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Activating JAK1 mutations contribute to multiple-step tumorigenesis by interacting with endogenous cytokine receptor complexes

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Only he can understand what a farm is, what a country is, he, who shall have sacrificed part of himself to his farm or country, fought to save it, struggled to make it beautiful. Only then will the love of farm or country fill his heart.

(Antoine de Saint-Exupéry)

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Abbreviations

ALL	Acute lymphoblastic leukemia
Ag	Antigen
ATP	Adenosine triphosphate
CHR	Cytokine-binding homology region
CIS	Cytokine inducible SH2 containing protein
CRF1(II)	Class I (II) cytokine receptor family
CRLF2	Cytokine Receptor-Like Factor 2
DBD	DNA-binding domain
EGF	Epithelial Growth Factor
Epo	Erythropoietin
ERK	Extracellular regulated kinase
ET	Essential thrombocythemia
FERM	4.1, erzin, radixin, moesin
FGF	Fibroblast Growth Factor
FNIII	Fibronectin III
GAS	Gamma-activated sequence
GH	Growth Hormone
GIST	Gastro-intestinal stromal tumors
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte and Macrophage Colony Stimulating Factor
HIES	Hyper-IgE syndrome
HNSCC	Head and neck squamous cell carcinoma
HOX	Homeobox
HSC	Hematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosomal entry site
ISRE	IFN-stimulated responsive element
ITDs	Internal tandem duplications
JAK	Janus kinase

JH	JAK homology
MAPK	Mitogen activated protein kinase
MPNs	Myeloproliferative neoplasms
NSCLC	Non-small cell lung carcinoma
NRTK	Non-receptor tyrosine kinase
PDGF	Platelet Derived Growth Factor
PI3K	Phosphoinositol-3'-kinase
PIAS	Protein inhibitor of activated STAT
Prl	Prolactin
PV	Polycythemia Vera
R	Receptor
R(T)K	Receptor (Tyrosine) Kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCID	Severe combined immunodeficiency disease
SH	SRC homology
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TAD	Transcriptional domain
TCR	T-cell receptor
TNF	Tumor Necrosis Factor
Tpo	Thrombopoietin
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular and Endothelial Growth Factor
WT	Wild type

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CHAPTER I

Introduction

1. Cytokines and their cognate receptors

1.1 Cytokines

Cytokines represent a diverse group of small soluble proteins that when secreted by one cell can act on the same cell, in an autocrine fashion, or on another cell, in a paracrine or endocrine fashion (1). Through binding to specific cell surface receptors, they initiate signals that are critical to a diverse spectrum of functions, including induction of immune responses, cell proliferation, differentiation, activation and apoptosis. Cytokine families have been described based on similarities in their three-dimensional structure. Members of the hematopoietic cytokine family, which includes most interleukins and hematopoietic colony stimulating factors, show little homology based on their amino acid sequence, but are thought to adopt a common tertiary protein folding of a 4-alpha-helix bundle conformation (**Figure 1.1**) with the four helices arranged in an up-up-down-down topology.

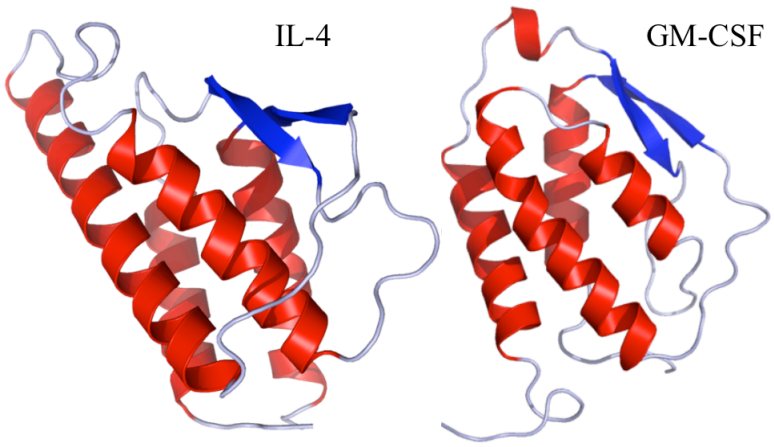

Figure 1.1 Thee-dimensional structures of 4-helix bundle cytokines. *Adapted from (2,3).*

1.2 Cytokines receptors

While cytokines show very little homology in their amino-acid sequence, their receptors share higher structural similarities within their extracellular domain. On the basis of common structural features the cytokine receptors are grouped into six major families: class I cytokine receptors (CRF1), class II cytokine receptors (CRF2), TNF receptors, IL-1 receptors (including Toll-like receptors), tyrosine kinase receptors and receptors for chemokines (1). Most hematopoietic cytokines binds to CRF1 receptors, while cytokines related to IL-10 and interferons (IFNs) bind to CRF2.

1.3 Class I cytokine receptors

Class I as well as class II cytokine receptors are single span membrane proteins with an N-terminal extracellular and C-terminal intracellular orientation. The extracellular segments of these cytokine receptors show a modular architecture that is composed of one or several cytokine-binding homology regions (CHR). The CHR module consists of two fibronectin-type III domains, also called CHR domains, connected by a linker. Both, D1 and D2, domains are composed of two anti-parallel β-sheets. The upper, N-terminal domain contains four conserved cysteine residues that form interstrand disulfide bonds and the lower, C-terminal domain has a conserved WSXSW motif. This basic CHR module is present in every class I cytokine receptor and for some receptors, such as the receptor for growth hormone (GH) or erythropoietin (Epo), a single CHR module is sufficient to mediate ligand binding and receptor activation (**Figure 1.2**). The cytokine-binding site for most of the CHR modules is at the apex of the elbow region, consisting mainly of the interstrand loops connecting the β-strands from the N- or C-terminal domains (1). However, many other class I cytokine receptors require additional domains, such as the Ig-like domain or additional membrane-proximal FnIII domains found in the gp130 receptor family (**Figure 1.3**).

The class I and II cytokine receptors have no intrinsic enzymatic activity, and they rely on members of the JAK family of tyrosine kinases to provide the phosphorylation potential. JAKs are therefore constitutively associated with a membrane proximal proline-rich BOX1 motif found in all CRF receptors (4). The paradigm for cytokine receptor activation is that cytokines induce signaling by bringing the cytoplasmic domains of two cell surface receptor subunits into close proximity. The ligand-induced receptor homo- or hetero- dimerization or a ligand-induced conformational change in the pre-associated receptor complexes instigates signaling and activates JAKs. JAKs then phosphorylate themselves and receptors they are associated with. The phosphorylated tyrosine residues in the receptors then serve as docking sites for the SH2 domain-containing proteins such as signal transducer and activator of transcription (STAT) proteins. Each class I or class II cytokine receptors is associated with a particular JAK that phosphorylates, after ligand binding, a specific set of STATs, thus assuring the specific signaling of each cytokine (5). Within the type I cytokine receptors, there are different cytokine receptor subfamilies that share similarities in the structure of their active cytokine receptor complexes and in the way their cognate cytokines bring the cytoplasmatic domains of their receptors subunits together.

1.3.1 Homodimeric class I cytokine receptors

The human erythropoietin receptor (EpoR) and the human growth hormone receptor (GHR) belong to homodimeric class I type cytokine receptor family. Their ectodomains consist of a single CHR module (composed of two CHR domains D1 and D2) pre-associated on the cell surface by contacts between the transmembrane domains (6,7) and probably other parts of the proteins within the homodimer (8) **(Figure 1.2)**. The C-terminal membrane proximal CHR domains (D2) of the two EpoRs are more distant in the crystal structure of the unliganded EpoR dimer than in the crystal structure of the liganded Epo/EpoR complex (9,10). This suggest that ligand-binding brings the transmembrane and cytoplasmic domains together by initiating a rearrangement or/and by a change in relative orientations of the CHRs in the preformed receptor dimer (11). This model can be further expanded to active complexes of other members of the homodimeric class I receptor family such as TpoR and PrlR.

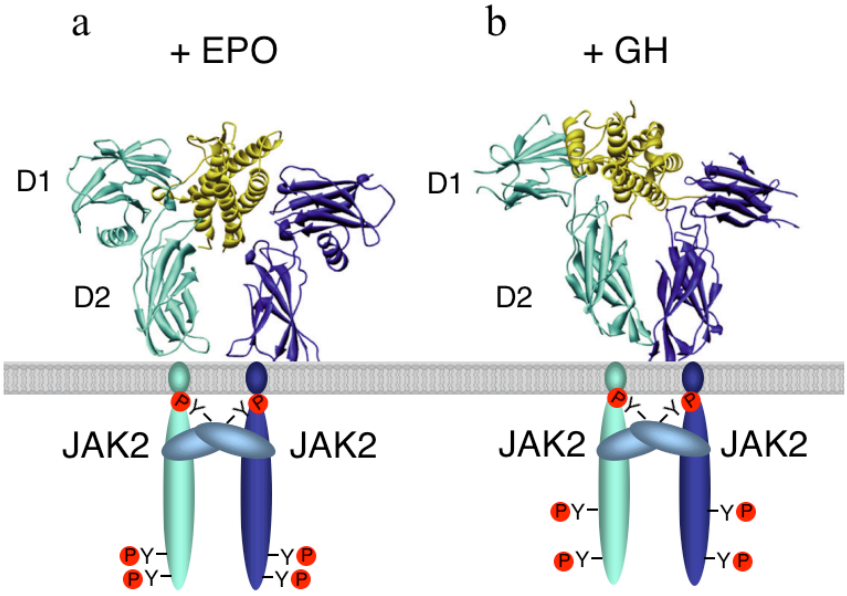

Figure 1.2 A schematic representation of the quaternary structure of the EpoR/Epo and GHR/GH complexes. *The two erythropoietin receptor (EpoR) (a) and growth hormone receptor (GHR) (b) molecules are shown in dark and light blue, Epo/GH in gold. Epo/GH induces a close dimer association of both the D1 and D2 domains so that their intracellular regions become substrates for phosphorylation by two activated JAK2 molecules. Adapted from (10).*

1.3.2 Non-homodimeric class I cytokine receptors

Most of the class I cytokine receptors do not signal through homodimerization, but form heterodimers (IL-4Rα, IL-7Rα, IL-9Rα with common γc), heterotrimers (IL-2 and IL-15 receptor complex) and if including ligands, even hexamers (IL-6/IL-6Rα/gp130), and dodecamers (GM-CSF/GM-CSFRα/βc), as shown by recent structural data. A main feature of these hetero-oligomeric receptor complexes is the use of a common, shared receptor subunit as a signal-transducing chain together with a cytokine-binding, specific α chain. The shared

receptors do not show significant affinity for cytokines, but in the presence of cytokine-binding specific α receptors they can form high-affinity cytokine receptor complexes that are capable of initiating intracellular cascades (12). When subgrouped by shared receptors, there are three major classes of heteroreceptor complexes in the class I cytokine receptor family: those that use γc, those that use gp130, and those that use βc (**Figure 1.3**).

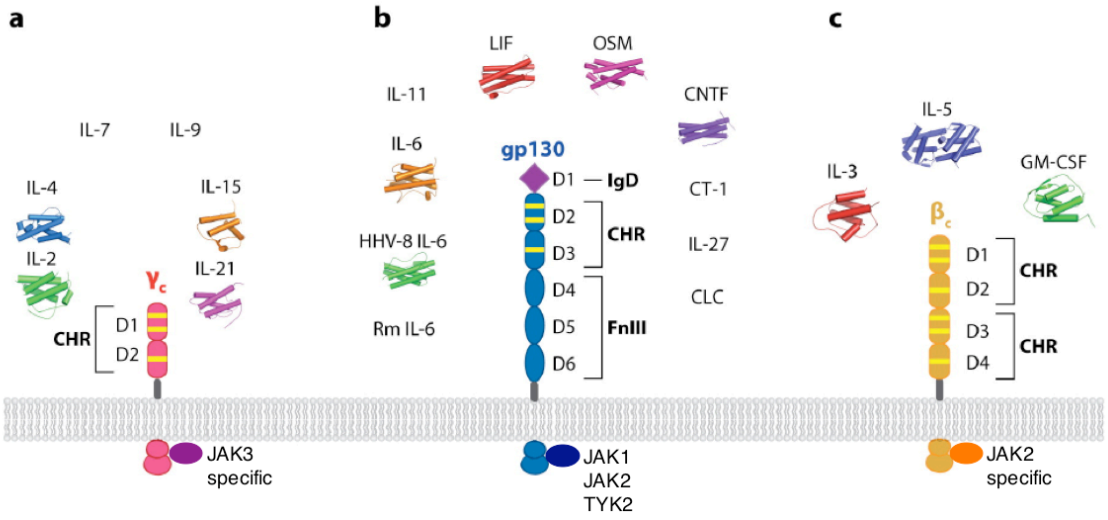

Figure 1.3 Diversity of shared cytokine-receptor interactions. *Shared cytokine receptors γc (a), gp130 (b), and βc (c) are represented schematically on a cell membrane. The respective interacting cytokines with known three-dimensional structures are shown with cylinder representations of the four-helix bundles. (Abbreviations: LIF, leukemia inhibitory factor; OSM, oncostatin-M; CNTF, ciliary neurotrophic factor; CLC, cardiotrophin-like cytokine; CHR, cytokine-binding homology region; HHV-8, human herpes virus; GM-CSF, granulocyte-macrophage colony-stimulating factor.) Adapted from (12).*

1.3.2a Gp130-related receptors

Gp130 is a founding member of the tall cytokine receptors and is the common signal-transducing receptor component for the gp130, or IL-6/IL-11, family of cytokines (**Figure 1.3b**) currently including ten members: IL-6, IL-11, LIF, ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin 1 (CT-1), cardiotrophin-like cytokine (CLC), IL-27 and two viral homologs of IL-6, one from HHV-8 and one from Rhesus macaque rhadinovirus (Rm IL-6). Almost all signaling functions of gp130 cytokines are mediated through a set of receptor complexes that are formed by combining gp130 with specific α or β receptor subunits. The extracellular part of gp130 is composed of six contiguous β-sandwich domains with a single Ig domain at the top (D1), followed by one CHR module (D2 and D3), and three fibronectin III-like domains (D4–D6) proximal to the cell membrane (13) (**Figure 1.3b**).

