Supplementary Materials for

G6PD-mediated increase in de novo NADP⁺ biosynthesis promotes antioxidant defense and tumor metastasis

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Sci. Adv. 8, eabo0404 (2022)
DOI: 10.1126/sciadv.abo0404

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Fig. S1. Expansion of total NADP(H) pool under oxidative stress conditions and its dependence on NADK1

(A to H, O, and P) MIA PaCa-2 (A to D, and O) or PANC-1 (E to H, and P) cells treated with control or NADK1 siRNA (A to H, O, and P) were cultured under matrix-attached (0 h) or -detached conditions (12 or 24 h) (A to D, and O), or treated with different doses of diamide (E to H, and P). Cells were assayed for protein expression (A and E), ROS content (B and F), the NADPH/NADP$^+$ ratio (C and G), NADP$^+$ and NADPH levels (normalized by total amount of proteins, D and H), and viability (O and P).

(I to K, M, and N) Control and NADK1-knockout PANC-1 cells were cultured under matrix-attached (-) or -detached (+) condition (24 h). Cells were assayed for protein expression (I), NADP$^+$ and NADPH levels (J), the NADPH/NADP$^+$ ratio (K), ROS content (M), and viability (N).

(L) PANC-1 cells treated with or without FK866 (0.16 μM) for 16 h were cultured for another 2 h in the presence of $^{13}$C$_3$-$^{15}$N-nicotinamide and assayed for the percentages of M+4 isotopomers of NAD$^+$ and NADP$^+$.

Data are mean ± SD (n =3) and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; two-way ANOVA for D, H, J, K, M, and N, unpaired Student’s t-test for the rest.
Fig. S2. G6PD expands the total NADP(H) pool by interacting with and activating NADK1 enzyme activity.

(A and B) G6PD mRNA and protein levels in PANC-1 cells treated with different doses of H$_2$O$_2$ (A) or diamide (B).

(C and D) Control and G6PD-knockdown PANC-1 cells were cultured under matrix-attached or -detached (24 h) conditions (C), or treated with or without 100 μM H$_2$O$_2$ (D). Cell lysates were incubated without antibody (-), with mouse control IgG, or anti-NADK1 antibody. Immunoprecipitates and whole cell lysates (WCL) were analyzed by western blot.

(E) G6PD enzymatic activity in PANC-1 cells treated with vehicle (-) or G6PD inhibitor (G6PDi-1, 80 μM).

(F to I) PANC-1 cells treated with vehicle (-) or G6PDi-1 (80 μM) and cultured under indicated conditions were assayed for NADP$^+$ and NADPH levels (F), the NADPH/NADP$^+$ ratio (G), ROS content (H), and viability (I).

(J and K) G6PDi-1 blocks formation of G6PD dimers and tetramers. HEK293T cells treated with vehicle (-) or G6PDi-1 (60 μM) were incubated with the indicated concentrations of DSS. Cell lysates were analyzed by western blot for G6PD (J) and p53 (K), with monomer, dimer, and tetramer of these proteins indicated. Endogenous p53 was used as a control for cross-linking.

(L) Interaction of human 6xHis-NADK1 and human G6PD pretreated with or without G6PDi-1 was analyzed by a pull-down assay with nickel affinity gel. The input and pulldown samples were assayed by western blot.

(M and N) Control and NADK1-knockdown MIA PaCa-2 cells were transfected with control or G6PD expression vectors and cultured under indicated conditions. Cells were assayed for the NADPH/NADP$^+$ ratio (M) and ROS content (N). Protein expression is shown in Fig. 2M.

(O) MIA PaCa-2 cells were transfected with control, Flag-G6PD, or Flag-G6PD$^{K171Q}$ plasmid as indicated. Cell lysates were incubated with an anti-Flag antibody. Immunoprecipitates and WCL were analyzed by western blot.

(P) MIA PaCa-2 cells transfected with control, Flag-G6PD, or Flag-G6PD$^{K171Q}$ plasmid were assayed for the NADPH/NADP$^+$ ratio. Protein expression is shown in Fig. 2P.

Data are mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; two-way ANOVA for F and P, unpaired Student’s t-test for the rest.
Fig. S3. G6PD mediated activation of NADK1 is major mechanism regulating NADK1 activity under matrix-detached condition.

(A to C) PANC-1 cells treated with control or G6PD siRNA were cultured and treated with or without NAMPT or NMNAT1 inhibitor as indicated. Cells were then assayed for protein expression (A), NAMPT activity (B), and NMNAT1 activity (C).

