Glucose-6-phosphate dehydrogenase is not essential for K-Ras-driven tumor growth or metastasis

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Running Title: G6PD is not essential for K-Ras tumor growth or metastasis

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Abstract

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is a major contributor to NADPH production and redox homeostasis and its expression is upregulated and correlated with negative patient outcomes in multiple human cancer types. Despite these associations, whether G6PD is essential for tumor initiation, growth, or metastasis remains unclear. Here we employ modern genetic tools to evaluate the role of G6PD in lung, breast, and colon cancer driven by oncogenic K-Ras. Human HCT116 colorectal cancer cells lacking G6PD exhibited metabolic indicators of oxidative stress but developed into subcutaneous xenografts with growth comparable to that of wild-type controls. In a genetically engineered mouse model of non-small-cell lung cancer driven by K-Ras G12D and p53-deficiency, G6PD knockout did not block formation or proliferation of primary lung tumors. In MDA-MB 231-derived human triple-negative breast cancer cells implanted as orthotopic xenografts, loss of G6PD modestly decreased primary site growth without ablating spontaneous metastasis to the lung and moderately impaired the ability of breast cancer cells to colonize the lung when delivered via tail vein injection. Thus, in the studied K-Ras tumor models, G6PD is not strictly essential for tumorigenesis and at most modestly promotes disease progression.

Statement of Significance: K-Ras driven tumors can grow and metastasize even in the absence of the oxidative pentose pathway, a main NADPH production route.
Introduction

The cofactor reduced nicotinamide adenine dinucleotide phosphate (NADPH) is required to fuel reductive biosynthesis and combat oxidative stress. Production of NADPH from its redox partner, NADP$^+$, is compartmentalized in eukaryotic cells [1]. In most mammalian cells, cytosolic NADP$^+$ reduction mainly occurs via three routes: malic enzyme 1 (ME1), isocitrate dehydrogenase 1 (IDH1), and the oxidative pentose phosphate pathway [2, 3]. The oxidative pentose phosphate pathway shunts glucose-6-phosphate from glycolysis to generate the nucleoside precursor ribose-5-phosphate (R5P) and two equivalents of NADPH; one by G6PD, which catalyzes the first and committed step, and one by phosphogluconate-6-phosphate dehydrogenase (PGD).

G6PD resides on the X-chromosome, and its loss is embryonic lethal in mice [4]. Hypomorphic mutations in G6PD, however, are prevalent and persist in humans as they convey partial resistance to malaria, despite sensitizing red blood cells to oxidative stressors [5, 6]. In line with tumors having high demands for NADPH and R5P, mounting evidence from the last two decades suggests G6PD may play an important role in tumorigenesis [7]. Overexpression of G6PD in subcutaneously implanted fibroblasts is sufficient to give rise to fibrosarcomas in nude mice [8]. Additionally, G6PD is often upregulated in solid tumors, and frequently correlates with negative patient outcomes [9-13]. This association is strongest in hepatocellular carcinoma, where patients with high G6PD expression fare dramatically worse in terms of survival [14, 15]. Accordingly, some have suggested that G6PD may be a therapeutic target for cancer [14, 16].

Despite these observations, few studies have directly interrogated the role of G6PD in cancer in vivo. As compensatory routes for producing NADPH and R5P are well annotated and broadly expressed in tumors [17], it is important to define the context in which G6PD is required for tumorigenesis. Prior attempts to address this relied mainly on RNA interference or pharmacological tools of questionable nature [18-20]. Recently, we knocked out G6PD in a variety of cancer cell lines using CRISPR/Cas9 gene editing [2]. G6PD-null lines proliferated somewhat more slowly than controls, with cell growth supported by ME1- and IDH1-mediated NADPH production.

What is unclear, however, is if this compensatory activity is sufficient to rescue G6PD loss in tumors, which often encounter oxidative stress in vivo. For example, lung tumors directly
interact with atmospheric oxygen and inflammatory cells—both of which can produce reactive oxygen species (ROS) [21]. Likewise, various stages of metastasis involve augmented ROS exposure, particularly during matrix detachment [22]. Conversely, antioxidants facilitate transit of metastatic cells through the highly oxidative blood compartment [23, 24]. Modern anticancer therapies, including ionizing radiation and chemotherapeutic agents, can also rely on ROS generation for antitumor activity [25, 26]. Data from several human cancer trials demonstrate that patients fare worse by multiple measures when standard therapies are supplemented with antioxidants [27, 28]. Finally, the master regulator of antioxidant defense, Nuclear factor erythroid 2-related factor 2 (NRF2) is an oncogene, and its negative regulator, Kelch-like ECH-associated protein 1 (KEAP1) is a tumor suppressor gene indicating a direct role of mitigation of oxidative stress in the evolution of human cancer [29]. Importantly, oncogenic K-Ras is known to activate the Nrf-2-mediated antioxidant program [30], and KEAP1 loss-of-function mutations are associated with and contribute to K-Ras driven lung tumor progression [31, 32]. Intriguingly, cancers with NRF-2/KEAP1 dysfunction have demonstrated an increased reliance on the oxidative pentose phosphate pathway [33, 34]. Taken together, these studies suggest that tumors are highly dependent on antioxidant defenses, with G6PD widely assumed to be a central player.