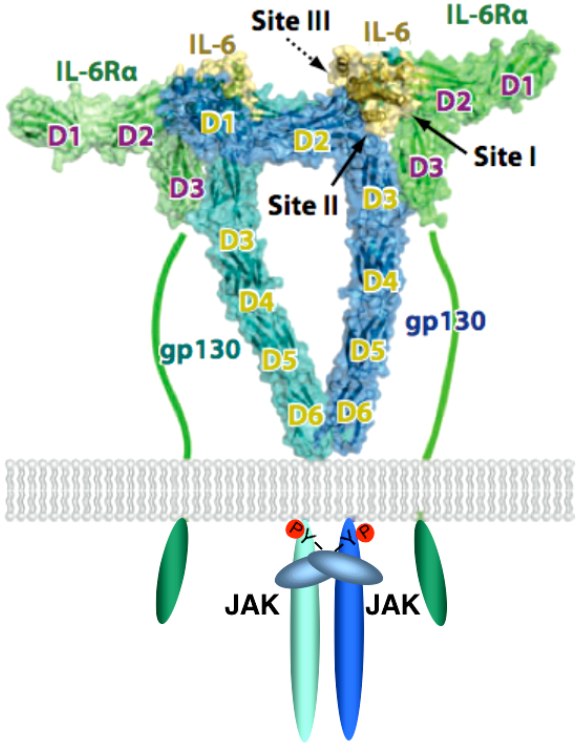

Figure 1.4 Schematic structure of human IL-6/IL-6Rα/gp130 hexameric complex *assembled from known crystallographic, biochemical and electron microscopic data. The model is depicted as elucidated from (14-16). IL-6 first engages IL-6Rα through a site I interaction to form a composite interface (site II) that recruits gp130. This trimeric structure can then engage a second trimer through two site III interfaces to form a productive signaling complex. Adapted from (12).*

Recently, a substantial amount of crystal structure data (14,17) and low-resolution electron microscopic (EM) three-dimensional reconstructions data (18,19) characterizing complexes involving gp130 were collected. The crystal structures established that gp130 cytokines first engage the elbow regions of the specific receptor chain (such as IL-6Rα) through a canonical site I interaction to form a composite interface (site II) that recruits the CHR domain of gp130 chain. This trimeric structure can then engage the Ig domain of the second trimer through two site III interfaces so that each gp130 contacts two different cytokines in an antiparallel fashion (12) (**Figure 1.4**). All gp130 cytokine receptor family members, including the non-shared members of the tall receptor family such as OSMR, use this basic assembly template (12).

I.3.2b Common β chain-related receptors

β_c is a type I transmembrane protein that serves as a shared signaling subunit for the receptors of IL-3, IL-5, and GM-CSF (**Figure 1.3c**), which are related cytokines involved in the regulation of hematopoiesis and inflammation (20,21). Although β_c does not measurably bind any of the ligands alone, its co-expression with cytokine-specific α receptors enhances the affinity of cytokine binding. The extracellular part of β_c has two contiguous CHR modules (**Figure 1.3c**) with features conserved among the class I cytokine receptors.

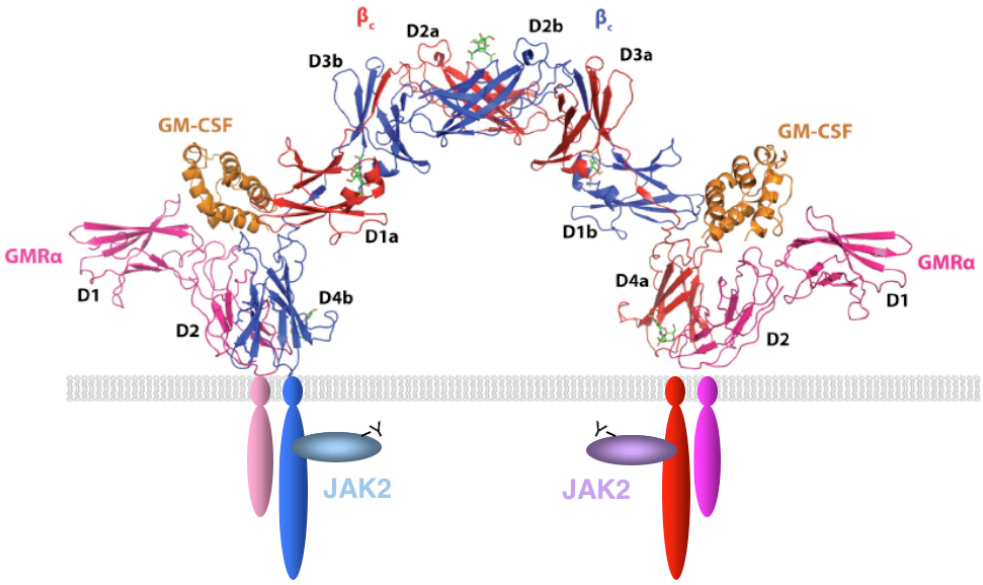

Figure 1.5 The 2:2:2 GM-CSF/GMRα/βc complex viewed from the side. *GMRα engages the cytokine GM-CSF via a canonical site I interaction, whereas the βc receptor engages site II on GM-CSF by using a composite cytokine-binding homology region (CHR) interface generated by domain 1 (D1) of one βc subunit and domain 4 (D4) of the second βc. Adapted from (22).*

The crystal structure of the unliganded extracellular domain of β_c shows an unusual, intertwined, strand-swapped, antiparallel homodimer (23). Furthermore, the recent crystal structure of the β_c in complex with the GM-CSFα receptor (GMRα) and GM-CSF shows that β_c engages the cytokine via a composite D1/D4 interface similar to site II in gp130, while the accessory receptor GMRα engages the cytokine via a site I–like interface (22) (**Figure 1.5**). GMRα does not engage a JAK kinase, leaving the JAK2-bound β_c as the sole carrier of the signal-transducing kinase (24). The asymmetric unit of the β_c/GMRα/GM-CSF complex consists of a 2:2:2 hexamer with the C termini of β_c too apart to reconcile how JAK kinases bound to β_c subunits of a single β_c dimer could be activated. Crystalographic data and site-directed mutagenesis strongly suggest that β_c signaling is mediated by two hexamers dimerized into a dodecameric structure (22). This atypical cytokine receptor-signaling paradigm can likely be extrapolated to explain also the activation mechanisms of the other β_c-family cytokines IL-3 and IL-5.

I.3.2c Common γ chain-related receptors

The common γ_c-dependent cytokines

γ_c serves as a shared signaling receptor for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (25) (**Figure 1.3a**). The biological importance of γ_c is illustrated by the fact that mutations in either γ_c or the associated JAK3 kinase can abolish the function of all γ_c-dependent pleiotropic cytokines playing crucial role in the immune system and thereby cause X-linked severe combined immunodeficiency diseases (X-SCID) (25,26). The γ_c-dependent cytokines play a key role in proliferation, differentiation and survival of lymphocytes.

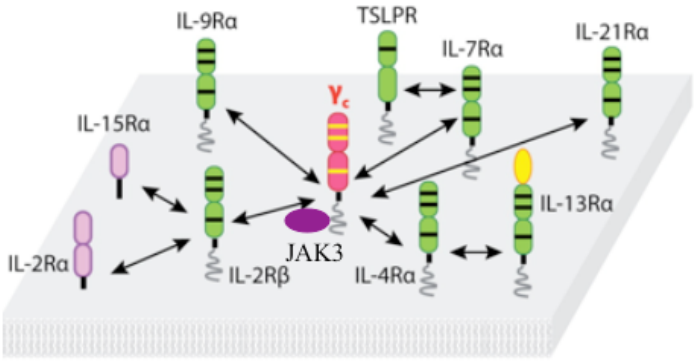

Figure 1.6 Diversity of shared γc chain-receptor interactions. *Shared γc receptor and its various receptor partners are depicted. These complexes are formed by the combination of ligand specific α and/or β chains with shared γc receptor subunit. (Abbreviation: TSLPR thymic stromal-derived lymphopoietin receptor.) Adapted from (12).*

The common γ_c-shared cytokine receptor complexes

IL-2Rβ, IL-4Rα and other α receptors such as IL-7Rα or IL-9Rα are functional and structural counterparts in their respective signaling complexes, and all form one of the two major signaling subunits in their γ_c receptor complexes. Although the nomenclature is confusing, IL-2Rβ is analogous to the α receptors for other γ_c cytokines, but because IL-2 has the additional nonstandard initiating receptor, IL-2Rα, IL-2Rβ is then referred to as the α receptor on the basis of the sequence of interactions with IL-2. The intracellular domains of IL-2Rβ, IL-4Rα and other α receptors such as IL-7Rα or IL-9Rα, possess the box 1 and box 2 motifs at the membrane-proximal region that constitute the binding sites for JAK1 (5). The cytokine binding–induced oligomerization of JAK1-preassociated IL-2Rβ or IL-4Rα with JAK3-preassociated γ_c will bring their intracellular domains into close proximity, inducing the activation and trans-phosphorylation of the JAK kinases (5) (**Figure 1.7**).

In contrast to type I IL-4R, IL-7R, IL-9R, and IL-21R signaling complexes, which heterodimerize cytokine-specific α subunits and γ_c, the IL-2 receptor (IL-2R) and IL-15R signaling complexes are heterotrimers composed of structurally unique α subunits and shared

IL-2Rβ and γ_c subunits (27) (**Figure 1.6**). Another interesting twist in the γ_c family is limited sharing of several α receptors including IL-2Rβ, IL-4Rα, and IL-7Rα to recognize different cytokines as well as different receptors (**Figure 1.6**). IL-4Rα heterodimerizes with γ_c to form type I IL-4R, and with IL-13Rα1 to form type II IL-4R. Type II IL-4R is also the functional receptor of IL-13 (28,29). IL-7Rα can also form a receptor heterodimer with TSLPR (thymic stromal-derived lymphopoietin receptor) to recognize TSLP (30).

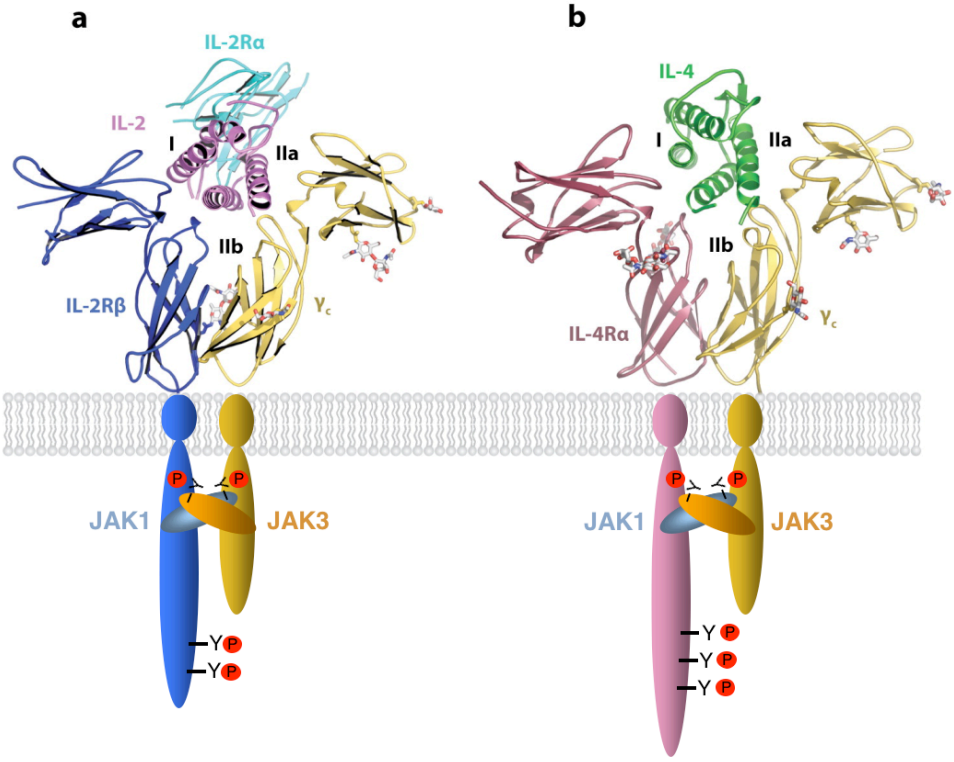

Figure 1.7 Schematic structure of IL-2 and IL-4 receptor complexes. *The first complex structure of γ_c to be solved was the IL-2/IL-2Rα/IL-2Rβ/γ_c quaternary complex (31), which is composed of one copy each of IL-2, IL-2Rα, IL-2Rβ, and γ_c. Viewed from the perspective of the cell membrane, IL-2Rα sits on top of IL-2, and receptors IL-2Rβ and γ_c form a Y shape in which IL-2 sits in the fork (a). The overall structural organization of the IL-4/IL-4Rα/γ_c ternary complex is very similar to that of the IL-2 quaternary complex with a 1:1:1 stoichiometry (32) except for the absence of a top-mounted IL-2Rα. In the IL-4/IL-4Rα/γ_c ternary complex, receptor IL-4Rα and γ_c form a Y-shape heterodimer that binds to IL-4 in the classical site I/site II paradigm (b). Adapted from (12).*

The IL-2 receptor complex assembly

There are three receptor chains for IL-2: IL-2Rα, IL-2Rβ, and γ_c, which form three different receptor complexes. The heterodimerization of IL-2Rβ and γ_c in the presence of IL-2 is necessary and sufficient for effective signaling. However a complex with three subunits, IL-2Rα, IL-2Rβ, and γ_c, is the high-affinity complex for IL-2 and is the receptor form found on activated T cells (25).

The IL-2/IL-2Rα complex represents the initiating step for formation of this quaternary signaling complex. From sequence analysis, IL-2Rα clearly lacks the signature

features of class I cytokine receptors and is unable of forming receptor-receptor contact with IL-2Rβ, therefore IL-2Rα's role is simply to capture free IL-2 and present it to IL-2Rβ and γ_c (31) (**Figure 1.8a**). After the capture of IL-2 by IL-2Rα, the delivery of IL-2 to IL-2Rβ in *cis* represents the second step in the formation of the quaternary IL-2 receptor complex and the binding interface between IL-2 and IL-2Rβ (site I) is formed (12) (**Figure 1.8a**).

The interaction of the IL-2/IL-2Rα/IL-2Rβ ternary complex with γ_c is the last step in formation of the IL-2 signaling complex on activated T cells. In the complex structure, two contact surfaces, a small one between IL-2 and γ_c and a larger one between IL-2Rβ and γ_c, form the interaction surface between IL-2/IL-2Rαβ and γ_c (12) (**Figure 1.8a**).

