analysis with DSF. Protein purity was estimated using SDS-PAGE.

4.6 Analysis of recombinant proteins (I, II,)

4.6.1 Thermal shift assay (TSA) by differential scanning fluorimetry (DSF) (I,II)

Thermal-shift assays were performed using a CFX96 real-time PCR cycler (Bio-Rad, Hercules, CA, USA) as described in (Vedadi et al. 2006) with proteins diluted in buffers (see Article I and Article II for details of the buffers and ligands). Sypro Orange (Molecular Probes, Thermo Fisher Scientific) was used as a probe. Samples

were heated at 1 °C per min from 4 °C to 95 °C and fluorescence readings were taken at each interval. To obtain the melting temperatures, the fluorescence data was normalized and fitted to the Boltzmann sigmoidal equation using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

4.6.2 Fluorometric nucleotide-binding assay with MANT-ATP (I)

Fluorescence resonance energy transfer (FRET) between the fluorescent ATP analogue 2´/3´-O-(N-methyl-anthraniloyl)-adenosine-5´-triphosphate (MANT–ATP, Jena Bioscience, Jena, Germany) and JAK was used to measure the binding of ATP to JAK JH2. The FRET between protein tryptophans and the fluorescent label was measured in a Quantamaster cuvette spectrofluorometer (Photon Technology International, Edison, NJ, USA) as described in (Niranjan et al. 2013). Dissociation constants (Kd) were obtained using GraphPad Prism (GraphPad Software) with the guidance and Excel layouts from Henrik Hammarén.

4.6.3 Kinase assay with radioactive ATP (I)

Radiolabeled (γ-^{32}P) ATP was used to assay the kinase activity of the recombinantly produced JAK JH2s. 10 μCi of (γ-^{32}P) ATP was added to the purified protein and the mixture was incubated in RT for 20, 40, 80 or 180 min until the reaction was stopped by adding SDS-PAGE sample buffer and boiling the sample at 95C°. The autophosphorylation was analyzed in SDS page gels that were exposed on film. Active tyrosine kinase EGFR was used as a positive control and a sample without γ^{32}P-ATP was used as a negative control.

4.6.4 Fluorescence Polarization Assay (I)

Fluorescent polarization assay was perforemd by Anniina Virtanen as described in Article I. Briefly, to determine the IC50 values for JAK JH2-ATP binding, ATP was titrated against a fluorescent tracer (Bodipy FL labeled JNJ-7706621). Proteins were used in concentrations that were determined by the Kd for the protein and the tracer. The fluorescent polarization values were measured with PerkinElmer Envision plate reader and analyzed with GraphPad Prism.

4.7 In vivo zebrafish model (I)

A majority of the in vivo zebrafish studies were conducted by Vilasha Bulleeraz under Prof. Alister Ward at the Deakin University School of Medicine, Centre for Molecular and Medical Research, Australia, as described in I. Briefly, in-crossed jak3+/− embryos were injected with in vitro transcribed capped mRNA encoding zebrafish JAK3 WT or M511I, I535F or M511+I535F mutants. At 5 dpf the fish were fixed and subjected to whole-mount in situ hybridization (WISH) with anti-sense rag1 probe and imaged and quantified as described in (Sertori et al. 2016).
The experiments done at Tampere University were conducted using 5 dpf embryos from lck:GFP zebrafish. The fish were injected with mRNA as described above and the rag1 expression was analyzed using RT-PCR.

4.8 Molecular dynamics simulations and analysis of trajectories (III)

The molecular simulation studies of JAK2 were carried out by our collaborators Ayaz Pelin under supervision of Y. Shan and D. E. Shaw at D. E. Shaw Research in New York, NY, USA, as described in Article III and Chapter 5.4. Briefly, X-ray structures of the JAK2 FERM-SH2 unit (McNally, Toms, and Eck 2016), JH1(Baffert et al. 2010) and the previously published model of the JH2-JH1 complex were used to construct the models (Shan et al. 2014). Addition of the EPOR was done by homology modelling based on the TYK2 and JAK1-bound IFNAR1 structures (Ferrao et al. 2016; Wallweber et al. 2014). Several different models of different JAK domain combinations where done by placing the various simulation systems in a cubic simulation with Na+ and Cl- ions at minimum distance of 10 Å between the protein surface and the edge of the simulation box. The missing loop regions and sidechain atoms were modeled using the software package Maestro (Schrödinger, LLC). The systems were parameterized using the CHARMM36 force field and the TIP3P water model. Production MD simulations were performed on the supercomputer Anton 2 and the lengths to hydrogen atoms were constrained using an implementation of M-SHAKE. The simulation trajectories were visualized and analyzed using Visual Molecular Dynamics (VMD) software and the images of protein structures were made using the PyMOL Molecular Graphics System (Schrödinger, LLC). All models went through a final restrained energy minimization using Maestro.

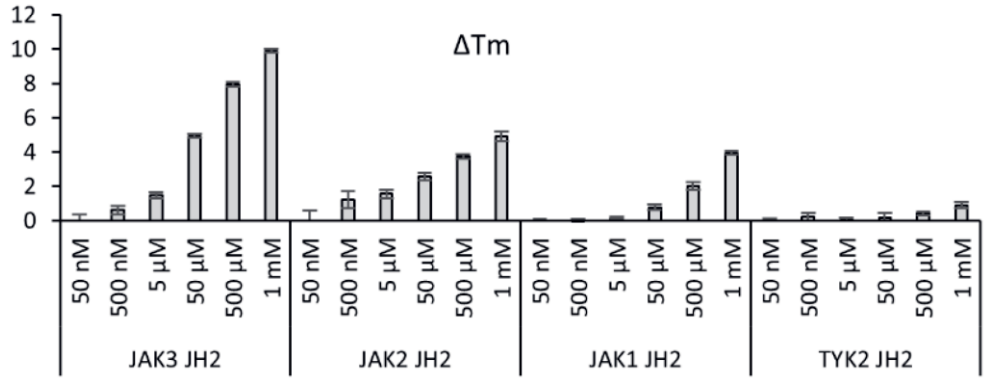
5 SUMMARY OF THE RESULTS

5.1 Biochemical characterization of JAK3 JH2 (I)

The structure of the isolated JAK1, JAK2 and TYK2 domains have been solved (for the schematic illustration of the JAK domains: see Figure 4 in Chapter 2.2.3). However, structural data is available only of the JAK3 kinase domain (JH1) (Boggon et al. 2005) and no previous studies have been conducted with the recombinant JAK3 JH2. This prompted us to express a C-terminally His-tagged JAK3 JH2 (residues 511–790-His_6) in insect cells and purify the construct with affinity chromatography followed by size-exclusion chromatography. The JAK3 JH2 stability was measured with thermal shift assay (DSF) that provides the melting temperature (Tm) of the protein. The Tm for JAK3 JH2 without ligands was 32 °C, which is lower in comparison to other JAK pseudokinase domains (Figure 8). The instability of JAK3 JH2 likely explains the lack of previous biochemical characterization of the recombinant protein.

The kinase-ATP affinities typically range from low micromolar to millimolar while the concertation in a cell is between one and five millimolar. Despite being saturated with the substrate in cells, knowledge of the kinase ATP affinity is important for the evaluation of the IC50 values of small molecule binders (more in Chapter 6.3). The ATP binding affinity of JAK3 JH2 was subsequently measured with three different methods: MANT-ATP tracer, DSF and fluorescent polarization competition assay. Each analysis showed that JAK3 JH2 binds ATP tighter in comparison to other JAKs (Figure 8). Moreover, addition of ATP increased the JAK3 JH2 Tm up to 11 degrees. Despite the tight binding of ATP, we did not detect any kinase activity for JAK3 JH2. After rigorous attempts to express and purify the construct with ATP-site mutations designed to block the nucleotide binding, we concluded that the stabilizing effect of ATP is crucial for maintaining the structural integrity of the unstable JAK3 JH2.

The pseudokinase domains of JAK1, JAK2 and TYK2 bind ATP with one cation present in the structure (reviewed in Hammaren, Virtanen, et al., 2015). However, we observed that the ATP-binding of JAK3 JH2 is independent of cations (divalent Mg^{2+}, Ca^{2+}, Mn^{2+} and monovalent K^{+}). In JAK3 JH2, the position that typically coordinates the cation (a conserved Asn171 in PKA, see Chapter 2.1.2) is reserved by a positively charged lysine (Lys652). This lack of the conserved Asn likely causes the distinct, cation-independent ATP-binding of JAK3 JH2 (Article I).



JAK JH2	Phospho transfer activity	Kd (MANT-ATP) [µM]	IC50(FP) [µM]	Tm_{Apo} [°C]	Tm_{ATP} [°C]	ΔTm (1 mM ATP) [°C]	Cations
JAK2	Weak	1.3 (*)	102	43	46	4.9	Yes
JAK1	No	3.1 (*)	110	46		3.9	Yes
TYK2	No	15 (**)	471	48	48	0.9	Yes
JAK3	No	0.5	16	32	41	9.9	No

Figure 8: Biochemical characterization of the JAK3 JH2 recombinant protein. Upper left panel: the DSF measurements with all the JAK pseudokinase domains. The y-axis indicates the fold change in Tm by addition of ATP (50 nM-1 mM). JAK3 JH2 Tm was measured without cations while $MgCl_2$ was added into the buffer of the other pseudokinase domains. The upper right panel shows the autophosphorylation assay using radiolabeled γ^{32}P-ATP. JAK3 JH2 does not show kinase activity. Active kinase EGFR serves as a positive control. The table below presents the combined results from all JAK JH2s. Data of the phosphotransferase activity, IC_{50} values (obtained from FP assay), Kd for MANT-ATP, melting temperatures and the cation dependency of the ATP binding are included. MANT-ATP Kd values for JAK1 and JAK2 are from Hammarén et al. 2015 (*), and the Kd for TYK2 was determined in the study of Min et al. 2015 (**). The figure is modified from Article I, the copyright of the original illustration Raivola et al. Front oncol. 2018 CC BY.

5.2 Different roles of the JAK1 pseudokinase domain in cytokine signalling (I/II)

JAK kinases form various complexes with each other and the receptors they associate. JAK1 is the most versatile in this respect as it can pair with all the other JAKs to induce different downstream effects (see Table 1). For our studies, we chose three JAK1-driven pathways with varying JAK partners: IFNγ (JAK1/JAK2), IFNα (JAK1/TYK2), IL-2 (JAK1/JAK3). First, only one of the corresponding JAKs was expressed in JAK-deficient fibrosarcoma cell lines (see Table 8). By detecting the activation of STAT1 or STAT5, we found that both JAKs are required for functioning cytokine signaling of each of the systems (Figure 9). The activation of the IFN systems strictly depended on both JAK1 and JAK2/TYK2. In the IL-2 system, however, JAK1 was able to induce STAT5 activation withouth JAK3, but the activation was unresponsive to IL-2 (Figure 9). This shows that the expression of JAK3 is crucial for cytokine-dependent signaling (Articles I and Article II).

Table 8: List of the used cell lines, related cytokine pathways and the mutations used to study the effect of αC and ATP-binding site mutants agains WT and constitutive JAK activation.The U4Cγβ cells are deficient of JAK1 and JAK3, γ2A of JAK2, and the 11.1 cells do not express TYK2. Mutants are homologous between each JAK, except JAK3 R657Q that is not homologous to the activating "VF" mutations in JAK1, JAK2 and TYK2.

Cell-lines	JAK	Cytokine pathways			Mutants			
		IFNγ	IL-2	IFNα	αC	ATP		Activating
U4Cγβ	JAK1	X	X	X	L633K	I597F	K622A	V658F
γ2A	JAK2	X			E592R	I559F	K581A	V617F
U4Cγβ	JAK3		X		E567E	I535F	K566A	R657Q
11.1.	TYK2			X	L653R	V603F	-	V678F

Next, we used similar experiment setup to study whether constitutively active JAK mutants can activate STATs, if the partnering JAK is not expressed in the cells (Article II). JAK2 V617F and the homolog mutants in JAK1 and TYK2 (the "VF" mutants) where transfected in JAK-deficient cell lines and the activity of the corresponding STAT was detected (Figure 9, bottom panel). Again, IL-2 signaling

differed from the IFN systems. JAK2 V617F (IFNγ) and TYK2 V678F (IFNα) activated STAT1 without JAK1 and JAK1 V658F was equivalently activating without JAK3 or JAK2 (in IL-2 and IFNγ systems, respectively). However, none of the activating JAK3 mutants (R657Q, M511I and L857Q) were able to activate STAT5 in JAK1-deficient cells (Article I and Article II). The following chapter further highlights the JAK1 dominance over JAK3, and the role of JAK1 JH2 in the signaling.

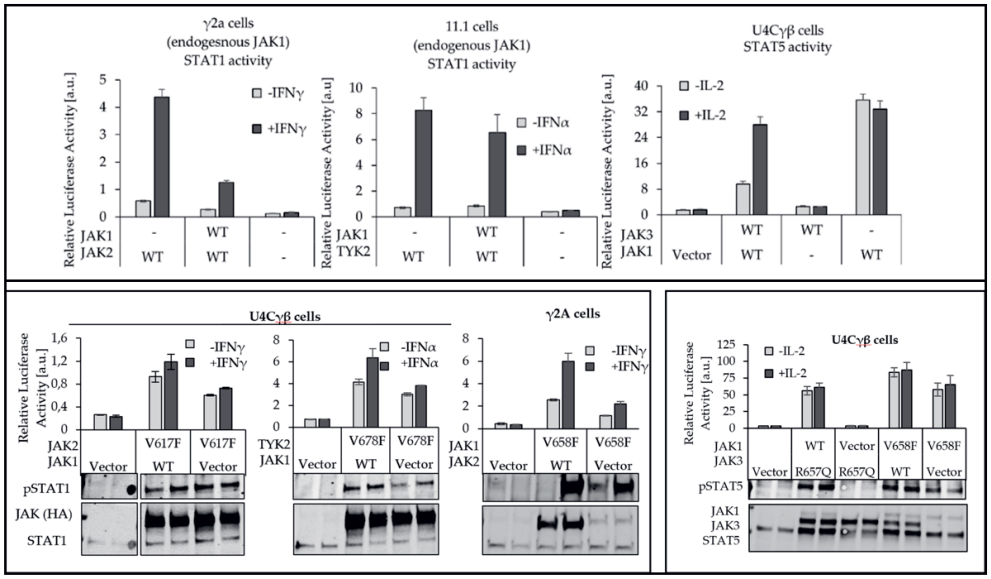


Figure 9. Top panel: a single JAK is not enough to provide functional IFNγ, IFNα or IL-2 signaling, but the pathways are dependent on both JAKs associalted to the cytokine complex. The transcriptional activity of STAT1 and STAT5 was detected as previously described. The γc signaling differs from the IFN systems in that JAK1 can induce JAK3 independent STAT5 activation, although the activation remains unresponsive to IL-2. JAK1-deficient U4Cγβ cells where used to show that no cytokine-induced STAT1 activation occurred in IFNα or IFNγ signaling, if only one TYK2/JAK2 were expressed, respectively (data not shown, see Article II). Bottom panel, left: constitutively active JAK2, TYK2 and JAK1 can induce STAT activation witouth JAK1 (in case of JAK2 and TYK2 mutants) or JAK2. Both the transcriptional STAT activity and the pSTAT-signals were detected as previsouly described. Right: the activation of the JAK3 R657Q-driven STAT5 is strictly dependent on JAK1. The figure is modified from Article II, copyright of the original illustration Raivola et al. Cancers 2019. CC BY.

5.3 Structure-based mutation analysis of the JAK pseudokinase domains

To study the biological characteristics of JH2, we used structure-based mutation approach together with known pathogenic JAK mutants. We were especially interested to find sites that could be modulated to reduce JAK hyperactivation. We modified the JH2 ATP-binding site and studied the effects in both wild type and pathogenic backgrounds. In addition, we mutated the JH2 αC-helix that was chosen based on the number of pathogenic mutations targeting this region (demonstrating its importance as an allosteric regulator) and previous studies showing that mutating the site inhibits constitutive JAK2 activation (Hammaren, Virtanen, Abraham, et al. 2019; Leroy et al. 2019). Homologous mutations were made into each member of the JAK-family, and the effects were analyzed in JAK-deficient fibrosarcoma cells (Table 8). Finally, we studied the inherent difference in JAK3, namely the lack of the "VF" mutation that is pathogenic in JAK2 (V617F) and JAK1 (V658F) and activating in cells when introduced to TYK2 (V678F). Together, these studies depict the molecular and biological differences between JAKs and their pseudokinase domains. These subtle differences may in part allow the flexible, but specific JAK-STAT signaling in the physiological state as well as contribute to the pathogenic JAK-activation.

5.3.1 Modulation of the ATP-binding sites (I/II)

To analyze the JH2 ATP-binding pockets, we took advantage of the previously studied JAK2 I559F mutation that was designed to cause steric hindrance and verifiably blocks the ATP binding and reduces constitutive JAK2 activation (Hammaren, Ungureanu, et al. 2015). We observed that the homologous JAK3 mutation I535F decreases constitutive STAT5 activation against R657Q and M511I backgrounds. The mutants target JAK3 JH2, and are recurrently mutated in T-ALL (Figure 10). In addition, I535F inhibited the activity of the JAK3 JH1 GOF mutant L857Q (Article I). Constitutive activation was similarly reduced when we mutated the conserved β3 sheet lysine (K556A) in the JAK3 JH2 ATP pocket, concurring with previous studies conducted with homologous JAK1 and JAK2 mutations (Hammaren et al., 2015). Interestingly, the JH2 mutations differ from the

conservative kinase negative (KN) mutations. The KN mutants target the JH1 ATP-binding site and completely abolish the kinase activity while the JH2 ATP-binding site mutants (and the JH2 αC-helix mutants described later) retain the cytokine responsiveness comparably to WT JAK (Article I and II).

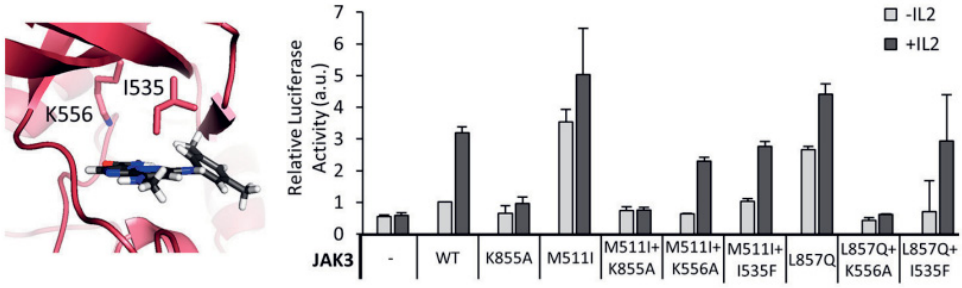


Figure 10: Mutations in the JAK3 JH2 ATP-binding site efficiently reduce JAK3 hyperactivation. JAK1 and JAK3 deficient U4Cγβ cells were transfected with JAK3 mutants, JAK1 WT and the SPI-Luc2 and renilla plasmids. The latter two were used to detect the STAT5 transcriptional activity (see Materials and Methods 4.3). The JH2 mutants reduce constitutive activation driven by either JH2 or JH1 mutants M511I and L857Q, respectively. The KN JAK3 K855A also reduces activation but the responsiveness to IL-2 is abolished. The figure is modified from Article I, the copyright of the original illustration Raivola et al. Front Oncol. 2018 CC BY.

Unlike the homologous mutations in other JH2 ATP-binding sites, JAK1 I597F did not reduce JAK1 or JAK3 hyperactivation, but rather increased basal STAT5 and STAT1 activation in WT background (Figure 11) (Article I and Article II). To study the biochemical properties of the JAK1 ATP-binding site, we produced and purified recombinant JAK1 JH2 with I597F or the β3 mutation K622A (see Table 8). Although the yield of the recombinantly produced mutants was typically smaller than the WT construct, we were able to analyze the stability and ATP-binding properties of the mutants with DSF. The mutants exhibited considerable difference in the melting temperatures; the K622A Tm was nine degrees higher than the JAK1 JH2 WT apo-form (53.3 ◦C and 44.3 ◦C, respectively) while the I597F mutant´s Tm was lower than the WT (40 °C). We concluded that I597F induces destabilization of the domain, which likely affects the JAK1 JH2 regulatory function. The K622A mutant, on the other hand, may stabilize JH2 and the entire JAK into the inactive conformation of (Article II).

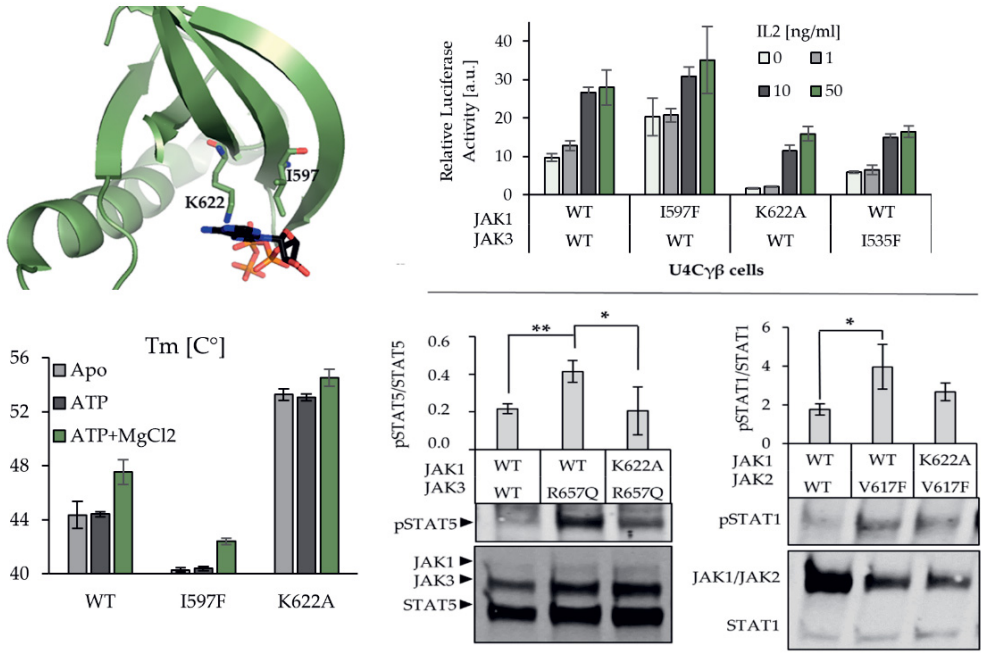


Figure 11: The JAK1 JH2 ATP-binding site mutants differently affect the activation of WT and constitutive active JAK-signaling. JAK1 K622A and JAK3 I535F reduce STAT5 activation but JAK1 I579F increases basal STAT5 activation. Top left panel: the N-lobe of JAK1 JH2 (PDB code 4L00) showing the ATP-binding site residues that were mutated. ATP is depicted as sticks. Top right panel: transcription activity of STAT5, measured with luciferase reporter. The U4Cγβ cells were transfected with full length JAK3 and JAK1 (mutant or JAK WT), and the analysis was done as previously described. P-values were calculated using two-tailed student`s t-test with unequal variances (* indicating $p<0.05$ and ** $p<0.001$), and no significant difference between the basal and IL-2 stimulated (50 ng/ml) cells was detected in JAK1 I579F transfected cells. Bottom left panel: melting temperatures (Tm, the y-axis) of the recombinant JAK1 JH2 constructs with and without ATP and/or MgCl2. Right: phospho-STAT analysis of cells transfected with JAK1 K622A and either the hyperactive JAK3 R657Q or JAK2 V617F. The picture is modified from Article II, the copyright of the original illustration Raivola et al. Cancers, 2019. CC BY.

5.3.2 Mutating the pseudokinase domain αC-helix reduces constitutive activation

Similarly to the ATP-binding site mutations discussed above, modulating the outer face of the JAK2 JH2 αC-helix can reduce both homodimeric (EPOR) and heterodimeric (IFNγ) JAK2 signaling (Hammaren, Virtanen, Abraham, et al. 2019;

Leroy et al. 2019). We extended the analysis of this site and mutated each JAK family member with homologous αC mutation. JAK1 L633K was chosen as a model mutation, since the equivalent JAK2 E592R mutation was previously shown to inhibit IFNγ (JAK1/JAK2) and EPO (JAK2/JAK2) signaling (Hammaren et al., 2019; Leroy et al., 2019). We evaluated the inhibitory effect of the mutant in cis, i.e. the αC mutation was introduced into full length JAK together with a pathogenic mutation. Either the activating single mutants or the double-mutated JAK constructs were transiently transfected into JAK-deficient fibrosarcoma cell lines, followed by the detection of the STAT activity. An effective reduction in the αC-mutated cells was detected when compared with the constitutively active single mutants (Figure 12).

Mutating the TYK2 JH2 ATP-binding site decreases the IFNα-induced STAT1 activation and slightly reduces the constitutive TYK2 V678F activation. However, the basal STAT activation was not reverted to the WT level and the αC mutation L653R was more potent in reducing the constitutive STAT1 activation (Figure 12, bottom panel) (Article II). This concurs with previous studies showing that the homologous JAK2 αC mutation more effectively reduces hyperactivation compared with the mutants that target the ATP-binding cleft (Hammaren, Virtanen, Abraham, et al. 2019).

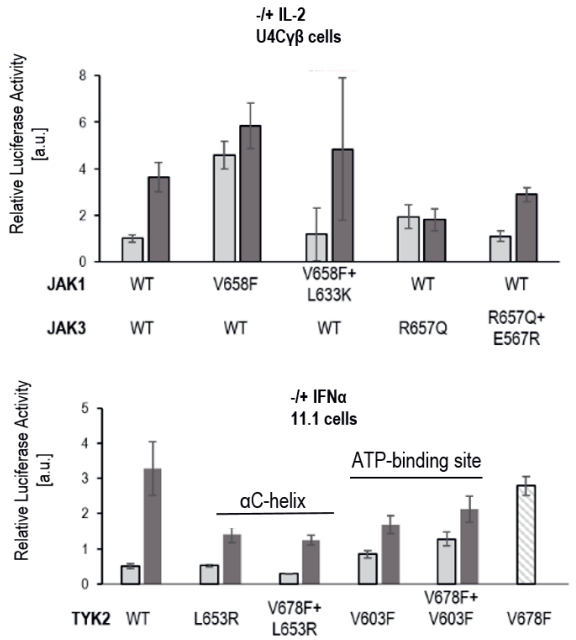


Figure 12. Homologous mutation in the JH2 αC-helix inhibits constitutive JAK activation in cis (JAK2 inhibition has been reported previously). Upper panel: The JAK1/JAK3-dependent IL-2 signaling is activated by pathogenic mutations in JAK1 or JAK3. The hyperactivation is reduced and cytokine responsiveness retained by introducing a second mutation that targets the outer face of the αC. Fibroblast cell line lacking both JAK1 and JAK3 was transfected with mutated or JAK WT and a luciferase reporter for detecting the STAT5 activity (described in Materials and Methods 4.3). Cells were starved or stimulated 5 h with IL-2 prior to the signal detection. Lower panel: Mutating the JAK JH2 αC inhibits the constitutive JAK signaling more efficiently compared with the ATP-binding site mutations. TYK2-deficinet fibroblast were transfected with WT or mutated TYK2 constructs and STAT1 (IFNα) responsive luciferase reporter. The L653R in the αC more efficiently reduces the activating V678F mutant compared with the V603F mutation that targets the ATP-binding site. The picture is modified from Article II, the copyright of the original illustration Raivola et al. Cancers 2019. CC BY.

5.3.3 Suppressing JH2 αC-helix mutation reveals differences in the activation mechanisms of JAK1-driven signalling pathways

After showing that modulation of the JH2 αC-helix can reduce the activation, we studied the effect of the JAK1 αC mutation L633K in three cytokine signaling pathways usding the JAK1-deficient U4Cγβ cells. L633K reduced both basal and cytokine stimulated STAT5 activation in the JAK1/JAK3-driven IL-2 signaling. The IFNγ signaling was not markedly altered in the L633K cells, and only a minor reduction in the basal STAT1 activity was detected. The mutation had no effect on the cytokine-induced activation (Figure 13), which is line with the previously described difference between IL-2 and IFNγ signaling showing that JAK1 is dominant in IL-2 signaling but less crucial for the IFNγ-induced STAT1 activation. The latter is more equally dependent on both JAK1 and JAK2 (Chapter 5.2 and 6.2.2.). JAK1 L633K also reduced the IFNα-induced activation although the effect was mainly seen in the IFN-stimulated cells (Article II). In conclusion, altering the JAK1 JH2 function strongly affects the JAK1/JAK3 signaling but has a lesser effect in the JAK1-driven IFN systems.

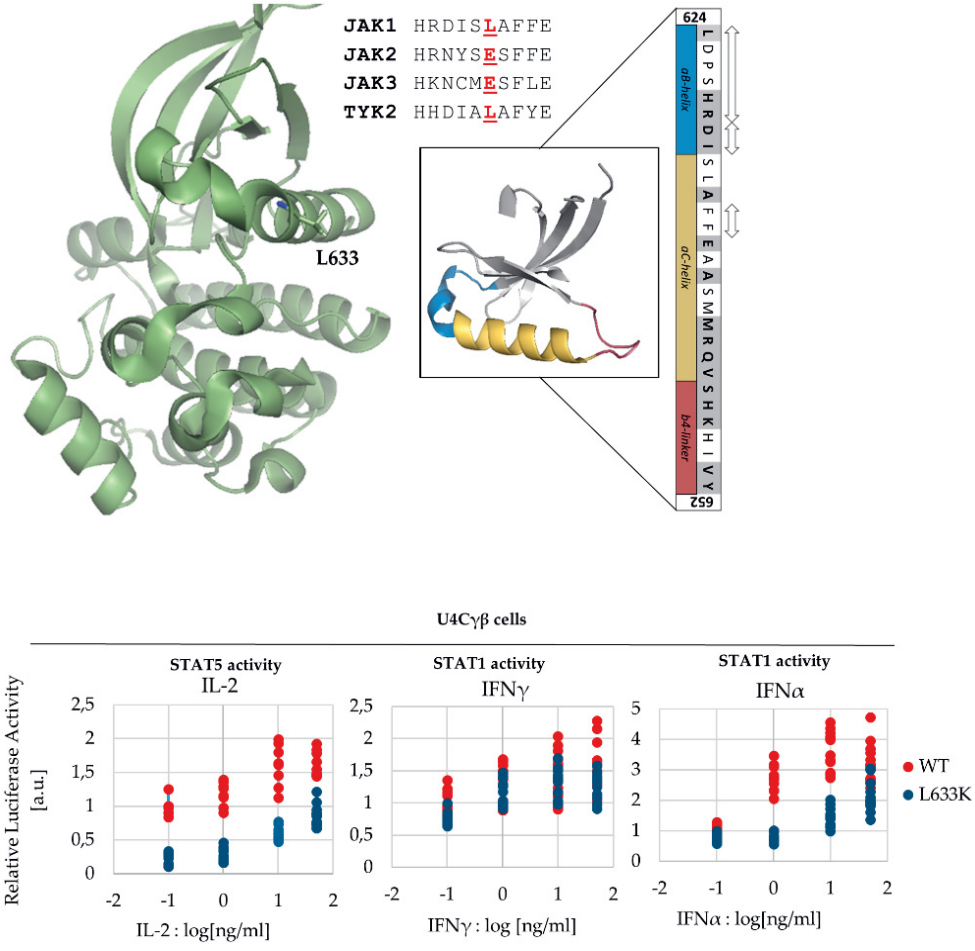


Figure 13: The effect of the JAK1 αC-helix mutant L633K. Upper panel: the structure of JAK1 JH2 (PDB code 4L00) with the L633 residue shown as sticks. Left panel: a close-up of the αC-helix (in yellow) and the linkers (in blue and red) attaching it to the β-sheets in the JH2 N-lobe. The primary sequence is also shown, and the known pathogenic mutations within the region are highlighted with gray. Below: the activation of STAT5 and STAT1 detected from JAK1-deficient cells expressing WT (red) or αC mutated (blue) JAK1. The relative luciferase activities were measured as described previously and in Chapter 4.3. The values are blotted in the y-axis against the cytokine amount used (x-axis), the latter of which are transformed to a logarithmic scale for clarity. The basal values where no cytokine was added is set to -1. The picture is modified from Article II, the copyright of the original illustration Raivola et al. Cancers, 2019. CC BY.

Next, we analyzed the inhibitory αC mutation in a system where the activating mutation is not within the same JAK, but introduced into the partnering JAK (i.e. the effect of the αC mutants in trans) (Figure 14). Supporting the concept that JAK1 is dominant over JAK3, JAK1 L633K reduces constitutive JAK3 activation, but the homologous JAK3 E567R does not inhibit constitutive JAK1 activation (Article II). Furthermore, we observed that JAK1 L633K is inefficient in reducing the hyperactivity of JAK2 V617F in the IFNγ system while the homologous JAK2 mutant E592R decreases JAK1 V658F-induced activation. We also tested the inhibitory effect of the TYK2 αC mutant L653R against constitutively active JAK1 V658F (data not shown, Article II). Altough the STAT1 activation of the JAK1 mutant was moderate, TYK2 L653R clearly decreased the cytokine-induced activation of the JAK1 V658F transfected cells. The basal STAT1 activation was not affected by the TYK2 mutation. Thus, the pseudokinase domains of JAK1 and TYK2, and especially the outer face of the αC, are likely important for the cytokine-induced activation of the IFNα system.

