Supplementary Materials and Methods

Reagents

The following reagents were used in this study as described:

Chemical	Company	Product #	[Stock]	[Final]	Application
Blasticidin	Sigma Aldrich	15205	10 mg/mL in diH_2O	4 µg/mL	Cell culture selection
Puromycin	Thermo Scientific	A1113803	10 mg/mL	3 µg/mL	Cell culture selection
Doxycycline	Clontech	631311	10 mg/mL in PBS	1 µg/mL	shRNA induction
Thapsigargin	Tocris	1138	100 µM in DMSO	40-100 nM	ER stressor
Trans-ISRIB	Tocris	5284	5 mM in DMSO	200 nM	ISR inhibitor
Etoposide	Sigma Aldrich	E1383	50 mM in DMSO	100 µM	Activator of apoptosis
N-acetylcysteine (NAC)	Sigma Aldrich	A9165	300 mM in diH_2O	≤ 5 mM	Antioxidant
E64-d	Cayman Chemical	13533	10 mg/mL in DMSO	10 µg/mL	Protease inhibitor
Pepstatin A (Pep)	EMD Millipore	516481	10 mg/mL in DMSO	10 µg/mL	Protease inhibitor
Cycloheximide (CHX)	Sigma	C7689	100 mg/mL in EtOH	100 µg/mL	Translation inhibitor

Cell lines

A table of cell lines used in this study is provided below. Annotation of lines is based on Cancer Cell Line Encyclopedia (Broad Institute). H2030 and PC9 cell lines stably express firefly luciferase construct as previously described (1). The patient derived xenograft cell line YLR086, harboring an EGFR L747-A750>P mutation and generated under HIC Protocol #111009228, was provided by Dr. Katerina Politi (Yale University, New Haven, CT) (2).

Cell Line	Cancer	Stage	Source
H2030	Adenocarcinoma	-	Lymph node
PC9	Adenocarcinoma	-	Lymph node
HCC-827	Adenocarcinoma	-	-
HCC-4006	Adenocarcinoma	-	Pleural effusion
H358	Bronchioalveolar adenocarcinoma	-	Primary
H441	Papillary adenocarcinoma	-	Pericardial fluid
H1975	Adenocarcinoma	-	-
H1650	Bronchioalveolar adenocarcinoma	IIIB	Pleural effusion
H1573	Adenocarcinoma	IV	Metastasis, soft tissue

H23	Adenocarcinoma	-	-
H1792	Adenocarcinoma	-	-
YLR086	Adenocarcinoma	-	Liver metastasis

shRNA sequences

Designation	shRNA Sequence (5'-3')
shCtrl	AGGCTATTACTCACCGTATTAT
shATF4-a (V2LHS_272113)	TCAAATCTTTCAGGTACTG
shATF4-b (V3LHS_302002)	AAACTAAAGGAATGATCTG
shASNS (V3LHS_410318)	TTGCTTTCACATTACAGCA

Quantitative Real Time-PCR

GUSB or *GAPDH* were used as housekeeping genes and relative quantification (RQ) values calculated via the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed below:

Target	Forward (5'-3')	Reverse (5'-3')
ASNS	GATGAACTTACGCACGCAGGGTTACA	CACTCTCCTCCTCGGCTTT
PSAT1	CGGTCCTGGAATACAAGGTG	AACCAAGCCCATGACGTAGA
SLC7A11	AGTCCCTGGAGTTATGCAGC	GTTGAGGTAAAACCAGCCAGC
MAP1LC3B	CGCCGCACCTTCGAACAA	CCGGGATTTTGGTTGGATGC
NFE2L2	CCCCAACACACGGTCCACA	AAATCCATGTCCTGCTGGGACG
HMOX1	ACTGCGTTCCTGCTCAACAT	GGGGCAGAATCTTGCACTTT
TRIB3	CGCCTTTTTCACTCGGACCC	GCTTCTTCCTCTCACGGTCA
DDIT4	CTAGCTGCGGCTTCTACGC	CCAAAGGCTAGGCATGGTGA
CCNB1	TCTGAGACAACTTGAGGAAGAGC	TGTTTCCAGTGACTTCCCGA
GUSB	CGCCCTGCCTATCTGTATTC	TCCCCACAGGGAGTGTGTAG
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Western blotting

Antibodies used for Western blotting are listed below:

Antibody	Company	Product #	Source	kDa	Dilution
ATF4	CST	11815	Rabbit mAb	50	1:500
GAPDH	CST	2118	Rabbit mAb	37	1:50000
p-eIF2α (S52)	Invitrogen	AHO1182	Rabbit	38	1:500
eIF2α	Invitrogen	44-728G	Rabbit	38	1:500
Tubulin	Sigma	T5168	Mouse mAb	50	1:10000
NRF2	CST	12721	Rabbit mAb	97-100	1:1000
LC3A/B-I/II	CST	12741	Rabbit mAb	14, 16	1:1000
CDC25C	CST	4688	Rabbit mAb	60	1:1000
Cyclin B1	CST	12231	Rabbit mAb	55	1:1000

p-AKT (S473)	CST	4060	Rabbit mAb	60	1:2000
p-AKT (T308)	CST	13038	Rabbit mAb	60	1:1000
AKT	CST	9272	Rabbit	60	1:1000
p-mTOR (S2448)	CST	5536	Rabbit mAb	289	1:1000
mTOR	CST	2983	Rabbit mAb	289	1:1000
p-P70S6K (T389)	CST	9234	Rabbit mAb	70-85	1:1000
p-S6 (S240/244)	CST	5364	Rabbit mAb	32	1:1000
S6	CST	2217	Rabbit mAb	32	1:1000
p-4E-BP1 (T37/46)	CST	2855	Rabbit mAb	15-20	1:1000
4E-BP1	CST	9644	Rabbit mAb	15-20	1:1000

Clonogenic assay and quantification

Cells were trypsinized, rinsed two times with PBS to remove residual amino acids, counted, and 500-1000 cells plated per well of 6-well plates in treatment media. Colonies were allowed to grow, changing media every 3-4 days, until they could be visualized (9-15 days depending on the cell line). To harvest, media was aspirated and plates rinsed with PBS, fixed in 70% EtOH for 30 minutes, and stained with 0.05% crystal violet in sterile water for 1 hr. Crystal violet was then removed and the plates gently rinsed in diH_2O. Plates were subsequently imaged on a Bio-Rad ChemiDoc Imaging System and quantified using ImageJ with the ColonyArea plug-in (3).

Viability assays

Cell viability was measured using resazurin (RD Systems # AR002). 500-1000 cells were plated per well of 96-well plates. At the time of analysis, media was removed, and 100 µL of diluted resazurin (1:10 in PBS) added to each well. Plates were incubated for 30 min at 37°C and 5% CO_2. Subsequent fluorescence was read at 560/610 nm (ex/em) using a Synergy MX Multi-Mode Microplate Reader (BioTek).

Bivariate cell cycle analysis

Cell cycle analysis was performed, following treatment of subconfluent cells, by measuring BrdU incorporation and concurrent DNA staining (7-AAD). An APC BrdU Flow Kit (BD Biosciences # 557892) was used and the recommend protocol followed. BrdU was pulsed at 10 µM in cell culture media for 1 hr. Cells were subsequently trypsinized, fixed, stained, and analyzed using an LSR II flow cytometer (BD Biosciences). Doublets were gated out by plotting APC signal height (y-axis) versus area (x-axis) using FlowJo® software (FlowJo, LLC) and the proportion of cells in each phase of the cell cycle determined.

Immunofluorescent staining for cleave caspase-3

Cells were plated on sterile glass coverslips in 6-well plates in treatment media. To harvest, media was removed and cells rinsed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized and blocked in 3% BSA in TBS with 0.3% Triton X-100 for 1 hr. Primary antibody

against cleaved caspase 3 (CST #9661) was used at 1:100 overnight at 4°C. Fluorescent secondary antibodies and DAPI were then used at 1:150 and 1:2000 respectively. Coverslips were mounted on slides using ProLong Gold (Invitrogen #P10144) and imaged using an inverted Olympus microscope or Keyence BZ-X microscope (Keyence). Images were quantified in ImageJ as the mean pixel intensity per cell (using DAPI mask) divided over the total number of cells in the sample. 100 μM etoposide treatment for 24 hr was used as a positive control.

Anchorage-independent growth assays

For anchorage-independent growth experiments, 100 μL of cells in media containing 0.5% methylcellulose (Sigma #M0512) were plated per well of non-adherent 96-well cell culture plates. Nutrients were replenished by adding 50 μL of fresh media with 0.5% methylcellulose every 3 days. To determine viability, 10 μL of undiluted resazurin was added directly to the cell culture media and the same viability procedure followed as described above.

Transwell migration assay

Cells were pre-treated under the indicated conditions and 2.5e4 cells plated in treatment media containing 0.2% FBS in transwell inserts (PET membrane with 8.0 μm pores, Falcon #353097) in 24-well plates. Treatment media with 10% FBS was used beneath the insert as a chemoattractant. After allowing cells to migrate for 24 hr, cells on the upper surface of the insert were removed using a cotton swab. Remaining cells on the lower surface of the insert were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then stained with DAPI, and membranes cut out using a razor blade and mounted on slides. Mounted membranes were imaged using a Keyence BZ-X microscope (Keyence) and the number of cells per membrane were quantified and normalized to area using ImageJ.

Scratch Assays

Wounds were created using a sterile pipette tip to scratch plated cells. Cells were then rinsed two times with PBS and incubated in the indicated treatment media. At the indicated times, images were taken using a Keyence BZ-X microscope (Keyence). Wound area was quantified using the MRI Wound Healing Tool available for ImageJ (4).

