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

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

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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

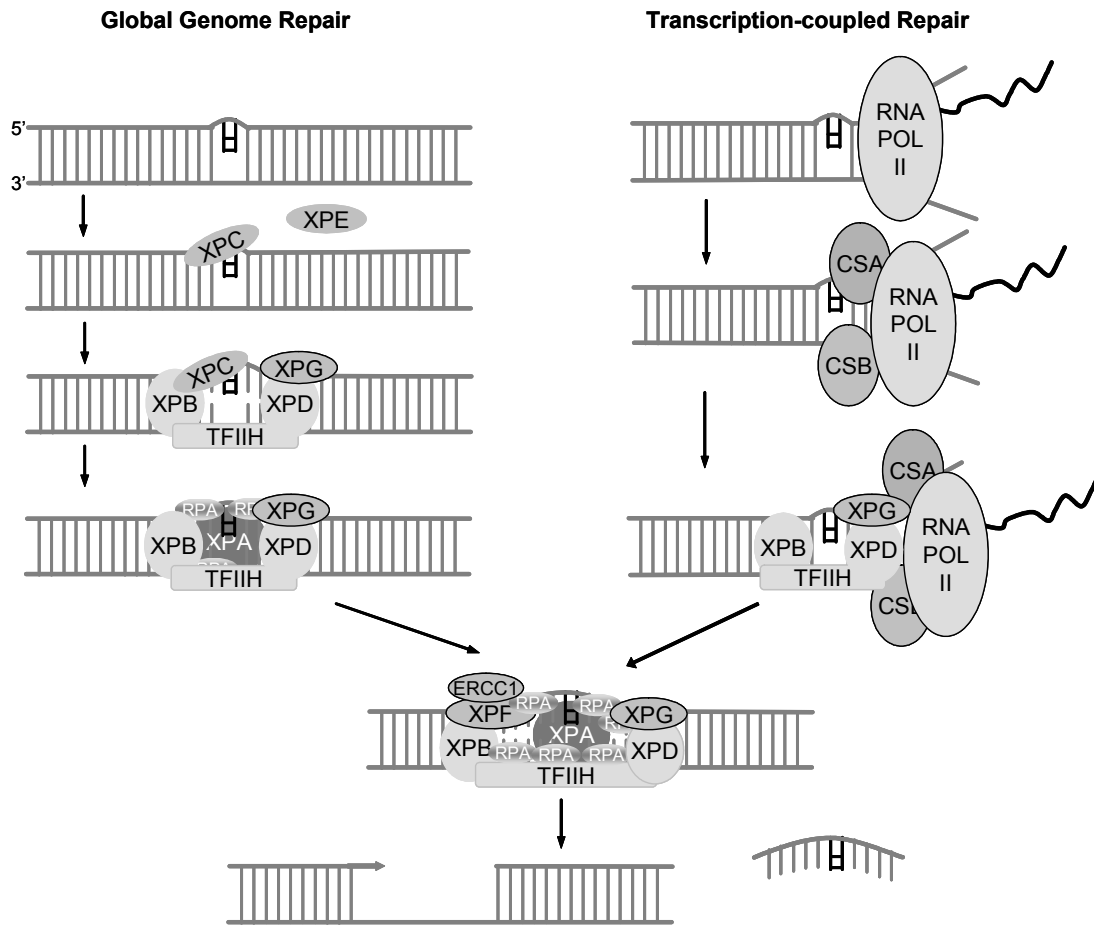


Figure 1. The nucleotide excision repair (NER) pathways: global genome repair (GGR; left panel) and transcription-coupled repair (TCR; right panel). DNA damage occurring anywhere in the genome can be repaired by the GGR pathway, but DNA lesions that occur on the transcribed strand of an active gene and 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 TFIID, which includes the two DNA helicases XPB and XPD is recruited. The endonuclease XPG is recruited simultaneously, or shortly thereafter. 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 TFIID (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 TFIID, 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; Figure 1).

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 sensing 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.

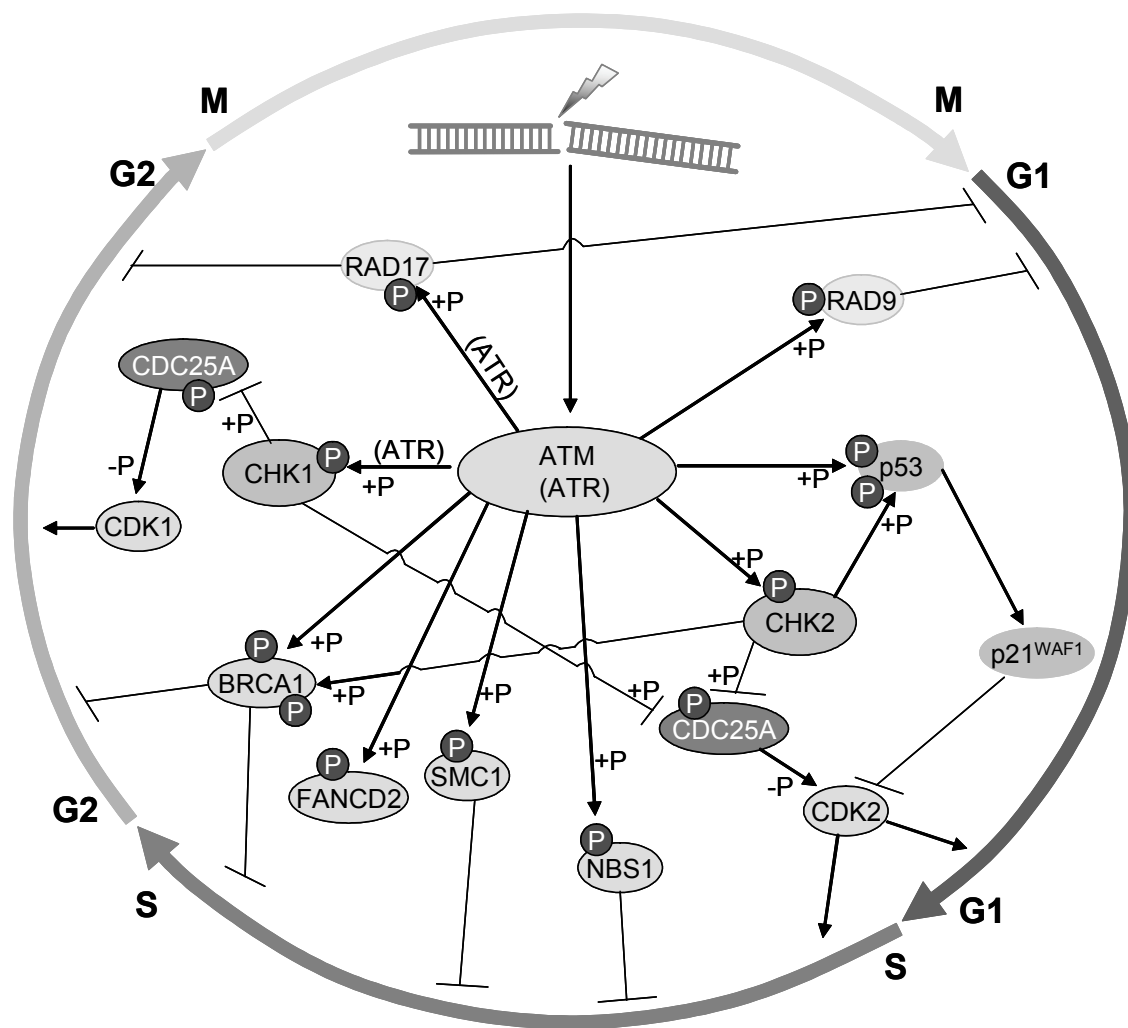


Figure 2. ATM/ATR-mediated activation of cell-cycle checkpoints in response to DNA double strand breaks. Arrows indicate stimulation; T-shaped lines mark inhibition. Phosphorylations are marked by '+P' and dephosphorylations by '-P'. Most of these pathways are regulated primarily by ATM, particularly 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).

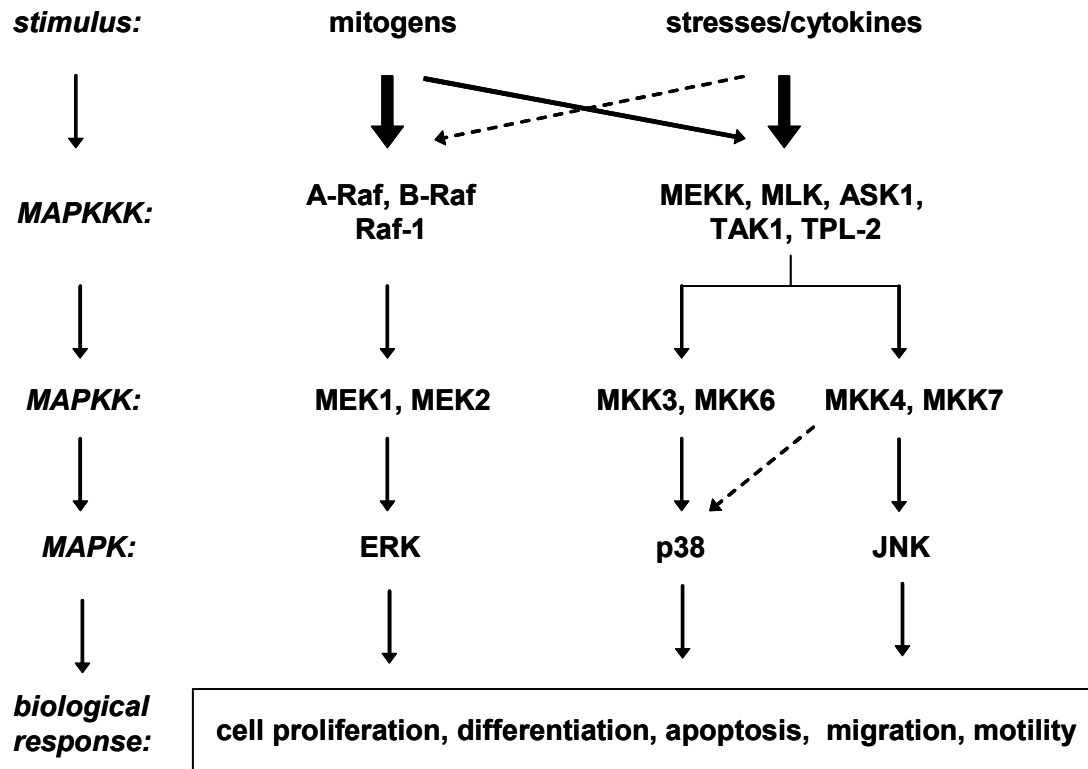


Figure 3. Schematic representation of the mammalian MAP kinase (MAPK) signal transduction pathways with its three types of MAPKs: extracellular signal regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38. MAPKs are activated by dual phosphorylation on Thr and Tyr by members of the MAPK kinase (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- α , β , γ , and δ . 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 MAPKKs 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).

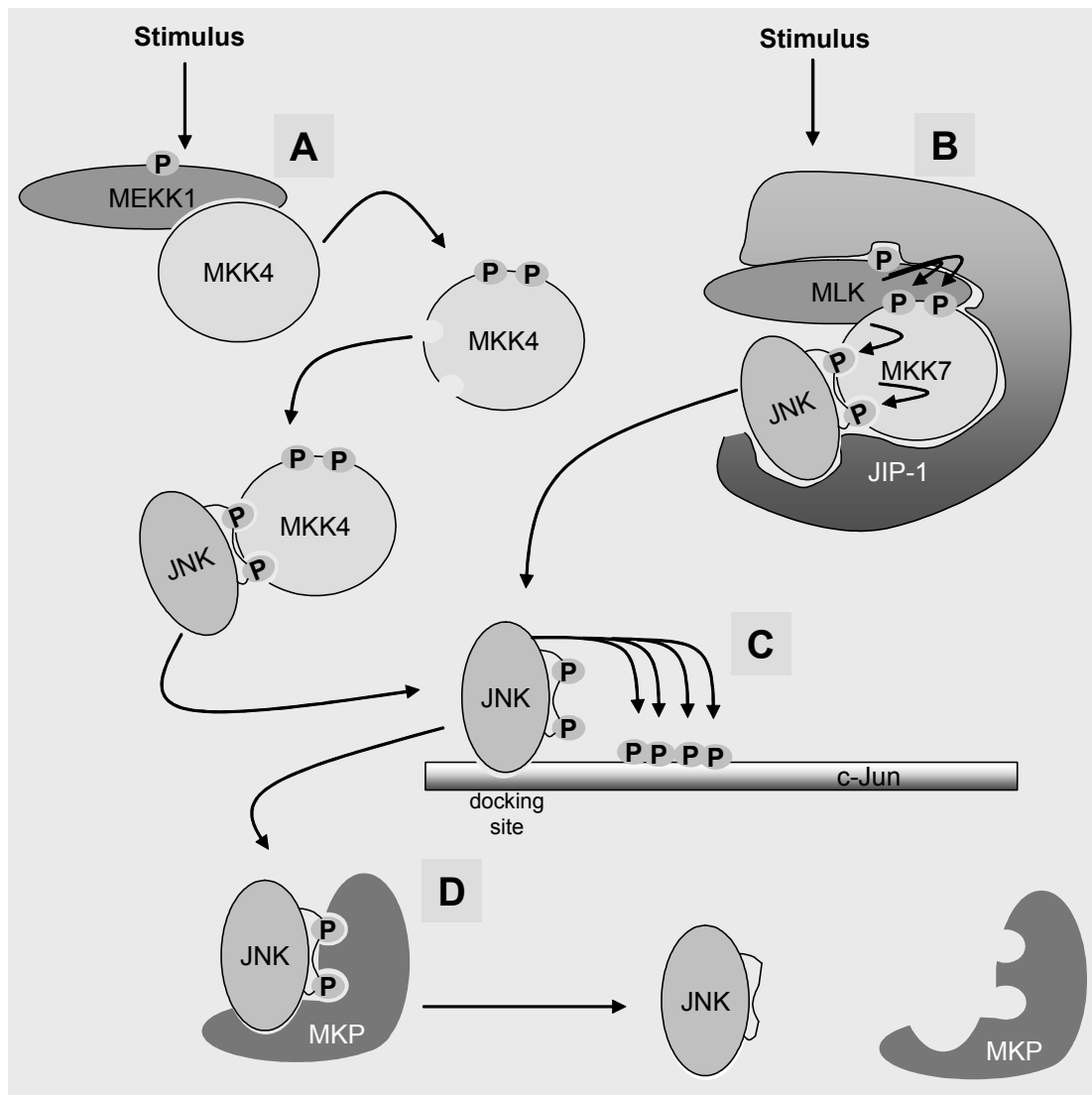


Figure 4. Examples of MAPK cascade signaling mechanisms (A) Sequential and specific interaction between members of a MAPK cascade. MEKK1 interacts with inactive MKK4 to form a MEKK1-MKK4 complex. MEKK1 subsequently phosphorylates and activates MKK4, resulting in dissociation of the complex. The free 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 ATFα) 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).

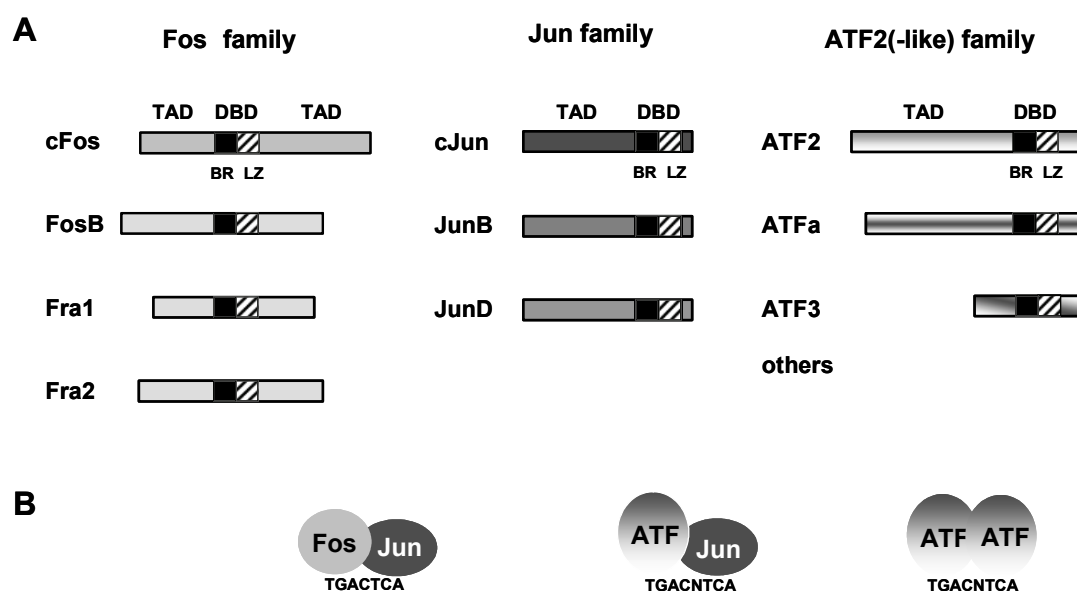


Figure 5. AP-1 transcription factors. The AP-1 family of transcription factors is a collection of homodimers and heterodimers composed of the indicated members of the Fos, Jun, and ATF protein families (A). These proteins share the same structural domains for dimerization and DNA binding: a leucine zipper (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(EIk1) 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.

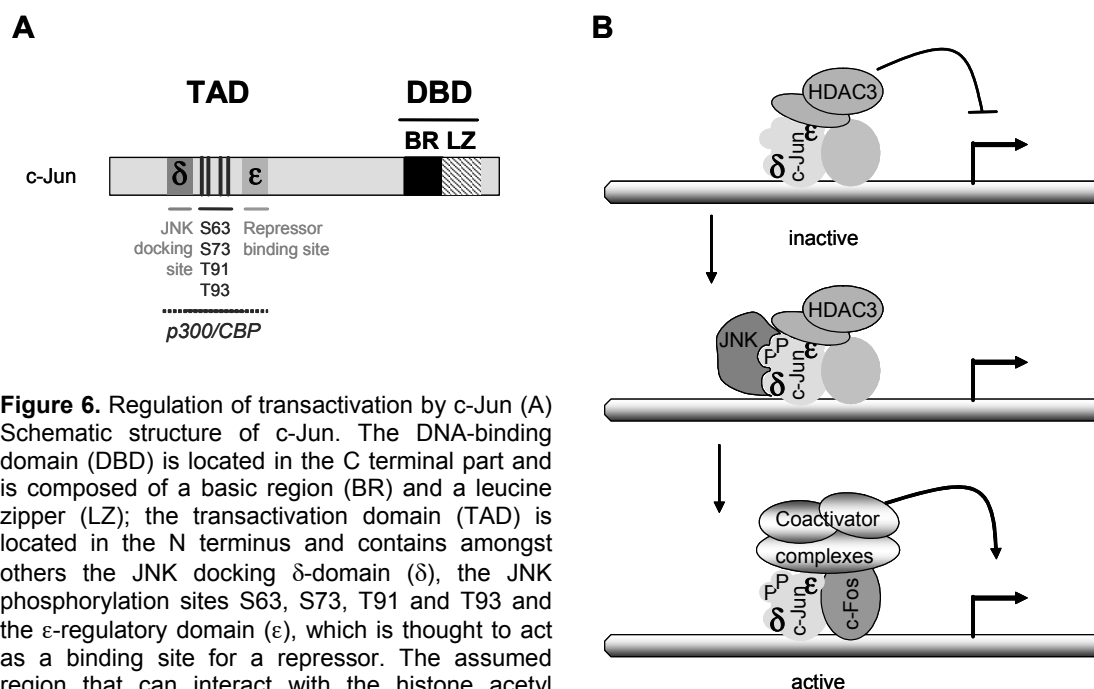


Figure 6. Regulation of transactivation by c-Jun (A) Schematic structure of c-Jun. The DNA-binding domain (DBD) is located in the C terminal part and is composed of a basic region (BR) and a leucine zipper (LZ); the transactivation domain (TAD) is located in the N terminus and contains amongst others 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 α , 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.

Reference List

- Adler,V., Fuchs,S.Y., Kim,J., Kraft,A., King,M.P., Pelling,J., and Ronai,Z. (1995). jun-NH2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells. *Cell Growth Differ.* 6, 1437-1446.
- Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P., and Karin,M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* 49, 729-739.
- Angel,P., Hattori,K., Smeal,T., and Karin,M. (1988). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* 55, 875-885.
- Angel,P. and Karin,M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* 1072, 129-157.
- Ataian,Y. and Krebs,J.E. (2006). Five repair pathways in one context: chromatin modification during DNA repair. *Biochem. Cell Biol.* 84, 490-504.
- Baan,B., van Dam,H., van der Zon,G.C., Maassen,J.A., and Ouwens,D.M. (2006). The role of c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase in insulin-induced Thr69 and Thr71 phosphorylation of activating transcription factor 2. *Mol. Endocrinol.* 20, 1786-1795.
- Bakiri,L., Lallemand,D., Bossy-Wetzel,E., and Yaniv,M. (2000). Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J.* 19, 2056-2068.
- Bandyopadhyay,S., Wang,Y., Zhan,R., Pai,S.K., Watabe,M., Iizumi,M., Furuta,E., Mohinta,S., Liu,W., Hirota,S., Hosobe,S., Tsukada,T., Miura,K., Takano,Y., Saito,K., Commes,T., Piquemal,D., Hai,T., and Watabe,K. (2006). The tumor metastasis suppressor gene Drg-1 down-regulates the expression of activating transcription factor 3 in prostate cancer. *Cancer Res.* 66, 11983-11990.
- Bartek,J. and Lukas,J. (2007). DNA damage checkpoints: from initiation to recovery or adaptation. *Curr. Opin. Cell Biol.* 19, 238-245.
- Bartels,C.L. and Lambert,M.W. (2007). Domains in the XPA protein important in its role as a processivity factor. *Biochem. Biophys. Res. Commun.* 356, 219-225.
- Behrens,A., Sibilio,M., and Wagner,E.F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.* 21, 326-329.
- Bekker-Jensen,S., Lukas,C., Melander,F., Bartek,J., and Lukas,J. (2005). Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. *J. Cell Biol.* 170, 201-211.
- Bergers,G., Graninger,P., Braselmann,S., Wrighton,C., and Busslinger,M. (1995). Transcriptional activation of the fra-1 gene by AP-1 is mediated by regulatory sequences in the first intron. *Mol. Cell Biol.* 15, 3748-3758.
- Bhoumik,A., Takahashi,S., Breitweiser,W., Shiloh,Y., Jones,N., and Ronai,Z. (2005). ATM-dependent phosphorylation of ATF2 is required for the DNA damage response. *Mol. Cell* 18, 577-587.
- Bogoyevitch,M.A. and Court NW (2004). Counting on mitogen-activated protein kinases--ERKs 3, 4, 5, 6, 7 and 8. *Cell Signal.* 16, 1345-1354.
- Bogoyevitch,M.A. and Kobe,B. (2006). Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol. Mol. Biol. Rev.* 70, 1061-1095.
- Bottone,F.G., Jr., Moon,Y., Kim,J.S., Alston-Mills,B., Ishibashi,M., and Eling,T.E. (2005). The anti-invasive activity of cyclooxygenase inhibitors is regulated by the transcription factor ATF3 (activating transcription factor 3). *Mol. Cancer Ther.* 4, 693-703.
- Brown,E.J. and Baltimore,D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev.* 14, 397-402.
- Brown,E.J. and Baltimore,D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev.* 17, 615-628.
- Buganim,Y., Kalo,E., Brosh,R., Besserglick,H., Nachmany,I., Rais,Y., Stambolsky,P., Tang,X., Milyavsky,M., Shats,I., Kalis,M., Goldfinger,N., and Rotter,V. (2006). Mutant p53 protects cells from 12-O-tetradecanoylphorbol-13-acetate-induced death by attenuating activating transcription factor 3 induction. *Cancer Res.* 66, 10750-10759.
- Casalino,L., De Cesare,D., and Verde,P. (2003). Accumulation of Fra-1 in ras-transformed cells depends on both transcriptional autoregulation and MEK-dependent posttranslational stabilization. *Mol. Cell Biol.* 23, 4401-4415.
- Chambard,J.C., Lefloch,R., Pouyssegur,J., and Lenormand,P. (2007). ERK implication in cell cycle regulation. *Biochim. Biophys. Acta* 1773, 1299-1310.
- Chang,L. and Karin,M. (2001). Mammalian MAP kinase signalling cascades. *Nature* 410, 37-40.
- Chen,R.H., Sarnecki,C., and Blenis,J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell Biol.* 12, 915-927.
- Chen,R.H., Joo,P.C., Curran,T., and Blenis,J. (1996). Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. *Oncogene* 12, 1493-1502.
- Choi,B.Y., Choi,H.S., Ko,K., Cho,Y.Y., Zhu,F., Kang,B.S., Ermakova,S.P., Ma,W.Y., Bode,A.M., and Dong,Z. (2005). The tumor suppressor p16(INK4a) prevents cell transformation through inhibition of c-Jun phosphorylation and AP-1 activity. *Nat. Struct. Mol. Biol.* 12, 699-707.

- Christmann,M., Tomicic,M.T., Origer,J., Aasland,D., and Kaina,B. (2006). c-Fos is required for excision repair of UV-light induced DNA lesions by triggering the re-synthesis of XPF. *Nucleic Acids Res.* 34, 6530-6539.
- Christmann,M., Tomicic,M.T., Aasland,D., and Kaina,B. (2007). A role for UV-light-induced c-Fos: Stimulation of nucleotide excision repair and protection against sustained JNK activation and apoptosis. *Carcinogenesis* 28, 183-190.
- Dard,N. and Peter,M. (2006). Scaffold proteins in MAP kinase signaling: more than simple passive activating platforms. *Bioessays* 28, 146-156.
- Davis,R.J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell* 103, 239-252.
- de Boer,J. and Hoeijmakers,J.H. (2000). Nucleotide excision repair and human syndromes. *Carcinogenesis* 21, 453-460.
- de Laat,W.L., Jaspers,N.G., and Hoeijmakers,J.H. (1999). Molecular mechanism of nucleotide excision repair. *Genes Dev.* 13, 768-785.
- Eferl,R. and Wagner,E.F. (2003). AP-1: a double-edged sword in tumorigenesis. *Nat. Rev. Cancer* 3, 859-868.
- Falck,J., Coates,J., and Jackson,S.P. (2005). Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* 434, 605-611.
- Farooq,A., Plotnikova,O., Chaturvedi,G., Yan,S., Zeng,L., Zhang,Q., and Zhou,M.M. (2003). Solution structure of the MAPK phosphatase PAC-1 catalytic domain. Insights into substrate-induced enzymatic activation of MKP. *Structure*. 11, 155-164.
- Fleischmann,A., Hafezi,F., Elliott,C., Reme,C.E., Ruther,U., and Wagner,E.F. (2000). Fra-1 replaces c-Fos-dependent functions in mice. *Genes Dev.* 14, 2695-2700.
- Garber,M.E., Troyanskaya,O.G., Schluens,K., Petersen,S., Thaesler,Z., Pacyna-Gengelbach,M., van de,R.M., Rosen,G.D., Perou,C.M., Whyte,R.I., Altman,R.B., Brown,P.O., Botstein,D., and Petersen,I. (2001). Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. U. S. A* 98, 13784-13789.
- Garrington,T.P. and Johnson,G.L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* 11, 211-218.
- Guo,Y.L., Baysal,K., Kang,B., Yang,L.J., and Williamson,J.R. (1998). Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. *J. Biol. Chem.* 273, 4027-4034.
- Hai,T. and Curran,T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. U. S. A* 88, 3720-3724.
- Hai,T., Wolfgang,C.D., Marsee,D.K., Allen,A.E., and Sivaprasad,U. (1999). ATF3 and stress responses. *Gene Expr.* 7, 321-335.
- Hai,T. and Hartman,M.G. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 273, 1-11.
- Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E., and Leder,P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* 55, 917-924.
- Hayakawa,J., Depatie,C., Ohmichi,M., and Mercola,D. (2003). The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. *J. Biol. Chem.* 278, 20582-20592.
- Hayakawa,J., Mittal,S., Wang,Y., Korkmaz,K.S., Adamson,E., English,C., Ohmichi,M., McClelland,M., and Mercola,D. (2004). Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. *Mol. Cell* 16, 521-535.
- Hess,J., Angel,P., and Schorpp-Kistner,M. (2004). AP-1 subunits: quarrel and harmony among siblings. *J. Cell Sci.* 117, 5965-5973.
- Higgins,J.P., Shinghal,R., Gill,H., Reese,J.H., Terris,M., Cohen,R.J., Fero,M., Pollack,J.R., van de,R.M., and Brooks,J.D. (2003). Gene expression patterns in renal cell carcinoma assessed by complementary DNA microarray. *Am. J. Pathol.* 162, 925-932.
- Hochedlinger,K., Wagner,E.F., and Sabapathy,K. (2002). Differential effects of JNK1 and JNK2 on signal specific induction of apoptosis. *Oncogene* 21, 2441-2445.
- Ip,Y.T. and Davis,R.J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr. Opin. Cell Biol.* 10, 205-219.
- Ishiguro,T., Nagawa,H., Naito,M., and Tsuruo,T. (2000). Inhibitory effect of ATF3 antisense oligonucleotide on ectopic growth of HT29 human colon cancer cells. *Jpn. J. Cancer Res.* 91, 833-836.
- Jazayeri,A., Falck,J., Lukas,C., Bartek,J., Smith,G.C., Lukas,J., and Jackson,S.P. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* 8, 37-45.
- Kallunki,T., Deng,T., Hibi,M., and Karin,M. (1996). c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* 87, 929-939.
- Kallunki,T., Deng,T., Hibi,M., and Karin,M. (1996). c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* 87, 929-939.
- Karin,M., Liu,Z., and Zandi,E. (1997). AP-1 function and regulation. *Curr. Opin. Cell Biol.* 9, 240-246.

- Kastan, M.B. and Bartek, J. (2004). Cell-cycle checkpoints and cancer. *Nature* 432, 316-323.
- Keyse, S.M. (2000). Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Curr. Opin. Cell Biol.* 12, 186-192.
- Kharbanda, S., Ren, R., Pandey, P., Shafman, T.D., Feller, S.M., Weichselbaum, R.R., and Kufe, D.W. (1995). Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature* 376, 785-788.
- Kolbus, A., Herr, I., Schreiber, M., Debatin, K.M., Wagner, E.F., and Angel, P. (2000). c-Jun-dependent CD95-L expression is a rate-limiting step in the induction of apoptosis by alkylating agents. *Mol. Cell Biol.* 20, 575-582.
- Kouzarides, T. and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. *Nature* 336, 646-651.
- Kumagai, A., Lee, J., Yoo, H.Y., and Dunphy, W.G. (2006). TopBP1 activates the ATR-ATRIP complex. *Cell* 124, 943-955.
- Kyriakis, J.M. and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* 81, 807-869.
- Lackinger, D., Eichhorn, U., and Kaina, B. (2001). Effect of ultraviolet light, methyl methanesulfonate and ionizing radiation on the genotoxic response and apoptosis of mouse fibroblasts lacking c-Fos, p53 or both. *Mutagenesis* 16, 233-241.
- Lam, M.H., Liu, Q., Elledge, S.J., and Rosen, J.M. (2004). Chk1 is haploinsufficient for multiple functions critical to tumor suppression. *Cancer Cell* 6, 45-59.
- Le Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F.X., Green, D.R., and Karin, M. (1999). Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol. Cell Biol.* 19, 751-763.
- Lees-Miller, S.P. and Meek, K. (2003). Repair of DNA double strand breaks by non-homologous end joining. *Biochimie* 85, 1161-1173.
- Lewis, T.S., Shapiro, P.S., and Ahn, N.G. (1998). Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* 74, 49-139.
- Liang, G., Wolfgang, C.D., Chen, B.P., Chen, T.H., and Hai, T. (1996). ATF3 gene. Genomic organization, promoter, and regulation. *J. Biol. Chem.* 271, 1695-1701.
- Liu, Y., Liu, Y., Yang, Z., Utzat, C., Wang, G., Basu, A.K., and Zou, Y. (2005). Cooperative interaction of human XPA stabilizes and enhances specific binding of XPA to DNA damage. *Biochemistry* 44, 7361-7368.
- Lu, D., Wolfgang, C.D., and Hai, T. (2006). Activating transcription factor 3, a stress-inducible gene, suppresses Ras-stimulated tumorigenesis. *J. Biol. Chem.* 281, 10473-10481.
- Mansouri, A., Ridgway, L.D., Korapati, A.L., Zhang, Q., Tian, L., Wang, Y., Siddik, Z.H., Mills, G.B., and Claret, F.X. (2003). Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to Fas ligand induction and cell death in ovarian carcinoma cells. *J. Biol. Chem.* 278, 19245-19256.
- Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80, 179-185.
- Matsuo, K., Owens, J.M., Tonko, M., Elliott, C., Chambers, T.J., and Wagner, E.F. (2000). Fos1 is a transcriptional target of c-Fos during osteoclast differentiation. *Nat. Genet.* 24, 184-187.
- Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., Gillieron, C., Boschert, U., Vial-Knecht, E., Martinou, J.C., and Arkinstall, S. (1997). Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J. Biol. Chem.* 272, 25238-25242.
- Mayr, B. and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* 2, 599-609.
- Mechta, F., Lallemand, D., Pfarr, C.M., and Yaniv, M. (1997). Transformation by ras modifies AP1 composition and activity. *Oncogene* 14, 837-847.
- Milde-Langosch, K. (2005). The Fos family of transcription factors and their role in tumorigenesis. *Eur. J. Cancer* 41, 2449-2461.
- Mitchell, J.R., Hoeijmakers, J.H., and Niedernhofer, L.J. (2003). Divide and conquer: nucleotide excision repair battles cancer and ageing. *Curr. Opin. Cell Biol.* 15, 232-240.
- Monje, P., Marinissen, M.J., and Gutkind, J.S. (2003). Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. *Mol. Cell Biol.* 23, 7030-7043.
- Morrison, D.K. and Davis, R.J. (2003). Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annu. Rev. Cell Dev. Biol.* 19, 91-118.
- Morton, S., Davis, R.J., McLaren, A., and Cohen, P. (2003). A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. *EMBO J.* 22, 3876-3886.
- Murphy, L.O., Smith, S., Chen, R.H., Fingar, D.C., and Blenis, J. (2002). Molecular interpretation of ERK signal duration by immediate early gene products. *Nat. Cell Biol.* 4, 556-564.
- Musti, A.M., Treier, M., and Bohmann, D. (1997). Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science* 275, 400-402.
- Nateri, A.S., Riera-Sans, L., Da Costa, C., and Behrens, A. (2004). The ubiquitin ligase SCFFbw7

