Regulation of alternative splicing by the p38 SAPK in response to stress

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A la meva família

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SUMMARY

SUMMARY

Cells have the ability to respond and adapt to environmental changes through activation of stress-activated protein kinases (SAPKs). Activation of the p38 SAPK is critical to elicit the early gene expression program required for cell adaptation to stress. Alternative splicing (AS) is a crucial mechanism for gene regulation that has been shown to be modulated in response to a wide range of extracellular stimuli. However, the mechanisms behind AS regulation and the functional consequences of differential isoform expression in stress conditions remain largely unknown.

In this study, we identified novel associations between p38 and the splicing machinery, providing mechanisms by which p38 regulates AS. Furthermore, characterization of protein isoforms produced by p38-dependent AS give an insight into the biological relevance of this regulation in adaptation to environmental stresses.

Altogether, the results of this thesis highlight a role for SAPK signaling pathways in adaptation to environmental changes through AS modulation.

SUMMARY

Les cèl·lules tenen l'habilitat de detectar i respondre a les fluctuacions en el seu entorn a través de l'activació de les proteïnes quinasa activades per estrès (SAPKs). L'activació de la SAPK p38 és essencial per activar el programa d'expressió gènica necessari perquè les cèl·lules s'adaptin als estímuls extracel·lulars. El processament alternatiu del pre-ARNm és un mecanisme de regulació gènica crucial que es regula en resposta a diferents canvis en l'ambient. No obstant, els mecanismes moleculars pels quals es regula, així com la funció de les diferents isoformes de les proteïnes que s'expressen en resposta a estrès no es coneixen.

En conjunt, els resultats presentats en aquesta tesis proporcionen una nova visió dels mecanismes pels quals p38 modula el processament alternatiu del pre-ARNm en resposta a estrès i posen en evidència la importància d'aquest mecanisme per l'adaptació de la cèl·lula a canvis en l'ambient extracel·lular.

PREFACE

PREFACE

Miss-regulation of signaling pathways in response to environmental changes has been associated to a high number of pathological processes and human diseases. For this reason, the understanding of the regulatory mechanisms that allow cells to respond and adapt to stress is a key aspect to study.

Upon stress, the expression of each gene in the genome is precisely regulated by several processes which modulate all steps of mRNA biogenesis. Remarkably, increasing evidence indicate that alternative splicing enhances the ability of cells to deal with environmental stresses.

In this thesis, we describe how stress modulates splicing and define a role of p38 over specific components of the splicing machinery to regulate their function. Together, our data demonstrate that p38 has a key function in the control of splicing during stress, resulting in a reciprocal regulation between signaling and splicing.

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1.1 MAPK pathways

Cells need to quickly sense and respond to changes in the extracellular environment in order to survive. In eukaryotic cells, Mitogen-Activated Protein Kinase (MAPK) cascades play a key role in the regulation of several aspects of the cellular physiology by converting extracellular signals into intracellular responses.

1.1.1 Organization of MAPK modules

MAPK pathways are organized in modules containing three sequentially activated protein kinases, MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). Activation of the MAPKKK usually involves interaction with small G-proteins or phosphorylation by protein kinases downstream from cell surface receptors (Cuevas et al., 2007). Once activated, the MAPKKKs directly phosphorylate and activate MAPKKs, which in turn activate MAPKs by dual phosphorylation of conserved threonine and tyrosine residues in the (T-X-Y) motif located in the activation loop (Canagarajah et al., 1997; English et al., 1999). One of the mechanisms providing specificity in signaling is the existence of docking sites in MAPKs that allow selective activation by MAPKKs (Bardwell and Thorner 1996). Moreover, additional substrate specificity is conferred by specific multi-domain scaffolding proteins (Meister et al., 2013). Upon activation, MAPKs phosphorylate specific substrates on serine or threonine residues followed by a proline, leading to modulation of diverse physiological processes.

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1.1.2 MAPK families

Eukaryotic cells possess multiple MAPK pathways that are activated by different stimuli, allowing cells to respond to divergent inputs and tightly regulate high complex processes.

There are three well-characterized subfamilies of MAPKs in multicellular organisms (Chang and Karin, 2001). These MAPKs include the ERKs (extracellular signal-regulated kinases 1/2), JNKs (Jun amino-terminal kinases) and p38 (Figure 1).
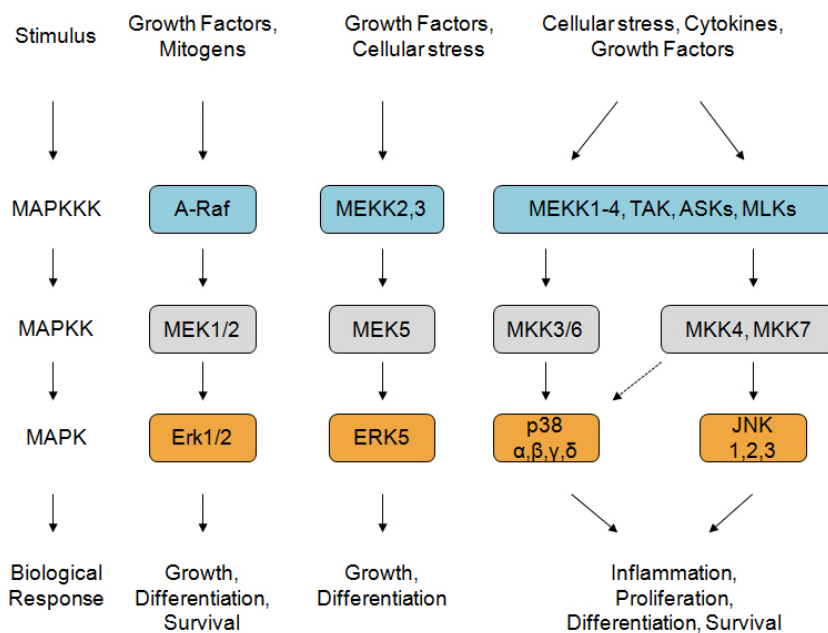
Figure 1. MAPK pathways
ERKs, JNKs, and p38 pathways respond to different types of stimuli and trigger different cellular responses. Representative signaling molecules are shown.

ERK components have a TEY motif in the activation segment and can be divided in two subfamilies, ERK1/2 and ERK5. ERK1/2 module is activated by growth factors and mitogens and induces cell growth and differentiation (McKay and Morrison 2007; Shaul

and Seger 2007) while ERK5 can be activated by mitogens as well as cellular stresses (Wang and Tournier, 2006).

On the other hand, JNK and p38 pathways are strongly stimulated by environmental stresses, therefore named Stress-Activated Protein Kinases (SAPKs). Both pathways are able to integrate a large number of extracellular stimuli, cooperating to elaborate an appropriate response depending on the cell type and stimulation. The JNK pathway is activated by ionizing radiation, heat shock, oxidative stress, osmostress and DNA damage, as well as inflammatory cytokines and growth factors. JNK family members are characterized by the presence of a TPY motif in the activation loop. The JNK module is composed by the MAPKKs MKK4 and MKK7, and the MAPKKKs MEKK1, MEKK4, MLK2, MLK3, ASK1, TAK1, and TPL2. The JNK pathway is involved in regulation of apoptosis, inflammation, cytokine production, and metabolism (Dhanasekaran and Reddy 2008; Huang et al. 2009; Rincon and Davis 2009). This MAPK was originally named as JNK by their ability to phosphorylate the transcription factor c-Jun, although it phosphorylates several substrates located not only in the nucleus but also in membranes, the cytoplasm and the cytoskeleton.

1.2 The p38 SAPK pathway

Activation of p38 kinases is mediated by dual phosphorylation at a TGY motif. p38 is the human homologous of the *Saccharomyces cerevisiae* Hog1 and complements Hog1 deficient yeast mutants (Han et al., 1994; Kumar et al., 1995). Due to the high conservation between yeast and mammals (Han J, Lee JD 1994; Moriguchi et al. 1996; Takekawa, Posas, and Saito 1997) characterization of the

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Hog1 SAPK pathway has significantly contributed to the understanding of the role of p38 in human.
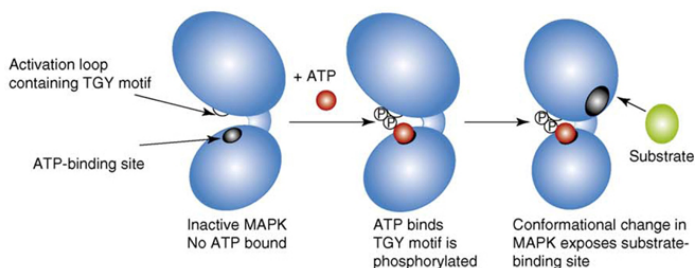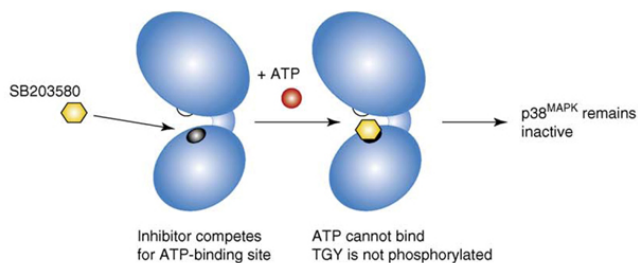
Figure 2. p38 activation and inhibition
p38 is activated in multiple steps. ATP is required for phosphorylation of the TGY motif on the activation loop. Once phosohorylated, there is a conformational change in the kinase, exposing a binding site for a substrate. Pyridinyl imidazole inhibitors (e.g. SB203580) compete for the ATP-binding site. Once the inhibitor is bound, ATP is blocked and phosphorylation of the TGY motif does not occur, leaving p38 inactive (Coulthard et al., 2009).

Mammalian p38 family include four different p38 isoforms, α, β, γ and δ (Nebreda and Porras, 2000). The four p38 SAPKs are encoded by different genes and are divided in two groups based on their similarity and tissue distribution. p38α and p38β are ubiquitously expressed, while p38γ and p38δ are differentially expressed depending on the tissue type. These two subgroups

have different susceptibilities to inhibition by pharmacological compounds. In fact, the use of different classes of inhibitors for the study of p38 functions allowed certain definition of the relative contribution of p38 isoforms to stress response (Coulthard et al., 2009). In this work we used the pyridinyl imidazole inhibitor SB203580, which preferentially inhibits p38α and p38β activity (Goedert et al., 1997) (Figure 2).

1.2.1 The p38 SAPK module

There are several MAP3Ks upstream p38, including ASK1, DLK, TAK, TAO1, TAO2, TPL2, MLK3, MEKK3, MEKK4, and ZAK1. MKK3 and MKK6 are the MAP2Ks that selectively activate p38 (Cuenda et al. 1996, 1997; Raingeaud et al. 1996). However, it has been shown that the JNK activator MKK4 can also activate p38 in specific cell types (Jiang et al. 1997). Moreover, other non-canonical mechanisms of p38 activation have been demonstrated through intrinsic p38 autophosphorylation (Avitzour et al., 2007; Levin-Salomon et al., 2008), which can be facilitated by direct interaction with TAB1 (Ge, 2002; De Nicola et al., 2013) or ZAP-70 (Mittelstadt et al., 2005; Salvador et al., 2005) in a cell type specific manner.

Upon activation, p38 translocates from the cytoplasm to the nucleus, where it activates specific substrates through docking-mediated interactions (Raingeaud et al. 1996) (Figure 3). Among the p38 targets there are transcription factors, chromatin remodeling molecules, protein kinases and RNA binding proteins. Nevertheless, it has been reported that p38 can also phosphorylate cytosolic proteins related to protein degradation and localization,

mRNA stability, endocytosis, apoptosis, cytoskeleton dynamics or cell migration (Trempolec et al., 2013).
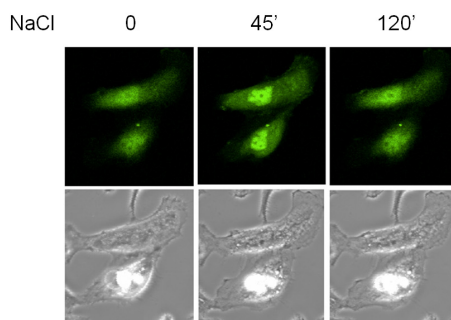
Figure 3. p38 translocates into the nucleus upon stress
In resting conditions, p38 localizes in the ctytoplasm. NaCl treatment induces phosphorylation and translocation of p38 to the nucleus. 2h after osmotic shock, p38 is relocalizes into the cytoplasm (Ferreiro et al., 2010a).

1.2.2 Regulation of the p38 pathway

Limited duration of MAPK signaling is essential to reach specific outcomes. Inactivation of p38 signaling is controlled by several phosphatases including PP2C (Ser/Thr phosphatase), PTP (Tyr phosphatase) (Takekawa et al, 2000; Takekawa et al, 1998) and members of the DUSP family, which can dephosphorylate both Tyr and Thr residues (Huang and Tan, 2012). Therefore, downregulation of p38 allows to achieve transient activation and to regulation of signal intensity.

Despite p38 activity is mainly controlled by phosphorylation and dephosphorylation mechanisms, there are additional regulatory mechanisms involved in the regulation of the pathway, including scaffolding and compartmentalization of their components, changes in protein expression and posttranslational modifications (Cuadrado and Nebreda 2010).

1.2.3 Activation of the p38 pathway

The p38 SAPK pathway is activated by environmental stresses such as high osmolarity, UV irradiation, heat shock, hypoxia, heat, oxidative stress, and inflammatory cytokines (Cuadrado and Nebreda, 2010). In this study, we have mainly worked with osmostress as a model to understand the effect of p38 activation on regulation of alternative splicing.

An increase in extracellular osmolarity results in a water flux out of the cell that leads to shrinking and intracellular dehydration. To maintain cellular integrity and homeostasis in these conditions, cells trigger adaptive responses to restore cell volume and preserve cytoskeletal architecture (Hoffmann et al., 2009; Koivusalo et al., 2009; Lang et al., 1998). The response to osmostress in mammalian cells is primarily mediated by the p38 SAPK pathway. Hyperosmotic shock induces recruitment of OSM, an actin binding scaffold protein that forms a complex with Rac, MEKK3 and MKK3 triggering activation of p38 (Uhlik et al. 2003). This model is supported by other studies that demonstrate that activation of p38 upon osmostress takes place close to the plasma membrane (Tomida et al. 2009).

1.2.4 Physiological processes regulated by p38

p38 has a pivotal role in regulation of cellular responses to different kinds of stresses, as well as in control of diverse physiological processes such as proliferation, differentiation and development of specific cell types (Kyriakis and Avruch, 2012). Remarkably, p38 regulates these processes through the coordination of essential physiological functions like cell cycle, survival and control of gene expression (Figure 4).

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In line with its role in regulation of essential biological functions, deregulation of p38 signaling pathway has been implicated in the development of many pathological conditions such as cancer, inflammation-related diseases and cardiovascular dysfunction (Cuenda and Rousseau, 2007).
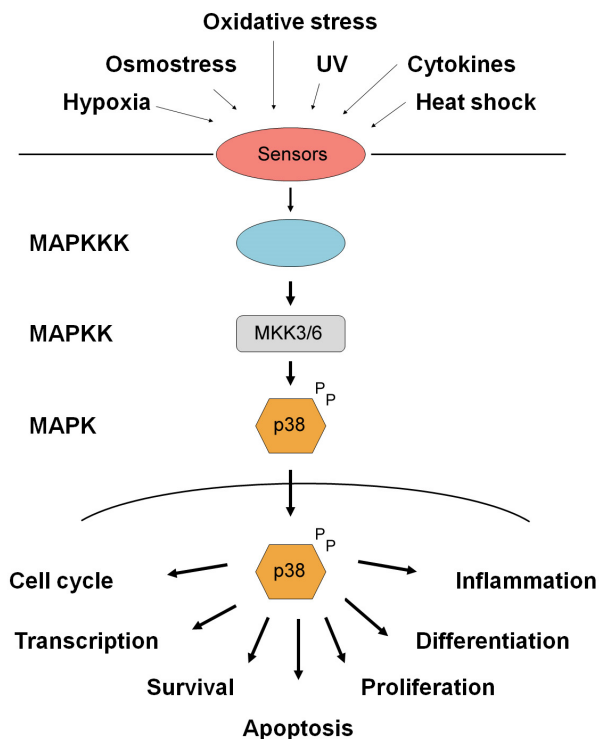
Figure 4. Adaptive responses regulated by p38
The p38 SAPK is activated in response to a wide variety of extracellular stimuli, which leads to the induction of adaptive responses including the modulation of cell cycle progression and the control of gene expression.

Cell cycle

p38 has been implicated in cell cycle delay induced by several stimuli and at different cell cycle checkpoints (Ambrosino and Nebreda, 2001; Bulavin and Fornace, 2004).

Different mechanisms have been described by which p38 can contribute at G1/S delay. One example is the direct phosphorylation of p57^{KIP2} CDK inhibitor by p38 after osmostresss (Joaquin et al., 2012a, 2012b). In addition, it has been recently reported that p38 phosphorylates retinoblastoma protein (RB) leading to an increase in its affinity towards the E2F transcription factor that results in repressed gene expression and delayed cell cycle progression (Gubern et al., 2016). p38 also phosphorylates CDC25A phosphatase upon osmostress, leading to protein degradation and therefore contributing to delaying G1/S transition (Goloudina et al., 2003). Moreover, p38 can delay G1/S transition by activation of the transcription factor p53, that results in the accumulation of the cyclin-dependent kinase inhibitor p21 (Kim et al., 2002; Kishi et al., 2001).

Interestingly, p38 has also been involved in the control of the G2/M transition. In response to DNA damage, p38 is able delay progression through G2/M by at least two different mechanisms: The activation and stabilization of p53 (Bulavin et al., 1999; Huang et al., 1999; She et al., 2001, 2000) and the inhibition of the phosphatase CDC25B (Bulavin et al., 2001). Moreover, a G2/M delay has been observed when cells are exposed to high osmolarity. Although the exact contribution of p38 in these conditions is not clear, it has been demonstrated that in the absence of p38 activity, cells significantly abrogate the G2 arrest caused by osmostress (Dmitrieva et al., 2002; Mavrogonatou and Kletsas, 2009).

Survival

Cells are continuously challenged by environmental stresses, thus they need to decide whether adapt or die depending on the type

and strength of stress. p38 plays a dual role in these decisions, as its activation is associated with survival or cell death depending on the duration of stimulus as well as on cell type.

p38 can induce apoptosis through several transcriptional and post-transcriptional mechanisms, like direct phosphorylation of proteins of the Bcl-2 family (Cai et al., 2006), down-regulation of survival pathways or up-regulation of pro-apoptotic proteins (Bulavin et al., 1999; Porras et al., 2004; Sarkar et al., 2002).

On the other hand, p38 has been shown to promote cell growth and survival through different mechanisms, including induction of antiapoptotic inflammatory signals, regulation of autophagy programs, or indirect regulation of survival genes (Thornton and Rincon, 2009).

Gene expression

Exposure to environmental stress often elicits extensive changes in gene expression, and the p38 pathway plays a key role in this process. Whole genome analysis have shown that upon different kinds of stresses, p38 mediates the expression of more than 60% of early-induced genes (Ferreiro et al., 2010b). Moreover, transcriptome analysis revealed that there is a clear enrichment in transcription factors among the p38-induced genes, suggesting that p38 coordinates a comprehensive program of gene expression for long-term adaptation to stress (Ferreiro et al., 2010b).