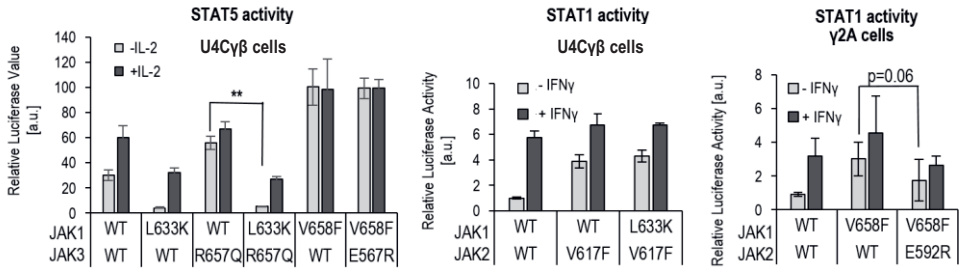


Figure 14: Effect of the JAK JH2 αC-helix mutants in contitutively active background (inhibition in trans). Left: JAK1 L633K effectively inhibits hyperactive JAK3 in the IL-2 system but the JAK3 homolog is not effective against JAK1 V658F-driven activation. JAK1 L633K is inefficient in reducing JAK2 V617F-induced STAT1 activation in the IFNγ system (middle). Right: The JAK2 αC-helix mutant reduces constitutive JAK1 activation in the IFNγ system. The graphs show the relative luciferase activities with and without the cytokine addition. The measurements were performed as described previously and in Chapter 4.3. The figure is from Article II (Figure 4), the copyright of the original illustration Raivola et al. Cancers, 2019. CC BY.

5.3.4 Analysis of the JAK2 V617F -homolog in JAK3 (II)

The valine to phenylalanine mutation in JAK1, JAK2 and TYK2 leads to a π-π stacking between the aromatic phenylalanine-residues and the formation of the FFV-triad that promotes the activation (Chapter 2.4.2). However, neither the valine nor the second phenylalanine required for the formation of the FFV triad are conserved in JAK3 JH2 (Figure 15). This drove us to use other pathogenic JAK3 mutations for studying the constitutively active JAK3 signaling. Intreagued by this specific feature, we constructed a JAK3 VF with two mutations. First, the M592F was introduced to simulate JAK1 V658F, accompanied with L590F that is homologous to JAK1 F636 and completes the FFV-triad. As expected, the JAK3 M592F+L570F double mutant was constitutively active (Figure 15). However, the JAK3 VF-driven STAT5 activation remained JAK1 dependent, differing from the other JAK VF mutations that can activate STAT without the partnering JAK (Figure 9, bottom panel). This shows that results we obtained previsouly are not biased by the fact that the R657 resides at the JH1-JH2 interface on the opposite side of JH2 to the VF site. Taken together, results with the non-homologous activating JAK3 mutation(s) in part validate the dominance of JAK1 over JAK3.

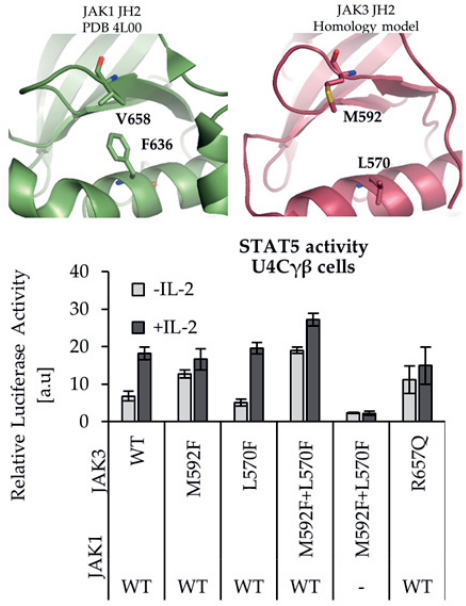


Figure 15: The FFV triad required for the activation of JAK1 V658F and homolog mutations in JAK2 and TYK2 is not conserved in JAK3 JH2. Upper panel: A schematic presentation of the JAK1 and JAK3 JH2 region near the αC-helix. The JAK1 V658 is methionine (M592) in JAK3, and JAK1 F636 is replaced by lysine (JAK3 L570). JAK1 F636 and homologs in JAK2 and TYK2 form a key interaction with JAK1 V658F. Bottom panel: the STAT5 activity measured from cells that were transfected with JAK1 WT (or vector) and JAK3 mutations. The partial reconstruction of the FFV-triad in JAK3 (M592F) increases the basal activation but the activity is further elevated when a double mutant (M592F + L570F) is introduced to complete the FFV-

triad. The JAK3 M592F + L570F is not activating in the absence of JAK1. The picture is modified from Article II, the copyright of the original illustration Raivola et al. Cancers, 2019. CC BY.

5.4 Molecular model of full-length JAK2 (III)

Due to the lack of full-length JAK-structures, we used long-timescale molecular dynamics (MD) simulations to model the possible states of the active and inactive JAK2 (Figure 16). MD simulations are the choice of approach when studying the dynamics of biomolecules at a high time resolution (nanoseconds or microseconds), which is not easy to achieve with current experimental techniques. The full-length JAK2 with EPOR was modelled basing on the resolved JH1-JH2 (Lupardus et al. 2014) and FERM-SH2 (Wallweber et al. 2014) structures and previous MD models (Shan et al. 2014). Initial simulations were done with minimum assumptions. In addition, the COSMIC database of known clinical mutation was used to construct the model of the active JAK2 dimer. Finally, the most biologically relevant models were chosen for extended analysis.

The modelled elongated conformation of the active JAK2, as well as the closed structure of the inactive JAK2 are concordant with the low-resolution EM data from JAK1 (Lupardus et al. 2011). The rearrangements that occurred in the simulations between the states were reversible, and the active monomer shifted back to the inactive form, likely illustrating the biological state where the dynamic equilibrium between the conformations allow the switching from inactive to active. Furthermore, we observed that majority of the known pathological JAK2 mutations enrich at the interfaces of the models, and provide a likely mechanism of action. Specifically, our study suggests that the constitutive activity of the V617F mutant arises from a dual effect of destabilizing the inactive conformation and stabilizing the active conformation.

To verify the models, we conducted functional analyses using point mutated JAK2 constructs together with cell-based assays. The mutated JAK2 constructs were transiently transfected into JAK2-deficient fibroblast cells (γ2A), followed by analysis of the phospho STAT1 (pSTAT1) levels of the cell lysates. The mutants were chosen based on the MD simulations and resided in the FERM or in the C-lobe of JH2. Importantly, these mutants have not been addressed in other studies. Together, our results give novel structural understanding of the JAK2 autoinhibition and activation.

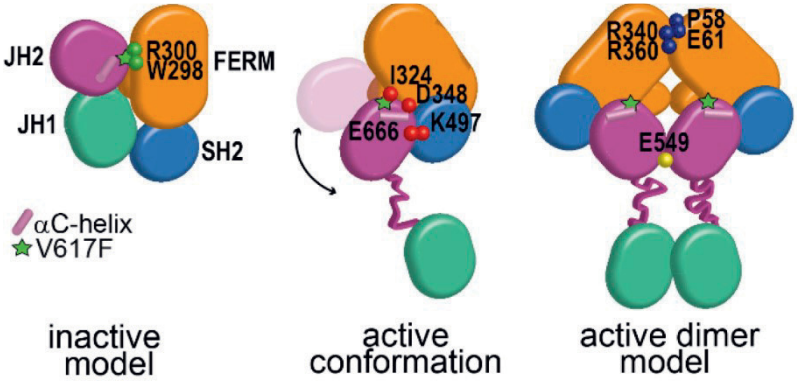


Figure 16: Schematic presentation of the different states of JAK2 activation; the inactive monomer and the active dimer (the active conformation of a monomeric JAK2 in the middle). MD simulations of full-length JAK2 with EPOR was verified with mutation analysis in cells and the mutations are tagged into the figure. The figure is from Article III (Pelin et al. 2020, submitted manuscript).

5.4.1 A structural model of full-length autoinhibited JAK2

Our model of the autoinhibited JAK2 monomer shows that SH2 interacts with JH1 near the catalytic cleft while the FERM F2 subdomain aligns with JH2, allowing the unit to interact with the membrane. The FERM F3 resides between JH1 and JH2 and completes the closed structure. SH2 also interacts with the JH2 αC helix and the β4 strand that are hotspots for clinical mutations (see Chapter 2.3.2).

In the model, V617(F) is at the interface between JH2 and the FERM F3. V617F and F595 from the FFV-triad share a hydrophobic cluster with the F285, W298, and L352 residues in the F3. We confirmed this cluster experimentally by showing that

W298A further increases the constitutive activity of the V617F mutant (Article III). In addition, we validated a salt bridge between FERM R300 and JH2 D620. The putative salt bridge was broken with the R300E mutation, and the mutant increased the V617F-driven STAT1 activation compared with the V617F single mutant. Neither W298A nor R300E where activating in the WT background, indicating that destabilizing the inactive conformation without stabilizing the active conformation may be insufficient to induce JAK2 WT activation.

The unique autophosphorylation of JAK2 S523 is observed in unstimulated conditions where it negatively regulates the JAK2 activation (Ungureanu et al. 2011). Our MD model showed that phosphorylated S523 interacts with residues in JH2 and the FERM F3 to maintain a closed four-domain structure that is generally considered as the state where JH2 holds the JH1 from transphosphorylation. The unphosphorylated S523 approaches the JH2 ATP γ-phosphate, plausibly getting set for autophosphorylation.

5.4.2 The active conformation of JAK2 and a model of its dimerization

To induce the active conformation, the V617F mutation was introduced into the previously modelled inactive monomeric JAK2. JAK2 V617F is thought to destabilize the JH2 structure (Shan et al., 2014; Silvennoinen & Hubbard, 2015) while reinforcing the hydrophobic FFV cluster that shifts the equilibrium towards the active conformation (Gnanasambandan et al. 2010). In our model, JH2 maintains contact with the FERM F3 but moves to occupy the space where JH1 resided in the inactive conformation. Although the FERM F3 interacts closely with JH2 in both the active and inactive models, the interfaces differ between the two. V617F interacts with I324 in the FERM F3 and P529 and F595 in JH2 (F595 is part of the FFV-triad). The FERM-JH2 interface was experimentally verified with the I324D+V617F double mutant that reduced the V617F-driven STAT1 activation.

In the model, several salt bridges surrounding the hydrophobic FFV cluster were detected that maintain maintain the F3-JH2 interface. Among these, the interaction between D348-R588 was confirmed in cells, as the D348K mutation reduced the

V617F activation (pSTAT1). JH2 interacts also with SH2 and allows the SH2-JH2 linker to dock between the FERM F3 and JH2. A salt bridge between the JH2 E666 and SH2 K497 was experimentally verified as before by mutating either of the residues (E666K or K497E). In addition, previous data shows that the E596A/R mutations suppresess the V617F activity, hence supporting the modelled formation of a K504-E596 salt bridge between SH2 and JH2 (Hammaren, Virtanen, Abraham, et al. 2019; Leroy et al. 2016, 2019).

Mutation data from COSMIC-database (Tate et al. 2018) guided the simulation of the active dimer model that was manually assembled. The FERM-membrane interaction and the assumed involvement of JH2 in the formation of the (assumed) symmetric dimer were also taken into consideration (Figure 17). During the simulation, a well-packed and highly stable dimer formed where large electrostatic interfaces occur between JH2-JH2 and FERM-FERM. Moreover, the majority of the mutations cluster at the interdomain interfaces of FERM, SH2 and JH2, or at the putative FERM F2-membrane interface (Article III). One-third of the COSMIC mutations that have no apparent mechanism of function locate at the dimerization interfaces, including a cluster on one side of the active monomeric model that likely reside at the dimerization interface of the active dimer.

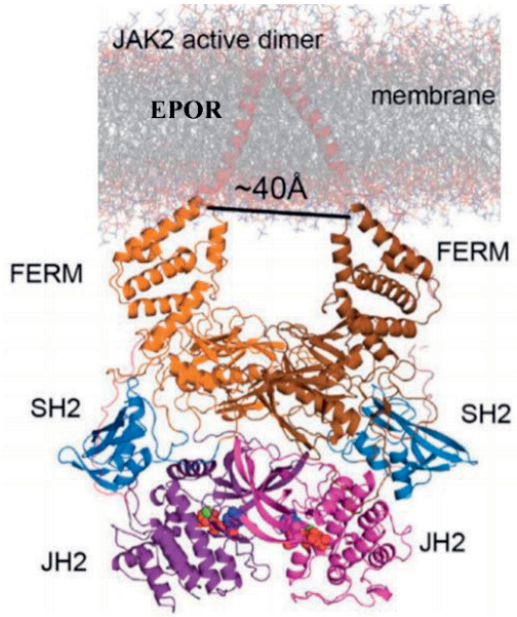


Figure 17: Schematic presentation of the active JAK2-EPOR dimer. JAK2 dimer was modelled without the kinase domain. The figure is modified from Article III (Pelin et al. 2020, and a version of the manuscript is available at bioRxiv (Pelin et al. 2019).

The JH2-JH2 dimer seen in our active dimer model exhibits a well-packed interface that is mainly covered by crossing of the N-lobe β-strands at the side opposite to the ATP-binding site. We found that the E549K mutation in the β1 inhibits the V617F driven phosphorylation of STAT1. Furthermore, previous studies have demonstrated that ATP binding to JH2 is required for the constitutive activity of V617F (Hammaren, Ungureanu, et al., 2015, Article I) and the MD simulations support these results and suggest that the ATP-binding maintains JH2 in a conformation that favors activation.

The FERM-FERM interaction primarily involves the F1 and F3 subdomains that, unlike the JH2-JH2 dimer that is rich with salt bridges, is weak and non-specific. The FERM-FERM dimer only contains a few salt bridges, among them interactions between E61 and R340/R360, and the adjacent P58. The interaction was confirmed by mutating the putative interface, and both the P58A/E61A and R340A/E360R double mutations reduced the V617F activity of JAK2.

The separation and geometry of the receptor chains of the model is concordant with the crystal structures and fluorescence resonance energy transfer (FRET) studies previously conducted with homodimeric JAK2 complexes (Livnah et al. 1996, 1998; Syed et al. 1998; de Vos, Ultsch, and Kossiakoff 1992; Waters, Brooks, and Chhabra 2014). Finally, sequence alignment and comparison of the active JAK2 dimer with the models of other JAKs shows that the interfaces are conserved among JAKs, concurring with the frequent heterodimerization between JAKs.

6 DISCUSSION

The JAK-STAT pathway is widely studied both at the mechanistic level (basic studies) and by taking a more applied approach, i.e. the development of kinase inhibitors. This thesis and the following discussion focuses on the JAK pseudokinase domain, how it regulates the activation of the kinase and how this information can be used to understand the pathogenesis of JAK-related diseases as well as to design novel more potent treatments against them. The chapter also considers the methodological difficulties and problems that rise from the incomplete knowledge about the biology within and surrounding the JAK-STAT pathways.

6.1 Function of the JAK pseudokinase domain

It is well established that the pseudokinase domain (JH2) of JAK kinases is not merely a decorative evolutionary remnant but the "other face" of the two-faced roman god Janus, actively participating in the inhibition and activation of the kinase domain. The interaction between JH2 and JH1 maintains the closed, inactive conformation that prevents the JH1 phosphorylation and the disruption of this JH2-JH1 module is a prominent mechanism for disease–causing mutations (Lupardus et al., 2014; Silvennoinen & Hubbard, 2015). However, the molecular details of how the switching between the active- and inactive states occur and especially how JH2 contributes to the cytokine-induced activation have remained elusive.

Our research, with several seminal works of others (Brooks et al. 2014; Ferrao, Wallweber, and Lupardus 2018; Haan et al. 2011; Leroy et al. 2016; Lupardus et al. 2011; Staerk et al. 2005; Toms et al. 2013; Wallweber et al. 2014) have elucidated the molecular mechanism of JAK activation. Results presented in this thesis show that ATP binding to JH2 is an important structural element that stabilizes the domain (Article I and II) and we suggest that the nucleotide binding alters the conformation of JH2, supporting the JAK activation (discussed further in Chapter 6.1.1.1). Our findings further illustrate that the JH2-JH1 interface is an important regulatory

region and modulating it can alter the JAK activity. The region includes the JH2 αC-helix (αC) that is homologous to the JH1 αC-helix. The dynamic movement of the αC is an important feature of protein kinases, as their activation is induced by the inward disposition of the helix, which enables the interaction between the αC G52 and the catalytic β3 K72 (see Chapter 2.1.2). Based on the mutagenesis studies presented in this thesis, also the αC helix in the JAK pseudokinase domain is important for both the activation and inhibition of the JAK-STAT pathway. The crystal structure of the TYK2 JH2-JH1 module (PDB 4OLI) shows that in the inactive state, the JH2 αC resides at a central place between JH1 and the JH2 ATP-binding site.

JH2 αC is also important in the pathogenic JAK activation. For example, the well-studed JAK2 V617F has been shown to rely on an activation circuit involving residues in the JH2 αC (Leroy et al. 2016), and to rigify and extend the helix without creating substantial changes in other parts of JH2 (Bandaranayake et al. 2012). The alterations in the JH2 αC conformation of the JAK2 V617F has been suggested to initiate the pathological activation, and recent result show that modulating the αC helix can reduce the V617F-driven dimerization (Hammaren, Virtanen, Abraham, et al. 2019; Leroy et al. 2019; Wilmes et al. 2020).

We established that mutations in the solvent-exposed face of the JH2 αC inhibit constitutive JAK activation more efficiently than the mutations designed to disrupt the ATP binding to JH2 (Article I and Article II). The observation is in line with other studies showing that modulation of the ATP site versus the αC have different mechanisms of action (Hammaren et al., 2019; Wilmes et al., 2020, Article II). Importantly, the inhibitory JH2 mutations used in our studies maintain the WT-like cytokine responsiveness while reducing the constitutive activation. Thus, they differ from the traditional kinase-negative (KN) mutations (targeting JH1) that completely abolish the signalling. Together, the biological and biochemical characteristics found in our studies suggest that the JAK pseudokinase domain is a suitable therapeutic target.

6.1.1 Differences in the JAK pseudokinase domains

In general, the members of the JAK family are homologous and the JAK-STAT signaling cascade conserved. However, the inherent differences within the JAK proteins and the receptor binding modes likely allow the flexible, cytokine-specific activation of the signaling. For example, the JAK2 JH2 has two autophosphorylation sites, S523 and Y570 that are not conserved in other JAKs. Somewhat surprisingly, it was shown that JAK2 JH2 posesses modest kinase activity that is regulated by the phosphorylation of S523 and Y570, and that contributes to the kinase activity of the entire JAK (Ungureanu et al. 2011). This differs form the JAK1, TYK2, and JAK3 JH2s that are catalytically inactive (inactivity of JAK3 JH2 was established in Article I) and from the conjecture that the pseudokinase domains serve solely as allosteric modulators. Structural analyses have depicted a small alpha helix in the JH2 activation loop (αAL) that is unique to the JAK JH2. The αAL of TYK2 and JAK1 has two salt bridges towards the αG in the C-lobe, making the αAL and αC rigid and intolerant to conformational changes, and blocks the proper positioning of the substrate ATP (Min et al. 2015). The JAK2 JH2 αAL has only one salt bridge that increases the flexibility and likely permits the substrate to bind correctly. This difference can in part explain the residual catalytic activity in JAK2 JH2 but not in other JAK pseudokinase domains. The conformation of the JAK3 JH2 αAL is not known due to the lack of structural data.

6.1.1.1 ATP binding properties of the JAK pseudokinase domains

To analyse the biochemical properties of JAK JH2s we combined our results with previous data from our laboratory, and found that the ATP-binding affinity and thermal stability vary among the JAK pseudokinase domains. The binding of the MANT-ATP to TYK2 JH2 is ~25-fold weaker compared with JAK3 JH2, the strongest ATP binder among the family. However, JAK3 JH2 has the lowest melting temperature, and the instablility of the domain likely explains the lack of previous studies with recombinant JAK3 JH2 (Min et al., 2015; Article I). However, we were unable to purify JAK3 JH2 with ATP-mutants, suggesting that the nucleotide binding has a critical role in maintaining the structural integrity of JAK3 JH2. The notion that JAK3 JH2 binds ATP with sub-micromolar affinity supports this hypothesis. In the absence of the mutant JH2 constructs, we could not biochemically

confirm that the JAK3 JH2 mutants block ATP binding. However, the homologous JAK2 mutant verifiably disturbs ATP binding to a recombinant JAK2 JH2 (Hammaren, Ungureanu, et al. 2015). In addition, the biological effects seen in cell-based assays with full-length JAK contructs were similar between the ATP-site mutated JAK2 and JAK3.

Classical kinases bind ATP with two divalent cations (Mg^{2+}) but the pseudokinase domains of JAK1, JAK2 and TYK2 have a non-conservative ATP binding mode with only one Mg^{2+} present in the structures (Hammaren, Virtanen, et al. 2015). This in mind, it was surprising to find that JAK3 JH2 binds ATP in Mg^{2+} independent manner (Article I). However, analasysis of the aminoacid sequence revealed that the inability to bind cations is likely caused by the lack of the conserved Asn171 (PKA) that is required to coordinate the cation (see Chapter 5.1).

The usage of non-identical recombinant JAK constructs and methodological difficulties in obtaining definite (biological) values for the ATP binding may in part skew the results described above and the topic is further discussed in Chapter 6.3. However, the variation between the JAK pseudokinases is remarkable and a similar trend was observed with various methods, making it justified to state that the JAK JH2s differ in their ATP-binding properties.

6.1.1.2 Varying mechanisms of the pathogenic JH2 mutations

There are various mechanisms of action for both the clinical and structure-based mutations. For example, although both the JAK2 V617F and JAK2 R683G/Sare locakted in the JH2 and case constitutive activation, the former is found in myeloid lineage cells, while the latter affects the B lymphoid lineage and causes lymphomas (Buchner et al., 2015; Tefferi, 2016). The R683G mutant is proposed to function in a manner that does not induce receptor dimerization while V617F activates via increased dimerization of the receptor complex (Hammaren et al., 2019; Leroy et al., 2016; Wilmes et al., 2020, Article III). R683 is located in the JH2-JH1 autoinhibitory interface (Shan et al. 2014), and R683S is thought to breake the autoinhibitory interface, release the kinase domans, and optimally expose the JH2 dimerization interface (Hammaren, Virtanen, Abraham, et al., 2019, Article III).

The study on Losdyck and colleagues showed that mice carrying the JAK3 JH2 mutation V674A develop lymphoblastic leukemia while the JAK3 L857P mutant in the JH1 αC induce the formation of lymphoid and myeloid leukemia. In addition, they found that the mutants exhibit different inhibition profiles between JAK1 and JAK3 specific inhibitors and that the activation of L857P is intendent of the receptor (Losdyck et al., 2015). These kinds of mechanistic differences may explain the differences in the phenotypes that occur among the JAK mutants.

6.1.1.3 The V617F site is conserved in JAK1 and TYK2, but lacking in JAK3 JH2

The valine to phenylalanine (V617F) mutation in JAK2 is prevalent in MPNs and leukemia, and the homologous JAK1 and TYK2 mutants are also activating. The VF mutant induces coupling of the Phe-Phe-Val residues in JH2, namely the forming the (FFV)-triad, and the interdomain stacking of the triad residues in JAK1, JAK2 and TYK2 lead to activation of the adjacent kinase domain (Toms et al. 2013). In JAK3 JH2, the valine is replaced by a methionine and the second phenylalanine (JAK1 F636) from the triad is also not conserved but occupied by a lysine L590 (Staerk et al. 2005). We verified that the entire FFV-triad has to be intact for VF-like hyperactivation, as the JAK3 M592F single mutant was only moderately activating when compared with the homologous VF mutations (Chapter 5.3.4, and Chapter 6.2). Interestingly, activation of the reconstituted JAK3 VF is dependent of JAK1, similar to the other studied JAK3 GOF mutants (Article I). On the contrary, JAK1, JAK2 and TYK2 VF exhibit lower but detectable STAT activation even without their heteromeric partner JAK (Article II).

Conformational changes in both the intra- and interdomain interactions are important for the VF-driven activation (Bandaranayake et al. 2012; Hammaren, Virtanen, Abraham, et al. 2019), and the lack of this pathogenic system indicates that JAK3 JH2 mutations have a distinct activation mechanism (the dimerization of kinases is discussed in Chapters 6.2 and 6.2.1). Moreover, the results suggest that the JAK1-dependent activation of the γc-(JAK1/JAK3) signaling is not an intrinsic property of JAK3, but stems from the receptor complex as an entity (discussed in Chapter 6.2).

6.1.1.4 Differenced between the ATP-binding site mutations

JH2 negatively regulates JAK activation, but the integrity of the domain is also important for the hyperactivation. Hence, disturbing the ATP-binding that stabilizes JH2 can be used to reduce JAK2 constitutive activity (Hammaren, Ungureanu, et al. 2015). We produced recombinant JAK1 pseudokinase domains where the ATP-binding site was mutated (K622A or I597F) and assayed the thermal stability (Tm) and affinity of the ATP-binding. According to our results, K622A strongly stabilizes JH2 and blocks the ATP binding while I597F destabilizes the domain in comparison to the WT control. In addition, the mutant does not completely block the ATP binding (Article II). Homologous JAK2 JH2 ATP-site mutations K677E and I559F do not alter the Tm of either the apo or the ATP-bound JH2 (Hammaren, Ungureanu, et al. 2015). We previously observed that full-length JAK1 I597F+V658F does not reduce the constitutive STAT activation in cells, which is in contrast to homologous mutations in JAK2, JAK3 and TYK2 (Article I). Moreover, the I597F mutant increases basal STAT activity in WT background. These studies highlight that depending on the modulation site even inside the same JAK region, the biochemical and biological effects can be vary, and even be opposing among JAKs.

Studies using structure-based JH2 mutants suggest that JAK phosphorylation is not always concordant with the STAT activation, and the following examples illustrate how similar modifications may lead to varying effects in JAKs, the understaning of which is important e.g. for drug development (discussed further in Chapter 6.2). The JAK JH2 ATP-binding site mutants have different JAK phosphorylation patterns although they suppress the constitutive STAT activation: JAK2 K581A increases JAK phosphorylation while the phosphorylation of the homologous JAK1 K622A mutant was weaker than WT JAK1 (Hammaren, Ungureanu, et al. 2015). In contrast, the JAK2 I559F mutant does not affect the phosphorylation of JAK2. All the homologous JH2 ATP-site mutations in JAK2, JAK3 and TYK2 reduce constitutive STAT activation (Articles I and II). Taken together, the phosphorylation of the kinase domain activation loop, and the activation of the downstream signaling is not straightforward. In our studies, we mainly detected the effects in the down-stream signaling (STATs), as it was considered the most biologically relevant denominator to assay at this point.

6.2 Models for inhibition and activation of JAKs

The activation of the seemingly simple JAK-STAT pathway is rather complex with several spatially separated interactions contributing along the cytokine, receptors, JAKs and STATs. A major question regarding the molecular mechanisms of JAK function is whether the autoinhibition of the kinase occurs in cis (between the domains of an individual JAK) or in trans (between JAK dimers/oligomers). For example, the crystallized TYK2 JH1-JH2 structure (PDB code 4OLI) depicted an in cis inhibition between the tandem kinase-pseudokinase domains. The structure exhibits an interaction among the αC-helix and the SH2-JH2 linker regions of JH2 and the hinge region and the N-lobe of JH1 (Lupardus et al. 2014; Shan et al. 2014). This indicates that JH2 regulates the activation by hindering the flexibility of the JH1 hinge region, the correct positioning of the αC helix and the catalytic elements. Furthermore, the KN TYK2 (D1023N) crystallized in the active conformation where the activation loop was extended (Lupardus et al. 2014), suggesting that the phosphorylation of the loop is not the sole determinant of kinase activation.

The inhibition in cis is further supported by the model of Shan et al. that was introduced concurrently but separately with the TYK2 JH1-JH2 structure (Shan et al. 2014). The model bases on MD simulations and mutational studies and depicts an autoinhibitory interaction between JAK2 JH2 and JH1, more specifically between the αC of both JH1 and JH2 (JH1 αC with the SH2–JH2 linker of JH2, and JH2 αC with the JH1 αD). Finally, the clinical mutation data strongly implies that the destabilization of the JH2-JH1 interaction site can result in cytokine-independent activation, lining with the in cis-JAK inhibition model. As an example, the JAK2 exon 12 mutations are thought to destabilize the JH2-SH2 linker, leading to the disruption of the JH2-JH1 interaction and constitutive activation (reviewed in Silvennoinen & Hubbard, 2015).

Contrasting the above-described JH1-JH2 structure, a small-angle X-ray scattering (SAXS) structure of JAK2 exhibited solely an elongated JH1-JH2 construct that does not correspond to the closely packed form that is considered a prerequisite for the in cis inhibition (Babon et al. 2014). In addition, studies showing the formation of preformed receptor dimers support the in trans inhibition (described in 2.2.1). Waters and colleagues studied the GHR system and used FRET-based assays with

MD simulation to build a model where, by hormone binding, a scissor-like movement of the receptor chains pulls the opposing JAK2 JH2s apart and allow the JH1s to contact each other (Waters et al. 2014). Of note, recent studies underpin models where the dimerization is strongly induced by cytokine binding and where the cytokine independent dimerization of EPOR is a putative mechanism for the pathogenic JAK2 V617F activation (Hammaren, Virtanen, Abraham, et al. 2019; Wilmes et al. 2020).

Our inactive monomer model presented in Article III describes an in cis inhibited JAK2 in a tightly packed four-domain conformation. The active dimer, on the other hand, forms via large electrostatic interaction site between the JH2-JH2 dimer (the FERM-FERM dimerization surface exhibits weaker interactions). The models correlate well with the low-resolution EM structure of the full-length JAK1 (Lupardus et al. 2011), and give insight into the mechanism of the V617F activation and how the hitherto noted autophosphorylation of JAK2 S523 directs the regulation of the kinase activity through strengthening the JH2-JH1 and FERM-JH2 interactions (Ungureanu et al., 2011, Article III). In the model, the JH2 β2 and β3 sheets, which are central to the JH2-JH1 interaction of the inactive JAK and to the JH2-JH2 dimerization within the active dimer, interact with the ATP-binding site. hence, we suggest that disruption of the JH2 ATP binding favors the inactive interactions within JH2 and disfavors the formation of the active conformation. These results are concordant with studies where modulating the ATP binding site did not disturb V617F-induced dimerization, but was shown to have an alternative mechanism of inhibition (Hammaren, Virtanen, Abraham, et al. 2019; Wilmes et al. 2020).

Although supporting data exist for both models, the in cis inhibition where the activation is initiated by the dimerization of the monomeric receptors is, in the light of the current knowledge, a likely mechanism for JAK activation. In this scenario, the molecular movements, although subtle, appear large enough to induce the distinct inactive and active states of the complex. However, as the JAK activity stems not only from JH1 and the JH1-JH2 interaction, the direct and indirect contribution of the FERM-SH2 module and the receptors needs to be considered, and in the absence of a high-resolution structure of the membrane-bound active JAK-receptor complex, constructing a holistic picture of the activation process is challenging.

6.2.1 Dimerization and allosteric activation of JAKs

The catalytic site is not per se responsible for the protein kinase function, but conformational changes together with protein dimerization are essential for signal transduction (Lavoie et al. 2014). Regarding the JAK-STAT signaling, the receptor dimerization allows the kinase domains to transphosphorylate. Recently, a crystal structure of the dimeric receptor-JAK2 FERM-SH2-modules revealed a receptor-mediated FERM-FERM dimer and suggested that interaction between the opposing FERM F3 and F2 subdomains is required to optimally position JAK2 for trans activation (Ferrao et al., 2018). The FERM-SH2 module is the main receptor-binding component but it alone is insufficient in inducing receptor dimerization, indicating that additional components are required for the event (Gauzzi et al. 1997). Indeed, several studies have shown that JH2 participates in the dimerization. In many kinases, the repositioning of the αC helix is important promoter of the dimeric packing, but the orientation of the dimer may vary between kinases (for example BRAF versus the asymmetric EGFR dimer) (Hu et al. 2015; Lavoie et al. 2014; Zhang et al. 2006). Interestingly, the structure of JAK1 JH2 showed a non-crystallographic dimer where the αC helix lies in the centre of the JH2-JH2 interface (Toms et al. 2013). In addition, the low resolution EM structures of the full-length JAK1 that exhibited a distinct open and closed forms, corroborating with the closely-packed inactive and the more open active conformatios that allows the activation of JH1s and is optimal for the JH2-JH2 interaction (Lupardus et al. 2011).

Activating mutations such as JAK2 V617F can induce cytokine-independent dimerization of the receptors and JH2s (Leroy et al., 2019; Hammaren, Virtanen, Abraham, et al., 2019; Wilmes et al., 2020, Article II and III). Recently, Liau and colleagues purified the full-length JAK1 WT and V658F proteins and showed that the mutation does not alter the affinity towards ATP or STAT5, the rate of auto- or dephosphorylation nor have an effect on the stability or the receptor binding capacity of JAK1 (Liau et al. 2019). These structural and kinetic studies strongly imply that the VF mutation mediates its effect allosterically rather than directly increasing the kinase activity (of JH1) (Sanz Sanz et al. 2014).

