IUBMB *Life*, 61(7): 697–706, July 2009

Critical Review

Nuclear Localization Signals and Human Disease

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Summary

In eukaryotic cells, the physical separation of the genetic material in the nucleus from the translation and signaling machinery in the cytoplasm by the nuclear envelope creates a requirement for a mechanism through which macromolecules can enter or exit the nucleus as necessary. Nucleocytoplasmic transport involves the specific recognition of cargo molecules by transport receptors in one compartment followed by the physical relocation of that cargo into the other compartment through regulated pores that perforate the nuclear envelope. The recognition of protein cargoes by their transport receptors occurs via amino acid sequences in cargo proteins called nuclear targeting signals. Both nuclear import and export of proteins are highly regulated processes that control, not only what cargo can enter and/or exit the nucleus, but also when in the cell cycle and in what cell type, the cargo can be transported. Deregulation of the nuclear transport of specific cargoes has been linked to numerous cancers and developmental disorders highlighting the importance of understanding the mechanisms underlying nucleocytoplasmic transport and particularly the modulation of the specific interactions between transporter receptors and nuclear targeting signals within target cargo proteins.
IUBMB *Life*, 61(7): 697–706, 2009

Keywords nuclear protein import; nuclear localization signal; karyopherins; nuclear transport receptors; Ran; signaling.

INTRODUCTION

A hallmark of a eukaryotic cell is the physical separation of the genetic material and its associated proteins in the nucleus from the translational machinery located in the cytoplasm. These two compartments are separated by the nuclear envelope, a double membrane that surrounds the nucleus (*1*). The presence of this physical barrier necessitates regulated mechanisms by which nuclear proteins, such as histones, transcription factors, and signaling molecules, can be imported into the nucleus and RNAs, associated proteins, and shuttling proteins can be exported to the cytoplasm. Transport mechanisms involve upwards of 200 evolutionarily conserved cellular factors that not only act as transporters but also form the channel in the nuclear envelope through which all transport occurs (*2, 3*). Proteins to be transported into or out of the nucleus are bound by transport receptors that recognize specific sequences in the cargo protein called nuclear targeting signals. The receptor-cargo complex is then translocated across the nuclear envelope and, following translocation, the complex dissociates resulting in the delivery of the cargo to its appropriate compartment.

Nuclear transport is a highly regulated process with controls that dictate both if and when a cargo can enter and exit the nucleus. Most mechanisms underlying the regulation of transport modulate the interaction of the transport receptors with their cargo proteins. Disrupting this regulation can result in many negative consequences to the cell and potentially to the entire organism. Here, we review the mechanisms of protein import via targeting signals and recent studies that have defined specific defects in the nuclear import of cargo proteins that have been connected to various human diseases.

OVERVIEW OF NUCLEOCYTOPLASMIC TRANSPORT

All macromolecular transport between the nucleus and cytoplasm occurs through large proteinaceous structures that perforate the nuclear envelope called nuclear pore complexes (NPCs) (*4–7*). Structural studies have revealed that the central core of the NPC has an eight-fold rotational symmetry (*7, 8*). Translocation occurs through a central channel and protruding on either side of this channel are the nuclear basket and cytoplasmic filaments, which serve as docking sites for many nucleocytoplasmic transport receptors and other regulatory proteins (*9*). NPCs are composed of more than 30 different proteins called nucleoporins (Nups), which are each present in from 8 to 32 copies per NPC (*9*). Many Nups contain phenylalanine-glycine (FG) repeat sequences. Although the details of translocation through

Received 23 January 2009; accepted 8 February 2009
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ISSN 1521-6543 print/ISSN 1521-6551 online
DOI: 10.1002/iub.194

the NPC have not yet been defined, it is accepted that these FG-Nups line the central channel of the pore and mediate transient interactions with transport receptors to facilitate movement across the nuclear envelope (*10*).

