Transcriptional regulation by the mammalian Stress-activated protein kinase p38

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A Mat

A mi familia

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SUMMARY

Regulation of transcription by Stress Activated Protein Kinases (SAPKs) is an essential aspect for adaptation to extracellular stimuli. In mammals, the activation of the p38 SAPK results in the regulation of gene expression through the direct phosphorylation of several transcription factors. However, how p38 SAPK regulates the proper gene expression program of adaptation to stress as well as the basic mechanisms used by the SAPK remains uncharacterized. The results displayed in this manuscript show that the p38 SAPK plays a central role in the regulation of gene expression upon stress, as up to 80% of the upregulated genes are p38 SAPK dependent. Moreover, we also observed that a specific set of genes were upregulated in response to each specific stimuli, and just a small set of genes were commonly up-regulated by several stresses, which involves mainly transcription factors. In addition, we observed that, to proper regulate gene transcription, the p38 SAPK is recruited to stress-induced promoters via its interaction with transcription factors. Additionally, p38 activity allows the recruitment of RNA polymerase II and the MAPKK MKK6 to stress-responsive promoters. The presence of active p38 SAPK at open reading frames also suggests the involvement of the SAPK in elongation. Altogether, the results showed in this manuscript establish the p38 SAPK as an essential regulator in the transcriptional response to stress, as well as define new roles for p38 in the regulation of transcription in response to stress.

La regulación de la transcripción por las Proteínas Quinasas activadas por Estrés (SAPKs) es un aspecto esencial para la adaptación a los estímulos extracelulares. En mamíferos, la activación de la SAPK p38 da lugar a la regulación de la expresión génica a través de la fosforilación de varios factores de transcripción. Sin embargo, cómo p38 SAPK regula el programa de expresión génica de adaptación al estrés así como los mecanismos utilizados por la SAPK permanece sin caracterizar. Los resultados presentados en este manuscrito muestran que p38 SAPK juega un rol central en la regulación de la expresión génica en respuesta a estrés, ya que hasta el 80% de los genes inducidos son dependientes de p38 SAPK. También observamos que en respuesta a cada tipo de estrés se induce un grupo de genes específicos, y sólo hay una pequeña respuesta de genes comunes a los diferentes tipos de estrés la cual engloba principalmente factores de transcripción. Además, hemos observado que para regular la transcripción, p38 se recluta a los promotores de respuesta a estrés a través de su interacción con factores de transcripción. Asimismo, la actividad de p38 permite el reclutamiento de la RNA Polimerasa II y de la MAPKK MKK6 a los promotores inducidos por estrés. La presencia de p38 activa en las regiones codificantes sugiere su participación durante la elongación. En conjunto, los resultados mostrados en este manuscrito establecen a p38 como un regulador esencial de la transcripción en respuesta a estrés, así como definen nuevas funciones de p38 en la regulación de la transcripción en respuesta a estrés

PREFACE

The identification of new biomarkers and potential targets for cancer therapies is one the main objectives in biomedical research nowadays. For that reason, understanding the molecular mechanisms that take place in the cell in response to stresses is a key point to study.

Studies in eukaryotes like *Saccharomyces cerevisiae*, as well as studies with human cell lines, provide important information on the mechanisms of cell regulation that are taking place in response to stress.

The present work results from the need to understand the mechanisms of regulation that take place under stress conditions and, to be able to recapitulate these mechanisms in the identification of possible targets for several diseases and cancer therapies as a consequence of stress stimulation.

Our results show that the p38 SAPK is an essential regulator of the transcriptional response to stress. The knowledge of the mechanisms used by the SAPK is relevant to understand how the appropriate pattern of genes are expressed under stress conditions, as deregulation of the appropriate pattern of gene expression leads to many diseases and cancers. In addition, identification and characterization of the various substrates of p38 might have a substantial impact on how clinical strategies will be addressed to treat cancer.

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INTRODUCTION

1. MAP Kinase pathways

In response to changes in the extracellular environment, cells have to coordinate their intracellular activities to maximize their chances of survival and proliferation. In eukaryotic cells, both unicellular and pluricellular, extracellular stimuli are converted to intracellular signals through multiple MAP kinase cascades. Exposure of cells to stress results in rapid activation of a family of highly conserved MAP kinases, known as SAPKs (stress-activated protein kinases). SAPK pathways are among the most widespread mechanisms in eukaryotic cell regulation. In fact, all eukaryotic cells possess multiple MAPK pathways, each of which is preferentially activated by distinct sets of stimuli, thereby allowing the cell to respond coordinately to multiple inputs.

Activation of the SAPKs results in the generation of a set of adaptive responses that lead to the modulation of several aspects of the cellular physiology essential for cell survival.

1.1. Modular organization

MAPK pathways are organized in modules containing at least three types of protein kinases, which transmit signals by sequential phosphorylation events in a hierarchical way (Figure 1). The activation of MAPK kinase kinase (MAPKKK) occurs by phosphorylation through an upstream protein kinase or through interaction with other proteins, a process that often involves small G-proteins. Once activated, the MAPKKKs phosphorylate and thereby activate MAPKKs on serine (Ser) and threonine (Thr) residues within a conserved part at the N-terminal lobe of the kinase domain. Subsequently, the MAPKKs phosphorylate MAPKs on a threonine (sometimes serine) and tyrosine (Tyr), residues within their activation loop for their full activation (Canagarajah et al, 1997). These

phosphorylation sites are separated by only one amino acid, thereby defining a tripeptide motif, Thr-X-Tyr. Historically, the nature of the amino acid X located between the two phosphor-acceptor sites has defined the different groups of MAPKs. The ability of specific MAPKKs to recognize different MAPKs depends in part on these distinct tri-peptide motifs. Therefore these motifs contribute to the specificity of the MAPK pathway activation.

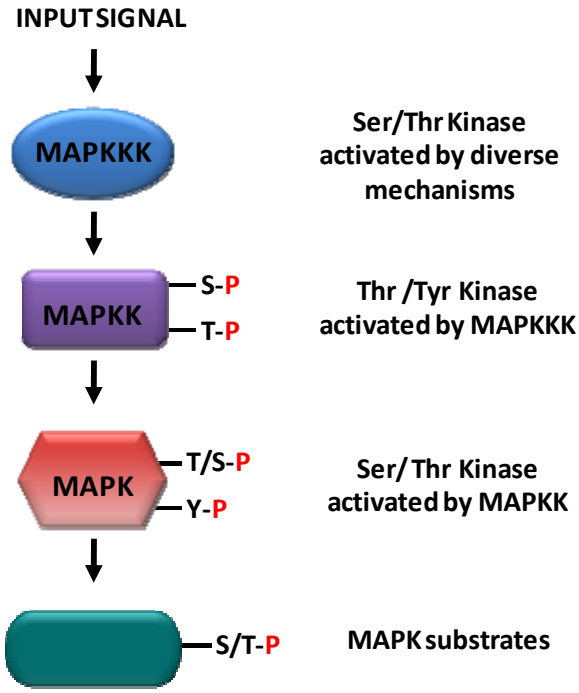


Figure 1. Schematic diagram of a MAPK pathway module. The core module of a MAPK pathway is composed of three kinases: MAPK kinase kinase, MAPK kinase and MAPK, which are sequentially activated by phosphorylation.

Upon activation, many MAPKs dissociate from their scaffolding molecules and MAPKKs, and translocate from the cytoplasm to the nucleus (Lenormand et al, 1998). However, a portion of activated MAPK remains in the cytoplasm to mediate cytoplasmic events (Reiser et al, 1999). Once localized into the nucleus, the MAPKs bind their target

molecules through docking-mediated interactions, followed by the recognition and phosphorylation of the Ser/Thr-Pro phospho-acceptor motif in their substrates.

Up to date, most of the reported substrates of the MAPKs are transcription factors, although MAPKs are also able to phosphorylate several substrates including other protein kinases, cytoskeleton associated proteins and ionic transporters (Chen et al, 2001).

1.2. Signalling specificity in MAPK pathways

Since different MAPK pathways within the same organism share protein kinases and phosphatases, there are numerous crosstalk nodes between these pathways. Given the complexity and diversity of MAPK regulation and function, cells must have mechanisms to maintain the specificity in order to avoid unwanted responses upon a stimulus, allowing at the same time that the proper response takes place. Scaffolding proteins play a key role in this signalling specificity. These proteins create a functional signalling module which promotes the interaction of the proper partners, allowing the coordinated and efficient activation and function of MAPK components in response to specific types of stimuli (Pawson & Scott, 1997). Importantly, it has been described that scaffolds not only physically link the MAPK cascade components, but also may allosterically regulate the phosphorylation events required for MAPK activation (Good et al, 2009).

Although a number of scaffold proteins have been implicated in the regulation of different MAPK signalling modules (Morrison & Davis, 2003), not many have been found to participate in the p38 MAPK cascade. One example of this is the protein osmosensing scaffold for MEKK3 (OSM) which forms a complex with Rac, MEKK3 and MKK3 in

the activation of the p38 SAPK in response to osmotic stress (Uhlik et al, 2003). OSM seems to be the mammalian counterpart of STE50 in *Saccharomyces cerevisiae,* which is required for the regulation of Hog1 SAPK upon osmostress (de Nadal et al, 2002).

1.3. The yeast Hog1 SAPK pathway

The existence of multiple MAPKs with distinct regulation and functions was found initially in the budding yeast *Saccharomyces cerevisiae (S. cerevisiae)*. In fact, this organism has five typical MAP kinases which are allocated in five distinct MAP kinase cascades. These MAPKs are required for several aspects of the yeast life as mating, sporulation, filamentous cell growth, cell wall integrity and maintenance of the appropriate turgor pressure (Hohmann, 2002).

A prototype of the MAPK family is the yeast *S. cerevisiae* high osmolarity glycerol 1 (Hog1) MAPK pathway. The Hog1 MAPK pathway, also called as stress-activated protein kinase (SAPK) pathway, is the best-characterized osmoresponsive system in eukaryotes and hence serves as a prototype osmosensing signalling pathway. For a single cell organism such as budding yeast a rapid adaptation to high osmolarity through Hog1 is a matter of life or death. Indeed a *hog1Δ* strain is not able to grow on a high osmolarity media (Figure 2). Activation of Hog1 SAPK pathway in response to an increase of the extracellular osmolarity results in the accumulation of intracellular osmolytes such as glycerol or ions in order to counterbalance the ensuing efflux of water from the cell (Gustin, Albertyn, Alexander & Davenport, 1998). This leads to a set of osmoadaptive responses, including metabolic regulation, cell cycle progression, translation and gene expression regulation (de Nadal et al., 2002; Sheikh-Hamad and Gustin, 2004).

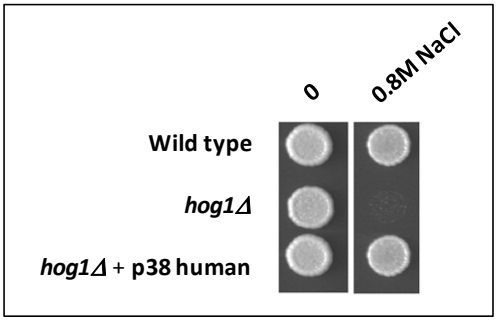


Figure 2. The Hog1 SAPK is essential for cell survival under stress conditions. A *hog1Δ* strain is unable to grow in medium with high concentration of NaCl. The functional conservation between the Hog1 and the p38 SAPK pathway is illustrated by the fact that the expression of the human p38 gene in the yeast *S.cerevisiae* rescued the defect in growth on hyper-osmolar media.

Two independent studies showed the mammalian p38 SAPK is the structural and functional homolog of the yeast Hog1 SAPK. The functional conservation between the Hog1 and the p38 SAPK pathway is illustrated in Figure 2 by the fact that the corresponding human gene complements the *hog1Δ* yeast mutants under a high concentration of sodium chloride in the media (Galcheva-Gargova et al, 1994; Han et al, 1994).

Schematically, this signalling pathway consists of two upstream independent branches that converge on the MAPKK Pbs2. The two branches are activated by completely different sensing mechanisms. One mechanism involves a two-component osmosensor (Sln1–Ypd1–Ssk1), which directly regulates the activity of the Ssk2 and Ssk22 MAPKKKs. The second sensing system is extremely complex and is not completely characterized. It involves the mucin-like proteins Hkr1 and Msb2, which likely act as osmosensors, activate Pbs2 in collaboration with a number of proteins, that include the transmembrane protein Sho1, Opy2, the small G-protein Cdc42, the PAK (p21-activated kinase) family kinase Ste20, Ste50 and the MAPKKK Ste11 (Chen and Thorner, 2007; Hohmann et al, 2007;

Tatebayashi et al, 2007; de Nadal et al, 2007). Once Pbs2 is activated, it phosphorylates and activates Hog1 SAPK (Figure 3).

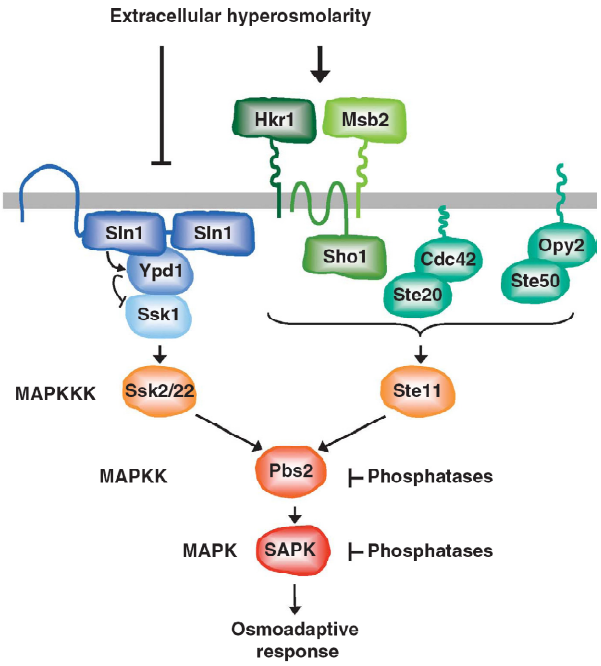


Figure 3. Schematic diagram of the yeast Hog1 SAPK pathway. Two independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs that converge on a common MAPKK, Pbs2. Under osmostress, activated Pbs2 phosphorylates Hog1, which induces a set of osmoadaptive responses (de Nadal & Posas, 2010).

2. Mammalian MAPK pathways

The basic structure of the MAPK signaling module has been conserved throughout evolution from yeast to mammals. However, mammalian MAPK pathways have been specialized in more complex processes required for the life of eukaryotic pluricellular organisms. Mammalian MAPKs control many biological processes such as development, cell differentiation, cell proliferation, cell death, as well as regulation of short-term changes required for adaptation to stress.

2.1. The four subfamilies of MAPKs in mammals

In multicellular organisms, there are four subfamilies of MAPKs. These MAPKs include the extracellular signal-regulated kinases, ERK1 and ERK2; the ERK5 MAPK which is distantly related to ERK1/2; the c-Jun NH2-terminal kinases, JNK 1, JNK 2, and JNK 3; and p38 SAPK comprising four isoforms, p38α, p38β, p38γ, and p38δ (Figure 4).

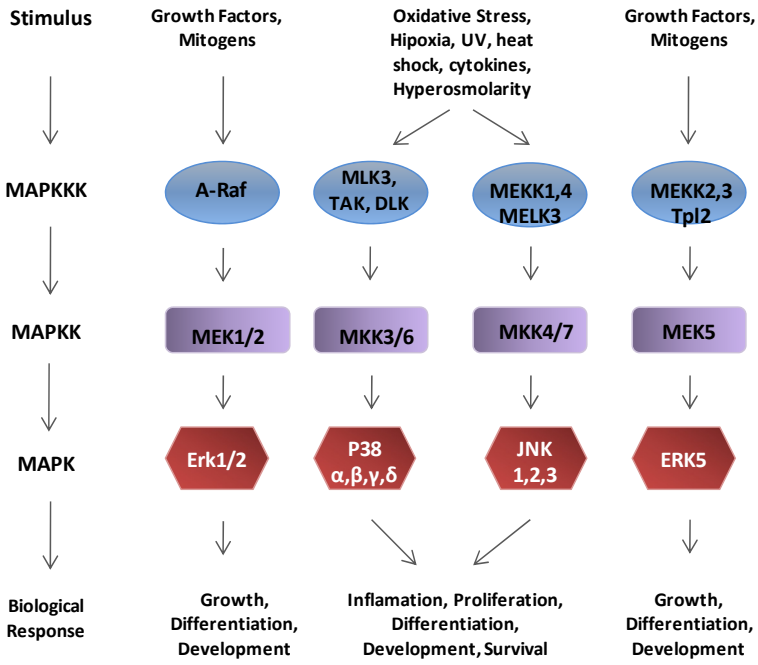


Figure 4. The mammalian MAP kinase pathways. There are four MAPK pathways in mammals: ERK1/2, p38 α, β, γ and δ, JNK 1,2,3 and ERK5.

2.2. ERKs, JNKs and ERK5 MAPKs

2.2.1. Extracellular signal-regulated kinases 1/2 (ERK1 and ERK2)

ERK1 and ERK2 are proteins of 43 and 41 kDa which are nearly 85% identical overall, with greater identity in the core regions involved in

substrate binding (Caffrey et al, 2000). Both are expressed in almost every cell, although their relative abundance in tissues is variable. ERK1 and ERK2 pathways can be activated by many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric guanine nucleotide-binding protein G protein-coupled receptors (GPCRs), transforming agents, and carcinogens. ERK1 and ERK2 are involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells.

Once the stimulus binds to the receptor, the MAPKKK upstream of the ERK1 and ERK2 pathway are the Raf isoforms (A-Raf, B-Raf and Raf-1) are activated, which subsequently activate a pair of closely related MAPKKs, MEK1 and MEK2 (Fabian et al, 1993; Stancato et al, 1997) which are responsible of the ERK1 and ERK2 activation.

Activated ERK1 and ERK2 phosphorylate numerous substrates in all cellular compartments, including various membrane proteins (CD120a, Syk, and Calnexin), nuclear substrates (NF-AT, Elk-1, MEF2, c- Fos, c-Myc, and STAT3), cytoskeletal proteins, and several Protein Kinases (Chen et al, 2001).

2.2.2. c-Jun amino-terminal kinases 1-3 (JNK1, JNK2 and JNK3)

JNK1-3, also known as SAPKα, SAPKβ and SAPKγ, exist as 10 or more spliced forms. They were originally named as JNK by their ability to phosphorylate the N-terminal of the transcription factor c-Jun. They were also identified as SAPKs because their activities strongly increase in response to different stresses such as cytokines, UV irradiation, growth factor deprivation, and agents that interfere with DNA and protein synthesis. JNK1-3 show more than 85% of similarity in their core catalytic domain. JNK1 and JNK2 are widely expressed, whereas JNK3 is

mainly found in the brain (Wagner & Nebreda, 2009). JNKs are involved in cytokine production, which mediate the inflammatory response and the immune system. They are also involved in stress-induced and developmentally programmed apoptosis as well as cell transformation.

Upon receptor binding, JNKs can be activated by the upstream MAPKKKs MEKK1,4, MLK3 and ASK1 which subsequently activate the MAPKKs MKK4 and MKK7. Unlike MEK1/2 which are specific to ERKs, MKK4 and MKK7 are not specific to JNKs since MKK4 can also phosphorylate p38α and p38δ isoforms in specific cell types and conditions (Jiang et al, 1997). JNKs phosphorylate several substrates located in membranes, in the cytoplasm, on the cytoskeleton, and also in the nucleus. Although there are many JNK substrates, its major target is the Jun family of transcription factors which are essential components of the Activation Protein 1 (AP-1), a dimeric transcription factor composed of Fos and Jun proteins.

2.2.3. Extracellular-signal-regulated kinase 5 (ERK5)

ERK5 is also termed BMK (big MAPK) because its size (100 kDa) nearly doubles the size of the other members of the MAPK family. It contains a catalytic domain homologous to those of ERK1/2 and a unique and very long carboxyl-terminal domain.

ERK5 is expressed in a variety of tissues and like ERK1/ 2 it is activated by a range of hormones, growth factors, cytokines as well as cellular stresses (Hayashi & Lee, 2004). It is involved in cell survival, cell differentiation, cancer (ERK5 is activated by Ras oncogene) and heart function (Wang & Tournier, 2006). In addition it has been shown that ERK5 has a key role in cardiovascular development and neural differentiation (Nishimoto and Nishida, 2006).

