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

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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

Mohamed Hamdi[1], Robert G. J. Vries[1], Herman E. Popeijus[1], Jan Schouten[1,4], Corina van der Burgt[1], Josephine Janssen[1], Mia Monkkonen[1], Erwin F. Wagner[2], Koichi Matsuo[2,3], and Hans van Dam[1]#

[1]Department of Molecular Cell Biology, Section Gene Regulation, Leiden University Medical Centre, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands, [2]Research Institute of Molecular Pathology (IMP), Dr. Bohr-Gasse 7, A-1030 Vienna, Austria, [3]Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[4]present address: Crucell, Archimedesweg 4, P.O. Box 2048, 2301 CA Leiden, The Netherlands.

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Corresponding author, telephone: +31-(0)71-5276133/6844, fax: +31-(0)71-5276284, e-mail: vdam@lumc.nl

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/m^2 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/m^2 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/m^2 UV was also detected in *jnk*1$^{+/-}$*jnk2*$^{-/-}$ 3T3 fibroblasts (data not shown). After exposure to 10 J/m^2 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 *jnk*1$^{-/-}$*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/m^2 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.

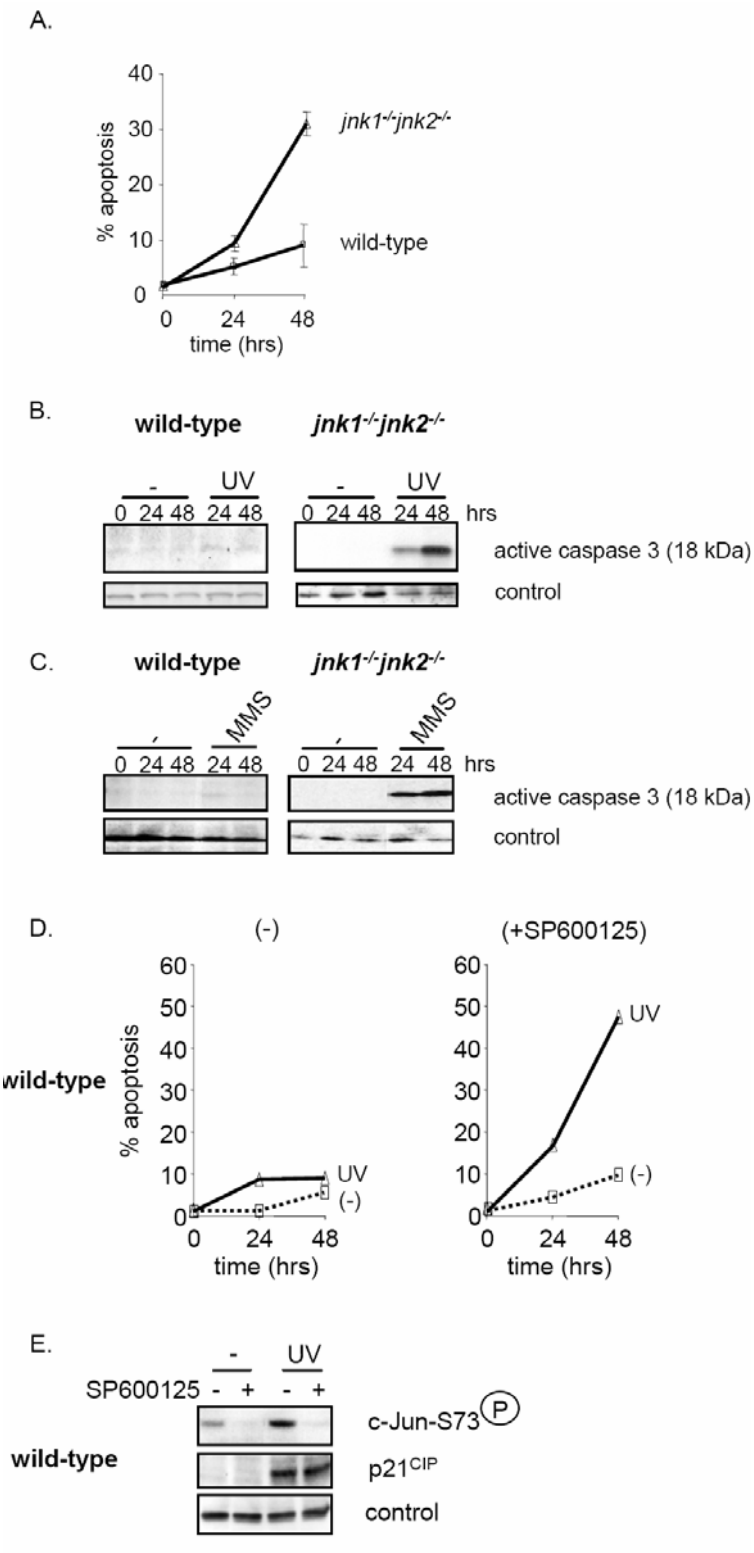

Figure 1. High levels of apoptosis in JNK-deficient fibroblasts after 10J UV or MMS

(A) Sub-confluent, proliferating cultures of wild-type and *jnk1*$^{-/-}$ *jnk2*$^{-/-}$ 3T3 fibroblasts were irradiated with 10 J/m^2 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/m^2 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 μM 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/m^2 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.

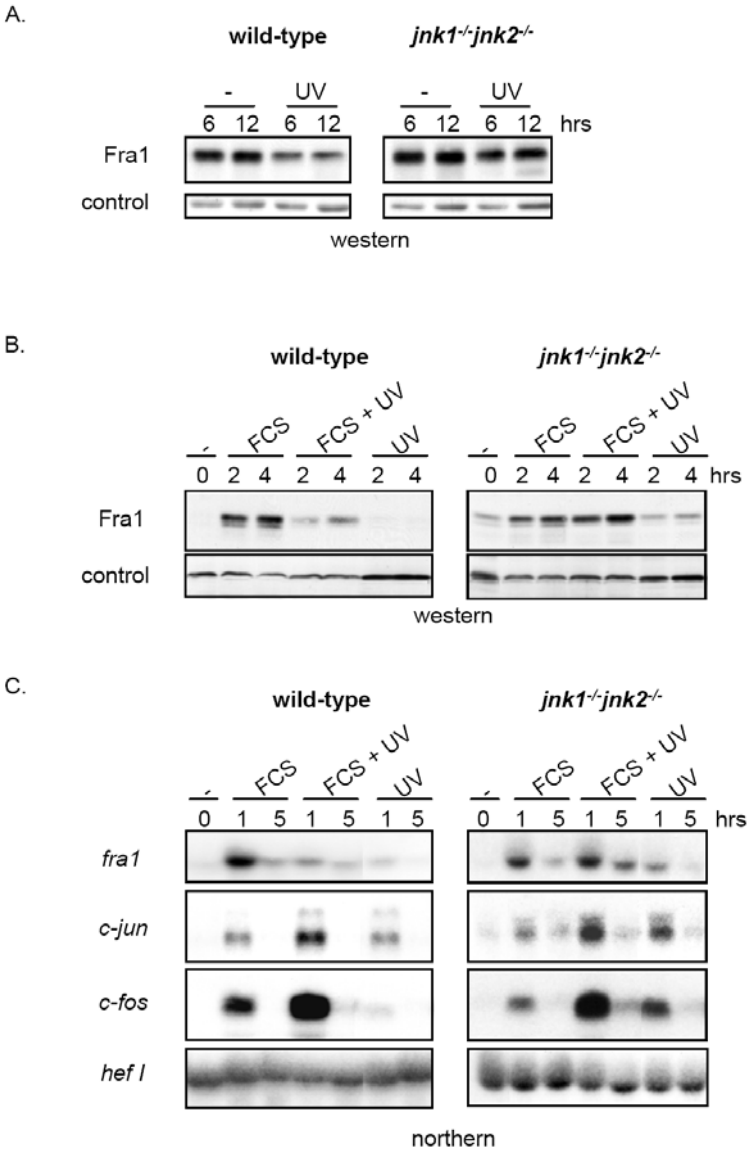

Figure 2. JNK-dependent inhibition of Fra1 expression by UV

(A) Sub-confluent, proliferating cultures of the wild-type and *jnk1*$^{-/-}$*jnk2*$^{-/-}$ cells shown in Figure 1 were irradiated with 10 J/m^2 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/m^2 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 I* was examined to verify equal loading.