Here we used CRISPR-mediated genetic knockout to assess the role of G6PD at various stages of K-Ras-driven tumorigenesis in vivo. Our goal is not to quantitate whether G6PD makes any contribution to cancer progression, but rather to determine its essentiality: Can K-Ras tumors initiate, grow and metastasize without G6PD? This focus is motivated by G6PD’s physiological essentiality, and thus its appeal as an anticancer target resting on it being particularly critical for cancer. We show G6PD loss has no impact on HCT116 xenograft growth, does not prevent tumor initiation and only slightly impairs growth of genetically engineered K-Ras$^{G12D/+;p53^{-/-}}$ (KP) lung tumors. G6PD knockout shows a modest effect on orthotopic xenograft growth and spontaneous metastasis to the lung of MDA-MB 231-derived breast cancer cells (although it somewhat more strongly impacts the ability of these cells to colonize the lung from the blood). Collectively, while not ruling out an essential role for G6PD in other contexts, including in other mutational backgrounds such as KEAP1 and in driving therapy resistance, these studies demonstrate that G6PD is not absolutely essential for K-Ras solid tumor growth or metastasis.
Materials and Methods

Cell lines, culture conditions and reagents

HCT116 and 293T cells were all originally obtained from ATCC (Manassas, VA). Generation of HCT116 clonal G6PD knockouts and MDA-MB 231:SCP28TR lines were previously described [2, 35]. Green-Go cells were a generous gift from Thales Papagiannakopoulos (NYU Langone Health, New York, New York). All cell lines (unless otherwise specified) were maintained in DMEM (CellGro 10-017, Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (F2442, Sigma-Aldrich, St. Louis, MO). Cell lines were routinely screened for mycoplasma contamination by PCR. LentiCRISPR v2 (52961) and pSECC (60820) were obtained from Addgene (Cambridge, MA). All primers were synthesized by IDT (Coralville, IA). Antibodies for Western blotting were used according to their manufacturer’s directions. Anti-β-actin (5125) was obtained from Cell Signaling Technologies (Danvers, MA). Anti-G6PD (ab993) was obtained from Abcam Inc. (Cambridge, MA). CoxIV antibody was obtained from Proteintech (11242-1-AP.). Standard laboratory chemicals were from Sigma.

Subcutaneous xenografts

These animal studies were approved and conducted under the supervision of the Princeton University Institutional Animal Care and Use Committee. For tumor growth studies, 7-wk-old female CD1/nude mice were bilaterally injected in the rear flanks with either HCT-116 wild-type (control) or G6PD-null cells (1×10^6 cells in 100 ml 1:1 PBS:Matrigel). Tumor growth measurements were taken biweekly using two caliper measurements [volume=1/2 (length×width^2)]. Animals were euthanized when tumors reached ~150 mm^3 or if they displayed any signs of distress or morbidity. Upon termination of the study, tumors were isolated immediately and snap-frozen in liquid nitrogen using a Wollenberger clamp to preserve metabolite levels. Isolated tumors were weighed, then ~10-mg tissue was disrupted using a cryomill and lysed in 1 ml ice-cold 40:40:20 acetonitrile:methanol:water (w/ 0.5% formic acid) followed by neutralization w/ 15% w/v ammonium bicarbonate. Solids were precipitated and supernatants were either utilized directly for LC-MS analysis (by HILIC LC-MS) or were dried and resuspended in water (for reversed-phase LC-MS).
Production of pSECC lentivirus

sgRNA sequences targeting murine G6pdx and non-coding region of DNA (intergenic control) were identified from the mouse GeCKOv2 sgRNA library [36]. The identified PAM sequences (Supplementary Table 1) were subcloned into the pSECC (Addgene, 60820) using the BsmBI restriction endonuclease (NEB R0580S). Virus was produced through PEI (MilliporeSigma, 408727) transfection of vectors and lentiviral packaging plasmids psPax2 and VSVG in 293T cells. Medium containing lentivirus was collected at 48 and 72 h and filtered through a PES filter (0.22 um, MilliporeSigma). Virus was concentrated by ultracentrifugation (~70,000g for 2 h at 4°C), and the resulting pellet was gently resuspended in an appropriate volume of HBSS (MilliporeSigma, 55037C). Virus was aliquoted and snap-frozen in liquid nitrogen before storing at -80°C. Viral titering was achieved using Green-Go cells and flow cytometry, as previously described [37].

