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AP-1 mediated retinal photoreceptor apoptosis is independent of N-terminal phosphorylation of c-Jun

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Abstract

Apoptosis is essential for retinal development but it is also a major mode of cell loss in many human retinal dystrophies. High levels of visible light induce retinal apoptosis in mice and rats. This process is dependent on the induction of the transcription factor AP-1, a dimeric complex composed of c-Fos and c-Jun/JunD phosphoproteins. While c-Fos is essential, JunD is dispensable for light-induced photoreceptor apoptosis. Here we show that N-terminal phosphorylation of c-Jun, the other main partner of c-Fos in induced AP-1 complexes is not required for programmed cell death during retinal development *in vivo* and is also dispensable for photoreceptor apoptosis induced by the exogenous stimuli 'excessive light' and *N*-nitroso-*N*-methylurea (MNU). Mice expressing a mutant c-Jun protein (JunAA) that cannot be phosphorylated at its N-terminus are apoptosis competent and their retina is not distinguishable from wild-type mice. Accordingly, Jun kinase, responsible for phosphorylation of wild-type c-Jun protein is at best only marginally induced by the apoptotic stimuli 'light' and MNU. Complex composition of light-induced AP-1 complexes is similar in wild-type and JunAA mice. This shows that the mutant c-Jun protein can be part of the DNA binding complex AP-1 and demonstrates that induction of the DNA binding activity of AP-1 after light insult does not depend on N-terminal phosphorylation of c-Jun. Our results suggest that transactivation of target genes by phosphorylated c-jun/AP-1 is not required for MNU- or light-induced apoptosis of photoreceptor cells. *Cell Death and Differentiation* (2001) **8,** 859–867.

Keywords: Apoptosis; AP-1; c-Jun; photoreceptor; phosphorylation; JunAA; light-induced degeneration

Abbreviations: AP-1, activator protein-1; ONL, outer nuclear layer; ROS, rod outer segment; RIS, rod inner segment; PE, pigment epithelium; JNK, Jun kinase; TUNEL, Terminal transferase-mediated dUTP nick end labeling; JNP, c-Jun N-terminal phosphorylation; MNU, *N*-methyl-*N*-nitrosourea

Introduction

Apoptosis is an essential process during development and differentiation. It serves a variety of specialized functions in tissue homeostasis or remodeling. Apoptosis removes unwanted cells which may be injured, misplaced or surplus in development and in mature tissues. This naturally occurring cell death is prominent in neurons of the retina.[1] If untimely activated or suppressed, apoptosis can cause severe diseases such as neurodegenerations, diseases of the bone marrow, cancer, virus infections or autoimmune diseases.[2,3] In inherited retinal degeneration in humans, apoptosis is considered the final common pathway of cell death leading to retinal degeneration and loss of vision.[4] The degenerative process is accelerated by light in many animal models representing the respective human condition[5,6] and light has been proposed to enhance progression and severity of major human retinal diseases such as age-related macular degeneration (AMD) and possibly some forms of retinitis pigmentosa (RP).[7–10] In these diseases retinal cells die by apoptosis.[11,12] Light not only enhances disease progression in inheriditary retinal degeneration but at overdose also induces apoptotic cell death in photoreceptors of vertebrates like mice and rats.[4,13–16] The light stimulus is absorbed by the visual pigment rhodopsin and converted into an intracellular death signal.[17] In mice, this process depends on the activation of the transcription factor AP-1[18,19] (and Wenzel *et al*,[56]). AP-1 is a dimeric complex consisting either of heterodimers of the Fos (c-Fos, FosB, Fra-1, Fra-2) and Jun (c-Jun, JunD, JunB) family of proteins or of homodimers of the Jun family of proteins.[20] The complex composition determines the biological function of AP-1 and different complexes may modulate expression of specific sets of target genes. AP-1 complexes containing c-Fos, for example, exhibit the strongest transactivation activity.[20] Activation of AP-1 has been implicated in apoptotic cell death in several systems with special emphasis on c-Jun.[21,22] In light-induced apoptosis of photoreceptors, induced AP-1 complexes mainly consist of c-Fos, JunD and c-Jun proteins.[18,19] Whereas JunD is dispensable,[23] c-Fos is essential for induction and/or execution of light-induced photoreceptor apoptosis.[23,24] Here, we focus on the analysis of c-Jun, the third member of induced AP-1 complexes. AP-1 mediated control and transactivation of target genes are at least partially regulated through N-terminal phosphorylation of c-Jun at serines 63 and 73 through Jun N-terminal kinases (JNKs).[25,26] Although mice lacking c-Jun are not viable and die at mid-gestation,[27,28] transgenic mice expressing only a mutant form of c-Jun that can not be phosphorylated at its N-terminus (JunAA) are viable and fertile.[29] In JunAA, the phosphorylation sites required for transactivation (serines 63 and 73) have been replaced by non-phosphorylatable alanines. JunAA mice are resistant to kainate induced

neuronal apoptosis and to epileptic seizures.[29] We used these transgenic animals to test whether c-Jun activation by N-terminal protein kinases would be involved in light-induced degeneration of retinal neuronal cells like photoreceptors. Mutant and wild-type mice were exposed to high intensities of white light and retinal morphology was analyzed at different timepoints after the end of illumination. Levels of activated JNK were determined, Jun kinase activity was tested and complex composition of induced AP-1 was analyzed. c-Jun N-terminal phosphorylation (JNP) plays at best a minor, non-essential role in light-induced photoreceptor apoptosis since retinal degeneration in transgenic JunAA mice was induced and proceeded similar as in wild-type mice. Furthermore, Jun kinase activity did not, or only marginally, increase after light exposure, and levels of phosphorylated (activated) JNK/SAPK did not increase after the application of the pro-apoptotic stimulus. JunAA mice were also sensitive to a chemical pro-apoptotic stimulus (*N*-methyl-*N*-nitrosourea, MNU) which also did not induce JNK over basal levels. In addition, DNA binding of AP-1 complexes was induced after light exposure to similar degrees in JunAA and wild-type mice and composition of induced complexes was identical in mice of both genotypes. This suggests that induction of AP-1 during light-induced photoreceptor apoptosis is not regulated through JNK-mediated N-terminal phosphorylation of c-Jun protein.

