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JAK-STAT pathway at 30: much learned, much more to do

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Abstract

The discovery of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway arose from investigations of how cells respond to interferons (IFNs), revealing a paradigm in cell signaling conserved from slime molds to mammals. These discoveries revealed mechanisms underlying rapid gene expression mediated by a wide variety of extracellular polypeptides including cytokines, interleukins and related factors. This knowledge has provided numerous insights into human disease, from immune deficiencies to cancer, and was rapidly translated to new drugs for autoimmune, allergic, and infectious disease, including Covid-19. Despite these advances, major challenges and opportunities remain.

Introduction

The molecular components of the JAK-STAT pathway were identified 30 years ago. We celebrate this birthday by providing a brief history (Figure 1) of how this discovery allowed us to understand mechanisms through which extracellular polypeptides drive physiological and pathological responses, including cellular and organismal development, proliferation, metabolism, infection, inflammation, and cancer. We also consider how this information has been leveraged to develop novel therapeutic modulators of the pathway. A wealth of new evidence has been obtained concerning how this pathway impacts the genome to induce and repress gene expression. In recognition of the many publications and reviews published since the birth of the JAK-STAT pathway, we now strive to synthesize the salient findings and concepts, hoping to summarize the current state of knowledge, aware that each major

Declaration of Interests

NIH holds a US patent related to JAK inhibitors and Dr. O'Shea receives royalty income.

sub-topic is itself worthy of a detailed review. Despite the extraordinary advances achieved so far, no shortage of profound questions remains. Indeed, some of the questions posed 30 years ago and revisited at year 20 (Stark and Darnell, 2012) are still being addressed, tremendously aided by contemporary technologies and perspectives.

In the beginning: a new paradigm in signal transduction

Discovered in 1957, interferon (IFN) was quickly recognized to rapidly induce a vital antiviral program. The discovery of the JAK-STAT pathway arose from a very simple but profound question: How do cells respond to IFN? More broadly, the challenge faced by investigators in pre-genomic era of the late 1980s and early 1990s was to elucidate mechanisms underlying how gene expression is mediated in response to a variety of exogenous signals. Receptor-mediated signal transduction via G protein coupled receptors and receptor tyrosine kinases was being probed using the tools of biochemistry and of molecular and cell biology, simultaneously with the identification of specific DNA binding proteins and an array of transcription factors. How the steps of signal transduction and transcriptional activation came together to provide a pathway from membrane-bound receptors to gene transcription remained a mystery at the time.

The discovery of reversible protein phosphorylation by Krebs and Fischer, coupled with the discovery of oncogenic serine/threonine and tyrosine kinases, sparked a race to discover new tyrosine kinases. Using the relatively new method of degenerate polymerase chain reaction to generate protein kinase libraries, a new family of tyrosine kinases was identified that included tyrosine kinase 2, (TYK2) (Firmbach-Kraft et al., 1990; Krolewski et al., 1990), Janus kinase 1 (JAK1), and JAK2 (Harpur et al., 1992; Wilks et al., 1991). The name JAK arose from the two-faced Roman god Janus, since this family of kinases has a characteristic two-faced structure with an amino-terminal kinase domain preceded by a regulatory pseudo-kinase domain. However, since the function of the JAK family of kinases was still elusive, the alternative definition of JAK as “Just Another Kinase” was also appropriate.

To celebrate the 20th anniversary of the discovery of the JAK-STAT pathway, Stark and Darnell (Stark and Darnell, 2012) wrote individual detailed accounts of the history of the work in their laboratories and in the laboratory of the co-discoverer Ian Kerr, allowing us to provide only a brief summary here. Through the pioneering efforts of many others, IFN was readily available as a reagent. The ability to produce cDNA libraries from mRNAs induced by IFN treatment allowed several IFN-induced genes to be found and thus the promoters and regulatory elements that governed their expression were identified (Levy et al., 1986). The Stark and Kerr labs used the promoter of an IFN-induced gene to drive the expression of a lethal marker that killed all cells that responded to IFN. Following extensive mutagenesis, a resistant cell clone was isolated, which through genetic complementation, yielded *TYK2* as the essential gene inactivated by the mutagen (Velazquez et al., 1992). The Darnell, Stark, and Kerr labs showed that the signal transducing agent was present in the cytoplasm and rapidly moved to the nucleus when cells were stimulated with IFN, enabling the Darnell lab to purify the first STATs (STAT1 and STAT2, along with IRF9), determine their partial amino acid sequences and, from this information, deduce enough of the coding sequence to enable cloning of the genes. Jim Darnell christened the factors that his lab discovered

"Signal Transducers and Activators of Transcription" (STATs) to signify their dual (in other words, two-headed?) functions and to emphasize how rapidly their functions were carried out (namely, *stat* in medical terminology) (Stark and Darnell, 2012).

With the wisdom of hindsight, the discovery of the JAK-STAT pathway was facilitated greatly by the choice of IFN as the initial target of investigation. Other factors that use this pathway had been discovered long before IFN, with growth hormone and prolactin being discovered 20–30 years earlier. However, focusing on IFNs was fortuitous since the gene expression they induce is very rapid and the pattern of antiviral genes is very distinct compared to the programs of gene expression regulating cell growth and differentiation.

Filling out the pathway

Using a set of cell lines deficient in each of the proteins required for both type I and type II IFN signaling, along with the power of genetic complementation, the Stark, Kerr, and Darnell labs and collaborators rapidly defined the broad outlines of JAK-STAT signaling. This information fueled an explosive effort by many labs that converged on the realization that JAK-STAT pathways are used by many cytokines, growth factors, and other ligands to regulate gene expression in target cells. We now know that nearly 60 cytokines including many interleukins, colony stimulating factors, hormone-like cytokines and growth factors use the JAK-STAT pathway as their predominant, requisite mode of initiating transcription.

With the completion of the human and other genomes, it became clear that there are only 4 members of the JAK family based on their distinctive structure. The final member of the JAK family identified was JAK3, which has more selective expression, unlike other family members (Figure 2). Consistent with its expression, JAK3 activation is restricted to a subset of cytokines that employ the common gamma chain (γc) (Johnston et al., 1994).

JAKs contain a carboxy terminal catalytic or kinase domain preceded by a pseudokinase (PK) or kinase-like domain, a feature that prompted the name of the family. JAKs are constitutively associated with the intracellular Box1 and Box2 domains of cytokine receptors via the amino-terminal band four point one ezrin radixin, moesin (FERM) domain and Src Homology 2 (SH2) domains (Figure 3). A detailed structure of a full-length JAK was remarkably challenging to obtain, and only recently has the structure of full-length JAK1 complexed with the intracellular domain of a cytokine receptor been obtained (Figure 3) (Glassman et al., 2022). This work revealed that JAK1 domains form an extended structural unit that facilitates the dimerization of the cytokine receptor/JAK complex via packing of the PK domains from the monomeric receptor/kinase complex. The regulatory function of the PK domain and its mechanistic biological impact will be discussed below.

Following the discovery of STAT1 and STAT2, additional members of the STAT family were rapidly discovered in library or functional screens. STAT3 was identified in response to IL-6 as a DNA-binding "acute phase response factor" that behaved like IFN-activated STAT1 and was found as both a homodimer and heterodimer with STAT1 (Akira et al., 1994; Zhong et al., 1994b). Of note, epidermal growth factor and other growth factors were also recognized as STAT3 activators. Correspondingly, while "JAK-STAT pathway" is a useful shorthand, JAKs are not required for STAT activation by receptor tyrosine kinases. Next, STAT4 was

identified based on homology to STAT1 and STAT2 (Zhong et al., 1994a) and was found to be predominantly expressed in lymphoid tissues and activated by IL-12, IL-23, and type I IFNs. A “mammary gland specific nuclear factor” activated by prolactin was identified and designated STAT5 (Gouilleux et al., 1994; Liu et al., 1995; Wakao et al., 1994), and further clarified as two *STAT5* genes, STAT5A and STAT5B, with a very high degree of homology that can be activated by factors such as IL-2, IL-3, GM-CSF, and IL-5. Lastly, STAT6 was identified and cloned as an IL-4-inducible factor (Hou et al., 1994).

These discoveries led to a coherent view of the JAK-STAT pathway (Figure 2). Cytokine-receptor ligation activates JAKs that are constitutively associated with cytokine receptors, leading to JAK auto/transphosphorylation, activation, and phosphorylation of tyrosine residues in the receptor tails. Receptor phosphorylation creates docking sites for latent, cytoplasmic STATs, which are recruited to the receptor complex via tyrosine-phosphate-binding SH2 domains and become phosphorylated themselves (Greenlund et al., 1994). The activated phosphorylated STATs homo- or heterodimerize via intermolecular SH2-phosphotyrosine interactions and acquire the ability to translocate to the nucleus and bind DNA regulatory elements to alter chromatin accessibility and induce gene transcription. While a preferential/dominant STAT can often be identified for a given cytokine, many cytokines have been reported to activate nearly all STATs in some circumstance or tissue, and all STATs have been shown to be activated by the major cytokines that have been extensively studied. This is not a trivial finding; given the large number of cytokines, the relative paucity of JAKs and STATs is inadequate to explain the assorted actions of so many diverse factors while still achieving specificity in signaling.

The JAK-STAT pathway is widely conserved amongst metazoans, from *Caenorhabditis elegans* and *Drosophila melanogaster* to vertebrates. *Drosophila* has three related cytokine-like ligands – Unpaired (Upd), Upd2 and Upd3, one cytokine-like receptor, Domeless (Dome) that leads to the activation of one JAK, termed Hopscotch (Hop) and one STAT transcription factor, Stat92E. *Dictyostelium discoideum* has four STAT proteins, but only in Bilateria is convergence of the entire pathway evident.

Even though the JAK-STAT pathway is a major component of cytokine signaling, other SH2 signaling molecules are also recruited, and pathways beyond STATs are engaged by cytokine receptors. These include Ras, MAP kinase, AKT, PI3K, and mTOR pathways (Silvennoinen et al., 1993; Yu et al., 1995). Based on proteomic analysis of IL-2-stimulated cells, 90% of the phospho-proteome of IL-2 activated T cells are JAK-dependent, with the remainder being mediated by SRC family kinase signaling, encompassing the production of phosphatidylinositol (3,4,5)-trisphosphate and AKT activity (Ross et al., 2016).

