Chapter

Gene Activation by the Cytokine-Driven Transcription Factor STAT1

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Abstract

Signal transducers and activators of transcription (STATs) are a family of cytokine-regulated transcription factors, which serve the dual role of external signal transduction and transcriptional activation. The founding member of this family, STAT1, is involved in a plethora of cellular processes, including interferon-dependent upregulation of various effector mechanisms in immune and non-immune cells to control bacterial, fungal and parasitic infections. In this chapter, we discuss the principles of STAT1-driven gene expression and focus on the clinical phenotypes of various human STAT1 mutations. In particular, we highlight the significance of sequence-specific DNA binding and intact nucleocytoplasmic shuttling for full transcriptional activation of interferon-driven target genes.

Keywords: signal transducer and activator of transcription (STAT), Janus kinase (JAK), DNA binding, cytokine signalling, gene expression, interferon, gain-of-function mutation

1. Introduction

The origins of the seminal discovery of the intracellular signal transmitters mediating cytokine signalling now date back nearly three decades. In the late 1980s, a group of researchers observed that signal transmission could be induced within minutes after stimulating cells with type I interferons (IFNs). Such speed, with which the signal generated at the plasma membrane-bound receptor was transduced to the nucleus, suggested the presence of only a few intermediate steps. Eventually, the only two players involved were identified, which are receptor-associated Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs) [1]. The STAT proteins comprise a family of evolutionary highly conserved transcription factors, which are thought to have evolved with the development of the first multicellular organisms [2, 3]. They are expressed in various metazoan animal species, including nematodes, insects and vertebrates [4–9]. In mammals, seven different STAT proteins have been identified, namely STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. The STATs are differentially activated by a variety of almost 50 extracellular signalling molecules, including interferons, interleukins, growth factors and hormones [10] and are involved in a plethora of biological processes, including cell differentiation, proliferation, development, immunity and apoptosis [11]. STAT1, the founding member of this

family, is the major signalling molecule activated by IFN-α/β and IFN-γ and plays a pivotal role in mediating immune responses to infectious pathogens. It is essential for the direct activation of various immune effector genes, including those coding for microbicidal molecules, antiviral proteins, antigen-presenting molecules, phagocytic receptors, chemokines and cytokines [12]. Not surprisingly, humans expressing STAT1 variants with nonsense or missense mutations often exhibit an increased susceptibility to bacterial and viral infections.

2. Design principles of the JAK/STAT signalling pathway

STAT signalling is a paradigm of a ligand-induced pathway from cell surface receptors to the transcriptional machinery, thereby connecting extracellular signals to the regulation of eukaryotic gene expression. The activation of STAT proteins and their mechanisms of action are unique in many ways: (i) This pathway represents one of the first examples of direct signalling to the nucleus without the involvement of second messengers, (ii) post-transcriptional modification by tyrosine phosphorylation is a hallmark feature of this pathway and, (iii) STATs make direct contacts with both membrane-bound receptors and DNA, thereby integrating cellular processes at the membrane to nuclear events. The basic model of STAT signalling depends on a cascade of tyrosine phosphorylation steps, as shown in **Figure 1** [11]. Binding of the ligand to its cognate cell surface receptor triggers the dimerization or multimerization of the transmembrane receptor subunits. Conformational changes in the receptor complex bring the non-covalently attached Janus kinases (JAKs) into close spatial proximity to each other and allow their trans-phosphorylation on specific tyrosine residues. As a result, the JAKs are activated and, in turn, phosphorylate specific tyrosine residues on cytoplasmic receptor domains, thereby creating docking sites for latent, cytoplasmic STAT molecules which bind through their src-homology-2 (SH2) domain. All mammalian STAT proteins bear a conserved signature tyrosine residue near their C-terminus, which becomes phosphorylated by the JAKs. Upon this modification, they dissociate from the receptor complex and immediately dimerise via reciprocal phosphotyrosine (pY)-SH2 domain interactions. With the exception of STAT2, all human STAT proteins form homodimers. In addition, STATs are frequently engaged in heterodimer formation, for example, STAT1:STAT2 and STAT1:STAT3, and the amount of heterodimeric STATs depends on the nature and concentration of the activating ligands.

Due to their large protein size (~180 kDa for the STAT1 dimer), nuclear import of activated STATs requires a carrier-facilitated transport process through the nuclear core complex. Nuclear trafficking of tyrosine-phosphorylated STAT is mediated by importin-α proteins [13]. Tyrosine-phosphorylated STAT1 dimers are translocated as cargo proteins by a mechanism depending on importin α-5:importin-β1 and the small G protein Ran [14–16], whereas nuclear import of STAT3, STAT5 and STAT6 appears to be mediated primarily by interaction with other members of the protein family of karyopherins [17, 18]. Once in the nucleus, the STAT proteins act as classical transcription factors and bind to specific regulatory DNA sequences to activate or repress the transcription of their target genes. All members of the STAT family bind to a palindromic consensus motif termed gamma-interferon activated sequence (GAS) (5′-TTCN_3GAA-3′). A notable exception is STAT2, which appears to be defective in DNA binding and instead associates with STAT1 and interferon-regulatory factor 9 (IRF9) in a ternary complex, which is called interferon-stimulated gene factor 3 (ISGF3) [19]. This transcriptionally active complex binds to a distinct direct repeat motif (5′-AGTTTCN_2TTTC-3′) termed interferon-α-stimulated response element (ISRE).

