**Supplemental Information**

Diet-Induced Unresolved ER Stress Hinders KRAS-Driven Lung Tumorigenesis

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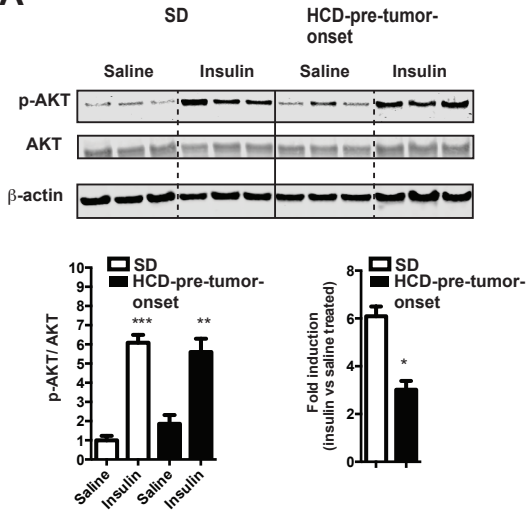
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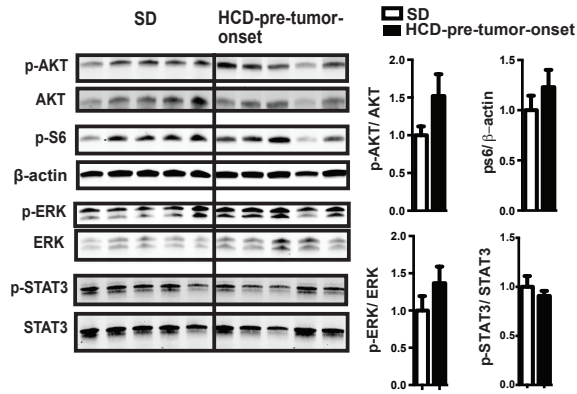
Supplemental Figures

