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2 Cyclocreatine suppresses prostate tumorigenesis through dual effects on SAM and
3 creatine metabolism

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ABSTRACT (174/175 words)

Prostate cancer is highly prevalent, being the second most common cause of cancer mortality in men worldwide. Applying a novel genetically engineered mouse model (GEMM) of aggressive prostate cancer driven by deficiency of PTEN and SPRY2 (Sprouty 2) tumour suppressors, we identified enhanced creatine metabolism within the phosphagen system in progressive disease. Altered creatine metabolism was validated in *in vitro* and *in vivo* prostate cancer models and in clinical cases. Upregulated creatine levels were due to increased uptake through the SLC6A8 creatine transporter and *de novo* synthesis, resulting in enhanced cellular basal respiration. Treatment with cyclocreatine (a creatine analogue that potently and specifically blocks the phosphagen system) dramatically reduces creatine and phosphocreatine levels. Blockade of creatine biosynthesis by cyclocreatine leads to cellular accumulation of S-adenosyl methionine (SAM), an intermediary of creatine biosynthesis, and suppresses prostate cancer growth *in vitro*. Furthermore, cyclocreatine treatment impairs cancer progression in our GEMM and in a xenograft liver metastasis model. Hence, by targeting the phosphagen system, cyclocreatine results in anti-tumourigenic effects from both SAM accumulation and suppressed phosphagen system.

INTRODUCTION

Prostate cancer is the most common cancer in men worldwide (Siegel *et al*, 2020). Around 20% of patients present with advanced disease at the time of diagnosis. Even for men found to have an early or indolent form of the disease, at least 20% of these patients will subsequently progress to a lethal and treatment-resistant form of prostate cancer (Lohiya *et al*, 2016). Therefore, identification and characterisation of genetic lesions that influence aggressive prostate cancer growth may provide new strategies to improve clinical management of disease progression. We have previously shown that genomic loss of the tumour suppressors PTEN and Sprouty2 (SPRY2) co-occur in progressive prostate cancers including in treatment resistance (Gao *et al*, 2012; Patel *et al*, 2018; Patel *et al*, 2013), and that concurrent loss of PTEN and SPRY2 leads to aggressive treatment-resistant disease (Gao *et al.*, 2012; Patel *et al.*, 2018; Patel *et al.*, 2013).

Oncogenic metabolic rewiring mediated by loss of tumour suppressors is one of the hallmarks of cancer (Hanahan & Weinberg, 2011). Identifying, understanding and targeting the metabolic vulnerabilities triggered by loss of tumour suppressors is an appealing approach for designing interventions to tackle cancer growth and invasion. Aggressive cancers have high energy demands and may experience nutrient restricting conditions during rapid proliferative stages of tumorigenesis (Fenouille *et al*, 2017). The phosphagen system refers to high energy storage compounds and associated biosynthesis enzymes (Patra *et al*, 2012; Wallimann, 1994; Wyss & Kaddurah-Daouk, 2000). Cancers of liver and breast exploit the phosphagen system, the cellular energy buffering system, to meet their high energy demands (Qian *et al*, 2012).

73 The phosphagen system comprises of creatine, a nitrogen amine that can be

74 phosphorylated by creatine kinases to form phosphocreatine. In addition to uptake of

75 creatine from the circulation, creatine can also be synthesised *de novo* from arginine

76 and glycine, with S-adenosyl methionine (SAM) serving as the methyl donor (Bera *et*

77 *al*, 2008). One of the well-characterised roles of creatine is to act as a cellular energy

78 buffer by rapidly transferring energy through a reversible reaction catalysed by

79 creatine kinases. Creatine kinases play a central role in the energy metabolism of

80 cells that have high and fluctuating energy requirements by catalysing the reversible

81 transfer of the phosphoryl group from phosphocreatine to ADP to generate ATP

82 (Kazak & Cohen, 2020). Phosphocreatine serves as an easily diffusible energy

83 storage metabolite to regenerate ATP on demand by cytosolic creatine kinase

84 isoforms.

85 The role of creatine metabolism in prostate cancer has not been fully explored.

86 Here, using clinically relevant human and murine prostate cancer models, we have

87 investigated the role of combined PTEN and SPRY2 deficiency in mediating

88 metabolic reprogramming of prostate cancer cells. Our data point to enhanced

89 creatine uptake and biosynthesis in progressive prostate cancer. Importantly, we

90 identified SAM utilisation as an additional mechanism of how cancer cells may use

91 creatine metabolism beyond fuelling cellular bioenergetics to maintain cell growth.

92

RESULTS

Concurrent loss of PTEN and SPRY2 mediates prostate cancer progression

Heterozygous and homozygous genomic deletions of the tumour suppressors *PTEN* and *SPRY2* occur in up to half of metastatic prostate cancers (Figure 1A) (Abida *et al*, 2019; Patel *et al.*, 2013). Using a murine model of prostate cancer, we have previously shown that heterozygous loss of *Pten* and *Spry2* cooperate to drive prostate cancer progression and treatment resistance (Patel *et al.*, 2018; Patel *et al.*, 2013). To investigate the functional co-operation of *Spry2* deficiency with homozygous deletion of *Pten*, we generated a prostate-specific *Pten* null mouse model with heterozygous or homozygous deletion of *Spry2* using a conditional Cre-loxP system driven by *Probasin-Cre* (Patel *et al.*, 2013). The overall survival of mice with complete deletion of *Pten* and *Spry2* (*Pten^{pc-/-} Spry2^{pc-/-}*) was significantly shorter than mice with deletion of *Pten* (*Pten^{pc-/-}*) alone, deletion of *Spry2* (*Spry2^{pc-/-}*) alone or *Pten* deletion with heterozygous loss of *Spry2* (*Pten^{pc-/-} Spry2^{pc-/+}*) (Figure 1B). As observed before, *Spry2* loss alone did not lead to tumour formation (Patel *et al.*, 2013). At the study endpoint, *Pten* null mice with single or double copy loss of *Spry2* had significantly higher prostate tumour burden compared to mice with *Pten* deletion alone (Figure 1C, EV1A). *Pten* and *Spry2* null (*Pten^{pc-/-} Spry2^{pc-/-}*) tumours were also highly proliferative with stronger Ki67 staining than the other genotypes (Figure 1D-E).

