# The Ins and Outs of STAT1 Nuclear Transport

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**There is an inherent elegance in being in the right place at the right time. The STAT1 transcription factor possesses regulatory signals that ensure its distribution to the right cellular location at the right time. Latent STAT1 resides primarily in the cytoplasm, and there it responds to hormone signaling through tyrosine phosphorylation by Janus kinases or growth factor receptors. After phosphorylation, STAT1 dimerizes, and this conformational change reveals a nuclear import signal that is recognized by a specific nuclear import carrier. In the nucleus, the STAT1 dimer dissociates from the import carrier and binds to specific DNA target sites in the promoters of regulated genes. STAT1 is subsequently dephosphorylated in the nucleus by a constitutively active tyrosine phosphatase, leading to its dissociation from DNA. A nuclear export signal of STAT1 appears to be masked when dimers are bound to DNA, but it becomes accessible to the CRM1 export carrier after dissociation from DNA. CRM1 binds STAT1 and transports the transcription factor back to the cytoplasm. Studies show that the regulatory trafficking signals that guide the nuclear import and export of STAT1 reside within its DNA binding domain. The location of these signals indicates that their function has coevolved with the ability of STAT1 to bind DNA and regulate gene expression. The nuclear import and subsequent recycling of STAT1 to the cytoplasm are integral to its function as a signal transducer and activator of transcription.**

## JAK/STAT Pathways

Cells respond to various stimuli with the activation of specific signal transduction pathways that lead to gene expression in the nucleus. Signal transducers and activators of transcription (STATs) are DNA binding transcription factors that were first identified as mediators of the interferon (IFN) signal pathway (*1, 2*). STATs remain the only characterized transcription factors that are activated by direct tyrosine phosphorylation (*3-6*). The identity of an IFN-stimulated tyrosine kinase responsible for STAT phosphorylation was originally revealed in a screen for genes that could correct the deficiency of an IFN-unresponsive cell line (*7*). This genetic complementation was achieved by transfection of a cDNA encoding the nonreceptor tyrosine kinase Tyk2, a member of the Janus kinase (JAK) family (*8, 9*). These discoveries were decisive contributions to our understanding of JAK/STAT signal transduction, a pathway that serves as a communication link between cell surface receptors and specific gene expression in the nucleus. The STAT molecules are now known also to be substrates of various oncogenic kinases and growth factor receptors, and they play critical roles in development, immune response, and proliferation. A number of comprehensive reviews on JAK/STAT signaling can provide the reader with detailed overviews of the pathway (*10-16*). In this Review, we discuss molecular mechanisms that regulate STAT cellular localization.

## STATs

STAT1 is the founder member of seven mammalian STATs. STAT homologs also exist in other eukaryotes, including *Xenopus*, *Danio*, *Drosophila*, and *Dictyostelium*, but are not present in yeast, indicating an evolutionary presence linked with multicellular organisms (*14, 17-19*). STATs share a similar structural arrangement of functional motifs, including a central DNA binding domain, a downstream Src homology 2 (SH2) domain, and a C-terminal tyrosine residue that is phosphorylated in response to tyrosine kinases (*3, 6, 20*). After tyrosine phosphorylation, the different STAT proteins homodimerize or heterodimerize through their phosphotyrosine and SH2 domain interactions. This dimerization alters the conformation of the STAT molecules and provides them with new properties. In the cytoplasm, activated STAT dimers gain the ability to bind nuclear import carriers that mediate their transport into the nucleus. More notably, STAT dimers gain the ability to bind specific DNA sequences in the promoters of responsive genes in the nucleus. The crystal structures of tyrosine-phosphorylated STAT dimers associated with DNA have been solved for STAT1 and STAT3, and the transcriptional activation of responsive genes appears to depend on the ability of STATs to recruit transcriptional coactivators (CBP/p300 or GCN5 or Mediator subunits) (*21-26*).

Targeted disruptions of STAT genes have revealed their critical roles in immunity and development. Animals deficient in STAT1 or STAT2 have an impaired response to IFNs and succumb to viral infections (*27-29*). Animals deficient in STAT4 lack specific responses to interleukin-12 and have impaired development of T helper 1 cells, whereas animals deficient in STAT6 lack specific responses to interleukin-4 and have impaired development of T helper 2 cells (*30-34*). The double knockout of STAT5a and STAT5b genes reveals defects in responses to prolactin and growth hormone with the consequences of defective mammary gland development, infertility, loss of male-specific liver gene expression, and hematopoietic defects (*35, 36*). Only disruption of the STAT3 gene leads to an early embryonic lethal phenotype (*37*). The subsequent generation of STAT3 tissue-specific knockouts demonstrates complex roles of STAT3 in proliferation, survival, and development. These loss-of-function phenotypes attest to the vital need for specific STAT-regulated gene expression. Moreover, continuous signaling by STAT proteins can elicit pathogenic effects, including immune dysfunction and uncontrolled cellular proliferation.

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Constitutive activation of STAT3 and STAT5 directly correlates with uncontrolled cell growth and appears to contribute to human neoplasias (*38-41*). Consistent with this, many promising medical therapeutics have targeted the JAK/STAT signaling pathway (*42*). Understanding the molecular mechanisms that have positive or negative effects on STAT function will contribute to the design of drugs for clinical intervention. Nuclear trafficking of STAT proteins is a critical aspect of their regulation and function.

