Functional studies of Tyk2 in IFNalpha signaling : a new interactor and an activating mutation

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Douglas Adams, *The Hitch Hikers's Guide to the Galaxy*

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List of abreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BMM	Bone marrow macrophages
β-TrCP	beta-transducin repeats-containing protein
CIS	cytokine-inducible SH2 domain-containing protein
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
DBD	DNA-binding domain
EBP	Epo-binding protein
Epo	erythropoietin
ERK	extracellular-regulated kinase
FERM	band 4.1-ezrin-radixin-moesin domain
FACS	fluoresence-activated cell sorting
FAK	focal adhesion kinase
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GAS	gamma-activated site
GH	growth hormone
HIES	Hyper IgE syndrome
IL	interleukin
IFN	interferon
IFNAR	interferon receptor
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
IRF	interferon response factor
Jak	Janus kinase
Jakmip1	Jak and microtubule interacting protein 1
JH	Jak homology
KL	kinase-like
KO	knock-out

LIF	leukemia inhibitory factor
MEF	mouse embryonic fibroblasts
MPD	myeloproliferative disorders
NK	natural killer cells
NLS	nuclear localisation signal
OSM	oncostatin M
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PIAS	protein inhibitor of activated STATs
Pot1	Partner of Tyk2 1
PTP	Protein tyrosine phosphatase
RANKL	receptor activator of nuclear κB ligand
RACE	rapid amplification of cDNA ends
SCID	severe combined immunodeficiency disease
SH2	Src-homology 2 domain
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signaling
SUMO	small ubiquitin-like modifier
TAD	transcriptional activation domain
TCPTP	T cell protein tyrosine phosphatase
TGFβ	transforming growth factor β
Th	T helper
TK	tyrosine kinase domain
TNF	tumor-necrosis factor
Tpo	thrombopoietin
VHR	Vaccinia H1-related phosphatase
WT	wild-type

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Résumé

Les récepteurs des cytokines à structure α-hélicoïdale sont pour la plupart composés de deux sous-unités transmembranaires associées aux protéine tyrosine kinases de la famille Janus (Tyk2, Jak1, Jak2 et Jak3). La fixation de la cytokine à son récepteur induit une dimérisation des sous-unités ce qui entraîne l'activation des Jak par transphosphorylation. Les Jaks ainsi activées phosphorylent les facteurs de transcription STAT (Signal Transducer and Activator of Transcription) qui transloquent dans le noyau. Les tyrosine kinases Jak présentent en position N-terminale un domaine FERM (4.1-ezrin-radixin-moesin), suivi par un domaine dit 'SH2-like' et deux domaines kinases: un domaine dit 'kinase-like' (KL) à fonction régulatrice et un domaine catalytique de type tyrosine kinase en position C-terminale. L'association au récepteur se fait par la région N-terminale. Le laboratoire s'intéresse particulièrement au récepteur de l'interféron de type I (IFNα/β) composé de deux chaînes, IFNAR1 et IFNAR2, et aux kinases Tyk2 et Jak1 auxquels elles s'associent respectivement.

La première partie de ma thèse a porté sur l'étude d'un nouveau partenaire de Tyk2, la protéine Pot1 (Partner of Tyk2) et de son rôle potentiel dans la voie de signalisation de l'IFNα. Plusieurs ADNc partiels de Pot1 avaient été isolés dans le laboratoire par criblage double-hybride utilisant comme appât le domaine FERM de Tyk2. Un ADNc complet de Pot1 avait été reconstruit donnant lieu à une protéine de 1003 acides aminés (aa). L'interaction entre cette protéine et les domaines FERM de Tyk2 et de Jak1 avait été confirmée par des expériences *in vitro*. L'analyse par northern blot de Pot1 montre une expression faible dans plusieurs tissus. L'étude de la localisation de la protéine Pot1 surexprimée avait montré une localisation cytoplasmique au niveau de vésicules non identifiées.

Un des aspects de mon travail sur Pot1 a été de déterminer si l'ADNc reconstruit dans le laboratoire, appelé 'lab cDNA', correspond à un vrai transcrit. En effet, les banques de données recensent plusieurs autres transcrits issus du gène *Pot1*, avec une portion 3' commune et identique au 'lab cDNA', mais une région 5' divergente. Ces transcrits donnent lieu à deux isoformes ne possédant pas les 200 aa ou 250 aa en position N-terminale par rapport à la protéine de référence codée par le 'lab cDNA'. De plus, l'unique protéine murine (mPot1) recensée dans les banques de données ne possède pas les 100 aa en N-terminal par rapport à la protéine humaine de référence. Pour analyser l'expression de différents transcrits, j'ai amplifié l'ADNc de cellules humaines HEK293T avec des primers me permettant de

distinguer les différents transcrits. Ces expériences ont permis de confirmer l'expression du transcrit correspondant au 'lab cDNA' et codant pour une isoforme de 1003 aa (112 kDa).

Par la suite j'ai étudié la localisation de la protéine Pot1 murine dans les cellules surexprimant mPot1. J'ai observé que mPot1 se trouve dans des vésicules cytoplasmiques, tout comme la protéine humaine, et semble être associée à la membrane de ces vésicules. Toutefois, nous ne pouvons pas écarter la possibilité de localisation inadéquate dû à la forte surexpression de mPot1. Il sera nécessaire de confirmer cette observation par l'analyse de la localisation de la protéine endogène. A ce jour les anticorps disponibles ne permettent pas sa détection.

Afin d'évaluer le rôle de Pot1 dans la voie de signalisation de l'IFNα, j'ai mesuré la réponse à l'IFNα de cellules déplétées en Pot1 par interférence à l'ARN. J'ai mesuré la phosphorylation des protéines STAT et l'induction d'un gène rapporteur. Ces expériences ont montré que, dans ce système, la diminution de l'expression de Pot1 n'a pas d'effet sur la signalisation par l'IFNα.

Afin d'éclairer la fonction de Pot1, de nouveaux interacteurs de Pot1 ont été recherchés par criblage double-hybride. Parmi les 14 protéines identifiées à haut niveau de confiance, nous nous sommes particulièrement intéressés à GIT1 (G protein-coupled receptor kinase interactor), une protéine adaptatrice impliquée dans de nombreux processus cellulaires, tels que l'internalisation de récepteurs, la signalisation induite par l'EGF (epidermal growth factor) et l'angiotensine II ainsi que la migration cellulaire. Afin d'analyser le rôle éventuel de GIT1 dans la signalisation par l'IFNα, j'ai mesuré plusieurs paramètres (internalisation des sous-unités du récepteur, phosphorylation des STAT, induction d'un gène rapporteur) dans des cellules surexprimant ou déplétées en GIT1. Les résultats obtenus ont écarté l'hypothèse d'une implication de GIT1 dans la réponse transcriptionnelle à l'IFNα.

La deuxième partie de mon travail a porté sur l'étude de la mutation V678F introduite dans la protéine Tyk2.Cette substitution est située dans le domaine KL et correspond à la mutation V617F de Jak2, décrite comme étant à l'origine de la *Polycythemia vera*. La *P. vera*, ou maladie de Vaquez, est une maladie myéloprolifération caractérisée par une hyperproduction d'érythrocytes et leur hypersensibilité à l'érythropoiétine (Epo). Il a été montré que la mutation V617F induit une augmentation de l'activité kinase de base de Jak2. De même, Jak2V617F induit une prolifération indépendante de facteurs de croissance des cellules murines BaF3, confirmant son potentiel transformant. Toutefois, il a été montré que

Jak2V617F nécessite la coexpression d'un récepteur homodimérique, tel que récepteur à l'Epo, pour exercer une activité transformante maximale.

Les questions que nous nous sommes posées sont les suivantes: 1) quel est l'effet de la substitution V678F sur l'activité catalytique de Tyk2? 2) quel est l'effet du mutant Tyk2V678F sur la signalisation par l'IFNα? 3) le mutant Tyk2V678F aurait-il un effet plus marquant ou délétère s'il est placé dans un contexte de récepteur homodimérique?

A cet effet, nous avons établi, à partir de cellules Tyk2-déficientes, des clones stables exprimant la protéine sauvage ou mutante. Nos résultats montrent que la mutation V678F augmente *in vivo* et *in vitro* la capacité de Tyk2 à s'auto-phosphoryler. Par la suite, j'ai mesuré la phosphorylation sur tyrosine des protéines STAT en réponse à l'IFNα. Aucune différence de niveau de phosphorylation de STAT1/2/5 n'a pu être décélée entre les cellules exprimant la protéine sauvage et les cellules exprimant le mutant Tyk2V678F. Par contre, j'ai mis en évidence une phosphorylation basale de STAT3 augmentée en présence de Tyk2V678F. Pour déterminer si cette phosphorylation basale de STAT3 corrèle avec une augmentation de son activité transcriptionnelle, j'ai analysé l'activité transcriptionelle de STAT3 à l'aide d'un gène rapporteur. Les résultats montrent que, dans les cellules exprimant le mutant Tyk2V678F, STAT3 possède une activité transcriptionelle augmentée.

Afin d'étudier l'effet du mutant Tyk2V678F placé dans un contexte de récepteur homodimérique, j'ai utilisé des cellules exprimant un récepteur chimérique comprenant l'ectodomaine du récepteur à l'Epo fusionné aux régions transmembranaire et cytoplasmique de la chaîne IFNAR1 (EpoR/R1). A l'aide de ces cellules, j'ai pu confirmer que l'expression du mutant Tyk2V678F engendre une phosphorylation basale de STAT3. De plus, dans ce contexte de récepteur homodimérique, suite à stimulation par le ligand Epo, le mutant Tyk2V678F induit une augmentation ultérieure de la phosphorylation de STAT1, STAT3 et STAT5.

Nous avons aussi voulu comparer directement le niveau de phosphorylation de Tyk2V678F et de STAT3 dans différents contextes de récepteurs. Les résultats obtenus montrent que la protéine Tyk2V678F est plus phosphorylée basalement dans les cellules exprimant le récepteur homodimérique EpoR/R1. Ceci est probablement la conséquence d'une transphosphorylation plus efficace de deux kinases mutantes juxtaposées. Par contre, la phosphorylation de STAT3 ne corrèle pas directement avec le niveau d'expression du mutant Tyk2V678F, ce qui suggère une absence de corrélation linéaire entre l'activation de Tyk2 et et de STAT3.

En conclusion, nous avons montré que la mutation V678F augmente l'activité catalytique de Tyk2. De plus, le mutant acquiert la capacité de phosphoryler STAT3 et ceci en absence du ligand. Cependant, le mutant Tyk2V678F n'affecte pas la réponse à l'IFNα en terme de phosphorylation de Jak1, STAT1 et STAT2. Ces résultats démontrent une interaction fonctionnelle étroite entre Tyk2 et STAT3. Etant donné que STAT3 constitutivement actif exerce des propriétés oncogéniques et que STAT3 est phosphorylé dans de nombreux cancers, il est possible de prédire aussi un rôle oncogénique pour Tyk2 constitutivement active.

Récemment, il a été suggéré qu'un polymorphisme de Tyk2, P1104A, pourrait être associé à la présence de tumeurs. Cette substitution est située dans le domaine tyrosine kinase au sein d'une hélice α présente uniquement dans les membres de la famille Jak. Nous avons introduit cette mutation dans Tyk2 et analysé son effet sur l'activité kinase. Les résultats montrent que le mutant Tyk2P1104A est incapable de s'autophosphoryler *in vitro*. Toutefois, en réponse à l'IFNα, aucune différence du niveau de phosphorylation de STAT1/2 /3 n'est décélée dans les cellules exprimant Tyk2P1104A par rapport aux cellules exprimant la protéine sauvage. Ces résultats suggèrent que la mutation P1104A abolit la capacité autophosphorylante de l'enzyme, mais n'affecte pas l'activité enzymatique induite par l' l'IFNα envers d'autres substrats *in vivo*. Ces résultats préliminaires devront être renforcés par des études plus approfondies de l'effet de la mutation P1104A sur la fonction que Tyk2 exerce au sein du récepteur de l'IFNα/β ainsi qu'au sein d'autres récepteurs de cytokines de la réponse immune.

Introduction

1. Cytokine signaling

Cytokines can be described as secreted polypeptides, acting as intercellular mediators with pleiotropic regulatory effects on all aspects of cell physiology. No short definition can encompass their diverse properties, thus cytokines can be best defined by the following general features (Vilcek, 2003):

- redundancy: different cytokines may have an overlapping spectrum of actions
- pleiotropy: cytokines may have multiple target cells and multiple actions
- synergism/antagonism: exposure of cells to two or more cytokines at a time may lead to qualitatively different responses
- constitutive production of cytokines is usually low or absent; cytokine production is regulated by various stimuli at the transcriptional and/or post-transcriptional level
- cytokine production is transient and the action radius is usually short (typical action is autocrine or paracrine)

The most widely accepted classification of cytokines is based on their structural features. Accordingly, there are several cytokine families, such as: α-helical, TNF (tumor-necrosis factor), TGFβ (transforming growth factor β), IL-1 (interleukin-1), chemokines. Here, I will focus on α-helical cytokines and their downstream signaling pathways.

1.1. α-helical cytokines and their receptors

The members of the α-helical cytokine family (also known as the hematopoietic growth factor family) are characterized by a similar four-helical bundle topology (Sprang, 1993). The first and second pair of helices in this fold are linked by long overhand connections packed against one side of the molecule. This topological feature ensures that the arrangement of helices in the bundle core resembles that of a classical antiparallel four-helix bundle in spite of the fact that the chain-sequential helices A and B, and C and D, are parallel (Fig. 1A). These cytokines bind receptors that, based on structural and evolutionary properties, can be grouped as class I receptors and class II receptors (see below).

1.1.1. Erythropoietin and interferon α: two representative α-helical cytokines

Given the large number of α-helical cytokines known to date (over 40), I will briefly describe below erythropoietin (Epo) and interferon α2b (IFNα2b), that I have used in my studies.

Human **Epo** has been co-crystallized with the extracellular region of EpoR (Epo binding protein, EBP) (Syed et al., 1998). Epo has an up-up-down-down four-helical bundle topology observed for other ligands of class I and class II cytokine receptors. It also contains two small antiparallel β-strands. The structure of human Epo complexed with EBP shows that one molecule of Epo binds two receptors (Fig. 1B). Furthermore, it was shown that the Epo receptor is a preformed dimer in the absence of Epo and that ligand binding induces a conformational change enabling activation of downstream signaling cascades (Livnah et al., 1999; Remy et al., 1999).

Both human **IFNα2b and IFNβ** (Fig. 1C) have been crystallized as zinc-mediated dimer (Karpusas et al., 1997; Radhakrishnan et al., 1996). However, experimental data (Radhakrishnan et al., 1996) and recent modelling of IFN binding to its receptor (Lamken et al., 2004) suggest that the active form of IFNα and IFNβ is monomeric. The topology of IFNα2b resembles the classical up-up-down-down four-helix bundle motif. However, IFNα2b is distinguished from other α-helical cytokines by the presence of a fifth helix in the molecular core. The presence of a similar length helix in the structure of IFNβ, IFNγ and IL-10, all of which bind class II cytokine receptors, suggests that this helix is a defining characteristic of the ligands of the class II receptors. The IFNα/β receptor complex is made of two subunits that are brought together after ligand binding. The mechanism of IFNα/β-receptor binding will be discussed in detail below (cf. IFNα/β signaling).

1.1.2. Class I and class II receptors

Both class I and class II receptors are transmembrane glycoproteins with a single membrane-spanning region of 20-25aa. These receptors generally contain one extracellular globular domain termed D200, made of two subdomains (SD100) of approximately 100aa (Bazan, 1990). The D200 of class I receptors contains 4 conserved cysteine residues in the N-terminal subdomain (SD100A or SD1) and a WSXWS box in the C-terminal subdomain (SD100B or SD2). Class II receptors share two cysteines with class I receptors and have an additional pair, but lack the WSXWS motif.

Functionally, the majority of class I and class II receptors are in the form of heterodimers. However, a small number of them function in the form of homodimers (receptors for Epo,

thrombopoietin (Tpo), prolactin (PRL), growth hormone (GH), granulocyte-colony stimulating factor (G-CSF)) or heterotrimers (ex. receptors for IL-2 and IL-15). Many of cytokine receptors can be grouped in subfamilies with shared components: one of the chains forming the heteromer is common to all members (some examples are given in Table 1).
Class I and class II cytokine receptors lack catalytic activity and they rely primarily, if not solely, on the Jak tyrosine kinases for initiation of signal transduction (Fig. 2).

Cytokine receptor class	Shared subunit	Ligands
class I	gp130	IL-6, IL-11, LIF, CT-1, CNTF, OSM, IL-27
	γ-chain (γc)	IL-2, IL-4, IL-7, IL-9, IL-15
	β-chain (βc)	IL-3, IL-5, GM-CSF
	IL-12Rβ1	IL-12, IL-23
	IL-4Rα	IL-4, IL-13
class II	IFNAR1, IFNAR2	type I IFNs
	IL-10R2	IL-10, IL-22, IL-26, type III IFNs
	IL-20R1	IL-19, IL-20, IL-24, IL-26
	IL-20R2	IL-19, IL-20, IL-24
	IL-22R1	IL-22, IL-20, IL-24

Table 1. Examples of receptor subunits shared among different ligands. LIF: leukemia inhibitory factor; CT-1: cardiotrophin-1; CNTF: ciliary neurotrophic factor; OSM: Oncostatin M; GM-CSF: granulocyte/macrophage-colony stimulating factor

1.2. The Jak/STAT signaling pathway

The simplest model of cytokine signaling holds that ligand binding to its cognate receptor induces functional association of the receptor subunits. These events bring together receptor-associated Jaks (Janus kinases), which are subsequently activated by transphosphorylation. Activated Jaks in turn phosphorylate the receptor cytoplasmic domain on specific tyrosine residues, which then serve as docking sites for the STATs (Signal transducer and activator of transcription). Once recruited to the receptor, STATs become phosphorylated by Jaks on a single tyrosine residue, then dissociate from the receptor, dimerize, translocate to the nucleus and bind GAS (gamma-activated site) or ISRE (IFN-stimulated response element) enhancer elements (Darnell et al., 1994; Kisseleva et al., 2002). However, 15 years have passed since the first description of this pathway and the present view is indeed of much higher complexity. I will try to summarize some aspects illustrating this.

1.2.1. The Jak tyrosine kinase family

The Jak family of tyrosine kinases comprises 4 vertebrate members: Jak1, Jak2, Jak3 and Tyk2. In *Drosophila* there is only one Jak kinase, Hoscotch (Hop). *C. elegans* and *Dictyostelium* lack the family, but they do express STATs, suggesting that the STATs arose in evolution before the Jaks (Yamaoka et al., 2004).

Jak1 and Jak2 were originally identified as orphan tyrosine kinases (and named JAK for just another kinase). All Jaks exhibit broad pattern of expression, except Jak3, whose expression is restricted to hematopoietic cells (Schindler et al., 2007). Genetic studies with IFNα-unresponsive cells first identified Tyk2 as a necessary component of the IFNα signaling pathway (Velazquez et al., 1992). Shortly thereafter other Jaks were shown to associate with various cytokine receptors (Table 2) and subsequently Jak knock-out mice have illustrated their essential and specific functions (Table 3).

The tyrosine kinase domains of Jak2 and Jak3 have been crystallized, but the entire three-dimensional structure of the protein has not been solved. This is no doubt partly because Jaks are large proteins of more than 1100 aa, with apparent molecular masses of 120-140 kDa. From the primary structure, conserved regions have been delimited between vertebrate and insect Jaks (Fig. 3A). Seven Jak homology (JH) domains were identified, numbered from the carboxyl to the amino terminus. The C-terminal JH1 region corresponds to the tyrosine kinase (TK) domain. JH2 shares high similarity with kinase domains, but it lacks motifs which are critical for catalytic activity. The presence of this kinase-like (KL) or pseudokinase domain with regulatory functions distinguishes the Jak family and gives them their present name (Jaks have two kinase domains just as the Roman god Janus has two-faces). The N-terminal region, encompassing segments JH3-JH7, is involved in receptor recognition and association. It is structurally divided into an SH2-like (JH3 and part of JH4) and a FERM (4.1-ezrin-radixin-moiesin, comprising the remaining JH4-JH7) domain. The N-terminal domain binds to a membrane-proximal region of cytokine receptors, often referred to as box1/2. The exact binding interfaces as well as the mechanism of receptor reorientation after ligand binding remain largely unknown. The Jak structure-function relation will be detailed later as well as their chaperone function in receptor processing and cell surface stability.

1.2.2. The STAT transcription factors

The seven members of the mammalian STAT family (STAT 1, 2, 3, 4, 5a, 5b and 6) range in size from 750 to 900aa. The STATs are activated as transcription factors through tyrosine phosphorylation which is catalyzed primarily by the Jaks. A summary of cytokine-specific

STAT induction is given in Table 2, and the phenotypes of STAT knock-out mice is outlined in Table 3.

The STATs feature several conserved domains (Fig. 3B). The structurally independent NH2 domain is followed by a coiled-coil domain that binds regulators. The DNA-binding domain (DBD) directs binding to GAS-containing enhancers and the adjacent linker assures the appropriate conformation between the DNA-binding and the dimerization domains. Reflecting its important role in receptor recruitment and dimerization, the SH2 domain is the most highly conserved domain. The tyrosine activation domain is positioned directly adjacent to the SH2 domain, precluding self (*i.e.* intramolecular)-association. The remaining carboxy-terminal residues, which vary considerably among STAT family members, constitute the transcriptional activation domain (TAD).

As mentioned before, after cytokine stimulation activated Jaks phosphorylate receptors and subsequently the STATs are recruited on the receptor chains. However, as new experimental data became available, additional elements have been incorporated in this model. First, in some cases STAT phosphorylation by Jaks can occur even in the absence of tyrosine residues in the receptors (Nadeau et al., 1999), pointing to the presence of potential recruitment site in the Jaks themselves (Barahmand-Pour et al., 1998; Fujitani et al., 1997; Nadeau et al., 1999). Second, although it was initially described that tyrosine phosphorylation led to STAT transcriptional activity, it later appeared that some STATs (1, 3, 4, 5) can be serine phosphorylated for maximal activity (Decker and Kovarik, 2000). The requirement for serine phosphorylation may be promoter-specific and/or cell-type dependent (at least for STAT3 and STAT5). Third, the original model assumed that the STATs were present in the cytoplasm as monomers brought together after tyrosine phosphorylation through their SH2 domains. However, subsequent evidence emerged suggesting that STATs exist in the cytoplasm as preformed inactive dimers, associating through their N-terminal regions (Mao et al., 2005; Stancato et al., 1996). Upon tyrosine phosphorylation the two proteins reorient into an antiparallel dimer, where the SH2 domain of one STAT binds to the phosphotyrosine of the other STAT. Fourth, functions other than transcriptional regulation have been proposed for STAT3 (Ng et al., 2006; Xu et al., 2007). Finally, it has also appeared that nonphosphorylated STAT1, STAT3 and STAT5 cycle constitutively between the cytoplasm and the nucleus and can be transcriptionally active (Chatterjee-Kishore et al., 2000; Iyer and Reich, 2007; Meyer et al., 2002a; Meyer et al., 2002b; Yang et al., 2005; Yang et al., 2007).

All these data suggest that, in spite of the extensive studies of the Jak/STAT signaling pathway, our understanding of the proximal signaling events induced upon cytokine binding are far from being fully understood.

Ligands	**Jak kinases**	**STATs**
IFN and IL-10 family		
IFNα/β	Tyk2, Jak1	STAT1, STAT2, STAT3, STAT4, STAT5, STAT6
IFN-γ	Jak1, Jak2	STAT1, STAT3
IFNλ	Tyk2, Jak1	STAT1, STAT2, STAT3, STAT4, STAT5
IL-10	Tyk2, Jak1	STAT3, (STAT1, STAT5)
IL-19	Jak1	STAT3
IL-20	Jak1	STAT3
IL-22	Tyk2, Jak1	STAT3, STAT1
IL-24	Jak1	STAT3, STAT1
IL-26	Tyk2, Jak1	STAT3, STAT1
gp130 and gp130-related family		
IL-6	Jak1, Jak2, Tyk2	STAT3, STAT1
IL-11	Jak1	STAT3, STAT1
OSM	Jak1, Jak2	STAT3, STAT1
LIF	Jak1, Jak2	STAT3, STAT1
G-CSF	Jak2	STAT3
CT-1	Jak1, Jak2	STAT3
Leptin	Jak1, Jak2	STAT4
IL-12	Tyk2, Jak2	STAT4, (STAT1, STAT3, STAT5)
IL-23	Tyk2, Jak2	STAT3, (STAT1, STAT4, STAT5)
IL-27	Jak1, Jak2	STAT1, STAT5, STAT3
γc family		
IL-2	Jak1, Jak3	STAT5, STAT3
IL-4	Jak1, Jak3	STAT6
IL-7	Jak1, Jak3	STAT5, STAT3
IL-9	Jak1, Jak3	STAT5, STAT3
IL-15	Jak1, Jak3	STAT5, STAT3
IL-21	(Jak1), Jak3	STAT3, STAT5, STAT1
IL-3 (βc) family		
IL-3	Jak2	STAT5
IL-5	Jak2	STAT5
GM-CSF	Jak2	STAT5
Single chain family		
EPO	Jak2	STAT5
GH	Jak2	STAT5, (STAT3)
PRL	Jak2	STAT5
TPO	Jak2, (Tyk2)	STAT5

Table 2. Cytokine specific JAK and STAT activation

Gene	Phenotype of null mice
Jak1	Perinatal lethality, defects in lymphoid development
Jak2	Embryonic lethality, failure of erythropoiesis
Jak3	SCID caused by cytokine-signalling defects from γc-containing receptors
Tyk2	Hypersensitivity to pathogens due to interferon- and IL-12-signalling defects
Stat1	Impaired type I and II interferon signalling, susceptibility to viral infections
Stat2	Impaired type I interferon signalling
Stat3	Embryonic lethality, impaired responses to pathogens, cell-survival defects
Stat4	Defects in Th1-cell differentiation, impaired IL-12 pathway
Stat5a	Defects in mammary-gland development, impaired prolactin signalling
Stat5b	Growth hormone pathway defects, defective NK-cell-mediated proliferation and cytolytic activity.
Stat5a/5b	No NK cells, impaired IL-2-induced T-cell proliferation
Stat6	Defects in TH2-cell differentiation, impaired IL-4/IL-13 pathway

Table 3. Physiological roles of Jaks and STATs

1.3. Signal termination/downmodulation

Of equal importance to the activation of a signaling pathway is its spatially and temporally coordinated attenuation. Several independent mechanisms are responsible for the negative regulatory control over the Jak/STAT pathway. Key regulators include the SOCS (suppressor of cytokine signaling) and PIAS (protein inhibitor of activated STATs) families, as well as various protein tyrosine phosphatases (PTP) (Greenhalgh and Hilton, 2001; Shuai and Liu, 2003; Wormald and Hilton, 2004). Also, availability of Jaks and STATs in different cell types can contribute to regulation of cytokine responses.

1.3.1. The SOCS family of cytokine-signaling repressors, the PIAS family of STAT inhibitors and Jak-STAT phosphatases

SOCS are the most thoroughly studied regulators of Jak/STAT signaling. SOCS are induced upon cytokine stimulation in a classical negative feedback loop. There are 8 members of this family: SOCS 1-7 and CIS (cytokine-inducible SH2-domain-containing protein), all of which are characterized by a central SH2 domain flanked by an N-terminal domain of variable length and sequence, and a C-terminal region containing a conserved motif called a SOCS box. The SOCS box is also found in other proteins and is involved in the formation of a

ubiquitin E3 ligase together with elongins B and C. The SOCS can exert their inhibitory function in different ways. The SH2 domain of SOCS1 binds directly to tyrosine phosphorylated Jaks resulting in direct inhibition of Jak activity (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). On the other hand, the inhibition of Jaks by SOCS3 requires binding of SOCS3 to the activated receptor (Sasaki et al., 2000). Instead of acting on Jaks, CIS seems to inhibit STAT activation by competing with STATs for binding to the receptor docking sites (Yoshimura et al., 1995). Finally, the involvement of SOCS proteins in the degradation of signaling components through the ubiquitin-proteasome pathway has also been suggested (Kamura et al., 1998). Importantly, induced SOCS proteins have been shown to downregulate signaling from several cytokines, like IFNγ (Brysha et al., 2001; Metcalf et al., 2000), IL-6 (Croker et al., 2003; Lang et al., 2003), IL-10 (Ding et al., 2003), IL-12 (Yamamoto et al., 2003), IL-4 (Losman et al., 1999), IL-9 (Lejeune et al., 2001) and IL-13 (Hebenstreit et al., 2005).

The mammalian **PIAS** family consists of 4 members: PIAS1, PIAS3, PIASx and PIASy, with a highly conserved RING-finger-like zinc-binding domain. PIAS1 and PIASy bind to STAT1, PIAS3 to STAT3 and PIASx to STAT4, all in a cytokine-dependent manner. PIAS1 and PIAS3 act by inhibiting the DNA-binding activity (Chung et al., 1997; Liu et al., 1998), whereas PIASx and PIASy seem to function as transcriptional co-repressor of STATs (Arora et al., 2003). It has been shown that PIAS proteins have a SUMO (small ubiquitin-like modifier) E3 ligase activity, suggesting a regulatory mechanism involving SUMO conjugation to STATs. Although it has been suggested that STAT1 can be SUMOylated, the exact role of this modification in the regulation of STAT activity remains to be clarified.

Several **phosphatases** have been implicated in Jak and STAT dephosphorylation. CD45 is a receptor PTP that is highly expressed in hematopoietic cells and can directly bind and dephosphorylate all Jaks (Irie-Sasaki et al., 2001). SHP1 and SHP2 are SH2-domain containing PTP. SHP1 is involved in Jak1 and Jak2 dephosphorylation, while SHP2 negatively regulatd Jak1. Jak2 and Tyk2 can also serve as substrates for PTP1B (Myers et al., 2001), and Jak1 and Jak3 for TCPTP (T-cell protein tyrosine phosphatase) (Simoncic et al., 2002). STATs can be inactivated by PTPs both in the cytoplasm and in the nucleus. TCPTP is involved in the dephosphorylation of both STAT1 and STAT3, in the cytoplasm and the nucleus. Recently, PTP-BL has been implicated in STAT4 and STAT6 dephosphorylation (Nakahira et al., 2007), whereas STAT5 was shown to be dephosphorylated by VHR (Vaccinia H1-related) dual-specific phosphatase (Hoyt et al., 2007).

1.3.2. Cytokine response modulation through availability of signaling components

An additional mechanism of modulation of cytokine responses in different cell types is represented by the differential availability of signaling components, ie receptors, Jaks and STATs (Coccia, 2006). One way to terminate membrane-initiated signal transduction is *via* transient or prolonged downmodulation of functional ligand binding sites. The cell surface level of a given receptor is the result of dynamic processes including internalization, degradation, recycling and replenishment, each of which can be affected by ligand binding. The extent by which the ligand influences each of these processes will depend on the intrinsic turnover of the receptor which itself may vary among different cell types. I will cite some examples from the IFNα/β signaling pathway. IFNα/β bind to their receptor complex made of two subunits, IFNAR1 and IFNAR2, which activate Tyk2 and Jak1, respectively (see below). Recent work in the lab has shown that IFNAR1 has a more dynamic turnover than IFNAR2 in all cell types analyzed (Daudi, Jurkat, HeLa, 293T) (Marijanovic et al., 2007). Both IFNα and IFNβ induce internalization and degradation of IFNAR1. On the other hand, IFNAR2 is recycled in response to IFNα but not IFNβ. Also, as part of their maturation program, monocyte-derived dendritic cells can modulate their responsiveness to type I IFNs partly through the downregulation of IFN receptor chains (Gauzzi et al., 2002; Severa et al., 2006). Interestingly, in addition to SOCS proteins acting as inhibitors of cytokine signaling and possessing E3 ubiquitin ligase activity, another ubiquitin ligase, β-TrCP2 (beta-transducin repeat-containing proteins), was shown to participate in ligand-induced degradation of several cytokine receptors, such as IFNAR1 (Kumar et al., 2004; Kumar et al., 2003), EpoR (Meyer et al., 2007), growth hormone (GH) (van Kerkhof et al., 2007) and prolactin (Li et al., 2004) receptors. The recruitment of β-TrCP2 onto these receptors appears to require phosphorylation of a serine-containing motif, $DSG(X)_{2+n}S$ (Fuchs et al., 2004). The identification of the Ser/Thr kinase(s) involved is presently object of intense investigations.

In contrast to the large number of class I and II cytokine receptors, only four Jaks are expressed in mammalian cells, implying the shared used of these kinases by different receptor complexes. Consequently, if receptor number exceeds the amount of available Jak, cross-interference patterns can be expected. Indeed, it was shown that titration of Tyk2 away from the IFNAR1 receptor chain *via* exogenously expressed Tyk2-interacting receptors can lead to reduced signaling from the endogenous receptor (Dondi et al., 2001). Cross-interference between different signaling pathway not utilizing Jaks has also been reported (Lee et al., 2007). RANKL (receptor activator of nuclear factor κB ligand) stimulates, whereas IFNβ

inhibits, differentiation of precursor cells into osteoclasts. It has been shown that RANKL overcomes IFNβ inhibitory effect by inducing ubiquitination and degradation of Jak1. Cells expressing low levels of Jak1 are thus refractory to the inhibitory effect of IFNβ and can proceed with osteoclastogenesis.

