Nucleolar AATF regulates c-Jun–mediated apoptosis

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ABSTRACT **The AP-1 transcription factor c-Jun has been shown to be essential for stress-induced apoptosis in several models. However, the molecular mechanisms underlying the proapoptotic activity of c-Jun are poorly understood. We identify the apoptosis-antagonizing transcription factor (AATF) as a novel nucleolar stress sensor, which is required as a cofactor for c-Jun–mediated apoptosis. Overexpression or down-regulation of AATF expression levels led to a respective increase or decrease in the amount of activated and phosphorylated c-Jun with a proportional alteration in the induction levels of the proapoptotic c-Jun target genes *FasL* and *TNF*-α. Accordingly, AATF promoted commitment of ultraviolet (UV)-irradiated cells to c-Jun-dependent apoptosis. Whereas AATF overexpression potentiated UV-induced apoptosis in wild-type cells, c-Jun–deficient mouse embryonic fibroblasts were resistant to AATF-mediated apoptosis induction. Furthermore, AATF mutants defective in c-Jun binding were also defective in inducing AP-1 activity and c-Jun–mediated apoptosis. UV irradiation induced a translocation of AATF from the nucleolus to the nucleus, thereby enabling its physical association to c-Jun. Analysis of AATF deletion mutants revealed that the AATF domains required for compartmentalization, c-Jun binding, and enhancement of c-Jun transcriptional activity were all also required to induce c-Jun–dependent apoptosis. These results identify AATF as a nucleolar-confined c-Jun cofactor whose expression levels and spatial distribution determine the stress-induced activity of c-Jun and the levels of c-Jun–mediated apoptosis.**

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INTRODUCTION

During the last decade several studies contributed to identifying nucleoli as stress biosensor organelles able to integrate and adjust cellular homeostasis to match the gravity of a given sensed stress (Andersen *et al.*, 2005; Mayer and Grummt, 2005; Pederson and Tsai, 2009; Stark and Taliansky, 2009). Indeed, apart from the more established role as a ribosome factory, the nucleolus plays a role in controlling cell cycle, aging, and maturation of small nuclear ribonucleoproteins. The nucleolus also acts as a safe to sequester or exclude molecules that under specific conditions are capable of operating in a different cellular compartment. For example, translocation of the transcription factor Hand1 from the nucleoli to the nucleus commits stem cells to differentiate (Martindill *et al.*, 2007). A similar translocation of the RNA helicase DDX21 upon c-Jun N-terminal kinase (JNK) activation facilitates its interaction with the nucleoplasmic AP-1 family transcription factor c-Jun (Westermarck *et al.*, 2002). It has become increasingly clear that further characterization of the functional roles of nucleolar proteins in regulating the activity of nucleoplasmic transcription factors will provide better understanding of how cellular stress responses are orchestrated (Mayer and Grummt, 2005).

Apoptosis-antagonizing transcription factor (AATF)/Che1 is an evolutionary conserved protein initially identified as a protein interacting with the Dlk/ZIP kinase, thereby interfering with the apoptotic

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Abbreviations used: AATF, apoptosis-antagonizing factor; AP-1, activating protein 1; ATF, activating factor, TNF α, tumor necrosis factor; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblast; RT, reverse transcriptase.

process (Page *et al*. 1999), and as an interacting factor of RNA polymerase I (Fanciulli *et al.*, 2000; Thomas *et al.*, 2000). The activation mechanism of AATF upon DNA damage resembles that of p53, even to the extent that activation of the DNA damage kinases ATM/ATR and Chk2 causes phosphorylation-mediated stabilization of AATF (Bruno *et al.*, 2006). The stabilized AATF has been reported to bind and activate *p53* and *p21* transcription. The subsequent increase in expression of p53 and cell cycle inhibitor p21 induce cell cycle arrest, preventing the replication of damaged DNA while cellular DNA-repair pathways are being activated. From previous reports AATF emerges as a Janus-faced stress sensor with ability to both induce cell cycle arrest and promote cell proliferation. The latter role is accomplished by the ability of AATF to bind to the retinoblastoma (Rb) protein, preventing Rb association with HDAC1 and the formation of a potent E2F inhibitory complex (Bruno *et al.*, 2002). In addition, AATF has the capacity to counteract Par4- and β-amyloid–induced apoptosis in neuronal cell models (Page *et al.*, 1999; Guo and Xie, 2004; Xie and Guo, 2004, 2006) and to promote cell survival upon endoplasmic reticulum stress via direct activation of the *Akt1* gene (Ishigaki *et al.*, 2010). However, it has not been demonstrated that AATF can also promote apoptotic cell death. This multifaceted property of AATF in regulation of proliferation is common to several critical proteins regulating cellular homeostasis, among which c-Jun is one of the best-studied examples.

AP-1 defines a family of dimeric basic leucine zipper (bZIP) transcription factors consisting of members of Jun, Fos, and activator transcription factor (ATF) subfamilies. The variation in dimerization partner preference, DNA-binding specificity, and posttranslational modifications provides a diverse array of AP-1 complexes that confers high flexibility in regulation of gene transcription. AP-1 dimers are key modulators of various processes, including cell proliferation, survival, and cell death (Devary *et al.*, 1991; Kasibhatla *et al.*, 1998; Le-Niculescu *et al.*, 1999; Dhanasekaran and Reddy, 2008; Herrlich *et al.*, 2008). Their activity is stringently controlled by interactions with regulatory and scaffolding proteins, as well as by posttranslational modifications (Herrlich *et al.*, 2008).

It is well established that AP-1 factors are involved in regulation of apoptosis, with early studies demonstrating that inhibition of c-Jun activity protected neuronal cells from apoptosis induced by nerve growth factor withdrawal (Ham *et al.*, 1995) and protected human monoblastic leukemia cells from apoptosis by exposure to various DNA-damaging agents (Verheij *et al.*, 1996). Furthermore, and relevant to this study, c-Jun–deficient fibroblasts have been shown to be resistant to apoptosis induced by ultraviolet (UV) irradiation (Shaulian *et al.*, 2000) or alkylating agents (Kolbus *et al.*, 2000). Accordingly, a prolonged and robust c-Jun activation precedes the onset of apoptosis in response to different types of stress signal (Kasibhatla *et al.*, 1998; Raivich, 2008). This suggests that key proapoptotic effectors must accumulate over time until a threshold level is exceeded to allow for a fully activated apoptotic machinery. Well-known proapoptotic genes induced by c-Jun are *TNFα*, *FasL*, and *Bim*, whose balance and amounts determine whether the final outcome will be cell survival or cell death (Devary *et al.*, 1991; Kasibhatla *et al.*, 1998; Le-Niculescu *et al.*, 1999; Dhanasekaran and Reddy, 2008; Herrlich *et al.*, 2008).

Although c-Jun is well established as a proapoptotic factor, our understanding of the molecular mechanisms underlying the proapoptotic ability of c-Jun is still poor, although significant advances have been made to identify transcriptional regulatory factors that determine c-Jun–mediated proliferation (Wulf *et al.*, 2001; Mialon *et al.*, 2005; Holmström *et al.*, 2008). Therefore the identification of regulatory molecules determining the proapoptotic functions of c-Jun will have broad ramifications, not least in terms of novel opportunities for therapeutic intervention in pathological conditions where c-Jun activity is deregulated.

Here we identify AATF as a nucleolar stress sensor whose expression and subnuclear localization are directly correlated to the level of c-Jun activation. We show that AATF interacts with c-Jun and promotes expression of phosphorylated c-Jun in response to several types of stress stimuli, thus identifying AATF as a direct c-Jun modulator. Moreover, we demonstrate that AATF is necessary for c-Jun–mediated induction of proapoptotic genes and for c-Jun–mediated apoptosis. Finally, we present data showing that stress-induced translocation of AATF from the nucleoli to the nucleus is followed by an increase in its binding to c-Jun and by c-Jun activation. Taken together, these observations suggest that AATF functions as a nucleolar stress sensor, which integrates stress stimuli into an appropriate c-Jun–mediated transcriptional response.

