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Stress mediated reprogramming of prostate cancer one-carbon cycle drives disease progression

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Abstract

One carbon (1C) metabolism has a key role in metabolic programming with both mitochondrial (m1C) and cytoplasmic (c1C) components. Here we show that Activating Transcription Factor 4 (ATF4) exclusively activates gene expression involved in m1C, but not c1C cycle in prostate cancer (PCa) cells. This includes activation of Methylenetetrahydrofolate dehydrogenase 2(MTHFD2) expression, the central player in the m1C cycle. Consistent with the key role of m1C cycle in PCa, MTHFD2 knockdown inhibited PCa cell growth, prostatosphere formation and growth of patient-derived xenograft (PDX) organoids. In addition, therapeutic silencing of MTHFD2 by systemically administered nanoliposomal siRNA profoundly inhibited tumor growth in a preclinical PCa mouse model. Consistently, MTHFD2 expression is significantly increased in human PCa and a gene expression signature based on the m1C cycle has significant prognostic value. Furthermore, MTHFD2 expression is coordinately regulated by ATF4 and the oncoprotein c-MYC, which has been implicated in PCa. These data suggest that the m1C cycle is essential for PCa progression and may serve as a novel biomarker and therapeutic target.

Significance

Here, we demonstrate that the mitochondrial, but not cytoplasmic, one-carbon cycle has a key role in prostate cancer cell growth and survival and may serve as a biomarker and/or therapeutic target.

Introduction

Cell proliferation requires energy, the availability of building blocks for new cellular components, and the ability to maintain cellular redox homeostasis (1). For building block generation and redox homeostasis, amino acid metabolism involving serine and glycine, and the carbon units that they provide, are essential (2). The 1C cycle mediates the folate-mediated transfer of 1C units from donor molecules, mainly serine, to acceptor molecules, such as purines, methionine and thymidylate; this is necessary for essential cellular processes including DNA synthesis, DNA repair, and the maintenance of cellular redox status.

Eukaryotic cells have complementary pathways for 1C metabolism in the cytosol and mitochondria comprising distinct serine hydroxymethyltransferases (SHMTs) and methylenetetrahydrofolate dehydrogenases (MTHFDs). While the cytoplasmic 1C pathway (c1C) prevails in non-proliferating somatic tissues, the mitochondrial pathway (m1C) is predominantly active in proliferating cells, as well as in cancer cells (3). In fact, the central player of the m1C cycle, methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), is overexpressed in many different tumor types (4). MTHFD2 is also critical during embryonic development (5), but is typically not expressed in normal adult tissues, except in highly proliferative cells, such as during T-cell lymphocyte activation (4,6).

While MTHFD2 is implicated in various cancers, little is known about its potential role in prostate cancer (PCa), which is the most frequently diagnosed noncutaneous cancer and the second most common cause of cancer death in men (7). The androgen receptor (AR) plays a key role in normal prostate growth, as well as in prostate carcinogenesis and progression. We previously found that AR signaling, a central driver of PCa, increased expression of activating transcription factor 4 (ATF4) (8). We have recently found that ATF4 has essential pro-survival functions in PCa cells *in vitro* and *in vivo* through direct activation of a broad range of genes including key metabolic pathways (9).

78 Here, we show that ATF4 directly and specifically regulates expression of genes

79 encoding m1C cycle enzymes in PCa cells. Among these, MTHFD2 is critical for

80 PCa growth *in vitro* and *in vivo*, and may serve as a novel therapeutic target. In

81 addition, the oncoprotein c-MYC interplays with ATF4 in regulating MTHFD2

82 expression establishing a new mode of action for c-MYC in PCa.

Materials and Methods

Cell culture

85 293T, RWPE1, LNCaP, DU145, and 22Rv1 cell lines were purchased from the

86 American Type Culture Collection (Rockville, MD). The VCaP, C4-2B, and LNCaP-c-

87 MYC cell lines were kind gifts from Dr. Frank Smit (Radboud University Nijmegen

88 Medical Centre, The Netherlands), Dr. Lelund Chung (Cedars-Sinai Medical Center,

89 CA), and Dr. Ian G. Mills (Oslo University Hospital, Norway), respectively. Cells were

90 routinely maintained in a humidified 5% CO_2 and 95% air incubator at 37°C. PCa

91 cells were cultured in RPMI 1640, and 293T cells in DMEM, containing 10% fetal calf

92 serum, 50 U/ml penicillin, 50 µg/ml streptomycin, and 4 mM L-glutamine (all

93 purchased from BioWhittaker-Cambrex). Where indicated, cells were treated with 30

94 nM Thapsigargin (Tg) (Sigma-Aldrich) for 5 h, unless stated otherwise. All cell lines

95 were used within 15 passages after reviving from the frozen stocks and routinely

96 tested and were free of mycoplasma contamination.

97

Ectopic expression of ATF4

99 ATF4 ORF entry clone was obtained from the Arizona State University plasmid

100 repository (HsCD00073682) and cloned into doxycycline-inducible pLIX_403

101 destination vector (Addgene #41395), a gift from Dr. David Root, through standard

102 gateway cloning procedure. Viruses were produced by transfecting HEK293T cells

103 with packaging (psPAX2), envelope (pMD2.G), and pLIX403-ATF4 plasmids, using

104 Lipofectamine 3000 reagent. LNCaP cells were then transduced with the harvested

105 lentivirus.

Cell proliferation and viability assays

107   Briefly, cells were reverse transfected using Lipofectamine RNAiMAX transfection
108   reagent (ThermoFisher) and plated into 96-well or 6-well plates. Cells in 6-well plates
109   were cultured for the indicated times, trypsinized, stained with trypan blue, and
110   counted using a hemocytometer. The data shown are representative of at least three
111   independent experiments performed in triplicate. Cells plated into the 96-well plates
112   were cultured for 48 hrs and cell viability was measured using the CCK-8 kit (Bimake,
113   Munich, Germany).

114   **Colony formation and prostatosphere assays**
115   Cells were trypsinized, seeded at a density of 5,000 cells per well into 6-well plates,
116   and cultured for 2-3 weeks. The cells were then fixed with methanol and stained with
117   0.4% crystal violet. Colonies were quantified by extracting crystal violet in 10% acetic
118   acid and measurement of absorbance at 590 nm. Prostatosphere assays were
119   performed as described previously (10). The data shown are representative of at
120   least two independent experiments performed in triplicate.

121   **Quantitative PCR**
122   RNA extraction, reverse transcription and quantitaive polymerase chain reaction
123   (qPCR) were performed as described previously (8). The values were normalized to
124   the relative amount of the internal standard *GAPDH*, *TBP,* or *ACTB*. Results
125   normalized to *GAPDH* are presented unless indicated otherwise. PCR primer
126   sequences are available upon request. The data shown are representative of at least
127   two independent experiments performed in triplicate.

128   **Western analysis**
129   Whole-cell extracts and Western analyses were performed by standard methods as
130   described previously (8). The antibodies used were: ATF4 (11815, Cell Signaling),
131   ATF4 (A5514, Bimake), ASNS (146811AP) (Proteintech); MTHFD2 (sc-390708),
132   GAPDH (sc-47274), β-Actin (sc-47778) (Santa Cruz Biotechnology). All antibodies
133   were used at a dilution of 1:1,000, except for MTHFD2 (1:100), GAPDH (1:5,000) and
134   β-Actin (1:2,000). The data shown are representative of at least two independent
135   experiments.

136 **Other methods**

137 Descriptions of the cell culture, RNA interference, chromatin immunoprecipitation

138 (ChIP), ChIP-Seq, patient-derived xenograft organoids, mitochondrial membrane

139 potential assay, nanoliposomal siRNA targeting in PCa xenografts,

140 immunohistochemistry (IHC), and bioinformatics analysis are available as

141 Supplementary Materials.

142 **Statistical analysis**

143 Mean and standard deviation values were calculated using Microsoft Excel software.

144 The potential effects were evaluated using Student's two-sided t-test unless indicated

145 otherwise. Values of $p < 0.05$ were considered as significant. Statistically significant

146 differences are denoted by *, **, and *** indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$,

147 respectively. Error bars indicate SEM.

148 # Results

149 **ATF4 specifically activates the mitochondrial one-carbon cycle in PCa**

150 To decipher novel ATF4 targets that may play essential roles in PCa, we performed a

151 ChIP-Seq experiment and identified ATF4 binding sites in LNCaP cells upon Tg

152 treatment. This analysis revealed 7488 ATF4 binding sites in close proximity (±1000

153 bp) to the transcription start sites of 5597 protein-coding genes **(Supp. Table 1)**.