Intranasal injection of lentivirus for lung tumorigenesis

These animal studies followed protocols approved by the Rutgers-CINJ Institutional Animal Care and Use Committee. KrasLSL-G12D/+ Trp53flox/flox mice aged 6–8 weeks were intranasally infected with ~200,000 plaque-forming units with pSECC lentivirus harboring sgRNA targeting intergenic control or G6pdx. Ten mice were infected per group. Mice were monitored and euthanized via cervical dislocation at 10 or 15 weeks post-lentiviral infection (5 mice per group per timepoint). Lungs were collected, weighed, and fixed in 10% buffered formalin solution for 24 h before being stored in 70% ethanol at 4°C.

Generation of knockout cell lines for mammary fat pad and tail vein assays

Generation of batch G6PD-null cells for mammary fat pad and tail-vein injection assays in the MDA-MB 231SCP:28TR background was achieved using the lentiviral CRISPR–Cas9 vector system lentiCRISPR v2 (Addgene #52961). Briefly, sgRNA sequences targeting exon-5 of human G6PD were designed using the crispr.mit.edu design tool. The identified PAM sequences or scrambled control sequence (Supplementary Table 1) were subcloned into the lentiCRISPR v2 using the BsmBI restriction endonuclease (NEB R0580S). Virus was produced through PEI (MilliporeSigma, 408727) transfection of vectors and lentiviral packaging plasmids...
psPax2 and VSVG in 293T cells. Medium containing lentivirus was collected after two days and filtered through a PES filter (0.22 μm, MilliporeSigma). Cells were transfected with virus containing scrambled control or targeting \textit{G6PD} and Polybrene (8 ug/mL, Invitrogen). Cells were split after 48 h into RPMI-media (10% FBS) containing puromycin (2 ug/mL) and cultured for 3 days. Cells were passaged once more in puromycin media prior to utilization in xenograft experiments. G6PD knockout was confirmed by western blotting.

\textbf{Mammary fat pad injections and monitoring}

These animal studies were approved and conducted under the supervision of the Princeton University Institutional Animal Care and Use Committee. Orthotopic xenograft experiments were conducted on eight-week-old female NOD-SCID gamma (NSG) mice. All of the mice were originally ordered from the Jackson Laboratory and breeding was conducted in a specific pathogen-free (SPF) barrier facility. Briefly, batch G6PD-knockout or control cells were generated as described above. On day of experiment, cells were trypsinized and resuspended in sterile PBS. Approximately 100,000 cells suspended in 10 µl PBS were injected into the mammary gland. Tumor growth measurements were taken weekly using two caliper measurements \(\text{[volume}=\frac{1}{2} (\text{length} \times \text{width}^2)\]}. Animals were euthanized when tumors reached \(~300 \text{ mm}^3\) or if they displayed any signs of distress or morbidity. Upon termination of the study, retro-orbital injection of luciferin was administered under anesthesia (ketamine/xylazine) just prior to euthanasia by cervical dislocation. Lungs were isolated immediately followed by bioluminescent imaging as previously described [38]. Lungs were then fixed in 10% buffered formalin solution for 24 h before being stored in 70% ethanol at 4°C.

\textbf{Tail vein injection and monitoring}

These animal studies were approved and conducted under the supervision of the Princeton University Institutional Animal Care and Use Committee. Tail-vein injection xenograft experiments were conducted on eight-week-old female athymic Nu/Nu mice. All of the mice were originally ordered from the Jackson Laboratory and breeding was conducted in a specific pathogen-free (SPF) barrier facility. Briefly, batch G6PD-knockout or control cells were generated as described above. On day of experiment, cells were trypsinized and resuspended in sterile PBS prior to injection into the tail vein (\(~45,000 \text{ cells in 100 } \mu\text{l PBS}\)). Live
bioluminescent imaging was conducted by retro-orbital injection of luciferin under anesthesia (ketamine/xylazine) followed by bioluminescent imaging as previously described [38]. Animals were euthanized if they displayed any signs of distress or morbidity. Upon termination of the study, lungs were isolated immediately and fixed in 10% buffered formalin solution for 24 h before being stored in 70% ethanol at 4°C.