Primary murine prostate cancer SP1 and SP2 cell lines were derived from two independent *Pten^{pc-/-} Spry2^{pc-/+}* double mutant tumours, and confirmed to have suppressed SPRY2 expression (Figure 1F, EV1B). As observed in murine tumours, SP1 and SP2 cells proliferated significantly faster than the P1 cells derived from a

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117   *Pten* null tumour (Figure 1G). Thus, concurrent loss of PTEN and SPRY2 tumour

118   suppressors enhances murine prostate tumorigenesis with increased cell

119   proliferation. Similarly, in PTEN deficient human PC3 prostate cancer cells, stable

120   knockdown of SPRY2 expression (CL1 and CL10) significantly promoted their soft

121   agar colony formation efficiency (Figure 1H-I, EV1C).

122

123   **PTEN and SPRY2 deficiency leads to increased creatine uptake through**

124   **SLC6A8**

125   We wished to test if there was evidence of metabolic rewiring in tumours driven by

126   concurrent loss of PTEN and SPRY2. Untargeted analysis of metabolites revealed

127   enriched creatine metabolism in $Pten^{pc-/-}$ $Spry2^{pc-/-}$ tumours as well as in SPRY2

128   deficient PC3 cells (Table S1, Figure EV2A). Targeted metabolomics analysis was

129   then carried out, and significant enrichment of creatine in SPRY2 deficient human

130   and murine prostate cancer cells was confirmed (Figure 2A-B).

131   To investigate if elevated levels of creatine is causative for enhanced tumour growth,

132   we tested the impact of creatine supplementation on tumour growth of *Pten* and

133   *Spry2* double deficient mice. We treated six months old $Pten^{pc-/-}$ $Spry2^{pc-/-}$ mice with

134   creatine for two months. Creatine treatment significantly increased the proliferation of

135   prostate tumours with enhanced Ki67 staining (Figure 2C-D, EV2B). Furthermore,

136   the creatine *SLC6A8* transporter was expressed at significantly higher levels in both

137   human and murine prostate cancer cells deficient for SPRY2 (Figure 2E-F). Cellular

138   creatine can be taken up via SLC6A8 as well as synthesised *de novo* from glycine

139   and arginine (EV2A). To further characterise the source of cellular creatine, we

140   stably knocked down SLC6A8 in SPRY2 deficient PC3 cells (Figure 2G). SLC6A8

141 deficiency significantly decreased cellular creatine levels in PC3 cells (Figure 2H).

142 Functionally, SLC6A8 deficiency significantly decreased soft agar colony formation

143 efficiency in SPRY2 deficient PC3 cells (Figure 2I), thus supporting the idea that

144 creatine uptake is required for SPRY2 deficient driven growth. In the MSKCC clinical

145 prostate cancer cohort (c-Bio portal) (Taylor *et al*, 2010), patients with high *SLC6A8*

146 expression were at risk of disease progression (Figure 2J), and expression of

147 *SPRY2* and *PTEN* negatively correlated with *SLC6A8* (EV2C-D). Collectively,

148 SLC6A8 mediated creatine uptake supports the growth of PTEN and SPRY2 double

149 deficient prostate cancer cells.

150

Cyclocreatine suppresses the phosphagen system

152 Cellular creatine and creatine kinases are components of the phosphagen system

153 (Ellington, 2001) which serves as a cellular energy buffering system to quickly

154 generate ATP on demand (Abida *et al.*, 2019). By efficiently storing high energy

155 phosphates as phosphocreatine, the phosphagen system may promote

156 tumorigenesis, especially in rapidly proliferating tumours with high energy demands.

157 Since creatine can stimulate mitochondrial respiration in certain tissues (Kazak *et al*,

158 2015), we investigated the effect of creatine on cellular respiration. Creatine

159 treatment significantly induced basal respiration in SPRY2 deficient PC3 cells (CL1

160 and CL10) (Figure EV2E-G). Consistent with creatine induced basal respiration, the

161 creatine kinase activity (namely generation of phosphocreatine and ADP from

162 creatine and ATP) was also elevated in SPRY2 deficient cells (Figure 3A). Despite

163 increased creatine kinase activity and creatine induced basal respiration, the cellular

164 ATP and phosphocreatine levels were not altered in SPRY2 deficient cells (Figure

165 3B-C).

166 Cyclocreatine is a creatine analogue that blocks the phosphagen system by

167 displacing creatine. We applied cyclocreatine treatment (1%) to investigate functional

168 effects of creatine metabolism. Cyclocreatine suppressed *in vitro* creatine kinase

169 activity (Figure 3A) and impaired *in vitro* proliferation of both human and murine

170 prostate cancer cells (Figure 3D-E). Furthermore, cyclocreatine treatment

171 significantly impaired the colony forming ability of PC3 cells (Figure 3F), along with

172 reduced cellular creatine and phosphocreatine levels (Figure 3G-H) while the ATP

173 levels were not altered (Figure 3I). Together these data suggest cyclocreatine

174 potently suppresses activities of the phosphagen system.

175

176 **Creatine metabolism regulates cellular S-adenosyl methionine (SAM) levels**

177 Consistent with the observation of enhanced creatine uptake in SPRY2 deficient

178 cells, in creatine proficient serum supplemented culture conditions, cyclocreatine

179 drastically reduced creatine uptake (Figure 4A). In addition, in serum-free culture

180 conditions, we observed enhanced creatine synthesis in SPRY2 deficient PC3 cells

181 (Figure 4A), which was accompanied by significantly higher levels of

182 phosphocreatine (Figure 4B). Treatment with cyclocreatine significantly reduced

183 cellular levels of creatine and phosphocreatine irrespective of SPRY2 status (Figure

184 4A-B), with both creatine uptake and biosynthesis suppressed. Collectively,

185 cyclocreatine treatment blocked creatine uptake, creatine biosynthesis and

186 phosphocreatine formation.

187   S-adenosyl methionine (SAM) is required for *de novo* creatine synthesis. In the

188   presence of enhanced cellular creatine synthesis, cellular SAM steady state level

189   may be reduced. We carried out targeted metabolomics analysis to study metabolic

190   changes associated with SPRY2 deficiency with and without cyclocreatine treatment.

191   In the absence of exogenous creatine, SPRY2 deficient cells (with enhanced

192   creatine biosynthesis) exhibited significantly reduced levels of SAM (Figure 4C left

193   side), consistent with increased SAM utilisation for creatine biosynthesis. We

194   therefore reasoned that blockade of creatine biosynthesis may reciprocally lead to

195   SAM accumulation. Indeed, cyclocreatine treatment significantly increased the

196   cellular level of SAM in PC3 cells regardless of SPRY2 status and culture condition

197   (Figure 4C right side, 4D). Consistent with a previous report (Schmidt *et al*, 2016),

198   SAM treatment decreased the growth of PC3 cells independent of SPRY2 status

199   (Figure 4E). Hence, in addition to the direct effects of reduced creatine levels,

200   cyclocreatine induced effects may result in part from the accumulation of SAM (due

201   to blockade of creatine biosynthesis).

