REVIEW

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# Regulation of T cell homeostasis by JAKs and STATs

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## Abstract

Regulation of T cell homeostasis is critical for maintaining normal immune function. An imbalance in T cell proliferation can result in disorders ranging from cancer and autoimmunity to immunodeficiencies. Full activation of T cells requires three sequential signals, where signal 3, which is delivered by multiple cytokines, regulates proliferation, differentiation, and survival/death. Signaling from cytokines through their receptors is primarily delivered by two molecular families, namely Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs). Invaluable knowledge about JAKs and STATs has arisen from studies of mice made genetically deficient in these molecules, analyses of tumor models, and studies of expression patterns by proteomics/genomics, which all have begun to define the role of JAKs and STATs in survival versus apoptosis. These findings also have suggested ways in which JAKs and STATs may be manipulated for therapeutic intervention in lymphoid-derived diseases. This review seeks to focus on the role of JAK tyrosine kinases and STAT transcription factors in mediating the lymphocyte life cycle and how they might be manipulated for therapeutic applications.

**Key words:** T cells, cytokines, Janus tyrosine kinases (JAK), signal transducers and activators of transcription (STAT), apoptosis, disease.

**Abbreviations:** a.a. – amino-acid, CaN – calcineurin, CsA – cyclosporine, DC – dendritic cells, $\gamma_c$ – common cytokine receptor γ chain, EPO – erythropoietin, FERM domain – Band 4.1, Ezrin, Radixin and Moesin, IFN – interferon, IL – interleukin, JAK – Janus tyrosine kinase, JH – JAK homological, MAb – monoclonal antibody, mTOR – mammalian target of rapamycin, Pias – protein inhibitor of activated STAT, PI3k – phosphatidyl inositol 3-kinase, PMSP – Proline Methionine Serine Proline (Pro-Met-Ser-Pro), PSP – Proline Serine Proline (Pro-Ser-Pro), RAPA – rapamycin, SP – Serine Proline (Ser-Pro), SCID – severe combined immunodeficiency disease, SH2 – src homology domain 2, SHP – SH2 containing tyrosine phosphatase, SOCS – suppressor of cytokine signaling, STAT – signal transducer and activator of transcription, TCGF – T cell growth factors, TCR – T cell receptor, Th – T-helper, Treg – T regulatory, PHA – phytohemagglutinin.

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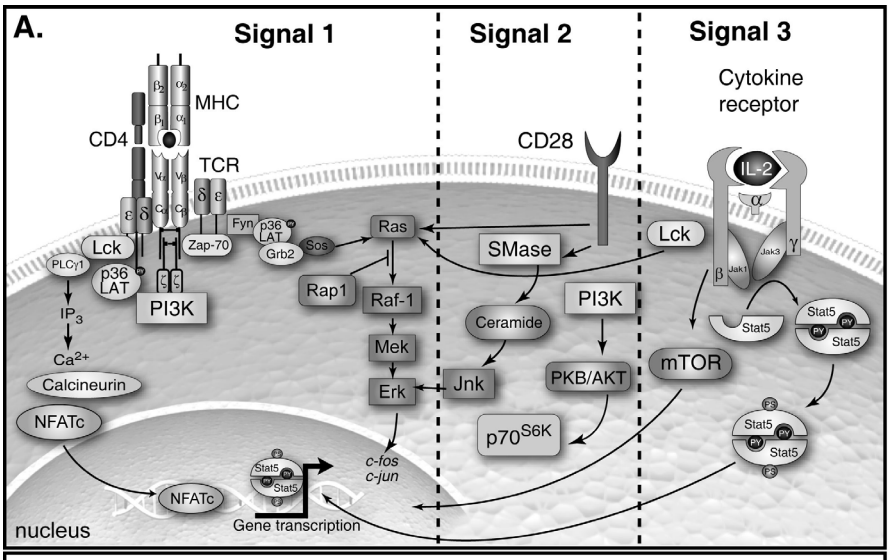
## OVERVIEW

Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs) regulate the entire hematopoietic process through cytokines binding to specific cell surface receptors. Although these receptors lack intrinsic intracellular catalytic domains, they share conserved elements within extracellular domains, such as a Trp-Ser-X-Trp-Ser (WSXWS) motif, with four paired cysteines predicting their homologous tertiary structures [11]. In T cells, JAK/STAT signaling coordinate unique events with "on" and "off" signals, allowing not only defensive plasticity but, equally important, regulating self-destruction through apoptosis. Intriguingly, apoptosis versus survival can be driven within the same cell by different cytokines: the α-helix bundle family (such as interferons) promotes cell death, whereas common γ chain ($\gamma_c$)-cytokines (such as interleukin (IL)-2, IL-7, IL-9, and IL-15) promote cell survival [10]. The current review presents information about JAK and STAT signaling in lymphoid cell growth, differentiation, and survival/apoptosis.

Full T cell activation requires at least three sequential and threshold-limiting signals [18], namely T cell receptor (TCR)/antigen followed by B7/CD28 signal induces the production T cell growth factors (TCGF),




**B.**

| Ligands | Jaks | Stats |
|---|---|---|
| **$\gamma_c$ family** | | |
| **IL-2, IL-9, IL-21** | Jak1, Jak3 | Stat1, Stat3, Stat5 |
| **IL-7, IL-15** | Jak1, Jak3 | Stat3, Stat5 |
| **IL-4** | Jak1, Jak3 | Stat6 |
| **IL-13** | Jak1, Jak2, Jak3, Tyk2 | Stat6 |
| **$\beta_c$ family** | | |
| **IL-3, IL-5** | Jak2 | Stat5 |
| **gp130 family** | | |
| **IL-6, NNT/BSF-3** | Jak1, Jak2 | Stat1, Stat3 |
| **IL-11** | Jak1 | Stat1, Stat3 |
| **OSM, LIF** | Jak1, Jak2, Tyk2 | Stat3, Stat5 |
| **IL-12** | Jak2, Tyk2 | Stat4 |
| **IL-23** | N/D | Stat4 |
| **IL-27** | Jak1, Jak2, Tyk2 | Stat1, Stat3, Stat4, Stat5 |
| **IFN family** | | |
| **IFN type I** | Jak1, Tyk2 | Stat1, Stat2 |
| **IFN type II** | Jak1, Jak2 | Stat1, Stat5 |
| **IL-10** | Jak1, Tyk2 | Stat3 |
| **IL-22** | N/D | Stat1, Stat3, Stat5 |
| **IL-28A, IL-28B** | N/D | Stat2, Stat3 |
| **IL-29** | N/D | Stat2, Stat3 |

