ATDC binds to KEAP1 to drive NRF2-mediated tumorigenesis and chemoresistance in pancreatic cancer

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SUPLEMENTARY FIGURES, FIGURES LEGENDS AND TABLES

Figure S1. Related to Figure 1

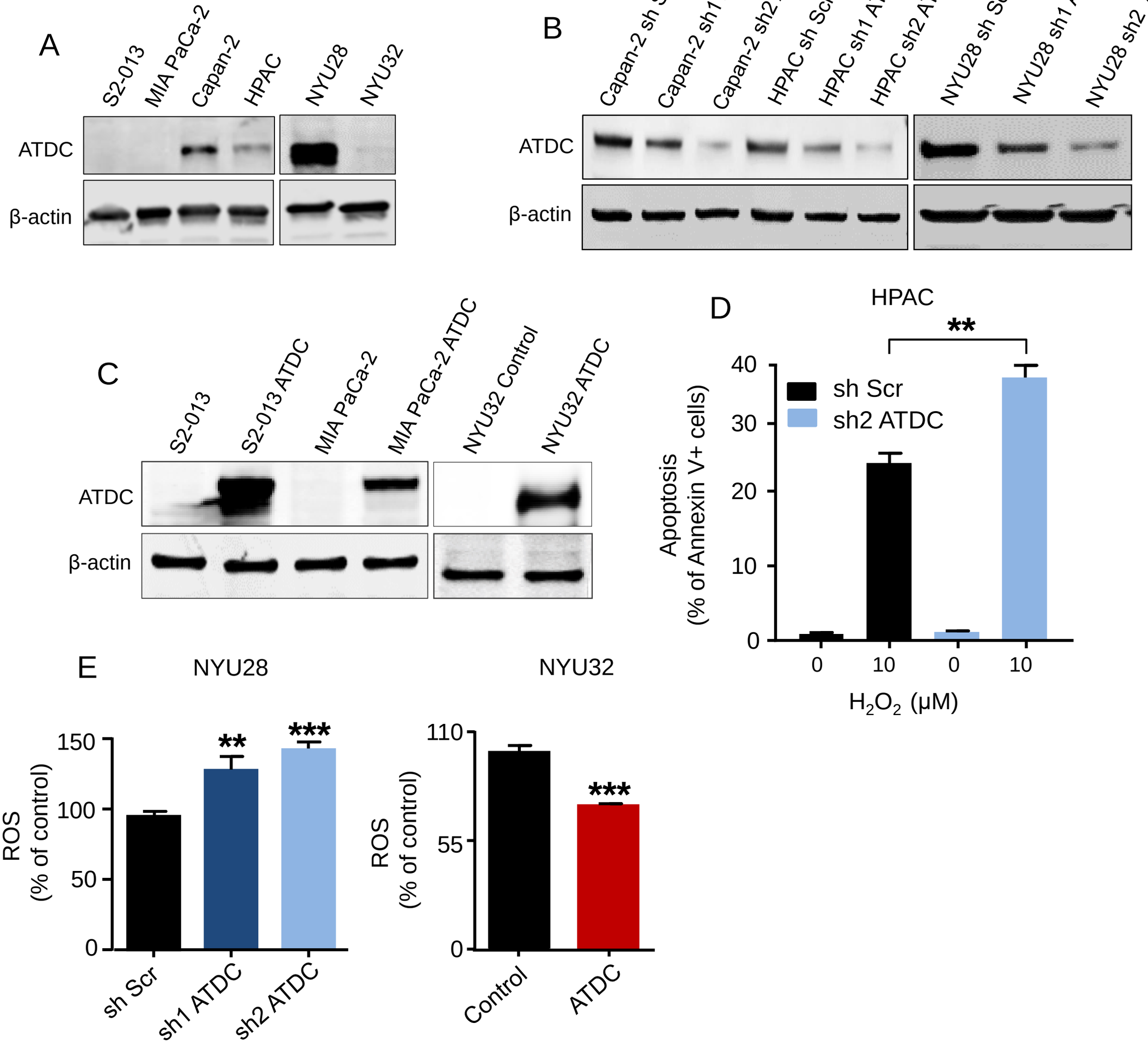
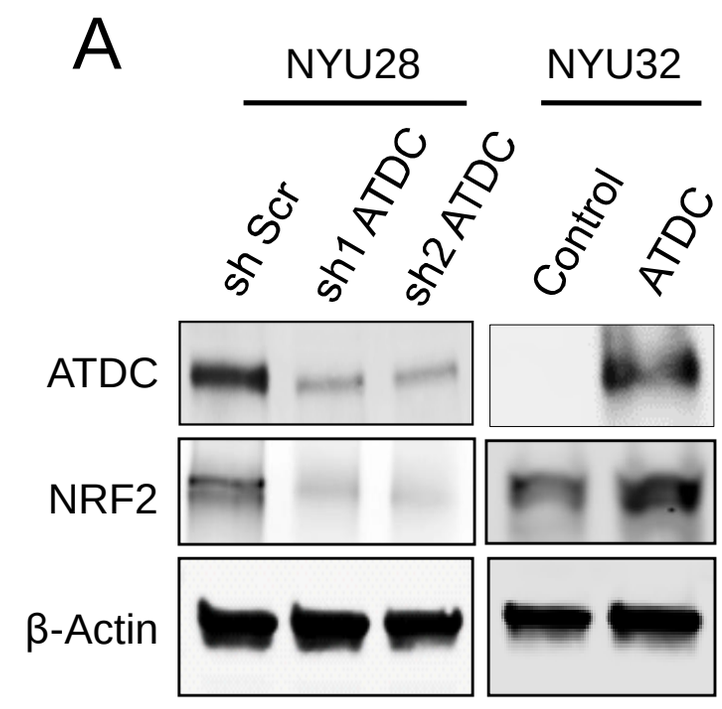
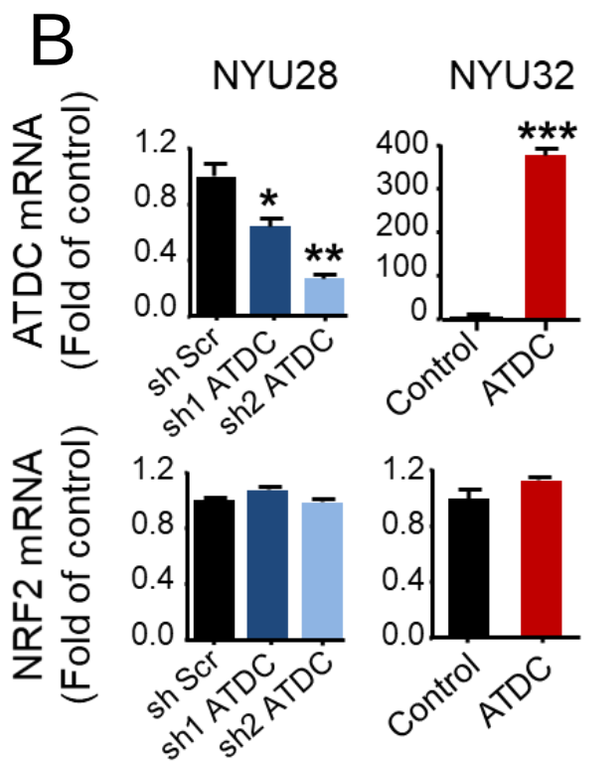
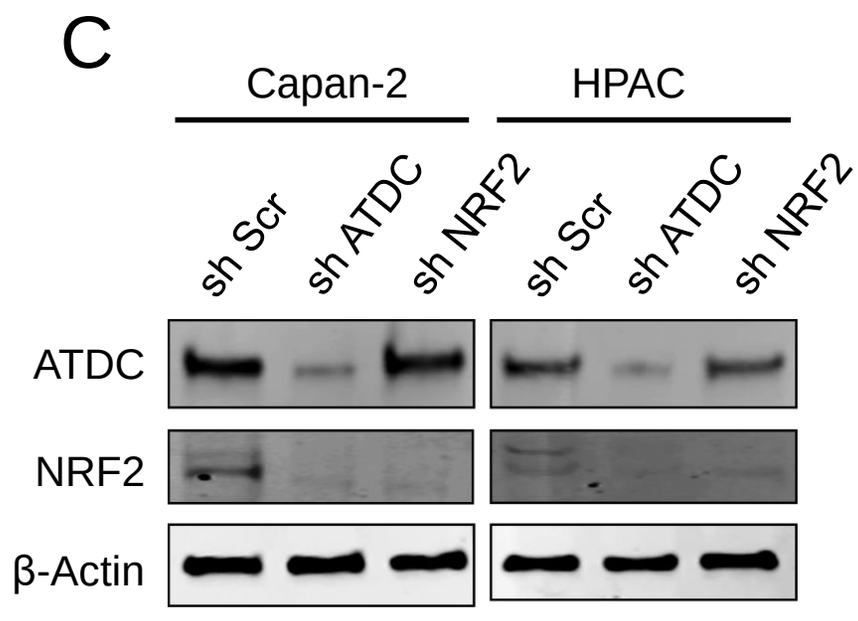


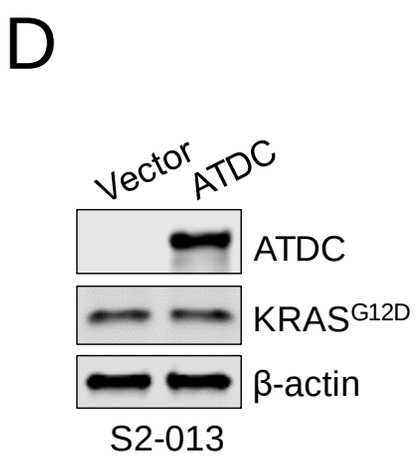
Figure S1. Related to Figure 1: (A) Western blots of ATDC protein levels in established cell lines (S2-013, MIA PaCa2, Capan2 and HPAC) and primary human PDA cell lines (NYU28 and NYU32). (B) Western blots of ATDC protein levels in isogenic cell lines expressing either a scramble shRNA (sh Scr) or sh ATDC. (C) Western blots of ATDC protein levels in isogenic cell lines overexpressing ATDC. (D) Apoptosis assays (Annexin V) performed in HPAC control cells (sh Scr) or cells expressing sh2 ATDC 72 hours after H_2O_2 treatment. (E) Quantification of ROS levels in NYU28 cells expressing sh ATDC or NYU32 overexpressing ATDC or control (vector) cells. All experiments were performed in triplicate. **p< 0.01; ***p <0.005; mean $\pm$ SEM.

