Inhibition of Melanoma Growth by Small Molecules That Promote the Mitochondrial Localization of ATF2


Abstract

Purpose: Effective therapy for malignant melanoma, the leading cause of death from skin cancer, remains an area of significant unmet need in oncology. The elevated expression of PKCε in advanced metastatic melanoma results in the increased phosphorylation of the transcription factor ATF2 on threonine 52, which causes its nuclear localization and confers its oncogenic activities. The nuclear-to-mitochondrial translocation of ATF2 following genotoxic stress promotes apoptosis, a function that is largely lost in melanoma cells, due to its confined nuclear localization. Therefore, promoting the nuclear export of ATF2, which sensitizes melanoma cells to apoptosis, represents a novel therapeutic modality.

Experimental Design: We conducted a pilot high-throughput screen of 3,800 compounds to identify small molecules that promote melanoma cell death by inducing the cytoplasmic localization of ATF2. The imaging-based ATF2 translocation assay was conducted using UACC903 melanoma cells that stably express doxycycline-inducible GFP-ATF2.

Results: We identified two compounds (SBI-0089410 and SBI-0087702) that promoted the cytoplasmic localization of ATF2, reduced cell viability, inhibited colony formation, cell motility, and anchorage-free growth, and increased mitochondrial membrane permeability. SBI-0089410 inhibited the 12-O-tetradecanoylphorbol-13-acetate (TPA)–induced membrane translocation of protein kinase C (PKC) isoforms, whereas both compounds decreased ATF2 phosphorylation by PKCε and ATF2 transcriptional activity. Overexpression of either constitutively active PKCε or phosphomimic mutant ATF2T52E attenuated the cellular effects of the compounds.

Conclusion: The imaging-based high-throughput screen provides a proof-of-concept for the identification of small molecules that block the oncogenic addiction to PKCε signaling by promoting ATF2 nuclear export, resulting in mitochondrial membrane leakage and melanoma cell death. Clin Cancer Res; 19(10); 2710–22. ©2013 AACR.

Introduction

The incidence of melanoma, the most deadly form of skin cancer, has steadily increased for the past 30 years. Our current understanding of the mechanisms underlying the initiation and progression of melanoma has led to the development of specific inhibitors targeting the major signaling pathways that are known to be deregulated in melanoma, including B-Raf, phosphoinositide 3-kinase (PI3K), and mitogen-activated protein/extracellular signal–regulated kinase (MEK; refs. 1–3). Although clinical trials of B-Raf and MEK inhibitors in melanoma have produced promising results, the development of resistance to such monotherapies represents a barrier that has yet to be overcome (4, 5).

ATF2, a member of the basic helix–loop–helix (HLH) family of transcription factors, is activated via phosphorylation by c-Jun N-terminal kinase or p38 in response to stimuli including stress and cytokines (6–8). ATF2 dimerizes with other members of the AP1 superfamily to activate the transcription of genes implicated in stress and DNA damage responses, growth, differentiation, and apoptosis (6–8). The genetic inactivation of ATF2 in melanocytes has been shown to abolish melanoma formation in the mutant N-Ras/mink1 mouse model (9), indicating an oncogenic role for ATF2 in melanocyte transformation. Conversely, a tumor-suppressor function for ATF2 was suggested by the increased incidence of papillomas (10) and mammary tumors (11) following the genetic inactivation of ATF2 in keratinocytes or mammary tissue.
Translational Relevance
The identification of new therapeutic modalities is among the most important priorities for improving cancer treatment. Among those is the need to halt oncogene addiction, which underlies tumor development, progression, and the development of resistance. We have recently shown the oncogenic addiction of melanoma to ATF2 through PKCe, which mediates its confined nuclear localization. Here, we have developed a platform to identify small molecules that impair the oncogenic function of ATF2 by permitting its translocation from the nucleus to the mitochondria, thereby enabling its tumor suppressor activities to facilitate melanoma cell death. Our initial screen identified and characterized 2 compounds that sensitized melanoma cells to death, offering a proof-of-concept for the therapeutic paradigm, whereby changing the subcellular localization of a protein, which can limit its oncogenic contributions and promote its tumor suppressor activities.

respectively. In our effort to understand the mechanisms underlying the opposing activities of ATF2, we discovered that the subcellular localization dictates the oncogenic or tumor-suppressor function of ATF2. Although its nuclear localization is required for oncogenic activity, ATF2 must be localized to the cytoplasm to conduct its tumor-suppressor function. Analysis of tissue microarrays (TMA) revealed that ATF2 exhibits cytosolic localization in basal cell carcinomas (BCC) or squamous cell carcinomas (SCC; ref. 10) but is primarily nuclear in melanoma tumors, consistent with the constitutive transcriptional activity of ATF2 in these tumors (12). Notably, the nuclear localization of ATF2 is associated with poor prognosis in patients with melanoma, suggesting that ATF2 localization might serve as a prognostic marker (12, 13).