Gene set enrichment analysis and pathway analysis

For gene set enrichment analysis (GSEA), ANOVA analysis was performed on indicated categories from either all TCGA LUADs (*n* = 489 tumors)(5), the TCGA Nature Core samples (*n* = 230 tumors and 45 matched normal tissues with exome sequencing), or the Director's Challenge Cohort of LUADs (DCC) (*n* = 442)(6), where appropriate, using Partek Genomic Suite® software (Partek, Inc.). Pre-ranked gene lists were then generated using directionally-corrected negative $\log_{10}(FDR)$ scores. The ATF4 target gene set used is from a

chromatin immunoprecipitation sequencing previously described (7) and includes ATF4-only targets and targets common to ATF4 and CHOP (*n* = 472 genes). GSEA was performed using default settings (8).

DAVID analysis of leading edge genes from the GSEA analysis was performed as previously described (9), and the resulting fold enrichment and negative $\log_{10}$(*FDR*) for select gene ontology categories plotted.

RNA sequencing and pathway analysis

Cells were treated as indicated in biological triplicate and total RNA extracted using an RNeasy kit (Qiagen #74106) including the DNase step. Samples were submitted to the Yale Center for Genome Analysis for RNA sequencing. RNA libraries were prepared using standard Illumina protocols. Raw 75-bp R1 single-end reads were mapped to the human genome (hg38) with Bowtie2 in local mode, which allows reads spanning exon-exon junctions to be mapped to one of the two exons independent of the transcriptome annotation. Uniquely mapped reads (cutoff by MAPQ>10) were counted to ENCODE gene annotation (v24) using featureCounts. Differential gene expression was performed with DESeq2. The VarianceStabilizationTransformation function was used to generate normalized gene abundances on a $\log_2$ scale. Genes without a count of at least one in at least two replicates were excluded. Differential gene expression and hierarchical clustering were then performed using Partek Genomic Suite® software (Partek, Inc.) to generate a heat map which includes genes significantly changed (*FDR* < 0.05 by Benjamini–Hochberg step-up method) by at least 1.5 fold in any comparison. All sequencing data are deposited in NCBI's Gene Expression Omnibus (GEO) under accession number GSE126232.

Subsequent analysis of all genes significantly changed (*FDR* < 0.05 by Benjamini–Hochberg step-up method) by at least 1.5 fold was performed using default settings in Ingenuity® Pathway Analysis software (Qiagen), allowing for the prediction of changes in upstream regulators and canonical pathways. Plotted upstream regulators were limited to genes, RNAs, and proteins.

CellROX assay

CellROX (Invitrogen #C10422) was used as a probe to measure the accumulation of ROS. Treated cells were incubated in cell culture with 5 μM CellROX for 30 min, trypsinized, and the resulting signal analyzed using an LSR II flow cytometer (BD Biosciences). Data was processed using FlowJo® (FlowJo, LLC).

Reverse phase protein arrays

Cells were treated in biological triplicate and cell pellets submitted to the MD Anderson Reverse Phase Protein Array Core Facility for analysis using recommended protocols. Partek Genomic Suite® software (Partek, Inc.) was used to standardize normalized $\log_2$ values to the mean and ANOVA analysis performed on indicated treatment comparisons. Proteins whose expression changed by at least 1.5 fold with *FDR* < 0.05 by

Benjamini–Hochberg step-up method were considered significantly changed. A heat map depicting representatives of these proteins was generated using Prism software (GraphPad). Results from RPPA analysis are included in Supplementary Table 4. For further analysis, this list was filtered for targets whose protein levels changed in the RPPA, but whose corresponding mRNA levels from the RNA sequencing data remained unchanged. Independently, Spearman correlation of *ASNS* expression in the TCGA with corresponding RPPA levels was performed for all LUAD samples with available RPPA data (n = 181) (5) in order to determine proteins that clinically correlate with *ASNS* expression. Finally, the initial targets that changed at the protein but not RNA levels in our experiments were plotted against the corresponding correlation between protein levels and *ASNS* expression from the TCGA.

Tumor RNA and asparagine concentration

Tumor tissue harvested for RNA extraction was incubated in RNAlater (Qiagen #AM7020) at 4°C overnight and stored at -80°C prior to processing. Tumor tissue harvested for LC-MS/MS analysis was flash-frozen and stored at -80°C. Approximately 50 mg of tumor tissue was homogenized in 400 µL acetonitrile:water (1:1) and spiked with 10 µL [1-13C]aspartate (1 mM). Lysates were then centrifuged at 4°C and the concentrations of asparagine and aspartate in the supernatant determined by LC-MS/MS. Aspartate and asparagine were baseline resolved (Asp: Rt = 2.5 min, Asn: Rt = 2.7 min) using a Hypercarb column (100x4, 5 µm) with a gradient (70% A to 20% A) elution of acetonitrile (A) and aqueous 12 mM ammonium acetate with 10 µM EDTA (B). MS/MS ESI (AB Sciex QTrap 6500) analysis was by single reaction monitoring mode at the negative ion transitions of 132/115 and 133/116 for aspartate and [1-13C]aspartate, and 131/114 for asparagine.

Supplementary Methods References

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