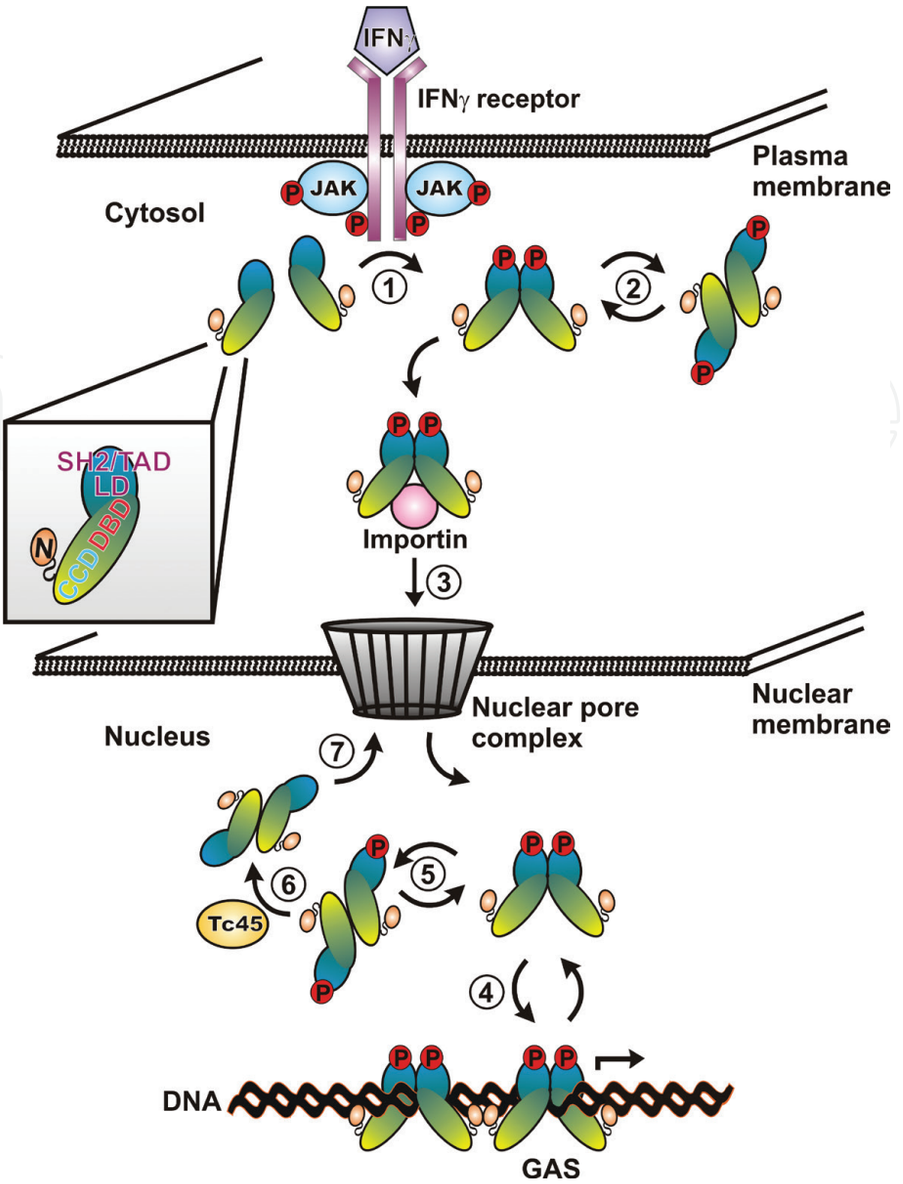

Figure 1.
Model of the interferon-γ-induced JAK/STAT signal pathway depicting the activation-inactivation cycle of STAT1. Binding of IFN-γ to the cell surface receptor triggers JAK-induced tyrosine phosphorylation of STAT1 (1). Through spontaneous dissociation and reassociation, the activated STAT1 molecules constantly oscillate between the parallel and antiparallel dimer conformation (2). Phosphorylated dimers are translocated to the nucleus via binding to importins (3) where they bind to gamma-interferon activated sequence (GAS) motifs, potentially polymerise and induce gene transcription (4). After a conformational shift (5), antiparallel dimers are susceptible to dephosphorylation by the nuclear phosphatase Tc45 (6) and thereafter exit the nucleus (7).

The kinetics of STAT-mediated gene transcription is strictly regulated in both the cytoplasmic and nuclear compartment. The inactivation of nuclear STAT proteins rapidly occurs following nuclear import and initiation of gene transcription. This process has been best studied for the founding member of this family, STAT1. When not contacting DNA, STAT1 dimers undergo a conformational change, which results in the exposure of the critical phosphotyrosine residue at position 701 [20–23]. In this conformation, STAT1 is highly susceptible to dephosphorylation by the nuclear isoform of the T-cell protein tyrosine phosphatase (Tc45) [24–26]. It has been demonstrated that Tc45 induced dephosphorylation is a prerequisite for STAT1 to exit the nucleus [27]. As shown for STAT1 and STAT3, nuclear export is assisted by the exportin CRM1 (chromosome region maintenance 1), which acts in a Ran-dependent manner [28–32]. Back in the cytosol, STATs can then participate in additional cycles of cytoplasmic re-activation, nuclear import

and inactivation, depending on the activation status of the cytokine receptor at the plasma membrane [33].

The rapid onset of JAK/STAT signalling is followed by subsequent decay, which includes the inactivation of cytokine receptors and JAKs, leading to a decrease in transcriptional activity. Apart from inactivation by nuclear phosphatases, STAT-mediated signal transduction is tightly controlled by additional negative regulators. A well-studied inhibitory mechanism involves the upregulation of proteins, collectively termed suppressors of cytokine signalling (SOCS) [34, 35]. These inhibitory proteins are components of a classical autoregulatory feedback loop, since most SOCS protein-encoding genes are well-established STAT targets. The SOCS proteins are generally expressed at low levels in resting cells but become rapidly induced after key stimulus exposure. The SOCS protein family comprises SOCS1–7 and the cytokine-inducible SH2-domain-containing protein (CIS), which counteract the JAK/STAT signalling by distinct ways. For example, SOCS1 binds to the catalytic subunit of the receptor-associated JAKs [36], while SOCS3 binds additionally to the receptor [37]. Both processes result in the inhibition of JAK activity and prevent further STAT activation. In contrast, CIS and SOCS2 have been proposed to suppress STAT activation by directly competing with STATs for binding to receptor docking sites [38, 39]. Another antagonistic mechanism involves protein inhibitors of activated STAT (PIAS) [40]. The family members of PIAS proteins are thought to directly bind to activated STAT dimers and show specificity as well as redundancy in their action: PIAS1, PIAS3 and PIASx bind to STAT1, STAT3 and STAT4, respectively [41–43], while PIASy interacts with STAT1 [44]. They employ distinct mechanisms to repress STAT-dependent gene transcription, for example, PIAS1 and PIAS3 block the DNA-binding activity to prevent STATs from binding to their target promoters. In contrast, PIASx and PIASy act as transcriptional co-repressors by recruiting other co-repressors, including histone deacetylases (HDACs) to stop the initiation of transcription. Interestingly, unlike other general negative regulators, PIAS1 does not counteract the entire STAT1-induced gene repertoire but rather selectively inhibits only a subgroup of genes [45].

