Protein Engineering of p38 MAP Kinase for Substrate Identification

Natalia Yasovich

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

Graduate Program in Biology
York University
Toronto, Ontario, Canada

December, 2012

Abstract:

Protein kinases play a central role in signal transduction pathways. Many studies have reported that MAPK pathways are implicated in growth, differentiation, apoptosis and immunity. p38 MAPK is one of the major groups within the MAPK superfamily. Recent studies indicate that the p38 MAPK signalling pathway is one of the major intracellular signalling pathways regulating myogenesis.

Identification of kinase substrates is a major goal of kinase biology. In order to identify p38 MAPK substrates, we have generated mutations in human p38α to create a structural distinction between the ATP binding site of p38 MAPK and that of other kinases in the cell in order to identify its substrates using modified nucleotide analogs. Identification of the relevant substrates will help to clarify the molecular mechanism underlying the role of p38 MAPK. Here, it is shown that engineered kinases, are able to use synthesized ATP analogs, which are typically not recognized by natural kinases. In particular, this work demonstrates that Leu87Ala/Thr106Gly p38 MAPK could be useful in a proteomic approach for identifying novel p38 MAPK substrates.

Acknowledgments:

I want to thank my supervisors, Dr. John C. McDermott and Dr. Michael K.W. Siu for the last two years of my academic life. I want to thank Dr. McDermott for his support, mentorship, encouragement and advice in every step of the way. I am also grateful to my supervisory committee, Dr. Mike Scheid for his time and helpful suggestions.

I would also like to thank Dr. Saridakis for her contribution and help with the computational analysis of the 3D structure of p38 MAPK.

I would like to thank to all McDermott lab members, but I must give special thanks to Nezeka Ali for mentoring me and teaching me all the skills to carry out my research, for being a guide and a friend. Also, a special mention to Christina Pagiatakis, Marianna Theodorou and Stephanie Wales. Your help, support, friendship and presence made the long hours in the lab fun. Many thanks to Catherine for keeping the lab an organized place for student's research.

I would like to thank to my parents who emphasized the value of education from early age, who supported and encouraged me in every step of my way.

Table of contents:

List of Tables:

List of Figures:

List of Abbreviations:

ADP	Adenosine diphosphate
ASK-1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
ATF-2	Activation transcription factor 2
BHLH	Basic helix-loop-helix
Bn	Benzyl
COS7	Cell line derived from monkey kidney tissue
cPe	Cyclopentyl
DMEM	Dulbecco's modified Eagle's medium
ERK	Extracellular signal-regulated kinase
Il-6	Interleukin 6
GM	Growth medium
HA	Human influenza Haemagglutinin
JNK	c-Jun N-terminal kinase
MAP3Ks	Mitogen activated protein kinase kinase kinase
MAP2Ks	Mitogen activated protein kinase kinase
MAPK	Mitogen activated protein kinase
MEF2	Myocyte enhancer binding factor 2
2MeBu	2MetylButyl
MLK-3	Mixed lineage kinase 3
MRF's	Muscle regulatory factors

NDPK	Nucleoside diphosphate kinase
PKC	Protein Kinase C
TAK-1	TGFβ activating kinase 1
TNFα	Tumor necrosis factor α
WT	Wild type

Chapter 1: Introduction

The role of kinases in cellular processes:

The eukaryotic protein kinases are one of the largest most functionally diverse gene families (1). The family of eukaryotic protein kinases consists of a total of 518 human protein kinases, a total of 478 protein kinases have been identified through analysis of human sequence sources (2). Protein kinases mediate most of the signal transduction in eukaryotic cells. This is done by modifications of substrate activity. Protein kinases also control many other biological processes, among those; metabolism, transcription, cell cycle progression, cytoskeletal rearrangement apoptosis and differentiation. Mutations and dysregulations in protein kinases play a major role in human disease (2,3). The eukaryotic protein kinases are homologous proteins. They are related by conserved sequence of kinase domains, also known as catalytic domains. The catalytic domain of the protein kinase serves three major roles: 1- binding and orientation of an adenosine triphosphate (ATP) or guanosine triphosphate (GTP) molecule in a complex with divalent cation (Mg^{+2} or Mn^{+2}), 2- binding and orientation of substrate, 3- transfer of the ɣ-phosphate group from ATP or GTP to the serine, threonine or tyrosine residue of the protein substrate (4). Most of the protein kinases belong to a single superfamily containing a eukaryotic protein kinase catalytic domain (1). The protein kinase family has two main subdivisions: the protein-serine/threonine kinases and the protein-tyrosine kinases (4). These two families can be devided into the following major families: CMGC- includes the cyclin-dependent kinases, mitogen activated protein kinases, glycogen synthase 3 family, Cdk-like kinase famliy; STE- homolouges of yeast

Sterile7, Sterile 11, Sterile 20 kinases; CK1- Casein kinase 1; AGC- includes the cyclic-nucloetide-dependent family (PKA and PKG), protein kinase C (PKC) family; CAMK - Calcium/calmodium-dependent protein kinases; TK- Tyrosine kinase; and TKL-Tyrosine kinase-like. Members of the same kinase family have the same domain structure (2,4).

Most protein kinases act in a network of kinases and other signalling effectors. They are characterized by autophosphorylation and phosphorylation by other kinases (3). Protein phosphorylation pathways regulate basic aspects of cell physiology, and are conserved throughout the eukaryotic evolution. Protein phosphorylation is a major regulatory mechanism. By adding the phosphate groups to substrate proteins, protein kinases direct the activity, localization and overall function of many proteins (4). The protein kinase family is stimulated by an extracellular signal, this stimulation results in intracellular signals that leads to activation of protein kinases, which in turn leads to phosphorylation of a specific substrate. Phosphorylation of the substrate alters the ability to interact with other molecules in the cell, what leads to diverse physiological responses (3). Protein phosphorylation also plays a critical role in intracellular communication during development, homeostasis and in the functioning of the nervous and immune system (2).

The regulation of eukaryotic protein kinases can occur on many levels, among those regulations; control of synthesis, posttranslational modification, binding of regulatory proteins, and the most common mechanism to regulate protein kinases activity is by phosphorylation key residues of the catalytic domain by other upstream protein kinase (3).

The biological impact of p38:

p38 MAPK has many physiological roles and it is involved in the control of many cellular pathways. Importantly, p38 MAPK has been shown to play a role in myogenesis and development. p38 MAPK is known to have a role in regulating gene expression at the transcriptional level (5). This section will focus on the roles of p38 MAPK and emphasize the importance of identification of its targets.

The role of p38 MAPK in myogenesis:

Myogenesis, the process of muscle cell formation is essential for normal development, and is also implicated in pathological conditions. In mammals, myogenesis begins shortly after gastrulation and lasts until the end of post-natal growth (6). Protein kinases play a major role in myogenesis. Mitogen activated protein kinase (MAPK) signalling is necessary for the maintenance of skeletal mass. Inhibition of these signalling cascades will cause muscle atrophy *in vitro* and *in vivo* (7). Skeletal myogenesis is a dynamic multistep process in which undifferentiated mononucleated myoblasts proliferate, withdraw from the cell cycle, and then differentiate and fuse to form the multinucleated mature muscle fibres, which are called myotubes (5). MAPK members regulate the formation of multinucleated myotubes, among those proteins is ERK1/2 pathway, which is necessary for muscle growth (7). Differentiation of skeletal muscle is a crucial step in development. Protein kinase C (PKC) is expressed in fetal muscle cells. This kinase mediates growth and differentiation (8). The process of myogenesis is regulated by signalling pathways in response to extracellular cues. Among these is the

p38 MAPK pathway. The process of skeletal myogenesis is controlled by muscle specific basic helix-loop-helix (bHLH) proteins, which are known as the muscle regulatory factors (MRF's) (9). These factors bind to specific DNA elements called E-boxes on muscle related gene promoters and activate transcription. The MRF family consists of MyoD, Myf5, MRF4 and myogenin. Those proteins ensure proper muscle differentiation. All the MRF members bind to specific DNA elements that are present in the promoters of many muscle-specific genes (10,11).

MyoD is considered to be a major transcription factor in muscle formation. MyoD triggers myoblast differentiation by activating the expression of myogenin. Myogenin is a muscle- specific transcription factor that induces myogenesis. Myogenin is necessary for the formation of myotubes (12).The other family of transcription factors that controls the expression of genes that contribute to initiation of the differentiation program and also maintance of the differentiated state is the Myocyte enhancer binding factor 2 (MEF2) (13).

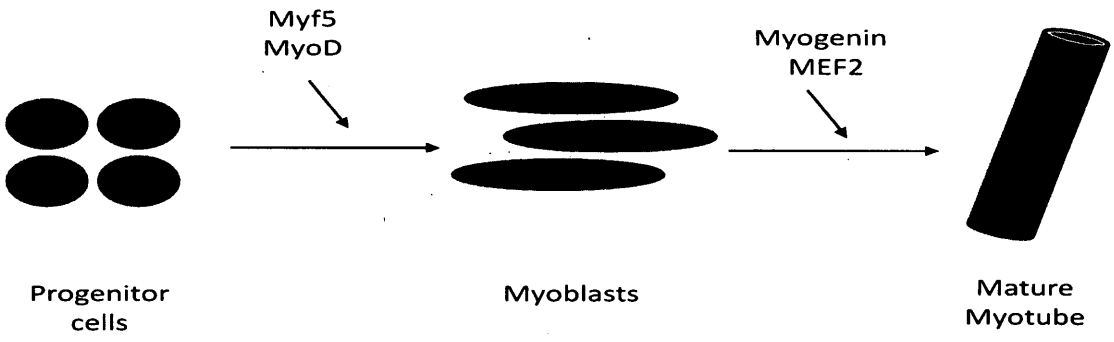

Figure 1: The stages of muscle development- myogenesis. Myogenesis strats from progenitor mesodermal cells, which are the control of Myf5 and MyoD form myoblasts. Myoblasts fuse and form the mature myotube, which the muscle fiber. This is done under the control of Myogenin and MEF2 family transcription factors.

p38 MAPK affects the activity of MRFs,which takes part in remodelling the chromatin at the muscle-regulatory specific regions. p38 MAPK plays a significant role in the signalling pathway of myogenesis and plays a role in several stages of the myogenic program (14). p38 MAP kinase activity increases during differentiation, and is also required for full myoblast differentiation and fusion (5).

Recent studies have shown that p38 MAPK is probably one of the major intracellular regulators of the myogenesis pathway(14). p38 MAPK activates myogenesis by phosphorylating the MEF2 proteins, to increase its transcriptional activity. It also increases the transcriptional activity of MyoD (15). Through MyoD and MEF2,which bind directly to the myogenin promoter, p38 MAPK controls myogenin gene expression (13). Therfore, p38 MAPK is a key player in the regulation of muscle differentiation.

p38 MAPK has four isoforms, but not all p38 isoforms lead to muscle specific gene expression. Interestingly, p38α is the isoform that has a main role in myogenesis (5). p38α not only promotes muscle differentiation and fusion, but is also a critical regulator of myoblast cell cycle exit. p38α also has the same effect on myoblast differentiation: mice lacking p38α failed to form myotubes (5). p38α is essential for embryonic development and deletion of the gene will result in embryonic death due to placental defects(16). Lack of other isoforms did not show any phenotypic changes(5).

All of this illustrates that the p38α isoform has a central role in myogenesis. Thus, it is of great interest to study the regulation of this kinase, and its downstream substrates.

p38 MAPK and inflammation:

There is a strong link between the p38 MAPK pathway and inflammation. There are several diseases that are thought to be regulated by p38 MAPK, among those are: Alzheimer's disease, rheumatoid arthritis and inflammatory bowel disease (17). The activation of the p38 MAPK pathway plays a role in the production of pro-inflammatory cytokines: IL-1β, tumor necrosis factor α (TNF-α), and interleukin 6 (IL- 6)(18). Studies have shown, that TNFα is a key regulator of myogenesis and muscle regeneration through it activation by p38 MAPK (19). IL-6 is a multifunctional cytokine that plays a major role in response to inflammation, and it leads to activation of p38 MAPK. Based on recent studies, p38 MAPK has also been implicated in having a role in T-cell cytokine production. p38 MAPK is a key regulator of pro-inflammatory cytokine biosynthesis at the transcriptional and translational level (21). A regulatory role for p38 MAPK in proliferation and differentiation of immune system cells has also been established (22).

Since p38 MAPK has been implicated in inflammatory responses and tissue remodeling, and since inflammatory cytokines have been shown to play an important role in airway inflamation, p38 MAPK has been implicated in having a role in pulmonary disease (23).This indicates that p38 MAPK activity is critical for normal immune and inflammatory response, and makes the p38 MAPK signalling pathway a promising target for autoimmune and inflammatory disease therapy.

p38 MAPK in cell cycle, senescence, apoptosis and tumoroginesis:

In eukaryotic cells, there are evolutionary conserved pathways that control the various checkpoints in the cell. In response to DNA damage, these checkpoints will delay progression to the next phase. This will give time for DNA repair. There are two main checkpoints in the cell: G1/S which will ensure that DNA synthesis is accurately completed, and G2/M, which will ensure that the division of the cell has been accurately completed (also called mitosis). p38 MAPK is involved in both checkpoints (16).

MAPK is required for spindle assembly checkpoint mechanism. This mechanism is conserved evolutionary and is required for accurate transition of the genetic material to the daughter cells. When p38 is activated it can arrest the cell cycle in the M phase, which will lead to disruption of spindle formation (34).

Apoptosis and arrest of cell growth is an important mechanism that will prevent cells from becoming cancer cells. In response to DNA double strand breaks, p38 MAPK is activated, which leads to G2/M cell cycle checkpoint by phosphorylating p53 (35). p53 can lead to apoptosis in case that there is damage to the DNA, and the cell will not continue to mitosis phase and will not replicate itself (16).The role of p38 MAPK in apoptosis is dependent on the cell type and the stimuli that are expressed by the cell (24). p38 MAPK can function upstream and downstream of caspases, which is a family of proteases that play an essential role in apoptosis and necrosis, in apoptosis (17,25). As well, the strength of the signal in the activation pathway is known to play a key role in the cellular response. There is evidence that strong p38 MAPK activation signal will lead to apoptosis, while lower levels of p38 MAPK activity will be associated with cell survival

(17). Activation of the p38 MAPK pathway is required for apoptosis induction in several different cells. p38 MAPK is known to phosphorylate and activate p53, which is a direct regulators of the apoptosis pathway (26). When p53 is activated in response to DNA damage and abnormal prolifarative signals, this leads to induction of apoptosis. This puts p38 in the picture of apoptosis regulation (27).

p38 MAPK also seems to have a role in tumorigenesis and senescence. A common feature of tumor cells is loss of senescence (27). It has been shown that p38 MAPK activity was responsible for senescence in response to telomere shortening, H_2O_2 exposure and chronic RAS oncogene signal. All of those signals are environmental stress, which will lead to activation of p38 MAPK. Activation of p38 MAPK will lead to activation of transcription factors, such as p53, that can lead to cell death (27).

p38 MAPK is activated in response to stimuli that cause cell cycle arrest, or cell death; this results is activation of its direct substrate MAP kinase-activated protein 2 (MAPKAP-K2). This will eventually lead to G2 phase delay (28). G2 phase in the cell cycle serves as a DNA damage checkpoint before the cell enters mitosis and divides into two identical cells. This checkpoint is important to ensure that DNA replication will be correct. p38 MAPK is involved in restraining uncontrolled cell proliferation. This leads to the idea that p38 MAPK acts as a tumor suppressor (29,30). One of the most common examples of p38 MAPK and tumorogenesis is rhabdomyosarcoma, a common tumor among children, where p38 MAPK inhibiting proliferation (30). The key role of p38 MAPK in tumoroginesis is to promote growth arrest and apoptosis.

p38 MAPK has been shown to promote cell death *in vitro* , but there are some cell lines, such as non-small lung tumors cells, where p38 MAPK has been shown to lead to survival and promote cancer cell growth. p38 MAPK was activated in human lung tumor samples compared to normal cells (17,31,32). The molecular mechanism that will determine whether p38 MAPK will be pro or anti-apoptotic has yet to be determined. It might be related to the cell type or the nature of p38 MAPK activating signal (33).

p38 MAPK is also participating in the G1/S phase of the cell cycle in response to osmotic stress and cellular senescence. It was also shown that p38α and p38γ are required for G2 cell cycle arrest that is induced by UV irradiation (17,36). The p38 MAPK pathway inhibits cell cycle progression, by inhibiting expression of cyclin D, and also by phosphorylating p53, which is a tumor suppressor. Defects in the p38 MAPK pathway can lead to cell cycle defects and increased tumorigenesis. Similarly, decreased p38 MAPK activity, by inhibition of MKK3 or MKK6, causes growth arrest defects in fibroblasts (37).

p38 MAPK in cardiovascular dysfunction:

Stress can activate cardiac hypertrophy, apoptosis and necrosis, which lead to ischemia of the heart. Stress will activate the stress-activated protein kinase pathway, among which is p38 MAPK. Ischemia in the heart will lead to activation of p38 MAPK. p38α is a dominant isoform that is found in the heart. It is yet unknown whether p38 MAPK in heart failure will rescue cardiac damage or will lead to heart failure; it seems that it also depends on the system studied. Studies with human hearts, at the end stage

failing hearts, were inconsistent with the observations made in mice hearts (38). Ischemia induced inhibition of p38 MAPK activity protects against necrosis and apoptosis. In another study, it was shown that activation of p38 MAPK in cardiac myocytes leads to phosphorylation of other proteins, and provides protection and improves cardiac function after myocardial infarction. It seems that p38 MAPK can promote specific remodeling processes in heart failure (39).Another study has shown that p38α MAPK activity is required for neointima formation after arterial injury and also for vascular smooth muscle cell prolifiration (40).

Understanding p38 MAPK targets could provide a better picture of how p38 MAPK functions and contribute to drug development and treatment of heart failure.

