

Hippo/Mst1 Stimulates Transcription of the Proapoptotic Mediator *NOXA* in a FoxO1-Dependent Manner

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Abstract

The proapoptotic protein Noxa, a member of the BH3-only Bcl-2 protein family, can effectively induce apoptosis in cancer cells, although the relevant regulatory pathways have been obscure. Previous studies of the cytotoxic effects of α -tocopheryl succinate (α -TOS) on cancer cells identified a mechanism whereby α -TOS caused apoptosis requiring the Noxa-Bak axis. In the present study, *ab initio* analysis revealed a conserved FoxO-binding site (DBE; DAF-16 binding element) in the *NOXA* promoter, and specific affinity of FoxO proteins to this DBE was confirmed by fluorescence anisotropy. FoxO1 and FoxO3a proteins accumulated in the nucleus of α -TOS-treated cells, and the drug-induced specific FoxO1 association with the *NOXA* promoter and its activation were validated by chromatin immunoprecipitation. Using siRNA knockdown, a specific role for the FoxO1 protein in activating *NOXA* transcription in cancer cells was identified. Furthermore, the proapoptotic kinase Hippo/Mst1 was found to be strongly activated by α -TOS, and inhibiting Hippo/Mst1 by specific siRNA prevented phosphorylation of FoxO1 and its nuclear translocation, thereby reducing levels of *NOXA* transcription and apoptosis in cancer cells exposed to α -TOS. Thus, we have demonstrated that anticancer drugs, exemplified by α -TOS, induce apoptosis by a mechanism involving the Hippo/Mst1-FoxO1-Noxa pathway. We propose that activation of this pathway provides a new paradigm for developing targeted cancer treatments. *Cancer Res*; 71(3); 946–54. ©2011 AACR.

Introduction

The total number of cells forming individual organs is determined by the balanced act of cell proliferation and cell death. For example, epithelial cells exhibit contact inhibition arising from cell–cell and cell–substratum interactions (1). However, tumors feature impaired mechanisms of contact inhibition, resulting in rampant cell proliferation. Thus, specific reactivation of these control mechanisms in cancer cells can promote inhibition of tumor progression associated with induction of apoptosis.

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The Hippo pathway controls organ size by inhibiting cell proliferation, and mutations of its constituents result in robust growth (2). The central mediator of the Hippo pathway is the mammalian sterile 20-like kinase-1 (Mst1), a mammalian homolog of the Hippo kinase from *Drosophila*, which promotes both proper exit from the cell cycle and apoptosis during development (3–5). In *Drosophila*, the loss of Hippo results in elevated transcription of cyclin E and the cell-death inhibitor d-IAP1, an antagonist of caspases. The Hippo pathway regulates transcription by the coactivator Yorkie (6), a homolog of the mammalian Yes-associated protein (YAP) and the transcription coactivator with PDZ domain (TAZ), which are sequestered by the 14-3-3 protein following their Hippo-dependent phosphorylation (7).

Evidence exists for another branch in the Hippo signaling pathway, originally identified in nematodes. It has been demonstrated that the Hippo/Mst1 kinase directly phosphorylates and activates the forkhead box proteins (FoxO), a conserved group of transcription factors with the forkhead DNA-binding domain (8). Under stress conditions, FoxO causes expression of proapoptotic genes (9), including *BIM* (10), *FASL* (11), and *TRAIL* (12). Expression of these genes has been shown to be stimulated in conjunction with a decrease in the Akt activity (13–15).

We have reported α -tocopheryl succinate (α -TOS) as a cancer cell-selective apoptosis inducer, which involves ROS accumulation followed by cell death (16, 17). Agents like α -TOS, members of a class of redox-silent mitocans from

the group of vitamin E (VE) analogues (18), have been reported as potentially highly intriguing anticancer drugs because of their greater specific activity for cancer cells (19–23). We have discovered that the mechanism of ROS production by α -TOS is based on displacement of ubiquinone from its site in the mitochondrial complex II (24). Our recent work points to the Bak channel being preferentially formed in cancer cells exposed to α -TOS and we also show that this process is modulated by Noxa, which is upregulated transcriptionally in a p53-independent manner (25). To date, the signaling pathway(s) causing transcriptional regulation of Noxa in response to anticancer drugs such as α -TOS have not been clarified.

In this communication, we describe the Hippo/Mst1-FoxO1-Noxa axis as a novel signaling pathway that controls apoptosis in cancer cells exposed to anticancer drugs. This pathway links the early events in cancer cells exposed to stressors like α -TOS and formation of a pore in the mitochondrial outer membrane, a hallmark of the intrinsic apoptotic machinery essential for efficient killing of cancer cells and tumor therapy by a host of different anticancer drugs.

Material and Methods

Cell culture

Jurkat T lymphoma cells and the p53^{-/-} H1299 non-small cell lung cancer cells were grown in the RPMI-1640 medium supplemented with antibiotics and 10% FBS. Jurkat cells at 5×10^5 cells per mL and the adherent H1299 cells at 70% to 80% confluence were treated with α -TOS (Sigma-Aldrich) dissolved in EtOH. Control cells received corresponding volumes of EtOH only ($\leq 0.1\%$, v/v).

Databases

Sequences of the tested genes including the *NOXA* promoter and the conserved DBE motif were obtained from *Ensembl* (26). Predictions of the *cis*-regulating elements were carried out using *cis-RED* (27).

