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Therapeutic Potential of Tailored Arrestins
A Peptide Mini-Scaffold Facilitates the Activation of JNK1 and JNK2 in vitro

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AFFIDAVIT

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ABSTRACT

Cellular communication is a fundamental property of every organism, allowing cells to respond to environmental and developmental changes in an appropriate way. Cells in eukaryotic organisms are constantly sending and receiving signals of different kinds such as hormones, growth factors or neurotransmitters that control cellular behavior through various pathways. This typically includes the phosphorylation and dephosphorylation of proteins as mediators of signaling. Deregulation of signaling pathways can have serious implications for cellular behavior, putting the whole organism at risk. Apoptosis, the programmed cell death, has become of special interest for signal transduction research because of its implications in the pathology of several diseases. A key player in apoptotic signaling is the highly conserved mitogen-activated protein kinase (MAPK), JNK. One of the regulators in the MAPK pathway are scaffold proteins (in the case of JNK, arrestin-3), which tether the pathway components therefore bringing them into closer proximity, promoting the activation of JNK.

My thesis aims to better describe the protein-protein interaction sites and binding affinities of the regulating scaffold protein arrestin-3, with pathway components and to reveal if a truncated version of arrestin-3 can facilitate the activation of JNK1/2. Therefore I applied *in vitro* binding assays to assess the binding affinity of peptides derived from arrestin-3 with the kinases. Furthermore, *in vitro* kinase activation assays using purified proteins were performed to assess the level of phosphorylation under varying peptide concentrations.

My results show that the first 25 amino acid residues of arrestin-3 are the critical binding element involved in the direct interaction with JNK1 and JNK2 and that they can also facilitate the activation of JNK1 and JNK2. My work gives a better understanding of arrestins role in signaling pathways and will hopefully contribute to the ultimate goal of custom designed arrestins to influence JNK signaling and therefore cellular fate.

ZUSAMMENFASSUNG

Zelluläre Kommunikation ist eine grundlegende Eigenschaft jedes Organismus, wodurch Zellen auf Veränderungen in deren Umwelt sowie deren Entwicklung angemessen reagieren können. Zellen in eukaryontischen Organismen senden und erhalten konstant unterschiedliche Signale wie zum Beispiel Hormone, Wachstumsfaktoren oder Neurotransmitter, welche das zelluläre Verhalten durch zugrundeliegenden Pfadwegen steuern. Dies beinhaltet typischerweise posttranslationale Proteinmodifikationen wie die Phosphorylierung und Dephosphorylierung als Mediatoren der Signalweiterleitung. Die Deregulierung von Pfadwegen kann ernste Implikationen für das zelluläre Verhalten haben, was ein Risiko für den gesamten Organismus darstellt. Die Apoptose, der programmierte Zelltod ist von besonderem Interesse aufgrund deren Rolle in zahlreichen pathologischen Zuständen. In der apoptotischen Signaltransduktion spielt die hoch konservierte mitogen-aktivierte Proteinkinase (MAPK), JNK eine wichtige Rolle. Ein Regulator in diesem Pfadweg ist das Gerüstprotein Arrestin-3, welches Komponenten des Pfadweges näher zusammenbringt und dadurch die Aktivierung von JNK fördert.

Das Ziel meiner Arbeit ist es Protein-Protein Interaktionsstellen und Bindeaffinitäten von Arrestin-3 mit Komponenten des JNK Pfadweges zu analysieren, sowie zu überprüfen ob die identifizierten Interaktionsstellen die Aktivierung von JNK1/2 fördern können. Für die Bestimmung der Bindeaffinitäten wurden *in vitro* Bindeexperimente mit aufgereinigten Proteinen durchgeführt sowie *in vitro* Aktivierungsexperimente um die Phosphorylierungslevels von JNK1/2 unter variierenden Peptidkonzentrationen zu bestimmen.

Meine Resultate zeigen, dass die ersten 25 Aminosäuren von Arrestin-3 kritisch für die Bindung mit JNK1 und JNK2 sind und sowohl auch die Aktivierung beider Kinasen als Minigerüstpeptid fördern. Meine Arbeit gibt ein besseres Verständnis über die Funktion von Arrestinen in der Signaltransduktion und trägt hoffentlich zum Ziel von maßgeschneiderten Arrestinen bei um dadurch den JNK Signalweg sowie das zelluläre Schicksal therapeutisch beeinflussen zu können.

CONTENTS

1. Introduction

1.1. Cellular Signaling

No cell lives in isolation; cellular communication is a fundamental property of all cells and shapes the function and abilities of every living organism (Berg, Tymoczko, & Stryer, 2002), (Lodish, 2008) . Throughout evolution, cells have become highly responsive towards environmental changes and capable to sense and respond to a large range of signals in a suitable way (Berg et al., 2002). Even the simplest life forms such as single-celled organisms established methods to communicate with each other or other organisms. For example, sexual mating in several eukaryotic microorganisms is promoted by the secretion of pheromones (O'Day & Horgen, 1981), (Lodish, 2008).

In animals and plants, extracellular signaling molecules unfold their functions within the organism, thereby controlling sugar, fat and amino acid metabolism, cell proliferation, apoptosis, differentiation as well as the synthesis and secretion of proteins. This fundamental property underlies a complex mechanism called cellular signaling, where environmental signals are communicated to internal responses through various pathways (Krauss, 2014).

1.1.1. Intercellular Communication

Cellular communications can take place on basis of (1) extracellular molecules: a cell releases molecules triggering a response in other cells. (2) Electric signals: nerve impulses can be conducted through chemical and electric synapses. In the latter, changes in membrane potential are a means of communication. (3) Gap junctions: the cytoplasms of two cells are in direct exchange through a channel. (4) Cell-cell interaction: cells interact via proteins embedded in their membrane (Krauss, 2014).

Intercellular communication usually starts with the synthesis of a signaling molecule as seen in **Figure 1**.

Synthesis: Most cells produce signals, but there are specialized cells that are dedicated to the synthesis of certain messenger molecules such as hormones. For example, β-cells produce the hormone insulin in the pancreas. However, the ability of cells to produce and release signaling molecules is regulated and only takes place in response to various signals (Krauss, 2014), (Berg et al., 2002).

Transport: Once the signal has been synthesized it has to reach its target cells. This can take place by diffusion or transportation via the circulatory system. Intercellular hormone signaling can be divided into three types of signaling (1) endocrine, (2) paracrine, and (3) autocrine. These different types of signaling describe the range of distances the signal travels to reach its target cell (Lodish, 2008), (Gomperts, Tatham, & Kramer, 2009).

(1) Signals in the endocrine system are excreted from cells at remote sites from their target cells. They reach their targets through the circulatory system (blood stream or lymphatic system in humans). Hormones such as insulin generally belong to the endocrine system (Lodish, 2008), (Krauss, 2014).

(2) In the paracrine system the messenger molecules, also referred to as local mediators, travel a shorter distance than in the endocrine signaling. The molecules are released in local distance to their targets and reach them through passive diffusion. An example for this type of signaling is the synaptic neurotransmission. Neurotransmitters released at the synaptic cleft transduce the signal to muscle or nerve cells. Beside neurotransmitters, many growth factors are responsible for the developmental regulation over short distances (Lodish, 2008), (Krauss, 2014).

(3) Autocrine signaling describes the signaling where receiving and releasing cells are of the same type. Some growth factors work in this way (Lodish, 2008).

Signaling: Intercellular signaling begins with the reception of a signal known as first messengers, which leads to the activation of specific intracellular

signaling cascades. This in turn leads to the transduction of the signal downstream to the level of DNA transcription or cytosolic proteins. Intracellular signaling pathways control all functions of the cell such as intermediary metabolism, cell division activity, morphology and the transcription program (Krauss, 2014).

The proteins that are responsible for the reception of the signal are referred to as receptors. Receptor activation due to a first messenger leads to its conformational change through which the signal can get relayed further downstream. Signals can be received on the extracellular site by cell-surface receptors or internally by receptors localized in the cytoplasm (nuclear receptors (NRs)). For the latter, the signal has to cross the plasma membrane to lead to their activation (Krauss, 2014). Non-polar signaling molecules such as steroid hormones or retinoids are able to diffuse through the plasma membrane and once inside the cell, bind intracellular receptors. The binding leads to direct interaction with DNA; hence nuclear receptors are classified as transcription factors, altering gene expression. (Berg et al., 2002), (Lodish, 2008), (Krauss, 2014). However, the majority of extracellular signaling molecules are too hydrophilic and too large in size to cross the membrane. In order to transmit the extracellular signal to the interior of the cell, cell surface receptors are required.

The regulation of cellular signaling is of utmost importance. Deregulation of signaling pathways can have serious implications for the development and health of the organism, putting it at risk. Almost all known diseases exhibit dysfunctional aspects in cellular signaling (Nature Biotechnology, 2000). The following mechanisms contribute to regulation in signaling networks: (i) degradation/inactivation of signaling molecules, (ii) increment / decrement of cell surface receptors, (iii) synthesis of signaling molecules through feedback loops (Krauss, 2014).

1.1.2. Membrane Receptors Relay the Signal from the Extracellular Space to the Cell Interior

Transmission of extracellular signals across the plasma membrane by receptor-mediated signaling, resulting in intracellular responses, is one of the most fundamental cellular processes (Lefkowitz & Shenoy, 2005).

The act of converting extracellular signals into physiological intracellular response is called signal transduction (Lodish, 2008). **Figure 1** shows a scheme of how signaling molecules, released by a specific cell, are released and lead to a physiological response in a cell equipped with the cognate cell-surface receptors.

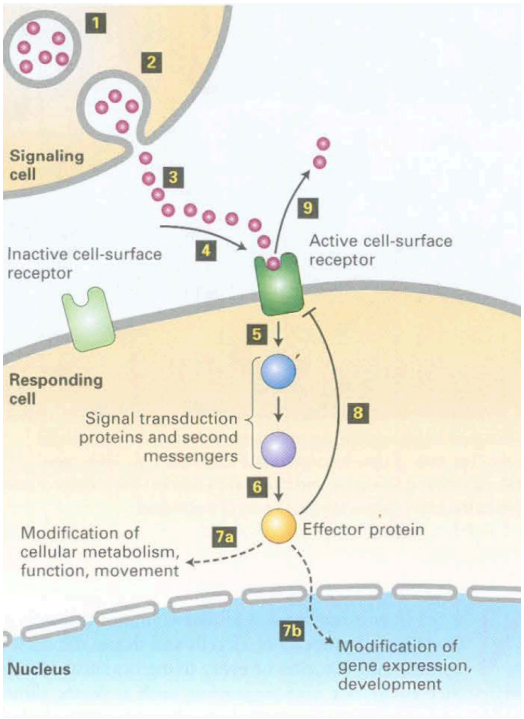


Figure 1: Simplified basic scheme of signal transduction. (1) A signal e.g. insulin is produced in response to high glucose levels and circulates through the bloodstream until it encounters a matching (4) membrane associated insulin receptor, which belongs to the highly conserved tyrosine kinases. (5) Upon agonist binding to the external domain of the receptor, auto phosphorylation of the receptor takes place, which leads to signal transduction through two cellular pathways, the MAP kinase signaling pathway and the PI-3K signaling pathway. (7) Depending on the signal and pathway, various cellular responses are triggered. Insulin regulates glucose homeostasis through metabolic enzymes and gene expression (Bevan, 2001), (Nelson, Lehninger, & Cox, 2008), (Lodish, 2008).

Signals can only exercise their effects if they are received. Signals such as light (e.g. rhodopsin), temperature, touch and chemicals (nitric oxide, lipids, peptides etc.) can be received by receptors, leading to their activation (Lodish, 2008). Many extracellular signaling molecules are produced in specific signaling cells and only trigger a response in cells, which are equipped with the matching cell surface receptors. They can be divided into three segments as seen in **Figure 2**: (1) the extracellular domain, which faces the extracellular space; (2) transmembrane domain; (3) intracellular domain, which faces the cytosol (Lodish, 2008).

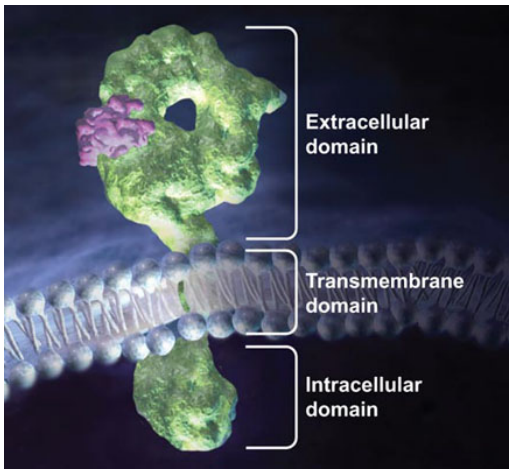


Figure 2: Cell surface receptor belonging to the family of HER receptors. Image from http://www.biooncology.com/research-education/her/overview/receptors (last access: 08th July 2015).

Signaling molecules, which are complementary to the structure of the ligand-binding domain in the extracellular domain of the receptor, are able to bind the receptor. This leads to the activation of the receptor and to the activation of cytosolic or membrane-attached proteins via the activated receptor. In order to achieve specificity in the communication, receptors generally only recognize a single molecule or a group of closely related molecules. This concept is often described in the analogy of a lock that can only be opened by a particular key (Lodish, 2008).

Ligand-receptor interaction results in conformational changes (tertiary as well as quaternary) in the receptor. However structural changes in the receptor are not sufficient enough to trigger an appropriate physiological response. The input from the ligand-receptor complex must be further relayed through intracellular signal transduction. An orchestra of signaling enzymes (second messengers, scaffold proteins and adaptor proteins) works together to achieve intracellular signal transduction.

