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Supplemental Information

***PARK2* Depletion Connects Energy
and Oxidative Stress to PI3K/Akt Activation
via PTEN S-Nitrosylation**

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SUPPLEMENTAL FIGURES

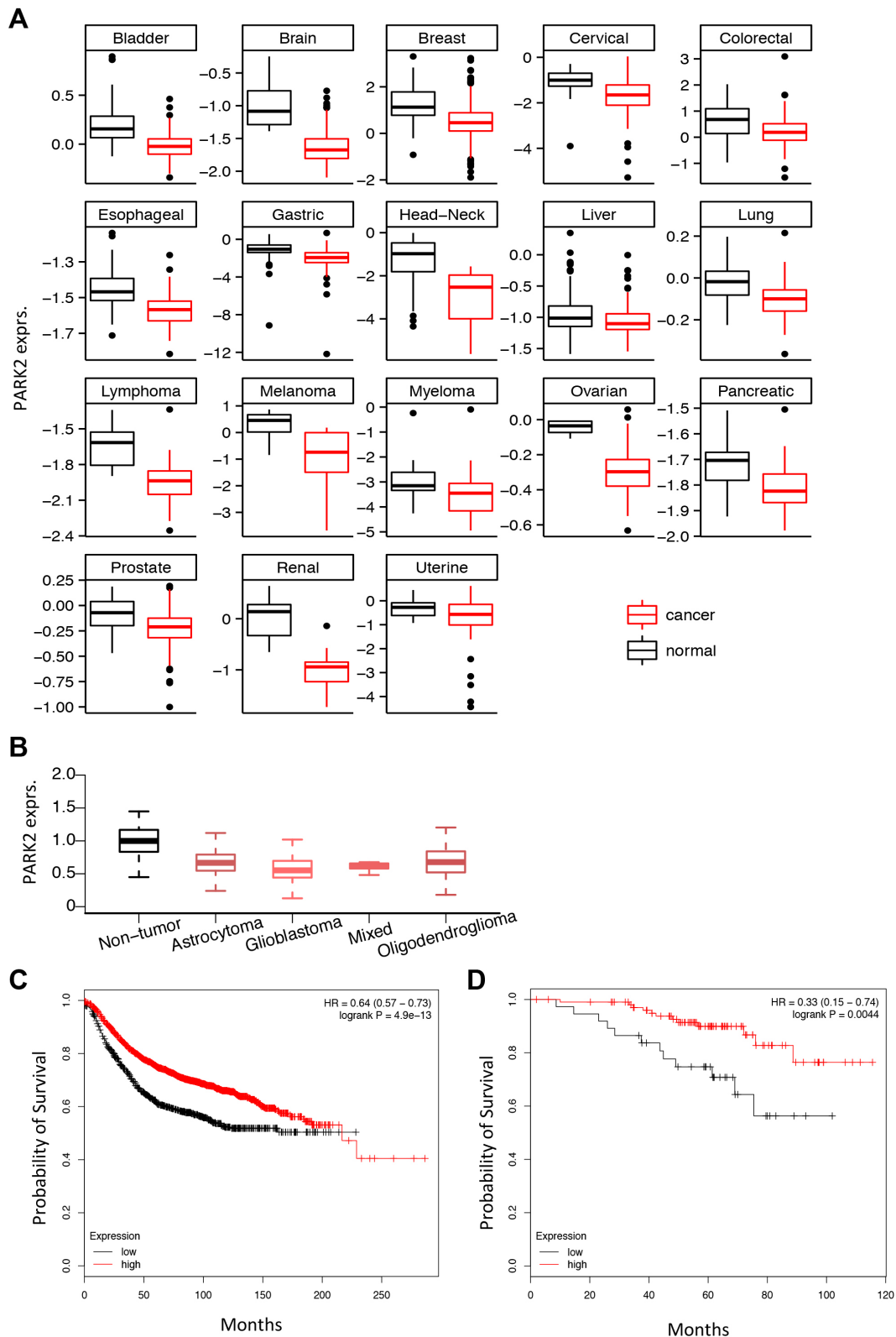


Figure S1, related to Figure 1. *PARK2* is significantly under-expressed across many tumor types and its loss is associated with poorer prognosis.

(A) Representative box-plots indicating significantly lower *PARK2* mRNA expression in cancerous versus corresponding normal tissue across many different tumor types including bladder; $p = 5.184 \times 10^{-11}$, brain; $p = 0.0003$, breast; $p = 3.103 \times 10^{-10}$, cervical; $p = 0.001$,

colorectal; $p = 0.016$, esophageal; $p = 1.145 \times 10^{-6}$, gastric; $p = 1.832 \times 10^{-5}$, head and neck; $p = 4.44 \times 10^{-5}$, liver; $p = 7.052 \times 10^{-5}$, lung; $p = 1.607 \times 10^{-6}$, lymphoma; $p = 1.251 \times 10^{-7}$, melanoma; $p = 0.036$, myeloma; $p = 0.015$, ovarian; $p = 0.0003$, pancreatic; $p = 0.005$, prostate; $p = 0.0003$, renal; $p = 4.803 \times 10^{-5}$, uterine; $p = 0.005$ (Oncomine database). 2-tailed t -test was used to evaluate differential expression of *PARK2* between cancer and normal tissues.