We recently found that the nuclear localization of ATF2 is dictated by its phosphorylation on threonine 52 (Thr52) by PKCe (14). Loss of Thr52 phosphorylation, as seen in several nontransformed or nonmalignant cell lines following exposure to genotoxic stress, is required to enable the nuclear export and translocation of ATF2 to mitochondria, where it reduces mitochondrial membrane potential and promotes apoptosis. Elevated levels of PKCe, found in the more advanced metastatic melanomas, prevent the nuclear-to-mitochondrial translocation of ATF2 that enable its tumor-suppressor function. Notably, the expression of peptides derived from ATF2 (amino acids 50–60 or 50–100) prevents the nuclear localization of ATF2 and sensitizes melanoma cells, but not melanocytes, to apoptosis (15–18). These effects were abolished by the mutation of the peptide at the PKCe phosphorylation site (Thr52; ref. 15), suggesting that the native peptide functions by competitively inhibiting PKCe association with/phosphorylation of endogenous ATF2. Taken together, these findings suggest that small molecule modulators of ATF2 localization could attenuate its oncogenic addiction to PKCe signaling, thereby enhancing its proapoptotic functions. Because the nuclear-to-cytosolic export of ATF2 also sensitizes mutant B-Raf-expressing melanoma cells to apoptosis, agents that promote the nuclear export of ATF2 are expected to represent a new therapeutic modality for drug-resistant melanomas.

Materials and Methods
Cell lines and culture conditions
HEK293T and NIH3T3 cells were obtained from American Type Culture Collection. Melanoma cell lines were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The melanoma cell lines UACC903 and 501Melwere were kindly provided by Drs. Gavin Robertson (Penn State University, Hershey, PA) and Ruth Halaban (Yale University, New Haven, CT), respectively. The cells were maintained at 37°C in a humidified 5% CO2 atmosphere and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 30 μM penicillin, 30 μg/mL streptomycin, and 2 mmol/L-glutamine (Gibco-Life Technologies). The human melanocytes (Hermes 3A) were maintained in 254 medium supplemented with 10% FBS and human melanocyte growth supplement (Gibco-Life Technologies). The Lenti-X Tet-Off Advanced lentiviral inducible expression system (Clontech Laboratories) was used to generate a stable UACC903 melanoma cell line that could induce the expression of GFP-ATF2. For this purpose, we used the Lenti-X Tet-Off Advanced lentiviral-inducible expression system, which requires the following 2 lentiviral constructs for tetracycline-controlled expression of ATF2: pLVX-Tet-Off Advanced (which is under G418 selection) and pLVX-Tight into which GFP-ATF2 was cloned (and which is under puromycin selection). The UACC903 melanoma cells were cotransduced with the 2 lentiviruses and selected by growth in G418- and puromycin-containing medium. The expression of GFP-ATF2 was repressed by the addition of the tetracycline analog doxycycline to the growth medium. The transfer of the cells into doxycycline-free medium then enabled the controlled expression of GFP-ATF2. Because melanocytes and melanoma cells are inherently resistant to G418 (19), we used fluorescence-activated cell sorting (FACS) to further enrich for GFP-ATF2–positive cells after withdrawal from doxycycline. The UACC903 GFP-ATF2 Tet-Off (hereafter referred to as GFP-ATF2Tet-Off UACC903) cell line was maintained in DMEM supplemented with 10% FBS, 500 ng/mL doxycycline, and 5 μg/mL puromycin, plus 1 mg/mL G418 to maintain the selection of stable lines.

Reagents
A 3,800 compound subset of the ChemBridge chemical library (ChemBridge) was provided by the Conrad Prebs Center for Chemical Genomics at our Institute. Dimethyl sulfoxide (DMSO), formaldehyde solution, crystal violet, Triton X-100 (TX-100), bovine serum albumin (BSA), and...
sucrose (PKc) were purchased from Sigma-Aldrich; the protein kinase C (PKC) inhibitor Go6850 was purchased from Calbiochem. MitoTracker Deep-Red FM was purchased from Invitrogen. Protease and phosphatase inhibitors cocktails (PhosSTOP and cOmplete, respectively) were purchased from Roche. The antibodies used were purchased as follows: ATF2 (#20F1), Stat3 (#9132), Cox IV (3E11), pATF2-T69/71 (#9225), pan-pPKC T514 (#9379), P38 (#9216), pP38 (#9212), pErk1/2 (#9106), Erk1/2 (#9102), PKC isoenzyme sampler kit (#9060), pAkt (#9271), and Akt (#9272) from Cell Signaling Technologies; PKCe (C15), ATF2 (N96 & C19), β-tubulin (G8), p53 (FL393), pan-PKC (H300), and GFP (B2) from Santa Cruz Biotechnology, Inc.; pS729 PKCe (44977G) from Invitrogen; HSP60 (clone 24) and β-catenin (clone 14) from BD Pharmingen; Actin (ACTN05) from Thermo-Fisher; p1/2 ATF2 antibodies were produced by PhosphoSolutions Inc.. Secondary antibodies were goat anti-rabbit Alexa-680 F(ab’)2, goat anti-rabbit Alexa-568 F(ab’)2, goat anti-mouse Alexa-488 F(ab’)2 (all Molecular Probes), and goat anti-mouse IRDye 800 F (ab’)2 (Rockland Immunochemicals).