The induction of p38-dependent gene expression observed in response to stress is achieved by regulation of multiple steps of the mRNA biogenesis (Figure 5).
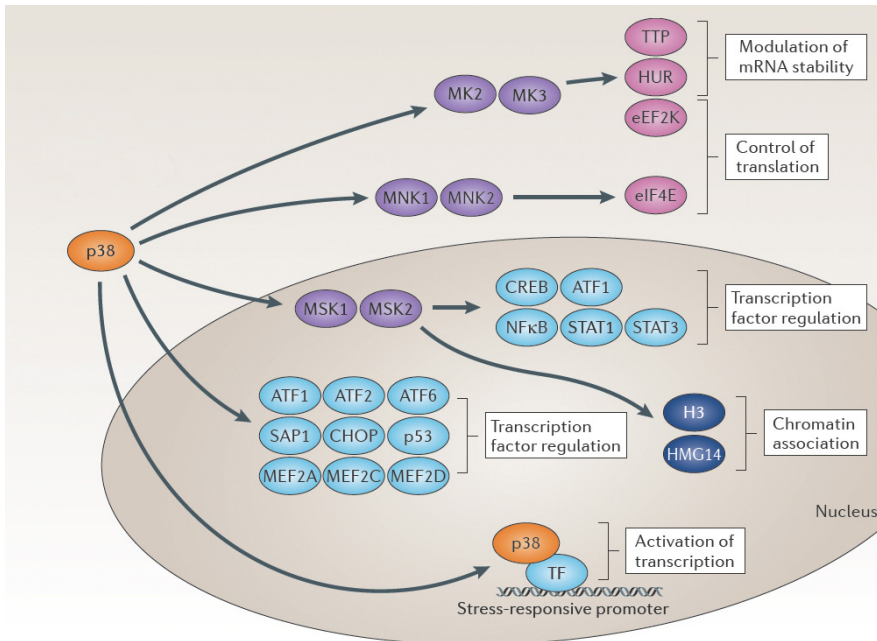
Figure 5. Control of gene expression by the p38 SAPK
Downstream targets of p38 include several kinases that are involved in the control of gene expression and nuclear proteins, such as transcription factors (TF) and regulators of chromatin remodeling (de Nadal et al., 2011).

Upon stress, p38 is recruited to chromatin via its interaction with specific transcription factors (Chow and Davis, 2006; Edmunds and Mahadevan, 2004), allowing recruitment of the RNA Pol II machinery to trigger initiation of gene expression (Ferreiro et al., 2010a). Moreover, p38 targets several transcription factors and chromatin remodeling factors, modulating its stability, localization as well as its interaction with DNA and other regulatory proteins. For instance, BAF60c is a subunit of the SWI/SNF complex that allows chromatin remodeling and transcriptional activation of muscle-specific promoters when is phosphorylated by p38 (Forcales et al., 2011). Transcription factors that have been reported to be directly phosphorylated by p38 include ATF1, ATF2,

ATF6, SAP1, CHOP, p53, MEF2C, MEF2D and MEF2A (Cuadrado and Nebreda, 2010). On the other hand, p38 activates the mitogen and stress-activated kinase 1 (MSK1) and MSK2, that in turn phosphorylates several transcription factors such as CREB, ATF1, NFκB, STAT1 and STAT3 (Wiggin et al., 2002; Zhang et al., 2001, 2004), as well as the nucleosomal proteins histone H3 and high-mobility-group 14 (HMG-14) (Arthur, 2008).

In addition to mRNA biogenesis, p38 also regulates mRNA stability and translation (Cargnello and Roux, 2011; Tiedje et al., 2014). For example, MK2 and MK3 kinases which act downstream of p38, regulate the stability of proinflammatory cytokine transcripts by phosphorylation of tristetraprolin (TTP), an AU-rich element (ARE)-binding protein that interact with AREs in cytokine mRNA 3' untranslated regions (3'UTRs) (Sandler and Stoecklin, 2008). In addition, p38 acts on some mRNAs through regulation of the RNA binding protein HuR (Farooq et al., 2009; Lafarga et al., 2009; Tiedje et al., 2012).

Furthermore, p38 activation leads to rapid adjustment of protein synthesis, targeting the MAPK signal-integrating kinase Mnk that catalyzes the phosphorylation of eukaryotic initiation factor 4E (EIF4E) (Lawson et al., 2013; Shveygert et al., 2010).

1.2.5 GADD45 in stress signaling

Multiple sensing mechanisms exist allowing a wide range of functions regulated by MAPKs. The Growth Arrest and DNA Damage-inducible 45 (GADD45) proteins are implicated as stress sensors that modulate the response of mammalian cells to genotoxic and physiological stress.

The GADD45 gene family encodes three related GADD45 proteins, GADD45α, β, and γ with similar functions and high sequence similarity (55%). However, the induction and the function of each protein member differ under diverse physiological conditions and depending on the cell types. GADD45γ is mainly detected in skeletal muscle, kidney and liver, GADD45β is principally expressed in the lungs, and GADD45α is predominantly expressed in kidney and skeletal muscle. Each GADD45-like gene is induced by a certain subset of environmental stresses. For instance, osmostress triggers GADD45β induction, while GADD45α is upregulated upon oxidative stress. Importantly, all three isoforms are highly induced by genotoxic agents (Takekawa and Saito, 1998).

The biologic effects of GADD45 are caused by its ability to facilitate protein–protein interactions as well as to directly affect protein conformation. GADD45 proteins can form protein dimers that play a key role in their functions. Crystallography and mutational analyses indicate that the central region of the protein is essential for dimerization (Schrag et al., 2008). In fact, Schrag et al. observed that most of the regions mapped in GADD45 important for the different functions are necessary for protein dimerization (Figure 6A).

GADD45 proteins have been implicated in the regulation of many cellular functions including DNA repair, cell growth arrest and apoptosis. Although GADD45 expression is usually associated with the induction of apoptosis, it also regulates pro-survival activities. Several reports have shown that modulation of these physiological processes by GADD45 can be mediated by regulation of SAPK pathways (Figure 6B).

A
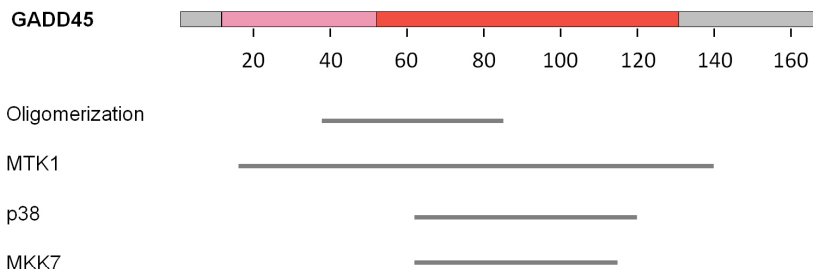
B
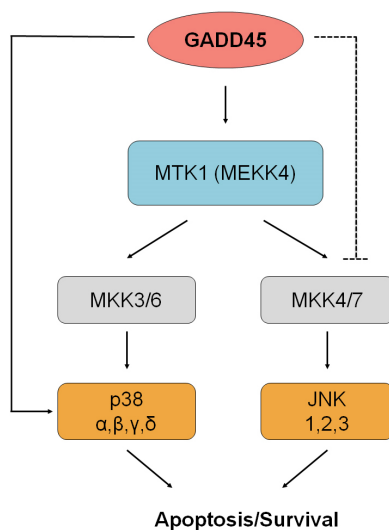
Figure 6. GADD45 protein domains
(A) GADD45 oligomerization domain was identified by nuclear magnetic resonance and crystallography. Putative interaction domains of GADD45 proteins with MTK1, p38, MKK7 were identified by mutational analysis. GADD45α protein domains encoded by constitutive exons 1 and 4 and alternative exons 2 and 3 are represented in the diagram by gray and color boxes respectively. (B) GADD45 mediates survival and apoptosis through regulation of the SAPK pathways. Arrows indicate activation, and dot lines inhibition (adapted from Tamura et al. 2012).

Particularly, all GADD45 isoforms can interact directly with MTK1 (MEKK4), resulting in apoptosis induction through activation of the JNK/p38 pathways. GADD45β interaction with MTK1 disrupts the MTK1 auto-inhibitory domain, allowing its dimerization and activation by autophosphorylation (Mita et al., 2002; Miyake et al., 2007; Takekawa and Saito, 1998). Moreover, GADD45α can activate p38 by direct interaction (Bulavin et al., 2003). On the other hand, GADD45β can play an anti-apoptotic function by suppression of JNK signaling through association and inhibition of the upstream kinase MKK7 (Papa et al., 2007) (Figure 6B). All these studies were performed using various extracellular stimuli in different cell types, suggesting that SAPK mediated modulation of stress by GADD45 is both cell type and stimulus specific.

1.3 Alternative splicing

Pre-mRNA splicing is the process by which intronic sequences are removed from primary transcripts and exonic sequences are spliced together to produce functional mRNAs. Alternative selection of exons (alternative splicing, AS) can generate multiple mRNAs and proteins from a single primary transcript, playing an important role in expanding genome complexity as well as in the generation of proteomic variety (Nilsen and Graveley, 2010). More than 95% of human genes undergo AS (Nilsen and Graveley, 2010), increasing the opportunities for gene regulation during diverse biological processes such as cell growth, cell death, and responses to environmental challenges (Braunschweig et al., 2013).

1.3.1 The catalytic steps of splicing

Correct processing of pre-mRNA molecules requires precise definition of exon-intron boundaries. The sequence elements required for exon-intron definition of the majority of mammalian introns (U2 type) include the 5' splice site (5'ss) at the intronic 5' boundary, the branch point (BP), the 3' splice site (3'ss) at the end of each intron and a polypyrimidine tract, a pyrimidine-rich stretch located between the BPS and the 3'SS (Burge et al. 1999) (Figure 7A).

Figure 7. Conserved sequence elements of mammalian U2 introns
The scheme includes the 5' and the 3' splice site, the polypyrimidine tract (Yn) and the branch point. The branch point adenosine is shown in bold. N represents any nucleotide, R a purine and Y a pyrimidine (adapted from Will & Lührmann 2011).

The process of intron removal from pre-mRNA consists of two transesterification reactions that occur in two sequential steps. In the first step of pre-mRNA splicing, the 2'-OH group of the branch point adenine nucleotide carries out a nucleophilic attack of the 3'-5' phosphodiester bond between the guanine nucleotide at the 5'-end of the intron and the 3'-end nucleotide of the 5'-exon, resulting in the release of the 5'-exon and formation of an intron lariat-3'-exon. In the second step, the 3′-OH group of the RNA nucleotide at the 3′-end of the 5′-exon attacks the phosphodiester bond at the 3' splice site of the lariat intermediate leading to joining of two exons and release of the intron lariat (Figure 8).
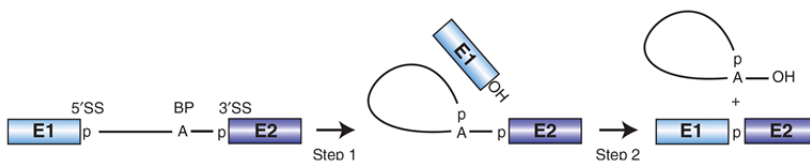
Figure 8. The two transesterification reactions of pre-mRNA splicing
The 5' splice site (5'ss), the branch boint (BP) and the 3' splice site (3'ss) are represented in the diagram (adapted from Will and Lührmann 2011).

1.3.2 The spliceosome

The splicing process is carried out by the spliceosome, a macromolecular machinery composed by five small nuclear ribonucleoprotein particles (snRNPs) and more than 200 auxiliary proteins. Two types of spliceosomes exist in mammalian cells. The major spliceosome is composed by U1, U2, U4, U5 and U6 snRNPs, and catalyzes the removal of the U2-type introns. In contrast, the minor spliceosome is composed by U11, U12, U4atac, U5 and U6atac snRNPs and catalyzes U12-type class of introns, that represent less than 5% of the total introns (Patel and Steitz, 2003).

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The spliceosome assembly cycle

The splicing machinery assembles on the RNA substrate in a stepwise manner and, progression through the splicing reaction is accompanied by extensive remodeling of several protein-protein and protein-RNA interactions.

Major spliceosome assembly begins with U1 snRNP recognition of the 5′ss via base pairing of U1 snRNA to the mRNA. In addition, the earliest assembly phase of the spliceosome also involves recognition of the BPS and 3'SS. The branch point binding protein (BBP)/SF1 bind to the BPS, while the 65 KDa and the 35 KDa subunits of the U2 snRNP auxiliary factor (U2AF) bind the pY tract and the AG acceptor site respectively. Moreover, SF1/BBP interacts with U2AF65, and the cooperative binding of these proteins leads to the formation of the early spliceosomal complex E. This complex facilitates assembly of U2 snRNP on the branch point region, a step that involves base-pairing interactions between a region of U2 snRNA known as the branch point recognition sequence (BPRS) and sequences in the precursor pre-mRNA flanking the branch point adenosine.

After the formation of the spliceosomal E complex, the U2 snRNP stably associates with the BP, leading to the formation of the A complex. Once the borders of the intron are defined, the precatalytic B complex is formed by recruitment of the pre-assembled U4/U6.U5 tri-snRNP. Subsequently, the U1 and U4 snRNPs are displaced with the help of the helicase Brr2, leading to the formation of the activated Bact complex. Catalytic activation by the DEAH-box RNA helicase Prp2, generates the B* complex, which catalyzes the first step of splicing. Conformational rearrangements lead to the formation of C complex, which

catalyzes the second step of splicing, the intron is excised and the exons are ligated together to form mRNA (Figure 9).
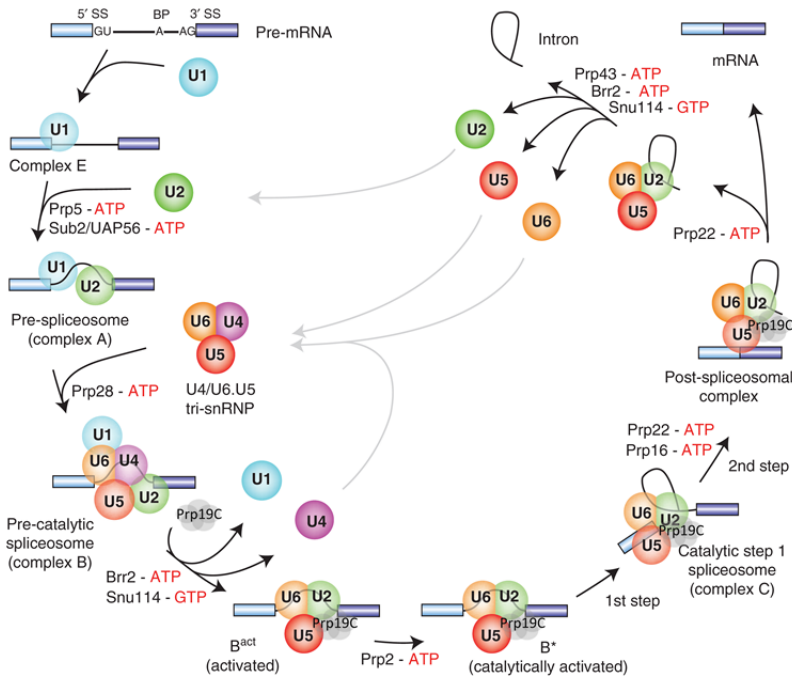
Figure 9. Stepwise assembly of the major spliceosome
The ordered interactions of the snRNPs (represented by circles) are shown. The stages at which DExH/D-box RNA ATPases/helicases Prp5, Sub2/UAP56, Prp28, Brr2, Prp2, Prp16, Prp22 and Prp43, or the GTPase Snu114, act to facilitate conformational changes are indicated. The Prp19C is recruited to the spliceosome before activation of B complex (adapted from Will and Lührmann 2011).

Spliceosome components

The core spliceosome is highly conserved between yeast and humans. In fact, the yeast spliceosome has been extensively studied by genetic approaches that allow the rapid identification of spliceosomal components and their interacting partners. Moreover, the structure of the yeast catalytically activated spliceosome has

recently been solved, elucidating the details of these interactions at the core of catalysis (Yan et al., 2016).

As mentioned above, the major spliceosome is composed of the conserved U1, U2, U4, U5 and U6 snRNPs. Each U snRNP contains the corresponding snRNA and a variable number of complex-specific proteins. Moreover, the U1, U2, U4, and U5 snRNPs all contain seven Sm proteins (B/B′, D3, D2, D1, E, F, and G), while U6 snRNP associates to SmL proteins (Will and Lührmann, 2011) (Figure 10).
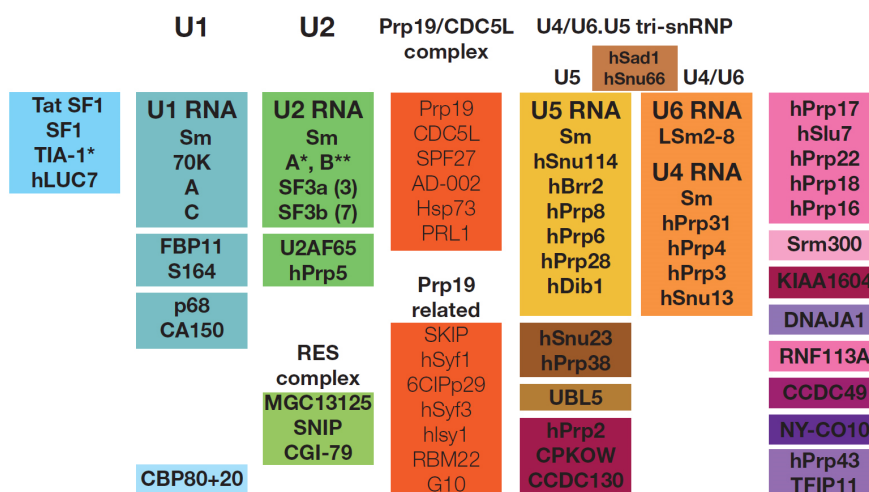
Figure 10 Evolutionary conserved human spliceosomal proteins
Proteins are grouped according to its association with different spliceosomal complexes (adapted from Stam et al. 2012).

In addition to the snRNPs, numerous non-snRNP proteins associate permanently or transiently with the spliceosome and play critical roles in the splicing process.

One of the most extensively studied non-snRNP group is the DEXD/H-box helicases family. DExH/D-box RNA ATPases/helicases facilitate the dynamic rearrangements during

the splicing cycle by the unwinding or annealing of RNA-duplexes and the weakening of RNA-protein interactions (Jankowsky et al., 2001). One example is the helicase Prp2, which dissociates the SF3a/b complex from U2, allowing BP to participate in the step I of reaction in the B* complex (Kim and Lin, 1996).

Another example of non-snRNP protein complex with an important function during splicing reaction is the conserved Prp19 complex (Prp19C), also known as NineTeen Complex (NTC). Rearrangements within the spliceosome facilitated by Prp19C has been demonstrated to be essential for its catalytic activation (Chanarat and Sträßer, 2013). The NTC/Prp19C and Prp19-associated proteins join the spliceosome together with the U4/U6.U5 tri-snRNP before unwinding of U4 from U6, and remain associated with the spliceosome during both steps of the splicing reaction (Fabrizio et al., 2009) (Figure 9). Additionally, the complex can be recruited through interaction with U2AF65. Interestingly, this interaction has been shown to occur co-transcriptionally and be stimulated by the phosphorylated carboxy terminal domain (CTD) of the RNA polymerase II (David et al., 2011).

There are also a large number of proteins associated with the human spliceosome that do not have an obvious counterpart in yeast and are not considered core spliceosomal components. These include, among others, serine–arginine repeat proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs), which have important functions in the regulation of AS (Wahl et al., 2009).