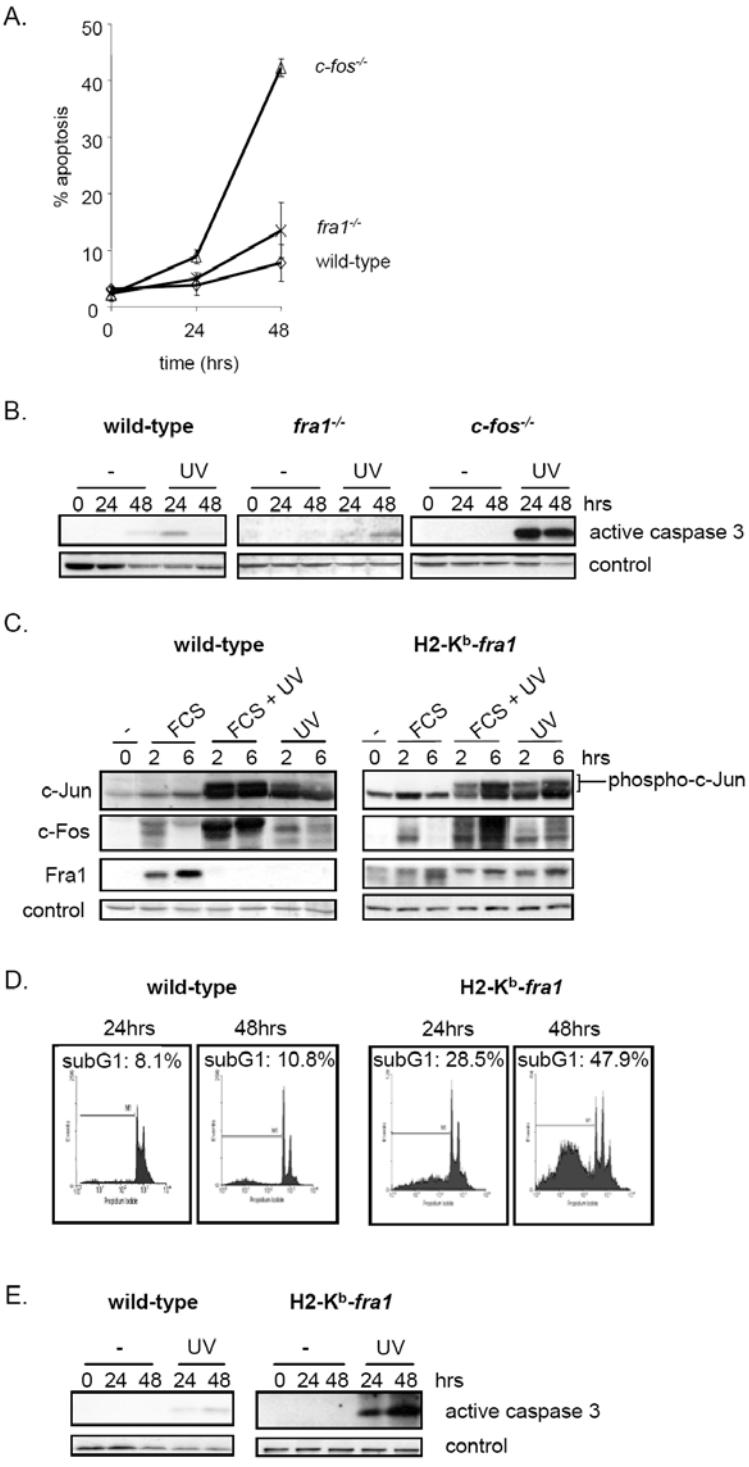

Figure3. Constitutive expression of Fra1 sensitizes mouse fibroblasts to UV-induced apoptosis