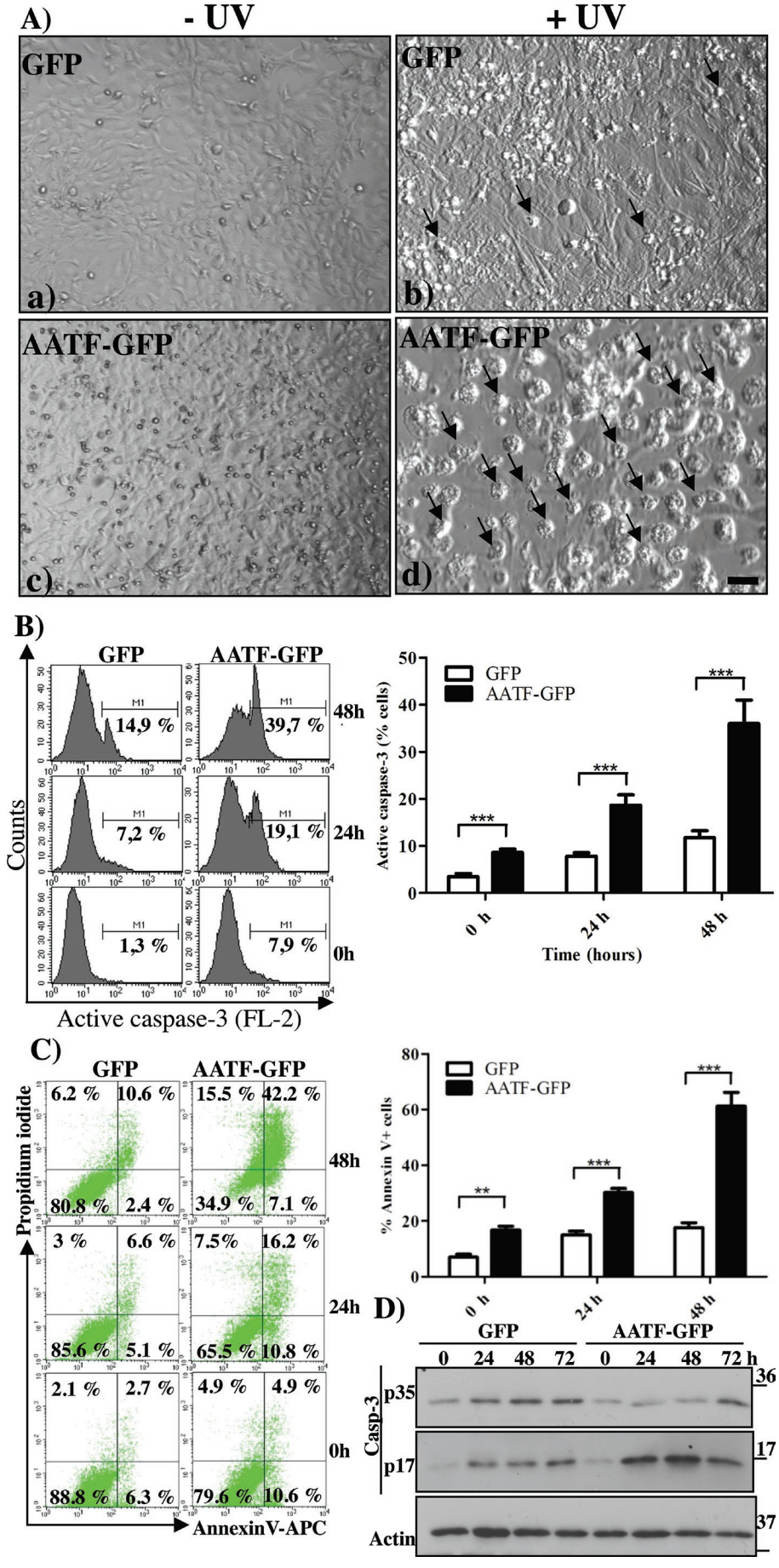
RESULTS

AATF expression regulates sensitivity to UV-induced cell death

AATF has emerged as a pleiotropic factor that modulates both cell proliferation and growth arrest by directly interacting with various components of the cell survival machinery (Fanciulli *et al.*, 2000; Bruno *et al.*, 2006). To investigate whether AATF affects the cellular response to a stress stimulus, we assessed the effects of AATF overexpression on UV-induced apoptosis in mouse embryonic fibroblast (MEF) cells and in HEK293 cells. Morphological analysis of UV-irradiated cells revealed that overexpression of AATF leads to a marked increase of detached and dead cells among both cell types (Figure 1A and Supplemental Figure S1A). To substantiate these findings, we analyzed UV-irradiated samples by flow cytometry for caspase-3 activation of green fluorescent protein (GFP)-positive cells (Figure 1B) and for double staining for annexin V and propidium iodide (PI) of GFP-positive cells (Figure 1C) to measure both early and later stages of apoptosis activation. Indeed, we found that overexpression of AATF induced a strong increase in the amount of caspase-3 activation (Figure 1B) and of annexin V–positive cells (Figure 1C). Similarly, UV-irradiated HEK293 cells overexpressing AATF-GFP that were ethanol-fixed and stained with PI for flow-cytometric analysis of dead cells showed a higher percentage of cells with a sub-G1 DNA content than did cells transfected with GFP empty vector (Supplemental Figure S1B). In accordance with these results, Western blot analysis showed a marked increase in the levels of activated caspase-3 (p17; Figure 1D) and in the cleavage of the caspase-3 substrate PARP-1 (unpublished data) in cells overexpressing AATF. Taken together, these results demonstrate that AATF has the capacity to markedly promote UV-induced apoptosis in both mouse and human cells of different origin.

AATF specifically regulates the activation of the c-Jun transcription factor

Given that a number of different studies identified c-Jun as a key regulator of UV-mediated apoptosis (Kasibhatla *et al.*, 1998; Kolbus *et al.*, 2000; Shaulian *et al.*, 2000), we investigated whether the proapoptotic role of AATF could be related to a differential activation of c-Jun. To this end, we exposed cells overexpressing AATF-GFP or GFP empty vector to UV irradiation and harvested them at the indicated time points (Figure 2A). To monitor the activity of c-Jun, we performed Western blot analysis using a c-Jun antibody recognizing phosphorylated serine 73 of c-Jun, a well-known site of c-Jun activation (Pulverer *et al.*, 1991) The blots revealed that in control cells, UV irradiation increased expression of phosphorylated



c-Jun, which was markedly amplified by AATF overexpression (Figure 2A), providing support for the assumption that AATF is involved in regulation of c-Jun activation. To test the c-Jun-specificity of AATF-mediated effect, we performed Western blotting for phosphorylated ATF-2, which also belongs to the AP-1 transcription factor family and is also activated and phosphorylated upon UV irradiation. The results showed that whereas UV irradiation promoted ATF-2 phosphorylation as expected, AATF overexpression had no further enhancing effect on ATF-2 activation (Figure 2A), indicating that the AATF-mediated effects are specific for c-Jun. Further backing for the postulation that AATF would regulate c-Jun activity was provided by experiments showing that down-regulation of AATF by small interfering RNA (siRNA) markedly reduced expression of phosphorylated c-Jun upon UV exposure (Figure 2B).