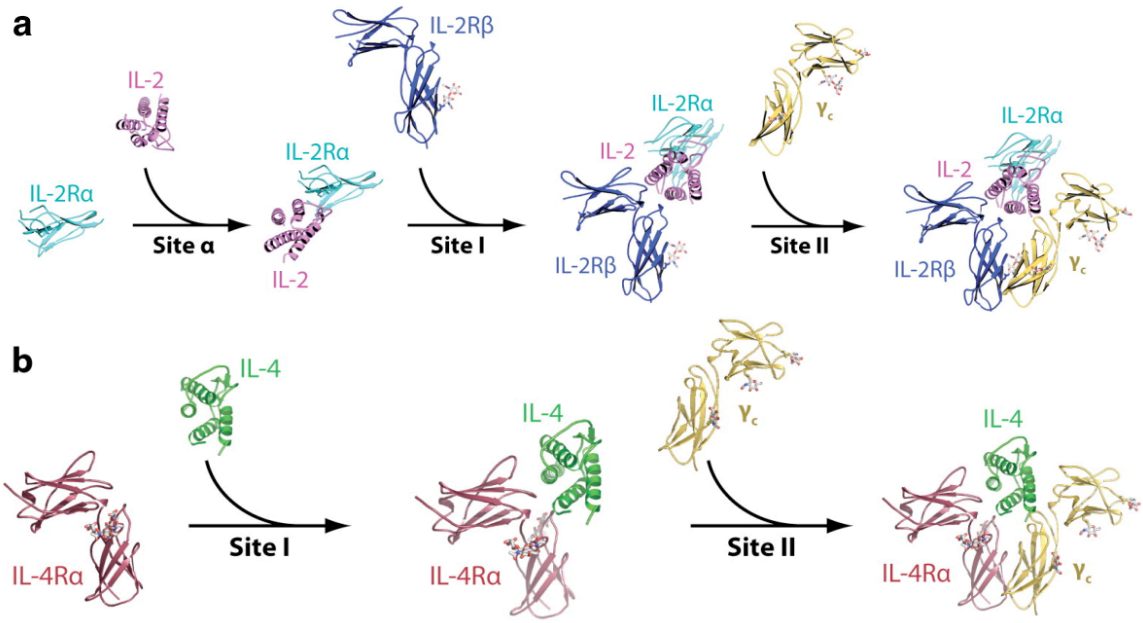

Figure 1.8 A sequential assembly pathways of the IL-2/IL-2Rα/IL-2Rβ/γ_c (a) and of the IL-4/IL-4Rα/γ_c (b). *Description of the figure is found in text above and below the figure. Adapted from (12).*

The IL-4 receptor complex assembly

There are two types of receptor complexes for IL-4 (**Figure 1.6**). Type I IL-4R is predominantly expressed on the surface of hematopoietic cells and consists of IL-4Rα and γ_c. It is a prototype of the classical heterodimeric complexes with shared γ_c such as IL-7Rα/γ_c, IL-9Rα/γ_c and IL-21Rα/γ_c. Type II IL-4R consists of IL-4Rα and IL-13Rα1 and is predominantly expressed on the surface of nonhematopoietic cells, and this receptor complex is also the functional receptor of IL-13 (29).

The first step in the formation of the IL-4/IL-4Rα/γ_c complex is the binding of IL-4 with IL-4Rα receptor though classical site I interface (**Figure 1.8b**). These interactions were first elucidated in the IL-4/IL-4Rα binary complex (33). After the initial binding of IL-4 with the

IL-4Rα receptor, the association of γ_c is also mediated through a composite interface: IL-4/γ_c and IL-4Rα/γ_c (**Figure 1.8b**).

1.3.3 Conclusion

In summary, both the IL-2 and IL-4 receptors show high degree of specificity for their cytokines, in contrast to γ_c's degeneracy. As a shared signaling subunit, the engagement of γ_c is the last step in the formation of functional signaling complexes (**Figure 1.8a,b**). On the basis of the structural information from the IL-2 quaternary complex and the IL-4 ternary complex, one can conclude that the preformed complexes of cytokines with the α receptors provide a composite binding site for γ_c that is composed of two interaction interfaces: cytokine/γ_c and α receptor/γ_c. Relatively nonspecific atomic interactions characterize the cytokine/γ_c binding interface. In contrast, the α receptor/γ_c binding interface is composed predominantly of specific (polar) interactions.

Overall, all the class I receptor complexes exhibit the basic core template in which the CHR of the cytokine-specific α receptors and the shared receptors engage the sides of the four-helix bundle in the typical site I/site II manner seen in homodimeric receptor complexes. The presence of an additional site III interacting Ig domain distinguishes gp130 from γ_c. In gp130, the heterodimers between gp130 and α receptors are nonproductive because in most cases the α receptors do not contain intracellular signaling motifs. Thus, site III is necessary to dimerize gp130, each of which is bound to a JAK, and signal. This stands in contrast to the γ_c family, in which the α receptors do contain intracellular signaling domains, and so the γ_c/α receptor heterodimers are productive: γ_c does not need to be dimerized.

2. The JAK/STAT pathway

One of the crucial mechanisms involved in regulation and coordination of cell proliferation, differentiation, migration or metabolism changes is protein tyrosine phosphorylation. Tyrosine phosphorylation modulates enzymatic activity and creates binding sites for the recruitment of SH2 domain-containing downstream signaling proteins. Enzymes that catalyze the transfer of gamma phosphate of ATP to hydroxyl group of tyrosine residues on protein substrates are protein tyrosine kinases. There are two classes of protein tyrosine kinases (TKs) present in cells: the receptor protein tyrosine kinases (RTKs) and the non-receptor protein tyrosine kinases (NRTKs) (34).

Identification of the conserved sequences and structural features that characterize PTKs (35) helped to elaborate strategies for discovery of new kinases. The polymerase chain reaction with low stringency probes was used by Wilks and his colleagues to identify several new members of PTK family (36). The two novel related cDNA sequences suggested a new family of non-receptor TKs (NRTKs) that was named Janus kinase (JAK), and the first two members JAK1 and JAK2. At first the name was referring to Just Another Kinase and later was changed to ascribe the acronym as 'Janus kinase', after the Roman god of gates, doors, beginnings and endings, also reflecting the fact that, the two kinases surprisingly appeared to have two kinase-like domains, which were reminiscent of Janus's double face (37). In the meantime, by screening a T cell library with a tyrosine kinase domain probe, Krolewski and his colleagues identified the third JAK TK gene, called TYK2 (38). The JAK family was completed by identification of JAK3 as a fourth member (39).

The importance of the JAK family members was completely understood when TYK2 was found to be the accessory molecule necessary for IFN-α signaling (40). The involvement of JAKs in cytokine signal transduction was extended to other cytokines and soon all known class I and class II cytokine receptors were associated to at least one JAK, demonstrating that JAKs are the key molecules allowing tyrosine phosphorylation and signal transduction by such receptors with no intrisic kinase activity (5).

Meanwhile, studies on transcriptional components of IFN signaling led to the discovery of the new family of transcriptional factors named STATs, for signal transducer and activator of transcription. Following the cloning of STAT1 and STAT2 (41), five additional STAT genes were identified (STAT3, STAT4, STAT6 and two genes coding the two variants of STAT5) and it became obvious that STAT proteins are activated in response to various cytokines. Therefore, in addition to JAKs activation, phosphorylation of latent STAT factors turned out

to be a common element in signal transduction through cytokines. The definitive proof of STAT factor-specific phosphorylation and activation by JAKs came shortly after (24), leading to description of the JAK/STAT pathway as the main cytokine receptor signaling pathway (5).

2.1 The Janus Kinase

2.1.1 Structure

The Janus family comprises four members: JAK1, JAK2, JAK3 and TYK2, identified in mammals, birds and fish, but JAK variants do exist also in lower species such as *Drosophila's* Hopscotch (42). In mammals, expression of these large 120 to 140 kDa kinases is ubiquitous, except for JAK3 with expression restricted to the hematopoietic lineages. The domain structure of the JAK family of PTKs was elaborated entirely based on the area of homology within the family members (37). Following the original nomenclature proposed for SRC-related proteins (SH domain for SRC-homologue domain) by Pawson and co-workers (43,44) homology domains for JAK family were named JH domains (e.g. JAK homology domains). Seven such domains, JH1 to JH7, have been defined (**Figure 2.1**).

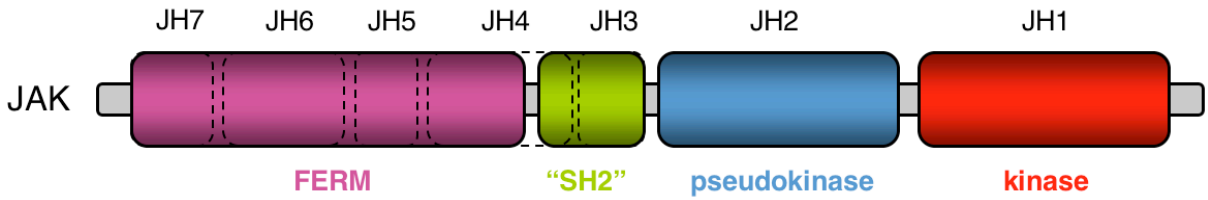

Figure 2.1 Schematic structure of Janus kinases (JAKs). *Seven JAK homology (JH) domains including the catalytically active kinase domain (JH1), the enzymatically inactive pseudokinase domain (JH2), the "SH2"-like domain (JH3, JH4), and FERM domain (JH5, JH6, JH7) form the JAK protein.*

The JH1 tyrosine kinase domain

The JH1 domain is the protein tyrosine kinase (TK) domain of JAK protein. It is located at the C terminus of the protein and contains all the typical features for a catalytic tyrosine kinase including the double tyrosine residues of the "KEYY" motif in the activation loop, the canonical glycine-rich motif in the nucleotide-binding loop and a conserved aspartic acid (D) residue involved in the phosphotransfer reaction in the catalytic loop (5). While there is no three-dimensional structure for a full size JAK protein, crystal structures of the TK domain have been solved for JAK1, JAK2 and JAK3 (45-47), each in an active conformation complexed with a specific inhibitor.

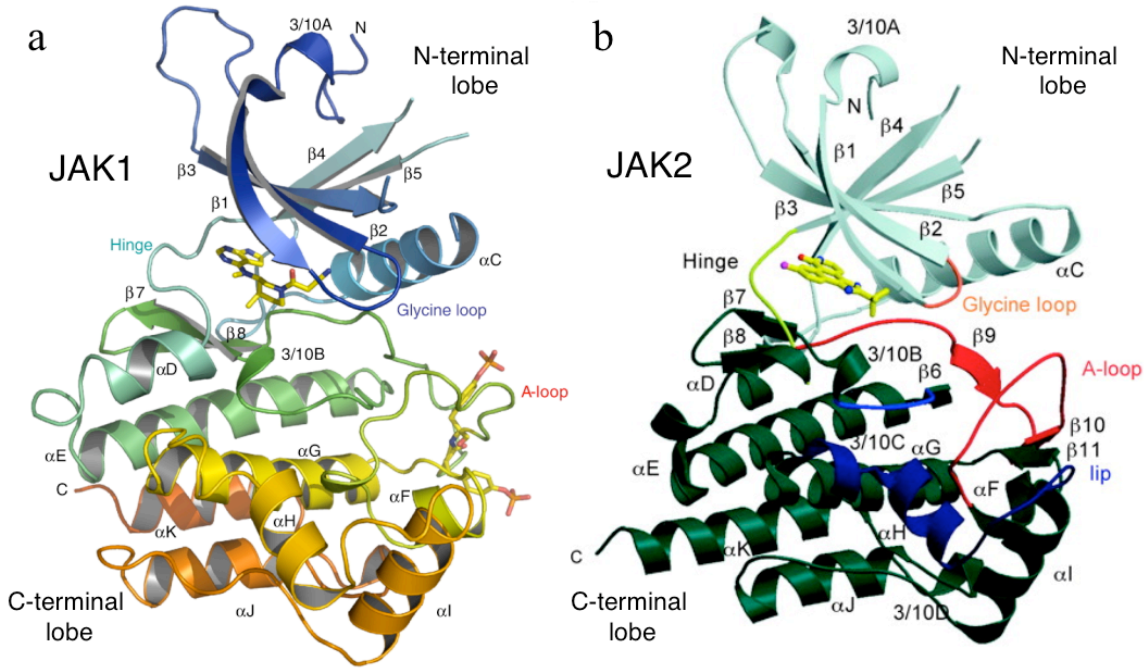

Figure 2.2 Ribbon representation of the crystal structure of the (a) JAK1 and (b) JAK2 TK domain in complex with specific JAK kinase inhibitors: CP-690,550 (47) and CMP6 (45), respectively. *The N-terminal lobe comprises a five-stranded antiparallel β-sheet (β1 to β5) and one α-helix (αC). The larger C-terminal lobe is predominantly helical with eight α helices (αD to αK) and three 3/10 helices (3/10B, 3/10C and 3/10D), with one main pair of antiparallel β-strands (β7–β8). Locations of the hinge region, glycine loop and activation loop (A-loop) are indicated. The bound CP-690,550 and P-Tyr residues 1034 and 1035 in JAK1 (a) and CMP6 in JAK2 (b) are presented in a ball-and-stick representation with carbon atoms in yellow, oxygen atoms in red, nitrogen atoms in blue and phosphorus atoms in orange. Adapted from (45) and (47).*

The JH1 crystal structures

The crystal structure of the JAK3 TK domain in complex with staurosporine was determined as first of the JAK protein family (46), followed by the crystal structure for the TK domain of JAK2 in complex with pan-JAK inhibitor (45) (**Figure 2.2b**). Recently, the crystal structures of the JH1 domain of JAK1 in complex with two pan-JAK inhibitors (CMP6 and CP-690, 550) (**Figure 2.2a**) were solved and compared with the crystal structures of the TK domain of JAK2 complexed with identical inhibitors (47). All described JAKs TK domain structures correspond to a typical structure of PTK domain shared by other tyrosine kinases comprising a N-terminal lobe composed of β-sheets (β1-5) and one α helix (αC), and a larger predominantly helical C-terminal lobe (8 α helixes) (45-47). Between the two lobes resides the substrate/ATP-binding cleft with a hydrophobic pocket, where ATP-competitive inhibitors were localized. In all of these structures, the TK domain appears to be in the open conformation, and as such represents the catalytically active conformation of the protein, with the activation loop displaced from the active site and with two Tyr residues at position Y1034 and Y1035 in human JAK1 (Y1007 and Y1008 for human JAK2) phosphorylated. The JAK1 TK domain shares 53%, and 50% sequence identity with JAK2 and JAK3, respectively;

therefore it is not surprising that there is a high degree of similarity in their overall structure with almost all residues within the ATP-binding site being identical between the three JAKs. The most significant differences in sequence are observed in the hinge region, glycin loop and activation loop (47). However the crystal structures of JAK1 and JAK2 TK domain with identical pan-JAK inhibitors showed high similarities in the binding mode of the ATP-competitive JAK inhibitors revealing Glu883 in the glycine loop and Phe958 in hinge region of JAK1 (corresponding to Lys857 and Tyr931 in JAK2) as unique and crucial residues that make direct contact with these inhibitors (**Figure 2.3**). Although these are crucial residues to interact with the inhibitors through their main-chain atoms, the sequence variability of JAK1 and JAK2, even being low, may contribute to a different plasticity of the hinge region and glycine loop between the two proteins, explaining the 3-4 fold difference in the binding affinity of the pan-JAK inhibitors to JAK1 and JAK2 (47).