Signaling enzymes are responsible for transmitting the signal, but also for ending it. They can be regulated through allosteric transitions in response to the binding of effector molecules or covalent modifications (Krauss, 2014). Post-translational modifications, such as the phosphorylation or dephosphorylation of signaling enzymes, play an important role in signal transduction. Protein kinases catalyze phosphorylation, whereas protein phosphatases catalyze dephosphorylation. These processes convert signaling enzymes into active or inactive states (Krauss, 2014).

Binding of the ligand to its cognate receptor results in two main cellular responses. These responses are changes in the activity of proteins and altered cellular transcription (Lodish, 2008). There are around 12 classes of cell surface receptors in eukaryotes which trigger various cellular responses (Lodish, 2008). The largest class of receptors is the class of G protein-coupled receptors (GPCRs) (Lefkowitz & Shenoy, 2005).

1.1.3. Cell Surface Receptors

G protein-coupled receptors

GPCRs, also known as seven-transmembrane domain receptors (7TMRs), are found on the surface of all cells of multicellular organisms. With more than 800 distinct GPCRs in the human genome, they represent the largest, most versatile and most ubiquitous of the several families of membrane receptors (Lefkowitz & Shenoy, 2005). GPCRs mediate fundamental biological functions of

mammalian cells and are involved in the visual, olfactory, and gustatory systems. The natural ligands are very diverse and range from neurotransmitters to hormones, ions, proteins and sensory stimuli (Lodish, 2008), (Kobilka, 2007).

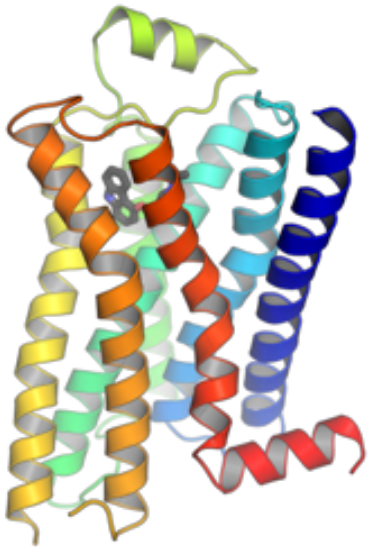


Figure 3: Schematic ribbon representation of a human G protein-coupled receptor (beta2-adrenergic-receptor-T4 lysozyme fusion protein) bound to the partial inverse agonist carazolol at 2.4 Å resolution (Cherezov et al., 2007).

These receptors play a role in the pathogenesis of many human diseases, and therefore are a prominent drug target. Around 40-60% of modern therapeutic drugs, such as antihypertensives and antipsychotics, target these receptors (Gomperts et al., 2009).

G protein-mediated signaling is highly conserved and all pathways share common characteristics:

- All GPCRs contain a structural signature of seven membrane-spanning domains, whereas the N-terminus is located in the extracellular space and the C-terminus is located in the cell (Kobilka, 2007).

- A small G protein, such as Ras, is coupled to the intracellular domain of

the receptor which works as a molecular switch between an active and inactive state, thus influencing downstream signaling of membrane-bound effector proteins (Lodish, 2008).

- Feedback regulation and desensitization of the signaling pathway (Lodish, 2008).

Mechanisms of G protein-mediated signaling

Ligand binding to the GPCR results in a conformational change and in the activation of a cognate G protein. Heterotrimeric G proteins are composed of three subunits referred to as G_{α}, G_{β} and G_{γ}, whereas the alpha and gamma subunit are attached to the membrane. In its inactive state, the G_{α} subunit is bound to guanosine diphosphate (GDP), whereas in its active state guanosine triphosphate (GTP) is bound to the subunit.

Activation of the receptor allows the binding to the $G_{\alpha\beta\gamma}$ hetero-trimer, resulting in the release of GDP from the G_{α} subunit. GTP binds to the “empty” site, resulting in a weaker interaction between the G_{α} and the G_{γ} and G_{β} subunits. G_{α} -GTP can now interact with various downstream effectors. Among these effectors are phospholipases and adenylyl cyclases (Lodish, 2008), (Pierce & Lefkowitz, 2001).

Down-regulation of GPCR-mediated signaling

A universal mechanism of receptors is desensitization. At some point receptor signaling needs to be reduced or terminated to ensure effective and precise signaling and ultimately homeostasis. Desensitization takes place shortly after the stimulation of the cell surface receptor and reduces or terminates signaling. This is a highly conserved mechanism in GPCR signaling. In the initial step, the GPCR gets phosphorylated by either second-messenger-dependent

protein kinases or through G protein-coupled receptor kinases (GRKs), which recognize the activated form of the receptor (Pierce & Lefkowitz, 2001). In addition to the receptor phosphorylation, β-arrestins are required which increase the desensitization. The underlying molecular mechanism that undermines the role of β-arrestins is that they have a high specificity for binding to phosphorylated activated GPCRs. This blocks the binding to G proteins and links the receptor to clathrin-coated pits for internalization by recruiting clathrin and adaptor protein 2 (AP2) (Luttrell & Lefkowitz, 2002). GRK phosphorylation determines the strength of β-arrestin binding to the receptor. This in turn influences the period until receptor degradation or recycling and is believed to be the general mechanism of receptor desensitization in all GPCRs (Pierce & Lefkowitz, 2001).

1.2. Role of Arrestins in Cellular Signaling

Arrestins are a small family of proteins that regulate cellular signaling, with an average size of around 45 kDa. Four subtypes are expressed in vertebrates. Two visual subtypes, arrestin-1 and arrestin-4, which are found in photoreceptors, have recently been linked to contributing to light adaptation (Lefkowitz and Shenoy 2005). The two non-visual arrestins, arrestin-2 and arrestin-3, also referred to as β-arrestin-1 and β-arrestin-2, are ubiquitously expressed with levels peaking in brain and spleen tissue. They serve as adaptors, scaffolds, and/or signal transducers, and connect activated receptors with diverse signaling pathways within the cell (Lefkowitz and Shenoy 2005).

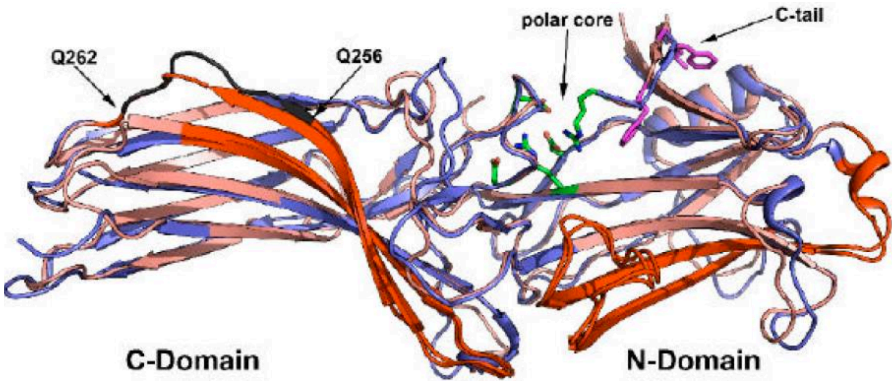


Figure 4: Crystal structure of arrestin-3 solved at 3.0 Å (Zhan, Gimenez, Gurevich, & Spiller, 2011)

Arrestin-1, the first identified arrestin subtype, was discovered on studies of the visual system in 1986. Rhodopsin, a prototypical GPCR, is localized in the outer segment of rod cells. With rod and cones being the primary recipients of visual stimulation, rhodopsin plays a fundamental role in the visual system (Lodish, 2008). Upon light absorption, a conformational change is triggered in rhodopsin, which leads to the indirect activation of cGMP phosphodiesterase (PDEase). Wilden et al. were first to mention that a purified 48-kDa sized protein arrests PDEase activation through photo-excited rhodopsin. Due to its arresting action of photo-excited rhodopsin signaling, the 48-kDa-sized-protein was termed arrestin and is now known as arrestin-1 (Wilden, Hall, & Kuhn, 1986).

1.2.1. Classical Roles of β-arrestins

Arrestins are of great interest because they not only bind specifically with hundreds of GPCR subtypes ubiquitously expressed in all animals, but also interact with several non-receptor signaling proteins (E. V. Gurevich & Gurevich, 2014). Both non-visual arrestins play a fundamental role in the regulation of cellular processes such as apoptosis, chemotaxis and protein ubiquitination by serving as multi-functional adaptor proteins. This puts the small group of arrestin proteins at the crossroads of cellular signaling pathways (Gurevich and Gurevich 2014), (Luttrell and Lefkowitz 2002).

For a certain, time the understanding of GPCR mediated signaling was governed by a classical paradigm. This paradigm suggests that ligand binding to the receptor only leads to a cellular response through the heterotrimeric G protein stimulation followed by the desensitization through GRKs and β-arrestins. However, a new signaling paradigm has emerged highlighting that β-arrestins not only mediate the desensitization of G protein signaling, but also act as signal transducers themselves as seen in **Figure 5** (Lefkowitz & Shenoy, 2005). Arrestins have been found to mediate a variety of receptor signaling and regulatory processes. They bind to a growing list of endocytic signaling proteins, therefore, making them multi-functional regulators of cellular signaling (Lefkowitz & Shenoy, 2005).

The discovery that β-arrestins play a larger role in signal transduction emerged when the molecular mechanisms by which β-arrestins link GPCRs to the clathrin-coated pits for internalization was discovered (Luttrell & Lefkowitz, 2002).

Upon internalization of the receptor, arrestin-2 and arrestin-3 re-direct the signaling to alternative pathways by interacting with non-receptor molecules. Growing numbers of proteins that interact with arrestins have rapidly been identified. β-arrestins have been identified as activators of all three mammalian main subfamilies of mitogen-activated protein kinases: ERK, JNK and p38, by tethering the signaling components (E. V. Gurevich & Gurevich, 2014).

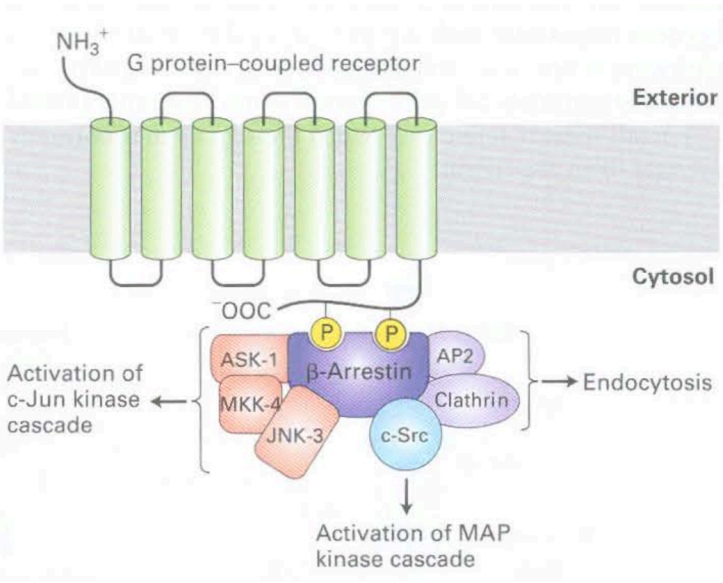


Figure 5: β-arrestin's role as independent signal transducer and mediator of endocytosis. Besides contributing to the termination of GPCR mediated signaling by binding to the phosphorylated activated receptor, they are mediators of signal transduction themselves by binding to a growing number of non-receptor binding partners. As depicted here (1) binding to elements of the endocytic system (clathrin, AP2), results in receptor internalization (2) activation of MAPK cascades results in the activation of transcription factors and protein kinases (Lodish, 2008), (V. V. Gurevich & Gurevich, 2004).

1.3. Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) are highly conserved serine/threonine kinases, belonging to the class of protein kinases, which constitute for the largest family of enzymes encoded by the human genome. They play critical roles in signal transduction by catalyzing the transfer of the gamma phosphate group of ATP to specific amino acid residues of substrate proteins (Bubici & Papa, 2014). As the name implies, serine/threonine kinases transfer the phosphate to serine and threonine amino acid residues on their targets. This stable post-translational modification of adding phosphate groups to amino acid residues, functions as a regulatory mechanism in signal transduction by influencing signaling protein activities. Depending on the target protein, the phosphorylation can either result in an activation or deactivation (Krauss, 2014).

Protein phosphatases are the opposing enzymes to protein kinases, which catalyze the removal of the added phosphate group. Together, protein kinases and phosphatases orchestrate the majority of intracellular pathways in a highly regulated fashion (Krauss, 2014). Therefore, MAPKs play a critical role in cellular processes such as proliferation, apoptosis, differentiation, development, and transformation, all of which influence cellular fate (E. V. Gurevich & Gurevich, 2014).

A highly conserved intracellular signaling pathway that consists of three protein kinases in sequence is known as the MAPK pathway (Krauss, 2014). The protein kinases successively phosphorylate and activate their downstream component. Thereby, extracellular and intracellular signals are transduced downstream to other kinases or to the level of transcription, leading to an altered gene expression (Krauss, 2014).
Mammalian MAPK pathways can be characterized into three main families:

(1) extracellular signal-regulated kinases (ERK) 1 and 2
(2) c-Jun N-terminal kinases/Stress-activated protein kinases (JNK/SAPK) 1, 2, and 3
(3) the p38/HOG1 MAPKs

The ERK1/2 pathway, generally activated by mitogens, is critically involved in proliferation and survival through G0 to G1 cell cycle regulation. JNK/SAPK and p38/HOG1 MAPKs are both activated due to environmental stress and inflammatory cytokines and can be referred to as stress-activated MAPKs. The activating signals for p38 and JNK show a great deal of diversity as well overlap with each other. (Krauss, 2014). JNK has been described as a mediator of apoptosis and is involved in cellular processes such as proliferation, apoptosis, cellular migration and differentiation (E. V. Gurevich & Gurevich, 2014).