Hammarén et al. showed that mutations at the outer face of the JAK2 JH2 αC reduce receptor dimerization and can hence inhibit the constitutive activation. It was

hypothesized that the mutants disrupt the JH2-JH2 dimer, which would suppress the formation of the active complex. The results presented in this thesis corroborate the model. We saw that homologous mutations in the JH2 αC of JAK1, JAK3 and TYK2 reduce constitutive JAK signaling (Article II). In addition, our experimentally confirmed MD model depicts a strong JH2-JH2 interaction as a characteristic feature of the active JAK2 complex (Article III). In a recent study of the TPOR, EPOR and GHR signaling systems, Wilmes and colleagues provided further insight about the homodimeric JAK2 activation mechanism (Wilmes et al. 2020). The study complements our MD model, the hypothesized role of the JH2-JH2 dimer in the JAK2 activation and the cytokine-induced dimerization of the monomeric receptors (Wilmes et al. 2020). Also in accordance to the work done in our laboratory, Wilmes et al. discovered that the V617F activation is predominantly induced via constitutive JH2-JH2 dimerization. Intriguingly, while the activating mutations in the SH2-JH2 linker and in the αC-helix induced dimerization, pathogenic mutations in the JH1-JH2 interface did not dimerize. This correlates with our studies suggesting that both dimerization and/or the release of the regulatory JH1-JH2 interaction may result in activation.

In conclusion, the current data strongly supports the model where the receptor bound JAK is inhibited in cis and activates in trans as the cytokine binds and induces dimerization. Furthermore, the pseudokinase domains likely play a crucial role in the dimerization, and the possible variation in the interaction among signaling systems should be studied: it is possible that the JH2-JH2 interaction brings about specificity to the JAK-signaling, comparably to the selective binding between the FERM-SH2 module and the receptors.

6.2.2 Function of JAKs within the signaling complexes

How can the four JAK family members mediate the signaling of ove 50 cytokines, each leading to unique biological effect? The binding modes vary between JAKs and receptors and specific STATs have stronger affinity towards designated receptor-JAK complexes (see Table 2). However, the underlying mechanism of JAK function within an individual signaling complex remains elusive. The studies of the different JAK-receptor complexes are complicated by cross-activation of STATs with other

kinases. Moreover, finding an experimental set up that enables the detection of an individual JAK in a biological setting is not an easy task. Analogue-sensitive JAK mutants, knock-out approaches, KN mutations, chimeric protein constructs and highly specific inhibitors have been used to elucidate JAK function but especially the molecular details of heteromeric (vs. homomeric JAK2-JAK2) JAK activation have remained largely unknown (Haan et al. 2011; Koppikar et al. 2012; Tvorogov et al. 2018).

IFNγ signaling complex, driven by JAK1 and JAK2, has been the topic of several studies both showing JAK1 as the primary driver of the STAT1 activation, and others suggesting that JAK2 dominates the signaling. In the work of Briscoe et al. KN JAK2 was shown unable to support the IFNγ-induced gene expression and to act as a dominant-negative inhibitor of the cytokine response (Briscoe et al. 1996). JAK1 KN cells sustained the activation altough the pSTAT1 was reduced and the antiviral response disturbed. This let the authors suggest a model where the JAK2 kinase activity is required predominantly for initiating IFNγ signaling and possibly for the phosphorylation of STAT1, whereas JAK1 phosphorylates the IFNGR1 and recruits STAT1 to the receptor. Our data supports this model of JAK2 more directly contributing to the STAT1 activation (Figure 18). We observed that, in contrast to IFNα and IL-2 systems, the JAK1 JH2 αC-mutant L633K did not effectively reduce IFNγ signalling while the homologous JAK2 E592R decreased the signaling and suppressed the constitutive JAK1 V658F activation (Chapter 5.3, Article II). Albeit we did not directly show the mechanism for L633K, previous studies using JAK2 E592R are in line with the hypothesis that the mutant hinders the dimerization and in this way inhibits the signaling (Hammaren, Virtanen, Abraham, et al. 2019). Regarding the possibility that the mutants disrupt the JH2-JH2 interaction, we conjectured that the dimerization interfaces between the JAK1 JH2 and JAK2 JH2 in the IFNγ system differ, since the modulation of the JAK1 αC had minor effect compared with JAK2.

Keil and colleagues studied mice where both of the JAK2 activation loop tyrosines were mutated (JAK2FF/FF) and found that the mutant completely abolishes the homomeric, EPOR-driven STAT5 phosphorylation. The mutant had no effect on the IFNγ driven STAT1 activation. Since the phosphorylation of JAK2 did not appear crucial for the activation, the heteromeric IFNγ activation was considered as

JAK1-dependent (Keil et al. 2014). Of note, the data did not include homologous JAK1 FF/FF mutants and was conducted in a mouse system, which might explain the differing results compared with the studies showing JAK2 as the initiator of the STAT1 activation (Briscoe et al., 1996; Eletto et al., 2016; Hammaren, Virtanen, Abraham, et al., 2019; Article II). Another study with conditional JAK1 KO-mice showed that somatic loss of JAK1 in the hematopoietic compartment leads to severe defects in the B-cell function and complete loss of the NK-cells (Kleppe et al. 2018). The same study designated the most downregulated genes in JAK1 deficient stem cells, including STAT1, STAT2 and multiple members of the IFN regulatory transcription factor family, and highlights the important role of JAK1. Interestingly, transfecting JAK2 V617F only modestly rescued the defects either in the myeloid or lymphoid lineage JAK1 KO cells. The authors concluded that the signaling between JAK1 and JAK2 is non-redundant and that absent JAK1 signaling attenuates self-renewal in JAK2 V617F MPN stem cells.

Recently, Mendoza et al. solved the structure of the hexameric IFNγ complex exhibiting a 2:2:2 stoichiometry for IFNγ, IFNGR1 and IFNGR2 (Mendoza et al. 2019). The high-affinity IFNGR1 binds JAK1 and JAK2 is bound to the IFNGR2. The tetramer forms solely in a cytokine-induced manner, as the low-affinity IFNGR2 binds to the preformed IFNγ-IFNGR1 complex. Strickingly, removal of the second copy of the IFNGR2 preserved normal STAT activation while the activation was decreased to 25% when both IFNGR2 were removed from the complex (2:2 IFNγ: IFNGR1). This indicates high sensitivity to the presence of the IFNGR2, and that the JAK2-bound receptor might be more directly contributing to the STAT1 activation. Assigning JAK2 as the driver of the STAT1 activation could function as a back-up mechanism to prevent overt signalling, as it would ensure that the STAT activation only occurs once the high-affinity state is reached. However, as mentioned above, the roles between JAK1 and JAK2 in this system are not yet explicitly established. Table 9 summarizes the studies described above and suggests an interpretation of the results regarding the roles of the JAK1/JAK2 in IFNγ signaling.

Table 9: Central findings of the selected IFNγ signaling studies with suggested interpretation of the results. The interpretations are highlighted with blue or yellow whether they support or contradict the hypothesis that JAK2 is the dominant in the IFNγ signaling, respectively. The result of Kleppe et al. mainly show that the functions of JAK1 and JAK2 are auxiliary and not identical, and is thus hihgiliighted as gray.

Study	Highlights	Interpretation of the results
Briscoe et al.	IFNγ signaling is sustained in KN JAK1 but not in KN JAK2 cells. JAK2 KN is a dominant-negative inhibitor of the pathway.	Function of JAK2 is more essential for STAT1 activation than JAK1.
Keil et al.	KN JAK2 mice embryonic fibroblast maintain STAT1 activation.	JAK2 deficiently does not abolish IFNγ signaling → JAK1 has complementary effects.
Mendoza et al.	STAT1 activation is highly sensitive to the concentration of the JAK2-bound IFNGR2.	The IFNGR2-JAK2 component has a more direct effect on the STAT1 activation.
Kleppe et al.	JAK1 KO mice have impaired lymphocyte function that is only partially rescued by JAK2 V617F.	In the IFNγ system, JAK1 and JAK2 have concomitant functions.
Hammarén et al.	The JAK2 JH2 αC mutation inhibits homodimeric EPOR signaling, but almost abrogates IFNγ signaling. The E592R mutant reduces EPOR dimerization.	JAK2 JH2 is strongly contributing to the activation of the IFNγ signaling, plausibly via JH2-JH2 dimerization.
Raivola et al (Article II)	Inhibitory JAK1 JH2 αC mutant has only weak/no effect in the IFNγ system (WTor JAK2 V617F). Homologous JAK2 mutant inhibits both WT and JAK1 V658F-driven IFNγ signaling.	JAK2 has more direct role in the STAT1 activation and the JH2 function/interfaces vary between JAK1 and JAK2 within the IFNγ complex (see Hammarén et al).

Unlike the somewhat ambiguous IFNγ system, our studies of the IL-2 signaling corroborate previous studies and together provide strong evidence that JAK1 dominantly activates STAT5 while JAK3 is required for fully functional, cytokine-responsive signalling of the γc system (Liu et al., 1997; Degryse et al., 2014; C. Haan et al., 2011; Article I; Article II). There are a few plausible reasons for the dominancy of JAK1. JAK1 is more potent in phosphorylating STAT5 than JAK3, and the phosphorylation of the JAK1 activation-loop tyrosines is essential for the regulation of the activity. The phosphorylation of JAK3 activation loop is not required for its catalytic function (Liu et al., 1997). Hence, JAK3 appears as an important regulatory component, where its kinase activity is perhaps not directly contributing to STAT phosphorylation. Moreover, the study of Smith and colleagues suggested that JAK3 plays a role in the induction of the second wave of IL-2 signalling, providing novel insights about the temporal regulation of JAK3 signaling (Smith et al., 2016). The authors proposed that the delayed signalling event drives the activation of additional signalling pathways and cell-cycle progression.

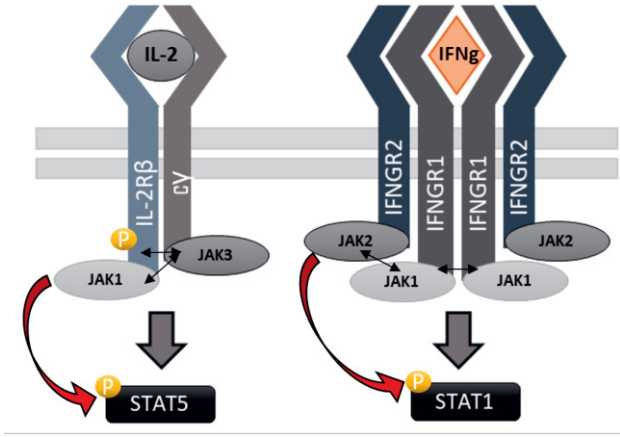


Figure 18: A schematic presentation of the IL-2 and IFNγ pathways and suggested order of the events. Black arrows indicate the interplay between the components and red arrows the transfer of the phosphate group. Left: Activation of the IL-2 signaling is driven by the activation of JAK1 and binding of STAT5 to its receptor (IL2Rβ). JAK3 plays important role in cross-activating and regulating the JAK1/STAT5 activation. Right: In the tetrameric IFNγ system, JAK1 and JAK2 more equally contribute to the STAT1 activation. The scheme illustrates a model where JAK1 drives the formation of the active IFNGR complex while JAK2 directly contributes to the IFNγ-driven STAT1 activation.The picture is modified from Article II, the copyright of the original illustration Raivola et al. Cancers, 2019. CC BY.

In parallel with the above-described pathways, we studied the IFNα pathway that was chosen to model a JAK1/TYK2 driven signaling (see Table 2). Both JAKl and TYK2 have been shown to be crucial for IFNα mediated STAT1 activation. Krishnan and colleagues observed that the phosphorylation of JAK1 and TYK2 is decreased when the JAK1 KN mutation is expressed in human osteosarcoma cells (Krishnan, Pine, and Krolewski 1997). Tranfection of TYK2 KN, however, led to the reduction of pTYK2 while pJAK1 levels remained non-affected. The more pronounced effect of JAK1 on the phosphorylation of TYK2 implies that JAK1 is dominating the activation of this pathway. Our results corroborate, as introducing the inhibitory JH2 αC mutant into JAK1 or TYK2 reduced the IFNα-induced STAT1 activation, but the basal activation of STAT1 was not affected. This suggests that both JAK1 and TYK2, and their pseudokinase domains, are important especially for the IFNα-induced STAT activation and for the formation of the active complex.

Refining the function of individual JAKs within a signalling system is difficult due to the noncatalytic functions of human JAKs, the number of possible signalling

complexes and experimental difficulties rising from the need to pin-point the causal connection between the two or more JAKs, receptor chains and STATs. In addition, cross talk between JAKs and other kinases such as the Src-kinase, the Ras-MAP kinase pathway and the PI3K-AKT signaling should be taken into account (Bruno et al. 2005; Soendergaard, Young, and Kopchick 2017). Fortunately, systematic studies using complementary methods allow us to form evidence-based models about the mechanism of individual cytokine complexes. The roles of JAKs have been mostly investigated in pathways that are driven by JAK1 together with a heteromeric JAK pair. These signaling systems include IL-2 (Degryse et al., 2014; C. Haan et al., 2011; Article I), IL-6 (Guschin et al. 1995; Rodig et al. 1998; Song et al. 2011), IFNα (Eletto et al., 2016; Krishnan et al., 1997; Article II) and IFNγ (Briscoe et al., 1996; Eletto et al., 2016; Hammaren et al., 2019; Article II). The ability of JAK1 to interact with all JAK family members makes it an especially interesting member. Together, these studies exemplify the versatility of the JAK signaling and why discovering the characteristics of the JAK-receptor complexes is crucial.

6.3 Illusion of specificity

Characterization of the biochemical and biological properties of the potential inhibitors is crucial for designing inhibitors against specific targets, such as a specific member of the JAK-family. Essential parameters for comparison of the potency and specificity include the binding properties of the compound (affinity, effect on the stability and the ATP-binding parameters in case of competitive compounds), and its effect on the kinase activity and phosphorylation of the kinase/downstream effectors. However, the compound binding does not necessarily lead to JAK inhibition, plausibly because the kinase activity is generally measured from the recombinant JH1 domain (or JH1-JH2 more recently), and the non-kinase function of the other domains is often disregarded. Moreover, the potency of inhibitors (e.g. tofacitinib) may differ between the cell-based assays and the in vitro results. This variation likely stems from the different experimental set-ups and additional regulator levels of the cell-based assays compared with the studies using isolated protein domains.

Few caveats in the current experimental methods hamper the development of JAK specific binders. To measure the binding affinities and inhibition of the kinase activity, several studies use ATP concentrations that are comparable to the cellular levels (1-5 mM) while others perform the kinase activity assays in [ATP] < 1 mM. If the coumpund is ATP competetive, the variation in the ATP amount causes divergence among the stated potencies and even in the detected (off-)targets of the inhibitor. Compounds that target kinases, which have a low affinity to ATP (i.e., high Kd) typically have more similar potency procile between biochemical and cell-based assays while the observed inhibition of high affinity kinases may vary significantly depending whether the measurement is done in vitro or in cells (Becher et al. 2013). Hence, standardizing the assay systems is essential for reliable and consistent results.

IC50 values are widely used to determine the enzyme inhibition, as they describe the compound concentration that inhibits 50 % of the kinase activity. Using the same, biologically more appropriate ATP concentration (1 mM) across all biochemical assays would unify the assay system. However, at high ATP the IC50 values are affected by the characteristics of the kinase constructs, i.e. which domains/amino acids are included, meaning that the data cannot be used to compare the potency of an inhibitor between different kinases. The issue is circumvented when the ATP concentration is optimized to the kinase´s K_mATP (K_m: the Michaelis–Menten constant), namely the amount of ATP where half of the binding-sites in the kinase are occupied. However, this data is incomparable with the one derived with cellular ATP amounts, which can be problematic considering the biological relevance of the binding properties (Hafenbradl, Baumann, and Neumann 2011).

A study of Thorarensen and colleagues exemplified how the assay conditions may skew the results. The authors stated that inhibition of JAK3 is sufficient to reduce the downstream STAT5 activation and concluded that the discrepancy between previous research arises from the usage of K_m rather than cellular ATP concentrations (Thorarensen et al. 2014). Indeed, the K_m ATP is lower for JAK3 compared with JAK1, which decreases the selectivity ratio between the two and biases the derived inhibition potencies. However, the inhibitors used in the sudy had relatively similar (30-fold) specificities between JAK1 and JAK3 that complicates the evaluation of the inhibition between the JAKs. The matter was pointed out by Thoma and colleagues, who used analogue-sensitive mutant JAK1 and JAK3 to

show that inhibition of JAK3 is not sufficient for STAT5 inhibition (Thoma, Drückes, and Zerwes 2014). Finally, stating JAK3 as an equal activator of the γc signalling contradicts the majority of studies showing JAK1 as the dominant STAT5 activator (discussed in previous chapters).

Despite the caveats described in this chapter, the importance of pre-clinical assays should not be dismissed as they influence the selectivity of the kinase-targeted compounds and are crucial for the proper evaluation of the potential drugs. Rather, the subject underlines how it is essential to understand the exact parameters measured and the restrictions of the assays. Conducting parallel experiments with various methods provide highly beneficial data about the properties of prospective drugs.

6.4 Towards more potent JAKinibs

As described in Chapter 2.4, only type I inhibitors are currently approved for JAK inhibition. These first-generation inhibitors exhibit good kinome-wide selectivity but are not specific among the JAK family (reviewed by Virtanen et al. 2019). My research focused on the JAK pseudokinase domain and how it can be modulated to alter the signaling with enhanced potency and efficacy. Recently, the screening of small compounds against JAK2 JH2 have led to the discovery of ATP-site ligands that induce changes in the V617F JH2 and promote the WT-like conformation (Puleo et al. 2017). Many of the compounds bind both JH1 and JH2 but structural analysis revealed that the binding modes differ between the domains. For example, a well characterized inhibitor JNJ-7706621 is aligned towards the C-lobe and does not fully enter the JH2 ATP-binding pocket. In JH1, the compound protrudes more deeply into the pocket. The same phenomenon is seen with AT9283 binding between JH2 and JH1 (McNally et al. 2019; Puleo et al. 2017). The steric hindrance created by the JH2 I559 and L579 residues in the JH2 ATP-binding pocket likely narrows the site, leaving the compound from fully entering the cavity (Figure 19). In contrast, the JH1 analogs are small aminoacids (A880 and V863) that leave more space for the substrate.

The above-described studies illustrate how the ATP-binding site ligands can modulate the JH2 conformation and disturb the constitutively active form. Moreover, they are in line with our studies showing that modulating the grooves of the interface between the FERM-SH2 and JH2-JH1 units can stabilize the inactive conformation. In conclusion, targeting regions outside the kinase domain is a feasible approach for drug development agasint hyperactive JAK signaling.

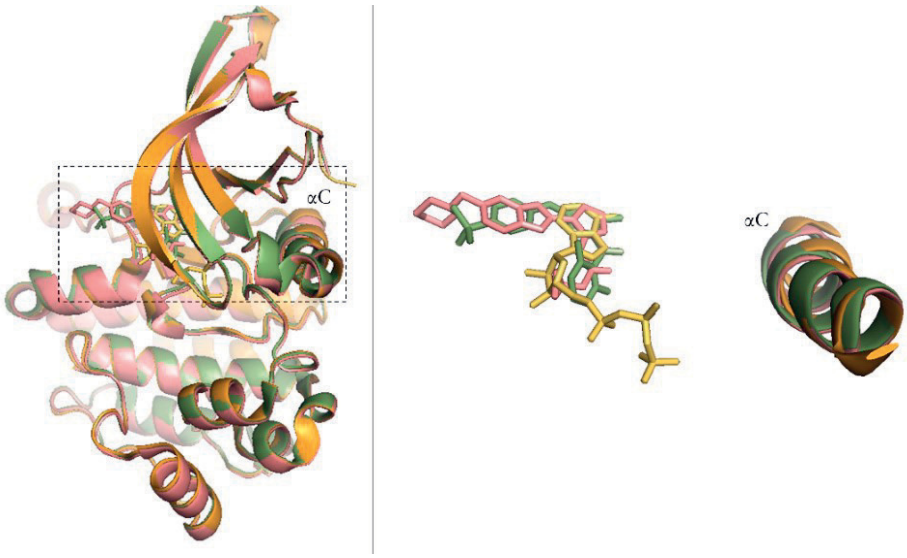


Figure19: Structures of the JAK2 pseudokinase domains bound with ATP (yellow: PDB 4FVQ), JNJ-7706621 (green: PDB 5USZ) and AT9283 (rose: PDB 5UT0). The colors of the ligands correspond to the color of each JAK2 JH2 structure. ATP binds deeper into the pocket than the compounds. Right: close-up of the boxed area of the left structure. Only the ligands and the αC-helix are shown.

Next generation JAK inhibitors aim to reduce off-target effects and provide solutions to the complications related to resistance and persistence. These issues may arise from acquired secondary mutations, increased heterodimerization and transactivation of JAKs (Meyer et al., 2015; Springuel et al., 2014). The heterodimerization is especially difficult to overcome with type I inhibitors that block JAK in its active state and may stabilize and protect the kinases from phosphatases (Tvorogov et al. 2018). Ruxolitinib is a type I inhibitor used to treat primary myelofibrosis and to reduce the symptoms, but it is unable to eradicate the disease. Hence, the treatment may be prolonged, if improvement does not occur before. Ruxolitinib stabilizes JAK2 into its phosphorylated form that, although the

kinase activity is inhibited, leads to accumulation of phosphorylated JAKs that are waiting in the active state to regain their kinase activity. Removal of the inhibitor can trigger a rapid and intensive relapse and rare cases of dose-related cytopenias, cytokine-related manifestations and even septic shock like syndrome collectively called the "discontinuation syndrome" have occurred related to the prolonged usage of the drug (Tvorogov et al. 2018). The unexpected results of the trans-activation of JAKs bound to ATP-competitive inhibitors highlight the interplay between the active region (JH1) and the allosteric sites of the kinase. The phenomenon is aslo encountered with other kinase inhibitors. When in their inactive state, BRAF and CRAF are thought to exist as monomers until their activating partner KSR1/2 binds. However, dimerization of BRAF and CRAF is sufficient to induce the active conformation and the assembly of the kinase R-spine. Inhibitors that bind to the BRAF ATP-binding pocket may induce the unintended formation of the BRAF/CRAF dimer where the ligand-bound kinase functions as a scaffold against the kinase, leading to constitutive proliferation (Poulikakos et al. 2010). The same effect occurrs with HER2 inhibitor that induces the formation of an active HER2-HER3 dimer (Claus et al. 2018). Targeting the dimerization interface or the usage of small molecule inhibitors that stabilize the kinase domain to its inactive, monomeric conformations could be beneficial to surmount the pitfalls of unintended pathway activation and resistance.

JAK kinases transmit signals for multiple pathways and their inhibition induces wider effects than obtained with biologics that mainly target a single pathway on a cell-surface level. Hence, inhibiting the function of a single JAK-kinase has been questioned, since while the side effects are minimized, the biological efficacy can be too narrow. Non-selective agents (i.e. pan-JAK inhibitors) have the advantage of suppressing several inflammatory cytokines, which promotes the inhibitory reaction. However, lowering the immune system extensively can predispose the organism to cancer immune evasion. Taken together, determining an optimal JAK selectivity profile is likely a key feature for refining the second-generation JAK inhibitors.

Combination therapies bring even more possibilities for fine-tuning the cytokine signaling. Currently tofacitinib together with methotrexate is used to treat patients who show inadequate responses to the primary methotextrate treatment (methotextrate: a general immunosuppressant having multiple mechanism of action).

The combination is as effective as methotrexate plus the biological TNF inhibitor adalimumab (Fleischmann et al. 2017). Using a combination of specific JAK inhibitors, e.g. against JAK1 and JAK3, could in the future provide more potency to the highly targeted treatments. Administrating the drugs together with other kinase inhibitors (e.g. Brcl/Abl and FLT3 (Sen and Grandis 2012)) or immunosuppression blockers (e.g. anti-PD-L1 (Shin et al. 2017; Xu et al. 2018)) could enhance the anticancer response by diminishing the proto-oncogene function (ABL) or by preventing the immune evasion (anti-PD-L1). In conclusion, currently available type I JAK inhibitors have brought relief to severe and difficult diseases. Still, they are not optimally effective nor without adverse effects.

7 CONCLUSIONS AND FUTURE ASPECTS

Not "Just another kinase", Janus kinases are now recognized as critical players within the cellular signalling network, driving the immune development and surveillance with other crucial functions. Although the JAK-STAT pathway appears as a simple cascade, it has proven to be a diverse and complex system where the precise molecular mechanisms remain partly unsolved even after decades of research. New structures of the domain modules and especially the recent success in gaining data from full-length JAKs have brought novel insight into the field. In addition, the supporting information using other advanced methods and molecular dynamic simulations has been valuable. Today, we recognize that JAK domains are interconnected in multiple ways and have other functions in addition to the one initially referred (e.g. the FERM-SH2 module being both a receptor-binding moiety and also directly interacting with the JH1-JH2 module, or JH2 negatively regulating JH1 as well as driving the JAK/receptor dimerization).

The dynamic movements of the JAK domains and the entire receptor complex, including the dimerization of the receptors and JAKs, allow the flexible signaling via dozens of cytokines. Recently, super-resolution imaging techniques have defined the dynamics of the JAK activation at a molecular level and allowed the construction of more precise activation models. However, comprehensive studies of the parallel JAK pathways using advanced and versatile methods are required and could bring insight into how, or if, the JAK function varies between the cytokine complexes. The more studied γc, IFNγ, IL-6, IFNα and some homomeric JAK2/JAK2 complexes (TPO/EPO) could be an approachable starting point. In addition, studying the temporal aspects (i.e. the initial versus delayed signalling events driven by JAKs) would be an interesting topic that has not been widely studied.

The clinical relevance of the JAK-STAT pathway is highlighted by the myriad of patient-derived JAK mutations that cause a range of severe diseases. The most prominent mutation JAK2 V617F was found 15 years ago but still a number of

studies focus on revealing how the mutation activates JAK signalling. Simultaneously, new patient-derived mutations are found that may use different mechanisms to alter the signalling. Fortunately, studying these mutants can help us to better understand both the physiological and pathogenic JAK signalling and to develop novel more efficient treatments agains the related diseases.

Cytokines, immunity and cancer development have a close-knit relationship, that bring interesting possibilities for treating autoimmune diseases and cancers that are not directly driven by JAK mutations. For example, JAK inhibitors are used in rheumatoid arthritis that is characterized by increased number of pro-inflammatory cytokines, but where no JAK mutatns have been detected. However, the efficacy of the current inhibitors is not optimal and the full potential of the JAK inhibition has not been harnessed regarding the range of diseases it may be effective against. Combination of kinase inhibitors and/or immunoblockers/inhibitors of transcription provides a host of new possibilities into the field.

Recent events with the COVID-19 pandemic have seriously affected the world in 2020 and the impact on public health, as well as social and economic well-being are wide and long lasting. The development of vaccines that introduce cells safely to the viral antigens and provide immunity has been a global effort that has led to significant results. Interestingly, also the JAK inhibitors ruxolitinib and baricitinib have gained interest as a possible treatment for COVID-infected patients. The anti-inflammatory effects of the JAK inhibitors can suppress the overt cytokine storm and related breakdown of the respiratory system, and again illustrates the wide possibilities of JAK-STAT inhibition (Anon 2020; Richardson, Corbellino, and Stebbing 2020).

To fulfil the expectations and potential described above, a detailed mechanistic understanding of the JAK signalling cascades and of the crosstalk occurring between JAKs and other cellular components is required. This work demonstrates how solving the mechanism of pathogenic and structure-based JAK mutations can bring insight into the function of JAKs in health and disease. Deciphering the JAK-STAT signaling will help to avoid the previous defects in the treatments and to diagnose, treat and prevent conditions that affect the quality of life of myriad of people.

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9 ORIGINAL PUBLICATIONS

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Hyperactivation of Oncogenic JAK3 Mutants Depend on ATP Binding to the Pseudokinase Domain

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Hyperactivation of Oncogenic JAK3 Mutants Depend on ATP Binding to the Pseudokinase Domain

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Janus kinase 3 (JAK3) tyrosine kinase has a central role in the control of lymphopoiesis, and mutations in JAK3 can lead to either severe combined immunodeficiency or leukemia and lymphomas. JAK3 associates with the common gamma chain (γc) receptor and functions in a heteromeric signaling pair with JAK1. In IL-2 signaling JAK1 is the effector kinase for STAT5 phosphorylation but the precise molecular regulatory mechanisms of JAK1 and JAK3 and their individual domains are not known. The pseudokinase domain (JAK homology 2, JH2) of JAK3 is of particular interest as approximately half of clinical JAK3 mutations cluster into it.

In this study, we investigated the role of JH2s of JAK1 and JAK3 in IL-2R signaling and show that STAT5 activation requires both JH1 and JH2 of JAK1, while both JH1 and JH2 in JAK3 are specifically required for the cytokine-induction of cellular signaling. Characterization of recombinant JAK3 JH2 in thermal shift assay shows an unstable protein domain, which is strongly stabilized by ATP binding. Unexpectedly, nucleotide binding to JAK3 JH2 was found to be cation-independent. JAK3 JH2 showed higher nucleotide binding affinity in MANT-ATP and fluorescent polarization competition assays compared to the other JAK JH2s. Analysis of the functional role of ATP binding in JAK3 JH2 in cells and in zebrafish showed that disruption of ATP binding suppresses ligand-independent activation of clinical JAK3 gain-of-function mutations residing in either JH2 or JH1 but does not inhibit constitutive activation of oncogenic JAK1. ATP-binding site mutations in JAK3 JH2 do not, however, abrogate normal IL-2 signaling making them distinct from JH2 deletion or kinase-deficient JAK3. These findings underline the importance of JAK3 JH2 for cellular signaling in both ligand-dependent and in gain-of-function mutation-induced activation. Furthermore, they identify the JH2 ATP-binding site as a key regulatory region for oncogenic JAK3 signaling, and thus a potential target for therapeutic modulation.

Keywords: JAK kinase, nucleotide binding, pseudokinase, cytokine, leukemia

INTRODUCTION

The family of Janus kinases (JAK1, JAK2, JAK3, and TYK2) are key mediators of cytokine signaling that regulate hematopoietic cell development, cellular metabolism and immune responses. Within the JAK family, JAK3 is unique in its restricted expression to hematopoietic lineages and specific interaction only with the common γ chain (γc) cytokine receptor (1). In cytokine signaling, JAK3 functions in a heterodimeric pair with JAK1, which binds to the cytokine specific receptor β chains. The JAK1-JAK3 pair transmit signals emanating from interleukin (IL)−2, −4, −7, −9, −15, and −21 that regulate development and activation of lymphoid lineage cells (2). The critical role of JAK3 in lymphoid cells is highlighted by the phenotypes of JAK3 activating mutations causing different types of leukemia and lymphomas (3–5), as well as by JAK3 deficiencies that cause severe combined immunodeficiency (SCID); a condition resulting from profound defects in mature T cells and B cells and innate lymphoid cells including NK cells (6–8).

JAKs share a conserved domain structure consisting of an N-terminal FERM domain and a Src homology 2 (SH2)-like domain that comprise the receptor-binding unit, followed by a pseudokinase domain (JAK homology 2, JH2) and a tyrosine kinase domain (JH1) (1, 9, 10). Signaling is initiated by cytokine binding to the extracellular domain of their respective receptors that induces receptor dimerization/oligomerization or a conformational shift juxtaposing the JAKs and allowing their activation through trans-autophosphorylation of the activation loop tyrosine residues. Following activation, JAKs phosphorylate tyrosine residues in the receptor chains leading to recruitment and subsequent phosphorylation of downstream effectors such as the signal transducers and activators of transcription (STATs) (1, 2, 10). In IL-2 receptor (IL-2R) signaling, JAK1 has been shown to play a dominant role in early cytoplasmic activation events where JAK1 is primarily driving STAT5 phosphorylation (11), while JAK3 is required for sustained activation during T-cell proliferation (11, 12). Both functions are dependent on the catalytic activity of the JH1 domains in JAK1 and JAK3, respectively. However, several aspects of IL-2R signal regulation are still elusive, especially with respect to the roles of the pseudokinase domains.

JAK kinases are constitutively associated with cytokine receptors and the activity of the JAK–STAT pathway needs to be tightly regulated at multiple levels to suppress signaling in the absence of cytokine stimulation and to allow rapid, transient activation upon stimulation (9, 10). Regulation relies primarily on control of the JH1 tyrosine kinase activity through intra- and intermolecular mechanisms (1, 9), and for the former, the function of JH2 is important, although still somewhat enigmatic. Genetic studies provide compelling evidence for the regulatory function of JH2 as disease-causing mutations strongly cluster in JAK JH2s (13, 14). The best characterized example is JAK2 where somatic mutations in JH2 (JAK2 V617F and >30 other mutations) result in constitutively active JAK2 and are responsible for approximately 80% of myeloproliferative neoplasms (MPN) and less frequently for different types of leukaemias including acute lymphoblastic leukemia (ALL), acute megakaryoblastic leukemia (AMKL), and acute myeloid leukemia (AML) (5, 14, 15). In JAK3, approximately half of all clinical mutations localize to JH2 (13, 16).