(B) *PARK2* mRNA expression across non-tumor and different histological subtypes of glioma specimens from the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) ($p = 0.0007$, one-way Anova analysis).

(C) Kaplan-meier survival plots of breast cancer patients stratified by tumors bearing *PARK2* low ($n=1786$, defined as below the 25th percentile) versus high ($n=5424$, defined as above the 25th percentile) mRNA expression and (D) lung adenocarcinomas with *PARK2* low ($n=127$) versus high ($n=376$) mRNA expression. Log-rank test was used to compare the respective survival curves (from left to right: $p = 4.9 \times 10^{-13}$, $p = 0.0044$). This analysis was performed using the KM plotter.

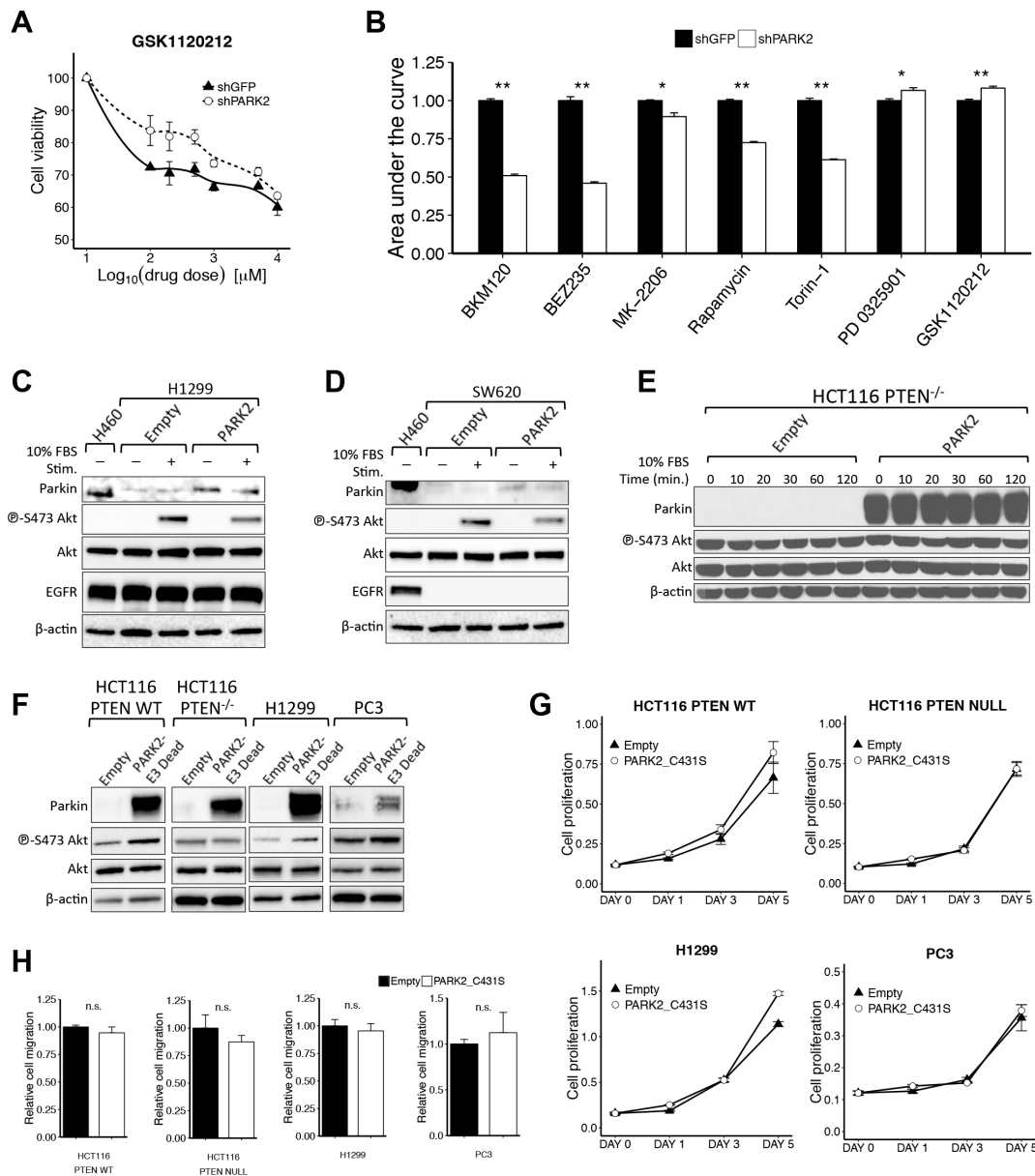


Figure S2, related to Figures 2 and 3. Tumor suppressor function of *PARK2* through regulation of PTEN-mediated Akt activation.