- antagonizes apoptotic JNK signaling. *Science* 303, 1374-1378.
- Nehme,A., Baskaran,R., Aebi,S., Fink,D., Nebel,S., Cenni,B., Wang,J.Y., Howell,S.B., and Christen,R.D. (1997). Differential induction of c-Jun NH2-terminal kinase and c-Abl kinase in DNA mismatch repair-proficient and -deficient cells exposed to cisplatin. *Cancer Res.* 57, 3253-3257.
- Nordstrand,L.M., Ringvoll,J., Larsen,E., and Klungland,A. (2007). Genome instability and DNA damage accumulation in gene-targeted mice. *Neuroscience* 145, 1309-1317.
- Ogawa,S., Lozach,J., Jepsen,K., Sawka-Verhelle,D., Perissi,V., Sasik,R., Rose,D.W., Johnson,R.S., Rosenfeld,M.G., and Glass,C.K. (2004). A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1-dependent gene networks required for macrophage activation. *Proc. Natl. Acad. Sci. U. S. A* 101, 14461-14466.
- Ouwens,D.M., de Ruiter,N.D., van der Zon,G.C., Carter,A.P., Schouten,J., van der,B.C., Kooistra,K., Bos,J.L., Maassen,J.A., and van Dam,H. (2002). Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO J.* 21, 3782-3793.
- Owens,D.M. and Keyse,S.M. (2007). Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* 26, 3203-3213.
- Papavassiliou,A.G., Treier,M., and Bohmann,D. (1995). Intramolecular signal transduction in c-Jun. *EMBO J.* 14, 2014-2019.
- Park,C.J. and Choi,B.S. (2006). The protein shuffle. Sequential interactions among components of the human nucleotide excision repair pathway. *FEBS J.* 273, 1600-1608.
- Passegue,E. and Wagner,E.F. (2000). JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. *EMBO J.* 19, 2969-2979.
- Petrini,J.H. and Stracker,T.H. (2003). The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends Cell Biol.* 13, 458-462.
- Potapova,O., Haghighi,A., Bost,F., Liu,C., Birrer,M.J., Gjerset,R., and Mercola,D. (1997). The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J. Biol. Chem.* 272, 14041-14044.
- Potapova,O., Basu,S., Mercola,D., and Holbrook,N.J. (2001). Protective role for c-Jun in the cellular response to DNA damage. *J. Biol. Chem.* 276, 28546-28553.
- Roos,W.P. and Kaina,B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol. Med.* 12, 440-450.
- Saxena,M. and Mustelin,T. (2000). Extracellular signals and scores of phosphatases: all roads lead to MAP kinase. *Semin. Immunol.* 12, 387-396.
- Schaeffer,H.J., Catling,A.D., Eblen,S.T., Collier,L.S., Krauss,A., and Weber,M.J. (1998). MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* 281, 1668-1671.
- Schaeffer,H.J. and Weber,M.J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* 19, 2435-2444.
- Schreiber,M., Baumann,B., Cotten,M., Angel,P., and Wagner,E.F. (1995). Fos is an essential component of the mammalian UV response. *EMBO J.* 14, 5338-5349.
- Schreiber,M., Poirier,C., Franchi,A., Kurzbauer,R., Guenet,J.L., Carle,G.F., and Wagner,E.F. (1997). Structure and chromosomal assignment of the mouse fra-1 gene, and its exclusion as a candidate gene for oc (osteosclerosis). *Oncogene* 15, 1171-1178.
- Schreiber,M., Kolbus,A., Piu,F., Szabowski,A., Mohle-Steinlein,U., Tian,J., Karin,M., Angel,P., and Wagner,E.F. (1999). Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev.* 13, 607-619.
- Shaulian,E., Schreiber,M., Piu,F., Beeche,M., Wagner,E.F., and Karin,M. (2000). The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell* 103, 897-907.
- Shaulian,E. and Karin,M. (2001). AP-1 in cell proliferation and survival. *Oncogene* 20, 2390-2400.
- Shaulian,E. and Karin,M. (2002). AP-1 as a regulator of cell life and death. *Nat. Cell Biol.* 4, E131-E136.
- Shiloh,Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer* 3, 155-168.
- Smits,V.A., Reaper,P.M., and Jackson,S.P. (2006). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Curr. Biol.* 16, 150-159.
- Sonobe,M.H., Yoshida,T., Murakami,M., Kameda,T., and Iba,H. (1995). fra-2 promoter can respond to serum-stimulation through AP-1 complexes. *Oncogene* 10, 689-696.
- Sugasawa,K., Ng,J.M., Masutani,C., Iwai,S., van der Spek,P.J., Eker,A.P., Hanaoka,F., Bootsma,D., and Hoeijmakers,J.H. (1998). Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol. Cell* 2, 223-232.
- Suzuki,T., Okuno,H., Yoshida,T., Endo,T., Nishina,H., and Iba,H. (1991). Difference in transcriptional regulatory function between c-Fos and Fra-2. *Nucleic Acids Res.* 19, 5537-5542.
- Syljuasen,R.G., Sorensen,C.S., Hansen,L.T., Fugger,K., Lundin,C., Johansson,F., Helleday,T., Sehested,M., Lukas,J., and Bartek,J. (2005). Inhibition of human Chk1 causes increased initiation

- of DNA replication, phosphorylation of ATR targets, and DNA breakage. *Mol. Cell Biol.* 25, 3553-3562.
- Tang, J.Y., Hwang, B.J., Ford, J.M., Hanawalt, P.C., and Chu, G. (2000). Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis. *Mol. Cell* 5, 737-744.
- Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000). A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.* 2, 110-116.
- Thoma, B.S. and Vazquez, K.M. (2003). Critical DNA damage recognition functions of XPC-hHR23B and XPA-RPA in nucleotide excision repair. *Mol. Carcinog.* 38, 1-13.
- Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Bar-Sagi, D., Jones, S.N., Flavell, R.A., and Davis, R.J. (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 288, 870-874.
- Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.* 4, 96-101.
- Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.* 8, 205-215.
- Turjanski, A.G., Vaque, J.P., and Gutkind, J.S. (2007). MAP kinases and the control of nuclear events. *Oncogene* 26, 3240-3253.
- Vallone, D., Battista, S., Pierantoni, G.M., Fedele, M., Casalino, L., Santoro, M., Viglietto, G., Fusco, A., and Verde, P. (1997). Neoplastic transformation of rat thyroid cells requires the *junB* and *fra-1* gene induction which is dependent on the *HMGI-C* gene product. *EMBO J.* 16, 5310-5321.
- van Dam, H., Duyndam, M., Rottier, R., Bosch, A., Vries-Smits, L., Herrlich, P., Zanetti, A., Angel, P., and van der Eb, A.J. (1993). Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO J.* 12, 479-487.
- van Dam, H. and Castellazzi, M. (2001). Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. *Oncogene* 20, 2453-2464.
- van, d.B., V, Jaspers, N.G., and Vermeulen, W. (2002). When machines get stuck--obstructed RNA polymerase II: displacement, degradation or suicide. *Bioessays* 24, 780-784.
- Ventura, J.J., Hubner, A., Zhang, C., Flavell, R.A., Shokat, K.M., and Davis, R.J. (2006). Chemical genetic analysis of the time course of signal transduction by JNK. *Mol. Cell* 21, 701-710.
- Wada, T. and Penninger, J.M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 23, 2838-2849.
- Wagner, E.F. (2001). AP-1--Introductory remarks. *Oncogene* 20, 2334-2335.
- Wagner, E.F. and Eferl, R. (2005). Fos/AP-1 proteins in bone and the immune system. *Immunol. Rev.* 208, 126-140.
- Weiss, C., Schneider, S., Wagner, E.F., Zhang, X., Seto, E., and Bohmann, D. (2003). JNK phosphorylation relieves HDAC3-dependent suppression of the transcriptional activity of c-Jun. *EMBO J.* 22, 3686-3695.
- Weitzman, J.B., Fiette, L., Matsuo, K., and Yaniv, M. (2000). JunD protects cells from p53-dependent senescence and apoptosis. *Mol. Cell* 6, 1109-1119.
- Wenzel, A., Grimm, C., Marti, A., Kueng-Hitz, N., Hafezi, F., Niemeyer, G., and Reme, C.E. (2000). c-fos controls the "private pathway" of light-induced apoptosis of retinal photoreceptors. *J. Neurosci.* 20, 81-88.
- Wijnhoven, S.W., Hoogervorst, E.M., de Waard, H., van der Horst, G.T., and van Steeg, H. (2007). Tissue specific mutagenic and carcinogenic responses in NER defective mouse models. *Mutat. Res.* 614, 77-94.
- Wisdom, R. (1999). AP-1: one switch for many signals. *Exp. Cell Res.* 253, 180-185.
- Wisdom, R., Johnson, R.S., and Moore, C. (1999). c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *EMBO J.* 18, 188-197.
- Wolfman, J.C., Palmby, T., Der, C.J., and Wolfman, A. (2002). Cellular N-Ras promotes cell survival by downregulation of Jun N-terminal protein kinase and p38. *Mol. Cell Biol.* 22, 1589-1606.
- Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. (1998). JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. *Genes Dev.* 12, 3369-3381.
- Yamamoto, K., Ichijo, H., and Korsmeyer, S.J. (1999). BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol. Cell Biol.* 19, 8469-8478.
- Yan, C., Lu, D., Hai, T., and Boyd, D.D. (2005). Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination. *EMBO J.* 24, 2425-2435.
- Yan, C., Lu, D., Hai, T., and Boyd, D.D. (2005). Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination. *EMBO J.* 24, 2425-2435.
- Yan, C. and Boyd, D.D. (2006). ATF3 regulates the stability of p53: a link to cancer. *Cell Cycle* 5, 926-929.
- Yang, J., Xu, Z.P., Huang, Y., Hamrick, H.E., Duerksen-Hughes, P.J., and Yu, Y.N. (2004). ATM and ATR: sensing DNA damage. *World J. Gastroenterol.* 10, 155-160.
- Yasuda, J., Whitmarsh, A.J., Cavanagh, J., Sharma, M., and Davis, R.J. (1999). The JIP group of mitogen-

activated protein kinase scaffold proteins. *Mol. Cell Biol.* 19, 7245-7254.

Yin,X., Dewille,J.W., and Hai,T. (2007). A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development. *Oncogene*.

Yoshioka,K., Deng,T., Cavigelli,M., and Karin,M. (1995). Antitumor promotion by phenolic antioxidants: inhibition of AP-1 activity through induction of Fra expression. *Proc. Natl. Acad. Sci. U. S. A* 92, 4972-4976.

You,Z., Chahwan,C., Bailis,J., Hunter,T., and Russell,P. (2005). ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol. Cell Biol.* 25, 5363-5379.

Young,M.R., Nair,R., Bucheimer,N., Tulsian,P., Brown,N., Chapp,C., Hsu,T.C., and Colburn,N.H. (2002). Transactivation of Fra-1 and consequent activation of AP-1 occur extracellular signal-regulated kinase dependently. *Mol. Cell Biol.* 22, 587-598.

Zachos,G., Rainey,M.D., and Gillespie,D.A. (2003). Chk1-deficient tumour cells are viable but exhibit

multiple checkpoint and survival defects. *EMBO J.* 22, 713-723.

Zenz,R. and Wagner,E.F. (2006). Jun signalling in the epidermis: From developmental defects to psoriasis and skin tumors. *Int. J. Biochem. Cell Biol.* 38, 1043-1049.

Zhang,C., Gao,C., Kawauchi,J., Hashimoto,Y., Tsuchida,N., and Kitajima,S. (2002). Transcriptional activation of the human stress-inducible transcriptional repressor ATF3 gene promoter by p53. *Biochem. Biophys. Res. Commun.* 297, 1302-1310.

Zhang,Y.W., Hunter,T., and Abraham,R.T. (2006). Turning the replication checkpoint on and off. *Cell Cycle* 5, 125-128.

Zhou,B.B. and Elledge,S.J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature* 408, 433-439.

Zou,L. and Elledge,S.J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 300, 1542-1548.

Chapter II

Induction of ATF3 by ionizing radiation is mediated via a signaling pathway that includes ATM, Nibrin1, stress-induced MAPkinases and ATF-2

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Induction of ATF3 by ionizing radiation is mediated via a signaling pathway that includes ATM, Nibrin1, stress-induced MAPkinases and ATF-2

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Exposure of human cells to genotoxic agents induces various signaling pathways involved in the execution of stress- and DNA-damage responses. Inappropriate functioning of the DNA-damage response to ionizing radiation (IR) is associated with the human diseases ataxia-telangiectasia (A-T) and Nijmegen Breakage syndrome (NBS). Here, we show that IR efficiently induces Jun/ATF transcription factor activity in normal human diploid fibroblasts, but not in fibroblasts derived from A-T and NBS patients. IR was found to enhance the expression of c-Jun and, in particular, ATF3, but, in contrast to various other stress stimuli, did not induce the expression of c-Fos. Using specific inhibitors, we found that the ATM- and Nibrin1-dependent activation of ATF3 does neither require p53 nor reactive oxygen species, but is dependent on the p38 and JNK MAPkinases. Via these kinases, IR activates ATF-2, one of the transcription factors acting on the *atf3* promoter. The activation of ATF-2 by IR resembles ATF-2 activation by certain growth factors, since IR mainly induced the second step of ATF-2 phosphorylation via the stress-inducible MAPkinases, phosphorylation of Thr69. As IR does not enhance ATF-2 phosphorylation in ATM and Nibrin1-deficient cells, both ATF-2 and ATF3 seem to play an important role in the protective response of human cells to IR.

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Introduction

Exposure of cells to genotoxic agents like ionizing radiation (IR) and UV light causes multiple types of cell damage. Both IR and UV cause DNA lesions, IR primarily causing DNA strand breaks, whereas UV light predominantly induces pyrimidine dimers and 6-4 photoproducts (Pfeiffer *et al.*, 1996). IR and UV treatment also trigger the generation of reactive oxygen species (ROS), which can cause lipid peroxidation, glutathione depletion and oxidation of proteins (Mer-

curio and Manning, 1999). The cell responds to this damage by activating multiple signaling pathways, resulting in the induction of genetic programs mediating the response to the afflicted damage.

One of the main protective responses to IR and UV is the induction of cell cycle arrest via the activation of cell cycle checkpoints. This enables cells to recognize and repair DNA and other damage before DNA synthesis or mitosis is continued (reviewed in Zhou and Elledge, 2000). An essential signaling enzyme in the activation of cell cycle arrest by IR or radiomimetic agents is the ATM kinase. In ATM-negative cells, IR cannot efficiently stabilize and activate transcription factor p53. This results in suboptimal and retarded induction of p53 target genes like p21^{CIP1/Waf1} and, as a consequence, inappropriate G1 arrest (Bar Shira *et al.*, 2002, reviewed in Rotman and Shiloh, 1999). In humans, homozygous mutation of the *Atm* gene causes ataxia-telangiectasia (A-T), a pleiotropic disease characterized by neurodegeneration, immunodeficiency, premature aging, increased cancer risk and extreme sensitivity to ionizing radiation (reviewed in Rotman and Shiloh, 1999). A second component of the IR response is the Nibrin1 (NBS1) protein, mutation of which causes Nijmegen Breakage Syndrome (NBS) (Varon *et al.*, 1998). Both A-T and NBS cells are hypersensitive to IR, show defects in radiation-induced Ckh2 kinase-dependent G2/M arrest as well as in S phase arrest, resulting in radioresistant DNA synthesis. Recent studies showed that the ATM kinase phosphorylates Nibrin1 and that the ATM and Nibrin1 proteins cooperate in mediating IR-induced S phase and G2/M arrest (Lim *et al.*, 2000; Wu *et al.*, 2000; Zhao *et al.*, 2000; Buscemi *et al.*, 2001; Kim *et al.*, 2002; Yazdi *et al.*, 2002).

The MAPK–AP-1 network represents another genotoxic stress-induced signaling pathway implicated in protection against cell damage. Treatment of cells with UV or chemical carcinogens activates the JNK/SAPK and p38 MAPKinases through receptor tyrosine kinases and/or cytoplasmic stress-sensors (Sachsenmaier *et al.*, 1994; Coffey *et al.*, 1995; Rosette and Karin, 1996; Wilhelm *et al.*, 1997). The receptor-dependent activation of JNK and p38 by UV occurs within 15 min, is independent of DNA damage and depends largely on UV-induced oxidative stress (Devary *et al.*, 1992, 1993;

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Sachsenmaier *et al.*, 1994; Knebel *et al.*, 1996; Gross *et al.*, 1999). JNK and p38 subsequently phosphorylate and thereby activate the transcription factors c-Jun, ATF-2 and TCF/elk1, which induce, among others, the promoters of the *c-jun*, *c-fos* and *atf3* genes (reviewed by Hai *et al.*, 1999; Shaulian and Karin, 2001). Together with the other Jun, Fos and (some) ATF family members (JunB, JunD, FosB, Fra-1, Fra-2, ATFa and ATF3) c-Jun, c-Fos and ATF-2 form homo- or heterodimeric complexes collectively called AP-1 transcription factors (Shaulian and Karin, 2001; van Dam and Castella, 2001). These AP-1 dimers can be divided into two distinct classes, Jun/Fos and Jun/ATF. Jun/Fos dimers preferentially bind to 7bp TGAGTCA motifs, the 'classical' AP-1 sites, whereas Jun/ATF (and ATF/ATF) dimers only efficiently bind to 8 bp TGACNTCA ATF-consensus sites. Depending on their composition, the cell type and the cellular context, AP-1 transcription factors can regulate cell proliferation, differentiation and apoptosis both positively and negatively (for reviews *Oncogene* 20(19), 2001). Studies with knockout fibroblasts indicate that the role of AP-1 in the response to genotoxic stress depends on the nature and dose of stress. c-Jun activation by low doses of UV allows mouse fibroblasts to exit from p53-imposed growth arrest after UV-C treatment (Shaulian *et al.*, 2000). However, upon exposure to high levels of UV-C or alkylating agents, c-Jun can induce apoptosis in these cells (reviewed by Shaulian and Karin, 2001). Under the same conditions, c-Fos protects mouse fibroblasts against treatment with UV or alkylating agents (Haas and Kaina, 1995; Schreiber *et al.*, 1995).

Relatively little is known about the role of the ATF family members in the cellular response to genotoxic stress, although ATF-2 has been reported to confer resistance to melanoma cells upon treatment with UV, IR or radiomimetic agents (Ronai *et al.*, 1998). The c-Jun/ATF-2 target gene *atf3* can regulate cell proliferation both positively and negatively (Allan *et al.*, 2001; Mashima *et al.*, 2001; Perez *et al.*, 2001) and its expression is strongly induced by both growth factors and stresses (reviewed by Hai *et al.*, 1999). ATF3 can act both as a repressor and as an activator of transcription, depending on its dimerization partner and the promoter context (Hsu *et al.*, 1992; Wolfgang *et al.*, 2000; Allan *et al.*, 2001). A recent report of Yan *et al.* (2002) suggests that ATF3 can modulate growth arrest and gene activation by p53.

In contrast to the effects of UV and a number of other genotoxic stresses, the effects of ionizing radiation on the various AP-1 family members, in particular on the ATF proteins, are hardly known. Here, we show that in primary human fibroblasts, IR efficiently induces Jun/ATF-2 and Jun/ATF3 activity via ATF-2-Thr69 + 71 phosphorylation and *atf3* gene induction, respectively. However, in contrast to UV, IR does not induce c-Fos and only slightly induces Fra1. The IR-induced signaling pathway to ATF-2 and *atf3* required functional ATM and Nibrin1 proteins, but not p53 and reactive oxygen species. In addition, the activation of ATF3 by IR was dependent on the p38 and JNK MAPkinases,

who phosphorylate ATF-2-Thr69 rather than ATF-2-Thr71 in response to X-rays. The absence of ATF3 induction and ATF-2-Thr69 + 71 phosphorylation after IR treatment of A-T and NBS cells might contribute to the extreme radiosensitivity of these cells.

Results

Selective induction of the AP-1 components c-Jun and ATF3 by ionizing radiation

AP-1 (Jun, Fos, ATF) transcription factors play an important role in the cellular responses to genotoxic stresses. However, the effects of IR on the various Jun/Fos and Jun/ATF complexes are hardly known, in contrast to the effects of, for instance, UV light. We therefore analyzed the effects of IR on c-Jun, Fos and ATF family members in primary human diploid fibroblasts. At 4–8 h after irradiation, the total levels of c-Jun and ATF3 were increased in the IR-treated cells, whereas enhanced c-Jun-Ser73 phosphorylation was already detected at 2 h (Figure 1). The increase in c-Jun-phosphorylation by IR was much weaker than the increase upon UV irradiation, which also resulted in mobility-shifted c-Jun (indicated by the two arrows) representative for c-Jun hyperphosphorylation. The levels of Fra1 only showed a very minor increase after IR compared to the mock treatment, whereas induction of c-Fos was only detectable upon UV irradiation. In contrast to their effects on c-Jun and ATF3, IR and UV did not significantly influence ATF-2 expression. However, the electrophoretic mobility of ATF-2 was reduced, probably because of changes in phosphorylation (see below). These results show that IR and UV induce different spectra of AP-1 in human fibroblasts,

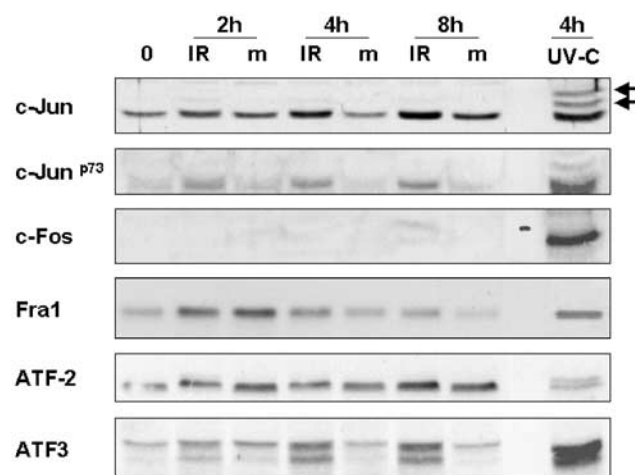


Figure 1 Differential effects of IR on AP-1 family members. Serum-starved primary human diploid fibroblasts derived from a healthy individual were nontreated (0), irradiated with 30 Gy IR, mock treated for IR (m) or irradiated with 10J/m² UV-C light (UV-C). Protein lysates were prepared 2, 4 and 8 h after treatment and subjected to gel electrophoresis followed by immunoblotting using the indicated antibodies

that is, IR induces mainly c-Jun/ATF3 activity, whereas UV induces both c-Jun/c-Fos and c-Jun/ATF3.

Distinct kinetics of ATF3 induction by IR and serum

As ATF3 was the most pronounced IR-induced AP-1 component in human diploid fibroblasts, we next investigated the mechanism of this induction. *atf3* mRNA levels were found to increase around 2 h after IR treatment and to peak at 6–8 h (Figure 2a). The *atf3* mRNA induction was found to resemble both in strength and kinetics the IR induction of *p21* and *gadd45* mRNA, two genes involved in IR-induced cell cycle arrest and apoptosis (Kastan *et al.*, 1992; El-Deiry *et al.*, 1994) (Figure 2a and data not shown). Compared to the induction of *atf3* by serum, which is maximal at 1 h, the induction of *atf3* by IR is relatively slow. IR induction of ATF3 was inhibited when cells were preincubated with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), an inhibitor of RNA polymerase II elongation (Tamm *et al.*, 1976; Zandomeni *et al.*, 1982), indicating that it is mediated predominantly at the level of transcription (Figure 2b).

IR-induction of ATF3 is not dependent on oxidative stress

Many ATF3-inducing agents have been reported to activate gene expression via ROS and/or oxidative stress (Hai, 1999; Mercurio and Manning, 1999). To investigate whether such molecules are also essential intermediates in *atf3* gene induction by IR, cells were preincubated with 30 mM of the active oxygen scavenger N-acetylcysteine (NAC). As depicted in Figure 3, NAC completely blocked the induction of ATF3 by H_2O_2 , an agent inducing high levels of oxidative stress, whereas the induction of ATF3 by UV was inhibited more than twofold. In contrast, NAC pretreatment did not at all prevent IR induction of ATF3. In fact, the oxygen scavenger enhanced the activation of ATF3 by IR. This suggests that IR induces ATF3 via a different mechanism than H_2O_2 and UV-C, independent of oxidative stress and ROS.

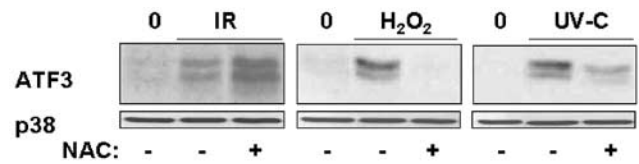


Figure 3 IR-induction of ATF3 is not inhibited by NAC. Primary WT human fibroblasts were incubated with (+) or without (–) 30 mM of NAC prior to treatment with 30 Gy of IR, 5 μ M H_2O_2 or 10 J/m² UV-C. Protein lysates were made 6 h after IR and ATF3 induction was determined by Western blot analysis. As a control for equal loading, the levels of p38 are depicted, which are not affected by the treatments

ism than H_2O_2 and UV-C, independent of oxidative stress and ROS.

ATF3 induction after IR requires ATM but not p53

To examine whether the ATM protein, a key player in regulation of the cellular response to double-strand DNA breaks is involved in induction of ATF3 after IR, ATF3 induction was analysed in human diploid A-T fibroblasts. Interestingly, these ATM-deficient cells did not show a detectable increase in *atf3* mRNA and protein after IR, while induction by UV was as strong as in wild-type (WT) fibroblasts (Figures 4a and b). These results indicate that the induction of ATF3 by IR, but not UV, requires functional ATM.

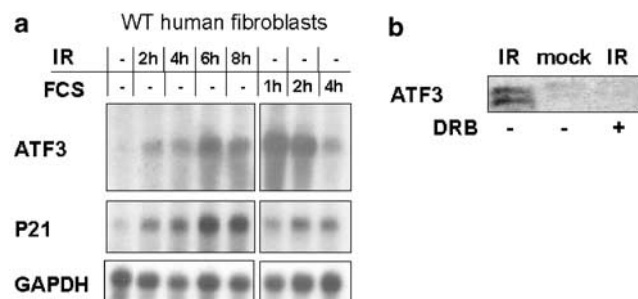


Figure 2 Prolonged ATF3 mRNA by IR. (a) Primary WT human fibroblasts were serum-starved and subsequently irradiated with 30 Gy IR or treated with 20% fetal calf serum (FCS). Induction of *atf3* and *p21*^{CIP1/Waf1} mRNA was analysed by Northern blot analysis. *gapdh* expression was examined as a control for equal loading. (b) ATF3 protein induction by IR in the absence (–) or presence (+) of the transcription inhibitor DRB. Primary WT human fibroblasts were treated with DRB 2 h prior to IR or mock treatment. At 6 h after IR, cell lysates were harvested and analysed by Western blotting

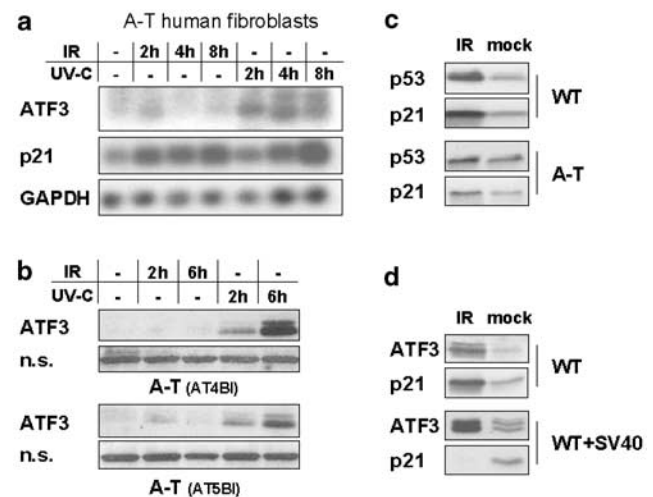


Figure 4 ATF3 induction after IR requires ATM but not p53. (a) Serum-starved AT4BI primary fibroblasts, derived from A-T patients, were treated with 30 Gy IR or 10 J/m² UV-C. Induction of *atf3* and *p21*^{CIP1/Waf1} mRNA was subsequently analysed by Northern blot analysis. *gapdh* expression was examined as a loading control. (b) Primary fibroblasts from two different A-T patients (AT4BI and AT5BI) were treated with IR or UV-C as described under (a). ATF3 protein levels were subsequently determined by Western blotting analysis. A nonspecific band bound by the ATF3 antibody (n.s.) is shown as a control for equal loading. (c) Western blot analysis showing p53 and p21 protein levels in WT and A-T human fibroblasts (A-T), described under (a) at 4 h (p53) or 8 h (p21) after IR or mock treatment (mock). (d) Primary human fibroblasts (WT) or their SV40-transformed derivatives (WT+SV40) were IR- or mock-treated and protein lysates were made after 8 h. ATF3 and p21 expression was subsequently determined by Western blotting analysis

The ATM kinase has also been found to be required for efficient IR-induced activation of the tumor suppressor protein p53, although residual induction of p53 and activation of the p53 target gene *p21* by IR can be detected in ATM-deficient cells approximately 2 h after irradiation (Khanna *et al.*, 1995; Jongmans *et al.*, 1997; reviewed in Abraham, 2001). As p53 is one of the transcription factors that can mediate *atf3* gene activation (Amundson *et al.*, 1999), we next examined the role of p53 in the IR induction of ATF3. Although to a lesser extent than in WT cells, p53 and p21 were still induced by IR in ATM-deficient cells, whereas ATF3 induction is completely abrogated (Figure 4a and c). This suggests that in human diploid fibroblasts, p53 activation is not sufficient for IR induction of ATF3. To further address the involvement of p53, we examined ATF3 induction in normal human fibroblasts transformed by SV40 T/t antigen, which functionally inactivates p53, and thereby inhibits the induction of p21 (Figure 4d). Importantly, ATF3 induction by IR was unaffected in these SV40-transformed cells (Figure 4d). From these experiments, we conclude that in human fibroblasts activation of ATF3 expression by IR requires functional ATM but not functional p53.

IR-induction of ATF3 is absent in NBS cells

Recent studies indicate that the ATM and the NBS protein Nibrin1 cooperate in the cellular response to IR. Indeed, similar as found for ATM-deficient cells, induction of ATF3 protein and mRNA by IR was absent in Nibrin1-deficient fibroblasts, whereas the induction of ATF3 by UV was not affected (Figure 5a and b). In contrast to ATF3, p21 was efficiently induced by IR in the NBS cells (Figure 5), indicating that p53 is sufficiently activated by IR in the absence of Nibrin1, which is in agreement with previous studies (Yamazaki *et al.*, 1998; Lim *et al.*, 2000). Thus, absence of ATM and Nibrin1 in human diploid fibroblasts does not cause a general defect in IR-induced gene expression, but rather specifically blocks IR-induced activation of the *atf3* gene.

ATF3 induction by IR involves phosphorylation of ATF-2-Thr69 + 71 by p38 and JNK

We next examined the role of the MAPkinases in the induction of ATF3 by IR. Western blot analysis with phospho-specific antibodies showed that IR predominantly activates p38 (Figure 6a). IR activated JNK only very weakly, whereas it did not further enhance the relatively high basal activity of ERK in the primary human fibroblasts. As the activation of p38 and JNK by IR was much less strong than their activation by UV (Figure 6a), we tested whether it is functionally relevant for ATF3 activation. For this purpose, cells were treated with two specific inhibitors of p38 and JNK, SB203580 and SP600125, respectively (Cuenda *et al.*, 1995; Bennett *et al.*, 2001). As shown in the right panel of Figure 6b, pretreatment with only the p38 inhibitor (lanes marked 'SB') did not inhibit, but in fact slightly enhanced ATF3

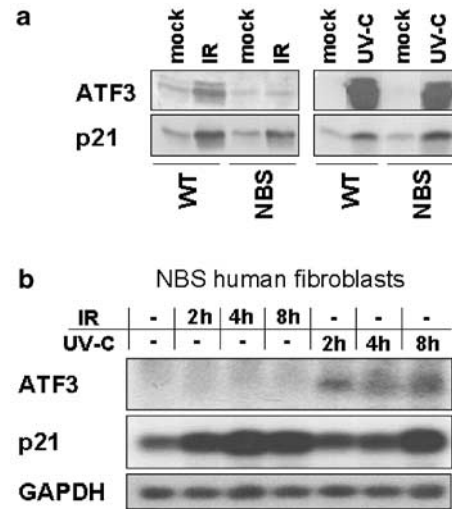


Figure 5 NBS cells are defective for ATF3 induction by IR. (a) Serum-starved normal primary fibroblasts (WT) or primary fibroblasts derived from NBS patients (NBS) were treated with 30 Gy IR, 10 J/m² UV-C or mock-treated as indicated. Protein lysates were prepared 8 h later, and ATF3 and p21 protein levels were determined by western blotting analysis. (b) Northern blot analysis of *atf3* and *p21*^{CIP1/Waf1} mRNA expression in the IR- or UV-C-induced Nibrin1-deficient fibroblasts described under (a). *gapdh* expression was examined as a control for equal loading

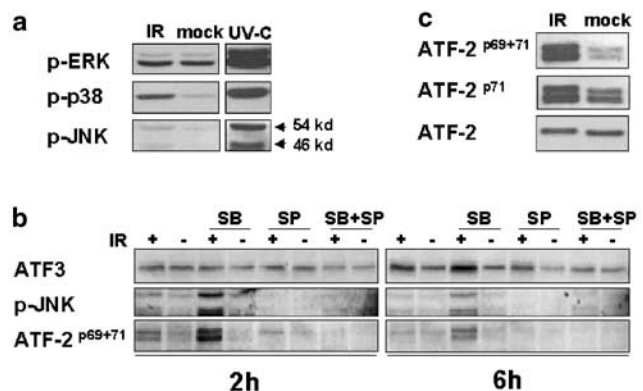


Figure 6 ATF3 induction by IR requires JNK and p38 MAP kinases. (a) Western blot analysis of the effects of IR and UV-C on ERK, SAPK/JNK and p38 phosphorylation in primary human fibroblasts. Cells were irradiated with IR, UV-C or mock-treated as described in Figure 2 and harvested after 2 h. The blots were probed with antibodies specific for the phosphorylated, active forms of the kinases. (b) Primary human fibroblasts were incubated with the p38 inhibitor SB203580 (SB), the JNK inhibitor SP600125 (SP) as indicated (for 60 min and 15 min, respectively) and subsequently IR- (+) or mock-treated (-). Protein extracts were prepared 2 and 6 h later and analysed for the levels of total ATF3, phosphorylated active JNK, and Thr69 + 71-phosphorylated ATF-2 by western blotting. (c) Western blot analysis of IR-induced ATF-2-Thr71 and ATF-2-Thr69 + 71 phosphorylation of the cells depicted under (a). The faster migrating band detected by the ATF-2 phospho-specific antibodies seems to represent a shorter, alternatively spliced ATF-2 product, which is not recognized by the C-terminal ATF-2 antibody (Georgopoulos *et al.*, 1992). For the specificity of the phospho-specific antibodies, see Ouwens *et al.* (2002)

induction by IR. However, besides inhibiting p38, SB203580 also strongly enhanced the activation of JNK by IR, as is most clearly visible at 2 h after IR treatment (Figure 6b, left panel). Pretreatment with only the JNK inhibitor (lanes marked 'SP') slightly reduced ATF3 induction, indicating that in the absence of SB203580 JNK only weakly contributes to ATF3 induction. However, the combination of the two inhibitors was found to completely block the activation of ATF3 by IR. We therefore conclude that IR induction of ATF3 requires both p38 and JNK activity.

p38 and JNK can activate both the *c-jun* and *atf3* promoters via Jun/ATF sites and a mechanism involving N-terminal phosphorylation of c-Jun/ATF-2 (Cai *et al.*, 2000; Shaulian and Karin, 2001; van Dam and Castella, 2001; C van der Burgt en H van Dam, unpublished results). Therefore, we next examined whether IR enhances p38- and JNK-dependent phosphorylation of ATF-2. As shown in Figures 6b and c, ATF-2-Thr69 + 71 phosphorylation is clearly increased after IR treatment of wild-type human diploid fibroblasts and like the induction of ATF3, the increase in ATF-2-Thr69 + 71 phosphorylation was inhibited by pretreatment of the cells with the combination of the p38-inhibitor SB203580 and the JNK-inhibitor SP600125. Please note that the presence of only SB203580 enhanced the phosphorylation of ATF-2 by IR, similar as was found for the phosphorylation of JNK (Figure 6b). These results thus show that IR induces both ATF3 expression and ATF-2-Thr69 + 71 phosphorylation in human diploid fibroblasts via p38 and JNK.

We recently found that the weak activation of p38 and JNK by growth factors is sufficient for ATF-2-Thr69 + 71 dual phosphorylation when Thr71 is already monophosphorylated by ERK (Ouwens *et al.*, 2002). As human diploid fibroblast contain relatively high levels of active, phospho-ERK (Figure 6a), which are not significantly elevated by IR, we examined the effects of IR on ATF-2-Thr71 monophosphorylation. As depicted in Figure 6c, the human fibroblasts contain significant levels of Thr71 monophosphorylated ATF-2, which were only mildly enhanced by IR and to a much lesser extent than the increase in Thr69 + 71 dual phosphorylation. Thus, the IR-induced stress kinases predominantly enhance ATF-2-Thr69 phosphorylation.

In summary, these results demonstrate that activation of JNK and p38 by IR is essential for IR-induced ATF3 gene induction and ATF-2-Thr69 + 71 dual phosphorylation, but not for ATF-2-Thr71 monophosphorylation.

No IR-induced ATF-2-Thr69 + 71 phosphorylation in A-T and NBS cells

Since the induction of ATF3 by IR was found to be dependent on ATM and Nibrin1, we subsequently examined whether these signaling components are also required for ATF-2-Thr69 + 71 phosphorylation by IR. As shown in Figure 7, no increase in ATF-2 Thr69 + 71 phosphorylation was detected in A-T and NBS fibroblasts (Figure 7). In contrast, UV treatment of the A-T

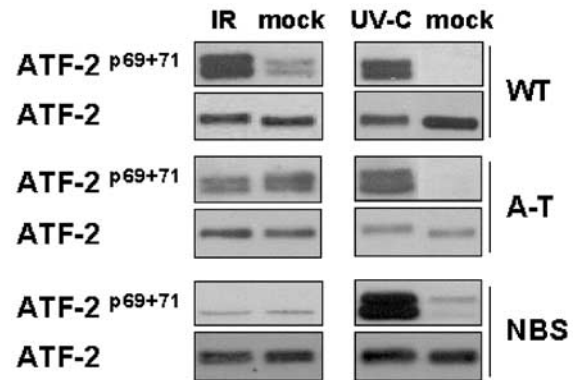


Figure 7 IR-induced phosphorylation of ATF-2 requires ATM and Nibrin1. Primary human fibroblasts and fibroblasts derived from ATM and NBS patients were treated with IR, UV-C or mock-treated as described in the previous figures. At 2 h after stimulation, cell extracts were prepared and analysed by Western blotting using either an antibody specific for Thr69 + 71-phosphorylated ATF-2 or an antibody recognizing total ATF-2

and NBS cells did result in strong phosphorylation of ATF-2-Thr69 + 71, demonstrating that these cells contain functional ATF-2 as well as JNK and/or p38. (Note that short exposures of films for the UV-treated samples are shown compared to the longer exposures for IR-treated samples.)