## Nuclear Trafficking

The nuclear membrane partitions the nuclear compartment from the cytoplasm and allows movement of macromolecules through discrete passageways referred to as nuclear pore complexes (NPCs) [reviewed in (*43-45*)]. The NPCs allow passive diffusion of small molecules, but restrict passage of molecules larger than about 50 kD to those that have localization signal sequences. These signal sequences are recognized by soluble shuttling carriers that transport large macromolecules into or out of the nucleus [reviewed in (*46-50*)]. The shuttling carriers for proteins are primarily members of the karyopherin β family, which includes distinct carriers responsible for nuclear import or export. Because the individual shuttling carriers were identified independently by several groups, they have been given various designations. We refer to the mammalian carriers involved in nuclear import as "importins," and those involved in export as "exportins." Importin and exportin carriers must interact with a critical transport effector component, the Ran guanosine triphosphatase (Ran GTPase) [reviewed in (*51*)]. Ran is primarily in a GTP-bound state in the nucleus and a GDP-bound state in the cytoplasm. Binding to Ran-GTP in the nucleus alters the conformation of the carriers, and this results in either importin release of cargo or exportin binding to cargo. There has been rapid progress in the characterization and solution structure of importins; however, the precise mechanisms of carrier-cargo movement through the NPC remain to be determined.

## Nuclear Import

Active transport of molecules through the nuclear pore requires the expression of a nuclear localization signal (NLS). The best characterized "classical" NLS signals are rich in the basic amino acids arginine and lysine and exist either as a single stretch of residues or as a bipartite sequence spaced by about 10 nonconserved residues (*52*). Classical basic NLS sequences are most commonly recognized by the family of importin α adaptors (Fig. 1). There are six characterized mammalian importin α adaptors that share similar structural features. They possess 8 to 10 Armadillo (Arm) repeats, each consisting of helices stacked to form a cylindrical superhelix. Shallow grooves formed by Arms 2 to 4 and by Arms 7 and 8 can bind the arginine-lysine residues of classical NLSs (*53, 54*). The C termini of importin αs are divergent, but are recognized by a specific export receptor in the nucleus, known as cellular apoptosis susceptibility (CAS) protein, that mediates their return to the cytoplasm (*55, 56*). The N termini of the importin αs contain a stretch of conserved residues recognized by importin β1, and the region is referred to as the importin β binding (IBB) domain. Importin β1 is the carrier that transports the importin α and its NLS cargo through the NPC to the nucleus. It contains repeated HEAT (huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, and Tor1 kinase) domain helices forming a superhelix that wraps around the IBB domain of importin α (*57*). Importin β1 binds to nucleoporins in the NPC, effecting translocation to the nucleus, where it associates with Ran-GTP, causing the release of importin α and NLS cargo. Importin β1 can also bind other adaptors and in some cases can directly translocate NLS-containing proteins.

## Nuclear Export

The exit of proteins from the nucleus shares many properties with the import process. Export requires the presence of a nuclear export signal (NES) on cargo destined for the cytoplasm, and also recognition of the NES by soluble carriers called exportins [reviewed in (*46, 49*)]. Some of the exportins are specific for particular cargos such as CAS for importin αs, whereas other exportins such as CRM1 (exportin chromosome region maintenance 1) exhibit a more general function. CRM1 recognizes a NES composed of a short sequence containing hydrophobic amino acids rich in leucine. CRM1 has a conserved Ran binding domain, and it binds Ran-GTP in the nucleus together with the NES cargo to form a stable ternary complex (Fig. 1). The complex is exported through the NPC and dissociates in the cytoplasm after the hydrolysis of Ran-GTP. The crystal structure of CRM1 remains to be solved, but much has been learned of its role in export with the discovery of a pharmacological inhibitor, leptomycin B (LMB) (*58*). LMB is an antibiot-

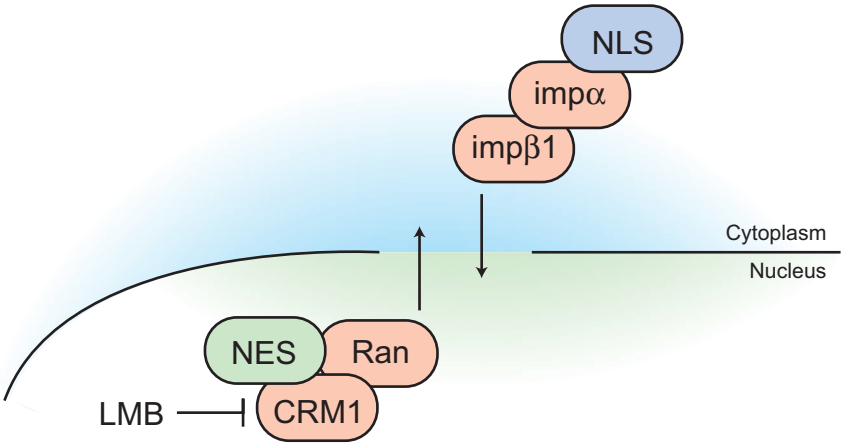


**Fig. 1.** Simple depiction of nuclear trafficking. Import: Members of the importin α (impα) family of adaptors recognize classical NLS sequences in proteins destined for nuclear translocation. Importin α is bound by the import carrier importin β1 (impβ1), which associates with nucleoporins in the NPC and mediates translocation. Export: The CRM1 exportin in association with Ran-GTP recognizes hydrophobic NES sequences in proteins and mediates their nuclear export. Leptomycin B (LMB) binds irreversibly to CRM1 and inhibits its activity.

ic that binds to CRM1 irreversibly, blocking its ability to bind to NES-containing cargo. The use of LMB has helped to identify targets of CRM1-mediated export, and it has proven useful in understanding the cellular distribution of many protein and RNA molecules.