NPCs allow passive diffusion of relatively small molecules (less than 40 kDa) (*11, 12*); however, most cargo proteins undergo active transport to access the nucleus (*13*). A large family of transport receptors, the karyopherin/importin-β (Kapβ) family of receptors, is responsible for selective recognition of the vast majority of cargoes, as well as the physical translocation of the cargoes through NPCs (*14*). There are more than 20 family members in mammals (*15–19*). Although sequence identity between family members is low, Kapβ receptors all assume a similar overall structure consisting largely of helical repeats, called HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast PI3-kinase TOR1) repeats (*20*). Most of the amino acid similarity shared between Kapβ receptors is confined to the N-terminal domain, which is involved in binding to a small GTPase, Ran (*21*). The central domain of Kapβ receptors interacts transiently with the FG-Nups of the NPC to translocate their cargoes into their appropriate cellular compartments (*5, 19, 22*).

The directionality of nuclear transport is imparted through modulation of compartment-specific interactions between transport receptors and their cargoes. Such mechanisms permit tight binding of the transport receptor to the cargo in the compartment where the cargo is picked up, with a subsequent shift to weak binding that facilitates release of the cargo in the destination compartment. The association-dissociation of the receptor-cargo complex is modulated by the molecular switch, Ran GTPase (*23*).

Ran, like all GTPases, cycles between two different states, a GTP-bound state, RanGTP, and a GDP-bound state, RanGDP. Because of the differential localization of Ran regulatory proteins, Ran is primarily in the GTP-bound state in the nucleus and in the GDP-bound state in the cytoplasm (*23*). The compartmentalization of the two forms of Ran ensures proper assembly and release of transport complexes. In the case of nuclear protein import, a Kapβ receptor recognizes and binds cargo in the cytoplasm in the absence of RanGTP (*19*) (Fig. 1). Following translocation into the nucleus, an import complex encounters RanGTP, which binds to the Kapβ receptor causing a decrease in affinity of the receptor for the cargo resulting in release of the cargo protein into the nucleus (*24, 25*). Conversely, export cargoes are recognized as part of an obligate trimeric complex consisting of the export cargo, a Kapβ export receptor, and RanGTP. Following export to the cytoplasm, RanGTP is converted to RanGDP causing dissociation of the export complex and delivery of the cargo (*13*).

Although nuclear import can occur through direct binding of a cargo to a Kapβ receptor, the best-studied system of nuclear import is the classical nuclear protein import system, which involves an adaptor protein, karyopherin/importin-α (*26*). In this system, a specific class of cargo proteins is recognized and bound by importin-α, which complexes with the Kapβ import receptor, importin-β95 (*3*) (Fig. 1, inset). This trimeric-import complex then translocates through the NPC and, once inside the nucleus, importin-β is bound by RanGTP causing dissociation of the importin-α/β complex (*25*).

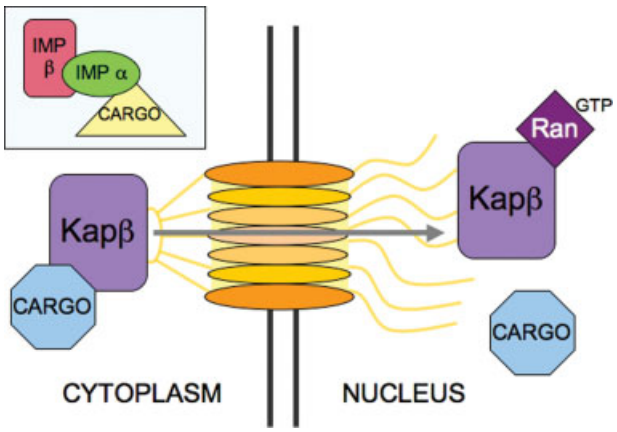

Figure 1. The nuclear protein import pathway. Cargoes destined to enter the nucleus are bound in the cytoplasm by a karyopherin-β (Kapβ) nuclear import receptor. Kapβ transiently interacts with phenylalanine-glycine (FG) repeat-containing Nups in the NPC to translocate bound cargo into the nucleus. Once inside the nucleus, Kapβ is bound by the small GTPase, RanGTP, which results in a decreased affinity of Kapβ for cargo and subsequent delivery of the cargo into the nucleus. Inset: In the case of classical nuclear protein import, cargoes are recognized by an adaptor protein, importin-α, which is then bound by the Kapβ family member, importin-β. This trimeric complex then translocates through the NPC into the nucleus where it is dissociated by RanGTP.

