Supporting Information

Schewe and Aguirre-Ghiso 10.1073/pnas.0800939105

SI Text

Methods. ***Reagents.*** Tunicamycin (Tm), Thapsigargin (Tg), DTT and Doxorubicin (Dox) were from Sigma. SB203580 was obtained from Calbiochem, and rapamycin was from LC Laboratories. SCIO-469 was kindly provided by Amit Verma (Albert Einstein College of Medicine, Bronx, NY).

Antibodies. Anti-ATF6α antibody was from Imgenex, anti-grp78 antibody was from BD. Anti-cleaved Caspase3, anti-Rheb, anti-*p*-Akt, anti-Akt, and anti-*p*-S6 ribosomal protein antibodies were from Cell Signaling Technology, and mouse anti-GAPDH antibody was from Calbiochem. Anti-β-tubulin antibody was from Abcam. HRP-conjugated mouse secondary antibodies and mounting media were from Vector Laboratories. HRP-conjugated anti-rabbit antibody was from Chemicon International. Alexa Fluor 488 or 568 anti-mouse antibodies were from Invitrogen.

ATF6α and XBP-1 siRNAs were from Santa Cruz Biotechnology (cat. nos. sc-37699 and sc-38627), and grp78/BiP and Rheb siRNAs were from Ambion (cat. nos. AM-16706 and AM-16708). The siRNA to p38 was from New England Biolabs (cat. no. N2004S). Scrambled siRNA from Ambion was used as negative control (cat no. AM-4611).

The sequence of the ATF6α-shRNA is 5′-TGCTGTTGACAGTGAGCGCGGAGACAGCAACGTATGATAATAGTGAAGCCACAGATGTATTATCATACGTTGCTGTCTCCTTGCCTACTGCCTCGGA-3′.

For the creation of stable cell lines, Phoenix A packaging cells were seeded at low density and incubated at 37°C overnight. They were then transfected with 2 μg of shRNA plasmid or with equal amounts of empty vector using Fugene 6 Reagent from Roche. Transfected Phoenix A cells were incubated at 32°C for 48 h, and the supernatant was collected and filtered. D-HEp3 cells ($2–3 \times 10^5$) were seeded onto six-well plates and incubated at 37°C overnight. Viral supernatant was added to each well together with Polybrene to a final concentration of 8 μg/ml (Sigma). The plate was spun at 1,500 rpm in a 5810R centrifuge from Eppendorf for 1 h at room temperature. D-HEp3 cells were incubated overnight at 32°C to allow viral infection. Medium was changed to standard medium for these cells (see above), and cells were incubated for another 48 h at 32°C. After that, infected cells were selected at a final concentration of 2.5 μg/ml puromycin, and knockdown was assayed by RT-PCR.

For ATF6α IF, $1–3 \times 10^4$ cells were plated on glass coverslips, transfected with ATF6α siRNA when applicable, and incubated 24 to 48 h. Cells were fixed in 3% PFA for 15 min on ice, washed, and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Coverslips were blocked with 3% normal goat serum (Sigma) for 30 min and then incubated with anti-ATF6α antibody (1:50) for 60 min, blocked again for 30 min and incubated with Alexa Fluor 488 or 568 antibodies (1:400) for 60 min. Nuclear counterstaining was performed with Hoechst-33342 (1:10,000; Invitrogen) for 2 min, and coverslips were mounted. For cleaved caspase-3 IF, the antibody was used at 1:200 and Alexa Fluor 488 anti-rabbit antibody at 1:1,000 as a secondary antibody. Nuclei were counterstained with Hoechst-33342.