Results

Light-triggered photoreceptor apoptosis in the absence of c-Jun N-terminal phosphorylation

c-Jun N-terminal phosphorylation (JNP) is not essential during mouse development. Transgenic mice expressing a mutant c-Jun protein that cannot be phosphorylated at its N-terminus by JNK/SAPK are phenotypically normal and show no obvious histological abnormalities.[29] Similarly, retinas of 4–6-week-old JunAA mice were indistinguishable from control wild-type mice in their morphological appearance (Figure 1A,D). Sequence analysis of PCR products amplified from retinal cDNA with c-*jun* specific primers showed that the mice exclusively expressed a mutant c-Jun protein containing base pair triplets encoding the amino acid alanine instead of serine at positions 63 and 73 of the protein (data not shown). Twenty-four hours after a 2 h exposure to 15 000 lux of white light, retinas of both wild-type (Figure 1E) and JunAA (Figure 1B) mice showed characteristic signs of retinal degeneration. The retinal pigment epithelium (PE) was swollen, rod outer segments (ROS) and rod inner segments (RIS) were disorganized and many photoreceptor nuclei in the outer nuclear layer (ONL) had condensed chromatin. In addition, the photoreceptor layer had thinned and some photoreceptor cells had already been eliminated. Ten days after light exposure, the ONL of both wild-type (Figure 1F) and JunAA

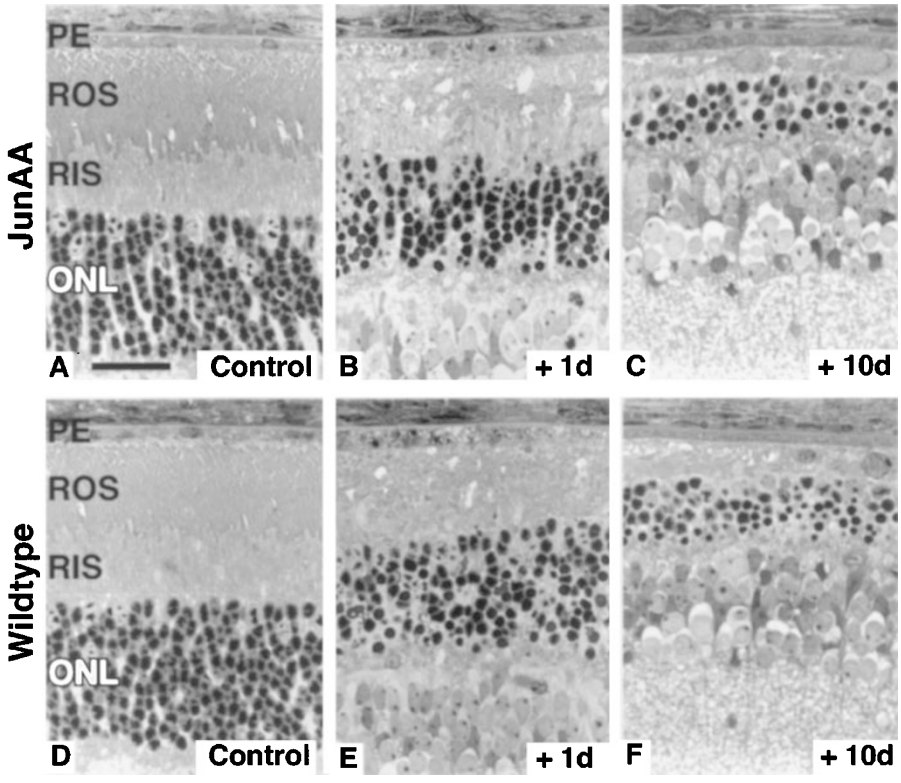

Figure 1 N-terminal phosphorylation of c-Jun is not essential for light-induced retinal degeneration. Morphology of the central inferior retinas of JunAA (**A–C**) or wild-type (**D–F**) mice was analyzed by light microscopy. (**A**, **D**) Retinas of control mice not exposed to light. (**B**, **E**) Retinas of mice 1 day after exposure to 15 000 lux of white light for 2 h. ROS and RIS are disorganized and disrupted. Many nuclei of the ONL are pycnotic indicating ongoing apoptosis. (**C**, **F**) Retinas of mice 10 days after exposure to 15 000 lux of white light for 2 h. Most cells have died and have been cleared from the ONL. Representative sections of 2–4 experiments are shown. PE: pigment epithelium. ROS: rod outer segment. RIS: rod inner segment. ONL: outer nuclear layer. Scale bar: 25 μm

(Figure 1C) mice was reduced to approximately four rows of nuclei. Most of the remaining nuclei were pycnotic as evidenced by the condensed chromatin. ROS and RIS were not distinguishable any more and were drastically reduced in size.

Two classical markers of apoptotic cell death are positive TUNEL staining of cell nuclei and internucleosomal fragmentation of genomic DNA leading to the characteristic DNA ladder formation after gel electrophoresis. Photoreceptor nuclei of both transgenic JunAA and wild-type mice stained positive by the TUNEL method (Figure 2A, B), whereas a control retina of unexposed wild-type mice did not show staining (Figure 2C). Furthermore, genomic DNA of both types of light exposed mice was cleaved internucleosomally as demonstrated by the DNA laddering shown in Figure 2D. Thus, although the presence of c-Fos[24] and the induction of AP-1 (Wenzel *et al*,[56]) is essential for light-induced photoreceptor apoptosis, N-terminal phosphorylation of c-Jun, a member of the light-induced AP-1 complexes[18] (Figure 5) is not required to start or execute this process.

Jun kinase activity and AP-1 complex composition after light exposure

Phosphorylation of Ser63 and Ser73 in wild-type c-Jun protein increases the transactivation ability of AP-1 and is mediated by Jun kinases (JNK/SAPK), members of the family of mitogen-activated protein kinases (MAPKs).[30] We tested JNK/SAPK activity in retinal extracts of wild-type mice before, and at 2 h after exposure to 120 min of 15 000 lux of white light, a timepoint when AP-1 DNA binding activity was already strongly induced but did not yet reach maximum levels[19] (see also Figure 4). JNK activity was judged by the ability of retinal extracts to phosphorylate a c-Jun-GST fusion peptide containing amino acids 1–79 of c-Jun protein. As shown in Figure 3A, light exposure did not significantly activate JNK/SAPK activity. In control experiments, we showed that our experimental conditions were not saturating: Increasing amounts of retinal extracts were incubated with the c-Jun-GST substrate peptide and the resulting signal intensities correlated well with the amount of extracts used (Figure 3B). As for wild-type mice, light did not induce JNK activity in JunAA mice (Figure 3C). Furthermore, light did not superinduce phosphorylation of JNK/SAPK over basal activity: levels of phospho–JNK/SAPK after light exposure remained similar to the levels before lights on (controls) in both wild-type and JunAA mice (Figure 3D). JunAA mice also did not down- or upregulate the levels of total JNK/SAPK protein, as shown by the immunodetection with an antibody recognizing both the phosphorylated and non-phosphorylated forms of JNK/SAPK (Figure 3D, lower panel). In contrast, treatment with UV light did induce phosphorylation in control 293 cells. Although the basal levels of phospho–JNK/SAPK were already quite high in the retina, a superinduction might still have been possible, especially of the p54 form (compare ratios of the p54 and p46 forms in lanes 1–4 with the ratio of the two forms in lane 6; Figure 3D, upper panel).