The three main MAPK pathways share a common structure, but differ in

their stimulating signals and outcomes (Sabio & Davis, 2014). In general, signaling through the MAPK modules is initiated through upstream activators, which catalyze the phosphorylation of the first kinase in the module. For example, in the ERK pathway (see **Figure 6**) RAF represents the MAPK-Kinase-Kinase (MAPKKK) in the cascade. Signal propagation happens through phosphorylation of the downstream kinases MEK1/2, a MAPK-Kinase (MAPKK) by RAF. This leads to the activation of the third kinase in the module, ERK. ERK represents the last kinase in the module and gets activated by dual phosphorylation. All MAPKs have a highly conserved Threonine-X-Tyrosine motif in the T-loop, whereas Threonine and Tyrosine are the target amino acid residues for phosphorylation. This activating mechanism is canonical for all MAPKs, whereas the amino acid residue between Threonine and Tyrosine varies between the MAPKs (Sabio & Davis, 2014). Once the MAPK is activated, it relays the signal to downstream targets in the cytosol such as transcription factors (TFs) or other protein kinases by phosphorylation into respective physiological responses (Krauss, 2014), (Morrison, 2012), (Shaul & Seger, 2007).

1.3.1. Scaffold Proteins

The existence of distinct MAPK signaling modules with similar architectures as shown in **Figure 6** raises the question how signaling in the pathways is organized and regulated (Krauss, 2014). Given the sheer amount of kinases at every level of the MAPK cascades and the wide range of signal sources (e.g.: Mitogens via GPCRs, growth factor signaling, environmental stressors, cytokine receptors etc.) that stimulate signaling, requires regulation. In addition to substrate specificity and binding affinities of the MAPKKKs and MAPKKs, specific signaling is achieved by bringing the single components of each module together, which make up a specific pathway as for example the ASK1-MKK7-JNK3 module, as seen in **Figure 6**.

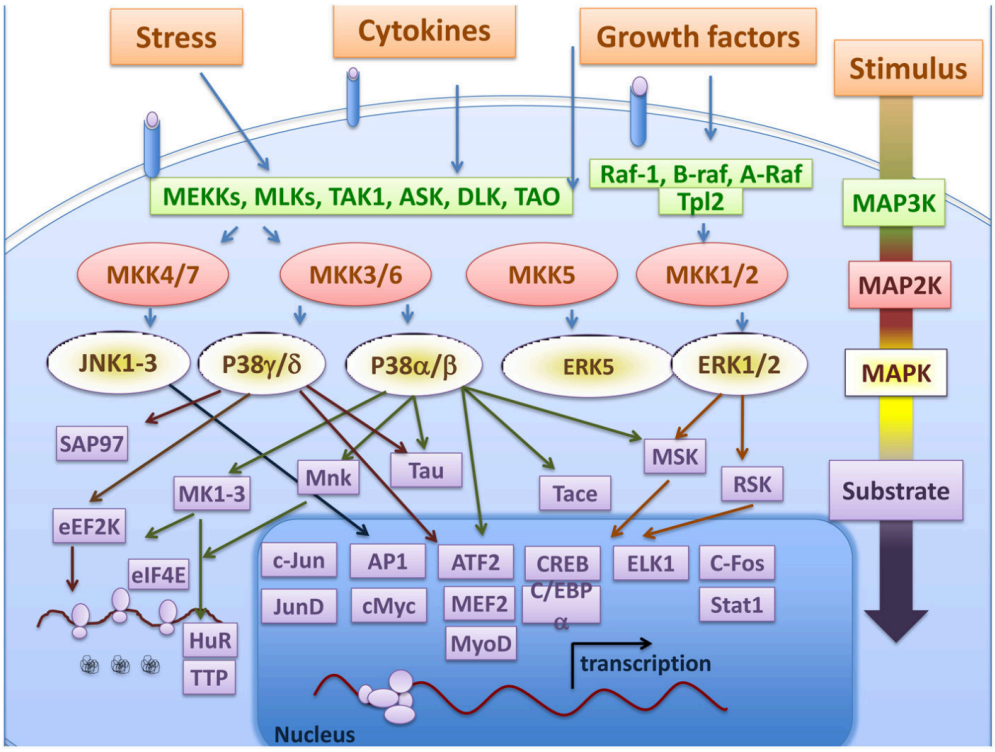


Figure 6: MAPK pathways sharing a common structure of three kinases in sequence: a MAPKKK, a MAPKK, and a MAPK. The three main MAPK ERK (extracellular signal-regulated kinases); JNK/SAPK (c-Jun N-terminal kinases/Stress-activated protein kinases) ; and p38 can be activated by various signals, resulting in the phosphorylation of transcription factors and proteins (Sabio & Davis, 2014).

These regulators of signal transduction pathways are referred to as scaffold proteins. Cells established these kinds of proteins to bring components of a certain signaling pathway (e.g.: MAPKKKs, MAPKKs and MAPKs in one of the MAPK pathways) into closer proximity, ensuring enhanced phosphorylation of the kinases. This ability of scaffold proteins enhances the specificity of the signal, hindering cross talk between the pathways but also influences the timing of the signaling (Krauss, 2014). However not only the ability to bind the components is of importance, but also the aspect of spatial orientation contributes to specific signaling. For example, arrestin-2 and arrestin-3 are both capable of binding the components of the ASK1-MKK4/7-JNK3 module, however only arrestin-3

facilitates the activation of JNK3. (Krauss, 2014), (E. V. Gurevich & Gurevich, 2014).

Transmembrane receptors often trigger the flow of cellular information through MAPK pathways stimulated by extracellular signals such as growth factors, oxidative stress, cytokines, hormones or UV radiation. Molecular switches such as Ras or Rac propagate the signal from the receptor to internal signaling cascades. Regulation of the cascade can be achieved at each level and signal transmission to other pathways may take place (Krauss, 2014).

1.3.2. JNK

The c-Jun N-terminal kinase (JNK) is one of the three main MAPK beside ERK and p38. The JNK signaling module is acknowledged as a critical regulator of cellular physiology such as apoptosis, proliferation and transformation (Zhan, Kaoud et al. 2013). JNKs are also referred to as stress-activated protein kinases (SAPKs) because environmental stressors such as ionizing radiation, heat shock, reactive oxygen species (ROS) and inflammatory cytokines lead to their activation. The stressors lead to misfolded proteins which accumulate and therefore trigger JNK or p38 signaling (Krauss, 2014).

Several MAPKKKs act upstream of JNK, which mirrors the broad range of signals that stimulate these kinases as seen in **Table 1** (Krauss, 2014). There are three different MAPKs in the JNK module: JNK1, 2 and 3 expressed by three different genes. Due to alternative splicing, 12 different isoforms are generated. Each of the three JNKs is expressed in mammals, however they differ in their expression profile. Whereas JNK3 expression is limited to the brain, JNK1 and JNK2 are ubiquitously expressed (E. V. Gurevich & Gurevich, 2014).

Table 1: Activating signals and kinase components of the JNK signaling module (Krauss, 2014).

Stimulating signals: reactive oxygen species, radiation, heat shock, DNA damage, cytokines	
MAPKKK	MEKK1-4, MLK2,3; TAO1, TAK1, ASK1
MAPKK	MKK4, MKK7
MAPK	JNK1, JNK2, JNK3
MAPK substrates	TFs: c-Jun, ATF-2, Stat3 cytoplasmic proteins

Signaling through the JNK cascade contributes to cellular homeostasis by regulating apoptosis, cellular proliferation, differentiation and migration (E. V. Gurevich & Gurevich, 2014). The JNK pathway exerts one of the major roles as mediators of apoptosis and has been associated with various diseases such as cancer, heart disease and inflammatory diseases (Weston & Davis, 2002).
Several links, specifically between the involvement of JNK3 and nerve diseases such as Parkinson's, Alzheimer's and Huntington's, have been established, making it a potential target for pharmaceutical treatment.

Targets of JNK are transcription factors (TFs) such as c-Jun, ATF-2, NF-ATc, JunD and protein kinases (Krauss, 2014). Protein kinases can also translocate to the nucleus where they activate TFs, thereby altering gene expression. Immediate early genes (IEGs) such as the well-characterized c-Fos or c-Jun genes are rapidly turned on as a response (Krauss and Ohio Library and Information Network. 2014).

C-Jun is a crucial JNK substrate and transcriptional activator protein. It is an element of the AP-1 early transcription factor and can interact with proteins of the b-ZIP family forming either homo- or hetero-dimeric basic leucine zipper transcription factors (see **Figure 7**), which are involved in many cellular processes. For its activation, c-Jun needs to be phosphorylated at Serine63 and Serine73, which is catalyzed by JNK (Wisdom, Johnson, & Moore, 1999).

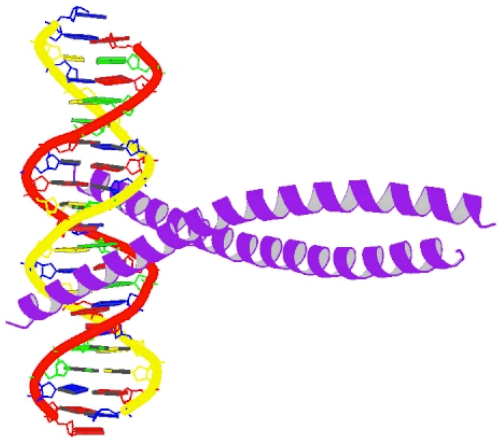


Figure 7: Schematic representation of the human c-Fos:c-Jun heterodimer. Together they form the activator protein 1 (AP-1), which influences life and death of cells. The heterodimers form a leucine zipper (violet), which interact with its basic amino acid region with the major groove of the DNA (Ameyar, Wisniewska, & Weitzman, 2003). Image taken from the Protein Data Bank, accession: (1FOS) showing the crystal structure at 3.05Å.

By regulating the transcription of Cyclin D1, a positive regulator in mitosis, c-Jun plays a major role in cell cycle progression in the G1 phase of mitosis (Wisdom et al., 1999). Furthermore the expressional levels of c-Jun influence p53 and p21 expression. Whereas in the absence of c-Jun, p53 and p21 levels are higher and vice versa, suggesting that upregulated c-Jun leads to an increased proliferation (Schreiber et al., 1999).

Signaling through the JNK module is triggered by activation of a MAPKKK in the JNK module (e.g. apoptosis signal regulated kinase 1 (ASK1)) as seen in **Table 1**. This leads to the phosphorylation and activation of the dual specific MKK4 and MKK7 which in turn leads to JNK activation by dual phosphorylation of the amino acid residues Threonine183 by MKK7 and Tyrosine185 by MKK4 in the Threonie-Proline-Tyrosine motif in the activation domain (Lopez-Bergami et al., 2007). Phosphorylation by only one of the two JNK upstream kinases leads to a partial activation, therefore both kinases are required to entirely activate JNK (E. V. Gurevich & Gurevich, 2014), (Lawler, Fleming, Goedert, & Cohen, 1998).

Depending on the environmental signal, the JNK pathway was shown to trigger apoptosis, stimulates cellular proliferation and transformation, therefore playing an important role in the decision making process of life or death (Weston and Davis 2007).

1.4. Role of Arrestins in JNK Activation

The first evidence that arrestin-3 interacts with components of the JNK module was provided by McDonald et al.. Specifically their experiments showed that arrestin-3 interacts with ASK1 and JNK3 (McDonald et al., 2000), (E. V. Gurevich & Gurevich, 2014).

Recently Zhan et al. showed in *in vitro* experiments that JNK3 in its fundamental conformation directly interacts with arrestin-3. Continuing experiments revealed that arrestin-3 directly interacts with JNK1, JNK2 and JNK3 as well as MKK4 and MKK7. arrestin-3 is able to simultaneously interact with the kinases in the JNK module (ASK1-MKK4-JNK1/2/3). Although all the four arrestin subtypes bind equally to the kinase components, only arrestin-3 acts as a true scaffold protein in the activation of JNK3 (E. V. Gurevich & Gurevich, 2014). Therefore, arrestin-3 brings them into closer proximity leading to an enhanced JNK activation and a more specific signaling. Studies with arrestin mutants, lacking receptor-binding function, showed that the scaffolding process takes place independently from receptor bound arrestin-3 (Gurevich and Gurevich 2014), (Zhan, Kaoud et al. 2013).

Crystal structures of arrestin-2 and arrestin-3 share a high similarity and 78% sequence identity. However the fact that arrestin-2 is able to bind the same kinase components as arrestin-3, but fails to activate JNK3 underpins that binding does not equal facilitation. This suggests that the orientation of the kinase components and their affinity to arrestins might play an important role for the latter (E. V. Gurevich & Gurevich, 2014). For a better understanding of the relationship between binding and scaffolding functions of arrestins, the

interactions of arrestin-3 with each kinase in the cascade need to be characterized quantitatively (E. V. Gurevich & Gurevich, 2014). *In vitro* experiments with purified proteins revealed that arrestin-2 exhibited a lower affinity towards MKK4 than arrestin-3. This in turn means that lower concentrations of arrestin-3 are required to perform the scaffolding function than compared to arrestin-2.