154 There was a significant overlap between these binding sites with those that were

155 previously identified in various tissues or cell lines in the Gene Transcription

156 Regulation Database (GTRD), suggesting effective capture of target sequences

157 **(Supp. Figure 1A, Supp. Table 2)**. To assess the potential functionality of

158 thebinding sites, we analyzed these data together with our data from global

159 transcriptomic and proteomic analyses upon siRNA-mediated ATF4 knockdown in

160 Tg-treated LNCaP cells (9). Among the genes identified by ChIP-Seq, 29 were

161 downregulated in both transcriptomic and proteomic analyses **(Figure 1A, Supp.**

162 **Table 3)**. In addition to well-established ATF4 target genes, such as Asparagine

163 Synthetase (ASNS) and Phosphoserine Phosphatase (PSPH), two 1C metabolism

164 genes, MTHFD2 and MTHFD1L were among these 29 genes. Another 1C

6

165   metabolism gene, SHMT2, also harbored an ATF4 binding site and was among the
166   downregulated genes in the microarray experiment **(Figure 1A)**.   Intriguingly,
167   although mammalian 1C metabolism is comprised of two parallel pathways (cytosolic
168   and mitochondrial) with almost identical core enzymatic capabilities (11), all three
169   identified ATF4-regulated 1C metabolism genes belong to the mitochondrial pathway
170   **(Figures 1B)**.   Notably, the identified ATF4 binding sites in the vicinity of m1C cycle
171   genes overlapped with ATF4 binding sites that have been reported in the Gene
172   Transcription Regulation Database, and were highly conserved among various
173   mammalian genomes **(Figure 1C)**.   In contrast, expression of two c1C metabolism
174   genes (SHMT1 or MTHFD1) was not affected; consistently, they did not harbor any
175   ATF4 binding sites near their TSS **(Supp. Figure 1B)**. Consistent with this
176   observation, in the great majority of the 16 distinct cancer types, *ATF4* or and its
177   target *ASNS* mRNA levels were highly correlated with m1C, but not c1C, enzyme
178   gene expression **(Supp. Figure 1C)**.   Moreover, analyses of the protein and mRNA
179   expression data from the Cancer Cell Line Encyclopedia dataset showed significant
180   correlations between ATF4 protein levels and expression of the m1C, but not c1C
181   enzymes **(Supp. Figure 2)**. In fact, in this dataset, after Sperm flagellar protein 2
182   (SPEF2) (its functional role in PCa, if any, is currently not known), *MTHFD2* is the
183   second gene with the highest correlation to protein levels of ATF4. Similarly, there
184   was a high correlation between *ASNS* and m1C cycle enzyme gene expression.
185   Evaluation of 1C metabolism gene expression in the Oncomine database revealed
186   that m1C pathway genes are more consistently upregulated in diverse cancers
187   compared to c1C genes **(Figure 1D)**.   Additionally, CRISPR-based cancer cell line
188   dependency profiles of the 1C metabolism genes showed a significant correlation
189   between the mitochondrial, but not cytosolic, members of the pathway **(Figure 1E)**.
190   These data suggested that ATF4 is involved in mediating m1C metabolism gene
191   expression in PCa.

192   We next examined 1C metabolism gene expression upon siRNA mediated
193   knockdown of *ATF4* in three independent PCa cell lines.   *ATF4*-specific siRNAs
194   effectively hindered expression of well-known ATF4 target genes such as
195   Phosphoglycerate Dehydrogenase (*PHGDH*), Phosphoserine Aminotransferase 1

196 (*PSAT1*) and *PSPH* in the LNCaP, VCaP, and 22Rv1 cell lines **(Figure 2A, and**
197 **Supp. Figures 3A-B)**, while Tg-mediated stimulation of ATF4 expression increased
198 their expression **(Figure 2B)**. Consistent with the ChIP-Seq experiment, m1C gene
199 expression for *SHMT2*, *MTHFD2,* and *MTHFD1L* were decreased upon *ATF4*
200 silencing and increased upon Tg-mediated ATF4 activation **(Figure. 2A-B, and**
201 **Supp. Figures 3A-B)**. In contrast, neither *ATF4* silencing nor *ATF4* induction
202 significantly affected expression of c1C cycle genes *SHMT1* and *MTHFD1* **(Figures.**
203 **2A-B, Supp. Figures 3A-B)**. Moreover, in a previously published RNA-Seq dataset
204 (12), treatment of LNCaP cells with Tunicamycin (Tm - an inducer of ER stress/ATF4
205 pathway through inhibition of N-linked glycosylation in the endoplasmic reticulum)
206 also resulted in the expression of m1C genes in an ATF4 dependent manner **(Figure**
207 **2C)**. We validated the Tm-induced upregulation of m1C genes in LNCaP and PC3
208 cell lines **(Supp. Figures 3C-D)**. In Tg-treated cells, ATF4 knockdown effectively
209 reduces MTHFD2 protein levels **(Figure 2D)**, and ectopic expression of ATF4
210 effectively rescues it back to control levels **(Figure 2E)**. In contrast, in non-stressed
211 LNCaP cells, knockdown of ATF4 does not alter MTHFD2 mRNA or protein levels, as
212 under these conditions, despite significant mRNA levels, ATF4 is not translated into
213 protein **(Figure 2F, bottom panel)**. However, ectopic ATF4 expression effectively
214 increased both mRNA and protein levels of MTHFD2. Interestingly, without
215 knockdown of endogenous ATF4 expression, ectopically introduced *ATF4* mRNA was
216 not detectable **(Figure 2F, upper panel)**. This was due to the downregulation of
217 endogenous ATF4 mRNA expression upon induction of ectopic ATF4 and suggested
218 an autoregulatory negative feedback loop by ATF4 on its transcription. Consistent
219 with these findings, ATF4 binding to the vicinity of the *MTHFD2* gene was verified by
220 ChIP and *ATF4* silencing abolished this interaction **(Figure 2G)**. Taken together,
221 these data establish ATF4 as a key regulator of the m1C, but not the c1C, cycle gene
222 expression.

223 **MTHFD2 is critical for PCa cell growth *in vitro* and *in vivo***
224 Since MTHFD2 has a key role in the m1C cycle (13) and was previously implicated in
225 cancer (4), we assessed whether it affects PCa cell growth. siRNA-mediated

226   *MTHFD2* silencing effectively reduced its mRNA and protein levels **(Figure 3A)**.
227   MTHFD2 knockdown was maximal (~80%) at 72 hours post-transfection and
228   remained significantly downregulated for more than one week, but returned back to
229   basal levels by day 12 **(Supp. Figure 4A)**. Short term MTHFD2 knockdown
230   significantly reduced the viability **(Supp. Figure 4B)** and long-term knockdown nearly
231   abolished both viability and colony formation ability of LNCaP, DU145, VCaP, and
232   22Rv1 cells **(Figures 3B-D)**. Furthermore, MTHFD2 knockdown significantly
233   hindered LNCaP and DU145 prostatosphere growth **(Figure 3E)**. However, viability
234   of a normal prostate epithelial cell line, RWPE1, was not affected by MTHFD2
235   knockdown **(Figure 3F)**.

236   To further evaluate the potential effects of MTHFD2 on PCa growth, organoids of
237   LuCaP patient-derived xenograft (PDX) models were used (14). Three of the six
238   analyzed LuCaP organoids expressed high levels of MTHFD2 expression **(Figure
239   3G)**. Three organoids, two with high expression and one with low expression, were
240   analyzed further. These three PDX models, LuCaP 35, LuCaP 96, and LuCaP 136,
241   express AR, lack PTEN expression, and were developed from lymph node
242   metastasis, localized PCa, and adenocarcinoma cells from ascites, respectively (14).
243   siRNA-mediated MTHFD2 knockdown effectively suppressed formation of LuCaP35
244   and LuCaP96 organoids that express high levels of MTHFD2 without inducing cell
245   death **(Figure 3G-H**; quantification is presented in **Supp. Figure 4C)**. On the other
246   hand, LuCaP 136 organoid that expresses very low levels of MTHFD2 was not affe
247   cted by MTHFD2 knockdown.

248   To assess the therapeutic potential of MTHFD2 inhibition *in vivo*, we performed
249   xenograft experiments as previously described(15,16). VCaP or 22Rv1 cells were
250   subcutaneously injected into male nude mice. Upon formation of palpable tumors,
251   empty nanoliposomes or those that carry MTHFD2-specific siRNA were administered
252   by intraperitoneal injection and tumor growth was monitored over time. Whereas
253   tumors continued to grow rapidly in mice injected with the empty nanoliposomes,
254   injection of nanoliposomes containing MTHFD2-specific siRNA dramatically inhibited
255   tumor growth in both models **(Figure 3I)**. Nanoliposomal siMTHFD2 delivery was

256 well-tolerated and did not result in any weight loss (**Supp. Figure 4D**) Together with

257 the findings from above, these data suggest that MTHFD2 is critical for PCa growth

258 and may serve as a novel therapeutic target.

