SUPPLEMENTAL INFORMATION (MS CD-11-0234)

Functional metabolic screen identifies 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4) as an important regulator of prostate cancer cell survival

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SUPPLEMENTARY FIGURES

Figure S1. Characterization of prostate cell lines (related to Figure 1)

A) Proliferation rate of prostate cancer cell lines grown in full medium (FM, DU145, LNCAP and PC3) or KGM medium supplemented with EGF and bovine pituitary extract (RWPE1) was measured by determining changes in cell mass. **B)** Proliferation rate of prostate cancer cell lines grown in lipoprotein deficient medium (LDM) was calculated as in A. **C)** Metabolomic analysis of prostate epithelial cells. The heat map shows differences in metabolite concentrations between prostate epithelial cells (RWPE1) and prostate cancer cells grown in FM or LDM. Colors indicate the metabolic pathway associated with each metabolite. Metabolite concentrations are provided in Supplementary Table 1. **D)** Cells were incubated in FM, LDM or KGM (RWPE1 only). After 1, 2 and 3 days, medium samples were collected and cell number was determined. Concentrations of the indicated metabolites were determined by GC-MS and used to determine the rates of metabolite uptake (decrease in medium concentration) or secretion (increase) relative to cell number by linear regression. All measurements were performed in three independent samples.

Figure S2. Histograms of population distribution and number of hits in siRNA screen targeting 222 metabolic genes and correlation between the phenotype and silencing efficiency of siRNAs and sensitivity of LNCaP and PC3 cells to PFKFB4 silencing in KGM medium (related to Figure 2)

A) Table listing the number of enzymes within each metabolic process that were targeted in the screen. **B)** Histogram plots of the effect of the 222 SMARTpools on caspase activity and cell mass relative to the median of the population. Green and red bars indicate the average value of negative and positive controls, respectively. **C-D)** DU145 cells were transfected with pooled and individual siRNA oligonucleotides targeting PRKAB1 (C) and PFKFB4 (D) or an unspecific control (siCtrl). Induction of caspase activity relative to cell mass (black bars) and loss of cell mass (grey bars) were determined 96 hours post transfection. The graph shows means and SD of triplicates expressed relative to siCtrl. Cells transfected in parallel were harvested 48 hours post-transfection and expression of the respective gene relative to β-actin was determined by qPCR (white bars). The graphs show mean and SD of duplicates expressed relative to siCtrl. Arrows indicate siRNA sequences used in subsequent experiments. **E)** RWPE1 cells were transfected with the originally used SMARTpools targeting PRKAB1 and PFKFB4, siRNAs targeting PLK1 and UBB were used as positive controls. Induction of caspase activity relative to cell mass (black bars) and loss of cell mass (grey bars) were determined 96 hours post transfection. The graphs show mean and SD relative to siCtrl of a representative experiment performed in triplicate. Cells transfected in parallel were harvested 48 hours post-transfection and expression of the respective gene relative to β-actin was determined by qPCR (white bars). The graph shows means and SD of duplicates expressed relative to siCtrl. **F)**

LNCaP and PC3 cells were transfected with the indicated siRNA pools. 24 hours later medium was changed to either LDM or KGM. Induction of caspase activity relative to cell mass was determined 96 hours post transfection. Graphs show means and SD of an experiment performed in triplicate.

Figure S3. PRKAB1 and PFKFB4 expression in a panel of cancer cell lines (related to Figure 3)

Cancer cell lines from different tissues were incubated in FM or LDM for 96 hours. Expression of PRKAB1 (above) or PFKFB4 (below) relative to β-actin was determined by qPCR. The graphs show mean and SD of triplicate measurements relative to the mean across the panel.

Figure S4. In vivo imaging of tumor growth (related to Figure 4)

Nude mice (nu/nu) were implanted with $1x10^6$ $PC3_{luc}$-TetOnPLKO-shPRKAB1 #70 **(A)** $PC3_{luc}$ -TetOnPLKO-shPFKFB4 #68 **(B)** or $PC3_{luc}$-TetOnPLKO-shPFKFB4 #64 **(C)** cells. For the in vivo imaging of tumor cells, mice were anesthetized and injected with luciferin (200 µl of a 15mg/ml solution). Bioluminescence measurements at day 12 were used to divide animals into the different treatment groups. Each group was composed of mice with a comparable tumor burden. Images show representative results for individual animals of the different treatment groups. Graphs show mean bioluminescence (p/sec) and SEM for each group. **D)** Correlation analysis between PRKAB1 expression in tumors at day 35 and tumor growth following doxycycline treatment, from A. **E)** Correlation analysis between PFKFB4 expression and tumor growth following doxycycline treatment, from C. In all cases, P values were determined using the student t-test and symbols represent * $p \leq 0.05$; ** $p \leq 0.005$.