Biphasic effect

Experiments with purified proteins confirmed that the formation of a ternary complex between MKK4, JNK3 and arrestin-3 takes places simultaneously. However, three existing states, depending on the protein concentration, can exist between these proteins as seen in **Figure 8** (E. V. Gurevich & Gurevich, 2014).
(1) In the absence of arrestin-3, signaling takes place however results in a low phosphorylation level of JNK3, whereas (2) in the presence of optimal arrestin-3 concentrations, signaling takes place with a high level of JNK3 phosphorylation. (3) Concentrations beyond the optimum result in incomplete scaffolds, where complexes either with arrestin-3 and JNK3 or arrestin-3 and MKK4 can be found, however the formation of the ternary complex does not take place. The outcome of this state is that there is no JNK3 phosphorylation (E. V. Gurevich & Gurevich, 2014).

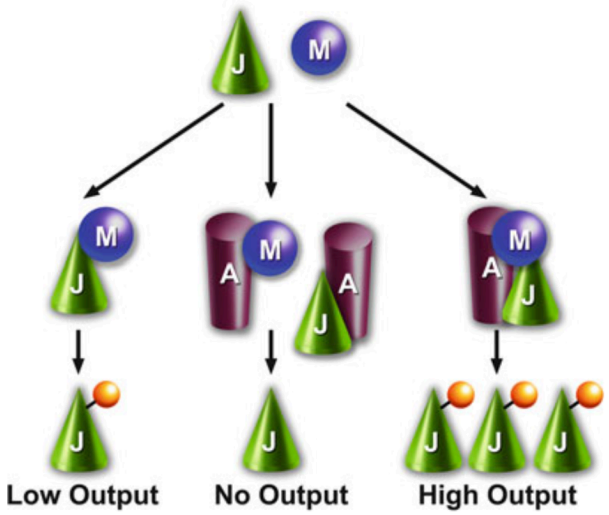


Figure 8: Three different states of JNK3, MKK4 and arrestin-3 exist as *in vitro* experiments with the purified proteins revealed (E. V. Gurevich & Gurevich, 2014) Low output JNK3 phosphorylation takes place in the absence of arrestin-3. No output can be observed at supraoptimal arrestin-3 concentrations. High output JNK3 phosphorylation takes place at optimal arrestin-3 concentrations, tethering JNK3 and MKK4, therefore promoting phosphorylation (E. V. Gurevich & Gurevich, 2014).

However in addition to Tyrosine phosphorylation by MKK4, JNK activation also requires the phosphorylation of the Threonine residue in the activation loop. Direct interaction assays revealed that MKK7 also directly interacts with arrestin-3 and further activation studies show that JNK3 phosphorylation by MKK7 is enhanced in the presence of optimal arrestin-3 concentration. This data is in accordance with those for MKK4 as **Figure 9** shows. The biphasic dependence of arrestin-3 can be observed for MKK4 as well as MKK7, however the optimal arrestin-3 concentrations vary and are around 10 times higher in the case of MKK7 (E. V. Gurevich & Gurevich, 2014).

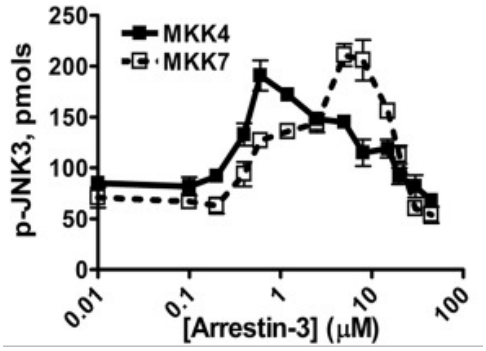


Figure 9: Biphasic dependence of arrestin-3 on the phosphorylation of JNK3α2 by MKK4 and MKK7 (Zhan, Kaoud, Kook, Dalby, & Gurevich, 2013).

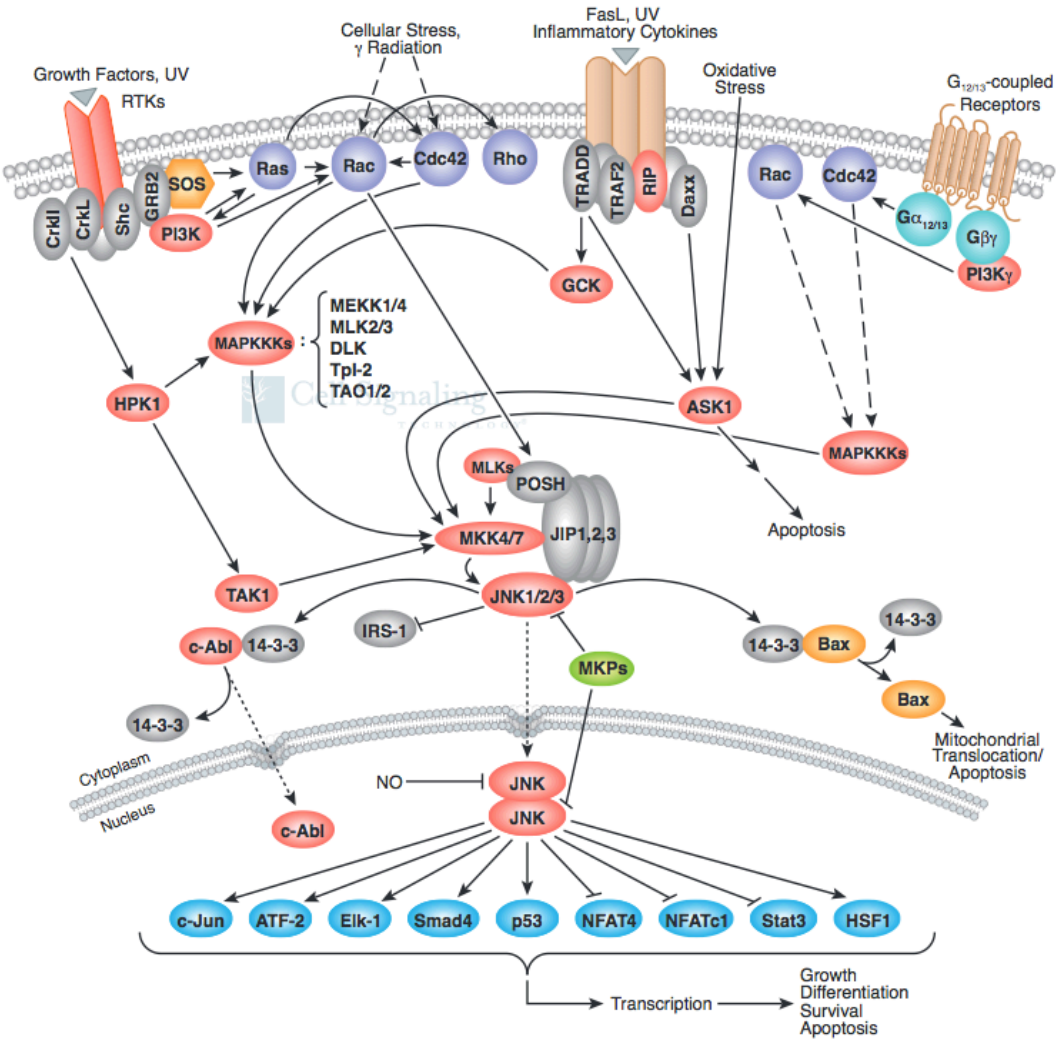


Figure 10: SAPK/JNK Signaling Cascades (Cell Signaling Technology, Inc)

Although arrestins bind dozens of non-receptor partners, the interaction sites for most signaling proteins remain unknown (Zhan, Perez, Gimenez, Vishnivetskiy, & Gurevich, 2014).

1.5. Proteins as Pharmacological Intervention

The majority of cellular functions (proliferation, apoptosis, differentiation etc.) are based on protein complexes interacting with other proteins through protein-protein interactions (PPIs) as for example the signal transduction cascade in GPCR mediated signaling. An agonist bound GPCR activates a heterotrimeric G protein followed by receptor phosphorylation through GRKs. Then arrestins bind to phosphorylated GPCRs, which results in the internalization of the receptor through clathrin-coated vesicles (Gurevich and Gurevich 2014).

Deregulation of PPIs plays a critical role for cellular physiology and pathology and constitutes as a promising target for pharmacological intervention (Jin, Wang, & Fang, 2014). As a matter of fact, almost all known diseases share malfunctions in signal transduction (Nature Biotechnology, 2000). Therapeutic relevant drugs are majorly based (>90%) on small molecules, which are quite straightforward in their development and delivery. However the downside for targeting PPIs with small molecules lie in their low affinity for big and flat protein surfaces. Furthermore, the precise structural elements of proteins that are involved in PPIs are often not identified, therefore putting specificity into question (E. V. Gurevich & Gurevich, 2014). Additionally many proteins in mammals are present in a disordered state and only upon the binding with an interaction partner do they fold into the right conformation (Sugase, Dyson, & Wright, 2007). Further limitations of small molecule drugs are that they only perform one function, therefore lacking additional alternatives (E. V. Gurevich & Gurevich, 2014).

By 2011, more than 100 protein drugs have been approved by the E.U. and USA with reports of great therapeutic successes. The majority of protein-based

therapeutics sales (48%) are owed to monoclonal antibodies, with Entanercept, an antibody targeting TNF-α, leading the list in 2010 (Voynov & Caravella, 2012). Therapeutic successes achieved through protein drugs encourage research and development for better, more specific protein-based therapeutics. The most realistic way of modulating PPIs in a desired manner is by relying on proteins themselves (E. V. Gurevich & Gurevich, 2014). Inserting recombinant proteins into cells that perform a specifically desired function e.g. linking a receptor with a signaling pathway of our choosing, would allow us to specifically influence cellular fate. This ability to consciously disrupt or enhance individual PPIs would give us an unprecedented leverage over the cell, essentially allowing us to tell the cell what to do and when to do it in a language it understands (E. V. Gurevich & Gurevich, 2014).

With technical and scientific advances in recombinant protein and DNA technology, the idea of custom-designed signaling proteins becomes a feasibility, rather than fiction. In order to selectively influence cellular fate by targeting PPIs through protein based therapeutics, a deeper understanding of the fine signaling meshwork is required. Therefore it is critical to decipher signaling pathways and identify protein elements involved in PPIs, which gives us a better mechanistic understanding and fundament for this venture (E. V. Gurevich & Gurevich, 2014).

Arrestins, as scaffold and adaptor proteins, are regulators of various cellular events by specifically interacting with proteins in the cell and are therefore of great interest for the development of protein-based therapeutics (Gurevich and Gurevich 2014). To date it has been shown that arrestins take over fundamental roles:

- Binding to hundreds of GPCRs and contribute to G protein desensitization
- Interacting with a rapidly growing number (162 proteins (Zhan et al., 2014)) of non-receptor binding proteins
- Contributing to receptor endocytosis
- Non-visual arrestins are ubiquitously expressed

- Signal transduction, which places them at important intersection of cellular communication where external and internal inputs are integrated into coherent behavior (Gurevich and Gurevich 2014), (Lefkowitz & Shenoy, 2005).

In a nutshell, arrestins are of great interest for pharmaceutical intervention for influencing unbalanced cellular signaling. With arrestins being one of the regulators of the MAPK pathways, they could give us the chance to influence cellular fate. Deregulation of the JNK signaling pathway plays a role in the pathogenesis of several diseases (E. V. Gurevich & Gurevich, 2014). Eventually this basic research is the precondition to develop new drugs to channel signaling into a desired direction.

My master thesis project focuses on the role of arrestins interacting with non-receptor signaling proteins, specifically within the JNK MAPK module.

1.5.1. Arrestin-3 Stimulates JNK1, JNK2 and JNK3 Activation Through Scaffolding

Recently, Gurevich et al. showed that arrestin-3 enhances JNK1/2/3 activation by directly binding to every single kinase in the module: ASK1, MKK4/7 and JNK1/2/3, therefore bringing them into closer proximity (Kook et al., 2013), (E. V. Gurevich & Gurevich, 2014). However the binding sites of arrestin-3 that are involved in the interactions are not well characterized and as a matter of fact the majority of binding sites of non-receptor partners on arrestin have not been identified yet (Zhan et al., 2014). Arrestin-3 as seen in **Figure 4** has a molecular weight of ~46 kDa and consists of 409 amino acids (E. V. Gurevich & Gurevich, 2014). Recently X.Zhan et al. revealed arrestin-3 interaction sites that mediate JNK3α2 binding and showed that arrestin-3 binds via multiple sites on both domains (Zhan et al., 2014). By designing maltose binding protein (MBP) fusion proteins of specified regions of arrestin-3, their binding affinities towards JNK3α2 were analyzed through *in vitro* binding assays as described in 2.3 *In vitro*

Binding Assay. The MBP-fusion proteins containing varying amino acid ranges (14-64) were selectively based on arrestin-3 elements, which are not involved in receptor binding. This led to the characterization and quantification of crucial binding sites of arrestin-3 and their affinities for interacting with JNK3α2 as seen in **Figure 12** (Zhan et al., 2014).

The results of the analyzed binding intensities clearly show that in total three of the MBP-fusion proteins bind to JNK3α2. One of the elements (T1) is located at the N-terminus and two elements (T3 & T6) are located at the C-terminus of arrestin-3. T1, which spans the first 52 N-terminal amino acids, exhibited the highest binding intensity (around 60% in comparison to the full length arrestin-3) for JNK3α2 out of the three elements. Compared to the binding of full-length arrestin-3, these results are quite remarkable and indicate that the N-terminus of arrestin-3 plays a critical role for JNK3α2 binding (Zhan et al., 2014).