1.3.3 The U2snRNP associated SF3B1 protein

The U2 snRNP plays a key role in pre-mRNA splicing catalysis. After formation of E complex, U2 snRNP is recruited to the branch region through the interactions with U2AF. U2 snRNA base pairs with nucleotides flanking the branch point, positioning the BP adenosine within the catalytic core of the spliceosome (Wahl et al., 2009)(Figure 11).
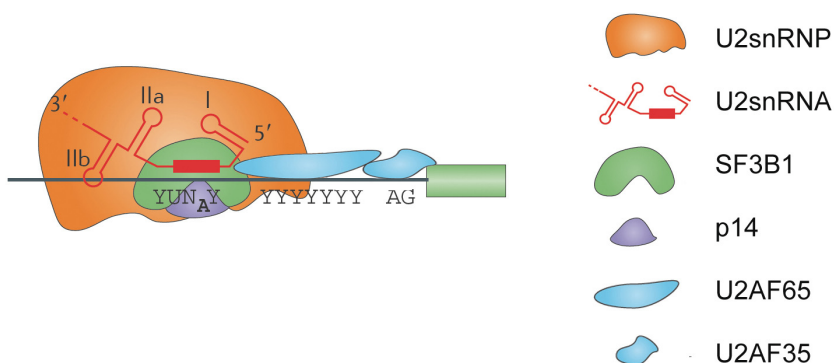
Figure 11. U2snRNP association to the branch point
U2 snRNP binding involves base-pairing interactions between U2 snRNA and nucleotides flanking the branch point. This base-pairing interaction is stabilized by the U2snRNP protein SF3B1 and the U2AF complex. U2AF65 subunit binds the polypyrimidine tract and associates with SF3B1, whereas U2AF35 subunit contacts the 3′ss. p14 subunit of SF3B complex interacts with the branch adenosine and with the SF3B1 (adapted from Bonnal, Vigevani, and Valcárcel 2012).

U2 snRNP consists of the U2 snRNA and several proteins, including Sm proteins, the stably associated U2-A′ and U2-B″ polypeptides, and the heteromeric protein complexes SF3A and SF3B. SF3A contains SF3a60, SF3a66 and SF3a120 subunits (Kramer, 1996) while SF3B is composed of at least eight subunits named SF3b155, SF3b130, SF3b145, SF3b49, SF3b10, SF3b14a

(or p14) SF3b14b and SF3b125, based on their molecular weight (Das et al., 2000; Krämer et al., 1999) .

SF3B1 (or SF3b115) is a central component of the U2 snRNP complex that plays a key role in stabilizing U2 snRNP recruitment (Corrionero et al., 2011; Gozani et al., 1998) (Figure 11). After formation of the A complex, SF3B1 remains associated with the spliceosome through activation and catalysis (Agafonov et al., 2011; Ilagan et al., 2013).

SF3B1 protein has an N-terminal domain involved in protein-RNA and protein-protein interactions, including p14 and U2AF65, and a C-terminal region consisting of 22 so-called HEAT domains (Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast PI3-kinase TOR1). Importantly, SF3B1 is phosphorylated in the N-terminal domain during spliceosome activation, and this phosphorylation is required for the transition to active splicing (Agafonov et al., 2011; Bessonov et al., 2010; Shi et al., 2006; Wang et al., 1998).

1.3.4 The Prp19 related protein SKIIP

SKIIP (SNW1 or SKIP) is a highly conserved Prp19 related protein, which is recruited to the U5 snRNP when the U1 and U4 snRNPs dissociate from the spliceosome (Makarov, 2002). Brr2 and Snu114 U5snRNP associated proteins directly interact with SKIIP, and it has been recently proposed that SKIIP association with Brr2 may be essential for its helicase activity and therefore for the unwinding of the U4/U6 snRNA duplex, a critical step in splicing catalysis.

On the other hand, SKIIP is required for loading of U2AF65 at particular genes, suggesting an additional function at earlier steps (Chen et al., 2011).

Beyond its role in splicing, SKIIP plays a critical function in transcription regulation. SKIIP has been reported as a coactivator of Notch and nuclear receptors, such as the vitamin D, retinoic acid, and androgen receptors (Folk et al., 2004). In addition, SKIIP associates with P-TEFb, c-myc, and Menin to activate the HIV-1 promoter (Brès et al., 2005, 2009).

In summary, these studies demonstrate that SKIIP plays a dual role in splicing and transcription regulation.

1.3.5 Regulation of alternative splicing

Splice site recognition is a flexible process that allows generation of different classes of AS patterns. The main types of AS found in eukaryotes are cassette exon, alternative selection of 5′ or 3′ splice sites, and mutually exclusive exons (Black, 2003; Kim et al., 2008) (Figure 12).

Alternative splice site selection can be regulated through many different mechanisms. Initial studies on AS regulation focused on early stages of spliceosome assembly. However, regulatory mechanisms exist allowing regulation of splice site choice at later stages of spliceosome assembly or even during splicing catalysis.
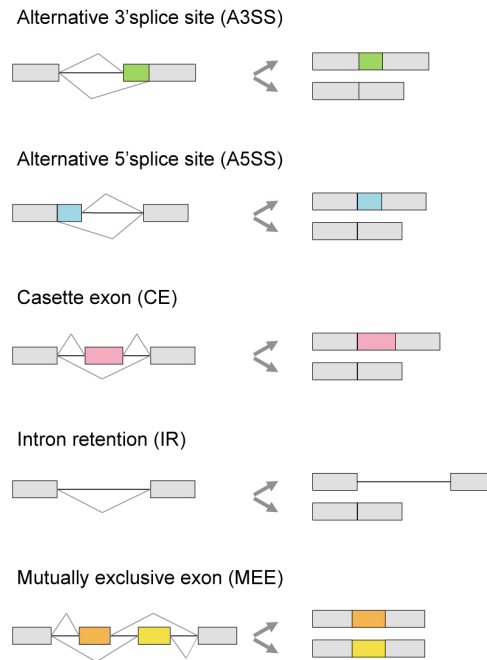
Figure 12. Types of alternative splicing
Major types of AS events are illustrated. Gray boxes indicate constitutive exons, while colored boxes indicate alternative spliced exons.

Due to the degenerate nature of the sequence elements recognized by the spliceosome, its recruitment to the pre-mRNA requires the action of *cis*-acting RNA sequence elements and *trans*-acting RNA binding proteins. *Cis*-acting elements include exonic splicing enhancers and silencers (ESEs and ESS) and intronic splicing enhancers and silencers (ISE and ISS) that are bound by *trans*-acting factors that can promote or repress the selection of the neighbouring splice sites. The best studied *trans*-acting factors are SR and hnRNP proteins.

SR proteins generally act as splicing enhancers when bound to exons but they can repress splicing when bound to introns. SR proteins contain one or two RNA recognition motifs (RRMs)

followed by a serine/arginine-rich sequence known as the RS domain, which is typically regulated by phosphorylation. In contrast, hnRNP proteins usually function as splicing silencers. However hnRNP proteins can activate splicing in some cases (Änkö, 2014; Busch and Hertel, 2013). Besides an RRM, most hnRNPs contain an RRM, a box of Arg-Gly-Gly repeats and additional glycine-rich domain. Post transcriptional modifications of SR and hnRNP proteins lead to changes in their activity and availability, and it can therefore influence the selection of particular splice sites.
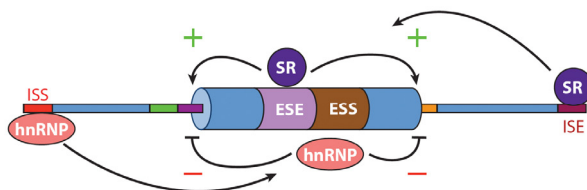
Figure 13. Positive and negative control of precursor messenger RNA (pre-mRNA) splicing by *cis*-acting intronic and exonic silencers and enhancers
Intronic and exonic splicing enhancers (ISE, ESE) and intronic and exonic splicing silencers (ISS, ESS*x*) are indicated (adapted from Lee & Rio 2015).

On the other hand a large set of alternative exons have been reported to be influenced by levels of the core spliceosomal components, adding an extra layer of complexity to regulation of AS (Papasaikas et al., 2015; Saltzman et al., 2011).

Although investigations on AS regulation have been mostly focused on understanding the influence of different splicing factors in the selection of specific splice sites, there is some growing evidence indicating that transcription and splicing are physically and functionally coupled and can influence each other. For instance,

variations in RNA Pol II elongation rates have been associated with changes in AS (Kornblihtt et al., 2013). Additionally, features in chromatin structure, including nucleosome density and epigenetic modifications can also influence AS decisions (Iannone and Valcárcel, 2013).

1.3.6 Identification of splicing regulators: The functional splicing network

Alternative splicing is a fundamental mechanism regulated in a large number of biological processes and human pathologies. Identification of *trans*-acting factors involved in this reaction in different situations represents a major challenge for understanding the molecular basis of AS regulation. Recently, functional splicing networks have been developed providing novel connections between different physiological responses and the spliceosome machinery.

In this thesis we have used a functional network reported by Papasaikas et al., 2015. For the generation of this network, >200 genes encoding core spliceosomal components, auxiliary splicing factors and other factors involved in RNA-processing were depleted from HeLa cells using siRNA libraries. Changes in 36 alternative splicing events (ASEs) relevant for cell proliferation and apoptosis were analyzed by RT-PCR and high-throughput capillary electrophoresis. The percentage of exon inclusion was calculated for each event, and a perturbation profile for every protein knockdown was generated by representing its impact across the 36 network ASEs. Functional interactions were determined based on quantitatively comparison of the similarities between the effects of knockdown for every pair of spliceosomal components (Figure 14A). Remarkably, the network captured a substantial number of

known physical and functional connections and was able to identify new ones (Figure 14B).

A
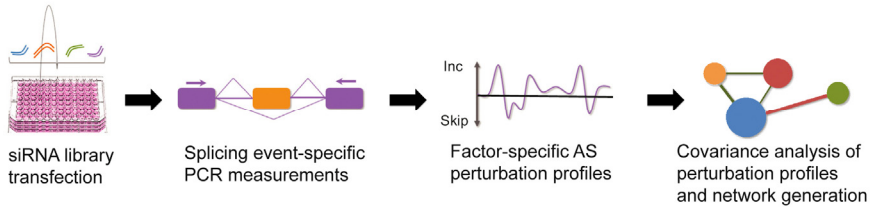
B
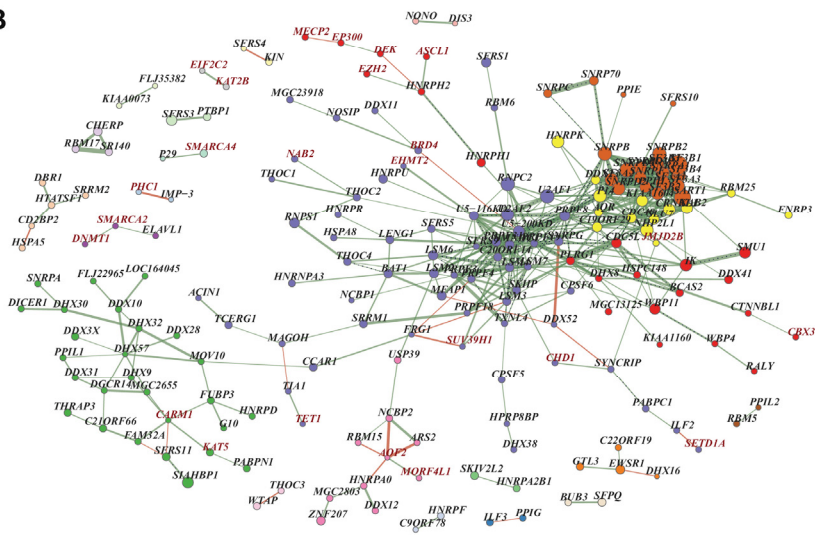
Figure 14. Functional Splicing Regulatory Network
(A) Flowchart of the pipeline for network generation. (B) Graphical representation of the reconstructed splicing network. Nodes represent splicing factors and lines between nodes represent either positive (green) or negative (red) functional connections. Node size is proportional to the overall impact of a given knockdown in the regulation of AS while line thickness indicates the strength of the functional interaction. Black dotted lines correspond to known physical interactions (Papasaikas et al., 2015).

Moreover, the effect of different pharmacological components was analyzed using the same experimental setting. Notably, the network was also able to link drug treatments that affect AS decisions to their known splicing factor targets. Thus, the use of

this methodology serves as a powerful experimental tool to identify possible targets within the splicing machinery of physiological perturbations that produce changes in AS.

1.4 Regulation of alternative splicing in response to stress

Modulation of AS has been reported to occur in response to many kind of different extracellular stimuli, including oxidative stress, heat shock, osmostress and ultraviolet radiation (Akaike et al., 2014; Biamonti and Caceres, 2009; van der Houven van Oordt et al., 2000; Shkreta and Chabot, 2015).

The regulatory processes controlling splicing modulation in response to heat shock and genotoxic stress have been extensively studied, while little is known about AS regulation in response to oxidative stress and osmostress. Heat shock induces a global inhibition of splicing, often mediated by the SR protein SRp38 (Biamonti and Caceres, 2009; Shalgi et al., 2014). Genotoxic stress leads to the regulation of the expression of different splicing factors. In addition, multiple spliceosomal components are regulated by different post-translational modifications that affect their activity and localization. Moreover, it has been reported that DNA damage disrupts the coupling of splicing with the transcription of genes involved in DNA repair, cell-cycle control and apoptosis (Shkreta and Chabot, 2015).

Remarkably, activation of p38 has been shown to modulate the activity of proteins involved in regulation of AS in different stress situations.

INTRODUCTION

For example, in response to osmostress and UV irradiation, p38 phoshorylates the heterogeneous nuclear ribonucleoprotein hnRNPA1, leading to its relocalization into the cytoplasm where it concentrates into stress granules (Guil et al., 2006) (Figure 15). Although the general effects on AS profiles of this translocation event are not described, it has been demonstrated that reduced nuclear hnRNP A1 levels can alter the AS pattern of a reporter transcript (van der Houven van Oordt et al., 2000).
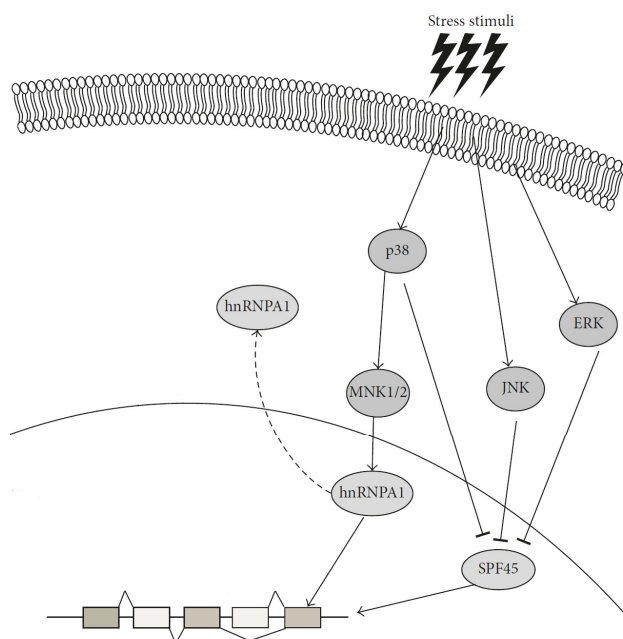
Figure 15. Regulation of the activity of splicing factors by MAPKs in response to cellular stress
p38, JNK and ERK inhibit SPF45 activity. p38 dependent phosphorylation of hnRNPA1 by Mnk1/2 leads to its cytoplasmic translocation (adapted form Naro and Sette 2013).

Interestingly, cell exposure to UV light leads to SPF45 phosphorylation by ERK, NK and p38 (Figure 15). The phosphorylation of SPF45 prevents exon 6 inclusion in the FAS

56

minigene, leading to the production of a dominant negative isoform of this death receptor. Moreover, SPF45 expression enhances the inclusion of the extra domain A (EDA) into fibronectin 1 (FN1) transcripts in a phosphorylation dependent manner (Al-Ayoubi et al., 2012).

Another example of RNA binding protein targeted by p38 is the Hu antigen R (HuR), which induces changes in Tra2β AS profiles in response to oxidative stress (Akaike et al., 2014).

In summary, there are evidences indicating that adaptive responses to changes in the environment lead to regulation of splicing profiles. In addition, several reports indicate that p38 is also involved in AS control. However, a complete view of the effects of p38 activation in modulation of AS is yet to emerge.

2. OBJECTIVES

2. OBJECTIVES

Our group is interested in understanding the mechanisms by which the p38 SAPK mediates stress adaptive responses. Because alternative splicing is strongly modulated in response to a wide range of extracellular stimuli, the aim of this project was to give insights into the role p38 in this process.

Specifically, the main objectives of this thesis were:

1. To determine the contribution of the p38 activation in regulation of alternative splicing upon stress.

2. To identify components in the spliceosome targeted by p38.

3. To study the physiological relevance of p38-mediated alternative splicing in stress adaptation.

3. EXPERIMENTAL PROCEDURES

3. EXPERIMENTAL PROCEDURES

Cell lines and transfection reagents

HeLa CCL-2 cells were purchased from the American Type Culture Collection (ATCC). 3T3 cell lines stably expressing JNK and p38 KTRs were provided by Dr. Covert. Cells were cultivated in Glutamax Dulbecco's modified Eagle's medium (Life technologies) supplemented with 10% Fetal bovine serum (Sigma) and antibiotics (penicillin 500 u/ml; streptomycin 0.5 mg/ml, Life technologies). Cell culture was carried out in cell culture dishes in a humidified incubator at 37°C under 5% CO_2.

Cells were treated with osmostress at the indicated NaCl concentrations for the appropriate times. When indicated, cells were pre-incubated with 10 µM SB203580 (Calbiochem) for 30 min.

Cells were transiently transfected with the indicated plasmids or small interference RNAs (siRNAs) using the FuGENE 6 transfection reagent (Promega) or Lipofectamine RNAiMAX (Invitrogen) respectively according to the manufacturer's directions for at least 48 h before treatments and harvesting.

RNA isolation and cDNA synthesis

Total RNA was isolated using the Qiagen RNeasy mini kit (Cat. No. 74104), with DNase digestion on column before elution using the RNase-free DNase Set (Cat.#79254).

First strand cDNA synthesis was set up with 500 ng of RNA, 50 pmol of oligo-dT (Sigma-Aldrich), 75 ng of random primers (Life Technologies), and superscript III reverse transcriptase (Life

Technologies) in 20 µl final volume, following manufacturer's instructions.

Splicing-sensitive microarray

HeLa cells were non-treated (c1) treated for 2h with 100 mM NaCl (c2) or pre-treated 30' with SB before 2h NaCl 100 mM treatment (c3). RNA from each experimental condition was isolated, reverse transcribed and labeled with Cy5 or Cy3 fluorochromes. For each assay a Cy5-labeled sample was combined with an equal amount of a Cy3-labeled sample and hybridized to custom a splicing sensitive microarray platform (Muñoz et al., 2009). A reciprocal experiment using switching fluorochromes was also carried out. Two independent microarray assays (µa) were performed: OSM µa (combining c1 with c2) and SB µa (combining c2 with c3). Exon-junction probes with a log2 (ratio) changes higher than 0.4 and changes in gene expression higher than 1.3, p-value < 0.01, z-score > 3 were considered.

Quantitative PCR (qPCR)

Primers for qPCR analysis were designed such that their annealing temperature was 60ºC, generating single amplification products in the range of 60-200 base pairs long. For analysis of alternatively spliced isoforms, one of the primers was chosen as overlapping an exon junction such that only upon hybridization to the correctly spliced mRNA the primer was able to produce an amplification product. Primer pairs used for qPCR measurements are listed in Table 1. qPCR amplification was carried out using 1 µl of 1:5 to 1:20 diluted cDNA with 5 µl of 2X SYBR Green Master Mix (Roche) and 4 pmol of specific primer pairs in a final volume of 10 µl in 384 well-white microtiter plate (Roche). qPCR mixes were analyzed in triplicates in a Light Cycler 480 system (Roche).

Semi-quantitative PCR

Primers for the analysis of AS were placed on constitutive exons flanking alternative regions and are listed in Table 2. GoTaq DNA Polymerase (Promega) was used for PCR amplification according to manufacturer's instructions. After 30 cycles of 15'' at 95°C, 30'' at 57°C and 1' at 72°C the products were analyzed on 2% agarose gels.

