Functional Metabolic Screen Identifies 6-Phosphofructo-2-Kinase/Fructose-2, 6-Biphosphatase 4 as an Important Regulator of Prostate Cancer Cell Survival

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INTRODUCTION

It is now widely accepted that cancer cells alter their metabolic activity to satisfy their increased needs for energy and biosynthetic precursors. Many cancers show increased glycolysis, a feature generally known as the Warburg effect, and the importance of biosynthetic processes such as lipid synthesis for cancer cell growth is increasingly recognized (1). In numerous studies, authors have shown that oncogenic signaling pathways regulate the expression and activity of metabolic enzymes to support macromolecule synthesis in cancer cells, which is required for their rapid proliferation (2). Alterations in metabolic activity can also protect cells from apoptosis (3). Furthermore, in cancer genome-sequencing studies, researchers have revealed that metabolic genes themselves can also be subjected to mutational alterations (4). Enzymes within the core metabolic pathways, including glycolysis, glutaminolysis, and fatty acid biosynthesis, have been shown to be essential in maintaining the balance between the use of glucose for energy generation and the synthesis of antioxidants. Targeting metabolic enzymes may therefore present new therapeutic opportunities.

RESULTS

Metabolic Characterization of Prostate Cell Lines

To identify metabolic weaknesses of prostate cancer cells, we first characterized the metabolic requirements of 222 metabolic enzymes, transporters, and regulators on the survival of 3 metastatic prostate cancer cell lines and a nonmalignant prostate epithelial cell line. This approach revealed significant complexity in the metabolic requirements of prostate cancer cells and identified several genes selectively required for their survival. Among these genes was 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4), an isoform of phosphofructokinase 2 (PFK2). We show that PFKFB4 is required to balance glycolytic activity and antioxidant production to maintain cellular redox balance in prostate cancer cells. Depletion of PFKFB4 inhibited tumor growth in a xenograft model, indicating that it is required under physiologic nutrient levels. PFKFB4 mRNA expression was also found to be greater in metastatic prostate cancer compared with primary tumors. Taken together, these results indicate that PFKFB4 is a potential target for the development of antineoplastic agents.

SIGNIFICANCE: Cancer cells undergo several changes in their metabolism that promote growth and survival. Using an unbiased functional screen, we found that the glycolytic enzyme PFKFB4 is essential for prostate cancer cell survival by maintaining the balance between the use of glucose for energy generation and the synthesis of antioxidants. Targeting PFKFB4 may therefore present new therapeutic opportunities.
androgen-independent prostate cancer cell lines, DU145 and PC3, and an androgen-dependent prostate cancer cell line, LNCaP. These were compared with the nontransformed prostate epithelial cell line RWPE1 (17). We first investigated glucose uptake, lactate secretion, and oxygen consumption in the 4 cell lines. All cell lines show high rates of glucose consumption and lactate secretion, indicating high glycolytic activity (Fig. 1A). DU145 and RWPE1 cells show the greatest rates of both glucose uptake and lactate secretion, which is consistent with their high proliferation rate (Supplementary Fig. S1A).

Basal oxygen consumption rate (OCR) was greatest in RWPE1 and LNCaP cells. PC3 cells showed a low basal OCR but increased oxygen consumption after treatment with the uncoupling agent FCCP. Only DU145 cells showed a complete loss of mitochondrial respiration (Fig. 1B), which is a common feature of cancer cells. We next investigated potential differences in the rates of lipid synthesis in the different cell lines. Pyruvate-dependent lipid synthesis in FM was similar in the 4 cell lines (Fig. 1C). The removal of lipoproteins from the growth medium (LDM) strongly increased the rate of lipogenesis (Fig. 1C) and reduced growth rates of the 3 prostate cancer cells (Supplementary Fig. S1A and S1B).

Many cancer cell lines depend on glucose for survival. In addition, glutamine has been shown to be a major anabolic substrate in some cancer cells (18). We therefore investigated the sensitivity of these cell lines to the depletion of glucose or glutamine. Remarkably, the withdrawal of glucose for 72 hours induced apoptosis in all prostate cancer cell lines in both FM and LDM (Fig. 1D). Only DU145 cells were affected by glutamine depletion (Fig. 1D). In contrast, RWPE1 cells were not affected by the withdrawal of either glucose or glutamine. This observation confirms that the prostate cancer cell lines are highly dependent on the availability of glucose for their survival.

The inhibition of fatty acid biosynthesis blocks the growth of prostate cancer cells in a xenograft model (19). We therefore investigated the dependency of these lines on de novo lipogenesis. Inhibition of lipid synthesis by treatment with inhibitors of fatty acid synthase or ATP-citrate lyase (C75 and SB204990, respectively) reduced viability of the prostate cancer cells only in the absence of lipoproteins (Fig. 1E). RWPE1 cells were sensitive to inhibition of lipid synthesis, indicating that their recommended growth medium (KGM) does not provide sufficient exogenous lipids for growth when de novo lipogenesis is blocked.

Next, we established differences in metabolite levels between the 3 prostate cancer cell lines and RWPE1 cells under the different aforementioned growth conditions described by using mass spectrometry (Supplementary Fig. S1C and Supplementary Table S1). The 3 prostate cancer cell lines showed greater intracellular levels of several glycolytic intermediates, including fructose-6-phosphate, phosphoenolpyruvate, and pyruvate. Interestingly, levels of adenine, a purine derivative required for nucleotide synthesis, were reduced in the prostate cancer cell lines, whereas the concentration of phosphoribosyl pyrophosphate, an intermediate in purine biosynthesis, was increased.

