# MAPKinase signaling and AP-1-regulated gene expression in cellular responses to DNA damage

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# Chapter I

## General Introduction

## DNA damage responses, MAPKinases and AP-1 transcription factors

The DNA of human cells is under constant attack by agents that can either directly damage one of its six billion bases or break the phosphodiester backbone on which the bases reside. For example, ionizing radiation (IR) induces double strand DNA breaks (Ataian and Krebs, 2006) and ultraviolet (UV) light induces cyclobutane-pyrimidine dimers and 6-4 photoproducts (Ataian and Krebs, 2006). Moreover, as a consequence of normal cellular metabolism reactive oxygen species (ROS) arise or can be created, which can cause both base damage and DNA breakage (Ataian and Krebs, 2006). To cope with this constant attack on their DNA and to overcome the different types of DNA lesions, organisms have developed a variety of different repair mechanisms. To enable cells to repair their DNA before DNA synthesis occurs or continues, mechanisms have evolved to halt cell-cycle progression in response to genotoxic stress. Finally, mechanisms exist to undergo programmed cell death if the damage is too severe. The cellular response to genotoxic stress is a very complex process. Damaged DNA has to be detected by sensors, which subsequently have to initiate a series of events including activation or inhibition of signal transduction pathways and transcription factors. The activated transcription factors induce expression of various types of genes, involved in DNA repair, cell cycle arrest and/or cell death, cytokine release and other recovery processes. Although we have a limited understanding of how the processes of cell-cycle arrest or apoptosis are coordinated with the process of DNA repair, such coordination must take place to optimize the outcome for the cell or the organism, e.g. protect the organism against the accumulation of undesirable mutations.

The purpose of this introduction is to give an overview of proteins and signaling pathways involved in the cellular response to genotoxic stress. The focus will be, in particular, on the MAPK pathways and the AP-1 family of transcription factors.

**DNA-repair pathways**

Cells have developed multiple specialized DNA-repair pathways to remove DNA lesions. Homologous recombination (HR) or non-homologous end rejoining (NHEJ) remove double strand DNA breaks (DSBs), e.g. caused by ionizing radiation (IR). Proteins involved in HR include amongst others Rad51, Rad52, Rad54, RPA, ATM and the MRN complex (MRE11-Rad50-Nbs1). Proteins required for NHEJ include for instance the DNA-dependent protein kinase (DNA-PK), XRCC4, and DNA ligase IV (reviewed by Lees-Miller and Meek, 2003; Shiloh, 2003; Nordstrand et al., 2007). Genetic disorders associated with these repair pathways include Ataxia-telangiectasia (A-T), Nijmegen breakage syndrome (NBS), and Ataxia-telangiectasia-like disease (ATLD). Ataxia-telangiectasia, caused by ATM deficiency, is characterized by cerebellar degeneration, which leads to severe, progressive neuromotor dysfunction, immunodeficiency, genomic instability, thymic and gonadal atrophy, predisposition to lymphoreticular malignancies and extreme sensitivity to ionizing radiation and DSB-inducing agents (Shiloh, 2003). Nijmegen breakage syndrome (NBS) is the result of mutations in Nbs1, which is an ATM substrate (see below). The NBS phenotype shows significant overlap with that of A-T with the important exception that NBS patients do not have cerebellar degeneration (Shiloh, 2003). A-T-like disease (ATLD), caused by defect in MRE11, resembles the A-T phenotype more closely, although the onset in ATLD patients is at later age and with slower progression (Shiloh, 2003).

Nucleotide excision repair (NER) removes lesions that distort the DNA double helix, interfere in base pairing and block DNA duplication and transcription. The most common examples of these lesions are the cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), the two major kinds of injuries induced by UV light (reviewed by de Boer and Hoeijmakers, 2000). The genetic disorders xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) are all associated with defects in nucleotide excision repair (reviewed by de Boer and Hoeijmakers, 2000). The clinical features of these syndromes differ considerably, varying from high cancer predisposition to aging symptoms. In short, CS and TTD patients do not show an elevated risk for skin cancers, whereas XP patients have a 1000-fold increased risk for skin cancers, and cells isolated from XP patients are more sensitive for UV-irradiation than wild type cells. The XP, CS and TTD syndromes are genetically complex, with

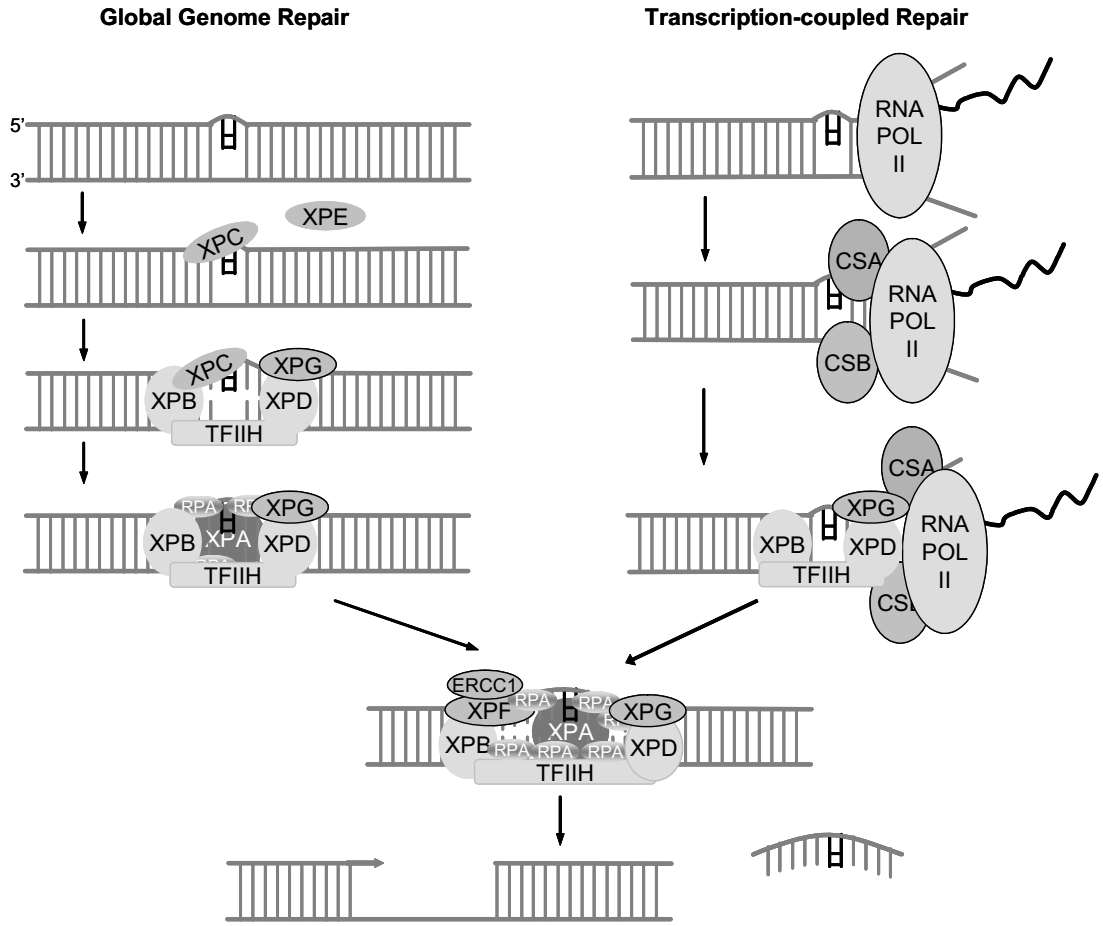


**Figure 1.** The nucleotide excision repair (NER) pathways: global genome repair (GGR; left panel) and transcription-coupled repair (TCR; right panel). DNA damage occurring anywhere in the genome can be repaired by the GGR pathway, but DNA lesions that occur on the transcribed strand of an active gene and block RNA polymerase II progression (right panel) can also be repaired by the TCR pathway. In the GGR pathway DNA lesions are detected by the protein XPC. For some types of damage (e.g. UV photoproducts), the XPE protein may assist. Subsequently, the basal transcription factor TFIIH, which includes the two DNA helicases XPB and XPD is recruited. The endonuclease XPG is recruited simultaneously, or shortly there after. During NER replication protein A (RPA) binds to the single-strand DNA to stabilise the open complex once the DNA is unwound around the site of the lesion and facilitates XPA binding. XPA replaces XPC, is believed to verify the presence of a lesion in an open DNA conformation and is crucial for the assembly of the repair machinery. Finally, the endonuclease ERCC1-XPF is recruited. Dual incisions are made in one strand flanking the lesion, the damaged oligonucleotide is released and the gap filled via template-dependent DNA polymerisation by the replication machinery. In the case of TCR, the site of DNA damage is identified when the lesion causes RNA polymerase II stalling during transcription. Such blocks trigger the recruitment of CSA and CSB, and subsequently TFIIH (XPB and XPD) and XPG. This protein complex is thought to play a role in unwinding the DNA around the lesion and in removing the stalled RNA polymerase to allow repair of the lesion (Adapted from Mitchell et al., 2003).

