CHARACTERIZATION OF A NOVEL NUCLEAR IMPORT MECHANISM BY NUCLEAR FACTOR OF ACTIVATED T-CELLS 5 (NFAT5)

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A thesis submitted in partial fulfilment of the requirements for

the degree of Master of Philosophy

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Certificate of originality

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December 2017

Abstract of thesis titled

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Abstract

Nuclear factor of activated T-cells (NFAT5), also known as osmotic response element-binding protein (OREBP) and Tonicity-responsive binding-protein (TonEBP), is a Rel-homology domain containing nuclear factor belongs to the NFAT family transcription factor. NFAT5 is a tonicity-regulated transcription factor, which orchestrates a genetic program that promotes the expression of heat shock proteins and transporters for organic osmolytes for the restoration of cellular homeostasis under hypertonic stress. Among other functions, NFAT5 is best known for its role in the protection of cells in the kidney inner medulla, which are constantly exposed to hypertonic stress due to urine-concentration mechanism. Under hypertonic stress, NFAT5 undergoes nuclear translocation, binds to its cognate DNA enhancer known as the osmotic response element (ORE) of its target genes, and induces gene transcriptions. Nuclear import and export of NFAT5 is essential for its activation and inactivation respectively. Nevertheless, the molecular mechanisms underlying NFAT5 nucleocytoplasmic trafficking remain elusive. Earlier findings suggested that nuclear import of NFAT5 is mediated by a putative novel nuclear import signal (NLS) composed of more than 60 amino acid residues, and requires importin-β as the only nuclear receptor for nuclear import. The aim of my study is to further understand the activities and function of the novel NLS, and to characterize the interaction between the NLS and importin-β. By preparing truncation mutants of the putative NLS and importin-β using bacterial overexpression and purification, I have characterized the interacting domain of the novel NLS with importin-β. Furthermore, using *in vitro* nuclear import assay, I have defined the sequence requirements and the factors required for nuclear import of NFAT5 for the first time.

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Chapter 1

Introduction

1.1 Cellular adaptation to changes in extracellular tonicity

Maintaining cellular water homeostasis is an important aspect for all cells to survive and thrive. In mammalian cells, a disturbance in water homeostasis will occur when cells are exposed to hypertonic or hypotonic environment, which leads to water efflux and water influx from the cells respectively. Either event will lead to cell death if it was allowed to proceed in an uncontrolled manner. Therefore, cells have developed a genetic program of water homeostasis that involves the activation of ion transporters such as sodium-potassium-chloride transporter and sodium-hydrogen exchanger (Bobulescu & Moe, 2006). These transporters are selectively activated by either cell swelling or shrinkage as a result to avoid excessive alternations in cell volume. In case of hypertonicity, activation of ion transporters results in intracellular accumulation of inorganic electrolytes to equalize increase in extracellular tonicity. However, since inorganic electrolytes are charged, long-term accumulation of electrolytes is deleterious to cell functions in long term. This is because increased charge density will inactivate enzymes, disturb membrane potential, denature proteins, and damage DNAs (Kültz & Chakravarty, 2001; Michea et al., 2000). Consequently, cells undergo a long term osmoadaptation process which inorganic osmolytes will be replaced by organic osmolytes (Burg & Ferraris, 2008). Organic osmolytes are composed of amino acids, sugars, methylamines and polyols (Yancey, 2005). Major organic osmolytes utilized by cells are sorbitol, myo-inositols, betaine and taurine respectively. Unlike inorganic electrolytes, organic osmolytes are neutral in charge. Therefore, long-term accumulation of organic osmolytes will not lead to a disturbance in cellular functions. Under hypertonicity, organic osmolytes are

accumulated inside cells by either intracellular synthesis or active transport. For example, sorbitol is synthesized from glucose by the enzyme aldose reductase (Garcia-Perez & Burg, 1991), whereas myo-inositol, betaine and taurine are transported into cells via their respective transporters (Peters-Regehr, Bode, Kubitz, & Häussinger, 1999). Under hypertonicity, the mRNA levels of these genes increases (Fontes, Teh, & Kobe, 2000; Ko, Ruepp, Bohren, Gabbay, & Chung, 1997; López-Rodríguez et al., 2004), followed by increased production of the enzymes and transporters. The concerted action of these proteins gradually leads to the accumulation of organic osmolytes (Takashi et al., 2004; Uchida, Yamauchi, Preston, Kwon, & Handler, 1993; Umenishi & Schrier, 2002). In the 5' regulatory region of these genes there is a specific enhancer known as the osmotic response element (ORE) (Ko et al., 1997), or the tonicity responsive element (TonE) (Miyakawa, Woo, Dahl, Handler, & Kwon, 1999), which is a potent enhancer that upregulates the transcriptional activity of these genes under hypertonic stress (Ferraris & García-Pérez, 2001; Fontes et al., 2000; Ko, Turck, Lee, Yang, & Chung, 2000).

1.2 NFAT5 is an tonicity-sensitive mammalian transcription factor

Nuclear factor of activated T cells-5 (NFAT5), also known as Tonicity-responsive binding-protein (TonEBP) or Osmotic Response Element Binding Protein (OREBP), is a Rel-homology domain containing protein and is a distant family member of the NFκB (Miyakawa et al., 1999). NFAT5 was identified as the cognate transcription factor the ORE or TonE by affinity purification (Ko et al., 2000) and yeast-1-hybrid screening (Miyakawa et al., 1999) respectively. On the other hand, NFAT5 was identified as a family member of the NFAT family by homology cloning (López-Rodríguez, Aramburu, Rakeman, & Rao, 1999). NFAT5 is a homodimer protein

(López-Rodríguez et al., 2001) and is ubiquitously expressed (López-Rodríguez et al., 1999). The first identified function of NFAT5 is to protect cells from hypertonic stress. In response to hypertonicity, NFAT5 undergoes nuclear translocation (Miyakawa et al., 1999), binds to the ORE/TonE, and mediates the transcription of genes involved in osmoadaptation (Miyakawa et al., 1999). Concordantly, NFAT5 is thought to protect cells in the kidney inner medulla, where cells are subjected to extreme hypertonicity due to urine concentration mechanisms. Interestingly, evidence suggested that hypertonicity also induces the expression of cytokine genes, including TNFα and lymphotoxin-β, via NFAT5 (López-Rodríguez et al., 2001), suggesting that NFAT5 may play other functional roles besides osmoadaptation.

1.3 Novel functional roles of NFAT5

Since mammals are well protected from hypertonic stress due to urine concentration mechanism, most of the anatomical sites except kidney inner medulla are maintained at near isotonicity by the surrounding blood and tissue fluids. Earlier studies did not provide clue regarding why NFAT5 is ubiquitously expressed. Nevertheless, emerging evidences suggested that NFAT5 is playing a broader role in physiology and pathology. Recent studies suggested that NFAT5 is regulated by extracellular signals besides hypertonicity. This is evidenced by the fact that in breast tumor cells, NFAT5 expression and activation were induced by integrin activation (Jauliac et al., 2002); Furthermore, NFAT5 has also been shown to regulate HIV-1 replications in monocytes (Ranjbar et al., 2006). More recent evidence suggested that hypertonicity is also present in tissue microenvironment and impact on the NFAT5-regulated functions. Accordingly, NFAT5 was shown to involve in dehydration-induced natriuresis (S. Chen et al., 2009), in autoimmune disease via the regulation of TH17

cells (Kleinewietfeld et al., 2013), as well as in the control of blood pressure via regulating macrophages activities (Machnik et al., 2009; Wiig et al., 2013). Together, these data suggested that NFAT5 is playing a much boarder functional role in physiology and diseases.

1.4 Structural organization of NFAT5

NFAT5 gene is 14-kb long and it consists of 16 exons (Dalski, Schwinger, & Zühlke, 2001). Four human NFAT5 isoforms have been identified, named NFAT5-a to d (Dalski et al., 2001). They were the splice variants of the same gene. The start site of isoform a, b as well as c is at exon 5, 3 and 1 respectively. Isoform d encodes the longest protein, which includes extra 18 amino acid residues from exon 2. The DNA-binding domain of NFAT5 exhibits significant homology to Rel-homology domain (RHD, and therefore, NFAT5 belongs to Rel-family of transcription factors. Members in this family include NFAT1-4, as well as the nuclear factor-kappa B (NF-κB). However, outside the DNA-binding domain, NFAT5 does not show sequence homology to other NFATs. Different from NFAT1 to 4, NFAT5 does not contain calcineurin-binding domain. Furthermore, other NFATs from complexes with Fos and Jun, whereas NFAT5 does not (Graef, Gastier, Francke, & Crabtree, 2001; López-Rodríguez et al., 1999). Therefore, NFAT5 is regarded as a distant member of the Rel protein family (Fig. 1.1). Besides RHD, NFAT5 consists of several structural domains, including a canonical nuclear export signal (NES, amino acid residues 1-19), a putative classical nuclear localization signal (cNLS, amino acid residues 199-216), auxiliary export domain (AED, amino acid residues 132-159), and a Rel-homology domain (RHD, amino acid residues 264-543). Within RHD there is a dimerization domain (DD, amino acid residues 370-433). In addition, NFAT5 also

contains three transactivation domains (AD1, amino acid residues 1-76; AD2, amino acid residues 1039-1249; and AD3, amino acid residues 1363-1476) (Fig. 1.2) (Do Lee, Colla, Sheen, Na, & Kwon, 2003; López-Rodríguez et al., 2001; Tong et al., 2006)

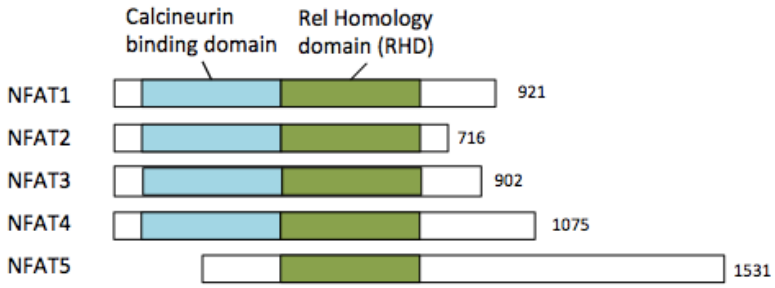


Figure 1.1 Structural differences among NFAT1-5

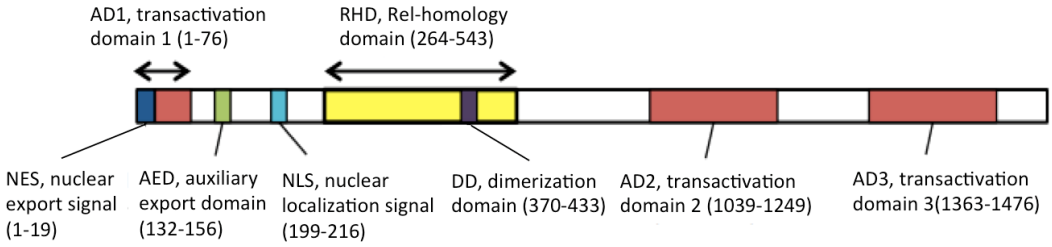


Figure 1.2 Structure of NFAT5

1.5 Regulation of NFAT5 activity by changes in tonicity

Activity of NFAT5 is regulated in response to changes in extracellular tonicities. NFAT5 activity is regulated in several levels including nuclear abundance, transcriptional activity, and rate of synthesis.

1.5.1 Regulation of nuclear abundance

NFAT5 undergoes continuous nucleocytoplasmic trafficking when cells are under isotonic condition (Tong et al., 2006). Therefore, under isotonic condition NFAT5 can be found in both nucleus and cytoplasm (Miyakawa et al., 1999). The canonical NES and cNLS regulate nuclear export and import of NFAT5 respectively under isotonic condition. Nucleocytoplasmic trafficking NFAT5 will be altered in response to change in extracellular tonicities. Under hypertonicity, NFAT5 undergoes a net nuclear import, resulting in accumulation of NFAT5 in nucleus. Whereas under hypotonicity, NFAT5 is subjected to nuclear export, resulting in reduced accumulation in the nucleus. (Bayliss, Littlewood, & Stewart, 2000; Fontes et al., 2000; Tong et al., 2006; Woo, Dahl, Handler, & Kwon, 2000). Unlike nuclear export under isotonic condition, which is dependent on the activity of the canonical NES, hypotonicity induced export of NFAT5 is regulated by the AED. The putative cNLS of NFAT5 is consisted of two clusters of basic amino acids, which is highly structurally similar to the canonical bipartite NLS (Dingwall & Laskey, 1991; Robbins, Dilwortht, Laskey, & Dingwall, 1991). However, functional analysis suggested that only the first cluster (amino acid 202-204) of the basic amino acids is critical to nuclear import of NFAT5. Site-directed mutagenesis of these three amino acid residues completely abolishes nuclear import of NFAT5 under both isotonicity and

hypertonicity. Hence, these evidences suggested that the cNLS of NFAT5 is functionally a monopartite NLS similar to that found in SV40 antigen (Tong et al., 2006). On the other hand, AED, which is critical to nuclear export activity under hypotonicity, does not show sequence homology to other proteins. However, AED alone could not direct nuclear export of heterologous protein (Tong et al., 2006), suggesting it is not a functional nuclear export signal per se, but requires collaboration with other sequences for nuclear export activity. On the other hand, post-translational modification of amino acid residues is important for nucleocytolasmic trafficking of NFAT5. In particular, amino acid residue Ser155 and Ser158 were sequentially phosphorylated and are critical for NFAT5 nuclear export under hypotonic condition (Xu et al., 2008). Besides, phosphorylation of Thr135 and Tyr143 by cyclin-dependent kinase 5 (CDK5) and c-Abl respectively contribute to nuclear import of NFAT5 under hypertonicity (Gallazzini et al., 2011; Gallazzini, Yu, Gunaratne, Burg, & Ferraris, 2010). Also, SHP-1 phosphatase is inhibited by hypertonic stress, resulting in the reduced dephosphorylation of Tyr143 of NFAT5 (Zhou, Gallazzini, Burg, & Ferraris, 2010). However, the mechanisms of how hypertonicity and hypotonicity stimulate these protein kinases and phosphatase are still unclear. On the other hand, evidence suggested that phospholipase C-γ1 (PLC-γ1) is participated in nuclear import of NFAT5 by interacting with phosphorylated Thr143 (Irarrazabal et al., 2010).

An alternative regulatory mechanism of NFAT5 nucleocytoplasmic trafficking has been suggested by a recent study. It is found that NFAT isoform a, the shortest isoform without NES, is able to anchor to plasma membrane through

myristoylation and palmitoylation at the Gly2 and Cys5 residue of NFAT isoform a at resting stage, but undergoes nuclear import under hypertonicity (Eisenhaber et al., 2011). The mechanism of how NFAT isoform a could detach from plasma membrane under hypertonic condition remains to be elucidated.

In addition to active nuclear import, nuclear abundance of NFAT5 under hypertonicity could be promoted by nuclear retention. A component of nuclear pore complex (NPC) named Nucleoporin 88 (Nup88) is involved in NFAT5 activity regulation through retaining NFAT5 in nucleus in IMCD3 cells (Andres-Hernando, Lanaspa, Rivard, & Berl, 2008). However, Nup88 does not exert its regulatory role on NFAT5 in HEK293 cells. This probably implies that the effect of Nup88 on NFAT5 activity is cell-type specific (Izumi et al., 2012). In addition, Izumi et al. (2012) show that NFAT5 nuclear import is reduced when DNA contact sites on NFAT5 is mutated, indicating that a disruption of DNA-transcription factor interaction may alter NFAT5 nuclear retention.

1.5.2 Regulation of transactivation activity

NFAT5 is consisted of three putative transactivation domains, all of them contribute to NFAT5 activity. Although each of the putative transactivation domain is able to enhance gene transcription activity of a heterologous DNA-binding domain, only one of them, transactivation domain (AD2), is involved in tonicity-dependent NFAT5 regulation, (Do Lee et al., 2003). The transactivation domains are phosphorylated constitutively under hypotonic condition. Ferraris, Williams, et al. (2002) suggested that that hypertonicity boosts phosphorylation of transactivation domain at C-terminal (TAD, amino acid 548-1531) composed of AD2 and AD3. On the other hand, Izumi et al. (2012) suggested that phosphorylation of transactivation domain including AD2 is not directly related

to changes in extracellular tonicity. Together these findings indicate that the phosphorylation level of NFAT5 at transactivation domains may play role in regulation of NFAT5 activity, but the underlying mechanisms as well as the exact phosphorylated site are still being investigated.