Numerous upstream MAPKKK to ERK5 have been described, such as COT, MEKK3 and MEKK2. On the other hand, MEK5 is the only MAPKK upstream to ERK5 reported to date since other MAPKKs do not appear to influence ERK5 activity (Nishimoto and Nishida, 2006). Several molecules have been identified as substrates of the ERK5 pathway including the myocyte enhancer factor 2 family of transcriptional factors (MEF2A, MEF2C and MEF2D), Sap1a (Ets-domain transcription factor) and SGK (serum and glucocorticoid-inducible kinase) (Hayashi & Lee, 2004; Kamakura et al, 1999; Kato et al, 1997).

3. The p38 SAPK pathway

The p38 MAPK pathway is also known as p38 SAPK (Stress Activated Protein Kinase) because this protein kinase can be activated by different stress stimuli. It plays a key role in the regulation of cellular responses to many types of stresses, but also in the regulation of proliferation, differentiation, survival and development of specific cell types.

3.1. The p38 SAPK isoforms

There are four p38 MAP kinases in mammals: α, β, γ and δ. Based on their similarity, sensitivity to pharmacological inhibitors, substrate specificity and expression pattern they can be divided into two groups: p38α and p38β on the one hand and p38γ and p38δ on the other. This is evident firstly from their amino-acid sequence identity: p38α and p38β are 75% identical, whereas p38γ and p38δ are 62% and 61% identical to p38α, respectively. Of note, p38γ and p38δ are more identical (70%) to each other. Secondly, their susceptibilities to inhibition at low concentrations by the compounds SB203580 and SB202190. *In vitro* and

in vivo assays demonstrated that only p38α and p38β are inhibited by these compounds, whereas p38γ and p38δ were completely unaffected by the drugs (Feaver et al, 1994). A third difference between these two subgroups of p38 MAPKs is related to the substrate selectivity of these kinases. For example the substrates MAPKAPK2, MAPKAPK3 and glycogen synthase are preferentially phosphorylated by p38α and p38β than by p38γ and p38δ (Cuenda & Rousseau, 2007). A fourth difference between these two subgroups is that while p38α and p38β are ubiquitously expressed, p38γ and p38δ appear to have a tissue specific expression pattern. Actually, p38γ is predominantly expressed in skeletal muscle whereas p38δ is enriched in lung, kidney, testis and pancreas (Ono & Han, 2000).

Among all p38 SAPK isoforms, p38α is the best characterised and it is expressed in most cell types. p38α was first isolated as a 38 kDa protein, which was rapidly tyrosine phosphorylated in response to lipopolysaccharides (LPS) stimulation (Han et al, 1993). The molecular cloning of the protein revealed that it was a MAP kinase family member (Han et al, 1994). Moreover, p38α (also termed RK and p40) was identified as an upstream kinase of MAP kinase-activated protein kinase-2 (MAPKAPK-2 or MK2) in IL-1 or arsenite-stimulated cells (Rouse et al, 1994). In addition, as mention before, p38α is the mammalian homologous of the yeast Hog1 MAPK (Galcheva-Gargova et al, 1994) (Figure 3). Three years after the identification of p38α, three more p38 SAPKs isoforms were described: p38β (also called SAPK2b) (Jiang et al, 1996), p38γ (also called SAPK3 and ERK6)(Mertens et al, 1996), and p38δ (also called SAPK4) (Goedert et al, 1997). All isoforms phosphorylate the Ser-Pro or Thr-Pro MAPK consensus motifs, but some substrate selectivity has been reported. For example, the microtubule associated protein Tau is a better substrate for p38γ and p38δ, while

MAPKAP kinase-2 (MK2) and MK3 are better substrates for p38α and p38β. Loss of p38β, p38γ or p38δ does not perturb normal development indicating that they are partially redundant. However, disruption of the p38α gene results in embryonic death because of placental defects, demonstrating that some functions performed by p38α cannot be compensated by other family members (Adams et al, 2000).

3.2. p38 SAPK activators

p38 SAPK can be activated by several stresses such as UV, hypoxia, hyperosmolarity, heat shock, oxidative stress, inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), DNA damage agents, the bacterial lipopolysaccharide (LPS), arsenic trioxide as well as different drugs like Anisomycin (Kyriakis J, 2001; Neuder et al, 2009). Among the different stimuli that can activate p38 SAPK, we have mainly worked with TNFα, anisomycin and osmostress.

3.2.1. Tumor necrosis factor α (TNFα)

TNFα is a cytokine involved in systemic inflammation and it belongs to a group of cytokines that are stimulated during the acute phase reaction. The primary role of TNFα is the regulation of immune cells. TNFα is able to induce apoptotic cell death, inflammation, and also to inhibit tumorigenesis and viral replication. Deregulation of TNFα production has been implicated in a variety of human diseases, including major depression (Dowlati et al, 2010), Alzheimer's disease (Swardfager et al, 2010) and cancer (Locksley et al, 2001). Binding of TNFα to its two receptors, TNF receptor type 1 and 2 (TNFR1 and TNFR2), results in the recruitment of signal transducers which activate distinct effectors. Through complex signalling cascades and networks, three main pathways can be activated:

*** Activation of NF-κB pathway:** Through different effectors, the IκB kinase (IKK) is activated, and this results in the phosphorylation of the inhibitory protein IκBα which normally binds to NF-κB transcription factor and retains it inactive in the cytoplasm. Phosphorylated IκBα is degraded, releasing NF-κB, which is therefore translocated to the nucleus. Once in the nucleus, NF-κB mediates the transcription of a vast array of genes involved in several aspects of cell survival (Baud and Michael Karin, 2001). The activation of the NFκB pathway is described in the following chapter and is illustrated in Figure 15.

*** Activation of the MAPK pathways**: TNFα binding to its receptors results in the activation of different MAPKKKs, as NIK, MEKK1 and ASK1. Next, these MAPKKKs induce a strong and transient activation of JNK and p38 SAPK pathways (Wajant et al, 2003) which modulate several biological processes.

*** Induction of death signaling**: TNFR1 is also involved in death signalling, however, the role of TNFα in cell death is minor compared to its overwhelming functions in the inflammatory process. Nevertheless, TNFα binding to its receptors results in the recruitment of the cysteine protease caspase-8. It is described that a high concentration of this caspase induces its autoproteolytic activation and subsequent cleaving of effector caspases, which leads the cell to apoptosis (Gaur & Aggarwal, 2003).

3.2.2. Anisomycin

Anisomycin is an antibiotic produced by *Streptomyces griseolus* which inhibits protein synthesis by binding to 60S ribosomal subunits and blocking peptide bond formation, thereby preventing elongation which results in polysome stabilization. It is also a strong activator of JNK and

p38 SAPKs in mammalian cells even used at a concentration that does not fully block protein synthesis (Grollman, 1967).

Although anisomycin provokes a strong and transient activation of the p38 SAPK little is known about the molecular mechanism underlying the effect of this drug. The ribotoxic effect of anisomycin results in the phosphorylation of the MAPKKKs MLK7 and ZAK and the MAPKKs MKK3/6 which subsequently activate p38 SAPK (Jandhyala et al, 2008; Wang et al, 2005).

3.2.3. High osmolarity

When eukaryotic cells are exposed to a hyperosmotic environment, they undergo an immediate process of shrinkage as a result of water efflux from the cell. To maintain cellular integrity and homeostasis under these conditions, cells restore their volume via the modulation of ion transport processes, as well as the production of specific hormones such as adrenomedullin, endothelin, guanylin and uroguanylin. This results in an increase of the transport across the cell membrane which alters the cellular osmolarity (Burg et al, 2007; Waldegger & Lang, 1998).

As explained above, the response to osmostress in mammals is primarily mediated by the p38 SAPK pathway. Osmostress signaling was initially described to involve the MAPKKK MEKK4/MTK1 (Takekawa et al, 1997). A more recent study showed that an osmotic shock induces the recruitment of the malcavernin/OSM protein to membrane ruffles, where it serves as a scaffold that brings together the MAPKKK MEKK3 with its upstream activator, the small GTPase Rac. It is believed that Rac recruitment to ruffle actin filaments mediates MEKK3 activation which subsequently activates MKK3 and p38. Altogether the mammalian Rac–OSM–MEKK3–MKK3 complex has been proposed to be the yeast counterpart of the CDC42–STE50–STE11–Pbs2 complex in *S. cerevisiae,*

and it regulates the p38 SAPK activity upon osmostress (Hilder et al, 2007; Uhlik et al, 2003) (Figure 5).

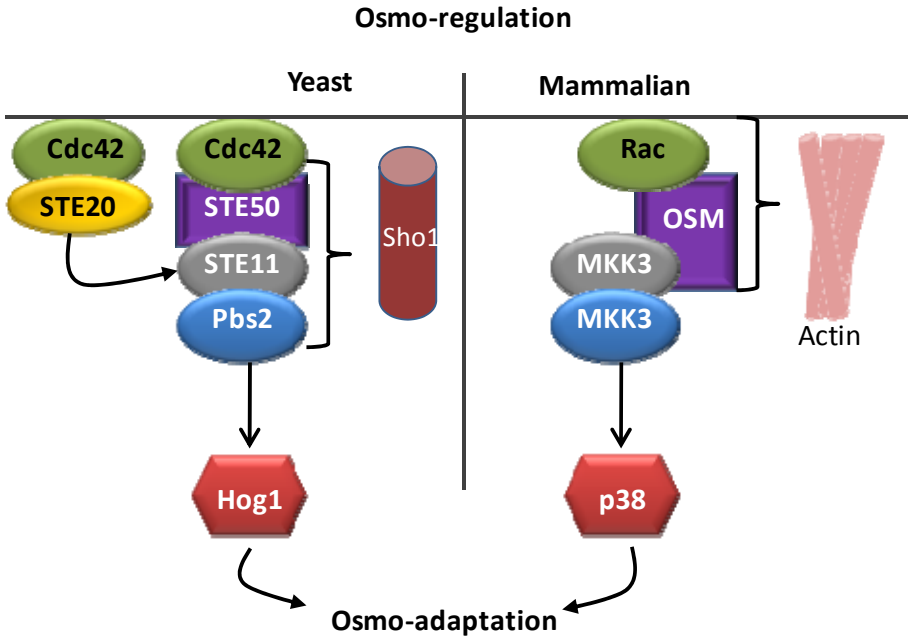


Figure 5. In mammalian cells, OSM is a scaffold for p38 activation by Rac, MEKK3 and MKK3. This complex is recruited to membrane ruffles after a hyperosmolar shock, similar to recruitment of STE50–STE11 to Cdc42 in yeast. Adapted from (Uhlik et al, 2003).

An independent work also demonstrated that the activation of the MAPKK MKK6 upon an osmotic shock takes place close to the plasma membrane (Tomida et al, 2009). These studies locate the putative mammalian osmosensor close to the plasma membrane. However the true nature of the mammalian osmosensor remains elusive to date. A mammalian ShoI hortholog has not been found but several transmenbrane mucin proteins similar to Hkr1 and Msb2 are known and have been proposed to participate to sense changes in osmolarity (de Nadal et al, 2007).

3.3. p38 SAPK Upstream kinases

Upstream of the p38 SAPK pathway, there are low molecular weight GTP-binding proteins from the Rho subfamily such as Rac1, Cd242, Rho and Rit (Marinissen et al, 1999) and also the heterotrimeric G-protein coupled receptors (Marinissen et al, 2001).

Downstream of these activators there are the MAPKKKs, which are the first components of the p38 SAPK pathway. It has been described that the MAPKKKs responsible for the activation of p38 SAPK are cell type and stimulus specific. Several MAPKKKs have been implicated in the regulation of p38 SAPK signalling, these include MLKs, ASK1, TAK1 and some members of the MEKK family (Cheung et al, 2003; Ichijo et al, 1997).

MKK3 and MKK6 (also called MEK3/6 and SKK3/6) are the major MAPKKs responsible for p38 SAPK activation. In fact, they are highly selective for p38 SAPKs and do not activate JNKs or ERK1/2 MAPKs (Feaver et al, 1994). The importance of the physiological function of these two kinases comes from knockout studies. Mice lacking either MKK3 or MKK6 yielded healthy mice (Lu et al, 1999) indicating that both proteins have redundant roles. However mice lacking both MKK3 and MKK6 are not viable, dying in mid-gestation with defects in the placenta and the development of the embryonic vasculature. This phenotype strongly resembled the one displayed by mice genetically lacking p38α, which suggest that MKK3 and MKK6 are epistatic to p38α (Brancho et al, 2003). These results have been further corroborated by the use of siRNAs specifically targeting MKK3 and MKK6. Silencing both MAPKKs disrupt p38 SAPK signalling upon most cell insults. However in some circumstances like ultraviolet radiation, MKK4, a known JNK activator, may contribute to p38α activation (Brancho et al, 2003). The major MAPKK required for p38 activation may not only be affected by the

stimuli, but also by cell type as their level of expression varies. For instance, MKK3 has been shown to be the major p38 activator in mesangial cells stimulated by transforming growth factor (Wang et al, 2002) while MKK6 appears to be the predominant isoform in thymocytes (Tanaka et al, 2002) (Figure 6).

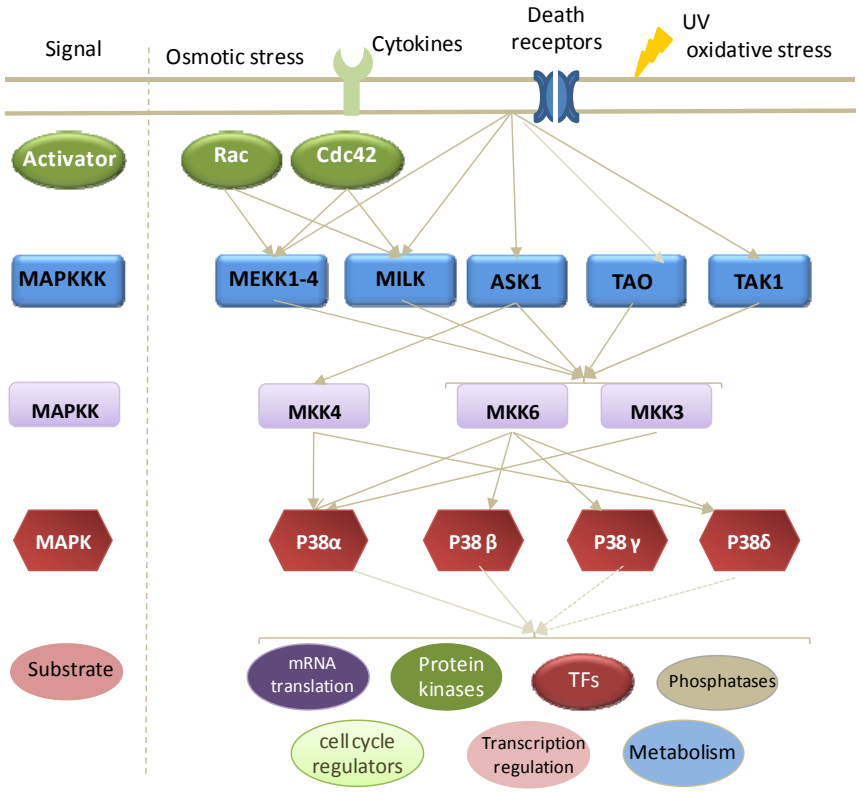


Figure 6. The p38 SAPK pathway. A variety of extracellular signals can activate the p38 SAPK pathway. These signals lead to the initiation of the MAPK phosphorylation cascade which results in the activation of the four p38 SAPK isoforms. The different p38 isoforms can activate several substrates implicated in different physiological functions.

3.4. Signalling through the p38 SAPK pathway

As we mentioned above, p38 MAPKs respond to a variety of extracellular stimuli. In response to one of these stimuli, G proteins activate the

MAPKKKs such as TAK1, ASK1/MAPKKK5, DLK/MUK/ZPK, MEKK4, and MLK, which phosphorylate and activate the MAPKK. Three MAPKKs are known: MKK6 (SKK3), which is able to phosphorylate the four p38 MAPK isoforms; MKK3, which phosphorylates the p38α, p38γ and p38δ isoforms. On the other hand, the MKK4, an upstream JNK kinase, can also phosphorylate the isoforms p38α and p38δ in specific cell types (Jiang et al, 1997).

The p38 SAPK is inactive in the non-phosphorylated state, becoming rapidly activated by MKK-dependent dual phosphorylation on Thr-Gly-Tyr motifs. This phosphorylation induces a conformational change on p38, enabling the binding of both, the ATP and the substrate. MKK3 and MKK6 typically phosphorylate p38 SAPK within a few minutes after exposure to the diverse activating stimuli.

Importantly, alternative mechanisms of activation of p38α (and probably p38β) have also been described. One is apparently specific to T-lymphocytes stimulated through the T cell receptor (TCR). This involves phosphorylation of p38α on Tyr323 by the TCR-proximal tyrosine kinases ZAP70 (ζ –chain associated protein kinase of 70 kDa), which leads to p38α autophosphorylation on the activation loop and, as a consequence, increases its kinase activity towards substrates (Salvador et al, 2005). Also an additional alternative pathway of p38α activation involves the TAK1-binding protein 1 (TAB1) have been described during myocardial ischaemia and in some functions of myeloid cells. TAB1 can bind to p38α, but not to other p38 SAPK family members, and induces p38α autophosphorylation in the activation loop (Ge et al, 2002). Finally, a third non-canonical MAPKK-independent mechanism for p38 SAPK activation has been proposed to operate upon down-regulation of the protein kinase Cdc7, which induces an abortive S-phase leading to p38α-mediated

apoptosis in HeLa cells. However, the underlying mechanism is unclear (Im & Lee, 2008).

In spite of MAPKs not having nuclear localization signals, upon stimulus, phosphorylated p38 SAPKs are translocated from the cytoplasm into the nucleus (Figure 7) (Lenormand et al, 1998).

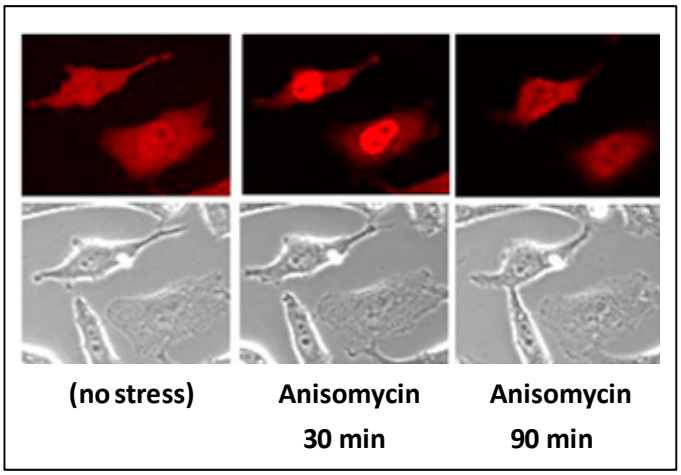


Figure 7. p38 SAPK is translocated into the nucleus under stress stimulus. The p38 SAPK is localized in the cytoplasm under basal conditions. After stress exposure, p38 is phosphorylated and translocated from the citoplasm to the nucleus where it can activate different substrates, performing the adaptive response in order to survival. Once that p38 has performed its function, it is dephosphorylated by phosphatases and it comes from the nucleus back to the cytoplasm.

Once localized into the nucleus, p38 SAPKs can activate a wide range of substrates through docking-mediated interactions that include transcription factors, coactivators and repressors, chromatin remodelling molecules, protein kinases and nuclear proteins, which mediates the adaptive responses to stress. The duration of phosphorylation is crucial to regulate the cellular response, and a sustained phosphorylation is frequently associated with induction of apoptosis (Tobiume et al, 2001). In contrast, transient phosphorylation can be associated with growth-factor-induced survival (Roulston et al, 1998). This suggests that p38 acts

as a biological switch that must be downregulated. Duration of signalling is controlled by phosphatases which interact with and inactivate p38 SAPK pathway. Among these phosphatases we find PP2C (Ser/Thr phosphatase) and PTP (Tyr phosphatase) (Takekawa et al, 2000; Takekawa et al, 1998). Moreover, the activity of the p38 SAPK can also be regulated by a family of dual-specificity phosphatases/MKP (DUSPs), which can dephosphorylate both Tyr and Thr residues (Keyse, 2000).