Design of primers

All primers were designed using the *GeneRunner* software (www.generunner.net/). The length of primers was between 18 and 23 nucleotides, the GC content was between 55% and 60%, and all primers were tested for secondary structure presence to avoid interfering structures. The following primers were used for PCR analysis: *NOXA*, forward: TGT AGT TGG CAT CTC CGC GC, reverse: CTC GAC TTC CAG CTC TGC TG; *MST1*, forward: CGC CGG CAG CTG AAA AAG TT, reverse: GCC CCA CAG TAC TCC ATA AC; *MST2*, forward: CAC CCA CCC CAC AAC CTA AA, reverse: CGA CAA CTT GAC CGG ATT CC; *FASL*, forward: AGC TGA GGA AAG TGG CCC AT, reverse: CTT CCC CTC CAT CAT CAC CA; *TRAIL*, forward: CCC TGC TGG CAA GTC AAG TG, reverse: GCT GCT ACT CTC TGA GGA CC; *BIM*, forward: GCA CAT TTC CCT CTG GCC TG, reverse: CCC ACG GGA GGC ATA CTT TC TG; and human ribosomal protein large (RPLP0) *P0*, forward: TCG ACA ATG GCA GCA TCT AC, reverse: ATC CGT CTC CAC AGA CAA GG.

RT-PCR and PCR analysis

Harvested cells were lysed in 1 mL of the TRIzol reagent (Invitrogen) and RNA was purified from the aqueous phase using the RNeasy Mini Kit (Qiagen). The purified RNA (0.5 μ g) was used for reverse transcription employing the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs). One microliter of cDNA was used for PCR analysis, which was performed using the PCR Master Mix (Promega) and the following conditions: initial denaturation 95°C for 2 minutes; cycles of 60°C for 30 seconds, 72°C for 30 seconds, and 95°C for 30 seconds; and the final extension at 72°C for 5 minutes.

Western blotting

Cells for the whole-cell analysis were washed with PBS and lysed in RIPA buffer, nuclear and cytosolic fractions were isolated using the Nuclear Extract Kit (Active Motif). Protein concentrations of individual samples were assessed using the EZQ kit (Molecular Probes). Eighty micrograms of whole-cell lysates, 30 μ g of nuclear extracts, or 100 μ g of cytosolic extract were applied to SDS-PAGE (Bio-Rad). After electrophoresis, proteins were blotted onto PROTEAN nitrocellulose transfer membranes (Sigma-Aldrich) using the Mini Trans-Blot Cell (Bio-Rad). For immunostaining, membranes were blocked and incubated with the primary antibody according to the manufacturer's protocol followed by incubation with a suitable secondary HRP-labeled antibody (Sigma-Aldrich). Immunodetection was conducted with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the MF-ChemiBIS bio-imaging system (DNR). The following antibodies were used: anti-Noxa IgG (Alexis), anti-FasL IgG (Abcam), anti-TRAIL IgG (Abcam), anti-FoxO1 IgG, anti-FoxO3a IgG, anti-phospho-Mst1 IgG (Thr183)/Mst2 (Thr180), anti-Mst1 IgG (all from Cell Signaling), anti-actin IgG, anti-lamin B IgG (both from Santa Cruz Biotechnology), and anti-phospho FoxO IgG (Invitrogen). The following antibodies were used for the ChIP analysis: anti-FoxO1 IgG, anti-GFP IgG (both from Santa Cruz Biotechnology), anti-FoxO3a IgG (Abcam), anti-RNA Pol II (Active Motif), rabbit nonspecific IgG (Active Motif), and anti-acetyl lysine IgG (Abcam).

Steady-state fluorescence and steady-state fluorescence anisotropy DNA-binding measurements

Fluorescence spectra were recorded using the PerkinElmer Life Sciences LS50B spectrofluorometer at 22°C with 0.6 μ mol/L protein and 0.6 μ mol/L dsDNA in 50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 1 mmol/L EDTA in a 10-mm cell. The excitation wavelength was 336 nm, the bandwidths were 5 nm for both excitation and emission. The following oligonucleotide containing the consensus FoxO-binding sequence were used to prepare samples of dsDNA: fluorescein (FLC)-labeled S1FLC, 5'-FLC-TTG GGT AAA CAA G-3'; and S2FLC, 5'-FLC-CTT GTT TAC CCA A-3'.

The steady-state fluorescence measurements were performed on a PerkinElmer Life Sciences LS50B fluorescence spectrometer at 22°C with 100 nmol/L dsDNA labeled with fluorescein at the 5' terminus of both strands (S1FLC/S2FLC). Increasing amounts of the recombinant FoxO DBD were added and the steady-state fluorescence anisotropy of fluorescein was

recorded (excitation, 494 nm; emission, 520 nm) at each FoxO-DBD concentration. K_d was calculated according to a recent report (28).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using a ChIP kit with enzymatic shearing (Active Motif). Briefly, nuclei isolated from 4×10^7 α -TOS-treated or control Jurkat cells were sheared for 10 minutes using the Enzymatic Shearing Cocktail, precleared by incubation with protein G beads to reduce the nonspecific background (10 μ L of pre-cleared chromatin was saved as input) and incubated overnight at 4°C on a rotator with anti-FoxO1 IgG, anti-RNA Pol II or anti-acetyl lysine antibody IgG and nonspecific rabbit IgG or anti-GFP IgG as a negative control. In the next step, the antibody complexes were precipitated by incubation with protein G beads and after several washes eluted with the solution of SDS and NaHCO₃. The nucleoprotein complexes were then de-cross-linked by an overnight incubation at 65°C and treated with RNase A and proteinase K. The resulting DNA molecules were purified using the DNA purification minicolumns, and 5 μ L of purified DNA was used for PCR analysis with the following primers: *DBE*, forward: 5'-TCC CTT CCC TGT TAC TGC CC-3', reverse: 5'-GCG GGA GGG GAA GGG TTT AA-3'; *transcription start site (TSS)*, forward: 5'-ACG TCA CCA GGG AAG TTC TC-3', reverse: 3'-AAC ACG AAC AGT

CCT GCA GG-5'; *distal site*, forward: 5'-TTG GCA AGG CTG GTC TCG AA-3', reverse: 5'-GTA GCC TTC CCA GCA ATC AG-3'. A standard PCR program with 35 cycles was used.