Numerous signaling molecules are implicated in the regulation of NFAT5 transactivation activity. These include ataxia telangiectasia mutated (ATM) kinase (Irarrazabal, Liu, Burg, & Ferraris, 2004), protein kinase A (PKA) (Ferraris, Persaud, Williams, Chen, & Burg, 2002) and heat shock protein 90 (HSP90) (Y. Chen et al., 2007) . In addition, Fyn and p38 (Ko et al., 2002) and phosphatidylinositol 3-kinase (PI3K) (Irarrazabal, Burg, Ward, & Ferraris, 2006) possibly regulates more upstream signaling cascade. The Fyn- as well as p38-signaling cascades probably play a critical role in NFAT5 activation because inhibition of these two proteins simultaneously abolished NFAT5 activation. The mechanism of how NFAT5 activity is regulated by different kinases remains to be explored.

1.6 Transportation across nuclear membrane

Small molecules such as sugars, ions as well as water molecules, transport across nuclear membrane by simple diffusion via nuclear pore complex (NPC). However, macromolecules that are larger than 40 kDa, such as proteins and RNAs, could only shuttle between cytoplasm and nucleus by active transport through the NPC (Allen, Cronshaw, Bagley, Kiseleva, & Goldberg, 2000). NPC is octagonal symmetrical and it is composed of nearly 30 distinct nucleophorins (Rout et al., 2000). It connects nucleus to the cytoplasm by allowing passage of macromolecules. Vertebrate NPC contains a central scaffold with diameter around 125 nm and height around 70 nm

spanning the nuclear envelope. On the cytoplasmic face of NPC there are eight filaments extend into cytoplasm. The nuclear face of NPC also consists of eight filaments. These filaments join at their end to create a basket-like structure. (Fahrenkrog & Aebi, 2003) In most cases, the transport of macromolecules across NPC requires the presence of nuclear transporters belong to the karyopherin family (Allen et al., 2000), which contains importin-α and importin-β proteins respectively. Most of the nucleoporins are characterized by the presence of phenylalanine-glycine (FG) repeats. FG repeats have been shown to interact directly with karyopherins. Such interaction is critical for nuclear import of molecules through NPC (Cronshaw, Krutchinsky, Zhang, Chait, & Matunis, 2002). Nuclear transporters shuttle between nucleus and cytoplasm through an import and export cycle (Fig. 1.3). Prior to nuclear import, macromolecules (cargos) are required to form complex with importin(s) through their nuclear localization signal (NLS). Subsequently, the importin-cargo complex will be transported through the nuclear pore (Melchior & Gerace, 1998). There are different types of NLSs. NLS can be short amino acid sequences such as the canonical NLS (cNLS), including the monopartite NLS found in SV40 T-antigen (Fontes et al., 2000) and the bipartite NLS found in nucleoplasmin (Kosugi et al., 2009), or can be composed of non-canonical large protein domain, such as the PY-NLS found in the FUS and hnRNP proteins (Zhang & Chook, 2012). Most of the cargos bearing non-canonical NLS complexed with members of the importin-β family directly and undergo nuclear import, whereas cargos bearing cNLS require the formation of heterotrimeric complex containing importin-α and importin-β for nuclear import (Melchior & Gerace, 1998). Under all circumstances, importin-β interacts with FG repeats of nucleoporins to mediate nuclear import. There is also a high concentration of RanGTP in the nucleus. RanGTP binds to importin-β, resulting

in conformational change and subsequent detachment of protein cargo. The RanGTP-bound importin-α leaves nucleus through via NPC by interacting with the CAS protein. In such way, importin-β can be returned to the cytoplasm to start another import cycle. In export cycle, exportins such as CRM1 binds to protein cargo as well as RanGTP and is exported back to the cytoplasm through NPC. There are also proteins that undergo nuclear import in the absence of karyopherins. For examples, nuclear import of Hsp70-ATP is mediated by a protein known as Hikeshi (Kose, Furuta, & Imamoto, 2012). Besides, nuclear import of certain ankyrin repeat proteins is mediated by RanGDP (Lu et al., 2014), whereas nuclear import of RanGTP is mediated by NTF2 (Ribbeck, Lipowsky, Kent, Stewart, & Görlich, 1998). Furthermore, β-catenin, a protein in the Wnt/β-Catenin pathway, undergo nuclear transport by direct nucleoporin binding (Fagotto, Glück, & Gumbiner, 1998).

1.7 Importin α and importin β family of proteins

There are three subfamilies in the importin α family, namely α1, α2 and α3 subfamily. The α1 subfamily contains importin α1, α2 and α8. The α2 subfamily is consisted of importin α3 and α4, whereas the α3 subfamily is consisted of importin α5, α6 and α7 respectively. These importin αs participate in nuclear import of different types of cargos. Importin α1 is found to interact with classical NLS, importin α2 can bind various types of NLS. Members from α2 subfamily specifically contribute to nuclear import of nuclear factor–kappa B (NF-κB) as well as Ran guanine nucleotide exchange factor (RanGEF) (Fagerlund, Kinnunen, Köhler, Julkunen, & Melén, 2005; Köhler et al., 1999). One of the well-known protein cargo of the α3 subfamily is phosphorylated signal transducer and activator of transcription 1 (STAT1) transcription factor (Fagerlund, Melen, Kinnunen, & Julkunen, 2002).

In mammals, there are over 20 members in importin β family. Different importin β

utilizes different nucleoporins, implying that unique transport pathways via NPC require specific importin βs (Suprapto, 2000). Importin β has the conserved multiple tandems helical repeats called HEAT repeats. They use this HEAT repeats in different ways. For example, importin β1 consists of 19 HEAT repeats, forming a compact super helix wraps around the N-terminal IBB domain of importin α. While 20 HEAT repeats of RanGTP bound importin β2 are organized into two contiguous arches. This arche structure of importin β2 make importin β2 more 'open' to its substrate (Chook & Blobel, 2001).

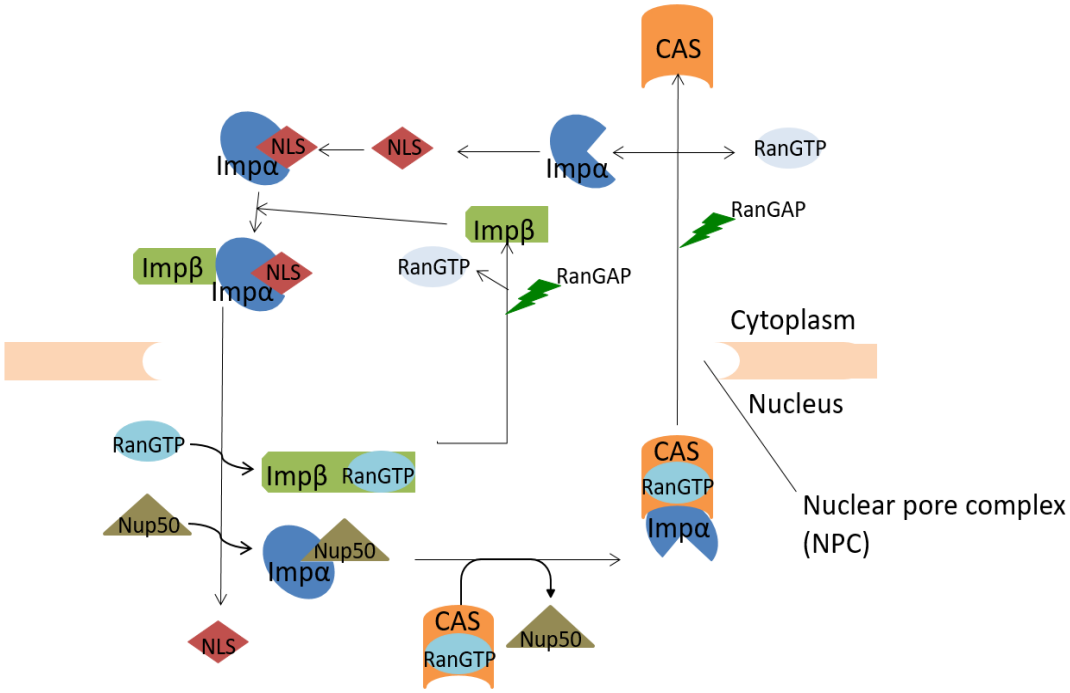


Figure 1.3 Nuclear import and export cycle of NLS-containing cargos

1.8 RanGTP gradient provides driving force of nuclear import and export

The directionality of trafficking of molecules is guided by the gradient of RanGTP down nucleus to cytoplasm. Ran is a Ras-like guanine nucleotide triphosphatase (GTPase) that is predominantly found in nucleus but it can diffuse freely across the nuclear envelope. Besides its involvement in nuclear transport, it is also found to contribute to post-mitotic nuclear assembly as well as mitotic spindle assembly (Joseph, 2006). Its activity depends on whether it is GDP- or GTP-bound. These two forms of Ran could be generated by two antagonistic proteins, Ran guanine nucleotide exchange factor (RanGEF) and GTPase activating protein (RanGAP) (Bischoff, Klebe, Kretschmer, Wittinghofer, & Ponstingl, 1994; Bischoff & Ponsting1, 1991). These two proteins locate in nucleus and cytoplasm respectively. RanGEF is also known as regulator of chromosome condensation 1 (RCC1). It is a chromatin-associated protein and the only known protein that is capable of guanine exchange in mammals (Yamada, Tachibana, Imamoto, & Yoneda, 1998). It catalyzes the exchange of GDP to GTP on Ran in nucleus. RanGTP formed then binds to importin β1 (Lounsbury & Macara, 1997; Raices & D'angelo, 2012), resulting in movements of the fifth and sixth HEAT repeats of importin β1. These rearrangement leads to the loss of FG repeat binding site on importin β1, causing the dissociation of importin β1 and nucleoporins (Bayliss et al., 2000) and dissociation of protein cargos and importins. Subsequently, the RanGTP-importin β heterodimer goes from nucleus to cytoplasm through NPC. For importin α, nuclear export is dependent to exportin CAS and RanGTP. Furthermore, RanGTP is involved in nuclear export of protein cargos in nuclear export signal (NES)-dependent manner (Richards, Carey, & Macara, 1997). In cytoplasm, RanGAP interacts with RanGTP, triggering the guanine exchange activity of Ran. As a result, RanGTP is converted to RanGDP. RanGAP is a

symmetric protein with an arginine finger. Arg74 is critical to insertion to Ran at nucleotide binding pocket. This helps to stabilize transition state during nucleotide exchange process (Ahmadian, Stege, Scheffzek, & Wittinghofer, 1997). Mammalian RanGAP is cytoplasmic. It exists in solution as dimer, which this dimeric protein is too large to pass via NPC by passive diffusion (Macara, 2001). Thus, it could only exert its effect on Ran in cytoplasm. RanGDP formed could be transported into nucleus by a cytosolic protein called nuclear transport factor 2 (NTF2). NTF2 is also called Ran-inactivating protein (Izaurralde, Kutay, von Kobbe, Mattaj, & Görlich, 1997). It only recognizes GDP-bound Ran. In addition, NTF2 appears as homodimer and each NTF2 binds one RanGDP. As a result, two RanGDPs are shuttled into nucleus per each transport cycle. Also, NTF2 is able to bind both RanGDP and FG repeats on nucleoporins at the same time (Stewart, Kent, & McCoy, 1998). Thus, RanGDP is docked at nucleoporins by NTF2, resulting in the consequent nuclear import of RanGDP through NPC.

1.9 Objectives of my project

Earlier findings suggested that NFAT5 nuclear import is mediated by a putative cNLS (a.a. 199-216) ($_{199}$-RKSRKRNPKQRPGVKRRD-$_{216}$) (Tong et al., 2006), which was identified by bioinformatics analysis. This is supported by the observation that alanine substitution of $_{202}$RKR$_{204}$, which are the core sequence of cNLS in other cargos, completely abolished nuclear import of recombinant NFAT5 under isotonic and hypertonic conditions (Tong et al., 2006). Despite that the whole putative cNLS sequence showed significantly homology to a bipartite nuclear localization signal, mutation of the second basic region of the signal, $_{213}$KRR$_{215}$, did not alter nuclear import activity (Tong et al., 2006). Therefore, it was concluded that the cNLS of

NFAT5 acts as a monopartite NLS with regard to activity.

Besides the identification of three critical amino acid residues in the putative cNLS, the mechanism underlying NFAT5 nuclear import has remained elusive. Whether the cNLS alone is sufficient for directing NFAT5 nuclear import has not been demonstrated. Furthermore, the nuclear import receptor(s) responsible for the process has not been elucidated. Therefore, the aim of my project is to clarify these unaddressed questions regarding the molecular mechanisms of NFAT5 nuclear import.

There are two objectives in my project:

1) To determine the minimal functional NLS of NFAT5 that could direct the nuclear import of cargos.
2) To characterize nuclear factors and receptor(s) responsible for NFAT5 nuclear import.

Chapter 2

Methodology

2.1 Cell culture

HeLa cells (American Type Culture Collection, Manassas, VA) were culture in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum and 10 U/mL Penicillin-Streptomycin.

2.2 NFAT5 subcellular localization studies and immunofluorescence assay

HeLa cells grown in 6-well plates to 80% confluence were transfected with 1ug of the plasmids expressing the proteins as indicated in each experiment using Lipofectamine (Invitrogen) according to the manufacturers' instructions. Transfected cells were incubated for 16 hours in complete growth medium before switching to medium with different osmolality. Hypotonic (250 mosmol/kgH2O), isotonic (300 mosmol/kg H2O), or hypertonic (450 mosmol/kg H2O) medium were prepared by supplementing NaCl to NaCl-deficient growth medium (minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2mM L-glutamine and 1X Non-Essential Amino Acid) to the desired osmolality. For immunofluorescence assay, cells were either maintained in isotonic medium, or treated with hypotonic or hypertonic medium for 30 and 120 minutes respectively. Cells were then fixed with w/v 4% paraformaldehyde on ice for 15 minutes, followed by incubation with 100% methanol at room temperature for 10 minutes. The cells were then incubated with blocking buffer (1% BSA, 22.5mg/ml glycine, 0.1% Tween 20 in PBS) at room temperature for 1 hour. Rabbit anti-FLAG antibodies (Cell signaling technology)

were added into incubation buffer (3% BSA in PBS) at a dilution 1:800, and incubated overnight at 4°C. After washing the slides three times with cold PBS, goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 (Invitrogen) was added into incubation with dilution 1:500 respectively. After 1 hour, nuclei of cells were stained by 4, 6-diamidino-2-phenylindole (DAPI). Cell images were obtained using Leica TCS SP8 confocal microscope.

2.3 Subcellular fractionation analysis

Control siRNA or Importin-β1 siRNA transfected HeLa cells were pretreated with either isotonic or hypertonic medium prior to harvest. Cells were gently washed with PBS for three times and the cells were pelleted by centrifugation at 1000 rpm and room temperature. The cell pellets were resuspended in 5 pellet-volume of Cytoplasmic Extract (CE) Buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, 1mM DTT, 1 mM PMSF, adjusted to pH 7.6) and followed by incubation of ice for 3 minutes. After centrifugation at 1000rpm for 4 min at 4°C, the supernatant with cytoplasmic proteins were kept in a new enppendorf. The pellets with nuclei were resuspended gently with 5 pellet-volume of NP40-free CE Buffer. The nuclei were spun down at 1000rpm for 4 min at 4°C and resuspended with 1 pellet-volume of Nuclear Extract (NE) Buffer (20 mM Tris Cl, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM PMSF, 25% (v/v) glycerol, adjusted to pH 8.0) by vortexing. The salt concentration of the resuspended mixture was adjusted to 400mM. 1 extra pellet-volume of NE buffer was added. The extract was incubated on ice for 10 minutes and vortexed every 2 minutes. Both cytoplasmic and nuclear extract were centrifuged at 13800rpm, 4°C for 10 minutes. The supernatant collected contains cytoplasmic

and nuclear proteins respectively. These extracts were then analyzed by SDS-PAGE and subsequent western blot analysis.