Immunoblotting

Cell lysates were prepared by lysis in radioimmunoprecipitation assay buffer (RIPA) buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.25% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris–HCl pH 7.4, freshly supplemented with phosphatase and protease inhibitors). Lysates were clarified by centrifugation and proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking with PBS containing 5% non-fat milk, the membranes were incubated with primary antibodies [p1S2-ATF2 (1:500); ATF2 (#20F1; 1:1,000)] in PBS containing 5% BSA and 0.1% Tween-20, at room temperature for 1 hour or at 4°C overnight. The membranes were washed and incubated for 1 hour at room temperature with goat anti-rabbit Alexa Fluor 680 F(ab’)2, or goat anti-mouse IRDye 800 F(ab’)2 diluted at 1:10,000. The blots were subjected to IR imaging with 2-color detection using an Odyssey Infrared Imaging System (LiCor Biosciences), and quantification of bands was conducted according to the manufacturer’s protocols.

Transfections

Plasmid DNAs were transfected using jetPrime reagent (Polypus Transfection Inc.) according to the manufacturer’s protocol.

Immunofluorescence

Cells were cultured on glass coverslips, subjected to the indicated treatments, and fixed with 4% formaldehyde in 100 mmol/L phosphate buffer, pH 7.4, for 30 minutes. After fixation, cells were permeabilized with PBS containing 0.4% TX-100 and 0.4% BSA for 20 minutes, and incubated for 1 to 18 hours with primary antibodies in staining buffer (0.1% TX-100/0.1% BSA in PBS). Cells were washed 4 to 5 times with washing buffer (0.2% TX-100/0.2% BSA in PBS), followed by a 3-hour incubation with goat anti-rabbit Alexa-568 and/or anti-mouse Alexa-488 F(ab’)2 in staining buffer, followed by 2 additional washes in washing buffer. The coverslips were then mounted with Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Fluorescence images were acquired using either an Olympus IX-71 inverted microscope equipped with a digital ORCA-ER camera (Hamamatsu) or with an Olympus Fluoview 1000 confocal microscope equipped with a CH350 CCD camera (Hamamatsu). All images were processed with Adobe Photoshop CS5 (Adobe Systems). Fluorescence images were evaluated and quantified using ImageJ software (NIH, Bethesda, MD).

Additional Methods can be found online in Supplementary Data.

Results

ATF2 translocation assay

We developed a cell-based imaging assay to monitor the subcellular localization of ATF2 by expressing GFP-ATF2 in melanoma cells. For this purpose, UACC903 cells were cotransduced with a tetracycline regulator lentivirus (pLVX Tet-Off Advanced) and a response lentivirus (pLVX-Tight puro) into which the GFP-ATF2 insert (GFP-ATF2Tet-Off) UACC903 cells; see Materials and Methods) was cloned. We confirmed using fluorescence microscopy that GFP-ATF2 expression was prevented in cultures maintained in the presence of 300 ng/mL doxycycline but was induced and readily detectable in the nuclei within 36 to 48 hours of cell transfer into doxycycline-free medium (Fig. 1A). We also monitored the expression of GFP-ATF2 by immunoblotting, because excessive GFP-ATF2 expression might interfere with the magnitude or kinetics of ATF2 export from the nuclei. As shown in Fig. 1, 24 to 48 hours after withdrawal of doxycycline, GFP-ATF2 was expressed at levels comparable with (within 1- to 5-fold as determined by immunoblotting analysis) the endogenous ATF2 levels (Fig. 1B). Taken together, these results show that in GFP-ATF2Tet-Off UACC903 melanoma cells, GFP-ATF2 was expressed at levels comparable with endogenous ATF2 and was similarly localized within the nucleus.