MTHFD2 expression is up-regulated in human PCa specimens

259

260 Both mRNA and protein expression of MTHFD2 were robust in the normal prostate

261 cell line RWPE1 and in all of the PCa cell lines tested, with some variability in the

262 level of expression **(Figure 4A)**. We next evaluated MTHFD2 expression in 24

263 human PCa specimens and their corresponding benign tissues from the same

264 patients using immunohistochemistry. MTHFD2 expression was significantly

265 increased in PCa compared to benign specimens **(Figure 4B)**. This observation was

266 verified by an independent tissue microarray cohort consisting of 860 PCa and 223

267 benign prostate specimens **(Figure 4C)**. In this large cohort, MTHFD2 expression

268 also correlated with the Gleason score indicating that it may have prognostic value.

269 Importantly, ATF4 and MTHFD2 protein expression was correlated in a sample

270 subset of this TMA **(Figure 4D)**. These data show that MTHFD2 expression is

271 significantly increased in PCa compared with normal tissue.

m1C gene expression signature is strongly associated with PCa
prognosis

272

273

274 To assess whether ATF4-regulated m1C cycle gene expression could serve as a

275 potential prognostic biomarker for PCa, we analyzed MTHFD2, SHMT2, MTHFD1L,

276 and MTHFD2L expression in five independent PCa cohorts in the Oncomine

277 database (17-21). MTHFD2 expression was consistently and significantly

278 upregulated in primary and metastatic PCa compared to benign samples **(Figure 5A)**.

279 SHMT2 was upregulated in three of the five cohorts **(Figure 5B)**. Only three cohorts

280 had expression data for MTHFD1L and two for MTHFD2L. MTHFD1L was

281 significantly upregulated in the primary and metastatic tumors while MTHFD2L was

282 upregulated in one of the two cohorts **(Supp. Figure 5)**. In addition, in the Cancer

283 Genome Atlas dataset, m1C metabolism gene expression, but not the cytosolic

284 counterparts, were more prominently upregulated in primary tumor samples **(Figure**

285 **5C)**. These data led us to evaluate whether m1C metabolism gene expression may

286   have prognostic value. Indeed, a gene expression signature consisting of the three

287   m1C cycle enzymes (MTHFD2, MTHFD1L, and SHMT2) was significantly associated

288   with recurrence-free survival in PCa patients **(Figure 5D)**. Taken together, these

289   observations suggest that activation of ATF4-regulated m1C metabolism could serve

290   as a prognostic biomarker in PCa.

291   **mTOR/ATF4 signaling regulates m1C expression in normal prostate but**

292   **not in PCa**

293   Previous work has shown that mTORC1 and PERK/eIF2A signaling regulate cell

294   metabolism by controlling ATF4 levels (22,23). To assess the impact of mTORC1

295   signaling on m1C metabolism in PCa, we investigated whether there is a correlation

296   between the expression of three ATF4-regulated m1C metabolism genes and those

297   that are specifically regulated by the mTORC1 signaling cascade. Genome-wide

298   transcriptional alterations were previously determined upon treatment of wild type or

299   ATF4 knockout human embryonic kidney (HEK) cells with Torin 1, a potent ATP-

300   competitive inhibitor of mTOR (22). We used these data to identify genes that are

301   exclusively regulated by mTORC1 signaling without input from the PERK/eIF2α/ATF4

302   cascade. Sixty genes were significantly deregulated by more than ±1.7-fold upon

303   Torin 1 treatment in both cell lines. Since some of these genes could still be

304   regulated and influenced by ATF4, whose expression is in fact also low in normal

305   cells, we further narrowed this list by filtering out known or potential ATF4 target

306   genes that were compiled from various public databases resulting in 38 genes as

307   ATF4 independent targets of mTOR signaling **(Figure 6A)**.

308

309   In the GTEX database, representing normal prostate samples, expression of majority

310   of the 38 genes were well correlated with m1C gene expression in the expected

311   direction (e.g. the genes inhibited by Torin 1 positively correlated with the investigated

312   genes) **(Figure 6A)**. However, such a correlation was absent in the TCGA dataset,

313   representing primary PCa. Consistently, there was a significant overlap between the

314   top 500 genes that correlated with MTHFD2 expression in the SEEK database that

315   contains 78 microarray-based PCa gene expression datasets, and the TCGA PCa

11

316   dataset (188 genes, **Figure 6B**). However, only 62 genes were shared between
317   GTEX and SEEK, GTEX and TCGA, or in all three datasets. These 62 genes were
318   not enriched for any specific signaling pathway, but contained genes for several
319   translation factors (EEF1B2, EIF2S2, EIF3J, EIF4EBP1), genes that encode proteins
320   involved in RNA transport (STRAP, THOC7, RAN), and the gene encoding
321   mitochondrial folate transporter SLC25A32 (**Supp. Table 4**). These results indicated
322   that m1C metabolism is distinctly regulated between normal prostate and PCa. In the
323   normal prostate, m1C metabolism appears to be primarily regulated by mTORC1
324   signaling, but in PCa another signaling pathway(s) may override this regulation.
325   Indeed, in LNCaP cells Tg-mediated induction of ER stress effectively supersedes
326   mTORC1-mediated MTHFD2 regulation; in contrast to basal conditions, mTORC1
327   inhibitor rapamycin failed to downregulate ATF4-regulated m1C metabolism gene
328   expression upon Tg treatment **(Figure 6C)**. These data suggest that m1C
329   metabolism is differentially regulated in PCa compared to normal prostate.

330   **c-MYC is a key mediator of m1C gene expression**

331   To assess which additional pathways could be involved in the regulation of m1C
332   metabolism, we performed gene set enrichment analysis on the top 500 genes that
333   correlate (Pearson r>0.4) with the m1C gene expression signature in the TCGA
334   dataset **(Supp. Table 5)**. As expected, genes involved in aminoacyl-tRNA
335   biosynthesis, 1C metabolism, cell cycle, and mitotic nuclear division were enriched
336   among the correlated genes **(Figure 6D)**. In addition, according to both ENCODE
337   and ChEA databases, genes that are associated with c-MYC were exceptionally
338   highly enriched (p=8.96e^{-90} in ENCODE, 8.6e^{-44} in ChEA). Indeed, c-MYC
339   expression itself significantly correlated with the m1C gene signature (r = 0.43).
340   Moreover, the 188 genes that correlated well with MTHFD2 in both SEEK and TCGA
341   databases **(Figure 6B)** were also highly enriched for c-MYC-mediated regulation
342   (p=1.15e^{-50} in Encode, p=1.18e^{-17} in ChEA) **(Supp. Table 4)**. These results
343   suggested that c-MYC may be involved in mediating the effects of m1C gene
344   expression in PCa.

345

346   We next determined whether m1C gene expression signature may be associated with
347   mutational events in PCa. Comparison of the mutation prevalence between the top
348   and bottom 180 samples based on the expression of the m1C gene expression
349   signature in the TCGA dataset revealed several enriched copy number alterations
350   and point mutations **(Figure 6E)**. Increased representation of 8p11 and 8p21-22
351   deletions, and c-MYC amplification among the signature-high group indicated that c-
352   MYC is involved in the regulation of m1C gene expression **(Figure 6E)** (24). 8p11
353   locus harbors the SFRP1 gene that encodes a Wnt signaling inhibitor that is
354   frequently inactivated in a variety of malignancies, including PCa, and has been
355   identified as an essential molecule in c-MYC-dependent transformation (25,26). In
356   the TCGA dataset, SFRP1 expression negatively correlated with those of *ATF4* (r=-
357   0.35), *ASNS* (r = -0.33) and m1C gene signature (r= -0.28) suggesting that loss of
358   SFRP1 could regulate m1C metabolism via ATF4 signaling. Similarly, loss of 8p21-
359   22 locus is one of the most frequent chromosomal aberrations in PCa and harbors
360   the *NKX3.1* gene that encodes a transcription factor, which acts as a tumor
361   suppressor by opposing c-MYC transcriptional activity (27). We further investigated
362   potential association of these mutations on m1C gene expression by assessing
363   expression of the three m1C genes in the mutated versus wild type samples in the
364   TCGA dataset **(Supp. Figure 6)**. All three m1C cycle genes were significantly
365   upregulated in all of the eight investigated mutant subgroups. Taken together, these
366   data suggest that mutational events in PCa could activate m1C gene expression
367   through c-MYC.
368
369   We next examined m1C gene expression at the protein level using the recently
370   reported proteomics data from 375 cancer cell lines (28). Since the number of
371   mitochondria per cell would vary among different cancer cell lines and can skew the
372   results, we used the expression of mitochondrial encoded proteins MT-CO1 and MT-
373   ATP8 to filter out proteins that correlate with m1C metabolism gene expression
374   simply due to varying numbers of mitochondria in the different cell lines. There was a
375   significant overlap between the expression of proteins that correlated with MTHFD2
376   and SHMT2 expression, and these did not coincide with mitochondrial proteins

377   **(Figure 6F; Supp. Table 6)**. In contrast, although almost one-fourth of the proteins

378   that correlated with MTHFD1L expression were mitochondrial proteins, MTHFD1L did

379   not correlate well with MTHFD2 or SHMT2 protein expression; this suggests that in

380   contrast to MTHFD1L, MTHFD2 and SHMT2 expression is regulated independently

381   from mitochondrial biogenesis. Interestingly, mRNA processing, splicing, transport,

382   and mitochondrial translation-related proteins were highly enriched among the 136

383   proteins that were shared between the MTHFD2 and SHMT2 groups **(Figure 6F)**.