2.4 Co-immunoprecipitation (Co-IP) of NFAT5 and importin β1

FLAG-OREBP1-581Δ1-131 was transfected into HeLa cells using Lipofectamine (Invitrogen) according to the manufacturers' instructions. 24 hours after transfection, the cells were lyzed with lysis buffer (50 mM Tris-HCl pH7.8, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100), followed by centrifugation at 13800rpm, 4°C for 10 minutes. The extracted proteins were in the supernatant. The extracted proteins were incubated with anti-FLAG beads at 4°C, overnight with shaking. Then the protein-bead mixture was washed 3 times with 1X TBS and the beads were boiled for 10 minutes to elute the captured proteins. The eluate was subjected to SDS-PAGE and analyzed by subsequent Western blot.

2.5 Recombinant plasmid construction

6 His no 3C-pETM was a gift from Dr. Zhao Yanxiang. For GST-tagged proteins, pGEX-4T-1 was used. Vector and PCR products of truncated forms of $NFAT5_{171-250}$, importin-β1 and AcGFP were digested by restriction enzyme at 37°C for 1 hour, followed by gel extraction using QIAEX II Gel Extraction Kit (Qiagen). Restriction-digestion products of vector and inserts were ligated in molar ratio 3:1 in the presence of ligase (NEB) at room temperature for 30 minutes. Ligation products were transformed into DH10B. Single colonies were picked and grown in LB broth with 100ug/mL ampicillin (Affymetrix) overnight at 37°C with shaking. The overnight bacterial cultures were subjected to DNA extraction using

PureLink Quick Plasmid Miniprep Kit (Invitrogen).

2.6 Protein overexpression

Extracted recombinant plasmids were transformed into BL21 (DE3). Single colonies were picked and grown in 500mL LB broth containing 100ug/mL ampicillin at 37°C with shaking for overnight. Isopropyl β-D-1-thiogalactopyranoside (IPTG; Affymetrix) was added into overnight bacterial culture to final concentration 0.5mM for induction of protein overexpression. 6 hours after induction, bacterial culture was harvested by centrifugation at 6,000rpm for 10 minutes. The pellet was resuspended in cold PBS with 10ug/mL lysozyme. The resuspended mixture was sonicated on ice, 10s on and 10s off, for 3 cycles, followed by addition of Triton-X100 (Affymetrix) to final concentration 1%. The mixture was incubated on ice for 30 minutes and then centrifuged at 18,000rpm at 4°C for one and a half hour. The supernatant was filtered using 0.22um filter. The filtered supernatant was subjected to affinity column.

2.7 Protein purification

For His-tagged proteins, HisTrap HP column (GE Healthcare) was used. HisTrap HP column was first washed by five-column volume (CV) of Mili-Q water, followed by equilibration with five CV of his binding buffer (20mM sodium phosphate, 500mM sodium chloride, 40mM imidazole, 10% glycerol). After injection of filtered supernatant from previous step, the column was washed with at least five CV of his binding buffer. Proteins were eluted using his elution buffer (20mM sodium phosphate, 0.5 sodium chloride, and 500mM imidazole). For GST fusion proteins, GSTrap 4B (GE Healthcare) was used. The GST binding

buffer was phosphate buffer saline (140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate bibasic, 1.8 mM Potassium phosphate monobasic, pH 7.4) (VWR Life Science) and GST elution buffer was made of 50 mM Tris-HCl, 10mM reduced glutathione, pH 8.0. The GST-fusion protein purification procedure was as same as that of his-tagged protein. His-importin-β1 was subjected to gel filtration using Superdex 200, 10/300 GL (GE Healthcare) after being eluted from HisTrap column. All proteins were concentrated using Amicon (Millipore).

2.8 Cytosol extraction

HeLa cells were grown in 175cm^2 tissue culture flasks. When cell density reached 80% confluence, cells were harvested by centrifugation at 1,500rpm for 5 minutes. For hypertonic and hypotonic cytosol extraction, cells were pretreated with hypertonic and hypotonic medium for 30 minutes respectively. Cell pellet was washed with washing buffer (10 mM HEPES, pH 7.3, 110 mM potassium acetate, 2mM Magnesium acetate, and 2 mM DTT), followed by centrifugation at 1,500 rpm for 5 minutes. Pellet was resuspended in equal volume of cold hypotonic lysis buffer (5 mM HEPES, pH 7.3, 10mM potassium acetate, 2 mM DTT, 1 mM PMSF, protease inhibitor) and incubated on ice for 10 minutes. 1.2uL of 10% Digitonin was added and the mixture was incubated on ice for further 5 minutes. Trypan blue was used to check if 90-95% of cells were permeabilized. The mixture was centrifuged at 1,500rpm for 5 minutes at 4°C. Supernatant was subjected to centrifugation at 4°C, 14,000rpm for 1 hour. Supernatant was kept as it contained the cytosol. The extracted cytosol was dialyzed against transport buffer (20 mM Hepes, pH7.3, 110 mM potassium acetate, 2 mM magnesium

acetate, 1 mM EGTA, 2mM DTT, 1 mM PMSF, protease inhibitor) using 3kDa MWCO dialysis tubing (Spectra/Por®). Dialysis was perform at 4°C for 16 hours. The dialyzed cytosol was concentrated using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore). Concentration was done when protein concentration of cytosol was about 10mg/mL.

2.9 In vitro permeabilized cell assay

The procedure was well-described by Cassany and Gerace (2008). In general, 12,000 HeLa cells were grown on each well of 10-well slide (Millipore). Before the assay, boundary was drawn to separate wells from each other using hydrophobic pen. The boundary was allowed to dry on ice for 15 minutes. Cells were then washed three times with cold transport buffer on ice, followed by permeabilization with 50ug/mL digitonin at room temperature for 5 minutes. Permeabilized cells were washed three times with cold transport buffer. Total volume of each transport reaction mixture was 25uL. The final concentrations of each component, if added, are as follow: 3uM His-tagged AcGFP fusion proteins, ATP-regenerating system (1mM ATP, 1mg/mL CP, and 15U/mL CPK), 0.1 mM GTP, 0.8 mg/mL WGA, 1.5uM His-importin-β1, 1.5uM GST-importin-α5, 2 mg/mL cytosol, 0.2mM GTPγS. For GTP hydrolysis dependence test, 2mM GTP and 2mM GDP were used. The prepared transport reaction mixture was added on each well, followed by incubation at 37°C for 30 minutes. After incubation, the cells were washed three times with cold transport buffer on ice and fixed with 4% paraformaldehyde on ice for 10 minutes. The cells were then permeabilized with 100% methanol for 5 minutes at room temperature. Nuclei were stained by 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI) in PBS for 5 minutes. Cells

were washed three times with PBS and the slide was air-dried. Coverslips were mounted on the glass slide by FluorSave™ Reagent (Millipore). The GFP and DAPI signal were observed using Leica TCS SPE.

2.10 In vitro pull down assay

40ug GST-NFAT5$_{171-250}$ was mixed with ATP-regenerating system (1mM ATP, 1mg/ml CP, and 15U/ml CPK), 0.1 mM GTP and 1X EDTA-free protease inhibitor (Roche). Hypertonic or hypotonic cytosol was added with final concentration 2mg/mL. Transport buffer was added to make the final volume up to 25uL. The mixtures were incubated at 37°C for 30 minutes. 120ug His-importin-β1 and 12uL Ni-NTA agarose (Qiagen) that prewashed with his-pull down incubation buffer (20mM sodium phosphate, 100mM sodium chloride, 40mM imidazole, 10% glycerol) were added into each reaction tube. For isotonic reaction mixture, GST-NFAT5$_{171-250}$, His-importin-β1 and Ni-NTA agarose were mixed without any pre-incubation. His-pull down incubation buffer was added to make the final volume of each pull down mixture to 250uL. The pull down mixtures were incubated at 4°C for overnight with shaking. The mixtures were centrifuged at 5000rpm, 4°C for 2 minutes. The flow through was discarded. The agarose was then washed three times with his-pull down incubation buffer. Proteins were eluted from agarose by boiling in the presence of 1X SDS dye for 10 minutes. The supernatant was analyzed by SDS-PAGE. The proteins in the SDS-PAGE gel were visualized by Coomassie Blue staining.

Chapter 3

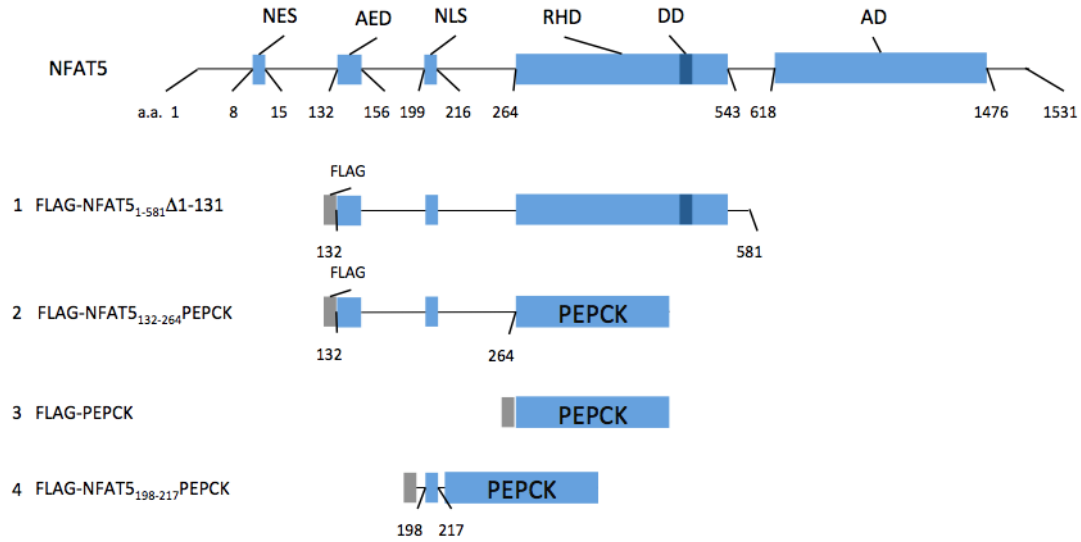
Results

3.1 Mapping of the minimal NFAT5 NLS required for nuclear import using heterologous protein cargo

Early findings from our laboratory suggested that a recombinant NFAT5 (FLAG-NFAT5$_{1-581}\Delta$1-131), which is a truncated from of NFAT5 devoid of both N-terminal NES and C-terminal transactivation domain, faithfully recapitulated nucleocytoplasmic trafficking properties of endogenous NFAT5 in response to changes in extracellular tonicity (Tong et al., 2006; Xu et al., 2008). This truncated recombinant NFAT5 contains three distinctive functional domains: an AED (a.a.132-156) essential for nuclear export under hypotonicity; a putative cNLS (a.a. 199-216), suggested by Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), essential for the nuclear import, and a Rel-homology DNA-binding domain (RHD) (a.a. 264 - 581) for DNA-binding (Lange et al., 2007; Sullivan, Kalaitzidis, Gilmore, & Finnerty, 2007; Tong et al., 2006). Earlier, by alanine-substitution analysis, our lab showed that amino acid residues $_{202}$RKR$_{204}$ of the putative cNLS are essential for nuclear import of FLAG-NFAT5$_{1-581}\Delta$1-131 (Tong et al., 2006). Deletion analysis was conducted to further decipher the minimal protein sequence requirement for NFAT5 nucleocytoplasmic trafficking. First, the RHD was substituted with cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK-C) to determine if RHD is dispensable for NFAT5 nucleocytoplasmic trafficking. PEPCK-C is an exclusively cytoplasmic protein, and has been used for the characterization of NLS activity of transcription factors (Theodore et al., 2008). The molecular weight of the new fusion protein (FLAG-NFAT5$_{132-264}$-PEPCK) is ~83 kDa (Fig. 3.1A), which precludes a passive nuclear transport mechanism. Another PEPCK fusion protein consisted of the

putative cNLS alone (FLAG-NFAT5$_{198-217}$-PEPCK) was also generated. As shown in Fig. 3.1B, both FLAG-NFAT5$_{1-581}\Delta$1-131 and FLAG-NFAT5$_{132-264}$-PEPCK were similarly subjected to nucleocytoplasmic trafficking in response to change in extracellular tonicity. Nevertheless, the replacement of RHD by PEPCK resulted in a modest but significant reduction in nuclear localization of recombinant protein under isotonic and hypertonic condition respectively, suggesting that RHD may contribute to, but not absolutely essential for, NFAT5 nuclear import. On the other hand, FLAG-NFAT5$_{198-217}$-PEPCK, which encoded fusion protein containing the putative cNLS (amino acid residues 199-216) and PEPCK, remains constitutively localized to the cytoplasm. As a control, FLAG-PEPCK was exclusively localized to the cytoplasm under all tonicities examined. Together these data suggested that amino acid residues 132-264 of NFAT5 confer tonicity-sensitive nucleocytoplasmic trafficking activity to a heterologous protein, whereas the putative cNLS *per se* is insufficient to function as a nuclear import signal to direct nuclear import of heterologous protein.

A


NES
AED
NLS
RHD
DD
AD
NFAT5
a.a. 1
8
15
132
156
199
216
264
543
618
1476
1531
FLAG
1 FLAG-NFAT5$_{1-581}$Δ1-131
132
581
FLAG
2 FLAG-NFAT5$_{132-264}$PEPCK
PEPCK
132
264
3 FLAG-PEPCK
PEPCK
4 FLAG-NFAT5$_{198-217}$PEPCK
PEPCK
198
217

B

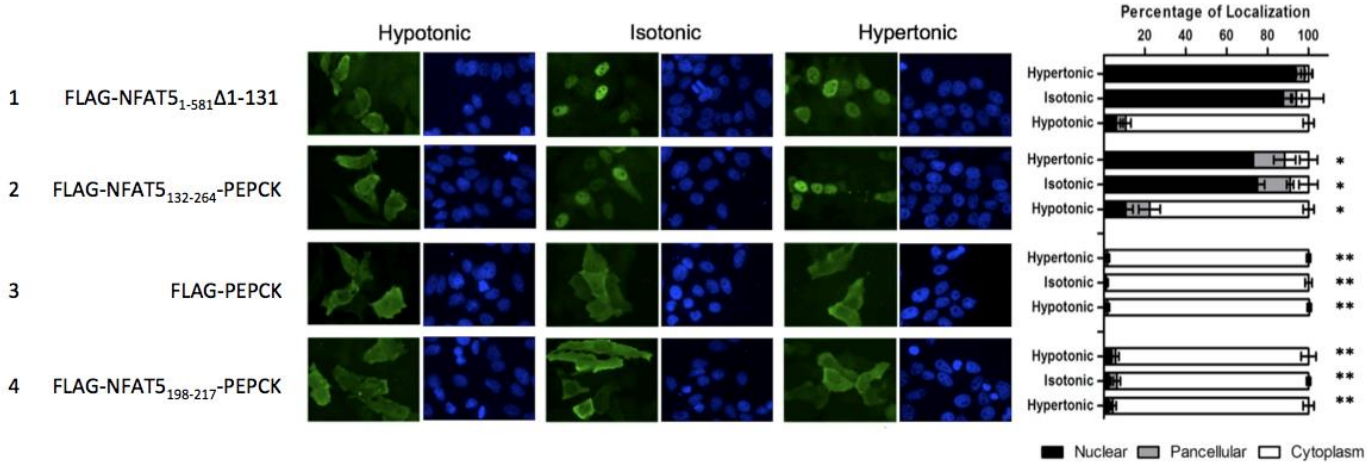
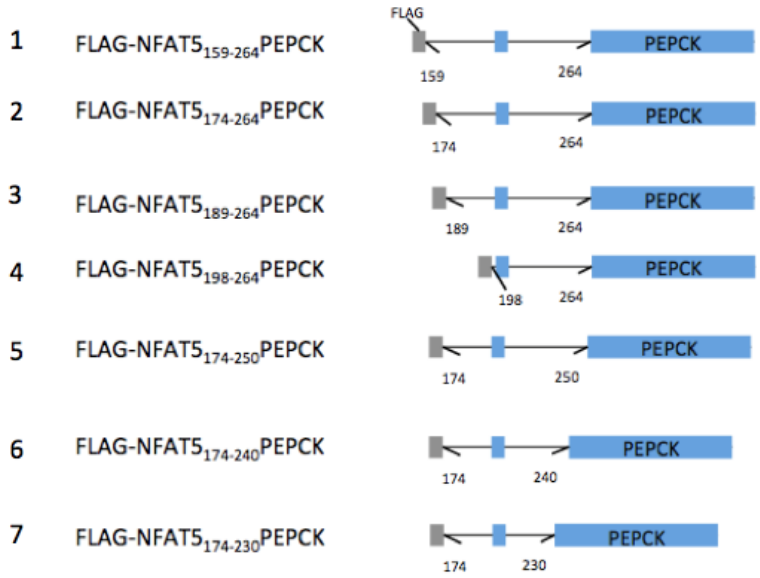

Hypotonic
Isotonic
Hypertonic
1 FLAG-NFAT5$_{1-581}$Δ1-131
2 FLAG-NFAT5$_{132-264}$-PEPCK
3 FLAG-PEPCK
4 FLAG-NFAT5$_{198-217}$-PEPCK
Percentage of Localization
20
40
60
80
100
Hypertonic
Isotonic
Hypotonic
*
**
Nuclear
Pancellular
Cytoplasm

Figure 3.1 Defining the nuclear import domain of NFAT5. A) Schematic diagram showing the domain organization of NFAT5, and different FLAG-tagged NFAT5-PEPCK recombinant plasmid. B) Nucleocytoplasmic trafficking of these recombinant NFAT5 proteins under hypotonic, isotonic and hypertonic conditions. HeLa cells were transfected with the indicated recombinant plasmids. 24 hours post transfection, cells were either left in isotonic medium, or switched to hypertonic or hypotonic medium for 2 hours, before fixation with 4% paraformaldehyde. Immunocytochemistry was conducted using FLAG antibodies and FITC-labeled anti-rabbit IgG. Cells were then analyzed by confocal microscopy. For quantification, 100 cells from each treatment were randomly selected for the analysis of subcellular localization. Signals were either scored as nucleus, cytoplasm, or pancellular, depending on the distribution of the signal.