NUCLEAR TARGETING SIGNALS

Recognition of cargoes by receptors depends on intrinsic signals within the amino acid sequence of cargo proteins. Cargoes destined to enter the nucleus contain nuclear localization signals (NLSs), whereas proteins that exit the nucleus contain nuclear export signals (NESs). Transport signals are specifically recognized and bound by transport receptors that translocate cargo to its appropriate compartment. Unlike endoplasmic reticulum- and mitochondrial-bound proteins whose N-terminal targeting signals are often cleaved following arrival at their destination organelle (*27*), nuclear targeting signals remain intact presenting the possibility of multiple rounds of nucleocytoplasmic transport.

In recent years, there has been a growing appreciation for the idea of regulated nuclear import and currently there is an increased interest in identifying novel NLS motifs and the import receptors that recognize and bind them.

Table 1
Examples of defined NLS sequences

		Reference
Classical NLSs		
SV40 large T-antigen	^{126}PKKKRKV^{132}	(*29, 30*)
Nucleoplasmin	^{155}KRPAATKKAGQAKKKK^{169}	(*28, 29, 30, 31*)
SRY	^{59}KRPMNAFIVWSRDQRRK^{75} ^{130}RPRRK^{135}	(*32, 33, 34*)
PY-NLSs		
hnRNP A1	^{270}SSNFGPMKGGNRFFRSSGPY^{289}	(*35*)
Hrp1	^{506}RSGGNHRRNGRGGRGGYNRRNNGYHPY^{532}	(*36*)
Other NLSs		
Nonclassical Imp-α NLSs		
Borna Disease Virus p10	^{5}LRLTLLELVRRLNGNG^{20}	(*37*)
PLSCR1	^{257}GKISKHWTGI^{266}	(*38*)
Ty1 Integrase	^{595}SKKRSLED^{602}...^{625}PPRSKKRI^{632}	(*39–41*)
Importin-β95 NLSs		
HIV-1 Rev	^{35}RQARRNRRRRWR^{56}	(*42, 43*)
HIV-1 Tat	^{48}GRKKRRQRRRAP^{59}	(*44*)
HTLV-1 Rex	^{1}MPKTRRRPRRSQRKRPPT^{18}	(*44*)
Kap121 NLSs		
Ste12	^{606}KSAKISKPLH^{615}...^{644}KNKEISMP^{651}	(*45, 46, 47*)
Pho4	^{144}KVTKNKS^{150}...^{157}KRRGKPGP^{164}	(*48*)
Yap1	^{10} TAKRS^{14}...^{49}KKKGSKTS^{56}	(*49, 50*)

Classical NLS Sequences

The most well studied NLS sequences are the classical NLS (cNLS) motifs (*3*). cNLS cargoes are recognized and bound by the transport receptor adaptor, importin-α. cNLSs can be monopartite or bipartite signals. Monopartite signals consist of a single stretch of basic amino acids comprised primarily of lysine (K) and arginine (R) residues (*28*). The prototypical monopartite NLS is the simian virus 40 (SV40) large T antigen NLS (^{126}PK**K**KRKV^{132}) (*29*) (Table 1). Amino acid substitution at the second lysine (bolded) completely abolishes nuclear import (*29*) highlighting the importance of this residue. Bipartite sequences contain two clusters of basic amino acids separated by a linker region that has been defined as 10–13 nonconserved amino acids (*3*). The archetypal bipartite NLS is found in the *Xenopus laevis* protein, nucleoplasmin (^{155}KRPAATKKAGQAKKKK^{169}) (*29, 31, 51*) (Table 1).