(A) Drug-response curve indicating that *PARK2* knockdown significantly mitigates the response to MEK inhibitor (GSK1120212) ($p = 0.03$, two-way Anova analysis).

(B) Area under the curve analysis of the dose-response curves presented in Figures 2E and 2SA indicating that *PARK2* knockdown selectively sensitizes HCT116 cells to inhibitors of the PI3K/Akt/mTOR, but not the MAPK pathway.

Immunoblotting analysis of

(C) H1299 (*EGFR* wild-type) and (D) SW620 (*EGFR* null) cells with ectopic Parkin expression close to the endogenous levels in H460 cells. Cells were subjected to 24 hour serum starvation before stimulation with 10% FBS for 10 minutes.

(E) EV and *PARK2*-overexpressing *PTEN* null HCT116 cells following 10% FBS stimulation. Cells were serum-starved for 24 hours prior to FBS stimulation,

(F) *PTEN*-wild-type (HCT116 *PTEN*^{+/+} and H1299) and *PTEN*-deficient (HCT116 *PTEN*^{-/-} and PC3) cells expressing EV or the E3 ligase-dead Parkin C431S mutant.

(G) Cell proliferation and (H) Cell migration assays of *PTEN*-wild-type and *PTEN*-deficient cells expressing EV or Parkin C431S. Statistical significance between proliferation curves was assessed using two-way ANOVA analysis (HCT116 *PTEN*^{+/+}; $p = 0.169$, HCT116 *PTEN*^{-/-}; $p = 0.947$, H1299; $p = 0.919$, PC3; $p = 0.751$), and between migration assays using 2-tailed

t-test (HCT116 *PTEN*^{+/+}; *p* = 0.392, HCT116 *PTEN*^{-/-}; *p* = 0.403, H1299; *p* = 0.633, PC3; *p* = 0.617). Data are represented as mean ±SEM (n.s.: not significant, **p* < 0.05, ***p* < 0.01).