**Tumor quantification and immunohistochemistry**

Fixed lungs were embedded in paraffin blocks and serial slides were prepared at a thickness of 4 µm on which immunohistochemistry (IHC), using a Ventana Immunostainer with staining against G6PD (Abcam, ab993), Ki67 (Abcam, ab15580) and hematoxylin and eosin (H&E) staining were conducted. Slides were imaged and analyzed at Rutgers Cancer Institute of New Jersey Biomedical Informatics shared resource using an Olympus VS120 whole-slide scanner (Olympus Corporation of the Americas) at 20X magnification or a Nikon Eclipse 80i microscope at 100× magnification. Scale bars are included for all representative images. A semi-autonomous method of quantification and designation of tumors was applied. Total lung area occupied by tumor was quantified from H&E-stained slides. Fractions of G6PD-null tumors were calculated from IHC slides. For quantification of Ki67, at least six representative images of entire lung lobes were obtained for each genotype and a minimum of 150 cells from each image at 100× magnification were scored.

**LC-MS analysis**

Metabolites were analyzed using a quadrupole-orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, Waltham, MA), coupled to hydrophilic interaction chromatography (HILIC) with LC separation on a XBridge BEH Amide column (Waters), or a stand-alone orbitrap (Thermo-Fisher Exactive) coupled to reversed-phase ion-pairing chromatography with LC separation on a HSS-T3 column (Waters). Both mass spectrometers were operating in negative ion mode and were coupled to their respective liquid chromatography methods via electrospray-ionization. Detailed analytical conditions have been previously described [39, 40]. Data were analyzed using the ElMaven software (v 0.2.4, Elucidata), with compounds identified based on exact mass and retention time match to commercial standards.
For metabolomics analysis, metabolites data were normalized to control condition and clustered using Cluster 3.0 software. Heatmaps were plotted using Java Treeview.

Statistics

Samples sizes are defined in each figure legend. Unless otherwise specified, results for biological replicates are presented as mean ± SEM. For tumor growth monitoring over time, statistical significance between conditions was calculated using a mixed-effects model for repeated measures followed by Dunnett’s post hoc analysis. For all other measurements, statistical significance was calculated using unpaired Student’s t-test when comparing two groups, and one-way ANOVA followed by Dunnett’s post hoc analysis when comparing three or more. All statistical calculations were performed using the software package GraphPad Prism 7.03.

Results

G6PD null xenografts exhibit oxidative stress phenotype but still grow rapidly

We first implanted three clonally isolated G6PD-null HCT116 K-Ras G13D colon cancer cell lines (ΔG6PD-1, ΔG6PD-2, ΔG6PD-3) subcutaneously into the lateral flanks of nude mice [41]. These cell lines were previously generated using CRISPR-Cas9-nickase editing [2]. Despite complete loss of G6PD expression (Figure 1A), each knockout clone still formed tumors. Moreover, the tumors grew at rates similar to those of the parental cell line (Figure 1B, Supplementary figure 1A) and of clonally isolated wild-type cells that escaped CRISPR mediated deletion (Cas9-ctrl) (Supplementary figure 1B-D).

To characterize the metabolic impact of G6PD loss in vivo, we analyzed water soluble metabolites from these tumors by liquid chromatography-mass spectroscopy (LC-MS). Several metabolites were consistently perturbed across G6PD-null tumors compared with controls (Figure 1C). As expected, the G6PD product, 6-phosphogluconate (6-pg), was significantly reduced in the knockout tumors (Figure 1D). Similarly, in accordance with our recent findings linking G6PD loss with impaired folate metabolism and associated thymidylate synthesis [2],...
levels of the thymidylate precursor deoxyuridine monophosphate (dUMP) were elevated in knockout tumors (Figure 1E). In addition, G6PD-null tumors displayed metabolic indications of oxidative stress. Though NADPH levels were maintained, NADP⁺ pools were elevated (Figure 1F). This imbalance led to an increase in the NADP⁺/NADPH ratio. Both the oxidized (GSSG) and reduced forms of glutathione were elevated in the knockout tumors (Figure 1G), and the GSSG/GSH ratio was maintained, suggesting these tumors may cope with oxidative vulnerability by increased production of the glutathione backbone. Thus, clonally selected G6PD-null cells formed subcutaneous tumors that appear to grow normally, but exhibit metabolic indicators of oxidative stress.

**G6PD is not essential for primary lung cancer tumorigenesis**

We next sought to examine the impact of G6PD loss in engineered primary K-Ras G12D, p53 null murine lung tumors. To this end, we utilized CRISPR/Cas9-mediated gene editing *in vivo* [37]. *Kras<sup>LSL-G12D/+</sup>* *Trp53<sup>lox/lox</sup>* (KP) mice were intranasally infected with lentivirus harboring the pSECC plasmid containing one of two different guide-RNA (sgRNA) targeting *G6pdx* (the murine gene encoding G6PD, mice henceforth referred to as sg*G6pdx*-1 and sg*G6pdx*-2) or a non-coding intergenic region of DNA (sgControl mice). In addition to expressing sgRNAs, the pSECC vector [37] expresses Cas9 and Cre-recombinase, resulting in Cre-mediated tumor initiation (via activation of mutant-*Kras* and deletion of *p53*) and target gene disruption in infected lung epithelia. Importantly, this approach avoids initially selecting for cells resistant to G6PD knockout *in vitro*, thereby providing a less biased readout on the functional importance of G6PD to *in vivo* tumorigenesis.