3.5. p38 SAPK substrates

p38 SAPKs have been reported to phosphorylate a broad range of proteins, both *in vitro* and *in vivo*. A large amount of the information about p38 SAPK substrates comes from the use of chemical inhibitors such as SB203580. However, this inhibitior only inhibits p38α and p38β, so it is not possible to distinguish between them or other kinases with similar potency. On the other hand, the recently discovered diaryl urea compound BIRB796, which not only inhibit p38α and p38β but also inhibits p38γ and p38δ at higher concentrations, provides a new tool for identifying physiological roles of these two p38 SAPK isoforms (Whitmarsh & Davis, 2000). Moreover, studies based on knockout mice for p38 isoforms has show some light in the field.

Among the p38 targets identified we find nuclear proteins like transcription factors, transcriptional regulators of chromatin remodelling or downstream protein kinases implicated in different processes. Moreover, p38 SAPK phosphorylates a heterogeneous collection of cytosolic proteins that regulate processes as diverse as protein degradation and localization, mRNA stability, endocytosis, apoptosis, cytoskeleton dynamics or cell migration (Figure 8).

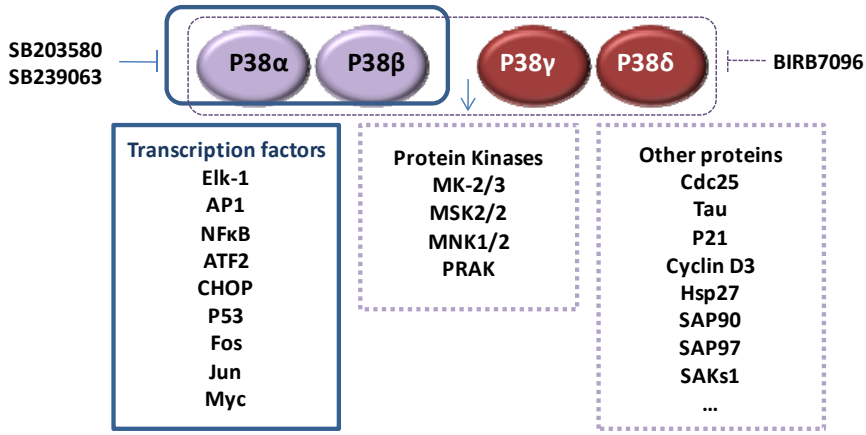


Figure 8. Different substrates activated by the p38 SAPK pathway. The two main groups of proteins that can be phosphorylated by p38 are transcription factors and protein kinases. Additional p38 substrates, including cell cycle regulators, metabolic enzymes and cytoskeletal proteins, have also been described. The different p38 isoforms can be inhibited by chemical inhibitors such as SB203580 which can inhibit p38α y p38β and BIRB796 which is able to inhibit the four p38 isoforms. Adapted from (Perdiguero et al, 2007).

The downstream activities attributed to these phosphorylation events are frequently cell-type-specific and include different physiological processes regulated by p38 SAPK which will be described below.

4. Physiological processes regulated by the p38 SAPK

There are several physiological processes regulated by p38 SAPK like stress adaptation, differentiation, proliferation, survival and development. Importantly, p38 regulates these processes through the regulation of essential molecular functions like cell cycle and control of gene expression.

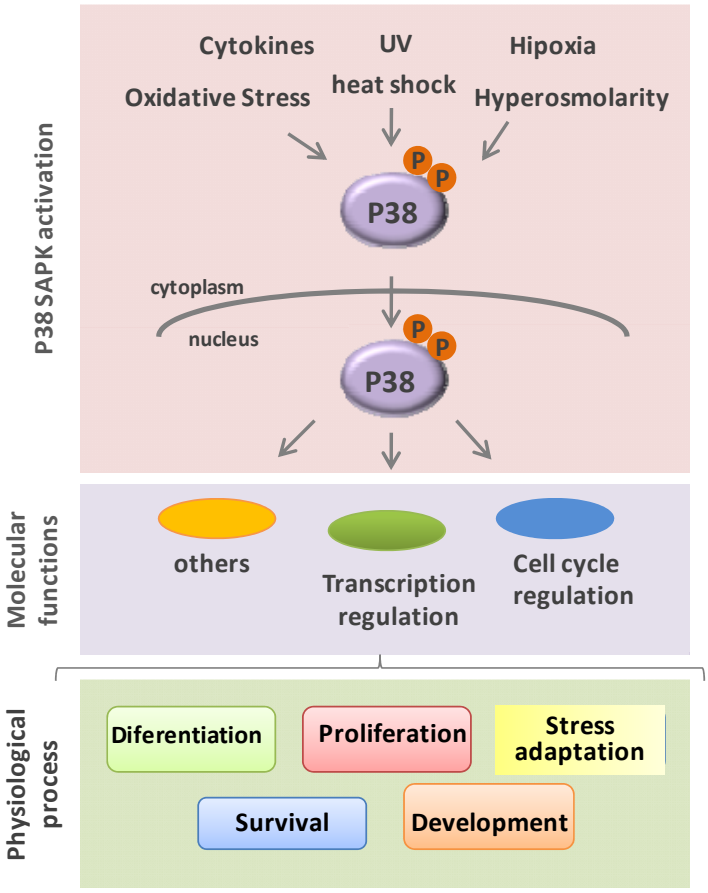


Figure 9. Molecular functions and physiological process regulated by p38 SAPK upon stress. Once p38 SAPK is phosphorylated, it concentrates into the nucleus where it regulates several molecular functions as cell cycle and transcription. This results in the modulation of several physiological processes as differentiation, proliferation, survival and development, required for adaptation to stress.

4.1. Stress adaptation

In response to changes in the intracellular environment, referred to as stress, cells require of rapid and efficient adaptive responses to adjust to such stress. These adaptive responses are mediated by the two SAPK pathways p38 and JNK and also by the ERK5 MAPK. In this PhD project we focussed in the p38 SAPK.

In order to mediate adaptation to stress, p38 SAPK regulates several molecular functions. Among them, the most importants are the regulation of cell cycle progression and the regulation of transcription. Cell cycle progression depends on the intracellular and extracellular stimuli. p38 has an essential role modulating cell cycle progression in response to changes in the extracellular environment. The regulation of cell cycle mediated by p38 SAPK is described below. In addition, cells can change the gene expression pattern to adapt to changes in their environment. Although all of the steps involved in gene expression can in principle be regulated, the regulation of transcription initiation by p38 SAPK is one of the most important points of control under stress conditions. Regulation of transcription by the p38 SAPK is described below.

4.2. Proliferation

It has been shown that depending on the cell type and stimulus, p38 SAPK can have either a positive or a negative role in cell proliferation. In general p38 SAPK activation is associated with anti-proliferative functions, as a suppressor of cell proliferation and tumorigenesis (Bulavin & Fornace, 2004), which seems to be mostly based on the ability of p38α SAPK to negative regulate cell cycle progression as well as to induce apoptosis. There are several examples in the literature supporting this role for p38. For instance, mouse embryonic fibroblasts (MEFs) display enhanced proliferation upon treatment with chemical inhibitors of p38α and β (Iwasa et al, 2003). Also, a MKK3 and MKK6 double knock out (KO), MEFs cells exhibited defects in growth arrest *in vitro* and also an increased tumorigenesis when compared to wild type (Brancho et al, 2003). In addition, genetic inactivation of the PPM1D/Wip1 phosphatase in mice, which can de-phosphorylate p38 SAPK, results in reduced

mammary tumorigenesis, which correlates with increased p38 SAPK activity levels (Bulavin & Fornace, 2004).

Moreover, several proteins that can potentially down-regulate p38α SAPK signalling have been found overexpressed in human tumours and cancer cell lines, like the phosphatases PPM1D/Wip1 and DUSP26/Mkp8 (Bulavin et al. 2002; Li et al. 2002; Yu et al. 2006) and the ASK1 inhibitors Gstm1 and Gstm2 (Dolado et al. 2007). Furthermore, some human tumours, such as hepatocellular carcinomas (Iyoda et al. 2003), show lower p38 SAPK activity levels than the corresponding non-tumourgenic tissues**.**

Also in cardiomyocytes, p38 supports the same role as a negative regulator of proliferation. p38 SAPK regulates the expression of genes required for mitosis in cardiomyocytes, including cyclin A and cyclin B. Inhibition of this SAPK promotes proliferation in cardiomyocytes as A and B cyclins and other cell cycle factors are upregulated (Engel et al, 2005).

In addition and also supporting the anti-proliferative role of p38, whole-genome expression profiles of Ras-transformed wild-type and p38α-deficient cells showed that 202 of genes involved in cell survival were regulated by p38, therefore, this signalling pathway has a function in the context of tumour suppression (Swat et al, 2011).

These results are all consistent with a tumour suppressor role of p38α. However, the picture is more complex than that. It is also known that, in particular cancer cell lines, p38 is also involved in cell migration and in key processes for cancer progression such as invasion and inflammation. Actually, there are also reports indicating that p38α can sometimes positively regulate proliferation. For example, p38 SAPK activity has been related with regulation of haematopoiesis (Platanias, 2003), and

several cancer cell lines as Chondrosarcomas, breast cancer cells and melanoma cells (Halawani et al, 2004; Neve et al, 2002; Recio & Merlino, 2002).

4.2.1. Regulation of cell cycle progression

Cell cycle deregulation is considered the main hallmark of cancer and it is normally associated with uncontrolled cell proliferation and checkpoint failure. In eukaryotic cells, cell cycle is divided in four phases: G1 (growth phase), S-phase (DNA synthesis), G2 (interphase) and M-phase (mitosis) (Figure 10).

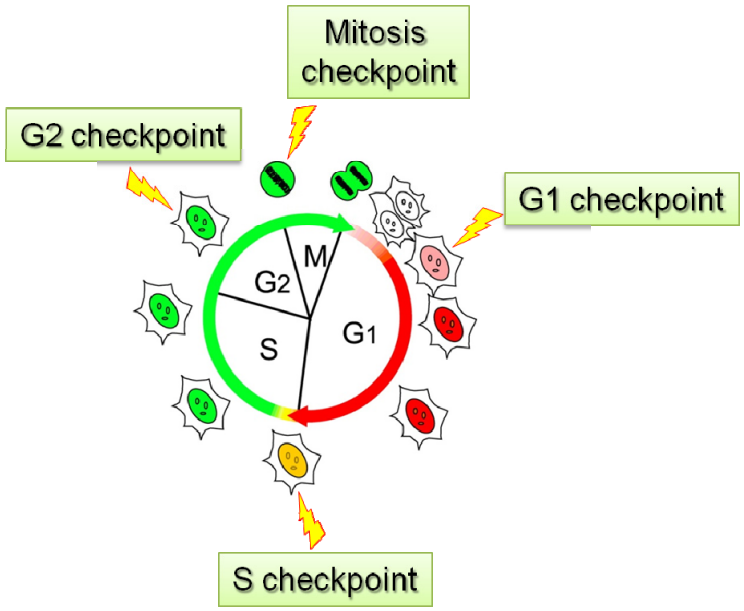


Figure 10. Overview of the different cell cycle checkpoints in eukaryotic cells There are different checkpoints throughout the cell cycle phases and the p38 SAPK has been shown to control the G1/S and G2/M checkpoints through different mechanisms.

Progression through the cell cycle is dependent on extracellular stimuli. In order to adapt to stress, p38 SAPK pathway regulates cell cycle

progression through the phosphorylation of specific target proteins. This regulation delays progression to the next phase of the cell cycle without adaptation. These points of control are known as checkpoints.

There are checkpoints in each phase of the cell cycle (Zhou & Elledge, 2000) and p38 SAPK might be regulating every checkpoint during cell cycle. However, to date, it has been shown that p38 regulates the G1/S and G2/M checkpoints (Bulavin & Fornace, 2004) and several mechanisms have been proposed to be involved in each case.

4.2.1.1. The role of p38 SAPK in the G1/S cell cycle checkpoint

It has been shown that p38 SAPK controls G1/S through regulation of different mechanisms and key proteins, as cyclins, phosphatases, inhibitors of cyclin-dependent kinases (CDKs) and also by the regulation of the tumor suppressor p53. For instance, it has been described that p38 can regulate the G1/S checkpoint by down-regulation of cyclin D1 (Lavoie et al. 1996). The p38 SAPK phosphorylates cyclin D which triggers its degradation by the proteosome (Casanovas et al. 2000, 2004). Cyclin D is also regulated at the transcriptional level through the transcriptional repressor HBP1 (Yee et al. 2004).

In addition, p38 regulates the cyclin-dependent phosphatase CDC25A, which can regulate the G1/S transition as well (Jinno et al, 1994). Upon osmostress, p38 SAPK can phosphorylate and promote CDC25A degradation contributing to the establishment of a G1/S checkpoint (Goloudina et al. 2003).

It has also been shown that p38 mediates the G1/S checkpoint by regulating several CDK inhibitors. Thus p38 up-regulates the CDK inhibitor p16INK4a (Bulavin et al. 2004; Wang et al. 2002), and stabilizes

the CDK inhibitors p21Cip1 and p27Kip1 (Cuadrado et al, 2009; Kim et al, 2002). On top of that p38 stabilizes the p21Cip1 mRNA by targeting the mRNA binding protein HuR which reinforces the G1/S checkpoint (Lafarga et al, 2009).

The p38 SAPK also mediates p53 phosphorylation which increases its stability and transcriptional activity and contribute to the p38α-mediated G1 cell cycle arrest in part through the induction of the CDK inhibitor p21Cip1 (Bulavin and Fornace 2004).

Therefore, p38 uses several mechanisms to control G1/S in response to several stresses.

4.2.1.2. The role of p38 SAPK in the G2/M cell cycle checkpoint

The p38α has been associated with G2/M cell cycle arrest or delay induced by a variety of stresses, including ultraviolet (UV) light, methylating agents, osmotic shock, γ-irradiation, chemotherapeutic drugs and immunoglobulin gene DNA recombination, inhibitors of topoisomerase II or histone deacetylases (Dmitrieva et al, 2002; Hunter & Karin, 1992; Manke et al, 2005; Mikhailov et al, 2005; Pedraza-Alva et al, 2006).

Two mechanisms have been proposed regarding the p38 SAPK regulation of the G2/M checkpoint, one involving p53 and the other involving the phosphatase CDC25B.

It has been shown that, upon stress, p38 SAPK phosphorylates and activates p53 tumor-suppressor protein, which induces a p53-dependent G2/M checkpoint (Bulavin et al, 1999). This leads to the transcriptional activation of p53-target genes such as Gadd45α, p21Cip1 and 14-3-3 (Yang et al, 2002). These proteins enforce a G2/M checkpoint by either

directly or indirectly inactivating cdc2/cyclinB complex which plays an important role in the G2/M transition (Hermeking et al, 1997).

The p38 SAPK can also induce a G2/M checkpoint through the phosphorylation and inhibition of the phosphatase CDC25B (Hunter & Karin, 1992). CDC25B dephosphorylates cdc2 and activates the CyclinB/cdc2 complex, which allows cell entry into mitosis. It has been proposed that phosphorylation of CDC25B inhibits its activity by promoting its association with 14-3-3 proteins, which sequester CDC25B in the cytoplasm (Lopez-Girona et al, 1999). While it was initially reported that p38 SAPK could directly phosphorylate CDC25B (Hunter & Karin, 1992), more recent studies have shown that p38 SAPK promoted the phosphorylation of CDC25B indirectly through the activation of MK2, a direct downstream target of p38 (Manke et al, 2005; Reinhardt et al, 2007). Interestingly, it has also been described that CDC25B can be phosphorylated by p38α directly in a specific site independently of MK2 (Lemaire et al, 2006).

Moreover, in cardiomyocites, p38 SAPK regulates G2/M checkpoint via the down-regulation of cyclins A and B expression, which contributes to the G2 cell cycle arrest (Engel et al, 2005).

Therefore, p38 regulates not only G1/S but also G2/M by different mechanisms.

4.3. Differentiation

The p38α SAPK is an important regulator of differentiation programmes in many cell types, including adipocytes, neurons, myocytes, and hematopoietic cells (Lluis et al. 2006; Nebreda and Porras 2000; Uddin et al. 2004).

In skeletal muscle differentiation, p38 has a key role in the regulation of the conversion of myoblasts to differentiated myotubes during myogenic progression (Cuenda & Cohen, 1999; Li et al, 2000). p38 regulates skeletal muscle differentiation by multiple mechanisms. One of the most important mechanisms is the regulation of specific transcription factors involved in tissue-specific differentiation, like the members of the myocyte enhancer factor-2 (MEF2) family, E47 and MyoD in skeletal muscle (Han et al, 1997; Ornatsky et al, 1999). Moreover, it has been reported that p38 promotes cell cycle exit prior to the onset of differentiation (Perdiguero et al, 2007).

Furthermore, during skeletal myogenesis, p38α SAPK regulates the recruitment of the SWI-SNF chromatin-remodelling complex to muscle-specific loci, possibly via MyoD and or its parner E47 (Lluis et al, 2006; Marinissen et al, 1999) thereby inducing transcription of the muscle specific genes. *In vivo* studies with mice have recently shown the important role of p38α in myoblast differentiation (Perdiguero et al. 2007). Importantly, in adipocytes, p38 phosphorylates the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ), which is critical for adipogenesis (Engelman et al, 1998). On the other hand, p38 also regulates differentiation of adult cardiomyocites allowing the maintenance of its differentiated state (Engel et al. 2005).

4.4. Survival

Although it has been shown that upon stress p38 SAPK mediates cell death, several studies have also found that activation of p38 SAPK by stress stimuli may not necessarily promote death, instead it enhances cell survival. For that reason, p38 SAPK have been implicated both as positive and negative regulator of cell survival.

There are different scenarios where survival instead of death is promoted by p38 activation.

On one hand several studies have described pro-survival roles for p38α. These can be mediated by the induction of cell differentiation or by anti-apoptotic inflammatory signals, such as the cytokine interleukin-6 (il-6), as well as by a quiescent state known as cancer dormancy that may be important for cancer cells to acquire drug resistance (Bulavin et al, 1999). In addition, p38α also mediates cell survival either by regulating autophagy programs through downstream targets, or by direct phosphorylation and inactivation of glycogen synthase kinase 3β (gsK3β), which results in the accumulation and activation of the transcription factor β-catenin (Malik & Roeder, 2005; Rincon, 2009).

On the other hand, p38 SAPK has been related to apoptosis. p38α induces apoptosis in normal and tumorigenic cell lines in response to various stimuli, including chemotherapeutic drugs (Olson & Hallahan, 2004), death receptor signals (Farley et al. 2006; Grethe and Porn-Ares 2006; Hou et al. 2002; Porras et al. 2004), UV irradiation (Bulavin et al. 1999), and also upon conditions that mimic tumorigenic environment (Fassetta et al. 2006; Porras et al. 2004). It also has been shown that, in some cases, apoptotic stimuli trigger p38α activation via secondary effects such as ROS production or the induction of DNA damage (Dolado et al. 2007).

Several mechanisms have been proposed to mediate the induction of apoptosis by p38α as the inactivation of the pro-survival proteins Bcl-2 and BclxL, the activation of the pro-apoptotic proteins BAD, Bim, Bax, and Bak, as well as de novo transcription of pro-apoptotic genes such as Fas, Bax, and Apaf-1. In addition, p38 can mediate the regulation of apoptotic pathways by the regulation of the transcription factors p53, E2F1, or STAT3 (Bulavin et al. 1999; Hou et al. 2002; Porras et al. 2004; Sanchez-Prieto et al. 2000). It has been recently published that p38α

contributes to apoptosis by phosphorylating the epidermal growth factor receptor (EGFR) and inducing its internalization (Zwang and Yarden 2006).

Other studies suggest different roles in cell survival for the p38α and p38β isoforms. For instance, in cardiomyocytes, Jurkat and HeLa cells, it has been described that p38α induces apoptosis, whereas p38β enhances survival (Nemoto et al, 1998).

4.5. Development

It has been described that the p38α SAPK has a key role in development. Several studies indicate that p38 null mice die during embryogenesis (Adams et al, 2000; Allen et al, 2000; Aouadi et al, 2006; Mudgett et al, 2000).

As p38α is specifically required for normal placental development, p38 null mice embryos die of suffocation and starvation (Adams et al, 2000). It has been shown that angiogenesis in p38α KO deficient mice was abnormal in the yolk sac and the embryo itself. This could be due to a massive reduction of the myocardium and malformation of blood vessels in the head region. However, this defect seems to be secondary to the insufficient oxygen and nutrient transfer across the placenta. When the placental defect is rescued, p38α KO embryos develop to term and are apparently normal, indicating that p38α is required for placental organogenesis (Adams et al, 2000). It is worth noting that p38 SAPK may play other unknown roles in development as mouse embryos grown ex vivo showed preimplantation problems when they are treated with the p38 αβ inhibitor (Maekawa et al, 2005).