Negative Regulation of Cytokine Signaling

Physiological negative regulation.—As with many biological settings, negative feedback systems are induced by a positive-acting stimuli; this is certainly the case for cytokines and interferons, and the suppressor of cytokine (SOCS family) of STAT-target genes is an outstanding example (Figure 2) (Linossi and Nicholson, 2015). Eight SOCS proteins are encoded in the human genome, including SOCS1-7 and CIS, which contain a central SH2 domain and a carboxy-terminal SOCS box. SOCS proteins interfere with

cytokine signaling by binding to cytokine receptors, competing with STATs, and inducing their ubiquitination and degradation via elongin BC and the E3 ligase Cullin5. SOCS1 and SOCS3 contain a short motif called the Kinase Inhibitory Region (KIR) that interacts with JAKs directly (JAK2, JAK1, and TYK2). The SOCS1 KIR inhibits JAK2 by obstructing the access of both ATP and substrate (Giordanetto and Kroemer, 2003) whereas SOCS3 interacts with JAK2 associated with gp130 (Kershaw et al., 2013). SOCS3 is preferentially induced by IL-6, whereas SOCS1 predominantly inhibits IFNs but also IL-12, IL-4/13, and IL-2 family of cytokines.

Other key negative feedback regulators are ubiquitin-specific peptidase 18 (USP18) and interferon stimulated gene 15 (ISG15) (Figure 2). ISG15 is a ubiquitin-like protein that is covalently conjugated to target proteins (ISGylation) to modulate the function of multiple host and viral proteins, and USP18 is a protease that deconjugates ISG15 from target proteins. Both are induced by interferons and are key negative regulators of type I IFN signaling (Figure 2). USP18 competes with JAK1 for binding to IFNAR2, and STAT2 is an essential adaptor that recruits USP18 to IFNAR2. Additionally, ISG15 non-covalently binds to USP18 to prevent its ubiquitination/degradation.

Negative regulators also target downstream of the JAK-STAT pathway (Figure 2). The protein inhibitor of activated STAT (PIAS) family comprises multiple members that act on STATs and other factors including NF-κB, and interfere with signaling via multiple mechanisms, such as blocking the DNA-binding activity of transcription factors, recruiting transcriptional co-repressors and promoting protein sumoylation (Shuai, 2006). Multiple phosphatases are reported to dephosphorylate STATs, including CD45, PTP1B, TC-PTP, PHLPP1, PTPRT, PRPRK, PTPN9, and PTPN2. Notably, SHP-1 and SHP-2 have both positive and negative effects on signaling.

Negative regulators are also conserved, as *Drosophila* have a single SOCS protein designated *Socs36E* and a single PIAS protein desginated *dPIAS.*

Negative regulation adapted by viruses.—The importance of STAT-mediated IFN-signaling as a fundamental component of antiviral defense is highlighted by the repeated evolution of viral anti-immune strategies that target STATs directly or indirectly. Mechanisms of STAT-directed IFN antagonism include targeted STAT degradation, sequestration, and cytoplasmic retention. Variations on these basic themes of STAT inhibition have been reinvented by many viruses to escape IFN antiviral responses. Paramyxovirus nonstructural proteins (V, C, and W) engage STATs directly and in combination with other factors. Parainfluenza viruses PIV5 and PIV2, and mumps virus use their V proteins to assemble STAT1 or STAT2-targeting ubiquitin ligase complexes to induce proteasomal degradation (Ulane and Horvath, 2002). Flaviviruses, including Dengue virus and Zika virus, target STAT2 for proteasome-dependent degradation (Ashour et al., 2009; Grant et al., 2016), and a porcine deltacoronavirus nsp5 protease can cleave STAT2 (Zhu et al., 2017).

The Henipaviruses, Nipah virus and Hendra virus, and measles virus bind STAT1 and STAT2 with their V proteins, preventing oligomerization, sequestration in cytosolic

aggregates, nuclear translocation, and import. Dimeric STAT1 nuclear import uses a unique binding site on importin 5, and the Ebola VP24 protein specifically blocks this interaction (Xu et al., 2014). Disruption of STAT nuclear import by karyopherin is also found in enteroviruses and rotaviruses (Wang et al., 2017a). The SARS-CoV-2 ORF6 protein interferes STAT import via the Nup98-Rae1 nuclear pore sub-complex (Miorin et al., 2020).

Other viruses can modulate STATs by sequestration in cytosolic aggregates or promote nuclear export. SARS-CoV-2 N protein can mediate STAT cytosolic retention (Mu et al., 2020) and both Henipavirus V and Chikungunya virus can actively export STATs via a CRM-1 export signal (Goertz et al., 2018).

STAT structure, complex formation, and impact on function

All the STATs share similar N-terminal, coiled-coil, DNA-binding, linker, SH2, and transactivation domains (Figure 4). In general, activated JAKs phosphorylate the conserved tyrosine residue of one STAT monomer, which then binds to the SH2 domain of another monomer, driving a conformational change that enables STATs to translocate into the nucleus to initiate the transcription of target genes. In this review, we use P-STAT to designate ‘canonical’ tyrosine phosphorylation (i.e., STAT1-Y701, STAT2-Y690, STAT3-Y705, STAT4-Y693, STAT5-Y694, SYAY6-Y641) and U-STAT to designate a STAT without tyrosine phosphorylation. Other residues may or may not be phosphorylated.

STATs can form a variety of hetero- or homodimers. STAT1, STAT3, and STAT5a have been shown to form homodimers in either a parallel or antiparallel conformation (Figure 4; STAT1 shown as an example). The DNA-binding domains (DBDs) of two P-STATs bind to DNA as a C-shaped clamp in the parallel conformation (Chen et al., 1998). However, in the antiparallel homo-dimer, the DBDs reciprocally interact through their coiled-coil domains (CCDs), preventing access to DNA (Zhong et al., 2005). Tyrosine phosphorylation regulates the equilibrium between these two conformations. For example, P-STAT1 is primarily parallel and U-STAT1 is primarily antiparallel, but low levels of the alternative conformations are also present. Notably, U-STAT3, U-STAT5 and U-STAT6 also have functional roles in the nucleus in regulating gene expression or heterochromatin formation. U-STAT3 competes with IκB and binds to NF-κB, which then with the help of importin-α3, brings the U-STAT3/p65/p50 complex to the nucleus to bind to a κB site for RANTES gene expression (Yang et al., 2007). Rather than initiating transcription, U-STAT5 functions as a tumor suppressor capable of repressing multiple oncogenes via heterochromatin formation (Hu et al., 2013b). U-STAT6, associated with p300, binds directly to the COX-2 GAS element to drive its expression in NSCLC cells (Cui et al., 2007).

The N-terminal domain is critical for regulating STAT homodimer formation and function. When P-STAT1 disengages from DNA, the N-terminal domain dimerizes and helps to expose the phosphorylated tyrosine as a target of the TC45 phosphatase (Chen et al., 2003; Wenta et al., 2008). Additionally, STAT tetramers form by the cooperative recruitment of pairs of homodimers via interactions between N-terminal domains. (Begitt et al., 2014; Lin et al., 2012; Vinkemeier et al., 1998). Such tetramers bind to adjacent DNA loci that combine high-affinity and low-affinity binding sites. (Mandal et al., 2011). Consequently, tetramers form more stable DNA complexes and have more contact with DNA than dimers.

Tetramer formation is essential for the complete transcriptional response of STAT1 to type II IFN (Begitt *et al.*, 2014) and of STAT3 to IL-6 (Zhang and Darnell, 2001). For STAT5, the tetramers not only directly induce gene expression but also indirectly regulate gene repression, presumably through the positive regulation of an intermediate repressor (Lin *et al.*, 2012; Mandal *et al.*, 2011).

Unlike the other STATs, which form homodimers, gene expression in response to IFNs I and III is driven by STAT1:STAT2 heterodimers that, together with IRF9, comprise ISGF3 (Figure 5). In P-ISGF3, the parallel conformation of the P-STAT1:P-STAT2 dimer is stabilized by reciprocal phosphotyrosine-SH2 interactions. In this conformation, the DBDs of the two STATs bind to DNA simultaneously, together with the DBD of IRF9 (Rengachari et al., 2018). In U-ISGF3, the two DBDs can still bind to DNA coordinately. Modular associations between STATs and IRFs can contribute to non-canonical complexes, including IRF9 in complex with variations of unphosphorylated STAT1 or STAT2 (Majoros et al., 2017). Recent evidence from electron microscopic and biochemical analyses reveals that U-STAT1 and U-STAT2 heterodimers adopt a very stable antiparallel conformation (Wang et al., 2021). It is clear though that U-STAT1:U-STAT2 and U-STAT2:IRF9 dimers are mutually exclusive; that is, IRF9 cannot bind to the U-STAT1:U-STAT2 dimer and U-STAT1 cannot bind to U-STAT2:IRF9. From this new information, the functional complex previously called “U-ISGF3” (Cheon et al., 2013) is now primarily the U-STAT2:IRF9 dimer and that U-STAT1 plays a more peripheral role. The U-STAT2:IRF9 complex drives the expression of the majority of ISGs in unstimulated cells and switches to an ISGF3 complex containing STAT1, STAT2 and IRF9 in response to IFN-I or IFN-γ stimulation (Platanitis et al., 2019).

Furthermore, functional Interferon-Stimulated Regulatory Elements (ISREs) that bind to U-STAT2:IRF9 are also present and functional in regions upstream of regulatory elements of a subset of NF-κB-driven genes, for example, *IL6* (Nan et al., 2018). It is important to stress that high levels of U-STAT2 and IRF9 are needed to drive substantial gene expression, as seen in cancer (discussed later in *Complex roles of JAKs and STATs in cancer*). The U-STAT1:U-STAT2 dimer also acts pervasively in an IFN-I-independent manner to inhibit STAT1 function in multiple signaling pathways, such as IFN-γ and IL-27 (Ho et al., 2016; Wang *et al.*, 2021). In this U-dimer, STAT2 binds to STAT1 through exceptionally strong interactions between the two N-termini. Since U-STAT2 is mainly located in the cytoplasm in unstimulated cells (Martinez-Moczygemba et al., 1997), it inhibits the phosphorylation and nuclear translocation of STAT1 in multiple signaling pathways (Wang *et al.*, 2021).

