Science Advances
AAAS

Supplementary Materials for

Activation of the NRF2 antioxidant program sensitizes tumors to G6PD inhibition

Hongyu Ding, Zihong Chen, Katherine Wu, Shih Ming Huang, Warren L. Wu, Sarah E. LeBoeuf, Ray G. Pillai, Joshua D. Rabinowitz, Thales Papagiannakopoulos*

*Corresponding author. Email: papagt01@nyumc.org

Published 17 November 2021, *Sci. Adv.* **7**, eabk1023 (2021)
DOI: 10.1126/sciadv.abk1023

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

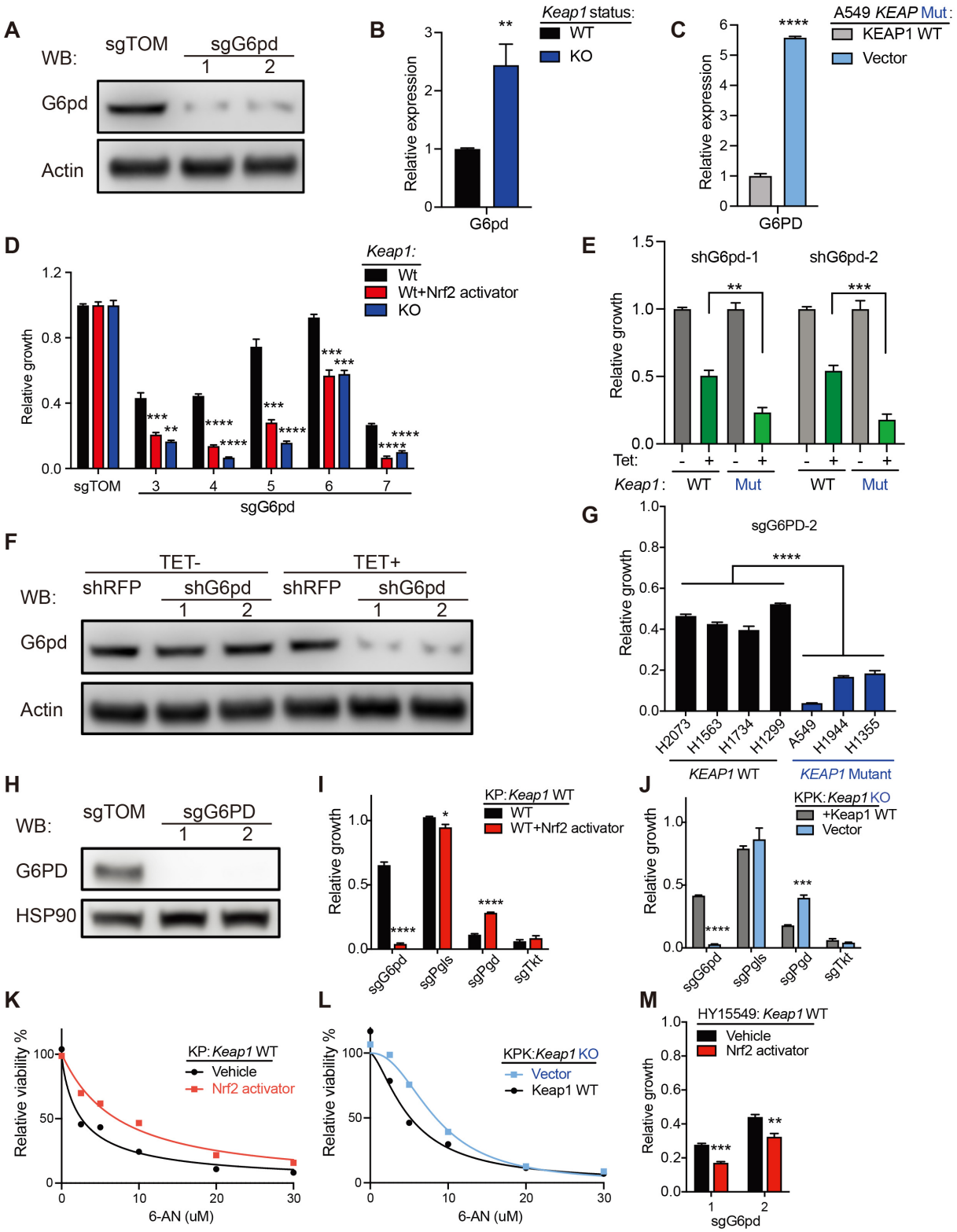

Fig. S1. G6PD is synthetical lethal with NRF2 activation and *KEAP1* mutations. (**A**) Western blot of KP (*Keap1* WT) cells with G6pd knock-out, TOM as non-targeting control. Actin is used as a loading control. (**B**) qPCR validation of G6pd in KP/KPK cells. Data of mRNA expression was normalized to Actin (n=4). (**C**) qPCR validation of G6PD in A549+*KEAP1* WT/A549+vector

cells. Data of mRNA expression was normalized to ACTIN (n=4). (**D**) Proliferation of KP (*Keap1* WT), KP treated with Nrf2 activator or KPK (*Keap1* KO) cells with G6pd knock-out using more sgRNA, normalized to TOM as non-targeting control (n=3). (**E**) Proliferation of KP (*Keap1* WT) or KPK (*Keap1* KO) cells with inducible G6pd knock-down, Tet stands for doxycycline treatment (n=3). (**F**) Western blot of KP (*Keap1* WT) cells with G6pd knock-down, shRFP as non-targeting control. Actin is used as a loading control. TET stands for doxycycline induced shRNA expression. (**G**) Proliferation of *KEAP1* WT or *KEAP1* mutant human cell lines with G6PD knock-out using one more sgRNA, normalized to non-targeting control (n=3). (**H**) Western blot of A549 (*KEAP1* mutant) cells with G6PD knock-out, TOM as non-targeting control. HSP90 is used as a loading control. (**I**) Proliferation of KP (*Keap1* WT) and KP+KI (KP treated with Nrf2 activator in the context of G6pd, Pgls, Pgd, or Tkt knock-out, normalized to TOM as non-targeting control (n=3). (**J**) Proliferation of KPK (*Keap1* KO) overexpressing vector or *Keap1* WT in the context of G6pd, Pgls, Pgd, or Tkt knock-out, normalized to TOM as non-targeting control (n=3). (**K**) Relative viability of KP (*Keap1* WT) and KP+KI (KP treated with Nrf2 activator) treated with 6-AN for 3days (n=3). (**L**) Relative viability of KPK (*Keap1* KO) overexpressing *Keap1* WT or Vector treated with 6-AN for 3days (n=3). (**M**) Proliferation of HY15549 (*Keap1* WT) and HY15549 treated with Nrf2 activator PDAC cells with G6pd knock-out, normalized to non-targeting control (n=3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