Disruptions of the other p38 SAPK isoforms (β, δ, γ) don't display any apparent phenotype. It has been shown that the KO of one isoform did not

affect the expression or activity of the others (Beardmore et al, 2005). Also the double KO for the p38 α and β isoforms are viable development when the placental defect was rescued, but, in this case, the embryo shows some defects related with inflammatory responses (O'Keefe et al, 2007).

Importantly, it has been shown that p38 SAPK plays a key role in the development in other organisms. For instance, in *Drosophila* embryos, p38 SAPK regulates the formation of the future anterior-posterior and dorsal-ventral axes (Suzanne et al, 1999). In zebrafish embryos, p38 SAPK is necessary for the proper cell division and the correct embryo development (Kuma et al, 2005).

4.6. Regulation of gene expression.

The regulation of gene expression is one of the most intensively studied areas in biology. The differential gene expression pattern in multicellular organisms constitutes the cell-type specificity. Deregulation of the appropriate pattern of gene expression has profound effects on cellular function leading to several diseases. However, in order to adapt to changes in their environment, cells change their pattern of gene expression.

In *S. cerevisiae*, genome-wide transcription studies revealed that a large number of genes (~ 7%) show significant but transient changes in their expression levels after a mild osmotic shock. Importantly, the p38 SAPK functional homolog, Hog1 SAPK, plays a key role in this global gene regulation (Posas et al, 2000).

Although all of the steps involved in gene expression can in principle be regulated, the regulation of transcription initiation is the most important point of control and will be furthered described in the following section.

p38 SAPK in the regulation of transcription under stress conditions (see below).

5. Regulation of Transcription

Transcription is a complex process responsible for the generation of a mature mRNA molecule using a sequence of DNA as a template. Transcription regulation of eukaryotic protein-coding genes is an orchestrated process that requires the concerted functions of multiple proteins, including sequence-specific DNA-binding transcription factors, general transcription factors, as well as coactivators and corepressors.

Several studies, from yeast to human, have shown that MAPKs regulate eukaryotic gene expression in response to extracellular stimuli through different mechanisms during the different stages of the transcription cycle (de Nadal & Posas, 2010; Whitmarsh, 2007; Yang et al, 2003)

5.1. DNA-dependent RNA polymerases

RNA polymerases (RNA Pol) are the enzymes that mediate transcription in collaboration with transcription factors and modification factors. RNA Pols catalyze the formation of the phosphodiester bonds that link the nucleotides together to form a linear chain of RNA.

Three different DNA dependent RNA polymerases perform transcription of eukaryotic genes: RNA polymerase I, II and III. The three polymerases are structurally similar to one another and share some common subunits, but they transcribe different types of genes. RNA polymerase I exclusively synthesizes ribosomal RNA genes, RNA polymerase II transcribes most of protein-coding genes, and the RNA polymerase III is

responsible for the transcription of tRNA genes, the 5S RNA ribosomal genes and the snRNAs.

The RNA Polymerase II is a large multisubunit enzyme composed of 12 different proteins encoded by the genes *RPB1* to *RBP12* (Myer & Young, 1998). All of them are essential, except for *RPB4* and *RPB7* (Woychik and Young, 1989). Rpb1 and Rpb2 subunits are the largest and the most evolutionarily conserved, and form a central core which contains the catalytic site of the enzyme. A unique feature of the RNA polymerase II is the C-terminal domain (CTD) of Rpb1, formed by multiple repeats of the heptapeptide sequence YSPTSPS. The number of these repeats increases with genomic complexity: 26 in yeast, 32 in *Caenorhabditis elegans*, 45 in *Drosophila*, and 52 in mammals. The major function of the CTD is to serve as a platform for a variety of factors (Phatnani & Greenleaf, 2006).

5.2. The eukaryotic transcription cycle

The transcription cycle can be divided into distinct steps: preinitiation, initiation, promoter clearance, elongation and termination.

The transcription cycle starts with the preinitiation complex (PIC) assembly at the promoter. The PIC includes the general transcription factors (GTFs) (Kornberg, 2001) which consist of TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and RNA Pol II, as well as several additional cofactors (Orphanides et al, 1996). The transcription factors are “general” because they are needed at nearly all promoters where RNA Pol II is recruited. They are designated as TFII for transcription factor and for polymerase II. RNA Pol II requires the GTFs because they allow the correctly positioning of the eukaryotic RNA polymerase at the promoter (Thomas & Chiang, 2006).

Different steps are involved in PIC formation and transcription initiation at a core promoter (Figure 11).

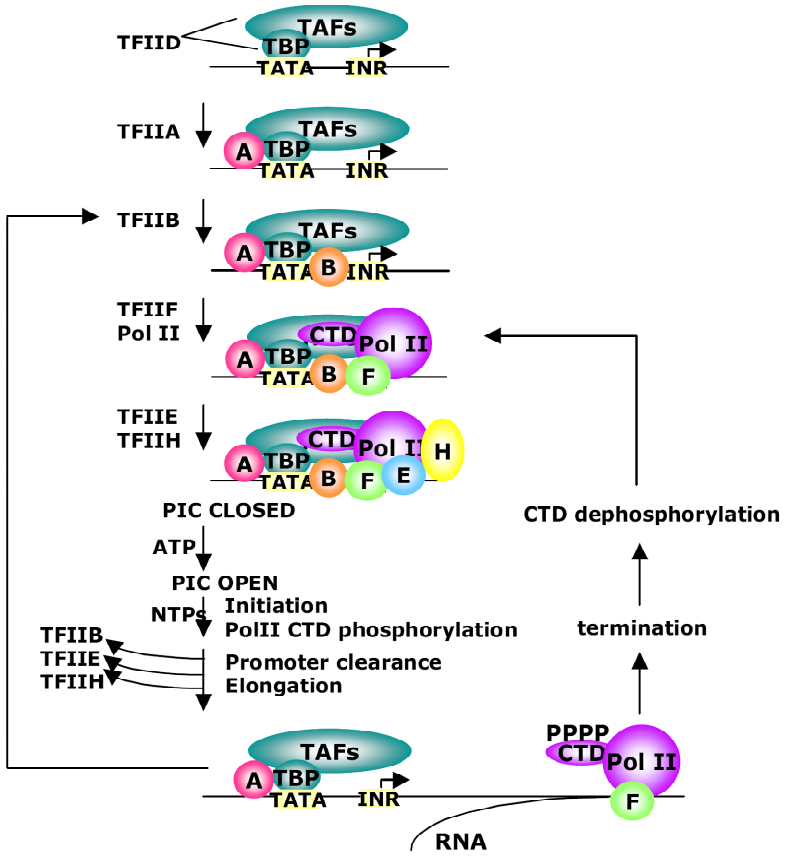


Figure 11. Model for a stepwise assembly and function of a preinitiation complex (PIC) (adapted from Martinez et al, 2002). The GTFs are designed as "A" for TFIIA, "B", for TFIIB and so on.

The first step of the PIC formation involves the binding of TFIID to the core promoter. TFIID is a stable complex composed of the TATA binding protein (TBP) and 14 distinct TBP-associated factors TAFs. The binding of TFIID to the core promoter is stabilized by TFIIA. TFIIB further stabilizes the TBP-TATA complex and allows the recruitment of TFIIF,

RNA Pol II, TFIIE and TFIIH. RNA Pol II has to bind the promoters at the transcription start site (TSS), which is typically located 25 nucleotides downstream from the TATA box. TFIID mediates this process as allow that RNA Pol II can gain access to the template strand. TFIIH is a complex of nine subunits, one them is the kinase Cdk7 (Kin28 in *S. cerevisiae*). During initiation or early elongation, Cdk7 phosphorylates the serine located at the fifth position (Ser5) of the CTD (carboxy-terminal domain) of Rpb1 (Feaver et al., 1994; Valay et al., 1995). This step facilitates promoter clearance by disrupting the CTD interactions with the components of the PIC, so the polymerase can then disengage from the cluster of general transcription factors. Importantly, a subset of GTFs remains at the promoter, working as a scaffold for the formation of the next transcription initiation complex (Fuda et al, 2009; Yudkovsky et al, 2000; Zawel et al, 1995). However, formation of the initiation complex and elongation of the transcript are more complex processes, as DNA is "trapped" into chromatin. Therefore, admission of the transcription initiation complex will be possible after modification and remodeling of the nucleosomal structure underlying the respective gene and its promoter.

During elongation, RNA Pol II, localized at the promoter, starts to synthesize short lengths of RNA by addition of the nucleoside triphosphates (NTPs) using base pairing complementarily with the DNA template. Also, during this phase, RNA Pol II is associated with several elongation factors which facilitate the different events of translational elongation. These proteins can modify the mRNA production at particular stages and in different ways during transcription. Some factors act directly on RNA Pol II, whereas others manipulate the chromatin environment (Fuda et al, 2009)

Efficient transcript elongation must overcome three blocks delimited by different checkpoints in order to assess whether the RNA Pol II is

correctly prepared for productive elongation, and allows rapid regulation of gene expression. The three blocks are: transcriptional pause, arrest and termination (Shilatifard et al, 2003; Uptain et al, 1997). Transcriptional pausing occurs when the RNA Pol II halts the addition of NTPs to the nascent RNA transcript for a time before resuming productive elongation on its own (Sims et al, 2004). Transcriptional arrest can be defined as an irreversible stop to RNA synthesis, whereby the RNA polymerase cannot resume productive elongation without accessory factors. During termination, the RNA polymerase and RNA transcript are released from the DNA, effectively ending the elongation stage of transcription. In addition, the phosphorylation status of the CTD determines the transition between initiation and elongation (Dahmus, 1996).

Once the polymerase II has begun elongating the RNA transcript, most of the general transcription factors are released from the DNA so that they are available to initiate another round of transcription with a new RNA polymerase molecule. Thus, transcription initiation and termination are interconnected and might influence in each other's efficiency.

5.3. Regulation of transcription initiation by MAPKs

Transcription initiation requires interactions between sequence-specific transcription factors and their regulatory sequences on the promoters of specific genes, as well as remodelling of the local chromatin structure by inducing histone modifications. All those steps can be controlled by MAPKs which modify the activity of the transcription factors, the chromatin remodelling activities and the RNA polymerases, and therefore, they can control which genes are transcribed and which are not (Orphanides et al, 1996) (Figure 12).

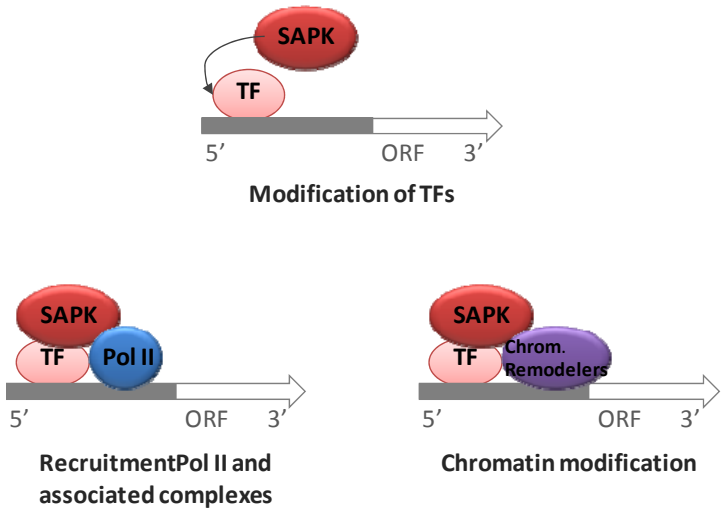


Figure 12. MAPKs regulate transcription initiation of stress genes through several mechanisms. MAPKs modulate initiation of transcription in response to signals by (1) Regulation of promoter-specific transcription factors; (2) stimulation of the recruitment of RNA Pol II and coactivators to the promoters; and (3) regulation of chromatin modifying activities. Adapted from (de Nadal & Posas, 2010).

5.3.1. Regulation of Transcription Factors by MAPKs

The most common regulatory mechanism driven by the MAPKs to modify gene expression, particularly for rapid alterations in transcription factor activity, involves phosphorylation and dephosphorylation of transcription factors (Hill & Treisman, 1995b; Hunter & Karin, 1992).

Transcription factors bind to proximal promoter elements and/or more distal regulatory sequences (i.e., enhancers and silencers) and modulate the rate of transcription of specific target genes in a concrete tissue and developmental stage or in response to physiological or environmental stimuli.

In order to efficiently phosphorylate their substrates, including transcription factors, MAPKs often directly interact with them via conserved docking sites. These docking sites are essential for efficient

phosphorylation and also impart specificity as they selectively bind to MAPK subtypes (Yang et al, 2003).

Phosphorylation by MAPKs regulate transcription factor activity through different mechanisms: (i) by controlling the length of time transcription factors spend in the nucleus; (ii) by controlling the transcription factor protein levels; and (iii) by regulating transcription factor DNA binding (Whitmarsh & Davis, 2000).

5.3.1.1. Intracellular localization

Many transcription factors permanently reside in the nucleus but others shuttle between the cytoplasm and the nucleus. MAPKs signaling pathways can either stimulate the translocation of transcription factors to the nucleus in order to stimulate their transcriptional activity or conversely, to stimulate the export of transcription factors from the nucleus to the cytoplasm and hence facilitate their inactivation. For example upon stimuli, ERK MAPK pathway targets the ERF repressor leading to its exclusion from the nucleus and also it positively regulates the transcriptional activity of a number of ETS-domain transcription factors (Sharrocks, 2002b).

5.3.1.2. Control of transcription factor protein levels

This control can be achieved by either modulating the transcription factor expression levels or the stability of the proteins. The regulation of the Activating Protein 1 (AP-1) TF and its family members c-Jun and Fos are well studied (Whitmarsh & Davis, 1996). For example, ERK5 and p38 MAPK have been implicated in the transcriptional regulation of one of these family members, c-Jun (Marinissen et al, 2001; Marinissen et al, 1999) by the phosphorylation and activation of the transcription factor MEF2C (Han et al, 1997). Like c-Jun, the c-Fos promoter contains a

number of elements that are targets for MAPK signaling (Whitmarsh & Davis, 1996). For instance, Elk1 and SAP1 transcription factors bind c-Fos promoter (Sharrocks, 2001) and are targets of MAPKs. Elk1 transcription factor can be phosphorylated and activated by ERK, JNK and p38 MAPKs while SAP1 is phosphorylated by ERK and p38, leading to enhanced transcription of the c-Fos gene (Sharrocks, 2002a).

Therefore, the regulation of c-Jun and c-Fos is highly complex and it requires the integration of multiple signaling pathways. In addition, the phosphorylation of transcription factors can affect their stability by regulating the covalent attachment of ubiquitin to lysine residues. However, its phosphorylation can promote their transcriptional activation or degradation.

One one hand, it has been shown that activated JNK phosphorylates c-Jun, JunB, ATF-2 and p53 transcription factors protecting them from degradation and therefore contributing to its transcriptional activation (Fuchs et al., 1997; Musti et al., 1997). Conversely, ERK and p38 pathways can phosphorylate the nuclear hormone receptors progesterone receptor (PR) and retinoic acid receptor-g2 (RARg2), resulting on their degradation (Shen et al., 2001; Gianni et al., 2002).

5.3.1.3. Regulation of DNA binding

MAPK can regulate the binding of transcription factors to gene promoters either positively or negatively by phosphorylation. This may occur by direct phosphorylation of the DNA-binding domain. For instance, the transcription factor TCFh1 is phosphorylated by JNK enhancing its binding to DNA (Kasibhatla et al, 1999). On the other hand, in response to growth factor stimulation, ERK phosphorylates the ribosomal transcription factor UBF preventing its interaction with DNA (Stefanovsky et al., 2001). Alternatively, DNA binding may be indirectly

regulated by phosphorylation. For example, the phosphorylation of Elk1 in its C-terminal Transcriptional Activation Domain (TAD) by MAPKs results in stimulation of DNA binding by the N-terminal ETS domain, apparently via an allosteric mechanism (Yang et al, 1999).

Therefore, MAPKs can regulate the transcription factor binding to gene promoters through several mechanisms.

5.3.2. Transcription factors regulated by p38 SAPK

There are several transcription factors that can be directly phosphorylated and activated by p38 SAP kinases (See Table 1). This suggests that depending on the promoter context, cell type and type of stress, p38 SAPK can function through a variety of structurally unrelated transcription factors.

Factor	Regulation	Pathway	References
MEF2A/C/D	Activation	Skeletal and cardiac muscle diff.; Cardiac hypertrophy	(Ornatsky et al. 1999; Yang et al. 999; Zhao et al. 1999)
E47	Activation	Heterodimerization Skeletal muscle diff.	(Lluis et al. 2005)
MRF4	Repression	Skeletal muscle differentiation	(Suelves et al. 2004)
BAF60	Activation	Skeletal muscle diff.	(Simone et al. 2004)
C/EBPβ, CHOP	Activation	Adipocyte diff., cellular stress	(Wang and Ron 1996; Engelman et al. 1998)
NFATc4	Inactivation	Downregulation of adipocyte differentiation	(Yang et al. 2002)
NFATc1, MITF	Activation	RANKL* signaling in osteoclast diff.	(Mansky et al. 2002; Matsumoto et al. 2004)
MafA/B, c-Maf	Activation	Lens differentiation	(Sii-Felice et al. 2005)
GATA-1	Activation	IL-9 expression in mast cells	(Stassen et al. 2006)
C/EBPε	Activation	Neutrophil diff.	(Williamson et al. 2005
PGC-1α	Activation	Metabolism, mitochondrial	(Puigserver et al. 2001; Akimoto et al. 2005)

		biogenesis	
Smad3	Activation	TGF-β signaling	(Furukawa et al. 2003; Wang et al. 2006)
p53	Activation	UV-induced cellular stress, G2/M checkpoint	(Bulavin et al. 1999; Huang et al. 1999; Pedraza-Alva et al. 2006)
HBP1	Stabilization	G1 arrest	(Yee et al. 2004)
STAT1/3/4/6	Activation	IFN-α, IFN-γ, IL-2, IL-4, IL-12, and IL-13 responses	(Goh et al. 1999; Gollob et al. 1999; Pesu et al. 2002; Ramsauer et al. 2002; Xu et al. 2003)
c-Fos	Activation	UV response, cellular stress	(Tanos et al. 2005)
NFATp	Activation	Nuclear export; Downregulation of calcium signaling	(Gomez del Arco et al. 2000)
ATF2	Activation	Cellular stress, insulin resistance	(Cuenda and Cohen 1999; Ouwens et al. 2002;
ATF6	Activation	ERb stress	Luo and Lee 2002)
Elk1, Sap-1, SRF, CREB	Activation	Cellular stress	(Whitmarsh et al. 1997; Yang et al. 1998; Bebien et al. 2003)
Usf-1	Activation	UV response, cellular stress	(Galibert et al. 2001; Corre et al. 2004)

Table 1. Transcription factors directly regulated by p38 SAPK. Adapted from (Perdiguero & Munoz-Canoves, 2008). Note:* Receptor activator of NF-κB ligand.

Among all the transcription factors that can be targets of p38 SAPK, Elk-1, AP-1 and NF-kB transcription factors have been studied thoroughly and are regulated by p38 SAPK under stress conditions (Baeza-Raja & Munoz-Canoves, 2004; Marais et al, 1993; Tanos et al, 2005).

5.3.2.1. The AP-1 Transcription Factor

The mammalian AP-1 transcription factor is a homodimer or heterodimer composed of a region of leucine proteins that belongs to Jun (JUN, JUNB and JUND) and Fos (FOS, FOSB, FRA1 and FRA2) families, and the

closely related activating transcription factors ATF and CREB subfamilies (Angel & Karin, 1991).

Jun proteins can form stable dimmers that bind the AP-1 DNA recognition elements, also known as TREs (Angel & Karin, 1991). However, Fos family proteins do not form stable dimmers but can bind DNA by forming heterodimers with Jun proteins that are more stable that Jun-Jun homodimers (Kouzarides & Ziff, 1988). In addition, Jun-ATF dimmers or ATF homodimers prefer to bind to the cAMP-responsive element (CRE) (Figure 13).