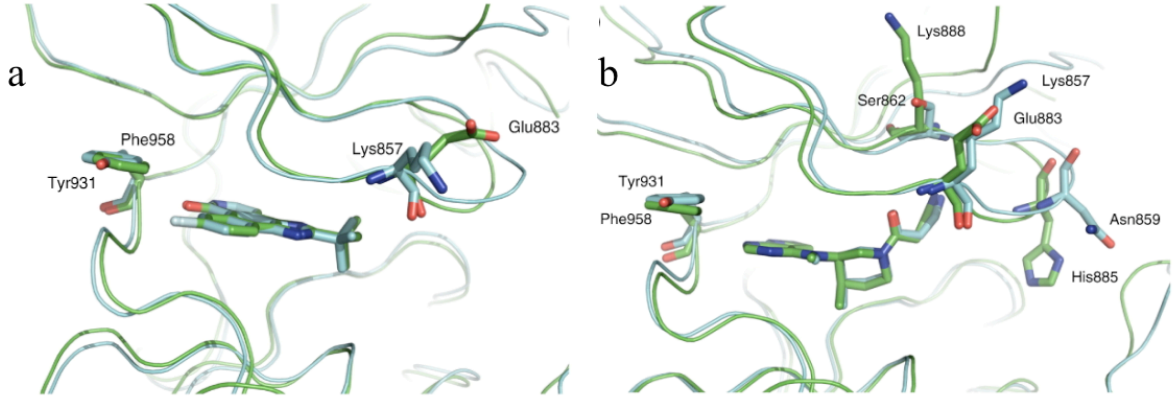

Figure 2.3 Comparison of the active-site regions of JAK1 and JAK2*. Superposition of JAK1 (green) and JAK2 (cyan) tyrosine kinase domain in complex with CMP6 (a) and CP-690,550 (b). Unique residues within 5 Å of the inhibitor are shown. Adapted from (47).*

The JH2 pseudokinase domain

The unique feature of JAKs within the TK family is the presence of a JH2 domain that follows JH1 domain at the C-terminal part of the protein. The sequence of the JH2 domain, also called kinase-like domain, is highly homologous to the JH1 kinase domain, but lacks several critical residues, such as the third glycine in the GXGXXG motif in TK domain and the nearly invariant aspartic acid involved in the phosphotransfer reaction (37). JH2 is therefore termed the pseudokinase domain and is considered catalytically inactive. Although the role of the pseudokinase domain is not fully understood, a computational model of the JH1 and JH2 domains based on crystal structure of the FGF receptor kinase has been proposed in which these two domains interact with each other, with JH2 domain inhibiting the activity of the JH1 domain via 2 interfaces (48) (**Figure 2.4**). The fact that deletion of the

pseudokinase domain led to the hyperactivation of JAK2 and JAK3 (49,50) supports the notion of JH2 domain playing an important role as a negative regulator of the JAK enzymatic activity. However, since the three-dimensional structure of the full JAK proteins is currently unknown, the precise mode of interactions between JH1 kinase and JH2 pseudokinase domain is still to be elucidated.

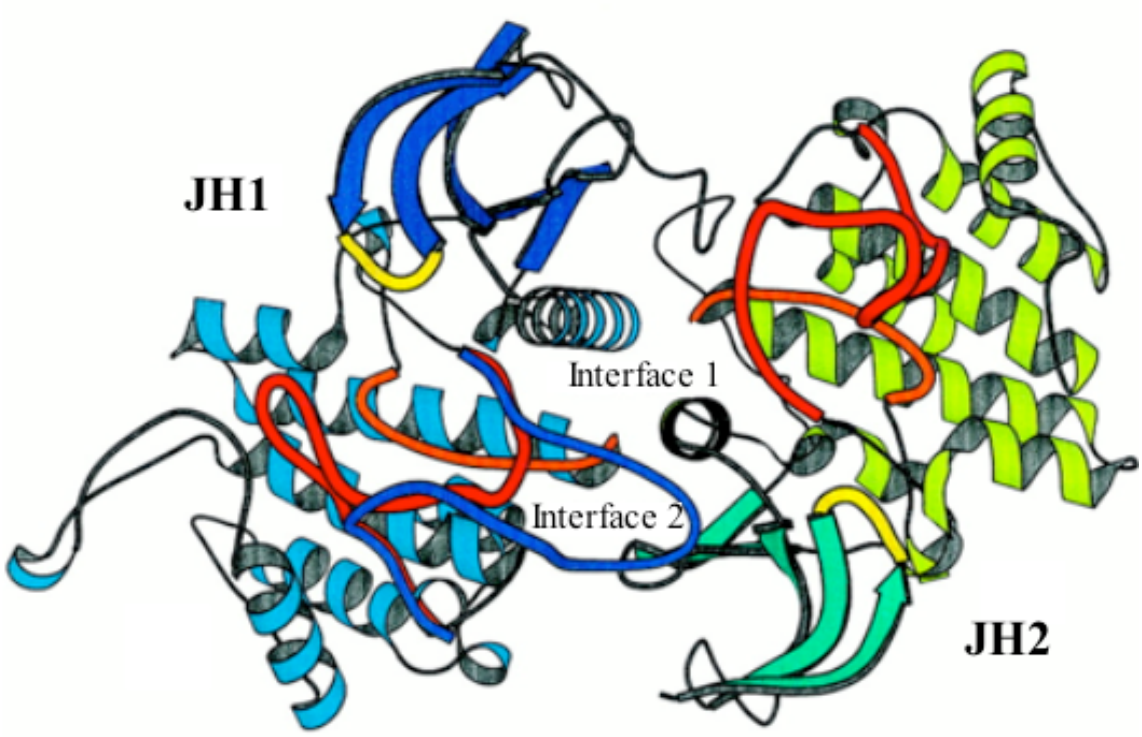

Figure 2.4 Predicted active versus inactive conformation of the JAK2 JH1-JH2 complex*. The JH2 domain is proposed to interact with JH1 domain via interface 1 and 2. It is predicted that in the inactive conformation of the protein, the interactions between the oscillating activation loop (red/blue) and the JH2 domain at interface 2 are unfavorable. Receptor-activation results in a conformational change that favors interactions between residues in the activation loop and interface 2. This stabilizes the open loop conformation (blue), allows phosphorylation of the critical tyrosine residues and therefore activation of the kinase activity. Adapted from (48).*

The SH2-like domain (JH3 and JH4)

The region immediately N-terminal to the pseudokinase domain composed of JH3 and JH4 segment, aligns well with known SH2 domains of other proteins (51). The role of this segment is not completely understood. However, several lines of evidence suggest that the whole N-terminal part of the JAK proteins, including SH2-like segment, is important for efficient JAK-mediated cell surface localization of the cytokine receptors (52-54). Therefore the SH2 domain plays a somewhat different role in JAK kinases than in other signaling molecules.

The FERM domain (JH5-JH7)

The N-terminal part of JAKs, spanning the JH5 to the JH7 domain, is called FERM (band – 4.1, ezrin, radixin, moesin) domain (55). The FERM domain was initially implicated in heterotypic interactions between JAKs and other kinases (56), but now it's clear that it essentially mediates JAKs association with the cytokine receptors. JAK proteins that lack their N-terminal domains (JH3-JH7) have been shown to be unable to bind their cognate receptors (57,58). Moreover, it seems that some residues, conserved among JAK members in the FERM domain and engaged in the hydrophobic interaction between the FERM domain and cytokine receptor membrane proximal region (BOX1 motif), are critical in the JAK-receptor interaction, since point mutations of the residues such as the Y107F in JAK1 have been shown to prevent kinase-receptor association (58). Several studies with chimeric JAKs indicate that FERM domain also determine the specificity of JAKs binding to distinct cytokine receptors (59-62). For example, a chimeric JAK2 with substituted JH6-JH7 segment of JAK3 could support IL-2-induced signal transduction, while wild-type JAK2 protein could not (61). Beside the role of JAKs as mediators of ligand-induced phosphorylation of cytokine receptors, JAKs are also important for efficient cell surface localization of their cognate receptors. This chaperone function of JAKs is also dependent on their intact FERM domain (52-54,63).

2.1.2 JAK family members

The analysis of chimeric JAKs described above suggests that it is not the C-terminal segment, but the N-terminal part of JAKs that significantly contributes to the binding specificity of JAKs to the distinct cytokine receptor complexes (51). Moreover, in contrast to many other tyrosine kinases that directly associate to their enzymatic substrates, JAK substrate specificity depends almost entirely on cytokine receptor complexes, which they are associated to, and on downstream signaling proteins recruited by these receptor complexes. Altogether, the specific association to distinct cytokine receptors explains why JAK gene knock-out studies have identified unique signaling defects in mice (**Table 2.1**).

JAK kinase	Size	Chromosomal position	Expression	JAK -/- mice	
				Cytokine affected	Knockout phenotype
TYK2	140 kDa	19p13,2	Ubiquitous	IL-12, modestly IFN-a	Impaired response of macrophages to LPS Modest effect on resistance to viral infections Viable
JAK1	135 kDa	1p31,3	Ubiquitous	LIF, IL-6 yc-dependent cytokines Type I and II IFNs, IL-10	LIF -/- like neurological lesions Impaired lymphopoiesis Perinathal lethality caused by impaired neurogenesis
JAK2	130 kDa	9p23-p24	Ubiquitous	Epo, Tpo IL-3, IL-5, GM-CSF IL-6, type II IFN	Impaired definitive erythropoiesis Epo -/-like phenotype Embryonic lethality caused by failure of erythropoiesis
JAK3	120 kDa	19p13	Myeloid Lymphoid	yc-dependent cytokines IL-2, IL-4, IL-7, IL-9, IL-15	Severe defects in lymphopoiesis (T-B-NK-) Severe combined immunodeficiency (SCID) yc -/- like phenotype Viable

Table 2.1 Properties of JAKs and JAK knockout mice phenotypes*. Adapted from (5,25,51,64).*

JAK1

JAK1 participates in the signal transduction of various cytokine receptor families regulating different cellular processes. JAK1-binding receptors include the common gamma chain (γc) receptor subfamily, where JAK1 associates to the specific cytokine-binding subunit of the receptor complex, usually called α chain, such as IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-21Rα (5,25). JAK1 also mediates signaling by the gp130 subfamily (IL-6R, LIFR, IL-11R) and the class II cytokine receptor complexes, where it associates to IFNAR2 and IFNGR1 chain in the type I and type II IFN receptor complexes, respectively. It also associates to the cytokine-binding subunit of the type III IFN and IL-10-related cytokine receptor complexes (13,65). In line with its pleiotropic character, JAK1-/- mice die after birth due to neuronal defects. This phenotype is probably caused by impaired IL-6, LIF and/or CNTF signaling in neurons (66).

JAK2

JAK2 is implicated in signaling by members of the class I homodimeric receptor family (e.g. EpoR, TpoR, GHR, PrlR, G-CSFR), the receptor family sharing βc (IL-3R, IL-5R and GM-CSFR), the gp130 sharing receptors and the IFN-γ receptor (67,68). Analogous to what has been observed in Epo -/- mice, the JAK2 deficient mice are dying at embryonic day 12 due to the failure of definitive hematopoiesis (68,69). Although, fetal liver cells from JAK2 -/- mice are unresponsive to Epo, Tpo or IL-3, these same cells are able to reconstitute peripheral lymphocytes in irradiated JAK3 -/- mice, indicating that JAK2 plays a critical role in transducing signals for cytokines such as Epo, Tpo, IL-3, GM-CSF and is not required for embryonic development of lymphoid cell lineages (67,68).

JAK3

JAK3 expression is essentially restricted to hematopoietic cells (25,70) and its expression in other tissues is still controversial. JAK3 is expressed at very low levels in immature hematopoietic cells, but its expression is dramatically up-regulated during terminal differentiation of these cells (71). JAK3 selectively associates with common γ chain (γc) and is thereby implicated in signaling by all receptor complexes sharing the common γ receptor subunit for signaling (e.g. IL-2R, IL-4R, IL-7R, IL-9R, IL-15R and IL-21R) (25,26). JAK3 -/- mice are viable but are defective in their response to IL-2, IL-4, IL-7 and IL-15 yielding severe combined immunodeficiency (SCID)-like phenotype (72) due to defects in T, B and NK cell development (25,26,73). This murine phenotype is particularly relevant for human disease, because humans with inactivating mutations in JAK3 have severe combined immunodeficiency (SCID) syndrome, which results from a profound T and NK cell defect (74). Similar immune deficiency appears in patients with mutations in γc (75) or in IL-7Rα (25,26,76). The fact that JAK3 and γc -/- mice have a similar phenotype suggests that JAK3 is not important for signaling through cytokine receptor complexes other than those using common γc chain and therefore plays a key role in γc-dependent lymphoid cells development.