at least seven complementation groups for XP (XPA to XPG), two for CS (CSA and CSB), and three for TTD (XPB, XPD, and TTD-A). The products of the XP genes are proteins involved in the different steps of NER, and comprise three damage-recognition proteins (XPA, XPC and XPE), two helicases (XPB and XPD), and two nucleases (XPF and XPG). The two helicases, XPB and XPD, are components of the basal transcription factor TFIIH, which has a dual role in NER and initiation of transcription (de Laat et al., 1999; Liu et al., 2005; Bartels and Lambert, 2007; Park and Choi, 2006; Thoma and Vasquez, 2003; Wijnhoven et al., 2007). Two types of NER can be distinguished: the repair of DNA damage over the entire genome, referred as global genome repair (GGR), and repair of DNA lesions in transcribed

strands of active genes, known as transcription coupled repair (TCR) (Figure 1) (reviewed by de Laat et al., 1999; Wijnhoven et al., 2007). GGR depends on recognition of the damage by the XPC protein (Sugasawa et al., 1998). The XPC protein recognizes helical distortions throughout the genome and is the first NER protein localized to the site of damage (Figure 1). In addition, the XPE protein may facilitate some specific subset of damage recognition (Tang et al., 2000). In contrast to GGR, TCR is initiated by the stalling of RNA polymerase II at a lesion on the transcribed strand of an active gene (Wijnhoven et al., 2007). RNA polymerase II stalling recruits the CSA and CSB proteins (Figure 1). CSA and CSB, together with the core NER components TFIIH (XPB and XPD) and XPG, are thought to play a role in removing the stalled RNA polymerase from the lesion and in recruiting the DNA repair machinery (van den Boom et al., 2002). Damage recognition by either mechanism initiates the subsequent stepwise assembly of the core NER factors (Mitchell et al., 2003). XPA verifies the damaged DNA in an open DNA conformation and is crucial in the assembly of the repair machinery. The NER components XPB, XPD, XPA, XPG, and XPF thus lie downstream of the recognition events specific for GGR or TCR and are shared by both NER pathways (Mitchell et al., 2003; Figure1).

**DNA damage sensor kinases ATM and ATR**

Cells respond to DNA damage by activating a complex network of checkpoint pathways. These pathways delay their cell-cycle progression to facilitate DNA repair and, if necessary, eliminate the hazardous damaged cells through induction of cell death. Both cell cycle checkpoints and DNA repair help to avoid accumulation of oncogenic mutations and play crucial protective roles in multi-step carcinogenesis. The sensoring checkpoint network must not only sense the damage, but also activate downstream cellular effector proteins. Activation of the phosphatidylinositol 3-kinase (PI3K) related kinases, ATM, ATR, and DNA-PK is the first step characterized to date in DNA-damage-induced signaling. Activation of these kinases can trigger cell-cycle arrest in G1, S or G2 phases, DNA repair and cell death (Bartek and Lukas 2007). ATM and DNA-PK seem to respond mainly to DSBs, whereas ATR also reacts to single-strand DNA and stalled DNA replication forks (Shiloh, 2003). Activation of the PI3K-related kinases involves their recruitment to DNA lesions through DNA-damage binding complexes containing Nbs1, ATRIP or Ku80 proteins. These proteins are required for direct interaction of respectively ATM, ATR, and DNA-PK with damaged DNA (Falck et al., 2005; You et al., 2005).

DSBs are recognized by the Mre11-Rad50-Nbs1 (MRN) complex (Petrini and Stracker, 2003). Rad50 is involved in DNA damage recognition and Nbs1 is required for recruitment of ATM to DSBs (Bartek and Lukas, 2007). The recruited ATM subsequently phosphorylates the C-terminal tail of histone H2AX, which in turn serves as a docking platform for the adaptor/mediator protein Mdc1. The binding of Mdc1 to phosphorylated H2AX and interaction with the Nbs1 subunit of the MRN complex allows a gradual spreading of H2AX phosphorylation (Bartek and Lukas, 2007). The spreading of phosphorylated H2AX enables additional proteins to interact with the DSB-flanking chromatin, such as 53BP1 and BRCA1 (Bekker-Jensen et al., 2005). The accumulation of ATM is important to boost phosphorylation of ATM targets: besides H2AX, ATM phosphorylates and activates a large group of proteins involved in cell cycle control, apoptosis and/or DNA repair, e.g. BRCA1, Nbs1, Chk1, Chk2, p53, MDM2 and SMC1 (Shiloh, 2003; Figure 2 and next section).

ATM and the MRN complex also play a role in DSB resection and formation of single-strand DNA, processes which allow DNA repair by HR and ATR-dependent signaling. DSB resection requires cyclin-dependent kinase (CDK) activity and is restricted to the S and G2 phase of the cell-cycle. After DSB resection, the single-strand DNA is coated and stabilized by replication protein A (RPA) (Bartek and Lukas, 2007). Coating of single-strand DNA with RPA also occurs in other situations. Zou and Elledge (2003) suggest a model in which any stimulus or stress that results in an abnormal stretch of single-strand DNA – such as an arrested replication fork after UV irradiation – triggers RPA coating of DNA.

During NER RPA binds to single-strand DNA and facilitates XPA binding (Park and Choi, 2006; Figure 1). RPA also facilitates the recruitment of the ATR-ATRIP complex (Zou and Elledge, 2003). Full activation of the ATR kinase requires TopBP1, a protein that is recruited to the single-strand DNA independent of the ATR-ATRIP complex (Kumagai et al., 2006). At this point ATR is competent to phosphorylate most of its downstream targets. ATR targets include p53, BRCA1, Rad17, Chk1 and Chk2 (Zhou and Elledge, 2000; Zou and Elledge 2003; Roos and Kaina, 2006; Figure 2). The Mitogen-Activated Protein Kinase (MAPK) pathway (see below) is also activated by ATM/ATR in response to genotoxic stress (Yang et al., 2004). It is, however, still not exactly clear how ATM/ATR activates MAPKs.