Modulation of STAT level was also shown to play an important role in cytokine response. For example, IFNγ and IL-6 can induce the phosphorylation of both STAT1 and STAT3, with IFNγ activating predominantly STAT1 and IL-6 activating STAT3. However, in STAT3-deficient MEFs (mouse embryonic fibroblasts) IL-6 mediates an IFNγ-like response *via* prolonged activation of STAT1 and induction of multiple IFNγ-inducible genes (Costa-Pereira et al., 2002). Similarly, IFNγ-treated $STAT1^{-/-}$ cells exhibit a much stronger and more prolonged STAT3 activation than WT cells (Qing and Stark, 2004). Also, studies with IFNα-treated THP-1 human monocytic cells expressing different amounts of STAT3 showed that this latter can suppress STAT1 homodimer-mediated gene activation (Ho and Ivashkiv, 2006). STAT3 is thought to exert this effect through the sequestration of STAT1 in the nucleus, thus preventing the formation of STAT1 homodimers. Other studies showed that antigen-specific CD8 T lymphocytes are rendered less sensitive to type I IFN-induced inhibition of proliferation by expressing lower STAT1 levels (Gil et al., 2006). Furthermore, NK (natural killer) cells express high level of STAT4, relative to STAT1. Thus these cells were shown to readily respond to IFNα by STAT4-mediated IFNγ production. However, during the course of a viral infection, STAT1 levels are augmented as a result of IFNα and IFNγ production, and subsequently the type I IFN response of the NK cell switches from a STAT4 to STAT1-predominant response (Miyagi et al., 2007). These results demonstrate how differential STAT1 expression can be used by immune cells to modify cytokine-mediated effects on T cell expansion and function.

2. Tyk2 in cytokine signaling

Tyk2 was shown to associate with the IFNAR1 chain of the IFNα/β receptor, the IL-10R2 chain of the IL-10 and IFNλ families and IL-12Rβ1 chain of the IL12 receptor family (Fig. 4). Tyk2 can also be activated by Tpo (Ezumi et al., 1995) and by IL-6 through interaction with gp130. Based on assays with cell lines, IL-6 was shown to signal mainly through Jak1, with Tyk2 and Jak2 having accessory roles, not clearly defined (Guschin et al., 1995). Surprisingly, PBMCs (peripheral blood mononuclear cells) from a recently described Tyk2-deficient patient was shown to have impaired response to IL-6, among others (Minegishi et al., 2006). The Tpo receptor, c-Mpl, was shown to activate Jak2 and Tyk2 *in vitro*, although only Jak2 is absolutely required for Tpo signaling (Drachman et al., 1999). Consistently, no defect in Tpo signaling was reported either in Tyk2 KO mice or in the Tyk2-deficient patient (see below). Altogether, these data suggest that the importance of Tyk2 in IL-6 signaling was underestimated, whereas Tpo *in vivo* does not need Tyk2.

2.1. Type I IFNs

The human type I IFN family comprises a large number of IFN subtypes: several αs, β, ω, ε, and κ, all of which bind to the same receptor complex (Pestka, 2007). There are 14 human genes that comprise the IFNα family and encode 12 distinct proteins

Interferon has been discovered 50 years ago as a substance that "interferes" with viral infection. Since then, IFNα has pioneered the field of cytokine research in many aspects (Billiau, 2006). IFN was the first cytokine to be purified to homogeneity, cloned, completely sequenced and produced in recombinant form. The IFN field has been an uncontested forerunner from the point of view of molecular mechanisms underlying production by cells and effects on cell physiology. In addition, IFN was the first cytokine reaching the stage of wide clinical application.

Virtually any cell can produce IFNα/β in response to viral and bacterial pathogens. Howevere, plasmacytoid dendritic cells are recognized as the most potent producers of type I IFNs. In addition to the inhibition of viral replication, type I IFNs exert a vast array of biological functions, the most pronounced being those affecting the immune system (Decker et al., 2005; Theofilopoulos et al., 2005). Some of the immunomodulatory effects of type I IFNs include: important contribution to the maturation and activation of dendritic cells; enhancing the production of IFNγ by Th1 (T helper 1) lymphocytes and NK cells, and

inducing antimicrobial genes, such as inducible nitric oxid synthase, in macrophages; increasing the vulnerability of infected cells to virus-induced apoptosis; reducing the rate of epithelial cell invasion by entero-invasive bacteria; enhancing the lethal effects of lipopolysaccharide during septic shock.

Despite all these advances, there are still many open questions concerning the biology of IFNα/β that await further investigations. An important issue yet to be solved is how a single receptor complex can generate such diverse physiological effects. Moreover, an open question concerns the subtle differences in action by different subtypes. Since there is no evidence for an absolute specialized function of the IFNs subtypes, a differential effect is defined as a lack of correlation between two specific activities (Uze, 2007). For example, IFNα2 and IFNβ exhibit similar antiviral potencies against VSV (vesicular stomatitis virus) replication on WISH cells; however, the IFNβ is much more potent than IFNα2 in inhibiting the proliferation of these cells (Jaitin et al., 2006).

There are other aspects of type I IFN signaling that are not fully clarified yet. For example, we are still far from understanding how exactly ligand-induced receptor dimerization and/or conformational changes induce activation of the associated Jak. Although many studies have analyzed the proximal signaling events induced by IFN-receptor binding, we still lack a comprehensive model of how each signaling component is activated. While it is accepted now that IFNα/β can activate signaling pathways other than Jak/STATs (Platanias, 2005; van Boxel-Dezaire et al., 2006), it is still unclear how much of them are cell type and/or IFN subtype specific. I will discuss here the initial events leading to IFN-induced Jak/STAT activation.

2.1.1. The type I IFN receptor

The receptor complex for type I IFNs consists of two chains, IFNAR1 and IFNAR2, which associate with Tyk2 and Jak1, respectively. IFNAR1 is a glycoprotein consisting of two extracellular cytokine binding domains (D200) and a cytoplasmic portion of ~100aa. The *IFNAR2* gene encodes three different isoforms: a soluble IFNAR2a, a short transmembrane IFNAR2b and a long transmembrane IFNAR2c (also IFNAR2-2) protein (de Weerd et al., 2007). The functional isoform is the long IFNAR2c. The short IFNAR2b lacks the intracellular domain implicated in Jak1 binding and STAT recruitment. This isoform is considered to be a negative regulator due to potential sequestering of the ligand. The soluble isoform is thought to have both a positive and a negative regulatory role.

As mentioned above, all type I IFNs engage the same receptor complex. Extensive structure-function analysis of the IFN-receptor subunits clearly showed that essentially the same binding sites on the receptor chains are engaged by IFNα2 and IFNβ, and possibly by all type I IFNs (Jaks et al., 2007; Lamken et al., 2004). Studies of the extracellular domains of IFNAR1 and IFNAR2 tethered on lipid bilayers showed that one IFN molecule is sandwiched between one IFNAR1 and one IFNAR2 chain. As no interaction between the receptor subunits could be observed, a pre-assembled complex on the plasma membrane, as proposed notably for EpoR, can be excluded. The key differences between IFNα2 and IFNβ in terms of receptor recognition are the affinities and the rate constants of their interaction with the receptor subunit (Fig. 5). While both IFNα2 and IFNβ bind IFNAR2 with higher affinity and faster kinetics than IFNAR1, IFNβ binds with higher affinity to both receptor chains than IFNα. It has been proposed that the differential affinity of the ligands towards IFNAR1 can account for the higher antiproliferative effect of IFNβ in the example cited above.

2.1.2. Jak and STAT activation by type I IFN

The ligand mediated bridging of the IFN receptor chains brings into close proximity their intracellular domains and the associated Jaks, *i.e.* Tyk2 and Jak1. Based on studies with the Tyk2-deficient 11,1 cell line reconstituted with a kinase inactive Tyk2 mutant (Tyk2 K930R), a temporal order of activation was proposed, whereby Jak1 is activated first and then it activates Tyk2 by transphosphorylation. However, Tyk2 'basal' kinase activity is still needed for full Jak1 activation. Interestingly, IFNα/β has the potential to activate all the STATs present in a given cell. However, the "classical" response to IFNα/β refers to activation of STAT1 and STAT2, binding of IRF9 to forme the trimeric transcriptional complex known as ISGF3 (IFN-stimulated gene factor 3). ISGF3 binds to ISRE elements in the promoter of target genes. IFNα/β can also induce the activation of STAT1 and STAT3 homodimers as well as STAT1:STAT3 heterodimers, which all bind to GAS containing sequences.

Numerous studies were undertaken to try to determine the exact mechanism and order of STAT activation upon IFN treatment. I will summarize schematically the principal results obtained by different groups and propose a general model of IFNα/β-induced STAT activation. These informations were gathered from a large number of published work performed on cell lines either stably or transiently transfected, in *in vitro* pulldown assays, with endogenous or chimeric receptors.

STAT1:

- needs the presence of STAT2 to be activated (in STAT2-deficient cells STAT1 is weakly phosphorylated by IFNα) (Leung et al., 1995);
- can bind to the phosphorylated Tyr466 containing motif on IFNAR1 independently of STAT2 (Li et al., 1997);
- forms constitutive dimers with STAT2 and STAT3 (Stancato et al., 1996)

STAT2: - does not need STAT1 to be activated (Improta et al., 1994);

- binds constitutively to an IFNAR2 region comprising aa418-422 *via* its N-terminal region (Nguyen et al., 2002);
- can bind to the phosphorylated Tyr466 containing motif of IFNAR1 (Li et al., 1997; Nadeau et al., 1999);
- needs membrane-proximal tyrosine residues on IFNAR2 to be activated (Nadeau et al., 1999);
- is not activated upon Epo stimulation of a chimeric EpoR/R1 receptor, consisting of the extracellular domain of the EpoR and the intracellular region of IFNAR1 (Marijanovic et al., 2006)

STAT3:

- co-precipitates with IFNAR1 in a phospho-dependent manner (Yang et al., 1996);
- associates to phosphorylated Tyr 527 and Tyr 538 on IFNAR1 and serves as an adaptor for PI3K (Pfeffer et al., 1997);
- its activation requires Tyr337 and Tyr512 on IFNAR2 (Velichko et al., 2002);
- can be activated by chimeric receptor complexes expressing only the intracellular part of IFNAR1 (EpoR/R1) (Marijanovic et al., 2006) or of IFNAR2 (EpoR/R2) (our unpublished results), and with all tyrosines on IFNAR1 and IFNAR2 mutated (unpublished results in (Nadeau et al., 1999));
- needs Tyk2 catalytic activity to be phosphorylated, as seen in Tyk2 K930R expressing cells (Rani et al., 1999), in bone marrow macrophages from Tyk2 knock-out mice (Karaghiosoff et al., 2000) and T lymphocytes from the Tyk2-deficient patient (Minegishi et al., 2006), cf. Physiological consequences of Tyk2 deficiency).

Since my work has partly adressed these issues, I will propose a general model in the discussion chapter.

2.1.3. Tyk2 as a chaperone

The concept that the Jaks may play functions in processes distinct from cytokine-induced signaling arose from the study of Tyk2-deficient cells (Gauzzi et al., 1997; Velazquez et al., 1995). These IFN-unresponsive cells express very low amounts of IFNAR1 protein, although the level of IFNAR1 mRNA is unchanged with respect to control 2fTGH cells. The level of IFNAR1 could be restored in these cells by expressing the N-terminal region (FERM and SH2-like domains) of Tyk2. Moreover, an intact kinase-like domain was found to be additionally required to reconstitute high affinity binding of IFNα (Gauzzi et al., 1997; Richter et al., 1998; Velazquez et al., 1995). It was subsequently shown that Tyk2 exerts a "chaperone" function towards IFNAR1, by reducing its constitutive internalization and degradation, thus stabilizing the receptor at the cell surface (Ragimbeau et al., 2003). Moreover, Tyk2 was shown to stabilize IL-10R2 (Ragimbeau et al., 2003) and TpoR (Royer et al., 2005) at the cell surface, but not IL-12Rβ (unpublished data in (Ragimbeau et al., 2003)).

The function of other Jaks in receptor traffic has also been studied and chaperone-like functions have been attributed to Jak1 and Jak2, but not Jak3. It was shown that Jak1 and Jak2 enhance OSMR (Radtke et al., 2002) and TpoR (Royer et al., 2005) expression at the cell surface, respectively. Furthermore, Jak2 was found to be required for Golgi processing and cell surface expression of the EpoR (Huang et al., 2001). On the other hand, Jak3 was found dispensable for γc expression, although the overexpression of Jak3 further enhanced γc level at the cell surface (Hofmann et al., 2004). Altogether, these findings suggest that Jak proteins can sustain expression of cognate receptors, but that properties intrinsic to each receptor will determine their degree of dependency on their tyrosine kinase partner.

Here below, I will give a brief overview of cytokines that have been described to depend on Tyk2 function for their actions.

2.1.4. Type I IFN and SLE

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by tissue damage resulting from the deposits of immune complexes and the presence of anti-nuclear antibodies. Several sets of data suggest an important pathogenic role for IFNs in SLE (Banchereau and Pascual, 2006; Crow, 2007). IFNα is present in particularly high levels in SLE patient serum. Therapeutic administration of IFNα to patients with viral infection or malignancy occasionally results in induction of typical lupus antibodies and, in some cases,

clinical lupus. This indicated that, given the appropriate genetic background and perhaps in the setting of concurrent stimuli, SLE could be induced or maintained by IFNα. Using microarray technology several groups have demonstrated that IFNα-induced genes are among the most prominent observed in peripheral blood cells of lupus patients. Additional data have proposed that a key pathogenic event in SLE can be a break in peripheral tolerance mechanisms after activation of myeloid dendritic cells in response to IFNα/β. Murine studies have also supported a role for type I IFN in SLE. For example, both New Zealand Black (NZB) and B6/lpr lupus-susceptible mice deficient in functional IFN receptor show significantly less severe manifestations of autoimmunity as well as decreased renal disease and improved survival. One significant question that remains to be answered is wheter the overexpression of IFNα is the primary abnormality contributing to development of disease or is it produced only after antibodies and immune complexes have formed. Available data suggest that IFNα can act both at the initiating as well as later steps.

Genetic contributions to variability among individuals in production and signaling of IFN have been suggested by recent investigations. A study performed to identify single nucleotide polymorphisms (SNPs) in a group of IFN pathway genes found statistically significant associations of IRF5 (IFN-response factor 5) and Tyk2 SNPs with a diagnosis of SLE (Graham et al., 2007; Sigurdsson et al., 2005). IRF5 is a gene encoding a transcriptional factor that has been implicated in TLR (Toll-like receptor) signaling and IFN production. Two SNPs have been found in Tyk2 leading to a V362F substitution in the N-terminus and to I684S in the KL domain. The functional consequence of these Tyk2 and IRF5 variants remains to be determined.

2.2. Cytokines utilizing the common IL-10R2: IL-10, IL-22, IL-26 and IFNλ

The IL-10 cytokine family comprises several viral and cellular homologs, including IL-19, IL-20, IL-22, IL-24 and IL-26. IL-28/29 (IFNλ) have been classified within this family. However, given their antiviral activity, which is by definition the common feature of all IFNs, it seems more appropriate to classify IFNλ as a distinct family (type III IFNs). The receptors for all of these cytokines are made of components belonging to the class II cytokine receptor family.

IL-10 is a cytokine with broad anti-inflammatory properties that result from its ability to inhibit the function of both macrophages and dendritc cells, including their production of pro-

inflammatory cytokines (O'Garra and Vieira, 2007). IL-10 has also been reported to suppress the differentiation of both Th1 and Th2 cells. A number of genes in all these cells are inhibited to some extent by IL-10 mediated STAT3 signaling, but the mechanisms responsible for the suppressive activities of IL-10 are still unclear. IL-10 is a noncovalent symmetric homodimer that binds to a receptor complex consisting of IL-10R1 and IL-10R2 chains, associated to Jak1 and Tyk2, respectively. While IL-10R2 is expressed on a broad range of cells, the expression IL-10R1 is restricted to leukocytes. The major downstream effector of IL-10 signaling is STAT3, which is recruited on phosphorylated tyrosine residues of IL-10R1 upon IL-10 treatment. In addition to STAT3, STAT1 and STAT5 can also be activated in response to IL-10, but their precise role in IL-10 signaling remains unclear (Dumoutier and Renauld, 2002). The IL-10R2 cytoplasmic domain lacks tyrosine residues.

IL-22 and IL-26 are IL-10 – related cytokines preferentially produced by Th17 and Th1 cells, respectively (Donnelly et al., 2004; Liang et al., 2006; Zenewicz et al., 2007). Their receptors contain the shared IL-10R2 subunit together with a specific IL-22R1 (for IL-22) or IL-20R1 (for IL-26) chain, both of which activate Jak1. Both IL-22 and IL-26 activate strongly STAT3, and to a lesser extent STAT1. In addition, IL-22 can activate ERK and p38 signaling pathways (Lejeune et al., 2002). In contrast to the IL-10R, the receptors of IL-22 and IL-26 have only been found on nonhematopoietic tissues such as colon, liver, lung and skin.

Type III IFNs are a novel family of antiviral cytokines. They comprise IFNλ1 (IL-29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B) (Uze and Monneron, 2007). The IFNλ receptor consists of a specific IFNLR1 chain (also termed IL28R) and the shared IL-10R2. Type III IFNs can induce the phosphorylation of STAT1, STAT2, STAT3 and STAT5 in lung carcinoma and colorectal adenocarcinoma cell lines (Dumoutier et al., 2003; Kotenko et al., 2003) and STAT4 in BW5147 T lymphoma cells (Dumoutier et al., 2004). Type III IFNs are the only cytokines other than IFNα/β that can induce activation of STAT2 and the formation of ISGF3 complex. However, while IFNLR1 was shown to be expressed in most organs, it is certainly not expressed in all cell types and appears to be subtly regulated. The restricted expression pattern of IFNLR1 and the ensuing restricted IFNλ responsiveness is a major difference with the type I IFN system.

2.3. Cytokines utilizing IL-12Rβ1: IL-12 and IL-23

IL-12 and IL-23, together with IL-27, define a family of related cytokines that play a central role in coordinating innate and adaptive immunity. IL-12 and IL-23 share a receptor subunit, IL-12Rβ1, whereas IL-27 binds to a specific chain termed WSX or IL-27R and the gp130 chain shared among receptors for the IL-6 cytokine family. All these receptors belong to class I cytokine receptors (Trinchieri, 2003; Watford et al., 2004; Watford et al., 2003).

IL-12 has a central role in promoting the differentiation of naive CD4+ T cells into mature Th1 effector cells and is a potent stimulus for NK cells and CD8+ T cells to produce IFNγ. The biologically active form of IL-12 is a p70 heterodimer composed of two disulfide-linked subunits, p35 and p40. The IL-12 receptor consists of two subunits, β1 and β2, which are homologous to gp130. The affinity of IL-12 for either chain alone is low, and co-expression of both subunits is required for high affinity ligand binding. IL-12p40 interacts with IL-12Rβ1 and IL-12p35 interacts with IL-12Rβ2. IL-12R expression is tightly regulated on T cells. IL-12Rβ2 expression is a determinant of Th phenotype commitment, since Th1 cell sexpress both subunits, but IL-12Rβ2 is lost in Th2 cells. IL-12Rβ2 can be upregulated by TCR and IFNα/β stimulation, and by IL-12 itself (Letimier et al., 2007). Furthermore, IFNγ upregulates the transcription factor T-bet, which in turn maintains IL-12Rβ2 chain expression. In contrast, the Th2-promoting cytokine IL-4 inibits IL-12Rβ2 expression. IL-12Rβ1 and IL-12Rβ2 chains are associated with Tyk2 and Jak2, respectively. Similarly to the IL-10R2 (see above), the intracellular domain of β1 has no tyrosines, so the STATs are recruited to phosphorylated β2 upon ligand stimulation. Although STAT1, STAT3, STAT4 and STAT5 have been reported to be activated by IL-12, STAT4 is the major specific player in IL-12 signaling.

IL-23 has a major role in promoting the development of a novel Th subset, namely Th17 (Bettelli et al., 2007; Zelante et al., 2007). IL-23 is also a heterodimer composed of the IL-12p40 and a p19 subunit. IL-23 receptor consists of IL-12Rβ1 and a specific IL-23R chain. Just like IL-12, IL-23 activates Tyk2 and Jak2 and STAT1, STAT3, STAT4 and STAT5. However, STAT4 is activated to a lesser extent by IL-23 than by IL-12, and STAT3 appears to play the major role in IL-23 signaling.

2.4. Physiopathological consequences of Tyk2 deficiency

The functional implication of Tyk2 in signaling pathways activated by the various cytokines mentioned above has been confirmed by studies performed in Tyk2 knock-out mice and by identification of a Tyk2-deficient patient.

2.4.1. Tyk2 knock-out mice

Initial studies reported a surprisingly mild phenotype of Tyk2 knock-out mice. $Tyk2^{-/-}$ mice are viable and fertile, with no overt development abnormalities, but with increased susceptibility to vaccinia virus and LCMV (lymphocytic choriomeningitis virus). IFNα response is impaired, but not abolished, whereas IL-12-driven Th1 differentiation is completely absent. In contrast to the finding in 11,1 cells (see above), IFNAR1 was normally expressed in MEFs lacking Tyk2. The absence of Tyk2 had no effect on Tpo, IL-6, LIF, IL-3 and G-CSF and, surprisingly, on IL-10 responses. Further characterization of $Tyk2^{-/-}$ mice revealed a critical role of Tyk2 in mediating LPS-induced endotoxin shock. A role of Tyk2 in allergic airway hypersensitivity was also described. Subsequently, Tyk2 was shown to be critical for optimal IL-10 – mediated signaling. Thus, Tyk2 likely plays a regulatory role broadly impacting innate and adaptive phases of both Th1- and Th2-mediated immune responses. In term of STAT activation, the phosphorylation of STAT3 was the most affected in $Tyk2^{-/-}$ mice, both in response to IL-12 and IFNα/β, suggesting a preferential interplay between Tyk2 and STAT3.

Tyk2 has also been implicated in the surveillance of B lymphoid tumors (Stoiber et al., 2004). It was shown that $Tyk2^{-/-}$ mice develop Abelson-induced B lymphoid leukemia/lymphoma with a higher incidence and shortened latency compared with control mice. The cell-autonomous properties of transformed cells were unaltered, but the high susceptibility of $Tyk2^{-/-}$ mice resulted from an impaired tumor surveillance. The increased rate of leukemia/lymphoma formation was linked to a decreased *in vitro* cytotoxic capacity of $Tyk2^{-/-}$ NK cells toward tumor-derived cells. The role of Tyk2 in tumor surveillance was confirmed in a model of Eμ-Myc transgenic mice where B lymphomas arise slowly and require an additional genetic alteration or hit, shown to be either overexpression of the anti-apoptotic protein Bcl-2 or alteration of p53-dependent signaling (Sexl, 2007). In the case of Bcl-2 overexpression as the second hit, the disease latency was found to be shorter in $Tyk2^{-/-}$ animals. However, Tyk2 deficiency did not have any impact on tumors that displayed impaired p53 signaling. These data suggest an important role for Tyk2 in tumor surveillance.

2.4.2. Human Tyk2 deficiency

Recently, a single patient with Tyk2 deficiency has been described (Minegishi et al., 2006). The patient exhibited broader and more profound immunological defects than could be anticipated from studies of $Tyk2^{-/-}$ mice. The signaling defects resulted in a complex clinical picture that included Hyper IgE Syndrome (HIES) and susceptibility to multiple infectious pathogens. The patient T lymphocytes displayed impaired response to IFNα/β, IL-12, IL-23, IL-10, and, unexpectedly, to IL-6. The response to IFN-γ was reduced compared with a healthy individual, presumably due to lower amount of STAT1. The patient's increased susceptibility to viral infections pointed out the critical role of Tyk2 in transmitting signals from the IFNα/β receptor. IFNα/β-induced phosphorylation of Jak1, STAT1, STAT2, STAT3 and STAT4 was completely abrogated in patient's T cells. Furthermore, the surface expression of IFNAR1 was decreased, confirming the role of Tyk2 in the proper trafficking of IFNAR1. The patient also suffered from atypical mycobacterial infections as a consequence of deficits in the IL-12 and IFNγ axis. Lack of IL-12 signaling resulted in impaired Th1 differentiation and IFNγ production. Concomitantly, and in concert with the absence of suppressive IL-10 signaling, Th polarization was skewed toward the Th2 phenotype, presenting exaggerated *in vitro* Th2 differentiation with increased production of IL-5 and IL-13. This led to another feature of the patient's disease - atopic dermatitis and elevated level of IgE. The patient cells showed also abrogated IL-23 signaling, but the significance of this impairement on Th17 maintenance was not examined. Another difference between humans and mice is the responsiveness to IL-6. While BMM (bone marrow macrophages) and EF from $Tyk2^{-/-}$ mice displayed normal IL-6 induced signaling, PBMCs from the Tyk2 deficient patient showed a partial Tyk2-dependence of IL-6 response. What is the exact role of Tyk2 in IL-6 signaling, and whether Tyk2 plays a role in the response to other cytokine utilizing the gp130 chain, remains to be elucidated.

In conclusion, these results establish the critical role of Tyk2 in humans. However, the clinical picture presented by this patient does not seem very common. In fact, the authors point out that this genetic lesion may be the determinant for only a subset of patients with autosomal-recessive (AR) HIES. In most sporadic and autosomal-dominant (AD) cases, these clinical manifestations are part of a multisystem disorder including abnormalities of the soft tissue, skeletal and dental systems. Interestingly, dominant-negative mutations of STAT3 have been found recently at the origin of sporadic and AD HIES (Holland et al., 2007; Minegishi et al., 2007; Renner et al., 2007). Defects in Tyk2 signaling seem to coincide well

with observed physiological defects in STAT3 activation. However, the consequences of STAT3 impaired function seem not to be restricted to the immune system, as observed for Tyk2, but extend to the whole organism. These findings confirm once more the preferential rapport of Tyk2 with STAT3.

3. Structure/function organization of the Jak kinases

3.1. The N-terminal region: FERM and SH2-like domains

The N-terminal part of the Jaks, necessary for receptor binding, is organized into a FERM domain followed by an SH2-like domain. The presence of these domains has been proposed after considerable computational modelling and is based on comparison of predicted secondary structure rather than sequence identity.

The **SH2-like** domain has been predicted to be functional, *i.e.* able to bind phospho-tyrosine motifs, in all human Jaks except Tyk2 (Al-Lazikani et al., 2001). In Tyk2, the presence of a His instead of a key Arg in the phosphotyrosine binding site is expected to impede phosphotyrosine binding. It is possible that another residue in the binding site coordinates a phosphotyrosine or that other still unknown factors serve to enhance binding. Alternate possibilities are that the Tyk2 SH2-like domain associates with a completely different class of targets and that its activity is not controlled by phosphorylation, or possibly, that it binds unphosphorylated tyrosines. However, the role of the Jak SH2 domain has not been completely clarified yet. The replacement of the key Arg by a Lys in the phosphotyrosine binding pocket of Jak1 SH2, which impairs the function of the SH2 domain but maintains its structural integrity, shows that the SH2 domain does not contribute to signaling via the Jak/STAT pathway in response to OSM, IL-6, IFNγ and IFNα (Radtke et al., 2005). Nevertheless, a structural role has been proposed for this domain since neither Jak1 nor Tyk2 deleted of the SH2 could support receptor binding *in vivo* (Radtke et al., 2005; Richter et al., 1998).

The **FERM** domain defines members of the band 4.1 superfamily, which includes cytoskeleton-associated proteins such as erythrocyte band 4.1, talin and the ERM protein family, the tumor-supressor merlin, some protein phosphatases, and FAK and Jak kinases. The moesin crystal structure shows that the FERM domain is composed of three structural modules (F1, F2 and F3) that together form a compact clover-shape structure. Each of the three lobes adopts a different fold. F1 is very smilar to ubiquitin, F2 is similar to the structure of acyl-CoA binding protein, F3 shares the fold of phosphotyrosine-binding (PTB) or pleckstrin-homology (PH) domains (Pearson et al., 2000). The FERM domain has been found to bind to the cytoplasmic region of transmembrane proteins in a large number of proteins. However, the FERM domain is large enough to accomodate binding sites for several partners and might have additional functions (Girault et al., 1999).

In the FAK (focal adhesion kinase), the FERM domain binds to transmembrane receptors such as integrin, but has also an autoinhibitory role. The crystal structure of the avian FAK containing the FERM, linker and kinase domain in an inactive, non-phosphorylated conformation shows the structural basis for this autoinhibition (Lietha et al., 2007). The three-lobed FERM domain bridges between the N- and C-lobes of the kinase domain, extending across the active site cleft. The disruption of the FERM/kinase interface does not affect FAK localization to focal adhesions but leads to increased basal kinase activity.

In Jak proteins, the FERM domain is involved in an ill-defined, non-covalent interaction with the membrane-proximal region of the cytokine receptors. It was suggested, based on the study of point mutations in the Jak3 FERM domain, that the FERM domain can regulate Jak kinase activity (Zhou et al., 2001). Furthermore, as proposed for the ERM proteins, the Jak FERM may act as a compact architectural unit with multiple binding surfaces. Jaks were reported to bind to a variety of signaling proteins (Yeh and Pellegrini, 1999) either constitutively or in a phosphorylation-dependent manner. Given that the FERM domain can mediate various interactions, it was of interest to find new interacting proteins of this domain. To this end, a yeast two-hybrid screen was performed in the laboratory using the Tyk2 FERM domain as bait to screen a cDNA library from Jurkat T cells. Two new proteins were identified and studied, Jakmip1 and Pot1. Part of my thesis work has consisted in characterizing Pot1. I will summarize the unpublished data obtained in the lab before my arrival in the Result section.

3.1.1. A new Tyk2 interacting protein: Jakmip1

Jakmip1 (Jak and microtubule interacting protein) is a 70kDa protein expressed mainly in lymphoid and neuronal tissues (Steindler et al., 2004). Jakmip1 colocalizes with microtubules and can affect their stability. Jakmip1 (alias Marlin-1) was independently found in a yeast two hybrid screen for partner of the neuronal $GABA_BR1$ (Couve et al., 2004). Recent studies in our lab showed that Jakmip1 is differentially expressed in different stages of T lymphocyte maturation (Libri, 2007). The expression of Jakmip1 in naive T cells is very low, augments in the central memory subset and even more in effector memory and effector T cells. Furthermore, it was shown that the cytotoxic capacity of CD8 T cells depleted of Jakmip1 was augmented. Altogether, these results suggest a negative regulatory role for Jakmip1 in T cell function, possibly through regulation of microtubule- dependent processes (migration, secretory vesicle transport). A functional link, if any, with Tyk2 and/or other Jaks has not been established yet.

3.2. The kinase-like domain as a sensor of ligand binding

The Jaks are characterized by a kinase-like (KL) or pseudokinase domain adjacent to the kinase domain. The KL domain is catalytically inactive and indeed it lacks several residues critical for tyrosine kinase activity. Although numerous studies have shed some light on its regulatory role in Jaks, we still lack a thorough understanding of its mechanism of functioning. Deletion studies with Jak2 and Jak3 (Saharinen and Silvennoinen, 2002; Saharinen et al., 2000) have suggested an autoinhibitory role for the KL domain. Deletion of Jak2 and Jak3 KL domain resulted in higher basal kinase activity and cytokine-independent STAT activation. However, cytokine stimulation did not increase further this STAT activation, suggesting the need for an intact KL domain for cytokine responsivness. Furthermore, a number of point mutations in the KL domain of Jak2 and Jak3 have been found in patients with leukemias and SCID, respectively, confirming the important regulatory role of this region (cf. Jak2V617F)

In contrast with these results, it was shown that Tyk2 deleted of its KL domain was catalytically inactive, both basally and in response to IFNα (Velazquez et al., 1995). Furthermore, this deletion mutant could not rescue high affinity IFN binding. These data suggest that the KL domain is likely involved in maintaining the configuration of the high-affinity receptor-kinase complex through its interaction with receptor components. Similar conclusions were obtained from analyses of several Tyk2 point mutants (Yeh et al., 2000). A screening was performed in order to identify mutations in the KL domain that alter Tyk2 kinase activity. Four point mutations were found that led to loss of high affinity IFNα binding, loss of catalytic activity and loss of IFNα-induced signaling. However, two of these mutants had high basal phosphorylation which did not increase upon IFNα stimulation.

Altogether, these results suggest that, in all the Jaks analyzed, the KL domain acts as a sensor of receptor-ligand binding. It serves to maintain the protein in a low-phosphorylated conformation and is needed for ligand-induced activation (Chen et al., 2000). The difference observed in the kinase activity after KL deletion between Jak2 and Jak3 on one side, and Tyk2 on the other side, could be due to differences in the constructs used in these studies (Tyk2 ΔKL is ~25aa shorter for the linker between KL and TK than the corresponding Jak2 construct). However, it is more likely that those discrepancies reflect inherent differences between these proteins.

3.2.1. Jak2V617F in *Polycythemia vera*

The critical regulatory role of the KL has come into attention with the discovery of a point mutation in Jak2 KL, V617F, in patients with *Polycythemia vera* (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). This myeloproliferative disorder is characterised by augmented erythrocyte production sometimes followed by platelet hyperproduction and myelofibrosis. Erythrocytes from these patients are hypersensitive to Epo and tend to form endogenous colonies in *in vitro* based assays (*in vitro* endogenous colony formation was the diagnostic tool for this disease). Based on structural modeling of the KL-TK interaction (Lindauer et al., 2001), the Val617 is thought to be located at one of the interfaces of KL-TK, keeping the TK inactive (Fig. 6). When overexpressed, Jak2V617F was shown to have higher basal catalytic activity than the wild-type (WT) protein and confers hypersensibility to Epo when compared with the WT protein. However, Jak2V617F still needs the coexpression of a cognate homodimeric receptor to promote factor-independent proliferation on BAF3 pro-B cells (Lu et al., 2005).

