SUPPLEMENTAL INFORMATION

SOD1 inhibition reduces non-small cell lung cancer by inducing cell death

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Inventory of Supplemental Information

Supplemental Data
Figure S1, related to Figure 1
Figure S2, related to Figure 2
Figure S3, related to Figure 3
Figure S4, related to Figure 5
Figure S5, related to Figure 6
Figure S6, related to all Figures (Schematic Summary)

Supplemental Methods

Supplemental References

SUPPLEMENTAL DATA

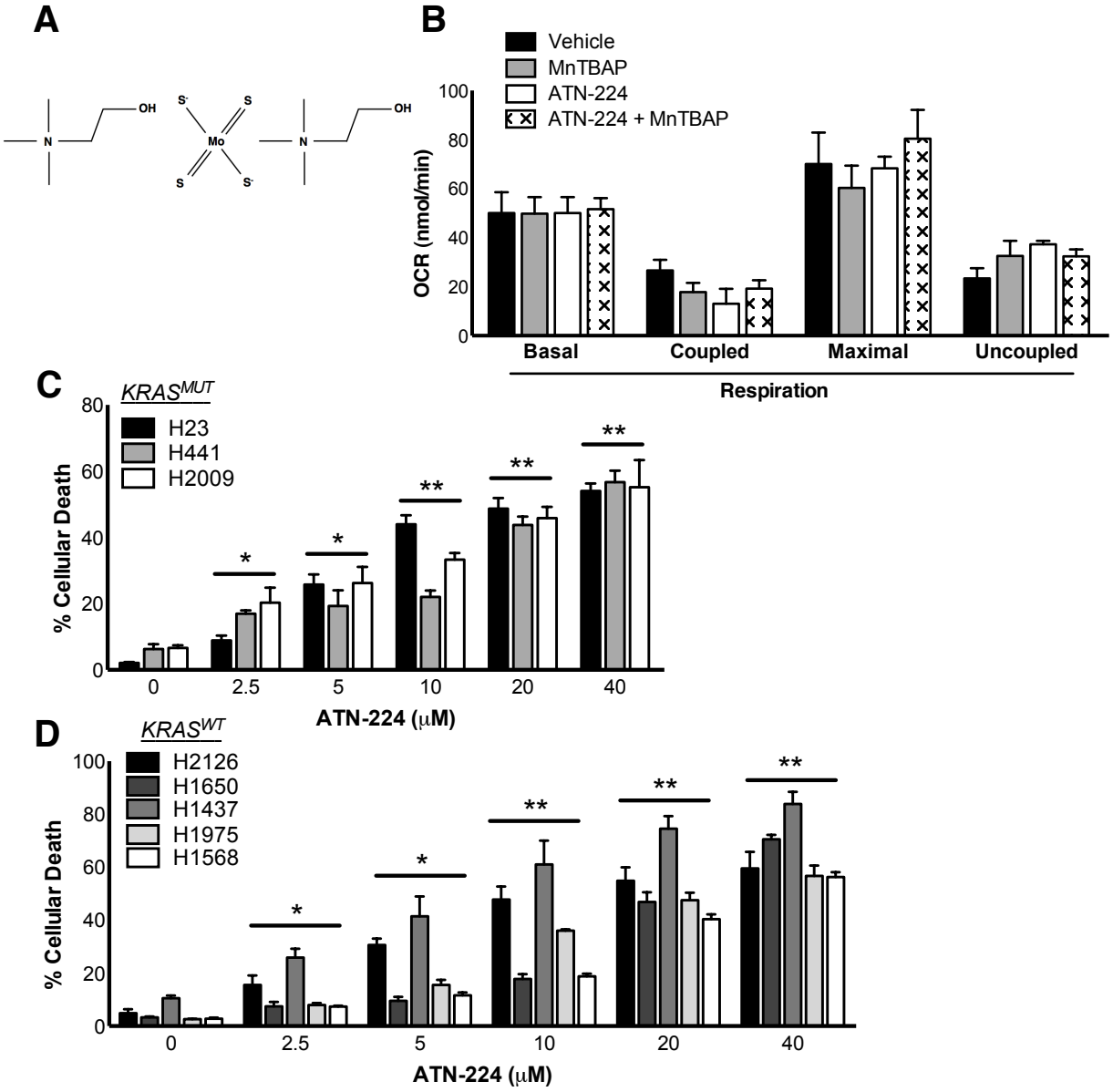

Figure S1, related to Figure 1. *The copper chelator ATN-224 selectively inhibits SOD1 and induces NSCLC cell death.* **(A)** Chemical structure of ATN-224. **(B)** Mitochondrial oxygen consumption rate (OCR) of A549 cells treated with 10μM ATN-224 alone or with 100μM of the SOD mimetic MnTBAP (16 hours) (n=3). **(C/D)** Mutant (C) and wildtype (D) *KRAS* human NSCLC cells were treated with increasing doses of ATN-224 and cell death (96h) was determined (both n=3). Statistical significane was determined by comparing 0μM ATN-224 with increasing concentrations. Date are mean ± SEM. *P<0.05. **P<0.01.