Despite that cNLS alone was insufficient to confer significant nuclear import activity to PEPCK-C, it is essential for NFAT5 nuclear import. This is because substitution of the three core residues ($_{202}$RKR$_{204}$) within the putative cNLS with alanine resulted in complete ablation of nuclear import activity of NFAT5 (Tong et al., 2006). Together our data suggested that a functional NFAT5-NLS might consist of protein sequences that comprised of, but not limited to, the cNLS. To further delineate the minimal essential sequence required for functional nuclear import activity, nested deletion of FLAG-NFAT5$_{132-264}$PEPCK from the N- and C-terminus was conducted respectively, and nucleocytoplasmic trafficking of the truncated proteins was analyzed. As shown in Fig. 3.2B, FLAG-NFAT5$_{159-264}$PEPCK became constitutively localized to the nucleus under hypotonic, isotonic and hypertonic conditions due to the removal of AED, which is responsible for nuclear export (Tong et al., 2006). Further deletion of amino acid (a.a) residues 159 to 173 from the amino terminus (FLAG-NFAT5$_{174-264}$PEPCK) did not affect nuclear localization of the fusion protein, suggesting that this region is not important for nuclear trafficking. Nevertheless, nuclear localization of the fusion protein was profoundly reduced when a.a. residue 174 to 188 (FLAG-NFAT5$_{189-264}$PEPCK) were removed, and it was completely abolished when a.a. residue 189 to 197 (FLAG-NFAT5$_{198-264}$PEPCK) were deleted. In the C-terminus, although removal of a.a. residue from 264-251 (FLAG-NFAT5$_{174-250}$PEPCK) did not affect nuclear trafficking of the fusion protein, deletion of a. a. residues from 250-241 (FLAG-NFAT5$_{174-240}$PEPCK) impaired nuclear localization of the fusion protein significantly. Further deletion of a. a. residues from 240-231 (FLAG-NFAT5$_{174-230}$PEPCK) resulted in exclusive cytoplasmic localization of the fusion protein. These data suggested that a. a. residue 174 to 250 of NFAT5 is the minimal NLS required for NFAT5 nuclear import. Sequence similarly search of this

sequence was conducted but did not reveal any known sequences that bear significant homology to it. Therefore, this sequence may represent a novel NLS that is only responsible for NFAT5 nuclear import. This protein domain was named as NFAT5-NLS (N-NLS) to differentiate it with the cNLS. These above experiments were conducted by Dr. Ting Ting Huang.

A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6 FLAG-NFAT5174-240PEPCK
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174
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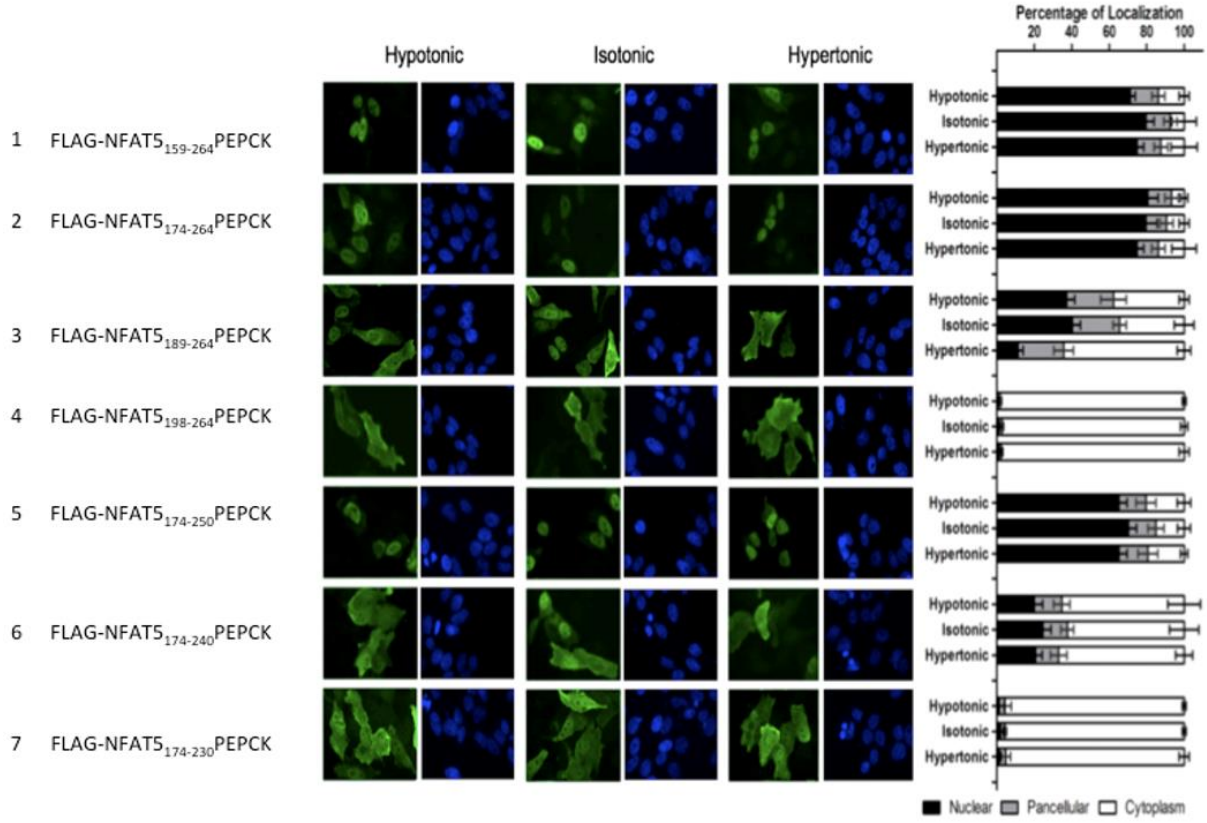
264
250
240
230

B


Hypotonic
Isotonic
Hypertonic
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2 FLAG-NFAT5174-264PEPCK
3 FLAG-NFAT5189-264PEPCK
4 FLAG-NFAT5198-264PEPCK
5 FLAG-NFAT5174-250PEPCK
6 FLAG-NFAT5174-240PEPCK
7 FLAG-NFAT5174-230PEPCK
Percentage of Localization
20 40 60 80 100
Nuclear
Pancellular
Cytoplasm

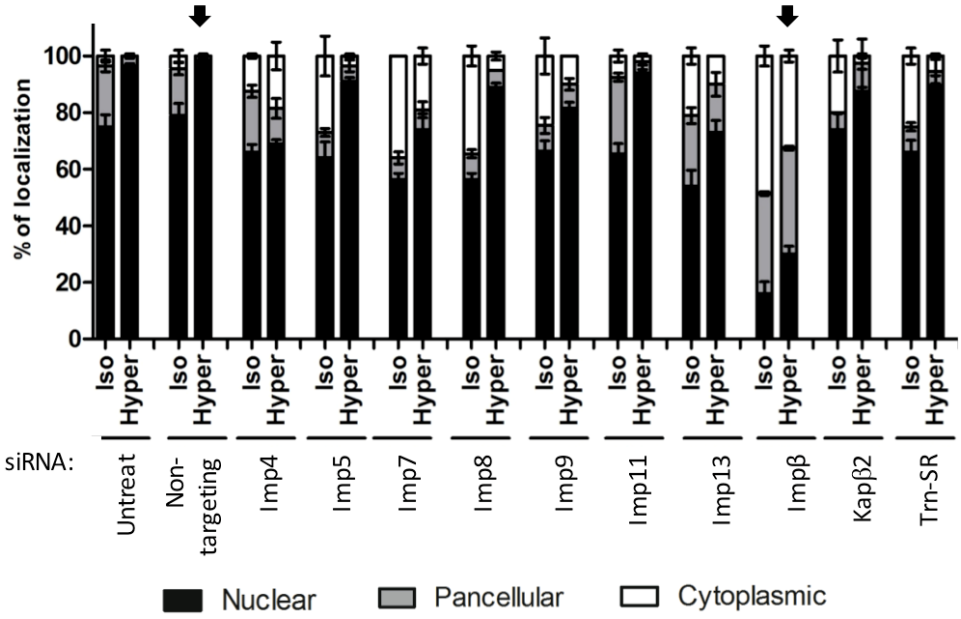
Figure 3.2 Characterization of minimal amino acid sequence of NFAT5 nuclear localization signal. A) Schematic representation of NFAT5-PEPCK recombinant construct. B) Subcellular localization of FLAG-PEPCK recombinant proteins. Recombinant plasmids were transfected into HeLa cells. After 24 hours, cells were either maintained in isotonic medium, or switched to hypertonic or hypotonic medium for 2 hours. Then, cells were subjected to paraformaldehyde fixation, followed by immunofluorescence analysis using FLAG antibody and FITC-labeled anti-rabbit IgG. Cells were analyzed by confocal microscopy. For quantification, 100 cells from each treatment were randomly selected for the analysis of subcellular localization. Signals were either scored as nucleus, cytoplasm, or pancellular, depending on the distribution of the signal.

3.2 Identification of nuclear transport receptor for NFAT5 nuclear import

The identification of a novel N-NLS in NFAT5 suggested that nuclear import of NFAT5 might be mediated by a novel nuclear receptor. To identify the cognate receptor for N-NLS, siRNA was used to knockdown members of the Kaps that are known to mediate nuclear import, including importin-β, Kapβ2, Imp4, Imp5, Imp7, Imp8, Imp9, Imp11, Imp13, Trn-SR respectively (Ullman, Powers, & Forbes, 1997), followed by determining the subcellular localization of FLAG-NFAT5$_{132-264}$PEPCK under different extracellular tonicity. Depletion of importin-β, but not other members of the Kaps family, markedly inhibited nuclear localization of the recombinant protein under both isotonic and hypertonic conditions (Fig. 3.3A). Representative image of the subcellular localization of the FLAG recombinant protein in response to importin-β knockdown was shown in Fig. 3.3B. Concordantly, subcellular fractionation analysis revealed that hypertonicity-induced nuclear accumulation of NFAT5 was diminished significantly in importin-β-depleted cells (Fig. 3.3C). Because importin-β was known to be involved nuclear transport of cNLS-containing cargo that heterodimerizes with nuclear import adaptor importin-α, siRNA knockdown analysis was carried out to determine if any member of the importin-α family (Importinα1, Importinα3, Importinα4, Importinα5, Importinα6, and Importinα7) (Pumroy & Cingolani, 2015), or another known nuclear import adaptor, Snurportin 1 (SNUPN) (Paraskeva et al., 1999), is involved in NFAT5 nuclear import. Nevertheless, depletion of these importins did not lead to a reduction of nuclear localization of FLAG-NFAT5$_{132-264}$PEPCK to a level comparable to importin-β knockdown (Fig 3.3D), suggesting that NFAT5 nuclear import is not dependent on any of these adaptors. On the other hand, co-immunoprecipitation with FLAG antibodies in cells expressing FLAG-OREBP$_{1-581}$Δ1-131 resulted in the presence of

endogenous importin β in the immunocomplex (Fig. 3.3E). Together these data suggested that members of the importin-α family does not involve in nuclear import of NFAT5. The above experiments were conducted by Dr. Ting Ting Huang.

A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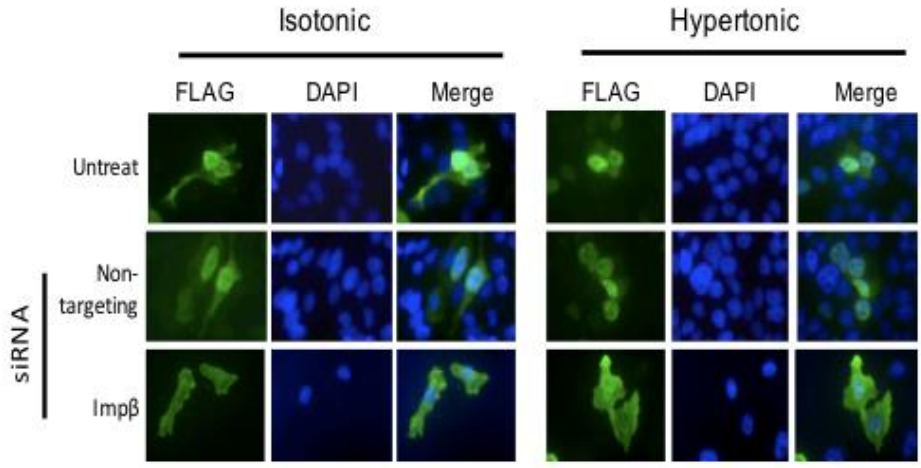
80
60
40
20
0
Iso
Hyper
siRNA:
Untreat
Non-targeting
Imp4
Imp5
Imp7
Imp8
Imp9
Imp11
Imp13
Impβ
Kapβ2
Trn-SR
Nuclear
Pancellular
Cytoplasmic