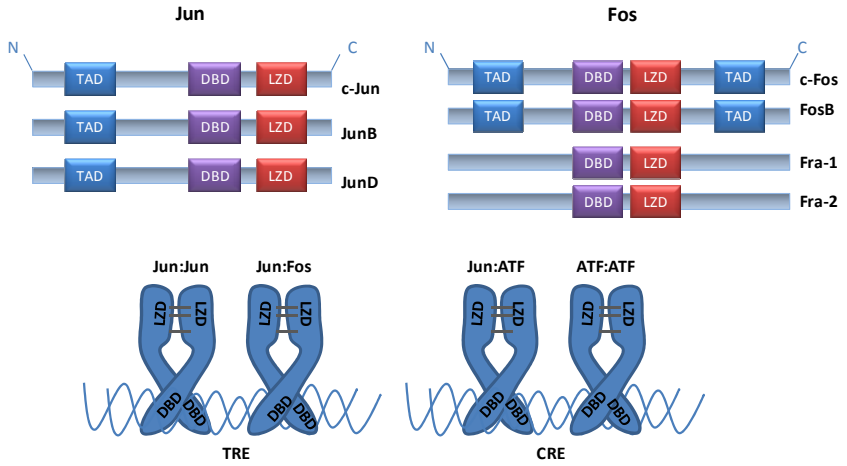


Figure 13. Schematic diagram showing the modular structures and dimerization and DNA binding properties of Jun and Fos proteins. TAD, transcription-activating domain; LZD, leucine-zipper domain; DBD, DNA binding domain. Adapted from (Reddy & Mossman, 2002).

It is well documented that the AP-1 transcription factor is regulated by MAPKs (Dhanasekaran & Premkumar Reddy, 1998). In effect, ERK and JNK MAPKs can directly regulate AP-1 transcription factor. Although p38 MAPKs do not activate AP-1 proteins directly, they can regulate jun and fos transcription by phosphorylating ATF-2, Elk1, SAP-1, and CCAAT enhancer binding proteins (C/EBPs), which then bind to the

promoter elements of jun and fos and regulate their transcription (Reddy & Mossman, 2002).

AP-1 binds to promoters of its target genes in a sequence-specific manner, and it activates or represses them. AP-1 proteins are implicated in the regulation of a variety of cellular processes including proliferation, survival, differentiation, growth, apoptosis, cell migration, and transformation. AP-1 factor can positively or negatively regulate a specific target gene depending on the abundance of the dimerization partners, dimer-composition and post-translational regulation. This last modification can facilitate the AP-1 ability to dimerize with other basic leucine zipper (bZIP) domain proteins, and its interaction with accessory proteins (Reddy & Mossman, 2002)

5.3.2.2. The Elk-1 Transcription Factor

Elk-1 (Ets-like protein 1) is a member of the Ets family of transcription activators. It is an essential component in the serum response ternary complex and it has been shown to regulate the expression of immediate-early response genes such as Zif268, MKP-1 (Sgambato et al, 1998), chemokine IL-8 (Li, 1999) and c-Fos (Hill & Treisman, 1995a). These genes are important regulators of gene transcription in response to stress. However, in some cases, Elk-1 does not require serum response factor to bind to DNA (Li et al.1999, 2000), and it can be bound in the absence of a stimulus (Sharrocks, 1995; Li et al., 1999, 2000).

The c-Fos promoter is one of the most well-studied promoters regulated by the Elk-1 TF (Sharrocks, 1995). In the c-Fos promoter, the Elk-1-binding site is constitutively occupied *in vivo* (Herrera et al., 1989). Elk-1 can be phosphorylated and activated by ERK, JNK and p38 MAPKs resulting in an enhanced SRE-dependent c-Fos expression (Whitmarsh & Davis, 1996). Moreover, phosphorylation of Elk-1 by MAPKs results in a

conformational change that enhances its DNA binding affinity (Sharrocks, 1995) (Yang et al, 1999) (Figure 14).

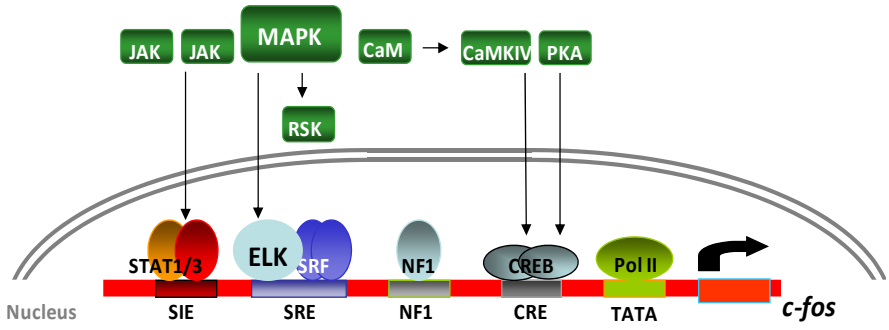


Figure 14. Schematic diagram of the c-Fos promoter. Depicted the locations of different DNA binding elements across the promoter, transcription factors as Elk-1 and interactions with different pathways and, among them, MAPKs pathways.

5.3.2.3. The Nuclear factor-kappa (NF-κB)

The Nuclear factor-kB (NF-kB) belongs to the *Rel* family of transcription factors that regulate expression of genes involved in immune and inflammatory responses (Baeuerle and Baltimore, 1996). NF-kB is a critical regulator of the immediate early pathogen response and it plays an important role in promoting inflammation and the regulation of cell proliferation and survival (Karin, cao, Li, 2002). In most cells, NF-kB is a complex whose predominant form is a heterodimer composed of p50 and RelA/p65 subunits, which is retained inactive at the cytoplasm through the binding to inhibitory proteins of the IkB family. The inactive NF-kB complex can be activated in response to a variety of stimuli, including viral and bacterial infections, exposure to proinflammatory cytokines, mitogens and growth factors, and stress-inducing agents (Karin, Ben-Neriah, 2000). Upon stimulus, IKK phosphorylates IKB, resulting in its poly-ubiquitination and subsequent degradation by the 26S proteasome. This results in the release of NF-kB from its inhibitor IkBα, allowing its nuclear translocation from the cytoplasm to the nucleus, where it binds to

DNA (Figure 15). On top of that, the NF-κB transcriptional activity can be further modulated through phosphorylation by TNFα responsive kinases, such as p38 SAPK (Baud & Karin, 2001) (Figure 15).

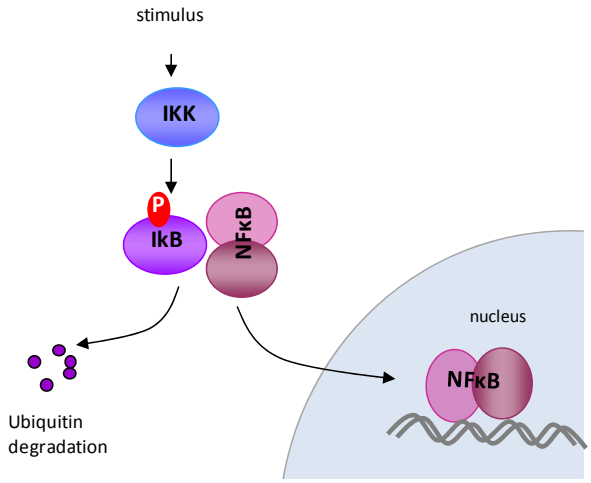


Figure 15. Activation of the NF-κB pathway upon stimulation. Activated IKK complex phosphorylates IkBα leading to its degradation by the proteasome. NFkB then translocates to the nucleus to activate its target genes.

It is worth noting that under specific conditions as differentiation, NF-kB activation can be dependent on p38 activity. In fact, p38 activation induces the activity of NF-kB by a dual mechanism, reducing IkBα levels thereby inducing NF-kB-DNA binding activity and, also, enhancing the transactivating activity of one of the NF-kB subunits (Baeza-Raja & Munoz-Canoves, 2004).

5.3.3. Regulation of RNA Pol II and TBP by MAPKs

It has been reported that a critical step to induce gene expression upon stress is the recruitment of the RNA Pol II complex to promoters. It has been shown that p38 SAPK and its yeast homologue Hog1 SAPK interact with the RNA Pol II holoenzyme (Alepuz et al. 2003; Proft et al. 2006). The functional relevance of the Hog1 and RNA Pol II interaction has been

further exemplified by the fact that artificial recruitment of Hog1 to DNA is able to induce gene expression upon stress (Alepuz et al, 2001). Importantly, the presence of active Hog1 in the promoters is necessary for RNA Pol II recruitment (Alepuz et al, 2003). Phosphorylation of the TATA binding protein (TBP) by p38 SAPK has been observed at NFκB- and AP-1-dependent promoters. This phosphorylation induces the affinity between TBP and the TATA box, enhancing the frequency of transcriptional initiation (Carter et al, 1999).

5.3.4. Regulation of coactivators and corepressors by MAPKs

The role of MAPKs in transcription is not only related to target transcription factors and the basal machinery to promoters. Increasing evidence suggests that coregulatory factors are also directly targeted by MAPK signaling pathways.

In *S. cerevisiae*, Hog1 regulates transcription through the regulation and recruitment to promoters of basic transcription complexes such as SAGA, mediator and SWI/SNF complex. Interestingly, mediator seems to be essential to induce gene expression under mild osmostress conditions, while SAGA is restricted to severe osmotic conditions (Zapater et al, 2007). In addition, the recruitment of SWI/SNF to promoters depends on the presence of Hog1 (Proft & Struhl, 2002). It has also been shown that Hog1 mediates the association of RSC complex, a member of the SWI/SNF family, to the ORFs of the osmo-responsive genes, for a proper gene expression upon osmostress (Mas et al, 2009).

In mammals, MAPKs also regulate the presence of coactivators and corepresors in promoters. It has been shown that mediator is recruited to ERK MAPK-responsive promoters after treatment with ERK activators (Zhang et al, 2008). In addition, p38 SAPK mediates the recruitment of

SWI-SNF chromatin remodeling complex to muscle-specific loci and this is prevented by p38 SAPK inhibitors (Marinissen et al, 1999).

There are more coactivators linked to MAPKs. For example, the coactivator CREB binding protein (CPB), which is recruited to DNA via transcription factors, is associated with and phosphorylated by ERK (Liu et al, 1999). The regulation of the coactivator PGC-1 is mediated by p38 SAPK trough its interaction with a PGC-1 repressor. In fact, activation of p38 SAPK enhances the activity of wild-type PGC-1 but not of a PGC-1 variant that no longer interacts with the repressor (Han et al, 1997).

5.3.5. Chromatin remodeling by MAPKs

Transcription in eukaryotic cells is influenced by the manner in which DNA is packaged. In resting cells, DNA is tightly compacted, packaged into chromatin. The nucleosome is the fundamental unit of chromatin and it is composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped (Figure 16).

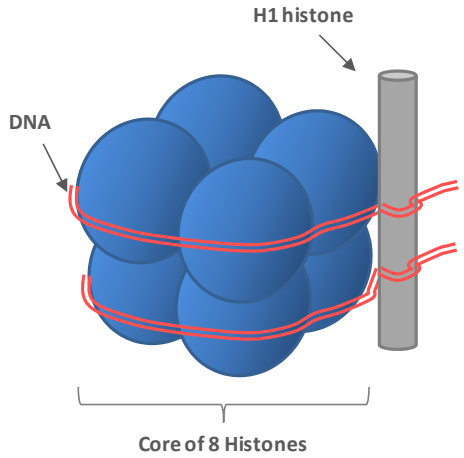


Figure 16. The nucleosome structure. The nucleosome consists on an octamer of the four core histones and the DNA wrapped around them. Each of the four histones share a very similar structural motif consisting of three alpha helices separated by loops.

Local chromatin architecture is now generally recognized as an important factor in the regulation of gene expression. During activation of gene transcription, this compact, inaccessible DNA is made available to DNA binding proteins via modification of the nucleosome.

Transcription is affected by chromatin structure, which is regulated by several protein factors that covalently modify histones or temporarily move or disassemble and reassemble nucleosomes (Li et al, 2007). Nucleosomes adopt canonical positions around promoter regions and more random positions in the interior of genes and are modulated to regulate DNA accessibility. Chromatin remodeling complexes utilize ATP hydrolysis to alter the histone-DNA contacts by unwrapping DNA transiently, forming DNA loops, sliding nucleosomes, displacing completely the histones from DNA or replacing histone subunits. This architecture of chromatin is strongly influenced by posttranslational modifications of the histones. There are at least eight distinct types of histone modifications: acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization (Kouzarides, 2007). These histone modifications can change the physical and chemical properties of the chromatin, alter the ability of other factors to interact with the chromatin, and also influence in the recruitment of many factors to the gene. Moreover, histones are modified at many sites. There are over 60 different histone residues where modifications have been detected. This huge array of modifications gives enormous potential for functional responses.

5.3.5.1. Histone modifications regulated by MAPKs

It has been described that several histone modifications are regulated by MAPKs. In fact, the histone modifications by MAPKs can be divided in two groups:

- **Direct modifications:**

MAPK signaling pathways phosphorylate histones in response to a variety of mitogenic and stress signals. For example, JNK phosphorylates the histone H2A isoform, H2AX, and this phosphorylation is required for apoptotic DNA fragmentation (Lu et al, 2006). Also, it has been described that in response to mitogenic or stress stimuli, the ERK and p38 downstream kinases, MSK1 and MSK2 kinases, phosphorylate the serine 10 of the histone H3 in immediate-early gene promoters (Clayton & Mahadevan, 2003; Clayton et al, 2000; Vicent et al, 2006).

- **Indirect modifications:**

It has been reported that MAPKs regulate the activity of acetyl transferases (HATs) and histone deacetylases (HDACs). These enzymes mediate the acetylation and deacetylation of transcription factors and histones respectively (Kuo & Allis, 1998).

It was originally thought that HATs act as co-activators by promoting chromatin relaxation through histone acetylation and HDACs act as co-repressors by removing acetyl groups from histones. However, a more complex picture has now emerged. The functions of these factors depend on the particular promoter context and their actions can be coordinated by signaling events to temporally regulate gene expression. In *S. cerevisiae,* Hog1 is essential for the recruitment of the Rpd3-Sin3 HDAC complex to these promoters (De Nadal et al, 2004). This leads to the modification of local chromatin structure by histone deacetylation, it increases the RNA Pol II recruitment and likely, it also increases the recruitment of additional coactivating factors to the promoters (De Nadal et al, 2004).

In mammals, MAPKs can directly interact with HATs and HDACs and this can lead to changes in the activities of both the MAPKs and the HAT/HDACs. For instance, the phosphorylation of ATF-2 by JNK promotes its intrinsic HAT activity (Kawasaki et al, 2000). Moreover,

activation of the mammalian JNK MAPK signaling pathway results in the acetylation of nucleosomes in the c-Fos promoter region, increasing gene induction (Alberts et al, 1998).

5.3.1. MAPKs as components of the transcription factor complex

One of the most remarkable discoveries about how MAPKs regulate gene expression is that MAPKs can be recruited to gene promoters as integral components of transcription complexes.

Several studies focused on the osmotic stress response pathway in *S. cerevisiae* have indicated such a role for Hog1 SAPK. Exposure of the yeast to hyperosmotic stress induces the activation and recruitment of Hog1 to distinct promoters by direct interactions with the DNA-bound transcription factors Hot1 and Sko1 (Alepuz et al, 2001; Proft & Struhl, 2002). This indicates that Hog1 itself might take part in the activation process. It is also shown that Hog1 phosphorylates the Hot1 transcription factor, although this is not a requirement for osmotic-stress induced gene transcription (Alepuz et al, 2003). This suggest that Hog1 must induce activation of gene expression by other mechanism than the phosphorylation of the transcription factor. In fact, the model proposed by the authors is that Hot1 transcription factor recruits Hog1 SAPK to phosphorylate additional transcription factors, chromatin remodeling complexes or nucleosomal components (Alepuz et al, 2003)

It is worth noting that the association of Hog1 to chromatin depends on its activity but not on its nuclear accumulation because the artificial increase of the amount of nuclear Hog1 by adding a nuclear localization signal on the MAPK did not result in enhanced chromatin association (Alepuz et al, 2001).

This scenario is not restricted to budding yeast. In *S. pombe*, activated Sty1 SAPK phosphorylates a number of targets including the transcription factor Atf1, the fission yeast homologue of mammalian ATF2 (Shiozaki & Russell, 1996; Wilkinson et al, 1996). As Hog1, activated Sty1 is recruited to Atf1-dependent genes in response to various stresses by association with Atf1 and its binding partner Pcr1 (Reiter et al, 2008).

Considering the conservation of MAPK pathways through evolution, it is highly likely that mammalian MAPKs are also components of the transcriptional machinery. Indeed ERK5 may have a direct role in transcriptional activation as it contains a transcriptional activation domain (Kasler et al, 2000).

In addition, several studies in mammalian cells have suggested that MAPKs are recruited to gene promoters as components of the transcriptional machinery. For instance, ERK1 MAPK and its downstream target MSK1 are recruited to genes through the interaction with the progesterone receptor (Vicent et al, 2006). In addition, ERK and MSK are recruited to c-Fos and Egr1 promoters, and its binding to the DNA is mediated by the Elk1 transcription factor (Zhang et al, 2008). Also, it has been shown that ERK1 and ERK2 are recruited to AP-1 reporter genes in a phorbol ester-dependent manner (Benkoussa et al, 2002).

This function is not only exclusive of ERK MAPKs. Recently, direct evidence of mammalian p38 SAPK recruitment to specific gene promoters *in vivo* has been demonstrated. For instance, it has been suggested that interferon (IFN)-γ mediates p38 SAPK recruitment to STAT1-regulated gene promoters (Ramsauer et al, 2002). In addition, during skeletal myogenesis, it has been shown that p38 SAPK is recruited to regulatory elements located in the promoters of the muscle-specific genes Myog and Ckm (Marinissen et al, 1999).

Taken together, these observations suggest that in response to stress p38 SAPK may be an integral component of transcriptional complexes and can regulate multiple aspects of transcriptional control at gene promoters.

5.4. Regulation of Transcription Elongation by MAPKs

Transcription elongation is a complex process involving different factors that regulate RNA Pol II progression and gene expression. Elongation begins when RNA Pol II is released from GTFs and travels into the coding region. This event signals the recruitment of the elongation machinery, which includes the factors involved in polymerization, mRNA processing, mRNA export, and chromatin function (Hahn, 2004).

Elongation is also a critical phase of transcription susceptible to regulation by MAPKs. Indeed, in *S. cerevisiae,* Hog1 behaves as a transcriptional elongation factor in osmostress-induced genes. It has been described that Hog1 interacts with elongating RNA Pol II (phosphorylated at serine 2 and 5 on the C-terminal domain), as well as with general components of the transcription elongation complex upon osmostress (Proft et al, 2006). Apart from its association with promoters, Hog1 is also present on the coding regions of osmostress-induced genes and it travels with the elongating RNA Pol II (Pascual-Ahuir et al, 2006; Proft et al, 2006). It is worth noting that the binding of Hog1 to the coding regions is independent on the promoter bound-specific transcription factors but it depends on the 3'UTR region of osmostress responsive genes. By uncoupling Hog1-dependent transcription initiation from transcription elongation it has been demonstrated that Hog1 at coding regions is essential for an increased association of RNA Pol II in ORFs, suggesting that it directly affects the elongation process (Proft et al, 2006).

Other yeast signaling kinases, such as Fus3 or PKA, have also been reported to associate with the coding regions of activated genes (Pokholok et al, 2006), which once again indicates that signaling kinases have a role on transcription beyond initiation. Similarly, the *S. pombe* Sty1 SAPK also associates to the coding region of stress-responsive genes (Reiter et al, 2008).

The key question whether mammalian MAPKs can behave as a transcriptional elongation factors remains unclear. We know that during transcription initiation, mammalian and yeast MAPKs work as components of transcription complexes, therefore, it is expected that both of them may have the same role during elongation. However, to date, there is no evidence of the p38 SAPK association with the elongating transcriptional machinery in mammals.

5.5. Post-transcriptional control by p38 SAPK

Before a gene transcript is ready to be transported out of the nucleus, it has to undergo three major processing events to produce the fully translatable mRNA. These events comprise the acquisition of a cap structure at the 5' terminus, the splicing out of introns within the body of the pre-mRNA, and the generation of a 3' end, usually modified by the addition of a poly (A) tail. In addition, the control of mRNA nuclear export and stability is an essential process to take into account in the post-transcriptional control. Although each of these reactions is a biochemically distinct process, they are interlinked and so, they influence on another's specificity and efficiency.

Up to date, the more well-studied post-transcriptional processes regulated by MAPKs are the regulation of alternative splicing and the control of mRNA stability.

- **Regulation of alternative splicing mediated by SAPKs**

Alternative splicing is a crucial mechanism for gene regulation and for generating proteomic diversity. It has been described that MAPKs play a role in the regulation of pre-mRNA splicing by influencing the subcellular distribution of splicing factors.