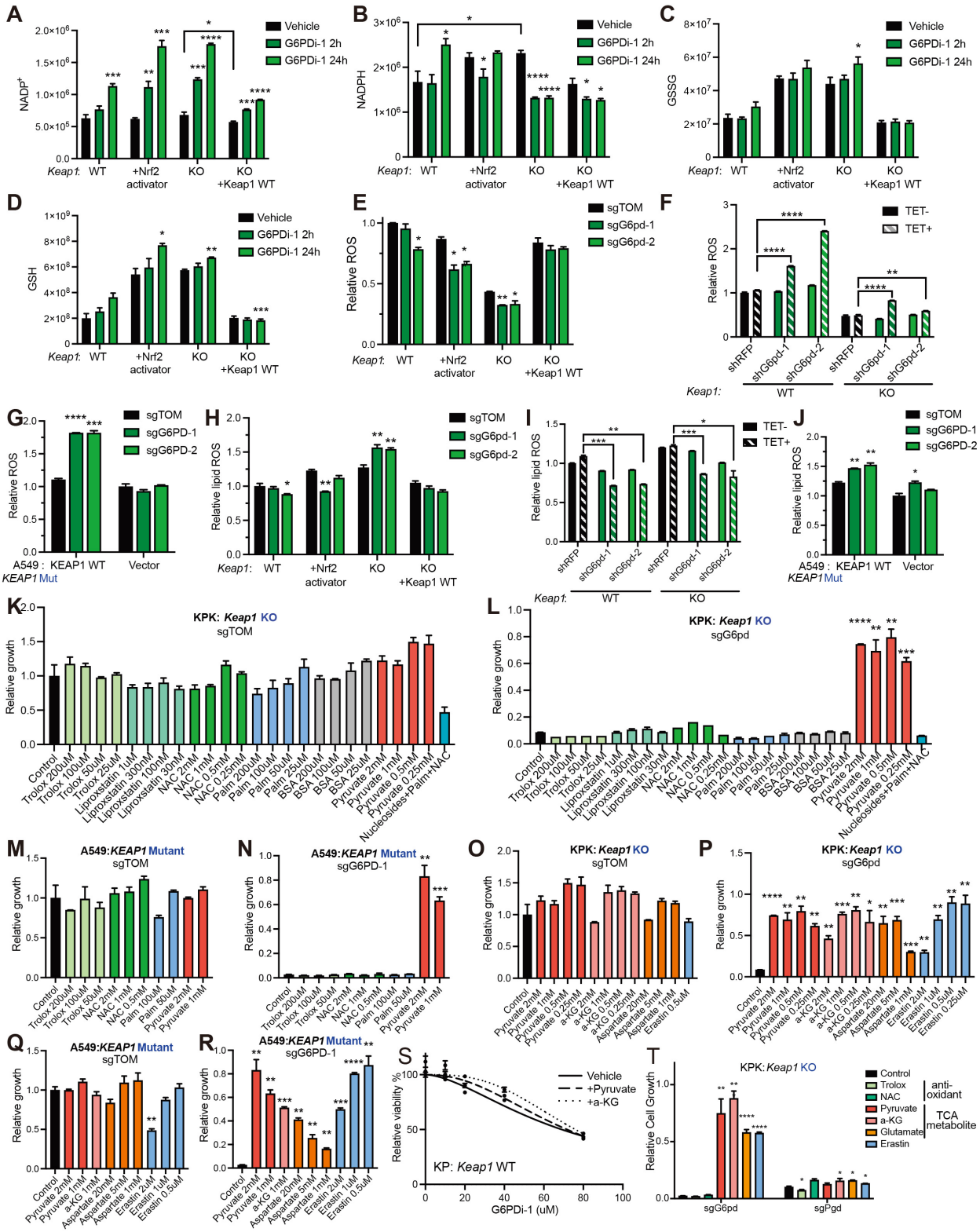

Fig. S2. G6PD loss or inhibition is rescued by central carbon metabolites but not antioxidants. (**A**) $NADP^+$ pool-size of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells treated with 50uM G6PDi-1 for 2h or 24h (n=3). (**B**) NADPH pool-size of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells treated with 50uM G6PDi-1 for 2h or 24h (n=3).

(**C**) GSSG pool-size of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells treated with 50uM G6PDi-1 for 2h or 24h (n=3). (**D**) GSH pool-size of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells treated with 50uM G6PDi-1 for 2h or 24h (n=3). (**E**) Relative ROS of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells with G6pd knock-out, data normalized to KP sgTOM group (n=3). (**F**) Relative ROS of KP and KPK with G6pd knock-down, data normalized to KP shRFP. (**G**) Relative ROS of A549+*KEAP1* WT and A549+vector with G6PD knock-out, data normalized to A549+vector sgTOM group (n=3). (**H**) Relative lipid ROS of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells with G6pd knock-out, data normalized to KP sgTOM group (n=3). (**I**) Relative lipid ROS of KP and KPK with G6pd knock-down, data normalized to KP shRFP (n=3). (**J**) Relative lipid ROS of A549+*KEAP1* WT and A549+vector with G6PD knock-out, data normalized to A549+vector sgTOM group (n=3). (**K** and **L**) Proliferation of KPK (*Keap1* KO) cells with TOM control (**K**) or G6pd KO (**L**) in media supplemented with different concentration of Trolox, Liproxstatin, N-acetyl-L-cysteine (NAC), Palmitic Acid (Palm, BSA conjugated), BSA (Control for Palm loading, fatty acid-free), Pyruvate and combination of 1X nucleoside mix (Sigma-Aldrich, ES-008), 100uM Palm and 0.5mM NAC. Data was normalized to KPK sgTOM control group (n=3). (**M** and **N**) Proliferation of A549 cells with TOM control (**M**) or G6PD KO (**N**) in media supplemented with different concentration of Trolox, Liproxstatin, N-acetyl-L-cysteine (NAC), Palmitic Acid (Palm, BSA conjugated), BSA (Control for Palm loading, fatty acid-free) and Pyruvate. Data was normalized to A549 sgTOM control group (n=3). (**O** and **P**) Proliferation of KPK (*Keap1* KO) cells with TOM control (**O**) or G6pd KO (**P**) in media supplemented with Pyruvate, dimethyl-2-oxoglutarate (a-KG precursor), aspartate and Erastin. Data was normalized to KPK sgTOM control group (n=3). (**Q** and **R**) Proliferation of A549 cells with TOM control (**Q**) or G6PD knock-out (**R**) in media supplemented with Pyruvate, dimethyl-2-oxoglutarate (a-KG precursor), aspartate and Erastin. Data was normalized to A549 sgTOM control group (n=3). (**S**) Relative viability of KP cells treated with G6PDi-1 and supplemented with 2mM Pyruvate and 2 mM a-KG for 3 days (n=4). (**T**) Proliferation of KPK (*Keap1* KO) cells with G6pd and Pgd knock-out in media supplemented with 50uM Trolox, 0.5mM N-acetyl-L-cysteine (NAC), 2mM Pyruvate, 2mM dimethyl-2-oxoglutarate (a-KG precursor), 6mM glutamate and 500nM Erastin. Data was normalized to KPK sgTOM (n=3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