B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DAPI
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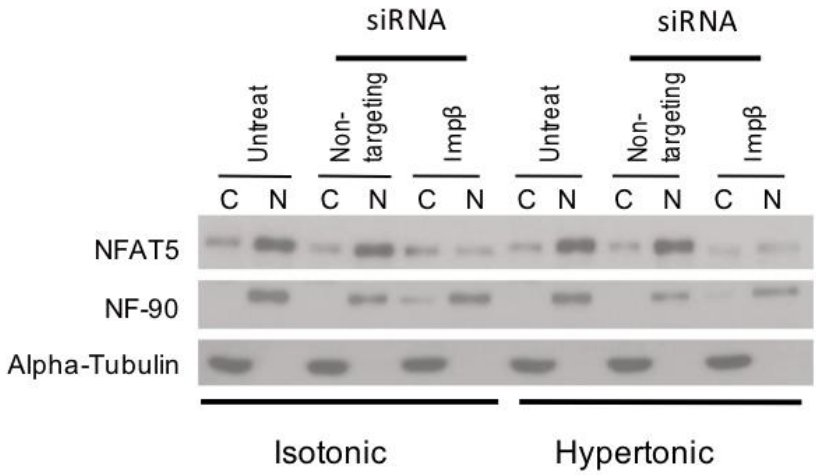
C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siRNA
Untreat
Non-targeting
Impβ
Untreat
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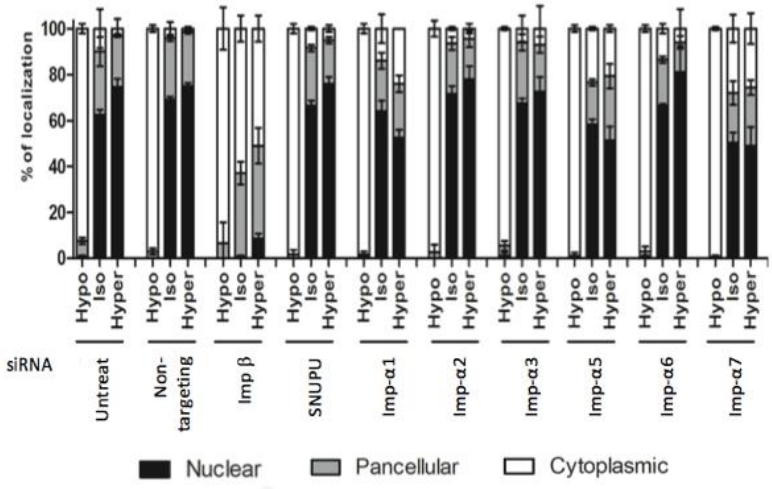
Impβ
C N C N C N C N C N C N
NFAT5
NF-90
Alpha-Tubulin
Isotonic
Hypertonic

D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60
40
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Hypo Iso Hyper
siRNA
Untreat
Non-targeting
Imp β
SNUPU
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Imp-α2
Imp-α3
Imp-α5
Imp-α6
Imp-α7
Nuclear
Pancellular
Cytoplasmic

E

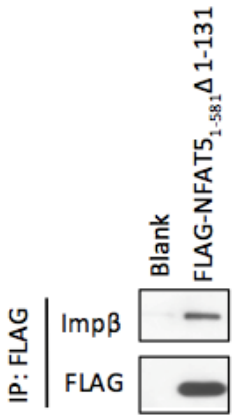

Blank
FLAG-NFAT5$_{1-581}$Δ1-131
IP: FLAG
Impβ
FLAG

Figure 3.3 Functional roles of importins in NFAT5 nuclear import. A) Functional assessment of Importin-β members in NFAT5 nuclear import. HeLa cells were transfected with the indicated siRNA for 48 hours. Subsequently, cells were either maintained in isotonic medium, or switched to hypertonic medium for another 2 hours. Finally, cells were fixed and immunofluorescence analysis was conducted using anti-FLAG antibody and FITC-labeled anti-rabbit IgG. siRNA against importin-β significantly reduced nuclear import of NFAT5, compared with non-targeting siRNA. *, $p<0.01$. For quantification, 100 cells from each treatment were randomly selected for the analysis of subcellular localization. Signals were either scored as nucleus, cytoplasm, or pancellular, depending on the distribution of the signal. B) Representative images Subcellular localization of exogeneous NFAT5 after knockdown of importin-β. C) Subcellular distribution of endogenous NFAT5 in response to depletion of importin-β. D) Effect of siRNA knockdown of different members of importin α subunit family in the subcellular distribution of FLAG-NFAT5. E) Co-immunoprecipitation of FLAG-NFAT5$_{1-581}$Δ1-131 and endogenous importin-β. Untreated HeLa cells or cells that were transfected with FLAG-NFAT5$_{1-581}$Δ1-131 were harvested. Subsequently, immunoprecipitation was conducted using anti-FLAG beads. The immunocomplex was subjected to western blotting analysis using antibodies against FLAG and importin-β respectively.

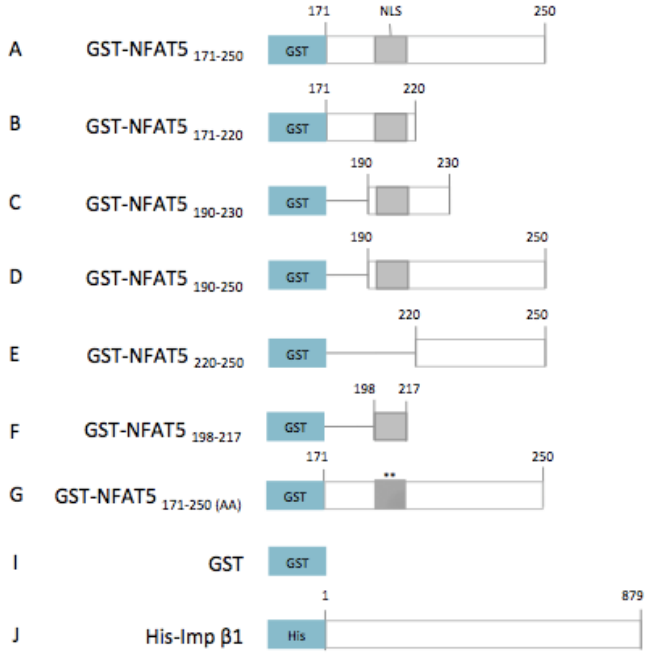
3.3 Bacterial expression and purification of recombinant importin-β and N-NLS for *in vitro* pull down analysis

The above data suggested that importin-β might interact with N-NLS to mediate nuclear import. To further substantiate if the two proteins interact directly and to define the interacting domain of N-NLS to importin-β, His-tagged importin-β and various protein fragments corresponding to different length of N-NLS, was well as the protein fragment corresponds to the cNLS (SV40 NLS), was expressed and purified from bacteria for in vitro pull-down analysis. Firstly, importin-β cDNA and N-NLS cDNAs correspond to different length of N-NLS were cloned into His-tagged and GST-tagged bacterial expression vector by in-frame insertion respectively (Fig. 3.4A). His-importin-β cDNA was transformed into BL21 bacteria and they were grown and induced with 0.5mM IPTG. Time point analysis suggested what recombinant protein corresponding to the size of the fusion protein became apparent after 6 hours of IPTG induction (Fig. 3.4B). Subsequently, a Ni-NTA affinity column was used to capture and purify the His-tagged fusion protein. As shown in Fig. 3.4C, after affinity capture and elution with excess imidazole, a highly purified protein species corresponds to the size of His-importin-β was identified.

Similarly, GST-NFAT5$_{171-250}$ cDNAs were transformed into BL21 cells and induced with 0.5mM IPTG. Time point analysis suggested what recombinant protein corresponding to the size of the GST-fusion protein becomes apparent after 6 hours of IPTG induction (Fig. 3.5A). Subsequently, the GST-fusion protein was purified by glutathione sepharose chromatography. After affinity capture and elution with excess glutathione, a highly purified protein species corresponds to the size of GST-NFAT5$_{171-250}$ was identified (Fig. 3.5B). Other GST-NFAT5 recombinant proteins were transformed in the BL21 cells and induced with IPTG using a similar protocol.

As shown in Fig. 3.5C, after affinity capture and elution with glutathione, highly purified protein species correspond to the size of different recombinant NLS proteins were identified. Together these data suggested that recombinant importin-β and NLSs were successfully produced and can be used for *in vitro* pull-down analysis.

A


A
GST-NFAT5 171-250
171
NLS
250
GST
B
GST-NFAT5 171-220
171
220
GST
C
GST-NFAT5 190-230
190
230
GST
D
GST-NFAT5 190-250
190
250
GST
E
GST-NFAT5 220-250
220
250
GST
F
GST-NFAT5 198-217
198
217
GST
G
GST-NFAT5 171-250 (AA)
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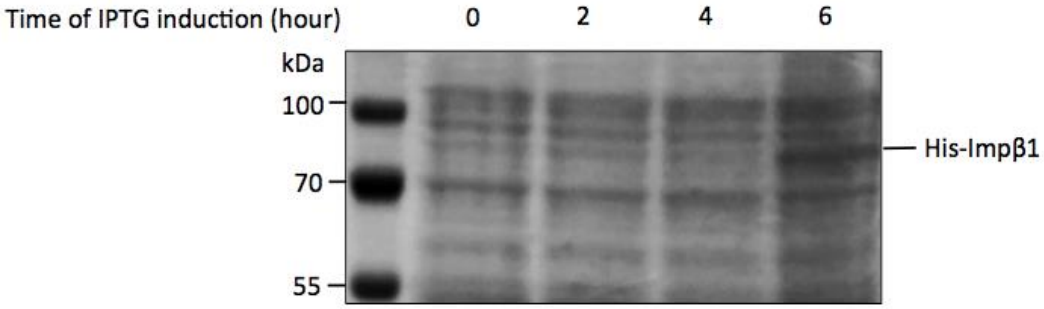
250
GST
I
GST
GST
J
His-Imp β1
1
879
His

B


Time of IPTG induction (hour)
0
2
4
6
kDa
100
70
55
His-Impβ1

C

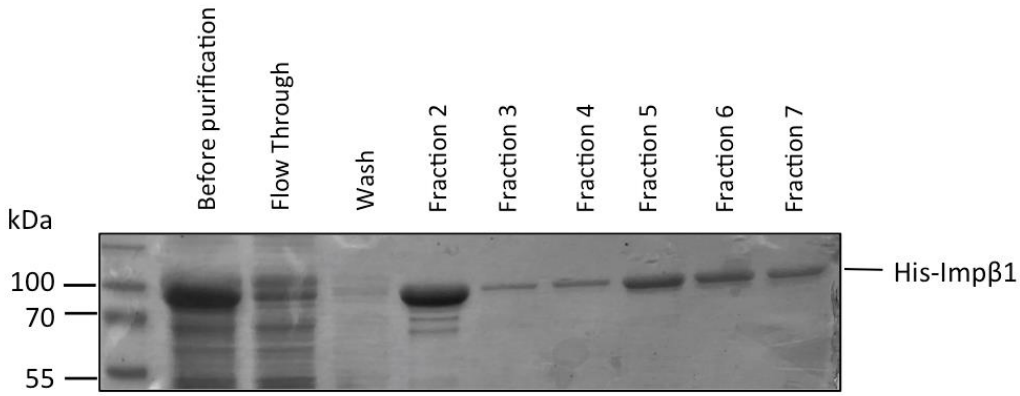
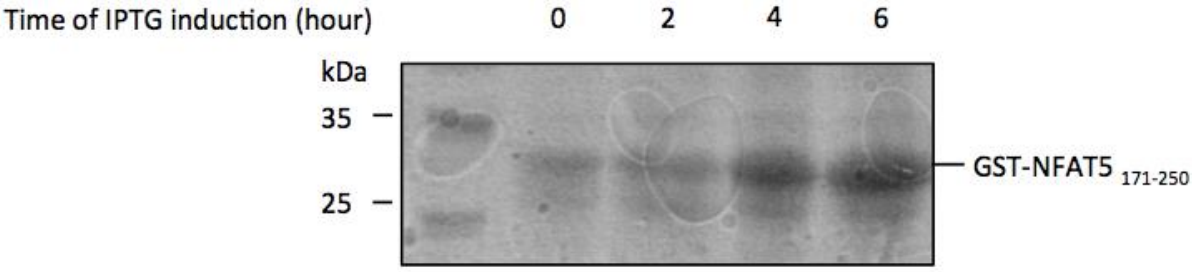
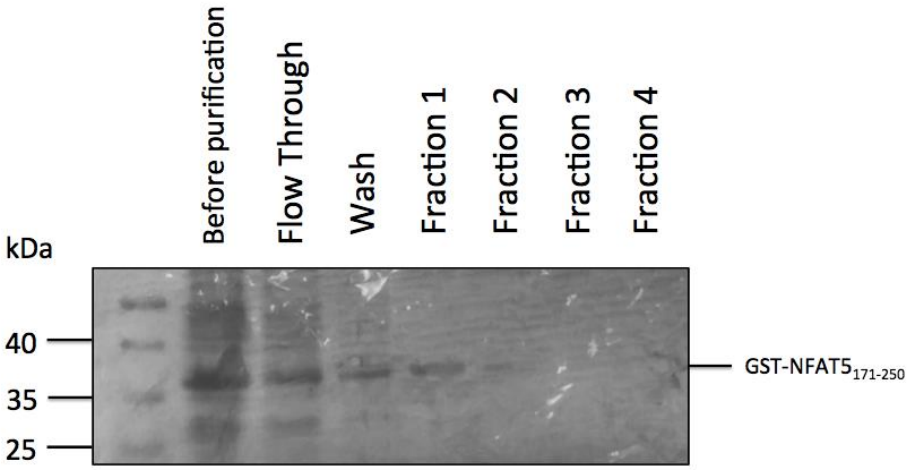

Before purification
Flow Through
Wash
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Fraction 7
kDa
100
70
55
His-Impβ1

Figure 3.4 Construction and purification of recombinant GST-NFAT5 and His-importin-β1. A) Schematic representation of different GST-fusion NFAT5-NLS proteins as well as His-importin-β1. Grey box represents the putative cNLS. B) Bacterial expression of His-importin-β1. Bacterial culture was collected at the indicated time points after the addition of IPTG. Bacteria were lysed and the protein extracts were subjected to SDS-PAGE analysis, followed by Coomassie Blue staining. C) Ni-NTA affinity column purification of His-importin-β1.

A


Time of IPTG induction (hour)
0
2
4
6
kDa
35
25
GST-NFAT5 171-250

B


Before purification
Flow Through
Wash
Fraction 1
Fraction 2
Fraction 3
Fraction 4
kDa
40
35
25
GST-NFAT5171-250

C

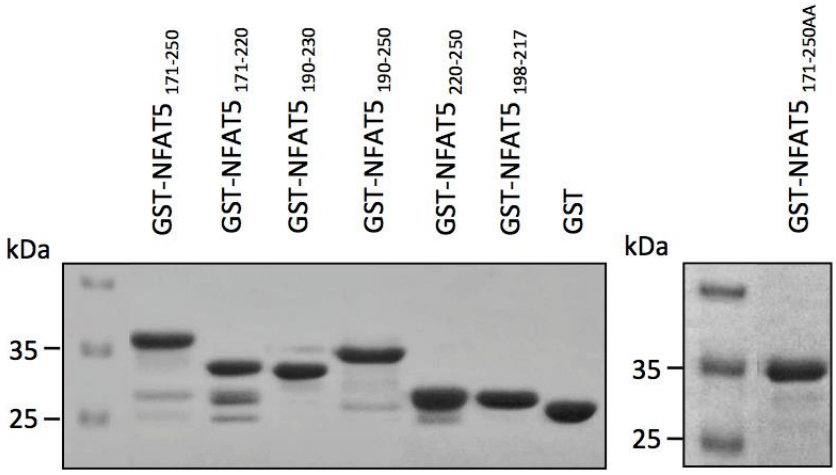

GST-NFAT5 171-250
GST-NFAT5 171-220
GST-NFAT5 190-230
GST-NFAT5 190-250
GST-NFAT5 220-250
GST-NFAT5 198-217
GST
GST-NFAT5 171-250AA
kDa
35
25
kDa
35
25

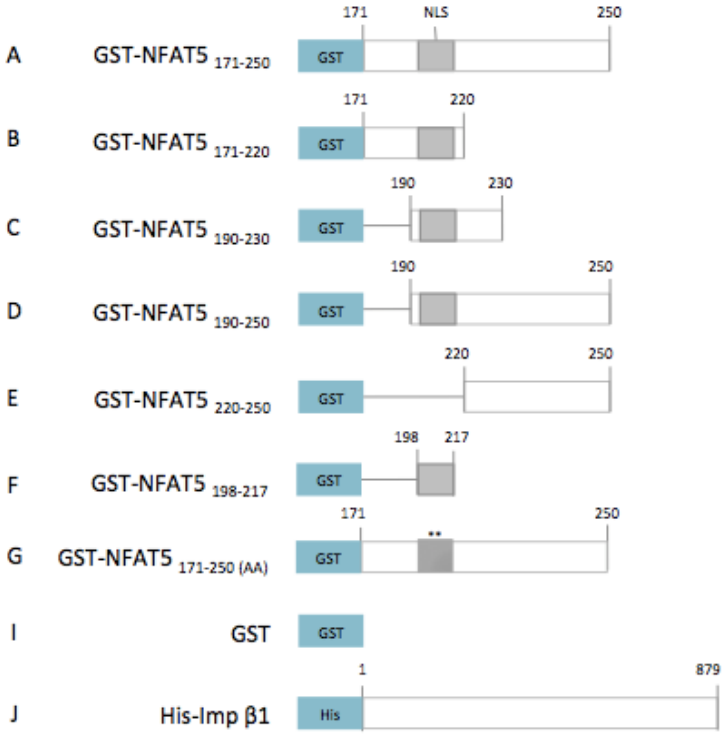
Figure 3.5 Purification of GST-NLS recombinant proteins. A) Bacterial expression of GST-NFAT5$_{171-250}$. Bacterial culture was collected at the indicated time points after the addition of IPTG. Bacteria were lysed and the protein extracts were subjected to SDS-PAGE analysis, followed by Coomassie Blue staining. B) Purification profile of GST-NFAT5 171-250. C) GST-NFAT5 proteins were purified using glutathione sepharose column. Purified proteins were subjected to SDS-PAGE analysis followed by Coomassie Blue staining. All purified proteins were expressed at the expected molecular weight.