Molecular regulation of p38 MAPK signalling:

MAPK families have a highly conserved signal transduction pathway,which combines different extracellular signals to intracellular responses (42).

There are three main MAPK families that have been characterized to date: extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 MAPK (41). All three MAPK families have different roles in the cells, but they all have a similar canonical pathway; activation of MAP kinases kinases kinase (MAP3Ks) is required to phosphorylate and activate MAP kinases kinase (MAP2Ks). The MAP2Ks are activating MAPK by dual phosphorylation of threonine and tyrosine residues on the activation loop. All the three main MAPKs have a specific sequence in their activation loop that is

recognized by MAP2K of the pathway (33). This spesificity gives the ability to a specific MAP2K to activate a particular MAPK selectively (37).

p38 MAPK is activated in response to cellular stress, such as UV irradiation, heat shock, high osmotic stress, protein synthesis inhibitors, proinflamatory cytokines and certain mitogens (19). It also, however, has a role in the immune response and plays an important role in cell survival and regulation of cell checkpoints (43). The activation of p38 MAPK is not only dependent on the stimulus, but also on the cell type (17). All the three MAPKs pathways have a cross talk between each other, and they can either activate or suppress each others activity. For example, ERK can activate JNK (44). On the other hand, stress activated JNK and p38 pathway suppress the survival-promoting activity of the ERK pathway (45,46). The MAPK pathways are regulated by several different signalling pathways, as well as by cross-talk within the MAPK family. The cross talk between the MAPK members contributes to decision of the cell whether to divide or terminally differentiate (47).

p38 MAPK has a role in regulating gene expression at the transcriptional level. Post-transcriptional regulation of inflammatory gene expression has also been linked with the p38 MAPK pathway. The p38 MAPK pathway is also involved in the induction of both cell cycle checkpoints G1/S (synthesis of the DNA) and G2/M (division of the cell): its role has been better established in the G2/M checkpoint. Studies have shown that p38 alpha and gamma are required for UV-induced G2 cell cycle arrest. p38 MAPK phosphorylates and activates p53, which leads to the induction of p53-dependent G2/M checkpoint, this will result in cell cycle arrest (48,49). The p38 MAPK pathway is an

important regulator of protein turnover. There is evidence that indicate that p38 MAPK activity is critical for normal immune and inflammatory response (16). Furthermore, p38α plays a central role in myogenesis, whereby myoblasts fail to differentiate to multinucleated myotubes in mice lacking this kinase (5).

As any many other protein kinases, the activation of p38 MAPK requires phosphorylation. This phosphorylation on the activation loop (Thr180 and Tyr182) leads to conformational changes and stabilizes it. It is suggested that Thr180 is required for catalysis, while Tyr182 is required for substrate recognition. These conformational changes will facilitate substrate binding (50). p38 MAPK is activated by MAP2Ks, which is activated by MAP3Ks (figure 2). The diversity and the regulatory mechanism of MAP3Ks allow for diverse responses to many different stimuli of the cell, and to integrate p38 MAPK activation with other signalling pathways (43). MAP3Ks that have been reported to activate the p38 MAPK pathway are TGF-β- activating kinase 1(TAK1), apoptosis signal-regulating kinase 1 (ASK-1) and mixed lineage kinase 3(MLK-3) (41). Activation of p38 is very specific, and occurs through MKK6 and MKK3, which are the MAP2Ks. There are also studies that show that MKK4 also activates p38 MAPK, but it does not lead to phosphorylation of all p38 MAPK isoforms. Interestingly, MKK4 usually leads to phosphorylation of JNK (35). Activation of MAP2Ks also requires phosphorylation. Their phosphorylation occurs at two conserved sites: serine151 and threonine155 (43).

p38 MAPK is controlled by phosphorylation and dephosphorylation of the activation loop, at Thr180 and Tyr182 residues (35). Protein phosphatases inactivate p38

MAPK pathway. Among those phosphatases are type-2 family phosphatases (PP2C) and protein tyrosine phosphatases (PTP) that dephosphorylate tyrosine residues and lead to inactivation (51). Unlike other kinases, p38 MAPK has no nuclear localization signal, and it is distributed throughout the nucleus and cytoplasm. In response to activation, p38 MAPK is translocated from the cytoplasm to the nucleus. This translocation does not require catalytic activity; it is induced by phosphorylation at Thr180 and Tyr182 (52). Phosphorylation of the p38 MAPK activation loop is critical for the nuclear translocation in response to DNA damage. This induces a conformational change and conformational reorganization, and stabilization of the activation loop, to facilitate substrate binding (23).

The ability of kinases to phosphorylate their substrates is due to the presence of docking domains, which are the binding domains of the substrate. The sequence of the docking domain seems to determine the binding specificity of the kinase to a substrate (53). p38 MAPK phosphorylates its substrates usually on the Ser-Pro or Thr-Pro motifs. There is also some evidence that indicates that p38 MAPK can bind to various proteins without phosphorylating them. This may be due to structural changes of the targets, changes to their subcellular localization or competition with their binding to other proteins (43). Very importantly, p38 MAPK have a large number of cytosolic proteins and transcription factors that it can phosphorylate; among those are the Bcl-2 family, MEF2 family, p53, cyclin D1, activating transcription factor 2(ATF-2) and many others. Thus it is evident that p38 MAPK plays a major role in protein regulation, and has a role in regulating gene expression at the transcription level.

Activation of the p38 MAPK pathway by cytokines will usually lead to cell differentiation, while activation of p38 MAPK pathway through environmental stress will lead to cell death. Those two outcomes discriminated by the strength of the signal (35). The strength of the signal, whether there is persistent stimulation of p38 MAPK, will determine whether p38 MAPK will lead to cell growth or cell arrest and death. It seems that enforced activation of p38 MAPK will lead to growth arrest, while activation by inflammatory cytokines leads to temporary activation of p38 MAPK, this might be due to activation of parallel pathways, such as JNK. It is still remains to be defined what leads to the two different outcomes when p38 MAPK is activated (30,43).

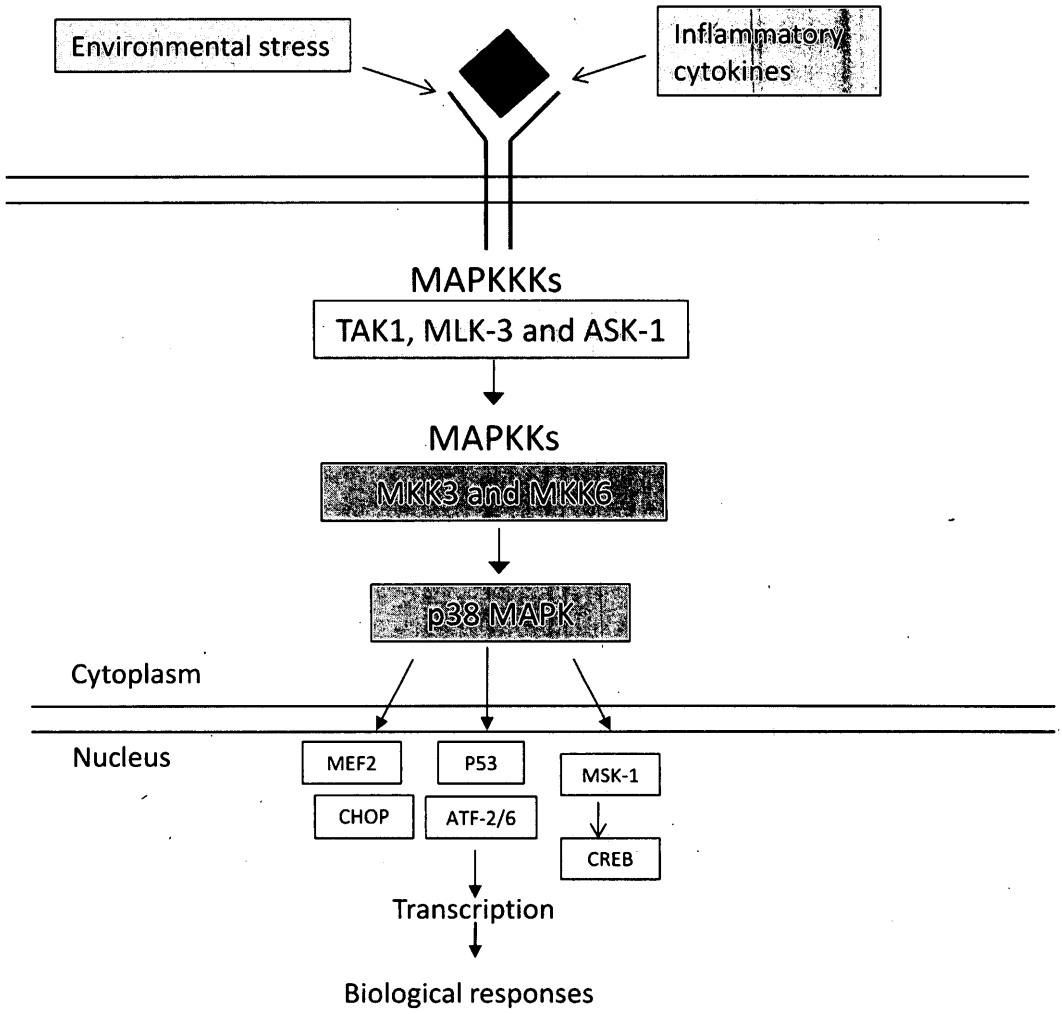

Figure 2: p38 MAPK pathway signalling.p38 MAPK is activated in response to stress signals outside the cell. This will lead to activation of MAP3K, which will phosphorylate and activate MAP2K, and it will in turn phosphorylate and activate p38 MAPK. Phosphorylation of p38 will cause to its translocation from the cytoplasm to the nucleus, which will lead to phosphorylation of many different substrates, which will lead to different biological responses.

p38 MAPK and its substrates:

p38 MAPK was identified in 1994. Since then, a number of its substrates have been also identified.The identification of physiological substrates was done by several ways; use of specific pyridinyl imidazole inhibitors, such as SB203580 and SB202190

(54,55), mutagenesis of amino acids at phosphorylation sites of the substrate (52), and use of knockout mice that are dificient for each of the p38 MAPK isoforms individually (55).Identification of p38 MAPK substrates is of widespread interest due to its important role in the cell regulationbut, so far, its substrates are incompletely characterized.

Among the substrates that were identified are: MAPKAP K2, 3 and 5. They are phosphorylated by p38 MAPK in response to inflammatory cytokines or environmental stresses that the cell is exposed to (23). p38 MAPK activation in response to cell cycle arrest or cell death will also lead to activation of MAPKAP K2 (28). MAPKAP K2 acts not only as a substrate, but also determines p38 MAPK cellular localization (23).p38-regulated/activated protein kinase (PRAK) is phosphorylated when the cell is stress induced (56). MAP kinase-interacting kinase 1 and 2 (MNK1/2) and Mitogen and stress activated kinase 1 (MSK1), both are activated in response to growth factors or environmental stress. Ribosomal S6 kinase-B (RSK-B), which phosphorylates transcription factor CREB (23).

p38 MAPK also phosphorylates transcription factors, such as p53,activation transcription factor 2 and 6 (ATF-2/6) (23,57), MEF2A and MEF2C (58). As mentioned before, p53 is pro-apoptotic and has a role in cell cycle checkpoints (26). MEF2A and MEF2C have a role in many cellular functions and in neurons, skeletal and cardiac muscle tissues. MEF2 plays a role in the differentiation of these cell types, and in neurons it also has a role against neuronal apoptosis. MEF2 is phosphorylated by p38 MAPK on Thr312 and Thr319 residues, which leads to its increased transcriptional activation (59). ATF-2 is phosphorylated by p38 MAPK at Thr69 and Thr71 residues. Upon activation,

interacts with regulatory proteins, such as the retinoblastoma (Rb) tumor suppressor. ATF-2 can contribute to global transcription and DNA damage response, and it also has oncogenic activities and can act as a tumor suppressor (57). Another transcription factor substrate is C/EBP homologous protein (CHOP). p38 MAPK phosphorylate CHOP on Ser^{78} and Ser^{81}, this results in increased transcriptional activity of CHOP. CHOP accumulates under stress conditions (60). p38 MAPK also phosphorylates Elk1 and SAP-1A (61). p38δ phosphorylates the cytosolic protein stathmin, which is involved in the regulation of microtubule dynamics (62). Tau, is a protein that is found in neurofibrillary lesions of Alzheimer's disease, is also phosphorylated by p38ɣ and p38δ. Phosphorylation of Tau leads to its ability to promote microtubule assembly (63).

As we can see, p38 MAPK has many different types of substrates that it can phosphorylate and activate. These substrates are activated by different stimuli and can have diverse effects within the cell. Knowing more about p38 MAPK substrates and understanding those that are already identified will provide a unified view of the p38 MAPK signalling pathway.

Structure of p38 MAPK:

The p38 MAPK family plays a key role in the cell-cycle regulation, inflammation and apoptosis (enhanced p38 MAPK activity leads to apoptosis, hence it can be used in cancer treatment). It belongs to a larger group of mitogen activated protein kinase (MAPK). (64).

p38 MAPK is approximately 38kDa protein. It has four isoforms, which are 60% identical at the amino acid level. Based on their expression pattern, substrate specificity and sensitivity to pharmacological inhibitors, they can be divided to two groups: p38 alpha and beta, and p38 gamma and delta (16). p38α is expressed in most cells, while other isoforms are more tissue specific.p38β is being expressed in brain cells, while p38γ is expressed in skeletal muscle cells, and p38δ in endocrine glands (23,65). In addition to differences in tissue specificity expression, p38γ and δ cannot phosphorylate all substrates, while p38α and p38β can phosphorylate all substrates (55). p38 MAPK is activated in response to ultraviolet radiation (UV), cellular stress, pro-inflammatory cytokines, TNFα and inteleukin-1 (Ile-1). p38 MAPK must be phosphorylated first in order to achieve maximum enzyme activity: p38 MAPK is phosphorylated by dual phosphorylation at the Thr180 and Tyr182 at the conserved motif Thr-Gly-Tyr, by an upstream kinase, MAP2K. The motif is located on the surface near the active site of p38 MAPK and exposed to solvents. This conserved motif of Thr-Gly-Tyr is called the activation loop, and it contains 12 amino acids (including Thr-Gly-Tyr). Phosphorylation of the activation loop leads to activation of p38 MAPK (66). The dual phosphorylation of p38 MAPK causes conformational changes of the folded protein, which enhances its access to substrate; together these factors increase the enzymatic activity of the substrate (23). There are specific MAP2Ks that were identified to phosphorylate p38 MAPK: MKK6 and MKK3 (66). Phosphorylation of p38 MAPK by MKK3 and MKK6 results in its phosphorylation and activation (41).

The structure of unphosphorylated p38 MAPK plays an important role in the regulation of the cellular cascade. Interestingly, a portion of the activation loop of unphosphorylated p38 MAPK blocks the substrate binding area by occupying the peptide binding channel with amino acids 170-178. This ensures that p38 MAPK is not active before its activation (66). Regulation of p38 MAPK signal transduction is important for the proper function of the cell, since activation of this pathway may lead to cell cycle arrest (67).

p38α and other MAP kinases have a docking groove, where activating kinases, inactivating phosphatases and substrates bind. This docking site, however, is not where the binding site for substrates, where they are being phophorylated by the kinase (66). Those docking domains form a binding site for the kinase to phosphorylate its substrate. Those interactions between MAPK and their substrates are necessary for signalling (33). The p38α catalytic ATP site is formed in a region between the N-terminal and the C-terminal domains of the protein. The ATP binding site is located on amino acids 106-112 (64). The ATP binding site is formed by the glycin-rich loop (67). The N-terminal domain creates a binding pocket for the adenine of the ATP ring, while the C-terminal domain contains the catalytic base, the binding sites and the phosphorylation loop (66).

Additional DFG (Phe residue of Asp-Phe- Gly) motif is known as a conserved motif, is buried in the hydrophobic pocket between the two lobes of the kinase. This motif enhances activation of the gatekeeper residue (Figure 3) (68).

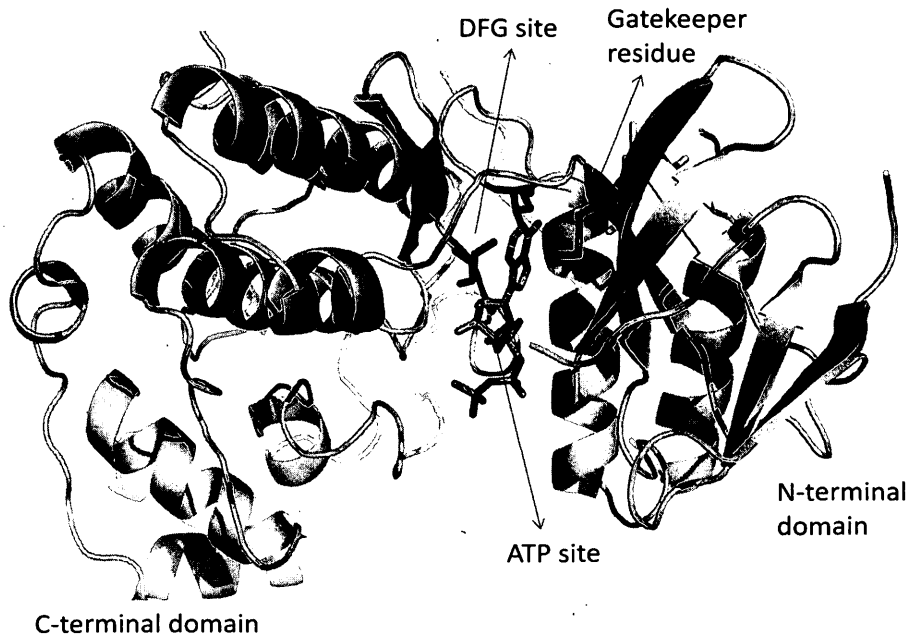

Figure 3: Computational 3D structure of p38 MAPK. The structure of p38 MAPK demonstrates the gatekeeper residue, DFG site and the ATP binding site, which is located between the C-terminal and the N-terminal domains. PDB ID: 1CM8.