**Fig. 1.** Involvement of JAKs and STATs in the activation of T cells. **A** – three signals of T cell activation include alloantigen engagement of TCR (signal 1) followed by CD28/CTLA4 binding (signal 2), which induces IL-2 and other cytokines' production, and IL-2 or other $\gamma_c$-cytokines' binding to their receptors (signal 3). **B** – cytokines engage unique combinations of JAKs and STATs in the $\gamma_c$-family, $\beta_c$-family, gp130-family, and IFN-family of cytokines. Unique sets of JAKs and STATs are activated by distinct receptors. IL – interleukin, BSF – B cell stimulating factor, NNT – novel neurotrophin, OSM – OncoSTATin M, IFN – interferon.

such as IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, and IL-27 [65, 102]. TCGFs bind to receptors that share a $\gamma_c$ associated with an affinity-conferring α-chain for each cytokine or, occasionally, with a β-chain (for IL-2 and IL-15) [4]. For example, IL-2 binding through the high-affinity IL-2Rα/β/$\gamma_c$ recruits JAK1 to IL-2Rβ and JAK3 to $\gamma_c$ chain [71] (Fig. 1A). Autoactivation of these kinases promotes tyrosine phosphorylation of the IL-2Rβ chain, thereby recruiting STAT5a and STAT5b [88]. After docking through SH2 domains to selected receptor phosphotyrosines, STAT5a/b are tyrosine- and serine-phosphorylated; they dissociate from their signaling receptor to form dimers, translocate to the nucleus, and then bind to promoter sites on multiple genes that control cell growth and differentiation in combination with other effector molecules. The unique patterns of how different cytokines activate JAKs and STATs are depicted in Fig. 1B.

## STRUCTURE AND FUNCTION OF JAKs AND STATs

JAKs have seven JAK homological (JH) domains, with JH1 localizing the kinase activity, JH2 harboring a pseudokinase, and the JH3-JH7 domains participating in JAK binding to its receptor (Fig. 2A). While the JH4 domain harbors a rather divergent SH2 domain, it has the necessary core structural residues as well as the critical arginine residue to coordinate the phosphate group, leaving no doubt that it is a bona fide SH2 domain [47]. The crystal structure of JAK3 with $Tyr^{981}$ that is present in the activation loop of the regulatory C-helix suggests that the loop active position is induced by phosphorylation [16]. There are seven STAT proteins, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [33, 72]. Each STAT consists of at least six domains, including an N-terminal domain, a coiled-coil domain, a DNA binding domain, a linker domain, an SH2 domain, and a transactivation domain (Fig. 2B).

Engagement of cognate cytokine receptors activates one or more of the four members of the JAK family (JAK1, JAK2, JAK3, and Tyk2). Following cytokine/receptor interaction, the ubiquitously expressed JAK1, JAK2, and Tyk2 as well as the inducible JAK3 become autoactivated. These tyrosine kinases range in molecular weight from 120–135 kDa, with their chromosomal locations mapped as reviewed earlier [33, 50]. JAKs' tyrosine phosphorylate their cytokine receptors, creating active docking sites for STATs (Fig. 3). There are very unique patterns of how individual cytokines recruit and activate JAKs and STATs among TCGF-, βc-, gp130-, and interferon (IFN)-families (Fig. 1B). Earlier models suggested that STAT monomers were recruited and activated by closely localized receptors bound with JAKs, whose tyrosine phosphorylated STATs, causing STAT disengagement and dimerization via SH2 domains, followed by some serine phosphorylation [36, 114]. Recent evidence complicated this model by suggesting that Src and other non-JAK tyrosine kinases phosphorylated tyrosine sites on STATs [123, 151]. There is also new evidence that STATs may exist as preformed dimers without tyrosine phosphorylation [149] and that these dimers even drive gene transcription [143].