In both wild-type and JunAA mice, light exposure induced AP-1 DNA binding with similar kinetics. At 2 h (Figure 4A, lane 2 and B lane 2) and especially at 6 h (Figure 4A, lane 3) and B lane 3) after a 2 h illumination, AP-1 activity was dramatically elevated as compared to unexposed controls (Figure 4A, lane 1 and B lane 1). Composition of induced complexes at 6 h after illumination was tested in supershift experiments using antibodies against c-Fos, JunD, c-Jun and JunB and an unrelated antibody as control. Ap-1 composition was identical in wild-type and JunAA mice. Induced complexes were mainly composed of c-Fos, JunD and c-Jun as evidenced by the formation of supershifts (* and ** in Figure 4) and/or the competition for the formation of AP-1/DNA complexes (in the case of α-JunD antibodies).

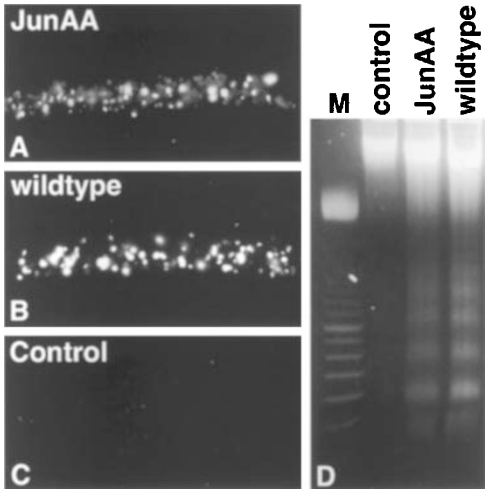

Figure 2 Apoptotic cell death of photoreceptors in the absence of N-terminal phosphorylation of c-Jun. JunAA or wild-type mice were exposed to 15 000 lux of white light for 2 h or kept in darkness. After a recovery period of 24 h in darkness, retinas were prepared for TUNEL staining (**A–C**) or for isolation and electrophoretic analysis of genomic DNA (**C**). (**A**) TUNEL positive photoreceptor nuclei in the ONL of light-exposed JunAA mice. (**B**) TUNEL positive photoreceptor nuclei in the ONL of light-exposed wild-type mice. (**C**) No TUNEL positive cells in unexposed control mice. Representative sections of 2–4 experiments are shown. (**D**) Internucleosomal DNA fragmentation of retinal DNA in JunAA and wild-type mice 24 h after the end of light exposure. Control: genomic DNA of unexposed mice

JNP after MNU treatment

MNU is a chemical agent that rapidly induces apoptotic cell death via methylation of genomic DNA[31,32] in a dose dependent way.[19,33,34] We used MNU to analyze the role of JNP during photoreceptor apoptosis induced by a light independent stimulus. A single intraperitoneal injection of MNU (50 mg/kg bodyweight) induced photoreceptor cell death in both wild-type and JunAA mice (Figure 5A–D). Twenty-four hours after injection, ROS and RIS were disorganized and the ONL contained many photoreceptor nuclei with condensed chromatin indicating ongoing apoptosis. However, MNU did not superinduce phosphorylation of JNK/SAPK over basal levels as judged by the amounts of phospho–JNK/SAPK in the retinas of salt or MNU injected wild-type (Figure 6A, lanes 1–6) and JunAA mice (Figure 6A, lanes 9 and 10). Extracts of untreated or UV treated 293 cells