TYK2

TYK2 was identified as a gene restoring the defective IFNα/β signaling in mutated cells (40). Subsequent studies suggested that TYK2 was also activated upon IL-6, IL-10 and IL-12 stimulation (5,26). Surprisingly, TYK2-/- mice show only subtle defects in IL-12 signaling and in their macrophage response to LPS. Moreover, the ability of TYK2-/- mice to respond to IFN-α/β, IL-10, IL-6 and to resist to viral infections is almost comparable with that of wild-type mice (77,78), suggesting that TYK2 might play a redundant role for cytokine receptors.

2.1.3 Genetic abnormalities in Janus kinase family

Loss-of-function JAK3 mutations

Spatial and temporal activation of tyrosine kinases is critical for ‘normal’ activation of their protein substrates in numerous signaling pathways. The activity of these kinases is normally tightly controlled, since prolonged and uncontrolled kinase activity in the wrong place or at the wrong time can have disastrous consequences, often leading to cell transformation and cancer (79).

In contrast to many other cases of dysregulated tyrosine kinases, the first genetic abnormality identified in the JAK protein family was not linked to their aberrant catalytic activation, but were loss-of-function mutations in JAK3 identified in the patients with severe combined immunodeficiency (SCID) syndrome (74). Impaired cytokine signaling plays a major role in the development of immunodeficiency in patients resulting in profound defects in T, NK cells and, to a lower extent, in B cells. Patients with loss-of-function mutations in γc (80) or in IL-7Rα (76) show a very similar phenotype, pointing to the family of cytokines that use JAK3-associating γc as a common receptor subunit for signaling. Mutations located in all domains of JAK3 have been identified up to now, without any hotspot. However, most of these mutations are located in the JH2, pseudokinase domain (26) and mainly affect protein expression. Interestingly, some JAK3 mutations affect different functions such as receptor binding, intracellular traffic or kinase activity (26).

JAK2-involving fusion proteins

The first evidence linking the JAK kinases and leukemogenesis came from studies in *Drosophila melanogaster* describing an activating point mutation as well as overexpression of the *Drosophila's* Janus kinase, Hopscotch, to cause hematopoietic neoplasia (81). This study suggested that aberrant activation of JAKs might be particularly relevant in pathogenesis of hematological malignancies in humans.

Indeed, in 1997 TEL-JAK2, the first aberrantly activated JAK fusion protein, was discovered in T-cell childhood acute lymphoblastic leukemia (T-ALL) patient (82). The t(9,12) chromosomal translocation resulted in a fusion protein between the kinase (JH1) domain of JAK2 and the oligomerization domain of TEL transcription factor, that led to dimerization of the TEL-JAK2 protein and constitutive activation of JAK2 and STAT5 (82). TEL-JAK2 was further described in one B-ALL child and one CML adult patient (83). Subsequently other fusion proteins involving JAK2 fused to PCM1 or BCR were described in atypical CML (84,85). Interestingly, up to date, no fusion protein involving other members of the JAK family were described, suggesting that JAK2 gene might be located in a specific part of the chromosome 9 prone to doublestrand DNA breaks and chromosomal translocation or that the chromosome 9 itself is a genetically unstable area of the genome.

The gain-of-function mutations in JAK2

Besides chromosomal translocation, a gain-of-function mutation is one of the most frequent mechanisms resulting in constitutive kinase activation.

As mentioned above, the first gain-of-function mutations in Janus kinase were found in the *Drosophila* model. Identification of the E695K mutation in the pseudokinase domain was the first evidence pointing to the JH2 domain playing role in activation of JAK kinase (81). Hopscotch carrying the E695K mutation resulted in hyperphosphorylation and constitutive activation of D-STAT, the STAT equivalent in fly, when expressed in Drosophila's cells. Moreover, the homologous mutation in the mouse JAK2 also induced an increased JAK2 phosphorylation and STAT5 activation in COS cells (86). This and another hyperactive Hopscotch mutant (G341E) were shown to cause malignant neoplasia of fly blood cells (87), a phenotype reminiscent of human leukemia.

In 2005, a clonal recurrent point mutation located in the pseudokinase domain of JAK2 leading to substitution of valine for phenylalanine at position 617 (V617F) has been reported in patients with myeloproliferative neoplasms (MPNs) (88-91). This mutation leads to constitutive tyrosine phosphorylation of JAK2, STAT5 and cytokine independent proliferation of the IL-3 dependent BaF3 cell line (88). Retroviral expression of JAK2 V617F in mouse hematopoietic cells resulted in a myeloproliferative disorder mimicking the phenotype described in MPNs patients (92). Since the initial discovery, the JAK2 V617F mutation has been described in 95% of polycythemia vera (PV) patients and 50-60% of patients with essential thrombocythemia (ET) and myelofibrosis (MF) (93).

The JAK2 V617F is the most prevalent JAK2 mutation in patients with myeloproliferative neoplasms (MPNs), but there are other mutations in JAK2 reported in JAK2 V617F negative PV patients (such as mutations in exon 12) and in patients with other hematological malignancies, especially acute leukemia (93). However, these other JAK2 mutations appear to have a very low patient incidence.

Mechanism of JAK2 V617F-mediated constitutive signaling

In contrast to fusion proteins involving tyrosine kinases such as TEL-JAK2 or BCR-ABL1, the JAK2 V617F is a weak gain-of-function mutation (94). Higher level of wild-type JAK2 expression can prevent constitutive JAK2 V617F mediated signaling (88), suggesting a competition for a limiting amount of JAK2-binding receptors. Indeed, the JAK2 mutant retains its ability to interact with cytokine receptors (95), and an intact FERM domain, which

mediates recruitment to cytokine receptors, is required for inducing transformation of hematopoietic cells (96).

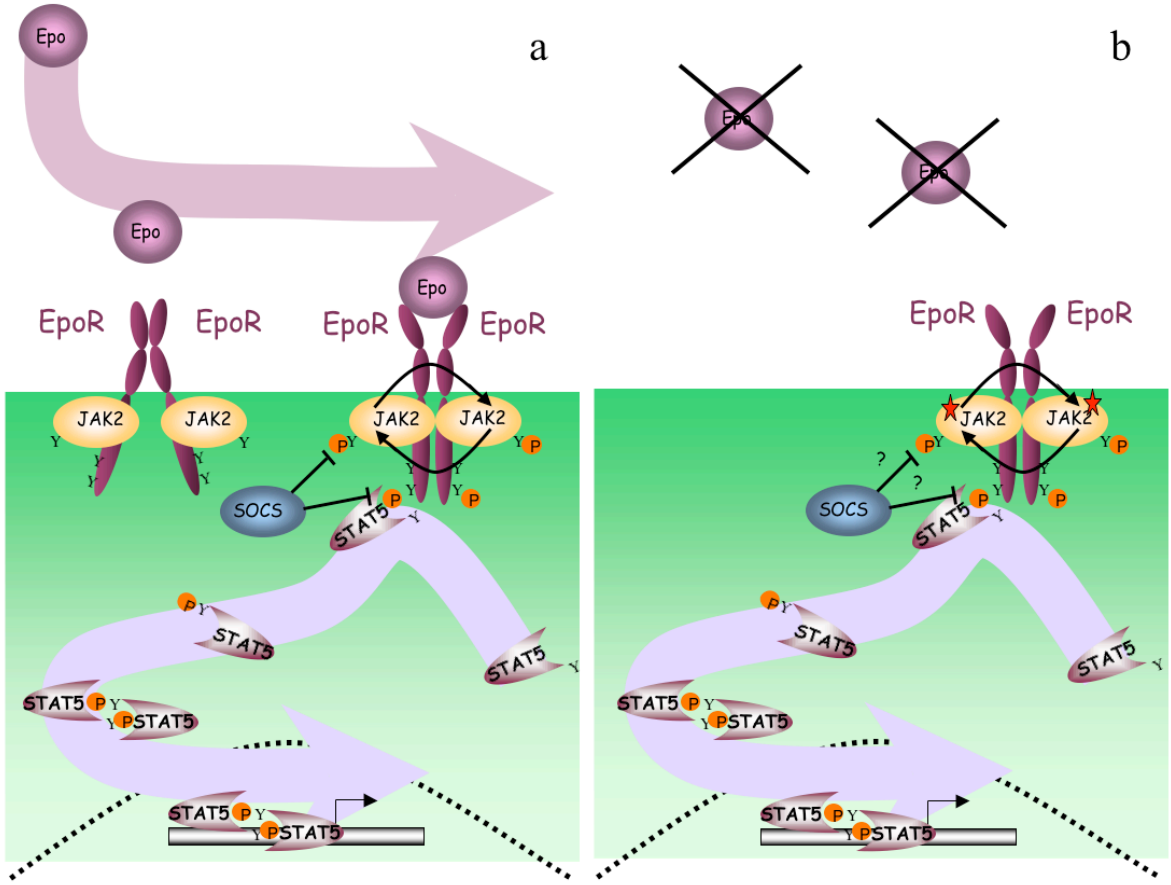

Figure 2.5 Schematic representation of the currently accepted mechanism of ligand-induced EpoR signaling and JAK V617F-mediated constitutive signaling of EpoR*. (a) In the absence of ligand, EpoR complex exists as a pre-associated homodimer with two JAK2 proteins associated to the cytoplasmic region of receptor chains. Epo binding induces conformation change allowing for cross-activation of wild-type JAK2 proteins. Subsequently receptor tyrosines are phosphorylated and recruit downstream signaling molecules such as STAT5. (b) In the absence of ligand, the JAK2 V617F associates to pre-formed EpoR dimers that provide a suitable scaffold for JAK2 V617F activation and by this way promote constitutive signaling by JAK2 V617F. Moreover, JAK2 V617F might be able to escape to the normal negative regulation mechanisms such as downregulation of phosphorylation by SOCS proteins.*

To allow for constitutive signaling, JAK2 V617F needs to associate to class I homodimeric cytokine receptors such as EpoR or TpoR. Association with these receptors can explain the lineage specific effect of the JAK2 mutant as well as the mechanism of JAK V617F-mediated constitutive signaling. Based on the currently accepted model, in the absence of ligand, the JAK2 V617F mutant associates to pre-formed EpoR or TpoR dimers, that provide a suitable scaffold for JAK2 V617F activation and in this way promote signaling (97,98) (**Figure 2.5**). Moreover, under physiological conditions, suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of Epo-induced signaling through interaction with both the EpoR and JAK2. In contrast to wild-type JAK2, SOCS3 failed to regulate JAK2 V617F so that its activation is actually potentiated in the presence of SOCS3 (99). This mechanism however

stays controversial since other group showed that JAK2 V617F does not overcome normal SOCS regulation (100).

The gain-of-function mutations in other JAKs

The presence of the JAK2 V617F mutation in MPNs has emphasized the oncogenic role of the JAK family and started an era of screening for the presence of mutations in JAKs in hematological malignancies.

It has been shown, that a homologous V617F mutation in JAK1 (V658F) and TYK2 (V678F), but not in JAK3, are also gain-of-function mutations (101). However, at the initiation of this thesis, no JAK1 or TYK2 mutations had been reported in human patients.

Considering the previous history of various loss-of-function mutations in JAK3 reported in patients with SCID-like syndromes, the recent discovery of activating point mutations in JAK3 in acute megakaryoblastic leukemia has been somehow surprising. All three JAK3 mutants, namely the A572A, V722I and P132T, exhibit constitutive JAK3 and STAT5 phosphorylation, when expressed in BaF3 cells (102).

2.2 Signal transducers and activators of transcription (STATs)

2.2.1 Structure and mechanisms of STAT activation

Signal transducer and activator of transcription (STAT) proteins were discovered as components of IFN-mediated gene transcription in the early 1990. Since then, seven members of the STAT family have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STAT proteins identified in mammals range in size from 750 and 850 amino acids and share 6 structurally and functionally conserved domains (**Figure 2.6**).

Figure 2.6 Schematic presentation of a STAT protein. *STAT proteins contain an N-terminal domain that is responsible for stabilizing interactions and cooperation between STAT dimers. The coiled-coil domain mediates interaction with other proteins on target promoters and is followed by a DNA-binding domain and the linker domain that are crucial for activation of transcription. Close to the C-terminal end, STAT proteins contain an SH2 domain that is the most highly conserved domain among STAT proteins. Through its capacity to bind to specific phosphotyrosine motifs, it plays a crucial role in the recruitment to the cytokine receptor and in the formation of STAT dimers. A transactivation domain (TAD) is the less conserved domain among STAT members and shows an autonomous transcriptional activity. All STATs are activated by tyrosine phosphorylation, however some can be also phosphorylated on serine residues in the TAD domain (103).*

STATs play an indispensable role in class I and class II cytokine receptor's signaling. Ligand binding to its receptor alpha subunit is the first step in the formation of a signaling-competent receptor complex, followed by subsequent receptor oligomerization. Class I and II cytokine receptors are devoid of intrinsic tyrosine kinase activity, therefore receptor oligomerization initiates the process of signaling by activation of receptor-associated Janus family tyrosine kinases (JAKs) through their phosphorylation in trans. Immediate targets of activated JAKs are the tyrosines in the cytoplasmic portion of the receptors. Phosphorylated tyrosines then serve as docking sites for SH2 domain-containing proteins such as STATs. In unstimulated cells, STATs are localized in the cytoplasm and they are recruited to the phosphotyrosine of the cytokine receptors. The recruitment of specific STAT isoforms is primarily dictated by the amino acid sequence surrounding the phosphorylated tyrosine in the cytokine receptor such as YXXQ for STAT3 for example. Also some cytokine receptors such as the IL-22 receptor can recruit STAT3 in a tyrosine-independent way (104). Once recruited to the receptors STATs are phosphorylated by JAKs on a single tyrosine residue in the C-terminal portion and form homo- and/or heterodimers through reciprocal interaction between the phosphotyrosine of one STAT and SH2 domain of another protein. Dimerized STATs translocate to the nucleus via

importins to induce target gene transcription by binding to specific regulatory elements (5,64,103) (**Figure 2.7**).

