NUCLEOCYTOPLASMIC LOCALIZATION OF MAPKS

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To my family and friends

NUCLEOCYTOPLASMIC LOCALIZATION OF MAPKS

by

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NUCLEOCYTOPLASMIC LOCALIZATION OF MAPKS

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Mitogen-activated protein kinases (MAPKs) comprise a family of protein-serine/threonine kinases, which participate in signal transduction pathways that control intracellular events. MAPKs are regulated by phosphorylation cascades, which are usually initiated by external stimuli including a variety of ligands. At least two upstream protein kinases are activated in series to lead to activation of a MAPK. The kinase that activates the MAPK is a MAPK kinase (MAP2K or MEK) and the kinase that phosphorylates the MAP2K is a MAP3K or MEK kinase (MEKK). Upon activation, MAPKs may translocate to the nucleus to phosphorylate nuclear targets. Previous findings from our laboratory showed that a constitutively active and nuclear form of the

MAPK ERK2 is sufficient for transformation of immortalized fibroblasts (Robinson MJ et al,1998). However the mechanisms of nuclear localization of MAPKs are still not fully understood clearly. Although most nucleocytoplasmic localization events require carrier proteins known as karyopherins (importins and exportins), ERK2 enters the nucleus of permeabilized cells even if these carrier proteins are missing. This is explained by direct binding to proteins in the nuclear pore complex (NPC). Similar to ERK2 targets, NPC proteins also contain Phe-Xxx-Phe (FXF) motifs.

My first aim in this project was to examine the roles of ERK2 residues that are crucial for FXF binding on nuclear localization of ERK2. Mutating these ERK2 residues decreased the nuclear import of ERK2 proteins in permeabilized cells. Secondly, the regulation of ERK2 nuclear export was analyzed. It was observed that ERK2 export occurs by two distinct processes; one energy-dependent and the other energy-independent. My final aim was analyzing the activation and nucleocytoplasmic trafficking of other MAPKs, JNK and p38.

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List of Tables and Figures

Abbreviations

ATP	Adenosine 5'-triphosphate
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Mono Phosphate
CD	Common Docking
CDK	Cyclin-Dependent Kinase
CRM1	Chromosomal Region Maintenance 1
D domain	Docking Domain
DED	Death Effector Domain
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetracetic acid
ERK	Extracellular Signal-Regulated Protein Kinase
FGF	Fibroblast Growth Factor
FBS	Fetal Bovine Serum
GAP	GTPase-Activating Protein
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GSK	Glycogen Synthase Kinase
GST	Glutathione-S-transferase
GTP	Guanosine 5'-triphosphate
HA	Hemagglutinin
HFF	Human Foreskin Fibroblast
IF	Immunofluorescence
JNK	c-Jun-N-terminal kinase
Kap	Karyopherin
KOH	Potassium Hydroxide
KSR	Kinase Suppressor of Ras
K_d	Dissociation Constant
LMB	Leptomycin B
LPA	Lysophasphatidic Acid
MAPK	Mitogen-Activated Protein Kinase
MAP3K	MAP Kinase Kinase Kinase
MAP2K	MAP Kinase Kinase
MEK	MAPK-ERK Kinase
MEKK	MEK Kinase
$MgCl_2$	Magnesium Chloride
MKP	Map Kinase Phosphatase
MLCK	Myosin Light Chain Kinase
MNK	MAPK-Interacting Kinase
MP1	MEK Binding Partner 1
MSK	Mitogen and Stress-Activated Kinase
NaCl	Sodium Chloride
$NaVO_3$	Sodium Orthovanadate

NES	Nuclear Export Sequence
NGF	Nerve Growth Factor
NLS	Nuclear Localization Sequence
NPC	Nuclear Pore Complex
NTF2	Nuclear Transport Factor 2
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma 12
PDGF	Platelet-Derived Growth Factor
PEA-15	Phosphoprotein Enriched in Astrocytes – 15 kDa
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PMA	Phorbol Myristate Acetate
RanGAP	Ran GTPase-Activating Protein
RanBP7	Ran Binding Protein 7
RCC1	Regulator of Chromose Condensation 1
REF	Rat Embryo Fibroblast
RNAi	RNA Interference
RSK	Ribosomal S6 Kinase
SDS	Sodium Dodecyl Sulfate
TB	Transport Buffer
Thio-P	Thiophosphorylated
TRITC	Tetramethylrhodamine B Isothiocyanate
WGA	Wheat Germ Agglutinin
WNK	With No Lysine (K)

Chapter 1: General introduction:

MAPKs

Many cellular activities are regulated by protein phosphorylation. Protein kinases are the enzymes that catalyze phosphorylation events, in which serine, threonine or tyrosine residues of the substrates are phosphorylated. Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases, which play critical roles in transducing signals from extracellular stimuli, including hormones, growth factors, and environmental stresses, throughout the cell. MAPKs are involved in many cellular regulation processes including cell proliferation, the control of gene expression, and programmed cell death (Pearson *et al*, 2001; Robinson *et al*, 1998; English *et al*, 1999; Chen *et al*, 2001; Brunet *et al*, 1995; Pouyssegur *et al*, 1993).

MAPK activity is regulated through a three-tiered kinase cascade composed of a MAPK, MAPK kinase (MAP2K, MKK or MEK) and a MAP2K kinase or MEK kinase (MAP3K or MEKK). MAP3Ks and MAPKs are serine/threonine, and MAP2Ks are dual specificity (serine/threonine and tyrosine) protein kinases. There are twenty MAPKs identified in mammals, and among them the best studied MAPKs are extracellular regulated protein kinases 1 and 2 (ERK1/2), three members of the c-jun N-terminal kinases (JNK1-3), four isoforms of p38 (α, β, γ, and δ), and ERK5. The other MAPKs include ERK3, ERK7 and NLK (Pearson *et al*, 2001; Robinson *et al*, 1998; Gupta *et al*, 1996; Kuida *et al*, 2004; Boulton *et al*, 1991).

MAPKs are activated only by MAP2Ks and each MAP2K can phosphorylate a few MAPKs, sometimes only one (Kosako *et al* , 1992; Crews *et al*, 1992, Wu *et*

al,1993). Although MAPKs can be activated by a small number of MAP2Ks, there are many MAP3Ks that can phosphorylate MAP2Ks (Figure 1-1).

Activation of MAPKs is achieved by phosphorylation on both tyrosine and threonine on the activation loop. MAPKs are phosphorylated first on tyrosine then threonine during the activation by MAP2Ks (Robbins and Cobb, 1992). Similarly, MAP2K activation occurs by phosphorylation on two residues in their activation loop. However, these residues on MAP2K are either serine or threonine but not tyrosine (Ahn and Seger, 1992; Chen, 1994).

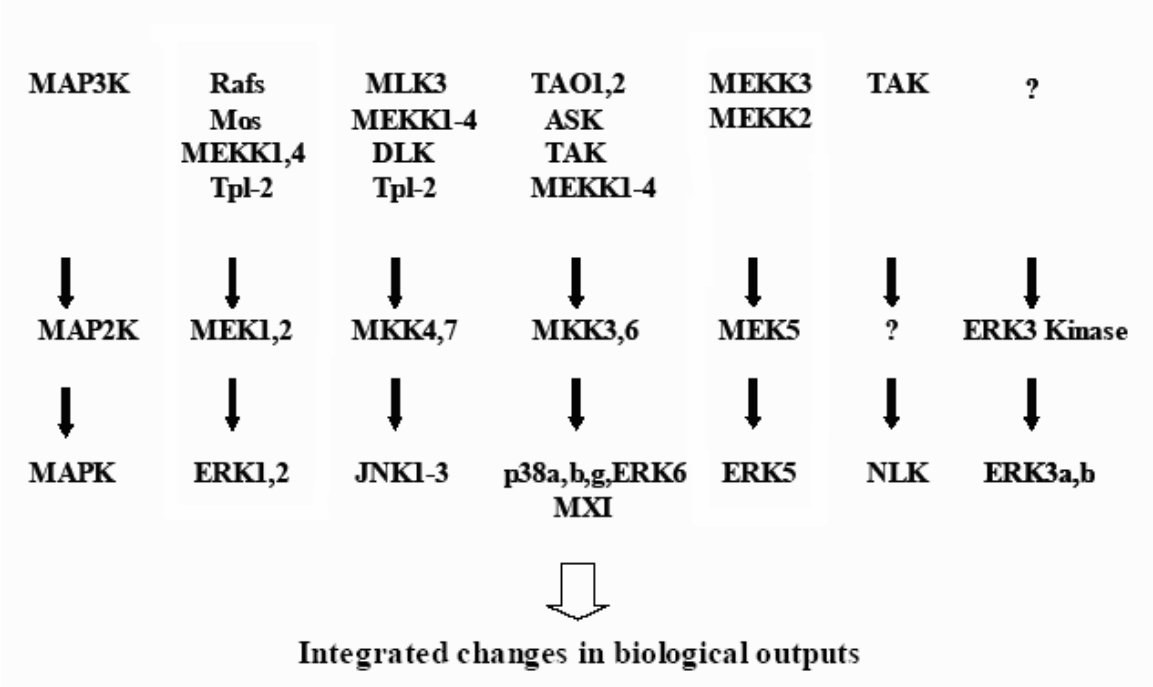


Figure 1-1. MAPK cascades. (Pearson, 2001)

ERK1/2

ERK1 and ERK2 are the best studied MAPKs, and 43 and 41 kDa respectively. They are 85% identical and most differences are outside the kinase core (Boulton,1991). Multiple stimuli including growth factors, phorbol esters, ligands for heterotrimeric G protein-coupled receptors, microtubule disorganization, and osmotic stress can activate ERK1 and ERK2. They are ubiquitously expressed in varying amounts depending on the cell type. The MAPK knockout phenotypes are summarized in table 1.1.

Tyrosine and threonine residues on the activation loop are separated by a glutamate, forming a TEY motif (Payne, 1991). Upon phosphorylation activity of ERK1 and ERK2 increases by more than 1000 fold (Prowse and Lew, 2000). MEK1 and MEK2 are the upstream kinases that can phosphorylate ERK1 and ERK2 (Kosako, 1992; Zheng, 1993). Substitutions of the two phosphorylation sites of MEK1 and MEK2 with acidic residues and deletions in the N terminus increase activities of these enzymes (Mansour *et al* ,1996). The combination of these mutations yields constitutively active MEK1 and MEK2 mutants.

ERK2 Structure

Structures of ERK2 in phosphorylated and unphosphorylated forms were solved (Zhang *et al* ,1994; Canagarajah *et al* ,1997) (Fig 1-2). ERK2 has two domains; the N-terminal domain is composed primarily of β strands and the C-terminal domain consists primarily of α helices. The interface of the N-terminal and C-terminal domains creates the proper active site (Wang *et al*, 1998; Goldsmith *et al*, 2004; Cobb *et al*, 1995).

A surface loop, L12 is also called the activation loop or phosphorylation lip as it contains the activating phosphorylation sites, T183 and Y185. Although activation of

some kinases requires a single phosphorylation, ERK2 must be dually phosphorylated on these two phosphoacceptor residues for full activation. If ERK2 is unphosphorylated, the phosphorylation lip blocks the binding site for protein substrates (Zhang *et al*, 1995; Canagarajah *et al,* 1997; Wang *et al*, 1998; Goldsmith *et al*, 2004).

In the unphosphorylated ERK2, the phosphorylation lip contacts the MAPK insert, an α-helical insert unique to MAPK and the cyclin-dependent kinases (CDKs). MAPK insert in ERK2 is comprised of two helices, and composed of about 50 residues. The MAPK insert is found in the C-terminal domain adjacent to the substrate docking surface. Upon dual phosphorylation, phosphorylation lip changes conformation and substrate binding is enhanced (Canagarajah *et al,* 1997; Wang *et al*, 1998; Goldsmith *et al*, 2004)

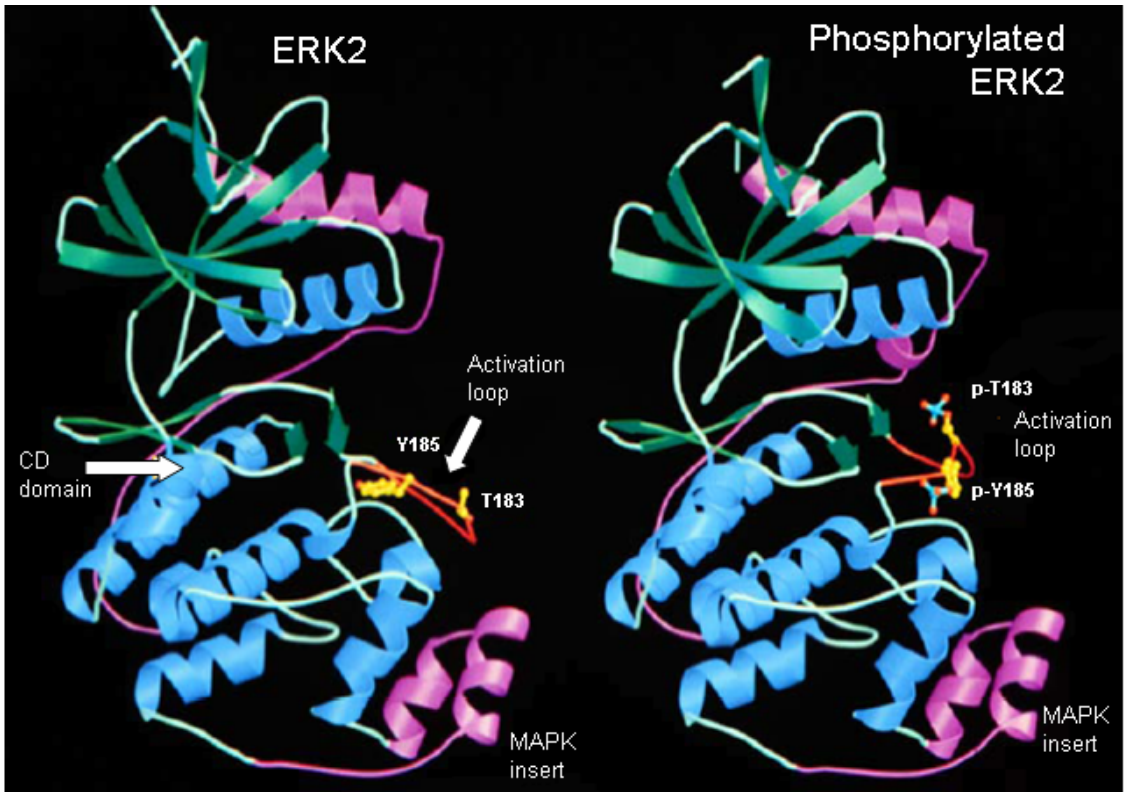


Figure 1-2: Structure of phosphorylated and unphosphorylated ERK2 (Canagarajah *et al*, 1997)

As in other protein kinases, lysine residue (K52) in the N-terminus is critical for orienting ATP; mutating this residue makes an inactive form of ERK2 (kinase dead) (Robinson *et al*, 1996).

ERK2 dimerizes in the dually phosphorylated form. A leucine-zipper and two ion pairs are critical for dimer formation. Mutating the leucine residues at positions 333, 336, 341 and 344 to alanine and introduction of charge repulsion to disrupt (H176E) creates a dimerization-deficient form of ERK2 (Khokhlatchev *et al*, 1998;).

p38

p38 was discovered by three different groups (Lee *at al* ,1994; Han *et al*, 1994; Rouse *et al*, 1994) as MAPKAP kinase-2 activator, a tyrosine phosphoprotein activated in response to LPS, and a target of an anti-inflammatory drug. The first identified member was p38α. cDNAs encoding the other members p38β, p38γ and p38δ were isolated shortly after (Stein *et al* 1997, Jiang *et al*,1996; Jiang 1997; Lechner *et al* , 1996)
p38 is identical 75% to p38β, 62% to p38γ, and 64% to p38δ. The phosphorylation motif in p38 is TGY, where a glycine separates the threonine and tyrosine residues. MEK3 and MEK6 are the major activators of p38 (Stein *et al* 1996, Han *et al* 1996).

Mxi2 is a splice isoform of p38α. It is identical to p38 from amino acids 1 to 280, but the last 80 residues of p38 are replaced with a unique 17 amino acid C terminus (Zervos ,1995). Mxi2 has some biochemical properties that distinguish it from other p38s. Its activity against p38 substrates is low, and it is not inhibited by pyridinyl imidazoles (Sanz, 2000). Crespo and colleagues found that Mxi2 binds to ERK1/2 directly; this interaction affects ERK1/2 nuclear signaling (Sanz-Moreno *et al*, 2003)

p38 can be activated by various physical and chemical stresses, including UV irradiation, DNA damage, hypoxia, ischemia/reperfusion ,hypo-osmolarity, TNFα, and interleukin-1 (IL-1) (Brewster,1993, Hazzalin 1996; Scott, 1998; Bogoyevitch, 1996; Kumar,1997; Lee *et al* , 1994).

Table 1-1. Summary of MAPK knockout phenotypes (Adapted from Kuida *et al*, 2004).

Genes	Summary of phenotypes
ERK1	Decreased T cell responses (Pages G *1999*)
ERK2	Lack of mesoderm differentiation (Yao Y *2003*) and defects in placenta (Hatano N 2003)
JNK1	Defects in inhibiting Th2 differentiation (*Dong C 1998*) in T cell activation and apoptosis of thymocytes (*Sabapathy 2001*)
JNK1	Less susceptible to insulin resistance (Hirosumi 2002)
JNK2	Defects in Th1 differentiation (*Yang 1998*)and T cell activation and apoptosis of thymocytes (*Sabapathy 1999*)
JNK1+JNK2	Neural tube closure defects (*Kaun 1999*,*Sabapathy 1999*)
JNK1+JNK2	Increased IL-2 production in T cells (*Dong 2000*)
JNK1+JNK2	Embryonic fibroblasts have increased resistance to UV-induced apoptosis (Tournier 2000)
JNK3	Resistance to kainate-induced neural damage (*Yang 1997*)
p38α	Placental angiogenesis defects (*Mudgett 2000*, *Adams 2000*)
p38α	Epo production defects (*Tamura 2000*)
p38ß or p38γ	No obvious phenotype (Kuida 2004)
ERK5	Cardiovascular development and angiogenesis defects (Regan 2002, Sohn 2002, *Yan 2003*)

JNK

The c-jun N terminal protein kinase (JNK), also known as stress-activated protein kinase (SAPK), is a member of the MAPK group that is activated in response to dual phosphorylation on threonine and tyrosine. This kinase participates in many stress responses, such as those induced by hypoxia, cold shock, and hyperosmolarity.

Initially JNK/SAPK was first purified as a 54-kDa protein kinase markedly activated after intraperitoneal injection of cycloheximide in the rat. (Kyriakis *et al*, 1990). It was also isolated as a protein kinase that binds stably to c-Jun and phosphorylates S63 and S73 in the c-Jun N terminus (Hibi *et al*, 1993). Three JNK genes (*JNK1*, *JNK2*, and *JNK3*) have been identified in humans; however, splice variants result in at least 10 isoforms (Gupta *et al*, 1996). All of these kinases contain the sequence TPY in their activation loops.

MEK4 and MEK7 are the kinases that activate the JNKs. The specificities of MEK4 and MEK7 are not as great as MEK1/2, because they can also activate p38 family members *in vitro*. (Meier *et al*, 1996).

Docking Domains for MAPK binding:

MAPKs phosphorylate serine/threonine residues followed by proline residues in their substrates. MAPKs can form tight complexes with their substrates through certain docking sites within the substrates. Docking site-MAPK interaction increases specificity of the interaction and efficient phosphorylation of the substrates. The first evidence for MAPKs can bind to their substrates with certain docking sites was acquired by studies on JNK and c-Jun interaction (Hibi *et al*, 1993; Kallunki *et al*, 1996). A domain which is

called delta domain was shown to be required for stable association between JNK and c-Jun.

D domain:

The docking or D domain resembles the delta domain found in c-Jun. The other name used for D domain is DEJL (Docking site for ERK and JNK, LXL), motif. This domain consists of a group of basic amino acids (lysine or arginine) usually located N- or C-terminal to a hydrophobic motif containing leucine, isoleucine, or valine residues separated by one residue. The D domain is present on many ERK1/2 substrates, including Elk-1 and c-Fos. This motif is not only found in substrates but also found in MAPK regulators such as MAP2Ks, MKPs, and scaffolding proteins (Kallunki *et al,* 1994; Bardwell and Thorner 1996; Yasuda *et al,* 1999; Sharrocks *et al,* 2000 and Tanoue *et al,* 2000).

The D domain can be recognized by ERK1/2, ERK5, JNK and p38 through interaction with their common docking (CD) domains (Enslen *et al,* 2000; Barsyte-Lovejoy *et al,* 2002 and Ho *et al,* 2003). The CD domain is an acidic cluster conserved in all MAPKs. In ERK2, aspartic acids at positions 316 and 319 create part of the binding site essential for D domains binding. The electrostatic interaction between these acidic residues in MAPKs and basic residues in the D domain are important for MAPK-substrate interaction, but not the only factor; hydrophobic residues in the D domain are also important.

FXF motif:

The other motif currently identified for MAPK binding is observed in several ERK1/2 substrates, including Elk1, c-Fos, SAP1, and phosphodiesterase 4D (Jacobs *et al*, 1999; Fantz *et al*, 2001; Galanis *et al*, 2001; MacKenzie *et al*, 2000 and Murphy *et al*, 2002). This motif has the an FXF consensus sequence and is named the FXF or DEF motif (Docking site for ERK, FXF). Although it serves as a docking site for ERK1/2 mainly, the FXF motif is also a docking site for p38α, but is not hought to bind to other MAPKs (Jacobs *et al*, 1999; Galanis *et al*, 2001). Some ERK1/2 interacting proteins bear both D domains and FXF motifs. Ahn and Goldsmith groups' collaborative study using hydrogen exchange mass spectrometry identified the regions of ERK1/2 required for interaction with FXF motif (Lee *et al*, 2004). Phosphorylation of ERK2 exposes these regions to bind to the FXF motif stronger (Figure 1-3).

MAPKs have cytosolic, nuclear, mitochondrial and membrane localized targets. For instance, ERK1/2 substrates include other kinases (e.g.: MAPKAP kinase-2, p90 ribosomal protein S6 kinase 2), proteins involving in cell adhesion (e.g.: paxilin), and transcription factors (e.g.: Elk1, c-Fos), which are in distinct parts of the cell (Ku *et al*, 2000; Gavin *et al*, 1999; Cano *et al*, 1996; Minden *et al*, 1994; Kortenjann *et al*, 1994). MAPKs can phosphorylate their substrates by localizing to certain sub-cellular compartments. The mechanism of nuclear localization of MAPKs, especially ERK1/2, is important for access to nuclear substrates such as those that are critical regulators of cell cycle progression and cellular proliferation. This subject will be described in more detail in the following sections. In order to understand what is known about MAPK nuclear

localization, a basic description of the nuclear transport mechanisms will be discussed next.

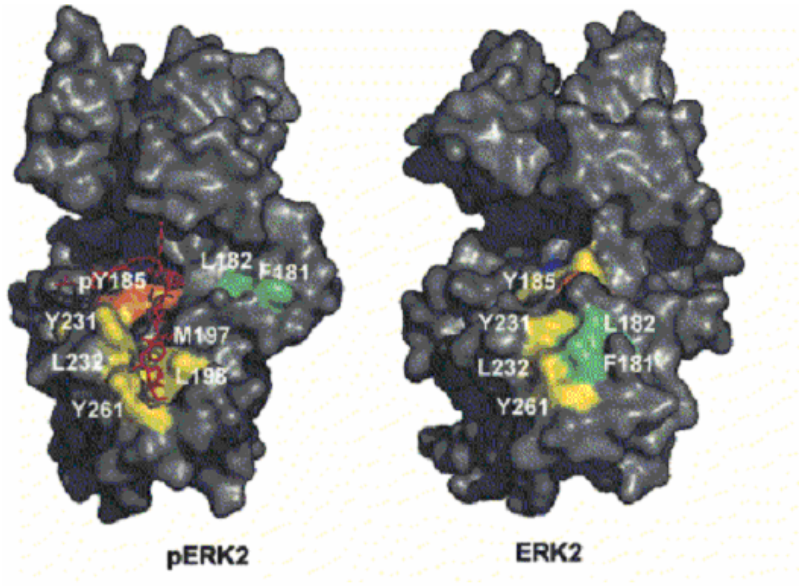


Figure 1-3 FXF Motif Interactions Are Enhanced by Phosphorylation and Activation of ERK2 (Lee *et al*, 2004) Activated ERK2 is on the left. The FXF peptide used in this study is shown in red.

Transport into the Nucleus

Nuclear Pore Complex (NPC):

The nuclear envelope (NE) is composed of a double membrane bilayer that separates the cytoplasm from the nucleus. Macromolecules can be transported between these compartments selectively through nuclear pore complexes (NPCs). The NPC is a large protein assembly with an estimated mass of ~66 MDa in fungi and ~125 MDa in vertebrates. The eightfold symmetrical NPC structure is composed of a central core embedded in the nuclear membrane, and extensions that make up the cytoplasmic

filaments and the nuclear baskets (Fig 1-3) (Reichelt et al 1990; Rout & Blobel 1993, Yang et al 1998; Chook and Blobel, 2001; Gorlich and Kutay, 1999).

The proteins that make up the NPC are called nucleoporins. The NPC is built from 30 different nucleoporins (Rout *et al*,2000; Cronshaw *et al*, 2002). Each NPC has multiple copies of nucleoporins. In this manner a large structure can be formed by a relatively small number of components. Only a few nucleoporins are located asymmetrically on either the nuclear face or the cytoplasmic face, while most of the nucleoporins are located symmetrically on both sides of the NPC. Some of the nucleoporins are essential for binding to transport factors, while some of them are essential for the proper structure of the NPC (Mosammaparast *et al,* 2004; Chook and Blobel, 2001; Gorlich and Kutay, 1999; Kuersten *et al*, 2001;).

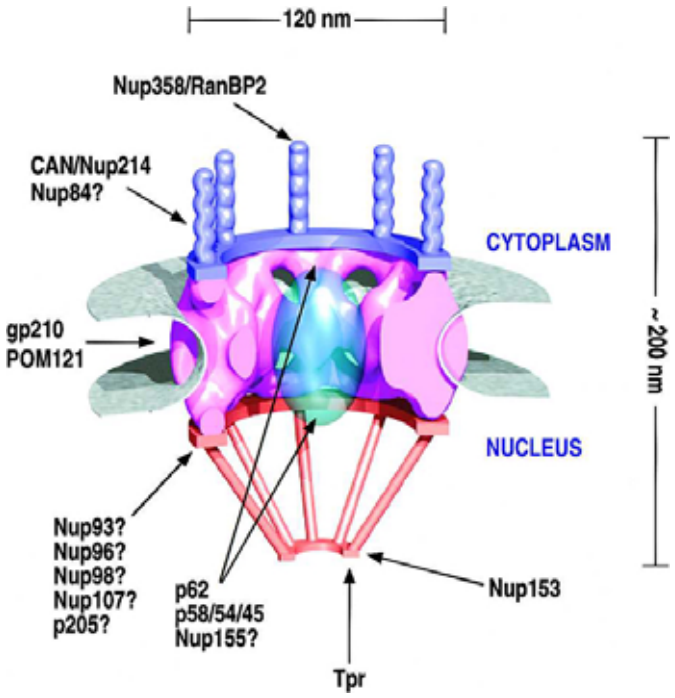


Figure 1-4 Nuclear Pore Complex Structure (Nakielny and Dreyfuss, 1999)

The number of NPCs per cell changes from cell to cell depending on several factors including cell size and proliferative activity. While there are no more than 200 NPCs in a yeast cell, the number increases to 3000–5000 in a proliferating human cell, and to 5×10^7 in a mature Xenopus oocyte (Rout and Blobel, 1993; Cordes et al, 1995; Nakielny and Dreyfuss*, 1999*).

Generally proteins smaller than 20-30 kDa are thought to pass through the NPC by passive diffusion, and proteins larger than 40 kDa must be specifically transported through the NPCs selectively. This selective transport is receptor-mediated, energy-dependent and triggered by specific transport signals. The first clue about transport signals was acquired during studies of the nuclear accumulation of nucleoplasmin. The tail of nucleoplasmin was found to be essential for nuclear import of the whole nucleoplasmin protein (Dingwall *et al,* 1982). Shortly after this study, the nuclear import signal of the simian virus 40 (SV40) large-T antigen was identified (Kalderon *et al,* 1984, Lanford and Butel, 1984). These arginine- and lysine-rich regions found in two different proteins were the first examples of classical nuclear localization signals (NLS).

A cell-based *in vitro* system developed by Adam and colleagues was a break through to study the nuclear import of a protein (Adam *et al*, 1990). This assay allows experimental manipulation, for example, to find the factors necessary for nuclear import of a certain protein. In this assay, cells are treated with digitonin, which selectively permeabilizes the cholesterol-rich plasma membrane but leaves the nuclear membrane intact. The cells are depleted of their soluble cytosolic contents. An import substrate which may be labeled with a fluorescent tag is added to the cells, and only when the factors required for import are added to the mixture, can nuclear import be observed The

transport of proteins and RNAs into the nucleus is usually achieved through proteins called karyopherins.

Karyopherins:

The karyopherins are transport factors that mediate the nucleo-cytoplasmic trafficking of proteins and RNAs. Karyopherins are an evolutionarily conserved family of relatively large proteins (95–145 kDa). Fourteen karyopherins have been identified in yeast and at least twenty karyopherins exist identified in mammalian cells. All the karyopherins consist of multiple HEAT repeats, which are essential for binding of karyopherins to the FG repeats of nucleoporins. The HEAT repeat is a helix–loop–helix structure, and named after the proteins in which it was first identified (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2a and TOR1) (Mosammaparast *et al,* 2004; Chook *et al,* 1999; Gorlich *et al,* 1999).

Karyopherins are also called importins and exportins depending on the direction of cargo transport. This nomenclature may be misleading as a karyopherin which can import one cargo might export another cargo as well. Importin 13 for example, can import the RNA-binding motif protein 8 and the MGN protein complex, but it can also export the translation initiation factor eIF1A (Mingot *et al*, 2001). Many of the cargoes for karyopherins and the direction of transports mediated by a specific karyopherins are not known. There are some proteins that do not require karyopherins to enter the nucleus. The Wnt pathway protein β-catenin contains armadillo repeats which resemble HEAT repeats, and can bind to nucleoporins directly (Fagotto *et al*, 1998). Smad2 can also pass

through the NPC without any karyopherins (Xu *et al*,, 2002). ERK2 is another example of a protein which can enter the nucleus by direct interaction with the NPC (Matsuyabashi *et al*, 2001; Whitehurst *et al*, 2002). In chapter 2 of this dissertation, we will focus on ERK2 residues that are important for nucleoporin interaction.

Nuclear Import-Export Cycle

The best understood nuclear import process is the import of proteins with classical NLSs. NLS-containing cargo is recognized by importin-α (karyopherin-α), then the karyopherin-α -cargo complex binds to importin-β (karyopherin-β) in the cytoplasm. After this complex passes through the NPC and enters the nucleus, RanGTP binds to the complex in the nucleus. This binding leads to the dissociation of the cargo from the importins and its release inside the nucleus *(*Nakielny and Dreyfuss, 1999; Chook *et al,* 1999; Gorlich *et al,* 1999; Kuersten *et al*, 2001).

Export occurs in the opposite direction of import and it involves cargo, karyopherin and another protein, Ran. Chromosomal region maintenance 1 (CRM1), also known as exportin-1, is a well characterized karyopherin that exports cargoes with a leucine rich nuclear export sequence (NES) out of nucleus. CRM1 can bind to these cargoes only in the presence of RanGTP. The affinity of CRM1 for its cargo increases upon RanGTP. This trimeric complex (cargo-CRM1-RanGTP) passes through the NPC. In the cytoplasm, RanGTP is converted to RanGDP. RanGDP has a decreased affinity for CRM1. Dissociation of Ran from CRM1 changes the conformation of karyopherin and the cargo is released in the cytoplasm (Gorlich *et al,* 1999; Kutay *et al,* 1997; Kuersten *et al*, 2001).

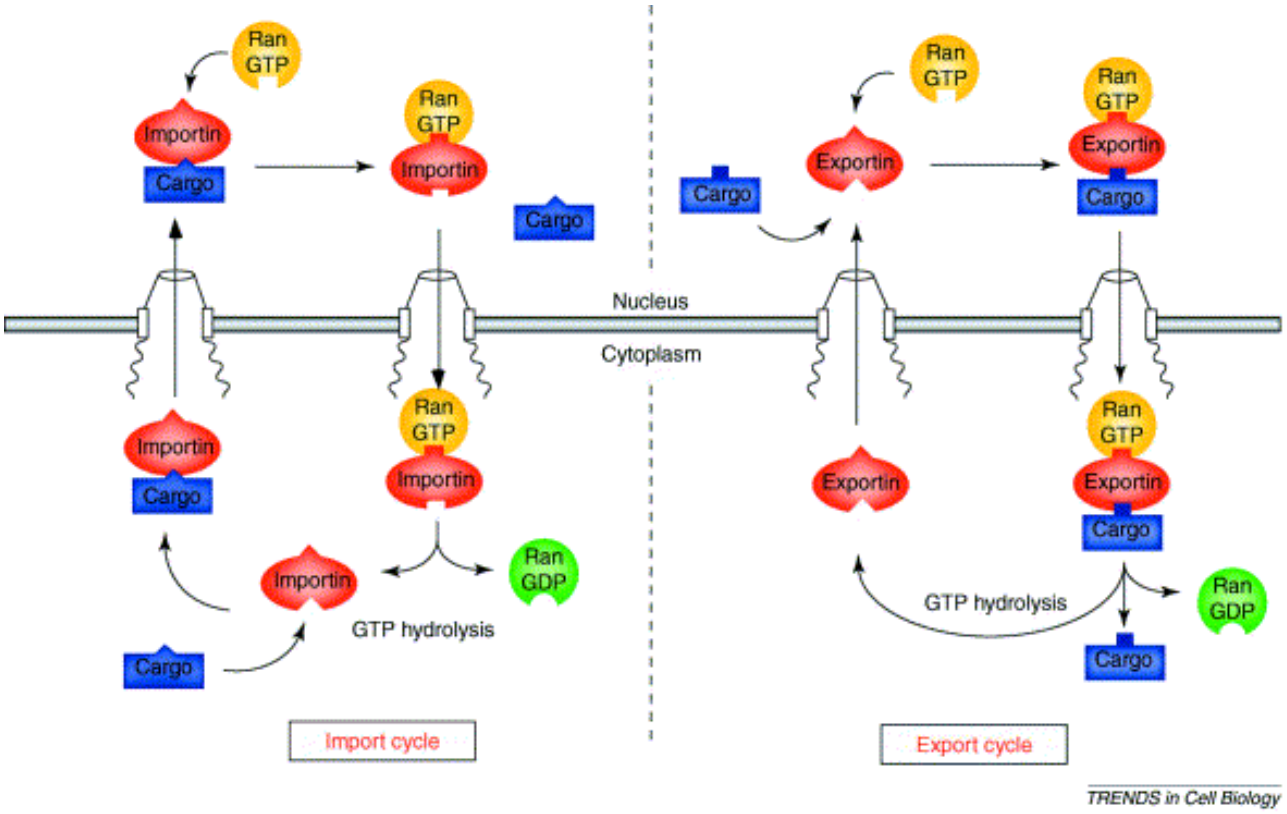


Figure 1-5: Nuclear import export cycle (Kuersten *et al*, 2001)

Ran is a Ras family GTPase. Ran is charged with GTP in the nucleus by the guanine nucleotide exchange factor (RanGEF) RCC1. In the cytoplasm, a Ran GTPase activating protein (RanGAP) stimulates GTP hydrolysis. As mentioned above, nuclear export factors bind to their cargoes in the presence of RanGTP, and the nuclear import factors can bind to their targets in the absence of RanGTP. RCC1 is on the chromosomes and strictly nuclear. Thus, Ran is mostly in the GTP-bound form in the nucleus, and in the GDP-bound form in the cytoplasm (Bischoff & Ponstingl *et al,* 1991; Ohtsubo *et al,* 1987; Bischoff *et al,* 1994; Mahajan *et al,* 1997).