The Val617 is conserved in Tyk2 and Jak1, but not in Jak3 where it is replaced by a Met. The corresponding Jak2 mutation in Tyk2 (V678F), and in Jak1(V658F) conferred higher basal phosphorylation to both proteins (Staerk et al., 2005). Just like Jak2 V617F, Tyk2V678F and Jak1V658F induced higher STAT activation as compared to the WT proteins.

So far, this was the only described gain-of-function mutant of Tyk2, so we sought to investigate more deeply its mechanisms of function (cf. Objectives)

3.3. The tyrosine kinase domain

The tyrosine kinase domains of Jak2 in complex with a pan-Jak inhibitor (Lucet et al., 2006) and of Jak3 in complex with a staurosporine analog (Boggon et al., 2005) have been recently crystallized, both in an active state (Fig. 7). They share similar features so I will discuss them here as the "Jak" kinase domain.

The Jak kinase domain exhibits the classical bilobed architecture conserved among the catalytic domains of all protein kinases. The small N-terminal lobe contains a 6-stranded anti-parallel β-sheet and a single helix (αC) which is involved in catalytic regulation in many protein kinases. The glycine-rich loop, or P-loop, which participates in coordination of the phosphate groups of ATP, is also found in the N-lobe. The large C-terminal lobe is predominantly helical, but contains two short strands of β-sheet within the activation loop. The two lobes are connected by a short linker termed the "hinge" region. The ATP-binding pocket is located between the two lobes and abutting the hinge. The so-called activation loop

with the critical regulatory tyrosines is located in the C-lobe. The phosphorylation of these tyrosines is required for the catalytic activation of the Jaks. However, there are some discrepancies between Jaks regarding the effect of each of these residues. It has been shown that the phosphorylation of Jak3 Y980 and Y981 activates and inhibits catalytic activity, respectively. On the other hand, phosphorylation of Jak2 Y1107 was required for the activation, whereas Y1008 had no effect at all on catalytic activity.

The architecture of the Jak kinase domain diverges significantly in two respects from other tyrosine kinases studied to date. Jak3 contains an additional helix inserted between the helices αG and αF in the C-lobe (termed αFG, Jak insertion loop or lip). This loop packs loosely against the base of the C-terminal lobe and is relatively mobile and solvent accessible. Although it is not found in other tyrosine kinases, it is well conserved in the vertebrate Jak family members. The function of this additional helix is unclear, but given that it appears to be present in all vertebrate Jak kinases, it is tempting to speculate that it could participate in intramolecular regulatory interactions with the KL or FERM domains.

Additionally, the conformation of the loop connecting the β2 and β3 sheets in the N-lobe is dramatically divergent. The loop folds "forward" leaving a small, mostly hydrophobic cleft exposed on the back of the N-lobe. As with the novel αFG helix, this cleft may represent a site of interdomain contact or of interaction with another regulator. Interestingly, residues involved in formation of this cleft and FG helix are respectively not conserved or are deleted in insect Jak; this may reflect differences in Jak regulation in invertebrates.

The role of the αFG loop in Tyk2 activity is particularly intriguing. When Tyk2 was cloned in Dr Pellegrini lab, among the partial cDNAs cloned from a HeLa library, one was found to lack 18nt at the beginning of exon 24. The corresponding protein lacks 6aa in the αFG loop and was found not to complement 11,1 cells. This indicates that Tyk2 deleted of the αFG loop is functionally impaired. Recently, a mutation (P1104A) was found in the αFG loop of human Tyk2 (Kaminker et al., 2007). A bioinformatic approach to distinguish cancer-associated missense mutations from common polymorphisms classified the P1104A substitution as a novel cancer-associated germ-line mutation that is likely to affect Tyk2 function. This mutation was found in 4 EST clones from 3 cancer libraries (expressed in breast, colon and stomach cancers), but not in ESTs from normal libraries. The authors propose that the mutation of the proline 1104 to alanine may precipitate an activated state of Tyk2, which may lead to an oncogenic phenotype. However, no experimental evidence were presented. I have introduced the P1104A mutation in Tyk2 and analyzed the function of the mutated protein.

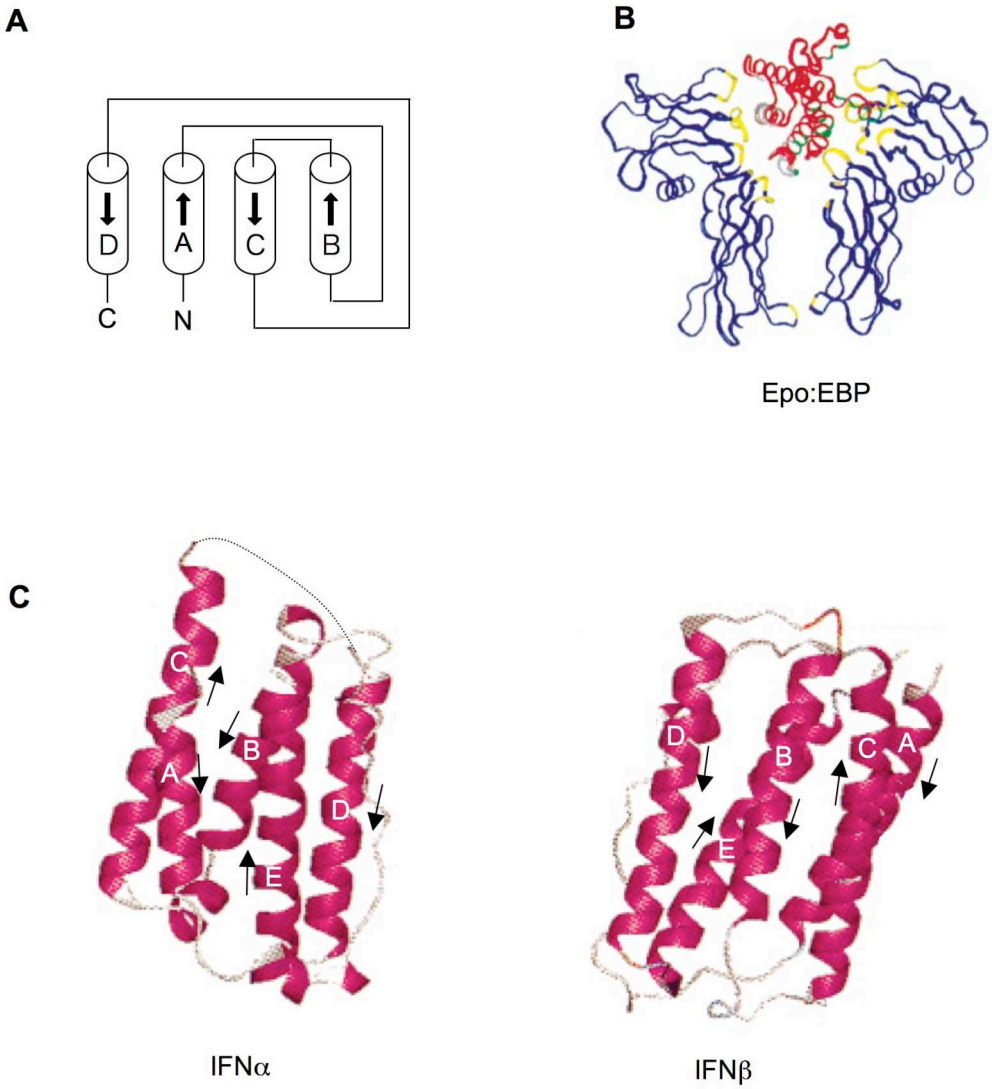


Figure 1. Epo and IFNα/β crystal structure

A) Schematic representation of α-helical cytokine fold (Sprang, 1993). The four helices are labelled A-D

B) Crystal structure of erythropoietin (Epo) in complex with the extracellular domain of its receptor (Epo-binding protein, EBP) (Syed et al., 1998). One molecule of Epo was shown to bind two receptor subunits

C) Crystal structure of IFNα (left) and IFNβ (right) (Karpusas et al., 1998). The core helix bundle is defined by helices A, B, C and E - helix A runs parallel with helix B and antiparallel with helices C and E. Helix D is part of the long loop connecting helices C and E

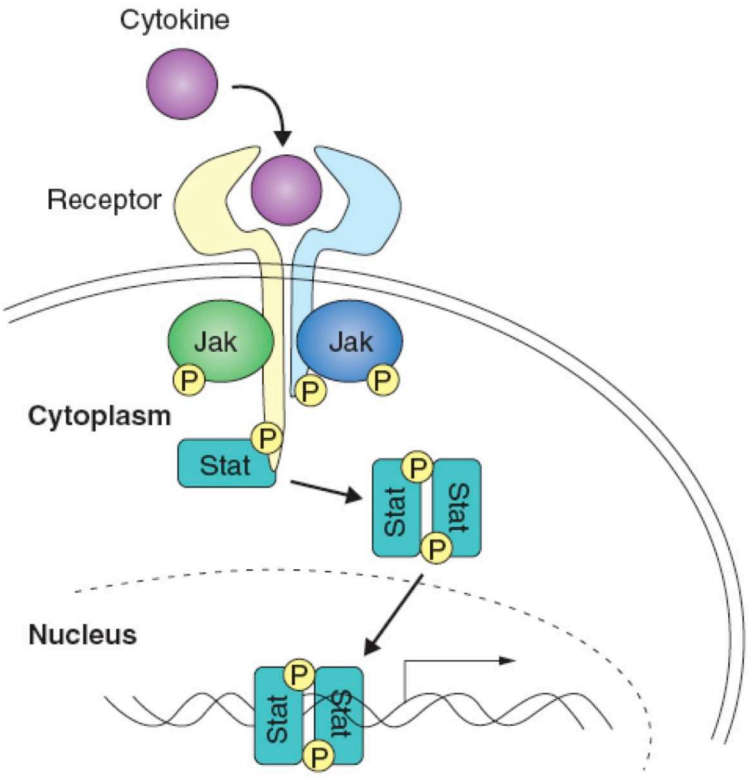


Figure 2. Schematic representation of the cytokine signaling pathway
After ligand binding, the two receptor chains are brought together, enabling the transphosphorylation of the associated Jaks. Activated Jaks in turn phosphorylate the receptor cytoplasmic domain on specific tyrosine residues, which then serve as docking sites for the STATs. Once recruited to the receptor, STATs become phosphorylated by Jaks on a single tyrosine residue, then dissociate from the receptor, dimerize and translocate to the nucleus (Yamaoka et al., 2004)

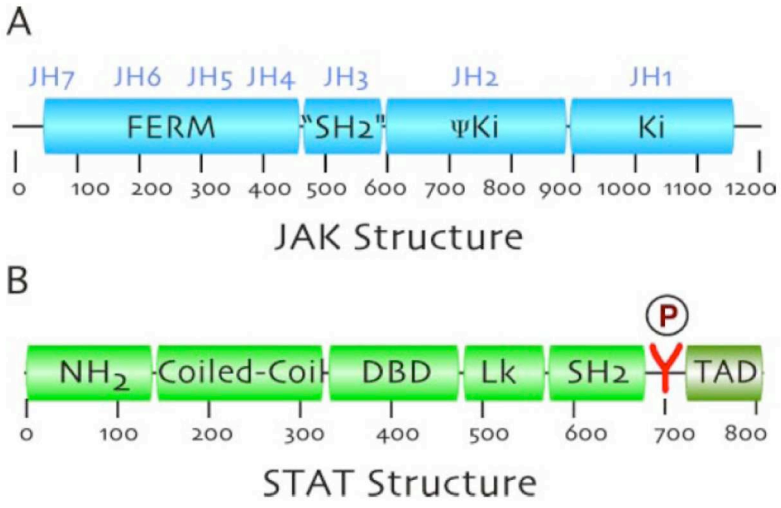


Figure 3. Domain organisation of Jaks and STATs
JH: Jak homology; FERM: band 4.1, ezrin-radixin-moesin domain; "SH2": SH2-like domain; ψKi: kinase-like or pseudokinase domain; Ki: tyrosine kinase domain; DBD: DNA-binding domain; Lk: linker; TAD: transactivation domain (Schindler et al., 2007)

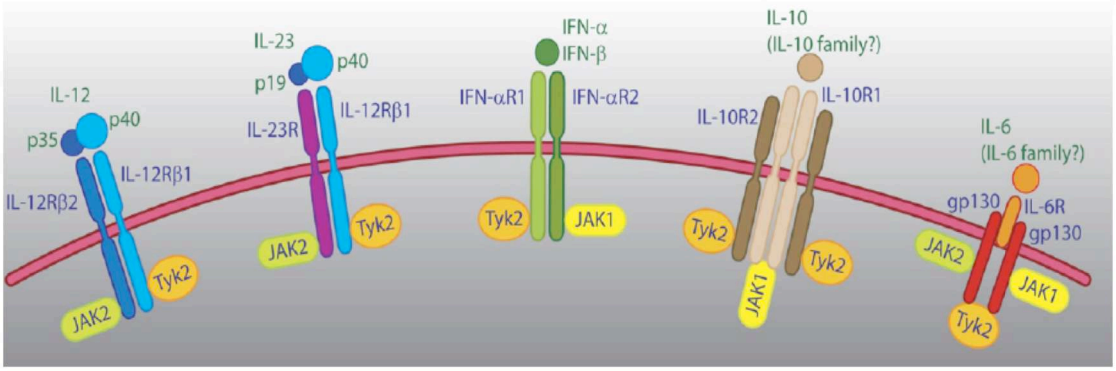


Figure 4. Receptor chains that bind Tyk2
Receptor chains that bind Tyk2: IFNAR1, IL-12Rβ1, IL-10R2 and gp130 (Watford and O'Shea, 2006). Among the IL-10 family, IL-10, IL-22 and IL-26, as well as IFNλ, utilize IL-10R2 and Tyk2. Among the IL-6 family, only IL-6 signaling has been found to depend on Tyk2.

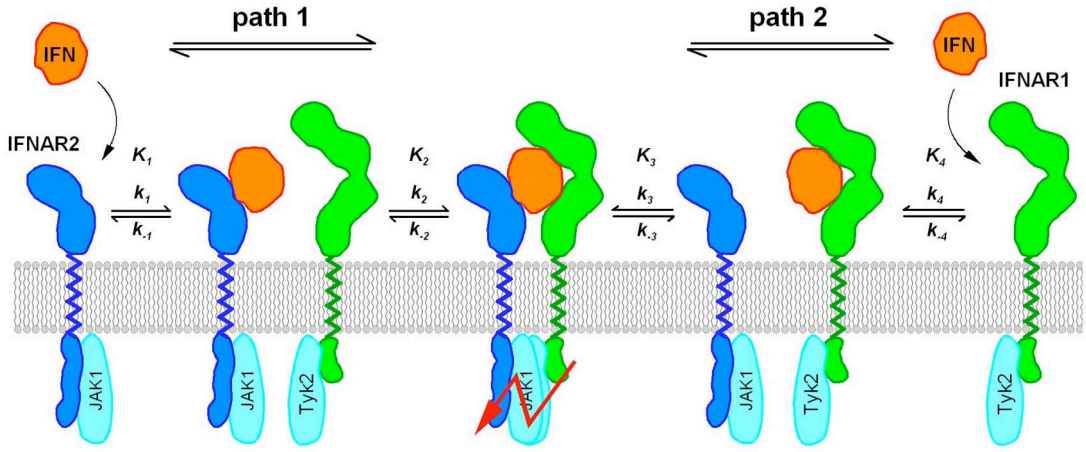


Figure 5. Two-step assembling of the IFN/receptor ternary complex.
After ligand binding to one of the receptor subunits, the second receptor subunit is recruited by lateral interaction on the membrane (Uze, 2007). Two assembling pathways are possible. Since the first step of ligand binding to one of the receptor subunits is rate-limiting, the population of the two pathways depends only on the association rate constants *k1* and *k4,* and the relative concentrations of IFNAR1 and IFNAR2. The ternary complex is in a dynamic equilibrium with the binary complexes, which is determined by the affinity constants *K2* and *K3*.

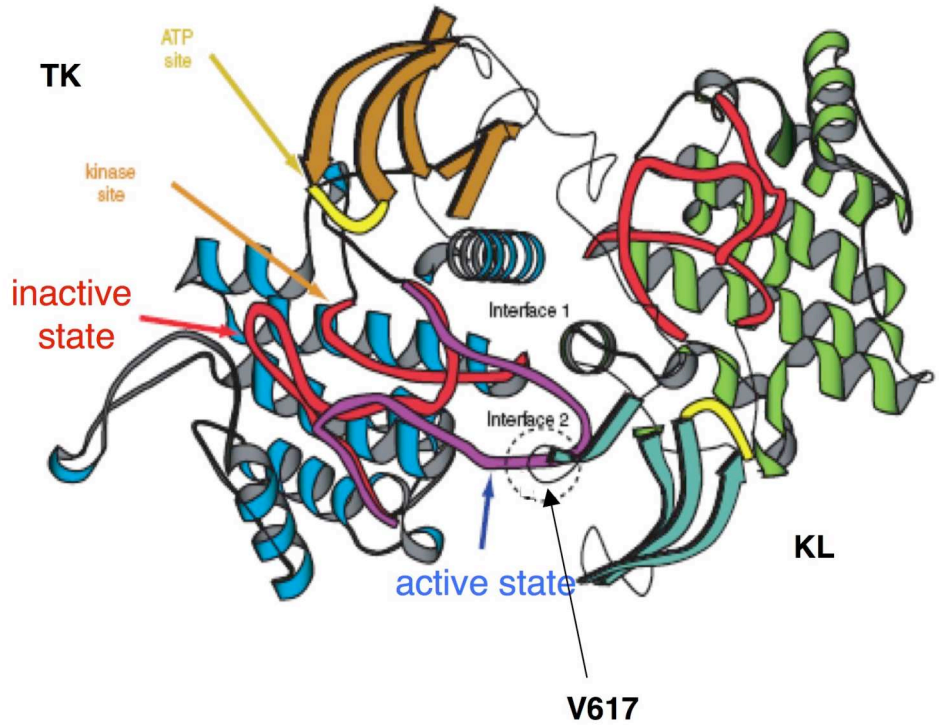


Figure 6. The model of KL-TK interaction.
The interaction between Jak2 kinase-like and tyrosine kinase domain was modelled based on the crystal structure of a dimer of the FGF receptor tyrosine kinase domain (Lindauer et al., 2001). The activation loop is depicted in red and purple when in the inactive and active state, respectively. The position of Val 617 in the KL-TK interface is indicated

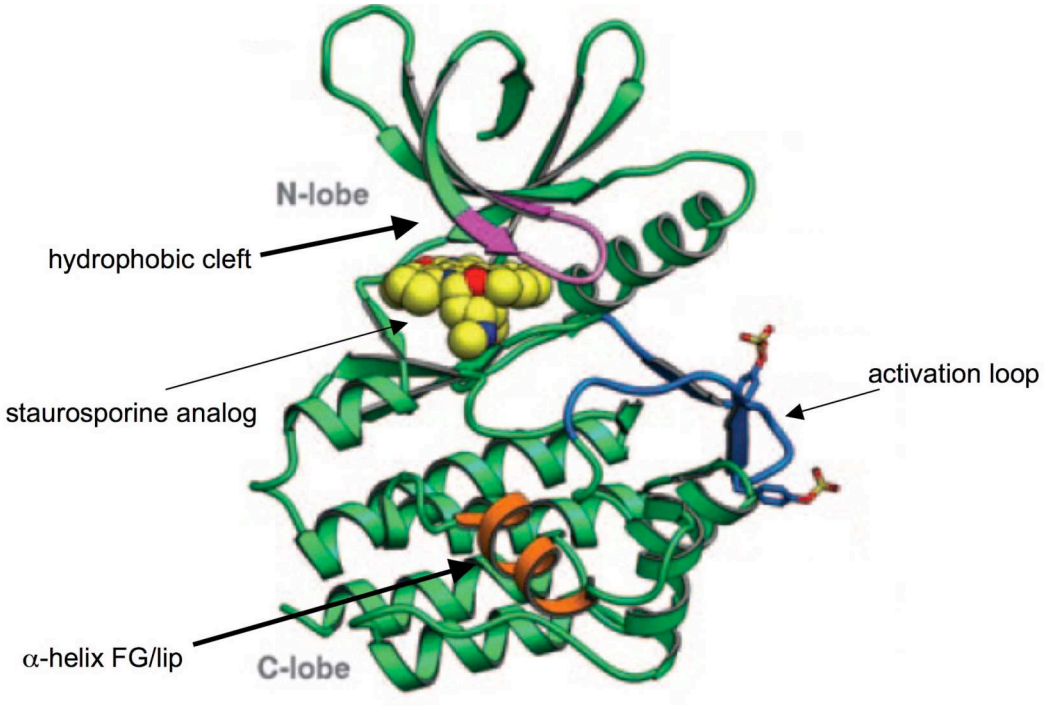


Figure 7. The crystal structure of Jak3 kinase domain.
Jak3 tyrosine kinase domain was crystallized in its active state in the presence of a staurosporine analog. The position of the FG helix in the C-lobe and of the hydrophobic cleft in the N-lobe are indicated (Boggon et al., 2005).

Objectives

The role of Pot1 in IFNα signaling

Pot1 is a newly identified protein, not described so far, that was isolated as an interactor of the Tyk2 FERM domain in a yeast two-hybrid screen. Studies undertaken to characterize Pot1 prior to my arrival in the lab will be summarized in the Results chapter. The main question I addressed when I joined the lab was whether Pot1 has a role in IFNα signaling.

The Tyk2V678F mutant in a homo- *vs* heterodimeric receptor context

Murine proB BaF3 cells are cytokine-dependent for survival and therefore represent a good cellular model to test the transforming potential of a protein involved in cytokine signaling. Jak2V617F was originally shown to confer cytokine-independent growth to BaF3 cells, and the same effect was later observed for the corresponding Jak1 and Tyk2 mutants (Staerk et al., 2005). As mentioned above, the Tyk2V678F mutant was found to be basally phosphorylated in BaF3 cells. Furthermore, using Tyk2-deficient 11,1 cells it was shown that the co-expression of Tyk2V678F, but not Tyk2 WT, with TpoR and STAT3 induced higher basal and Tpo-induced transcriptional response of a STAT3 reporter gene.

The V678 residue is located in the regulatory KL domain, which has been extensively studied in the lab. For these reasons we were interested to analyze its effect on IFNα signaling. Additionally, it was shown that Jak2V617F requires co-expression of a cognate homodimeric receptor in order to confer cytokine-independent growth to BaF3 cells (Lu et al., 2005). Tyk2 is implicated in responses to a subset of helical cytokines. Indeed, *via* its N-terminal domain Tyk2 can associate not only to IFNAR1, but also to IL-10R2 and IL-12Rβ1. As mentioned above, these two latter receptor subunits are components of several heteromeric receptor complexes. In these, Tyk2 is involved in heterotypic catalytic exchanges with either Jak1 or Jak2. On the other hand, Jak2 is the only Jak family member found in homodimeric class I cytokine receptors (eg Epo, Tpo, Prolactin, GH), where it functions in homotypic catalytic exchanges. Based on these premises, we also asked what would be the functional effect of Tyk2V678F placed in a homodimeric receptor context.

The effect of the P1104A on Tyk2 activity

So far, there has been only one report of a Tyk2 alteration in a human disease, namely the Tyk2 deficiency causing HIES (see above). A Tyk2 point mutation, P1104 to A, has been found in human cancer tissues and predicted to activate Tyk2 (Kaminker et al., 2007). The proline 1104 is located in the Jak insertion loop or "lip" in the TK domain, a short helix found only in Jaks likely to be a critical Jak-specific regulatory helix. Therefore, we were interested to analyze this mutation for the following reasons:

1) P1104A may represent a potential activating mutation activating mutation whose behaviour can be compared with that of the V678F

2) its study could give us further insight into the precise role of the "lip" on Tyk2 catalytic functioning

3) it may shed some light on pathological consequences of dysregulated human Tyk2 activity

Materials and methods

Cell lines

Tyk2 deficient 11,1 cells and 11,1 cells expressing the EpoR/R1 chimeric protein were described before (Marijanovic et al., 2006; Pellegrini et al., 1989). 293T cells, 2fTGH, 11,1 and 11,1-derived cells were culture in Dulbecco's modified Eagle medium (Gibco) supplied with 10% foetal calf serum and the appropriate antibiotic for selection. Cells transfected with Tyk2WT, Tyk2V678F and Tyk2P1104A were selected in 400μg/ml of geneticin G418 (Gibco) to obtain neo^R clones.

siRNA and plasmids

The sequences of Pot1 siRNA and Sc control as are follows (with the corresponding reverse strand):

siPot1-1623: 5'-UGCUCAGUUACGAAGGCAGdTdT-3'

Sc control: 5'-GCUAGGACGACAGUAUGCUdTdT-3'

The **pG-SUPER** vector was obtained from D. Vignjevic (Institut Curie, Paris). To insert shRNA into the pG-SUPERvector, the following sequences were used:

1623 forward

5'GATCCCCTGCTCAGTTACGAAGGCAGTTCAAGAGACTGCCTTCGTAACTGAGCATTTTTGGAAA3'

1623 reverse

5'AGCTTTTCCAAAAATGCTCAGTTACGAAGGCAGTCTCTTGAACTGCCTTCGTAACTGAGCAGGG-3'

Sc forward

5'GATCCCCGCTAGGACGACAGTATGCTTTCAAGAGAAGCATACTGTCGTCCTAGCTTTTTGGAAA-3'

Sc reverse

5'AGCTTTTCCAAAAAGCTAGGACGACAGTATGCTTCTCTTGAAAGCATACTGTCGTCCTAGCGGG-3'

The mix of 4 siRNAs targeting Pot1 (*siPot1-D*) and GIT1 (*siGIT1*) were purchased from Dharmacon as SMARTpool reagents. The Ctrl-Q siRNA was from QIAgen. The sequence of the siRNA targeting *Jakmip1* is 5'−CGAGCUGUUAGAAUUCAGAdTdT-3'

Pot1 cDNA reconstructed in the lab was clones into the pcDNA4 vector with HA and V5 tags at the N- and C-terminus, respectively (**hPot1-V5**). The murine Pot1 (IMAGEclone 5066612) is cloned in the pCMV-SPORT6 vector (**mPot1**).

For the yeast two-hybrid screen, the 2,9 kb Pot1 fragment was amplified from hPot1-V5 with primers containing SpeI and PacI restriction sites. The fragment was cloned into the pB27 bait vector (LexA binding domain) and sequenced. The expression of the fusion protein was confirmed by Western blot.

Human GIT1 and GIT1R39A in pBK-CMV vector were obtained from N. Vitale (Université Louis Pasteur, Strasbourg).

Human Tyk2 with a C-terminal VSV tag was cloned into the pRc/CMV vector (referred to as **Tyk2WT**). **Tyk2V678F** in pMX-IRES-CD4 retroviral vector was obtained from S. Constantinescu (Ludwig Institute for Cancer research, Brussels). A BspEII - BstXI fragment of 2kb containing the mutation was introduced into previously digested Tyk2WT.

The C3310G mutation generating the **P1104A** substitution was introduced in Tyk2 WT by site-directed mutagenesis with the plasmids:

MG22 (containing the BstXI restriction site)

5'-ATGCTGCCACCCTCCTGGAGATCTGCTTTG-3' together with

MG23 5'-GGAATTTCGTGGGGGCGCTCTGGCTGGAGT-3'

and *MG24* 5'-ACTCCAGCCAGAGCGCCCCCACGAAATTCC-3'

together with *MG28* (containing the XbaI restriction site)

5'-CAACAGATGGCTGGCAACTAGAAGGCACAG-3'.

The resulting amplicons of 900bp and 400bp, respectively, were mixed and reamplified with MG22 and MG28 to yield a 1,3kb fragment. This fragment was digested with BstXI and XbaI and introduced into prevously digested Tyk2WT. The plsmids obtained were subsequently sequenced.

Transfections were performed using Lipofectamine2000 (Invitrogen) or Fugene6 (Roche) to transfect siRNA or plasmids, respectively. The transfections were performed azccording to the manufacturer's recommendantions.

SDS-PAGE and Western blot

For Western blots, cells treated with IFNα (Wellcome Reaserch Laboratories, UK) or Epo (gift from P. Mayeux, Institut Cochin, Paris, France) were lysed in RIPA buffer containing 200mM NaCl, 50mM Tris pH8, 1% NP40, 0,5% deoxycholate, 0,05% SDS, 2mM EDTA, freshly supplied with 1mM sodium-vanadate, 1mM sodium-fluoride, 10mM PMSF and 3μg/ml aprotinine, leupeptine and pepstatine each. Typically 30-60μg of lysate were analyzed by SDS-PAGE followed by Western blot. The following Abs were used: anti-Pot1 AP452 (generated by Eurogentech), anti-Tyk2 T10.2 (generated in the lab), anti-phosphorylated -YY1054/55-Tyk2 (Calbiochem), anti-Jak1 (UBI), anti-phosphorylated-YY1022/23-Jak1 (Biosource), anti-IFNAR1 64G12 (gift from P . Eid, Paris), anti-STAT1 (Upstate), anti-phosphorylated-Y701-STAT1 (Cell Signaling), anti-STAT2 (Upstate), anti-phosphorylated-Y689-STAT2 (Upstate), anti-STAT3, anti-phosphorylated-Y705-STAT3 (Cell Signaling), anti-STAT5 (Santa Cruz), anti-phosphorylated-Y694-STAT5 (Cell Signaling), anti-GIT1 (Santa Cruz and BD Transduction Laboratories), antiphosphotyrosine 4G10 (Upstate).

Tyk2 immunoprecipitation and *in vitro* kinase assay

Cells were lysed in 50mM Tris pH6,8, 0,5% NP40, 200mM NaCl, 10% glycerol, 1mM EDTA, 1mM sodium-vanadate, 1mM sodium-fluoride, 10mM PMSF and 3μg/ml aprotinine, leupeptine and pepstatine each. Tyk2 was immunoprecipitated using the rabbit serum R5 (generated in the lab). One μl of serum was used for 1mg of lysate in 300-400μl. After ON incubation, 30μl of 50% proteinA-sepharose was added for one additional hour at 4° with rotation. The precipitates were washed three times with 50mM Tris pH6,8, 400mM NaCl, 0,5%TritonX-100, 1mM EDTA, then once with 50mMTrispH6,8, 200mM NaCl, and once with the kinase buffer (50mM Hepes pH7,4, 10mM $MgCl_2$). The beads were resuspended in 10μl kinase buffer. The *in vitro* kinase reactionwas performed with 50μM ATP at 30° for 5' in a total volume of 20μl. The reaction was stopped by adding 10μl of 3xSB and boiling at 95° for 5'. The reaction products were detected by Western blot using the anti-phospho-tyrosine Ab 4G10 (Upstate).

Luciferase reporter assay

The following luciferase reporter plasmids were used in Pot1 knock-down experiments: ISG54-luc, IRF1-luc (from T. Decker, University of Vienna, Austria), SIE-luc (from I. Behrmann, University of Luxembourg, Luxembourg), rPAP-luc (from J. Tavernier, Ghent

University, Belgium), 3xLy6E-luc (from V. Poli, University of Turin, Italy)), NFkB-luc (R. Weil, Institut Pasteur, Paris, france). Twenty-four hours after transfection cells were plated in 96-well plate (Dutcher) and 16h later treated with different doses of IFNα, hIL-6 (Serono), IFNγ or TNFα as indicated on the figure. Cells were lysed in 0,5M KPO4 pH7,8, 0,1% TritonX-100 and 1mM DTT, and luciferase activity was measured after adding luciferase assay buffer (0,1mM EDTA, 20mM Tricine, 1,07mM Mg-carbonate, 2,67mM Mg-sulfate, 33,3mM DTT, 270μM coenzyme-A (Sigma), 530μM ATP (Sigma), 470μM D-Luciferine (Sigma)) on a Microplate Luminometer (EG&G Berthold). All experiments were done in triplicate.

For the analysis of STAT3 activity in Tyk2V678F-expressing cells, STAT3-luc (Pathway profiling luciferase system 5, Clontech) and *Renilla* luciferase (Promega) were used. Eight hours after transfection cells were plated in a 96-well plate and 16h later luciferase activity was measured using the Dual-Glo luciferase assay system (Promega) according to manufacturer's recommendations. The values for the reporter gene were normalized by the values for the control *Renilla* luciferase. Each experiment was done in triplicate.

PCR

Total RNA was isloated using the RNeasy mini kit (QIAGEN). Reverse transcription was performed with 1μg RNA using random primers with M-MLV reverse transcriptase (Invitrogen). For the PCR to amplify the 5' end of Pot1, the following primers were used:

MG1 5'-GGAGTTGCCTGAGATTCAGAATGAGAGGGAGC-3'

MG2 5'-AGAGATGGTCAGGTCTGGAGCTCGACC-3'

MG3 5'-CGGGCTTGATGGTTGTGCTTCTTTGCTGAGGG-3'

MG4 5'-ACTGCCAACAGTGACTACATCTGGAGGGTGG-3'

H2 5'-GAGACACATCAGACCTAGAGC-3'

H3 5'-CTCCAGAAGACCAGTCCTTAAAGAC-3'

The DNA polymerases used in different experiments were: *Pfu* DNA polymerase (Stratagen), Platinum *Taq* DNA Polymerase (Invitrogen) and *Vent* DNA Polymerase (New England Biolabs).