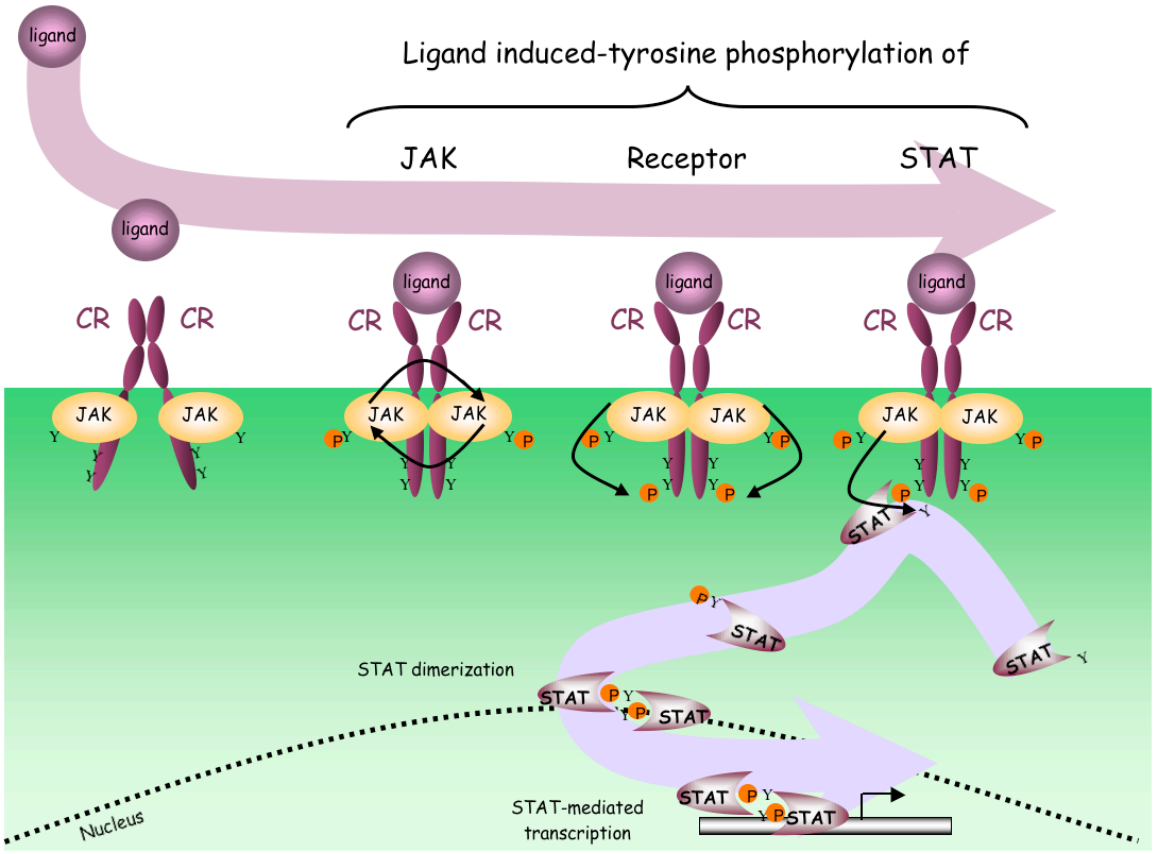

Figure 2.7 The JAK/STAT signaling pathway*. Sequential tyrosine phosphorylations triggered by cytokine-receptor interaction. Receptor dimerization allows transphosphorylation and activation of Janus kinases (JAKs). This is followed by phosphorylation of receptor tyrosine residues and by the recruitment of the signal transducers and activators of transcription (STAT) proteins through their SRC-homology-2 (SH2) domains. STAT tyrosine phosphorylation then occurs. Dimerization of activated (tyrosine phosphorylated) STATs is followed by their accumulation in the nucleus to drive gene transcription.*

STAT dimerization is a prerequisite for DNA binding. The preferential binding affinity of the distinct cytokine-activated STATs to the natural site of genes depends on the composition of the STAT dimeric complex. STAT1, 3, 4, 5 and 6 homodimerize. STAT1 and 2, and STAT1 and 3 can form heterodimers, and several STATs can form tetramers (105). All STATs recognize a consensus oligonucleotide sequence termed a GAS (γ activated site) element ($TT(N)_5AA$) via the DNA binding domain (DBD). Only STAT6, which binds optimally to $TT(N)_6AA$ sequences, and the complex formed of STAT1, STAT2 and IRF-9 after IFN-α/β stimulation, that binds the IFN-α/β-stimulated response element (ISRE, $AGTT(N)_3TTTC$), prefer different optimal binding sites (105). As a consequence, despite the limited number of STATs to mediate signal transduction of over 50 cytokine receptor complexes a significant specificity is achieved by activating specific STATs: stimulation by IFNα/β is characterized

by the formation of STAT1/STAT2/IRF-9 complex, IL4 and IL-13 stimulates STAT6 and the IL-12 family activates STAT4. Moreover, as in case of STAT1, 3 and 5 that are activated by many different cytokines, the specificity of the signal is achieved also by activation of other signaling pathways such as ERK/MAPK and PI3K/AKT pathway in conjunction with STATs (5). Finally, serine phosphorylation within the TAD domain, that is required for full transcriptional potency of STAT1, 3, 4 and STAT5, provides a mechanism to modulate intensity of the cytokine-induced STAT activation (64).

STATs are the major substrates of cytokine receptor-associated JAK tyrosine kinases, but can be activated through other mechanisms. Receptors with intrinsic tyrosine kinase activity (RTKs) including PDGFR, EGFR, RET and FLT3 can activate STATs without involvement of JAKs, either directly or indirectly through receptor-associated SRC family of kinases (64). However, activation of STATs by the RTKs seems to be restricted to specific types of cells. Up to date, no gain-of-function mutations in STAT genes have been reported, suggesting that constitutive activation of STAT is a secondary event in most cancer cases. On the other hand, loss-of-function mutations in the STAT3 gene have been described in most of the patients with type I HIES, hyper-IgE syndrome (106,107). These mutations in DBD, SH2 and TAD domain of STAT3 were shown to be responsible for defects in TH17 development and impaired IL-22 signaling, two features associated with pathology of this primary immunodeficiency syndrome (108).

STATs	Chromosomal position	STAT -/- mice	
		Cytokine affected	Knockout phenotype
STAT1	2q32.2	Type I and II IFNs	Defective IFN-dependent immune responses High susceptibility to bacterial/viral infestions Increased susceptibility to tumors
STAT2	12q13.3	Type I IFN (and type III IFNs)	Defective type I IFN-dependent immune responses Impaired lymphopoiesis Perinathal lethality caused by impaired neurogenesis
STAT3	17q21.2	yc-dependent cytokines IL-2, IL-7, IL-9, IL-15, IL-21 IL-6, IL-11, OSM, LIF, IL-10 EGF, G-CSF, Tpo, GH	Impaired T-cell proliferation in response to IL-6 and IL-2 Impaired IL-10-mediated anti-inflamatory responses in macrophages Defective wound healing in skin Embryonic lethality
STAT4	2q32.2	IL-12	Impaired TH1 differentiation
STAT5	17q21.2	IL-2, IL-7, IL-9, IL-15 IL-3, IL-5, GM-CSF G-CSF, Epo, Tpo, GH, Prl	Infertility in females Fetal anemia Reduced number of NK cells Impaired IL-2-induced T cell proliferation
STAT6	12q13.3	IL-4, IL-13	Impaired TH2 differentiation Defective IgE class switch

Table 2.2 Properties of the human STATs and the STAT knockout mice phenotypes*. Adapted from (64,109).*

The contributions of individual STAT proteins to normal cytokine signaling and development have been studied in various cell culture systems. The best models illustrating the physiological role of STAT proteins *in vivo* are STAT knock-out mice (**Table 2.2**).

2.2.2 STAT proteins and oncogenesis

Beyond various roles of STAT proteins in normal cellular and physiological processes (**Table 2.2**), these proteins are also known to participate in cellular transformation and oncogenesis. Particularly, constitutive activation of STAT1, STAT3 and STAT5 has been demonstrated in a number of malignant cell lines and human cancers (109-112). Current data from primary cancers and tumor-derived cell lines, shows that persistent STAT3 activation is frequently associated with a transformed phenotype in solid tumors, whereas STAT5 constitutive activation is commonly found in leukemias and lymphomas (110). The primary causes of such sustained STAT activation are quite variable. However, the most common routes to persistent activation of STATs in cancers include: (i) overexpression of ligand or/and growth factor receptor and (ii) dysregulation of tyrosine kinase activity (**Figure 2.9**).

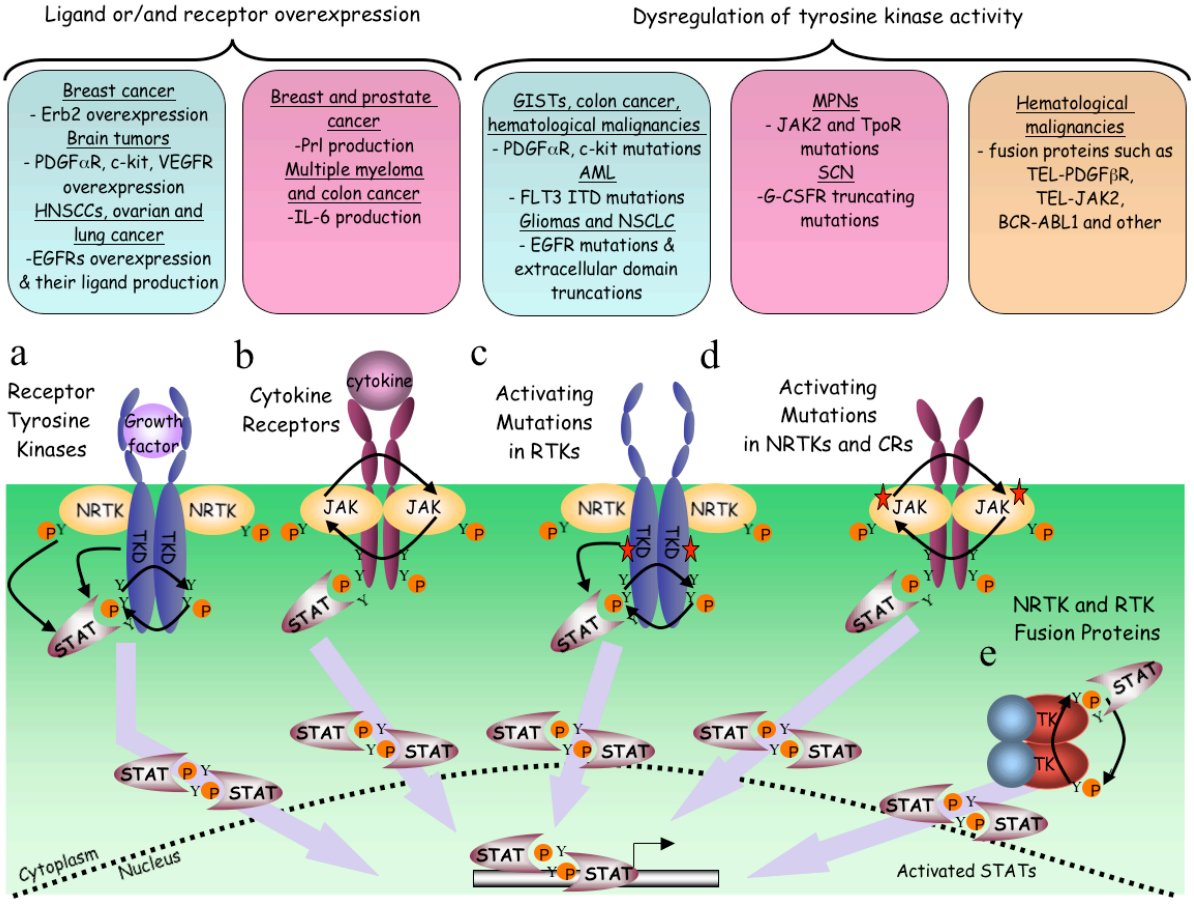

***Figure 2.9 Various routes to persistent STAT activation in cancer**. The primary causes of the sustained STAT activation are potentially quite variable. However, the most common routes to persistent activation of STATs in cancers include: (a, b) overexpression of ligand, or/and growth factor receptor and (c, d, e) dysregulation of tyrosine kinase activity. Some examples are depicted based on the references listed in the text above and below the figure.*

Autocrine or paracrine growth factor production was described in various solid tumors. The overexpression of several EGF receptors and production of their cognate ligands has been reported as a bad prognostic factor in ovarian cancer (113). In head and neck cancers, TGFα and EGFR are overexpressed resulting in constitutive STAT3 activation (114). Prolactin expression mediates persistent activation of JAK2 and STAT5 in breast and prostate cancers (115). In multiple myeloma, constitutive activation of STAT3 is at least in part mediated by IL-6 and by overexpression of its receptor (116). The basis of RTKs overexpression is not understood, but may involve the loss of internalization and turnover that normally follows ligand-binding. Increased expression of PDGFαR, c-kit and VEGFR in brain tumors (117) and Erb2/HER2 receptor in breast cancer is thought to be responsible for STATs activation and to be a critical step in the formation of these cancers (115) (**Figure 2.9a, b**). Moreover, non-receptor tyrosine kinases such as JAK or SRC, which associate with growth factor receptors, may also accumulate to abnormal levels and thus promote STAT hyperactivation in malignant cells (118).

Excessive tyrosine kinase activity in human malignancies is perhaps the most common mechanisms for constitutive phosphorylation and activation of STAT transcription factors. Numerous gain-of-kinase function mutations, truncations and fusion proteins involving receptor (RTKs) or non-receptor tyrosine kinases (NRTKs) have been described as a primary cause of constitutive STATs activation in human cancers. Truncations of the extracellular part of receptor and mutations in the tyrosine kinase domain of EGFR resulting in constitutive activation of STAT3 were identified in patients with glioblastoma and non-small cells lung carcinoma (119,120). Activating mutations of PDGFαR and c-kit are in part responsible for constitutive STAT1 and STAT3 activation in gastro-intestinal cancers (GISTs) (121). FLT3 receptor tyrosine kinase mutations in the activation loop or involving internal tandem duplications (ITDs) are present in approximately 30% of AML patients and are crucial STAT5-activating genetic events in these patients (122) (**Figure 2.9c**). Frequently found in hematological malignancies, the TEL-PDGFβR, TEL-JAK2 and BCR-ABL1 are results of specific genomic translocations producing a STAT5-activating fusion proteins with a tyrosine kinase domain of non-RTKs or RTKs and a dimerization-mediating part of the other protein partner (111) (**Figure 2.9e**).