The U-STAT1:U-STAT2 dimer not only suppresses multiple P-STAT1 homodimer functions but also inhibits ISGF3 formation. Although we know that robust stimulation with IFN-I drives the formation of P-STAT2, key molecular events that regulate U-STAT2 function are just now being revealed, including the recent discovery of two important regulatory phosphorylations of STAT2 on T387 and T404 (Figure 5). The phosphorylation of T387 favors the formation of U-STAT1:U-STAT2 heterodimers at the expense of tyrosine-phosphorylated heterodimers and ISGF3 (Wang et al., 2017b). On the other hand, T404 is phosphorylated by IKKi or TBK1, disrupting the very stable U-STAT1:U-STAT2 dimer, facilitating the response to IFN-I and dramatically increasing the affinity of ISGF3 affinity

for ISREs. Phosphorylation of T404 also facilitates the binding of U-STAT2 to STING, inhibiting the ability of STING to drive the production of IFN-I (Wang et al, submitted). Further research will expand our knowledge of the mechanisms underlying these novel non-canonical functions of U-STAT2 and of the range of roles that U-STAT2 plays in biology.

Complex formation also affects nucleocytoplasmic shuttling of STATs (Reich and Liu, 2006). This occurs through nuclear pore complexes and is regulated by interactions with transporter proteins of the karyopherin-β family (i.e. importins and exportins). STAT nuclear trafficking is not always dependent on tyrosine phosphorylation, and the regulation of nuclear trafficking is diverse among different STATs. The dynamic redistribution of STAT1 and STAT2 between the cytoplasm and the nucleus is coordinate with their gain of ability to bind DNA. Nuclear trafficking of STAT1 homodimers is dependent on tyrosine phosphorylation where importin-α5 recognizes a conditional nuclear localization signal (NLS) in the P-STAT1 dimer. The constitutive NLS of IRF9 facilitates nuclear localization of the U-STAT2:IRF9 complex. Additionally, nuclear export of STAT2 is independent of its phosphorylation status and is mediated by a nuclear export signal (NES) in its carboxyl terminus. The STAT3 NLS is in its coiled-coil domain and nuclear localization of STAT3 is independent of tyrosine phosphorylation but dependent on recognition by importin-α3. The regulation of nuclear trafficking of STATs is complex and much remains to explore.

STATs and transcriptional regulation

Once STAT complexes arrive in the nucleus, they accomplish all the tasks associated with transcriptional regulation: engagement of chromatinized target genes, binding response elements, and recruitment of transcriptional co-activators that can recruit and activate RNA Pol II (Au-Yeung and Horvath, 2018).

STAT regulation of ISGs.—Induction of ISG transcription is typically fast, considering the necessity of a quick response to viral infection. This corresponds with an increased transcription rate, fast mRNA processing and degradation rates, and little or no evidence for post-transcriptional control (i.e. microRNA, translation) (Jovanovic et al., 2015). RNA polymerase II (RNA Pol II) recruitment to ISGs *de novo* is stimulated by IFN, but pause-release mechanisms are also used in ISG regulation (Patel et al., 2013), sometimes in association with a TBP-free $TAF_{II}130$/GCN5 transcription complex (Paulson et al., 2002). Here, phosphorylation of its C-terminal domain by pTEFb via BRD4 results in the release of the RNA Pol II-associated repressors NELF and DSIF, enabling ISG mRNA elongation (Patel *et al.*, 2013). Coordinately with RNA Pol II recruitment, STAT2 transactivation domain engages Mediator via MED14 and MED17 subunits (Lau et al., 2003) and enables STAT2 to activate RNA Pol II.

Additionally, after IFN treatment, there is an increase in chromatin accessibility at promoters and gene bodies at or near ISGF3 binding sites (Mostafavi et al., 2016) as well as recruitment of SWI/SNF nucleosome remodeling complex subunits. BRG1, an ATPase subunit of the SWI/SNF nucleosome remodeling complex (or mammalian BAF/pBAF complex), interacts with STAT2 to promote transcription. Both RVB1 and RVB2,

components of SRCAP, TIP60, URI and INO80 chromatin remodeling complexes also associate with STAT2 (Gnatovskiy et al., 2013). Interference with RVB1 prevents Pol II recruitment to ISG promoters, but the exact role is unclear. Additionally, chromatin remodelers can influence histone acetylation, as knockdown of BAF47 leads to decreased histone H4 acetylation at ISG promoters (Cui et al., 2004). Likewise, acetylated chromatin can enhance bromodomain recruitment, bringing BRG1 to ISGF3-proximal histones.

H2A.Z nucleosomes play a regulatory role in ISG transcription, as shRNA interference with H2A.Z results in greater ISGF3 occupancy, increased ISG mRNA production, and created a more potent antiviral state leading to more robust inhibition of virus replication. Correspondingly, ISGF3 arrival induces H2A.Z loss from the promoter, and inhibition of either the HAT GCN5 or the bromodomain protein BRD2 disrupts both H2A.Z removal and ISG transcription. It is important to note that well known coactivators including CBP, HDACs, and RVB1 are dispensable for H2A.Z removal but strictly needed for ISG transcription. Along with H2A.Z remodeling, histone H3.3 is deposited in the gene body, and genes with this mark are transcriptionally sensitized due to prior activation.

Both histone acetylation and deacetylation activities are required for ISG transcription activation. Multiple control points contribute to acetylation-based regulation of interferon/cytokine signaling, as acetylation and deacetylation target the chromatin and transcriptional apparatus and many proteins beyond STATs themselves (Wieczorek et al., 2012). Multiple STATs associate with histone acetyltransferases (HAT), as STAT2 associates with CBP/p300 and GCN5 and p300 associates with STAT1, STAT3, STAT5 and STAT6 (Bhattacharya et al., 1996; Paulson et al., 1999). Interference with either GCN5 or CBP inhibits ISG transcription, but H2A.Z eviction from ISG promoters depends only on GCN5, not CBP, demonstrating specificity rather than redundancy in HAT function and reflecting the potential for multiple regulatory outcomes (Au-Yeung and Horvath, 2018). Likewise, ISG transcription is blocked by both interference with HDAC proteins and HDAC inhibitors (Chang et al., 2004; Nusinzon and Horvath, 2003), as RNA Pol II recruitment to ISG promoters is impaired by HDAC inhibitors. In addition, HDAC inhibition may create an acetylation sink for BRD4, restricting its availability for STAT co-activation (Marie et al., 2018). The importance of both BRD4 and HDACs to ISG regulation may enable cross-regulation due to reversible sequestration of BRD4 on hyperacetylated versus hypoacetylated chromatin.

STATs and other transcription factors.—Under physiological conditions, cells receive multiple concurrent signals that activate multiple signaling pathways. A classic example is toll-like receptor (TLR) plus a cytokine signal (such as IFN-γ) that activate the NF-κB and JAK-STAT pathway, respectively. Multiple observations have been made in the literature describing the interaction between STATs and other transcription factors, including regulation of transcription factor synthesis, regulation of transcription factor activity, and direct or indirect interactions within the genome that affect transcription (Farlik et al., 2010; Platanitis and Decker, 2018). Most notably, some key genes in an immune response require synergy between multiple pathways, namely NK cells require synergistic IL-18 (NF-κB) and IL-12 (STAT4) for maximal IFN-γ expression (Takeda et al., 1998).

Cooperativity and antagonism between STATs.—Whether it is from two different signals, or the same, different STATs can synergize or antagonize each other to affect transcriptional output. These cross-talks can occur by physical interaction, serine phosphorylation of the transactivation domain, cooperative DNA binding, and synthesis of specific signaling inhibitors. STAT1 and STAT3 represent clear examples whereby crosstalk can occur in either a synergistic or antagonistic manner. Synergy mediated by IL-2 and IL-12 leads to greater IFN-γ expression due to serine phosphorylation of STAT1 and STAT3 (Gollob et al., 1999). Antagonism between these STATs are commonly described between interferons and STAT3-activating cytokines, which have biological consequences as seen in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or STAT1 gain-of-function (GOF) patients where an exaggerated STAT1 response leads to chronic mucocutaneous candidiasis (CMC), an immune response dependent on STAT3 (Break et al., 2021; Liu et al., 2011). In many cases, STAT1 and STAT3 are activated by many of the same cytokines (i.e. IL-6, IL-21, and IL-27). Therefore, the mechanisms underlying antagonizing effects of these transcription factors is more elusive. Efforts have been made to try to address this conundrum and in the case of IL-6 and IL-27, STAT3 is responsible for transcriptional output, whereas STAT1 is the driver of specificity (Hirahara et al., 2015). With IL-21 signaling, STAT1 and STAT3 have partially opposing roles in $CD4^+$ T cells, even though both are critical for IL-21-mediated gene transcription (Wan et al., 2015).

STAT1 and STAT4 can also antagonize each other. For example, IL-12 and IL-18 antagonizes IFN-α signaling by reducing STAT1 transcript levels as well as type I IFN transcriptional output (Wiedemann et al., 2021). However, in response to type I IFNs both STAT1 and STAT4 are activated in NK and CD8 T cells (Nguyen et al., 2002); yet STAT1 antagonizes STAT4, specifically by impairing IFN-γ production and proliferation in NK cells and CD8 T cells, respectively. This effect is modified by alteration of levels of each STAT during viral infection. STAT4 levels are initially high in comparison to STAT1, but as STAT1 protein levels increase during infection, STAT1 antagonistic functions begin to take hold presumably to limit potential damaging responses.

Furthermore, crosstalk occurs between ISGF3 and GAF after IFN-I induction. When IFN-I signaling is impaired with STAT2 or IRF9 deficiency, induction of negative regulators such as USP18 is lost, resulting in a prolonged GAF transcriptional response (Gothe et al., 2022). In principle, the antagonistic actions of STATs can help explain specificity of cytokine signaling; however, this is an area that clearly needs further investigation to truly understand the logic and mechanisms.

Genomic views of cytokine action

Technological advances facilitated new discoveries through the investigation of cytokine and interferon action. The ability to comprehensively define the entire transcriptome induced by cytokines and interferons began with microarray technology (Aberger et al., 2001; Chatterjee-Kishore et al., 2000; Ramana et al., 2002) but was quickly supplanted with massively parallel sequencing platforms. These efforts revealed that cytokines and interferons induce thousands of genes, including the expected genes participating in host defense responses, but also genes encoding proteins that regulate cell cycle and metabolism.