FACS

Cells were detached with PBS supplied with 5mM EDTA and subsequently resuspended in FACS buffer (3% FCS in PBS). Cells were incubated with the appropriate antibody (AA3 to detect IFNAR1 gift from L. Runkel, CD118 to detect IFNAR2, BD Transduction Laboratories), washed twice with FACS buffer and incubated either with anti-mouse antibody conjugated with biotin for 30' on ice followed by 10' of incubation with streptavidin-PE, or with anti-mouse IgG Ab coupled with phycoerythrin (Jackson ImmunoResearch Laboratories). Cells were analyzed on FACSCan or FACSCalibur cytometers (BD Bioscience).

Immunofluorescence studies

To analyse the subcellular localization of the murine Pot1 protein, cells were plated on coverslips and transfected with mPot1 (see above). Forty-eight hours after transfection cells were fixed with 2% para-formaldehyde, permeabilized with 0,1% TritonX-100 and incubated with ant-Pot1 Ab AP452, followed by Alexa594-coupled anti-rabbit Ab (Molecular Probes).

Results

Pot1: a new Tyk2 interacting protein

Previous data

Before my arrival in the laboratory, a yeast two-hybrid screen had been performed by M. Algarté, a post-doc in the laboratory. In this screen, the N terminal region of human Tyk2, containing the FERM domain (aa 1-450) was used as a bait to screen a cDNA library from the human T cell line Jurkat. Among the positive clones obtained, 6 contained overlapping sequences (Fig.8A), encoding portions of the C-terminal region of a novel protein, that was provisionally named Pot1 (acronym of Partner of Tyk2 1). Since then, several members of the laboratory (J. Ragimbeau, M. Oloomi, T. Yeh, C. Steindler, L. Zhi) had variably contributed to the study of Pot1. I will summarize below the studies that had been perfomed and the information and tools concerning Pot1 that were made available to me when I joined the laboratory.

In order to map the 5'end of the Pot1 transcript(s), a RACE (rapid amplification of cDNA ends) experiment was performed. A putative ATG was found which corresponded to the initiation codon of the murine orthologue transcript present at that time in the database. However, different human IMAGE clones were found that extended further at the 5' end. Thus, a Pot1 cDNA was reconstructed (4.6 kb) by ligating those clones (Fig. 8B). The protein encoded by this cDNA contains 1003 aa, has a predicted molecular weight of 112 kDa and no known functional or structural domains, except for two coiled-coil regions (the sequence of the cDNA reconstructed in the lab is given in Annex). A northern blot analysis of poly A+ mRNA from several human tissues was performed, using as probe the 5' region of the cDNA. Several faint ubiquitously expressed Pot1 transcripts were detected at 9.5, 7.5 and 4.4 kb (Fig. 9).

To analyze the interaction between Pot1 and Tyk2, a GST-pulldown assay was performed. After *in vitro* transcription and translation, the ^{35}S-labeled Pot1 product was incubated with the FERM domain of Tyk2 or Jak1 purified from bacteria as GST fusion proteins (Fig. 10). The results showed that Pot1, but not the ^{35}S-labeled β-gal control, bound to the FERM domain of both Tyk2 and Jak1. Attempts to coimmunoprecipitate endogenous Tyk2 and Pot1

were unsuccessfull. This may be due to a number of reasons: - the low level of cellular Pot1; - the fact that Pot1 is only partially extracted in lysis buffers containing non ionic detergents (NP40 or Triton) and may reside partly in raft microdomains; - the modest quality of the rabbit antisera raised against Pot1. Indeed, out of 10 Abs generated against Pot1, only one (AP452) directed to aa 425-440 weakly detects the endogenous protein in direct Western blot (see below).

The possibility that Pot1 can be phosphorylated on tyrosine residues was examined. For this, Pot1 was transiently co-expressed with Tyk2, Jak1 or Lck in 293T cells. Pot1 was immunoprecipitated, analyzed by western blot with anti-phopshotyrosine Abs and found to be indeed tyrosine phosphorylated (Fig. 11A). Moreover, endogenous Pot1 was found to be tyrosine phosphorylated in Jurkat cells treated with pervanadate, an inhibitor of protein tyrosine phosphatases (Fig. 11B). These results represent an indirect demonstration that Pot1 can interact and be phosphorylated by tyrosine kinases, such as the Jaks.

The antibodies raised against Pot1 did not detect the endogenous protein in immunofluorescence studies on human fibrosarcoma cells. Thus, to examine the subcellular localization of Pot1, cDNAs encoding the full-length (hPot1-V5), the C-terminal (aa 510-1003) or the N-terminal (aa 1-486) regions of Pot1, tagged at the C end with the V5 epitope, were transiently or stably expressed in the fibrosarcoma cell line HT1080. The staining pattern suggested that the full-length Pot1 as well as the C-terminal region are localized in cytoplasmic vesicles (Fig. 12). The nature of the vesicles positive for Pot1 could not be identified. These vesicles were not stained with markers specific for the endoplasmic reticulum, the Golgi complex, lysosomes or mitochondria. The N-terminal region strongly stained the nucleoli, presumably due to the unmasking in the truncated protein of a putative nuclear localization signal (NLS) present in this region (56 - RRDPFRKRKLGGRAKKVR - 73).

To assess the role of Pot1 in IFNα signaling, a functional assay was utilized based on the co-expression of Pot1 expression vectors and a reporter plasmid containing the luciferase reporter under the control of an IFNα-inducible promoter (ISG54). Expression of the full-length Pot1 (Fig. 13) or of the N-terminal region (data not shown) did not affect IFNα-induced luciferase expression. However, expression of the C-terminal region of Pot1 reduced IFNα-induced luciferase activity. This result suggested that the ectopically expressed C-terminal region of Pot1 could interfere with IFNα-induced transcriptional activation. These

data were indicative of the involvement of Pot1 in IFN signaling, but could also have resulted from the mere sequestration of Tyk2 away from the IFN receptor.

The different aspects of my work on Pot1 have focused on :

1) further characterization of the protein
2) analysis of Pot1 transcripts
3) subcellular localization of the murine orthologue (mPot1)
4)functional analysis of the role of Pot1 and its interactor GIT1 in IFNα signaling.

Database analyses of Pot1 mRNA transcripts

A putative Pot1 cDNA had been constructed in the lab, as discussed above. However, the alignement of this sequence with the reference mRNA sequences found in the NCBI database strongly suggested the existence of several Pot1 transcripts (Fig. 14). For convenience, I will use the term "lab cDNA" when referring to the sequence cloned in the laboratory. Figure 14 shows the Pot1 transcripts and Fig. shows the encoded proteins. These latter will be named from their four initial aa residues.

The "lab cDNA" is 4.6 kb and contains 13 exons with the first ATG located in exon I. The encoded protein (MPWP) is 1003 aa long and is the longest among all isoforms. The other transcripts are identical to the lab cDNA starting from exon VI, but diverge in their 5' UTR and translation initiation site. The encoded proteins are 217aa (MPME) and 250aa (MYSP) shorter than the MPWP. The MPME protein has 6 unique aa at the N-terminal region and is otherwise identical to MPWP. The short AK022954 mRNA sequence encompasses exon XIII, giving rise to a predicted 307aa protein (MAKL).

Compared to the Pot1 protein predicted from the "lab cDNA" (MPWP), the murine Pot1 orthologue lacks the first 100aa at the N-ter. Its initiation ATG codon is present in the lab cDNA sequence and was found as the starting ATG in the 5' RACE experiment (MVRS).

Mapping of Pot1 transcripts

To determine whether the lab cDNA is indeed a *bona fide* transcript in cells, I performed RT-PCR analyses. I used a reverse primer encompassing the splice junction between exons VI and VII (MG1), combined with different forward primers (MG2, MG3 and MG4). Figure 14 shows the position of the primers and Table 3 gives the size of the fragments amplified from the different database transcripts. Total RNA from 293T cells was reverse transcribed with random hexanucleotide primers and the cDNA obtained was subjected to PCR. MG1 and

MG2 amplified a 600 bp band whose identity was confirmed by sequencing to be the 608 bp band present in the “lab cDNA” (Fig. 16). Amplification with primers MG1 and MG3 gave rise to two bands. The smaller one had the same size as the one amplified from hPot1-V5 and was confirmed to be the 431 bp from transcripts BC040285 and "lab cDNA". The other band is presumably the 511 bp from transcript BC042613.

primers	expected size (bp)			
	full-length	NM_022757	BC042613	BC040285
MG1+MG2	608	no	no	562
MG1+MG3	431	3730	511	431
MG1+MG4	no	no	2707	no
H2+H3	2305	2305	2305	2305

Table 4. Expected size of the fragments amplified from different Pot1 transcripts

The expected 3.7 kb fragment from trancript NM_022757 (table) could not be amplified. Intriguingly, the 2.3 kb 3’ fragment could not be amplified from 293T cDNA, although it should be common to all transcripts. This could be explained by the length of the fragment or an inefficient PCR. Indeed, the same fragment was successfully amplified using as template hPot1-V5 plasmid DNA, though with a much lower yield than the smaller bands.

Our main concern was to determine whether the cDNA reconstructed in the lab is indeed expressed *in vivo*. The results obtained suggest that it is. However, further experiments are needed to corroborate these findings and the expression of the different transcripts needs to be studied in different cell lines. It should be noted that the AP452 Ab reveals a number of bands on western blot (see below "Functional studies of Pot1" and Fig. 19B).

Subcellular localisation of the murine Pot1

The predicted murine protein is 120 aa shorter than our human full-length Pot1 (Fig. 15). An IMAGE clone (5066612) encoding this mPot1, cloned in the pCMV-SPORT6 vector, was purchased and overexpressed transiently in 293T cells.

The human peptide (aa 423-440) which had been used to raise the anti-Pot1 polyclonal AP452 serum is 90% identical to the murine sequence and, indeed, AP452 Abs recognized mPot1 in western blot. As shown in Fig. 17, the expression level of the transfected mPot1 was

considerably higher than that of the transfected human Pot1-V5 or of the endogenous protein. The murine Pot1 protein has an apparent molecular weight of 115kDa in Western blot instead of the expected 97kDa.

To analyze its subcellular localization, mPot1 was transfected in Tyk2-deficient 11,1 cells or in the parental 2fTGH cells and visualized by wide-field immunofluorescent microscopy. Figure 18 shows that, in both cell lines, mPot1 stains cytoplasmic vesicular structures or organelles, possibly associated to membranes. However, given its high level of expression, the protein may be mislocalized.

Functional studies of Pot1

To investigate the potential role of Pot1 in IFNα signaling, we used the RNA interference approach. A 21 bp oligonucleotide was chosen that targets a central region of Pot1 (as shown in Fig. 15). This oligo was named "1623" according to the number of the starting target nucleotide (referred to as siPot1-1623). As a control, we designed a "scrambled" oligo (Sc), that contains the same nucleotides but in a non-specific or scrambled order. This oligo displayed less than 16 bp identity to all mRNA sequences annotated in the NCBI database. This degree of identity should not to induce aspecific, off-target, mRNA recognition and degradation (cf. Materials and Methods for sequences). The efficiency of knock-down of the exogenous and endogenous proteins was 50-80 %, as shown in Fig. 19. As stated above and shown in Fig. 19B, several bands are detected with the Pot1-specific Ab AP452. We consider the 112kDa band to represent endogenous Pot1 since it is specifically diminished by siRNA.

Additionally, we constructed a vector (pG-SUPER) co-expressing GFP and the 1623 (pG-SUPER1623) sequence or the Sc (pG-SUPERSc) sequence, in the form of a short-hairpin RNA (shRNA). This shRNA is processed in the cell, yielding an siRNA which downmodulates Pot1 expression. Although the knock-down efficiency with these vectors is not as high as with the oligos, the advantage is that transfected GFP positive cells can be monitored. We planned to use pG-SUPER vector to monitor highly transfected cells. However, subsequently we also commercial oligos that diminished Pot1 expression with a higher efficiency (see below, siPot1-D).

We initially analyzed the effect of Pot1 depletion on IFNα signaling using a luciferase reporter assay. 293T cells were co-transfected with siPot1-1623 and the firefly luciferase gene under the control of the ISRE from the ISG54 gene, an IFNα-inducible gene (ISG54-luc). Forty hours after tansfection, cells were treated with increasing doses of IFNα for 8 hr. As

shown in Fig. 20, knock-down of Pot1 resulted in a remarkable diminution of IFNα-induced luciferase activity, which was around 20% of that of the Sc control.

Since Pot1 had been shown to interact also with Jak1, we also analyzed the effect of Pot1 knock-down on IFNγ and IL-6 transcriptional responses. To this end, 293T cells were cotransfected with siPot1-1623 or the Sc oligo and an IFNγ (IRF1-luc) or IL-6 (rPAP-luc) luciferase reporter. As shown in Fig. 21, Pot1 knock-down diminished the IFNγ response to 25% of the Sc control. On the other hand, the IL-6 response was barely affected (Fig. 22). Two other IL-6 reporters were used, SIE-luc and 3xLy6E-luc, with results comparable to those obtained with the rPAP-luc reporter (data not shown). To assess the specificity of the effect of Pot1 depletion on the Jak/STAT pathway, we monitored the response to TNFα using a NFκB luciferase reporter. Unexpectedly, the TNFα response was reduced to 50% in Pot1-depleted cells (Fig. 23). Altogether, these results suggested that Pot1 is required for IFNα, IFNγ and TNFα, but not IL-6, signaling.

Next, we set to identify which step of the signaling pathway was affected by Pot1 depletion. Since Tyk2 is needed for stable IFNAR1 surface expression, one possibility was that Pot1 depletion affected IFNAR1 expression. Therefore, I analyzed IFNAR1 and IFNAR2 surface level by FACS in cells transfected with pG-SUPER vector, pGSUPER-1623 or pGSUPER-Sc, after gating on GFP-positive cells. No difference was observed in IFNAR1 and IFNAR2 surface levels in cells expressing pG-SUPER, pGSUPERr-Sc or pGSUPER-1623 (Fig. 24A). I also analyzed Jak1, Tyk2 and STAT1/2/3 phosphorylation in Pot1-depleted cells. As positive control, I used an siRNA directed against Tyk2 (siTyk2). As shown in Fig. 24B, STAT1 and STAT3, but not STAT2, phosphorylation were abolished in Tyk2-depleted cells. No evidence of Pot1 involvement in these processes could be obtained, as there was no difference in the profile of STAT tyrosine phosphorylation induced by IFNα between cells transfected with Pot1 siRNA or the Sc control.

To improve the efficiency of Pot1 knock-down, we purchased a mixture of four oligos targeting Pot1 (siPot1-D). The knock-down efficiency was higher than the one obtained with oligo 1623 (Fig. 25A). In parallel, we used two other oligos as controls, one targeting Jakmip1 (siJakmip1), a protein that is not expressed in 293T cells, and one designed by QIAgen to have minimal off-target effects (Ctrl-Q). We also used siTyk2 as positive control. To our surprise, it appeared that cells transfected with the Sc oligo exhibited a considerably higher IFN-induced luciferase activity as compared with cells transfected with the other two control oligos (Fig. 25B). The knock-down of Pot1 had no effect when compared to the

siJakmip1 and Ctrl-Q control cells. As expected, Tyk2 knock-down abolished IFNα-induced luciferase activity.

These data also undermined the results obtained from the study of the IFNγ, IL-6 and TNFα induced responses in Pot1 knock-down cells. Although the experiments were not repeated with proper controls, it is likely that the Sc oligo exerts an off-target also on these reporters.

Identification of Pot1 interactors by yeast two-hybrid screen

Another approach that we used in order to shed light on Pot1 function was to identify putative interactors. To this end, a yeast two-hybrid screen was performed by Hybrigenics, S.A. Since Pot1 has no conserved domains that could be preferentially used for the screen, we chose as bait the sequence corresponding to the 97 kDa MVRS protein isoform (Fig. 15). A lymphoid T cell line (CEMC7) cDNA library was screened. We cloned the 2.9 kb cDNA fragment into the pB27 vector provided by Hybrigenics (cf. Materials and Methods). The screen yielded a multitude of interacting proteins, classified according to an "interaction confidence", as shown in Table 4. No components of the IFNα, or more generally Jak/STAT, signaling pathway were present among the 71 proteins found with significant confidence. These latter proteins could not be functionally grouped, though many of them appeared to be functionally associated to small GTPases, either through GAP (GTPase-activating protein) or GEF (GTP-exchange factor) activities. The list of all the proteins found is given in the Annex. Of note, Pot1 was found to interact with itself. The best Pot1 interactors are two yet undescribed proteins. Among the 12 proteins with a high or good interaction confidence, GIT1 (G protein-receptor coupled kinase-interactor 1) was considered the most interesting candidate.

Score	clones	proteins
A : very high confidence in the interaction	29	2
B : high confidence in the interaction	23	4
C : good confidence in the interaction	21	8
D : moderate confidence in the interaction - "hard to find ones" or potential false positives	76	57
total	149	71
not significant	119	
total	268	

hKIAA0998
simil. to BZW1
CROP
GIT1
RINT1
cyclinB1
Pot1
ARFIP2
GIT2
Cep63
hPP1553
hSEC5L1
TALDO1
ZNF277

Table 5. Proteins found in the two-hybrid screen performed by Hybrigenics using Pot1(MVRS) as bait

GIT1

The first cloning of GIT1 originated from studies of signaling throughr the β_2-adrenergic receptor (β_2AR), which is a G protein-coupled receptor (Premont et al., 1998). Ligand-binding to β_2AR leads to the activation of heteromeric G proteins and also to the recruitment of G protein-coupled receptor kinases (GRKs) to the membrane. GRKs promote the inactivation of the receptors, subsequent sequestration of the receptors from the cell surface and their resensitization through dephosphorylation. GIT1 was cloned in a yeast two-hybrid screen using GRK2 as bait and was found to decrease β_2AR function when overexpressed. It was shown that GIT1 exerts this role through its GAP activity towards ARF1. ARFs form a family of small GTPases thought to function primarily as GTP-dependent regulators of vesicular trafficking. More recently, ARFs have been implicated in the regulation of the actin cytoskeleton and the organization of focal adhesions (Turner et al., 2001). By promoting GTPase activity of ARF1, GIT1 blocks β_2AR internalization and thus impedes receptor resensitization. Subsequent studies showed that GIT1 and its homologue GIT2 are the best GAPs for ARF6, but they can also stimulate GTP hydrolysis by ARF1, ARF2, ARF3 and ARF5 (Vitale et al., 2000). GIT1 overexpression was shown to regulate the internalization of numerous receptors, all of which internalize through the clathrin-coated pit pathway (Claing et al., 2000; Lahuna et al., 2005).

GIT1 was also found to interact with β-PIX, a GEF associated with PAK (p21-activated serne-threonine kinases) (Bagrodia et al., 1999). Since then, numerous studies have addressed the function of GIT1 in focal adhesions formation. GIT1 was found to interact with the multidomain scaffolding protein paxillin and FAK and to have a complex role in regulating protrusive activity and cell migration (Manabe et al., 2002; Zhao et al., 2000).

Another aspect of GIT1 activity has emerged with findings that it can mediate the activation of PLCγ and MEK1-ERK1/2 by angiotensin II and epidermal growth factor (EGF) (Haendeler et al., 2003; Yin et al., 2004). It is thought to function as an adaptor protein in these signaling pathways. Interestingly, GIT1 was not found to be substrate for the serine-threonine kinase GRK2 (Premont et al., 1998), but was found to be tyrosine phosphorylated in a cell cycle and adhesion-dependent manner (Bagrodia et al., 1999), as well as in response to angiotensin II and EGF (Haendeler et al., 2003).

Based on these data and, given that another member of the same family, GIT2, was also found to interact in yeast with Pot1, we asked whether GIT1 could play a role in the IFNα-dependent internalization of the receptor complex and/or in IFNα-induced signaling.

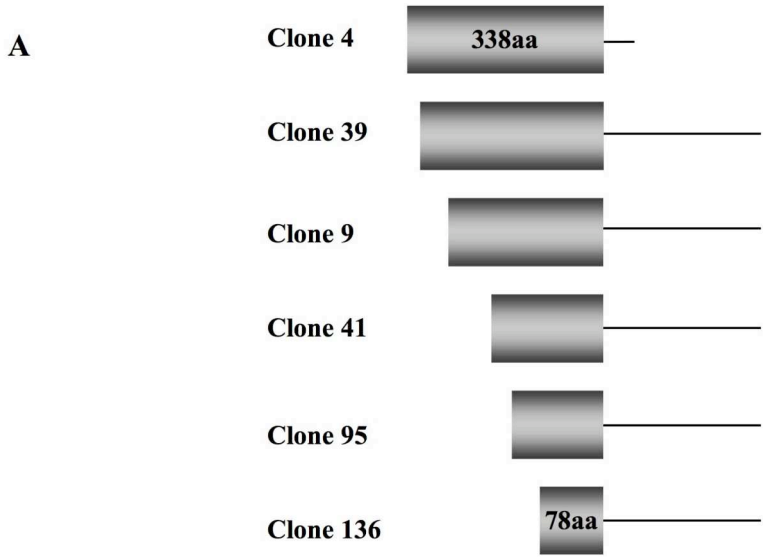
The role of GIT1 in IFNα signaling.

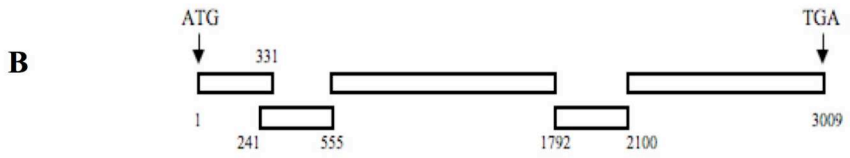
To analyze the potential role of GIT1 in IFNα signaling, I monitored IFNα-induced events after GIT1 knock-down or overexpression. For knocking down, I used a mixture of 4 commercial siRNA oligos targeting GIT1, with the Sc oligo (see above) as control. Figure 26 (left panel) shows an efficiency of knock-down of 80 %. For the overexpression, we obtained from N. Vitale (Strasbourg) cDNAs encoding WT GIT1 or the GAP-deficient mutant, R39A. The GIT1 R39A mutant has no GAP activity and was shown not to affect receptor internalization (Lahuna et al., 2005). Figure 26 (right panel) shows that both the WT and mutant GIT1 are well expressed in transiently transfected 293T cells.

First, I analyzed by FACS the effect of GIT1 overexpression on the down regulation of the IFNα receptor subunits in 293T cells treated with IFNα. As shown in Fig. 27A, the overexpression of GIT1 or of the GAP-deficient mutant did not affect IFNα induced down regulation of IFNAR1 and IFNAR2. The same assay was performed in GIT1-depleted 293T cells. As shown in Fig. 27B, there was no effect of GIT1 siRNA on IFNα-induced receptor internalization as measured by FACS. To assess whether GIT1 had a role in downstream signaling, I monitored the effect of GIT1 depletion on IFNα-induced STAT1 and STAT2 phosphorylation or luciferase production. There was no difference in the level of IFN-induced STAT phosphorylation (Fig. 28) or luciferase production (Fig. 29) in GIT1-depleted cells compared to control cells.

Altogether, these data showed no evidence of a role of GIT1 in IFNα induced Jak/STAT signaling at least in this cell system.

These negative observations led me to slowly change direction of my studies and I chose to focus more on the biochemical and functional study of Tyk2 point mutants, as described below.





1-331	PCR on IMAGE clone 3175912 (GenBank BE218504)
241-555	PCR on IMAGE clone 1288470 (GenBank AA761473)
555-1792	digestion of IMAGE clone 1288470
1792-2100	RT-PCR from Jurkat T cells
2100-3009	the longest cDNA clone obtained from the screen

Figure 8. Pot1 yeast clones and cDNA reconstruction.
A) Six overlapping clones obtained in the two-hybrid screen with Tyk2 FERM domain;
B) Reconstruction of Pot1 cDNA in the lab ("lab cDNA")

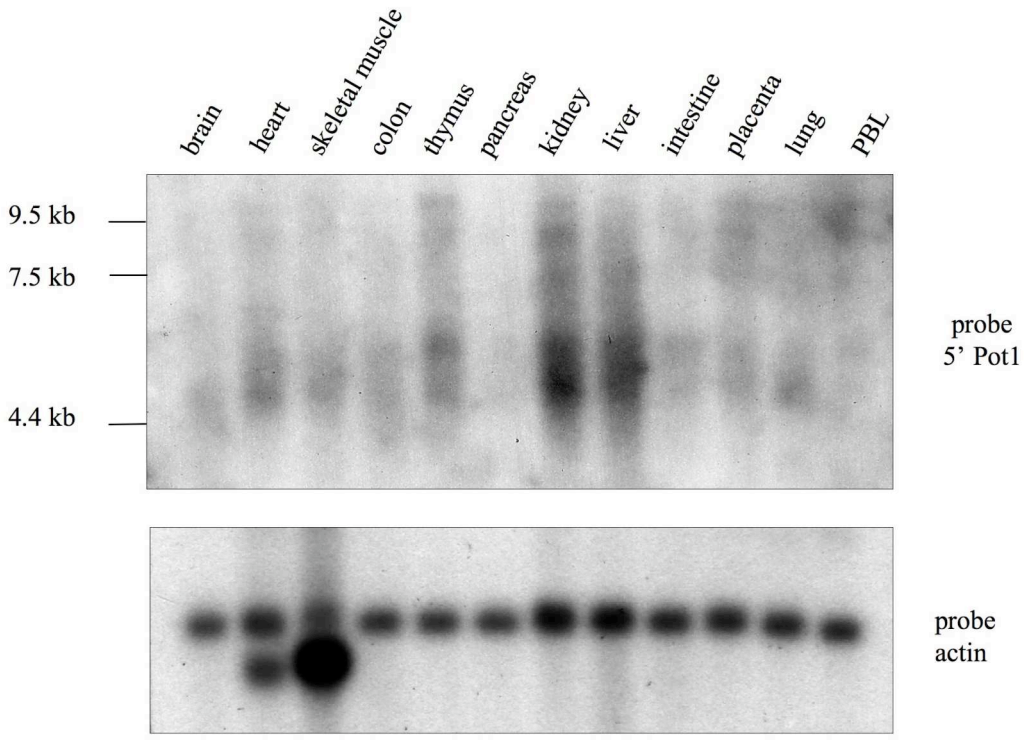


Figure 9. Northern blot analysis of Pot1mRNA expression.
Poly A+ mRNA from tissues as indicated was analyzed with a probe covering the 5' region of the cDNA. PBL: peripheral blood lymphocytes

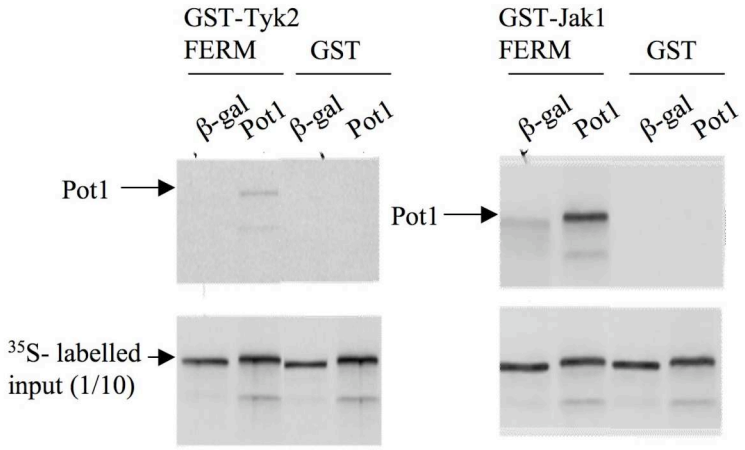


Figure 10. *In vitro* interaction of Pot1 and the FERM domain of Tyk2 and Jak1.
After *in vitro* transcription and translation, ^{35}S-labeled Pot1 was incubated *in vitro* with either the FERM domain of Tyk2 (left panel) or of Jak1 (right panel) expressed as GST fusion proteins. *In vitro* translated and transcribed β-galactosidase (β-gal) was used as control.

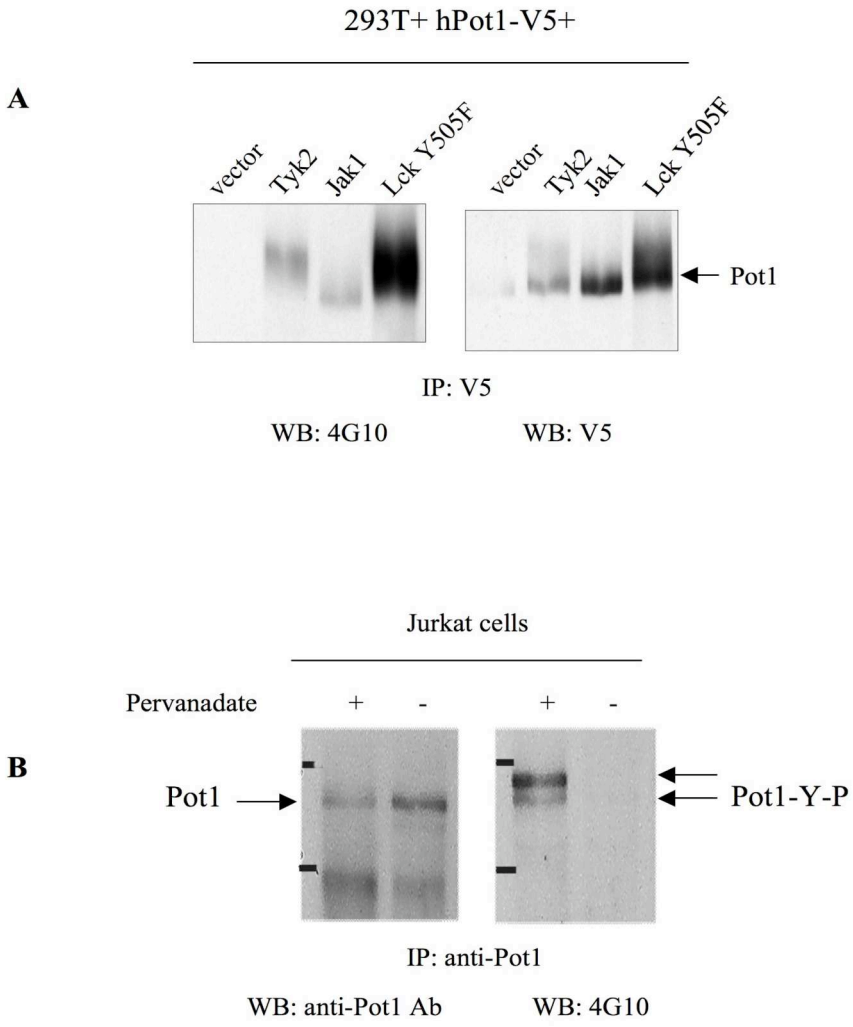


Figure 11. Study of Pot1 tyrosine phosphorylation.
A) 293T cells were co-transfected with hPot1-V5 and Tyk2, Jak1 or a constitutively active form of Lck, as indicated. Pot1 was immunoprecipitated with anti-V5 Ab. The tyrosine phosphorylation of Pot1 was analyzed by Western blot with the anti-phospho-tyrosine 4G10 mAb. The membrane was re-probed for total Pot1 levels.
B) Jurkat T cells were treated or not with pervanadate. Cell lysates were immunoprecipitated with anti-Pot1Abs and analyzed with the anti-phospho-tyrosine 4G10 mAb (right). The membrane was stripped and reblotted with anti-Pot1 Ab (left).