Identification of hit compounds in a high-throughput screen

We conducted a pilot study to determine whether the GFP-ATF2Tet-Off UACC903 system was amenable for the high-throughput screening of small molecules that promote ATF2 nuclear export. For this purpose, we selected approximately 3,800 compounds from the ChemBridge compound library, containing representatives of different scaffolds and structures. The cells were plated in 12 384-well plates. Each plate included 32 wells treated with vehicle (0.2% DMSO) as negative controls and 32 wells treated with the PKC inhibitor Go6850 (5 μmol/L) as a positive control, whereas the remaining wells were treated with the library compounds (single compound, 10 μmol/L each well).
determine if GFP-ATF2 localization was affected by the compounds, we measured the ratio between the mean nuclear intensity and the mean cytoplasmic intensity of GFP fluorescence (Fig. 1C). Although in DMSO-treated cells, GFP-ATF2 was mainly localized to the nucleus, in G6850-treated cells, GFP-ATF2 was found both in the nucleus and in the cytosol (Fig. 2A and B). The average Z-value (20) for this screen based on the positive and negative control wells was 0.47 and ranged between 0.4 and 0.56 between plates. As noted in the Materials and Methods, the assay was designed to minimize influence of the autofluorescence of the positive control compound G6850. It is, however, possible that residual G6850 compound fluorescence might have artificially increased the reported Z-value beyond the true assay window. As expected, most compounds were inactive and GFP-ATF2 localization was mostly nuclear, similar to that observed in the DMSO-treated cells. The automated analysis initially identified 20 wells with activity more than 50%, but further examination revealed that 14 of 20 hits were due to auto-fluorescence or other artifacts, leaving 6 potential hits (0.16% hit rate). Two of 6 compounds failed the PAINS cheminformatics filter (for known Promiscuous and Assay-Interfering Nuisance compounds). The remaining 4 compounds were selected for further analysis using independently ordered powders, which were confirmed in a set of dose-dependent assays (Fig. 2C and data not shown).

Validation of hit compounds with endogenous ATF2

To confirm that the 4 hits identified in the GFP-ATF2Tet-Off UACC903 pilot screen are able to promote the cytoplasmic localization of endogenous ATF2, we ordered new batches of the compounds and tested their effects on endogenous ATF2 translocation in WM793 melanoma cell lines by immunofluorescence microscopy (Fig. 3A). The cells were incubated in the presence of the compounds for either 6 or 24 hours. The images were analyzed and quantified using ImageJ software, which confirmed that treatment with hit 3 (SBI-0089410; N-[2-(1-adamantyl)ethyl]-4-([(dimethylamino)sulfonyl]benzamide) or hit 4 (SBI-0087702; N-[(4-methoxy-1-naphthyl)methyl]-2-(4-methoxyphenyl)ethanamine), but not the other 2 hits (hits 1 and 2), promoted cytoplasmic/mitochondrial localization of endogenous ATF2 (Fig. 3B). Partial colocalization between GFP-ATF2 and mitochondria (using HSP60 as surrogate marker of mitochondrial localization) was observed (Fig. 3A, arrowheads) following treatment with both hits, thereby confirming their ability to promote ATF2 mitochondrial localization. SBI-0089410 and SBI-0087702 represent low-molecular weight compounds that do not seem to be promiscuous based on a SciFinder search of each structure. These compounds do not contain undesirable or reactive functional groups, and thus represent possible starting points for a hit-to-lead effort, and also illustrate that synthetically tractable hits can be obtained from a screen. Notably, the nuclear-to-cytoplasmic ratio of endogenous ATF2 was reduced to the same degree as seen for GFP-ATF2 in 0087702-treated cells as compared with control, DMSO-treated cells (Fig. 3B)]. Similar results were also observed in UACC903 melanoma cells (Fig. 3B). Interestingly, the effect of SBI-0087702 on ATF2 localization was detected only after incubation for 24 hours, whereas SBI-0089410 induced earlier translocation (after 6 hours), which was not sustained at later time points, suggesting that each of these compounds affects ATF2 translocation via a distinct mechanism/target(s). Consistent with the immunostaining data, subcellular biochemical fractionation revealed increased ATF2 in the purified VDAC1-containing mitochondrial fractions, as well as in the β-tubulin–containing cytosolic fraction of WM793 cells after 24-hour treatment with SBI-0087702. Similar increase of ATF2 in the mitochondrial fraction was observed in cells treated for 6 hours with SBI-0089410 (Supplementary Fig. S1A and S1B).

Notably, the treatment of UACC903 cells with DMSO, SBI-0089410, SBI-0087702, or G6850 did not affect the nuclear compartmentalization of p53 (Supplementary Fig. S1C), a transcription factor that partially localizes at the mitochondria following oxidative stress (21). Furthermore, neither SBI-0089410 nor SBI-0087702 affected the localization of Stat3 and β-catenin, under conditions that induced ATF2 translocation (Supplementary Fig. S2), suggesting that these compounds do not have pleiotropic effect on other transcription factors that can be also localized at the mitochondria.