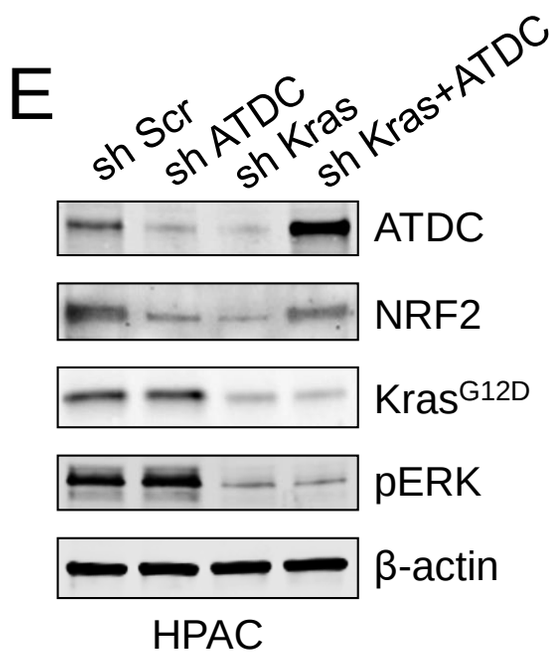
Figure S2. Related to Figure 2











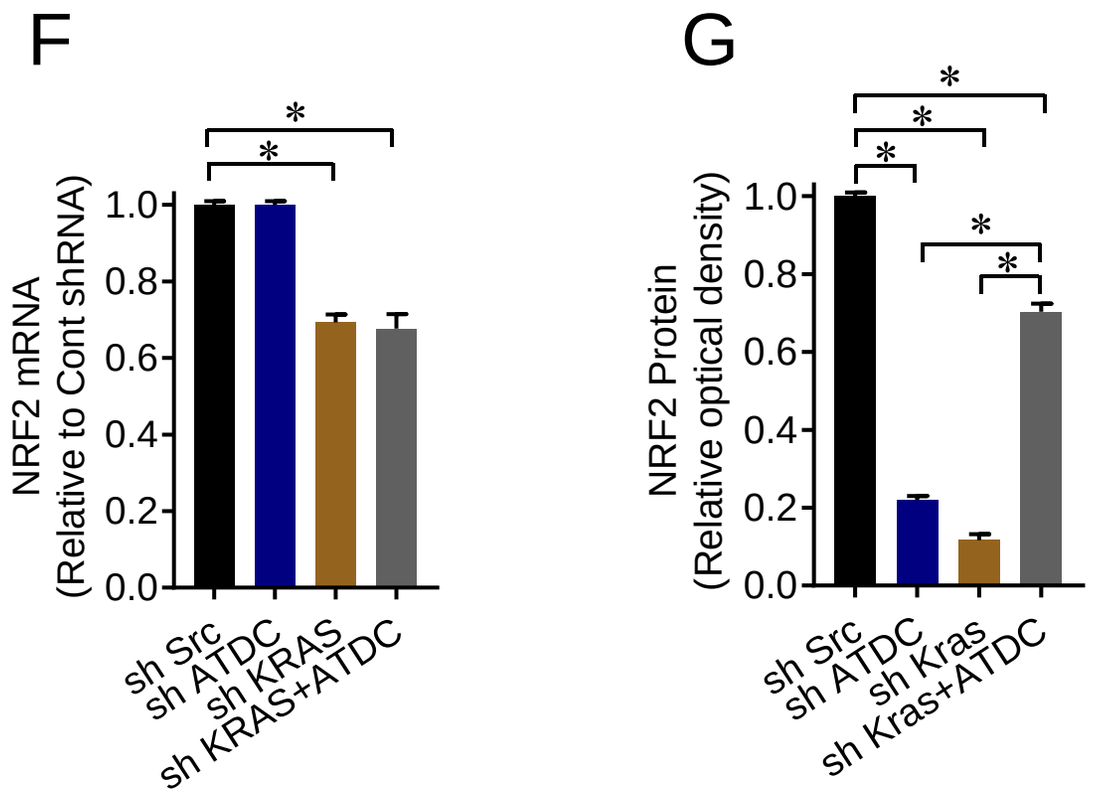
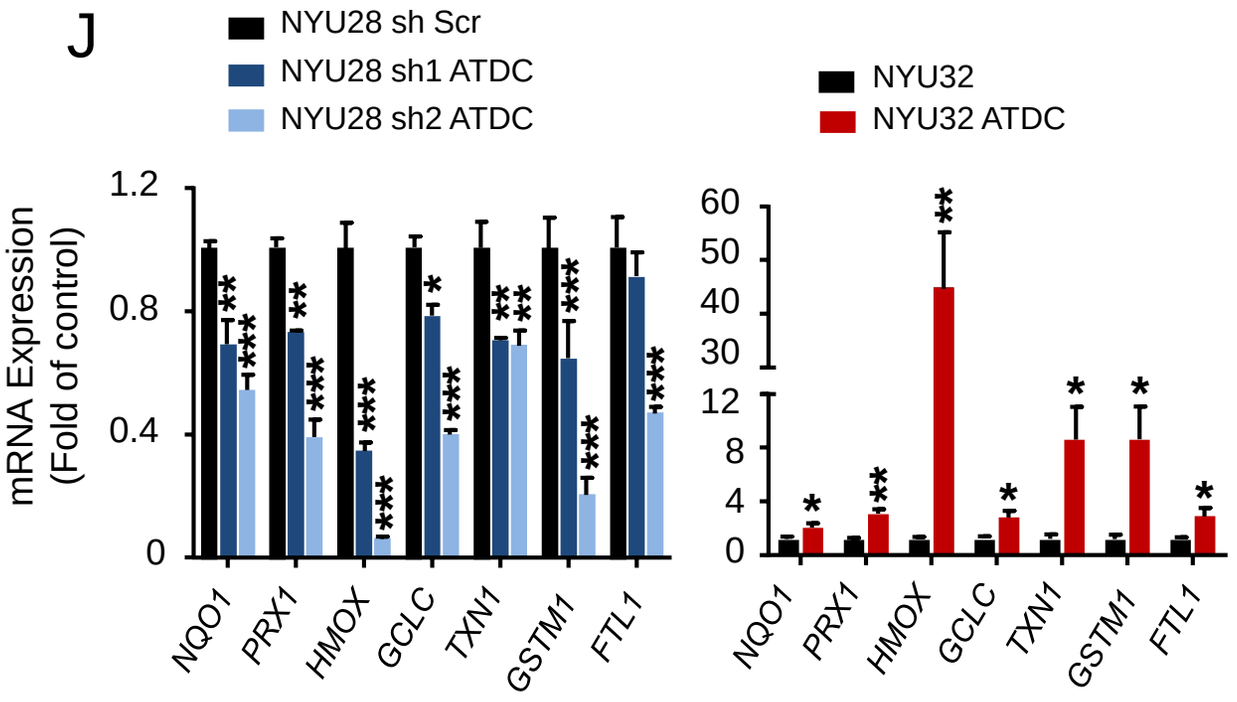
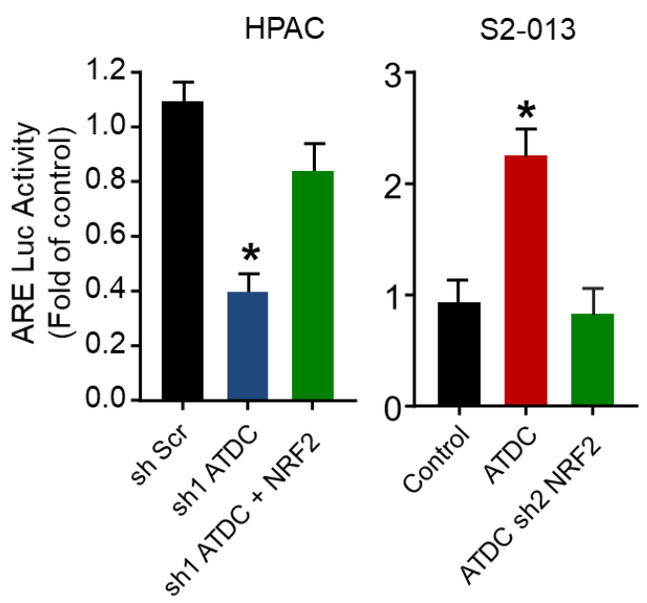
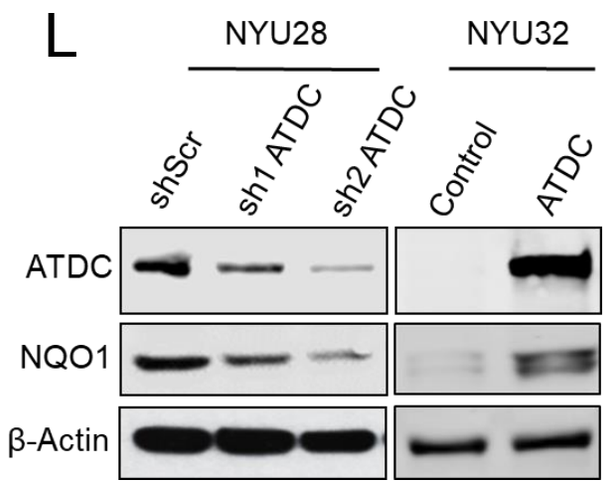


Figure S2. Related to Figure 2







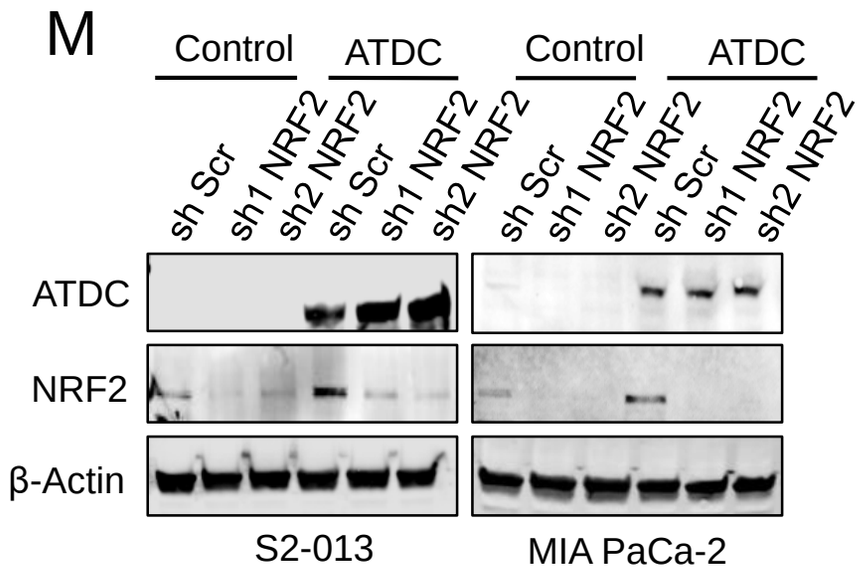
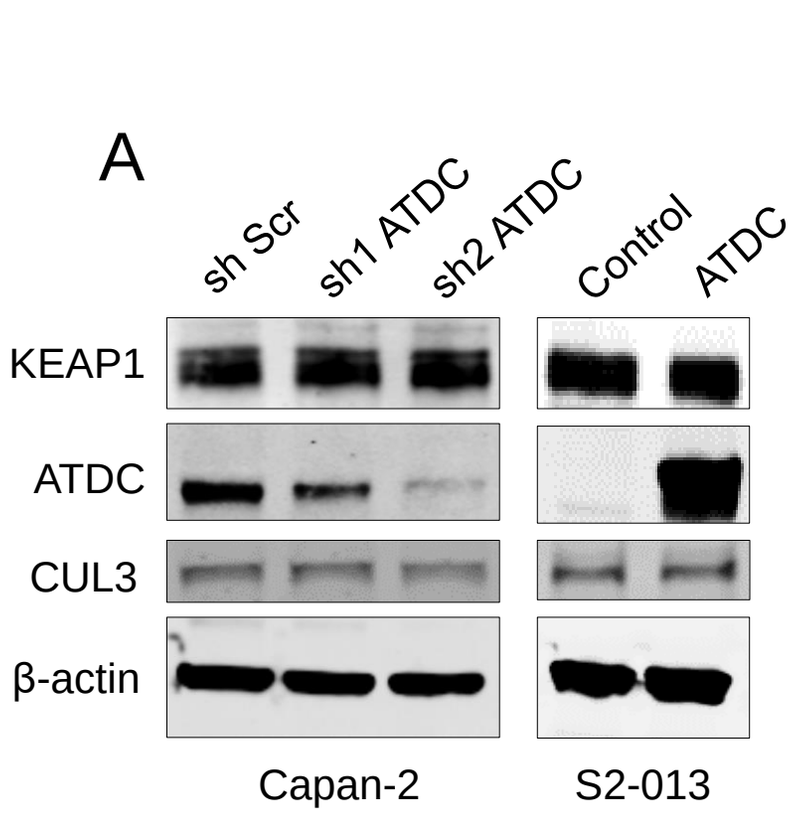
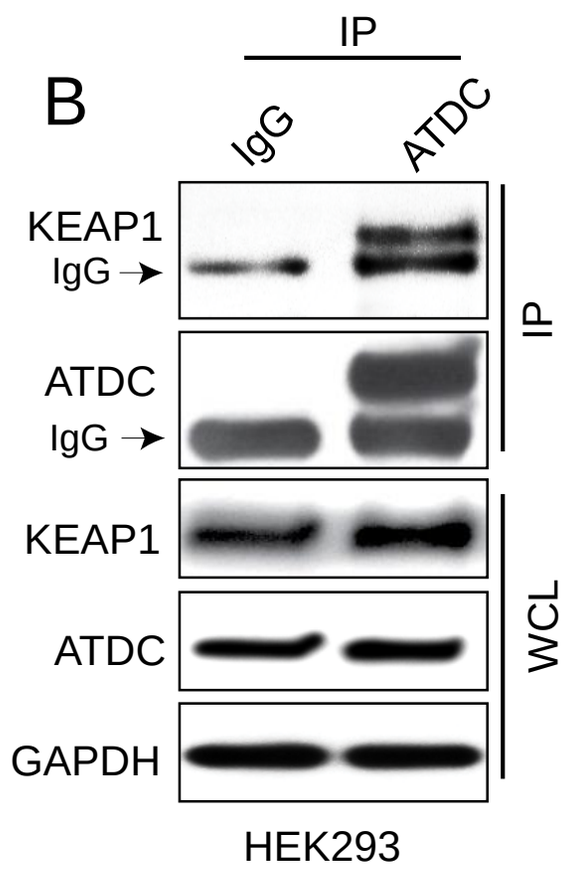
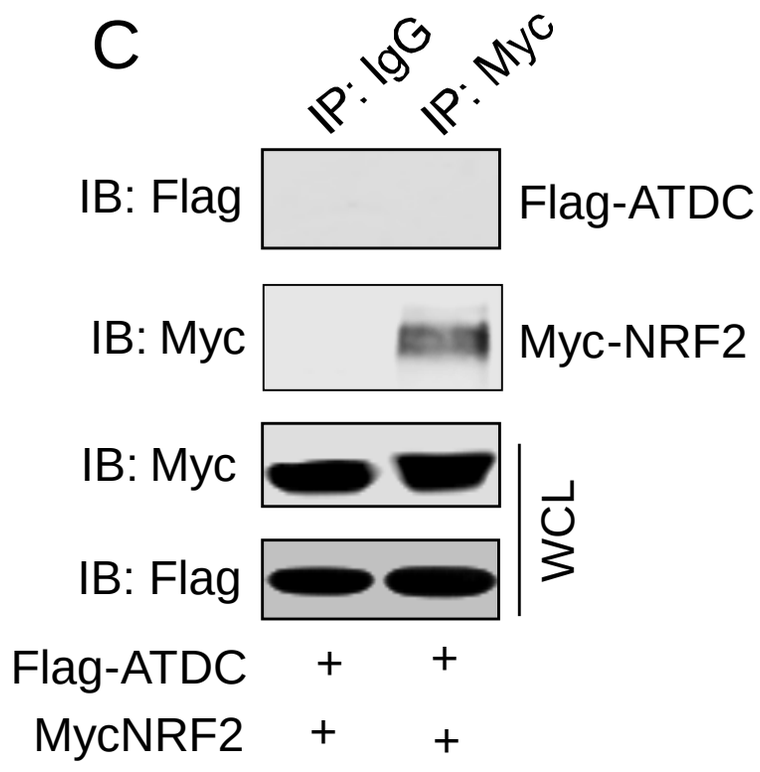


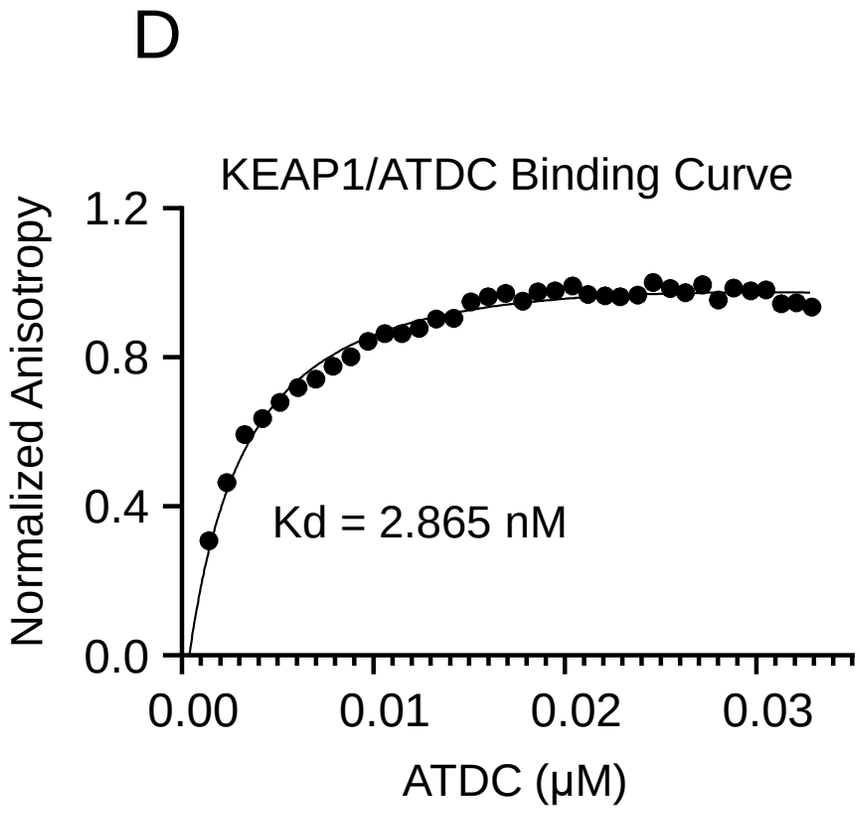
Figure S2. Related to Figure 2: (A) Western blots of ATDC or NRF2 protein levels in NYU28 cells expressing sh ATDC or NYU32 cells overexpressing ATDC. β-actin was used as a loading control. (B). ATDC and NRF2 mRNA levels in NYU28 or NYU32 cells described in A. (C) ATDC and NRF2 protein levels in Capan-2 or HPAC cells with knockdown of ATDC or NRF2. β-actin was used as a loading control. (D) ATDC and $KRAS^{G12D}$ protein levels in S2-013 cells overexpressing ATDC. β-actin was used as a loading control. (E) ATDC, NRF2, $KRAS^{G12D}$, pERK protein levels in HPAC cells with knockdown of ATDC, KRAS or ATDC rescue. β-actin was used as a loading control. (F) NRF2 mRNA levels in HPAC cells were described in E measured by RT-PCR. (G) NRF2 protein levels in HPAC cells were described in E measured by western blot. (H, I) Immunofluorescence staining of ATDC (green), NRF2 (red), DAPI (blue) in S2-013 cells overexpressing ATDC (H) or HPAC cells expressing sh ATDC (I). Insets indicate magnified views of staining patterns. White arrows indicate the nuclear staining of NRF2. Scale bar: 50 μm. (J) RT-PCR assay to determine the effect of ATDC knockdown (NYU28 cells) or overexpression (NYU32 cells) on mRNA expression of the NRF2-regulated genes (NAD(P)H Quinone Dehydrogenase 1, NOQ1; Peroxiredoxin 1, PRDX1; Hemoxygenase 1, HMOX1; Glutamate-Cysteine Ligase Catalytic Subunit GCLC; Thioredoxin, TXN1; Glutathione S-Transferase Mu 1, GSTM1; and Ferritin light chain, FTL). (K) ARE-Luc reporter activity assessed in HPAC cells expressing sh ATDC, sh ATDC+NRF2, or S2-013 cells overexpressing ATDC with or without subsequent knockdown of NRF2. (L) ATDC or NQO1 protein levels in NYU28 cells expressing sh ATDC or NYU32 expressing ATDC. β-actin was used as a loading control. (M) ATDC and NRF2 protein levels in S2-013 or MIA PaCA2 cells overexpressing ATDC with or without subsequent knockdown of NRF2. All experiments were performed in triplicate. *p< 0.05; **p< 0.01; ***p <0.005; mean ± SEM.