202

203   **Effects on cyclocreatine treatment on *in vivo* prostate carcinogenesis**

204   To investigate the tumour suppressive effects of cyclocreatine, we treated prostate

205   tumour bearing $Pten^{pc-/-}$ $Spry2^{pc-/-}$ mice with 1% cyclocreatine for one month.

206   Cyclocreatine treatment significantly decreased tumour cell proliferation with reduced

207   Ki67 staining (Figure 5A-B), while the trend for reduced tumour weights did not reach

208   statistical significance (Figure 5C). Regardless of the tumour genotype, the process

209   of cancer metastasis is metabolically demanding for both energy and biomass. We

210   therefore applied a liver metastasis model with splenic injection of PC3M cells to test

211   the impact of manipulation of the SAM-creatine mediated metabolism by

212   cyclocreatine in metastasis. Treatment with 1% cyclocreatine for a month drastically

213   decreased liver metastatic burden of injected PC3M prostate cancer cells (Figure

214   5D-E).

215   Overall, our data highlighted the importance of creatine metabolism in prostate

216   cancer and identified a potential role of SAM utilisation during creatine biosynthesis

217   as an additional control of cancer growth (Figure 6).

DISCUSSION

Metabolic adaptations in cancers (including prostate cancer) may contribute to progressive tumour growth. Understanding oncogenic metabolic alterations will highlight relevant tumour metabolic vulnerability to inform novel strategies to overcome cancer progression. Hence, we investigated the metabolic changes observed in prostate cancer driven by clinically-relevant deficiency of PTEN and SPRY2. Building on previous research (Patel *et al.*, 2018; Patel *et al.*, 2013), combined loss of the tumour suppressors PTEN and SPRY2 was found to further enhance tumour growth and progression. Here we show that creatine metabolism (uptake and biosynthesis) supports the growth of PTEN and SPRY2 deficient prostate cancer while cyclocreatine mediated blockade of the phosphagen system suppressed proliferation.

Diet is a major source of creatine. Circulating creatine can enter cells via the bi-directional SLC6A8 creatine transporter. PTEN and SPRY2 deficient cells have upregulated SLC6A8 expression to maintain elevated cellular creatine levels required for enhanced proliferation. In addition, combined PTEN and SPRY2 deficiency also promotes creatine biosynthesis to sustain cell growth. We find that PTEN and SPRY2 deficient cells are able to utilise exogenous creatine to stimulate basal respiration, and exhibit elevated creatine kinase activity.

We have previously demonstrated that PTEN and SPRY2 deficiency drives prostate cancer progression through enhanced HER2 signalling (Gao *et al.*, 2012). HER2 mediated oncogenic signals can promote mitochondrial creatine kinase stability to enhance cellular bioenergetics (with increased phosphocreatine and cellular ATP levels) (Ben-Sahra & Puissant, 2018; Kurmi *et al*, 2018). Although we have not

11

242 directly investigated the expression status of creatine kinases, we reason that the

243 observed increased creatine kinase activity in PTEN and SPRY2 deficient prostate

244 cancer cells is likely a result of altered expression of creatine kinases. However, we

245 did not detect alterations in the overall levels of phosphocreatine and ATP in PTEN

246 and SPRY2 deficient cells, which may reflect dynamic flux of the phosphagen

247 system and tightly regulated nature of cellular energy homeostasis. Together and in

248 support of recent reports (Kazak & Cohen, 2020; Kazak *et al*, 2019; Tang *et al*,

249 2015), our data is consistent with a functional role of creatine metabolism beyond

250 energy buffering and ATP generation. Importantly, by blocking creatine uptake and

251 biosynthesis using cyclocreatine, we identified SAM utilisation as a potential

252 mechanism whereby changes in creatine metabolism may indirectly influence cancer

253 growth. The functional and clinical significance of altered SAM metabolism warrants

254 future investigations. Our data implicate cyclocreatine treatment in suppressing both

255 creatine uptake and biosynthesis, although it remains to be clarified whether the

256 observed effect on creatine biosynthesis represents a consequence of reduced

257 creatine utilisation due to functional inhibition of creatine kinases.

258 The concept of resistance training is being considered as means to tackle ADT-

259 induced loss of lean body mass. The idea of creatine supplement is studied as a

260 component of ongoing lifestyle intervention clinical trial in prostate cancer (Fairman

261 *et al*, 2019). Our observation of increased cellular proliferation in tumours from

262 *Pten*$^{pc-/-}$ *Spry2*$^{pc-/-}$ mice following creatine supplement for only eight weeks highlights

263 the need to assess creatine supplement in patients with caution.

264 Overall, we show that SPRY2 and PTEN double deficient prostate cancer cells

265 sustain growth by engaging creatine metabolism to stimulate cellular respiration and

266 regulate SAM levels. Furthermore, blockade of the phosphagen system produces

267   anti-tumorigenic effects regardless of the status of SPRY2. Therapy such as

268   cyclocreatine that targets the phosphagen system may bring about anti-tumour

269   effects through suppressed creatine metabolism and accumulation of cellular SAM.

270

271 **METHODS**

272 **Mice**

273 All the animal experiments were carried out with ethical approval from the University

274 of Glasgow under the revised Animal (Scientific Procedures) Act 1986 and the EU

275 Directive 2010/63/EU (PPL P5EE22AEE). The ARR2Probasin-Cre (Pb-Cre), *Pten*^{flox},

276 *Spry2*^{flox} (Patel *et al.*, 2018; Patel *et al.*, 2013) mice have been previously described,

277 and applied to generate the *Pten*^{pc-/-} *Spry2*^{pc-/-} experimental cohort. All mice in this

278 study were on a mixed background and were genotyped by Transnetyx™ using PCR

279 analysis of ear notch tissue. Mice were housed at 19°C to 23°C with a 12-hour light-

280 dark cycle, and were fed a conventional diet with mains water ad libitum. They were

281 housed in an enriched environment, with igloos, cardboard tubes and chewing sticks.

282 Genotype-specific male mice were handled and aged until experimental time-points

283 or ethically approved clinical endpoints (including palpable tumour with maximum

284 diameter of 1.2 cm).

285 To investigate the effects of creatine supplement on GEMM prostate tumour growth,

286 creatine (40 mg daily) (Sigma #C0780) was administered via gavage of 1% creatine

287 solution for two months. Similarly, 1% cyclocreatine (w/v) (2- imino-1-

288 imidazolidineaceticacid) (Sigma #377627) was supplied in drinking water *ad libitum*

289 for one month.