SBI-0089410 and SBI-0087702 induce apoptosis and inhibit melanoma cell growth in an ATF2-dependent manner

To determine whether SBI-0089410 or SBI-0087702 affected melanoma cell growth, we measured the colony formation of 501Mel cells. Notably, although both compounds reduced colony formation, SBI-0087702 exhibited the greatest effect, reducing the number of colonies by approximately 90% (Fig. 4A). The inhibition of colony formation is likely an outcome of the cytotoxicity of these compounds. To verify that the cell death observed following treatment with SBI-0089410 and SBI-0087702 was mediated by ATF2 translocation, we examined whether overexpression of a constitutively nuclear ATF2 mutant (ATF2N) could rescue cell death. Indeed, ATF2N-stably transduced UACC903 cells exhibited reduced degrees (up to 50%) of cell death upon treatment by SBI-0089410 or SBI-0087702, compared with control empty vector–transduced cells (Fig. 4B and C). Furthermore, similar to their effect on UACC903 cells (which harbor mutated B-Raf and wild-type N-Ras), SBI-0089410 and SBI-0087702 induced apoptosis in WM1346 melanoma cells (which harbor wild-type B-Raf and mutated N-Ras), which was dependent on ATF2 (Fig. 4B and C). Notably, the combined treatment of PLX4032-resistant melanoma cell lines (501Mel and UACC903) with low dose (2 μmol/L) PLX4032 and 10 μmol/L of either of the 2 compounds suppressed their viability and colony-forming ability compared with PLX4032 alone (see data later). Importantly, the 2 compounds did not induce apoptosis in...
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A

Phosphate contrast

GFP

B

UACC903 GFP-ATF2

Anti-GFP

GFP-ATF2

Anti-ATF2 (N96)

ATF2

Actin

Ponceau

C

DAPI

ATF2

Overlay

Nuclear Metrics:

Cell 1

Cell 2

Area

817

1070

Int.

148

146

Cyt I

17

16

Nuc I

267

195

Create subpopulation

GFP Int > Threshold

Well statistics:

Mean, SD

Count

Published OnlineFirst April 15, 2013; DOI: 10.1158/1078-0432.CCR-12-2689
melanocytes, even when used at a higher concentration (20 μmol/L; Supplementary Fig. S3D), although in human foreskin (BJ) fibroblasts, they induced moderate levels of apoptosis (Supplementary Fig. S3D) relative to the melanoma cell lines. Treatment with SBI-0089410 (6 hours) resulted in an increased G2–M phase population, whereas SBI-0087702 (24 hours) reduced the G2–M population and increased sub-G1 (dead cell) content (Supplementary Fig. S3E), indicating that both compounds affect melanoma cells by distinct, albeit ATF2-dependent, mechanisms. Interestingly, the ability of ATF2T52E to attenuate the effect of SBI-0087702 was also accompanied by changes in cell morphology; ATF2T52E-expressing cells seemed more rounded and compact than empty vector–expressing cells (Supplementary Fig. S4A).

We next examined the effect of these 2 compounds on migration, a major phenotype of melanoma cells. Both SBI-0089410 and SBI-0087702 inhibited the motility of WM1346 cells across a porous membrane in a Boyden chamber assay. Notably, the expression of ATF2T52E in the WM1346 cells effectively reversed the inhibitory effect of SBI-0089410 and to lesser extent the effect of SBI-0087702 (Fig. 4D and Supplementary Fig. S4A–S4C). These results show that both compounds promote the apoptosis of melanoma cells and inhibit their migration in an ATF2-dependent manner.

SBI-0089410 and SBI-0087702 perturb mitochondrial membrane integrity and promote leakage

We previously showed that cytoplasmic ATF2 promotes cell death by binding to protein complexes at the mitochondrial membrane, resulting in reduced mitochondrial membrane potential, increased mitochondrial leakage, and subsequent apoptosis (14). Consistent with its effects on ATF2 localization, we found that SBI-0087702 compromised mitochondrial membrane potential and promoted mitochondrial leakage in WM793 and UACC903 cells (Fig. 5A and B and Supplementary Fig. S5, respectively). SBI-0089410 induced a transient decrease in mitochondrial membrane potential at 6 hours, which is consistent with its transient effect on ATF2 cytoplasmic localization.

To verify that the decrease in mitochondrial membrane potential observed following treatment with SBI-0089410 and SBI-0087702 was mediated by ATF2 translocation, we examined whether the overexpression of ATF2T52E (the constitutively nuclear ATF2 mutant) would prevent mitochondrial leakage (Fig. 5C). Indeed, the ectopic expression of ATF2T52E effectively attenuated the loss of mitochondrial membrane potential induced by both SBI-0089410 and SBI-0087702 but not by the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which promotes mitochondrial leakage in an ATF2-independent fashion.

To determine the potential of SBI-0089410 and SBI-0087702 to inhibit tumor growth, we assessed their ability to inhibit 3-dimensional (3D), anchorage-free growth using the spheroid growth assay (Fig. 4E and F). We found that after 8 days, SBI-0089410 and SBI-0087702 inhibited spheroid growth by more than 70% and 90%, respectively. These results suggest that these compounds are stable enough to affect long-term growth and are able to penetrate into 3D structures, characteristics required for effective therapy.