Supplemental Figure 1
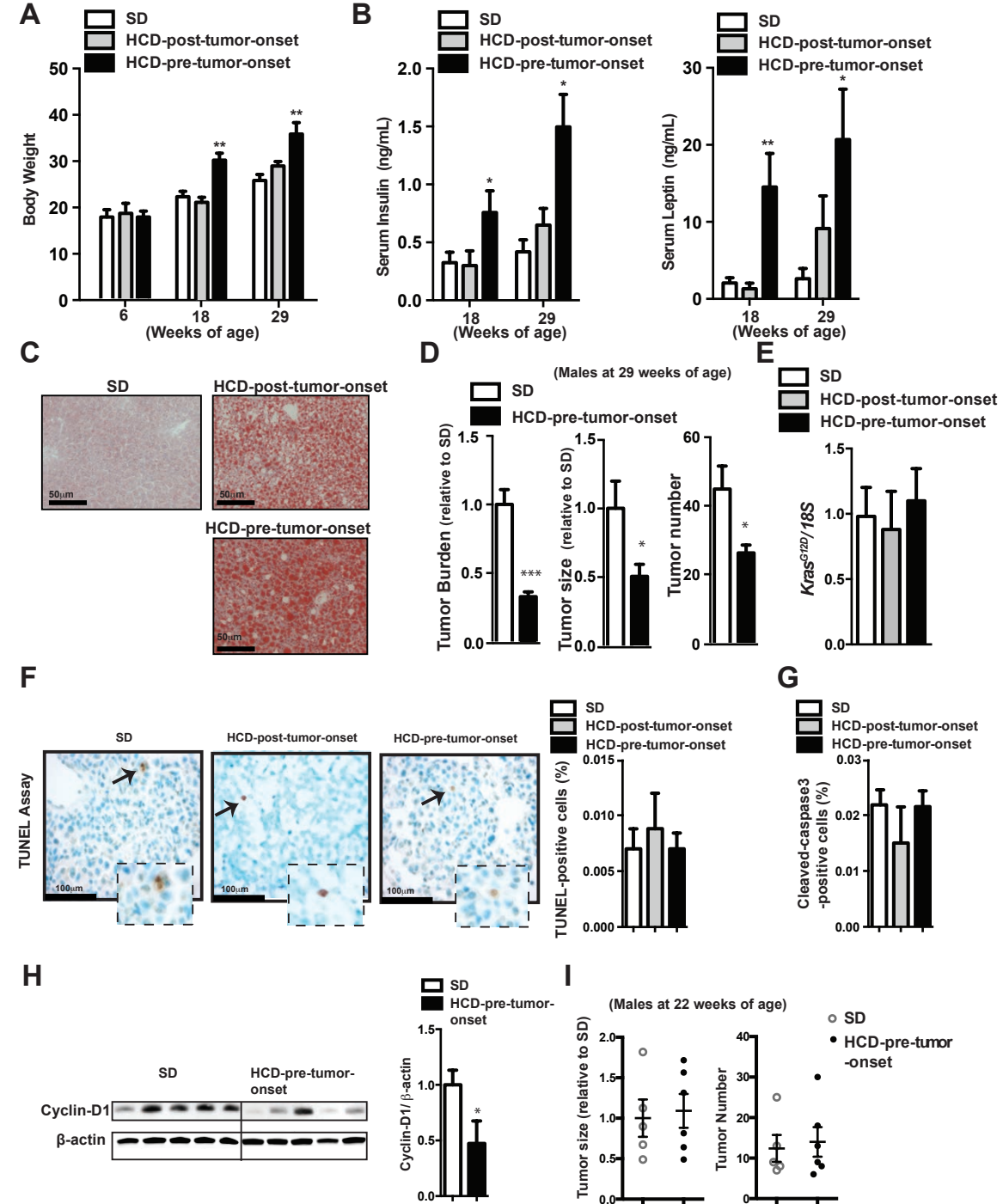
Supplemental Figure 1, related to Figure 1. Effect of chronic HCD feeding on KRAS-driven lung tumorigenesis. In **(A-H)** SD, HCD-post-tumor-onset and HCD-pre-tumor-onset $Kras^{G12D}$ mice were treated as shown in Figure 1A. **(A)** Body weight, **(B)** serum leptin and insulin levels and **(C)** liver Oil-Red-O staining from age-matched 18- and 29-weeks-old SD, HCD-post-tumor-onset and HCD-pre-tumor-onset $Kras^{G12D}$ mice (females; n=5-7 mice/ group). **(D)** Quantification of tumor burden, number and size in 29-week-old SD and HCD-pre-tumor-onset $Kras^{G12D}$ mice (males; n=5-6/ group). **(E)** mRNA level of $Kras^{G12D}$ in micro-dissected lung tumors and **(F)** representative images of lung tumor sections stained for TUNEL assay (in brown TUNEL positive cells) and relative quantification histograms of 29 weeks-old SD, HCD-post-tumor-onset and HCD-pre-tumor-onset $Kras^{G12D}$ mice (females; n=5-7 mice/ group), **(G)** quantification of cleaved-caspase-3 positive cells and **(H)** immunoblots from total cell lysates of micro-dissected tumors of 29 weeks-old SD- and HCD-pre-tumor-onset $Kras^{G12D}$ mice (males; n=5 mice for group). **(I)** Quantification of tumor burden and tumor number in 22 weeks-old SD- and HCD-pre-tumor-onset $Kras^{G12D}$ mice (males; n=5 mice for group). Error bars represent s.e.m. Statistical analyses were done using one-way ANOVA (Tukey's post test). *$P<0.05$, **$P<0.01$, ***$P<0.001$.
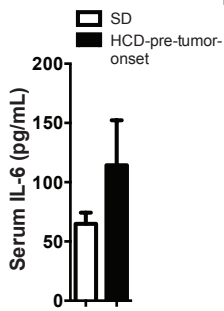
Supplemental Figure 2 (part A)

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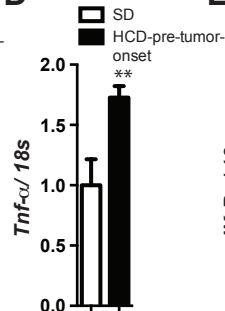
Supplemental Figure 2 (part B)

Supplemental Figure 2, related to Figure 2. Unresolved ER-stress in lung tumors of HCD-pre-tumor-onset *Kras*[G12D] **mice.** **(A)** Immunoblot and quantification of pAKT relative to total AKT in micro-dissected lung tumors of 29-week-old HCD-pre-tumor-