MAPK nuclear localization

Although ERK1/2 do not possess any identified NLS or NES, they can shuttle between nucleus and cytoplasm. When phosphorylated ERK2 proteins were microinjected into the cytoplasm of quiescent rat embryo fibroblast (REF52) cells, the phosphorylated proteins rapidly translocated into the nucleus (Khokhlatchev *et al,* 1998). Unphosphorylated proteins also entered the nucleus but were exported quickly in five minutes, showing that ERK2 can enter nucleus without activation.

Transport factors that are required for the nuclear import of a protein can be found using i*n vitro* reconstitution assay explained above. Because mot of the cytosol is washed away, only factors that are required for nuclear import of the protein of interest will reconstitute the nuclear localization of the protein. Two different groups used this technique to identify the factors required for nuclear entry of GFP-ERK2 (Matsuyabashi *et al,* 2001; Whitehurst *et al,* 2002). Interestingly, GFP-ERK2 did not require any transport factors to enter the nucleus. In those experiments ERK2 was shown to bind to nucleoporins directly, suggesting that this binding was the mechanism for entry of ERK2 into the nucleus without the need for cytosolic factors. Indeed, addition of karyopherin-β and NTF2/p10 to the reaction did not increase but rather inhibited the import of GFP-ERK2. This might be due to competition for the same sites on nucleoporins by both ERK2 and karyopherins. Wheat germ agglutinin (WGA), which binds to *N*-acetyl glucosamine residues on nucleoporins and inhibits traffic through nuclear pore, also inhibited ERK2 entry into the nucleus in the reconstitution assay (Whitehurst *et al,* 2002). Similar to ERK2, some other proteins including Smad2 and β-catenin can bypass the

requirement for karyopherins to enter the nucleus. They perform this by direct binding to the NPC.

Question remaining was the role of the kinase activity of ERK2 on the import process. Different experiments showed that kinase activity is not required for ERK2 nuclear import. Phosphorylated kinase dead ERK2 (K52R) accumulated in the nucleus similar to the wild type protein (Khokhlatchev *et al*, 1998). *In vitro* reconstitution assays using wild type and kinase dead ERK2 gave similar results for import into the nuclei of digitonin-permeabilized cells. In these *in vitro* experiments, other ERK2 mutants including a dimerization defective (L4EH176E) and phosphorylation site mutant (T183A), were shown to behave similarly to wild type ERK2 in their nuclear import (Whitehurst *et al,* 2004).

If ERK2 can bind to nucleoporins, there must be motifs in the nucleoporins that ERK2 binds. In fact, most of the nucleoporins contain multiple FXF repeats that can be recognized by ERK2. If ERK2 residues that are crucial for FXF binding can be mutated, decreased nuclear import of ERK2 might be observed. As mentioned in an earlier section, ERK2 residues crucial for binding an FXF peptide from Elk1 were identified by Ahn and Goldsmith using hydrogen exchange mass spectrometry. Elk-1 is a substrate of ERK2 (Lee *et al,* 2004). Several residues on phosphorylated ERK2 were identified to be protected from the solvent, which is an indication of the interaction between those residues and the peptide (Figure 1-3). Mutations in these residues decreased phosphorylated-ERK2 binding to nucleoporins. In a later study, Dimitri and colleagues tested the behavior of these mutants by over-expressing them in HeLa cells. Over-expressed HA-ERK2 proteins, either wild type or mutants for FXF binding, were found

in the nucleus of the cells (Dimitri *et al*, 2005). The interaction between ERK2 and nucleoporins was not completely lost upon mutation of putative FXF binding residues. This suggests that over expressed proteins might still accumulate in the nuclei of cells via binding of FXF motifs to the impaired sites. The use of these mutant proteins in the *in vitro* import reconstitution assay might be a better way to test their role in the nuclear import. The results in our experiments show that mutations in ERK2 residues crucial for FXF motif binding decrease the apparent rate of nuclear import of ERK2 *in vitro.* This is explained in detail in chapter 2.

Role of MEK1 in ERK1/2 nuclear export

Export of ERK1/2 out of the nucleus is as intriguing as the import process. ERK1/2 do not have any identified leucine rich NES. On the other hand, MEK1, the MAP2K that activates ERK1/2, has a well identified NES (Fukuda *et al,* 1996). MEK1 is suggested to be one of the cytoplasmic anchoring proteins for ERK1/2 (Fukuda *et al,* 1997). In the inactive state, MEK1 can sequester ERK1/2 in the cytoplasm, but upon stimulation of cells, ERK1/2 are activated and their affinity for MEK1 decreases.

Other than cytoplasmic anchoring, MEK1 is also suggested to play a role in export of ERK1/2 by a piggyback mechanism. When ERK2 was injected into the nucleus, it was rapidly exported from the nucleus by coinjected wild-type MEK1. But injection of MEK1 lacking its NES could not export ERK2 (Adachi *et al*, 2000). Although this study shows that MEK1 can export ERK2 from the nucleus, it does not prove that MEK1 is an export mechanism in cells. Moreover, according to another study from the same group, ERK2 could be exported from the nucleus in the *in vitro* reconstitution assay

(Matsuyabashi *et al,* 2001). As explained in chapter 3, ERK may be exported from the nucleus by an energy-independent and an energy-dependent mechanism. The energy requirement comes at least in part from CRM1 which plays an important role. ERK2 export is not dependent on interaction with MEK1, as an ERK2 mutant that cannot bind to MEK1 is still exported from nucleus. To what extend MEK1 contributes to ERK2 export is still uncertain.

To identify the possible effect of ERK2 localization on cellular phenotype, a fusion protein composed of MEK1 and ERK2 (ERK2- MEK1) was made and expressed in cells (Robinson *et al*, 1998). The ERK2-MEK1 fusion localized primarily to the cytoplasm and was excluded from the nucleus of the cells. Mutation of the leucine residues in the NES of MEK1 to alanine (ERK2-MEK1-LA) inactivated the MEK1 NES. While ERK2-MEK1 stays in the cytoplasm, ERK2-MEK1-LA localized strongly in the nucleus. The fusion proteins, NES- containing and NES-deficient, caused different phenotypes. The ternary complex factor Elk-1 was phosphorylated in 293 cells that expressed the nuclear fusion protein, ERK2-MEK1-LA, but not in the cells expressing the cytoplasmic protein, ERK2-MEK1. In PC12 cells, ERK2-MEK1 but not ERK2-MEK1-LA promoted differentiation of the cells. The nuclear fusion protein also transformed NIH3T3 cells, while the cytoplasmic form did not have such an effect. These experiments showed that ERK2 activation may lead to different phenotypes depending on the cellular localization (Robinson *et al*, 1998).

Roles of Karyopherins in MAPK Signaling:

The role of karyopherins in MAPK signaling was first shown in yeast. HOG1, the p38 homolog in yeast can be activated by osmotic stress and activated HOG1 translocates into the nucleus. However the nuclear accumulation of HOG1 requires the karyopherin-β homolog NMD5, as HOG1 could not localize to nucleus of the cells without NMD5 (Ferrigno *et al*, 1998). HOG1 could still be activated in these cells. The defect in HOG1 nuclear localization is also not a nonspecific phenotype due to defect in any karyopherin, because HOG1 still localized to the nucleus in cells with karyopherin-α or karyopherin-β mutations. Export of HOG1 from the nucleus required the activity of the CRM1 homolog XPO1 (Ferrigno *et al*, 1998).

One study in *Drosophila* showed that *Drosophila* importin 7 (DIM-7) can interact with activated *Drosophila* ERK (D-ERK) (Lorenzen *et al*, 2001). When the gene encoding DIM-7 was deleted, the nuclear localization of active D-ERK was decreased, indicating a role of DIM-7 in nuclear import of D-ERK.

ERK1/2 can enter the nucleus without a requirement for any transport factors, but other MAPKs may require karyopherins to enter the nucleus. NMD5 in yeast and DIM-7 in *Drosophila* are homologs of each other. In mammals, the closest homolog of these proteins is the karyopherin Ran Binding Protein 7 (RanBP7 or Importin 7) (Gorlich *et al*, 1997). RanBP8 is another mammalian karyopherin with strong sequence similarity to NMD5 and DIM-7. Although RanBP7 and RanBP8 are specialized for different cargoes, it is not known whether they can compensate for each other in the nuclear import of specific proteins (Gorlich *et al*, 1997; Dean *et al,* 2001).

Chapter 2: Mutations in ERK2 binding sites affect nuclear entry

Abstract:

The MAPK ERK2 can enter and exit the nucleus by an energy-independent process that is facilitated by direct interactions with nuclear pore proteins. Several studies also suggest that the localization of ERK2 can be influenced by carrier proteins. Using import reconstitution assays, I examined a group of ERK2 mutants defective in known protein interactions to determine structural properties of ERK2 that contribute to its nuclear entry. ERK2 mutants defective in binding to substrates near the activesite or to basic/hydrophobic docking (D) motifs were imported normally. Several ERK2 mutants defective in interactions with FXF motifs displayed slowed rates of nuclear import. The import-impaired mutants also showed reduced binding to a recombinant C-terminal fragment of nucleoporin 153 which is rich in FXF motifs. In spite of the deficit revealed in some mutants via reconstitution assays, all but one of the ERK2 mutants accumulated in nuclei of stimulated cells in a manner comparable to the wild type protein; however, the mutant most defective in import remained in the cytoplasm. These results further support the idea that direct interactions with nucleoporins are involved in ERK2 nuclear entry and that multiple events contribute to the ligand dependent relocalization of these protein kinases.

Introduction:

The nuclear localization of the MAP kinases (MAPKs) ERK1 and ERK2 has been intensely explored because their regulated nuclear accumulation has been linked to their ability to change cell programs such as differentiation and proliferation (Chen et al, 1992; Lenormand et al, 1993; Robinson *et al,* 1998; Wu *et al,* 2000). The altered localization of these kinases has also been reported in disease (Nakopoulou *et al*, 2005).

The first studies of ERK1/2 localization indicated that ligands and other stimuli caused their nuclear translocation and that phosphorylation, which activates the protein kinases, is sufficient to promote their redistribution (Chen *et al*, 1992). An early indication that regulation of nuclear localization might be more complex came from microinjection studies with unphosphorylated ERK2 (Khokhlatchev *et al* 1998). Protein injected into the cytoplasm of fibroblasts rapidly entered the nucleus and then redistributed to the cytoplasm, suggesting that phosphorylation is not essential for nuclear entry but might be involved in nuclear retention.

Two laboratories used import reconstitution to show that unphosphorylated ERK2 enters the nucleus by a process distinct from the well characterized carrier-mediated mechanisms (Matsubayashi et al, 2001; Robinson *et al*, 2002). Nuclear uptake of ERK2 requires neither energy nor carrier proteins and has been proposed to occur by the binding of ERK2 directly to nuclear pore proteins. Two different nucleoporins (Nups), Nup153 and Nup214, were shown to bind to ERK2 in vitro. More recently a second import mechanism was found to occur with active, phosphorylated ERK2 (pERK2) but not with

the unphosphorylated protein; this second import process requires energy and cytosolic factors (Ranganathan *et al,* 2006).

Studies of ERK2 export using reconstitution in permeabilized cells concluded that, independent of its phosphorylation state, the export of ERK2 can occur by at least two processes. One process is carrier- and energy-independent and is consistent with the capacity of the Nup interaction to mediate bidirectional movement of ERK2, both into and out of the nucleus. Substantiation for the facilitated import and export mechanism came from studies using fluorescence recovery after photobleaching, which supported the conclusion that the nuclear entry and exit of ERK2 was rapid and did not require energy (Burack *et al* 2005). The second export process is active and is dependent on the export carrier CRM1 (Ranganathan *et al,* 2006). A role for CRM1 had already been deduced from the ability of the CRM1 inhibitor leptomycin B to increase the nuclear concentration of ERK1/2 (Adachi et al, 2000).

A variety of studies have suggested the importance of ERK1/2 binding proteins in determining their subcellular localization. The existence of a labile nuclear anchoring protein was inferred from a kinetic analysis of ERK1/2 activation and nuclear retention (Lenormand *et al* ,1998). Mxi2, an unusual splice variant of p38 MAPK, promotes nuclear translocation and retention of ERK1/2 (Sanz-Moreno *et al,2003* ; Casar *et al* 2006). MEK1/2, the upstream activators of ERK1/2, have been implicated in their cytoplasmic retention (Adachi *et al*, 2000). Over-expressed ERK2 accumulates in the nucleus even when not phosphorylated (Robinson *et al*, 2002; Rubinfeld,et al, 1999); when over-expressed, ERK2 is assumed to exceed MEK1/2 in concentration, resulting in an excess of free ERK2 to accumulate in the nucleus.

The small non-catalytic protein enriched in astrocytes of 15 kDa (PEA-15) was identified in several contexts including a screen for reversion of a Ras phenotype (Danziger *et al* 1995; Ramos *et al* ,2000; Condoelli *et al,* 1998). Its mechanism of action included binding to ERK1/2 and sequestering them in the cytoplasm (Danziger *et al* 1995). Using import reconstitution, PEA-15 was shown to bind to ERK2 preventing its nuclear import. Pull down experiments suggested that PEA-15 competed with Nups for binding to ERK2 (Whitehurst *et al,* 2004). Two-hybrid tests and an NMR study of ERK2-PEA-15 association suggested that PEA-15 binds to a short helical region of ERK2 known as the MAPK insert (residues 241-272) (Whitehurst *et al,* 2004; Hill et al 2002). In aggregate these and other findings suggest that the distribution of ERK1/2 is controlled not only by import and export processes but also by the presence of binding proteins with a range of affinities in both the cytoplasm and the nucleus (Lenormand et al, 1998; Casar *et al*, 2006: Danziger *et al,* 1995; Fukuda *et al*, 1997). In this study I examined the behaviors of a series of ERK2 mutants defective in known protein interactions to gain further insight into mechanisms of ERK2 nuclear localization.

Materials and Methods:

Constructs and Recombinant Proteins– His6-tagged GFP-ERK2 (rat), pERK2 and MEK1R4F were expressed and purified as described (Ranganathan *et al,* 2006; Robbins *et al*, 1993). The following His6GFP-ERK2 mutants were described (Whitehurst *et al,* 2002): K52R, T183A,L333A/L336A/L341A/L344A/H176E (referred to as L4A/H176E), D316A/D319A, Y261N, and Δ241- 272 (deletion of the MAPK insert). Rhodaminelabeled bovine serum albumin (BSA) coupled to a synthetic nuclear

localization sequence (NLS) was described previously (Whitehurst *et al,* 2002). Additional ERK2 mutants were generated by site-directed mutagenesis using the Quikchange kit (Stratagene).

Cell Culture – HeLa, HEK293, and BJ cells (human foreskin fibroblasts immortalized with h-TERT (Ramirez *et al*, 2004)) were grown on coverslips for 24 h in Dulbecco's modified Eagle's medium at 37oC in 10% CO2 supplemented with 10% fetal bovine serum and 1% L-glutamine and, in early experiments, 100 units/ml penicillin/streptomycin. BJ cells were incubated in serum-free medium for 2 h prior to use in import assays with pERK2.

Transfection–HEK293 and HeLa cells were transfected with Myc-ERK2 constructs using FuGENE 6 following the manufacturer's protocol (Roche Applied Science). After 48-72 h, cells were lysed in Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Samples were resolved by SDS-PAGE and analyzed by immunoblotting with appropriate antibodies after transferring to nitrocellulose membranes. Anti-Myc was from the National Cell Culture Center.

Import Assays--Import and export assays were as described (Whitehurst *et al*, 2002; Ranganathan *et al*, 2006). Cells were washed once in transport buffer (TB - 20 mM Hepes-KOH pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM ethylene glycol-bis (β-aminoethylether)-N,N,N',N'tetraacetic acid (EGTA) and 2 mM dithiothreitol (DTT)) and permeabilized for 5 min with 70 μg/ml digitonin. Import assays were for 15 min or as indicated in 40 μl TB with the following: 10 mg/ml BSA, and

either 0.8 μM recombinant ERK2 substrate (wild type or mutant GFP-ERK2 or pERK2) or 0.14 μM tetramethylrhodamine B isothiocyanate (TRITC)- NLS-BSA. In the indicated experiments dialyzed HeLa cytosol (2.5 mg/ml) was included as a source of transport factors along with an energy regenerating system containing 1 mM ATP, 1 mM GTP, 5 mM phosphocreatine and 20 U/ml creatine phosphokinase. After addition of 0.25 ml TB, cells were either fixed or used for export assays as described next. To examine export, pre-loaded cells were placed in 40 μl TB with 10 mg/ml BSA for 30 min or as indicated. The reaction was terminated with 0.25 ml TB. Cells were fixed for 10 min in 3% paraformaldehyde; coverslips were mounted with polymount.

p-ERK2 was detected by immunofluorescence in fixed cells that had been repermeabilized in 0.5% Triton-X 100 for 10 min. Cells were blocked in 1X Tris-buffered saline (TBS), 0.1% Tween-20, and 10 mg/ml BSA for 30 min at room temperature and incubated with the pERK1/2 antibody (Sigma) at 1:300 for 16 h at 4^{o} C; the secondary antibody, Alexa-546 anti-mouse (Molecular Probes), was used at 1:3000 for 1 h. Washes between incubations were performed 3X with TBS and 0.05% Tween-20 for 10 min.

Fluorescence Microscopy–Fluorophores were visualized by fluorescence microscopy using a Zeiss Axioskop 2-plus microscope and a Hamamatsu digital CCD camera (C4742-95). Exposures for all conditions within an experiment were kept the same; fluorescence intensity was quantified using Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.).

Binding Experiments–ERK2 binding to the C-terminal fragment of nucleoporin Nup153 was as described (Whitehurst *et al,* 2002). GST-Nup153c or GST were incubated

for 30 min with glutathione-agarose equilibrated in import buffer containing BSA (10 mg/ml) at 4^{o} C. Wild type and mutant His6-ERK2 proteins (0.2 μM) were added and mixed at 4^{o} C for another 2 h. Beads were washed 3 times with 1 M NaCl, 0.1% Triton X-100 in import buffer. Samples were resolved by electrophoresis in 10% polyacrylamide gels in SDS and transferred to nitrocellulose. Antiserum Y691 was used to detect ERK1/2 (Boulton *et al*, 1991). pERK2 was detected with monoclonal anti-MAPK antibody against activated ERK1/2 (Sigma) used at a 1:1000 dilution for immunoblotting and 1:200 for immunofluorescence.

Results:

*Known binding sites on ERK2: T*he behavior of ERK2 mutants, unphosphorylated and phosphorylated, were examined using reconstitution assays in permeabilized BJ cells in order to explore the role of ERK2 interactions in its nuclear entry. BJ cells are human foreskin fibroblasts immortalized with human telomerase (Ramirez *et al*, 2004). Two regions of ERK2 have been identified as docking sites for substrates and regulators that lie outside of the protein kinase active site. One region includes acidic residues (common docking (CD) site) and a hydrophobic groove on the C-terminal domain; this region binds to basic/hydrophobic docking (D) motifs in interacting proteins (Sharrocks *et al,* 2000; Tanoue *et al*, 2000; Liu *et al*, 2006; Zhou *et al*, 2006). The other region includes residues in a-helix G and the MAPK insert and binds substrates with FXF motifs (also called DEF motifs), as well as PEA-15 (Whitehurst *et al*, 2004; , Jacobs *et al*, 1999; Lee *et al*, 2004).

The behavior of the mutants in these two docking regions and nearer the active site of ERK2 were characterized (Fig. 1). Results of import assays are summarized in Table 1.

Import of ERK2 mutated in the putative Nup binding site:A former graduate student, Angelique Whitehurst, previously showed that PEA-15 blocks binding of ERK2 to Nups (Whitehurst *et al*, 2004). Thus, I tested the idea that ERK2 mutants that displayed reduced binding to PEA-15 might also interact less well with Nups. In a screen for ERK2 mutants with reduced binding to interactors, I found that ERK2 Y261N showed reduced binding to PEA-15 (Robinson *et al*, 2002; Whitehurst *et al*, 2004). This residue lies within the MAPK insert, residues 241-272 of ERK2 (Fig. 1). Deletion of the MAPK insert also reduced binding to PEA-15 and resulted in a defect in ERK2 import (Whitehurst *et al*, 2004).

The import of these MAPK insert mutants over a longer time course were further characterized in the absence (Fig. 2A) and presence (Fig. 2C) of cytosol, to provide import factors, and an energy regenerating system, to maintain sufficient GTP for the Ran-GTP cycle. Under these conditions, a defect in import of GFP-ERK2 Y261N relative to wild type ERK2 was also observed. GFP-ERK2 Δ241-272 displayed a slower rate of import than wild type ERK2 or the point mutant at all times and under both conditions. Quantitation indicated that Y261N was imported better in the presence of cytosol and energy, reaching ~60% of the intensity of wild type ERK2 after 2 h (Fig. 2B, D).

Nups contain numerous FXF motifs. FXF motifs are present in a number of ERK2 substrates and bind directly to ERK2. Previously studies proposed that the Nup binding site was also the site for binding to FXF motifs of substrates (Whitehurst *et al*, 2004).

Hydrogen-deuterium exchange studies demonstrated the location of this site; a peptide from Elk-1 containing an FXF motif bound to pERK2 between the MAPK insert and a-helix G (Lee *et al*, 2004). An examination of the crystal structures of ERK2 and pERK2 suggested that phosphorylation of ERK2 may affect the accessibility of this hydrophobic binding site. Based on the structure, residues that might interact with the Nup FXF motif in ERK2 were predicted; some were different from the pERK2 residues contacting Elk-1. These residues and ones revealed by the hydrogen-deuterium exchange study were mutated and tested in import assays. Both GFP-ERK2 I196A/M197A and N199A/S200A, predicted to interfere with Nup binding to ERK2, were impaired for import (Fig 3A). Quantification of fluorescence intensity indicated close to 50% inhibition of nuclear entry for each mutant (Fig. 3C). Combinations of mutations did not further reduce import (data not shown). Residues Y231and L232 on pERK2 were protected when bound to the Elk1 peptide. However, the behavior of the Y231A/L232A mutant in unphosphorylated ERK2 was not different from that of wild type ERK2 (Fig. 3B).

Following in vitro phosphorylation (Fig. 4E) import of these mutants in the absence of energy and cytosol were examined to determine if mutations that impaired import of the unphosphorylated proteins had a similar effect on the pERK2 mutants. Although import of ERK2 Y231A/L232A was comparable to wild type ERK2 in the unphosphorylated state, import of pERK2 Y231A/232A was decreased (Fig. 4A,B). This would be expected if pERK2 binds Nups in a manner similar to substrate binding (Lee *et al*, 2004). The opposite occurred for ERK2 N199A/S200A. Import of its unphosphorylated form was reduced, while pERK2 N199A/S200A was imported like wild type pERK2 (Fig. 4C,D). Decreased import was observed for both unphosphorylated

and phosphorylated forms of ERK2 Y261N or Y261A (Fig. 4A,B, data not shown) and ERK2 I196A/M197A (Fig. 4C,D) and consistent with the idea that these residues are involved in Nup binding independent of phosphorylation state. Other pERK2 mutants, K52R, the dimerization mutant L4A/H176E, and D316A/D319A, were imported like wild type pERK2 (Fig. 4A,B). Together these results support the idea the Nups bind in the same vicinity of ERK2 as FXF motif-containing substrates, although the exact binding orientation may vary with ERK1/2 phosphorylation state.

His6-ERK2 and the GFP-ERK2 fusion proteins behave similarly in import assays, despite the size difference (Whitehurst *et al*, 2002). As a control for experiments here, I compared import of His6-ERK2 mutants using immunofluorescence. The behavior of pERK2 mutants in import assays was similar whether His6-tagged or GFP fusion proteins were examined (data not shown). To test for dephosphorylation of ERK2 and mutants by the residual phosphatase activities in the import assays, pERK1/2 antibodies were used to immunoblot the pERK2 proteins following incubation with permeabilized cells. Incubation of the phosphorylated proteins with digitonin permeabilized cells for the time required for import assays revealed little difference in wild type or mutant pERK2 immunoreactivity ((Ranganathan *et al*, 2006), data not shown), indicating that the proteins are not dephosphorylated during import assays.

Nucleoporin binding is decreased in the import-defective mutants: Because ERK2 binds to nucleoporins directly, decreased import might result from decreased nucleoporin binding. The results above suggest that some overlapping and some unique residues of ERK2 and pERK2 interact with Nups. To determine if this may be the case, binding of

unphosphorylated (Fig. 5A) and phosphorylated (Fig. 5B) GFP-ERK2 proteins to a C-terminal fragment of Nup153 were tested. Binding of GSTNup153c to unphosphorylated mutant ERK2 proteins was decreased relative to binding to wild type ERK2 roughly in proportion to the decrease in their import. pY231A/L232A binds to GSTNup153c less well than wild type pERK2. These results are consistent with the idea that the decreased import of certain ERK2 mutants is a result of their decreased interaction with the nuclear pore complex.

As an additional test of this idea, increasing concentrations of GST-Nup153c were added to the import assay. As GST-Nup153c concentration was increased, the import of GFP-ERK2 decreased. In contrast, addition of GST alone had no detectable effect on import (Fig. 5C).

Import of ERK2 mutated in the D domain docking site: Proteins with D motifs may influence the localization of ERK2 by anchoring it in one compartment or escorting it through the nuclear pore (Fukuda *et al*, 1997). Because ERK1/2 have no CRM1 consensus nuclear export sequence, CRM1-dependent export may occur by a piggyback mechanism, for example. Import of the ERK2 mutant D316A/D319A which is defective in interactions with D motifs were previously tested (Whitehurst *et al*, 2004). This mutant was imported nearly as well as wild type ERK2 in the unphosphorylated (not shown) and the phosphorylated state (Fig. 4A,B). D319 was originally identified in Drosophila ERK2 in a screen for mutations that increased activity in a tyrosine kinase pathway (Brunner *et al*, 1994). The adjacent residue E320 (E322 in human ERK2) was recently found mutated in a human cancer cell line, and its mutation was reported to result in constitutive activation of ERK2 (Arvind *et al*, 2005). Thus, Daryl Goad, another graduate student in

our laboratory, prepared and tested this mutant in the import assay. ERK2 E320K and pERK2 E320K were imported indistinguishably from wild type ERK2 (Fig. 6A). In the unphosphorylated state ERK2 E320K had a significantly higher specific activity (15-20-fold) towards a model substrate than wild type ERK2 (Fig. 6B). As in the earlier report (Arvind *et al*, 2005), ERK2 E320K was found to have a greater mobility than ERK2 in denaturing gels. Because pERK2 E320K had a specific activity similar to wild type pERK2 under our conditions, Daryl and I did not characterize its activity further.

*Import of ERK2 mutated in the vicinity of the substrate binding site:*The import of ERK2 with mutations near the protein substrate binding pocket of the active site were evaluated. ERK2 P224G, P227G, and W190A were tested. Mutation of either P227 or W190 was shown to have a significant impact on catalytic activity, causing a ~5-fold increase in Km and greater than a 30-fold increase in kcat/Km for the substrate Elk1 (Zhang *et al*, 2003). These mutants were each imported similarly to wild type ERK2 in either the unphosphorylated (Fig. 7A,B) or the phosphorylated (Fig. 7C,D) state.

*Import of pERK2 mutants in the presence of energy and cytosol:*Phosphorylated ERK2 can also enter the nucleus by an energy-dependent mechanism not available to the unphosphorylated protein (Ranganathan *et al*, 2006). Although the relevant carriers have not yet been identified, this process is assumed to be dependent on cytosolic import carriers and not direct interactions with Nups. Thus, we also examined import of the pERK2 mutants in the presence of cytosol and an energy-regenerating system. All of the mutants were imported nearly as well as wild type ERK2 (Fig. 8A,B). Minimal defects were observed for pERK2 L231A/L232A, Y261A, and I196A/I197A.

Evaluation of RanBP7 in MAPK import: Nuclear transport factors influence the localization of MAPKs in other organisms. In Drosophila, importin7 (dim-7), interacts with fly ERK2 (Lorenzen *et al*, 2001). Mutation of the gene encoding dim-7 caused decreased nuclear localization of Drosophila ERK2. To determine if mammalian MAPKs may be imported via RanBP7, the human homolog of Dim7, RNAi strategy was used to knock down RanBP7 expression (Fig. 9A). The endogenous MAPKs ERK1/2, JNK, and p38 were activated normally by UV and PMA in cells in which RanBP7 was knocked down (Fig. 9B). No obvious changes in nuclear localization of any of these MAPKs were observed (Fig. 9C-E). Thus, the energy-dependent import of ERK2 and the other MAPKs tested apparently takes place through the actions of some other import carrier.

Localization of ERK2 mutants in cells: Heterologous expression of ERK2 generally results in greater accumulation of ERK2 in the nucleus than observed with endogenous protein (Rubinfeld *et al*, 1999). The distribution of ERK2 mutants that showed decreased import in vitro were examined. The majority of the mutants were found throughout the cell and in the nucleus as was wild type ERK2 upon expression in HeLa or HEK293 cells in the absence of stimuli (data not shown), as reported previously for some of the mutants (Robinson *et al*, 2002 ; Dimitri *et al*, 2005). The single exception was ERK2 Δ241-272 (MAPK insert deletion); a reduced nuclear concentration of this ERK2 mutant was observed in most of the cells, in a few cases appearing as nuclear exclusion (Fig. 10).

Discussion:

The nuclear localization of ERK1/2 is highly regulated. Reconstitution of import and export in permeabilized cells were used to explore the mechanisms involved and to isolate the individual steps to elucidate their contributions to the behavior observed in intact cells. Import and export in several cell types were analyzed using reconstitution assay. Although ligand induced localizations of ERK1/2 are not identical in BJ and cell lines frequently used for localization studies (e.g., HeLa, Ref52), few distinctions among cell types have been observed in the import/export reconstitution experiments performed thus far. BJ cells were selected for the majority of these studies because they are immortalized in a defined manner; they are diploid; and, in contrast to several standard cell lines, different ligands induce highly distinct pERK1/2 localization patterns in BJ cells (Whitehurst *et al*,2004). Thus, a greater number of regulated steps can be discerned in this cell system.

It was previously found that ERK2 in the unphosphorylated state enters the nucleus by an energy-independent mechanism facilitated by direct interaction with nucleoporins (Matsubayashi *et al*, 2001; Whitehurst *et al*,2002). This appears to be the primary entry mechanism for the unphosphorylated protein and a significant import mechanism for pERK2 as well. Consistent with this idea, Nup153c inhibits the nuclear uptake of both unphosphorylated ERK2 and pERK2. Furthermore, nucleoporin binding is decreased for several ERK2 mutants that show a nuclear import defect in reconstitution assays.

From an examination of the crystal structures of ERK2, it seemed possible that unphosphorylated and phosphorylated forms of ERK2 might bind somewhat differently

to hydrophobic sequences such as the FXF-rich motifs of Nups. Results of import and binding assays suggest that this may be the case (Table 1). Mutations in residues in the insert itself appeared to affect import of both unphosphorylated and pERK2, while other residues predominantly affect import of the unphosphorylated or the phosphorylated kinase. In this regard, it is also recently found that these ERK2 mutants bind less well to FXF motifs from the transcription factor substrate ERF (Polychronopoulos *et al*, 2006). ERF contains four FXF motifs. One of these motifs binds better to unphosphorylated ERK2, while a second motif displays the selectivity for pERK2 that has often been found for FXF motifs. Together these results suggest that there is more than one mode through which ERK2 may interact with FXF motifs: the Elk peptide mode revealed by hydrogendeuterium exchange using pERK2 (Lee *et al*, 2004), which is likely to be the common mode of interaction with protein substrates, and a second mode accessible primarily in the low activity state of ERK2.

In spite of the fact that mutations that are defective in import using reconstitution assays can be identified, these mutations have little impact on the localization of ERK2 in cells. The ERK2 mutants that show impaired import are all imported to a significant extent; what is measured is a slowed rate of import. The permeabilized cell assays allow the discrimination of relatively subtle differences in protein properties. Overexpression of ERK2 usually results in its nuclear accumulation in cells. This phenomenon has been attributed to overwhelming the capacity of cytoplasmic anchoring proteins and may also suggest a wealth of low affinity nuclear binding sites for ERK1/2. If this is the case, it is perhaps not surprising that overexpressed ERK2 mutants are usually found in the

nucleus. The kinetics of import of mutants expressed at levels less than or equal to the endogenous protein might reveal defects not observed in the overexpression assay.

Perhaps more puzzling is the observation that ERK2 Δ241-272 showed an increased cytoplasmic localization. This mutant is imported in permeabilized cells, although it is the most impaired in reconstitution assays. From earlier experiments this mutant interacts less well with MEK1/2 and is not phosphorylated by MEKs (Robinson *et al*, 2002). Considerable evidence suggests that MEKs are key cytoplasmic anchoring proteins (Fukuda *et al*, 1997). Thus, ERK2 Δ241-272 protein would accumulate in the nucleus due to weakened cytoplasmic binding. This ERK2 deletion mutant is similar to a newly identified ERK1 splice form, ERK1c. The ERK1c splice form lacks an additional 12 residues, 11 of which are C-terminal to the residues deleted in the ERK2 mutant (Aebersold *et al*, 2004). Interestingly, ERK1c is localized to the Golgi. Further exploration of the localization mechanism of this protein may lead to some different ideas about why ERK2 accumulates in the nucleus as a default state.

Analysis of import of the deletion mutant and ERK2 Y261N in the presence and absence of energy revealed an unanticipated difference in import rate relative to wild type ERK2. Previously no difference in import of wild type, unphosphorylated ERK2 in the presence or absence of energy were found. While the difference in behavior of ERK2 Y261N is small, it seems possible that it may reflect a subtle difference in import mechanism that was observed because of the slowed kinetics.

A major component of nuclear entry of pERK2 is energy-dependent. Studies of ERK2 homologs in other organisms suggested that RanBP7 might be the relevant carrier in mammalian cells. However, RNAi of RanBP7 had no effect on the localization of

ERK2 or other MAPKs under stimulated conditions. It is possible that the assay conditions selected were inappropriate to measure the function of this protein in MAPK import. Alternatively, one or more other carriers may be the active import carrier in mammalian cells whose existence was inferred from our earlier transport studies (Ranganathan *et al*, 2006).

Table 1. Import of ERK2 and mutants.

Mutation	ERK2-E	pERK2-E	pERK2+E	Mutant type
ERK2	100	100	100	Wild type
Y261N or A	35	35	75	FXF binding
I196/M197A	45	75	80	FXF binding
N199/S200A	55	100	100	FXF binding
Y231/232A	100	45	75	FXF binding
Δ241-272	25	--	--	- MAPK insert
D316/319A	90	95	100	D motif binding
E320K	100	100		D motif binding
W190A	100	95	90	Substrate site
P224G	95	80		Substrate site
P227G	95	90		Substrate site
K52R	100	100		Inactive
L_4A/H176E	100	100		Monomeric

Table 2-1: Summary of *in vitro* import assays. Values shown are import in 20 min relative to wild type ERK2 or pERK2 rounded to the nearest 5 percent based on analyzing 100 cells for each condition. -E, without energy and cytosol; +E, with energy and cytosol.