### STAT1 Nuclear Import

Nuclear trafficking of molecules can also be regulated conditionally. Cytoplasmic anchoring, phosphorylation, or masking can affect the accessibility or function of signal sequences (*59, 60*). The conditional presence of a transcription factor in the nucleus can have profound effects on cell cycle progression or specific biological responses. The STAT proteins are directly regulated by tyrosine phosphorylation and, in the cytoplasm, they act as molecular messengers that respond to extracellular stimuli. STATs dimerize through SH2-phosphotyrosine interactions and bind DNA targets in the nucleus to regulate gene expression.

Because the STAT family members closely resemble each other, often lessons learned from one member can apply to other family members. For this reason, we initially focused on the nucleocytoplasmic localization of STAT1. Latent STAT1 resides primarily in the cytoplasm as a soluble protein that is freely diffusible, although a minor fraction is present in the nucleus, and a minor fraction has been reported in membrane raft preparations (*61*). Experimental evidence indicates that unphosphorylated STAT1 is not anchored in the cytoplasm, because it can translocate to the nucleus if a basic NLS is added to the protein (*62*). FLIP (fluorescence loss in photobleaching) and FRAP (fluorescence recovery after photobleaching) analyses on cytoplasmic STAT1 reveal that it diffuses throughout the cytoplasm with high mobility (*63*). However, in response to an activating cytokine such as IFN-γ, latent STAT1 is recruited through its SH2 domain to phosphorylated residues on the receptor where associated JAKs phosphorylate STAT1 on tyrosine 701 (*64*). After tyrosine phosphorylation, STAT1 forms a stable dimer and quickly accumulates in the nucleus. The cellular redistribution can be demonstrated by fluorescent microscopy of cells expressing STAT1 fused to GFP (green fluorescent protein) (Fig. 2). This marked shift in STAT1 localization begins within seconds and is maximal within 15 to 30 min. Mutations that prevent tyrosine phosphorylation (*65, 66*) or STAT dimerization (*67, 68*) inhibit translocation to the nucleus in response to cytokines. It is clear that tyrosine phosphorylation mediates a switch-like response leading to this rapid STAT1 nuclear import. This switch involves the recognition of STAT1 dimers by at least one member of the importin α adaptor family, importin α5 (*69*). Importin α5 recognizes only tyrosine-phosphorylated STAT1 dimers, consistent with a gain-of-function response for STAT1 nuclear import.

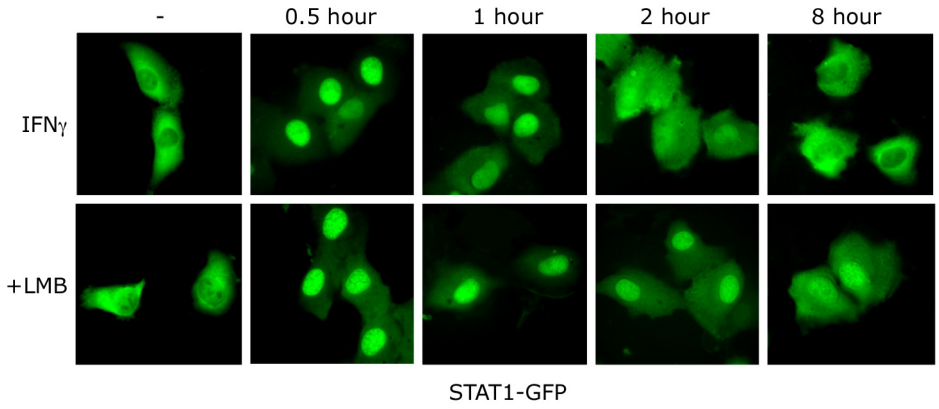


**Fig. 2.** Kinetics of STAT-GFP nuclear localization. **Top panels**: STAT1-GFP was expressed in cells that lack endogenous STAT1. The cells were untreated (-) or pulse-treated with IFN-γ for 30 min and maintained in cycloheximide. Time after initial addition of IFN-γ is indicated. Localization was visualized by fluorescence microscopy. **Bottom panels**: Cells were pretreated with leptomycin B (+LMB) for 20 min followed by IFN-γ, as in top panels. Figure is a modified version of previous publication (*62*).

A truncated importin α5 molecule that lacks the IBB domain and cannot be transported across the NPC by importin β1 acts as a dominant interfering molecule, binding to STAT1 dimers but inhibiting their nuclear accumulation. These investigations also showed that STAT1 nuclear translocation is Ran dependent (*70*). Introduction of Ran mutations or microinjection of Ran antibodies that block function prevented STAT1 nuclear transport in response to IFN.

