Transcriptional switch by activating transcription factor 2-derived peptide sensitzes melanoma cells to apoptosis and inhibits their tumorigenicity

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The notorious resistance of melanoma cells to drug treatment can be overcome by expression of a 50-aa peptide derived from activating transcription factor 2 (ATF250–100). Here we demonstrate that ATF250–100 induced apoptosis by sequestering ATF2 to the cytoplasm, thereby inhibiting its transcriptional activities. Furthermore, ATF250–100 binds to c-Jun N-terminal kinase (JNK) and increases its activity. Mutation within ATF250–100 that impairs association with JNK and the inhibition of JNK or c-Jun expression by RNA interference (RNAi) reduces the degree of ATF250–100-induced apoptosis. In contrast, TAM67, a dominant negative of the Jun family of transcription factors, or JunD RNAi attenuates inhibition of melanoma’s tumorigenicity by ATF250–100. We conclude that inhibition of ATF2 in concert with increased JNK/Jun and JunD activities is central for the sensitization of melanoma cells to apoptosis and inhibition of their tumorigenicity.

Response of melanomas to treatment and inhibition of their notorious metastatic potential are key for effective therapy (1). Common to advanced melanomas are impairments of their death cascade pathways (2,3). Such impairments include down-regulation of FAS receptor through transcriptional silencing (4) or aberrant trafficking (5), poor expression of apoptotic protease activating factor 1 (Apaf1; ref. 6), expression of decay tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL; ref. 7), or overexpression of tumor necrosis factor receptor-associated factor 2 (TRAF2; ref. 8). Over 60% of melanomas contain activating mutations within B-Raf (9), whereas 15% harbor N-Ras mutation, resulting in constitutive activation of the mitogen-activated protein kinase (MAPK) pathway (10). MAPK has been implicated in transcriptional activation of cAMP-response-element binding protein (11), c-Fos (12) and the melanocyte transcription factor microphthalmia (13), which affects melanoma cell pigmentation and viability (14). Furthermore, MAPK/extracellular response kinase has also been implicated in the activation of matrix metalloproteinase-9 (15), B-3 integrins (16), and Janus kinase (JAK)/signal transducers and activators of transcription (17).

Activating transcription factor 2 (ATF2) is phosphorylated by the stress kinases and MAPK/extracellular response kinase and is involved in the cellular response to stress (18–19). Transcriptionally active ATF2 binds to ATF/cAMP response element target sequences as a heterodimer with c-Jun (20) and activates a large set of genes including tumor necrosis factor α, transforming growth factor β, IL-6, IFN-γ, IFN-β, cyclin A, and E-selectin (21–24).

Our earlier studies indicated that ATF2 has a key role in melanoma’s acquisition of resistance to apoptosis after treatment with various DNA-damaging agents (24, 25). Interference with ATF2 function via the use of dominant negative forms, or by overexpression of a short ATF2-derived peptide (ATF250–100), resulted in the sensitization of this resistant tumor to a variety of chemical and physical treatments (26, 27). Expression of the ATF250–100 inhibits endogenous ATF2 and activates c-Jun, resulting in inhibition of melanoma tumorigenicity and metastatic potential in vivo (27). Important support for the role of ATF2 in melanoma progression comes from tissue array analysis, showing a significant correlation between nuclear localization of ATF2 in melanoma tumors and poor prognosis (28).

Materials and Methods

Cell Culture and Derivation of Stable Cell Line. The mouse melanoma cell line SW1 and 293T human embryonic kidney cells were maintained as described in ref. 27.