During this time, it became possible to comprehensively define STAT binding sites throughout the genome by using chromatin immunoprecipitation sequencing (ChIP-seq) (Robertson et al., 2007) archived in relevant databases (http://cistrome.org/db/#/). This work revealed tens of thousands of cytokine-induced genomic binding sites. At the time, the significance of this binding was unclear. That is, while much work had been devoted to enumerating conventional, protein-coding genes, we now know that much of the genome is transcribed in cell- and activation-specific manners, and that microRNAs and long noncoding RNAs (lncRNAs) exceed the number of conventional genes. Unsurprisingly, STATs have major effects on all these transcripts, including *Ifngas-1* and others (Hu et al., 2013a; Witte and Muljo, 2014).

STAT-Regulated Enhancers.—Enhancers can be operationally defined as regions of DNA that in their genomic, cellular, and organismal context interact with proteins that augment the likelihood of transcription of one or more genes through cis-regulatory mechanisms. Along with STAT ChIP-seq, antibodies directed against chromatin modifications or the histone acetyltransferase p300 were used in concert with STAT-deficient activated cells to decipher the genomic impact of STATs. Newer methodologies for measuring genomic accessibility, including assessment of small numbers of cells or even single cells, enable us to map cells activated *in vivo* without extensive manipulation and expansion *in vitro*. This has further accelerated understanding the genomic impact of STATs.

It has become clear that the binding of STATs to the genome has multiple consequences. Diseases associated with a transcriptomic “interferon signature” (i.e. Interferonopathies) are associated with an interferon epigenomic signature that primes cells for subsequent responses (Barrat et al., 2019). Cytokines like IFN-γ and IL-4 can mutually inhibit epigenomic and transcriptomic alterations induced by each cytokine alone. Thus, STATs can mediate induction and repressive epigenomic and transcriptomic effects (Piccolo et al., 2017).

STATs can have broad impacts on active enhancers (Figure 6). They can influence proximal histone modifications and are not replaced by the action of lineage-defining transcription factors (LDTFs) (Vahedi et al., 2012). STATs can also participate in the formation of latent enhancers, regions that acquire accessibility via LDTFs but become active after cellular stimulation (Ostuni et al., 2013). On the other hand, STATs can drive accessibility in regions that lack LDTF-binding motifs. Though, LDTFs can be enriched in these regions by presumed non-canonical interactions and/or association with STATs (Sciume et al., 2020). That is, for unmarked (latent) enhancers, the canonical roles of LDTFs and STATs vis-à-vis accessibility can be inverted. This calls into question a “chicken and egg” scenario for the temporal primacy of LDTFs as “pioneer factors” over STATs, as is the case for STATs and T-bet in NK cells (Sciume *et al.*, 2020). Consistent with the primacy of STATs over LDTFs in establishing enhancer recognition, JAK inhibition prevents histone marking and PU.1 recruitment (Ostuni *et al.*, 2013). Thus, interdependence of LDTFs and STATs in both directions provides yet another layer of combinatorial variability contributing to specificity of gene programs.

Key genes controlling cell type-specific function tend to be regulated by clusters of enhancers densely occupied by transcription factors, coactivators, RNA polymerase II, and elongation factors (Vahedi et al., 2015; Whyte et al., 2013), a structure designated super- or stretch-enhancers (SE) (Figure 6). In B cells, the balance between STAT5 and other transcription factor network at SE is key and act as a molecular switch to govern proliferation, survival, and differentiation of progenitors (Katerndahl et al., 2017).

In summary, STATs can deliver their effects via multiple context dependent mechanisms—direct transcriptional induction/repression, epigenetic conditioning, or both. On a genome-wide scale, activated STATs contribute significantly to the modulation of epigenetic processes.

Mitochondrial localization of STATs

In addition to their prominent role in the cytoplasm and nucleus, a small fraction of STAT1, STAT3, and STAT5 (5–10% of the total) is found in the mitochondria where it functions independent of cytoplasmic signaling and nuclear gene activation. Mitochondrial STAT3 is studied the best, as it directly contributes to Ras-induced transformation by regulating respiration, redox homeostasis, and mitochondrial transcription (Carbognin et al., 2016; Garama et al., 2015). In Ras-transformed cells, ATP levels are decreased, and the activities of Complexes II and V of the electron transport chain are diminished in the absence of STAT3 (Gough et al., 2009). Tyrosine 705 phosphorylation, which is crucial for canonical nuclear functions of STAT3, is not needed to drive its mitochondrial functions but phosphorylation of STAT3 on S727 seems to be needed instead (Peron et al., 2021). Access of STATs to the mitochondria, as well as to other cellular organelles, including the endoplasmic reticulum (Avalle et al., 2019), and their potential organellar functions in cellular physiology remains an open and active research area.

In vivo functions of JAKs and STATs

As expected, gene targeting of mice provided great insights into actions of JAKs, STATs and related molecules; however, mutations and variants very quickly revealed relevance to human disease (Figure 2) Consequences of germline mutations in the JAK-STAT pathway vary widely in severity (Table I), but the salient clinical findings are summarized below.

JAK mutations.—The critical role of JAKs was first shown in humans with severe combined immunodeficiency (SCID). Mutations of *IL2RG*, which encodes gc, underlies X-linked SCID. Because JAK3 associates with γc, JAK3 autosomal recessive loss-of-functions (LOF) mutations were also sought and found to be a cause of SCID (Macchi et al., 1995; Russell et al., 1995) and confirmed in mouse models (Park et al., 1995; Thomis et al., 1995). Notably, these patients and mice are otherwise normal, indicative of JAK3's very specific function compared to other JAKs.

TYK2-deficient mice are viable and have selective impairment of cytokine responses that include loss of IFN and IL-12/23 family cytokine responses and thus increases susceptibility to infections. TYK2 deficiency in humans is associated with bacterial and viral infections, as well as allergic disease (Minegishi et al., 2006). A LOF variant of *TYK2* in humans

(P1104A) is associated with reduced incidence of autoimmune disease and increased risk of tuberculosis.

JAK2 has essential roles for many cytokines including hematopoietic growth factors. JAK2-deficient mice die *in utero* with bone marrow failure (Neubauer et al., 1998; Parganas et al., 1998). In a classic series of papers, JAK2 GOF mutations were identified in patients with myeloproliferative neoplasms (MPN), including polycythemia vera, primary myelofibrosis and essential thrombocytopenia (Kralovics et al., 2005; Zhang et al., 2012). The most common mutation is V617F located in the JAK2 PK domain, which results in constitutive activity and cytokine-independent proliferation (Hammaren et al., 2015); other mutations also associated with MPN include R683G, R683S, and R683T. The first evidence of JAK GOF mutations were revealed by the Drosophila Tumorous-lethal (Tum-l) mutation in the kinase-like domain of the hopscotch locus, which resulted in malignant neoplasia of blood cells (Hanratty and Dearolf, 1993; Harrison et al., 1995; Luo et al., 1995). The structure of a full-length JAK now provides detailed insights into the mechanism by which PK mutations lead to cytokine-independent signaling, with GOF mutations residing within the core of the PK interdimer interface, enhancing packing complementarity to facilitate ligand-independent activation (Glassman *et al.*, 2022).

In keeping with the many cytokines that rely on this kinase, JAK1-deficient mice die perinatally with impaired organogenesis and dwarfism (Rodig et al., 1998). In humans, a JAK1 LOF mutation was associated with recurrent mycobacterial infection, developmental delay, and early-onset metastatic bladder carcinoma (Eletto et al., 2016). JAK1 GOF mutations lead to an autoinflammatory-related disease and hyperimmunoglobulin E syndrome (HIES) (Del Bel et al., 2017; Gruber et al., 2020). Genome-wide association studies (GWAS) have revealed polymorphisms of *JAK1* in juvenile idiopathic arthritis, multiple sclerosis, autoimmune thyroid disease, C-reactive protein levels, lymphocyte and eosinophil counts.

STAT mutations.—Although, STAT1 is activated by many cytokines, the predominant phenotype of STAT1 knockout mice and mutations in humans is susceptibility to intracellular pathogens such as bacteria and viruses. But in addition, STAT1 is important for hematopoietic precursor function (Li et al., 2022). STAT1-GOF patients have autoimmunity and increased susceptibility to fungal and viral infection; however, the molecular mechanisms underlying STAT-GOF mutations is very incomplete. GOF mutations may favor STAT1's parallel phosphorylated state, however, these observations have yet to be confirmed by detailed structural analysis. Notably, these "accidents of nature" provide substantial opportunities to link structure and function.

Germline STAT2 LOF mutations in mice and humans confer susceptibility to viral infections. However, some STAT2 mutations do not affect phosphorylation or activation; rather, they prevent STAT2 from recruiting USP18 to the IFN receptor subunit, IFNAR2. These STAT2 mutations can be associated with severe interferonopathy due to defective negative regulation of type I interferon signaling (Gothe *et al.*, 2022).

Germline deletion of STAT3 is lethal in mice because of its importance in placental function. Conditional knockouts have revealed broad roles of STAT3 in multiple tissues (Levy and Lee, 2002), with important roles in innate and adaptive immunity, stem cells, wound healing, tissue repair, and cancer. Dominant negative LOF mutations of *STAT3* underlie hyperimmunoglobulin E (Job's) syndrome, a primary immunodeficiency syndrome also associated with craniofacial, skeletal and vascular abnormalities (Holland et al., 2007; Minegishi et al., 2007). STAT3 GWAS variants are linked with inflammatory bowel disease, multiple sclerosis, type 2 diabetes mellitus, and myocardial infarction and patients with germline STAT3 GOF mutations exhibit lymphoproliferation and poly-autoimmunity (Milner et al., 2015).

STAT4 knockout mice have impaired IFN-γ production and increased susceptibility to *T. gondii* and other pathogens that rely on a type 1/IFN-γ response. *STAT4* LOF mutations in humans are associated with susceptibility to fungal infections and viral infections. *STAT4* variants are associated with lupus, rheumatoid arthritis (RA), and Sjogren's syndrome with the presence of the risk allele being associated with more severe disease (Hasni et al., 2021; Remmers et al., 2007). Moreover, a *STAT4* coding variant (Glu128Val) doubles the risk of RA (Saevarsdottir et al., 2022).

Adjacent in the genome, STAT5A and STAT5B are >90% identical. *Stat5a* knockout mice have loss of prolactin-mediated mammary gland development, whereas *Stat5b*-deficient mice have impaired sexually dimorphic growth. *Stat5a/b* double-deficient mice die within a few weeks of birth, are infertile and have defective mammary gland development, as well as hypocellular bone marrow. Homozygous *STAT5B* LOF mutations in humans result in growth failure, facial dysmorphism, and autoimmunity. Activating somatic mutations in hematopoietic progenitors can also cause nonclonal hypereosinophilia, eczema, urticaria, and diarrhea. *STAT5A* variants are linked with obesity, whereas *STAT5B* GWAS reveal links to lymphocyte and neutrophil counts, asthma and allergy, and bone density.