*Nature of the STAT1 conditional NLS.* Several studies have surveyed the effects of mutations on STAT1 nuclear import. Mutations within a small region of the DNA binding domain have been found to disrupt nuclear import in response to cytokine stimulation. A single mutation in leucine 407 (L407A) or a double mutation in lysines 410 and 413 (KK410/413AA) produce STAT1 molecules that become tyrosine-phosphorylated after IFN-γ treatment but remain in the cytoplasm (*68, 71*). Further analyses determined that neither of these STAT1 mutations were able to bind to importin α5. More important, although the KK410/413AA mutant cannot bind DNA, the tyrosine-phosphorylated L407A mutant still retains its ability to dimerize and bind DNA, indicating that its defect in import is specific to the lack of importin α5 recognition. The structure of the NLS formed in STAT1 may be even more intricate, because a study reported the N terminus to be required for cytokine-induced nuclear transport (*72*).

The interaction of tyrosine-phosphorylated STAT1 dimers with importin α5 appears distinct from the interaction of classical basic NLSs with importin α5. Analyses of importin α5 truncations indicate that STAT1 interacts with the C terminus of importin α5 containing Arms 8 to 10 (residues 425 to 538), whereas classical NLS sequences associate with Arms 2, 3, 4, 7, and 8 (residues 80 to 450) (Fig. 3, bottom) (*53, 54, 69*). Further evidence that the binding site for STAT1 is distinct from classical NLSs is the fact that both STAT1 dimers and a classical NLS can associate with importin α5 simultaneously in vivo (*69*). These results indicate that STAT1 may have an unconventional NLS.

Importin α5 binds directly to STAT1 dimers and not to a

bridging adaptor. This was demonstrated by analyzing the interaction of proteins isolated from bacteria, which do not possess nuclear trafficking mechanisms. A direct interaction was observed in vitro between importin α5 and tyrosine-phosphorylated STAT1, but not with unphosphorylated STAT1. Furthermore, the direct interaction requires STAT1 dimerization, because STAT1 molecules that are phosphorylated but unable to dimerize as the result of an SH2 domain mutation do not bind importin α5 (*68*). The key event that triggers the conditional appearance of the NLS function in STAT1 is dimerization, mediated by tyrosine phosphorylation. This is consistent with the observation that artificially dimerized STATs can accumulate in the nucleus (*39, 73*).

The requirement for leucine at residue 407 is certainly atypical of classical NLS character. This finding, together with the fact that STAT1 dimers bind to an unconventional carboxyl region of importin α5, rather than to the central Arm repeats, indicates that the NLS is unusual. Conventional NLS sequences can function autonomously; however, this region of the STAT1 DNA binding domain (amino acids 369 to 436) linked to GFP does not promote nuclear transport (*62*). In fact, this region of STAT1 has the opposite effect because of the presence of an overlapping NES (discussed below). In addition, the NLS does not appear to be created in the dimer by the juxtaposition of residues from each STAT1 monomer, because a wild-type STAT1 can rescue nuclear import by heterodimerization with a STAT1 mutant L407A (*68*). This result indicates that dimerization produces a conformational change that allows the NLS to become functional. The nature of the NLS may be complex, encompassing additional residues or domains of STAT1. One report indicates that the N terminus is required for IFN-induced nuclear transport, which suggests that the tyrosine-phosphorylated dimers express multiple surfaces needed for interaction with importin α5 (*72*). Future cocrystal structures of STAT1 dimers with importin α5 may reveal unique conformational interactions.

The data indicate that a STAT1 dimer enters the nucleus with importin α5 bound to its DNA binding domain. Occupancy of this domain by importin α5 might prevent STAT1 recognition of DNA, but experiments designed to evaluate this possibility showed that specific target DNA sequences effectively compete with importin α5 for binding to STAT1 dimers (*68, 71*). The ability of STAT1 dimers to bind target DNA with higher affinity than importin α5 may contribute to appropriate targeting of STAT1 to specific genes in the nucleus. The released importin α5 can be recycled to the cytoplasm by the CAS exportin (*55, 56*). The binding site of CAS to the importin α5 C terminus overlaps with the binding site of STAT1 dimers, and for this reason the recycling mechanism of importin α5 may be directly coupled to STAT1 release and binding to DNA.

*Additional aspects of STAT1 nuclear trafficking.* Although it is clear that unphosphorylated STAT1 resides predominantly in the cytoplasm, there is an apparent constitutive level of STAT1 in the nucleus (Fig. 2). Publications with immunostaining describe varied levels of nuclear STAT1 from undetectable to substantial. Because differences in staining can depend on the specific STAT1 antibody, it may be more reliable to use low expression of tagged STAT1 for localization studies.

