

Methylation-mediated silencing of protein kinase C zeta induces apoptosis avoidance through ATM/CHK2 inactivation in dedifferentiated chondrosarcoma

島田, 英二郎

<https://hdl.handle.net/2324/4784510>

出版情報 : Kyushu University, 2021, 博士 (医学), 課程博士
バージョン :

権利関係 : Public access to the fulltext file is restricted for unavoidable reason (2)



**Methylation Mediated Silencing of Protein Kinase C Zeta Induces Apoptosis Avoidance
through ATM/CHK2 Inactivation in Dedifferentiated Chondrosarcoma**

**Eijiro Shimada¹, Yoshihiro Matsumoto¹, Makoto Nakagawa¹, Yosuke Susuki², Makoto Endo¹,
Nokitaka Setsu¹, Toshifumi Fujiwara¹, Keiichiro Iida¹, Akira Nabeshima¹, Kenichiro Yahiro¹,
Atsushi Kimura¹, Takeshi Hirose^{1,3}, Masaya Kanahori¹, Ryunosuke Oyama¹, Yoshinao Oda²,
Yasuharu Nakashima¹**

¹Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka, 812-8582, Japan

²Department of Anatomic Pathology, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka, 812-8582, Japan

³Division of Cellular Signaling, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, 104-0045, Japan

[Corresponding author]

Yoshihiro Matsumoto

Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University

Address: 3-1-1, Maidashi, Higashi-ku, Fukuoka, Fukuoka, 812-8582, Japan

Phone: +81-92-642-5488

Email: ymatsu@ortho.med.kyushu-u.ac.jp

ORCID: 0000-0002-0039-2855

22 Abstract

23 **Background** Dedifferentiated chondrosarcoma (DDCS) is an aggressive bone tumour with poor
24 prognosis and no effective treatment. Because changes in DNA methylation play critical roles in DDCS,
25 we explored the roles that DNA methylation plays in oncogenesis to potentially identify an effective
26 epigenetic treatment.

27 **Methods** We identified genes downregulated in DDCS vs. conventional chondrosarcoma (CCS) due
28 to DNA methylation using *in silico* analysis. The results were validated in DDCS clinical samples, and
29 the molecular functions of the genes of interest were investigated in multiple chondrosarcoma cell
30 lines (NDCS-1, SW1353, and OUMS-27). The therapeutic effect of decitabine, a DNA
31 methyltransferase inhibitor, was evaluated *in vitro* and *in vivo*.

32 **Results** PRKCZ was specifically downregulated by DNA methylation in DDCS. Overexpression of
33 PRKCZ decreased the proliferation of NDCS-1 and SW1353 cells. PRKCZ directly bound to and
34 activated ATM, which was followed by phosphorylation of CHK2 and subsequent apoptosis.
35 Decitabine increased PRKCZ expression through de-methylating the promoter region of *PRKCZ*,
36 which activated the ATM/CHK2 pathway and inhibited cell proliferation by inducing apoptosis.

37 **Conclusions** Increased DNA methylation and reduced expression of PRKCZ prevents apoptosis via
38 inactivation of the ATM/CHK2 pathway in DDCS. Decitabine-induced expression of PRKCZ
39 represents a promising therapy for DDCS.

40

41 **Background**

42 Chondrosarcoma, the second most common primary sarcoma of bone, is characterised by the
43 production of a cartilage matrix. Chondrosarcoma is divided into several subtypes: conventional (CCS),
44 dedifferentiated (DDCS), mesenchymal, and clear-cell.¹ The genetic characteristics differ for each
45 subtype. Mutations in isocitrate dehydrogenase (IDH) 1 or 2 are characteristic markers for CCS and
46 DDCS, but not for the other subtypes.^{1,2} By contrast, the presence of a fusion gene is common for the
47 mesenchymal subtype, but not for the other subtypes.¹ DDCS, which has a high-grade dedifferentiated
48 component occurring adjacent to a low-grade chondrogenic component, comprises ~10% of
49 chondrosarcoma.³ The prognosis for patients with DDCS is much poorer than for those with other
50 chondrosarcoma subtypes; the five-year survival rates for patients with DDCS, CCS, mesenchymal,
51 and clear-cell are 0–13%, 70%, 48%, and 100%, respectively.⁴⁻⁶ Despite the poor prognosis, there is
52 no established and efficacious systemic therapy for DDCS other than clinical resection.² Hence, there
53 is an urgent need for more effective systemic treatment for this cancer.