Several studies indicated that the duration of c-Jun activation may be critical as a determinant for the functional outcome of the stress response. Specifically, it has been shown that while transient c-Jun activation is often associated with suppression of UV-induced cell death by inducing cell cycle arrest, prolonged c-Jun activation is likely to trigger apoptosis (Devary *et al.*, 1991; Shaulian *et al.*, 2000; Shaulian and Karin, 2001, 2002). To determine whether AATF expression was able to maintain the activation of c-Jun for an extended time, thereby targeting cells to cell death, we irradiated cells overexpressing AATF (Figure 2C) or cells depleted of AATF by siRNA (Figure 2D)

FIGURE 1: AATF overexpression enhances UV-induced apoptosis. (A) Representative phase contrast images of MEF cells transfected with GFP or AATF-GFP and subjected to 20-μJ UV irradiation. At 48 h after irradiation, cell morphology was examined by inverted light microscopy (scale bar, 150 μm). Arrows indicate apoptotic cells. (B) MEF cells transfected and treated as in A were harvested at the indicated time points for detection of caspase-3 activation by flow cytometry. Cells were gated for the GFP population, and the percentage of cells positive for active caspase-3 is indicated. Right, histogram showing the averages of at least three independent experiments (mean ± SEM). (C) MEF cells treated as in A were double-stained for annexin V-APC and PI, and the GFP transfected population was analyzed by flow cytometry. Representative pictograms are shown. Right, histogram summarizing the results from five independent experiments (mean ± SEM). (D) Western blot analysis of active caspase-3 cleavage from matching samples treated as in A. Actin serves as loading control.

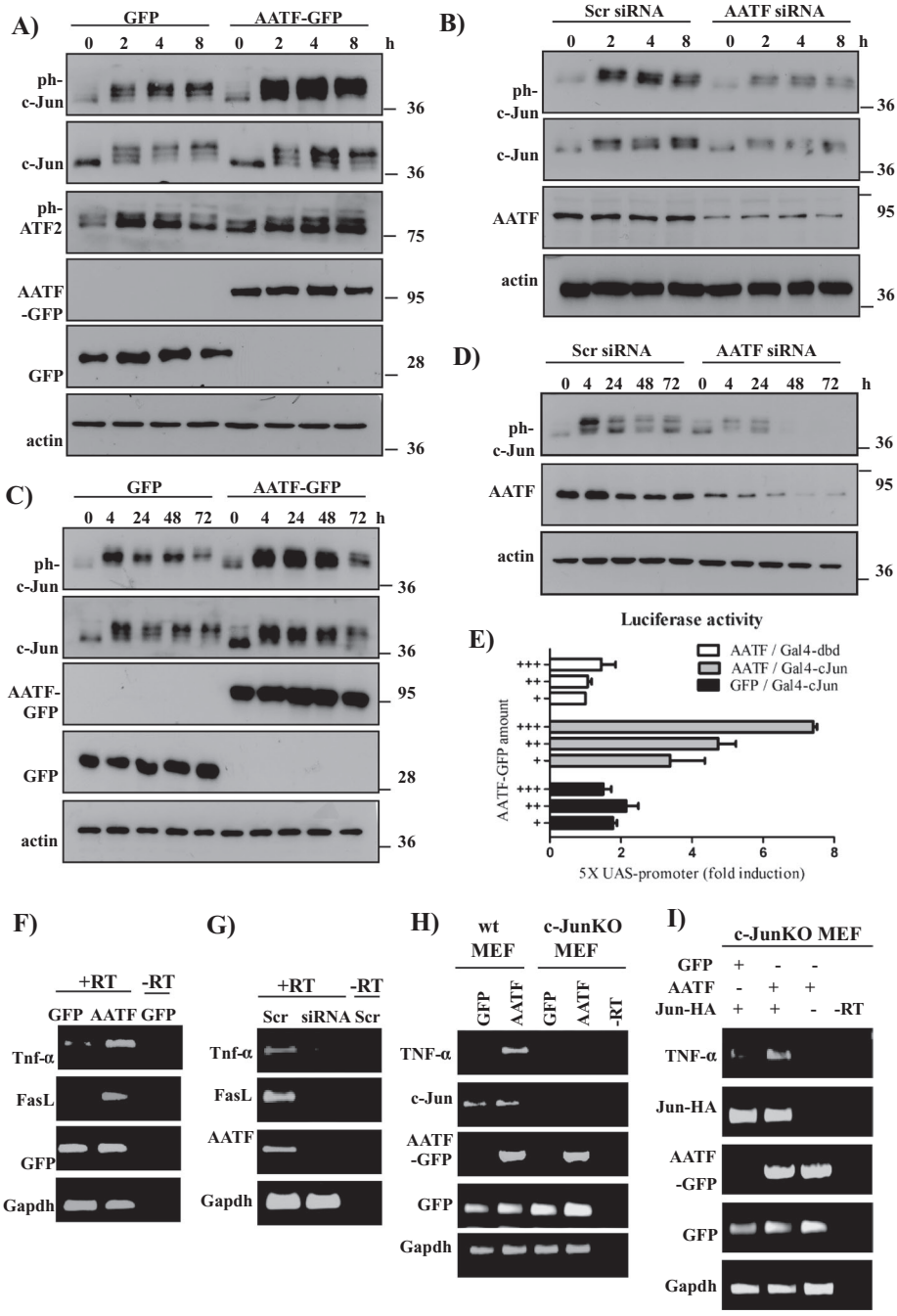


FIGURE 2: AATF expression levels regulate c-Jun activation upon UV exposure. (A) HEK293 cells were transfected with GFP empty vector or AATF-GFP and subjected to 100-μJ UV irradiation. Cells were harvested at the indicated time points and loaded on a gel for Western blot analysis of the indicated proteins. (B) HEK293 cells were transfected with AATF siRNA to down-regulate AATF expression levels or with a scrambled RNA as a control and subjected to 140-μJ UV irradiation. Cells were harvested at the indicated time points and loaded on a gel for Western blot analysis of the indicated proteins. (C) Samples were prepared as in A and harvested at the indicated late time points of the UV response for Western blot analysis of the indicated proteins. (D) Samples treated as in B were prepared and harvested at the indicated late time points of the UV response. (E) HEK293 cells were transfected with GFP empty vector or increasing amount of AATF-GFP, together with a β-galactosidase plasmid, a 5X-UAS-DNA-binding-domain-Gal4 expression construct (Gal4-DBD), or a 5X-UAS-Gal4-cJun (Gal4-cJun) expression construct and a reporter pf2-Luc construct. The ratio of luciferase activity vs. β-galactosidase activity in cell lysates was measured 24 h after transfection. Shown are mean values ± SEM of three experiments, each performed in triplicate. (F) HEK293 cells transfected with AATF-GFP or GFP empty vector were UV irradiated (100 μJ) and harvested after 12 h to analyze the induction of the proapoptotic c-Jun target genes *TNF-α* and *FasL* by semiquantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene, and GFP shows the transfection efficiency. The –RT sample corresponds to a nontransfected cell mRNA sample retrotranscribed in the absence of transcriptase enzyme as a negative control. (G) HEK293 cells transfected with AATF siRNA or Scr siRNA were UV-irradiated (140 μJ) and harvested after 12 h to analyze the induction of the proapoptotic c-Jun target genes *TNF-α* and *FasL* by semiquantitative RT-PCR. The –RT sample corresponds to the scrambled mRNA sample retrotranscribed in the absence of transcriptase enzyme as a negative control. (H) Wild-type MEF and c-JunKO MEF cells were transfected with GFP or AATF-GFP and analyzed by semiquantitative RT-PCR for the expression of the proapoptotic c-Jun target gene TNF-α. GAPDH was used as a housekeeping gene, and AATF-GFP and GFP show similar transfection efficiency. (I) c-JunKO MEF cells were transfected with GFP and cJun-HA (lane 1) or AATF-GFP and cJun-HA (lane 2) or a mock plasmid and AATF-GFP (lane 3). The expression levels of the transfected constructs and of TNF-α was verified by RT-PCR.

and harvested samples up to 3 d after UV exposure. As shown in Figure 2C, AATF overexpression was able to maintain high levels of phosphorylated c-Jun for at least 2 d (Figure 2C), whereas AATF down-regulation almost completely abrogated c-Jun phosphorylation on the activation site Ser-73 (Figure 2D). Taken together, these results demonstrate that AATF promotes an increase in the amount of c-Jun phosphorylated on transactivating Ser-73 (Pulverer *et al*., 1991). Based on the long-term effect of AATF on c-Jun activation, it is plausible that AATF would participate in c-Jun–mediated cellular processes, such as proapoptotic signal transduction.