Following lentivirus delivery to the lung, mice were monitored and euthanized at 10 and 15 weeks post-infection, with the latter timepoint nearing the predefined humane endpoint due to substantial filling of the lung with tumor (Supplementary figure 2A) [42]. Lungs were collected, weighed and fixed for histological analysis. Strikingly, a significant fraction of lung tumors from sg*G6pdx*-1 and sg*G6pdx*-2 mice were G6PD-deficient (Figure 2A-C, Supplementary figure 2A). The fraction of G6PD-null tumors (out of the total) was stable between 10 and 15 weeks post-infection (Figure 2B-C), despite significant tumor progression (Supplementary figure 2B). Similarly, no differences in lung size (Supplementary figure 2C-D), tumor burden (% total tumor...
cross-sectional area) (Figure 2D) or total tumor count (Figure 2E) were observed between sgG6pdx-1,-2 mice and controls at 10 weeks post-infection, with a slight decrease seen in tumor burden at 15 weeks. The cross-sectional area distributions of wild-type and G6PD-null tumors were well aligned in these mice (Supplementary figure 2E). Moreover, at both time points, proliferation as measured by Ki67 staining was not significantly different between wild-type and G6PD-null tumors (Supplementary figure 2F-G). Taken together, while not ruling out some role for G6PD in accelerating tumor growth, these data show that G6PD is not essential for primary KP tumor formation or growth in the lung.

**G6PD loss minimally impacts growth in and spontaneous metastasis from mammary fat pad**

Having established that G6PD is not essential for K-Ras mutant tumor initiation or growth, we aimed to examine whether G6PD enables metastatic spread. We employed the MDA-MB 231:SCP28 human K-Ras G13D triple negative breast cancer cell line [43], with constitutive luciferase expression [35], in a breast cancer model of spontaneous metastasis. These cells were infected with lentiviral vectors harboring CRISPR/Cas9, puromycin resistance genes and sgRNAs targeting G6PD (sgG6PD-1 and sgG6PD-2) or a scrambled guide RNA (sgControl). Western blot analysis after puromycin selection of a pooled population indicated significant G6PD depletion (Figure 3A).

Implantation of these batch-knockout cells in the mammary fat pad of NOD-SCID gamma (NSG) mice gave rise to tumors modestly smaller than those derived from control cells (Figure 3B, Supplementary figure 3A). This difference was detectable at 7 days post-implantation, before tumors were palpable, by live bioluminescence imaging (Supplementary figure 3B-C). Subsequent growth rate (k), as measured by exponential fitting, was not significantly different (Figure 3B, Supplementary figure 3D). In addition, there was not strong selection against the G6PD-deficient cells as tumors were predominantly G6PD deficient as identified by immunohistochemical staining at endpoint (IHC) (Figure 3C). Thus, while G6PD supports some step of MDA-MB 231:SCP28TR subcutaneous tumor growth, most likely initial
cell survival and implantation efficiency, K-Ras mutant triple negative breast cancer tumors can form despite genetic G6PD deletion.

We next examined the potential for spontaneous metastasis to the lungs in these mice. Bioluminescent imaging of the lungs immediately after euthanasia showed no difference in metastatic burden between mice bearing control and sgG6PD tumors (Figure 3D). Furthermore, though highly variable between individuals, histological analysis of the microscopic metastatic nodules present (no macroscopic nodules were detected) revealed a similar metastatic tumor burden in the lungs across groups (Figures 3E-F). However, despite G6PD wild-type cells representing a nearly undetectable fraction of the original injected pooled populations of sgG6PD cells (and a minor portion of the primary tumors), a significant fraction of metastatic tumors arose from G6PD wild-type cells (Figures 3E-F). This suggests spontaneous metastasis selects against G6PD loss during spread to the lung. Thus, G6PD likely facilitates one or more stages of metastatic spread, but nevertheless is not strictly essential for metastasis.

