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Regulation of Nuclear-Cytoplasmic Transport of STAT5a Transcription Factor

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Ha Youn Shin

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ABSTRACT OF THE DISSERTATION

Regulation of Nuclear-Cytoplasmic Transport of STAT5a Transcription Factor

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Signal Transducer and Activator of Transcription 5 (STAT5) is a transcription factor that is critical for various biological processes including hematopoiesis, liver metabolism and mammary gland development. However, persistent activation of STAT5 has been causally linked to human leukemia and solid tumor formation. Since STAT5 is important for normal physiological functions and aberrant activity can promote cancer, accurate regulation of STAT5 is essential. As a transcription factor, STAT5 must gain entry into the nucleus to act, and one means of controlling its activity is to regulate its cellular localization. This study explores the nuclear trafficking mechanisms of STAT5a that may provide information to stimulate or inhibit its actions.

Classically, proteins that are actively transported through nuclear pore complexes in the nuclear membrane have a short stretch of basic amino acids called the nuclear localization signal (NLS). The classic NLS can be recognized by transport receptor called importins that mediate nuclear import. In this study we provide evidence that STAT5a has an unconventional large NLS that functions within the conformation of a coiled-coil domain. With the use of both *in vitro* binding and *in vivo* functional assays, we demonstrate that STAT5a nuclear import is mediated by the importin-α3/β1 system. The integrity of the STAT5a NLS is required for the transcription of β-casein milk protein gene in response to prolactin, and the ability of STAT5a to synergize with the glucocorticoid receptor. The glucocorticoid receptor localizes to the nucleus following prolactin treatment, and this nuclear accumulation is dependent on STAT5a nuclear import.

STAT5a continuously shuttles between nucleus and cytoplasm independent of tyrosine phosphorylation, and live cell imaging with photobleaching assays reveal that STAT5a nuclear export is mediated by both the conventional Crm1 exportin and by a Crml-independent pathway. A Crm1-dependent nuclear export signal (NES) was identified in the STAT5a amino-terminal domain, and a Crm1-independent NES in the DNA-binding domain of STAT5a. These findings provide insight to fundamental mechanisms of STAT5a nuclear trafficking and cooperation with glucocorticoid receptor, and provide a basis to design clinical therapeutics to target STAT5a activity in human disease.

DEDICATION

To my parents, Chang Sik Shin and Hong Myung Ok

and

my husband, Bon Young Suh

and

my son, Juvin Suh

with LOVE

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ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ARM	Armadillo
ATCC	American Type Culture Collection
β-gal	Beta-galactosidase
BSA	Bovine serum albumin
CAS	Cellular apoptosis susceptibility
CC	Coiled-coil
cDNA	Complementary deoxyribonucleic acid
cFLIP	Cytoplasmic fluorescent loss in photobleaching
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CIS	Cytokine-inducible SH2-containing protein
CML	Chronic myeloid leukemia
COX2	Cyclooxygenase-2
CRM1	Chromosome region maintenance 1
DBD	DNA-binding domain
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
ds	Double-strand
dsODN	Double-stranded oligonucleotides
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-tetraacetic acid
EPO	Erythropoietin
FBS	Fetal bovine serum

FL	Fluorescent intensity
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Interferon-γ activated site
GDP	Guanosine 5’ diphosphate
GEF	Guanine-nucleotide exchange factor
GFP	Green fluorescent protein
GH	Growth hormone
GM-CSF	Granulocyte macrophage colony stimulating factor
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
GTP	Guanosine 5’ triphosphate
HC	Hydrocortisone
HEAT	Huntingtin elongation factor 3
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IβB	Importin-β1 binding
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Imp	Importin
IPTG	Isopropyl thio-β-D-galactoside
IRF	Interferon regulatory factor
ISGF-3	Interferon stimulated gene factor-3
ISRE	Interferon stimulated response element
JAK	Janus kinase
KCl	Potassium chloride
kDa	Kilodalton
LB	Luria-bertani
LMB	Leptomycin B

LMP2	Low molecular mass polypeptide 2
µg	Microgram
µl	Microliter
MBP	Maltose binding protein
mg	Milligram
MGF	Mammary gland factor
ml	Milliliter
mm	Millimeter
mM	Milli molar
NaCl	Sodium chloride
NaF	Sodium fluoride
NES	Nuclear export signal
NK	Natural killer cell
nFLIP	Nuclear fluorescent loss in photobleaching
ng	Nanogram
NLS	Nuclear localization signal
NP-40	Nonidet-P40
NTF2	Nuclear transport factor 2
NPC	Nuclear pore complex
Nup	Nucleoporin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLSCR1	Phospholipid scramblase 1
PMSF	Phenylmethylsulphonyl fluoride
PRL	Prolactin
PRL-R	Prolactin receptor

pY	Phosphotyrosine
RNA	Ribonucleic acid
RNAi	RNA interference
ROI	Region of interest
RT	Room temperature
PTD	Protein transduction domain
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
pY	Phosphorylated tyrosine
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecylsulfate
SH2	Src homology 2
SHP	Src homology 2 domain-containing phosphatase
siRNA	Short interfering ribonucleic acid
SOC	Super optimal broth with catabolite repression
SOCS	Suppressor of cytokine signaling protein
SREBP2	Sterol regulatory element binding protein-2
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TAD	Transcriptional activation domain
TBS	Tris buffered saline
TKR	Tyrosine kinase receptor
Tris	Tris (hydroxymethyl) aminomethane
TPO	Thrombopoietin
Tween 20	Polyoxyethylene-sorbitan-monolaurate
wt	Wild-type

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I would also like to thank my friends who have made the past few years a pleasant experience. Last, I would like to dedicate this dissertation to my family, especially to my parents, my husband, and my lovely son. Without their unconditional support and love, I could not have completed this work.

Chapter 1

Introduction

The immediate response of cells to extracellular stimuli is essential to survive environmental changes. Eukaryotes orchestrate the multiple independent cell signals that induce various physiological effects, including cell proliferation, differentiation, and apoptosis. The secretion of cytokines or hormones from single cells or endocrine glands initiates the signaling pathway that regulates cellular responses. The progression of this cell signaling is mediated by cytoplasmic proteins that transmit signals from plasma membrane to nucleus, resulting in the induction of specific gene expression. Such cytoplasmic proteins include a family of transcription factors named Signal Transducer and Activator of Transcription (STAT).

In this dissertation, I have focused on one member of the STAT family, STAT5a. STAT5a is tyrosine-phosphorylated in response to hormone stimulation and forms a dimer. STAT5a gains the ability to bind target DNA sequence with the tyrosine-phosphorylated dimer and induced the gene expression. STAT5a plays a critical role in hematopoiesis, liver metabolism, and mammary gland development. Aberrant continual activation of STAT5a leads to various cancers, and therefore its activity must be tightly regulated. For this reason, my research has been focused on one of the mechanisms that regulate STAT5a, the nuclear trafficking of STAT5a.

1. The JAK-STAT signaling cascade

Cytokine signals regulate a variety of important biological functions related to immune response, cell growth, and development. Over the past 30 years, cytokine-induced signal transduction has been extensively studied, and one of the critical pathways is the Janus Kinase (JAK)-STAT pathway. A simple schematic of canonical STAT activation is shown in Figure 1.

Following cytokine binding to cell surface receptors, receptor-associated JAKs are activated and trans-phosphorylate tyrosine residues on receptors at cytoplasmic tails as well STATs that are recruited to the receptor. JAK1, JAK2, JAK3 and TYK2 are the four JAKs that have been identified in mammals. They are non-receptor tyrosine kinases of approximately 120-130 kDa in molecular weight and share similar domain arrangements (1, 2). JAK1, JAK2, and TYK2 are ubiquitously expressed, whereas JAK3 expression is restricted to lymphoid cells (3). JAKs are constitutively associated with distinct receptors that are expressed in specific tissues. Cytokines bind individual receptors that are dependent on receptor engagement, JAKs and specific downstream STAT molecules.

JAKs phosphorylate receptors at specific tyrosine residues and provide docking sites for STATs. Recruited STATs are tyrosine-phosphorylated and form dimers through the interaction of reciprocal SH2 domains and phosphorylated tyrosine residues (4). Following entrance into the nucleus, STATs bind target DNA sequences in responsive genes.

Cumulative studies have shown that unphosphorylated STATs also play a critical role in specific gene expressions. Latent STATs have been crystalized (5, 6), and it reveals that STATs can form a dimer without tyrosine-phosphorylation. In addition, an increasing number of studies have shown that unphosphorylated STATs can enter the nucleus (7-9) and function to regulate gene expressions (10-12).

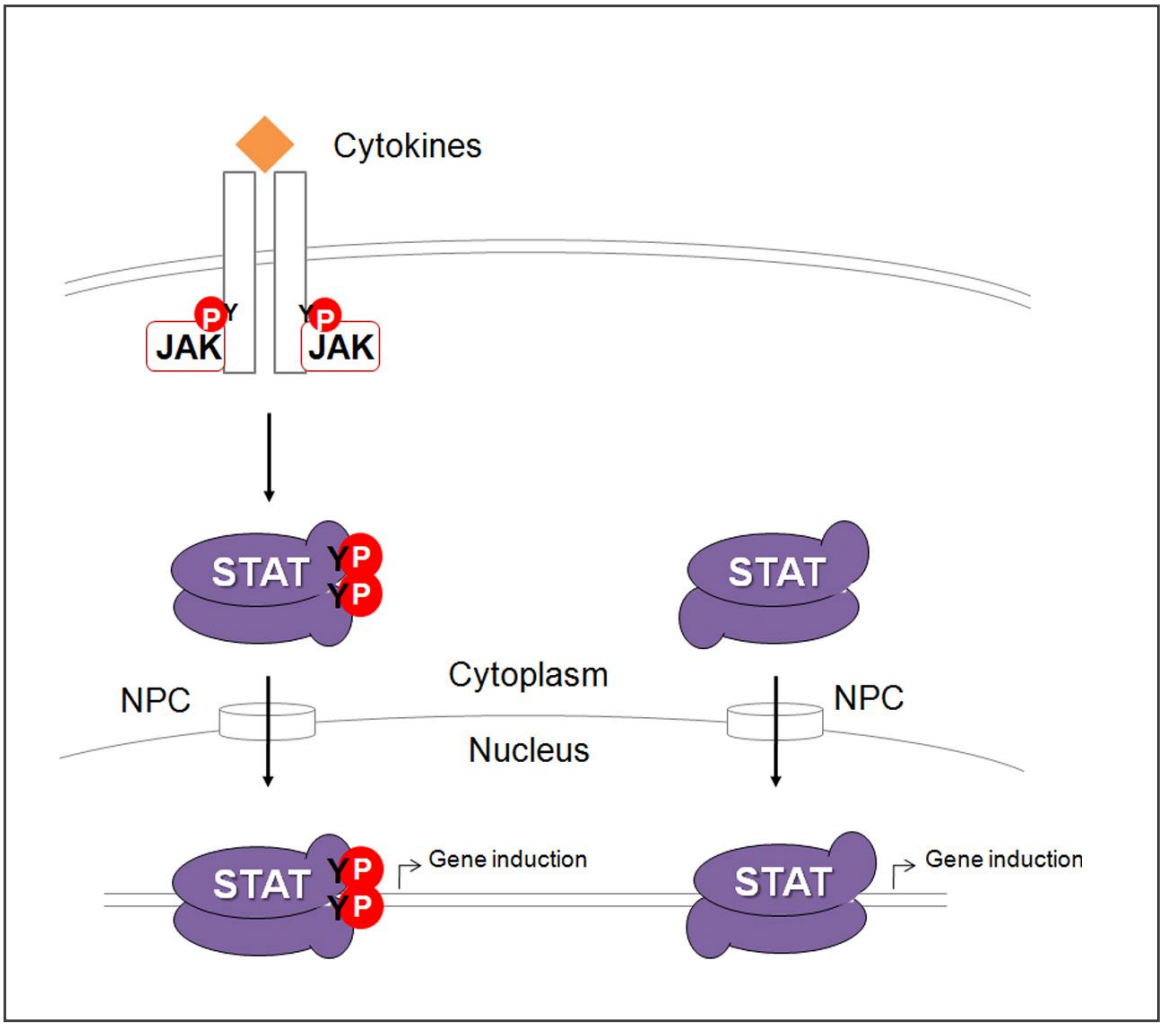


Figure 1: JAK-STAT signaling pathway.

A schematic of the JAK-STAT signaling pathway. Cytokine binding to cell surface receptors activates receptor-associated JAKs. JAKs phosphorylate the receptors as well as STAT molecules. Tyrosine-phosphorylated STATs form parallel dimers and gain the ability to bind specific genes in the nucleus. The induction of gene expression promotes various cellular responses. Unphosphorylated STATs exist as anti-parallel dimers and can enter the nucleus.

2. The STAT family: Domain, Structure, Biological functions

There are seven members of the STAT family in mammalian cells including STAT1, 2, 3, 4, 5a, 5b, and 6 (13). Each STAT protein responds to distinct cytokine signals and induces the transcription of specific genes that can elicit different biological effects. STAT1 and STAT2 are founding members of STAT family that are tyrosine-phosphorylated in response to interferon (IFN) stimulation and are involved in immune responses (14, 15). STAT proteins appear to have diverged from a single gene through successive duplications into three genetic loci. In human, STAT1 and STAT4 map in chromosome 2 (16, 17), and STAT2 and STAT6 map in chromosome 12 (18, 19). STAT3, STAT5a and STAT5b are located at chromosome 17 (20, 21).

STAT proteins consist of 750-850 amino acids and share similar structures and functional domains (Figure 2) (22). The amino-terminal domain is followed by the coiled-coil domain that consists of four α-helices and is responsible for protein-protein interactions (23). The central DNA-binding domain is composed of several β-barrel sheets and is critical for STAT binding to target DNAs (24). The Src homology 2 (SH2) domain associates with the phosphorylated tyrosine residues on the receptor or other STAT proteins to form homodimers or heterodimers (25). The carboxyl terminal transcriptional activation domain interacts with several co-activators to facilitate the transcription of genes (26). A specific tyrosine residue is phosphorylated in response to cytokine stimulation (27).

The crystal structure of STAT1 has been solved in both an unphosphorylated state and a DNA-bound tyrosine-phosphorylated state (Figure 3) (5, 25). Unphosphorylated STAT1 was found to form dimers or tetramers. Each monomer associates with an anti-parallel configuration so that the N-terminal coiled-coil domain and the DNA-binding domain of each monomer are close to each other, whereas carboxyl terminal SH2 domains are far apart. Following tyrosine-phosphorylation, STAT1 switches to a parallel configuration so that SH2 domains from each monomer associate with phosphorylated tyrosine residues, and the coiled-coil domains and DNA-binding domains are far apart resulting in exposure of DNA binding sites.

Figure 2: Functional domains of STATs.

STAT proteins vary from 750-850 amino acids in length and share a similar domain arrangement; a coiled-coil domain (CC), a DNA binding domain (DBD), a Src homology 2 (SH2) domain, a specific phosphorylated tyrosine (pY), a transcriptional activation domain (TAD).

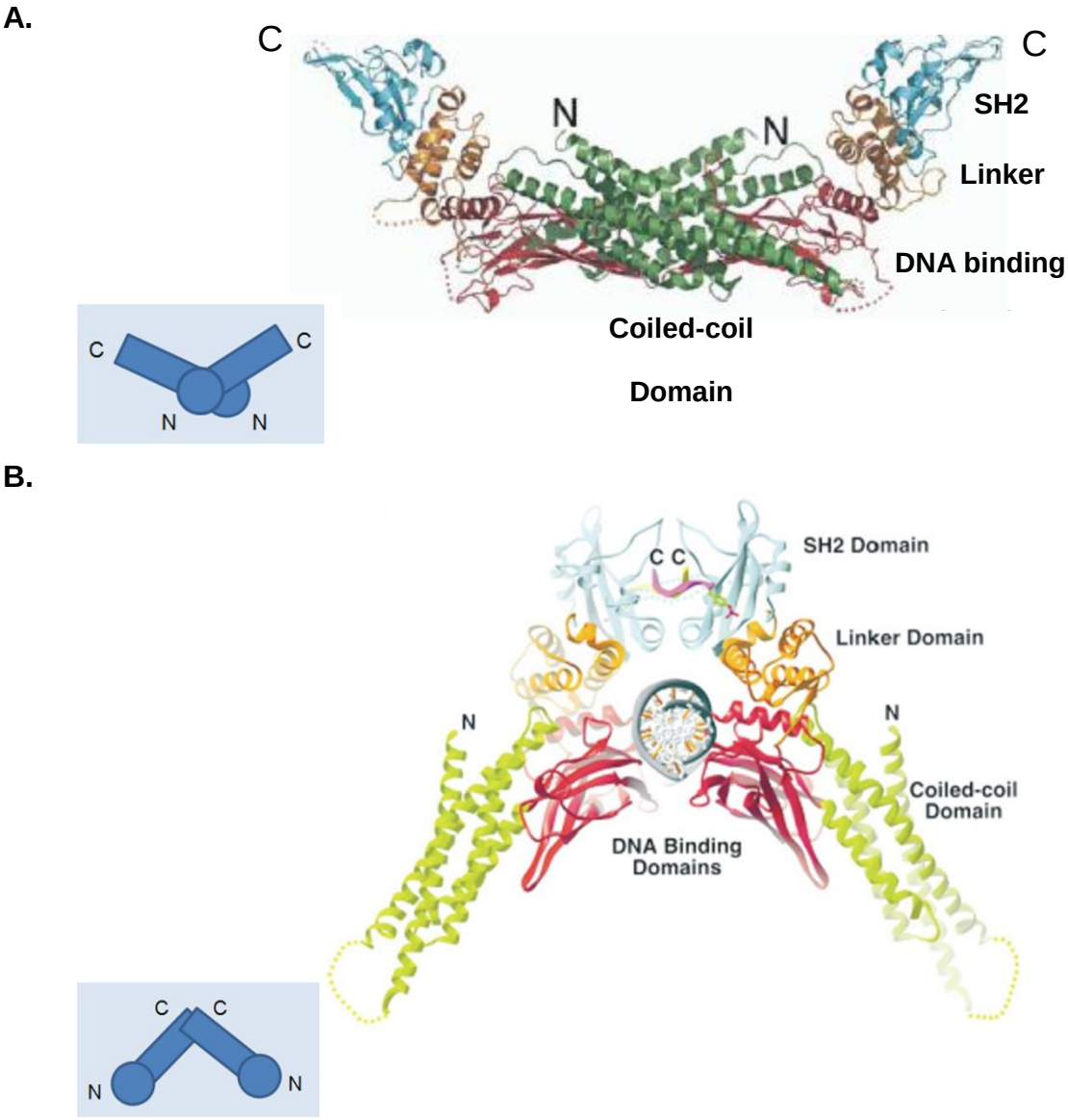


Figure 3: The crystal structure of STAT1 dimers in a ribbon diagram.

A. Structure of unphosphorylated STAT1 core dimer. A coiled-coil domain is shown in green, a DNA-binding domain in red, a linker in orange, and a SH2-domain in blue (5) (Protein Data Bank ID Code 1YVL). Inset shows simple anti-parallel conformation of monomers .

B. Structure of tyrosine-phosphorylated STAT1 core dimer binding to DNA. A coiled-coil domain is indicated as yellow, a DNA-binding domain as red, a linker domain as orange, a SH2 domain as light blue, and the DNA as gray (25) (Protein Data Bank ID Code 1BF5). Inset shows parallel conformation of monomers.

Although the STAT family shares similar domain organizations, each STAT has distinct biological functions. Studies with knockout mice have shown specific loss-of function phenotype *in vivo* (Table 1) (28, 29). Animals lacking STAT1 or STAT2 gene are susceptible to virus infections due to impaired IFN signaling (30-32). STAT3 deficient mice exhibit early embryonic lethality, and tissue specific conditional knockouts are defective in normal development, including cell proliferation, differentiation, and apoptosis (33). STAT4 null mice have impaired T_H1 cell differentiation due to the loss of IL-12 receptor signaling (34), whereas STAT6 null mice have defective T_H2 cell differentiation due to the impaired IL-4 induced responses (35). STAT5a and STAT5b are identical in ~95% of protein sequence. STAT5a/b double knockout mice are perinatal lethal and have impaired T cell differentiation and severe anemia (36). STAT5a null female mice show defective prolactin (PRL) dependent mammary gland development (37), whereas STAT5a null male mice exhibit impaired prostate gland development (38). By contrast, STAT5b knockout mice are fail to respond to growth hormone (GH) stimulation (39).

Table 1: Phenotypes of mice deficient in individual STAT genes.

Targeted STAT	Phenotype of STAT null mice
STAT1	Defective response to interferons, succumb to viral infection and tumors
STAT2	Defective response to interferons
STAT3	Embryonic lethality, tissue specific knockouts exhibits susceptibility to pathogens
STAT4	Impaired T_H1 cell differentiation due to the defective IL-12 signaling
STAT5a	Defective prolactin signaling results in impaired mammary gland development
STAT5b	Defective growth hormone pathway, natural killer (NK) cell-mediated proliferation and cytolytic activity
STAT5a/b	Perinatal lethality, severe anemia, defective T-cell proliferations
STAT6	Defective T_H2 cell differentiation due to the impaired IL-4 and IL-13 signalings

(Modified from reference 29)

3. Fraternal twins: STAT5a and STAT5b

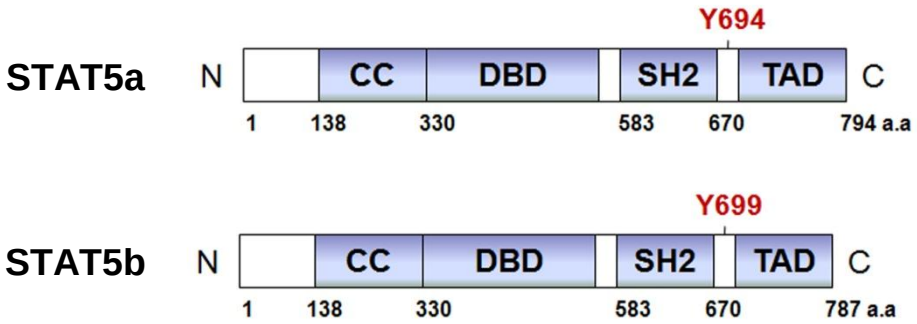
STAT5 was originally found as a transcription factor in the mouse mammary gland that is responsible for the transcription of milk protein gene in response to PRL (40). After STAT5 cDNA was isolated from sheep mammary glands, it was named as mammary gland factor (MGF) (41, 42). Subsequently, an isoform of STAT5 was identified at a separate gene locus, and MGF was renamed as STAT5a and its isoform was named as STAT5b (21, 43, 44). Genes encoding these two isoforms are located in the same chromosome, and appear to be derived from a single gene by duplication.

Human STAT5a and STAT5b consist of 794 a.a. and 787 a.a. respectively, and exhibit 95% of amino acids sequence homology (43). Similar to other STATs, STAT5 shares similar functional domains, including an amino terminal domain, a coiled-coil domain (CC), a DNA-binding domain (DBD), a SH2 domain, a transactivation domain (TAD), and a specific tyrosine residue, Y694 for STAT5a and Y699 for STAT5b, that is phosphorylated in response to various cytokines (Figure 4A) (21, 42). The carboxyl terminal TAD domain shows the high level of sequence variation between STAT5a and STAT5b, indicating that it leads distinct biological effects (43). The crystal structure of an unphosphorylated STAT5a dimer has been solved (Figure 4B), and it is a similar anti-parallel configuration like unphosphorylated STAT1 (6).

1) Activation of STAT5

STAT5 tyrosine-phosphorylation is stimulated by a variety of cytokines or hormones such as IFNs, IL-2, IL-3, IL-5, IL-7, IL-15, erythropoietin (EPO), thrombopoietin (TPO), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), growth hormone (GH), and prolactin (PRL) (45). STAT5 follows the classic STAT activation pathway (Figure 5). Peptide hormones such as prolactin (PRL) stimulate the cell surface receptor and associated JAK2 is activated resulting in tyrosine-phosphorylation of receptors as well as STAT5a in the cytoplasm. Tyrosine-phosphorylated STAT5a forms dimers and gains the ability to bind to target DNA consensus in the nucleus. The STAT5a binding consensus sequence found to be a

A.



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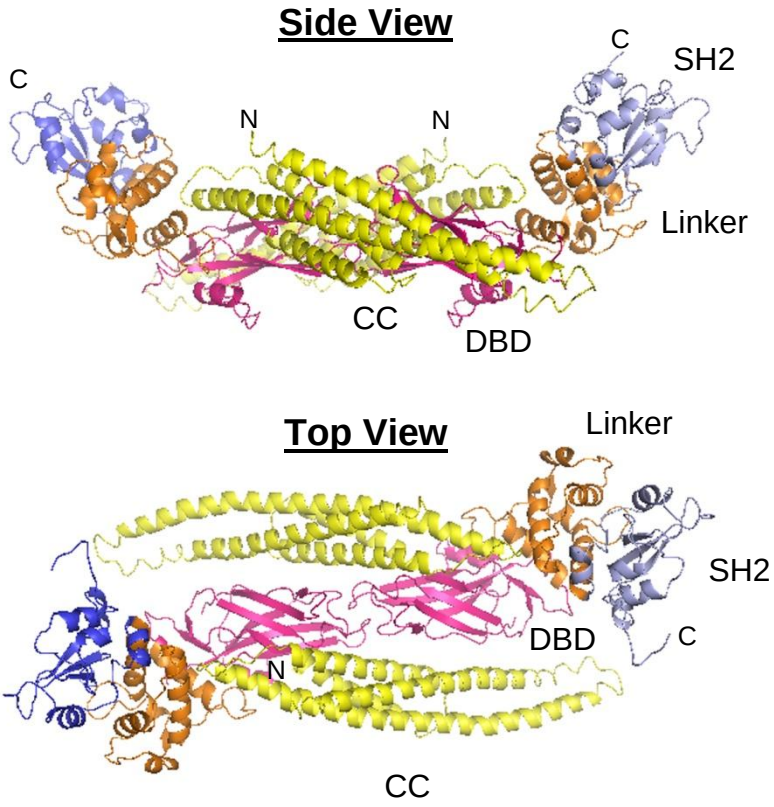


Figure 4: STAT5 functional motifs and the unphosphorylated STAT5a dimer in the ribbon diagram.