3. Structure and conformations of dimeric STATs

Mammalian STATs are composed of 750–850 amino acids, and their molecular weights range between 80 and 113 kDa. Biochemical, genetic and structural studies have revealed that STAT family members share the same modular architecture with six conserved functional domains organised in three independently folded structural units (**Figure 2**). The characteristic domains are the amino-terminal domain (ND) involved in protein-protein interactions and the core fragment which is composed of a coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (LD) and SH2 domain, followed by the carboxy-terminal transactivation domain (TAD) [46, 47]. The N-domain and the transactivation domain are connected to the core fragment through short flexible linkers, of which the C-terminally

Figure 2.
Domain structure of STATs. STAT proteins are composed of conserved functional domains: ND: amino-terminal domain, CCD: coiled-coil domain, DBD: DNA-binding domain, LD: linker domain, SH2: src-homology-2 domain, TS: phosphotyrosyl tail segment with the specific phosphorylation site, TAD: transactivation domain.

located one harbours the conserved tyrosine residue in a stretch referred to as the phosphotyrosyl tail segment (TS). The overall similar structure and high conservation among the STAT proteins reflect similarities in activation, dimerization and DNA binding. The two most conserved structures are the N-domain and the SH2 domain, both of which facilitate protein interactions, for example, dimerization [48], binding of the transcriptional co-activator CBP/p300 [49] or binding to the receptor. The transactivation domain is intrinsically unstructured and undergoes folding transition upon interaction with transcriptional co-activators [50]. Its residues vary considerably among the STAT family members affording divergent ways to activate individual target genes.

The first crystallographic analyses of STAT proteins revealed the structure of the core fragments of phosphorylated STAT1 and STAT3 lacking the N-domain and parts of the transactivation domain [46, 51] (**Figure 3A–C**). Details of the STAT-DNA interaction showed substantial similarities among the various members of the STAT protein family. The crystal structures of unphosphorylated STAT1 (**Figure 3D–F**) and STAT5A dimers, as well as N-domain dimers of STAT4, greatly extended our general knowledge about the STAT proteins [48, 52, 53]. The original concept of latent STATs existing as cytoplasmic monomers [54] was soon challenged by a growing body of compelling evidence arguing for dimeric conformation prior to stimulus exposure. Crystallographic analyses of STAT1 and STAT5A

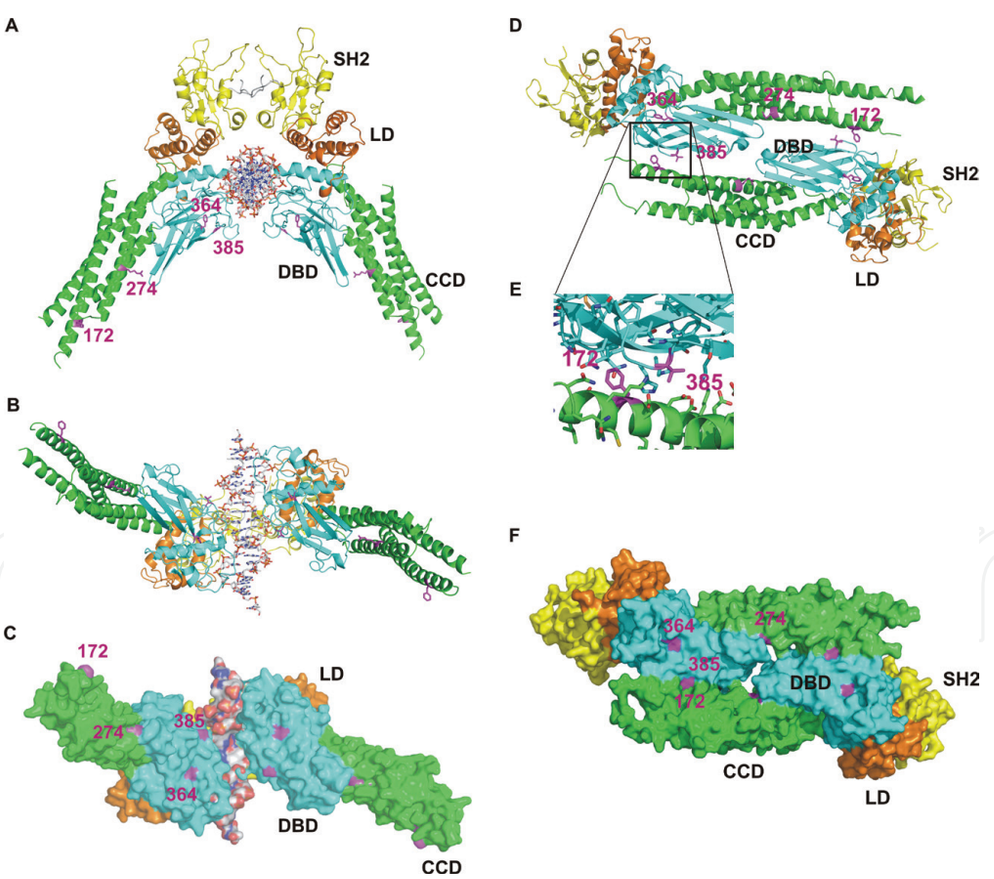

Figure 3.
Localisation of selected gain-of-function mutations within the STAT1 dimer. (A–C): Crystal structure of dimeric STAT1 in parallel conformation bound to DNA including the positions of some important site chains which, when mutated, cause an inborn error. The images show a ribbon diagram (A, B, D, E) or a molecular surface structure (C, F) of a STAT1 core fragment in parallel (A–C) and antiparallel dimer conformation (D–F), respectively. The enlarged section of the ribbon diagram demonstrates the spatial orientation of the residues F172 and T385 (coloured in magenta) in the interdimeric CCD:DBD interface. The selected GOF mutation sites F172, R274, F364 and T385 are marked in magenta. The STAT1 domains are coloured according to the panel in **Figure 2**. *CCD: coiled-coil domain. DBD: DNA-binding domain. LD: linker domain. SH2: src-homology-2 domain.*