Transfection and siRNA experiments

FoxO1 and FoxO3a proteins were knocked down by transfecting Jurkat or H1299 cells with specific siRNAs (Santa Cruz Biotechnology) using the transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's protocol. Protein levels were determined by Western blotting and experiments performed following 72 hours of siRNA exposure. The Mst1 protein was knocked down by transfection with 4 specific siRNAs (Thermo Scientific) into Jurkat cells employing the DharmaFECT 4 reagent (Thermo Scientific). In Jurkat cells, *NOXA* mRNA levels were quantified 4 hours after α -TOS addition and in the H1299 cells after a 6-hour exposure to the drug.

Results

α -TOS triggers transcription of proapoptotic genes

RT-PCR analysis revealed that the Jurkat cells responded to the drug stressor by an increase in the transcripts of several genes positively regulating apoptosis, e.g., *NOXA*, *FASL*, and *TRAIL* (Fig. 1A). The kinetics of expression of *NOXA*, *FASL*, and *TRAIL* paralleled each other, suggesting that similar

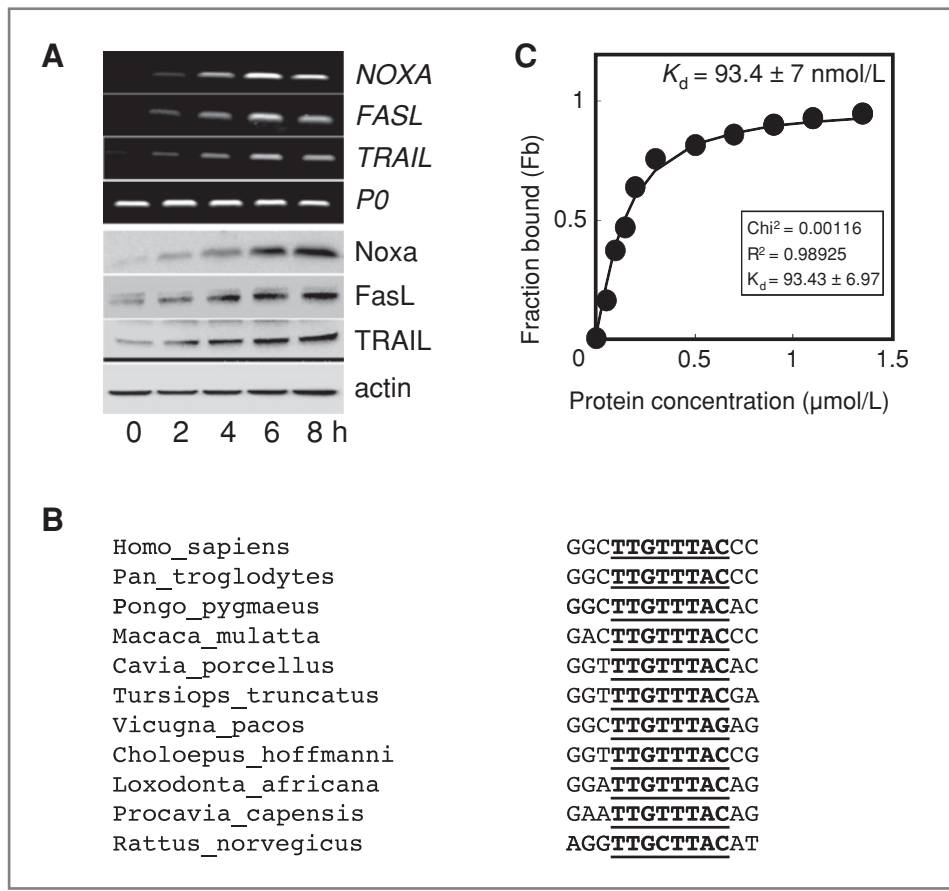


Figure 1. α -TOS stimulates transcription of proapoptotic genes. A, Jurkat cells were exposed to 50 μ mol/L α -TOS and assessed by RT-PCR for the levels of the *NOXA*, *FASL*, and *TRAIL* mRNA, and by Western blotting for the expression of the Noxa, FasL, and TRAIL proteins. *P0* was used as a house-keeping gene for RT-PCR and actin as a loading control for Western blotting. The images shown are representative of 3 independent experiments. B, sequence alignments of part of the *NOXA* promoter from different mammalian species containing the conserved DBE. C, the DBD fragment of the recombinant FoxO protein was incubated at increasing levels with the fluorescein-labelled DBE of the *NOXA* promoter, and binding was assessed by fluorescence anisotropy. The K_d value and statistical significance were calculated using the ORIGIN software.

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mechanisms may be involved in their regulation. The levels of the Noxa, FasL, and TRAIL proteins correlate with the mRNA data (Fig. 1A).