(A) Sub-confluent, proliferating cultures of wild-type, *c-fos*$^{-/-}$ and *fra1*$^{-/-}$ 3T3 fibroblasts were irradiated with 10 J/m^2 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/m^2 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

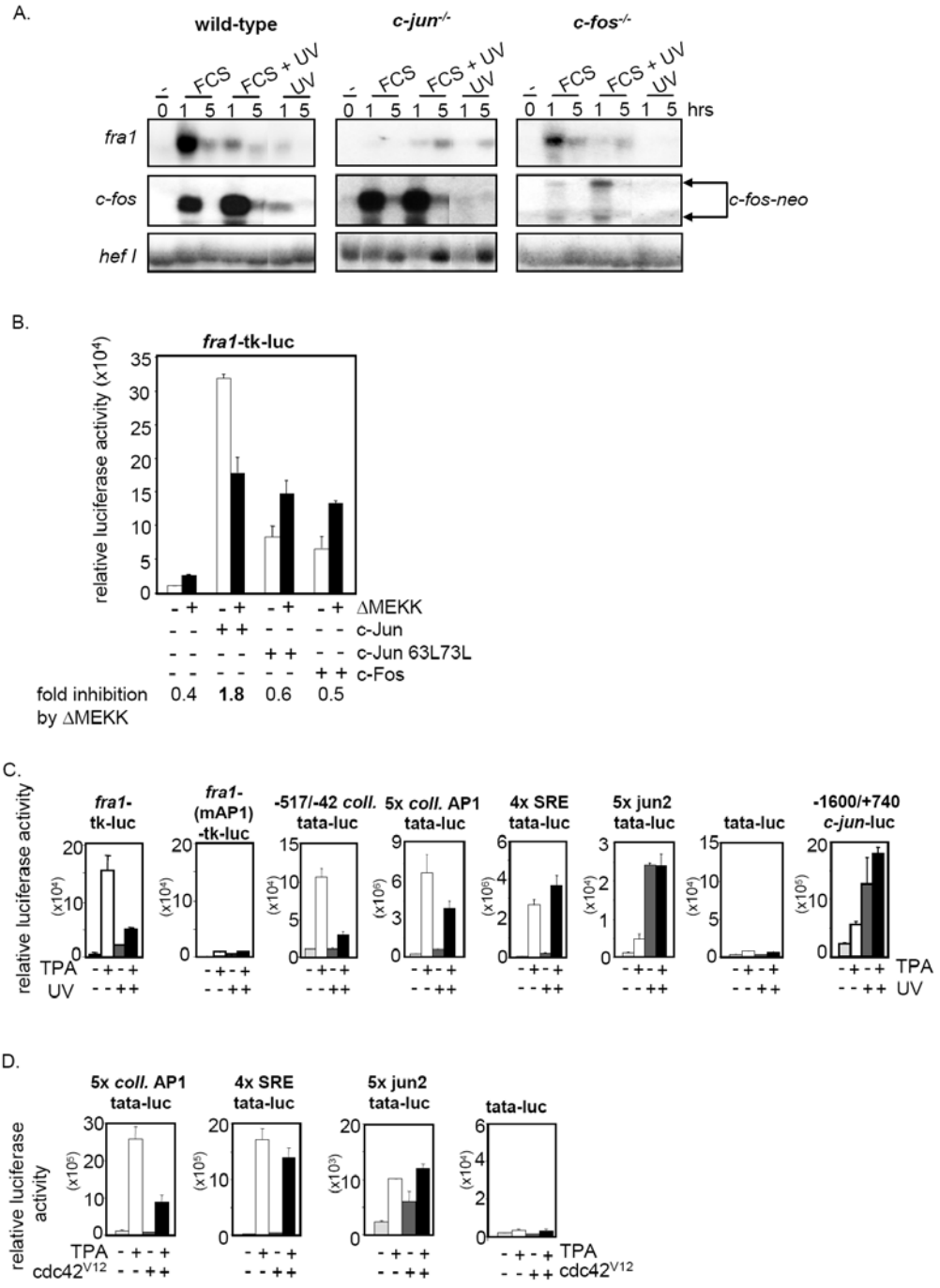

Figure 4. Inhibition of c-Jun-dependent *fra1* transcription by UV and stress-induced JNK
(A) Confluent, serum-starved wild-type, *c-jun*$^{-/-}$ and *c-fos*$^{-/-}$ 3T3 cells were irradiated with 10 J/m^2 UV or mock-treated, and subsequently stimulated with 20% FCS. Total RNA isolated at 1 and 5 hours 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/m^2 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 (ΔMEKK). In contrast, trans-activation by c-Fos was not inhibited via ΔMEKK. Moreover, ΔMEKK 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 ΔMEKK 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 ΔMEKK 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 $c\text{-}Jun^{AA}$ 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 $c\text{-}Jun^{AA}$ 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/m^2 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.