Importin-α is the adaptor protein required for cNLS-cargo nuclear import. Co-crystal structures of both *S. cerevisiae* and *M. musculus* importin-α have been solved in complex with various NLS peptides (*52–59*). The three-dimensional structures reveal that NLS peptides bind specifically in two binding grooves created by flexible armadillo (ARM) motifs in the central domain of importin-α (*54, 55*). The binding pockets are lined with evolutionarily conserved tryptophan and asparagine residues, which are surrounded by acidic, negatively charged amino acids. These grooves interact specifically with the basic, positively charged residues of the cNLS through hydrophobic and electrostatic interactions (*3*). Between these two binding pockets is a linker-binding region that interacts with the NLS peptide backbone (*52, 55, 57*). Changes to specific residues within each of these three regions of importin-α disrupt the nuclear localization and binding of cNLS-cargoes to importin-α (*60, 61*).

Although cNLS motifs likely mediate the majority of nuclear protein import (*36*), it is critical to note that there are many additional transport routes facilitated by nonclassical transport pathways. Hence, there are likely many unidentified and undefined NLS motifs that interact with various transport receptors.

Proline-Tyrosine NLS (PY-NLS) Sequences

Recently, the first new consensus sequence for an NLS to be defined in twenty years was identified (*35*). This new class of NLS motifs is recognized by a Kapβ transport receptor, karyopherin-β2 (Kapβ2)/transportin in humans (*35*). Co-crystal structures of Kapβ2 in complex with an NLS peptide from its best-characterized cargo protein, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), reveal that the negatively-charged NLS binding domain of Kapβ2 interacts with a long (>30 amino acids) structurally disordered basic sequence containing a proline (P) and tyrosine (Y) in hnRNP A1 (Table 1). Chook et al. defined the PY-NLS as a consensus sequence containing a hydrophobic or basic region followed by an arginine (R)/lysine (K)/histidine (H) then a proline and tyrosine (R/K/H-$X_{(2-5)}$-PY) (*35, 62*). Using these guidelines, the human proteome was searched and 81 potential cargoes for Kapβ2 were identified,

some of which they demonstrated interact directly with Kapβ2 *in vitro* (*35*). A follow-up study provided evidence that the PY-NLS motif is conserved in budding yeast in the RNA binding protein, Hrp1 (Table 1), and also that the PY-NLS functions as a nuclear targeting signal *in vivo* (*36*).

Other NLS Motifs

Importin-α recognizes both classical and a growing group of nonclassical NLS sequences. Most of the identified nonclassical NLSs that are recognized by importin-α are hydrophobic in nature and include sequences from the Borna Disease Virus p10 protein (*37*) and the *S. cerevisiae* Phospholipid Scramblase 1 (PLSCR1) (*38*) (Table 1). Although classical bipartite NLSs are defined as two stretches of basic amino acids separated by a 10–13 amino acid linker region, recently the Ty1 integrase protein in *S. cerevisiae,* which contains a nonclassical, long bipartite NLS (*39, 40*) (Table 1), has also been shown to be recognized by classical import machinery (*41*).

Another emerging class of NLSs includes both the cytomegalovirus UL40 protein NLS (*63*) and the human STAT-1 (signal transducers and activators of transciption-1) NLS (*64*) which both contain clusters of basic and hydrophobic residues that do not follow each other in the linear amino acid sequence but are dependent upon importin-α for nuclear import. The UL40 protein folds in such a way to form a domain in the three-dimensional structure of the protein that allows binding to importin-α (*63*). In contrast, STAT-1 forms a homodimer and residues from each monomer contribute to form an NLS that interacts with importin-α (*64*). Both the UL40 and STAT-1 NLS motifs suggest that a number of NLS sequences, and likely other targeting signals, may not always exist as simple linear sequences.