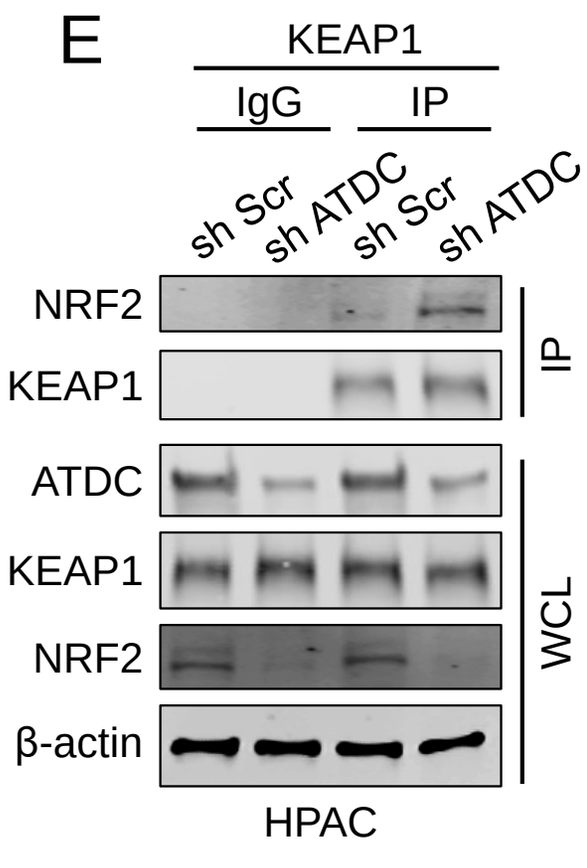
Figure S3. Related to Figure 3

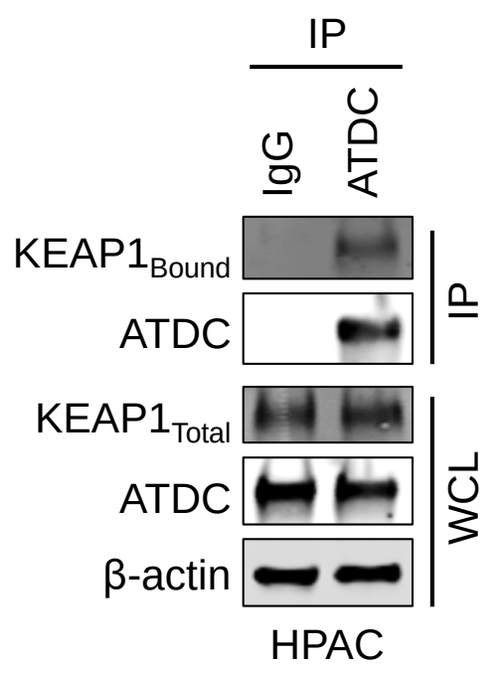


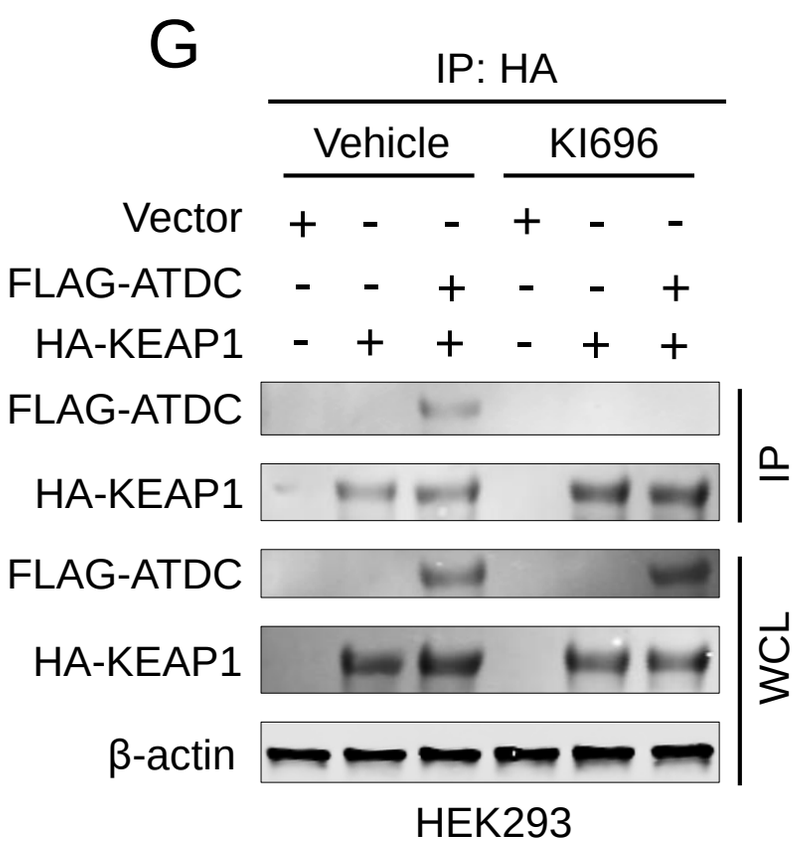


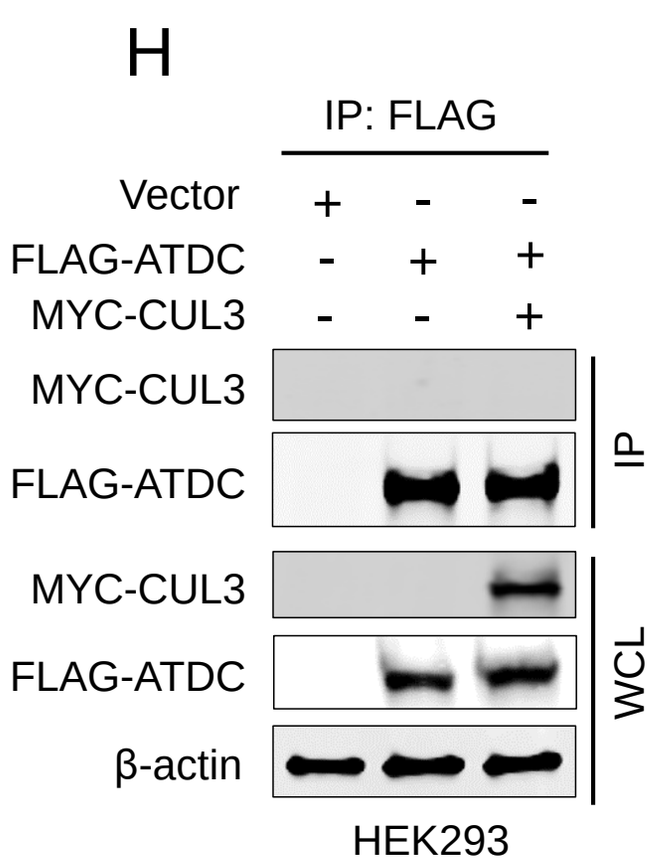


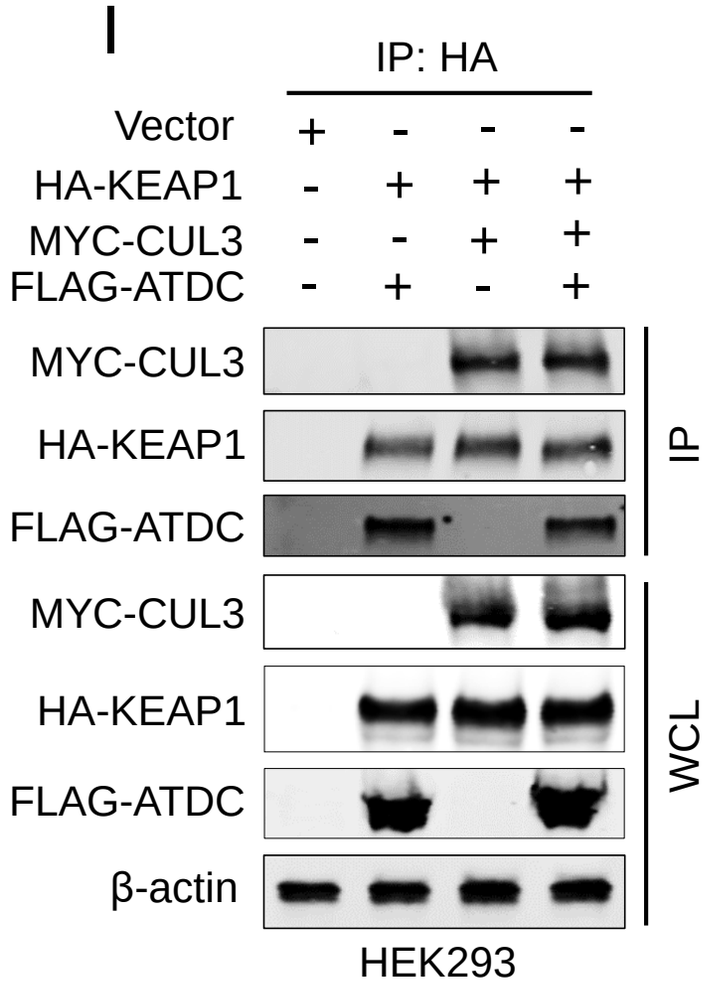












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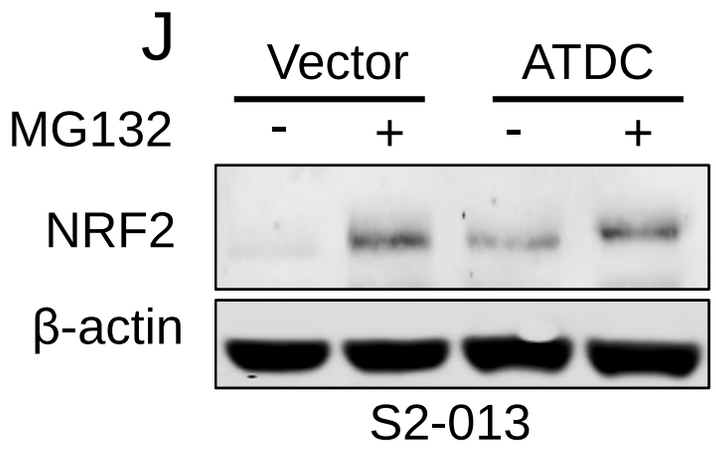
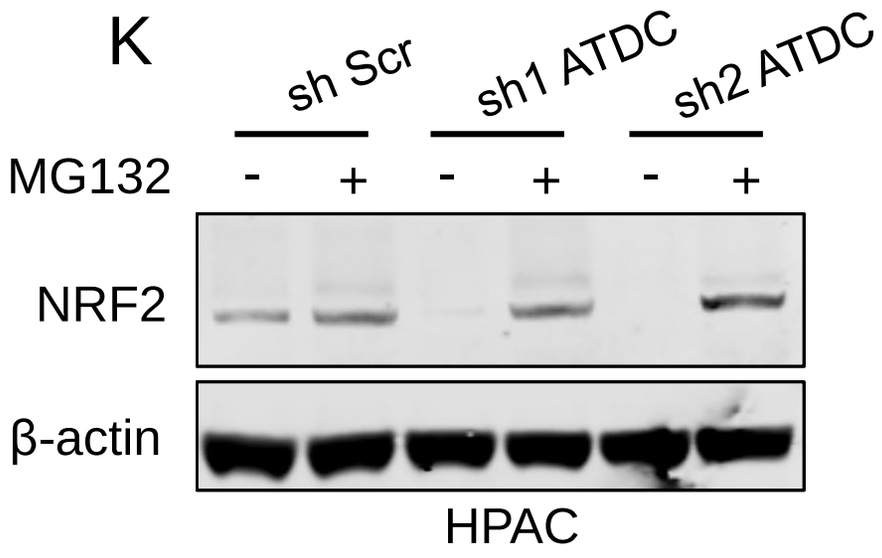
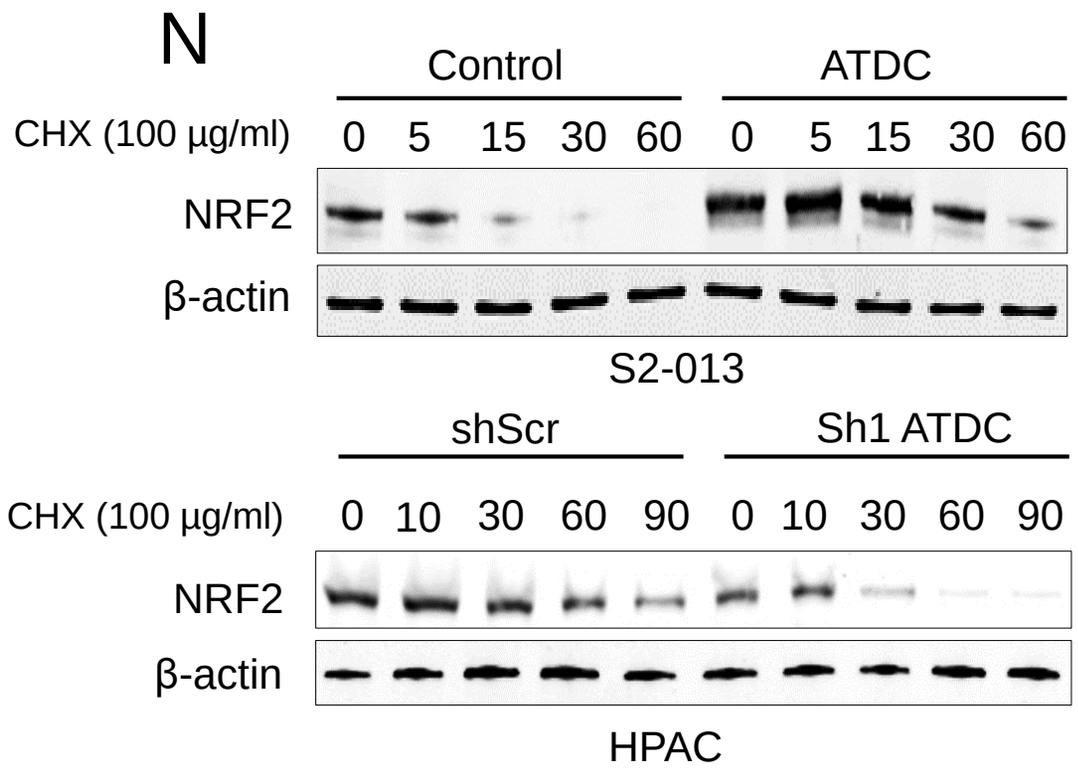
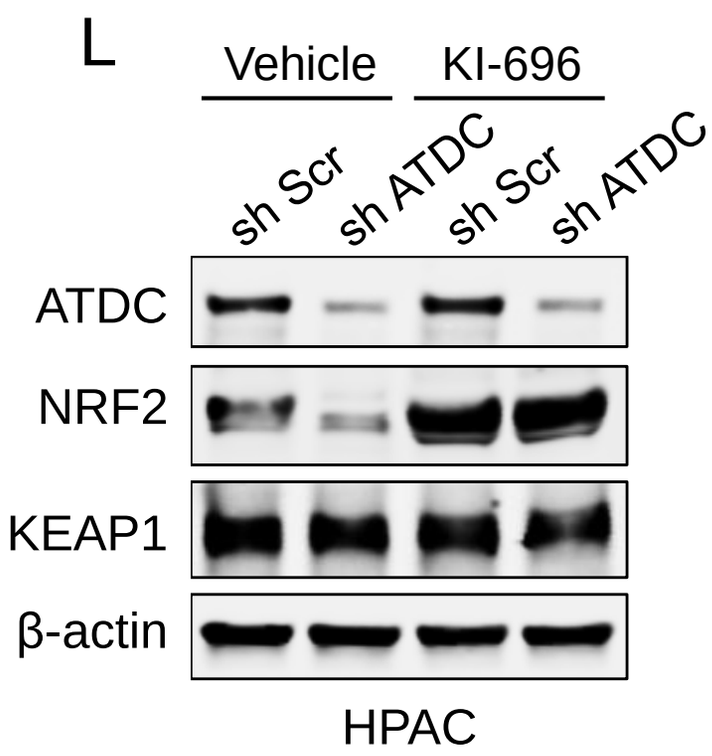
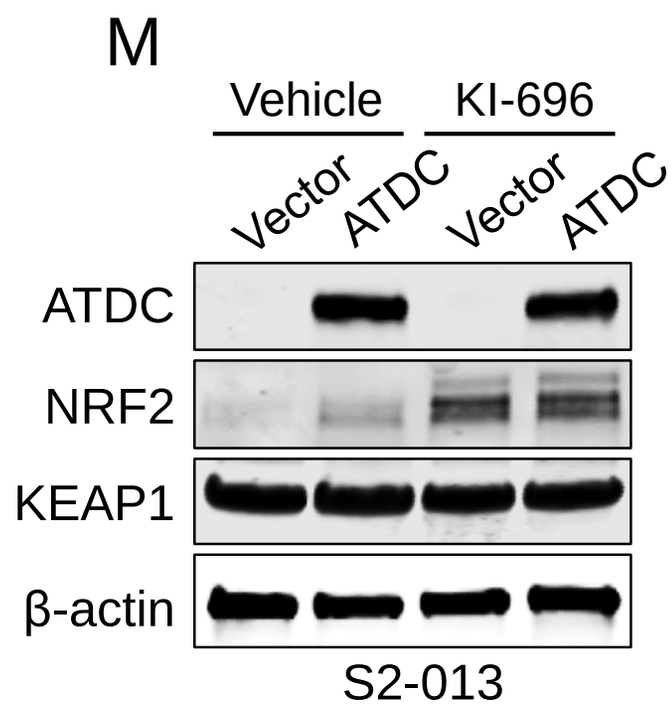










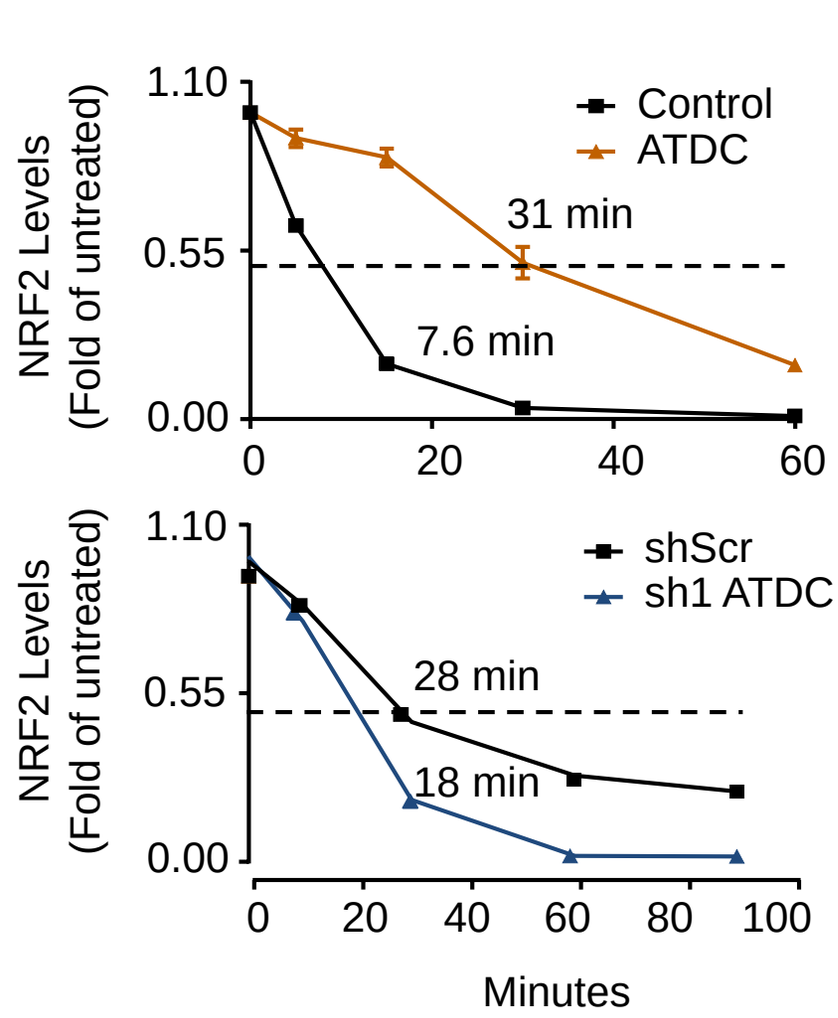
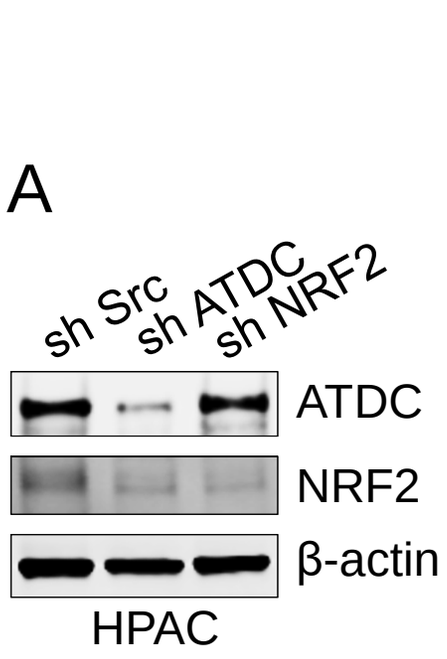
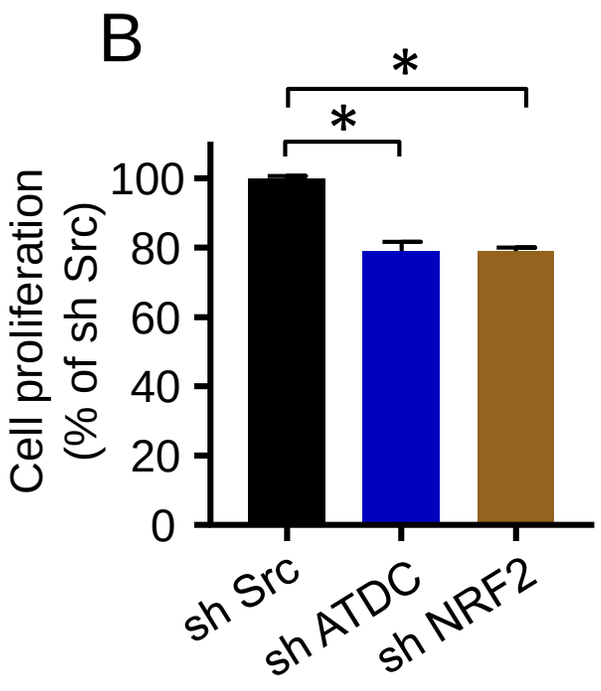
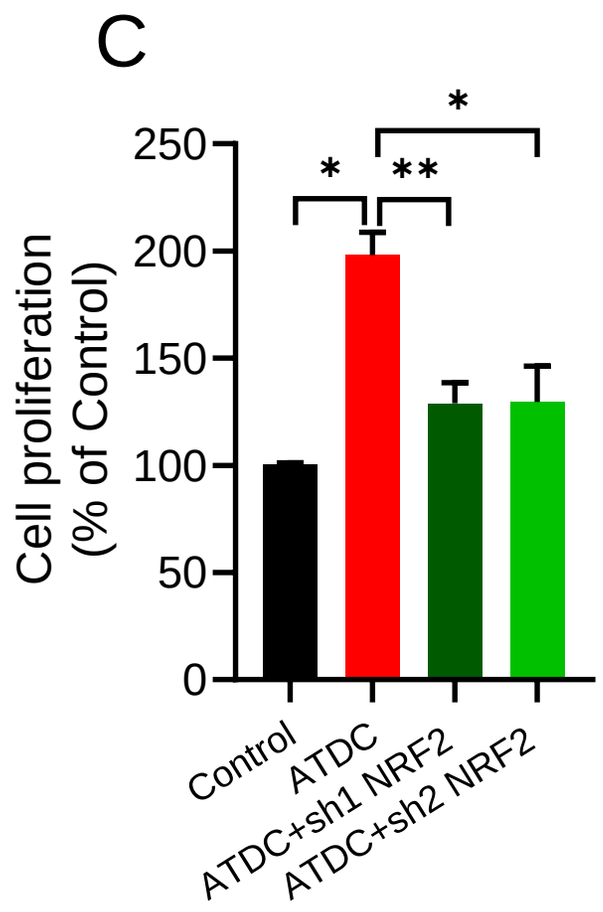


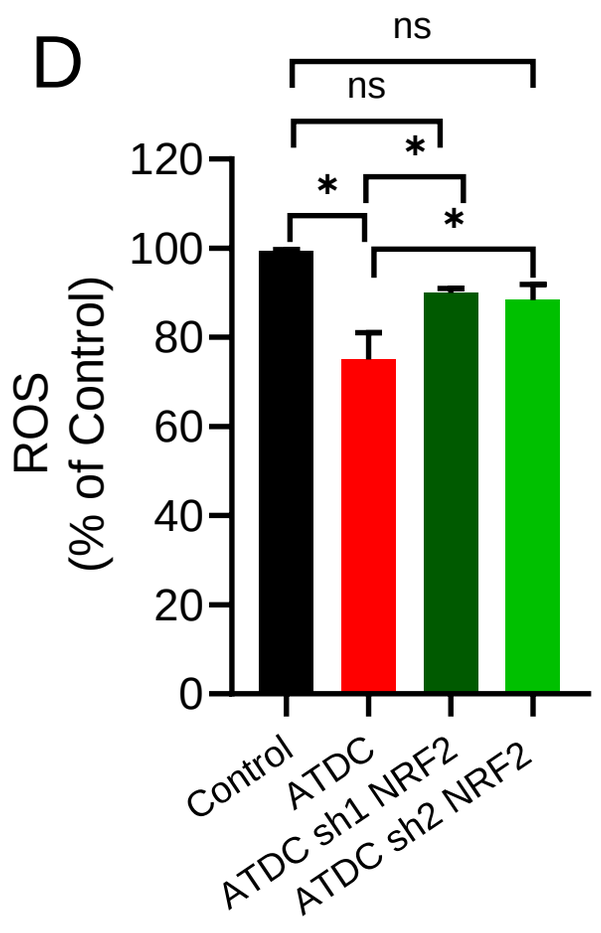
Figure S3. Related to Figure 3: (A) Western blots of KEAP1, CUL3 and ATDC protein levels in Capan-2 cells expressing sh ATDC or S2-013 cells overexpressing ATDC. β-actin was used as a loading control. (B) Cell lysates from HEK293 cells transfected with ATDC were subjected to IP with ATDC antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with KEAP1 and ATDC antibodies, demonstrating endogenous KEAP1 binds to ATDC. (C) Cell lysates from HEK293 cells transfected with FLAG-ATDC and MYC-NRF2 were subjected to IP with MYC antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with FLAG and MYC antibodies, demonstrating ATDC does not bind to NRF2. (D) Fluorescence anisotropy curve to determine the dissociation constant (Kd) for KEAP1/ATDC binding using purified KEAP1 and ATDC protein. Data points correspond to the average of five independent anisotropy measurements. (E) Cell lysates from HPAC cells ± sh ATDC were