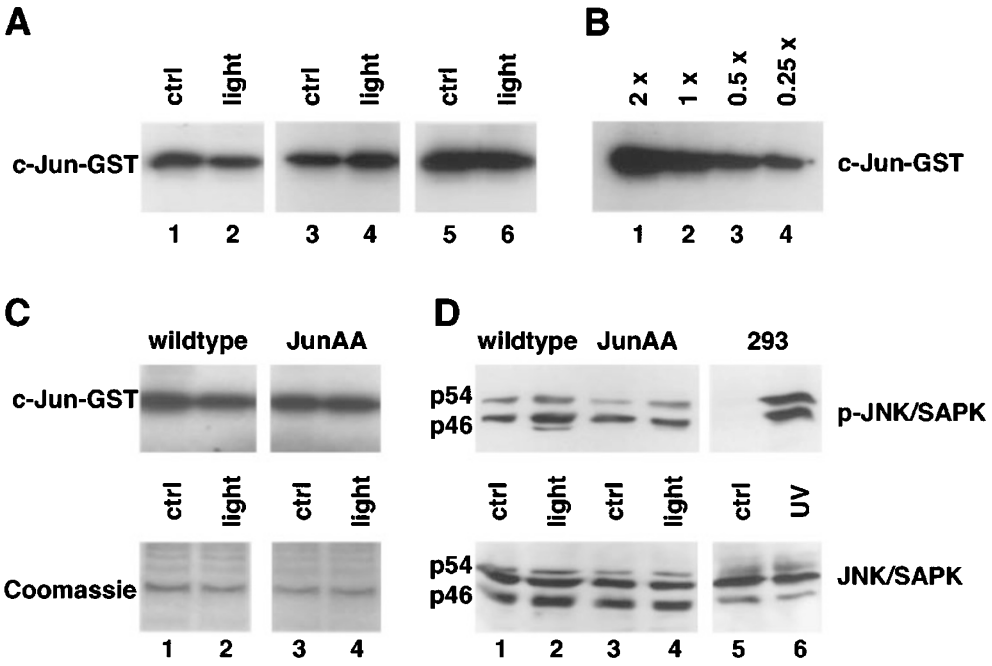

Figure 3 JNK/SAPK in retinal extracts. (**A**) Wild-type mice were exposed to 15 000 lux of white light for 2 h and sacrificed after a recovery period of 2 h in darkness (lanes 2, 4 and 6; 'light'). Controls were kept in darkness at all times during the experiment (lines 1, 3 and 5; 'ctrl'). 0.5 retina equivalents were incubated with c-Jun-GST as substrate for JNK/SAPK in the presence of $\gamma[^{32}P]$ATP. Reactions were resolved by polyacrylamide gel electrophoresis and exposed to X-ray film. Results of three independent experiments are shown (**B**) Control incubations with decreasing amounts of retinal extracts (two retina equivalents, lane 1: one retina equivalent, lane 2; 0.5 retina equivalents, lane 3; 0.25 retina equivalents, lane 4). (**C**) Wild-type (lanes 1 and 2) and JunAA (lanes 3 and 4) mice were kept in darkness (lanes 1 and 3; 'ctrl') or were exposed to 15 000 lux of white light for 2 h and sacrificed after a recovery period of 2 h in darkness (lanes 2 and 4; 'light'). 0.5 retina equivalents were incubated with c-Jun-GST as substrate for JNK as above. As control for quality and quantity of extracts used for the kinase assay, supernatants after the JNK assay were run on a 10% denaturing polyacrylamide gel and proteins stained with Coomassie blue (bottom panel). Representative panels of two experiments are shown (**D**) 20 μg of retinal extracts (lanes 1–4) used for the JNK assays in (**C**) or 15 μg of control extracts from untreated (lane 5) or UV treated (lane 6) human kidney 293 cells were resolved on 10% denaturing polyacrylamide gels and blotted on nitrocellulose membrane for Western blotting. Antibodies specific for phosphorylated (active) JNK/SAPK (top panel) or for all forms of JNK/SAPK (bottom panel) were used to detect total or active JNK/SAPK. Representative gels of 2–4 experiments are shown

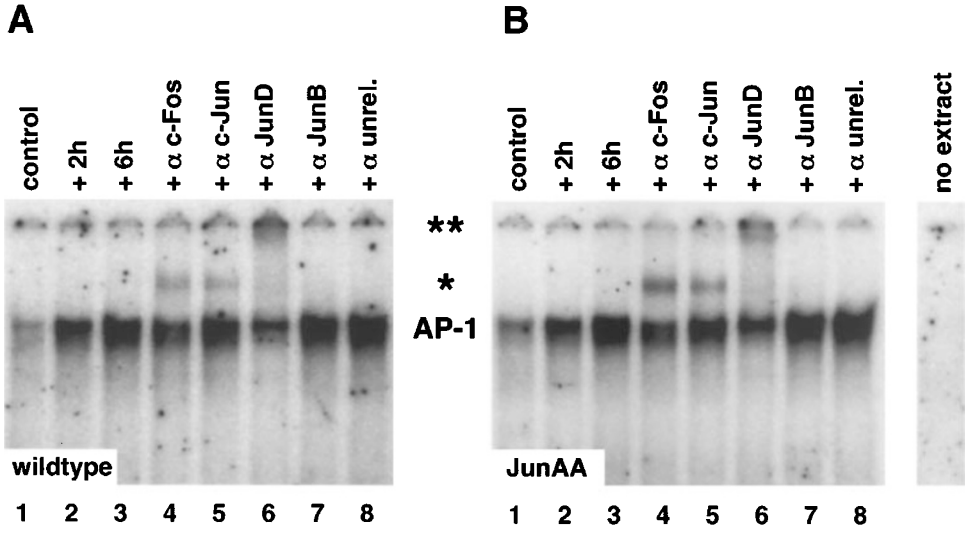

Figure 4 AP-1 induction and complex composition. Wild-type (**A**) or JunAA (**B**) mice were kept in darkness (lane 1; 'control') or were exposed to 15 000 lux of white light for 2 h and analyzed after a recovery period of 2 h (lane 2; '+ 2 h') or of 6 h (lanes 3–8; '+ 6 h'). Retinal nuclear extracts were incubated with ^{32}P-labeled oligonucleotide presenting an AP-1 binding site in the absence (lanes 1–3) or presence (lanes 4–8) of antibodies specific for proteins of the Fos or Jun families of proteins or for an unrelated protein as indicated. Complexes were resolved by polyacrylamide gel electrophoresis. Positions of AP-1/DNA complexes ('AP-1') and of complexes supershifted by antibodies (* and **) are indicated. (**) also indicates start of gel. Control incubation without nuclear extract did not produce a signal. Representative panels of two experiments are shown

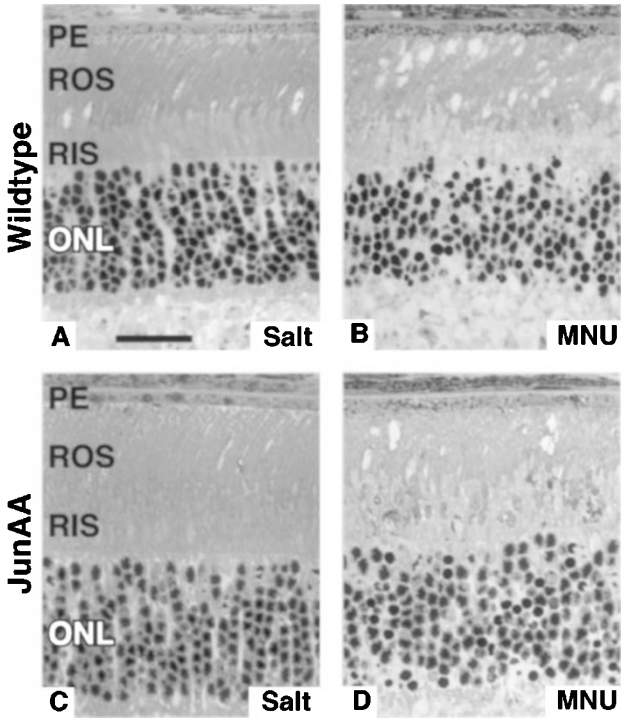

Figure 5 MNU-induced photoreceptor apoptosis in the absence of JNP. Wild-type (**A, B**) or JunAA (**C, D**) mice were intraperitoneally given a single dose of salt (0.9%; **A**, **C**) or MNU (1 mg/ml in 0.9% NaCl; 50 mg/kg bodyweight; **B, D**). After a recovery period of 24 h, mice were sacrificed and eyes prepared for morphological analysis. MNU induced photoreceptor cell death in both wild-type and JunAA mice as indicated by the nuclei with condensed chromatin in the ONL and the disorganization and vesiculations of ROS and RIS. Representative panels of two experiments are shown. Scale bar: 25 μm. Abbreviations as in Figure 1

served as controls for induction of JNK phosphorylation by an exogenous stimulus (Figure 6A, B, lanes 7 and 8). MNU treatment did not alter levels of total JNK/SAPK in wild-type or JunAA mice (Figure 6B). This strongly suggests that JNP – as for light induced cell death – is not important for apoptosis induction by MNU.