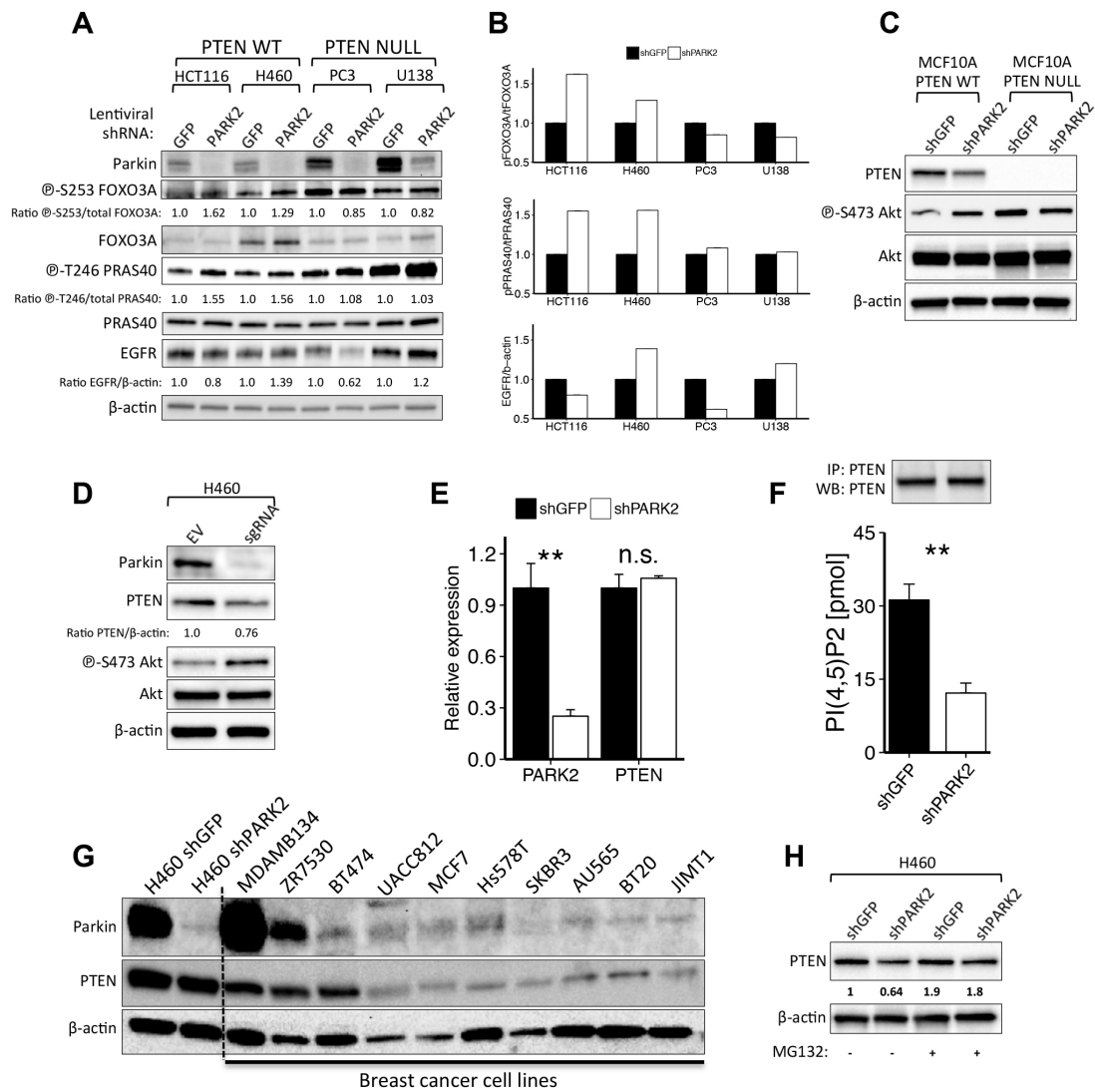


Figure S3, related to Figure 4. The role of *PARK2* depletion in *PTEN*-mediated Akt activation.

(A) Immunoblotting analysis and (B) corresponding densitometry plot of *PTEN* WT and deficient cell lines for PI3K/Akt activation markers.

Immunoblotting analysis of

(C) H460 cells transfected with control or CRISPR/Cas9-plasmids targeting the *PARK2* gene and

(D) MCF10A *PTEN* WT and isogenic null cell lines,

(E) Quantitative real-time PCR for *PARK2* and *PTEN* (*PARK2*; $p = 0.007$, *PTEN*; $p = 0.52$) and

(F) *PTEN* activity assay between shGFP and shPARK2 H460 cells ($p = 0.008$, 2-tailed t -test). *PTEN* was ectopically expressed equally between shGFP and shPARK2 H460 cells.

Immunoblotting analysis of

(G) A panel of breast cancer cell lines ($n=10$) showing a statistically significant reciprocal correlation between Parkin and *PTEN* protein expression following densitometry analysis (Correlation coefficient: 0.79, $p = 0.007$, Pearson's correlation). shGFP and shPARK2 H460 cells were used as control for Parkin expression.

(H) shGFP and shPARK2 H460 cells with or without treatment with 10 μ M MG132 for 6 hours. Data are represented as mean \pm SEM (n.s.: not significant, * $p < 0.05$, ** $p < 0.01$, 2-tailed t test).