revealed unphosphorylated protomers self-assembled in a head-to tail alignment where the SH2 domains project from opposite ends of the dimer (**Figure 3D–F**). In this antiparallel conformation, the extended dimeric interface is formed by reciprocal interactions between the coiled-coil domain of one protomer and the DNA-binding domain of its partner protomer (CCD:DBD) [52, 53].

Despite significant efforts, it is still unclear whether a stable dimerization of unphosphorylated monomers is common to all STATs. In addition to unphosphorylated homodimers, experimental data indicate the existence of STAT1:STAT2 and STAT1:STAT3 heterodimers prior to cytokine stimulation [55, 56]. In contrast to the antiparallel alignment in the absence of stimulation, activation of the JAK/STAT signal pathway results in the formation of dimers in parallel conformation, where the tyrosine-phosphorylated protomers arrange themselves in a highly symmetric head-to-head alignment held together by reciprocal phosphotyrosine (pY)-SH2 domain interactions [46, 51] (**Figure 3A–C**). In this conformation, homotypic STAT dimers bind to DNA without the need of further protein-protein interactions between the two protomers. It has been shown that tyrosine-phosphorylated STAT1 must first become dephosphorylated by nuclear phosphatases to be capable of leaving the nucleus to participate in additional rounds of a cytosolic reactivation and nuclear deactivation [27]. In the parallel conformation, the two phosphorylated tyrosine residues are buried in the opposing SH2 domain pockets, thereby protecting STAT1 from being dephosphorylated. By the conformational rearrangement of the STAT1 dimer from a parallel to an antiparallel alignment, the critical phosphotyrosine residues are subjected to enzymatic dephosphorylation [23].

The molecular details of the transition between the parallel and antiparallel STAT1 dimer conformation are still controversial. Initially, a model was proposed in which the N-terminal domains dimerise and keep the dimer partners held together, while the monomer's core domains rotate around each other after phosphotyrosine-SH2 disjunction [22]. The alternative mechanism, widely accepted today, describes the conformational reorientation as achieved by spontaneous dissociation of the tyrosine-phosphorylated dimer into isolated monomers and their reassociation in antiparallel alignment [20, 57]. Studies indicate that STAT1 constantly oscillates between the two dimer conformations, and the abundance of each conformer is determined by the level of tyrosine phosphorylation. While unphosphorylated molecules exist in monomeric or dimeric, antiparallel conformation, there is a conformational equilibrium between phosphorylated dimers in parallel and antiparallel alignment [20].

4. Nucleocytoplasmic shuttling and transcriptional activity

Initially, STAT proteins were considered to function as latent transcription factors, which translocate to the nucleus and induce gene transcription exclusively in response to stimulus exposure. However, STAT1 and STAT3 were found to be present in the nucleus independent of tyrosine phosphorylation [33, 58, 59]. It became evident that, in contrast to phosphorylated STATs that are actively transported into the nucleus, the nuclear import of unphosphorylated STATs is facilitated by direct interactions with protein components constituting the nuclear pore complex [28]. This carrier-free translocation follows the concentration gradient across the nuclear envelope and, as facilitated diffusion, does not depend on metabolic energy. Thus, STAT proteins are constantly shuttling between the cytoplasmic and nuclear compartment, irrespective of their activation status. For some STAT family members, for example, STAT1, STAT2, STAT3 and STAT6, it has been shown that they play important roles in facilitating gene expression in the absence of stimuli and tyrosine phosphorylation [60–64]. In principle, unphosphorylated STAT1 is able to bind to

DNA, but its binding activity is 200-fold lower than that of activated STAT1 and, therefore, may not have any physiological relevance as a DNA-binding protein without the recruitment of cofactors [20]. In line, unphosphorylated STAT1 was found in a complex with interferon-regulatory factor 1 (IRF1) to bind DNA at the promoter of the *low molecular mass polypeptide 2* (*LMP2*) gene, which contains overlapping binding sites for both transcription factors [65]. The *LMP2* gene encodes a component of the 20S proteasome and is induced by stimulation of cells with IFN-γ, but it is also expressed at low levels in the absence of cytokines [66]. From these experiments, it became clear that unphosphorylated STAT1 functions as a transcription factor, since it is directly responsible for constitutive *LMP2* expression, but it is rapidly replaced by activated STAT1 dimers in response to IFN-γ stimulation [65].

More recently, it was reported that, in comparison to STAT1-deficient knockout animals, the pathogene *Listeria monocytogenes* grows less robustly in knockin mice expressing the $Y^{701}F$ mutant, which is unable to be tyrosine phosphorylated, indicating a potential contribution of unphosphorylated STAT1 to innate antibacterial immunity [67]. Due to a positive autoregulatory loop of STAT1 signalling, expression of unphosphorylated STAT1 molecules is greatly increased after activation of STAT1. While the phosphorylation in response to IFNs lasts only for several hours, newly synthesised unphosphorylated STAT1 persists for several days and results in enhanced signal transduction when the cells are thereafter re-exposed to low doses of IFN [68].