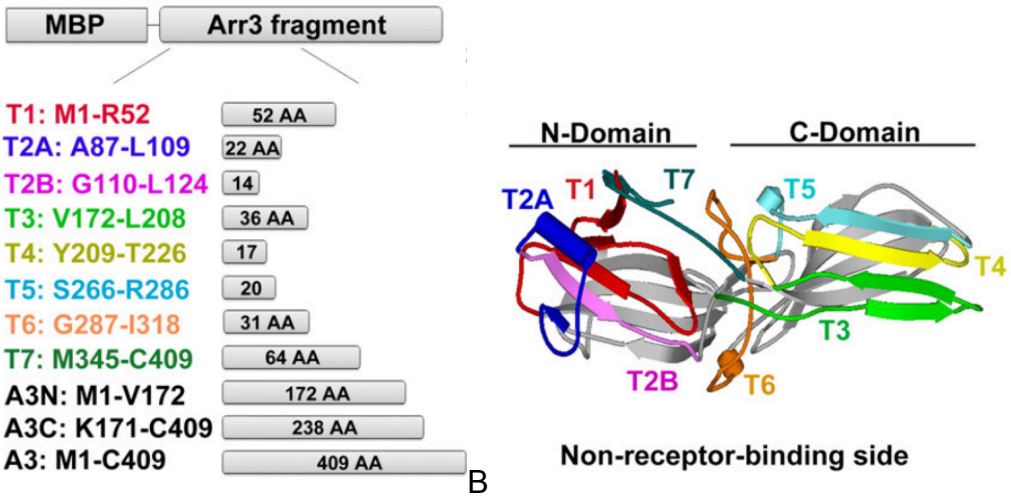


Figure 11: (A) Schematic representation of arrestin-3 regions that were used for creating the MBP-fusion proteins for testing their affinities toward JNK3α2 in *in vitro* experiments. (B) Arrestin-3 regions used for the MBP-fusion proteins are highlighted in the basal conformation of arrestin-3,

depicted from the view of the non-receptor binding side (PDB ID 3P2D) (Zhan et al., 2014), (E. V. Gurevich & Gurevich, 2014).

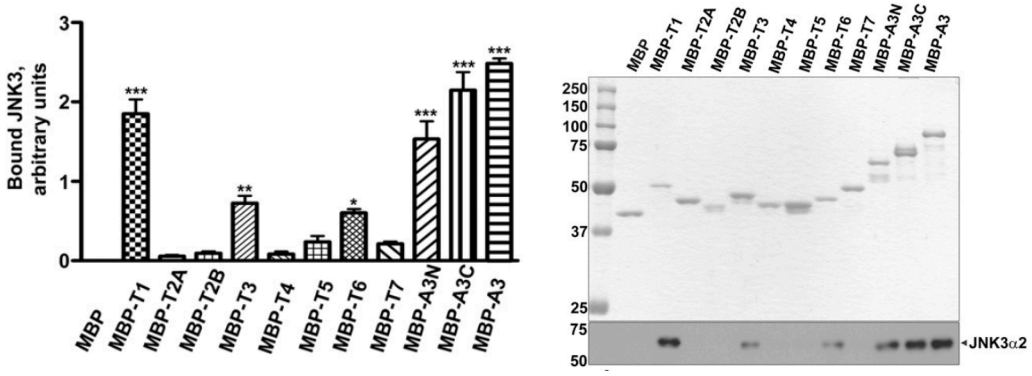
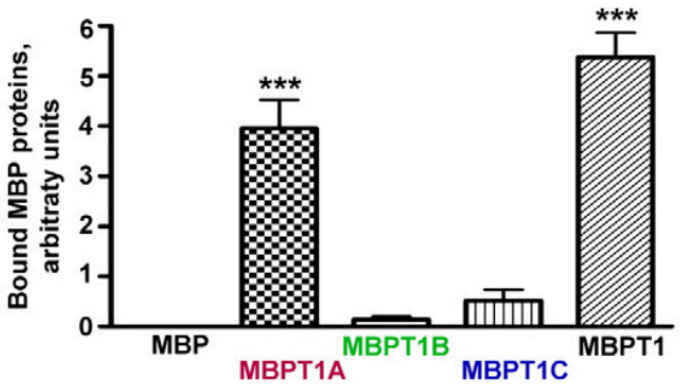


Figure 12: Multiple elements of arrestin-3 are involved in the interaction with JNK3α2 as the quantified western blot results from the *in vitro* binding assays reveal. One of the elements (T1) is located at the N-terminus and two elements (T3 & T6) are located at the C-terminus of arrestin-3. (Zhan et al., 2014).

1.5.2. T1A

Based on T1, three additional MBP-fusion proteins were constructed as shown in **Figure 13**. It was revealed that the first 25 amino acids (T1A) still exhibit binding to JNK3α2. In comparison to T1 (52 N-terminal amino acids), T1A's binding intensity is around ~20% lower (see **Figure 13**) but still higher than those observed in T3 and T6. As it appears, T1A is the main binding element of arrestin-3 in JNK3α2 binding (Zhan, Perez et al. 2014). Further evidence, that the three elements (T1A, T3, T6) are involved in JNK3α2 binding, stem from competition assays, where Zhan et al. showed that with higher peptide concentrations, less arrestin-3 binds to the MAPK (Zhan et al., 2014).

A



B

Figure 13: The first 25 N-terminal amino acid residues of arrestin-3 are the primary binding element in JNK3α2. (A) The structures of the three elements based on T1 are highlighted and the arrestin-3 spanning regions are schematically shown (PDB ID 3P2D) (B) Quantification of binding assays based on western blots (Zhan et al., 2014).

Recent findings by my colleagues from the laboratory (already submitted but not published yet) show that T1A promotes the activation of JNK3α2 *in vitro* as well as *in vivo*. JNK3 is one of the three JNK isoforms, which is primarily expressed in neurons, heart and testes (E. V. Gurevich & Gurevich, 2014). JNK1/2 on the other hand such as arrestin-3 are ubiquitously expressed. However T1A's ability to promote their activation has yet to be determined. Hence the core question of my thesis: Is T1A, a 25 N-terminal amino acid residue of arrestin-3 capable of promoting JNK1 and JNK2 activation?

In order to get to the bottom of this question, it was first and foremost fundamental to evaluate T1A's binding affinity for JNK1 and JNK2. To assess and characterize their direct interactions, *in vitro* binding assays with purified proteins as described in 2.3 *In vitro* Binding Assay were performed. *In vitro* experiments were of benefit for my project for several reasons: (1) *in vitro* assays are controlled systems, whereas in cell based systems several unidentifiable factors may interfere with direct binding results. (2) Limitations in co-transfection and (3) complications in controlling the expression levels of each component (E. V. Gurevich & Gurevich, 2014).

Therefore MBP-fusion proteins, containing the full-length arrestin-3 and those containing the T1A region, were purified from *Escherichia coli (E. coli)* as described in 2.2 Expression and Purification of Recombinant MBP-fusion Proteins. The purified proteins were utilized to assess and compare the binding affinities of T1A and arrestin-3 for JNK1 as well as JNK2 respectively as described in 2.3 *In vitro* Binding Assay.

Once the binding affinities were characterized and quantified as seen in 3.1 Results *in vitro* Binding Assay, the next step was to assess if T1A was able to facilitate the phosphorylation of the MAPKs, JNK1 and JNK2 as previously reported with arrestin-3 (Kook et al., 2013).

1.5.3. Identification of Further Arrestin-3 Binding Sites

In order to further identify arrestin-3 regions that might contribute to JNK interaction, new MBP-fusion constructs were designed, based on previously published data by X Zhan et al. (Zhan et al., 2014). The data seen in **Figure 12** shows that in addition to the T1 region, the T6 region of arrestin-3 is a promising element in JNK binding. Therefore the T6 region, which spans 32 amino acids on the C domain, was separated into 2 shorter regions:

Arrestin-3 T6 region spans 32 amino acids:
GLALDGKLKHEDTNLASSTIVKEGANKEVLGI

- T6A containing 14 amino acids: GLALDGKLKHEDTN
- T6B with 18 amino acids: LASSTIVKEGANKEVLGI

The two aspartic acids (highlighted in red), which are involved in the formation of the polar core, are part of the T6A region.

1.5.4. Aims

The major aim of my thesis is to assess the binding affinities of purified T1A with the MAPKs JNK1 and JNK2 as well as determine if T1A can facilitate the activation of JNK1 and JNK2 by its upstream kinase MKK4 *in vitro*.

2. Experimental Procedures

2.1. MBP-T6A and MBP-T6B Fusion Protein Constructs

The T6 element spans the regions G287-I318 of arrestin-3. This region was cleaved into 2 smaller regions: T6A and T6B. The two shorter MBP-fusions were used to evaluate their binding affinities towards JNK1 and JNK2. The following amino acid sequence describes the region of T6, whereas the two aspartic acids (red-marked Ds), responsible for the formation of a polar core, are part of the T6A region:

T6: GLALDGKLK HEDTNLASSTIVKEGANKEVLGI

To purify the two fusion proteins, constructs containing the T6A and T6B peptides of arrestin-3 were sub-cloned into pMal-p2T and inserted into *Escherichia coli* (*E. coli*) strain BL21-codon plus (DE3)-RIL. Forward and reverse primers with overlapping regions were ordered from Eurofins Scientific.

1. T6A: GLALDGKLKHEDTN

Forward primer:

ACTG GAATTCGCGGGCCTCGCTCTAGATGGGAAGCTCAAGCACGAG

EcoRI

Reverse primer:

ACTGACAAGCTTTCAGTTGGTGTCCTCGTGCTTGAGCTTCCC

HindIII

2. T6B: LASSTIVKEGANKEVLGI

Forward primer: ACTGGAATTC GCG CTGGCCTCGAGCACCATTGTG
Reverse primer: ACTGACAAGCTTTCATATCCCCAGCACCTCCTTGTTGGC

PCR

The T6A and T6B regions were amplified via PCR with an elongation time of 10 seconds, a Tm of 68°C and 40 cycles. No template DNA was needed since the primer regions are overlapping. The ingredients of the PCR are shown in **Table 2**.

Table 2: Ingredients for the amplification of the DNA inserts T6A and T6B.

	PCR1 (100 µl)	**PCR2**
Primer	4 µl eT6Af	1µl eT6Bf
Primer	4µl eT6Ar	1µl eT6Br
dNTP (5mM)	4µl	4µl
10x Buffer	10µl	10µl
BSA	1µl	1µl
Vent DNA Polymerase	1µl	1µl
H2O	76µl	81µl
Restriction Enzymes	EcoRI HindIII	EcoRI HindIII

Cleaning of PCR products

After the confirmation of the right amplified PCR products via 2% agarose gel electrophoresis, the PCR products were purified utilizing the Zymo Research Kit (DNA Clean & Concentrator^{TM}-5). First, 500 µl of DNA binding buffer was added to the 100 µl PCR sample and the mixtures loaded onto the Zymo-Spin column in a collection tube. The tubes were centrifuged at >10.000g for 30 seconds and the flow through were discarded. 200 µl of DNA washing buffer was added to the column and centrifuged for 30 seconds at >10.000g. This step was performed twice. The column was transferred to a new collection tube and the DNA was eluted with DNAse free water.

Restriction digest

The restriction digest was performed for 1 hour at 37°C and the inserts/vectors were cut with the restriction enzymes EcoRI and HindIII (New England Biolabs).

Table 3: Ingredients for the insert restriction digestion.

	Volume in µl
10x Cut Smart Buffer	5
EcoRI	1
HindIII	1
DNA	20
H2O	23

Table 4: Ingredients for the vector restriction digestion.

	Volume in µl
10x Cut Smart Buffer	5
ExoRI	2
HindIII	2
DNA	4
H2O	37

After the digestion, the samples were loaded onto a 2% agarose gel and run for 30 minutes at 100 V to confirm the presence of the insert and vector.

Ligation of inserts and vector

Vector and insert were combined as seen in **Table 5** (Quick Ligase, 2x Quick Ligase Reaction Buffer (New England Biolabs)). After combining the ingredients, the ligation took place at RT for 10 minutes. After 10 minutes the tubes were put on ice for 2 minutes and added to the *E. coli* strain BL21-codon plus (DE3)-RIL and incubated on ice for 30 minutes. The incubated cells were then spread on lysogeny broth (LB) agar plates and incubated overnight at 37°C. After the incubation, three single cultures were picked per plate and put into 15 ml falcon tubes with 5 ml LB growth medium (1000x Ampicillin). The falcon tubes with the cultures were incubated overnight at 37°C, 225 rpm.

Table 5: Ingredients for Ligation of Insert into Vector

	T6A and T6B inserts	Neg. Control
2xBF [µl]	5	5
pMAL [µl]	1 µl	pMAL
Insert [µl]	4 µl of the respective insert	4 µl of H2O (neg. control)
Ligase [µl]	1 µl	1 µl

MiniPrep (Short Protocol Zyppy™ Plasmid Miniprep Kit (Zymo Research)) - in order to extract and purify plasmid DNA

The overnight cultures were spun down at 3500 rpm for 10 minutes and the liquid LB media was discarded. 600 µl of TE Buffer (pH 8.0; Amresco) were added and re-suspended and transferred to reaction tubes. 100 µl of 7xLysis Buffer was added, mixed and incubated at RT for 2 minutes. 350 µl of neutralization buffer was added after 2 minutes, the tubes were centrifuged at 13.000 rpm for 1 minutes and the supernatant was added to filtration tubes. Centrifugation at 13.000 rpm for 30 seconds was performed and 200 µl of Endo Wash Buffer was added and centrifugation at 13.000 rpm took place again. Two further washing steps were performed with 400 µl of Zyppy Wash Buffer to remove the ethanol. The supernatant was discarded. A new collection tube was used and 30 µl of elution buffer was directly added to the filter and incubated at RT for 1 minute followed by centrifugation at 13.000 rpm for 30 seconds. The respective DNA concentrations were measured utilizing UV-Vis spectrophotometry at a wavelength of 260 nm (Thermo Scientific NanoDrop 2000c). To measure the DNA concentrations, 2 µl of eluent were directly added to the cuvette station.