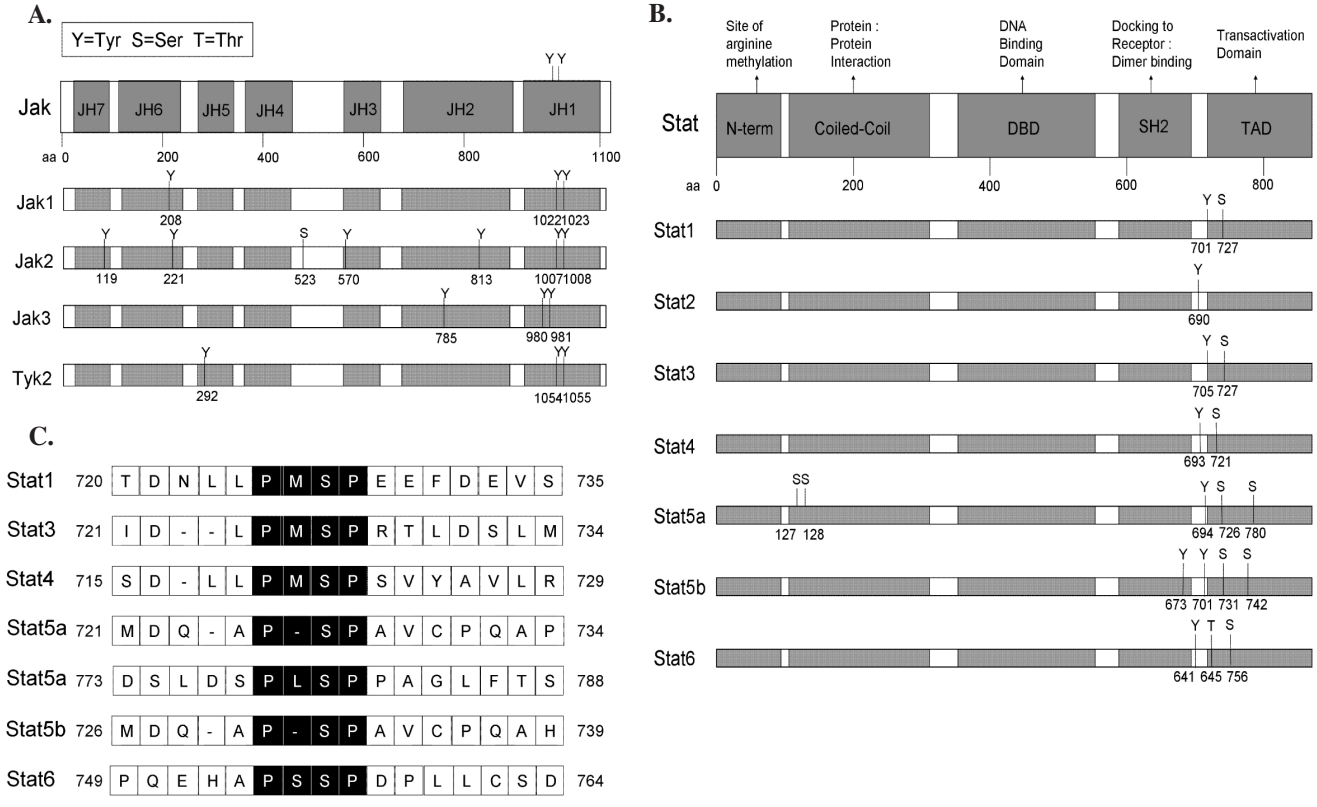



**Fig. 2.** Schematic model of JAK and STAT structure. **A** – the JAKs share seven regions of JAK homology (JH) domains denoted JH1-JH7. The JH1 domain harbors the tyrosine kinase and conserved Tyr-Tyr (YY)-motif within the autoactivation loop. The JH2 domain contains the pseudokinase that regulates kinase activity and binds substrates. The JH3-JH7 domains are critical for receptor association. **B** – the STATs share conserved domains, namely an amino terminal domain (promoting STAT tetramerization), a coiled-coil domain (promoting protein-protein interaction), a DNA binding domain (DBD), a linker domain, an SH2 domain (reciprocally binding with a phosphorylated tyrosine (pY) dimer-partner), and a transactivation domain (TAD – recruiting and promoting transcriptional activity). **C** – STATs (except STAT2) have cytokine-regulated serine phosphorylation sites in TAD. This serine (S) was mapped to a Pro-Met-Ser-Pro (PMSP) motif in STAT1, STAT3, and STAT4, to a Pro-Ser-Pro (PSP) motif in STAT5a and STAT5b, and to a SP motif in STAT6.

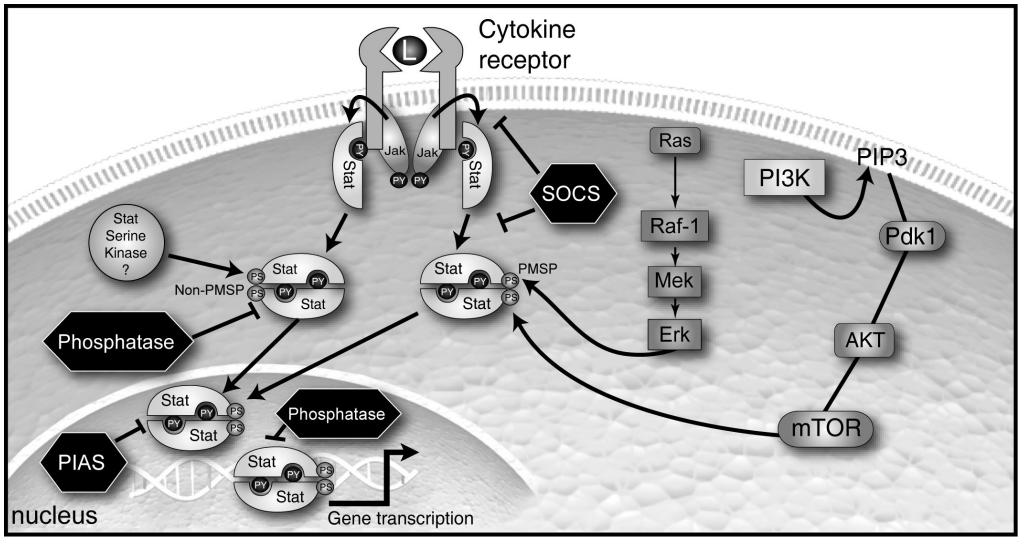



**Fig. 3.** Model of ligand-induced JAK/STAT signaling pathway. Following ligand (L)-induced receptor dimerization/oligomerization, JAK tyrosine kinases autophosphorylate each other then receptor tyrosine residues to create docking sites for the SH2 domain containing STATs. Ligand binding also results in activation of other signaling pathways, such as Ras-Raf-Mek-Erk and PI3k-Pdk1-Akt-mTor serine kinase cascades, that have been shown to phosphorylate the serine residue of the PMSP-motif of STAT1, STAT3, and STAT4. STAT5a, STAT5b, and STAT6 void of the conserved PMSP motif (Non-PMSP STAT) may be regulated by a yet-to-be-identified proline-directed serine kinase. This unknown kinase might also be competent to phosphorylate the consensus PMSP motif as indicated by the arrow and question mark. STATs would then translocate through the nuclear pore, associate with other factors, and bind IFN-stimulated response element (ISRE) or IFN-γ-activated sequence (GAS) sites to regulate gene transcription. Three families of molecules regulate JAK/STAT pathways, namely suppressor of cytokine signaling (SOCS1-7) and CIS; T cell protein tyrosine phosphatase (TC-PTP), including the SH2-domain containing phosphatase-2 (SHP-2) and CD45; and protein inhibitor of activated STAT (Pias), including Pias1, Pias3, PiasX, and PiasY. See details in the text.