290 CD-1 Nudes 6-8 weeks old male mice were used to study the extent of liver

291 metastases following splenic injection of PC3M cells (five million cells per injection

292 into the spleen of each experimental mouse). 1% cyclocreatine (w/v) (2- imino-1-

293 imidazolidineaceticacid) (Sigma #377627) in drinking water was started one week

294 after tumour cell implantation *ad libitum* for one month. The ARRIVE guidelines were

295   used to design and execute experiments. Matastasis burden in the liver was

296   obtained histologically as previously described (Patel *et al.*, 2018).

297

Cell Lines

299   Human PC3 and PC3M cells were purchased from ATCC. PC3M cells are

300   metastasis-derived variant of human prostate cancer PC3 cells. Cell lines were

301   mycoplasma negative and authenticated by LGC standards. P1 murine prostate

302   cancer cells (RRID:CVCL_VQ82) were derived from a prostate tumour from a mouse

303   bearing $Pten^{pc-/-}$ tumour at 14 months. Similarly. SP1 and SP2 cell lines

304   (RRID:CVCL_VQ84; RRID:CVCL_VQ85) were generated from two $Pten^{pc-/-}$

305    $Spry2^{pc+/-}$ tumour bearing endpoint mice at 6 and 8 months, respectively. Cell lines

306   were routinely grown *in vitro* in media (RPMI for PC3 and PC3M and DMEM for P1,

307   SP1 and SP2) supplemented with 10% FBS and 2 mM glutamine.

308   All siRNA and plasmid transfection experiments in PC3 cells were performed using

309   nucleofection (Lonza-kit V)(see supplementary information for details). For stable

310   SPRY2 KD, a 19-mer SPRY2 target sequence (5′-AACACCAATGAGTACACAGAG-

311   3) (QIAGEN) was used and for the control a 19-mer NSi control sequence (QIAGEN)

312   was used. Stable expression of ShSPRY2 and ShNSi in human PC3 cells was

313   achieved using a plasmid pTER+ to insert the sequence of interest and selected

314   using zeocin (300 μg/ml).

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LR, RP, CAF, OJS and HYL designed research; RP, LR, CAF, JF, EM, TZ, DW, GM, and DS, performed research; LR, CAF, RP, LKR and HYL analysed and reviewed data; and LKR, CAF, RP and HYL wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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393 **Figure legends**

394 **Figure 1. Concomitant PTEN and SPRY2 deficiencies drive prostate cancer**

395 **progression**

396 (A) *PTEN* and *SPRY2* genomic alterations in metastatic prostate cancer patients

397 (SU2C/PCF Dream Team, PNAS 2019).

398 (B) Kaplan–Meier plot for overall survival of indicated mice (*p<0.05, *Pten^{pc-/-} Spry2^{pc-/-}*

399 compared to *Pten^{pc-/-}*; Log-rank Mantel-Cox test).

400 (C) Non-cystic prostate tumour weights from indicated mice at clinical endpoints (n=7

401 mice for each group; mean values ± SD are shown; *p<0.05 compared to *Pten^{pc-/-}*; 1-

402 way ANOVA with Tukey's multiple comparison test).

403 (D) Representative H&E and Ki67 images of prostate tumour sections from *Pten^{pc-/-}*,

404 *Pten^{pc-/-} Spry2^{pc-/+}* and *Pten^{pc-/-} Spry2^{pc-/-}* mice (n=5 mice for each group; scale bar =

405 100 μm).

406 (E) IHC quantification of Ki67 in prostate tumours as indicated (n=5 mice for each

407 group; mean values ± SD are shown; *p<0.05 compared to *Pten^{pc-/-}*; 1-way ANOVA

408 with Tukey's multiple comparison test).

409 (F) Relative mRNA levels of *Spry2* in primary murine prostate cancer cells, as

410 indicated. P1 derived from *Pten^{pc-/-}*; SP1 and SP2 derived from two independent

411 *Pten^{pc-/-} Spry2^{pc-/+}* prostate tumours (n=3 independent experiments; mean values ±

412 SD are shown; *p<0.05 compared to P1; 1-way ANOVA with Dunnett's multiple

413 comparison test).

414   (G) Growth of indicated cells (n=3 independent experiments; mean values ± SEM

415   are shown; *p<0.05 compared to P1; 2-way ANOVA with Tukey's multiple

416   comparison test).

417   (H) Relative mRNA levels of *SPRY2* in PC3 human prostate cancer cell lines as

418   indicated. Nsi is PC3 cells with stable expression of non-silencing vector control; CL1

419   and CL10 are PC3 clones with stable knockdown (KD) of SPRY2 (n=3 independent

420   experiments; mean values ± SD are shown; *p<0.05 compared to Nsi; 1-way

421   ANOVA with Dunnett's multiple comparison test).

422   (I)   Soft agar colony quantifications of the indicated cells (data shown are

423   representative of n=3 independent experiments; mean values ± SD are shown;

424   *p<0.05 compared to Nsi; 1-way ANOVA with Dunnett's multiple comparison test).

425

426   **Figure 2. Increased cellular creatine sustains elevated growth rate of SPRY2**

427   **deficient cells**

428   (A-B) Relative cellular creatine levels in human (A) and murine (B) prostate cancer

429   cells (n=3 independent experiments; mean values ± SD are shown; *p<0.05

430   compared to Nsi and P1, respectively; unpaired t-test).

431   (C) Representative H&E and Ki67 images of prostate tumour sections from $Pten^{pc-/-}$

432    $Spry2^{pc-/-}$ mice treated for two months with vehicle (VC) or 1% creatine. (n=5 mice for

433   each group; scale bar = 100 μm).

434   (D) IHC quantification of Ki67 in prostate tumours from $Pten^{pc-/-}$ $Spry2^{pc-/-}$ mice treated

435   for two months with vehicle (VC) or 1% creatine (n=5 mice for each group; mean

436   values ± SD are shown; *p<0.05; Mann-Whitney test).

20

437 (E) Relative levels of creatine transporter, *Slc6a8* mRNA in murine prostate cancer

438 cells (data shown are representative of n=3 independent experiments; mean values

439 ± SD are shown; *p<0.05 compared to P1; 1-way ANOVA with Dunnett's multiple

440 comparison test).