Approximately 40% of patients with severe congenital neutropenia (SCN) carry mutations in the gene enconding the G-CSF receptor that truncate part of the cytoplasmic domain of the receptor (123). Acquisition of these mutations is associated with constitutively active STAT5

and the development of AML and myelodysplastic syndrome (MDSs) (124). Lastly, as already mentioned, a single somatic activating mutation V617F in the JAK2 tyrosine kinase identified in 95% of polycythemia vera (PV) patients and 50% of essential thrombocythemia (ET) patients, and has been associated with constitutive STAT5 and STAT3 activation (88). Moreover, some JAK2 V617F-negative patients carry an activating mutation in thrombopoietin receptor (TpoR) mimicking STAT activation-mediated phenotype of JAK2 V617F-positive patients (125) (**Figure 2.9d**). In contrast to leukemic fusion proteins such as BCR-ABL1 or TEL-JAK2 that have been shown to activate STATs directly without the need for receptor activation or SRC/JAK as mediators, the JAK2 V617F mutant needs association with class I homodimeric cytokine receptors such as EpoR or TpoR as a scaffold to activate STAT proteins constitutively (96).

2.2.3 Negative regulation of STAT signaling

Since STAT activation is included in many biological processes such as anti-microbial defense, hematopoiesis, differentiation and proliferation, it is therefore not surprising that numerous regulatory layers exist to modulate such signaling pathway.

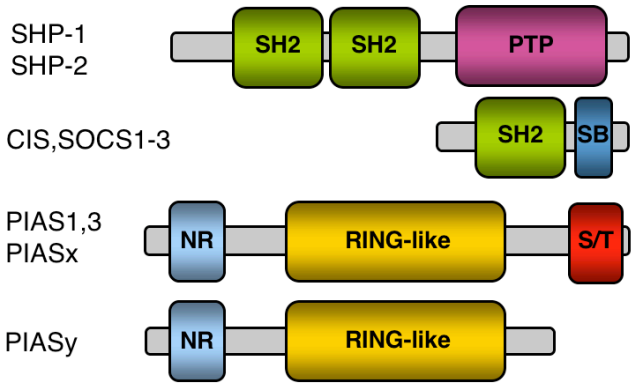

Figure 2.11 Schematic representation of proteins that regulate the JAK/STAT signaling pathway. *(Abbreviations: PTP, protein-tyrosine phosphatase domain; SB, SOCS Box; NR, LXXL nuclear receptor interaction motif; RING-like, zinc binding domain; S/T, serine/threonine rich region.)*

Tyrosine phosphatases

There are several types of negative regulator of STAT proteins in the cell cytoplasm. Tyrosine dephosphorylation of receptors or associated kinases by SHP1 and SHP2 have been found to limit further STAT tyrosine phosphorylation (126). Mutations in the phosphatase recruitment site of cytokine receptors prolong cytokine-induced activation of JAK/STAT pathway. Such a mutation occurs in EpoR in humans and results in familial erythrocytosis (127). Recently, T cell specific phosphatase (TC-PTP) has been identified as a specific phosphatase regulating γc-dependent cytokine-induced JAK1 and JAK3 activation (128). These and many other

observations confirm the importance of phosphatases in the regulation of JAK/STAT pathway.

SOCS

The SOCS family comprises a group of at least eight cytokine-inducible SRC homology 2 (SH2)-domain containing proteins (CIS), but only four members, CIS, SOCS1, SOCS2 and SOCS3 have been studied more extensively (126). SOCS proteins inhibit the catalytic activity function of JAK kinases by competing with substrates either by binding to JAKs directly or indirectly. The SH2 domain of SOCS1 recognizes and binds the phosphorylated tyrosine residue located in the activation loop of the JAK kinase domain and inhibits its catalytic activity by preventing the access of JAK substrates (129,130). In contrast to SOCS1, the SH2 domain of SOCS3 binds preferentially to phosphorylated tyrosine of receptors rather than to JAKs (130,131). The SH2 domain of CIS associates with STAT5-binding sites of various types of receptor and inhibits the activation of STAT5 competitively by masking the STAT5-binding sites on cytokine receptors. Notably, proteins of the SOCS family can also mark their targets for degradation by the ubiquitin pathway (130,132).

SOCSs, as well as other negative regulators of STAT signaling, do not only play important role in physiological processes but might also control tumor incidence and progression. Indeed, SOCS1 expression is frequently impaired in hepatocellular carcinomas (133), suggesting that similar genetic or epigenetic alterations of SOCS genes participate on carcinogenesis of other cancers as well.

PIAS

All above-mentioned negative regulators function in the cytoplasm. PIAS or “Proteins that Inhibit Activated STATs” are negative nuclear regulators that block DNA binding of activated STAT in nucleus. PIAS is a family of conserved proteins including PIAS1, PIAS3, PIASy and PIASx that inhibit STAT1, STAT3, STAT1 and STAT4-mediated transcriptional activation respectively (126). PIAS proteins regulate transcription through several mechanisms, including blocking the DNA-binding activity of transcription factors, recruiting transcriptional co-repressors or co-activators, and promoting protein sumoylation (132). PIAS1 and PIAS3 bind STATs to inhibit association with DNA, whereas PIASx and PIASy inhibit transcription by a still unknown mechanism. A critical role of PIAS3 in IL-6-induced STAT3 activation has been demonstrated in multiple myeloma cells (134).

3. Acute lymphoblastic leukemia

3.1 Definition of hematopoiesis

Hematopoiesis corresponds to the process of blood cells formation, starting from pluripotent hematopoietic stem cells (HSC). This process takes place essentially in the bone marrow where complex signals from the microenvironment drive HSC asymmetric division to form other stem cells and early progenitors. These progenitors produce all hematopoietic cells found in blood through many steps of differentiation and proliferation.

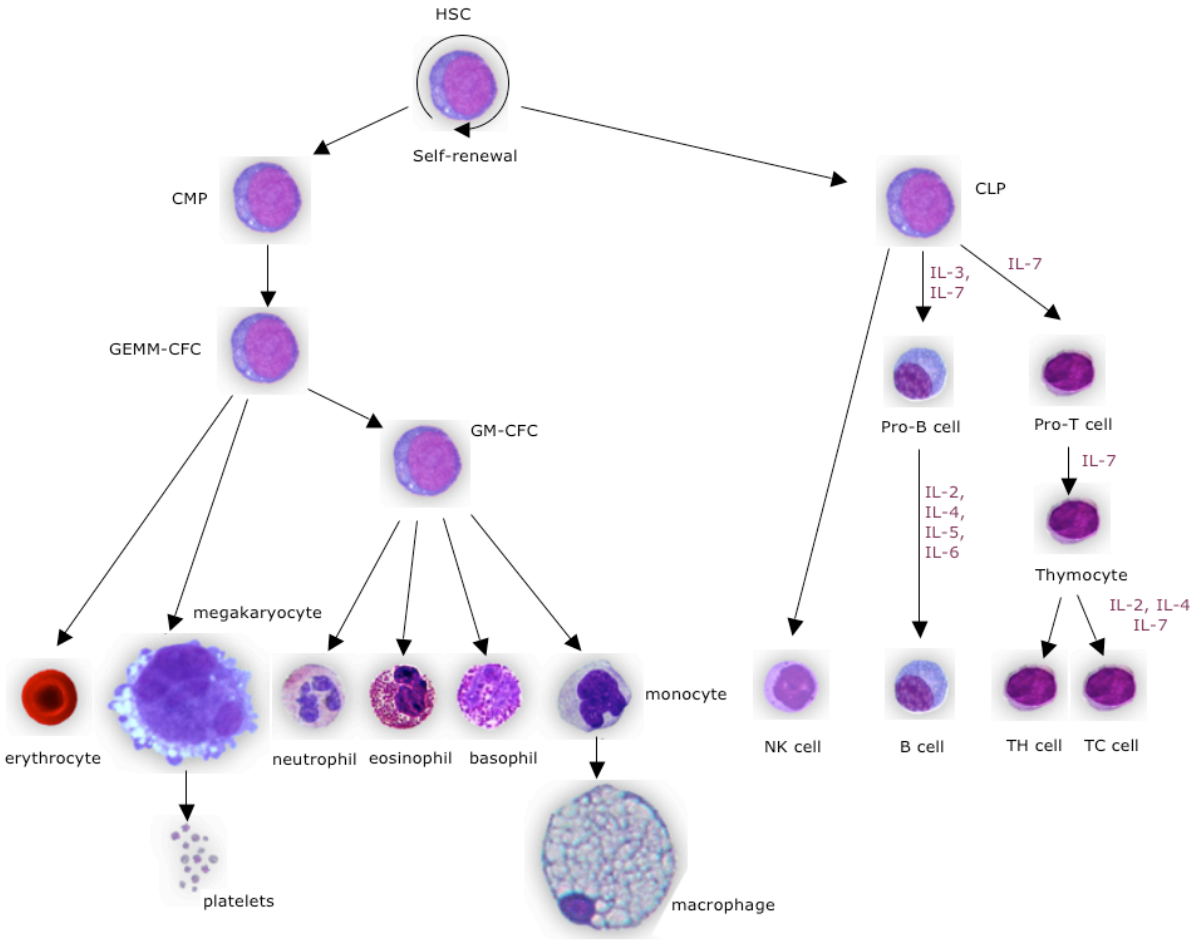

Figure 3.1 Schematic representation of normal hematopoiesis*. The different steps of blood cell development are shown, initiated by the hematopoietic stem cell (HSC) and ending with differentiation of mature cells. Proliferation, survival and differentiation of cells at each step of their development are controlled by set of complex signaling provided by cytokines and other growth factors. The cytokines are shown at the steps of lymphocyte development they support/promote. Adapted from (135,136).*

The commitment of a particular progenitor towards the myeloid (common myeloid progenitor - CMP) or lymphoid (common lymphoid progenitor - CLP) lineage depends on signals that are very poorly understood. Cells committed to the myeloid lineage differentiate into red blood cells, monocytes/macrophages, neutrophils, eosinophils, basophils/mast cells and

megakaryocytes/platelets. Cells committed to the lymphoid lineage differentiate into NK cells, B- and T-lymphocytes (**Figure 3.1**).

3.2 Definition of acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is an aggressive neoplastic disorder that originates in a single lymphoid progenitor committed to the B- or T-cell lineage. Following somatic genetic modifications, the progenitor acquires the capacity to proliferate independently of any growth signals and to escape apoptosis (137). This leads to uncontrolled proliferation and accumulation of leukemic cells (lymphoblasts) in the bone marrow, blood and other tissues resulting in the suppression of normal hematopoiesis (**Figure 3.1**).

Acute lymphoblastic leukemias account for 11% of all leukemia cases. ALL is the most common childhood malignancy. Recent decades have witnessed tremendous advances in treatment of the disease in children resulting in a cure rate of childhood ALL of 70-80%. Despite improvements in the treatment of childhood ALL, relapsed ALL makes a major contribution to the morbidity and mortality of childhood cancer (138). In contrast to pediatric patients, the majority of adult ALL patients are not cured. Current treatment involving a scheduled sequence of multiagent, multicycle chemotherapies provides an overall survival between 25 and 40% of adults patients (138). The T-ALL is a heterogeneous group of diseases comprising several clinico-biological entities and several classifications for T-ALL have been proposed.

3.3 T-ALL

3.3.1 Definition of T-ALL

Similar to other types of leukemia, T-ALL is caused by genetic alterations in thymocytes leading to a variety of changes, including loss of cell cycle arrest, unlimited self-renewal capacity, impaired differentiation, hyperproliferation and clonal expansion of maturating T cells. T-ALL accounts for approximately 15% of pediatric and 25% of adult ALL cases (139). T-ALL is fatal for 30% of pediatric and the majority of adult T-ALL patients (138). An improvement in these outcomes by refining schedules or escalating doses of existing chemotherapeutic agents is highly limited by toxicity. Conventional drugs used in T-ALL target DNA directly (anthracyclines, cyclophosphamide) or inhibit nucleic acid synthesis (cytarabine, methotrexate), some block protein synthesis by hydrolyzing an amino acid essential for leukemic cell growth (asparaginase) or by interfering with the mitotic apparatus

(vincristine) (140). These are all nonspecific drugs that often produce adverse cytotoxic effects in various normal tissues. Therefore, novel targeted therapies are urgently required in T-ALL to improve cure rates and reduce short- and long-term toxicities.

To identify targets of new therapies, a thorough understanding of the genetic lesions contributing to leukemogenesis and to resistance to therapy is required. Several microarray-based techniques for the genome-wide analysis of DNA copy number alteration or loss-of-heterozygosity (LOH) together with genome-wide sequencing have become standard tools in the study of cancer genomics (141). Recent studies of global gene expression in T-ALL have not only enabled the classification into prognostically and therapeutically important subgroups (**Figure 3.2**), but they have also led to the identification of novel genes whose expressions are altered in T-ALL and which can be used for targeted therapies (140).

3.3.2 Classification based on immunophenotype

T-ALL form a heterogeneous group of diseases comprising several clinico-biological entities. Several classifications for T-ALL have been proposed. The maturation of the bone marrow-derived T-cell precursors (pro-T-cell) through different stages can be recognized by sequential expression of T-cell receptor gene transcripts but also by cell surface antigens (**Figure 3.2**). Since the different T-ALL subtypes correspond to the differentiation arrest of lymphoblasts at specific stages of the T-cell development, ALL can be immunophenotypically classified according to the degree of T-cell maturation. The antigen-expression profiles of leukemic lymphoblasts parallel the normal stage of T-cell differentiation and maturation as well. According to the degree of T-cell maturation starting with CLP (common lymphoid progenitors), pro-T cells (committed T cells), double CD4 and CD8 negative pre-T cells, double positive, early and ending with late cortical T cells and mature single positive T cells, several ALL subgroups can be recognized (140) (**Figure 3.2**).