## JAKs/STATs AND LYMPHOID CELL SURVIVAL

*Lessons from JAK knockouts*

Selective gene deletion of JAKs and STATs has provided insight into their role in development and function. Most JAK1$^{-/-}$ mice died perinatally and exhibited profound defects in lymphoid cell development, most likely owing to the disruption of JAK1-dependent signaling to cytokine/growth factor receptor subfamilies, namely IFNs, TCGFs (i.e. IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), and gp130 subunit cytokines (i.e. IL-6, IL-11, LIF, OSM, CNTF, and CT-1) [105]. Although surviving JAK1$^{-/-}$ mice have a 260-fold reduction in thymocyte numbers, the distribution of CD4$^+$ and CD8$^+$ cells remains similar to that of wild-type mice [104]. Thus it appears that JAK1 is required for early but not late stages of thymocyte maturation. Because JAK1-deficient T cells fail to respond to TCGFs (e.g. IL-2 IL-7, IL-9), they are destined to undergo apoptosis. Furthermore, JAK1 deficiency also blocks B cell differentiation from pro-B to pre-B cells, resulting in a significant deficit of mature B cells in JAK1$^{-/-}$ mice [105]. Overall, the inability of JAK1-deficient T and B cells to respond to positive survival signals leads to reduced numbers of thymocytes, pre-B cells, and mature T and B lymphocytes, thereby producing a severe combined immunodeficiency disease (SCID) phenotype even though other hematopoietic lineages are not affected [105].

Making mice deficient in JAK2 rapidly results in embryonic lethality that is likely caused by a lack of erythropoiesis [89, 96]. Indeed, JAK2 regulates erythropoiesis through multiple hematopoietic factors, including erythropoietin (EPO), thrombopoietin, IL-3, and IL-5 [96]. The effect of JAK2 in the function of mature lymphocytes is less clear. To address this question, sublethally irradiated mice were reconstituted by retroorbital injection with JAK2-deficient fetal liver cells. Indeed, JAK2-deficient T cells responded well to signal 1/2 and 3, whereas B cells were activated by anti-IgM or lipopolysaccharide stimulation [88]. Thus JAK2 was not mandatory for the survival and function of lymphoid progenitors.

A very different picture emerged when examining the function of JAK3. Most importantly, JAK3 is expressed exclusively in lymphoid tissues (namely in T, B, NK, and monocytic cells) displaying the highest binding fidelity to the $\gamma_c$ chain [20]. JAK3 has been characterized and reported under a number of different names, including p116 [62] and L-JAK [53, 58, 63]. Although a low level of JAK3 is constitutively expressed in B, NK, and monocytic cells, TCR engagement is needed to induce JAK3 expression in naïve T cells. JAK3 promoter activity mapped to a 267-bp fragment contained binding sites for STATs, Sp-1, AP-1, and Ets proteins [6]. Engagement of the B cell receptor or cross-linking of CD40 elevated the expression of JAK3 in B cells [122]. Similarly, exposure to IL-2 increased JAK3

protein expression in NK cells. JAK3$^{-/-}$ dendritic cells (DCs) developed normally and, in fact, their survival was enhanced as confirmed by the decreased expression of pro-apoptotic proteins. Because JAK3$^{-/-}$ DCs had normal antigen uptake and expression of co-stimulatory proteins and produced more IL-12, JAK3 may have a regulatory function in DCs [140].

JAK3 activation is critical for development and survival of T and B cells. Blockade of $\gamma_c$ chain with monoclonal antibodies (MAbs) induced rapid T cell apoptosis *in vitro* and extended the survival of mouse islet allografts [73]. In similar fashion, blockade of $\gamma_c$ chain by MAbs in a murine pro-B cell line, BAF3, promoted apoptosis, which correlated with up-regulation of Fas ligand, and subsequently down-regulated the expression of anti-apoptotic Bcl2 protein [73]. It also has been reported that JAK3-deficient T cells increased the expression of Bax and reduced the expression of Bcl2, suggesting common pathways by which JAK3/$\gamma_c$ regulates T cell function [135]. Direct evidence for the role of JAK3 in the function of T and B cells has been provided by JAK3- or $\gamma_c$-deficient mice, as manifested by SCID syndrome [78, 94, 106]. Indeed, JAK3-deficient patients have a significant reduction in the number of circulating T and NK cells. Additionally, B cells are not fully functional, presumably due to the severe absence of T-helper (Th) cytokines [71]. Spontaneous mutations of JAK3 in patients have been reported in all seven domains. A single amino-acid (a.a.) substitution at position 100 from tyrosine to cysteine in the JH7 domain prevented kinase-receptor association [23]. Similarly, a change at a.a. position 481 from glutamine to glycine in the JH3 domain reduced phosphorylation levels of JAK3 and STAT5 in response to IL-2 [24]. Spontaneous mutation at a.a. position 759 from cysteine to arginine in the JH2 domain resulted in basal levels of constitutively phosphorylated JAK3 [24]. Although SCID-like patients inevitably succumbed to opportunistic infections, the retroviral-JAK3-gene transfer to SCID mice dramatically increased the number of T and B cells, thereby restoring their antigen-specific immunity [21]. Similarly, bone marrow transplantation in SCID patients restored T cell activity and partially re-established B and NK cell functions [104]. Re-implantation of autologous hematopoietic stem cells transfected with $\gamma_c$ reconstituted the normal numbers of functional T and NK cells in $\gamma_c$-deficient patients [27]. These clinical cases and the findings from JAK3-deficient mice clearly confirmed the fundamental role of JAK3 tyrosine kinase in T, B, and NK cells.

A different picture emerged in Tyk2$^{-/-}$ mice displaying only reduced responses to IFN-$\alpha/\beta$ and IL-12 [112]. Furthermore, the IFN-$\gamma$-induced T cell response was reduced in Tyk2$^{-/-}$ mice after infection with lymphocytic choriomeningitis virus [57]. IFN-$\alpha$ signaling requires Tyk2 to drive the translocation of a nuclear protein, Daxx, which is possibly involved in apoptosis and transcriptional repression in B cell growth arrest [111]. In contrast to pro-inflammatory cytokine signaling, Tyk2 has recently been shown to be an important regulator for the signaling and expression of the immunosuppressive cytokine IL-10 [110].

*Lessons from STAT knockouts*

STATs are an evolutionarily conserved family of proteins that play diverse roles in embryonic cell development, differentiation, proliferation, migration, survival, and apoptosis. They have traditionally been categorized into two groups. The first group, comprising STAT2, STAT4, and STAT6, is utilized by a few selected ligands that appear to be unique to mainly specialized T and B cell functions. The second group, containing STAT1, STAT3, STAT5a, and STAT5b, displays more diverse functions, protecting lymphocytes against apoptosis and driving cell-cycle progression. Mice made deficient in STATs have greatly defined the role of STATs in lymphocytes, as discussed below.