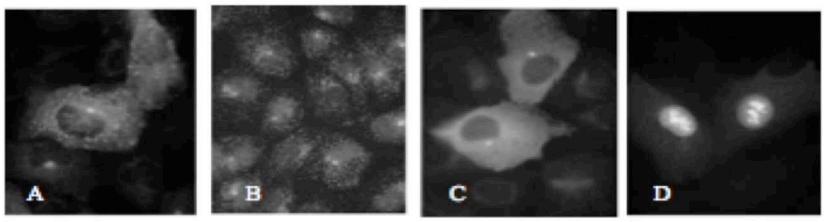


Figure 12. Subcellular localization of Pot1 analyzed by immunofluorescence microscopy using the anti-V5 mAb in HT-1080 cells
A) Cells transiently transfected with hPot1-V5
B) A clone stably expressing exogenous Pot1
C) Cells transiently expressing the C-terminal region of Pot1
D) Cells transiently expressing the N-terminal region of Pot1

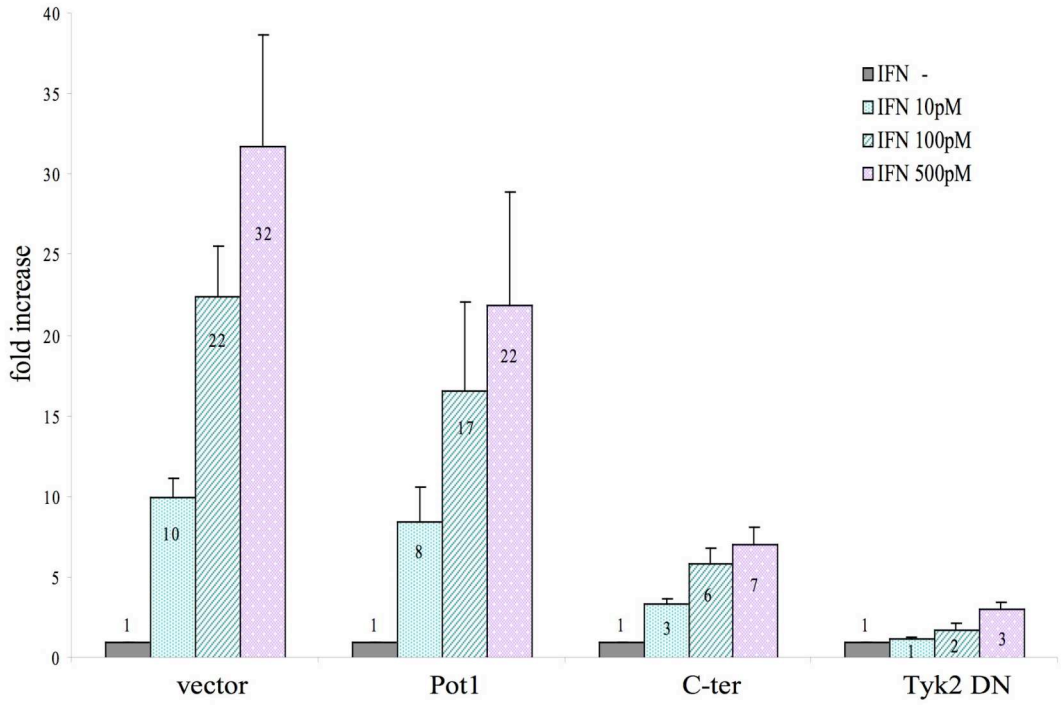


Figure 13. Effect of Pot1 overexpression on IFNα-induced luciferase activity.
293T cells were transfected with the indicated plasmid and ISG54 (ISRE-Luci reporter). 36 h post-transfection, cells were stimulated for 8 h with different concentrations of IFN-α2. Luciferase activity was measured using a Microplate luminometer. Tyk2 DN: a dominant-negative mutant of Tyk2 (K930R/Y1054F/Y1055F)

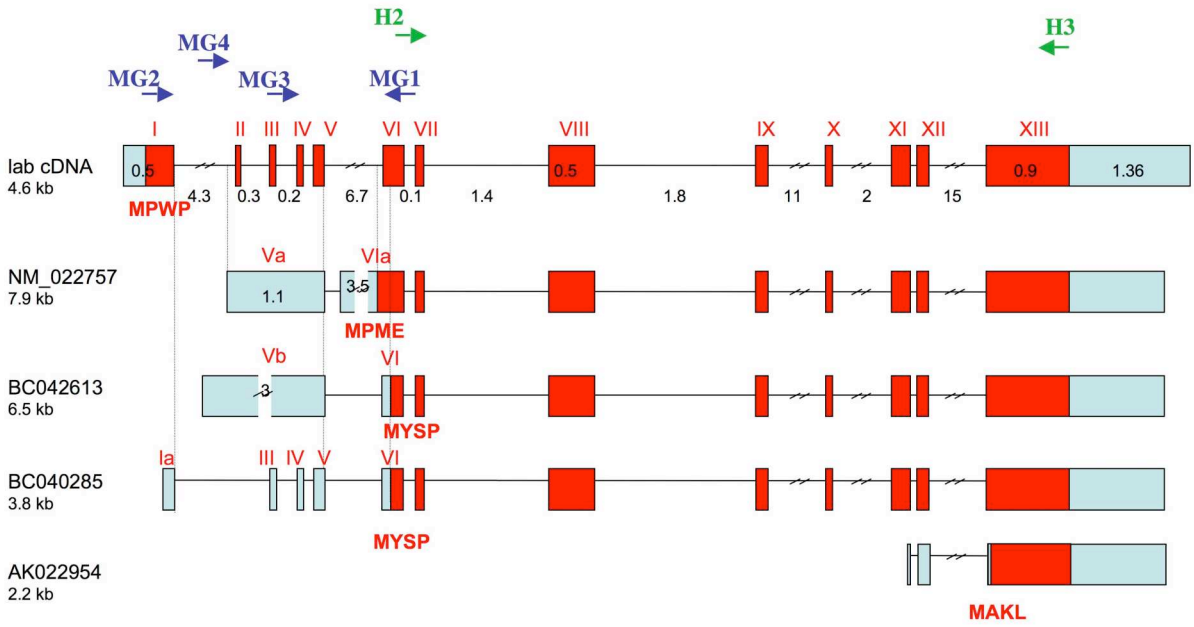


Figure 14. Comparison of the exon-intron structure of the 'lab cDNA' and transcripts annotated in the NCBI database.
The exons are represented as boxes, numbered with roman numbers, and depicted in red and blue for coding and non-coding exons, respectively. The name and size of the transcript are given on the left. The first four amino-acids corresponding to the protein encoded by the transcript are given below the starting exon of each transcript.
MG1-MG4, H2 and H3 are primers used for mapping different Pot1 mRNAs.

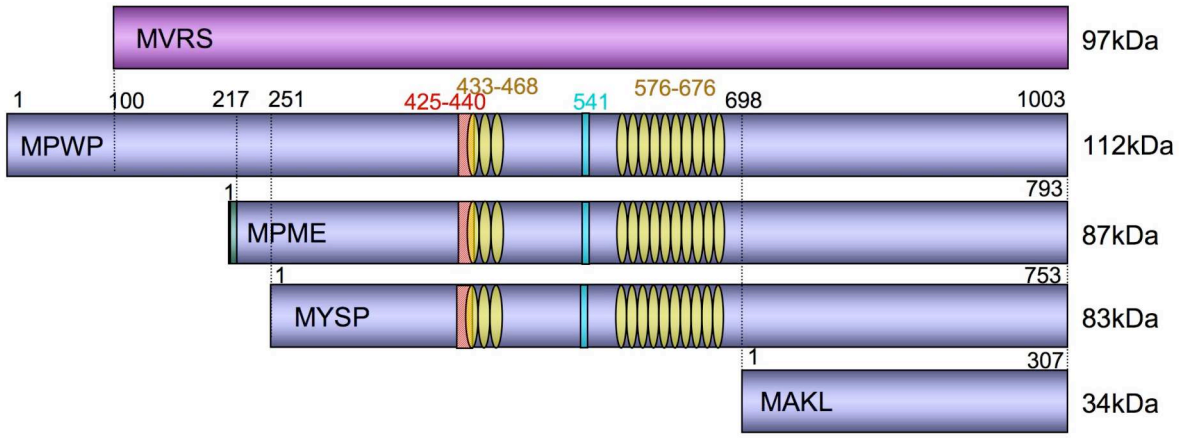


Figure 15. Pot1 protein isoforms.
The murine protein is in violet. The human isoforms are depicted in blue. The coiled coils, the AP452 antibody target region and the siRNA target region are depicted in yellow, red and light blue, respectively. The predicted molecular weights are given on the right.

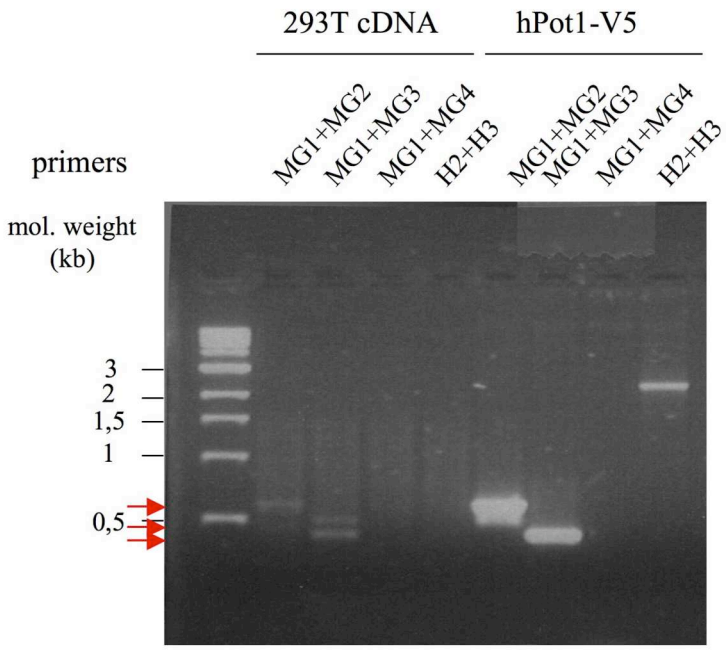


Figure 16. Mapping Pot1 mRNA isoforms.
Total mRNA from 293T cells was isolated, reverse transcribed and subjected to PCR with primers as indicated. 1/10 of the PCR reaction was analyzed on a 2% agarose gel. Red arrows show the amplified fragments of 608, 511 and 431 bp.

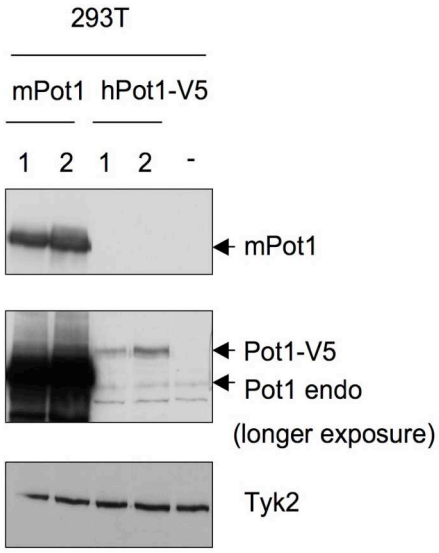


Figure 17. Detection of the murine Pot1 protein (mPot1).
293T cells were transfected with 1 or 2 μg of the indicated plasmid. Fourty-eight hr later, cells were lysed and the amount of human and murine Pot1 was analyzed by SDS-PAGE and Western blot using anti-Pot1 AP452 Ab. Two different exposure times are shown. The murine protein migrates at 115kDa instead of the expected 97kDa.

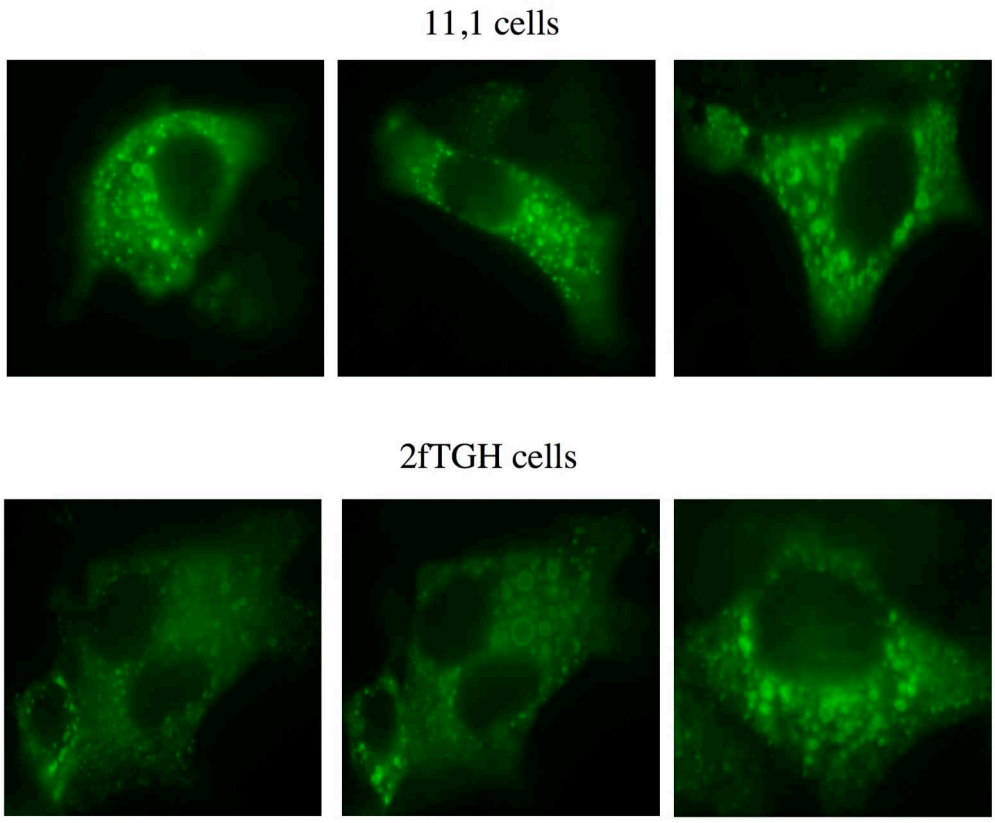


Figure 18. Subcellular localization of murine Pot1 (mPot1).
Tyk2-deficient 11,1 cells or the parental 2fTGH cells were transiently transfected with mPot1. Forty-eight hours later, cells were fixed, permeabilized and stained for mPot1 using the AP452 Abs and Alexa594-coupled anti-rabbit as secondary Abs

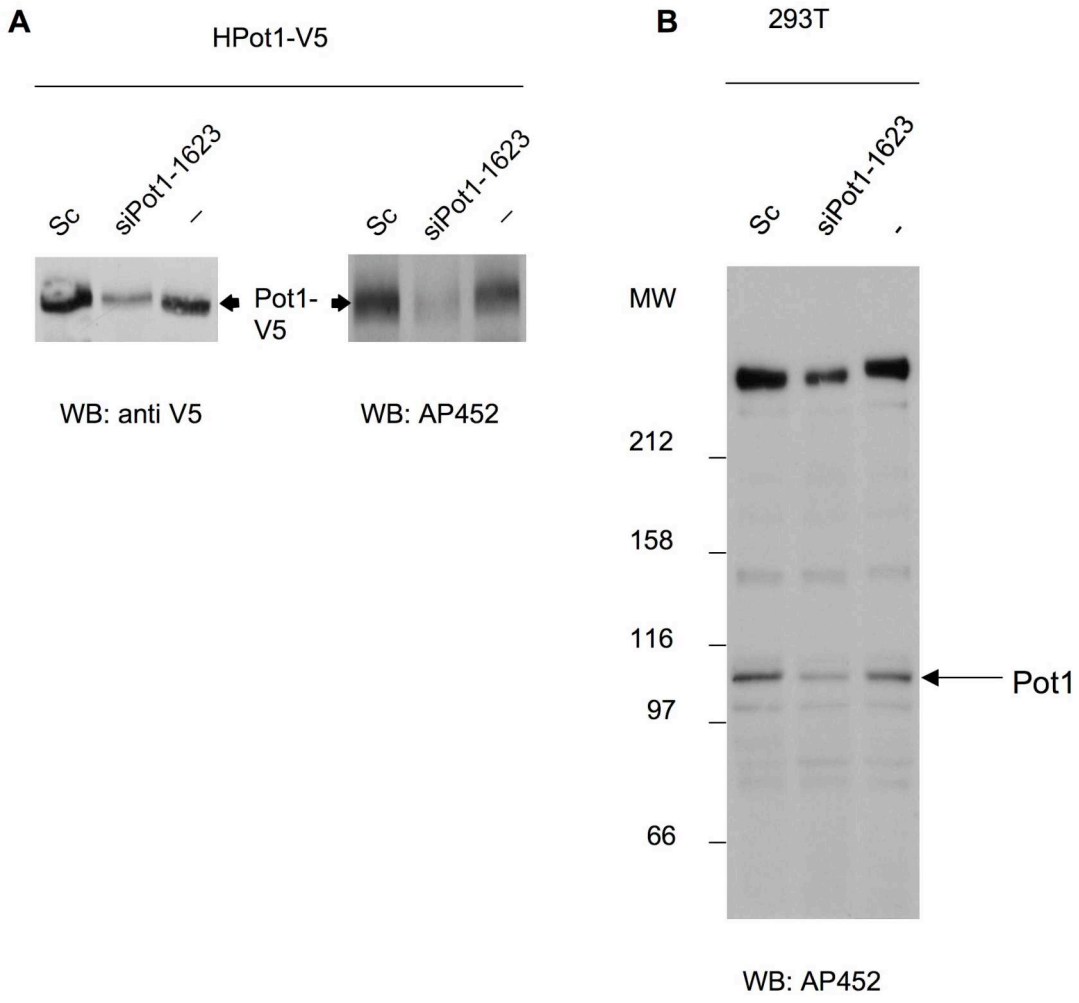


Figure 19. Efficiency of Pot1 knock-down
A) HT1080 cells stably expressing Pot1-V5 (HPot1-V5) were transfected with Pot1 siRNA 1623 or the Sc as control. Forty eight hr after transfection, cells were lysed and the level of Pot1 was analyzed by western blot using anti-V5 or anti-Pot1 Ab AP452.
B) Pot1 knock-down efficiency was analyzed in 293T cells as described in A). The lysates were analyzed on a 7% SDS-PA gel followed by western blot and detection by anti-Pot1 AP452 Ab.

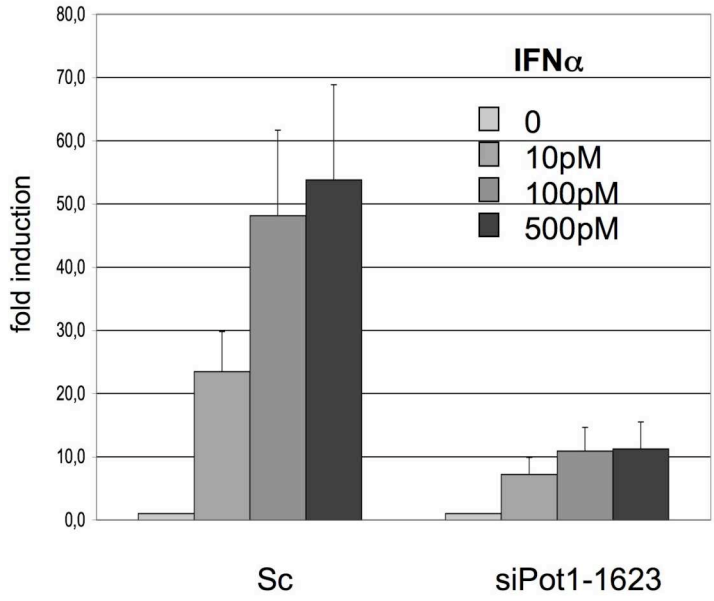


Figure 20. Effect of Pot1 knock-down on IFNα-induced luciferase activity.
293T cells were transfected with Pot1 siRNA 1623 or the Sc as control as indicated, together with the IFNα reporter plasmid ISG54-luc. Forty hours after transfection cells were treated for 8 h with different doses of IFNα as indicated and luciferase activity was measured. The mean of 9 different experiments are represented with standard deviations.

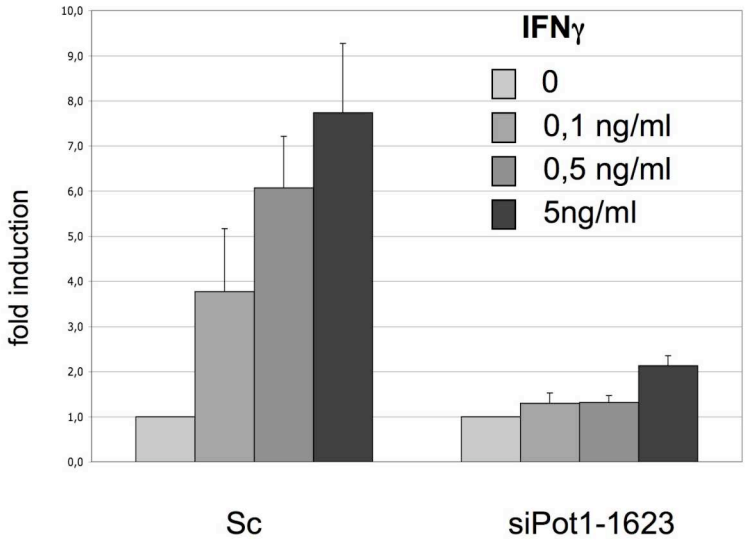


Figure 21. Effect of Pot1 knock-down on IFNγ-induced luciferase activity.
293T cells were transfected with Pot1 siRNA 1623 or the Sc as control as indicated, together with the IFNγ reporter plasmid IRF1-luc. Forty hours after transfection cells were treated for 8 h with different doses of IFNγ as indicated and luciferase activity was measured. The mean of 3 different experiments are represented with standard deviations.

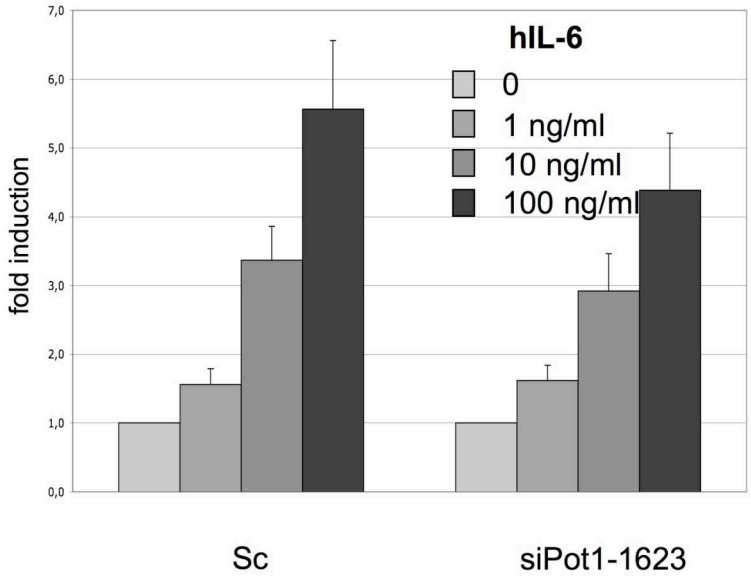


Figure 22. Effect of Pot1 knock-down on IL-6-induced luciferase activity.
293T cells were transfected with Pot1 siRNA 1623 or the Sc as control, as indicated, together with the IL-6 reporter plasmid rPAP-luc. Forty hours after transfection, cells were treated for 8 h with different doses of hyperIL-6 as indicated and luciferase activity was measured. The mean of 3 different experiments are represented with standard deviations.

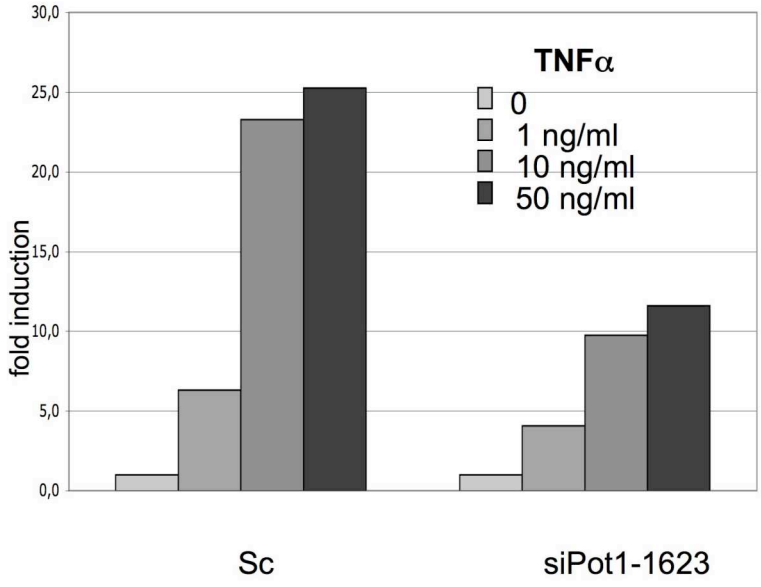


Figure 23. Effect of Pot1 knock-down on TNFα-induced luciferase activity.
293T cells were transfected with Pot1 siRNA 1623 or the Sc as control as indicated, together with the TNFα reporter plasmid NF-κB-luc. Forty hours after transfection cells were treated for 8 h with different doses of TNFα as indicated and luciferase activity was measured. The figure shows one representative experiment.

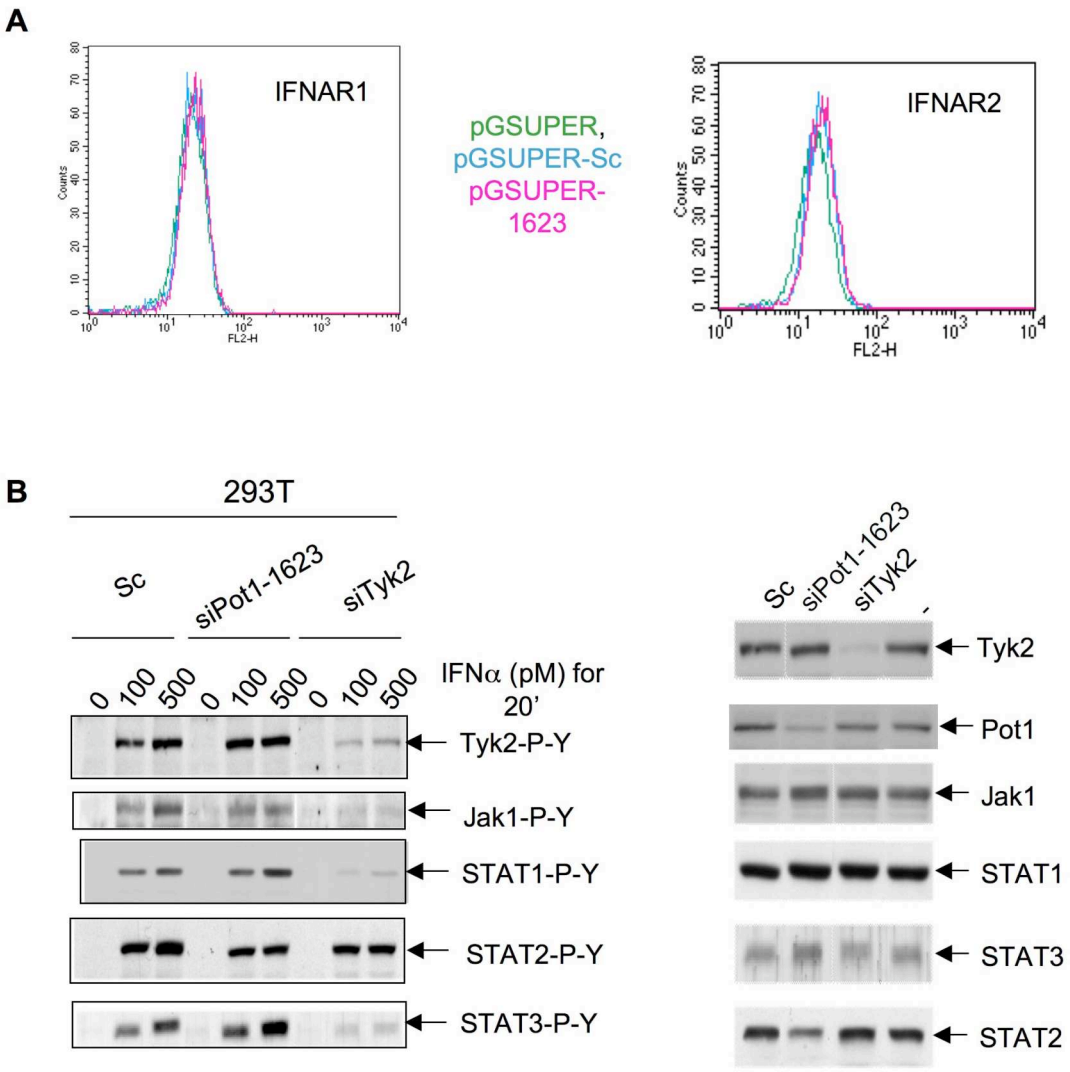


Figure 24. Effect of Pot1 depletion on IFNAR1 and IFNAR2 surface expression and IFNα-induced STAT phosphorylation

A) Pot1-depleted cells express the same level of IFNAR1 and IFNAR2 at the cell surface. 293T cells were transfected with pG-SUPER empty vector, the pG-SUPER containing the shRNA Pot1 1623 or the Sc control, as indicated. IFNAR1 and IFNAR2 surface levels were measured by FACS on GFP-positive gated cells.

B) IFNα-induced signaling in Pot1-depleted cells. 293T cells were transfected with siPot1-1623, the Sc as control or the siTyk2. Forty eight hr after transfection, cells were treated with different doses of IFNα for 20 min, as indicated. The phosphorylation state of signaling components was measured with phospho-specific antibodies. The membranes were subsequently re-probed for the total protein level.

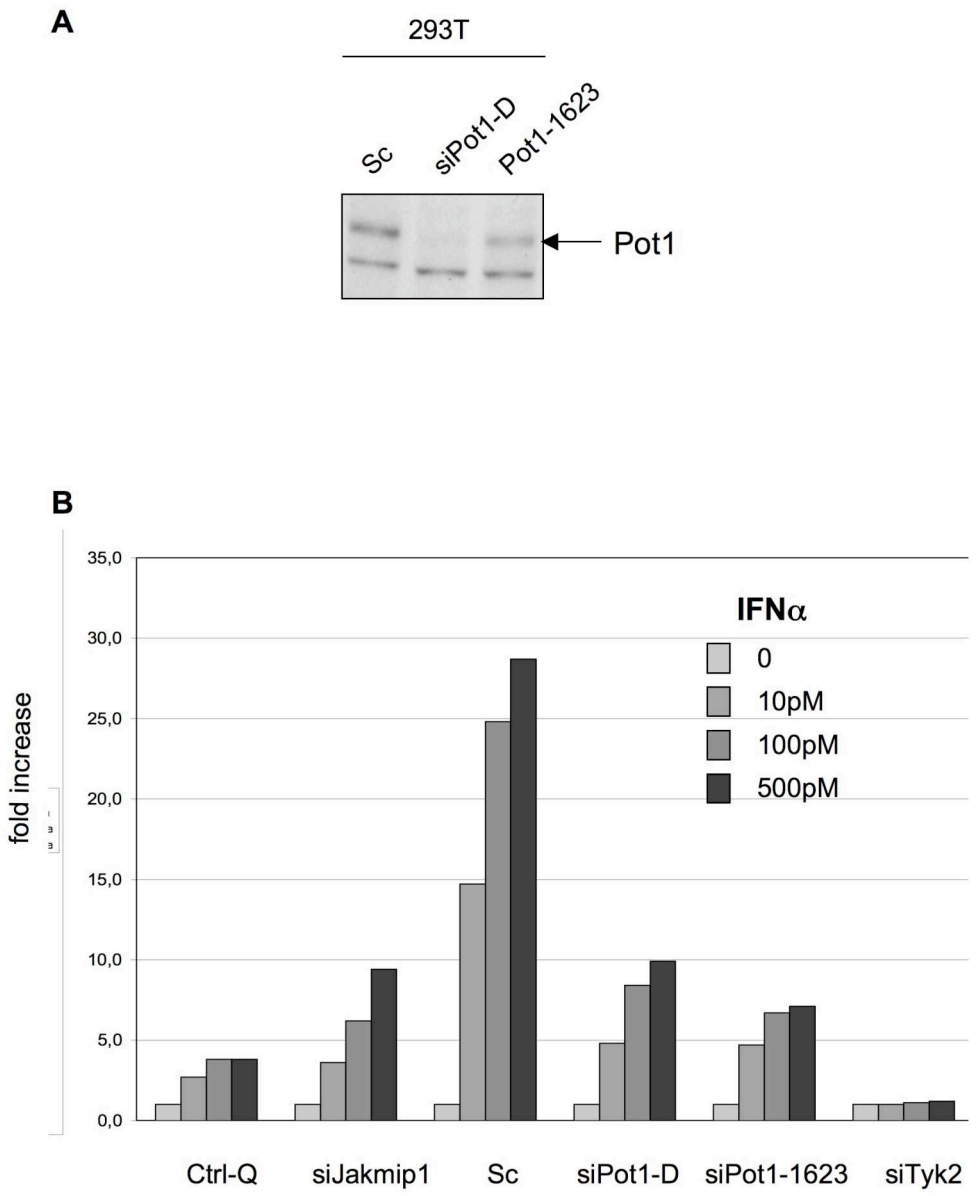


Figure 25. Effect of Pot1 knock-down on IFNα-induced luciferase activity with different siRNA

A) Pot1 knock-down efficiency. 293T cells were transfected with the Sc oligo, siPot1-1623 or siPot1-D mix. 48 hr after transfection the amount of Pot1 in the cells was analyzed by Western blot.

Effect of Pot1 depletion on IFNα-induced luciferase production. 293T cells were transfected with different siRNA as indicated, together with the IFNα reporter plasmid ISG54-luc. Forty hr after transfection, cells were treated with different doses of IFNα as indicated for 8 hr and luciferase activity was measured.