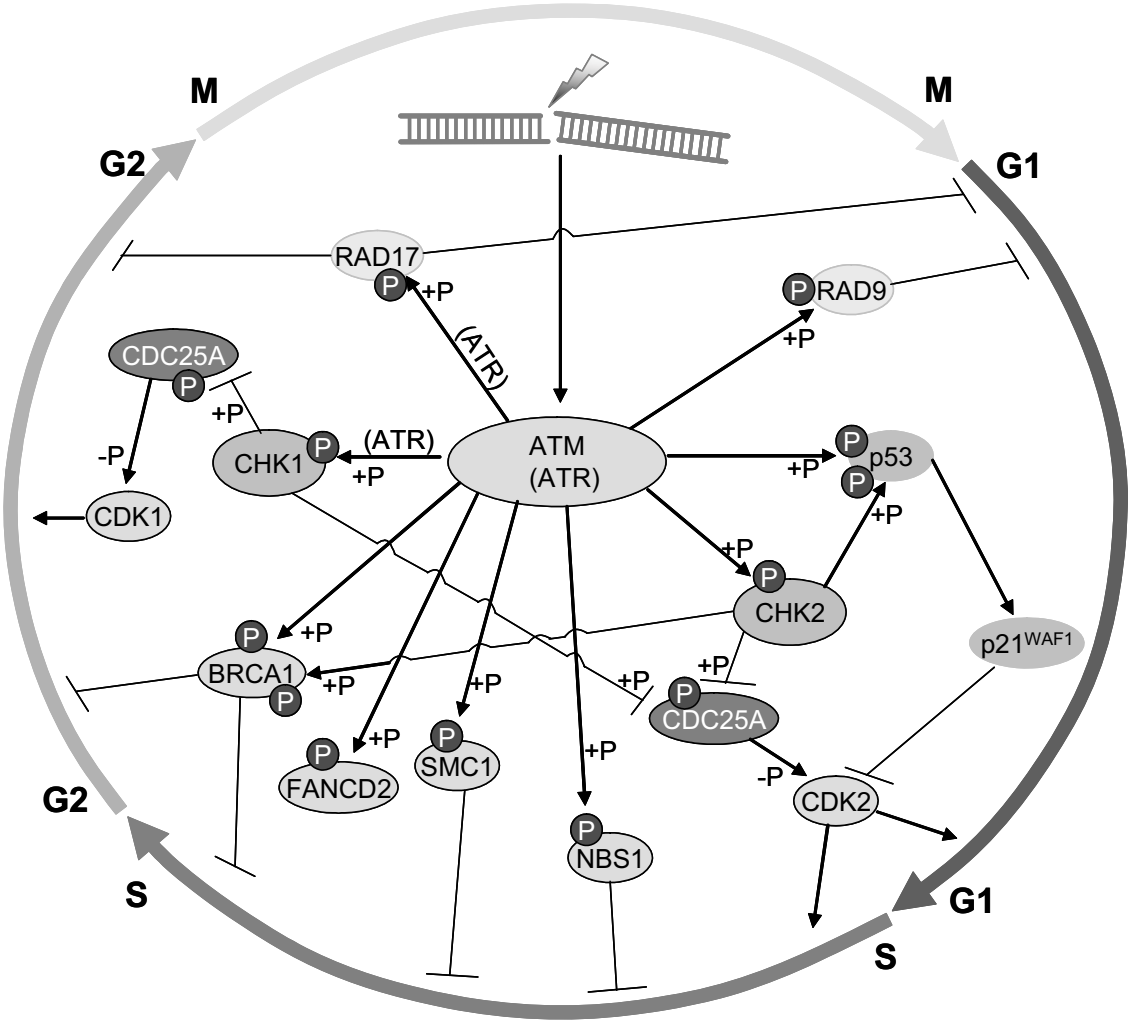


**Figure 2.** ATM/ATR-mediated activation of cell-cycle checkpoints in response to DNA double strand breaks. Arrows indicate stimulation; T-shaped lines mark inhibition. Phosphorylations are marked by '+P' and dephosphorylations by '-P'. Most of these pathways are regulated primarily by ATM, particularly in the early stage of the DNA-damage response, with ATR probably becoming important at later stages to maintain activation of these pathways. However, the pathways activated via RAD17 and CHK1 phosphorylation are primarily under ATR control. In some cases, the mechanism by which the cell-cycle machine is disturbed is not clear, but ATM-mediated phosphorylation of the indicated proteins is important for these processes (Adapted from Shiloh, 2003).

**ATR/ATM, Chk1 and Chk2 in DNA damage-induced cell cycle arrest and apoptosis**

The genetically most vulnerable period of the cell cycle, the DNA-synthesis (S) phase, is protected against DNA damage by four checkpoints: the replication-independent G1/S checkpoint, the replication-independent intra-S-phase checkpoint, the replication-dependent S-phase checkpoint, and the replication-dependent G2/M checkpoint (also known as S/M checkpoint), which prevents mitotic entry when DNA is incompletely replicated (Figure 2) (Kastan and Bartek 2004). After ionizing radiation (IR), ATR and ATM each contribute to early delay in M-phase entry but ATR regulates a majority of the late phase (2-9 h post-IR). Double deletion of ATR and ATM eliminates nearly all IR-induced delay, indicating that ATR and ATM cooperate in the IR-induced G2/M-phase checkpoint (Brown and Baltimore, 2003). Recent studies showed that ATM regulates ATR recruitment and Chk1 activation only in the S and G2 phases of the cell cycle upon IR (Jazayeri et al, 2006).

ATR and Chk1 also mediate the responses induced by stalled DNA replication and, in contrast to ATM and Chk2, are essential for early embryonic development and cell viability in mice, apparently because they also maintain chromosomal integrity in S phase under non-stressed conditions. The S phase or replication checkpoint controlled by ATR and Chk1 monitors the progress of DNA replication forks, and delays the firing of later replication origins when active replication forks are stalled due to collisions with damaged or abnormally structured DNA (Syljuasen et al., 2005; Zhang et al 2006). In $ATR^{+/-}$ mice a small decrease in survival and increase in tumor incidence was observed (Brown and Baltimore, 2000).

To elicit G1, S-phase and G2–M cell-cycle blocks Chk1 and Chk2 phosphorylate amongst others p53 and cdc25A, which can inhibit Cdk1- and Cdk2-dependent cell cycle progression (Figure 2). Via p53, E2F1 and PML Chk2 can also activate apoptosis. Other potential functions of Chk1 include replication fork stabilization, chromatin remodelling and DNA (recombination) repair (Kastan and Bartek, 2004). Chk1 is haploinsufficient in embryonic mouse cells and conditional Chk1 heterozygosity resulted in three distinct haploinsufficient phenotypes that can contribute to tumorigenesis: inappropriate S phase entry, accumulation of DNA damage during replication, and failure to restrain mitotic entry (Lam et al., 2004). $Chk1^{-/-}$ somatic avian DT40 B-lymphoma cells show slightly enhanced levels of spontaneous apoptosis but are viable. However, these cells fail to arrest in G2/M and are hypersensitive to killing by ionizing radiation and, in addition, fail to maintain viable replication forks when DNA polymerase is inhibited (Zachos et al., 2003). Recent studies suggest that Chk1 phosphorylation occurs at localized sites of DNA damage, that phosphorylated Chk1 does not stably associate with chromatin and that constitutive immobilization of Chk1 on chromatin results in a defective DNA-damage-induced checkpoint arrest (Smits et al., 2006).

**Mitogen-Activated Protein Kinase (MAPK) pathways**

Signal transduction pathways in eukaryotic cells integrate input from distinct types of extracellular stimuli, including growth factors and cytokines, as well as environmental factors such as ionizing radiation, UV light and chemical mutagens. Signaling pathways thereby regulate complex biological responses such as cell proliferation, differentiation and cell death. An important group of signal transducing molecules are the MAPKs. MAPKs regulate diverse cellular activities, e.g. gene expression, cell cycle progression, and metabolism, but also motility, survival and apoptosis, and differentiation (Chang and Karin, 2001; Schaeffer and Weber, 1999). MAPKs are activated by a protein kinase cascade (Figure 3): the MAPK is activated through phosphorylation by a MAPK Kinase (MAPKK), which is itself activated by phosphorylation by a MAPKK Kinase (MAPKKK) (Schaeffer & Weber 1999). Three distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 kinases (Schaeffer & Weber 1999; Figure 3).