onset or SD-mice after 10' of treatment with 15U/kg of insulin or saline (n=3/group). **(B)** Immunoblots from total cell lysates of micro-dissected lung tumors (values normalized to total protein levels or β-actin levels), **(C)** Serum IL-6 levels, **(D-E)** mRNA levels in micro-dissected tumors of 29-week-old HCD-pre-tumor-onset and SD mice. **(F)** Heat maps of the genes encoding for chaperones enriched in a genome-wide expression profiling illustrating the changes in gene expression between micro-dissected tumors of 29-week-old HCD-pre-tumor-onset and SD mice (expression levels shown are representative of each replicate from micro-dissected lung tumors). Red signal denotes higher expression relative to the mean expression level within the group and blue signal denotes lower expression relative to the mean expression level within the group. **(G)** mRNA levels in micro-dissected tumors of 29-week-old HCD-pre-tumor-onset and SD mice. **(H)** mRNA levels, **(I)** immunoblots from whole lysate and relative quantification, **(J)** immunoblots from cytosolic and nuclear fraction of lysates and relative quantification, **(K and L)** mRNA levels, from tumor-free lung tissue of 29-week-old HCD-pre-tumor-onset and SD mice. **(M and O)** Immunoblots and relative quantification and **(N,P and Q)** mRNA levels from lysate of micro-dissected tumors of 29-week-old HCD-post-tumor-onset and SD mice. **(R and U)** mRNA levels, **(S)** mRNA ratio, and (**T**) protein carbonylation in micro-dissected tumors of 29-week-old HCD-pre-tumor-onset and SD mice. In **(B-U)** mice were treated as shown in Figure 1A (males; n=5-6/ group). Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test or using one-way ANOVA (Tukey's post test). *P<0.05, **P<0.01.
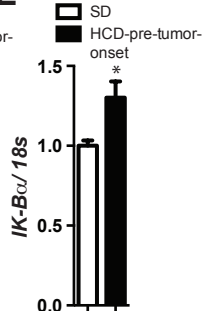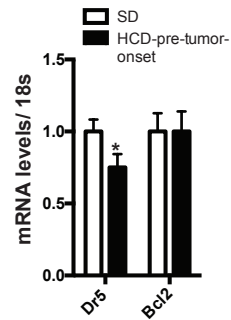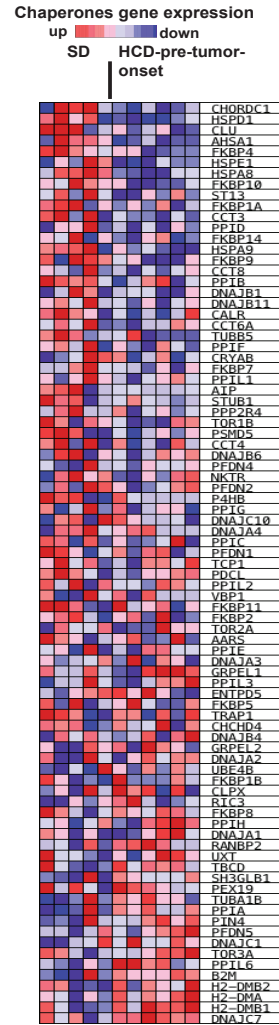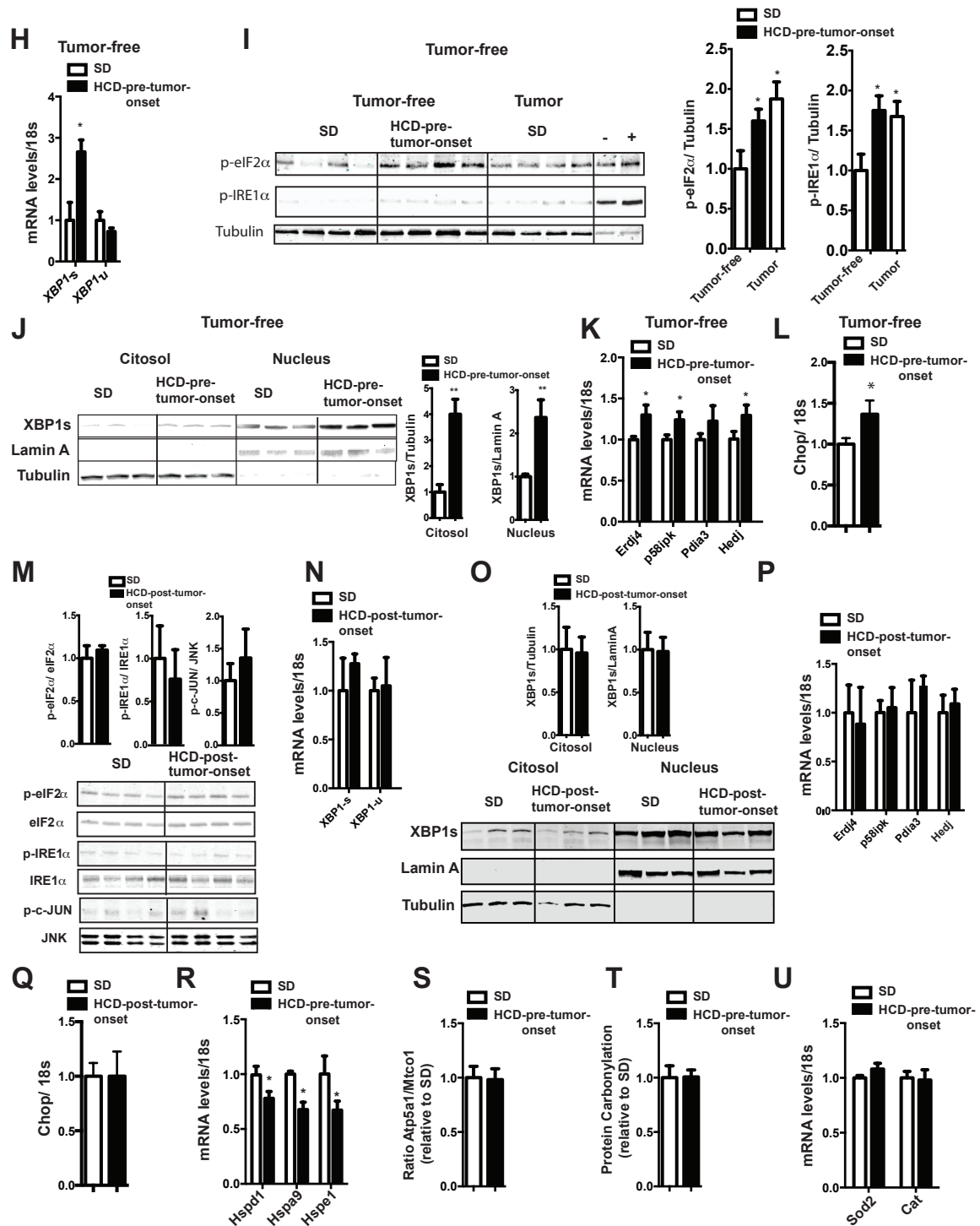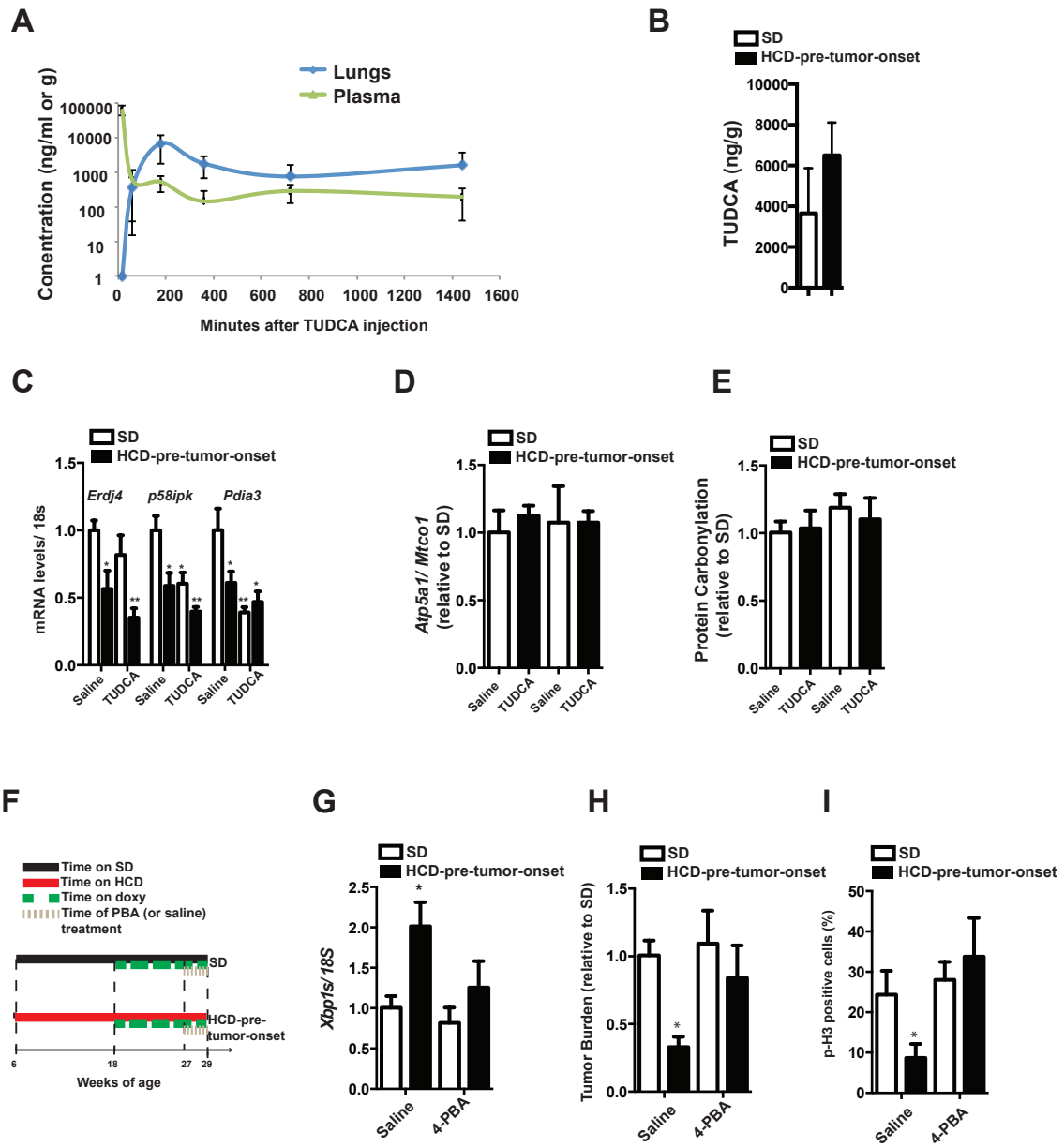
Supplemental Figure 3