3.4 Interaction between importin-β and NFAT5 N-NLS

To determine if N-NLS is associated directly with importin-β, *in vitro* pull-down analysis was conducted. Agarose beads coupled to His-importin-β efficiently pulled-down GST-NFAT5$_{171-250}$, which corresponds to the minimal N-NLS sequence (FLAG-NFAT5$_{174-250}$PEPCK) that is required for directing nuclear import of PEPCK (Fig. 3.2), but not GST control (Fig. 3.6B, lane 1 vs lane 7). The interaction between GST-NFAT5$_{171-250}$ and His-importin-β1 was disrupted in the presence of excessive RanGTP (RanQ69LGTP) (40mM) (Fig. 3.6B, lanes 9-10). Together these data suggested that N-NLS directly interacts with importin-β1 in a RanGTP-sensitive manner. Next, deletion mutants of GST-NFAT5$_{171-250}$ were examined for their interaction with importin-β1. As shown in Fig. 3.6B, deletion-mapping analysis suggested that removal of amino acid residues N-NLS from the C-terminus (GST-NFAT5$_{171-220}$, Fig. 3.6A, lane B) does not lead to a significant reduction in its affinity to His-importin-β1 (Fig. 3.6B, lane 2). Similarly, removal of amino acid residues from the N-terminus (GST-NFAT5$_{190-250}$, Fig. 3.6A, lane D) does not significantly reduce its affinity to His-importin-β1 (Fig. 3.6B, lane 5). However, removal of amino acid residues from both N- and C-terminus (GST-NFAT5$_{190-230}$, Fig. 3.6A, lane C) significantly diminished its affinity to His-importin-β1 (Fig. 3.6B, lane 3). Further deletion of GST-NFAT5$_{190-230}$ from the N-terminus to remove the putative cNLS (GST-NFAT5$_{220-250}$, Fig. 3.6A, lane E) abolished its ability to interact with His-importin-β1 (Fig. 3.6B, lane 4). Together these data suggested that the putative cNLS sequences together with the amino acid sequence distal or proximal of it are important for the interaction with His-importin-β. Concordant with this observation, alanine substitution of R202 and K203 (basic amino-acid cluster of the putative cNLS) of GST-NFAT5$_{171-250}$ (GST-NFAT5$_{171-250}$AA, Fig. 3.6A, lane G) completely abolished

its interaction with His-importin-β (Fig. 3.6B, lane 11 and 12). To further demonstrate the requirement of proximal and distal domains of the N-NLS for the interaction with His-importin-β1, a GST-fusion protein containing only the putative cNLS region (GST-NFAT5$_{198-217}$, Fig 3.6A, lane F) was used. However, unexpectedly, this recombinant protein also interacts with importin-β (Fig. 3.6B, lane 6). Together these data suggested that the minimal putative cNLS is sufficient for its interaction with importin-β1. However, it is unclear at present why a slightly extended NLS (GST-NFAT5$_{190-230}$) weakened its interaction with importin-β, whereas a more extensive NLS covering the proximal and distal region of NLS (GST-NFAT5$_{171-250}$) led to a stronger interaction with importin-β1.

A


A
GST-NFAT5 171-250
171
NLS
250
GST
B
GST-NFAT5 171-220
171
220
GST
C
GST-NFAT5 190-230
190
230
GST
D
GST-NFAT5 190-250
190
250
GST
E
GST-NFAT5 220-250
220
250
GST
F
GST-NFAT5 198-217
198
217
GST
G
GST-NFAT5 171-250 (AA)
171
250
GST
I
GST
GST
J
His-Imp β1
1
879
His

B

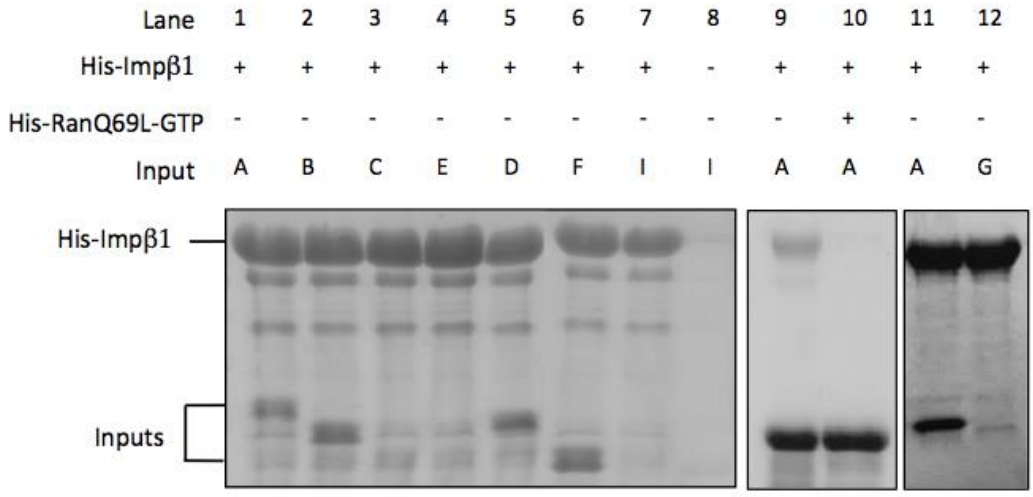
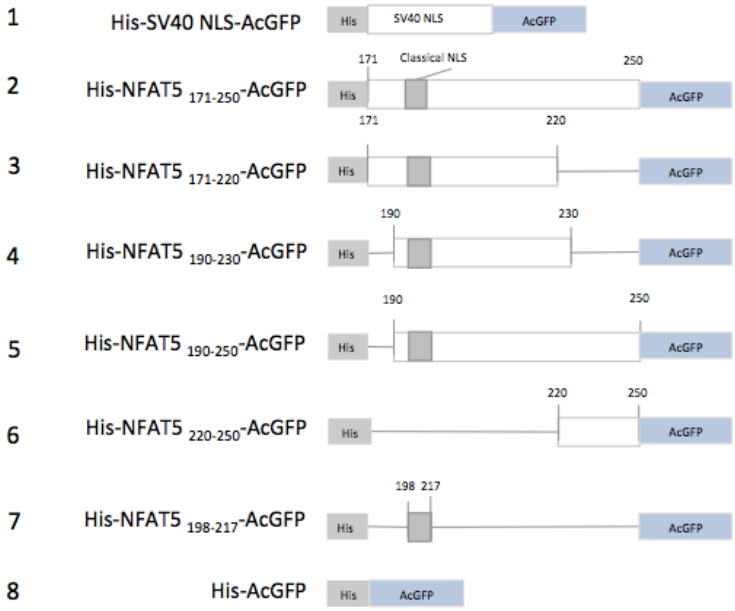

Lane 1 2 3 4 5 6 7 8 9 10 11 12
His-Impβ1 + + + + + + + - + + + +
His-RanQ69L-GTP - - - - - - - - - + - -
Input A B C E D F I I A A A G
His-Impβ1
Inputs

Figure 3.6 In vitro pull down analysis of GST-NFAT5 recombinant proteins with and His-importin-β1. A) Schematic representation of different GST-fusion NFAT5-NLS proteins as well as His-importin-β1. B) The *in vitro* pull down analysis. For lane 1-8 His-importin-β1 was immobilized on Ni-NTA agarose followed by the addition of GST-NFAT5 mutants. The column was then washed and eluted with excess imidazole. For lane 9 and 10, glutathione sepharose was used to capture GST-$NFAT5_{171-250}$ followed by the addition of His-importin-β1 and His-RanQ69L-GTP. After washing, proteins were eluted by the addition of excess imidazole. Proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining.

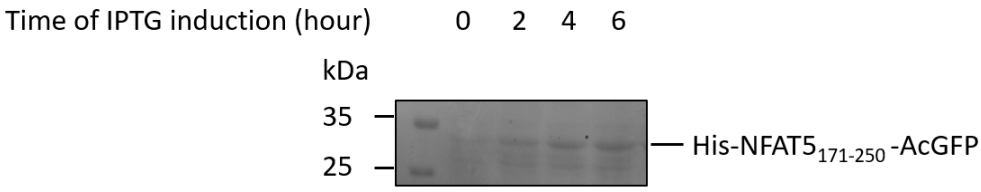
3.5 Preparation of recombinant NFAT5-AcGFP proteins for in vitro nuclear import assay

The above finding has suggested that the putative cNLS region ($NFAT5_{198-217}$) is the minimal amino acid sequences required for the interaction with importin-β1. On the other hand, the PEPCK nuclear translocation assay suggested that the minimal amino acid sequences $NFAT5_{174-250}$ is required for directing nuclear import of a heterologous protein. To reconcile the discrepancy between these two observations, and to further define the function of importin-β1 in NFAT5 nuclear import, as well as to characterize the function of importin-β1-NLS interaction, *in vitro* nuclear import assay was carried out. In preparation for this assay, His-NFAT5-NLS-AcGFP proteins that contains various fragments of N-NLS were generated by in frame insertion of the respective cDNA into the 3' end of His-epitope tag, followed by an in-frame insertion of monomeric GFP (AcGFP) cDNA to the 3' end of the N-NLS sequence (Fig 3.7A). As a positive control for nuclear import, a cNLS (SV40-NLS) was also fused with the AcGFP in a similar manner (Fig. 3.7A). Recombinant importin-β was prepared as described in Fig. 3.4B. BL21 bacteria transformed with His-$NFAT5_{171-250}$-AcGFP cDNAs were induced by 0.5mM IPTG. Time point analysis suggested what recombinant protein corresponding to the size of the fusion protein becomes apparent after 6 hours of IPTG induction (Fig. 3.7B). Other His-NFAT5-AcGFP mutants were transformed and grown using a similar protocol, and they were expressed after IPTG induction (data not shown). Subsequently, Ni-affinity chromatography was used to capture and purify the fusion protein. As shown in Fig. 3.7C, after affinity capture using Ni-NTA column and eluting with excess imidazole, highly purified protein species correspond to the size of the predicted His-NFAT5-NLS-AcGFPs were identified.

A


1
His-SV40 NLS-AcGFP
His
SV40 NLS
AcGFP
2
His-NFAT5 171-250-AcGFP
171
Classical NLS
250
171
220
3
His-NFAT5 171-220-AcGFP
4
His-NFAT5 190-230-AcGFP
190
230
5
His-NFAT5 190-250-AcGFP
190
250
6
His-NFAT5 220-250-AcGFP
220
250
7
His-NFAT5 198-217-AcGFP
198 217
8
His-AcGFP

B


Time of IPTG induction (hour) 0 2 4 6
kDa
35
25
His-NFAT5171-250-AcGFP

C

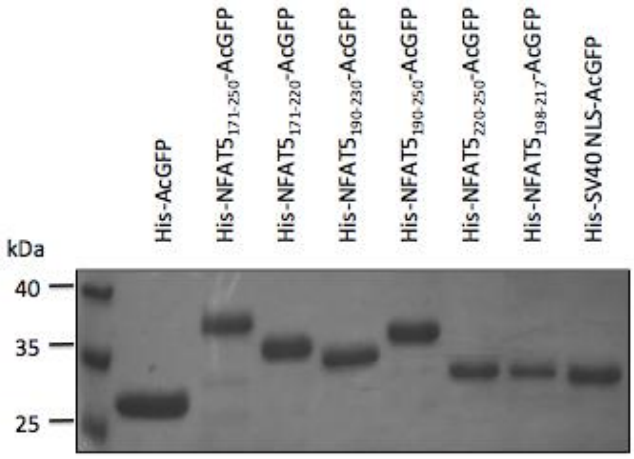

His-AcGFP
His-NFAT5171-250-AcGFP
His-NFAT5171-220-AcGFP
His-NFAT5190-230-AcGFP
His-NFAT5190-250-AcGFP
His-NFAT5220-250-AcGFP
His-NFAT5198-217-AcGFP
His-SV40 NLS-AcGFP
kDa
40
35
25

Figure 3.7 Preparation of recombinant proteins for in vitro nuclear import assay. A) Schematic representation of different NFAT5-AcGFP reporter constructs. B) Bacterial expression of His-NFAT5$_{171-250}$-AcGFP. 500 µl of bacterial culture was collected at different time points after addition of IPTG. Cell lysates were analyzed by SDS-PAGE and visualized by Coomassie Blue staining. C) SDS-PAGE analysis of purified recombinant proteins. Recombinant His-NFAT5-AcGFP proteins were purified using HisTrap column. All purified proteins were subjected to SDS-PAGE analysis and visualized by Coomassie Blue staining.

3.6 *In vitro* permeabilized cell assay to determine the activity of a functional N-NLS

In vitro nuclear transport assay was conducted using digitonin-permeabilized HeLa cells. In this assay, cells were first depleted with cytoplasmic contents (including the nuclear receptors, energy, and other cytoplasmic factors required for nuclear import). However, because these cells still retain an intact nuclear membrane, proteins can undergo nuclear import via nuclear pore complex. Therefore, *in vitro* nuclear transport assay measures if a cargo can be transported into the nucleus by supplementing defined factors, in a controlled manner (Cassany & Gerace, 2008). To test if this system is working properly, first, nuclear import of His-NFAT5$_{171-250}$-AcGFP and His-SV40-AcGFP proteins was determined by replenishing cytosols. In the presence of cytosol, ATP-regeneration mixture, and RanGTP, increased green fluorescence was detected in the nucleus of HeLa cells when His-NFAT5$_{171-250}$-AcGFP and His-SV40-AcGFP proteins were added to the reaction respectively. (Fig. 3.8, lane 1), whereas His-AcGFP which does not contain any NLS failed to enter nucleus (Fig. 3.8, lane 1). These data suggested that both His-NFAT5$_{171-250}$-AcGFP and His-SV40-AcGFP each contains a function NLS. Nuclear entry of His-NFAT5$_{171-250}$-AcGFP and His-SV40-AcGFP was abolished in the absence of cytosol (Fig. 3.8, lane 3), ATP-regenerating mixture (Fig. 3.8, lane 2), or in the presence of nuclear pore inhibitor wheat germ agglutinin (WGA) (Finlay, Newmeyer, Price, & Forbes, 1987) (Fig. 3.8, lane 4). These data suggested that the activities of these two NLSs are energy-dependent, and required nuclear receptors. In addition, the NLSs travelled into the nucleus via nuclear pore complexes. Earlier studies suggested that elevated level of cytoplasmic RanGTP disrupts interactions between NLS-containing proteins and importin-β (Melchior, Paschal, Evans, & Gerace, 1993). Concordantly, the

presence of high concentration of GTP, or GTPγS (a non-hydrolysable GTP analogue) abrogated nuclear entry of His-NFAT5$_{171-250}$-AcGFP and His-SV40-AcGFP respectively (Fig. 3.8, lane 5 and 6). These data suggested that, similar to His-SV40-AcGFP, His-NFAT5$_{171-250}$-AcGFP enters nucleus in a GTP-sensitive and importin-dependent manner.