Constructs. ATF250–100 was cloned in frame into an hemagglutinin (HA)-penetratin pcDNA3 vector as described (26). The 5’-Jun2-Luc and 5’-12-o-tetradecanoylphorbol 13-acetate response element (TRE)-Luc constructs are described elsewhere (27). Substituting mutations in ATF250–100 amino acids MT (to AA at positions 51 and 52), RNDS (to AAAA at amino acids 59–62), or VIVA (to RRRR at amino acids 63–66) were generated by oligonucleotide-directed mutagenesis (QuickChange, Stratagene). Wild-type and mutant forms of ATF250–100 were cloned into BamHI/NotI sites of pGEX-4T vector, resulting in GST fusion constructs. Cloning of JNK into the BamHI/NotI sites on the pEF-Flag generated pEF-Flag JNK and its mutant counterpart pEF-Flag JNK-APF. The production of ATP-pocket mutant pcDNA HA-JNK-αs3; JNK-DLD was described in ref. 29. DNA sequencing combined with Western blotting confirmed the integrity and expression of each construct. Transfections were carried out by using Lipofectamine Plus (Invitrogen), and stably expressing clones were selected as described in ref. 27.

RNAi in SW1 Cells. Nineteen-mer oligonucleotides based on sequences derived from the corresponding target transcripts were used to mediate suppression of gene transcription through RNAi as follows: murine JNK1/2 (accession nos. AB005663 and AAD22579, respectively) within nucleotides 379–397 AGAATGTCCTACCTTCTCT, murine c-Jun (accession no. BC002081) within nucleotides 983–1002 CGCAGCAGTTGCAAACGTT, and murine JunD (accession no. X15358) within nucleotides 984–1003 GTCCCTAGCCACGTCAACA. Each of these oligos was cloned into the BglII/HindIII sites of the pRETRO-SUPER (pRS) vector (30).

Protein Half-Life Measurements. SW1 cells that stably express empty vector (SW1 pcDNA) or pcDNA-ATF250–100 (SW1-ATF250–100) were treated with cycloheximide (60 mg/ml) for the indicated time periods.

Abbreviations: ATF2, activating transcriptional factor 2; HA, hemagglutinin; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; pKs, pRETRO-SUPER; RNAi, RNA interference; TRE, 12-o-tetradecanoylphorbol 13-acetate response element.

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Transcriptional Analysis. Reporter constructs and luciferase assays were carried out as described in ref. 27.

Apoptosis. Cells were treated with 10 μg/ml anisomycin for 36 h, and apoptosis was measured as described in ref. 4.

Retroviral Infection. Packaging of retroviral constructs (pRS containing RNAi oligos) was carried out in human 293T cells as described in ref. 31. SW1 cells (1.5 × 10^7) were infected for 12 h in the presence of 4 μg/ml polybrene.

In Vitro Protein-Binding Assay. 293T cells transfected with the respective JNK plasmids were lysed in binding buffer (20 mM Tris, pH 7.5/150 mM NaCl/1 mM EDTA/1 mM EGTA/0.5% Nonidet P-40/1 mM NaVO_{3}/1 mM DTT supplemented with protease inhibitors) and then incubated (500 μg per assay) in bacterially expressed and purified GST-ATF2 wild-type and mutant forms bound to glutathione beads for 2 h at 4°C. The glutathione beads were blocked with BSA (3 mg/ml in PBS) for 2 h before use. Bead-bound material was then washed (three times with 0.5 M LiCl), eluted, and separated on SDS/PAGE, followed by immunoblotting.

Western Blot and Immunohistochemistry Analysis. Cell lysates (50–150 μg) were resolved on 10% SDS/PAGE, transferred to nitrocellulose, and blotted according to standard protocols. The antibodies used were polyclonal anti-ATF2 directed to the C-terminal peptide (C-19) and anti-c-Jun (SC-45) (both from Santa Cruz Biotechnology), anti-HA (Babco, San Francisco), anti-JNK 1/2/3 (Affinity BioReagents, Golden, CO). Antibody–antigen complexes were detected with the ECL system (Amersham Pharmacia). Membranes were also scanned with Bio-Rad PhosphorImager using BIOANALYST software for quantification. When applicable, the x-ray films were scanned and relative band intensity was determined with a GS-800 calibrated densitometer (Bio-Rad). Immunohistochemistry analysis and confocal microscopy were carried out as described in refs. 27 and 32.