Since Noxa, a BH3-only Bcl-2 family protein (28), was found to promote formation of the Bak channel in cancer cells exposed to α -TOS (29), we further focused here on the regulation of *NOXA* transcription. We found that this process, although previously shown to be regulated mainly by p53 (29), was under our conditions regulated in a p53-independent manner. We also observed decreased mRNA levels for other transcription factors suggested to regulate *NOXA* transcription, that is, c-Myc and p73 (data not shown) during α -TOS treatment, indicating that in our system transcription of the *NOXA* gene may also involve other factor(s) (30).

Ab initio analysis of NOXA promoter reveals the presence of a DAF-16-binding element

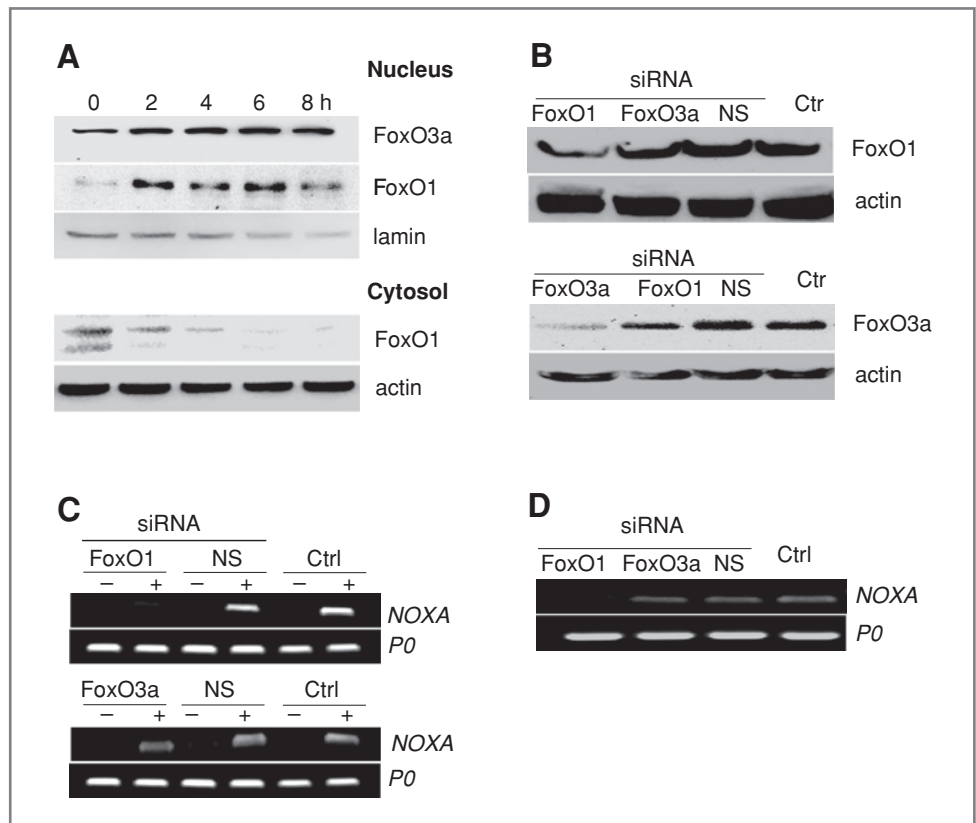
To identify elements that may regulate transcription of the *NOXA* gene, we performed *ab initio* analysis of the human *NOXA* promoter, employing *Ensembl* and the *cis*-Regulatory Element Database (*cis-RED*; ref. 31). This resulted in the identification of a conserved DBE, which is constrained in this position of the *NOXA* gene in at least 11 different mammalian species (Fig. 1B). This DBE is present in the human *NOXA* promoter with the + strand sequence TTG TTT AC localized on chromosome 18 in the position 57,566,785 to 57,566,792, implicating regulation by FoxO proteins (32).

To assess the affinity of the DBE within the *NOXA* promoter for the forkhead box DNA-binding domain (DBD), fluorescence anisotropy was measured using a fluorescently labelled DNA sequence from the part of the *NOXA* promoter carrying the DBE titrated with the recombinant DBD common to FoxO proteins. On the basis of this assay, we calculated the value of $K_d = 93.4 \pm 7$ nmol/L (Fig. 1C) for the interaction of the FoxO protein with the DBE. This indicates high-affinity binding, similar to that of the well established DBE from the *insulin receptor* promoter used as a reporter (28).

The FoxO1 protein regulates NOXA transcription

Because several reports suggested a role for the FoxO1 (14, 33) and FoxO3a proteins in regulating apoptosis (10–12), we tested their nuclear translocation as a marker of activation in cancer cells exposed to α -TOS, analyzing nuclear and cytosolic fractions by Western blotting. We observed barely detectable levels of the FoxO1 protein in the nucleus of control Jurkat cells, whereas a significant pool of the FoxO3a protein was detected in the control cell nuclei (Fig. 2A). The FoxO1 protein translocated to the nucleus within a 2-hour exposure of the cells to α -TOS, and the levels of the protein remained relatively unchanged over the period of exposure. On the other hand, nuclear levels of the FoxO3a protein gradually increased during the treatment. We also observed decreasing levels of FoxO1 in cytosolic fraction of Jurkat cell during α -TOS treatment (Fig. 2A), whereas the total level of the protein remained