5. Serine phosphorylation and its effect on transcriptional activity

Apart from canonical, tyrosine phosphorylation STAT-driven transcription is also regulated by a number of posttranslational modifications, including serine phosphorylation and sumoylation. All STATs except STAT2 can become phosphorylated at least at one serine residue embedded in a proline-rich sequence in the C-terminal transactivation domain [69]. An evolutionary conserved phosphorylation site includes Ser 727 in STAT1 and STAT3, Ser 721 in STAT4, Ser 725 in STAT5A, Ser 730 in STAT5B and Ser 756 in STAT6 [70, 71]. The maximal transcriptional activity and biological effects of at least STAT1, STAT3, and STAT4, and possibly also STAT5A, require both tyrosine and serine phosphorylation [70, 72–75]. This was corroborated by infection experiments with mice unable to become phosphorylated at STAT1 Ser 727. Mice expressing STAT1 $S^{727}A$ died when challenged with high doses of the intracellular pathogen *Listeria monocytogenes*, whereas 80% of the wild-type littermates survived [72]. In the case of STAT1, a variety of signals cause the phosphorylation of Ser 727 and multiple candidate serine/threonine kinases have been implicated in this process. For example, interferons induce serine phosphorylation in addition to prior tyrosine phosphorylation. It was reported that, for canonical serine phosphorylation, STAT1 needs to be assembled into chromatin-bound transcriptional complexes and that the responsible kinase itself is similarly associated with DNA [76]. In this light, it was proposed that STAT1 recruits the cyclin-dependent kinase 8 (CDK8) to IFN-γ-driven target genes, which provides the kinase activity [77].

The need for serine phosphorylation to enhance transcriptional activity of STAT1 varies with different target genes and cell types, suggesting a complexity, which is not yet fully understood [72, 78]. It is hypothesised that STAT1 serine phosphorylation provides an extra supply of IFN-γ-induced gene products that are critical for full protection against pathogens [72]. Signals such as bacterial lipopolysaccharide (LPS) or mediators of inflammation and cellular stress exclusively stimulate the phosphorylation of serine 727, independent of tyrosine phosphorylation [79]. This modification takes place in the cytosol and involves the p38 mitogen-activated protein kinase (MAPK) pathway [80, 81]. It has been proposed that the

biological relevance of serine phosphorylation is to prime STAT1 for an increased transcriptional response once IFN-γ provides the stimulus for tyrosine phosphorylation [78], as observed for macrophage activation.

An important paper from the Vinkemeier lab demonstrated that the activity of STAT1 is inhibited by conjugation to small ubiquitin-like modifier (SUMO), which is added to Lys 703 [82]. Although only a small fraction of the intracellular, unphosphorylated STAT1 pool is sumoylated, the modification by SUMO interferes with the formation of paracrystalline arrays in the nucleus, which sequester activated STAT1 molecules. SUMO conjugation diminishes the activity of STAT1 by interference with tyrosine phosphorylation and, in addition, solubilizes the highly dynamic paracrystals in cytokine-stimulated cells [83].

6. Mechanisms of STAT1 DNA binding

Sequence-specific DNA binding of STAT proteins is a prerequisite for their function as cytokine-driven transcriptional regulators. STATs interact with high-affinity binding sites on DNA through their DNA-binding domain, which shows the general architecture of an immunoglobulin fold [46, 47]. The crystal structure of DNA-bound STAT1 revealed that the transcription factor engages in several interactions with the phosphodiester backbone of the DNA, but makes relatively few base-specific contacts, for example, through the residues Asp 460 and Lys 336 in the major and through Glu 421 in the minor groove [46]. Each STAT protomer binds to a half-site of a palindromic consensus motif termed gamma-interferon activated sequence (GAS). STAT1, STAT3, STAT4 and STAT5 bind to half-sites spacing two or three base pairs (5′-TTCN$_{2\text{-}3}$GAA-′3), while STAT6 favours a four nucleotide spacer within the palindrome [84, 85]. The nucleotides between and around the core palindrome impart some level of specificity.

The implication of impaired DNA-binding activity became evident, when STAT1 mutants were engineered by substituting numerous residues within the DNA-binding domain that potentially make contacts with the phosphodiester backbone of the DNA. The introduction of positive, negative or neutral charges was intended to increase or reduce electrostatic interactions between STAT1 and DNA, respectively. A STAT1 mutant termed STAT1 DNA^{minus}, in which negative charges were introduced by substituting aspartic acid (Asp) for valine (Val) at position 426 (Val^{426}Asp) and threonine at position 427 (Thr^{427}Asp), was normally tyrosine phosphorylated in response to IFN-γ but essentially lost its DNA-binding activity [27]. The STAT1 DNA^{minus} mutant was highly susceptible to inactivation by the nuclear phosphatase Tc45 and failed to accumulate in the nucleus. In contrast, the mutant STAT1 DNA^{plus} (Thr^{327}Arg; Val^{426}His; Thr^{427}His) was capable of strongly binding to both specific GAS sites and nonspecific DNA sequences with nearly equal affinity. This mutant lost its discrimination for GAS sites and, due to its hindered dissociation from DNA, resisted dephosphorylation, resulting in prolonged nuclear accumulation [27]. These and additional experiments indicated that DNA binding determines the accumulation of STAT1 in the nucleus. This assumption was corroborated by the finding that high-affinity DNA binding reduces the dissociation rate of STAT1 dimers from DNA and impairs the interdimeric exchange of their protomers in the presence of DNA [57].

In contrast to STAT1 DNA^{plus}, substitution of two glutamic acid residues (Glu^{411}Ala; Glu^{421}Lys) generates a double mutant, which maintains the discrimination between GAS and nonspecific sites. However, STAT1-DNA complexes are stabilised independent of the nucleotide sequence, leading to persistent and enhanced tyrosine phosphorylation and prolonged nuclear accumulation [86]. The presence of negatively charged residues at these positions is critical for the release of STAT1

from DNA. IFN-γ-induced expression of reporter genes and endogenous target genes is dramatically reduced in cells expressing STAT1 DNA^{plus}, STAT1 $Glu^{411}Ala$ or STAT1 $Glu^{421}Lys$ [27, 86]. In summary, these data revealed the significance of sequence-specific DNA binding and fast dissociation from DNA for efficient STAT1-mediated gene regulation. An impaired dissociation of STAT1 from genomic DNA not only interferes with the continuous search for GAS sites but also prevents fast nucleocytoplasmic shuttling and full transcriptional activity of STAT1.