Tumorigenesis. SW1 cells expressing wild-type or mutant forms of the ATF2 peptide alone or in combination with TAM67, JunD RNAi, c-Jun RNAi, or control pRS were trypsinized, resuspended in PBS, and injected s.c. into 6- to 7-wk-old mice in the lower flank. Tumor growth was monitored as described (27).

Immunokinase Assays. Purified GST or GST fusion proteins were incubated in buffer (20 mM Mops, pH 7.2/25 mM β-glycerophosphate/5 mM EGTA/1 mM sodium orthovanadate/1 mM DTT) supplemented with 75 mM MgCl_{2}/0.5 mM cold ATP/10 mCi (1 Ci = 37 GBq) [γ-32P]ATP at 30°C for 15 min. Samples were separated on SDS/PAGE, transferred to a membrane that was then stained with Ponceau, and autoradiographed.

Results

Expression of ATF2^{50–100} Increases Cytoplasmic Localization of ATF2. Melanoma cells that express ATF2^{50–100} (SW1-ATF2^{50–100}) exhibit a marked decrease in ATF2-dependent transcription as shown in refs. 26 and 27 (see Fig. 4b). Under normal conditions, ATF2 localizes in the nucleus of advanced human or mouse melanoma cells as shown in ref. 28 and Fig. 1. However, expression of ATF2^{50–100} caused a substantial increase in cytoplasmic localization of ATF2 (Fig. 1 and Fig. 6, which is published as supporting information on the PNAS web site) due to inhibition of nuclear import, because addition of an inhibitor of nuclear export did not affect the level of ATF2 localization (data not shown). Minimal mutations within ATF2^{50–100} MT, and RNDS designed to abolish JNK binding and p38 binding, respectively, failed to inhibit ATF2 nuclear localization to the degree seen with wild-type ATF2^{50–100} (Fig. 6). This finding suggests that ATF2^{50–100} association with JNK/p38 is important for retention of ATF2 within the cytoplasm, which consequently impairs ATF2 transcriptional activities. SW1-ATF2^{50–100} melanoma cells also exhibited accumulation of c-Jun within the cytoplasm (Fig. 7, which is published as supporting information on the PNAS web site). In both cases, the ATF2 peptide appears to change also the morphology of the cells. Changes in the cellular distribution of ATF2 in SW1-ATF2^{100} melanoma cells provide important insight into mechanisms underlying the changes observed in ATF2 and c-Jun transcriptional activities.