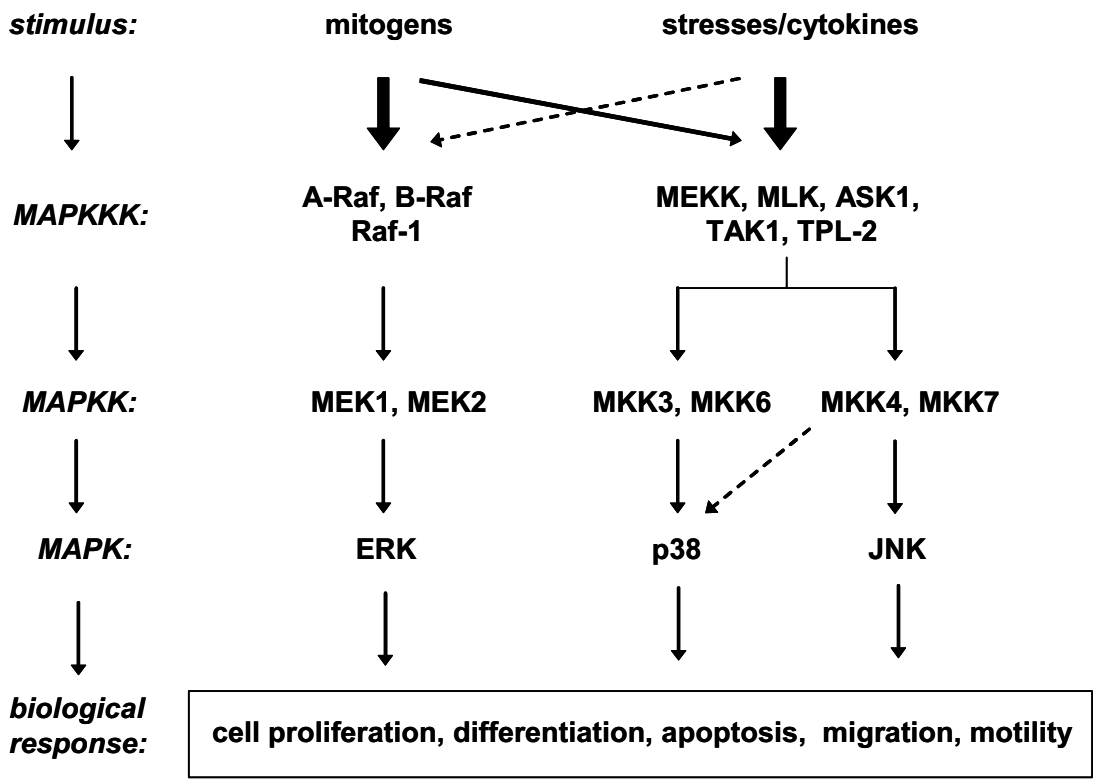


**Figure 3.** Schematic representation of the mammalian MAP kinase (MAPK) signal transduction pathways with its three types of MAPKs: extracellular signal regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38. MAPKs are activated by dual phosphorylation on Thr and Tyr by members of the MAPK kinase (MAPKK) group of protein kinases, of which under normal conditions MEK1 and MEK2 activate the ERK family members, MKK4 and MKK7 activate the JNK family, and MKK3 and MKK6 activate the p38 family. The MAPKK are activated by phosphorylation by the indicated MAPKK kinases (MAPKKKs). For further details, see text.

**The ERK signaling cascade**

The ERK cascade plays an important role in the transmission of proliferation and differentiation signals involved in normal development and tumorigenesis. This cascade consists of Raf (MAPKKK), MEK (MAPKK) and ERK (MAPK) kinases (Figure 3) (Davis, 2000; Shaulian and Karin, 2001). In mammalian cells, there are three Raf proteins (A-Raf, B-Raf, and Raf-1 (C-Raf)), two MEK proteins (MEK1 and MEK2), and 8 ERK family members, of which ERK1 and ERK2 are studied most intensively (for review see Bogoyevitch and Court 2004). In most cell types ERK 1 and 2 are strongly activated by growth factors and phorbol esters and to a (much) lesser extent by heterotrimeric G protein-coupled receptors, cytokines, DNA damaging agents and osmotic stress, and microtubule disorganization (Lewis et al., 1998). Upon activation ERK translocates to the nucleus where it phosphorylates and activates numerous substrates, including AP-1 transcription factors and regulators of the AP-1 transcription factors (see below) (Chen et al., 1992; Shaulian and Karin, 2001).

**The JNK and p38 signaling cascades**

The JNK and p38 MAPKs and their upstream MAPKKs and MAPKKKs play important roles in cell proliferation, cell survival and differentiation and in DNA damage responses. The JNK family consists of JNK1, JNK2, and JNK3, the latter of which is mainly expressed in neuronal tissues. The p38 family consists of p38-$\alpha$, $\beta$, $\gamma$, and $\delta$. JNK is activated by the MAPKKs MKK4 and MKK7, whereas

p38 MAPK is activated by MKK3, MKK4, and MKK6 (Davis, 2000; Shaulian and Karin, 2001). The MAPKKKs upstream of MKK3-7 include TAK1 and members of the ASK1, MEKK, and MLK families (Davis, 2000) (Figure 3). The JNK and p38 cascades are activated in response to various physical and chemical stresses, such as oxidative stress, UV irradiation, hypoxia, ischemia, and various cytokines including interleukin-1 and tumor necrosis factor alpha (Kyriakis and Avruch, 2001; Davis, 2000). JNK and p38 are also activated in response to growth factor deprivation, other DNA-damaging agents, and, to a lesser extent, some G protein-coupled receptors, serum, and growth factors (Kyriakis and Avruch, 2001; Davis, 2000). Like ERK, JNK and p38 translocate from the cytoplasm to the nucleus following stimulation (Turjanski et al., 2007; Baan et al., 2006).

**Mechanisms for MAPK signal specificity and efficiency**

Scaffold proteins organize MAPKs and their upstream activators into specific cascade modules, which are assumed to increase the specificity of MAPK activation and function (Figure 4). The scaffold protein JIP1 organizes JNK1/2, MKK7 and the MAPKKK MLK1 into a specific signaling cassette (Yasuda et al., 1999). MP1, another scaffold protein, interacts with ERK1 and MEK1, thereby potentiating ERK activation (Schaeffer et al., 1998). Numerous other scaffold proteins have been described (see for review Morrison and Davis, 2003; Dard and Peter, 2006). Sequential physical interactions between members of a given cascade also ensure specific MAPK activation (Figure 4). JNK1/2 is bound by the N-terminal extension of MKK4, which also interacts with the catalytic domain of MEKK1. Each interaction is disrupted upon activation of the downstream kinase (Xia et al., 1998). Once phosphorylated by MAPKKs, MAPKs show enhanced kinase activity for their substrates. All MAPKs recognize similar phosphorylation sites composed of serine or threonine followed by a proline, and the amino sides that surround these sites further increase the specificity of recognition by the catalytic pocket of the MAPK (Tanoue et al., 2000).

Another mechanism for maintaining signal specificity and efficiency is the interaction of MAPKs with their substrates and regulators through high-affinity docking sites (Figure 4). In the case of c-Jun, a short sequence that precedes the JNK phosphorylation sites is recognized by an interaction surface on JNK, outside its catalytic pocket (Kallunki et al., 1996). Similar interactions involving distinct phosphorylation and docking sites determine substrate recognition by other MAPKs (Tanoue et al., 2000).

Importantly, the duration and magnitude of the MAPK activation is essential for the final outcome (Marshall, 1995; Wada and Penninger, 2004; Chambard et al., 2007; Owens and Keyse, 2007). For instance, hyperactivation of a pathway may produce lethal effects. Therefore, molecular mechanisms exist that ensure an accurate intensity of signaling and a precise moment of activation. As phosphorylation of both the threonine and the tyrosine within the activation loop is necessary for MAPK activity, dephosphorylation of either is sufficient for inactivation. In the cell, inactivation of MAPKs by dephosphorylation of (one of) these residues can be established by a family of protein phosphatases, the MAPK phosphatases (MKPs) (Farooq and Zhou, 2003; Saxena and Mustelin, 2000; Figure 4). As multiple MKPs are transcriptional targets of the MAPKs, MKPs are part of a negative feedback mechanism. MKPs are divided into tyrosine-specific phosphatases, serine/threonine-specific protein phosphatases, and the dual-specificity (threonine/tyrosine) protein phosphatases (Farooq and Zhou, 2003; Saxena and Mustelin, 2000). Work using a variety of model organisms has demonstrated that all three groups of protein phosphatases can regulate MAPKs in vivo (Keyse, 2000; Saxena and Mustelin, 2000). However, the largest group dedicated to MAPK regulation are the dual-specificity MKPs. At this moment 13 dual-specific MKPs are described (for review Farooq and Zhou, 2003).