A. Linear diagrams of STAT5a and STAT5b functional motifs with the number corresponding to STAT5 amino acids.

B. Top panel, a side view of unphosphorylated STAT5a dimer in a ribbon diagram. The coiled coil domain (CC) is shown in yellow, the DNA-binding domain (DBD) in hot pink, the linker domain in orange, and the Src homology 2 (SH) domain in blue (6) (Protein Data Bank ID code 1Y1U). Lower panel, a top view of unphosphorylated STAT5a dimer in a ribbon diagram.

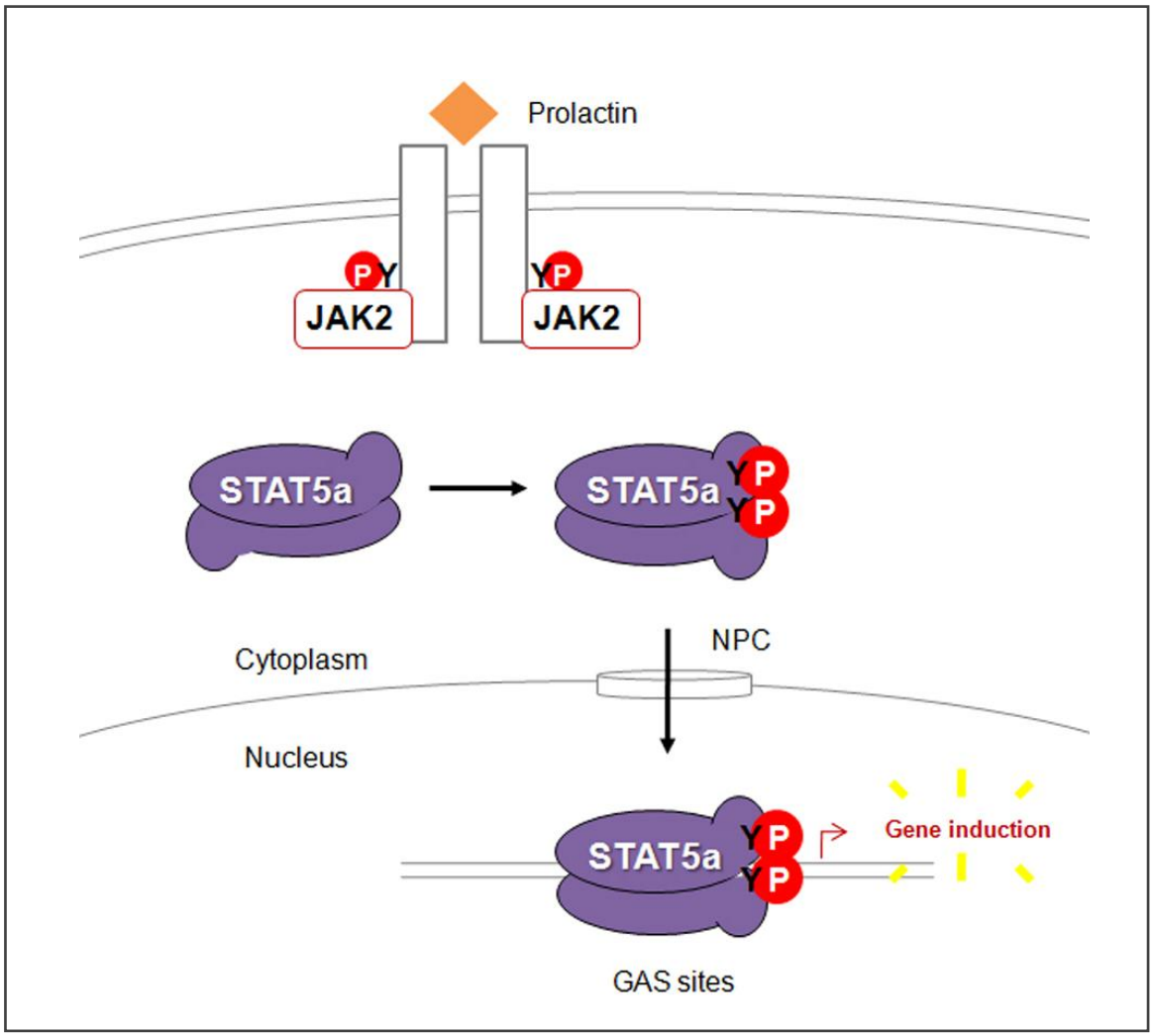


Figure 5: STAT5a signaling pathway in response to PRL.

A schematic of STAT5a activation in response to PRL stimulation. After PRL binds to PRL receptors, JAK2 phosphorylates tyrosine residues on PRL receptors as well as STAT5a. Tyrosine-phosphorylated STAT5a forms parallel dimers and gains the ability to bind to consensus GAS sites in β-casein promoter to induce the gene expression.

GAS site that is comprised of TTCNNNGAA. STAT5a binding to the target promoter sequence induces the transcription of specific gene expression like the β-casein milk protein gene (46).

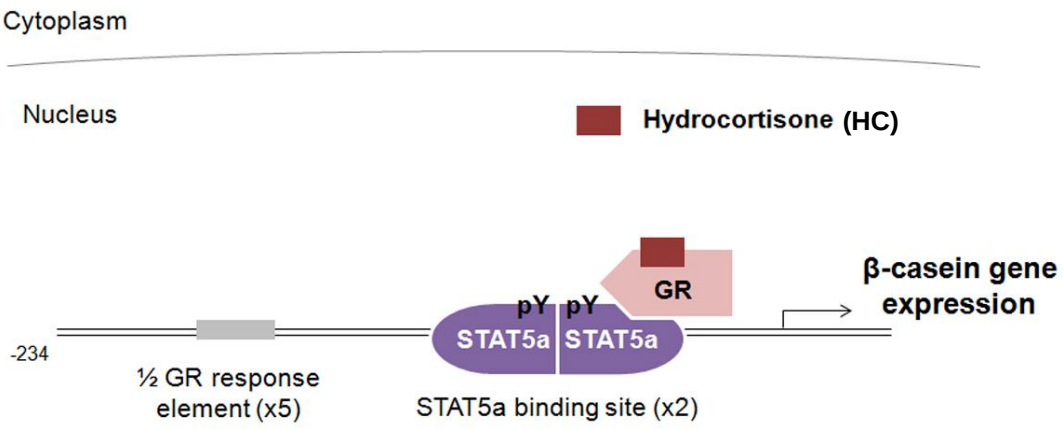
Although STAT5a and STAT5b show a high level of sequence homology, they have both redundant and distinct functions (47). Loss of function studies in mice showed both STAT5a and STAT5b play a critical role in normal development, including cell proliferation and differentiation. However, STAT5a is more specific to the mammary gland development and lactogenesis. STAT5b plays a more important role in body growth in response to GH and natural killer (NK) cell development.

2) The crosstalk between STAT5 and other transcription factors

Cumulative studies have shown that there is cross talk between seemingly unrelated signaling pathways that modulate STAT5 functions. STAT5a was originally found to be needed for the transcription of milk protein gene in response to PRL, but further studies have shown that STAT5a enhances the transcription of β-casein gene by the use of several steroid hormone receptors as co-activators (48).

One of the best characterized hormone receptors is the glucocorticoid receptor (GR) (49, 50). GR is a ligand binding transcription factor that is activated by glucocorticoid or its derivatives, such as dexamethasone (Dex) or hydrocortisone (HC). Latent GR is localized in the cytoplasm, but ligand-binding induces its nuclear localization, leading to target gene expression. GR is critical for many biological functions, including mammary gland development and lactation (51). A number of studies have shown that PRL-stimulated STAT5a and ligand-bound GR synergize to induce the transcription of β-casein gene (Figure 6) (52-54). Activated STAT5a dimers or tetramers interact with ligand-bound GR, and bind to STAT5a binding sites in the β-casein gene promoter as a complex to enhance the gene expression. However, the detailed molecular mechanism of STAT5a and GR cooperation remains to be determined. Immunofluorescence and cell fractionation studies have revealed that PRL-stimulated STAT5a induces the nuclear translocation of un-liganded GR (55, 56). Conversely, Dex-treated GR increases the nuclear accumulation of unphosphorylated STAT5a. Since PRL does not induce the

A.



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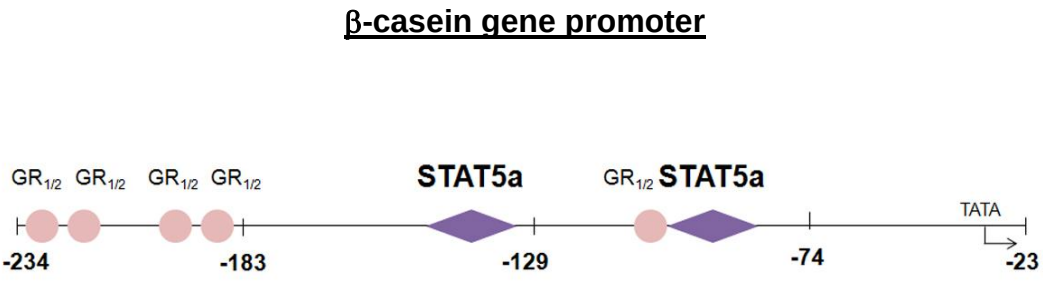


Figure 6: The cooperation of STAT5a and GR to synergize the transcriptional induction of β-casein gene.

A. A schematic represents the functional interaction between PRL-mediated STAT5a signaling and HC-mediated GR signaling. PRL stimulated STAT5a physically associates with HC ligand-bound GR, and synergizes the transcription of β-casein gene.
B. A linear depiction of the human β-casein gene core promoter. The proximal core promoter contains two STAT5a binding sites and five half palindromic GR sites.

nuclear translocation of GR in the absence of STAT5a, activated STAT5a may interact with GR and affect the nuclear import or vice versa.

Increasing studies have shown that STAT5a uses several other steroid hormone receptors as a co-activator, including estrogen receptor, progesterone receptor, mineral corticoid receptor, and androgen receptor to enhance or reduce various cellular responses (48, 57, 58). A recent study also found that STAT5b and GR physically interact and synergize normal growth and sexual maturation of hepatocytes (59). Cross-talk of independent signaling pathways appears to provide the robust and profound effects on the transcriptional function of STAT5.

3) Negative regulation of STAT5

One of the critical mechanisms that regulate STAT5 function is tyrosine-phosphorylation and dephosphorylation. Dephosphorylation of STAT5 negatively regulates gene expression to prevent the constitutive activity of tyrosine-phosphorylated STAT5 (60, 61). Evidence has shown that STAT5 can be inactivated by dephosphorylation with several phosphatases in the nucleus and in the cytoplasm. Members of the protein tyrosine phosphatase (PTP) family such as SHP-2 and PTP-1B are known to dephosphorylate STAT5 in the cytoplasm (60, 62), whereas TC-PTP dephosphorylates STAT5 in the nucleus (63). A recent study reported that a dual-specificity phosphatase VHR can selectively dephosphorylate the IFN-stimulated STAT5 in the nucleus (64).

Another possible mechanism of STAT5 inactivation is by protein degradation. One study has shown that tyrosine-phosphorylated STAT5 is polyubiquitinated and degraded in the nucleus through proteasome-dependent protein degradation (65). It provides the evidence that nuclear STAT5 is stabilized after proteasome inhibitor MG132 treatment by cell fractionation assays. However, I did not observe the prolonged STAT5a nuclear accumulation with the treatment of MG132 in living cells that express STAT5a-GFP (data not shown). Suppressor of cytokine signaling (SOCS) proteins are also known to interfere with activation of STAT5 (47). SOCS proteins are rapidly induced after cytokine stimulation and inhibit phosphorylation of STATs (66). One of the SOCS family members, Cytokine-inducible SH2 domain containing (CIS) protein is reported to inhibit

STAT5 activation by directly binding to the PRL, EPO, IL-3, and IL-2 receptors (67, 68). In addition to dephosphorylation or protein-degradation, export of STAT5 from the nucleus might be another possible mechanism that down regulates STAT5 functions. Our previous studies have shown that STAT5a is continuously exported out of the nucleus in living cells (8). STAT5a export to the cytoplasm may shut down the STAT5a activity as well as to recycle STAT5a back to the cytoplasm.

4) STAT5 and cancer

STAT5 regulates a number of important biological functions that are related to normal development. However, aberrant persistent activity of STAT5 leads to oncogenesis. Studies have revealed that STAT5 is constitutively tyrosine-phosphorylated and is present in the nucleus of cancer cells, including breast cancer, prostate cancer, and leukemia (69-71). One of the well known causes of persistent STAT5 activation is the constitutive activation of JAKs. The TEL-JAK2 fusion proteins are often formed by chromosomal translocation in leukemia (72, 73). The oligomerization domain of ets leukemia (TEL) protein is fused to the catalytic domain of JAK, which results in constitutive JAK kinase activity. A point mutation in JAK also induces the persistent JAK activity. Substitution of valine for phenylalanine at 617 a.a. of JAK2 (JAK2V617F) results in constitutive phosphorylation of JAK (74). This activating mutant of JAK leads to continuous STAT5 signaling, and promotes leukemia. Since deregulation of STAT5 leads to many cancers, tight regulation of STAT5 is essential. One means of STAT5 regulation is nuclear and cytoplasmic transport of STAT5.

4. Nuclear transport

The nucleus of eukaryotic cells is separated from the cytoplasm by the nuclear envelope, but the two compartments maintain close communication. The nuclear envelope is a double layer membrane with 100-150 nm in diameter of nuclear pore complexes (NPCs) that serve a gate for the bidirectional exchange of macromolecules (Figure 7) (75-78). The NPC is a large cylindrical complex comprised of 30 different proteins named nucleoporins (Nup). Approximately one third of Nups contain Phe-Gly

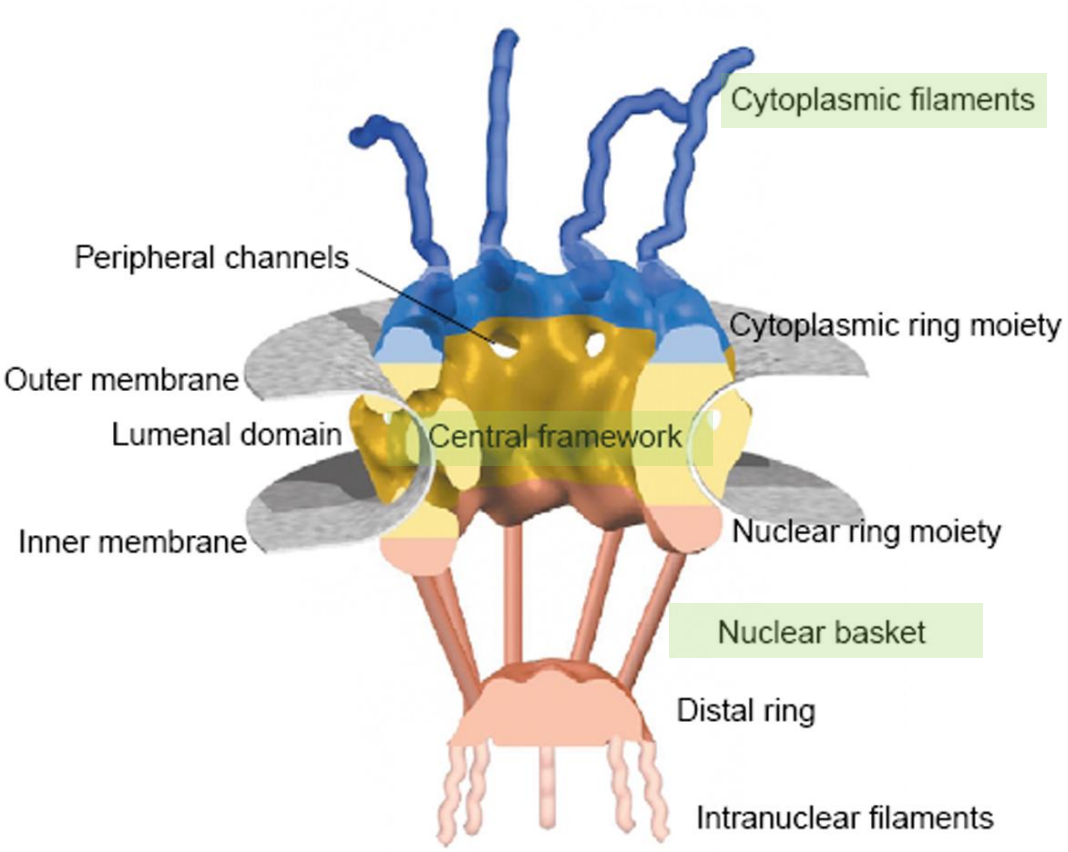


Figure 7: A schematic diagram of nuclear pore complexes (NPCs)

The NPC is composed of three main structures: cytoplasmic filaments, a central core, and a nuclear basket. Nucleoporins (Nups) are organized into the cylindrical shape with octagonal symmetry (79).

(FG) repeats that line the central channel of NPCs and provide the selectivity of protein passage. NPC allows passive diffusion of small molecules, but large molecules over 40-60 kDa need the help of transport receptors for active transport.

The most well characterized transport receptors belong to a karyopherin-β family, also called importin or exportin depending on the direction of cargo transport. The direction of protein transport is determined by concentration of the Ras-related GTPase, Ran (80, 81). GTP bound Ran is more abundant in the nucleus, whereas GDP bound Ran is more prominent in the cytoplasm. The guanine nucleotide exchange factor (GEF) in the nucleus and the GTPase activating factor (GAP) in the cytoplasm control the relative concentration of GTP bound Ran or GDP bound Ran in cells. Ran-GTP binding to importins releases importins from cargo in the nucleus, whereas Ran-GTP binding to exportins enforces the association of exportins and cargo.

1) Nuclear import

The nuclear transport of proteins is initiated by the recognition of signal sequences in cargo by the transport receptor (82). This specific sequence is called a nuclear localization signal (NLS). The classic NLS is a short stretch of basic amino acids that are either as a monopartite or bipartite sequence, particularly rich in lysine or arginine (83). The monopartite sequence usually consists of 4-8 basic amino acids, and it was first found in SV40 large T antigen (PKKKRK) (84, 85). The bipartite sequence is comprised of two basic stretches separated by 10 amino acids, and was found in nucleoplasmin (KRPAATKKAGQAKKKK) (86). The basic NLS is recognized directly or indirectly by the family of karyopherin-β transport receptors, also known as importins that facilitates the nuclear import through NPCs (87-89). The classic nuclear import pathway is shown in Figure 8. The classic import receptor consists of a heterodimer that includes an adapter protein importin-α and a carrier protein importin-β1. The importin-α recognizes the basic NLS sequence in cargo, and associates with the importin-β1 (90). Subsequently, the importin-β1 carries the importin-cargo complex into the nucleus. In the nucleus, Ran-GTP binds to importin-β1 resulting in the dissociation of importin complex from cargo (80).

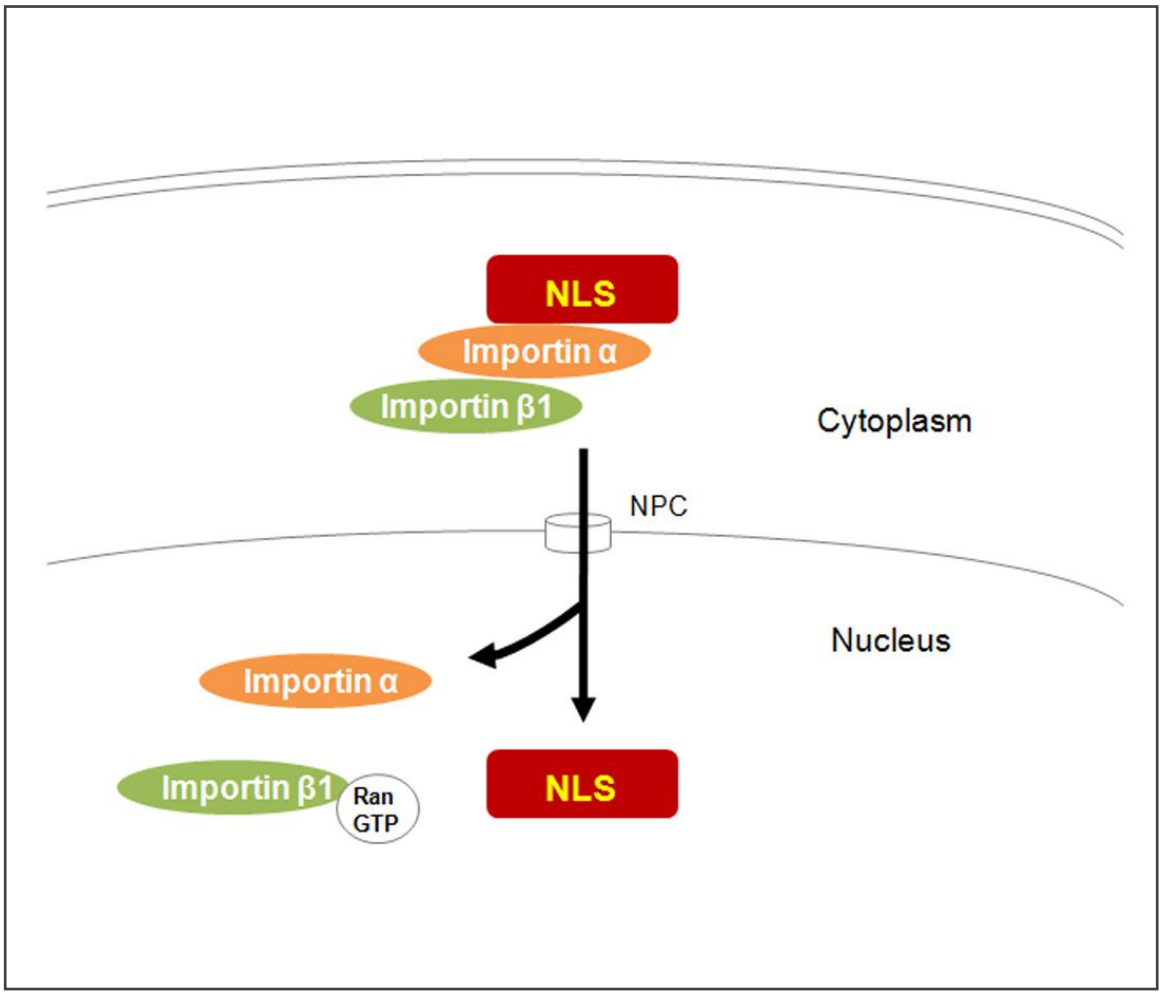


Figure 8: Classic nuclear import pathway.

A schematic diagram that represents classic nuclear import. The basic NLS containing cargo is recognized by the adapter importin-α, and links to importin-β1 carrier to facilitate the nuclear transport through NPCs. Following translocation into the nucleus, Ran-GTP binds to importin-β1 and leads to the release of importin complex from cargo.

The importin-αs are ~60 kDa proteins that are highly conserved from yeast to human (90, 91). There are six members in the human importin-α family, including importin-α1, α3, α4, α5, α6, and α7. The members of the importin-α family share similar functional domains (Figure 9). They have an amino terminal importin-β1 binding (IβB) domain that interacts with importin-β1. In the absence of importin-β1, the IβB binds to an internal NLS for autoinhibition (92). Importin-αs have ten central tandem armadillo (ARM) repeats. Each ARM is comprised of three α-helices, and the ARMs are responsible for binding to an NLS sequence (93). The classic NLS binds to ARM 2-4 and ARM 6-8. The carboxyl terminal domain has an NES that binds to Cellular apoptosis susceptibility (CAS) exportin in the nucleus which transports importin-α back to the cytoplasm.

Importin-β1 is a ~95 kDa protein that is mostly composed of 19 HEAT repeats. Each HEAT repeat consists of two anti-parallel α-helices (94). Human importin-β1 directly or indirectly binds to the NLS, and transport cargo into the nucleus. Importin-β1 binds to Phe-Gly (FG) repeats in Nups of NPCs (95). Following the nuclear translocation, Ran-GTP binds to importin-β1 resulting in the dissociation of importin complex from cargo (96).

2) Nuclear export

The mechanism of nuclear export shares similar features with nuclear import, but works in a reverse way. Nuclear export requires a nuclear export signal (NES) and an export carrier (Figure 10). The best characterized NES consensus is a leucine-rich hydrophobic sequence (LxxxLxxLxL) that can be recognized by export transporter chromosome region maintenance 1 (Crm1) (97, 98). Crm1 forms a ternary complex with Ran-GTP and cargo protein in the nucleus (99-101). Following transport to the cytoplasm, nucleotide exchange of Ran-GTP to Ran-GDP leads to the release of cargo from exportin (102). A specific inhibitor of Crm1, leptomycin B, is an anti-fungal antibiotic that directly binds to Crm1 and covalently modifies the critical cysteine residue on Crm1 resulting in the inhibition of export function of Crm1 (103, 104). Therefore, it has been a useful tool to assess the role of Crm1 in export of various proteins. Recent studies have

A.

B.

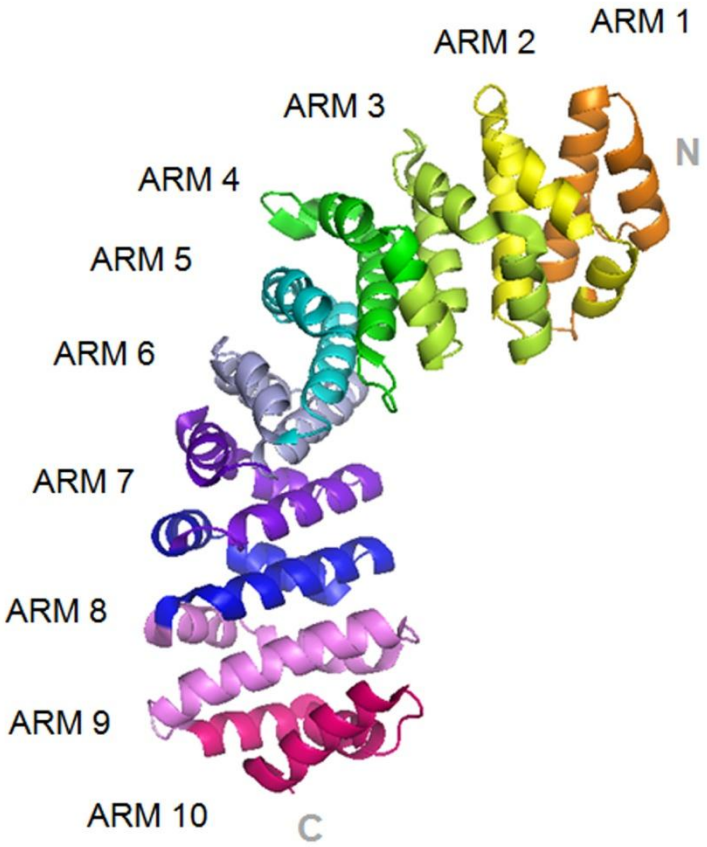


Figure 9: Importin-α functional domains and the structure in ribbon diagram.