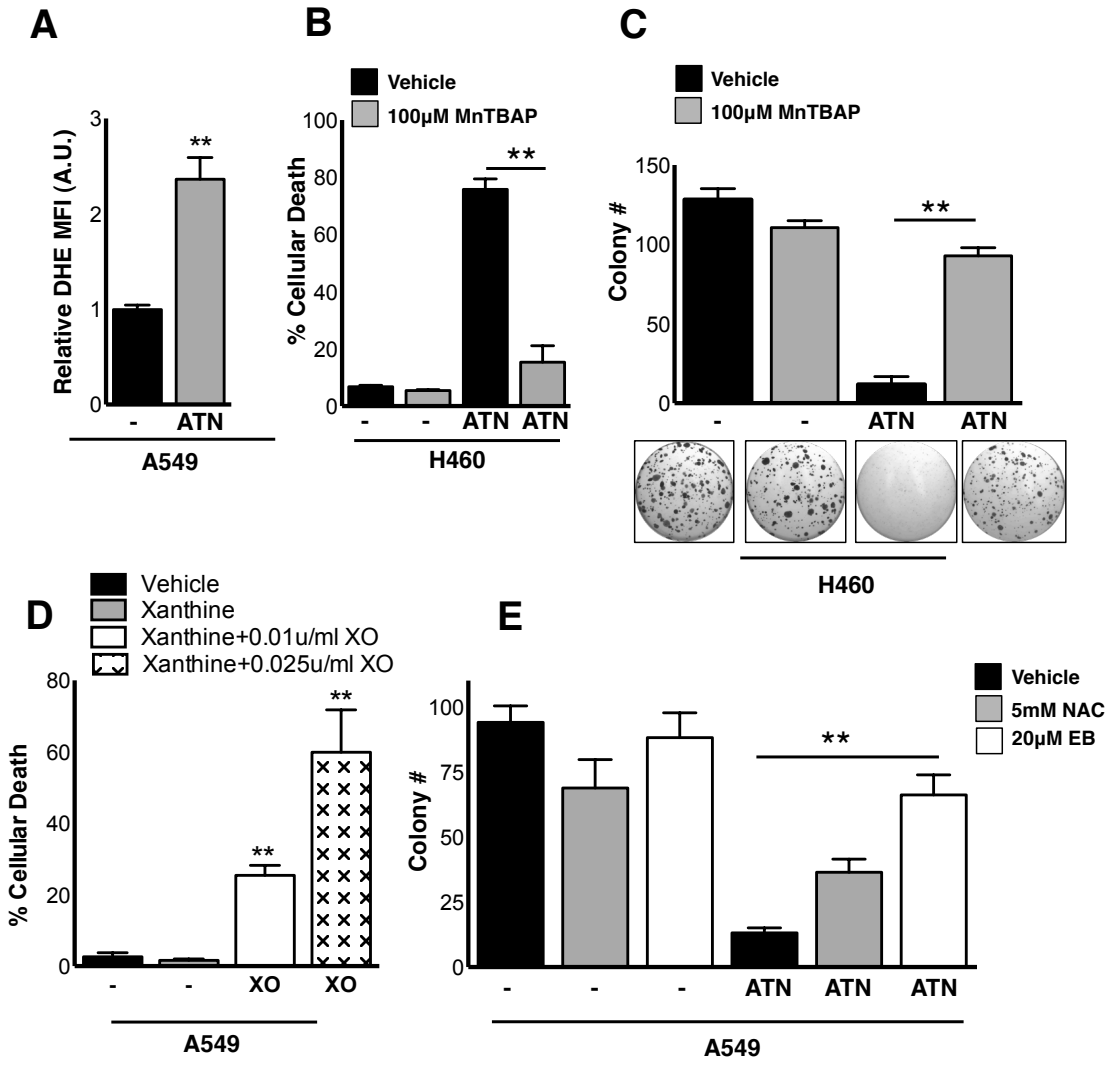

Figure S2, related to Figure 2. *ATN-224-induced cell death and anchorage-independent growth impairment are mediated through an increase in intracellular superoxide and H_2O_2.* **(A)** A549 cells were treated with 10μM ATN-224 and superoxide levels were determined by DHE (5μM) staining (24h) (n=3). **(B/C)** H460 (*KRAS*^{mut}) human lung NSCLC cells were treated with 10μM ATN-224 alone or with 100μM MnTBAP and (B) cell death (96h) or (C) anchorage-independent growth (3 weeks) were determined. Images show colony formation in 96 well plates (n=3, n=8). **(D)** A549 cells were treated with 1mM xanthine and varying doeses of xanthine oxidase and cell death was determined (48h) (n=3). **(E)** Analysis of soft agar colonies of A549 cells treated with 10μM ATN-224 alone or with either 5mM NAC or 20μM ebselen (3 weeks) (n=7). Data are represented as mean ± SEM. *P<0.05. **P<0.01.