Plasmids

pCDNA3 (Invitrogen), pEFmlink-MKK6^{DD} (with the two phosphorylation sites Ser 207 and Thr 211 in the activation loop of MKK6 changed to Glu) has been described (Alonso et al., 2000), pCDNA3-3HA-p38α was provided by Dr. Engelberg (The Hebrew University of Israel, Jerusalem), T7-tag expression vectors and pJ609-T7-SF3B1 were provided by Dr. Valcárcel (Centre for Genomic Regulation, Barcelona), pON3-SF3B1 was provided by Dr. Vilardell (Molecular Biology Institute of Barcelona).

The GST-SF3B1 Nter and Cter were obtained using the full-length pON3-T7-SF3B1 as a template by PCR and subcloned into the *BamHI/XhoI* sites of the pGEX-6P-1. GST-SF3B1 Nter 5A was generated by sequential site-directed mutagenesis of the GST-SF3B1 Nter WT vector. In this mutant construct, T142, T207, T224, Thr303, T328 were mutated to A. GST-SKIIP WT was obtained by cloning SKIIP in the *BamHI/XhoI* sites of the pGEX-6P-1 vector in-frame of the GST N-terminal tag. GST-SKIIP 3A was generated by sequential site-directed mutagenesis of GST-SKIIP WT. In this mutant construct, T180, S224 and S232 were mutated to A. T7-SKIIP WT and T7-SKIIP 3A were generated by PCR amplification using SKIIP WT and SKIIP 3A as a template. The obtained PCR product was cloned into the *XbaI/BamHI* restriction sites of the

pcGT7 expression vector. HA-SKIIP was generated by subcloning SKIIP from GST-SKIIP WT in the *BamHI/XhoI* sites of the pCDNA3-3HA pcDNA vector. GADD45α[B] and GADD45α[S] constructs were generated by PCR amplification using cDNA from HeLa cells subjected to stress as a template. GADD45α[B] and GADD45α[S] were purified from an agarose gel and cloned in the *EcoRV/XhoI* sites of the pcDNA3 vector. Primer pairs used for PCR amplification are listed in Table 3.

Minigene constructs

DYRK1A and MIB1 minigenes were obtained by overlap PCR and were cloned into the *EcoRI/XhoI* restriction sites of pcDNA3. A GADD45α minigene was constructed by PCR amplification of the indicated region between exon 1 and exon 4 and inserted into the pcDNA3 *EcoRV/XhoI* restriction sites. Primer pairs used for PCR amplification are listed in Table 3.

Immunoprecipitation assays and western blot

After the indicated treatments, cells were washed with ice-cold PBS and scraped into 500 µl of IP/lysis buffer (10 mM Tris HCL pH 7.5, 1% NP40, 2 mM EDTA, 50 mM NaF, 50 mM b-glycerophosphate, 1 mM Sodium Vanadate, supplemented with the protease inhibitors 1 mM PMSF, 1 mM Benzamidine, 200 µg/ml Leupeptine and 200 µg/ml Pepstatine). The lysates were cleared by microcentrifugation. For immunoprecipitation assays, 10% of the total lysate was retained as input. The remainders were subjected to immunoprecipitation with either 50 µL T7-Tag Antibody Agarose (Millipore) or 50 µl sepharose-protein A beads (GE Healthcare, 50% slurry equilibrated in IP buffer) coupled to specific antibodies by mixing overnight at 4°C. Immune complexes were collected by brief centrifugation and washed rapidly five times with IP buffer.

Immunoprecipitates, input samples or total lysates were subjected to PAGE–SDS and western blotting.

Antibodies

The antibodies used are as follows: mouse monoclonal anti-a-Tubulin (Sigma, S9026), mouse monoclonal anti-GAPDH (Santa Cruz, sc-32233), rabbit polyclonal anti SF3B1 (Abcam ab39578), rabbit polyclonal anti-SKIIP (Santa Cruz sc-30139), rabbit polyclonal anti-SYNCRIP (Cell Signaling, #8588), mouse monoclonal anti-hnRNPA1, mouse monoclonal anti-Flag (Sigma, S2220), rabbit polyclonal anti-p38α SAPK (Santa Cruz, sc-535), rabbit polyclonal anti-T7 epitope tag antibody, rabbit polyclonal anti-Brr2 (Bethyl Laboratories, A303-454A), rabbit polyclonal anti-Snu114 (Gentex, GTX115222). Rabbit polyclonal anti-GADD45α (Santa Cruz, sc-792). Mouse monoclonal anti-HA was house made from the 12CA5 and 9E10 hybridomas, respectively. Rabbit-polyclonal anti-SPF45 was provided by Dr. Valcárcel (Centre for Genomic Regulation, Barcelona).

siRNAs

The siRNA used are as follows: ON-TARGETplus siRNA Human HNRPA1 ORF (Dharmacon, J-008221-11), SMARTpool: ON-TARGETplus SNW1 siRNA (Dharmacon, L-012446-00-0005), ON-TARGETplus Non-targeting siRNA #2 (Dharmacon, D-001810-02-05), SF3B1 Stealth siRNA (Invitrogen, 1299001 HSS146415), Stealth RNAi siRNA Negative Control (Invitrogen). Custom siRNAs designed against the caggtctctccaaagtgaatgaact sequence of the SKIIP 3'UTR region (stealth SKIIP 774) and its scrambled control (stealth scrambled 774) were ordered from Invitrogen.

EXPERIMENTAL PROCEDURES

Network analysis

After the indicated treatments, RNA from HeLa cells was extracted and reverse transcribed to cDNA as described above. PCR reactions for every splicing event analyzed were carried out using primer pairs listed in Table 4. HTCE measurements for the different splicing isoforms were performed in 96-well format in a Labchip GX Caliper workstation (Caliper, Perkin Elmer) using a HT DNA High Sensitivity LabChip chip (Perkin Elmer). Data values were obtained using the Labchip GX software analysis tool (version 3.0). Data analysis was performed as described in Papasaikas et al. 2015.

Expression and purification of recombinant proteins

Escherichia coli bacteria were at 37°C until they reached an OD600 of 0.5 absorbance units. At this point, GST-tagged proteins were induced for 4h by adding 1 mM IPTG and switching the culture temperature to 25°C. After induction, cells were collected by centrifugation and resuspended in 1/50 volume of STET 1X buffer (100 mM NaCl, 10mM Tris–HCl pH 8.0, 10mM EDTA pH8.0, 5% Triton X-100 supplemented with 2 mM DTT and the 1 mM PMSF, 1 mM Benzamidine, 200 mg/ml Leupeptine and 200 mg/ml Pepstatine). Cells were lysed by ice-cold brief sonication and cleared by high speed centrifugation. GST-fused proteins were pulled down from supernatants with 300 µl of gluthatione-sepharose beads (GE Healthcare, 50% slurry in equilibrated with STET) by mixing 45 min at 4°C. The gluthatione-sepharose beads were collected by brief centrifugation and washed four times in STET buffer and two times in 50 mM Tris-HCl pH 8.0 buffer supplemented with 2 mM DTT. The GST-fused proteins were then eluted in 200 µl of 50 mM Tris-HCl pH 8.0 buffer supplemented with

2 mM DTT and 10 mM reduced gluthatione (Sigma) by mixing for 45 min at 4ºC and stored at 80°C.

Saccharomyces cerevisiae yeast transformed with the LEU2 selectable pON3-SF3B1 vector were grown to mid-log phase in 50 ml of SD Leu- and collected by brief centrifugation at 4ºC. Protein was extracted in buffer A (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 15 mm EDTA, 15 mm EGTA, 2 mm DTT, 0.1% Triton X-100, 1 mm PMSF, 1 mm benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin). SF3B1 was pulled down from supernatants with 50 µl sepharose-protein A beads (GE Healthcare, 50% slurry equilibrated in IP buffer) coupled to SF3B1 antibody. Beads were washed once with lysis buffer A, 3 times with buffer A + 0.5 M NaCl and two times with 50 mM Tris pH8 + 2 mM DTT.

In vitro p38 kinase assay

GST-p38α was activated *in vitro* in a small volume (15 µl/assay) by mixing with GST-MKK6[DD] in 1x kinase assay buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl2, 2 mM DTT) in the presence of 100 µM cold ATP in the presence or the absence of 10 µM SB203580 (Calbiochem) for 20 min at 30°C. In all, 15 µl of the activated GST-p38α was used to phosphorylate *in vitro* either eluted GST-fused proteins or immunoprecipitates from mammalian expressed proteins. The reactions were carried in 1x kinase assay buffer in the presence of 1 µCi/assay of radiolabelled 32P-γ-ATP (3000 Ci/mmol from Perkin-Elmer) in a final volume of 40 ml/assay for 20 min at 30°C. Reactions were stopped by adding SB 5x (250mM Tris-HCl pH 6.8, 0.5M DTT, 10% SDS, 20% glycerol, 0.5% Bromophenol Blue) and boiling at 100°C for 5 min. Phosphorylated proteins were subjected to PAGE-SDS and Coomassie blue

stained or transfer blotted onto a PVDF membrane and exposed to BIOMAX XAR films (KODAK) or phosphorimager.

Mass spectrometry analysis

Sample preparation. For identification of SF3B1 phosphorylation sites, GST-SF3B1 Nter was phosphorylated in vitro and subjected to SDS-PAGE. Subsequently, the phosphorylated protein band were excised from Coomassie stained gels. Gel bands were destained with 40% ACN/100 mM ABC, reduced with dithiothreitol (2 M, 30min, 56ºC), alkylated in the dark with iodoacetamide (10 mM, 30 min, 25ºC), dehydrate with ACN and digested with 0.7 µg of trypsin (Promega, cat # V5113) overnight at 37ºC. After digestion, peptide were extracted and cleaned up on a homemade Empore C18 column (3M, St. Paul, MN, USA).

For identification of SKIIP associated proteins, T7-SKIIP pull down assays were performed in biological triplicates as described above. The beads with the immuno-precipitated proteins were washed with ammonium bicarbonate (3x, 200 mM NH4HCO3) and re-suspended in 60 µl of 6M Urea + 200 mM NH4HCO3. Then the sample was reduced by adding 10 µl of 10 mM DTT + 200 mM NH4HCO3 (1h, 37 ºC) and alkylated by adding 10 µl of 20 mM IAA + 200 mM NH4HCO3 (30 min, RT). Samples were diluted prior trypsinization (o/n, 37 ºC) and the digestion reaction was stopped with formic acid (5% final concentration). Tryptic peptides were desalted with C18 columns and re-suspended in 10 µL of H2O + 0.1% formic acid.

LC-MSMS sample acquisition. Samples were analyzed using a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, USA) coupled to a nano-LC (Proxeon,

Odense, Denmark) equipped with a reversed-phase chromatography 2-cm C18 pre-column (Acclaim PepMap-100, Thermo; 100 µm i.d., 5 µm), and a 25-cm C18 analytical column (Nikkyo Technos, 75 µm i.d., 3 µm). Chromatographic gradients started at 3% buffer B with a flow rate of 300 nL/min and gradually increased to 7% buffer B in 1 min and to 35% buffer B in 60 min. After each analysis, the column was washed for 10 min with 90% buffer B (Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile). The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.5 kV and source temperature at 200 °C. Ultramark 1621 was used for external calibration of the FT mass analyzer prior the analyses. The background polysiloxane ion signal at m/z 445.1200 was used as lock mass. The instrument was operated in data-dependent acquisition (DDA) mode, and full MS scans with 1 microscan at resolution of 60 000 were used over a mass range of m/z 350−1500 with detection in the Orbitrap. Auto gain control (AGC) was set to 106, dynamic exclusion was set at 60 s, and the charge-state filter disqualifying singly charged peptides for fragmentation was activated. Following each survey scan, the 10 most intense ions with multiple charged ions above a threshold ion count of 5000 were selected for fragmentation at normalized collision energy of 35%. Fragment ion spectra produced via collision-induced dissociation (CID) were acquired in the linear ion trap, AGC was set to 3·104 and isolation window of 2.0 m/z, activation time of 30 ms, and maximum injection time of 250 ms were used. All data were acquired with Xcalibur software v2.2.

Data Analysis. Acquired data were analyzed using the Proteome Discoverer software suite (v1.4, Thermo Fisher Scientific), and the

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Mascot search engine (v2.5, Matrix Science) was used for peptide identification. Data were searched against the human protein database derived from the SwissProt database plus common contaminants (April 2016; 20,200 sequences). A precursor ion mass tolerance of 7 ppm was used, and up to three missed cleavages were allowed. The fragment ion mass tolerance was set to 0.5 Da, and oxidation (M), acetylation (Protein N-term) and phosphorylation (STY) were defined as variable modifications, whereas carbamidomethylation (C) was set as fixed modification. The identified peptides were filtered by FDR < 5%. Average area of the three most intense peptides per protein as calculated by Proteome Discoverer was used as quantitation indicator and a Student's t-test was performed between the 3 replicates of each state to pinpoint differentially abundant proteins.

Proteomics analyses were performed at the CRG/UPF Proteomics Unit of the Centre de Regulació Genòmica (CRG), Universitat Pompeu Fabra (UPF), 08003 Barcelona, which is part of the "Plataforma de Recursos Biomoleculares y Bioinformáticos del Instituto de Salud Carlos III (PT13/0001)".

Time-lapse microscopy analysis
Cells were seeded at 50.000 cells/well onto 12 well Glass Bottom Plates (Cellvis) coated with 10 µg/ml fibronectin (Millipore). The next day, media were changed to imaging media (DMEM without phenol red with 1% FBS) at least 1 hr prior to imaging. Cells were imaged with a Nikon Eclipse Ti fluorescence microscope controlled by the NIS elements AR software. Temperature (37°C), CO_2 (5%), and humidity were held constant during the experiments. Five blank positions (media only) were imaged for every channel and used to flat field the rest of the images. Flatfielding, image

registration, object identification and cell tracking were performed using custom software as described in Regot et al., 2014. Quantification and graphics were performed in Matlab.

Primers

Table 1. Primer pairs used for qPCR analysis

GAPDH	GAPDH GE F	TCACCAGGGCTGCTTTTAAC
	GAPDH GE R	TGGAAGATGGTGATGGGATT
c-FOS	cFOS GE F	AAAAGGAGAATCCGAAGGGA
	cFOS GE R	GCAACCCACAGAGTACCTAC,
ATF3	ATF3 GE F	CAAGTGCATCTTTGCCTCAA
	ATF3 GE R	CCACCCGAGGTACAGACACT
DYRK1A^S	DYRK1A e10a_e11 F	CGTTCCTGTCTAAGGAAATGTG
	DYRK1A e11 R	TTACCCAAGGCTTGTTGTCC
DYRK1A^{GE}	DYRK1A e11 F	CCTTTCATGTAGCCCCTCAA
	DYRK1A e11 R	ACTGTGGCCAACCTCCATAG
MIB1^S	MIB1 e6_e7a F	TCCAAGTGGCAATAGCAAAG
	MIB1 e7.2 R	CTTCAGCCCATTCTCCATGT
MIB1^{GE}	MIB1 e6 F	GGATTGCAGATTGGTGACCT
	MIB1 e6 R	TCAATGCCACAAACAGTTCC
GADD45α^S	GADD45A e1_e3 F	GAGAGCAGAAGACCGAAAGC
	GADD45A e3 R	CAGCGTCGGTCTCCAAGA
GADD45α^{S2}	GADD45A e1.2 F	GCCTGTGAGTGAGTGCAGAA
	GADD45A e1_e4 R	CATTGAGATGAATGTGGATTCTTT
GADD45α^B	GADD45A e2_e3 F	GCCAAGCTGCTCAACGTC
	GADD45A e3 R	CAGCGTCGGTCTCCAAGA
GADD45α^{GE}	GADD45A e1.2 F	GCCTGTGAGTGAGTGCAGAA
	GADD45A e1 R	GCCGAGAATTCCTCCAAAGT
mDYRK1A^S	T7	TAATACGACTCACTATAGGG
	DYRK1A e10a R	CAGGAACGTCATGAACCTCT
mDYRK1A^{GE}	DYRK1A e11 F	CCTTTCATGTAGCCCCTCAA
	Sp6	TATTTAGGTGACACTATAG
mMIB1^S	MIB1 e7a F	CCCATTTTGAGGTTTAGCTTTG
	Sp6	TATTTAGGTGACACTATAG
mMIB1^{GE}	T7	TAATACGACTCACTATAGGG
	MIB1 e6 R	TCAATGCCACAAACAGTTCC
mGADD45α^S	GADD45A e1_e3 F	GAGAGCAGAAGACCGAAAGC
	Sp6	TATTTAGGTGACACTATAG
mGADD45α^B	GADD45A e2_e3 F	GCCAAGCTGCTCAACGTC
	Sp6	TATTTAGGTGACACTATAG
PKM1	PKM1 F	GAGGCAGCCATGTTCCAC
	PKM1 R	TGCCAGACTCCGTCAGAACT
PKM2	PKM2 2F	GAGGCTGCCATCTACCACTT
	PKM2 R	CTGCCAGACTTGGTGAGGAC
MADD^{e16inc}	MADD e16 F	TTTGGGCTAAATACTCTAATGGAGA
	MADD e17R	GGGGTGATGGAGGTTCTCTT
MADD^{GE}	MADD e18 F	CAGTTCCTGAAGGAGGTGGT
	MADD e18 R	GCATCGTCCTCTGACTGCAC

Table 2. Primer pairs used for semi-quantitative PCR analysis

DYRK1A	DYRK1A e9 F	CCCCGCCATGGAGCAGTCTC
	DYRK1A e11.2 R	ACCCTTGGCCTGGTCCGGTT
MIB1	MIB1 e6 F	GGATTGCAGATTGGTGACCT
	MIB1 e7 R	GCATCTCCACTTCGGACAAT
GADD45α	GADD45A e1 F	AATATGACTTTGGAGGAATTC
	GADD45A e4 R	TCACCGTTCAGGGAGATTAATC

Table 3. Primer pairs used for cloning minigenes and expression vectors

DYRK1A minigene	EcoRI e10 F	CCGGAATTCgtggctcatcggggacaagc
	240-240 i10 R	aataggttcatctatagagtcgagtcattc
	240-240 i10 F	ctgaatgactcgactctatagatgaacctatttaagg
	XhOI e11 R	CCGCTCGAGggcctggtccggttacccaag
MIB1 minigene	EcoRI e6 F	ccggaattcgtgagcagaatggcaacagg
	284-227 i7 R	cctgcttctcctcttgaaggaaaaatagtg
	284-227 i7 F	gagaagcaggagaatcatgcagtga
	XhoI i7 R	CCGCTCGAGgcacgagtatgtctaggtgcaa
GADD45α minigene	EcoRV e1 F	CAGCGATATCagtttgcaatatgactttggaggaat
	XhOI e4 R	GCCGCTCGAGtttctgtaatccttgcatcagtgta
GST-SF3B1 Nter	Nter F	CAGCGGATCCatggccaaaatcgccaaaacgca
	Nter R	GCCGCTCGAGCTAattcttgattttcaacaacagc
GST-SF3B1 C-ter	Cter F	CAGCGGATCCctgttgttgaaaatcaagaat
	Cter R	GCCGCTCGAGCTAcatgacggcctggattacatg
GST-SKIIP WT	GST-SKIIP F	AGCGGATCCatggcgctcaccagctttttacctgc
	GST-SKIIP R	GCCGCTCGAGctattccttcctcctcttccttgcct
T7-SKIIP WT	T7-SKIIP F	AGATGGGTTCTAGAatggcgctcaccagctttttacctgc
	T7-SKIIP R	GAAGTTCTCAGGATCCctattccttcctcctcttccttgcct
pcDNA3 GADD45α	eGADD45α F	AGCGATATCAtgactttggaggaattctcgg
	eGADD45α R	GCCGCTCGAGTcaccgttcagggagattaatc