441 (F) Relative levels of creatine transporter, *SLC6A8* in human prostate cancer cells

442 (n=3 independent experiments; mean values ± SD are shown; *p<0.05 compared to

443 Nsi; 1-way ANOVA with Dunnett's multiple comparison test).

444 (G) Relative levels of *SLC6A8* mRNA in PC3 Nsi and SPRY2 KD CL10 with SLC6A8

445 KD (n=3 independent experiments; mean values ± SD are shown; *p<0.05 compared

446 to Nsi CTRL & #p<0.05 compared to respective CTL cells; 1-way ANOVA with

447 Tukey's multiple comparison test).

448 (H) Relative cellular creatine levels in PC3 Nsi and SPRY2 KD CL10 with SLC6A8

449 KD (n=3 independent experiments; mean values ± SD are shown; *p<0.05 compared

450 to Nsi CTRL & #p<0.05 compared to respective CTL cells; 1-way ANOVA with

451 Tukey's multiple comparison test).

452 (I) Number of soft agar colonies of PC3 Nsi and SPRY2 KD CL10 with SLC6A8 KD

453 (data shown are representative of n=3 independent experiments; mean values ± SD

454 are shown; *p<0.05 compared to Nsi CTRL & #p<0.05 compared to respective CTRL

455 cells; 1-way ANOVA with Tukey's multiple comparison test).

456 (J) Kaplan–Meier plot for progression-free survival in MSKCC, Cancer Cell 2010

457 (Taylor *et al.*, 2010) prostate cancer dataset showing cases with high (more than z-

458 score= 1.8; n=6) or low (less than z-score= 1.8; n=74) expression of *SLC6A8*; log-

459 rank Mantel-Cox test.

21

460

Figure 3. Phosphagen system in SPRY2 deficient cells

(A) Creatine kinase activity (conversion of creatine and ATP to phosphocreatine and ADP) in control and 1% cyclocreatine (CC) treated PC3 Nsi and SPRY2 KD clones CL1 and CL10 (n=3 independent experiments; mean values ± SD are shown; *p<0.05 compared to Nsi; #p<0.05 comparing 1% CC treated with untreated; 2-way ANOVA with Holm-Sidak's multiple comparison test).

(B-C) Relative levels of cellular ATP (B) and phosphocreatine (C) in PC3 Nsi and SPRY2 KD clones CL1 and CL10 (n=3 independent experiments; mean values ± SD are shown).

(D-E) *In vitro* growth of human PC3 (E) and murine (F) cells treated with control (CTRL) or 1% cyclocreatine (CC) (n=3 independent experiments; mean values ± SD are shown; **p<0.005, ***p<0.0005 comparing CC treatment with respective CTRL cells; unpaired t-test).

(F) Soft agar colony quantifications of PC3 Nsi and SPRY2 KD clones (CL1 and CL10) treated with control or 1% cyclocreatine (CC) containing medium (data shown are representative of n=2 independent experiments; mean values ± SD are shown; *p<0.05 compared to Nsi CTRL & #p<0.05 compared to respective CTRL cells; 1-way ANOVA with Tukey's multiple comparison test).

(G-I) Relative levels of cellular creatine (H), phosphocreatine (I) and ATP (J) in control (CTRL) or 1% cyclocreatine (CC) treated PC3 Nsi and SPRY2 KD clones CL1 and CL10 (n=3 independent experiments; mean values ± SD are shown;

22

482   *p<0.05 compared to Nsi CTRL & #p<0.05 compared to respective CTL cells; 1-way

483   ANOVA with Tukey's multiple comparison test).

484

485   **Figure 4. Cyclocreatine treatment induces growth arrest and SAM**

486   **accumulation**

487   (A-B) Relative levels of cellular creatine (A) and phosphocreatine (B) in PC3 Nsi and

488   SPRY2 KD clone CL10 grown in either control or 1% cyclocreatine (CC) containing

489   serum free medium (SFM) or medium with 10% serum (FM) (n=3 technical

490   replicates; mean values ± SD are shown; *p<0.05 compared to Nsi CTRL & #p<0.05

491   compared to respective CTL cells; 2-way ANOVA with Tukey's multiple comparison

492   test).

493   (C) Relative levels of cellular SAM in PC3 Nsi and SPRY2 KD clone CL10 grown in

494   either control or 1% cyclocreatine (CC) containing serum free medium (SFM) or

495   medium with 10% serum (FM) (n=3 technical replicates; mean values ± SD are

496   shown; *p<0.05 compared to Nsi CTRL & #p<0.05 compared to respective CTL

497   cells; 2-way ANOVA with Tukey's multiple comparison test).

498   (D) Relative levels of cellular S-adenosyl methionine (SAM) PC3 Nsi and SPRY2 KD

499   clones (CL1 and CL10) treated as indicated (n=3 independent experiments; mean

500   values ± SD are shown; #p<0.05 compared to respective CTL cells; 1-way ANOVA

501   with Tukey's multiple comparison test).

502   (E) Growth of indicated cells in the presence of 200 µM S-adenosyl methionine

503   (SAM) relative to respective cells treated with control medium (n=3 independent

23

504   experiments; mean values ± SD are shown; *p<0.05 compared to respective CTRL

505   cells; Multiple t-tests using Holm-Sidak method).

506

507   **Figure 5. Cycocreatine treatment and *in vivo* prostate cancer progression**

508   (A) Representative Ki67 images of prostate tumour sections from $Pten^{pc-/-}$ $Spry2^{pc-/-}$

509   mice treated for one month with vehicle (VC) or 1% cyclocreatine (n=5 mice for each

510   group; scale bar = 100 μm).

511   (B) IHC quantification of Ki67 in prostate tumours from $Pten^{pc-/-}$ $Spry2^{pc-/-}$ mice treated

512   for two months with vehicle (VC) or 1% cyclocreatine (CC) (n=5 mice for each group

513   mean values ± SD are shown; *p<0.05; Mann-Whitney test).

514   (C) Non-cystic prostate tumour weights from $Pten^{pc-/-}$ $Spry2^{pc-/-}$ mice treated for one

515   month with vehicle (VC; n=5) or 1% cyclocreatine (CC; n=6); mean values ± SD are

516   shown).

517   (D) Representative images of PC3M liver metastases in CD-1 nude mice treated with

518   control or 1% cyclocreatine (CC) water (*ad libitum*) for one month (n=5 mice for each

519   group; arrows indicate liver metastases).

520   (E) Percentage PC3M liver metastases burden in CD-1 nude mice treated with

521   control or 1% cyclocreatine (CC) water (*ad libitum*) for one month (n=5 mice for each

522   group; mean values ± SEM are shown; *p<0.05; Mann-Whitney test).