Given that c-Jun phosphorylation promotes c-Jun–mediated transcriptional activity, we next studied whether the AATF-mediated stimulation of c-Jun is dependent on c-Jun dimerization or the DNA-binding property. To this end, we used a luciferase-based reporter assay with constructs that have the leucine zipper domain of c-Jun replaced with the DNA-binding domain of the yeast transcription factor Gal4. These constructs are thus independent of c-Jun dimerization and c-Jun DNA binding (Weiss *et al*. 2003). We compared the activity of a construct consisting of only the Gal4 DNA-binding domain (5XUAS-Gal4-DBD) to the activity of the same construct whose Gal4 DNA-binding domain was coupled to the

c-Jun transactivation domain (Gal4–c-Jun). Whereas AATF increased the transcriptional activity of Gal4–c-Jun in a concentration-dependent manner, AATF overexpression did not influence Gal4-luciferase activity in cells transfected with Gal4-DBD (Figure 2E). Equal expression of AATF in both Gal4–c-Jun– and Gal4-DBD–transfected cells was confirmed by Western blot analysis (unpublished data). The strong effect of AATF on the activity of Gal4–c-Jun reporter plasmid demonstrates that AATF exerts its effect by stimulating c-Jun transactivation capacity.

To rule out any p53-mediated effects of AATF, we performed additional experiments using two different cell lines that do not express p53—the prostate cancer cell line PC3 and the K562 cell line. In both PC3 cells (Supplemental Figure S2 and unpublished data) and K562 cells (unpublished data), AATF overexpression strongly induced c-Jun activation upon UV irradiation, demonstrating that the AATF-mediated stimulation of c-Jun activation does not require the presence of p53 and that this property is not specific to a cell type (Supplemental Figure S2 and unpublished data).

To explore whether AATF-mediated regulation of c-Jun was specific to UV irradiation, we also induced c-Jun activation in HEK293 cells by treatment with the proinflammatory cytokine tumor necrosis factor α (TNFα; Supplemental Figure S3A) or by serum withdrawal (Supplemental Figure S3B). In both cases, overexpression of AATF strongly enhanced the activation of c-Jun, implying that AATF-mediated stimulation of c-Jun extends beyond the UV response and is also highly relevant for stimuli related to normal cell physiology.

To confirm that the AATF-mediated increase in phosphorylated and activated c-Jun is reflected in the regulation of endogenous c-Jun target genes, we analyzed GFP and AATF-GFP transfected cells 12 h after UV treatment for expression of the well-established c-Jun–targeted genes *TNF*α and *FasL* by semiquantitative reverse transcription RT-PCR. Expression of both genes was strongly up-regulated in cells overexpressing AATF-GFP as compared with the GFP empty vector–transfected cells (Figure 2F). Conversely, down-regulation of AATF expression levels by siRNA significantly reduced both TNFα and FasL mRNA expression in UV-treated cells (Figure 2G). These results verified that the AATF-mediated activation of c-Jun upon UV treatment was mirrored in an increased transcriptional activity of c-Jun and consequently of its target genes.

We next examined whether the observed AATF-mediated induction of endogenous c-Jun target genes was c-Jun dependent, which would demonstrate the specificity and interdependence of the novel transcriptional complex c-Jun–AATF. For this purpose, we focused on TNF-α and determined the induction of TNF-α mRNA by semiquantitative RT-PCR in wild-type MEF cells and in c-JunKO MEF cells. Overexpression of AATF-GFP alone, without any other stress stimuli, was sufficient to induce *TNF*-α expression in wild-type MEF cells in the absence of any stress signal (Figure 2H). To further prove that both AATF and c-Jun are necessary to induce the endogenous *TNF*-α gene, we reintroduced c-Jun (c-Jun–hemagglutinin [HA]) in c-JunKO MEFs with or without AATF and verified the induction of *TNF*-α by semiquantitative RT-PCR. In c-JunKO MEFs, overexpression of c-Jun-HA only (Figure 2I, lane 1) or only AATF (Figure 2I, lane 3) could not induce the expression of *TNF*-α. In contrast, the combined reintroduction of both AATF and c-Jun (Figure 2I, lane 2) had the maximal effect on *TNF*-α induction, further confirming the c-Jun–specific capacity of AATF to regulate expression of the c-Jun target gene *TNF*α, which was chosen for validating the c-Jun requirement.

Taken together, these results show that AATF has the capacity to specifically induce c-Jun transactivity and thereby stimulate the transcription of c-Jun target genes. Furthermore, we showed that the induction of c-Jun–targeted genes is dependent on the concomitant presence of both AATF and c-Jun, proving that AATF induction of *TNF*-α transcription is indeed c-Jun dependent.

AATF promotes apoptosis in a c-Jun–dependent manner

A prolonged and sustained activation of the c-Jun transcription factor has been associated with an increased induction of apoptosis upon various stress stimuli. Given that the previous results demonstrate a proapoptotic role for AATF upon UV irradiation (Figure 1) and a stimulatory effect of AATF on c-Jun transcriptional activity (Figure 2), we next examined whether AATF-mediated enhanced apoptosis is dependent on its newly identified functional cooperation with c-Jun.

To this end, we compared the effect of AATF overexpression on UV-induced apoptosis in wild-type (wt) MEFs and in c-Jun–deficient MEFs (c-JunKO). Flow cytometry analysis of annexin V and propidium iodide double-stained cells gated for the GFP-transfected population showed that AATF overexpression was able to strongly promote apoptosis in the wt MEFs but not in the c-JunKO MEF cells (Figure 3, A and B). These results prove that the potent enhancing effect of AATF in the apoptotic response is dependent on and mediated through a direct regulation of c-Jun activity.

UV irradiation promotes interaction of c-Jun with AATF and induces its nucleoplasmic translocation

To examine whether AATF-mediated regulation of c-Jun phosphorylation involves physical interaction between AATF and c-Jun, we lysed untreated or UV-exposed HEK293 cells and subsequently subjected them to c-Jun or AATF immunoprecipitation and immunoblotted for AATF or c-Jun, respectively. Whereas low-stoichiometry interaction between endogenous AATF and c-Jun was observed in untreated cells, their interaction was dramatically increased already 15 min after UV treatment (Figure 4, A and B). To confirm the antibody-binding specificity, we performed c-Jun immunoprecipitation from lysates of cells transfected with AATF siRNA or a control scrambled RNA and verified the specificity of the antibody and of the AATF–c-Jun interaction (Figure 4C).

The subcellular sequestration of proteins and the consequent variability in the composition of multiprotein signaling complexes are used to modulate the strength and kinetics of a pathway. AATF was previously described as a nuclear protein that can also be detected in the nucleolus and the cytoplasm (Fanciulli *et al.*, 2000; Thomas *et al.*, 2000; Andersen *et al.*, 2002, 2005). We therefore hypothesized that, similar to another c-Jun activator—DDX21 (Westermarck *et al.*, 2002)—also the subcellular localization of AATF could be affected by UV irradiation and could provide a mechanistic explanation for the stimulus-specific interaction with c-Jun.