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Supplemental Figure 3, related to Figure 3. Treatment with chemical chaperones reverses the anti-tumor effect of HCD feeding on KRAS-driven lung tumors. (A) TUDCA pharmacokinetics in plasma and lung of mice after a single intraperitoneal injection of TUDCA (dose= 250mg/kg). **(B)** TUDCA accumulation in dissected tumors of HCD-pre-tumor-onset and SD mice after 3 hours after the last injection of 5 days of

treatment. (C) mRNA levels, **(D)** mRNA ratio and **(E)** protein carbonylation, from microdissected tumor of HCD-pre-tumor-onset and SD mice treated with TUDCA or saline.   **(F)** Time table of mouse treatments, **(G)** mRNA levels, **(H)** tumor burden and **(I)** tumor proliferation in HCD-pre-tumor-onset and SD mice treated as shown in **(F)** (males; n=4-5/ group). Error bars represent s.e.m. Statistical analyses were done using one-way ANOVA (Tukey's post test). *P<0.05, **P<0.01.

Supplemental Figure 4
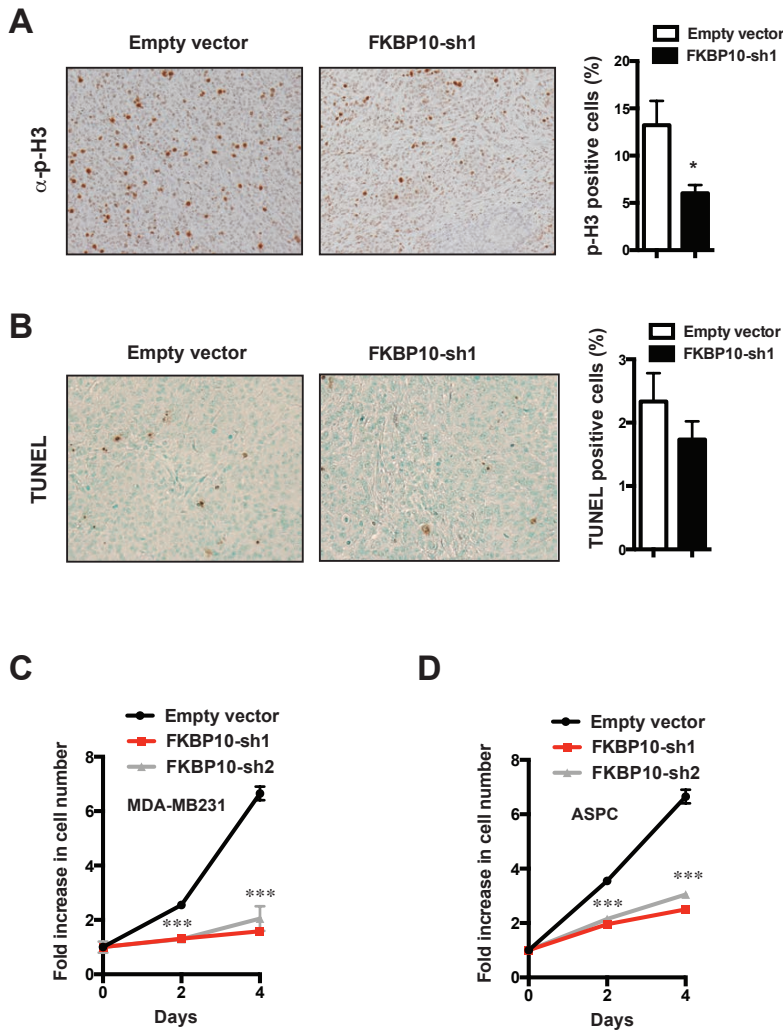
Supplemental Figure 4, related to Figure 4. Unraveling FKBP10 as a novel therapeutic target for KRAS-driven lung cancer. **(A)** Representative images of tumor sections stained with anti-p-H3 (p-H3 positive cells in dark-brown) and histograms indicating % of p-H3 positive cells/tumor cells (n=5 for group). **(B)** Representative images of tumor sections stained for TUNEL assay (in brown TUNEL positive cells) and relative quantification histograms indicating % of TUNEL positive cells/tumor cells (n=5 for group). **(C)** Anti-proliferative effect of FKBP10 knockdown in MDA-MB231 (cell line from human mammary adenocarcinoma) and **(D)** ASPC (cell line from human pancreatic ductal adenocarcinoma) cells. Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test or using one-way ANOVA (Tukey's post test). *P<0.05, ***P<0.001.

Supplemental Methods.

Mouse generation and studies