Early studies on the DNA binding of STATs revealed that their binding sites can extend over two or more adjacent consensus motifs and that activated STAT1, STAT3, STAT4 and STAT5 dimers can interact in homotypic fashion to polymerise on such DNA sequences, that is form tetramers or even higher order oligomers [48, 87–91]. GAS motifs linked in tandem orientation have been identified in various STAT-driven target genes, including, for example, those encoding IFN-γ [90], interleukin 2 receptor α (IL-2Rα) [87, 88], perforin [92], cytokine-inducible SH2-containing protein (CIS) [93], α2-macroglobulin (α2-M) [91], and glycosylation-dependent cell adhesion molecule 1 (GlyCam1) [94]. The synergistic recognition of DNA, generally referred to as cooperative DNA binding, is mediated by the conserved N-terminal domain of the STAT proteins. It was shown that deletion or mutation of the STAT N-terminus abolishes cooperative dimer-dimer interactions [48, 89–91, 95].

Analysis of the crystal structure of the N-domain dimer has indicated an extensive interface involving interactions between hydrophobic residues [96]. Targeted mutagenesis has revealed an orthologue residue (Phe 77 in STAT1 and Phe 81 in STAT5A/B, respectively) as physiologically most relevant for the oligomerisation of STAT proteins [95, 96]. Substitution of alanine for the critical phenylalanine in STAT1 does not overtly affect DNA binding on a single GAS site in response to IFN-γ but severely impairs tetramerisation [95, 97]. The cooperative binding of STAT1 dimers strongly increases their DNA-binding affinity [89]. Such interactions do not require high-affinity GAS sites linked in tandem orientation. *In vitro* studies have revealed the recruitment of multiple STAT1 dimers to a single GAS site adjacent to low-affinity or even GAS-unrelated sequences [95]. Thus, N-domain interactions of DNA-bound STAT1 molecules greatly expand the repertoire of potential STAT1-regulated IFN-γ target genes. An *in silico* analysis revealed that although single GAS sites frequently occur in the mouse genome, GAS sites linked in tandem orientation are rare and may not be enriched in IFN-γ target genes [97]. Paradoxically, a genome-wide transcriptional analysis revealed that STAT1-mediated cooperative DNA binding is indispensable for IFN-γ signalling, since the IFN-γ response is essentially lost in murine cells expressing the STAT1 $F^{77}A$ mutant [97]. Furthermore, cooperativity-deficient STAT1 $F^{77}A$ showed a pervasive promoter recruitment defect at GAS-containing IFN-γ-driven but not IFN-α/β-driven genes. It has, therefore, been proposed that STAT1 tetramerisation or polymerisation originates from a GAS site and then proceeds with loose additional requirements for adjacent sequences [97]. Infection experiments have highlighted the physiological relevance of STAT1 tetramerisation. *Listeria monocytogenes* infection of STAT1 $F^{77}A$-expressing mice revealed a severe defect in antibacterial immunity. The STAT1 cooperativity-deficient animals succumbed more easily than their wild-type littermates [97].

The crystal structure of the unphosphorylated STAT1 dimer indicates that the dimerisation interface is composed of the phenylalanine residue 172 in the coiled-coil domain of one protomer, which reciprocally is inserted into a pocket in the DNA-binding domain of its partner molecule. This pocket is created by the residues Q340, L383, G384, T385, H406, L407 and Q408 [52]. Several GOF mutations have been identified at this interdimeric interface [98, 99]. Studies on the molecular basis of these point mutations underscore the paramount importance of the conformational shift for STAT1-driven gene expression [98]. The critical phenylalanine

residue 172 is in close proximity (7 Å) to the threonine residue 385 at the surface of the partner protomer (**Figure 3E**). Its substitution impedes the reciprocal interactions between the coiled-coil and DNA-binding domain of the two interacting STAT1 molecules, thereby negatively affecting the stability of the antiparallel dimer conformation. Shifting the conformational equilibrium towards the parallel conformation buries the critical tyrosine residue 701 inside the SH2 domain and, thus, prevents access for nuclear phosphatases.

Another interesting phenotype was reported from the targeted mutagenesis of the phenylalanine residue 364. The crystal structure indicates that this residue locates in the centre of the DNA-binding domain and contributes to the gross structural alignment of this domain (**Figure 3**). The $F^{364}A$ mutant is characterised by tyrosine hyper-phosphorylation in response to stimulation of cells with IFN-γ, most likely because of its hampered interaction with the Tc45 phosphatase and, in addition, a significantly reduced DNA-binding activity. The data suggest that Phe 364 is crucial for stabilising the antiparallel dimer and, in addition, for recognising DNA-binding sites. When assessed for its ability to induce endogenous STAT1-regulated genes, the mutant unexpectedly shows a well-preserved transcriptional activity. Remarkably, it appears that a shift in the equilibrium towards the parallel dimer conformation can compensate for a critical impairment in high-affinity DNA binding, restoring nearly full transcriptional activity at IFN-γ-induced target genes [21].

7. Phenotypes of patients with inborn errors of human STAT1

Regulation of the JAK-STAT signalling pathway is most critical, and dysfunctions in this pathway are associated with various immune disorders and cancer. Either the loss of STAT transcriptional activity or uncontrolled constitutive, prolonged activity can have devastating effects. Recent progress in the genetic dissection of various human infectious diseases has shed light on the inborn errors of human STAT1-mediated immunity. In the latest version of the publicly accessible section of the Human Gene Mutation Database (HGMD), more than 40 missense/nonsense mutations in the *Stat1* gene have been listed as disease-causing mutations. These include mutations with (i) autosomal recessive (AR) complete STAT1 deficiency, (ii) AR partial STAT1 deficiency, (iii) autosomal dominant (AD) STAT1 deficiency and (iv) AD gain-of-function activity. Biallelic and even monoallelic loss-of-function (LOF) mutations have been associated with lethal or milder pathogenesis of intramacrophagic bacterial and viral diseases. In addition, STAT1 LOF mutations have been identified in the rare syndrome of Mendelian susceptibility to mycobacterial disease (MSMD), which is characterised by infections with weakly virulent mycobacteria in otherwise healthy individuals. Patients with immunological defects caused by gain-of-function (GOF) mutations suffer from autoimmunity and recurrent or persistent infections of nails, skin and mucous membranes with the opportunistic yeast pathogen *Candida albicans*, referred to as chronic mucocutaneous candidiasis (CMC) [100–102] (**Table 1**).