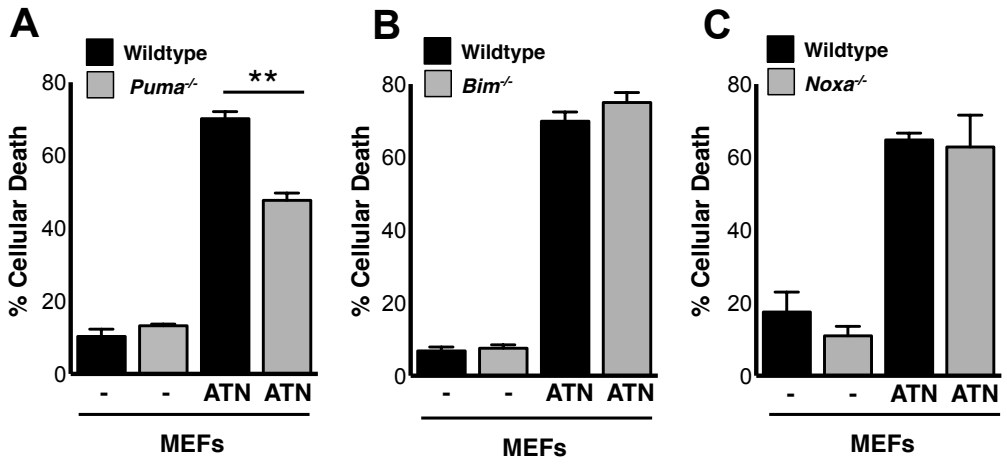

Figure S3, related to Figure 3. *ATN-224 does not function through the BH3-only proteins BIM and NOXA alone.* **(A/B/C)** Cell death analysis (48h) using PI staining of immortalized wildtype or (A) *Puma*$^{-/-}$ MEFs, (B) *Bim*$^{-/-}$ MEFs or (C) *Noxa*$^{-/-}$ MEFs treated with 5μM ATN-224. Data are represented as mean ± SEM. *P<0.05. **P<0.01.

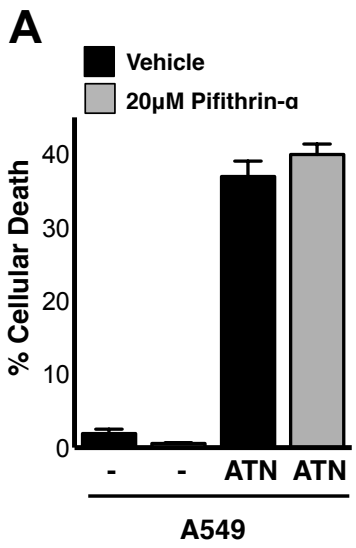

Figure S4, related to Figure 5. *ATN-224 induces cell death independent of P53*. **(A)** A549 cells (*KRAS*^{onc}, *TP53* wildtype) were treated with 10μM ATN-224 alone or with 20μM of the P53 inhibitor pifithrin-α and cell death (96h) was determined (n=6). Data are represented as mean ± SEM.