54 Several retrospective reports describe the activity of a variety of sarcoma-focussed cytotoxic agents,
55 including adriamycin and ifosfamide, in combination with surgical resection of DDCS; however, the
56 prognosis following such approaches remains generally poor.⁷ Several candidate molecularly targeted
57 medicines, such as mTOR inhibitor,⁸ CDK4/6 inhibitor, multi-kinase inhibitor,⁹ and Hedgehog
58 pathway inhibitor,^{10,11} have been proposed for the treatment of chondrosarcoma, including DDCS;
59 however, their clinical efficacy has been unsatisfactory. Consequently, alternative approaches other
60 than cytotoxic agents and molecularly targeted medicines are needed.

61 Mutation of IDH induces DNA methylation due to excessive accumulation of 2-hydroxyglutarate.^{12,13}
62 Aberrant DNA methylation, which is detected in various types of cancer, regulates the biological
63 behaviour of cancers by suppressing the transcription of specific genes.¹⁴ In addition, a multi-omics
64 molecular profiling study of patients with chondrosarcoma revealed that DNA hyper-methylation is
65 associated with worse prognosis of chondrosarcoma.¹⁵ Accordingly, inhibitors of mutant IDH exert

tumour-suppressive activity in chondrosarcoma *in vitro* and *in vivo*, which is concomitant with DNA demethylation.¹⁶⁻¹⁸ For the same reason, DNA methyltransferase inhibitors (DNMTis) were thought to suppress chondrosarcoma growth; however, whether DNMTis inhibit or exacerbate oncogenicity remains controversial.¹⁹⁻²³ In addition, no studies have focused on the activity of DNMTis in DDCCS. In this study, by analysing comprehensive data sets from patients with chondrosarcoma, we identified genes downregulated by DNA methylation in DDCCS relative to CCS. In particular, we identified *PRKCZ*, which encodes the atypical PKC isoform protein kinase C zeta,²⁴ as one of the genes of interest. The role *PRKCZ* plays in oncogenesis varies depending on the tumour histology²⁵⁻³²; no previous studies have reported a relationship between *PRKCZ* and the oncogenesis of DDCCS. Hence, we investigated the biological roles of *PRKCZ* in DDCCS. In addition, we evaluated the therapeutic efficacy of decitabine, a well-known DNMTi, on DDCCS *in vitro* and *in vivo*.

77

78 **Methods**

79

80 **Bioinformatic analysis**

81 Gene expression, methylation, and clinical data of chondrosarcoma were collected from Gene
82 Expression Omnibus (National Center for Biotechnology Information,
83 <https://www.ncbi.nlm.nih.gov/geo/>) and ArrayExpress (European Bioinformatics Institute,
84 <https://www.ebi.ac.uk/arrayexpress/>). Values were subjected to quantile normalization and divided into
85 two groups: CCS and DDCCS. The methylation difference, the fold changes, and standard errors were
86 estimated by fitting a linear model for each gene using the R package *limma*. When processed data
87 were available, they were analysed with GEO2R (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>).
88 Thresholds were as follows: adjusted P value, 0.05; methylation difference 0.2 for hypermethylation;
89 log fold change -0.5 for hypoeexpression.

90

91 **Whole-genome bisulfite sequencing (WGBS) library construction & sequencing**

92 Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Integrity
93 of genomic DNA was checked by agarose gel electrophoresis, and gDNA was quantified using Quant-
94 IT PicoGreen (Invitrogen, Carlsbad, CA, USA). WGBS libraries were prepared using Accel-NGS
95 Methyl-Seq DNA library kit (Swift BioSciences). Briefly, 200 ng of genomic DNA was fragmented
96 on a Covaris LE220 focused-ultrasonicator (Covaris, Woburn, MA, USA) to a target peak size of 400-
97 700 bp. The fragmented DNA was then subjected to bisulfite conversion using the EZ DNA
98 Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). The bisulfite-treated ssDNA fragments
99 were repaired, and truncated adapters 1 and 2 were ligated to the 3' and 5' ends of the fragments,
100 respectively. The truncated adapter-ligated DNA was amplified with an indexed primer to complete
101 the WGBS libraries with full-length adapters. The final libraries were then quantified by qPCR
102 according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina
103 Sequencing platforms), and quality was checked using TapeStation DNA ScreenTape D1000 (Agilent).
104 Sequencing was performed on the NovaSeq6000 platform (Illumina, San Diego, CA, USA).