384   These proteins were also enriched for harboring a nearby c-MYC binding site in their

385   genes (p = $1.23e^{-13}$) further indicating that c-MYC is a prominent player in the

386   regulation of m1C metabolism at the protein level **(Figure 6G)**.

387

388   **Under stress conditions, ATF4 counterbalances c-MYC loss to drive m1C gene**

389   **expression.**

390   A recent study has identified an intricate regulation of protein synthesis through c-

391   MYC-ATF4 cooperation, where c-MYC was involved in the regulation of EIF4EBP1

392   expression in an ATF4 dependent manner (29). We thus considered the possibility

393   that ATF4 and c-MYC may bind to adjacent or neighboring sites and coordinately

394   regulate m1C cycle gene expression. Intriguingly, data from ENCODE indicated that

395   all three ATF4-regulated m1C genes also harbor c-MYC binding sites that are in

396   close proximity, in fact almost overlapping, with the identified ATF4 binding sites

397   **(Supp. Figure 7)**. Moreover, the analysis of a previously published ChIP-Seq

398   experiment in LNCaP cells revealed c-MYC binding sites that overlap with those of

399   ATF4 that we have identified in the m1C genes and the EIF4EBP1 gene **(Figure 7A)**

400   (30). c-MYC binding to these sites was enriched upon its ectopic expression.

401   Furthermore, analysis of publicly available datasets revealed that m1C gene

402   expression was modulated upon ectopic expression or CRISPRi-mediated inhibition

403   of c-MYC **(Figures 7B-C)**. In these datasets, modulation of m1C gene expression

404   could not be attributed to an alteration upon cellular stress as in contrast to the report

405   by Tameria et al, indicators of ER stress (such as DDIT3, HERPUD1, ERLEC1,

406   PDIA4, PDIA6) were not deregulated by c-MYC induction or knockdown (29) **(Supp.**

407   **Figures 8A-B)**. We verified the effect of c-MYC on MTHFD2 expression in LNCaP

408 cells, where siRNA-mediated c-MYC knockdown resulted in a significant decrease in
409 MTHFD2 at both mRNA and protein levels **(Figure 7D).**

410

411 We next investigated whether ATF4 and c-MYC could synergistically regulate
412 MTHFD2 expression in PCa cells. To that end, we modulated the levels of the two
413 transcription factors by siRNA-mediated knockdown and assessed MTHFD2
414 expression, under basal and Tg-treated conditions. Under non-stressed conditions,
415 c-MYC knockdown effectively reduced mRNA and protein levels of MTHFD2, while
416 ATF4 knockdown had a slight effect **(Figure 7E)**. In contrast, under Tg-induced
417 stress conditions, MTHFD2 expression was clearly dependent on ATF4 as Tg
418 induction, in a remarkable fashion, completely inhibited c-MYC expression, and ATF4
419 knockdown effectively decreased MTHFD2 levels **(Figure 7E)**. These data suggest
420 that, along with ATF4, c-MYC is a key component of the regulatory network that
421 affects m1C gene expression.

422

423 Discussion

424 Metabolic reprogramming is critical for cancer cell growth and dissemination (31-33).
425 Some key determinants of this cellular rewiring have been established, but there is an
426 urgent need to identify the molecular mechanisms at play that can be targeted for
427 novel therapeutic approaches. We have recently found that ATF4 is critical for PCa
428 growth *in vitro* and *in vivo* (9). We now show that ATF4 makes significant
429 contributions to metabolic reprogramming of PCa cells by significantly increasing
430 m1C cycle gene expression (MTHFD2, MTHFD1L and SHMT2), without affecting
431 expression of their cytoplasmic counterparts (MTHFD1 and SHMT1). In particular,
432 we found that ATF4-driven deregulation of MTHFD2 expression promotes PCa cell
433 proliferation *in vitro* and *in vivo*. Consistently, a gene expression signature based on
434 the m1C cycle has significant prognostic value for PCa progression.

435 Previous studies have shown that ATF4 regulates metabolic pathways connected to
436 amino acid uptake, tRNA synthesis, and transport (34,35). In particular, ATF4 is a
437 major regulator of the serine biosynthesis pathway that is upregulated and is

438   associated with poor prognosis in various cancers (2,36-38). Conversion of serine

439   into glycine and formate is catalyzed by the m1C cycle enzymes in three metabolic

440   steps **(Figure 1B)**; by concurrently regulating these two cascades, ATF4 enables *de*

441   *novo* synthesis of purines, thymidylate, and glutathione which are essential for rapidly

442   proliferating cells.

443   It is currently not known what could be the benefit for cancer cells to preferentially use

444   the m1C cycle, rather than the cytosolic counterpart. The products of the m1C cycle,

445   formate and glycine, are transported to the cytosol, where formate is metabolized

446   back to 10-Formyltetrahydrofolate (CHO–THF) to serve as a substrate for purine

447   synthesis (39). Although the c1C cycle could also drive purine synthesis by yielding

448   CHO–THF, the m1C pathway provides the dominant flux for purine synthesis (40).

449   However, neither formate nor glycine treatment was able to recover the viability of

450   PCa cells upon MTHFD2 knockdown **(Supp. Figure 9)** suggesting that m1C

451   metabolism plays other essential roles beyond supplying the building blocks for

452   nucleotide biosynthesis.

453   One possibility in this regard is the potential contribution of m1C cycle to the energy

454   and redox demands of proliferating cells by generation of ATP and NADH/NADPH

455   (3,41). In the m1C cycle, the reaction catalyzed by MTHFD2 is a significant source of

456   NADH, which can be used in oxidative phosphorylation to generate 2.5 ATPs, and the

457   reaction catalyzed by MTHFD1L itself generates an ATP molecule giving an overall

458   yield of 3.5 ATPs per cycle (3). Moreover, NADH production by the m1C cycle could

459   contribute to PCa development by enhancing the antioxidant defense of cancer cells

460   (42,43). Consistently, inhibition of MTHFD2 and SHMT2 expression has been

461   reported to disturb redox homeostasis and impair cell survival under hypoxic

462   conditions in colorectal cancer and glioma, respectively (42,44). However, N-acetyl

463   cysteine, a potent antioxidant, failed to rescue viability of PCa cells upon MTHFD2

464   knockdown suggesting that altered redox homeostasis may not be the primary reason

465   for MTHFD2 knockdown-mediated cell death **(Supp. Figure 9)**.

466 MTHFD2 also participates in the formation of formylmethionyl transfer RNA (fMet)
467 that is required for the initiation of protein synthesis in the mitochondria, thereby
468 regulating mitochondrial protein translation **(Figure 1B)**, suggesting that its inhibition
469 may impair mitochondria genesis and/or biology. However, in preliminary experiments
470 we did not observe any decrease in the number of mitochondria or mitochondrial
471 membrane potential upon MTHFD2 knockdown **(Supp. Figures 10A-C)**;
472 nevertheless, it is still possible that the growth advantage that is contributed by
473 MTHFD2 to PCa cells is due to its effects on mitochondrial homeostasis.

474 Previous research has shown that the key regulator of protein synthesis mTORC1
475 can activate ATF4 through mechanisms distinct from its canonical induction by stress
476 cascades (22,45). However, according to a recent study, only a very small subset (61
477 genes) of ATF4 regulated genes are actually induced by both mTOR/ATF4 and
478 PERK/ATF4 cascades (46). All three mitochondrial 1C cycle enzymes (MTHFD2,
479 SHMT2, and MTHFD1L) were among these genes, but not the two cytosolic
480 counterparts (MTHFD1 and SHMT1). This study was performed on normal mouse
481 embryonic fibroblasts suggesting that ATF4-mediated metabolic regulation is not
482 specific to cancer cells.