**G6PD facilitates seeding of the lung from the blood**

Since the blood compartment is relatively oxidative, we hypothesized that metastatic cells undergoing transit through the blood and/or subsequent colonization of the lung may rely most heavily on G6PD activity. To more directly examine this possibility, we regenerated batch-knockout MDA-MB 231:SCP28TR cells lacking G6PD (Figure 4A) and injected them into the tail vein of athymic nu/nu mice. Intravital bioluminescent imaging over six weeks (Figure 4B-C) demonstrated that mice injected with sgG6PD cells generally had lower metastatic burden in the lungs than mice that received control cells. This was supported by terminal histological analysis, where lungs from mice injected with sgG6PD cells had lower total tumor counts (Figure 4D-E), with a trend towards lower overall tumor area. Strikingly, histological staining for G6PD demonstrated the majority of tumors that formed from injection of the sgG6PD cells arose from wild-type cells (Figure 4D-E). Since G6PD wild-type cells represented a very small fraction of the initial batch-knockout populations (Figure 4A), this suggests that G6PD was important for transit through the blood and/or seeding of the lungs. Thus, though not strictly required, G6PD markedly supports lung colonization in this setting.
Discussion

Tumors evolve in harsh metabolic environments and invest heavily in systems that combat oxidative stress [44]. Studies in both mice and humans have demonstrated that enhanced antioxidant buffering promotes tumor growth and progression [23, 24, 27, 28, 45, 46]. NADPH is used to reduce oxidized glutathione and thioredoxin and thus maintains endogenous antioxidants. However, the importance of different NADPH-producing enzymes for cancer in vivo remains poorly understood.

Here, we analyze the essentiality of G6PD and by extension, the oxidative pentose phosphate pathway, in tumorigenesis using several mouse models of K-Ras-driven cancer. Having pursued the discovery of G6PD small molecule inhibitors [47], we hoped that G6PD would be important for in vivo tumorigenesis. Initially, we tested this possibility using the K-Ras mutant human colorectal cancer line HCT116. Clonal G6PD-null HCT116 cells are relatively difficult to generate due to poor single cell outgrowth. Furthermore, such cells grow at a moderately reduced rate in vitro [2]. Nevertheless, they showed no impairment of subcutaneous tumorigenesis. The strong subcutaneous growth of these tumors might reflect adaptive events that occurred during in vitro passage or the subcutaneous milieu being particularly permissive for oxidative stress-sensitive cells.

Accordingly, we elected to assess G6PD’s importance in a model that avoids in vitro selection and involves a medically important oncogenesis location. To this end, we tested lung tumorigenesis in a genetically engineered KP mouse model. Here, G6PD knockout occurs simultaneously with oncogene induction and strong selection against G6PD knockout cells should lead to decreased tumor burden and/or near absence of knockout tumors. This model is immunocompetent and thus tumors must deal with reactive oxygen species made by immune cells. Moreover, the tumors must cope with the lung’s high oxygenation. Despite all of these factors, we observed many knockout tumors, and these tumors grew relatively normally during the interval from 10 to 15 weeks after infection, with longer-term analyses impossible due to animals reaching the defined humane endpoint. Thus, surprisingly, G6PD is not essential for K-Ras tumor initiation or growth.

In search of a setting where G6PD function might be critical, we evaluated the impact of its loss in metastasis. Metastasis in epithelial cancers is a complex, multi-step process involving
the detachment of cells from primary tumors, survival during transit through lymphatics and/or blood, and eventual colonization and proliferation in distant tissues [48]. Using a model of K-Ras mutant triple negative breast cancer, G6PD knockout somewhat slowed primary site growth in the mammary fat pad, but the extent of growth reduction would likely offer only a brief survival benefit. Surprisingly, despite loss of G6PD, cells from these tumors still migrated to the lung and formed pulmonary metastases. Because of the impairment in primary tumor growth and the lack of statistically significant changes in metastasis, it remains unclear what steps, if any, in metastasis may have been impacted in this model. Injection of the same cells via the tail-vein, revealed that G6PD supports, but is not absolutely essential for colonization of the lung from the blood. These studies were small and not designed to evaluate G6PD’s role in advanced metastatic disease. Nevertheless, they establish the capacity for G6PD deficient cells to form tumors and spontaneously metastasize.

Despite previously demonstrating G6PD is often dispensable for cancer cell growth in culture, we were nonetheless surprised this phenotype largely holds in vivo, where nutrients are scarcer and sources of ROS abound. How might tumors overcome G6PD loss to grow and metastasize? One potential mechanism is by augmenting flux through alternative NADPH production pathways: ME1, IDH1, or folate metabolism. Such compensatory NADPH production must be paired also with an alternative source of ribose-phosphate, likely the non-oxidative pentose phosphate pathway. In addition, Ras-driven tumors are avid nutrient scavengers [49]. By acquiring lipids and/or nucleosides from the microenvironment or circulation, cancer can alleviate demand for products of the G6PD reaction. To decipher the root of this unfortunate robustness, further mechanistic studies are required.