Table 4. Primer pairs used for semi-quantitative PCR analysis of network alternative splicing events

CHEK2 forward	CAGCTCTCAATGTTGAAACAGAA
CHEK2 reverse	CAGCCAAGAGCATCTGGTAA
MCL1 forward	AGACCTTACGACGGGGTTGG
MCL1 reverse	ACCAGCTCCTACTCCAGCAA
FN1EDB forward	GGCCTGGAGTACAATGTCAGT
FN1EDB reverse	CATGGTGTCTGGACCAATGT
FN1EDA forward	CTCAGAATCCAAGCGGAGAG
FN1EDA reverse	AACATTGGGTGGTGTCCACT
NOTCH3 forward	CGTCAGTGTGAACTCCTCTCC
NOTCH3 reverse	CTCAGGCACTCATCCACATC
CCNE forward	ATCCTCCAAAGTTGCACCAG
CCNE reverse	ATGATACAAGGCCGAAGCAG

RAC1 forward	TGCCAATGTTATGGTAGATGGA
RAC1 reverse	CTTTGCACGGACATTTTCAA
MSTR1 forward	GCTTTACACTGCCTGGCTTT
MSTR1 reverse	GCCACCAGTAGCTGAAGACC
PKM2 forward	GCTGGAGAGCATGATCAAGAA
PKM2 reverse	CAACATTCATGGCAAAGTTCA
MAP3K7 forward	GAATCTGGACGTTTAAGCTTGG
MAP3K7 reverse	TGACCAGGTTCTGTTCCAGTT
MINK1 forward	CCTCAGAGGACCTCATCTATCG
MINK1 reverse	CGTGAGAGGCTGGAAGGAC
NUMB forward	CTCCCTGTGCTCACAGATCA
NUMB reverse	CGGACGCTCTTAGACACCTC
CASP2 forward	GCTCTTTGACAACGCCAACT
CASP2 reverse	GGCATAGCCGCATATCATGT
BCL2L1 forward	CAGTAAAGCAAGCGCTGAGG
BCL2L1 reverse	AAGAGTGAGCCCAGCAGAAC
APAF1 forward	ATGCGACATCAGCAAATGAG
APAF1 reverse	CTCTGCAATCAGCCACCTTT
FAS forward	GAACATGGAATCATCAAGGAATGCAC
FAS reverse	AGTTGGAGATTCATGAGAACCTTGG
CFLAR forward	TTCAAGGAGCAGGGACAAGTTAC
CFLAR reverse1	GAATGATTAAGTAGAGGCAGTTCCA
CFLAR reverse2	TCCCATTATGGAGCCTGA
OLR1 forward	GGCATGGAGAAAACTGTTACCTATTTTCCTC
OLR1 reverse	CACTGTGCTCTTAGGTTTGCCTTCTTCTG
MAP4K3 forward	GGCCAAGTGAAATTTGATCC
MAP4K3 reverse	TTGCACCTAAAAAGTAACCACCT
PAX6 forward	CCGGCAGAAGATTGTAGAGC
PAX6 reverse	CTCCCGCTTATACTGGGCTA
CASP9 forward	CGGGCAGGCTCTGGATCTC
CASP9 reverse1	CCAGCACCATTTTCTTGG
CASP9 reverse2	TGGTCTTTCTGCTCGACA
GADD45A forward	AATATGACTTTGGAGGAATTC
GADD45A reverse	TCACCGTTCAGGGAGATTAATC
CCND1 forward	CTGAGGAGCCCCAACAACT
CCND1 reverse1	CTTCGCCCAGAAACTCTACG
CCND1 reverse2	AGGTCCACCTCCTCCTCCT
MAP4K2 forward	GAGCTGACAGCGTCTGTGG
MAP4K2 reverse	GCGAGTCTTATCTCTCAGTTTGG
LMNA forward	ACCCCGCTGAGTACAACCT
LMNA reverse	GGCATGAGGTGAGGAGGAC
H2AFY forward	AAGCAGGGTGAAGTCAGTAAGG
H2AYF reverse	CCAGGACAGCTTCCACAAAC
STAT3 forward	CCCCATACCTGAAGACCAAG
STAT3 reverse	ACTCCGAGGTCAACTCCATGTCAA
BMF forward	CTCAGCCGACTTCAGCTCTTC
BMF reverse	CCCCGTTCCTGTTCTCTTCT
BIM forward	ATGGCAAAGCAACCTTCTGA
BIM reverse	TCAATGCATTCTCCACACCA
BIRC5 forward	GAGGCTGGCTTCATCCACT
BIRC5 reverse	TCTCCGCAGTTTCCTCAAAT
DIABLO forward	ACCATGGCACAAAACTGTGA
DIABLO reverse	TCCTATGATCACCTGCCACA

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SMN forward	ATAATTCCCCCACCACCTCCC
SMN reverse	TTGCCACATACGCCTCACATAC
PHF19 forward	CCTGCTCACACCTTGGTTCT
PHF19 reverse inc	CACCAAACCCATCTCCACTT
PHF19 reverse inc 2	AGGCGCTATCTGTCTCCAAA
PHF19 reverse skip	CCGCAGTAGCAGTAGCATTG

4. RESULTS

4. RESULTS

4.1 p38 regulates stress-specific alternative splicing

In response to osmostress, activated p38 leads to transcription regulation of a set of osmoresponsive genes (Ferreiro et al., 2010b). In order to investigate additional mechanisms by which p38 regulates gene expression, changes in splicing patterns where analyzed upon osmostress.

4.1.1 p38 contributes to alternative splicing modulation in response to stress

HeLa cells were treated with NaCl (100 mM) for 2h in order to activate p38. In addition, cells were pre-treated during 30 min with the p38 inhibitor SB203580. Osmostress (OSM) induced p38 activation, as shown by an increase on mRNA accumulation of cFOS and ATF3 osmoresponsive genes. Of note, the chemical inhibitor SB203580 impaired expression of both genes, indicating that it was able to prevent p38 activity (Figure 16A). Thus, we confirmed that in our experimental conditions p38 is activated and induces a transcriptionally response that it is greatly impaired in the presence of SB203580, as it has been previously described (Ferreiro et al., 2010b).

A

B

C

OSM and SB		
BUB1B	FN1	MYBL2
CD44	**cFOS**	**NFKBIA**
CDC25C	GADD45A	PDCD4
CDC40	RNPC2	PTK2
CLK1	ITGB1	RBM15
CROP	MAP2K1	SCUBE2
CUG-BP	MAPK10	SPTAN1
DYRK1A	MDM2	SWAP2
FAP	MIB1	TNFAIP3
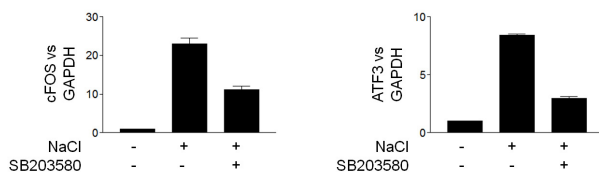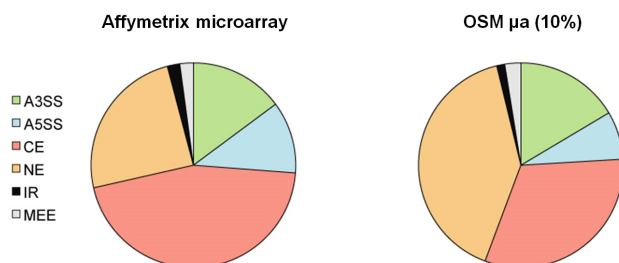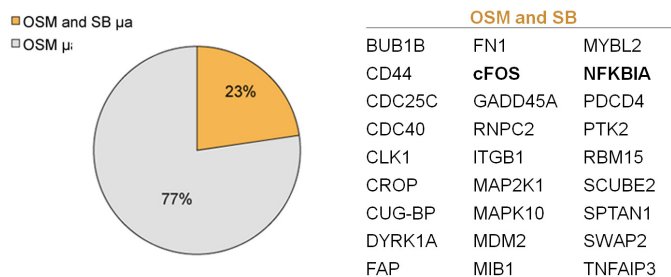
Figure 16. The p38 SAPK modulates a subset of stress-induced splicing events

(A) OSM induced p38-dependent transcription. HeLa cells were stimulated with 100 mM NaCl for 2h and treated with 10 µM SB203502 30 min prior to NaCl addition. Total mRNA was extracted and analyzed by qPCR. Expression of p38-stress induced genes (*c-FOS* and *ATF3*) relative to GAPDH was represented as fold change over non treated cells. Data represent the mean and standard deviation of technical triplicates. (B) *Left.* Pie chart shows the distribution of AS categories included in the microarray platform (A3SS, alternative 3' splice site, A5SS, alternative 5' splice site, CE, cassette exon, NE, novel exon, IR, intron retention, MEE, mutually exclusive exon). *Right.* Distribution of AS changes comparing NaCl *versus* non treated cells (OSM µa) (C) Impact of p38 activation in OSM induced AS was assessed by microarray analysis comparing NaCl treated cells *versus* NaCl treated cells in the presence of SB203502 (SB µa). Pie chart shows percentage of OSM µa positive genes affected in SB µa, listed under the diagram.

To analyze the role of p38 in splicing, RNA from non-treated, stressed cells, and stressed cells in the presence of SB203580 was analyzed using a custom splicing-sensitive microarray. This platform contained exon and exon-junction probes covering 1920 AS events in 491 genes encoding splicing factors and cancer related proteins (Figure 16B) (Muñoz et al., 2009).

Two independent microarray assays (μa) were performed in order to identify p38 mediated AS events. First, all changes induced by osmostress were determined comparing stressed with non-stressed cells (OSM μa). Osmostress affected 207 AS events in 119 genes, triggering changes in all the categories of events included in the platform (Figure 16B). Thus, osmostress causes a strong effect in AS.

Next, p38-dependent AS events were identified comparing stressed cells in the presence or the absence of SB203580 (SB μa). The p38 inhibitor affected 23% of the genes that displayed AS changes in stress conditions, and only two of them (*cFOS* and *NFκB*) were also transcriptionally regulated (Figure 16C).

Collectively, these results indicate that in response to osmostress, cells change the regulation of AS and that p38 regulates a subset of specific AS changes beyond regulation of total gene expression.

4.1.2 DYRK1A, MIB1 and GADD45α AS changes are dependent on p38

AS events in *DYRK1A*, *MIB1* and *GADD45α* genes were selected from the list of p38-regulated candidates for further analysis. AS changes were validated by semi-quantitative RT-PCR using primers flanking constitutive exons (Table 2). In addition, quantification of stress-induced splicing isoforms was assessed by

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qPCR using oligonucleotides covering exons and exon junctions normalized to the total gene expression levels (Table 1).

DYRK1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A) is a proline- and arginine-directed Ser/Thr kinase involved in regulation of several cellular processes like mRNA splicing, transcription, cell survival and neuronal development (Guo et al., 2010; Gwack et al., 2006; Qian et al., 2011; Tejedor and Hämmerle, 2011; Vona et al., 2015). While in basal conditions the distal 5'ss of exon 10 was preferentially selected (DYRK1A[B]), osmostress induced the selection of the proximal 5'ss generating a novel mRNA isoform (DYRK1A[S]). Regulation of this event is dependent on p38 activity, as is strongly prevented by addition of SB203580 (Figure 17A). The change on 5'ss choice results in a shift in the reading frame that introduces a premature termination codon (PTC).

MIB1 (mindbomb E3 ubiquitin protein ligase 1) is a large multi-domain RING-type E3 ligase that has been related to Notch signaling pathway (Itoh et al., 2003) and centriole biogenesis (Villumsen et al., 2013). Osmostress induced the use of a downstream 3'ss splice site acceptor in exon 7, generating a longer isoform (MIB1[S]) that was partially reduced in the presence of the p38 inhibitor (Figure 17B). The selection of the proximal 3'ss leads to inclusion of an in-frame PTC and is predicted to reduce MIB1 mRNA levels.
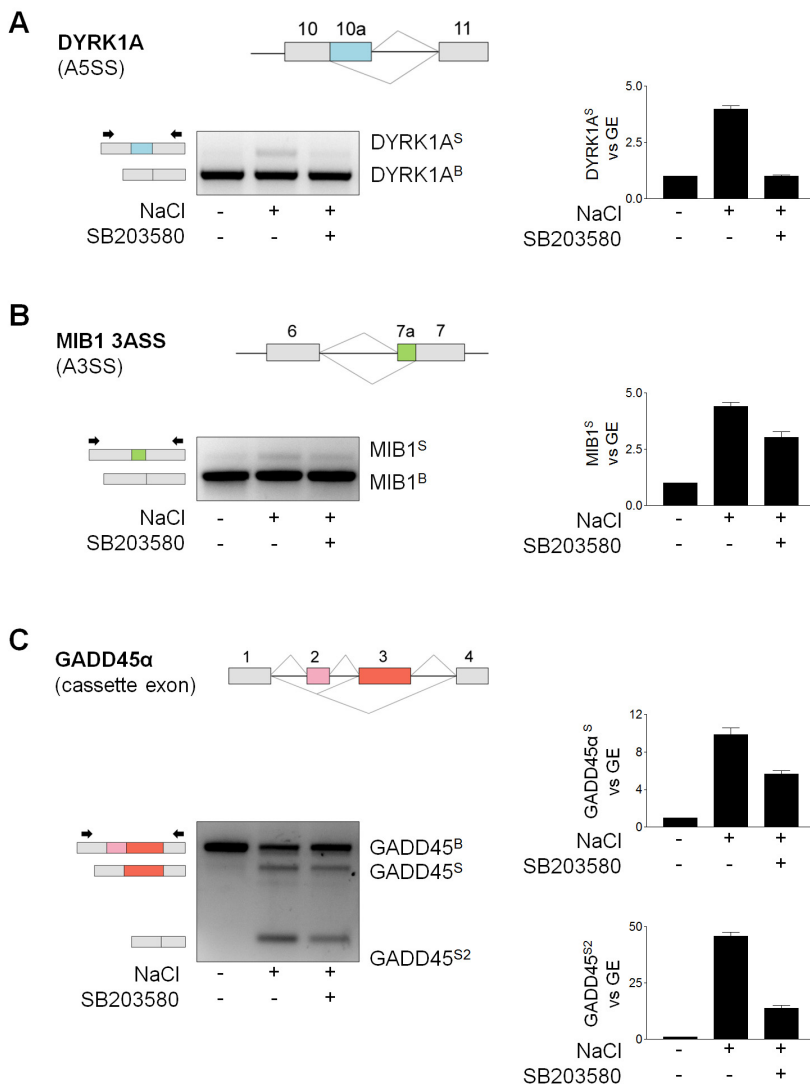
Figure 17. p38 regulates DYRK1A, MIB1 and GADD45α AS upon stress

Schematic diagrams of AS events in *DYRK1A* (A), *MIB1* (B) and *GADD45α* (C) genes. Schemes include constitutive exons (gray boxes) alternative exons (colored boxes) and introns (black lines). HeLa cells were treated with 100 mM NaCl for 2h in the absence or the presence of SB203502. Changes in AS were assessed by semi-quantitative RT-PCR with primers represented by arrows. Levels of stress induced isoforms (S) and total gene expression (GE) were determined by qPCR using primers targeting exon-junctions and constitutive exons respectively. Relative expression of stress isoforms was represented as fold change over non treated cells. Data represent the mean and standard deviation of technical triplicates.

As mentioned in the introduction, GADD45α (Growth Arrest and DNA damage-inducible 45 alpha) plays a key role in DNA damage repair, cell cycle control and regulation of apoptosis in stress conditions. *GADD45α* gene comprises four exons, two constitutive exons (1 and 4) and two cassette exons (2 and 3). Although microarray analysis only contained probes for the detection of changes in exon 2 inclusion levels, variations in both alternative exons were assessed by PCR amplification comprising exons 1 and 4. In non treated cells, full length mRNA isoform was preferentially expressed (GADD45α^B). Interestingly, two splicing variants appeared upon osmostress, generated either by the skipping of exon 2 (GADD45^S) or the skipping of both exon 2 and 3 (GADD45α^{S2}). Incubation of HeLa cells with SB203580 reduced the expression of both GADD45α^S and GADD45α^{S2} (Figure 17C), indicating that these AS events may be dependent on the p38 SAPK. Skipping of exon 2 in GADD45α^S maintains the reading frame and results in a 34 amino acids deletion, while skipping of exon 2 and 3 in GADD45^{S2} triggers inclusion of a PTC. Based on the potential interest of this isoform, we focused on of GADD45^S for the study of p38 mediated AS.

4.1.3 Activation of p38 modulates minigene AS

To further confirm our results, we constructed DYRK1A, MIB1 and GADD45α reporter minigenes. For the analysis of DYRK1A A5SS event, the region from exon 10 to 220 bp upstream 3'ss of exon 11 was cloned including a 5 kb deletion of intron 10. The minigene construct for the study of MIB1 A3SS event included the DNA segment harboring exon 6 to 140 nt upstream intron 7, with a 11 kb truncation of intron 6. The GADD45α minigene comprised the

whole gene-coding region between exon 1 and the terminal exon 4 (Figure 18A).

HeLa cells were transfected with the minigene vectors and subjected to osmostress under the conditions described above. Minigene (m) splicing profiles were assessed by qPCR using exon-juntion primers combined with plasmid-specific primers (T7 or SP6) (Table 1). Upon stress, all minigenes showed changes in splicing in the same direction than the corresponding endogenous genes (Figure 18B). Taken together, these results indicate that our minigene reporters contain *cis*-elements important for p38 regulation of AS.

To prove that regulation of AS was directly mediated by p38 activation, minigene constructs were cotransfected with p38 and the constitutively active MAPKK upstream p38 (MKK6^{DD}) (Takekawa et al., 1998). The AS regulation of GADD45α, DYRK1A and MIB1 minigenes under ectopically expression of MKK6^{DD} was similar to the observed upon stress (Figure 18C).

Therefore, minigene assays validated the implication of p38 in regulation of AS profiles and constitute a valuable tool for the study of the molecular mechanism underlying this regulation.
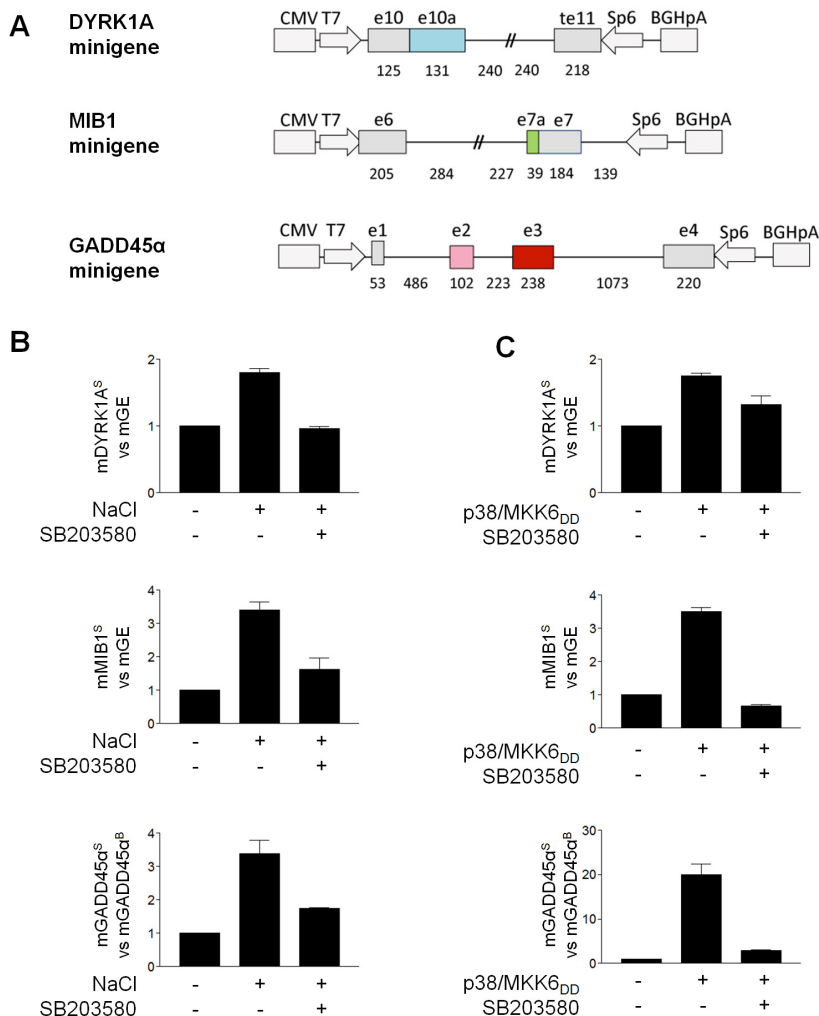
Figure 18. Activation of the p38 modulates DYRK1A, MIB1, and GADD45α minigene AS
(A) Schematic diagram of the minigenes used in transient transfection assays. Length of exons and introns included in the minigenes is indicated under the diagrams. (B) HeLa cells transiently transfected with the minigene constructs were treated for 2h with 100 mM NaCl pre-treated or not with SB203580 for 30 min. Minigene (m) AS isoforms and total minignene expression was assessed by qPCR. OSM effect on minigene AS was normalized to that in non treated cells, which was assigned a value of 1. Data represent the mean and standard deviation of technical triplicates. (C) HeLa cells were co-transfected with p38, MKK6^{DD} and the minigenes constructs. Effect of p38 activation on minigene AS was assessed by qPCR as in (B).