Besides importin-α-interacting cargo, the Kapβ family of import receptors recognizes a wide variety of nuclear targeting signals in various cargo proteins (*16, 65*). Many Kapβ receptors have specific cargoes that they are responsible for transporting; however, either the sequences that are critical for binding to the import receptor are not defined or, when the sequence is defined, no linear amino acid consensus emerges (*19*). The exceptions to this include cargoes that are recognized and bound by either importin-β95 or Kap121. Examples of importin-β95 cargoes include HIV-1 Rev (*42*), HIV-1 Tat (*44*), and HTLV-1 Rex (*66*) (Table 1) which all contain long NLSs typically rich in arginine (R) residues. A subset of cargo bound by Kap121 contain a general bipartite structure comprised of two clusters of nonsequential basic amino acids separated by anywhere from 7 up to about 40 amino acids. Kap121 cargoes include, but are not limited to, the transcription factors, Ste12 (*45–47*), Pho4 (*67*), and Yap1 (*49, 50*) (Table 1). As more cargoes are identified for specific Kapβ receptors, it is likely that additional consensus sequences for new classes of NLS motifs will emerge.

REGULATION OF NUCLEAR PROTEIN IMPORT

Nucleocytoplasmic transport is a highly regulated process involving the intricate interplay of cell signaling molecules, transport receptors, and cargo proteins. Such regulation is critical for the modulation of gene expression through transcription factors, including tumor suppressors and oncoproteins, in response to specific stimuli. Because the rate of nuclear import of a cargo is directly related to the binding affinity of the import receptor for its cargo (*68–70*), the regulation of nuclear transport can be achieved by modulating the binding affinity of the receptor for the NLS-cargo either through physically blocking this interaction or by modification to the NLS region that either promotes or occludes binding by the import receptor.

Deregulation of nuclear protein import can be deleterious resulting in any number of diseases. Global changes to the nuclear import machinery are likely not compatible with life; however, there are at least three ways the interaction between an import receptor and an NLS-cargo has been documented to be disrupted and consequently result in disease: 1) mutation to or altered expression of an import receptor or nucleoporin; 2) an amino acid substitution within a critical residue in the NLS of the cargo itself; and, 3) modification or deregulation of a signaling factor that regulates NLS recognition either by prohibiting or promoting nuclear import. Specific examples of each of these scenarios have been identified and are discussed below.

ALTERATION TO NUCLEAR TRANSPORT MACHINERY

One point of regulation of nuclear transport that has potential to cause disease is the modulation in the expression of specific import receptors and/or Nups. In eukaryotes, certain transport receptors are responsible for the import of specific cargoes. Furthermore, some cargoes, such as β-catenin or the STAT family, take specific paths through the NPC by interacting with only a subset of Nups (*71*). Therefore, the presence or absence of a particular import receptor or Nup may determine whether or how efficiently a specific cargo can enter the nucleus. Such a mechanism allows for tissue- and cell-specific expression of different import receptors and Nups that regulate the transport of specific cargoes only when necessary (*72*). Alterations to this system, or mutation to the import receptors themselves, have obvious potential negative effects on the transport of key cargoes into the nucleus.

Deregulation of the appropriate expression levels of different import receptors has been linked to various diseases as well. Overexpression of both importin-α and Kapβ receptors has been detected in colon, breast, and lung cancers (*73, 74*). Specifically, karyopherin α2 (KPNA2) overexpression is suggested to be a potential prognostic marker for both melanomas and breast cancers (*74, 75*) consistent with increased nuclear import.

Changes to the import receptors themselves can also cause detrimental effects to the cell. There are few known links between mutations to transport machinery and specific diseases. However, a truncated form of Kpna1 isolated in the breast cancer cell line, ZR-75-1, has been linked to a defect in p53 nuclear import (*76*) resulting in constitutive cytoplasmic localization of p53 with repression of genes involved in apoptosis and

increased expression of those involved in cell proliferation (*77–80*). Such a truncation could, therefore, cause cells to divide uncontrollably as occurs in tumorigenesis.

ALTERATION TO THE NLS-CARGO

Nuclear import can be disrupted by changes within the NLS motif itself. Clearly, if the import receptor does not recognize the NLS-cargo, then that cargo will remain in the cytoplasm, which could be deleterious if the nuclear role is critical for proper cell function. A recent example where loss of nuclear localization of a key developmental protein has been linked to disease is in Swyer syndrome where developmental defects include male-to-female sex reversal (*81, 82*).