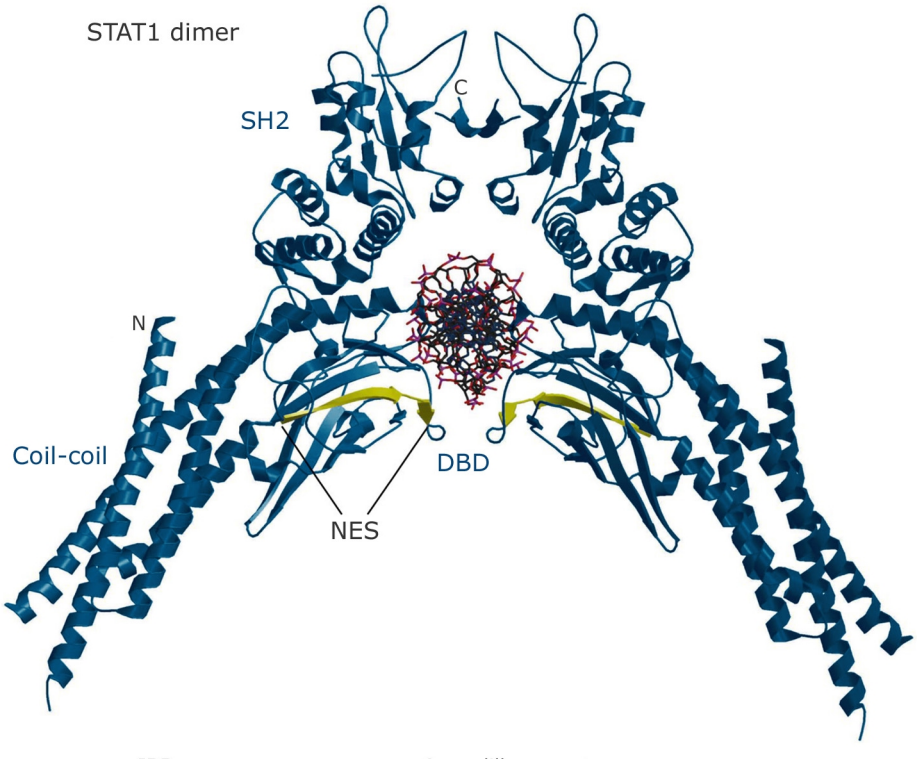


**Fig. 3.** STAT1 NES within the DNA binding domain (DBD). **Top**: Location of NES in the crystal structure of tyrosine-phosphorylated STAT1 dimers bound to DNA (amino acids 136 to 710) (*88*). The DNA in the center is oriented perpendicular to the page. The NES (amino acids 400 to 409) is shown in yellow indicated on one monomer. The ribbon diagram was prepared for a previous publication (*62*). **Bottom**: Diagrammatic representation of importin α5 with the importin β1 binding (IBB) domain and Armadillo helical repeats. Regions of association with classical NLS and STAT1 dimers are indicated (*53, 54, 69*).

The process that leads to low levels of constitutive nuclear STAT1 appears to be distinct from the process described above for tyrosine-phosphorylated dimers, because mutations of the STAT1 phosphotyrosine Y701 or the SH2 domain do not ablate the nuclear pool (*74*). The N-terminal region of STAT1 reported to be necessary for nuclear localization of phosphorylated STAT1 is not necessary for STAT1 constitutive nuclear appearance (*72*). Analyses with FLIP indicate that the nuclear pool does not rapidly exchange with the cytoplasm (*63*). In addition, treatment of cells with LMB, an inhibitor of CRM1 exportin, does not lead to nuclear accumulation of unphosphorylated

STAT1, indicating that the majority of STAT1 does not shuttle in and out of the nucleus (*68, 75*) (Fig. 2). One study has shown that the constitutive nuclear STAT1 pool can be enhanced by "trapping" STAT1 in the nucleus after microinjection with specific antibody (*76*). The kinetics and the mechanism that governs apparent unphosphorylated STAT1 movement to the nucleus remain to be resolved.

Unphosphorylated STAT1 does not appear to bind importins specifically in vitro (*68, 69, 71*). However, it is possible that unphosphorylated STAT1 is carried across the NPC by association with a non-STAT protein. A dimer of unphosphorylated STAT1 in a complex with interferon regulatory factor 1 (IRF-1) has been described to recognize a specific overlapping STAT/IRF-1 target site in the low molecular mass polypeptide 2 (LMP-2) gene (*74*). The constitutive expression of LMP2 and other genes appears to reflect a possible role for unphosphorylated STAT1 in the nucleus (*77*). Interaction of STAT1 with other NLS-containing proteins such as IRF-1 may result in low levels of nuclear STAT1. Alternatively, a weak NLS in unphosphorylated STAT1 may be responsible for nuclear presence, but the affinity for importins may be too low for specific detection, or unphosphorylated STAT1 may interact with an as yet uncharacterized import mechanism. Although STAT1 SH2 domain or phosphotyrosine 701 are not necessary for constitutive nuclear presence, our studies indicate that a functional DNA binding domain is necessary (*62*).

Recent reports have described the possibility of nuclear translocation of transcription factors through association with growth factor receptors or receptor fragments (*78*). An analogous model for STAT1 nuclear import suggests that translocation is mediated by an NLS in the IFN-γ cytokine associated with its receptor (*79, 80*). If a mutation is introduced in the putative NLS of IFN-γ, the cytokine loses its ability to stimulate STAT1 nuclear accumulation. However, this loss-of-function mutation may not allow the cytokine to function normally in receptor binding or activation, and for this reason additional studies are needed. It is also not clear that IFN-γ must be internalized to function; earlier studies indicate otherwise. In addition, agents such as the tyrosine phosphatase inhibitor pervanadate lead to JAK activation and do not require ligand-receptor signaling for STAT1 tyrosine phosphorylation or nuclear import. As described above, the STAT1 L407A mutation can be accurately tyrosine-phosphorylated by the IFN-γ-receptor complex, but it does not translocate to the nucleus because it cannot bind importin α5. These latter observations clearly indicate that STAT1 has an intrinsic NLS that can impart nuclear localization independent of association with ligand-receptor complexes.