JAK3 JH2 has two interesting and unique features compared to the other JAKs: it is the only JH2 harboring both activating and inactivating clinical mutations, and secondly it is the only JH2 where the prototypic JH2 gain-of-function mutation (JAK2 V617F, JAK3 homolog M592F) does not cause hyperactivation (17). Some inhibitory SCID mutations result in truncated proteins, but several mutations cause amino acid changes in either the C- and N-lobes of JH2 (e.g., E481G, del482-596, R582W, G589S, C759R). More recently, several activating JAK3 JH2 mutations (e.g., M511I, A572V, R657Q, V722I) have been found in acute myeloid and lymphoblastic leukemia (AMKL, T-ALL, AML, natural killer cell lymphoma, acute megakaryoblastic leukemia, T-cell prolymphocytic leukemia, juvenile myelomonocytic leukemia and natural killer T cell lymphoma) (3, 4, 18). Although genetic information from patients provide compelling evidence for the importance of JH2 regulatory function, they do not provide molecular explanations of the underlying mechanisms of normal or pathogenic JH2 function.

JAK JH2s show significant sequence homology with classical protein kinases but several conserved functional residues or motifs are missing or altered. Most importantly, the HRD motif, including the catalytic base aspartate (D) is replaced by HGN resulting in a presumably catalytically inactive kinase (hence "pseudokinase") (19–21). Structural and biochemical analysis of JAK1, JAK2 and TYK2 JH2s have shown that despite the sequence variations, the JH2 domains adopt a typical two lobed kinase-fold able to bind ATP (22–24). JAK2 and TYK2 JH2 structures have been solved with ATP and they show a non-canonical binding mode with ATP complexed to only a single cation instead of the usual two (23, 25). In addition, the canonical ionic interaction between the β3 lysine (K556 in JAK3 JH2; K72 in the archetypal cAMP-dependent protein kinase PKA) and αC (E91 in PKA, alanine in JAK JH2s) is replaced by a β3 lysine–aspartate interaction to the DFG motif (DPG in JAK JH2s) (19, 23, 25–27).

Early biochemical studies suggested a dual regulatory or "switch" function for JH2 where the domain is required for maintaining JAKs inactive in the absence cytokine stimulation, as well as mediating cytokine-induced signaling (27–31). Recent structural information from JAK2 (structural model) and TYK2 (crystal structure) provide molecular basis for the JH2 inhibitory function and show that JH2 interacts with the hinge-region of JH1 and stabilizes the JH1 in an inactive conformation (27, 32). Most gain-of-function JAK2 disease mutations localize in this interface and are predicted to disrupt the JH1–JH2 autoinhibitory interaction resulting in constitutive activation (27, 28, 33). The nature of the activating function of JH2 in cytokine signaling and in oncogenic JAK3 mutations is currently unknown.

MATERIALS AND METHODS

Protein Expression and Purification

Expression: JAK3 JH2 (residues 511–790) was cloned into the pFASTBAC1 vector (Invitrogen) with a C-terminal His_6 tag and expressed as a fusion protein in *Spodoptera frugiperda* (*Sf*9) cells.

For protein expression, cells were infected with 10% (v/v) virus supernatant, grown for 48 h and collected by centrifugation.

Ni-NTA purification: Cell pellets containing JH2-His fusion protein were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20% (v/v) glycerol, 0.5 mM TCEP and 20 mM imidazole, supplemented with protease inhibitors cocktail, lysed using a cell disruptor (Sonics) and clarified by centrifugation for 1 h at 10,000 g. The supernatant was incubated for 2 h with prewashed Ni-NTA beads (Qiagen) with gentle rotation at 4 °C. The beads were extensively washed with a buffer supplemented with 40 mM imidazole, and the fusion protein was eluted with 150 mM imidazole buffer. Fractions containing the fusion protein were pooled and buffer-exchanged by several dilution-concentration cycles using 10 K Amicon Ultra centrifugal filters.

Size exclusion chromatography: Ni-NTA purified, buffer-exchanged and concentrated proteins were loaded into Superdex 75 10/300 GL column (GE Healthcare Life Sciences) equilibrated with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20% (v/v) glycerol and 0.5 mM TCEP. The fractions including the protein were collected and concentrated for further analysis.

Differential Scanning Fluorimetry

Protein concentration of 3 μM was used for the measurement of the melting temperature (Tm). Reactions were done in the buffer used in the SEC. Addition of other components (ATP, salts) were taken into account by including 2X buffer, if needed. Signal arising from the fluorescent dye Life Technologies SYPRO^{TM} Orange Protein Gel Stain, 4X working solution from 5000X concentrate in DMSO) was measured with a conventional Real-Time PCR system (Bio-Rad CFX), 1°C per min from 4 to 96°C. Results were transferred and normalized in Microsoft Excel and the Tm was derived from the sigmoidal function using GraphPad Prism version 5.02 for Windows, GraphPad Software, La Jolla California USA, https://www.graphpad.com/.

MANT-ATP Binding Assay

The steady-state fluorescence of MANT-labeled ATP was measured as described in Mysore et al. (34). Briefly, the fluorescently labeled MANT (2′/3′-(N-methyl-anthraniloyl)-ATP was titrated against 1.5 μM recombinant JAK3 JH2. In addition, the protein was titrated as 0, 0.05, 0.25, 1.25, and 6.26 μM against 0.25 μM MANT-ATP. FRET signal was measured with QuantaMaster PTI Fluorometer and the data was analyzed using GraphPad Prism version 5.02 and Microsoft Excel. Protein titrations were done in duplicate and in MANT-ATP titration in triplicate.

Fluorescence Polarization Assay

Fluorescence polarization measurements were performed in black 384-well plates (ProxiPlate-384 F Plus, PerkinElmer) at sample volume of 5 μl/well on PerkinElmer Envision plate reader using 480 nm excitation and 535 nm emission filters. Fluorescent tracer, Bodipy FL labeled JNJ-7706621 [compound 5 in (35)] was used at 1.5 nM concentration. Recombinant JAK1 JH2 553-836-His, JAK2 JH2 503-827-His, JAK3 JH2 511-790-His, and TYK2 JH2 564-876-His proteins were used at concentrations (2 nM JAK1, 150 nM JAK2, 1 μM JAK3, 20 nM TYK2) dependent on protein-tracer dissociation constants. The assays were performed in a buffer consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 20% glycerol, 0.01% Brij-35, and 2 mM DTT. ATP was titrated at concentration range of 5 nM−5 μM and fluorescence polarization values obtained were fitted against log[ATP] in GraphPad Prism to yield IC50 values. Assays were performed in triplicate.

Plasmids and Mutagenesis

C-terminally HA-tagged full length JAK1, JAK3, and STAT5 cloned into pCIneo mammalian expression plasmid were used for transfections. Mutations into JAK1 and JAK3 were introduced by site-directed mutagenesis using QuikChange-protocol with specifically designed primers. The method was also used for the domain deletion constructs.

Cell Culture, Transfection, and Immunoblotting

JAK1 and JAK3 deficient U4C fibrosarcoma cells stably expressing IL-2Rγ and -β receptors were kindly provided by Dr. Claude Haan, (Life Sciences Research Unit, Signal Transduction Laboratory, University of Luxembourg, Luxembourg, L-1511, Luxembourg) and Hans-Günter Zerwes (Novartis Institute for Biomedical Research). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% pen strep and 1% glutamine and antibiotics: puromycin, zeozin and blasticidin for maintaining the IL-2Rγ and -β expression.

Transient transfections were done with full-length or mutant human JAK1-HA (75 ng), human JAK3-HA (75 ng) and human STAT5A-HA (1.5 ng) using FuGENE HD (Promega) according to the manufacturers' instructions. After 34 h cells were starved in serum-free medium overnight and stimulated for 15 min with 100 ng/ml of human IL-2. After stimulation, cells were lysed into Triton-X cell lysis buffer and centrifuged, and the supernatant used for SDS/PAGE and immunoblotting. Blots were double-stained with phosphospecific antibodies (P-Stat5 (Y694), Cell Signaling Technology and anti-phospho-JAK1 (Tyr1022/Tyr1023), Millipore) and anti-HA (Aviva Systems Biology OAEA00009) and detected with a mix of IRDye-labeled secondaries. The STAT5 (HA) signals were used for normalization of phospho-STAT5 levels. Blots were quantified using a LI-COR Odyssey CLx imaging system. A minimum of three independent experiments were performed for each condition.

JAK3 Homology Modeling

A JAK3 homology model was generated using the SWISS-MODEL server with the TYK2 structure (Protein Data Bank identification 4OLI) as the modeling template. Graphical presentations were generated using the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA).

Luciferase and Dual Luciferase Assays

STAT5 transcriptional activity was assessed by measuring the luciferase expression (SPI Luc 2) driven by a STAT5

responsive promoter from growth hormone regulated serine protease inhibitor, as described previously (27). U4Cγβ cells were transfected with indicated DNA constructs including SPI-Luc2 and β-galactosidase reporter plasmids. The latter was co-transfected as an internal transfection control. Transient transfections were done in 96 well plates with FuGENE HD (Promega) according to manufactures instructions. 42 h after transfection, cells were stimulated (in starvation media) or starved for 5 h after which luciferase assays were analyzed using the dual luciferase reporter assay system (Promega) according to manufactures instructions. Luciferase values were measured with EnVision 2104 Multilabel Reader (Perkin Elmer). The results are presented as relative luciferase activity (RLU) corresponding to the firefly luciferase light emission values divided by renilla luciferase light emission values.

In addition to Renilla luciferase (pRLTk; Promega), the β-galactosidase reporter was also used, and similar results were obtained with both reporters. Briefly, cells were washed twice with PBS and 20 μl of 1X Luciferase lysis buffer was added to the wells. 10 μl of the lysate was used for luminescence measurement with 75 μl luciferase reagent. β-galactosidase signal was measured by adding 50 μl of ONPG per well and the absorbance signal values were used for normalization of the luciferase luminescence values.

In vivo Studies in Zebrafish

Embryos derived from *jak3*$^{+/-}$ in-crosses were injected with 100 pg/nl *in vitro* transcribed capped mRNA encoding zebrafish JAK3 wild-type (WT), and M511I, I535F and M511+I535F mutants. At 5 dpf they were fixed and subjected to whole-mount *in situ* hybridization (WISH) with anti-sense *rag1* probe and imaged and quantified as described (36), with only *jak3*$^{-/-}$ embryos, as determined by post-WISH genotyping, used in the analysis. In other experiments, embryos from *lck*::GFP zebrafish were injected and rag1 expression analyzed using RT-PCR.

RESULTS

Both JH1 and JH2 in JAK1 and JAK3 Are Required for Functional IL-2–STAT5 Signaling

In order to investigate the functional role of JAK3 JH2 in IL-2R signaling, we used the human fibrosarcoma U4Cγβ cell line that stably expresses IL-2Rγ and IL-2Rβ but lacks expression of JAK1 and JAK3 (11). A functional IL-2R signaling cascade was reconstituted by ectopic expression of JAK1 and JAK3, which allowed analysis of JAK1/JAK3 mutants. Optimization of experimental conditions showed that expression of JAK1 alone induced strong basal activation of STAT5-driven luciferase reporter SPI-Luc2 (27), which was, however, unresponsive to IL-2 stimulation (**Supplementary Figure 1A**). Expression of JAK3 alone showed no basal STAT5 transcriptional activity and no responsiveness to IL-2. IL-2-induced STAT5 activation was achieved only by expression of both JAK1 and JAK3, which is in line with previous studies (3, 11, 37).

Immunoblot analysis of STAT5 Y694 phosphorylation (pSTAT5) from U4Cγβ cells transfected with JAK1 and JAK3 concurred with STAT5 transcriptional activity showing IL-2-independent phosphorylation by JAK1 alone, lack of pSTAT5 by JAK3 alone and requirement for both JAK1 and JAK3 for IL-2-responsiveness (**Supplementary Figure 1B**). Collectively these results imply JAK1 as the immediate downstream effector kinase and JAK3 as a regulator/activator in the IL-2R signaling complex, thus confirming previous reports (3, 11, 37). However, the underlying molecular mechanisms and regulatory domains in JAK1–JAK3 heterodimer signaling are still largely unknown and, in this context, we wanted to analyze specifically the roles of JH2s.

In order to investigate the roles of different JH domains we produced deletion constructs for JH1 and JH2 domains in JAK1 and JAK3 (**Figure 1**) In accordance with the critical role of JAK1 kinase activity in IL-2R–STAT5 signaling (see above), deletion of JH1 in JAK1 (JAK1 ΔJH1) abolished both basal and IL-2-induced pSTAT5 and STAT5 reporter activity. Similarly, deletion of JH1 in JAK3 abolished IL-2-inducibility but retained basal activity mediated by JAK1. Interestingly, deletion of JH2 in JAK1 also completely abrogated IL-2R signaling activity, and in JAK3 deletion of JH2 or both JH1 and JH2 prevented IL-2-inducibility without affecting basal activity (**Figures 1B,C**). These results indicate that, in addition to the kinase domains, the pseudokinase domains in JAK1 and JAK3 are also required for IL-2R signaling.

Next, we extended our studies on the regulatory function of JH2 to clinical JAK3 mutations. As the structure of JAK3 JH2 has not been solved, we produced a homology model based on the TYK2 JH2-JH1 structure (Protein Data Bank (PDB) code: 4OLI, see **Figure 2A**) (27). We analyzed pathogenic inactivating mutant C759R found in SCID patients as well as activating JAK3 mutants M511I and R657Q in JH2 and L857Q in JH1 (list of mutations; see **Table 1**). The activating mutants are found in T-ALL patients and reported to be constitutively active in cellular assays and result in leukemic phenotypes in mouse models (3). Mutations were studied in the IL-2–STAT5 pathway in U4Cγβ cells co-expressed with wild-type JAK1. The JAK3 C759R inhibited basal and IL-2-induced STAT5 activation as efficiently as the kinase-inactive JAK3 JH1 mutation K855A (**Figure 2B**). JAK3 M511I and R657Q showed similar, cytokine-independent STAT5 activation as the JH1 L857Q mutant (**Figures 2B,C**, **Supplementary Figure 2C**).

Most activating JAK3 mutants have been shown to depend on JAK1 for signaling (3), and we sought to define the JAK1 domains required for constitutive IL-2 signaling. Activating JAK3 mutants were transfected with either wild-type JAK1, JAK1 ΔJH2 or JAK1 ΔJH1 into U4Cγβ cells. All tested JAK3 mutants residing in either JH1 or JH2 were found to depend on normal JAK1 activity and showed increased basal STAT5 activation only in the presence of full-length JAK1 (**Figure 2B**, **Supplementary Figure 2A**); deletion of either JH1 or JH2 of JAK1 completely abrogated signaling as measured by pSTAT5 and STAT5 luciferase assays (**Figure 2C**, **Supplementary Figures 2B,C**). Taken together, these data show that the analyzed oncogenic JAK3 JH2 and JH1 mutations require both JH1 and JH2 domains of JAK1 for constitutive signaling.

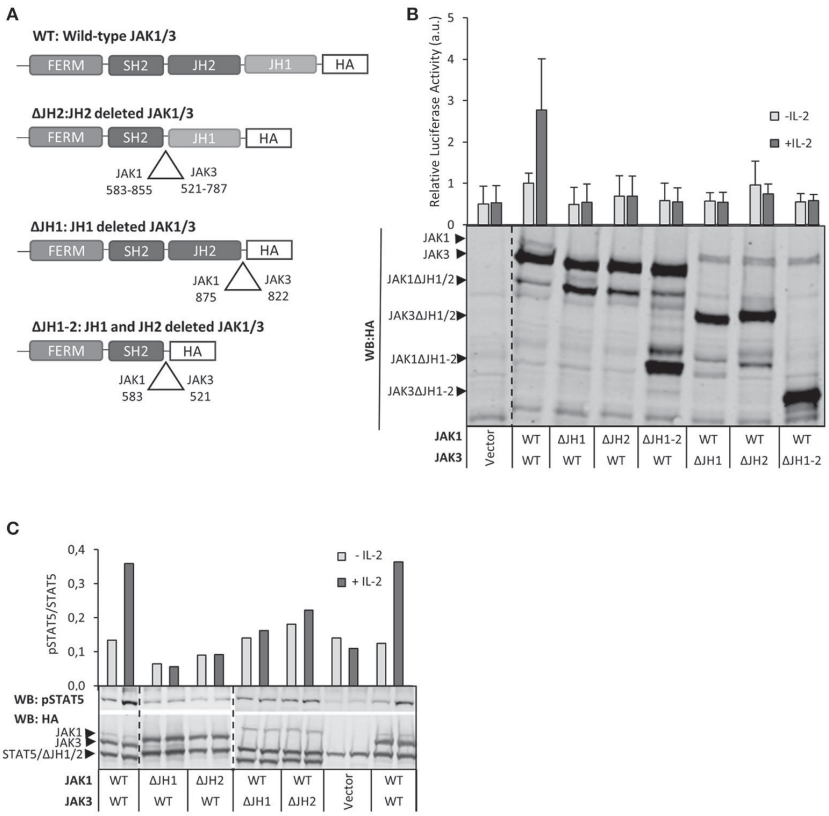


FIGURE 1 | Both JH1 and JH2 in JAK1 and JAK3 are required for functional IL-2 signaling. **(A)** Schematic presentation of JAK1 and JAK3 wild-type (WT) and domain deletion constructs. **(B)** Both JH1 and JH2 of JAK1 and JAK3 are required for IL-2 dependent STAT5 activation. U4Cγβ cells were transfected with JAK1 WT, JAK3 WT and domain deletion constructs together with STAT5 responsive reporter SPI-luc2 (28), either with Renilla or pRL-TK control vector (see Materials and Methods) and starved/stimulated. Similar results were obtained with both control vectors. Data shows averages and SD from normalized values combined from three independent experiments, each having triplicate samples. The expression of JAK1/JAK3 constructs was analyzed by anti-HA Western blotting. **(C)** IL-2-induced pSTAT5 requires both JH1 and JH2 in JAK1 and JAK3. STAT5 phosphorylation and expression of JAK1, JAK3 and STAT5 were detected from cell lysates from transfected U4Cγβ cells by immunoblotting with HA- and pTyr694 STAT5 antibodies. Similar results were obtained in three independent experiments.

Characterization of Recombinant JAK3 JH2 Shows a Domain Capable of Tight, Cation-Independent Nucleotide Binding

Pseudokinases are generally considered to be inactive kinases but an estimated 30–40% of pseudokinases, including JAK1, JAK2 and TYK2 JH2, bind nucleotides (21). The function of JAK3 JH2 has not been analyzed and for biochemical characterization recombinant JAK3 JH2 (residues 511–790 with C-terminal His_6-tag) was expressed in insect cells and purified with affinity chromatography followed by size-exclusion chromatography (**Supplementary Figures 3A,B**). The protein was analyzed by differential scanning fluorimetry (DSF) for its ability to bind adenosine nucleotides (**Figure 3A**). The melting temperature (Tm) of JAK3 JH2 (apo) was as low as 32.4 ± 0.2°C, indicating inherent lability of the domain but Tm was significantly increased by addition of ATP (ΔTm 8°C with 0.2 mM ATP, 12°C with 1 mM ATP). Supplementing the buffer with either Mg^{2+}, Mn^{2+} or Ca^{2+} (in the form of 2 mM of their chloride salts) alone or with ATP did not have a significant effect on the Tm. Adding the monovalent cation salt KCl did not affect the Tm, which was expected as the buffer contained monovalent NaCl. As other JAK JH2s have been shown to require salts to bind ATP, we tested all JAK JH2s with 500 μM ATP with, or without $MgCl_2$ (**Supplementary Figure 4A**) and confirmed, that the Tm of JAK1, JAK2 and TYK2 JH2s increase only if $MgCl_2$ is added with ATP, while JAK3 JH2 has higher ΔTm without Mg^{2+} (ATP only). The cation-independency of ATP binding was confirmed by adding 2 mM of the chelating agent EDTA to the buffer, which did not affect the Tm for JAK3 JH2 ATP (**Figure 3B**).

Measuring the affinity of JAK3 JH2 binding to the fluorescent ATP-analog MANT [2′-(3′)-O-(N-methylanthraniloyl)]-ATP

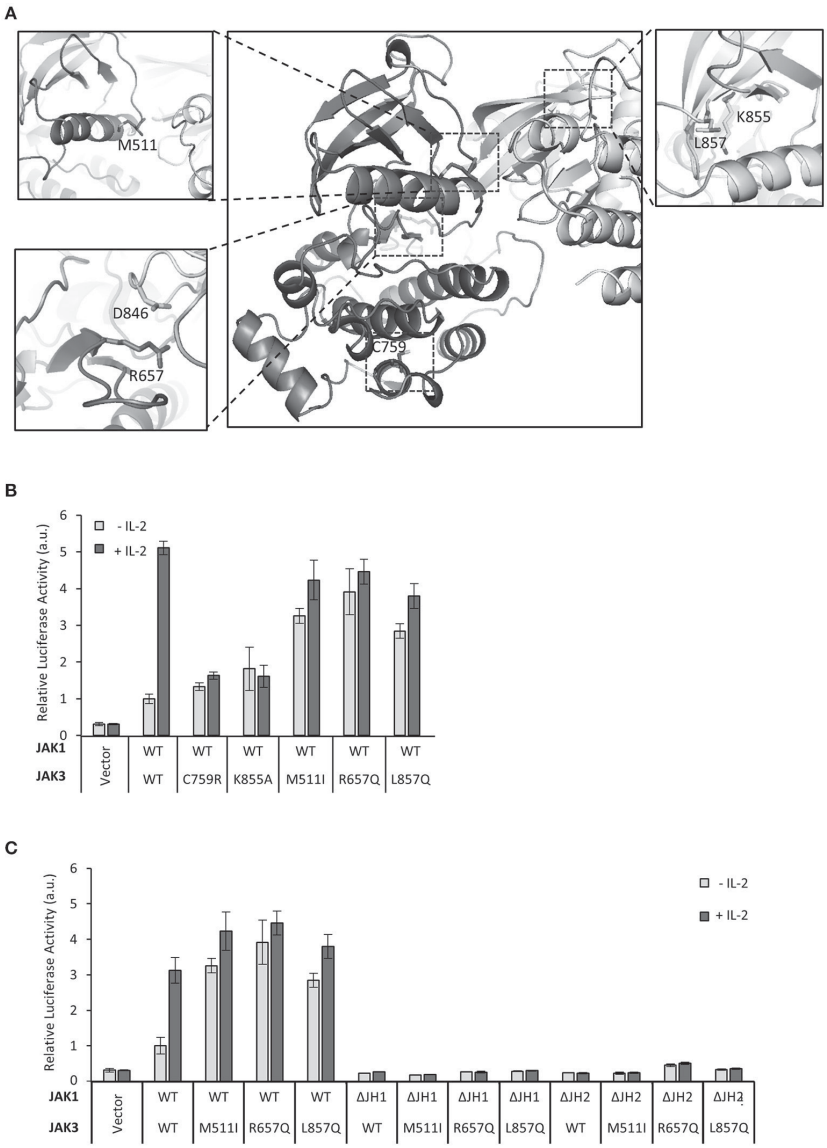


FIGURE 2 | Mutations in JAK3 JH2 can alter the function of the IL-2 pathway. **(A)** Localizations of the JAK3 mutants used in this article. Homology model of JAK3 JH2-JH1 (based on the TYK2 structure in Protein Data Bank (PDB) code: 4OLI) and the JAK3 JH1 structure [PDB code: 1YVJ (38)] were aligned with the JH1-JH2 structure of TYK2 (PDB code: 4OLI). JH2 is shown in red and JH1 in gray. Close-ups of the mutations are shown in subpanels. C759 is shown in the main picture. In the lower left panel, the (possible) interaction pair R657–D864 (in JH1) is shown. In the right panel, ATP was aligned from ATP-bound form of PKA [PDB code: 1ATP (39)]. **(B)** JAK3 JH2 C759R mutation efficiently inhibits IL-2 signaling and activating mutations in JAK3 JH2 and JH1. Luciferase assay of STAT5 activation in U4Cγβ cells transfected with JAK1 WT and JAK3 point mutations, SPI-luc2 reporter and β-galactosidase control. Values were normalized to JAK1 WT and JAK3 WT transfected sample Errors bars show SD of triplicate samples. Similar results were obtained using pRL-TK as a control. **(C)** Activating JAK3 mutants rely on both JH1 and JH2 of JAK1 for constitutive downstream signaling. SPI-luc2 reporter was used to detect the activation of STAT5 in U4Cγβ cells. Cells were transfected with WT or domain deletion JAK1 and WT or constitutively active JAK3 mutants. β-galactosidase plasmid was used as an internal control for the transfection efficiency. Values were normalized to WT JAK1 and JAK3. Error bars are SD of triplicate samples.

TABLE 1 | Mutations used in this study.

JAK	Mutation	Domain	Effect	References
JAK3	M511I	JH2	Found in T-ALL patients, constitutively active	(3)
	R657Q	JH2	Found in T-ALL patients, constitutively active	(3)
	L857Q	JH1	Found in T-ALL patients, constitutively active	(3)
	C759R	JH2	Found in SCID patients, inhibits JAK3 kinase activity and reduces STAT5 activity	(31)
	K855A	JH1	Catalytic lysine, kinase dead	
	K566A	JH2	Homologous to K855A, disrupts ATP binding to JH2	
	I535F	JH2	Homologous to JAK2 I559F, disrupts ATP binding to JH2	(24)
JAK1	V658F	JH2	Homologous to JAK2 V617F, constitutively active	(17)
	I535F	JH2	Homologous to JAK2 I559F, disrupts ATP binding to JH2	(24)

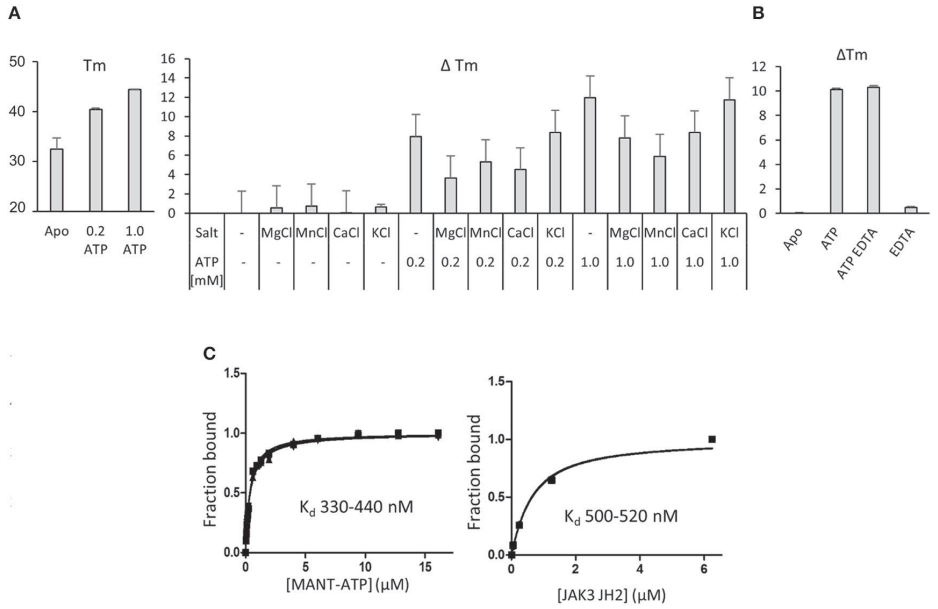


FIGURE 3 | Nucleotide binding to recombinant JAK3 JH2. **(A)** JAK3 JH2 binds ATP without cations. The effect of ATP on Tm of JAK3 JH2 is shown in the left panel. The following graph shows changes in the melting temperature (ΔTm) in JAK3 JH2 in the presence of different cations and ATP. **(B)** Chelating agent does not affect ATP-induced increase on Tm of JAK3 JH2. DSF analysis performed in the presence of ATP (1 mM) and chelating agent EDTA (2 mM). **(C)** MANT-ATP binding assay with recombinant JAK3 JH2. Both protein and MANT-ATP were titrated and both experiments gave similar Kd in the sub-micromolar range. Left panel: titrating MANT-ATP to 1.5 μM JAK3-JH2. Kd range 330–440 nM. Right panel: titrating JAK3 JH2 to 0.25 μM MANT-ATP, Kd 500 nM. Protein concentrations were 0; 0.05; 0.25; 1.25 and 6.25 μM. In both figures, the Y-axis is the fraction of ligand-bound protein in relation to the total protein amount ([PL/Ptot]). The MANT-ATP titration was repeated three times and all experiments are plotted in the lower left panel. Protein titration was repeated twice and both experiments are blotted in the figure.

showed that JAK3 JH2 binds MANT-ATP with a dissociation constant (Kd) of 0.4 μM (**Figure 3C**). Similar values were obtained by titration of the ligand to a constant amount of protein and *vice versa*. IC50 values was determined using fluorescent polarization assay (FP), by competition assay, where ATP competes with ATP pocket binding tracer. The IC50 values for JAK JH2s were compared, and the IC 50 for JAK3 JH2 was 16 μM (without $MgCl_2$), indicating tighter ATP-binding affinity for JAK3 JH2 compared to other JAK JH2s (**Supplementary Figure 4B**, **Table 2**). Approximation of the JAK3 JH2 Kd was further with DSF, where 50 nM−1 mM ATP was titrated to 3 μM protein. The value was higher than measured with MANT-ATP, namely 40.4 ± 97.1 μM (**Supplementary Figure 4C**). However, the variation was wide and, as there was no saturation seen when the values were fitted, no Kd was obtained for other JAK JH2s

(**Supplementary Figure 4C**). The differences in the magnitude of the Kd and IC50 values stems from the methods used to evaluate the affinity, e.g., the fact that MANT-ATP uses modified ATP that may affect the binding properties of the molecule. However, both the MANT-ATP and FP methods showed that JAK3 JH2 binds ATP tighter compared to other JAK JH2s, and the binding is cation-independent. Furthermore, the effect of ATP binding to the stability of JAK3 JH2 was greater compared to other JAK JH2s (**Supplementary Figure 4C**, **Table 2**).

Finally, we investigated the binding of ADP and AMP to JAK3 JH2 (**Supplementary Figure 4D**). ADP showed a significantly reduced ΔTm compared to ATP, and AMP showed no effect on Tm indicating that JAK3 JH2 does not bind AMP and prefers ATP over its de-phosphorylated counterparts. An autophosphorylation assay with radiolabeled ATP did not show incorporation of ^{32}P into JAK3 JH2, suggesting that JAK3 JH2 does not possess catalytic activity (**Supplementary Figure 4E**).

Mutating the JAK3 JH2 ATP-Binding Site Reverts the Oncogenic JAK3 Hyperactivation

Given that JAK3 JH2 has a functional nucleotide-binding site, we wanted to analyze the functional role of ATP binding to JAK3 JH2 in IL-2 signaling. The JAK3 JH2 model identified two residues in the ATP-binding pocket conserved between JAKs (**Figure 2A**) and suited for mutagenesis. We thus mutated I535 in the β2-sheet of the JH2 N-lobe to a bulkier phenylalanine to sterically inhibit ATP binding. The corresponding JAK2 mutation (JAK2 I559F) has been shown to inhibit ATP binding without affecting the stability of JAK2 JH2 (24). We also mutated the conserved JAK3 JH2 β3 lysine K556, to alanine. The activities of the mutant JAK3 were analyzed in U4Cγβ cells with STAT5-responsive reporter and pSTAT5 analysis. Neither mutation disrupted responsiveness to IL-2 stimulation and the signaling properties resembled wild-type JAK3 (**Figure 4**).

Inhibition of ATP binding to JH2 by mutations or small molecular weight (smw) inhibitors has been demonstrated to abrogate pathogenic hyperactivation of JAK2 and cytokine signaling via TYK2, respectively (23, 24). To assess the effect of JH2 nucleotide binding in constitutively active JAK3, we constructed double mutants consisting of an activating mutant (M511I, R657Q, or L857Q; see **Figure 2**) and either of the ATP binding mutants (I535F or K556A). The constructs were transiently transfected and analyzed for STAT5 activation (SPI-luc2 reporter) and pSTAT5 (immunoblotting) (**Figures 4A,B,D**, **Supplementary Figure 5A**). In both assays the JH2 ATP-binding site mutations reduced STAT5 activation back to or below wild-type JAK3 levels while retaining cytokine-responsiveness. The sole exception was JAK3 L857Q+K556A, which showed strongly reduced activation by cytokine as well. The more conservative sterically hindering ATP-binding site mutation I535F, however, resulted in wild-type-like STAT5 activation in the L857Q context. IL-2-responsiveness was also regained in M511I+I535F/K556A and R657Q+I535F/K556A (**Figures 4A,B**). Notably, these results are distinct from complete removal of JAK3 JH2 (ΔJH2, see **Figure 1**) or removal of JH1 kinase activity by the JAK3 JH1 β3 lysine-to-alanine mutation JAK3 K855A, which resulted in unresponsiveness to cytokine, even when combined with the activating M511I mutation (**Figure 4D**).