Table 6: Results of the DNA measurement at 260 nm for sequencing.

Clones	Concentration [ng/µl]
pMAL–T6A Clone ♯1	35.6
pMAL–T6A Clone ♯2	31.2
pMAL–T6A Clone ♯3	36.8
pMAL–T6B Clone ♯1	30.9
pMAL–T6B Clone ♯2	36.7
pMAL–T6B Clone ♯3	32.5

Sequencing

Sample probes were sent to GenHunter Sequencing and the results analyzed with Applied Biosystems Sequence Scanner V 1.0. The DNA sequence was converted into an amino acid code with the online tool ExPASy translation (http://web.expasy.org/translate/). The sequencing results and the ExPASy translated amino acid code of T6A clones and T6B clones are shown below.

NNNNNNNNNGNNTGNCNTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTN
NAAGANGCGCAGACTAATTCGAGCTCGAACAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGAAG
GACTCTGGTTCCGCGTGGATCCCCGGAATTCGGCCTCGCTCTAGATGGGAAGCTCAAGCACGAGGACACCAACTG
AAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC
AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGC
CTGAATGGCGAATGGCAGCTTGGCTGTTTTGGCGGATGAGATAAGATTTTCAGCCTGATACNNATTAAATCTTAAC
GCCCAAGCGGCCTGATACTCATAGAATTTTGCC

Figure 14: DNA-sequencing results of pMAL-T6A

5'3' Frame 2
XXXXXXRTAVINAASGRQTVDEAXXXAQTNSSSNNNNNNNNNNNLGIEGRTLVPRGSPEFGLALDGKL
KHEDTN Stop KLGTGRRFTTS Stop LGKPWRYPT Stop SPCSTSPFRQLA Stop Stop RRGPHRSPFPTVAQP

Figure 15: Region of the amino acid sequence that includes the T6A region (highlighted in blue). Translated from the DNA-sequence in **Figure 14**.

NNNNNNNNGGGTTGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTGA
AAGACGCGCAGACTAATTCGAGCTCGAACAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGAAGG
ACTCTGGTTCCGCGTGGATCCCCGGAATTCCTGGCCTCGAGCACCATTGTGAAGGAGGGTGCCAACAAGGAGGTG
CTGGGGATATGAAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTT
AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
AGTTGCGCAGCCTGAATGGCGAATGGCAGCTTGGCTGTTTTGGCGGATGAGATAAGATTTTCAGCCTGATACCGA
TTAAATCATAACGCAGAACGGTCTGATA

Figure 16: DNA-sequencing results of pMAL-T6B

Figure 17: Region of the amino acid sequence that includes the T6B region (highlighted in blue). Translated from the DNA-sequence in **Figure 16**.

Expression of MBP-T6A and MBP-T6B

After making sure that the clones contained the plasmid with the T6A and the T6B construct, the plasmids were introduced into the *E. coli* strain BL21-codon plus (DE3)-RIL (1 µl of pMAL T6A & pMAL T6B were transferred to the cells) and inoculated on ice for 20 minutes. The cells containing the respective plasmid were spread on LB-agar plates (1000x ampicillin) and inoculated overnight at 37°C. The next day 2-3 colonies were picked from both plates and transferred into 5 ml LB (ampicillin 5 µl = 1000x) media and grown for approximately 6 hours. After 6 hours of growth, the grown cultures in 5 ml of LB media were transferred to 80 ml LB (80 µl ampicillin) and incubated overnight at 30°C, 225 rpm. On the following day, 10 ml of each of the 80 ml overnight cultures were transferred (T6A & T6B) to 4 x 1.1L, respectively, ending up with 8 x 1.1 L in total. After an incubation period of 3 hours at 30°C and 225 rpm the OD was measured. Ideally the OD_{600} should have been at around 0.6 to 0.8. However the OD_{600} was 1.4, meaning, that there was an overgrowth of the culture. Nonetheless, the proteins were purified. Therefore the shaker flasks were put on ice for 30 minutes and 1 mM of IPTG (1.1 ml to 1.1 l) was added. The flasks were then put on the shaker for overnight incubation at 25°C. The next

day, the cells were spun down at 6000 rpm for 10 minutes and the purification was preceded in the same way as described in 2.2 Expression and Purification of Recombinant MBP-fusion Proteins.

2.2. Expression and Purification of Recombinant MBP-fusion Proteins

For the expression of MBP-T1A, MBP-T6A, MBP-T6B, MBP-arrestin-3 and MBP itself, constructs of the *E. coli* strain BL21-codon plus (DE3)-RIL were used. Constructs for MBP-T1A, MBP-arrestin-3 and MBP were already available, however constructs for MBP-T6A and MBP-T6B were made according to 2.1 MBP-T6A and MBP-T6B Fusion Protein Constructs.

For the expression, three 15 ml falcon tubes were filled with 3 ml of LB/Ampicillin (1:500) and *E. coli* cells containing the respective plasmids. They were stored at -80°C and used for inoculation. The cells were grown in the shaker for 5 hours at 37°C. The pre-cultures were transferred to 1.1L LB/Ampicillin (1:733) and overnight cultures at 30°C and 220 rpm were grown to an OD_{600} of 1.5-2.0. The next day, the cells were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 5-6 hours. The cells from each 1.1L batch were pelleted (6000 rpm for 3 x 10 minutes, 4°C, Sorvall SLA-3000 rotor) and re-suspended in 45 ml lysis buffer in the presence of lysozyme (50 mM Tris pH 8.0, 5 mM EGTA, 2 mM Benzamidine (BA), 4mM DTT and 1mM PMSF and 3 mg/liter of lysozyme). After an incubation of 60 minutes on ice, the cells were sonicated (3 x 15 s; 80% amplitude; Sonic Dismembrator Model 500, Fisher) and centrifuged (6000 rpm for 60 minutes, 4°C, Sorvall SLA-3000 rotor).

The supernatant was filtrated and injected into a liquid chromatography (LC) system (General Electrics) with an MBP column (Sigma Aldrich) that has been equilibrated with a pre-cooled (4 °C) equilibration buffer (50 mM HEPES 7.3, 150 mM NaCl, 2 mM BA). The precooled (4 °C) supernatant ran through the LC

system with a flow through of 2 ml/minute. Afterwards the MBP-proteins were eluted with precooled (4 °C) elution buffer (50 mM Maltose, 150 mM NaCl, 50 mM HEPES 7.3, 2 mM BA) from the column and after that the proteins were run through a gravity based desalting column to clean up the proteins (General Electrics PD-10 Desalting Column).

Desalting of proteins

The desalting procedure was performed at 4 °C. First the columns were equilibrated with the same equilibration buffer as used in the LC. The protein samples were added and the eluted proteins were collected for protein measurement.

2.3. *In vitro* Binding Assay

Utilizing purified proteins in *in vitro* binding assays, excluding any interfering protein or complex, was applied to reveal the intensity of direct binding events between bait proteins and prey proteins. The binding assays were performed in a pull-down dependent fashion, utilizing purified MBP-fusion tagged proteins as bait (MBP-arrestin-3, MBP-T1A, MBP-T6A, MBP-T6B and MBP by itself) and respectively purified His-JNK1 and His-JNK2 as prey proteins. The purified His-JNK1 and His-JNK2 proteins were a generous gift by Dr. Kevin Dalby from the University of Texas at Austin. The splice versions JNK1α1 with around 46 kDa and JNK2α2 with around 54 kDa were utilized. MBP by itself was used as negative control, whereas MBP-arrestin-3 was utilized as positive control.

Incubation

For the incubation, 25 µl of 50% amylose resin slurry (New England Biolabs) was transferred to reaction tubes, incubated with 5 µg of the MBP-fusion tagged proteins (bait protein) and filled up to 75 µl with buffer (50 mM Hepes pH 7.3, 150 mM NaCl). The reaction tubes were placed on a shaker for 120 minutes at 4 °C.

After the first incubation with the bait protein, 5 µg of prey protein (either His-JNK1 / His-JNK2) diluted in buffer was added to the tubes, resulting in a final volume of 100 µl. The tubes were incubated for 60 minutes at 4 °C on a shaker.

Washing and elution

The incubated protein samples were transferred to 0.65 µM centrifugal filter tubes (Millipore Ultrafree®-MC and –CL Centrifugal Filter Units 0.65 µM) and washed three times with 300µl of precooled 4 °C washing buffer (50 mM Hepes pH 7.3, 150 mM NaCl) ; each washing step was performed at 5.000 rpm for 10 seconds. It was critical for the stability of the proteins to perform the washing steps quickly. Finally the proteins were eluted with 100µl elution buffer (50 mM maltose, 50 mM Hepes pH 7.3, 150 mM Nacl).

Following the pull-down, a 90% methanol precipitation at 14.000 rpm for 10 minutes was performed. The spun down proteins were re-suspended in 30µl 2xLaemelli SDS sample buffer (Sigma) and subjected to SDS-PAGE. 8µl of the total reaction sample was added to gels that were later subjected for western blot analysis and 12µl of the total reaction sample was used for gels that were later subjected to Coomassie Blue staining. The polyvinylidene difluoride (PVDF) membranes (Millipore) for western blot analysis were incubated overnight at 4°C, respectively with anti-JNK1 and anti-JNK2 antibody (1:1000) (Cell Signaling).

2.4. *In vitro* Kinase Activity Assay of His-JNKs by ac-MKK4 in the Presence of T1A

The activation assay was established to evaluate the effect of the T1A peptide on the phosphorylation levels of the MAPKs JNK1, and JNK2, by one of its upstream kinases MKK4 in a T1A concentration dependent fashion *in vitro*. Therefore T1A concentrations ranging from 0 to 30 µM as seen in **Table 7** were used for this assay.

First a master mix (MM) as seen in **Table 8** was made and 13 reaction tubes were prepared and labeled. The reactions were performed at final concentrations

of 50 nm active MKK4 (ac-MKK4) and 0.5μM final concentrations of His-JNK1 or His-JNK2 respectively.

Therefore 16 μl of the MM were transferred to each tube and 2 μl of the respective T1A concentration was added to the tubes. With 18 μl volume, the tubes were quickly spun down and incubated in a 30°C water bath for 15 minutes. To initiate the phosphorylation of JNK by ac-MKK4, 2 μl of ATP (0.5 mM) was added and the reaction tubes were mixed for 10 seconds. The phosphorylation was aborted after 10 seconds, by adding 20 μl 2xLaemelli SDS sample buffer (Sigma). 8μl of total reaction sample from each tube was added to polyacrylamide gels (10%) and SDS-PAGE was performed. Next a protein transfer to PVDF membranes was performed. The membranes were incubated with primary anti-phosphorylated-JNK antibody (Cell Signalling) 1:1000. The membranes were incubated overnight at 4°C and developed the next day.

Table 7: Range of T1A concentrations used for the activation assay

T1A concentrations [μM]	0	0.02	0.05	0.1	0.2	0.5	1	2	5	8	10	20	30

Table 8: Ingredients for master mix used for *in vitro* activation assays.

	Stock concentrations	**Amount for Master Mix for 15 reactions [μl]**	**Final Concentration Per Tube (20 μl total in RXN tube)**
Kinase Buffer (10X) ; RT	10 x	30	10 mM Hepes-Na, pH 7.3, 100 mM NaCl, 5mM MgCl2
DTT (dilute to 0.1M)	0.1M	15	5 mM
Ac-MKK4	0.65 mg/ml	1.1	50 nM
His-JNK1 /His-JNK2	2.53 mg/ml -> 52.7 μM	2.85	0.5 μM
T1A	Serial Dilutions ranging from 0.2 μM to 300 μM	-	-
H2O		191.05	-
Total		240	

2.5. Data Quantification

The x-ray films yielded from all western blot analysis were digitalized utilizing the ChemiDoc™ imaging system (Bio-Rad) and Quantity One 1-D Analysis Software.

3. Results

3.1. Results *in vitro* Binding Assays

Out of the three JNK isoforms (JNK1, JNK2 and JNK3), JNK1 and JNK2 are ubiquitously expressed, as well as arrestin-3 which has previously been shown to directly interact with JNK1 and JNK2 (Kook et al., 2013) (E. V. Gurevich & Gurevich, 2014). Utilizing purified proteins for *in vitro* binding assays revealed that T1A, a truncated version of arrestin-3 (first 25 N-terminal amino acids), directly interacts with JNK1α1 as well as JNK2α2 (see **Figure 19**, **Figure 20**).

Quantification independently repeated experiments revealed that MBP-T1A (45.5) exhibits a slightly weaker binding to His-JNK1α1 than MBP-arrestin-3 (54) as seen in **Figure 19**.

The opposite can be observed for binding to His-JNK2 as seen in **Figure 20**. MBP-T1A exhibits a binding affinity greater than 2 fold compared to MBP-arrestin-3.

In both cases, no unspecific interaction of the negative control, MBP, was observed (see **Figure 19**, **Figure 20**).