### STAT1 Nuclear Export

In response to IFN-γ, tyrosine-phosphorylated STAT1 dimers rapidly accumulate in the nucleus. However, nuclear presence is transient and STAT1 redistributes to the cytoplasm after several hours (*62, 81, 82*) (Fig. 2). The effect of the CRM1 inhibitor LMB was evaluated to determine whether the CRM1 exportin mediates the return of STAT1 to the cytoplasm. Treatment of cells with LMB drastically inhibited STAT1-GFP relocalization to the cytoplasm, indicating that CRM1 is the major exportin for STAT1 (*62, 75, 83*) (Fig. 2). In these studies, LMB alone had no effect on the cellular distribution of unphosphorylated STAT1, indicating that unphosphorylated STAT1 does not shuttle constitutively between nucleus and cytoplasm via CRM1 activity. Because tyrosine phosphorylation is necessary for dimerization and inducible STAT1 nuclear import, we evaluated the phosphorylation state of STAT1 during import and export (*62*). The results indicated that STAT1 was dephosphorylated in the nucleus and that this dephosphorylation correlated with export. Dephosphorylated STAT1 dissociates from specific DNA targets, and this dephosphorylation and dissociation correlate with export from the nucleus.

Protein tyrosine phosphatases (PTPs) comprise a large family of enzymes with diverse roles in signaling and development (*84, 85*). Recent studies have identified a PTP that appears to be able to dephosphorylate STAT1 in the nucleus (*86*). The nuclear form of the T cell PTP (TCPTP) known as TC45 was shown to dephosphorylate STAT1 dimers in vitro. In addition, cells from a TCPTP null animal were found to be partially defective in STAT1 dephosphorylation, displaying prolonged tyrosine phosphorylation after IFN treatment. These effects may also reflect increased JAK activity (*87*).

*Nature of the STAT1 NES.* NES sequences recognized by CRM1 are rich in leucine residues, but do not conform to a strict consensus. Because leucine is the most prevalent amino acid in proteins, there is a high probability of detecting motifs that resemble NESs but do not function like NESs (*47*). Scanning the primary sequence of STAT1 reveals more than two dozen leucine-rich NES candidates. Furthermore, an NES detected in the primary sequence may be buried in the native structure of STAT1 and not accessible to CRM1. If such a sequence is studied as a peptide outside of its normal context, it may demonstrate export activity, but this may not contribute to the true export regulation of the native protein. Studying peptide behavior is useful, but only to confirm a characterized NES in the protein. For these reasons, we used experiments with an in vitro CRM1 binding assay in the presence of Ran to determine its recognition site in the unphosphorylated STAT1 molecule. The studies indicated that CRM1 recognizes a region in the DNA binding domain of STAT1 between amino acids 369 and 436 (*62*). Analyses of this region linked to GFP and the effect of site-directed mutations demonstrated CRM1-dependent nuclear export of a functional NES located within residues 399 to 410. Two other reports described the presence of additional NES motifs in the N terminus of STAT1 (*75, 83*). The NES functions of these motifs (amino acids 197 to 205 and 302 to 320) were evaluated by using isolated peptides fused to GFP. However, we did not detect CRM1 binding to this region of STAT1, and for this reason the mechanisms by which these motifs contribute to STAT1 export remain to be more fully characterized.

*Regulation of STAT1 NES by DNA binding.* The location of the NES that we identified is within the domain of STAT1 that contributes to specific DNA binding (*20, 88*). A ribbon diagram representing the crystal structure of a tyrosine-phosphorylated STAT1 dimer bound to DNA is presented in Fig. 3 (*88*). The position of this NES is shown in yellow on one of the STAT1 monomers; this location suggests that the NES may be masked when STAT1 is bound to DNA. In the crystal structure, the side chains of L400, F404, L407, and L409 within this NES are buried and their surface accessibility is predicted to be limited when STAT1 is associated with DNA. This led to the hypothesis that the NES function is conditional; that is, when STAT1 dimers are bound to DNA, CRM1 cannot gain access to the NES. Conversely, when nuclear STAT1 is not bound to DNA, it

is a target of CRM1-mediated export.

To test this hypothesis, we evaluated the behavior of a mutated STAT1 protein that can be tyrosine-phosphorylated but cannot bind DNA (*62*). In contrast to wild-type STAT1, the DNA binding mutant did not accumulate in the nucleus after IFN-γ treatment due to efficient nuclear export mediated by CRM1. These and other experiments support a model in which the NES is masked when tyrosine-phosphorylated dimers are bound to DNA, but after dephosphorylation the STAT1 proteins are released from DNA and are now accessible to CRM1 binding and export into the cytoplasm. Regulated nuclear export of STAT1 may serve to help silence the pathway when a stimulating signal has ceased, or it may contribute to reactivation of the pathway by returning STAT1 to the cytoplasm to respond to receptor-kinase signals. The NES motifs described by others have not been proposed to be regulated conditionally, but they may contribute to STAT1 constitutive export (*75, 83*).