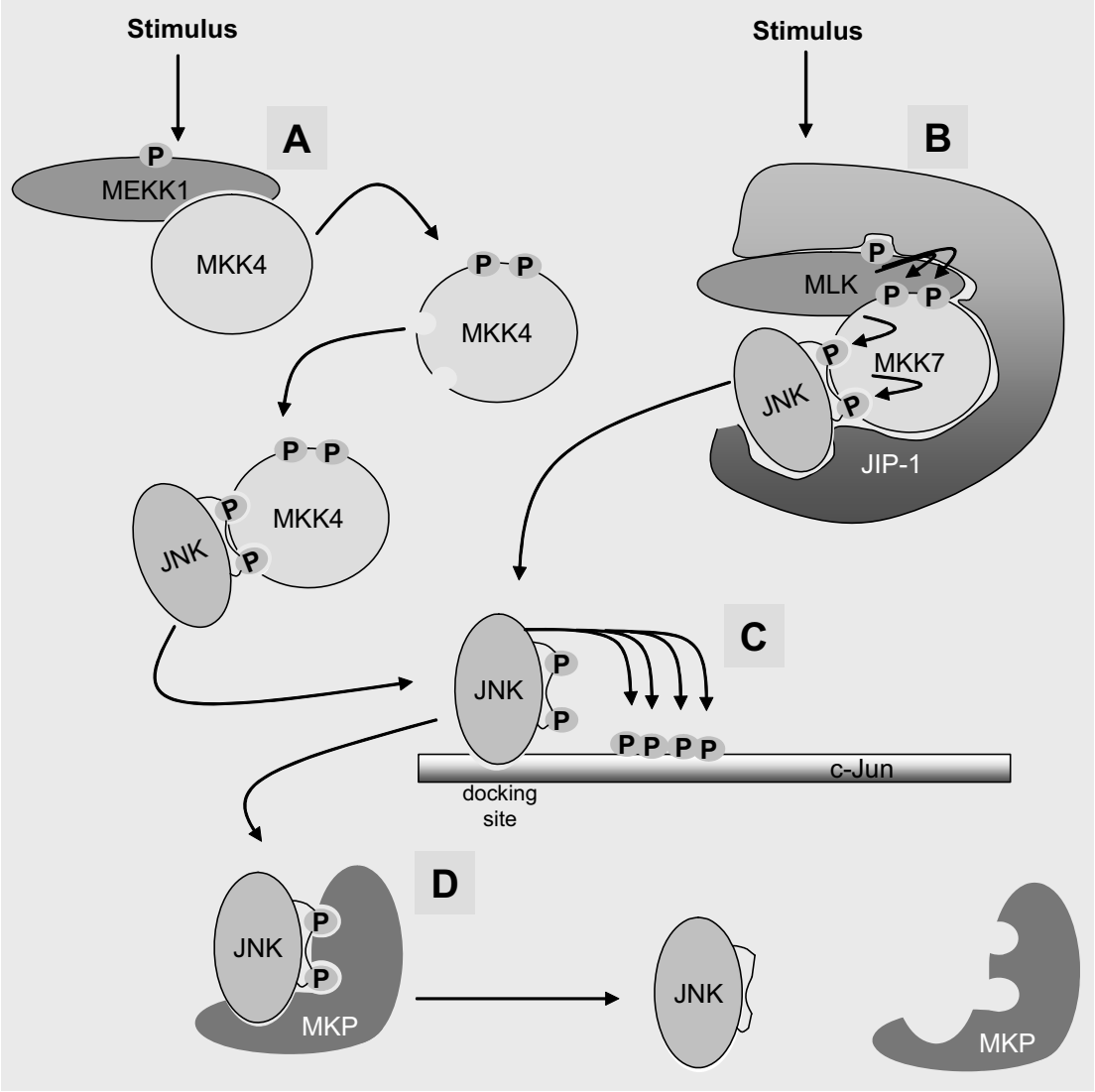


**Figure 4.** Examples of MAPK cascade signaling mechanisms (A) Sequential and specific interaction between members of a MAPK cascade. MEKK1 interacts with inactive MKK4 to form a MEKK1-MKK4 complex. MEKK1 subsequently phosphorylates and activates MKK4, resulting in dissociation of the complex. The free and active MKK4 then specifically interacts with JNK. Once JNK is activated, the MKK4-JNK complex is disrupted and the active JNK is now able to phosphorylate downstream effectors (Chang and Karin, 2000). (B) Scaffold mechanism for generation of specificity in MAPK activation and function. The JIP-1 scaffold brings the MAPKKK MLK1, the MAPKK MKK7 and the MAPK JNK together in a specific signalling module. This complex allows MLK1 to phosphorylate MKK7 and activated MKK7 on its turn to phosphorylate JNK. Once JNK is activated, the scaffold complex is disrupted and the active JNK is now freed to phosphorylate downstream effectors (Chang and Karin, 2000). (C) The JNK docking site of c-Jun determines its specific phosphorylation at Ser 63, Ser 73, Thr 91, and Thr 93. A substrate lacking a functional docking site, such as JunD, can be phosphorylated by JNK that is recruited through the DNA-binding domain (DBD) of c-Jun or JunB (Chang and Karin, 2000). (D) Dephosphorylation of JNK by MAPK phosphatases (MKPs). Active JNK is bound by MKP. JNK-MKP complex formation allows dephosphorylation of JNK by the catalytic domain of the MKP and concomitant release of inactive JNK (Farooq and Zhou, 2004). Image adapted from Chang and Karin, 2001.

**The AP-1 family of transcription factors**

The AP-1 family of transcription factors is a group of homodimers and heterodimers composed of bZip (basic region-leucine zipper) proteins that belong to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1, and Fra2), and ATF (ATF-2, ATF3, and ATFa) subfamilies (Angel and Karin, 1991; Karin et al., 1997; Wisdom, 1999; Figure 5). The basic region harbors the actual DNA-contact surface, whereas the leucine zipper enables the formation of homo- and heterodimeric complexes with other bZip proteins, which is essential for DNA binding (van Dam and Castellazzi, 2001). In vitro, c-Jun proteins can form homodimers that bind specifically to the 7 bp DNA motifs 5'-T(G/T)A(G/C)TCA-3', also known as TREs (TPA response elements) (Angel et al., 1987; van Dam and Castellazzi, 2001). Fos proteins do not form stable homodimers, but can bind to TREs by forming heterodimers with Jun proteins, which are much more stable than Jun homodimers (Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Figure 5B). ATF proteins form homodimers as well as heterodimers with Jun proteins. In contrast to Jun/Fos heterodimers, these ATF containing dimers only efficiently bind to the 8 bp DNA motifs 5'-T(G/T)ACNTCA-3', of which the TGACG variants can act as CREs (cAMP responsive elements) (van Dam and Castellazzi, 2001) (Figure 5B).

Depending on the cell type, Jun, Fos and ATF family members can play crucial roles in in vitro cell proliferation, cell survival and differentiation, in particular c-Jun and JunD. Knockout studies have shown that several AP-1 components have critical roles in embryonic development (Wagner and Eferl, 2005; Zenz and Wagner, 2006). Moreover, multiple AP-1 components have oncogenic activity in rodent or chicken model systems and/or show elevated expression in human cancer cells, including c-Jun, Fra1 and ATF3 (van Dam and Castellazzi, 2001; Wagner, 2001; Eferl et al. 2003). Both in the absence or presence of DNA damage, AP-1 components can regulate cell cycle progression and cell survival through functional interactions with components of the RB and p53 pathways, including cyclin D1, p53, p16 and p21 (Shaulian and Karin, 2002; Hess et al., 2004; Choi et al., 2005; Yan et al., 2005).