Interestingly, tail vein injection of G6PD-null cells exaggerates the importance of G6PD for colonization of the lung relative to spontaneous metastasis of cells from established G6PD-deficient tumors. Multiple factors may account for this discrepancy, including differences in host immune status (NSG vs. nude mice), selection and/or adaptation during orthotopic tumor growth, migration through lymph vasculature rather than the blood, or differences in cellularity of migrating metastases (single cell vs. circulating tumor microemboli or “clumps” of cells). Though this study and others support the notion that adequate antioxidant buffering is important
for cancer cell survival in the blood, spontaneous metastases may be somewhat less vulnerable to oxidative stress.

Is there a role for G6PD inhibition in the treatment of cancer? While our analysis is not comprehensive, it argues against G6PD being a preferred target for monotherapy of K-Ras driven solid tumors. Although we saw some effects of G6PD loss on metastatic spread, given the importance of G6PD to normal physiology, other approaches to limiting metastasis seem likely to be more promising. That said, like many other metabolic enzymes, G6PD is likely to be particularly important in the context of specific tumor type or genetic events, such NRF2 activation or KEAP1 loss [33, 34]. Identification of tumors that are particularly sensitive to G6PD loss is an important ongoing area of investigation. Moreover, both conventional cancer treatments (e.g. ionizing radiation, chemotherapeutics) and immunotherapy often rely on the production of oxidative stress (and the resulting DNA damage) for their activity. Thus, it is easy to envision how G6PD inhibitors might synergize with other anticancer therapies.
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Author contributions

J.D.R. and J.M.G. conceived the study. J.M.G., M.E., J.Y.G., E.W., Y.K., and J.D.R. designed the experiments. J.M.G., M.E., J.Y.G., V.B., T.L. and J.W. conducted the experiments. J.M.G., and J.D.R. wrote the paper with input from the other authors.
References


Figure Legends

Figure 1. Growth and metabolomics of G6PD-null subcutaneous xenografts

(A) Western blots of clonal G6PD knockout cells (ΔG6PD) in the HCT116 background. (B) Growth of subcutaneous tumors from HCT116 wild-type and ΔG6PD cells (mean ± SEM, n = 10). (C) Water-soluble metabolites levels from HCT116 wild-type and ΔG6PD subcutaneous tumors. For each condition, individual biological replicates are shown, normalized to wild-type tumors and analyzed in parallel by LC-MS. (D) Relative changes in 6-phosphogluconate (6-pg) levels in ΔG6PD subcutaneous tumors (mean ± SEM, n = 10, one-way ANOVA). (E) Relative changes in dUMP levels in ΔG6PD subcutaneous tumors (mean ± SEM, n = 10, one-way ANOVA). (F) Relative changes in NADP⁺, NADPH and NADP⁺/NADPH in ΔG6PD subcutaneous tumors (mean ± SEM, n = 10, one-way ANOVA). (G) Relative changes in glutathione species (GSH = reduced, GSSG = oxidized) and GSSG/GSH in ΔG6PD subcutaneous tumors (mean ± SEM, n = 10, one-way ANOVA). TIC = total ion count. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, # = p = 0.085, n.s. = not significant.

Figure 2. Histology of lung tumors from mice infected with pSECC targeting G6PD

(A) Representative immunohistochemistry (IHC) staining for G6PD (top panel) and hematoxylin and eosin (H&E) (bottom panel) staining of serial sections from primary lung tumors generated from pSECC infection in KP mice. Black arrow = G6PD wild-type, white arrow = G6PD-null. (B-C) Fraction of tumors staining null for G6PD by IHC in sgG6pdx-1 and sgG6pdx-2 mice, calculated from percentage tumor area (B) and tumor count (C) (individual replicates shown, bars represent mean, Student’s t-test). (D-E) Quantification of primary lung tumors by H&E in sgControl, sgG6pdx-1 and sgG6pdx-2 mice; (D) total tumor burden as a percent of cross-sectional lung area, (E) total tumor count (individual replicates shown, bars represent mean, one-way ANOVA). * = p < 0.05, n.s. = not significant.
Figure 3. Growth and metastasis from mammary fat pad by G6PD-null triple negative breast cancer tumors