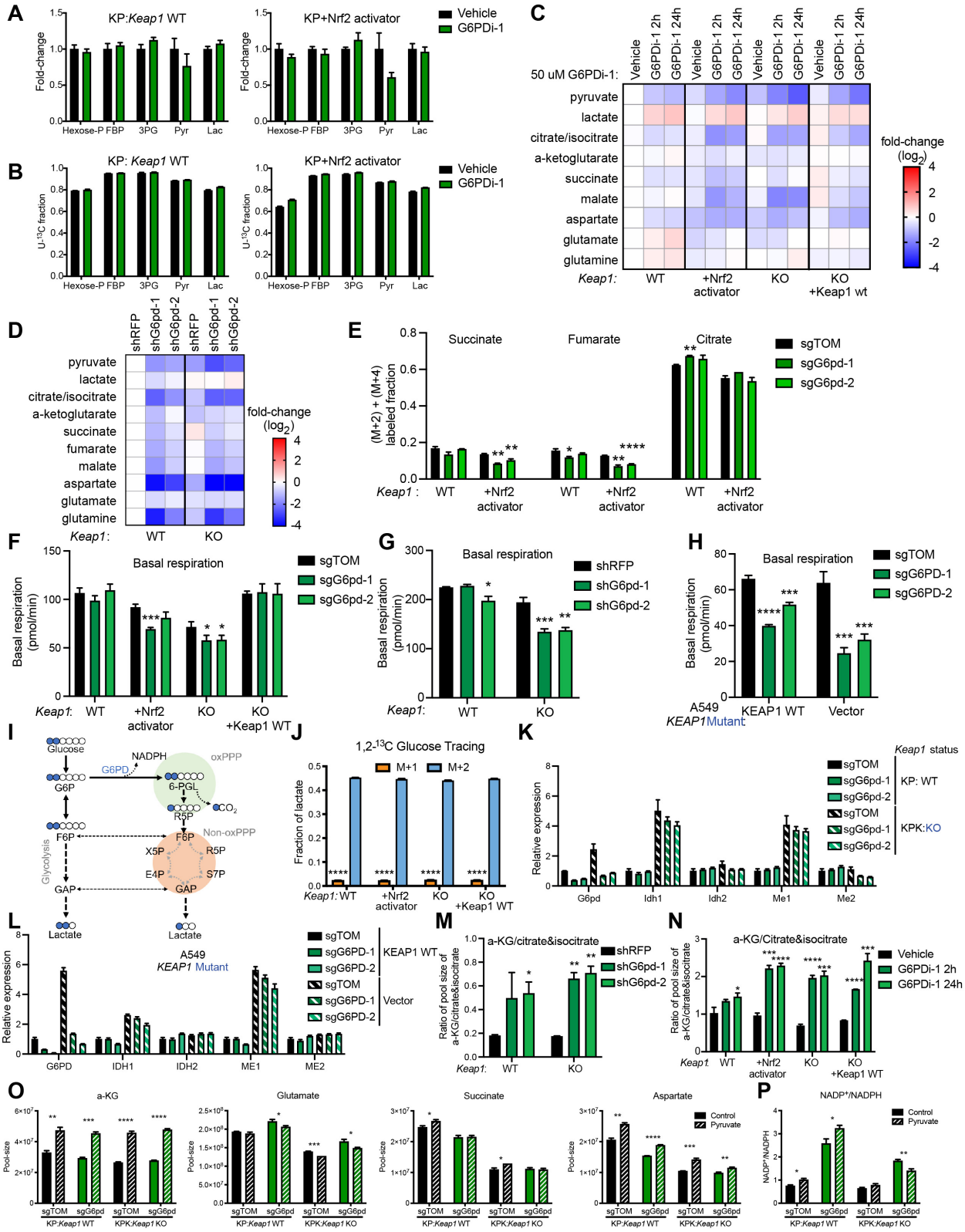

Fig. S3. G6PD disruption leads to an impairment of central carbon metabolism. (A) Pool sizes of glycolytic intermediates upon 3h treatment of 50uM G6PDi-1 in KP and KP+Nrf2 activator cells. Hexose-P: hexose phosphate, FBP: Fructose 1,6-bisphosphatase, 3PG: 3-

Phosphoglyceric acid, Pyr: Pyruvate, Lac: Lactate. (**B**) ^{13}C-incorporation from U-^{13}C-glucose to glycolytic intermediates upon G6PDi-1 inhibition in KP and KP+Nrf2 activator cells. Cells were pre-treated with 50uM G6PD-i1, and U-^{13}C-glucose tracing was conducted for 20 min. (**C**) Heatmap of pool sizes of TCA cycle metabolites in KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells treated with 50uM G6PDi-1 for 2h or 24h. Data was normalized to KP Vehicle group and calculated as $\log_2$(fold-change) (n=3). (**D**) Heatmap of pool sizes of TCA cycle metabolites in KP and KPK cells with G6pd knock-down. Data was normalized to KP shRFP group and calculated as $\log_2$(fold-change) (n=3). (**E**) Mass isotopomer analysis of TCA cycle metabolites (succinate, fumarate and citrate) in KP and KP+KI cells cultured for 8 hr with U-^{13}C-glucose (n=3). (**F**) Basal respiration of KP, KP+Nrf2 activator, KPK+vector and KPK+*Keap1* WT cells with G6pd knock-out measured by Seahorse (n=5). (**G**) Basal respiration