A. Importin-α consists of an N-terminal importin-β1 binding domain (IβB), ten central tandem armadillo (ARM) repeats that are responsible for NLS binding, and a carboxyl terminal CAS binding domain.

B. Each ARM repeat is composed of three helix turn helix α-helices.

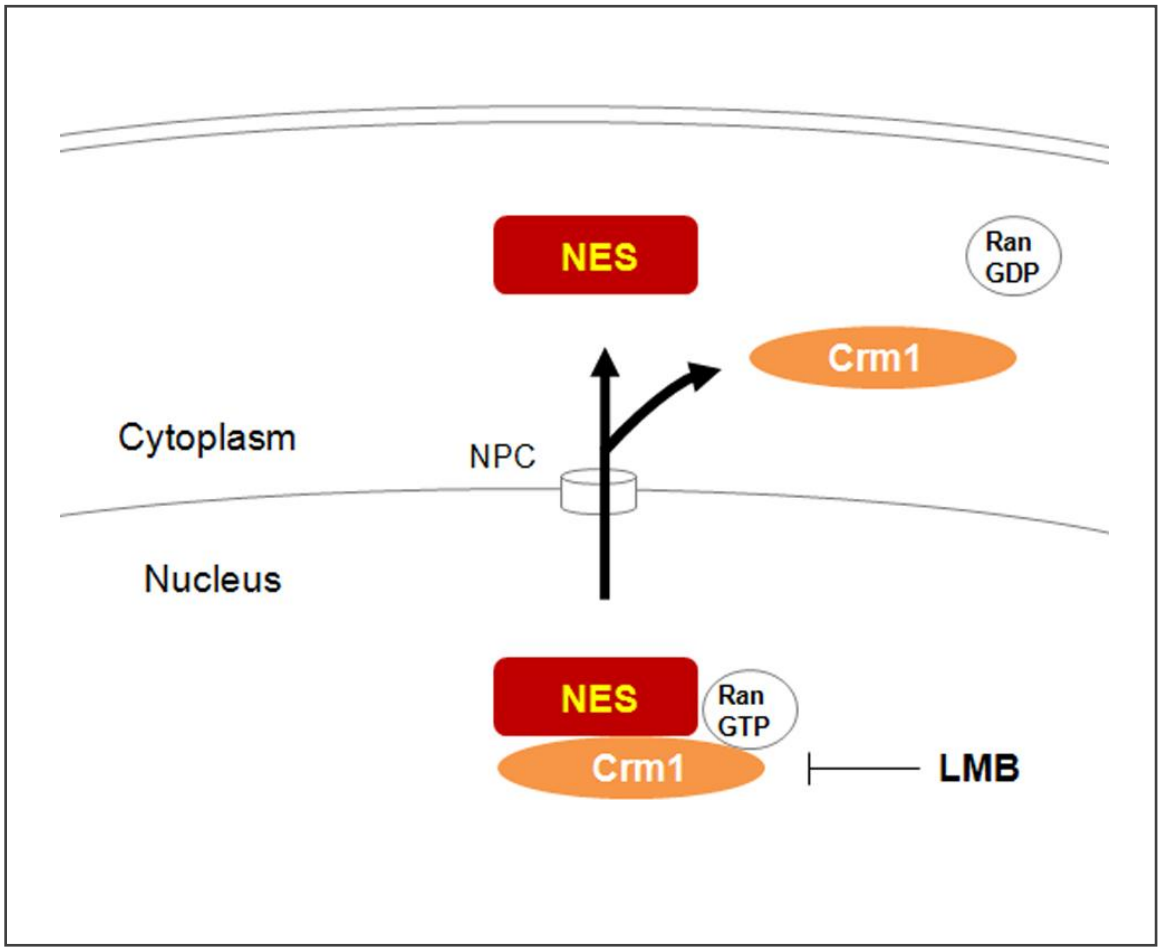


Figure 10: Nuclear export pathway.

A simple schematic of nuclear export pathway. Proteins in the nucleus that contain an NES are recognized by Crm1 exportin bound to Ran-GTP, and exported to the cytoplasm as a ternary complex. Nucleotide exchange of Ran-GTP to Ran-GDP releases the Crm1 from cargo. Leptomycin B (LMB) is a specific Crm1 inhibitor.

identified additional export mechanisms that are mediated by other exportins, including exportin-4, 6, 7, importin-13, and calreticulin (Table 2). Exportin-4, 6, and 7 are identified to be novel exportins that function in higher eukaryotes (105-109). Importin-13 mediates both import and export (110). Molecular chaperone calreticulin is recently identified to have an ability to export glucocorticoid receptor and thyroid receptor-α1 (111-113). The sequence similarity between various exportins is low, and often restricted to amino terminal Ran-GTP binding motif. Each exportin appears to recognize distinct NES sequences.

Table 2: Karyopherin-β that mediates the nuclear export.

Function	Karyopherin-β	Cargo
Export	Exportin-1 (Crm1)	Hydrophobic NES (ex. LxxxLxxLx)
	Exportin-2 (CAS)	Importin-a
	Exportin-t	tRNA
	Exportin-4	Eukaryotic initiation factor 5A (eIF-5a), Smad 3
	Exportin-5	MicroRNA precursors
	Exportin-6	Profilin-actin complex
	Exportin-7	p50RhoGAP
Export/ Import	Importin-13	eIF-1A

To execute its function as a transcription factor, latent STAT proteins in the cytoplasm must gain access to the nucleus. Since STAT proteins are large molecules over 90 kDa, it needs active transport facilitated by transport receptors rather than passive diffusion. STAT5 is critical for both normal development and cancer formation. Understanding the nuclear trafficking mechanism of STAT5 will provide knowledge of the fundamental mechanism that regulates STAT5 and provides ideas to design the therapeutics for STAT5 related disease.

5. Nuclear trafficking of STAT family

Current studies have shown that each STAT factor shows different properties of cellular localization (Figure 11) (114, 115). For example, STAT1 nuclear import is conditional, dependent on tyrosine-phosphorylation (116), whereas STAT3, 5, and 6 nuclear import is continous, independent of tyrosine-phosphorylation (7-9). The nuclear transport of STAT family members is briefly described in the following sections.

1) Founding members, STAT1 and STAT2

Latent STAT1 resides predominantly in the cytoplasm (Figure 11) (116). Following tyrosine-phosphorylation in response to IFN treatment, a conformational STAT1 NLS is recognized by import-αs and transported into the nucleus. Since STAT1 mutations that lack the ability of tyrosine-phoshosphorylation or dimerization are localized in the cytoplasm even after IFN stimulation, STAT1 nuclear import appears to be conditional and dependent on tyrosine-phosphorylation and parallel dimerization. STAT1 NLS function resides in the DNA binding domain and leucine 407 was found to be critical for the nuclear import. However, sequences of the region do not confer NLS function to another protein. This unconventional NLS is recognized by the importin-α5 adapter protein through ARM 8-9 regions and transported into the nucleus. STAT1 is dephosphorylated in the nucleus and exported out of the nucleus and redistributed in the cytoplasm. STAT1 nuclear export is mediated by Crm1 exportin and Crm1-dependent NES was found in the DNA binding domain (117). STAT1 NES appears to be accessible only after STAT1 is dephosphorylated and dissociated from DNA. Since STAT1 nuclear trafficking only occurs after IFN stimulation and both STAT1 NLS and NES are in the DNA binding domain, one of the key determinants for the regulation of STAT1 nuclear trafficking are tyrosine-phosphorylation and DNA binding.

Similar to STAT1, STAT2 is also primarily localized in the cytoplasm (118). However, the regulation of STAT2 nuclear trafficking is distinct from STAT1 in that STAT2 continuously shuttles between nucleus and cytoplasm independent of tyrosine-phosphorylation. It is associated with a non-STAT factor, the interferon regulatory factor 9 (IRF-9) that is responsible for nuclear import of unphosphorylated STAT2.

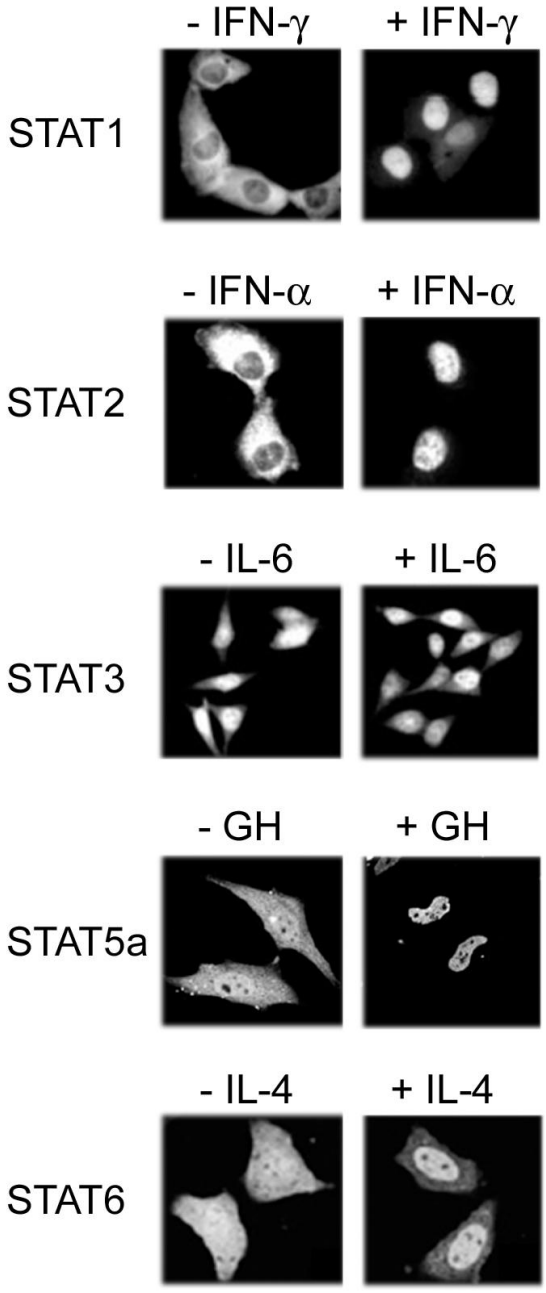


Figure 11: Cellular localization of GFP tagged STATs.

STAT proteins show distinct cellular localization. Unphosphorylated STAT1 and STAT2 are predominantly in the cytoplasm and accumulate in the nucleus following IFN stimulation. STAT3 shows a nuclear presence with or without IL-6 treatment. STAT5a and STAT6 are present both in the nucleus and cytoplasm and accumulate in the nucleus after growth hormone (GH) or IL-4 treatment, respectively. (Modified from references 7, 8, 116 and 118)

Evidence shows that following LMB treatment STAT2 accumulates in the nucleus without IFN stimulation. STAT2 in cells lacking IRF-9 or a STAT2 mutant lacking IRF-9 binding ability were not able to be translocated into the nucleus. IRF-9 has an NLS in its DNA binding domain, and it appears that STAT2:IRF-9 heterodimers are transported into the nucleus by importin-α/β1 system through the recognition of an IRF-9 NLS. STAT2 nuclear export is continuous and mediated by Crm1 exportin. STAT2 Crm1-dependent NES was found in the carboxyl terminus of STAT2. Since latent STAT2 is primarily in the cytoplasm although unphosphorylated STAT2 nuclear import is constitutive, STAT2 NES appears to dominate a STAT2 NLS. Following IFN stimulation, both STAT1 and STAT2 are tyrosine-phosphorylated and form a heterodimer. Since STAT2 is constitutively bound to IRF-9, STAT1, STAT2, and IRF-9 form a multimeric complex which is also called IFN stimulated gene factor 3 (ISGF-3) (119). Subsequently, this oligomer is transported into the nucleus by importin-α5/β1 transport receptors through the STAT1 conditional NLS.

2) STAT3, STAT5 and STAT6

Unlike STAT1 and STAT2, GFP-tagged STAT3, STAT5a and STAT6 are in the nucleus even without cytokine treatment (Figure 11) (7-9). Live cell imaging also demonstrated that nuclear import of these STAT proteins is continuous independent of tyrosine-phosphorylation. Deletion analyses identified several residues in the coiled-coil domain of these STATs that are required for nuclear import, although the critical sequence in each STAT was different from each other. The sequence necessary for STAT nuclear import is not a classic NLS like sequence. The functional NLS appears to be a larger protein conformation of the coiled-coil domain. Although each STAT has distinct sequences in this region, STAT3 and STAT6 nuclear import are both mediated by importin-α3. Following tyrosine-phosphorylation, STAT3, STAT5a and STAT6 form parallel dimers that accumulate in the nucleus. The more prominent nuclear accumulation of tyrosine-phosphorylated STAT5a and STAT6 is due to DNA binding.

The nuclear export of STAT3, STAT5a and STAT6 is also continuous and independent of tyrosine-phosphorylation. Live cell imaging with photobleaching

techniques showed that nuclear export of these STATs are constitutive. Evidence suggests both Crm1 and non-Crm1 exportins are responsible for export.

Although studies have shown that STAT5a nuclear import is constitutive and independent of tyrosine-phosphorylation, the detailed mechanism of STAT5a nuclear trafficking needs to be determined. Several residues (LQINQTFE) in the coiled-coil domain of STAT5a were found to be critical for the nuclear import, but this sequence does not contain any basic amino acids. I investigated whether these several amino acids are sufficient to function as an NLS and if STAT5a nuclear import is mediated by importin-α/β1 system. Since STAT5a nuclear export is continuous, it may contain an NES that can be recognized by exportins. The role of Crm1 in STAT5a nuclear export was investigated. STAT5a plays a critical role in cell proliferentiation and differentiation, and its aberrant persistent activation promotes the cancer formation. Thereby, STAT5a needs to be tightly regulated. For this reason, my research goal is to elucidate the mechanism that regulates STAT5a nuclear trafficking to provide knowledge needed to develop nuclear import inhibitors.

6. Targeting STATs for cancer therapy

Since aberrant continual activation of STATs promotes many tumors and human malignancies, elucidation of mechanisms that deregulate STATs may provide a basis to design novel therapeutics. Extensive studies have put time and effort in targeting different steps of STAT signaling to inhibit STAT functions (Figure 12) (120). In addition to the down regulation of specific STAT gene expression, several steps of STAT signaling pathway have been targeted that include antibodies to block cytokine receptor (121), tyrosine kinases (122, 123), JAK tyrosine-phosphorylation (124), dimer formation (125), non-specific nuclear translocation (126), and DNA binding (127). Although several elegant strategies have been develop to target STATs, there are also some challenges and side effects (Table 3) (128).

Inappropriate activation of STATs can initiate from constitutive stimulation of cytokine receptors. Indeed, high levels of autocrine and paracrine prolactin hormones are found in human prostate cancers and results in continual activation of prolactin receptors (129). It suggests that the receptor itself can serve as a target for therapeutics. Inhibition

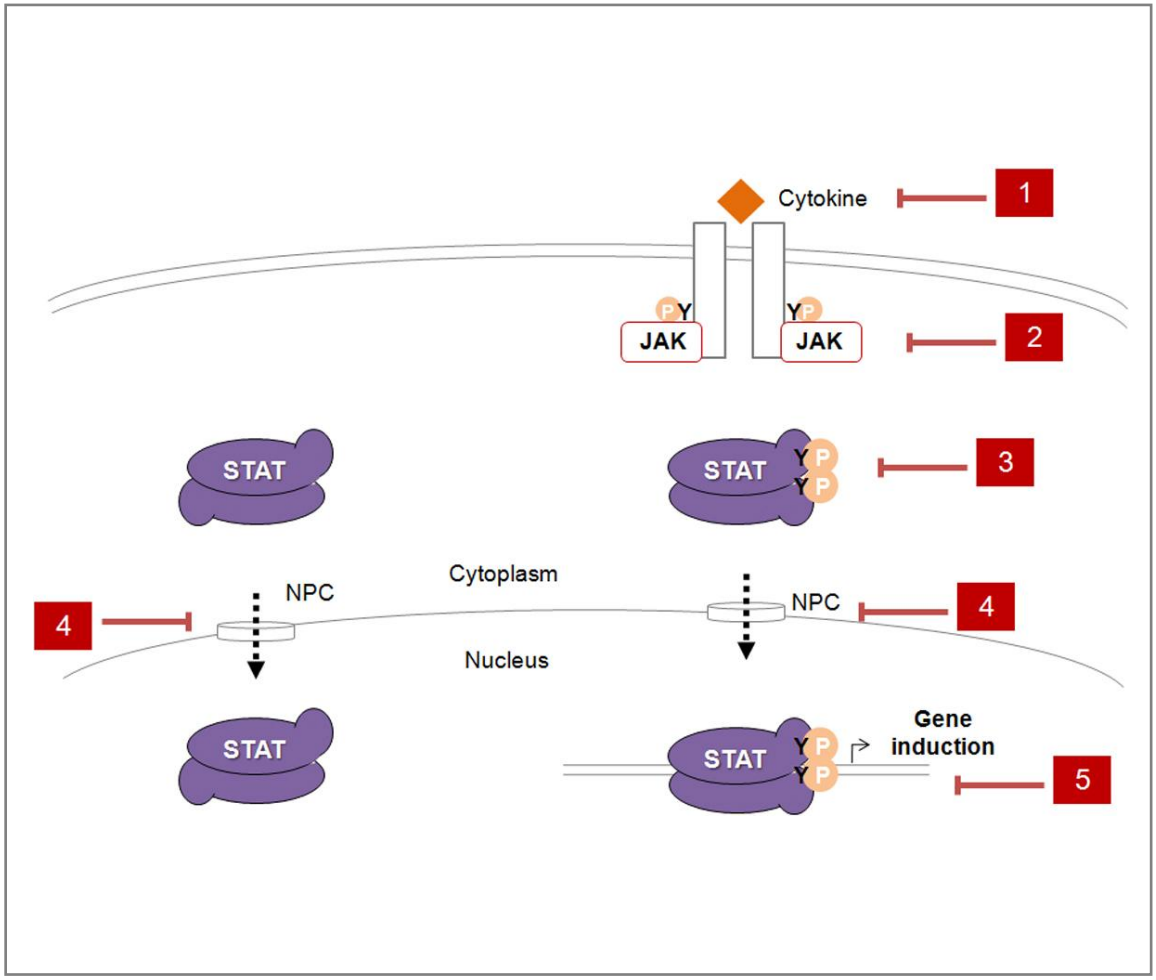


Figure 12: Targeting STAT functions

A conceptual model of targeting STAT protein functions. In addition to down regulation of endogenous STAT gene expressions, STATs can negatively regulated by targeting 1) cytokine binding to receptor, 2) tyrosine kinase, 3) dimer formation, 4) nuclear translocation, 5) DNA binding.

Table 3: Strategies to inhibit STAT signaling

Strategy	Targets	Examples
Inhibit tyrosine phosphorylation	EGFR agonism TKR activity JAK activity SFK activity	Cetuximab, panitummumab, Gefitinib, erlotinib, lapatinib, AG490, LS-104, ICNB1824, CEP-701, Dasatinib, AzD0530, bosutinib
Inhibit dimerization	SH2 domain	Oligopeptides designed from EGFR, gp130, and other receptor or pY-containing peptides; peptide aptamer G-quartet oligonucleotides; small-molecule peptidomimetrics
Inhibit nuclear transport	Importin α3, α5, α7 Importin-β Exportin-1	Karyostatin 1A Leptomycin B and Ratjadone A
Inhibit DNA binding	DNA binding sites	dsODN decoy; peptide aptamer
Natural products	Unspecified	Guggulsterone, Honokiol, Curcumin, Resveratrol, Flavopiridol, Cucurbitacin

(modified by Reference 128)

of the persistent activation of cytokine receptors can be achieved by the use of receptor antagonists or antibodies that can specifically bind to receptors and inhibit the access of activating ligand. A well known example of such antagonist is the monoclonal antibody trastuzumab (Herceptin) that blocks the EGF receptor family and is being used for the treatment of breast cancers (121). Although several other monoclonal antibodies (ex. cetuximab, panitumumab) have been developed to target EGF receptors, clinical studies show that many patients become resistant to EGF receptor inhibitor treatment (130-132). Another approach to to target STAT activity is the receptor associated tyrosine kinases or cytoplasmic tyrosine kinases that phosphorylate STATs. For example, AG490 is a specific JAK2 inhibitor and is reported to inhibit leukemic cell growth (124). Other JAK inhibitors called Jakinibs have also been developed. Tofacitinib is known to inhibit JAK1 and JAK3, and Ruxonitinib is the first FDA proved JAK inhibitor that blocks JAK1 and

JAK2 (133). Imatinib mesylate (Gleevec) blocks the BCR-ABL kinase and is being used for the treatment of chronic myeloid leukiema (CML) (122, 123). Several other JAK inhibitors (ex. LS-104, INCB1824, and CEP-701) have been developed, but show modest efficacy and cause gastrointestinal toxicity in clinical trials (134-136).

Several groups have developed phosphorylated tyrosine (pY)-containing peptides to inhibit the dimer formation of STAT3 (137, 138). The pY^{705} peptide interacts with STAT3 SH2 domains and prevents STAT3 homodimerization. However, peptide inhibitors have poor membrane permeability and metabolic stability. Recently, an orally bioavailable small molecule inhibitor, BP-1-102, has been developed (139). It is a STAT3 SH2 domain ligand that blocks STAT3 binding to phosphorylated tyrosines of activated IL-6R/gp130 which results in inhibiting STAT3 phosphorylation and dimerization. This STAT3 inhibitor is reported to inhibit growth, survival, and invasion in STAT3-dependent tumor cells. Activated STATs induce gene expression in the nucleus. For the inhibition of STAT binding to target DNA, double-stranded oligonucleotides (dsODN) have been designed to mimic the DNA consensus in the promoter of STAT responsive gene (127, 140, 141). dsODN decoy competes for active STAT dimers and is expected to inhibit specific STAT responsive gene expressions. STAT3-specific dsODN decoys have been reported to reduce tumor cell growth in mouse xenograft models. Although the preclinical studies showed that there is no adverse effect with this dsODN decoy injection, the present formulation is easily degraded in serum and not appropriate for the systemic injection (141). Peptide aptamers that prevent STAT3 DNA binding *in vitro* have been found in a modified yeast two-hybrid screen (142, 143). However, these peptides showed poor membrane permeability and stability. A number of natural products (ex. Guggulsterone, honokiol, curcumin, resveratrol) have been found to repress STAT3 activation and inhibit tumor cell growth, but since they are also inhibitory to various cellular events, it is ambiguous if the effect is specific to STAT3 (144-146).

Prevention of STAT nuclear transport would provide another means to inhibit STAT functions. However, the detailed molecular mechanism of STAT nuclear trafficking is yet to be understood. Several sequences necessary for STAT1, STAT3, STAT5a, and STAT6 nuclear import are defined, but they are not sufficient to function as an NLS (7-9, 116). Tyrosine-phosphorylated STAT1 nuclear import is mediated by

importin-α5, and STAT3 and STAT6 nuclear import is dependent on importin-α3. To date, no small molecule inhibitors of importin-α3 and import-α5 have been found. Recently, importin-β inhibitor Karyostatin 1A has been identified, but its effect on STAT nuclear import needs to be evaluated (126). Inhibitors of Crm1 exportin (ex. Leptomycin B and Ratjadone A) have been identified, but since they are general export inhibitors, they will effect all proteins that are actively exported (104, 147).

Targeting the individual steps of STAT activation pathway is critical to inhibit STAT5a functions. Increasing studies have shown that unphosphorylated STATs as well as phosphorylated STATs also function in nuclear gene expression (10-12, 148). Targeting the nuclear trafficking mechanisms of STATs will be more beneficial to inhibit both tyrosine-phosphorylated and unphosphorylated STAT functions. For this reason, my study focuses on the elucidation of STAT5a nuclear trafficking mechanism.

Chapter 2

Materials and Methods

Cell cultures and reagents

HeLa and Cos-1 cells were obtained from American Type Culture Collection (ATCC), and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS). T47D cells (ATCC) were cultured in RPMI1640 with 10% FBS, and MCF-7 cells (a gift from Dr. Todd Miller, Stony Brook University) were grown in DMEM with 10% FBS. DNA transfections were performed using *Trans*IT-LT1 (Mirus) transfection reagent following the manufacturer's instructions. HeLa cells were treated with 10 nM of leptomycin B (LMB; a gift from Barbara Wolff-Winiski, Novartis Research Institute). Cos-1 cells were stimulated with 10 ng/ml of epidermal growth factor (EGF; Sigma Aldrich) after serum starvation. T47D and MCF-7 cells were stimulated with human recombinant prolactin (PRL; PBL Biomedical Laboratories) and hydrocortisone (HC; Sigma-Aldrich) at 1 μg/ml each after serum starvation.

Plasmid constructs

Human full-length STAT5a cDNA and deletion constructs amplified by polymerase chain reaction (PCR) were cloned into mammalian expression vectors pcDNA3 (Invitrogen), pEF1/V5-His (Invitrogen), pCGN (Addgene), or bacterial expression vector pMAL-c4X (New England Biolabs) to express STAT5a, or STAT5a proteins tagged with V5, HA or maltose-binding protein (MBP). Full length or deletion mutants of STAT5a were linked to a monomeric form of enhanced green fluorescent protein (GFP) (149), glutathione S-transferase (GST)-2GFP, or SV40 large-T antigen NLS-GST-2GFP (84) to visualize the cellular localization of STAT5a in the fluorescence microscopy. Human importin-αs or β1 deletion mutants lacking the importin-β1 binding

(IBB) domain were cloned into pGEX-KG for bacterial expression and purification as described previously (7). The β-casein gene promoter driven luciferase reporter gene was a gift from Dr. David Waxman (Boston University School of Medicine), and the β-galactosidase (β-gal) gene was purchased from Promega. Human full length glucocorticoid receptor (GR) cDNA (Origene) amplified by PCR was subcloned into pEF1/V5-His (Invitrogen) to create V5 tagged GR.