(A) Western blot analysis of G6PD in MDA-MB 231:SCP28TR-luciferase expressing cells treated with lentiviral CRISPR-Cas9 targeting scrambled control (Control) or G6PD (G6PD-1 and G6PD-2), just prior to implantation in the mammary fat pads of NSG-mice. (B) Growth of orthotopic tumors in mammary fat pad from cells depicted in (A) (mean ± SEM, n ≥ 6). Dashed line represents exponential curve fitting. (C) Representative H&E and IHC staining for G6PD at low- and medium-magnification of orthotopic xenografts isolated from the mammary fat pad. (D) Photonic flux of lungs isolated from NSG-mice bearing the orthotopic mammary fat pad xenografts. Mice were injected with luciferin just prior to euthanasia (mean ± SEM, n ≥ 6, one-way ANOVA). (E) Representative images of spontaneous metastatic lung tumors; (left) H&E staining of lungs with metastatic nodules, (middle) IHC staining of adjacent slide for G6PD in lung tumors, (right) IHC for G6PD at higher magnification. Black arrow = wild-type tumor, white arrow = knockout tumor. (F) Quantification of spontaneously arising metastatic lung tumors from mice bearing the mammary fat pad tumors depicted in B-E; percentage tumor area and count were calculated from H&E, G6PD wild-type or null status were assigned based on staining for G6PD (individual replicates shown, bars represent mean, one-way ANOVA). n.s. = not significant. Experiment was repeated twice with similar results.

Figure 4. Colonization of lungs by G6PD-null triple negative breast cancer cells from blood

(A) Western blot analysis of G6PD in MDA-MB 231:SCP28TR-luciferase expressing cells treated with lentiviral CRISPR-Cas9 targeting scrambled control (Control) or G6PD (G6PD-1 and G6PD-2), just prior to injection into the tail vein of nude mice. (B) Representative live bioluminescence imaging from mice at 9 weeks after injection of cells depicted in (A). (C) Photonic flux as determined by live imaging over time for each group depicted in (A) (mean ± SEM, n ≥ 7, mixed-effects model for repeated measures). Fluxes are normalized to baseline signal detected immediately after tail vein injection; higher photonic flux correlates with higher metastatic burden. (D) Representative images of lung tumors at 9 weeks post-tail vein injections; (left) H&E staining of lungs tumors, (middle) IHC staining of adjacent slide for G6PD, (right) IHC for G6PD at higher magnification. Black arrow = wild-type tumor, white arrow = knockout tumor.
tumor. (E) Quantification of lung tumors at 9 weeks post-tail vein injection. Percent tumor area and count were calculated from H&E, G6PD wild-type or null status was assigned based on staining for G6PD (individual replicates shown, bars represent mean, one-way ANOVA). * = p < 0.05, ** = p < 0.01, **** = p < 0.0001, † = p = 0.066. Experiment was repeated twice with similar results.
Figure 1.
Figure 2.

A. G6PD

B. C.

knockout fraction (% area)

weeks: 10 15 10 15

sgG6pdx-1 vs sgG6pdx-2

D. E.

tumor count

weeks: 10 15 10 15

sgControl vs sgG6pdx-1 vs sgG6pdx-2

1 mm
Figure 3.

A. 

<table>
<thead>
<tr>
<th>sgRNA</th>
<th>Control</th>
<th>G6PD-1</th>
<th>G6PD-2</th>
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<tbody>
<tr>
<td>MDA-MB 231:SCP28</td>
<td>G6PD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CoxIV</td>
<td></td>
<td></td>
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</table>

B. 

![Graph showing tumor volume (mm³) over days for different sgRNAs.]

C. 

<table>
<thead>
<tr>
<th>sgRNA</th>
<th>Control</th>
<th>sgG6PD-1</th>
<th>sgG6PD-2</th>
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<tr>
<td>H&amp;E</td>
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<td></td>
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<tr>
<td>5 mm</td>
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<td></td>
</tr>
<tr>
<td>H&amp;E</td>
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<tr>
<td>G6PD</td>
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<tr>
<td>100 µm</td>
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</table>

D. 

![Graph showing photon count (10⁷/sec) for different sgRNAs.]

E. 

<table>
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<tr>
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<th>Control</th>
<th>G6PD-1</th>
<th>G6PD-2</th>
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<tr>
<td>10 µm</td>
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</table>

F. 

![Graph showing tumor area (%) and tumor count for different sgRNAs.]

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Figure 4.

A. MDA-MB 231:SCP28

<table>
<thead>
<tr>
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<th>CoxIV</th>
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<tr>
<td>G6PD-2</td>
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</table>

B. sgRNA

sgControl  sgG6PD-1  sgG6PD-2

C. photon counts / sec (fold-change)

D. H&E  G6PD

E. wild-type  ΔG6PD

<table>
<thead>
<tr>
<th>sgRNA</th>
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<th>tumor area (%)</th>
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Glucose-6-phosphate dehydrogenase is not essential for K-Ras-driven tumor growth or metastasis

Jonathan M. Ghergurovich, Mark Esposito, Zihong Chen, et al.

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