In order to investigate the mechanism of suppression in JAK3 oncogenic signaling by the JH2 ATP-binding mutants, we analyzed phosphorylation of the JAK1 activation loop tyrosine residues. The JH2 ATP binding mutation in JAK3 double mutants reduced JAK1 activation loop phosphorylation compared to activating mutations alone (**Supplementary Figure 5C**).

The effect of JH2 ATP binding on JAK3 oncogenic mutants was also analyzed using zebrafish (*Danio rerio*). Expression of *rag1*, an IL-2Rγc/JAK3-dependent marker for mature lymphoid cells, was analyzed in JAK3-deficient embryos injected with mRNA encoding JAK3 WT, M511I, I535F or the combined M511I + I535F mutant mRNA as described (36). Each produced a range of *rag1* expression, but both M511I and I535F elicited a small but reproducible enhancement in the mean *rag1* expression compared to WT, which was significantly reduced in the ATP binding deficient M511I + I535F double mutant (**Supplementary Figure 6**). A similar reduction in *rag1* expression was observed with the double mutant compared to M511I in qPCR experiments performed on embryos from transgenic *lck*::GFP zebrafish injected with JAK3 WT, M511I and M511I + I535F mRNA (data not shown). In contrast, the presence of the I535F mutation did not ablate the induction of *rag1* expression by JAK3 WT in either case (**Supplementary Figure 6** and data not shown).

Together, the data indicate that disrupting ATP binding to JAK3 JH2 preserves IL-2-responsiveness but decreases constitutive STAT5 activation, probably by reducing the activating potential of JAK3 to JAK1.

JAK3 JH2 ATP-Binding Mutations Do Not Inhibit Activation by Pathogenic JAK1 Mutations

We have previously shown that JH2 ATP-binding mutations suppress activating mutations in homomeric cytokine receptor signaling in the same JAK molecule (24). Using the IL-2–JAK1/JAK3 signaling system, we wanted to study the effect of JH2 ATP binding in heterodimeric JAK signaling by assessing whether inhibition of ATP binding in one JAK JH2 could suppress activation by hyperactivating mutations in the other JAK in *trans*. We co-transfected JAK1 JH2 ATP mutant I597F (corresponding to JAK3 I535F) with JAK3 activating mutation M511I, or JAK3 JH2 I535F mutant with the activating JAK1 T-ALL-associated mutation V658F (analogous to the most prevalent MPN-inducing JAK2 mutation V617F) (40). Similar amounts of wild-type and mutated JAK3 and JAK1 proteins were expressed as quantified by western blotting (**Supplementary Figure 5B**).

JAK1 JH2 mutant I597F, when co-transfected with JAK3 M511I, did not reduce (hyper)activation and thus did not restore cytokine responsiveness (**Figure 5A**). In the reverse experimental setting, the JH2 ATP pocket mutation JAK3 I535F could not reduce the activation of constitutively active JAK1 V658F. Together these data suggest that JH2 ATP-site mutations exert their direct suppressing function in *cis* and cannot suppress

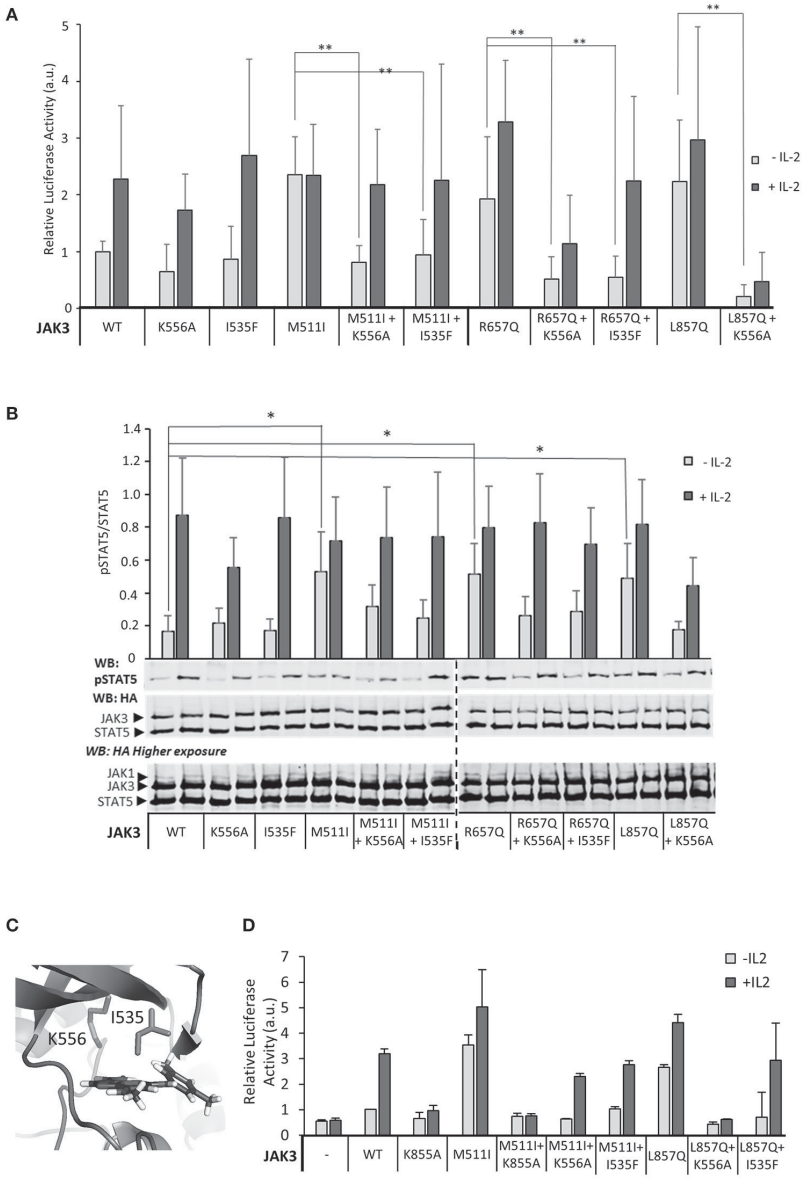


FIGURE 4 | Inhibition of ATP binding to the pseudokinase domain of JAK3 reverts pathogenic, ligand-independent JAK3 signaling, while retaining cytokine-responsiveness. **(A)** JAK3 JH2 ATP-binding site mutations suppress activating JAK3 mutants. Luciferase assay was performed in U4Cγβ cells transfected with JAK1 and the indicated JAK3 mutants using β-galactosidase as a transfection control. The results are from three independent experiment, each having triplicate samples ($n = 9$, expect for K556A $n = 7$). Similar results were obtained with pRL-TK control. Data is normalized to the basal wild-type sample, errors are presented as SD. Statistical analysis of the activating mutations, and their corresponding double mutants were done with two-tailed *t*-test using unequal variances. $^{**}p < 0.001$. **(B)** Representative Western blot of the transiently transfected U4Cγβ cell lysates showing the pSTAT5 signal. HA-tagged STAT5 was co-transfected with HA-tagged JAK1 and JAK3 and the lysates were blotted with anti-HA and anti-pSTAT5. Lower panel: Quantitative pSTAT5/STAT5 signal analysis. Activation of JAK3 signaling by M511I, R657Q and L857Q is significant ($^{*}p < 0.05$). Data is average of five experiments. **(C)** The localization of the mutated JAK3 JH2 ATP-binding residues. Homology model of JAK3 JH2 (based on TYK2 structure from PDB, code: 4OLI), ATP is shown in gray. **(D)** Kinase-inactive JAK3 K855A abolishes IL-2 signaling in the presence of the activating JAK3 mutation M511I. Luciferase assay with STAT5-responsive promoter and with pRL-TK control.

TABLE 2 | Comparison of JAK JH2s.

JAK JH2	Phospho transfer activity	Kd (MANT-ATP)* (References) [μM]	IC50(FP)* [μM]	Tm_{Apo} [°C]	Tm_{ATP} [°C]	ΔTm (1 mM ATP) [°C]	Cations
JAK2	Weak	1.3 (24)	102	43	46	4.9	Yes
JAK1	No	3.1 (24)	110	46		3.9	Yes
TYK2	No	15 (23)	471	48	48	0.9	Yes
JAK3	No	0.5	16	32	41	9.9	No

**Kd (MANT-ATP) and IC50 values are shown with $MgCl_2$ for JAK1, JAK2 and TYK2 and no $MgCl_2$ added for JAK3 JH2.*

activation caused by mutations in the other JAK of a heteromeric JAK pair. Mutants disrupting the JH2 ATP-binding possibly function by lowering the kinase activity of JH1 though lowering the stability of the JH2 αC, which interacts with JH1 (24, 32).

To further investigate JAK1–JAK3 interplay on IL-2R, we analyzed the loss-of-function JAK3 SCID mutant C759R (see **Figure 2A**). The JAK3 C759R mutation completely abrogated signaling of wild-type JAK1 but still failed to inhibit signaling by the hyperactive JAK1 V658F (**Figure 5B**). Comparable JAK1 and JAK3 expression levels between the mutants and wild-type were confirmed by immunoblotting the samples (**Supplementary Figure 5B**).

DISCUSSION

Although JAKs have been intensively studied since their discovery in the late 1980s and early 1990s, many of their molecular regulatory mechanisms are still not fully understood. This notion is particularly true for JAK3, which has nonetheless become a relevant drug target. Importantly, critical information of its regulation is lacking, and for example, structural data exists only for the JAK3 JH1 domain (41). In this study, we focused on characterization of JAK3 JH2 and its function in the context of both normal IL-2R signaling and oncogenic signaling mediated via activating JAK3 mutations. We found that in addition to the well-established functions of the respective JH1s, the JH2s of JAK3 and JAK1 are also required for IL-2 signaling and for hyperactivation of JAK1 and JAK3 oncogenic mutants. Biochemical analysis of recombinant JAK3 JH2 showed that the domain binds ATP but differs from other JAK JH2s in terms of a lower initial Tm, more robust stabilizing effect by ATP, and cation-independency of the nucleotide binding. Furthermore, our cellular signaling analysis suggests that disrupting the ATP-binding site of JAK3 JH2 can inhibit activation caused by JAK3 gain-of-function mutations.

Our DSF results indicate that JAK3 JH2 binds ATP in a cation-independent manner. In canonical protein kinases such as PKA, Mg-ATP binds in the cleft between the N and C lobes and the two Mg^{2+} ions are coordinated by an aspartic acid in the conserved DFG motif (21). However, pseudokinases are known to possess non-canonical nucleotide binding modes and JAK2 and TYK2 JH2s bind ATP with a single cation coordinated by an upstream asparagine (N678 in JAK2 JH2) (19). Interestingly, this asparagine is conserved also in JAK1, JAK2 and TYK2 JH2s, as well as in the zebrafish (danio rerio) JAK3, but is replaced by a positively charged lysine (K652) in JAK3 JH2 (**Figure 6**). This difference can explain the cation-independency of ATP binding in JAK3 as it would replace the need for a positively charged cation in coordinating the negatively charged phosphates of ATP. A few other pseudokinases with cation-independent ATP-binding modes have been characterized (19, 21). Of these, MLKL, STRADα, and EphB6 are catalytically inactive, while CASK has been reported to possess phosphotransfer activity (21, 42). Furthermore, ULK4 has been shown to bind nucleotides without cations, but its kinase activity has not been characterized (21). MLKL, STRADα, EphB6 and CASK have divergent sequences in place of the conventional cation-binding DFG motif, namely GFE, GLR, RLG, GFG (all conserved between human and mouse), respectively. In all JAK JH2s, this motif is DPG. In addition, the residue corresponding to JAK3 K652 varies between the above-mentioned proteins: human MLKL has conserved the typical Asn, while ULK4 has a Lys residue similar to JAK3 JH2 and CASK, EphB6 and STRADα all have different residues (Cys, Ser and His, respectively, see **Figure 6**). These examples illustrate the highly divergent mechanisms for ATP binding that have evolved in pseudokinases (19, 21).

Characterization of recombinant JAK3 JH2 showed an unstable protein with low Tm, but 3-, 6- and 30-fold tighter MANT-ATP binding compared to JAK2, JAK1, and TYK2 JH2s, respectively. A similar trend was also seen in IC50 values derived from a fluorescence polarization competition assay in which a fluorescently-labeled small molecule probe is competed off with unlabeled ATP. In addition, the effect of ATP on the protein stability was considerably larger in JAK3 than in other JAKs (**Table 2**). Based on the high ATP-binding affinity and lack of kinase activity in JAK3 JH2 (**Supplementary Figure 4E**), we speculate that ATP binding has a mostly structural role in JAK3 JH2. Furthermore, failure to produce recombinant JAK3 JH2 with mutations designed to disrupt ATP binding (and the success in production of the JAK3 JH2 with the activating R657Q mutation) also indirectly support the notion of a stabilizing effect for ATP (**Supplementary Figure 3C**).

To assess the effects of ATP binding to JAK3 JH2 on cellular signaling, we constructed mutations into the JH2 ATP binding pocket of full-length JAK3 targeting residues I535 and K556 (see **Figures 2**, **4**). JAK3 JH2 ATP-binding site mutations

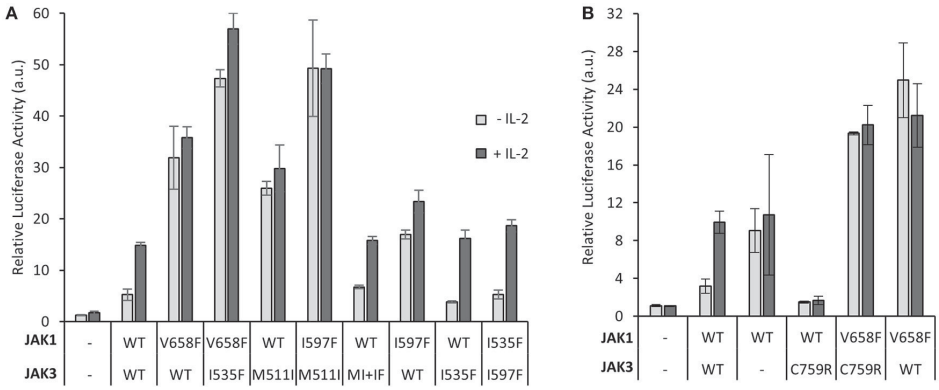
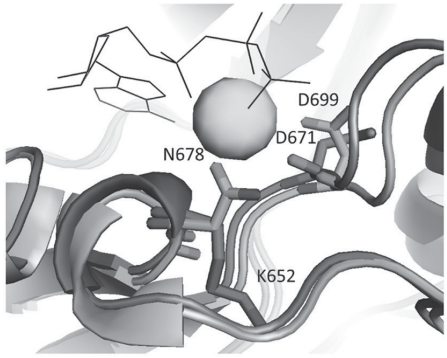


FIGURE 5 | Disrupting the JAK3 JH2 nucleotide binding site inhibits JAK3-induced, but not JAK1-induced, hyperactivation, and *vice versa*. **(A)** Constitutive activation of STAT5 caused by oncogenic JAK3 mutations are suppressed only by ATP-binding site mutations in JAK3 and not its dimerization partner JAK1. Luciferase assay of U4Cγβ cells transiently transfected with JAK1, JAK3, SPI-luc2 and pRL-TK. Results are average from two separate experiments each with triplicate or duplicate (I597F+I535F and I597F+WT JAK3) samples. The results are normalized to the JAK1 WT and JAK3 WT sample. Error bars show the SD. **(B)** Inhibitory JAK3 SCID mutation C759R does not reduce oncogenic hyperaction of JAK1-mutant. Luciferase assay in U4Cγβ cells transiently transfected with JAK1, JAK3, SPI-luc2, and pRL-TK. Error bars are SD of triplicate samples and the data is representative of three independent experiments. **Supplementary Figure 5B** shows immunoblotting for protein expression levels.



	K652	DFG
JAK3 Homo sapiens	NVSARKVLLAREG	DPG
JAK3 Mus musculus	NVSARKVLLAREG	DPG
JAK3 Danio rerio	NICAKNLLLVREG	DPG
JAK1 Homo sapiens	NVCTKNLLLAREG	DPG
JAK1 Mus musculus	NVCTKNLLLAREG	DPG
JAK2 Homo sapiens	NVCAKNILLIREE	DPG
JAK2 Mus musculus	NVCAKNILLIREE	DPG
TYK2 Homo sapiens	NVCGRNILLARLG	DPG
TYK2 Mus musculus	NVCGRNILLARLG	DPG
CASK Homo sapiens	DVKPHCVLLASKE	GFG
CASK Mus musculus	DVKPHCVLLASKE	GFG
MLKL Homo sapiens	KIRSSNFLVTQGY	GFE
MLKL Mus musculus	NISSSSFLVAGGY	GFE
EPHB6 Homo sapiens	SLSAHSVLVNSHL	RLG
EPHB6 Mus musculus	ALSARSVLVNSHL	RLG
STRADA Homo sapiens	SVKASHILISVDG	GLR
STRADA Mus musculus	SVKASHILISTDG	GLR
ULK4 Homo sapiens	DISPRKILLEGPG	DNG
ULK4 Mus musculus	DLSPQKILLEGPG	DSG

FIGURE 6 | Left: Homology model of JAK3 JH2 aligned with the crystal structure of JAK2 [PDB code: 4FVQ (25)]. JAK3 is shown in red and JAK2 in orange. ATP is shown as black shits and the Mg^{2+} present in the JAK2 JH2 structure as a green sphere. **Right**: An alignment of human and mice sequences of JAKs and several other pseudokinases for Mg^{2+} coordination, JAK3 K652, which is conserved as N in other JAKs, and is important for Mg^{2+} coordination, is highlighted. Also the canonical DFG motif are compared and show variation between the pseudokinases.

efficiently suppressed ligand-independent STAT5 activation by JAK3 ALL-mutants R657Q, M511I in JH2, as well as L857Q in JH1 (**Figure 4**). The suppressing effect of the I535F mutant was further studied in the zebrafish model, where the double mutant (M511I + I535F) induced lower *rag1* expression compared to the M511I mutant (**Supplementary Figure 6** and data not shown). In contrast to the kinase-inactive form of JAK3 (K855A), which lowered both ligand-independent basal activity and IL-2 induced signaling, the JH2 ATP-binding site mutations only suppressed ligand-independent STAT5 activation, while retaining IL-2-dependent STAT5 activation. The only exception to this was the double mutant L857Q + K556A, which showed lowered signaling capacity for as of yet unknown reasons.

Based on homology modeling the JAK3 JH2 gain-of-function mutations R657Q and M511I reside in the autoinhibitory JH1–JH2 interface (see **Figure 2** for suggested JH2–JH1 interaction pair between JAK3 R657 and D865), which, when disrupted, is likely to cause increased JAK3 JH1 basal tyrosine kinase activity (27, 32). This, in turn activates JAK1, which finally acts as the effector kinase phosphorylating STAT5 (3, 43) and leading to ligand-independent activation of the IL-2-STAT5 pathway. Our results presented here corroborate this hypothesis as we show

that functionally sound JAK1 (including both JH1 and JH2) is required for STAT5 activation also by clinical gain-of-function JAK3 mutations (3, 43).

In contrast to the JH2 mutations, the L857Q, which is located next to the catalytically important K855 in JH1 (see **Figure 2**) and thus unlikely to disrupt the JH1–JH2 interaction, might activate JH1 more directly by e.g., repositioning the JH1 C-helix or the nearby DFG motif into a more active conformation. Our data shows that L857Q is still dependent on JAK1 JH1/JH2, thus the mutation does not seem to result in sufficiently high JAK3 JH1 kinase activity to lead to JAK1-independent phosphorylation of STAT5 as has been suggested for a proline-mutation of the same residue: L857P (43). Interestingly, our results also show that L857Q is suppressed by JAK3 JH2 ATP-binding site mutations, thus showing clear commonalities in activation mechanisms of JAK3 JH2 and JH1-borne gain-of-function mutations. Molecular dynamics simulations of JAK2 JH2 have suggested that ATP binding stabilizes the N lobe of C helix (αC) in JH2 (24), and this is likely to be the case for JAK3 as well. Whether this stabilization causes strengthening of the JH2-JH1 interaction (and thus suppression of gain-of-function mutations), or whether ATP binding to JH2 stabilizes a conformation of JH2 that enables a distinct activating interaction involved in ligand-independent activation of JAKs, is currently not unequivocally solved (24).

In addition to activating JAK3 mutations, we studied the SCID-related loss-of-function mutation JAK3 C759R. JAK3 C759R has been shown to be deficient in phosphorylating STAT5 and STAT3 and lacks kinase activity, but to be constitutively phosphorylated on the JH1 activation loop (JAK3 Y980-981) and bind to its receptor IL-2Rγ (31). JAK3 JH2 homology modeling suggests that mutation of C759 on α-helix H (see **Figure 2C**) to the bulkier and cationic Arg is likely to cause severe disruption of the JH2 C-lobe. Indeed, JAK3 C759R showed strongly reduced STAT5 activation and the effect was comparable to JAK3 ΔJH2 or kinase-dead JAK3 K855A.

Currently it is not clear, whether inhibition of JH1 by JH2 occurs in *cis* or *trans* in physiological JAK dimers, and data supporting both hypotheses has been presented (9). However, the interplay between JAK1 and JAK3 is evident in hematological malignancies, and cells transformed by activating JAK1 mutations become resistant to JAK inhibitors by acquiring activating mutations in JAK3 and *vice versa* (44). Considering the tight JAK1–JAK3 interplay on IL-2R, we studied the possible cross-regulation between JAK1 and JAK3 JH2s and observed that mutating the JAK1 JH2 ATP binding site could not inhibit activation caused by JAK3 M511I, which was also the case in the reversed experimental setting (JAK3 JH2 ATP-site mutant transfected with constitutively active JAK1 V658F). These data suggest that any suppressing effect caused by disrupting the JAK3 JH2 ATP-binding site is exerted only in *cis* and not between JAK3 and JAK1. However, as noted before, JAK1 is the dominant effector in STAT5 activation, and thus the overexpression of JAK1 in our experimental setup may compensate for any inhibitory effects of a JAK3 JH2 ATP binding mutant. Furthermore, in our experimental approaches we have used rational mutagenesis and deletion/recombinant constructs, and although the constructs have been designed based on best available information they are not natural proteins and thus our experimental systems bear inherent limitations.

We also tested the JAK1–JAK3 interaction by co-transfecting the inhibitory JAK3 SCID mutant C759R with JAK1 V658F. Again, JAK1 dominancy was apparent and JAK3 C759R could not reduce the activation of constitutively active JAK1 V658F even though it did inhibit IL-2-induced activation of wild-type JAK1. Together, these data support a model in which JAK3 acts as a regulating initiator kinase mainly responsible for activation of JAK1, which subsequently phosphorylates downstream targets. If JAK1 is activated by other means, like constitutively activating JAK1 mutations, any activating functions of JAK3 become unnecessary. Notably, the reverse is not true and even mutationally activated JAK3 relies on JAK1 for effector functions.

The vast majority of JAK inhibitors currently in use or development target the kinase domain of JAKs in its active, DFG-in, conformation, and they often suffer from poor selectivity (45). Novel JAK3 inhibitors have been developed that bind to a JAK3-specific, non-catalytic cysteine (C909) in the JH1 domain, thus enabling significant selectivity toward JAK3 (41, 46). Recently, an ATP binding competitive inhibitor against JH2 of TYK2 was shown to have high specificity and efficacy in IL-23 and type I IFN signaling (47). Our results suggest that the JAK3 JH2 ATP pocket may also be a relevant therapeutic target in diseases caused by JAK3 hyperactivation.

ETHICS STATEMENT

All experiments performed were approved by the Deakin University Animal Welfare Committee.

AUTHOR CONTRIBUTIONS

JR, AV, and VB planned and performed the experiments (AV contributed to the FP measurement and VB performed the *in vivo* experiments). HH devised and planned the experiments. JR and HH analyzed the data and drafted the manuscript. OS and AW supervised the work and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00560/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Hyperactivation of oncogenic JAK3 mutants depend on ATP binding to the pseudokinase domain

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Supplementary Figures

Figure S 1: JAK1 mediates cytokine-independent activation of STAT5, but both JAK1 and JAK3 are required for IL-2-dependent STAT5 activation

(A) STAT5 activity when varying amounts of JAK1 and JAK3 were transfected in U4Cγβ cells. STAT5 responsive promotor SPI-luc2 and pRL-TK control plasmid were also included. Values under the bars show the amount of plasmid transfected in ng per 96-well plate well.

(B) Immunoblots of U4Cγβ cells transfected with wild-type HA-tagged JAK3 and/or JAK1 and STAT5. Blots were labelled with HA and pSTAT5 antibodies. The ratio of the pSTAT5/STAT5 (HA) signal is shown as a bar chart.

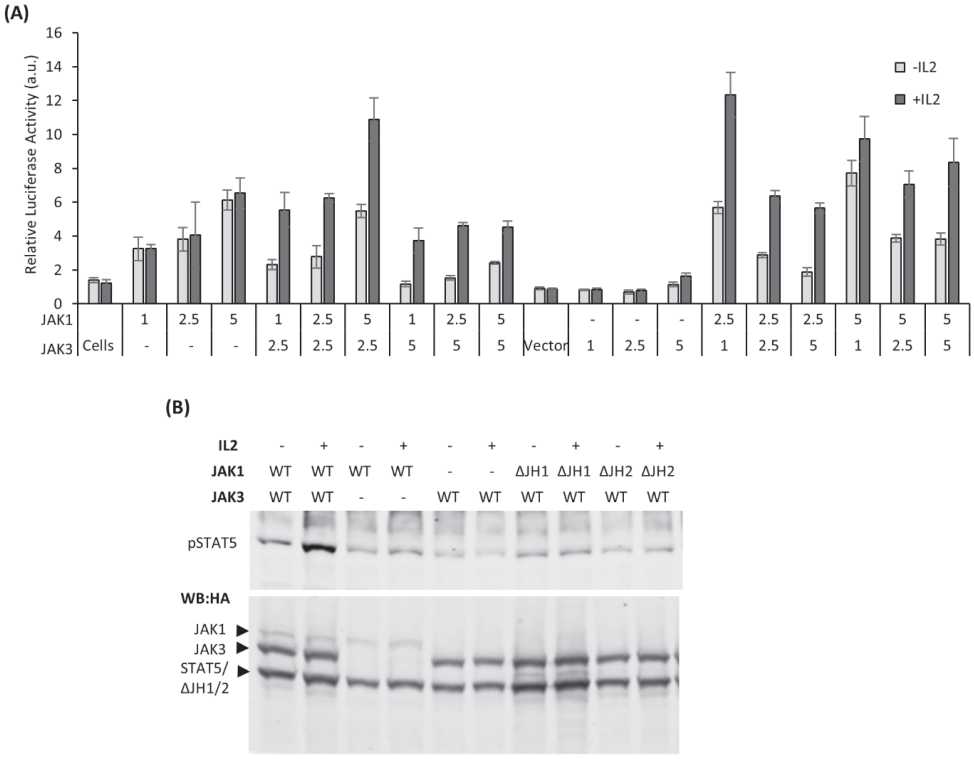


Figure S 2: Expression levels of the JAK1 and JAK3 mutants

(A) Constitutively active JAK3 mutants cannot signal without JAK1. STAT5-responsive promoter with activating JAK3 mutants without JAK1 were transfected into U4Cγβ cells.
(B) Western blot of the JAK1 domain deletions with JAK3 activating mutants. Cells were transiently transfected with different HA-tagged JAK1, JAK3 and STAT5 constructs, and detected with HA and pSTAT5 antibodies.
(C) Expression analysis of JAK1 domain deletions. JAK1ΔJH1 and ΔJH2 are same size with STAT5 and to see the expression of the constructs, the cells were transfected with the JAK1 constructs with and without STAT5 and labelled with HA antibody. The expression of full-length JAK1, JAK3 and STAT5 are shown as a control.

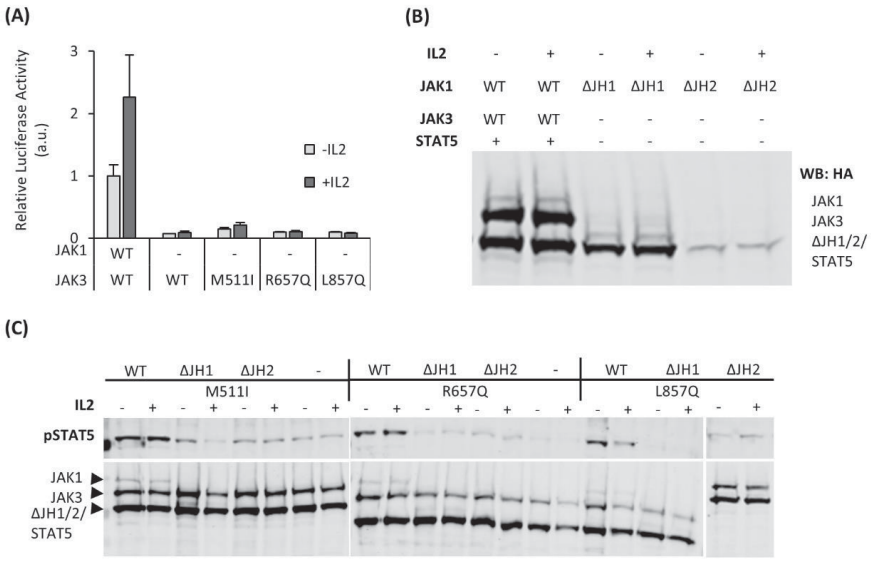


Figure S 3: Purification and kinase activity of the recombinant JAK3 JH2

(A) Purification of the recombinant JAK3 JH2 (511-790 C-terminal His). Elution (UV-signal) profile of the SEC purification. Peak 2 includes the fraction with JAK3 JH2 and peak 1 the void.

(B) Fractions corresponding to the peak 2 in the UV detector were collected and run to 12% SDS-PAGE gel. The size of the band in the gel corresponds to JAK3 JH2 (33 kDa). Also shown SDS-PAGE from the Ni-NTA affinity purification

(C) Ni-NTA purification of the Mutant JAK3 JH2 constructs. From right to left, wild type JAK3 JH2, ATP-binding site mutants I535F and K556A and activating mutant R657Q. Three elutions from the beads are shown. Gels of I535F and R657Q include also un-bound (UB) sample. Right: List of JAK3 JH2 mutants designed to inhibit ATP-binding. No protein was obtained from the mutants.

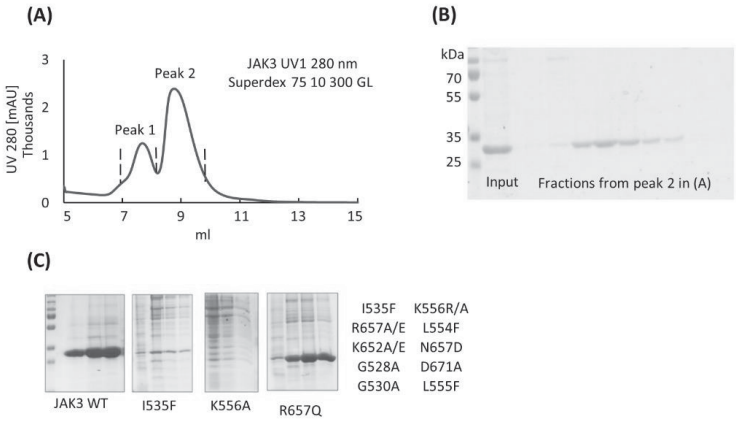


Figure S 4: Analysing the ATP binding of recombinant JAK3 JH2

(A) Comparison of the cation dependency of JAK JH2s with DSF. Melting temperature of different JAK JH2s was measured with or without ATP and MgCl2. 3 μM protein was used with 500 μM ATP and the ΔTm was compared to ATP + 1 mM MgCl2. Errors are SD from triplicate samples.

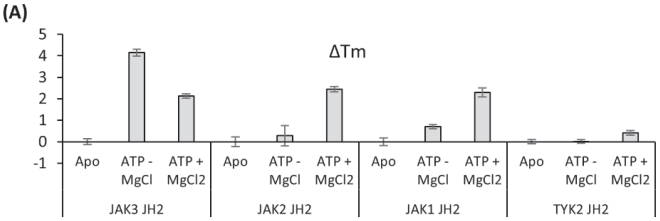
(B) IC50 values of JAK JH2 ATP binding. ATP was competed with fluorescently labelled tracer, which binds to the JH2 ATP binding pocket and the binding curves for all JAK JH2s were determined by ATP titration, with and without MgCl2. All proteins were run in triplicates and the data with SD errors are shown.

(C) Left panel: ATP titrationin DSF with all JAK JH2s. Effect of ATP to the melting temperature is presented as ΔTm values. Errors are SD from triplicate samples. Right panel: Kd value for JAK3 JH2 was determined from the ATP titration curve shown in the left panel.

(D) JAK3 JH2 binds preferentially ATP. Nucleotide titration with ATP, ADP and AMP (0.2, 0.5, 1 mM concentrations) and the stability of the protein was analyzed with DSF. All bar graphs in (A), (B) and (C) show average ΔTm from duplicate samples. Error bars represent SD.