Studying the structural and functional impact of STAT1 missense mutations has greatly contributed to our current understanding of cytokine-regulated transcriptional activity. To decipher the underlying molecular mechanisms of the known STAT1-mediated immunodeficiencies, the pathogenic amino-acid substitutions need to be assigned to the structural changes they induce in the context of intra- and intermolecular interactions [112]. When mapped to the crystal structure, it becomes evident that the occurrence of disease-causing mutations is unevenly distributed among the different STAT1 domains. It appears that the preferential

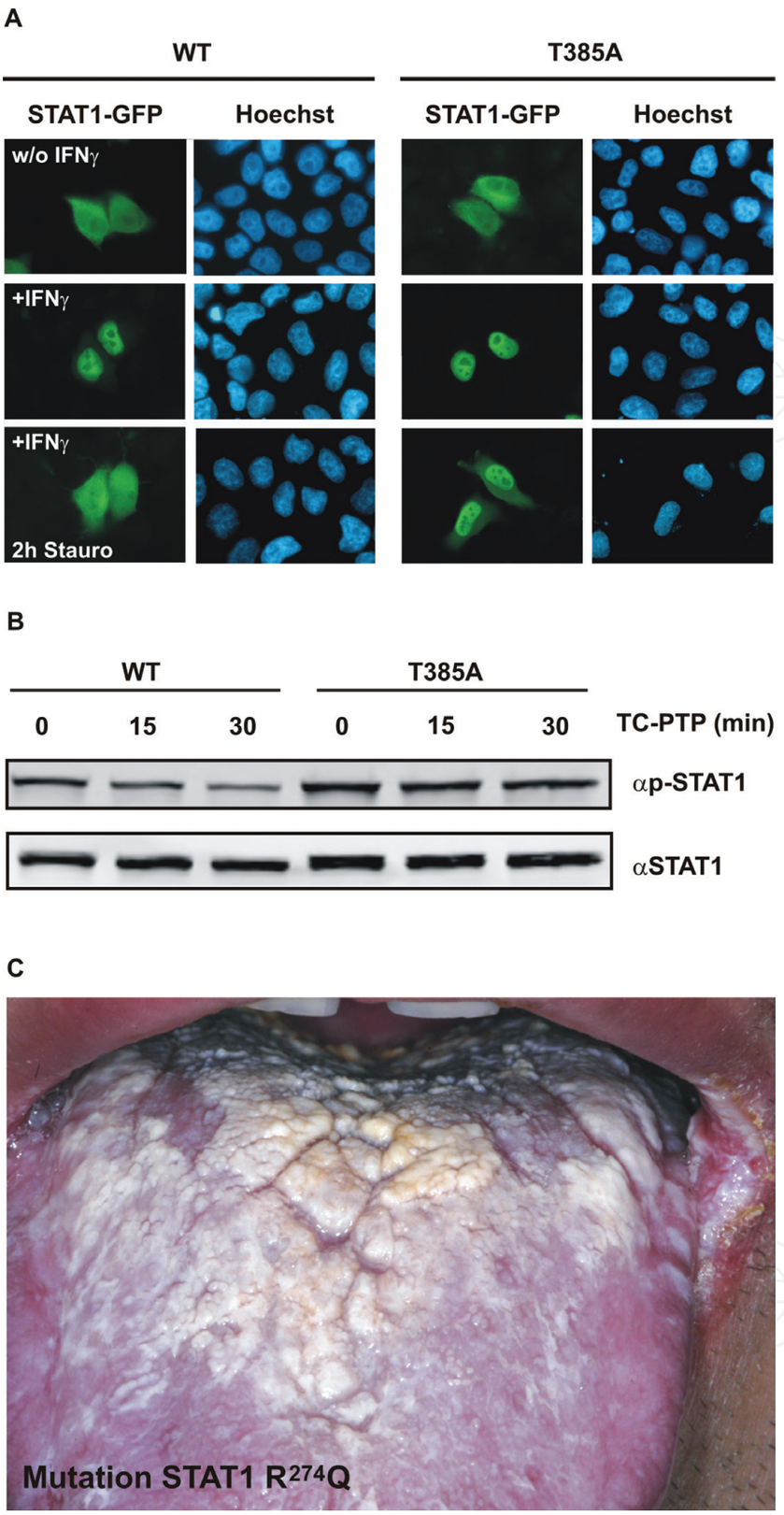

Figure 4.
Phenotype of clinically relevant STAT1 GOF mutations. (A, B): Characterisation of the STAT1 mutation $T^{385}A$. (A) Localisation of fusion proteins of green-fluorescent protein-tagged STAT1 in reconstituted U3A cells expressing recombinant wild-type STAT1 or STAT1 $T^{385}A$. Exposure to the kinase inhibitor staurosporine resulted in the loss of IFN-γ-induced nuclear accumulation of wild-type STAT1, whereas, in contrast, the STAT1 point mutant showed a significantly prolonged nuclear residence. (B) Impaired tyrosine dephosphorylation of the GOF mutant, as shown by Western blotting. Incubation of protein extracts from STAT1-reconstituted U3A cells with the STAT1-specific nuclear phosphatase TC-PTP (Tc45) led to rapid dephosphorylation of wild-type STAT1, while the level of tyrosine phosphorylation was prolonged in the $T^{385}A$ mutant. (C) Intraoral chronic mucocutaneous candidiasis in a patient carrying the heterozygous STAT1 GOF mutation $R^{274}Q$.