Fig. 2-1

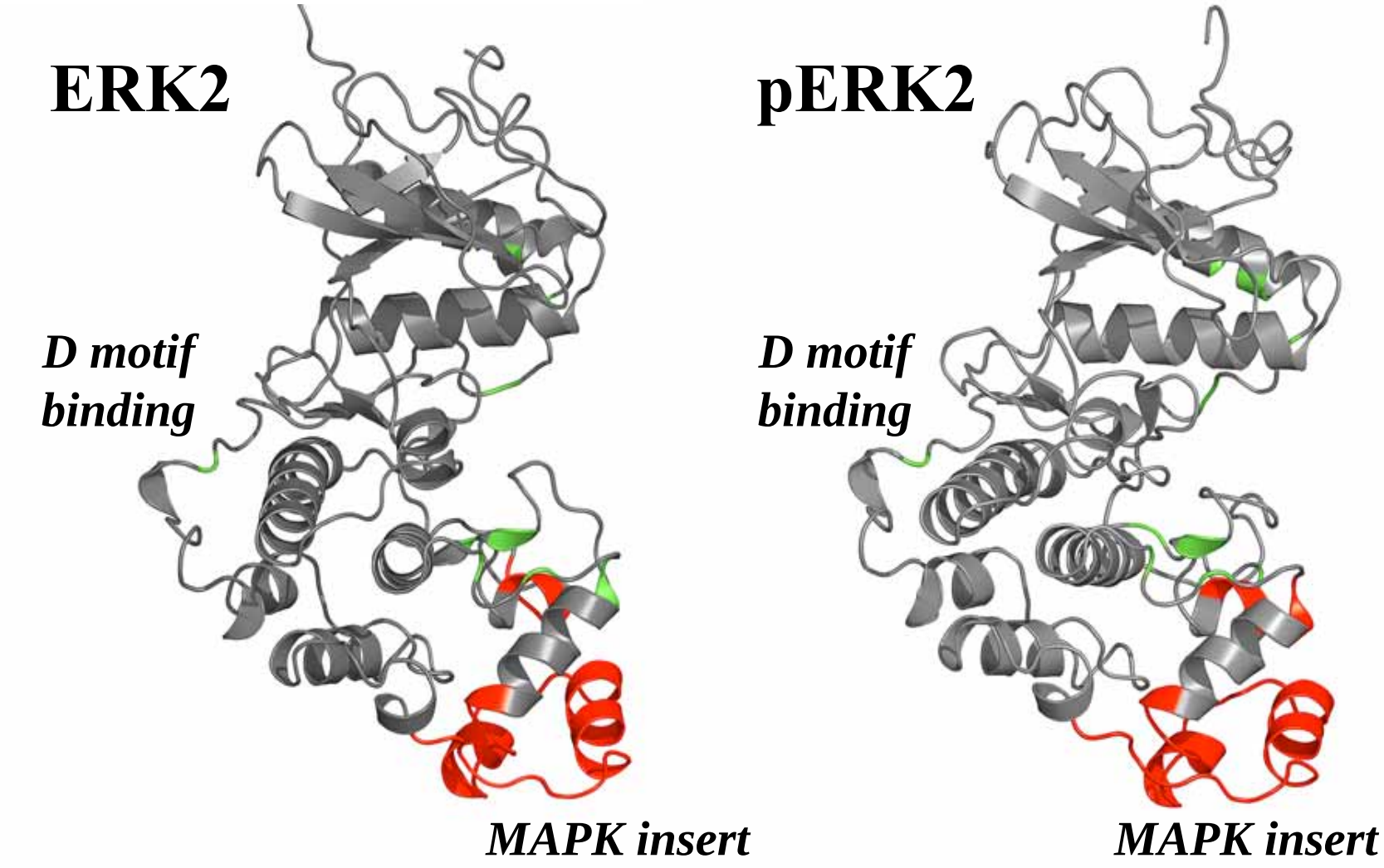


Figure 2-1. Structure of ERK2. Comparison of unphosphorylated (ERK2) and phosphorylated ERK2 (pERK2). Similar views of ERK2 and pERK2 with residues mutated for import assays highlighted. Red, mutated residues that reduced import; green, mutated residues that did not affect import.

Fig. 2-2A

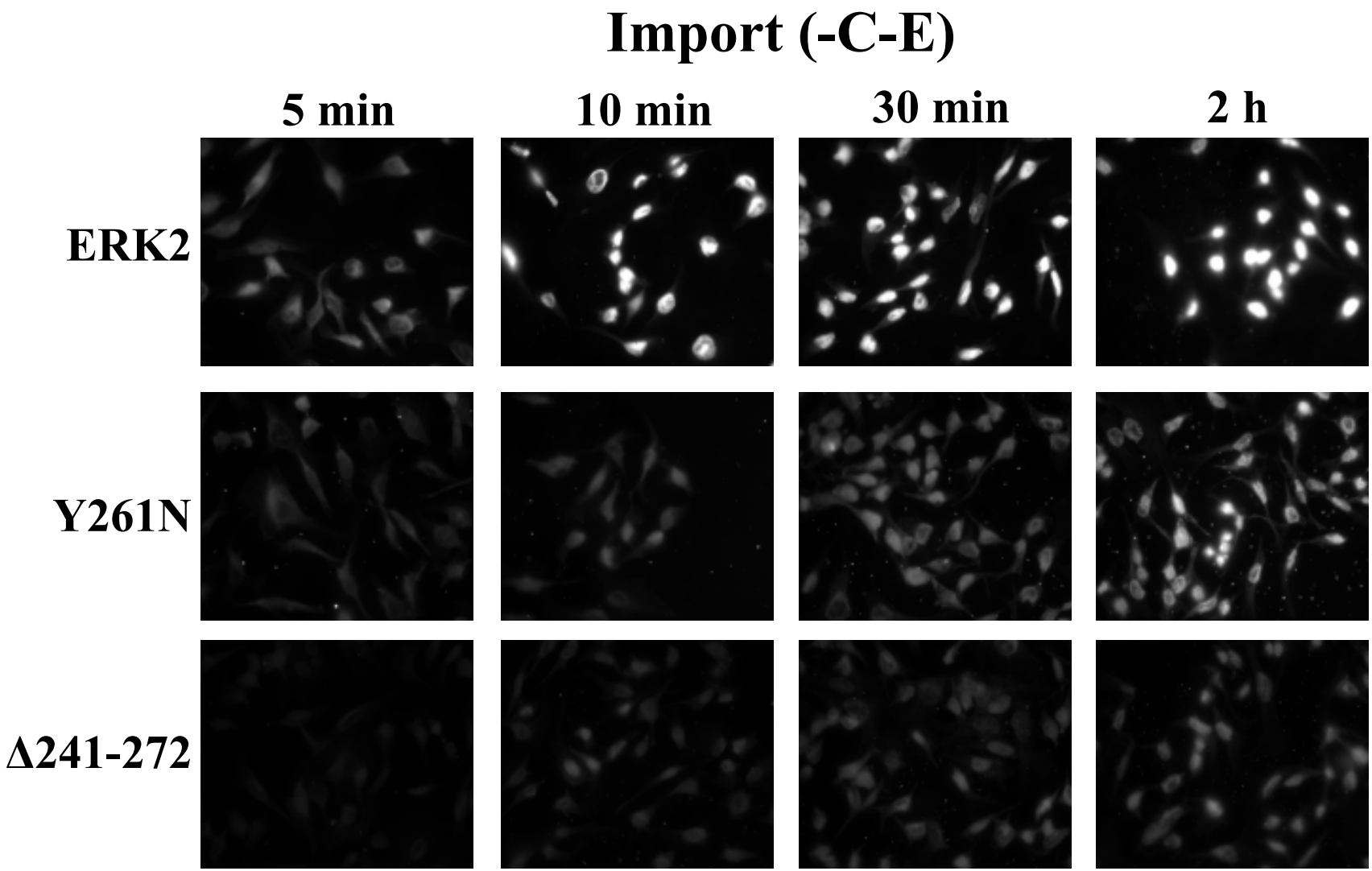


Fig. 2-2B

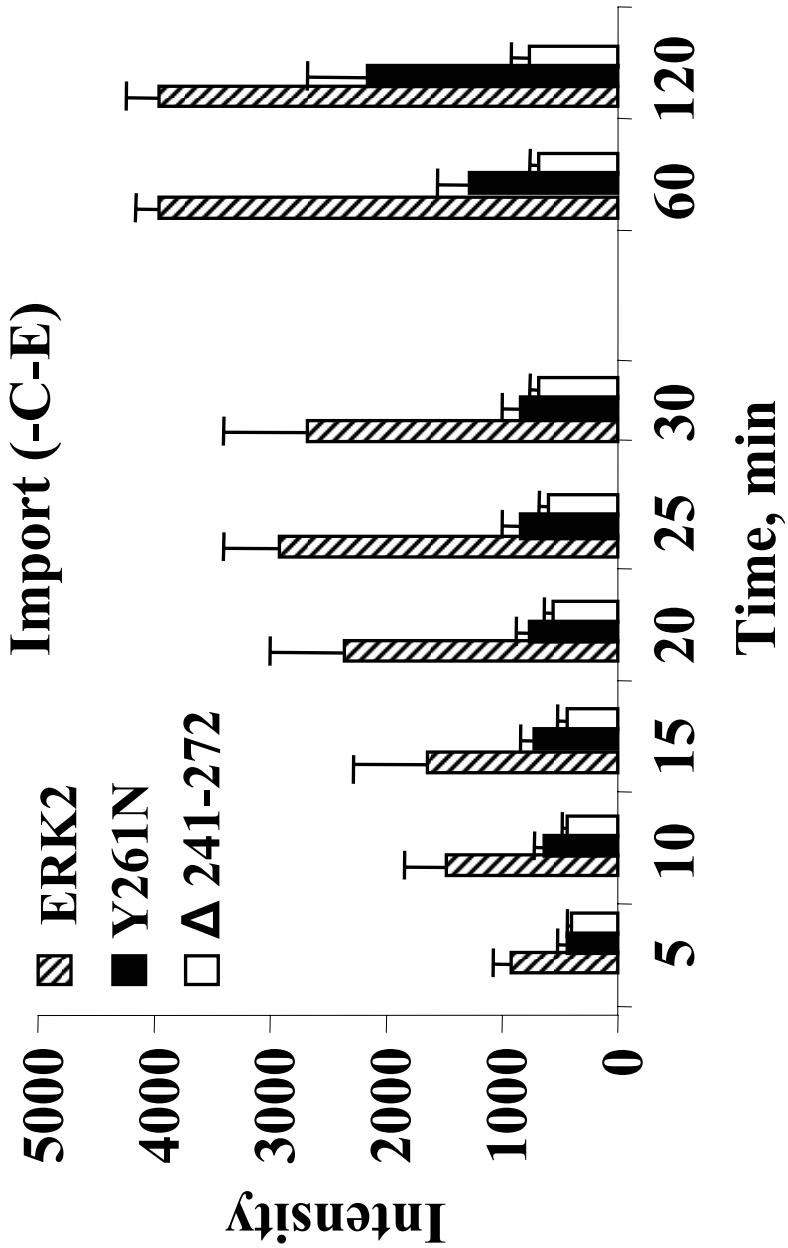

Import (-C-E)
ERK2
Y261N
Δ 241-272
Intensity
5000
4000
3000
2000
1000
0
5
10
15
20
25
30
60
120
Time, min

Fig. 2-2C

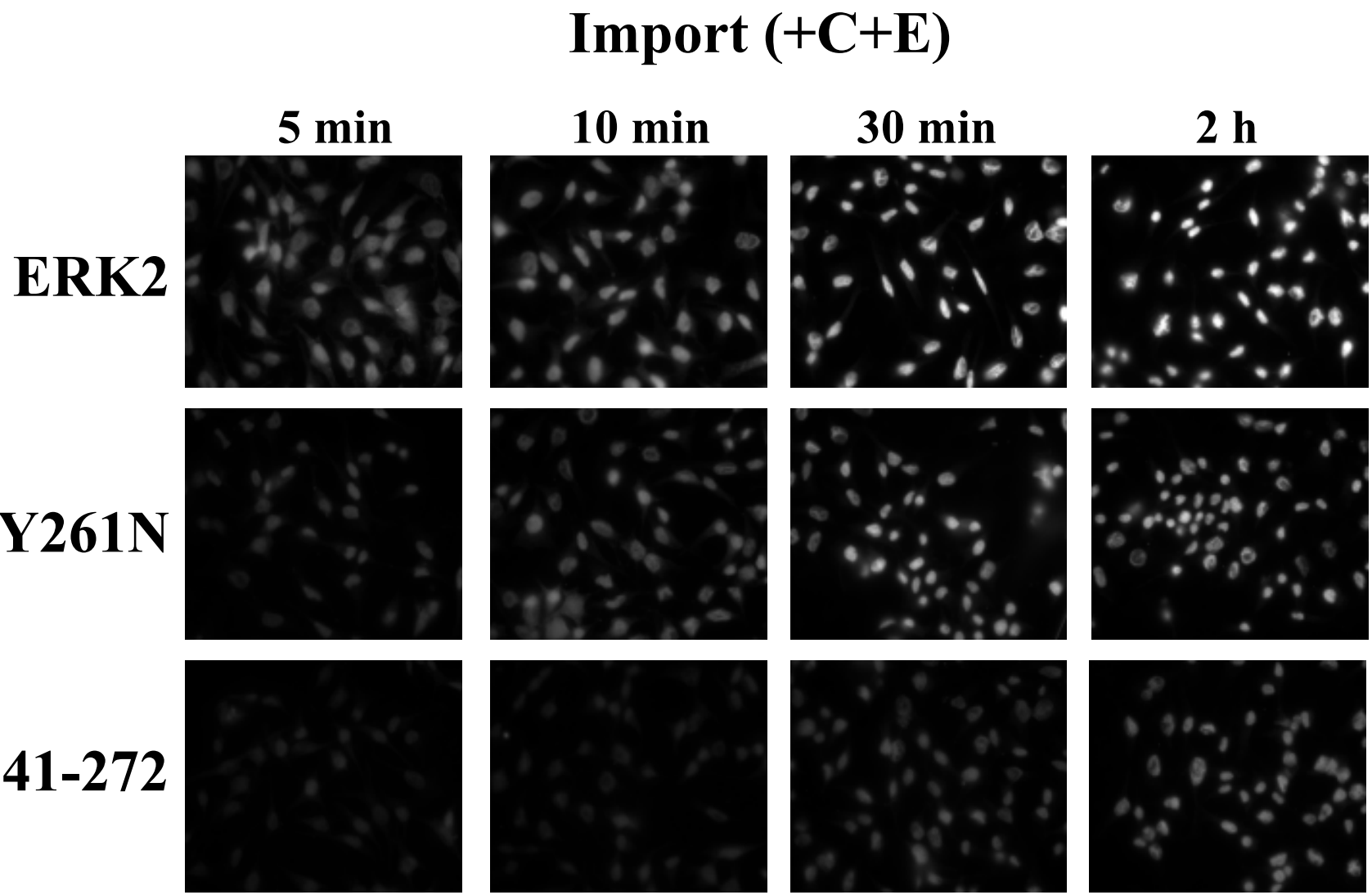


Fig. 2-2D

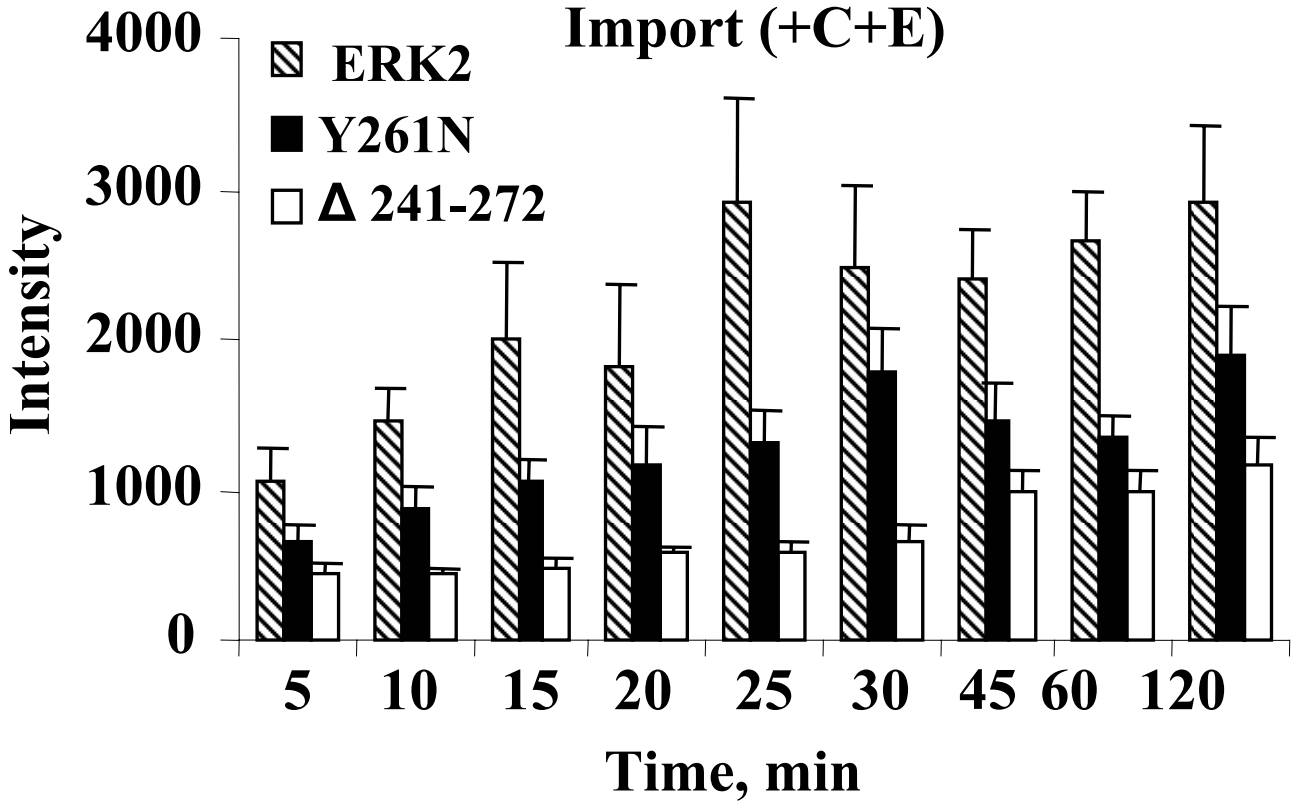


Figure 2-2. Time courses of import of ERK2 and mutants. GFP-ERK2 and mutants Y261N and Δ241-272 were imported for up to 2 h in transport buffer without (**A**) and with (**C**) cytosol and energy (-C-E, +C+E). Fluorescent images of nuclei at selected time points are shown. **B**. and **D**. Quantitation of fluorescence intensity within the nuclei of experiments shown in **A**. and **C**.

Fig. 2-3A

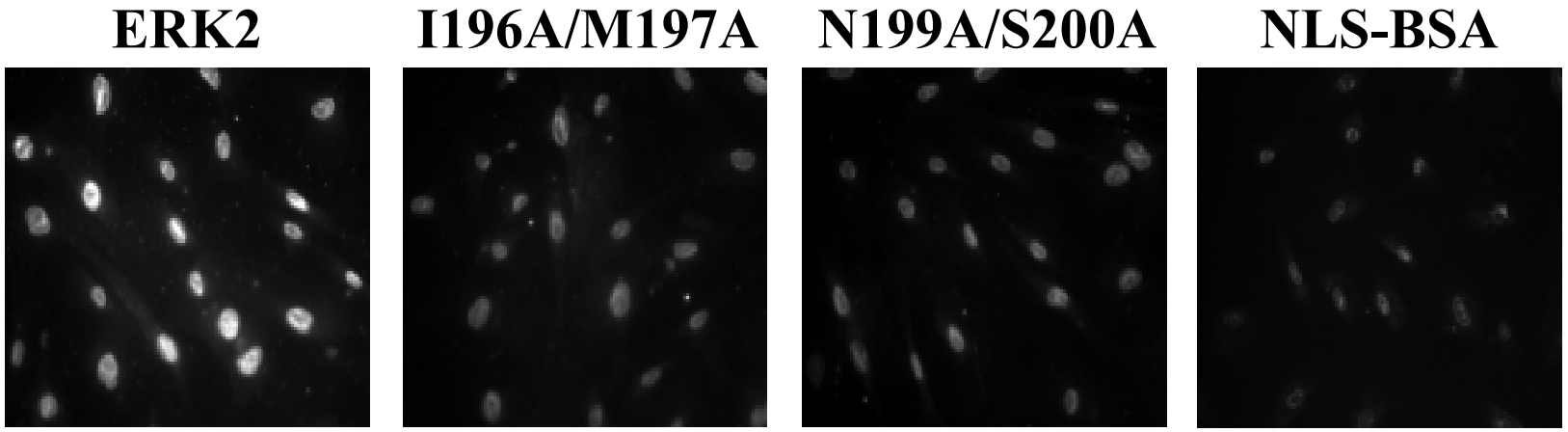


Figure 2-3. Import of ERK2 proteins mutated in the putative FXF binding site. **A.** and **B.** Import of the indicated mutants was performed for 20 min in transport buffer without added energy or cytosol. Import of rhodamine-labeled NLS-BSA with cytosol and energy for 20 min is shown as a control in **A**. Fluorescence of cells not incubated with GFP-ERK2 is shown as a control in **B**. **C.** Quantitation of fluorescence intensity within the nuclei of experiments shown in **A**. and **B**.

Fig. 2-3B

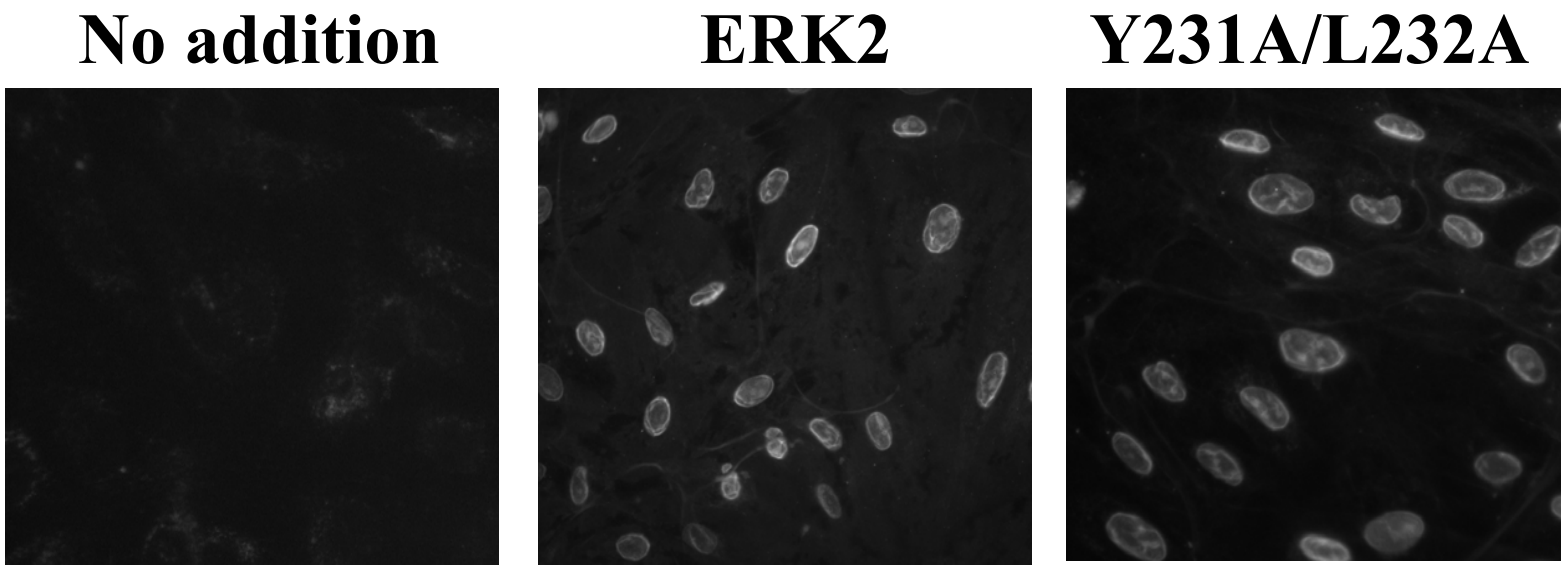


Fig. 2-3C

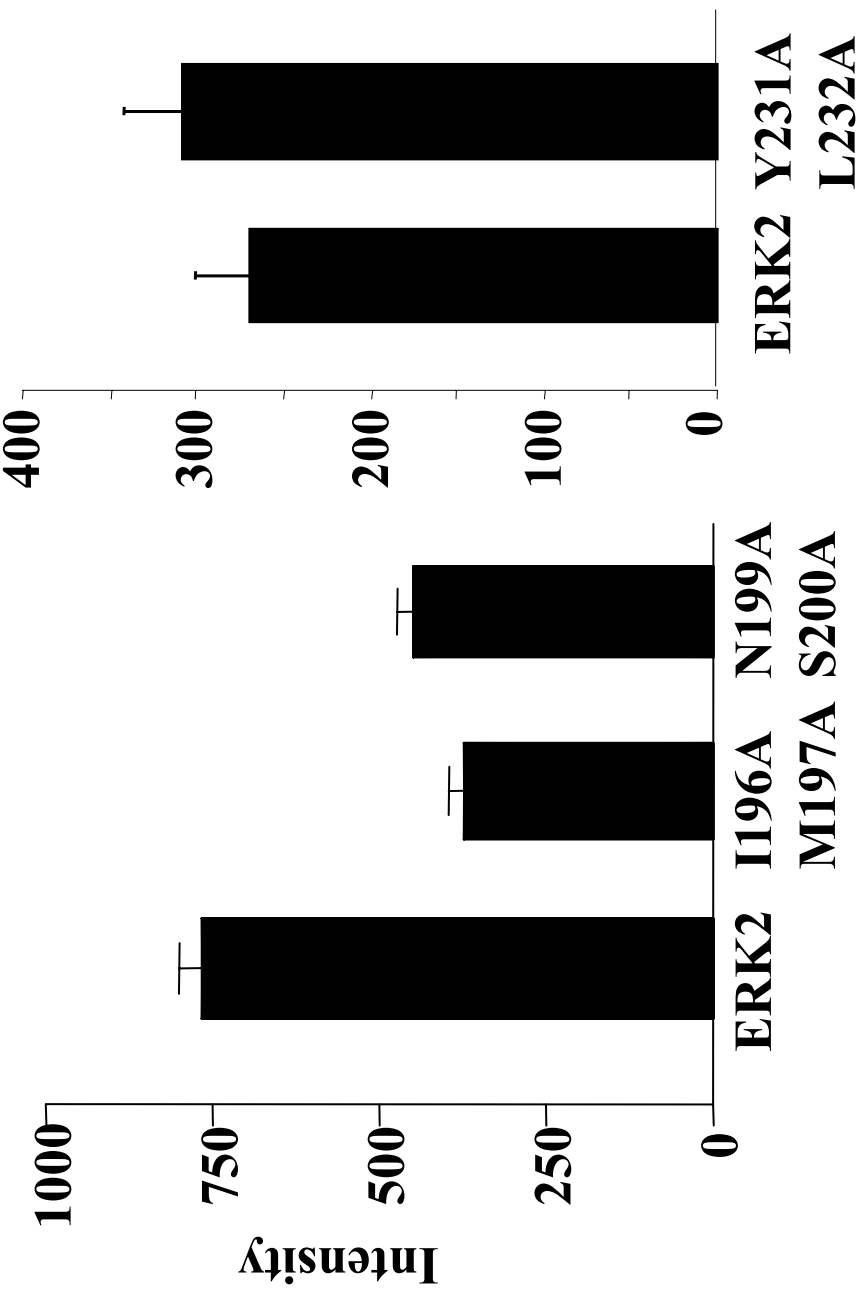

Intensity
1000
750
500
250
0
ERK2
I196A
M197A
N199A
S200A
400
300
200
100
0
ERK2
Y231A
L232A

Fig. 2-4A

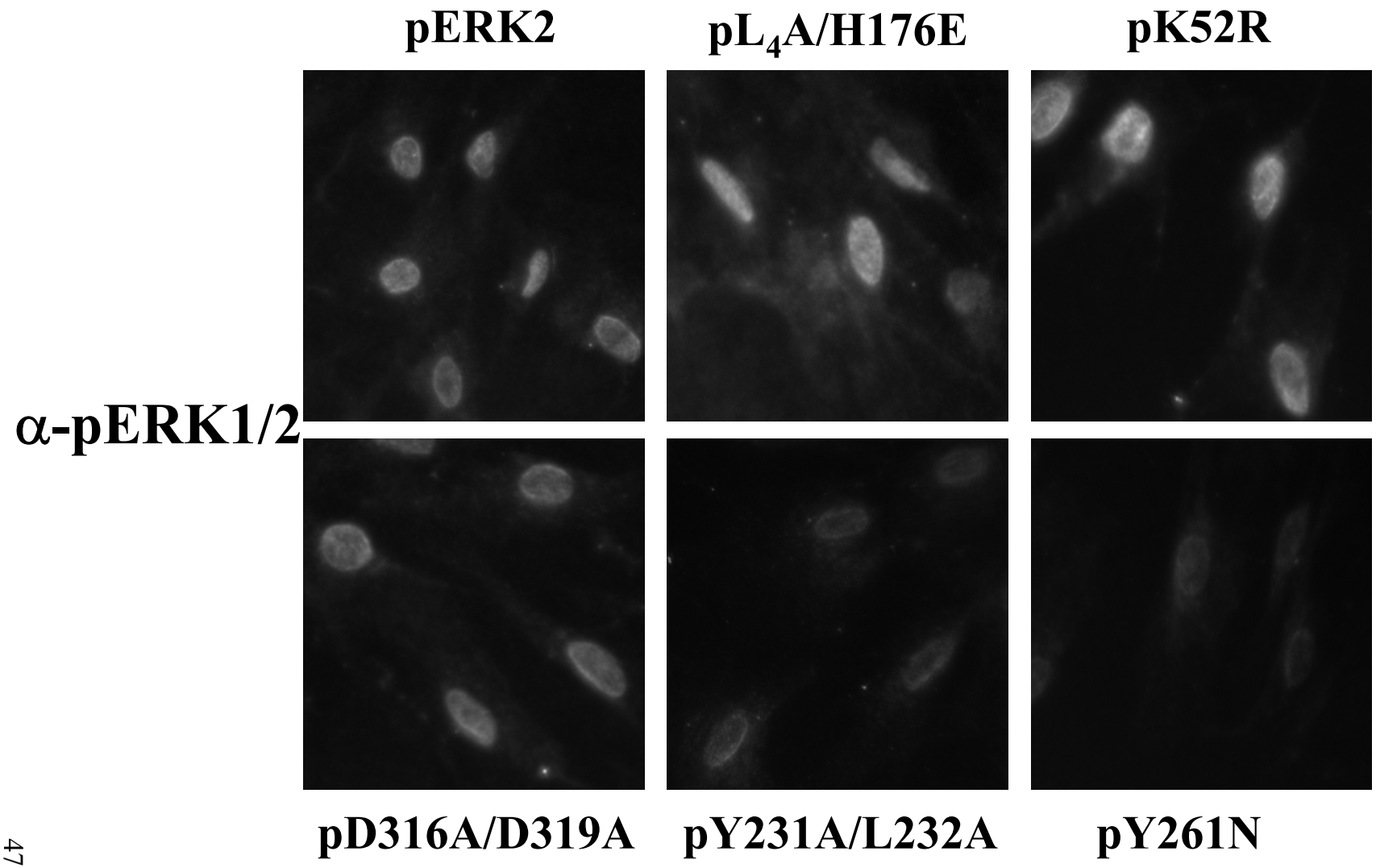

pERK2
pL4A/H176E
pK52R
α-pERK1/2
pD316A/D319A
pY231A/L232A
pY261N

Fig. 2-4B

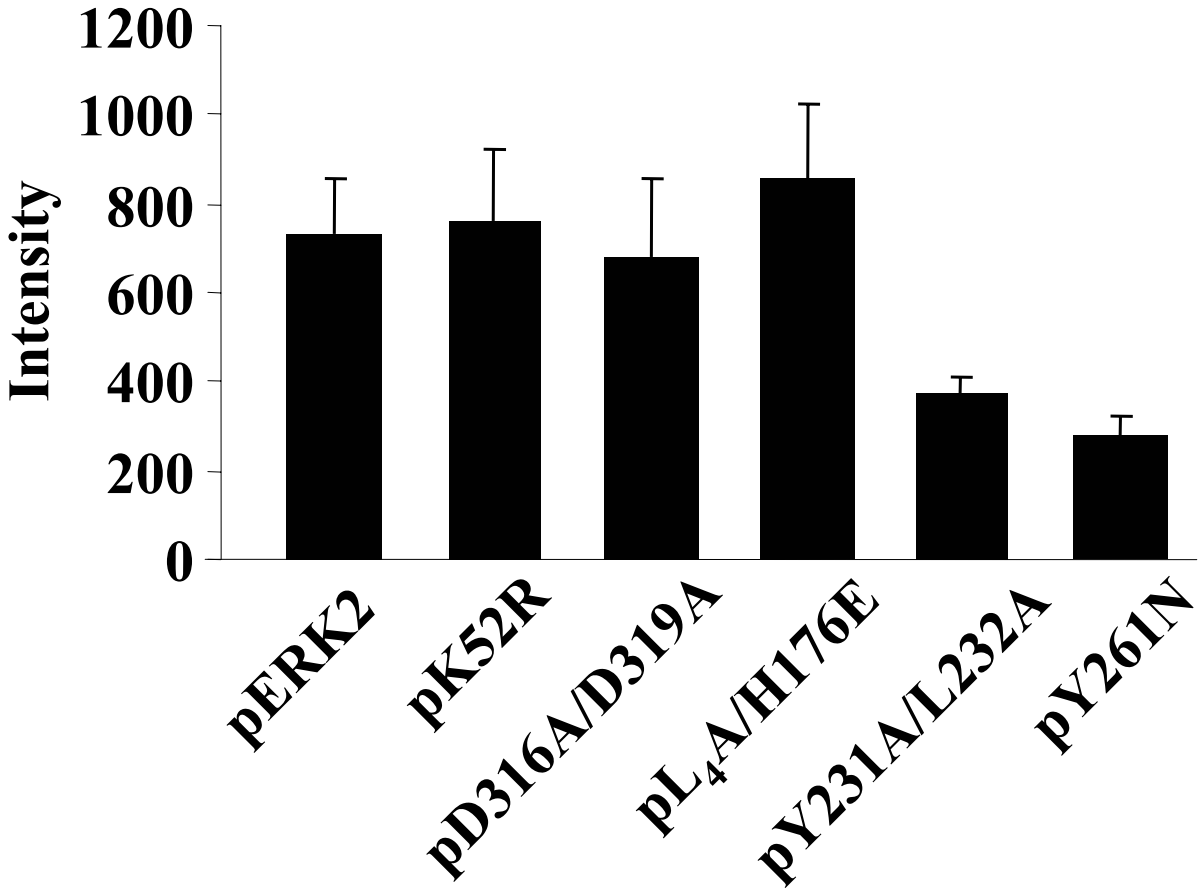

Intensity
1200
1000
800
600
400
200
0
pERK2
pK52R
pD316A/D319A
pL4A/H176E
pY231A/L232A
pY261N

Fig. 2-4C

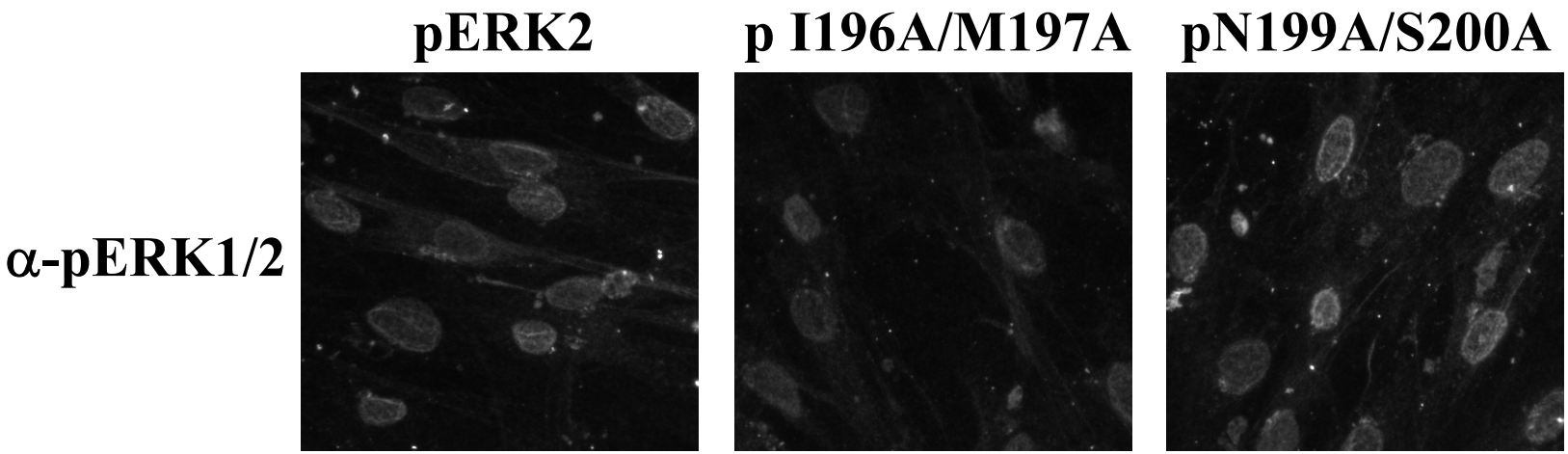


Fig. 2-4D

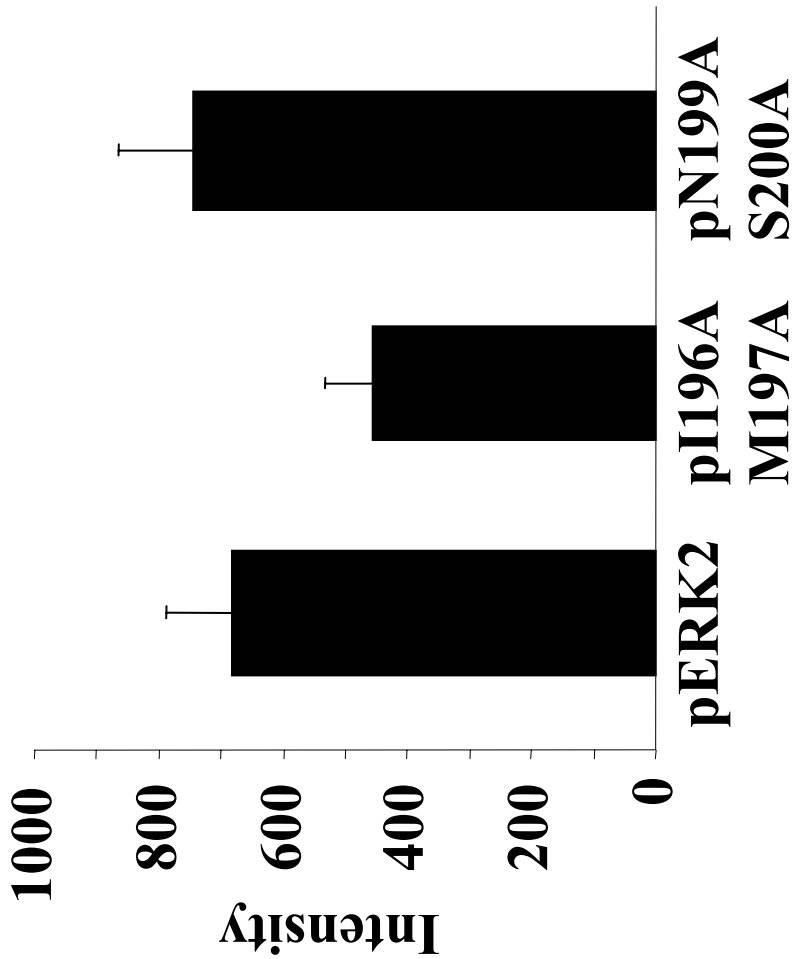

Intensity
1000
800
600
400
200
0
pERK2
pI196A
M197A
pN199A
S200A

Fig. 2-4E

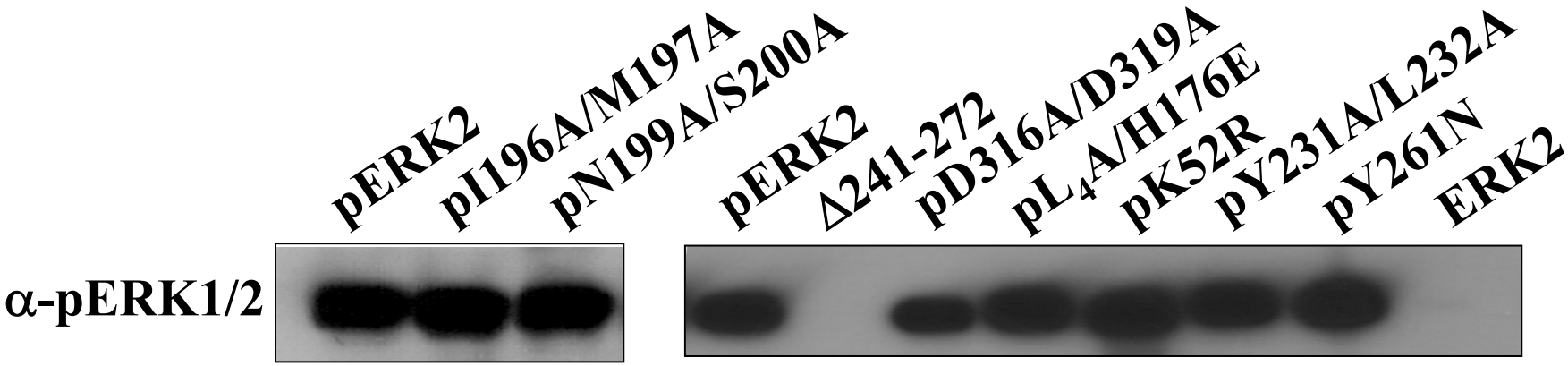


Figure 2-4. Import of pERK2 and mutants. **A.** and **C.** Import of ERK2 and the indicated ERK2 mutants phosphorylated in vitro was performed for 20 min in transport buffer without added energy or cytosol. **B.** and **D.** Quantitation of fluorescence intensity within the nuclei of experiments shown in **A**. and **C**. **E**. pERK2 blots of in vitro phosphorylated ERK2 and mutants. ERK2 Δ241-272 cannot be phosphorylated by MEK1 (8). The last lane contains unphosphorylated ERK2.