We also measured the uptake and secretion rates of metabolites that could be readily detected by mass spectrometry (Supplementary Fig. S1D). Overall, the uptake rate for most amino acids did not show significant differences between cell lines or conditions. However, the rate of glycine and isoleucine uptake and glutamate secretion was markedly greater in the 3 prostate cancer cells compared with RWPE1. Increased glutamate secretion has been previously reported in cancer cells (20).

Taken together, the metabolic characterization of these cell lines revealed that despite some heterogeneity in glucose and oxygen usage, the high dependence on glucose is a unifying feature of the 3 prostate cancer cell lines, whereas the non-malignant RWPE1 cells may have the ability to use alternate substrates. Furthermore, reliance on lipid synthesis, which has been previously described for prostate cancer cells in a xenograft model (19), is only observed when they are grown in lipid-depleted conditions.

### Functional Metabolic Screen

Using a custom library of siRNA oligonucleotides targeting 222 metabolic enzymes, transporters, and regulators located within the core glucose metabolism (see Supplementary Fig. S2A and Supplementary Discussion), we performed a functional screen to identify metabolic genes required for proliferation and survival of prostate cancer cell lines. Because these cells only showed sensitivity to inhibition of lipogenesis in LDM (Fig. 1E), we used both FM and LDM growth conditions for the screen. Induction of apoptosis (caspase activity) and reduction in total protein amount (cell mass) in response to siRNA transfection was measured (Supplementary Fig. S2B and Supplementary Table S2). We used the apoptosis z-scores to perform a 2-way cluster analysis (Fig. 2A), which revealed considerable overall differences between the cell lines and conditions whereas the different serum conditions for each cell line clustered together. Importantly, the results from the 3 prostate cancer cell lines were clearly separated from the results of the nonmalignant RWPE1 cell line. A detailed discussion of the primary screen results is provided in the Supplementary Discussion.

siRNA sequences that caused apoptosis (z-score ≥2.5) or a loss of cell mass (cell mass ≤0.6 relative to control) in at least 2 of the cancer cell lines but had no effect in RWPE1 cells (Table 1) were selected for validation by the use of 4 individual siRNA sequences (Supplementary Table S3). The same 18 genes were also used to perform a gene set-enrichment analysis with 2 public expression datasets from prostate cancer (GSE3325 and GSE6752). This analysis showed that these genes tended to be overexpressed in samples from metastatic prostate cancer compared with samples from primary sites (Fig. 2B). This observation is particularly interesting because the 3 prostate cancer cell lines used for this study were all originally isolated from metastases.

Only 2 genes compromised the viability of all 3 prostate cancer cell lines (Fig. 2C and D). Silencing of PRKABI induced apoptosis in both FM and LDM conditions whereas silencing of PFKFB4 caused apoptosis in LDM only. Importantly, in several independent studies, investigators show that expression of these 2 genes is increased in prostate cancer metastasis when compared with primary tumors (Fig. 2E). PRKABI codes for the β1 subunit of AMP-activated protein kinase (AMPK), a kinase that regulates metabolic processes in response to low energy levels (21). PFKFB4 is an isomerase of PFK2 and has been originally identified in testes (22). PFK2
### Table 1. List of genes targeted by siRNA pools that induce apoptosis or reduce cell mass in at least two cancer cell lines but not in RWPE1

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>RWPE1</th>
<th>DU145</th>
<th>LNCaP</th>
<th>PC3</th>
<th>Cell mass (fold of negative control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitate hydratase 1, soluble</td>
<td>ACO1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acyl-CoA synthetase short-chain family member 2</td>
<td>ACSS2</td>
<td>-0.23</td>
<td>-0.13</td>
<td>1.57</td>
<td>0.74</td>
<td>1.67</td>
</tr>
<tr>
<td>ATPase inhibitory factor 1</td>
<td>ATPIF1</td>
<td>-0.58</td>
<td>0.09</td>
<td>-0.07</td>
<td>-0.46</td>
<td>7.61</td>
</tr>
<tr>
<td>Choline phosphotransferase 1</td>
<td>CHPT1</td>
<td>0.22</td>
<td>-0.03</td>
<td>4.19</td>
<td>6.81</td>
<td>3.38</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>CS</td>
<td>0.99</td>
<td>-0.12</td>
<td>-0.39</td>
<td>3.64</td>
<td>1.52</td>
</tr>
<tr>
<td>Glutathione peroxidase 4</td>
<td>GPX4</td>
<td>-0.23</td>
<td>8.34</td>
<td>2.09</td>
<td>-0.51</td>
<td>2.11</td>
</tr>
<tr>
<td>Similar to aconitase 2</td>
<td>LOC441996</td>
<td>1.22</td>
<td>4.46</td>
<td>1.56</td>
<td>1.70</td>
<td>6.99</td>
</tr>
<tr>
<td>Malate dehydrogenase 1, NAD (soluble)</td>
<td>MDHI</td>
<td>0.90</td>
<td>0.25</td>
<td>-0.63</td>
<td>4.15</td>
<td>2.79</td>
</tr>
<tr>
<td>Diphosphomevalonate decarboxylase</td>
<td>MVD</td>
<td>0.56</td>
<td>7.66</td>
<td>6.80</td>
<td>0.16</td>
<td>2.09</td>
</tr>
<tr>
<td>3-Oxoadic CoA transferase 2</td>
<td>OXCT2</td>
<td>0.19</td>
<td>-0.35</td>
<td>6.04</td>
<td>1.48</td>
<td>0.67</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isozyme 3</td>
<td>PDK3</td>
<td>0.55</td>
<td>0.16</td>
<td>0.87</td>
<td>3.14</td>
<td>0.83</td>
</tr>
<tr>
<td>6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4</td>
<td>PKFB4</td>
<td>1.36</td>
<td>1.48</td>
<td>2.87</td>
<td>1.09</td>
<td>2.92</td>
</tr>
<tr>
<td>AMP-activated kinase, α-2 catalytic subunit</td>
<td>PRKAA2</td>
<td>-0.60</td>
<td>2.75</td>
<td>1.35</td>
<td>13.44</td>
<td>4.61</td>
</tr>
<tr>
<td>AMP-activated kinase, β-1 noncatalytic subunit</td>
<td>PRKAB1</td>
<td>0.65</td>
<td>12.98</td>
<td>6.57</td>
<td>16.89</td>
<td>3.96</td>
</tr>
<tr>
<td>Monocarboxylic acid transporter 4</td>
<td>SLC16A3</td>
<td>2.06</td>
<td>7.10</td>
<td>5.49</td>
<td>1.63</td>
<td>2.99</td>
</tr>
<tr>
<td>Mitochondrial carrier; dicarboxylate transporter</td>
<td>SLC25A10</td>
<td>0.83</td>
<td>1.51</td>
<td>1.55</td>
<td>3.47</td>
<td>3.79</td>
</tr>
<tr>
<td>Carbohydrate response element binding protein</td>
<td>WBSCR14</td>
<td>-0.43</td>
<td>0.39</td>
<td>-0.17</td>
<td>1.27</td>
<td>2.72</td>
</tr>
</tbody>
</table>