Identification of kinase substrates:

Protein kinases play an important role in many cell signalling pathways. Identification of the direct substrates of each kinase is still a challenge, due to the fact that all kinases use ATP to phosphorylate their substrates. There are a few methods that are being used by researchers to identify substrates of protein kinases. One of the techniques is a use of inhibitors. Since the inhibitors bind to the ATP binding site, it also prevents from the kinase to phosphorylate it substrates, hence this is not the preferred technique for substrate identification.For this reason a new technique was developed by K. Shokat in 1997(69). In this technique, specific kinases of interest are engineered to accept an ATP analog that has bulkier substituents synthesized on the N6 position, which will not be

developed to identify direct substrates of many kinases. This approach was used in a recent study to identify new substrates of PKA (71).

A recent technique has been developed to identify substrates. In this technique 5'-4-fluorosulphonyl-benzoyladenosine (FSBA) is used in a whole-cell lysate. FSBA is an ATP analog that inhibits the kinase by occupying the ATP binding site. This technique uses FSBA to inhibit all endogenous kinases in the lysate. Once they are inhibited, any unbound inhibitor is removed and the kinase of interest is added to the cell lysate. To visualize phosphorylation of the substrates of the kinase of interest, γP^{32}-ATP is added, any labelling that occurs is due to the kinase. Further the substrates are identified by mass spectrometry analysis (72).

Substrate identification using ATP binding pocket mutations:

The hallmark of using Shokat's technique is that products of phosphotransfer reactions with the modified kinase are specifically labelled. This method allows for identification of substrates of a specific kinase, and subsequently to determine their role in intracellular signal transduction. Importantly, this method is based on the observation that all protein kinases have a structurally similar ATP binding domain that comes into close contact with the N6 position of ATP (70).

This method of engineering kinases has been used by other groups to identify new substrates of different kinases, including CDK1 (73), Rous sarcoma virus (69), ERK2 (74) and JNK (75).

Identification of the relevant substrates of a medically important kinase can help to clarify the molecular mechanism in diseases and foresee side effects of treatment with kinase inhibitors (76).

Therefore, it is of great interest to identify kinase substrates; our current study will specifically focus on human p38α MAPK. p38α MAPK has a central role in myogenesis, also p38 MAPK signalling pathway a promising target for autoimmune and inflammatory disease therapy. This makes p38 MAPK an interesting target to identify its substrates.

In order to properly engineer the mutant kinase, there are specific criteria that need to be met. Firstly, the kinase should be able to accept an ATP analog that cannot be used by the wild type kinase. Secondly, it should be able to use ATP analog with high catalytic efficiency and finally should have the same substrate specificity as the wild type kinase (77). More specifically, the engineered kinase will accept the ATP analog by modification of the ATP binding pocket (73), and thus mutated kinase will prefer to use the ATP analog as the phosphor donor for substrate phosphorylation.

Modification of the ATP binding pocket is achieved by mutating the amino acid at the gate keeper residue, which is the residue within the ATP binding site of a kinase that controls the accessibility of the substrate. The gatekeeper residue is conserved as a large hydrophobic pocket, and mutation of this residue to Alanine or Glycine creates a new pocket that can be uniquely accessed by an ATP analog (78). Since the size of the gatekeeper residue affects the accessibility of the hydrophobic pocket mutation at this residue creates additional space that will expend the ATP binding pocket and will allow an ATP analog to bind, while still allowing the mutated kinase to retain its natural

substrate specificity (79). Additional mutation to the conserved DFG (Phe residue of Asp-Phe- Gly) is known to enhance activation of the gatekeeper residue in p38 (76).

Once the mutation is generated in the kinase, it is necessary to find a compatible ATP analog, which will be utilized by themodified protein.

In order to trace substrates phosphorylated by the modified kinase radioactive tagging of ATP analog with gamma P^{32} is used to identify direct substrates of a kinase. The kinase will catalyze the transfer of the gamma phosphate from ATP into the substrate. This will allow specifically label substrate of a specific kinase, what will allow identifying direct substrates of the kinase of interest in a pool of other kinases (figure 3) (76). Radioactive labelling allows us to track the substrates, only those proteins that are direct substrates of that kinase will become radiolabeled.

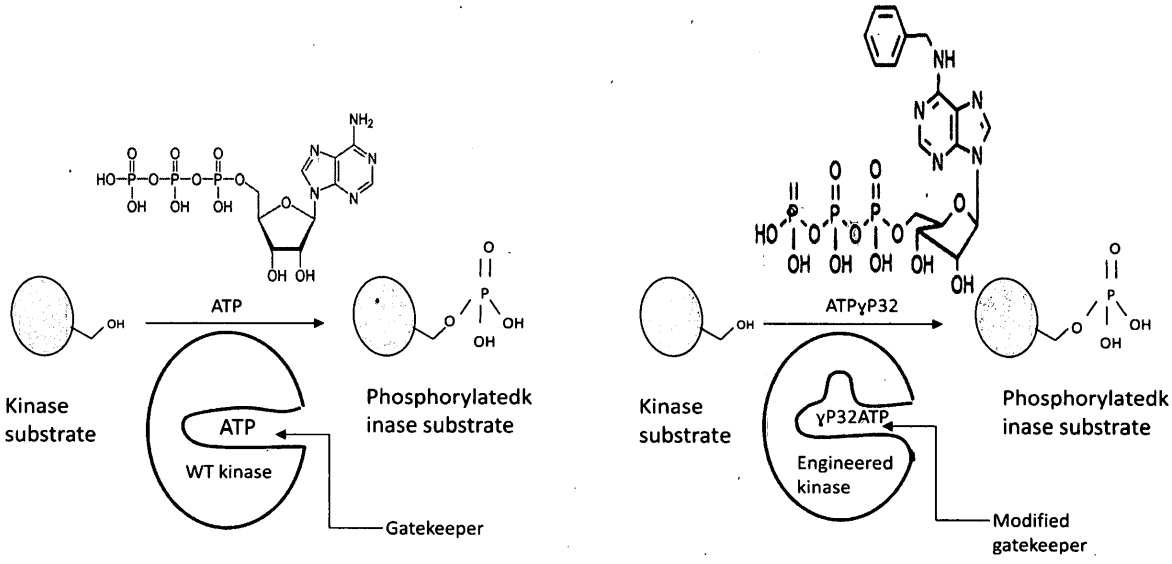

Figure 4: The approach to engineer a kinase to accept ATP analog. Smaller amino acid is generated as a point mutation at the gatekeeper residue of the kinase, which allows the kinase to accept an ATP analog, that have additional chemical group synthesized on the N6 position.

Chapter 2: Statement of Purpose:

Recent studies have shown that p38 MAPK plays an important role as a key regulator of pro-inflammatory cytokine biosynthesis, which makes p38 MAPK a promising target for autoimmune and inflammatory disease therapy. p38 MAPK also plays a central role in myogenesis and embryonic development. Also, based on recent studies, there is a possible indication that p38 MAPK can act as tumor suppressor. All of these roles make it of great interest to study the regulation of p38 MAPK and its downstream substrates. There are some substrates that are already known, but many still remain unknown. Identification of the full inventory of direct substrates of kinases is a key factor in determining their roles in intracellular signal transduction.

The long term goal of these studies is to identify novel substrates of human p38α. Identification of the relevant substrates of medically important kinases can help to clarify molecular mechanisms in disease and foresee side effects of treatments with kinase inhibitors.

First, we wanted to generate mutations of p38 MAPK without losing its biological function. For this purpose we have generated mutations at the gatekeeper residue. The purpose of generating mutations at the gatekeeper is to use them as tools for the identification of physiological substrates. This will distinguish the substrate preference of p38 MAPK from the rest of the cellular kinases in terms if ATP usage. The mutation was generated at the gate keeper residue, which controls the accessibility of the kinase to the substrate. The mutation was a smaller amino acid, which will create an additional pocket were an ATP analog can bind that cannot be accepted by wild type kinase.

The next step was to engineer our kinase ATP binding pocket, so that p38 MAPK will be able to accept and bind ATP analogs. Based on the 3D structure of p38 MAPK we made an additional mutation that will modify the ATP binding pocket. Once mutations were generated, we wanted to test whether synthesized ATP can be accepted by these mutations. For this purpose, we have tested different ATP analogs (that were purchased from Biolog Life Science). The ATP analogs used were: N6-Bn-ATP, N6-(2MethylButyl)-ATP and N6-Cyclopentyl-ATP. Our mutations, which are genetically engineered, should be able to use the more bulky synthesized ATP analog, which is typically not recognized by natural endogenous kinases. All protein kinases use ATP as a cofactor to phosphorylate their targets, that is why the identification cannot be done easily, and that is where the ATP analog plays a role. Lastly, our purpose was to use radiolabelled γP^{32}ATP in order to transfer a radioactive label to the substrate when the kinase uses the γ P^{32} ATP in the catalytic reaction for identification of p38 MAPK substrates. By marking the substrates of engineered kinase with gamma-phosphate group of ATP analog, it can be specifically labelled in a complex mixture of other wild type kinases. The radioactive labelling allows it to track the substrates. Only the proteins that are direct substrates of p38 MAPK will become radiolabeled.

Therefore, the criteria that need to be met for engineered kinase are: 1) the mutated kinase should be expressed in the cell content, 2) the engineered kinase should accept ATP analogs, 3) the kinase should be able to phosphorylate a known substrate, 4) the wild type p38 MAPK does not accept ATP analog.

Based on this initial approach, one can continue with identification of new substrates. A recent approach is to couple the transfer of the phosphate group to the identification of the substrate using mass spectrometry.

Chapter 3: Materials and Methods:

Antibodies and plasmids:

The following antibodies were used: α-Actin (cat. # SC-1616) and α-dsRED (cat. # G2307) were purchased from Santa Cruz biotechnology. Anti HA- 125CA5 supernatant was obtained from Developmental Studies Hybridoma bank. p38 (cat. # 9212), pp38 (cat.# 9211) and pATF-2 (cat. # 9224) were purchased from Cell Signaling Technology. ATF-2 Fusion Protein was purchased from Cell Signaling Technology. Anti HA affinity matrix was purchased from Roche (cat. # 11815016001)

PMT2 and HA-p38 we got from David Cox. MKK6

Cell culture:

The COS7 cell line, which is derived from the African Green Monkey kidney were cultured in growth media (GM) which contains high-glucose Dulbecco's modified Eagle's medium (DMEM), 10% FBS (HyClone), 1% penicillin-streptomycin and 1% L-Glutamine (Gibco) at 37 °C and 5% CO_2. Cells were passaged at 60-70% confluency. (For details see appendix)

Transient Transfection:

Cells were seeded 24 hours before transfection. Transient transfection of cells was performed based on the calcium- phosphate precipitation method. Cells were allowed to recover in GM 16-18 hours after transfection. dsRED signal was confirmed 24 hours later and cells were harvested with 1X cell lysis buffer. (For details see appendix)

Western Blot analysis:

Total cell lysate were harvested with 1X cell lysis buffer (see appendix) and supplements (1mM phenylmethylsulfonyl (PMSF) and 1mM Na_3VO_4).

Equal volumes of protein for each sample (15µl) were resolved by SDS-PAGE on 10% gels. Proteins were transferred electrophoretically to Immobilin-FL membranes (Millipore). Primary antibodies were diluted in appropriate buffer in the following ratios: Actin (1:2000), dsRED (1:2000), HA (1:20), p38 (1:3000), pp38 (1:3000) and p ATF2 (1:1500), and blots were incubated overnight in 4°C. Blots were incubated in secondary antibodies at room temperature. ECL (GE) was added, and blots were exposed to film. (For more details refer to appendix).

Immunoprecipitation and *in vitro* kinase assay:

Total cell lysate was prepared in 1X cell lysis buffer. Anti-HA beads were added to the total cell lysate and incubated overnight at 4°C. The pellets were washed 2 times with 1X cell lysis buffer and twice with 1X kinase buffer (based on Kinase Assay Kit from Cell Signaling Technology). The following was added to the pellet: 250µM ATP or ATP analog, 2µg/ul ATF2 fusion protein (purchased from Cell Signaling Technologies) and 1X kinase buffer for a total volume of 40µl. The reaction was incubated for 30min at 30°C. The reaction was stopped by adding 4X Lummeli sample buffer. Samples were boiled for 5 minutes at 95°C. Samples were run on 10% SDS-PAGE and western blot analysis was done to detect the proteins of interest.

6XHN NDPK protein purification and ATP analog labeling:

BL21 6xHN NDPK was purified on a column with Ni-Nta (nickel-nitriloacetic acid column) beads. Samples with the eluted fraction were resolved on a 15% SDS-PAGE gel and coomasie stained to assess protein levels and purity. The fractions with the highest NDPK levels were dialysed against three changes of HKG buffer (see appendix). This was used for ATP labelling.

165µg of purified 6xHN NDPK was added to a column with Ni-Nta beads. The column was washed with 1X PBS. 1mci of γP32 was added to the column. ADP analog was added to the column. γP^{32} ATP analog was eluted from the column. The eluate was used for radioactive *in vitro* kinase assays. The procedure for "hot" *in vitro* kinase assay was the same as the "cold" (for details see appendix).

Mutagenesis:

The mutations were constructed by polymerase chain reaction (PCR) with two sets of primers cloned into p38 MAPK. The various site mutations of p38 were generated using Quickchange II Site directed mutagenesis kit (Stratagene). Mutations were sequence verified (For details see appendix).

Chapter 4: Results:

Generated mutations of p38 MAPK:

We have generated mutations of wild type (WT) human p38α MAPK by site directed mutagenesis. Firstly, mutation at the gatekeeper residue (Thr106) was generated. Threonine was exchanged to a smaller amino acid Glycine or Alanine. Additional mutation was done at the conserved DFG motif, L167, which is a conserved motif in p38 MAPK that enhances the activity of the gatekeeper residue. Leucine was exchanged to Alanine. The clones were screened for correct mutation by sequencing at the core facility. The following mutations were introduced into the DNA sequence: 1) T106G, 2) T106A, 3) T106G + L167A, 4) T106A + L167A.

T106G:

GAGGAATTCAATGATGTGTATCTGGTGACCCATCTCATGGGGGCAGAT
GAGGAATTCAATGATGTGTATCTGGTGGGCCATCTCATGGGGGCAGAT

T106A:

GAGGAATTCAATGATGTGTATCTGGTGACCCATCTCATGGGGGCA
GAGGAATTCAATGATGTGTATCTGGTGGCCCATCTCATGGGGGCA

+L167A:

GCTGTGAATGAAGACTGTGAGCTGAAGATT-CTGGATTTTGGACTGGCT
GCTGTGAATGAAGACTGTGAGCTGAAGATTGC-GGATTTTGGACTGGCT

Over-expression of MKK6 activates WT p38 and p38 mutations:

Once mutations in p38 MAPK were generated, we wanted to confirm that p38 MAPK had not lost its catalytic activity, and the mutant forms of p38 MAPK are still able to be

activated by MKK6. This was tested by exogenously expressing COS7 cells with WTp38 and p38 mutations. PMT2, which is the vector p38 is cloned in, was used as a control. The results demonstrated in figure 4, indicate that the mutations that were generated did not affect the ability of p38 being activated by MKK6.The results show that the generated mutations are phosphorylated at the same level as the WTp38, based on the pp38 immunoblot. MKK6 is a physiological upstream activator of p38 MAPK. Cells were co-transfected with WTp38 or p38 mutations with the addition of MKK6. This was to test whether the generated mutations have changed the activity of p38 MAPK, or they are still phosphorylated by the biological activator of p38 MAPK. This was a preliminary screening to confirm that we can use our mutations for further experiments.

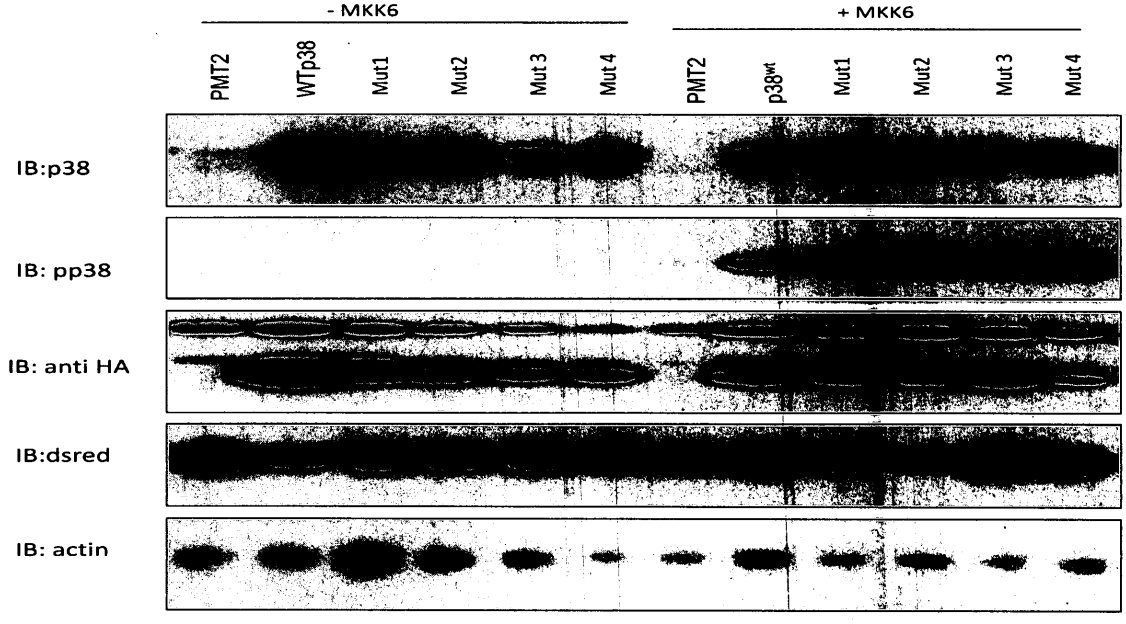

Figure 5:Over-expression of MKK6 can activate p38 mutations. Western blot analysis in COS7 cells to over-expression of WTp38 and the 4 generated mutations of p38. PMT2 was used as an empty vector control, HA was used as a control to detect exogenously expressed p38, dsRED was used as transfection efficiency control, and Actin was used as a loading control. Phospho p38 was used to detect phosphorylated p38 after co-transfection with MKK6.
The p38 mutations are as follows: Mut 1- T106G, Mut2- T106A, Mut3-T106G +L167A, Mut 4- T106A+ L167A.