105

106 **Data processing and methylation profile calling**

107 Sequence quality of paired-end sequencing reads (151 bp) generated from WGBS libraries was verified
108 with *FastQC*. Before analysis, *TrimGalore* was used to remove adapter sequences. The cleaned reads
109 were aligned to the *Homo sapiens* genome (hg38) using *Bismark*. Mapped data (BAM file format)
110 were sorted and indexed using *Samtools*. Methylation level was called with the R package *methyKit*.
111 Methylation of the promoter region was called for 1000 bases before and after the transcription start
112 site of each gene. WGBS data are available at GEO (accession number: GSE185124).

113

114 **Targeted bisulfite sequencing**

115 Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). Bisulfite treatment of genomic

116 DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research). After the bisulfite
117 treatment, methylated cytosine (C) of CpG remained as C, whereas non-methylated C was converted
118 into uracil. Polymerase chain reaction (PCR) was performed with TaKaRa EpiTaq™ HS (Takara Bio,
119 Kusatsu, Japan). Primers are listed in Supplementary Table S1. Following agarose gel electrophoresis,
120 the bands of PCR products were cut out of the gel and purified using the QIAquick Gel Extraction Kit
121 (Qiagen). PCR products were inserted into pUC19 DNA (Takara Bio) and cloned using *E. coli* DH5α
122 Competent Cells (Takara Bio). Sequencing reactions were performed in a BioRad DNA Engine Dyad
123 PTC-220 Peltier Thermal Cycler using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing
124 Kits (Applied Biosystems). Single-pass sequencing was performed on each template. Fluorescently
125 labeled fragments were purified from unincorporated terminators by ethanol precipitation. The
126 samples were resuspended in distilled water and subjected to electrophoresis on an ABI 3730xl
127 sequencer (Applied Biosystems). Assays were performed in five clones to calculate the methylation
128 percentage.

129

130 **Reagents**

131 Decitabine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Chloroquine diphosphate
132 was purchased from FUJIFILM Wako (Osaka, Japan). Dulbecco's phosphate-buffered saline (PBS)
133 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Decitabine and chloroquine
134 diphosphate was dissolved in PBS. For proliferation assays, decitabine or PBS were administered at
135 the time of seeding. For DNA extraction, RNA extraction, protein extraction, and cell cycle assays,
136 decitabine or PBS were administered at 0 and 48 hours after seeding. Cells were harvested 96 hours
137 after seeding. Chloroquine diphosphate was administered at a concentration of 400 μM for 3 hours.

138

139 **Cell lines and culture**

140 Each cell line was maintained at 37 °C in an atmosphere of 5% CO₂. NDCS-1 (CVCL_EJ23) was

kindly provided by Dr. Akira Ogose³³ and maintained in Roswell Park Memorial Institute 1640 (RPMI 1640, Thermo Fisher Scientific). OUMS-27 (CVCL_3090) was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). SW1353 (CVCL_0543) was obtained from the American Type Culture Collection (Manassas, Virginia, USA). OUMS-27 and SW1353 were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific). Each medium was supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin. OUMS-27 and SW1353 was confirmed with STR DNA fingerprinting. NDCS-1 was not registered in the database. All cell lines were used after confirmation of non-contamination with *Mycoplasma* using the EZ-PCR Mycoplasma detection kit (Biological Industries, Kibbutz Beit-Haemek, Israel).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RNA of cell line was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and RNA of frozen tissue was extracted using TRIzol (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using a Prime-Script™ RT reagent Kit (Takara Bio). RT-qPCR was performed on a LightCycler 2.0 (Roche, Basel, Switzerland) with TB Green Premix Ex Taq II (Takara Bio). mRNA levels of protein kinase C zeta (*PRKCZ*), ataxia telangiectasia mutated (*ATM*), collagen type II alpha 1 chain (*COL2A1*), aggrecan (*ACAN*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were measured; relative expression was determined by the delta-delta Ct method. Assays were performed in triplicate. Primers are listed in Supplementary Table S1.