	Domain/ region affected	Main cellular phenotype	Predominant clinical phenotype	References (incomplete)
AR complete STAT1 deficiency	ND, SH2	No STAT1-dependent response to IFN-α/β, IFN-γ, IFN-λ, IL-27	Life-threatening intracellular bacterial (mostly mycobacteria) and viral (mostly herpes) diseases	[103–105]
AR partial STAT1 deficiency	ND, CCD, TS	Impaired STAT1-dependent response to IFN-α/β, IFN-γ, IFN-λ, IL-27	Mild intracellular bacterial (mostly mycobacteria) and viral (mostly herpes) diseases	[106, 107]
AD LOF STAT1 disorder	CCD, DBD, SH2, TS	Impaired STAT1-dependent response to IFN-γ, IL-27	Mendelian susceptibility to mycobacterial disease (MSMD)	[108, 109, 116]
AD GOF STAT1 disorder	CCD, DBD	Enhanced STAT1-dependent response to IFN-α/β, IFN-γ, IFN-λ, IL-27, IL-6, IL-21, impaired IL-17-mediated T-cell immunity	Chronic mucocutaneous candidiasis (CMC) Autoimmunity	[99, 110, 111]

AD, autosomal dominant; AR, autosomal recessive; CCD, coiled-coil domain; DBD, DNA-binding domain; GOF, gain-of-function; LOF, loss-of-function; ND, amino-terminal domain; SH2, src-homology-2 domain; TS, tail segment.

Table 1.
Inborn errors of human STAT1-mediated immunity.

localisation of LOF mutations is less well defined as compared to the more restricted localisation of GOF mutations. The critical LOF substitutions are widely scattered in the coiled-coil, DNA-binding and SH2 domains, as well as in the tail segment. In contrast, the GOF mutations identified so far are exclusively clustered in the coiled-coil and DNA-binding domains. The crystal structure of tyrosine-phosphorylated, DNA-bound STAT1 shows that the coiled-coil domain prominently protrudes outward [46]. Therefore, it was initially postulated that the coiled-coil domain may function as a docking site for transcription factors and coactivators to cooperatively facilitate STAT1-dependent gene transcription and that the GOF mutations in this domain may enhance these interactions. Typically, pathogenic GOF mutations cluster in structural areas of the STAT1 protein, which markedly affect the regulation of its transcriptional activity. As described above, the conformational rearrangement from the parallel to the antiparallel dimer conformation is a prerequisite for the inactivation of STAT1 and requires, as a *conditio sine qua non,* the dissociation into monomers. Monomeric STAT1 spontaneously reassociates into either the parallel or antiparallel conformation. In the parallel alignment, STAT1 regains its DNA-binding activity with the chance of participating in another round of transcriptional initiation of target genes. It appears that the antiparallel conformation of the STAT1 dimer is the substrate for the highly active Tc45 phosphatase, which rapidly dephosphorylates the critical tyrosine residue 701 followed by nuclear export of the now transcriptionally inactive molecule. As described above, the formation of the antiparallel dimer is facilitated by reciprocal interactions between the coiled-coil and the DNA-binding domain and, thus, it is not surprising that mutations in this critical area affect the stability of this complex.

Experimental data revealed that the two point mutants STAT1 $F^{172}W$ and $T^{385}A$ are resistant against dephosphorylation by the Tc45 phosphatase, which results in

prolonged nuclear accumulation in cells exposed to IFN-γ (**Figure 4A**, **B**) [113]. The resulting phenotype is associated with enhanced tyrosine phosphorylation in response to various cytokine stimuli, including IFN-α, IFN-γ, IL-6, IL-21 and IL-27 [23, 110, 113–115]. The functional characterisation of the disease-causing interface mutations has demonstrated sequence-specific requirements for differential gene expression of endogenous IFN-γ target genes. The expression of genes with a classical GAS consensus-binding motif, including *interferon-regulatory factor 1* (*irf1*), *guanylate-binding protein 1* (*gbp1*) and *monokine induced by interferon gamma 1* (*mig1*), is virtually unaffected by these mutations. In contrast, transcription of genes, such as *CXC motif chemokine 10* (*cxcl10*) and *monocyte chemotactic protein 1* (*mcp1/cxcl9*) is greatly increased, which have a "one-and-a-half-GAS" element in their promoters, that is, half of the palindromic motif adjacent to the GAS site [113]. The superiority of the STAT1 mutants as transcriptional activators appears to directly reflect their enhanced binding as tetramers to these "one-and-a-half-GAS" sequences. The increased DNA binding for these mutants contributes to their pathologically elevated level of tyrosine phosphorylation. However, numerous disease-associated GOF mutations are not directly located at the surface of the interdimeric interface but rather disturb the gross architecture of this interface (**Figure 3**).

A prominent and well-studied example is the GOF mutation resulting from the exchange of the arginine 274 residue in the coiled-coil domain (**Figure 4C**). A change in the local structure induced by this substitution may impair the stability of the antiparallel dimer. Consistently, a genome-wide expression profile indicated that the pathogenic $R^{274}Q$ mutation increased the IFN-γ-induced transcriptional activity of STAT1 but overall retained its sequence specificity [98]. In other words, the mutation increases the expression rate of STAT1 target genes but does not dramatically change its repertoire. However, the molecular mechanisms of this GOF mutation are not fully understood. In particular, it is unclear whether this missense mutation affects the kinetics of JAK-induced phosphorylation or, alternatively, its dephosphorylation rate.

8. Concluding remarks

Activation at the IFN receptor and sequence-specific, cooperative DNA binding are key features of STAT1 signal transduction. Cytoplasmic and nuclear activities are functionally coupled by repetitive cycles of STAT1 phosphorylation and dephosphorylation. This highly dynamic activation-inactivation circuit is controlled by a shift between the parallel and antiparallel dimer conformers. Missense mutations in either the coiled-coil or DNA-binding domain destabilising the antiparallel dimer critically interfere with the equilibrium between the two conformers. Genetic variations affecting this intradimeric interface result in enhanced cytokine-induced gene expression and cause severe immunodeficiencies in heterozygous mutation carriers.

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