Total RNA was extracted by using TRIzol from Invitrogen. PCR primers were from IDT, sequences were: ATF6α 5′-TGA TGA GCT GCA ATT GGA AGC AGC-3′ and 5′-ACC ACA GTA GGC TGA GAC AGC AAA-3′, GAPDH 5′-CGT CAT GGG TGT GAA CCA TGA G-3′ and 5′-GTA GAC GGC AGG TCA GGT CCA-3′, XBP-1 5′-CCT TGT AGT TGA GAA CCA GG-3′ and 5′-GGG GCT TGG TAT ATA TGT GG-3′, Rheb 5′-GAT ATC ATT TGG GTC AGA GCT CCC-3′ and 5′-CAG TCA AGG TCT AGG GAT TTG AGG-3′.

For *in vivo* Luc assays, cells transfected with the Luc constructs were inoculated onto CAMs of 9- to 10-day-old chicken embryos from Charles River (1.5×10^6 cells/CAM). At 24 or 48 h later, excised CAMs were homogenized in passive lysis buffer from the Promega kit using the Bullet Blender from Next Advance. Lysates were then frozen at −20°C, centrifuged at 14,000 rpm in a 5415R Eppendorf microcentrifuge for 10 min on the day of analysis, and processed.

For the CAM growth/survival assay, cells were detached with 2 mM EDTA in PBS, washed, and inoculated on CAMs (D-HEp3 500.000 cells/CAM and T-HEp3 150,000 cells/CAM). Cell numbers were quantified by using a hemocytometer at 7 days after excision, mincing, and collagenase treatment.

Results. ATF6α confers a survival advantage to D-HEp3 cells upon genotoxic, ER, and nutritional stress *in vitro*. We have previously shown that D-HEp3 cells are highly resistant to Tm and glucose deprivation (10). The facts that ATF6α has its predominant role in regulating genes essential for coping with ER stress, and that D-HEp3 cells have higher levels of ATF6α suggested a potential prosurvival function of ATF6α in these cells. We down-regulated ATF6α mRNA (Fig. 3*A*) and protein levels (Fig. 3*B*). As expected, this resulted in enhanced sensitivity of D-HEp3 cells to Tm (Fig. S1*C*) as measured by Trypan blue exclusion test. In agreement with published data that UPR activation causes resistance to TOPO II inhibitors (ref. 10 and references within) ATF6α down-regulation conferred pronounced sensitivity to Doxorubicin (Fig. S1*C*). Finally, whereas no significant difference in cell viability could be observed after ATF6α knockdown at a dose of 5 mM glucose (data not shown), 1 mM glucose resulted in the induction of significantly more death in D-HEp3 cells after ATF6α knockdown (Fig. S1*C*). All in all, these data point at ATF6α as a regulator of D-HEp3 survival in response to ER and genotoxic stress but also to glucose restriction, a more physiological source of stress that might be encountered *in vivo*.