Fig. 2-5A

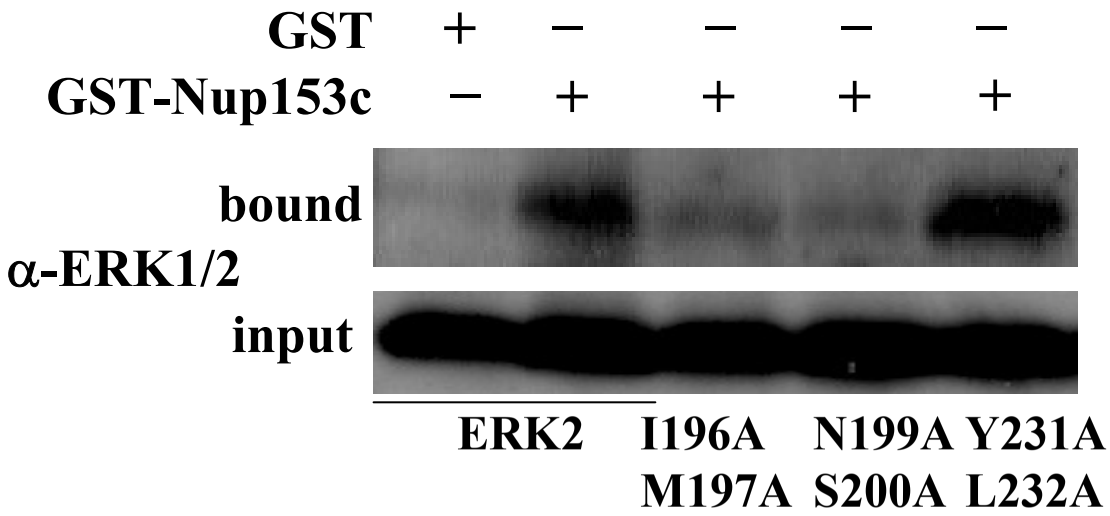


Figure 2-5. In vitro binding of ERK2 and mutants to nucleoporin. **A.** Binding of His6-ERK2 proteins to GST-Nup153c or GST. Bound and input proteins were detected with anti-ERK antibody Y691. One of three similar experiments is shown. **B.** Binding of phosphorylated His6-ERK2 and pERK2 Y231A/L232A to GST-Nup153c. Bound and input proteins were detected with anti-pERK antibodies. One of three similar experiments is shown. **C.** Import of ERK2 in the presence of increasing concentrations of GST or GST-Nup153c. Fluorescent images of nuclei at selected time points are shown.

Fig. 2-5B

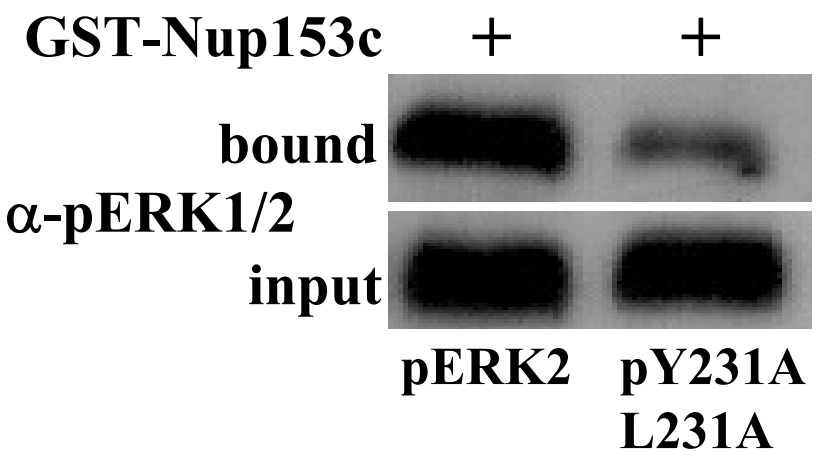


Fig. 2-5C

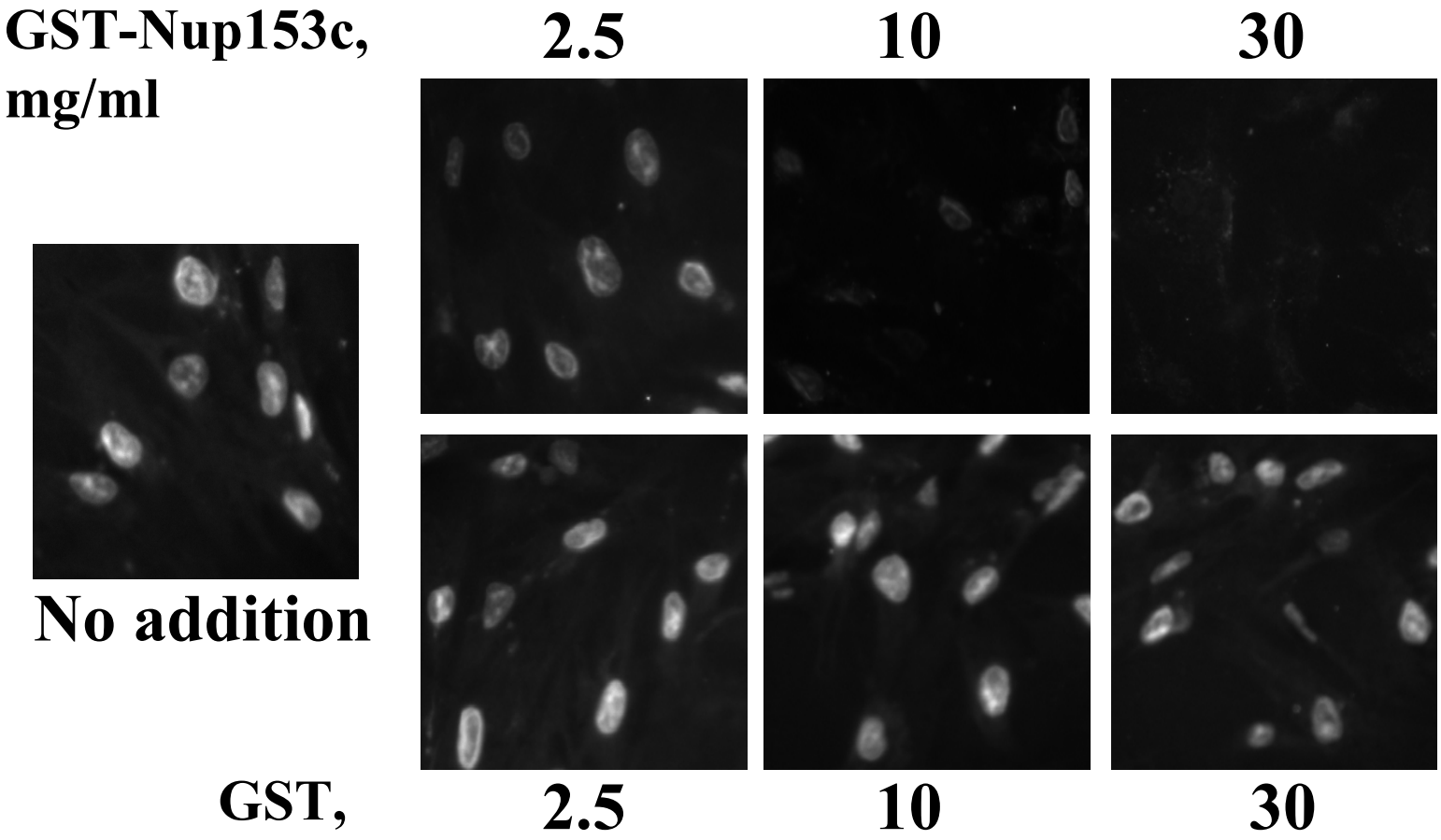


Fig. 2-6A

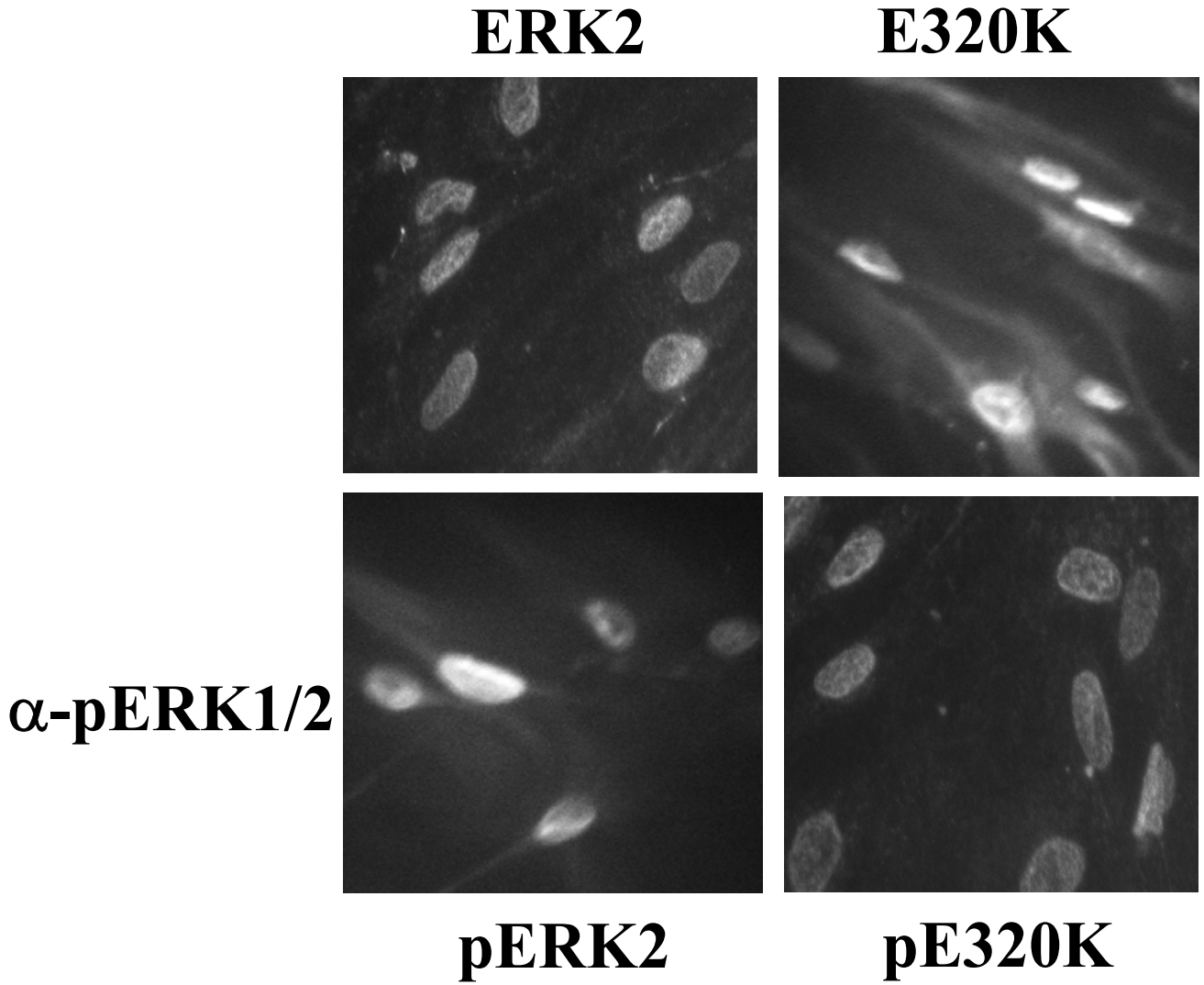


Fig. 2-6B

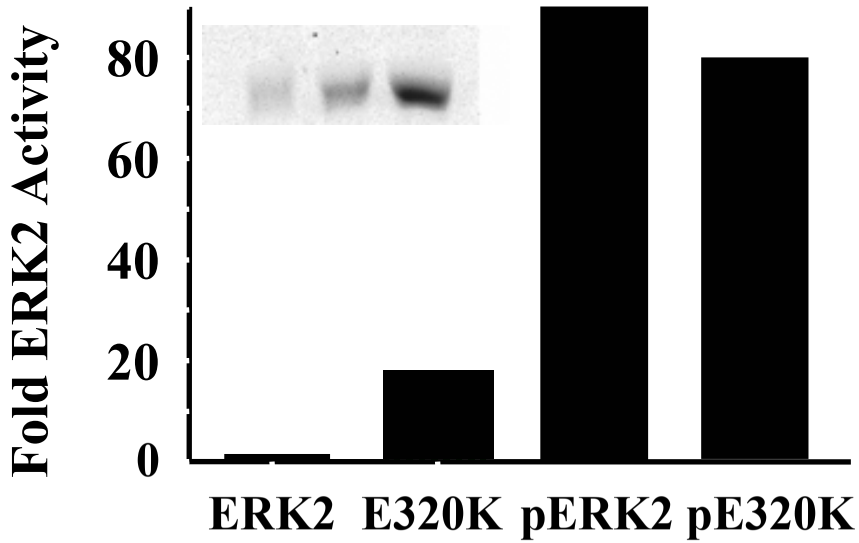


Figure 2-6: ERK2 E320K is imported like wild type ERK2. **A.** Import of ERK2 E320K and pERK2 320K detected as GFP fluorescence and with anti-pERK. **B.** Comparison of protein kinase activity of ERK2 and ERK2 E320K with and without phosphorylation by MEK1 in vitro. Activity was measured using myelin basic protein as substrate and is shown relative to unphosphorylated ERK2. Inset shows Coomassie blue stain of 0.75, 1.5, and 3 mg of ERK2 E320K.

Fig. 2-7A

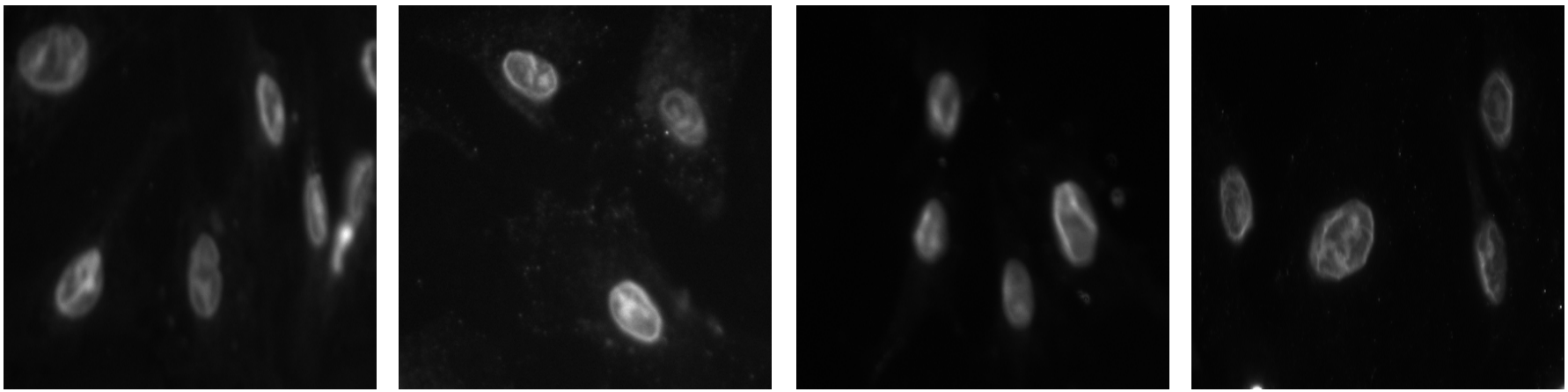


Figure 2-7. Import of ERK2 proteins mutated to affect the protein substrate binding site. Import of the indicated ERK2 mutants either unphosphorylated (**A.**) or phosphorylated (**C.**) was performed for 20 min in transport buffer without added energy or cytosol. **B.** and **D.** Quantitation of fluorescence intensity within the nuclei of experiments shown in **A**. and **C**. The lower panel of **D**. shows pERK2 blots of in vitro phosphorylated ERK2 and mutants.

Fig. 2-7B

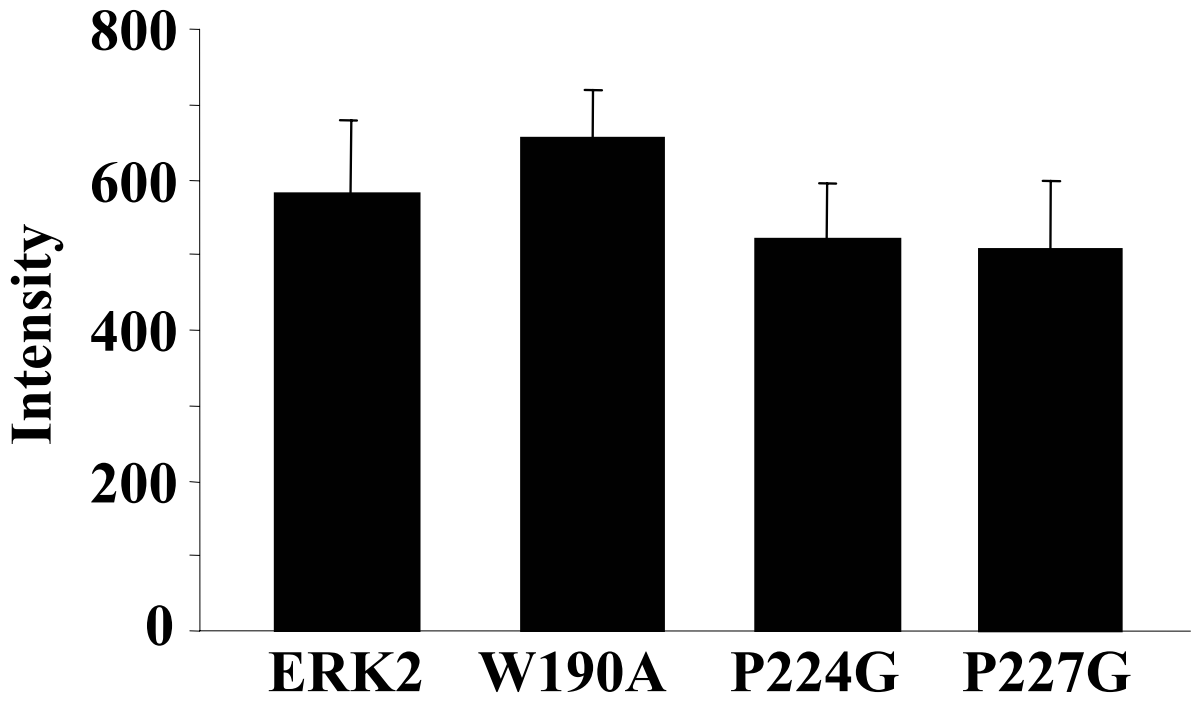


Fig. 2-7C

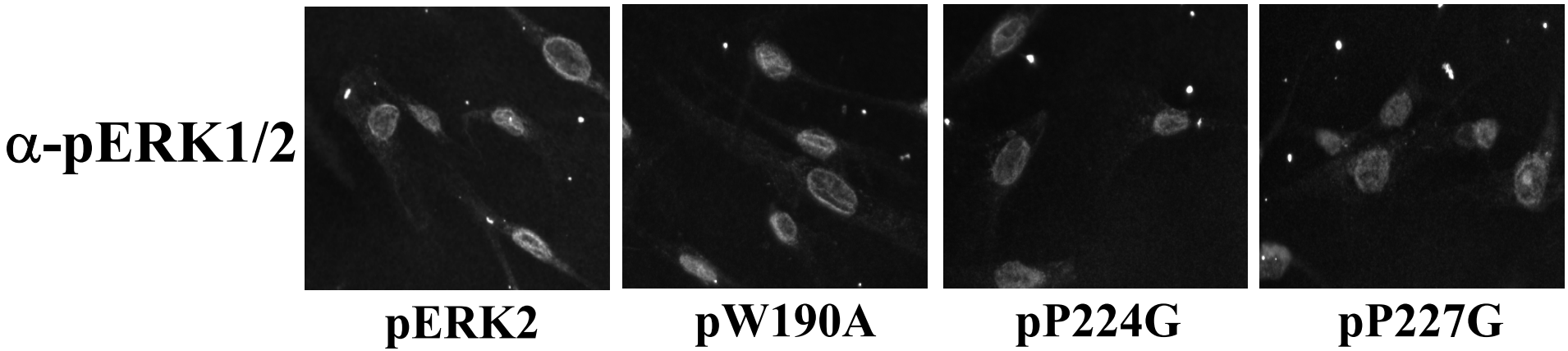
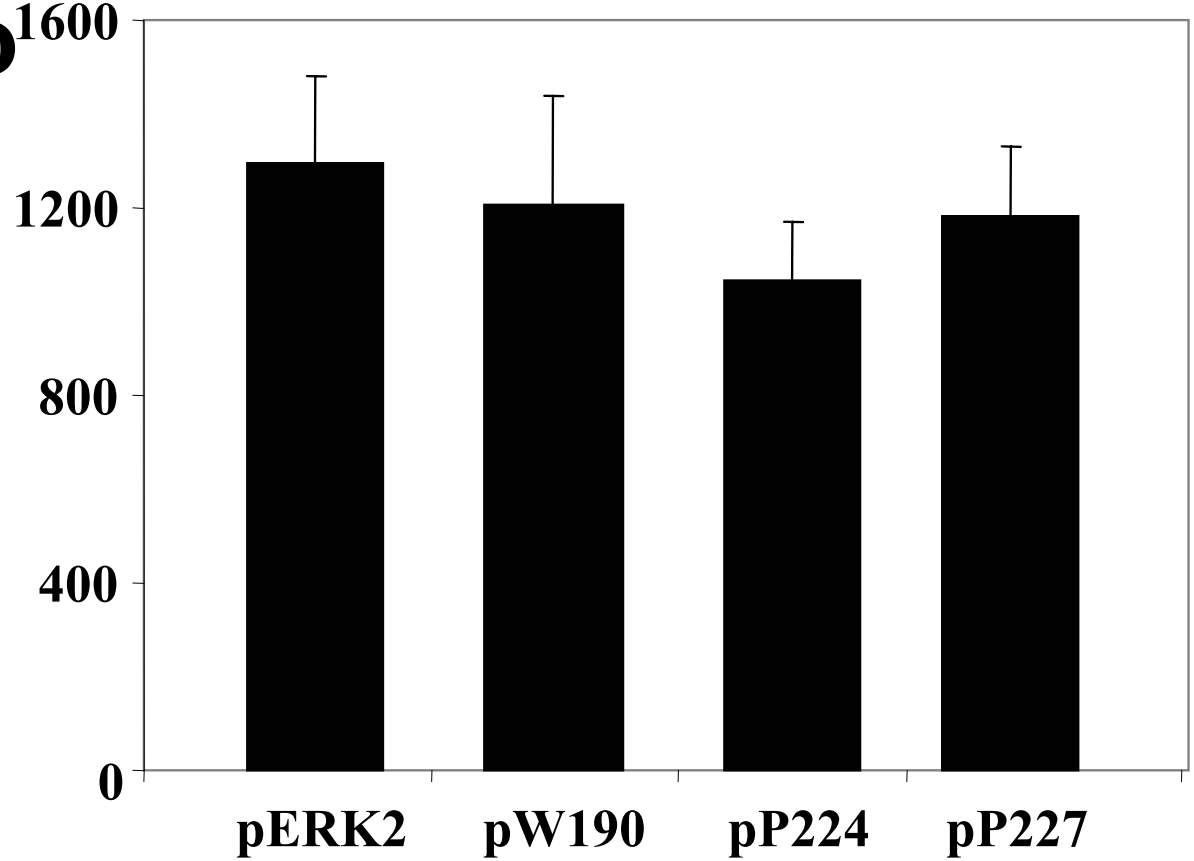


Fig. 2-7D


1600
1200
800
400
0
pERK2
pW190
pP224
pP227

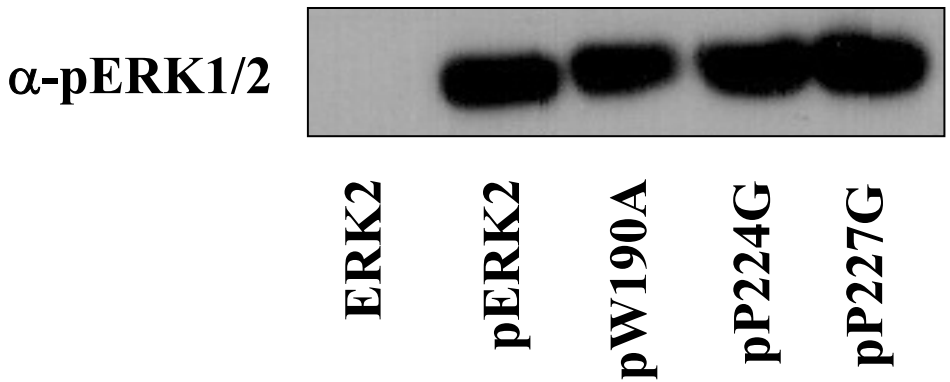

α-pERK1/2
ERK2
pERK2
pW190A
pP224G
pP227G

Fig. 2-8A

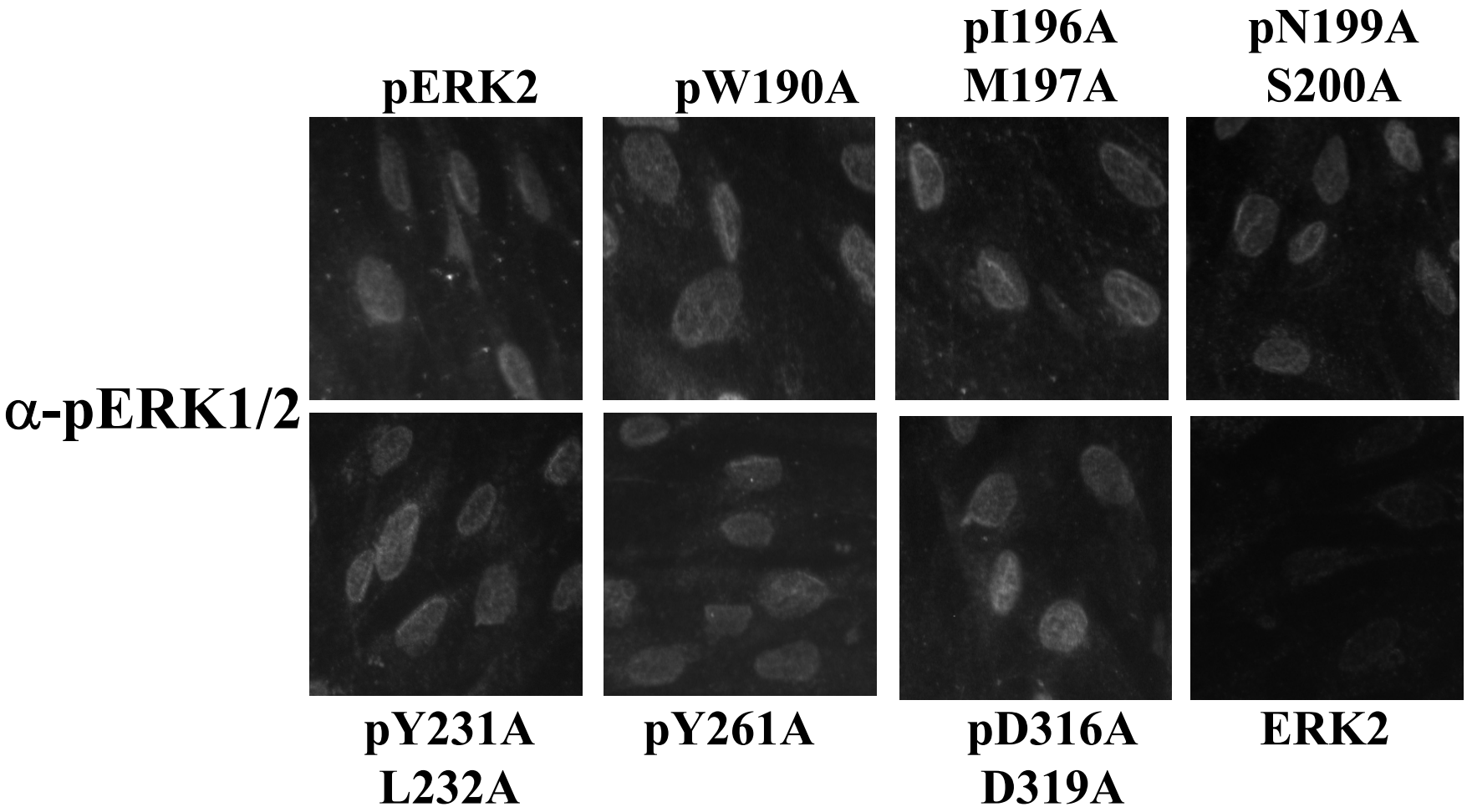


Figure 2-8. Import of selected pERK2 binding mutants in the presence of energy and cytosolic factors. **A.** Import of the indicated pERK2 mutants was performed for 20 min in transport buffer with energy and cytosol (+C+E). **B.** Quantitation of fluorescence intensity within the nuclei of experiments shown in **A**.