In summary, these data identify both ATF-2 and ATF3 as targets of the ATM and Nibrin1-signaling pathway.

Discussion

Exposure of cells to genotoxic agents leads to activation of multiple signaling pathways and transcription factors, which mediate the induction of transient or irreversible growth arrest, programmed cell death and/or the induction of detoxifying enzymes. The mechanisms by which different types of DNA-damaging agents activate these signaling enzymes and gene expression programs are still poorly understood. In this study, we have investigated the effects of IR on transcription factor AP-1 components and AP-1-activating MAPkinases. We found that, in primary human fibroblasts, IR activates Jun/ATF transcription factor activity rather than Jun/Fos activity, and that this activation involves both Thr69 + 71 phosphorylation of ATF-2 and *de novo* synthesis of c-Jun and ATF3. The p38 enzymes appear to be the main MAPkinases mediating Jun/ATF activation by IR in human diploid fibroblasts, as the JNK family members are only very weakly activated under normal conditions. These results are in line with previous studies showing that IR also very inefficiently activates JNK/SAPkinases in mouse embryo fibroblasts (Liu *et al.*, 1996; Shaulian and Karin 1999). The fact that c-Fos is efficiently induced by genotoxic stresses like UV and the alkylating agent MMS, but not by IR, could mean that c-Fos, which protects fibroblasts against UV light and MMS (Haas and

Kaina, 1995; Schreiber *et al.*, 1995), is not involved in protection against IR-induced damage. In addition, the absence of c-Fos induction suggests that IR induces AP-1 activity via different stress sensors and signaling enzymes than UV and other genotoxic stresses. Indeed, we found that IR, unlike UV and H₂O₂, does not induce ATF3 expression via the generation of ROS and/or oxidative stress. Moreover, the activation of ATF-2 and ATF3 by IR, but not UV, was found to require ATM and Nibrin1, two essential signaling enzymes in the cellular response to IR.

The ATF3 induction via ATM and Nibrin1 was expected to be dependent on p53, since (a) the induction of ATF3 by IR in mouse thymus cells requires p53 (Amundson *et al.*, 1999) and (b) ATM and Nibrin1 are involved in the activation of p53 by IR in fibroblasts (reviewed by Rotman and Shiloh, 1999; Girard *et al.*, 2002 and references therein). However, in contrast to ATF3, activation of p53 and its downstream target *p21^{CIP/Waf1}* by IR could still be detected in A-T cells and was not significantly affected in Nibrin1-deficient fibroblasts. Moreover, IR still efficiently induces ATF3 in WT human fibroblasts after inactivation of p53 by SV40 T/t. This suggests that activation of ATF3 by p53 is cell-type-specific. In addition, it shows that IR induction of ATF3 rather than IR induction of *p21^{CIP/Waf1}* is strictly dependent on ATM and Nibrin1.

Studies with specific inhibitors showed that induction of ATF3 by IR in human fibroblasts is associated with p38- and JNK-dependent dual phosphorylation of ATF-2-Thr69 + 71, two residues in the ATF-2 transactivation domain that enhance ATF-2's transactivating capacity upon phosphorylation (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995; Kawasaki *et al.*, 2000). ATF-2 can activate the *atf3* promoter by binding to its Jun/ATF site, an element that is essential for *atf3* activation upon exposure to various JNK and p38 stimuli (reviewed in Hai *et al.*, 1999; Cai *et al.*, 2000; H van Dam, unpublished results). Although activation of p38 and, in particular, JNK by IR is relatively weak in fibroblasts, these MAPkinases were found to be essential for ATF-2 phosphorylation and ATF3 gene induction. Interestingly, IR predominantly induced Thr69 phosphorylation, whereas Thr71 was already significantly phosphorylated prior to IR treatment. As monophosphorylation of ATF-2-Thr71 in nonirradiated cells appears to be mediated by ERK (J Kool, unpublished observations), the activation of ATF-2 by IR resembles the recently described two-step activation of ATF-2 by growth factors, which also involves two different MAPKs (Ouwens *et al.*, 2002).

The fact that ATF3 induction but not *p21^{CIP/Waf1}* induction is strictly dependent on ATM and Nibrin1, suggests that the absence of ATF3 induction in cells from AT and NBS patients might contribute to the radiosensitivity of these cells. A-T and NBS cells show a very similar phenotype in the sense that they are both defective in IR-induced S phase and the G2/M phase arrest (Lim *et al.*, 2000; Zhao *et al.*, 2000; Wu *et al.*, 2000; Buscemi, 2001; Kim *et al.*, 2002; Yazdi *et al.*, 2002). Interestingly, a recent report of Yan *et al.* (2002)

links ATF3 induction to p53-regulated growth arrest and gene activation. These authors showed that ATF3 can bind to p53 both *in vitro* and *in vivo* and thereby inhibit the transcription activation of the *mmp-2* gene by p53. Moreover, they showed that the prolonged G2/M arrest mediated by p53 after IR is abrogated in cells stably transfected with an ATF3 expression vector. ATF3 might therefore be required for proper regulation of p53-dependent gene expression and/or cell cycle arrest. Understanding the role of ATF3 in the IR-induced stress response might thus help to understand the aberrant response of A-T and NBS cells to IR and the complex phenotype of these genetic disorders.

Materials and methods

Cells and cell culture

The primary human foreskin fibroblasts used in this study have been described: VH10 and VH10 cells transformed with SV40 (Klein *et al.*, 1990), A-T fibroblasts AT4BI and AT5BI, and NBS fibroblasts 79RD27 (Jaspers *et al.*, 1988). Cells were grown on DMEM (Life technologies, Inc, Paisley, UK) supplemented with 8% FBS (Life Technologies, Inc), penicillin and streptomycin.

Before irradiation or serum stimulation of primary fibroblasts, cells were grown to full confluence, after which the medium was replaced with DMEM containing 0.5% serum. Cells were subsequently kept on low serum for 3 days prior to treatment. SV40-transformed cells were irradiated, while growing on 8% FBS medium when dishes were 80–90% confluent. For ionizing irradiation, cells were irradiated with 30 Gy of X-ray in medium at room temperature at a dose rate of 2 Gy/min (200 kV, 4 mA, Andrex Smart 225 source). For UV-C irradiation, a 30 W germicidal lamp (TUV; Philips Electronic Instruments Inc, Eindhoven, The Netherlands) was used with a dose rate of 0.5 J/m²s. Prior to UV treatment, the medium was collected, cells were washed once with PBS (10 mM Na₂HPO₄/0.14 mM NaCl, pH 7.4), and the PBS was removed before irradiation at room temperature. After irradiation, cells were refed with the collected medium.

Chemicals

DRB and NAC were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA), SB20358 from Calbiochem (San Diego, CA, USA) and SP600125 from Biomol Research Laboratories Inc. (Plymouth meeting, PA, USA).

RNA isolation and Northern analysis

RNA was isolated with the SV total RNA isolation system of Promega Corporation (Madison, WI, USA). After RNA isolation, an additional acid phenol extraction was performed. Northern analysis using as probes *p21^{CIP/Waf1}* and *gapdh* has been described (van Laar *et al.*, 1995). As *atf3* probe, a 709 bp *NaeI*–*XbaI* fragment was used.

Western analysis and antibodies

For preparation of protein extracts, cells were washed once with ice-cold PBS and subsequently lysed on ice in ice-cold buffer containing 10 mM Tris pH7.5, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate containing protease and phosphatase inhibitors (van Dam

et al., 1993). Lysates were cleared by centrifugation. Protein (20 µg) was loaded on SDS-PAGE gels. Antibodies used are Fra1 (R-20), c-Jun (H79), ATF-2-C19, p38-N20, p21-C19, p53-DO1 and ATF3-C19 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), c-Fos (06-341) from Upstate (Charlottesville, VA, USA), phospho-specific ATF-2-Thr71, ATF-2 Thr69 + 71, ERK-Thr202/Tyr204 and PhosphoPlus® p38 MAP Kinase (Thr180/Tyr182) from Cell Signalling Technology, Inc. (Beverly, MA, USA) and Anti-ACTIVE® JNK pAb pTPpY from Promega Corporation (Madison, WI, USA). As second antibodies, goat-anti-rabbit antibodies labeled with horseradish peroxidase were used. Proteins were made visible using enhanced chemoluminescence. For blots

incubated with ATF-2-Thr69 + 71 antibodies, we used Lumi-Light^{plus} Western blotting substrate from Roche Diagnostics, Basel, Switzerland.

Acknowledgements

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References

- Abraham RT. (2001). *Genes Dev.*, **15**, 2177–2196.
- Allan AL, Albanese C, Pestell RG and LaMarre J. (2001). *J. Biol. Chem.*, **276**, 27272–27280.
- Amundson SA, Bittner M, Chen Y, Trent J, Meltzer P and Fornace Jr AJ. (1999). *Oncogene*, **18**, 3666–3672.
- Bar-Shira A, Rashi-Elkeles S, Zlochover L, Moyal L, Smorodinsky NI, Seger R and Shiloh Y. (2002). *Oncogene*, **21**, 849–855.
- Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM and Anderson DW. (2001). *Proc. Natl. Acad. Sci. USA*, **98**, 13681–13686.
- Buscemi G, Savio C, Zannini L, Micciche F, Masnada D, Nakanishi M, Tauchi H, Komatsu K, Mizutani S, Khanna K, Chen P, Concannon P, Chessa L and Delia D. (2001). *Mol. Cell. Biol.*, **21**, 5214–5222.
- Cai Y, Zhang C, Nawa T, Aso T, Tanaka M, Oshiro S, Ichijo H and Kitajima S. (2000). *Blood*, **96**, 2140–2148.
- Coffer PJ, Burgering BM, Peppelenbosch MP, Bos JL and Kruijer W. (1995). *Oncogene*, **11**, 561–569.
- Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR and Lee JC. (1995). *FEBS Lett.*, **364**, 229–233.
- Devary Y, Gottlieb RA, Smeal T and Karin M. (1992). *Cell*, **71**, 1081–1091.
- Devary Y, Rosette C, DiDonato JA and Karin M. (1993). *Science*, **261**, 1442–1445.
- el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wiman KG, Edward Mercer W, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW and Vogelstein B. (1994). *Cancer Res.*, **54**, 1169–1174.
- Girard PM, Riballo E, Begg AC, Waugh A and Jeggo PA. (2002). *Oncogene*, **21**, 4191–4199.
- Gross S, Knebel A, Tenev T, Neininger A, Gaestel M, Herrlich P and Bohmer FD. (1999). *J. Biol. Chem.*, **274**, 26378–26386.
- Gupta S, Campbell D, Derijard B and Davis RJ. (1995). *Science*, **267**, 389–393.
- Haas S and Kaina B. (1995). *Carcinogenesis*, **16**, 985–991.
- Hai T, Wolfgang CD, Marsee DK, Allen AE and Sivaprasad U. (1999). *Gene Expr.*, **7**, 321–335.
- Hsu JC, Bravo R and Taub R. (1992). *Mol. Cell. Biol.*, **12**, 4654–4665.
- Jaspers NGJ, Gatti RA, Baan C, Linssen PCML and Bootsma D. (1988). *Cytogenet. Cell. Genet.*, **49**, 259–263.
- Jongmans W, Vuillaume M, Chrzanowska K, Smeets D, Sperling K and Hall J. (1997). *Mol. Cell. Biol.*, **17**, 5016–5022.
- Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace Jr. AJ. (1992). *Cell*, **71**, 587–597.
- Kawasaki H, Schiltz L, Chiu R, Itakura K, Taira K, Nakatani Y and Yokoyama KK. (2000). *Nature*, **405**, 195–200.
- Khanna KK, Beamish H, Yan J, Hobson K, William R, Dunn I and Lavin MF. (1995). *Oncogene*, **11**, 609–618.
- Kim ST, Xu B and Kastan MB. (2002). *Genes Dev.*, **16**, 560–570.
- Klein B, Pastink A, Odijk H, Westerveld A and van der Eb AJ. (1990). *Exp. Cell. Res.*, **191**, 256–262.
- Knebel A, Rahmsdorf HJ, Ullrich A and Herrlich P. (1996). *EMBO J.*, **15**, 5314–5325.
- Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH and Kastan MB. (2000). *Nature*, **404**, 613–617.
- Liu ZG, Baskaran R, Lea-Chou ET, Wood LD, Chen Y, Karin M and Wang JY. (1996). *Nature*, **384**, 273–276.
- Livingstone C, Patel G and Jones N. (1995). *EMBO J.*, **14**, 1785–1797.
- Mashima T, Udagawa S and Tsuruo T. (2001). *J. Cell. Physiol.*, **188**, 352–358.
- Mercurio F and Manning AM. (1999). *Oncogene*, **18**, 6163–6171.
- Ouwens DM, de Ruiter ND, van der Zon GC, Carter AP, Schouten J, van der Burgt C, Kooistra K, Bos JL, Maassen JA and van Dam H. (2002). *EMBO J.*, **21**, 3782–3793.
- Perez S, Vial E, van Dam H and Castellazzi M. (2001). *Oncogene*, **20**, 1135–1141.
- Pfeiffer P, Gottlich B, Reichenberger S, Feldmann E, Daza P, Ward JF, Milligan JR, Mullenders LH and Natarajan AT. (1996). *Mutat. Res.*, **366**, 69–80.
- Ronai Z, Yang YM, Fuchs SY, Adler V, Sardana M and Herlyn M. (1998). *Oncogene*, **16**, 523–531.
- Rosette C and Karin M. (1996). *Science*, **274**, 1194–1197.
- Rotman G and Shiloh Y. (1999). *Oncogene*, **18**, 6135–6144.
- Sachsenmaier C, Radler-Pohl A, Zinck R, Nordheim A, Herrlich P and Rahmsdorf HJ. (1994). *Cell*, **78**, 963–972.
- Schreiber M, Baumann B, Cotten M, Angel P and Wagner EF. (1995). *EMBO J.*, **14**, 5338–5349.
- Shaulian E and Karin M. (1999). *J. Biol. Chem.*, **274**, 29595–29598.
- Shaulian E and Karin M. (2001). *Oncogene*, **20**, 2390–2400.
- Shaulian E, Schreiber M, Piu F, Beeche M, Wagner EF and Karin M. (2000). *Cell*, **103**, 897–907.
- Tamm I, Hand R and Caligiuri LA. (1976). *J. Cell. Biol.*, **69**, 229–240.
- van Dam H and Castellazzi M. (2001). *Oncogene*, **20**, 2453–2464.
- van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smith L, Herrlich P, Zantema A, Angel P and van der Eb AJ. (1993). *EMBO J.*, **12**, 479–487.

- van Dam H, Wilhelm D, Herr I, Steffen A, Herrlich P and Angel P. (1995). *EMBO J.*, **14**, 1798–1811.
- van Laar T, Steegenga WT, Jochemsen AG, Terleth C and van der Eb AJ. (1995). *Biochem. Biophys. Res. Commun.*, **217**, 769–776.
- Varon R, Vissinga C, Platzer M, Cersaletti KM, Chrzanowska KH, Saar K, Beckmann G, Seemanova E, Cooper PR, Nowak NJ, Stumm M, Weemaes CM, Gatti RA, Wilson RK, Digweed M, Rosenthal A, Sperling K, Concannon P and Reis A. (1998). *Cell*, **93**, 467–476.
- Wilhelm D, Bender K, Knebel A and Angel P. (1997). *Mol. Cell. Biol.*, **17**, 4792–4800.
- Wolfgang CD, Liang G, Okamoto Y, Allen AE and Hai T. (2000). *J. Biol. Chem.*, **275**, 16865–16870.
- Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G, Livingston DM and Weaver DT. (2000). *Nature*, **2000**, 405, 477–405, 482.
- Yamazaki V, Wegner RD and Kirchgesner CU. (1998). *Cancer Res.*, **58**, 2316–2322.
- Yan C, Wang H and Boyd DD. (2002). *J. Biol. Chem.*, **277**, 10804–10812.
- Yazdi PT, Wang Y, Zhao S, Patel N and Lee EY and Qin J. (2002). *Genes Dev.*, **16**, 571–582.
- Zandomeni R, Mittleman B, Bunick D, Ackerman S and Weinmann R. (1982). *Proc. Natl. Acad. Sci. USA*, **79**, 3167–3170.
- Zhao S, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y and Lee EY. (2000). *Nature*, **2000**, 405, 473–405, 477.
- Zhou BB and Elledge SJ. (2000). *Nature*, **408**, 433–439.

Chapter III

**DNA damage in transcribed genes induces apoptosis via the JNK pathway
and the JNK-phosphatase MKP-1**

Oncogene (2005) 24, 7135–7144

DNA damage in transcribed genes induces apoptosis via the JNK pathway and the JNK-phosphatase MKP-1

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The nucleotide excision repair (NER) system consists of two subpathways, global genome repair (GGR) and transcription-coupled repair (TCR), which exhibit distinct functions in the cellular response to genotoxic stress. Defects in TCR result in prolonged UV light-induced stalling of RNA polymerase II and hypersensitivity to apoptosis induced by UV and certain chemotherapeutic drugs. Here, we show that low doses of UV trigger delayed activation of the stress-induced MAPkinase JNK and its proapoptotic targets c-Jun and ATF-3 in TCR-deficient primary human fibroblasts from Xeroderma Pigmentosum (XP) and Cockayne syndrome (CS) patients. This delayed activation of the JNK pathway is not observed in GGR-deficient TCR-proficient XP cells, is independent of functional p53, and is established through repression of the JNK-phosphatase MKP-1 rather than by activation of the JNK kinases MKK4 and 7. Enzymatic reversal of UV-induced cyclobutane pyrimidine dimers (CPDs) by CPD photolyase abrogated JNK activation, MKP-1 repression, and apoptosis in TCR-deficient XPA cells. Ectopic expression of MKP-1 inhibited DNA-damage-induced JNK activity and apoptosis. These results identify both MKP-1 and JNK as sensors and downstream effectors of persistent DNA damage in transcribed genes and suggest a link between the JNK pathway and UV-induced stalling of RNAPol II.

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Introduction

Mammalian cells exhibit multiple biological responses to genotoxic stress, including cell cycle checkpoints,

DNA repair, and apoptosis. Inactivation of these responses may result in genomic instability and cell transformation, as well as modifications of therapeutic sensitivity. Bulky DNA-helix distorting lesions such as UV-induced photolesions and cisplatin-induced DNA adducts can be removed by the nucleotide excision repair (NER) system. Deficiencies in NER lead to enhancement of DNA-damage-induced cell killing and mutagenesis. Two distinct NER subpathways can be distinguished: global genome repair (GGR), which repairs DNA damage throughout the genome, and transcription-coupled repair (TCR), which repairs DNA lesions in the transcribed strand of active genes. The DNA repair factors XPC and XPE are exclusively involved in GGR, whereas the CSA and CSB proteins only play a role in TCR. Other NER proteins, such as XPA, XPB, and XPD, function in both TCR and GGR (reviewed by Berneburg and Lehmann, 2001; Mitchell *et al.*, 2003). Genetic defects in these NER genes can cause the human diseases Xeroderma pigmentosum (XP) and Cockayne Syndrome (CS). XP patients are very sensitive to UV light, undergo progressive degeneration of the skin, and show a 1000-fold increased risk for skin cancer, mainly on sun-exposed parts of the body. CS patients, who die at young age, show more pleiotropic features, including neurological degradation, but not increased skin cancer (reviewed by van Steeg and Kraemer, 1999; de Boer and Hoeijmakers, 2000). Importantly, in contrast to TCR-proficient cells, TCR-deficient cells already undergo apoptosis at very low doses of UV light, and, in addition, are sensitive to the chemotherapeutic drugs cisplatin and irinotecan/Irofulven (McKay *et al.*, 1998, 2001; van Oosten *et al.*, 2000; Jaspers *et al.*, 2002; Spivak *et al.*, 2002). This sensitivity of TCR-deficient cells is associated with prolonged UV-induced inhibition of transcription due to prolonged stalling of RNA polymerase II (RNAPol II) (Rockx *et al.*, 2000; Berneburg and Lehmann, 2001; Svejstrup, 2002).

In addition to DNA lesions, UV light and other genotoxic agents can generate reactive oxygen species (ROS), which result in lipid peroxidation, protein oxidation, and glutathione depletion (Mercurio and Manning, 1999). The cell can respond to these types of stress by activating various signaling cascades, including

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the p53, NF- κ B, ATR, and MAPkinase (MAPK) pathways. Depending on the cell type and the genotoxic stress, the combined action of these signaling pathways can trigger transient or prolonged cell cycle arrest or apoptosis (Martindale and Holbrook, 2002; Shaulian and Karin, 2002; Brancho *et al.*, 2003; Todd *et al.*, 2004). The MAPK family member JNK/SAPK is able to control apoptosis both positively and negatively, depending on the cell type, cellular context, and the stress signal. Proapoptotic substrates of JNK comprise both transcription factors, like the transcription factor AP-1 component c-Jun, and components of the apoptosis-executing machinery, like the proapoptotic Bcl-2-related proteins (Lei *et al.*, 2002; Shaulian and Karin, 2002; Weston and Davis, 2002; Lei and Davis, 2003). Antiapoptotic JNK targets include JunD and the survival gene *cIAP-2* (Lamb *et al.*, 2003). In addition to c-Jun and JunD, JNK can activate and/or induce the expression of various other AP-1 components involved in cell proliferation and survival, like ATF-2, ATF-3, and c-Fos (Hai *et al.*, 1999; Shaulian and Karin, 2001; van Dam and Castellazzi, 2001). Interestingly, recent studies indicate that c-Jun and ATF-2 can control the cellular response to DNA damage by regulating the expression of various DNA repair genes, including *ERCC3*, *XPA*, *RAD23B*, and *MSH2* (Hayakawa *et al.*, 2003, 2004; MacLaren *et al.*, 2004).

JNK activity in the cell is tightly controlled by both protein kinases and protein phosphatases. Various types of stimuli activate JNK through phosphorylation by the dual specificity kinases MKK4 or MKK7 (Morrison and Davis, 2003; Wada *et al.*, 2004). In contrast, mitogens and stress stimuli can inactivate JNK through induction of the expression of JNK phosphatases, which include serine/threonine phosphatases, tyrosine phosphatases, and dual specificity (threonine/tyrosine) phosphatases (Camps *et al.*, 2000; Keyse, 2000; Farooq and Zhou, 2004). Activation of the dual specificity JNK-phosphatase MKP-1 seems to be involved in the cellular defense against H₂O₂- and UV-induced apoptosis (Franklin *et al.*, 1998; Xu *et al.*, 2004), whereas inhibition of MKP-1 can potentiate JNK-related apoptosis (Guo *et al.*, 1998; Desbois-Mouthon *et al.*, 2000; Sanchez-Perez *et al.*, 2000; Mizuno *et al.*, 2004).

In this study, we investigated the signaling route by which UV-induced DNA damage induces apoptosis in TCR-deficient human fibroblasts. We identified ATF-3, c-Jun, and JNK as important sensors of DNA damage in the transcribed strand of active genes, at UV doses that are cytotoxic for TCR-deficient fibroblasts, but not for TCR-proficient fibroblasts. Activation of ATF-3, c-Jun, and JNK at these UV doses occurs with delayed kinetics and is mediated by inhibition of MKP-1 expression rather than by activation of the JNK kinases MKK4 and 7. Importantly, removal of UV-induced cyclobutane dimers (CPDs) by CPD photolyase abrogated both the inhibition of MKP-1 and the activation of JNK, c-Jun, and ATF-3, while inhibition of JNK activation by ectopic expression of MKP-1 strongly suppressed DNA-damage-induced apoptosis. These results reveal a new functional link between the MKP-

1-JNK pathway and persistent DNA damage in transcribed genes.

Results

Low doses of UV induce ATF-3, c-Jun, and JNK activity in TCR-deficient primary human fibroblasts

TCR-deficient human fibroblasts including XPA, XPD, and CSB cells show high levels of apoptosis after irradiation with 5–20 J/m² UV-C, whereas TCR-proficient fibroblasts mostly survive at these UV doses (Queille *et al.*, 2001). In addition, TCR-deficient cells show delayed transcriptional activation of the AP-1 target genes collagenase 1 and metallothionein IIA under these conditions (Blattner *et al.*, 1998). Since both AP-1 transcription factors and their upstream activators, the MAPKs, can regulate programmed cell death, we examined the role of these UV-inducible factors in the apoptotic response of TCR-deficient primary human fibroblasts. As shown for serum-starved cells in Figure 1, we found ATF-3, c-Jun, JNK, and p38 to be selectively activated in TCR-deficient cells after low doses of UV: 16 h after irradiation with 3 or 5 J/m² UV-C, the levels of ATF-3 were enhanced in XP cells that are both TCR and GGR deficient (XPA and XPD), whereas ATF-3 induction was only observed with 20 J/m² in wild-type cells or GGR-deficient XP cells (XPC and XPE) (Figure 1a). Moreover, XPA and XPD fibroblasts showed enhanced levels of the phosphorylated, active forms of c-Jun, JNK, and p38 after irradiation with 5 and/or 20 J/m² UV-C, while none or much less activation of phospho-c-Jun (Thr91), phospho-JNK, and phospho-p38 was detected in the XPC, XPE, or normal fibroblasts (Figure 1a). No clear difference was seen in the activation of ERK between TCR-deficient and -proficient primary human fibroblasts.

We also tested activation of the stress-induced MAPK pathways in primary fibroblasts from CS patients. These cells are only TCR deficient and somewhat less sensitive for UV than TCR + GGR-deficient cells because the relative slow GGR machinery can partially repair UV-induced lesions in transcribed genes (de Boer and Hoeijmakers, 2000; Queille *et al.*, 2001). As shown in Figure 1b, ATF3, c-Jun, JNK, and p38 activation became detectable between 5 and 10 J/m² UV in CSA and CSB cells, but not in GGR-deficient XPC cells. These results thus indicate that persistent DNA damage in transcribed genes results in activation of the JNK pathway.

The observed activation of ATF-3, c-Jun, and JNK in TCR-deficient cells was unlikely to be merely a late, secondary effect of cell death, as in XPA fibroblasts the levels of apoptosis were higher at 5 J/m² UV than at 20 J/m² whereas the levels of phospho-JNK and phospho-c-Jun were higher at 20 J/m² (Figure 2). Moreover, JNK pathway activation by low dose UV could already be detected between 8 and 24 h after treatment, whereas significant increases in the levels of apoptotic cells could only be detected after 48 h (Figures 2 and 5a;

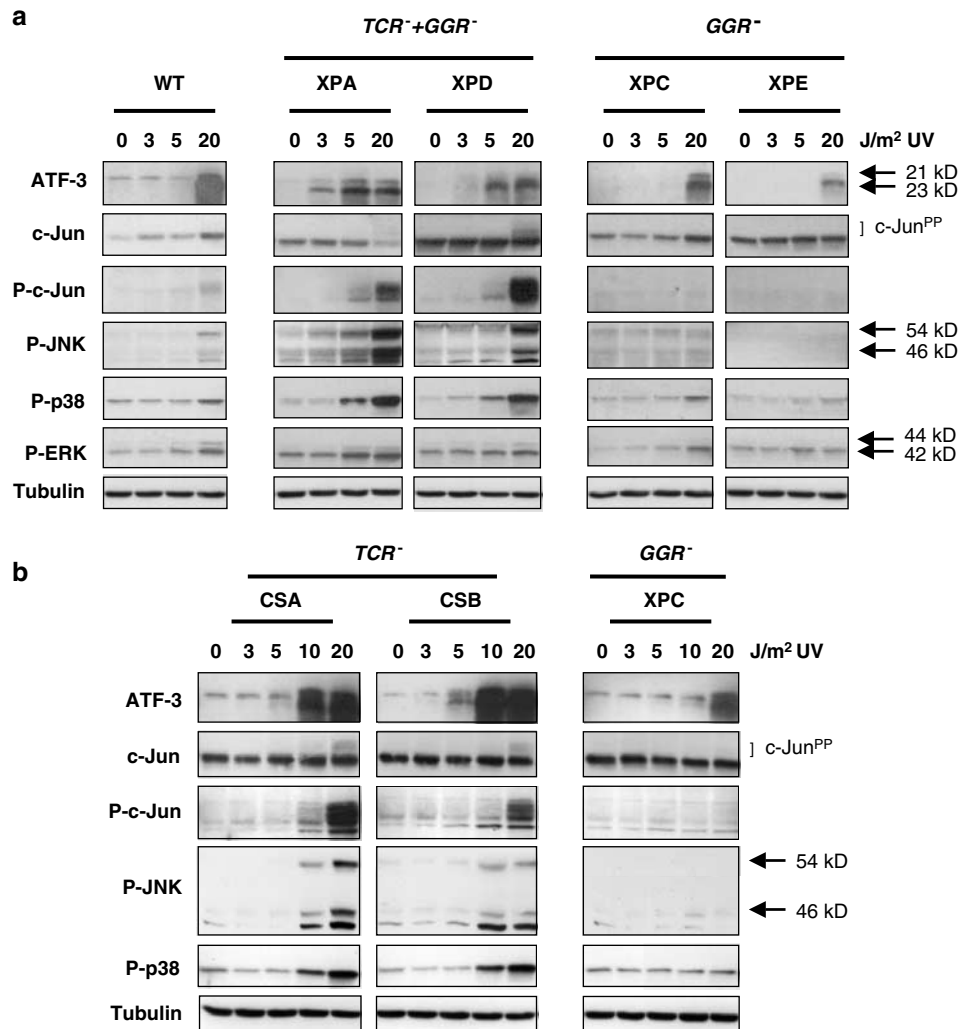


Figure 1 Activation of the JNK pathway in TCR-deficient human fibroblasts. Confluent cultures of primary human diploid fibroblasts from a normal individual (WT) or patients with the indicated XP and CS complementation groups were serum-starved (0.5%) for 3 days and irradiated with the indicated doses of UV-C or mock-treated (0). After 16 h, protein extracts were prepared and analysed by SDS/PAGE and immunoblotting with the indicated antibodies. Tubulin levels were examined as a control for equal loading. Human fibroblasts examined under (a) are: VH12 (WT), XP25RO (XPA), XP3NE (XPD), XP8CA (XPC), and XP2RO (XPE); under (b): CS3BE (CSA), CS1AN (CSB), and XP21RO (XPC). (The phospho-c-Jun antibody only recognizes Thr91-phosphorylated c-Jun; c-Jun^{PP} = hyperphosphorylated, mobility shifted c-Jun.)

data not shown). The relatively low levels of apoptosis in XPA cells after 20 J/m² UV are presumably due to strong inhibition of transcription at this UV dose (Queille *et al.*, 2001), preventing the expression of proapoptotic genes. In line with this, XPA cells contain relatively low amounts of ATF-3 and c-Jun protein and mRNA after high doses of UV (Figures 2 and 3).

In summary, these results suggest that the JNK pathway is involved in the apoptotic response of TCR-deficient cells.

Low doses of UV light induce apoptosis and JNK pathway activation in XPA cells independently of functional p53

DNA damage in transcribed genes can result in activation of p53 (Blattner *et al.*, 1998), a transcription

factor that can regulate DNA repair and induce apoptosis (Ford and Hanawalt, 1995; Smith *et al.*, 2000). p53 can participate in a stress-inducible regulatory network that also includes JNK, c-Jun, and ATF-3 (Kannan *et al.*, 2001; Shaulian and Karin, 2002; Yan *et al.*, 2002). We therefore examined whether low doses of UV induce cell death and/or JNK pathway activation in TCR-deficient human fibroblasts via p53. For this purpose, we compared SV40-immortalized XPA and normal human fibroblasts in which p53 is nonfunctional due to its association with SV40T antigen. As shown in Figure 3a, SV40T-expressing XPA fibroblasts (XPA/SV) show high levels of apoptosis after irradiation with 1–5 J/m² UV-C, indicating that low dose UV-induced apoptosis in these TCR-deficient human fibroblasts does not require functional p53, in line with the previous

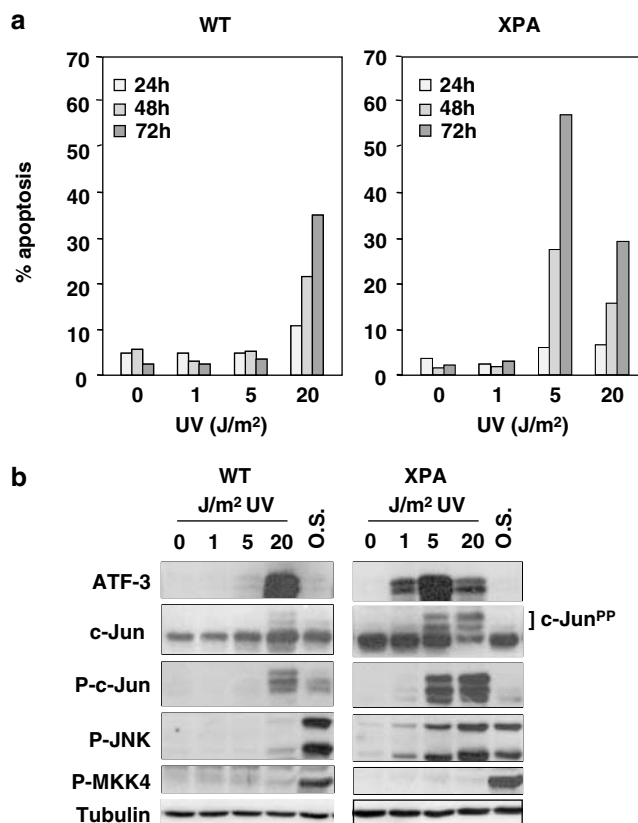


Figure 2 Apoptosis and JNK-c-Jun pathway activation in XPA and control primary human fibroblasts. **(a)** Subconfluent, proliferating cultures of XPA and normal human fibroblasts were irradiated with the indicated doses of UV-C or mock-treated (0) and harvested for FACS analysis after 24, 48, and 72 h. The level of apoptosis represents the percentage of cells in sub-G1. **(b)** Western analysis of the experiment described under **(a)**. Protein extracts were prepared 24 h after UV irradiation and analysed by SDS/PAGE and immunoblotting with the indicated antibodies. Tubulin levels were examined as a control for equal loading. As a control for MKK4 activation, parallel cultures were treated for 15 min with 0.5 M sodium chloride (osmotic stress: O.S.)

studies (McKay *et al.*, 2001). Moreover, after irradiation with 1 and 5 J/m² UV-C, high levels of phosphorylated active JNK, c-Jun, and, to a lesser extent, p38 were detected in the XPA/SV cells but not in wild-type cells (WT/SV; Figure 3b). Likewise, at 1 J/m² the amounts of ATF-3 protein and mRNA were only induced in the XPA/SV cells (Figure 3b and c). These data show that in XPA cells both the apoptotic response and activation of the JNK pathway by low dose UV do not require functional p53.

Apoptosis and JNK pathway activation via accumulation of UV-induced cyclobutane pyrimidine dimers

About 75% of the total amount of UV-induced DNA damage comprises CPDs (de Boer and Hoeijmakers, 2000). To verify that UV-induced DNA damage rather than effects on other cellular targets like

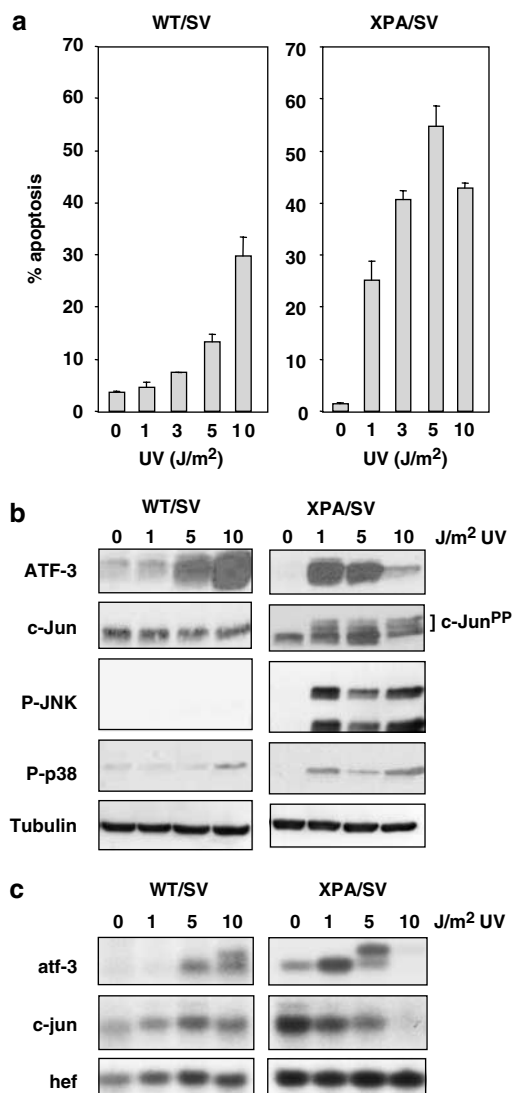


Figure 3 Apoptosis and JNK pathway activation in SV40T-immortalized XPA and control human fibroblasts. **(a)** Subconfluent, proliferating cultures of SV40T-immortalized human diploid fibroblasts from a control individual (WT/SV) or an XPA patient (XPA/SV) were irradiated with increasing doses of UV-C and harvested for FACS analysis 48 h later. The level of apoptosis represents the percentage of cells in sub-G1. **(b, c)** The immortalized XPA and control fibroblasts analysed under **(a)** were irradiated with 1, 5 or 10 J/m² UV-C or mock-treated (0). After 24 h, protein extracts and mRNA were prepared for immunoblot **(b)** and Northern blot **(c)** analysis. *hef-1* mRNA expression was determined to verify equal loading **(c)**

cell membrane receptors triggers the apoptosis-associated JNK pathway activation in UV-irradiated XPA cells, we examined immortalized human XPA cells ectopically expressing CPD photolyase (Nakajima *et al.*, 2004). This enzyme can revert CPDs upon illumination with visible light (photoreactivation (PR)). As depicted in Figure 4a, removal of UV-induced CPDs by photoreactivation strongly reduced the induction of apoptosis by low doses of UV in XPA cells: 3.9-fold at 1 J/m² and 3.8-fold at 3 J/m². (The residual

apoptosis after photoreactivation is most likely due to UV-induced 6-4-photoproducts, which are not converted by CPD photolyase.) Importantly, CPD removal almost completely abrogated the activation of ATF-3, JNK, and p38 (Figure 4b, right panel).