CCSP-rtTA/Tet-op-K-ras mice (FVB/SV129 mixed background) were generated as previously described (Konstantinidou et al., 2009; Konstantinidou et al., 2013). Mice were housed in groups of 4-5 with food (either a standard chow rodent diet or the high-calorie (HC) diet (D12331 from Research Diets, New Brunswick, NJ, USA) and water available *ad libitum* in light- and temperature-controlled environments. Drinking water has been supplemented with doxycycline at the concentration of 200 µg/mL for the length of time indicated in the figure legends. TUDCA (Calbiochem) resuspended in saline at the concentration of 100 mg/mL (or saline for the controls) was administered by

intraperitoneal injection (250mg/kg at 8am and 250mg/kg at 8pm). 4-PBA (Calbiochem) was administered at the same time points (500 mg/kg for 8am and 8pm, total 1g/kg/day) by oral gavage. Controls for PBA, received the same volume of vehicle by gavage of the PBA-treated mice. Tumor burden was assessed by digital quantification of the area occupied by tumors in the left lobe compared to unaffected tissue using NIH ImageJ (v1.42q) software. Tumor number was assessed by microscope analysis of lung sections stained with H&E and defined as the average of the number of adenomas present in three adjacent sections of the left lobe. Tumor size is defined as the ratio between tumor area arbitrary values (using NIH ImageJ (v1.42q) software) and tumor number in the left lobe. Fed hormones/metabolites levels were determined by collecting tail blood from mice that were without food for 3 hours. Fasted hormones/metabolites levels were assessed in mice provided only with water *ad libitum* and without food for the indicated period. Time of day at which blood was collected was the same between groups. Tail vein blood was assayed for glucose levels using a standard glucometer (Fisher Scientific, Morris Plains, NJ). Serum was collected by centrifugation and assayed for leptin (Crystal Chem. Inc., Downers Grove, IL) and insulin (Crystal Chem. Inc.) levels using commercially available kits. Xenograft experiments were performed by subcutaneous inoculation of 10^6 cells into 8-week-old male SCID mice. Tumor volumes were calculated every three days using the formula: (length X width^2) /2.