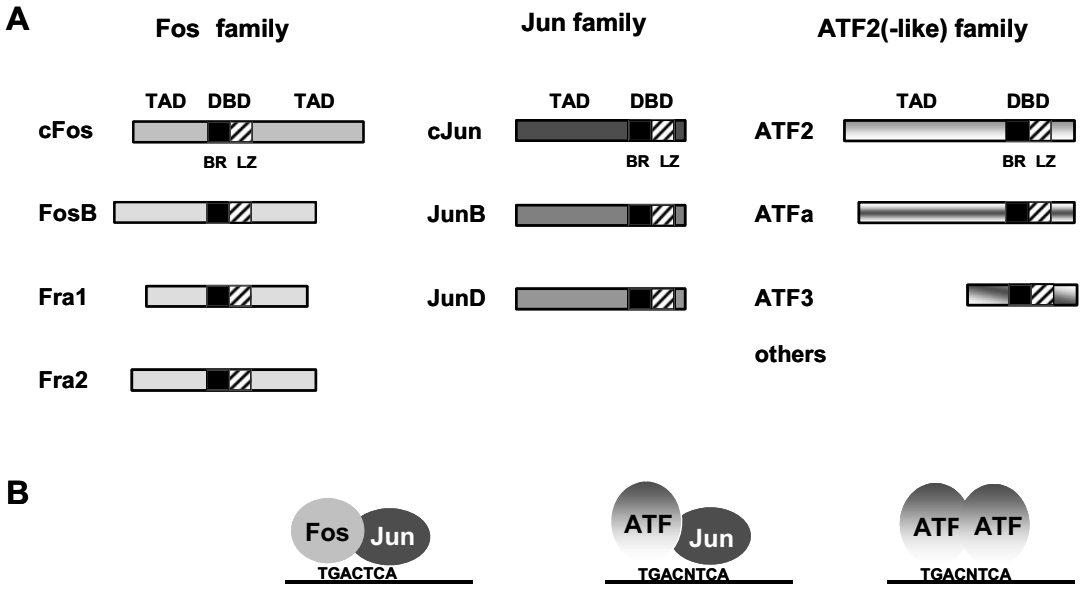


**Figure 5.** AP-1 transcription factors. The AP-1 family of transcription factors is a collection of homodimers and heterodimers composed of the indicated members of the Fos, Jun, and ATF protein families (A). These proteins share the same structural domains for dimerization and DNA binding: a leucine zipper (LZ) and a basic region (BR). The leucine zipper mediates dimerization, which is essential for DNA-binding, whereas the basic region harbors the actual contact surface for the preferred binding site (B). The main characteristic of the AP-1 complexes in the cell is their heterogeneity in dimer composition. This heterogeneity is caused by the fact that multiple Fos, Jun, ATF proteins can be expressed at the same time. For further details, see text.

**MAPKs regulate transcriptional activity and expression of AP-1 transcription factors**

The relative levels and phosphorylation states of each of the individual AP-1 proteins are under tight control of multiple signaling cascades, including the various MAPKs (Karin et al., 1997; Garrington and Johnson, 1999). The Jun family members contain a single transactivation domain (TAD) located N-terminal to the bZip domain (Figures 5 and 6A). JNK members of the MAPK family regulate c-Jun and JunD TADs via phosphorylation of Ser 63 and Ser 73 of c-Jun and Ser 90 and 100 of JunD (Karin et al., 1997). In addition, c-Jun-Thr 91 and -Thr 93 are phosphorylated by JNK (Figure 6A) (Papavassiliou et al., 1995; Morton et al., 2003). JNKs phosphorylate c-Jun very efficiently, JunD less efficiently, but they do not phosphorylate JunB, as JunB lacks the evolutionary conserved phosphorylation sites present in c-Jun and JunD (Kallunki et al., 1996).

The N-terminal phosphorylation of Ser63 and Ser73 of c-Jun is thought to enhance its transcriptional activity. Most models proposed that phosphorylation facilitates the interaction of signal responsive transcription factors with the basal transcriptional machinery or with transcriptional co-activators, including the histone acetyl transferases p300 and CBP (Treisman, 1996; Mayr and Montminy, 2001; Figure 6). More recent studies provide evidence that in the absence of JNK signaling a repressor activity that is associated with HDAC3 inhibits c-Jun. Phosphorylation of c-Jun by JNK relieves this repression, thereby enhancing the transcriptional activity of c-Jun, suggesting that unphosphorylated c-Jun can stimulate transcription efficiently if the repressor is dissociated (Figure 6B) (Weiss et al., 2003; Ogawa et al., 2004). However, the activity of the c-Jun transactivation domain, is likely also to depend on (the transactivation domain of) its dimer partner and/or the promoter context.

Interestingly, using c-junAA knock-in mice, in which Ser 63 and 73 were mutated to alanines, evidence was provided that c-Jun phosphorylation is dispensable for mouse development (Behrens et al., 1999). However, primary JunAA fibroblasts derived from these mice showed proliferation defects when grown at 20% oxygen and reduced levels of apoptosis after high doses of UV (Behrens et al., 1999). These JunAA cells also contained less AP-1 transcriptional activity, although this was only measured using a multimeric TRE-dependent luciferase reporter plasmid (Behrens et al., 1999). Paradoxically, studies by other groups showed that c-JunAA can, in fact, also sensitize mouse fibroblasts and other cell types to UV light and other DNA-damaging agents (Wisdom et al., 1999; Potapova et al., 2001). This suggests that the actual effect of JNK-dependent c-Jun phosphorylation on c-Jun activity not only depends on the c-Jun dimer-partner and the promoter-context but also on the cellular context and experimental conditions, both with respect to c-Jun-dependent gene activation and c-Jun-dependent phenotypes. In this respect it is also important to note that JNK-dependent phosphorylation of c-Jun can control c-Jun ubiquitination and degradation (Musti et. al 1997; Nateri et al., 2004).

Upon activation by stress-stimuli JNK and p38 can enhance ATF-2-dependent transactivation by phosphorylation of both Thr 69 and Thr 71. In contrast, in various cell types insulin, EGF, and serum activate ATF-2 via a two-step mechanism: ERK phosphorylates Thr 71 of ATF-2 and subsequently Thr 69 is phosphorylated by p38 or JNK (Ouwens et al., 2002; Baan et al., 2006). As growth factors are often very poor activators of JNK and p38, cooperation of ERK and p38 or JNK is essential for efficient activation of ATF-2.

The C-terminus of c-Fos contains an ERK-dependent transactivation domain, which is phosphorylated by ERK at multiple residues resulting in increased transcriptional activity (Monje et al., 2003). c-Fos contains also ERK phosphorylation sites that regulate c-Fos turnover (Chen et al., 1996). Efficient phosphorylation of the c-Fos C-terminus by ERK stabilizes c-Fos for several hours (Murphy et al., 2002). Fra1 and Fra2 lack the potent transactivation domains present in both c-Fos and FosB. However, Fra1 can be activated by ERK and this activation requires Fra1-Thr 231 (see below and Young et al., 2002). Similar to c-Fos, ERK-dependent phosphorylation results in Fra1 stabilization (Casalino et al., 2003).

In addition to posttranslational activation, activation of AP-1 components occurs at the level of transcription.

Transactivation of the c-fos promoter depends on the SRF/TCF(Elk1) transcription factor complex. Upon stress stimuli JNK phosphorylates TCF, whereas ERK kinase activates the SRF/TCF complex after stimulation by growth factors and TPA (Treisman, 1994).

A key feature of most AP-1 components is that they are under positive and/or negative transcriptional control of AP-1 itself. fra1 and fra2 transcription can be regulated by Jun/c-Fos dimers and once transcribed, Fra1 can positively regulate its own transcription by binding to the AP-1 site in the first intron (Bergers et al., 1995; Schreiber et al., 1997; Sonobe et al., 1995; Matsuo et al., 2000; Casalino et al., 2003). Furthermore, c-jun and atf3 expression is regulated via Jun/ATF binding sites in their promoters (Angel et al., 1988; van Dam et al., 1993; Liang et al., 1996).

In summary, the relative levels and activities of Jun, Fos, and ATF proteins present in cells at a given time strongly depend on the types of stimuli the cell has received. As a consequence, the relative levels and composition of the various AP-1 dimers (Jun/Fos, Jun/Fra, Jun/ATF, and ATF/ATF) is stimulus dependent as well and MAPK signaling plays an important role in regulating these AP-1 dimers. As the Jun/Fos(Fra) and Jun/ATF complexes control different sets of target genes by binding to different DNA sequence motifs (Figure 5B) (Hai and Curran, 1991; van Dam and Castellazzi, 2001), the MAPK-AP-1 module represents a cellular network with large flexibility to regulate and integrate multiple distinct stimuli.