of KP and KPK cells with G6pd knock-down measured by Seahorse (n=5). (**H**) Basal respiration of A549+*KEAP1* WT and A549+vector cells with G6PD knock-out cells measured by Seahorse (n=5). (**I**) Schematic of 1,2-^{13}C-glucose tracing, M+2 lactate is derived from glycolysis, M+1 lactate is derived from PPP. (**J**) Mass isotopomer analysis of lactate in KP or KPK cells cultured with 1,2-^{13}C-glucose tracing for 2h (n=3). (**K** and **L**) qPCR validation of G6PD, IDH1, IDH2, ME1 and ME2 expression in KP/KPK (**K**) and A549+*KEAP1* WT/A549+vector (**L**) cells with G6PD knock-out. Data of mRNA expression was normalized to ACTIN (n=4). (**M**) Idh activity of KP and KPK cells with G6pd knock-down. Proxy Idh activity was calculated as ratio of a-KG/citrate&isocitrate pool size. Data was normalized to KP shRFP (n=3). (**N**) Idh activity of KP and KPK cells treated with 50uM G6PDi-1 for 2h or 24h. Proxy Idh activity was calculated as ratio of a-KG/citrate&isocitrate pool size ratio. Data was normalized to KP sgTOM (n=3). (**O**) Pool sizes of a-KG, glutamate, succinate, and aspartate in KP and KPK sgTOM or sgG6pd cells cultured for 2 days with 2mM pyruvate (n=3). (P) NADP+/NADPH ratio of KP and KPK sgTOM or sgG6pd cells cultured for 2 days with 2mM pyruvate (n=3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