4.1.4 Regulation of DYRK1A, MIB1 and GADD45α AS events is independent of hnRNPA1 and SPF45

p38 has been reported to phosphorylate hnRNPA1 and SPF45 splicing factors (Al-Ayoubi et al., 2012; van der Houven van Oordt et al., 2000). Thus, we wondered whether the p38-mediated AS events identified in the microarray could be regulated through these spliceosomal components. To test this possibility, hnRNPA1 and SPF45 were knocked down from HeLa cells by siRNA transfection, and reduction in protein levels was confirmed by western blot analysis (Figure 19A). hnRNPA1 and SPF45 depleted HeLa cells were subjected to osmostress, and AS changes in p38-dependent events were assayed by qPCR. The decreased expression of hnRNPA1 and SPF45 did not trigger significant changes in AS under basal conditions. Moreover, depletion of hnRNPA1 and SPF45 did not abolish induction of DYRK1A, MIB1 and GADD45α stress isoforms upon NaCl treatment (Figure 19B and C). These results indicate that regulation of DYRK1A, MIB1 and GADD45α AS upon stress is not dependent on the function of hnRNPA1 and SPF45, and suggest that other spliceosomal components could be involved in p38 mediated AS.
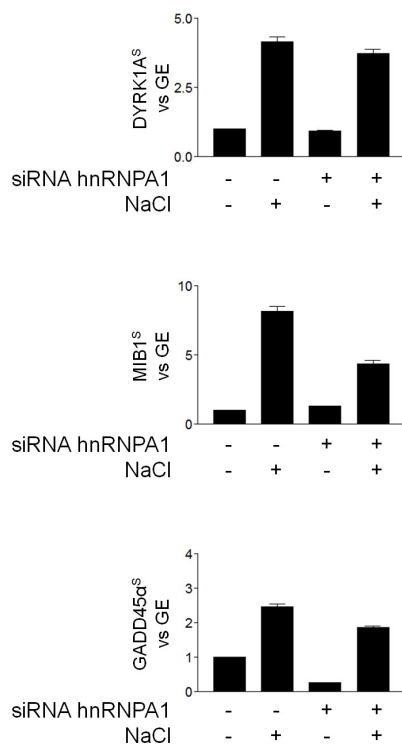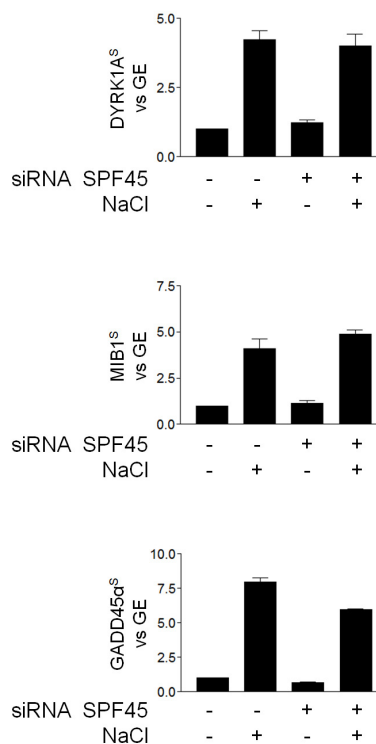
Figure 19. Depletion of hnRNPA1 and SPF45 does not prevent changes in DYRK1A, MIB1 and GADD45α AS upon stress
(A) Western blot analysis of HeLa cells treated with control, hnRNPA1 or SPF45 siRNAs. (B) siRNA-treated HeLa cells were stimulated for 2h with NaCl 100 mM. AS patterns were analyzed by qPCR as in Figure 17.

4.2 The function of U2snRNP upon stress

4.2.1 Network analysis reveals functional connections between osmostress and the U2snRNP complex

To identify novel targets in the splicing machinery for the p38 SAPK we took advantage of the functional splicing network reported by Papasaikas et al. 2015 in which connections between splicing factors are established based on the effects of protein knockdowns in 36 AS events. Time-course experiments were performed to capture the clearest effects of osmostress on splicing (data not shown), and two NaCl treatment time points (3 and 6 h) were selected for generation of network perturbation profiles (Figure 20A).

The perturbation profiles upon osmostress resulted in functional associations with U2snRNP components (Figure 20B), being snRNPA1, SF3B4 and SF3B1 the strongest connections between osmostress treatments and splicing factors. In addition, we extended the network analysis including drug treatments known to affect AS patterns (Papasaikas et al., 2015). Osmostress appear to be closely related to spliceostatin A, a splicing inhibitor that targets SF3B1 (data not shown) (Kaida et al., 2007).

Thus, splicing network analyses suggested that changes in AS observed in osmostress conditions could be mediated by modulation of the U2snRNP function.
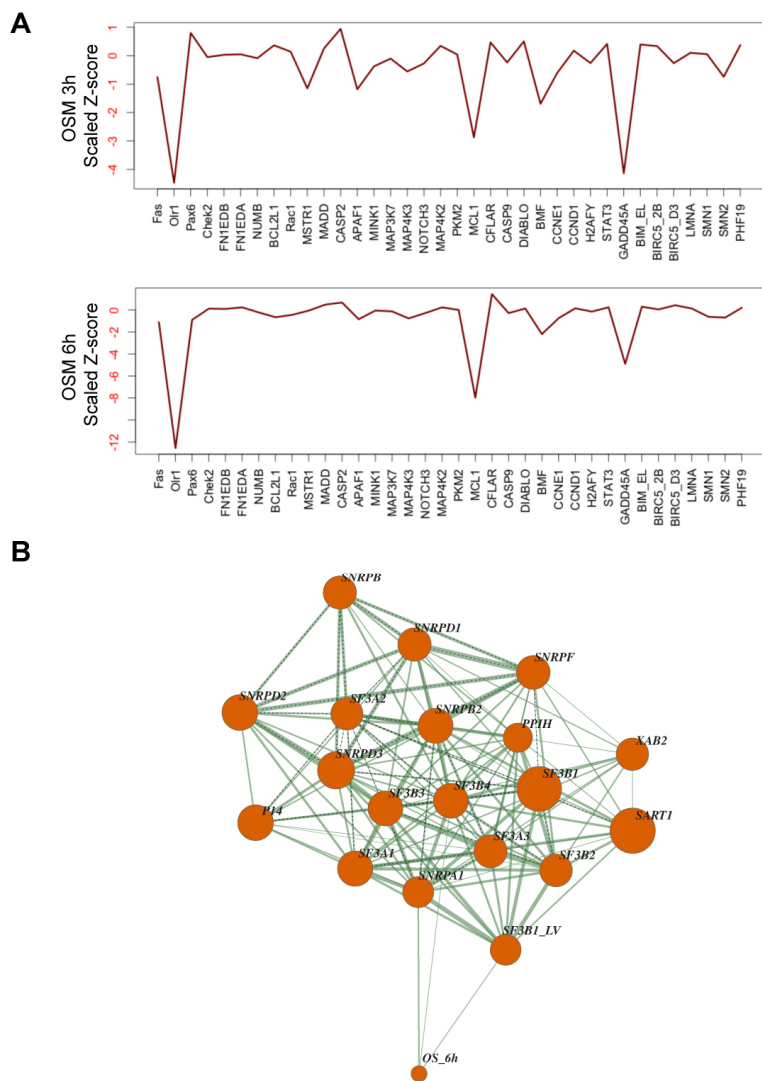
Figure 20. Functional connections between osmostress and the U2snRNP complex

(A) HeLa cells were treated for 3 h or 6 h with NaCl 100 mM. AS changes across 36 network events were analyzed by semi-quantitative RT-PCR and capillary electrophoresis to generate the osmostress (OSM) perturbation profiles. Results correspond to scaled Z-score changes from three biological replicas. Positive and negative Z-scores indicate an increase in exon inclusion or skipping, respectively. (B) Functional links between U2snRNP components knockdowns and 6 h OSM induced splicing, derived from the splicing network analysis.

4.2.2 SF3B1 is phosphorylated *in vitro* by p38 in the N-terminal domain

We next asked whether the U2snRNP complex could be targeted by p38. Taking into account that OSM associates with spliceostatin A in the network, and that SF3B1 is a central component of U2snRNP tightly regulated by phosphorylation (Bessonov et al., 2010; de Graaf et al., 2006), we focused on the study of SF3B1 as a potential target for p38.

The N-terminal region of SF3B1 contains p14 and U2AF-binding domains and the C-terminal region consists of 22 non-identical, tandem HEAT repeats (Figure 21A). To determine whether SF3B1 can be directly phosphorylated by p38, we performed *in vitro* kinase assays using activated p38α in the absence or the presence of SB203580. SF3B1 expressed and purified from yeast was used as a substrate since its expression in bacteria was too toxic. p38α was able to specifically phosphorylate full length SF3B1, and the phosphorylation was fully prevented by SB203580 (Figure 21B).

SF3B1 protein contains 38 putative S/TP MAPK consensus sites distributed along the protein. To characterize SF3B1 phosphorylation by p38, two truncated protein variants were generated; the N-terminal (comprising from aa 1 to 508) and the C-terminal (comprising from aa 508 to 1230). The N-terminal SF3B1 fragment was strongly phosphorylated by p38 in *in vitro* kinase assays, whereas the C-terminal fragment was not phosphorylated at all (Figure 21C). Phosphorylation at T142, T207, T244, T303 and T328 residues were detected by mass spectrometry analysis.

A SF3B1 (1304 aa; 155 KDa)

B

C

D
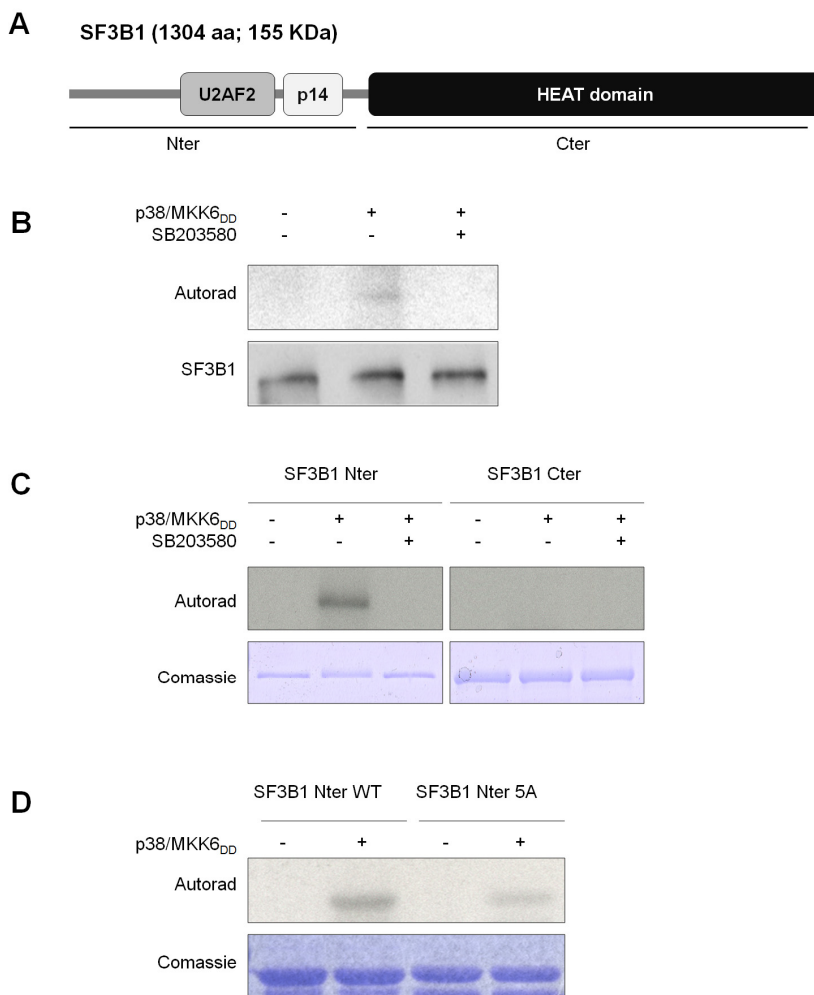
Figure 21. The p38 SAPK phosphorylates SF3B1 *in vitro*
(A) Schematic representation of SF3B1 protein domains. The N-terminal region (Nter) is involved in interactions with U2AF65 and p14, whereas the C-terminal region (Cter) includes 22 HEAT repeats. (B) *In vitro* kinase assays were performed by incubation of SF3B1 purified from *Saccharomyces cerevisiae* and activated p38α in the absence or the presence of SB203580. Phosphorylated SF3B1 was detected by autoradiography and total SF3B1 was detected by Western blot. (C) Purified GST-SF3B1 Nter (1-508) and GST- SF3B1 Cter (508-1230) were assayed *in vitro* with activated p38. Phosphorylation of SF3B1 fragments was detected by autoradiography and total protein was visualized by Comassie staining. (D) GST-SF3B1 WT and GST-SF3B1 5A (T142A, T207A, T244A, T303A, T328A) were assayed *in vitro* with activated p38.

Notably, simultaneous mutation of the five residues to alanine (SF3B1 Nter 5A) significantly prevented p38 phosphorylation compared to wild type (SF3B1 Nter WT) (Figure 21D).

4.2.3 Depletion of SF3B1 affects p38 dependent AS regulation

To investigate whether modulation of SF3B1 activity could affect p38 dependent AS, we depleted SF3B1 in HeLa cells (Figure 22A) and DYRK1A, MIB1 and GADD45α AS was analyzed after NaCl treatment. While DYRK1A^S was strongly induced by osmostress in control cells, SF3B1 knockdown significantly prevented the regulation of 5' splice site selection upon NaCl treatment. In clear contrast, depletion of SF3B1 greatly induced the expression of MIB1 and GADD45α stress isoforms even in the absence of stress, thus resulted in MIB1^S/MIB1^B and GADD45α^S/GADD45α^B similar ratios between stress and non stress conditions (Figure 22B).
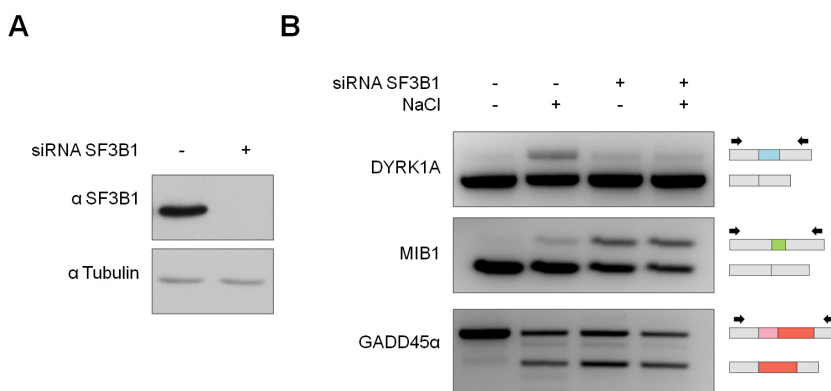
Figure 22. SF3B1 depletion influences regulation of p38-dependent AS events
(A) HeLa cells were transfected with control siRNAs or siRNAs against SF3B1, and SF3B1 protein levels were analyzed by Western blot. (B) HeLa cells were treated during 3 h with 100 mM NaCl after transfection of control siRNA or siRNA against SF3B1. DYRK1A, MIB1 and GADD45α AS was analyzed by semi-quantitative RT-PCR.

Therefore, variations in SF3B1 activity display differential effects on specific splice targets, not only by altering 3' splice site recognition but also influencing 5' splice site selection upon stress. The sensitivity of DYRK1A, MIB1 and GADD45α AS regulation to a loss of function of SF3B1 suggested a role of the U2snRNP complex in regulation of p38 dependent AS upon stress.

We are presently working to assess the effect of SF3B1 phosphorylation on p38-dependent AS regulation in stress conditions by expression of the phosphomimetic and non-phosphorylatable versions of SF3B1. However, the low expression levels achieved by transfection of SF3B1 expression vectors in HeLa cells have been a limitation. We are currently testing alternative expression systems as well as different cell lines to reach proper SF3B1 expression levels. These experiments might help to uncover the impact of SF3B1 phosphorylation in the regulation of AS in stress conditions.

4.3 p38 modulates SAPK pathway activation upon stress through SKIIP mediated GADD45α alternative splicing

4.3.1 MKK6[DD] expression functionally connects p38 with SKIIP and SYNCRIP

To find new spliceosomal components targeted by p38, we performed a second functional network analysis by hyperactivating p38 via expression of the constitutively active kinase MKK6 (MKK6[DD]). HeLa cells were co-transfected with p38α and MKK6[DD] expression vectors, and splicing profiles of the 36 events included in the network were assessed by PCR. The MKK6[DD] perturbation profile was generated by analysis of exon inclusion triggered by p38α compared to GFP transfected control cells (Figure 23A). Direct activation of p38 induced changes in the AS profiles of several genes. The strongest modification was in GADD45α exon 2 skipping, as we previously observed upon osmostress (Figure 23B).