Fig. 2-8B

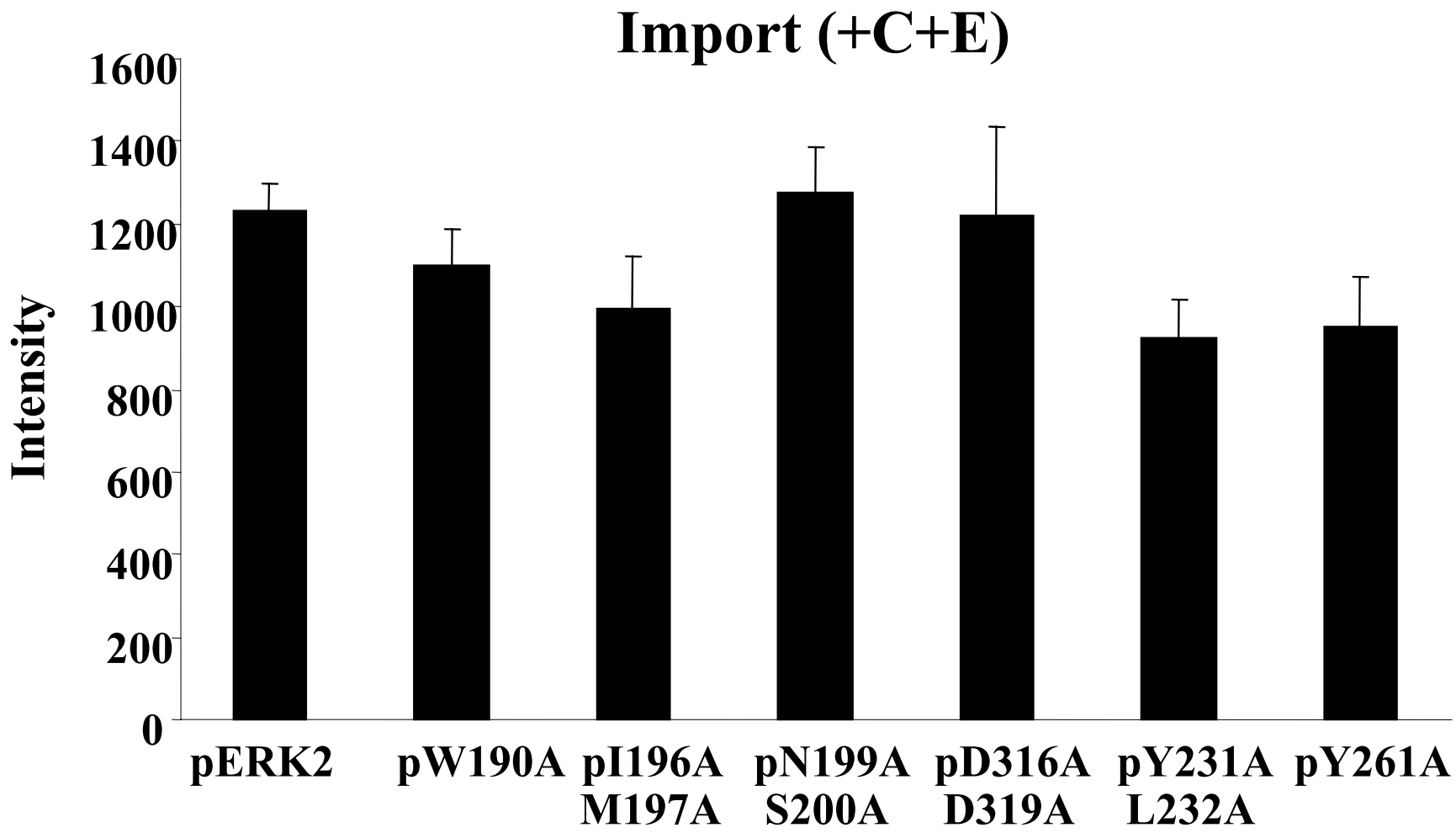

Import (+C+E)
Intensity
1600
1400
1200
1000
800
600
400
200
0
pERK2
pW190A
pI196A M197A
pN199A S200A
pD316A D319A
pY231A L232A
pY261A

Fig. 2-9A

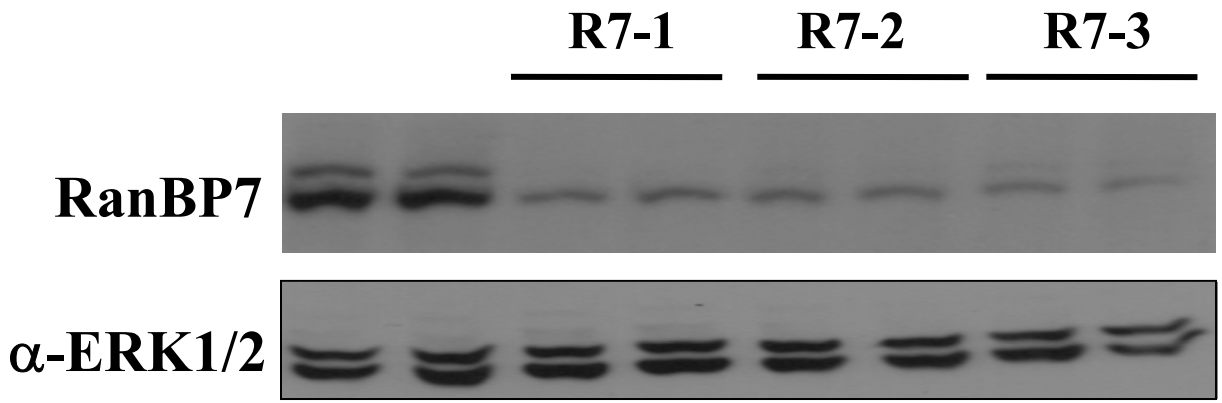


Figure 2-9: Effect of RanBP7 RNAi on nuclear localization of MAPKs. **A**. Immunoblot of RanBP7 and ERK1/2 in HeLa cells transfected with three dsRNA oligonucleotides (R7-1, -2, and -3). **B.** Activation of ERK1/2, JNK and p38 MAPKs by PMA or UV in the cells in transfected with RanBP7 dsRNA oligonucleotides. Activation of the MAPKs was detected by immunoblotting with the indicated antipMAPK antibodies. Immunolocalization of pERK1/2 (**C**), pp38 (**D**) and pJNK (**E**) with anti-pMAPK antibodies in HeLa cells in which RanBP7 has been suppressed with dsRNA oligonucleotides.

Fig. 2-9B

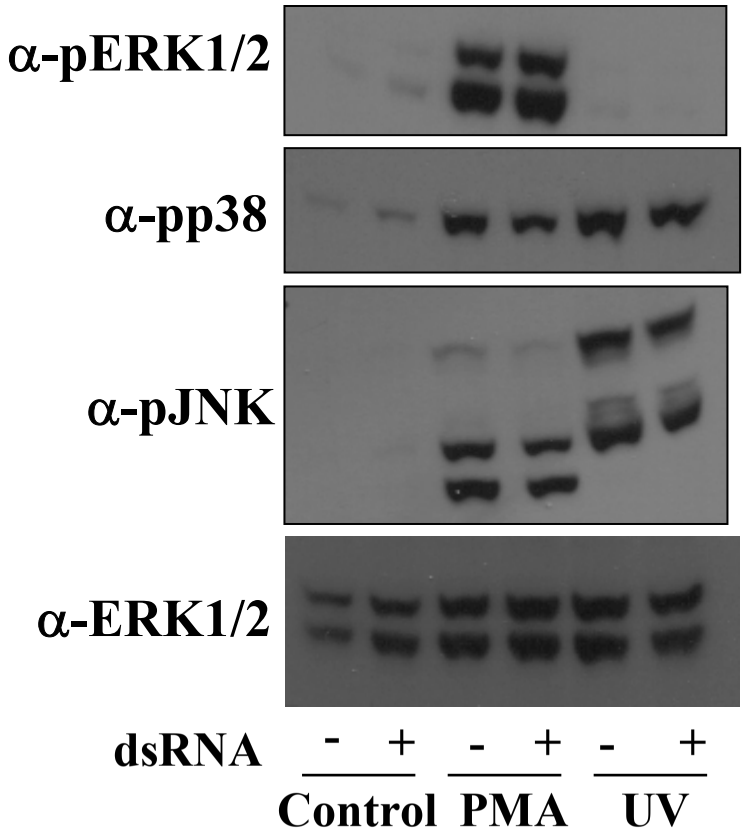


Fig. 2-9C

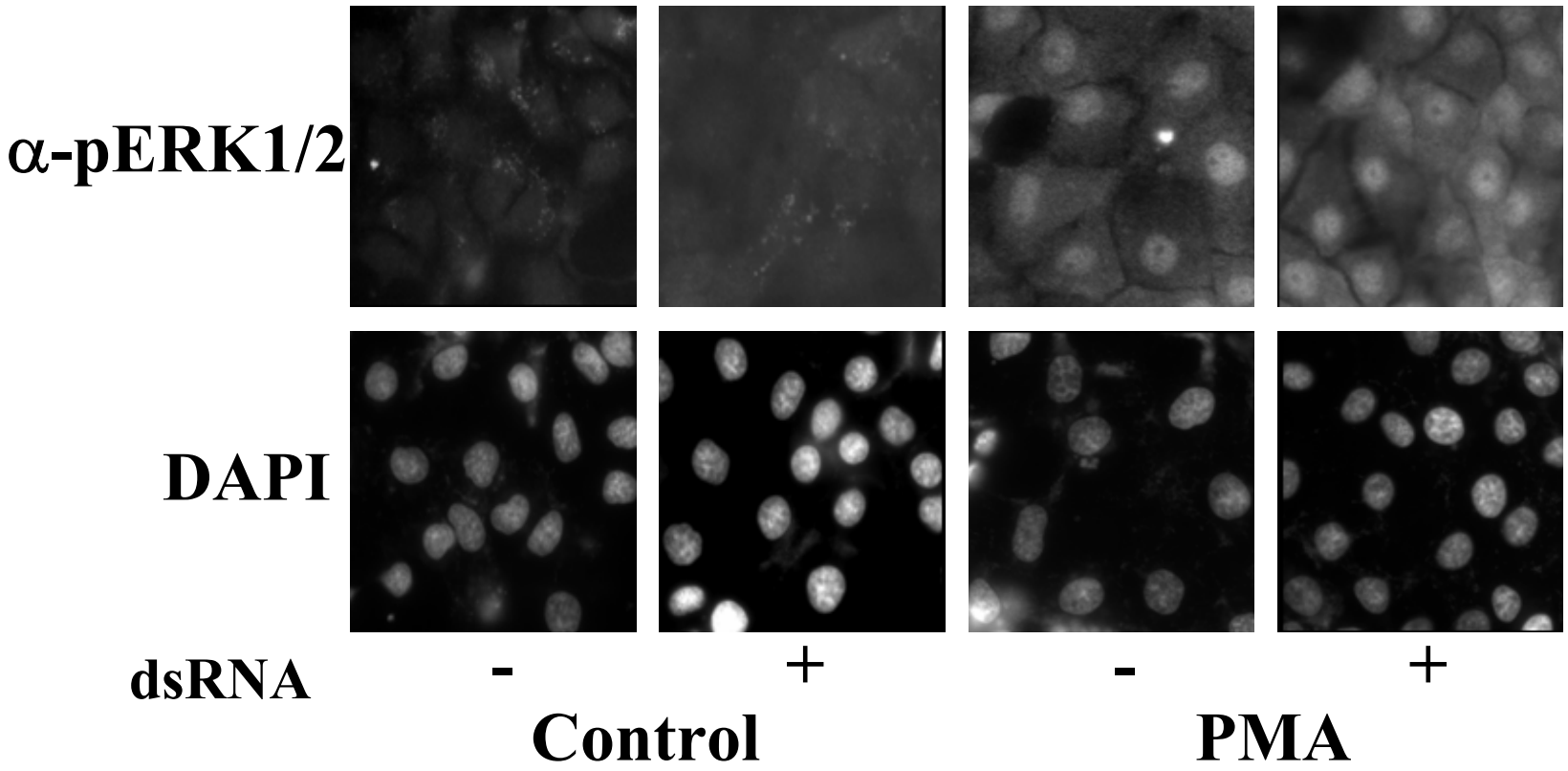


Fig. 2-9D

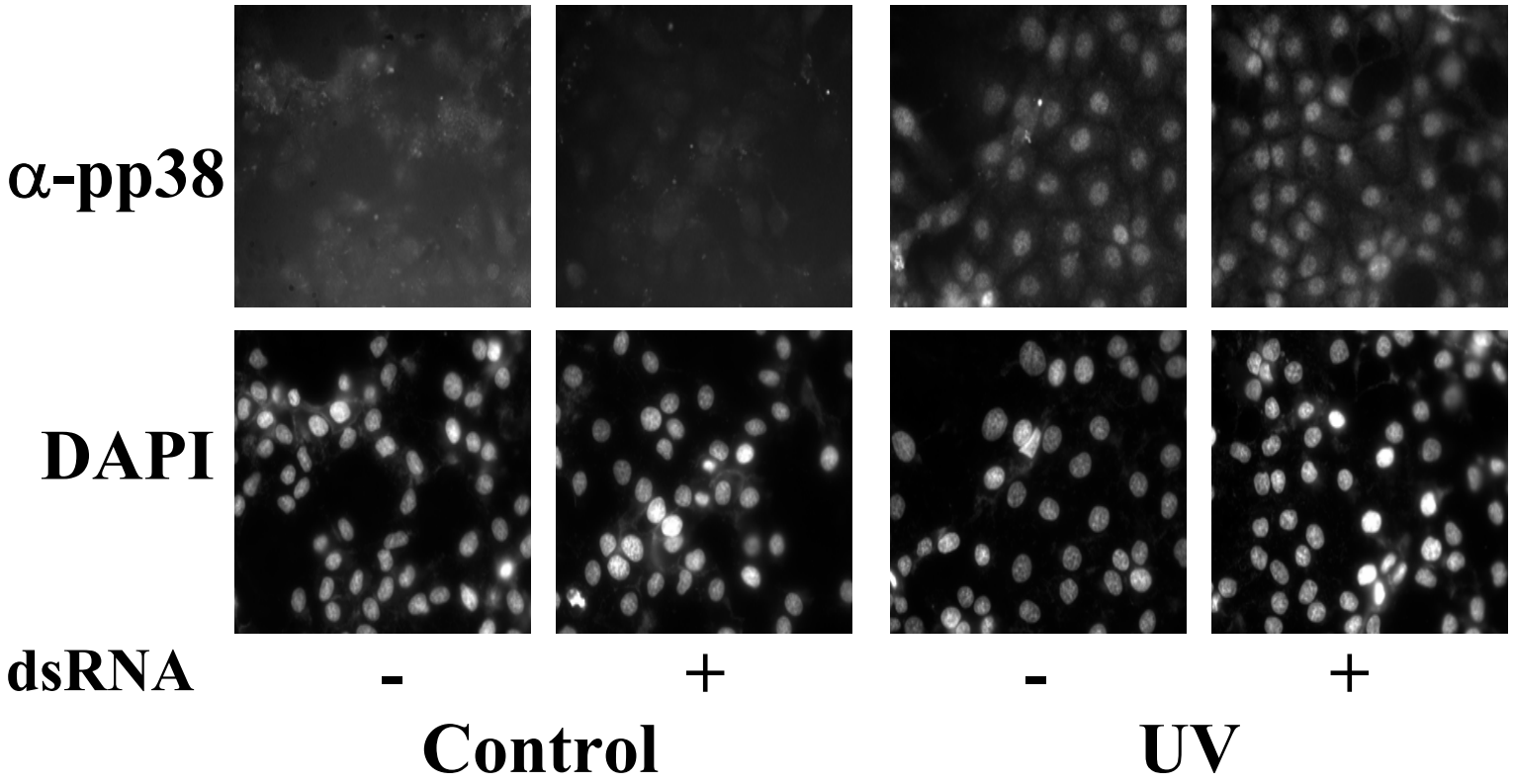


Fig. 2-9E

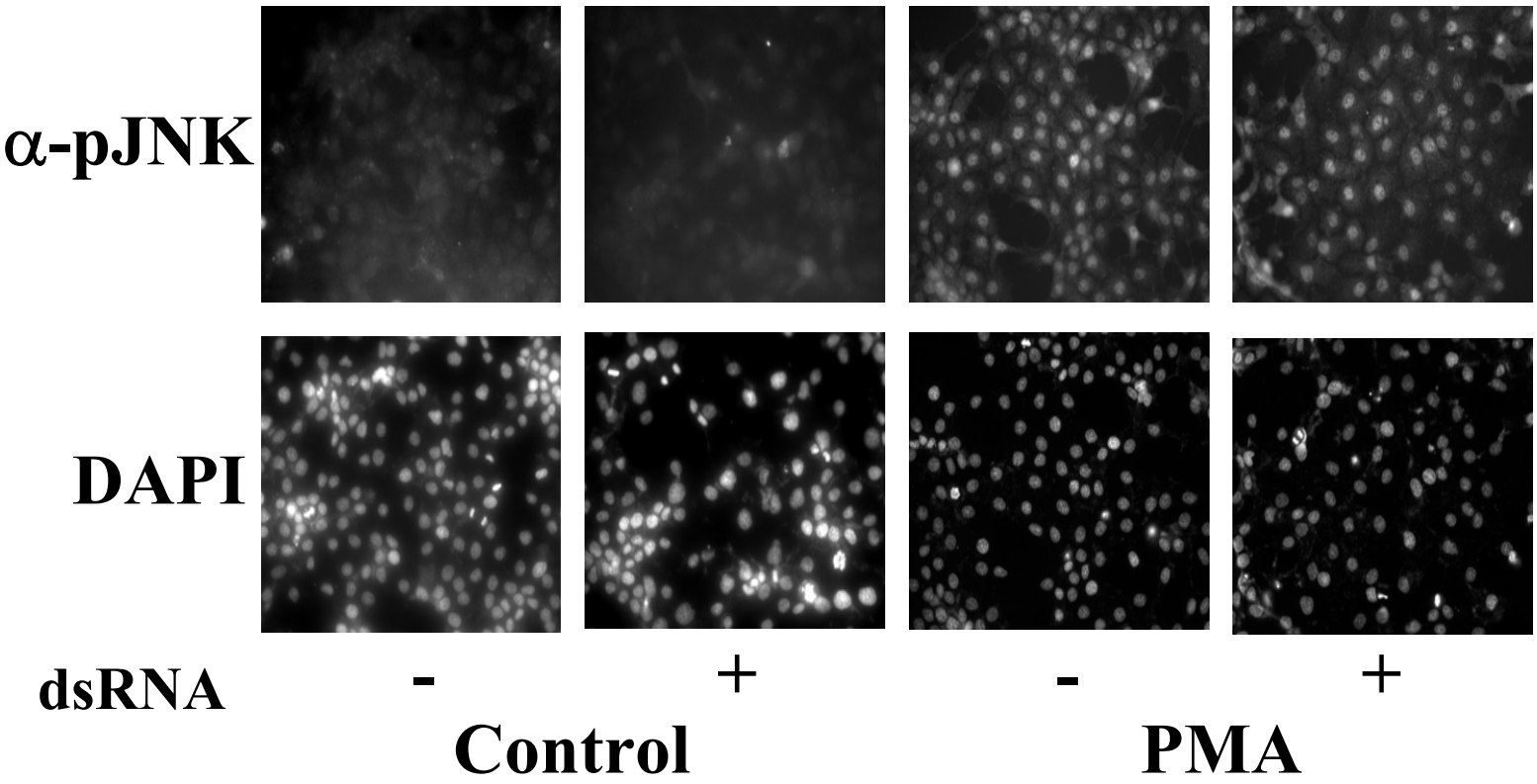


Fig. 2-10

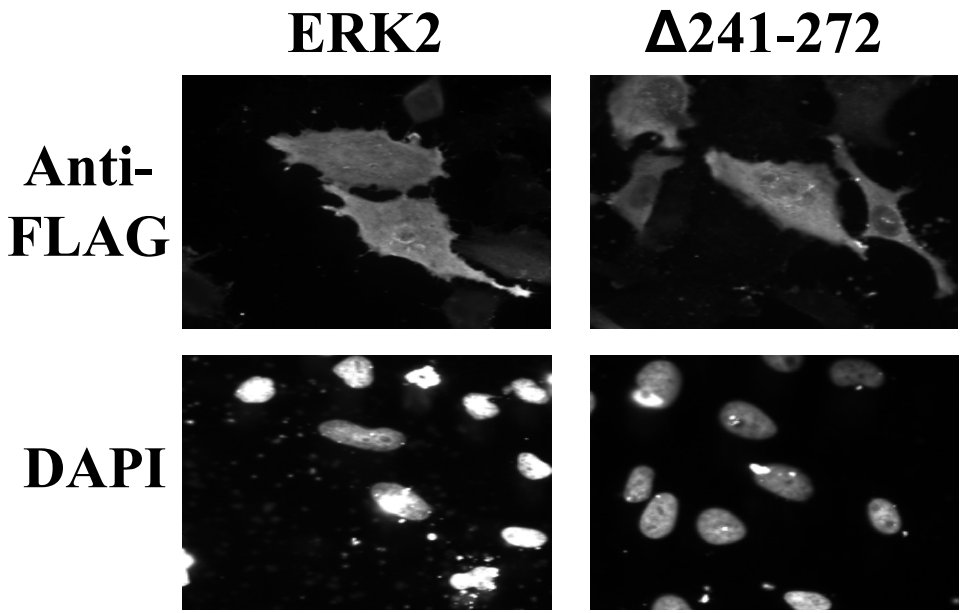


Figure 2-10. Expression of ERK2 and ERK2 Δ241-272 in HeLa cells. The heterologously expressed ERK2 proteins were detected with antibodies to the Flag epitope tag. Stain of nuclei in the bottom panels.

CHAPTER 3: The Nuclear Localization of ERK2 Occurs by Mechanisms Both Independent of and Dependent on Energy

Abstract:

The mitogen-activated protein (MAP) kinases ERK1 and ERK2 often accumulate in the nuclei of stimulated cells to mediate changes in transcription. The mechanisms underlying stimulus-dependent redistribution of these kinases remain unclear. In this study, a permeabilized cell reconstitution assay in HeLa cells and human foreskin fibroblasts was used to explore the processes by which ERK2 enters and exits the nucleus. Our laboratory previously reported that entry of unphosphorylated ERK2 into the nucleus occurs by facilitated diffusion not requiring cytosolic transport factors. This current work shows that export, like import, can occur by an energy- and carrier-independent mechanism. An energy-dependent mechanism of ERK2 export can also be distinguished, mediated at least in part through the exportin CRM1. Import and export of thiophosphorylated, active ERK2 were also examined. Import of active ERK2 is significantly enhanced by the addition of exogenous transport factors and an energy regeneration system. These studies support a model in which multiple constitutive and regulated processes control the subcellular distribution of ERK2.

Introduction:

Many signaling events are controlled by acute changes in the activities of proteins due to covalent modifications. The appropriate localization of regulatory molecules and the enzymes that modify them is crucial for coordinated cellular responses. The subcellular localization of ERK1/2 is tightly regulated. ERK1/2 affect regulatory processes in cell compartments including the cytoskeleton, the plasma membrane, and the nucleus. The stimulus-induced nuclear uptake of ERK1/2 is essential for some of the phenotypic programs to which they contribute including differentiation, transformation, and other events requiring altered transcription or other nuclear functions. Improper localization of ERK1/2 has been found in certain cancers, highlighting the importance of elucidating the mechanisms regulating their localization.

Chen *et al.* (1992) first observed that inactive ERK2 is present in the cytoplasm of resting cells and that upon stimulation, a fraction of total ERK2 accumulates in the nucleus; similar observations were subsequently reported by others with ERK1 and ERK2 (Leonard *et al,* 1993; Brunet *et al,* 1999; Gonzales *et al,* 1993). Microinjection of thiophosphorylated ERK2 (thio-pERK2) into the cytoplasm of REF52 cells resulted in its rapid translocation to the nucleus (Khokhlatchev *et al*, 1998), suggesting that activation of ERK2 is sufficient for its nuclear localization. In support of this finding, inhibition of ERK2 phosphorylation by the MEK inhibitor PD98059 reduced ERK2 nuclear localization (Lenormand *et al* 1998).

Activation of ERK1/2 does not ensure their nuclear localization. Endogenous inactive ERK1/2 are also found in the nucleus, and active ERK1/2 are observed in the cytoplasm (Cheng *et al,* 1996; Menice *et al,* 1997; Whitehurst *et al,* 2004).

Microinjection of unphosphorylated ERK2 into the cytoplasm of fibroblasts led to its nuclear localization within 2 min and redistribution to the cytoplasm by 5–10 min, suggesting that ERK2 can rapidly enter and exit the nucleus in unstimulated cells (Khokhlatchev *et al,* 1998). Overexpressed inactive ERK2 accumulates in the nucleus and has been used to assay for factors that promote cytoplasmic retention (Rubinfield *et al,* 1999; Robinson *et al,* 2002). These later findings suggest that nuclear localization of ERK1/2 can be uncoupled from their activation.

Import reconstitution assays with unphosphorylated ERK2 showed that ERK2 enters the nucleus by an energy- and carrier-independent facilitated mechanism (Matsubayashi *et al,* 2001; Whitehurst *et al,* 2002). Direct interactions with nuclear pore proteins were inferred from pull-down assays. More recently, fluorescence recovery after photobleaching was used to confirm the energy-independent movement of ERK2 into the nucleus (Burack *et al* 2005). Fluorescence resonance energy transfer demonstrated that ERK2 interacts with MEK1 in the cytoplasm. This finding supports earlier suggestions that MEK1 is involved in cytoplasmic anchoring of ERK2 (Fukuda *et al* 1997). Phosphorylation of ERK2 releases it from a MEK1 complex, presumably increasing the free pool of ERK2 available to enter the nucleus by the facilitated, energy-independent mechanism. Together these studies suggest an important role for anchoring proteins including MEK1 in the subcellular redistribution of ERK2.

In addition to cytoplasmic anchoring, MEK1 has been proposed to cause nuclear export of ERK2. MEK1 has a nuclear export sequence (NES) that enables its export via the export receptor CRM1 (Fukuda *et al,* 1997; Adachi *et al,* 2000). A MEK1 mutant in which the NES was disrupted caused nuclear retention of microinjected ERK2, whereas

wild-type MEK1 enhanced cytoplasmic localization of ERK2 (Fukuda *et al,* 1997; Adachi *et al,* 2000). Thus, it was proposed that inactive ERK2 can be exported from the nucleus by association with MEK1. Leptomycin B (LMB), an inhibitor of CRM1-mediated export, reduced loss of ERK2 from the nucleus, suggesting that ERK2 export is CRM1- and energy-dependent. Several studies have shown that the localizations of ERK2 and MEK1 are sensitive to LMB (Whitehurst *et al,* 2002; Adachi *et al* 2000, Whitehurst *et al,* 2004; Yao *et al* 2001). Because the dually phosphorylated form of ERK2 does not bind MEK1, it was suggested that the active form of ERK2 is not exported from the nucleus (Adachi *et al,* 2000).

It is not clear whether the behavior of MEK1 is consistent with a role in ERK1/2 export. MEK1 has been found primarily in the cytoplasm of cells. Disabling its NES causes its nuclear accumulation, suggesting that it shuttles between the two compartments (Adachi *et al,* 2000; Zeng *et al,* 1994; Jaaro *et al,* 1997). However, no significant translocation of MEK1 into the nucleus has been detected upon stimulation (Burack *et al,* 2005; Zeng *et al,* 1994). Thus, the question remains as to whether quantities of MEK1 that enter the nucleus or the kinetics of its entry or exit are sufficient to drive ERK2 export.

In aggregate, these studies suggest that there is continuous nuclear-cytoplasmic shuttling of the free pool of ERK2 by facilitated diffusion. The active form binds to proteins in the nucleus and, as a consequence, is less readily exported. Anchoring ERK2 in different compartments of the cell determines the size of the pool available for relocalization.

We analyzed import and export in separate reconstitution assays to gain further insight into the mechanisms of ERK2 nuclear transport. Our results identify additional regulated events that must be added to the current model.

Materials and Methods:

Constructs and Recombinant Proteins- GFP-ERK2, thio-pERK2, MEK1, all with His_6 tags, CRM1, Ran, p10/NTF2, karyopherin-β1, karyopherin-α2, and rhodamine-labeled bovine serum albumin (BSA) containing a synthetic nuclear localization sequence (NLS) was prepared as described before (Whitehurst *et al, 2002*).

Cell Culture-HeLa cells and BJ fibroblasts were grown on coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum(FBS), 1% L-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C under 10% CO_2. Cells were treated with 10–20 nM (5.5–11 ng/ml) LMB (LC Laboratories) for 2–6 h prior to use in import assays. Cells were starved by changing the media to DMEM without serum for 2-4 hours.

RNAi-CRM1 expression was reduced with RNAi as described in Elbashir *et al,* 2001. Small interfering RNA oligonucleotides generated toward three CRM1 sequences (CRM1-1, 5'-ATACGTTGTTGGCCTCATT-3'; CRM1-2, 5'-ATATGTTGTTGGTATCTGA-3'; CRM1-3, 5'-TTACTCATCTGGATTATGT-3') were synthesized by the UT Southwestern core facility. HeLa cells were grown on coverslips to 30–50% confluence in DMEM media with FBS but without antibiotics. 100 nM CRM1-1, CRM1-2, or CRM1-3 double-stranded-RNA oligonucleotides were added, and cells were grown for 36-48 hours. CRM1 antibody (BD Transduction Laboratories) or ERK1/2

antibody were used in western blots to verify that CRM1 expression was reduced without affecting ERK1/2 expression.

Import and Export Assays- Import and export assays were performed as described before (Whitehurst *et al,* 2002 ; Kehlenbach *et al* ,1998). Unless otherwise indicated, experiments were performed at least three times in duplicate coverslips. Cells were washed in transport buffer (TB) (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 2 mM dithiothreitol), and permeabilized with 70 μg/ml digitonin in TB for 5 min. The import reaction was performed in a 40-μl reaction mix that contained one or more of the following components as specified in the figure legends: TB with 10 mg/ml BSA; 0.8 μM recombinant substrate (GFP-ERK2 or thio-pERK2); 0.14 μM TRITC-NLS-BSA; HeLa cell cytosol dialyzed against TB (2.5 mg/ml or as specified); energy-(ATP/GTP regenerating system consisting of 1 mM GTP, 1 mM ATP, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase); 4 units of apyrase; 0.5 mg/ml wheat germ agglutinin (WGA); and recombinant transport factors kapα2 (0.5 μM), kapβ1 (0.25 μM), Ran (2 μM), and p10/NTF2 (0.4 μM). Import was for 15 min unless otherwise specified and was terminated with 0.25 ml of TB. Cells were fixed in paraformaldehyde or used for export assays. For export assays, cells that had been preincubated with import substrate were transferred to a 40-μl reaction mix that contained TB with 10 mg/ml BSA and one or more of the following as specified in the figure legends: cytosol, energy, WGA at the same concentrations as above. The reaction was terminated with 0.2 ml of TB. Cells were fixed in 3% paraformaldehyde for 20 min on ice, and coverslips were mounted using Aqua PolyMount (Polysciences, Inc.).

Indirect immunofluorescence –For endogenous ERK1/2 localization, cells were fixed in 3.7% paraformaldehyde for 10 minutes and washed 3X with 1X TBS. Cells were then permeabilized with 0.5% Triton X-100 for 10 min and washed once with 1X TBS and twice with 1XTBST. Blocking was performed in TBST (1X tris buffered saline, 0.1% Tween 20, 10 mg/ml BSA) for 30 min at room temperature. The pERK1/2 antibody (Sigma) was used at 1:300 for 12-18 h at 4 °C, and the Alexa Fluor 546 anti-mouse secondary antibody (Molecular Probes) was used at 1:3000 for 1 hour at room temperature. For detection of thiophosphorylated ERK import *in vitro*, procedure is similar with slight modification. The cells were fixed in 3% paraformaldehyde for 20 min on ice and the rest of the protocol is same with endogenous ERK1/2 localization above.

Fluorescence Microscopy-GFP, TRITC, and Alexa Fluor fluorophores were visualized by fluorescence microscopy using a Zeiss Axioskop 2 Plus microscope, and images were acquired using a Hamamatsu digital CCD camera (C4742-95). Exposures for all conditions within an experiment were constant, and fluorescence intensity within the nucleus was quantified using Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.).

Results:

The amount of ERK1/2 amount in the nucleus increases after inhibition of CRM1 by Leptomycin B or siRNA of CRM1: Starving cells for 3 hours was enough to decrease the amount of active ERK1/2 to a level almost undetectable by immunoblotting. Stimulation with EGF or PMA increases the nuclear ERK2 levels (*data not shown*). This increase is using antibodies against total ERK1/2 or phosphorylated ERK1/2.

When HeLa cells were pre-incubated with LMB to inhibit CRM1, there was an increase in nuclear ERK1/2 amount (figure 3-1), consistent with the previous findings (Adachi *et al,* 2000; Whitehurst *et al*, 2004). We observed similar results in EGF stimulated HeLa cells as well (data not shown). In the case of BJ fibroblast cells, where EGF and PMA stimulation result in distinct localization patterns (cytosolic vs nuclear p-ERK respectively), LMB treatment increased the amount of active and total ERK1/2 levels in the nucleus in response to both stimuli (data not shown and Whitehurst *et al*, 2004).

To demonstrate that the results observed in LMB treated cells are not due to a nonspecific action of the drug, RNAi was used to knockdown CRM1 in cells. I designed three different siRNA oligonucleotides. All three of the oligonucleotides effectively decreased CRM1 expression in the cells 36-48 hours after treatment (figure 3-2A). In the CRM1 knockdown cells, ERK1/2 was still activated in response to PMA or EGF, indicating that CRM1 is dispensable for the activation of ERK1/2 by the upstream kinases (Fig 3-2B).

There was more nuclear ERK1/2 in HeLa cells if CRM1 was knocked down. This is the case for both activated ERK (Fig 3-3A) and total ERK (Fig 3-3B). This result was

similar to what was seen in LMB treated cells, in which LMB treatment increased the nuclear localization of ERK1/2 (Fig 3-1).

In vitro export of GFP-ERK2 occurs through the NPC: My collaborator Aarati Ranganathan modified the import reconstitution assay that was explained in the second chapter to analyze the factors that might be required for the export of ERK2. In this *in vitro* export assay, digitonin permeabilized cells were used. GFP-ERK2 was initially imported into the nuclei of these cells for 15 minutes. For export, cells were incubated in transport buffer containing cytosol and energy (+C+E) or lacking both (-C-E). The transport mixture with cytosol and energy (+C+E) promoted a higher rate of export compared to cells in transport buffer alone (-C-E) (Ranganathan *et al*, 2006) . This result implies a role of some cytosolic factors in the export process.

To show that export occurs through nuclear pore complex, WGA was added to block nuclear transport through the nuclear pore complex. WGA binds to carbohydrate moieties on nucleoporins which prevents interactions of other proteins with nucleoporins (fig 3-4). If WGA was added before the import reaction, no fluorescent signal in the nucleus could be detected indicating that GFP-ERK2 is no longer imported. In the absence of WGA, GFP-ERK2 could be detected in the nucleus of the cells (fig 3-4, cells in the second lane). If WGA was added after import but before the export process, the GFP-ERK2 signal could still be detected in the nucleus of the cells. On the other hand, untreated cells showed a small amount of signal in the nucleus as a result of export (cells at the bottom). This confirms that GFP-ERK2 export occurs through nuclear pore complex and WGA can block both the export and import processes.

The role of CRM1 in export: As export of GFP-ERK2 occurs without cytosol and energy, but is enhanced by cytosol and energy, it seemed likely that there are two different pathways for export. We used both RNAi and LMB in *in vitro* export assays to understand the role of CRM1 in ERK2 export.

In the cells in which CRM1 was knocked down (CRM1 RNAi+), there was a decrease in the export of GFP-ERK2 (fig 3-5B) in the presence of of cytosol and energy (+C+E). Quantification of the fluorescent light showed that the decrease was almost 30% at 15 minutes and 45% at 30 minutes of export (fig 3-5C). However, there was no significant decrease in export with the absence of energy and cytosol (-C-E). Although CRM1 knockdown may have slightly decreased export, there was no clear difference between the control cells and the siRNA-treated cells (fig 3-5D and data not shown). For the NLS-BSA import, we did not see any difference between control and cells with decreased CRM1 (fig 3-5 E and F).

Similar experiments were performed with LMB. As was the case with CRM1 RNAi experiments, decreased export of GFP-ERK2 was observed in LMB-treated samples with cytosol and energy (+C+E) in the export assay. Little or no difference was observed in export of GFP-ERK2 without cytosol and energy (-C-E) (fig 3-6).

Thus, CRM1 plays a role in the energy-dependent export of ERK1/2, but ERK1/2 can still be exported by an alternative energy-independent mechanism.

Disscussion:

We have used import and export assays in permeabilized cells to evaluate mechanisms of ERK2 entry into and exit from the nucleus. Much of the previous work directed at understanding these processes employed overexpressed proteins that may behave differently from endogenous proteins or small molecule inhibitors that may block the functions of unrelated components in the cell.

The following model best captures the current findings with ERK2 and is likely to apply also to ERK1. ERK2 in the unphosphorylated state enters the nucleus by an energy-independent mechanism facilitated by direct interaction with nucleoporins (Matsuyabashi 2001, Whitehurst 2002). An effect of transport factors and energy has not been detected, suggesting that this is the primary entry mechanism for the unphosphorylated protein. Entry is very rapid in reconstitution assays and as reported using photobleaching in intact cells (Burack 2005). Proteins that bind ERK2 in the cytosol inhibit its entry by tethering it at sites distant from the nuclear membrane or, like PEA-15, by blocking its ability to interact with nucleoporins (Whitehurst *et al*, 2004). ERK2 can also exit the nucleus by an energy-independent process as suggested previously (Matsuyabashi *et al*, 2001), presumably by the same nucleoporin-mediated mechanism. Thus, there is a constant exchange of ERK2 between the cytoplasm and the nucleus. In the case of export, a second process dependent on energy and transport factors including CRM1 also exists. Like unphosphorylated ERK2, phosphorylated ERK2 can enter and exit the nucleus by an energy- and carrier-independent mechanism. However, a significant component of entry of phosphorylated ERK2 requires energy. Energy appears to increase the rate and extent of nuclear uptake. This process presumably promotes the nuclear uptake of active ERK2

selectively over the unphosphorylated form. Interactions specific for the phosphorylated form of ERK2 would then be expected to enhance retention of the active form in the nucleus.