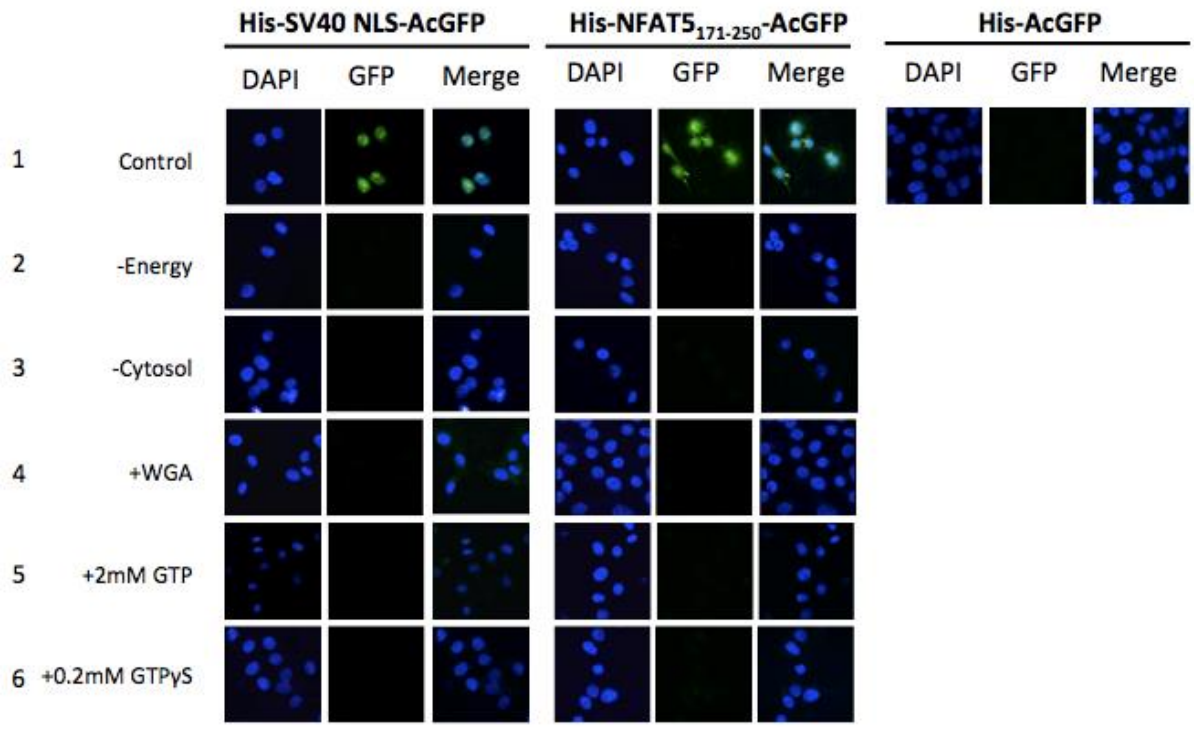


Figure 3.8 ***In vitro*** **permeabilized cell assay.** Nuclear import of His-SV40 NLS-AcGFP, His-NFAT5$_{171-250}$-AcGFP, and His-AcGFP was determined using digitonin-permeabilized HeLa cells. Lane 1, cells were supplemented with cytosol, ATP-regenerating system, and RanGTP. Lane 2, cells were only supplemented with cytosol and RanGTP. Lane 3, cells were only supplemented with ATP-regenerating system and RanGTP. Lane 4, cells were supplemented with cytosol, ATP-regenerating system, RanGTP and WGA. Lane 5, cells were supplemented with cytosol, ATP-regenerating system, and RanGTP and 2mM GTP. Lane 6, cells were supplemented with cytosol, ATP-regenerating system, and 0.2mM GTPγS. Transport assay was carried out for 30 min at 37°C, cells were fixed with paraformaldehyde, stained with DAPI and images were taken using confocal microscopy.

Next, nuclear transport assay was conducted using defined factors. As expected, His-SV40-AcGFP (Fig. 3.9, lane 1) or His-NFAT5$_{171-250}$-AcGFP (Fig. 3.9, lane 6), when added to the permeabilized HeLa cells in the absence of exogenous factors, was unable to enter the nucleus. In the presence of RanGTP, nuclear transport factor 2 (NTF2), ATP-regenerating mixture, Imp α5, and Imp β1, both recombinant proteins translocated into the nucleus respectively (Fig. 3.9, lane 3 and 8), and was abolished by the addition of WGA (Fig. 3.9, lane 2 and 7). In agreement with existing notion that nuclear import of cNLS requires ternary complex formation with importin-α and importin-β, nuclear accumulation of His-SV40-AcGFP was abolished in the absence of importin-α5 (Fig. 3.9, lane 4) or importin-β1 (Fig. 3.9, lane 5). On the other hand, nuclear import of His-NFAT5$_{171-250}$-AcGFP was abolished in the absence of importin-β1 (Fig. 3.9, lane 10) but not importin-α5 (Fig. 3.9, lane 9). Therefore, these data suggested that, unlike nuclear import of cNLS-containing proteins, NFAT5 nuclear import is mediated by an importin-α-independent, but importin-β-dependent mechanism.

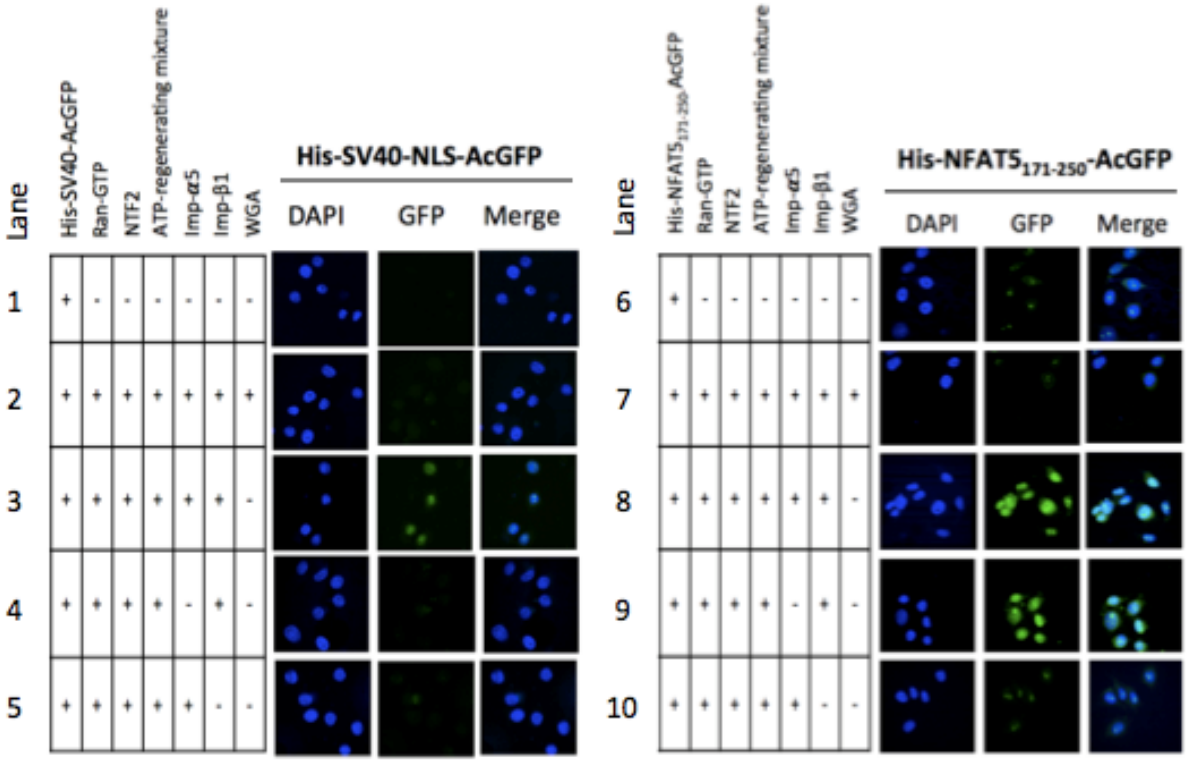
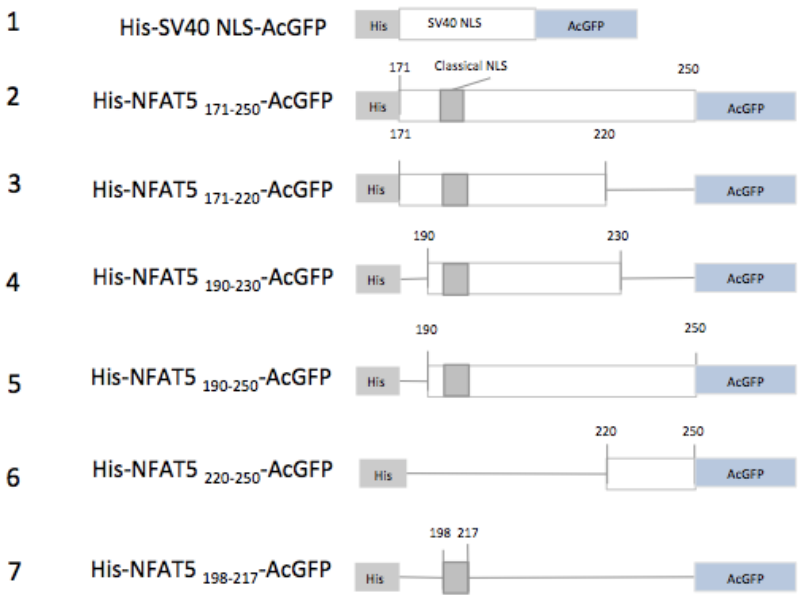


Figure 3.9 *In vitro* permeabilized cell assay using defined nuclear transport factors. Nuclear import of His-SV40 NLS-AcGFP and His-NFAT5$_{171-250}$-AcGFP was determined using digitonin-permeabilized HeLa cells supplemented with individual factor as specified. Transport assay was carried out for 30 min at 37°C, cells were fixed with paraformaldehyde, stained with DAPI and images were taken using confocal microscopy.

To further define the sequence requirement for importin-β1-mediated NFAT5 nuclear import, deletion mutants of His-NFAT5$_{171-250}$-AcGFP were generated and analyzed by *in vitro* nuclear transport assay using defined factors (RanGTP, NTF2, ATP-regenerating mixture, and importin-β1). As a control, nuclear signal was not detected when His-SV40-AcGFP was tested due to the absence of importin-α5 (Fig. 3.10B, lane 1), whereas His-NFAT5$_{171-250}$-AcGFP was subjected to nuclear import in the presence of imp-β1 (Fig. 3.10B, lane 2). Deletion of amino acid residues 221-250 from the C terminus (His-NFAT5$_{171-220}$-AcGFP) (Fig. 3.10B, lane 3) did not appreciably affect importin-β1-mediated nuclear import. This observation is consistent with the *in vitro* pull down assay, which showed that a similar NLS region (GST-NFAT5$_{171-220}$) is associated with importin-β1 (Fig. 3.6). Similarly, His-NFAT5$_{190-250}$-AcGFP (Fig. 3.10B, lane 5) was subjected to nuclear import, concordant with the *in vitro* pull down analysis using GST-NFAT5$_{190-250}$ (Fig. 3.6). On the other hand, data from the *in vitro* pull down analysis suggested that the affinity of NLS composed of amino acid residues 190-230 (GST-NFAT5$_{190-230}$) with importin-β1 was reduced significantly (Fig. 3.6). Concordantly, His-NFAT5$_{190-230}$-AcGFP (Fig. 3.10B, lane 4) also failed to undergo nuclear import. Similarly, nuclear accumulation of the His-NFAT5$_{220-250}$-AcGFP, which does not contain the putative cNLS, was also abolished (Fig. 3.10B, lane 6). Finally, recombinant protein bearing the putative cNLS (His-NFAT5$_{198-217}$-AcGFP) alone (Fig. 3.10B, lane 7) did no undergo nuclear entry. This is in contrary to the data obtained from the *in vitro* pull down assay, which showed that the putative cNLS (GST-NFAT5$_{198-217}$) is sufficient to interact with importin-β1. Collectively, these data suggested that NFAT5$_{171-220}$ or NFAT5$_{190-230}$ acts as a functional NLS for NFAT5 nuclear import. Consistent with the *in vitro* pull down analysis, these findings suggested that NLS activity requires the

presence of the putative cNLS, together with protein sequences either proximal or distal to the cNLS core. Importantly, the cNLS core alone, although capable of interacting with importin-β1, failed to induce nuclear import activity.

A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His
SV40 NLS
AcGFP
2
His-NFAT5 171-250-AcGFP
171
Classical NLS
250
3
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220
4
His-NFAT5 190-230-AcGFP
190
230
5
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6
His-NFAT5 220-250-AcGFP
7
His-NFAT5 198-217-AcGFP
198 217

B

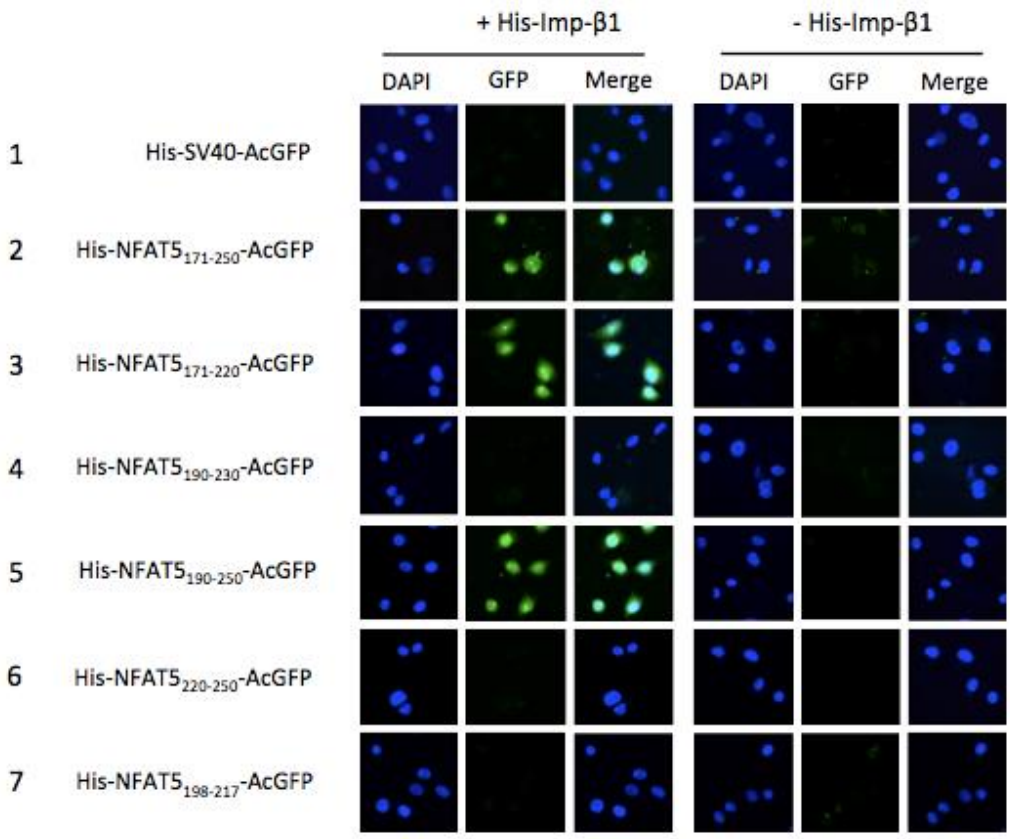
+ His-Imp-β1
- His-Imp-β1
DAPI
GFP
Merge
1 His-SV40-AcGFP
2 His-NFAT5171-250-AcGFP
3 His-NFAT5171-220-AcGFP
4 His-NFAT5190-230-AcGFP
5 His-NFAT5190-250-AcGFP
6 His-NFAT5220-250-AcGFP
7 His-NFAT5198-217-AcGFP

Figure 3.10 *In vitro* permeabilized cell assay using His-NFAT5-AcGFP mutants. A) Schematic representation of different NFAT5-AcGFP reporter constructs. B) Results of *in vitro* permeabilized cell assay using His-NFAT5-AcGFP mutants. Permeabilized HeLa cells were used to determine the nuclear import activity of the indicated recombinant proteins. The reaction was supplemented with RanGTP, NTF2, and ATP-regenerating mixture, in the presence of absence of importin-β1. Transport assay was carried out for 30 min at 37°C, cells were fixed with paraformaldehyde, stained with DAPI and images were taken using confocal microscopy.