Mammalian gender is determined by the presence or absence of a dominant gene located on the Y chromosome called SRY (sex-determining region of the Y chromosome) (*83, 84*). SRY is one of many transcription factors required during early development for proper testicular formation in XY males. Mutations in SRY result in male-to-female sex reversal also known as Swyer syndrome. This gonadal dysgenesis results in an XY female with external feminine genitalia but a lack of formation of both ovaries and testes (*85*).

A number of sex-reversing mutations have been documented in the DNA-binding domain of SRY, called the high-mobility group (HMG) box (*86, 87*). Interestingly, two NLS motifs at either end of the SRY HMG box have also been characterized (*32*) (Table 1) and, recently, mutations that impact both of these NLS motifs were identified in four Swyer syndrome females (*88, 89*). In normal individuals, SRY is recognized and bound by its import receptor, importin-β1, which transports SRY to the nucleus to activate testes-specific genes (Fig. 2A). However, the mutations identified in the Swyer syndrome females result in decreased nuclear localization of SRY leading to lower activation of testes-specific genes required for proper XY male testes formation (*81, 82*).

At the moment, Swyer syndrome is the only disease known where changes within the NLS sequence have been linked to a human disease. However, as new classes of NLS motifs are defined, it is likely that the pathology of various diseases will be attributed to the mislocalization of nuclear proteins due to direct changes within the NLSs of key nuclear proteins.

ALTERATION TO THE MASKING OF NLS MOTIFS FROM THEIR IMPORT RECEPTORS

One of the key mechanisms utilized by the cell to regulate the nuclear import of specific cargo proteins involves physically modulating the interaction between an NLS-cargo and its import receptor. This mode of regulation can be achieved either through intermolecular or intramolecular masking of the NLS. In both cases, the NLS of the cargo protein can be masked or blocked, inhibiting interaction with an import receptor. To be imported into the nucleus, the physical block must be removed to allow recognition and binding by the import receptor. Deregulation of these processes can result in constitutive nuclear import or cytoplasmic retention of specific NLS-cargoes, which has obvious implications for the localization of many oncoproteins and tumor suppressors that are subject to extensive regulatory mechanisms including dynamic intracellular localization.

Intermolecular Masking of an NLS

Intermolecular masking of an NLS occurs when an import receptor is unable to bind the NLS of the cargo protein because the NLS in the cargo is masked by a second macromolecule. This masking is regulated by upstream signaling factors that either permit or prohibit the masking of the NLS, which ultimately affects the localization of the cargo protein. The best-characterized example of intermolecular masking mechanisms is the regulation of the nuclear import of the transcription factor, nuclear factor-κB (NF-κB).

NF-κB is a transcription factor that is involved in immune responses, inflammatory responses, and tumorigenesis (*90–92*). The roles that NF-κB plays in cell proliferation and preventing apoptosis, as well as the mechanisms that regulate these activities, are well defined. NF-κB is held in an inactive form in the cytoplasm by an inhibitor protein, inhibitor of κB (IκB), which binds to and masks the NLS of NF-κB preventing its nuclear import (*93–95*) (Fig. 2B). In response to cellular stress, IκB is phosphorylated by the inhibitor of κB kinase (IKK) complex leading to degradation of IκB (*93, 96*). The loss of IκB causes the NLS of NF-κB to be revealed allowing nuclear import via importin-α and subsequent transcription of NF-κB target genes (*95, 97*).

Loss of control of cellular localization of NF-κB can have tumorigenic effects. In fact, deregulation of NF-κB localization has been linked to breast, ovarian, colon, pancreatic, and thyroid cancers, as well as Hodgkin's lymphoma (*73*). In cancerous cells, NF-κB is predominantly localized to the nucleus due to the hyperphosphorylation of IκB, which results in degradation of IκB leading to the unregulated nuclear import of NF-κB (*90*) causing the up-regulation of IκB-dependent anti-apoptotic and pro-cellular proliferation target genes.

Intramolecular Masking of an NLS

Intramolecular masking of an NLS to modulate cargo/import receptor interactions can occur in one of two ways: 1) the addition of a post-translational modification, such as phosphorylation, within or proximal to the NLS of a cargo protein; or 2) the cargo protein can assume an inhibitory conformation that masks the NLS.