Western blot analysis of *in vitro* kinase assay with N6-Bn-ATP shows that generated p38 mutations can accept an ATP analog and phosphorylate a known substrate (ATF2):

Using the p38 MAPK mutations, an *in vitro* kinase assay was done with an ATP analog, N6-Bn-ATP, to confirm whether mutations of p38 MAPK can use the ATP analog to phosphorylate a known substrate of p38, ATF-2. To isolate exogenously expressed WTp38 and p38 mutations, p38 MAPK was immunoprecipitated with HA beads in order to pull down only exogenous p38 MAPK which is tagged with HA.COS7 cell line was used. WTp38 and p38 mutations were co- transfected with MKK6. The analog that was used is N6-Bn-ATP.This analysis helped us determine whether ATP analog can be used as a phosphate source by mutations of p38 MAPK.

The results demonstrated in figure 5 have shown that mutation to the gatekeeper residue (Thr106 to Gly) was able to phosphorylate ATF-2 more efficiently than Thr106 to Ala. Enhancement of that mutation with L167A was also able to phosphorylate the substrate, but it was not by far more efficient phosphorylation than mutation just to the gatekeeper residue, which was a surprise. Surprisingly WTp38 can also accept ATP analog and phosphorylate ATF-2.

It seems that exchange to amino acid Glycine is better than to Alanine. Exchange to Glycine seems to phosphorylate ATF-2 more efficiently, which might be an indication to the fact that this mutation is able to accept ATP analog better.

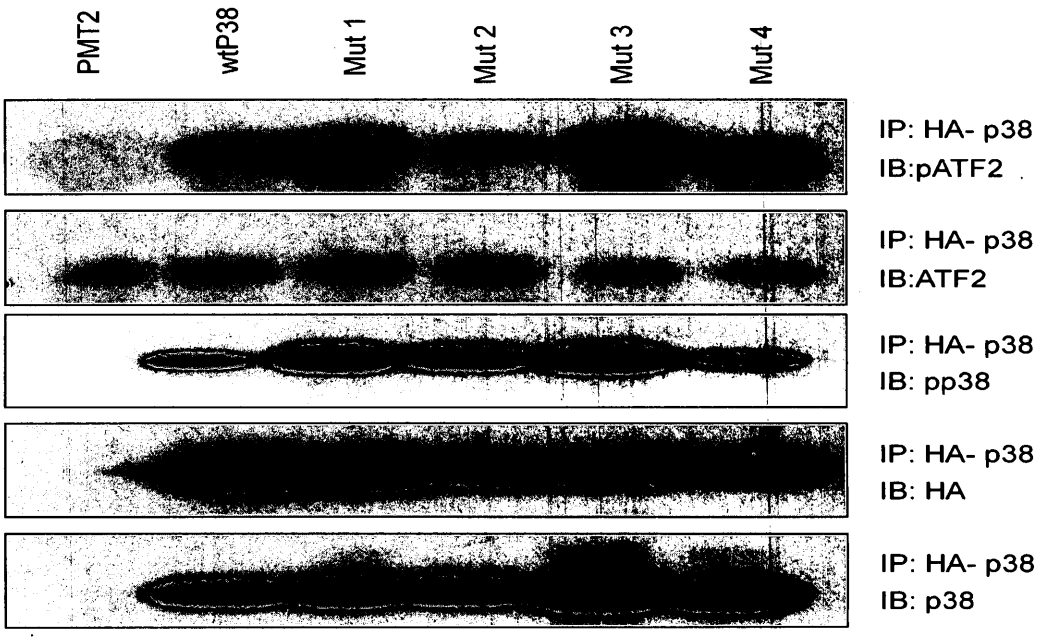

Figure 6: *In vitro* kinase assay with mutations of p38 and ATP analog shows that generated mutations can phosphorylate known substrate ATF-2. Western blot analysis in COS7 cells of an *in vitro* kinase assay with N6-Bn-ATP. The kinaseassay was done with ATF2. All the conditions were activated by co-transfection with MKK6.
PMT2 was used as a control, HA was used as a control to detect exogenously expressed p38. ATF-2was used to detect total ATF2 levels, while pATF-2 was to show phosphorylation by activated p38. pp38panel was to show that MKK6 successfully activated p38, and p38 was to show total p38 levels in thecell lysate. The p38 mutations are as follows: Mut 1- T106G, Mut2- T106A, Mut3-T106G +L167A, Mut 4- T106A+ L167A.

Additional ATP analogs were tested in *in vitro* kinase assay with mutations of p38 MAPK:

Once we have generated mutation in p38 MAPK, it is necessary to find a compatible ATP analog, which will be utilized by the modified protein. Previous studies that were done on identification of new substrates, have tried few analogs with this labelling technique (69, 74), to determine which analog will chemically match best, this will result in better

catalytic efficiency and better substrate phosphorylation.Different analogs were tried to test which analog will be the most compatible with p38 mutations (Fig 7). For this purpose we have used two additional analogs:N6- (2MethylButyl)-ATP and N6-Cyclopentyl-ATP from Biolog Life Science company. All three analogs were tested in "cold" *in vitro* kinase assay with ATF-2.

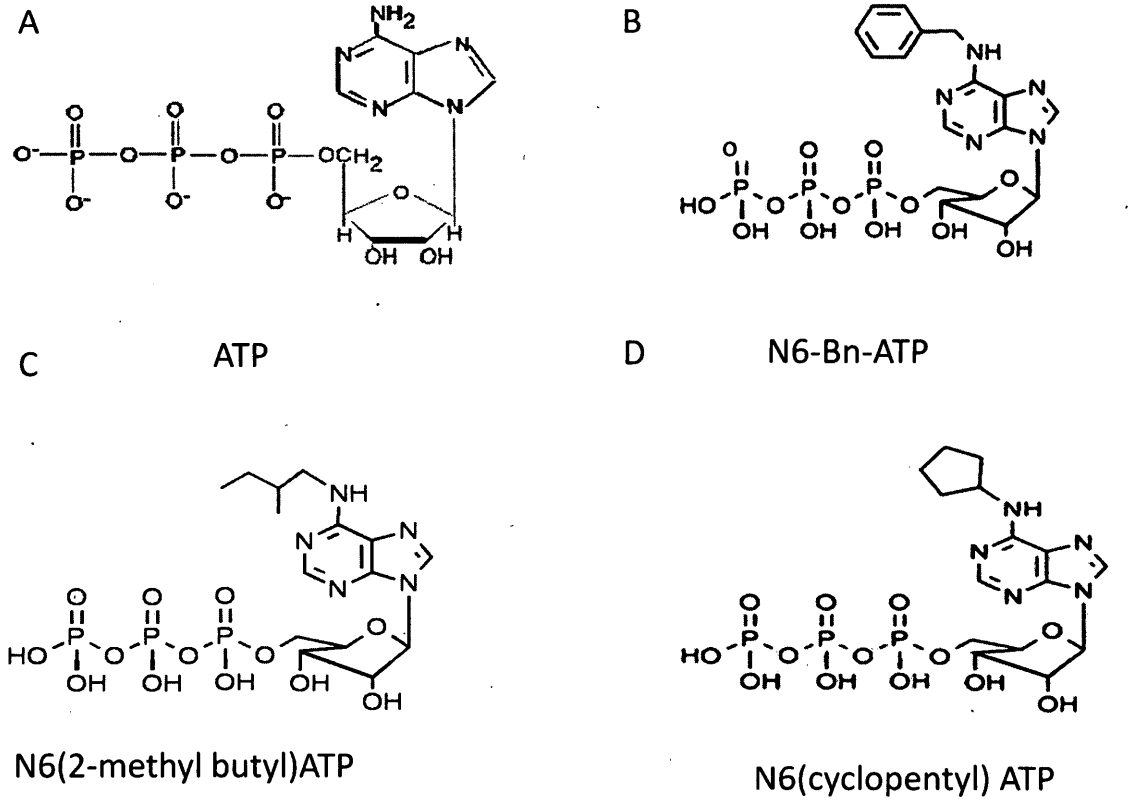

Figure 7: Structural comparison of ATP and ATP analogs. A. Chemical structure of ATP. B. Chemical structure of N6-Bn-ATP. C. Chemical structure of N6(2-methyl butyl)ATP. D. N6(cyclopentyl)ATP. The benzyl group that is found at the N6 position of the adenine ring was synthesized to a bigger chemical group. Structure was adopted from Biolog Life Sciences.

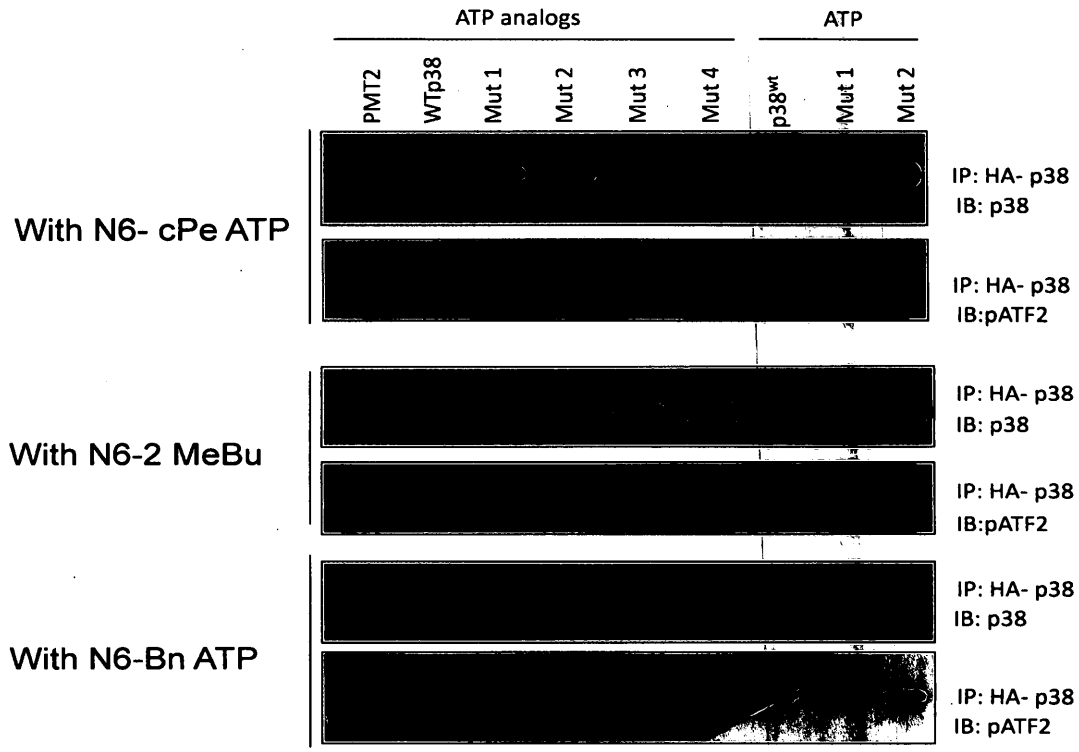

Figure 8: Additional ATP analogs in *in vitro* kinase assay do not phosphorylate ATF-2 with the generated mutations.

Western blot analysis of in vitro kinase assay of WTp38 and mutation of p38 co-transfected with MKK6 in COS7 cells. PMT2 was used as control. The blots were probed with pATF2 to detect whether activated p38 mutations by MKK6 can phosphorylate known substrate in an assay with ATP analogs. The assay was done with different analogs. Blots were also probed with p38, to detect levels of total p38 in the lysate after immunopercipitation.

The p38 mutations are as follows: Mut1- T106G, Mut2- T106A, Mut 3- T106G +L167A, Mut 4- T106A+L167A.

These results demonstrate that the generated mutations of p38 MAPK are not accepting ATP analogs very efficiently. The reason for that can be that the amino acid Threonine is not a very big amino acid, and exchange of this amino acid to Glycine or Alanine does not open up the binding pocket of the substrates as much, hence the ATP analog does not bind in this pocket as efficiently and cannot be used to phosphorylate substrates of p38 MAPK. In other studies that used this technique, usually the gatekeeper residue is a

bigger amino acid, like Methionine, and exchange of this type of amino acid to a smaller one will make a difference in the size of the ATP binding pocket.

Analysis of the three dimensional structure of p38 demonstrated that there is additional mutation that can be done in order to open ATP binding pocket further (Fig 9A, 9B, V. Saridakis).

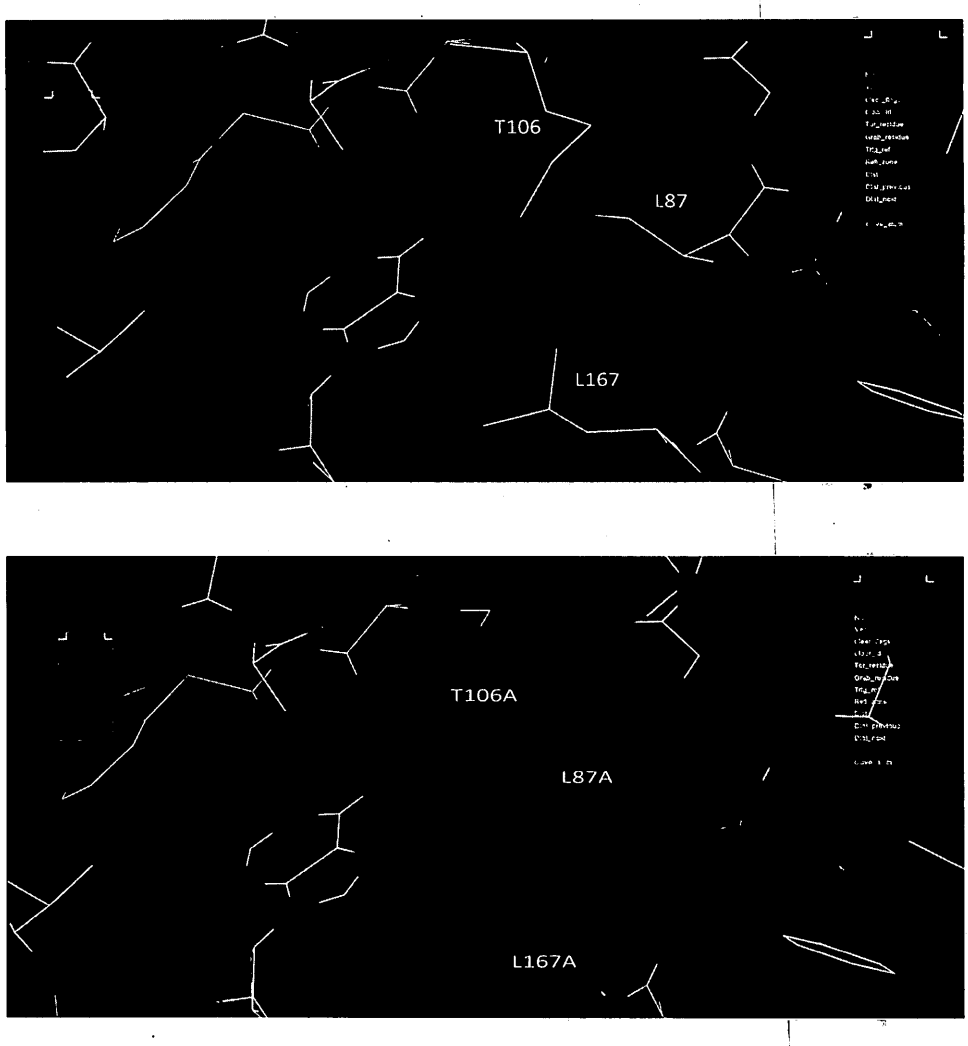

Figure 9: Hypothetical computational analysis of p38 demonstrates that additional mutation to L87 residue will increase the ATP binding pocket size. Computational structure of p38 was tested. Based on the structure T106 and L167 that were already mutated are residues that need to be mutated to a smaller amino acid to open the ATP binding site. Though, the hypothetical computational analysis shows that L87 residue is possible to be on the way to increase the ATP binding site (A). (B) Hypothetical computational analysis that shows how the ATP binding site will look like if all those three residues mutated to Alanine.

Additional mutation of p38 MAPK to L87 residue was generated:

Based on the computational analysis with the help of Dr. Saridakis, we have generated another mutation at the L87 residue; the amino acid was exchanged to Alanine.

An additional 4 mutations were introduced into the DNA sequence. Table 1 demonstrates all mutations that were generated in p38 MAPK.

Table 1: All the mutations that were introduced into the p38 MAPK DNA sequence

Mut 1	T106G
Mut 2	T106A
Mut 3	T106G+L167A
Mut 4	T106A+L167A
Mut 5	L87A+T106G
Mut 6	L87A+T106A
Mut 7	L87A+T106G+L167A
Mut 8	L87A+T106A+L167A
Mut 9	L87A

The clones were screened for correct mutation by sequencing at the core facility.