Site-directed mutagenesis

Site-directed mutagenesis of STAT5a was carried out with *Pfu Turbo* DNA polymerase (Stratagene) with targeted oligonucleotides. The protocol was modified from the instruction manual of Stratagene QuikChange II Site-directed Mutagenesis Kit. Two complementary oligonucleotides were synthesized with the desired mutation in the middle of each primer. In a 50 μl reaction volume, 50 ng of plasmid DNA, 125 ng of each primer, 0.2 mM dNTP mix, 1x *Pfu Turbo* reaction buffer, and 2.5 unit of *Pfu Turbo* were mixed and applied to 20 cycles of thermal cycle of 30 seconds 95℃, 1 minute 55℃ and 18 minute 68℃. The parental DNA template was digested with 1 unit of *Dpn*I at 37℃ for 4 hours to select the newly synthesized DNA containing the expected mutation. The resulting product was transformed into RapidTrans™ TAM1 competent *E. coli* (Active Motif) and plasmids were purified from single bacterial colonies. The DNA sequence was verified to screen the desired mutation in the plasmid.

Plasmid transformation

RapidTrans™ TAM1 competent *E. coli* (Active Motif) or BL21 codon plus *E. coli* (Stratagene) were used for the plasmid transformation. 2.5 μl of plasmid DNA/ligation was added to 25 μl of thawed competent E.coli and incubated on ice for 30 minutes. After competent cells were heat-shocked at 42°C for 30 seconds, the cells were replaced on ice for 2 minutes. Followed by adding 200 μl of Super optimal broth with catabolite repression (SOC) to the reaction, the cells were grown at 37°C with shaking at 225-250 rpm for 1 hour. The transformations were plated on LB agar plates containing 50 μg/ml of ampicillin or kanamycin and incubated overnight at 37°C.

DNA transfection into mammalian cells

Cells were plated 24 hours prior to transfection to obtain 50% confluency at the time of transfection. DNA transfections were performed using *Trans*IT-LT1 transfection reagent (Mirus) following the manufacturer's instructions. The desired amount of transfection reagent was added into serum-free media and mixed by gentle pipetting. After incubation at room temperature for 5 minutes, the desired amount of plasmid DNA was added to the diluted *Trans*IT-LT1 reagent. Following the 15 minutes of incubation at room temperature, the *Trans*IT-LT1 reagent/DNA complex mixture was added dropwise to the cells. After 24-72 hours of transfection, cells were harvested and assays were performed.

The amount of DNA and *Trans*IT-LT1 transfection reagent used were:

Culture vessel	Total DNA (μg)	*Trans*IT-LT1 reagent (μl)
12-well plate	0.5	1.5
6-well plate/35 mm dish	1.5	4.5
100-mm dish	3.0	9.0

GST and MBP fusion protein purification from bacteria

For GST-fusion protein expression, plasmid encoding GST-importin-αs or β was transformed into BL21 codon plus *E. coli* (Stratagene). A single transformant was inoculated into 10 ml LB with ampicillin for overnight culture. The 10 ml bacterial growth culture was subcultured in 800 ml LB with ampicillin and grown at 37℃. Once the absorbance at 600 (A_{600}) reached 0.4~0.6., 0.2 mM IPTG was added and incubated at 25℃ for overnight to induce the bacterial protein expression. For the purification, cells were harvested and incubated in pre-chilled lysis buffer (1% Triton X-100, 50 mM EDTA, 1 mM PMSF, and 1x bacterial protease inhibitor cocktail in PBS) containing 1 mg/ml of lysozyme on ice for 30 minutes. After sonication (total 30 cycles with 10 sec purse at level 4 and 20 sec rest), cell lysates were centrifuged at 12,000 rpm for 30 min at 4℃ to remove cell debris. The supernatant was collected and incubated with glutathione beads (Sigma) at 4℃ for 2 hours with gentle agitation. The beads were washed three times with cold lysis buffer. Bound proteins were eluted from beads with 40 mM glutathione in 10 mM TE buffer and subsequently dialyzed twice with cold dialysis

buffer (20 mM HEPES pH8.0, 50 mM NaCl, 1 mM EDTA, 15% glycerol, 0.2 mM PMSF and 1 mM β-mercaptoethanol).

For MBP-fusion protein expression, plasmid encoding MBP-STAT5a(1-330) was transformed into BL21 codon plus *E. coli* (Stratagene) and a single bacterial colony was inoculated in 1 ml LB with ampicillin for overnight culture. 1 ml bacterial growth culture was innoculated in 100 ml LB with ampicillin at 37℃ until A_{600} reached 0.4~0.6. Bacterial protein expression was induced by adding 0.2 mM IPTG with 4 hours of incubation at 30℃. Cells were harvested and lysed with cold column buffer (20 mM Tris pH7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT) with 1 mg/ml of lysozyme on ice for 30 minutes. After sonication, cell lysates were centrifuged at 12,000 rpm for 30 min at 4℃ and the supernatant was used as a source of MBP-STAT5a(1-330) proteins for the *in vitro* importin binding assay.

In vitro importin binding assay

STAT5a-V5 was transiently transfected into COS-1 cells, and cells were harvested and lysed with pre-chilled lysis buffer [50 mM Tris-HCl pH8.2, 5mM EDTA, 280 mM NaCl, 0.5% Nonidet P-40, 1mM PMSF, 1x mammalian protease inhibitor cocktails (Sigma)]. 500 μg of cellular proteins were used for each importin binding assay. Anti-V5 antibody was immobilized on the protein G beads overnight at 4℃. STAT5a-V5 was captured with anti-V5 antibody coated protein G beads for 2 hours at 4℃, and then incubated with 15 μg of purified GST-importin-αs or β1 proteins for an additional 2 hours at 4℃ with gentle agitation. Immunocomplexes were eluted with SDS sample buffer and analyzed by Western blot using anti-GST and anti-STAT5a antibodies.

To determine the domain of importin-α3 that directly binds to STAT5a, both bacterially expressed STAT5a and importin deletions were used in the assay. Bacterially expressed MBP-STAT5a(1-330) was immobilized on amylose resin (New England Biolabs) in pre-chilled Column Buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT, 1mM PMSF, and 1x bacterial protease inhibitor cocktails (Sigma)] with 0.1% CHAPS (Sigma) at 4℃ for an hour. 15 μg of purified GST-importin-α3 and deletions were incubated with STAT5a protein immobilized beads at 4℃ for an additional 2 hours with gentle agitation. Bound protein complexes were detected by

Western blot using anti-GST antibody, and the amount of STAT5a bound to resin was examined by Ponceau S staining.

Western blot analysis and antibodies

pcDNA3-STAT5a or HA-STAT5a was transiently expressed in T47D or MCF7 cells respectively. After 24 hours of serum starvation, cells were treated with human prolactin for 1 hour or 16 hours. Cells were lysed with SDS sample buffer and subjected to 8% SDS-PAGE. The separated proteins were transferred to the nitrocellulose membrane (Fisher Scientific) and blocked with 5% non-fat dry milk in TBS. The membrane was incubated with primary antibodies in 5% milk in TBS-Tween 20 (TBS-T: 20 mM Tris pH 7.5, 137 mM NaCl, 0.05% Tween 20) for 1 hour at room temperature. To detect the tyrosine-phosphorylated STAT5a, the phospho STAT5a antibody was diluted in 5% BSA in TBS-T at 4℃ overnight. The membrane was washed three times with TBS-T, and incubated with secondary antibody in TBS-T for 1 hour at room temperature. Followed by the membrane wash, proteins were detected by enhanced chemiluminescence system or Odyssey infrared imaging system (Li-COR Bioscience, Lincoln, NE).

For the western blot analyses, rabbit anti-STAT5a antibody (sc-1081, Santa Cruz Biotechnology), anti-phospho STAT5 (Tyr694) antibody (#9351, Cell signaling), anti-HA antibody, and anti-GST antibody (sc-33613, Santa Cruz Biotechnology) were used as primary antibodies. Alexa Fluor 680-labeled (A21109, Invitrogen) or horseradish peroxidase-conjugated (NA934V, Amersham Bioscience) anti-rabbit IgG was used as secondary antibody for chemiluminescence or infrared imaging system, respectively.

Direct imaging of GFP fusion proteins

Cells were seeded on glass coverslips and transfected with STAT5a constructs linked to GFP. After 24 hours of transfection, cells were serum starved for a day, and untreated or treated with LMB for an hour. Cells were rinsed with cold PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Fluorescence of GFP-tagged protein was visualized by Zeiss LSM 5 pascal confocal microscope using a 40x oil objective. GFP was excited at 488 nm using an argon laser, and emission was collected

with a 505-530 nm filter. Images were obtained using Zeiss LSM Image Browser program and presented using Adobe Photoshop.

Immunofluorescence assay

MCF-7 human breast cells were plated on glass coverslips and co-transfected with GR-V5, human prolactin receptor (hPRL-R), HA-STAT5a or HA-STAT5a(Δ142-149). After 24 hours of transfection, cells were serum starved for a day, and untreated or treated with PRL or HC for an hour. Cells were washed with cold PBS and fixed with 4% paraformaldehyde for 10 min, followed by the permeabilization in 0.5% Triton X-100 for 5 min. Cells were blocked with 3% BSA in PBS for an hour at room temperature. Subsequently, cells were incubated with primary antibodies for 3 hours and then incubated with secondary antibodies conjugated with FITC or Texas Red. Rabbit anti-HA antibody (sc-805, Santa Cruz) and mouse anti-V5 antibody (sc-58052, Santa Cruz) were used as primary antibodies, and anti-rabbit conjugated with FITC (A10526, Molecular Probes) and anti-mouse conjugated with Texas Red (T862, Molecular Probes) were used as secondary antibodies. Immunofluorescence of cells was observed by a Zeiss LSM 5 pascal confocal microscope using a 40x oil objective. Microscopic images were obtained and nuclear and cytoplasmic fluorescence in cells were quantified using Zeiss LSM Image Browser program. Images were presented by Adobe Photoshop and graphically plotted by GraphPad Prism software.

Live cell imaging

HeLa cells were plated on 35 mm glass-bottom tissue culture dishes (Mattek Corporation), and transfected with STAT5a-GFP constructs. After 24 hours of serum starvation, cells were treated with or without LMB for an hour. Cells were maintained at 37℃ and 5% CO_2 for entire live cell imaging using the Zeiss Tempcontrol 37-2 Digital and CTI Controller 3700. For the fluorescence loss in photobleaching (FLIP), a region of interest (ROI) in the cytoplasm was continuously bleached every 60 seconds with 100% power of an argon laser at 488 nm for 50 min. The time lapse images were acquired with the Zeiss LSM 510 META NLO two-photon laser scanning microscope system using a 63x or 40x oil objectives. The excitation wavelength used for GFP was 488 nm and

emission was collected with a 505 nm filter. Images were captured and analyzed using LSM Image Browser and presented with Adobe Photoshop. Nuclear and cytoplasmic fluorescence intensities in a ROI were quantified using LSM Image Browser program, and analyzed by curve-fitting and graphically depicted using GraphPad Prism software.

Luciferase reporter assay

T47D human breast cells that express endogenous GR and PRL-R were plated in 6-well plates and co-transfected with β-casein promoter-luciferase reporter, β-galactosidase, and pcDNA3-STAT5a wild type or import mutation. After 24 hours of transfection, cells were split into 12-well plates for duplication and serum starved for 16 hours. Cells were untreated or treated with 1 μg/ml of PRL or/and HC for 16 hours. Firefly luciferase (Promega) and luminescent β-galactosidase (Clontech) were measured following the manufacturer's instructions. Firefly luciferase values were normalized to luminescent β-galactosidase values to compensate variations of transfection efficiency.

RNA interference (RNAi)

Hela cells were seeded on the coverslips in 6-well plates and 160 pmole of short interfering RNAs (siRNA) specific for human importin-α3 or β1 (Qiagen) were transfected using X-tremeGENE siRNA transfection reagent (Roche). Vimentin siRNA was used as a negative control. Following 24 hours of siRNA transfection, cells were transfected with STAT5a-GFP. Cellular localization of STAT5a-GFP was observed after 24 hours by confocal microscopy. Total RNA was isolated with TRIzol reagent (Invitrogen), and cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison). RT-PCR was performed using specific primers for importin-α3, β1 or GAPDH as an internal control. Sequences of importin-α3 and β1 primers were described previously (7). Image J software was used to quantify the endogenous importin-α3 or β1 mRNA levels.

Chapter 3

Characterization of STAT5a Nuclear Import

Abstract

STAT5a is a transcription factor that has dual functions. In the cytoplasm, it senses the extracellular cytokine or hormone stimulation, and in the nucleus it induces the specific gene expression. For this reason, precise cellular localization of STAT5a within cells is essential for its normal biological functions. Indeed, persistently active and nuclear localized STAT5a promotes cancer, including prostate cancer, breast cancer, and leukemia. Since STAT5a is a 94 kDa large molecule, it needs carrier proteins to be actively transported to the nucleus. In this chapter, I described how STAT5a nuclear import is regulated in molecular basis.

Analysis of the crystal structure and the cellular localization of STAT5a truncation mutants identified an unconventional large NLS in the coiled-coil domain and several critical residues that function in the conformation of an NLS. Both *in vitro* importin binding assays and *in vivo* functional assays with importin siRNAs demonstrated that the nuclear import of STAT5a is mediated by the classical importin-α3/importin-β1 system. The intact STAT5a NLS is required for the transcriptional induction of β-casein milk protein gene and for synergy with glucocorticoid receptor. These findings elucidate the fundamental mechanism of STAT5a nuclear import and will contribute to the design of small molecule inhibitors to block STAT5a functions in disease.

Results

1. STAT5a coiled-coil domain functions as an unconventional NLS.

Fluorescence imaging of STAT5a tagged with monomeric EGFP (STAT5a-mGFP) was used to determine the STAT5a functional NLS. Our previous study demonstrated that STAT5a nuclear import is continuous, independent of tyrosine-phosphorylation (8) (Figure 13, top panel). In addition, deletion of a small region in the coiled-coil domain of STAT5a inhibited the nuclear import function of STAT5a.

To determine if this small peptide is sufficient to function as an NLS, I linked this peptide to GFP and evaluated its cellular localization. Since a peptide linked to GFP proteins are small and can passively diffuse into the nucleus, it must be linked to a larger molecule normally excluded from the nucleus. For this reason, I linked the peptide corresponding to 142-149 a.a. to a larger protein containing glutathione S-transferase and two tandem repeats of GFP (GST-2GFP). The GST-2GFP construct alone does not have an NLS and does not enter the nucleus (Figure 13, bottom panel). However, a well known monopartite NLS from SV40 large T antigen efficiently leads this GST-2GFP protein into the nucleus, therefore this GST-GFP construct is useful to evaluate nuclear import ability of STAT5a proteins. The amino terminal domain with the coiled-coil domain of STAT5a (1-330 a.a.) linked to GST-2GFP was able to promote the nuclear import of this fusion construct, although it is not strong as the T antigen NLS. However, the peptide of 142-149 a.a. alone was not sufficient enough to provide NLS function to GST-2GFP construct. The results indicate that a.a 142-149 are necessary for STAT5a nuclear import, but are not sufficient to function as an NLS.

Since STAT5a 142-149 a.a. may be a part of larger domain that functions as an NLS, I evaluated the cellular localization of serial truncations of STAT5a coiled-coiled domain tagged with GST-2GFP. The crystal structure of unphosphorylated STAT5a dimer has been solved (6) and reveals that the STAT5a coiled-coil domain is composed of four α-helices. Amino acids 142-149 needed for STAT5a import are located in the first α-helix of the coiled-coil domain. To define the domain of STAT5a that serves as an

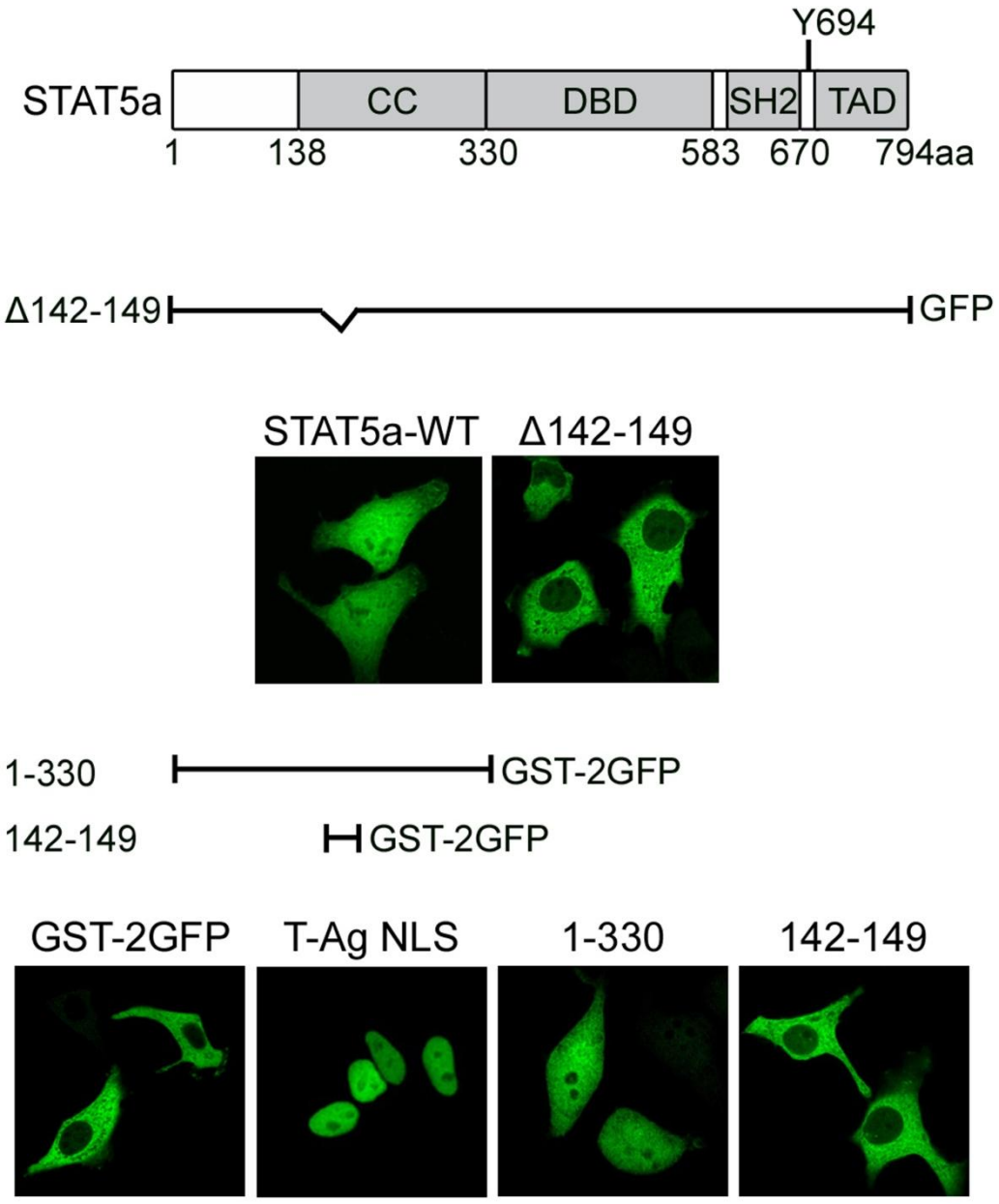


Figure 13: Identification of a region in STAT5a required for nuclear import.

Top panel, linear depiction of STAT5a functional motifs, including the coiled-coil domain (CC), DNA-binding domain (DBD), Src-homology 2 (SH2) domain, transcriptional activation domain (TAD), and specific phosphorylated tyrosine residues 694 (Y694). Linear diagram of STAT5a 142-149 a.a. deletion construct linked to GFP. Cellular localization of STAT5a full length (WT) and deletion mutant visualized in fluorescence microscopy. *Lower panel*, linear diagrams and fluorescence images of STAT5a deletion mutants linked to GST-2GFP. Numbers indicates STAT5a amino acids. Fluorescence images of GST-2GFP and SV40 T antigen NLS (T-Ag-NLS) linked to GST-2GFP are shown.

NLS, each α-helix was evaluated for its contribution to nuclear import of STAT5a (Figure 14A).

The cellular localization of STAT5a fragments revealed that the first α-helix (138-190 a.a.) containing 142-149 a.a. is not sufficient to import GST-2GFP construct to the nucleus. A STAT5a fragment containing both the first and second α-helices (138-256 a.a.) is also not sufficient for nuclear import. However, a fragment containing first, second, and third α-helices shows nuclear accumulation in nearly 50% percent of cells. Complete nuclear import in entire cell population is only seen with all four α-helices of the STAT5a coiled-coiled domain. Fluorescence intensities in nucleus and cytoplasm were quantified by LSM Image Browser program and statistically analyzed by two-tailed Test (Figure 14B). The data indicate that the entire coiled-coil domain of STAT5a is required for the complete nuclear import, and it also suggests that STAT5a NLS is not a conventional short stretch of basic amino acids, but an unconventional large NLS.

Results indicate that STAT5a has an unconventional NLS that functions in the conformation of extended coiled-coil domain. It also applies to STAT3 NLS (Figure 15). The sequence (150-162 a.a.) needed for STAT3 import is in the coiled-coil domain (7), but not sufficient to function as an NLS. To compare the functional NLS of STAT3 with STAT5a, I linked the serial truncations of the STAT3 coiled-coil domain to GST-2GFP. I found that only the entire coiled-coil domain of STAT3 confers complete NLS function to GST-2GFP protein. STAT3 is different from STAT5a in that nuclear accumulation was only seen with all four α-helices of coiled-coil domain, not the gradual nuclear accumulation with each additional α-helix like STAT5a.

2. Identification of critical residues in the STAT5a NLS

To more closely examine the property of STAT5a NLS, the sequence of the coiled-coil domain was evaluated to determine if it contains any of classic lysine or arginine-rich basic residues. Although STAT5a 142-149 a.a. (LQINQTFE) in the first α-helix are needed for nuclear import, they do not possess any basic residues. However, the sequence contains the charged residue glutamic acid (E) that is exposed on the surface of STAT5a and may be accessible to importins. For this reason, I evaluated its contribution to the nuclear import. Mutation of glutamic acids to alanine (E149A) showed inhibition

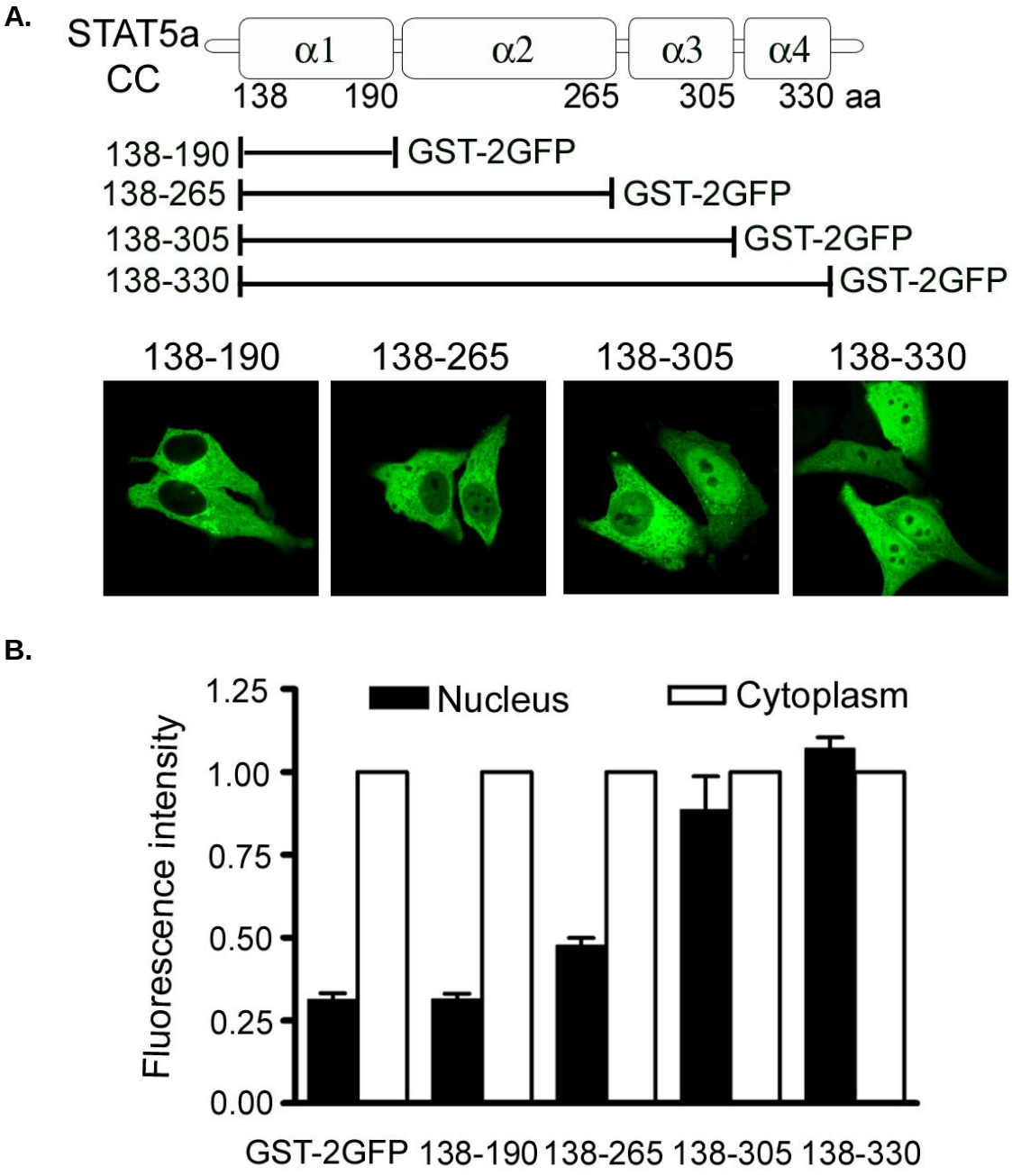


Figure 14: STAT5a coiled-coil domain and nuclear import.