**STAT1 DNA Binding Coordinates with Cellular Localization**

The DNA binding domain of STAT1 appears to have coevolved with signal sequences that conditionally regulate its cellular localization (Fig. 4). The coevolution of NLS sequences within the DNA binding domains of many transcription factors appears to be a common event (*89, 90*). Although residues critical to STAT1 import are resident within the DNA binding domain, this region alone cannot mediate import. The regulated nuclear import mediated by importin α5 binding is contingent on STAT's ability to dimerize. Tyrosine phosphorylation in the cytoplasm functions to serve as a molecular switch that mediates STAT1 dimerization in such a way as to alter its conformation to express an NLS. This dimerization is also required for specific target DNA binding. In the nucleus, specific DNA targets successfully compete with importin α5 for association with STAT1 dimers. When STAT1 is bound to DNA, its NES is masked and not accessible to CRM1 binding for export. However, after STAT1 dephosphorylation in the nucleus by a constitutive PTP, STAT1 dissociates from the DNA. The NES in the DNA binding domain of unphosphorylated STAT1 in the nucleus now becomes accessible to CRM1-mediated export, and STAT1 is returned to the cytoplasm. STAT1 has thereby evolved an elegant mechanism of nuclear trafficking that involves (i) a conditional NLS dependent on STAT1 dimerization through tyrosine phosphorylation, and (ii) a conditional NES dependent on dephosphorylation and STAT1 dissociation from DNA (Fig. 4). Future studies are still needed to address mechanisms that regulate basal levels of nuclear STAT1 and the possible function of N-terminal export signals.

Negative modulators of STAT function include the family of

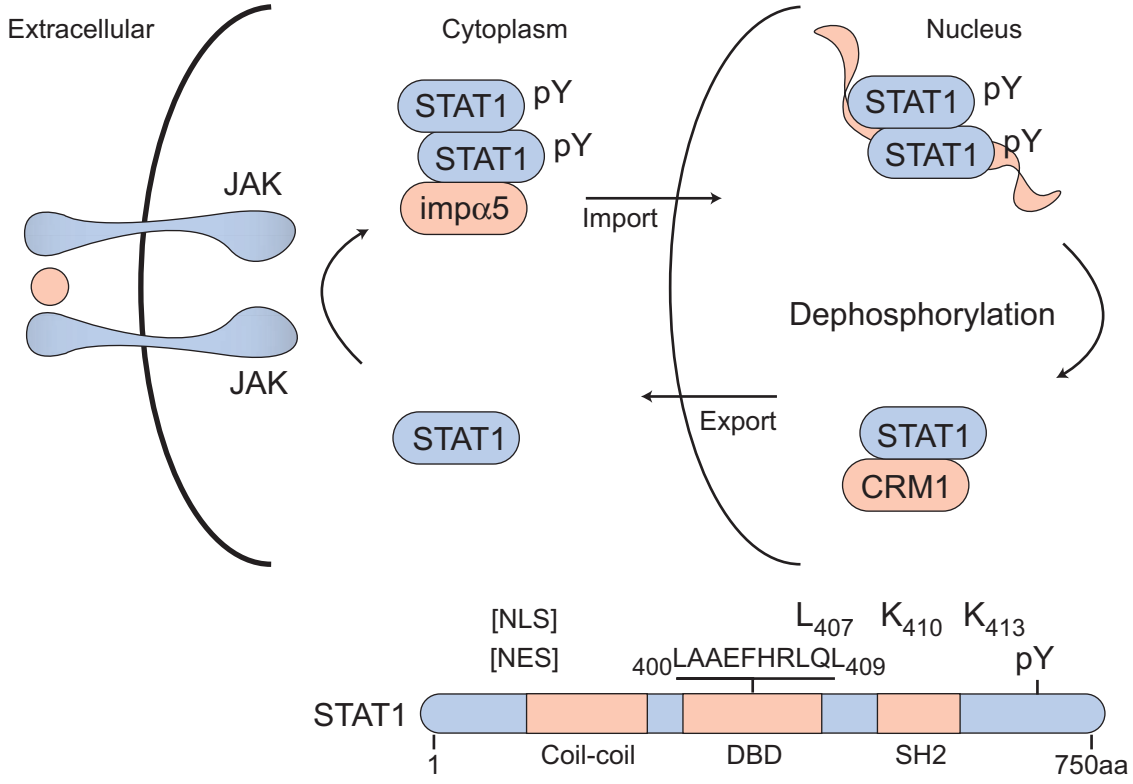


**Fig. 4.** Conceptual model of STAT1-regulated nuclear import and export. **Top**: STAT1 nuclear import can be mediated by importin α5 (impα5) binding to STAT1 tyrosine-phosphorylated (pY) dimers in the cytoplasm. In the nucleus, target DNA sequences successfully compete with importin α5 for binding to STAT1 dimers. The NES of STAT1 is masked when the dimers are bound to DNA. Dephosphorylation by a nuclear phosphatase releases STAT1 from DNA, and the NES becomes accessible to CRM1-mediated export. **Bottom**: Linear diagram of STAT1 showing the location of the NES within the DNA binding domain and several amino acids that are required for NLS function within the DNA binding domain (*62, 68, 71*).

protein inhibitors of activated STATs (PIAS) (*91*). These proteins reside in the nucleus and can bind tyrosine-phosphorylated STAT dimers, resulting in an inhibition of STAT binding to DNA. Members of the PIAS family have been found to function as SUMO-1 (small ubiquitin-related modifier 1) ligases, and although STAT1 appears to be sumoylated, this modification does not play an apparent role in gene induction by STAT1 dimers (*92*). For this reason, sumoylation does not as yet appear to influence nuclear trafficking. However, binding to PIAS proteins may influence STAT localization. STAT1 has been shown to be modified by arginine methylation, and cell-permeable inhibitors of methylation increase STAT interaction with PIAS1 and decrease STAT interaction with TCPTP, the nuclear phosphatase (*93*). Although the effect of methylation on STAT1 nuclear trafficking has not been evaluated directly, future studies may indicate an influence on STAT nuclear presence due to effects on PIAS or TCPTP interactions.