Consistent with the role of STAT6 in IL-4 and IL-13 signaling, STAT6-deficient mice fail to mount type 2 immune responses and impaired parasite elimination and reduced allergic disease. Germline *STAT6* GOF mutations are associated with severe, treatment resistant allergic disease with eosinophilia (Sharma et al., 2022). GWAS links with *STAT6* include allergy, asthma, and eosinophilia.

Mutations in negative feedback regulators.—Homozygous deficiency of SOCS1 in mice is lethal with hypersensitivity to IFN-γ, and SOCS1-deficient humans exhibit early onset autoimmune and allergic disease (Hadjadj et al., 2020). A major consequence of USP18 and ISG15 deficiency in humans is severe, systemic interferonopathy (Alsohime et al., 2020; Martin-Fernandez et al., 2020; Martin-Fernandez et al., 2022; Meuwissen et al., 2016; Speer et al., 2016; Zhang et al., 2015).

Complex roles of JAKs and STATs in cancer

Constitutive activation of JAKs and STATs in cancer was quickly recognized shortly after the discovery of the pathway, particularly in the setting of viral or oncogene-mediated transformation (Meydan et al., 1996; Migone et al., 1995). Subsequently, there are many

examples of overexpression and hyperactivation of JAKs and STATs associated with cancer. However, cancers evade immune cell killing, abrogating IFN-γ signaling by inactivating *JAK1* mutations (Zaretsky et al., 2016) and altering immune cell function in the tumor microenvironment. Thus, the roles of cytokines and the JAK-STAT pathway in cancer and the tumor microenvironment is anything but simple – and a particular challenge to review in just a few paragraphs.

The simplest scenarios relate to GOF mutations. A Tel-JAK2 fusion protein associated with leukemia was identified early on (Lacronique et al., 1997), followed by the identification of other JAK2 fusion partners (e.g. PCM1, SEC1A, PAX5). Additionally, generation of a constitutively active version of STAT3 with two cysteine substitutions in the SH2 domain that causes spontaneous dimerization, binding to DNA, and transcriptional activation results in cellular transformation (Bromberg et al., 1999). This remarkable insight anticipated the common mutations in the SH2 domains of STAT3 and STAT5 often seen in hematopoietic malignancies (Tate et al., 2019); (https://cancer.sanger.ac.uk/cosmic). One common mutation, STAT5 N642H, is sufficient to drive leukemogenesis in mice (Kollmann et al., 2021).

With improvements in sequencing technology, a more comprehensive understanding of JAK/STAT germline and somatic GOF mutations or amplifications has been achieved (Tate *et al.*, 2019) (https://cancer.sanger.ac.uk/cosmic). In fact, mutations of all JAKs and STATs are common but are seen predominantly in hematopoietic/lymphoid malignancies. For example, STAT1 is a tumor promoter in leukemia development (Kovacic et al., 2006). In B cell acute lymphocytic leukemia (ALL), STAT5- and ERK-activating mutations are commonly found but, interestingly, the two pathways are antagonistic (Chan et al., 2020; Katerndahl *et al.*, 2017). STAT5 antagonizes NF-κB and IKAROS by opposing regulation of shared target genes; that is, super-enhancer enrichment for STAT5 binding is associated with an opposing network of transcription factors, including PAX5, EBF1, PU.1, IRF4 and IKAROS. Additionally, STAT5B-N642H is a frequently occurring driver mutation that promotes aggressive T cell leukemia/lymphoma, by which this mutation causes a structural conformation yielding a STAT5B that is resistant to dephosphorylation (de Araujo et al., 2019). Moreover, patients with a high ratio of active STAT5 to NF-κB or IKAROS had more-aggressive disease, but deletion of divergent pathway components also accelerates leukemogenesis – a more nuanced view of transformation.

While mutations of JAKs and STATs occur in nonhematopoietic tumors, including tumors affecting breast, lung, and large intestine, this is relatively uncommon; nonetheless, this does not mean that the pathway is irrelevant for solid tumors. Beyond mutations, activation of STAT3 and STAT5 is very frequent in many human cancers, both solid and liquid, and overexpression of STATs are associated with poor prognosis. STATs, especially STAT3 and STAT5, can directly regulate genes that promote proliferation (cell cycle genes like MYC) and oppose cell death (BCL2, BCLXL, MCL1, BIRC5). Additionally, STAT3 and STAT5 can promote stem cell-like characteristics, metastatic potential, and alter metabolism by canonical and non-canonical mechanisms. This includes lipid metabolism, which promotes tumor growth, chemoresistance and alter immune cell function (Zhang et al., 2020). Beyond activation of tyrosine phosphorylation, the action of STAT3 can also be mediated by

serine phosphorylation. In a lung adenocarcinoma model, Ras dependent STAT3 serine phosphorylation is an important driver (Alhayyani et al., 2022).

In addition to cell intrinsic actions (i.e. role of JAKs and STATs in tumor cells themselves), STAT3 in particular has a role in inflammation, wound repair, and fibrosis. The role of non-malignant cells in the tumor microenvironment and stromal cancer-associated fibroblasts can drive development and progression of tumors by alterations in extracellular matrix production, angiogenesis, and metabolic milieu.

Importantly, IFNs have complex roles in cancer. A recent review summarizes evidence on how cancer cells control their own synthesis of IFN-I and how these cells control responses to the IFN-I that is present in the tumor microenvironment (Cheon et al, under review). In addition, the importance of IFN-I as a regulator of the immune system in cancer has been well reviewed by others recently (Snell et al., 2017; Yu et al., 2022). Namely, cancer cells minimize the pro-apoptotic and anti-growth activities of high concentrations of IFN-I and maximize the pro-tumor effects of low concentrations of this important cytokine. In many tumors, STAT1 and STAT2 lacking tyrosine phosphorylation (U-STATs) and IRF9 are expressed constitutively at high levels, forming U-ISGF3, which drives the expression of most ISGs, some of which encode important pro-tumor proteins. The levels of U-ISGF3 are low in normal cells but are increased by IFN-I (Cheon et al., 2021; Cheon *et al.*, 2013). In many cancers, high levels of U-ISGF3 correlate with resistance to DNA damage by inducing the expression of “Interferon-Related DNA Damage resistance gene Signature”, or IRDS (Weichselbaum et al., 2008). In lung cancer cells, high levels of U-STAT2 promote an aggressive phenotype and confer resistance to cisplatin-induced cell death (Nan *et al.*, 2018).

PD-L1, often expressed on the surfaces of tumor cells and well recognized for its importance in inactivating cytotoxic T cells, also has important tumor cell-intrinsic activities, including dampening acute responses to cytotoxic high levels of IFN-I and sustaining the expression of the low levels of IFN-I that benefit tumors (Cheon *et al.*, 2021).

Fortunately, many strategies are being employed to optimize immuno-oncologic therapies, but in this short discussion, it should be very clear that the roles of cytokines and the JAK/STAT pathway in cancer are anything but simple.

Therapeutic targeting of the JAK/STAT pathway

JAK inhibitors.—Unsurprisingly, the discovery of JAKs and strong genetic evidence demonstrating their indispensable role provoked consideration of JAKs as therapeutic targets (Russell *et al.*, 1995). Ten jakinibs are now approved to treat a wide, and still expanding, variety of disorders driven by both innate and adaptive immune mechanisms (Table II).

Tofacitinib was the first jakinib used *in vivo* using a model of allograft rejection (Changelian et al., 2003). However, ruxolitinib, a JAK1/JAK2 inhibitor, was the first jakinib to receive approval for treatment of MPN based on the discovery of JAK2 GOF mutations. Fedratinib and pacritinib are also approved for MPN. Ruxolitinib is also approved for acute and chronic graft-versus-host disease and topical ruxolitinib is approved for atopic dermatitis and vitiligo. Tofacitinib was the first jakinib approved for immune-mediated disease, including

multiple forms of arthritis and inflammatory bowel disease. Baricitinib is approved for the treatment of RA, alopecia areata and COVID-19. Several other jakinibs have now been approved for other forms of immune-mediated arthritis, allergic disorders, and inflammatory bowel disease. Oclacitinib is approved to treat allergic dermatitis in dogs. Currently, numerous clinical trials are ongoing for systemic lupus erythematosus, Sjogren syndrome, sarcoidosis, psoriasis, hidradenitis suppurativa, asthma and malignancies.

Most of the adverse events observed so far can be explained by the known mechanism of action of specific cytokines with infections, anemia, and cancer, and the safety profile is largely comparable to biologics. Additional adverse interactions include increased risk of thromboembolic events, alteration in lipids, and cardiovascular complications.

Selective JAK inhibitors were developed with the motivation to interfere with the function of fewer cytokines, increase specificity, and ideally fewer adverse effects. These include JAK1 inhibitors (upadacitinib, filgotinib, and abrocitinib), TYK2 inhibitors (ropsacitinib/PF-06826647 and NDI-031407 (Gracey et al., 2020)) and deucravacitinib, an allosteric JAK2 inhibitor that targets the PK domain (Burke et al., 2019). Deucravacitinib is approved for that treatment of plaque psoriasis. An allosteric JAK1 inhibitor that targets the PK domain has also been generated (https://doi.org/10.1038/s41589-022-01098-0). In addition, topical and inhaled jakinibs are being generated to reduce side effects associated with systemic administration. Targeted protein degradation is also now an option for generation of kinase inhibitors including JAK1 (Kavanagh et al., 2022).

STAT inhibitors.—Various strategies have been employed to develop STAT inhibitors, including interfering with SH2 mediated dimerization, DNA binding, or transactivation and inducing STAT degradation. Multiple inhibitors are in clinical trials, but no agents have been approved at this time.

SOCS mimetics.—SOCS peptidomimetics have shown efficacy in immune and tumor preclinical models (Ahmed et al., 2015; La Manna et al., 2018).

Challenges and opportunities ahead

We hope that our summary has convinced the reader that the discovery of the JAK-STAT pathway 30 years ago brought a wealth of information that directly impacts human health – including treatment of COVID-19. Yet, it would be misleading to not consider the challenges ahead – particularly since the JAK-STAT pathway is a tractable tool that provides many opportunities for future investigation. In the brief section below, we reflect on a few topics, recognizing that we are just touching the tip of the iceberg – the reader may well come to different conclusions regarding priorities going forward.