These results thus indicate that both apoptosis and JNK pathway activation can be triggered by prolonged presence of CPDs on transcribed genes and subsequent stalling of RNA pol II.

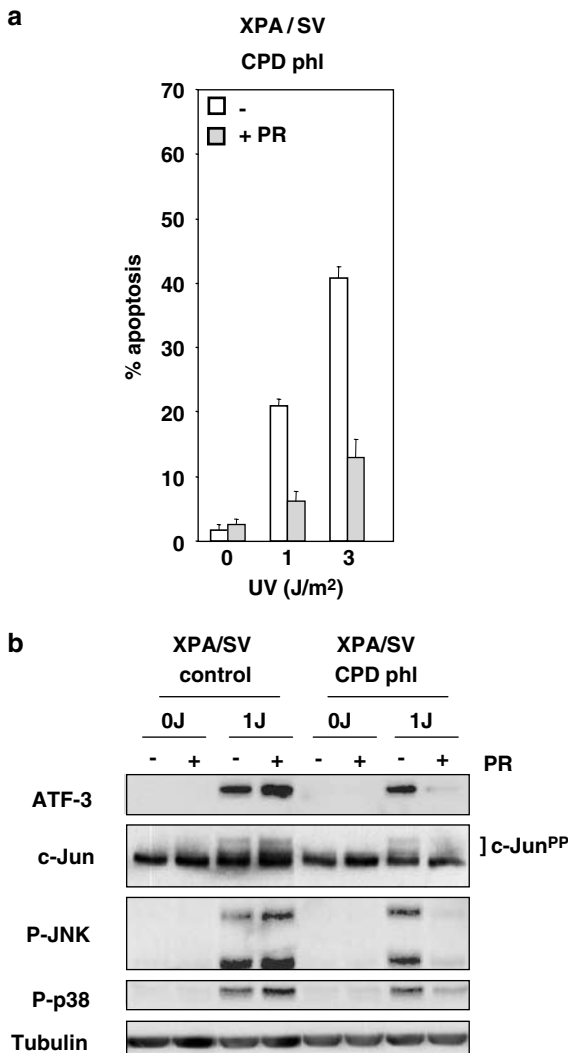


Figure 4 UV-induced apoptosis and JNK pathway activation through nonrepaired cyclobutane pyrimidine dimers (CPD). (a) SV40T-immortalized XPA cells expressing CPD photolyase (XPA/SV CPD phl) were irradiated with 1 or 3 J/m² UV-C or mock-treated (0). Subsequently, cells were treated for 1 h with photoreactivating light or nontreated ($\pm$ PR) and harvested for FACS analysis 47 h later. The level of apoptosis represents the percentage of cells in sub-G1. (b) Immortalized XPA cells expressing CPD photolyase (XPA/SV CPD phl) or control cells only containing the empty vector (XPA/SV control) were irradiated with 1 J/m² UV-C (1J) or mock treated (0J), and subsequently treated for 1 h with photoreactivating light or non-treated ($\pm$ PR). After 23 h, protein extracts were prepared for analysis by SDS/PAGE and immunoblotting with the indicated antibodies

DNA-damage-induced JNK activation through a CPD-mediated decrease of MAPK phosphatase-1

We next examined the mechanism by which DNA damage induces JNK activation in XPA cells. A multitude of stimuli including osmotic stress activate JNK through phosphorylation and activation of the JNK kinases MKK4 (SEK) and MKK7 (Morrison and Davis, 2003; Wada *et al.*, 2004). However, we did not observe MKK4 or MKK7 activation by low doses of UV, neither in primary nor in SV40T-immortalized cells (Figures 2 and 5a, data not shown), although activation of MKK4 and 7 activity by osmotic stress could be detected (Figure 2, data not shown). In contrast, as depicted in Figure 5a, we observed a significant decrease in the expression of MKP-1, a dual-specificity MAPK phosphatase that preferably dephosphorylates p38 and JNK (Franklin and Kraft, 1997; Li *et al.*, 2001). Importantly, reduction in the protein levels of MKP-1 in SV40T-immortalized XPA cells could already be observed 8 h after 1 J/m² UV, a time point at which JNK phosphorylation was only starting to increase.

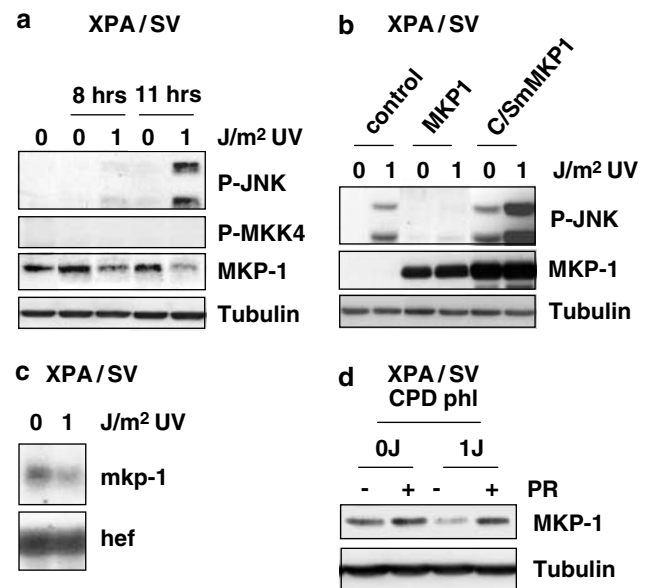


Figure 5 Low dose UV activates JNK in XPA cells via inhibition of MKP-1 expression. (a, c) SV40T-immortalized XPA fibroblasts were irradiated with 1 J/m² UV-C or mock-treated (0), and harvested after 8 and 11 h for immunoblot analysis (a), or after 8 h for Northern blot analysis (c). HEF-1 mRNA expression was determined to verify equal loading (c). (b) SV40T-immortalized XPA cells were transduced with lentiviruses containing wild-type MKP-1, the catalytically inactive dominant-negative mutant C/SmMKP-1, or an empty control virus. Transduction efficiency was 95–100% as verified by FACS determination. Next, the three cultures were irradiated with 1 J/m² UV-C or mock-treated (0), and harvested after 16 h for immunoblot analysis. Please note that in the control cells the endogenous MKP-1 is not visible because a relatively short exposure is shown. (d) SV40T-immortalized XPA cells expressing CPD photolyase (XPA CPD phl) were irradiated with 1 J/m² UV-C (1J) or mock treated (0J), and subsequently treated for 1 h with photoreactivating light or nontreated ($\pm$ PR). After 7 h, protein extracts were prepared for immunoblot analysis

We next examined whether inactivation of MKP-1 activity in XPA cells can cause JNK activation in the absence of UV-induced DNA damage. For this purpose, we inhibited the function of the endogenous MKP-1 protein via ectopic expression of the catalytically inactive dominant-negative MKP-1 mutant C/SmMKP1, introduced by a lentiviral vector (Bazuine *et al.*, 2004; Slack *et al.*, 2001). As depicted in Figure 5b, expression of the dominant-negative MKP-1 mutant resulted in increased levels of active phospho-JNK, but did not interfere with the activation of JNK by low dose UV. In contrast, overexpression of wild-type MKP-1 did not increase the basal activity of JNK and strongly inhibited JNK activation by UV. From these results we conclude that inhibition of JNK-phosphatase activity can cause JNK activation in the XPA cells. Subsequent studies showed that irradiation with low doses of UV results in a decrease in the levels of *mkp-1* mRNA (Figure 5c) and that enzymatic removal of CPDs by CPD photolyase abrogates the UV-induced decrease in MKP-1 expression (Figure 5d).

In summary, accumulation of UV-induced cyclobutane dimers causes a decrease in the expression of the JNK-phosphatase MKP-1 in XPA cells, which subsequently results in enhancement of JNK activity.

Ectopic expression of MAPK phosphatase-1 inhibits both DNA-damage-induced JNK activity and DNA-damage-induced apoptosis in XPA cells

The JNK pathway can trigger and/or enhance programmed cell death in various cell types (Davis, 2000). We therefore examined whether inhibition of JNK affects low dose UV-induced apoptosis in XPA cells. For this purpose, we ectopically expressed either wild-type MKP-1, which can dephosphorylate both JNK, p38, and ERK, or an MKP-1 mutant that only efficiently binds to JNK (m(JNK)MKP-1) (Slack *et al.*, 2001). As shown in Figure 6a, we found the JNK-restricted MKP-1 mutant to be more efficient in inhibiting UV-induced JNK activation than wild-type MKP-1, whereas the (weak) activation of p38 and ERK by UV was not significantly inhibited by either of the two MKP-1 constructs. Moreover, the JNK-specific MKP-1 mutant did not have any effect on the proliferation of nonirradiated XPA cells, while, as reported previously (Li *et al.*, 1999; Engelbrecht *et al.*, 2003), wild-type MKP-1 slightly reduced the proliferation of these cells, also after low dose UV irradiation (data not shown). Importantly, the JNK-restricted m(JNK)MKP-1 efficiently inhibited the induction of apoptosis by 1 J/m² UV in the XPA cells (Figure 6b), showing that JNK is an essential component of the DNA-damage-induced apoptotic program in these cells. In line with this, expression of the catalytically inactive dominant-negative MKP-1 mutant C/SmMKP1 not only induced JNK activity but also further enhanced the apoptotic response of XPA cells to low doses of UV.

In conclusion, our results demonstrate that upon DNA damage in transcribed genes down-regulation of MKP-1 causes activation of the JNK pathway, which

subsequently plays an important functional role in DNA-damage-induced apoptosis.

Discussion

DNA repair and DNA-damage-induced signaling play key protective roles in cancer development and cancer therapy (Mitchell *et al.*, 2003). The NER system, a repair pathway for DNA-helix distorting lesions like UV-induced photolesions exhibits a critical function in both processes, as deficiencies in NER lead to enhancement of DNA-damage-induced mutagenesis and cell killing. Several studies indicate that the two NER subpathways, GGR and TCR, differentially control mutagenesis and programmed cell death, because TCR-deficient cells are much more sensitive to UV-induced apoptosis and certain chemotherapeutic drugs than TCR-proficient XP cells (McKay *et al.*, 1998, 2001; Jaspers *et al.*, 2002; Spivak *et al.*, 2002). In this study, we identified the JNK pathway as one of the critical sensors and downstream effectors of persistent DNA damage in transcribed genes. We showed that low doses of UV light activate JNK, c-Jun, and ATF-3 in TCR-deficient human fibroblasts (XPA, XPD, CSA, and CSB), but not in TCR-proficient cells (XPE and XPC). Moreover, inhibition of JNK activity was found to strongly reduce low dose UV-induced apoptosis in XPA cells.

Previous studies showed that JNK signaling can regulate apoptosis both positively and negatively, depending on the cell type, cellular context, and the nature and dose of treatment (Davis, 2000; Chang and Karin, 2001). Strong and sustained JNK activation is predominantly associated with induction or enhancement of apoptosis, whereas transient JNK activation can result in cell survival (Chang and Karin, 2001; Lamb *et al.*, 2003). In line with the presumed proapoptotic function of sustained JNK activation, we found the low dose UV-induced apoptotic response in XPA fibroblasts to be preceded by delayed and long-lasting JNK activation. Moreover, this sustained JNK activation was accompanied by prolonged phosphorylation and activation of the endogenous JNK substrate c-Jun, a component of transcription factor AP-1 that can act as a proapoptotic effector of JNK (Bossy-Wetzel *et al.*, 1997; Behrens *et al.*, 1999).

The delayed phosphorylation and activation of JNK in XPA and other TCR-deficient cells is accompanied by inhibition of the JNK-phosphatase MKP-1 (this manuscript, our unpublished observations). Various studies indicate that MKP-1 mainly exhibits antiapoptotic activity. For instance, conditional over expression of MKP-1 can repress the rapid activation of JNK and p38 in response to high doses of UV and subsequently impede apoptosis (Franklin *et al.*, 1998). Moreover, the nonapoptotic DNA-damaging agent *trans*-platin induces MKP-1 expression and activates JNK only transiently, while its apoptotic isomer *cis*-platin which cannot activate MKP-1 induces more sustained JNK activation (Sanchez-Perez *et al.*, 1998, 2000).

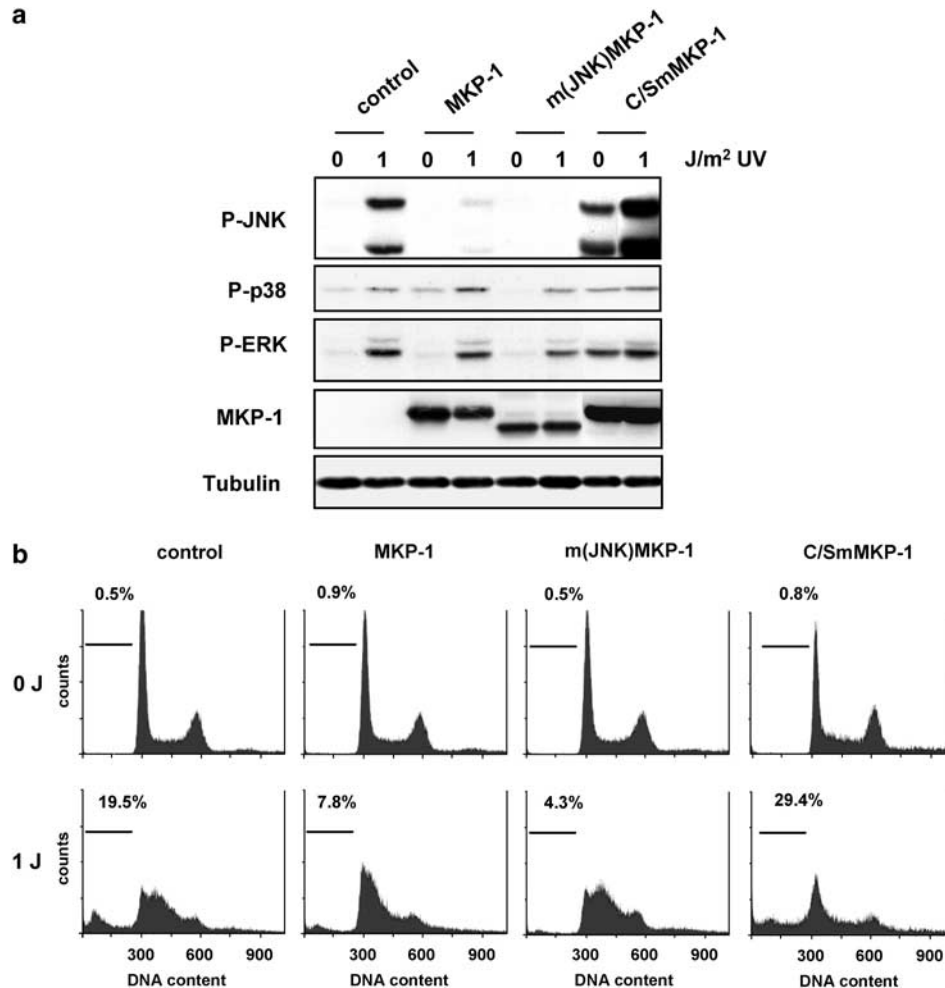


Figure 6 Ectopic expression of MKP-1 in XPA cells inhibits apoptosis by low dose UV. XPA cells were transduced with lentiviruses containing wild-type MKP-1, the JNK-restricted mutant m(JNK)MKP-1, the catalytically inactive dominant-negative mutant C/SmMKP-1, or an empty control virus. Transduction efficiency was 95–100% as verified by FACS determination. Next, the three cultures were irradiated with 1 J/m² UV-C or mock-treated (0), and harvested after 16 h for immunoblot analysis (**a**) or after 24 h for FACS analysis (**b**). The percentage of apoptotic (sub-G1) cells is indicated in the graphs. Please note that for the control cells in the Western blot (**a**), the endogenous MKP-1 is not visible because a relatively short exposure is shown

Importantly, MKP-1 inhibition and/or prolonged JNK activation does not appear to be sufficient to induce apoptosis in TCR-deficient fibroblasts, as we found JNK activation by ectopic expression of dominant-negative MKP-1 to enhance low dose UV-induced apoptosis, but not to induce high levels of cell death by itself (Figure 6). Moreover, inhibition of JNK via over expression of JNK-specific MKP-1 did not completely inhibit low dose UV-induced apoptosis in the XPA fibroblasts. This indicates that persistent UV-induced DNA damage also causes activation of other proapoptotic pathways and/or inhibition of prosurvival factors (see model in Figure 7). Such proapoptotic factors may be for instance death and cytokine receptor family members like CD95/Fas (Herrlich *et al.*, 1999; Martindale and Holbrook, 2002). Although low doses of UV are unlikely to activate cytokine or death receptors in a direct manner, in contrast to high doses of UV (Sachsenmaier *et al.*, 1994;

Rosette and Karin, 1996), these receptors might be activated in an autocrine fashion, since XPA cells secrete cytokines upon treatment with low doses of UV (Herrlich *et al.*, 1999). The accumulation of proliferating XPA cells in S phase after 1 J/m² UV (Figure 6b) suggests that replication fork stalling might contribute to UV-induced apoptosis in TCR-deficient cells. However, at higher doses of UV (3–5 J/m²), these XPA cells mainly arrest in G1, whereas the levels of apoptosis are further enhanced (Figure 3a, our unpublished observations). Moreover, low doses of UV also induce apoptosis in serum-starved G1-arrested primary XPA and CSB fibroblasts (our unpublished observations), indicating that replication fork stalling is not essential for cell death. p53 does not seem to contribute strongly to the induction of apoptosis by low dose UV in TCR-deficient fibroblasts (McKay *et al.*, 2001). In agreement with this finding, we found low dose UV-induced JNK-dependent apoptosis also to occur in XPA cells that have been

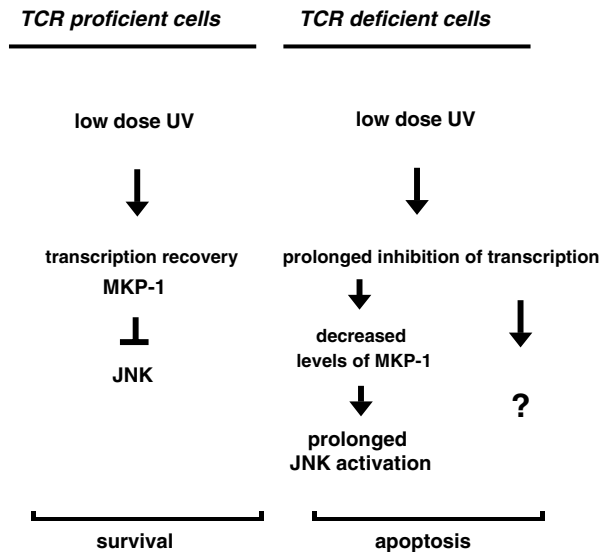


Figure 7 Proposed model for the role of MKP-1 and JNK in DNA-damage-induced apoptosis in TCR-deficient human fibroblasts. In TCR-proficient cells, UV-induced damage in transcribed genes is rapidly repaired, resulting in recovery of MKP-1 transcription and inhibition of JNK activity. In TCR-deficient cells, damage in transcribed genes persists, resulting in prolonged inhibition of transcription and, as a consequence, reduced levels of MKP-1. MKP-1 underexpression subsequently results in delayed and prolonged JNK activation. In addition to JNK activation, prolonged transcription inhibition results in activation of other, as yet unknown, proapoptotic pathways and/or inhibition of pro-survival factors (?)

immortalized by SV40T, a protein that functionally inactivates p53.

To our knowledge this is the first study that provides a functional link between DNA damage in transcribed genes, JNK activation, and JNK-dependent apoptosis. In fact, so far the studies addressing the role of DNA damage in the activation by JNK by genotoxic stress have provided ambiguous data. However, the delayed activation of JNK by low doses of UV in TCR-deficient fibroblasts shown in this study is clearly due to the prolonged presence of unrepaired DNA in transcribed genes: (1) it does not occur in cells with normal DNA repair capacity or in GGR-deficient, TCR-proficient XPC and XPE cells; (2) it is not observed upon activation of a functional DNA repair enzyme (CPD-photolyase) in XPA cells. Interestingly, we found the mechanism by which persistent DNA damage triggers delayed activation of the JNK pathway to be different from the mechanism by which osmotic stress or high doses of UV trigger rapid JNK activation. The latter stimuli rapidly activate the JNK kinases MKK4 and MKK7, which phosphorylate and activate JNK activity within 15 min (this manuscript, our unpublished observations). Moreover, high doses of UV rapidly induce the expression of MKP-1, which subsequently suppresses the UV-induced JNK activity between 60 and 120 min after irradiation (Liu *et al.*, 1995; our unpublished observations). In contrast, low dose UV does not significantly activate MKK4 and MKK7, and can cause

enhanced JNK activity via inhibition of *mkp-1* mRNA expression. It should be noted here that inhibition of MKP-1 might not be sufficient to cause JNK activation in TCR-deficient cells and that also other JNK phosphatases might be downregulated by persistent DNA damage. Although we found dominant-negative MKP-1 to activate JNK in the absence of another stimulus, this catalytically inactive mutant has an intact JNK-binding domain (Alessi *et al.*, 1993) and therefore could also block dephosphorylation of JNK by other phosphatases.

It remains to be established how persistent DNA damage suppresses *mkp-1* mRNA levels. MKP-1 suppression is unlikely to be the result of JNK activation, as pharmacological inhibition of JNK with SP600125 did not inhibit the repression of MKP1 by low dose UV (our unpublished observations). Interestingly, in TCR-deficient cells, UV-induced DNA damage has been found to cause sustained inhibition of transcription, in particular of genes with large transcription units (McKay *et al.*, 1998). This appears to be due to the inability of the elongating RNAPol II complex to bypass UV-induced lesions on the DNA template, which subsequently leads to partial, UV dose-dependent depletion of the initiating form of RNAPol II (Rockx *et al.*, 2000; Svejstrup, 2002). Since the *mkp-1* gene is not very large (3.1 kb), in contrast to *atf-3* (15 kb), inhibition of MKP-1 expression by low doses of UV may be due to reduced transcription initiation rather than by reduced transcription elongation.

The role of the MAPK family members p38 and ERK in DNA-damage-induced apoptosis in TCR-deficient cells remains still unclear. Preliminary experiments using the p38 inhibitor SB203580 indicate that p38 does not play a significant role in the induction of apoptosis. Inhibition of ERK activation via the inhibitor U0126 also does not seem to affect UV-induced apoptosis in the human XPA fibroblasts (M Hamdi and H van Dam, unpublished results), suggesting that ERK might play an auxiliary role. Finally, it remains to be established whether JNK-dependent apoptosis in XPA cells involves its transcription factor substrates c-Jun and ATF-2, and/or its target genes *atf-3* and *c-fos*. Interestingly, induction of ATF-3 can accelerate apoptosis in certain cell types (Mashima *et al.*, 2001), whereas c-Fos is antiapoptotic in UV-treated mouse embryo fibroblasts (Schreiber *et al.*, 1995; Blattner *et al.*, 2000).

In summary, our results provide a novel link between DNA damage hypersensitivity, and the MKP-1–JNK pathway and suggest that MKP-1 might be an interesting target for therapeutic intervention in combination with DNA-damaging agents.

Materials and methods

Cells and cell culture

The primary and SV40T-immortalized human foreskin fibroblasts used in this study have been described (Abrahams *et al.*, 1988, 1992, 1998; Klein *et al.*, 1990; Rockx *et al.*, 2000; Nakajima *et al.*, 2004). Cells were grown on DMEM (Life

technologies, Inc., Paisley, UK) supplemented with 9% FBS (Life technologies, Inc.), penicillin, and streptomycin.

For UV-C irradiation, a 30 W germicidal lamp (TUV; Philips Electronic Instruments Inc., Eindhoven, The Netherlands) was used. Prior to UV treatment, culture medium was collected, dishes were washed once with PBS (10 mM Na₂HPO₄/0.14 mM NaCl, pH 7.4), and the PBS was removed before irradiation at room temperature. After irradiation, cells were refed with the collected medium. For photoreactivation, cells were irradiated for 1 h with a 30 W 450 nm emitting light tube (Philips Electronic Instruments, Inc.) at RT, while mock-treated cells were kept in dark at RT for the same period.

FACS analysis

For quantitation of sub-G1 apoptotic cells, adhering and floating cells were collected via trypsinization and centrifugation. After resuspending in PBS, cells were fixed in 70% ethanol, washed in PBS, stained with 7.5 µM propidium iodide (PI) containing 50 µg/ml RNase A, and analysed by flow cytometry (FACS-Calibur, Becton Dickinson). FACS results were analysed with WinMDI 2.8 software.

Western analysis and antibodies

For preparation of protein extracts, cells were washed once with ice-cold PBS and subsequently lysed on ice in ice-cold buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) containing protease and phosphatase inhibitors (van Dam *et al.*, 1993). Lysates were cleared by centrifugation. Routinely either 20 or 30 µg of protein were loaded on SDS-PAGE gels. Antibodies used are c-Jun-H79, ATF3-C19, and MKP-1-M18 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), ERK-Thr202/Tyr204, SEK/MKK-4-Thr223, and PhosphoPlus® p38 MAP kinase (Thr180/Tyr182) from Cell Signalling Technology, Inc. (Beverly, MA, USA), Anti-ACTIVE® JNK pAb pTPpY from Promega Corporation, and tubulin from Sigma. The monoclonal antibody against threonine 91-phosphorylated c-Jun was kindly provided by Dr A Behrens, Cancer UK, London (Nateri *et al.*, 2004).

References

- Abrahams PJ, Houweling A, Cornelissen-Steijger PDM, Jaspers NGJ, Darroudi F, Meijers CM, Mullenders LHF, Filon R, Arwert F, Pinedo HM, Natarajan APT, Terleth C, van Zeeland AA and van der Eb AJ. (1998). *Mut. Res-DNA Repair*, **407**, 189–201.
- Abrahams PJ, Houweling A and van der Eb AJ. (1992). *Cancer Res.*, **52**, 53–57.
- Abrahams PJ, van der Kleij AAM, Schouten R and van der Eb AJ. (1988). *Cancer Res.*, **48**, 6054–6057.
- Alessi DR, Smythe C and Keyse SM. (1993). *Oncogene*, **8**, 2015–2020.
- Bazuine M, Carlotti F, Tafrechi RSJ, Hoebe RC and Maassen JA. (2004). *Mol. Endocrinol.*, **18**, 1697–1707.
- Behrens A, Sibilia M and Wagner EF. (1999). *Nat. Genet.*, **21**, 326–329.
- Berneburg M and Lehmann AR. (2001). *Adv. Genet.*, **43**, 71–102.
- Blattner C, Bender K, Herrlich P and Rahmsdorf HJ. (1998). *Oncogene*, **16**, 2827–2834.
- Blattner C, Kannouche P, Litfin M, Bender K, Rahmsdorf HJ, Angulo JF and Herrlich P. (2000). *Mol. Cell. Biol.*, **20**, 3616–3625.

RNA isolation and Northern analysis

RNA was isolated with the SV Total RNA isolation system (Promega) followed by an additional acid phenol extraction. Northern analysis including the *c-jun*, *atf-3*, and *hef-1* probes has been described previously (van Dam *et al.*, 1989, 1990; Kool *et al.*, 2003). As *mkp-1* probe, the *EcoRI* insert of p3CH134 containing the mouse *mkp-1* cDNA (Lau and Nathans, 1985) was used.

MKP-1 constructs and lentivirus transduction

cDNAs of MKP-1, C/SmMKP1, and m(JNK)MKP-1 (Keyse and Emslie, 1992; Alessi *et al.*, 1993; Slack *et al.*, 2001) were a kind gift of Dr S Keyse (Dundee, Scotland, UK) and recloned into the lentiviral vector pRRL-cPPT-CMV-IRES-GFP-PRE-SIN (also named pLV-CMV-IRES-GFP; Carlotti *et al.*, 2004). The viruses were produced as described previously (Carlotti *et al.*, 2004). Briefly, the lentiviral cDNA construct was cotransfected overnight with three helper plasmids encoding HIV1 gag/pol, HIV1 rev, and VSV-G envelope into 293T cells. The medium was refreshed and viruses were harvested after 48 and 72 h, passed through 0.45 µm filters and stored at –80°C. Virus was quantitated by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation, New York, USA). For transduction, viral supernatants were overnight added to subconfluent cell cultures in fresh medium supplemented with 8 µg/ml polybrene (Sigma). The next day, the medium was replaced. Transduction efficiency was 95–100% as verified by FACS analysis (IRES-GFP).

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- Bossy-Wetzel E, Bakiri L and Yaniv M. (1997). *EMBO J.*, **16**, 1695–1709.
- Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA and Davis RJ. (2003). *Genes Dev.*, **17**, 1969–1978.
- Camps M, Nichols A and Arkinstall S. (2000). *FASEB J.*, **14**, 6–16.
- Carlotti F, Bazuine M, Kekarainen T, Seppen J, Pognonec P, Maassen JA and Hoebe RC. (2004). *Mol. Ther.*, **9**, 209–217.
- Chang LF and Karin M. (2001). *Nature*, **410**, 37–40.
- Davis RJ. (2000). *Cell*, **103**, 239–252.
- de Boer J and Hoeijmakers JHJ. (2000). *Carcinogenesis*, **21**, 453–460.
- Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoel MJ, Bertrand F, Caron M, Atfi A, Cherqui G and Capeau J. (2000). *Endocrinology*, **141**, 922–931.
- Engelbrecht Y, de Wet H, Horsch K, Langeveldt CR, Hough FS and Hulley PA. (2003). *Endocrinology*, **144**, 412–422.
- Farooq A and Zhou MM. (2004). *Cell. Signal.*, **16**, 769–779.
- Ford JM and Hanawalt PC. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 8876–8880.

- Franklin CC and Kraft AS. (1997). *J. Biol. Chem.*, **272**, 16917–16923.
- Franklin CC, Srikanth S and Kraft AS. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 3014–3019.
- Guo YL, Kang BB and Williamson JR. (1998). *J. Biol. Chem.*, **273**, 10362–10366.
- Hai T, Wolfgang CD, Marsee DK, Allen AE and Sivaprasad U. (1999). *Gene Expression*, **7**, 321–335.
- Hayakawa J, Depatie C, Ohmichi M and Mercola D. (2003). *J. Biol. Chem.*, **278**, 20582–20592.
- Hayakawa J, Mittal S, Wang Y, Korkmaz KS, Adamson E, English C, Omichi M, McClelland M and Mercola D. (2004). *Mol. Cell*, **16**, 521–535.
- Herrlich P, Bender K, Knebel A, Bohmer FD, Gross S, Blattner C, Rahmsdorf HJ and Gottlicher M. (1999). *CR Acad. Sci. III*, **322**, 121–125.
- Jaspers NGJ, Raams A, Kelner MJ, Ng JMY, Yamashita YM, Takeda S, McMorris TC and Hoeijmakers JHJ. (2002). *DNA Repair*, **1**, 1027–1038.
- Kannan K, Amariglio N, Rechavi G, Jakob-Hirsch J, Kela I, Kaminski N, Getz G, Domany E and Givol D. (2001). *Oncogene*, **20**, 2225–2234.
- Keyse SM. (2000). *Curr. Opin. Cell Biol.*, **12**, 186–192.
- Keyse SM and Emslie EA. (1992). *Nature*, **359**, 644–647.
- Kool J, Hamdi M, Cornelissen-Steijger PDM, van der Eb AJ, Terleth C and van Dam H. (2003). *Oncogene*, **22**, 4235–4242.
- Klein B, Pastink A, Odijk H, Westerveld A and van der Eb AJ. (1990). *Exp. Cell Res.*, **191**, 256–262.
- Lamb JA, Ventura JJ, Hess P, Flavell RA and Davis RJ. (2003). *Mol. Cell*, **11**, 1479–1489.
- Lau LF and Nathans D. (1985). *EMBO J.*, **4**, 3145–3151.
- Lei K and Davis RJ. (2003). *Proc. Natl. Acad. Sci. USA*, **100**, 2432–2437.
- Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, Bar-Sagi D and Davis RJ. (2002). *Mol. Cell Biol.*, **22**, 4929–4942.
- Li CH, Hu YH, Mayr M and Xu QB. (1999). *J. Biol. Chem.*, **274**, 25273–25280.
- Li J, Gorospe M, Hutter D, Barnes J, Keyse SM and Liu YS. (2001). *Mol. Cell Biol.*, **21**, 8213–8224.
- Liu YS, Gorospe M, Yang CL and Holbrook NJ. (1995). *J. Biol. Chem.*, **270**, 8377–8380.
- MacLaren A, Black EJ, Clark W and Gillespie DAF. (2004). *Mol. Cell Biol.*, **24**, 9006–9018.
- Martindale JL and Holbrook NJ. (2002). *J. Cell. Physiol.*, **192**, 1–15.
- Mashima T, Udagawa S and Tsuruo T. (2001). *J. Cell. Physiol.*, **188**, 352–358.
- Mckay BC, Becerril C and Ljungman M. (2001). *Oncogene*, **20**, 6805–6808.
- Mckay BC, Ljungman M and Rainbow AJ. (1998). *Oncogene*, **17**, 545–555.
- Mercurio F and Manning AM. (1999). *Oncogene*, **18**, 6163–6171.
- Mitchell JR, Hoeijmakers JHJ and Niedernhofer LJ. (2003). *Curr. Opin. Cell Biol.*, **15**, 232–240.
- Mizuno R, Oya M, Shiomi T, Marumo K, Okada Y and Murai M. (2004). *J. Urol.*, **172**, 723–727.
- Morrison DK and Davis RJ. (2003). *Ann. Rev. Cell Dev. Biol.*, **19**, 91–118.
- Nakajima S, Lan L, Kanno S, Takao M, Yamamoto K, Eker APM and Yasui A. (2004). *J. Biol. Chem.*, **279**, 46674–46677.
- Nateri AS, Riera-Sans L, Da Costa C and Behrens A. (2004). *Science*, **303**, 1374–1378.
- Queille S, Drougard C, Sarasin A and Daya-Grosjean L. (2001). *J. Invest. Dermatol.*, **117**, 1162–1170.
- Rockx DAP, Mason R, van Hoffen A, Barton MC, Citterio E, Bregman DB, van Zeeland AA, Vrieling H and Mullenders LHF. (2000). *Proc. Natl. Acad. Sci. USA*, **97**, 10503–10508.
- Rosette C and Karin M. (1996). *Science*, **274**, 1194–1197.
- Sachsenmaier C, Radlerpohl A, Zinck R, Nordheim A, Herrlich P and Rahmsdorf HJ. (1994). *Cell*, **78**, 963–972.
- Sanchez-Perez I, Martinez-Gomariz M, Williams D, Keyse SM and Perona R. (2000). *Oncogene*, **19**, 5142–5152.
- Sanchez-Perez I, Murguia JR and Perona R. (1998). *Oncogene*, **16**, 533–540.
- Schreiber M, Baumann B, Cotten M, Angel P and Wagner EF. (1995). *EMBO J.*, **14**, 5338–5349.
- Shaulian E and Karin M. (2001). *Oncogene*, **20**, 2390–2400.
- Shaulian E and Karin M. (2002). *Nat. Cell Biol.*, **4**, E131–E136.
- Slack DN, Seternes OM, Gabrielsen M and Keyse SM. (2001). *J. Biol. Chem.*, **276**, 16491–16500.
- Smith ML, Ford JM, Hollander MC, Bortnick RA, Amundson SA, Seo YR, Deng CX, Hanawalt PC and Fornace AJ. (2000). *Mol. Cell Biol.*, **20**, 3705–3714.
- Spivak G, Itoh T, Matsunaga T, Nikaido O, Hanawalt P and Yamaizumi M. (2002). *DNA Repair*, **1**, 629–643.
- Svejstrup JQ. (2002). *Mol. Cell*, **9**, 1151–1152.
- Todd DE, Densham RM, Molton SA, Balmano K, Newson C, Weston CR, Garner AP, Scott L and Cook SJ. (2004). *Oncogene*, **23**, 3284–3295.
- van Dam H and Castellazzi M. (2001). *Oncogene*, **20**, 2453–2464.
- van Dam H, Duyndam M, Rottier R, Bosch A, De Vries-Smits L, Herrlich P, Zantema A, Angel P and van der Eb AJ. (1993). *EMBO J.*, **12**, 479–487.
- van Dam H, Offringa R, Meijer I, Stein B, Smits AM, Herrlich P, Bos JL and van der Eb AJ. (1990). *Mol. Cell Biol.*, **10**, 5857–5864.
- van Dam H, Offringa R, Smits AMM, Bos JL, Jones NC and van der Eb AJ. (1989). *Oncogene*, **4**, 1207–1212.
- van Oosten M, Rebel H, Friedberg EC, van Steeg H, van der Horst GTJ, van Kranen HJ, Westerman A, van Zeeland AA, Mullenders LHF and de Gruijl FR. (2000). *Proc. Natl. Acad. Sci. USA*, **97**, 11268–11273.
- van Steeg H and Kraemer KH. (1999). *Mol. Med. Today*, **5**, 86–94.
- Wada T, Joza N, Cheng HYM, Sasaki T, Kozieradzki I, Bachmaier K, Katada T, Schreiber M, Wagner EF, Nishina H and Penninger JM. (2004). *Nat. Cell Biol.*, **6**, 215–226.
- Weston CR and Davis RJ. (2002). *Curr. Opin. Genet. Dev.*, **12**, 14–21.
- Xu QH, Konta T, Nakayama KJ, Furusu A, Moreno-Manzano V, Lucio-Cazana J, Ishikawa Y, Fine LG, Yao J and Kitamura M. (2004). *Free Radic Biol. Med.*, **36**, 985–993.
- Yan CH, Wang H and Boyd DD. (2002). *J. Biol. Chem.*, **277**, 10804–10812.