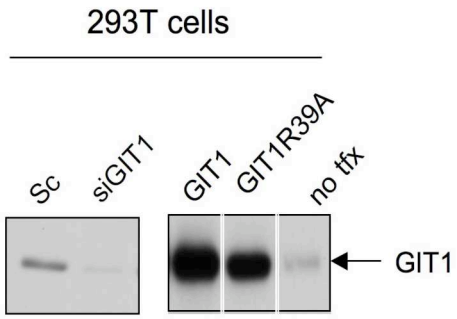


Figure 26. Efficiency of GIT1 knock-down and overexpression.
293T cells were transfected with a siRNA targeting GIT1 (siGIT1) or the control siRNA (Sc) (left panel) and plasmids encoding either WT GIT1 or the R39A mutant (right panel). The membrane was probed with anti-GIT1 Abs

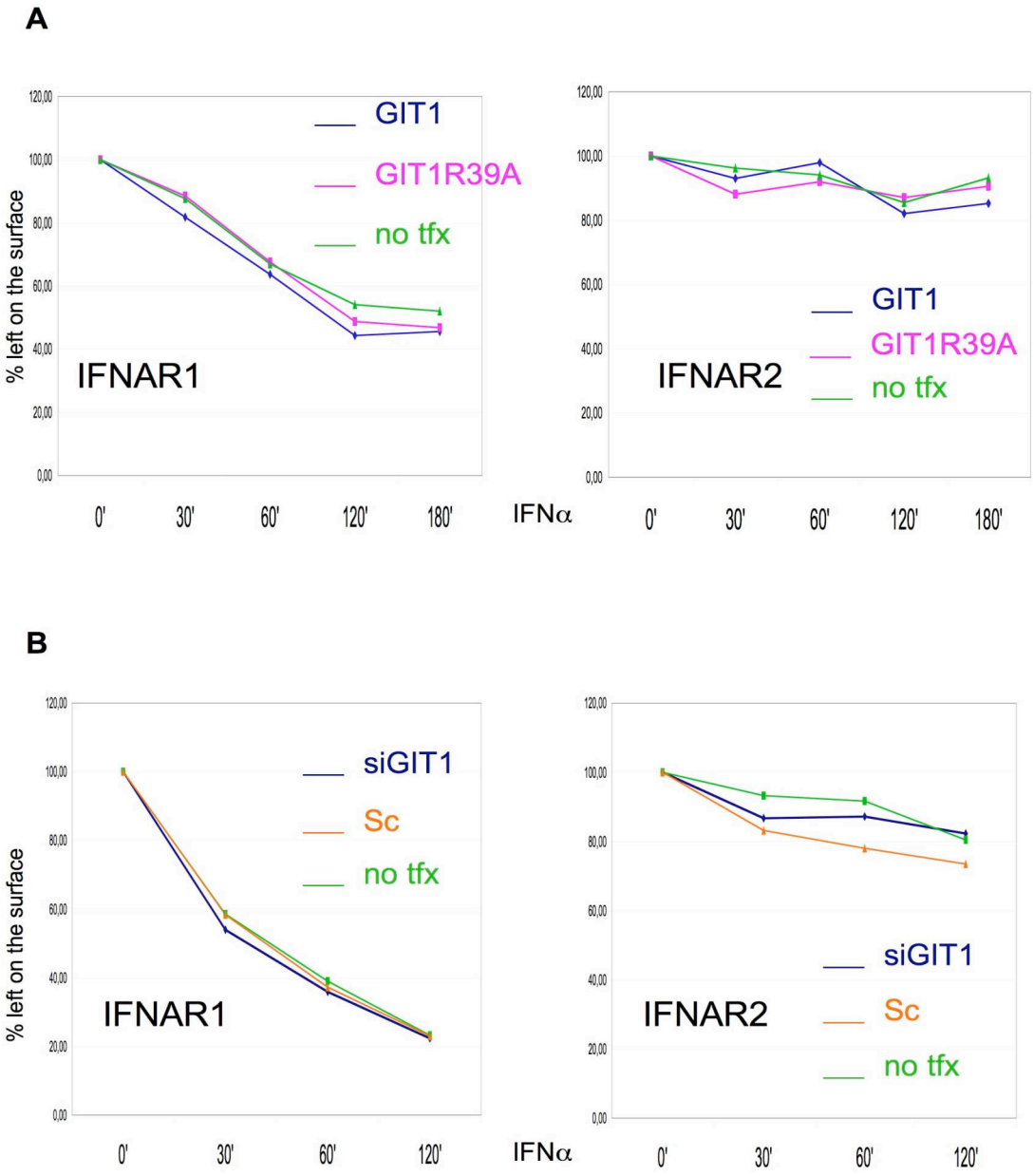


Figure 27. Effect of GIT1 overexpression or depletion on IFNα-induced receptor surface down regulation.

A) GIT1 overexpression; B) GIT1 depletion. Cells were transfected as indicated and 45 hr after transfection treated with 500 pM IFNα for the indicated times, with (B) or without (A) cycloheximide. The surface levels of IFNAR1 and IFNAR2 were monitored by FACS.

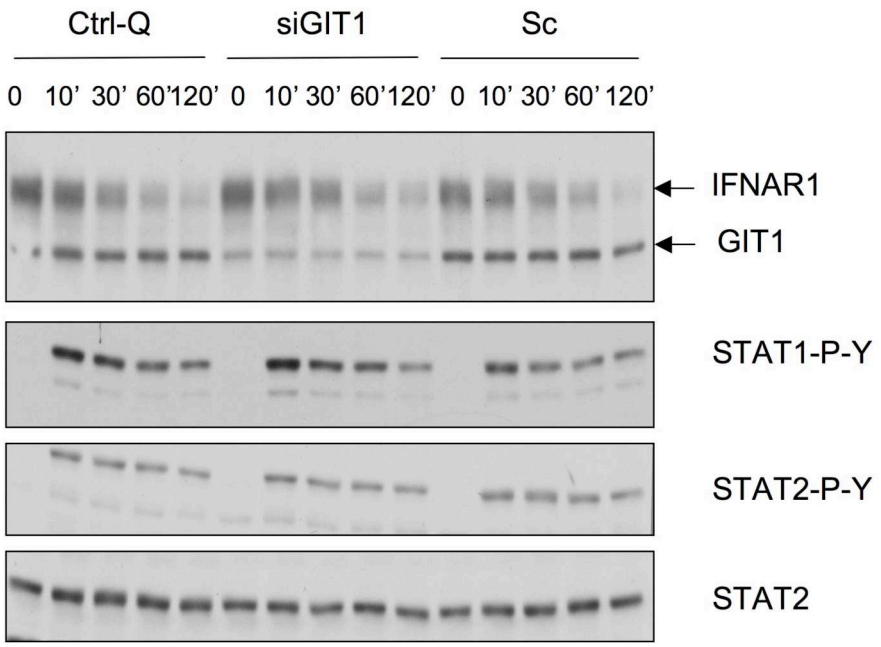


Figure 28. Effect of GIT1 knock-down on IFNα-induced STAT phosphorylation
293T cells were transfected with a siRNA targeting GIT1 (siGIT1) or the control siRNA (Sc and Ctrl-Q). Forty-eight hr after transfection cells were treated with 500 pM IFNα for the indicated time and the phosphorylation of signaling components was analyzed by western blot with phospho-specific antibodies.

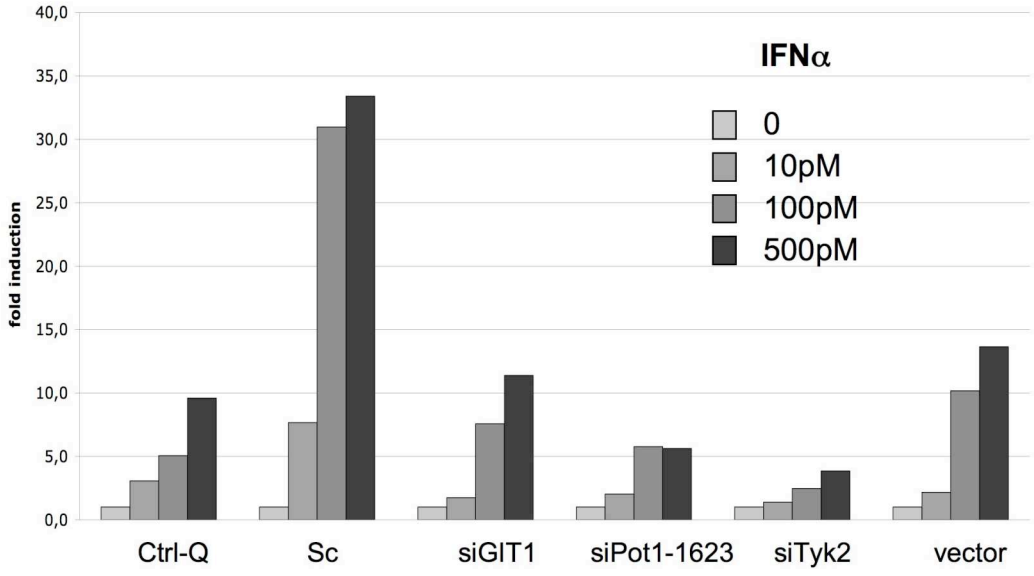


Figure 29. Effect of GIT1 knock-down on IFNα-induced luciferase reporter activity.
293T cells were co-transfected with the siRNA or control vector as indicated together with the IFNα reporter ISG54-luciferase. The assay was performed as described above.

Tyk2 mutations

V678F, an activating mutation of Tyk2

Tyk2V678F basal phosphorylation in vivo and in vitro

To analyze the basal phosphorylation state of the Tyk2 V678F mutant, we transiently transfected Tyk2 WT or V678F in Tyk2 deficient 11,1 cells and Jak1 deficient U4C cells. As shown in Fig. 30A, in 11,1 transfectants the phosphorylation on the activation loop of the V678F mutant was readily detected compared to that of the WT. Similar level of phosphorylation was achieved in U4C cells, suggesting that Jak1 is not required for Tyk2 V678F basal phosphorylation.

To test whether this high phosphorylation content is a consequence of increased basal catalytic activity of the mutant enzyme, we derived neo^R clones that expressed equivalent levels of the WT and mutant protein and performed cold *in vitro* kinase assay. Tyk2 WT and V678F were immunoprecipitated and subjected to an *in vitro* kinase assay, in the presence or absence of ATP. The reaction products were analyzed by Western blot with anti-phospho-tyrosine and subsequently with anti-Tyk2 Abs. As shown in Fig. 30B and C for one representative clone, the basal autophosphorylation activity of mutant V678F was 7-fold higher than that of WT. The quantification shows the mean of 5 different experiments with standard deviations.

The V678F mutant leads to basal STAT3 phosphorylation but normal IFNα induced signaling

To test the functional consequence of this potential gain-of-function mutation, basal and IFN-induced phosphorylation levels of Jak/Stat proteins were monitored in dose response and kinetic analyses in WT and V678F-expressing cells. Interestingly, the phosphorylation levels of Jak1, STAT1/2/5 were equivalent in the two cell lines (Fig. 31). However, only in V678F-expressing cells STAT3 and V678F were basally phosphorylated, with a further increase upon IFNα stimulation (Fig. 31, lanes 1 and 5, left panel, and lanes 1 and 6, right panel). The surface expression of IFNAR1 was equal in both clones. The same results were obtained with three other V678F clones (data not shown).

While the V678F mutant appeared dysregulated, it did not affect STAT1/2/5 activation nor it increased IFNα sensitivity. However, it did gain the capacity to phosphorylate endogenous

STAT3 in the absence of cytokine stimulation. We therefore asked whether this basally phosphorylated STAT3 was transcriptionally active. For this, WT- and V678F-expressing cells were transiently transfected with a STAT3-responsive luciferase reporter and 24 hr later luciferase activity was measured. A 3.5 fold increase in luciferase activity was observed in V678F-expressing cells (Fig. 32), indicating that the phosphorylated STAT3 is indeed transcriptionally active.

Analysis of the Tyk2V678F mutant placed in a homodimeric receptor complex

As mentioned above, Tyk2 is found associated only to receptor subunits that form heteromeric complexes (cf. Introduction). Conversely, Jak2 is the only Jak family member able to transduce signals from homodimeric receptors. Therefore, we sought to test whether the Tyk2V678F mutant gained further function when placed in an homodimeric receptor context, instead of the native IFN receptor context. For this, we used 11,1-derived cells expressing the chimeric EpoR/R1-Flag receptor that contains the EpoR ectodomain fused to the transmembrane and the intracellular regions of IFNAR1 (Fig. 33). These cells were reconstituted with equal level of either WT Tyk2 or the V678F mutant. First, we measured the basal auto-phosphorylation capacity in an *in vitro* kinase assay. The mutant showed about 14 fold increased activity with respect to the WT protein (Fig. 34). Basal and Epo-induced Jak/Stat phosphorylation profiles were monitored in dose response and kinetic analyses. Only in V678F-expressing cells, STAT3 and Tyk2V678F were basally phosphorylated (Fig. 35A). An effect was also observed on the level of Epo-induced STAT1 phosphorylation, which was higher in V678F-expressing cells than in control cells. Three other EpoR/R1-derived V678F clones were tested with similar results, one of which is shown in Fig. 35B. Basal STAT3 and Epo-induced STAT1 and STAT5 phosphorylation were higher in the V678F-expressing clone, in spite of its lower Tyk2 level as compared to the WT clone.

Equivalent basal STAT3 phosphorylation level in 11,1 and EpoR/R1 clones

The above data showed that the V678F mutant has the propensity to phosphorylate STAT3, but not STAT1/2. In order to test whether a direct correlation exists between Tyk2 and STAT3 phosphorylation, we compared the various clones generated throughout this study in the same blot (Fig. 36). The data showed that the WT protein was never detectably phosphorylated, while the V678F mutant was always phosphorylated, but remarkably more in the EpoR/R1 clones. STAT3 was more phosphorylated in V678F-expressing clones than in WT cells. However, its phosphorylation level did not seem to parallel that of V678F, since it

was roughly equivalent in all V678F-expressing clones, whether derived from 11,1 or EpoR/R1 cells. These data suggest that the correlation between Tyk2 and STAT3 phosphorylation status is not linear, since higher basal Tyk2 phosphorylation, as found in EpoR/R1 clones, does not necessarily lead to higher STAT3 phosphorylation.

Analysis of the Tyk2P1104A mutant

Impaired *in vivo* auto/transphosphorylation of Tyk2 P1104A

The P1104A mutation is located in the lip segment of the TK domain and potentially could dysregulate Tyk2 function. Indeed, it was suggested to precipitate an activated state of Tyk2 (Kaminker et al., 2007). Thus, we sought to analyze the effect of this mutant by comparing it with the WT and V678F. Tyk2-deficient 11,1 cells we transiently transfected with Tyk2 WT, Tyk2P1104A, Tyk2V678F or empty vector and total lysates were analyzed by Western blot. While the level of expression of the three exogenous proteins was comparable, their level of tyrosine phosphorylation on the activation loop differed remarkably (Fig. 37). When the membrane was overexposed, a weak phosphorylation of the WT protein could be detected, whereas the V678F mutant was very robustly phosphorylated. On the other hand, no evidence of phosphorylation of the P1104A mutant could be obtained, even after prolonged exposure of the membrane.

From the analysis of the V678F mutant it appeared that STAT3 is a preferential substrate of this mutant. Thus, we compared the tyrosine phosphorylation level of STAT3 in cells overexpressing WT, P1104A or V678F proteins. As shown in Fig. 37, in the presence of WT, STAT3 was more phosphorylated than in 11,1 cells. As shown above, the presence of the V678F mutant induced higher STAT3 basal phosphorylation when compared to the WT-expressing cells. On the other hand, the level of STAT3 phosphorylation in the presence of Tyk2 P1104A was similar to the one observed in the absence of Tyk2. Altogether, these results confirm the correlation between Tyk2 and STAT3 phosphorylation states and show that overexpressed Tyk2P1004A is unable to autophosphorylate or phosphorylate STAT3 *in vivo*.

Tyk2 P1104A rescues IFNα signaling

When overexpressed, the phosphorylation of WT is believed to result from the elevated concentration of intracellular molecules which *trans*-phosphorylate each other on the activation loop and consequently shift to an activated state (Gauzzi et al., 1996; Ragimbeau et al., 2003). The absence of phosphorylation of the overexpressed P1104A mutant pointed to a catalytic impairment. To assess whether the P1104A mutant can still rescue IFN signaling in 11,1 cells, we derived neo^R clones that expressed equivalent levels of WT and P1104A proteins and monitored the phosphorylation of Tyk2, Jak1 and STAT1/2/3. As shown in Fig. 38, the IFNα induced activation profiles were comparable in the two sets of clones. The basal

phosphorylation level of STAT3 may be variable with culture conditions and indeed it was not systematically observed in all stable clones. Fig. 38 shows that STAT3 is less phosphorylated in the P1104A than in the WT clone (left panel, lanes 1 and 5), in accordance with the transient transfection.

Tyk2P1104A cannot auto/transphosphorylate itself in vitro

The results described above indicate that the basal auto/trans-phosphorylation activity of Tyk2 P1104A is impaired. Nevertheless, upon IFN stimulation Tyk2P1104A is as phosphorylated as the WT protein, suggesting an intact induced catalytic activity. To test this hypothesis, we compared the *in vitro* autophosphorylation potential of Tyk2 WT and P1104A proteins from cells treated or not with IFNα. Tyk2 WT and P1104A were immunoprecipitated and subjected to an *in vitro* kinase assay in the presence or absence of ATP, as described above for the V678F mutant. The reaction products were analyzed by Western blot with anti-phospho-tyrosine and subsequently with anti-Tyk2 Abs. As shown in Fig. 39, in the absence of IFN the WT protein was phosphorylated when incubated with ATP (lanes 1 and 2), whereas the P1104A mutant was not (lanes 5 and 6). After IFN treatment, the phosphorylation of the WT protein augmented remarkably in the presence of ATP (lanes 3 and 4). Surprisingly, although the level of phosphorylation of the WT and P1104A proteins was equivalent in the cells after IFNα stimulation (lanes 3 and 7, samples without ATP), the phosphorylation of the P1104A mutant did not increase further in the presence of ATP *in vitro* (lane 8).

Altogether, these results demonstrate that the P1104A substitution in the « lip » of the TK domain impairs the basal as well as the IFN induced auto/trans-phosphorylation activity of the enzyme measured *in vitro*. Whether this is due to an overall impaired catalytic activity of the P1104A mutant or whether the P1104A itself is a poor substrate of autophosphorylation remains to be determined.

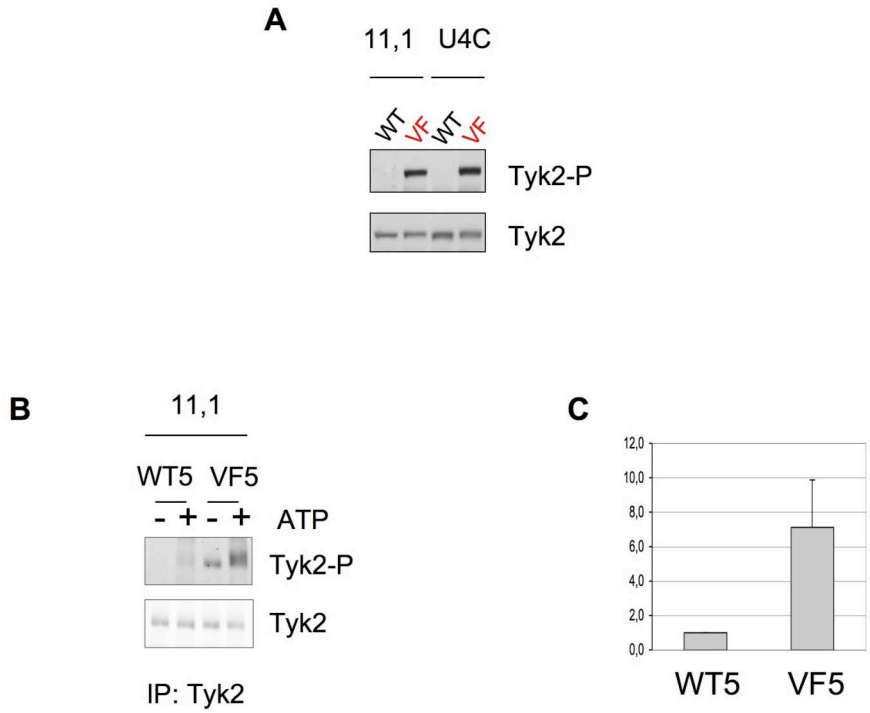


Figure 30. *In vivo* and *in vitro* autophosphorylation of Tyk2V678F

A) Tyk2V678F basal phosphorylation. 11,1 or U4C cells were transiently transfected with Tyk2 WT or the V678F mutant. Cell lysates were analyzed 48 hr later by Western blot with an Ab directed to the phosphorylated tyrosines (Tyr1054-55) of the activation loop. The membrane was stripped and re-probed for total Tyk2.

B) *In vitro* kinase assay. Tyk2 from 11,1-derived clones was immunoprecipitated and subjected to an *in vitro* kinase assay in the presence or not of 50μM ATP for 5 min at 30°C. The phosphorylation of Tyk2 was analyzed by Western blot with thr anti-phospho-tyrosine 4G10 mAb. The membrane was stripped and re-probed for total Tyk2.

C) Results from 5 different *in vitro* kinase assays were quantified as follows: the amount of phosphorylated Tyk2 was normalized by total Tyk2 protein. The values for the sample without ATP were substracted from the sample with ATP and this was considered as the *in vitro* reaction. For each experiment, the values for the V678F were normalized by the WT (fold increase) and the mean of the fold increases obtained from the 5 experiments is represented with the standard deviations (SD).

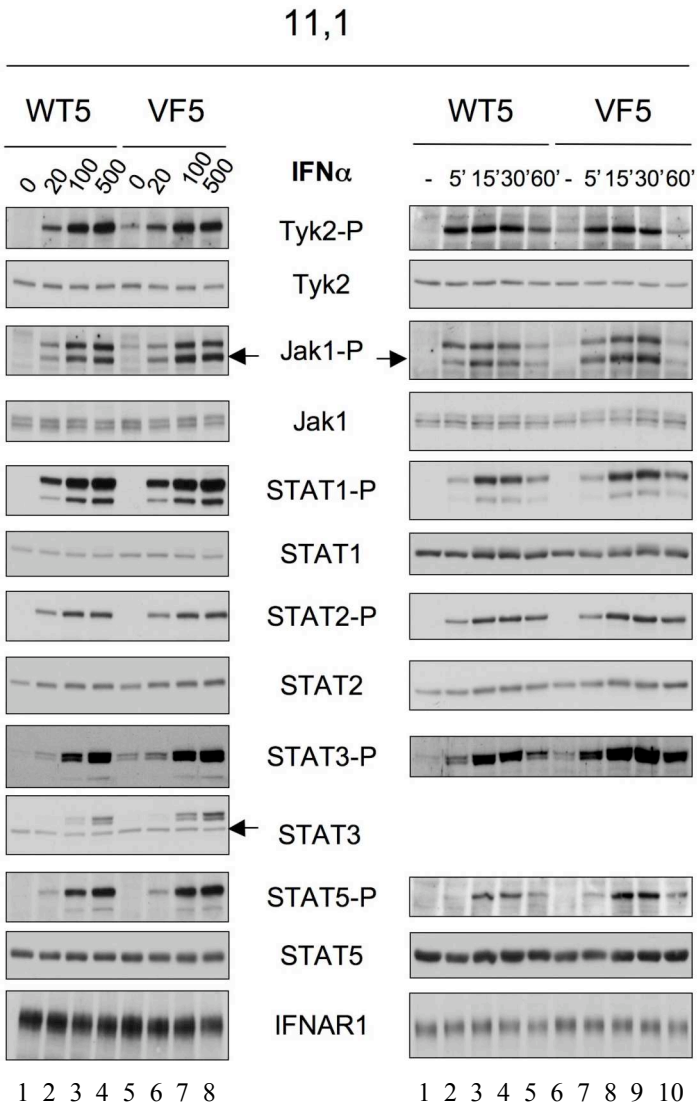


Figure 31. IFNα-induced signaling in 11,1-derived WT and V678F clones.
WT or V678F-expressing cells were treated with different doses of IFNα for 15 min (left panel, doses in pM) or with 100 pM IFNα for different times (right panel). The phosphorylation of the signaling components was analyzed by Western blot with phospho-tyrosine specific Abs. The membranes were subsequently re-probed for total protein level.

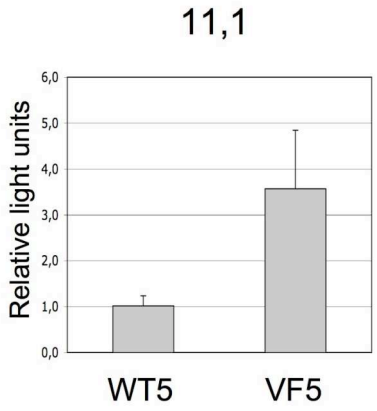


Figure 32. Basal transcriptional activity of STAT3 in WT and V678F clones
WT or V678F-expressing clones were transfected with a STAT3-reporter luciferase gene together with *Renilla* luciferase as transfection control. 24h afer transfection the luciferase activity was analyzed with Dual-Glo luciferase system (Promega). The values for STAT3-induced luciferase were normalized by the *Renilla* luciferase and the mean of three experiment is shown with SD.

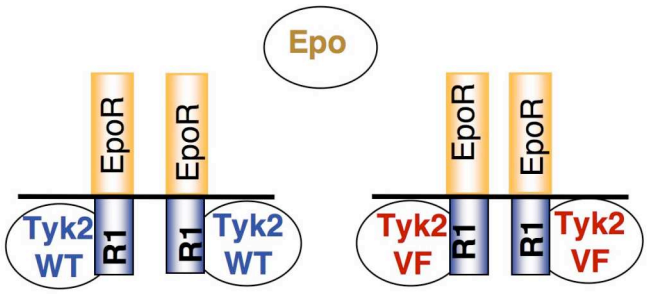


Figure 33. Schematic representation of the EpoR/R1 chimeric receptor associated with either WT Tyk2 or the V678F mutant.

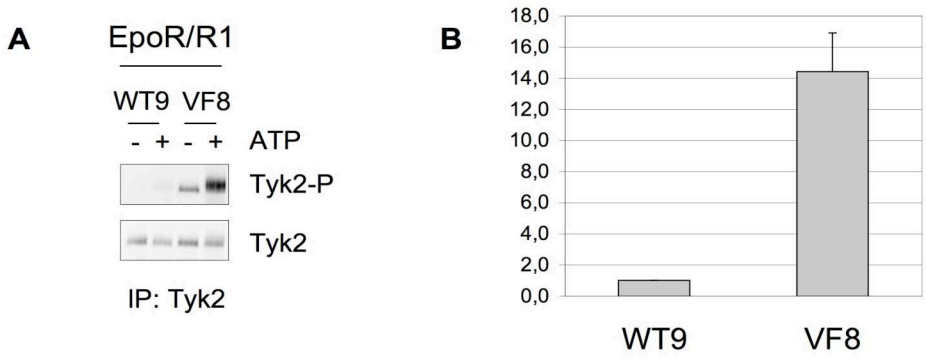


Figure 34. Tyk2 WT and V678F *in vitro* kinase activity in EpoR/R1-derived clones. The assay was performed (A) and quantified (B) as described for 11,1 clones (Fig. 30).

A

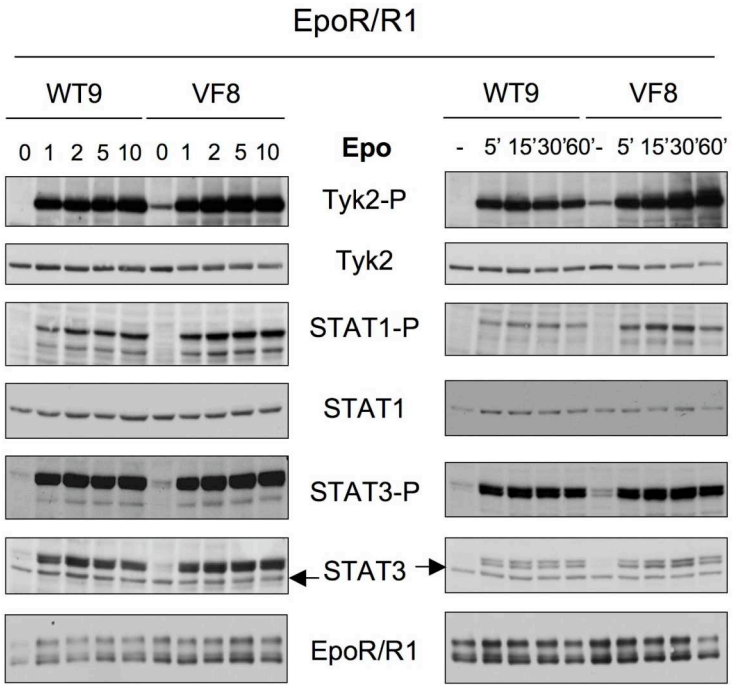


Figure 35. Epo-induced signaling in EpoR/R1-derived WT and V678F clones.
A) WT- or V678F-expressing cells were treated with different doses of Epo for 15 min (left panel; doses in U/ml) or with 2 U/ml of Epo for different times (right panel). The phosphorylation of the signaling components was analyzed by Western blot with phospho-specific antibodies. The membranes were subsequently re-probed for the total protein.
B) (see next page) Epo-induced phosphorylation was similarly analyzed in another pair of clones

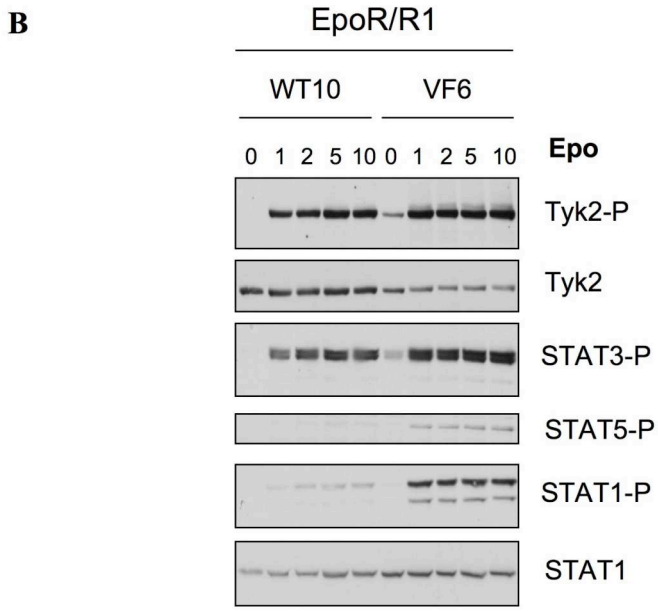


Figure 35 *continued*

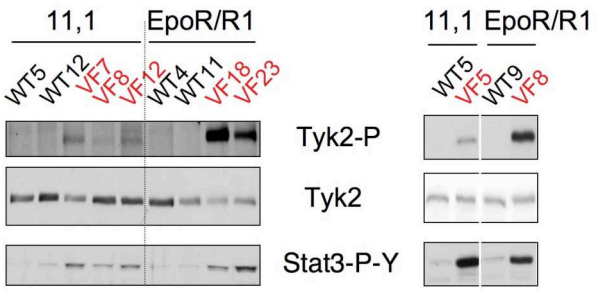


Figure 36. Basal Tyk2 phosphorylation in WT- and V678F-expressing 11,1 and EpoR/R1 clones.
The phosphorylation level in untreated cells was analyzed by Western blot with phospho-tyrosine specific Abs. The membrane was re-probed for total Tyk2 level.

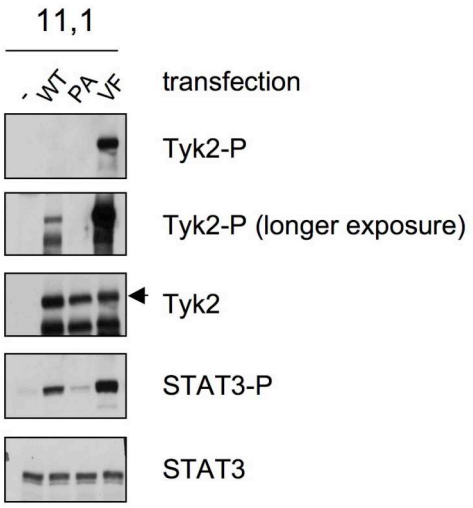


Figure 37. Basal phosphorylation of Tyk2 WT, P1104A and V678F.
11,1 cells were transiently transfected as indicated and analyzed by Western blot with specific anti-phospho-tyrosine Abs specific for Tyk2 and STAT3. The membrane was stripped and re-probed for total Tyk2.

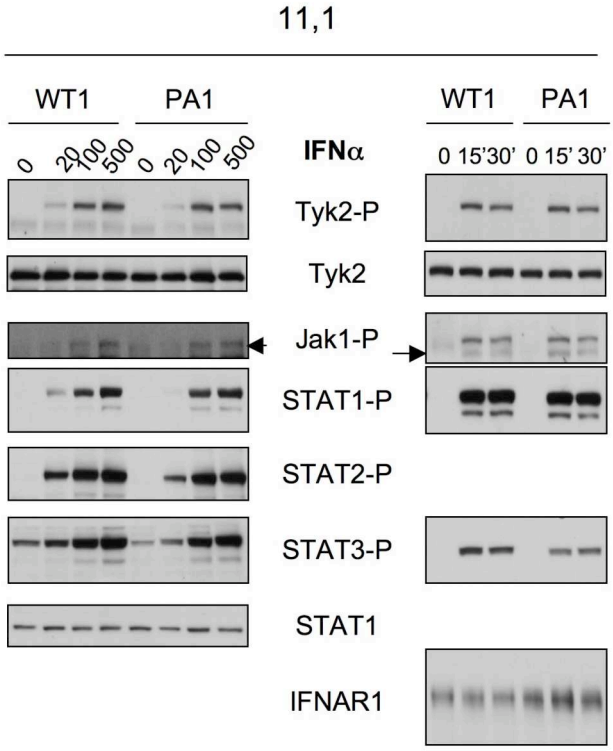


Figure 38. IFNα-induced signaling in 11,1-derived WT and P1104A stable clones.
WT- or P1104A-expressing cells were treated with different doses of IFNα for 15 min (left panel; doses in pM) or with 100 pM of IFNα for different times (right panel). The phosphorylation of the signaling components was analyzed by Western blot with phospho-tyrosine specific Abs. The membranes were subsequently re-probed for total protein content.