The MKK6[DD] perturbation profile pointed out a possible association of p38 with several splicing factors involved in different steps of the pre-mRNA splicing reaction. One of the connections was established with the DBR1 (lariat debranching enzyme) which specifically hydrolyzes 2-prime-to-5-prime branched phosphodiester bonds at the branch point of excised lariat intron RNA (Kim et al., 2000). MKK6 [DD] was also linked to C22ORF19 (THOC5) which is part of the conserved THO/TREX complex, that plays an important role in mRNA 3′ processing of immediate-early genes induced by extracellular stimuli (DD Tran et al., 2014).
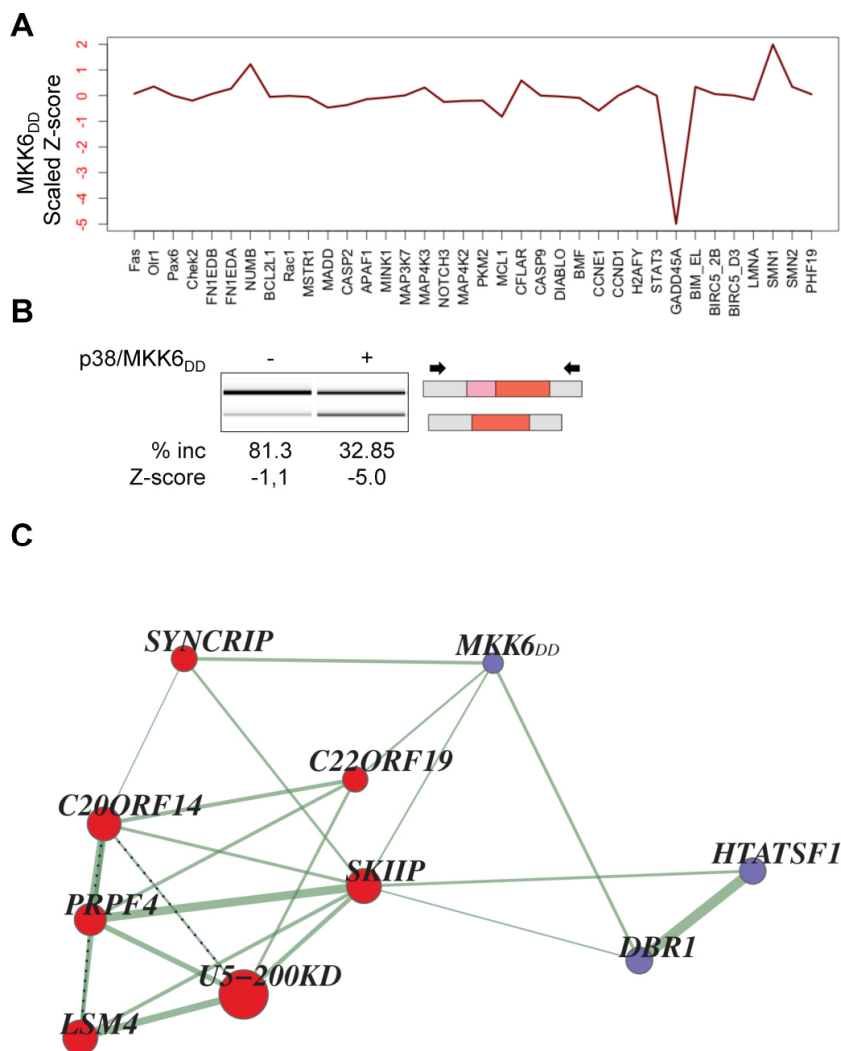
Figure 23. MKK6^{DD} is functionally associated to SKIIP and SYNCRIP
(A) The perturbation profile of AS changes induced by p38α and MKK6$_{DD}$ co-expression. Positive and negative scaled Z-scores indicate increased exon inclusion or skipping respectively. (B) GADD45α AS was assessed by semi-quantitative RT-PCR followed by capillary electrophoresis. % exon 2 inclusion and Z-score values are indicated. (C) Lines connecting RNA processing factors and MKK6$_{DD}$ expression indicate similar effects on the profile of AS changes by protein knockdown or by p38 activation.

In addition, activation of p38 was linked to SYNCRIP and SKIIP, both related to U5snRNP components in the network. SYNCRIP

(heterogeneous nuclear riboprotein Q) is an RNA binding protein that has been reported to have multiple functions in mRNA metabolism, including regulation of mRNA splicing as well as stability control, transport, and translation (Bannai et al., 2004; Blanc et al., 2001; Chen et al., 2008; Weidensdorfer et al., 2009). SKIIP (SNW domain-containing protein 1) functions as an splicing factor and as a transcriptional coactivator (Ambrozková et al., 2001; Chen et al., 2011). Interestingly the depletion of SYNCRIP and SKIIP affected GADD45α AS, while C22ORF19 and DBR1 did not (Table 5). However, only SKIIP contains putative phosphosites for p38 (SP/TP) and was therefore selected as a potential target for p38.

Table 5. Effects of protein knockdowns on GADD45α AS.
GADD45α % exon 2 inclusion, p-values and Z-score values are indicated.

Gene Symbol	% inc GADD45a	stdev	pvalue	z-score
control	98.525	2.086	0.353	0.325
C22ORF19	89.861	17.561	0.973	0.444
DBR1	85.571	2.222	0.609	-0.723
SYNCRIP	42.077	46.503	0.002	-4.240
SKIIP	49.608	43.756	0.005	-5.413

4.3.2 p38 phosphorylates SKIIP

To examine whether SKIIP could be directly phosphorylated by p38, GST tagged SKIIP was purified from bacteria and subjected to an *in vitro* kinase assay. Activated p38α was able to phosphorylate SKIIP (Figure 24A). SKIIP protein contains three putative MAPK consensus sites, all of them located in the SNW1 domain. The potential phosphosites T180, S224 and S232 were mutated to alanine and phosphorylation of the SKIIP 3A (which contains the three mutations) was assayed *in vitro*. Mutation of these three sites strongly reduced the *in vitro* phosphorylation of SKIIP by p38 (Figure 24A).

A
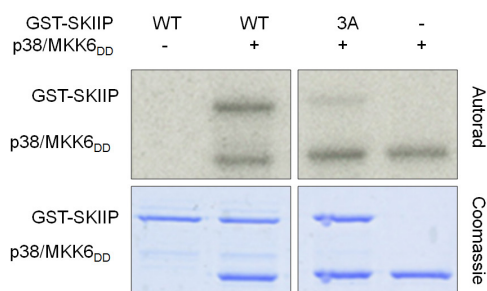
B
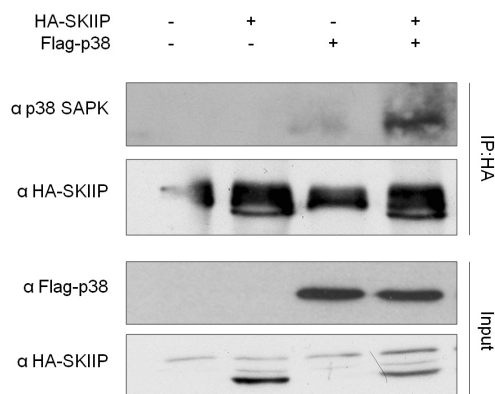
Figure 24. SKIIP is targeted by p38
(A) Wild type GST-SKIIP WT and GST-SKIIP 3A (T180, S224 and S232 mutated to alanine) were purified from *E.coli* and subjected to an *in vitro* kinase assay using activated p38α. (B) SKIIP interacts with p38 *in vivo.* HA-SKIIP and Flag-p38α were expressed in HeLa cells, immunoprecipitated with anti-Flag or anti-HA coupled sepharose beads and analyzed by Western blot with anti-HA and anti-p38α antibodies.

Then, we tested whether p38 was able to interact with SKIIP *in vivo*. We performed immunoprecipitation experiments using extracts from HeLa cells expressing Flag-tagged p38α and HA-tagged SKIIP. Binding of Flag-p38 was detected when HA-SKIIP was immunoprecipitated from cell extracts (Figure 24B). Taken together these results indicate that SKIIP is a suitable substrate for p38.

4.3.3 SKIIP is essential for p38 regulation of GADD45α alternative splicing upon stress.

We next assessed whether p38 could mediate AS through SKIIP regulation. GADD45α AS was tested after siRNA SKIIP knockdown and p38 activation. While in control cells GADD45^S expression was induced by p38, SKIIP depletion totally suppressed MKK6^{DD} dependent GADD45α exon 2 skipping (Figure 25A).

We then tested whether GADD45α AS regulation upon stress was similar to p38 direct activation. In SKIIP depleted HeLa cells subjected to NaCl, GADD45^S expression was prevented (Figure 25B). Thus, osmostress and MKK6^{DD} induce identical AS changes. In contrast, DYRK1A and MIB1 AS was not affected by SKIIP depletion (data not shown). These results demonstrate that SKIIP is necessary for GADD45α AS regulation by p38, and suggest that SKIIP might be involved in the regulation of a subset of p38 dependent AS events upon stress.

A

B
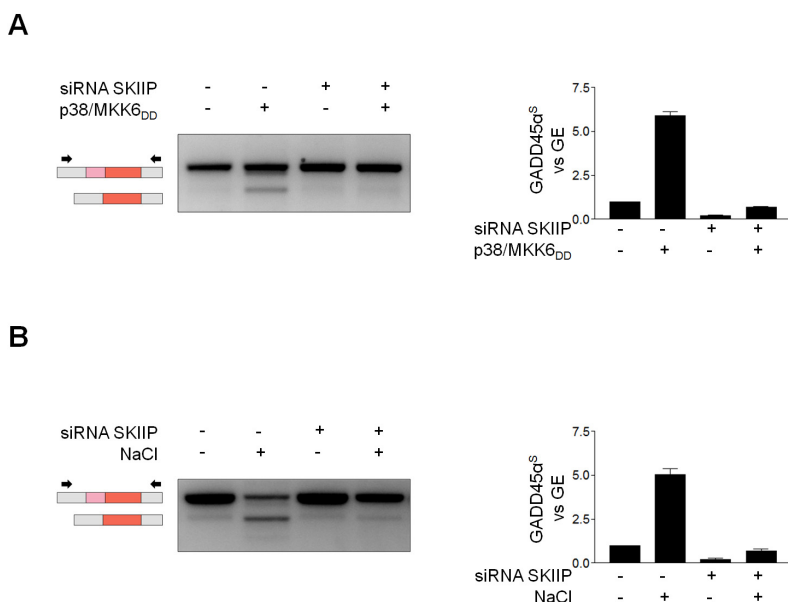
Figure 25. SKIIP is essential for p38 dependent GADD45α^S induction upon stress
(A) HeLa cells were treated with scambled or SKIIP stealth siRNA and transfected with an empty vector or with MKK6^{DD} and p38 expression vectors. GADD45α AS was assessed by semi-quantitative RT-PCR and qPCR. (B) HeLa cells were treated for 2h with 100 mM NaCl after scrambled or SKIIP stealth siRNA transfection. GADD45α AS was analyzed as in (A).

4.3.4 GADD45α alternative splicing regulation is dependent on SKIIP phosphorylation

To assess the relevance of T180, S224 and S232 SKIIP phosphosites in the regulation of stress dependent AS, siRNA resistant T7 SKIIP WT and T7 SKIIP 3A were expressed in HeLa cells in parallel to the depletion of endogenous SKIIP (Figure 26A). Cells were subjected to osmostress and induction of GADD45^S was measured by qPCR. Induction of GADD45α exon 2 skipping upon osmostresswas strongly impaired in SKIIP knockdown cells as previously demonstrated. Expression of T7-SKIIP WT in SKIIP depleted cells was sufficient to restore GADD45α^S induction, while

expression of T7 SKIIP 3A was unable to induce GADD45α^S upon osmostress (Figure 26B). Therefore, regulation of GADD45α AS upon stress depends on the phosphorylation of SKIIP at the T180, S224 and S232 phosphosites.
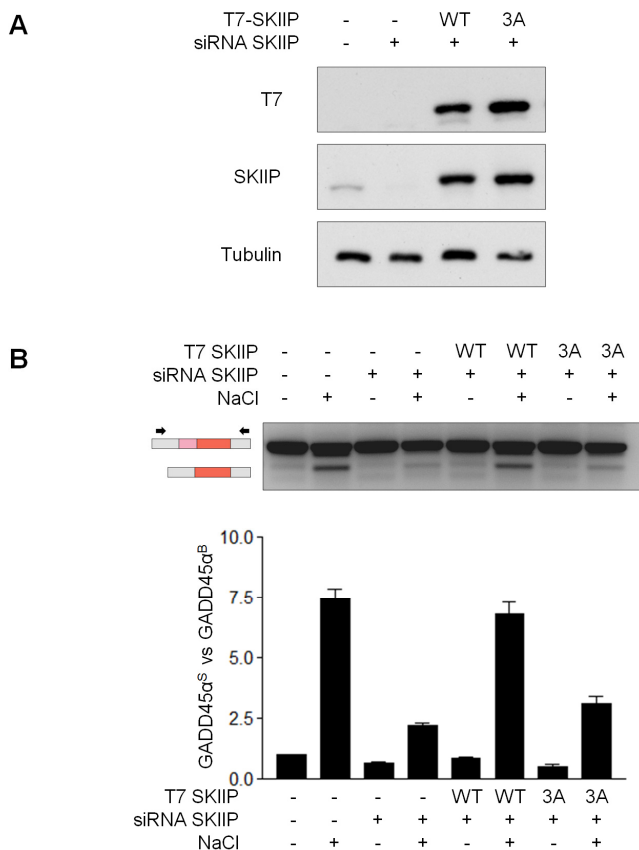
Figure 26. Regulation of GADD45α upon stress depends on SKIIP T180, S224 and S232 SKIIP phosphosites.
(A) HeLa cells were transfected either with an empty, T7-SKIIP WT or a T7-SKIIP 3A expression vectors. After 24h, cells where transfected with stealth siRNAs targeting the SKIIP 3'UTR or its corresponding scrambled siRNA. Cells were harvested 72h after siRNA transfection and protein extracts were analyzed by Western blot. (B) Endogenous SKIIP knockdown and T7-SKIIP WT and 3A expression was performed as in A. Cells were stimulated for 2h with 100 mM NaCl and the relative expression of GADD45α^S was analyzed by semi-quantitative PCR and qPCR. Data represent the mean and standard deviation of two independent experiments.

4.3.5 Analysis of SKIIP interaction with spliceosomal components upon stress

In order to understand the molecular mechanism behind SKIIP mediated GADD45α AS regulation, we assessed the association of SKIIP with its known spliceosomal interactors.

Snu114 and Brr2 have been reported to bind SKIIP through its SNW1 protein domain (Sato et al., 2014), that includes T180, S224 and S232 p38 phosphosites. Thus, we tested whether interaction between Brr2 or Snu114 was affected upon stress. The T7-SKIIP WT and T7-SKIIP 3A mutant were transfected in HeLa cells, and co-immunporecipitation experiments were performed after NaCl treatment.
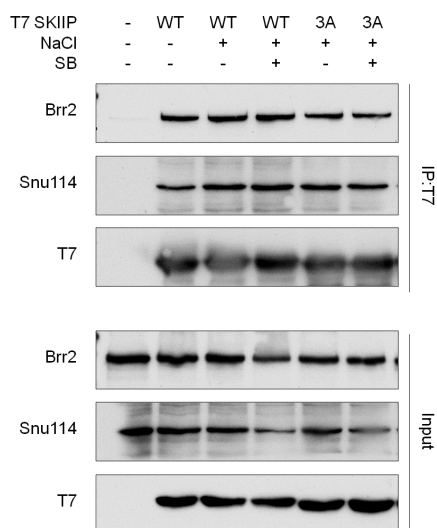
Figure 27. Association of SKIIP with Brr2 or Snu114 is not affected upon stress
HeLa cells were transfected either with an empty, a T7-SKIIP WT or a T7-SKIIP 3A expressing vector. Cells were stressed with NaCl 100 mM and harvested after 1h. Interaction between SKIIP and spliceosomal components was assessed by T7 pull down assays followed by Western blot using antibodies against Brr2 and Snu114.

Osmostress had no significant effect in SKIIP association with Brr2 or Snu14, and mutation of the three phosphosites had no effect in the interactions assessed (Figure 27). Therefore, the role of the phosphorylation of SKIIP upon stress is not mediated by changes in the association with Snu114 and Brr2 splicing factors.

4.3.6 SYNCRIP functionally and physically associates with SKIIP and it is required for GADD45α AS regulation by p38

In order to gain insights into the mechanisms how SKIIP modulates AS, we performed mass spectrometry analysis to identify SKIIP-interacting proteins involved in splicing. HeLa cells were transfected with T7-SKIIP WT, and T7 pull down assays were performed as described above. Immunoprecipitated SKIIP associated proteins were digested with trypsin and subjected to mass spectrometry analysis (Table 6). Consistent with previous reports, we identified core spliceosomal components related to U5nsnRNP, PRP19 complex and U2snRNP (Ambrozková et al., 2001; Chen et al., 2011; Sato et al., 2014; Wahl et al., 2009). Moreover, a novel association between SKIIP and the hnRNP protein SYNCRIP was detected. We are currently studing whether association between SKIIP and its identified binding partners is affected upon stress.

Considering the functional connection between MKK6[DD], SKIIP and SYNCRIP in the splicing network (Figure 23), and the physical interaction between SKIIP and SYNCRIP detected by mass spectrometry, we asked whether SYNCRIP could be also involved in GADD45α AS regulation upon stress.

RESULTS

Table 6. Identification of SKIIP associated proteins related to splicing
T7-SKIIP WT was expressed in HeLa cells, and SKIIP associated proteins
were immunoprecipitated with T7 antibody and subjected to mass
spectrometry analysis. Same pull down assay was performed with non
transfected cells as specificity control. The table contains identified SKIIP
associated proteins related to splicing. Number of unique peptide
sequences is indicated for each protein.

Accession	Gene Symbol	Σ# Unique Peptides	Family
O75643	Brr2 (U5-200KD)	51	U5 snRNP
Q15029	Snu114 (EFTUD2)	33	U5 snRNP
Q96DI7	SNRNP40	7	U5 snRNP
O94906	PRPF6 (C20ORF14)	11	U5 snRNP
O43660	PLRG1	9	PRP19 complex
Q99459	CDC5L	28	PRP19 complex
Q9UMS4	PRPF19	22	PRP19 complex
O43172	PRPF4	12	U4/U6 snRNP
Q8WWY3	PRPF31	9	U4/U6 snRNP
Q9Y4Z0	LSM4	3	LSm
Q01081	U2AF35	8	17S U2 snRNP associated
P26368	U2AF65	3	17S U2 snRNP associated
O60506	SYNCRIP (hnRNPQ)	36	Spl. Factor

SYNCRIP was knocked down from HeLa cells using siRNA (Figure
28A) and GADD45α AS was analyzed after activation of p38 by
osmostress treatment. GADD45α^S expression upon stress was
abolished by SYNCRIP depletion (Figure 28B), indicating that its
activity is necessary for p38-mediated GADD45α AS.

In summary, we found that SYNCRIP, a new binding partner for
SKIIP, is involved in p38 mediated GADD45α AS regulation.

A

B
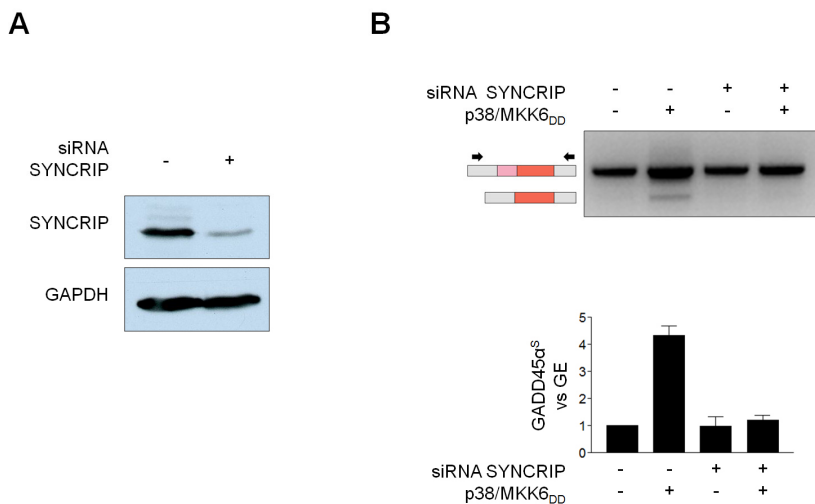
Figure 28. SYNCRIP is required for GADD45α AS regulation by p38
(A) HeLa cells were transfected with control or SYNCRIP siRNA. After 72h, SYNCRIP protein amount was determined by Western blot. (B) MKK6^{DD} and p38 were expressed for 24h in SYNCRIP siRNA transfected HeLa cells. GADD45a AS was evaluated by semi-quantitative RT-PCR and qPCR.