Figure 2. FoxO proteins translocate to the nucleus in cancer cells after α -TOS treatment and are responsible for *NOXA* transcription. A, Jurkat cells were exposed to 50 μ mol/L α -TOS and 30 μ g of the nuclear fractions assessed for the presence of FoxO1 and FoxO3a proteins by Western blotting, with lamin B used as the loading control. 100 μ g of cytosolic fraction was assessed for FoxO1 protein. B, H1299 cells were transfected with FoxO1, FoxO3a, or nonsilencing (NS) siRNA and the expression of FoxO1 and FoxO3a protein evaluated by Western blotting. H1299 (C) and Jurkat cells (D) were transfected with FoxO1, FoxO3, or NS siRNAs, treated with α -TOS for 4 hours (Jurkat cells) or for 6 hours (H1299 cells), and the *NOXA* mRNA levels detected by RT-PCR. The images shown are representative of 3 independent experiments.



unchanged (data not shown). To further define the role of FoxO1 and FoxO3a proteins in the regulation of the *NOXA* gene, we knocked down the individual FoxO proteins with specific siRNAs and assessed *NOXA* mRNA levels in the α -TOS-exposed Jurkat and p53^{-/-} H1299 cells. Figure 2B shows that the RNA interference (RNAi) approach reduced the levels of the individual FoxO proteins. We next tested the effect of the FoxO siRNAs on the expression of the *NOXA* gene. The results clearly show that only FoxO1 siRNA decreased the *NOXA* mRNA levels, in both the p53^{-/-} H1299 (Fig. 2C) and Jurkat cells (Fig. 2D).

The FoxO1 protein binds to the DBE in the *NOXA* promoter and causes its activation

To establish if the FoxO1 proteins are enriched in the *NOXA* promoter on the newly identified DBE after α -TOS treatment, we performed ChIP assays in Jurkat cells exposed to α -TOS for 4 hours. A significant increase in the FoxO1 protein levels bound to this *NOXA* promoter region was observed after α -TOS treatment (Fig. 3A). These results confirm that the FoxO1 protein is activated to directly interact with the DBE in the *NOXA* promoter after α -TOS exposure and suggest that this promotes the induction of the *NOXA* gene transcription in cells exposed to the VE analogue. In support of this, we found RNA polymerase II (RNA Pol II) bound to the *NOXA* gene transcription starting site (TSS) in Jurkat cells exposed to α -TOS (Fig. 3B) while, interestingly, RNA Pol II was detected to bind in the distal part of the *NOXA* promoter in control Jurkat

cells. This suggests that RNA Pol II associates with the *NOXA* promoter in the resting cells and that an activation signal is needed to promote initiation of transcription of the *NOXA* gene, such as acetylation-induced chromatin opening promoted by FoxO proteins. In addition, we also tested the presence of the FoxO3a in the *NOXA* gene promoter using ChIP assay, but we observed only weak signal with nearly the same intensity in control and α -TOS-treated cells (Supplementary Fig. S1).

We next used a FoxO activity marker to assess possible changes in the chromatin structure associated with the FoxO1 activity in the *NOXA* promoter. Thus, ChIP assays were carried out using an antibody against acetyl lysine, which represents an epigenetic modification on histone proteins and proteins involved in transcription commonly occurring on active promoters (34) and is also associated with the FoxO activity (35). We monitored 3 regions in the *NOXA* promoter using specific primers (the region around the TSS, the DBE, and within the distal part, some -1,000 bp from the TSS) for lysine acetylation in cells treated with α -TOS (Fig. 3D). In control cells, we detected a low signal only for the DBE region-specific primers, and observed an increase in the signal in α -TOS-treated cells for the DBE- and TSS-specific primers. No signal was observed using primers specific for the distal region (Fig. 3C). These results indicate that the region around TSS (some -500 bp) of the *NOXA* gene promoter may represent an active part of the promoter which functions in the regulation of the *NOXA* gene transcription.