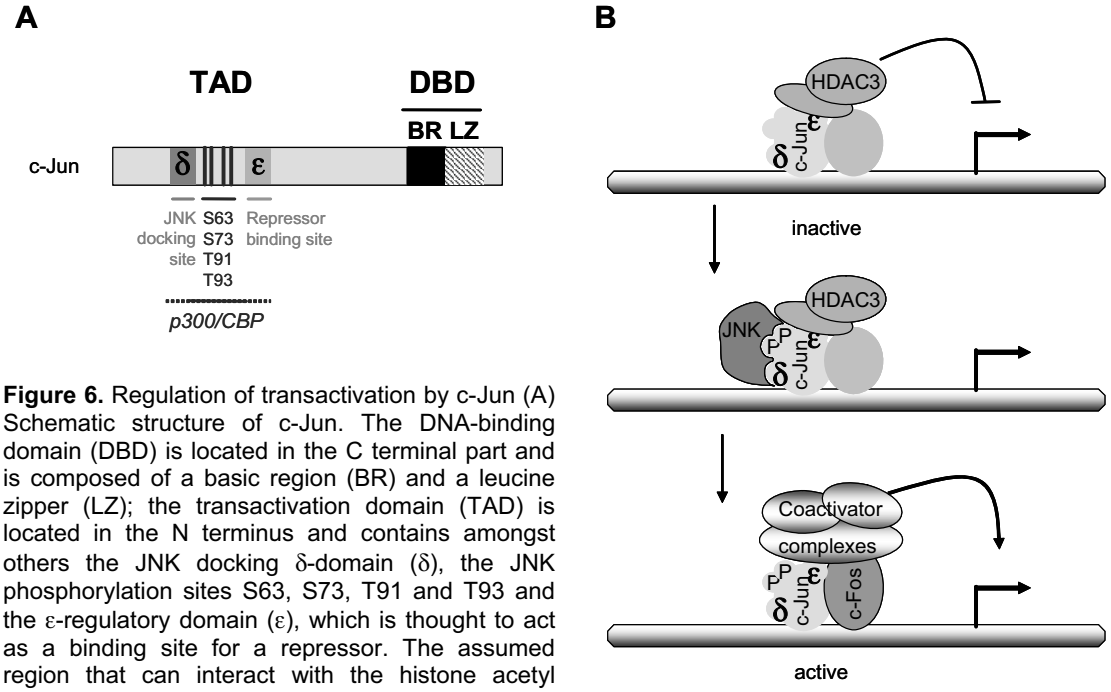


**Figure 6.** Regulation of transactivation by c-Jun (A) Schematic structure of c-Jun. The DNA-binding domain (DBD) is located in the C terminal part and is composed of a basic region (BR) and a leucine zipper (LZ); the transactivation domain (TAD) is located in the N terminus and contains amongst others the JNK docking δ-domain (δ), the JNK phosphorylation sites S63, S73, T91 and T93 and the ε-regulatory domain (ε), which is thought to act as a binding site for a repressor. The assumed region that can interact with the histone acetyl transferases p300 and CBP is also indicated.
(B) Model for relief of HDAC3-dependent repression on c-Jun target genes. JNK-dependent phosphorylation of c-Jun results in dissociation of the HDAC3-containing repressor complex. This then allows binding of a co-activator complex, which might be accompanied by a change in the c-Jun dimer partner, and which results in transcriptional activation (Weiss et al., 2003; Ogawa et al., 2004).

**Overlap and differences between Fos and Fra proteins**

The Fos family members c-Fos and FosB contain transactivation domains (TADs) at both their N- and C-termini. As mentioned above, the potent C-terminal TAD is absent in Fra1 and Fra2 (Milde-Langosch, 2005). However, the relative weak N-terminal domain is conserved in all four Fos family members. This explains why in most assays the Fra proteins have been found to be much weaker transactivators than the Fos proteins. Initial studies in fact showed that Gal4DBD-Fra1TAD fusion proteins could not activate Gal4-

dependent reporters and suggested that Fra1 was a negative inhibitor of AP-1 activity (Suzuki et al., 1991; Yoshioka et al., 1995). However, later studies demonstrated that the C-terminus of Fra1 does have transactivation potential, e.g. in JB6 cells (Young et al., 2002). In these cells, a Gal4DBD-Fra1 fusion protein containing the Fra1 C-terminal domain plus most of its bZip domain was activated in an ERK-dependent manner. Mutation analysis showed that the activation required ERK-dependent phosphorylation of Thr231. A corresponding GAL4-Fra2 fusion protein was not activated suggesting that this ERK-dependent activation was unique to the C-terminal transactivation domain of Fra1 (Young et al., 2002).

Interestingly, the target genes of c-Fos and Fra1 appear to overlap. In c-fos$^{-/-}$ mice osteoclast differentiation can be restored by expression of a Fra1 transgene, or by inserting the fra1 gene in the c-fos locus. In addition, Fra1 can substitute for c-Fos during light-induced apoptosis of retinal photoreceptors when cloned in the c-Fos locus (Matsuo et al., 2000; Fleischman et al., 2000). However, these studies with transgenic mice suggest that Fra1 also has c-Fos-independent gene-regulating functions (Matsuo et al., 2000).

Ectopic (over)expression studies showed that wild type Fra1 was able to transform rat fibroblast resulting in anchorage-independent growth in vitro and tumor development in mice (Bergers et al., 1995). Several studies also suggest a role for Jun/Fra1 and Jun/Fra2 complexes in oncogenic transformation induced by viral and cellular oncogenes. Levels of Jun/Fra1, rather than of Jun/Fos, are upregulated in cells transformed by Ras and other oncogenic stimuli (Mechta et al., 1997; Vallone et al., 1997). Overexpression of c-Jun and Fra1 in NIH3T3 cells induces a Ras-like transformed phenotype (Mechta et al., 1997). Interestingly, the low susceptibility to transformation by active Ras of human fibroblasts compared to rat fibroblasts was the result of the weak induction of Fra1 by Ras in human fibroblast and – in contrast – a much stronger Ras-induced upregulation of Fra1 in rat fibroblasts (Kakumoto et al., 2006). In fact, although, initially c-Jun and c-Fos were thought to be the primary candidate AP-1 transcription factor targets responsible for misregulation in carcinogenesis, detailed analysis of human cancer by gene array and proteomics now indicates that Fra1 may be a more universal target. Elevated Fra1 mRNA and protein have been detected in multiple tumors as well as transformed cell lines, including breast, colon and lung (Young and Colburn, 2006).

**JNK and AP-1 in DNA-damage-induced cell cycle arrest and apoptosis**

As described above, both JNK and AP-1 are strongly activated by various types of genotoxic agents and have been implicated in apoptosis as well as survival signaling. The actual role of JNK and AP-1 in DNA-damage induced apoptosis appears to be dependent on the cell type and the nature and dose of the genotoxic agent (Ip and Davis, 1998; Wisdom, 1999; Davis, 2000; Chang and Karin, 2001; Shaulian and Karin, 2001; Bogoyevitch and Kobe, 2006). A large amount of studies demonstrated that JNK has pro-apoptotic activity. For instance, knock-out of both the jnk 1 and jnk 2 genes caused a defect in the mitochondrial death-signaling pathway in primary mouse embryo fibroblasts (MEFs), including failure to release cytochrome c after irradiation with high doses of UV (Tournier et al., 2000). Moreover, both jnk1$^{-/-}$ mouse fibroblasts and JunAA MEFs – cells in which JNK-phosphorylation sites Ser63 and 73 of c-Jun are mutated to alanines – were found to be less sensitive to apoptosis upon relatively high doses of UV (Hochedlinger et al., 2002; Behrens et al., 1999). Other studies showed that JNK can inhibit the anti-apoptotic function of Bcl2 and Bcl-XL (Maundrell et al., 1997; Yamamoto et al., 1999). JNK phosphorylation of the c-Jun N-terminus can result in enhanced expression of pro-apoptotic c-Jun-dependent genes, such as Fas-ligand (Le-Niculescu et al., 1999; Kolbus et al., 2000).

Although jnk1$^{-/-}$ mouse fibroblasts showed reduced apoptosis upon high doses of UV, they were more sensitive to tumor necrosis factor-alpha (TNF-α) and sorbitol-induced cell death (Hochedlinger et al., 2002). Moreover, in mouse fibroblasts exposed to moderate doses of UV, JNK-induced c-Jun stimulates cell-cycle re-entry rather than inducing

apoptosis (Shaulian et al., 2000). T98G human glioblastoma cells expressing high levels of non-phosphorylatable c-Jun(S63A,S73A) showed enhanced sensitivity to apoptosis induced by DNA-damaging agents (Potapova et al., 2001). Importantly, multiple studies indicate that transient JNK activation mainly results in cell survival, while more sustained JNK activation predominantly mediates death signaling (Ventura et al., 2006; Christmann et al., 2007; Mansouri et al., 2003; Wolfman et al., 2002; Guo et al., 1998). Additional pro- and anti-apoptotic substrates of JNK that have been identified include Mcl-1, Bad, Bim, Bmf, RXR$\alpha$, cMyc, p53, and Foxo4 (for review Bogoyevitch and Kobe, 2006).

c-Fos can also exhibit both pro- and anti-apoptotic functions. The induction of c-Fos by UV protects mouse fibroblasts against UV-induced apoptosis (Schreiber et al., 1995), presumably by facilitating recovery from UV induced DNA damage (Christmann et al., 2006 and 2007). However, in other cells c-Fos can have a pro-apoptotic function, for instance during light-induced apoptosis of retinal photoreceptors in mice (Wenzel et al., 2000).