subjected to IP with a KEAP1 antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with KEAP1 and NRF2 antibodies. (F) Cell lysates from HPAC cells were subjected to IP with IgG or ATDC antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with KEAP1 and ATDC. The $KEAP1_{bound}$ and $KEAP1_{total}$ bands were quantified by densitometry. (G) After pretreatment of KI-696 (1 μM, 6 hrs), cell lysates from HEK293 cells transfected with FLAG-ATDC and HA-KEAP1 were subjected to IP with HA antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with FLAG and HA antibodies. (H) Cell lysates from HEK293 cells transfected with FLAG-ATDC and MYC-CUL3 were subjected to IP with FLAG antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with FLAG and MYC antibodies. (I) Cell lysates from HEK293 cells transfected with FLAG-ATDC, HA-KEAP1, and MYC-CUL3 were subjected to IP with HA antibody. Immunocomplexes and WCL were resolved by SDS-PAGE and subjected to western analysis with FLAG, HA, and MYC antibodies, demonstrating ATDC does not affect KEAP1 bind to CUL3. (J, K) Effect of MG132 treatment (10 μM, 6 hrs) on NRF2 protein levels in S2-013 cells overexpressing ATDC (J) and HPAC cells expressing sh ATDC (K). (L, M) Effect of KI-696 treatment (1 μM, 6 hrs) on NRF2 protein levels in HPAC cells expressing sh ATDC (L) and S2-013 cells overexpressing ATDC (M). (N) NRF2 protein levels in S2-013 cells overexpressing ATDC or HPAC cells expressing sh ATDC after cycloheximide (CHX) (10 μg/ml) treatment. (O) NRF2 protein levels in N were quantitated by densitometry and normalized to the 0 hour time point. All experiments were performed in triplicate.