Figure S5. Glycolysis rate after PFKFB3 silencing and sensitivity to PFKFB4 silencing following glucose removal, metabolomic analysis after PFKFB4 silencing, oxaditive stress gene expression in vivo after PFKFB4 silencing and PFKFB4 expression after co-silencing of PFKFB3 and PFKFB4 (related to Figures 5 and 6)

A) Glycolytic rate after PFKFB3 silencing in RWPE1 and DU145 cells. Glycolytic flux was measured by determining the rate of release of tritium from 5-^{3}H-glucose relative to cell mass. Graphs show means and SD of an experiment performed in triplicate. **B)** Induction of caspase activity relative to cell mass was determined 96 hours following PFKFB4 depletion in DU145, LnCAP and PC3 cells in the presence or absence of glucose for 72 hours. Graphs show means and SEM of three independent experiments. C**)** Metabolomic analysis of prostate cells after PFKFB4 silencing. The heat map shows differences in concentrations of pentose phosphate pathway metabolites between prostate epithelial cells (RWPE1) and prostate cancer cells (DU145, LNCAP and PC3). **D)** mRNA gene expression of catalase (CAT), DNA-damage-inducible protein GADD153 (CHOP), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and thioredoxin (TRX) in vivo following doxycycline treatment in tumors recovered after $PC3_{luc}$-TetOnPLKO-shPFKFB4 #64 injection at day 35. **E)** PFKFB4 mRNA gene expression after co-silencing of PFKFB3 and PFKFB4 in DU145 and PC3 cells. In all cases, P values were determined using the student t-test.

SUPPLEMENTARY MATERIAL AND METHODS

Table outline cell line origin and method of authentication

Cell line	*Organ*	*ATCC*	*Morphology*	*Disease*	*Sex*
MCF7	Breast	HTB-22	Epithelial	adenocarcinoma	Female
MDA-MB-231	Breast	HTB-26	Epithelial	adenocarcinoma	Female
T-47D	Breast	HTB-133	Epithelial	invasive ductal carcinoma	Female
HCT116	Colon	CCL-247	Epithelial	colorectal carcinoma	Male
DLD1	Colon	CCL-221	Epithelial	colorectal adenocarcinoma	Male
SW620	Colon	CCL-227	Epithelial	colorectal adenocarcinoma	Male
U87	Brain	HTB-14	Epithelial	glioblastoma	Male
NCI-H23	Lung	CRL-5800	Epithelial	adenocarcinoma; non-small cell lung cancer	Male
EKVX	Lung		Epithelial	adenocarcinoma; non-small cell lung cancer	Male
NCI-H358	Lung	CRL-5807	Epithelial	bronchioalveolar carcinoma; non-small cell lung cancer	Male
NCI-H520	Lung	HTB-182	Epithelial	squamous cell carcinoma	Male
U2-OS	Bone	HTB-96	Epithelial	osteosarcoma	Female
OVCAR-4	Ovary		Epithelial	adenocarcinoma	Female
OVCAR-5	Ovary		Epithelial	adenocarcinoma	Female
SKOV-3	Ovary	HTB-77	Epithelial	adenocarcinoma	Female
BXPC3	Pancreas	CRL-1687	Epithelial	adenocarcinoma	Female
HPAF-II	Pancreas	CRL-1997	Epithelial	adenocarcinoma	Male
AGS	Stomach	CRL-1739	Epithelial	gastric adenocarcinoma	Female
MKN-45	Stomach		Epithelial	adenocarcinoma	Female
DU145	Prostate	HTB-81	Epithelial	prostate carcinoma/brain metastasis	Male
PC-3	Prostate	CRL-1435	Epithelial	prostate adenocarcinoma/bone metastasis	Male
LNCAP	Prostate	CRL-1740	Epithelial	left supraclavicular lymph node metastasis	Male
RWPE-1	Prostate	CRL-11609	Epithelial	normal	Male