As mentioned previously, an important function of AP-1 appears to be regulation of the p53/RB growth suppressor pathway. Multiple AP-1 components can control the activity and/or expression levels of p53, p21, cyclin D1, p16, and Arf (Schreiber et al., 1999,; Shaulian et al., 2000; Weitzman et al., 2000; Wisdom et al., 1999; Bakiri et al., 2000; Passegue and Wagner, 2000). Nevertheless, c-Jun and c-Fos can also regulate cell proliferation and cell death in cells lacking functional p53 (Potapova et al., 2001; Lackinger, 2001).

Like c-Fos, the expression of ATF3 is low or not detectable in most cell types, but rapidly induced by a wide-range of stimuli, including growth factors and genotoxic agents (Hai et al., 1999; Hai and Hartman, 2001). Moreover, also ATF3 has been found to act both as a negative or positive regulator of cell cycle progression and cell death. For instance, ATF3$^{-/-}$ MEFs show enhanced proliferation and are partially protected against UV-induced apoptosis, whereas ectopically overexpressed ATF3 can induce apoptosis (Lu et al., 2006). Moreover, ATF3 can suppress Ras-mediated tumorigenesis in mouse fibroblasts, which is in part due to binding of ATF3 to the cyclin D1 promoter resulting in repression of cyclin D1 transcription (Lu et al., 2006). Both in MEFs and in lung cancer cells ATF3 was shown to interact with the p53 protein thereby augmenting p53 function by preventing p53 ubiquitination and degradation (Yan et al., 2005). On the other hand, the atf3 gene was shown to be a direct target of p53 upon exposure of HCT116 human colorectal carcinoma cells to UV (Zhang et al., 2002). Mutant p53 can attenuate TPA-induced expression of ATF3 and thereby protect SKOV3 ovarian cancer cells from TPA-induced cell death (Buganim et al., 2006).

As mentioned above, ATF3 can also enhance cell proliferation and in most cancers ATF3 levels actually appear to be increased (Hai and Hartman, 2001; Yin et al., 2007). Using various cell lines and xenograft injection models ATF3 has been demonstrated to be either a tumor suppressor or an oncogene (Ishiguro and Nagawa, 2000; Bottone et al., 2005; Bandyopadhyay et al., 2006; Lu et al., 2006). This suggests that ATF3 has a dual role in cancer development. In fact, a recent study shows that ATF3 enhances apoptosis in untransformed MCF10A mammary epithelial cells, while protecting and enhancing the motility of transformed MCF10CA1a cells, the aggressive MCF10A derivatives (Yin et al., 2007). These results provide a correlative argument that it is advantageous for the malignant cancer cells to express ATF3.

**Role of AP-1 in DNA repair**

Emerging evidence reveals involvement of AP-1 transcription factors in the regulation of expression of key players in DNA repair pathways. Upon treatment of certain human tumour cell lines with cisplatin JNK, c-Jun and ATF-2 can enhance DNA repair and cell survival (Kharbanda et al., 1995; Adler et al., 1995; Potapova et al., 1997, 2001; Nehme et al., 1997; Hayakawa et al., 2003). In at least one cell type, cisplatin treatment resulted in binding of phospho-ATF-2 and phospho-c-Jun to promoters of genes involved in DNA repair, including ERCC1, ERCC3, XPA, RAD23B, MSH2, RAD50 and ATM. This binding was blocked by specific inhibitors of JNK (Hayakawa et al., 2004). Upon IR, ATM was found to phosphorylate the C-

terminus of ATF-2 causing co-localization of ATF-2 with Rad50, Nbs1 and Mre11 (Bhoumik et al., 2005). Moreover, inhibition of ATF-2 expression was found to decrease recruitment of Mre11 to these DNA damage foci, resulting in reduced activation of ATM, Chk1, and Chk2, in abrogation of S-phase checkpoints, and in impaired radioresistance (Bhoumik et al., 2005).

Yan and Boyd (2006) suggest that dysfunction of ATF3 impairs p53-mediated DNA damage responses, including p53-induced cell cycle arrest and p53-dependent activation of repair genes, allowing MEFs to be readily transformed by oncogenes. Consistent with this notion ATF3 expression is downregulated in human renal and lung cancer (Garber et al., 2001; Higgins et al., 2003). Finally, in mouse embryo fibroblasts c-Fos appears to stimulate nucleotide excision repair by counteracting the inhibition of XPF transcription by UV, thereby facilitating recovery from UV induced DNA damage (Christmann et al., 2006 and 2007).

**Introduction to this thesis**

Understanding the molecular mechanisms of the cellular processes initiated by genotoxic stress is of great importance. The work presented in this thesis has focused on the role of Mitogen Activated Protein Kinases (MAPKs) and their major downstream targets, the AP-1 transcription factors, in particular the AP-1 components ATF3, Fra1, c-Jun, ATF-2 and c-Fos. DNA damaging agents such as ionizing radiation (IR), ultraviolet light (UV), and the anti-cancer drug cisplatin were already known to cause activation of MAPKs and AP-1 transcription factors in various cell types. However, the upstream signaling events in the activation and the actual effects of these agents on the various AP-1 sub-classes and target genes (e.g. c-Jun/Fos versus c-Jun/ATF-dependent genes) were still largely unknown. Furthermore, for most genotoxic agents and cell types the specific roles of AP-1 components and AP-1 dimers in DNA damage responses remained to be established.

**Chapter II** provides information on the signaling pathways involved in the activation of the AP-1 components ATF-2 and ATF3 in the response of primary human fibroblasts to IR. The data show that ATM and NBS1 – two protein kinases essential for the cellular response to IR-induced double strand breaks (DSBs) – are required for IR-induced phosphorylation of ATF-2-Thr69 and Thr71 and induction of ATF3. This activation also involves the MAPKs p38 and JNK, which phosphorylate ATF-2-Thr69 and 71, and can induce mRNA and protein levels of ATF3.

In **chapter III** the AP-1 components c-Jun and ATF3, the MAPK JNK and the MAPK-phosphatase MKP-1 are identified as important sensors of UV-induced-DNA damage in transcribed genes. In transcription-coupled repair (TCR)-deficient human fibroblasts low doses of UV were found to activate JNK via inhibition of MKP-1 expression. Removal of UV-induced cyclobutane pyrimidine dimers abrogated the inhibition of MKP-1 and the activation of JNK, c-Jun, and ATF3. Ectopic expression of MKP-1 inhibited JNK activation and suppressed low dose UV-induced apoptosis.

**Chapter IV** describes the functions of the MAPKs JNK and ERK and the AP-1 components ATF3 and Fra1 in the cisplatin and UV response of T98G glioblastoma cells, human tumor cells that are relatively insensitive to cisplatin. JNK, but not ERK is found to be required for the induction of ATF3 by cisplatin, whereas both JNK and ERK are required for the induction of Fra1. RNA interference experiments show that ATF3 acts as an anti-apoptotic JNK target in these cells, whereas Fra1 seems to act as a pro-apoptotic effector of both JNK and ERK. In addition, it is shown that ATF3 and Fra1 have opposite effects on cisplatin-induced S phase arrest.

**Chapter V** shows that Fra1 also can exhibit a pro-apoptotic function in UV-irradiated fibroblasts, in particular at relatively low UV doses at which JNK is anti-apoptotic. Furthermore, this chapter reports an as yet unknown function of JNK: repression of the transactivating activity of c-Jun/Fos(-like) dimers, mediated via hyper-phosphorylation of the c-Jun transactivation domain. Intriguingly, high and prolonged JNK activity as induced by various types of genotoxic agents was found to inhibit activation of c-Jun/Fos-dependent genes like fra1, but not c-Jun/ATF-dependent genes like c-jun and atf3. This further emphasizes that c-Jun/Fos(-like) and c-Jun/ATF dimers and their respective target genes can exhibit opposite functions in DNA damage responses.

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