The factors required to mediate the steps we have identified are present in unstimulated cells following permeabilization. Residual amounts of transport proteins are present, but factors that are dependent on stimulation, due to either covalent modification or induction, are not required for these processes. Additional regulation is expected to be introduced under stimulated conditions. Active ERK2 binds kinetochores, topoisomerase II, and many transcription factors, as well as an unidentified newly synthesized protein (Lenormand *et al*,1998; Zecevic *et al*, 1998; Shapiro *et al,* 1999; Sharrocks *et al*, 2000), all of which may serve to sequester active ERK2 from the readily exchanging pool.

Energy-dependent import mediated via importins requires direct binding of cargo proteins through their NLS to import factors. ERK2 has no obvious NLS. Thus, as for export, the energy-dependent process of import of phosphorylated ERK2 may be mediated by binding to other NLS-containing proteins; phosphorylation of ERK2 would be expected to enhance interaction with such proteins. It is also possible that phosphorylation unmasks an otherwise silent NLS in ERK2.

The energy-dependent component of ERK2 export is mediated, at least in part, by CRM1. Thus far, we have been unable to show any direct interaction between ERK2 and CRM1 (data not shown). It is possible that binding of ERK2 to other karyopherin-β family members bypasses the need for an NES. There is precedent for direct interactions of MAPKs with transport factors. The *Drosophila* ERK (D-ERK) binds to the homolog of

importin7 (DIM-7) (Lorenzen *et al*, 2001), and the yeast p38 MAPK HOG1 binds to a karyopherin-β family member, NMD5 (Ferrigno *et al*, 1998).

It seems more likely that ERK2, which lacks a recognizable NES, requires binding to other NES-containing proteins to be exported via the classical export machinery. One such protein is MEK1. These results support a role for MEK1 as a cytoplasmic anchor, as originally proposed (Fukuda *et al*, 1997), but do not yet provide a clear picture of its significance in ERK2 export. MEK1 is present in the nuclei of permeabilized cells and might participate in the active export process as it has a leucine-rich NES and is exported via CRM1. The results suggest that MEK1 does not enter the nucleus readily when complexed to ERK2, nor is it apparently exported as readily as is ERK2 in the presence of energy and cytosolic factors. Reduced MEK1 binding does not detectably decrease ERK2 export. Nevertheless, these findings are insufficient to rule out a role for MEK1 in the CRM1-dependent component of ERK2 export.

In summary, active ERK2 enters the nucleus by two processes; one is energy-dependent, and the other is energy-independent. The energy-dependent process is enhanced by transport factors. The energy-independent process is facilitated by interaction of ERK2 with nucleoporins. Two processes, one energy-dependent and one energy-independent, also appear to mediate export of ERK2 from the nucleus. It seems likely that differential regulation of these processes would be sufficient to achieve a range of stimulus-induced localizations such as have been reported in different cell types in response to different ligands.

Figure 3-1

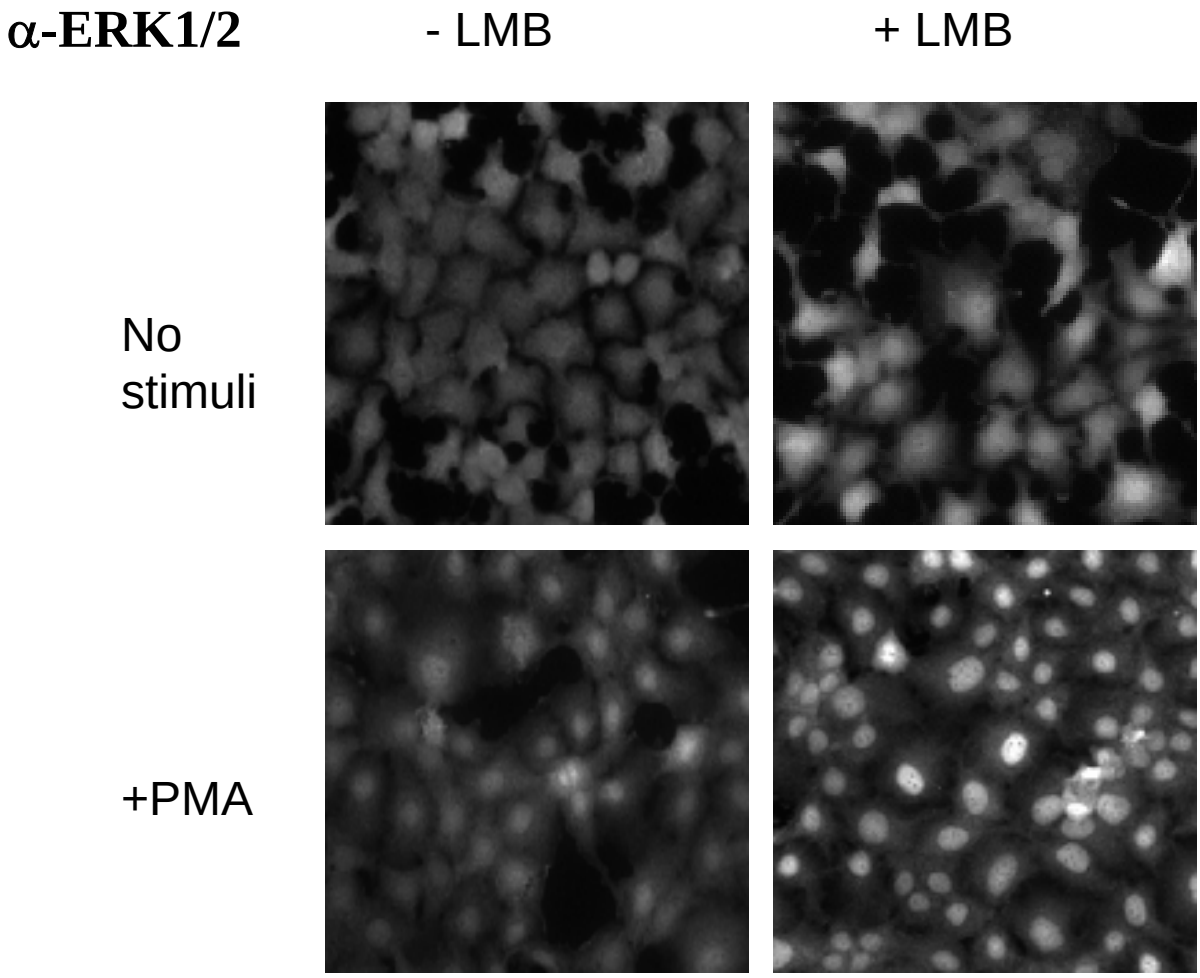


Figure 3-1 Leptomycin B treatment increases the amount of nuclear ERK1/2 as detected with immunofluorescence. Hela cells growing on coverslips were starved for 3 hours in DMEM medium containing no FBS or L-glutamine. After 3 hours of starvation ERK1/2 can be detected in both cytosol and nuclei of the cells. The cells that were treated with PMA showed more nuclear localization of ERK1/2. This increase in nuclear localization of ERK1/2 was even greater for cells which were treated with LMB for 2 hours prior to PMA induction. Anti-ERK1/2 antibody Y691 was used for the immunofluorescence.

Figure 3-2

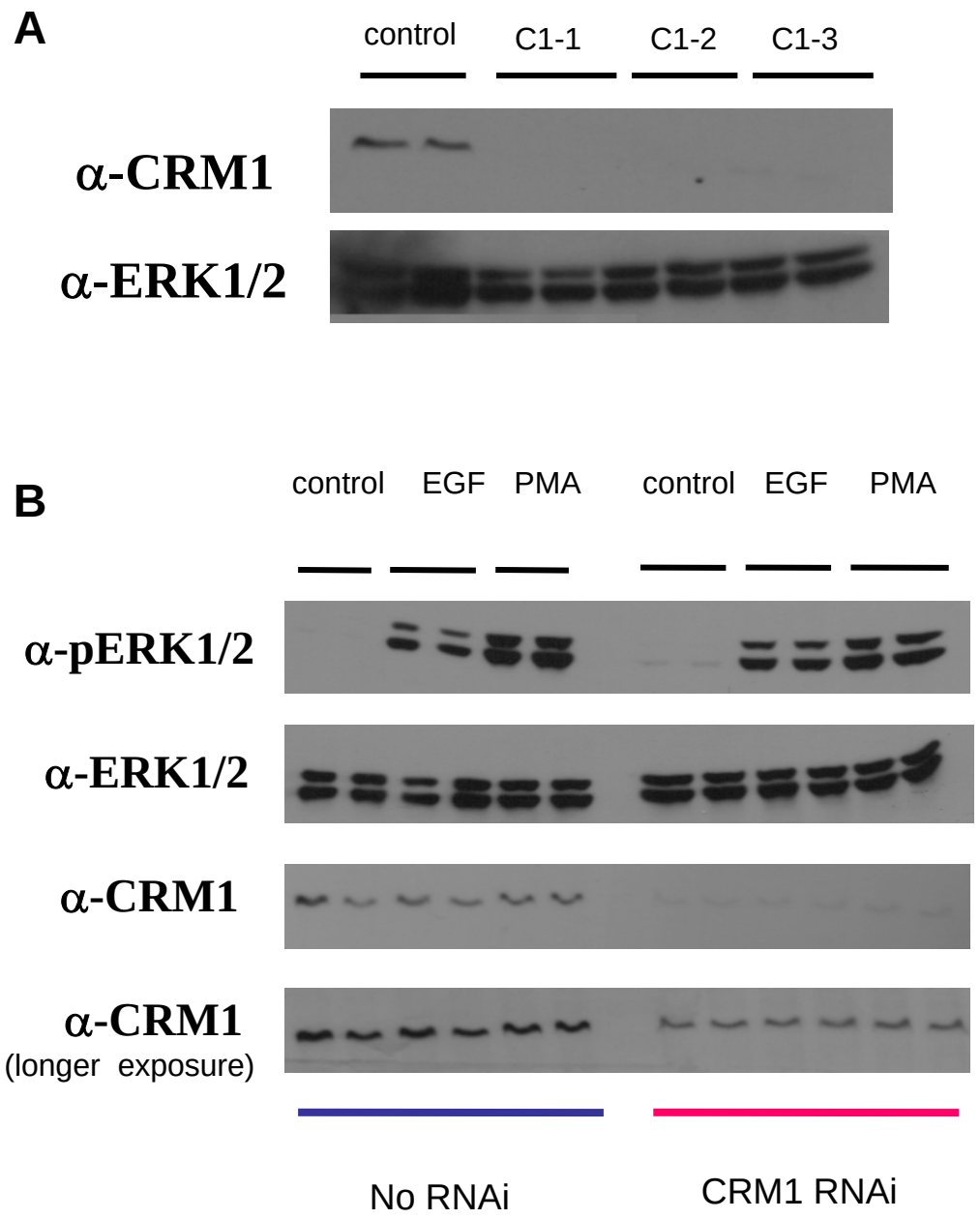


Figure 3-2 CRM1 can be knocked down by RNAi A, Small interfering RNA oligonucleotides were generated toward three CRM1 sequences. HeLa cells were grown on coverslips to 30–50% confluence without antibiotics and they were treated with 100 nM CRM1-1, CRM1-2, or CRM1-3 double-stranded-RNA oligonucleotides. Cells were harvested 24-36 hours after treatment with siRNAs. All 3 oligonucleotides decreased CRM1 levels, but ERK2 levels remained unchanged. **B**, EGF or PMA stimuli lead to phosphorylation of ERK1/2. In cells that were treated with siRNAs against CRM1, ERK1/2 could still be phosphorylated in response to EGF or PMA. Not all the CRM1 was knocked down as we could detect signals with CRM1 antibody in longer exposures of immunoblots (lower blot).

Figure 3-3

A

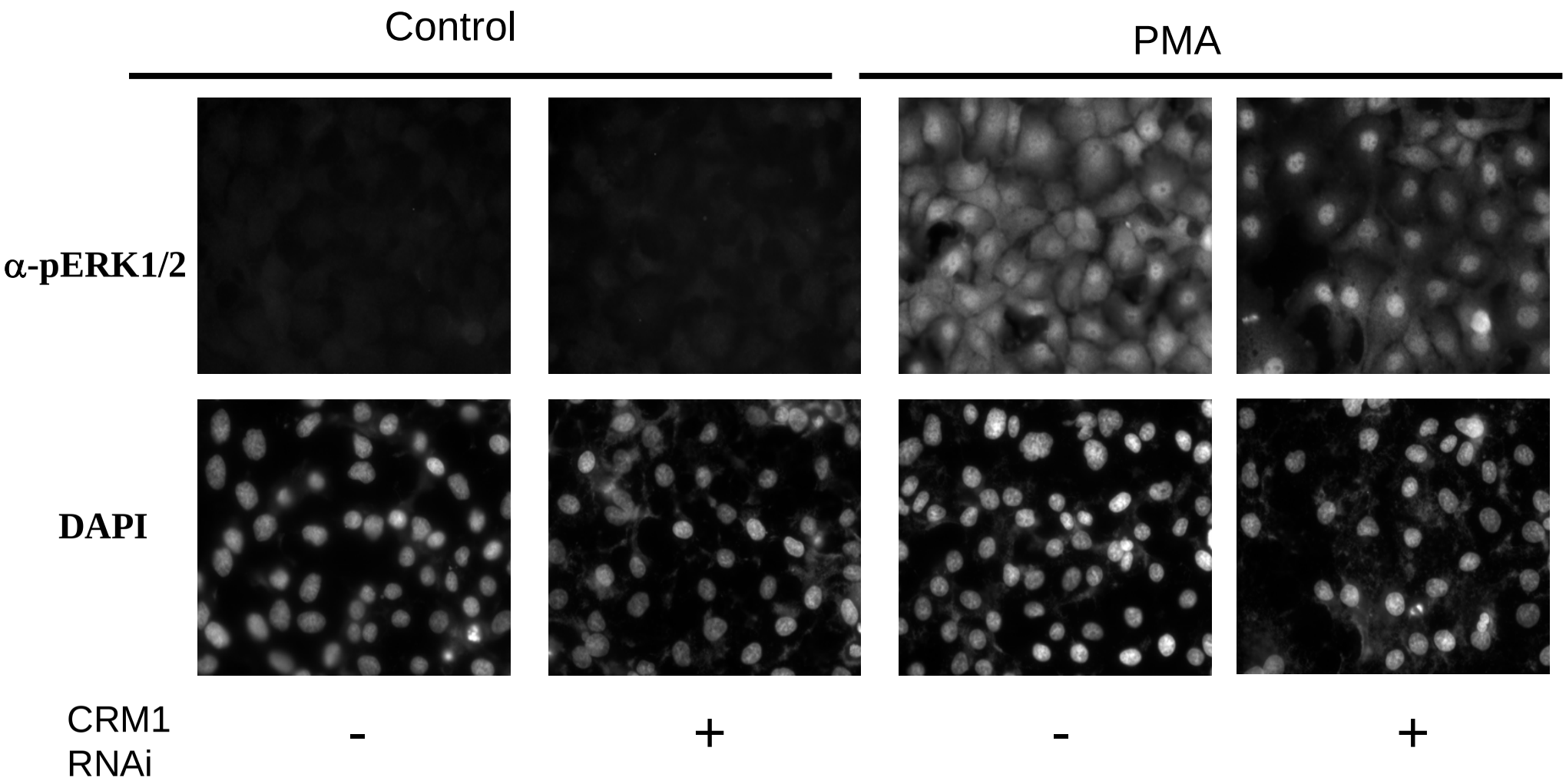


Figure 3-3

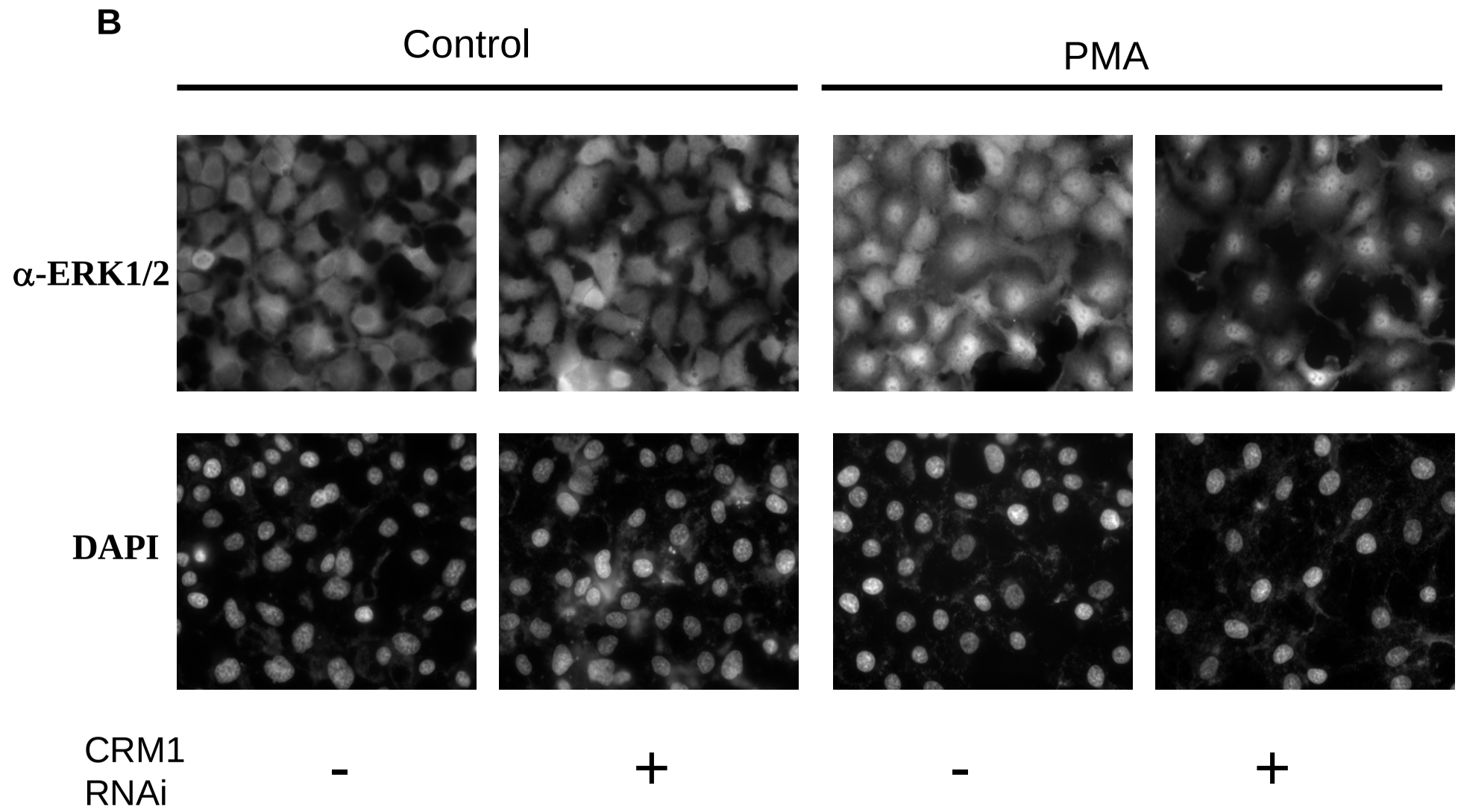


Figure 3-3 CRM1 knockdown increases ERK1/2 levels in the nucleus. PMA treatment increases nuclear ERK1/2 levels. Cells that were treated with siRNA against CRM1 showed higher nuclear localization of both (A) p-ERK1/2 and (B) total ERK1/2 compared to untreated cells. In the cells that were not stimulated, p-ERK could not be detected.

Figure 3-4

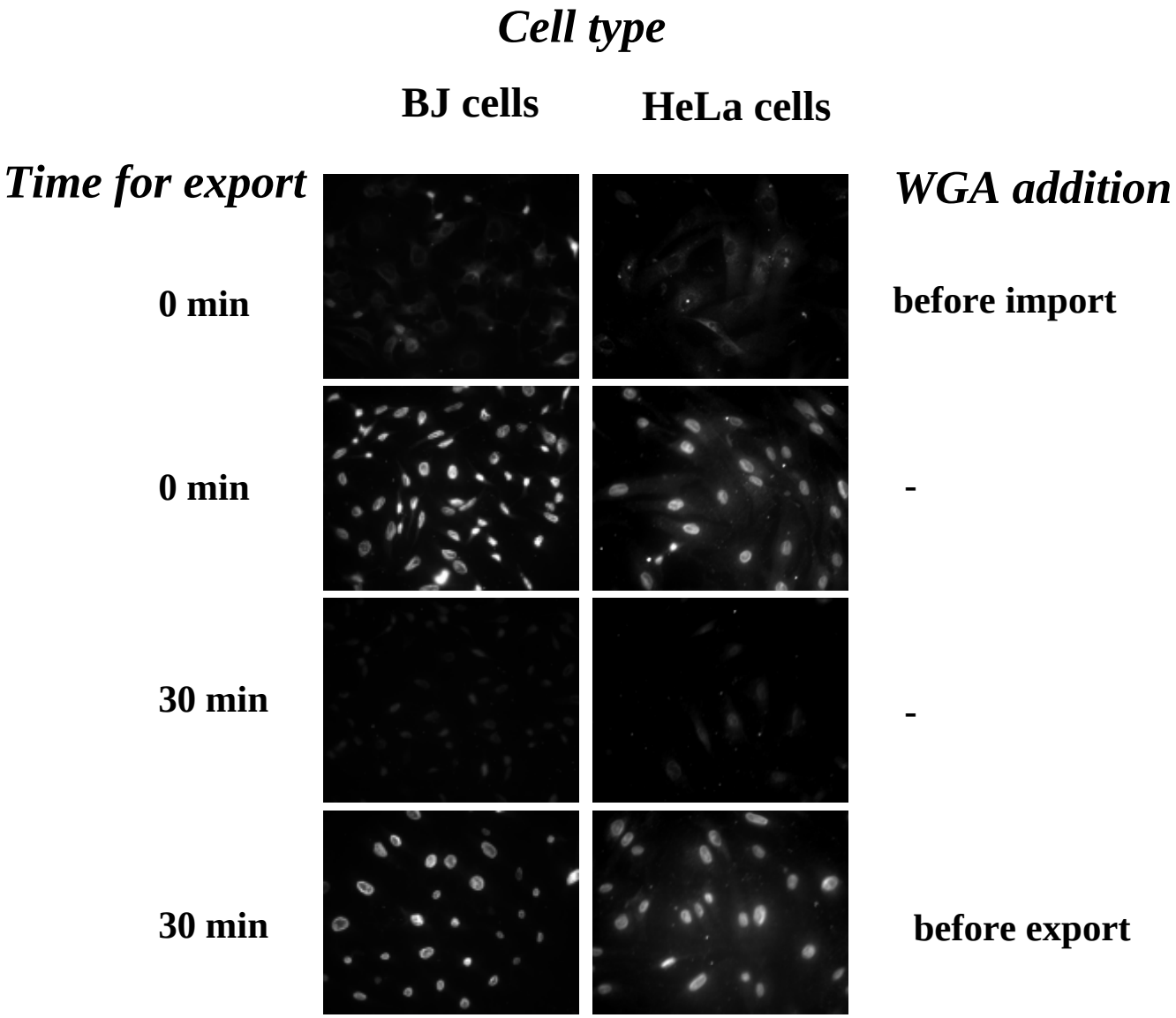
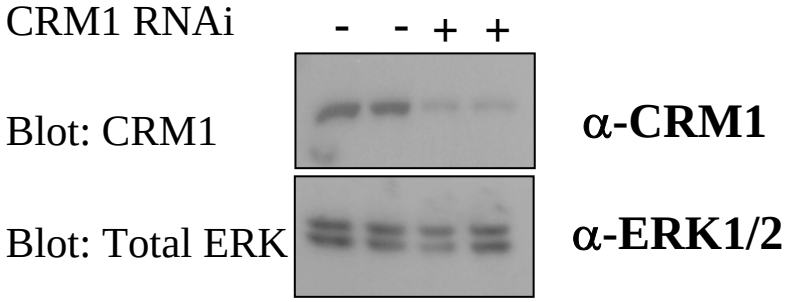


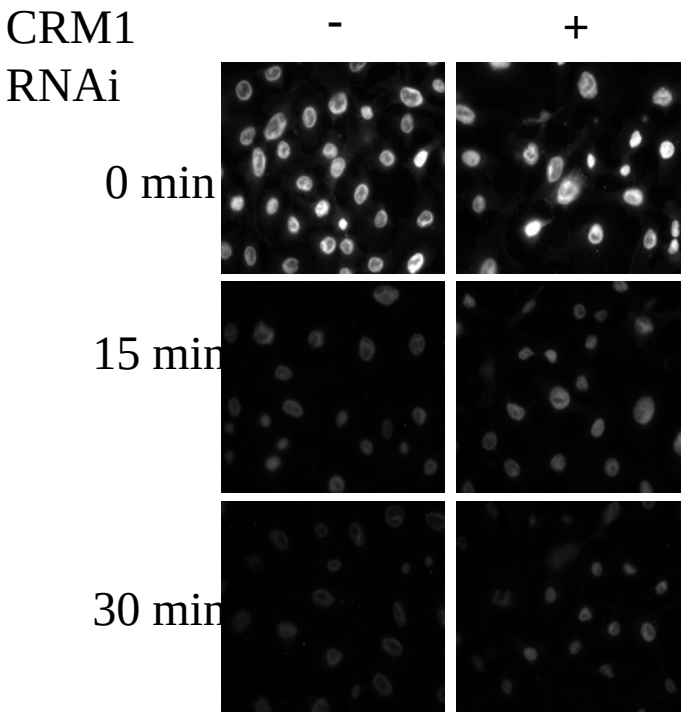
Figure 3-4: Export of GFP-ERK2 occurs through the nuclear pore complex. Import of GFP-ERK2 was for 20 min in transport buffer with or without WGA in BJ or HeLa cells. 30-min export assays were performed on cells incubated in transport buffer containing energy and 2.5 mg/ml cytosol in the presence or absence of WGA added before import or before export. Aarati Ranganathan did the import and export assays.

Figure 3-5

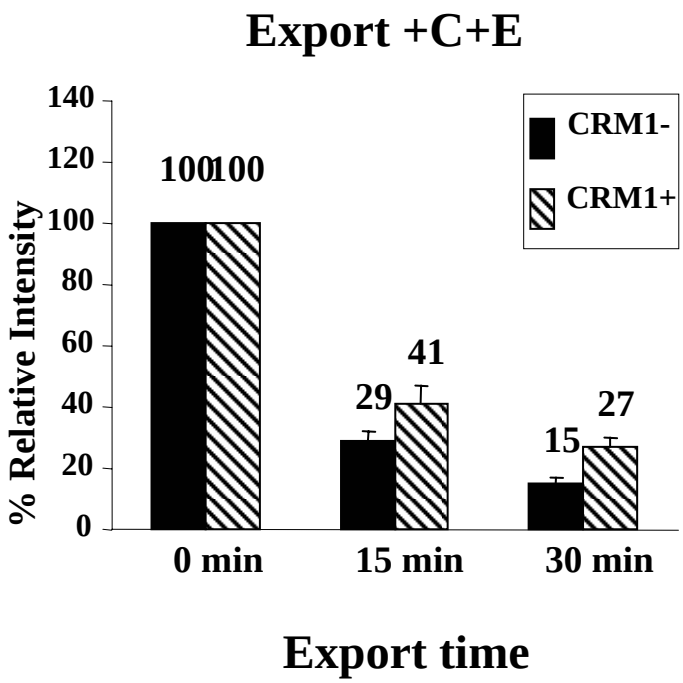
A


CRM1 RNAi
- - + +
Blot: CRM1
α-CRM1
Blot: Total ERK
α-ERK1/2

B


CRM1 RNAi
-
+
0 min
15 min
30 min

C


Export +C+E
140
120
100
80
60
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% Relative Intensity
100100
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CRM1-
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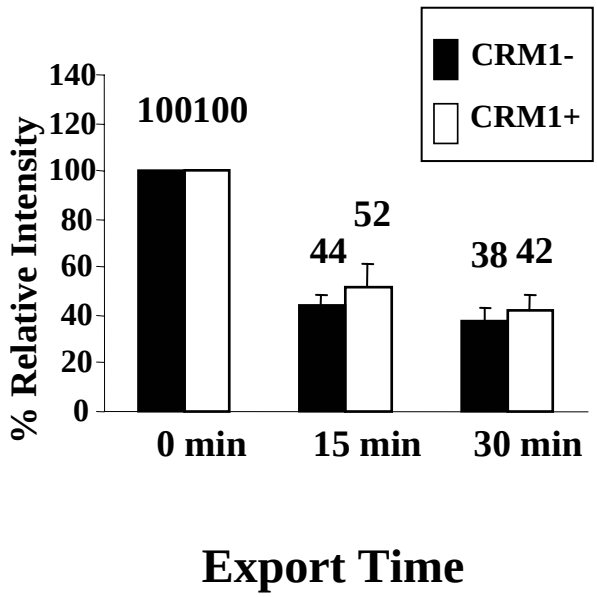
Export -C-E
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CRM1-
CRM1+
0 min
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Export Time

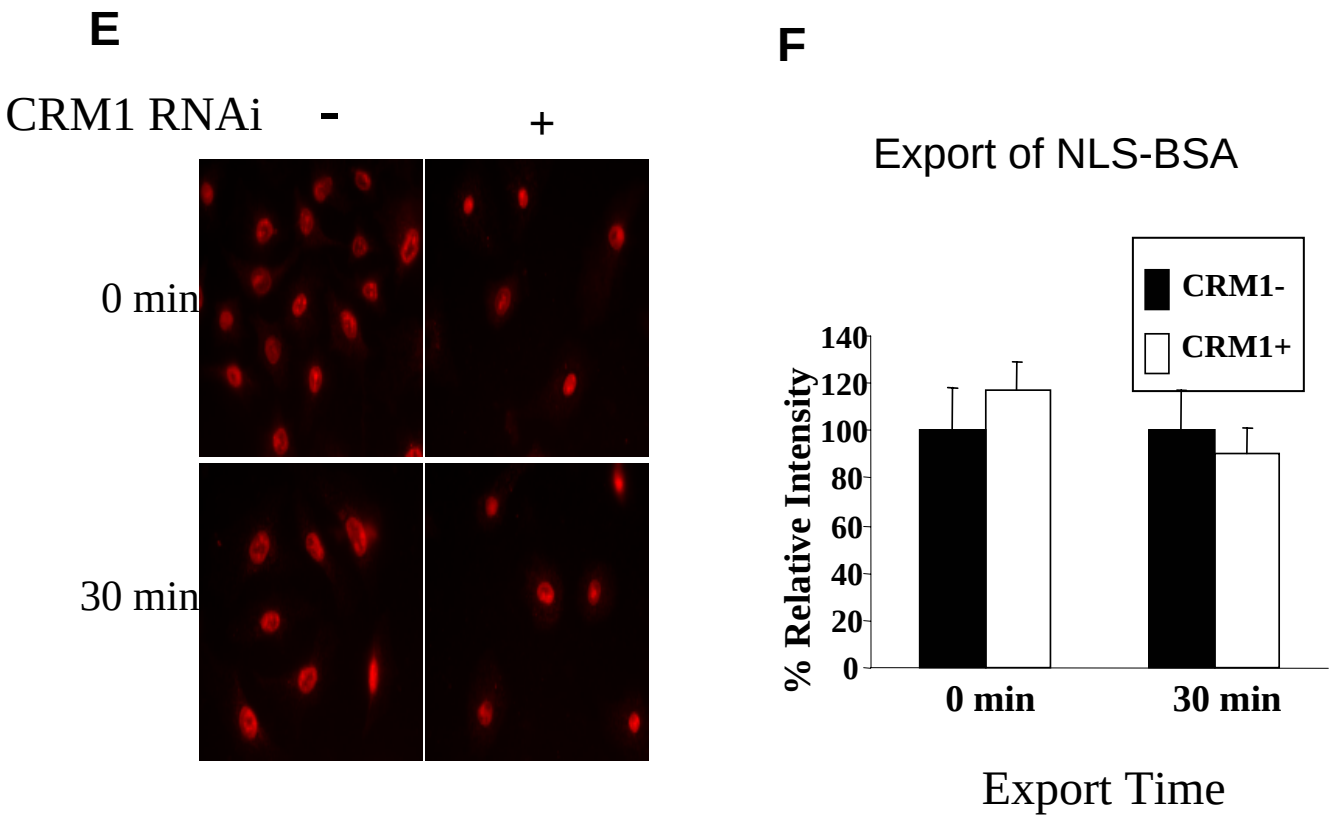


Figure 3-5 Effect of CRM1 knockdown on the export of GFP-ERK2. *A*, CRM1 was knocked down in HeLa cells. The knockdown was detected by Western blot in HeLa cell lysates. Total ERK blots are also shown as a control. *B*, GFP-ERK2 was imported for 15 min in HeLa cells and exported for the indicated times. Import was in transport buffer without added factors, and the export mixture contained cytosol and energy. *C*, fluorescence intensity of GFP-ERK2 quantified from two independent export experiments as in *panel B* in HeLa cells. *D*, Export of GFP-ERK2 was applied in transport buffer without cytosol or energy and fluorescence intensity of GFP-ERK2 in HeLa cell nuclei was quantified. The import assay was in transport buffer without added factors similar to *panel B*. E, export assay of NLS-BSA was in HeLa cells treated or untreated with siRNAs against CRM1. NLS-BSA was imported for 15 min in transport buffer with cytosol and energy. Export was in the presence of cytosol and energy for the indicated times and the fluorescence intensity of NLS-BSA remaining in the nucleus was quantified in *F* (+C+E; cytosol and energy , -C-E; no cytosol or energy but transport buffer only). I did the treatment of HeLa cells with siRNA against CRM1 and Aarati Ranganathan did the import and export assays

Figure 3-6

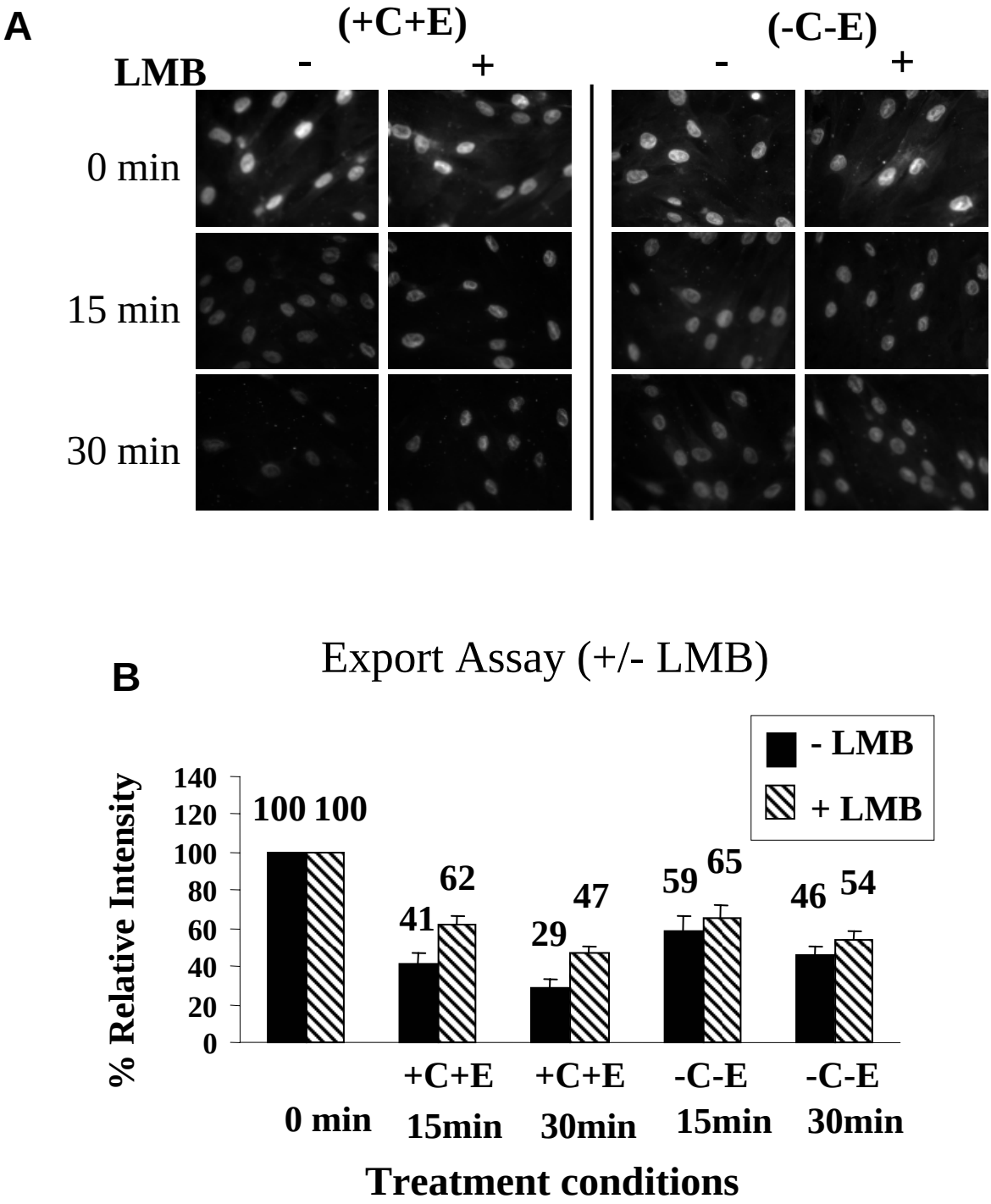


Figure 3-6 Export of GFP-ERK2 in control (–) and LMB-treated (+) HeLa cells. *A*, We treated HeLa cells and cytosol that was used for export with 20 nM LMB for 5.5 h. GFP-ERK2 was imported for 15 min in transport buffer and exported in transport buffer either including cytosol and energy (+*C*+*E*) or not (–*C*-*E*) for the indicated times. Fluorescence intensity of GFP-ERK2 in HeLa cell nuclei was quantified in *B*. I did the treatment of HeLa cells with LMB and Aarati Ranganathan did the import and export assays.