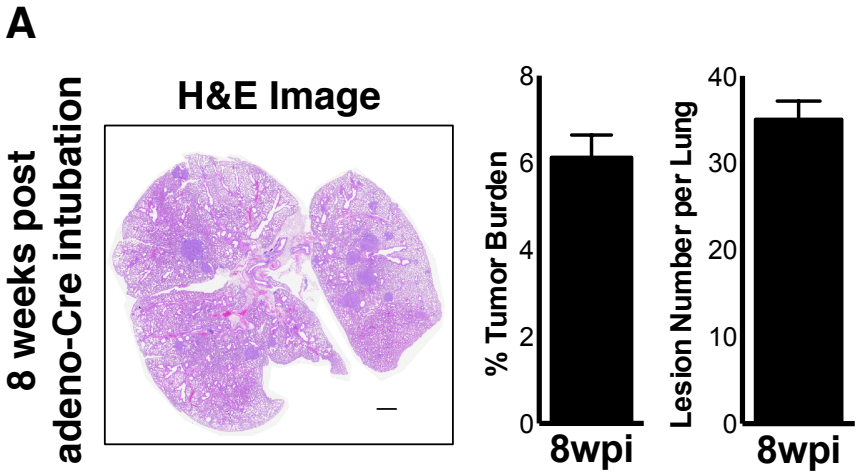

Figure S5, related to Figure 6. *ATN-224 reduces tumor burden in a clinically relevant KP mouse model.* **(A)** Representative H&E image, tumor burden and lesion number quantifications of lungs isolated from KP mice 8 weeks post adeno-Cre intubation (10^7 PFU) (n=4). Tumor burden was calculated by averaging the tumor area from H&E-stained whole lung sections (n=4). Scale bar = 1mm.