Cell line	*Growth media*	*Where obtained*	*Authenti-cation method*	*Date of authentication*	*Used after authen. (frozen vial)*
MCF7	DMEM:F12+10% FBS	LRI Cell Services	STR	27/03/2011	<6 months
MDA-MB-231	DMEM:F12+10% FBS	LRI Cell Services	STR	02/09/2011	<6 months
T-47D	DMEM:F12+10% FBS	LRI Cell Services	STR	27/04/2009	<6 months
HCT116	DMEM+10%FBS	LRI Cell Services	STR	04/02/2011	<6 months
DLD1	RPMI+10%FBS	LRI Cell Services	STR	20/09/2011	<6 months
SW620	RPMI+10% FBS	LRI Cell Services	STR	19/06/2009	<6 months
U87	DMEM+10%FBS	LRI Cell Services	STR	24/04/2009	<6 months
NCI-H23	RPMI+10% FBS	LRI Cell Services	STR	20/08/2010	<6 months
EKVX	RPMI+10% FBS	LRI Cell Services	DNA FP	<6 months	<6 months
NCI-H358	RPMI+10% FBS	ATCC	ND	01/10/2008	<6 months
NCI-H520	RPMI+10% FBS	LRI Cell Services	STR	30/11/2011	<6 months
U2-OS	DMEM+10%FBS	LRI Cell Services	STR	26/05/2011	<6 months
OVCAR-4	RPMI+10% FBS	LRI Cell Services	STR	17/03/2011	<6 months
OVCAR-5	RPMI+10% FBS	LRI Cell Services	STR	26/10/2010	<6 months
SKOV-3	RPMI+10% FBS	LRI Cell Services	STR	08/12/2008	<6 months
BXPC3	RPMI+10% FBS	ATCC	STR	19/06/2009	<6 months
HPAF-II	DMEM+10%FBS	ATCC	STR	29/06/2009	<6 months
AGS	DMEM+10%FBS	LRI Cell Services	STR	19/06/2009	<6 months
MKN-45	RPMI+10% FBS	LRI Cell Services	STR	19/06/2009	<6 months
DU145	RPMI+10% FBS	LRI Cell Services	STR	09/05/2011	<6 months
PC-3	RPMI+10% FBS	LRI Cell Services	STR	25/11/2011	<6 months
LNCAP	RPMI+10% FBS	ATCC (clone FGC)	ND	15/08/2005	<6 months
RWPE-1	KGM	ATCC	ND	03/12/2007	<6 months

List of Abbreviations

DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
KGM	Keratinocyte serum-free medium (Gibco) supplemented with EGF and bovine pituitary extract (KGM)
ND	Not determined
STR	Short tandem repeat profiling
DNA FP	DNA fingerprinting

RNA extraction, reverse transcription and RT-qPCR

Cells were grown or reverse-transfected in 6-well plates using 50 nM of siRNA. 48 hours after transfection, total RNA was isolated using an RNeasy kit (Qiagen). For tissue RNA extraction from tumor material, total RNA was extracted using Trizol Reagent (GibcoBRL) following the manufacturer's protocol. 1000 ng of total RNA was used for first strand cDNA synthesis with SuperScript II Reverse Transcriptase and oligo dT primers (Invitrogen). Real time PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems) using Quantitect primers (Qiagen) in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). All reactions were performed at least in duplicate. The relative amount of all mRNAs was calculated using the comparative CT method after normalization to β-actin or β-2-microglobulin (B2M).

Cell lysis and immunoblotting

Cells were lysed in Triton lysis buffer (1% Triton X100, 50 mM Tris pH 7.5, 300 mM NaCl, 1 mM EGTA, 1mM DTT, 1 mM NaVO4, Protease-Inhibitor-Cocktail and Phosphatase-Inhibitor-Cocktail (Roche)) and snap frozen in liquid nitrogen. Proteins were separated on SDS-PAGE and blotted onto PVDF membrane (Immobilon). Membranes were blocked with 3 % BSA, incubated with antibody solutions and signals were detected by incubation with HRP-conjugated secondary antibodies followed by chemiluminescent detection using ECL-reagent and exposure to Hyperfilm-ECL (Amersham).

Gene expression analysis

Data were analyzed using the open source bioinformatics platform Bioconductor.

The raw Affymetrix .CEL files relating to the Varambally dataset were downloaded from the NCBI's Gene Expression Omnibus (GSE3325) and probeset expression measures were calculated using the Robust Multichip Average (RMA) default method from the "Affy" package. The normalized CodeLink expression data from the Chandran study were downloaded from the Gene Expression Omnibus (GSE6752). The "limma" package was used to assess differential expression between replicate groups. A Benjamini and Hochberg adjusted p-value of <0.05 was considered significant. Genes passing this threshold and showing an increase in expression in metastasis relative to primary tumours were represented in heatmaps. A directional mean-rank gene-set enrichment test (limma's geneSetTest function) was used to assess whether interesting sets of genes were associated with differential expression in the published datasets. This function computes a p-value to test the hypothesis that a selected set of genes tend to be more highly ranked in terms of their t-statistics, than those of a randomly selected set of genes after 10000 simulations.