NOTE: For each gene, the effect of gene silencing on apoptosis (z-score) and cell mass (fold of negative control) is indicated. Results that passed the filtering criteria used for hit identification (apoptosis z-scores > 2.5 or cell mass < 0.6) are marked in bold.
Figure 1. Metabolic profiling and characterization of reliance on de novo lipid synthesis of prostate cell lines. **A**, glucose uptake (left) and lactate (right) secretion in 3 prostate cancer cell lines (DU145, LNCaP, and PC3) and a nonmalignant prostate epithelial cell line (RWPE1). **B**, OCR of cells in the presence or absence of either 10 μM FCCP (gold bars) or 0.2 μg/mL oligomycin (purple bars). Total protein concentration was used for normalization. **C**, de novo pyruvate-dependent lipid synthesis rates of cells incubated in FM or LDM. **D**, glucose and glutamine dependency of cells incubated in FM or LDM. Induction of apoptosis was determined 72 hours after incubation in the indicated medium (CM, cell mass). **E**, cell mass assay after 72 hours of treatment with increasing concentrations of the fatty acid synthase inhibitor C75 (left) or the ATP-citrate lyase inhibitor SB204990 (right) in FM or LDM. The significance of the difference between FM and LDM was tested for the 2 greatest concentrations of each inhibitor. All graphs show mean and SD of 3 independent replicates (*P ≤ 0.05; **P ≤ 0.005, Student t test).
Figure 2. Identification of metabolic enzymes required for prostate cancer cell survival. A, functional viability profiling of prostate epithelial cells. Prostate cell lines were transfected with Dharmacon SMARTpool siRNAs targeting 222 different metabolic genes in either FM (KGM for RWPE1, FM or LDM for cancer cell lines). Loss of viability was determined by measuring caspase activity and cell mass. The dendrogram shows a 2-way clustering of apoptosis z-scores for the different cell lines and medium conditions. B, genes for which silencing caused a significant induction in apoptosis or a loss of cell mass in at least 2 prostate cancer cell lines but has no effect in RWPE1 were used to perform a gene set--enrichment analysis (i.e., GSEA) using published datasets from human prostate cancer (GSE3325 and GSE6752). P-values represent the enrichment score of all probe sets representing these genes and indicate increased expression in samples from metastatic disease compared to primary site tumors. C, Venn diagrams showing the number of siRNA sequences that induce apoptosis in each cancer cell line in FM or LDM (z-score $\geq 2.5$) but have no effect in RWPE1. D, Venn diagram showing the number of siRNA sequences that caused a reduction in cell mass in FM or LDM (cell mass $\leq 0.6$ relative to siCtrl) but have no effect in RWPE1. E, P values and fold-change showing increased expression of PRKAB1 and PFKFB4 in metastatic prostate cancer compared with primary site tumors. Data are publicly available in Oncomine, and references are provided in the Supplementary Methods.
is a bifunctional enzyme that regulates the levels of fructose-2,6-bisphosphate (Fru-2,6-BP), a strong allosteric activator of the most important regulatory enzyme of glycolysis phosphofructokinase 1 (PFK1).

We confirmed that the efficiency of mRNA depletion correlated with the biological effect for each individual siRNA sequence targeting these 2 genes (Supplementary Fig. S2C and S2D) and that silencing of either gene had no effect in nonmalignant RWPE1 cells despite efficient depletion of the respective mRNA (Supplementary Fig. S2E). Because RWPE1 cells are grown in the presence of supplements, including epidermal growth factor, we investigated whether the ability to tolerate depletion of PRKAB1 or PFKFB4 was determined by the growth medium. DU145 cells did not tolerate culture in KGM (data not shown). However, both LNCaP and PC3 cells were still sensitive to PRKAB1 and PFKFB4 depletion under the medium conditions used to grow the nonmalignant RWPE1 cell line (Supplementary Fig. S2F).

Dependency on PRKAB1 and PFKFB4 for Cancer Cell Survival Is Isoform Specific and Not Restricted to Prostate Cancer Cells

We analyzed whether the requirement for PRKAB1 in prostate cancer cell survival was specific to this isoform. Transfection with siRNAs targeting PRKAB2, the second isoform of the AMPK β subunit, did not induce apoptosis in any of the prostate cancer cell lines (Fig. 3A), although both isoforms PRKAB1 and PRKAB2 were efficiently depleted (Fig. 3B). In contrast, silencing of PRKAB1 was detrimental to the 3 prostate cancer cells but had no effect in RWPE1 cells.