Chapter 4

Discussion

Environmental stressors, such as extremes of temperature, oxidative insults, and hypoxia, elicit distinctive genetic programs in mammalian cells that aim to restore cellular homeostasis to the original state or reprogram the cells for survival in a new environment. Among others, hypertonic (high Na^+) stress is one of these environmental stressors that perturbs cellular homeostasis, leading to DNA damage, cell cycle arrest, and disruption of membrane potential, etc (Burg, Ferraris, & Dmitrieva, 2007). Nuclear Factor of Activated T-cells (NFAT5) is the only known tonicity-regulated transcription factor that orchestrates a transcriptional program for cellular adaptation to hypertonic stress in mammals. NFAT5 is a member of the NFAT family transcription factors (Ko et al., 2000; López-Rodríguez et al., 1999; Miyakawa et al., 1999). Upon activation by hypertonicity, NFAT5 promotes the expression of various organic osmolyte transporters, resulting in replacement of deleterious intracellular Na^+ with non-perturbing organic osmolytes and restoration of cellular homeostasis under hypertonic stress (Burg et al., 2007). NFAT5-dependent osmoadaptive response was once considered only relevant to the survival of kidney medullary cells and pulposus cells of the intervertebral disc, which are exposed to hypertonic environment. However, most recent findings revealed that, besides osmoadaptation, Na^+-NFAT5 signaling is also playing novel functional role in regulating epithelial chemokine production for establishing an antibacterial defense zone in inner medulla (Berry et al., 2017). More importantly, emerging evidence further suggested that hypertonicity is indeed present in various tissue microenvironments (Go, Liu, Roti, Liu, & Ho, 2004; Jantsch et al., 2015; Machnik et al., 2009; Wiig et al., 2013). Importantly, in these compartments, Na^+-NFAT5

signaling influences various physiological and disease processes, ranging from blood pressure regulation (Machnik et al., 2009), inflammation (Jantsch et al., 2015), to autoimmune disorders (Kleinewietfeld et al., 2013), via regulating the expression of a variety of cytokines and growth factors. Therefore, NFAT5 is a potential therapeutic target in these diseases.

How elevated Na^+ signal is transduced to and activates NFAT5 remains largely elusive. However, it is clear that under hypertonic conditions, NFAT5 activity is augmented by increased abundance and nuclear availability, as well as enhanced transactivation activity respectively (Cheung & Ko, 2013). In contrast, NFAT5 undergoes nuclear export and inactivation under hypotonic conditions. Among others, increased nuclear availability is the primary gateway for NFAT5 activation: NFAT5 undergoes nuclear import and accumulation under hypertonic stress; whereas it undergoes nuclear export upon hypotonic challenge (Dahl, Handler, & Kwon, 2001; Tong et al., 2006; Woo et al., 2000). A number of kinases have been identified and implicated in the nuclear import of NFAT5 (Gallazzini et al., 2011; Gallazzini et al., 2010; Irarrazabal et al., 2010). However, a universal hypothesis on how NFAT5 nucleocytoplasmic trafficking is regulated, the exact NFAT5 domain(s), nor the nuclear receptor(s) involved in this process, have not been demonstrated directly. Earlier findings from Dr. Ko's laboratory have identified the essential amino acid residues and protein domains, as well as the protein kinases involved in the nuclear import and export of NFAT5 (Cheung & Ko, 2013; Tong et al., 2006; Xu et al., 2008). Although these studies have identified several critical residues involved in NFAT5 nuclear import, a NFAT5 nuclear import domain which could mediate nuclear import of a heterologous protein has not been identified. Towards this goal, Dr. Ko's laboratory has conducted deletion mapping and mutation analysis of the NFAT5 gene,

and identified a novel amino acid domain (N-NLS) that could direct nuclear import of a heterologous protein PEPCK. The N-NLS is comprised of 77 amino acids, including a region that shows high similarly to the classical nuclear localization signal. Although mutation of the predicted core residues (a.a. 202-204) according to the consensus sequence of classical nuclear localization signal abolished nuclear import of NFAT5, this classical nuclear localization-like region failed to mediate nuclear import of heterologous protein. More importantly, nuclear receptors for classical nuclear localization signals, namely, the importin-α and importin-β heterodimer respectively, do not involved in nuclear import of NFAT5. Instead, data from Dr. Ko's laboratory suggested that N-NLS utilizes importin-β as the sole nuclear receptor for nuclear import. My project, therefore, is to extend these previous findings by further characterizing N-NLS with respect to its activity and mechanism of action.

To substantiate these observations further, I attempted to purify recombinant N-NLS and confirm its interaction with importin-β. To facilitate purification, GST-tagged N-NLS and its deletion mutants were generated, and they were successfully expressed and purified from bacteria (Figure. 35C). Similarly, His-tagged importin-β was also successfully expressed and purified (Figure. 3.4C). *In vitro* interaction analysis revealed that GST-tagged N-NLS (GST-NFAT5$_{171-250}$) is able to pull down His-tagged importin-β, suggesting that N-NLS directly interacts with importin-β *in vitro*. Subsequently, deletion analysis was conducted to further define the essential region within N-NLS required for importin-β interaction. Although cell-based nuclear import assay suggested that NFAT5$_{171-250}$ is the minimal domain required for directing nuclear import of heterologous protein (Figure 3.2B), deletion-mapping analysis of *in vitro* interaction studies showed that that shortened protein fragments

within this region interacts with importin-β respectively (Figure 3.6B). More intriguingly, although this minimal domain interacts with importin-β, inclusion of amino acid sequences proximal and distal to this region abolished the interaction of this fragment with importin-β. Nevertheless, further lengthening of the protein sequences to the C-terminal (Figure 3.6B, lane D) restored its interaction with importin-β. This observation could be explained by the fact that truncated domain may not present the correction conformation required for the interaction. Furthermore, although the minimal cNLS-like sequence interacts with the importin-β, full-length N-NLS may need to interact with importin-β at multiple sites to elicit nuclear import activity. Despite these discrepancies, consistent with earlier findings, mutation of the three core amino acid residues $_{202}$RKR$_{204}$ completely abolished the interaction between importin-β and NFAT5$_{171-250}$, suggesting that these three amino acid residues are critically important for the interactions between N-NLS and importin-β.

To further understand the underlying mechanism of NFAT5 nuclear import, nuclear import activity of N-NLS was evaluated using in vitro nuclear import assay in the presence of defined factors. First of all, different fragments of NFAT5$_{171-250}$ were fused in frame with AcGFP respectively. They served as reporters to allow the tracking of the fusion protein using confocal microscopy in real time. When cells were deprived of cellular factors after permeabilization by detergent, replenishment of cytosolic extracts, RanGTP, and ATP are required to direct nuclear import of NFAT5$_{171-250}$-AcGFP fusion protein into the nucleus (Figure 3.8, lane 1), suggesting that nuclear entry of NFAT5 requires cytosolic factors, and is a RanGTP- and an energy-dependent process. On the other hand, nuclear import of NFAT5$_{171-250}$-AcGFP was inhibited by nuclear pore inhibitor, excess GTP, or non-hydrolysable GTP,

suggesting that the protein enters nucleus via nuclear pore, and is dependent to a GTP gradient similar to classical nuclear import pathway (Figure 3.8, lane4 -6). To understand the mechanism further, *in vitro* nuclear import assay was conducted in the presence of individual protein factors. Specifically, individual purified importins were tested for its ability to direct the fusion protein. Similar to the observation earlier, importin-β alone, but not importin-α, is able to direct nuclear import of NFAT5$_{171-250}$-AcGFP in the presence of RanGTP, NTF2 and energy (Figure 3.9, lane 9). This is clearly distinctive from the classical nuclear import pathway, because SV40-NLS-AcGFP protein undergoes nuclear import only in the presence of both importin-α and importin-β (Figure 3.9, lane 3). These observations are consistent with the observation from the *in vitro* pull-down analysis.

To determine the protein domains responsible for *in vitro* nuclear import by importin-β, deletion mutants of NFAT5$_{171-250}$-AcGFP were generated. Interestingly, however, although the minimal region composed of the putative cNLS alone (a.a. residues 198-217) is sufficient (Fig. 3.6B, lane F) and essential (Fig. 4A, lane E) for the interaction of this protein domain with importin-β significantly, it is not sufficient for directing nuclear import of cargoes (Fig. 3.10). The classical NLS-like region, together with protein region either proximal or distal to this region, are required are required for nuclear import activity of the N-NLS. In contrast, the NLS-like region, the distal region, or proximal region alone failed to mediate nuclear import (Figure 3.10, lane 4, 6 and 7). These observations are clearly different from the results of *in vitro* pull down assay, which showed that the classical NLS-like region forms stable complex with importin-β (Figure 3.6B, lane 6).

Earlier studies showed that importin-β, by itself, mediate nuclear import of a number of proteins. These include THrP, SREBP2, human immunodeficiency virus (HIV)

Rev and Tat, cyclin B1, Smad-3, SREBP-2, parathyroid hormone-related protein, and snurportin1 respectively (Harel & Forbes, 2004). Structural analysis has been conducted on several proteins (THrP, SREBP2, and snurportin1) (Cingolani, Bednenko, Gillespie, & Gerace, 2002; Lee et al., 2003; Wohlwend, Strasser, Dickmanns, & Ficner, 2007) and confirmed that importin-β interacts with unique nuclear import sequences on these proteins. These data also suggested that importin-β is a flexible protein that interacts with structurally heterogeneous NLS sequences through different regions on the protein and through distinct binding modes (Forwood et al., 2010). Therefore, it is possible that although importin-β interacts with different regions within the N-NLS, a strong interaction is required to induce nuclear import activity of importin-β. Alternatively, interaction between N-NLS and importin-β at multiple sites may induce changes in the conformation of importin-β that is required for nuclear import. Therefore, it is likely that N-NLS interacts with importin-β in a distinctive manner.

Although NFAT5 is the only known transcription factor that could carry out nucleocytoplasmic trafficking in response to the change in extracellular tonicity, it is found that hypertonic stress could also induce the nuclear import of several transcription factors such as signal transducer and activator of transcription 1 (STAT1), STAT3 (Gatsios et al., 1998) and activator protein-1 (AP-1) (Irarrazabal et al., 2008). However, the nuclear import of STAT1 and STAT3 is importin α/β-dependent (Melén et al., 2003; Ushijima et al., 2005). Both AP-1 and NFAT5 contribute to cellular osmoadaptation and both of them undergo nuclear import in importin β-dependent manner (Irarrazabal et al., 2008; Tong et al., 2006), suggesting that importin β-mediated nuclear import might help to differentiate hypertonic stress from other stimuli such as UV and oxidant stress. On the other hand, Riddick and

Macara (2007) suggest that direct importin β-mediated nuclear import is faster and less energy-consuming than importin α/β nuclear import. Since long-term exposure to hypertonic stress is harmful to cells, importin β-dependent and importin α-independent nuclear import of NFAT5 and AP-1 might facilitate the cellular osmoadaptation, leading to increasing survival rate of cells that are always subjected to hypertonic condition such as cells in kidney inner medulla.

Immunofluorescence (IF), *in vitro* pull down assay and *in vitro* permeabilized cell assay were used together to investigate nucleocytoplasmic trafficking of NFAT5. IF is one of the most commonly used methods to observe the subcellular localization of molecules. It is of high specificity and sensitivity because of the use of specific antibodies. Also, each time of IF allows detection of multiple molecules and thus, it could be used to examine colocalization of molecules. However, in IF, cells are fixed before staining. As a result, the real-time tracing of molecular trafficking is not allowed. Also, the fixatives might cause damage to lipid components of cells and cross-linking of epitops, which might affect the experimental results.

In vitro pull down assay is an easily handled method to determine the direct interaction of proteins. Bacterial cells are used for protein overexpression. Therefore, the amount of proteins used in each time of pull down is large comparing with immunoprecipitation. Furthermore, mutation could be introduced to target protein easily by site-directed mutagenesis, and truncated forms of proteins could be generated and used in in-vitro pull down as well. However, truncated and mutated proteins generated might have different conformation comparing with wild type proteins. Also, post-translational modification is lacked in bacterial cells and hence, the interaction between proteins might be different from what observed in in-vivo system.

In-vitro permeabilized cell assay is a well-established method developed over 15 years ago by Adam, Marr, and Gerace (1990).It is widely used to characterize the direct contribution of factors to the nuclear import of specific cargos and the mechanism of nuclear transport. Fluorescence tags on protein cargos allow the visualization of protein cargo. However, the concentration of digitonin used needs to be optimized. Inappropriate concentration of digitonin increases the chance of incomplete plasma membrane permeabilization or nuclear envelope permeabilization. Also, digitonin-permeabilized cells are more easily washed away from the glass slide. The bacterial expressed fluorescent-tagged protein cargo might have different structure comparing with the protein found in in-vivo system and thus, the results might be different from that obtained from in-vivo experiments.

Besides, live cell imaging is widely used in cell biology research. It allows the *in-vivo* real-time detection of trafficking of molecules such as proteins and chromosomes (Jensen, 2013). In live cell imaging, recombinant proteins with fluorescent tags and live cell dyes are used to visualize the target molecules. Fluorescence resonance energy transfer (FRET) and fluorescence resonance energy transfer (FRET) could be used to detect protein-protein interactions. In FRET, recombinant proteins with tags could be expressed in cells. If two proteins interact or become spatially close to each other, the energy emitted from a protein would be absorbed by its interacting proteins, resulting in the excitation of the fluorescent tag on the interacting proteins (Song, Madahar, & Liao, 2011). As a result, the co-localization of a protein and its interacting partners could be determined. On the other hand, the dynamic of a protein could be examined using fluorescence recovery after photobleaching (FRAP). To examine the nuclear import, a region in nucleus could be bleached by a fine beam of laser. The recovery rate of the fluorescence signal of the

bleached region reflects the rate of the protein to travel from cytoplasm to nucleus (Carisey, Stroud, Tsang, & Ballestrem, 2011). However, the transport of the cells might induce stress to cells, the laser used in live cell imaging might cause apoptosis in live cells. Also, the results of live cell imaging are easily affected by temperature, humidity of the culture chamber, and the intensity of laser and other factors. Therefore, by comparing the pros and cons of the above methods, live cell imaging was not adopted in this project.

Chapter 5

Future work

Although in this study I have defined the amino acid sequences of a functional nuclear import signal of NFAT5, as well as defining importin-β as the nuclear receptor for its nuclear import, the underlying mechanism of how NFAT5 undergoes nuclear import, and the discrepancy between the amino acid sequences required for N-NLS nuclear activity and N-NLS/importin-β interaction remains unsolved. To further understand the underlying mechanism of N-NLS nuclear import activity, structural analysis of the N-NLS/importin-β protein complexes will be helpful. This could be done by crystallization of the protein complexes, followed by X-ray diffraction analysis for structure elucidation. The crystal structure will define the interaction domains between N-NLS and importin-β at molecular level, as well as providing insights into whether importin-β undergoes structural changes upon binding to full N-NLS versus N-NLS fragments. Such information will definitely enhance our understanding on the molecular mechanism of NFAT5 nuclear import.

NFAT5 undergoes nuclear export under hypotonicity, mediated by a putative auxiliary export domain (AED) (Tong et al., 2006; Xu et al., 2008). Interestingly, although the AED is essential for hypotonicity-mediated NFAT5 nuclear export, this domain failed to direct nuclear export of a heterologous protein. A relevant question is whether AED requires the collaboration with N-NLS for nuclear export. In other transcription factors, NLS activity could be modulated by adjacent protein domain (Okamura et al., 2000), or by another protein (Karin & Ben-Neriah, 2000). Under these circumstances a change in NLS activity is usually associated with post-translational modifications of the NLS, or of a nearby domain, resulting in a

conformational change of the NLS structure, or the affinity the NLS to its binding partner. My data suggested that nuclear import of NFAT5 can be accomplished solely by importin-β. However, whether N-NLS is also involved in nuclear export remains elusive. Therefore, it would be important to determine if AED is working in conjunction with N-NLS to mediate nuclear import, and if these sequences undergo post-translational modifications upon hypotonic challenge.

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