(D and E) PANC-1 cells were transfected with control or Flag-NADK1 plasmids were serum-starved overnight and treated with 100 ng/ml EGF for 10 min (D), treated with 2 μM AKT inhibitor MK2206 for 2 h (D) or 100 ng/ml PMA for 1 h (E), or cultured under matrix-attached or -detached conditions (12 h) (D and E). Flag-NADK1 was immunoprecipitated with anti-Flag antibody. Immunoprecipitates and whole cell lysates (WCL) were analyzed by western blot.

(F to H) Control and G6PD-knockdown PANC-1 cells were transfected with Flag-NADK1 and cultured under matrix-attached or -detached conditions (12 h) as indicated. Cells were assayed for protein expression (F). Flag-NADK1 was affinity purified (G) and assayed for enzymatic activity (H).

Data are mean ± SD (n = 3) and are representative of three independent experiments. ***P < 0.001; two-way ANOVA for B and C.
Fig. S4. Several other NADPH-regenerating enzymes are unable to enlarge NADP(H) pools. (A to D) mRNA (A and C) and protein (B and D) levels of major NADPH-regenerating enzymes in PANC-1 (A and B) and MIA PaCa-2 (C and D) cells cultured under matrix-attached (0 h) or -detached conditions (12 or 24 h).

(E to I) PANC-1 cells treated with control, IDH1, or ALDH1L2 siRNA were cultured as indicated and assayed for protein expression (E), the NADPH/NADP⁺ ratio (F), ROS content (G), viability (H), and NADP⁺ and NADPH levels (I). The dashed lines in (F) to (I) indicate the values for G6PD-knockdown cells under the same assay conditions.

(J to L) PANC-1 cells harboring control, G6PD, IDH1, or ALDH1L2 expression vectors were assayed for protein expression (J), the NADPH/NADP⁺ ratio (K), and NADP⁺ and NADPH levels (L).

Data are mean ± SD (n = 4 for A and C, and 3 for the rest) and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; two-way ANOVA for I, K and L, unpaired Student’s t-test for the rest.
Fig. S5. Endogenous G6PD is critical for maintaining redox homeostasis.

(A) Western blot analysis of control and G6PD-knockdown PANC-1 cells cultured in the presence or absence of 1 mM of NAC.

(B to D) The NADPH/NADP⁺ ratio (B), ROS content (C), and viability (D) of control and G6PD-knockdown cells were cultured under matrix-attached (0 h) or -detached conditions (12 or 24 h) in the presence or absence of 1 mM of NAC.

(E) G6PD enzymatic activity in PANC-1 cells that were cultured in the presence of vehicle, G6PDi-1 (80 μM), and/or NAC (1 mM).

(F to H) PANC-1 cells were cultured under matrix-attached or -detached conditions in the presence of vehicle, G6PDi-1 (80 μM), and/or NAC (1 mM). Cells were assayed for the NADPH/NADP⁺ ratio (F), ROS content (G), and viability (H). These experiments were done together with those shown in fig. S2, E to I.

(I) Western blot analysis of control and G6PD-overexpressing MIA PaCa-2 cells treated with or without of H₂O₂ (50 μM).

(J to L) Control and G6PD-overexpressing MIA PaCa-2 cells were cultured under matrix-attached or -detached conditions in the presence or absence of H₂O₂ (50 μM). Cells were assayed for the NADPH/NADP⁺ ratio (J), ROS content (K), and viability (L).

Data are mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; unpaired Student’s t-test.
Fig. S6. G6PD is transcriptionally activated by TAp73 under various redox stress conditions.

(A to D) Control and G6PD-overexpressing PANC-1 cells were treated with or without p73 siRNA and cultured under indicated conditions. Cells were assayed for protein expression (A), the NADPH/NADP⁺ ratio (B), ROS content (C), and viability (D).

(E to G) HCT116 (E), HepG2 (F), and PC3 (G) cells treated with control or p73 siRNA were cultured as indicated and assayed for protein expression.

(H to N) HMEC cells treated with control or p73 siRNA (H to K), or HMEC cells harboring control or TAp73 expression vector (L to N), were cultured as indicated. Cells were assayed for protein expression (H and L), G6PD mRNA levels (I), ROS content (J and M), and viability (K and N).

(O to R) PANC-1 (O), HCT116 (P), HepG2 (Q), and PC3 (R) cells treated with control or p73 siRNA were exposed to different dose of H₂O₂ and assayed for protein expression.

(S and T) HCT116 cells treated with control or p73 siRNA were exposed to different doses of diamide for 48 h and assayed for protein expression (S) and ROS content (T).

Data are mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; unpaired Student’s t-test.
Fig. S7. NADK1 and G6PD modulate anchorage independent growth.