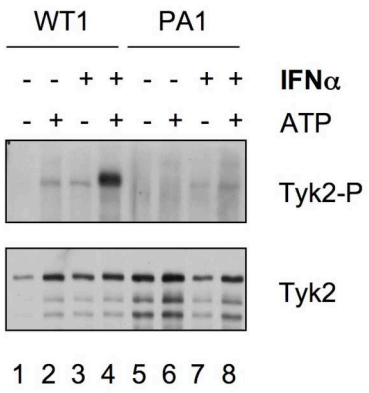


Figure 39. Tyk2 WT and P1104A *in vitro* kinase activity.
WT and P1104A-expressing clones were left untreated or treated with 500 pM IFNα for 10 min. Tyk2 was subsequently immunoprecipitated and subjected to an *in vitro* kinase assay in the presence or not of 50 μM ATP for 5 min at 30°C. The phosphorylation state of Tyk2 was analyzed by Western blot with an anti-phospho-tyrosine 4G10 mAb.

Discussion

Pot1

Pot1/Tyk2 interaction

Pot1 was identified in a yeast two-hybrid screen using the FERM domain of Tyk2 as bait. Six positive clones with overlapping Pot1 sequences were obtained. This finding represented a promising indication of a robust interaction of the Tyk2 bait with the C-terminal portion of Pot1, at least in yeast cells. Next, the interaction between the two partners was confirmed in a GST pulldown assay.

In a separate two-hybrid screen performed by Hybrigenics, Pot1 itself was used as bait. Surprisingly, among the many interactors, no members of the Jak family were found. One possibility that may explain this result is the low abundance of Tyk2 mRNA. In this screen Pot1 itself was found as an interactor and this points to its capacity to dimerise or oligomerise, possibly through the coiled coil regions.

Another possibility is that the Pot1 isoform (MVRS) which was used as bait (Fig. 15) cannot interact with Tyk2. Pot1 (MVRS) may adopt a fold that prevents Pot1 C-terminal region from binding Tyk2. This hypothesis is unlikely because the longest isoform of Pot1 (MPWP) which was used in the GST pulldown assay was shown to interact with the FERM domain of Tyk2.

At the present time, the question of the interaction between Tyk2 and Pot1, through their FERM and C-terminal regions respectively, remains open. It should be said that the only partner of Tyk2 that has been successfully co-immunoprecipitated with endogenous Tyk2 is Jakmip1, in Jurkat T cells (Steindler et al., 2004). In spite of much progresses on the function of Jakmip1 in T lymphocytes, the physiological relevance of this interaction remains unclear.

Pot1 isoforms and localization

In the human and murine genomes, Pot1 exists as a single gene that gives rise to several mRNA transcripts differing in their 5' regions and translation initiation site. It is thus conceivable that the protein isoforms diverge in their expression patterns. It would be of interest to analyze the possible cell-type specific expression of these isoforms.

Human Pot1 is localized in cytoplasmic vesicles of unknown nature. Interestingly, the murine Pot1 seemed to be associated to the membrane of vesicles. However, these results have to be

analyzed cautiously because all pertained to overexpressed Pot1. Although it is not always the case, overexpressed proteins can be aberrantly localized in the cell. The generation of higher quality antibodies will eventually facilitate the localization of the endogenous protein in cells.

Pot1 is not implicated in the IFNα-induced transcriptional response in 293T cells

In spite of the interaction between Tyk2 and Pot1 in the two-hybrid screen, we did not observe any effect of Pot1 depletion on IFNα-induced Jak-STAT signaling in 293T cells, in terms of STAT1/2/3 phosphorylation and induction of an ISGF3-responsive reporter. It is possible that the functional relevance of the Pot1-Tyk2 interaction is cell-type and/or ligand specific. In this sense, it would be of interest to analyze the role of Pot1 in the response to other cytokines that utilize Tyk2 and in other cell types. Alternatively, Pot1 could link Tyk2 to other cellular processes distinct from cytokine-induced signaling.

The "scrambled" control oligo that we initially used in the knock-down experiments led to a dramatic increase IFNα response. We do not know how the Sc exerts this effect. It has been reported that siRNAs can induce IFN production, mimicking antiviral response (Judge et al., 2005; Sledz et al., 2003). We believe that this is not the case in our system for the following considerations: 1) we never observed basal STAT phosphorylation in cells transfected with the Sc, 2) STAT1, whose expression augments in response to IFN, was present in similar amounts in Sc, Pot1-depleted and Tyk2-depleted cells 48 hr after transfection. Furthermore, the response to IFNα was measured as fold increase over the unstimulated basal value, and this increase was reproducibly higher in cells expressing the Sc oligo in respect to other controls. It was also reported that siRNA oligos can induce unpredictable off-target effects on certain mRNAs (Scacheri et al., 2004). This may be the case for the Sc oligo.

Pot1 interacting proteins

To get a possible hint on Pot1 function, we searched for proteins that can interact with Pot1. A yeast two-hybrid screen was performed. Given the number and diversity of Pot1 interactors that emerged from the screen, it is conceivable that Pot1 may function as a scaffold or adaptor protein in many different processes. Since the two "best" Pot1 interactors are undescribed proteins, we chose to focus on GIT1. This GTPase-activating factor for the ARF family of small GTPases has been implicated in diverse cellular processes (cf. Results). We investigated the potential involvement of GIT1 in IFNα signaling, but did not detect any effect of GIT1 knock-down or of its overexpression on IFN-induced Jak/STAT signaling events. We have

not analyzed the possibility that Pot1 plays a role in some GIT1-mediated processes, like cell migration or vesicle trafficking.

Tyk2V678F

The regulatory role of the KL domain

Among tyrosine kinases, the KL domain is a unique feature of Jaks. The regulatory role of this region has been extensively studied through deletion and point mutants. Jak2 and Jak3 deleted of the KL were found to have increased basal catalytic activity, which could not be augmented further after ligand stimulation (Chen et al., 2000; Saharinen and Silvennoinen, 2002; Saharinen et al., 2000). These observations, together with studies with Tyk2 point mutants (see below), led to the conclusion that the KL domain has both a positive and negative regulatory role: it keeps the kinase in a low phosphorylated state, but is required for ligand-induced activation (Chen et al., 2000; Yeh et al., 2000).

A number of pathology-associated mutations have been found to map in this domain, stressing further its importance for the maintenance of proper catalytic activity of Jaks. Fig. 40 shows a comparison of the naturally occurring or artificially generated point mutations found and/or analyzed in different Jaks reported to the corresponding Tyk2 residue (in black) (referenced in (Argetsinger et al., 2004; Chen et al., 2000; Choi et al., 2007; Feener et al., 2004; Hayashi et al., 2006; Luo et al., 1997; Mella et al., 2001; Mercher et al., 2006; Roberts et al., 2004; Sandberg et al., 2004; Schnittger et al., 2006; Shaw et al., 2003; Sigurdsson et al., 2005; VonDerLinden et al., 2002; Yeh et al., 2000)). One immediate observation is the abundance of naturally-occurring mutations found in the KL compared with the TK domain (not taking into account deletions and STOP mutations). Particularly interesting is the cluster of mutations between the T668 and I684, which may represents a putative "hot spot" region. In their model of the Jak2 JH1-JH2 (ie KL-TK) structure, Lindauer *et al.* predicted this 20aa region to be one of the interaction interfaces of the KL and TK domains (Tyk2 corresponding sites: S660 to P681) (Lindauer et al., 2001). This model fits well with the mutations identified so far and can be useful for future studies. However, only solving the crystal structure of an intact Jak or at least of the TK and KL domains together will help us better understand the general mechanism of functioning of the Jaks as well as possible differences among them.

Tyk2 loss-of-function mutations

To investigate more thoroughly the role of the KL domain in Tyk2, a mutagenesis screening was performed in the laboratory in order to identify potential gain- and loss-of-function mutations (Yeh et al., 2000). For this, a library of human Tyk2 cDNAs containing randomly

generated point mutations in the KL domain was transfected in 11,1 cells. These cells contain an integrated reporter: the bacterial guanine phosphoribosyltransferase gene (gpt) controlled by an IFNα/β-responsive promoter from the human gene 6-16.

After transfection of the randomly mutagenized Tyk2, cells expressing potential gain-of-function Tyk2 mutants were selected for survival in HAT, without added IFN. No positive colonies could be obtained from this type of selection (see below). IFNα-unresponsive cells, *ie* expressing loss of function mutants, were selected for survival in 6TG and IFNα. cDNAs from surviving colonies were sequenced for mutations in the KL domain. Four single point mutants with impaired complementing ability were analyzed: V584D, G596V, H669P and R856G (Fig.). All four mutants were shown to lack *in vitro* catalytic activity, both basal and IFN-induced. However, two of these mutants, V584D and G596V (N lobe of the KL domain), were not or weakly phosphorylated basally and were further phosphorylated *in vivo* after IFNα treatment. On the other hand, the H669P and R856G mutants (C lobe of the KL domain) exhibited high basal phosphorylation levels that did not augment in response to IFNα.

An E to K substitution (E782K), also mapping in the C lobe of the KL domain, was introduced in human Tyk2 (Shaw et al., 2003). It corresponds to the murine Tyk2E775K mutation that occurs naturally in the B10.Q/J strain of mice. The phenotype of Tyk2E782K was in all traits identical to that of the R856G mutant.

These results highlighted the central regulatory role that the KL domain plays: it keeps Tyk2 in a basal, low phosphorylated state and at the same time it maintains Tyk2 sufficiently flexible to 'sense' changes induced on the receptor upon ligand binding.

Tyk2V678F: a gain-of-function phenotype

As stated above, no gain-of-function Tyk2 KL mutants could be isolated in this potent screen. Nevertheless, Staerk *et al.* (Staerk et al., 2005) studied a mutant form of Tyk2 which they described as potentially activated. The mutation, V678F, corresponds to the Jak2V617F mutation that is implicated in the pathogenesis of *Polycythemia vera.* Indeed, sensitive methods allowed the detection of this mutation in more than 95% of patients with *Polycythemia vera* and in 50-60% of patients with essential thrombocythemia or idiopathic myelofibrosis (Campbell and Green, 2006). It is now thought that Jak2V617F may not the primary and only event leading to *Polycythemia vera* (Nussenzveig et al., 2007). Rather,

another genetic alteration may precede it and the cells that subsequently acquire Jak2V617F will gain a proliferative advantage leading to disease development (Dupont et al., 2007).

Jak2V617F was shown to possess an increased basal catalytic activity and to confer cytokine-independent growth to murine proB BaF3 cells (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). The V617 is conserved in Tyk2 and Jak1, but not in Jak3 where a methionine is found. To assess the effect of the corresponding substitution on Tyk2, Jak1 and Jak3 activity, Staerk *et al.* analyzed whether Tyk2V678F, Jak1V658F and Jak3M592F could confer cytokine-independent growth to BaF3 cells (Staerk et al., 2005). BaF3 cells were transduced with a retroviral vector encoding for the WT or the mutant proteins and the CD4 marker (Tyk2 and Jak3) or the GFP marker (Jak1). Transduced cells were appropriately sorted and monitored for growth in the absence of cytokines. The results showed that Tyk2V678F and Jak1V658F, but not Jak3M529F, could confer cytokine-independent growth to BaF3 cells. The Tyk2V678F mutant was found to be basally phosphorylated in BaF3 cells. These Tyk2V678F-expressing cells exhibited basal STAT5 phosphorylation and enhanced IGF1-dependent activation of Erk1/2 and Akt when compared to cells expressing the WT. Furthermore, using Tyk2-deficient 11,1 cells it was shown that the co-expression of Tyk2V678F, but not Tyk2 WT, with TpoR and STAT3 induced higher basal and Tpo-induced transcriptional response on a STAT3 reporter gene. Altogether, these results suggest that the Tyk2V678F mutation leads to a gain-of-function.

Tyk2V678F has no effect on IFNα-induced signaling, but leads to basal STAT3 phosphorylation

Tyk2V678F on signaling through the endogenous IFN receptor. Moreover, we analyzed the behavior of Tyk2V678F placed in a homodimeric receptor context, by introducing it in 11,1 cells expressing the chimeric EpoR/R1 receptor. The need for the expression of a cognate homodimeric receptor in Jak2V617F-mediated transformation will be described below.

Our results show that Tyk2V678F indeed exhibits a higher basal kinase activity than the WT protein *in vitro*. In spite of this gain-of-function, the Tyk2V678F mutant had no gain of function effect on IFNα-induced phosphorylation of Jak1, STAT1, STAT2 and STAT5. An effect was observed only on STAT3, which was found to be basally phosphorylated in V678F-expressing cells and more so after IFNα treatment. This basal STAT3 phosphorylation correlated with its transcriptional activity, which was higher in V678F- than WT-expressing cells, confirming in a more physiological context previous observations made by Staerk *et al*. (Staerk et al., 2005).

These results suggest that Tyk2V678F does not lead to cellular hypersensitivity to IFNα, as opposed to Jak2V617F, which confers hypersensitivity to Epo. The absence of an effect of the Tyk2V678F on IFNα-induced signaling also explains why no gain-of-function mutants of Tyk2 were found in the screen performed by Yeh *et al.* and described above. Indeed, this screen used as reporter an ISGF3-binding promoter, expected to be insensitive to STAT3 activation.

Preferential Tyk2-STAT3 interaction

Overall, our results argue for a preferential interaction ofTyk2 with STAT3. Numerous evidence point to STAT3 as a privileged substrate of Tyk2. Human cells expressing a kinase-dead Tyk2 mutant (Rani et al., 1999), as well as MEFs and bone marrow macrophages from Tyk2 KO mice (Karaghiosoff et al., 2000), exhibit some residual STAT1 and STAT2 phosphorylation in response to IFN, whereas STAT3 phosphorylation is fully abrogated. A recent analysisof the transforming potential of the Jaks in BaF3 cells showed that all autonomous cell lines resulting from Jak-promoted transformation presented basal activation of STAT5, Akt and ERK1/2. Interestingly, only Tyk2-expressing cells additionally exhibited STAT3 phosphorylation (detailed below) (Knoops et al., 2007). Furthermore, the recent discovery that, in humans, Tyk2 deficiency (Minegishi et al., 2006) and STAT3 dominant-negative mutations (Holland et al., 2007; Minegishi et al., 2007; Renner et al., 2007) have enough overlapping physiological consequences to be classified as the same disease (Hyper-IgE syndrome, cf. Introduction) supports the hypothesis of a preferential interplay between Tyk2 and STAT3.

The question that remains to be answered is the mechanistic basis of this interaction. One possibility is that STAT3 is recruited *via* the IFN receptor. It was reported that STAT3 and IFNAR1 can be co-precipitated in Daudi cells after IFNα stimulation (Pfeffer et al., 1997; Yang et al., 1996). However, our attempts to co-immunoprecipitate IFNAR1 or the chimeric EpoR/R1 with STAT3 have failed (data not shown). This could be due to the transient nature of the interaction or to the lower expression of IFNAR1 in fibroblasts as compared to Daudi cells. Another possibility is the occurrence of a direct interaction between Tyk2 and STAT3. One candidate STAT3-recruitment residue is found in Tyk2 (Y1176), which is located in a consensus STAT3 binding motif (YXXQ) (Shao et al., 2004). Previous studies have analyzed the effect of substituting the Y1176 to F in Tyk2. It was shown that this Y1176F mutation has no effect on Tyk2 basal or IFN-induced kinase activity *in vivo* and *in vitro* (Gauzzi et al., 1996). The Y1176F mutant behaved like the WT protein in the 11,1 complementation assay

described above, excluding an impairment in IFN-induced STAT1 and STAT2 phosphorylation. However, the phosphorylation level of STAT3 was not assessed. Therefore, we analyzed the capacity of this mutant to phosphorylate basally STAT3 by transiently overexpressing the Y1176F mutant or the WT protein in 11,1 cells. When overexpressed, WT Tyk2 is phosphorylated on the activation loop, presumably due to efficient transphosphorylation of the overproduced protein. Basal phosphorylation of STAT3 is also observed (cf. Fig. 37 in results for the P1104A mutant). Similar results were obtained upon overexpression of the Y1176F mutant (data not shown), arguing against a role of this tyrosine residue as STAT3 recruitment site. The possibility that STAT3 and Tyk2 interact directly through another residue/motif, in a phosphorylation- dependent or -independent manner, cannot be excluded and needs further investigation.

The prerequisite of a homodimeric receptor for Jak2V617F-mediated transformation

Jak2 is needed for signaling through many class I receptors that form heteromeric complexes, eg receptors for IFNγ, IL-12, IL-23, IL-3, IL-5. Jak2 is also the only Jak required for signaling through homodimeric receptor complexes, like EpoR, TpoR, GHR and G-CSFR. Jak2V617F was initially reported to be able, by itself, to confer cytokine-independent growth to BaF3 cells (see above). However, Lu et al. reported somewhat different observations (Lu et al., 2005). They showed that Jak2V617F promoted cytokine-independent growth only when in the presence of a cognate homodimeric receptor, like EpoR and TpoR. They also showed that STAT5, the major downstream effector of Epo-Jak2 signaling, was phosphorylated basally only in cells co-expressing Jak2V617F and EpoR. Furthermore, mutated EpoRs that could not bind either Jak2 or STAT5 could not fully promote Jak2V617F-mediated STAT5 phosphorylation.

Taken together, the above data suggest that Jak2V617F can promote cytokine-independent growth to BaF3 cells, but much more efficiently when co-expressed with a cognate homodimeric receptor. The homodimeric receptor is thought to act as a scaffold, bringing together two mutated enzymes and providing binding sites for downstream effectors.

Tyk2V678F placed in a homodimeric receptor context confers ligand hypersensitivity

We showed that STAT3 is basally phosphorylated in Tyk2V678F-expressing cells in the physiological context of the heterodimeric IFN receptor. However, we wanted to analyze the possible contribution of a homodimeric receptor to Tyk2V678F functioning. To this end, we analyzed Epo-induced signaling in EpoR/R1-expressing cells reconstituted with Tyk2 WT or

V678F. We showed that, like in 11,1 cells, Tyk2V678F was basally phosphorylated and evoked the tyrosine phosphorylation of STAT3, while STAT1 and STAT5 were not basally phosphorylated. On the other hand, Epo-induced phosphorylation of STAT1 and STAT5 was higher in V678F- than in WT-expressing cells. These data show that, when Tyk2V678F is placed in a homodimeric receptor context, the two juxtaposed mutant enzymes can confer ligand hypersensitivity.

These findings are in line with the observations that the amount of Jak2V617F expressed in *Polycythemia vera* patients correlates with the severity of the disease. Indeed, homozygosity for the V617F mutation plays an important role in the myeloproliferative disorders and likely results from mitotic recombination. In *Polycythemia vera* the prevalence of blood cells that are homozygous for the Jak2 mutation increases with time, presumably owing to a proliferative or survival advantage of mutant progenitor cells (Dupont et al., 2007; Scott et al., 2006). Homozygosity for V617F is associated with more marked changes in the expression of the downstream target genes than is heterozygosity for V617F. This reflects increased signaling mediated by a double dose of V617F. The results we have obtained with Tyk2V678F in EpoR/R1 cells can be regarded as a proof-of-principle for this effect.

The effect of Tyk2V678F on STAT3 basal phosphorylation is not linear

We have compared the basal phosphorylation level of Tyk2V678F and STAT3 in transfected 11,1 *vs* EpoR/R1 cells. As expected, in the absence of ligand, Tyk2V678F was more phosphorylated in EpoR/R1 expressing cells than in 11,1 cells, presumably because of preformed EpoR/R1 dimers which bring together two mutant enzymes and facilitate their transphosphorylation. However, the extent of STAT3 phosphorylation was comparable in EpoR/R1 and 11,1 cells, suggesting that the correlation between Tyk2 and STAT3 is not linear. One possibility is that the overall configuration of the EpoR/R1-Tyk2 complex does not provide additional STAT3 binding sites. This could be due to the overall configuration of the artificial chimeric receptor that partially impedes STAT3 accessibility.

A general model of IFN-induced STAT activation

Many studies have addressed the question of the exact order and mechanism of STAT activation upon type I IFN stimulation (cf. Introduction). Based on published data and on the results I obtained, I propose a general model of STAT activation as follows:

Several evidence indicate that an absolute requirement for STAT2 phosphorylation in response to IFN is the presence of the IFNAR2 cytoplasmic region (IFNAR2cyt). The most

compelling evidence for this is that in EpoR/R1 cells STAT2 is not phosphorylated in response to Epo. It is likely that STAT2 is constitutively associated to IFNAR2, as suggested by several studies. On the other hand, STAT1 appears to require the presence of STAT2, possibly through their constitutive dimerization. However, STAT1 can be also activated in the presence of IFNAR1cyt and Tyk2, as seen in EpoR/R1 clones and in STAT2-deficient cells, although not as efficiently as in the presence of STAT2. These observations argue for the existence of two pathways leading to STAT1 activation in response to IFNα. The major pathway employs preformed STAT1-STAT2 heterodimers pre-associated to IFNAR2, whereas the minor pathway utilizes IFNAR1 and Tyk2. This minor pathway could lead to IFNα-induced STAT1 homodimers. As for STAT3, most studies indicate that it needs Tyk2 activity to be phosphorylated. Nonetheless, STAT3 can also be activated in the presence of only IFNAR2cyt and Jak1 (as seen in cells expressing the chimeric EpoR/R2 receptor, our unpublished results and (Pattyn et al., 1999)), suggesting a more complex mechanism of activation. These divergent observations can be reconciled should the receptor chains be considered as a complex. In that sense, STAT3 would need two receptor chains and two active kinases, with some preference for Tyk2. How does it exactly become activated in one case or the other (IFNAR1 and Tyk2 or IFNAR2 and Jak1) remains to be determined.

Jak2V617F- and Tyk2V678F-mediated STAT5 activation

The activation of STAT5 by type I IFN has been reported, though STAT5 is not thought to be a major downstream effector of the IFNα/β response (Matikainen et al., 1999; Meinke et al., 1996; Uddin et al., 2003). It was shown that STAT5, together with STAT3, participates in the mitogenic effect induced by IFNβ in murine $STAT1^{-/-}$ T lymphocytes (Tanabe et al., 2005). Also, STAT5 was shown to mediate IFN resistance of melanoma cells since knocking-down STAT5 led to prolonged IFNα-mediated STAT1 phosphorylation (Wellbrock et al., 2005).

In lymphoid BaF3 cells, STAT5 is the major downstream effector of Jak2 in response to Epo and is thought to drive the Jak2V617F-induced cytokine-independent growth of BaF3 cells. Previous studies with the Tyk2V678F mutant expressed in BaF3 showed that STAT5 was basally phosphorylated in these cells, just like in cells expressing Jak1V658F and Jak2V617F, but not in cells expressing the corresponding WT proteins. In human fibrosarcoma 11,1 cells stably expressing Tyk2V678F, we did not observe any basal STAT5 phosphorylation. Several possibilities can account for this difference. First, the level of STAT5 expression may differ in the two cell types. If more abundant in BaF3 cells, STAT5 could be more readily available for

phosphorylation by Tyk2. Second, a receptor present in BaF3 but not in 11,1 cells may provide a recruitment site for STAT5. Third, there can be a species-specific ability of the murine, but not human, STAT5 to be recruited on Tyk2. A possibility that needs to be tested is whether Tyk2V678F can induce basal STAT5 phosphorylation in human B cells.

Physiological consequences of constitutively active Tyk2

An interesting issue that stems from our study is the potential patho-physiological consequence of an activating Tyk2 mutation. Here we showed that a gain-of-function mutation of Tyk2 (Tyk2V678F) leads to basal STAT3 phosphorylation with no effect on IFNα-induced ISGF3-mediated signaling. Thus, it is conceivable that a constitutively active Tyk2 may be involved in the same pathologies as a constitutively active STAT3. STAT3 is implicated in many cellular processes and the correct functioning of many organic systems. In mice, STAT3 deficiency leads to embryonic lethality, whereas in humans dominant-negative STAT3 mutations lead to a complex multisystem disorder (Hyper-IgE syndrome, cf. Introduction). On the other hand, constitutive STAT3 activation has been linked to cancer progression.

STAT3 implication in tumor development

STAT3 has been found constitutively phosphorylated in many cancers (Bromberg and Darnell, 2000; Inghirami et al., 2005; Levy and Inghirami, 2006). It has been shown that STAT3 is required for v-Src induced transformation (Bromberg et al., 1998). To mimic constitutive STAT3 activation, Bromberg et al. generated a Cys-mediated STAT3 homodimer (STAT3C) that was shown to bind to DNA and activate transcription spontaneously (Bromberg et al., 1999). The STAT3C protein in immortalized fibroblasts caused cellular transformation scored by colony formation in soft agar and tumor formation in nude mice. More recently, STAT3C has been used in knock-in study to assay its transforming potential in mice (Poli, 2007). $STAT3^{c/c}$ mice die of myocarditis, whereas $STAT3^{c/wt}$ mice develop a multiple myeloma-like syndrome with age. $STAT3^{c/c}$ MEFs were shown to upregulate a subset of STAT3 target genes, to display increased growth capacity and survive indefinitely after confluence. Furthermore, to assess STAT3 contribution to mammary tumor development, the group of V. Poli crossed $STAT3^{-/-}$ and $STAT3^{c/c}$ mice with MMTV-Her2 Neu transgenic mice (NeuT). NeuT mice overexpress the oncogenic form of ErbB-2 and develop multifocal mammary adenocarcinomas at high multiplicity. STAT3C was shown to enhance ErbB-2-mediated tumorigenesis even in heterozygosity, proving for the first time the

STAT3 oncogenic properties *in vivo*. Altogether, their studies suggest that STAT3 enhances tumorigenesis by allowing cells to evade apoptosis and by increasing adhesion-independent growth and migration capacity with potential involvement in metastasis. Another important role of STAT3 in tumor progression is its involvement in tumor-induced immunosuppression (Yu et al., 2007). Tumor cells can generate a global shut-down of immune-stimulating molecules, such as co-stimulatory molecules and cytokines, in the tumor microenvironment. STAT3 was found to have an important role in these processes since it is a potent negative regulator of Th1 cell-mediated inflammation and an activator of many genes that are crucial for immune suppression. Furthermore, a STAT3 inhibitor was shown to reverse immune tolerance and enhance immune response in malignant glioma cells (Hussain et al., 2007). Taken together, these data argue for a crucial role of STAT3 in tumor progression. Thus, it is conceivable that a constitutively activating mutation of Tyk2, leading to phosphorylation of STAT3, could also contribute to tumor progression.

Tyk2 association with cancer

Several lines of evidence support the view that Tyk2 could have a role in cancer progression. The ability of Tyk2V678F to confer cytokine-independent growth to BaF3 cells point to its oncogenic potential. Recently, Knoops *et al.* assayed the oncogenic properties of overexpressed Jaks in BaF3 cells using a two-step selection system involving IL-9 (Knoops et al., 2007). Cells transfected with a defective IL-9 receptor acquired IL-9 responsiveness during a first step of selection and progress after a second selection step to autonomously growing tumorigenic cells. Knoops *et al.* demonstrated that overexpression of any of the Jak proteins promotes the transformation of BaF3 cells into cytokine-independent cell lines with constitutive STAT5, Akt and Erk1/2 activation. Interestingly, constitutive activation of STAT3 was observed in all Tyk2-overexpressing autonomous clones, sometimes in Jak2- and never in Jak1 or Jak3-overexpressing clones. Also, a fusion protein between the oligomerization domain of the translocated Ets leukemia (TEL) protein fused to the tyrosine kinase domain of Tyk2 (TEL-Tyk2) can confer cytokine-independent growth to BaF3 cells to a similar extent as the corresponding TEL-Jak1, TEL-Jak2 and TEL-Jak3 constructs (Lacronique et al., 2000). Finally, comparison of the protein expression profiles of three normal breast cell lines and seven breast cancer cell lines have identified ten differentially expressed proteins. Among these, Tyk2 was shown to be more expressed in cancer than in normal cell lines (Song et al., 2007). It was shown by western blot that the expression of Tyk2 in normal cells was between 15-40% of its expression in cancer cells. Taken together, these

data corroborate the hypothesis that a constitutively active and/or overexpressed Tyk2 can contribute to tumor progression, either by promoting cell growth or by suppressing anti-tumor immune response.

Tyk2 and tumor surveillance

Given the shared usage of Tyk2 in cytokine signaling in immune cells (IL-12, IL-23, IL-10 etc), Tyk2 is likely to play a protective role against tumor development. Indeed, it has been shown that $Tyk2^{-/-}$ mice develop Abelson-induced B cell leukemia/lymphoma with a higher incidence and shortened latency than WT mice. This effect is thought to occur *via* impaired NK cell-mediated cytotoxicity. The two hypotheses that both loss-of-function and gain-of-function Tyk2 phenotypes can lead to tumor development, are not in opposition. An intact Tyk2 function is needed for the normal functioning of the immune system and for tumor rejection (of note, one common complication of the HyperIgE syndrome is the development of lymphomas (Grimbacher et al., 2005; Kashef et al., 2006; Leonard et al., 2004)). On the other hand, an hyperactive Tyk2 could promote tumor growth through a different mechanism, possibly also involving defective anti-tumor response. In all cases, the only way that physiological consequences of an activating Tyk2 mutation can be better understood is through the generation of transgenic mice.

Tyk2P1104A

With the objective of discriminating between SNPs that contribute to tumor progression from mutations that are consequence of genetic instability in cancer, Kaminker *et al.* generated a computational program that predicts cancer-associated germ-line mutations (Kaminker et al., 2007). To test the program, they performed mass spectrometry genotyping for 65 predicted cancer variants over a collection of 128 tumor tissue samples. From this analysis, they identified a C to G change, leading to a missense P1104 to A variant in Tyk2, which was present in four independent tumor tissues. This variant was classified as a germ line change since it was also identified in the matched normal samples. The authors proposed this variant to be a novel mutation since they did not find it in other normal samples and it was reportedly absent from SNP databases (dbSNP, Seattle SNP project and SNP500Cancer project). However, it is now present in the NCBI dbSNP database (SNP ID: rs34536443). Also, the HapMap (Haplotype Mapping) genome project reports the presence of this SNP in a European, but not Asian or sub-Saharan African, population. The frequency of the C allele is very low (0,017) and no C/C homozygous genotypes are reported.

The P1104 is located in the "lip" of the tyrosine-kinase domain, a characteristic feature of the vertebrate Jaks. The P1104 residue lies in the substrate-binding groove of Tyk2 and closely packs (in a ring-stacking interaction) with the conserved W1067 residue tied to the activation loop. P1104 also contacts the ring of the neighboring P1105. Any disruption of this tightly packed trio of aa might affect the catalytic activity of Tyk2 by either disrupting the substrate-binding groove or the conformation of the activation loop. (Fig. 41).

The P1104A mutation was predicted by Kaminker *et al.* to alter Tyk2 catalytic activity, but no functional studies were performed. We introduced this substitution in Tyk2 and tested its functional effects. We analyzed Tyk2 auto/transphosphorylation potential *in vitro* with or without IFNα stimulation. The results obtained showed that Tyk2P1104A is unable to phosphorylate itself. However, after IFNα treatment, the level of phosphorylation of Jak1 and STAT1/2/3 in P1104A-expressing cells were the same as in WT-expressing cells. Furthermore, the extent of phosphorylation of the P1104A mutant on the activation loop tyrosines was comparable to that of the WT protein. This could be partly explained by the activation of of Jak1 which is juxtaposed to Tyk2 and can phosphorylate Tyk2 and the STATs. However, previous studies of a *bona fide* catalytically dead mutant of Tyk2 suggested that Jak1 alone cannot account for the normal IFNα response. Indeed, Tyk2 K930R, mutated in the ATP-binding pocket (the classical kinase inactivating mutation), shows a strikingly

different behaviour than P1104A. Tyk2K930R can be weakly phosphorylated on the activation loop tyrosines *in vivo* after IFNα stimulation (Gauzzi et al., 1996). However, the phosphorylation of Jak1 and STAT1/2/3 are severly impaired in K930R-expressing cells when compared to the WT ((Rani et al., 1999) and our unpublished results).

Taken together, these data suggest that the P1104A variant is not a typical 'kinase-dead' mutant. Rather, the defective auto/transphosphorylation points to impaired catalytic activity towards certain substrates. Alternatively, Tyk2P1104A may be catalytically active, but may not be a good (auto)phosphorylation substrate. These two possibilities are not mutually exclusive and will be tested by *in vitro* kinase assays using different exogenous substrates. It would also be interesting to express the P1104A mutant in EpoR/R1-expressing cells in order to measure the signaling capacity of two juxtaposed P1104A mutants in response to Epo.

As mentioned above, Tyk2P1104A variant is thought to predispose to cancer due to altered catalytic activity. What is the exact effect of this mutation on Tyk2 functioning remains unclear at present, but data obtained so far point to a partial loss-of-function rather than the proposed gain-of function. Hence, Tyk2P1104A mutant could participate in tumor progression through impaired tumor surveillance (see above). The very low frequency of the C allele reported in the HapMap project and the lack of homozygosity for the P1104A mutation may indicate a more severe physiological consequence than predicted from studies will cell lines. Alternatively, the P1104A mutation may represent a recently acquired polymorphism in the European population that did not have time to propagate. Generation of transgenic mice can provide us with a better insight into the physiopathological consequences of the Tyk2P1104A mutation.