For instance, it has been described that upon osmotic stress, p38 SAPK phosphorylates the heterogeneous ribonucleoprotein A1 (hnRNP A1), and as a result, the hyperphosphorylated protein is relocalizated in the cytoplasm. This change in hnRNP A1 localization can alter the alternative splicing pattern of some transcripts (Guil et al, 2006; van der Houven van Oordt et al, 2000).

- **Control of mRNA stability by SAPKs**

Control of mRNA turnover is an essential step in the regulation of gene expression in eukaryotes. Moreover, the degradation of each mRNA is influenced by the environment surrounding the cell. The connection between the environment and changes in the half-lives of mRNAs is regulated by the activity of the SAPKs and their substrates. In fact, the regulation of the stability of target mRNAs in response to different stimuli has been described as one of the mechanisms used by SAPKs to control gene expression. Several works indicate that p38 SAPK has a key role in the regulation of the stability of target mRNAs.

For instance, it has been shown that p38 SAPK regulates the binding of the destabilizing factor tristetraprolin (TTP) to AU-rich elements (ARE) in the 30-untranslated regions of cytokine mRNAs, either directly or via the downstream kinase MK2 (Hitti et al, 2006; Ronkina et al, 2008; Sandler and Stoecklin, 2008). Another example is that the K-homology splicing regulator protein (KSRP), a RNA binding protein, needs to be

phosphorylated by p38 in order to regulate myogenic transcripts (Briata et al, 2005). This links SAPK activity and mRNA stabilization.

In addition, p38 stabilizes survival motor neuron (SMN) mRNA through the binding of RNA binding protein HuR to the SMN ARE (Farooq et al, 2009). Similarly, p38 SAPK can also induce p21Cip1 mRNA stabilization, also via phosphorylation of the RNA binding protein HuR, in response to ionizing radiation (Lafarga et al, 2009).

Therefore, MAPKs play a key role in mRNA biogenesis.

OBJECTIVES

Research in our group is focused on understanding the mechanisms by which the stress-activated protein kinase p38 and its yeast relative Hog1 regulate adaptive responses to stress.

Because one of the most important responses controlled by the p38 SAPK is the regulation of gene expression, the aim of this thesis project was to give insight into the mechanisms by which p38 regulates gene transcription in response to different types of stress.

The specific objectives of this PhD Thesis were:

(1) To study the transcriptome mediated by p38 SAPK upon different stresses and its dynamical control upon stress.

(2) To characterize the molecular mechanisms by which p38 SAPK regulates transcription upon stress.

RESULTS and DISCUSSION I

Ferreiro I, Joaquin M, Islam A, Gomez-Lopez G, Barragan M, Lombardia L, et al. Whole genome analysis of p38 SAPK-mediated gene expression upon stress. BMC Genomics. 2010 Mar 1;11:144.

Figure S1. TNFα gene Network. Immune Response. Immunological Disease. Connective Tissue Disorder

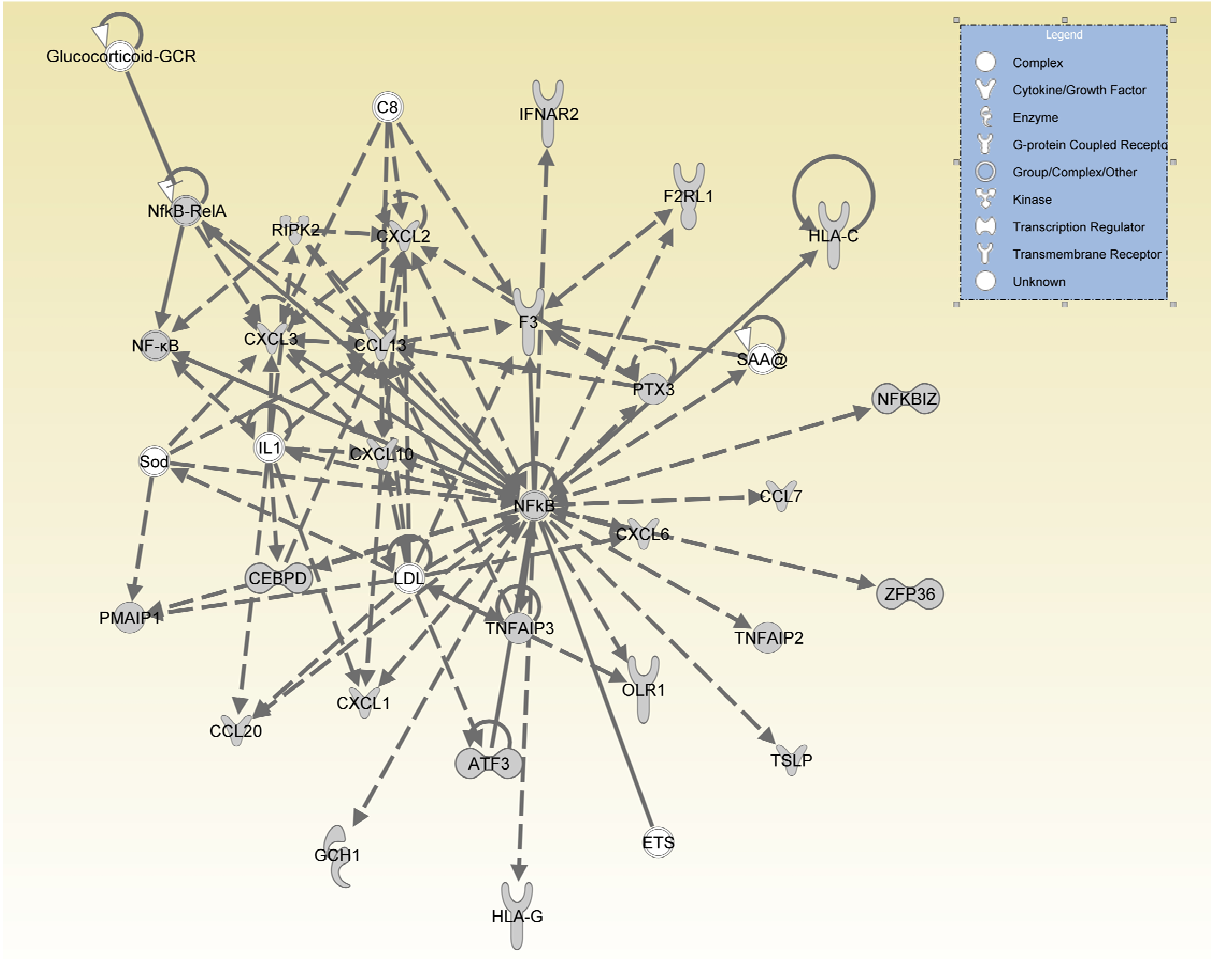


Figure S2. Anisomycin gene Network. Gene Expression and Development

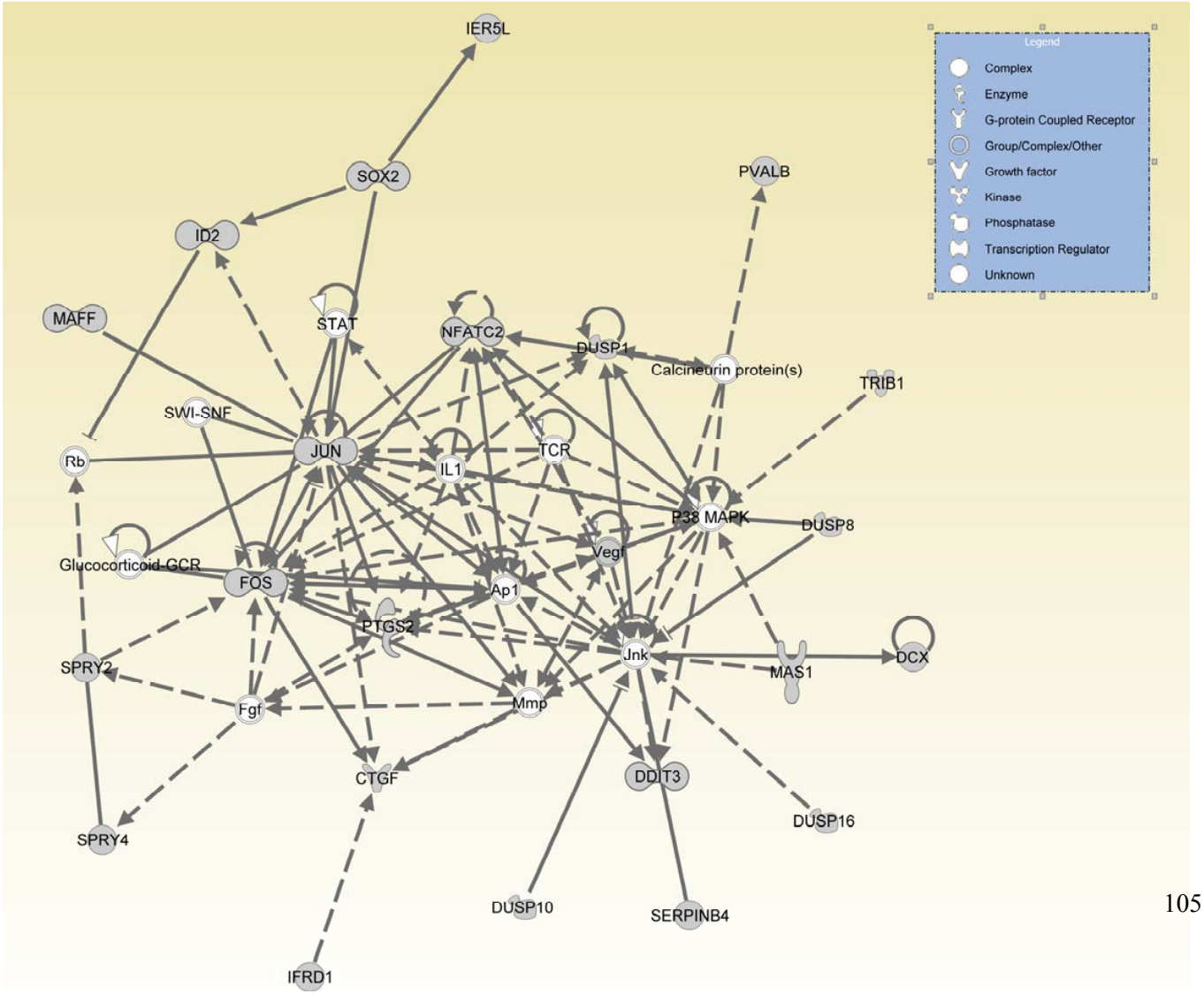
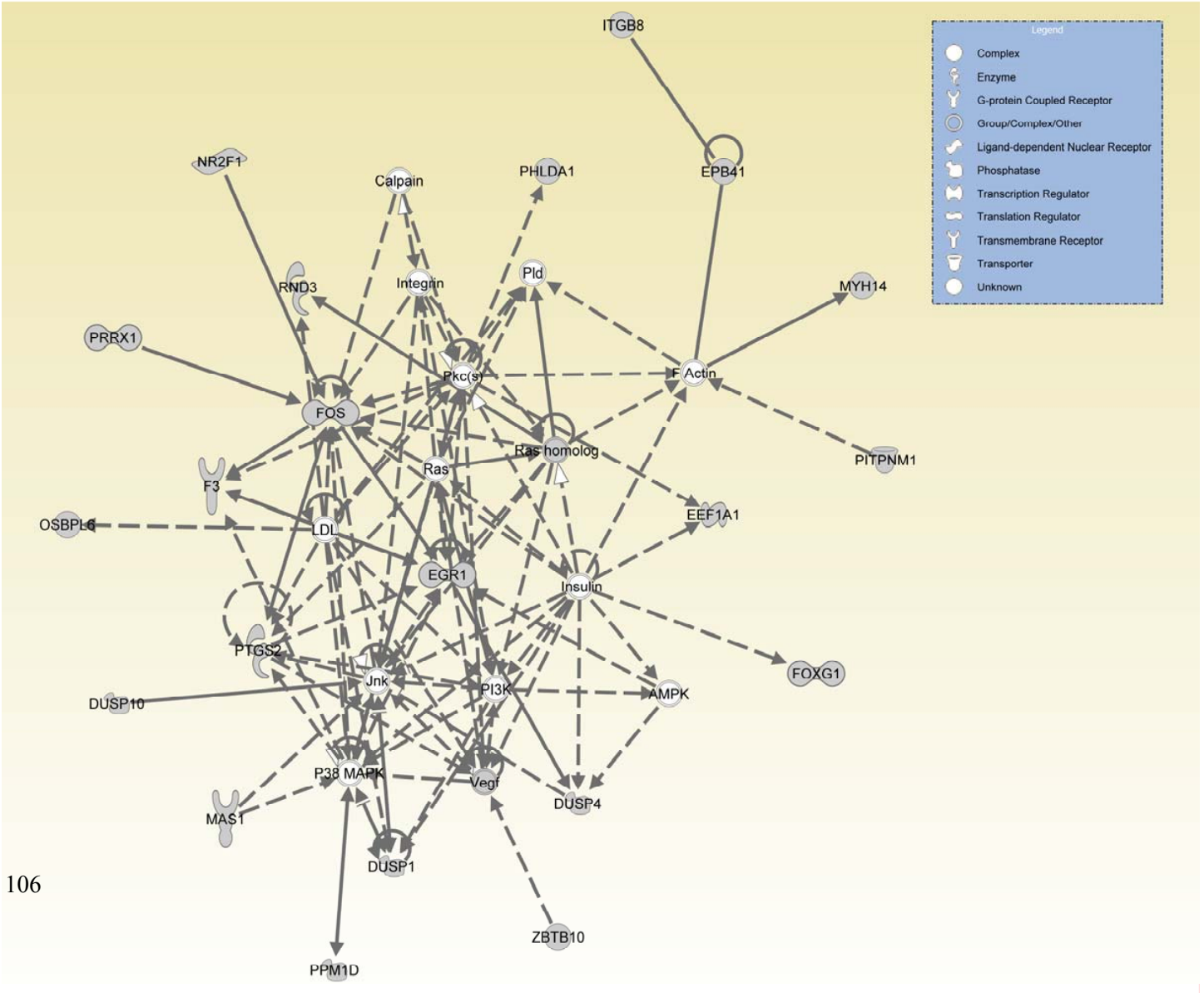


Figure S3. NaCl (2h) gene Network. Cell Cycle. Amino Acid Metabolism. Post-Translational Modification



Whole genome analysis of the p38 SAPK-mediated gene expression upon stress

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BMC Genomics 2010, 11:144

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As mentioned previously in the introduction, cells have the ability to respond and adapt to environmental changes. The activation of specific mitogen-activated protein kinases (MAPKs) triggers the adaptive responses aiming to cell survival under different environmental challenges. Several molecular and physiological processes are affected during these adaptive responses and among them regulation of gene expression is widely affected.

Over the past 18 years the p38 SAPK pathway has demonstrated to be an important mediator of stress responses (Han et al, 1993; Kyriakis J, 2001). However, how the p38 SAPK pathway regulates the expression of the appropriate genetic program allowing cells to respond to those particular stresses remains unclear.

Studies on the Hog1 SAPK had shed some light on how MAPKs might regulate transcriptional response to stress. Genome-wide transcription studies in *S. cerevisae* have revealed that after an osmotic shock, almost 7% of the genome suffers significant changes in their expression levels. Moreover, up to 70% of those genes are dependent on the Hog1 SAPK activity. All together, these results seem to indicate that Hog1 has a key

role in reprogramming the whole gene expression pattern in response to an external stimulus (Posas et al, 2000).

Up to date, the whole genome transcriptional studies on p38 SAPK that have been performed in mammals have focused on monitoring long-term transcriptional changes induced by individual stimulus. In fact, neither comprehensive genome transcription studies describing the involvement of p38 SAPK on inmediate stress-induced genes during time nor comparative analyses of the transcriptome under different stimuli have been reported to date.

The goal of the work presented here was to gain a deeper understanding on the regulation of gene expression by p38 SAPK in cells under different kinds of stress. To achieve this objective, we performed whole genome microarray analyses on mouse embryonic fibroblast (MEFs) cells treated with different p38 SAPK activators: NaCl, the cytokine tumor necrosis factor α (TNFα), and the protein synthesis inhibitor anisomycin. We also analyzed the contribution of p38α, the major p38 family member present in MEFs, to the overall transcription response using p38α knockout MEFs. To analyze the contribution of p38α and p38β, similar experiments were done using the chemical inhibitor SB203580.

Activation of p38α SAPK shows different kinetics depending on the external insult

p38 can be activated by a variety of extracellular stimuli such as osmotic shock, anisomycin, inflammatory cytokines such as TNFα, UV radiation, hypoxia and DNA damaging agents used in chemotherapy (e.g.: doxorubicin, cisplatin, etc). To analyze the kinetics of activation of p38 in response to different stimuli, we analysed by western-blot p38 phosphorylation levels by using antibodies that recognize the phosphorylated and active form of p38 after stimulation. We tested three

different stimuli that activate the SAPK: TNFα, a proinflamatory cytokine released at the sites of cell damage; the protein synthesis inhibitor anisomycin (but used at a concentration that does not fully block protein synthesis (Grollman AP, 1967)); and high osmolarity. We found that p38 SAPK showed distinct activation profiles depending on the stimulus: while TNFα caused a rapid increase in p38 activity followed by a quick down-regulation, anisomycin induced a more prolonged activation, decreasing slowly after. On the other hand, treatment with NaCl resulted in the most prolonged activation and a very slow decrease over time.

The different kinetics of p38 SAPK activation observed upon the different stimuli might be the consequence of the different upstream mechanisms by which the SAPK is activated. As described in the introduction different stimuli activate specific receptors and stress sensors which result in p38 SAPK activation. Importantly, the three stimuli did not induce p38 SAPK activation in p38α -/- MEFs at detectable levels, indicating that these cells represent a useful tool to study the loss of function of p38α SAPK. Also, we confirmed that the chemical inhibitor SB203580 did not prevent p38α phosphorylation but it inhibited p38 SAPK activity, as the phosphorylation of the p38 SAPK downstream substrate MK2 was impaired.

Analysis of the microarray data

To analyse the changes in gene expression mediated by p38 SAPK in response to different stimuli, we performed a whole genome microarray analysis. We analyzed wild type and p38α knockout MEFs, treated with TNFα, anisomycin or NaCl. Treatments were performed with different timings: 45 min for TNFα and anisomycin, and 2h for NaCl. To assess the relevance of p38 in gene induction upon stress we undertook two different approaches; the first one was based on the use of p38α knockout MEFs,

and alternatively we treated wild type MEF cells with the p38αβ inhibitor SB203580 prior to the addition of the stress.

The statistical robustness of the gene expression data obtained was guaranteed by taking into account the following criteria:

- On average, every gene was covered by at least two different probes.
- Duplicates of each sample were performed in all cases.
- The Pearson correlation coefficient (PCC) analysis was used to test the correlation between replicates, for all the probes found on the microarray. The PCC is a measure of the correlation (linear dependence) between two variables X and Y, giving values between +1 and −1 inclusive. Values of 1 or -1 imply that a linear equation describes the relationship between X and Y perfectly while a value of 0 implies that there is no linear correlation between the variables. All samples showed a high correlation coefficient between the two replicates with most correlation values higher than 0.9, indicating that there was a high reproducibility between the experimental duplicates.
- The quality of the microarray data was also diagnosed using the Bioconductor package arrayQualityMetrics that provides a report with diagnostic plots for one or two colour microarray data. With the quality metrics analysis we can assess reproducibility, identify apparent outlier arrays and compute measures of signal-to-noise ratio. The quality of the microarray data was determined by MA plots; that is, a graphic representation of the distribution of the red/green intensity ratio ('M') plotted by the average intensity (”A”). In addition, the reproducibility and homogeneity among experiments was assessed by Box plots and Density plots,

respectively. All these analyses showed a high quality for both the array data and the experimental duplicates.

- Log2 ratio average of two independent hybridizations per biological condition was selected; subsequently, the Agilent probe-Ids were converted to unique ensemble gene-Ids.
- In order to minimize the number false positives obtained, we set up 2.5-fold induction cut off. This is a more astringent threshold compared with the 2-fold induction set up employed by most of the published microarrays analyses.
- A few representative stress-regulated genes were selected to validate the results of the microarrays, and their mRNA levels were tested by RT-PCR. As expected, the tendency of regulation observed by RT-PCR confirmed the changes in expression levels found in the microarrays.