Discussion

Exposure to excessive levels of visible light induces DNA binding activity of AP-1 in the retina.[18,19] Induced complexes contain mainly c-Fos, JunD and c-Jun.[18] AP-1 induction and complex composition are not dependent on JNP as assayed in transgenic mice expressing a mutant, non phosphorylatable c-Jun protein (JunAA) instead of the wild-type protein. In fact, the applied apoptotic stimulus did not induce JNK/SAPK, the kinases responsible for JNP. Furthermore, JunAA mice show normal sensitivity for light damage, suggesting that JNP is not required for execution of photoreceptor apoptosis induced by light. Similarly, JNP is also not important for apoptosis induction by the DNA methylating agens MNU.

xThe activities of newly formed or pre-existing AP-1 components are modulated mainly through phosphorylation reactions at several sites in the protein. Phosphorylation of a cluster of amino acids located close to the basic region in c-Jun for example influences DNA binding.[35,36] Phosphorylation at N-terminal serines at positions 63 and 73, which are located within the transactivation domain increases the ability of c-Jun/AP-1 homodimers or heterodimers (with c-Fos) to activate gene expression of AP-1 target genes.[37,38] The responsible enzymes for this type of AP-1 activation have been identified in the so called c-Jun (JNK) or stress-activated protein kinases (SPAK) (reviewed in[30,39]). JNK/SAPK signaling and thus JNP has been implicated in cellular stress response and apoptosis.[39] c-Jun itself has been found to have dual roles in apoptosis and to be important on one hand for the defense against apoptotic stimuli and on the other hand for the execution of apoptosis: livers of c-Jun knock-out mice show increased apoptosis of hepatoblasts and hematopoietic cells and primary c-Jun-mutant hepatocytes have a lower survival rate *in vitro*.[28,40] On the other hand, transgenic mice expressing the JunAA protein are resistant against kainate induced neuronal apoptosis.[29] Thus, c-Jun can have critical roles in opposite cellular processes. Little is known about the role of c-Jun in the retina. In a xenograft approach, Herzog and coworkers showed that the protein might be dispensable for developmental cell death in the retina.[41] The morphologically normal appearance of the retinas in JunAA mice supports this conclusion and shows that at least the N-terminal phosphorylation of c-Jun is dispensable for normal retinal development in mice *in vivo*. This is further supported by our observation of equal levels of the visual pigment rhodopsin in photoreceptors of transgenic (0.54 nmol per eye) and wild-type mice (0.52 nmol per eye). In contrast, c-Fos, another member of AP-1 complexes influences normal retinal development: c-*fos*$^{-/-}$ mice have 20–25% less rhodopsin and about 20% fewer rod photoreceptors than their wild-type counterparts.[42] Nevertheless, c-Jun has been found to be transiently expressed around post natal day P11 in retinal glial cells during the degenerative process in *rd* (retinal degeneration) mice[43] and c-*jun* gene expression is induced during retinal ischemia/reperfusion.[44] This suggests a role for c-Jun in disease processes in both the developing and adult retina.

High levels of light induce photoreceptor apoptosis that involves upregulation of the DNA binding activity of AP-1.[18,19] Similarly, light exposure upregulates expression of c-*fos* and c-*jun* genes in the retina.[45] Upregulation of AP-1 is essential since suppression of AP-1 activity by the activation of glucocorticoid receptor protects against light damage (Wenzel *et al*,[56]). In contrast, the same treatment did not protect against photoreceptor apoptosis induced by MNU (Wenzel *et al*,[56]). Although MNU treatment induces AP-1 DNA binding activity in the retina with a timecourse similar to light exposure,[19] this result suggests that AP-1 activity may not be essential for MNU induced retinal apoptosis. This conclusion was supported by our data showing that cell death induced by MNU in JunAA mice was comparable to wild-type mice and by experiments showing that lack of c-Fos did not prevent cell death although it prevented AP-1 activation by MNU.[19] Evidence from other laboratories even suggests that AP-1 might be

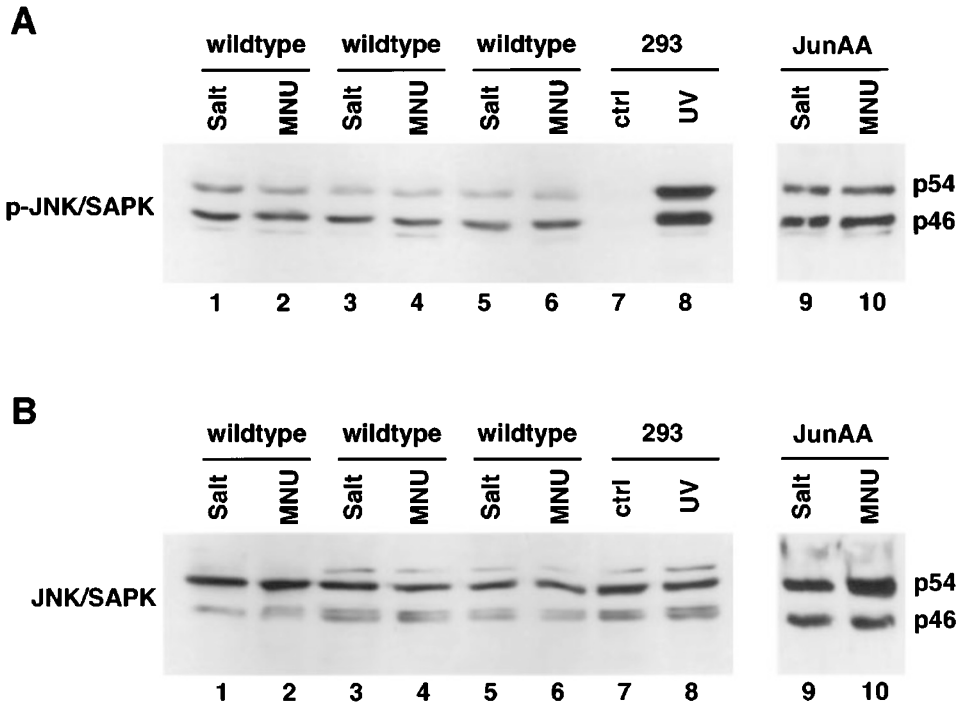

Figure 6 Lack of induction of JNK/SAPK phosphorylation by MNU were. (**A**) Cell extracts prepared from wild-type (lanes 1–6) and JunAA (lanes 9 and 10) mice 2 h after injection of salt (0.9%, lanes 1, 3, 5 and 9) or MNU (50 mg/kg bodyweight, lanes 2, 4, 6 and 10) were tested for the presence of phosphorylated (active) JNK/SAPK by Western blotting as described in legend to Figure 3. Extracts (15 μg) from untreated (lane 7) or UV treated (lane 8) human kidney 293 cells served as controls. (**B**) Extracts were tested for the presence of total JNK/SAPK. Three independent experiments with wild-type and one representative (out of two) experiment with JunAA mice are shown

involved in the defense against MNU and that lack of correct AP-1 activity may even facilitate MNU-induced photoreceptor apoptosis.[46,47]