SBI-0089410 and SBI-0087702 inhibit melanoma cell growth and Thr52 phosphorylation of ATF2 in a PKCε-dependent manner

In cell viability assays, treatment with 10 μmol/L SBI-0087702 was sufficient to inhibit the growth of 501Mel cells by more than 90% (Fig. 5D, left). Significantly, the ectopic expression of a constitutively active form of PKCε (PKCεCA) prevented the cell death induced by SBI-0087702 treatment, suggesting that this compound mediates its effect on ATF2 translocation and cell viability via inhibition of PKCε (Fig. 5D, right). Because PKCε-mediated phosphorylation of ATF2 on T52 controls its subcellular localization, we next investigated whether SBI-0089410 and SBI-0087702 affect the phosphorylation of endogenous ATF2 at Thr52. Lysates from DMSO–, SBI-0089410–, or SBI-0087702–treated NIH3T3 cells were subjected to immunoblot analysis for altered phosphorylation on T52, as well as on T69/71, which are the sites for JNK/p38 phosphorylation. SBI-0089410, and to a lesser degree SBI-
0087702, reduced the level of ATF2 phosphorylation on T52 (SBI-0089410 exhibited comparable effects to that of the PKC inhibitor G06850; Fig. 6A). Interestingly, both G06850 and SBI-0087702 also attenuated the degree of ATF2 phosphorylation on T69/71, indicative of the effect of these compounds on JNK/p38-mediated phosphorylation of ATF2. Unlike the broad PKC inhibitor G06850, which also reduced the total ATF2 expression level, the selected hits were more selective and did not exhibit such an effect (Fig. 6A). We further tested whether SBI-0089410 might elicit similar effects on ATF2 phosphorylation in UACC903 cells (Fig. 6B). Indeed, SBI-0089410 inhibited ATF2 phosphorylation on Thr52 but to a lesser degree on Thr69/71. In addition, SBI-0089410 inhibited p38 phosphorylation. The inhibitory effect on phosphorylation was transient, peaking at 6 hours after incubation with SBI-0089410. This transient effect on ATF2 phosphorylation is consistent with the effect on ATF2 translocation (Fig. 3) and on mitochondrial leakage (Fig. 5 and Supplementary Fig. S5). Notably, SBI-0089410 and SBI-0087702 did not inhibit other key signaling molecules including Erk1/2, Akt, pan-PKC (Supplementary Fig. S6A), suggesting that these compounds do not affect signal transduction pathways that are central in melanomagenesis. Because SBI-0089410 and SBI-0087702 affected ATF2 phosphorylation by PKCe but not the phosphorylation of PKC itself, we set to assess their possible effect on the recruitment of PKC to cellular membranes. Interestingly, SBI-0089410, but not SBI-0087702, inhibited the recruitment of PKCe and other PKC isoforms to the membrane within 3 minutes of stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA; Fig. 6C and D and Supplementary Fig. S6B–S6E). At 10 μmol/L, SBI-0089410 inhibited PKC translocation, although this effect was stronger with...
increasing doses (10–50 μmol/L) or at lower TPA concentrations (Fig. 6C and Supplementary Fig. S6E). Taken together, these results suggest that SBI-0089410 and SBI-0087702 inhibit ATF2 phosphorylation on Thr52, thus preventing ATF2 nuclear translocation, and that the effect of SBI-0089410 is mediated by impairing PKC-to-membrane translocation.