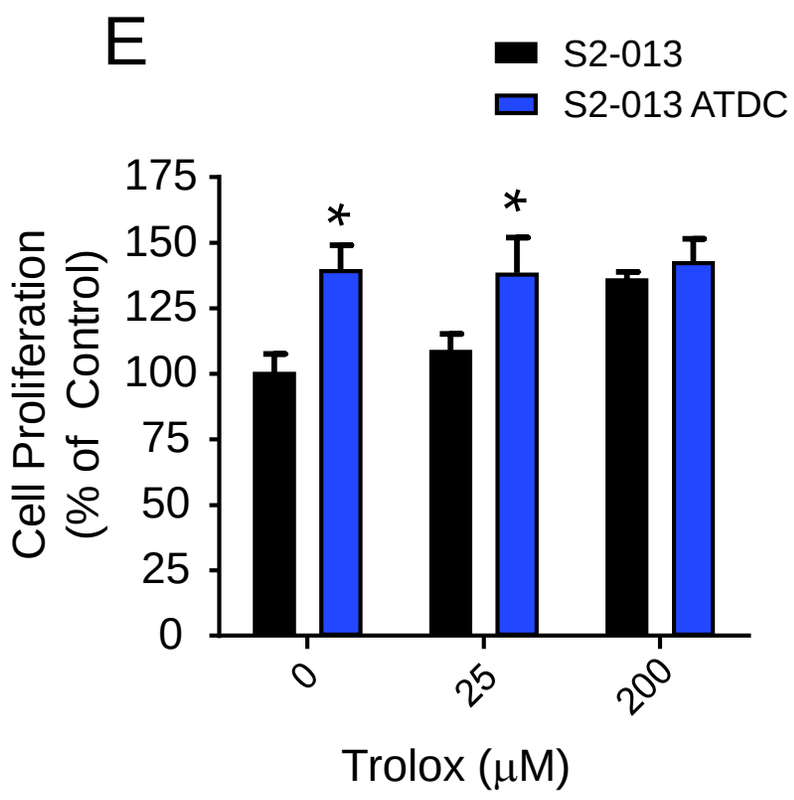
Figure S4. Related to Figure 4

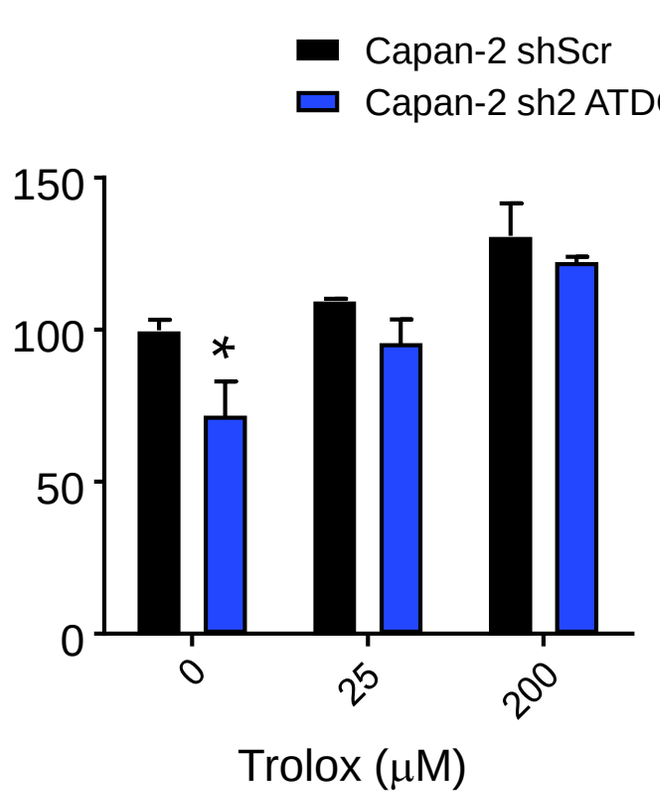


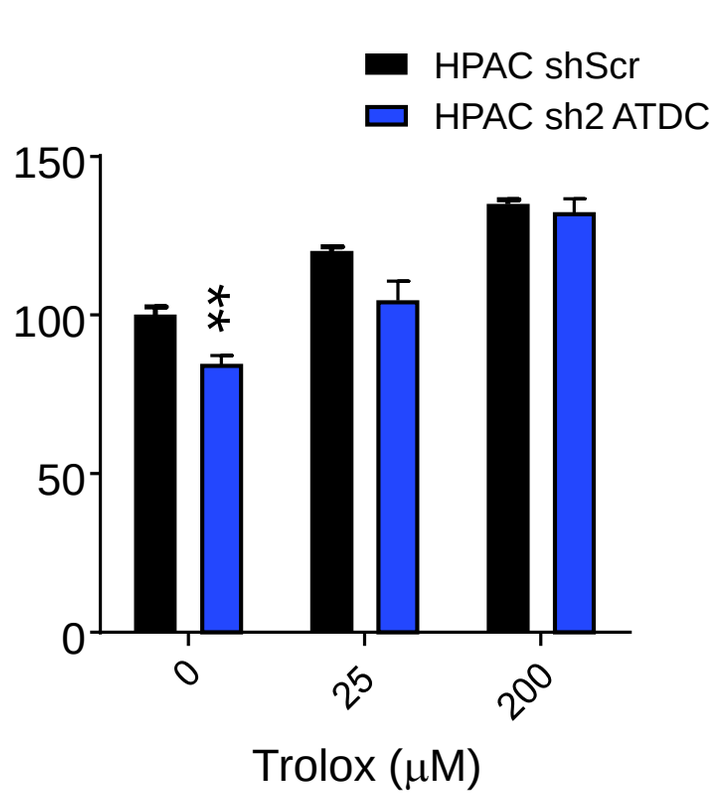


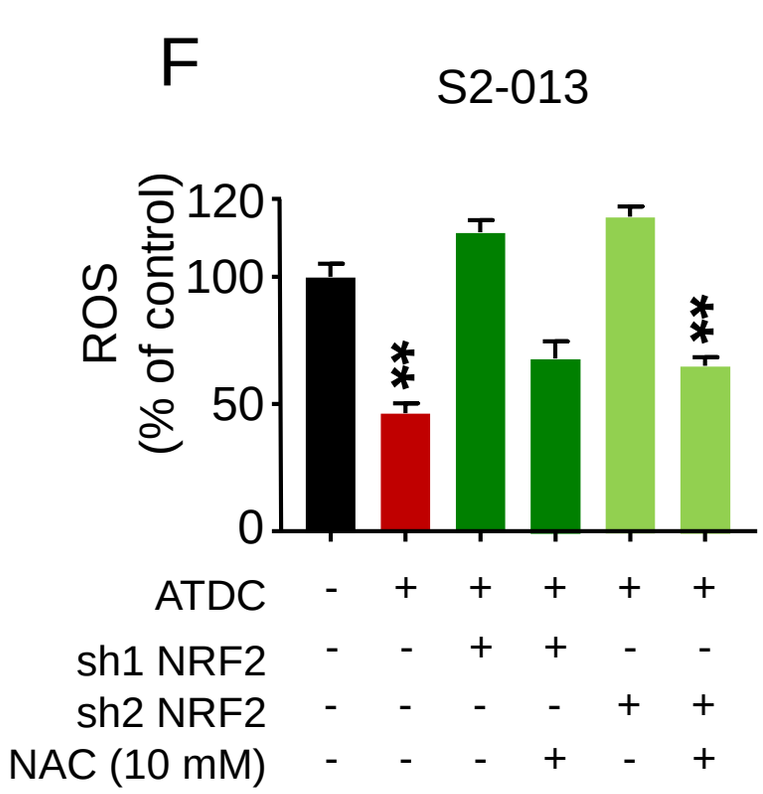


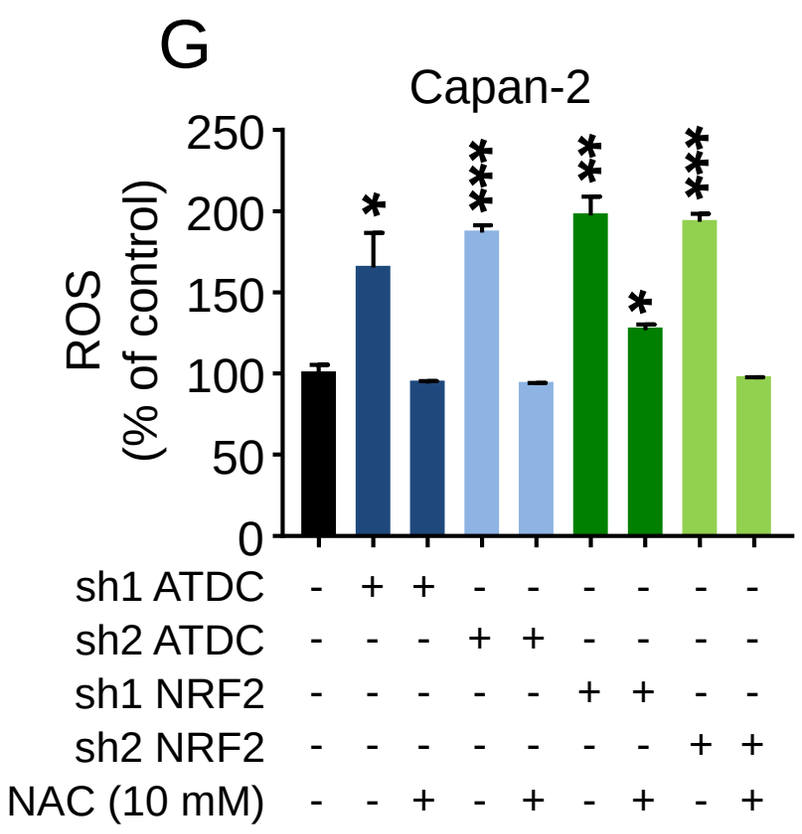












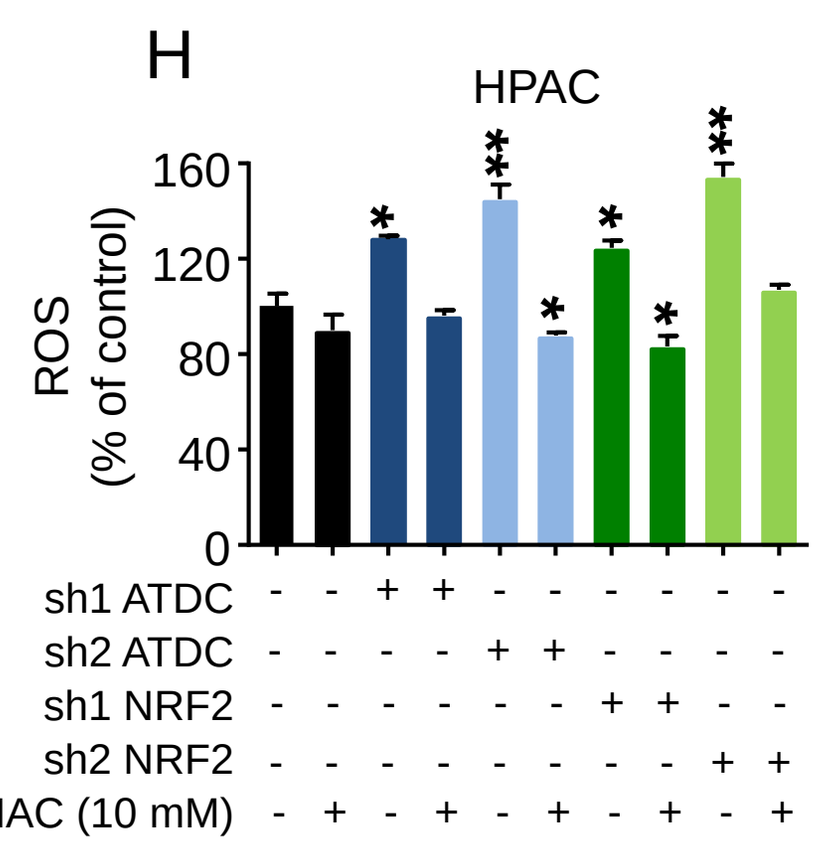
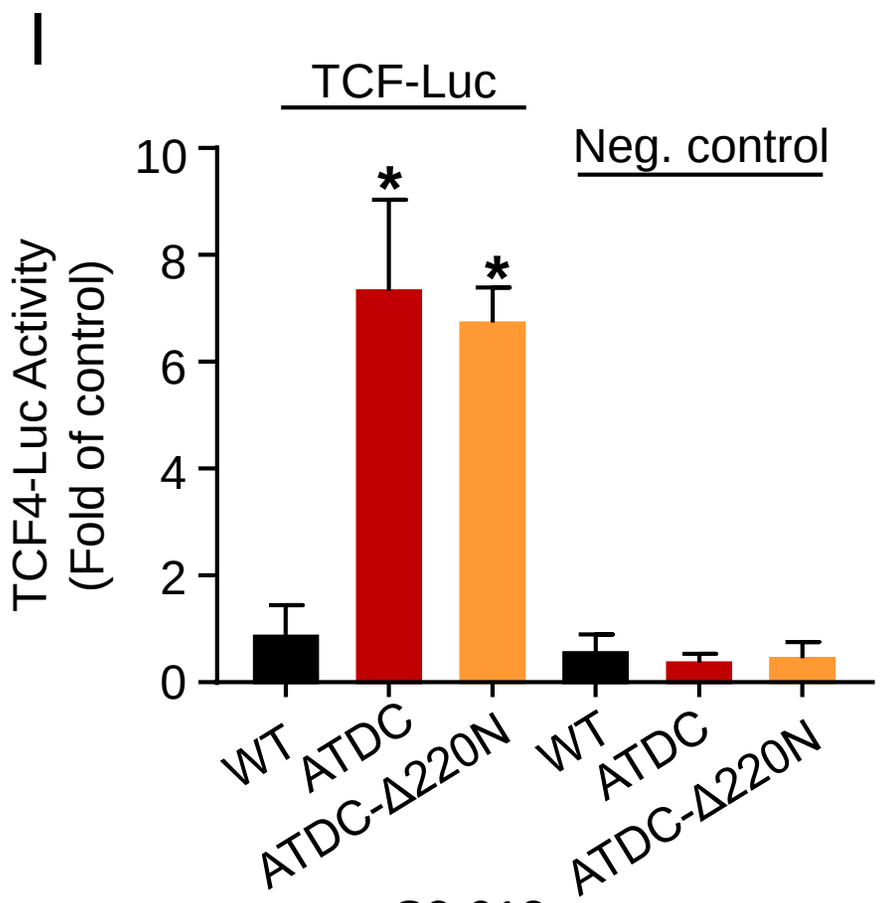