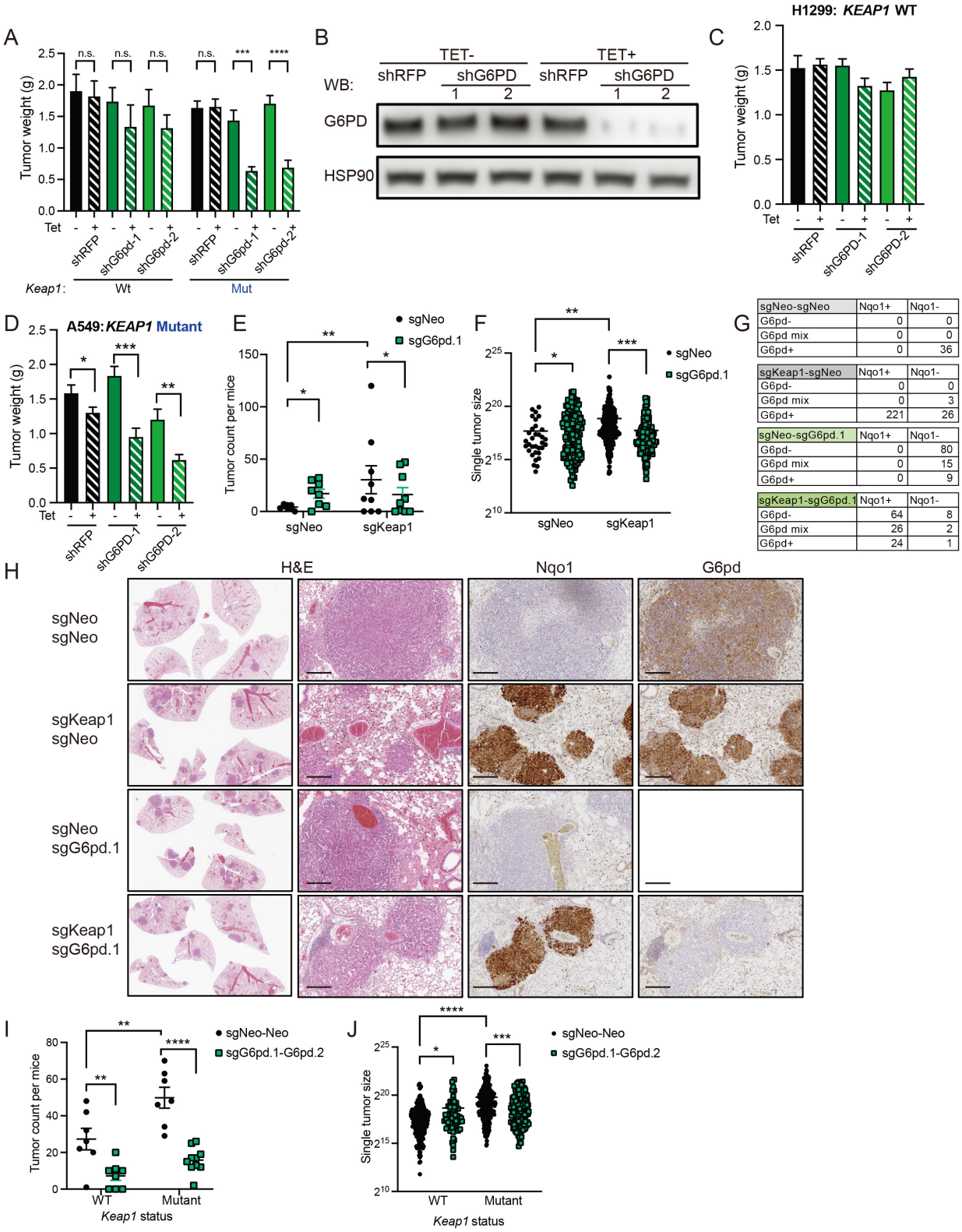

Fig. S4. G6PD is required for *KEAP1* mutant LUAD. (**A**) Tumor weight of subcutaneous KP and KPK tumors with inducible hairpin against G6pd in animals receiving doxycycline diet or control diet. TET stands for doxycycline diet which activate shRNA expression (n=7). (**B**) Western