Oncomine data were from the following studies:

(1-6)

Glycolytic rate

Glycolytic rate was determined by measuring the conversion of 5-^{3}H-glucose to ^3H_2O. Cells were incubated with 10 μCi of 5-^{3}H-glucose (Perkin-Elmer Life Sciences) at 37°C for 1 h. After incubation, the reaction was stopped with 0.2 N HCl, and ^3H_2O was separated from 5-^{3}H-glucose by diffusion in an airtight container. Diffused and undiffused counts were measured using a scintillation counter and compared with controls of 5-^{3}H-glucose and ^3H_2O alone. Results were normalized to cell mass.

In vivo imaging

Quantification of tumor growth was performed by bioluminescent imaging (IVIS Spectrum Bioluminiscence) and Living Image 4.1 (Caliper Lifesciences). For the bioluminescence imaging, mice were anesthetized and imaged at approximately 10-15 minutes after luciferin injection (SC, 200 µl of 15mg/ml solution), which assures a linear range for bioluminescence.

TetOnPLKO lentiviral vectors

The following sequences were used to generate TetOnPLKO lentiviral vectors.

shPFKFB4 #64:

forward

5'CCGGGCCAACATCGTGCAAGTGAAACTCGAGTTTCACTTGCACGATGTTGGCTTTTTG

reverse

5'AATTCAAAAAGCCAACATCGTGCAAGTGAAACTCGAGTTTCACTTGCACGATGTTGGC,

shPFKFB4 #68:

forward

5'CCGGGACGTGGTCAAGACCTACAAACTCGAGTTTGTAGGTCTTGACCACGTCTTTTTG

reverse

5'AATTCAAAAAGACGTGGTCAAGACCTACAAACTCGAGTTTGTAGGTCTTGACCACGTC

shPRKAB1 #70

forward

5'CCGGGCCTGGCTATGGAACTAAATACTCGAGTATTTAGTTCCATAGCCAGGCTTTTT

reverse

5’AATTAAAAAGCCTGGCTATGGAACTAAATACTCGAGTATTTAGTTCCAT
AGCCAGGC.

shPRKAB1 #71

forward
5’CCGGCCTCACCAGAAGCCACAATAACTCGAGTTATTGTGGCTTCTGGTG
AGGTTTTT

reverse
5’
AATTAAAAACCTCACCAGAAGCCACAATAACTCGAGTTATTGTGGCTTCT
GGTGAGG

shCtrl

forward

5’CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTT
AGG

reverse

5’AATTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTT
AGG.

SUPPLEMENTARY DISCUSSION

Selection of genes for a targeted siRNA screen

The main metabolic features of tumor cells known to date are increased glucose and glutamine uptake and metabolism and the use of the products of their metabolism for the synthesis of lipids, amino acids and nucleotides required for cell growth and proliferation (reviewed in (7)).

In order to investigate the importance of the enzymes involved in these processes for the proliferation and survival of prostate cancer cell lines we obtained an siRNA library consisting of Dharmacon SMARTpools targeting all glycolysis, TCA cycle, pentose phosphate pathway and glutamine metabolism enzymes (Fig. S2A). The library also targets the enzymes involved in shuttles and anapleurotic reactions required for maintaining glycolysis and the TCA cycle running, as well as the enzymes that catalyze the initial steps in several biosynthesis pathways that start from glycolysis and TCA cycle metabolites. Since it has been suggested that both fatty acid and cholesterol synthesis pathways are deregulated in prostate cancer, the targeted library targets a large proportion of the enzymes in these processes. We also included siRNAs targeting enzymes within the ROS metabolism and hexosamine pathways as well as several metabolic regulators like AKT, HIF and AMPK. We chose not to include siRNAs targeting components of the electron transport chain due to the large number of genes involved.

Discussion of primary screen results

The cancer cell lines used in this study convert a significant proportion of glucose into lactate, which ranges from 50% (LNCaP and PC3) to almost 100% (DU145), based on the fact that they secrete approximately 1 or 2 molecules of lactate, respectively, per molecule of glucose taken up (Figure 1A). Despite their high glycolytic rate, three of the four cell lines consumed detectable amounts of oxygen and showed an increase in oxygen consumption after treatment with an uncoupling agent. DU145 was the only cell line completely deficient for oxygen consumption (Figure 1B) suggesting that these cells meet most of their energetic requirements through glycolysis.

Overall, the small number of siRNAs targeting glycolytic genes that showed an effect in the cancer cell lines is rather surprising. This may be due to insufficient silencing, at least in some cases. Another possible explanation could be that most glycolytic enzymes have several isoforms and depletion of one enzyme might be compensated by another isoform.