Light-induced AP-1 complexes are composed mainly of c-Fos, JunD and c-Jun.[18,19] Of these, c-Fos is essential,[24] but JunD is dispensable[23] for the induction and execution of light-triggered apoptosis. Our experiments with the third main member of induced AP-1 complexes, c-Jun, showed that JNP was also not essential for the apoptotic process. Furthermore, neither JNK/SAPK activity nor phosphorylation (activation) of JNK/SAPK increased after light exposure over basal levels (Figure 3). The presence of rather high levels of activated JNK/SAPK in the retina suggests that JNK/SAPK may be required for other functions in the retina. Targets of JNK are mostly transcription factors and include activating transcription factor (ATF-2),[48] and ETS-containing factors such as Elk1[49] besides c-Jun. Other proteins with functions that are regulated by JNK-mediated phosphorylation include insulin receptor substrate 1[50] and Bcl-2.[51] JNK seems also to be required for the stress-activated mitochondrial death pathway and the absence of JNK prevents cytochrome *c* release in mouse fibroblasts.[52]

It is important to note that light-induced AP-1 complexes in JunAA mice contained c-Jun protein (Figure 4). Thus, the mutant form of c-Jun (JunAA) was not replaced by a redundant member of the Jun family of proteins in induced AP-1 complexes. Thus, our data show that light exposure induced AP-1 complexes containing c-Jun protein, but not the signaling cascade needed for an increased transactivation of AP-1 target genes. Therefore, transactivation of AP-1 target genes may not be the primary role of light-induced AP-1 complexes. This interpretation is supported by a recent report showing that Fra-1, another member of the Fos family of proteins can functionally substitute for c-Fos.[53] In contrast to c-Fos, however, Fra-1 does not have a known transactivation domain.[54] Our results suggest that not AP-1 mediated activation but rather AP-1 dependent repression of target genes might be of importance during light-induced photoreceptor apoptosis. Future experiments are designed to test this hypothesis.>

Materials and Methods

Animals

All experiments conformed to the ARVO statement for care and use of animals in research and to the guidelines of the Veterinary Authority of Zurich. Wild-type or JunAA mice (genetic background: 129SV/BP6) were raised in dim cyclic light (12 : 12 h; 60 lux at cage level). Mice were kept for at least 10 days under these conditions before used in the experiments.

Light exposure and retinal morphology

Mice were dark adapted overnight (16 h) and their pupils were dilated with 1% Cyclogyl and 5% Phenylephrine 45 min before exposure to diffuse, white fluorescent light (TLD36 W/965 tubes, Philips; ultraviolet-impermeable diffuser) in cages with reflective interior.

After light exposure, mice were either kept in darkness until retinal morphology was analyzed or until retinas were isolated for extract preparation. For morphological analysis of retinal tissue, enucleated eyes were fixed in 2.5% glutaraldehyde and embedded in Epon 812.[19]

TUNEL staining and DNA ladder formation

Terminal transferase-mediated dUTP nick end labeling was done essentially as described.[19] Briefly, retinal tissue was fixed in 2% paraformaldehyde for 2 h at 4°C and embedded in paraffin. The '*in situ* cell death detection kit' (Roche diagnostics, Rotkreuz, Switzerland) was used with minor modifications to perform staining on 0.5 μm sections. Genomic DNA was prepared from isolated retinas by phenol-chloroform-isoamylalcohol extraction as described.[19] Twenty μg of total DNA was separated on 1.5% agarose gels and stained with ethidium bromide.

Amplification and sequencing of c-jun

Retina preparations, RNA isolations and cDNA synthesis were done as described.[17,45] PCR amplification of c-*jun* was done on cDNA representing 12 ng of total retinal RNA using the upstream primer 5′-GAT CCT AAA ACA GAG CAT GAC-3′ and the downstream primer 5′-GAA GTT GCT GAG GTT GGC G-3′. Amplification was for 35 cycles at 94°C (30 s, denaturation), 55°C (45 s, annealing) and 72°C (45 s, extension). Products were purified using QIAquick PCR purification kit (Qiagene, Hilden, Germany) and sequenced by Microsynth (Microsynth, Balgach, Switzerland).

Jun kinase assay

Retinas were isolated through a slit in the cornea and immediately frozen in liquid nitrogen. For extract preparation, two retinas from separate mice were pooled and homogenized in 250 μl JEB (25 mM HEPES pH 7.7, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 20 mM beta-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM PMSF, 10 μg/ml of aprotinin and leupeptin). Extracts were centrifuged at 23 000 $\times$ *g* for 15 min at 4°C, supernatants were collected and 250 μl of buffer JEB was added. One hundred and twenty-five μl of 20 mM HEPES (pH 7.7) / 2 mM $MgCl_2$ was added to 125 μl of extract (0.5 retina equivalents), incubated for 10 min on ice and centrifuged for 10 min at 23 000 $\times$ *g* at 4°C. The cleared lysate was mixed with 5 μg c-Jun(1–79)-GST agarose (StressGen, Victoria, Canada) and rotated slowly for 4 h at 4°C. The mixture was centrifuged for 10 s, the supernatant saved for control electrophoresis (see below) and the pellet washed four times with 500 μl of HBIB (20 mM HEPES pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Triton X-100). Pellet was resuspended in 30 μl of JNK buffer (20 mM HEPES pH 7.7, 20 mM $MgCl_2$, 20 mM beta-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM Na_3VO_4, 2 mM DTT), 20 μM ATP and 10 μCi ^{32}P-γ ATP and incubated for 40 min at 30°C. The mixture was centrifuged for 10 s and the pellet washed once with 500 μl of HBIB. The pellet was resuspended in 40 μl sample buffer (62 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.002% bromphenol blue), incubated 5 min at 95°C, centrifuged and the supernatant was run on a 15% SDS-polyacrylamide gel. The dried gel was exposed to X-ray film.

Electrophoresis of the supernatants after the incubation with the c-Jun(1–79)-GST substrate was done as control for quality and quantity of the extracts used. Fifty μl of the extracts were mixed with SDS loading buffer and proteins resolved on 10% polyacrylamide SDS gels. Gels were fixed for 10 min with 80% methanol / 10% acetic acid, stained for 1 h with Coomassie Blue and destained for 16 h with 80% methanol / 10% acetic acid.

MNU injections

MNU (*N*-methyl-*N*-nitrosourea) was freshly dissolved in 0.9% of sterile NaCl at a concentration of 10 mg/ml. Mice were intraperitoneally injected with 50 mg/kg MNU and sacrificed 2 h (for Western blotting) or 24 h (morphology) thereafter. Eyes were prepared for morphological analysis as described above or retinas were removed through a slit in the cornea and stored at -70°C until use.