Immunoblotting

Immunoblots were performed according to standard procedures in RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.5, 1% NP40, 1% Deoxycholate, 0.1% SDS) and supplemented

with phosphatase and protease inhibitors (SIGMA). Samples were resolved on EZ-Run™ 10% Protein Gel Solution (Fisher Scientific), blotted on Protran® membrane 0.2 μm (Whatman) and developed with Li-Cor imaging System. The following antibodies were used: p-AKT^{Ser473}, total AKT, p-ERK1/2$^{Thr202/Tyr204}$, total ERK1/2, p-eIF2α, eIF2α, p-STAT3, STAT3, p-S6, p-c-Jun, Cyclin D1 and P58IPK (Cell Signaling); β–Actin (Sigma); XBP1-s, p-IRE1α, IRE1a and Lamin A and Tubulin (Abcam); total JNK (Santa Cruz); PDIA3 (GeneTex Antibodies); FKBP10 (ProteinTech).

Immunohistochemistry

Mouse lungs and tumors were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. IHC procedure was performed as previously described (Konstantinidou et al., 2009; Konstantinidou et al., 2013), using a commercial antibody against p-H3 (Millipore) and cleaved-caspase-3 (Cell Signaling). To detect apoptosis in mouse lung tissue we used the ApopTag plus peroxidase in situ apoptosis detection kit (Millipore). Methyl-green was used as counterstaining in TUNEL assay. Hematoxylin was used as conterstaining in IHC for p-H3 and cleaved-caspase-3. In order to quantify the % of p-H3 and TUNEL-positive cells, a total of 200 cells were scored/slide for at least 3 replicates.

In order to determine FKBP10 expression in human paraffin embedded lung samples we used the following procedure. Sections of 3-5 μm thick were deparaffinized and the detection of antigens has occurred in automated manner with DAKO PT Link using ENVISION™ FLEX TARGET RETRIEVAL SOLUTION LOW pH (50X) (DAKO) at a temperature of 98°C. After 70 minutes of treatment, sections were treated with 3% hydrogen peroxide and incubated for 30 minutes with unconjugated anti-mouse IgG at

RT. Subsequently sections were incubated for 1 hour at RT with mouse Anti-FKBP65 antibody (1:750, BD Transduction Laboratories™). The staining was completed using ENVISION FLEX ™/HRP (DAKO), as detection system; 3,3- diaminobenzidine-hydrogen peroxide was used as chromogen. Then, the slides were counterstained with Meyer's hematoxylin for 1 minute, dehydrated in a graded series of alcohol, treated with xylene, and coverslipped. Immunohistochemical staining was semi-quantitatively assessed by considering the "percentage of positive tumor cells" (range 0-100%).

Quantitative real-time PCR

RNA was extracted using a RNA extraction kit (Qiagen). Complementary DNA was generated by Superscript II (Invitrogen) and used with SYBR Green PCR master mix (Applied Biosystem) for quantitative real time PCR (q-RTPCR) analysis. mRNA contents were normalized to *18-S*. Sequences of deoxy-oligonucleotides primers used are outlined here: *Erdj4* 5'CCCCAGTGTCAAACTGTACCAG and 5'AGCGTTTCCAATTTTCCATAAATT; *p58ipk* 5'CAGTTGATGGTGACCCCGAT and 5'GTCTTGCGGCAGTAAAGTCC; *Pdia3* 5' TGTTGGAACTGACGGACGAA and 5'GGCGAAGAACTCGACTAGCA; *Hedj* 5'AGGAGCGAAGAGAACTGGACT and 5'AAATGACTCCAATCCCCAGCC; *Xbp1-s* 5'CTGAGTCCGCAGCAGGT and 5'TGTCAGAGTCCATGGGAAGA; *Xbp1-u* 5'CTGAGTCCGCAGCACTCAGA and 5' TCAGAGTCCATGGGAAGATGTTC; *Chop* 5'ACCTGAGGAGAGAGTGTTCCA and 5'CAAGGTGAAAGGCAGGGACT; *18S* 5'ACCGCAGCTAGGAATAATGGA and 5'GCCTCAGTTCCGAAAACCA; *Dr5* 5'GGAGCTCTGCTTGTCTGGAA and 5'CGTGCTAGATGTCTGTCGGT; *Bcl2* 5'TTCTTTGAGTTCGGTGGGGTC and

5'TGGGGCCATATAGTTCCACAA; *Hspd1* 5'TCAGTCCATTGTCCCTGCTC and 5'AACCAGCGTGCTTAGAGCTT; *Hspa9* 5'CAAGGGTGCAGTGGTTGGTA and 5'GGGGTAGTTCTGGCACCTTC; *Hspe1* 5'TGAAAGGAGTGCTGCCGAAA and 5'TTCCTTTCCCTCCTGACCCC; *Sod2* 5'GCCTCCCAGACCTGCCTTAC and 5'GTGGTACTTCTCCTCGGTGGCG; *Cat* 5'AGGACCGTGTTTGGTTGCTT and 5'CCGCTGGCGCTTTTATTGT. All assays were performed using an Applied Biosystems Prism 7900HT sequence detection system. For each mRNA assessment, quantitative RT-PCR analyses were repeated at least 3 times.