In nontreated cells, AATF was almost exclusively localized in the nucleolus, where it indeed colocalized with nucleolin (Figure 4D and Supplemental Figure S4A) and with B23 (unpublished data), two well-known nucleolar markers. However, UV irradiation induced redistribution of AATF to the nucleoplasm, with a time-dependent increase in the number of cells showing a complete loss of AATF from the nucleoli (Figure 4D), in line with a previous observation that identified AATF as a nucleolar protein (Moore *et al.*, 2011). The release of AATF from the nucleolus upon UV irradiation led us to study whether nucleoplasmic endogenous AATF in UV-treated cells would colocalize with endogenous c-Jun. Indeed, a clearly distinct compartmentalization of AATF and c-Jun could be observed under nonstimulated conditions, in which most of the c-Jun signal was coming from the nucleoplasm, whereas AATF displayed a highly specific nucleolar signal. In contrast, confocal images of UV-irradiated cells demonstrated the

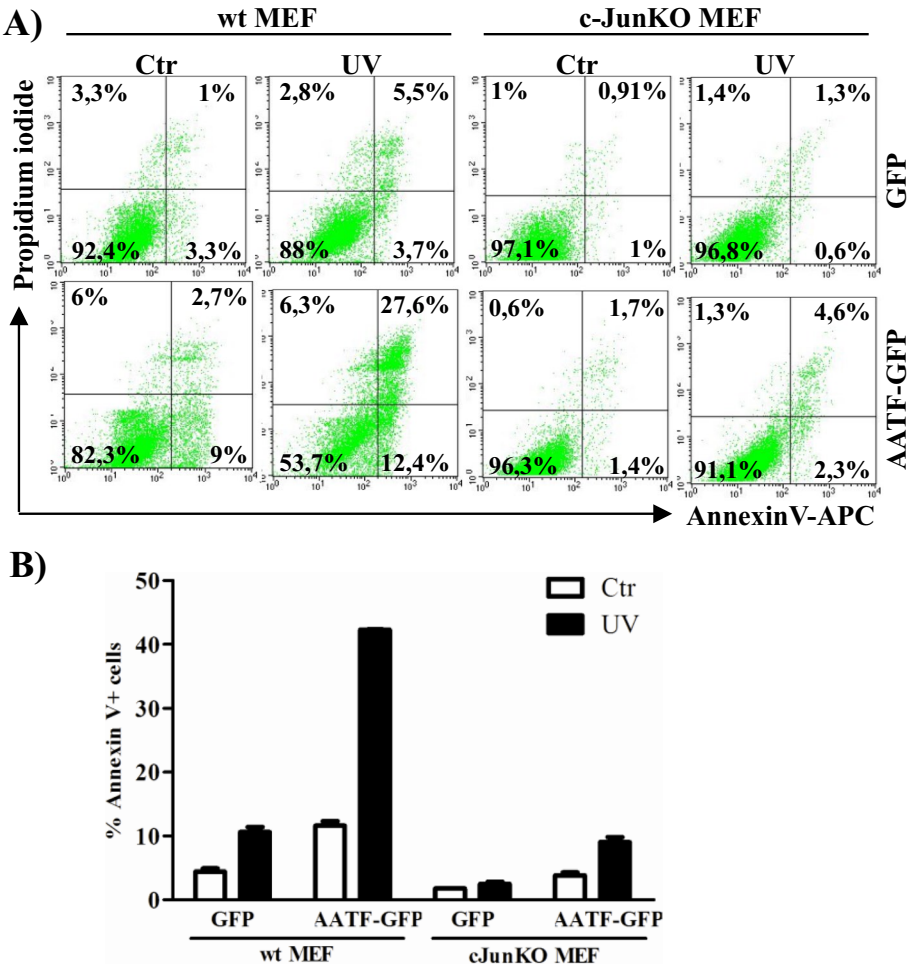


FIGURE 3: AATF induces apoptosis in a c-Jun–dependent manner. (A) Wild-type MEF and c-JunKO MEF cells were transfected with AATF-GFP or GFP empty vector and subjected to 15-μJ UV irradiation. After 48 h cells were collected and stained for annexin V-APC and (PI for flow cytometry analysis of apoptotic cells. Representative FACS pictograms of the GFP-selected population are shown. (B) The quantification of four independent experiments performed as in A is shown as a percentage of annexin V–positive cells before and 48 h after UV irradiation (mean ± SEM).

concomitant presence of AATF and c-Jun in nuclear dot-like structures (Figure 4E; higher-magnification of images of c-Jun-AATF colocalization are provided in Supplemental Figure S4B).

Taken together, these results indicate that in unstimulated cells, AATF interaction with c-Jun may be limited by AATF nucleolar localization and nucleolar sequestration of AATF may serve as a concrete constraint for c-Jun binding and activation in the absence of proapoptotic signals.

AATF domains required for stimulatory activity and interaction with c-Jun

The localization studies described here strongly suggest that the capacity of AATF to potentiate UV-induced apoptosis is mediated by its physical interaction with c-Jun and its subsequent capacity to stimulate c-Jun transcriptional activity. To verify that physical interaction between AATF and c-Jun is truly required for these phenotypes, we identified the domains of AATF necessary for binding to c-Jun. For this purpose, we constructed the following deletion mutants: an N-terminal fragment encoded by exons I–IV (amino acids 1–198), an N-terminal fragment encoded by exons I–X of AATF (amino acids 1–494), and a C-terminal fragment encoding exons V–XIII (amino acids 199–526). In addition, we also used an AATF isoform lacking exons IV–V (Δ125–246). Coimmunoprecipitation analysis revealed that the first half of AATF (amino acids 1–198) is not able to bind to endogenous c-Jun, whereas both the longer N-terminal fragment of AATF (1–454) and the C-terminal fragment of AATF (199–526) retain the ability to associate to c-Jun (Figure 5A). These results indicate that the central fragment of AATF (amino acids 199–454) contains the primary binding site for c-Jun.

We next examined the effect of the different AATF deletion mutants on the stimulatory transcriptional activity of c-Jun, using a luciferase-based AP-1 reporter assay. Although both the C-terminal-fragment AATF(199–526) and the AATF(Δ125–246) strongly induced AP-1 activity, neither of the N-terminal -truncated isoforms, AATF(1–199) and AATF(1–454), was able to do so (Figure 5B). These results revealed that the C-terminal tail of AATF (amino acids 456–526) is necessary to stimulate c-Jun transcriptional activity and uncovered the presence of a potential AATF-inhibitory domain located within amino acids 125–246. Indeed, the lack of these amino acids in the isoform AATF(Δ125–246) or in the C-terminal-fragment AATF(199–526) strongly enhanced AATF-mediated AP-1 promoter inducibility when compared with the full-length AATF(1–526) (Figure 5B).

Confocal images of the localization of these deletion mutants also showed that the C-terminal domain of AATF is necessary for the nucleolar localization. The N-terminal fragment of AATF(1–198) exhibits a completely cytoplasmic localization, whereas full-length AATF(1–526) and the C-terminal fragment of AATF(199–526) display nucleolar localization (Supplemental Figure S5). Of interest, the N-terminal fragment AATF(1–454) loses nucleolar localization but retains nuclear localization. These results imply that the ability of AATF(1–454) to bind to c-Jun and to localize in the nucleoplasm is not sufficient to stimulate c-Jun activity and that the C-terminal tail of AATF (amino acids 456–526) is required for proper AATF functionality.