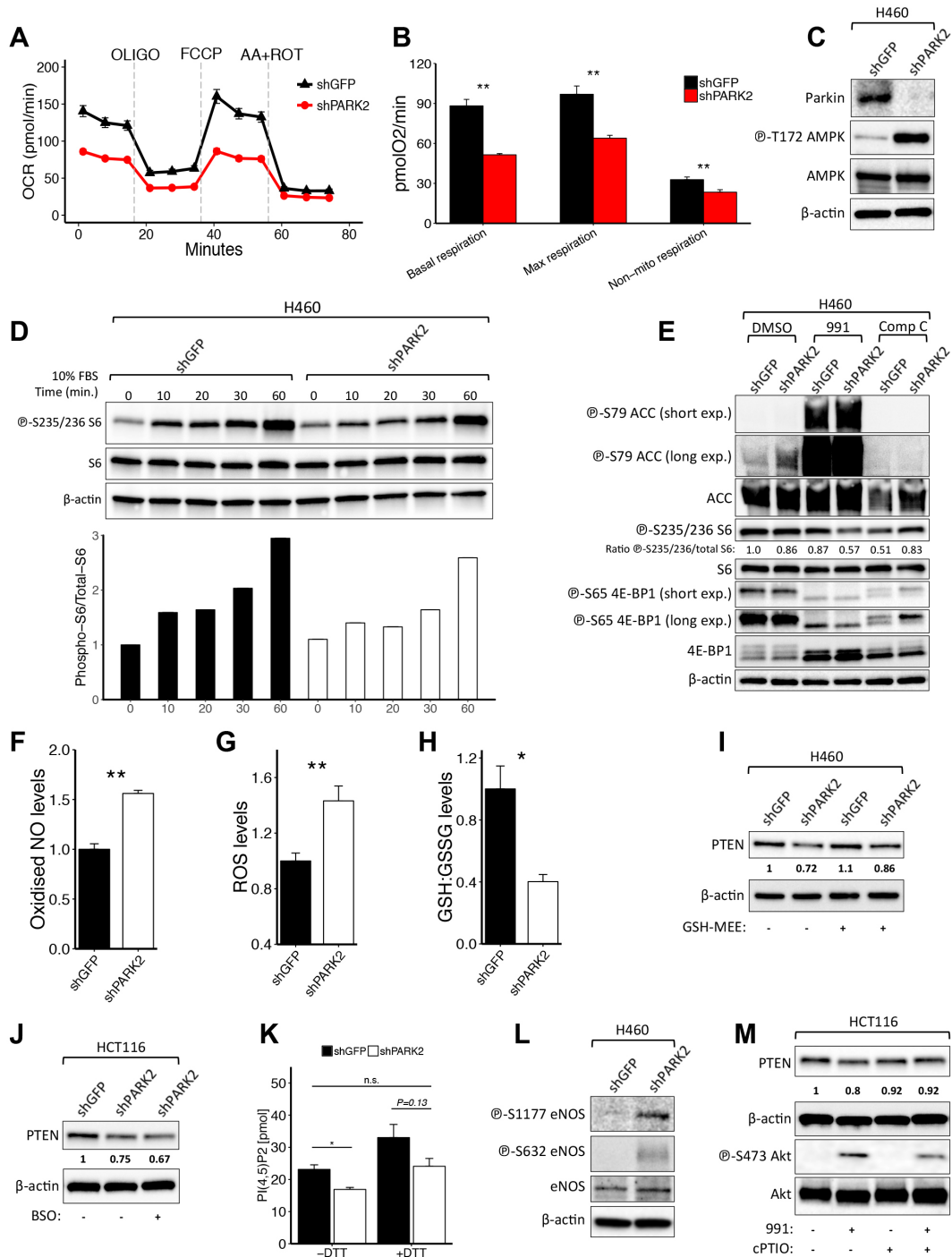


Figure S4, related to Figures 4 and 5. PARG2 depletion enhances energy depletion-mediated oxidative and nitrosative stress.

(A) Seahorse analysis of oxygen consumption rate (OCR) in shGFP and shPARK2-expressing H460 cells, following sequential injection of oligomycin, FCCP and antimycin A/rotenone (n=6).

(B) Oxygen consumption rates of basal ($p = 2.1 \times 10^{-5}$), maximal ($p = 5 \times 10^{-4}$) and non-mitochondrial respiration ($p = 0.006$) of shGFP and shPARK2 H460 cells.

Immunoblotting analysis of

(C) Phospho-AMPK (T172), between shGFP and shPARK2 H460 cells.

(D) Phospho-S6 (S235/236) following 10% FBS stimulation (top), with densitometric analysis of immunoblotting (bottom), and

(E) AMPK and mTORC1 activation markers following treatment with DMSO, AMPK activator 991 (20 μ M for 5 hours) and AMPK inhibitor Compound C (20 μ M for 5 hours).

- (F) Oxidized nitric oxide (NO) levels upon *PARK2* depletion in H460 cells ($p = 9 \times 10^{-4}$).
- (G) Reactive oxygen species (ROS) levels following 2 hours menadione treatment (20 μ M) ($p = 0.023$), and
- (H) GSH:GSSG levels between shGFP and shPARK2 H460 cells ($p = 0.018$).
- Immunoblotting analysis of
- (I) shGFP and shPARK2 H460 cells with or without treatment with 5mM glutathione reduced ethyl ester (GSH-MEE) for 24 hours,
- (J) shGFP and shPARK2 HCT116 cells with or without treatment with 500 μ M BSO for 16 hours.
- (K) PTEN activity of shGFP or shPARK2 HCT116 cells in the absence (-) or presence (+) of DTT (50 mM) [shGFP (-DTT) vs. shPARK2 (-DTT); $p = 0.02$, shGFP (+DTT) vs. shPARK2 (+DTT); $p = 0.13$, shGFP (-DTT) vs. shPARK2 (+DTT); $p = 0.75$].
- Immunoblotting analysis of
- (L) shGFP and shPARK2 H460 cells,
- (M) HCT116 cells following treatment with the allosteric AMPK activator 991 (20 μ M) for 5 hours with or without co-treatment with cPTIO (100 μ M) for the same period. Data are represented as mean \pm SEM (n.s.: not significant, * $p < 0.05$, ** $p < 0.01$, 2-tailed t test).