(A and B) Western blot analysis of PANC-1 treated with different doses of H$_2$O$_2$ (A) or diamide (B).

(C) PANC-1 cells with individual knockdown of NRF2, PIRH2, E2F1, TAp73, G6PD, and control cells (Fig. 8J) were assayed for soft-agar colony formation. Shown are representative images. Quantification is depicted in Fig. 8L.

(D to F) Protein expression (D), soft agar colony formation (E), and adherent growth (F) of control and E2F1-knockdown PANC-1 cells treated with or without 1 mM NAC.

(G) MIA PaCa-2 cells were transduced a G6PD expression plasmid and NADK1 shRNA as indicated and assayed for soft-agar colony formation (related to Fig. 8O). Protein expression is shown in Fig. 8M.

(H to K) Control and G6PD-knockdown PANC-1 cells were treated with or without 1 mM. Cells were analyzed for protein expression (H), adherent growth (I), and soft-agar colony formation (J and K).

(L and M) Control and NADK1-knockdown PANC-1 cells were assayed for adherent growth (L) and soft-agar colony formation (M; related to Fig. 8Q). Protein expression is shown in Fig. 8P.

Data are mean ± SD (n = 3) and are representative of three independent experiments. **$P < 0.01$, ***$P < 0.001$; two-way ANOVA for E, F, and K, unpaired Student’s t-test for the rest.
Fig. S8. G6PD promotes metastatic colonization of pancreatic cancer cells.

(A to C) PANC-1 cells transfected with control shRNA, G6PD shRNA #1, G6PD shRNA #2 (which targets 3’-UTR of G6PD mRNA), and a shRNA #2-resistant form of G6PD (which expresses only the ORF) as indicated. Cells were cultured as indicated and assayed for protein expression, G6PD enzyme activity (A), and NADP+ and NADPH levels (B), or cultured in soft-agar medium for colony formation (C).

(D to F and K to M) Half a million of PANC-1/GFP cells with or without G6PD knockdown (D to F) or MIA PaCa-2/GFP cells with or without G6PD overexpression (K to M) were inoculated into immunodeficient mice via tail vein injection. Eight weeks later, mice were analyzed for tumor nodules in the liver by GFP imaging (D and K) and H&E staining (E and L; scale bar 120 μm), and for tumor burden in the lung by western blot analysis of GFP (F and M). No tumors were detected in the liver. (F) and (M) are related Fig. 9, G and N, respectively.

(G to J) Lung tissues of mice injected with control or G6PD-knockdown PANC-1 cells were analyzed by IHC for the expression of G6PD (G and H) and NADK1 (I and J). Shown are representative images of tumor nodules formed by control cells as well as relatively large and small tumor nodules formed by G6PD-knockdown cells (G and I), and quantification of G6PD or NADK1 staining in these tumor populations (H and J). Red arrows indicate tumors. Scale bar, 40 μm.

Data are mean ± SD (n = 3 for A, B, and C) of a representative of three independent experiments or as indicated. ***P < 0.001; two-way ANOVA.
Fig. S9. G6PD and NADK1 do not modulate cell migration and invasion in pancreatic cancer cells.

(A to E) PANC-1 cells with (+) or without (-) G6PD or NADK1 knockdown by siRNAs were analyzed for protein expression (A), cell migration with representative images (B) and quantification (C) shown, and cell invasion with representative image (D) and quantification (E) shown. Scale bar, 80 μm.

(F and G) PANC-1 cells treated with (+) or without (-) FK866 (0.16 μM) were assayed for cell migration (F) and cell invasion (G).

(H and I) NADP+ and NADPH levels (H) and viability (I) of PANC-1 cells that were cultured under matrix-attached or -detached condition (24 h) in the presence or absence of 0.16 μM FK866. Data are mean ± SD (n = 3 for H and I, 4 for E and G, and 10 for C and F) and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not statistical significant; two-way ANOVA for C, E, and H, unpaired Student’s t-test for the rest.
Fig. S10. Expression of G6PD and its upstream regulators in primary and metastatic pancreatic cancers and their correlation with metastasis and survival of patients.

(A and B) mRNA levels (a.u) of G6PD (A) and PIRH2 (B) in primary and metastatic tumors of pancreatic cancer patients from GEO database (GSE63124).

(C to F) Pearson correlation coefficient analysis for the expression of indicated protein pairs: NRF2 (a.u. determined in Fig. 10B) and p73 (a.u. determined in Fig. 10D) (C, n = 46), NRF2 and G6PD (a.u. determined in Fig. 10A) (D, n = 46), E2F1 (a.u. determined in Fig. 10C) and G6PD (E, n = 43), and PIRH2 (a.u. determined in Fig. 10E) and G6PD (F, n = 45).