A. Schematic of STAT5a coiled-coil domain with four α-helices and constructs used in the cellular localization analyses. Representative fluorescence images of STAT5a coiled-coil domain fragments linked to GST-2GFP.

B. Statistical analyses of nuclear and cytoplasmic fluorescence intensity in cells expressing STAT5a proteins. Fluorescence intensity was measured in the nucleus and cytoplasm of 10 cells that express individual constructs by LSM Image Browser program and analyzed statistically by two-tailed test. Nuclear fluorescence (black bar) normalized to cytoplasmic fluorescence (white bar) is shown.

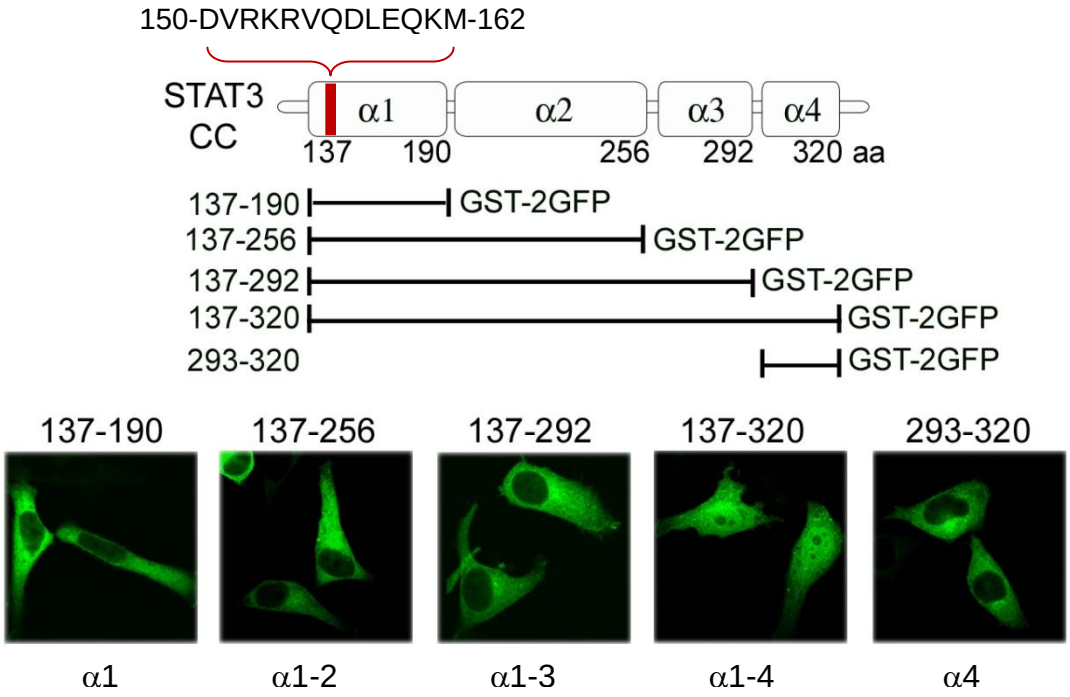


Figure 15: STAT3 coiled-coil domain functions as an unconventional NLS.

Top panel, linear diagrams of STAT3 coiled-coil helices and STAT3 fragments linked to GST-2GFP. The red line indicates a sequence necessary for STAT3 nuclear import. *Lower panel*, fluorescence images of STAT3 coiled-coil domain fragments linked to GST-2GFP. The first α-helix (α1), the first to second α-helices (α1-α2), the first to third α-helices (α1-α3), or the fourth helix (α4) of STAT3 coiled-coil domain were not sufficient to function as an NLS. Only entire coiled-coil domain (α1-α4) of STAT3 provides the complete NLS function to GST-2GFP.

of nuclear import of STAT5a coiled-coil domain in more than 80% of cells in culture (Figure 16A). Thus, it appears that E149 plays a major role in STAT5a nuclear import.

A potential bipartite basic sequence was found in the second α-helix of STAT5a (R241, K242/R257, R258), and I evaluated its contribution to STAT5a import by alanine substitutions. The R241A, K242A/R257A, R258A mutant abrogated the nuclear import of STAT5a coiled-coil domain in about 30% of cell populations. It is not clear that why only a subpopulation of cells showed the inhibition of nuclear import, but it may suggest that this region plays an additive or auxiliary role for STAT5a import. Alanine substitution of charged residues in the third and fourth helices did not identify any critical residues (Figure 17), but it is possible that the third and fourth helices are needed to retain the conformation of the coiled-coil domain structure. Comparison of the protein sequence in fourth α-helix of STAT5a with STAT1 and STAT3 found a conserved isoleucine in STAT5a and STAT3, but not in STAT1. Since STAT5a nuclear import is independent of tyrosine phosphorylation-similar to STAT3, whereas STAT1 nuclear import is dependent on tyrosine-phosphorylation, I tested the influence of alanine mutation in this conserved isoleucine 320 (I320). A single I320A mutation had no evident effect on STAT5a import, but combined with mutations in the first and second α-helices it abrogated the nuclear import of STAT5a coiled-coiled domain in the total population of cells.

The locations of the residues found to be essential for STAT5a nuclear import are indicated in the crystal structure of STAT5a (6) (Figure 16B). The four α-helices of the coiled-coil domain are indicated as yellow and locations of essential residues for STAT5a NLS are indicated as red. Side chains of E149 and R241, K242/R257, R258 are exposed on the same surface of the STAT5a coiled-coil domain. Although the side chain of I320 in the fourth α-helix is embedded, it may interact with third α-helix and may contribute to maintenance of the conformation of coiled-coil domain.

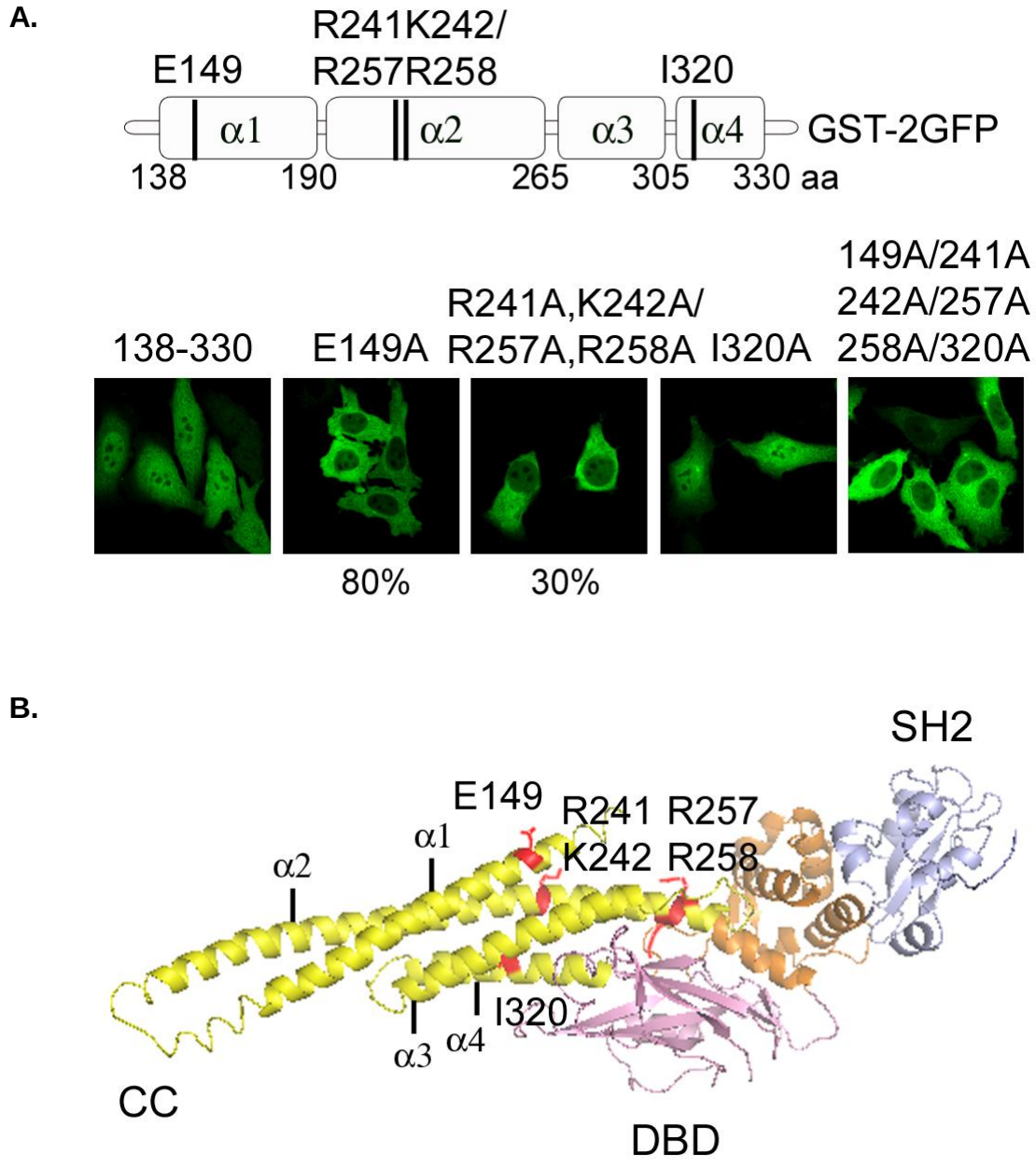


Figure 16: Identification of residues critical for the STAT5a NLS.

A. *Top panel*, position of alanine replacements in STAT5a coiled-coil domain. Fluorescence images of STAT5a coiled-coil domain linked to GST-2GFP with single or combined mutations. Images represent more than 90% of cell populations unless indicated as 80% for E149A and 30% for R241, K242/R257, R258.

B. Location of critical residues for STAT5a NLS in a ribbon diagram of the crystal structure of STAT5a coiled-coil domain (Protein Data Bank ID code 1Y1U). E149 in the first α-helix (α1), basic residues (R241, K242/K257, R258) in the second α-helix (α2), and I320 in the fourth α-helix (α4) are indicated as red.

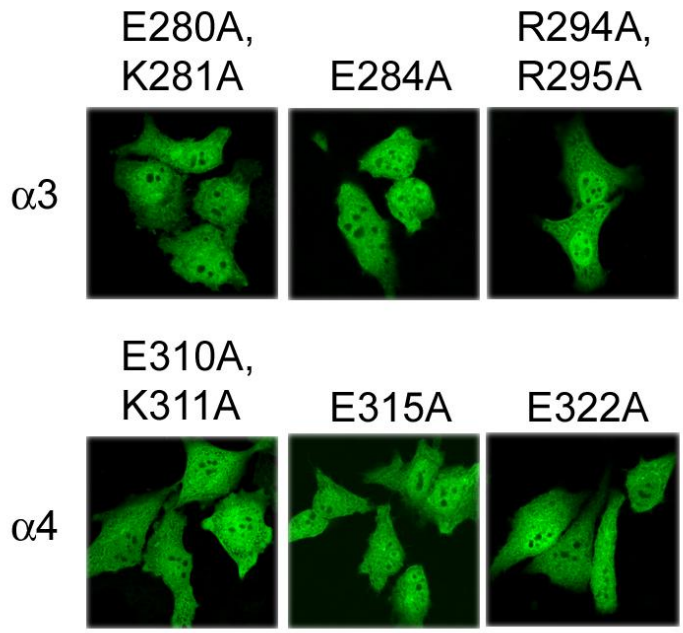


Figure 17: Alanine substitution of charged residues in the third and fourth α-helices of STAT5a coiled-coil domain.

Top panel, fluorescence images of alanine substitiutions in charged residues (E280A/K281A, E284A, and R294/R295A) in the third α-helix of the STAT5a coiled-coil domain tagged with GST-2GFP. *Lower panel*, Images of mutations in charged residues (E310A/K311A, E315A, and E322A) in the fourth α-helix are shown.

3. STAT5a nuclear import is dependent on the importin-α3/β1 system.

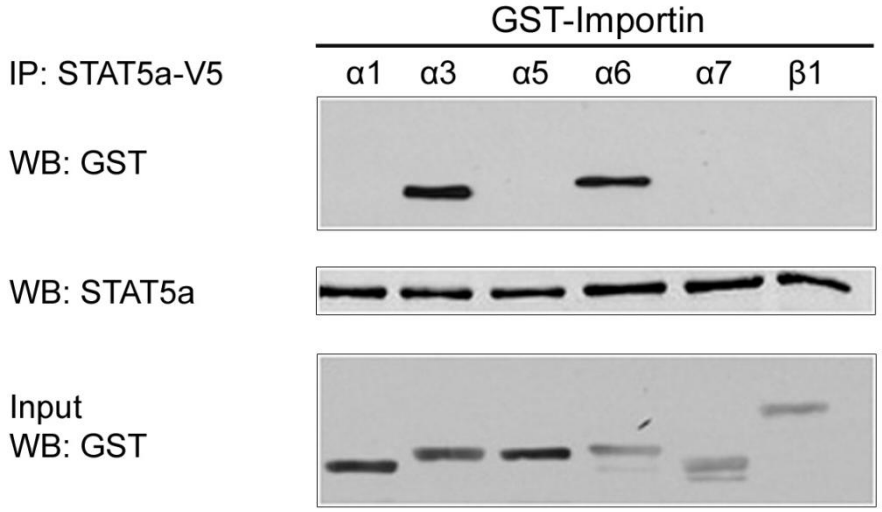
Active transport of large proteins through the NPC is facilitated by carrier proteins of the karyopherin-β family (87). Importin-β1 is a primary karyopherin-β transporter that binds the NLS of cargo protein directly or indirectly via the adapter protein of importin-α family. Importin-α adapter directly recognizes the NLS and forms a heterodimer with importin-β1. The importin-α/β1 dimer mediates the nuclear import of cargo protein. There are six family members in importin-α family that directly recognize the NLS (90).

To determine if any of the importin-α family members physically associate with STAT5a, *in vitro* binding assays were carried out using importins and STAT5a (Figure 18A). Mammalian cells were transfected with V5 tagged STAT5a and whole cell extracts were used as a source of STAT5a protein. STAT5a-V5 was immunoprecipitated with V5 antibody coated protein G agarose beads, and incubated with bacterially expressed GST tagged importin family members. STAT5a bound importins were detected by Western blot using anti-GST antibody. STAT5a was found to bind both importin-α3 and importin-α6. Since importin-α3 is ubiquitously expressed, whereas importin-α6 is limited to testes, importin-α3 appears to be a primary adapter that recognizes STAT5a NLS (150).

To investigate if tyrosine-phosphorylated STAT5a shows a similar importin binding pattern, an *in vitro* binding assay was performed using STAT5a from EGF treated cells (Figure 18B). STAT5a-V5 was immunoprecipitated from EGF treated cells, and incubated with purified GST-importins. Results show that STAT5a from EGF treated cells were bound to importin-α3, importin-α6, and importin-β1. Importin-β1 binding may indicate that tyrosine-phosphorylated STAT5a has an additional ability to recognize importin-β1.

To examine the functional role of defined importins in STAT5a nuclear import, I investigated the cellular localization of STAT5a after knockdown importin-α3 or β1 in cells by RNA interference. siRNA duplex specific to importin-α3, importin-β1, vimentin control were transfected into cells expressing STAT5a-GFP, and cellular localization of STAT5a was analyzed by fluorescence microscopy. The knockdown efficiency of corresponding siRNA in cells was evaluated by measuring endogenous importin-α3 or

A.



B.

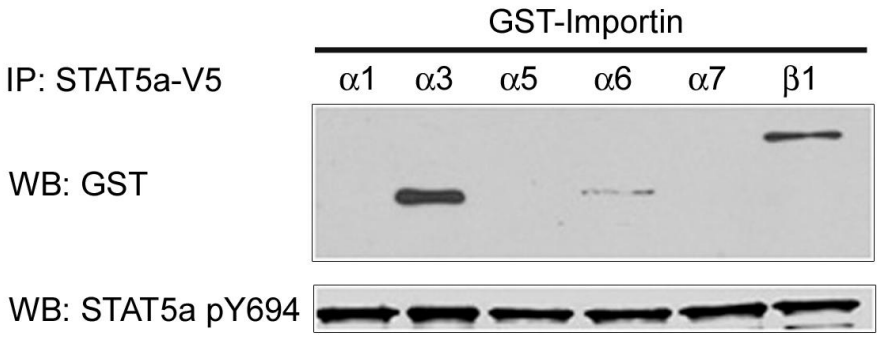


Figure 18: STAT5a interacts with importin-α3 *in vitro*.

A. STAT5a-V5 expressed in Cos-1 cells was immunoprecipitated using V5 antibody coated protein G beads, and incubated *in vitro* with bacterially expressed GST-importins. Western blot identified importins bound to STAT5a with anti-GST antibody, and the amount of STAT5a bound to resin with anti-V5. Ten percent of purified importin inputs are shown in the bottom panel.

B. Cells were transfected with STAT5a-V5 and treated with EGF to activate STAT5a by tyrosine-phosphorylation. STAT5a was collected on agarose G beads, and incubated with purified importins. Importins bound to STAT5a were analyzed by Western blot using anti-GST antibody. The levels of tyrosine-phosphorylated STAT5a bound to resin were detected by anti-phosphotyrosine 694 (pY694) antibody.

importin-β1 mRNA levels by RT-PCR. There was a significant inhibition of STAT5a nuclear accumulation in cells that were treated with individual importin-α3 siRNAs or pooled importin-α3 siRNAs (Figure 19). Cells treated with pooled importin-β1 siRNAs also showed the inhibition of STAT5a nuclear localization, whereas there was no effect of vimentin siRNA control (Figure 20). Together with the importin binding assay, results suggest that STAT5a nuclear import is mediated by the importin-α3 - importin-β1 heterodimer.

To more closely investigate the interface between STAT5a and importin-α3, a domain of importin-α3 that binds to STAT5a was determined. Importin-αs consist of an amino-terminal importin-β1 binding domain followed by central 10 tandem armadillo (ARM) repeats (93). Co-crystal structures of importin-α with classic NLS reveal that basic NLSs are able to bind two regions of importin-α, ARM 2-4 and ARM 6-8. To determine the region of importin-α3 that directly binds to the unconventional STAT5a NLS, I performed *in vitro* binding assay using both bacterially expressed STAT5a and importin-α3 fragments. MBP tagged STAT5a 1-330 a.a. was immobilized on amylose resin, and incubated with bacterially purified GST-importin-α3 or GST-importin-α1 as a control (Figure 21). STAT5a bound importins were identified by Western blot. Only importin-α3 directly bound to STAT5a, but not importin-α1.

To further define the domain of importin-α3 that binds to STAT5a, an *in vitro* binding assay was performed with STAT5a and importin-α3 truncations. STAT5a was found to bind to two independent regions of importin-α3, ARM repeats 1-4 and 7-10 (Figure 22). Further deletions of importin-α3 narrowed down the region that binds to STAT5a, ARM repeats 2-4 and ARM repeats 8-10, however there is some binding to ARM repeats 7-9 (Figure 23). Collectively, *in vitro* binding assays using mammalian and bacterial expression systems and *in vivo* functional assay using siRNAs showed that STAT5a nuclear import appears to be mediated by importin-α3/β1 pathway.

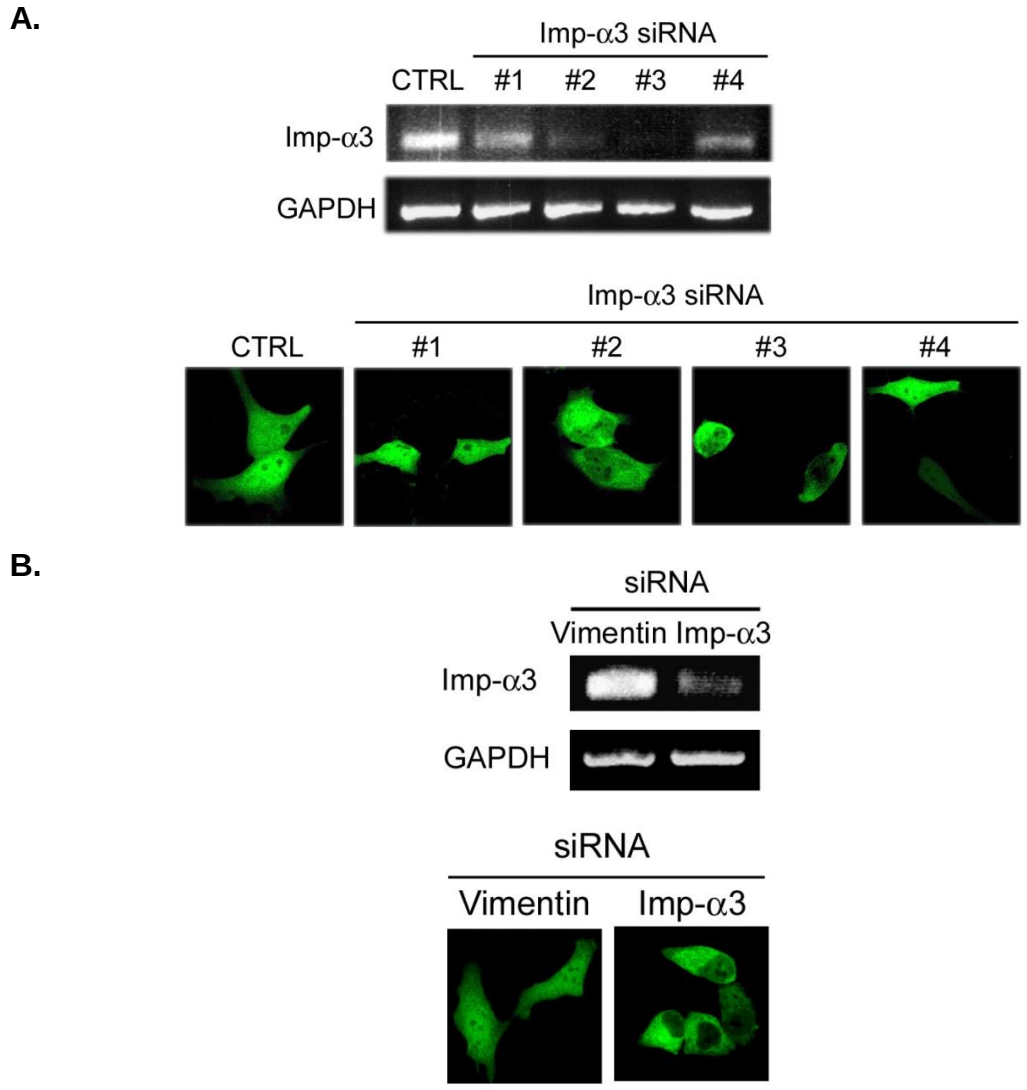


Figure 19: The effect of importin-α3 knockdown in STAT5a nuclear import.
HeLa cells were transfected with four independent siRNAs or pooled siRNAs targeting importin-α3 for 24-48 hours, and then transfected with STAT5a-GFP. Vimentin siRNA was used as a negative control.

A. *Top panel*, the effect of four individual importin-α3 siRNAs on endogenous importin-α3 mRNA detected by RT-PCR. The amount of GAPDH was quantified as an internal control. *Lower panel*, cellular localization of cells expressing STAT5a-GFP transiently transfected with individual importin-α3 siRNAs. Nuclear accumulation was only inhibited in cells treated with importin-α3 siRNAs that reduced the endogenous importin-α3 levels, siRNA #2 and #3.

B. *Top panel*, pooled importin-α3 siRNAs reduced the endogenous importin-α3 mRNA levels by ~80%. *Lower panel*, fluorescence images of cells expressing STAT5a treated with pooled importin-α3 siRNAs. Inhibition of nuclear accumulation was seen in 10% of cultures transfected with importin-α3 siRNAs.

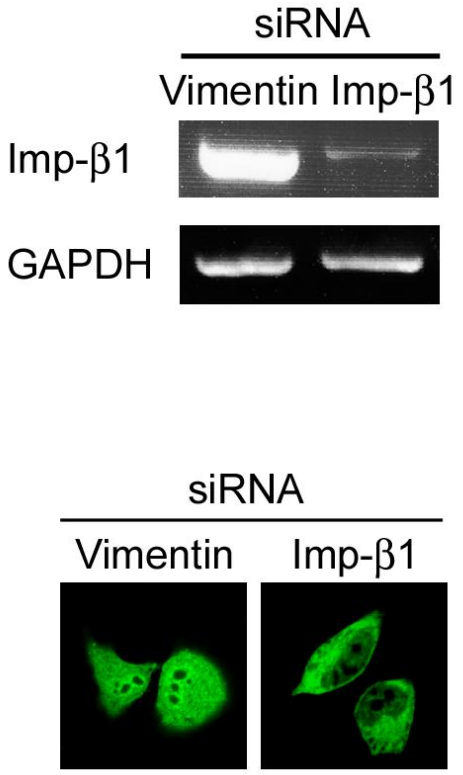


Figure 20: The effect of importin-β1 knockdown in STAT5a nuclear import.

HeLa cells were transfected with pooled importin-β1 siRNAs for 48 hours followed by transfection with STAT5a-GFP. *Top panel*, the effect of importin-β1 or vimentin siRNA on endogenous importin-β1 mRNA analyzed by RT-PCR. *Lower panel*, cellular localization of STAT5a-GFP expressing cells treated with importin-β1 siRNAs. Cytoplasmic localization was seen in 30% of cultures transfected with importin-β1 siRNAs.

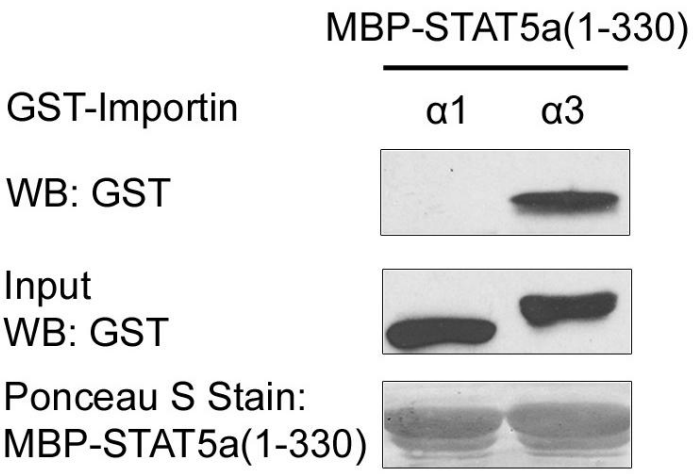


Figure 21: STAT5a directly binds to importin-α3.