Expression profiling

Gene expression profiles were obtained from micro-dissected lung tumors of 29-week old mice. For total RNA extraction we used a RNA isolation kit (Qiagen) followed by DNase digestion and cleanup according to the manufacturer's instructions. Microarray was performed by UTSW Microarray Core facility (http://microarray.swmed.edu) using Illumina MouseWG-6 v2.0 Expression BeadChips (Illumina, NC, USA). The Selected enriched groups of genes where analyzed by Gene Set Enrichment Analysis (GSEA) (Huang da et al., 2009). The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE56260.

shRNAs, virus production and transduction

The pLKO lentiviral vectors encoding shRNAs targeting *FKBP10* were purchased from Open Biosystems. Clones IDs were the following: 1) TCRN0000053928, 2) TCRN0000053929, 3) TCRN0000053930, 4) TCRN0000053931, 5) TCRN0000053932.

We produced recombinant lentiviruses by transfecting 293T cells, using TransIT-293 tranfection reagent (Mirus), with pMD2G (VSV-G protein), pCMV-dR8.74 (lentivirus packaging vector) and lentiviral constructs.

Cell lines

Human cell lines A549, H596, H1650, ASPC and MDA-MB231 were from the Hamon Center cell line repository (UT Southwestern Medical Center). All cell lines were DNA-fingerprinted for provenance (PowerPlex 1.2 Kit; Promega) and Mycoplasma-free (e-Myco Kit; Boca Scientific).

Carbonylated protein determination.

Levels of carbonylated proteins in lysates from tumors were determined by ELISA (Enzo Life Sciences) following manufacturers' instructions.

Pharmacokinetics assessments.

Analytical processing of plasma samples: 100µl of plasma were mixed with 200µl of methanol containing 0.15% formic acid and 37.5 ng/ml n-benzylbenzamide internal standard (IS). The samples were vortexed 15 sec, incubated at room temp for 10 min and spun twice at 16,100 x g 5 min 4ºC in a standard microcentrifuge. TUDCA levels were then monitored by LC-MS/MS using an AB/Sciex (Applied Biosystems, Foster City, CA) 4000 Qtrap mass spectrometer coupled to a Shimadzu (Columbia, MD) Prominence LC. The compound was detected using electrospray ionization with the mass spectrometer in

positive MRM (multiple reaction monitoring) mode by following the precursor to fragment ion transition 522.4 (M + Na+) → 486.4.

<u>Analytical processing of lung and tumor samples</u>: samples from lungs or tumors were prepared by homogenizing tissue in a three (Fig S3A) or six (Fig S3B)- fold volume of PBS100 µl of homogenized tissue solution were mixed with 25 µl of 12.5% perchloric acid (PCA). The samples were vortexed 15 sec and centrifuged at 16,100 x g for 5min at 4ºC in standard microcentrifuge tubes. Aliquots of 75 µl were neutralized with 75 µl of 350 mM KOH and 75 µl of 175 mM KHCO3. These samples were vortexed 15 sec and centrifuged again at 16,100 x g for 5 min at 4ºC in standard microcentrifuge tubes. Aliquots of 180µl of supernatant were put into HPLC vials with inserts and spiked with 0.9 µl of 1 ng/µl n-benzylbenzamide IS vortexed and analyzed by QTRAP 4000 LC-MS/MS system as described for plasma.

Supplemental References

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc *4*, 44-57.
Konstantinidou, G., Bey, E.A., Rabellino, A., Schuster, K., Maira, M.S., Gazdar, A.F., Amici, A., Boothman, D.A., and Scaglioni, P.P. (2009). Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. Cancer Res *69*, 7644-7652.
Konstantinidou, G., Ramadori, G., Torti, F., Kangasniemi, K., Ramirez, R.E., Cai, Y., Behrens, C., Dellinger, M.T., Brekken, R.A., Wistuba, II*, et al.* (2013). RHOA-FAK is a required signaling axis for the maintenance of KRAS-driven lung adenocarcinomas. Cancer discovery *3*, 444-457.