Structural and cell biology JAKs, STATs and cytokine signaling.—The structural discoveries pertaining to JAK1 and STATs are illuminating but equally striking is the paucity of structural information regarding other JAKs complexed with cytokine receptors and other STATs. With the advances in imaging modalities, we clearly have a great deal to learn. Using more sophisticated tools to analyze early events in cytokine signaling, following STAT activation from membrane to the nucleus and regulation of transcription is likely

to be revealing, as the canonical model of STAT phosphorylation inducing dimerization that activates nuclear translocation does not take into consideration the elaborate dynamic movements of STATs through nuclear pores in both the cytokine-activated and steady states. STAT protein associations with diverse organelles is yet to be fully appreciated functionally and physically. Capitalizing upon the many mutations in JAKs and STATs identified in human immune and malignant disorders and deciphering their mechanisms of action is likely to a useful strategy.

Towards a more complete understanding of STAT-mediated chromatin modifications and transcriptional regulation.—Dynamic modifications of complex enhancer structure, enhancer transcription, lncRNAs, histone modifications and chromatin looping and interactions in topologically associating domains (TADs) afford many opportunities for precise tissue-specific, temporal and dynamic regulation of gene expression (Figure 6). The impact of cytokines and STATs on these events is becoming clearer (Kanno et al., 2012; Platanitis et al., 2022). Furthermore, while STATs are prime examples of signal-dependent transcription factors, the documented actions of nonphosphorylated STAT1 illustrate its constitutive actions beyond a role as a signal-dependent transcription factor. How U-STAT1 modifies chromatin and the three-dimensional genome will be of interest and the extent to which other STATs have this capacity needs to be explored.

An alternative model that explains the function of complex enhancers beyond chromatin looping and the proximity of enhancers and promoters is the phenomenon termed liquid-liquid phase separation (LLPS) (Banani et al., 2017; Hnisz et al., 2017; Hyman et al., 2014). Occupancy of transcription factors at defined regulatory regions with concomitant recruitment of coactivators and transcriptional machinery complexes with intrinsically disordered regions may trigger demixing and condensate formation. STATs appear to participate in the process (Figure 6) and are endowed with characteristics conducive to demixing: high valency (ability to heterodimerize; phosphorylation) and nucleation perhaps initiated by high STATs density at enhancers (Zamudio et al., 2019). Detailed understanding of the structural and functional contributions of STATs to biophysical aspects of phase separation will be important, again taking advantage of germline and somatic GOF STAT mutations. Parenthetically, an area that has not been pursued in detail is the role of nuclear JAK2 and its ability to phosphorylate histones (Dawson et al., 2009; Dawson et al., 2011).

Achieving cell, tissue, and stimulus-specific programs.—Perhaps the greatest challenge is to understand how signals generated by a vast array of cytokines are interpreted in cells that use a limited cassette of JAKs and STATs. There is no simple response to this challenge at this time, but we will offer some brief reflections that may be considered. Deciphering how cell signaling provides specific instructions is not a problem unique to cytokines; rather, given its simplicity, the JAK-STAT pathway may have many attributes that can resolve this fundamental biological conundrum.

A first consideration is specificity and regulation of expression for signaling components. JAKs and STATs are broadly expressed, but some have more selectivity, e.g., JAK3, TYK2, and STAT4 are preferentially in immune cells. STATs also directly and dynamically regulate their own expression, STAT1 being a particularly good example. Thus, in the course of

infection, STAT4 initially mediates type I IFN signaling but later STAT1 predominates (Nguyen *et al.*, 2002). Along with cytokine receptors, the dynamic expression of JAKs, STATs, SOCS proteins and other downstream elements need to be considered in analysis of output. In this respect, both the strength and quality of the signaling can evolve during development or acutely during infections.

Second, STAT activation dynamics have been studied, but most cytokines activate more than one STAT family member with different amplitudes of activation, rates of phosphorylation and dephosphorylation, nuclear translocation and DNA binding. These measures are often not comprehensively and rigorously determined over time and improved techniques for quantitatively assessing these changes are needed. Notably, engineered cytokines and cytokine antagonists have been generated that alter these variables and are likely to be useful tools (Ren et al., 2022; Spangler et al., 2015; Yen et al., 2022). Formation of STAT heterodimers has been suggested as another way to explain specificity but is often overlooked (Delgoffe and Vignali, 2013). Notably, while genomic analysis of motifs bound by STATs always reveals the canonical palindromic motifs, a substantial proportion of STAT-bound regions depart from the classical motif. How STATs are recruited in the absence of a cognate DNA binding motif remains to be elucidated. What underlies binding to noncanonical sites?

Third, as mentioned, many stimulus-specific STAT post-translationally modified states can occur that influence intracellular trafficking, DNA binding and transcriptional potential; however, these modifications are typically not comprehensively and dynamically measured (Myers and Gottschalk, 2022). Almost no information has been provided regarding post-translational modifications of JAKs in response to different stimuli over time. This is an important topic that needs further investigation.

Finally, the diversity of cytokine receptors allows considerable variability in their engagement of pathways as discussed above (e.g., MAPKs, PI3K, etc.). During developmental, inflammatory, or other events, cells are not impacted by a single cytokine; multiple exogenous, endogenous and inducible signals participate in the outcome. Multiple SDTFs, LDTFs, and inducible TFs beyond STATs are also deployed. In this respect, STATs coordinate with other factors cooperating and antagonist pathways. Added to this is the complexity of enhancers and opportunities for dense binding of multiple factors.

Although complex, context-specific regulation is needed to achieve specificity, an obvious solution to achieving specific interpretations of signals relates to combinatorial logic. While this may seem like a daunting proposal, the generation of multiomic tools now facilitates comprehensive measurement of changes over time, with minimal manipulation of single cells responding to physiologic and relevant pathologic challenges in vivo. In short, there’s lots to do – but the tools are so much better now. Based on precedent, more sophisticated knowledge of JAKs and STATs will unquestionably offer many opportunities for new drugs with greater specificity. If you’re not already working on the JAK-STAT pathway presently, you should be thinking about doing so.

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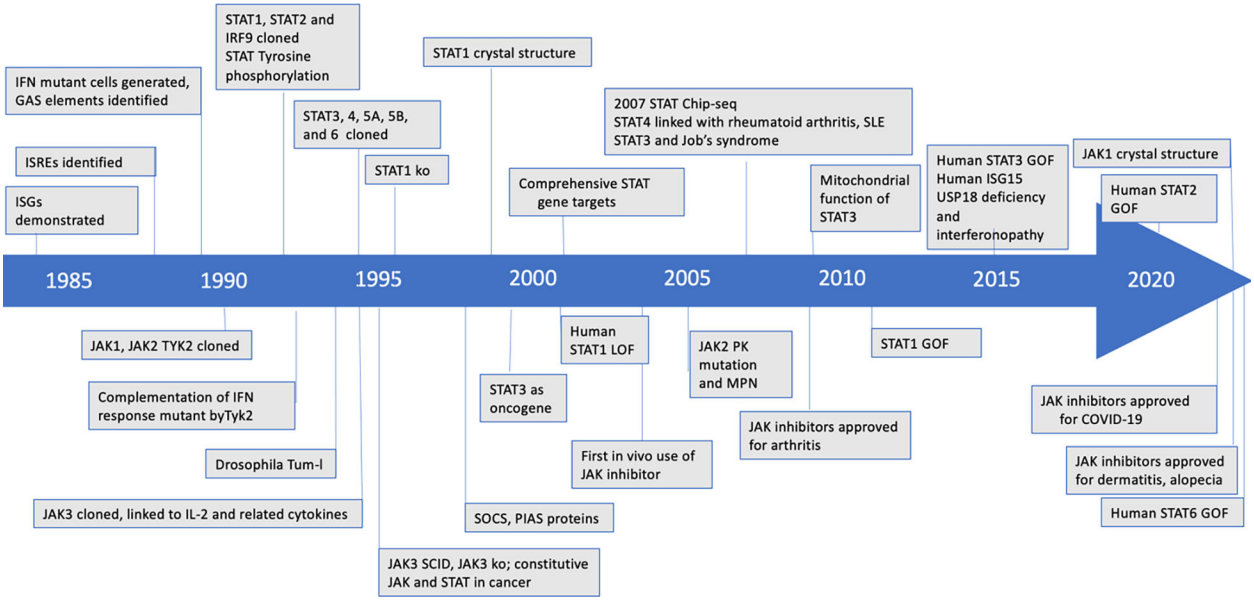

Figure 1. JAK-STAT Timeline.

This timeline highlights various milestones in understanding the JAK-STAT pathway since 1984. GAS, gamma activated sequence; IFN, interferon; ISGs, IFN stimulated genes, ISRE, interferon-sensitive response element; SCID, severe combined immune deficiency; KO, knockout; SLE, systemic lupus erythematosus; PK, pseudokinase; MPN, myeloproliferative neoplasms; GOF, gain-of-function; SOCS, suppressor of cytokine signaling; PIAS, protein inhibitor of activated STAT, Tum-l, tumorous lethal.