Bacterially expressed MBP tagged STAT5a(1-330) was immobilized on the amylose resin, and incubated with bacterially purified GST tagged importin-α3 or importin-α1 as a control. Importins bound to STAT5a were eluted from beads and analyzed by Western blot using anti-GST antibody. Ten percent of purified importin inputs used in binding assays are shown in the middle panel. The amount of STAT5a protein bound to resin was detected by Ponceau S staining.

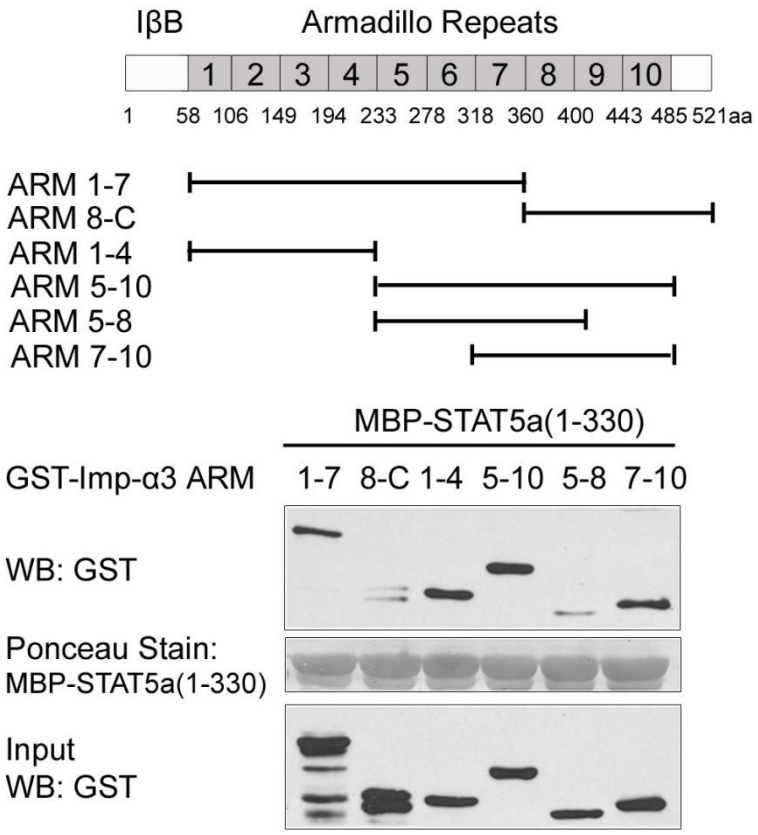


Figure 22: Identification of a domain of importin-α3 that binds STAT5a.

Top panel, diagram of importin-α3 functional domains that includes importin-β1 binding domain (IβB), 10 tandem ARM repeats with the linear depiction of importin-α3 fragments used in the binding assay. *Lower panel*, bacterially expressed MBP-STAT5a(1-330) was immobilized on amylose resin, and incubated with bacterially purified GST-importin-α3 deletion mutants. Importin-α3 truncations bound to STAT5a was identified by Western blot using anti-GST. The input levels of STAT5a were monitored by Ponceau S staining. Ten percent of purified GST-importin-α3 fragment used in the study was detected by anti-GST.

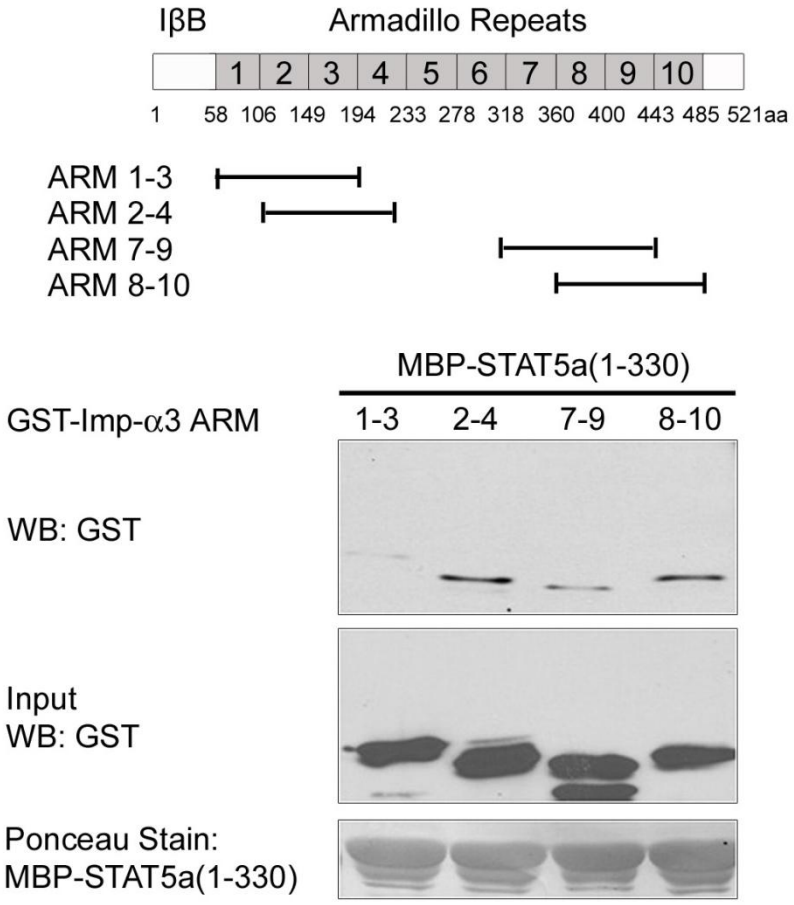


Figure 23: STAT5a binds to two independent regions of importin-α3.

Top panel, linear diagram of importin-α3 functional motifs with the constructs of importin-α3 truncations. *Lower panel*, bacterially expressed MBP-STAT5a(1-330) was collected on amylose resin, and incubated with purified importin-α3 fragments. Importin-α3 truncations bound to STAT5a was identified by anti-GST. The input levels of importin-α3 fragments are shown in middle panel and the resin bound STAT5a was examined in the bottom panel.

Following nuclear translocation of the NLS cargo by importins, the cargo dissociates from the importin complex and binds to target DNA to induce gene expression. Dissociation of the NLS cargo from importins is dependent on the Ran GTPase (96). There is a relatively higher level of the GTP-bound form of Ran in the nucleus. When Ran-GTP binds to importin-β1, it facilitates the dissociation of importin complex from the NLS cargo. To determine if Ran plays a classical role in STAT5a nuclear import, cellular localization of STAT5a was observed with Ran wild type or with constitutively active Ran mutant Q69L that is maintained in a GTP bound state (151, 152) (Figure 24). Cells were co-transfected with YFP tagged STAT5a and CFP-tagged Ran wild type or Ran Q69L mutant. STAT5a was found to localize in the cytoplasm in the presence of Ran Q69L mutant, indicating that RanQ69L mutants inhibit the nuclear import of STAT5a. RanQ69L remains in a GTP bound state and is presumed to constitutively bind to importin-β1 and inhibit complex formation. This constitutive binding prevents the ability of importin-β1 to bind importin-α and transport its cargo into the nucleus.

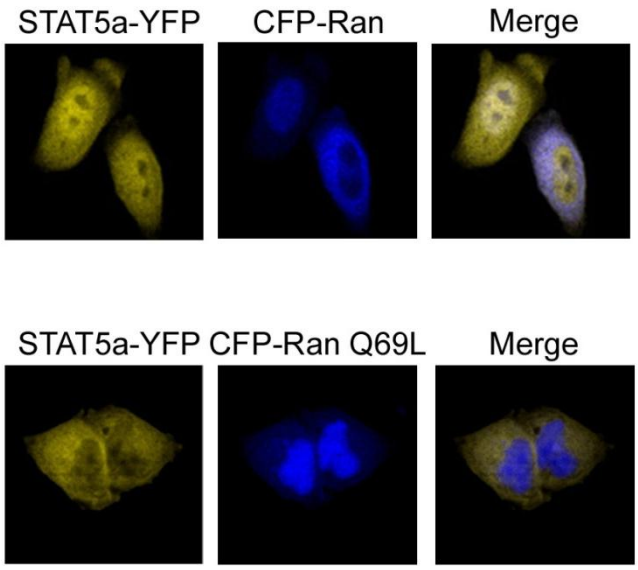


Figure 24: STAT5a nuclear-cytoplasmic transport is dependent on Ran.

HeLa cells were co-transfected with STAT5a-YFP and CFP-Ran wild-type (top) or Q69L mutant (bottom). Cellular localization of STAT5a and Ran were analyzed by fluorescence microscopy.

4. STAT5a nuclear import is necessary for transcription of the β-casein gene as well as synergy with the glucocorticoid receptor.

STAT5a plays a critical role in mammary epithelial cell differentiation and alveologenesis (43). During lactation, the prolactin hormone activates STAT5a by tyrosine-phosphorylation, and induces the β-casein milk protein gene with the cooperation with GR (50, 153). The synergistic effect of STAT5a and GR allows maximal β-casein gene expression. The GR is a ligand-binding transcription factor that is activated by glucocorticoid or derivatives such as dexamethasone or hydrocortisone (48, 52, 53, 55, 56, 154).

To test the effect of the STAT5a import mutant Δ142-149 on transcription of the β-casein gene, I measured the luciferase reporter gene activity regulated by the β-casein gene promoter (Figure 25). STAT5a wild type or import mutant Δ142-149 was transfected into human breast cells with the luciferase reporter gene regulated by β-casein gene promoter. Cells were stimulated with PRL or/and hydrocortisone (HC) after serum starvation. Results show that PRL activated STAT5a induced the transcriptional activity of β-casein gene, but activation of the STAT5a import mutant did not result in β-casein gene induction. The STAT5a import mutant can be tyrosine-phosphorylated in response to PRL and has DNA binding ability *in vitro* (Figure 27A) (8).

To determine the effect of the STAT5a import mutant on synergy with the GR, cells expressing STAT5a wild type or import mutant Δ142-149 were co-stimulated with PRL and HC. HC treatment alone had no effect on the transcriptional induction of β-casein gene with STAT5a. However, co-treatment of PRL and HC enhanced the transcription of β-casein gene with STAT5a wild type. STAT5a import mutant did not induce the β-casein gene in response to PRL alone or with HC. Results suggest that STAT5a nuclear import is necessary for cooperation with the GR in transcriptional induction of the β-casein gene.

Although STAT5a and GR synergize to induce gene expression, the detailed molecular mechanism of their cooperation remains to be determined. Several studies showed that STAT5a and GR can physically associate (53, 55). For this reason, I

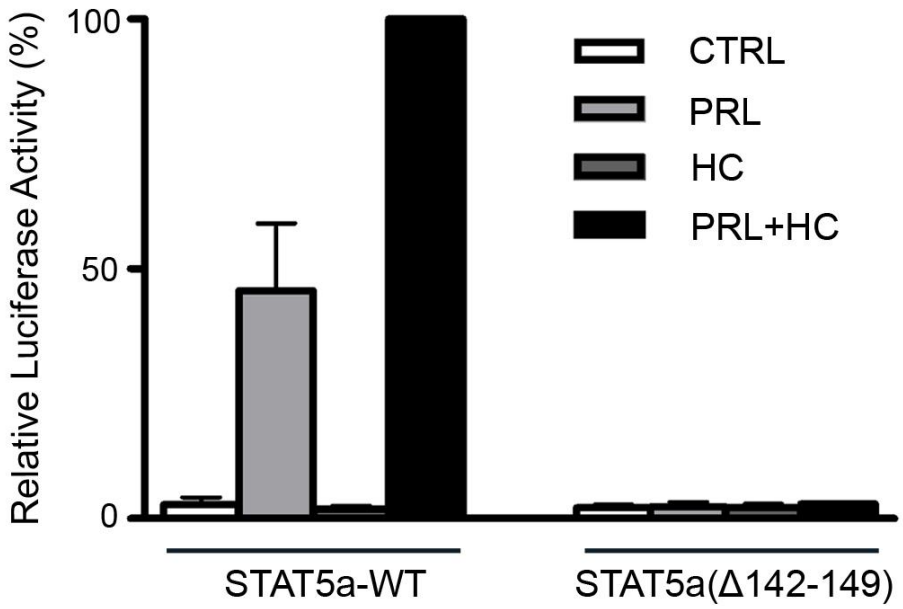


Figure 25: STAT5a nuclear import is necessary for the transcription of the β-casein gene promoter and synergy with GR.

T47D human breast cells were transfected with luciferase reporter under the control of β-casein gene promoter, β-gal as a control, and STAT5a wild type (WT) or import mutant Δ142-149. Followed by serum starvation, cells were untreated or treated with PRL or/and HC for 16 hours to activate STAT5a or GR. The level of β-casein gene promoter activity was evaluated by luciferase assay and normalized with β-gal activity.

examined the effect of STAT5a import on the nuclear accumulation of GR (Figure 26A, left panel). Immunofluoresence assays were performed with human breast cells expressing GR-V5 and HA-STAT5a wild type or import mutant. Cells were stimulated with PRL or HC to activate STAT5a or GR respectively. Without any hormone treatment, STAT5a enters the nucleus and GR is primarily localized in the cytoplasm. After PRL treatment, STAT5a prominently accumulates in the nucleus due to its DNA binding ability, and GR also shows the nuclear presence with STAT5a in a significant number of cells. HC treatment induced the nuclear accumulation of GR as well as STAT5a. Quantitative nuclear and cytoplasmic fluorescence intensities of STAT5a and GR in response to PRL or HC were analyzed by LSM Image Browser and statistically plotted with two-tailed test (Figure 26B). Results suggest that PRL-stimulated STAT5a may interact with GR in the cytoplasm and induce its nuclear transport as a binding partner. In a similar manner, HC-stimulated GR can interact with STAT5a in the cytoplasm and promote the nuclear import of both GR and STAT5a.

To assess the effect of the STAT5a import mutant on cellular localization of GR in response to PRL or HC, the STAT5a import mutant Δ142-149 was used in the immunofluorescence assay (Figure 26A, right panel). Without any hormone treatment, both the STAT5a import mutant and GR remained predominantly in the cytoplasm. PRL treatment did not influence the cellular localization of either STAT5a or GR, as expected. However, HC treatment promotes the nuclear accumulation of both GR and the STAT5a import mutant. These data suggest that ligand-bound GR can physically associate with STAT5a (Δ142-149) in the cytoplasm and induce the nuclear transport of STAT5a (Δ142-149) even though STAT5a (Δ142-149) lacks independent nuclear import ability. Collectively, results suggest that activated STAT5a or GR can influence the nuclear import of its associated partner.

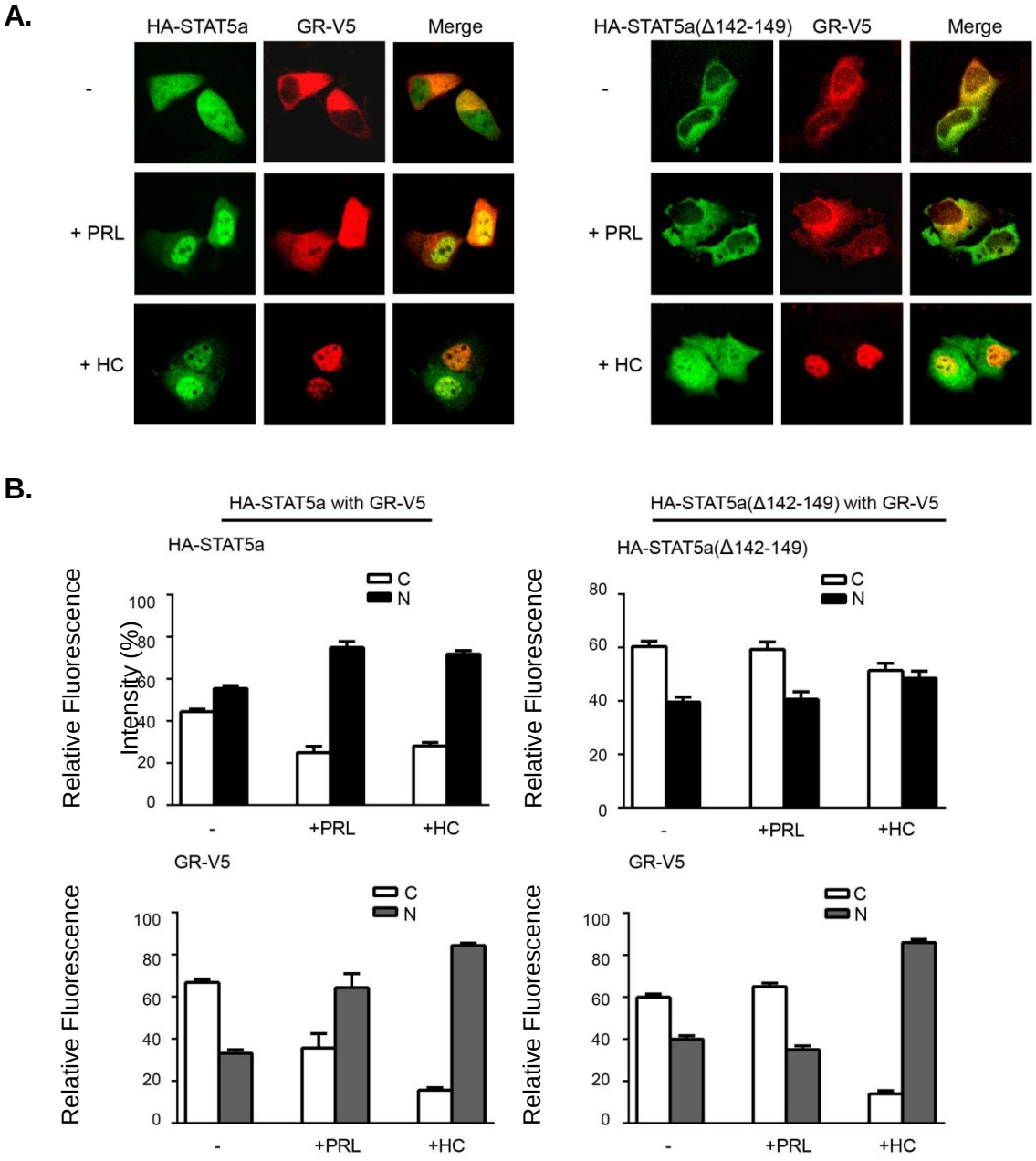
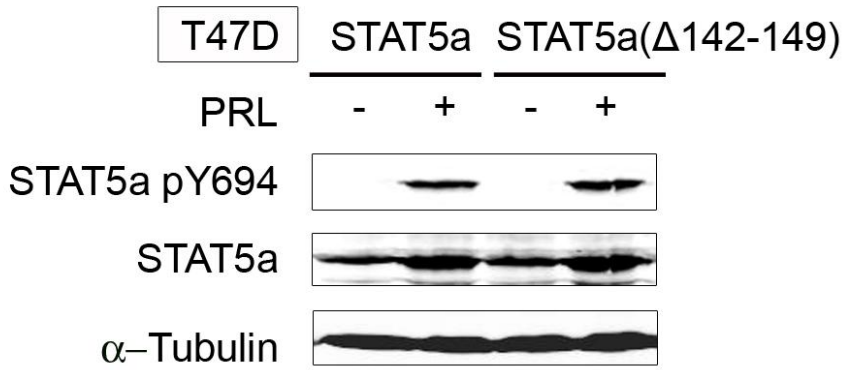


Figure 26: Activated STAT5a induces the nuclear import of GR.

A. MCF-7 human breast cells were transfected with human prolactin receptor, GR-V5, HA-STAT5a wild type or import mutant. After serum starvation, PRL or HC was added to cells an hour to stimulate STAT5a or GR. Immunofluorescence of HA-STAT5a or GR-V5 was visualized by fluorescence microscopy using either FITC- or Texas red-conjugated secondary antibody.

B. Quantitative nuclear and cytoplasmic fluorescence of STAT5a and GR in images of Fig. 19A (above) was measured by LSM Image Browser in multiple cells. Relative nuclear (white) and cytoplasmic (dark) fluorescence intensities of HA-STAT5a with GR is plotted on the left panel and HA-STAT5a(Δ142-149) with GR on the right panel.

A.



B.

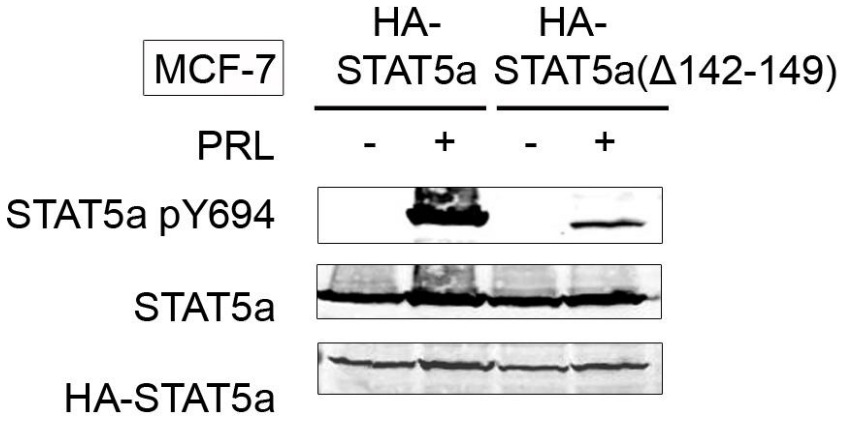


Figure 27: Tyrosine phosphorylation of STAT5a import mutant.

A. T47D cells were transfected with pcDNA3-STAT5a or pcDNA-STAT5a(Δ142-149), and cells were untreated or treated with PRL for 16 hours following serum starvation. Cellular lysates were obtained and used to analyze the tyrosine-phosphorylated levels of STAT5a proteins by Western blot using anti-STAT5 phosphotyrosine 694 (pY694) antibody. The levels of STAT5a and α-tubulin expression are shown in the bottom panel.

B. MCF-7 cells were co-transfected with human prolactin receptor, HA-STAT5a or HA-STAT5a(Δ142-149). After serum starvation, cells were untreated or treated with PRL for an hour. Western blots of cellular lysates with anti-STAT5a pY694, anti-STAT5a, and anti-HA-STAT5a.

Chapter 4

Characterization of STAT5a Nuclear Export

Abstract

Latent STAT5a continuously shuttles between nucleus and cytoplasm. Following the induction of gene expression, STAT5a is negatively regulated to prohibit the continual gene expression. One means of negative regulation is nuclear export of STAT5a and recycling dephosphorylated STAT5a back to the cytoplasm. One of the best characterized nuclear export pathways is dependent on Crm1/exportin1. In this chapter, I describe how STAT5a nuclear export is regulated.

Live cell imaging assays show that STAT5a nuclear export is continuous, independent of tyrosine-phosphorylation. Deletion analyses identified a Crm1-dependent nuclear export signal (NES) in the amino-terminal domain of STAT5a. The Crm1 exportin inhibitor, leptomycin B, delays the nuclear export of STAT5a, but export still continues. This result suggests the existence of another export pathway that is independent of Crm1. A Crm1 independent NES was found to be functional in the DNA binding domain of STAT5a. Since the Crm1 independent NES resides in the DNA-binding domain, it may be masked and not functional when STAT5a is bound to the target DNA.

Results

1. STAT5a has a Crm1-mediated NES in the amino terminal domain.

Unphosphorylated STAT5a continuously shuttles between nucleus and cytoplasm (8). Following tyrosine-phosphorylation, STAT5a accumulates in the nucleus due to the DNA binding. Evidence indicates that it is dephosphorylated in the nucleus by nuclear tyrosine phsophatase, TC-PTP (63) and regains shuttling kinetics of unphosphorylated STAT5a. The advantage of continous shuttling may be a rapid response to activation (import) or a mechanism to turn off signaling (export).

The best characterized nuclear export is mediated by carrier protein Crm1, also known as exportin1 (97, 101). Leptomycin B (LMB) is an anti-fungal antibiotic that covalently binds to Crm1 and interferes with its export function (103, 104). To determine if STAT5a nuclear export is facilitated by Crm1, I treated cells expressing STAT5a-GFP with LMB. Cells treated with LMB showed a significant nuclear accumulation of STAT5a, indicating that STAT5a nuclear export is mediated by Crm1 (Figure 28).

To identify the region necessary for Crm1-dependent STAT5a export, the cellular localization of STAT5a fragments tagged with GFP was evaluated by fluorescence microscopy. I found that an amino terminal fragment 1-138 a.a. of STAT5a responded to LMB treatment. STAT5a 1-138 a.a. (Figure 29, top panel) was predominantly localized in the cytoplasm, and became nuclear only after LMB treatment and inhibition of STAT5a export. The classic NES sequence that is recognized by Crm1 is a leucine rich hydrophobic sequence (97, 155). I generated alanine substitutions of L119A and L133A in STAT5a 1-138 a.a. to evaluate the effect on export. The double mutation inhibited STAT5a nuclear export, indicating that these leucine residues are critical for Crm1-mediated nuclear export. With the further deletion analyses, I found that amino acids 118-138 of STAT5a sufficiently functions as a Crm1-dependent NES. I attached STAT5a 118-138 a.a. to a nuclear protein to determine if defined STAT5a region has ability to export a nuclear protein. As a nuclear construct, I used the well characterized SV40 large T antigen NLS linked to GST-2GFP (NLS-GST-2GFP). This construct is prominently localized in the nucleus and useful to study the export function of STAT5a fragment

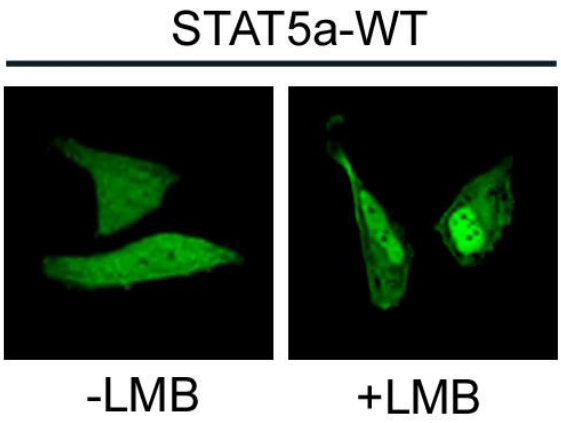


Figure 28: Role of Crm1 in STAT5a nuclear export

Fluorescence images of STAT5a-GFP in HeLa cells with or without LMB treatment. STAT5a-GFP expressing cells were untreated or treated with 10 nM of LMB for an hour, and cellular localization was visualized the by fluorescence microscopy.