L87A+T106G:

GTCTGTTGGACGTTTTTACACC
GTCTGGCGGACGTTTTTACACC

TATCTGGTGACCCATCTCATG
TATCTGGTGGGCCATCTCATG

L87A+T106A:

GTCTGTTGGACGTTTTTACACCTT
GTCTGGCGGACGTTTTTACACCTT

TATCTGGTGACCCATCTCATGGG
TATCTGGTGGCCCATCTCATGGG

L87A+T106G+L167A:

TGGTCTGTTGGACGTT
TGGTCTGGCGGACGTT

ATGGTCTGGCGGACGT
ATCTGGTGGGCCATCT

AGCTGAAGATTCTGGATT
AGCTGAAGATTGCGGATT

L87A+T106A+L167A:

GTGATTGGTCTGTTGGACGTTTT
GTGATTGGTCTGGCGGACGTTTT

ATCTGGTGACCCATCTCAT
ATCTGGTGGCCCATCTCAT

GAAGATT-CTGGATTTTGGACTGGCTC
GAAGATTGC-GGATTTTGGACTGGCTC

Western blot analysis of *in vitro* kinase assay with additional generated mutations of p38 and ATP analogs and ATF-2 as a substrate:

A "cold" in vitro kinase assay was done with the additional mutations of p38 MAPK and all the three analogs, in order to confirm whether the new mutation has changed the chemical structure of the ATP binding pocket, and whether this will allow an ATP analog to bind better and phosphorylate known substrate of p38 MAPK. All the three analogs were used to determine which analog can bind to the generated ATP binding pocket. As demonstrated in figure 10, it is observed that the addition of the new mutation L87A has changed the chemical structure of the ATP binding pocket and together with the gatekeeper residue mutation to T106G can very efficiently use an ATP analog to phosphorylate known substrate of p38 MAPK. Based on the results, we can see that N6-

2MeBu-ATP is binding better to the ATP binding pocket. Even though we can see that other analogs are also phosphorylating ATF-2 (they are also accepted at the ATP binding pocket) N6-cPe-ATP and N6-Bn-ATP can also be accepted by WTp38, and N6-2MeBu-ATP is not. This makes it a more promising analog for the identification purpose. Surprisingly, all the three mutations did not seem to have an ability to phosphorylate ATF-2, despite the fact that from the computational analysis it seemed that the combination with the three mutations would open the ATP binding pocket to its maximum for ATP analog to be accepted. The *in vitro* kinase assay shows that the interaction between the ATP analog and the ATP binding site is not only based on size but also structural changes that are occurring with the protein.

Regular ATP was used as a control to see whether an ATP analog can bind almost as efficiently to mutated p38 MAPK as regular ATP, which based or the results in figure 9 it was confirmed.

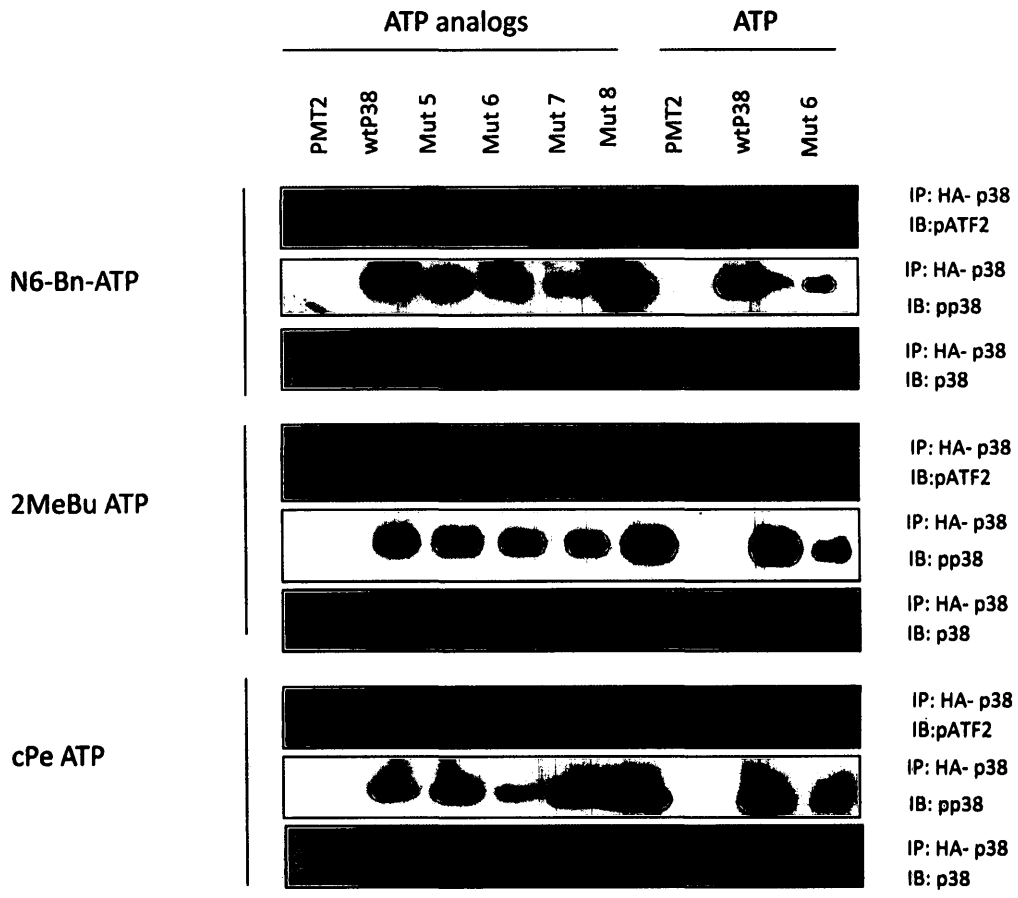

Figure 10: In vitro kinase assay with new p38 mutations and ATP analogs have demonstrated that N6-2MeBu-ATP analog is being utilized better than the other analogs by the new mutations and can phosphorylate know substrate of p38 ATF-2. Western blot analysis of in vitro kinase assay of WTp38 and mutations of p38 co-transfected with MKK6 in COS7 cells. PMT2 was used as control. The blots were probed with pATF2 to detect whether activated p38 mutations by MKK6 can phosphorylate known substrate in an assay with ATP analogs. Different analogs were tested to confirm which one uses the analog best. Blots were also probed with p38, to detect levels of total p38 in the lysate after immunopercipitation, and phsopho p38 used to detect levels of activated p38 by MKK6.
The p38 mutations are as follows: Mut5- L87A+T106G, Mut6- L87A +T106A, Mut 7- L87A+T106G+L167A, Mut8- L87A+T106A+L167A.

Western blot analysis of *in vitro* kinase assay of L87A mutation by itself with ATP analogs:

Based on the structural analysis of p38 and the additional mutation to L87 residue, we wanted to confirm whether L87A by itself can phosphorylate known substrate of p38-ATF-2. An in vitro kinase assay with the L87 mutation, ATP analogs and ATF-2 as a

substrate was done. For comparison L87 mutation with the gatekeeper residue and L87 mutation with gatekeeper and conserved motif was also analysed on the western blot. As demonstrated in figure 11, the results showed that L87A by itself can bind N6-Bn-ATP and N6-cPe-ATP analogs and phosphorylate ATF-2, but N6-2MeBu-ATP does not bind to L87A and does not phosphorylate the substrate. This is an interesting result, which indicates that L87A does have an effect in binding ATP analogs, as it shows that the addition of this mutation has made structural changes that enabled ATP analogs to bind to the ATP binding site of mutated p38 and phosphorylate ATF-2. It is interesting that the additional mutation of L87A to the gatekeeper residue T106G leads to better binding with N6-2MeBu-ATP analog, but L87A by itself cannot bind. This is not the case with the other two analogs.

Regardless, those results show that L87A does contribute to the chemical changes at the ATP binding site and lead to better binding of ATP analogs.

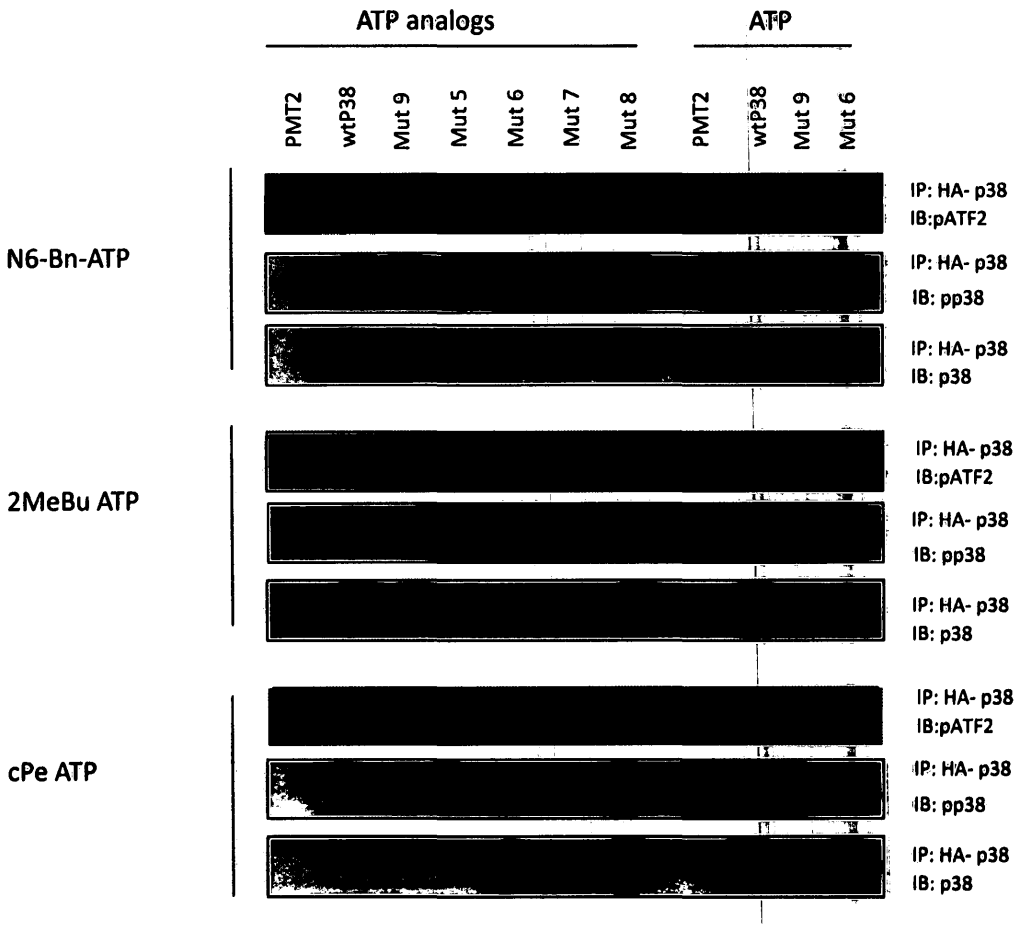

Figure 11: In vitro kinase assay with new p38 mutations and L87 mutation by itself and ATP analogs demonstrate that L87 mutation modifies analog binding to the ATP binding site. Western blot analysis of in vitro kinase assay of p38wt and mutations of p38 co-transfected with MKK6 in COS7 cells. PMT2 was used as control. The blots were probed with pATF2 to detect whether activated p38 mutations by MKK6 can phosphorylate known substrate in an assay with ATP analogs. Different analogs were tested. Blots were also probed with p38, to detect levels of total p38 in the lysate after immunopercipitation, and phsopho p38 used to detect levels of activated p38 by MKK6.
The p38 mutations are as follows: Mut5- L87A+T106G, Mut6- L87A +T106A, Mut 7- L87A+T106G+L167A, Mut8- L87A+T106A+L167A, Mut9- L87A.

Evolutionary sequence alignment of p38 MAPK:

Based on our results, we have done an evolutionary sequence alignment of p38 MAPK. We wanted to confirm whether L87 residue is conserved among the species. This is an indication that the residue is important if it is conserved through evolution. This alignment shows that p38 MAPK is conserved about 90% among the species, starting from drosophila to human. We can observe that L87 is conserved residue among the species. As well, the gatekeeper residue (T106) and L167 are conserved residues evolutionary. The TGY motif, which is the activation loop, is also conserved. This is an indication that all of those residues have an important role in p38 MAPK, and they haveremained conserved through evolution.

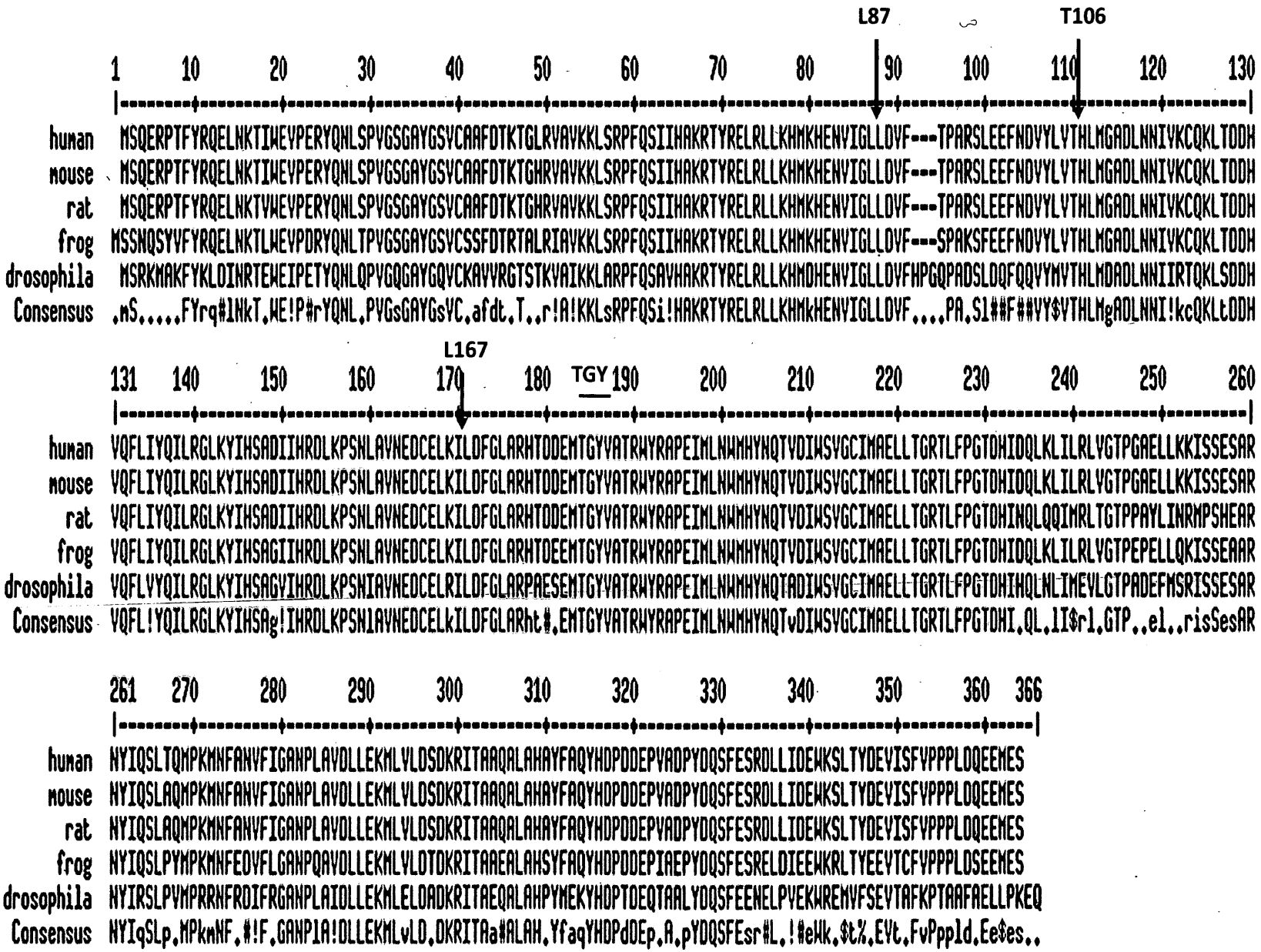
L87
T106
L167
TGY
human
mouse
rat
frog
drosophila
Consensus

Figure 12: Evolutionary sequence alignment of p38. Sequences were aligned using Multalin program. The gatekeeper residue at T106 and the conserved motif at L167 are conserved among the species. The TGY motif is also conserved among the species, this is p38 is phosphorylated by MKK6/3. L87 is also conserved among the species.

Sequence alignment of p38 MAPK and other kinases:

Once we have done the evolutionary sequence alignment, we have done also an alignment of other protein kinases, this is to confirm whether L87 residue is conserved among other kinases as well. The alignment results showed that this residue is not conserved among other kinases. This residue is only conserved between JNK and p38 MAPK, which makes sense due to the fact that those two MAPKs are in the same sub-group.