There are 4 genes that code for different PFK2 isoforms (PFKFB1-4), and these isoforms differ in their tissue-specific expression as well as their kinetic properties (23). One of the splice variants of the PFKFB3 isoform is induced by mitogens and hypoxia and is overexpressed in tumors (24, 25) and is required for Ras-induced tumorigenesis (26). Expression of PFKFB4 is induced in response to hypoxia (27) and in some types of cancer (28–30). We compared the effects of silencing of PFKFB3 and PFKFB4 on prostate cancer cell survival in LDM. Depletion of PFKFB4 selectively induced apoptosis in the 3 cancer cell lines but not in RWPE1 (Fig. 3C). In contrast, silencing of PFKFB3 did not impair survival of any of the prostate cell lines, although both isoforms were efficiently depleted (Fig. 3D–F).

We next investigated whether the requirement of PRKAB1 and PFKFB4 for cancer cell survival is specific to prostate cancer cells. PRKAB1 silencing induced apoptosis in a variety of cancer cell lines from different tissues in both FM and LDM (Fig. 3G). Silencing of PFKFB4 reduced the viability of the majority of the cell lines cultured in LDM, whereas the effect in FM was observed in a smaller number of cell lines. Sensitivity to silencing did not correlate with the level of expression across the different cell lines (Supplementary Fig. S3). Depletion of PRKAB2 or PFKFB3 had only minor effects, indicating that the specific dependency on PRKAB1 and PFKFB4 extends to cancer cell lines from a variety of tissues.

PFKFB4 Is Required for Tumor Growth

An important aspect of the metabolic adaptations of cancer cells is to support the growth and survival under the conditions encountered by a growing tumor in vivo. We therefore studied whether depletion of PRKAB1 or PFKFB4 affects the ability of prostate cancer cells to form tumors in nude mice. For this purpose, we generated lentiviral inducible expression constructs encoding short hairpin RNAs (shRNA) targeting the expression of PRKAB1 and PFKFB4 (TetOnPLKO).

Transduction of PC3 cells with these constructs resulted in efficient depletion of mRNA and protein for both genes after treatment with doxycycline (Fig. 4A and B) and led to a significant reduction in cell viability (Fig. 4C and D).

To detect live tumor cells in vivo, PC3 cells expressing luciferase (PC3luc) were generated and transduced with lentiviruses coding for inducible shRNA sequences. These cells were then injected subcutaneously into the dorsal flank of immunocompromised mice (nu/nu). At 12 days after injection, animals with similar tumor burden (as detected by luminescence analysis) were divided into 2 groups, and one group was treated with doxycycline. Results show a moderate reduction in tumor volume in doxycycline-treated animals injected with PC3luc-TetOnPLKO-shPRKAB1 cells compared with the untreated control group (PC3luc-TetOnPLKO-shB1 #70; Fig. 4E). However, we did not observe significant differences in bioluminescence intensity in the doxycycline-treated mice compared with untreated controls (Supplementary Fig. S4A).

Histologic analysis of the tumors showed a small increase in cells with apoptotic morphology compared with the untreated controls (Fig. 4I). When we investigated the efficiency of gene depletion, we observed a 50% to 60% reduction in PRKAB1 mRNA levels after treatment with doxycycline (Fig. 4F) but failed to find a correlation between tumor size and PRKAB1 mRNA expression (Supplementary Fig. S4D). Although we do not rule out the possibility that the level of PRKAB1 gene depletion was not sufficient to prevent tumor growth, it is also possible that PC3 cells can tolerate the depletion of this gene under the physiological metabolite levels provided by the in vivo situation.

In contrast, treatment with doxycycline prevented the growth of PC3 cells expressing an inducible shRNA targeting PFKFB4 (PC3luc-TetOnPLKO-shB4 #68; Fig. 4G). In vivo detection of bioluminescence also confirmed a dramatic loss of viable tumor cells after treatment with doxycycline (Supplementary Fig. S4B), suggesting that ablation of PFKFB4 mRNA not only prevents tumor formation but also induces tumor regression. Notably, we were only able to recover one small tumor from the treated cohort. Histologic analysis of this tumor showed an increase in cells with apoptotic morphology compared with the untreated controls (Fig. 4J).

Experiments in which we used PC3 cells that expressed a second, less-efficient, shRNA sequence targeting PFKFB4 (PC3luc-TetOnPLKO-shB4 #64; Fig. 4H) also showed a significant reduction in tumor volume and in viable tumor cells upon treatment with doxycycline (Supplementary Fig. S4C). When we investigated the efficiency of gene depletion, we observed a 50% to 60% reduction in PFKFB4 mRNA levels after treatment with doxycycline (Fig. 4I). Remarkably, we observed a positive correlation between tumor growth and PFKFB4 mRNA expression (Supplementary Fig. S4E). Taken together, these results demonstrate that PFKFB4 is required to support the survival of prostate cancer cells both in vitro and in vivo.
Figure 3. Dependency on PRKAB1 and PFKFB4 for cancer cell survival is isoform specific and not restricted to prostate cancer. 

A, cells were transfected with pooled siRNAs targeting PRKAB1 or PRKAB2, and induction of apoptosis was measured after 96 hours of culture in either KGM (RWPE1) or LDM. PLK1 silencing was used as positive control.

B, lysates from cells transfected as in A were analyzed for expression of PRKAB1 and PRKAB2.

C, cells were transfected with pooled siRNAs targeting PFKFB3 or PFKFB4, and induction of apoptosis was measured after 96 hours culture in either KGM (RWPE1) or LDM. PLK1 silencing was used as positive control.