STAT1$^{-/-}$ mice showed increased mortality in the presence of otherwise harmless pathogens [82]. STAT1$^{-/-}$ lymphocytes had decreased rates of apoptosis coinciding with reduced levels of caspases 1 and 11 [70]. Although STAT2$^{-/-}$ mice developed in normal fashion, they were susceptible to viral infections and their T cells poorly responded to IFN-$\alpha/\beta$ [99]. Under different conditions, STAT2 was proactive or prevented the apoptosis of T cells in response to IFNs [55]. In contradistinction, STAT3$^{-/-}$ mice died in embryonic development between days 6 and 7 [121]. Constitutive expression of active STAT3 was identified in numerous malignancies, including leukemias, lymphomas, breast carcinoma, multiple myeloma, as well as head, neck, brain, lung, and prostate cancers [10]. However, specific deletion of STAT3 in T cells caused severely impaired proliferative and anti-apoptotic response to IL-6 and somewhat impaired response to IL-2 in T cells [120]. Because anti-apoptotic Bcl2 protein was normally expressed in STAT3-deficient T cells, STAT3 promoted survival independent of Bcl2. It is well established that STAT4 is important in the differentiation of naïve T cells into IL--2/IFN-$\gamma$-producing Th1 cells [14]. Although Th1--dependent functions were impaired in STAT4-deficient mice, including production of IFN-$\gamma$ and NK cell cytotoxicity, naïve STAT4$^{-/-}$ T cells displayed increased differentiation into IL-4/IL-10-producing Th2 cells [56]. Thus, STAT4s acts as a potent differentiation transcription factor regulating T cell destination.

STAT5a/b transcription factors play very unique roles in T cells. STAT5a and STAT5b are activated not only by $\gamma_c$-cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), but also by IL-3, IL-5, various growth factors, prolactin, growth hormone, and EPO [95]. Selective STAT5a knockout in female mice caused a defective lobulo-alveolar development resulting in deficient milk production in response to prolactin [76]. In contrast, exclusive STAT5b$^{-/-}$ mice showed a retarded growth profile similar to Laron dwarfism (defective growth hormone receptor function) [126]. Although no defects in

immune activity were apparent in STAT5a$^{-/-}$ or STATb$^{-/-}$ mice, double ΔNSTAT5a/b$^{-/-}$ mice exhibited severely impaired immune functions, predominantly in T cells. In particular, ΔNSTAT5a/b$^{-/-}$ T cells failed to proliferate in response to IL-2 [83] but remained able to produce cytokines (less IL-2 and more IFN-γ) in response to signals 1/2. Defective response to signal 3 correlated with reduced levels of cyclins A, D2, D3, E, and Cdk6 [83]. In fact, STAT5a/b were required for the protection of mature T cells from apoptosis [13, 28]. Lack of IL-2-driven CD4$^+$CD25$^+$ natural T regulatory (Treg) cells in ΔNSTAT5a/b$^{-/-}$ mice disturbed the homeostasis of self tolerance, leading to autoimmune diseases affecting multiple organs with lymphocytic infiltration found in bone marrow, colon, liver, and kidneys [115]. New findings with mice made completely STAT5a/b deficient showed comparable but more profound phenotypes than the ΔNSTAT5a/b$^{-/-}$ mice [144].

We recently explored the role of STAT5a and STAT5b in T and B cells during allograft rejection [116]. Activated ΔNSTAT5a/b$^{-/-}$ T cells produced cytokines but failed to proliferate and instead entered apoptosis. Apoptosis of T cells correlated with increased expression of multiple proapototic genes (Bok, Blk, Bax, Bcl10, Nip3Myd88, Fadd), as revealed by microarray analysis [150]. Furthermore, only activated STAT5a/b--deficient T cells had decreased expression of antiapoptotic Bcl2 and induced proapoptotic Bak mRNA. Similar experiments revealed that STAT5a/b deficiency had no effect on B cell function, including cell proliferation and immunoglobulin class switching by monitoring levels of alloantigen-specific IgM, IgG1, IgG2a, IgG2b, and IgG3. This finding was confirmed *in vivo*, where pure ΔNSTAT5a/b$^{-/-}$ T cells failed to mediate rejection, whereas a mixture of ΔNSTAT5a/b$^{-/-}$ T and B cells rejected heart allografts in a delayed fashion after adoptive transfer to SCID mice. Because ΔNSTAT5a/b$^{-/-}$ T cells produced cytokines within 24 h after activation and prior to entering apoptosis, these cytokines activated B cells to produce donor-specific antibodies, resulting in primary heart allograft rejection. These observations were supported by our *in vitro* studies [13]. Inhibition of STAT5a/b protein expression by phosphorothioate-2'-*O*-methoxyethyl oligodeoxynucleotides (antisense-ODN) induced apoptosis in 70% of phytohemagglutinin (PHA)-activated T cells [13]. Indeed, because activated T cells rely on the TCGF-driven JAK3-STAT5a/b "survival" pathway, even transient activation of STAT5a/b in IL-2-deficient mice by exogenous IL-2 increased the number of CD25$^+$CD4$^+$ Treg cells [3]. Constitutively active STAT5a/b were observed in tumor cell lines [28, 133] and correlated with elevated transcription of anti-apoptotic genes with promoters that had binding for STAT5a/b, such as c-myc, bcl-x, bcl2, and pim-1 [77, 80].

STAT6 proved to be important in differentiation into Th2 cells and antibody production in B cells. Although STAT6$^{-/-}$ mice displayed no apparent defects, they were deficient in IL-4/STAT6-mediated MHC class II expression, immunoglobulin class switching from IgM to IgE, and the generation of Th2 cells [56]. Production of Th2 cytokines, such as IL-4, IL-5, and IL-10, was almost completely blocked in the STAT6$^{-/-}$ mice following infection with the parasite *N. brasiliensis* [113]. Interestingly, constitutive expression of active negative STAT5a induced Th2 differentiation in the absence of IL-4/STAT6 signaling in IL-4Rα-deficient T cells [154].