Western blotting

Whole-cell protein extracts were obtained using CellLytic M (Sigma-Aldrich) containing protease inhibitor cocktail tablets (cOmplete™; Roche) and phosphatase inhibitor cocktail (PhosSTOP™; Roche). Western blot analysis was performed as described previously.³⁴ Antibodies and their dilutions

are listed in Supplementary Table S2. Semi-quantitation was performed on CS Analyzer 3·0 (ATTO, Tokyo, Japan).

Clinical samples

Clinical samples were obtained from 17 DDCS patients and nine CCS patients who underwent surgical resection between 1982 and 2018 at the Graduate School of Medical Science, Kyushu University, Fukuoka, Japan. In each case, a diagnosis was made on the basis of histologic features. All patients provided written informed consent before undergoing the study procedures. The Institutional Review Board at Kyushu University approved the use of human specimens for this study (approval number: 27-420). This study was conducted in accordance with the Declaration of Helsinki. The demographics of patients are listed in Supplementary Table S3.

Immunohistochemistry (IHC) staining

All samples were fixed in 4% paraformaldehyde (FUJIFILM Wako) and embedded in paraffin. After being deparaffinized in xylene and dehydrated in a graded ethanol series, sections were pretreated with TE buffer (10 mM Tris-HCl and 1 mM EDTA) in an autoclave at 98 °C for 25 min, and then incubated with primary antibody at 4 °C overnight. Anti-PRKCZ antibody (HPA021851, Sigma-Aldrich, St. Louis, MO, USA) was used as the primary antibody. Samples were incubated with Dako EnVision Dual Link System-HRP (Agilent Technologies, Santa Clara, CA, USA), visualized using the diaminobenzidine substrate system (FUJIFILM Wako), and counterstained with diluted hematoxylin. All images were captured a BZ-X800 microscope (Keyence, Osaka, Japan) and the percentage of positive cells were calculated by Hybrid Cell Count Software (Keyence). Staining intensity was classified into four classes in each of the cartilaginous and dedifferentiated components. Evaluation of intensity was performed by two bone oncologists who were blinded to the clinical information.

191 **Vector construction and transfection**

192 The full-length cDNA of PRKCZ was generated from cDNA of HEK293 cell line by PCR and gel
193 extraction. Primers are listed in Supplementary Table S1. The PRKCZ overexpression vector was
194 created by inserting full-length PRKCZ cDNA into pCDH-EF1-MCS-IRES-Puro (System Biosciences,
195 Palo Alto, CA, USA). GFP-tagged PRKCZ vector was created by inserting full-length *PRKCZ* cDNA
196 into pEGFP-N1-FLAG (Addgene, Totton, UK). Plasmids without inserts were used as control
197 vectors. Plasmids were transfected into cells using Lipofectamine3000 (Invitrogen).

198

199 **Lentiviral vector production and infection**

200 Vectors were co-transfected with Lentivirus Package plasmid mix (System Biosciences) into 293TN
201 cells (System Biosciences) using Lipofectamine3000 (Invitrogen). Culture supernatants containing
202 lentivirus particles were collected 48 h post-transfection. The supernatant was concentrated using
203 PEG-it Virus Precipitation Solution (System Biosciences), and the concentrated supernatant was used
204 to infect target cells. Cells were plated at a density of 5×10^4 cells per well in 24-well plates
205 containing culture medium supplemented with 10% FBS, without antibiotics. After incubation for 24
206 h, the cells were transfected with lentivirus particles using the TransDux Virus Transduction Reagent
207 (System Biosciences). Two days after transfection, puromycin was added to the medium to eliminate
208 uninfected cells, and single clones were picked.