Mapping transcriptional programs that are affected by SBI-0087702

To measure the effects of the 2 compounds on ATF2 transcriptional activities, we conducted transcription-based reporter assays and a quantitative PCR (qPCR) analysis of known ATF2 transcriptional target genes. Using an ATF2-targeted (Jun2) promoter-luciferase assay system, we evaluated how SBI-0089410 and SBI-0087702 affect the transcriptional activity of ATF2 (Fig. 6E). Consistent with the cytoplasmic/mitochondrial translocation effects of SBI-0089410 and SBI-0087702 on ATF2, these compounds reduced the transcriptional activity of ATF2 in UACC903 cells at 24 hours by approximately 47% and 56%, respectively. Notably, the effect of the compound was more pronounced than a PKCε peptide inhibitor, which reduced ATF2 transcriptional activity by approximately 24%. Assessment of the Jun2-luciferase activity in another melanoma cell line, WM793, revealed that SBI-0089410 and SBI-0087702 similarly reduced ATF2 transcriptional activity at 24 hours by approximately 30% and 58%, respectively, whereas the PKCε peptide inhibitor reduced ATF2 transcriptional activity by approximately 37% (Supplementary Fig. S6F). Evaluation of the effects of these compounds on the activity of the c-Jun–targeted TRE promoter did not reveal inhibition but rather slightly increased c-Jun activity (Supplementary Fig. S6G). To further define the specificity of these compounds, we examined the effects of SBI-0089410 and SBI-0087702 on the transcriptional activities of the heat shock response element (HSRE) and androgen receptor (AR), which are representative of independent transcriptional regulatory elements. As shown in Supplementary Fig. S6H and S6I, neither of the 2 compounds caused notable changes in the transcriptional activity from these unrelated promoters.
In comparing the effect of the 2 compounds on ATF2 translocation, phosphorylation and sensitization of melanoma cells to apoptosis, SBI-0087702 seemed to be superior. Therefore, we further characterized the transcriptional changes elicited by this compound. To this end, we subjected WM793 cells treated with either DMSO or 10 μmol/L SBI-0087702 for 24 hours to microarray-based expression analysis [see details in the Materials and Methods; data deposited in Gene Expression Omnibus (GEO) accession # GSE43135]. The top 3 functional networks upregulated in response to SBI-0087702 (Fig. 6F) were as follows: (i) lipid/smolecule/vitamin and mineral metabolism, (ii) cellular development, and (iii) carbohydrate/small molecule/lipid metabolism. Notably, 2 of 3 major networks that were upregulated are associated with lipid metabolism, consistent with the increase seen in mitochondrial mass (Supplementary Fig. S5B). The top 3 downregulated clusters were: (i) tumor morphology/cell growth and proliferation/development, (ii) cell morphology, and (iii) cellular movement. Here, all of the 3 major networks affected by SBI-0087702 are associated with cell morphology and growth, consistent with the effects elicited by this compound on the migratory behavior of the melanoma cells (Fig. 4 and Supplementary Fig. S4). The corresponding top 20 up- and downregulated genes are shown in Supplementary Tables S1 and S2.

Discussion

Oncogene addiction is the phenomenon, whereby the survival of cancer cells depends on constitutive oncogenic...
PKCε, which confers its oncogenic addiction and attenuates its role in genotoxic stress-induced cell death (14). We therefore sought to establish an imaging-based high-throughput screen to identify small molecules capable of inducing the nuclear export of ATF2 in melanoma cells. We report here on the establishment and the results of such proof-of-concept screen. The GFP-ATF2T52E (JAC903) cell line was used to conduct the initial screen of 3,800 compounds, from which we identified 2 small molecules that promote cytoplasmic/mitochondrial localization of ATF2 in melanoma cells. The compounds were confirmed to reduce mitochondrial membrane potential and to have concomitant effects on melanoma growth and colony

In melanoma, ATF2 is largely confined to the nucleus, predominantly due to its constitutive phosphorylation by PKCε, which confers its oncogenic addiction and attenuates its role in genotoxic stress-induced cell death (14). We therefore sought to establish an imaging-based high-throughput screen to identify small molecules capable of inducing the nuclear export of ATF2 in melanoma cells. We report here on the establishment and the results of such proof-of-concept screen. The GFP-ATF2T52E (JAC903) cell line was used to conduct the initial screen of 3,800 compounds, from which we identified 2 small molecules that promote cytoplasmic/mitochondrial localization of ATF2 in melanoma cells. The compounds were confirmed to reduce mitochondrial membrane potential and to have concomitant effects on melanoma growth and colony

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formulation. Importantly, the effects of the compounds could be partially blocked by the overexpression of a constitutively active form of PKCe or a phosphomimic ATF2 mutant that is constitutively active transcriptionally. These observations strongly suggest that the compounds mediate their inhibitory effects through PKCe and ATF2.

Interestingly, SBI-0089410 reduced ATF2 phosphorylation on Thr52 by a similar degree as the PKCe inhibitor Gö6850, suggesting that this compound directly affects PKCe or its phosphorylation of ATF2. SBI-0087702 was effective in inhibiting ATF2 Thr52 phosphorylation, albeit to lesser degree compared with SBI-0089410, suggesting that its mechanism of action might be at least partially independent of the PKCe–ATF2 axis. Consistent with this possibility, the duration of the effects of these 2 compounds were different. SBI-0087702 elicited sustained ATF2 phosphorylation effects of the compounds through PKC