(E) Autophosphorylation assay of recombinant JAK3 JH2. 0.05 μM of radioactively labelled γ^{32}P-ATP (10 μCi/μl) was incubated 20, 40, 80 or 180 min with 0.6 μM JAK3 JH2 or with a positive control (0.3 μM of active tyrosine kinase, EGFR, supplemented with 20 mM $MgCl_2$). A sample without γ^{32}P-ATP was used as a negative control. The faint bands in JAK3 JH2 + ATP (indicated as arrowheads) are the same size as in the EGFR and thus considered to be caused by impurities (other kinase) in the sample. No phosphotransferase activity was detected in the negative control.

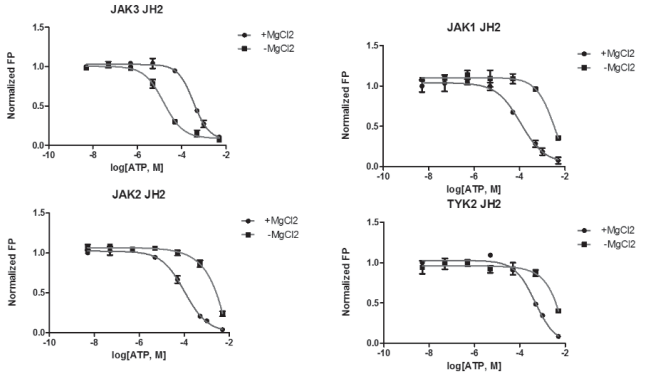
(A)



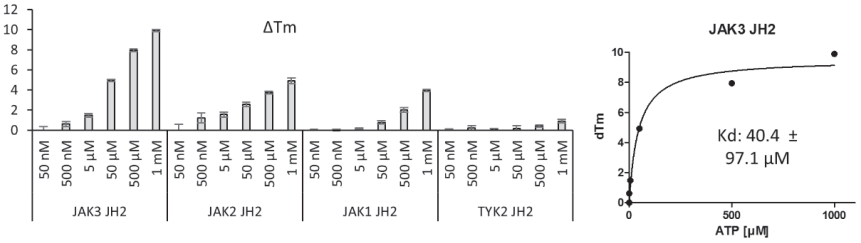
(B)

IC50 [ATP, µM]

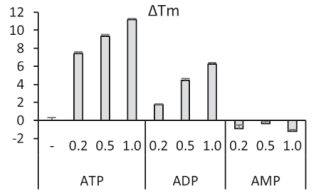
JH2	JAK1	JAK2	JAK3	TYK2
+MgCl2	110	102	343	471
-MgCl2	> mM	> mM	16	> mM



(C)



(D)



(E)

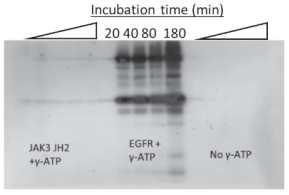


Figure S 5: Whole blots from Figure 4 and 5 with pJAK1 analysis

(A) Representative whole blots form the pSTAT5 analysis of the JAK3 ATP binding, activating, and double mutants shown in Figure 4.

(B) Analysis of JAK1 phosphorylation. pJAK1 antibody was used to detect pJAK1 status with different JAK3 mutants. Lower panel: Quantitative analysis from the blots done by dividing the pJAK1 signal with HA-signal from HA-tagged JAK1.

(C) Representative blots from experiments used in Figure 5. HA-tagged JAK1 and JAK3 expression levels were detected with HA-antibody. Dotted lines between blots indicate separate gels.

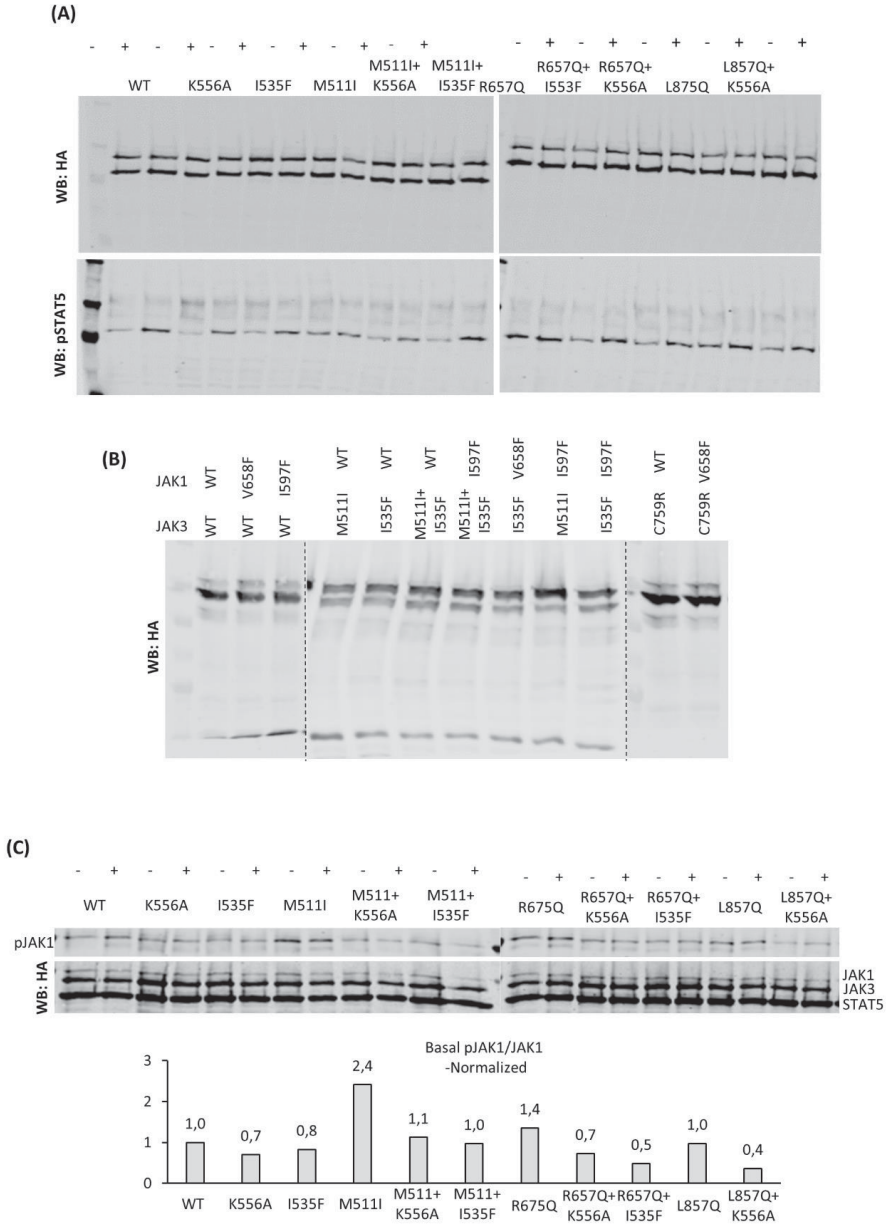
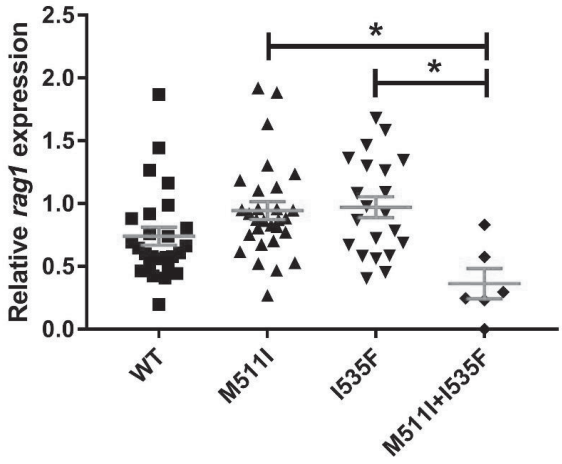

(A)
WT
K556A
I535F
M511I
M511I+ K556A
M511I+ I535F
R657Q
R657Q+ I553F
R657Q+ K556A
L875Q
L857Q+ K556A
WB: HA
WB: pSTAT5
(B)
JAK1
JAK3
WT
V658F
I597F
M511I
I535F
M511I+ I535F
C759R
WB: HA
(C)
WT
K556A
I535F
M511I
M511+ K556A
M511+ I535F
R675Q
R657Q+ K556A
R657Q+ I535F
L857Q
L857Q+ K556A
pJAK1
WB: HA
JAK1
JAK3
STAT5
Basal pJAK1/JAK1 -Normalized
1,0
0,7
0,8
2,4
1,1
1,0
1,4
0,7
0,5
1,0
0,4

Figure S 6: Mutation of JAK3 JH2 ATP site ablates the effects of activating mutation in vivo.

Relative expression of the IL-2Rγc/JAK3-dependent lymphoid marker *rag1* at 5 dpf in *jak3*$^{-/-}$ mutant embryos injected with mRNA encoding zebrafish JAK3 wild-type (WT) (n=29) and mutants M511I (n=26), I535F (n=21) and M511I+I535F (n=6), as indicated. Data is presented as mean ± SEM, with statistical significance indicated (* $p < 0.05$).



PUBLICATION II

Characterization of JAK1 Pseudokinase Domain in Cytokine Signaling

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Article

Characterization of JAK1 Pseudokinase Domain in Cytokine Signaling

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Abstract: The Janus kinase-signal transducer and activator of transcription protein (JAK-STAT) pathway mediates essential biological functions from immune responses to haematopoiesis. Deregulated JAK-STAT signaling causes myeloproliferative neoplasms, leukaemia, and lymphomas, as well as autoimmune diseases. Thereby JAKs have gained significant relevance as therapeutic targets. However, there is still a clinical need for better JAK inhibitors and novel strategies targeting regions outside the conserved kinase domain have gained interest. In-depth knowledge about the molecular details of JAK activation is required. For example, whether the function and regulation between receptors is conserved remains an open question. We used JAK-deficient cell-lines and structure-based mutagenesis to study the function of JAK1 and its pseudokinase domain (JH2) in cytokine signaling pathways that employ JAK1 with different JAK heterodimerization partner. In interleukin-2 (IL-2)-induced STAT5 activation JAK1 was dominant over JAK3 but in interferon-γ (IFNγ) and interferon-α (IFNα) signaling both JAK1 and heteromeric partner JAK2 or TYK2 were both indispensable for STAT1 activation. Moreover, IL-2 signaling was strictly dependent on both JAK1 JH1 and JH2 but in IFNγ signaling JAK1 JH2 rather than kinase activity was required for STAT1 activation. To investigate the regulatory function, we focused on two allosteric regions in JAK1 JH2, the ATP-binding pocket and the αC-helix. Mutating L633 at the αC reduced basal and cytokine induced activation of STAT in both JAK1 wild-type (WT) and constitutively activated mutant backgrounds. Moreover, biochemical characterization and comparison of JH2s let us depict differences in the JH2 ATP-binding and strengthen the hypothesis that de-stabilization of the domain disturbs the regulatory JH1-JH2 interaction. Collectively, our results bring mechanistic understanding about the function of JAK1 in different receptor complexes that likely have relevance for the design of specific JAK modulators.

Keywords: JAK; STAT; cytokine; cytokine receptor; cancer; inflammation

1. Introduction

Janus kinases (JAK1–3 and Tyrosine Kinase 2, TYK2) are non-receptor tyrosine kinases that play a critical role in cell signaling via type I/II cytokines and interferons (IFNs) [1]. JAKs are constitutively bound to the intracellular part of the receptors that dimerize after ligand binding, thus enabling the transphophorylation and activation of JAKs and subsequent phosphorylation and translocation of STATs (signal transducer and activator of transcription proteins) into nucleus to regulate transcription. Structurally all JAKs consist of four domains; the N-terminal FERM (4.1-band, ezrin, radexin, moiesin) domain that together with the SH2-like domain (SH2) compose the main receptor binding moiety,

followed by the C-terminal JAK-homologue (JH) 2 and JH1 domains. JH1 is an active tyrosine kinase while JH2 has an important role in regulating both basal and cytokine-induced activation [2–5]. Some residues required for the phosphotransferase reaction from ATP to a substrate are not conserved in JH2, such as altered Gly-rich region and lack of catalytic Asp, hence giving the domain "pseudokinase domain" designation [6]. Despite the absent or low catalytic activity, JH2s regulates JAK activity through allosteric mechanisms. For example, JH2 forms an autoinhibitory interaction with JH1 by binding to the hinge region and stabilizing the inactive conformation of JH1 [7]. Furthermore, the ATP binding to JH2 stabilizes the domain and nucleotide binding is critical for pathogenic activation of JAK2 [8,9]. In TYK2, a small ATP pocket-binding compound has been shown to efficiently decrease the activation [10]. In addition, the SH2-JH2 linker is an important regulative region that controls the activity of JAKs [4].

JAK1, as well as other JAK family members, harbor pathogenic gain-of-function mutations (GOFs) that cause acute lymphoid and -myeloid leukemia (ALL and AML, respectively). JAK1 mutations are found in varying types of cancers; e.g., 9% of hepatocellular carcinoma patients have been found to have JAK1 mutations [10]. In addition to GOFs, JAK loss-of-function mutations (LOFs) have been identified in JAK3, which cause severe combined immune deficiency (SCID); a disease resulting a depletion of B-cells and complete loss of T- and NK-cells [11,12]. A majority of the pathogenic mutants clusters in JH2 highlighting the regulative role of the domain [10]. The most common mutation, JAK2 V617F also resides in JH2. JAK2 V617F and accounts for ~95% of patients with polycythaemia vera and about 50% of patients with essential thrombocytosis and primary myelofibrosis [13–15]. Mechanistically, the mutation stabilizes JAKs the αC-helix (αC) in the N-lobe of JH2, and induces cytokine independent dimerization of the receptors, possibly via JH2-dimerization [16,17]. The αC resides in the JH1-JH2 interface but also lines the ATP-binding pocket. The region is important in cytokine-induced activation of kinases, and modulating it with mutations can inhibit constitutive activation of JAKs [17–19]. However, the mechanisms of function for many mutations is yet unknown.

Homologous mutation to JAK2 V617F in JAK1 (V658F) causes acute lymphoblastic leukemia [20]. In addition to the JAK2 V617F and its homologues, a distinctive cluster of GOFs resides in the JH1-JH2 interface and these mutations disrupt the autoinhibitory interaction between the domains. For example, JAK1 R724E has one of the highest incidence rates among the JAK1 pathologic mutations. It resides in the N-lobe of JH2 (similarly to homologous JAK2 R683S) and interacts with JH1. Other JAK1 mutants in the JH1-JH2 interface are A634D and L653F [21,22].

Currently there are three JAK JH1-targeted inhibitors in clinical use, but recently targeting JAK JH2s with small molecular compounds has gained interest as a potential treatment for constitutively active cytokine signaling [10,23,24]. The unique mode of ATP binding in JH2 may allow increased specificity over other eukaryotic protein kinases [25].

Although the general principles of the JAK-STAT signaling are well established, the underlying differences between the function of JAKs and their individual domains in different cytokine signaling pathways are not fully defined. Moreover, the unbalanced number of JAKs (four) versus the cytokine pathways they transduce (over fifty) raises the question about the mechanisms that allow the versatile function of JAKs. For example, JAK1 is employed by the IL-2, IL-4, IL-10, and gp130 (including e.g., IL-6) receptor families as well as type I and type II interferons (e.g., IFNα and IFNγ, respectively). Intrigued by these questions, we set to study the role of JAK1 and particularly its JH2 in various signaling pathways. Our results show that JAK1 has varying roles in different receptor complexes, and that JH2 mediates important allosteric regulation of the JAK activity.

2. Results

2.1. JAK1 Is Dominant STAT Activator in IL-2, but not in IFNγ and IFNα Signaling

To investigate the role of individual JAKs in heterodimeric receptor complexes, we focused on IL-2, IFNγ, and IFNα receptor systems. These pathways utilize JAK1 but consists of different JAK

dimers: IL-2-signaling is driven by JAK1-JAK3, IFNγ by JAK1-JAK2, and IFNα by JAK1-TYK2. First, we studied how dependent the signaling systems are to the presence of an individual JAK. For this, we used cell lines that lack specific JAK expression. JAK1 and JAK3 deficient U4Cγβ-, JAK2 deficient γ2A- or TYK2 deficient 11.1 human fibroblast cells were transiently transfected with one or both JAKs relevant to the pathway, and the expression of JAKs and STATs was detected from the cell lysates. In addition, the phosphorylation of STAT1 (pSTAT1) and STAT5 (pSTAT5) was assayed. Of note, HA-STAT5 was transfected into the U4Cγβ cells, since endogenous STAT5 could not be detected with this method.

All of the studied cytokine receptor pathways required the expression of two different wild-type (WT) JAKs for the cytokine-dependent activation of STAT (Figure S1A,B). In IFNγ and IFNα systems, STAT1 activation required the expression of both JAK1 and JAK2 or TYK2, respectively. In the IL-2 receptor complex both JAK1 and JAK3 were also required for cytokine dependent signaling; JAK1 alone activated STAT5 but the activation was unresponsive to cytokine while JAK3 could not induce STAT5 phosphorylation (even with IL-2) without the presence of JAK1 (Figure S1C). Together these results confirm the previous findings that cytokine induced signaling requires heterodimerization of JAK WT [26,27] as well as validated the experimental system.

Next, we studied the JAK dependency of the three signaling systems in the context of hyperactive mutations. JAK2 V617F mutant or homologous mutations in JAK1 and TYK2 (see Table 1) were transfected in JAK1 deficient cells either alone or with the relevant partner JAK. Interestingly, we observed that unlike in the WT-setting, JAK2 V617F and homologous mutations in JAK1 and TYK2 could induce (reduced) pSTAT1 even in the absence of the partnering JAK. However, the presence of the partner JAK increased the activation (Figure 1A). JAK1 V658F also activated STAT5 independently of JAK3 in the IL-2 system (Figure 1A [9]). As JAK1, JAK2 and TYK2 are roughly same size (~130 kDa) they appear as a single band in the western blot (with less intensive band when only one JAK is transfected with the vector).

Table 1. Mutations used in this study qualified as loss-of-function mutations (LOFs) or gain-of-function mutations (GOFs) based on the shown effects (-, designates as neutral).

JAK	Mutation	Effect	Short Description.
JAK1	L633K	LOF	At the solvent exposed face of the JH2 αC-helix, homologous to the JAK2 E592R.
	I597F	GOF/-	Residing JH2 ATP-binding site and designed to inhibit ATP binding. Homologous to JAK2 I559F.
	K622A	LOF	Removes conserved β3 lysine in JH2. Designed to inhibit ATP binding. Homologous mutations shown to inhibit hyperactivation in JAK2 and JAK3.
	V658F	GOF	Homologous to JAK2 V617F and TYK2 V678FF. Resides in the JH2 β4-β5 loop and potentially disturbs the SH2-JH2 linker and causes cytokine independent activation. Mutation in JAK1 or JAK2 cause ALL.
JAK2	E592R	LOF	At the solvent exposed face of the JH2 αC-helix, shown to inhibit JAK2 V617F-driven dimerization of EPOR [19].
	I559F	LOF	In the JH2 β2. Designed to sterically inhibit ATP binding and shown to inhibit ATP binding in recombinant JH2 [8].
	V617F	GOF	Homologous to JAK1 V658F and TYK2 V678F.
TYK2	L653R	LOF	At the solvent exposed face of the JH2 αC-helix, homologous to the JAK2 E592R.
	V603F	LOF	At the ATP, binding pocket of JH2, designed to inhibit ATP binding. Homologous to JAK2 I559F.
	V678F	GOF	Constitutive active mutation in JH2. Homologous to JAK1 V658F and JAK1 V617F.

Table 1. *Cont.*

JAK	Mutation	Effect	Short Description.
JAK3	E567R	LOF	Resides at the solvent exposed face of the JH2 αC-helix. Homologous to the JAK2 E592R.
	I535F	LOF	At the ATP binding pocket of JH2 and homologous to JAK2 I559F.Shown to inhibit constitutive JAK3 activation [9].
	R657Q	GOF	Activating mutation found in ALL patient. Resides in the JH1-JH2 interface.
	M592F	GOF	Homologous to constitutively active JAK1 V658F, JAK2 V617F and TYK2 V678F.
	L570F	-	Mutation designed to create the WT state as in JAK1, JAK2 and TYK2 (F595 in JAK2). Stacks with the mutated JAK2 V617F and enables the hyperactivation via the FFV-triad formation (see text).
	M592F + L570F	GOF	Double mutant designed to create a complete FFV-triad into JAK3 (see above).

In contrast, the pathogenic JAK3 R657Q residing in the JH2-JH1 interface was strongly dependent on the presence of JAK1 WT, shown as abolished STAT5 activation when JAK1 was not transfected into the U4Cγβ cells (Figure 1B [9]). Of note, equal amounts of DNA for each JAK were transfected although the JAK1 HA-signal is consistently weaker than JAK3 HA. As we have previously noticed that overexpression of JAK1 quickly increases the activation status of the basal STAT5 [9], and to keep the experimental set-up reliable, we did not want to increase the expression of JAK1 despite the weaker bands obtained with the immune labeling. In addition, the proper expression of JAK1 is evident also from the IL-2 responsive STAT5 activation, which cannot be induced without JAK1.

JAK3 R657Q was chosen as a representative activating JAK3 JH2 mutation because JAK3 lacks the residue homologous to JAK2 V617 that is present in other JAK JH2s (see Table 1). Furthermore, JAK3 has a leucine in the JH2 αC that in all other JAKs is occupied by a phenylalanine (Phe) (Table 1). This Phe is part of the so-called FFV-triad (Phe-Phe-Val) that is important for JAK2 V617F activation. The residue stack with the V617F, rigidify the αC and interacts with the SH2-JH2 linker via (JAK2) Phe 575, as well as alters the ATP-site cleft, leading to hyperactivation [28–30]. We were interested whether the difference in JAK3 FFV-triad (or the usage of non-homologous activating mutation) is causing the lack of STAT-activation in the absence of JAK1. Hence, we reconstituted the FFV-triad by double-mutating JAK3 at M592F (mimicking the JAK2 V617F) and at L570F to introduce the phenylalanine present in JAK1 WT (Phe F636) and JAK2 (Phe 595) (Figure 1C,D). The JAK3 M592F + L570F caused constitutive activation of STAT5 in the presence of JAK1 WT, but not in the absence of JAK1 WT. The single mutants were also analyzed for their STAT5-activation potential, and while JAK3 M592F showed some hyperactivation, L570F did not affect the STAT5 activation. The unaltered STAT activation in response to L570F was expected, as it simulates the WT-situation in JAK1 and JAK2. These results suggest that the dominance of JAK1 in the IL-2 system is not an intrinsic feature of JAK3, but rather a property of the IL2R complex.

Next, we compared the roles of JAK1 JH1 and JH2 in the IL-2 and IFNγ signaling. U4Cγβ cells were transiently transfected with JAK1 constructs where either JH1 or JH2 was deleted (Figure 1E). We have previously shown that in IL-2 signaling both JAK1 JH1 and JH2 are crucial for STAT5 activation (Figure 1E) [9]. However, in the IFNγ system JH1 deleted JAK1 maintained IFNγ responsiveness albeit the inductivity was considerably lowered, while JH2 was critical for any cytokine responsiveness (Figure 1E). This observation is in line with the study of Eletto et al., where JAK1 JH2, but not JH1, was found to be essential in IFNγ signaling, while both JH1 and JH2 were crucial for IFNα signaling [31].

Based on these results, the role of JAK1, and its JH2, is different between IL-2 and IFNγ (or IFNα) systems. In IL-2 signaling, JAK1 dominantly mediated the STAT5 phosphorylation and the activation requires both JH1 and JH2 domains. In the IFNγ and IFNα signaling, STAT1 activation requires both JAK1 and JAK2/TYK2, and IFNγ signaling shows to be dependent specifically on the pseudokinase domain of JAK1.

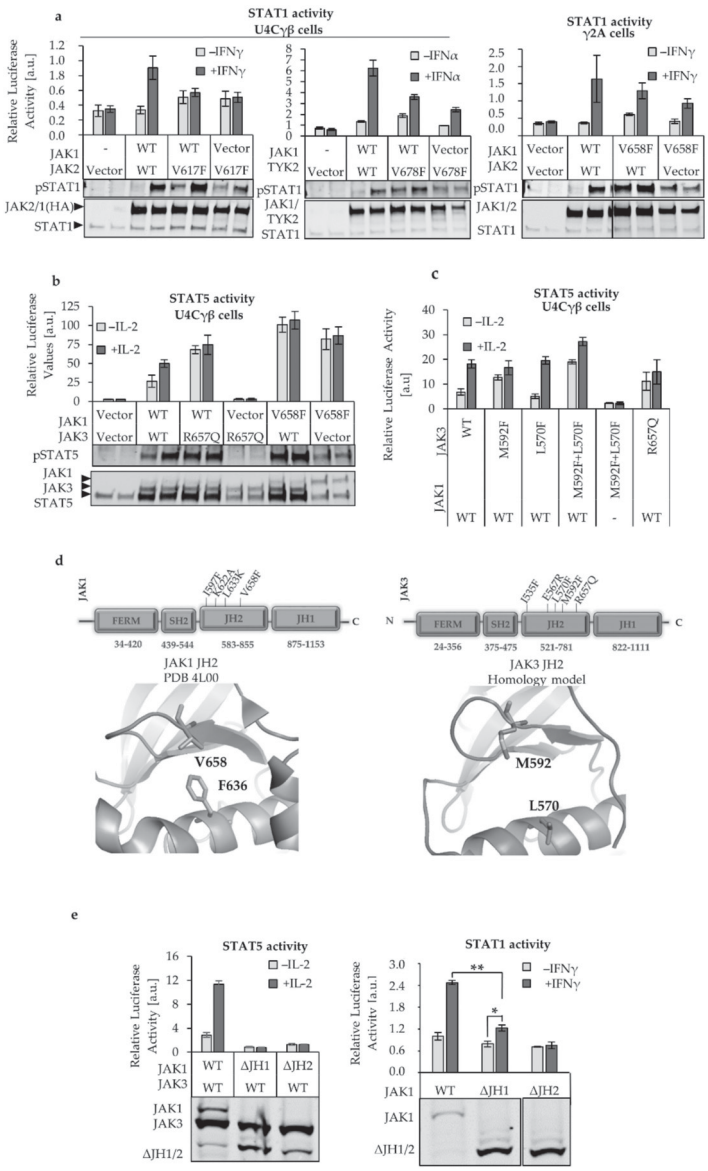

Figure 1. Comparison of JAK JH2s. (**a**) Janus kinases (JAK1, JAK2 and TYK2) hyperactive mutants can activate STAT1 without the partner JAK. pSTAT1 analysis and the transcriptional activity of STAT1 were detected from U4Cγβ and γ2A cells that were transfected for 24 h with JAK2 V617F, TYK2 V678F or JAK1 V658F. Wild-type (WT) partner JAK or vector was co-transfected with the activating mutants. Cells were starved overnight, and left untreated or stimulated with 100 ng/mL interferon γ (IFNγ) and interferon α (IFNα) for 20 min after which the pSTAT1 was detected by immunolabeling. For the STAT1 transcriptional activity detection, IRF-GAS or ISRE-luc plasmids) were co-transfected for 43 h with renilla plasmid (pRL-TK) (see Materials and Methods). Cells were stimulated or starved for 5 h, and the luciferase activity was measured. The values were divided with the renilla values to reduce the effects the possible differences in the transfection efficiency might have. Errors are SD of triplicates. Below are representative immunoblots of whole-cell lysates from U4Cγβ and γ2A cells transiently transfected with full-length hemagglutinin (HA)-tagged JAK mutants with or without JAK WT, as indicated. The cell lysates were immunolabeled with pSTAT1 (STAT1 Y701 phosphorylation), HA and

STAT1 antibodies. The experiment was repeated twice with similar results. (**b**) JAK3 R657Q cannot induce STAT5 activation without JAK1. U4Cγβ cells were transfected with JAK WT or hyperactive JAK1 V658F or JAK3 R658Q and left untreated or stimulated with IL-2 (100 ng/mL) for 5 h. STAT5 specific SPI-Luc 2 plasmid was used for the detection of STAT5 transcriptional activity and the pRL-TK was used as a control. Errors are SD of triplicates. Below: Whole cell lysates from transiently transfected U4Cγβ cells were labelled with pSTAT5 (phosphorylation at Y694) and HA antibodies. HA-tagged STAT5 was transfected with JAK-HA constructs. Blot is a representative from three experiments. (**c**) Reconstituted JAK2 V617F homolog in JAK3 cannot signal without JAK1. JAK3 M592F, L570F, and double mutant were transiently transfected with JAK1 WT or vector. The U4Cγβ cells were starved and/or stimulated with 100 ng/mL IL-2, and the activity of the STAT5 responsive SPI-Luc vector measured as described above. (**d**) Illustration of JAK1 V658, F636 and homologous mutations in JAK3 pseudokinase domain (JH2) with schematic presentation of the four domains and approximate location of the JH2 mutations. Also the amino acid range for domains (according to the UniProt database) are shown in the scheme below each domain. Structures were visualized with PyMol using JAK1 JH2 structure (PDB 4L00) and JAK3 JH2 homology models (modelled based on TYK2 structure; Protein data bank 4OLI). (**e**) JAK1 JH2 is critical for IL-2 and IFNγ signaling. U4Cγβ cells were transfected with full-length JAK1 or with JAK1 JH1 or JH2 deletions. STAT5 and STAT1 responsive Luc-vectors were used as described before to detect the IL-2 and IFNγ responsiveness of the constructs. Errors are SD of triplicates, and *p*-values according to two-tailed student *t*-test (*—indicating $p < 0.05$ and **—$p < 0.001$). Expression of the HA-tagged, unstimulated JAK1 (and JAK3 in the IL-2 system) was confirmed by immunolabeling the whole cell lysates with HA-antibody. The band below the JAK1 WT and JAK3 bands in the left side panel WT/WT sample is due unspecific binding of the HA antibody.

2.2. L633K in the Outer Face of JAK1 JH2 aC-helix Inhibits WT and Hyperactive IL-2, IFNγ, and IFNα Signaling at Variable Degrees

We continued the analysis of JAK1 JH2 with available information from other JAK JH2s and focused on regions that have been identified to allosterically regulate JAK activation. We focused on the hydrophilic outer face of the JH2 αC and introduced a mutation in JAK1 that corresponds to JAK2 E592R (Figure 2A). JAK2 E592R inhibits hyperactivation in JAK2 and reduces V617F-driven dimerization [17].

αC-helix is an essential conserved region in protein kinases, and in the active kinase conformation the N-terminus of the αC-helix typically interacts with the activation loop phosphate. Furthermore, the conserved β3 Lys^{72} (numbering based on Protein Kinase A, PKA) couples the ATP phosphates to the αC-helix [25]. The clinical relevance of this region in pathogenic JAK1 signaling was evaluated by searching for patient-derived mutations that cluster in the area. Based on the COSMIC database [32] together with a literary research by Hammarén et al., the JAK1 JH2 αC-helix and the surrounding αB and β4 linkers were found to be highly mutated in human cancers. Based on mutations from Hammarén et al. [10], 11 out of the 29 residues (38%) were mutated and with all COSMIC mutations included, total of 16 mutations could be depicted in this region (Figure 2A, mutated residues shown as grey-shaded, bolded letters in the sequence).

To test the effect of the αC modulation in cell-based assays, JAK1 L633K was transfected into U4Cγβ cells with STAT1 or STAT5 responsive luciferase reporters and the normalized luciferase values were compared to cells transfected with JAK1 WT. L633K reduced the cytokine responsiveness in JAK1-JAK3 driven IL-2 signaling and JAK1-TYK2 driven IFNα signaling (Figure 2B). In addition, the basal STAT5 activity in L633K transfected cells was reduced compared to WT. On the contrary, JAK1 L633K did not markedly affect the IFNγ responsiveness (driven by JAK1-JAK2), but slightly reduced the basal STAT1 activity. Basal STAT1 activity profile correlated with the reduced pSTAT1 in L633K transfected cells (Figure S2A,B). Homologous mutations JAK2 E592R and TYK2 L653R also reduced the cytokine responsiveness as measured with STAT1 responsive IRF-GAS- and ISRE-luciferase reporters (Figure S2C).