209

210 **Knockdown**

211 Small interfering RNA (siRNA) and negative control siRNA were purchased from Thermo Fisher
212 Scientific. siRNA sequences were as follows: siPRKCZ#1 (#s11129), sense 5'-
213 GGACUUUGACCUGAAUCAGA(dTdT) -3', antisense 5'- UCUGAUUAGGUCAAAGUCC(dTdT) -
214 3'; siPRKCZ#2 (#s11128), sense 5'- CGUUCGACAUCACCGA(dTdT) -3', antisense 5'-
215 UCGGUGAUGAUGUCGAACG(dGdG) -3; siATM (#s42813), sense 5'-

216 GGAAAUCAGUAGUUUGGUC(dTdT) -3', antisense 5'- CACCAAACUACUGAUUUUCC(dTdTG)-3.
217 siRNA (50 nM) was transfected into cells with Lipofectamine3000 (Thermo Fisher).

218

219 **Proliferation assay**

220 Cells were seeded at 1.0×10^3 cells per well in 96-well plates. Four hours after seeding, the cell
221 viability in each well was measured as a standard value. After that, relative cell viability was monitored
222 every 24 h for 72 h using the CellTiter-Glo Luminescent Cell Viability kit (Promega, Madison, WI,
223 USA).

224

225 **Cell cycle analysis by flow cytometry**

226 Cells were harvested with trypsin, washed with PBS, and fixed in ice-cold 70% ethanol at 4 °C for 30
227 min. The fixed cells were washed with PBS and incubated with propidium iodide and RNase
228 (Immunostep S.L, Salamanca, Spain) for 15 min. Flow cytometric analyses were performed on a BD
229 Accuri C6 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and cell cycle
230 analyses were performed with ModFit (Verity Software House, Topsham, ME, USA).

231

232 **Colony formation assay**

233 Assays were performed in six-well plates. Cells were seeded at a density of 500 cells/well and cultured
234 for 7-14 days. The cells were stained by addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-
235 diphenyltetrazolium bromide (FUJIFILM Wako), followed by incubation for 1 h. Colonies were
236 counted by naked eye.

237

238 **Xenograft transplantation experiments**

239 Male four-week-old BALB/cAJcl-nu/nu mice were purchased from CLEA Japan (Tokyo, Japan) and
240 maintained in a pathogen-free environment with a 12-h dark/light cycle in the animal facility at Kyushu

University. Three to five animals were housed per cage, and all animals were fed and watered ad libitum. Animal health was checked at least twice a week to visually assess appearance behavior, faces, and other conditions. The mice were anesthetized using a mixed cocktail of midazolam (Fuji Pharma, Tokyo, Japan), butorphanol tartrate (Meiji Seika Pharma, Tokyo, Japan), and medetomidine hydrochloride (Kyoritsu Seiyaku Corp., Tokyo, Japan). Cells (1.0×10^6) were dissolved in 100 μ l Matrigel (Corning, Corning, NY, USA) and injected subcutaneously (s.c.) into the trunks of mice on day 0. Tumour size was measured weekly by calipers in a blinded manner, and the tumour volume was calculated using the following formula: (major axis of tumour) $\times$ (minor axis of tumour)² / 2. When conducting experiments with the drug, 14 days after tumour injection, mice were randomly divided into two groups based on a random number table and injected intraperitoneally (i.p.) twice per week with 100 μ l of 200 μ g/ml decitabine or 100 μ l PBS. The Institutional Review Board at Kyushu University approved the use of xenograft models for this study (approval number: A20-339-0). All experiments met the National Institutes of Health guide for the care and use of Laboratory animals. Although sample sizes were calculated using a significance criterion of $\alpha = 0.05$ to achieve 80% statistical power, the sample sizes were kept as small as possible for the sake of animal welfare.

256

257 **Antibody array analysis**

Each cell line was cultured in a 15-cm culture dish to 80% confluence. Whole-cell protein extracts were obtained using Cell Lysis Buffer (R&D Systems, Minneapolis, MN, USA) containing protease inhibitor cocktail tablets (cOmplete™; Roche). Protein concentration in cell lysates was measured by the Bradford method. Antibody array analysis was performed using Proteome Profiler Human Phospho-Kinase Array Kits (ARY003B; R&D Systems). Semi-quantitation was performed on CS Analyzer 3.0 (ATTO)

264

265 **Immunoprecipitation**

Whole-cell protein extracts were obtained as described in the Western blotting section. Anti-GFP mAb-Magnetic Beads (Medical & Biological Laboratories, Nagoya, Japan) or rat IgG2a-Magnetic Beads (Medical & Biological Laboratories) were added to the extracts and incubated at 4 °C for 1 h. Incubated lysates were placed on a magnetic rack, and the supernatant was removed. The beads were then washed three times with cold wash buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% NP-40). Proteins were resuspended with 1× LDS sample buffer (Invitrogen) and boiled at 90 °C for five min.