blot of A549 cells with G6PD knock-down, shRFP as non-targeting control. HSP90 is used as a loading control. TET stands for doxycycline treatment activating shRNA expression. (**C**) Tumor weight of subcutaneous H1299 (*KEAP1* WT) with inducible hairpin against G6PD in animals receiving Tet diet or control diet. TET stands for doxycycline diet which activate shRNA expression (n=8). (**D**) Tumor weight of subcutaneous A549 (*KEAP1* mutant) with inducible hairpin against G6PD in animals receiving Tet diet or control diet. TET stands for doxycycline diet which activate shRNA expression (n=6). (**E**) Quantification of tumor count per mice in KPC mice after infection with pUSEC lentiviruses against Neo-Neo, Keap1-Neo, Neo-G6pd, Keap1-G6pd (n=8 for sgNeo-Neo and sgKeap1-Neo, n=9 for sgNeo-G6pd.1 and sgKeap1-G6pd.1). (**F**) Quantification of individual tumor size in KPC mice after infection with pUSEC lentiviruses against Neo-Neo, Keap1-Neo, Neo-G6pd, Keap1-G6pd. Tumor size is measured as total pixel area in QuPath software (n=8 for sgNeo-Neo and sgKeap1-Neo, n=9 for sgNeo-G6pd.1 and sgKeap1-G6pd.1). (**G**) Contingency tables demonstrating the IHC staining result of each tumor in KPC mice after infection with pUSEC lentiviruses against Neo-Neo, Keap1-Neo, Neo-G6pd, Keap1-G6pd. (**H**) Representative H&E and IHC staining of serial sections from lung tumors of mice 12 weeks after infection with pUSEC lentiviruses against Neo-Neo, Keap1-Neo, Neo-G6pd, Keap1-G6pd. First panels, overall lung tumor burden based on H&E staining; second panels, higher-magnification H&E staining of representative tumors; third panels, Nqo1 IHC analyses; fourth panels, G6pd IHC analyses. Nqo1 works as the marker of *Keap1* mutation and Nrf2 activation. Note that *Keap1* mutant group have higher staining of G6pd, proving that G6pd is *KEAP1/NRF2* pathway substrate. Scale bars, 200um. (**I**) Quantification of tumor count per mice in KPC or KPKC mice after infection with pUSEC lentiviruses against Neo-Neo, G6pd.1-G6pd.2 (n=7 for *Keap1* WT, n=8 for *Keap1* mutant sgNeo-Neo, n=10 for *Keap1* mutant sgG6pd.1-G6pd.2). (**J**) Quantification of individual tumor size in KPC or KPKC mice after infection with pUSEC lentiviruses against Neo-Neo, G6pd.1-G6pd.2. Tumor size is measured as total pixel area in QuPath software (n=7 for *Keap1* WT, n=8 for *Keap1* mutant sgNeo-Neo, n=10 for *Keap1* mutant sgG6pd.1-G6pd.2). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Detailed statistics analysis is presented in Table S3.