Western blotting

Protein extracts from retinas were prepared in JEB as described above. Protein concentrations were determined by the Bradford (Bio-Rad, Hercules, USA) assay. Twenty μg total protein were resolved on 10% SDS-polacrylamide gels and blotted onto nitrocellulose membrane with standard methods. Total JNK/SAPK or active (phosphorylated) JNK/SAPK were detected using specific antibodies (#9251 for p-JNK/SAPK; #9252 for total JNK/SAPK; antibodies from New England Bioloabs, Beverly, MA, USA). Detection was with a secondary, HRP-conjugated antibody against rabbit IgG (#sc-2004; SantaCruz Biotechnology, Santa Cruz, USA) and proteins visualized using the Renaissance Western Blot Detection Kit (PerkinElmer Life Sciences, Emeryville, USA). As controls served cell homogenates from untreated and UV-treated 293 cells from human kidney (#9253; New England Biolabs, Beverly, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously.[18] Briefly, one retina was homogenized in 400 μl of 10 mM HEPES-KOH pH 7.9, 1 mM β-mercapto-ethanol, 1 mM DTT in the presence of protease inhibitors. After incubation on ice for 10 min the homogenate was vortexed for 10 s and centrifuged. The pellet was resuspended in 50 μl of 20 mM HEPES-KOH pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM β-mercapto-ethanol, 1 mM DTT in the presence of protease inhibitors and incubated on ice for 10 min. Cellular debris was removed by centrifugation at 23 000 $\times$ *g* for 30 min at 4°C. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, USA) with BSA as standard.

EMSAs were performed as described previously.[55] Briefly, two oligonucleotides were annealed to form a double stranded DNA coding for an AP-1 specific (5′-AAG CAT GAG TCA GAC AC-3′) DNA binding sequence (TPA response element, TRE). The annealed oligos were end-labeled using polynucleotide kinase (Boehringer Mannheim, Germany) and γ[^{32}P]ATP (Hartmann Analytic GmbH, Braunschweig, Germany). For EMSA, 2–5 μg (5 μl) protein of nuclear extract were incubated on ice for 20 min with 19 μl of 5 mM $MgCl_2$, 0.1 mM EDTA, 0.75 mM DTT, 7.5% glycerol, 0.05% NP-40 containing 24 μg BSA and 2 μg poly d (I-C) (Boehringer Mannheim, Germany). Radiolabeled oligonucleotide (1 μl) was added and incubation was continued for another 20 min. Where appropriate, 3 μl antibodies (α-c-Fos: sc052; α-c-Jun: sc045; α-JunD: sc074; α-JunB: sc046; α-EPO (unrelated antibody): sc7956; all antibodies from Santa Cruz Biotechnology, Santa Cruz, USA) were added and incubation continued for 50 min on ice. Protein/DNA complexes were resolved at 150 V on a 6% native polyacrylamide gel using 0.25 $\times$ TBE as running buffer and visualized on X-ray film.