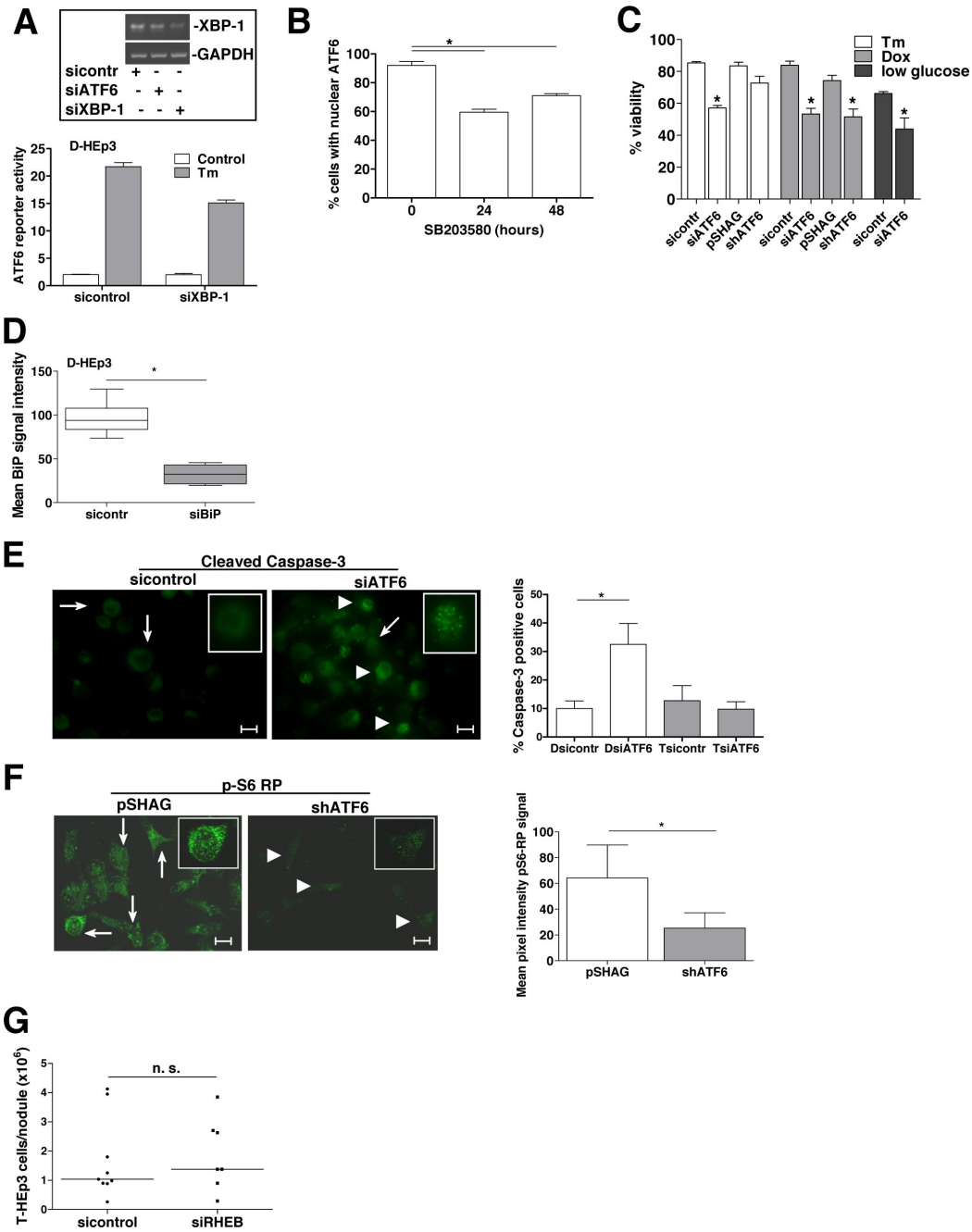

Fig. S1. Additional mechanism analysis on ATF6 signaling. (*A*) RT-PCR showing the knockdown of XBP-1 but not ATF6α mRNA (*Upper*). The 5xATF6-GL3 activity (full serum) in D-HEp3 cells with siRNA-mediated knockdown of XBP-1 under basal conditions or treated with 5 μg/ml Tm for 6 h (*Lower*). (*B*) Percentage of D-HEp3 cells with nuclear ATF6α treated with 10 μM SB203580 for 24 and 48 h as measured by counting $\approx$200 cells stained by IF in duplicate experiments, *, $P < 0.05$. (*C*) ATF6α confers a survival advantage to different types of stress *in vitro*. D-HEp3 cells with and without knockdown of ATF6α were subjected to a 24-h treatment with Tm 10 μg/ml or Dox 4 μg/ml or glucose restriction for 72 h at 1 mM. Viability was assessed by using TB exclusion. *, $P < 0.05$. (*D*) Quantification of mean BiP pixel intensity in three independent Western blot experiments down-regulating ATF6α by siRNA in D-HEp3 cells by using ImageJ (96.35 $\pm$ 7.65 for sicontrol, 32.37 $\pm$ 4.26 for siATF6α. *, $P < 0.0001$). (*E*) IF for cleaved (active) caspase-3 in D-HEp3 cells with siRNA-mediated knockdown of ATF6α (*Right*) and a control siRNA (*Left*) recovered from the CAM 24 h after inoculation. The *Insets* show a cell positive (*Right*) and negative (*Left*; note the pale background signal) for cleaved Caspase-3 in higher-magnification view. (Scale bars: 40 μm.) Cells positive for active caspase-3 are indicated by arrowheads and cells negative by arrows. The graph on the right shows a quantification of positive cells counting $\approx$300 cells per group in triplicate. *, $P = 0.01$. (*F*) IF for *p*-S6 ribosomal protein in D-HEp3 cells with stable expression of ATF6α shRNA (*Right*) or the pSHAG control vector (*Left*). The *Insets* show a cell with high fluorescence (*Left*) and low fluorescence (*Right*) in higher-magnification view. (Scale bars: 40 μm.) Cells with high fluorescence are indicated by arrows and cells with low fluorescence by arrowheads. The graph on the right shows a quantification of mean pixel intensity by using ImageJ in 100 cells of two independent experiments (64.14 $\pm$ 2.55 for pSHAG empty and 25.36 $\pm$ 1.18 for shATF6α. *, $P < 0.0001$). (*G*) T-HEp3 cells with or without Rheb knockdown proliferated at similar rates on CAM for 7 days.