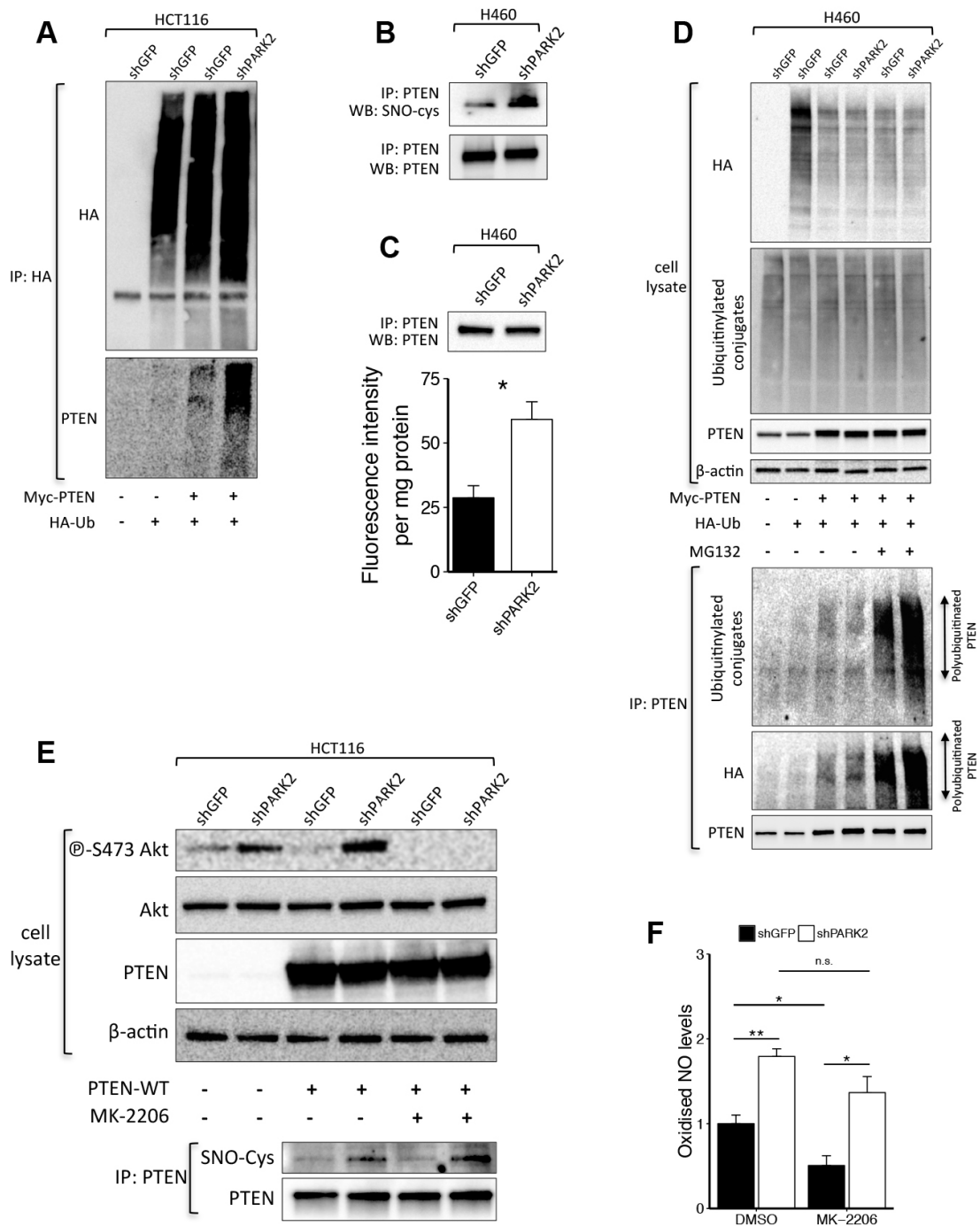


Figure S5, related to Figure 6. *PARK2* depletion promotes *PTEN* inactivation by S-nitrosylation and ubiquitination.

(A) Immunoblotting analysis of anti-HA immunoprecipitates (IP) derived from HA-ubiquitin (Ub) and Myc-tagged *PTEN* transfected shGFP and shPARK2 HCT116 cells.

(B) Immunoblotting analysis of Anti-PTEN immunoprecipitates (IP) derived from MYC-tagged transfected *PTEN* shGFP and shPARK2 H460 cells.

(C) Fluorometric measurement of S-nitrosylated PTEN between shGFP and shPARK2 H460 cells ($p = 0.021$).

(D) Immunoblotting analysis of whole-cell lysates and anti-PTEN immunoprecipitates (IP) derived from HA-ubiquitin (Ub) and Myc-tagged *PTEN* transfected shGFP and shPARK2 H460 cells. Where indicated cells were treated with MG132 (10 μ M) for 6 hours before collection.

(E) Immunoblotting analysis and anti-PTEN immunoprecipitates derived from WT PTEN-expressing shGFP and shPARK2 HCT116 cells following 24 hour treatment with Akt inhibitor MK-2206 (100 nM).

(F) Oxidized nitric oxide (NO) levels of HCT116 cells following 24 hour treatment with DMSO or Akt inhibitor MK-2206 (100 nM) [shGFP (DMSO) vs. shPARK2 (DMSO); $p = 0.004$, shGFP (MK-2206) vs. shPARK2 (MK-2206); $p = 0.018$, shGFP (DMSO) vs. shGFP (MK-2206); $p = 0.033$, shPARK2 (DMSO) vs. shPARK2 (MK-2206); $p = 0.112$]. Data are represented as mean \pm SEM (n.s.: not significant, * $p < 0.05$, ** $p < 0.01$, 2-tailed t test).