NF-ATs (nuclear factor of activated T-cells) were originally identified as a family of four transcription factors that are involved in interleukin-2-mediated T-cell activation (*98*). NF-AT localization is highly regulated and many studies have sought to identify the mechanisms underlying this regulation. Studies of NF-AT2, for example, have defined two NLS motifs

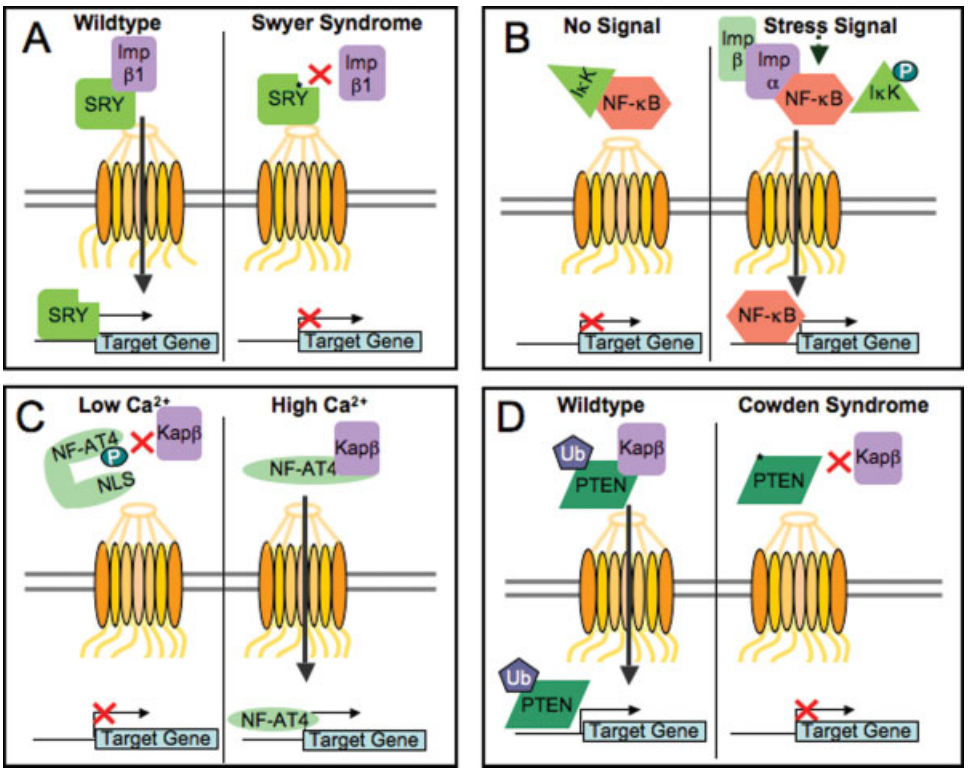

Figure 2. Modes of regulating nuclear import of key transcription factors. See text for details of each pathway. A: Nuclear import of the developmental protein, SRY. During normal development, the NLS of SRY is recognized by Impβ1 and escorted into the nucleus where SRY-dependent genes are upregulated. In the case of Swyer syndrome individuals, the NLS of SRY is mutated resulting in decreased recognition by the import receptor and, consequently, less import of SRY into the nucleus decreasing transcription of target genes. B: Nuclear import of NF-κB. In the absence of an extracellular stimulus, NF-κB is anchored in an inactive form in the cytoplasm through its interaction with IκB. Following an extracellular stress signal, IκB is phosphorylated and degraded, resulting in the unmasking of the NLS of NF-κB, which allows binding by Imp-α/β receptors, translocation of NF-κB into the nucleus, and up-regulation of target genes. C: Nuclear import of NF-AT4. Under conditions where intracellular calcium levels are low, NF-AT4 is phosphorylated at a site distal from the NLS causing the protein to assume a conformation which blocks access to the NLS. In this conformation, NF-AT4 remains in the cytoplasm as the NLS is not available to interact with a Kapβ receptor. When intracellular calcium levels rise, NF-AT4 is dephosphorylated causing a conformational change that reveals the NLS. This change allows binding to a Kapβ receptor followed by nuclear import and subsequent upregulation of target genes. D: Nuclear import of PTEN. Although the exact mechanism is not fully understood, in normal cells, PTEN is ubiquitinated to allow binding of a Kapβ, nuclear import, and transcription of target genes. In Cowden syndrome patients, lysine residues within PTEN are mutated resulting in a loss of ubiquitination and consequent loss of interaction with Kapβ resulting in exclusion of PTEN from the nucleus.