Figure 3-7

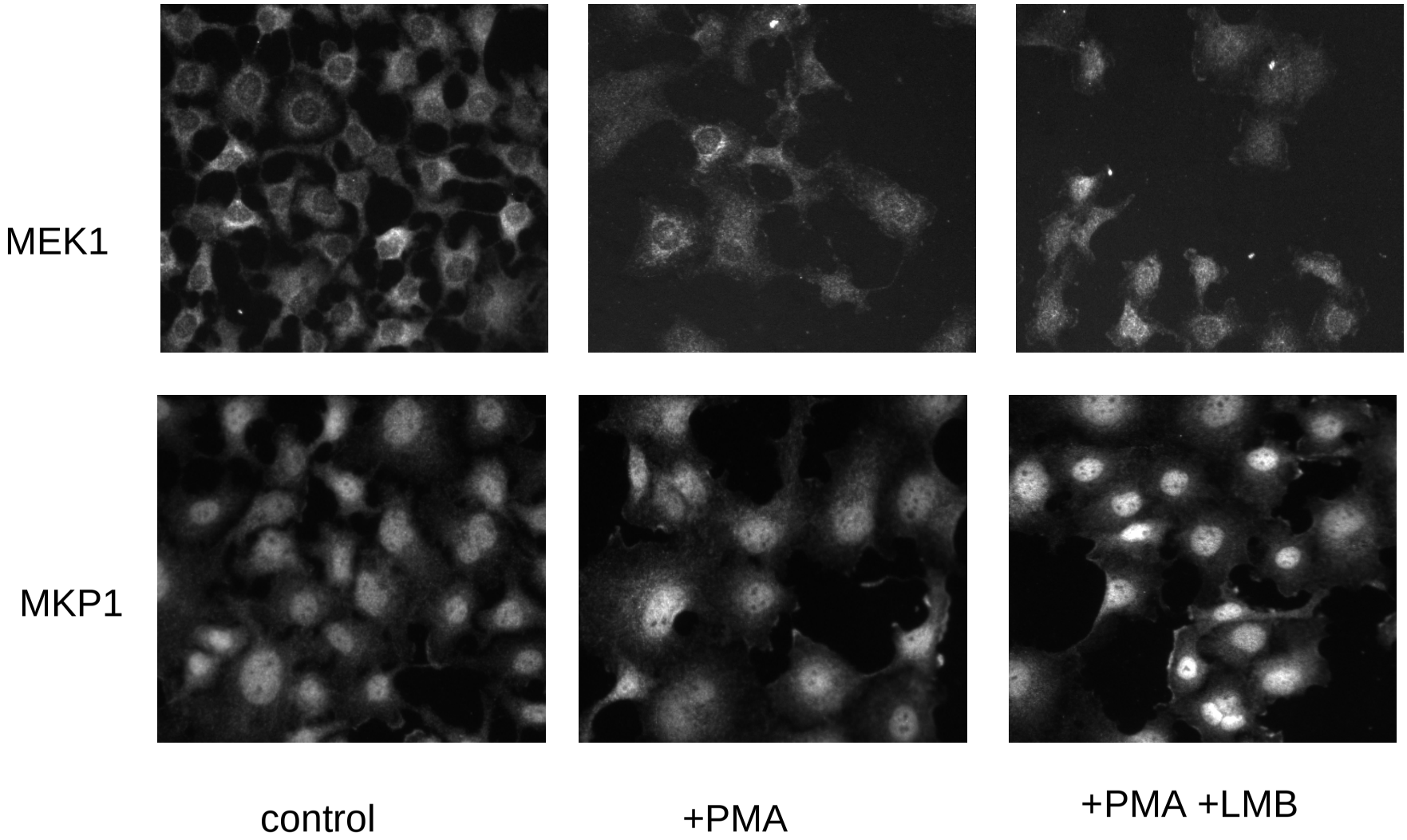


Figure 3-7: LMB treatment increases levels of MEK1 and MKP-1 in nucleus. Both MEK1 and MKP-1 contain strong NESs. Inhibition of CRM1 with LMB increases nuclear localization of p-ERK, total ERK as well as MEK1 and MKP-1.

Chapter 4: Nuclear Localization of JNK and p38 MAPKs

Abstract:

JNK and p38 MAPKs are among the well studied MAPKs. However, the processes that are regulating their nuclear localization are not known at a molecular level. As explained in the second and third chapters of this dissertation, ERK1/2 can bind to the nuclear pore complex proteins directly and can enter the nucleus without any cytosolic factor or energy added during *in vitro* import assays. Also, ERK1/2 in the HFF cells was differentially localized depending on the stimuli (Whitehurst *et al*, 2004). While activated ERK1/2 can remain in the cytosol upon EGF induction, a greater portion of ERK1/2 activated upon PMA treatment localizes into the nucleus. In this study, I asked whether such a distinct localization pattern could be observed for other MAPKs as well. Furthermore, we searched for the factors that might be regulating the import of JNK and p38 into the nucleus. The results showed that knockdown of certain karyopherins has no observed effect on JNK and p38 localization. However, but change in the microtubule dynamics might be affecting the nuclear import of JNK. Finally, the role of the p38 splice variant Mxi2 on the nuclear import of GFP-ERK2 was examined.

Introduction:

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases, which are regulated by three-tiered phosphorylation cascades. MAPKs are activated by an upstream kinase (MAP2K), and the kinase that phosphorylates the MAP2K is a MAP3K. MAPKs are activated by dual phosphorylation on their threonine and tyrosine residues on their activation loop.

The c-Jun N-terminal kinases (JNKs) and p38 are also known as stress-activated protein kinases reflecting their roles in stress responses. JNK and p38, like ERK1/2, have substrates at different locations throughout the cell. Both JNK and p38 can be activated by stresses such as hyperosmolarity, UV irradiation, hypoxia, and cold shock (Zanke *et al*, 1996; Shrode et al,1997; Iordanov *et al*, 1998; Hashimoto, 1999; Foltz, 1998; Seko *et al*, 1997). Three genes encode JNK (JNK1, JNK2, and JNK3), but the total number of JNK isoforms is at least 10 due to alternative splicing (Gupta et al, 1996)

Experiments with *in vitro* reconstitution assays demonstrated that ERK1/2 may enter the nucleus without requirement for any transport factors. ERK1/2 can enter by binding to nucleoporins directly through FXF motifs on the nucleoporins. Mutations in ERK2 residues that are critical for FXF binding strongly decrease nuclear import. Addition of karyoperins but not JNK or p38 in the transport buffer decreases the nuclear import of ERK2, suggesting that there is no competition for nucleoporin binding between ERKs and JNK or p38 (Whitehurst *et al*, 2002).

It is thought that JNK and p38 require cytosolic components to be imported into the nucleus. Karyopherin NMD5 is essential for nuclear localization of the p38 homolog HOG1 in yeast (Ferrigno *et al*, 1998). In mammals the NMD5 homolog is RanBP7,

which mediates nuclear import ribosomal proteins, histone H1, the glucocorticoid receptor, the intracellular reverse transcription complex of human immunodeficiency virus type 1 (Gorlich *et al*, 1997; Jakel, 1998; Jakel 1999; Freedman, 2004; Fassati, 2003). Possible functions of RanBP7 in MAPK import in mammals has not previously evaluated.

Mxi2 is a splice isoform of p38α , and it can bind to ERK1/2 directly (Sanz-Moreno et al, 2003). Mxi2 is identical to p38 except for the last 80 residues, which are replaced with different 17 amino acid residues (Zervos ,1995). Mxi2 has low activity against p38 substrates, and is not inhibited by pyridinyl imidazoles (Sanz, 2000).

In this chapter, I examined the activation and localization of JNK and p38 in HeLa and human foreskin fibroblast (HFF) cells. I also searched for possible roles of RanBP7 and microtubule stability in the import of activated JNK and p38. Finally the effect of p38 homolog Mxi2 on the import of ERK2 was tested by *in vitro* import assay.

Materials and Methods:

Cell Culture – HeLa, HFF and Ref52 cells were grown on coverslips for 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 100 units/ml penicillin/streptomycin at 37^{o}C in 10% CO_2. Cells were incubated in serum-free medium for 2 h prior to treatment with different stimuli to test for JNK and p38 activation.

Immunoblotting– Cells were lysed in Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.5 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl

fluoride). Samples were resolved by SDS-PAGE and analyzed by immunoblotting with appropriate antibodies after transferring to nitrocellulose membranes.

Import and Export Assays. Cells were washed once in transport buffer (TB - 20 mM Hepes-KOH pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM ethylene glycol-bis (β-aminoethylether)-N,N,N',N'tetraacetic acid (EGTA) and 2 mM dithiothreitol (DTT)) and permeabilized for 5 min with 70 μg/ml digitonin. Import assays were performed as described in chapters 2 and 3 of this dissertation. In experiments using cytosol and energy to import Mxi2, dialyzed HeLa cytosol (2.5 mg/ml) was included as a source of transport factors along with an ATP-GTP regenerating system containing 1 mM ATP, 1 mM GTP, 5 mM phosphocreatine and 20 U/ml creatine phosphokinase. After addition of 0.25 ml TB, cells were fixed for 10 min in 3% paraformaldehyde; coverslips were mounted with polymount.

Fluorescence Microscopy–Fluorophores were visualized by fluorescence microscopy using a Zeiss Axioskop 2-plus microscope and a Hamamatsu digital CCD camera (C4742-95). Exposures for all conditions within an experiment were kept the same; fluorescence intensity was quantified using Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.).

Results:

JNK activation in different cell lines was tested first. In HeLa cells, JNK was activated within 10 minutes after EGF, PMA, sorbitol, UV or NaCl treatment and remained active for at least 40 minutes (Fig 4-1 B). Some isoforms of JNK were activated by certain stimuli. The 48 kDa isoform of JNK was activated by all the stimuli. On the

other hand, a 54 kDa isoform was activated only by sorbitol and NaCl but not by PMA in the first 10 minutes.

JNK activation can also be detected in HFF cells after treatment with PMA, EGF, NaCl or sorbitol (Fig 4-1 A). While I observed 3 different bands corresponding to JNK in HeLa cells, only two species were resolved in HFF cells. I could not determine which gene products these bands correspond to , because the antibodies that were used cannot distinguish among JNK gene product.

p38 is activated by sorbitol and NaCl. PMA can slightly activate p38 but no activation of p38 was observed after EGF treatment (Fig 4-2A). However, UV can activate JNK, p38 and ERK1/2. Activation of JNK, p38 and ERK1/2 was observed within 15 minutes of UV stimulation, but JNK was activated even more at longer times (Fig 4-2B). ERK1/2 activation ended within 60 minutes of UV stimulation but JNK and p38 remained active for at least 2 hours (Fig 4-2B and Fig 4-4). UV induced JNK and p38 activation leads to accumulation of these active proteins into the nucleus within 5 minutes (fig 4-4).

ERK1/2 show distinct localization patterns in HeLa and HFF cells. In HeLa cells activation of ERK1/2 almost always leads to their nuclear localization. On the contrary, activated ERK1/2 may remain largely in the cytosol or accumulate in the nucleus depending on the stimuli in HFF cells (Whitehurst et al, 2004).

When I compared the localization of active JNK in HeLa cells and HFF cells, most of the activated JNK localized to the nucleus of HeLa cells (fig 4-3A). Active JNK could also localize to the nucleus in HFF cells, but some remained in the cytoplasm depending on the stimulus (fig 4-3B). Differential JNK localization depending on the

stimuli can be observed clearly in HFF cells treated with EGF. Active JNK was observed primarily in the cytoplasm in these EGF treated cells.

Geldanamycin is a natural product produced by *Streptomyces hygroscopicus*. Geldanamycin can bind in the ATP binding pocket of Hsp90, which is a ubiquitous molecular chaperone critical for the folding and activity of multiple proteins. One client of Hsp90 is mixed-lineage kinase 3 (MLK3), a MAP3K that can activate the JNK and p38 pathways (Merritt et al, 1999; Rana et al,1996; Zhang et al, 2004). I treated HeLa cells with geldanamycin to inhibit Hsp90 function and used sorbitol to activate JNK. Active JNK remained in the cytoplasm of some cells treated with geldanamycin (Fig 4-6A). On the other hand, active JNK was observed in the nucleus of the cells that are not treated with geldanamycin and no nuclear exclusion was observed.

Treatment of cells with taxol, which affects microtubule dynamics (Schiff, 1980; Yvon, 1999), also leads to exclusion of the activated JNK from the nucleus of some cells as well (Figure 4-6A). Neither taxol nor geldanamycin alone activated JNK in this experimental system (Figure 4-6B). I did not observe any difference in the localization of active p38 and ERK1/2 after taxol or geldanamycin treatment (data not shown).

RanBP7 knockdown does not inhibit nuclear localization of JNK and p38

The karyopherins are transport factors that mediate the nucleo-cytoplasmic trafficking of proteins and RNAs. Karyopherin NMD5 in yeast was shown to be important for HOG1 MAPK nuclear localization (Ferrigno *et al* ,1998). HOG1 is the homolog of p38 in yeast. I used RNAi to knockdown RanBP7, which is the NMD5 homolog in mammals. Oligonucleotides against RanBP7 successfully reduced protein levels in HeLa cells (Fig 2-9A). Based on Different exposures of the immunoblots,

RanBP7 was reduced by 75-80%. However unlike the results observed in yeast, no change in JNK or p38 nuclear localization was detected in mammalian cells when RanBP7 was knockdown (Figure 4-5). Similarly, I did not observe any difference after Karyopherin beta1 knockdown (data not shown). It is still possible that karyopherins play roles in nuclear import of MAPKs; however, I was not able to show their roles in this experimental system.

Mxi2 increases import of GFP-ERK2

The p38 homolog Mxi2 can bind to ERK1/2 **(**Sanz-Moreno *et al*, 2003**).** Previous work by Crespo's work suggested that Mxi2 increased the nuclear localization of ERK1/2. Therefore, I used *in vitro* import assays to test this idea. Mxi2 was used in increasing concentrations in the transport buffer without any cytosolic factors and GFP-ERK2 import was examined (Fig 4-7 A and B). Increasing the Mxi2 concentration in this system in the absence of cytosol and energy did not increase GFP-ERK2 import; instead, but it had the opposite effect. The import of GFP-ERK2 into the nucleus decreased in assays containing Mxi2.

Because Mxi2 nuclear import might require cytosol and energy, omitting these factors from the import assay might account for the observed results. To test this possibility I used cytosol and energy to import Mxi2 into the nucleus (figure 4-7 C and D). GFP-ERK2 levels in the nucleus were higher in the assays containing Mxi2 than the ones without Mxi2 (figure 4-7 C and D). Unfortunately, I could not detect the increase in the nuclear Mxi2 levels in this import assay, because the antibody against Mxi2 was not useful for immunofluorescence (data not shown). Mxi2 might enhance ERK1/2 signaling by increasing the nuclear levels of ERK1/2.

Discussion:

In this study, the nuclear localizations of p38 and JNK were examined in response to several stimuli. Possible mechanisms that are regulating these processes were considered.

There are different JNK isoforms (Gupta *et al*, 1996) and different stimuli may be able to activate each isoform separately. In the cell types that I used, the hypertonic environment was the best activator. Sorbitol and NaCl not only activated all JNK isoforms more than the other stimuli, but they activated JNK isoforms more rapidly as well. There may be different pools of different isoforms and the activation of each pool might be dependent on the upstream signal specificity.

Activated MAPKs can localize to distinct parts of the HFF cells. HFFs are diploid cells which are immortalized with h-TERT but not transformed. Activated MAPKs strongly localize to the nucleus of HeLa and other transformed cells. There are several possibilities for this difference; the cytoplasmic anchoring proteins might be altered, scaffolding proteins might be different in primary cells and transformed cell, or the import and export processes might be affected after transformation.

The expression of cytoplasmic MAPK anchoring proteins has been suggested to have an impact on diseases (Condorelli *et al*,1998). PEA15, for example, can anchor ERK1/2 in the cytoplasm. Release of the MAPK from the anchor may facilitate its nuclear entry(Ramos *et al*, 2000; Hill *et al*, 2002). If anchoring proteins are more abundant than MAPKs, the phosphorylation of MAPKs may not be coupled to nuclear

localization, and active MAPKs may remain in the cytosol. However, in some cases a signal that leads to both phosphorylation of MAPK and conformation changes in an anchoring protein may decrease the affinity between those two.

The rate of MAPK export from the nucleus might cause MAPK localization differences between HFF and HeLa cells. A greater nuclear localization of ERK1/2 and JNK is observed after LMB treatment of EGF-treated HFFs cells. Without LMB, most of the active ERK1/2 and JNK stays in the cytoplasm of EGF-treated HFF cells. The increase in nuclear active JNK and ERK1/2 levels after export inhibition suggests that MAPKs shuttle between the nucleus and cytosol after activation by EGF. As explained in chapter 3, ERK1/2 can be exported from the nucleus by both energy dependent and independent mechanisms. For the energy-dependent part CRM1 plays a role. CRM1 may bind to ERK1/2 directly or use another protein as a bridge. In the latter scenario, ERK1/2 would bind to a protein with NES in the nucleus and it can be exported in a piggyback manner. Such a nuclear partner for JNK and p38 might be more abundant in HFF cells than in HeLa cells; thus, the nuclear export rate in HeLa cells would be less. This currently unknown export factor might be a transcription factor, phosphatase, another kinase for example.

The roles of karyopherins in MAPK nucleocytoplasmic localization is a topic that is not well studied in mammalian cells. ERK1/2 can bind to the NPC directly and can enter the nucleus without requirement for any cytosolic factors *in vitro*. ERK1/2 binds to proteins possessing FXF motifs and several nucleoporins contain these FXF repeats. In yeast, karyopherin NMD5 is essential for HOG1 nuclear import. However, when I knocked down RanBP7, the mammalian homolog of NMD5, no defect in nuclear import

of JNK or p38 was observed. Several explanations might be relevant. First is the relative number of karyopherins in yeast and mammals. The number of mammalian karyopherins is greater than that of yeast. Some karyopherins may be able to compensate for a missing one. RanBP8 is a good candidate to compensate for RanBP7. It shows strong homology to NMD5. Double knockdowns for RanBP7 and RanBP8 should be tested for JNK and p38 nuclear localization. However, the lack of antibodies against RanBP8 makes this experiment challenging. It is also possible that karyopherins might play a role in MAPK signaling after certain types of stimuli. One could broaden the types of stimuli tested after the RanBP7 and RanBP8 knockdown to get a more complete understanding of roles of karyopherins in MAPK signaling.

In this part of my studies, I searched for the regulation of JNK and p38 nuclear import. Different stimuli may lead to distinct localization of JNK in HFF cells but not in HeLa cells. Moreover, the knockdown of certain karyopherins had no observed effect on JNK or p38 localization. Finally, the p38 homolog Mxi2 was shown to enhance the nuclear import of ERK2. The nuclear entry of Mxi2 itself requires cytosolic factors.

Figure 4-1

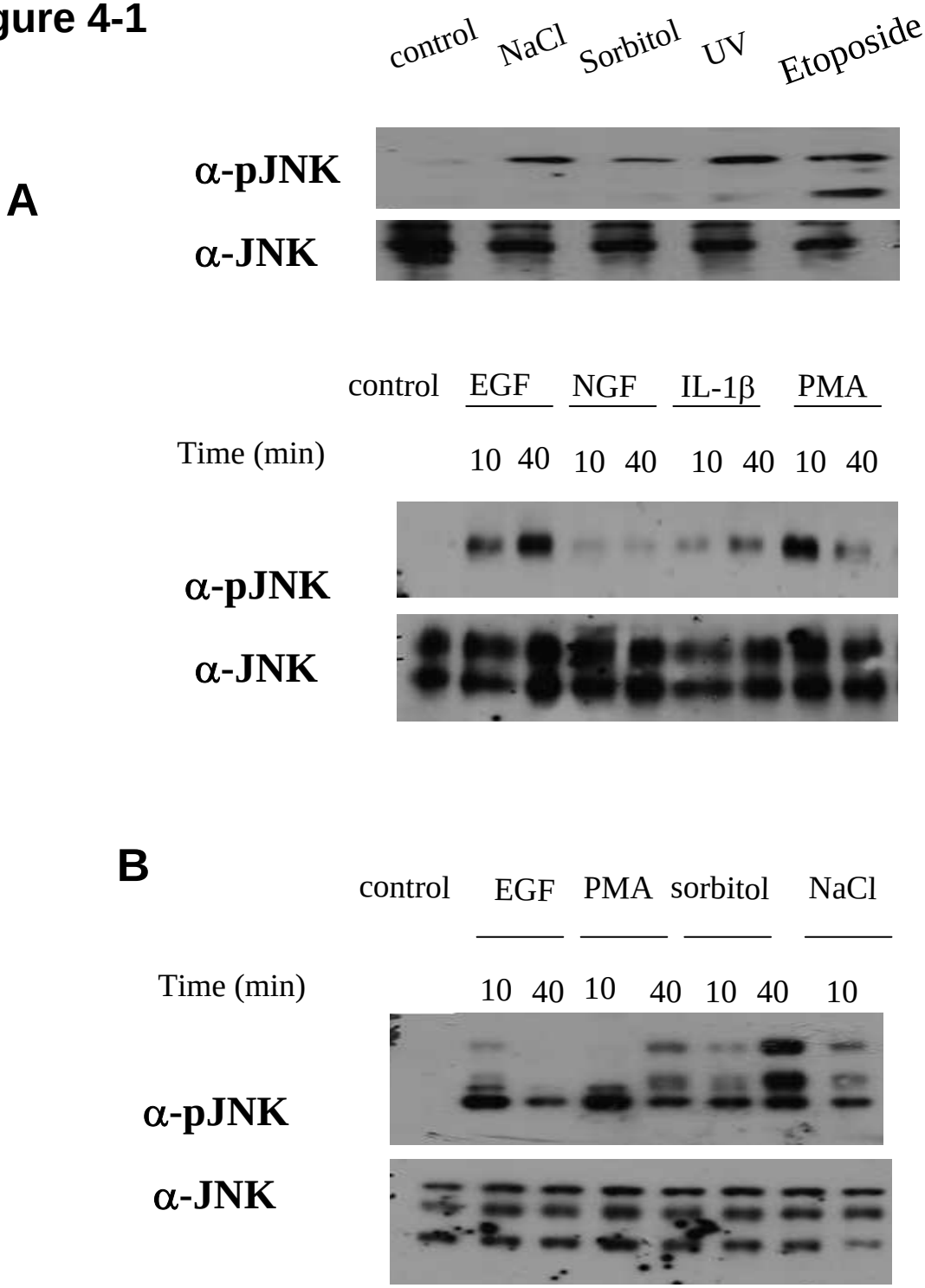


Figure 4-1: Activation of JNK by different stimuli: JNK activation was tested by immunoblotting after different stimuli. **A** JNK is activated in HFF cells by NaCl, sorbitol and DNA damaging agents. EGF and PMA can also activate JNK in this cell type within 10 minutes. JNK activation by NGF was not detected in this cell type over 40 minutes. **B** The same activators that activate JNK in HFF cells also activate JNK in HeLa cells. In this cell type, three different bands corresponding to JNK proteins were detected with the antibodies. The same antibodies detected only two bands in the HFF cells.

Figure 4-2

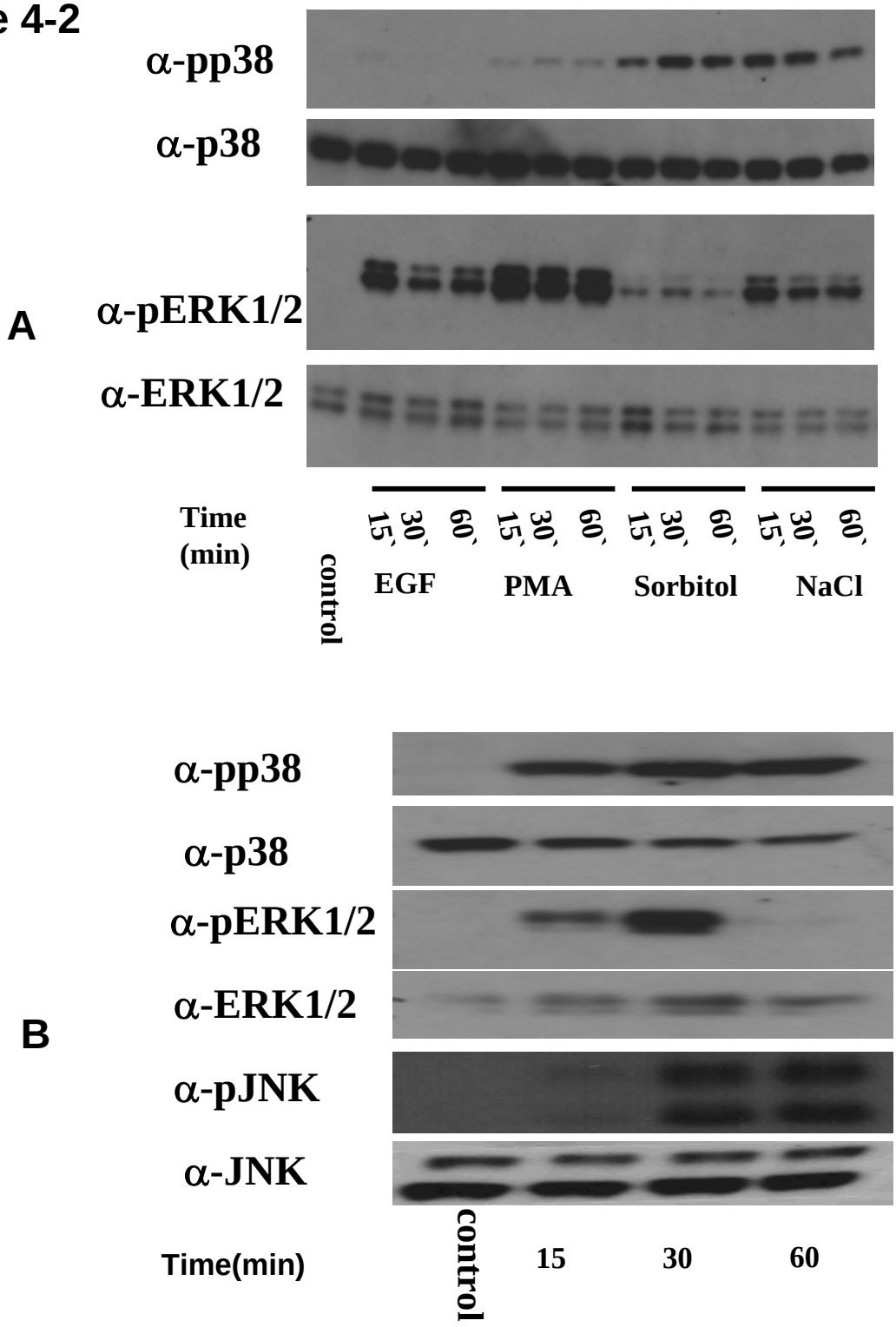


Figure 4-2: Activation of p38 by different stimuli: A p38 is activated within 15 minutes by NaCl and Sorbitol. EGF or PMA did not activate p38 in this cell type over the time tested. ERK1/2 are activated by EGF and PMA showing they are functional. **B** UV activates ERK1/2, p38 and JNK. P38 and ERK1/2 are activated within 15 minutes. ERK1/2 activation ceases within 60 minutes, while p38 remains active during that time. JNK is slightly activated within 30 minutes after UV treatment and remains active more than 60 minutes.

Figure 4-3

A

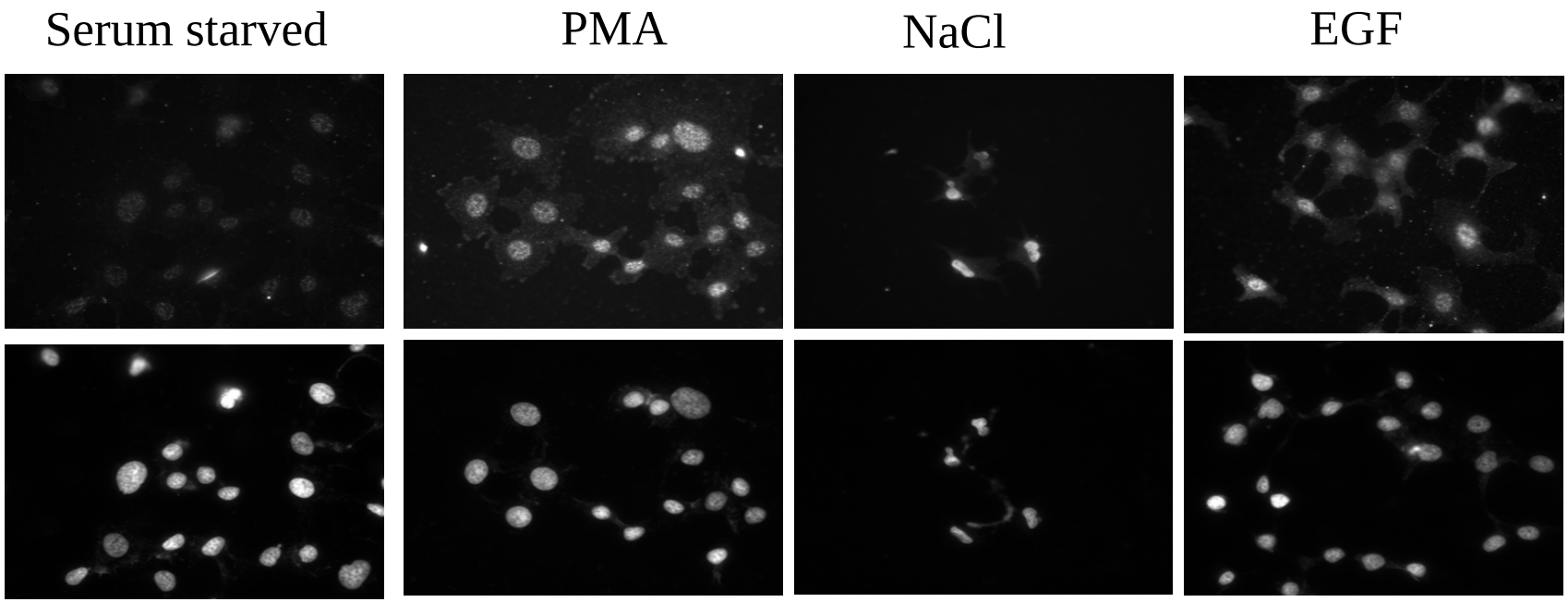


Figure 4-3

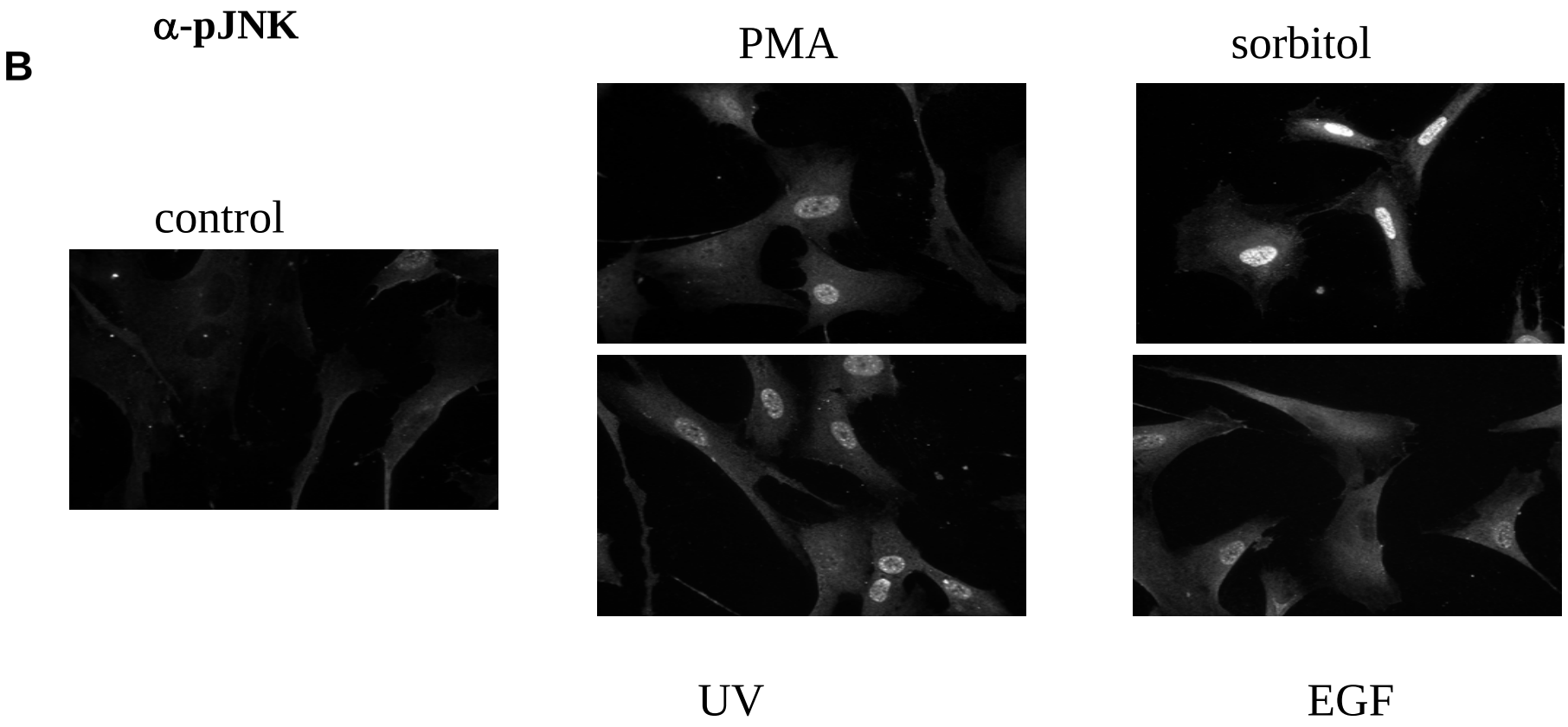


Figure 4-3: JNK localization in HeLa and BJ cells: A. Activated JNK localizes to the nucleus in HeLa cells. **B**. In HFF cells activated JNK localizes to the nucleus or remains in the cytosol depending on the activator.