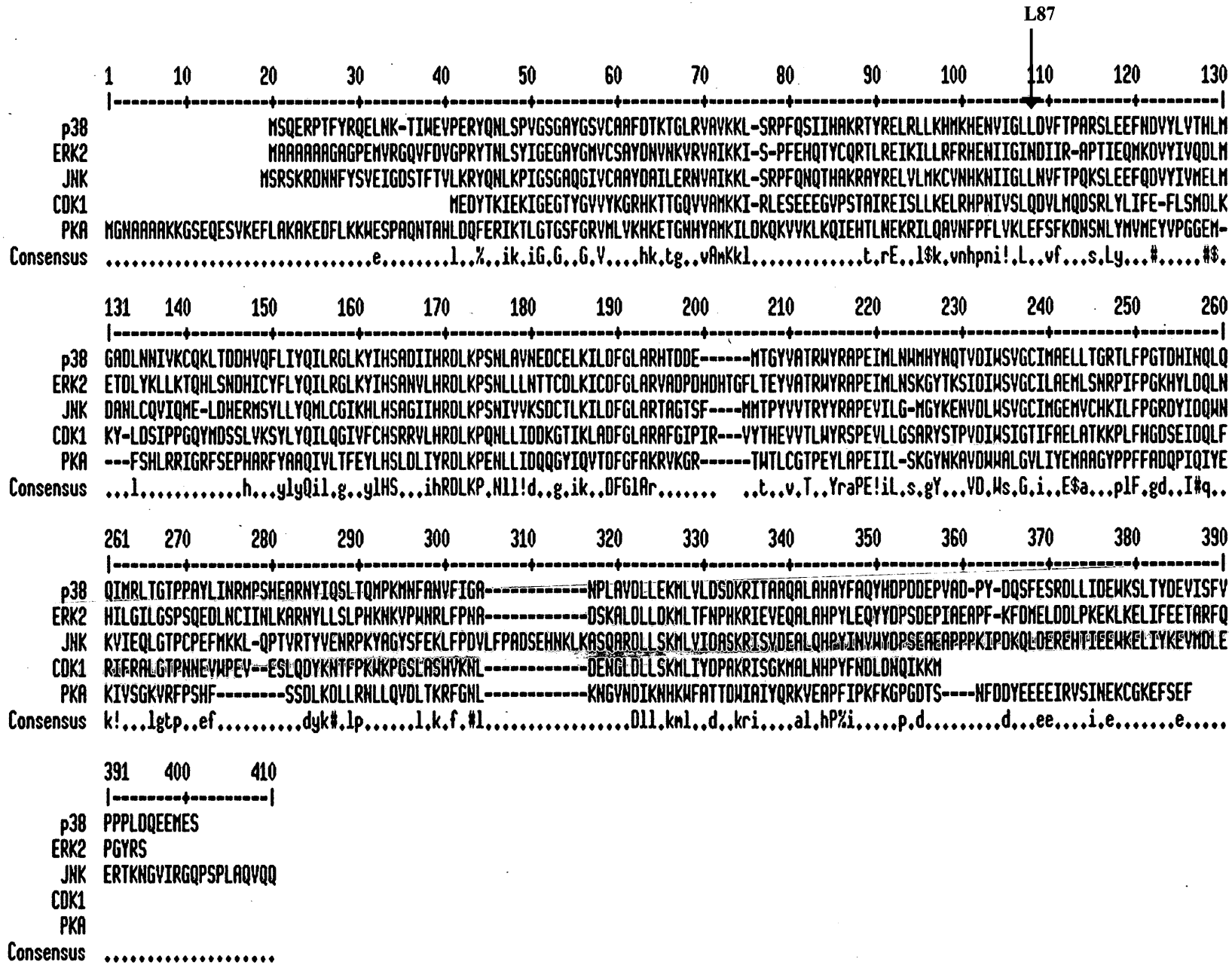
L87
1 10 20 30 40 50 60 70 80 90 100 110 120 130
p38 MSQERPTFYRQELNK-TIWEVPERYQNLSPVGSGAYGSVCAAFDTKTGLRVAVKKL-SRPFQSIIHAKRTYRELRLLKHMKHENVIGLLDVFTPARSLEEFNDVYLVTHLM
ERK2 MAAAAAAGAGPEMVRGQVFDVGPRYTNLSYIGEGAYGMVCSAYDNVNKVRVAIKKI-S-PFEHQTYCQRTLREIKILLRFRHENIIGINDIIR-APTIEQMKDVYIVQDLM
JNK MSRSKRDNNFYSVEIGDSTFTVLKRYQNLKPIGSGAQGIVCAAYDAILERNVAIKKL-SRPFQNQTHAKRAYRELVLMKCVNHKNIIGLLNVFTPQKSLEEFQDVYIVMELM
CDK1 MEDYTKIEKIGEGTYGVVYKGRHKTTGQVVAMKKI-RLESEEEGVPSTAIREISLLKELRHPNIVSLQDVLMQDSRLYLIFE-FLSMDLK
PKA MGNAAAAKKGSEQESVKEFLAKAKEDFLKKWESPAQNTAHLDQFERIKTLGTGSFGRVMLVKHKETGNHYAMKILDKQKVVKLKQIEHTLNEKRILQAVNFPFLVKLEFSFKDNSNLYMVMEYVPGGEM-
Consensus ....e........l..%..ik.iG.G..G.V....hk.tg..vAmKkl.............t.rE..l$k.vnhpni!.L..vf...s.Ly...#.....#$.
131 140 150 160 170 180 190 200 210 220 230 240 250 260
p38 GADLNNIVKCQKLTDDHVQFLIYQILRGLKYIHSADIIHRDLKPSNLAVNEDCELKILDFGLARHTDDE------MTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHINQLQ
ERK2 ETDLYKLLKTQHLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCDLKICDFGLARVADPDHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLN
JNK DANLCQVIQME-LDHERMSYLLYQMLCGIKHLHSAGIIHRDLKPSNIVVKSDCTLKILDFGLARTAGTSF----MMTPYVVTRYYRAPEVILG-MGYKENVDLWSVGCIMGEMVCHKILFPGRDYIDQWN
CDK1 KY-LDSIPPGQYMDSSLVKSYLYQILQGIVFCHSRRVLHRDLKPQNLLIDDKGTIKLADFGLARAFGIPIR---VYTHEVVTLWYRSPEVLLGSARYSTPVDIWSIGTIFAELATKKPLFHGDSEIDQLF
PKA ---FSHLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQGYIQVTDFGFAKRVKGR------TWTLCGTPEYLAPEIIL-SKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYE
Consensus ...l............h...ylyQil.g..ylHS...ihRDLKP.Nll!d..g.ik..DFGlAr..... ..t..v.T..YraPE!iL.s.gY...VD.Ws.G.i..E$a...plF.gd..I#q..
261 270 280 290 300 310 320 330 340 350 360 370 380 390
p38 QIMRLTGTPPAYLINRMPSHEARNYIQSLTQMPKMNFANVFIGA----------NPLAVDLLEKMLVLDSDKRITAAQALAHAYFAQYHDPDDEPVAD-PY-DQSFESRDLLIDEWKSLTYDEVISFV
ERK2 HILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA----------DSKALDLLDKMLTFNPHKRIEVEQALAHPYLEQYYDPSDEPIAEAPF-KFDMELDDLPKEKLKELIFEETARFQ
JNK KVIEQLGTPCPEFMKKL-QPTVRTYVENRPKYAGYSFEKLFPDVLFPADSEHNKLKASQARDLLSKMLVIDASKRISVDEALQHPYINVWYDPSEAEAPPPKIPDKQLDEREHTIEEWKELIYKEVMDLE
CDK1 RIFRALGTPNNEVWPEV--ESLQDYKNTFPKWKPGSLASHVKNL----------DENGLDLLSKMLIYDPAKRISGKMALNHPYFNDLDNQIKKM
PKA KIVSGKVRFPSHF--------SSDLKDLLRNLLQVDLTKRFGNL----------KNGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPGDTS----NFDDYEEEEIRVSINEKCGKEFSEF
Consensus k!...lgtp..ef.....dyk#.lp.....l.k.f.#l.....Dll.kml..d..kri....al.hP%i.....p.d.....d...ee....i.e.....e.....
391 400 410
p38 PPPLDQEEMES
ERK2 PGYRS
JNK ERTKNGVIRGQPSPLAQVQQ
CDK1
PKA
Consensus .....

Figure 13: Sequence alignment of p38 MAPK and other kinases. Sequences were aligned using Multalin program. The alignment was done to confirm whether L87 residue is conserved among other kinases as well. The alignment shows that this residue is conserved only among JNK and p38.

Chapter 5: Discussion:

Protein kinases play a central role in signal transduction pathways. There are many studies that reported that MAPK pathways are implicated in many pathological conditions, including cancer.

Recent studies indicate that p38α MAPK plays a role in regulation of inflammation pathways, which indicates that p38 MAPK activity is critical for normal immune and inflammatory responses. p38 MAPK also plays a role in cell cycle regulation and apoptosis, and can act as tumor suppressor. Furthermore, recent studies indicate that it also has a role in cardiovascular dysfunction. The most studied role of p38α MAPK is its central role in myogenesis and embryonic development. It seems that the p38 MAPK pathway and all the roles it plays in the cell can be used as potential targets for therapeutic intervention. Better understanding of p38 MAPK signalling cascades can help uncover important targets for drug design(76).

In this study, we have generated mutations of human p38α and engineered its ATP binding pocket to accept and bind synthesized ATP analogs, which will enable us to identify substrates. ATP analogs are synthesized and have an additional group on the N6 position, what makes them bulkier. ATP analogs cannot be accepted by wild type p38 MAPK, and can only be accepted by the engineered kinase. Due to those structural changes, the substrates that will be specific to only that kinase can accept ATP analogs. Firstly, we generated a mutation at the gatekeeper residue (Thr106) of p38 MAPK. Mutation of the Thr106 residue in p38 MAPK to an alanine or glycine should yield the space required to accommodate an ATP analog. Our results have shown that this mutation

did not affect p38 MAPK biological application and can still be phosphorylated and activated by its upstream kinase MKK6 (fig. 5). p38 MAPK is activated only after phosphorylation by MKK3 or MKK6. In our study, we have used MKK6 for phosphorylation and activation of p38 MAPK. Figure 4 show that we have total levels of p38 MAPK in the lysates. Co-transfection of wild type p38 or mutations of p38 with MKK6 has shown phosphorylated p38. With no MKK6 there is no phosphorylation of p38, when MKK6 co-transfected with p38 there is phosphorylation of p38 MAPK. This was an indication that the mutation we have generated did not affect p38 MAPK biological activity, and it can still be activated by MKK6. The next step was to engineer the kinase ATP binding pocket. We have generated additional mutations (additional to Thr106), at the conserved DFGmotif (L167) that is supposed to enhance gatekeeper residue binding activity. We have confirmed that the addition of this mutation (L167) did not affect the biological activity of p38 MAPK (fig. 5), we can observe that MKK6 is able to activate p38 MAPK with those mutations. Once this was confirmed, we wanted to confirm that the mutations introduced to p38 MAPK are able to accept ATP analogs. For this purpose we were using N6-Bn-ATP analog (this was based on previous studies that showed that this analog can be used for substrates identification). To screen the mutations of gatekeeper residue with the additional mutation to DFG motif within p38 to confirm they allow the use of the bulky ATP analog, we did a non-radioactive *in vitro* kinase assay, based on phosphorylation of known p38 MAPK substrate: ATF-2 (Fig. 6).

It is necessary to find a compatible ATP analog, which will bind to the generated mutation, but will not bind to the wild type p38 MAPK. For this purpose we have tested

additional ATP analogs: N6-(2Methylbutyl)-ATP, and N6-cyclopenthyl-ATP (all the analogs were purchased from Biolog Life Sciences), as well as N6-Benzyl-ATP. Those three analogs were chosen based on previous studies that were done with engineered kinases and substrate identification. For the identification of Rous sarcomavirus tyrosine kinase substrates, that was done by Shah et al., N6-Cyclopenthyl-ATP analog showed to have best catalytic efficiency. For identification of CDK1 and ERK2 N6-Bn-ATP was used, due its high catalytic efficiency (73,73). N6-2MeBu-ATP was also tested because it is a smaller analog. p38 ATP gatekeeper residue is relatively small amino acid (Thr), unlike other kinases that have bigger amino acid at the gatekeeper residue, like isoleucine in ERK2, or methionine in JNK, that is why smaller analog might have better catalytic efficiency. N6-2MeBu-ATP is the smallest synthesized analog among all the three that were tested. Our results of *in vitro* kinase assay in comparison with ATP analogs or ATP have demonstrated that our generated mutations did not phosphorylate ATF-2 efficiently, from this can be concluded that the ATP analogs did not bind well with the generated ATP pocket (Fig. 8). We observed that ATP is able to phosphorylate ATF-2 with higher catalytic activity than ATP analogues. We have concluded from that, that the mutations that were introduced to p38 MAPK might not have enough space in the pocket to accept ATP analogues.

To generate a p38 MAPK that might be more capable of utilizing ATP analogs, we introduced an additional mutation at L87, based on computational analysis of p38 MAPK structure. The computational analysis indicated that L87 residue is part of the ATP binding pocket, and changing this residue to a smaller amino acid (alanine) might

increase the size of the ATP binding pocket (fig. 9). To screen these mutations of gatekeeper residue with the additional mutation L87 within p38 MAPK to confirm they allow the use of the bulky ATP analog, we did an *in vitro* kinase assay, based on phosphorylation of known p38 MAPK substrate, ATF-2. Our results of *in vitro* kinase assay have shown that there is phosphorylation of ATF-2 by activated p38 MAPK with the additional mutation L87 and the gatekeeper residue mutation. It seems that the L87 mutation in combination with the gatekeeper residue mutation has resulted in structural changes to the ATP binding site. Specifically, figure 9 shows that L87A/T106G mutation is able to utilize ATP analogues and phosphorylate ATF-2. We have compared p38 MAPK mutations in kinase assays with ATP or ATP analogues. The fact that ATP is able to phosphorylate p38 MAPK mutations should not be a concern, since previous published studies have shown that generated kinase mutation is able to use ATP analog and ATP in similar manner (82). This result is important because it shows that we were successfully able to generate p38 MAPK mutation that can be used for substrate identification in a whole cell lysate. It seems that we have identified a residue that can increase ATP binding pocket size and allow ATP analogues to bind more efficiently, without losing its catalytic activity. This new discovery can help with the identification of new substrates of p38α.

Our results of *in vitro* kinase assay have shown that N6-2MeBu-ATP is the analog that can phosphorylate ATF-2, from this can be concluded that there is better binding to the generated p38 MAPK mutation of L87A+T106G; it has the best catalytic efficiency. This was the analog that was used by p38 MAPK mutation, but was not used by wild type

p38 MAPK. The other analogs were also been able to phosphorylate the generated mutations, but they were also used by the wild type p38 MAPK (fig. 10).

From all the six generated mutations, the one that worked the best was T106G+L87A. Those two mutations have generated an ATP binding pocket that can accept synthesized ATP analog, but cannot be used by wild type p38 MAPK. Based on our results, T106A+L87A can also accept ATP analogs to phosphorylate a known substrate, but not as efficiently as the mutation to the gatekeeper residue when T106 is exchanged to Gly. Our results also showed, based on *in vitro* kinase assay, that the additional mutation to the DFG motif (L167) that is supposed to enhance ATP binding site activity did not do that; it was not phosphorylating ATF-2 efficiently, which implies that this double mutation did not accept ATP analog (fig. 6). Surprisingly, all the three mutations (T106+ L167+L87) were not able to accept the ATP analog and phosphorylate ATF-2 either (fig. 10). This maybe because the structural changes lead to chemical changes, that will interfere with the ATP binding. Another reason might be because the L167 residue is located farther from the ATP site (fig 9A), hence it doesn't have much effect on the ATP binding pocket. From this, was concluded that T106G+L87A is the mutation that can accept ATP analog, without losing its biological function. This p38 MAPK mutation can be used further for substrate identification.

We have radioactively labelled N6-2MeBu-ADP with γP^{32}. The enzymatic labelling of N6-2MeBu-ADP with $\gamma P^{32}$was done with NDPK. Radioactive labelling allows us to track the direct substrates of the kinase in question. Our result of radiolabeled γP^{32} N6-2MeBu-ATP was not successful. Based on our results of *in vitro* kinase assay,

γP^{32} N6-2MeBu-ATP did not phosphorylate ATF-2 (data not shown). There is a possibility that NDPK was not catalytically active. If NDPK is not catalytically active, it is unable to transfer phosphor from γP^{32}ATP to analog ADP, what will result that we are unable to visualize p38 MAPK substrates on autoradiography. This is a possibility due to the fact that we have tested and seen that the analog and the mutations of p38 MAPK that were generated are working in a "cold" assay, and able to phosphorylate ATF-2. Labelling approach is good for visualising substrate phosphorylation, but it is hard to identify the phosphorylated proteins. The way to identify proteins is mass spectrometry.

The next step to do for substrate identification will be mass spectrometry. Mass spectrometry analysis is a very common technique for protein analysis and identification. This proteomics technique has a potential in identifying new signalling targets to give insight into the mechanism of cellular control (83). In this technique, the proteins in the complex are separated on SDS-PAGE gel and then stained either by coomassie or silver staining, the radiolabeled silver-stained band are excised. The excised proteins then go through proteolysis (usually with trypsin). Mass spectrometry is followed to analyse the peptides, by peptide separation on 2D-gel electrophoresis (83). Phosphorylation of cellular homogenate by γP^{32}(analog) ATP will lead to a mixture of highly phosphorylated proteins. The γ-thiol-phosphate of ATP serves as the kinase transferable tag, which allowsto purify proteins from tryptic digestion (73).This method relies on the unique ability of the mutated kinase to utilize and use the ATP analog, any nonspecific utilization of the ATP analog by other kinases in the cell will increase the false-positive substrate identification (79). To identify direct substrates of the mutated kinase, thiophosphorylated

substrate peptides captured on a solid-phase agarose, then released by oxidative hydrolysis, this will yield phosphorylated peptides (73). The newly phosphorylated proteins are isolated and identified by mass spectrometry. The next step of analysis is database searching and statistical use of software to interpret mass spectrometry data, which will give a list of proteins that were present in the initial sample (84, also reviewed by 85). The aquired data from mass spectrometry is analysed by Mascot sequence alignment algorithm (73).From this list of proteins, we can identify substrates that are already known and also those that are not yet known. Those substrates that are not known will need further molecular analysis to understand their role in the cell and the molecular mechanism.Once substrates identified, there is further investigation need to done to understand their role in the pathway of p38 MAPK and their characterization. As well, it is important to establish whether these "new" substrates are physiological substrates of p38 MAPK.

In conclusion, in our current study, despite the fact that we were not able to identify new substrates, we have identified a residue (L87) in ATP binding pocket, which will allow binding of synthesized ATP analogs. L87 residue is a conserved residue evolutionarily (Fig. 11). We have followed the four criteria that need to be met to engineer a kinase successfully; we have mutated p38 MAPK that is expressed in the cell context, we have engineered the p38 MAPK ATP binding site to accept ATP analogs, p38 MAPK was able to phosphorylate a known substrate ATF-2 with ATP analogs, and finally wild type p38 MAPK does not accept ATP analog: N6-2MeBu-ATP. We have

successfully mutated p38 MAPK to accept synthesized ATP analog. This generated mutation of p38 MAPK can be further used for substrate identification.