## REGULATION OF JAKs AND STATs

For many kinases, their catalytic activity is regulated by phosphorylation of residues within the activation loop of the kinase domain. Upon ligand stimulation, two adjacent tyrosines within the activation loop undergo phosphorylation and display distinct regulatory effects of JAK kinases. For example, mutagenesis showed that phosphorylation of tyrosine at position 1007 (Y$^{1007}$) but not at position 1008 (Y$^{1008}$) in JAK2 was required for its catalytic activity [40]. In JAK3, phosphorylation of Y$^{980}$ positively regulates its catalytic activity, whereas phosphorylation of Y$^{981}$ negatively regulates its catalytic activity [153]. Simultaneous mutations of the two homologous tyrosines of Y$^{1054}$ and Y$^{1055}$ in TYK2 prevented ligand-induced activation of this kinase [45]. Although it is not yet well understood how phosphorylation of corresponding tyrosines in JAK1 may affect its activity, it has been shown that phosphorylation of Y$^{1023}$ is at a much higher level than phosphorylation of Y$^{1022}$ [131].

In addition to the tyrosine residues within the kinase domain, phosphorylation sites in other regions of the JAKs have also been identified (Fig. 2A). Among the four JAK kinases, autophosphorylation of JAK2 is currently best characterized. Two groups have reported that Y$^{221}$ in the FERM domain (Band 4.1, Ezrin, Radixin and Moesin) and Y$^{570}$ in the JH2 domain are autophosphorylated and have a potential regulatory effect [5, 39]. Mutagenesis experiments revealed that phosphorylation of Y$^{221}$ slightly increased JAK2 catalytic activity, while phosphorylation of Y$^{570}$ significantly decreased its activity. A recent study has identified another tyrosine (Y$^{119}$) in the FERM domain of JAK2 as a site of phosphorylation. Phosphorylation of Y$^{119}$ is implicated in regulating the dissociation of JAK2 from its receptor in a receptor-specific manner [43]. Using a functional proteomics approach, Y$^{208}$ in JAK1 and Y$^{292}$ in TYK2 were found to be phosphorylated in IFN-α signaling. However, it is not clear whether these two tyrosines have a regulatory effect on kinase activity [152]. Recently, serine (S) phosphorylation of JAK2 has also been reported. Phosphorylation of S$^{523}$ in JAK2 is stimulated by growth hormone and epidermal growth factor and is proposed to function as a feedback mechanism to dampen the activation of JAK2 [81]. Furthermore, in leptin receptor signaling, phosphorylation of S$^{523}$ inhibited JAK2 activity and that inhibition was independent of Y$^{570}$ phosphorylation [52]. Phosphorylation

of $Y^{813}$ in JAK2 did not affect JAK2 activity, but was required for binding to the adaptor protein SH2-Bβ, resulting in enhancement of JAK2 activity. The corresponding $Y^{785}$ in JAK3 is phosphorylated in response to IL-2 and is also important for binding to SH2-Bβ [67]. Interestingly, when we mutate all previously reported tyrosine phosphorylation sites in JAK3 ($Y^{785}$, $Y^{980}$, and $Y^{981}$), we do not observe complete loss of tyrosine phosphorylation. Ongoing studies seek to identify these novel regulatory tyrosines in human JAK3.

STATs have phosphoacceptor sites with highly conserved tyrosine (Y) and serine (S) residues (Fig. 2B) [36]. One of the serine sites, mapped to a Pro-Met-Ser--Pro (PMSP) motif in STAT1, STAT3, and STAT4, was shown to regulate gene transcription in positive or negative fashion [33, 135, 148] (Fig. 2C). In T cells, cytokine-independent signals such as TCR have driven phosphorylation of $S^{727}$ in STAT1 and STAT3 [90, 136], whereas CD28 ligation increased phosphorylation of $S^{727}$ in STAT1 [44]. Similarly, in B cells, the cross-linking of FcγRIIa stimulated phosphorylation of $S^{727}$ in STAT3 [68]. Several serine kinases phosphorylate the PMSP motif, which represents a consensus mitogen-activated protein kinase phosphorylation sequence [138], as documented by co-precipitation of Erk1/2 with PMSP motif of STATs [34], leading to subsequent inhibition of such co-precipitation by Mek1/2 inhibitors [31]. The non-cell--surface-receptor-activation of Erk1/2 by phorbol esters that utilize PKC-Raf intermediates also increased $S^{727}$ phosphorylation in STAT3 [66, 90]. Recent reports have promoted the involvement of phosphatidyl inositol 3-kinase (PI3k) [44] and mammalian target of rapamycin (mTOR) [145] in serine phosphorylation on STATs; previous reports suggested this role for both p38 and Jnk (Fig. 3) [31, 74]. However, although IL-12--induced sustained activation and nuclear translocation of STAT4 is conditional upon its tyrosine and serine phosphorylation [8], both PI3k and Erk1/2 inhibitors failed to prevent IL-12-induced serine phosphorylation on STAT4. In T cells, IL-12-induced phosphorylation of $S^{721}$ was abrogated by mutation of $Y^{693}$ on STAT4, preventing transcriptional activity [129]. Although p38α and its upstream activator MKK6 were involved in phosphorylation of $S^{721}$ on STAT4, the latter was not required for proliferation, but rather for IFN-γ production by Th1 cells [84]. Thus the PMSP motif as well as phosphorylation of tyrosine and serine residues regulate the function of STATs.

A slightly different Pro-Ser-Pro (PSP) motif, serving as a phosphoacceptor site, was mapped to STAT5a and STAT5b (Fig. 2C). The PSP regulatory site seems to function distinctly from the PMSP site, as the PSP motif proved to be insensitive to the inhibitors of PI3k, mTOR, and Mek [61, 86, 132]. As proposed, two sites must be phosphorylated on STATs to enable their binding to importin-α protein and translocation through the nuclear pore and binding to proper DNA sequences such as IFN-stimulated response element (AGTT-TNNNTTTCC) or IFN-γ-activated sequence (TTC-NNNGAA). Simultaneous binding of other transcription factors to respective DNA sequences promotes gene transcription. An unknown serine kinase controls TCGF-induced serine phosphorylation on STAT5a/b and STAT3 [86]. Phosphorylation of the PSP motif of STAT5a and STAT5b was not essential for DNA binding or transcriptional activation [142]. Recent studies showed that the epidermal growth factor receptor ERBB4 was required for $S^{779}$ phosphorylation on STAT5a, thereby stabilizing ERBB4/STAT5a interaction for efficient STAT5a-induced gene expression [32]. Two other $S^{127}$ and $S^{128}$ sites on STAT5a were needed for ERBB4-dependent phosphorylation of $Y^{694}$, documenting that three serines regulate ERBB4-mediated activation of STAT5a. Additionally, phosphorylation of residues $S^{725}$ and $S^{779}$ on STAT5a cooperatively suppressed prolactin-stimulated transcription [141].