Figure 4-4

A

α-pJNK

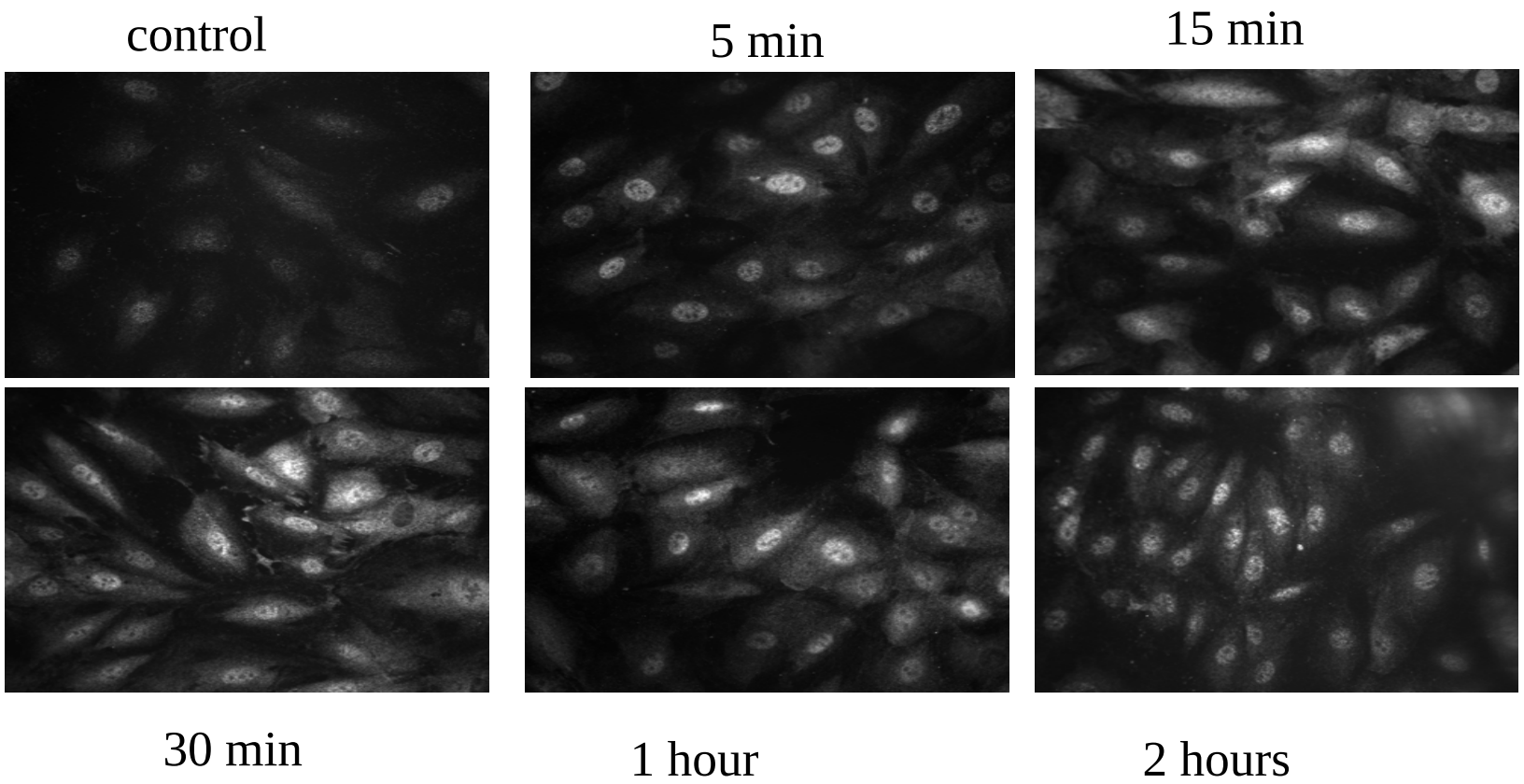


Figure 4-4

B

α-pp38

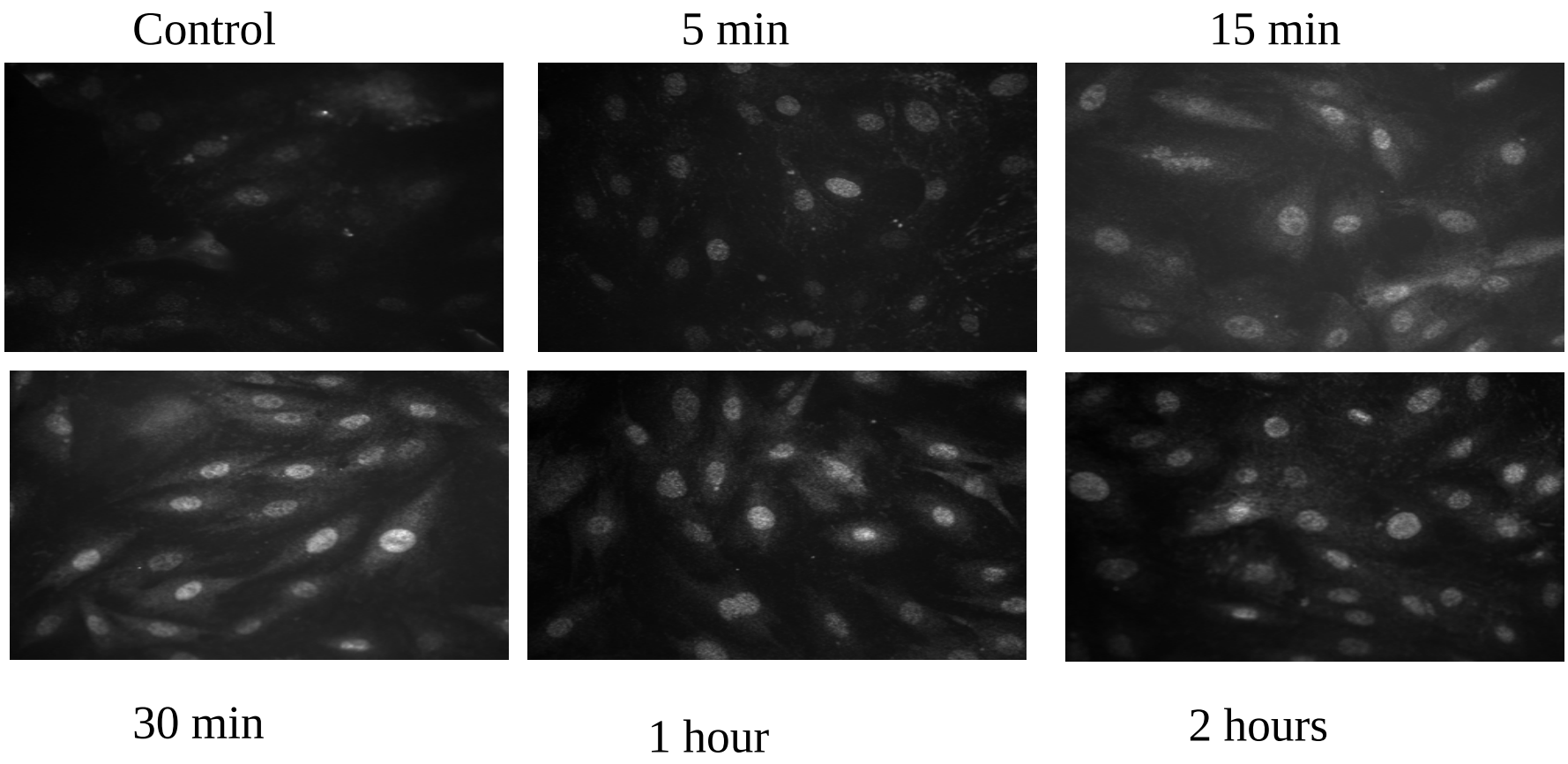


Figure 4-4: The amount of active JNK and p38 in the nucleus increases after UV treatment: A. JNK and **B.** p38 localizes to the nucleus after UV treatment. More active p38 is observed in the nucleus after 30 minutes. Both active JNK and p38 is observed in the nucleus 2 hours after UV treatment.

Figure 4-5

A

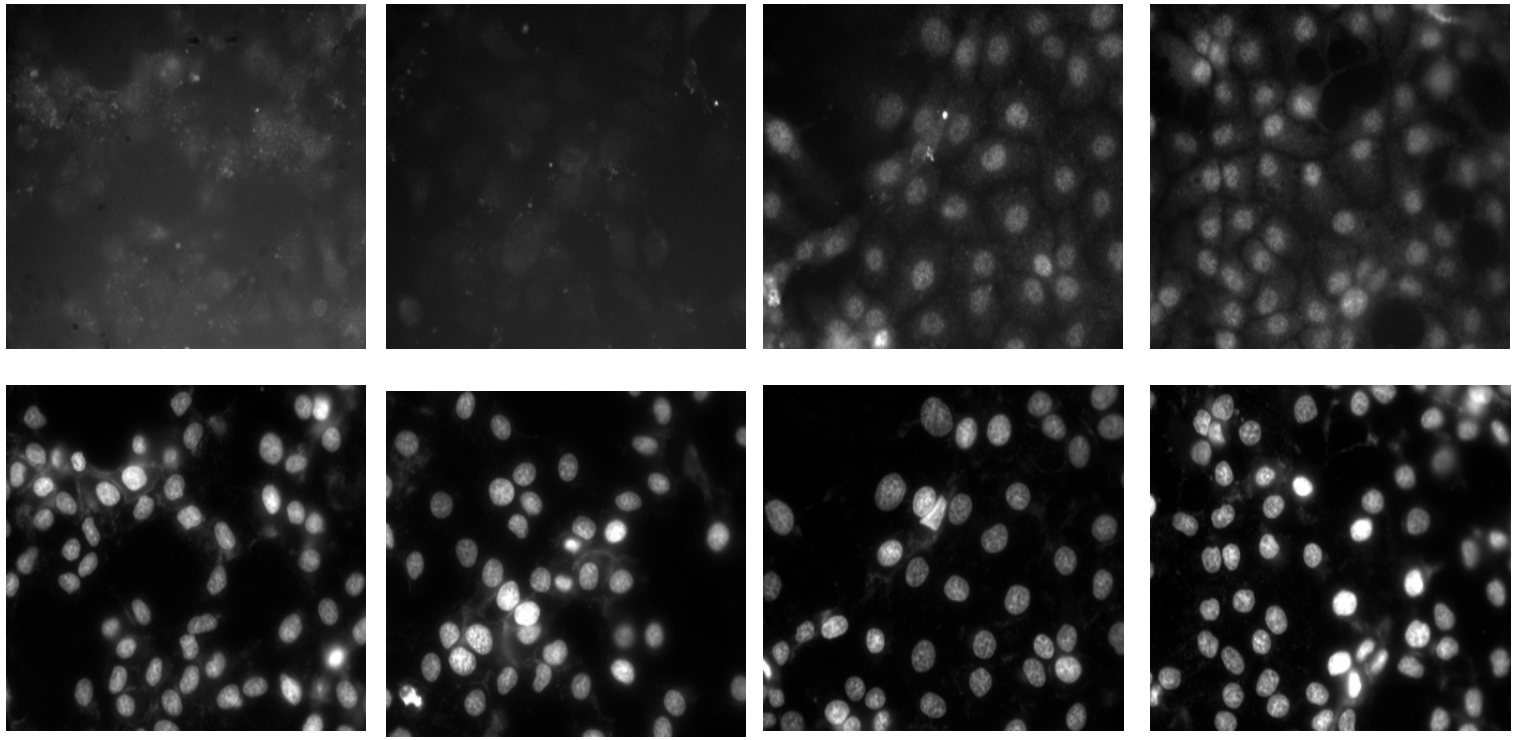


Figure 4-5

B

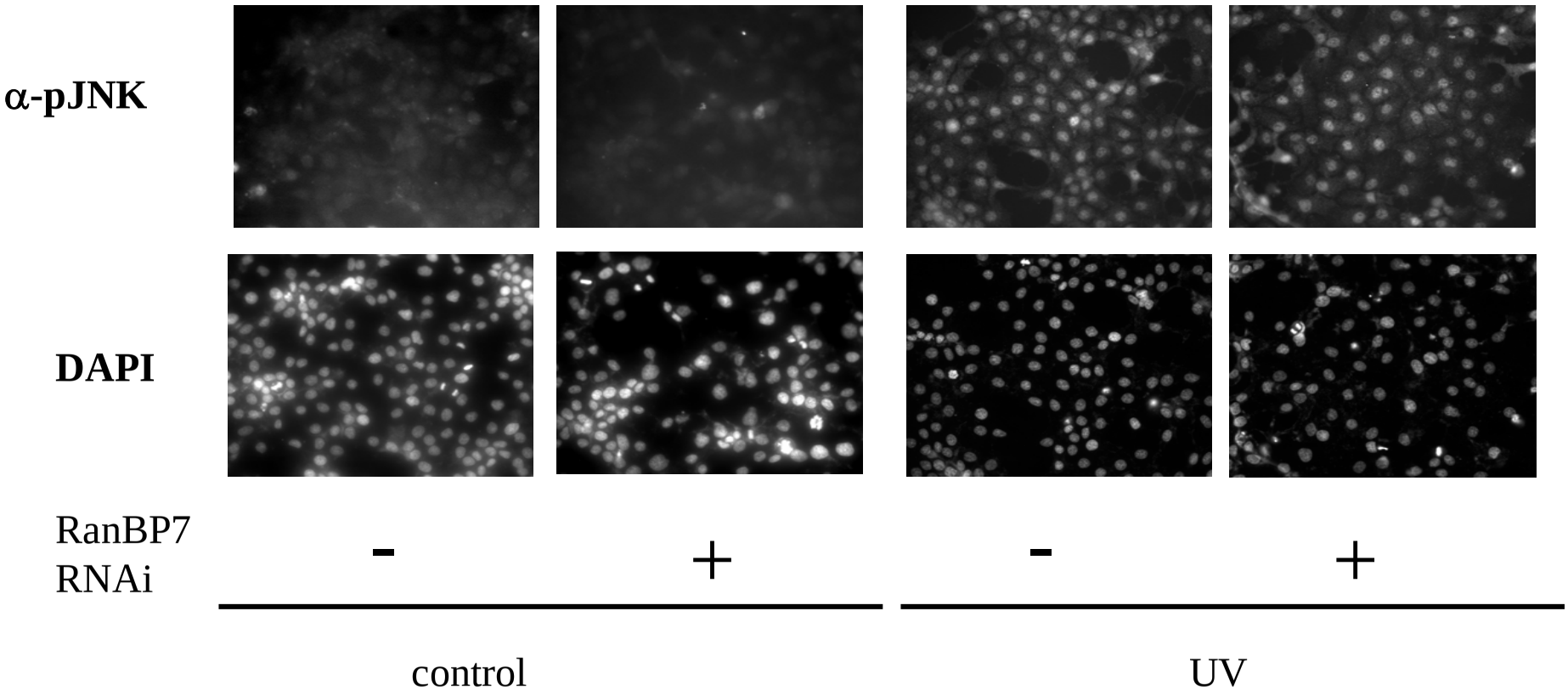


Figure 4-5: RanBP7 RNAi: **A.** active p38 and **B.** active JNK localizes to the nucleus after UV treatment in HeLa cells which are treated with siRNA oligonucleotides against RanBP7.

Figure 4-6

A

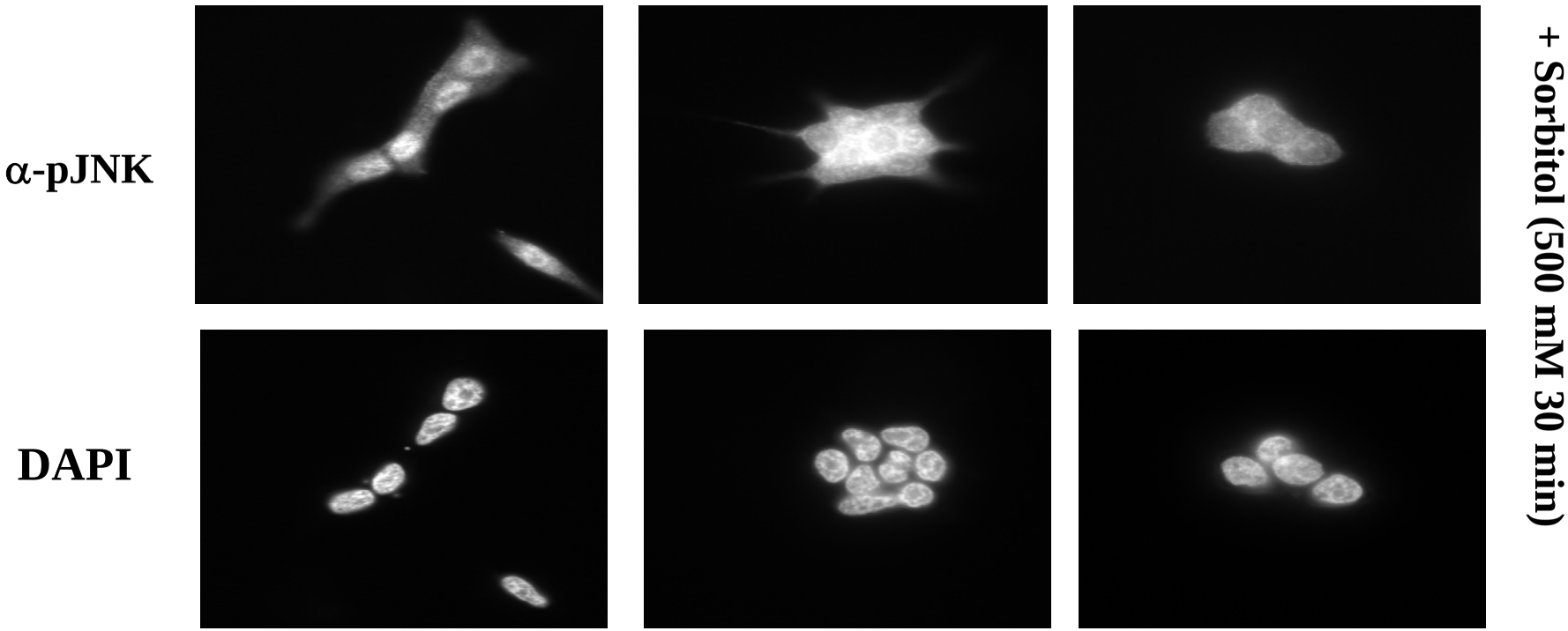

α-pJNK
DAPI
+ Sorbitol (500 mM 30 min)
control
+Geldanamycin
+Taxol

Figure 4-6

B

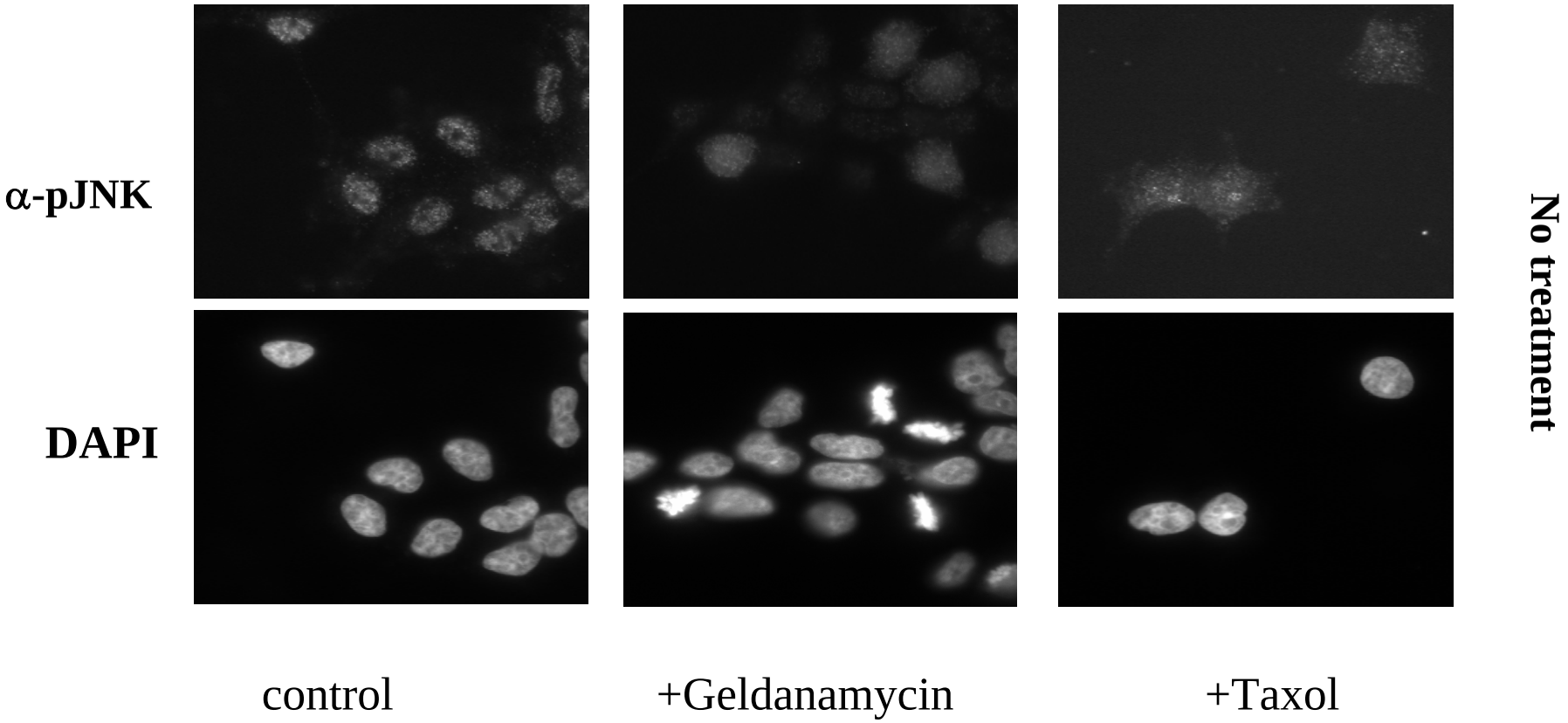
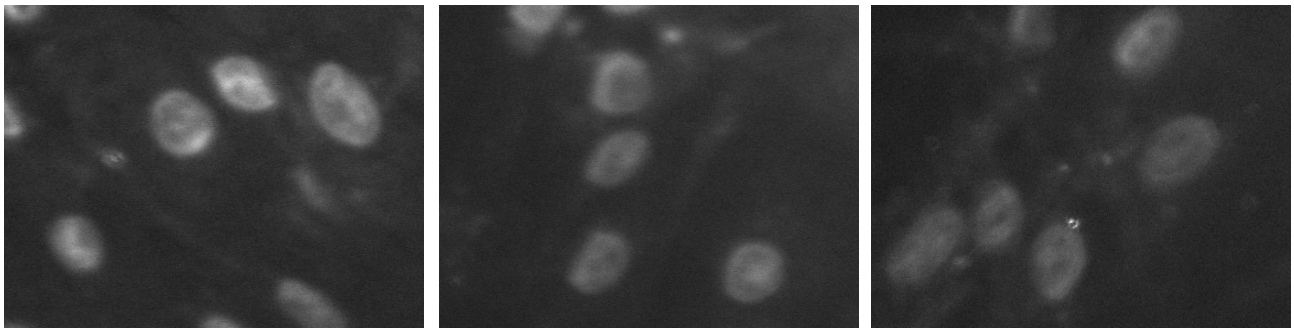


Figure 4-6: Geldanamycin and taxol treatment of HeLa cells: A. JNK localization into the nucleus is blocked after suppression of microtubule dynamics by taxol or HSP90 inhibition by geldanamycin. **B** Neither taxol nor geldanamycin activate JNK in this cell type, but they alter the localization of the activated JNK.

Figure 4-7

A



GFP-ERK2

GFP-ERK2
+ Mxi2 (5X)

GFP-ERK2
+ Mxi2 (50X)

B

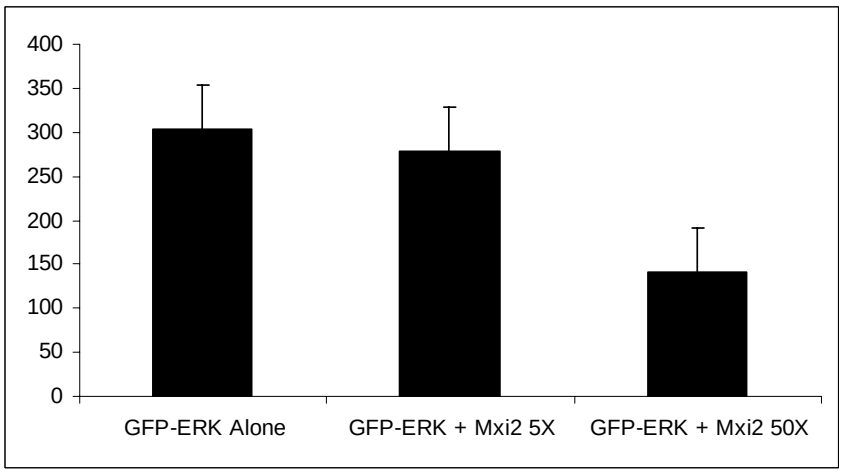
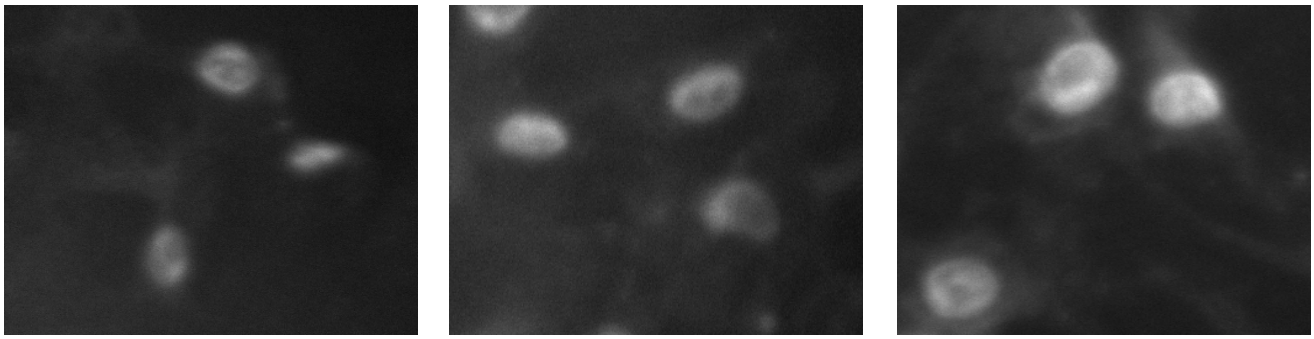


Figure 4-7

C



No cytosol
no Mxi2

No cytosol
+Mxi2

+ cytosol
+ Mxi2

D

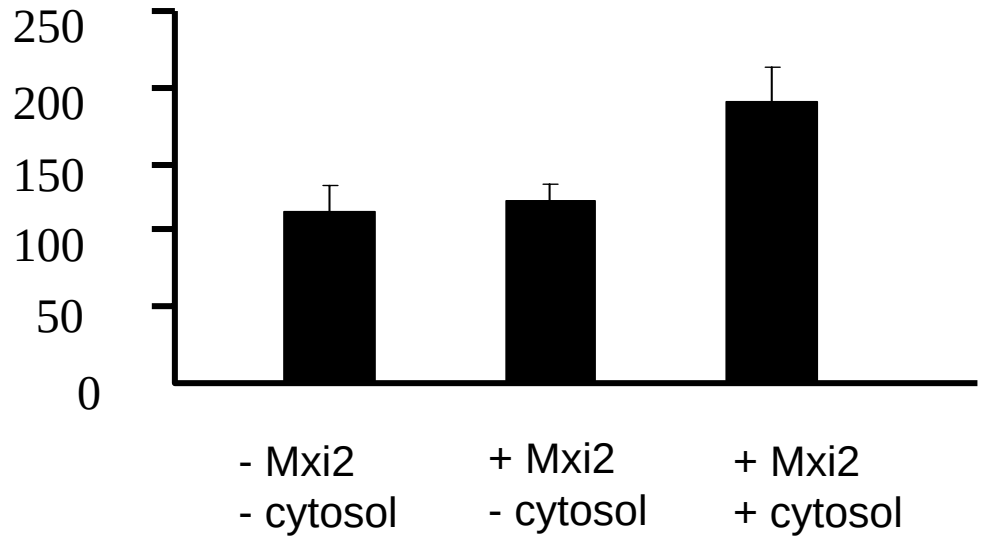


Figure 4-7 The effect of Mxi2 on ERK1/2 localization: A-B Mxi2 decreases import of GFP-ERK2 into the nucleus when the import assay is done without cytosol and energy. **C-D** Nuclear GFP-ERK2 is increased when cytosol and energy are used for the import of Mxi2.

CHAPTER 5: Discussion and Future Prospects

In this study, I analyzed the mechanism of MAPK import into the nucleus. It was previously shown that ERK2 enters the nucleus without any requirement for cytosolic factors in the *in vitro* import assay (Whitehurst *et al*, 2002; Matsubayashi *et al*, 2001). My studies using the same technique do not contradict with the previous studies but add more details into the big picture. As described in the second chapter of the dissertation, certain ERK2 residues that are important for FXF motif binding are highly important for ERK2 nuclear entry. As there are FXF motifs on the nucleoporins, altering ERK2-FXF motif interaction can decrease nuclear import. Mutations can alter import of both active and inactive ERK2 depending on the conditions.

When the ERK2 mutants that have decreased FXF binding were expressed in cells, they did not behave differently from the wild type in terms of nuclear localization. Only the MAPK insert mutant showed a clear difference and most of the expressed proteins remained in the cytoplasm. There are possible reasons for the difference between *in vitro* system and cellular expression:

1) Nuclear localization of ERK2 apperantly occurs by multiple mechanisms one of which is direct binding to the nuclear pore complex and another of which employs interactions with other proteins. The proteins binding to ERK2 and carrying it into the nucleus are not tested during *in vitro* import assay because cytosol was omitted. However, in the overexpression studies these interactions may be sufficient to overcome the defect in nucleoporins binding. The i*n vitro* system focuses on ERK2-FXF interaction.

2) One can control the time for nuclear import during *in vitro* studies but not for the expressed proteins. One cell might start protein synthesis earlier than another cell. Although they may express the same amount of protein, the protein expressed earlier maybe more highly concentrated in the nucleus.

3) Overexpression in cells might alter the export process as well. While the mutants and wild type ERK2 proteins can be exported in the *in vitro* system, in the intact cell wild type ERK2 might be exported more readily. Such an effect on export could not be distinguished from an effect on import.

Aarati and I showed that ERK2 can be exported from the nucleus by two distinct mechanisms, energy-dependent and energy-independent. The karyopherin CRM1 plays a role in the energy-dependent mechanism, while energy independent mechanism most likely occurs by direct interaction of ERK2 with the nucleoporins. Interestingly, energy dependence and independence also distinguishes two processes in active ERK import. However, the role of karyopherins in this process remains to be investigated. Motor proteins may also be strong candidates for playing part in energy dependant process of ERK2 nuclear localization. It is possible that motor proteins may bind to MAPKs directly or by using scaffold proteins like JIPs or cytoplasmic anchoring proteins like PEA-15 (Kelkar *et al*, 2005). Moreover, the interaction between MAPKs and motor proteins might be essential to regulate the functions of the motor proteins (Gdalyahu *et al*, 2004; Kashina *et al*, 2005). The proper interaction between motor proteins and MAPKs is currently analyzed in neuronal cells, where the motor proteins key components of axonal transport.

The mechanism of action of karyopherins in MAPK nuclear export is not clear. The role of CRM1 in ERK2 export was shown before, but it was suggested to occur in a piggyback process in which MEK1 binds to inactive ERK2 and exports it out of the nucleus(Adachi *et al*, 2000; Fukuda *et al*, 1997). However, Aarati's and my studies showed that ERK2 mutants that are deficient in MEK1 binding can still be exported from the nucleus. Thus, how CRM1 mediates ERK2 export is not clear. The role of CRM1 in the export of other MAPKs has not been studied extensively. In one experiment, I observed increased nuclear localization of endogenous active JNK in the nucleus of BJ cells after the addition of LMB. However I did not observe similar results in HeLa cells. LMB treatment had no effect on the nuclear levels of JNK in HeLa cells. It is possible that karyopherins might regulate MAPK nucleocytoplasmic traffic depending on the stimuli and cell type.

Although we could not show any direct interaction between CRM1 and ERK2, this does not rule out the possibility that they interact directly. Otherwise they must interact using another protein as a scaffold. Piggyback export of ERK2 by using MEK1 is still possible, but we could not relate it to the function of CRM1. ERK2 proteins that can no longer bind to MEK1 can still be exported *in vitro* (Ranganathan *et al*, 2006). In this export process, ERK2 can bind to other factors including Map Kinase phosphatases, which may also contain NESs. Some of the proteins in MAPK signaling with NESs are listed in table 5-1.

Protein	*NES*
MEK1	1 STRONG
MEK2	1 STRONG
MKP-3	1 MODORATE 2 WEAK
MKP-2	WEAK
MKP-10	NONE
MKP-7	WEAK
MKP-9	1 MODERATE 1 WEAK
MKP-1	VERY WEAK
MKP-4	1 MODERATE 1 WEAK

Table 5.1: NESs in some proteins involved in MAPK signaling (NetNES 1.1 server at http://www.cbs.dtu.dk/services/NetNES was used for predictions)

In Drosophila and yeast, certain karyopherins have essential functions for MAPKs signaling (Ferrigno *et al*, 1998; Lorenzen *et al*, 2001). I used an RNAi strategy to search for the possible roles of these karyopherins in mammalian MAPK signaling. Among the possible candidates, only knockdown of CRM1 had effect on MAPK import. The knockdown of RanBP7, RanBP8, or Karyopherin beta1 did not seem to alter ERK1/2, JNK or p38 nuclear localization. However, RNAi does not get rid of all of the protein and it is possible that the remaining karyopherins might still be sufficient for import of

MAPKs. Also karyopherins may compensate for one another. When one of them is knocked down, another karyopherin may still bind to MAPKs and perform the function. Using multiple dsRNA oligonucleotides targeting different karyopherins in the same cell is one way to test this hypothesis. Interactions between MAPKs and karyopherins might be tested in another screen. Moreover, each karyopherin can be used in the *in vitro* reconstitution assay and its impact on the MAPKs import can be analyzed.

It was shown by a previous graduate student that activation of ERK1/2 does not always lead to nuclear localization (*Whitehurst et al* 2004). EGF and PMA can both activate ERK1/2 in BJ cells, but activated proteins can be observed in the nucleus after PMA treatment, but not after EGF treatment. I used this cell type to understand whether activation of JNK could also be uncoupled from its nuclear localization in some cases. In HeLa cells, activated JNK always localized into the nucleus following all stimuli tested. However, activated JNK remained in the cytoplasm in some of the EGF-treated cells, confirming the signal specific activation and localization of JNK also occurs in this cell type. This question should be reevaluated in different untransformed cells with more stimuli to determine the generality of this behavior.

The mechanisms that localize MAPK to other locations in the cell rather than to the nucleus should be studied. Very little is known about the localization of MAPKs to mitochondria. Several signaling pathways in mitochondria can be regulated by MAPK signaling. The p38 activation is required for Bax translocation to mitochondria in human keratinocytes (Van Laethem *et al*,2004). As activated ERK1/2 can interact with several mitochondrial proteins, ERK1/2 signaling might have greater role in mitochondria

functioning than currently known (Zhu *et al*, 2003). The link between cell proliferation and cell death will be better understood as mechanisms of MAPK function in mitochondrial signaling events are defined.

The role of cytoskeletal proteins in the MAPK localization is also a poorly understood area. An intact cytoskeleton is essential for MAPK signaling events as a certain pool of MAPKs is associated with the microtubules in the cell. The microtubule disrupting agents might alter the interaction of some pools of MAPKs with their anchoring proteins on microtubules.

It is found that small ubiquitin-related modifiers (SUMO) modification may regulate degradation, activity and intracellular localization of the targeted proteins. Some of the proteins that are phosphorylated by MAPKs might be also modified with SUMO as well (Yang *et al*, 2003, Yang *et al* 2006, Utsubo-Kuniyoshi *et al*, 2007). Whether SUMO conjugation to a protein involved in MAPK signaling might regulate MAPK localization to certain organelles is another further research.

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Vitae

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