D, efficiency of PFKFB3 mRNA depletion by siRNA transfection.

E, efficiency of PFKFB4 mRNA depletion by siRNA transfection.

F, lysates from cells transfected as in C were analyzed for expression of PFKFB4.

G, cancer cell lines from different tissues were transfected with siRNAs targeting PRKAB1, PRKAB2, PFKFB3, or PFKFB4. Caspase activity normalized to cell mass is displayed as fold change over siCtrl in FM or LDM. PLK1 and UBB1 were used as positive controls. All graphs show mean and SD of 3 independent replicates (*P < 0.05; **P < 0.005, Student t test).
PFKFB4 Regulates the Levels of the Allosteric Activator Fru-2,6-BP in Prostate Cancer Cells

Our results shown so far have identified PFKFB4 as a gene selectively required to support the viability of prostate cancer cells. PFK2 isoforms play an important role in controlling the distribution of metabolites between glycolysis and the pentose phosphate pathway (Fig. 5A). The metabolic properties of the different PFK2 isoforms and their effect on prostate cell survival are summarized in Fig. 5B. To investigate the role of PFKFB4 in prostate cancer metabolism and survival, we first analyzed expression of PFKFB3 and PFKFB4 in the different prostate cell lines. Expression of PFKFB3 was greatest in DU145 cells, but LNCaP and PC3 showed similar levels of PFKFB3 expression compared with the noncancer cell line RWPE1 (Fig. 5C). In contrast, PFKFB4 expression was consistently greater in the 3 cancer cell lines in FM and was further increased after incubation with LDM (Fig. 5C).

PFK2 regulates the steady-state concentration of Fru-2,6-BP. Although the PFKFB3 isoform has a greater kinase activity and strongly favors the formation of Fru-2,6-BP, resulting in enhanced glycolytic rate (31), PFKFB4 is more balanced, with the phosphatase activity being slightly stronger [32 (Fig. 5B)]. We investigated whether levels of this metabolite are affected by silencing of PFKFB3 or PFKFB4 in the 4 prostate cell lines. Silencing of PFKFB3 caused a substantial reduction in Fru-2,6-BP levels in RWPE1 cells and a decrease in their glycolytic rate (Supplementary Fig. S5A). However, silencing of PFKFB3 did not affect the concentration of this metabolite in any of the prostate cancer cell lines (Fig. 5D). Depletion of PFKFB4 caused a significant increase in Fru-2,6-BP levels in each of the 3 prostate cancer cells but only had a minor effect in RWPE1 cells (Fig. 5D). The small reduction in Fru-2,6-BP levels after PFKFB4 depletion in RWPE1 cells could be caused by compensation by other PFK2 isoforms or caused by differential regulation of the relative kinase/phosphatase activity in these cells. In addition, the fold induction of apoptosis after silencing of PFKFB4 was decreased in glucose-depleted medium (Supplementary Fig. S5B).

These results show differences in the control of Fru-2,6-BP levels between RWPE1 and the glucose-dependent prostate cancer cells and suggest that PFKFB4 supports their viability by preventing the accumulation of this important allosteric regulator.
**PFKFB4 Maintains Cellular Redox Balance in Prostate Cancer Cells**

Accumulation of Fru-2,6-BP could lead to decreased availability of metabolic intermediates for the pentose phosphate pathway (Fig. 5A). One of the main functions of the oxidative arm of the pentose phosphate pathway is the generation of NADPH for biosynthetic processes and for the maintenance of cellular redox balance. We found that silencing of PFKFB4 leads to a decrease in the concentration of several pentose phosphate pathway metabolites (Supplementary Fig. SSC) and therefore investigated whether ablation of PFKFB4 affects redox balance in prostate cancer cells.

Silencing of PFKFB4 in DU145 cells resulted in a significant decrease in the levels of NADPH (Fig. 6A). NADPH is required to maintain cellular stores of reduced glutathione (GSH), an important antioxidant that prevents the accumulation of reactive oxygen species (ROS). PFKFB4 silencing caused a reduction in the GSH/oxidized glutathione (GSSG) ratio in DU145 cells (Fig. 6B). NADPH is also important to provide the reducing power for anabolic reactions, such as the de novo synthesis of fatty acids. Indeed, silencing of PFKFB4 in LDM caused a significant reduction in lipid synthesis in the 3 prostate cancer cell lines (Fig. 6C).

We also investigated the effect of PFKFB4 depletion on ROS accumulation. PFKFB4 silencing had no effect on ROS levels in RWPE1 cells (Fig. 6D). In contrast, we observed a strong increase in ROS levels upon PFKFB4 depletion in the cancer cell lines (Fig. 6E). This induction was comparable with ROS levels caused by depletion of superoxide dismutase 2 (SOD2), a major mitochondrial ROS detoxifying enzyme.

Of importance, the differential sensitivity to PFKFB4 silencing in the prostate cell lines is not caused by lower expression of the ROS-detoxifying enzymes catalase and SOD2, which are expressed at similar or higher levels compared with RWPE1 (Fig. 6F). We also investigated the expression of several genes involved in the oxidative stress response and observed greater levels of thioredoxin, an important antioxidant involved in ROS detoxification, in PFKFB4-depleted xenograft tumors (Supplementary Fig. S6). Furthermore, treatment with the chemical antioxidant 4-hydroxy-TEMPO, a SOD2 mimic, fully rescued viability after PFKFB4 silencing in the 3 prostate cancer cell lines (Fig. 6G), demonstrating that...
PFKFB4 silencing compromises viability of prostate cancer cells by decreasing their ability to manage ROS accumulation. Finally, we hypothesized that the detrimental effect of Fru-2,6-BP accumulation in response to PFKFB4 silencing could be rescued by cosilencing PFKFB3, the PFK2 isoform that strongly favors formation of Fru-2,6-BP. Indeed, cosilencing of PFKFB3 significantly reduced apoptosis after PFKFB4 depletion in DU145 and PC3 cells (Fig. 6H) and blocked the induction of ROS levels (Fig. 6I), while not affecting PFKFB4 silencing efficiency (Supplementary Fig. S5E). This finding strongly suggests that PFK2 activity is crucial to control the balance between glycolytic flux and redox regulation by the pentose phosphate pathway.