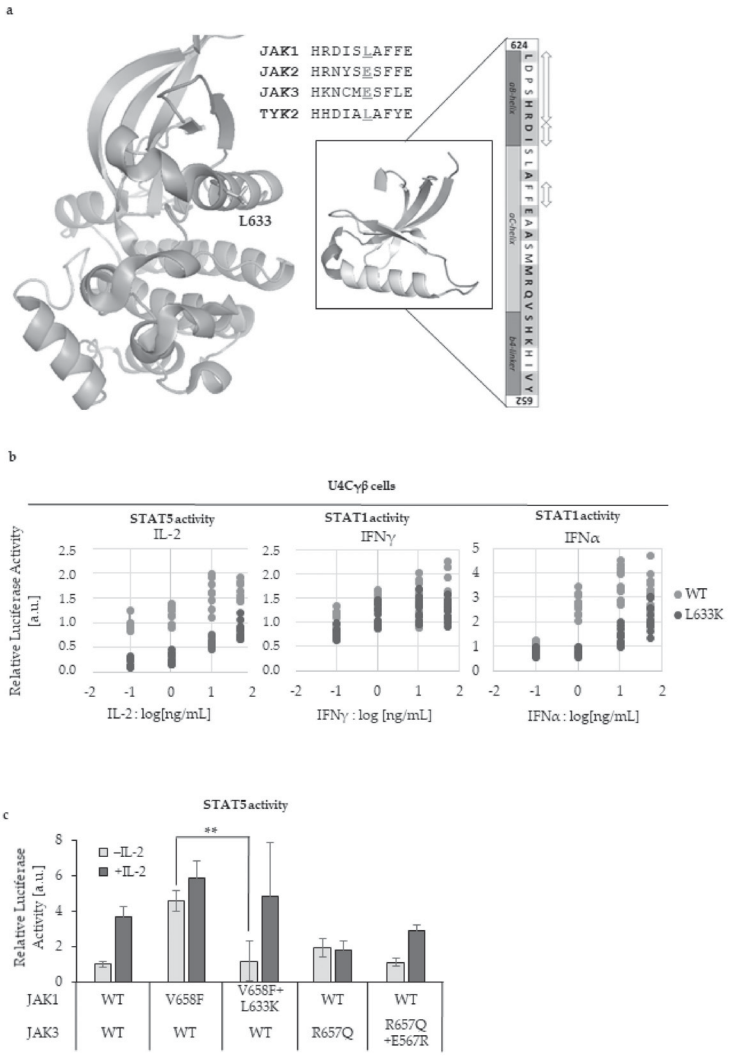

Figure 2. Analysis of the JAK1 JH2 αC-mutation. (**a**) JAK1 JH2 (PDB 4L00) structure is shown with the L633 in the αC-helix. The sequence around JAK1 L633 in JAK family is shown. **Right:** Mutations in the JAK1 αC-region including the adjacent αB-helix and the β4-linker. The mutations (shaded residues with bold letters) are derived from the review of Hammarén et al. 2018 [17] and the Catalogue of Somatic Mutations in Cancer (COSMIC)-database [32]. Deletions are shown as arrows. (**b**) Mutation in the JAK1 JH2 αC inhibits JAK1 driven IL-2 and IFNα signaling, but has lesser effect in the IFNγ-induced signaling. JAK1 WT (shown as blue dots) or JAK1 L633K (red dots) were transiently transfected with STAT1 and STAT5-responsive luciferase plasmids as described previously. The U4Cγβ cells were then starved or stimulated with increasing amounts of cytokine, and the STAT-activation was detected. All 12 replicas are presented as dots in a logarithmic axis showing the cytokine amount versus the relative luciferase activity. Basal state is set to −1. (**c**) JAK1 L633K and homologous mutation in JAK3 JH2 αC reduces hyperactivation in *cis*. JAK1 and JAK3 mutations were studied with (100 ng/mL) and without IL-2 stimulation in U4Cγβ cells that lack JAK1 and JAK3. In comparison to the activating mutations, the double mutants had reduced basal activity and responded to cytokine stimulation similarly as JAK WT transfected cells. STAT5 activity was studied with SPI-Luc luciferase system as described previously. The errors are SD of two separate experiments both having triplicate samples (n = 6). Two-tailed t-test was performed and **—indicates p-value <0.001.

Since JAK1 L633K reduced IL-2-driven STAT activation, we were interested whether the mutation can inhibit constitutively activated JAKs in cis, as shown with JAK2 [17]. We created double mutants of JAK1 and JAK3, where the JAK1 L633K was combined with hyperactivating JAK1 V658F. In addition, similar mutations in JAK3 JH2 were tested (JAK3 E567R + R658Q, see Table 1). Both double mutants reduced the hyperactivation back to WT levels but retained the IL-2 inductivity (Figure 2C). Similar reduction of hyperactivation has been shown with homologous mutations in JAK2 [8,17] and were seen with TYK2 (Figure S2C). Of note, more fluctuation in the STAT activation levels was observed in the JAK1 L633K + V658F double mutant, shown as larger errors seen in Figure 2C. However, the reduction from JAK1 V658F transfected cells was significant ($p < 0.001$).

2.3. Characterization of ATP Binding to JAK1 JH2

Next, we set to compare the inhibitory potential between the αC-mutant and another allosteric region of JH2, namely the ATP-binding site. First, we showed that in addition to JAK2 I559F and JAK3 I535F mutations that have previously been shown to inhibit ATP binding and JAK hyperactivation, [8,9] also homologous TYK2 V603F inhibits hyperactive TYK2 V678F in the IFNα system (Table 1, Figure S2D). The mutation was originally designed to create steric hindrance in the pocket and have been veritably shown to inhibit ATP binding into JAK2 JH2 [8]. We introduced a mutation in JAK1 JH2 ATP-site, JAK1 I597F, which is homologous to the above-mentioned JAK mutants. In addition, another ATP site mutant, JAK1 K622A was chosen as its homolog has been shown to inhibit JAK2 and JAK3 hyperactivation in cis [8,9]. This highly conserved lysine (Lys72 in PKA) is critical in making a salt bridge to the conserved Glu (91 in PKA) in the αC, and is required for coordinating nucleotide binding of multiple kinases and pseudokinases [33].

We have previously noted that JAK1 I597F is unable to inhibit hyperactive IL-2 signaling, contrasting the effect of the homologous mutants in JAK2 and JAK3 [8,9]. Here, we found that JAK1 I597F increased basal STAT5 activity and pSTAT5 in WT background, although to a lesser extent than hyperactive JAK1 and JAK3 mutants (Figure 3A,B). Furthermore, the IL-2 induction was disturbed in comparison to JAK1 WT, and although some increase was apparent in the STAT5 transcriptional activity assay, JAK1 I597F could not significantly respond to IL-2 addition ($p = 0.12$ between the basal vs. IL-2, 50 ng/mL). The pSTAT5 analysis of the mutant showed more variability, but also in this setting both the increased basal activity and the disturbed cytokine responsiveness were detected (Figure 3A,B). Mutation of the conserved lysine K622 in the JAK1 JH2 ATP-binding site (Table 1) to alanine reduced the cytokine induced STAT activation, thus correlating with the behavior of the JAK2 [8] and JAK3 homologs (Figure 3B).

To decipher the cause for the untypical behavior of the JAK1 JH2 ATP-site mutant I597F, we produced and purified JAK1 JH2s with I597F or K622A mutation. GST-tagged proteins were bound to glutathione sepharose, eluted by digestion with Tobacco Etch Virus TEV Protease and further purified in size-exclusion chromatography (SEC) (Figure 3C). The JAK1 JH2 mutants were then analyzed in differential scanning fluorimetry (DSF) with and without ATP. JAK1, JAK2, and TYK2 JH2s have been shown to bind ATP in the presence of divalent cations while JAK3 JH2 binds ATP without cations [8,9] and thus proteins were analyzed with and without $MgCl_2$. Figure 3C shows the melting temperatures (Tm), as well as the change in the melting temperatures (dTm) relative to the WT JH2 apo-form (protein that does not bind any ligands). As expected, ATP binding did not increase the Tm in K622A. However, the mutant showed significantly increased thermal stability. Compared to the WT JH2 Tm (44 °C), the I597F mutant showed reduced Tm of 40 °C, and addition of 1 mM ATP and 1 mM MgCl2 increased the melting temperature by 2 °C. The increase was only slightly differed from the observed 3 °C increase in WT JH1. The Tm for apo-form WT, I597F, and K622A were 44.3 °C, 40.3 °C, and 53.3 °C, respectively.

Taken together, our cell-based and recombinant protein assays suggest that the JAK1 JH2 ATP-site differs from other JAKs in that the I597F cannot efficiently block ATP binding and shows increased basal activity and no inhibition with hyperactivating mutants. These unexpected effects to the signaling may be due to reduced stability of the mutated JH2 with I597F. This hypothesis is supported by data

from JAK1 K622A that shows increased thermal stability and reduced signaling, probably stemming from the stabilizing interaction towards the αC that locks the whole domain in an inhibitory position.

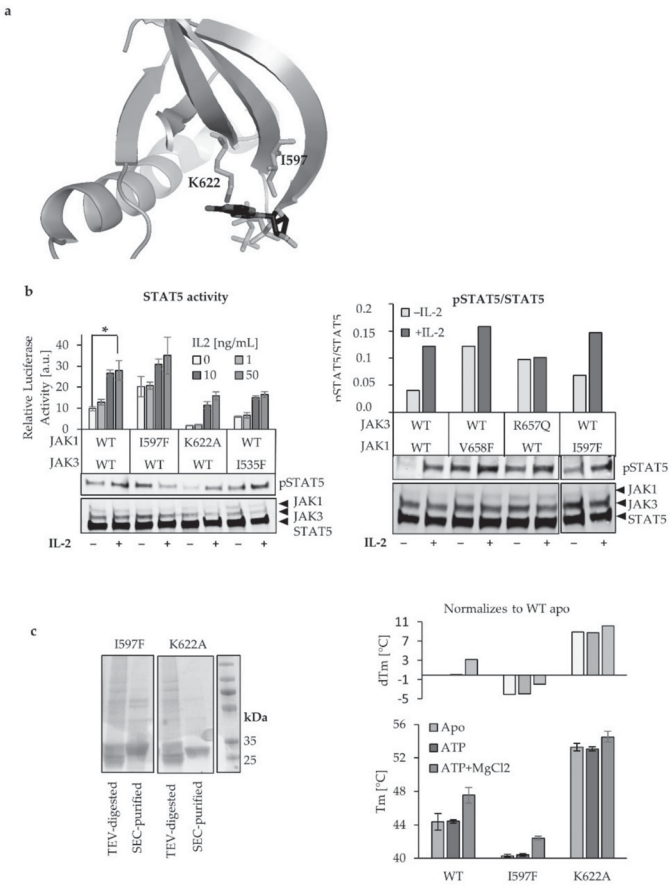

Figure 3. Characterization of the JAK1 JH2 ATP-binding site mutants. (**a**) Illustration of the JAK1 JH2 ATP-binding pocket, including the αC-helix of (PDB 4L00). The mutated residues K622 and I597 are shown, as well as ATP. (**b**) JAK1 I597F slightly increases the basal STAT5 activity and is responding to IL-2. JAK1 K622A shows reduced but cytokine-responsive STAT activation. STAT5 responsive luciferase system was used as previously described in U4Cγβ cells transfected with JAK1 and JAK3 JH2 ATP-site mutants or JAK WT. The errors are SD triplicate samples. **Below**: pSTAT5- and HA- labeled cell lysates from basal, and cytokine treated cells. Two-tailed *t*-test was performed and $p < 0.05$ is indicated as *. **Right**: comparison of JAK1 I597F with WT and hyperactive JAK1 and JAK3. Immunoblots from whole-cell lysates were labelled with HA (JAK1/JAK3/STAT5) and pSTAT5 antibodies to detect the pSTAT5/STAT5 ratios for basal and IL-2 stimulated (100 ng/mL) cells. (**c**) Recombinant JAK1 JH2 mutants vary in their stability. Differential scanning fluorimetry (DSF) analysis of size-exclusion chromatography (SEC)-purified JAK1 JH2 proteins show that JAK1 I597F has lower Tm compared to WT, while K622A increases Tm. **Left**: the SDS page gels with JAK1 JH2 elutions before- and after SEC purification. The size of the JAK1 JH2 is ~34 kDa. Right: DSF analysis showing protein Tm −/+ ATP and −/+ MgCl2. Errors are SD ($n = 6$). Graph above presents the dTm that is normalized to the wild-type JAK1 JH2 in its apo-form (protein that does not bind any ligands).

2.4. JAK1 L633K Inhibits Hyperactive JAK3 but Does not Inhibit Hyperactive JAK2 and TYK2

To further depict the role of JAK1, and its JH2 in IL-2 and IFNγ pathways, we studied the inhibition potential of the JH2 mutations towards the partner JAK (inhibition in trans). The JAK1 L633K was first transfected with active JAK3 R657Q into U4Cγβ cells and the pSTAT5 as well as the activity of endogenous STAT5 were detected (Figure 4A, Figure S3A). Correlating with the results presented above, JAK1 L633K had a strong inhibitory effect toward JAK3, while in the opposite experimental layout the αC mutated JAK3 E567R was unable to reduce JAK1 V658F hyperactivation. In the IFNγ system, however, JAK1 L633K was unable to effectively reduce JAK2 V617F hyperactivation (Figure 4B, Figure S2B). This observation supports the previous results showing that JAK1 mutants have less effect in the IFNγ-driven STAT1 activation when compared to the IL-2 induced STAT5 activation (see Figure 2B). Reduction of the IFNγ signaling occurred both in basal and in IFNγ stimulated cells when the JAK2 αC-mutant E592R was co-transfected with activating JAK1 V658F. However, the variation in the STAT1 activity was large, and the JAK2 E592R driven inhibition was not significant (two-tailed *p*-value 0.06). Similarly, large variation was detected also in IL-2 system with homologous JAK1 mutant although in the IL-2 system a distinct inhibition was observed (Figure 4C, Figure S2C).

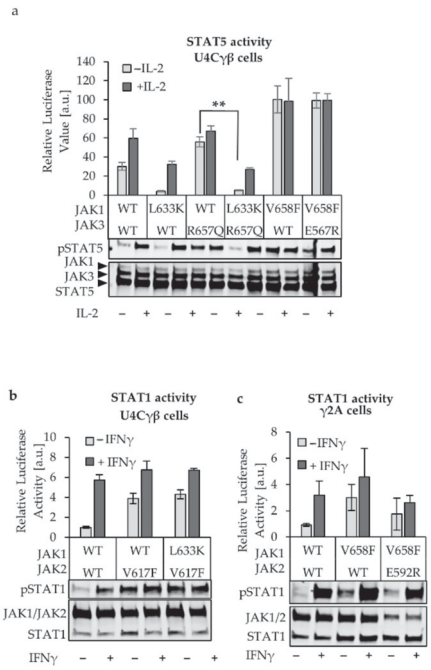

Figure 4. JAK1 L633K effect in trans. (**a**) JAK1 L633K reduces hyperactive JAK3 R657Q while homologous JAK3 E567R cannot reduce JAK1 V658F-driven activation. Both the transcriptional activity and the phosphorylation status of STAT5 were studied as previously described. Normalized SPI-Luc luciferase signal shows averages and SD of six experiments. HA- and pSTAT5 antibodies were used to detect the expression of JAKs and the STAT5/pSTAT5 ratio of non-stimulated and stimulated (100 ng/mL IL-2) cells. **—indicates $p < 0.001$ (two-tailed *t*-test) (**b**) JAK1 L633K does not efficiently inhibit JAK2 V617F driven STAT1 activation. JAK1 WT or L633K were transiently transfected with JAK2 V617F, and STAT1 (IFNγ) responsive IRF-GAS plasmid was used to detect the STAT1 activity as described earlier. Errors are SD of triplicate samples. Below: whole cell lysates from unstimulated (basal-state) cells transfected with JAK1 and JAK2 and labelled with HA and pSTAT1 antibodies. The blots are representative of three experiments. (**c**) JAK2 E592R inhibits JAK1 V658F-driven STAT1 activation. JAK2 WT or E592R were transiently transfected with JAK1 V658F and STAT1 (IFNγ) responsive IRF-

GAS plasmid into γ2A cells that lack JAK2, and the STAT1 activity was detected as above. Errors are SD of six replicas. Below: whole cell lysates from basal-state and stimulated (100 ng/mL IFNγ) cells that were transfected with JAK1 and JAK2 and labelled with HA and pSTAT1 antibodies.

In the IFNα system, JAK1 L633K did not show inhibition of the co-transfected TYK2 V678F (Figure S2C), and vice versa, TYK2 L653R at the JH2 αC did not show inhibition in the JAK1 V658F-driven basal pSTAT1 (Figure S2D). However, TYK2 L653R reduced the IFNα-induced activation (Figure S2D). In Figure 2B JAK1 L633K was previously shown to affect the IFNα-induced activation but not the basal STAT1 activation. This could indicate that TYK2 and JAK1 JH2 αC-helices are important for cytokine induced STAT1 activation, but not necessarily in maintaining the low basal activation state in the IFNαR complex.

2.5. JH2 Mutations in JAK Heterodimer Partners Show a Cumulative Inhibitory Effect

After establishing that JAK1 L633K in the αC strongly inhibits IL-2 signaling, and that JAK1 K622A at the ATP site also reduced hyperactivation in cis, we wanted to compare the inhibition potential between the two regulatory JH2 sites in trans. JAK1 JH2 ATP site mutant K622A reduced but did not abrogate hyperactivation in the JAK3 R657Q background (Figure 5A) and the mutation was similarly non-efficient against the JAK2 V617F driven STAT1 activation. In the work of Hammarén et al., the JAK2 JH2 ATP-site mutant I559F was found to lower the kinase reaction catalysis rates (k_{cat}) [17], which could explain the reduction in the pSTAT-values shown also with JAK1 K622A. However, the results indicate that ATP mutants have lesser inhibitory effect in comparison to the effect driven by the αC mutants.

Modulating JAK JH2s can reduce constitutively active JAK signaling, but unlike the kinase-dead mutations that target the JH1 active site and completely abolish the signaling, JH2 mutants tend to maintain the cytokine responsiveness [8,9,17]. Thus, we were interested to see the cumulative effect of the JH2 mutants and transfected JAK1 and JAK3, both carrying a homologous αC mutation into U4Cγβ cells (Figure 5B). IL-2 titration shows that the STAT5 activity is abrogated when the αC-helix of both JAKs is mutated, and the same is true regarding the pSTAT5 status. Interestingly, when both of the JAK1 and JAK3 JH2 ATP-binding sites are mutated, the IL-2 response is severely diminished but the cells maintain their responsiveness to the cytokine (Figure 5B, right side).

To be noted, the expression levels of the JAK3 mutants are slightly reduced in comparison to the JAK3 WT in the Figure 5B western blot, although this kind of variation in the expression levels was not generally observed between the JAK3 mutants. Moreover, the expression levels between the αC and ATP mutants are similar, and the results further supported by the transcriptional activity assay (luciferase assay above the western blot). Thus, the results can be considered representative in showing that the αC mutant combination abolishes IL-2 signaling, while ATP mutant maintains a IL-2 inductivity, albeit reduces the STAT activation.

As suggested by Hammarén et al., the disruption of the dimerization interaction between JH2 domains can be mediated by the in the αC and mutants in this region may result in complete loss of signaling. The JH2 ATP-binding site, on the other hand, likely affects the kinase activity in cis via regulating JH1 and thus have a weaker inhibitory potential [17].

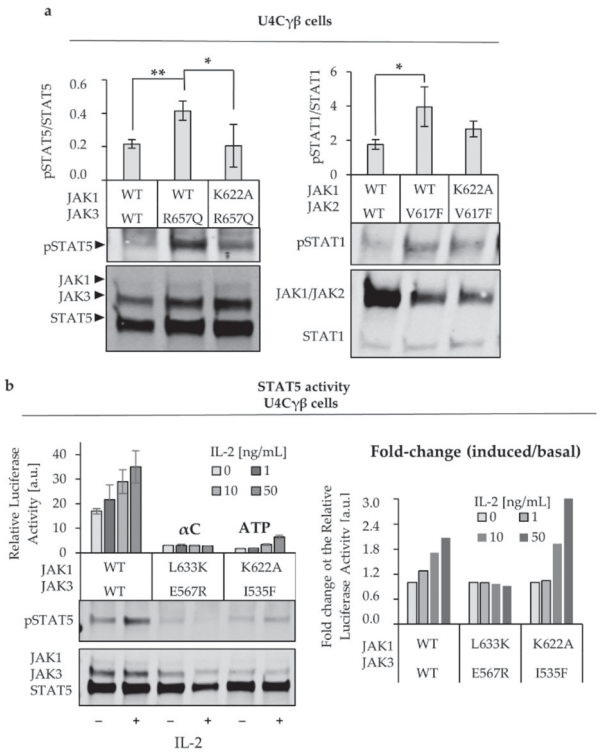

Figure 5. Characterizing the cumulative effect of the JAK JH2-mutants. (**a**) JAK1 K622A had variable reduction potential towards hyperactive JAK3 R657Q or JAK2 V617F. pSTAT1/5 was detected from cells transfected with HA-JAK1 and JAK3 or JAK2. The lysed cells were blotted to a membrane and labelled with HA, STAT1, and pSTAT1/5 antibodies as prescribed previously. Errors are SD of triplicate experiments. Two-tailed student *t*-test was performed between the WT and K622A in JAK3 R657Q background and indicates as * ($p < 0.05$) or ** ($p < 0.001$). (**b**) JAK1 and JAK3 with homologous JH2 αC mutations were co-transfected into U4Cγβ cells, which show abolishes IL-2 signaling. Similar setting with JAK1 and JAK3 ATP-site mutants can weakly respond to stimulation with 100 ng/mL of IL-2. STAT5-responsive plasmid was transfected as previously with JAK1 and JAK3 αC and ATP site mutants and after 43 h an increasing amount of cytokine was added into cells or the cells were only starved. Relative luciferase values of triplicates were detected and error are SD. **Below**: pSTAT5 analysis of cells transfected with JAK1 and JAK3 and treated, or not, with 50 ng/mL of IL-2. **Right**: the values shown in left were normalized to the basal values for each mutant (and WT) pair to show the fold change between the unstimulated and stimulated cells.

3. Discussion

JAK1 is the most widely employed JAK by a variety of cytokine receptors but the underlying molecular mechanisms of JAK1 regulation are still largely elusive. One interesting question is whether JAK1 function is conserved between the different receptor systems, and in this work, we investigated the role of JAK1 in IL-2, IFNγ, and IFNα signaling.

JAK1 null mice die perinatally and the newborn mice display a strong reduction of thymocytes, highlighting its importance in immunological and hematological functions [34]. Accordingly, somatic JAK1 GOF mutations are found in 10–20% of T-cell acute lymphoblastic leukemia (T-ALL) patients [35]. Recently, conditional JAK1 knock-out (KO) mice were developed, and RNA sequencing from isolated hematopoietic stem cells showed that the genes most affected by the loss of JAK1 were STAT1, STAT2, and multiple members of the IFN regulatory transcription family, confirming the important role of

JAK1 in regulating these factors [36]. Moreover, it was shown that loss of JAK1 leads to decreased IFNγ sensitivity ex vivo. Interestingly, constitutive JAK2 (V617F) activation could not rescue the defects in the JAK1 KO stem cells, which is supporting our observation of non-redundant functions of JAK1 and JAK2 in IFNγ signaling.

The focus of our study was on the JAK1 pseudokinase (JH2) domain, where we compared its function in IL-2, IFNγ, and IFNα signaling. Our results show that in the tetrameric IFNγR-complex both JAK1 and JAK2 are required for STAT1 activation while in IL-2 signaling JAK1 functions plays a dominant role in STAT phosphorylation. Interestingly, in IL-2 signaling both JH1 and JH2 in JAK1 were indispensable for signaling while the JH2 domain in JAK1 was crucial for IFNγ induced STAT1 activation, and deletion of JH1 only reduced stimulation (Figure 1). Thus, our data supports the model suggested by Briscoe et al. where JAK2 kinase activity is required predominantly for initiating signaling and possibly for the phosphorylation of STAT1, whereas JAK1 phosphorylates the IFNγR1 and recruits STAT [37]. Eletto et al. further suggest that JAK1 JH2 domain is required for interaction with JAK2 and conclude that this interaction, rather than JAK1 kinase activity, is mandatory for JAK2 activation after IFNγ stimulation leading to STAT1 phosphorylation [31]. Furthermore, our studies specify that the outer face of the αC-helix in JAK1 JH2 is important in allosteric regulation of the IFNγ (and more strongly the IL-2) signaling. However, evidence exists also for a dominant role of JAK1 in STAT activation at IFNγ, and kinase-dead JAK1 was found to abolish phosphorylation of JAK2 while kinase-dead JAK2 only lowered JAK1 phosphorylation (in both cases the pSTAT1 signal was abolished) [38]. In a recent study JAK1 JH2 αC mutations A639F, E637R, F636A, F575A were shown to almost completely abolish IFNγ signaling, while similar mutations in JAK2 only reduced the cytokine responsiveness [39]. However, also in this study, the above-mentioned JAK1 mutants had the potential to inhibit JAK3 in trans, in line with our observation. Lastly, Keil et al. have reported an important scaffold function of kinase-dead JAK2 in a mouse model of IFNγ and concluded that JAK1 is the main activator of STAT1 [40]. Our data shows a distinct difference between the dominance of JAK1 in IL-2 and IFNγ systems and indicate that JAK2 JH2 mutants have greater potential to alter the IFNγ system than JAK1. However, we cannot unequivocally point JAK2 as a dominant similarly to JAK1 in IL-2. In conclusion, the interplay between heteromeric receptors and heteromeric JAK pairs involves intricate and receptor specific regulation.

Although the structures of the JAK1, JAK2, and TYK2 FERM-SH2 domains have been solved with erythropoietin receptor (EPOR), leptin receptor (LEPR), and interferon receptors [41–43], the dimerization mechanism of JAKs and their cognate receptors is not yet fully resolved. In both JAK2 structures, FERM-FERM interactions between JAK2-molecules were apparent, but the residues contributing them varied slightly in EPOR and LEPR [44]. In addition to the FERM-FERM dimerization, also a JH2-JH2 interaction has been suggested [45]. A plausible model for the activation of JAKs is loosening of the JH1-JH2 interface that opens the conformation of the full-length JAK, allowing transphosphorylation of the adjacent JH1s [45]. The model is supported by the electron microscope images of the full-length JAK1 where "closed" (inactive) and "open" (active) conformations were observed [46]. More direct evidence of the JH2 dimerization came from the study of Hammarén et al., who showed that mutating the JAK2 JH2 αC reduces constitutive receptor dimerization driven by the V617F mutation [17]. Since the JAK1 αC-mutant used in this article is homologous to the JAK2 E592R studied by Hammarén et al., we hypothesize that also in JAK1 the mutant reduces dimerization of the JAK JH2s and thus the receptors. Together, these observations allowed us to suggest a model where JAK1 drives the oligomerization of IFNγ-receptor (IFNγR) complex, while JAK2 is the initiator and main contributor in the activation of STAT1 (Figure 6).

Individual JAKs show high specificity to distinct cytokine receptors. However, the study of Koppikar et al. showed that under some circumstances, conserved JAK pairing can be circumvented. They observed that long-term treatment with ruxolitinib results in resistance of JAK2 hyperactivation (persistence) that is caused by transphophorylation of JAK2 by JAK1 or TYK2 which allows constitutive STAT5 activation typically driven by homodimeric JAK2 [47]. Ruxolitinib is type I inhibitor targeting

the active conformation of the kinase. Interestingly, the persistence could be overcome by applying type II inhibitor, which binds to the inactive JAK and locks it in the unphosphorylated form [48].

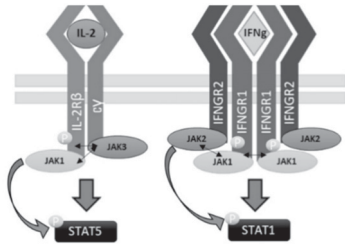

Figure 6. Illustration of the suggested activation cascades in IL2R and IFNγR systems. **Left**: Binding of IL-2 induces dimerization of the receptor subunits, possibly via JAK1 and JAK3 JH2-JH2 and FERM-FERM interaction allowing JAK1/3 transphosphorylation and activation leading to activation of STAT5 by JAK1. **Right**: In IFNγR JAK2 mediates STAT1 phosphorylation while JAK1 contributes by phosphorylating the IFNGR1, hence creating a docking site for the STAT1 and strengthening the oligomerized complex conformation.

Recently, Tvorogov et al. showed that ruxolitinib induces dose-dependent pJAK2, which can cause life-threatening cytokine-rebound syndrome (due to re-activation of STAT) when the drug is withdrawn [49]. Again, the effect was not apparent when the JAK2 V617F expressing cells were treated with type II inhibitor. These studies show the importance to consider the trans-activation properties between JAKs as well as the active vs. inactive conformation of the protein, even if the ATP transferase is inhibited. Thus, the phosphorylation status of drug inhibited JAK2 appears to be critical for the development of persistence. In conclusion, in the JAK-receptor complex both kinetic and structural characteristics appear to be critical determinants in activation of JAK-STAT signaling. Future studies are required to depict the exact mechanism of the receptor complex activation (dimerization and phosphorylation).

In line with previous studies in IL-2 signaling, we showed that JAK1 is dominant over JAK3, and JAK3 is incapable of inducing STAT activation in the absence of JAK1 [9,26]. Our data suggests that JAK3 does not directly phosphorylate STAT5 but is an important regulator of the cytokine responsive STAT5 activation, most likely through modulating the activation potential of JAK1. To study the roles of JAK3 and JAK1 more thoroughly, we reconstituted the JAK2 V617F homolog into JAK3 (M592F + L570F). Here, we showed that JAK2 V617F and homologous activating mutants in JAK1 and TYK2 could induce low levels of activation in the absence of the partner JAK, while in the WT context expression of both JAKs was crucial for functional signaling. However, the JAK1:JAK3 pair presents an exception as JAK1 WT could induce STAT5 activation in JAK3-deficient cells, although the activation was unresponsive to IL-2 (Figure 1). Interestingly, even with the V617F simulating, constitutively active JAK3 mutant M592F + L570F remained JAK1 dependent for STAT5 activation.

The apo structure of JAK1 JH2 has been solved but its regulatory function has not been investigated in detail. We focused on the allosteric JAK1 JH2 regions and their role in different signaling pathways and found that mutating residue L633 in the solvent-exposed face of αC-helix inhibits JAK1-driven signaling. The L633K mutant resides in the regulative JH1-JH2 interface and recently, a homologous JAK2 E592R mutant was shown to inhibit constitutive signaling in EPOR and IFNγR systems [17,50]. The L633K mutation was most effective in inhibiting IL-2 signaling, in basal and cytokine-induces signaling. In addition, JAK1 L633K effectively reduced STAT activation in a background where JAK1 or JAK3 were constitutively active (inhibition both in cis and in trans).

Lastly, we introduced two mutations to disrupt ATP binding to JAK1 JH2. The JAK1 I597F mutation, homologs of which were shown to inhibit constitutive activation in JAK2 and JAK3 and TYK2, did not inhibit JAK1, JAK2, or JAK3 driven hyperactivation, but even increased the basal STAT5 activation. Interestingly, the mutation decreased the stability of the JH2 recombinant protein, and did

not inhibit ATP binding. Another JAK1 JH2 ATP-site mutant, K622A inhibited STAT activation in both WT and JAK1 V658F context, and biochemical studies showed that the mutant stabilizes the JH2. To be noted, all tested JH2 mutants differed from the kinase-dead (JH1) mutants in that they maintained the cytokine inductivity. Combining the cell-based and recombinant protein derived data support mechanism of regulation where stabilizing JH2 inhibits hyperactivation while de-stabilizing further activates JAKs.

Taken together, we have obtained detailed information of the JAK1 JH2 and identified differences in JH2 function in different cytokine receptor pathways. These results underline the importance of thorough understand of the mechanism of JAK signaling as a means to create safer and more efficient inhibitors.

4. Materials and Methods

4.1. Plasmid Constructs and Mutagenesis

Full-length human JAKs were previously cloned in pCIneo expression vector by using SalI-NotI restriction sites. Full-length human STAT5A was in pXM vector. JAKs and STAT5A were C-terminally HA tagged. Site-directed mutagenesis was performed with QuikChange (AgilentTechnologies, Santa Clara, CA, USA), according to the manufacturer's instructions, and verified by using Sanger sequencing (see the primers that were used in Table 2). For luciferase reporter assays, firefly luciferase reporter constructs for STAT5 (SPI-Luc) or STAT1 (IRF-GAS/ISRE for IFNγ and IFNα respectively) were used together with a constitutively expressing renilla luciferase plasmid.