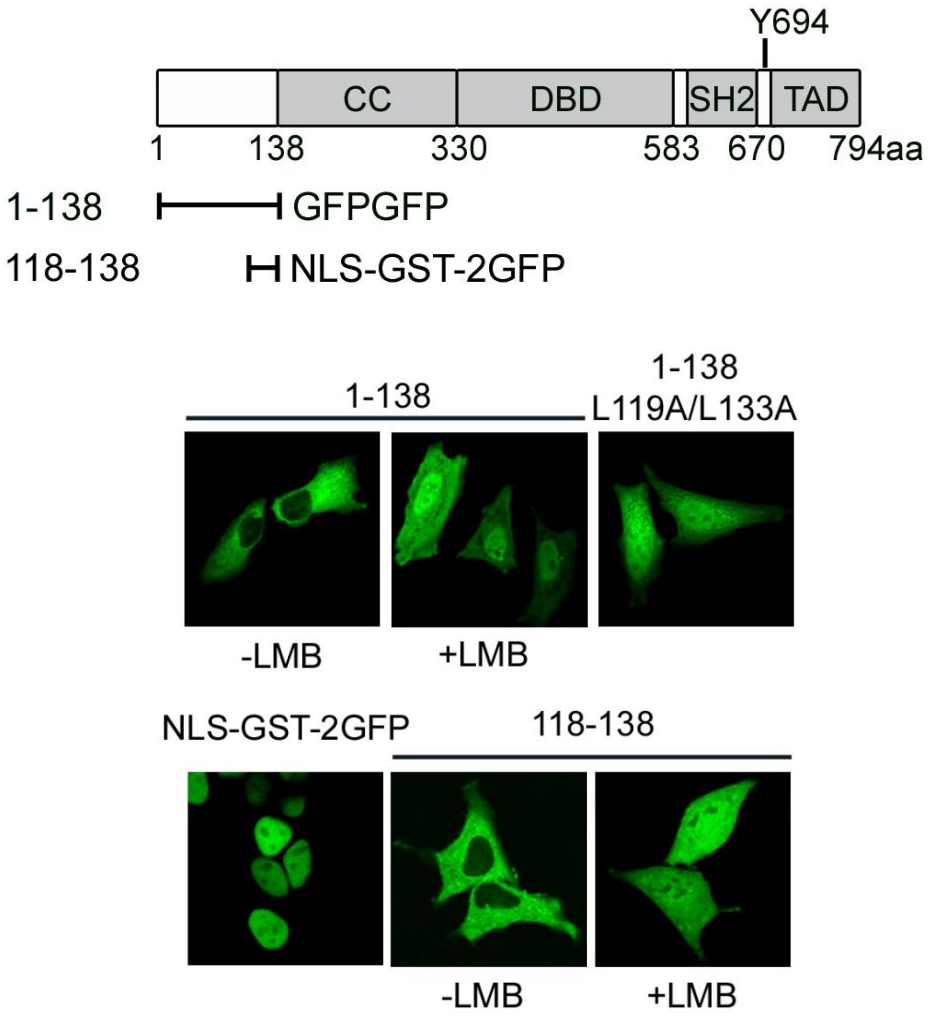


Figure 29: The Crm1-dependent NES function in the amino terminal domain of STAT5a.

Top panel, linear diagrams of STAT5a functional domains and STAT5a deletion constructs. *Middle panel*, fluorescence images of STAT5a(1-138) fragment linked to two tandem repeats of GFP with or without LMB treatment; L119A/L133A double mutation in STAT5a(1-138)-GFPGFP. *Bottom panel*, fluorescence images of SV40 large T antigen NLS linked to GST-2GFP (NLS-GST-2GFP); STAT5a (118-138) fragment linked to NLS-GST-2GFP with or without LMB treatment.

(Figure 29, lower panel). Results showed that STAT5a 118-138 a.a. linked to NLS-GST-2GFP localizes the protein to the cytoplasm. Following LMB treatment, the protein accumulates in the nucleus. Data suggest that STAT5a 118-138 a.a. has a clear ability to direct the nuclear protein to the cytoplasm, and this export function is mediated by Crm1 exportin. The function of this STAT5a NES was also demonstrated in the full length STAT5a by introducing an internal deletion of 118-138 a.a. or the site-directed mutations L119A/L133A (Figure 30).

STAT5a nuclear export may play a role in negative regulation of STAT5a. To assess the role of STAT5a nuclear export on a well known STAT5a function, the influence of the STAT5a NES mutant was evaluated for transcriptional induction of the β-casein gene and its ability to synergize with the glucocorticoid receptor (GR). Since the STAT5a NES mutant, L119A/L133A, accumulates in the nuclues, this mutant should be still able to induce the β-casein gene expression and to cooperate with GR. Although its overall activity was much lower than wild type STAT5a, it did stimulate the gene expression (Figure 31A). The STAT5a NES mutant was tyrosine-phosphorylated similar to the wild-type in response to prolactin (PRL) (Figure 31B).

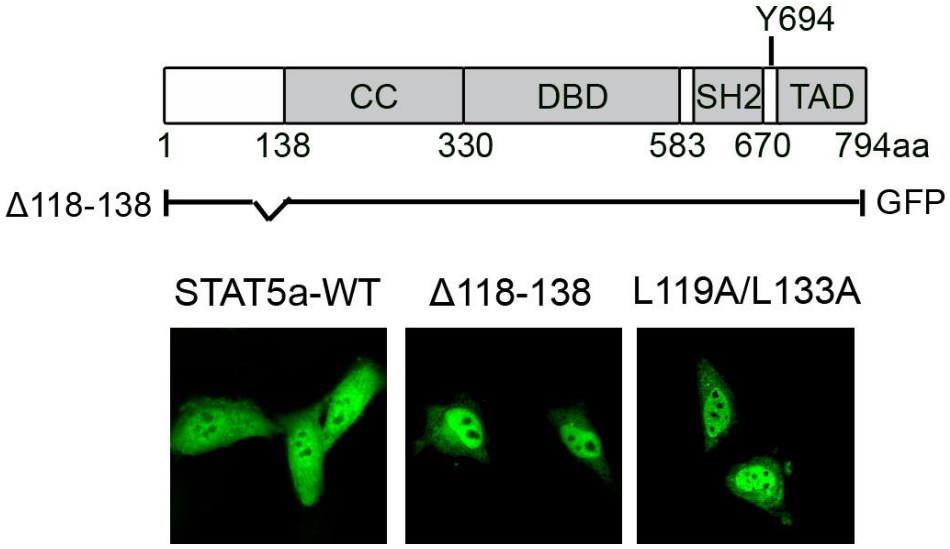
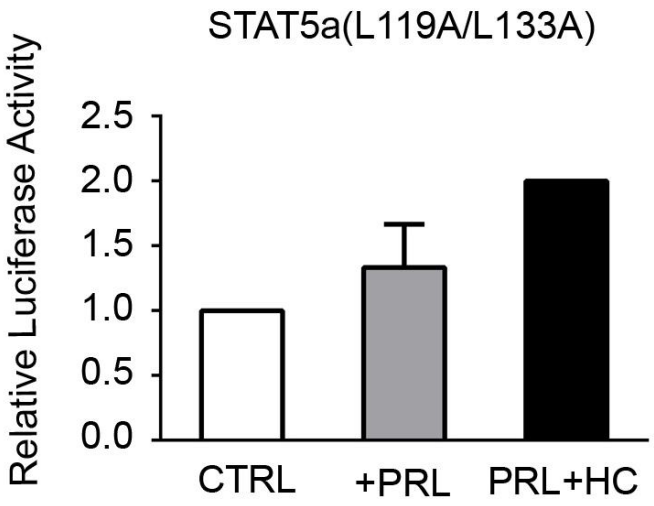


Figure 30: Cellular localization of Crm1-dependent NES deletion or site-directed mutations with a full length STAT5a.

Top panel, a diagram of STAT5a functional motifs with the NES deletion construct. Lower panel, fluorescence images of STAT5a-GFP wild type, Δ118-138 internal deletion, and L119A/L133A double mutation within full length STAT5a.

A.



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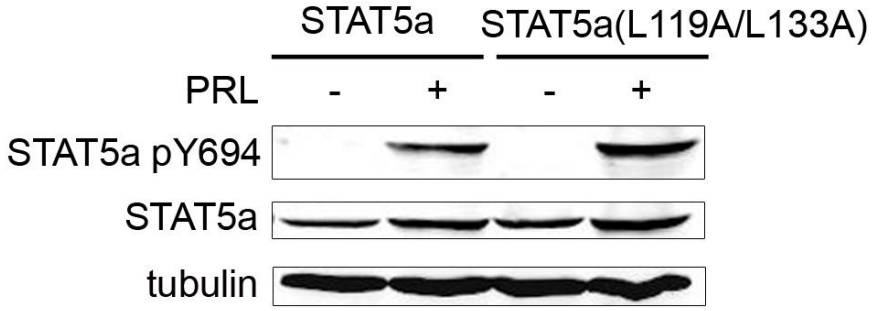


Figure 31: The effect of STAT5a Crm1-dependent NES mutant on the transcriptional induction of β-casein gene expression and synergy with GR.

A. T47D cells were transfected with β-casein promoter-luciferase construct, β-gal, and STAT5a (L119A/L133A). Cells were treated with PRL alone or with PRL and HC after serum starvation. Luciferase results were normalized to the β-gal value.

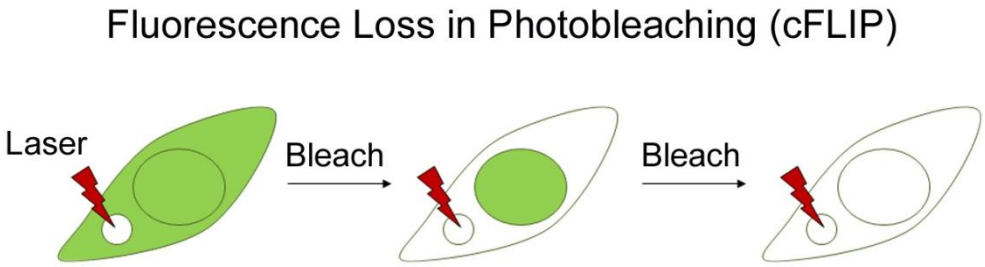
B. The level of tyrosine-phosphorylated STAT5a (L119A/L133A) was evaluated by Western blot with the anti-STAT5a pY694 antibody. STAT5a expression levels were analyzed with anti-STAT5a antibody. Tubulin levels were monitored as a loading control.

2. Evidence supporting the existence of an additional exportin in STAT5a nuclear export

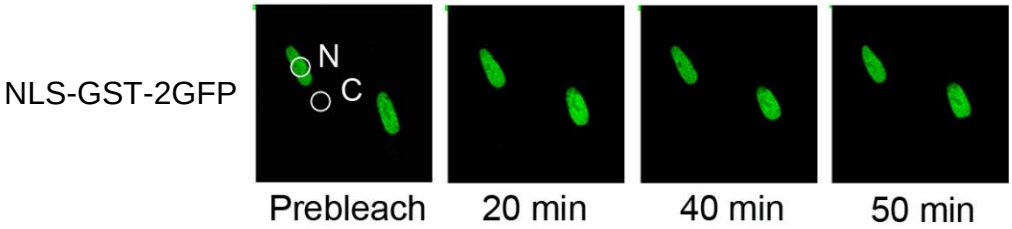
Imaging of fixed cells only provides information of protein cellular localization in a static window. To investigate the temporal and spatial dynamics of STAT5a nuclear export, I used the live cell imaging and technique of cytoplasmic Fluorescence Loss in Photobleaching (cFLIP). Live cells were maintained at 37℃ and express STAT5a-GFP. A small region of cytoplasm was continuously subjected to high intensity laser. STAT5a molecules that rapidly move in the cytoplasm and pass through the path of laser will be photobleached which results in loss of cytoplasmic fluorescence. If STAT5a is exported out of the nucleus, the nuclear fluorescence will also decrease over time (Figure 32A). To control for photobleaching non-specific effects, I performed the cFLIP assay with the nuclear construct, NLS-GST-2GFP. Since NLS-GST-2GFP does not have any export signal, it predominantly remained in the nucleus and showed a steady nuclear fluorescence intensity even after 50 min of continuous photobleaching by cFLIP (Figure 32B). Data indicate that any fluorescence loss occurs during cFLIP assay is not due to the experimental artifacts, but due to the result of protein photobleaching in the small region.

Following the continuous photobleaching in a small region of cytoplasm (cFLIP) in cells expressing STAT5a-GFP, cytoplasmic fluorescence intensity rapidly disappeared (Figure 33A). With additional times of photobleaching, nuclear fluorescence was completely lost by 40 minutes. Results suggest that STAT5a is moving fast in the cytoplasm and continuously exported out of the nucleus. To compare the nuclear export rate of STAT5a in LMB-treated and untreated cells, STAT5a-GFP fluorescence in nuclear and cytoplasmic compartments of an individual cell is graphed following cFLIP (Figure 33B). LMB treatment delayed the nuclear export rate as expected due to the inhibition of Crm1, but STAT5a-GFP nuclear continued (nuclear fluorescence loss) (Figure 33B). The half-time of nuclear fluorescence decrease in multiple cells was calculated by curve-fitting analyses when cytoplasmic fluorescence was at a low baseline level (Figure 33C). LMB was found to delay the half-time of nuclear fluorescence loss by 1.8 fold. Similar to LMB treated cells, the STAT5a NES mutant, Δ118-138, showed a delayed nuclear export rate (Figure 34). Collectively, live cell imaging suggests the role

A.



B.



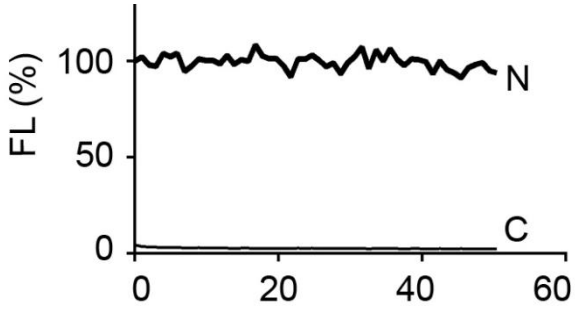


Figure 32: Cytoplasmic Fluorescence Loss in Photobleaching (cFLIP)

A. In cells expressing fluorescence tagged proteins, a small region in cytoplasm is continuously bleached with the high intensity laser which results in photobleaching of molecules passing through the path of the laser. If the fluorescent proteins are exported from the nucleus, nuclear fluorescence will decrease with time. The rate of fluorescence loss in the cytoplasm and nucleus is monitored with time to evaluate the movement of fluorescent proteins in cells.

B. Cytoplasmic FLIP was performed with cells expressing the nuclear construct, SV40 large T Ag NLS-GST-2GFP. The high intensity laser was continuously subjected to a small region in the cytoplasm (C) of cells. The loss of fluorescence intensity in the cytoplasm and nucleus was monitored with time and plotted in graph.

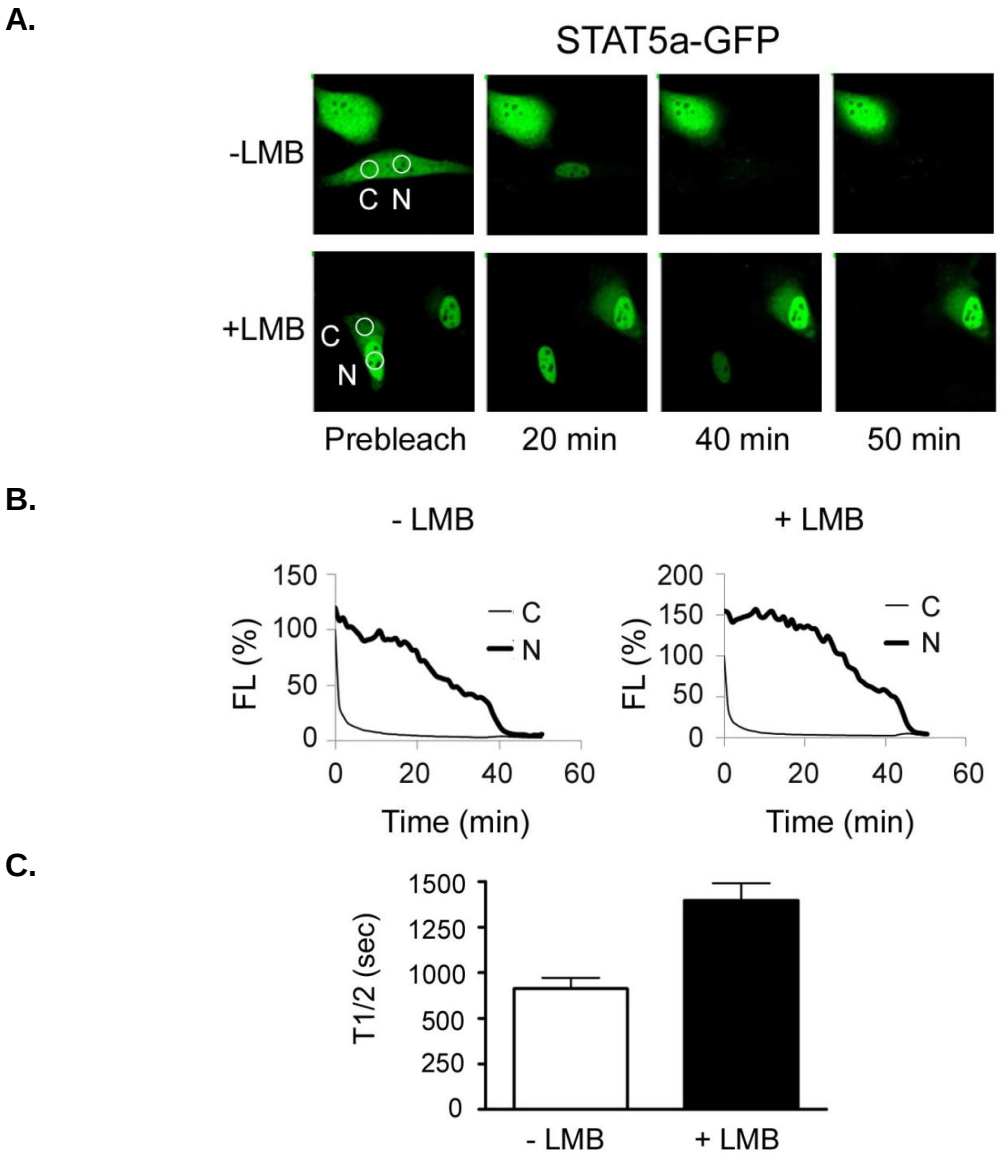


Figure 33: Evidence supporting the additional NES in STAT5a.

Cytoplasmic FLIP was performed in cells expressing STAT5a-GFP in the presence or absence of LMB treatment. High intensity laser was continuously subjected to a small region (C) of cytoplasm.

A. Time lapse fluorescence images of LMB treated or untreated cells.
B. Loss of fluorescence intensity (FL%) in the cytoplasm (C) and nucleus (N) of an individual photobleached cell with and without LMB was measured by LSM Image Brower and graphically plotted with time. Data represent three independent experiments.
C. The half-time (T1/2) of nuclear fluorescence decay was quantified by curve-fitting analyses for multiple LMB treated or untreated cells after the cytoplasmic fluorescence of photobleached cells decreased to basal level.

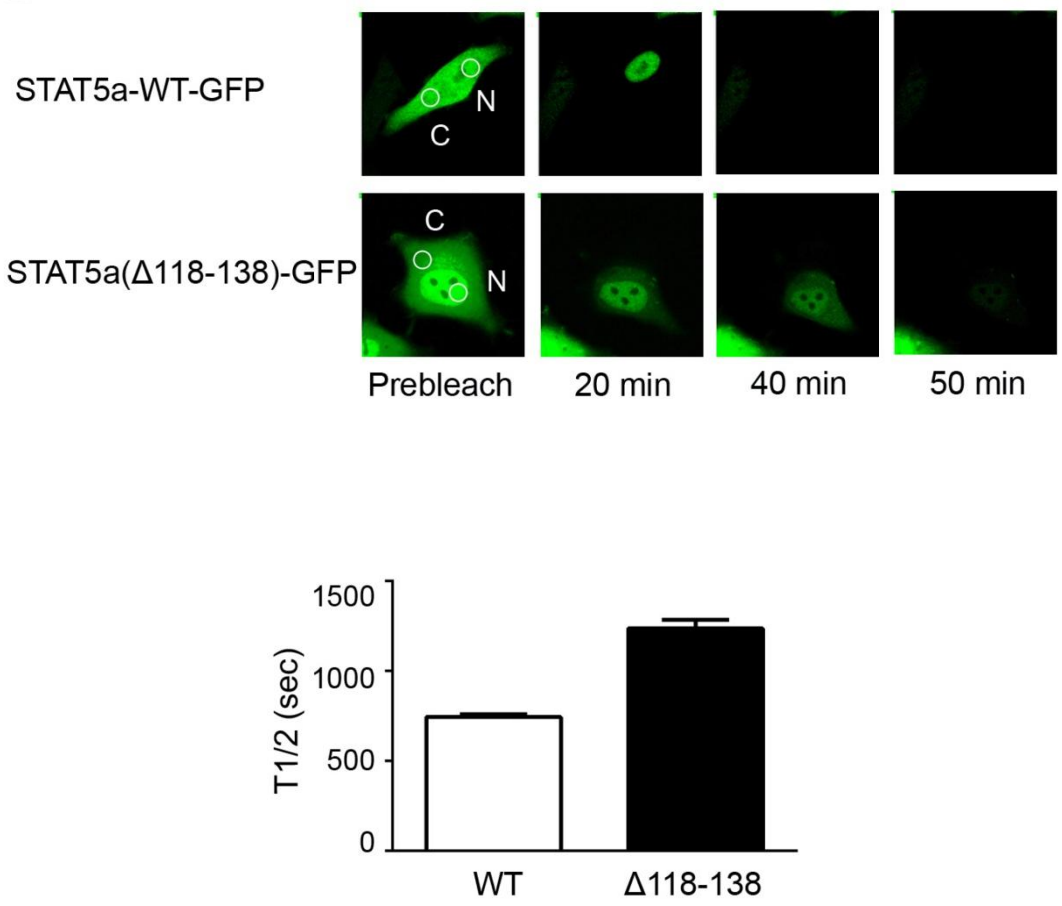


Figure 34: Cytoplasmic FLIP of STAT5a Crm1-mediated NES mutant.

Top panel, continuous high intensity laser was subjected to a small region in cytoplasm (C) of cells expressing wild type STAT5-GFP or STAT5a(Δ118-138)-GFP. *Lower panel*, the half-time (T1/2) of nuclear fluorescence decay was evaluated with curve-fitting analyses and plotted in graph.

of Crm1 in STAT5a nuclear export, but also provides evidence of an additional exportin independent of Crm1 since export was continuous even in the presence of LMB.

3. STAT5a has a Crm1-independent NES in the DNA-binding domain.

To charaterize the Crm1-independent NES of STAT5a, cellular localization of additional STAT5a fragments was evaluated (Figure 35). STAT5a 1-330 a.a. clearly localized in the nucleus although this fragment contains both Crm1 dependent NES and an NLS. Data suggests that the NLS within the coiled-coil domain is dominant to the Crm1-dependent NES within the amino-terminal domain at steady state. The carboxyl terminal fragment 331-794 a.a. localizes to the cytoplasm. This could be due to the lack of an NLS or the function of an NES. To determine if this domain contains a functional NES, further deletions of this region were linked to the nuclear protein, NLS-GST-2GFP. The NLS-GST-2GFP showed prominent nuclear localization, but when STAT5a 331-583 a.a. or 403-474 a.a. of STAT5a was linked to NLS-GST-2GFP, the protein was exported to the cytoplasm (Figure 36). The nuclear export mediated by these fragments is independent of Crm1 since the export was not influenced by LMB treatment. Results indicate that STAT5a has a Crm1-independent NES in the DNA binding domain as well as a Crm1-dependent NES in the amino terminal domain. It remains to be determined if Crm1-independent NES is masked when STAT5a binds to DNA.

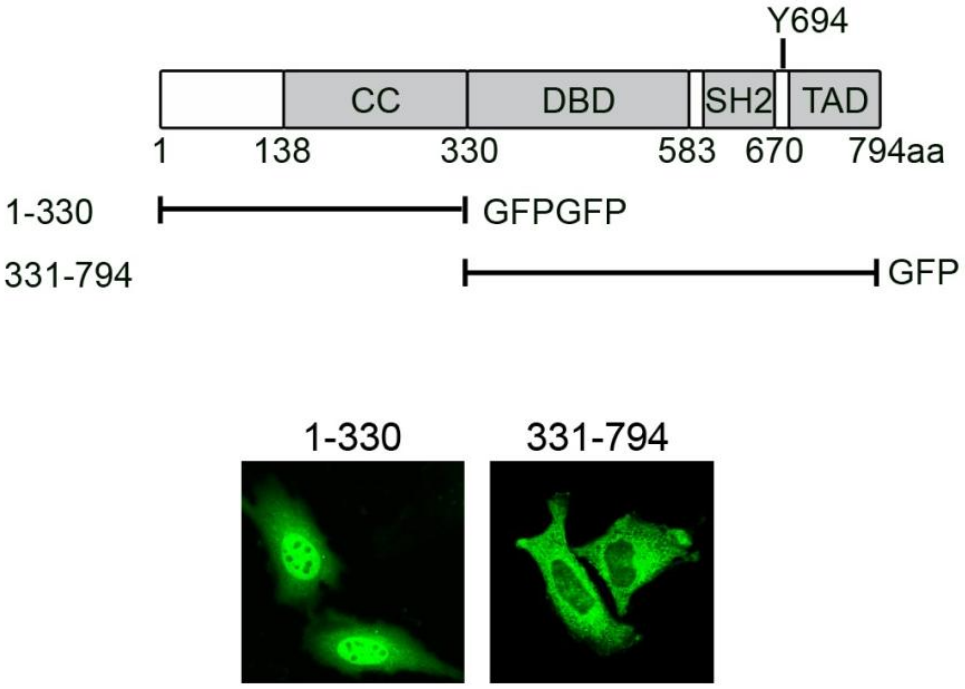


Figure 35: Cellular localization of STAT5a fragments tagged with GFP.