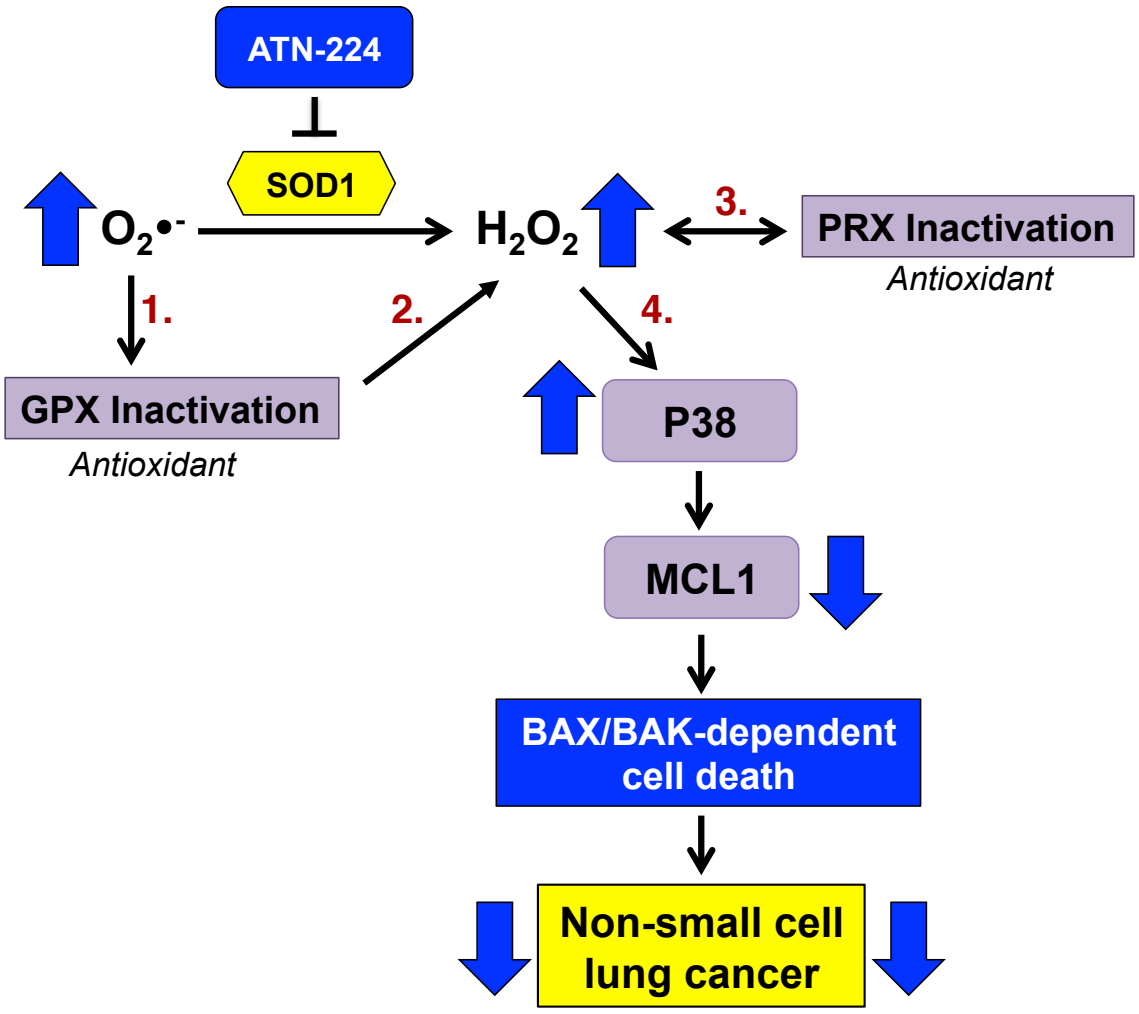

Figure S6. *Schematic Summary.* ATN-224-induced SOD1 inhibition leads to (1.) superoxide stress and subsequent oxidation and inhibition of the antioxidant enzymes glutathione peroxidases (GPXs). As a result (2.) intracellular H_2O_2 concentrations rise, which leads to (3.) hyper-oxidation and resulting inactivation of peroxiredoxins (PRXs), which also metabolize H_2O_2 to water. Thus, PRX inhibition reinforces the increase in H_2O_2. The resulting abundance in H_2O_2 (4.) activates P38 MAPK-mediated MCL1 down-regulation, which causes BAX/BAK-dependent programmed lung cancer cell death and dampening of NSCLC tumorigenicity.

SUPPLEMENTAL METHODS

Cell culture

Cells were incubated at 37°C and maintained at 5% CO_2. Human lung adenocarcinoma H460 (*KRAS* mutant, *TP53* wildtype) cells were obtained from ATCC and grown in RPMI 1640 media (Cellgro) plus 10% fetal bovine serum (GemCell), 5% penicillin and streptomycin (Cellgro), 5% L-glutamine (Gibco) and 5% HEPES buffer (Cellgro). Wildtype (H1437, H1650, H1568, H1975, H2126) and mutant (H23, H441, H2009) *KRAS* NSCLC cells were a kind gift of Dr. Ralph DeBerardinis (UT Southwestern, Dallas, TX) and were cultured in supplemented RPMI 1640 media (see above). *Bim*$^{-/-}$ MEFs and their wildtype controls were simian virus 40 transformed. Wildtype, *Puma*$^{-/-}$, and *Noxa*$^{-/-}$ MEFs were E1A and RAS transformed. These MEFs were a kind gift of Dr. Andreas Strasser (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). MEFs were generated and cultured as previously reported (1).

ROS measurements

For superoxide measurements cells were incubated in 5μM dihydroethidium (DHE, Invitrogen) for 1 hour. Mean fluorescence intensity (MFI) was measured by flow cytometry.

Mitochondrial oxygen consumption rate measurement.

Mitochondrial oxygen consumption rate (OCR) was measured using the Extracellular Flux Analyzer (XF24, Seahorse Bioscience) according to the manufacturer's protocol. Basal mitochondrial respiration was measured by subtracting the OCR values after treatment with 1μM antymicin A and 1μM rotenone (Sigma). Coupled respiration was determined by treatment with 1μM oligomycin A (Sigma), by the subtraction of oligomycin A values from basal respiration. Maximal respiration was determined by treatment with 2μM of the uncoupling agent FCCP (Sigma). OCR was measured 16 hours after drug treatment and normalized to cell number.

SUPPLEMENTAL REFERENCES

1. Brunelle, J.K., Shroff, E.H., Perlman, H., Strasser, A., Moraes, C.T., Flavell, R.A., Danial, N.N., Keith, B., Thompson, C.B., and Chandel, N.S. 2007. Loss of Mcl-1 protein and inhibition of electron transport chain together induce anoxic cell death. *Mol Cell Biol* 27:1222-1235.