523

524   **Figure 6. Schematic representation of creatine metabolism in prostate cancer**

525   **and how cyclocreatine (CC) treatment suppresses prostate carcinogenesis.**

24

526

Expanded View Figure Legends

Figure EV1. Concomitant PTEN and SPRY2 deficiencies in prostate tumours

(A) Representative prostate tumour images from the indicated mice (n=7 mice for each group; scale bar = 1 cm).

(B) Western blot showing Sprouty2 expression in murine P1, SP1 and SP2 cells (data shown are representative of n=3 independent experiments; GAPDH is used a loading control).

(C) Western blot showing Sprouty2 expression in PC3 human prostate cancer cell lines as indicated human (data shown are representative of n=4 independent experiments; HSC70 is used as a loading control).

537

Figure EV2. Creatine metabolism in prostate cancer

(A) Schematic of creatine biosynthesis and metabolism: Creatine can be synthesised from glycine and arginine. Alternatively, creatine can be taken up by cells from circulation through the creatine transporter SLC6A8. Cellular creatine can be converted to phosphocreatine, which may be utilised as cellular energy buffer generating ATP upon demand.

(B) Non-cystic prostate tumour weights from $Pten^{pc-/-}$ $Spry2^{pc-/-}$ mice treated for two months with vehicle (VC; n=5) or 1% creatine (n=6) (mean values ± SD are shown).

(C-D) Correlation analyses of *SLC6A8* with *SPRY2* (C) and *PTEN* (D) mRNA in MSKCC, Cancer Cell 2010 (Taylor *et al.*, 2010) prostate cancer dataset.

548   (E-G) Oxygen consumption rate in PC3 Nsi (E), SPRY2 KD CL1 (F) and SPRY2 KD