Table 2. Primer sequences for mutations used in this study:

	Fw	Rev
JAK1ΔJH2 (583–855)	atcctcaagaaggatctgaaaccagcaactgaagtggacccc	cttcagttgctggtttcagatccttcttgaggatccgatcg
JAK1Δ JH1-HA (583–1153)	ctgaaaccagcaactgaagtgtacccatacgatgttccagattacgcttag	ctaagcgtaatctggaacatcgtatgggtattcagttgctggtttcagatccttctt
JAK1 L633K	cagggatatttccaaggccttcttcgaggc	gcctcgaagaaggccttggaaatatccctg
JAK1 I597F	gagaacacacttctattctgggaccctgatgg	cccagaatagaagtgtgttctcgtgcctctcc
JAK1 V658F	ctatggcgtctgtttccgcgacgtggag	ctccacgtcgcggaaacagacgccatag
JAK1 K622A	gaagataaaagtgatcctcgcagtcttagaccccagccacagg	cctgtggctggggtctaagactgcgaggatcacttttatcttc
JAK2 E592R	gcacacagaaactattcacggtctttctttgaagcagc	gctgcttcaaagaaagaccgtgaatagtttctgtgtgc
JAK2 V617F	atggagtatgtttctgtggagacgagaatattctgg	tcgtctccacagaaacatactccataatttaaaacc
JAK2 I559F	ggccaaggcacttttacaaagttttttaaaggcgtacgaagagaagtagg	cctacttctcttcgtacgcctttaaaaaactttgtaaaagtgccttggcc
TYK2 V603F	cacaaggaccaacttctatgagggccgcc	ggcggccctcatagaagttggtccttgtg
TYK2 L653R	ccatgacatcgcccgggccttctacgagacagccagcc	cgtagaaggcccgggcgatgtcatggtgactagggtcc
TYK2 V678F	gcatggcgtctgtttccgcggccctga	tcagggccgcggaaacagacgccatgc
JAK3 I535F	ggtccttcaccaagttttaccggggctgtcgc	gcgacagccccggtaaaacttggtgaaggacc
JAK3 L570F	ggagtcattctttgaagcagcgagcttgatgagcc	ctcgctgcttcaaagaatgactccatgcagttcttgtgc
JAK3 M592F	ggcgtgtgctttgctggagacagcaccatggtgcagg	gtctccagcaaagcacacgccgtggagcagcacgagatgccgg
JAK3 E567R	gcacaagaactgcatgcgttcattcctggaagc	gcttccaggaatgaacgcatgcagttcttgtgc
JAK3 R657Q	aaggtgctcctggctcaggaggggctgatggg	cccatcagccccctcctgagccaggagcacctt

4.2. Mammalian Cell Culture

JAK1 and JAK3 deficient U4Cγβ, JAK2-deficient γ2A, TYK2 deficient 11.1. human fibrosarcoma cells were cultured according to standard culturing conditions in Dulbecco modified Eagle medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma, St Louis, MO, USA), 2 mmol/L L-glutamine (Lonza), and antibiotics (0.5% penicillin/streptomycin; Lonza). For transfection, cells were seeded on 24- or 96-well tissue-culture plates and transfected with FuGENE HD (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. Cells were transfected for 48 h and, where needed, cytokine stimulated in starvation medium without FBS for 15 min (for immunoblotting) or 5 h (for reporter assays), unless otherwise specified, with human recombinant IL-2 (PeproTech, Rocky Hill, NJ, USA), IFNγ (PeproTech) or IFNα (PeproTech).

4.3. Cell Transfection and Immunolabeling

Human fibroblast cell lines U4Cγβ, γ2A, and 11.1 (deficient in JAK1 and JAK3, JAK2 or TYK2, respectively) were transiently transfected using Fugene HD reagent with different human JAK-hemagglutinin (HA) constructs in pCIneo vector (75 ng for each per 24 well plate well). If two JAK constructs were transfected simultaneously, equal amounts of DNA were used. If STAT5 phosphorylation was studied, 2 ng or HA-tagged STAT5 was co-transfected. After 48 h transfection, cell were stimulated (if needed) for 20 min and washed with cold phosphate buffered saline (PBS). Triton X-100 lysis buffer with protease and phosphatase inhibitors (2 mM vanadate, 1 mM phenylmethanesulfonyl fluoride, 8.3 μg/mL aprotinin, and 4.2 μg/mL pepstatin) was used to lyse the cells. Whole cell lysates were spun for 20 min at 16,000 g at 4 °C, and the resulting supernatants were run on 4–15% Mini-PROTEAN®TGX™ Precast Gels (BioRad, Irvine, CA, USA). Immunoblots were blocked with bovine serum albumin (BSA) and incubated with primary antibodies for HA Tag (1:2000, OAEA00009, Aviva Systems Biology, San Diego, CA, USA), phosphorylated STAT1 (pSTAT1; 1:1000, #7649, Lot1 Cell Signaling), STAT1 (1:1000, 610116, BD Biosciences, San Jose, CA, USA), or phosphorylated STAT5 (pSTAT5; Cell Signaling, #4322, Lot9), and with a mixture of goat anti-rabbit and goat anti-mouse DyLight secondary antibodies (both from Thermo Fisher Scientific, Waltham, MA, USA). Blots were scanned with an Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA), and immunoblot signals were quantified with Image Studio software (LI-COR Biosciences) by manually assigning bands and dividing the phosphorylation (pSTAT1 or pSTAT5) signal values with the expression (STAT1 or HA) signals.

4.4. Luciferase and Dual Luciferase Assays

STAT5 transcriptional activity was assessed by measuring the luciferase expression (SPI-Luc 2) driven by a STAT5 responsive promoter, as described previously [2]. For STAT1 transcription activity detection, either IRF-Gas or ISRE-Luc plasmids (30 ng) were transfected instead of SPI-Luc 2 (transfected with 15 ng plasmid). These are specific for IFNγ and IFNα stimulated activation of STAT1, respectively [2,51]. U4Cγβ, 11.1, or γ2A cells were transfected with indicated DNA constructs including the STAT-reporter and with 15 ng renilla plasmid (pRL-TK). The latter was co-transfected as an internal transfection control. Transient transfections were done in 96 well plates with FuGENE HD (Promega) according to manufactures instructions. Then, 42 h after transfection, cells were stimulated (in starvation media) or starved for 5 h after which luciferase assays were analyzed using the dual luciferase reporter assay system (Promega) according to manufactures instructions. Luciferase values were measured with EnVision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA, USA). The results are presented as relative luciferase activity (arbitrary units: a.u.) corresponding to the firefly luciferase light emission values divided by renilla luciferase light emission values.

4.5. Recombinant Protein Production and Purification

JAK1 JH2 constructs spanning residues 561–852 of human JAK1, of the wild-type sequence or either I597F or K622A mutations were sub-cloned into pFastBac vector for expression as N-terminal glutathione-S-transferase (GST) fusion proteins with a tobacco etch virus (TEV) proteins. Constructs were expressed in High Five insect cells (Thermo Fisher Scientific) using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. After protein expression (10% P3 virus, 48 h, 27 °C), the cells were collected by centrifugation, resuspended in lysis buffer containing 50 mM Tris HCl pH 8.0, 10% Glycerol, 500 nM NaCl, 1 mM TCEP supplemented with phosphatase and protease inhibitors (100 mM sodium orthovanadate, 100 mM PMSF, 10 μg/mL pepstatin A), and lysed by applying four freeze-thaw cycles. The lysates were clarified by centrifugation and incubated 1.5 h with GSH-coupled resin beads (Protino Glutathione Agarose 4B). Beads were washed and the protein detached by digesting with a tobacco etch virus (TEV) protease (overnight at 4 °C). The flow-through was concentrated and run on a Superdex 200 gel filtration column equilibrated

in final buffer (20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 4 mM DTT and 0.02% CHAPS). The eluted peak was concentrated and stored at −80 °C.

4.6. Differential Scanning Fluorometry (DSF)

Thermal-shift experiments were carried out in 96-well PCR plates in a final volume of 25 µL with the following reagent concentrations: 6x Sypro Orange (Molecular Probes, cat. no. S6551), 3 µM protein Ni-NTA eluate, 1 mM MgCl2, 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 4 mM DTT and 0.02% CHAPS, and 1 mM ATP. Reactions were heated in a real-time CFX96 PCR cycler (Bio-Rad) at 1 °C per min from 4 °C to 95 °C with a fluorescence read every 1 °C. Fluorescence data were then normalized to represent unfolding curves, which were fitted to a Boltzmann sigmoidal equation with GraphPad Prism to obtain average Tm values with errors as SD.

5. Conclusions

Our studies show that JAK1 has varying roles in IL-2, IFNα, and IFNγ systems. Our results demonstrate that the pseudokinase domain (JH2) of JAK1 is an important regulatory region and modulating it can either down, or up-regulate JAK1-driven signaling. Specifically the outer face of the JH2 αC-helix was effective in reducing wild-type and constitutive STAT activation, although the potency dependent on the JAK and the related signaling complex. Furthermore, we highlighted biochemical and biological differences between the ATP-binding sites of JAK JH2s, which can be beneficial in design novel JAK modulators targeting outside the conserved kinase domain.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/1/78/s1, Figure S1. Both JAKs are required for cytokine responsive STAT activation in IFNα, IFNγ, and IL-2 systems: Figure S2. Modulating JH2 domain can suppress WT and hyperactive JAKs. (**a**) JAK1 L633K reduces basal pSTAT1 as seen with STAT-activity assay in Figure 2B; Figure S3. Effects of the αC-mutants in trans; Figure S4. Whole blots for figures.

Author Contributions: J.R. designed and performed the experiments and wrote the article, T.H. designed and cloned the recombinant JAK1 JH2 constructs and assisted in composing the article, O.S. supervised the work and critically read and edited the article. All authors have read and agreed to the published version of the manuscript.

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Supplementary: Figure 1

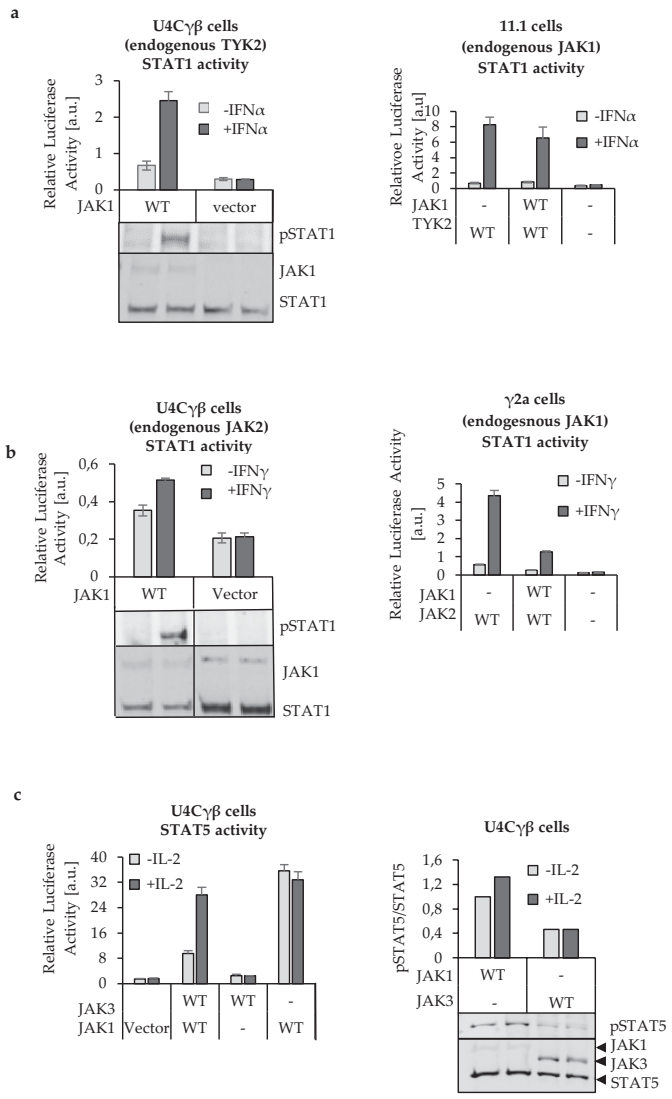


Figure S1. Both JAKs are required for cytokine responsive STAT activation in IFNα, IFNγ and IL-2 systems. (a) U4Cγβ or 11.1 cells were transiently transfected with WT JAK1 or TYK2, respectively, or with vector-only sample. IFNa responsive ISRE-Luc reporter was co-transfected to detect the STAT1 activation and pRK-TL. Neither JAK1-deficient U4Cγβ cells or TYK2-deficient 11.1 cells could respond to IFNa stimulation without transfecting the JAK that was not endogenously expressed (indicated in the headings). Errors are SD of triplicates. Cell lysates from U4Cγβ cells were immunolabeled with HA (JAK1), STAT1 and pSTAT1. The pSTAT1 status correlated with the activity data. (b) U4Cγβ cells or JAK2-deficient γ2A cells were transiently transfected with JAK1 or JAK2, respectively. IRF-GAS, IFNγ specific luciferase plasmid was used to detect STAT1 transcriptional activity and the values normalized with pRL-TK values. Errors show the SD of triplicate samples. Cell lysates from U4Cγβ cells were immunolabeled with HA (JAK1), STAT1 and pSTAT1 and the pSTAT1 status correlated with the luciferase data. (c) U4Cγβ cells were used to analyse the IL-2 systems similarly as above. Spi-Luc2 vector was used to detection of the STAT5 transcriptional activity. Right: an immunoblotted cell lysates and analysis of the pSTAT5/STAT5 ratios -/+ IL-2 with only JAK1 or JAK3 transfected. For pSTAT5 analysis, HA-tagged STAT5 was co-transfected.

Supplementary Figure 2

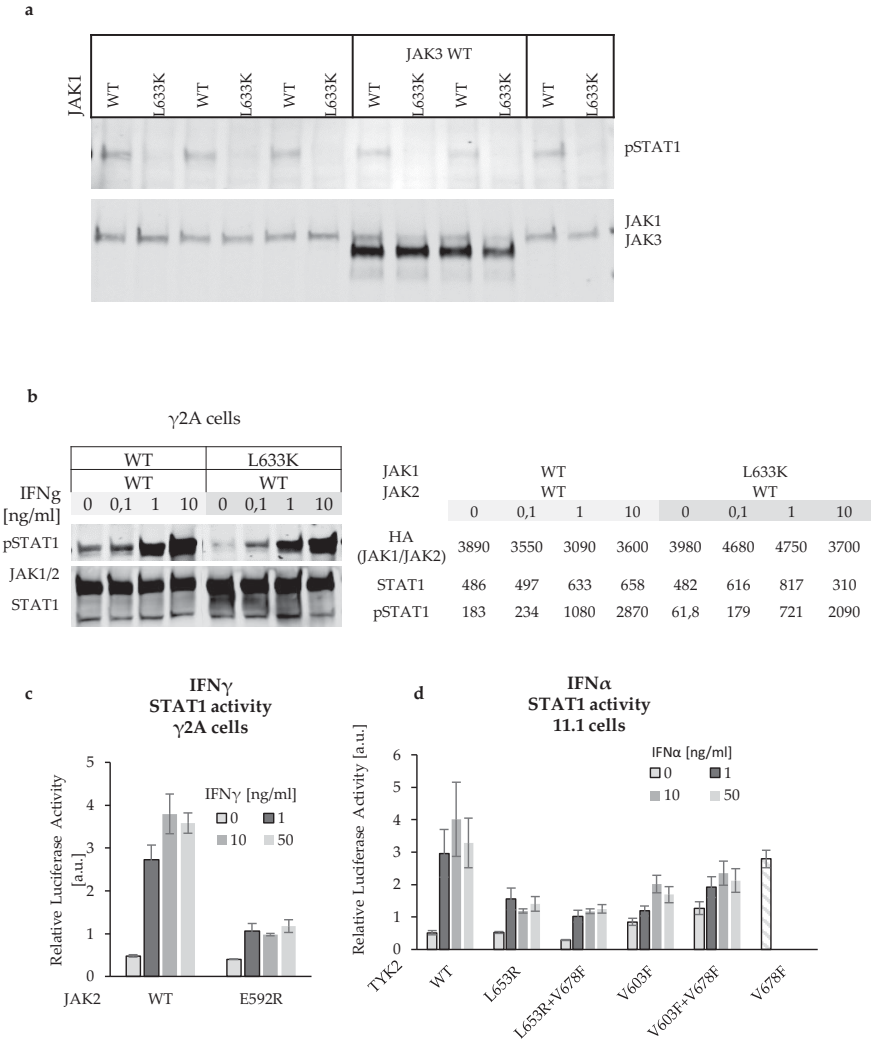


Figure S2. Modulating JH2 domain can suppress WT and hyperactive JAKs. (a) pSTAT1 analysis and expression test for Figure 2. JAK1 L633K reduces basal pSTAT1 as seen with STAT-activity assay in Figure 2B. (b) JAK1 L633K reduces basal, but not (effectively) the IFNγ stimulated pSTAT1. The effect seen with JAK1-deficient U4Cγβ cells was also apparent in JAK2- deficient γ2A cells (in JAK1-overexpression system). Cells were transfected with WT JAK2 and the L633K JAK1 and titrated with increasing amount of IFNγ. (c) JAK2 homolog strongly reduces IFNγ signaling. IRF-GAS plasmid was used to detect STAT1 activity. Average of triplicate samples (with SD) are shown. Data correlates with previous reports [Hammarén et al. 2019] (d) Homologous TYK2 JH2 αC (L653R)- and ATP-site (V603F) mutations used in this work inhibit IFNα response and constitutive activity *in cis*. ISRE-Luc plasmid was co-transfected with pTK-RL to detect relative IFNα-driven STAT1 activity. Errors are SD of triplicate samples.

Supplementary Figure 3

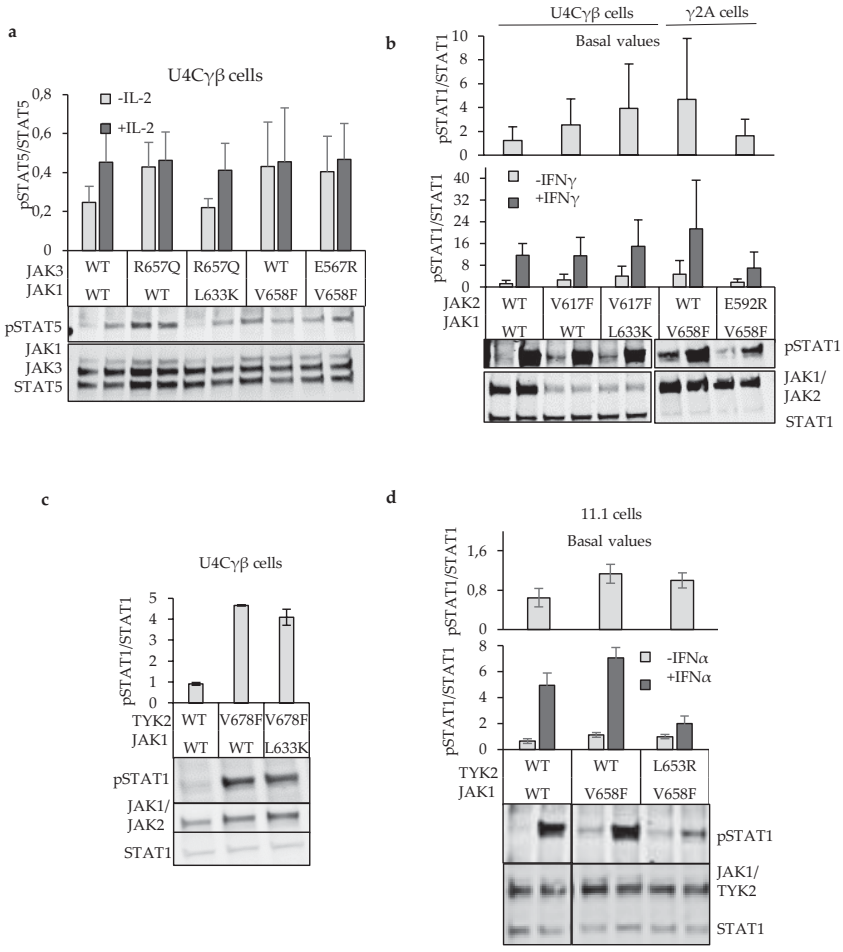
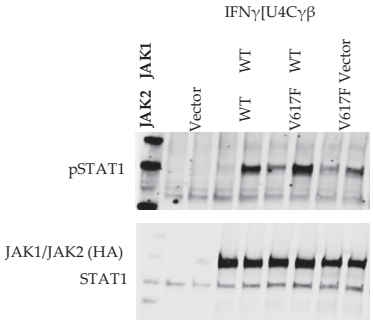
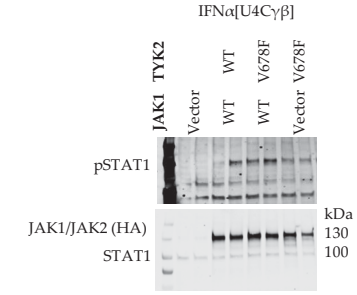


Figure S3. Effects of the αC-mutants in trans. (a) JAK1 L633K effectively inhibits JAK3-driven constitutive pSTAT5, while JAK3 homolog have no effect towards JAK1 V658F. HA-tagged JAK1, JAK3 and STAT5 were transiently transfected into U4Cγβ fibroblasts and stimulated with IL-2 or only starved. pSTAT5/STAT5 ratios were analyzed and the graph shows the averages and errors as SD of triplicate samples. (b) U4Cγβ or γ2A cells were transiently transfected with JAK1 L633K or homologous JAK2 mutant and their inhibitory potential was tested against V617F or homologous JAK1 mutant -/+ IFNg stimulation. Cell lysates were labelled with HA, STAT1 and pSTAT1 and the graph shows the pSTAT1/STAT1 average and SD from triplicate samples. The upper graph shows only the basal values from the lower graph. (c) U4Cγβ cells were transfected with TYK2 V678F and with WT JAK1 or JAK1 L633K. pSTAT1 from the un-stimulated cell lysates were analyzed, but no reduction of V678F driven hyperactivation could be detected. Errors are SD of triplicate samples. (d) TYK2- deficient 11.1 fibroblast cells were transiently transfected with JAK1 V658F and L653R TYK2, or WT JAKs, and the pSTAT1 analyzed from IFNa stimulated (or basal) cell lysates. The graph shows the average of triplicate samples with SDs. Only the basal values are shown in the upper graph.

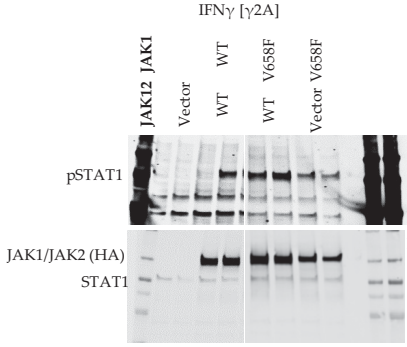
Blots from Figure 1a



JAK2	Vector		WT		V617F		V617F	
JAK1	Vector		WT		WT		Vector	
HA (JAK1/JAK2)	10,6	71,6	2500	2220	2470	2770	2100	1690
STAT1	212	152	318	373	393	497	537	519
pSTAT1	24,2	49	74,5	762	276	1070	279	417
pSTAT1/STAT1	0,11	0,32	0,23	2,04	0,70	2,15	0,52	0,80

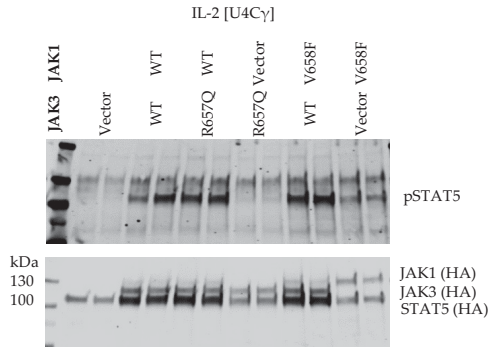


TYK2	Vector		WT		V678F		V678F	
JAK1	Vector		WT		WT		Vector	
HA (JAK1/TYK2)	12	29,7	1420	1090	1710	1080	1330	686
STAT1	136	128	135	153	200	141	155	90,1
pSTAT1	8,96	27,3	59,2	194	167	181	86,8	67,3
pSTAT1/STAT1	0,07	0,21	0,44	1,27	0,84	1,28	0,56	0,75



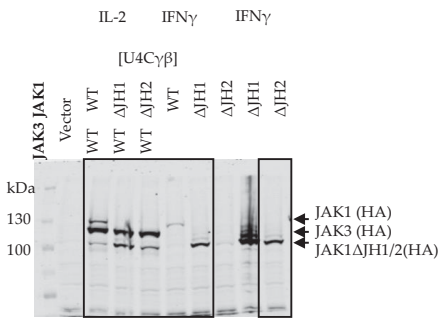
JAK1	Vector		WT		V658F		V658F	
JAK2	Vector		WT		WT		Vector	
HA (JAK1/JAK2)	0,878	3,43	1300	1380	2020	1670	1030	803
STAT1	379	6,6	33,4	196	236	339	267	114
pSTAT1	86,9	33,5	98,8	67,6	158	63,3	144	99,3
pSTAT1/STAT1	4,36	0,20	0,34	2,90	1,49	5,36	1,85	1,15

Blots from Figure 1b



JAK3	Vector		WT		R657Q		R657Q		WT		Vector	
JAK1			WT		WT		Vector		V658F		V658F	
JAK3	1,13	0,208	131	88,6	128	112	114	106	164	137	-6,03	-4,57
JAK1	-0,841	-0,239	34,9	25,6	25,8	47,3	17,9	16,3	69,3	43,1	51,8	49,2
STAT5	156	118	377	372	580	412	162	168	496	504	165	142
pSTAT5	26,3	18	131	307	332	333	34,3	45,4	321	409	130	117
pSTAT5/STAT5	0,17	0,15	0,35	0,83	0,57	0,81	0,21	0,27	0,65	0,81	0,79	0,82

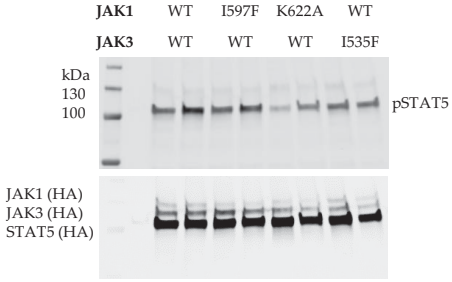
Blots from Figure 1c

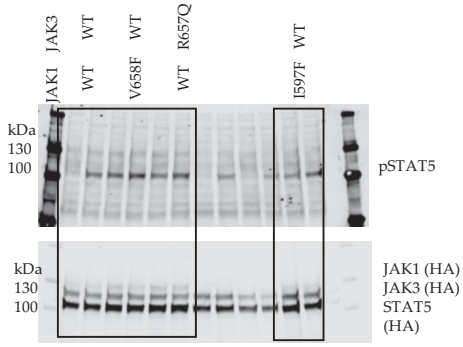


JAK1	WT	ΔJH1	ΔJH2	WT	ΔJH1	ΔJH2
JAK3	WT	WT	WT	-	-	-
JAK1 ΔJH1/2	58,8*	460	165	17,9	575	504
JAK3 (HA)	1190	725	867	23,8	48,8	41,2
JAK1 WT (HA)	178	28,3	57,2	72,7	17,2	18,8

* Unspecific band

Blots from Figure 3b

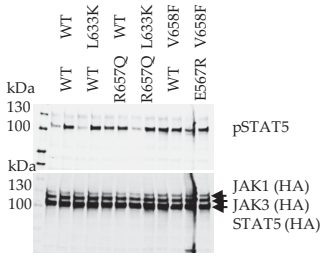




JAK1	WT		I597F		K622A		WT	
JAK3	WT		WT		WT		I535F	
JAK1 (HA)	753	701	535	400	367	331	763	542
JAK3 (HA)	1100	1200	1480	1140	859	674	745	954
STAT1	8530	8570	8490	8580	10200	7020	11500	6840
pSTAT1	10100	13300	9140	11600	3100	7640	9580	8660
pSTAT1/ STAT1	1,2	1,6	1,1	1,4	0,3	1,1	0,8	1,3

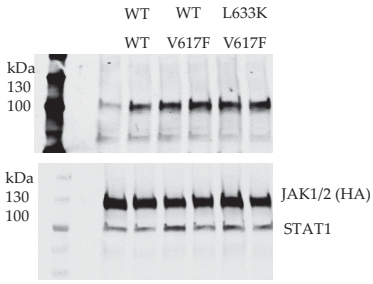
JAK3	WT		WT		R657Q		WT	
JAK1	WT		V658F		WT		I597F	
STAT5	1200	1240	1790	1540	1700	1790	1710	1450
JAK3	446	366	464	457	431	461	735	608
JAK1	108	92	130	123	150	127	93,9	94,3

Blots from Figure 4a



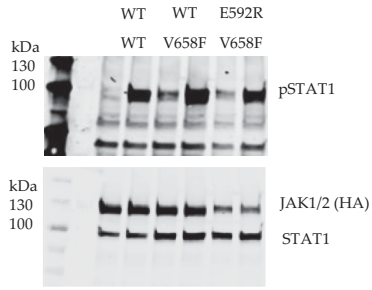
JAK1	WT		L633K		WT		L633K		V658F		V658F	
JAK3	WT		WT		R657Q		R657Q		WT		E567R	
JAK1	255	404	361	449	387	295	255	503	576	543	2500	795
JAK3	1030	1340	1130	1530	1250	1100	830	1330	1090	868	4080	1200
STAT5	6610	9010	10900	13200	7810	7730	7420	10900	9320	7240	7480	8350
pSTAT5	1890	6810	1410	8530	4610	5860	1670	7920	7290	6870	5640	7750
pSTAT5/STAT5	0,29	0,76	0,13	0,65	0,59	0,76	0,23	0,73	0,78	0,95	0,75	0,93

Blots from Figure 4b



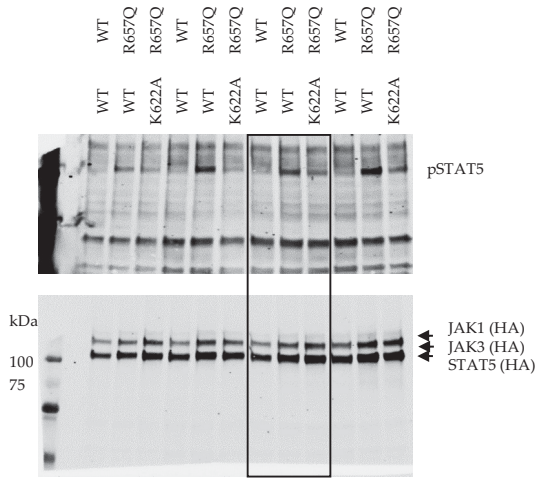
JAK2	WT		V617F		V617F	
JAK1	WT		WT		L633K	
JAK1/JAK2 (HA)	2800	2390	2120	2120	3100	1980
STAT1	539	440	650	718	801	745
pSTAT1	176	406	571	725	686	907
pSTAT1/STAT1	0,33	0,92	0,88	1,01	0,86	1,22

Blots from Figure 4c

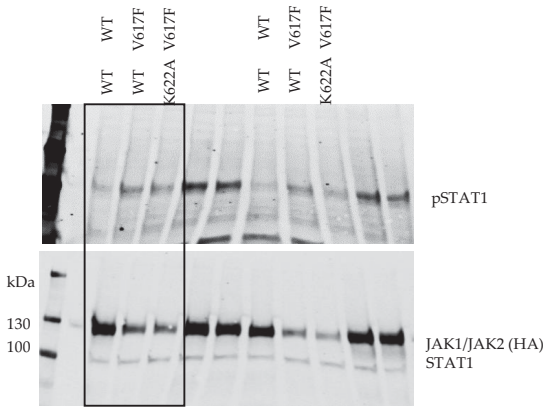


JAK1	WT		V658F		V658F	
JAK2	WT		WT		E592R	
JAK1/JAK2 (HA)	1810	1780	1650	1660	575	611
STAT1	714	772	1370	1620	1010	1190
pSTAT1	0,16	2,41	0,24	1,91	0,19	0,83

Blots from Figure 5a

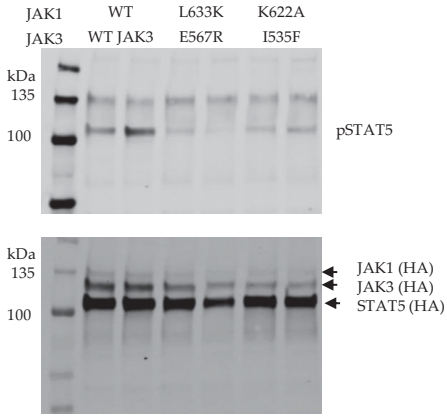


JAK1	**WT**	**R657Q**	**R657Q**	**WT**	**R657Q**	**R657Q**	**WT**	**R657Q**	**R657Q**	**WT**	**R657Q**	**R657Q**
JAK3	**WT**	**WT**	**K622A**	**WT**	**WT**	**K622A**	**WT**	**WT**	**K622A**	**WT**	**WT**	**K622A**
STAT5	541	514	263	386	285	183	276	349	218	301	199	145
pSTAT5	90,5	188	62,6	87,9	144	79,2	72,9	167	73,1	63,3	89,3	24,7
JAK1	9,17	12,9	16,7	12,3	26,4	18,5	15,1	25,4	21,8	17,8	35,9	54,4
JAK3	53	79,7	116	71,7	126	97,6	68,6	111	129	87,4	170	170



JAK2	**WT**	**V617F**	**V617F**	**WT**	**V617F**	**V617F**
JAK1	**WT**	**WT**	**K622A**	**WT**	**WT**	**K622A**
HA (JAK1/JAK2)	484	100	62,6	319	75,9	40
STAT1	18,2	13,1	18,8	13,4	12,7	8,21
pSTAT1	33,3	48,3	38,3	25,2	64	15,4
pSTAT1/STAT1	1,8	3,7	2,0	1,9	5,0	1,9

Blots from Figure 5b



JAK1	**WT**		**L633K**		**K622A**	
JAK3	**WT JAK3**		**E567R**		**I535F**	
JAK1	59,9	44,8	26,2	19,3	21,4	21
JAK3	206	196	125	68,2	75,7	68,8
STAT5	1440	1550	1190	468	1350	1120
pSTAT5	697	1160	247	120	327	405
pSTAT5/STAT5	0,5	0,7	0,2	0,3	0,2	0,4

PUBLICATION III

Structural models of full-length JAK2 kinase

Ayaz P., Hammarén H.M., Raivola, J. Abraham B.G., Sharon D., Hubbard S.R., Silvennoinen O., Shan Y., Shaw D.E.

Submitted manuscript.