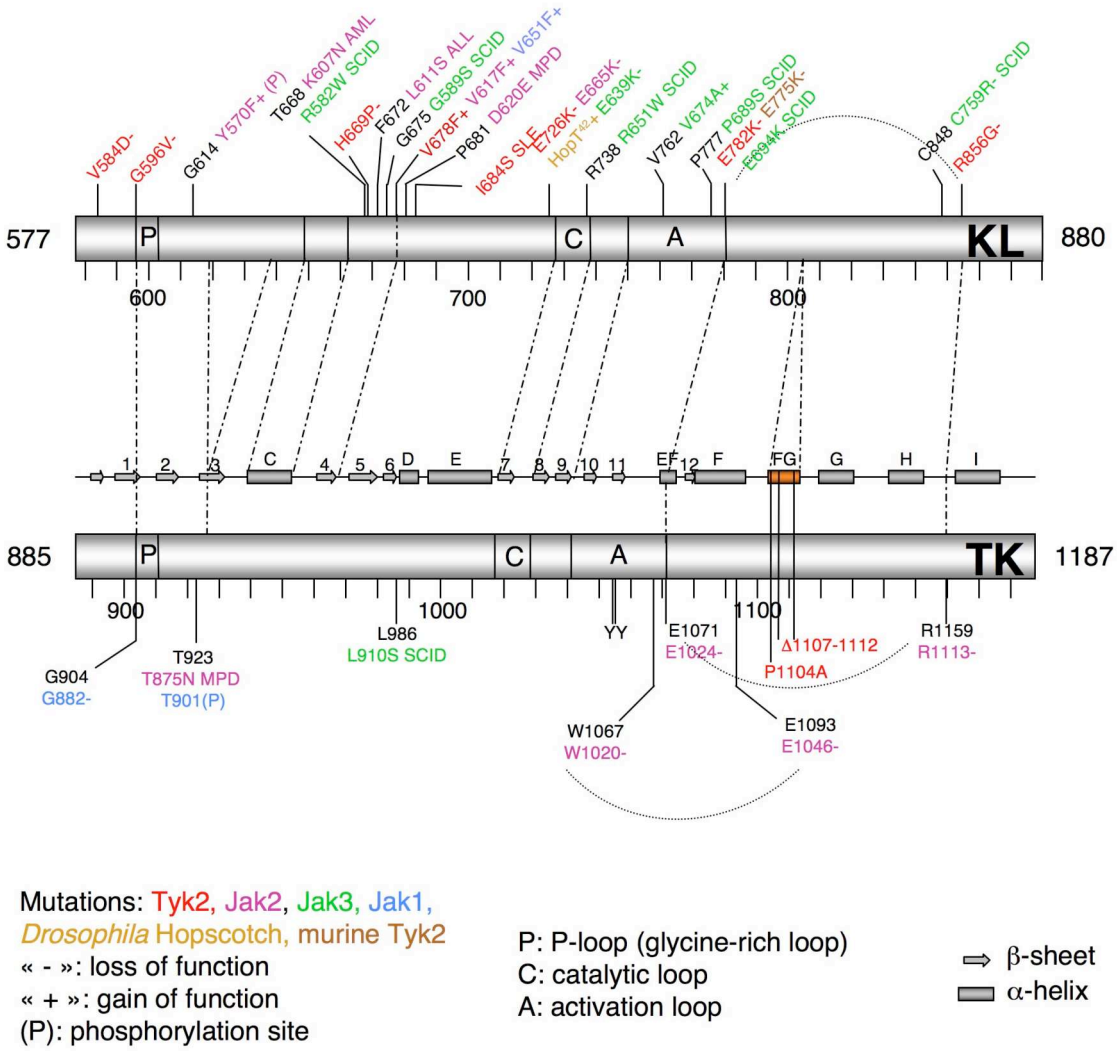


Figure 40. Comparison of naturally occurring or artificially generated point mutations in the KL and TK domains that alter Jak catalytic activity.

The "-" or "+" symbols refer to a loss-of-function or gain-of-function phenotype, respectively, when the mutations were analyzed. When the mutations were found in a disease, the name of the disease is indicated. The symbol (P) indicates a phosphorylation site. The dotted half-circles indicate hydrogen bonds thought to be important for the structural integrity of the protein (between residues E782 and R856 in KL; E1071 and R1159, and W1067 and E1093 in TK). The Δ18nt deletion refers to a HeLa Tyk2 cDNA isolated in the lab and encoding for a non functional protein (cf. Introduction). AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MPD: myeloproliferative disorders; SLE: systemic lupus erythematosus; SCID: severe combined immunodeficiency disease

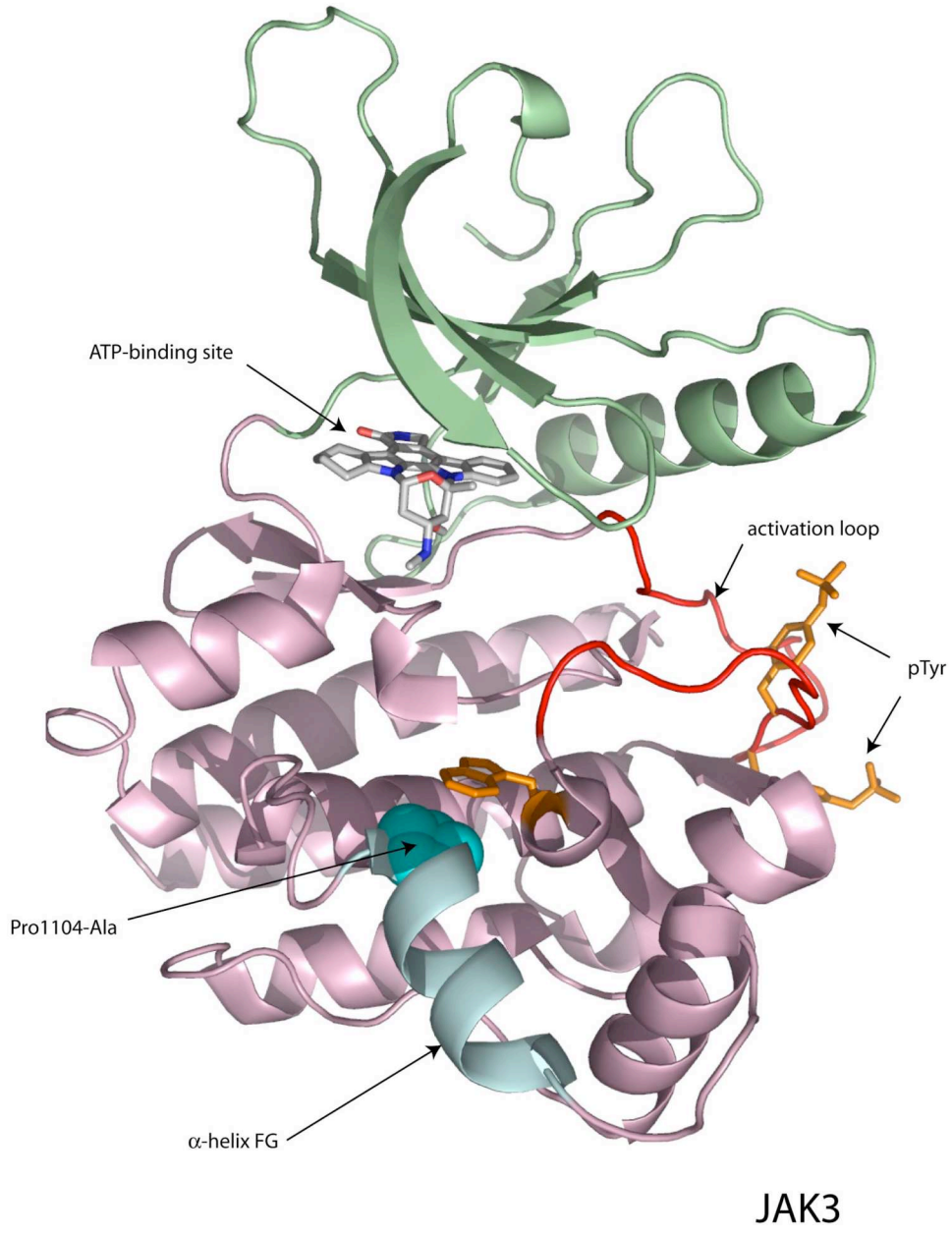


Figure 41. The position of P1104A in Jak3 crystal structure
The position of the P1104 residue within the a-helic FG (light blue) in the C-lobe (from P. Alzari). Based on the solved structure of the TK domain of Jak3

Perspectives

Pot1

Pot1 was identified as a Tyk2 interactor in a yeast two-hybrid screen. One limit of this method is that it employs an artificial system that does not provide the physiological and cellular context where the interaction may take place. Although the finding of 6 overlapping clones in the screen is strongly supportive of an interaction of Pot1 with Tyk2, this interaction still needs to be confirmed in a cellular system. The same applies to the interaction of Pot1 with GIT1. To confirm these interactions, we need high quality Abs that would enable us to coimmunoprecipitate Pot1 with Tyk2 or GIT1. High quality Abs would also permit us to analyze more in-depth the subcellular localization of the endogenous protein.

So far, knocking down Pot1 expression by siRNA has shown no indication that Pot1 plays a role in IFNα signaling. RNA interference is a powerful method to rapidly and easily diminish protein expression, but it has its own limits. One disadvantage of this approach is the possible off-target effect, as we have observed for the Sc siRNA (Scacheri et al., 2004). Another limit of RNA interference is that the expression of the target protein may be diminished, but not completely abrogated. The residual amount of the protein could make it difficult to clearly observe an effect of the knock-down. We do not believe this to be the case for Pot1, since the use of two different sets of siRNAs with different knock-down efficiencies yielded similar results.

It is possible that the functional meaning of the Pot1-Tyk2 interaction could be revealed in a different cell and/or cytokine receptor context. However, it is also possible that Pot1 has no role in the immediate cytokine-induced signaling, but links Tyk2 to other cellular processes. In this sense, it would be of interest to analyze the role of Pot1 in cellular processes that involve GIT1, like cell migration.

Tyk2V678F

We show here that the V678F mutation localized in the KL domain augments the basal Tyk2 autophosphorylation activity. The V678F mutation was shown to have a more prominent effect on Tyk2 functioning when placed in a homodimeric receptor context – Tyk2V678F is more phosphorylated basally and confers Epo hypersensitivity to cells expressing an artificial EpoR/R1 receptor. It would be interesting to coexpress Tyk2V678F and the corresponding

Jak1 mutant, V658F, to analyze if the coexpression of the two mutants leads to IFNα hypersenstivity.

The work on Tyk2V678F also revealed the preferential interplay between Tyk2 and STAT3. The possibility that Tyk2 and STAT3 interact directly in a phosphorylation-dependent or independent manner can be investigated by coimmunoprecipitation of the two proteins. It is also conceivable that Tyk2 has a preference towards STAT3 as a substrate, rather than STAT1 or STAT2. This could be analyzed *in vitro* with substrates corresponding to STAT1, STAT2 or STAT3 phosphorylation sites.

Another aspect of the study of Tyk2V678F mutant would be to analyze the effect of this mutant on IFNα response in other cell types, for example lymphocytes. The levels of STATs may vary among different cell-types, which can play an important role in cytokine response (Costa-Pereira et al., 2002; Gil et al., 2006; Ho and Ivashkiv, 2006; Miyagi et al., 2007; Qing and Stark, 2004). If more expressed, STATs can be more readily available for phosphorylation by Tyk2 (cf. Discussion). Also, Tyk2V678F could have an effect on non-STAT pathways that are induced by IFNα in a cell-type specific manner (Platanias, 2005; van Boxel-Dezaire et al., 2006).

It would also be interesting to analyze the effect of Tyk2V678F on the signaling induced by other cytokines that utilize Tyk2, like IL-10, IL-12, IL-23. Additionally, microarray analyses of the genes specifically expressed in Tyk2V678F-containing cells should provide useful data on the gene expression profile of these cells. These analyses could yield a comprehensive picture of the potential physiopathological consequences of an activating mutation in Tyk2. Studies with knock-in mice would be even more helpful in that sense, although Tyk2 does not seem to have a role as important in mice as in humans.

In regard to the KL domain, the model of the interaction between the KL and TK domain by Lindauer et al. has proven to be quite accurate (Lindauer et al., 2001). However, solving of the crystal structure of the two kinase domains is required for more in-depth understanding of their interaction and of Jak functioning.

Tyk2P1104A

The P1104A mutation has an interesting effect on Tyk2 functioning. Tyk2P1104A cannot autophosphorylate itself *in vitro*, but, surprisingly, this impairement has no consequence on IFN-induced signaling. This suggests that the autoinhibited basal state of Tyk2P1104A is relieved upon IFNα stimulation, most likely as a consequence of Jak1 activation. To test the

hypothesis that a fully competent kinase is needed for Tyk2P1104A activation, we analyzed the activation of Tyk2P1104A in a homodimeric context, where two mutant proteins are juxtaposed. For this, Josiane Ragimbeau, who is pursuing this project, made use of 11,1-derived cells expressing the chimeric homodimeric EpoR/R1 receptor that she transiently transfected with Tyk2 WT or the P1104A mutant. Preliminary data show that the Epo-induced phosphorylation of Tyk2P1104A on activation loop tyrosines is extremely weak when compared to that of the WT (data not shown), presumably due to the inability of Tyk2P1104A for transphosphorylation. These data suggest that the autophosphorylation capacity of Tyk2P1104A is impaired *in vivo* as well as *in vitro*.

An important issue that remains to be addressed is whether Tyk2P1104A is truly impaired in its catalytic activity or, due to the mutation, represents a bad substrate for autophosphorylation. This will have to be analyzed by assaying the *in vitro* kinase activity of Tyk2P1104A towards an exogenous substrate. The best substrates would be the kinase-inactive TK domain of Tyk2, containing the K930R mutation in the ATP-binding pocket so that it cannot to be activated *in vitro* (TK-KR), and the TK-KR with the P1104A mutation (TK-KR/PA). If Tyk2 WT phosphorylates TK-KR and TK-KR/PA to the same extent *in vitro*, it means that Tyk2P1104A is as good a substrate for autophosphorylation as the WT protein. On the other hand, if Tyk2 WT cannot phosphorylate TK-KR/PA as well as TK-KR, it means that the activation loop of Tyk2P1104A cannot be phosphorylated as a consequence of the mutation. Tyk2P1104A activity can be similarly analyzed after IFNα treatment.

The proline 1104 is conserved among vertebrate Jaks and is located in the Jak-specific αFG loop or "lip". The results obtained so far suggest that this loop somehow regulates the autophosphorylation capacity of Tyk2. It is possible that the "lip" interacts directly with the phosphorylated activation loop, as suggested by Kaminker et al. (Kaminker et al., 2007). The "lip" could help stabilize the activation loop in its active conformation, or enable its correct positioning for autophosphorylation. Another possibility is that the "lip" interacts with the regulatory KL domain and that the P1104A mutation disrupts this interaction. This interaction can be analyzed by coimmunoprecipitations of the Tyk2 KL domain with the WT TK domain or the TK domain containing the P1104A mutation.

Since the P1104A substitution is a human polymorphism presumed to be associated with cancer, it would be of interest to analyze its presence in different tumors. More generally, the possible link of the P1104A polymorphism with human pathologies could be uncovered by advanced genetic studies.

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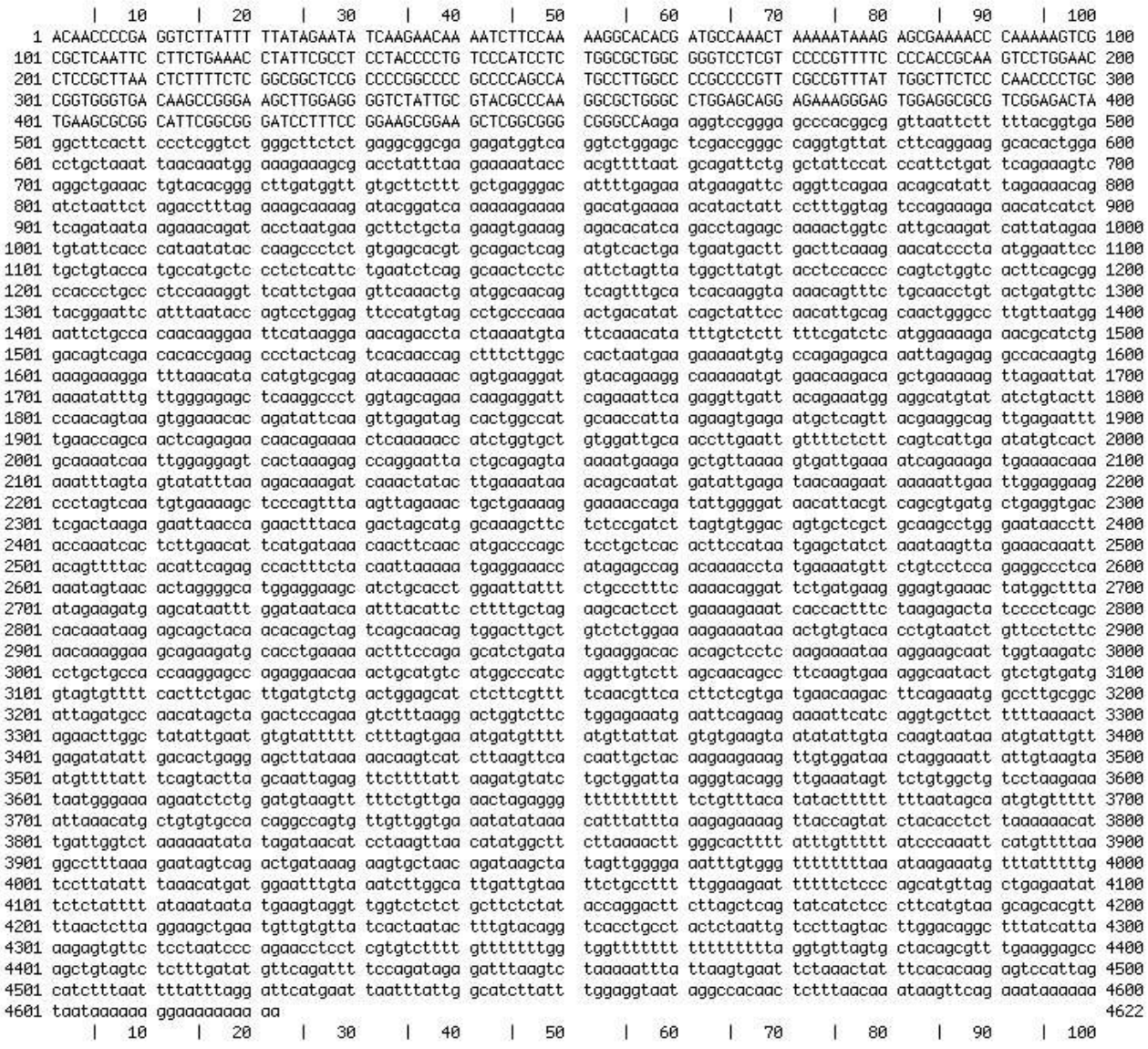
Annex

Pot1 cDNA reconstructed in the lab



List of Pot1 interacting proteins

name	n° of clones	score	function
hKIAA0998	15	A	unknown; tubuline tyrosine-ligase family
similar to basic leucine zipper and W2 domains1(BZW1)	17	A	unknown; BZW1 regulates translation initiation
CROP (cisplatin resistance-associated overexpressed protein)	4	B	RNA splicing, apoptosis, response to stress
GIT1 (G-protein coupled receptor kinase interactor 1)	9	B	ARF GAP, paxillin binding, actin cytoskeleton
RINT1 (RAD50-interacting protein)	5	B	radiation-induced checkpoint control
cyclinB1	5	B	regulation of cell cycle
ARFIP2 (ADP-ribosylation factor interacting protein 2 (arfaptin 2))	3	C	cross-talk between Rac and Arf small GTPases (Arf1 and Arf6)
Pot1	3	C	interaction via the second coiled-coil
FLJ13386 (centrosome protein Cep63)	3	C	unknown; chromosome segregation ATPases (SMC domain)
GIT2 (isoform2)	3	C	

hPP1553/CDK5RAP3 (CDK5 regulatory subunit associated protein3)	2	C	cell proliferation
hSEC5L1 (Sec5-like protein)	2	C	component of the exocyst, binds to RalA
TALDO1 (transaldolase1)	2	C	pentose phosphate pathway
ZNF277 (zinc finger protein 277)	3	C	unkknown; nuclear receptor, DNA binding
RARA (retinoic acid receptor alpha)	1	D	steroid receptor, transcription factor
cyclin B2	1	D	cell cycle control
ALDH1A2 (aldehyde dehydrogenase family 1, member A2)	1	D	retinoic acid synthesis
AP2A1 (adaptor-related protein complex 2, alpha 1 subunit)	1	D	AP2 complex, clathrin-coated pit
ARHGEF18 (rho/rac guanine nucleotide exchange factor (GEF) 18)	1	D	Rho-specific guanine nucleotide exchange factor
AXOT (MARCH7 membrane-associated ring finger (C3HC4) 7)	1	D	ubiquitin ligase complex, RING domain
BAT3 (HLA-B associated transcript-3)	4	D	apoptosis control, heat shock protein regulation
BITE (centrosome protein Cep70, p10 binding protein)	1	D	unknown; chromosome segregation ATPases (SMC domain)

FLJ20424 (C14orf94 chromosome 14 open reading frame 94)	1	D	unknown
CGTHBA (C16orf35 chromosome 16 open reading frame 35)	3	D	unknown
CSE1L (CSE1 chromosome segregation 1-like (yeast), isoform a)	1	D	importin-alpha receptor export activity
DKFZP434F054 (WDR24, WD repeat domain 24)	1	D	unknown; adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly
DKFZP434I116	1	D	unknown
DOCK10 (dedicator of cytokinesis 10)	1	D	unknown; guanine nucleotide exchange factor
EIF3S10 (eukaryotic translation initiation factor 3, subunit 10 theta)	3	D	translation initiation
FAM38A (family with sequence similarity 38, member A)	5	D	unknown
FBXO18 (F-box protein, helicase, 18)	1	D	ubiquitin ligase, DNA repair
FHOD1 (formin homology 2 domain containing 1)	1	D	actin cytoskeleton rearengment, GTPase-binding
FLJ32115	2	D	unknown; isomerase activity
FP3235 (Queuine tRNA-ribosyltransferase 1 (tRNA-guanine transglycosylase) (QTRT1))	1	D	

GTF3C3 (general transcription factor IIIC, polypeptide 3)	1	D	RNA polymerase III transcription factor activity
HIC (MDFIC, MyoD family inhibitor domain containing)	1	D	transcriptional regulation of viral genome expression
IQGAP1 (IQ motif containing GTPase activating protein 1)	2	D	RasGAP, cytoskeleton remodelling, Cdc42 binding
KIAA0685	1	D	unknown; SIT4 phosphatase-associated protein (G1 cyclin transcription)
KIAA1007 (CNOT1, CCR4-NOT transcription complex, subunit 1)	1	D	regulation of transcription
KIF11 (kinesin family member 11)	1	D	chromosome positioning, centrosome separation and establishing a bipolar spindle during cell mitosis
LGALS3BP (lectin, galactoside-binding, soluble, 3 binding protein)	1	D	galectin, cell-cell and cell-matrix interactions; macrophages and NK cells
RLTPR (RGD, leucine-rich repeat, tropomodulin and proline-rich containing protein)	2	D	unknown
MACF1 (microtubule-actin crosslinking factor 1)	1	D	actin and microtubule binding domains; TGN transport
MAP3K4 (mitogen-activated protein kinase kinase kinase 4)	1	D	activates CSBP2 and JNK MAPK pathways, but not the ERK pathway
MCPR (ANAPC1, anaphase promoting complex subunit 1)	1	D	anaphase-promoting complex (APC), E3 ubiquitin ligase
MDN1 (midasin homolog (yeast))	1	D	AAA ATPase, protein folding; in the nucleus

MYO1G (myosin IG)	1	D	phagocytosis and vesicular transport
NAG (neuroblastoma-amplified protein)	1	D	unknown
NGFRAP1 (NADE, nerve growth factor receptor (TNFRSF16) associated protein 1)	1	D	induces apoptosis
NUCB2 (nucleobindin 2, NEFA)	1	D	DNA and calcium binding
NUP205 (nucleoporin 205kDa)	2	D	nuclear pore assembly, nuclear transport
PKD1 (polycystic kidney disease 1)	1	D	polycystin-1
POLR1A (polymerase (RNA) I polypeptide A)	2	D	transcription
RANBP5 (RAN binding protein 5)	1	D	nuclear transport, member of the importin beta family
RRN3 (RNA polymerase I transcription factor homolog; TIF-IA)	1	D	homolog of yeast RRN3
SEC24C (SEC24 related gene family, member C)	1	D	ER to Golgi binding; COPII
SIN3A (SIN3 homolog A, transcription regulator)	1	D	transcriptional repression
SPTBN1 (spectrin, beta, non-erythrocytic 1)	2	D	spectrin, cytoskeleton, cell shape, organelles organization and molecular traffic

SRGAP2 (SLIT-ROBO Rho GTPase activating protein 2)	1	D	
STXBP1 (syntaxin binding protein 1; Munc 18-1)	1	D	vesicle transport and secretion
TIP120A (CAND1, cullin-associated and neddylation-dissociated 1)	1	D	modulates ubiquitin-ligase activity
U5-200KD (ASCC3L1, activating signal cointegrator 1 complex subunit 3-like 1)	1	D	helicase; splicing
USP13 (ubiquitin specific protease 13 (isopeptidase T-3))	1	D	cysteine-protease, deubiquitination
VPS28 (vacuolar protein sorting 28)	2	D	ESCRT-I complex, cell surface receptor sorting in MVB/late endosomes
WDR3 (WD repeat domain 3)	1	D	various; nuclear
XPO4 (exportin 4)	1	D	nuclear export
ADH5 (alcohol dehydrogenase 5 (class III), chi polypeptide)	1	D	glutathione-dependent formaldehyde dehydrogenase
PSAP (prosaposin)	1	D	saposin precursor
MTMR12 (myotubularin related protein 12)	1	D	adaptor subunit in a complex with an active PtdIns(3)P 3-phosphatase
chromosome 16 clone RP11-?	1	D	???

chromosome 7	2	D	???

Abstract

Functional studies of Tyk2 in IFNα signaling: a new interactor and an activating mutation

Tyk2 is a member of the Jak family of tyrosine kinases (Jak1, Jak2, Jak3 and Tyk2), which are indispensable components of α-helical cytokine signaling cascades. Receptors for α-helical cytokines are mostly made of two transmembrane subunits that associate with Jaks. Ligand bridging of two receptor chains brings together the associated Jaks, enabling their activation by transphosphorylation. Activated Jaks phosphorylate the STATs (Signal Transducer and Activator of Transcription) which translocate into the nucleus to drive gene expression. The Jaks have an N-terminal FERM (band 4.1-ezrin-radixin-moesin) domain, followed by an "SH2-like" domain and two kinase domains: a kinase-like (KL) domain and the catalytic tyrosine-kinase domain. The FERM and SH2-like domains are necessary for receptor binding. The KL domain has no catalytic activity, but plays an important regulatory role. The laboratory is particularly interested in the type I interferon (IFNα/β) receptor, made of two subunits IFNAR1 and IFNAR2, which bind Tyk2 and Jak1, respectively.

During the first part of my thesis, I analyzed a new Tyk2 interacting protein, Pot1 (Partner of Tyk2). Pot1 was isolated in a yeast two-hybrid screen using the Tyk2 FERM domain as bait. To assess the role of Pot1 in IFNα signaling, I monitored IFNα-induced response in Pot1-depleted cells by measuring STAT phosphorylation and the induction of a reporter gene. These experiments showed that, in this system, Pot1 depletion had no effect on IFNα-induced signaling. A two-hybrid screen was performed with Pot1 as bait. Among the 14 proteins found with high interaction confidence, we focused on GIT1 (G protein-coupled receptor kinase interactor 1), an adaptor protein implicated in a number of cellular processes, like cell migration, receptor internalization and EGF and angiotensin II signaling. To analyze the role of GIT1 in IFNα signaling, I monitored IFNα-induced receptor internalization, STAT phosphorylation and the induction of a reporter gene in GIT1-depleted cells. The results obtained allow us to exclude a role for GIT1 in type I IFN signaling.

During the second part of my thesis, I analyzed the effect of the V678F substitution on Tyk2 function. This mutation, located in the KL domain, corresponds to the V617F mutation of Jak2 found at the origin of *Polycythemia vera*. To study the effect of the V678F mutation on Tyk2 activity, I reconstituted Tyk2-deficient cells with Tyk2 WT or the V678F mutant and monitored IFNα-induced response. Our results show that the V678F mutation augments basal Tyk2 kinase activity measured *in vitro*. This gain-of-function leads to an increase of the basal STAT3 phosphorylation level, but has no effect on IFNα-induced Jak1 and STAT1/2/5 phosphorylation.

As opposed to Jak2, Tyk2 has been implicated only in signaling *via* heterodimeric receptor complexes. Interestingly, it has been shown that Jak2V617F needs the coexpression of a cognate homodimeric receptor to fully exert its transforming activity in the BaF3 cellular model system. Therefore, we analyzed the effect of Tyk2V678F on signaling *via* an artificial homodimeric receptor. To this end, we used Tyk2-deficient cells that express a chimeric receptor containing the extracellular domain of erythropoietin receptor fused to the intracellular region of IFNAR1. These cells were stably reconstituted with Tyk2WT or the V678F mutant. In this context, Tyk2V678F confers ligand hypersensitivity as seen by STAT1/3/5 phosphorylation. Moreover, the ensemble of these data point to STAT3 as a preferred substrate of Tyk2.

Résumé

Les récepteurs des cytokines à structure α-hélicoïdale sont pour la plupart composés de deux sous-unités transmembranaires associées aux tyrosine kinases de la famille Janus (Tyk2, Jak1, Jak2 et Jak3). La fixation du ligand au récepteur induit une dimérisation des sous-unités ce qui entraîne l'activation des Jak par transphosphorylation. Les Jaks ainsi activées phosphorylent les facteurs de transcription STAT (Signal Transducer and Activator of Transcription), induisant leur translocation dans le noyau. Les tyrosine kinases Jak présentent en position N-terminale un domaine FERM (4.1-ezrin-radixin-moesin), suivi par un domaine dit SH2-like et deux domaines kinases: un domaine kinase-like (KL) à fonction régulatrice et un domaine catalytique de type tyrosine kinase en position C-terminale. L'association aux récepteurs se fait par la région N-terminale. Le laboratoire s'intéresse particulièrement au récepteur de l'IFNα (interféron α) composé de deux chaînes, IFNAR1 et IFNAR2, auxquelles s'associent Tyk2 et Jak1 respectivement.

La première partie de ma thèse a porté sur l'étude d'un nouvel interacteur de Tyk2, la protéine Pot1 (Partner of Tyk2) et de son rôle potentiel dans la voie de signalisation de IFNα. Pot1 a été isolée dans le laboratoire par un criblage double-hybride utilisant comme appât le domaine FERM de Tyk2. Afin d'évaluer le rôle de Pot1 dans la voie de signalisation de l'IFNα, j'ai mesuré la réponse à l'IFNα de cellules déplétées en Pot1 en évaluant la phosphorylation des STATs et l'induction d'un gène rapporteur. Ces expériences ont montré que, dans ce système, la diminution de l'expression de Pot1 n'a pas d'effet sur la signalisation par l'IFNα. Un criblage double-hybride a été effectué avec Pot1. Parmi les 14 protéines identifiées à haut niveau de confiance, nous nous sommes particulièrement intéressés à GIT1 (G protein-coupled receptor kinase interactor), une protéine adaptatrice impliquée dans de nombreux processus cellulaires, tels que l'internalisation de récepteurs, la signalisation induite par l'EGF et l'angiotensine II ainsi que la migration cellulaire. Afin d'analyser le rôle évantuel de GIT1 dans la signalisation de l'IFNα, j'ai mesuré plusieurs paramètres (internalisation des sous-unités du récepteur de l'IFNα, phosphorylation des STAT, induction d'un gène rapporteur) dans des cellules sur-exprimant ou déplétées en GIT1. Les résultats obtenus ont montré que GIT1 n'avait pas de rôle dans la réponse à l'IFNα.

La deuxième partie de mon travail a porté sur l'étude de la mutation V678F introduite dans la protéine Tyk2. Cette substitution, située dans le domaine KL, correspond à la mutation V617F de Jak2, décrite comme étant à l'origine de maladies myeloprolifératives. Pour étudier l'effet de cette mutation de Tyk2, sur la signalisation induite par l'IFNα, nous avons établi, à partir de cellules Tyk2-déficientes, des clones stables exprimant la protéine sauvage ou mutante. Étant donné que Tyk2 se trouve associée uniquement à des récepteurs de type heterodimérique, nous avons aussi analysé le mutant Tyk2V678F associé à un récepteur de type homodimérique afin de se placer dans une situation analogue à celle de Jak2. En effet, dans le cas de maladies myeloprofiératives, il a été montré que Jak2 a besoin de la coexpression d'un récepteur homodimérique, par exemple du récepteur à l'érythropoïétine (Epo), pour avoir une activité transformante maximale. A cet effet, j'ai utilisé un récepteur chimérique comprenant l'ectodomaine du récepteur à l'Epo associé aux régions transmembranaire et cytoplasmique de la chaîne IFNAR1 (EpoR/R1). Nos résultats montrent que la mutation V678F augmente *in vivo* et *in vitro* la capacité de Tyk2 à s'auto-phosphoryler, cela plus fortement dans le cas du récepteur homodimérique. De plus, le mutant acquiert la capacité de phosphoryler STAT3 en absence de ligand. Cependant, la mutation Tyk2V678F n'affecte pas la réponse à l'IFNα en terme de phosphorylation de Jak1, STAT1 et STAT2. Ces résultats montrent une interaction fonctionnelle étroite entre Tyk2 et STAT3.