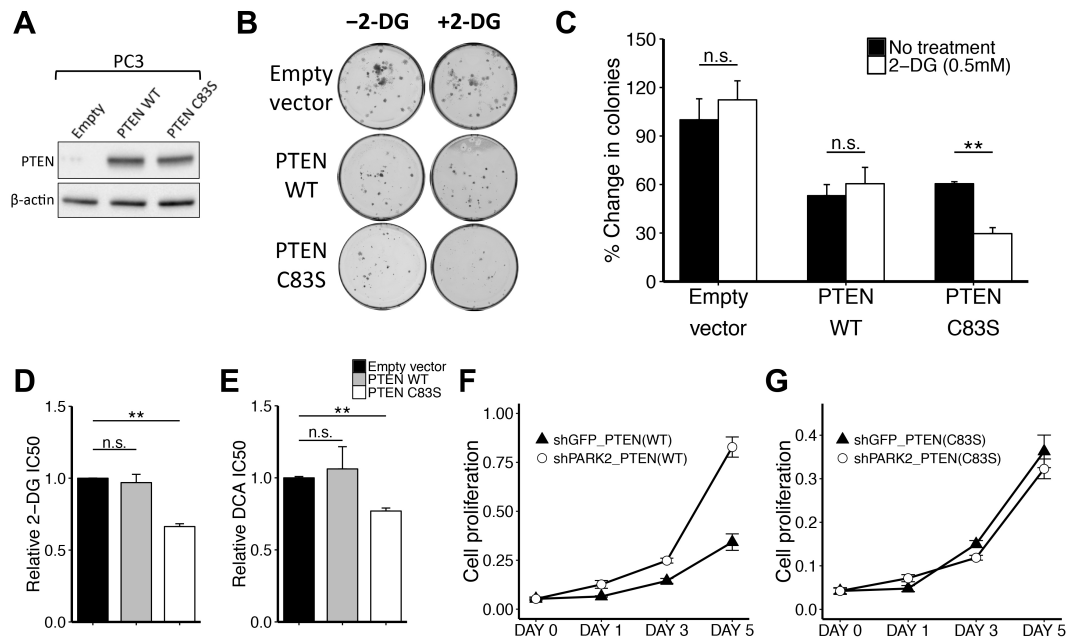


Figure S6, related to Figure 6. PTEN S-nitrosylation supports cell survival and proliferation under conditions of energy deprivation.

(A) Immunoblotting analysis of whole-cell lysates of PC3 cells expressing empty-vector, WT or S-nitrosylation-resistant (C83S) mutant *PTEN*.

(B-C) Clonogenic assays performed in 2-DG-treated (0.5 mM) or untreated PC3 cells expressing empty-vector (EV), wild-type (WT) or S-nitrosylation-resistant (C83S) mutant *PTEN* [EV (-DTT) vs. EV (+DTT); $p = 0.52$, *PTEN* WT (-DTT) vs. *PTEN* WT (+DTT); $p = 0.577$, *PTEN* C83S (-DTT) vs. *PTEN* C83S (+DTT); $p = 0.001$].

(D) 2-DG and (E) DCA IC₅₀ values for PC3 cells expressing empty-vector, WT or C83S mutant *PTEN* (2-DG IC₅₀: EV vs. *PTEN* WT; $p = 0.622$, EV vs. *PTEN* C83S; $p = 6.9 \times 10^{-5}$, DCA IC₅₀: EV vs. *PTEN* WT; $p = 0.708$, EV vs. *PTEN* C83S; $p = 5.3 \times 10^{-4}$).

Cell proliferation assays of shGFP or shPARK2 PC3 cells co-transfected with (F) WT or (G) C83S mutant *PTEN* (*PTEN* WT: shGFP vs. shPARK2; $p = 4.7 \times 10^{-5}$, *PTEN* C83S: shGFP vs. shPARK2; $p = 0.512$, 2-way Anova). Data are represented as mean \pm SEM (n.s.: not significant, * $p < 0.05$, ** $p < 0.01$, 2-tailed *t* test).