This shows that T1A's binding affinity for His-JNK2 is greater than for JNK1, which is in accordance with recent reports for arrestin-3 interaction with JNK1 and JNK2 (Kook et al., 2013) (E. V. Gurevich & Gurevich, 2014).

The data suggests that T1A is a critical binding element for ubiquitously expressed JNK1 and JNK2 interaction, which implies that T1A mimics the functions of arrestin-3 to a certain extent.

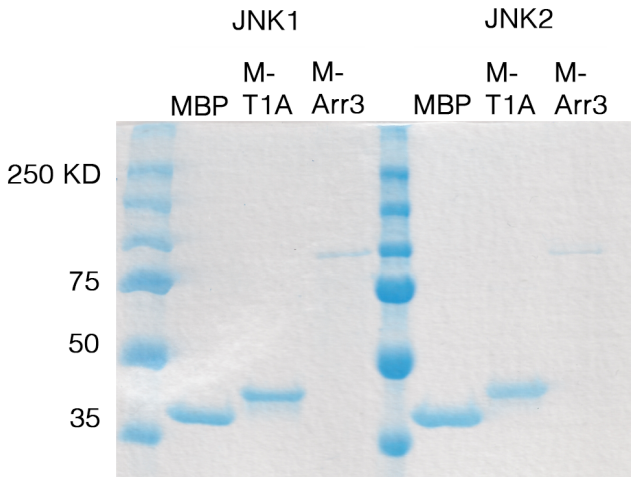


Figure 18: SDS-PAGE gel stained with Coomassie Blue shows the eluted bait proteins for the binding assays as seen in **Figure 19** and **Figure 20**.

3.1.1. Results *in vitro* Binding Assay: JNK1α1

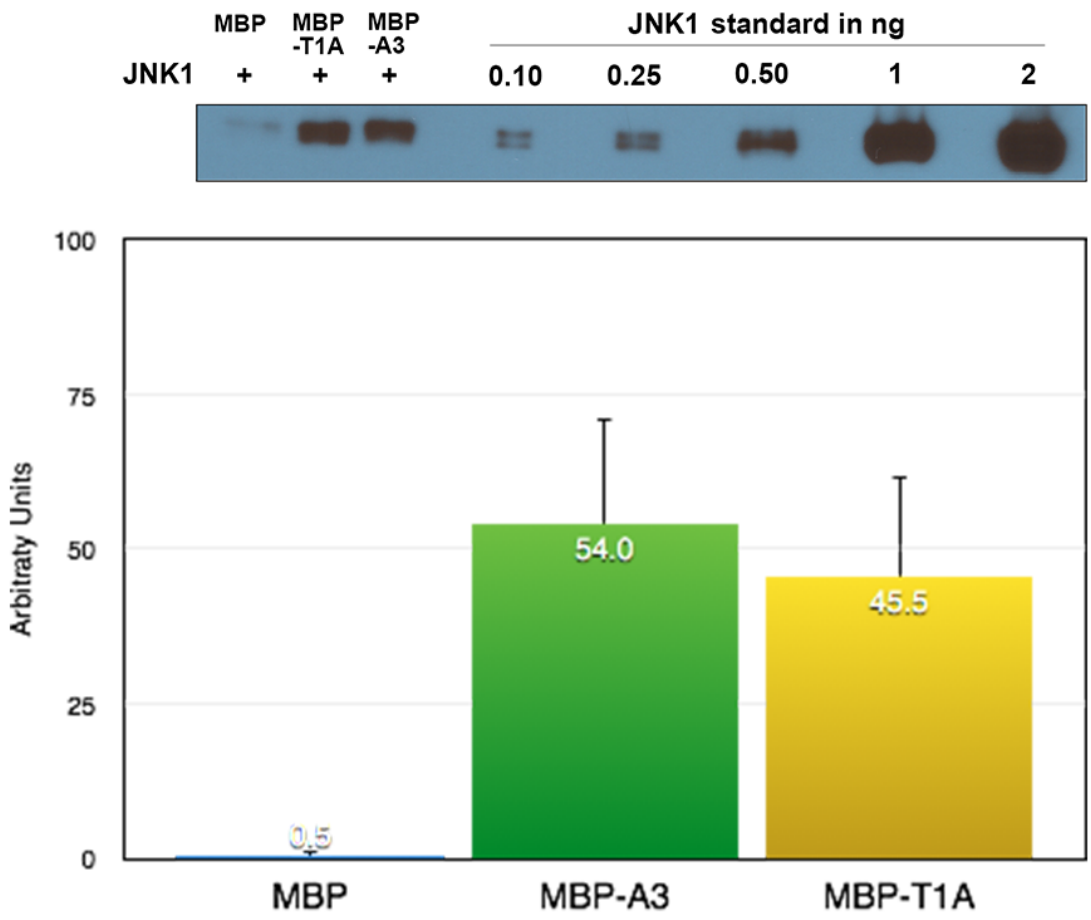


Figure 19: MBP-T1A and MBP-arrestin-3 bind JNK1 *in vitro* with similar affinity. The quantity of retained MBP, MBP-arrestin-3 and MBP-T1A was analyzed by Western blot with anti-JNK1 specific antibody. Bar graphs visualize the quantification analysis. Representative SDS-PAGE gels are depicted in **Figure 18**.

3.1.2. Results *in vitro* Binding Assay: JNK2α2

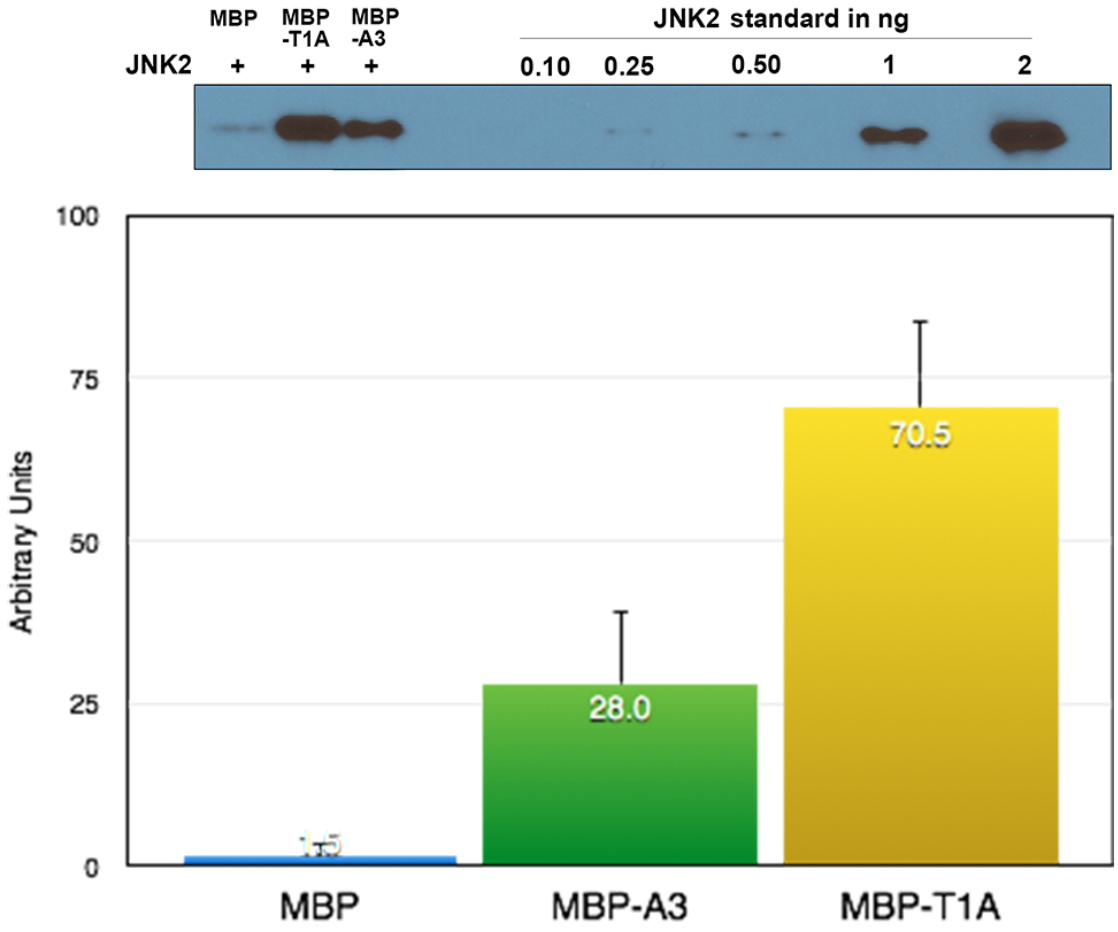


Figure 20: MBP-T1A and MBP-arrestin-3 bind His-JNK2 *in vitro* with MBP-T1A exerting an observable higher intensity than MBP-arrestin-3. The amount of bound MBP-arrestin-3 and MBP-T1A bound to His-JNK2 was analyzed by Western blot with anti-JNK2 specific antibody. Representative SDS-PAGE gels are depicted in **Figure 18** and bar graphs visualize the quantification analysis.

3.2. Results *in vitro* Kinase Activation Assay

Arrestin-3 has been shown to facilitate the activation of JNK1 and JNK2 through the ASK1-MKK4/7-JNK1/2 module *in vivo* (Kook et al., 2013). To examine if the truncated version of arrestin-3 (T1A) shows a similar behavior, kinase activation assays with reconstructions of the MKK4-JNK1/2 module with purified proteins were performed.

The data, as seen in **Figure 21** and **Figure 22**, suggest that T1A promotes the activation of ubiquitously expressed JNK1 and JNK2 by their upstream kinase, MKK4 *in vitro*. A biphasic effect of varying T1A concentrations on the phosphorylation levels of JNK1 and JNK2 can be observed. In both JNK isoforms, the phosphorylation level is enhanced at lower concentrations of T1A, whereas high T1A concentrations appear to have a drastically reducing effect. This observation is in agreement with previous reports, where arrestin-3 was shown to have a similar biphasic effect on the phosphorylation levels of JNK3α2 by its upstream kinases MKK4 and MKK7 (Zhan et al., 2013).

The highest phosphorylated JNK1 levels can be observed at a T1A concentration of 0.1 μM, whereas the highest phosphorylated JNK2 levels can be observed at a T1A concentration of 0.5 μM. Previous experimental data show that the optimal arrestin-3 concentration, promoting the phosphorylation of JNK3 by MKK4, are at 0.6 μM (Zhan et al., 2013). Furthermore the Gurevich laboratory reported that the optimal T1A concentration facilitating the activation of JNK3α2 by MKK4 was observed as high as 0.1 μM as shown in **Figure 23**. This suggests, that the truncated version (T1A) of arrestin-3 is able to scaffold and promote the phosphorylation of all three JNK isoforms.

3.2.1. Results *In vitro* Kinase Activation Assay: JNK1α1

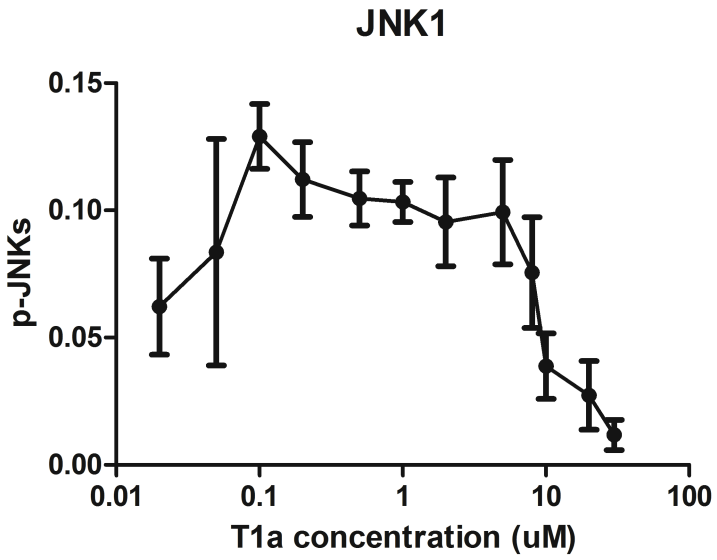
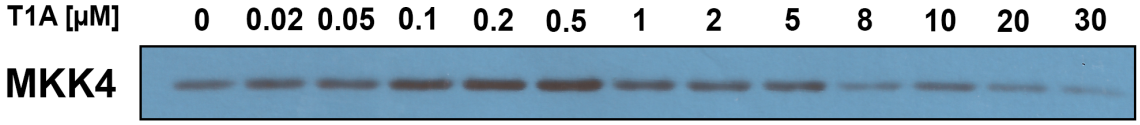


Figure 21: T1A facilitates the phosphorylation of purified JNK1α1 by MKK4. Western blot results show that JNK1α1 phosphorylation levels peak at an optimum level of T1A (0.1 μM) and wind down at supraoptimal T1A concentrations. The bell shaped curves can be explained by a biphasic dependence of T1A.