272

273 **Statistical analysis**

Equality of data variances was assessed by the F-test. When the variances had equality, Student's t-test was applied; otherwise, the Welch's t-test was applied for two-group comparisons. P values less than 0.05 were considered significant. Data in graphs are given as means ± standard deviation (S.D.). All statistical analyses were performed with R (The R Foundation for Statistical Computing, Vienna, Austria).

279

280 **Results**

281

282 ***PRKCZ* is epigenetically silenced in DDCS**

To determine which candidate genes are epigenetically silenced in DDCS, we applied a bioinformatic approach. We began by analysing the E-MTAB-7263 and E-MTAB-7264 datasets from Array Express.¹⁵ To generate this dataset, DNA methylation and gene expression in chondrosarcoma were analysed using array-based assays. We compared methylation status between CCS (n=73) and DDCS (n=16). Methylation near the transcription start site was significantly higher for 74 genes, and mRNA levels were significantly lower for 681 genes in DDCS vs. CCS (Figure 1a left and middle); 17 of these genes overlapped (Figure 1b). To further narrow down the candidate genes, we analysed the GSE12532 dataset from Gene Expression Omnibus.³⁵ To generate this dataset, gene expression in

291 chondrosarcoma was analysed using array-based assays. Again, we compared CCS (n=12) and DDCS
292 (n=3). mRNA levels of 54 genes were lower in DDCS than in CCS (Figure 1a right). After these
293 processes, the candidates were narrowed down to four genes (Figure 1b). Of these, two had CpG island
294 near the transcription start sites: *PRKCZ* and *COL11A2* (Supplementary Figure S1). To determine
295 which genes to investigate, we performed whole-genome bisulfite sequencing (WGBS) for two cell
296 lines: NDCS-1 and OUMS-27. NDCS-1 was established from a DDCS tumour and is characterised by
297 rapid growth and xenografts that present with a dedifferentiation component and a cartilage component.
298 OUMS-27 was established from a CCS tumour and is characterised by relatively slow growth and
299 stable expression of a differentiated chondrocyte phenotype (Supplementary Figure S2). The detailed
300 data for the WGBS analysis is presented in Supplementary Table S4. *PRKCZ* was hypermethylated in
301 NDCS-1 relative to OUMS-27 (Figure 1c). Therefore, we decided to target *PRKCZ* for further
302 investigation.

303

304 ***PRKCZ* expression levels correlate with the prognosis for DDCS patients**

305 Methylation and expression levels of *PRKCZ* in DDCS and CCS were validated with the datasets used
306 *in silico* analysis (Figure 2a). We observed no differences in the methylation or expression of *PRKCZ*
307 amongst the histological grades of CCS (Figure 2b). To verify the correlation between *PRKCZ*
308 expression and the prognosis of chondrosarcoma, we examined the difference in overall survival
309 between patients with high and low *PRKCZ* expression using the E-MTAB-7264 dataset described
310 above. DDCS patients with lower-than-median *PRKCZ* had a significantly lower survival rate (Figure
311 2c). By contrast, CCS patients exhibited no differences in survival rate as a function of *PRKCZ*
312 expression (Figure 2d). These results suggest that a lower expression level of *PRKCZ* correlates with
313 a poor prognosis for patients with DDCS, but the expression level of *PRKCZ* does not correlate with
314 the tumour histological grade or prognosis for patients with CCS.

315

316 **Immunohistological analysis of PRKCZ in DDCS**

317 To determine whether protein expression of PRKCZ was reduced in DDCS, we performed IHC
318 staining of clinical samples of DDCS. The semi-quantitative assessment was performed using
319 immunoreactive scores (IRS).³⁶ The dedifferentiated components had significantly lower IRS relative
320 to the cartilaginous components (Figure 3a and 3b). The expression levels of PRKCZ in clinical
321 samples were validated using RT-qPCR (Supplementary Figure S3). These results suggest that PRKCZ
322 protein expression decreased in the dedifferentiated components in DDCS.