To complete our characterization of AATF functional domains, we examined whether the different abilities of the AATF deletion mutants to stimulate c-Jun transcriptional activity mirrored their capacity to enhance apoptosis in a c-Jun–dependent manner. We therefore overexpressed the different AATF mutants in wild-type and c-JunKO MEF cells and determined the amount of cell death by fluorescence-activated cell sorting (FACS) analysis of annexin V/PI–stained cells, selecting for the GFP-positive population. In accordance with the AP1 reporter analysis, the AATF(199–526) and AATF(Δ125–246) deletion mutants possessing the strongest stimulating property (as in Figure 5B) also exhibited the most robust induction in apoptosis in wt and, importantly, not in c-JunKO MEF cells (Figure 5C). In contrast, both the N-terminal-truncated mutants AATF(1–198) and AATF(1–454), which were not able to stimulate AP-1 transcriptional activity, were also incapable of promoting apoptosis.

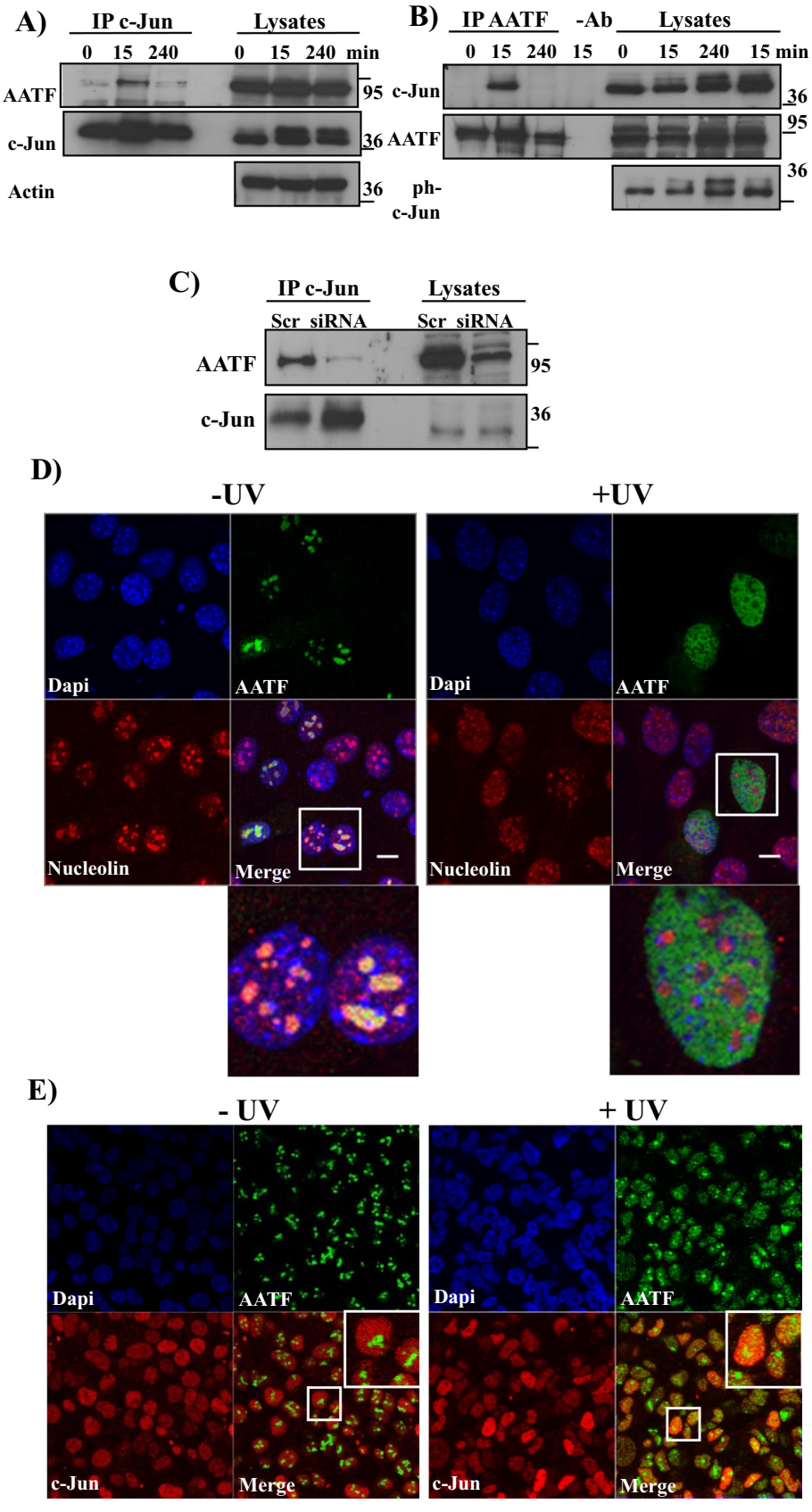


FIGURE 4: Endogenous AATF interacts and colocalizes with c-Jun after UV exposure. (A, B). HEK293 cells were UV irradiated and harvested at the indicated time points. Cell lysates were immunoprecipitated with c-Jun (A) or AATF (B) antibodies and immunoblotted for AATF (A) or c-Jun (B) to detect their association. Reblotting for c-Jun (A) and AATF (B) shows that an equal amount of protein was immunoprecipitated from each cell lysate. Actin serves as loading control. (C) AATF expression levels were either down-regulated by siRNA (siAATF) or not (scrambled RNA) in HEK293 cells. c-Jun was immunoprecipitated from cell lysates, and c-Jun association specificity was examined by Western blotting with AATF antibody. (D) MEF cells were transfected with AATF-GFP and UV irradiated. After 4 h cells were fixed and stained for nucleolin (red) and DAPI (blue) to visualize the nucleus (scale bars, 10 μm). The merge and magnification of representative cells show a strong colocalization (yellow) of AATF-GFP and nucleolin in the control condition (–UV; left). (E) HEK293 cells were irradiated, fixed after 4 h, and stained for AATF (green), c-Jun (red), and DAPI (blue) (scale bars, 10 μm). The insets in the merge correspond to a magnification of the area indicated by the square and show increased colocalization (yellow) of AATF and c-Jun in the UV-irradiated sample.

As Figure 5D summarizes, our data demonstrate that AATF enhances UV-induced apoptosis exclusively by its functional interaction with c-Jun and through its C-terminal tail. Furthermore, these results strongly suggest that the rate of nucleoplasmic relocalization of AATF determines its proapoptotic activity and function as a c-Jun cofactor.

DISCUSSION

Our study identifies AATF as a required activator and interacting partner of the AP-1 transcription factor c-Jun and suggests that the spatiotemporal regulation of AATF during stress can adjust the magnitude and duration of c-Jun activation. Of importance, we demonstrate that in the absence of c-Jun expression, AATF loses its capacity to induce c-Jun–targeted proapoptotic genes and consequently loses its proapoptotic activity. AATF is the first c-Jun endogenous regulator identified to be necessary for targeting and allowing for c-Jun–mediated apoptosis.

c-Jun is essential for cell adaptation to many environmental changes and oncogenic transformation. The specificity of a given biological response to the variety of stimuli that regulate c-Jun seems often to be the outcome of signal-specific multiprotein complexes whose variety in composition and activity allows for a high flexibility in c-Jun target gene regulation. Related to c-Jun–mediated oncogenic effects, we previously demonstrated that another nucleolar c-Jun interaction protein, TopoI, enhances c-Jun–mediated epidermal growth factor receptor expression and cancer cell proliferation