Taken together, these results implicate PFKFB4 as an important regulator of prostate cancer cell survival. Our results support a model in which PFKFB4 is required to maintain the balance between energy production and cellular redox control (Fig. 6J).

**DISCUSSION**

In this study, we have applied a functional genetic approach to investigate metabolic adaptations that differentiate prostate cancer cell lines from nonmalignant prostate epithelial cells. This strategy proved to be well-suited for the identification of metabolic processes that are essential for prostate cancer cell survival. Overall, the overlap between the 4 cell lines used in this study was relatively small. This finding is most likely attributable to the diversity of the cell lines used. The 3 prostate cancer cell lines differ in the mutation...
**Figure 6.** PFKFB4 is required to maintain NADPH levels and cellular redox balance in prostate cancer cells. **A,** DU145 cells were transfected with siRNA targeting PFKFB4 and grown in LDM for 60 hours. NADPH levels were determined. Graphs show means and SEM of 8 independent experiments. **B,** DU145 cells were transfected with siRNAs and grown in LDM for 60 hours. Intracellular levels of reduced GSH and GSSG were determined. Graphs show means and SD of 2 independent experiments, each performed in triplicate. **C,** lipid synthesis rate was determined in cells transfected as indicated in FM or LDM. Graphs show means and SD of 3 independent experiments. **D,** lipid synthesis rate was determined in cells transfected as indicated in FM or LDM. Graphs show means and SD of 3 independent experiments. **E,** ROS levels in DU145, LNCaP, and PC3 cells incubated in LDM after depletion of PFKFB4 as in panel E. Graphs show means of 2 independent experiments. **F,** prostate cells were grown in FM or LDM for 24 hours. SOD2 and catalase (CAT) mRNA levels were determined by quantitative real-time PCR. Graphs show means and SEM of 3 independent experiments. **G,** prostate cancer cells were depleted of PFKFB4 and treated with the indicated concentrations of 4-hydroxy-TEMPO (TEMPOL). Induction of apoptosis was measured after 96 hours of culture in LDM. Graphs show means and SEM of 3 independent experiments. **H,** cells were transfected as indicated and induction of apoptosis was measured after 96 hours of culture in LDM. PLK1 silencing was used as positive control. Graphs show the means and SEM of 3 independent experiments. **I,** ROS levels in cells treated as in panel H. Graphs show the means and SEM of 3 independent experiments. **J,** schematic model shows the metabolic effects of PFKFB4 silencing in prostate cancer cells. In all cases, P values were determined by use of the Student t test; *P ≤ 0.05; **P ≤ 0.005.
status of cancer genes and have been isolated from metastases in different organs. Notably, LNCaP is the only androgen-dependent cell line in this study. Despite this diversity, the effect of siRNA mediated gene silencing of the panel of genes used in this study clearly separated the cancer cell lines from the nonmalignant line, indicating that the different cancer cell lines share common metabolic features.

We only found 2 genes that showed similar effects in all cancer cell lines. This may be attributable to the stringent selection criteria but could also indicate redundancy between different genes within each pathway. Interestingly, both of these genes, PRKAB1 and PFKFB4, are expressed at greater levels in metastatic prostate cancer compared with primary site tumors in public data from human prostate cancer.

PRKAB1 codes for one of the isoforms of the β subunits of AMPK. AMPK is generally considered to be part of a tumor suppressor pathway (21). Loss of an AMPK upstream kinase LKB1 decreases the latency of prostate lesions in PTEN+/− mice (35). However, certain cancers may depend on AMPK to downregulate energy-consuming processes under conditions of nutrient starvation. Despite the strong effect of PRKAB1 silencing on cancer cell survival in vitro, depletion of this gene only had a minor effect on tumor growth in vivo, suggesting that PRKAB1 could be dispensable under conditions encountered by prostate cancer cells in vivo.

The second gene identified in our study was PFKFB4, an isoform of PFK2 (22). We found that silencing of PFKFB4 not only selectively induced apoptosis in prostate cancer cells but also completely blocked tumor growth in vivo. Expression of PFKFB4 has been shown to be increased in breast, colon, gastric, and prostate cancer (28, 30). Our analysis of public microarray data showed that PFKFB4 expression is greater in metastatic prostate cancer compared with primary site tumors. Indeed, the 3 prostate cancer cell lines used in this study, which originate from metastases, showed increased expression of PFKFB4 compared with nonmalignant RWPE1 cells.

Although the core structure is highly conserved between the different PFK2 isoforms, the relative kinase and phosphatase activity of these bifunctional enzymes shows large variation [Fig. 5B (23)], and all PFK2 isoforms can be subject to posttranslational modifications, which can also modulate the relative activities of their catalytic domains (36). We found that silencing of PFKFB4 increased levels of Fru-2,6-BP levels selectively in the prostate cancer cells, suggesting that it was mainly functioning as a fructose-2,6-bisphosphatase. This increase in Fru-2,6-BP, an allosteric activator of the master regulator of glycolysis PFK1, should divert glucose 6-phosphate towards the glycolytic pathway, thereby depleting the pentose phosphate pathway. This would explain why prostate cancer cells showed lower NADPH and reduced glutathione levels after PFKFB4 silencing, resulting in enhanced oxidative stress and cell death (see model in Fig. 6).