Figure 6. SBI-0089410 and SBI-0087702 inhibit melanoma cell growth and ATF2 transcriptional activity, and affect the expression of ATF2 target genes in a PKCe-dependent manner. A, NIH3T3 cells were incubated with DMSO, 10 µmol/L SBI-0089410 (410) for 6 hours, 10 µmol/L SBI-0087702 (722) for 24 hours, or with 10 µmol/L of the PKCe inhibitor Gö6850. Anisomycin was then added for 30 minutes to prevent de novo protein synthesis and to activate cellular stress responses. Cells were lysed in RIPA buffer and analyzed by Western blotting with the indicated antibodies. Protein bands were quantified using ImageJ software (numbers below). B, SBI-0089410 (410) transiently inhibited ATF2 and p38 phosphorylation in UACC903 melanoma cells. Cells were pretreated with SBI-0089410 for 3 to 9 hours, before stimulation with anisomycin. SBI-0089410 inhibited ATF2 phosphorylation at Thr52 and p38 phosphorylation with maximum activity after 6 hours incubation. C, SBI-0089410 inhibited the TPA-induced membrane translocation of PKCe. WM793 cells were incubated in the presence of SBI-0089410 or vehicle (DMSO) for 6 hours, then stimulated with 100 nmol/L TPA for 3 minutes, washed in cold PBS, lysed in lysis buffer, and the membrane and cytosol fractions were isolated according to the protocol detailed in Materials and Methods. The different fractions were analyzed by Western blotting using the antibodies indicated. D, WM793 cells were treated with compounds and TPA as in C and subsequently fixed with 4% formaldehyde before processing for immunofluorescence using anti-PKCε primary and fluorescence isothiocyanate (FITC)-conjugated goat anti-rabbit–immunoglobulin G (IgG) secondary antibodies. Imaging was conducted using an Olympus IX-71 fluorescent microscope. Arrowheads point to PKCe staining at the plasma membrane and the insets show enlarged region near the plasma membrane. Scale bar, 10 µm. E, luciferase assay evaluation of the effects of SBI-0089410 and SBI-0087702 on ATF2 transcriptional activity. UACC903 cells were stably transfected with a secreted 3X Jun2–(Gaussia) Luciferase construct. The cells were then transiently transfected with pCMV-Cypridina luciferase (normalization control), followed by treatment with DMSO, 10 µmol/L SBI-0089410, 10 µmol/L SBI-0087702, or with 10 µmol/L PKCe translocation peptide inhibitor (PKCe-i). At the indicated time points (0, 1, 6, 24 hours – T0, T1, T6, T24), Cypridina and Cypridina luciferase activities were measured, and Cypridina luciferase activity was normalized to Cypridina luciferase activity. The graph represents the mean Jun2-luciferase activities ± SD relative to DMSO of 3 replicate experiments. F, Ingenuity Pathway Analysis of gene expression changes observed upon treatment with SBI-0087702 identifies main clusters that are up- or downregulated. The lists of top 20 genes for each cluster are provided in Supplementary Tables S1 and S2. The original data were deposited into GEO (GSE43135).
translocation, whereas the effect of SBI-0089410 was more rapid but transient, perhaps due to its reduced stability or alternate cellular target. Likely, SBI-0089410 and SBI-087702 elicit their effect on ATF2 and melanoma cells through different pathways. Consistent with the gene expression profiling, SBI-087702 reduces the expression of genes associated with growth and proliferation, whereas increasing mitochondrial lipid biosynthesis, corresponding to the inhibition of melanoma growth and tumorigenicity seen in cultured cells. Arguably, each of the compounds identified in this screen exhibit a profile seen for G6850, pointing to a more selective effect. Among the 2 selected compounds that were characterized here, SBI-0087702 exhibits the more desired profile with respect to ATF2 translocation, phosphorylation, and melanoma sensitization to apoptosis.

Although we do not yet understand the mechanism by which the small molecules might induce ATF2 nuclear export and function at the mitochondria, several possibilities exist. The compounds might inhibit PKCζ activity (as seems to be the case for SBI-0089410), or they might activate a protein phosphatase, which would increase T52 dephosphorylation, thereby promoting ATF2 nuclear export. Alternatively, the compounds might modify ATF2 to facilitate its interaction with nuclear export factors. We cannot exclude the possibility that the compounds might elicit more indirect and global effects; for example, by influencing the function of import/export proteins in general. However, we note that the hit validation testing funnel was designed to exclude hits that affect ATF2 localization influencing the function of import/export proteins in general. This approach can be readily adapted to evaluate other transcription factors that elicit opposing functions dependent on their subcellular localization.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: T. Varsano, E. Lau, C.A. Hassig, Z.A. Ronai
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Acknowledgments
The authors thank Drs. Meenhard Herlyn, Ruth Halaban, and Gavin Robertson for the melanoma cell lines used in this study. The authors also thank Dr. Edward Mosonov and the Cell-Imaging Facility for help with cell sorting.

Grant Support
This study was supported by NIH grant CA099961 (to Z.A. Ronai) and a Melanoma Research Alliance grant (to Z.A. Ronai). E. Lau was supported in part by NIH T32 grant CA11949.

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Received August 15, 2012; revised March 13, 2013; accepted March 28, 2013; published OnlineFirst April 15, 2013.

References
RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. Cancer Cell 2010;18:683–95.
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