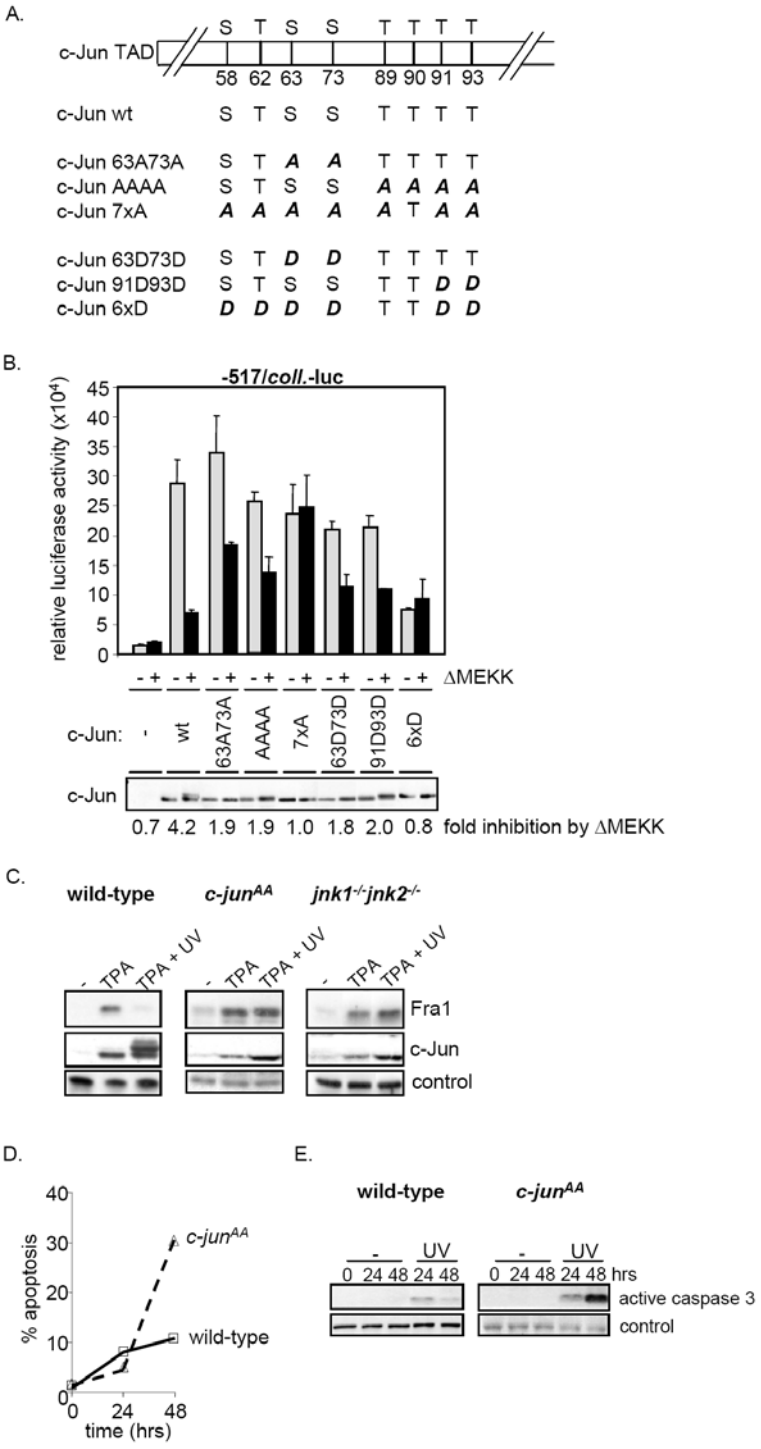

Figure 5. N-terminal phosphorylation of c-Jun is required for inhibition of collagenase and Fra1 expression by stress-stimuli

(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/m^2 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/m^2 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 ΔMEKK. 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/m^2 UV-C) in human and mouse fibroblasts, and not at doses above 30 J/m^2 (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.