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References

1. Young RW (1984) Cell death during differentiation of the retina in the mouse. J. Comp. Neurol. 229: 362 – 373
2. Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456 – 1462
3. Gorman AM, McGowan A, Oneill C and Cotter T (1996) Oxidative Stress and Apoptosis In Neurodegeneration. J. Neurol. Sci. 139: 45 – 52
4. Reme CE, Grimm C, Hafezi F, Marti A and Wenzel A (1998) Apoptotic cell death in retinal degenerations. Prog. Retin. Eye Res. 17: 443 – 464
5. LaVail MM, Gorrin GM, Yasumura D and Matthes MT (1999) Increased susceptibility to constant light in nr and pcd mice with inherited retinal degenerations. Invest. Ophthalmol. Vis. Sci. 40: 1020 – 1024
6. Naash ML, Peachey NS, Li ZY, Gryczan CC, Goto Y, Blanks J, Milam AH and Ripps H (1996) Light-induced acceleration of photoreceptor degeneration in transgenic mice expressing mutant rhodopsin. Invest. Ophthalmol. Vis. Sci. 37: 775 – 782
7. Simons K (1993) Artificial light and early-life exposure in age-related macular degeneration and in cataractogenic phototoxicity. Arch. Ophthalmol. 111: 297 – 298
8. Taylor HR, Munoz B, West S, Bressler NM, Bressler SB and Rosenthal FS (1990) Visible light and risk of age-related macular degeneration. Trans. Am. Ophthalmol. Soc. 88: 163 – 173
9. Cideciyan AV, Hood DC, Huang Y, Banin E, Li ZY, Stone EM, Milam AH and Jacobson SG (1998) Disease sequence from mutant rhodopsin allele to rod and cone photoreceptor degeneration in man. Proc. Natl. Acad. Sci. USA. 95: 7103 – 7108
10. Cruickshanks KJ, Klein R and Klein BE (1993) Sunlight and age-related macular degeneration. The Beaver Dam Eye Study. Arch. Ophthalmol. 111: 514 – 518
11. Xu GZ, Li WW and Tso MO (1996) Apoptosis in human retinal degenerations. Trans. Am. Ophthalmol. Soc. 94: 411 – 430
12. Wong P (1994) Apoptosis, retinitis pigmentosa, and degeneration. Biochem. Cell. Biol. 72: 489 – 498
13. Noell WK, Walker VS, Kang BS and Berman S (1966) Retinal damage by light in rats. Invest. Ophthalmol. 5: 450 – 473
14. Organisciak DT, Darrow RM, Barsalou L, Darrow RA, Kutty RK, Kutty G and Wiggert B (1998) Light history and age-related changes in retinal light damage. Invest. Ophthalmol. Vis. Sci. 39: 1107 – 1116
15. LaVail MM, Gorrin GM and Repaci MA (1987) Strain differences in sensitivity to light-induced photoreceptor degeneration in albino mice. Curr. Eye Res. 6: 825 – 834
16. Gorgels TG, van Norren D (1995) Ultraviolet and green light cause different types of damage in rat retina. Invest. Ophthalmol. Vis. Sci. 36: 851 – 863
17. Grimm C, Wenzel A, Hafezi F, Yu S, Redmond TM and Remé CE (2000) Protection of Rpe65-deficient mice identifies rhodopsin as mediator of light-induced retinal degeneration. Nature Genet. 25: 63 – 66
18. Hafezi F, Marti A, Grimm C, Wenzel A and Remé CE (1999) Differential DNA binding activities of the transcription factors AP-1 and Oct-1 during light-induced apoptosis of photoreceptors. Vision Res. 39: 2511 – 2518
19. Wenzel A, Grimm C, Marti A, Kueng-Hitz N, Hafezi F, Niemeyer G and Remé CE (2000) c-fos controls the 'private pathway' of light-induced apoptosis of retinal photoreceptors. J. Neurosci. 20: 81 – 88
20. Curran T and Franza Jr BR (1988) Fos and Jun: the AP-1 connection. Cell 55: 395 – 397
21. Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M and Rubin LL (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron. 14: 927 – 939
22. Bossy-Wetzel E, Bakiri L and Yaniv M (1997) Induction of apoptosis by the transcription factor c-Jun. EMBO J. 16: 1695 – 1709
23. Hafezi F, Grimm C, Wenzel A, Abegg M, Yaniv M and Reme CE (1999) Retinal photoreceptors are apoptosis-competent in the absence of JunD/AP-1. Cell Death Differ. 6: 934 – 936
24. Hafezi F, Steinbach JP, Marti A, Munz K, Wang ZQ, Wagner EF, Aguzzi A and Reme CE (1997) The absence of c-fos prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration in vivo. Nat. Med. 3: 346 – 349
25. Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J and Woodgett JR (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369: 156 – 160
26. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M and Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76: 1025 – 1037
27. Johnson RS, van Lingen B, Papaioannou VE and Spiegelman BM (1993) A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. Genes Dev. 7: 1309 – 1317
28. Hilberg F, Aguzzi A, Howells N and Wagner EF (1993) c-jun is essential for normal mouse development and hepatogenesis. Nature 365: 179 – 181
29. Behrens A, Sibilia M and Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat. Genet. 21: 326 – 329
30. Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. 270: 16483 – 16486
31. Tominaga Y, Tsuzuki T, Shiraishi A, Kawate H and Sekiguchi M (1997) Alkylation-induced apoptosis of embryonic stem cells in which the gene for DNA-repair, methyltransferase, had been disrupted by gene targeting. Carcinogenesis 18: 889 – 896
32. Wurdeman RL, Douskey MC and Gold B (1993) DNA methylation by N-methyl-N-nitrosourea: methylation pattern changes in single- and double-stranded DNA, and in DNA with mismatched or bulged guanines. Nucleic Acids Research 21: 4975 – 4980
33. Nambu H, Yuge K, Nakajima M, Shikata N, Takahashi K, Miki H, Uyama M and Tsubura A (1997) Morphologic Characteristics Of N Methyl N Nitrosourea Induced Retinal Degeneration In C57bl Mice. Pathology International 47 (6): 377 – 383
34. Nakajima M, Yuge K, Senzaki H, Shikata N, Miki H, Uyama M and Tsubura A (1996) Photoreceptor apoptosis induced by a single systemic administration of N-methyl-N-nitrosourea in the rat retina. Am. J. Pathol. 148: 631 – 641
35. Nikolakaki E, Coffer PJ, Hemelsoet R, Woodgett JR and Defize LH (1993) Glycogen synthase kinase 3 phosphorylates Jun family members in vitro and negatively regulates their transactivating potential in intact cells. Oncogene 8: 833 – 840
36. Boyle WJ, Smeal T, Defize LH, Angel P, Woodgett JR, Karin M and Hunter T (1991) Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell 64: 573 – 584
37. Smeal T, Binetruy B, Mercola DA, Birrer M and Karin M (1991) Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature 354: 494 – 496
38. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E and Woodgett JR (1991) Phosphorylation of c-jun mediated by MAP kinases. Nature 353: 670 – 674
39. Leppa S and Bohmann D (1999) Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. Oncogene 18: 6158 – 6162
40. Eferl R, Sibilia M, Hilberg F, Fuchsbichler A, Kufferath I, Guertl B, Zenz R, Wagner EF and Zatloukal K (1999) Functions of c-Jun in liver and heart development. J. Cell Biol. 145: 1049 – 1061
41. Herzog KH, Chen SC and Morgan JI (1999) c-jun Is dispensable for developmental cell death and axogenesis in the retina. J. Neurosci. 19: 4349 – 4359
42. Kueng-Hitz N, Grimm C, Lansel N, Hafezi F, He L, Fox DA, Reme CE, Niemeyer G and Wenzel A (2000) The retina of c-fos-/- mice: electrophysiologic, morphologic and biochemical aspects. Invest. Ophthalmol Vis. Sci. 41: 909 – 916
43. Rich KA, Zhan Y and Blanks JC (1997) Aberrant expression of c-Fos accompanies photoreceptor cell death in the rd mouse. J. Neurobiol. 32: 593 – 612
44. Otori Y, Shimada S, Morimura H, Ishimoto I, Tohyama M and Tano Y (1997) Expression of c-fos and c-jun mRNA following transient retinal ischemia: an approach using ligation of the retinal central artery in the rat. Surv. Ophthalmol. 42: S96 – S104
45. Grimm C, Wenzel A, Hafezi F and Reme CE (2000) Gene expression in the mouse retina: the effect of damaging light. Mol. Vis. 6: 252 – 260
46. Ivanov VN and Nikolic-Zugic J (1997) Transcription factor activation during signal-induced apoptosis of immature CD4(+)CD8(+) thymocytes. A protective role of c-Fos. J. Biol. Chem. 272: 8558 – 8566
47. Dosch J and Kaina B (1996) Induction of c-fos, c-jun, junB and junD mRNA and AP-1 by alkylating mutagens in cells deficient and proficient for the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) and its relationship to cell death, mutation induction and chromosomal instability. Oncogene 13: 1927 – 1935

48. Gupta S, Campbell D, Derijard B and Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 267: 389 – 393
49. Whitmarsh AJ, Shore P, Sharrocks AD and Davis RJ (1995) Integration of MAP kinase signal transduction pathways at the serum response element. Science 269: 403 – 407
50. Aguirre V, Uchida T, Yenush L, Davis R and White MF (2000) The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J. Biol. Chem. 275: 9047 – 9054
51. Yamamoto K, Ichijo H and Korsmeyer SJ (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
52. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA and Davis RJ (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 288: 870 – 874
53. Fleischmann A, Hafezi F, Elliott C, Reme CE, Ruther U and Wagner EF (2000) Fra-1 replaces c-fos-dependent functions in mice. Genes Dev. 14: 2695 – 2700
54. Matsuo K, Owens JM, Tonko M, Elliott C, Chambers TJ and Wagner EF (2000) Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. Nat. Genet. 24: 184 – 187
55. Marti A, Jehn B, Costello E, Keon N, Ke G, Martin F and Jaggi R (1994) Protein kinase A and AP-1 (c-Fos/JunD) are induced during apoptosis of mouse mammary epithelial cells. Oncogene 9: 1213 – 1223
56. Wenzel A, Grimm C, Seeliger N, Jaissle G, Hafezi F, Kretschmer R, Zreuuer E and Remé CE (2001) Photoreceptor apoptosis prevented by activation of the glucocorticoid receptor. Invest. Ophthalmol. Vis. Sci., in press