### Are All STATs the Same?

Because all STATs function in cell surface signaling to the nucleus, their cellular distribution between the cytoplasm and nucleus must be regulated. Dimerization through tyrosine phosphorylation serves as an activating switch to stimulate nuclear import and DNA binding, and in this respect the behavior of STAT1 in response to IFN serves as a paradigm for other members of the STAT family. However, studies indicate that there are aspects of STAT cellular distribution that distinguish family members. The STAT3 protein is tyrosine-phosphorylated by the largest number of characterized cytokines, growth factors, and oncogenic tyrosine kinases, and in response, phosphorylated dimers localize to the nucleus. However, unphosphorylated STAT3 exhibits chiefly nuclear presence (*94*). Reports vary in the literature as to the localization of unphosphorylated STAT3, but STAT3 appears to have a constitutive NLS that is not dependent on tyrosine phosphorylation. A domain that functions in import, either directly or by interaction with an NLS-containing protein, may reside within the STAT3 N terminus (*95*). STAT3 has also been reported to interact with other factors such as EZI and c-Jun; this may influence nuclear presence (*96, 97*). Although STAT3 is primarily nuclear, recently it has been reported to possess three NES elements that can function as isolated peptides (*98*). This study also showed that inhibition of CRM1-mediated export by LMB enhanced the constitutive nuclear accumulation of STAT3, indicating that STAT3 shuttles continuously in and out of the nucleus. An additional connection between receptor signaling and STAT3 nuclear accumulation has been reported through endocytosis of the epidermal growth factor receptor (*99*).

In a manner similar to STAT3, unphosphorylated STAT5b has been found to shuttle in and out of the nucleus (*100*). In response to LMB inhibition of nuclear export, STAT5b accumulates in the nucleus. An N-terminal region of STAT5b is proposed to be responsible for its constitutive nuclear import, possibly directly or by association with other factors such as Nmi or the glucocorticoid receptor (*101, 102*). As for STAT3, several NESs have been identified in STAT5b as isolated peptides linked to GFP. Therefore, both STAT3 and STAT5 appear to have constitutive NLS and NES functions that promote shuttling of unphosphorylated factors.

STAT2 is clearly cytoplasmic in an unphosphorylated state and localizes to the nucleus in response to IFN-α after tyrosine phosphorylation and dimerization with STAT1. This nuclear import of tyrosine-phosphorylated STAT2 is mediated by the binding of importin α5 to STAT2-STAT1 heterodimers (*68*). Additionally, STAT2 is unique among the STATs in that it is associated constitutively with a non-STAT protein, interferon regulatory factor-9 (IRF-9), and therefore a trimeric complex of STAT1-STAT2-IRF-9 (ISGF3) associates with a specific DNA target in the nucleus (*103, 104*). Our recent studies have shown that unphosphorylated STAT2 localizes to the cytoplasm as the result of a dominant NES in a domain distinct from that of STAT1 (*105*).

The differences and commonalities in the mammalian STATs are also reflected evolutionarily in various STAT homologs of *Dictyostelium*, Dd-STATa and Dd-STATc (*106, 107*). Dd-STATa resides in the cytoplasm in a latent state; in response to cyclic adenosine monophosphate (cAMP) stimulation of a G protein-coupled receptor, it is tyrosine-phosphorylated and accumulates in the nucleus, similar to mammalian STAT1. It must gain an NLS function to translocate to the nucleus. LMB treatment alone does not promote nuclear accumulation, indicating that Dd-STATa does not constitutively shuttle. In comparison, Dd-STATc is different from STAT1 because it appears to shuttle constitutively in and out of the nucleus. Dd-STATc contains a constitutive NLS in its N terminus and a constitutive NES adjacent to its DNA binding domain. In response to the DIF activator, it becomes tyrosine-phosphorylated and accumulates in the nucleus. This nuclear accumulation appears to be due to masking of the NES, possibly when the dimer is bound to DNA, similar to mammalian STAT1. As with mammalian STATs, the individual Dd-STATs have evolved distinct features that regulate their nuclear localization.

STAT proteins transmit signals into the cell in response to various stimuli at the cell surface. It is not surprising that regulatory mechanisms have evolved to control their cellular localization on the basis of their state of activation and their competence to bind DNA. The ability of STATs to sense external stimuli and undergo dynamic localization allows them to be sensitive and rapid transducers of cell surface signaling. Future molecular, structural, and genetic investigations will lead to a better understanding of the mechanisms, signal sequences, and specific transport carriers that effect the cellular distribution of the STAT family members.

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