549   CL10 (G) treated as indicated (n=4 independent experiments; mean values ± SEM

550   are shown; *p<0.05 compared to CTRL; 2-way ANOVA with Tukey's multiple
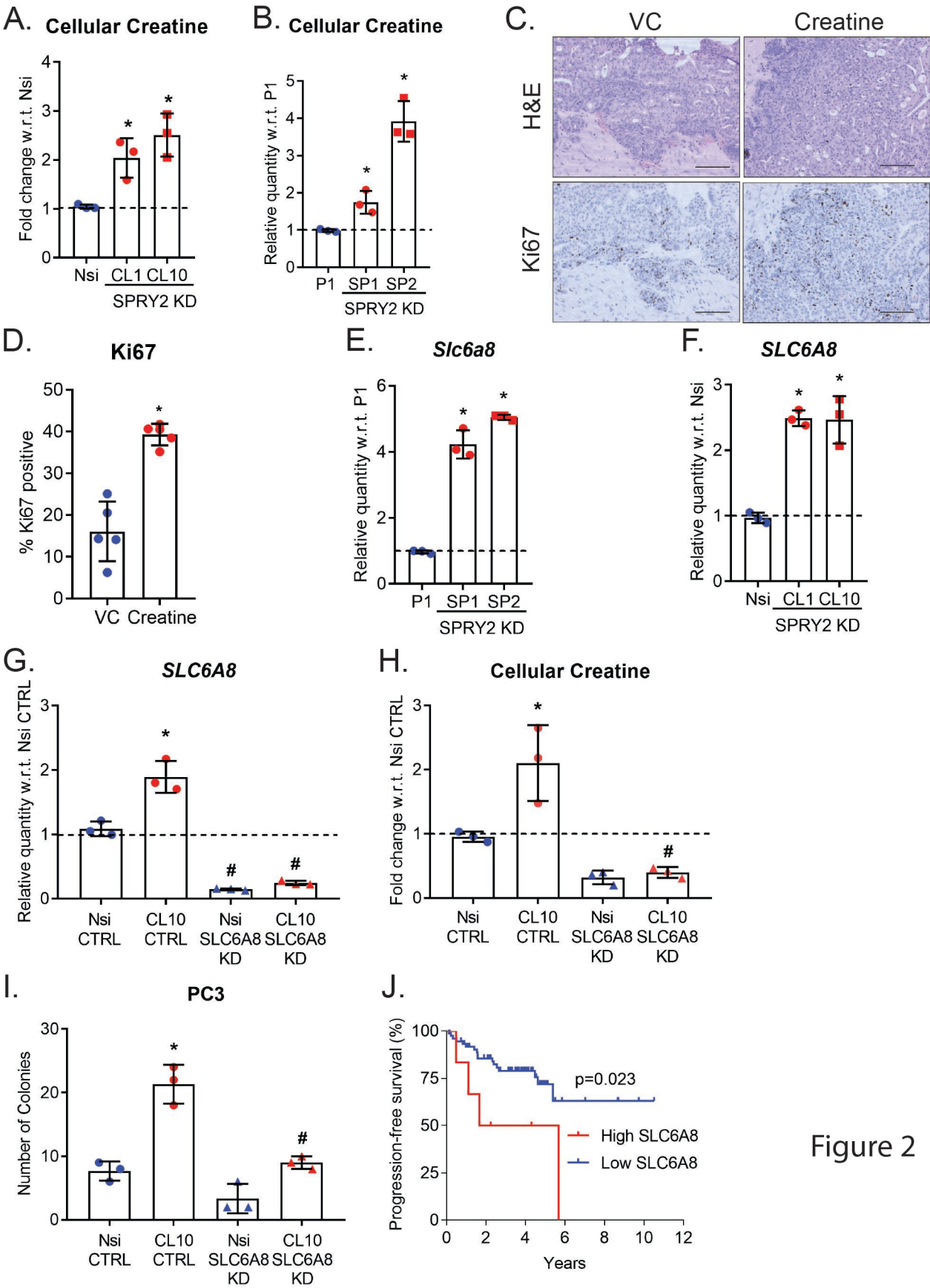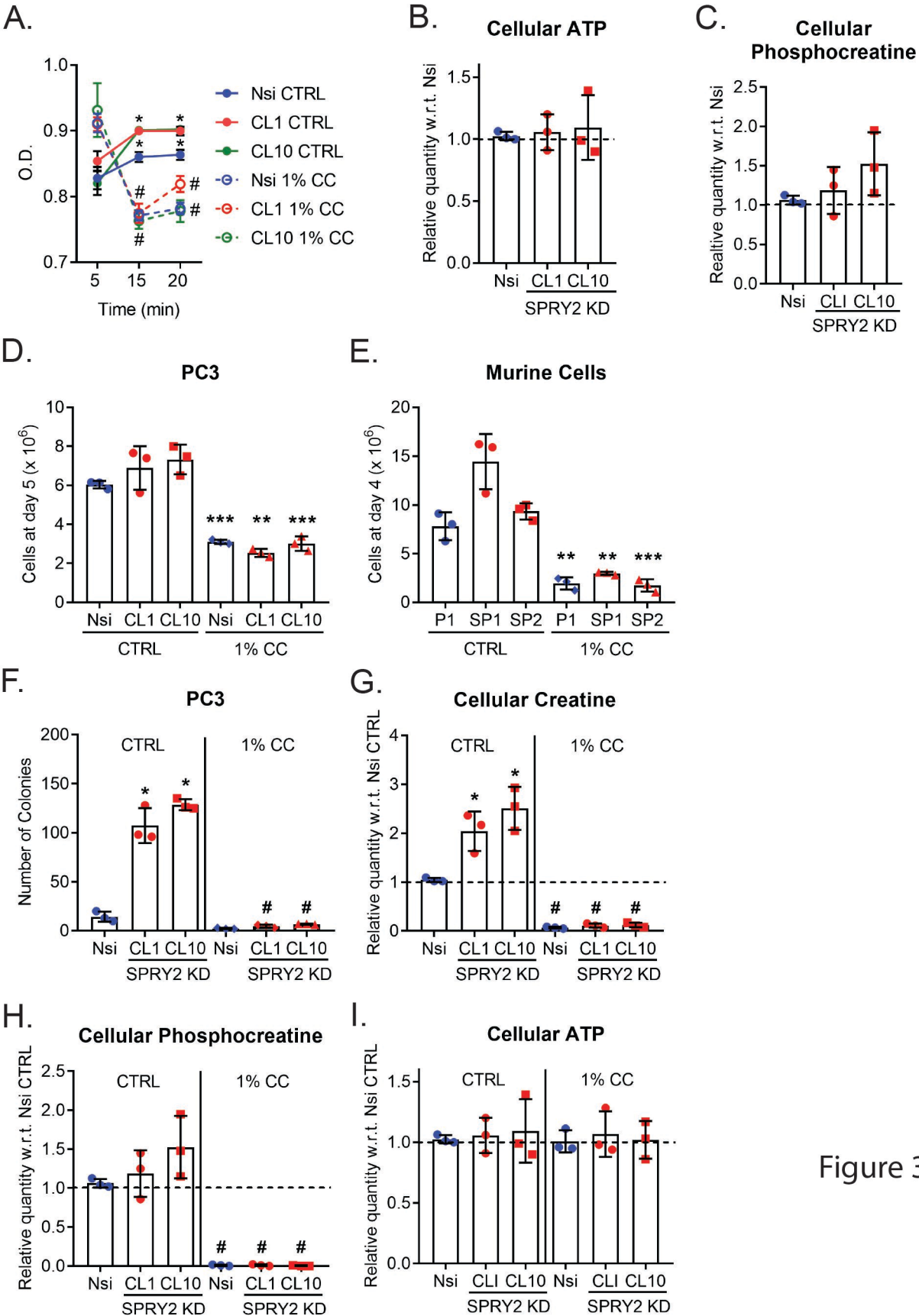
551   comparison test).
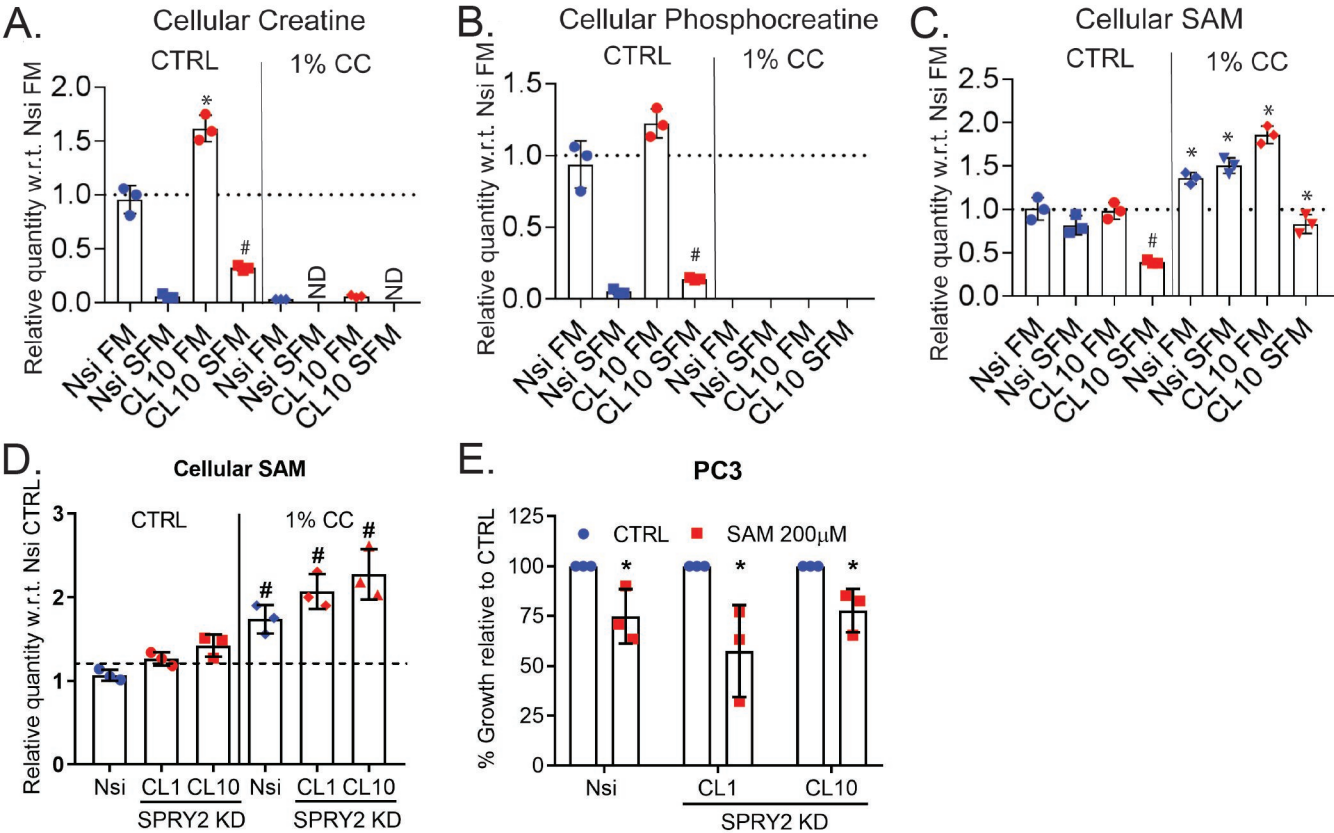
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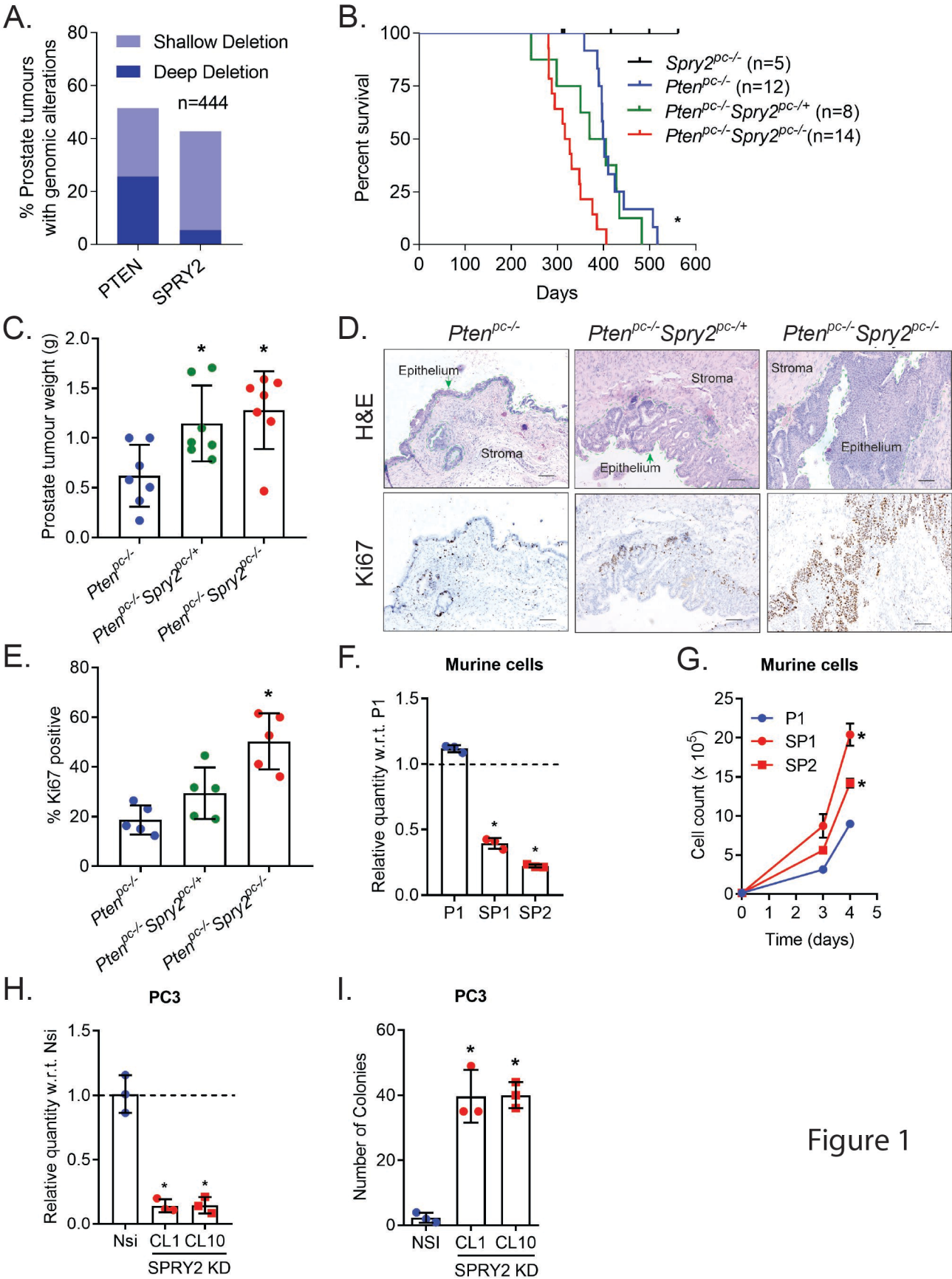
Figure 1