323

324 **PRKCZ decreases the proliferation rate in a DDCS cell line**

325 For *in vitro* analysis, we used the SW1353 cell line in addition to the NDCS-1 and OUMS-27 cell lines
326 used for WGBS analysis. SW1353 was characterised by rapid growth, no expression of differentiated
327 chondrocyte genes, and xenografts that presented with a dedifferentiated tissue structure despite the
328 cell line being derived from a CCS tumour (Supplementary Figure S2). Therefore, we concluded that
329 the SW1353 phenotype more closely matches that observed in DDCS tumours. PRKCZ was expressed
330 at a lower level in NDCS-1 and SW1353 relative to the level observed in OUMS-27 (Figure 4a and
331 4b).

332 To investigate the biological functions of PRKCZ in DDCS, we forced expression of PRKCZ in
333 NDCS-1. Elevated expression levels of PRKCZ were confirmed by RT-qPCR and western blotting in
334 three independent clones (Figure 4c and 4d). Overexpression of PRKCZ significantly inhibited
335 proliferation (Figure 4e). To determine why the proliferation rate was reduced, we performed cell cycle
336 analysis. We found that overexpression of PRKCZ resulted in the expansion of a sub-G1 fraction
337 relative to control cells (Figure 4f, Supplementary Figure S4a), and the expression levels of apoptosis-
338 related proteins such as cleaved caspase 3 and cleaved poly (ADP-ribose) polymerase (PARP)
339 increased (Figure 4g). These results suggest that overexpression of PRKCZ in NDCS-1 induced
340 apoptosis, which was followed by inhibition of cell proliferation. Overexpression of PRKCZ in NDCS-

1 also inhibited colony-forming ability (Supplementary Figure S4b). To confirm the effects from the overexpression of PRKCZ in NDCS-1 cells *in vivo*, we injected PRKCZ-overexpressing and control NDCS-1 cells into the trunk of BALB/cAJcl-nu/nu mice and periodically measured the volume of the resultant tumours (n=3 per group). All PRKCZ-overexpressing NDCS-1 cells grew significantly more slowly than control NDCS-1 cells, consistent with the results *in vitro* (Figure 4h). Forced expression of PRKCZ in SW1353 also produced the same results; that is, overexpression of PRKCZ induced apoptosis, which was followed by the inhibition of cell proliferation *in vitro*, and the tumour exhibited slow growth *in vivo* (Supplementary Figure S5). Because OUMS-27 expressed PRKCZ endogenously, we knocked down PRKCZ in this cell line using siRNA. The effects of siRNA on PRKCZ expression were confirmed by RT-qPCR and western blotting 48 h after siRNA transfection (Figure 4i and 4j). Notably, PRKCZ-knockdown cells exhibited no difference in proliferation rates relative to cells transfected with scrambled control siRNA (Figure 4k). In addition, cell cycle analysis revealed no difference in the sub-G1 fraction between knockdown and control cells (Figure 4l, Supplementary Figure S4c).

PRKCZ induces apoptosis by activating ATM/CHK2 pathway

We next investigated the molecular mechanism of apoptosis induced by overexpression of PRKCZ. Because PRKCZ has kinase activity, we used a human phospho-antibody array (Human Phospho-Kinase Array, Proteome Profiler Array Kit) to semi-quantitatively determine the phosphorylations of many proteins in parallel. Compared to control NDCS-1, all PRKCZ-overexpressing NDCS-1 clones had higher phosphorylation of CHK2 (Figure 5a), a well-known activator of apoptosis pathway.³⁷ Therefore, we further investigated the relationship between PRKCZ and CHK2. The upstream pathway of apoptosis induction by phosphorylation of CHK2 is as follows: once the MRE11–RAD50–NBS1 (MRN) complex senses double-strand breaks of DNA, ATM is recruited to chromatin, where it is activated; there, it promotes activation of CHK2, which can lead to apoptosis depending on the level