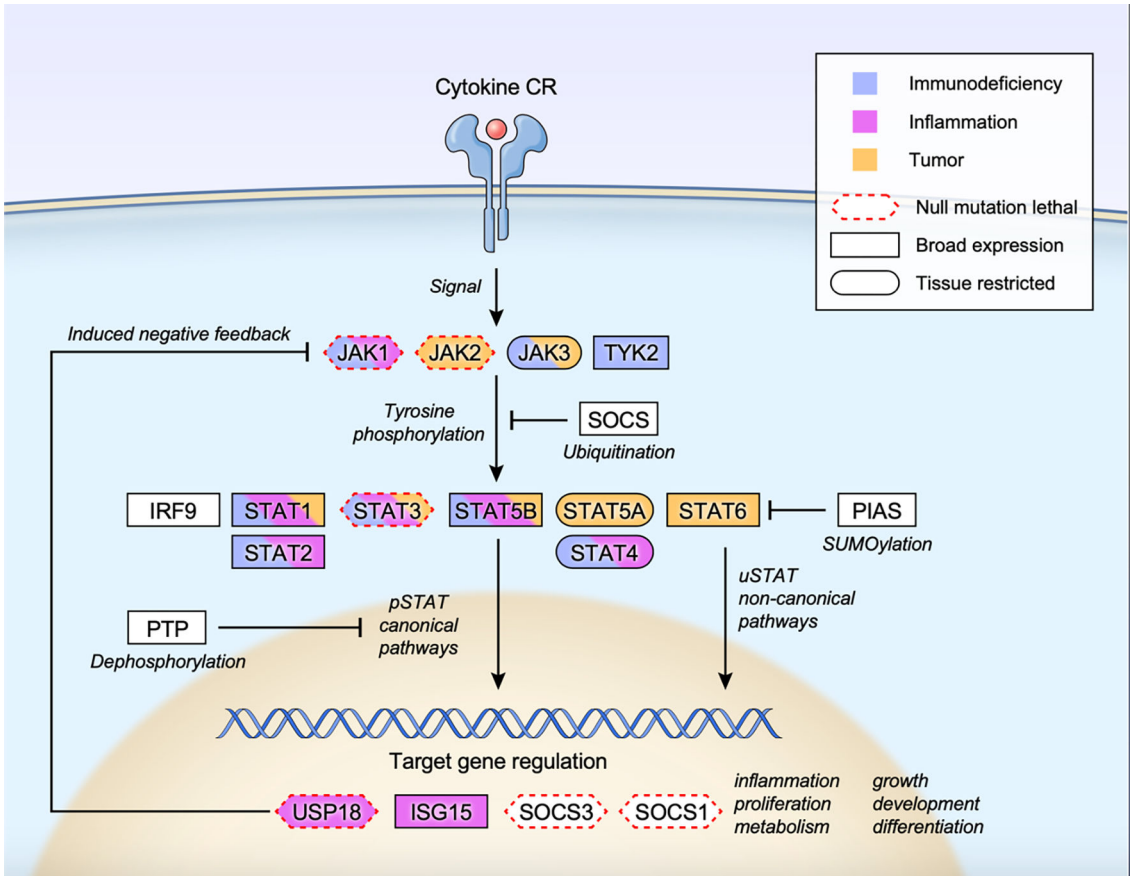

Figure 2. Overall scheme of JAK STAT signaling.

Cytokine-receptor engagement transmits signal to tyrosine phosphorylate and activate receptor associated JAKs, which subsequently phosphorylate and activate STATs. Tyrosine phosphorylated STATs (pSTATs) form dimers, translocate to the nucleus, bind to target DNA sequences, and regulate gene expression (pSTAT canonical pathways). Unphosphorylated STATs also form dimers, enter the nucleus and regulate transcription (uSTAT non-canonical pathways). Multiple inducible negative feedback systems are in place to constrain the signaling circuit that include SOCS (suppressor of cytokine signaling) family proteins, PIAS (protein inhibitor of activated STAT) family proteins, PTP (protein tyrosine phosphatase), USP18 and ISG15. Human monogenic mutations that lead to gain-of-function and/or loss-of-function phenotypes are color coded as immunodeficiency (blue), inflammation (pink) or tumor (orange) for each constituent gene. Genes whose null mutation in mice leads to lethality is marked with red dotted border (Mouse Genome Informatics; http://www.informatics.jax.org). Tissue expression pattern of each protein is also marked as broad expression (square border) or tissue restricted (oval border) (The human protein atlas; https://www.proteinatlas.org/search).

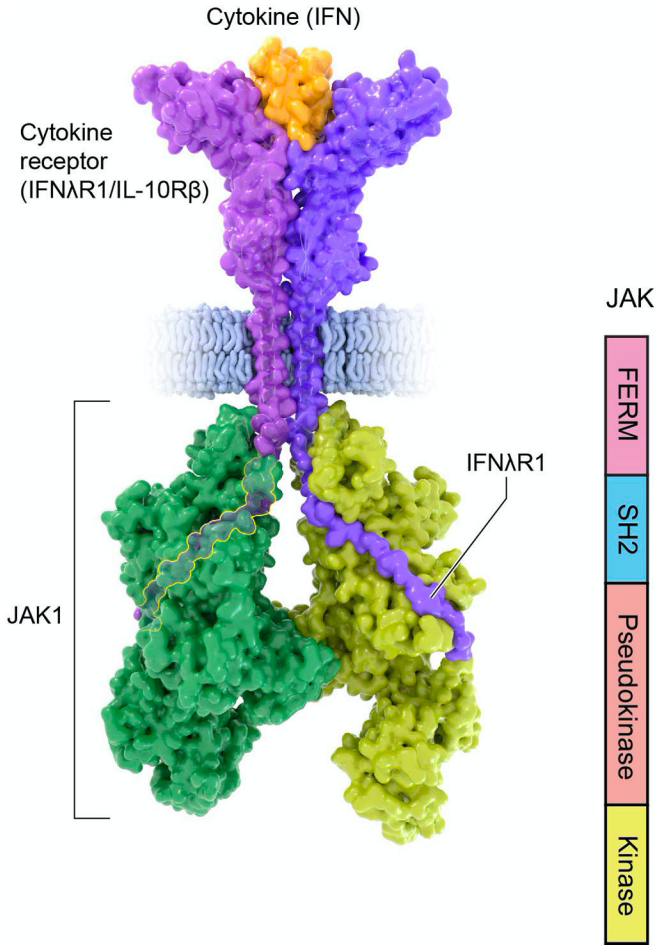

Figure 3. Structure of Janus kinases.

Structure of activated Janus Kinase dimer (green; PDB 7T6F) complexed with the intracellular domain of matured IFNlR1/IL-10Rb (purple) bound to IFN-l (orange) (PDB 5T5W). Of note, the intracellular portion of the receptor binds JAK1 FERM and SH2 domains through N-terminal Box1 and C-terminal Box2 motifs. The JAK kinase-like or pseudokinase domain promotes dimerization of the cytokine receptor/JAK complex.

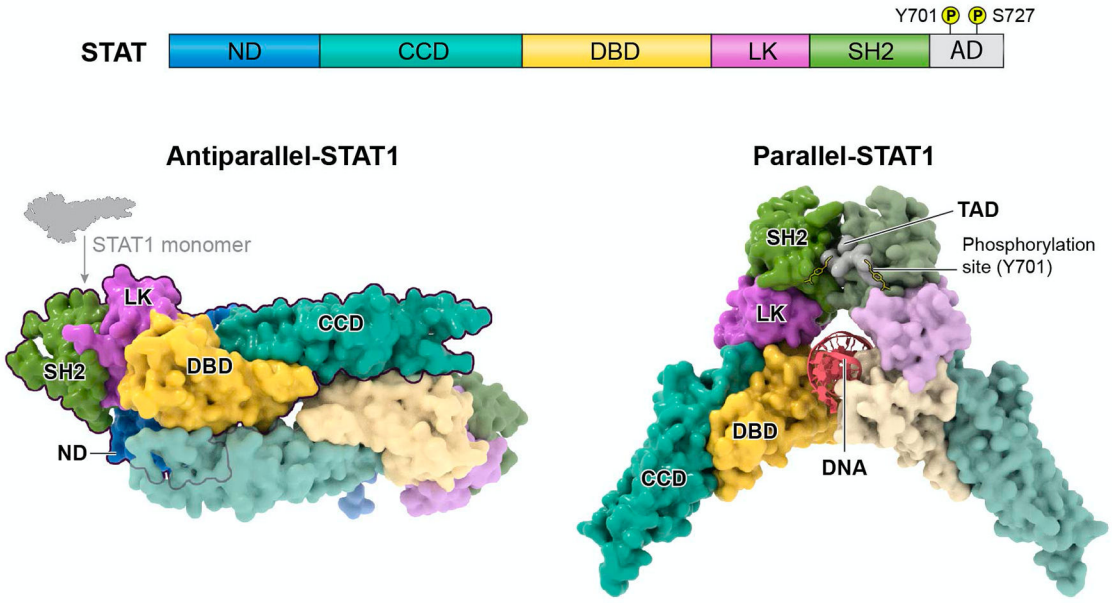

Figure 4. STAT domains and structure.

STAT proteins contain the following domains: N-terminal domain (ND), coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (LK), Src Homology 2 domain (SH2), and transactivation domain (AD) (upper panel). Key phosphorylated tyrosine and serine amino acids reside within the AD for STAT dimerization and functional modulation. Below are the structures of STAT1 dimers, specifically, in its unphosphorylated anti-parallel conformation (PDB 1YVL) and phosphorylated parallel conformation bound to DNA (PDB 1BF5). Notably, the antiparallel confirmation prevents the DBD from interacting with DNA through reciprocal interaction with the opposing dimer's CCD. Like STAT1, parallel structures of homodimers bound to DNA have been shown for STAT3 (PDB 1BG1) and STAT6 (PBD 4Y5W) and anti-parallel confirmation of STAT dimers have been described for STAT3 (PDB 6TLC) and STAT5a (PDB 1Y1U).

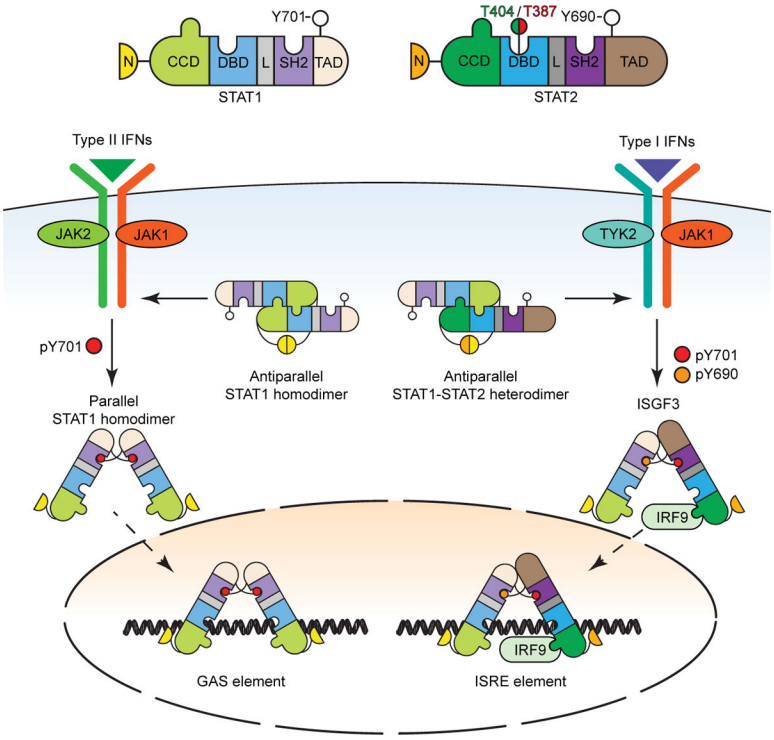

Figure 5. Conformational changes of STAT1 and STAT2 complexes.