Activation of p38 by different stimuli leads to regulation of different sets of genes

Gene expression analysis showed that activation of p38 upon each stress type (osmostress, anisomycin or TNFα) resulted in the upregulation of a specific set of genes. This stress-specific response might be a result of the different activated members of the p38 pathway, the different kinetics of p38 activation along with the parallel activation of other signaling pathways as well as the differential regulation of co-activators, co-repressors and the phosphatase activity (Figure 17).

We also found that there was a limited gene expression overlapping between two stresses (27 genes were activated by osmostress and anisomycin treatment and 23 were activated by anisomycin and TNFα) and we only found a core of 37 genes that were up-regulated by the three stimuli.

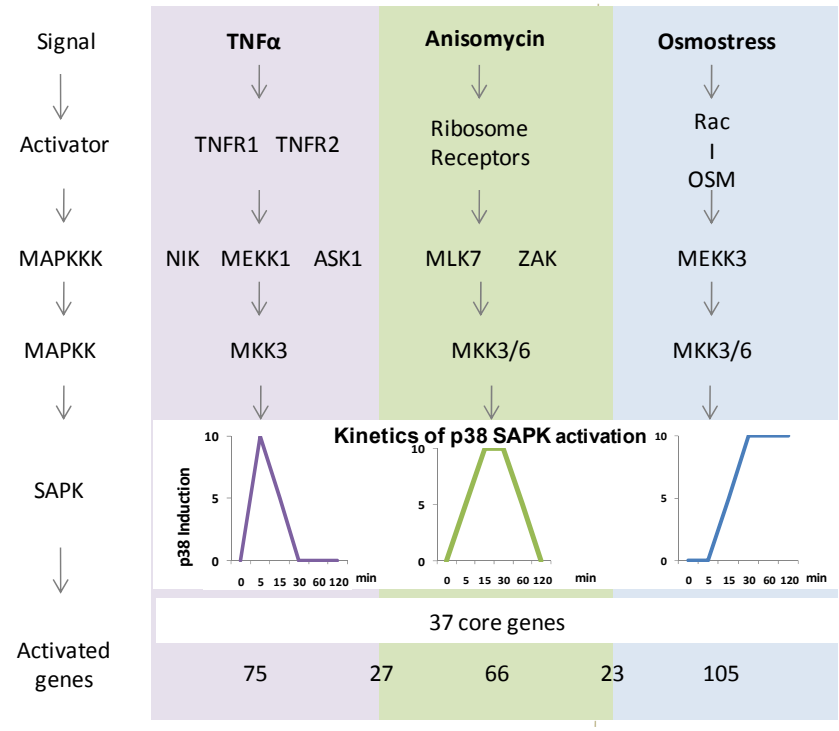

Figure 17. Relation between the stimuli used, the kinetics of p38 SAPK activation and the number of up-regulated genes. Different members of the MAPK pathway are activated upon the different stresses (osmostress, anisomycin or TNFα). This results in different kinetics of p38 SAPK activation, and specific genes upregulated for each stress type, although there are some set of genes common for two or the three stress types.

In yeast, whole genome analyses have shown that a large number of genes are induced in response to stress. This last group of genes is called environmental stress response genes (ESR) in *S. cerevisiae* and core ESR (CESR) in *S. pombe*. The ESR/CESR group includes a number of genes whose transcription is increased in response to DNA damage, heat shock, osmostress or oxidative stress among others (Gasch, 2000, Chen, 2003, Causton, 2001). Although such an ESR/CESR group has not been reported in mammals, we have found a set of 37 genes that are up-regulated upon the three types of stress tested. This group of genes might have a relevant role in the global cell adaptation response to stress.

The regulation of gene expression upon stress is highly p38 SAPK-dependent, as the p38 SAPK dependency ranged between 60% and 88% depending on the treatments. Similar results were obtained with both approaches (p38α KO MEFs and the inhibitor SB203580), although the p38 dependency in the up-regulated genes using the inhibitor appeared greater than in the p38α KO MEFs. A possible explanation could be that p38α KO cells have been adapted to the lack of p38 and may regulate gene expression through other stress activated pathways.

In addition, from 22 to 36 genes were down-regulated at least 4 fold upon the different stresses and this response was also highly depending on the p38 SAPK.

Altogether these results highlight the central role for p38 SAPK in the transcriptional response to stress. It also suggests that, in order to mediate the stress responses, p38 SAPK pathway collaborates with distinct stimulus-specific pathways and different transcription factors.

Analysis of the function of induced genes shows major stimulus-specific responses and a restricted common response to all treatments

To better understand the role of the genes induced in response to each stimulus, we analyzed the regulated genes on the basis of Gene Ontology (GO) categories and looked for the selective enrichment of genes associated with particular cellular components, molecular functions and biological processes. In addition, we performed an Ingenuity Pathway Analysis (IPA), which makes use of a bibliographic database to enable the identification of relevant gene networks and their associated functions.

As anticipated, the gene set up-regulated by TNFα was significantly enriched for genes associated with the immune, inflammatory and stress

responses. Several chemokines and cytokines genes were also up-regulated upon TNFα. TNFα treatment also up-regulated the expression of NFATc1, Pax, FosL1, IRF1 and LHX5, transcription factors which have been related with immune responses, inflammation and chemotaxis (Figure 18). This result was expected as TNFα is a cytokine involved in inflammation and immune responses. Moreover, TNFα strongly induced the up-regulation of chemokines and cytoquines that, once secreted to the media, may trigger a paracrine cell response leading to the activation of other signalling pathways and gene expression programs. Accordingly, the most relevant gene network induced by TNFα was related to the immune response and immunological disease functions.

After anisomycin treatment, several chemokine and cytokine genes were up-regulated, but to a lesser extent than with TNFα, showing a partial overlap in the responses to both stimuli. Remarkably, a large number of the anisomycin up-regulated genes were related with transcription factor activities and stress response. Consequently the most relevant gene network induced after anisomycin treatment was related to the regulation of cell differentiation and gene expression. Treatment with anisomycin also resulted in the transcriptional induction of SOX2, Edr-1, ID1, ID4, FosB, DDIT3 transcription factors. These transcription factors are involved in the regulation of mRNA biosynthesis of ribosomal proteins, so they might be required to induce the transcriptional machinery (Figure 18). Moreover, inhibitors of DNA binding (ID) family of transcription factors (ID1, ID4,) have been specifically up-regulated upon anisomycin. This family can inhibit the DNA binding and transcriptional activation and it also plays a role in cell growth and differentiation. Up-regulation of this family might be necessary to assess the correct response to the stress signals.

The genes induced by osmotic stress were enriched for those associated with RNA biosynthesis pathways, cell migration, apoptosis and the regulation of proliferation, and fitted with a gene network associated with the control of cell cycle and posttranslational modification. Again, there were some overlaps between responses as the anisomycin-up-regulated genes were also enriched for components of RNA biosynthesis pathways, whereas all the stresses showed some enrichment for regulators of cell migration, proliferation and apoptosis. Treatment with osmostress also induced a specific set of transcription factors as Mafk, n-Myc, Foxg1, Foxc1, Hoxc12 and Runx2, which are involved in regulation of solute carriers and ion transporters (Figure 18). The regulation of these processes is essential during stress adaptation in order to overcome the changes in osmolarity between the extra-cellular environment and the intracellular medium.

Not surprisingly, the main enriched functional grouping across all three stresses was for genes encoding transcription factors. Each treatment induced the up-regulation of a specific set of transcription factors, suggesting once again that their regulation could be a key process to build up the appropriate gene response for cell adaptation to a particular stress. In addition, all three treatments strongly induced a core of 37 common genes whose expression showed a high dependency on p38 SAPK activity. Importantly, 5 out of these 37 genes were transcription factors. This set of genes includes genes encoding the ubiquitously expressed AP-1 family members c-Jun and c-Fos, as well as Myc, Egr-1 and Maff. Interestingly, some of these genes have previously been reported to be p38 pathway targets (Yang et al, 2003). For example, p38 may regulate c-Jun transcription via MEF2 and ATF2, two direct substrates of p38, whereas the c-Fos and Egr-1 can be regulated by other p38 substrates, such as

ternary complex factors (TCFs) (Cuenda & Rousseau, 2007; Kyriakis J, 2001).

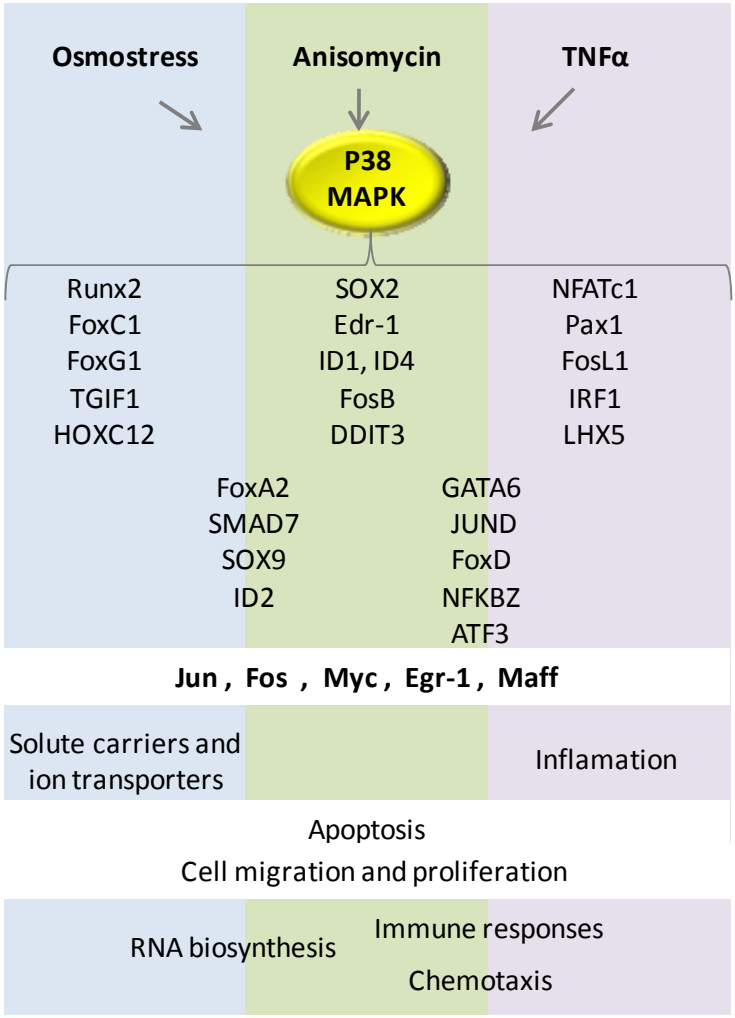

Figure 18. p38 SAPK mediates the transcriptional response to stress. The p38 SAPK pathway is activated by different stresses and leads to the phosphorylation of a number of transcriptional regulators that can orchestrate a gene expression program, including the up-regulation of many transcription factor genes. The transcription factor genes listed in the white box were up-regulated by all the stresses to some extent. The other transcription factor genes have a shadow color coded with the stress type. There are examples of genes that are up-regulated by one or more stress types. Functional categories of genes that were enriched are depicted at the bottom of the figure. (The functional categories listed in the white box were enriched in all the stresses). Adapted from (Whitmarsh, 2010).

It has previously been reported that the Dual Specificity Phosphatases (DUSP) family are p38 targets (Keyse, 2000). According to this, we have observed that activation of the p38 SAPK pathway by the three stress types might promote the expression of several DUSP genes. It is worth noting that although several DUSP genes are induced upon the three type of stresses tested, different sets of DUSPs genes are induced depending on the stimulus. These results suggest that the up-regulation of these phosphatases can differentially contribute to the different kinetics of stress response mediated by the stimuli. In agreement with our results, whole genome microarray analyses performed by (Gazel et al, 2008) have also shown that p38 SAPK pathway regulates the expression of DUSP family genes.

As already mentioned in the introduction, p38 SAPK controls the stability of several mRNAs through a mechanism that requires the activity of MK2 and the presence of AU-rich elements (AREs) at the 3' UTR of specific mRNAs. A high-throughput study showed that the stability of up to 42 mRNAs bearing ARE sequences were enhanced upon treatment of the human monocytic cell line THP-1 with LPS in a p38 SAPK dependent manner (Frevel et al, 2003). When comparing Frevel's results with ours, we found an overlapping of 12 genes (e.g. Tnfaip3, Gro1, Gro2, Gch1, PTGS2/COX2, Ccl2, Cxcl10, Junb, Csf1, Irf1, Pmaip1 and Cited2). Notably, a number of these genes were up-regulated by TNFα, which suggests that different p38 SAPK activators may contribute differently to mRNA stabilization. Moreover, we have seen that osmostress strongly up-regulates the ARE binding protein tristetraprolin (TPP) which suppress inflammation by accelerating the decay of cytokine mRNAs upon p38 SAPK activation (Sandler & Stoecklin, 2008). However, further experimental work is necessary to assess the contribution of TTP on gene transcription and mRNA stabilization in response to stress.

To date, few whole genome studies on p38 SAPK regulated genes have been reported. All of them have focused on long-term transcriptional changes using just TNFα as a p38 SAPK activator. Therefore, because of the different experimental conditions, our results are different to these other works. For instance it has been observed that cell treatment with TNFα for a long time (5h) resulted in the induction of just 58 genes, 12 of which were dependent on p38 SAPK (Viemann et al, 2004). In contrast, a 45 minutes TNFα treatment produced a strong up-regulation of 144 genes in a p38 SAPK dependent manner. This difference is consistent with our results showing that SAPK dependency decreases over time upon osmostress treatment. Therefore, the transcriptional up-regulation after 5h of cell stimulation observed by Vienmman and collaborators might correspond to a late cytokine response, driven by secondary signalling events rather than by TNFα itself whereas we might have evaluated the primary TNFα response after stress. Accordingly, the work of Zer reached similar conclusions after evaluating the transcriptional response to TNFα treatment at longer times (Zer et al, 2007).

Importantly, whole genome-wide studies on p38 SAPK transcriptionally regulated genes using osmostress or anisomycin as p38 SAPK activators have not been reported to date. Therefore, the results shown in this article have shed some light on how p38 SAPK regulates transcription upon different stresses. Overall, these results suggested that most of the genes are regulated in a stress-specific manner to mediate the specific response to each stress type. However, there is also a core set of genes fully dependent on p38 SAPK that might be essential cell adaptation to stress.

Gene Ontology analysis of p38 SAPK-mediated gene expression in response to osmostress

To obtain the big picture of how changes in transcription trigger the cellular responses to stress, we analysed the dynamic response of the genes up-regulated by osmotic stress in a time dependent manner. Interestingly, we found that the gene-expression profiles changed significantly over time upon osmostress treatment and, moreover, the dependence on p38 SAPK decreased over time.

Although it is possible that those effects might be specifically related to osmotic stress, studies in different cell types at longer time points after stimulation with TNFα showed that fewer genes are p38 SAPK dependent at later times (Viemann et al, 2004; Zer et al, 2007). In addition, we have found that there is little overlap in the set of genes up-regulated at early and late time points. These results highlight the importance of p38 SAPK for the early transcriptional responses to stress, whereas other signaling pathways also become important at later stages of the adaptation process.

The temporal changes in the transcriptional profiles in response to osmotic stress can be linked to specific cellular responses. For example, short exposures to osmotic stress (45 minutes) led to the up-regulation of solute carriers and ion transporters to allow cells to rapidly counteract the differences in osmotic pressure between the inside of the cell and the environment. The regulation of ion transport across the membrane would allow the cell to adapt to osmotic imbalances. Mid-late exposures (2 hours) results in the up-regulation of genes encoding for transcription factors and genes involved in ribosome biogenesis, which indicates the importance of new protein synthesis processes during adaptation. However, after 8 hours of cell exposure to osmostress most of the genes expressed were involved in cell survival regulation, cell growth, and

response to stress. Indeed we observed the up-regulation of many cytokine and chemokine genes, which resembled a TNFα-like response.

At this late time we also observed the up-regulation of several protein kinases and phosphatases. This could be important to down-regulate p38 SAPK signaling as well as to shut down other signalling pathways activated at earlier times which are no longer needed. The finding of novel protein kinases might reflect that new signaling pathways, might be required for long-term cell adaptation.

It is worth noting that phosphatase activity is up-regulated over time, most of the phosphatases are up-regulated at early times, and they remained highly expressed along time (DUSP1 and DUSP 4). Others, such as DUSP14, have been specifically up-regulated at later times. All together those results suggest that regulation of phosphatases is an important event to down-regulate osmostress-induced signaling pathways.

Importantly, the enrichment for transcription-factor genes increased over time, whereas p38 SAPK dependency decreases. This suggests that the primary wave of up-regulated transcription factors plays a key role on cell adaptation to osmostress, which is mainly driven by the activation of the p38 SAPK. In addition, other osmostress-activated signaling pathways are activated at later times and regulate the expression of several transcription factors which contribute to the long-term stress adaptation (Figure 19).

These results indicate that cells require the expression of an extensive genetic program for long-term adaptation to stress. The regulation of transcription factors by p38 SAPK at early times seems to play a key role to build up the gene expression network for osmostress response over time.

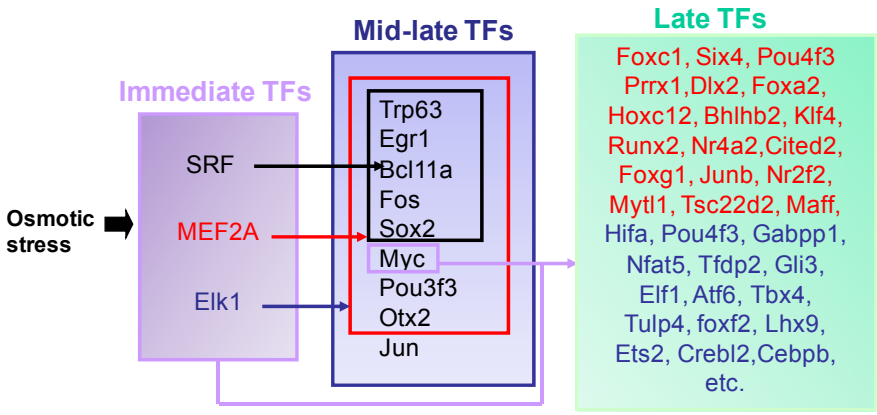

Figure 19. A complex transcription factor network. Upon osmostress, the enrichment for transcription-factor genes increases over time. This results in a complex transcription factor network, in which, the immediate TFs are the responsible for the activation of the mid-late ones and subsequently, these mediate the activation of the late TFs.

My personal contribution to this work was focused on the biological experiments. I have also been involved in the discussion and analysis of the results described in this article.

RESULTS and DISCUSSION II

Ferreiro I, Barragan M, Gubern A, Ballestar E, Joaquin M, Posas F. The p38 SAPK is recruited to chromatin via its interaction with transcription factors. J Biol Chem. 2010 Oct 8;285(41):31819-31828.

The p38 SAPK Is Recruited to Chromatin via Its Interaction with Transcription Factors

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The whole genome analysis presented in the first article of this manuscript gave us an overview of the genes regulated upon stress. Our results showed that a high percentage of the genes regulated upon these conditions were p38 dependent (Ferreiro et al, 2010b). However, the basic molecular mechanisms underlying p38 SAPK regulation of gene expression remained unknown.

In yeast, the activation of Hog1 SAPK upon osmostress affects the transcription process at different levels; 1) the direct modification of transcription factor activities by phosphorylation; 2) induces the recruitment of the kinase to the stress-responsive promoters, and 3) regulates the recruitment of RNA Pol II and transcriptional co-activators and co-repressors to stress-responsive promoters (Alepuz et al, 2003; de Nadal & Posas, 2010; Proft et al, 2006).

Despite the strong structural and functional similarities of yeast and mammalian MAP kinases (Sheikh-Hamad & Gustin, 2004), there are some fundamental aspects on how MAPKs regulate gene expression that remain elusive. The aim of this work was to study whether some of the transcriptional regulation mechanisms identified in yeast were conserved in mammalian cells.

To address this issue, we studied the basic mechanisms used by p38 SAPK to regulate transcription in response to different types of stress: osmotic shock anisomycin or TNFα. We assessed the expression of c-Fos, Cox2 and Interleukin 8 (IL8) genes as models since they were among the most upregulated and p38 dependent upon stress (Ferreiro et al, 2010a).