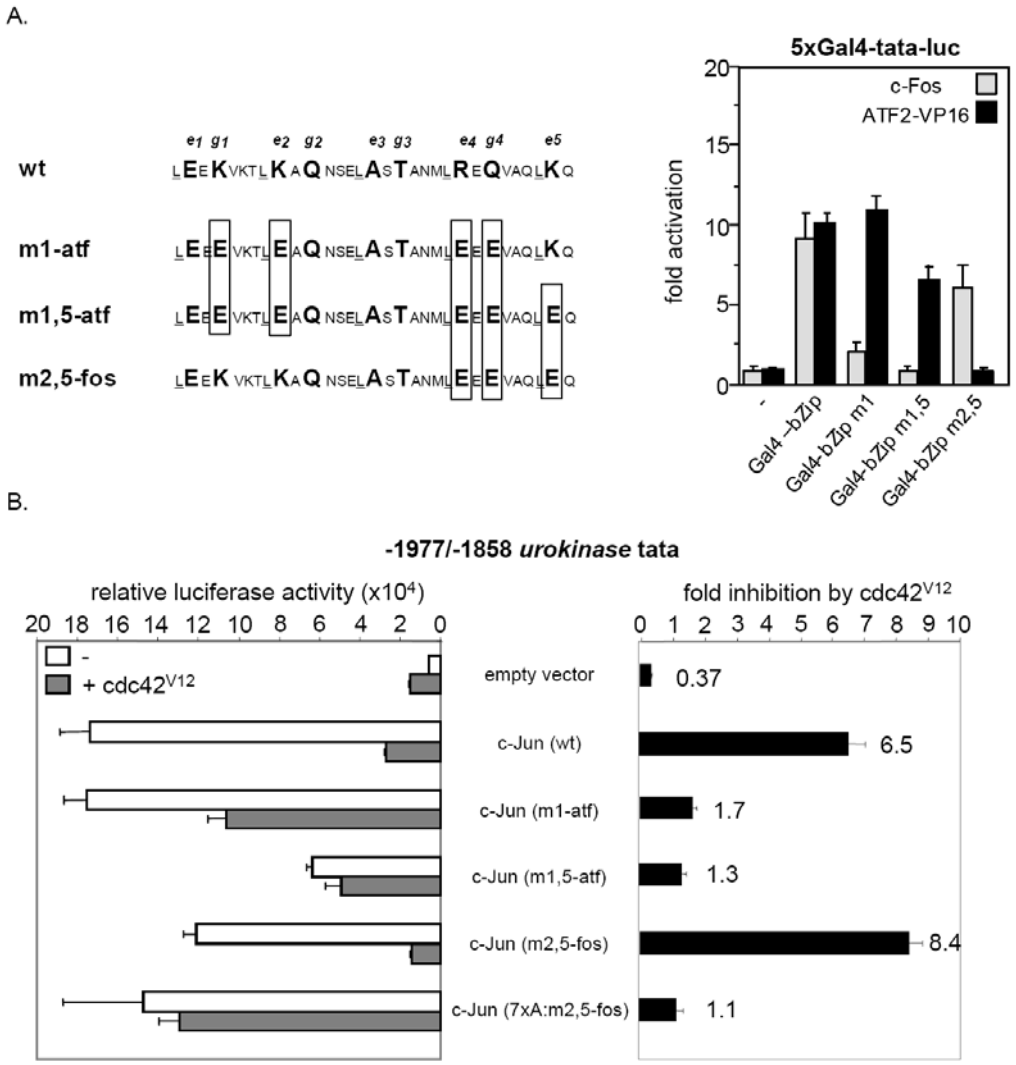

Figure 6. N-terminal c-Jun-phosphorylation differently affects transactivation by c-Jun/Fos and c-Jun/ATF-2

(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-repressable *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).

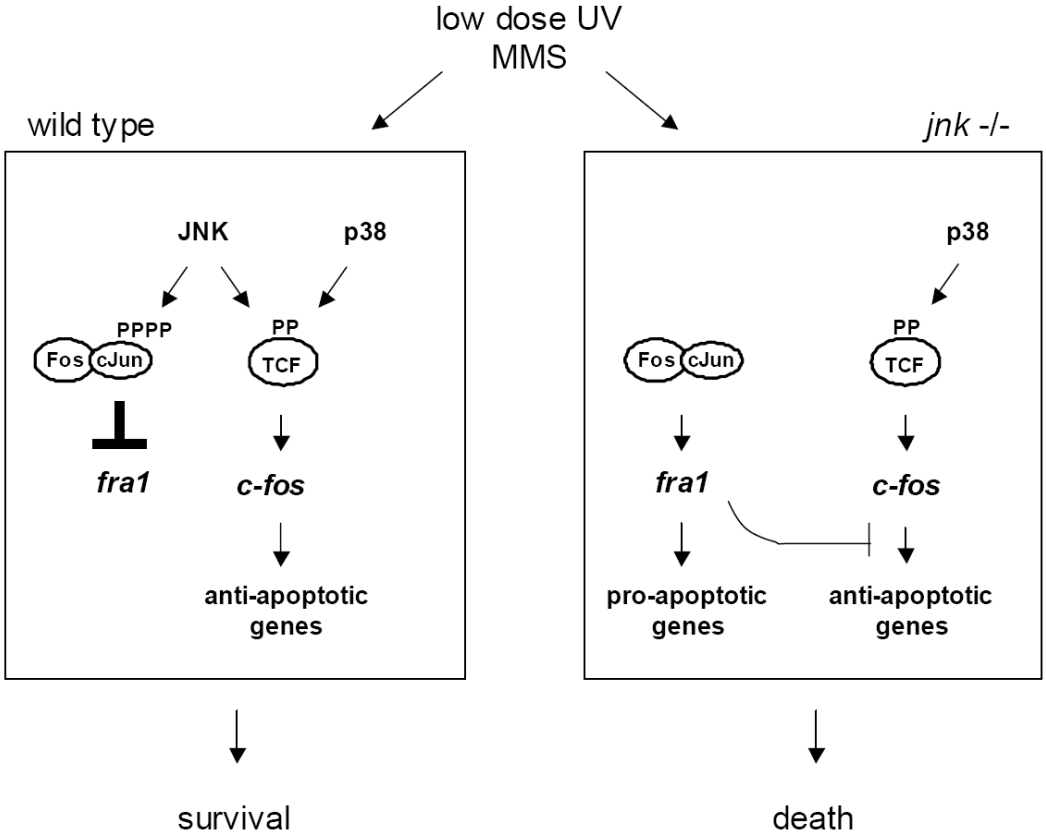

Figure 7. Proposed model for the role of JNK, phospho-c-Jun and Fra1 in the cellular response to low levels of DNA damage
In wild-type fibroblasts (left box), UV and MMS inhibit the growth factor-dependent expression of *fra1* through phosphorylation of c-Jun by JNK. The expression of *c-fos* is induced 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 hyper-phosphorylation 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 hyper-phosphorylation 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 μ g/ml RNAse A, and analysed by flow cytometry (FACS-Calibur, Becton Dickenson). 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 plasimids 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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