A similar role in the regulation of cellular ROS levels has been demonstrated for TIGAR, a p53-inducible protein with fructose-2,6-bisphosphatase activity similar to PFK2 (37). Furthermore, degradation of PFKFB3 by the action of the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C)-Cdh1 has been shown to promote antioxidant formation in neurons by enhancing the activity of the pentose phosphate pathway (38). Interestingly, PFKFB3 expression can be induced by ROS after glucose starvation in an AMPK-dependent manner (39), indicating that this enzyme can be both upstream and downstream of oxidative stress.

Induction of lipid biosynthesis increases cellular demand for NADPH and the dependency of prostate cancer cells on PFKFB4 was particularly pronounced under conditions of lipoprotein starvation in vitro. The increased biosynthetic demand of cancer cells renders them highly dependent on NADPH generation by the pentose phosphate pathway and the importance of ROS detoxification for tumorigenesis has been demonstrated recently (40). The dramatic effect of PFKFB4 depletion on tumor growth in vivo suggests that the balance between glycolysis and pentose phosphate pathway is crucial to support tumor cells under physiologic conditions. Furthermore, depletion of PFKFB4 prevented the induction of lipid synthesis, required for growth of cancer cells, possibly by reducing NADPH availability.

Other enzymes have been shown to play a role in preventing oxidative stress by balancing glycolytic and pentose phosphate pathway flux. Depletion of TIGAR inhibits the pentose phosphate pathway and induces oxidative stress and autophagy in cancer cells under metabolic stress (37, 41). In contrast, reduction of glycolytic flux by inhibition of PKM2 is required to enhance antioxidant production by increasing pentose phosphate pathway activity in response to oxidative stress (42).

In summary, this study demonstrates the usefulness of functional screens to identify metabolic weaknesses in cancer cells and highlights the importance of metabolic regulation to maintain the balance between bioenergetics and antioxidant production for cancer cell survival. Targeting this balance may also provide novel strategies for cancer therapy.

**METHODS**

**Cell Culture and Reagents**

RWPE1 and LNCaP (clone FGC) were obtained from the American Type Culture Collection. All other cell lines were from the LRI Cell Services. RWPE1 cells were grown in Keratinocyte serum-free medium (Gibco) supplemented with epidermal growth factor and bovine pituitary extract (KGM). All other cell lines were grown in RPMI, DMEM, or DMEM/F12 supplemented with 10% fetal calf serum (FM; PAA Laboratories) or 1% fetal bovine lipoprotein-deficient serum (LDM; Intracel), glutamine, and penicillin/streptomycin. A full description of the additional cell lines used, where they were obtained from, and the method and date of cell line authentication is detailed in the Supplementary Methods.

Polyclonal PRKAB1 (A4856) and monoclonal PRKAB2 (clone 2G9) antibodies were from Sigma-Aldrich. Antibodies for PFKFB4 were from Abgent (AP8154C). All other primary antibodies were from Cell Signaling. Horseradish peroxidase-conjugated anti-GAPDH and anti-β-actin antibodies were from Abcam and Sigma-Aldrich, respectively. ATP-citrate lyase inhibitor SB204990 was provided by GlaxoSmithKline (Stevenage). All other reagents were from Sigma.

**Glucose Uptake, Lactate Secretion, and Oxygen Consumption**

Glucose and lactate concentrations in media incubated with or without cells were determined by the use of glucose and lactate assay kits from BioVision. Cell mass was used for normalization. Oxygen consumption was measured with the BD Oxygen Biosensor Systems (OBS) from BD Bioscience with 1 × 10⁶ cells in 200 μL of medium in the presence or absence of 10 μM FCCP or 0.2 μg/mL oligomycin. Oxygen consumption rate was normalized to protein concentration.
**Lipid Synthesis**

Cells were incubated in medium containing 2.5 μCi/mL [2-14C]-pyruvate (166 μM final concentration) or 10 μCi/mL [1-13C] acetate (85 μM final concentration, Perkin Elmer) for 4 hours. After washing 3 times in PBS, cells were lysed in 0.5% Triton X-100. Lipids were extracted by successive addition of 2 mL of methanol, 2 mL of chloroform, and 1 mL of H2O. Phases were separated by centrifugation before the organic phase was dried and used for scintillation counting. Results were normalized to cell mass.

**Metabolite Extraction and Analysis by Mass Spectrometry**

Cells were grown in 6-well plates, washed with warm PBS, and frozen in liquid nitrogen. Intracellular metabolites were extracted by addition of a prewarmed 70% (v/v) ethanol aqueous at 75°C. 13C-labeled biomass was added to serve as internal standard for metabolite analysis. The cell mixture was centrifuged, the supernatant collected, and dried. Metabolites were dissolved in ultrapure water. For the analysis of extracellular metabolites, the medium was rapidly collected and frozen in liquid nitrogen. Internal standards (norvaline and glutaric acid) were added before drying.

Intracellular metabolites were analyzed by ion-pairing ultrahigh-performance liquid chromatography-tandem mass spectrometry (43). Extracellular metabolites were analyzed by gas chromatography-time of flight-mass spectrometry after off-line methoxyamination and just-in-time N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide derivatization. Metabolite quantification for both intracellular and extracellular metabolites was obtained by means of calibration curves of a mixture of standard compounds (44). Metabolite data were analyzed by an in-house MATLAB 2010 (Mathworks) application for peak integration, internal standard correction, and absolute quantification of metabolites. Absolute amounts were normalized to cell number or cell mass in silencing experiments.