483 Furthermore, our analysis on the GTEx and TCGA datasets suggested that in PCa
484 tumors cascades other than mTORC1/ATF4 signaling are also involved in regulation
485 of the m1C cycle **(Figure 6A)**. In particular, c-MYC-associated gene expression was
486 highly correlated with m1C gene expression in PCa. Together with the other data we
487 present here, this suggested that ATF4 and c-MYC may coordinately regulate m1C
488 gene expression. Consistent with this, the ATF4 binding sites in the vicinity of m1C
489 enzymes from our ChIP-Seq analysis coincide with those of c-MYC that were
490 identified earlier **(Figure 7A)** (30). Furthermore, both mRNA and protein levels of
491 MTHFD2 were inhibited upon c-MYC knockdown. These observations are intriguing
492 as c-MYC is an established oncoprotein for PCa, and we have recently identified it as
493 a downstream target and mediator of the IRE1-XBP1s arm of the unfolded protein
494 response (UPR) (10,47). Our data thus establish a new role of c-MYC in modulating

495   the UPR and a potential novel mode of crosstalk between the IRE1-XBP1s and

496   PERK-eIF2α-ATF4 signaling.

497   Based on our findings herein and recently published studies, we suggest the

498   following model (Figure7F): Under normal conditions, ATF4 regulates m1C gene

499   expression, whereas c-MYC is involved in regulating both m1C and c1C gene

500   expression.   Under conditions of some types of stress, c-MYC expression is

501   downregulated, and ATF4 can compensate for this to sustain the expression of the

502   m1C cycle gene expression, whereas c1C gene expression remains low. However,

503   in tumors, various mechanisms, such as c-MYC gene amplification and activation of

504   the IRE1/XBP1 cascade, can keep c-MYC expression high resulting in further

505   elevated levels of m1C gene expression, which will satisfy the metabolic needs of the

506   cancer cell in cooperation with UPR-mediated ATF4 signaling.   These data thus

507   establish that UPR activation can induce m1C cycle by promoting both ATF4 and c-

508   MYC expression. There may be other points of interaction of c-MYC with the UPR in

509   PCa to establish autoregulatory mechanisms, such as c-MYC heterodimerization with

510   XBP1s to activate the IRE1-XBP1s pathway, which then activates c-MYC expression,

511   as observed in breast cancer cells (48,49) (for a review, see (50)). Thus there

512   appears to be feedback loops that are likely to be responsive to environmental cues

513   and determine the outcome of the interactions between ATF4 and c-MYC signaling,

514   which converge on activation of m1C expression.

515

516   In summary, our findings establish an interplay between ATF4 and c-MYC to drive

517   m1C cycle gene expression as a critical component for PCa growth. As exemplified

518   by the dramatic tumor inhibitory effects of MTHFD2 targeting *in vivo* and the robust

519   prognostic value of the m1C gene signature, future work should further evaluate the

520   m1C cycle as a potential biomarker and/or therapeutic target in PCa.

521

522   Acknowledgments

References

527 1.   Locasale JW, Cantley LC. Metabolic flux and the regulation of mammalian cell
528   growth. Cell metabolism **2011**;14:443-51

529 2.   Locasale JW. Serine, glycine and one-carbon units: cancer metabolism in full
530   circle. Nat Rev Cancer **2013**;13:572-83

531 3.   Meiser J, Vazquez A. Give it or take it: the flux of one-carbon in cancer cells.
532   FEBS J **2016**;283:3695-704

533 4.   Nilsson R, Jain M, Madhusudhan N, Sheppard NG, Strittmatter L, Kampf C*, et
534   al.* Metabolic enzyme expression highlights a key role for MTHFD2 and the
535   mitochondrial folate pathway in cancer. Nat Commun **2014**;5:3128

536 5.   Di Pietro E, Sirois J, Tremblay ML, MacKenzie RE. Mitochondrial NAD-
537   dependent   methylenetetrahydrofolate   dehydrogenase-
538   methenyltetrahydrofolate cyclohydrolase is essential for embryonic
539   development. Molecular and cellular biology **2002**;22:4158-66

540 6.   Vazquez A, Tedeschi PM, Bertino JR. Overexpression of the mitochondrial
541   folate and glycine-serine pathway: a new determinant of methotrexate
542   selectivity in tumors. Cancer Res **2013**;73:478-82

543 7.   Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin
544   **2020**;70:7-30

545 8.   Sheng X, Arnoldussen YJ, Storm M, Tesikova M, Nenseth HZ, Zhao S*, et al.*
546   Divergent androgen regulation of unfolded protein response pathways drives
547   prostate cancer. EMBO Mol Med **2015**;7:788-801

548 9.   Pallmann N, Livgard M, Tesikova M, Zeynep Nenseth H, Akkus E, Sikkeland J*,
549   et al.* Regulation of the unfolded protein response through ATF4 and FAM129A
550   in prostate cancer. Oncogene **2019**;38:6301-18

551 10. Sheng X, Nenseth HZ, Qu S, Kuzu OF, Frahnow T, Simon L*, et al.* IRE1alpha-
552   XBP1s pathway promotes prostate cancer by activating c-MYC signaling. Nat
553   Commun **2019**;10:323

554 11. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease.
555   Cell metabolism **2017**;25:27-42

556 12. Luhr M, Torgersen ML, Szalai P, Hashim A, Brech A, Staerk J*, et al.* The
557   kinase PERK and the transcription factor ATF4 play distinct and essential roles
558   in autophagy resulting from tunicamycin-induced ER stress. J Biol Chem
559   **2019**;294:8197-217

560 13. Tibbetts AS, Appling DR. Compartmentalization of Mammalian folate-mediated
561   one-carbon metabolism. Annu Rev Nutr **2010**;30:57-81

562 14. Nguyen HM, Vessella RL, Morrissey C, Brown LG, Coleman IM, Higano CS*, et
563   al.* LuCaP Prostate Cancer Patient-Derived Xenografts Reflect the Molecular
564   Heterogeneity of Advanced Disease an--d Serve as Models for Evaluating
565   Cancer Therapeutics. Prostate **2017**;77:654-71

566 15. Jin Y, Wang L, Qu S, Sheng X, Kristian A, Maelandsmo GM*, et al.* STAMP2
567   increases oxidative stress and is critical for prostate cancer. EMBO Mol Med
568   **2015**;7:315-31
569 16. Jin Y, Qu S, Tesikova M, Wang L, Kristian A, Maelandsmo GM*, et al.*
570   Molecular circuit involving KLK4 integrates androgen and mTOR signaling in
571   prostate cancer. Proceedings of the National Academy of Sciences of the
572   United States of America **2013**;110:E2572-81
573 17. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS*, et al.*
574   Integrative genomic profiling of human prostate cancer. Cancer cell
575   **2010**;18:11-22
576 18. Vanaja DK, Cheville JC, Iturria SJ, Young CY. Transcriptional silencing of zinc
577   finger protein 185 identified by expression profiling is associated with prostate
578   cancer progression. Cancer Res **2003**;63:3877-82
579 19. Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K*, et al.* Gene
580   expression profiling identifies clinically relevant subtypes of prostate cancer.
581   Proceedings of the National Academy of Sciences of the United States of
582   America **2004**;101:811-6
583 20. LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V*, et al.*
584   Comprehensive gene expression analysis of prostate cancer reveals distinct
585   transcriptional programs associated with metastatic disease. Cancer Res
586   **2002**;62:4499-506
587 21. Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C*, et al.* Gene
588   expression correlates of clinical prostate cancer behavior. Cancer cell
589   **2002**;1:203-9
590 22. Park Y, Reyna-Neyra A, Philippe L, Thoreen CC. mTORC1 Balances Cellular
591   Amino Acid Supply with Demand for Protein Synthesis through Post-
592   transcriptional Control of ATF4. Cell Rep **2017**;19:1083-90
593 23. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M*, et al.* An integrated
594   stress response regulates amino acid metabolism and resistance to oxidative
595   stress. Molecular cell **2003**;11:619-33
596 24. Xing C, Ci X, Sun X, Fu X, Zhang Z, Dong EN*, et al.* Klf5 deletion promotes
597   Pten deletion-initiated luminal-type mouse prostate tumors through multiple
598   oncogenic signaling pathways. Neoplasia **2014**;16:883-99
599 25. Cowling VH, D'Cruz CM, Chodosh LA, Cole MD. c-Myc transforms human
600   mammary epithelial cells through repression of the Wnt inhibitors DKK1 and
601   SFRP1. Molecular and cellular biology **2007**;27:5135-46
602 26. Zheng L, Sun D, Fan W, Zhang Z, Li Q, Jiang T. Diagnostic value of SFRP1 as
603   a favorable predictive and prognostic biomarker in patients with prostate
604   cancer. PloS one **2015**;10:e0118276
605 27. Anderson PD, McKissic SA, Logan M, Roh M, Franco OE, Wang J*, et al.*
606   Nkx3.1 and Myc crossregulate shared target genes in mouse and human
607   prostate tumorigenesis. J Clin Invest **2012**;122:1907-19
608 28. Nusinow DP, Szpyt J, Ghandi M, Rose CM, McDonald ER, 3rd, Kalocsay M*, et
609   al.* Quantitative Proteomics of the Cancer Cell Line Encyclopedia. Cell
610   **2020**;180:387-402 e16