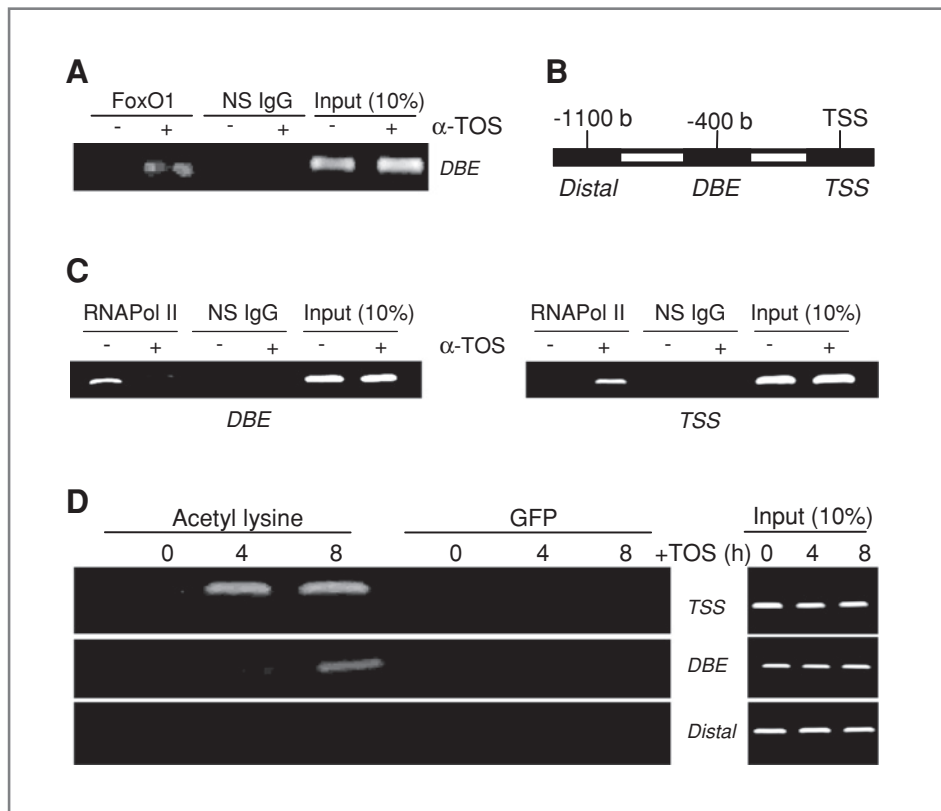


Figure 3. FoxO1 protein binds to and activates the *NOXA* promoter. A, cross-linked and sheared chromatin from control and α -TOS-treated Jurkat cells (50 μ mol/L, 4 hours) was immunoprecipitated with anti-FoxO1 IgG or nonspecific (NS) rabbit IgG. The precipitates were then probed for the presence of DNA fragments containing the DBE from the *NOXA* gene promoter using primers specific for this region. Pre-cleared and de-cross-linked chromatin diluted 10-fold was used as the loading control. B, Jurkat cells were treated with 50 μ mol/L α -TOS for 4 hours and assessed by the ChIP method for binding of RNA Pol II to the TSS and DBE sites in the *NOXA* promoter. C, Jurkat cells were treated with α -TOS (50 μ mol/L) and assessed for lysine acetylation in the *NOXA* promoter by the ChIP method. Anti-GFP IgG was used as a negative control. The images shown are representative of 3 independent experiments. Panel D indicated the position of the Distal and DBE sites and TSS in the *NOXA* promoter.

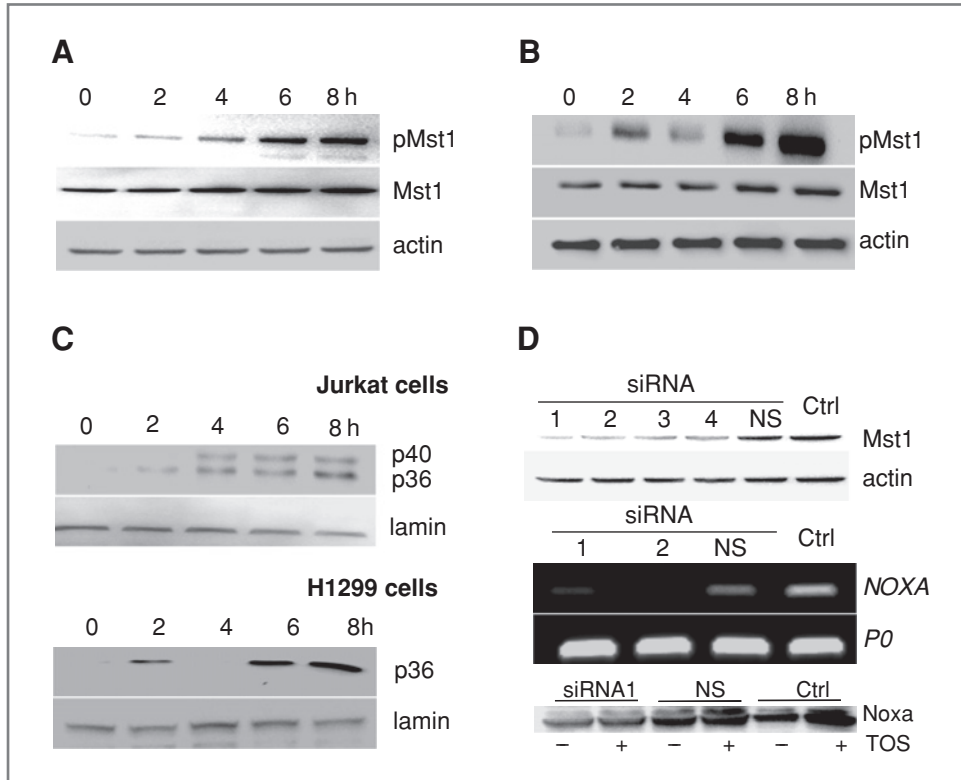


Figure 4. Hippo/Mst kinase is activated in cancer cells exposed to α -TOS and modulates Noxa expression. (A) H1299 and (B) Jurkat cells were exposed to 50 μ mol/L α -TOS and assessed for phosphorylation of the Hippo/Mst kinase on threonine 183 and for total Mst1 protein levels by Western blotting. C, Jurkat and p53^{-/-} H1299 cells were exposed to 50 μ mol/L α -TOS for the time periods shown and the nuclear fraction assessed for the p40 and p36 fragments of activated Mst1 kinase by Western blotting with antibody against the Mst1 protein phosphorylated on threonine 183. D, four different Mst1-specific siRNAs were tested for their ability to decrease Noxa level in Jurkat cells. Parental Jurkat cells or cells pretreated with two different Mst1-specific or NS siRNA were exposed to 50 μ mol/L α -TOS for 4 hours and analyzed for *NOXA* mRNA levels by RT-PCR. Jurkat cells treated with the Mst1-specific siRNA1 or NS siRNA and parental cells were tested for Noxa protein level after 4 hours of treatment by α -TOS using Western blotting. The images shown are representative of 3 independent experiments.