A different picture emerged after analysis of STAT6 [132], which exerts its biological activity following IL-4- and IL-13-induced stimulation of T cell growth and especially differentiation into Th2 cells [107, 130]. When PHA-primed T cells are re-challenged with IL-4 or IL-13, STAT6 displayed phosphorylation of $S^{756}$ localized in the conserved Serine Proline (SP) motif that is distinct from the PMSP and PSP motifs (Fig. 2C). Consequently, $S^{756}$ in the SP region of STAT6 must be differently regulated in comparison with the PMSP region in STAT1, STAT3, and STAT4, as well as the PSP region in STAT5a/b. Published work revealed that STAT6 cycles between active and inactive forms regulated by the serine-threonine phosphatase PP2A [139]. It is suggested that phosphorylation of threonine at position 645 ($T^{645}$) uncoupled the tyrosine kinases responsible for activating $Y^{641}$, which blocked STAT6 oligomerization. Our recent results showed that IL-4 regulated the phosphorylation of $S^{756}$ localized in the transactivation domain of STAT6, but that $Y^{641}$ phosphorylation (or dimerization) and the IRS/PI3k pathway were not required for this process [132, 137]. We propose that another residue distinct from $S^{756}$ and proximal to the $Y^{641}$ and $T^{645}$ sites may be involved in STAT6 function. The phosphomimic variants of $T^{645}$ mutated to glutamic acid or to aspartic acid ablated STAT6 activation, whereas substitutions of $T^{645}$ to glycine, asparagine, alanine, valine, or phenylalanine showed no effects. Thus, the same conserved serine site plays distinct functional roles in STATs (Fig. 3C), showing further regulatory complexity among STATs.

The negative regulation of JAK/STAT signaling occurs at several levels of signaling (Fig. 3). A family of SH2-domain containing proteins called suppressor of cytokine signaling (SOCS) displayed eight members, namely CIS and SOCS1-SOCS7 [48]. SOCS proteins were induced by cytokines and blocked JAKs/STATs activation by a classical negative-feedback loop [46]. Through SH2-binding domains, SOCS could bind to tyrosine-phosphorylated sites on cytokine receptors or JAKs blocking JAK/STAT activity. Another SOCS box domain constantly attaches such complexes to the

Elongin B/C/Cullin-2/E3 ligase that is a part of the ubiquitination system for degradation in proteosomes. SOCS may act via distinct mechanisms. For example, SOCS1 was shown binding directly to tyrosine phosphorylated JAKs [38], SOCS3 to the activated cytokine receptor itself [91], and CIS to the STAT receptor docking sites [146]. The importance of SOCS regulation was confirmed in knockout mice. SOCS1$^{-/-}$ mice displayed lymphopenia, increased apoptosis of lymphoid organs, and impaired IFN-γ signaling [2, 87], whereas SOCS3$^{-/-}$ mice exhibited an embryonic death [79]. In the opposite situation, an over-expression of SOCS1 induced apoptosis of cells with involvement of active ubiquination [42]. SOCS1 over-expression in T cells reduced the total number of thymocytes and increased the apoptosis of mature T cells in a similar fashion as observed in JAK3- or $\gamma_c$-deficient mice [42]. These observations suggest that regulation by SOCS of JAK/STAT signaling contributes to T and B cell function.

The second family of regulatory molecules for JAKs/STATs function involves tyrosine phosphatases (Fig. 3). Selective deletion of phosphatases impaired T and B cell function related to T cell protein tyrosine phosphatase, the SH2-domain containing phosphatase-2 [147] and CD45 [22]. For example, the CD45 tyrosine phosphatase positively regulated signal 1 (e.g. p56 Lck and p59 Fyn activation) but negatively regulated signal 3 (JAKs/STATs). Cytokine stimulation of cell lines derived from CD45$^{-/-}$ mice displayed elevated tyrosine phosphorylation of JAKs/STATs [51]. So far, the activating ligand for CD45 is not known, but such a molecule may be used to regulate the immune response.

The third family of regulatory proteins, called Pias (protein inhibitor of activated STAT), most likely bind to dimerized STATs (Fig. 3). So far, four members have been identified, namely Pias1, Pias3, PiasX, and PiasY. Whereas Pias1 interferes with STAT1 function, Pias3 regulates STAT3 DNA binding [30]. In similar fashion, PiasX and PiasY down-regulate gene transcription induced by STAT4 and STAT1, respectively [7, 75]. These and as yet not discovered regulatory mechanisms of JAKs/STATs function may be explored to improve allograft survival and induction of transplant tolerance.

The unique specificity in response to cytokines by JAKs and STATs derives from multiple aspects of their responses, including positive and negative regulators by kinases/phosphatases, SOCS, and Pias proteins as well as the interplay of homo- or hetero-dimers of STATs and binding of other transcription factors. Multiple tyrosine and serine phosphorylation sites within STATs are involved in regulatory functions. As recently proposed, PP2A sites retain STAT6 in the cytoplasm [132] and deactivate nuclear STAT6 localization prior to its shuttling back to the cytoplasm [139]. It is also proposed that STATs are able to constantly shuttle between the cytoplasm and nucleus regardless of their phosphorylation status [103]. Furthermore, SUMO-ylation may be involved in the regulation of the STAT life cycle, impacting lymphocyte development [108].

## THERAPEUTIC TARGETING OF JAKs AND STATs

### *JAK3 inhibition and immune suppression*

Considering that JAK3 has a central role in T cells and a limited pattern of expression in lymphoid tissue, targeted inhibition of JAK3 may provide immune suppression without side effects compared with current therapies. Current clinical immunosuppressive regimens that target phosphoryl-transferase enzymes in immune-competent cells are dominated by cyclosporine (CsA) or FK 506 to inhibit the calcineurin (CaN) phosphatase and rapamycin (RAPA) that blocks the serine--threonine kinase mTOR [1, 37, 54, 134]. Inhibition of CN disrupts Tcell progression through early $G_1$, while RAPA disrupts the later $G_1$-S cell cycle phase [1, 54, 134]. However, the ubiquitous expression profiles of both enzymes have limited the efficacy of these drugs, which overtly yield adverse side effects, including nephrotoxicity and neurotoxicity for CaN inhibitors and myelosuppression and hyperlipidemia for RAPA [37].