611 29. Tameire F, Verginadis, II, Leli NM, Polte C, Conn CS, Ojha R*, et al.* ATF4
612   couples MYC-dependent translational activity to bioenergetic demands during
613   tumour progression. Nature cell biology **2019**;21:889-99
614 30. Barfeld SJ, Urbanucci A, Itkonen HM, Fazli L, Hicks JL, Thiede B*, et al.* c-Myc
615   Antagonises the Transcriptional Activity of the Androgen Receptor in Prostate
616   Cancer Affecting Key Gene Networks. EBioMedicine **2017**;18:83-93
617 31. Faubert B, Solmonson A, DeBerardinis RJ. Metabolic reprogramming and
618   cancer progression. Science **2020**;368
619 32. Yoshida GJ. Metabolic reprogramming: the emerging concept and associated
620   therapeutic strategies. Journal of experimental & clinical cancer research : CR
621   **2015**;34:111
622 33. Sun H, Zhou Y, Skaro MF, Wu Y, Qu Z, Mao F*, et al.* Metabolic
623   Reprogramming in Cancer Is Induced to Increase Proton Production. Cancer
624   Res **2020**;80:1143-55
625 34. Wang Y, Ning Y, Alam GN, Jankowski BM, Dong Z, Nor JE*, et al.* Amino acid
626   deprivation promotes tumor angiogenesis through the GCN2/ATF4 pathway.
627   Neoplasia **2013**;15:989-97
628 35. DeNicola GM, Chen PH, Mullarky E, Sudderth JA, Hu Z, Wu D*, et al.* NRF2
629   regulates serine biosynthesis in non-small cell lung cancer. Nature genetics
630   **2015**;47:1475-81
631 36. Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K*, et al.*
632   Functional genomics reveal that the serine synthesis pathway is essential in
633   breast cancer. Nature **2011**;476:346-50
634 37. Sun L, Song L, Wan Q, Wu G, Li X, Wang Y*, et al.* cMyc-mediated activation of
635   serine biosynthesis pathway is critical for cancer progression under nutrient
636   deprivation conditions. Cell research **2015**;25:429-44
637 38. Vie N, Copois V, Bascoul-Mollevi C, Denis V, Bec N, Robert B*, et al.*
638   Overexpression of phosphoserine aminotransferase PSAT1 stimulates cell
639   growth and increases chemoresistance of colon cancer cells. Molecular cancer
640   **2008**;7:14
641 39. Lane AN, Fan TW. Regulation of mammalian nucleotide metabolism and
642   biosynthesis. Nucleic Acids Res **2015**;43:2466-85
643 40. Ducker GS, Chen L, Morscher RJ, Ghergurovich JM, Esposito M, Teng X*, et*
644   *al.* Reversal of Cytosolic One-Carbon Flux Compensates for Loss of the
645   Mitochondrial Folate Pathway. Cell metabolism **2016**;23:1140-53
646 41. Shin M, Momb J, Appling DR. Human mitochondrial MTHFD2 is a dual redox
647   cofactor-specific methylenetetrahydrofolate
648   dehydrogenase/methenyltetrahydrofolate cyclohydrolase. Cancer Metab
649   **2017**;5:11
650 42. Ye J, Fan J, Venneti S, Wan YW, Pawel BR, Zhang J*, et al.* Serine catabolism
651   regulates mitochondrial redox control during hypoxia. Cancer Discov
652   **2014**;4:1406-17
653 43. Martinez-Reyes I, Chandel NS. Mitochondrial one-carbon metabolism
654   maintains redox balance during hypoxia. Cancer Discov **2014**;4:1371-3
655 44. Ju HQ, Lu YX, Chen DL, Zuo ZX, Liu ZX, Wu QN*, et al.* Modulation of Redox
656   Homeostasis by Inhibition of MTHFD2 in Colorectal Cancer: Mechanisms and

657   Therapeutic Implications. Journal of the National Cancer Institute
658   **2019**;111:584-96

659 45.   Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1
660   induces purine synthesis through control of the mitochondrial tetrahydrofolate
661   cycle. Science **2016**;351:728-33

662 46.   Torrence ME, MacArthur MR, Hosios AM, Valvezan AJ, Asara JM, Mitchell JR*,*
663   *et al.* The mTORC1-mediated activation of ATF4 promotes protein and
664   glutathione synthesis downstream of growth signals. Elife **2021**;10

665 47.   Koh CM, Bieberich CJ, Dang CV, Nelson WG, Yegnasubramanian S, De
666   Marzo AM. MYC and Prostate Cancer. Genes Cancer **2010**;1:617-28

667 48.   Zhao N, Cao J, Xu L, Tang Q, Dobrolecki LE, Lv X*, et al.* Pharmacological
668   targeting of MYC-regulated IRE1/XBP1 pathway suppresses MYC-driven
669   breast cancer. J Clin Invest **2018**;128:1283-99

670 49.   Sheng X, Nenseth HZ, Qu S, Kuzu O, Frahnow T, Simon L*, et al.* IRE1α-
671   XBP1s pathway promotes prostate cancer by activating c-MYC signaling.
672   Nature Communications **2019**;10:323-34

673 50.   Jin Y, Saatcioglu F. Targeting the Unfolded Protein Response in Hormone-
674   Regulated Cancers. Trends Cancer **2020**;6:160-71

675

Figure Legends

Figure 1: ATF4 binds to and regulates expression of mitochondrial but not cytoplasmic 1C metabolism genes. **A.** Venn diagram depicting the number of genes affected by the different analyses (microarray gene expression in yellow, mass spectrometry (MS) in blue, and ChIP-Seq in light red) and their intersections, upon ATF4 knockdown. **B.** A simplified diagram showing c1C and m1C metabolic pathways. **C.** ChIP-Seq analyses of ATF4 target genes reveal strong binding sites in the vicinity of genes that encode m1C cycle enzymes. The GTRD (Gene Transcription Regulation Database) lane shows the number of ATF4 peak calls and their location based on this database. Conservation track shows conservation among vertebrate genomes (phastCons scores). **D.** Summary of 1C metabolism gene regulation in cancer versus normal tissues in the Oncomine database (Top 5% threshold). Darker color in each box indicates larger number of studies and thus more significant association. **E.** Diagram showing the correlation of cancer cell line dependency profiles between m1C metabolism genes and their functionally relevant neighbors. In contrast to the others, ATF4-regulated m1C cycle genes are more strongly linked to each other (red lines). m1C and c1C cycle genes are represented by blue and red boxes, respectively. The numbers in the ovals on the connectors show Pearson correlation coefficients between the linked genes. The red lines have a Pearson correlation coefficient that is greater than 0.15.

Figure 2: ATF4 induces expression of mitochondrial but not cytoplasmic 1C cycle enzymes. **A.** siRNA-mediated ATF4 knockdown decreases expression of serine synthesis and m1C cycle enzyme gene expression, but not that of c1C cycle. LNCaP cells were transfected with either control or two independent ATF4-specific siRNAs, treated with Tg (300 nM for 5 h), and were analyzed by qPCR. The efficiency of ATF4 knockdown was confirmed by western analysis as shown in the inset. **B.** Expression of genes encoding serine synthesis and 1C cycle enzymes were analyzed by qPCR upon treatment of LNCaP cells with Tg (30 nM) for the indicated time points. Inset shows ATF4 protein levels upon Tg treatment in the time course experiment. **C.** Data extracted from a RNA-sequencing experiment that was

707   published previously (12). LNCaP cells were transfected with non-targeting siRNA

708   (siCTRL) or two distinct ATF4-targeting siRNAs. Cells were then either treated with

709   DMSO as control or tunicamycin (2.5 μg/mL for 18 hrs). RNA was isolated and

710   subjected to RNA-sequencing. **D.** ATF4 knockdown decreases MTHFD2 protein

711   expression in multiple PCa cell lines. Indicated cell lines were transfected with either

712   control siRNA or two independent siRNAs targeting ATF4. Cells were treated with Tg

713   (30 nM, 5hr), harvested, and used in Western analysis. **E.** Downregulation of m1C

714   gene expression upon ATF4 knockdown is rescued by ATF4 re-expression.