Chapter IV

ATF3 and Fra1 have opposite functions in JNK- and ERK-dependent DNA damage responses

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ATF3 and Fra1 have opposite functions in JNK- and ERK-dependent DNA damage responses

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ABSTRACT

JNK and ERK MAP kinases regulate cellular responses to genotoxic stress in a cell type and cell context-dependent manner. However, the factors that determine and execute JNK- and ERK-controlled stress responses are only partly known. In this study, we investigate the roles of the AP-1 components ATF3 and Fra1 in JNK- and ERK-dependent cell cycle arrest and apoptosis. We show that the anti-cancer drug cisplatin or UV light activates both JNK and ERK in human glioblastoma cells lacking functional p53. Inhibition experiments of JNK or ERK activities revealed that the ERK pathway strongly promotes cisplatin- and UV-induced apoptosis in these glioblastoma cells. Furthermore, JNK but not ERK is required for ATF3 induction, and both ERK and JNK are necessary for post-transcriptional induction of Fra1 in response to cisplatin or UV. Knock-down of ATF3 and Fra1 results in increased and decreased cisplatin-induced apoptosis, respectively, indicating that ATF3 is an anti-apoptotic JNK effector and Fra1 is a pro-apoptotic ERK/JNK effector. Knock-down experiments also revealed that ATF3 and Fra1, respectively, enhance and reduce S-phase arrest through differential modulation of the Chk1–Cdk2 pathway. Thus, we identify novel reciprocal functions of ATF3 and Fra1 in JNK- and ERK-dependent DNA damage responses.

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1. Introduction

To protect against accumulation of undesirable mutations induced by DNA damage, cells activate programs to initiate cell cycle arrest or cell death. Low levels of DNA damage cause transient cell cycle arrest, allowing cells to repair DNA before

cell cycle progression. Persistent cell cycle arrest or apoptosis is induced after high doses of DNA damage, resulting in elimination of highly damaged cells. The mitogen-activated protein (MAP) kinase super-family, including JNK (Jun-N-terminal kinase), p38, and ERK (extracellular signal-regulated kinase) subgroups, can execute DNA damage responses by

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regulating cell cycle progression and apoptosis. The onset, magnitude and persistence of activation of MAP kinases depend on cell type, cellular context, and nature and dose of the agent.

The anti-cancer drug cisplatin and UV light are genotoxic agents that generate bulky DNA-helix distorting lesions inducing replication fork stalling and stalling of RNA polymerase II [1]. These genotoxic agents alter activities of MAP kinases both via MAP kinase-kinases (MKKs) and MAP kinase-phosphatases (MKPs) [2,3]. For example, these agents induce sustained JNK activity by activating JNK kinases MKK4 or MKK7 and inhibiting JNK phosphatase MKP-1 expression [2,4,5]. In general, strong and persistent JNK activation following cell stress enhances apoptosis after DNA damage, whereas weak and transient JNK activation enhances cell survival [4,6]. ERK signaling can also activate both pro- and anti-apoptotic functions after genotoxic stress, but the relevant ERK substrates are as yet unknown [7–9]. As for JNK substrates, c-Jun has been shown to act as a pro- and anti-apoptotic JNK substrate upon DNA damage [10,11].

The Jun, Fos, and ATF families of proteins act as downstream effectors of MAP kinases by forming AP-1 transcription factor complexes [2,9,12,13]. Based on DNA-binding specificities, AP-1 complexes can be classified into two groups: Jun/Fos dimers, which preferentially bind to the 7 bp “classical” AP-1 motif (TGAG/CTCA), and Jun/ATF and ATF/ATF dimers, which efficiently bind only to 8bp ATF-sites (TGACNTCA). Gene-regulatory activities of AP-1 components thus depend on their dimer-partner [14–16]. MAP kinases alter the transcriptional activity, protein stability and/or mRNA levels of AP-1 components, such as c-Jun, ATF2, ATF3, and Fos family members c-Fos and Fra1 [15,17–21].

In various cell types, MAP kinases and AP-1 complexes regulate cell cycle progression and cell survival through modulation of components of the retinoblastoma 1 (RB) and p53 tumor suppressor pathways. For instance, JNK, c-Jun, c-Fos, Fra1, and ATF3 can control the levels and/or activities of cyclin D1, p53, and/or the cell cycle inhibitors p16-INK4A, p14-ARF, and p21-CIP [13,22–25]. p53, p16-INK4A and other components of the RB pathway are frequently mutated or absent in human cancer cells, yet little is known about the roles of AP-1 in DNA damage responses in the absence of p53 and p16-INK4A.

Here we used T98G human glioblastoma cells, which lack both functional p53 and p16-INK4A, and in which c-Jun controls cell survival after DNA damage [11]. These cells are relatively resistant to cisplatin [26]. Based on recent reports that Fra1 expression is altered in cisplatin-resistant tumor cells [27,28] and that ATF3 is induced during UV-induced apoptosis in human fibroblasts with defects in p53 and nucleotide excision repair genes [5], we analyzed expression and function of Fra1 and ATF3, which form Jun/Fos- and Jun/ATF-type dimers with c-Jun, respectively. We show that DNA damage induces the anti-apoptotic effector ATF3 in a JNK-dependent manner, whereas Fra1 is induced as a pro-apoptotic effector requiring both JNK and ERK in T98G glioblastoma cells. Moreover, we demonstrate that ATF3 and Fra1 differently affect cisplatin-induced S-phase accumulation and Tyr15-phosphorylation of the S-phase regulatory kinase Cdk2.

2. Material and methods

2.1. Cell culture

T98G glioblastoma cells [26] were grown on DMEM supplemented with 9% FBS, penicillin and streptomycin. For UV-C irradiation, a 30W germicidal lamp was used. Prior to UV-treatment, culture medium was collected, dishes were washed once with phosphate-buffered saline (PBS) and PBS was removed before irradiation at room temperature. After irradiation, cells were fed again with the collected medium. Treatment with U0126 was performed at 25 μ M as described [29].

2.2. Western analysis and antibodies

To prepare protein extracts, cells were washed twice with ice-cold PBS and lysed on ice in ice-cold buffer containing 10 mM, Tris pH 7.5, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate containing protease and phosphatase inhibitors [30]. Routinely either 20 or 30 μ g of protein were loaded on SDS-PAGE gels. Antibodies used are Fra1 (R-20), ATF3 (C19), cyclin A (H-432), cyclin E (C19), Cdk2 (M2) and Chk1 (FL-476) from Santa Cruz Biotechnology, phospho-specific ERK-Thr202/Tyr204 and JNK-Thr183/Tyr185 (monoclonal) from Cell Signaling Technology, CDK2-Tyr15 from Chemicon, Chk1-Ser317 from Bethyl and active caspase 3 from Pharmingen.

2.3. RNA isolation and Northern analysis

RNA was isolated using the SV Total RNA isolation system (Promega) followed by an additional acid phenol extraction. Northern analysis including the *atf3*, *fra1* and *hef1* probes has been described [31–33].

2.4. Cell counting and FACS analysis

For the analysis of cell proliferation, viable cells were quantified with a CASY TT Cell Counter + Analyser (Schaefer System) according to the manufacturer's instructions. To quantitate G1, S and G2/M phase cells and sub-G1 apoptotic cells both adhering and floating cells were collected via trypsinization and/or centrifugation. After resuspension in PBS, cells were fixed in 70% ethanol, washed in PBS, stained with 7.5 μ M propidium iodide (PI) containing 50 μ g/ml RNase A, and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). FACS results were analyzed with WinMDI 2.8 software.

2.5. Lentivirus constructs and transduction

For RNAi experiments, the following oligomers were inserted into BglIII/HindIII-digested pSuper [34]: *atf3* sh1 forward 5'-GAGCGACGAGAAAGAAAT-3' and reverse 5'-ATTCTTTCTCGTCGCCCTC-3'; *atf3* sh2 forward 5'-GCAGCTGCAAAGTGCCGAA-3' and reverse 5'-TTCGGCACTTTGCAGCTGC-3'; *fra1* sh1 forward 5'-AGGCCTTGTGAACAGATCA-3' and reverse 5'-TGATCTGTTTCAAGGCCT-3', *fra1* sh2 forward 5'-CTGGAAGATGAGAAATCT-3' and reverse 5'-AGATTTCTCATCTCCAG-3'. Subsequently, the H1-RNAi cassettes of pSuper were inserted

into the pRRL-CMV-GFP lentiviral backbone [35] upstream of the CMV-GFP cassette using XhoI and PstI restriction sites. Inserts were verified by sequencing. Details on lentivirus production and transduction, including a description of the m(JNK)MKP-1 construct have been described [5,36].

2.6. Statistics

Quantitative values are represented as the mean $\pm$ S.E.M. of at least three experiments. Statistical comparisons were made using an unpaired two-tailed Student's t-test. A P-value <0.05 was considered to be significant.

3. Results

3.1. Cisplatin and UV light induce JNK, ERK, ATF3 and Fra1 followed by cell cycle arrest and apoptosis

To understand the functions of JNK and ERK in response to genotoxic stress, we exposed T98G human glioblastoma cells to cisplatin or UV light. Active, phosphorylated JNK and ERK were induced within 4–6 h by both agents, becoming detectable at 50 μ M of cisplatin and 10 J/m² UV (Fig. 1A and B). Cisplatin induced the two potential target proteins of JNK and ERK signaling, ATF3 and Fra1, in a dose-dependent manner (Fig. 1A). UV also induced ATF3 and Fra1 at a low dose (10 J/m²) (Fig. 1B). No further induction of ATF3 protein was observed at higher UV doses (30 and 60 J/m²), presumably reflecting UV-induced stalling of RNA polymerase II (Fig. 1B and D) [5]. Since Fra1 protein levels were increased and *fra1* mRNA was repressed by cisplatin and UV, Fra1 induction is most likely due to post-translational stabilization (Fig. 1C and D). The levels of DNA damage induced by these doses of cisplatin and UV are within the same range: 200 μ M cisplatin induces 1 DNA lesion in 5.4 kb [26] whereas 30 J/m² UV induces 1 DNA lesion in 5.3 kb (Mullenders, personal communication).

We next examined the effects of cisplatin and UV radiation on T98G cell proliferation and survival. Essentially complete inhibition of proliferation was observed already at 50 μ M cisplatin or 10 J/m² UV (Fig. 2A and B). At these low doses, only approximately 20% of the cells became apoptotic and active caspase 3 levels were relatively low (Fig. 2C and D). Upon exposure to higher doses of cisplatin or UV, numbers of apoptotic cells increased to 30–40% and caspase 3 was strongly activated (Fig. 2C and D). Analysis of cell cycle progression showed that the non-treated cells gradually accumulated in G1 as a result of contact inhibition. By contrast, cells treated with 50 μ M cisplatin or 10 J/m² UV mainly accumulated in S-phase (Fig. 2E and F). Cisplatin- or UV-treated cells showed elevated levels of the S-phase markers cyclin E, cyclin A and Cdk2 (Fig. 2G and H). Based on these results, we used 50 μ M cisplatin and 10 J/m² UV to determine the roles of JNK, ERK, Fra1 and ATF3 in cell cycle arrest and apoptosis in T98G cells.

3.2. ERK rather than JNK pathways are critical for cisplatin- and UV-induced apoptosis in T98G cells

Previous studies showed that cisplatin and UV light can induce prolonged JNK activation through transcriptional inhi-

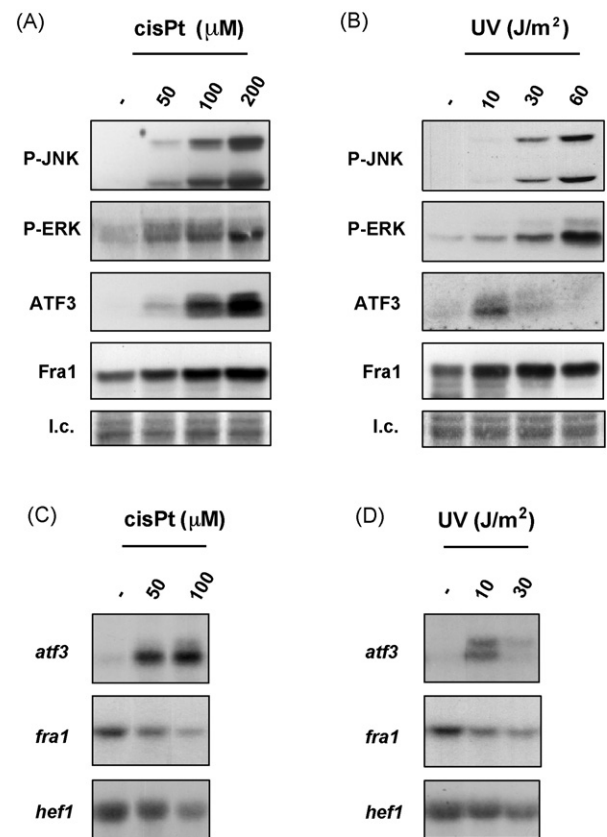


Fig. 1 – Activation of JNK, ERK, ATF3 and Fra1 by cisplatin and UV in T98G cells. (A and B) Immunoblot analysis of T98G glioblastoma cells treated with the indicated doses of cisplatin (cisPt) for 6 h (A) or UV light for 4 h (B). Ponceau S staining of part of the filter is shown as a loading control (l.c.). (C and D) Northern blot analysis of T98G cells 5 h after cisplatin (C) or UV (D) treatment. *hef1* mRNA expression was determined as loading control.

bition of MKP-1 [4,5]. We therefore used a lentiviral vector expressing the JNK-restricted mutant of MKP-1 m(JNK)MKP-1 (hereafter called mMKP-1) to analyze the role of JNK in T98G cell cycle arrest and cell death. mMKP-1 efficiently blocked cisplatin- and UV-induced accumulation of phosphorylated, active JNK, but did not affect induction of phosphorylated ERK (Fig. 3A and B). Importantly, ATF3 and Fra1 induction by cisplatin was suppressed by mMKP-1 (Fig. 3A). mMKP-1 also reduced both basal and UV-induced levels of all detectable Fra1 isoforms (Fig. 3B). In contrast to the drastic effects of mMKP-1 on AP-1 components, the induction of apoptosis by cisplatin or UV was mildly inhibited by mMKP-1 expression (Fig. 3C), and the levels of active caspase 3 were also mildly reduced (Fig. 3D). Similar results were obtained with the JNK-inhibitor SP600125 (data not shown). Thus, JNK signaling is required for efficient expression of ATF3 and Fra1, and partially contributes to cisplatin- or UV-induced apoptosis in T98G cells.

To investigate the role of the ERK pathway in DNA damage responses, we used U0126, a specific inhibitor of the ERK-kinase MEK. As shown in Fig. 4A and B, U0126 com-

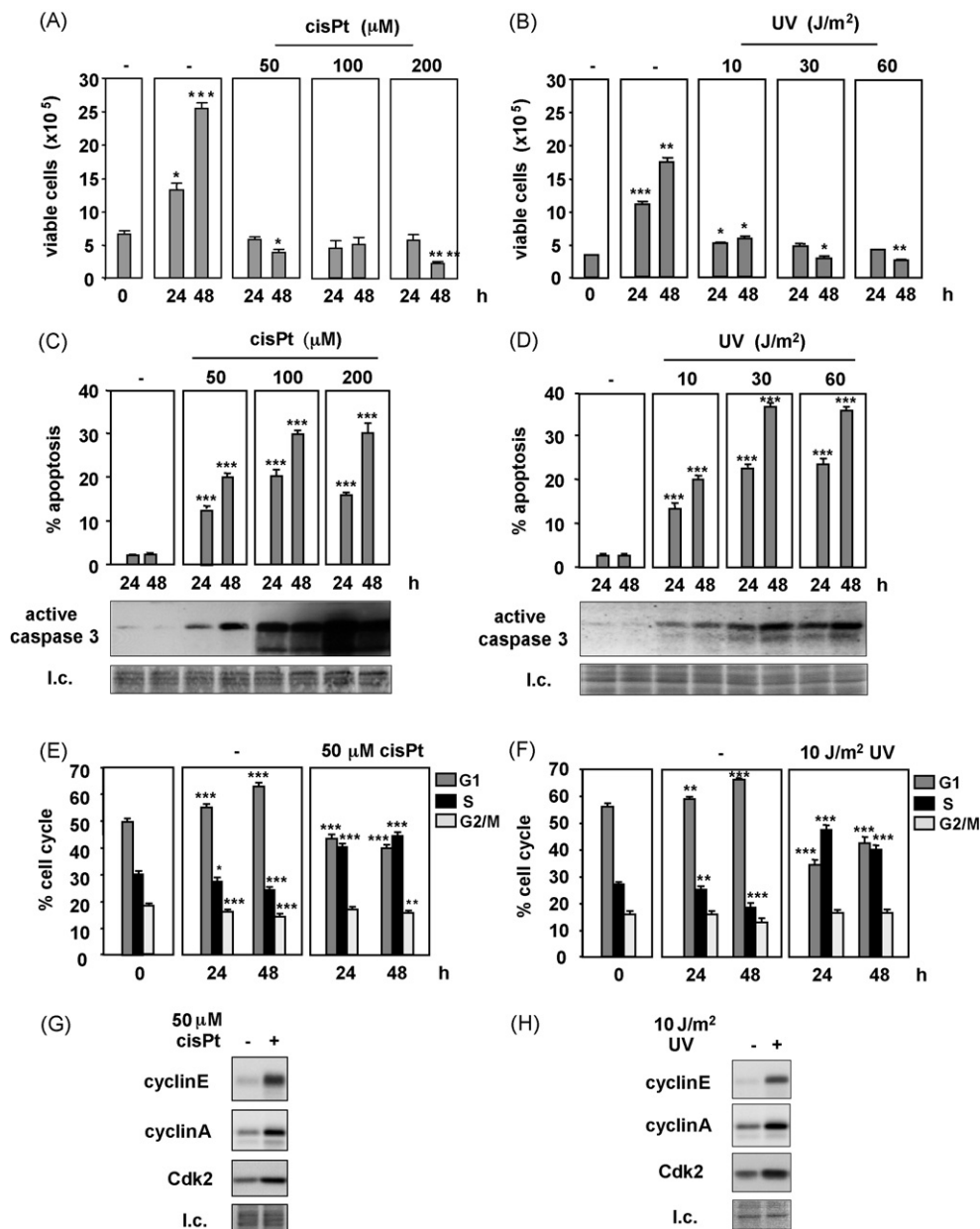


Fig. 2 – Cisplatin- and UV-induced cell cycle arrest and apoptosis in T98G cells. (A and B) Cell number of subconfluent cells treated with cisplatin (A) or UV (B). *P* values were determined relative to the *t*=0 control. **P*<0.05, ***P*<0.01, ****P*<0.001. (C and D) The percentage of cells in sub-G1 (% apoptosis) after cisplatin (C) or UV (D) treatment. *P* values were determined relative to the 24 h non-treated control. (E and F) Cell cycle profile. *P* values were determined relative to the G1, S, or G2/M values at *t*=0. (G and H) Immunoblot analysis of cells treated for 24 h. Ponceau S staining of part of the filter is shown as a loading control (l.c.).

pletely blocked cisplatin- and UV-induced accumulation of phosphorylated, active ERK. While ATF3 induction was not suppressed by U0126, this inhibitor blocked accumulation of the hyper-phosphorylated, slow migrating form of Fra1 in response to cisplatin and UV (Fig. 4A and B, note that different Fra1 isoforms are separated) [19,37]. As *fra1* mRNA was not induced by cisplatin and UV (Fig. 1), these genotoxic agents appear to enhance levels of Fra1 protein through MEK-ERK-dependent phosphorylation and stabilization, a mech-

anism previously identified for various oncogenic stimuli [19,20].

Importantly, inhibition of MEK-ERK by U0126 significantly suppressed both cisplatin- and UV-induced apoptosis (Fig. 4C). Consistently, U0126 blocked the induction of active caspase 3 by cisplatin (Fig. 4D). These results demonstrate that the ERK pathway rather than the JNK pathway mainly promotes cisplatin- and UV-induced cell death in T98G glioblastoma cells.

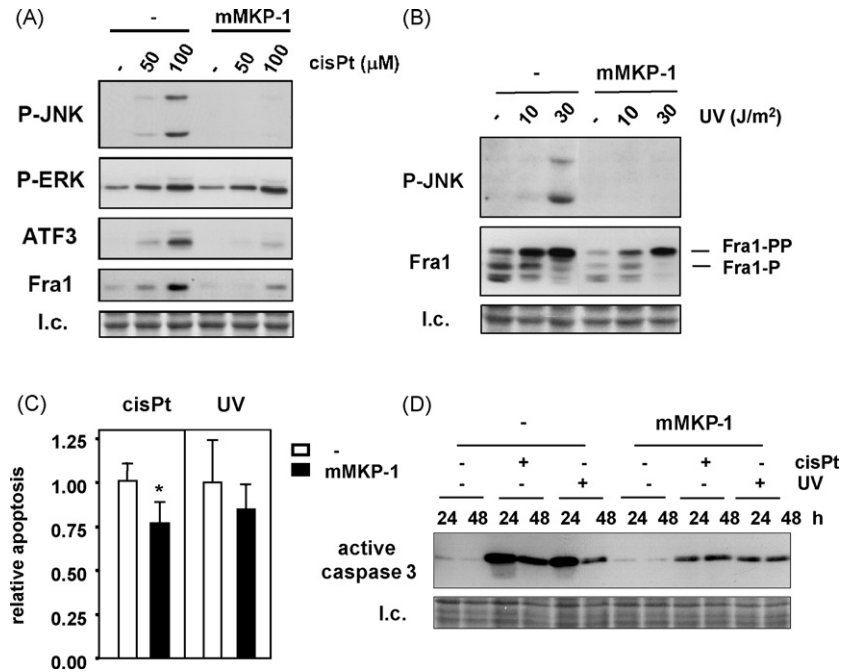


Fig. 3 – Inhibition of cisplatin- and UV-induced JNK activity. (A and B) T98G cells were transduced with a lentiviral vector expressing the JNK-restricted mutant mMKP-1 or a control vector. Transduction efficiency was 95–100% as verified by FACS analysis. Sub-confluent, proliferating cultures were subsequently treated with the indicated doses of cisplatin (A) or UV (B) and harvested for immunoblot analysis after 6 h. Ponceau S staining of part of the filter is shown as a loading control (I.C.). (C and D) The same cultures were treated with 50 μ M cisplatin or 10 J/m² UV and harvested for FACS (C) and immunoblot (D) analysis after 24 h (C and D) and 48 h (D). The percentage of apoptotic (sub-G1) cells measured for the control vector at 24 h was set at 1.0 (C). P values were determined relative to the empty vector control (–) in the same panel. *P = 0.0019, n = 4.

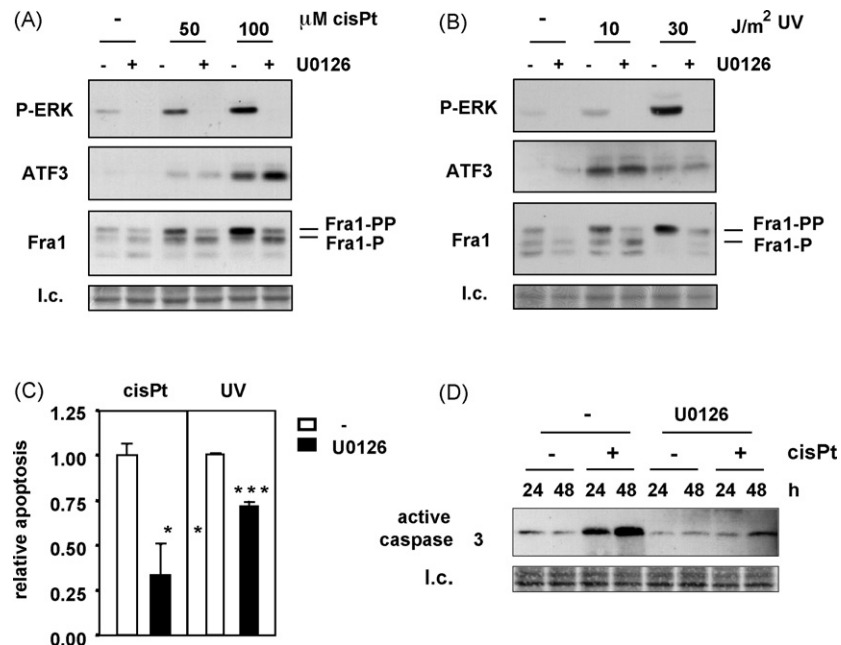


Fig. 4 – Inhibition of cisplatin- and UV-induced ERK activity. (A and B) T98G glioblastoma cells were treated with the indicated doses of cisplatin (A) or UV (B) in the presence or absence of the MEK inhibitor U0126. Protein extracts were prepared after 6 h and analyzed by immunoblotting. Loading control (I.C.). The slower migrating MAPK-phosphorylated forms of Fra1 [19,37] are indicated as Fra1-P and Fra1-PP. (C and D) Sub-confluent, proliferating cultures of T98G glioblastoma cells were treated with 50 μ M cisplatin or 10 J/m² UV in the presence or absence of U0126 and harvested for FACS (C) and immunoblot (D) analysis after 24 h (C and D) and 48 h (D). The percentage of apoptotic (sub-G1) cells measured in the absence of U0126 at 24 h was set at 1.0 (C). P values were determined relative to non-U0126 treated cells (–) in the same panel. **P = 0.0013, n = 4; ***P = 0.0008, n = 3.

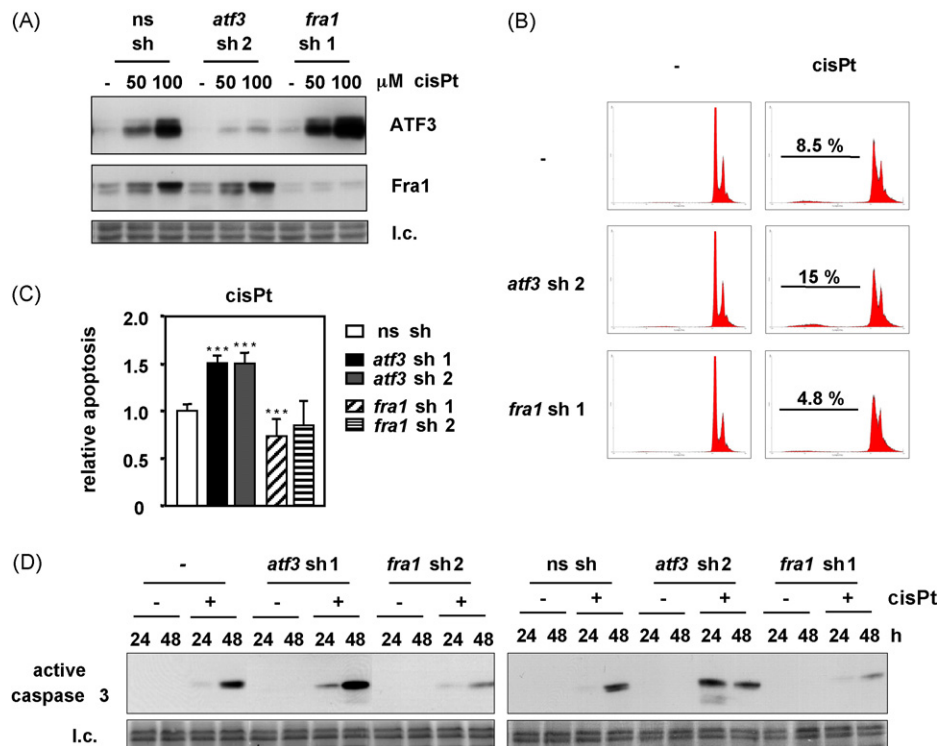


Fig. 5 – Opposing functions of ATF3 and Fra1 in cisplatin-induced apoptosis. T98G cells were transduced with lentiviral vectors expressing the indicated *atf3* or *fra1* short hairpins, a non-specific short hairpin (ns) or the empty control vector (–). The cultures were subsequently treated with 50 μ M cisplatin unless indicated otherwise and harvested for immunoblot analysis after 6 h (A), or after 24 and 48 h (D), and for FACS analysis after 24 h (B and C). (B) An example of the FACS profiles obtained; the percentage of apoptotic (sub-G1) cells is indicated in the graphs. (C) The percentage of apoptotic (sub-G1) cells measured for the non-specific hairpin (ns) at 24 h was set at 1.0. P values were determined relative to the ns sh control. *** $P < 0.001$.

3.3. ATF3 is anti-apoptotic and Fra1 is pro-apoptotic in response to cisplatin

Our results suggest that the JNK- and ERK-target Fra1 rather than the JNK-target ATF3 may be critical for the apoptotic response of T98G cells. To examine the roles of Fra1 and ATF3 directly, we constructed lentiviral vectors expressing short hairpin RNAs (shRNA) that target these proteins for suppression. For each protein, two independent viral vectors were generated to test reproducibility, and non-specific (ns) short hairpin served as controls. The *atf3*- and *fra1*-shRNA constructs specifically reduced levels of ATF3 and Fra1 upon treatment with cisplatin (Fig. 5A). In the absence of DNA damage, these constructs did not significantly affect cell survival and cell proliferation (Fig. 5B). Strikingly, however, knock-down of ATF3 and Fra1 had the opposite effects on cisplatin-induced apoptosis. Knock-down of ATF3 increased the levels of apoptotic cells and active caspase 3, whereas knock-down of Fra1 reduced these (Fig. 5C and D). Since both ATF3 and Fra1 were induced in a JNK-dependent manner by cisplatin and UV, we conclude that ATF3 acts as an anti-apoptotic effector of DNA damage-induced JNK signaling, whereas Fra1 functions as a pro-apoptotic JNK (and ERK) target in T98G cells.

3.4. ATF3 and Fra1 differently affect cisplatin-induced Chk1 and Cdk2 phosphorylation and S-phase arrest

Since apoptosis can be triggered by defects in cell cycle control, we next analyzed the effects of ATF3 and Fra1 on cisplatin-induced cell cycle arrest. As shown in Fig. 6A, knock-down of ATF3 reduced cisplatin-induced S-phase arrest in T98G cells, whereas knock-down of Fra1 enhanced it. To obtain clues on the mechanism by which ATF3 and Fra1 reciprocally control cell cycle arrest, we analyzed the levels and phosphorylation state of the S-phase regulatory kinase Cdk2. Cisplatin treatment can induce S-phase arrest by inhibition of Cdk2 activity, which is mediated by phosphorylation of Cdk2-Tyr15 by the DNA damage-induced kinase Chk1 [38]. Intriguingly, we found that ATF3 knock-down strongly reduced the cisplatin-induced activation of Chk1 (as measured by Chk1-Ser317-phosphorylation) and phosphorylation of Cdk2-Tyr15 (Fig. 6B). By contrast, knock-down of Fra1 slightly enhanced phosphorylation of Chk1 and Cdk2-Tyr15 (Fig. 6B and C). On the other hand, the levels of cyclin E and cyclin A were not affected by Fra1 and ATF3 (Fig. 6C). Together, these results show that ATF3 and Fra1 have opposing functions in cisplatin-induced S-phase arrest and apoptosis presumably through activation and inhibition of the Chk1-Cdk2 DNA damage response pathway, respectively (Fig. 7).

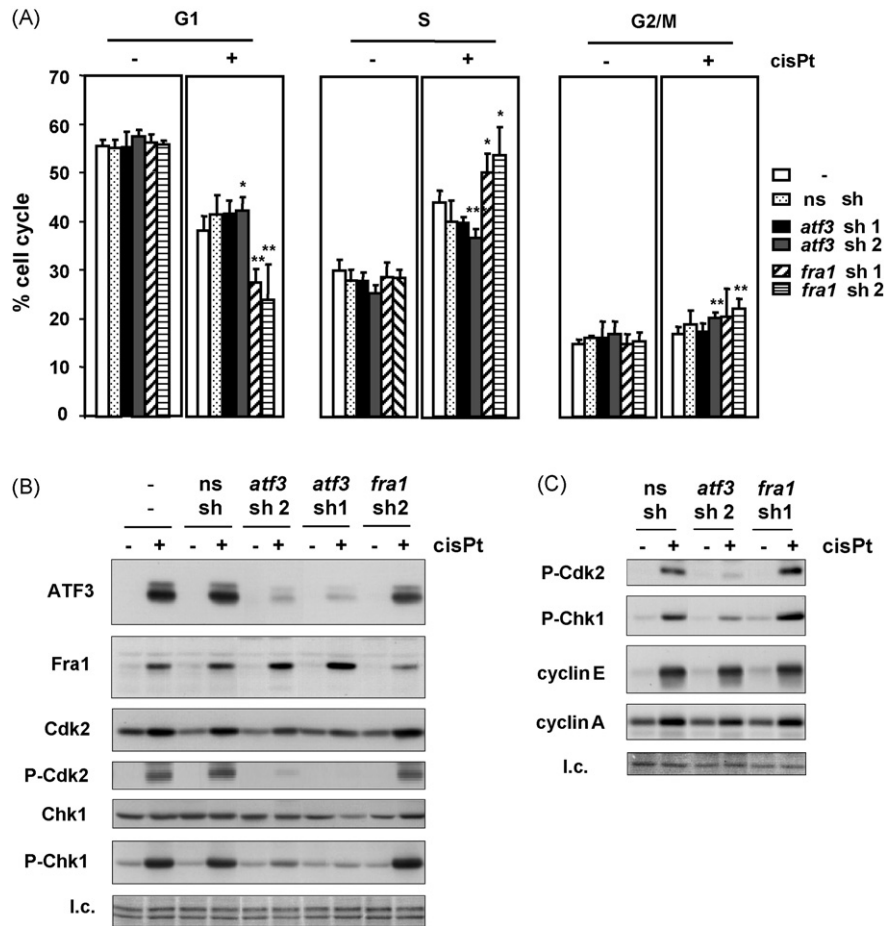


Fig. 6 – Opposing functions of ATF3 and Fra1 in cisplatin-induced S-phase arrest and Cdk2 phosphorylation. T98G cells were transduced with lentiviral vectors expressing the indicated *atf3* or *fra1* short hairpins, a non-specific short hairpin (ns) or the empty control vector (–). The cultures were subsequently treated with 50 μ M cisplatin and harvested after 24 h for FACS analysis (A) or immunoblot analysis (B and C). (A) Phase shifts upon cisplatin treatment: non-specific sh (ns) $42 \pm 4\%$ G1 and $40 \pm 4\%$ S-phase; *fra1* sh1: $28 \pm 3\%$ G1 and $50 \pm 4\%$ S-phase; *fra1* sh2: $24 \pm 7\%$ G1 and $54 \pm 6\%$ S-phase. P values were determined relative to empty vector controls (–) in the same panel. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Loading control (l.c.).