(G and H) Correlation of NRF2 mRNA expression (Reads Per Kilobase Million (FPKM)) and G6PD mRNA expression (FPKM) (G), and correlation of p73 mRNA expression (FPKM) and G6PD mRNA expression (Log10 FPKM) (H) were analyzed from human protein atlas database. n=176.

(I to N) Survival curves of pancreatic cancer patient with high and low expression of indicated gene from human protein atlas database. The number of subjects included in each group and P value are also shown. N is as indicated, *** P < 0.001; unpaired Student’s t-test for A and B.
|                          | NRF2 | PIRH2 | E2F1 | p73 | G6PD | NADK1 |
|--------------------------|------|-------|------|-----|------|-------|
| Expression cut off (FPKM)| 22.94| 2.74  | 4.57 | 0.18| 10.1 | 10.96 |
| 5 years survival rate (high)| 7%  | 42%  | 22% | 24% | 20%  | 25%   |
| 5 years survival rate (low)| 37% | 18%  | 32% | 42% | 46%  | 27%   |
| P value                  | 0.047| 0.007| 0.000| 0.035| 0.045| 0.173 |
| Gene   | Forward primer                           | Reverse primer                           |
|--------|-----------------------------------------|-----------------------------------------|
| 6PGD   | 5’-GACATCATCATTGACGGAGGAAA-3’           | 5’-GGGCCACGCTTCTTTGTTC-3’               |
| Actin  | 5’-CATGTACGTGGCTATCCAGGC-3’             | 5’-CTCCTTAATGTCACGCACGAT-3’             |
| ALDH1L1| 5’-GCCTGGCTTCTGGTGCTCTC-3’              | 5’-GCCACGTCGCTTGGTTC-3’                |
| ALDH1L2| 5’-TAACACATAACACAAGACAGAT-3’            | 5’-ATATTCATTAGAGCTCTCTCA-3’             |
| FTL    | 5’-CAGCCTGGTCAATTGTATCTC-3’             | 5’-GCCAATTCCCGGAAGAAGTG-3’             |
| G6PD   | 5’-CGAGGCCGTCACCAAAGAC-3’               | 5’-GTAGTGGTCGATGCGGTAGA-3’             |
| GAPDH  | 5’-ACAACCTTGTATCGTGGAAGG-3’             | 5’-GCCATCACGCACAGTTTC-3’               |
| GCLC   | 5’-GGAGACACAGGATAGGGAGTT-3’             | 5’-CCGGCGTTTCGACATGTTG-3’              |
| GCLM   | 5’-CATTTACAGCCTTACTGGGAGG-3’            | 5’-ATGCAGTCAAATCTGGTGGCA-3’             |
| GPx2   | 5’-GGTAGATTTCTAACGTCCGGG-3’             | 5’-TGACAGTCTCCTGATGTTCCA-3’             |
| IDH1   | 5’-TGTGGTAGAGATGCAAAGAGA-3’             | 5’-TTGGTGACTTTGGTCTGTTGGTG-3’           |
| IDH2   | 5’-TACAACACACGGAGTGCCA-3’               | 5’-CAACACAAGCACCAGAGAGA-3’             |
| ME1    | 5’-CTGCTGACACGGACACCCTC-3’              | 5’-GATCCTCCTAGCTGGTGAAGGAAG-3’         |
| ME2    | 5’-ATATACACCGAGGTGGTCT-3’               | 5’-CATCAGTCTACAACACAGCCTT-3’           |
| NQO1   | 5’-GAAGAGCACTGATCGTACTGGC-3’            | 5’-GGATACGAAAGTTTCAGCAGG-3’            |
| PIRH2  | 5’-TGTTGAATTTGTAGGATTGGTCC-3’           | 5’-CAACAGGGATGTGTAATGGT-3’             |
| PRX1   | 5’-CCACGGGAGATCATTGCTTTTCA-3’           | 5’-AGGTTGATTTGACCCATGCTAGAT-3’         |
| PRX6   | 5’-GTGACAGCTGCTGGTGTTGTT-3’             | 5’-CTGGGGTGAGCACCCTTTT-3’              |
| TAp73  | 5’-CGGGCCATGCCTGTTTACA-3’               | 5’-TGTTCTCCTGTTGAAGTCCCT-3’            |
| TRX    | 5’-GTGAAGCAGATCGAGAGCAAG-3’             | 5’-CTGGGCTGAGAAAGTCAACTA-3’            |