References:

1. Hanks, S. K., and Quinn, A. M. (1991). Protein Kinase Catalytic Domain Sequence Database: Identification of Conserved Features of Primary Structure and Classification of Family Members. Methods in Enzymology, 200: 38-62.

2. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The Protein Kinase Complement of the Human Genome. Science, 298: 1912-1934.

3. Kostich, M., English, J., Madison, V., Gheyas, F., Wang, L., Qiu, P., et al. (2002). Human members of the eukaryotic protein kinase family. Genome Biology, 3: 1-12.

4. Hanks, S. K., and Hunter, T. (1995). Protein Kinases 6: The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J, 9: 576-596.

5. Perdiguero, E., Ruiz-Bonilla, V., Gresh, L., Hui, L., Ballestar, E., Sousa-Victor, P., et al. (2007). Genetic analysis of p38 MAP kinases in myogenesis: fundamental role of p38alpha in abrogating myoblast proliferation. EMBO J, 26: 1245-1256.

6. Cossu, G., De Angelias, L., Borello, U., Berarducci, B., Buffa, V., Sonnino, C., et al. (2000). Determination, diversification and multipotency of mammalian myogenic cells. Int.J.Dev.Biol, 44, 699-706.

7. Shi, H., Scheffler, J. M., Zeng, C., Pleitner, J. M., Hannon, K. M., Grant, A. L., et al. (2009). Mitogen-activated protein kinase signaling is necessary for the maintenance of skeletal mass. Am J Physiol Cell, 296, C1040-C1048.

8. Fabrizio, F., Willems, D., Osada, S.-i., Ohno, S., Wetsel, W. C., Molinaro, M., et al. (1996). The Inhibition of Differentiation Caused by TGFb in Fetal Myoblasts Is Dependent upon Selective Expression of PKCu: A Possible Molecular Basis for Myoblast Diversification during Limb Histogenesis. Dev Biol, 180, 156-164.

9. Valdez, R. M., Richardson, J. A., Klein, W. H., and Olson, E. N. (2000). Failure of Myf5 to Support Myogenic Differentiation without Myogenin, MyoD, and MRF4. Dev Biol, 219, 287-298.

10. Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., et al. (1991). Functional Activity of Myogenic HLH Proteins Requires Hetero-Oligomerization with E12/E47-like Proteins In Vivo. Cell, 66, 305-315.

11. Lluis, F., Perdiguero, E., Nebreda, A. N., and Munoz-Canoves, P. (2006). Regulation of skeletal muscle gene expression by p38 MAP kinases. Trends in Cell Biol, 16, 36-44.

12. Bismuth, K., and Relaix, F. (2010). Genetic regulation of skeletal muscle development. Exp Cell Res, 316, 3081-3086.

13. Wang, H., Xu, Q., Xiao, F., Jiang, Y., and Wu, Z. (2008). Involvement of the p38 Mitogen-activated Protein Kinase alpha, beta and gamma isoforms in Myogenic Differentiation. Mol Biol Cell, 19, 1519-1528.

14. Keren, A., Tamir, Y., and Bengal, E. (2006). The p38 MAPK signaling pathway: A major regulator of skeletal muscle development. Mol Cell Endocrinol, 252, 224-230.

15. Perdiguero, E., Ruiz-Bonilla, V., Serrano, A. L., & Munoz-Canoves, P. (2007). Genetic Deficiency of p38 alpha Reveals its Critical Role in Myoblast Cell Cycle Exit. Cell Cycle, 6, 1298-1303.

16. Thronton, T. M., and Rincon, M. (2009). Non-Classical p38 Map Kinase Functions: Cell Cycle Checkpoina and Survival. Int.J.Biol.Sci, 5, 44-52.

17. Zarubin, T., and Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. Cell research, 1, 11-18.

18. Guan, Z., Buckman, S. Y., Pentland, A. P., Templeton, D. J., and Morrison, A. R. (1998). Induction of Cyclooxygenase-2 by the Activated MEKK1-->SEK1/MKK4 --> p38 Mitogen-activated Protein Kinase Pathway. J Biol Chem, 273, 12901–12908.

19. Choi, T. G., Lee, J., Ha, J., and Kim, S. S. (2011). Apoptosis signal-regulated kinase 1 is an intracellular inducer of p38 MAPK-mediated myogenic signalling in cardiac myoblasts. Biochim Biophys Acta , 1813, 1412-1421.

20. Johnson, C., Han, Y., Hughart, N., McCarra, J., Alpini, G., and Meng, F. (2012). Interlukin-6 and its receptor,key players in hepatobiliary inflammation and cancer. Transl Gastrointest Cancer , 1, 58-70.

21. Lu, H.-T., Yang, D. D., Wysk, M., Gatti, E., Melman, I., Davis, R. J., et al. (1999). Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. EMBO J, 18, 1845-1857.

22. Lee, J., Laydon, J., and McDonnell, P. (1994). Identification and characterization of a novel protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature , 372, 739-746.

23. Lee, J. C., Kumar, S., Griswold, D. E., Underwood, D. C., Votta, B. J., and Adams, J. L. (2000). Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology , 47, 185-201.

24. Porras, A., Zuluaga, S., Black, E., Valladares, A., Alvarez, A. M., Ambrosino, C., et al. (2004). p38 alpha Mitogen-activated Protein Kinase Sensitizes Cells to Apoptosis induced by Different Stimuli. Mol Biol Cell, 15, 922-933.

25. Huang, S., Jiang, Y., Li, Z., Nishida, E., Mathias, P., Lin, S., et al. (1997). Apoptosis Signaling Pathway in T Cells Is Composed of ICE/Ced-3 Family Proteases and MAP Kinase Kinase 6b. Immunity, 6, 739–749.

26. Sanchez-Prieto, R., Rojas, J. M., Taya, Y., and Gutkind, S. J. (2000). A Role for the p38 Mitogen-activated Protein Kinase Pathway in the Transcriptional Activation of p53 on Genotoxic Stress by Transcriptional Activation of p53 on Genotoxic Stress by Chemotherapeutic Agents. Cancer Res, 60, 2464–2472.

27. Wang, W., Chen, J. X., Liao, R., Deng, Q., Zhou, J. J., Huang, S., et al. (2002). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol Cell Biol, 22, 3389-3403.

28. Manke, I. A., Ancho, N., Lim, D., Stewart, M. Q., Elia, A. E., and Yaffe, M. B. (2005). MAPKAP Kinase-2 Is a Cell Cycle Checkpoint Kinase that Regulates the G2/M Transition and S Phase Progression in Response to UV Irradiation. Mol Cell, 17, 37-48.

29. Bulavin, D. V., and Fornace, A. J. (2004). p38 MAP Kinase's Emerging Role as a Tumor Suppressor. Adv Cancer Res, 92, 95-118.

30. Puri, P. L., Wu, Z., Zhang, P., Wood, L. D., Bhakta, K. S., Han, J., et al. (2000). Induction of terminal differentiation by constitutive activation of p38 MAP kinase in human rhabdomyosarcoma cells. Genes Dev, 14, 574-584.

31. Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., et al. (2001). Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature, 411, 102-107.

32. Greenberg, A. K., Basu, S., Hu, J., Yie, T.-a., Tchou-Wong, K. M., Rom, W. N., et al. (2002). Selective p38 Activation in Human Non–Small Cell Lung Cancer. Am. J. Respir. Cell Mol Biol, 26, 558–564.

33. Junttila, M. R., Li, S.-P., and Westermarck, J. (2008). Phosphate-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J, 22, 954-965.

34. Takenaka, K., Moriguchi, T., and Nishida, E. (1998). Activation of the Protein Kinase p38 in the Spindle Assembly Checkpoint and Mitotic Arrest. Science, 280, 599-602.

35. Wood, D. C., Thornton, T. M., Sabio, G., Davis, R. A., and Rincon, M. (2009). Nuclear Localization of p38 MAPK in response to DNA damage. Int J Biol Sci.,5, 428-437.

36. Wang, Y., Huang, S., Sah, V. P., Ross, J., Brown, J. H., Han, J., et al. (1998). Cardiac Muscle Cell Hypertrophy and Apoptosis Induced by Distinct Members of the p38 Mitogen-activated Protein Kinase Family. J Biol Chem, 273, 2161-2168.

37. Brancho, D., Tanaka, N., Laeschke, A., Ventura, J.-J., Kelkar, N., Tanaka, Y., et al. (2003). Mechanism of p38 MAP kinase activation in vivo. Genes Dev, 17, 1969-1978.

38. Lemke, L. E., Bloem, L. J., Fouts, R., Esterman, M., Sandusky, G., and Vlahos, C. J. (2001). Decreased p38 MAPK Activity in End-Stage Failing Human Myocardium: p38 MAPK alpha is the Predominant Isoform Expressed in Human Heart. J Mol Cell Cardiol, 33, 1527-1540.

39. Hoover, H. E., Thuerauf, D. J., Martindale, J. J., and Glembotski, C. C. (2000). aB-crystallin Gene Induction and Phosphorylation by MKK6-activated p38. J Biol Chem, 275, 23825-23833.

40. Proctor, B. M., Jin, X., Lupu, T. S., Muglia, L. J., Semenkovich, C. F., and Muslin, A. J. (2008). Requirement for p38 mitogen-activated protein kinase activity in neointima formation after vascular injury. Circulation, 118, 658-666.

41. Enslen, H., Raingeaud, J., and Dvis, R. J. (1998). Selective Activation of p38 Mitogen-activated Protein (MAP) Kinase Isoforms by the MAP Kinases MKK3 and MKK6. J Biol Chem, 273, 1741-1748.

42. Adams, R. H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Klein, R., et al. (2000). Essential Role of p38alpha MAP kinase in Placental Cardiovascular Developmentcental but Not Embry. Mol Cell, 6, 109-116.

43. Cuadrado, A., and Nebreda, A. R. (2010). Mechanism and functions of p38 MAPK signaling. Biochem J, 429, 403-417.

44. Pedram, A., Razandi, M., and Levin, E. R. (1998). Extracellular Signal-regulated Protein Kinase/Jun Kinase Cross-talk Underlies Vascular Endothelial Cell Growth Factor-induced Endothelial Cell Proliferation. J Biol Chem, 273, 26722–26728.

45. Li, S.-P., Junttila, M. R., Han, J., Kahari, V.-M., and Westermarck, J. (2003). p38 Mitogen-activated Protein Kinase Pathway Suppresses Cell Survival by Inducing Dephosphorylation of Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase1,2. Cancer Res, 63, 3473–3477.

46. Shen, Y. H., Godlewski, J., Zhu, J., Sathyanarayana, P., Leaner, V., Birrer, M. J., et al. (2003). Cross-talk between JNK/SAPK and ERK/MAPK Pathways: sustained activation of JNK blocks ERK activation by mitogenic factors. J Biol Chem , 278, 26715–26721.

47. Zhang, W., and Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res, 12, 9-18.

48. Ambrosino, C., and Nebreda, A. R. (2001). Cell cycle regulation by p38 MAP kinases. Biol Cell , 93, 47-51.

49. Molnar, A., Theodooras, A. M., Zon, L. I., and Kyriakis, J. M. (1997). Cdc42Hs, but Not Rac1, Inhibits Serum-stimulated Cell Cycle Progression at G1/S through a Mechanism Requiring p38/RK. J Biol Chem, 272, 13229-13235.

50. Askari, N., Beenstock, J., Livnah, O., and Engelberg, D. (2009). p38 is active in vitro and in vivo when monophosphorylated on Thr180. J Biol Chem, 283, 2497-2504.

51. Takekawa, M., Maeda, T., and Saito, H. (1998). Protein phosphatase 2Ca inhibits the human stress-responsive p38 and JNK MAPK pathways. EMBO J,17, 4744-4752.

52. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., et al. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem, 270, 7420-7426.

53. Tanoue, T., Adachi, M., and Nishida, E. (2000). A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat Cell Biol, 2, 110-116.

54. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., et al. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS letters, 364, 229-233.

55. Cuenda, A., and Rousseau, S. (2007). p38 MAP-kinase pathway regulation, function and role in human diseases. BBA , 1773, 1358-1375.

56. New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., et al. (1998). PRAK, a novel protein kinase regulated by the p38 MAP kinase. EMBO J ,17, 3372–3384.

57. Morton, S., Davis, R. J., and Cohen, P. (2004). Signalling pathways involved in multisite phosphorylation of the transcription factor ATF-2. FEBS J, 572, 177-183.

58. Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., Padova, F., et al. (1999). Regulation of the MEF2 Family of Transcription Factors by p38. Mol Cell Biol, 19, 21-30.

59. Cox, D. M., Du, M., Marback, M., Yang, E. C., Chan, J., Siu, M. K., et al. (2003). Phosporylation Motifs Regulating the Stability and Function of Myocyte Enhancer Factor 2A. J Biol Chem , 278, 15297-15303.

60. Wang, X. Z., and Ron, D. (1996). Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. Science, 272, 1347-1349.

61. Janknecht, R., and Hunter, T. (1997). Convergence of MAP kinase pathways on the ternary complex factor Sap-1a. EMBO J,16, 1620–1627.

62. Parker, C. G., Hunt, J., Diener, K., Mcginley, M., Soriano, B., Kesller, G. A., et al. (1998). Identification of Stathmin as a Novel Substrate for p38 Delta. Biochem Biophys Res Commun, 249, 791-796.

63. Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A., and Cohen, P. (1997). Phosphorylation of microtubule-associated protein tau by strees-activated kinases. FEBS Letters, 69, 57-62.

64. Perry, J. J., Harris, R. M., Moiani, D., Olson, A. J., and Tainer, J. A. (2009). p38alpha MAP Kinase C-Terminal Domain Binding Pocket Characterized by Crystallographic and Computational Analyses. J Mol Biol, 391, 1-11.

65. Jiang, Y., Gram, H., Zhao, M., Liguo, N., Gu, J., Feng, L., et al. (1996). Characterization of the Structure and Function of the Fourth Member of p38 Group Mitogen-activated Protein Kinases, p38d. J Biol Chem, 272, 30122–30128.

66. Wilson, K. P., Fitzgibbon, M. J., Caron, P. R., Griffith, J. P., Chen, W., McCaffrey, P. G., et al. (1996). Crystal Structure of p38 Mitogen-activated Protein Kinase. J Biol Chem, 271, 27696-27700.

67. Haar, E. T., Prabakhar, P., Liu, X., and Lerpe, C. (2007). Crystal Structure of the p38alpha MAPKAP kinase 2 Heterodimer. J Biol Chem, 282, 9733-9739.

68. Huang, D., Zhou, T., Lafleur, K., Nevado, C., and Caflisch, A. (2010). Kinase selectivity potential for inhibitors targeting the ATP binding site: a netwrork analysis. Bioinformatics, 26, 198-204.

69. Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997). Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. PNAS, 94, 3565-3570.

70. Kumar, V. N., Eblen, S. T., and Weber, M. J. (2004). Identifying specific kinase susbstrates through engineered kinases and ATP analogs. Methods, 32, 389-397.

71. Schauble, S., King, C. C., Darshi, M., Koller, A., Shah, K., and Taylor, S. S. (2007). Identification of ChChd3 as a Novel Substrate of the cAMP-dependent Protein Kinase (PKA) Using an Analog-sensetive Catalytic Subunit. J Biol Chem, 282, 14952-14959.

72. Knight, J. D., Tian, R., Lee, R. E., Wang, F., Beauvais, A., Zou, H., et al. (2012). A novel whole-cell lysate kinase assay identifies substrates of the p38 MAPK in differentiating myoblasts. Skelet Muscle, 2, 1-12.

73. Blethrow, J. D., Glavy, J. S., Morgan, D. O., and Shokat, K. M. (2008). Covalent capture of kinase-specific phosphopeptides reveals Cdk1- cyclin B substrates. PNAS , 105, 1442-1447.

74. Eblen, S. T., Kumar, V. N., Shah, K., Henderson, M. J., Watts, C. K., Shokat, K. M., et al. (2003). Identification of Novel ERK2 Substrates through Use of an Engineered Kinase and ATP analogs. J Biol Chem, 278, 14926-14935.

75. Habelhah, H., Shah, K., Huang, L., Burlingame, A. I., Shokat, K. M., and Ronai, Z. (2001). Identification of New JNK Substrate Using ATP Pocket Mutant JNK and a Corresponding ATP analogs. J Biol Chem, 276, 18090-18095.

76. Koch, A., and Hauf, S. (2010). Strategies for the identification of kinase substrates using analog-sensetive kinases. Euro J Cell Biol, 89, 184-193.

77. Liu, Y., Shah, K., Yang, F., Witucki, L., and Shokat, K. M. (1998). Enginnering Src family protein kinases with unnatural nucleotide specificity. Chemistry and Biology , 5, 91-101.

78. Knight, Z. A., and Shokat, K. M. (2007). Chemical Genetics: Where Genetics and Pharmacology Meet. Cell , 128, 425-430.

79. Carlson, S. M., Chouinard, C. R., Labadorf, A., Lam, C. J., Schmelzle, K., Fraenkel, E., et al. (2011). Large-Scale Discovery of ERK2 Substartes Identifies ERK-Mediated Transcriptional Regulation by ETV3. Sci Signal, 4, 1-10.

80. Amutha, B., and Pain, D. (2003). Nucleoside diphosphate kinase of Saccharomyces cerevisiae, Ynk1p: localiztion to the mitochondrial intermembrane space. Biochem J, 370, 805-815.

81. Lascu, I., and Gonin, P. (2000). The Catalytic Mechanism of Nucleoside Diphosphate Kinases. J Bioenerg Biomembr, 32, 237-245.

82. Ulrich, S. M., Kenski, D. M., and Shokat, K. M. (2003). Engineering a "methionine clamp" into Src family kinases enhances specificity toward unnatural ATP analogues. Biochemistry, 42, 7915-7921.

83. Lewis, T. S., Hunt, J. B., Aveline, L. D., Jonscher, K. R., Louie, D. F., Yeh, J. M., et al. (2000). Identification of Novel MAP Kinase Pathway Signalling Targets by Functional Proteomics and Mass Spectrometry. Mol Cell , 6, 1343-1354.