**siRNA Screen and Data Analysis**

Cells were reverse-transfected with 25 nM of Dharmacon SMARTPools in 96-well plates using Dharmafect 1 reagent. After 24 hours, culture medium was replaced with fresh medium. Then, 72 hours later, caspase 3/7 activity was measured with a fluorescent substrate (Invitrogen). Cells were then fixed with trichloroacetic acid and stained with Sulforhodamine B assay (Sigma-Aldrich) to determine total protein content (cell mass). Two independent screens were performed, each with triplicate transfections.

Caspase 3/7 activity was normalized to cell mass. Normalized caspase activity and cell mass values were then normalized to the median value of all samples within each plate (plate centering), and the average of the 6 replicates was calculated. Histograms determined by normalized caspase activity and cell mass data are shown in Supplementary Figure S2B. Z-scores were calculated from normalized caspase activity values using the median absolute deviation (45) as recommended for small-scale screens (46). Z-scores are provided in Supplementary Table S2. Cell mass results were expressed relative to the average of the negative controls and are provided in Supplementary Table S2. A 2-way cluster analysis was generated using Pearson correlation (centered) and average linkage clustering with Cluster 3.0 and Java TreeView (1.1.3).

Gene expression data were downloaded from Gene Expression Omnibus and analyzed using Gene Set Enrichment Analysis (see Supplementary Methods). Normalized expression data and P values for PRKAB1 and PFKFB4 were downloaded from Oncomine (Compendia Bioscience). Citations for individual studies are included in the Supplementary Methods.

**siRNA Screen Validation**

Cells were transfected with the original SMARTPool or the 4 individual siRNA oligonucleotides. siGENOME nontargeting controls 1–4, RISC-free siRNA, and a SMARTPool targeting PLK1 were used as negative and positive controls, respectively. A gene was considered validated if at least 2 individual siRNA reduced cell mass to less than 70% of the average of the negative controls or increased normalized caspase activity more than 1.8-fold.

**Generation of Doxycycline-Inducible shRNA Cell Lines**

shRNA sequences targeting PFKFB4 or PRKAB1 were cloned into the TetOnPLKO lentiviral vector (Addgene; refs. 47 and 48). Lentiviruses were produced by cotransfecting HEK 293T with the shRNA plasmid and the packaging plasmids pCMVΔR8.91 (gag-pol) and pMD.G [VSV-G glycoprotein (49)]. Supernatants containing lentiviruses were collected 72 hours after transfection, mixed with polybrene (8 μg/mL), and used to infect PC3 cells. Fresh medium-containing puromycin (3 μg/mL) was added after 24 hours, and cells were selected for at least 48 hours before being used in experiments.

**Xenograft Experiments**

PC3 cells stably expressing the ecotropic retrovirus receptor were generated by transfection with the pWZLneoEcoR vector. To generate PC3 expressing luciferase cells, the retroviral pBabe-Luciferase vector (a generous gift from M. Murillo, LRI) was packaged in Phoenix cells and used to infect the PC3 EcoR-neo cells. PC3neo cells were then infected with lentiviral vectors and selected as described previously.

Nude mice (nu/nu) were injected subcutaneously with 10⁶ PC3neo-TetOnPLKO-PRKAB1 cells (sequence 70) or PC3neo-TetOnPLKO-PFKFB4 (sequences 68 and 64) cells into the dorsal flank. After 12 days, animals were subdivided into 2 experimental groups, a doxycycline-treated group and a nontreated group. Bioluminescence imaging was used to split mice with similar tumor burden equally into the different treatment groups. For induction of shRNA expression, mice were treated with doxycycline (doxycycline diet, 0.2 g/kg food pellets, TD.98186; Harlan Laboratories), and tumor growth was followed over the course of 23 days. Tumor volume was determined by use of the ellipsoidal volume formula: 1/2 × length × width². All animal experiments were performed according to U.K. Home Office guidelines.

**Determination of Fru-2,6-BP Levels**

Cells were homogenized in 0.1 M NaOH and 0.1% Triton X-100, heated to 80°C for 10 minutes, and centrifuged for 5 minutes. The supernatant was neutralized with acetic acid in 20 mM HEPES. Fru-2,6-BP was determined as previously described (50). Protein concentration was used for normalization.

**Determination of NADPH Levels and GSH/GSSG Ratio**

For detection of NADPH, cells were lysed in buffer containing 20 mM nicotinamide, 20 mM NaHCO3, and 100 mM Na2CO3. Cleared supernatants were incubated for 30 minutes at 60°C. Then, 20 μL of supernatant was mixed with 160 μL of NADP-cycling buffer (100 mM Tris-Cl, pH 8.0; 0.5 mM thiazolyl blue; 2 mM phenazine ethosulfate; 5 mM EDTA; 1.3 IU glucose-6-phosphate dehydrogenase). After a 1-minute incubation in the dark at 30°C, 1 mM glucose-6-phosphate was added, and the change in absorbance at 570 nm was measured every 30 seconds for 4 minutes at 30°C. Protein concentration was used for normalization. The GSH/GSSG ratio was determined with a kit (Biovision).

**Determination of ROS Levels**

ROS levels were determined by incubating cells for 30 minutes at 37°C in medium with 3 μM CM-H2DCFDA (Molecular Probes) followed by fluorescence-activated cell-sorting analysis. 4′,6-Diamidino 2-phenylindole (i.e., DAPI) was added before analysis to exclude dead cells.
Disclosure of Potential Conflicts of Interest

A. Schulze provides minor consultancy work for Astra Zeneca.

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Functional Metabolic Screen Identifies 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4 as an Important Regulator of Prostate Cancer Cell Survival

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