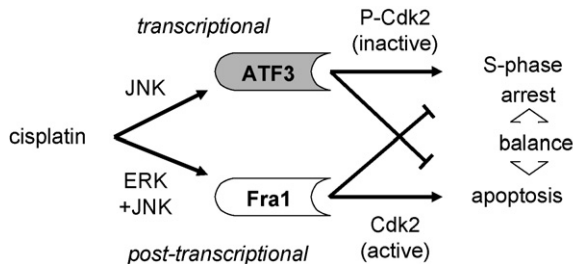


Fig. 7 – A model describing the roles of JNK, ERK, ATF3 and Fra1 in the cellular response to cisplatin. Cisplatin induces ATF3 (JNK-dependent) and Fra1 (ERK- and JNK-dependent). ATF3 and Fra1 counteract each other to balance and switch S-phase arrest and apoptosis through distinctly modulating Tyr15-phosphorylation of Cdk2.

4. Discussion

AP-1 transcription factors regulate cell proliferation, survival and death in response to numerous mitotic and stress stimuli. In the case of c-Jun and ATF3 this regulation can be mediated through the tumor suppressor proteins p53 and p16-INK4A [13,22,24,25,39]. Here we demonstrate that the AP-1 components ATF3 and Fra1 control DNA damage responses in human cancer cells lacking functional p53 and p16-INK4A. Knock-down of ATF3 in T98G glioblastoma cells increased levels of cisplatin-induced apoptosis, whereas knock-down of Fra1 reduced it. In addition, ATF3 and Fra1 showed opposite effects on cisplatin-induced S-phase arrest. In this respect it is worth noting that Fra1 expression is altered in cisplatin-resistant tumor cells [27,28].

Previous studies showed that ATF3 and Fra1 either inhibit or enhance cell survival and malignancy depending on cellular context [25,27,40–46]. In certain types of cells, a common

target of these AP-1 components is cyclin D1, which regulates G1-phase progression and can trigger apoptosis when overexpressed [40,41,47]. However, we did not detect significant changes in cyclin D1 levels following knock-down of ATF3 or Fra1 in T98G cells (data not shown). Instead, knock-down of ATF3 and Fra1, respectively, reduced and increased S-phase arrest upon cisplatin treatment. Moreover, the cisplatin-induced phosphorylation of Chk1-Ser317 and Cdk2-Tyr15 was strongly decreased upon ATF3 knock-down, whereas knock-down of Fra1 rather enhanced Chk1 and Cdk2 phosphorylation. Such enhanced Chk1-dependent phosphorylation of Cdk2 inhibits Cdk2 activity, thereby triggers S-phase arrest [38], enhances BRCA2-dependent DNA repair [48], and stimulates Foxo1-induced apoptosis [49]. Furthermore, Chk1 is required for mammalian homologous recombination repair [50]. As cell cycle arrest allows cells to repair DNA before cell cycle progression, our results suggest that AP-1 can regulate both cisplatin-induced S-phase arrest and apoptosis via Chk1 and Cdk2 (Fig. 7). The levels of cyclin E and cyclin A, other putative ATF3 and/or Fra1 target genes, were not or much less affected.

Both ATF3 and Fra1 may dimerize with c-Jun to regulate DNA damage-responses. Consistently, c-Jun controls expression of both pro- and anti-apoptotic genes [13] and can protect cells against DNA damage accumulation [51]. Furthermore, overexpression of c-Jun mutants that cannot be phosphorylated by JNK sensitizes T98G cells to cisplatin and other DNA damaging agents [11,26]. c-Jun/ATF2 dimers can control expression of DNA repair enzymes in a JNK-dependent manner [52,53], while the DNA repair factor XPF (also known as ERCC4) is an essential transcriptional target of the c-Jun dimer-partner and Fra1 homologue c-Fos after UV-irradiation [54,55]. Importantly, c-Jun/ATF3 and c-Jun/ATF2 dimers only bind to 8 bp ATF/CRE sites, whereas c-Jun/c-Fos and c-Jun/Fra1 dimers prefer 7 bp 'classical' AP-1 sites. Moreover, while Fra1 does not homodimerize, ATF3 homodimers repress transcription, whereas c-Jun/ATF3 heterodimers activate [17]. Therefore, ATF3 and Fra1 might control cisplatin-induced S-phase arrest and apoptosis by differently regulating c-Jun-dependent DNA repair genes.

We found that induction of Fra1 protein by cisplatin and UV required both JNK and ERK pathways. Since *fra1* mRNA accumulation was inhibited rather than activated, Fra1 induction may be mediated via post-translational modification of Fra1 protein. Previous studies showed that phosphorylation by ERK protects Fra1 against proteosomal degradation [19,20,37]. Along these lines, inhibition of the ERK pathway mainly blocked cisplatin and UV-induced accumulation of the hyper-phosphorylated, slow migrating form of Fra1. By contrast, JNK inhibition reduced both basal and stress-induced levels of all detectable Fra1 isoforms. Therefore, JNK appears to predominantly control basal level expression of Fra1 ([21]; our unpublished observations).

In conclusion, our results show that ATF3 acts as an anti-apoptotic JNK target in glioblastoma cells lacking functional p53 and p16-INK4A, whereas Fra1 acts as a pro-apoptotic effector of DNA damage-induced JNK and ERK signaling. These observations explain why JNK inhibition has ambivalent effects on programmed cell death, even when its kinase activity is strongly induced. Therefore, ATF3 and Fra1 may be

useful in monitoring effects of anti-cancer therapy. Alternatively, inhibition of ATF3 activity might enhance apoptosis in cancer cells with defects in the p53 pathway.

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REFERENCES

- [1] S.G. Chaney, A. Sancar, DNA repair: enzymatic mechanisms and relevance to drug response, *J. Natl. Cancer Inst.* 88 (1996) 1346–1360.
- [2] C.R. Weston, R.J. Davis, The JNK signal transduction pathway, *Curr. Opin. Genet. Dev.* 12 (2002) 14–21.
- [3] M. Camps, A. Nichols, S. Arkinstall, Dual specificity phosphatases: a gene family for control of MAP kinase function, *FASEB J.* 14 (2000) 6–16.
- [4] I. Sanchez-Perez, M. Martinez-Gomariz, D. Williams, S.M. Keyse, R. Perona, CL100/MKP-1 modulates JNK activation and apoptosis in response to cisplatin, *Oncogene* 19 (2000) 5142–5152.
- [5] M. Hamdi, J. Kool, P. Cornelissen-Steijger, F. Carlotti, H.E. Popeijus, B.C. van der, J.M. Janssen, A. Yasui, R.C. Hoebe, C. Terleth, L.H. Mullenders, H. van Dam, DNA damage in transcribed genes induces apoptosis via the JNK pathway and the JNK-phosphatase MKP-1, *Oncogene* 24 (2005) 7135–7144.
- [6] L. Chang, M. Karin, Mammalian MAP kinase signalling cascades, *Nature* 410 (2001) 37–40.
- [7] X. Wang, J.L. Martindale, N.J. Holbrook, Requirement for ERK activation in cisplatin-induced apoptosis, *J. Biol. Chem.* 275 (2000) 39435–39443.
- [8] A. Mandic, K. Viktorsson, T. Heiden, J. Hansson, M.C. Shoshan, The MEK1 inhibitor PD98059 sensitizes C8161 melanoma cells to cisplatin-induced apoptosis, *Melanoma Res.* 11 (2001) 11–19.
- [9] P. Dent, A. Yacoub, P.B. Fisher, M.P. Hagan, S. Grant, MAPK pathways in radiation responses, *Oncogene* 22 (2003) 5885–5896.
- [10] R. Wisdom, AP-1: one switch for many signals, *Exp. Cell Res.* 253 (1999) 180–185.
- [11] O. Potapova, S. Basu, D. Mercola, N.J. Holbrook, Protective role for c-Jun in the cellular response to DNA damage, *J. Biol. Chem.* 276 (2001) 28546–28553.
- [12] E.F. Wagner, AP-1—introductory remarks, *Oncogene* 20 (2001) 2334–2335.
- [13] E. Shaulian, M. Karin, AP-1 as a regulator of cell life and death, *Nat. Cell Biol.* 4 (2002) E131–E136.
- [14] E. Shaulian, M. Karin, AP-1 in cell proliferation and survival, *Oncogene* 20 (2001) 2390–2400.
- [15] H. van Dam, M. Castellazzi, Distinct roles of Jun: Fos and Jun: ATF dimers in oncogenesis, *Oncogene* 20 (2001) 2453–2464.
- [16] L. Bakiri, Y. Takada, M. Radolf, R. Eferl, M. Yaniv, E.F. Wagner, K. Matsuo, Role of heterodimerization of c-Fos and Fra1 proteins in osteoclast differentiation, *Bone* 40 (2007) 867–875.

- [17] T. Hai, C.D. Wolfgang, D.K. Marsee, A.E. Allen, U. Sivaprasad, ATF3 and stress responses, *Gene Expr.* 7 (1999) 321–335.
- [18] L.O. Murphy, S. Smith, R.H. Chen, D.C. Finger, J. Blenis, Molecular interpretation of ERK signal duration by immediate early gene products, *Nat. Cell Biol.* 4 (2002) 556–564.
- [19] L. Casalino, D. De Cesare, P. Verde, Accumulation of Fra-1 in ras-transformed cells depends on both transcriptional autoregulation and MEK-dependent posttranslational stabilization, *Mol. Cell Biol.* 23 (2003) 4401–4415.
- [20] E. Vial, C.J. Marshall, Elevated ERK-MAP kinase activity protects the FOS family member FRA-1 against proteasomal degradation in colon carcinoma cells, *J. Cell Sci.* 116 (2003) 4957–4963.
- [21] J.J. Ventura, N.J. Kennedy, J.A. Lamb, R.A. Flavell, R.J. Davis, c-Jun NH(2)-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor, *Mol. Cell Biol.* 23 (2003) 2871–2882.
- [22] W. Jochum, E. Passegue, E.F. Wagner, AP-1 in mouse development and tumorigenesis, *Oncogene* 20 (2001) 2401–2412.
- [23] M. Ameyar-Zazoua, M.B. Wisniewska, L. Bakiri, E.F. Wagner, M. Yaniv, J.B. Weitzman, AP-1 dimers regulate transcription of the p14/p19ARF tumor suppressor gene, *Oncogene* 24 (2005) 2298–2306.
- [24] B.Y. Choi, H.S. Choi, K. Ko, Y.Y. Cho, F. Zhu, B.S. Kang, S.P. Ermakova, W.Y. Ma, A.M. Bode, Z. Dong, The tumor suppressor p16(INK4a) prevents cell transformation through inhibition of c-Jun phosphorylation and AP-1 activity, *Nat. Struct. Mol. Biol.* 12 (2005) 699–707.
- [25] C. Yan, D. Lu, T. Hai, D.D. Boyd, Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination, *EMBO J.* 24 (2005) 2425–2435.
- [26] O. Potapova, A. Haghighi, F. Bost, C. Liu, M.J. Birrer, R. Gjeriset, D. Mercola, The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin, *J. Biol. Chem.* 272 (1997) 14041–14044.
- [27] K. Macleod, P. Mullen, J. Sewell, G. Rabiasz, S. Lawrie, E. Miller, J.F. Smyth, S.P. Langdon, Altered ErbB receptor signaling and gene expression in cisplatin-resistant ovarian cancer, *Cancer Res.* 65 (2005) 6789–6800.
- [28] K. Nakatani, M. Nakamura, K. Uzawa, T. Wada, N. Seki, H. Tanzawa, S. Fujita, Establishment and gene analysis of a cisplatin-resistant cell line, Sa-3R, derived from oral squamous cell carcinoma, *Oncol. Rep.* 13 (2005) 709–714.
- [29] D.M. Ouwens, N.D. de Ruiter, G.C. van der Zon, A.P. Carter, J. Schouten, B.C. van der, K. Kooistra, J.L. Bos, J.A. Maassen, H. van Dam, Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38, *EMBO J.* 21 (2002) 3782–3793.
- [30] H. van Dam, M. Duyndam, R. Rottier, A. Bosch, L. Vries-Smits, P. Herrlich, A. Zantema, P. Angel, A.J. van der Eb, Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein, *EMBO J.* 12 (1993) 479–487.
- [31] H. van Dam, R. Offringa, I. Meijer, B. Stein, A.M. Smits, P. Herrlich, J.L. Bos, A.J. van der Eb, Differential effects of the adenovirus E1A oncogene on members of the AP-1 transcription factor family, *Mol. Cell Biol.* 10 (1990) 5857–5864.
- [32] E. Passegue, W. Jochum, A. Behrens, R. Ricci, E.F. Wagner, JunB can substitute for Jun in mouse development and cell proliferation, *Nat. Genet.* 30 (2002) 158–166.
- [33] J. Kool, M. Hamdi, P. Cornelissen-Steijger, A.J. van der Eb, C. Terleth, H. van Dam, Induction of ATF3 by ionizing radiation is mediated via a signaling pathway that includes ATM, Nibrin1, stress-induced MAPkinases and ATF-2, *Oncogene* 22 (2003) 4235–4242.
- [34] T.R. Brummelkamp, R. Bernards, R. Agami, A system for stable expression of short interfering RNAs in mammalian cells, *Science* 296 (2002) 550–553.
- [35] F. Carlotti, M. Bazuine, T. Kekarainen, J. Seppen, P. Pognonec, J.A. Maassen, R.C. Hoeben, Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes, *Mol. Ther.* 9 (2004) 209–217.
- [36] D.N. Slack, O.M. Seternes, M. Gabrielsen, S.M. Keyse, Distinct binding determinants for ERK2/p38alpha and JNK map kinases mediate catalytic activation and substrate selectivity of map kinase phosphatase-1, *J. Biol. Chem.* 276 (2001) 16491–16500.
- [37] M.C. Gruda, K. Kovary, R. Metz, R. Bravo, Regulation of Fra-1 and Fra-2 phosphorylation differs during the cell cycle of fibroblasts and phosphorylation in vitro by MAP kinase affects DNA binding activity, *Oncogene* 9 (1994) 2537–2547.
- [38] J. Bartek, C. Lukas, J. Lukas, Checking on DNA damage in S phase, *Nat. Rev. Mol. Cell Biol.* 5 (2004) 792–804.
- [39] J. Kawachi, C. Zhang, K. Nobori, Y. Hashimoto, M.T. Adachi, A. Noda, M. Sunamori, S. Kitajima, Transcriptional repressor activating transcription factor 3 protects human umbilical vein endothelial cells from tumor necrosis factor-alpha-induced apoptosis through down-regulation of p53 transcription, *J. Biol. Chem.* 277 (2002) 39025–39034.
- [40] F. Mechta, D. Lallemand, C.M. Pfarr, M. Yaniv, Transformation by ras modifies AP1 composition and activity, *Oncogene* 14 (1997) 837–847.
- [41] A.L. Allan, C. Albanese, R.G. Pestell, J. LaMarre, Activating transcription factor 3 induces DNA synthesis and expression of cyclin D1 in hepatocytes, *J. Biol. Chem.* 276 (2001) 27272–27280.
- [42] T. Mashima, S. Udagawa, T. Tsuruo, Involvement of transcriptional repressor ATF3 in acceleration of caspase protease activation during DNA damaging agent-induced apoptosis, *J. Cell Physiol.* 188 (2001) 352–358.
- [43] S. Perez, E. Vial, H. van Dam, M. Castellazzi, Transcription factor ATF3 partially transforms chick embryo fibroblasts by promoting growth factor-independent proliferation, *Oncogene* 20 (2001) 1135–1141.
- [44] E. Vial, E. Sahai, C.J. Marshall, ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility, *Cancer Cell* 4 (2003) 67–79.
- [45] N.V. Shirsat, S.A. Shaikh, Overexpression of the immediate early gene fra-1 inhibits proliferation, induces apoptosis, and reduces tumorigenicity of c6 glioma cells, *Exp. Cell Res.* 291 (2003) 91–100.
- [46] M.G. Hartman, D. Lu, M.L. Kim, G.J. Kociba, T. Shukri, J. Buteau, X. Wang, W.L. Frankel, D. Guttridge, M. Prentki, S.T. Grey, D. Ron, T. Hai, Role for activating transcription factor 3 in stress-induced beta-cell apoptosis, *Mol. Cell Biol.* 24 (2004) 5721–5732.
- [47] O. Kranenburg, A.J. van der Eb, A. Zantema, Cyclin D1 is an essential mediator of apoptotic neuronal cell death, *EMBO J.* 15 (1996) 46–54.
- [48] F. Esashi, N. Christ, J. Gannon, Y. Liu, T. Hunt, M. Jasin, S.C. West, CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair, *Nature* 434 (2005) 598–604.
- [49] H. Huang, K.M. Regan, Z. Lou, J. Chen, D.J. Tindall, CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage, *Science* 314 (2006) 294–297.
- [50] C.S. Sorensen, L.T. Hansen, J. Dziegielewska, R.G. Syljuasen, C. Lundin, J. Bartek, T. Helleday, The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair, *Nat. Cell Biol.* 7 (2005) 195–201.

- [51] A. MacLaren, E.J. Black, W. Clark, D.A. Gillespie, c-Jun-deficient cells undergo premature senescence as a result of spontaneous DNA damage accumulation, *Mol. Cell Biol.* 24 (2004) 9006–9018.
- [52] J. Hayakawa, C. Depatie, M. Ohmichi, D. Mercola, The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair, *J. Biol. Chem.* 278 (2003) 20582–20592.
- [53] J. Hayakawa, S. Mittal, Y. Wang, K.S. Korkmaz, E. Adamson, C. English, M. Ohmichi, M. McClelland, D. Mercola, Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress, *Mol. Cell* 16 (2004) 521–535.
- [54] M. Schreiber, B. Baumann, M. Cotten, P. Angel, E.F. Wagner, Fos is an essential component of the mammalian UV response, *EMBO J.* 14 (1995) 5338–5349.
- [55] M. Christmann, M.T. Tomicic, J. Origer, D. Aasland, B. Kaina, c-Fos is required for excision repair of UV-light induced DNA lesions by triggering the re-synthesis of XPF, *Nucl. Acids Res.* 34 (2006) 6530–6539.

Chapter V

Inhibition of *fra1* by the JNK-Jun pathway can protect cells against genotoxic stress

Inhibition of *fra1* by the JNK-Jun pathway can protect cells against genotoxic stress

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The mitogen-activated protein kinases JNK (SAPK) regulate the balance between cell proliferation and cell death in response to growth factors and stresses. JNK signaling can reach the nucleus via the transcription factor AP-1, which includes Jun/Fos and Jun/ATF dimers. Here we report a novel protective role of JNK and AP-1 in stress responses. JNK-deficient fibroblasts were prone to apoptosis upon exposure to either low doses of ultraviolet light (UV), or the alkylating agent methyl methanesulfonate (MMS). The increased sensitivity of JNK-deficient cells to these agents was associated with elevated expression of the Fos family member Fra1, whereas activation of JNK by stress-stimuli in wild-type cells inhibited the activity of the *fra1* promoter/enhancer. Fibroblasts expressing a non-repressable *fra1* transgene showed hyper-sensitivity to low doses of UV similar to JNK-deficient cells, indicating that inhibition of *fra1* expression represents an anti-apoptotic JNK function. The repression of *fra1* by JNK required both phosphorylation of c-Jun and the Jun/Fos-binding sites of the *fra1* enhancer. Transactivation studies using c-Jun mutants with modified phosphorylation sites and dimerization specificity showed that hyper-phosphorylation of c-Jun by JNK inhibits transactivation by c-Jun/Fos, but not by c-Jun/ATF dimers. These results indicate that inhibition of *fra1*, and possibly other Jun/Fos-dependent genes, by JNK and phospho-c-Jun is a mechanism for protection against genotoxic stress.

Introduction

Exposure of mammalian cells to DNA-damaging agents induces responses that protect the organism against the accumulation of undesirable mutations. Low doses of genotoxic stress can result in cell cycle arrest, which allows cells to repair the damage. In contrast, larger amounts of damage may induce apoptosis, which seems beneficial to the organism for eliminating non-repairable cells (for review Zhou and Elledge, 2000; Norbury and Hickson, 2001). Execution of these stress- and DNA-damage responses requires multiple signaling pathways

involving JNK (for c-Jun N-terminal kinases, also known as SAPK for stress-activated protein kinases) and AP-1 transcription factors (Wisdom, 1999; Shaulian and Karin, 2001; Weston and Davis, 2002). AP-1 refers to Jun/Fos heterodimers, Jun homodimers, and Jun/ATF heterodimers, which are composed of Jun (c-Jun, JunB and JunD), Fos (c-Fos, Fra1 and Fra2) and ATF (ATF2, ATFa, CREBP-1 and ATF3) family members (Chang and Karin, 2001; Weston and Davis, 2002). Jun/Fos and Jun/Jun dimers preferentially bind to the heptamer consensus, 5'-TGA(G/C)TCA-3' found, for example, in the *fra1* and

collagenase genes. In contrast, Jun/ATF dimers prefer the octamer consensus 5'-T(T/G)ACNTCA-3' found in *c-jun*, *atf3* and other genes. JNK can phosphorylate multiple AP-1 family members and phosphorylation of c-Jun by JNK at serines (Ser) 63 and 73 and threonines (Thr) 91 and 93 is believed to enhance the stability and transactivation potential of c-Jun (Binetruy et al., 1991; Pulverer et al., 1991; Musti et al., 1997; Weiss et al., 2003).

JNK and AP-1 regulate cell fate such as proliferation, survival and death in response to a variety of growth and stress-stimuli (Weston and Davis, 2002; Kyriakis and Avruch, 2001; Wagner, 2001). Depending on the cell type and the nature of the stimulus, JNK can establish both pro- and anti-apoptotic signaling during early brain development in mice as well as in cultured cells (Kuan et al., 1999; Sabapathy et al., 1999; Shaulian and Karin, 2001; Weston and Davis, 2002). In mouse embryo fibroblasts (MEFs), JNK1 and JNK2 mediate programmed cell death induced by high doses of UV light (60 J/m² UV-C or more), which involves the mitochondrial death-signaling pathway and the pro-apoptotic Bcl-2-related proteins Bax and Bak (Tournier et al., 2000; Lei et al., 2002). Moreover, JNK activation followed by c-Jun and ATF2 phosphorylation can result in expression of death receptor ligands (Faris et al., 1998; Le Niculescu et al., 1999; Kolbus et al., 2000). In contrast to its pro-apoptotic effects, JNK can protect MEFs against TNF- α and sorbitol-induced apoptosis (Hochedlinger et al., 2002; Tournier et al., 2000). JNK-dependent cell survival in response to TNF- α appears to be mediated via JunD and the survival gene *cIAP-2* (Lamb et al., 2003).

Most studies on the role of JNK and AP-1 in response to DNA damaging agents use high doses that severely inhibit transcription in general (Mayne and Lehmann, 1982; McKay et al., 1998; Tornaletti and Hanawalt, 1999). Here, however, we have examined the role of JNK and AP-1 upon exposure to relatively low amounts of DNA damaging agents. JNK-deficient fibroblasts showed enhanced apoptosis after exposure to low doses of UV or MMS. This increased sensitivity of JNK-deficient cells correlated with elevated expression of the AP-1 target gene *fra1*, while in wild-type cells stress-induced JNK was found to inhibit

the *fra1* enhancer through its Jun/Fos binding sites. Fibroblasts expressing a non-repressable *fra1* transgene showed hyper-sensitivity to low doses of UV. Our data further show that JNK specifically inhibits the transactivation of Jun/Fos-, but not Jun/ATF2-dimers via hyperphosphorylation of c-Jun. This indicates that inhibition of *Fra1* expression by phosphorylated c-Jun is a mechanism for JNK-dependent protection of cells against low doses of genotoxic stress.

Results

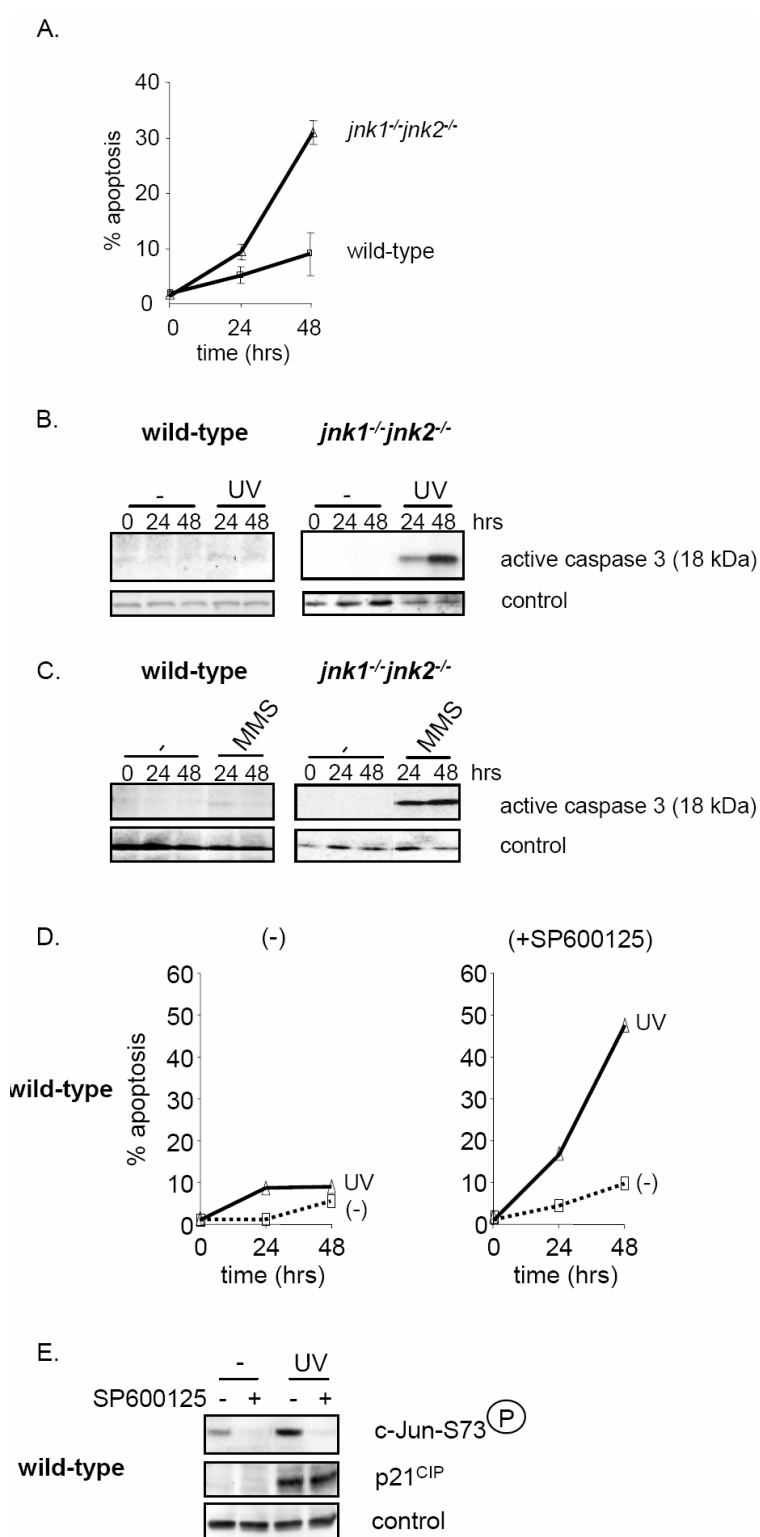
JNK protects mouse fibroblasts against low doses of UV and MMS

To examine the role of JNK and AP-1 at low levels of DNA damage, we first analyzed the induction of apoptosis in wild-type and *jnk1^{-/-}jnk2^{-/-}* 3T3 fibroblasts (Hochedlinger et al., 2002), which will further be referred to as wild-type and *jnk1^{-/-}jnk2^{-/-}* cells. At 10 J/m² UV, the levels of apoptosis were less than 10% in the wild-type cells, but more than three-fold higher in the *jnk1^{-/-}jnk2^{-/-}* cells (Fig. 1A). Enhanced apoptosis at 10 J/m² UV was also detected in *jnk1^{+/-}jnk2^{-/-}* 3T3 fibroblasts (data not shown). After exposure to 10 J/m² UV, the *jnk1^{-/-}jnk2^{-/-}* cells expressed higher amounts of active caspase 3 than the wild-type cells, consistent with the increased levels of apoptosis (Fig. 1B). Similar results were obtained after treatment of these wild-type and *jnk1^{-/-}jnk2^{-/-}* cells with MMS, an alkylating agent that, like UV, induces DNA damage and activates JNK (Fig 1C).

The enhanced apoptosis in fibroblasts lacking JNK activity suggests that JNK plays an anti-apoptotic role in response to DNA damage. We next analyzed the effect of the JNK-inhibitor SP600125 in the wild-type cells. In line with the data obtained from *jnk1^{-/-}jnk2^{-/-}* cells, this compound enhanced apoptosis at 10 J/m² UV more than four-fold (Fig. 1D). To verify the inhibitory effect of SP600125 under these conditions, we analyzed JNK-dependent c-Jun phosphorylation. As expected, SP600125 suppressed the basal and UV-induced phosphorylation of c-Jun-Ser73, whereas UV-induction of the p21^{CIP/Waf1} control was unaffected (Fig. 1E). These results show that JNK has an anti-apoptotic function in 3T3 fibroblasts at low doses of UV or MMS.

Figure 1. High levels of apoptosis in JNK-deficient fibroblasts after 10J UV or MMS

(A) Sub-confluent, proliferating cultures of wild-type and *jnk1^{-/-}jnk2^{-/-}* 3T3 fibroblasts were irradiated with 10 J/m² UV, and harvested at the indicated time points. The level of apoptosis was measured by the percentage of cells in sub G1, determined by FACS analysis. Two independent clones of each genotype were analyzed with very similar outcome. Only one of them is shown. (B-C) Western blot analysis showing active caspase 3 expression in the wild-type and *jnk1^{-/-}jnk2^{-/-}* cells shown under A after treatment with 10 J/m² UV (B), or 0.5 mM MMS (C). The control is a background band. (D) The wild-type 3T3 cells were treated as described under A, in the presence or absence of 10 μ M of the JNK inhibitor SP600125. (E) The wild-type cells were treated as described under (D). After 6 hours, cell extracts were prepared and were analyzed by western blotting.



JNK-dependent cell survival is associated with inhibition of *fra1* expression

Since cells lacking c-Jun or c-Fos exhibit altered apoptotic responses to genotoxic stress (reviewed in Shaulian and Karin, 2001), JNK may exert its anti-apoptotic

function at low doses of UV and MMS through alteration of AP-1-dependent gene expression. Interestingly, upon treatment with these genotoxic agents the *jnk1^{-/-}jnk2^{-/-}* cells specifically showed altered expression of *fra1*, a Jun/Fos target gene. First, UV diminished the Fra1 protein levels in growing wild-type fibroblasts, but only

marginally in the *jnk1^{-/-}jnk2^{-/-}* cells (Fig. 2A). Second, UV inhibited the induction of Fra1 protein and mRNA by serum (Fig. 2B and C) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA, Fig. 5C) in wild-type, but not in *jnk1^{-/-}jnk2^{-/-}* cells. In sharp contrast to *fra1*, UV further enhanced the serum-induction of *c-jun* and *c-fos* mRNAs in wild-type and *jnk1^{-/-}jnk2^{-/-}* cells (Fig. 2C). Similar results were obtained with experiments using MMS instead of UV (data not shown). Thus, JNK appears to be required to inhibit the growth factor-dependent expression of *fra1* upon UV-treatment, while it is dispensable for the induction of *c-jun* and *c-fos*.

We subsequently examined whether the inhibition of *fra1* expression might play a role in JNK-dependent cell survival. *fra1^{-/-}* 3T3 fibroblasts (Schreiber et al., 1999) were more resistant to apoptosis than *c-fos^{-/-}* 3T3 cells (Schreiber et al., 1995), which show enhanced apoptosis and active caspase 3 expression after UV

or MMS (Figure 3A and 3B; data not shown). This is consistent with the view that inhibition of Fra1, but not c-Fos, can be anti-apoptotic upon DNA damage. We next examined the possibility that forced expression of *fra1* might enhance UV-induced apoptosis. For this purpose, we made use of 3T3 cells expressing a *fra1* transgene under the control of the MHC class I promoter (H2-K^b-*fra1*) (Jochum et al., 2000). UV failed to repress the expression of Fra1 in these cells, whereas the activation of JNK, c-Jun and c-Fos was similar to the wild-type cells (Fig. 3C, data not shown). Strikingly, the H2-K^b-*fra1* expressing cells showed much higher levels of apoptosis and active caspase 3 expression than the wild-type cells after 10 J/m² of UV (Fig 3 D and E). Together, these results indicate that JNK protects cells against low doses of genotoxic stress, at least in part, by downregulating growth factor-dependent *fra1* expression.

Figure 2. JNK-dependent inhibition of Fra1 expression by UV

(A) Sub-confluent, proliferating cultures of the wild-type and *jnk1^{-/-}jnk2^{-/-}* cells shown in Figure 1 were irradiated with 10 J/m² UV or mock-treated (-) and incubated for 6 or 12 hours prior to western analysis. (B,C) Confluent, serum-starved cultures of the wild-type and *jnk1^{-/-}jnk2^{-/-}* cells shown in Figure 1 were irradiated with 10 J/m² UV or mock-treated, and subsequently stimulated with 20% FCS. After the indicated time points, protein or RNA was isolated for western (B) or northern blot (C) analysis. Expression of *hef 1* was examined to verify equal loading.