715   Doxycycline inducible LNCaP-ATF4 cells were transfected either with scrambled

716   siRNA or an ATF4-specific siRNA targeting the 5'-UTR of the gene (siATF4); 4 days

717   later cells were treated with Tg (30 nM for 5h). Doxycycline-mediated induction of

718   ATF4 effectively restored MTHFD2 levels as analyzed by qPCR. **F.** Under non-

719   stressed conditions, despite its high mRNA expression, ATF4 is not translated to a

720   significant protein level, hence its knockdown fails to down-regulate MTHFD2 levels.

721   Doxycycline-inducible LNCaP-ATF4 cells were transfected either with scrambled

722   siRNA or two independent siRNAs targeting the open reading frame (siATF4 #1) or

723   5'-UTR (siATF4 #2) of the ATF4 mRNA. At the same time with the transfection, cells

724   were treated with indicated amounts of doxycycline to induce ATF4 expression and

725   processed after 48 hrs. Note that ectopic ATF4 expression (upon Dox treatment)

726   effectively upregulates MTHFD2 levels while downregulating endogenous ATF4

727   levels (detected by UTR specific primers). ASNS expression, a well-characterized

728   ATF4 target gene, is shown as a reporter of ATF4 activity. #: p<0.001; ^: p<0.01; *:

729   0.05; ns: non-significant. **G.** Individual ChIP analysis verified ATF4 binding to the

730   intronic region (chr2:74,426,212-74,426,487 – hg19) of the MTHFD2 gene identified

731   in the ChIP-Seq experiment. LNCaP cells were transfected with either scrambled

732   siRNA or ATF4-specific siRNA, treated with vehicle or Tg, and ChIP assay was

733   performed using an ATF4-specific antibody.

734   **Figure 3: MTHFD2 knockdown inhibits growth of PCa cells, PDX-derived**

735   **organoids, and tumor xenografts.** **A.** MTHFD2 knockdown efficiency was

736   determined in LNCaP and DU145 cells that were transfected with either scrambled

737   siRNA or two independent MTHFD2-specific siRNAs for 48 hours by both qPCR and

738   Western analyses. **B.** LNCaP, VCaP, or 22Rv1 cells transfected with control or

739   MTHFD2-specific siRNAs were cultured for the indicated times, and cell numbers

740   were determined by trypan blue staining. *p < 0.01. MTHFD2 knockdown hinders

741   colony **(C-D)** and prostatosphere **(E)** formation ability of PCa cells. Indicated cells

742   were transfected with control siRNA or MTHFD2-specific siRNAs and cultured for two

743   weeks. Colonies formed were stained and quantified as described in Materials and

744   Methods. Prostatospheres were pictured and counted under a light microscope;

745   representative areas are presented. **F.** Viability of normal prostate cells is not

746   affected by MTHFD2 knockdown. RWPE1 cells were transfected with either control

747   siRNA or two independent MTHFD2-targeting siRNAs. After 48 hours, relative cell

748   viability and MTHFD2 expression were determined using the CCK8 assay and qPCR,

749   respectively. **G.** MTHFD2 expression and knockdown efficiency in various LuCaP

750   organoids were assessed by Western analysis. **H.** MTHFD2 knockdown significantly

751   decreased LuCaP organoid formation without affecting cell death, in LuCaP 35 and

752   LuCaP 96 that express MTHFD2, but not in LuCaP 136, which does not express

753   MTHFD2. **I.** Nanoliposomal systemic delivery of MTHFD2-specific siRNA profoundly

754   inhibited the growth of VCaP and 22Rv1 xenograft tumors *in vivo*. VCaP and 22Rv1

755   cells were implanted subcutaneously in male nude mice. Once tumors were

756   palpable, mice (n = 5 per group) were given either empty nanoliposomes or

757   MTHFD2-specific siRNA as described in Materials and Methods. Tumor volumes

758   were measured at the indicated time points.

759   **Figure 4: MTHFD2 expression is increased in PCa. A.** Basal levels of MTHFD2

760   mRNA and protein were determined in various PCa cell lines and the normal prostate

761   cell line by qPCR and Western analysis, respectively. **B.** MTHFD2 expression was

762   analyzed by immunohistochemistry in matched benign prostate and PCa specimens

763   from 24 patients. Representative images and quantification of staining is shown. **C.**

764   Tissue microarrays with normal prostate (n = 223) and primary prostate tumors

765   (n = 860) were analyzed by immunohistochemistry. Middle panel shows increased

766   MTHFD2 expression with increasing Gleason grade of the samples. Representative

767   images and quantification of staining intensity are shown. **D.** In a subset of the

768   samples used for IHC analysis shown in Figure 5C , the correlation of ATF4 and

769   MTHFD2 staining scores is depicted. r denotes Pearson correlation between the two

770   stainings.

771   **Figure 5: Mitochondrial 1C gene expression is deregulated in PCa and a gene**

772   **expression signature derived from it is strongly associated with PCa**

773   **prognosis. A-B.** Expression of MTHFD2 and SHMT2 is upregulated in primary

774   and/or metastatic PCa compared to benign samples. Expression data were retrieved

775   from the Oncomine database. 1: Singh et al. (21), 2: Taylor et al. (17), 3: Vanaja et al.

776   (18), 4: Lapointe et al. (19), 5: La Tulippe et al. (20), N: Normal, P: Primary, M:

777   Metastatic. **C.** m1C but not c1C cycle genes are upregulated in primary PCa samples

778   in the TCGA dataset. **D.** A gene expression signature consisting of the three ATF4-

779   regulated m1C cycle genes (MTHFD2 + MTHFD1L + SHMT2) is significantly

780   associated with recurrence-free survival in the TCGA dataset. PCa samples were

781   ordered based on the expression of the signature genes and a Kaplan-Meier graph

782   was drawn based on the survival data for top and bottom ~36% of the samples.

783   **Figure 6: Mitochondrial 1C cycle gene expression is differentially regulated in**

784   **benign and PCa tissues. A.** m1C cycle gene expression correlates with ATF4

785   independent target genes of mTORC1 signaling in normal prostate, but not in PCa

786   samples. Pearson correlation coefficients between indicated genes were calculated

787   in the GTEx dataset containing 106 normal prostate tissue samples and TCGA

788   database containing 426 primary PCa samples. Genes in green and red were up and

789   down-regulated, respectively, upon Torin 1 treatment. **B.** Distinct sets of genes

790   correlate with MTHFD2 in normal and PCa samples. Venn diagram shows the

791   number of shared genes that correlate with MTHFD2 in the SEEK, TCGA, or GTEx

792   datasets. In each dataset, the top 500 genes that correlate with MTHFD2 expression

793   were included in the analyses. **C.** ER stress-mediated induction of ATF4 overrides

794   mTORC1/ATF4-mediated MTHFD2 expression. LNCaP cells were treated with 30

795   nM Tg (for 8 hours) and/or 100 nM Rapamycin for 24 hours and expression of m1C

796   cycle genes was determined by qPCR. Bottom panel shows Western analysis

797   verifying the activity of the compounds. **D.** Enrichment analysis of top 500 genes that

798 correlate with MTHFD2 expression in the TCGA dataset. **E.** Prevalence of various

799 mutations among the top and bottom 180 (~36 %) samples in the TCGA dataset

800 based on the expression of the m1C cycle gene signature. **F.** Venn diagram showing

801 proteins that correlate with the three m1C cycle enzyme expression and two

802 mitochondrial DNA encoded proteins (MT-CO1 and MT-ATP8) (r>0.5). **G.** Geneset

803 enrichment analysis of 136 proteins that were shared between the MTHFD2 and

804 SHMT2 groups identified in **F.**

805 **Figure 7: c-MYC co-occupies ATF4 response elements and regulates m1C cycle**

806 **gene expression.** **A.** c-MYC ChIP-Seq analyses in LNCaP cells revealed co-

807 localization of ATF4 binding sites that were identified in the proximity of m1C cycle

808 genes and EIF4EBP1. The data were obtained from GSE73994 in which Dox-

809 inducible LNCaP-c-MYC cell line was used. Orange triangles show the location of

810 ATF4 binding sites identified in our ChIP-Seq analysis (Figure 1D). Please note that

811 for all four genes, in contrast to other nearby sites, the c-MYC binding at the ATF4

812 target site was relatively more increased upon c-MYC expression. **B.** CRISPRi-

813 mediated inhibition of *MYC* expression downregulates m1C cycle enzyme genes and

814 *EIF4EBP1* expression in the 22Rv1 PCa cell line. The data were obtained from

815 GSE142808. **C.** Results of a microarray study showing induction of m1C enzyme

816 gene and *EIF4EBP1* expression upon ectopic *c-MYC* expression in LNCaP cells.