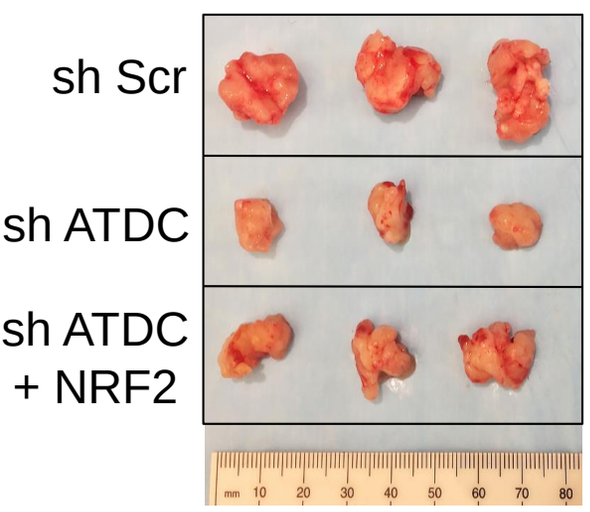
Figure S4. Related to Figure 4

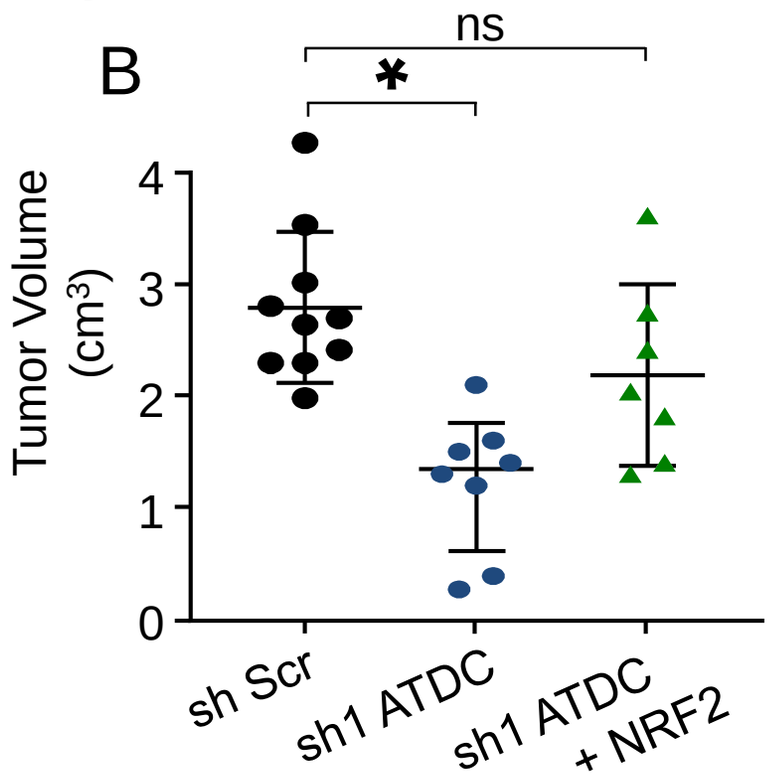


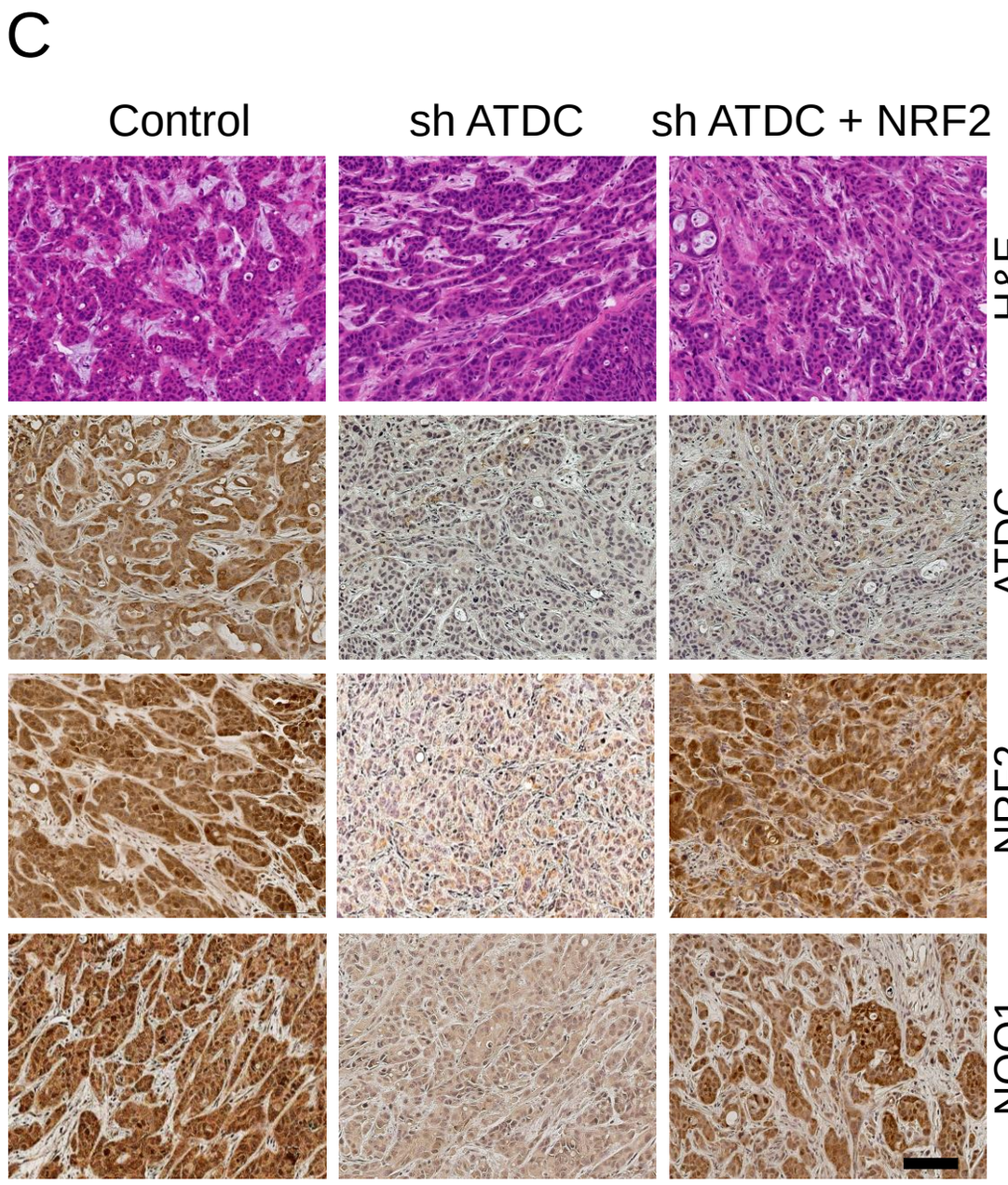
S2-013

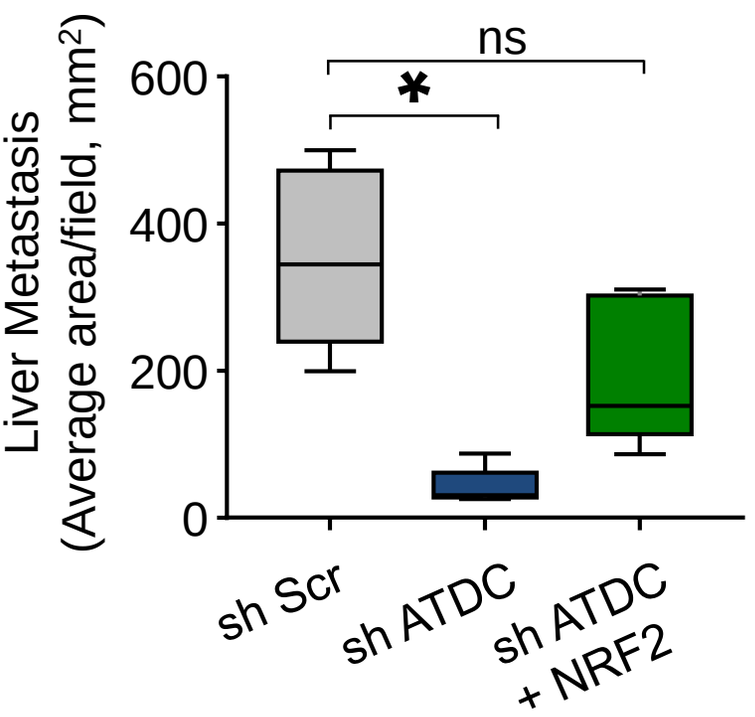
Figure S4. Related to Figure 4: (A) Western blots of ATDC or NRF2 protein levels in HPAC cells expressing sh ATDC or sh NRF2. β-actin was used as a loading control. (B) Cell proliferation assays in HPAC cells expressing sh ATDC or sh NRF2. (C, D) Cell proliferation (C) and ROS levels (D) in S2-013 cells expressing ATDCor ATDC+sh NRF2. (E) Cell proliferation in control and ATDC-overexpressing S2-013 cells and Capan2 or HPAC cells with control or ATDC knockdown. Cells were treated with increasing concentrations of Trolox (0-200 μM). (F) Quantification of ROS levels in S2-013 cells overexpressing ATDC, with or without sh NRF2 in the presence or absence of NAC. (G, H) Quantification of ROS levels in Capan-2 (G) or HPAC cells (H) expressing sh ATDC or sh NRF2 in the presence or absence of NAC. (I) TCF4-Luc reporter activity was assessed in S2-013 cells overexpressing ATDC or ATDC-Δ220N. All experiments were performed in triplicate. *p< 0.05; **p< 0.01; ***p <0.005; mean ± SEM.