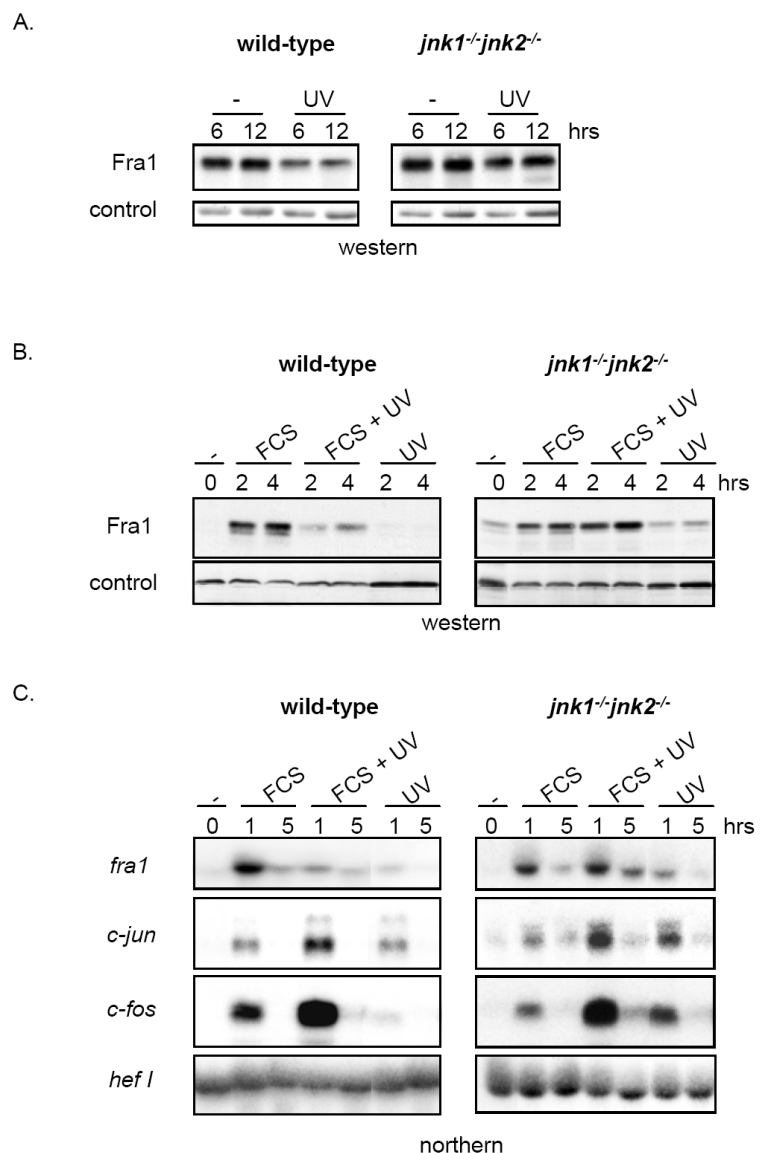
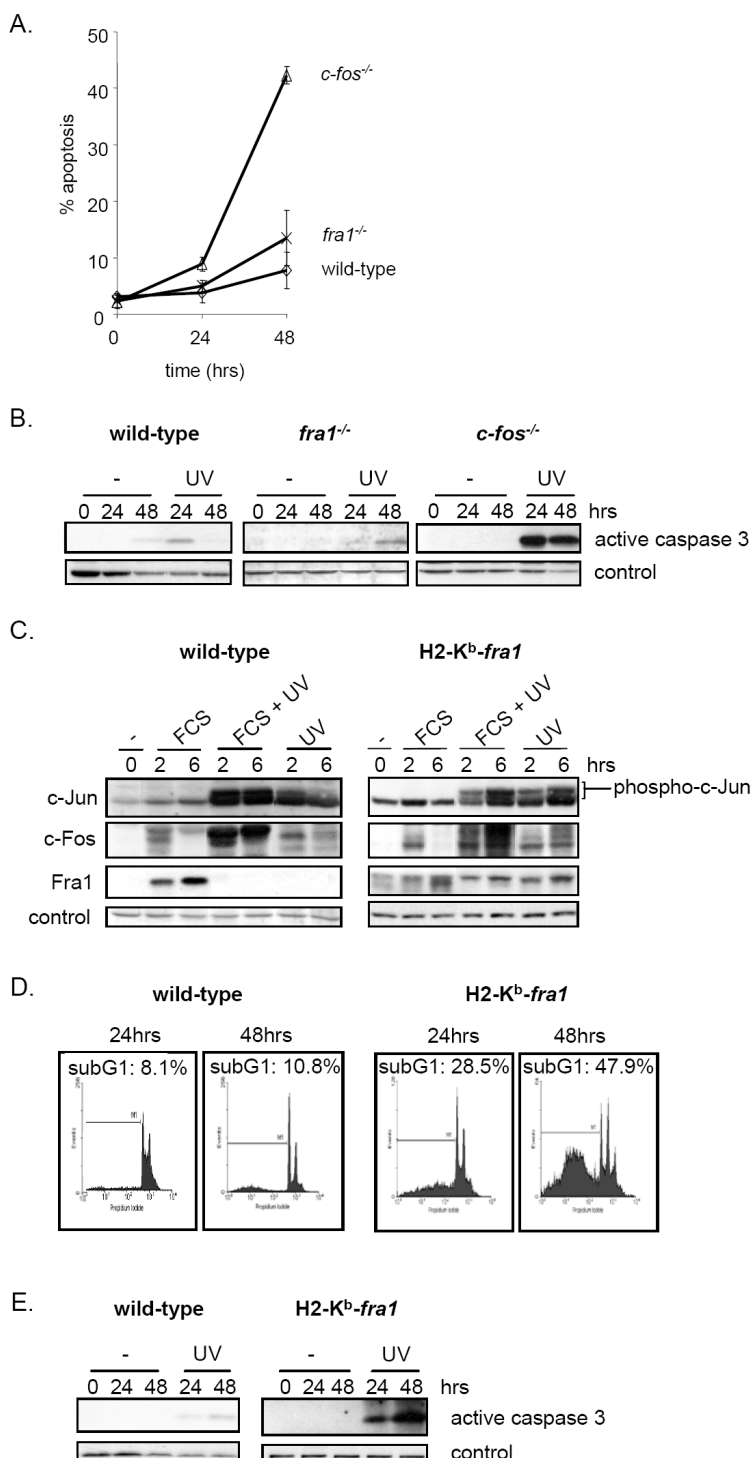


Figure3. Constitutive expression of Fra1 sensitizes mouse fibroblasts to UV-induced apoptosis

(A) Sub-confluent, proliferating cultures of wild-type, *c-fos*^{-/-} and *fra1*^{-/-} 3T3 fibroblasts were irradiated with 10 J/m² UV, and harvested at the indicated time points. The level of apoptosis was measured by the percentage of cells in sub G1, determined by FACS analysis. The wild-type control cells shown are the littermate control of the *fra1*^{-/-} cells, which showed the same levels of apoptosis as the *c-fos*^{+/+} and H2K^b-*fra1* control cells. (B) Western blot analysis showing active caspase 3 expression in the cells described under A. The control is a background band recognized by the antibody. (C) Confluent, serum-starved 3T3 cells derived from H2K^b-*fra1* transgenic mice or control mice were irradiated with UV or mock-treated, and subsequently stimulated with 20% FCS. Cell extracts were analyzed by western analysis. The control is a background band recognized by the Fra1 antibody. (D) Sub-confluent, proliferating cultures of wild-type 3T3 fibroblasts and 3T3 cells derived from H2K^b-*fra1* transgenic mice, were irradiated with 10 J/m² UV, and harvested at the indicated time points. The level of apoptosis was measured by the percentage of cells in sub G1, determined by FACS analysis. (E) Western blot analysis showing active caspase 3 expression in the cells described under D. The control is a background band recognized by the antibody. Two independent clones of H2K^b-*fra1* expressing cells were analyzed with very similar outcome. Only one of them is shown.



Inhibition of *fra1* by JNK is mediated through c-Jun

To obtain further clues on JNK-dependent cell survival, we examined the mechanism of *fra1* inhibition by UV. As growth-factor-dependent expression of *fra1* requires both c-Jun and c-Fos (Bergers et al., 1995; Schreiber et al., 1997), we first examined whether the inhibition of *fra1*

also requires AP-1. As depicted in Figure 4A, the weak serum-induction of *fra1* mRNA in *c-fos*^{-/-} cells was efficiently inhibited by UV, indicating that the presence of c-Fos is not essential for *fra1* inhibition by UV. In *c-jun*^{-/-} cells serum-induction of *fra1* mRNA was hardly detectable, but upon UV treatment *fra1* expression appeared to be increased (Fig. 4A). We next analyzed the role of c-Jun

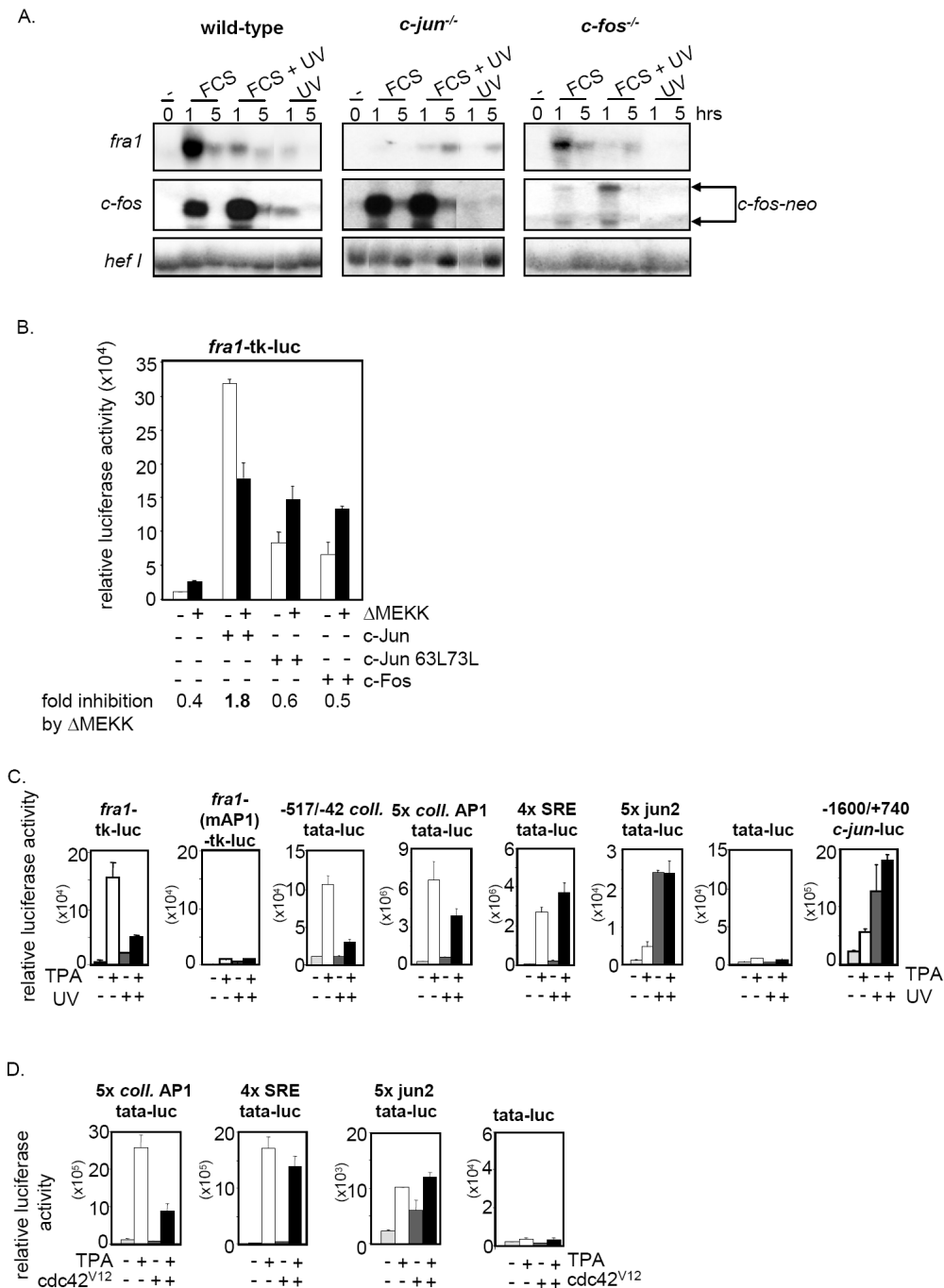


Figure 4. Inhibition of c-Jun-dependent *fra1* transcription by UV and stress-induced JNK
 (A) Confluent, serum-starved wild-type, *c-jun*^{-/-} and *c-fos*^{-/-} 3T3 cells were irradiated with 10 J/m² UV or mock-treated, and subsequently stimulated with 20% FCS. Total RNA isolated at 1 and 5 hours was analysed by Northern blot analysis. The arrows indicate the transcripts resulting from the *c-fos*-neo targeting vector expressed under control of the endogenous *c-fos* promoter in *c-fos*^{-/-} cells. The wild-type control cells shown are the littermate control of the *c-fos*^{-/-} cells, which showed the same *fra1* and *c-fos* expression patterns as the *c-jun*^{+/+} control cells. (B) F9 cells were transfected with the Fra1-B-TK luciferase reporter (containing the AP1 enhancer element of the *fra1* promoter in front of a minimal TK promoter), and pMT2-ΔMEKK or pMT2-empty (-), and pRSV-c-Jun-HA, pRSV-c-Jun63L73L-HA, pRSV-c-Fos or the empty RSV vector (-). Cell extracts were prepared after 5 hours. (C) HeLa tk⁻ cells were transfected with the indicated luciferase reporter constructs and treated with 30 J/m² UV and/or 100 nM TPA as indicated. Cell extracts for luciferase activity were isolated 8 hours later. Depicted values represent the mean ± standard deviation. (D) HeLa tk⁻ cells were transfected with the indicated luciferase reporter constructs in the absence or presence of pMT2-cdc42^{V12} or pMT2-empty. Sixteen hours later TPA was added for another 8 hours.

and c-Fos on the inhibition of *fra1* via transient transfection assays in F9 cells, which lack endogenous c-Jun and c-Fos, but do express JunD and the other Fos-family members (van Dam et al., unpublished observations). As shown in Figure 4B, trans-activation of the *fra1*-promoter/enhancer by c-Jun was inhibited when JNK was activated via constitutively-active MEKK (Δ MEKK). In contrast, trans-activation by c-Fos was not inhibited via Δ MEKK. Moreover, Δ MEKK failed to inhibit activation of the *fra1* promoter/enhancer by a c-Jun mutant with serine to leucine (Leu or L) substitutions at JNK-phosphorylation sites (Fig. 4B, c-Jun 63L73L). These results suggest that c-Jun, rather than c-Fos, is the critical mediator for inhibition of *fra1* by stress-induced JNK.

Inhibition of c-Jun-dependent gene expression by UV and JNK has not been described so far, and might be specific to 3T3 fibroblasts or to *fra1*. We, therefore, examined *fra1* repression in other cell types and tested other Jun/Fos- or Jun/ATF-dependent promoter/enhancers. Inhibition of *fra1* was not restricted to 3T3 cells, as UV and MMS down-regulated the serum- or TPA-induced expression of Fra1 in the human tumor cell lines HeLa and T98G, as well as in primary and SV40-virus transformed human fibroblasts (data not shown). Transient transfections in HeLa cells showed that UV inhibits the activity of the *fra1* promoter/enhancer in an AP-1-dependent manner (Fig. 4C). Activation of JNK through UV (Fig. 4C) or constitutively-active cdc42 (cdc42^{V12}, Fig. 4D) also suppressed TPA-induction of the prototypical Jun/Fos-dependent collagenase I promoter/enhancer (-517/42 *coll.*) and the minimal Jun/Fos-dependent 5x*coll*.AP1 enhancer (Fig. 4C and D). In contrast, serum- or TPA-induction of the *c-jun* and *c-fos* promoters and the minimal Jun/ATF (5xjun2)- or SRF/elk1 (4xSRE)-dependent enhancers were not inhibited by concomitant JNK activation (Fig. 4C and D; data not shown). Thus, these data indicate that stress-induced JNK suppresses growth factor-induced activation of Jun/Fos-controlled promoters in multiple cell types.

Inhibition of c-Jun-induced transactivation via hyper-phosphorylation of the c-Jun N-terminus

To examine whether phosphorylation of c-Jun by JNK can result in transcriptional inhibition of Jun/Fos-dependent genes, we analyzed c-Jun mutants in which the JNK phosphorylation sites Ser63, Ser73, Thr91, Thr93 as well as other potential sites were replaced by alanine (Ala or A, Fig. 5A). Upon transient transfection in F9 cells, transactivation of the collagenase promoter by the Ser(63+73)Ala mutant was clearly less inhibited by Δ MEKK than the activation by wild-type c-Jun, whereas the 7xA mutant was not inhibited at all (Fig. 5B). Since the inhibition was also attenuated with c-Jun AAAA in which Thr 89, 90, 91, and 93 are mutated, not only Ser63 and Ser73 but also the more C-terminal Thr residues seem to mediate inhibition of the collagenase promoter by stress-treatment.

To test whether hyper-phosphorylation of the c-Jun N-terminus is sufficient to suppress the ability of c-Jun to activate the collagenase promoter, we examined c-Jun mutants in which the various JNK phosphorylation sites were replaced by aspartic acids (Asp or D, Fig. 5A). These Asp-substitutions create pseudo-phosphorylated c-Jun molecules that exhibit gain-of-function properties (Papavassiliou et al., 1995; Treier et al., 1995; Watson et al., 1998; Bakiri et al., 2000). In contrast to the Ala mutants, Asp mutants with 4 or more substitutions were already weak activators of the collagenase promoter under non-stressed conditions, both in F9 cells and in HeLa cells (Fig. 5B, data not shown). This indicates that hyper-phosphorylation of c-Jun creates molecules which have lost the capacity to efficiently transactivate the collagenase promoter.

Since transient transfection experiments suggested that the inhibition of the c-Jun-induced *fra1* enhancer by Δ MEKK was also dependent on c-Jun Ser63 and Ser73 (Fig. 4B), we further examined the role of these Ser residues using 3T3 fibroblasts derived from c-Jun^{AA} mice, which express c-Jun 63A73A instead of c-Jun (Behrens et al., 1999). In line with the transfection assays, TPA induction of the endogenous *fra1* was not inhibited by UV in c-Jun^{AA} cells (Fig. 5C). We therefore conclude that stress-induced

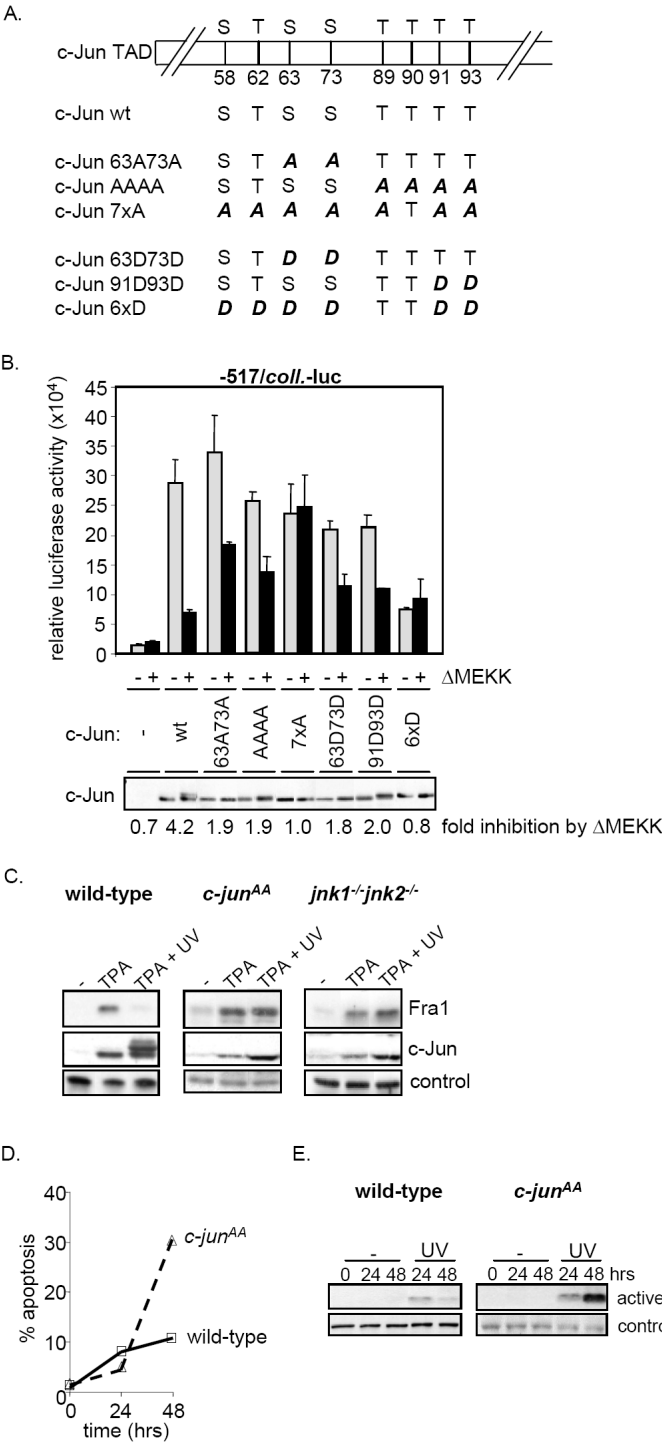
JNK inhibits expression of both collagenase and *fra1* via N-terminal phosphorylation of c-Jun.

We subsequently used these c-Jun^{AA} cells to examine the involvement of c-Jun-Ser(63+73) phosphorylation in the control of apoptosis at low doses of UV. Like the *c-fos*^{-/-} and *jnk1*^{-/-}*jnk2*^{-/-} cells (Figs 1A and 3A), the c-Jun^{AA} cells showed high

levels of apoptosis and active caspase 3 upon treatment with 10 J/m² UV (Fig. 5D and E). Thus, c-Jun-phosphorylation, especially of Ser63 and Ser73, appears to mediate the anti-apoptotic function of JNK in mouse fibroblasts at low doses of UV.

Figure 5. N-terminal phosphorylation of c-Jun is required for inhibition of collagenase and Fra1 expression by stress-stimuli

(A) Schematic representation of the transactivation domain (TAD) of c-Jun showing its N-terminal phosphorylation sites and the various Ala (A) and Asp (D) mutants used. (B) F9 cells were transfected with the -517/+63 collagenase luciferase reporter and expression vectors for MEKK and the (HA-tagged) c-Jun mutants described under A. After 5 hours, cell extracts were prepared, of which one part was used for western blot analysis (lower panel) with antibodies against the HA-tag. (C) Confluent, serum-starved wild-type, *c-jun*^{AA}, and *jnk1*^{-/-}*jnk2*^{-/-} 3T3 fibroblasts were irradiated with 10 J/m² UV or mock-treated, and subsequently stimulated with 100nM TPA. Cell extracts were prepared 2 hours later and analysed by western analysis. The control is a background band recognized by the Fra1 antibody. (D and E) Wild-type and *c-jun*^{AA} cells were irradiated with 10 J/m² UV and analyzed for apoptosis as described in Figure 1.



N-terminal c-Jun-phosphorylation specifically inhibits Jun/Fos but not Jun/ATF dimers

The results obtained with the c-Jun mutants were intriguing, since previous studies had linked phosphorylation of Ser63 and Ser73 to activation, rather than inhibition of c-Jun-inducible promoters. For instance, Gal4-c-Jun and c-Jun-GHF1 fusion proteins activate Gal4- and GHF1-dependent promoters more strongly after phosphorylation (Binetruy et al., 1991; Devary et al., 1992; Weiss et al., 2003). As described above, we found only a subset of c-Jun-regulated promoters to be inhibited by stress-induced JNK, controlled by Jun/Fos- rather than by Jun/ATF dimers. Therefore, the inhibitory effect of c-Jun phosphorylation is likely to be dimer-specific. To further explore the specificity of inhibition, we examined the effects of c-Jun and JNK on the -1977/-1858 urokinase enhancer, which contains both a c-Jun/ATF2 and a c-Jun/c-Fos site (De Cesare et al., 1995). To activate this enhancer in a dimer-specific manner, we used c-Jun leucine zipper mutants that heterodimerize preferentially with either c-Fos- or ATF2-like proteins (van Dam et al., 1998; Huguier et al., 1998). While wild-type c-Jun heterodimerizes with both Fos and ATF families of proteins, c-Jun(m1-atf) and c-Jun(m1,5-atf) heterodimerize much more efficiently with ATF proteins than with Fos proteins (Fig. 6A). Conversely, c-Jun(m2,5-fos) binds to Fos more efficiently than to ATF proteins. In addition, while c-Jun and c-Jun(m1-atf) can bind to DNA as homodimers, c-Jun(m1,5-atf) and c-Jun(m2,5-fos) cannot (data not shown). Interestingly, both in HeLa cells (Fig. 6B) and F9 cells (not shown) only the c-Jun molecules that can dimerize with c-Fos, namely c-Jun wild-type and c-Jun(m2,5-fos), were strongly inhibited by *cdc42^{V12}* or Δ MEKK. Moreover, in all experiments, the inhibition of the Fos-specific mutant was stronger than the inhibition of wild-type c-Jun, and the inhibition was still totally dependent on the presence of the JNK-phosphorylation sites (Fig. 6B c-Jun 7xA(m2,5-fos)). In summary, these data indicate that stress-induced JNK specifically represses transactivation by c-Jun molecules heterodimerized with Fos proteins.

Discussion

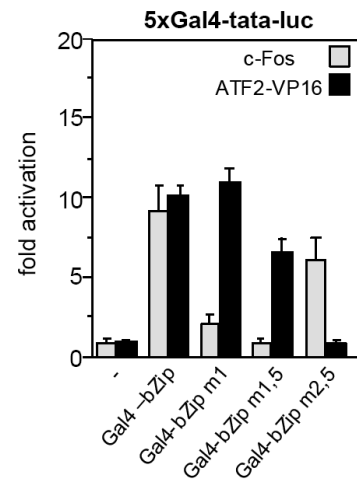
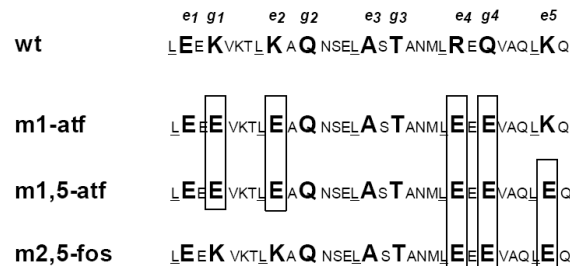
Cells can respond to genotoxic stress by inducing transient or prolonged cell cycle arrest, or by triggering apoptosis. The JNK proteins can mediate apoptosis at high levels of stress in various cell types, including MEFs and 3T3 cells (Shaulian and Karin, 2001; Weston and Davis, 2002; Hochedlinger et al., 2002; M. Hamdi, H. van Dam and B. van de Water, unpublished observations). Here, we show that JNK proteins protect 3T3 cells against low doses of genotoxic agents. Similar double-edged control of apoptosis by JNK may occur in human T cells and mesangial cells (Chen et al., 1996; Guo et al., 1998). Importantly, higher levels of genotoxic stress result in stronger and more sustained JNK activation, which seems to be a prerequisite for sufficient activation of pro-apoptotic JNK substrates. Moreover, high levels of DNA damage can severely inhibit transcription elongation (Mayne and Lehmann, 1982; Tornaletti and Hanawalt, 1999). In fact, the cell cycle regulator *p21^{CIP/Waf1}* is only efficiently induced at relatively low, sub-lethal levels of UV (10 J/m² UV-C) in human and mouse fibroblasts, and not at doses above 30 J/m² (McKay et al., 1998; M. Hamdi unpublished observations). This suggests that the anti-apoptotic functions of JNK may only predominate when low levels of DNA damage allow transcription of *p21^{CIP/Waf1}* and/or other growth arrest genes.

JNK seems to control apoptosis at least in part via transcription factor AP-1. JNK can regulate the activity of Jun/Fos and Jun/ATF complexes, which control various genes that are implicated in cell death (Shaulian and Karin, 2001; Lamb et al., 2003). For instance, N-terminally phosphorylated c-Jun (phospho-c-Jun) can enhance stress-induced apoptosis by activating the Fas-ligand gene (Behrens et al., 1999; personal communication; Le Niculescu et al., 1999; Kolbus et al., 2000). However, phospho-c-Jun can also protect cells against DNA-damaging agents (Wisdom et al., 1999; Potapova et al., 2001). Here we show that this protection is most likely due to transcriptional inhibition of the mitogen-dependent expression of *fra1* and putative other Jun/Fos-controlled genes. Interestingly, this inhibition only seems to be efficient when four or more residues in the c-Jun transactivation domain are

phosphorylated by JNK. This suggests that the role of phospho-c-Jun in the control of cell proliferation and cell death is

dependent on its relative phosphorylation state.

A.



B.

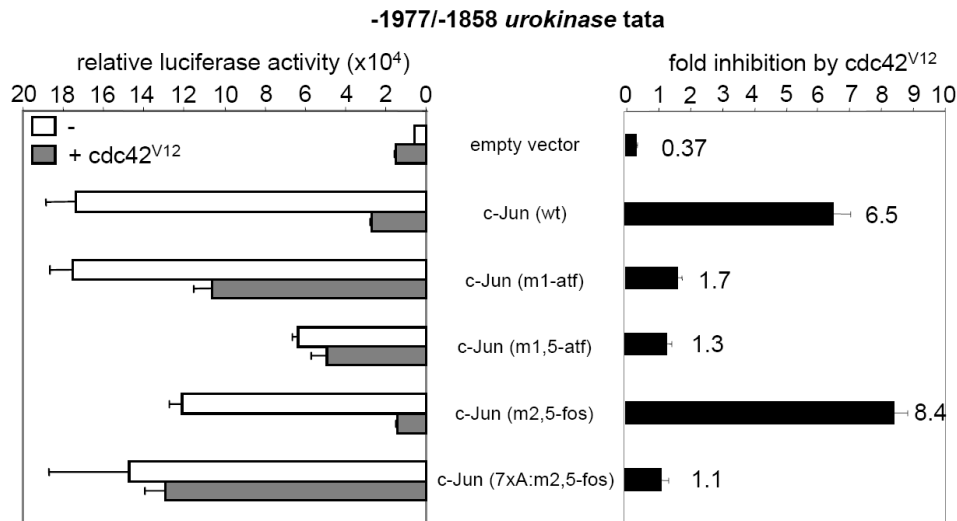


Figure 6. N-terminal c-Jun-phosphorylation differently affects transactivation by c-Jun/Fos and c-Jun/ATF-2

(A) c-Jun mutants with altered Jun/Fos and Jun/ATF2 dimerization specificities. Left panel: amino acid sequences of the wild-type and mutant leucine zipper domains in which the mutated *e* and *g* residues are indicated by the boxes. Right panel: The interaction of the mutants with c-Fos and ATF2 as measured by mammalian-one-hybrid analysis in F9 cells (van Dam et al., 1998). (B) HeLa tk⁻ cells were transfected with the -1977/-1858 *urokinase* TATA luciferase reporter and expression vectors for cdc42^{V12} and the indicated (HA-tagged) c-Jun mutants. Sixteen hours after transfection cell lysates were prepared. The fold inhibition by cdc42^{V12} on the activity of the different c-Jun constructs is shown in the right panel.

How could inhibition of *fra1* and possibly of other c-Jun/c-Fos-dependent genes support cell survival after genotoxic stress? First, it may be important that only a subset of c-Fos target genes is inhibited by JNK, because c-Fos can protect fibroblasts against UV and MMS-induced cell death (Schreiber et al., 1995;

Lackinger et al., 2001; this manuscript). We indeed found that transactivation by JunB/c-Fos was not significantly repressed upon JNK activation (C. van der Burgt, J. Jansen and H. van Dam, unpublished observations), presumably because JunB is a poor JNK substrate. Protective activity of c-Fos might therefore be exerted in

combination with non-c-Jun partner proteins. Moreover, Fra1 seems to have completely different functions in fibroblasts than c-Fos, as in contrast to *c-fos*^{-/-} cells, *fra1*^{-/-} cells show neither UV-hypersensitivity nor prolonged UV-induced S phase arrest (M. Hamdi, unpublished observations). In addition, Fra1 does not activate all c-Fos-inducible promoters, and even represses the c-Fos-induced activation of these genes when expressed at sufficient levels (Yoshioka et al. 1995). Since we found that a non-UV-repressible *fra1* transgene increases the sensitivity of 3T3 cells to low doses of UV, Fra1 may inhibit c-Fos-dependent anti-apoptotic genes. Alternatively, Fra1 may induce pro-apoptotic genes (Figure 7). Interestingly,

the survival gene *cIAP-2* has recently been identified as a JNK- and AP-1-controlled gene that can mediate cell survival in response to TNF- α (Lamb et al., 2003). However, we found the levels of cIAP-2 not to be affected by UV treatment (C. van der Burgt and M. Hamdi, unpublished observations).

It remains to be analyzed how N-terminal c-Jun phosphorylation specifically inhibits Jun/Fos-, but not Jun/ATF transcriptional activity. Co-precipitation assays indicate that the interaction between c-Jun and c-Fos is not significantly altered upon phosphorylation of c-Jun by JNK (M. Hamdi and H. van Dam, unpublished observations).

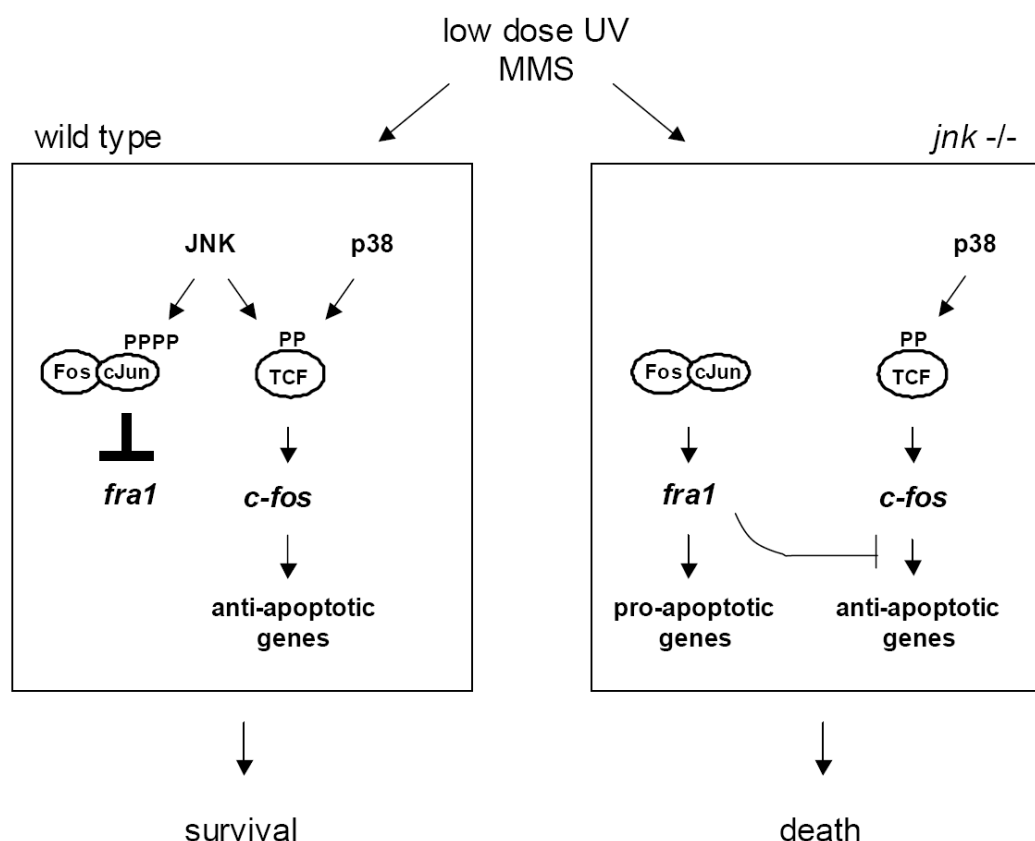


Figure 7. Proposed model for the role of JNK, phospho-c-Jun and Fra1 in the cellular response to low levels of DNA damage

In wild-type fibroblasts (left box), UV and MMS inhibit the growth factor-dependent expression of *fra1* through phosphorylation of c-Jun by JNK. The expression of *c-fos* is induced through phosphorylation of TCF/elk1 by JNK or p38 MAPkinases. In JNK-deficient cells (right box), Fra1 expression is not inhibited, leading to the activation of putative Fra1-dependent, pro-apoptotic genes, and to the inhibition of c-Fos-dependent, anti-apoptotic genes.

It is reported that phosphorylation of Thr91 and Thr93 can influence the phosphorylation state of Thr231, Thr239, Ser243 and Ser249, which are located just upstream of c-Jun DNA-binding domain

(Papavassiliou et al., 1995). c-Jun hyperphosphorylation in the context of Jun/Fos dimers might therefore reduce the on-rates or enhance the off-rates on their target promoters. However, mutation of all four

C-terminal phosphorylation sites into alanines did not affect the inhibition of c-Jun dependent transactivation of the *fra1*- or collagenase promoter/enhancers by JNK (J. Jansen, C. van der Burgt and H. van Dam, unpublished observations). Importantly, various co-activators and co-repressors have been found to interact with the c-Jun transactivation domain in a Ser63 and Ser73-dependent manner, including the histone-acetyl-transferases CBP and p300 (Bannister et al., 1995; Goodman and Smolik, 2000), TAF7 (Munz et al., 2003) and TGIF (Pessah et al., 2001). As CBP/p300 also interact with the transactivation domain of c-Fos (Bannister and Kouzarides, 1995; Goodman and Smolik, 2000), N-terminal hyperphosphorylation of c-Jun might differentially affect the affinity of c-Jun/Fos and c-Jun/ATF dimers for specific co-activators or, alternatively, increase the affinity for specific co-repressors. In this respect it is interestingly to note that the adenovirus E1A protein has been found to selectively interfere with the stimulatory effect of p300/CBP on Jun/Fos- but not Jun/ATF2-dependent promoters (Offringa et al., 1990; Hagmeyer et al. 1993; Duyndam et al., 1999).

It is tempting to speculate about the *in vivo* relevance of JNK-Jun mediated protection in response to genotoxic stress. It is known that at least some therapeutic agents sensitize cancer cells to DNA damage-induced apoptosis while inhibiting JNK and c-Jun-phosphorylation (Potapova et al., 2001; Zhong et al., 2003). Regardless of the mechanisms, our findings raise the issue that JNK and phospho-c-Jun support cell survival by inhibiting a subset of AP-1 target genes in the presence of low doses of genotoxic agents.

Materials and methods

Cells and cell culture

3T3 fibroblasts derived from *jnk1*^{-/-}, *jnk2*^{-/-}, *c-jun*^{-/-}, *c-fos*^{-/-}, *fra1*^{-/-}, *c-jun*-63A73A, H2-K^b-*fra1* and control mouse embryos have been described previously (Schreiber et al., 1995, 1999; Behrens et al., 1999; Jochum et al., 2000; Hochedlinger et al., 2002). Fibroblasts and HeLa tk- cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 9% fetal calf serum (FCS), 100 µg/ml penicillin and 100 µg/ml streptomycin. F9 cells were cultured in F12-DMEM (1:1) containing 9% FCS, penicillin, streptomycin, and 0.1mM β-

mercaptoethanol. For UV-C irradiation, a 30W germicidal lamp (TUV; Philips Electronic Instruments Inc, Eindhoven, The Netherlands) was used with a dose rate of 0.5J m⁻² sec⁻¹. Prior to UV-treatment, the medium was collected, cells were washed once with phosphate buffered saline (PBS) and PBS was removed before irradiation at room temperature. After irradiation, cells were refed with the previously collected medium.

Chemicals

TPA and MMS were obtained from Sigma; the JNK inhibitor, SP600125, from Biomol Research Laboratories, Inc.

FACS analysis

For quantitation of sub-G1 apoptotic cells, adhering and floating cells were collected via trypsinization and centrifugation. After resuspending in PBS, cells were fixed in 70% ethanol, washed in PBS, stained with 7.5 µM propidiumiodide (PI) containing 50 µg/ml RNase A, and analysed by flow cytometry (FACS-Calibur, Becton Dickinson). FACS results were analysed with WinMDI 2.8 software.