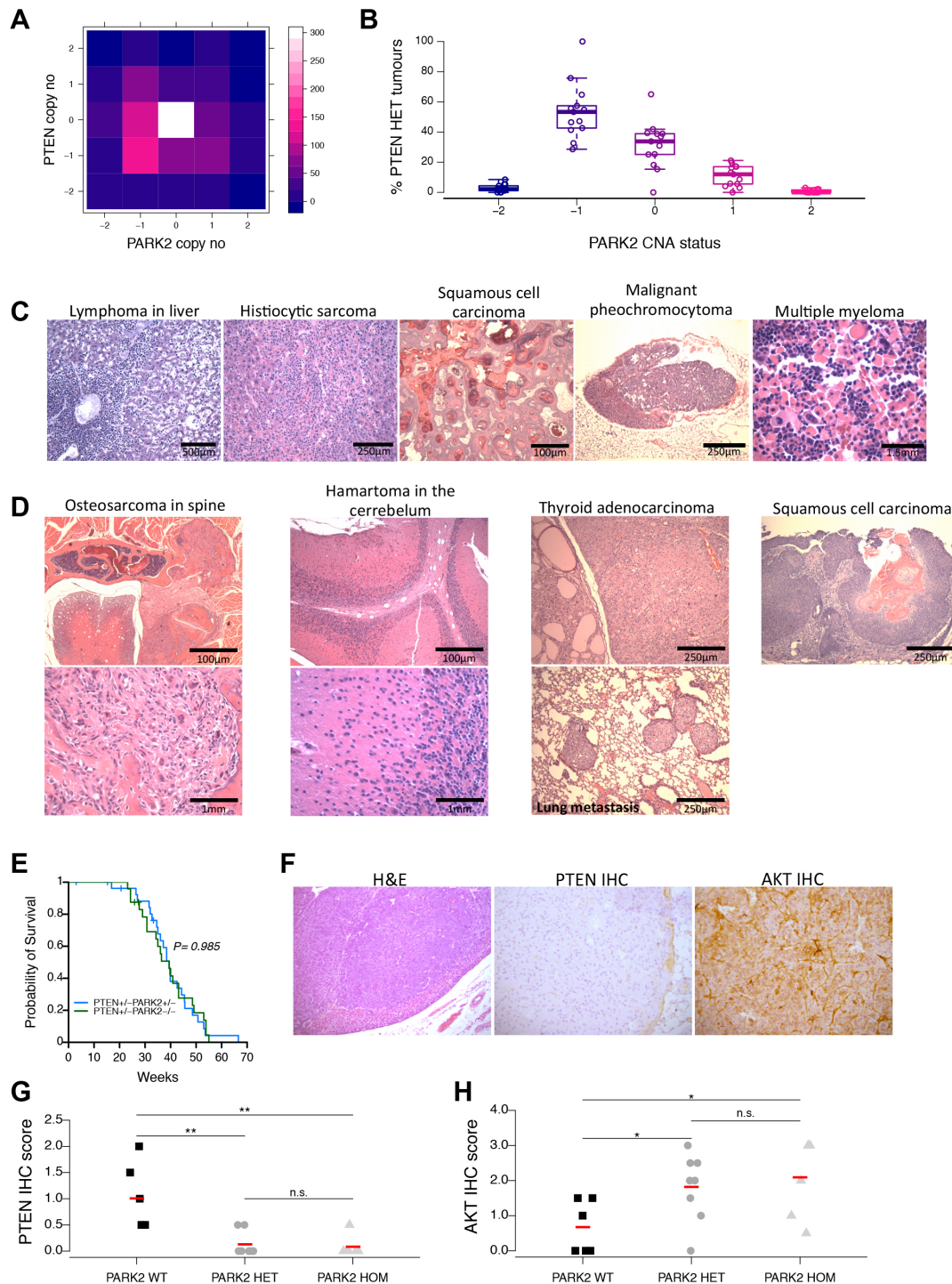


Figure S7, related to Figure 7. *PARK2* loss and *PTEN* loss display striking cooperativity to promote tumorigenesis in vivo.

(A) Image-plot indicating the co-existence of *PARK2* and *PTEN* copy number alterations (CNAs) across 995 cell lines from the Cancer Cell Line Encyclopedia (CCLE). The color panel indicates the number of cell lines for each combination and x- and y-axis indicate the CNA status of *PARK2* or *PTEN* genes, respectively.

(B) Box-plot indicating the fraction of *PARK2* CNAs of primary tumors or cancer cell lines with heterozygous deletion of *PTEN*. This analysis was performed on 1,953 specimens across 13 different cancer types from the TCGA database. Each point represents the percentage of a different tumor type with the respective copy number alteration in *PARK2*. A point was also added to represent the fraction of *PARK2* CNAs in cancer cell lines with heterozygous deletion of *PTEN* as reported in the CCLE database e.g. 17/312 (5.4%) have homozygous

deletion, 145/312 (46.5%) have heterozygous deletion, 79/312 (25.3%) have retention, 66/312 (21.2%) have 1-copy gain and 5/312 (1.6%) have 2-copy number gain of the *PARK2* gene.

(C) Representative H&E-stained sections of various tumor types found in *Pten*^{+/-} mice with heterozygous and

(D) homozygous *Park2* deletion. Scale bar is 0.1-1.5 mm.

(E) Kaplan–Meier survival plot of *Pten*^{+/-} mice bearing heterozygous (blue) (n=27) or homozygous (green) (n=24) *Park2* deletion, indicating that there is no statistical difference in survival between the two mouse cohorts (p = 0.985, Log-rank test).

(F) Representative H&E (x100), PTEN (x200) and AKT (x200) stained sections of an adrenal pheochromocytoma found in a *Pten*^{+/-} *Park2*^{+/-} mouse.

(G) Quantification of IHC scores of PTEN and (H) AKT protein expression in mouse tissue sections from tumor lesions found in *Pten*^{+/-} mice with WT, HET or HOM deletion of *Park2* (PTEN IHC: *Park2* WT vs. HET p = 0.003, *Park2* WT vs. HOM p = 0.007, AKT IHC: *Park2* WT vs. HET p = 0.033, *Park2* WT vs. HOM p = 0.027, 2-tailed t-test).

SUPPLEMENTAL TABLES

Table S1. Related to Figure 1. Number and type of tumors used to report the frequency of *PARK2* DNA copy number loss from the TCGA dataset. Table S1 is provided as a Microsoft Excel file.

Table S2. Related to Figure 1. Number and type of tumors that showed significantly lower *PARK2* mRNA expression compared to their normal counterparts. Table S2 is provided as a Microsoft Excel file.