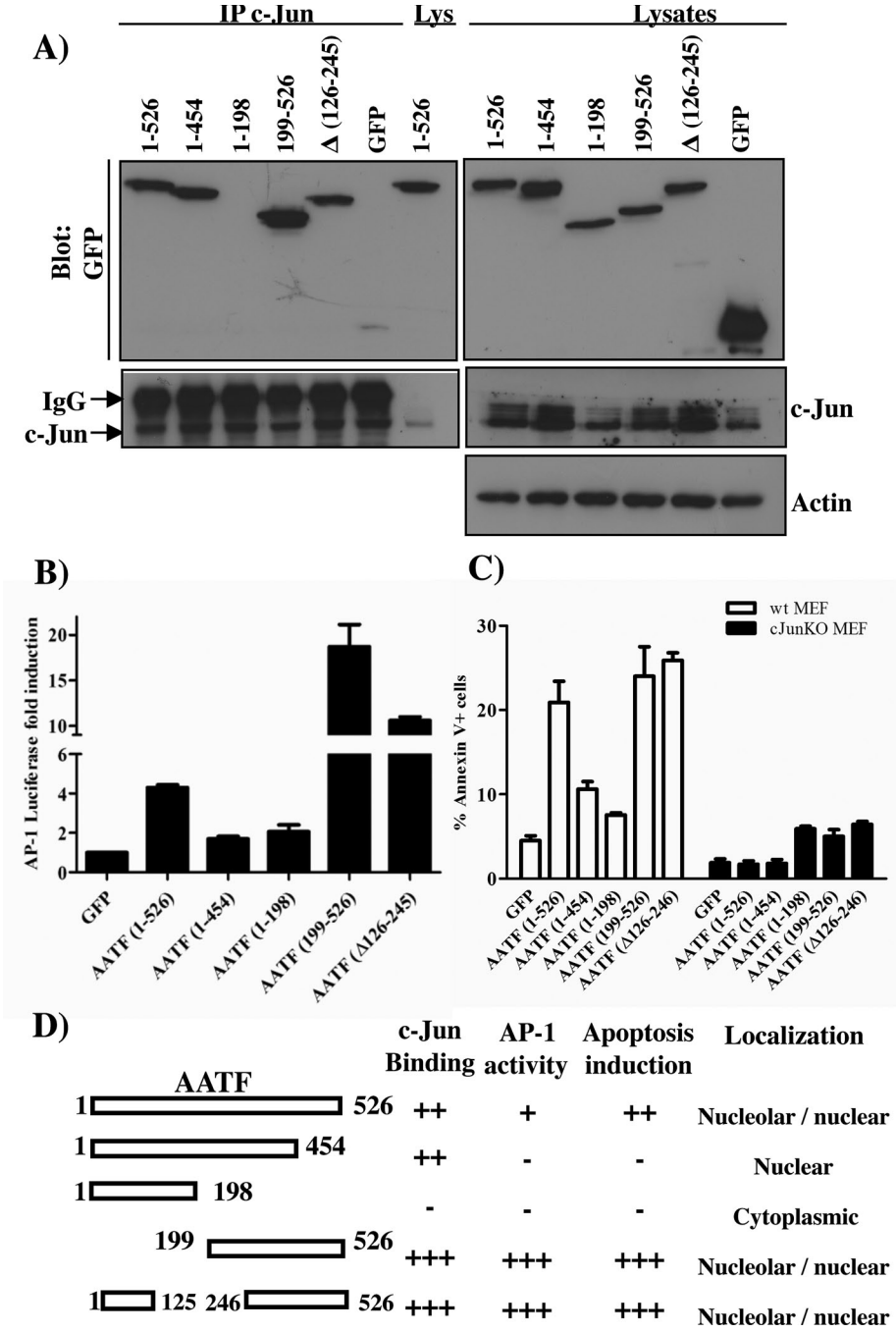


FIGURE 5: AATF domains required for stimulatory activity and interaction with c-Jun. (A) HEK293 cells were transfected with the indicated AATF-GFP deletion constructs, and cell lysates were immunoprecipitated with c-Jun antibody and immunoblotted with GFP and c-Jun antibodies (left). Immunoblotting cell lysates with GFP and actin shows equivalent expression of all AATF deletion proteins (right). (B) The same AATF constructs as in A were transfected in HEK293 cells together with β-galactosidase and AP-1-promoter-luciferase reporter plasmids. The ratio between the luciferase activity and the β-galactosidase activity for each construct is shown in the recapitulative histogram (mean ± SEM; $n \geq 3$). (C) The same AATF constructs as in A were transfected into wt MEF cells and c-JunKO MEF cells. The following day, cells were collected and labeled with annexin V-APC and PI for flow cytometry analysis of apoptotic cells. The histogram summarizes the results from four independent experiments (mean ± SEM). (D) Recapitulative scheme of the AATF deletion mutants used, showing relative binding to cJun, ability to stimulate AP-1 promoter, ability to induce apoptosis, and subcellular localization (shown in Supplemental Figure S5).

(Mialon *et al.*, 2005). On the other hand, here we show that AATF recruitment to c-Jun–containing complexes provides a spatiotemporal switch that adjusts the duration and strength of c-Jun activation in response to apoptotic stimuli. In support of this hypothesis, we show that the interaction kinetics of endogenous AATF and c-Jun proteins during the UV response displays a dynamic pattern in which an interaction is evident, especially during the early phases of the UV response, and seems to precede a persistent increase in expression of phosphorylated c-Jun. AATF may therefore tag the amount of c-Jun that can be activated, thereby being the limiting or permissive factor in the sequence of events leading to c-Jun–mediated apoptosis.

Previous studies showed that UV-mediated c-Jun activation induces the transcription of a wide range of genes, including those that encode for the proapoptotic TNFα and Fas-L (Kasibhatla *et al.*, 1998; Le-Niculescu *et al.*, 1999; Herr *et al.*, 2000; Dhanasekaran and Reddy, 2008). Our study on AATF identifies the first regulator of the proapoptotic functions of c-Jun and shows it acting by directly regulating c-Jun transcriptional activation and, thereby, the induction of TNFα and Fas-L. Indeed, AATF overexpression potentiated c-Jun activation and c-Jun–mediated induction of TNFα. Moreover, AATF overexpression was sufficient to induce TNFα expression in absence of stress signal and was dependent on the concomitant presence of both AATF and c-Jun. These results demonstrate that c-Jun transcriptional activity and proapoptotic functions are proportional to the amount of AATF available for interaction with c-Jun in the nucleoplasm.

Protein sequestration is an obvious modality to control regulatory factors whose presence in a given protein complex determines its signal-specific activity. The nucleolar sequestration of AATF and its specific release upon UV-mediated stress indicate that sequestration is the main modus operandi in determining the amount of AATF targeting to c-Jun. An increasing number of proteins have been found to recolocalize upon stress signals into different subnuclear domains (Carmo-Fonseca *et al.*, 2000; Pederson and Tsai, 2009; Stark and Taliansky, 2009; Boulon *et al.*, 2010). In addition to the example described in this study, UV stress can also induce the redistribution of a number of proteins from the nucleolus to the nucleoplasm, such as ARF, TIFI-A (Mayer *et al.*, 2005; Szymański *et al.*, 2009), B23 (Wu *et al.*, 2002), nucleolin (Yang *et al.*, 2008), and DDX21 (Westermarck *et al.*, 2002).