We identified 18 siRNA pools that caused apoptosis or loss of cell mass in at least two prostate cancer cell lines but were dispensable for the non-malignant RWPE1 cells (Table 1).

One gene required for survival of DU145 and PC3 cells is SLC16A3. This gene codes for MCT4, one of four proton-linked monocarboxylate transporters that shuttle pyruvate and lactate across the plasma membrane (MCT1-4). Lactate secretion is a critical step during aerobic glycolysis to prevent accumulation of cytoplasmic lactate and to regulate intracellular pH. MCT4 expression is induced by hypoxia (8) and MCT4 has been shown to be important for the survival of glycolytic tumors (9). Due

to its kinetic properties, MCT4 is the main transporter involved in the secretion of glycolytic lactate (10). The high rate of lactate secretion in prostate cancer cell lines, particularly in DU145, could explain their dependency on this transporter for survival.

DU145 and PC3 cells were also sensitive to depletion of mevalonate decarboxylase (MVD), an enzyme in the cholesterol biosynthesis pathway. Induction of cholesterol biosynthesis genes has been associated with progression to androgen independence in prostate cancer (11) and the use of inhibitors of cholesterol biosynthesis has been discussed in the treatment of the disease (12).

The siRNA targeting OXCT2 caused a loss of cell mass in the three prostate cancer cell lines without inducing apoptosis. OXCT2 is an isoform of the enzyme 3-oxoacid CoA-transferase, which is involved in utilization of acetoacetyl generated during the oxidation of fatty acids and the degradation of valine, leucine or isoleucine.

We were able to validate the results for SLC16A3, MVD and OXCT2 using independent siRNA sequences (Table S3 and data not shown). However, in the case of SLC16A3, we did not observe a good correlation between the efficiency of silencing and the biological effect (data not shown).

We also made a number of additional interesting observations. LNCaP and PC3 cells were sensitive to silencing of different isoforms of aldolase, ALDOC and ALDOA, respectively (Tables S2) suggesting the possibility of isoform compensation. Furthermore, silencing of PFKL (an isoform of PFK1, the rate limiting enzyme in glycolysis) and phosphoglycerate kinase 1 (PGK1) induced apoptosis exclusively in

DU145, while LNCaP and PC3 cells seem to be relatively insensitive to the ablation of glycolytic genes. This is consistent with our observation that DU145 cells have the highest rate of glucose uptake and lactate secretion (Fig. 1A) and have low mitochondrial activity (Fig. 1B). LNCaP and PC3 cells were sensitive to silencing of citrate synthase (CS), a TCA cycle enzyme, as well as malate dehydrogenase 1 (MDH1) and solute carrier family 25 member 10 (SLC25A10), two genes required for the shuttling of NAD^+ across the mitochondrial membrane (Tables S2). Interestingly, both cell lines exhibit lower rates of glucose uptake and lactate secretion and retain functional mitochondria.

One group of genes that showed interesting results in the primary screen is glutathione peroxidases (GPX1-7). We found that silencing of GPX4 strongly induced apoptosis in LNCaP and PC3 cells while silencing of GPX1, GPX3, GPX5 or GPX6 reduced cell mass in either LNCaP or PC3 cells (Tables S2). In contrast, GPX7 was detrimental to all cell lines apart from LNCaP (Table S2). These results suggest that different cell lines rely on different anti-oxidant systems. However, only GPX4 passed the stringent cut-off in more than two cell lines.

Several of the enzymes targeted by our siRNA library, such as hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDHA) and glutaminase (GLS), have been implicated in cancer cell survival by previous studies. Silencing of some of these genes reduced viability in one or more prostate cell lines but none of them passed our stringent cut-off in more than one cell line (Table S2). This could indicate that there is considerable diversity in the metabolic wiring of cancer cells from different tissues.

We were also surprised by the limited effect of silencing of genes involved in fatty acid biosynthesis. Several studies have shown that tumor cells re-activate lipid biosynthesis, a process otherwise restricted to the liver and adipose tissue in adults (13). Fatty acid synthase (FASN), a key lipogenic enzyme is overexpressed in several tumor types including prostate and small molecule inhibitors targeting FASN have shown anti-tumorigenic activity (14). We observed that the three prostate cancer cell lines were relatively insensitive to silencing of lipogenic enzymes including FASN and ATP-citrate lyase (ACLY) (Tables S2). However, RWPE1 cells showed increased apoptosis following depletion of these genes. This finding is consistent with our observation that RWPE1 cells are highly sensitive to inhibition of FASN and ACLY using chemical inhibitors (Figure 1E).

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