817 The data were obtained from GSE73917. **D.** *MYC* knockdown effectively

818 downregulates m1C cycle enzyme genes and *EIF4EBP1* expression. LNCaP cells

819 were transfected with siCTRL or sic-MYC (an siRNA pool), and 48 hrs later

820 processed for qPCR and Western analyses. **E.** Under stress conditions ATF4

821 counterbalances the c-MYC loss to drive m1C gene expression. LNCaP cells were

822 transfected with control, ATF4 and/or c-MYC specific siRNAs, and 48 hrs later treated

823 with DMSO or 30 nM Tg for 5 or 24 hrs. ATF4, MYC and MTHFD2 expressions were

824 determined by qPCR. Under the same conditions, protein levels of c-MYC, ATF4 and

825 MTHFD2 were measured by Western analysis. **F.** Schematic depiction of m1C and

826 c1C cycle gene expression under normal prostate and in PCa. Under normal

827 conditions, ATF4 protein expression is high enough only under transient stress

828 conditions and it specifically induces m1C expression, whereas c-MYC drives both

829   c1C and m1C expression upon transient growth-promoting signals. In tumors, there

830   is chronic stress that activates ATF4 signaling and m1C gene expression. Likewise,

831   various chronic signals, such as proliferative signaling and gene amplification,

832   increase c-MYC expression in PCa tumors that results in higher levels of m1C and

833   c1C gene expression. Having input from both ATF4 and c-MYC, m1C gene
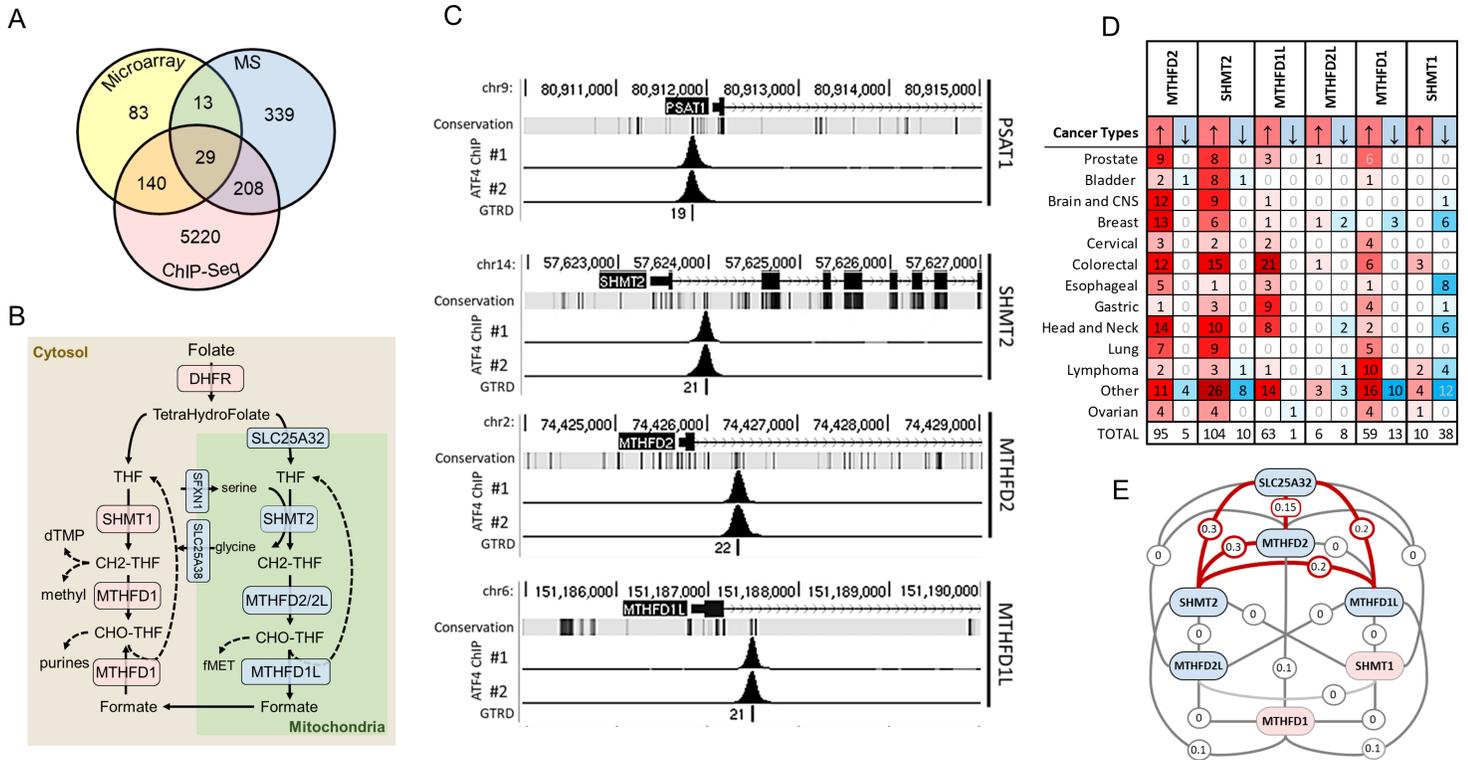
834   expression is markedly higher than that of the c1C cycle in PCa.

Figure 1

Figure 2
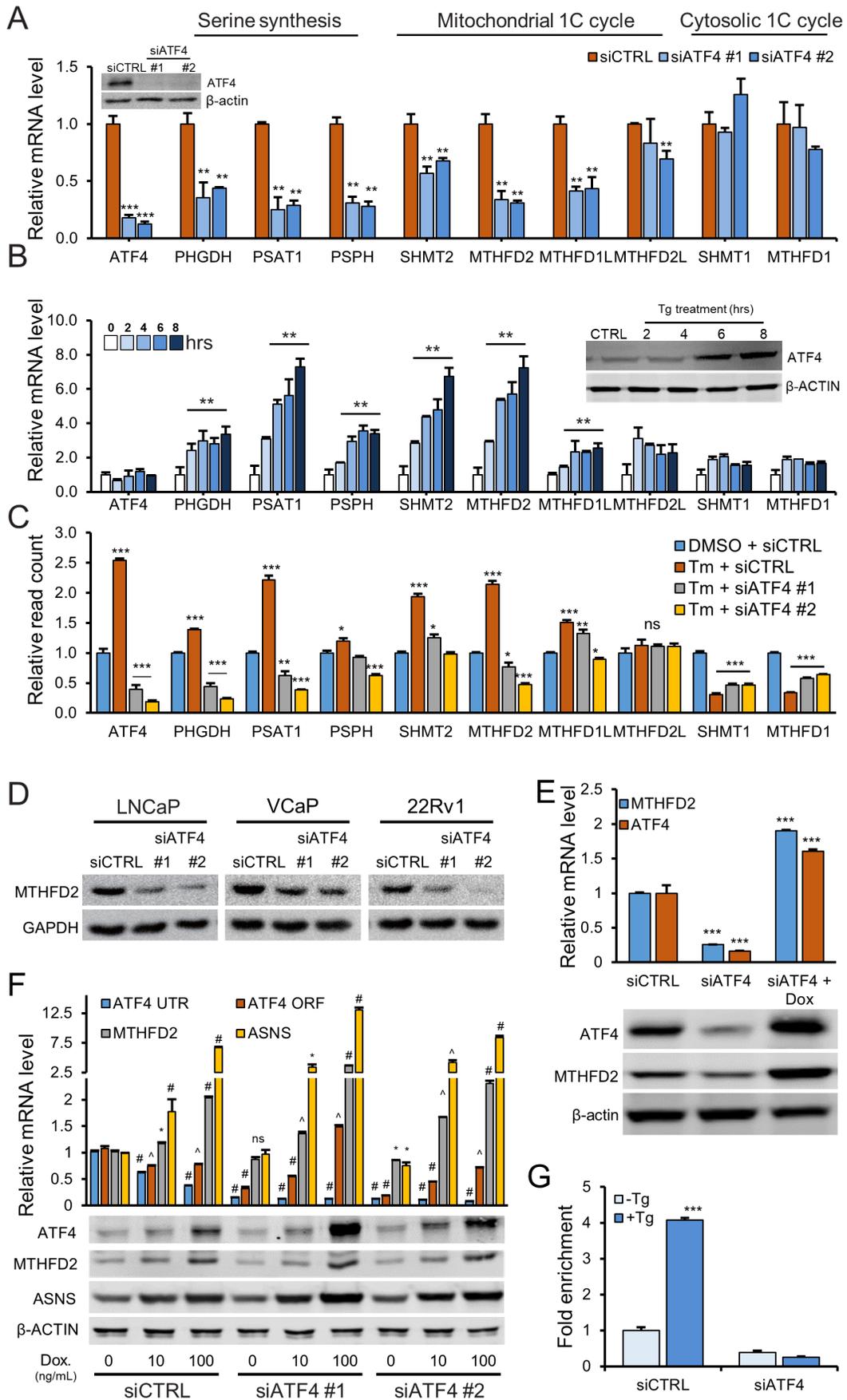
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7