4.2.7 GADD45α^S expression upon stress impairs activation of JNK and p38

GADD45 proteins have been implicated in the control of many cellular responses, including activation of SAPK pathways. To elucidate the biological relevance of GADD45α AS regulation, SAPK dynamics were analyzed in the presence of different GADD45α splicing isoforms. For this purpose, we used a highly sensitive system based on kinase translocation reporters (KTR) established by Regot et al., 2014. The KTR technology is based on constructs that change their nucleocytoplasmic shuttling upon phosphorylation. This event can be followed by fluorescence microscopy and allows quantitative and simultaneous measurements of diverse kinases activities at single cell resolution.
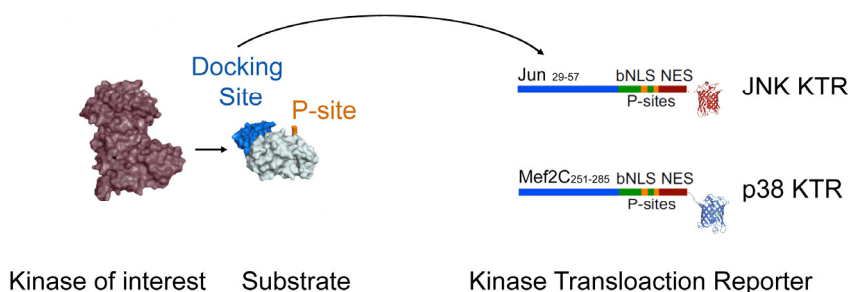
Figure 29. Kinase translocation reporter (KTR) technology
Schematic diagram of KTRs design. KTRs include a docking site and a phosphorylation site for the kinase of interest, a nuclear localization signal (NLS), a nuclear export signal (NES) and a flourochrome. In resting conditions, the reporter localizes in the nucleus. Upon activation, the kinase phsophorylates the KTR leading to its cytoplasm translocation by inhibition of the NLS and the activation of the NES. KTR cytoplasm/nuclear ratio is used as readout of kinase activity. Combination of KTRs fused to p38 KTR-mCerulean and JNK KTR mRuby allows simultaneous activity measurements of both SAPKs (adapted from Regot et al. 2014).

3T3 cell lines stably expressing JNK and p38 KTRs provided by Dr, Markus W. Covert (Figure 29) were transiently transfected either with GADD45α^B or GADD45α^S expressing vectors (Figure 30A). Kinase activities were monitored upon osmostress treatment by fluorescence time-lapse microscopy. Osmostress induced similar activation of JNK in control and GADD45α^B cells, while cells expressing GADD45α^S showed a reduced JNK activation upon NaCl treatment (Figure 30B). The p38 SAPK also presented an altered activation dynamics depending on the expression of different GADD45 isoforms. Of note, cells overexpressing GADD45α^S showed lower p38 induction upon NaCl treatment than control.

A

B
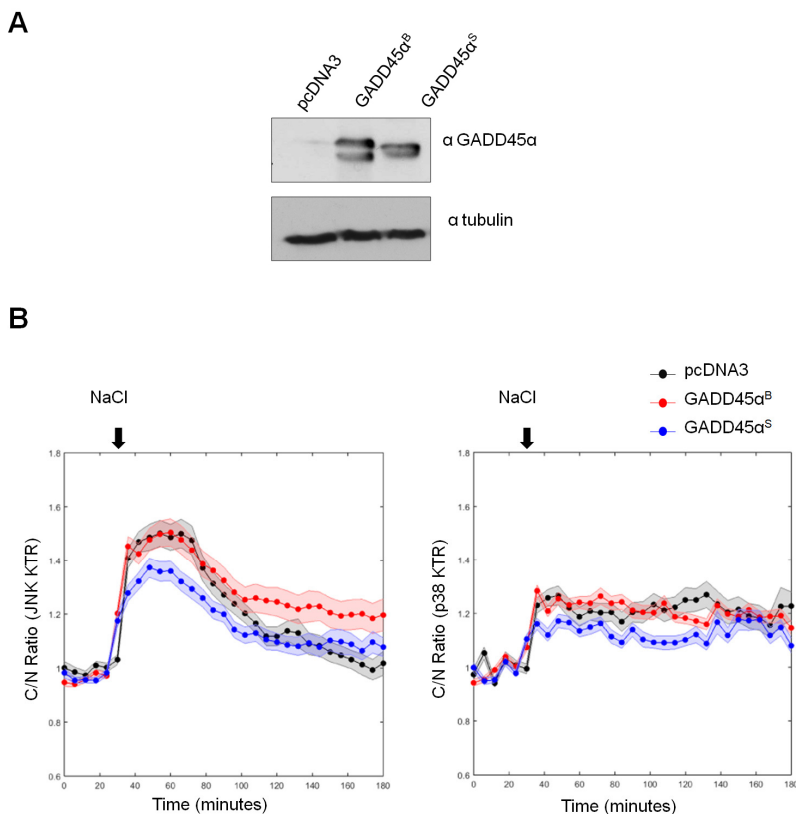
Figure 30. Activation of the JNK and p38 pathways is impaired by expression of GADD45α^S

(A) GADD45α^B and GADD45α^S isoforms were cloned in a pcDNA3 vector and transfected in 3T3 cell lines stably expressing JNK and p38 KTRs obtained from Regot et al., 2014. GADD45 protein expression was assessed by Western blot (B) After GADD45α^B and GADD45α^S transfection, 3T3 cells were stimulated with NaCl 100mM, imaged, and quantified as described in Regot et al 2014. C/N refers to cytoplasmic over nuclear intensities. Data are represented as the mean ± SD from 54, 44 and 53 cells transfected with pcDNA3, GADD45α^B and GADD45α^S respectively.

This suggested that activation of both p38 and JNK pathways are affected by induction of GADD45α^S. Altogether, these results indicate expression of GADD45α stress isoform could be part of a negative feedback regulation.

5. DISCUSSION

5. DISCUSSION

5.1 Impact of p38 activation in alternative splicing upon osmostress

A number of evidences indicate that alternative splicing is regulated in response to a broad range of extracellular signals through activation of different signaling pathways (Lynch, 2007). The aim of this thesis was to investigate the role of p38 in alternative splicing regulation. To this end, we used osmostress as experimental model, which is a strong activator of p38 (Kumar et al 1995). In contrast to other stresses such as heat shock or genotoxic stress, the mechanisms controlling AS in response to osmostress are poorly understood.

The p38 SAPK plays a major role in regulation of gene expression upon osmostress (De Nadal and Posas, 2015), but a general picture defining its impact in modulation of AS profiles remains unknown. Here, we identified a set of AS events that are regulated in response to p38 activation. In addition to identify AS in genes already known to be regulated by p38, such as *FN1* (Al-Ayoubi et al., 2012) and *CD44* (Robbins et al., 2008), we were also able to identify regulation in new genes involved in cell cycle, splicing processing and signaling. Gene expression levels of these genes did not change upon stress, suggesting a role for p38 in the regulation of these processes through modulation of AS.

Generally, our results clearly demonstrate that there is a strong, general modulation of alternative splicing when cells are subjected to osmotic stress. Splicing-sensitive microarray analysis showed that all types of AS events were affected by NaCl treatment, with a

DISCUSSION

higher representation of novel exons. Moreover, during the process of validation of the microarray results, we discovered additional regulation of multiple non-annotated alternative splicing events, suggesting that the response to osmostress activates a highly specific splicing profile. In addition, we did not detect a significant increase in intron retention, suggesting that osmostress induces switches in splice site selection rather than a generalized inhibition of splicing.

We have studied in detail three different AS events regulated by p38, which includes *DYRK1A, MIB1* and *GADD45α*. These examples provide compelling evidence that induction of stress specific splicing-isoforms is directly mediated by p38 activation.

The switch of 5' splice site selection in DYRK1A upon osmostress leads to the inclusion of a premature stop codon (PTC). This truncated isoform (DYRK1A^S) could either be targeted by the NMD pathway or translated into a protein that differs from its canonical counterpart in the carboxyl-terminal tail. The C-terminal region of DYRK1A contains a histidine-rich segment that is necessary for its accumulation in nuclear speckles (Alvarez et al., 2003). Therefore, translation of the isoform lacking the histidine-rich domain is expected to affect localization and activity of the kinase.

p38-dependent splicing regulation of MIB1 also leads to the transcription of a PTC-containing isoform (MIB1^S) and might be degraded by the NMD. MIB1 has been previously reported to be inactivated in a p38-independent manner upon stress (Villumsen et al., 2013), supporting the hypothesis that the change in splicing pattern observed upon p38 activation could lead to the degradation of the transcript.

Finally, we also described two p38-dependent AS events in the GADD45α gene. GADD45α^S encodes a protein isoform with a 34 amino acids deletion, while Gadd45α^{S2} transcript contains a PTC that might lead to its degradation. The biological function of GADD45α^S expression will be further discussed below.

Altogether, our results suggest a role for p38 in the regulation of diverse physiological processes through direct regulation of alternative splicing.

5.2 Mechanisms by which p38 modulates alternative splicing

Environmental insults alter splicing decisions through multiple molecular mechanisms, including post-transcriptional modifications of splicing factors that affect their cellular localization or their interactions, regulation of the expression of spliceosome components and modulation of splicing factors that mediate the coupling between splicing and transcription (Dutertre et al., 2011).

Phosphorylation represents an important regulatory process for both core components and auxiliary splicing factors (Naro and Sette, 2013). Splicing factors that are targeted by p38 have been previously identified. For example, activation of the p38 pathway induces hnRNP A1 phosphorylation and export from the nucleus into the cytoplasm (van der Houven van Oordt et al., 2000). However, the general effects of this regulation on AS modulation remain unknown.

In this thesis, we addressed the question of how p38 regulates splicing through a functional approach. We hypothesized that correlating AS changes induced by depletion of splicing factors and

changes induced by p38 activation could help us to unravel which proteins mediate p38 effects on AS. To this purpose, we studied the effects of the activation of p38 in the functional splicing network described by Papasaikas et al., 2015. Activation of the p38 pathway by two different means, osmostress and expression of an activated MKK6 protein, revealed two new connections between p38 and the splicing machinery through the U2snRNP complex and the Prp19-related protein SKIIP.

5.2.1 The function of the U2snRNP complex upon stress

Network analysis resulted in a strong association between osmostress and U2snRNP components. It was previously shown that limited downregulation of different spliceosome core components did not trigger a general effect of splicing inhibition but specifically modulates different AS changes (Papasaikas et al., 2015). Therefore the similar AS pattern between osmostress and the knockdown of U2snRNP associated proteins suggest that the U2snRNP function could be affected upon stress.

In vitro kinase assays showed that p38α phosphorylates SF3B1, an essential subunit of the U2snRNP. SF3B1 is tightly controlled by phosphorylation during spliceosome activation (Agafonov et al., 2011; Bessonov et al., 2010; Wang et al., 1998), and the phosphosites involved in this reaction are mapped at the N-terminal region of the protein (Girard et al., 2012). It is worth noting that all the p38-phosphosites in SF3B1 differ from the ones previously identified in the spliceosome B active complex, suggesting a regulatory mechanism independent of the catalytic activation of the spliceosome. In addition, the T244 phosphorylation site targeted by p38 is necessary for the interaction of SF3B1 with the nuclear

inhibitor of protein phosphatase 1 (NIPP1) (Boudrez et al., 2002), which mediates the interaction between SF3B1 and the protein phosphatase-1 (PP1)(Tanuma et al., 2008).

Combining observation of the *in vitro* kinase assays and analysis of splicing pattern of GADD45α, MIB1 and DYRK1A upon SF3B1 knockdown, we suggest diverse mechanisms upon which SF3B1 could mediate osmotress-induced splicing changes.

On one hand, our experiments show that SF3B1 knockdown and osmostress influenced the splicing pattern of GADD45α and MIB1 in the same way. SF3B1 mediates the recruitment of U2snRNP to the branch point adenosine by interacting with U2AF and with the intronic RNA on both sides of the BP (Gozani et al., 1998). The SF3B1 N-terminal region interacts with U2AF65 (Gozani et al., 1998) and p14 (Will et al., 2001). Hence, we hypothesize that p38 phosphorylation of SF3B1 could interfere with the association of these proteins promoting differential recognition of GADD45 and MIB1 alternative 3' splice sites.

On the other hand, SF3B1 knockdown prevented the stress-induced regulation of DYRK1A exon 10 5'ss. SF3B1 regulates Bcl-x exon 2 5'ss selection through direct binding to a RNA motif within exon 2 (Massiello et al., 2006), and this regulation is dependent on PP1 activity. Thus, it is formally possible that the phosphorylation of SF3B1 by p38 could alter its *trans*-acting activity. Moreover, it would be interesting to explore whether SF3B1 phosphorylation by p38 could affect 5'ss selection through modulation of SF3B1 binding to NIPP1.

However, further investigations are needed to prove that phosphorylation of SF3B1 mediates p38 dependent AS regulation.

DISCUSSION

We are currently testing whether the mutation of p38-phosphosites has a direct effect in the regulation of GADD45α, MIB1 and DYRK1A AS upon osmostress. Moreover, genome wide analysis should allow further characterization of the splice sites modulated by osmostress through SF3B1.

5.2.2 The role of SKIIP in p38-mediated alternative splicing

Another important observation that emerged from the splicing network analysis was the connection between p38 and SKIIP, a spliceosomal component related to the Prp19C.

Our results demonstrate that SKIIP is essential for p38-dependent skipping of GADD45α exon 2 upon stress. SKIIP was already known to be required for intron removal of a set of introns in cell cycle related genes encoding for sororin (Lelij et al., 2014) or the cyclin-dependent kinase inhibitor p21 (Chen et al., 2011). However, a direct role in regulation of alternative splicing has not yet been demonstrated in mammals. Our data suggest that SKIIP, in addition to its well defined role in constitutive splicing, might also be required for the regulation of alternative splicing regulation of a subset of the p38-dependent events upon osmostress.

In vitro kinase assays showed that p38α phosphorylates SKIIP. Moreover, we found that SKIIP and p38 interact *in vivo*, suggesting that SKIIP is a *bona fide* p38 target. Mutagenesis analyses revealed that p38α phosphorylates SKIIP at T180, S224 and S232 phosphosites, which are located within the SNW1 domain of the protein. This domain contains the highly conserved motif SNW1KD, and it is essential for interaction with several transcriptional

regulators and the splicing factors Brr2 and Snu114 (Folk et al., 2004; Sato et al., 2014).

Remarkably, mutation of the three p38 phosphosites of SKIIP completely abolished expression of the Gadd45α^S isoform upon stress, suggesting that SKIIP phosphorylation is essential for the regulation of a set of p38-dependent AS events. We hypothesize that SKIIP phosphorylation could modulate its association to spliceosomal components, favoring selection of specific splice sites. Thus, SKIIP phosphorylation upon stress could either favor recognition of the 3' splice site of exon 3 or inhibit recognition of exon 2 of GADD45α.

Considering that the SNW1 domain of SKIIP binds both Brr2 and Snu114, we assessed whether these associations were altered upon stress depending on SKIIP phosphorylation. Co-immunoprecipitation experiments proved that neither Brr2 nor Snu114 altered their association of SKIIP after phosphorylation at T180, S224 and S232.

It is worth noting that the splicing network analysis also indicated that SYNCRIP (alternatively known as hnRNPQ1) was connected to p38 activation and was also involved in GADD45α AS regulation upon stress. In agreement with the functional connection between SKIIP and SYNCRIP in the splicing network, our mass spectrometry experiments demonstrated a physical association between these two proteins. SYNCRIP contains three RNA recognition motifs (RRMs) in its central region (Mourelatos et al., 2001), and it is known to be involved in the regulation of alternative splicing through direct binding to the SMN pre-mRNA (Chen et al., 2008). Therefore, it would be very interesting to explore whether

SYNCRIP is recruited to GADD45α RNA to regulate its AS and whether this association is modulated by SKIIP phosphorylation by p38.

Collectively, splicing network analysis uncovered two novel connections between p38 activation and spliceosomal components. The fact that the two treatments led to the identification of different target complexes could be the result of their diverse strength and kinetics of p38 activation. Osmostress leads to a strong and fast activation of p38, while expression of the constitutive active MKK6 triggers a weaker but sustained activation of the p38 kinase. Therefore, it could be either possible that the SAPK targets several components of the spliceosome upon stress, or alternatively that depending on the nature and the intensity of the stress conditions, independent mechanisms acting on U2snRNP and SKIIP could be activated.

Of note, since SF3B1 plays a key function in regulation of genes important for cancer progression and SKIIP has been reported to specifically regulate genes involved in apoptosis, both proteins are now considered promising targets for antitumor drugs (Bonnal et al., 2012; Sato et al., 2014). Therefore, the implication of p38 in the regulation of these spliceosomal components in splicing deserves further exploration.

5.3 The induction of the stress-responsive GADD45α isoform modulates SAPK signaling

GADD45α regulates many cellular functions such as cell cycle control, apoptosis and stress signaling (Tamura et al., 2012). Previous studies showed that expression of the GADD45α protein isoform lacking exon 2 (Gadd45α^S) differentially regulates cell

proliferation and cell cycle transition in response to arsenic treatment (Zhang et al., 2009). However, the role of the GADD45α[S] isoform in additional biological processes was not known.

Several studies reported that activation of signaling pathways can be modulated through alternative splicing changes on genes encoding signaling proteins. For instance, alternative splicing regulation of Mnk2 transcripts influences the p38 SAPK pathway (Maimon et al., 2014), and expression of different isoforms of MKK7 modulate the JNK signaling response (Martinez et al., 2015). In response to environmental stresses, GADD45 proteins directly interact with the upstream kinase MTK1, inducing apoptosis through the p38/JNK pathways (Takekawa and Saito, 1998). Thus, we investigated whether expression of Gadd45α[S] upon osmostress could be involved in regulation of SAPK signaling. Analysis of activation of p38 and JNK pathways in the presence of Gadd45α[S] indicate that this isoform has a negative regulatory effect on the activation of both pathways.

The interaction of GADD45 proteins with the upstream kinase MTK1 occurs in its N-terminus (Takekawa and Saito, 1998). The lack of 34 aminoacids in the central region of the protein resulting from exon 2 skipping disrupts the interaction domains required for the association of GADD45 with MTK1. The reduced activation of p38 and JNK in cells expressing GADD45α[S] suggests that this isoform could be acting as a negative feed-back loop to down-regulate the activity of the SAPKs signaling pathways. Further experiments are needed to understand the physiological implications of this regulation. A possible role of a negative regulation of SAPK signaling pathways through GADD45α AS could be to control apoptosis induction in response to different

kinds of stress. In fact, we have observed that GADD45α^S is preferentially expressed under low concentrations of NaCl. These observations suggest that GADD45α^S could have an anti-apoptotic function through restriction of SAPK activation in situations of mild stress.

In summary, the results of this study unraveled a new role of p38 in stress-response through regulation of alternative splicing. This provides new evidence on how extracellular stimuli can be converted into transcriptome changes. p38-mediated splicing regulation can lead to rapid and specific gene expression changes that might provide an advantage for cell response to stress and maintenance of cellular homeostasis.

6. CONCLUSIONS

6. CONCLUSIONS

1. Osmostress induces specific changes in alternative splicing.

2. The p38 SAPK is involved in modulation of a set of alternative splicing events upon stress.

3. Network analysis reveals functional connections between p38 and the spliceosomal components SF3B1 and SKIIP.

4. Regulation of p38-dependent alternative splicing upon osmostress is altered by a loss of function of SF3B1, which is phosphorylated by p38.

5. SKIIP and SYNCRIP are essential for p38-dependent GADD45α alternative splicing regulation in stress conditions.

6. SKIIP is directly phosphorylated by p38 at T180, S224 and S232, and phosphorylation at these sites is necessary for GADD45α alternative splicing regulation upon stress.

7. Stress induced GADD45α protein isoform impairs activation of JNK and p38 upon stress.

7. REFERENCES

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