In summary we found that in response to different stresses, the p38 SAPK is recruited to stress responsive genes via its interaction with specific transcription factors, allowing the recruitment of RNA Pol II to those promoters. In addition, we observed that p38 is recruited to the coding regions of stress-induced genes under these conditions, suggesting that it might be playing a role in transcription elongation

The p38 SAPK is recruited to osmostress-induced genes

Upon osmostress, p38 is transiently phosphorylated and accumulated into the nucleus. Importantly, at the concentration of NaCl tested, osmostress did not induce the phosphorylation of other MAPKs, such as JNK or ERK. Consequently, the outcome observed upon osmostress resulted only from p38 activation.

It has been shown that the yeast Hog1 SAPK regulates transcription of osmostress-induced genes by directly binding to their promoters (Alepuz et al, 2003; de Nadal & Posas, 2010). These findings prompted us to wonder whether kinase recruitment could also be established in higher eukaryotic cells in response to stress. In mammals, ERK MAPK and other signaling kinases are also known to be recruited to gene promoters upon stimuli (Benkoussa et al, 2002; Lawrence et al, 2008; Vicent et al, 2006; Zhang et al, 2008). Moreover, the p38 SAPK is recruited to specific gene promoters during skeletal myogenesis (Simone et al, 2004). Indeed, we observed that p38 SAPK is recruited at the c-Fos promoter upon osmostress, anisomycin and TNFα treatments. Interestingly, the activity of

p38 SAPK is essential for its recruitment since the addition of SB203580 prevents it. These results indicate that p38 SAPK itself might be a component of the transcription factor complex, probably playing an important role in the regulation of transcription initiation.

In addition to its association with promoters, the *S. cerevisiae* kinase Hog1 has also been found on coding regions of osmostress-induced genes, traveling with the elongating RNA Pol II (Pascual-Ahuir et al, 2006; Pokholok et al, 2006; Proft et al, 2006). It has also been shown that in *S. Pombe* the Sty1 MAPK was recruited at the coding regions of stress-responsive genes (Reiter et al, 2008). Given the high degree of conservation of MAPK functions along eukaryotes, we assessed whether the p38 SAPK was recruited at the coding regions of c-Fos, Cox2 and IL8 genes during the stress response. We found that upon stress p38 is recruited at the ORFs of these genes. Moreover, this recruitment is dependent on the p38 activity itself, as it is abolished by treatment with the p38αβ inhibitor SB203580. These results suggest that, as in yeast, p38 SAPK is traveling with the RNA Pol II along the ORF, traveling with the elongating machinery as the nascent mRNA is synthesized (Proft et al, 2006).

We found also that p38 SAPK is not the only member of the signaling cascade present in the stress-responsive genes. In fact, the upstream p38 MAPKK, MKK6, was also detected in the gene promoters when p38 was there. This suggests the existence of an active signaling module comprising both the MAPK and the MAPKK. Probably, MKK6 plays a role in maintaining p38 SAPK phosphorylation during transcription. Similar mechanisms have been described for other kinases such as the ERK1 MAPK as it associates to chromatin together with the MAPKK MEKK1 (Lawrence et al, 2008). This result suggests that it could be a

general strategy to ensure the kinase activities at the gene-promoters during the transcriptional response to stress.

Recruitment of RNA Pol II at stress-responsive promoters requires p38 SAPK activity

As pointed out in the introduction, RNA polymerases are enzymes that mediate transcription in collaboration with transcription and modification factors. Transcription initiation starts when RNA Pol II and the basal transcription machinery bind at gene promoters and form the so-called transcription initiation complex.

It has been described that SAPKs interact with RNA Pol II in yeast and mammals. Also, the yeast Hog1 facilitates the recruitment of the RNA Pol II holoenzyme to promoters, to promote transcription initiation of osmoresponsive genes (Alepuz et al, 2003). The presence of the activated p38 SAPK at the c-Fos promoter could be an essential step in mammalian cells for recruiting the RNA Pol II to this promoter. Based on the functional conservation between yeast and mammalian SAPKs, we asked ourselves if the recruitment of the activated p38 SAPK to c-Fos promoter was a necessary step to recruit RNA Pol II to this promoter.

ChIP analysis in HeLa cells showed that RNA Pol II is recruited to the c-Fos promoter after NaCl addition and consequently, c-Fos transcription was induced. This result is consistent with previous data on genome-wide transcription analysis upon stress which showed how c-Fos gene expression was highly induced after NaCl addition (Ferreiro et al, 2010b). As previously mentioned, the recruitment of RNA Pol II to stress-induced promoters requires p38 activity; this is in agreement with the results of the genome-wide transcriptional analyses that showed how c-Fos transcription is also abolished in the presence of the p38αβ inhibitor (Ferreiro et al, 2010b).

Promoter-bound p38 SAPK drives gene transcription

We showed that the presence of p38 at the promoters of stress-responsive genes is necessary to induce gene expression, facilitating the recruitment of the Pol II complex to these promoters. However, it is still not known whether the selective recruitment of the p38 SAPK to the promoters is enough to induce transcriptional activation.

In yeast, it has been shown that when the Hog1 SAPK is artificially tethered to a promoter, the presence of the kinase is sufficient to recruit the RNA Pol II complex and induce transcription upon osmostress (Alepuz et al, 2003). To address whether the mammalian p38 recruitment to the promoters is sufficient to induce gene transcription, we used a recombinant Lex A-p38 SAPK which includes the LexA DNA binding domain fused to the p38 SAPK. We observed that the artificial binding of p38 SAPK to Lex A promoter was sufficient to activate transcription in a stress-regulated manner. Moreover, the increase of transcription only was observed under stress conditions or when MKK6 was over-expressed. Importantly, the transcription response was blocked when using the p38αβ inhibitor, showing that the activity of the SAPK is essential to induce transcription. Overall, these results show that the binding of active p38 SAPK to a promoter is sufficient to drive gene transcription.

Recruitment of p38 at the c-Fos promoter requires the transcription factor Elk1

It is well known that MAPKs have no defined DNA binding domain, suggesting that its binding to gene promoters might not be direct. Likewise, no DNA binding domain has been described in the yeast Hog1 and however, the kinase is recruited to chromatin through its interaction with transcription factors (Alepuz et al, 2001). In that case, transcription factors act as nuclear anchors for the SAPK to the chromatin by engaging

stable interactions with them (Reiser, 1999). Given the functional similarities found between yeast Hog1 and mammalian p38 SAPK, we hypothesized that the binding of p38 to chromatin might be mediated as well by its association to transcription factors.

The c-Fos promoter is regulated by a broad range of basal and inducible transcription factors in response to different stimuli (Hill & Treisman, 1995a). Among them, the Elk-1 transcription factor, an essential component in the serum response element (Sharrocks, 2001), can be phosphorylated at multiple sites by ERK, JNK and p38 MAPKs (Marais et al, 1993; Price et al, 1996). In addition, Elk-1 transcription factor is up-regulated in response to stress (Yang & Sharrocks, 2006). Based on these observations, we decided to study the relationship between p38 and Elk1, and how these proteins might contribute to the regulation of c-Fos gene expression.

We found that both proteins were able to interact with each other, which suggested that Elk1 might be a substrate for the p38 SAPK. Indeed Elk1 was phosporylated upon stress in a p38 SAPK dependent manner. These results encouraged us to study whether Elk1 might be responsible for p38 recruitment to the c-Fos promoter. By knocking down endogenous Elk1 levels using a specific RNA interference, we observed that c-Fos expression was significantly compromised upon osmostress. However, the fact that c-Fos expression was not totally suppressed suggests that either an Elk1-independent mechanism or other members of the Elk1 family might be also playing a role in the regulation of c-Fos expression.

Importantly, the ChIP assays showed that p38 recruitment to the c-Fos promoter was impaired in the absence of Elk1 protein upon osmostress. Notably Elk1 binding to the c-Fos promoter was abolished when p38 was inactivated with a specific inhibitor. Therefore Elk1 and p38 SAPK recruitment to chromatin is interdependent and the Elk1 transcription

factor acts as an anchor for p38 SAPK recruitment to the chromatin. This observation resembles the interdependency observed in yeast between Hog1 SAPK and the transcription factor Hot1; as they both are essential elements at the Hot1-osmoresponsive promoters in order to induce transcription (Alepuz et al, 2003) (Figure 20).

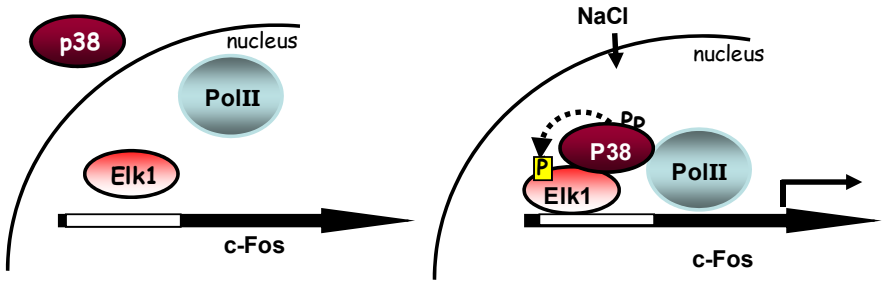

Figure 20. Model of p38 SAPK regulation of c-Fos promoter upon osmostress. In response to stress, p38 is phosphorylated and then translocates from the cytoplasm into the nucleus where it regulates transcription of the stress-induced genes, such as c-Fos. Once into the nucleus, p38 binds at the c-Fos promoter via Elk1 transcription factor and mediates RNA Pol II recruitment at the promoter.

Overall, these results indicate that upon stress, p38 SAPK phosphorylates the transcription factor Elk1, and binds to c-Fos gene promoter through its interaction with this transcription factor, which acts as an anchor for the SAPK to chromatin.

Recruitment of active p38 SAPK to chromatin is a general mechanism

To address whether the recruitment of p38 SAPK to the stress-responsive gene promoters via transcription factors was a general mechanism of gene expression regulation, we extended our study to other p38-dependent genes u-regulated by stress, such as Cox2 and Interleukin 8 (IL8).

We knew from the whole genome analysis (Ferreiro et al, 2010b) that upon osmostress p38 regulates Cox2 transcription. In addition, we found that p38 was recruited to Cox2 promoter in response to osmostress.

It is known that the Cox2 gene expression is not regulated by Elk1 albeit it can be activated by both NFκB and AP1 transcription factors (Kang, Smith, 2007). Based on these observations, we addressed whether Cox2 transcription upon osmostress was regulated by NFκB or AP1 transcription factors.

We observed that osmostress did not induce IkB degradation, and consequently NFκB remained inactive in the cytoplasm. On the other hand, p38 activation upon osmostress directly up-regulates AP1 transcriptional activity (Tanos, Marinissen, Coso, 2005). Considering all these data, we focused on the putative role of AP1 transcription factor in p38 recruitment at the Cox2 promoter. We found that targeting the c-Jun protein, a component of the dimeric AP1 transcription factor, with specific siRNAs, reduced Cox2 gene transcription upon osmostress. Correspondingly p38 recruitment at the Cox2 promoter was also compromised.

As pointed out in the introduction p38 activation occurs in response to several stimuli. To determine whether p38 recruitment to responsive gene promoters via interaction with a transcription factors was not limited to osmostress, we analyzed the binding of p38 to the IL8 gene promoter in response to TNFα. TNFα strongly mediates NFκB and p38 SAPK signaling and induces IL8 expression (Schieven, 2005). We observed that upon TNFα the p38 SAPK was recruited to the IL8 promoter. In addition, NFκB inactivation prevented the recruitment of the p38 to IL8, imparing the transcription of the IL8 gene completely. These results show that the recruitment of p38 SAPK to the IL8 promoter upon stress requires the NFκB transcription factor.

Here we show that regardless the stress used to activate p38 (osmostress, or treatments with either TNFα or anisomycin) p38 is recruited to stress-responsive promoters via its interaction with transcription factors. Anchoring an active p38 to specific gene promoters mediates the recruitment of RNA Pol II and transcription initiation. Moreover, the presence of p38 SAPK at the ORFs of stress-induced genes suggests that p38 SAPK might be regulating the mRNA elongation process. Taken together, SAPK recruitment to target genes appears to be a general mechanism for regulation of gene transcription that has been conserved from yeast to mammals (Figure 21).

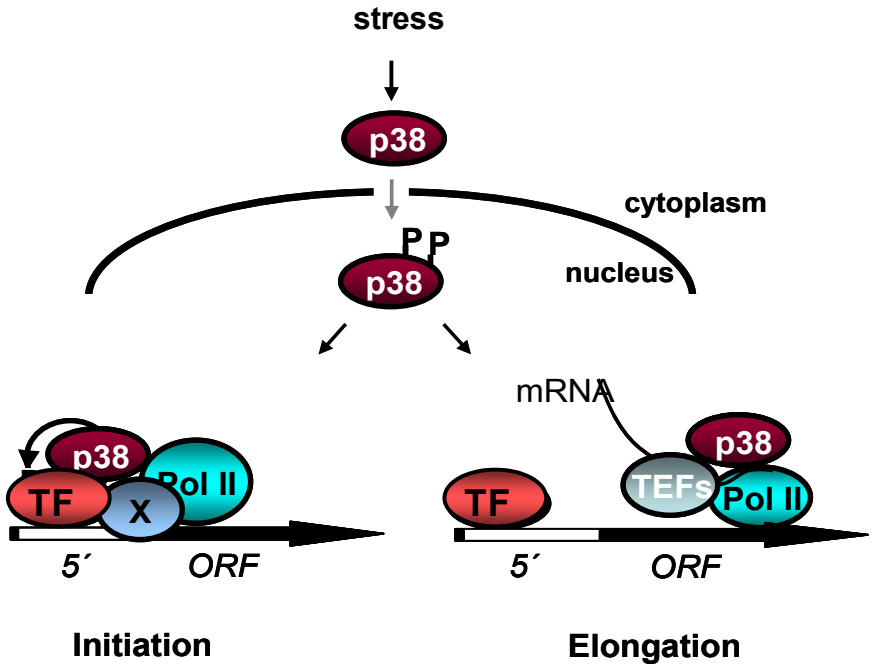

Figure 21. Model for the p38 SAPK regulation of transcription upon stress. p38 SAPK plays a role on the transcription regulation of stress-induced genes at initiation and elongation through phosphorylation of transcription factors and direct binding at promoters and ORFs of stress-responsive genes

Personal contribution to this work: I have been involved in the design and carrying out most of the experiments described in this article. Moreover, I have also contributed to the discussion and writing of the article.

GLOBAL DISCUSSION

Adaptation to stress requires efficient and rapid responses to maximize cell survival, and the regulation of gene expression by MAPKs is essential in this process. Therefore, understanding the role of p38 SAPK in the regulation of gene expression upon stress was a key aim of this PhD project.

Studies with the yeast Hog1 SAPK have shed some light on the transcription regulation by MAPKs in response to stress. Indeed, the key role of Hog1 on transcription is illustrated by several genome-wide transcriptional studies which revealed that a large number of genes (5-7% of the yeast genome approximately) showed significant expression changes in response to osmotic shock in a Hog1-dependent manner (Posas et al., 2000; Rep et al., 2000; Gasch et al., 2000; Causton et al., 2001; O'Rourke and Herskowitz, 2004). In addition, the key role of Hog1 regulating transcription upon osmostress has been clearly demonstrated in several works (Alepuz et al., 2001; Alepuz et al., 2003; de Nadal et al., 2003; Proft and Struhl, 2002).

Whole-genome expression analysis showed that the treatment with distinct stresses leads to a complex gene pattern response specific for a given stress with a restricted set of overlapping genes that involves an important set of transcription factors. Also we observed that most of these genes are highly p38 dependent. Furthermore, we found that the dependency of SAPK varies depending on the time the cells are subjected to osmostress and the number of transcription factors induced increased over time. This indicates that cells require a program of new gene expression for long-term adaptation to stress, and the transcription factors regulated by p38 SAPK might be critical for either cell adaptation or preparation for continuous extra-cellular changes.

It has been described that the most frequent regulatory mechanism driven by MAPKs to modify gene expression is the phosphorylation and

activation of transcription factors (Hill & Treisman, 1995b; Hunter & Karin, 1992). In fact, numerous transcription factors and their co-regulatory proteins were identified as targets of p38 SAPK pathway (Table 1).

The mechanisms underlying gene expression regulation by p38 SAPK have not yet been elucidated beyond the modification of transcription factors, and it has been proposed that MAPKs regulate gene expression by using a broad range of molecular mechanisms (Whitmarsh, 2007). We observed that upon stress, p38 SAPK regulates transcription through its recruitment to gene promoters via its interaction with transcription factors which function as anchors to the chromatin for the SAPK. Moreover, the RNA Pol II and the MKK6 are also recruited to stress-responsive promoters and this association depends on the presence of the active p38 SAPK.

Therefore, the strong up-regulation of transcription factors mediated by p38 that occurs upon stress (Ferreiro et al, 2010b) seems to have a key role in the mechanism of transcription regulation mediated by p38 upon stress, since the p38 SAPK needs to interact with transcription factors to be recruited to promoters (Ferreiro et al, 2010a).

In *S. cerevisiae* is well described that upon osmotic shock Hog SAPK mediates the recruitment of different co-activators to gene promoters and ORFs to properly induce transcription. For instance SAGA, mediator and SWI/SNF complex are targeted to osmostress-induced genes in a Hog1-dependent manner for proper transcriptional outcome (De Nadal et al, 2004; Zapater et al, 2007). In addition, it has been shown that Hog1 SAPK interacts with RSC to mediate its recruitment to the ORF of osmoresponsive genes during elongation (Mas et al, 2009). To date, in mammals, it only has been reported that p38 SAPK mediates the recruitment of SWI-SNF chromatin remodeling complex to promoters

(Marinissen et al, 1999) but no other co-activator has been reported to be recruited by p38. Taking into account the structural and functional conservation between Hog1 and p38 SAPK, it could be interesting to study if the p38 SAPK is also mediating the recruitment of several coactivators to promoters and to coding regions of the stress-induced genes to properly regulate transcription.

In addition, a previous work from our laboratory in *S. cerevisiae* showed that Hog1 mediates the recruitment of a histone deacetylase complex to stress-responsive genes to mediate gene expression (De Nadal et al, 2004). In mammals there are examples of some relation between MAPKs and HAT/HDAC activity (Alberts et al, 1998) (Kawasaki et al, 2000), however whether actelase/deacetylase activity is necessary to regulate stress-induced gene expression has not been defined yet. Preliminary results from our laboratory suggested that deacetylase activity might be necessary for transcription regulation of stress-induced genes and that p38 SAPKs plays a key role in this regulation. Although further experiments would be required to confirm it, our results suggest that regulation of gene expression by SAPKs is conserved through evolution from yeast to mammals.

Posttranslational modifications of histones play an important role in transcription and they can also influence the recruitment of many factors to gene. Thus, it would be also interesting to study if p38 is regulating histone modifications to regulate transcription upon stress.

The results presented in this PhD project established that p38 SAPK is an essential regulator of the transcriptional response to stress. In addition, the knowledge of the mechanisms used by this SAPK to regulate transcription is important to understand how the appropriate pattern of genes is expressed under stress conditions, as deregulation of the appropriate

pattern of gene expression has profound effects on cellular functions and many diseases.

In conclusion, the results obtained in this PhD project have shed light on the mechanisms by which the p38 SAPK regulates gene expression upon different stress types.

CONCLUSIONS

The following conclusions can be drawn from the results of the articles presented in this PhD thesis:

1. The kinetic of p38α SAPK activation in response to external insults depends on the stimuli.
2. Activation of p38 SAPK by three different stimuli leads to the regulation of different sets of genes, with only a small core of overlapping genes.
3. From 60 to 80% of the induced stress-responsive genes are dependent on p38 SAPK activity.
4. Upon osmostress, the dependency of the stress-induced genes to p38 SAPK decreased over time.
5. The set of genes upregulated by the three different stimuli are enriched in different functions and molecular processes associated with each stimulus, except for the upregulation of transcription factors that are common for the three stresses.
6. p38 SAPK regulates different genes during time upon osmostress.

7. Upon stress, p38 SAPK associates to and regulates c-Fos, Cox2 and IL8 promoters.
8. p38 SAPK is recruited to the coding regions of c-Fos, Cox2 and IL8 genes after stress treatment.
9. Association of the SAPK to stress-responsive genes depends on its catalytic activity.
10. Recruitment of RNA Pol II to stress induced promoters requires the p38 SAPK catalytic activity.
11. Tethering active p38 SAPK to promoters is sufficient for drive gene transcription.

12. Recruitment of p38 SAPK to promoters via its association with transcription factors is a general mechanism employed by SAPKs to regulate gene expression that has been conserved throughout evolution.

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