Figure 2

Figure 3

A. Cellular Creatine

B. Cellular Phosphocreatine

C. Cellular SAM

D. Cellular SAM

E. PC3

Figure 4

A. $Pten^{pc-/-}Spry2^{pc-/-}$

VC

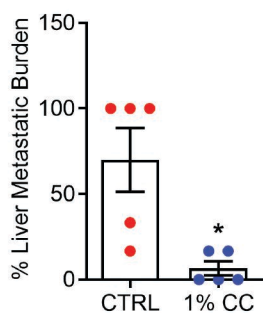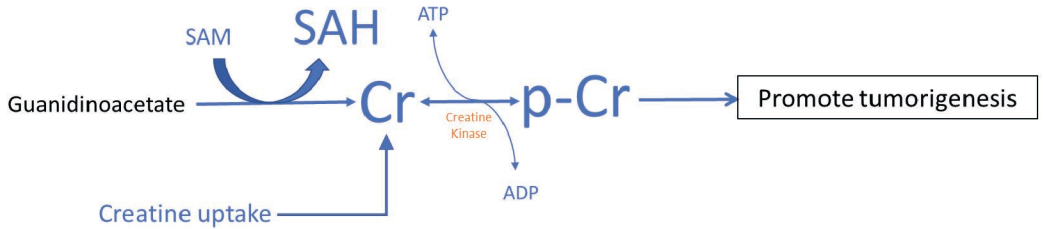1% CC
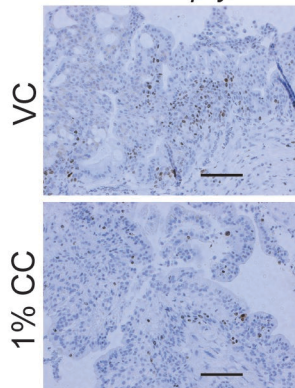
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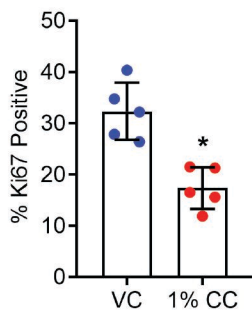
C.
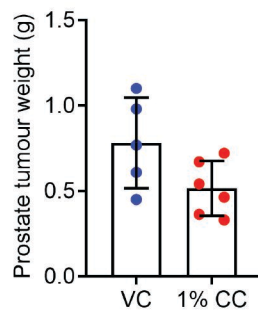
D. Prostate cancer liver metastases model

CTRL

1% CC

E.

Figure 5

Figure 6