A diagram of STAT5a functional domains and STAT5a deletion constructs. Cellular localization of STAT5a fragments, 1-330 a.a. or 331-794 a.a., tagged with GFP was visualized with fluorescence microscopy.

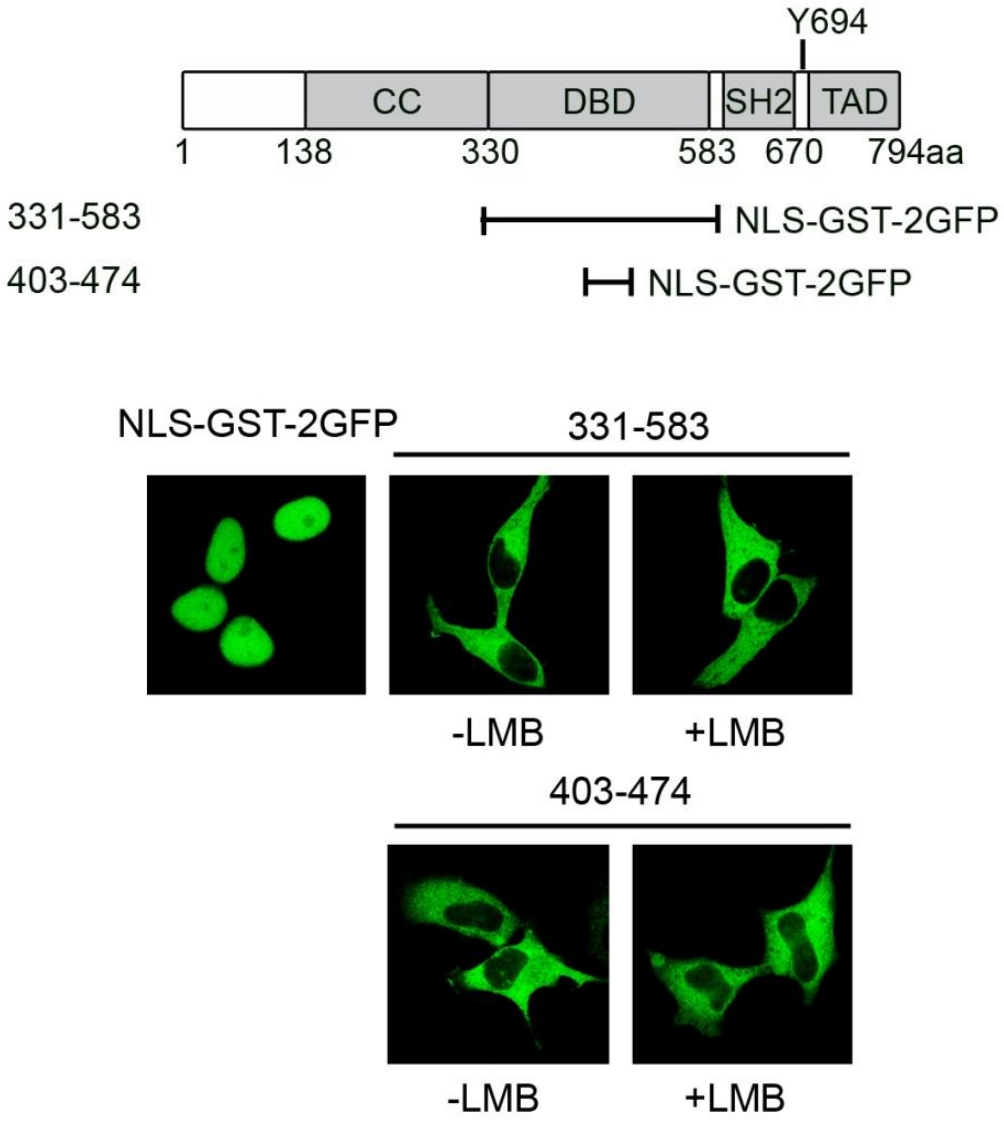


Figure 36: A Crm1-independent NES in the DNA binding domain of STAT5a.

A linear diagram of STAT5a functional motifs and STAT5a constructs. Fluorescence images of SV40 large T Ag NLS-GST-2GFP (NLS-GST-2GFP) and STAT5a fragments, 331-583 a.a. or 403-474 a.a. linked to NLS-GST-2GFP with or without LMB treatment.

Chapter 5

Discussion

STATs are dual function transcription factors that sense cytokine or hormone signaling in the cytoplasm and transmit those signals to responsive genes in the nucleus. For this reason, precise cellular localization of STAT is essential for its biological functions. Although tyrosine-phosphorylated STATs are well known to form dimers and bind to target DNA in the nucleus for gene expression, an increasing number of studies indicate that unphosphorylated STATs also function in nuclear gene expression. Unphosphorylated STAT1 is known to associate with IRF-1, and promote the continual expression of low molecular mass peptide 2 (LMP2) gene (10). Unphosphorylated STAT3 interacts with NF-kB, and induces the transcription of NK-kB responsive genes (148). Unphosphorylated STAT6 promotes the constitutive gene expression of cyclooxyginase-2 (COX-2) (12). In addition, STAT3, STAT5, and STAT6 nuclear import are continuous independent of tyrosine-phosphorylation (7-9). Although the biological function of unphosphorylated STAT5 remains to be determined, accurate nuclear trafficking is essential for both tyrosine-phosphorylated and unphosphorylated STAT5 functions.

Green fluorescence protein (GFP) has been useful to visualize the cellular localization of various proteins. However, since GFP is a small protein that can passively diffuse through NPCs, it is not appropriate to study the active nuclear transport of protein fragments. For this reason, I linked STAT5a fragments with larger GFP containing constructs that are restricted in the cytoplasm or in the nucleus, GST-2GFP or NLS-GST-2GFP. This approach provided clear-cut criteria and allowed me to identify the functional NLS or NESs of STAT5a. I found that STAT5a has an unconventional NLS in the extended coiled-coil domain, and STAT5a has dual NESs; one that is Crm1-dependent

and one that is Crm1-independent. This finding provides fundamental understanding of STAT5a nuclear trafficking.

STAT5a has an unconventional NLS that functions in the coiled-coil domain.

Studies have shown that STAT3, STAT5a, and STAT6 are continuously transported into the nucleus independent of tyrosine-phosphorylation (7-9). Deletion analyses identified several residues critical for nuclear import of these STATs. Defined critical residues were in the coiled-coil domain, but they were not a classic NLS sequence. These sequences are different between each STAT. For example, a sequence necessary for STAT3 nuclear import is comprised of thirteen amino acids that contains a few basic amino acids (150 DVRKRVQDLEQKM 162) (7). A critical sequence defined in STAT5a is eight non-basic amino acids (142 LQINQTFE 149) (8), whereas in STAT6 a short stretch of five amino acids (136 RLQHR 140) is necessary (9). Internal deletion of these sequences in full length STATs show cytoplasmic localization due to the inhibition of STAT nuclear import. However, those peptides were not sufficient to function as an NLS which means that they were not able to direct a heterologous protein into the nucleus (Figure 13 and unpublished data). Data suggest that an NLS of STAT3, STAT5a, and STAT6 may function in a larger structure or more stable conformation.

To determine the functional NLS region of STAT5a, I evaluated the cellular localization of larger STAT5a fragments containing 142-149 a.a., located in the coiled-coild domain and found to be required for nuclear import. I linked STAT5a fragments with cytoplasmic protein, which is GST-2GFP. This protein is ~80 kDa in molecular weight and thereby big enough not to passively diffuse through NPCs. In addition, the GST-2GFP construct does not have any NLS sequence and cannot enter the nucleus. With these features, GST-2GFP is useful to study the NLS function of STAT5a fragments. The crystal structure of unphosphorylated STAT5a dimer has been solved, and it reveals that the coiled-coil domain (138-330 a.a.) is comprised of four α-helices (6). To identify which region of STAT5a coiled-coil domain has an NLS function, I linked serial truncations of STAT5a coiled-coil domain to GST-2GFP and evaluated its cellular localization. I observed the gradual nuclear accumulation by adding each helix of STAT5a coiled-coil domain to GST-2GFP. Subsequently, I found that only the entire

coiled-coil domain provides the complete NLS function to GST-2GFP (Figure 14). It also applied to STAT3. I found that the STAT3 NLS only functioned within the entire coiled-coil domain (Figure 15). Although the conformation of entire coiled-coil domain is required for the efficient function of an NLS, there are several critical residues that contribute for the NLS function. With targeted mutations, I found that a glutamic acid (E) 149 in the first α-helix plays a major role, and a bipartite like sequence (241 RK-KRR 258) in the second α-helix and an isoleucine (I) 320 in the fourth α-helix play lesser role in STAT5a nuclear import (Figure 16). The effect of I320 mutation was only apparent in the combination with other mutations.

The crystal structures of both unphosphorylated STAT5a and unphosphorylated STAT3 have been solved (6, 156), and the coiled coil domain of both STAT5a and STAT3 appears to be accessible to importin carrier proteins. Similar to STAT1 (5), unphosphorylated STAT5a forms a homodimer, and the β-barrels of the DNA binding domain of each monomer are in the close proximity to each other, but the coiled-coil domain is exposed to the surface (Figure 4B). Following tyrosine-phosphorylation, STAT5a is expected to form a dimer through the interaction of reciprocal SH2 domains with phosphotyrosines like STAT1 and STAT3 (25). In this parallel configuration, the coiled-coil domain of each monomer is far apart from each other and exposed to the surface. Based on this structural information, the coiled-coil domain of both unphosphorylated and tyrosine-phosphorylated STAT5a appears to be accessible to importins. For this reason, the accurate regulation of nuclear trafficking is critical for the function of both unphosphorylated and tyrosine-phosphorylated STAT5a.

Our results show that both STAT5a and STAT3 have an unconventional large NLS whose conformation is important for its function. Although basic NLS sequences are best characterized, an increasing number of studies have reported non-typical NLSs. A hydrophobic NLS (GKISKHWTGI) that is recognized by importin-α was found in phospholipid scramblase 1 (PLSCR1) (157), a proline-tyrosine (PY)-NLS was identified in hnRNPs and is directly recognized by karyopherin-β2/Transportin (158). A basic helix-loop-helix NLS comprised of 120 a.a. was found in sterol regulatory element binding protein-2 (SREBP2), and is known to directly bind to importin-β1 (159). Studies show that NLSs are more diverse than previously characterized.

STAT5a nuclear import is mediated by importin-α3/β1 system.

Detection of *in vivo* binding between NLS-containing cargo and importins is technically difficult since importins transiently bind to thousands of molecules. For that reason, I developed an *in vitro* binding assay to determine the interaction between STAT5a and importins. Assays with unphosphorylated STAT5a expressed in either mammalian cells or bacterial cells showed the primary interaction with the importin-α3 adapter protein (Figure 18 & Figure 21). *In vitro* binding assays with a STAT5a NLS mutant and importin-α3 will further clarify the interface between STAT5 and importins. Tyrosine-phosphorylated STAT5a in mammalian cells was also found to bind to importin-β1, indicating that tyrosine-phosphorylated STAT5a has an additional function to directly bind to the importin-β1 carrier protein. It may due to the different structural configuration between unphosphorylated STAT and tyrosine-phosphorylated STAT (5, 6, 25). The *in vivo* role of both importin-α3 and importin-β1 was determined by silencing endogenous importin-α3 and β1 using siRNAs. Significant number of STAT5a expressing cells showed the inhibition of nuclear import, indicating that STAT5a nuclear import is mediated by importin-α3/β1 system (Figure 19 & Figure 20). Besides importins, it was reported that the nuclear import of tyrosine-phosphorylated STAT3 and STAT5 was dependent on GTPase activating protein, MgcRacGAP, and that a dominant negative N17Rac1 inhibited nuclear import of both STAT3 and STAT5 (160). However, we found no evidence of the negative effect of N17Rac1 in the nuclear import of either unphosphorylated or tyrosine-phosphorylated STAT5a (unpublished observation, Chen & Reich).

To obtain more detailed information about the interface between STAT5a and importins, I investigated the domain of importin-α3 that binds to STAT5a. I performed the *in vitro* binding assays using both bacterially expressed STAT5a and importin-α3 fragments containing various ARM repeats. Results showed that STAT5a directly binds to two independent regions of importin-α3, ARM 2-4 and ARM 7-10 (Figure 23). Co-crystal structure of classic monopartite or bipartite basic NLS with importins has been solved, and it showed that the basic NLS binds to the major ARM 2-4 sites and minor ARM 6-8 sites in anti-parallel direction (93). Although STAT5a has an unconventional

NLS, it does bind to ARM 2-4, but also binds to a more extensive region ARM 7-10. The latter region might provide more extensive surface for STAT5a binding or it is needed for the larger structure to maintain the conformation of importins. The basic NLS is known to interact with importins through an array of tryptophan and asparagine residues (88). If we test importin-α3 that has targeted mutations in specific tryptophan and asparagine residues within ARM 2-4 and ARM 7-10, it will provide more concrete information about the interface between STAT5a and importins. Since two independent regions of importin-α3 interact with STAT5a, it may suggest that one importin-α3 binds two STAT5a monomers, unphosphorylated STAT5a dimers, or tyrosine-phosphorylated dimers.

Similar to STAT5a, STAT3 and STAT6 nuclear import are primarily mediated by the importin-α3/β1 receptors (7, 9). However, STAT3, STAT5a, and STAT6 bind different regions of importin-α3. STAT3 binds importin-α3 with ARM 1-8 (7), and STAT5a with ARM 2-4 and ARM 7-10 (Figure 23). STAT6 binds to ARM 5-6 of importin-α3 (unpublished data, Chen & Reich). Co-crystal structure of importin-α3 and individual STATs will provide more definitive understanding of the interface between importins and STATs. This information will contribute to design the specific inhibitors of individual STATs.

STAT5a cooperates with GR to synergize the transcription of β-casein gene.

The regulation of biological effects is generally exerted through the combination of multiple signaling pathways. Accumulated studies have shown that the crosstalk between STAT5 and steroid hormone receptor signaling stimulates or inhibits specific STAT5 functions (48). Steroid hormone receptors are transcription factors that belong to a nuclear receptor super family (51). They are activated by hormonal ligands, and involved in growth, differentiation, and homeostasis. One of the best characterized steroid hormone receptors that cooperates with STAT5 is the glucocorticoid receptor (GR) (53). GR is activated by glucocorticoid or its derivatives, including dexamathasone or hydrocortisone (HC). Upon ligand binding, GR dissociates from heat shock proteins, forms a dimer, and binds to target DNA, or it is recruited as a coactivator for specific gene expression. GR is known to interact with STAT5a or STAT5b, and enhances the β-

casein gene transcription in mammary glands, or the normal postnatal growth in hepatocytes, respectively (53, 59).

To determine the role of STAT5a nuclear import in the transcription of β-casein gene and its ability to synergize with GR, I assessed the effect of STAT5a NLS mutant in human breast cells. I found that neither β-casein gene activation nor synergy with GR was observed with STAT5a NLS mutant, STAT5a (Δ142-149) (Figure 25). Our data suggest that STAT5a nuclear import is required for both β-casein gene transcription and the cooperation with GR. In addition, it also demonstrates that 142-149 a.a. of STAT5a is necessary for nuclear import and plays a role in the biological function of STAT5a.

Although STAT5a is known to synergize with GR, the detailed mechanism of their cooperation needs to be determined. Previous studies demonstrated a physical interaction between STAT5 and GR (53), but it is not clearly understood if the interaction takes places in the nucleus or in the cytoplasm. Immunofluorescence assay and cell fractionation assays have shown that activated STAT5 induces the nuclear import of GR and liganded GR transports STAT5 into the nucleus (55, 56). GR has two NLSs that can be recognized by importins; one in the ligand binding domain and another in the DNA binding domain (161). Our study demonstrated that activated GR induces the nuclear transport of the STAT5a NLS mutant (Figure 26). Our data suggest that STAT5a and GR can associate in the cytoplasm, and each can induce the partner's import.

STAT5a has two NESs: a Crm1-dependent NES and a Crm1-independent NES

In addition to positive regulation of STAT5, negative regulation is also important to maintain the normal function of STAT5. Negative regulation of STAT5 is known to be dependent on tyrosine phosphatases and suppressors of cytokine signaling (SOCs) (45, 47). Several tyrosine phosphatases are reported to dephosphorylate STAT5 in the cytoplasm and in the nucleus, including PTP-1B, TC-PTP, and VHR (62-64). Another possible means to negatively regulate STAT5 function is export STAT5 from the nucleus. Our studies demonstrated that STAT5a has two NESs that are Crm1-dependent and Crm1-independent.

STAT5a has a Crm1-dependent NES in its amino terminal domain. Our studies have shown that Crm1 exportin inhibitor LMB reduces the nuclear export of both

STAT5a full length and STAT5a amino terminal domain (Figure 28 & Figure 29). Amino acids 118-138 in the amino terminal domain of STAT5a found to be a functional NES that can be recognized by Crm1 exportin. Although STAT5a NES does not exactly follow the leucine rich Crm1-NES consensus (LxxxLxxLxL), it contains several leucines. Two leucines, L119 and L133, are critical residues for the NES function (Figure 29 & Figure 30). However, live cell imaging with a photobleaching assay (cFLIP) provides the evidence that STAT5a has an additional NES that is independent of Crm1. Although the nuclear export rate of cells treated with LMB was delayed, STAT5a export was continuous (Figure 33). Deletion analyses found an additional NES in the DNA-binding domain that is independent of Crm1 (Figure 36). Since this additional NES is in the DNA-binding domain, it is possible that it only functions when STAT5a does not bind to DNA.

The additional exportin that mediates STAT5a nuclear export independent of Crm1 remains to be determined. An increasing number of studies have shown that there are additional exportins besides Crm1. Exportin-4, 6, 7 are reported to mediate the nuclear export of several eukaryotic transcriptional or translational factors (105-108). Importin-13 has both import and export functions. Importin-13 can mediate the nuclear import of RNA-binding protein RBM8, but it can also mediate the nuclear export of translation initiation factor 1A (eIF-1A) (110). Chaperone molecule calreticulin is recently reported to mediate the nuclear export of GR and thyroid receptor-α1 (112, 113). One approach to identify a candidate exportin that mediates STAT5a export can be performed with a screen using *in vitro* STAT5a binding assays. The STAT5a Crm1-independent NES can be linked to a resin and incubated with mammalian cell extracts. The NES bound proteins can be isolated from total cell extracts and identified using a mass spectrometry. The *in vivo* function of identified exportins can be determined by silencing endogenous exportins by siRNAs. Emerging studies have identified dual NESs in other eukaryotic proteins. GR and thyroid receptor-α1 were found to have both Crm1-dependent and Crm1-independent NESs (112, 113). To date, no consensus sequence was found in these Crm1-independent NESs. Although it is not known why these proteins have evolved to have dual NESs, it suggests the possibility that each NES plays a role in distinct biological functions or they cooperate to enhance the nuclear export ability.

Our studies have demonstrated that STAT5a nuclear import and export are continuous independent of tyrosine-phosphorylation, and tightly regulated by active transport mechanisms. The conceptual model of STAT5a nuclear trafficking mechanisms from my studies is shown in Figure 37. STAT5a has an unconventional NLS that functions in the extensive coiled-coil domain, and importin-α3/β1 mediates STAT5a import. STAT5a has two NESs that can be recognized by Crm1 or other exportins. STAT5a nuclear import and export regulate STAT5a functions in positive or negative way to maintain its normal functions. Our results provide a fundamental understanding of dynamic STAT5a nuclear trafficking.

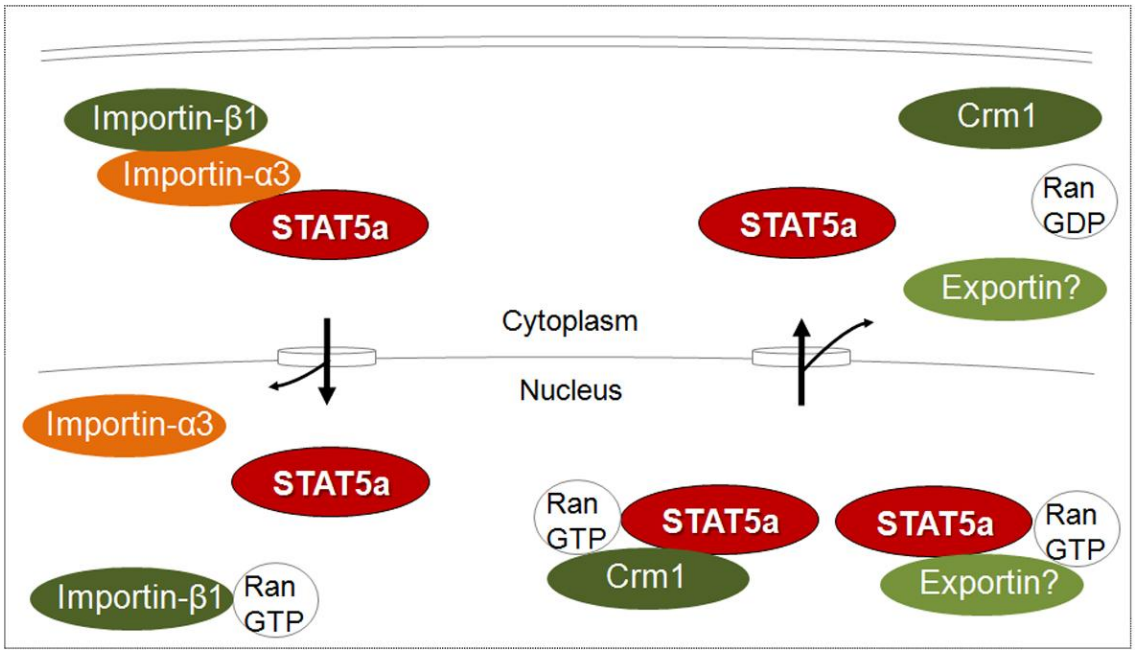


Figure 37: A brief model of STAT5a nuclear trafficking

STAT5a has an unconventional NLS that can be recognized by importin-α3 adapter protein. Importin-β1 carrier proteins transport STAT5a-importin complex into the nucleus. In the nucleus, Ran-GTP binds to importin-β1 and it leads to the dissociation of importin-cargo complex. Crm1 or additional exportin recognize NESs in STAT5a and form a complex with Ran-GTP. Trimer of STAT5a:exportin:Ran-GTP is transported out of the nucleus.

Targeting STAT5 in disease

Targeting the STAT activation pathway is important to inhibit many STAT functions, but an increasing number of studies have also shown that unphosphorylated STATs play a role in nuclear gene expression (148). Accurate mechanisms of STAT nuclear trafficking need to be determined, since a strategy to inhibit STAT nuclear transport might be beneficial to suppress both tyrosine-phosphorylated and unphosphorylated STAT functions.

For the inhibition of STAT5a nuclear import, peptide aptamers can be screened using STAT5a coiled-coil domain. Peptide aptamers are short peptide sequences that can be selected from peptide library to bind to a specific protein domain (47). A yeast two hybrid system can be used to screen peptide aptamers against STAT5a coiled-coil domain from a random peptide library. The conformation of selected peptide can be stabilized in the scaffold protein, such as bacterial thioredoxin A (47). In this way, peptide aptamers that specifically bind to STAT5a coiled-coil domain can be identified and used for the inhibition of STAT5a nuclear import.

Our results show that STAT5a nuclear import is mediated by importin-α3/β1 system. If small molecule inhibitors of importin-α3 or importin-β1 exist, it may be useful to suppress STAT5a functions. However, since importin-α3 and importin-β1 mediate the nuclear import of thousands of molecules, inhibition of these importin factors would be deleterious to cells. Indeed, I observed cell death when I knocked down importin-α3 or β1 using siRNAs in cells. For this reason, a minimal importin-α3 fragment that contains STAT5a binding sites but does not bind to importin-β1 carrier protein may be a more effective tool to target specific STAT5a functions. It may compete with endogenous importin-α3 and interfere with STAT5a nuclear import. Since peptide inhibitors are known to be inefficient in cell permeability, it may be more beneficial to link peptide inhibitor with protein transduction protein (PTD). A simple PTD is comprised of nine arginine residues and allows the introduction of extracellular proteins into the cells (162). This approach will compensate current challenges of peptide inhibitors.

Recent studies have shown that high-throughput screens can effectively identify small molecule inhibitors of specific proteins. With this approach, one of the nonpeptide

STAT3 inhibitor Stattic was identified (163). A fluorescent-labeled pY-containing oligopeptide derived from the IL-6 receptor signaling subunit gp130 was used to screen STAT3 binding factors from 17,000 compounds molecular library. Stattic is reported to inhibit phosphorylation of STAT3 as well as dimerization and nuclear translocation of activated STAT3, although we observed that Stattic is not a specific inhibitor of STAT3 (unpublished observation, Cimica & Reich). By using this high throughput screening, we may identify the specific small molecule inhibitor of STAT5a nuclear import. From our studies, we know STAT5a-GFP shows the nuclear presence, but deletion of 142-149 a.a. in STAT5a shows cytoplasmic localization due to the inhibition of nuclear import. We can use cells that stably expressing STAT5a-GFP as a system to screen the inhibitor of STAT5a nuclear import. Cells expressing STAT5a 142-149 a.a. internal deletion mutant can be used as a negative control. Any compound that inhibits STAT5a import will induce the cytoplasmic localization of STAT5a-GFP expressing cells. Recently, more than 195,000 compounds molecular library was completed by the Scripps Research Institute Molecular Screening Center sponsored by NIH (128). High throughput fluorescent assays are expected to identify inhibitors of STAT5a nuclear import or STAT5a nuclear export to inhibit STAT5a functions in human disease.

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