ATF2^{50–100} Association with JNK Is Required for Sensitization of Melanoma Cells to Apoptosis. c-Jun expression is increased in SW1-ATF2^{50–100} melanoma cells as shown in ref. 27 and Fig. 2a, because of an increase in the c-Jun half-life from 150 min to 210 min (Fig. 2b). Because JNK is implicated in the regulation of c-Jun stability (33) and ATF2^{50–100} contains the consensus amino acid domain required for JNK binding (N.J., unpublished work), we tested whether JNK plays a role in mediating ATF2^{50–100} effects. In vitro binding assays revealed specific association between ATF2^{50–100} and endogenous JNK, employing ATF2^{1–115} as a control (Fig. 2c). Mutant ATF2^{50–100} that was altered in the two amino acids important for JNK association (MT-AA) was deficient in JNK binding, whereas the mutant deficient in p38 binding (RNDS-AAAA) or in β-sheets formation (VIVA-RRRR, expected to disturb ATF2^{50–100} proper
Fig. 2. **ATF2** associates with JNK and requires its binding to sensitize melanoma to apoptosis and inhibit tumorigenicity. (a) Expression of the **ATF2** peptide increases expression of c-Jun. Protein extracts prepared from SW1 cells that stably express empty vector (control) or wild-type HA-**ATF2** peptide (**ATF2** peptide) were subjected to immunoblot analysis by using antibodies to phosphorylated c-Jun followed by reprobing with control c-Jun antibodies. The membrane was reprobed with antibodies to β-actin. (b) The half-life of c-Jun increased in melanoma cells expressing **ATF2**. The half-life of c-Jun was monitored with cycloheximide, which was added to control or **ATF2**-expressing cells for increasing time periods. Proteins were then subjected to immunoblot analysis by using antibodies to c-Jun. Numbers on the bottom of each image indicate relative change in level of expression based on a densitometry analysis normalized to background control with the QUANTIFY ONE program. Values represent the absolute densitometric values for each band. (c) JNK2 associates with **ATF2**. In vitro binding assays were carried with bacterially expressed and purified GST-**ATF2** wild-type or mutant peptide incubated with extracts of 293T cells expressing Flag-tagged JNK2 for 2 h at 4°C. The beads were washed, and eluted material was subjected to immunoblot analysis with antibodies to Flag. (Lower) Coomassie blue staining reflecting the quantity of proteins used for the reaction. Relative change was quantified and normalized per binding to ATF2(21–100), a commonly used substrate for JNK, p38, based on densitometry. (d) Mutations within either the phosphoacceptor sites (APF) or the ATP pocket (DLD) of JNK reduces JNK association with **ATF2**. An in vitro binding reaction was carried out as indicated above, except that the GST peptides were incubated with extracts from 293T cells expressing Flag-tagged JNK2 for 2 h at 4°C. The beads were washed, and eluted material was subjected to immunoblot analysis with antibodies to Flag. (Lower) Coomassie blue staining reflecting the quantity of proteins used for the reaction. Relative change was quantified and normalized per binding to ATM(21–100) and normalized per binding to ATM(21–100) and normalized per binding to ATM(21–100). Numbers reflect quantification of phosphorylation based on PhosphorImager analysis. (e) JNK binding and, to a lesser degree, p38 binding are required for sensitization of melanoma cells to apoptosis by **ATF2**. SW1 cells expressing the wild-type or mutant forms of **ATF2** were analyzed to determine the basal degree of apoptosis by using fluorescence-activated cell sorter analysis. (f) JNK but not p38 is required for the ability of **ATF2** to inhibit melanoma growth in vivo. SW1 cells expressing **ATF2** in its wild-type or mutant forms were injected s.c. into C3H mice (groups of six mice per experimental condition), and tumors were excised and analyzed to determine size and weight after 21 d.

**JNK Is Important for the Sensitization of Melanoma Cells to Apoptosis by **ATF2**.** RNAi against the two major forms of JNK, JNK1/2 (termed pRS-JNK) were used to assess the role of JNK in mediating the activities of **ATF2** peptide. Expression of pRS-JNK reduced the levels of JNK by 50% (Fig. 3a), and the degree of tumor suppression by 50% (Fig. 2b). These data suggest that the association of **ATF2** with JNK but not with p38 is required for inhibition of melanoma growth in vivo.

**Expression of any of the different mutant **ATF2** listed above also failed to increase the expression of c-Jun (data not shown), suggesting that association with JNK is required for stabilization of c-Jun.**

Mutations within the ATP pocket of JNK (DLD) or its phosphoacceptor sites (APF at amino acids 183–185) were sufficient to cause a 3-fold decrease in JNK association with **ATF2**(21–100), suggesting that the active or conformationally intact forms of JNK associate with the peptide (Fig. 3d). **ATF2**(21–100) also binds p38 even more robustly than with JNK in a conformation-dependent manner, because association was abolished by each of the mutations described above (Fig. 2c).

Immunokinesis assays with c-Jun as a substrate revealed that **ATF2**(21–100) increased basal JNK but not p38 kinase activity (Fig. 2f). **ATF2**(21–100) did not alter JNK activity after anisomycin treatment, but it attenuated activation of p38 kinase (Fig. 2f). These data suggest that the increase in c-Jun expression and activities in response to **ATF2**(21–100) could be attributed to changes in JNK, whereas decreased **ATF2** activity could be attributed, in part, to decreases in p38 activity after treatment with anisomycin.