The Hippo/Mst1 kinase regulates FoxO1 activation

The FoxO1 protein has been reported as a substrate for several kinases (36), which are responsible for its regulation. Of these, we detected activation (phosphorylation) of the Hippo/Mst1 kinase in Jurkat as well as H1299 cells within 2 hours following addition of α -TOS (Fig. 4A and B). We also observed the appearance of the p36 and p40 fragments of the Hippo/Mst1 protein in the nuclear fraction of Jurkat cells exposed to α -TOS (Fig. 4C). Formation of the cleaved protein fragments has been documented to occur during activation of the kinase and is important for its proapoptotic functions (37, 38). Surprisingly, we were able to detect only one active kinase fragment in the nucleus of H1299 cells (Fig. 4C). This indicates a cell type-specific Hippo/Mst1 kinase processing.

We next tested the effect of lowering the Hippo/Mst1 protein levels by 2 different Mst1-specific siRNAs on the *NOXA* gene expression in Jurkat cells. We observed a significant decrease in the Noxa mRNA as well as protein levels in cells exposed to α -TOS for 4 hours (Fig. 4D). Furthermore, we did not detect any *MST2* mRNA in Jurkat cells using primers specific for this homologue of the Hippo/Mst1 kinase (data

not shown). These results indicate that the Hippo/Mst1 kinase is likely to be the main protein kinase responsible for stimulating *NOXA* gene transcription in Jurkat cells exposed to α -TOS.

To further examine the direct involvement of the Hippo/Mst1 kinase in FoxO1 activation, nuclear extracts of Jurkat cells treated with α -TOS were assessed for the levels of FoxO1 protein phosphorylated at serine 212, which is specifically phosphorylated by the Hippo/Mst1 kinase (33). Using Western blotting, increased levels of the pFoxO1 protein were detected in the nuclear fraction of Jurkat cells but only low levels were detected in the cytosolic fraction (Fig. 5A). These results are consistent with the aforementioned data (Fig. 2A) on the levels of total FoxO1 protein detected in the nucleus of α -TOS-treated cells. We also detected inhibition of FoxO1 translocation into the nucleus of Jurkat cells when the expression of the Hippo/Mst1 protein was knocked down (Fig. 5B). Moreover, the Hippo/Mst1 siRNA suppressed the levels of the FoxO1 protein phosphorylated on serine 212 in α -TOS-treated cells (Fig. 5C). Finally, differences in cellular viability were analyzed after treating Jurkat cells with α -TOS for 8 hours following their pretreatment with the Hippo/Mst1 siRNA. Figure 5D

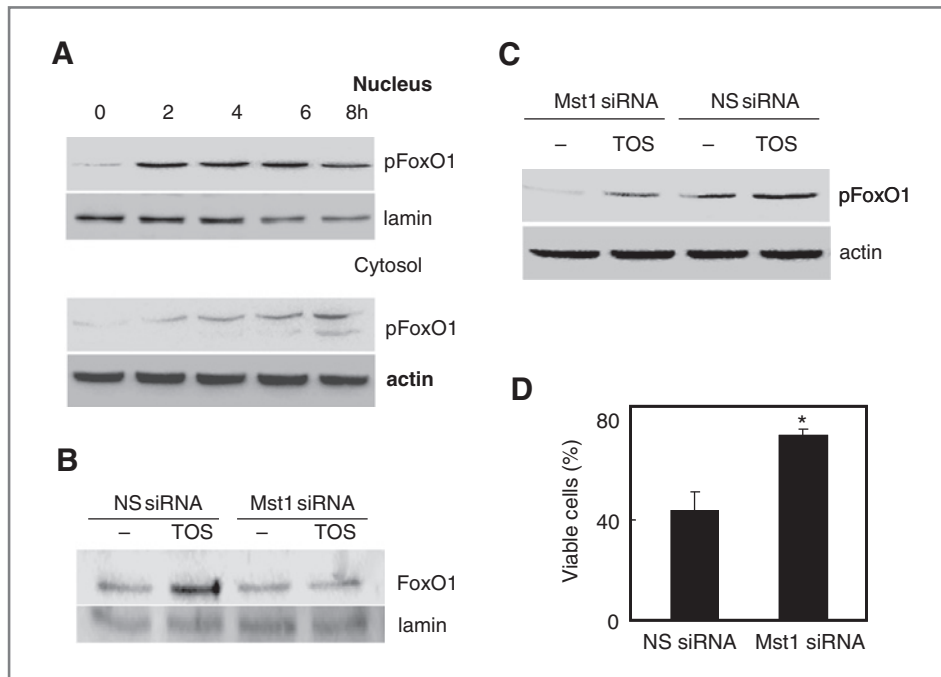


Figure 5. The Hippo/Mst1 kinase phosphorylates FoxO1. A, Jurkat cells were exposed to 50 $\mu\text{mol/L}$ α -TOS and assessed for Mst1-specific phosphorylation of the FoxO1 protein on serine 212 by Western blotting in the nuclear and cytosolic fraction. Jurkat cells were pretreated with Mst1 or nonsilencing (NS) siRNA, exposed to 50 $\mu\text{mol/L}$ α -TOS for 4 hours, and assessed for the levels of the FoxO1 protein in the nuclear (B) and phosphorylated FoxO1 protein on serine 212 in the whole cell lysate (C). D, Jurkat cells pretreated with Mst1-specific or NS siRNA were exposed to 50 $\mu\text{mol/L}$ α -TOS for 8 hours and assessed for viability using the Trypan blue dye-exclusion method. The images in the panels A to C are representative of 3 independent experiments; the data in panel D are derived from 3 independent experiments and are presented as mean values \pm SD. *, significant difference between the control and treated cells with $P < 0.05$.

shows that knocking down the Hippo/Mst1 protein significantly increased the fraction of live cells compared with control cells exposed to the VE analogue after using NS siRNA.