Consistent with the observation that subnuclear distribution and c-Jun interaction determine the capacity of AATF to affect c-Jun functions, we show that AATF mutants defective in c-Jun binding were also defective in inducing AP-1 reporter activity and c-Jun–mediated apoptosis. All results are in accordance with the hypothesis

that AATF operation is regulated by sequestration, which then determines the degree of c-Jun interaction and in turn the magnitude of c-Jun–mediated apoptosis. Of interest, in addition to AATF, our previous studies showed that also two other nucleolar c-Jun–interacting proteins, DDX21 and TopoI, translocate from the nucleolus to the nucleoplasm upon UV stress (Mao *et al.*, 2002; Westermarck *et al.*, 2002; Mialon *et al.*, 2005; Holmström *et al.*, 2008), and, moreover, DDX21 binding to c-Jun is also promoted by UV (Westermarck *et al.*, 2002). Moreover, our preliminary results indicate that DDX21 helicase activity, which is required for c-Jun activation, is also involved in UV-mediated inhibition of HEK293 cell survival (unpublished data). Taken together, these results strongly imply that c-Jun activation and function in response to proapoptotic stimuli are determined by nucleolar cofactors that upon stress stimulus are recruited to c-Jun and permit the formation of a transcriptionally active c-Jun complex.

c-Jun activity has been described as regulated by different posttranslational modifications, such as phosphorylation events, acetylation by p300 (Vries *et al.*, 2001), and sumoylation by Ubc9 (Bossis *et al.*, 2005). Similarly, AATF stability and promoter-binding capability are controlled by posttranslational modifications, such as phosphorylation by the ATM-ATR kinases. These kinases are well-known regulators of stress responses and of safeguard regulators, among which p53 is also a target gene of AATF (Bruno *et al.*, 2006). The positioning of AATF between two general master switch regulators provides a new level for synchronous regulation of c-Jun and p53. It is indeed tempting to speculate on the existence of a c-Jun–p53–AATF "stress trio" in which the action of one determines the interactions and promoter-binding affinities of the other two. From this perspective, the presence of interdependent stress sensors able to communicate and regulate each other may explain their Janus-faced behavior. The combinatorial use of different proteins ensures proper integration of a variety of stimuli, allowing careful fine tuning of cellular responses. The amount of interacting AATF determines the amount of c-Jun available for phosphorylation and the consequent *life-to-death* switch of the c-Jun transcriptional complex.

MATERIALS AND METHODS

Cell culture reagents and transfection materials

HEK293 cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, and penicillin G (100 U/ml)/streptomycin (100 µg/ml) growth medium. c-Jun$^{-/-}$ mouse embryo fibroblasts (c-JunKO MEF) and the corresponding wt MEF were described previously (MacLaren *et al.*, 2004; Holmström *et al.*, 2008). MEF cells were transfected using Lipofectamine LTX reagent (Invitrogen, Grand Island, NY) according to manufacturer's protocol. HEK293 cells were pelleted and resuspended in Opti-MEM I (Life Technologies) and electroporated at 220 V and 975 µF. Transfected cells were plated and harvested after 24–48 h for further analysis. AATF was down-regulated in HEK293 or MEF cells using AATF siRNA or scrambled siRNA (Qiagen, Hilden, Germany) transfected with Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions. The luciferase-based reporter assays were performed using the Luciferase Assay System kit purchased from Promega (Fitchburg, WI) according to the manufacturer's protocol.

Plasmids

AATF was cloned from a mouse muscle tissue into enhanced GFP vector. More information about the cloning procedures is available upon request. The HA-tagged c-Jun constructs Gal4-cJun-wt, Gal4-cJun-AA, and Gal4-cJun-Δ have been described elsewhere (Westermarck *et al.*, 2002; Weiss *et al.*, 2003).

Immunofluorescence labeling

Cells grown on coverslips were fixed in 3% (vol/vol) paraformaldehyde and permeabilized with 0.2% (vol/vol) Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature. Nonspecific binding was blocked by incubation in 2% bovine serum albumin in PBS with 0.2% Triton X-100 for 40 min at room temperature. Cells were subsequently stained for 2 h with primary antibodies, after which coverslips were rinsed three times with PBS and stained for 45 min with fluorescently labeled secondary antibodies (Alexa 488 goat anti-mouse or Alexa 568 goat anti-rabbit; Molecular Probes, Invitrogen). Cells were washed three times in PBS before mounting in 4′,6-diamidino-2-phenylindole (DAPI)/Vectashield. Images were collected using a Zeiss LSM confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with argon and helium–neon lasers.

UV irradiation and cell death analysis

Cells were grown until they reached 80–90% confluency, rinsed with PBS, and exposed to UVC irradiation. Medium was replaced immediately after UV exposure. The range of intensity of the UVC was 15–20 µJ for MEF cells and 100–140 µJ for HEK293 cells, as indicated in the text. Samples to be analyzed by flow cytometry were harvested, rinsed with PBS, and resuspended in 350 µl of annexin V binding buffer (2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 35 mM NaCl, 0.6 mM $CaCl_2$) containing 0.5 µl of annexin V-APC (BD PharMingen, Franklin Lakes, NJ) and 5 µg/ml propidium iodide (Sigma-Aldrich, Saint Louis, MO). After 10 min of incubation on ice and in the dark, samples were directly analyzed using FACSCalibur by gating on GFP-positive cells (FL-1). Caspase-3 activity within the GFP-transfected population was analyzed by FACSCalibur flow cytometer (BD PharMingen) by labeling cells with PE-conjugated monoclonal active caspase-3 antibody according to manufacturer's instructions. Both adherent and floating cells were collected for all cell death analysis.

Immunoprecipitation and antibodies

HEK293 cells were lysed in IP buffer (50 mM HEPES, pH 7,4, 140 mM NaCl, 5 mM EDTA, 0.4% NP40, 0.1% Triton, 10 mM pyrophosphate, 1 mM dithiothreitol [DTT], 5 mM sodium orthovanadate, and a protease inhibitor cocktail [Roche Diagnostics, Basel, Switzerland]) for 20 min on ice, followed by centrifugation at 10,000 × *g* for 10 min at 4°C. Lysates were precleared using Sepharose beads, followed by immunoprecipitation using antibodies against c-Jun, AATF, or GFP. Immunocomplexes were captured on protein G–Sepharose beads and washed four times in 20 mM HEPES (pH 7.4), 2 mM EDTA, 100 mM NaCl, 0.4% NP40, and 1 mM DTT and finally resuspended in Laemmli sample buffer.

The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): mouse anti-AATF (used at 1:500), rabbit anti–c-Jun (1:1000), and rabbit nucleolin (1:500). In addition, the following antibodies were used: rabbit phS73 c-Jun (used at 1:1000; Cell Signaling Technology, Danvers, MA) mouse anti-actin (1:1000; Sigma-Aldrich), rabbit phJNK (Cell Signaling Technology), and rabbit GFP (Living Colors, Clontech, Mountain View, CA). The secondary antibodies (used at 1:10,000 to 1:20,000) were purchased from Southern Biotechnology (Birmingham, AL).

RT-PCR

Total RNA was purified from cells using TRIzol (Invitrogen) following manufacturer's instructions. From 1 to 2 µg of RNA was reverse transcribed with random primers (Promega) using reverse transcriptase purchased from Fermentas (Burlington, Canada), according to the

manufacturer's instructions. From 1 to 2 µl of the cDNA was amplified in 25 µl of PCR volume using Taq Polymerase (Fermentas). After amplification, the cDNAs were separated on 2% agarose gels and visualized by staining with ethidium bromide. The following primer pairs were used: mouse TNF-α: 5′-CCA CCA CGC TCT TCT GTC TA-3′, 5′-GTG GGT GAG GAG CAC GTA GT-3′; mouse FasL: 5′-CTGTGGCTACCGGTGGTATT-3′, 5′- GCTGGTTGTTGCAA-GACTGA-3′; human Tnfα: 5′-ACCTCCTCTCTGCCATCAAG-3′, 5′-CCAAAGTAGACCTGCCCAGA-3′; glyceraldehyde-3-phosphate dehydrogenase: 5′-ACCACAGTCCATGCCATCAC-3′, 5′- TACAGC-AACAGGGTGGTGGA-3′.

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