3.2.2. Results *In vitro* Kinase Activation Assay: JNK2α2



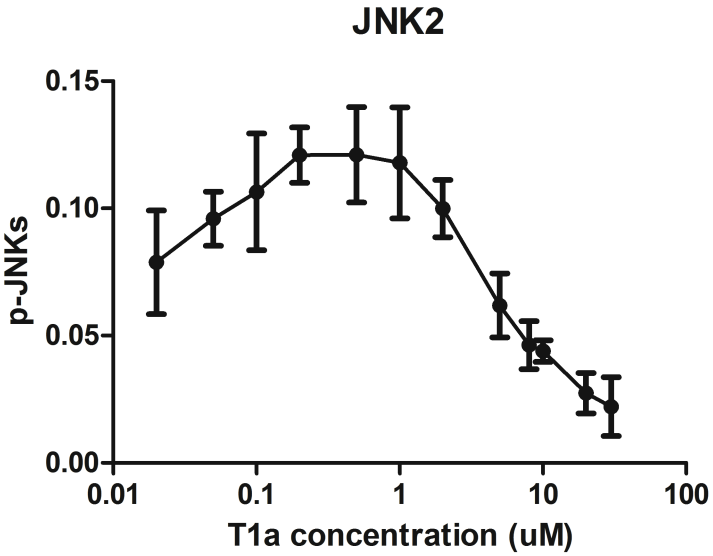


Figure 22 : T1A facilitates the phosphorylation of purified JNK2α2 by MKK4. Western blot results show that JNK2α2 phosphorylation levels peak at an optimum level of T1A (0.5 μM) and wind down at supraoptimal T1A concentrations. The bell shaped curves can be explained by a biphasic dependence of T1A.

3.2.3. T1A Facilitates the Activation of JNK3 by MKK4

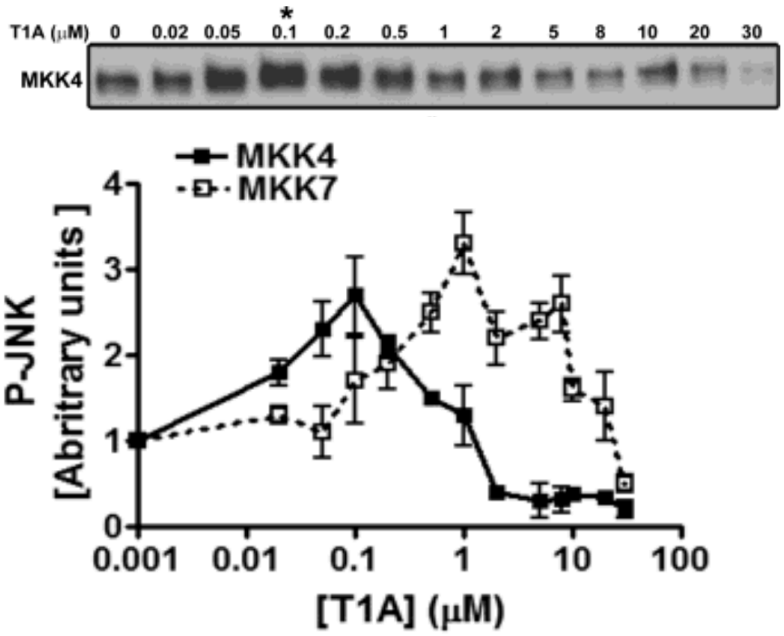


Figure 23: T1A promotes JNK3α2 phosphorylation by its upstream kinase MKK4. The optimal T1A concentration levels are around 0.1 μM, which is in accordance with the observed data for

JNK1 phosphorylation (see **Figure 21**). The data shown in this figure (**Figure 23**) was recently gathered by my colleagues from the Gurevich laboratory and are not mine. The data is part of a submitted publication that has not been published yet.

3.3. Results *in vitro* Binding Assays in the Presence of ATP

ATP, by binding proteins in a non-covalent fashion influences protein conformation (Ramirez-Alvarado, Kelly, & Dobson, 2010). As it appears, the presence of physiological ATP (1mM) and $MgCl_2$ (2mM) concentrations modulates the direct interaction of arrestin-3 with JNK3 as well as the interaction of arrestin-3 with MKK4. The experiments leading to this observation were first discovered on the interaction of arrestin-3 with JNK3 and were repeated by my colleague in the Gurevich laboratory, David J. Marcus. In addition to the interaction of arrestin-3, he also tested the effect of ATP on the interaction of T1A on JNK3 as well as arrestin-3 and T1A on MKK4. **Figure 24** and **Figure 25** reveal that the presence of ATP enhances the binding affinity of arrestin-3 to JNK3 by around 2 fold, whereas a limiting binding affinity of T1A towards JNK3 can be observed in the presence of ATP.

Analyzing the effect of ATP on the interaction of arrestin-3 and MKK4 shows that the presence of ATP drastically reduced the direct interaction as **Figure 25** shows.

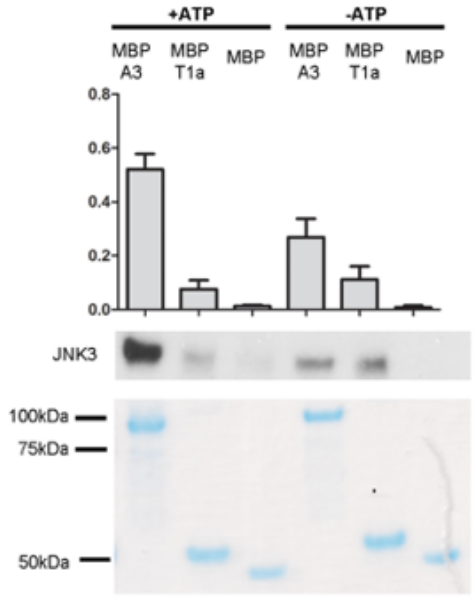


Figure 24: Physiological ATP concentrations enhance the direct interaction of arrestin-3 and JNK3. Data gathered by my colleague David J. Marcus from the Gurevich laboratory.

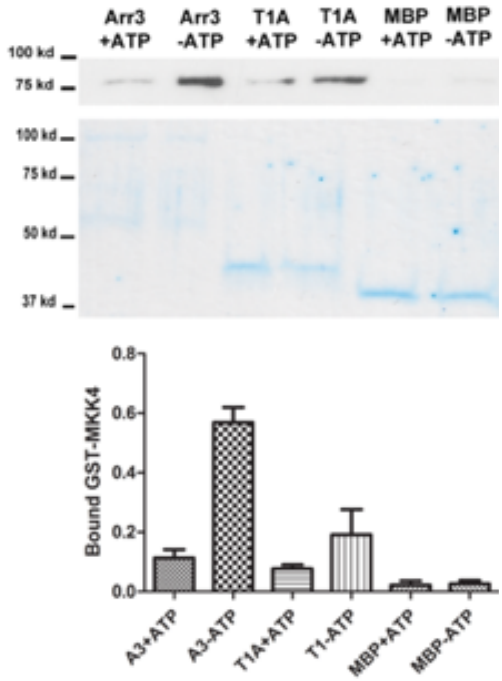


Figure 25: ATP modulation of arrestin-3 and T1A binding to MKK4. Data gathered by my colleague David J. Marcus from the Gurevich laboratory.

3.4. Summary Results

- MBP-T1A binds His-JNK1α1 with similar affinity as MBP-arrestin-3 *in vitro*
- MBP-T1A binds HisJNK2α2 with higher affinity than MBP-arrestin-3 *in vitro*
- The first 25 N-terminal amino acids of arrestin-3 are crucial for its binding to His-JNK1
- T1A facilitates the activation of His-JNK1 α1 *in vitro*
- T1A facilitates the activation of JNK1 & JNK2 *in vitro*
- ATP modulates arrestin-3 binding to His-JNK3
- ATP modulates arrestin-3 binding to MKK4
- The fusion proteins MBP-T6A (14 amino acids) and MBP-T6B (18 amino acids) show hardly any binding to JNK 2/3 and MKK7 compared to MBP-T6 (32 amino acids)

4. Discussion

4.1. Binding Assay

Previously it has been reported that the first 25 amino acid residues of arrestin-3 are the critical binding element involved in the direct interaction of arrestin-3 with JNK3α2. My data suggests that they are also the critical binding element for ubiquitously expressed JNK1 and JNK2. The results of the binding assays show that the amount of T1A retained by His-tagged JNK2 is higher than those retained by JNK1. This shows that the T1A's affinity for JNK2 is greater than for JNK1, which is in accordance with the recent reports for arrestin-3 interaction with JNK1 and JNK2 (Kook et al., 2013) (E. V. Gurevich & Gurevich, 2014). This suggests that T1A mimics the functions of arrestin-3 to a certain extent.

A limiting factor of the utilized *in vitro* binding assay is that the MAPKKK ASK1 is not available in purified form (Zhan et al., 2015). Therefore the reconstruction of the *in vitro* MAPK scaffold was incomplete. Future experiments aiming to identify T1A's effect on the ASK1-MKK4/7-JNK module can only be performed via cell-based assays.

4.2. T1A Facilitates the Activation of JNK1 and JNK2 *in vitro* Through the Upstream Kinase MKK4

The data shows that the first 25 amino acids of arrestin-3 can modulate the activity of ubiquitously expressed JNK1 and JNK2 *in vitro*. The data shows that the presence of optimal T1A concentrations enhances the phosphorylation level of JNK1 and JNK2 by one of their upstream kinases MKK4. All JNKs are activated by their upstream kinases MKK4 and MKK7, however the ability of a truncated version of arrestin-3 to facilitate JNK activation has never been reported before. The data suggests that T1A serves as a scaffold, facilitating the phosphorylation of JNK1 and JNK2 by MKK4 by directly binding the components JNK1, JNK2 and MKK4, bringing the phosphorylation targets into closer proximity of MKK4.

A biphasic dependence of T1A can be observed for JNK1 and JNK2 respectively. The decline of phosphorylated JNK can be explained due to an oversaturation of scaffolding protein. Once a certain level is reached, the scaffolding protein arranges the components of the JNK module (here MKK4 & JNK1/2), but runs out of kinases to bind, therefore decreasing the overall phosphorylation level.

My work demonstrated that T1A, a truncated version of arrestin-3, not only is the critical binding element for the ubiquitously expressed MAPKs JNK1 and JNK2, but also facilitates the activation of JNK1 and JNK2 through the upstream MAPKKs MKK4 as the data suggests (**Figure 21**; **Figure 22**). T1A recruits JNK1

and JNK2 and leads to its activation by the upstream kinase MKK4 as my gathered experimental data shows. By mimicking arrestin-3, this mini-scaffolding influences cellular fate.

4.3. ATP Modulates the Binding Affinity of Arrestin-3 to JNK3 and MKK4

Proteins interact with arrestins in two states receptor-bound arrestin and basal arrestin (E. V. Gurevich & Gurevich, 2014). At least 162 proteins have been identified as arrestin-binding partners, which compete with each other. However the affinity for receptor-associated arrestin and basal arrestin differs, therefore some proteins would rather bind to receptor-bound arrestin than to the basal arrestin and vice versa, but also proteins that do not distinguish in affinity between the two forms have been described (E. V. Gurevich & Gurevich, 2014). Arrestin-3 as a scaffold protein can only bind 4 to 6 proteins at a time (E. V. Gurevich & Gurevich, 2014), therefore one might ask which protein interacts with arrestin?

The data in **Figure 24** and **Figure 25** shows that physiological ATP concentrations modulate the binding of arrestin-3 to JNK3 as well as to MKK4. However in case of arrestin-3 binding to JNK3, ATP enhances the binding affinity, whereas the opposite is true for arrestin-3 binding to MKK4. The non-covalent modification of ATP binding to proteins influences protein conformation which suggests that the conformational state of arrestin-3 in the presence of ATP prefers to directly interact with JNK3 rather than MKK4 (Ramirez-Alvarado et al., 2010).

4.4. *In vitro* Binding Assays of MBP-T6A/MBP-T6B Interaction with JNK2/3 or MKK7

Unfortunately no interaction between MBP-T6A/MBP-T6B and JNK2/3 or MKK7 could be observed. It is very likely that the arrestin-3 region T6 was separated at

the wrong site, meaning that certain amino acids for either MBP-T6A or MBP-T6B were missing which are crucially involved in binding.

5. Future Perspectives

Future goals are going to evaluate if MKK7, the upstream MAPKK of JNK, can promote phosphorylation of JNK1 and JNK2 on the Threonine amino acid residue under the presence of T1A.

It is indispensable to mention that in addition to the performed *in vitro* experiments, *in vivo* experiments are required to foster the acquired findings.
The obvious advantage of *in vitro* assays is that it is a controlled system in which the influence of other factors can be ruled out. However a setback is that the MAPKKK, ASK1 cannot be purified, therefore having an incomplete JNK MAPK module (Zhan et al., 2015).

In summary, my work provides first evidence that a truncated version of arrestin-3 (T1A) directly binds to both ubiquitously expressed JNK isoforms JNK1 and JNK2 and promotes the phosphorylation by their upstream kinase MKK4. In comparison to previous reports, my work shows that the optimal T1A concentrations for JNK1 and JNK3 phosphorylation by their upstream kinase MKK4 are very similar. A surprising difference, in the binding affinity of the truncated version (T1A) as to the full-length arrestin-3, was discovered since the truncated version exhibits a higher binding affinity (>2 fold) to JNK2 than the full-length arrestin-3.

T1A's size (25 amino acids) paves the way to design small molecule mimics that can be used as tools for targeted manipulation of pro-apoptotic JNK signaling in cells.

Arrestin's involvement in G protein desensitization, receptor endocytosis

and signal transduction as scaffold proteins, places them at an important intersection of cellular communication where external and internal inputs are integrated into coherent behavior (E. V. Gurevich & Gurevich, 2014).

My work, which gives a better understanding of arrestin's role in signaling pathways contributes to the ultimate goal of constructing protein scaffolds that can selectively couple specific receptors with the signaling pathways, that we choose. Therefore arrestins are a great proving ground for the development of entirely new types of drugs.

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7. List of Figures

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