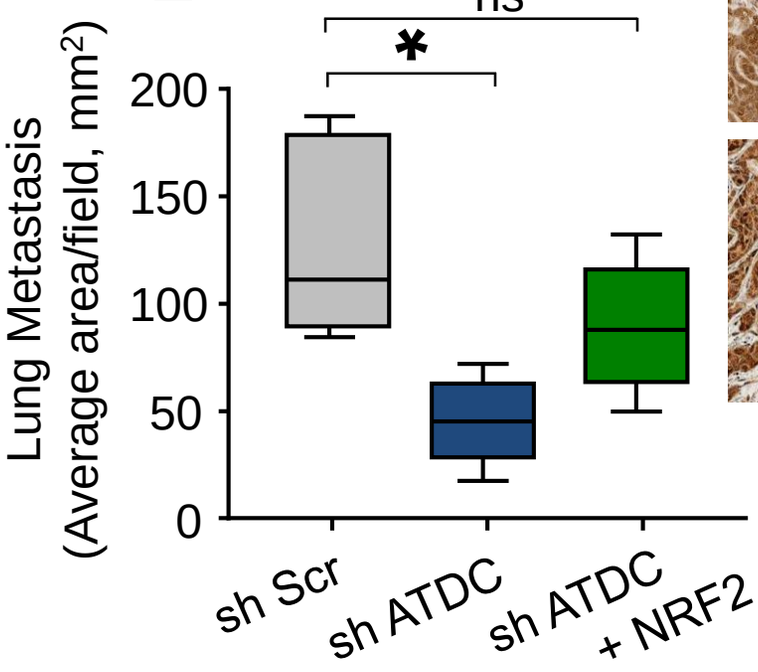
Figure S5. Related to Figure 5

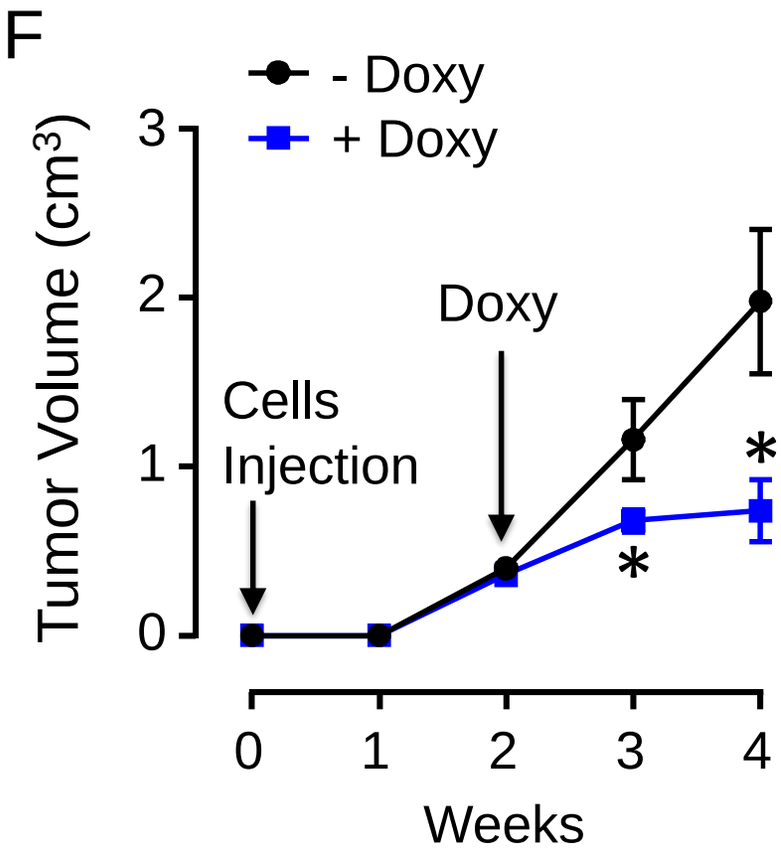


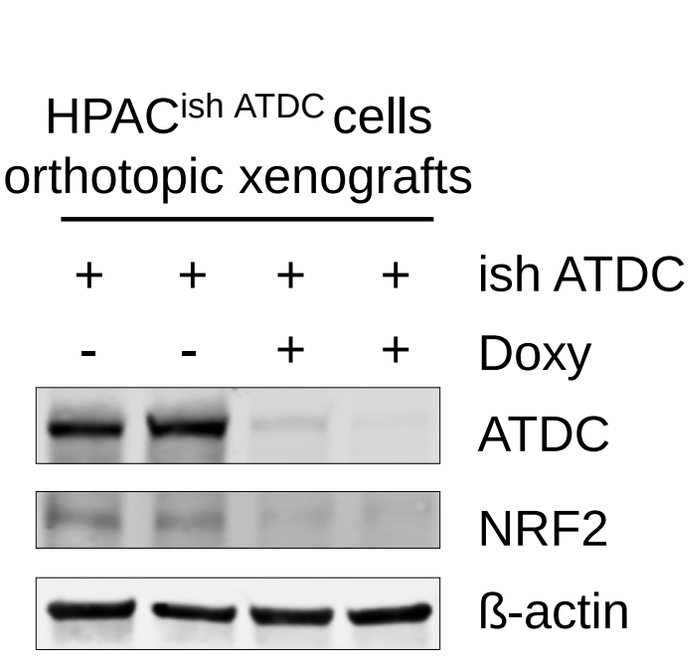


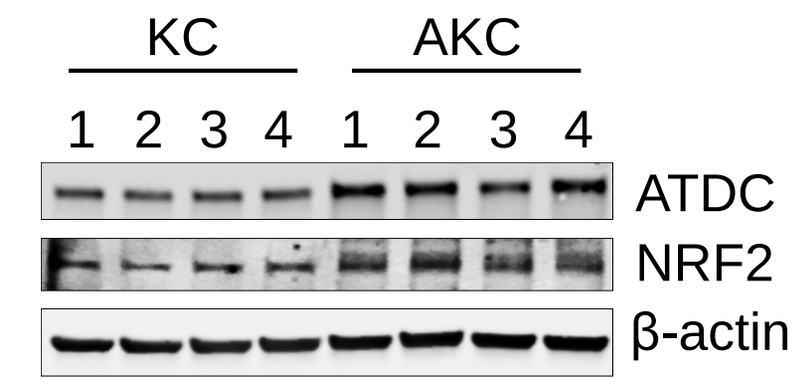


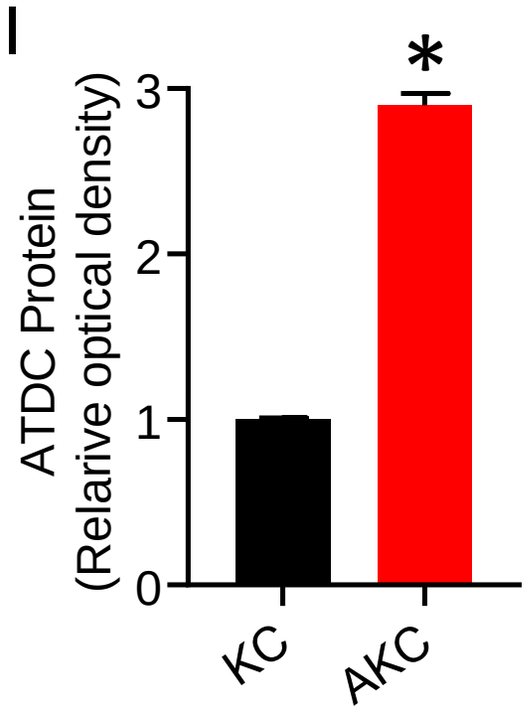












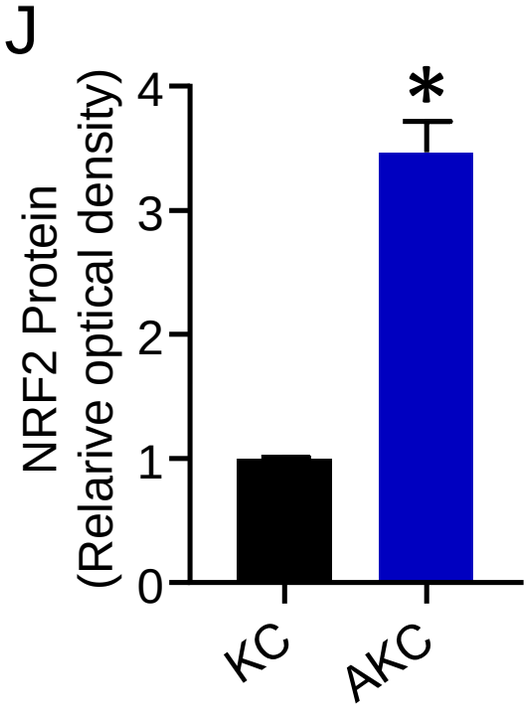


Figure S5. Related to Figure 5

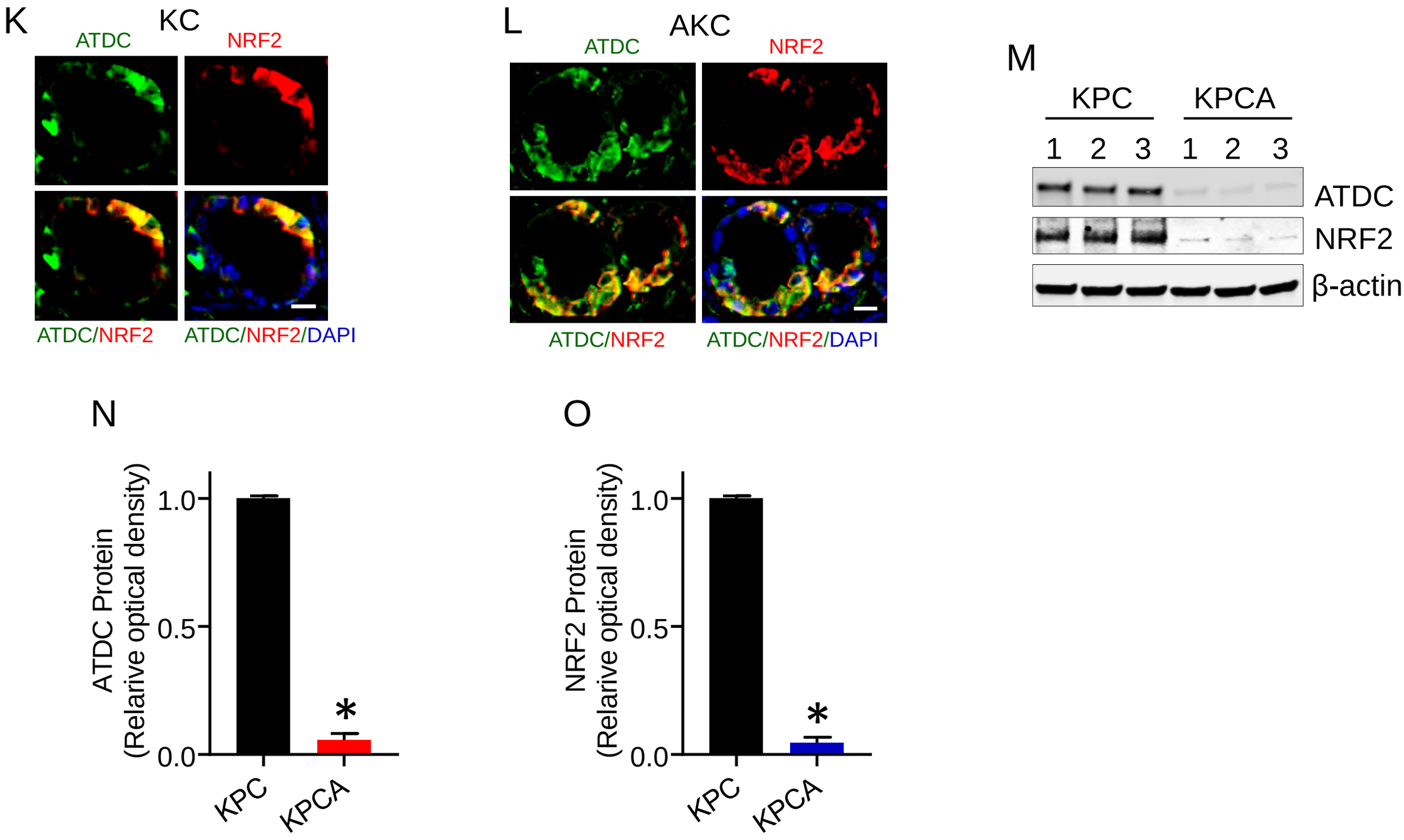
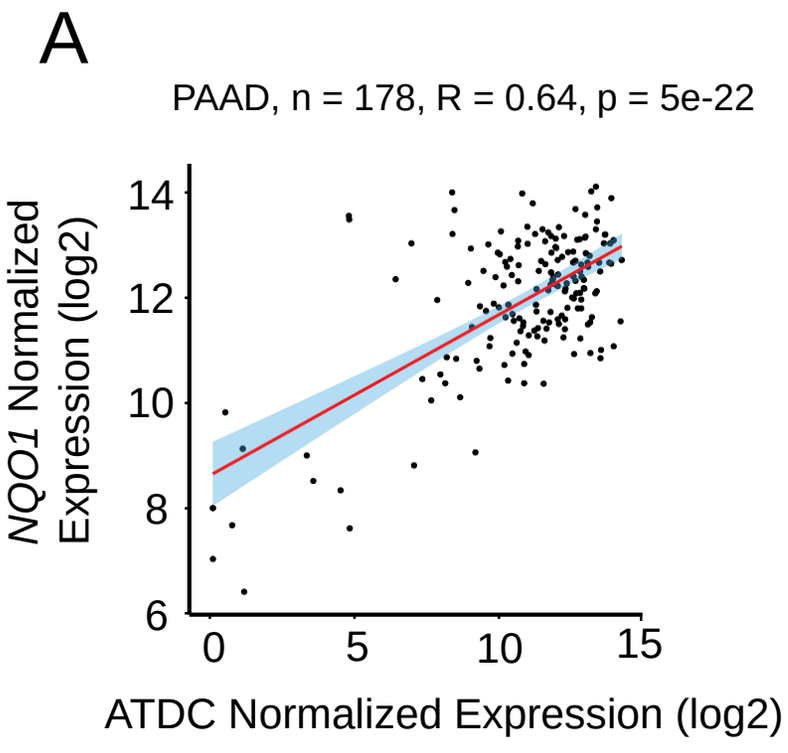
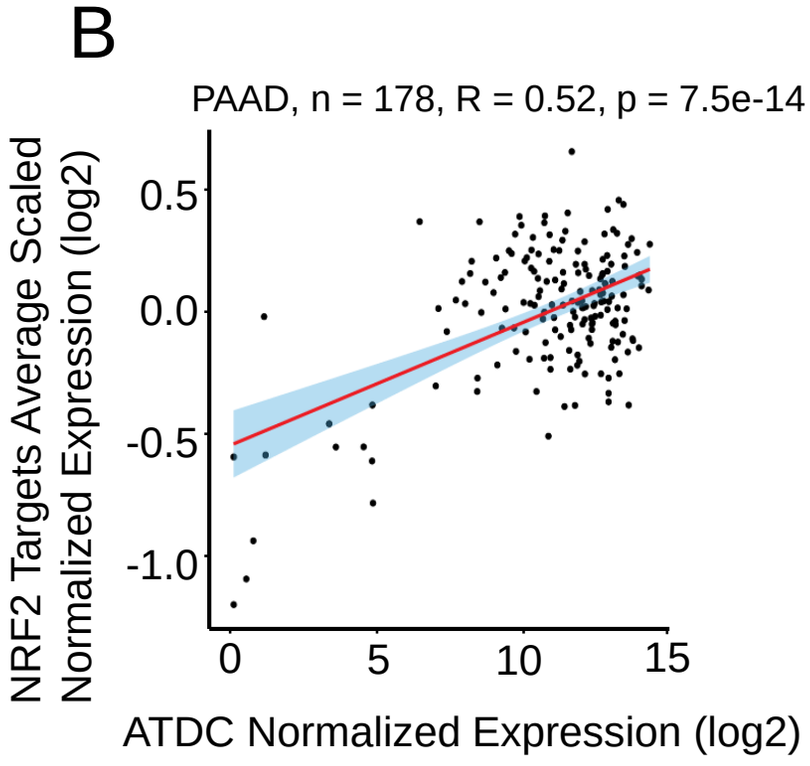
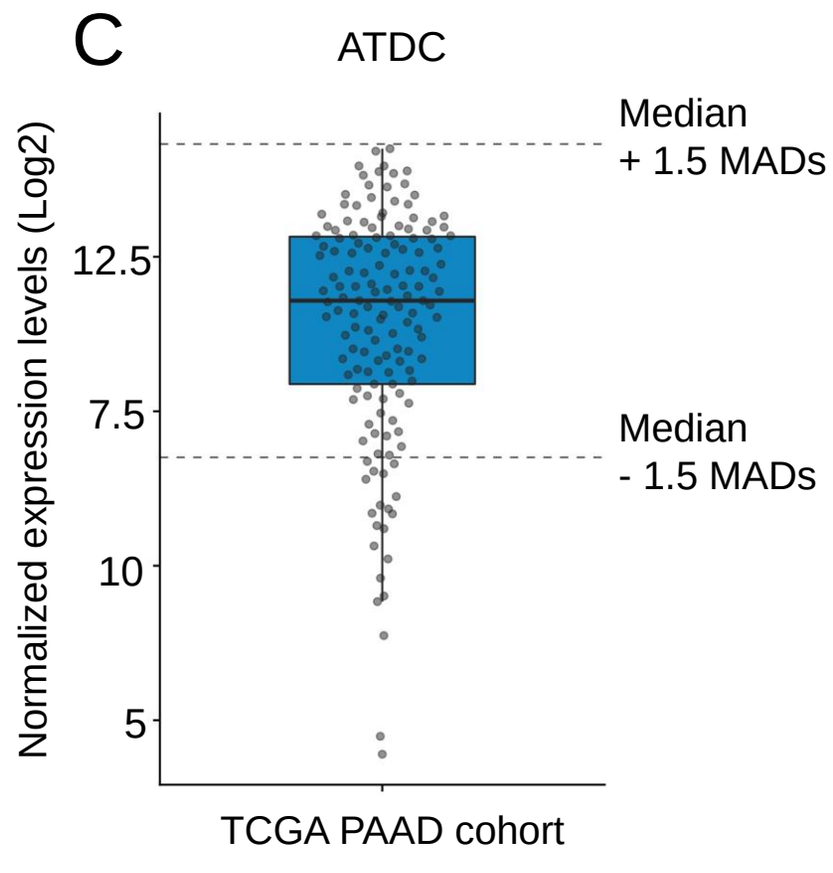