that are phosphorylated to maintain NF-AT2 in its inactive, cytoplasmic form through blocking recognition by an import receptor (*99*). Studies of NF-AT4, however, have identified an NLS that is not directly phosphorylated but instead an upstream phosphorylation domain exists that is key to the regulation of NF-AT4 cellular localization (*100*). When NF-AT is phosphorylated in this upstream domain, the NLS of NF-AT4 folds over and interacts with the phosphate group, which, in turn, masks the NLS from its import receptor (*99, 100*) (Fig. 2C). Mutation within the upstream phosphorylation domain results in constitutive nuclear localization of NF-AT4 leading to subsequent up-regulation of NF-AT4 target genes (*100*).

The localization of NF-ATs is strictly dependent upon the intracellular concentration of calcium. In resting cells, intracellular calcium levels are low resulting in the cytoplasmic retention and hyperphosphorylation of NF-ATs (*99, 101*). Upon T-cell activation, high levels of intracellular calcium cause an increase in the level of the phosphatase, calcineurin. Calcineurin binds to and dephosphorylates NF-ATs resulting in their nuclear import (*101*). In the case of NF-AT2, the NLS motifs are unmasked by removal of the negatively charged phosphate allowing for recognition and binding by an import receptor. Dephosphorylation of the upstream regulatory domain of NF-AT4 results in a conformational change that exposes the NLS

and allows recognition by its corresponding import receptor (Fig. 2C). Association of these NF-AT NLS motifs with their import receptors then allows translocation into the nucleus and up-regulation of T-cell activation target genes.

Although there are no defined diseases specifically linked to the uncontrolled localization of NF-ATs, it is possible that altered localization of NF-ATs does contribute to various diseases as a consequence of upstream regulators.

ALTERATIONS TO COVALENT MODIFICATIONS OF NLS MOTIFS

Unlike intramolecular masking which involves a covalent modification that inhibits nuclear import of cargo proteins in the cytoplasm, covalent modifications, such as ubiquitination, can also facilitate, and are even required in some cases for, the nuclear import of these cargoes. A well-characterized example of such a mechanism occurs with the tumor suppressor, PTEN (phosphatase and TENsin homolog on chromosome 10).

PTEN was originally identified as a tumor suppressor gene (*102, 103*) whose loss of function is linked to many cancers and inherited cancer predisposing syndromes (*102, 104*). The primary function of PTEN is at the plasma membrane where it is involved in the conversion of the lipid second messenger phosphatidylinositol biphosphate3 (PIP_3) to phosphatidylinositol biphosphate2 (PIP_2) (*105*). Although no NLS has been defined in PTEN, it is also found in the nucleus in various cell lines and tissues (*106*). Recently, amino acid substitutions at two lysine residues (K13 and K289) within PTEN have been linked to Cowden syndrome (*107*), an autosomal dominant disease that leads to high susceptibility to various cancers (*108*). Although these PTEN mutants retain catalytic activity, PTEN is excluded from the nucleus in Cowden syndrome patient tissues (*107*). A recent study shows that these lysines are specifically mono-ubiquitinated and that this modification is essential for PTEN nuclear import although the exact mechanism is undefined (Fig. 3D).

CONCLUSIONS

With new NLS motifs and nuclear import pathways being increasingly defined, defects in cargo nuclear targeting and localization are likely to be revealed as a basis for human disease. Furthermore, as we understand the mechanisms of nuclear import better, we may be able to target nuclear import as a therapeutic approach and also design new or more effective therapies for human diseases.

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