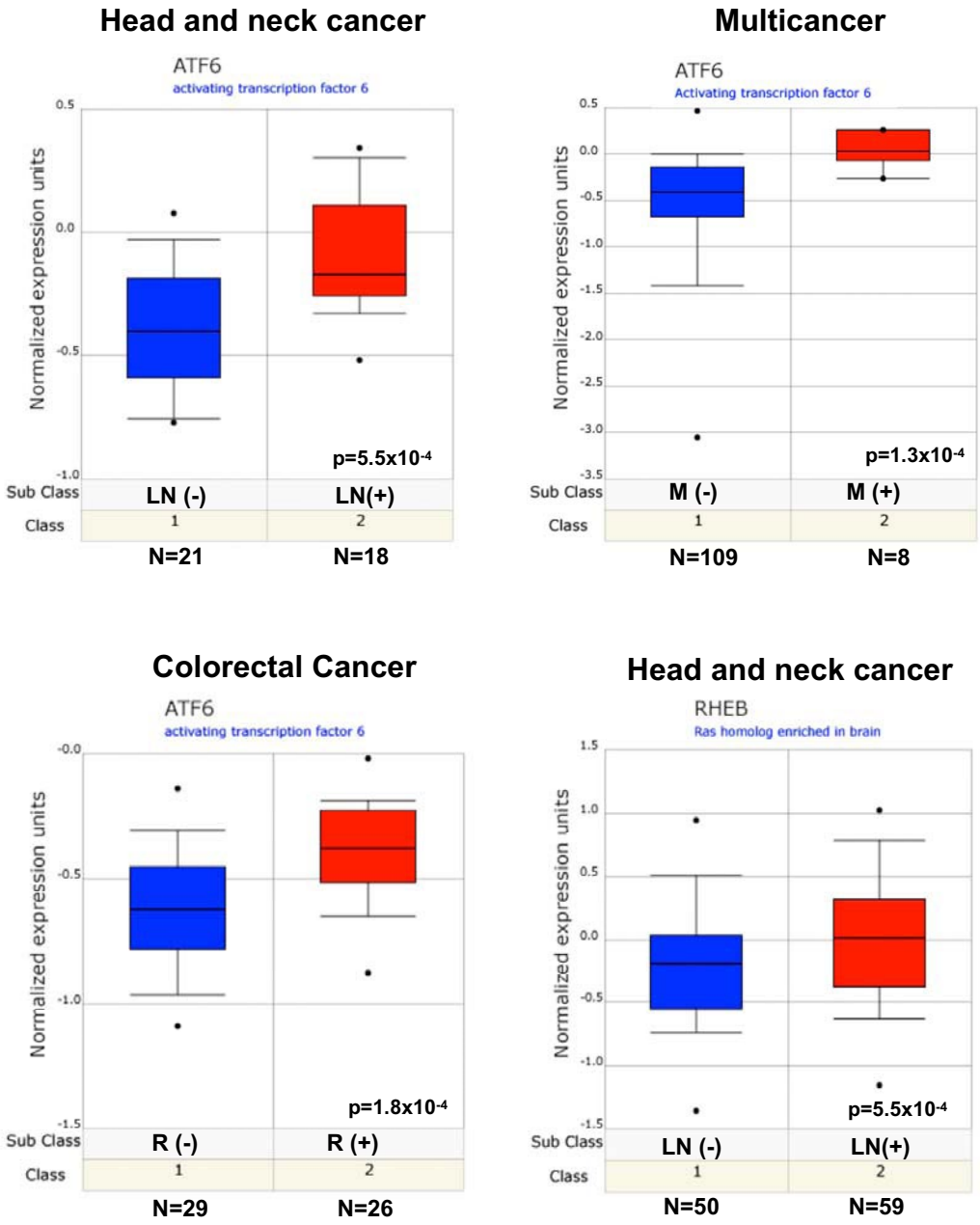

Fig. S2. Compiled patient data from Oncomine (13) on ATF6 as a prognostic indicator in head and neck cancer (14), colon cancer (16), and in a multicancer study (15) as well as Rheb in head and neck cancer (29). N is the number of patients for each condition. LN, lymph node; M, metastasis; R, relapse.

Table S1. Representative changes in gene expression regulated by ATF6α as determined by gene array analysis

Gene	Gene locus	Accession no.	-Fold induction/repression	Function
Activating transcription factor 6	ATF6	NM_007348	10.25*	
Protein folding				
Heat shock protein 90-kDa β (Grp94), member 1	HSP90B1	AI684643	1.71	Chaperone
DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9	NM_012328	1.64	Chaperone
Protein degradation				
Ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)	UBE2E1	AI742722	1.93	Ubiquitinylation of proteins
F-box and leucine-rich repeat protein 13	FBXL13	NM_145032	1.88	Ubiquitin cycle
SEC11 homolog C (*S. cerevisiae*)	SEC11C	BF055352	1.59	Signal peptide processing
OTU domain, ubiquitin aldehyde binding 2	OTUB1	AI656232	1.56	Ubiquitin iso-peptidase
Protein secretion				
Secretogranin II (chromogranin C)	SCG2	NM_003469	2.96	Secretory granule protein