84. Xue, L., Wang, W.-H., Iliuk, A., Hu, L., Galan, J. A., Yu, S., et al. (2012). Sensetive kinase assay linked with phosphoproteomics for identifying direct kinase substrates. PNAS , 109, 5615-5620.

85. Gingras, A.-C., Gstaiger, M., Raught, B., and Aebersold, R. (2007). Analysis of protein complexes using mass spectrometr. Nature Reviews Mol Cell Biol, 8, 645-654.

Appendix:

Molecular Techniques

Cell Culture:

Reagents:

1X Dulbecco's PBS (1L, pH 7.4: NaCl 8gr, KCL 0.2 gr Na_2HPO_4 $7H_20$ 1.44 gr, KH_2PO_4 0.24 gr)

Versine (0.2 gr EDTA in 1L 1X PBS)

Trypsin (0.125 Trypsin EDTA (Gibco) diluted in Versine)

Growth Medium (GM): DMEM supplemted with 1% Penicillin-streptomycin and 1% L-Glutamine and 10% Fetal Serum Albumin (FBS)

Freezing Medium: Growth medium supplemented with 10% DMSO

Methods:

Thawing cells:

1. Remove the vial of frozen cells from -80°C and thaw in 37°C water bath
2. Mix 1ml of the cells with 5ml of growth medium in 15ml falcon tube
3. Centrifuge for 5min at 1500 rpm to pellet the cells
4. Aspirate the supernatant from the falcon tube
5. Add 10 ml growth medium to the cells, pipette up and down for proper mixing and plate the cells in 10cm plate

Passing cells:

1. Aspirate the media from 10cm plate
2. Wash cells in 1X PBS

3. Add 1ml 0.125 % Trypsin incubate for 1 min in 37°C
4. Flick the plate to detach the cells
5. Add cells to a new culture plate in a ratio of 1:6 containing growth medium
6. Incubate cells at 37°C and 5% CO_2 overnight

Freezing cells:

1. Follow steps 1-4 for passing cells
2. Pellet the cells in a falcon tube in 5ml growth medium with centrifugation at 1500 rpm for 5 min
3. Aspirate the media and add freezing media to the falcon tube, pipette up and down for better suspension
4. Aliquot 1ml of cell in freezing vials
5. Store the cells in -80°C until further use

Transient transfection of mammalian cells with DNA:

Reagents:

2X HEBS (pH 7.15: 280mM NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes)

Filter, sterilize and store in -20°C

2.5 M $CaCl_2$ (36.75gr $CaCl_2$ in 100ml ddH_2O), filter, sterilize and store in -20°C

Method:

1. Seed cells 24 hours prior to transfection at 60-70% confluency
2. Re- feed cells with growth medium three hours before transfection
3. Add 0.5ml 2X Hepes into sterile culture tubes

4. To prepare DNA- $CaCl_2$ mixture, add ddH_2O to 25μg DNA to reach 450 μl, and add 50μl $CaCl_2$, mix gently
5. Add DNA- $CaCl_2$ drop wise into 2X Hepes tube on the vortex with low speed
6. Add the final mixture to the cell culture drop wise
7. After 16-18 hours wash cells twice with 1X PBS and add growth medium
8. Check dsRED signal after 24 hours and then harvest cells

Harvesting cells:

Reagents:

1X Dulbecco's PBS (1L, pH 7.4: NaCl 8gr, KCL 0.2 gr, Na_2HPO_4 $7H_20$ 1.44 gr, KH_2PO_4 0.24 gr)

1x Cell lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1Mm EGTA, 1%Triton, 2.5 mM sodium pyrophosphate, 1mM β- glycerophosphate, 1mM Na_3VO_4, 1μg/ml Leupeptin)

Method:

1. Remove media and add cold 1x PBS. Wash twice with 1xPBS
2. Remove PBS and add 0.5ml 1x Cell Lysis buffer plus 1mM phenylmethylsulfonyl (PMSF) and 1mM Na_3Vo_4 to each 10cm plate, and incubate on ice for 5 min
3. Scrape cells off the plate and transfer to eppendorf tube
4. Sonicate lysates on ice 4 times for 5 seconds each
5. Microcentrifuge for 10 min at 4°C at 16000xG, and transfer the supernatant to a new tube. Store in -80°C

Immunoprecipitation and *in vitro* kinase assay:

Reagents:

Anti HA affinity matrix

1x Cell lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1Mm EGTA, 1%Triton, 2.5 mM sodium pyrophosphate, 1mM β- glycerophosphate, 1mM Na_3VO_4, 1ug/ml Leupeptin)

1x Kinase buffer (25mM Tris pH 7.5, 5Mm β- glycerophosphate, 2mM DTT, 0.1 mM Na_3VO_4, 10mM $MgCl_2$)

ATF-2 Fusion protein

10 mM ATP/ 10mM ATP analog

Methods:

1. Add 20µl of Anti Affinity HA beads to 200µl of cell lysate
2. Incubate overnight with gentle rocking at 4°C
3. Microcentrifuge cell lysate at 16,000xG for 1 min at 4°C
4. Wash pellet twice with 0.5 ml 1x Cell lysis buffer
5. Aspirate buffer from the tube and microcentrifuge at 16000xG for 1 min at 4°C
6. Wash pellet twice with 0.5 ml 1x Kinase buffer
7. Aspirate buffer from the tube and microcentrifuge at 16000xG for 1 min at 4°C
8. Re-suspend pellet in 40µl 1x Kinase buffer, and add it to the kinase reaction 250µM ATP/ATP analog and 1mg of ATF-2 fusion protein
9. Incubate for 30 min at 30°C
10. Terminate reaction by adding 13.3µl 4x SDS Sample Buffer and vortex

Western Blotting:

Reagents:

1.5M Tris-HCL, pH 8.8

1M Tris-HCL, pH6.8

10% Ammonium Persulphate (APS)

Temed

TBST (10X TBS: 80gr NaCl, 24.2 gr Tris, pH 7.6) - 1X TBS + 0.5% Tween-20

Transfer Buffer (For 1L: 3gr Tris, 14.4 gr Glycine, 20% Methanol)

Methods:

1. Prepare resolving and stocking solutions, cast the gel according to western blotting techniques.
2. Load 15µl per well and load 5µl marker on 10% SDS-PAGE gel
3. Run the gel at 100V for approximately 1.5 hours
4. Transfer the proteins to Immobilin-FL membranes (Millipore) for 1 hour at 100V
5. After transfer wash the membranes with PBS or TBST buffer at room temperature
6. Block membranes for 1 hour at room temperature in 5%milk in PBS or TBST buffer with gentle rocking
7. Dilute primary antibodies in the appropriate buffer and incubate over-night at 4°C
8. Wash membranes 3 times for 5 min in PBS or TBST
9. Incubate membrane with secondary antibody (1:3000) for 2 hours with gentle rocking at room temperature
10. Wash membranes 3 times for 10 min in PBS or TBST

11. Add Enhanced Chemiluminescence (ECL) to each membrane, expose to a film to detect proteins

6XHN- NDPK protein purification:

Reagents:

Terrific Broth (for 1L: in 900 ml of ddH_2O -tryptone 12gr, yeast extract 24 gr, glycerol 4ml. Add 100 ml of sterile solutions of 0.17M KH_2PO_4, 0.72M K_2HPO_4)

Carbencillian 100μg/ml

1mM IPTG (2.383 gr in 10 ml ddH_2O)

1M Imidazole (34.04 gr on 0.5L ddH_2O)

Binding buffer (50 mM Tris pH 7.5, 500Mm NaCl, 5mM Imidazole and 1:100 protease inhibitors)

Wash Buffer (50 mM Tris pH 7.5, 500mM NaCl, 30mM Imidazole)

Elution Buffer (50mM Tris pH 7.5, 500mM NaCl, 250mM Imidazole)

HKG buffer (20mM Hepes pH 7.4, 250mM KCL, 20% (v/v) glycerol)

Ni-Nta agarose beads (purchased from Qiagen, cat. # 30210)

Methods:

1. 6x HN -NDPK was grown overnight with shaking at 37°C in 100 ml Terrific broth (TB) culture with 100μg/ml carbencillian
2. 100ml of TB was added to 1L of fresh TB culture. When the OD_{600} reading reaches 1, the culture is stimulated with 1mM IPTG
3. The culture was incubated overnight at 16°C with shaking

4. Cells were pelleted by centrifugation at 5000rpm for 10 min and resuspended in 60 ml of binding buffer
5. Cells were lysed by sonication at 30% amplitude, 2sec on 3sec off, for a total of 6 min.
6. Insolubles were pelleted by centrifugation at 1800rpm for 30 min at 4°C , and the supernatant was bound to 5ml Ni-Nta beads at 4°C for 20 min with gentle rocking
7. The binding supernatant was flown through the column 2-3 times
8. Ni-Nta beads were washed with 10x column volumes with wash buffer
9. 6XHN-NDPK was then eluted from the column in 5ml fractions with elution buffer
10. Samples with the eluted fraction were separated by 15% SDS-PAGE and coomasie stained to assess protein levels and purity
11. The fractions with the highest NDPK levels were dialyzed against three changes of HKG buffer. Fractions were stored at -80°C

ATP analog labelling:

Reagents:

Ni-Nta agarose beads

1X Dulbecco's PBS (1L, pH 7.4: NaCl 8gr, KCL 0.2 gr, Na_2HPO_4 $7H_20$ 1.44 gr, KH_2PO_4 0.24 gr)

Hepes buffered saline (HBS): (150mM NaCl, 100mM Hepes pH 7.4)

1mci of ɣP32 ATP purchased from Perkin Elmer (6000Ci/mmole 150mCi/ml)

10mM ADP analog purchased from BioLog Life Sciences

5mM $MgCl_2$ (50.825gr in 0.25L ddH_2O)

Gamma P^{32}-ATP (purchased from Perkin Elmer, cat. # NEG035C001MC)

Methods:

The following was adapted from Current Protocols in Molecular Biology 18.11.8.

1. To a Bio- disposable column, add 200µl of 1:1 slurry Ni-Nta beads
2. Add 1ml of HBS and allow the buffer to drain until level with the top of the beads
3. Add 165µg purified 6xHN-NDPK. Bind NDPK to the beads for 20 min by gentle rocking at 4°C
4. Wash the column with 1X PBS buffer
5. 1µci of γP^{32} with 200µM cold ATP (as a carrier) was added to the reaction diluted in 1x PBS
6. Wash 5 times with 1X PBS
7. 80nmole of N6-ADP analog was added to the column and diluted in 1X PBS + 5mM $MgCl_2$
8. Elute with 250µl of 1xPBS containing 5mM $MgCl_2$. Store aliquots in -80°C

Mutagenesis:

Reagents:

Primers ordered from Sigma Aldrich

PFU ultra DNA polymerase enzyme (Agilent, cat. # 600380)

10mM dNTP's purchase Cell Signaling Techologies

10x Buffer purchased from Stratagine

Mineral Oil

DpnI purchased from New England BioLabs, cat. # RO176S

Methods:

The set-up of the reaction was done based on Quickchange II Site directed mutagenesis kit (Stratagene)

1. Prepare mixture of primers, DNA template, 10x buffer, dNTP's, ddH_2O and PFU ultra enzyme for a total volume of the reaction 50µl as per kit instructions
2. Add 40µl of mineral oil to the top of the sample
3. Amplify samples using PCR
4. PCR-amplified samples were then aliquoted without the mineral oil. 1µl of DpnI was added
5. Incubate at 37°C for 1 hour
6. DNA samples were then transformed in 50µl of BL21 E.Coli bacteria
7. Incubate for 30 min on ice
8. Heat shock samples for 1 min at 42°C, place the reaction on ice for 2 min to recover
9. Add 200µl of LB +0.1% glucose per sample, and incubate for 30 min at 37°C
10. Spread 50µl on agar plate and grow plate overnight at 37°C

Primers used for mutagenesis:

Forward T106A: 5'- GATGTGTATCTGGTGGCCCATCTCATGGGGG- 3'

Antisense T106A: 5'-CCCCCATGAGATGAGATGGGCCACCAGATACACATC-3'

Forward T106G: 5'- ATGATGTGTATCTGGTGGGCCATCTCATGGGGGCAG-3'

Antisense T106G: 5'-CTGCCCCCATGAGATGGCCCACCAGATACACATCAT-3'

Forward L167A:

5'-TGAAGACTGTGAGCTGGAAGATTGCGGATTTTGGACTGGCT-3'

AntisenseL167A:

5'-AGCCAGTCCAAATCCGCAATCTTCAGCTCACAGTCTTCA-3'

Forward L87A:

5'-CATGAAATGTGATTGGTCTGGCGGACGTTTTTACACCTGCAA-3'

Antisense L87A:

5'-CTTGCAAGGTGTAAAAACGTCCGCCAGACCAATCACATTTTCATG-3'

p38 primers used:

Forward 1: 5'- ATGTCTCAGGAGAGGCCCA-3'

Forward 3: 5'- GGATTTTGGACTGGCTCG-3'

Forward 5: 5'- TAGATGAGTGGAAAAAGCC-3'

Supplementary data:

Purification of NDPK:

For the procedure of labelling ATP analogs, purification of nucleoside diphosphate kinase (NDPK) protein was needed. NDPK is an enzyme that catalyzes the exchange of phosphate groups, it will catalyze the phosphorylation reaction of ADP→ATP. The enzyme phosphorylates itself on a conserved histidine residue at the catalytic site, and then it transfers the phosphate (80). ATP or GTP is used as a donor, while ADP or GDP is a good substrate for pyruvate kinase. The reaction is active as a complex with Mg^{+2}. The nucleoside diphosphate binds to the free enzyme, while

nucleoside triphosphate binds to the phosphorylated enzyme. ATP is generated if NDPK is incubated with phosphoramidate and ADP (81).

For the purpose of our study NDPK was used to label gamma P^{32}ADP analog and transform it into labelled ATP. Labelled ATP will transfer the gamma phosphor into the substrate once the substrate is phosphorylated by p38.

NDPK was purified from bacteria through an Ni-Nta column and was stained by commasie blue to assess protein levels and purity. Those fractions that had the highest NDPK levels were dialyzed. This was used for ATP labelling.

NDPK was used to label ADP analog with γP^{32} to γP^{32} ATP analog, and that was used for "hot" *in vitro* kinase assay as the phosphate source for substrate phosphorylation with mutations of p38.

NDPK was also identified by mass spectrometry after it was stained, to make sure we have the right protein product (this is due to the important purpose NDPK serves).

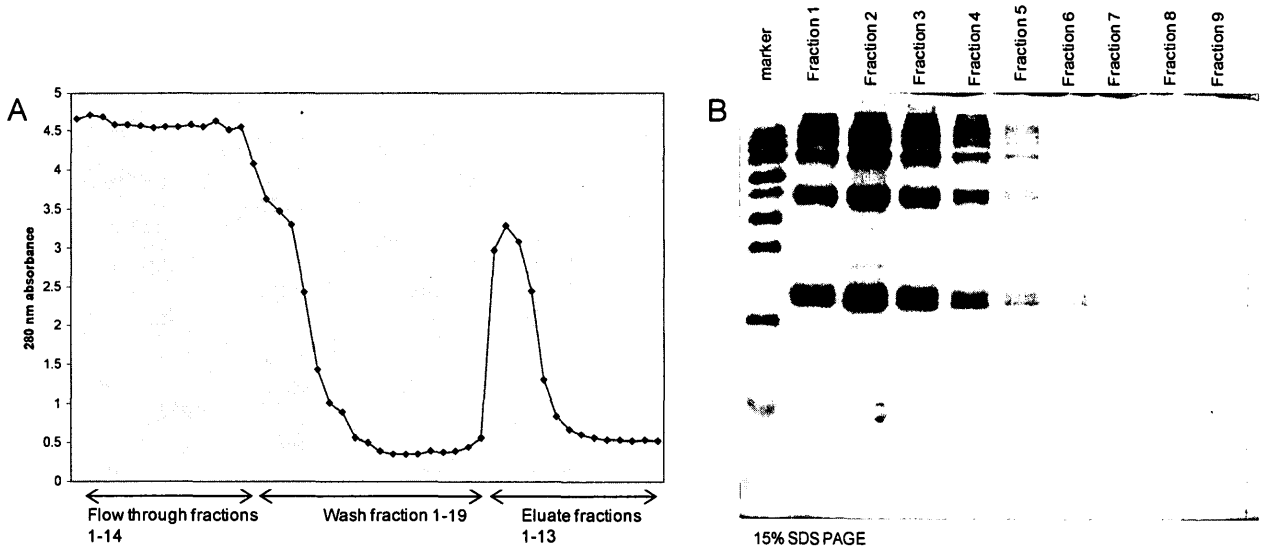

Figure14:6XHN-NDPK purification on Ni-Nta column. Fractions were eluted from the column. (A) All fractions werecollected and their OD was measured to asses that fractions were washed and the elution was with NDPK protein. (B) Eluate samples were separated by 15% SDS- page and commasie stained to

assesprotein levels and purity. Thosefractions that were the highest NDPK levels were dialyzed and used for ATP labeling. In our case fraction 2 had thehighest protein levels, and that was used for ATP labeling.

```
  1 MSSQTERTFI AVKPDGVQRG LVSQILSRFE KKGYKLVAIK LVKADDKLLE
 51 QHYAEHVGKP FFPKMVSFMK SGPILATVWE GKDVVRQGRT ILGATNPLGS
101 APGTIRGDFG IDLGRNVCHG SDSVDSAERE INLWFKKEEL VDWESNQAKW
151 IYE
```

Figure 15: Identification of NDPK. Tandem mass spectrometry of excised spot from figure 12, fraction 2 was sent for identification since it showed to be the highest level of protein purity. The sequence above shows that it was identified as Ynk1p, which is Nucleoside diphosphate kinase of *Saccharomyces cerevisiae*. Matched peptides shown in bold red.