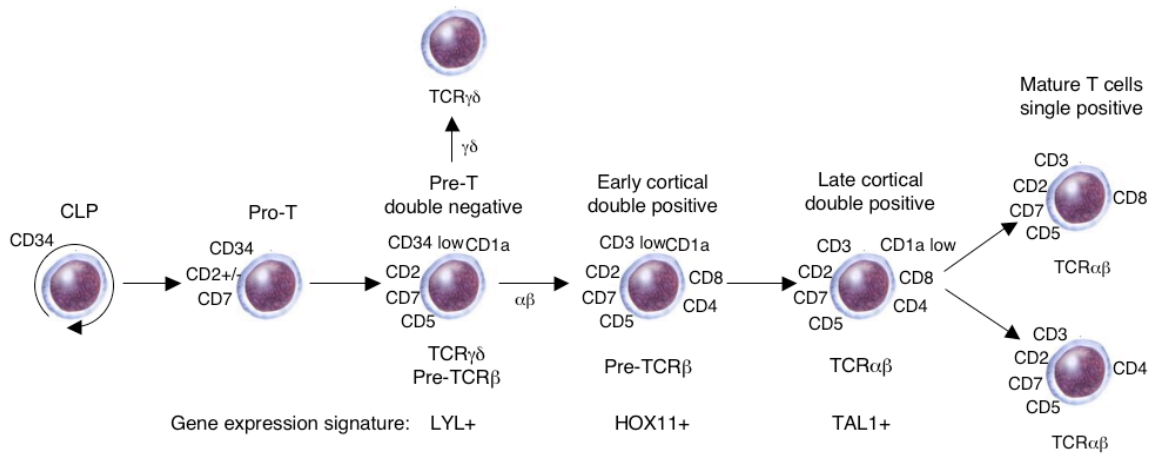

Figure 3.2 Schematic stages of T-cell development and their association to different T-ALL subtypes. *When common lymphoid progenitors (CLP) enter into thymus, they are still able to differentiate into B, T, NK or dendritic cells. The immature subgroup or pro-T-ALL corresponds to lymphoblast derived from committed pro-T-cells that express CD34 and CD7 (and maybe CD2) with all TCR genes in germline configuration The commitment to the T-cell lineage is followed by downregulation of CD34, upregulation of CD1a and CD5 but not CD4 or CD8. These cells also undergo rearrangement of genes coding for TCRD and TCRG. Therefore the pre-T-ALL subgroup corresponds to lymphoblast derived from the early committed pre-T-cells that are double negative for CD4 and CD8. As these cells mature, they begin to express CD4 and CD8, and thereby become double-positive T-cells that are characterized by CD7, CD2 and CD5 on top of CD4 and CD8 expression, with rearrangement of genes coding for TCRB that allows for formation of the pre-TCR complex with low levels of CD3. The differentiation arrest of lymphoblasts at this stage of maturation is specific for early cortical T-ALL. Productive rearrangement of TCRA results in the production of TCRα molecules, which combine with TCRβ proteins to form TCRαβ, which in turn combines with CD3 and moves to the surface of double-positive T cells. The differentiation arrest of lymphoblasts at this stage of maturation is specific for late cortical T-ALL. Signaling from the mature TCRαβ mediates further selection procedures leading to a repertoire of non-self reactive, MHC-restricted, Ag-specific mature T-cells (positive and negative selection). Only about 2% of all double-positive T-cells survive and proceed though a final step of differentiation into either CD4+ and MHC class II restricted or CD8+ and MHC I restricted T-cells. Adapted from (140,142).*

3.3.3 Genetic alterations in T-ALL development

Altogether, the genetic and functional data from in vivo and in vitro analysis of T-ALL transformation suggest that a stepwise alteration of at least four specific pathways is required for thymocytes to become fully malignant (139) (**Figure 3.3**). These pathways can be associated with different classes of genetic alterations: (i) alterations that impair differentiation; (ii) alterations providing self-renewal capacity; (iii) alterations that affect cell cycle; (iv) alterations that provide proliferative and survival advantages. Each of these pathways represents a potential target for therapeutic intervention (**Figure 3.4**). The genetic alterations within the same group are mutually exclusive, while alterations between different groups are additive.

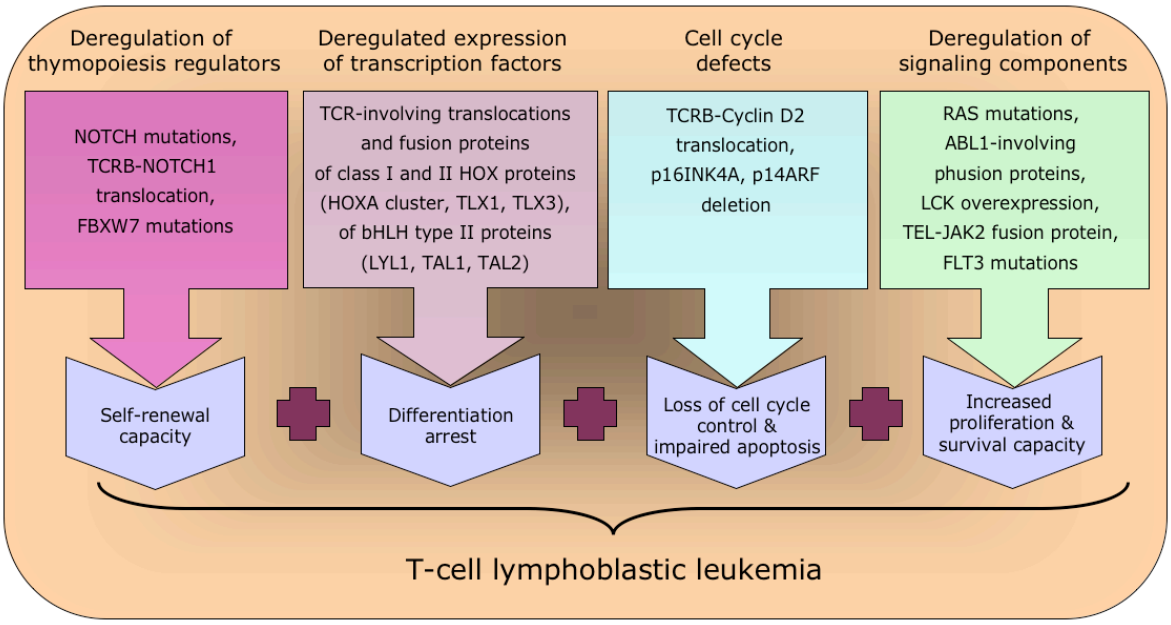

Figure 3.3 Multiple-step T-cell leukemogenesis *depicted based on elucidations from (137,139,140,143). Multiple hits cooperating in the generation of T-ALL affect signaling pathways involved in regulation of self-renewal process, T-cell differentiation and maturation, control of cell cycle and proliferation. Genetic alterations within the same group are mutually exclusive, while alterations between different groups are additive.*

Alterations providing self-renewal capacity

Unlimited self-renewal potential is one of the hallmarks of all cancers (144). The NOTCH signaling has been shown to be an important regulator of hematopoietic stem cell maintenance (self-renewal) and plays a critical role in T-cell lineage commitment of pluripotent progenitors and the subsequent assembly of the pre-T-cell receptor in immature thymocyte development (145,146). Activating NOTCH1 mutations resulting in constitutive NOTCH signaling have been identified in more then 50% of T-ALL samples (147). In addition, heterozygous missense mutations in the FBXW7 gene coding for a protein mediating NOTCH1 degradation have been identified in 15% of T-ALL (148). NOTCH1 mutations were identified in all major molecular subtypes of T-ALL consistent with the notion that these mutations are relatively early events in T-cell leukemogenesis, occurring in immature T-lineage cells (pro-T cells) or uncommitted pluripotent marrow progenitors (CSP) (147,149).

Impaired differentiation caused by transcription factor defects

Key steps of lymphocyte development are controlled by several transcriptional regulators. T-ALL is mainly associated with the deregulated expression of normal, non-chimeric transcription factor proteins. This deregulation often results from chromosomal rearrangements juxtaposing promoter and enhancer elements of T-cell receptor genes (TCRA,

TCRB, TCRG, TCRD) to a small number of developmentally important transcription factors. Among these, the class A (such as E2A protein) and class B (such as LYL1, TAL1, TAL2) proteins of basic helix-loop-helix (bHLH) family and the class I, and class II (TLX1, TLX3 also known as HOX11, HOX11L2) homeobox (HOX) proteins are major players in T-cell ontogeny as well as in T-cell leukemogenesis. Aberrant expression of transcription factors involving translocation of TCR loci is found in about 35% of T-ALL and blocks the differentiation at various stages of T-cell maturation. Deregulation of critical transcription factors alters the gene expression programs that regulate thymic differentiation. Therefore distinct gene-expression signatures corresponding to aberrant activation of specific transcription factors are associated with leukemic cells with recognized stages of thymocyte differentiation. For example, T-ALL cells expressing the oncogene LYL (LYL1+ T-ALL) have a signature corresponding to more immature thymocytes, HOX11+ (also known as TLX1+) T-ALL cells correspond to early cortical and TAL1+ T-ALL cells to late cortical thymocytes (**Figure 3.2**). These aberrations are important as events initiating the leukemogenesis, but the fact that they are often detectable years before the onset of leukemia suggest that other cooperating genetic or epigenetic lesions are required to full transformation of the T-cell progenitor (139).

Cell cycle defects

Cell cycle progression is tightly regulated at various checkpoints in order to maintain genomic integrity of the cell and to obtain an adequate balance between proliferation and differentiation. Inactivation of CDKN2A and CDKN2B, 2 genes located in close proximity on chromosome region 9p21, is the most common genetic defect in T-ALL (150,151). The CDNK2A gene encodes both p16 and p14, both potent inhibitors of cell cycle, although using different mechanisms: p16 inhibits RB1 phosphorylations whereas p14 activates TP53 (152). Inactivation of CDKN2A and CDKN2B by homozygous deletion has been described to be present in 65% and 23% of T-ALL samples, respectively (151).

Alterations that provide proliferative and survival advantages

Signals generated upon activation of the mature T-cell receptor (TCR$\alpha\beta$)/CD3 complex by an MHC bound antigen are critical for T-cell survival and proliferation. As the TCR is devoid of intrinsic kinase activity, it recruits multiple tyrosine kinases such as LCK, FYN, ZAP70 and ABL1 that play a central role in initiation of signaling cascades from Ag-stimulated TCR. In T-ALL, a number of genes encoding tyrosine kinases are activated either by gene fusion, as a

consequence of chromosomal rearrangement or by mutations. Besides the rare cases of LCK overexpression, several variants of the ABL1-fusion proteins such as BCR-ABL1, TEL-ABL1, EML1-ABL1 and NUP214-ABL1 were identified in T-ALL patients (137,153,154).
The Ag-binding to TCR leads to activation of multiple survival signaling pathways including the PI3K and the RAS-MAPK pathway. These and other signaling cascades regulate transcription of various genes encoding growth factors such as IL-2, which support clonal expansion of Ag-reactive T-cells. Deregulation of these pathways is common in T-ALL cells and provides uncontrolled proliferation signals. Mutations in RAS genes have been described in various malignancies. Activating mutations in N-RAS have been detected in 4-10% of pediatric T-ALL (155,156), while other studies indicate that RAS is highly activated in 50% of T-ALL suggesting a key role for RAS in T-ALL pathogenesis (157). Activation of PI3K, another key component of the TCR signaling pathway, is opposed by PTEN phosphatase. Consequently, inactivating mutations of PTEN results in the uncontrolled activation of the AKT pathway and proliferation in T cells (158).

Genetic defect	Target	Potential therapy	Rationale
Self-renewal capacity			
NOTCH1 mutations FBXW7 mutations	NOTCH1 hyperactivation	γ-secretase inhibitors	Inhibition of γ-secretase cleavage
Transcription factor defects			
TAL1 translocation	TAL1 overexpression	HDAC inhibitor	Gene silencing caused by overexpression of TAL1 is opposed by inhibition of HDAC activity
Cell cycle defects			
CDKN2A/CDKN2B deletion	CDKN2A/B inactivation	CDK inhibitors	CDKN2A inactivation causes activation of cyclin-CDK complexes
Proliferative and survival advantage			
ABL1-involving fusion proteins	ABL1 hyperactivation	ABL1 kinase inhibitors	Inhibition of ABL1 kinase activity
LCK translocation	LCK hyperactivation	SRC/ABL1 kinase inhibitor	Inhibition of LCK and its downstream targets
RAS mutations	RAS hyperactivation	Farnesyltransferase inhibitor	Inihibition of a post-translational modification of RAS proteins
PTEN mutations & deletion	PI3K hyperactivation	PI3K inhibitors	Opposition of PI3K function by PTEN is replaced by PI3K inhibitors
FLT3 duplication	FLT3 hyperactivation	FLT3 inhibitors	Inhibition of FLT3 tyrosine kinase activity
TEL-JAK2 fusion protein	JAK2 hyperactivation	JAK2 inhibitors	Inhibition of JAK2 tyrosine kinase activity

Figure 3.4 Overview of new molecular targets for T-ALL therapy *based on elucidations from (139,140,143).*

CHAPTER II

Objectives

Cytokines are known to play an important role in tumorigenesis as autocrine or paracrine growth factors (159). However, the efficacy of most anti-tumor treatments targeting cytokines turned out to be elusive, partly because tumor cells tend to acquire cytokine-independent growth properties. The goal of my PhD thesis was to contribute to a better understanding of the mechanisms underlying this process.

Various observations in humans and mice pointed to IL-9 as a factor promoting oncogenesis (160-163). Several *in vitro* models associating acquired IL-9 responsiveness with malignant transformation of lymphoid cells have been developed in our laboratory.

One of these models stemmed from the study of IL-3-dependent BaF3 cells transfected with a defective IL-9 receptor. These cells poorly responded to IL-9, however, prolonged culture with IL-9 allowed for the selection of IL-9-responding BaF3 phe116/9 cells. After cytokine withdrawal, those cells, in contrast to parental cells, gave rise to autonomous BaF3 cells that had cytokine-independent JAK1 and STAT5 phosphorylation and were highly tumorigenic *in vivo* (164).

This *in vitro* model represented a suitable and original system to study the mechanisms involved in multiple-step process of spontaneous tumorigenesis associated with constitutive STAT5 activation. Taking advantage of the microarray approach, Laurent Knoops showed during his PhD thesis that the first step of selection, when cells acquire IL-9 responsiveness, is associated with spontaneous overexpression of JAK1 (165), which renders BaF3 phe116 cells hypersensitive to IL-9, thus conferring a selective growth advantage. However, further selection of these cells towards autonomous clones with constitutive activation of STAT5 involved a second, unknown genetic hit that remained to be identified.

Therefore, when I started my PhD project, my objectives were the following:

(1) To identify the second genetic event involved in transformation of the phe116/9 cells to highly tumorigenic, autonomous BaF3 cells with constitutive STAT5 activation.

(2) If I would be able to identify this transforming event, to address its relevance for human cancer, and particularly for hematological malignancies.

(3) To study the biochemical processes controlled by this second hit with the hope of highlighting new potential avenues for therapeutic applications.