Intersectin 1 (SH3 domain protein)	ITSN1	AF114488	1.83	Endocytosis, formation of clathrin-coated vesicles
Component of oligomeric Golgi complex 3	COG3	BC038953	1.63	Intracellular protein transport
Metabolism and transport				
Peroxisome proliferator-activated receptor-γ	PPARG	NM_015869	2.33	Response to nutrients, lipid metabolism
ATP-binding cassette, sub-family A (ABC1), member 13	ABCA13	NM_152701	3.25	Transport
Solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	NM_006931	1.80	Glucose transporter
Solute carrier family 41, member 1	SLC41A1	AW439816	−1.88	Cation transport
Signal transduction				
Rho GTPase activating protein 5	ARHGAP5	N50119	2.48	Regulation of RHO GTPases
Ras homolog enriched in brain	RHEB	NM_005614	2.37	Activator of mTOR
Transforming growth factor, β-receptor III	TGFBR3	NM_003243	2.24	TGF signaling pathway
SMAD family member 3	SMAD3	AI475033	1.94	TGF signaling pathway
SAR1 gene homolog B (*S. cerevisiae*)	SAR1B	NM_016103	−1.93	Small GTPase mediated signal transduction

Genes listed are examples of genes up- or down-regulated >1.5-fold ($P < 0.05$) by knockdown of ATF6α .
*ATF6α appears to be changing the most because its down-regulation is the strongest (10.25-fold).