Western analysis and antibodies

After rinsing twice with ice-cold PBS, cell lysates were prepared from 60 or 90 mm dishes in 200 µl of ice-cold Fos-Ripa buffer containing protease and phosphatase inhibitors (van Dam et al., 1993), in SDS-sample buffer, or in luciferase lysis buffer (see below). Proteins were separated on 10% or 15% polyacrylamide gels and transferred to Immobilon (Millipore). Blots were stained with Ponceau S before blocking to verify equal loading and appropriate protein transfer. Filters were incubated with antibodies and stripped as described (Ouwens et al., 2002). The antibodies used were: Fra1 (R-20), c-Jun (H79), p21 (C-19) (all from Santa Cruz Biotechnology), c-Fos (06-341) (Upstate), phospho-specific c-Jun-Ser73 (New England Biolabs), active caspase 3 CM1 (Pharmingen), monoclonal anti-HA-tag antibody 16B12 (BabCO), monoclonal anti-myc-tag antibody 9E10 (Evan et al., 1985), goat-anti-rabbit and goat-anti-mouse IgG-HRP conjugate (Promega).

RNA analysis

RNA isolation and Northern blotting analysis were performed as described (van Dam et al., 1989). The *c-jun*, *c-fos*, *fra1*, and *hef-1* probes used have been described in (van Dam et al., 1990; Offringa et al., 1990; Passegue et al., 2002).

Transient transfection and plasmid constructs

For luciferase reporter assays cells 3T3 and F9 cells were transfected via the calcium phosphate method (Graham and van der Eb, 1973) and HeLa tk- cells via the DEAE-

dextrane method (Offringa et al., 1990). Luciferase activity was determined according to the manufacturer's protocol (Promega). As controls for transfection efficiency and proper expression of effector proteins, aliquots of the same lysate from which luciferase activity was determined were analyzed by western blotting.

The luciferase reporter constructs -517/+63 collagenase pGL3, -1600/+740 c-jun pGL3, Fra1-AP1-tk-Luc and Fra1-(mAP1)-tk-Luc have been described (Duyndam et al., 1999; Bergers et al., 1995). To create the pGL3# reporters tata, 4xSRE-tata, 5xjun2-tata, and 5xcollAP1-tata (also known as 5xcollTRE-tata), the XhoI/SmaI fragments of the corresponding pBL-tata-CAT 5 constructs (Jonat et al., 1990; van Dam et al., 1993; unpublished results) were replaced by the XhoI/blunted BamHI fragment of pGL3 basic (Promega). The pGL3# reporters -517/-42-collagenase-tata and -1977/-1858-urokinase-tata were subsequently constructed by replacing the BamHI-KpnI fragments of -517/-42 collagenase-TK-CAT4 (Angel et al., 1987) and -1977/-1858-uPA-TK-CAT4 (De Cesare et al., 1995) with the BamHI-KpnI fragment of tata-pGL3#.

The effector plasmid pRSV-c-Fos (Offringa et al., 1990) has been described. The plasmids pRSV-c-Jun-HA and pRSV-c-Jun-63L73L-HA were constructed by replacing the HpaI-KpnI fragment of pRSV-c-Jun and pRSV-c-Jun-LL (Radler-Pohl et al., 1993) with that of pCMV-c-Jun-HA. The pCMV-c-Jun-HA expression vector and the corresponding Ala and Asp mutants (wt, 63A73A, 63D73D, 91D93D, AAAA, 7xA and 6xD) were kindly provided by D. Bohmann and M. Musti (Musti et al., 1997, Papavassiliou et al., 1995, Treier et al., 1995). HA-tagged versions of 63A73A, 63D73D, 91D93D and AAAA were constructed by replacing the HpaI-KpnI fragments with the corresponding fragment of pCMV-c-Jun-HA. The leucine zipper mutant encoding plasmids pCMV-c-Jun-m1-J/A-*zip*-HA, -m1,5-J/A-*zip*-HA and -m2,5-J/F-*zip*-HA were created by replacing the PstI-KpnI fragments of pCMV-c-Jun-HA (wt TAD), c-Jun (7xA) and c-Jun (6xD) with the corresponding fragments of pRSV-c-Jun-m1-HA, -m1,5-HA and -m2,5-HA (van Dam et al., 1998; to be published elsewhere). pMT2, pMT2-cdc42^{V12} and pMT2-ΔMEKK were kindly provided by A. Hall.

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References

- Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H.J. and Herrlich, P. (1987) 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. *Mol. Cell Biol.*, **7**, 2256-2266.
- Bakiri, L., Lallemand, D., Bossy-Wetzel, E. and Yaniv, M. (2000) Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J.*, **19**, 2056-2068.
- Bannister, A.J. and Kouzarides, T. (1995) CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO J.*, **14**, 4758-4762.
- Bannister, A.J., Oehler, T., Wilhelm, D., Angel, P. and Kouzarides, T. (1995) Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. *Oncogene*, **11**, 2509-2514.
- Behrens, A., Sibilio, M. and Wagner, E.F. (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.*, **21**, 326-329.
- Bergers, G., Graninger, P., Braselmann, S., Wrighton, C. and Busslinger, M. (1995) Transcriptional activation of the fra-1 gene by AP-1 is mediated by regulatory sequences in the first intron. *Mol. Cell Biol.*, **15**, 3748-3758.
- Binetruy, B., Smeal, T. and Karin, M. (1991) Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature*, **351**, 122-127.
- Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature*, **410**, 37-40.
- Chen, Y.R., Wang, X., Templeton, D., Davis, R.J. and Tan, T.H. (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.*, **271**, 31929-31936.
- De Cesare, D., Vallone, D., Caracciolo, A., Sassone-Corsi, P., Nerlov, C. and Verde, P. (1995) Heterodimerization of c-Jun with ATF-2 and c-Fos is required for positive and negative regulation of the human urokinase enhancer. *Oncogene*, **11**, 365-376.
- Devary, Y., Gottlieb, R.A., Smeal, T. and Karin, M. (1992) The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell*, **71**, 1081-1091.

- Duyndam, M.C., van Dam, H., Smits, P.H., Verlaan, M., van der Eb, A.J. and Zantema, A. (1999) The N-terminal transactivation domain of ATF2 is a target for the co-operative activation of the c-jun promoter by p300 and 12S E1A. *Oncogene*, **18**, 2311-2321.
- Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.*, **5**, 3610-3616.
- Faris M, Kokot N, Latinis K, Kasibhatla S, Green DR, Koretzky GA, and Nel A. (1998) The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. *J Immunol.* **160**, 134-144.
- Goodman, R.H. and Smolik, S. (2000) CBP/p300 in cell growth, transformation, and development. *Genes Dev.*, **14**, 1553-1577.
- Graham, F.L. and van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, **52**, 456-467.
- Guo, Y.L., Baysal, K., Kang, B., Yang, L.J. and Williamson, J.R. (1998) Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor- α in rat mesangial cells. *J. Biol. Chem.*, **273**, 4027-4034.
- Hagmeyer, B.M., Konig, H., Herr, I., Offringa, R., Zantema, A., van der, E.A., Herrlich, P. and Angel, P. (1993) Adenovirus E1A negatively and positively modulates transcription of AP-1 dependent genes by dimer-specific regulation of the DNA binding and transactivation activities of Jun. *EMBO J.*, **12**, 3559-3572.
- Hochedlinger, K., Wagner, E.F. and Sabapathy, K. (2002) Differential effects of JNK1 and JNK2 on signal specific induction of apoptosis. *Oncogene*, **21**, 2441-2445.
- Huguier, S., Baguet, J., Perez, S., van Dam, H. and Castellazzi, M. (1998) Transcription factor ATF2 cooperates with v-Jun to promote growth factor-independent proliferation in vitro and tumor formation in vivo. *Mol. Cell Biol.*, **18**, 7020-7029.
- Jochum, W., David, J.P., Elliott, C., Wutz, A., Plenk, H., Jr., Matsuo, K. and Wagner, E.F. (2000) Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. *Nat. Med.*, **6**, 980-984.
- Jonat, C., Rahmsdorf, H.J., Park, K.K., Cato, A.C., Gebel, S., Ponta, H. and Herrlich, P. (1990) Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell*, **62**, 1189-1204.
- Kolbus, A., Herr, I., Schreiber, M., Debatin, K.M., Wagner, E.F. and Angel, P. (2000) c-Jun-dependent CD95-L expression is a rate-limiting step in the induction of apoptosis by alkylating agents. *Mol. Cell Biol.*, **20**, 575-582.
- Kuan, C.-Y., Yang, D. D., Samanta Roy, D.R., Davis, R.J., Rakic, P. and Flavell, R.A. (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22**, 667-676.
- Kyriakis, J.M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.*, **81**, 807-869.
- Lackinger, D., Eichhorn, U. and Kaina, B. (2001) Effect of ultraviolet light, methyl methanesulfonate and ionizing radiation on the genotoxic response and apoptosis of mouse fibroblasts lacking c-Fos, p53 or both. *Mutagenesis*, **16**, 233-241.
- Lamb, J.A., Ventura, J.J., Hess, P., Flavell, R.A. and Davis, R.J. (2003) JunD mediates survival signaling by the JNK signal transduction pathway. *Mol. Cell*, **11**, 1479-1489.
- Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, Bar-Sagi D, and Davis RJ. (2002) The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. *Mol Cell Biol.* 2002 **22**, 4929-4942.
- Le Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F.X., Green, D.R., and Karin, M. (1999) Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol. Cell Biol.*, **19**, 751-763.
- Mayne, L.V., and Lehmann, A.R. (1982) Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. *Cancer Res.*, **42**, 1473-1478.
- McKay, B.C., Ljungman, M., & Rainbow, A.J. (1998) Persistent DNA damage induced by ultraviolet light inhibits p21waf1 and bax expression: implications for DNA repair, UV sensitivity and the induction of apoptosis. *Oncogene*, **17**, 545-555.
- Munz, C., Psichari, E., Mandilis, D., Lavigne, A.C., Spiliotaki, M., Oehler, T., Davidson, I., Tora, L., Angel, P. and Pintzas, A. (2003) TAF7 (TAFII55) Plays a Role in the Transcription Activation by c-Jun. *J. Biol. Chem.*, **278**, 21510-21516.
- Musti, A.M., Treier, M. and Bohmann, D. (1997) Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science*, **275**, 400-402.
- Norbury, C.J. and Hickson, I.D. (2001) Cellular responses to DNA damage. *Annu. Rev. Pharmacol. Toxicol.*, **41**, 367-401.
- Offringa, R., Gebel, S., van Dam, H., Timmers, M., Smits, A., Zwart, R., Stein, B., Bos, J.L., van der, E.A. and Herrlich, P. (1990) A novel function of the transforming domain of E1a: repression of AP-1 activity. *Cell*, **62**, 527-538.
- Ouwens, D.M., de Ruiter, N.D., van der Zon, G.C., Carter, A.P., Schouten, J., van der, B.C., Kooistra, K., Bos, J.L., Maassen, J.A. and van Dam, H. (2002) Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO J.*, **21**, 3782-3793.

- Papavassiliou, A.G., Treier, M. and Bohmann, D. (1995) Intramolecular signal transduction in c-Jun. *EMBO J.*, **14**, 2014-2019.
- Passequé, E., Jochum, W., Behrens, A., Ricci, R. and Wagner, E.F. (2002) JunB can substitute for Jun in mouse development and cell proliferation. *Nat. Genet.*, **30**, 158-166.
- Pessah, M., Prunier, C., Marais, J., Ferrand, N., Mazars, A., Lallemand, F., Gauthier, J.M. and Atfi, A. (2001) c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 6198-6203.
- Potapova, O., Basu, S., Mercola, D. and Holbrook, N.J. (2001) Protective role for c-Jun in the cellular response to DNA damage. *J. Biol. Chem.*, **276**, 28546-28553.
- Pulverer, B.J., Kyriakis, J.M., Avruch, J., Nikolakaki, E. and Woodgett, J.R. (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature*, **353**, 670-674.
- Radler-Pohl, A., Sachsenmaier, C., Gebel, S., Auer, H.P., Bruder, J.T., Rapp, U., Angel, P., Rahmsdorf, H.J. and Herrlich, P. (1993) UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. *EMBO J.*, **12**, 1005-1012.
- Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M. and Wagner, E.F. (1999) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech. Dev.*, **89**, 115-124.
- Schreiber, M., Baumann, B., Cotten, M., Angel, P. and Wagner, E.F. (1995) Fos is an essential component of the mammalian UV response. *EMBO J.*, **14**, 5338-5349.
- Schreiber, M., Poirier, C., Franchi, A., Kurzbauer, R., Guenet, J.L., Carle, G.F. and Wagner, E.F. (1997) Structure and chromosomal assignment of the mouse fra-1 gene, and its exclusion as a candidate gene for oc (osteosclerosis). *Oncogene*, **15**, 1171-1178.
- Schreiber, M., Wang, Z.Q., Jochum, W., Fetka, I., Elliott, C. and Wagner, E.F. (2000) Placental vascularisation requires the AP-1 component fra1. *Development*, **127**, 4937-4948.
- Shaulian, E. and Karin, M. (2001) AP-1 in cell proliferation and survival. *Oncogene*, **20**, 2390-2400.
- Tornaletti, S. and Hanawalt, P.C. (1999) Effect of DNA lesions on transcription elongation. *Biochimie*, **81**, 139-146.
- Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Bar-Sagi, D., Jones, S.N., Flavell, R.A. and Davis, R.J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*, **288**, 870-874.
- Treier, M., Bohmann, D. and Mlodzik, M. (1995) JUN cooperates with the ETS domain protein pointed to induce photoreceptor R7 fate in the Drosophila eye. *Cell*, **83**, 753-760.
- van Dam, H., Offringa, R., Smits, A.M., Bos, J.L., Jones, N.C. and van der Eb, A.J. (1989) The repression of the growth factor-inducible genes JE, c-myc and stromelysin by adenovirus E1A is mediated by conserved region 1. *Oncogene*, **4**, 1207-1212.
- van Dam, H., Offringa, R., Meijer, I., Stein, B., Smits, A.M., Herrlich, P., Bos, J.L. and van der Eb, A.J. (1990) Differential effects of the adenovirus E1A oncogene on members of the AP-1 transcription factor family. *Mol. Cell Biol.*, **10**, 5857-5864.
- van Dam, H., Duyndam, M., Rottier, R., Bosch, A., Vries-Smits, L., Herrlich, P., Zanema, A., Angel, P. and van der Eb, A.J. (1993) Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO J.*, **12**, 479-487.
- van Dam, H., Huguier, S., Kooistra, K., Baguet, J., Vial, E., van der Eb, A.J., Herrlich, P., Angel, P. and Castellazzi, M. (1998) Autocrine growth and anchorage independence: two complementing Jun-controlled genetic programs of cellular transformation. *Genes Dev.*, **12**, 1227-1239.
- Wagner, E. F. (2001) *Oncogene*, **20**, 2333-2498.
- Watson, A., Eilers, A., Lallemand, D., Kyriakis, J., Rubin, L.L. and Ham, J. (1998) Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J. Neurosci.*, **18**, 751-762.
- Weston, C.R. and Davis, R.J. (2002) The JNK signal transduction pathway. *Curr. Opin. Genet. Dev.*, **12**, 14-21.
- Weiss C, Schneider S, Wagner EF, Zhang X, Seto E, and Bohmann D. (2003) JNK phosphorylation relieves HDAC3-dependent suppression of the transcriptional activity of c-Jun. *EMBO J.* **22**, 3686-3695.
- Wisdom, R. (1999) AP-1: one switch for many signals. *Exp. Cell Res.*, **253**, 180-185.
- Wisdom, R., Johnson, R.S. and Moore, C. (1999) c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *EMBO J.*, **18**, 188-197.
- Yoshioka, K., Deng, T., Cavigelli, M. and Karin, M. (1995) Antitumor promotion by phenolic antioxidants: inhibition of AP-1 activity through induction of Fra expression. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 4972-4976.
- Zhong X, Li QQ, and Reed E. (2003) SU5416 sensitizes ovarian cancer cells to cisplatin through inhibition of nucleotide excision repair. *Cell Mol Life Sci.* **60**, 794-802.
- Zhou, B.B. and Elledge, S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, **408**, 433-439.

Chapter VI

Summary and discussion

Samenvatting

Organisms have evolved elegant ways to protect their genome from harmful agents to maintain genomic integrity and preserve their genetic information. Environmental factors like ultraviolet light (UV) and ionizing radiation (IR) can induce DNA damage, hence create and accumulate undesirable mutations and cause loss of genetic information. Furthermore, DNA damage may also be incited as a consequence of normal cellular processes: by errors arising during DNA replication or by free radicals that are generated during cellular metabolism. To overcome this attack on their DNA, mechanisms have developed to repair DNA, to halt cell-cycle progression to enable DNA repair before DNA synthesis is initiated or resumed, and – if necessary – to induce permanent cell arrest or apoptosis.

Understanding the molecular mechanisms and cellular processes in response to genotoxic stress is of great importance. The work presented in this thesis has focused on the role of Mitogen Activated Protein Kinases (MAPKs) and AP-1 transcription factors. MAPK signaling pathways regulate cellular activities such as cell proliferation, cell survival, cell motility and migration via a multitude of downstream targets, which include amongst others the Jun, Fos and ATF components of the AP-1 transcription family. Based on their dimer composition various AP-1 sub-classes can be discriminated, Jun/Fos, Jun/Fra, Jun/ATF, Jun/Jun, and ATF/ATF, which regulate distinct sets of target genes due to differences in DNA binding site preference. When this work was initiated the upstream signaling events through which genotoxic agents activate MAPKs, and the actual effects of these agents on distinct AP-1 sub-classes and target genes were still largely unknown. Moreover, for most genotoxic agents and cell types the roles of specific AP-1 dimers in DNA damage responses remained to be established.

A key feature of most AP-1 components is that their promoters are themselves under positive and/or negative transcriptional control by AP-1 dimers. Chapter II showed that treatment of human fibroblasts with IR leads to phosphorylation of c-Jun and ATF-2 and induction of the mRNA and protein levels of ATF3, a potential c-Jun/ATF-2 target gene. Although IR also induces the tumor suppressor p53, a known activator of the *atf3* promoter, the presence of functional p53 was not essential for IR-induced ATF3 expression. The use of fibroblasts derived from patients suffering from the hereditary diseases Ataxia-telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) showed that both ATF-2 phosphorylation and ATF3 induction by IR is dependent on the presence of functional ATM and Nbs1. Furthermore, use of chemical inhibitors indicated that the activation of ATF-2 and the induction of ATF3 by IR was dependent on JNK and p38 MAPKs. These data suggest that IR induces ATF3 in human fibroblasts via a signaling pathway involving ATM, Nbs1, stress-induced MAPKs and ATF-2. Both A-T and NBS cells are defective in IR-induced S- and G2/M-phase arrest, and show increased radiosensitivity. The inability of these cells to properly activate ATF-2 and ATF3 after IR might contribute to this phenotype.

DNA damage induced by UV is repaired by the nucleotide excision repair (NER) pathway and mutations in components of this pathway can cause the human disease Xeroderma pigmentosum (XP). Two types of NER can be distinguished: global genome repair (GGR) and transcription-coupled repair (TCR). Analysis of TCR-deficient human fibroblasts identified ATF3, c-Jun, and JNK as important sensors of UV-induced damage in transcribed genes (chapter III); in these cells low doses of UV strongly induced (delayed) phosphorylation and activation of JNK and c-Jun, and enhanced the expression of ATF3. In contrast, TCR-proficient cells irradiated with the same low UV doses neither showed JNK and c-Jun activation, nor ATF3 induction. The non-repaired UV-induced cyclobutane pyrimidine dimers (CPDs) in TCR-deficient cells were found to cause inhibition of the expression of MAPK phosphatase 1 (MKP-1), an inhibitor of JNK; removal of these CPDs by activation of CPD photolyase abrogated both the inhibition of MKP-1, the phosphorylation and activation of JNK and c-Jun, and the induction of ATF3. Importantly, ectopic expression of MKP-1 inhibited JNK activation and strongly suppressed low dose UV-induced apoptosis, showing that JNK is pro-apoptotic under these conditions. These results therefore reveal a functional link between DNA damage in transcribed genes and the MKP-1–JNK pathway.

In mouse embryo fibroblasts prolonged activation and phosphorylation of both JNK and c-Jun has been implicated in the onset of apoptosis after UV irradiation. TCR-deficient cells are more sensitive to UV-irradiation and initiate apoptosis at lower doses of UV than wild type cells. Therefore, it is conceivable that the enhanced UV-sensitivity of TCR-deficient human fibroblasts is at least in part caused by the activation and phosphorylation of JNK, c-Jun and their downstream targets, including pro-apoptotic c-Jun-dependent genes. The role of ATF3 is intriguing in this context. ATF3 is induced by both growth factors and stresses and

has been reported to affect cell death and cell cycle progression both positively and negatively, depending on the cell type and type and dose of stimulus. In chapter II we showed that ATF3-induction by IR appears to correlate with cell survival, while in UV-irradiated NER-deficient fibroblasts ATF3 induction correlates with cell death. Several studies in NER-proficient cell types reported that ATF3 is pro-apoptotic upon UV-induction. However, knock-down of ATF3 in TCR-deficient SV40-transformed XPA and CSB human fibroblasts seems to have no obvious effects on low dose UV-induced apoptosis (M. Hamdi, H.P. Popeijus and H. van Dam, unpublished observations). It is therefore possible that the pro-apoptotic and pro-survival functions of ATF3 counteract each other in TCR-deficient cells. Alternatively, ATF3 might directly or indirectly control the expression of one or more DNA repair genes. Further investigation will be necessary to elucidate the role of ATF3 activation in repair-deficient and repair-proficient cells. Since ATF3 has been reported to activate p53 by blocking Mdm2-dependent degradation, it will be interesting to examine the effect of ATF3 on the levels of p53 and the p53-target genes p21, Puma, Noxa and XPC and DDB2 – genes that respectively induce cell cycle arrest, apoptosis and DNA repair. In fact, in T98G human glioblastoma cells lacking functional p53 and p16-INK4A ATF3 has an anti-apoptotic function after treatment with cisplatin (chapter IV). In these cells (one of the) pro-apoptotic function(s) of ATF3 might be lost due to p53 mutation.

The AP-1 component Fra1 was found to act as a pro-apoptotic effector upon UV- or cisplatin-induced DNA damage (chapters IV and V). Under these conditions, Fra1 might be involved in the induction of pro-apoptotic genes, or, alternatively, counteract the activation of anti-apoptotic genes (chapter V, figure 7). Further investigation is needed to elucidate the role of Fra1 in UV- and cisplatin-induced apoptosis. Interestingly, in T98G glioblastoma cells ATF3 and Fra1 also have opposite effects on cisplatin-induced S phase arrest (chapter IV). It remains to be established whether ATF3 and Fra1 mediate these (opposite) functions as ATF3/ATF, ATF3/Jun and/or Fra1/Jun dimers. An intriguing possibility is both ATF3 and Fra1 need to dimerize to c-Jun to exhibit these functions. Like ATF3, c-Jun and Fra1 can affect cell cycle progression and cell survival both positively and negatively, depending on cell type and nature and dose of stimulus. c-Jun can control multiple pro- and anti-apoptotic genes and, in addition, has been reported to be able to regulate several DNA repair genes, in particular upon JNK-dependent phosphorylation. Thus, it will be important to examine whether knock-down of Fra1 and ATF3 influences the expression of (c-Jun-dependent and c-Jun-independent) genes involved in apoptosis, survival and DNA repair. Unfortunately, efforts to examine the role of c-Jun via RNA interference in the human cell types described in chapters II-IV were hampered by the fact that the resulting c-Jun knock-down cells already died or ceased to proliferate in the absence of DNA damage (H.P. Popeijus, F. Carlotti, M. Hamdi, J. Janssen and H. van Dam, unpublished observations.)

In human glioblastoma cells UV and cisplatin induce Fra1 predominantly through activation of ERK MAPKs, which phosphorylate and stabilize the pre-existing Fra1 protein. In contrast, in 3T3 mouse embryo fibroblasts the Fra1 protein levels are inhibited rather than induced by UV treatment, presumably because ERK activity is not induced by UV in these cells (chapter V; M. Hamdi and H. van Dam, unpublished observations). Importantly, in both glioblastoma and 3T3 cells fra1 mRNA levels were inhibited by UV irradiation, in particular in the presence of growth factors (chapter IV and V). This UV-induced inhibition of fra1 mRNA was not observed in 3T3 fibroblasts derived from JNK1+2 knock-out mice or c-jun knock-out mice and thus appeared to be dependent on the presence of c-Jun as well as on the activation of JNK. Subsequent studies in fibroblasts derived from c-jun, junB and c-fos knock-out and knock-in mice, as well as analysis of a large set of c-Jun gain- and loss-of function and domain-swap mutants, revealed an as yet unknown function of JNK: inhibition of the transactivating capacity of c-Jun/Fos(-like) dimers (Chapter V; M. Hamdi, R. Vries, M. Monkonen, T. Zuiverloon, C. van der Burgt, J. Janssen, K. Matsuo and H. van Dam, unpublished observations). This inhibitory function of JNK is mediated by hyperphosphorylation of Ser63, Ser73, Thr 91 and Thr93 in the c-Jun transactivation domain and dependent on the ability of c-Jun to dimerize with Fos-family members. In line with this, 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 and collagenase, but not c-Jun/ATF-dependent genes like c-jun and atf3. This further emphasizes the opposite functions that c-Jun/Fos(-like) and c-Jun/ATF dimers and their target genes can exhibit in DNA damage responses. Intriguingly, in the context of Gal4-c-Jun fusion proteins and c-Jun/ATF-2 dimers phosphorylation of c-Jun Ser63 and Ser73 by JNK is thought to enhance its transcriptional

activity by decreasing the interaction with the histone deacetylase HDAC3 and/or increasing the interaction with histone acetylases like CBP and p300. It remains to be established whether c-Jun/Fos(-like) dimers can still efficiently bind to DNA and/or interact with co-activators and chromatin-remodeling factors upon hyper-phosphorylation of the c-Jun transactivation domain. Such experiments are expected to reveal novel insights into the mechanisms by which MAPKs regulate c-Jun-dependent gene activation.

With respect to cancer therapy, it is important to note that elevated levels of c-Jun, Fra1 and ATF3 have been detected in multiple human tumors as well as human cancer cell lines, including breast, colon and lung. It will therefore be of interest to examine whether the enhanced levels of these AP-1 components affect the efficiency of genotoxic anti-cancer drugs, and whether such effects are dependent on the integrity of the p53 pathway. Moreover, combination therapy of genotoxic agents and agents that can stabilize Fra1 protein levels might have anticancer therapeutic potential in the future.

In conclusion, along the growing complexity of understanding AP-1 regulation and function are the observations that point to its ability to regulate DNA repair pathways. Future studies will hopefully further delineate the important role of the AP-1 transcription factors in cellular growth control following DNA damage.

Samenvatting

Omgevingsfactoren zoals ultraviolet licht (UV) en ioniserende straling kunnen DNA-schade veroorzaken, wat kan resulteren in accumulatie van ongewenste mutaties en verlies van genetische informatie. DNA-schade kan ook een gevolg zijn van normale biologische processen, zoals fouten veroorzaakt bij het repliceren van het DNA of door reacties met zogenaamde *reactive oxygen species* (ROS), zuurstofradicalen en -ionen die ontstaan als gevolg van normale metabole reacties. Organismen hebben elegante oplossingen gevonden om hun genoom te beschermen tegen schadelijke stoffen, wat nodig is om de genomische integriteit te waarborgen en de genetische informatie te behouden. Om de aanslagen op DNA tegen te gaan zijn mechanismen ontstaan om (a) DNA te repareren, (b) de celcyclus stop te zetten zodat het beschadigd DNA gerepareerd kan worden voordat DNA replicatie plaats vindt en (c), indien noodzakelijk, de celcyclus permanent stil te zetten of geprogrammeerde celdood te induceren.

Het ontrafelen van de moleculaire mechanismen van de processen die het gevolg zijn van DNA-schade is van groot belang. In het onderzoek beschreven in dit proefschrift lag de nadruk op de rol van de zogenaamde *Mitogen Activated Protein Kinases* (MAPKs) en de AP-1 transcriptie factoren, met name de AP-1 componenten ATF3, Fra1, c-Jun, ATF-2 en c-Fos. De MAPK signaal-transduktie routen reguleren en coördineren diverse cellulaire activiteiten zoals celproliferatie, celcyclus arrest, geprogrammeerde cel-dood (apoptose), en celbeweeglijkheid en migratie. Om deze processen aan te kunnen sturen, moeten de MAPKs ondermeer een aantal AP-1 componenten fosforyleren en/of activeren. Als gevolg van hun eigenschap om dimere complexen te vormen, zijn er meerdere AP-1 subklassen te onderscheiden: Jun/Fos, Jun/Fra, Jun/ATF, Jun/Jun en ATF/ATF. Deze AP-1 dimeren reguleren verschillende sets van genen, doordat ze verschillen in hun bindingsactiviteit voor specifieke DNA sequenties. Toen met dit onderzoek begonnen werd, waren de moleculaire mechanismen waarmee DNA-beschadigende agentia de verschillende MAPK signaal-transduktie routen kunnen activeren voor een groot deel nog onbekend. Ook waren de effecten van DNA-beschadigende agentia op de verscheidene AP-1 subklassen en de door hen aangestuurde genen nog grotendeels onduidelijk. De (mogelijk celtype-specifieke) functies van de diverse AP-1 dimeren in DNA-schade responsen moesten ook nog worden onderzocht.

Hoofdstuk II van dit proefschrift geeft informatie over de signaal-transduktie route waarmee ioniserende straling (IR) de AP-1 componenten ATF-2 en ATF3 activeert in primaire humane fibroblasten. De resultaten tonen aan dat voor de door ioniserende straling-geïnduceerde fosforylering van ATF-2 en de daarop volgende inductie van ATF3, de ATM en Nbs1 eiwitten nodig lijken te zijn, twee enzymen die essentieel zijn voor de reparatie van DNA na ioniserende straling. Ook de MAPKs p38 en JNK, welke ATF-2 fosforyleren en ATF3 mRNA kunnen induceren, blijken betrokken te zijn bij de aktivatie van ATF2 and ATF3 door ioniserende straling, maar aanwezigheid van functioneel p53 tumor suppressor eiwit, een belangrijke andere target van IR, lijkt niet essentieel. Aktivatie van ATF2 en ATF3 zou een belangrijke bijdrage kunnen leveren aan het mechanisme waarmee humane cellen zich kunnen beschermen tegen de door ioniserende straling veroorzaakte dubbelstrengs DNA breuken.

Het *nucleotide-excisie reparatie* (NER) systeem kan DNA repareren wat beschadigd is door UV licht en mutaties in de eiwitten van het NER systeem kunnen de humane ziekte *Xeroderma pigmentosum* (XP) veroorzaken. Er kunnen twee typen NER worden onderscheiden: reparatie van DNA-schade over het hele genoom ('*global genome repair*': GGR) en reparatie van DNA-schade in getranskribeerde genen, de zogenaamde *transkriptie-gekoppelde reparatie* (TCR). Door UV-bestraalde fibroblasten van humane TCR-deficiënte en GGR-deficiënte patiënten te vergelijken (**hoofdstuk III**), konden de AP-1 componenten ATF3, c-Jun, het MAPK JNK en het JNK-fosfatase MKP-1 geïdentificeerd worden als belangrijke sensoren van DNA-schade in getranskribeerde genen. De niet-gerepareerde DNA-schade in de TCR deficiënte cellen resulteerde bovendien in een verlaging van het MKP-1 mRNA. Een belangrijk resultaat was ook dat ectopische expressie van MKP-1 niet alleen de door UV-licht geïnduceerde JNK aktivatie remde, maar tevens de door UV-geïnduceerde apoptose onderdrukte. Dit laat zien dat JNK onder deze omstandigheden een pro-apoptotische functie

heeft en onthullen tevens een functionele link tussen DNA-schade in getranskribeerde genen en de MKP-1-JNK route.

Hoofdstuk IV beschrijft de functies van MAPKs JNK en ERK en de AP-1 componenten ATF3 en Fra1 in T98G glioblastoma cellen behandeld met UV licht of met het cytostaticum cisplatinum. De effecten van cisplatinum op DNA replicatie en transkriptie zijn in een aantal opzichten vergelijkbaar met de effecten van UV. T98G glioblastoma cellen, afkomstig van een humane hersen tumor, zijn relatief ongevoelig voor cisplatinum. Zowel JNK als ERK MAPKs werden in T98G cellen geactiveerd door cisplatinum en UV, maar alleen ERK activatie lijkt een belangrijke rol te spelen bij de inductie van apoptose door deze agentia. De resultaten laten verder zien dat JNK en niet ERK betrokken is bij de inductie van ATF3 door cisplatinum, maar dat zowel JNK als ERK betrokken zijn bij de inductie van Fra1. In tegenstelling tot de activatie van ATF3, vindt de activatie van Fra1 door UV en cisplatinum niet plaats op het nivo van mRNA accumulatie, maar neemt de hoeveelheid fra1 mRNA juist af. Het reeds aanwezige Fra1 eiwit wordt echter gestabiliseerd door UV en cisplatinum-geïnduceerde ERK-afhankelijke fosforylering. Remming van ATF3 en Fra1 inductie met behulp van kleine interfererende RNAs laat zien dat ATF3 na cisplatinum behandeling functioneert als een anti-apoptotisch target-eiwit van JNK en dat Fra1 eerder opereert als een pro-apoptotisch verlengstuk van zowel JNK als ERK. Tevens blijken ATF3 en Fra1 in T98G glioblastoma cellen tegenovergestelde effecten te hebben op de door cisplatinum geïnduceerde celcyclus (S-phase) arrest. Een mogelijke verklaring voor deze effecten is dat ATF3 en Fra1 tegengestelde effecten hebben op door AP-1 gereguleerde DNA herstel enzymen.

Hoofdstuk V laat zien dat Fra1 ook een pro-apoptotische functie heeft in UV bestraalde muize-fibroblasten, met name bij lage dosis UV, wanneer JNK een anti-apoptotische werking heeft. Verder wordt in dit hoofdstuk een tot nu toe onbekende functie van JNK aangetoond, namelijk het vermogen om de transkriptionele activiteit van c-Jun/Fos dimeren te remmen. Deze remming wordt bewerkstelligd door JNK-afhankelijke hyper-fosforylering van Ser 63, Ser 73, Thr 91 en Thr 93, gelegen in het c-Jun transaktivatie domein. Transaktivatie door c-Jun mutanten die geen dimeren kunnen vormen met Fos of Fra eiwitten wordt niet geremd na JNK activatie. In overeenstemming met deze resultaten resulteert relatief sterke en langdurige activatie van JNK (zoals vaak plaats vindt na behandeling met DNA-beschadigende agentia) in remming van een aantal c-Jun/Fos afhankelijke genen, zoals fra1, maar niet in remming van c-Jun/ATF afhankelijke genen, zoals atf3 en c-jun zelf. Het is nog onduidelijk of de door JNK-gemedieerde hyperfosforylering van het c-Jun transaktivatie domein resulteert in verstoring van binding van c-Jun/Fos dimeren aan DNA of in verstoring van de interactie van c-Jun/Fos met c-Jun/Fos-specifieke co-aktivatoren en/of chromatine remodeling factoren. De resultaten in dit hoofdstuk ondersteunen echter het idee dat c-Jun/Fos en c-Jun/ATF dimeren en door hen gereguleerde genen zoals fra1 en atf3 tegenovergestelde functies, kunnen hebben in DNA-schade responsen.

Curriculum Vitae

De auteur van dit proefschrift werd geboren in Beni Said in Marokko op 1 september 1974. Na het behalen van het Gymnasium diploma aan het Bredero College in Amsterdam werd in 1994 begonnen met de studie Medische Biologie aan de Universiteit van Amsterdam. Tijdens de doctoraal fase werd een stage gedaan op de afdeling Medische Microbiologie van het Academisch Medisch Centrum te Amsterdam onder supervisie van Dr. S.A.J. Zaat en een stage bij het Centrum voor Biomembranen en Lipide Enzymologie binnen de faculteit Scheikunde van de Universiteit Utrecht onder begeleiding van Dr. K.N.J. Burger. In 1999 werd begonnen met het promotie onderzoek op de afdeling Moleculaire Cel Biologie van de Universiteit Leiden onder leiding van Dr. ir. J.A.F. van Dam en Prof. Dr. C.P. Verrijzer. Na vertrek van Prof. C.P. Verrijzer naar Erasmus MC (Rotterdam) onder leiding van Prof. Dr. P. ten Dijke. In 2005 werd begonnen als post-doctoraal onderzoeker in de groep van Prof. Dr. R. Versteeg op de afdeling Humane Genetica van het Academisch Medisch Centrum te Amsterdam.