Mutation in **ATF2**(21–100) within the JNK binding site (MT-AA) abolished the peptide’s ability to sensitize melanoma cells to apoptosis (Fig. 2e), suggesting that JNK association is required for this sensitization. Mutation within the p38-binding site (RNDS-AAAA) also reduced the sensitization of the cells to apoptosis, albeit to a lesser degree compared with the mutation within the **JNK-binding site** (Fig. 2g). These data suggest that the association with JNK and, to a lesser degree, p38 is required for the sensitization of melanoma cells to **ATF2**-induced apoptosis.

Although SW1 cells produce aggressive tumors in mice (27, 34), a substantial inhibition of tumor growth was seen during expression of wild-type and p38 mutant **ATF2**(21–100). In contrast, mutation within the **ATF2**(21–100) JNK-binding site decreased the degree of tumor suppression by 50% (Fig. 2h). These data suggest that the association of **ATF2**(21–100) with JNK but not with p38 is required for inhibition of melanoma growth in vivo.
Expression of pRS-Jun decreased the degree of basal apoptosis (Fig. 3d) and inhibited cell growth (data not shown). Expression of pRS-Jun decreased the degree of basal apoptosis from 40% to ~20% (Fig. 3e). pRS-Jun does not affect TRE-dependent transcription and inhibits TRE-dependent activities in cells expressing ATF20–100 or ATF250–100. SW1 cells that constitutively express control or ATF20–100 were infected with pRS-JunD or pRS-JNK or with both. Cells were transfected with TRE-Luc constructs. Proteins were prepared 48 h after infection, and Western blot analysis was performed with antibodies to JunD. β-actin was used as a loading control. (c) Suppression of c-Jun expression affects the degree of basal apoptosis induced in ATF20–100-expressing cells. SW1 cells were infected with pRS-c-Jun or control vectors 48 h later. Twenty hours later, cells were subjected to anisomycin treatment, and the degree of apoptosis was monitored by fluorescence-activated cell sorter analysis. (f) pRS-c-Jun does not affect TRE-dependent transcription in ATF250–100-expressing cells. SW1 cells were infected with the pRS control pRS-c-Jun, followed by cotransfection of ATF20–100 or control vectors with TRE-Luc 48 h later. Twenty hours later, proteins were prepared and assayed for luciferase activity. Values depict absolute luciferase activity.

c-Jun Is Important for the Sensitization of Melanoma Cells to Basal Apoptosis. To directly assess the role of c-Jun in the sensitization of melanoma expressing ATF20–100 to apoptosis, we generated c-Jun RNAi constructs (termed pRS-Jun) that efficiently decrease the expression of endogenous c-Jun expression (Fig. 3d) and inhibited cell growth (data not shown). Expression of pRS-Jun decreased the degree of basal apoptosis (Fig. 3e). pRS-Jun results in efficient inhibition of JunD expression. SW1 cells that constitutively express control or wild-type ATF20–100 were infected with control or pRS-JunD constructs. Proteins were prepared 48 h after infection, and Western blot analysis was performed with antibodies to JunD. β-actin was used as a loading control. (d and e) Inhibition of JunD expression increases Jun2-mediated transcription and inhibits TRE-dependent activities in cells expressing ATF20–100; SW1 cells that constitutively express control or ATF250–100 were infected with pRS control or pRS-JunD. Forty-eight hours after infection, the cells were transfected with Jun2-luc or TRE-Luc. Proteins were prepared 20 h later and assayed for luciferase activity. (d) Inhibition of TRE-mediated transcription is enhanced after inhibition of both JNK and JunD expression. SW1 cells that constitutively express control or ATF250–100 were infected with pRS-JunD or pRS-JNK or with both. Cells were transfected with TRE-Luc 48 h after infection, and proteins were prepared for luciferase assays after an additional 20 h. A portion of the same extracts was used to verify inhibition of JNK and JunD expression by Western blots. (e) Suppression of JunD transcription inhibits sensitization of the ATF2 peptide-expressing melanoma to apoptosis after anisomycin treatment. SW1 cells were infected with pRS-JunD or control pRS followed by transfection of the ATF2 peptide or control vectors 48 h later. Twenty hours later, cells were subjected to anisomycin treatment, and the degree of apoptosis was monitored by fluorescence-activated cell sorter analysis after an additional 24 h.
that c-Jun affects transcriptional targets that are not regulated by TRE.