JAK3 inhibitors such as tyrphostin AG490 [12], prodigiosin PNU156804 [117], dimethoxyquinazoline JANEX-1 [125], and CP-690,550 [29] blocked allograft rejection. However, lack of selectivity to JAK3 versus JAK2 may produce undesirable effects: AG490 had similar effects on both kinases, whereas PNU156804 was slightly more effective toward JAK3 than JAK2. Although CP-690,550 was much more effective to JAK3 than JAK2 and caused no apparent metabolic abnormalities, CP-690,550 treatment of cynomolgus monkeys was associated with anemia in some recipients, which was most likely related to JAK2 inhibition [17, 29]. We recently tested a Mannich base compound, NC1153, for its ability to inhibit JAK3. In *in vitro* assays, NC1153 preferentially inhibited JAK3 compared with JAK2 and several other kinases [118, 119]. When tested *in vivo*, a short-term 14-day therapy with NC1153 extended the survival of rat kidney allografts, whereas a continuous 90-day therapy induced transplantation tolerance. The combination of NC1153 with CsA produced potent therapeutic synergism, whereas NC1153 alone was neither nephrotoxic nor affected hematopoiesis and lipid metabolism. In fact, NC1153 did not increase CsA--induced nephrotoxicity, but addition of C1153 allowed a lowering of CsA doses. We also showed that NC1153 was not metabolized by the cytochrome P450 3A4 isoform, the primary metabolizing enzyme of CsA and RAPA. Overall, we propose that a selective JAK3 inhibitor may provide very unique clinical benefits for transplant patients by avoiding toxicities produced by CaN and mTOR inhibitors.

Our present results suggest that blockade of JAK3 not only prevents allograft rejection, but also induces donor-specific transplantation tolerance [119]. There are two phases in tolerance induction, namely elimination of donor-specific Tcell clones and generation of Treg cells. Although blockade of JAK3/STAT5 induces

apoptosis of T cells, the same signaling is needed for the generation of Treg cells. Thus, maneuvering between selective deletion of alloreactive T and B cells and promoting Treg cells may require targeting regulatory molecules such as SOCS, phosphatases, and Pias or other pro- or anti-apoptotic molecules.

*STAT5 and survival mechanisms in tumor models*

Supportive evidence for the role of STAT5a/b as survival factors is clearly evident in lymphoid tumors. Whereas STAT proteins are unphosphorylated and inactive in quiescent cells, constitutively active STATs such as STAT5 are commonly observed in a number of malignancies, including HTLV-1, *Src*, v-Abl, Epstein-Barr virus-transformed cell lines, and some patient lymphocytes. [19, 25, 28, 60, 85, 124, 133, 148]. These finding support the notion that STAT5 promotes cytoprotective (or anti-apoptotic) gene transcription that could include possible STAT5 target genes c-*myc*, *bcl-x*, *bcl2*, [77] and *pim-1* [80]. Others have reported that transgenic mice expressing a variant of the IL-2Rβ devoid of STAT5a/b binding sites within an IL-2Rβ-null background possess lymphocytes with impaired proliferative and prosurvival responses to IL-2 [41].

Additionally, fusion tyrosine kinases, including Bcr/Abl, Tel/Abl, Tel/JAK2, Tel/PdgfβR, Tel/Trkc(L), and Npm/Alk, arising from reciprocal chromosomal translocations have also been linked to acute and chronic leukemias and non-Hodgkin's lymphoma [15, 64]. One unifying factor is their shared use of constitutively activated STAT5 [35, 49, 93]. Interestingly, although JAK2 is not proposed to have a critical role in T cell development, a Tel-JAK2 fusion [t(9;12)(p24;p13) chromosomal translocation] detected in childhood acute T cell leukemia has been associated with active STAT5 and an increase in the number of $CD8^+$ T cells [26]. Likewise, the Npm/Alk fusion gene, formed by the t(2;5) translocation in anaplastic large-cell lymphoma, results in hyperactive STAT5 function that is partially corrected by a dominant-negative STAT5 [92].

*STAT5: impaired function in immunodeficiencies*

Hyperactive forms of STAT5 are found in transformed lymphocytes, but are there diseases that might result if STAT5 activity were "shut off"? Indeed, lymphocytes harvested from mice bearing subcutaneously introduced mammary adenocarcinoma tumors are immune compromised, unable to generate cellular and humoral responses. Purified T and B cells from these mice have a marked decrease in STAT5a and STAT5b protein levels [100]. In contrast, no change in the expressions of STAT1, STAT3, and STAT6 was detected [100]. Similarly, immune-compromised HIV-1 infected patients that display T cell dysfunction with increased rates of apoptosis displayed lower levels of STAT5a/b and STAT1 protein [101]. Whether or not STAT5 down-regulation is a direct consequence of the virus is unclear and requires further investigation. However, we did observe that co-culturing of normal PHA-activated human T cells with a dual-tropic HIV isolate, but not with the M-tropic strain, resulted in decreased expression of both STAT5a and STAT5b [101].

The basic mechanism by which STAT5a/b protein level is reduced remains unclear. Messenger RNA levels were not significantly affected, suggesting the involvement of other regulatory pathways [59, 97]. Several areas that could be investigated to answer this question would include studying ubiquitylation, which was reported to regulate STAT1 [59] and proteosome targeting by viruses [97, 98, 127, 128]. It is tempting to speculate that disease progression may provoke protease degradation of the STATs by cleaving their C-terminal transactivation domain [9]. One 25-kDa partially purified STAT5 protease has been reported that is competent to generate carboxyl-terminally truncated isoforms of STAT5 present in myeloid and not lymphoid progenitor cells [69]. Other mechanisms by which infectious agents might uncouple immune response via these pathways have been put forth by Selliah et al. in which some HIV strains (NL4-3), but not others (HIV-1 IIIB), inactivated JAK3, subsequently promoting T cell death [109]. Thus, as opposed to therapeutic strategies seeking to uncouple JAK/STAT activity, finding methods to recapitulate active STAT5 might have significant therapeutic potential for other types of patients.

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