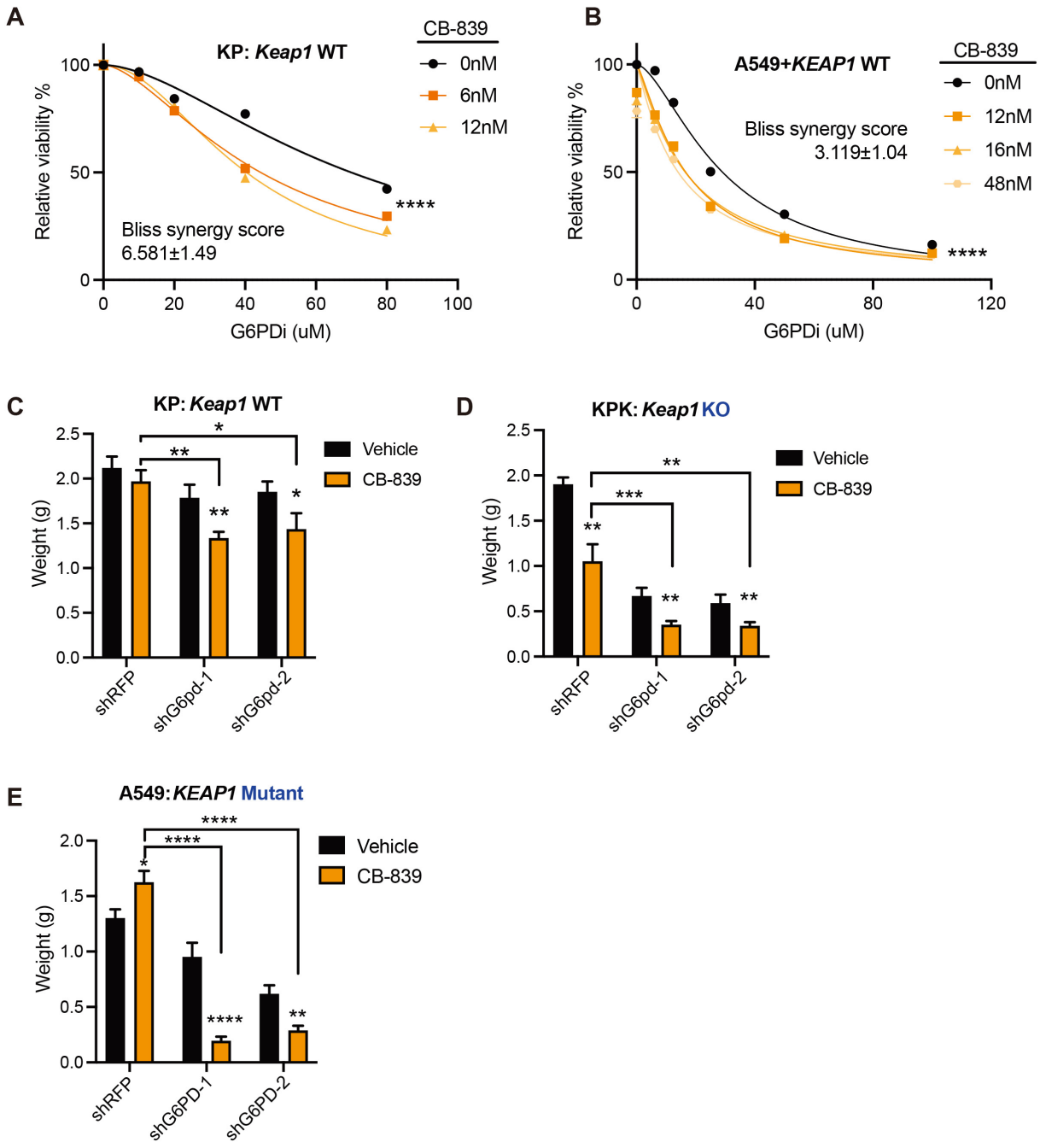

Fig. S5. Inhibition of G6PD and glutaminase synergize to attenuate *KEAP1* mutant LUAD. (**A**) Relative viability of KP (*Keap1* WT) cells cultured with 0, 6, 12nM CB-839 and treated with G6PDi-1 for 3 days. Data was normalized to CB-839 0nM and G6PDi-1 vehicle group (n=4). (**B**) Relative viability of A549+*KEAP1* WT cells cultured with 0, 12, 16, 48nM CB-839 and treated with G6PDi-1 for 3 days. Data was normalized to CB-839 0nM and G6PDi-1 vehicle group (n=3). (**C**) Tumor weight of subcutaneous KP tumors with inducible hairpin against G6pd in animals treated with either CB-839 or vehicle (n=6). (**D**) Tumor weight of subcutaneous KPK tumors with inducible hairpin against G6pd in animals treated with either CB-839 or vehicle (n=6 for shRFP and shG6pd-1, n=8 for shG6pd-2). (**E**) Tumor weight of subcutaneous A549 shRFP control and

shG6PD-1/2 tumors with inducible hairpin against G6PD in animals treated with either CB-839 or vehicle (n=6). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Detailed statistics analysis is presented in Table S3.

Dataset S1. (Separate file)

Table S1. Full list of enrichment of genes according to difference between KP+KI vs. KP.

Table S2. Primers sequences.

Table S3. Summary of tumor growth statistics analysis