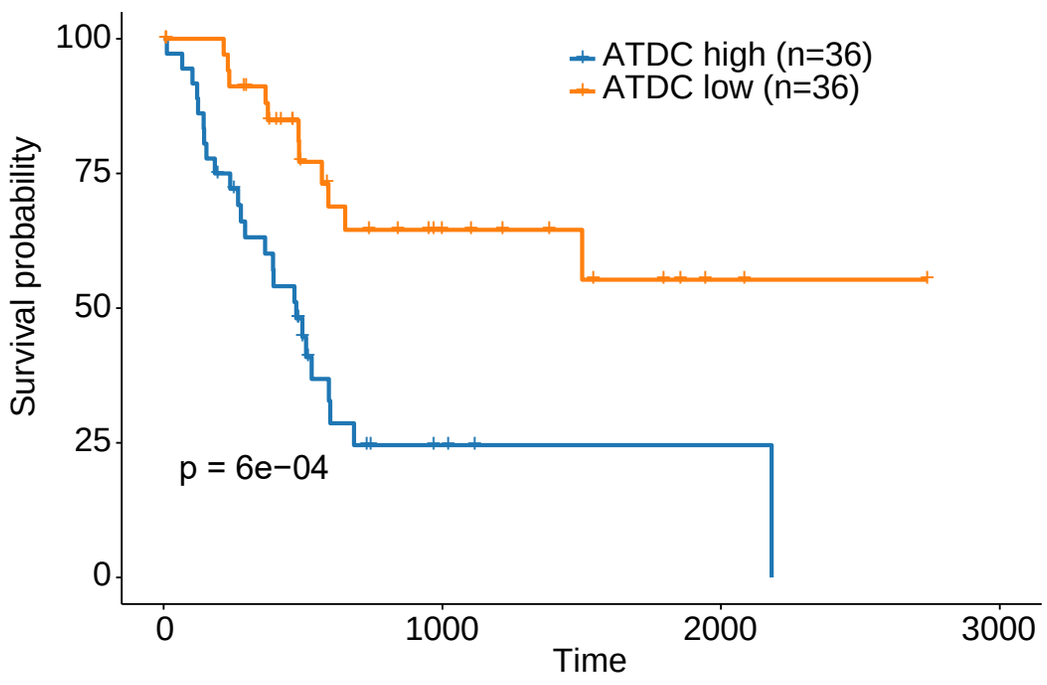
Figure S5. Related to Figure 5: NSG mice were implanted orthotopically with HPAC expressing sh Scr, sh ATDC, sh ATDC+NRF2, or ish ATDC (n= 10/group). (A) Representative images of tumors harvested at the end of the study. (B) Measurement of tumor volume in the aforementioned groups depicted as scatter plots with error bars denoting SEM. (C) Hematoxylin and eosin (H&E) staining and immunohistochemical staining of ATDC, NRF2, and NQO1 in tumors. (D) Quantification of liver metastases in all groups. (E) Quantification of lung metastases in all groups. (F) Measurement of tumor volume in established HPAC$^{ish\ ATDC}$ xenograft with or without doxycycline (doxy) treatment imaged by ultrasound (n= 10/group). (G) ATDC or NRF2 protein levels in HPAC$^{ish\ ATDC}$ tumor with or without doxy treatment. β-actin was used as a loading control. (H) ATDC or NRF2 protein levels in pancreatic tissues from 3 month-old KC or ATDC; KRAS^{G12D}; p48-Cre (AKC) mice. β-actin was used as a loading control. (I, J) Densitometric quantification of ATDC (I) or NRF2 (J) protein levels of H. (K, L) Representative co-immunofluorescent images showed co-expression of ATDC (green) with NRF2 (red) in PanIN1 lesions from KRAS^{G12D}; p48-Cre (KC) mice (K) or ATDC; KRAS^{G12D}; p48-Cre; ATDC (AKC) mice (L). Scale bars, 20 μm. (M) ATDC or NRF2 protein levels in pancreatic tissues from 3 month-old KPC or KRAS^{G12D}; p53$^{-/+}$; Pdx1-Cre; ATDC$^{-/-}$ (KPCA) mice. (N, O) Densitometric quantification of ATDC (N) or NRF2 (O) protein levels of M. β-actin was used as a loading control. All experiments were performed in triplicate. *p< 0.05; mean ± SEM.

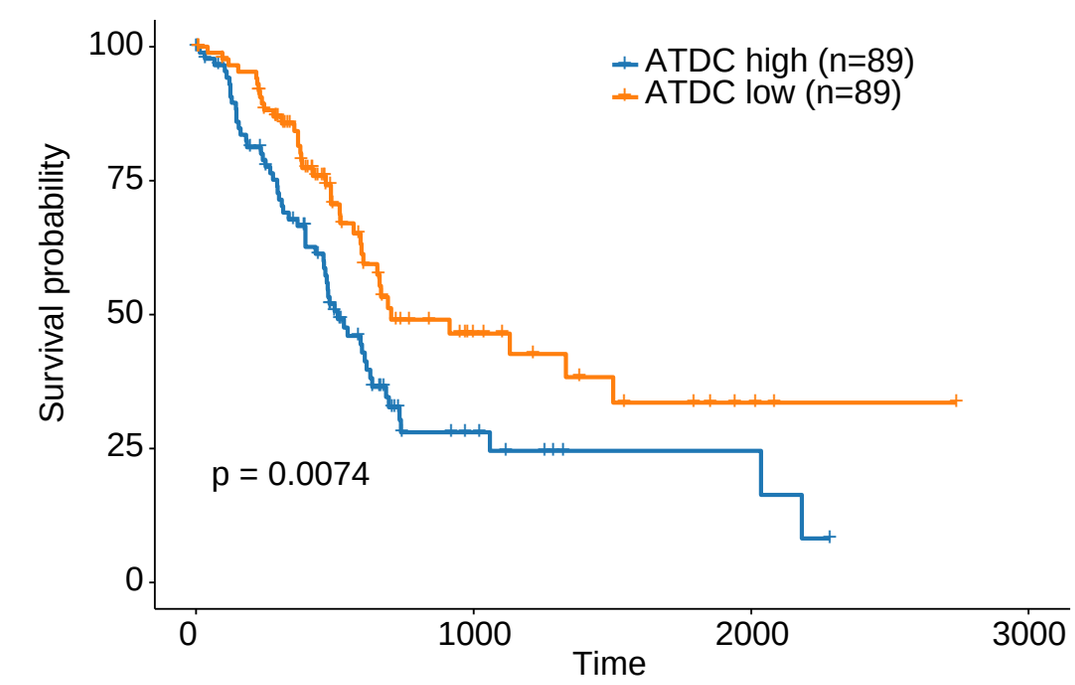
Figure S6. Related to Figure 6

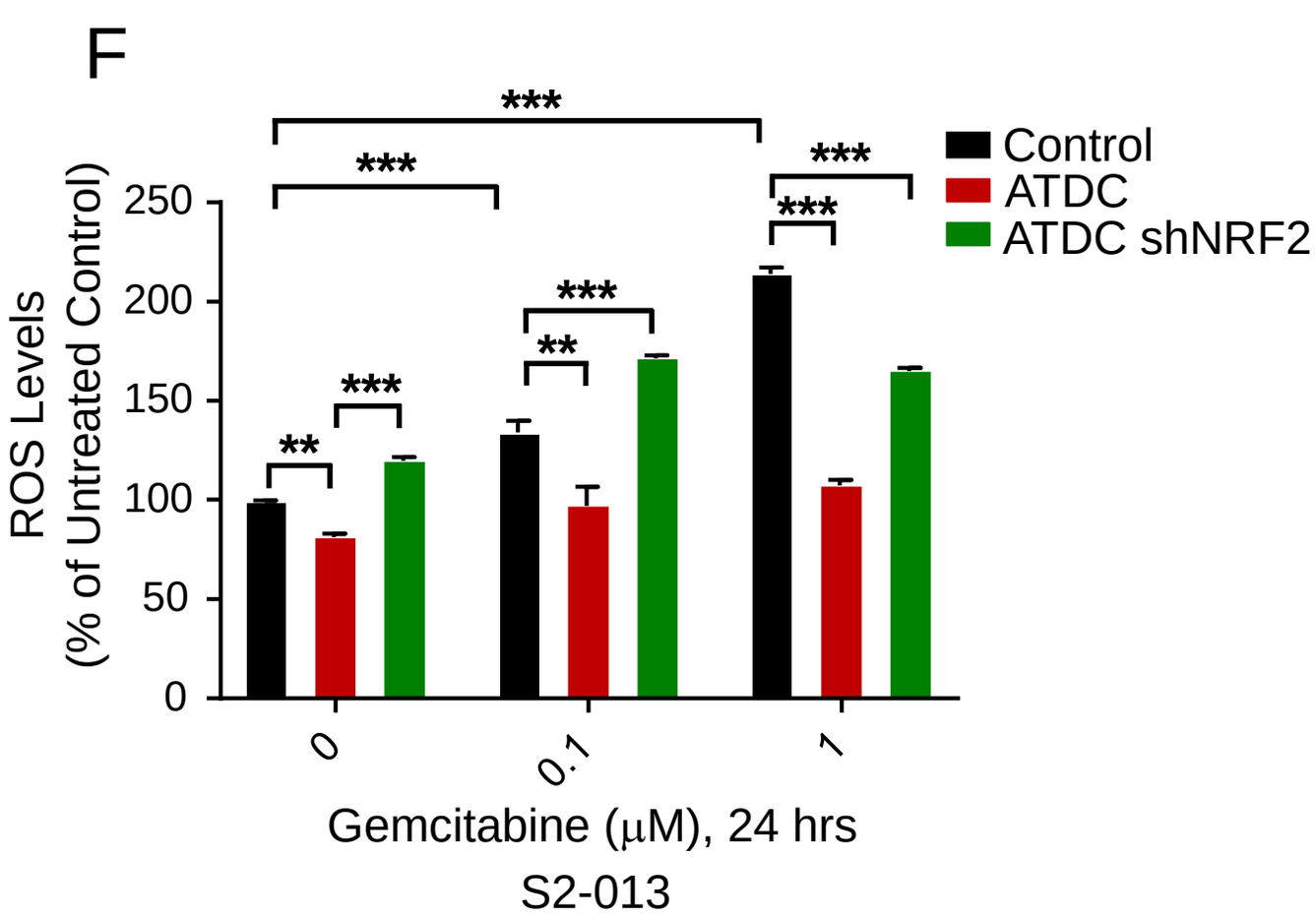












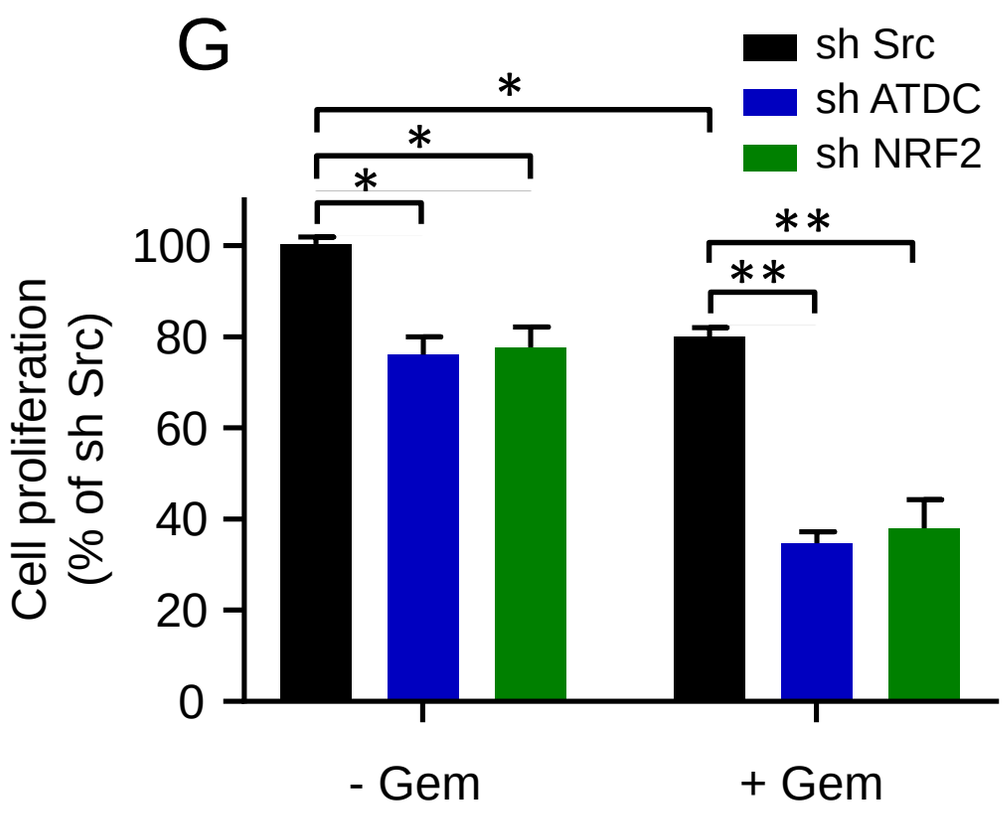


Figure S6. Related to Figure 6: (A-B) Correlation between the expression of ATDC (A) and NQO1 (A) or NRF2 core target genes (B) in TCGA PAAD cohort. (C) Top 20 versus bottom 20 percentile comparison. (D-E) Relative survival comparison between patients with ATDC-high and ATDC low expression levels. Data were obtained from TCGA PAAD dataset. (F) ROS levels in control and ATDC-overexpressing S2-013 cells with or without shNRF2 expression, 24 hours after GEM treatment. (G) Cell proliferation in HPAC cells expressing sh ATDC or sh NRF2 24 hours after GEM treatment. All experiments were performed in triplicate. *p< 0.05; **p< 0.01; ***p <0.005; mean ± SEM.

Supplementary Table 1. IC50 of Gemcitabine or 5FU Treatment in S2-013 or HPAC cells

	Gemcitabine	5FU
	IC_{50}	IC_{50}
S2-013 Vector	0.83±0.02 nM	3.236±0.12 μM
S2-013 ATDC	5.40±0.03 nM**	10.75±0.45 μM**
HPAC sh Control	0.256±0.003 μM	9.252±0.67 μM
HPAC shATDC	0.016±0.002 μM**	2.557±0.11 μM**

** p<0.01 vs Vector
** p<0.01 vs sh Control

Supplementary Table 2. Comparison of endogenous ATDC expression, ROS level, invasion capacity, and gemcitabine resistance in MiaPaCa2, HPAC, and Capan2 cells

	MiaPaCa2	HPAC	Capan2
ATDC (Relative optical density)	0	1.00±0.01***	1.56±0.14***
ROS (Fold vs MiaPaCa2)	1.00±0.06	0.67±0.02***	0.41±0.04***
Invasion (% of MiaPaCa2)	100.0±0.07	142.3±5.89**	189.6±6.0**
Gemcitabine (IC_{50}, μM)	0.035±0.004	0.25±0.03**	0.79±0.06**

** p<0.01 vs MiaPaCa2
*** p<0.001 vs MiaPaCa2

Supplementary Table 3. Real-Time PCR Assays

GENE	**TaqMan primer (human)**
Trim29 (ATDC)	Hs00988448_m1
NRF2 (NFE2L2)	Hs00975961_g1
beta-actin	Hs01060665_g1
GAPDH	Hs02786624_g1
NQO1	Hs01045993_g1
PRDX1	Hs00602020_mH
HMOX	Hs01110250_m1
GCLC	Hs00155249_m1
TXN	Hs00828652_m1
FTL	Hs00830226_gH