See Figure 4 for definitions of the STAT structural domains. Structural insights indicate that the STAT1 homodimer and the STAT1:STAT2 heterodimer each adapt two conformations, termed parallel and antiparallel. The parallel dimers are stabilized by interactions between the SH2 domains and the phosphorylated tyrosine residues, whereas the antiparallel dimers are stabilized by ND interactions. In addition to the tyrosine phosphorylations, two newly discovered phosphorylations of T387 and T404 of STAT2 regulate U-STAT1:U-STAT2 heterodimer stability. See the text for details.

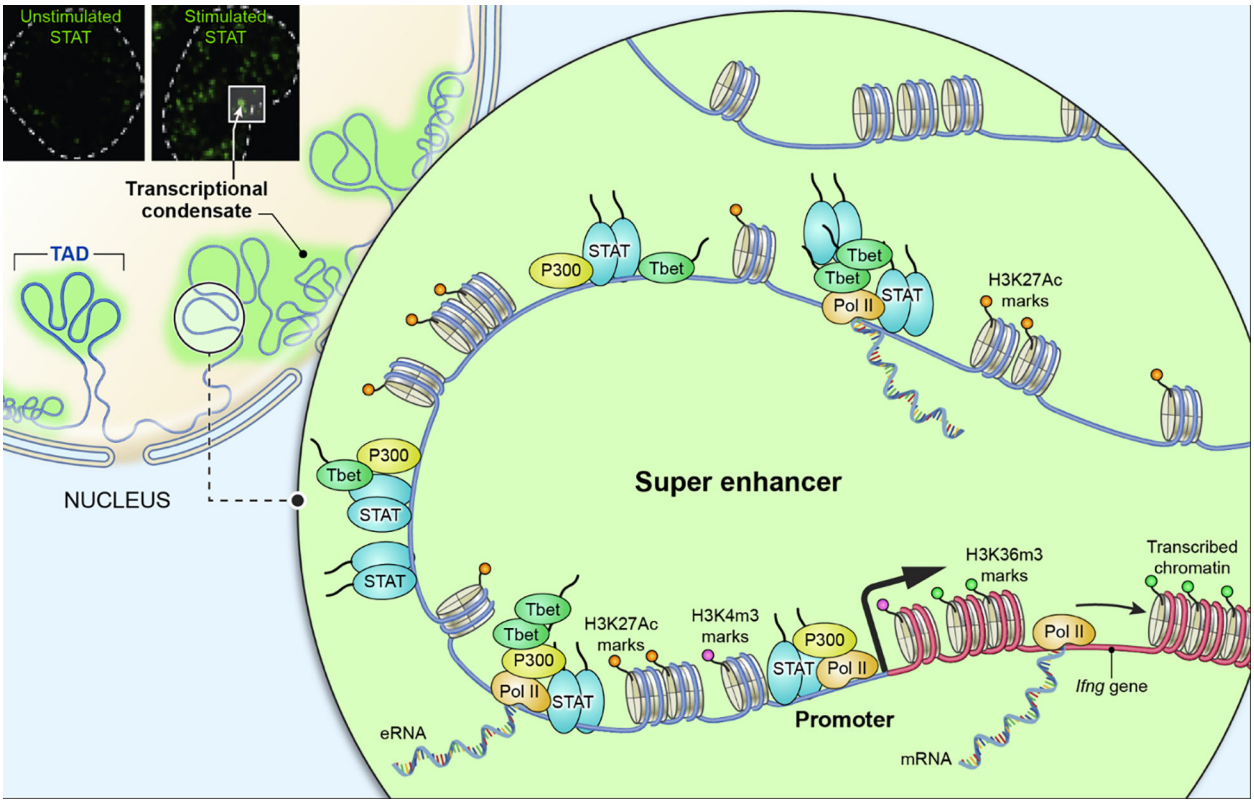

Figure 6. STAT and chromatin organization.

STATs bind to DNA at epigenetically marked promoters and enhancers of target genes to regulate transcription. Distinct histone epigenetic marks are observed at promoters (H3K4 trimethylation), enhancers (H3K27 acetylation) and pol II transcribed region (H3K36 trimethylation). As an example, the *Ifng* locus is depicted, which is regulated by a super enhancer composed by multiple tandem enhancer elements occupied by and enriched for STAT, T-bet, as well as p300, in response to cytokine stimulation. Confocal microscopy (upper left corner) documents signal dependent aggregation of STAT transcriptional nuclear condensates (Zamudio *et al.*, 2019). Super enhancers, transcriptional condensates, and topologically associating domains (TADs) may all converge into a functional unit establishing a physical platform to regulate dynamic transcription of target genes.

Table I.

JAK STAT pathway genetics and in vivo functions in mice and humans

	Knockout phenotype	Mendelian Disorders	Somatic GOF mutations	GWAS links
JAK1	Perinatal lethality Impaired responses to multiple cytokines including type I and II IFNs, IL-2, IL-4, IL-10, IL-27	LOF: Mycobacterial disease, warts parasitic and fungal infection Progressive T lymphopenia, increased IgG and IgA levels, GOF: atopy, autoimmunity	Acute lymphoblastic leukemia Acute myeloid leukemia	JIA, MS, autoimmune thyroid disease, CRP levels, acute myeloid leukemia, lymphocyte and eosinophil counts, body mass and height
JAK2	Embryonic lethal, absence of definitive hematopoiesis		Myeloproliferative neoplasms: Polycythemia vera, Essential thrombocythaemia Acute lymphoblastic leukemia Acute myeloid leukemia Chronic myelogenous leukemia	SLE, allergy, eczema, AS, RA, T1DM, Psoriasis, IBD, cholesterol level, eosinophil basophil platelet erythrocyte count, body height
JAK3	Defective T and B cell development, Impaired responses to IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 immunodeficiency	LOF: T-B+NK- SCID	Myeloproliferative neoplasms Acute lymphoblastic leukemia Myelomonocytic leukaemia NK Lymphoma/Leukemia T cell Lymphoma/Leukemia	
TYK2	Bacterial and viral infection Impaired responses to type I interferon, IL-12, IL-23 and IL-10	LOF: Mycobacterial disease and infections with other bacteria and viruses		SLE, RA, JIA, psoriasis, AS, IBD, MS, T1DM, T2DM, COVID-19, mycobacterial susceptibility, platelet and lymphocyte count,
STAT1	Bacterial and viral infection	LOF: Mycobacterial disease, viral infections GOF: Autoimmunity Mucocutaneous candidiasis, Invasive fungal infections, viral susceptibility	T cell Lymphoma-Leukemia Other hematopoietic and solid organ cancers	SLE, JIA, reticulocyte count, eosinophil and neutrophil count, brain measurement, body height
STAT2	Impaired type I IFN responses Viral susceptibility	LOF: Severe viral infections mitochondrial abnormalities,	Hematopoietic and solid organ cancers	Psoriasis, psoriatic arthritis, diabetic retinopathy, lymphocyte count, height
STAT3	Impaired placental development, defective signaling by multiple cytokines	LOF: Hyperimmunoglobulin E recurrent infection syndrome GOF: Multisystem autoimmune disease	NK Cell Lymphoma-Leukemia T cell Lymphoma -Leukemia B cell lymphoma Myelodysplastic syndrome Solid organ tumors (Head and neck, breast, skin, gastrointestinal, neural cancers)	eczema, IBD, MS, SLE, T2DM, Mean corpuscular volume, CRP, blood pressure, mosquito bite reaction,
STAT4	Impaired IL-12 and IL-23 signaling, and type 1 immune responses	LOF: fungal disease	Hematopoietic and solid organ cancers	SLE, RA, Sjogren's syndrome
STAT5A	Defective mammary gland development and impaired GM-CSF signaling		Leukemia Lymphoma Solid organ tumors (Head and neck, breast, skin, gastrointestinal, neural, prostate cancers)	Asthma, cholesterol, bone density, obesity
STAT5B	Impaired growth, liver gene expression, innate lymphocyte homeostasis	Growth hormone insensitivity, immunodeficiency, autoimmunity atopy	Acute and chronic lymphocytic leukemia Lymphoma Large granular lymphocytic leukemia Solid organ tumors (Head and neck, breast, skin, gastrointestinal, neural, prostate cancers)	Asthma, allergy, cholesterol level, neutrophil, eosinophil, and lymphocyte count, bone density, blood pressure, obesity

	Knockout phenotype	Mendelian Disorders	Somatic GOF mutations	GWAS links
			Atopy, autoimmunity, eosinophilia	
STAT6	Impaired IL-4 and IL-13 signaling, and type 2 immune response	GOF: treatment resistant allergic disease	B Cell Lymphoma Follicular lymphoma Other hematopoietic and solid organ cancers	Asthma, allergy, eczema,eosinophil count, IgE levels, mosquito bite size,

AS- ankylosing spondyloarthritis; CRP-C-reactive protein, GOF – gain of function; IBD – inflammatory bowel disease, JIA – juvenile idiopathic arthritis; LOF – loss of function; MS – multiple sclerosis; RA- rheumatoid arthritis; SCID – severe combined immunodeficiency; SLE-systemic lupus erythematosus, T1DM – type 1 diabetes mellitus; T2DM-type 2 diabetes mellitus; (https://www.ebi.ac.uk/gwas)

Table II.

Approved JAK inhibitors

Indication	Drug
Rheumatoid arthritis	Tofacitinib, Baricitinib, Upadacitinib, Filgotinib, Peficitinib
Psoriatic arthritis	Tofacitinib, Upadacitinib
Juvenile arthritis (> 2 yrs)	Tofacitinib
Ankylosing spondyloarthritis	Tofacitinib, Upadacitinib
Ulcerative Colitis	Tofacitinib, Filgotinib,
Atopic Dermatitis	Baricitinib, Upadacitinib, Abrocitinib, Delgocitinib (topical), Ruxolitinib (topical), Oclacitinib (dogs)
Alopecia Areata	Baricitinib
Graft Versus Host Disease	Ruxolitinib
Myeloproliferative neoplasms	Ruxolitinib, Fedratinib, Pacritinib
Covid-19	Baricitinib
Vitiligo	Ruxolitinib (topical)
Plaque Psoriasis	Deucravacitinib