**JunD Is Required for TRE-Dependent Transcription and Sensitization of Melanoma Cells Expressing ATF2^250–100 to Apoptosis.** Although inhibition of JNK or c-Jun appears to play a key role in sensitizing SW1-ATF2^250–100 to anisomycin-induced apoptosis, the expression of TAM67, a dominant negative form of the Jun family of transcription factors, was efficient in mediating such inhibition (27). Because TAM67 is equally potent in inhibiting JunD (35, 36), we tested the role of JunD by using RNAi that efficiently inhibit its tumorigenic potential. At least three distinct changes seen in response to TAM67 expression. These results directly support the role of JunD in mediating the effects of ATF2^250–100.

**JunD Inhibits Tumorigenicity of SW1 Melanomas in C3H Mice.** Further assessment of the role of JunD in mediating the effect of ATF2^250–100 was carried out in vivo. First, we evaluated the effect of TAM67 expression on tumor growth of SW1 cells implanted in C3H mice. As we described before, expression of ATF2^250–100 in SW1 melanoma cells suppressed almost completely their ability to produce tumors, compared with SW1 control vectors, which produced tumors of \( \approx 500 \) mm\(^3\) within 25 d after injection (Fig. 4). However, expression of TAM67 in SW1-ATF2^250–100 melanoma cells abolished the peptide’s suppressive effect on tumorigenesis, increasing tumor size from 15 mm\(^3\) to \( > 750 \) mm\(^3\) (Fig. 5a).

Because TAM67 equally affects c-Jun and JunD, we next assessed the possible role of each transcription factor on tumorigenesis by employing SW1-ATF2^250–100 melanoma cells stably expressing RNAi to the respective transcription factors. Stable expression of pRS-JunD, but not pRS-c-Jun, attenuated the ATF2^250–100-mediated inhibition of SW1 tumorigenicity (Fig. 5b). Furthermore, inhibition of JunD caused a \( > 16 \)-fold increase in the tumor size of SW1-ATF2^250–100 cells (from 18.7 to 315 mm\(^3\) ), similar to the changes seen in response to TAM67 expression. These results directly support the role of JunD in mediating the ATF2^250–100 inhibition of melanoma tumorigenicity and confirm that the effects elicited by TAM67 are primarily mediated through JunD.

**Discussion**

This work provides insight into mechanisms underlying the ability of ATF2^250–100 to sensitize melanoma to apoptosis and inhibit its tumorigenic potential. At least three distinct changes are elicited in advanced melanoma cells that express the ATF2 peptide (model outlined in Fig. 5c). First, ATF2^250–100 increases ATF2 localization within the cytoplasm. The mechanism of this
effect may involve the peptide’s affect on a protein kinase(s) that could regulate ATF2 translocation. Consistent with these findings, the observation that melanoma patients that exhibit cytoplasmic localization of ATF2 also had better prognoses (28).

Second, ATF2<sup>50–100</sup> associates with JNK and increases its basal kinase activity, resulting in higher levels of c-Jun expression and phosphorylation. Yet, neither TRE-Luc activity nor the induction of apoptosis after anisomycin treatment, depended on c-Jun, suggesting that c-Jun regulates a yet-identified subset of genes that contribute to the sensitization of ATF2<sup>50–100</sup>-expressing melanomas to apoptosis. Consistent with these findings, c-Jun was previously shown to be capable of inducing apoptosis in nontransformed cells (27, 39), explaining the increase in Jun phosphorylation and stability. Such binding may also out-compete JNK phosphorylation of other substrates and/or protect JNK from protein phosphatases that would otherwise limit its activities.

Third is the activity of JunD, which was found to play a central role in regulating different stress responses, and Ruth Halaban for helpful comments. This work was supported by National Cancer Institute Grants CA99961 and CA51995 and by a Sharp Foundation grant to Z.R.