Discussion

α -TOS represents a class of anticancer agents that selectively induce apoptosis in cancer cells involving accumulation of ROS (16, 18, 39), causing an increase in the Noxa protein levels in a p53-independent process and triggering Bak channel formation in the mitochondrial outer membrane. Since Noxa is the only Bcl-2 family protein regulated by α -TOS (25), we studied here its regulation in the context of apoptosis induced by the agent.

To determine which factors are involved in the regulation of *NOXA* transcription, we performed *ab initio* analysis of the *NOXA* promoter and found a constrained DBE, which is conserved in a number of mammalian species. Because we did not find any reports on FoxO protein binding to the DBE-containing region in the *NOXA* promoter, we believe this is the first example which clearly identifies a specific role for the FoxO1 protein in regulating the *NOXA* promoter. These results are in agreement with recent reports demonstrating activation of *NOXA* transcription in a FoxO3 DBD-dependent manner in endothelial cells (40), and can also be reconciled with the role of FoxO proteins in apoptosis induction by oxidative stress or chemotherapeutic agents (9, 10, 36, 41). With regard to this, we have also demonstrated fast Hippo/Mst1 kinase activation induced by hydrogen peroxide which led to the FoxO1 protein phosphorylation at serine 212 and its nuclear localization, followed by increased *NOXA* gene transcription (Supplementary Fig. S2). These pioneer observations firstly linked ROS-induced *NOXA* gene transcription to the Hippo/

Mst1 kinase signaling. Although we observe activation of the Hippo/Mst1 protein in response to α -TOS as well as hydrogen peroxide, its mechanism, which may include direct or indirect effect, is unclear at this stage and will be a subject of further investigation.

Thus far, only the FoxO3a protein has been reported to regulate the *NOXA* gene transcription (10, 40). Using siRNA, we observed in our system that the *NOXA* gene transcription is modulated specifically by the FoxO1 protein. One possible explanation for the differences with other studies is that the expression of FoxO proteins may vary depending on the cell type (41). The exact molecular mechanism of FoxO-dependent *NOXA* transcription has been only partially decoded. Several papers have reported that FoxO proteins promote acetylation of histone proteins in the target promoters via recruitment of the CBP/p300 acetyl transferase, facilitating (RNA Pol II) binding to TSS (35, 36). We observed an increase in lysine acetylation in the *NOXA* promoter following FoxO1 activation in Jurkat cells treated with α -TOS (Fig. 3D) and also detected translocation of RNA Pol II from the DBE region in the *NOXA* promoter to its TSS (Fig. 3C). Similar results demonstrating presence of RNA Pol II in distal parts of the *NOXA* promoter in control cells were also published by others (42). This suggests that the FoxO1 protein can stimulate transcription of *NOXA* via acetylation of the histone proteins in the *NOXA* promoter. Interestingly, ChIP analysis of Jurkat cells treated with α -TOS also showed increased p53 protein binding following FoxO1 binding to the *NOXA* promoter (data not shown), which indicates that the FoxO-dependent acetylation of the *NOXA* promoter may also allow binding of other transcription factors. To this end, a possible cross-talk between FoxO, p53 and other factors (e.g., ATFs) is a focus of current FoxO research (36, 43), and such cross-talk may fine-tune activation of the

NOXA gene transcription following FoxO1 binding to its promoter in cancer cells exposed to anticancer drugs. However, since α -TOS triggers FoxO-dependent *NOXA* upregulation in the p53^{-/-} H1299 cells, the p53 transcription factor is not required for the regulation of the FoxO-Noxa pathway, at least in some systems.

Our observations are supported by other recent reports demonstrating Hippo/Mst1-dependent apoptosis induction, largely due to the involvement of the FoxO1 protein (33, 35). Studies identifying other proapoptotic genes regulated by the Hippo/Mst1 kinase are scarce. However, we have also observed transcriptional upregulation of the *FASL* and *TRAIL* genes and their protein products, which have been reported as alternative FoxO targets (11–13). Therefore, the *FASL* and *TRAIL* genes are probably additional targets for the Hippo/Mst1-FoxO1 pathway in Jurkat cells, indicating that the Hippo/Mst1-FoxO1 axis may represent a potent tumor suppressor system. We observed no changes in the *Bim* mRNA or protein levels in cancer cells treated with α -TOS (25). This is rather surprising because *BIM* transcription has been reported to be linked to the activation of FoxO proteins (10). Therefore, it would appear that transcription of *BIM* is not regulated by the Hippo/Mst1-FoxO1 pathway in cancer cells exposed to the VE analogue. The *BIM* promoter may be also epigenetically deregulated in the Jurkat cells, as it was found to be constitutively expressed (data not shown).

Taken together, we have established a novel tumor suppressor pathway based on the activation of the Hippo/

Mst1-FoxO1-Noxa axis and we also found that this pathway regulates transcription of additional proapoptotic genes (e.g., *FASL* and *TRAIL*). On the basis of this study, we propose a new molecular mechanism by which cancer cell-selective agents such as α -TOS trigger the Hippo/Mst1-dependent expression of the BH3-only protein Noxa that, in turn, causes mitochondrial permeabilization and apoptosis (25). To translate these findings into the context of tumor therapy, a study has been initiated to verify the Hippo/Mst1-FoxO1-Noxa pathway in an experimental model of cancer.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

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