of stress.³⁷ Hence, we investigated the protein and phosphorylation levels of the factors upstream of CHK2. The phosphorylation levels of ATM and CHK2 were higher, with no change in total protein expression, in the PRKCZ-overexpressing NDCS-1 cells relative to the mock-transfected cells (Figure 5b and Supplementary Figure S6a). In the SW1353 cells, PRKCZ-overexpressing clones also exhibited higher phosphorylation levels of ATM and CHK2 relative to the control cells (Supplementary Figure S6b). To confirm whether phosphorylation of ATM induced apoptosis in chondrosarcoma, we administered chloroquine diphosphate, which induces the phosphorylation of ATM.³⁸ Consistent with reports using other cell lines, chloroquine induced the phosphorylation of ATM, which was followed by apoptosis (Supplementary Figure S6c).

To elucidate the physical interaction between PRKCZ and proteins involved in ATM/CHK2 pathway, we constructed a GFP-tagged PRKCZ vector, transfected it into NDCS-1, and established a single clone (Figure 5c left). Immunoprecipitation with anti-GFP antibody confirmed that PRKCZ interacted with ATM, RAD50, and MRE11 (Figure 5d left). To further confirm these interactions in other cells, we transiently transfected GFP-tagged PRKCZ into HeLa cells (Figure 5c right) and performed immunoprecipitation as described above for NDCS-1. The results were consistent with those obtained with NDCS-1 (Figure 5d right). Moreover, a computational prediction of PRKCZ phosphorylation sites identified several predicted sites in ATM (Supplementary Figure S7). Together, these results suggest that PRKCZ phosphorylated ATM by physical interaction.

384

385 **DNA methylation inhibitor increases PRKCZ expression**

386 Our results strongly implied that in DDOS, expression of PRKCZ is suppressed by DNA methylation, and that this reduction in PRKCZ expression enhances cell proliferation. We then tested the effect of decitabine, a DNMTi, on PRKCZ expression, proliferation, and apoptosis. We confirmed the effects of decitabine on demethylation using bisulfite sequencing (Figure 6a) and decided to increase the concentration of decitabine to 10 μ M because the methylation rate was low. As expected, decitabine-

391 treated NDCS-1 cells expressed higher levels of PRKCZ relative to PBS-treated cells (Figure 6b and
392 6c). Furthermore, in decitabine-treated NDCS-1, proliferation was slower, and more apoptosis was
393 observed (Figure 6d, 6e, and Supplementary Figure S8a). Consistent with our observations in PRKCZ-
394 overexpressing NDCS-1, western blotting of decitabine-treated NDCS-1 revealed more
395 phosphorylation of ATM and CHK2, and higher expression of apoptosis-related proteins (Figure 6c).
396 Colony formation was inhibited by decitabine (Supplementary Figure S8b). To confirm the effects of
397 decitabine on NDCS-1 *in vivo*, we injected NDCS-1 cells into the trunk of BALB/cAJcl-nu/nu mice.
398 Starting two weeks after injection, mice were injected i.p. twice per week with 100 μ L of 200 μ g/mL
399 decitabine or 100 μ L of PBS (n=6 per group) (Figure 6f). The tumour volume was significantly smaller
400 in the decitabine-treated mice relative to the PBS-treated mice, which was consistent with our
401 observations from the PRKCZ-overexpressing NDCS-1 cells (Figure 6g–h). We confirmed that
402 PRKCZ was expressed at higher levels in the tumours from decitabine-treated mice relative to the
403 PBS-treated mice (Supplementary Figure S8c). Decitabine also induced PRKCZ expression, which
404 was followed by ATM/CHK2 activation and apoptosis in SW1353 cells (Supplementary Figure S9a–
405 h). By contrast, decitabine did not induce PRKCZ expression or inhibit the growth of OUMS-27 cells
406 (Supplementary Figure S10).

407 To determine whether the effect of decitabine was due to the expression of PRKCZ, we administered
408 decitabine to NDCS-1 along with siRNA against PRKCZ. Twenty-four hours after transfection with
409 50 nM siRNA, the medium was changed, and 10 μ M decitabine was administered for another two days.
410 The effect of knockdown of PRKCZ was confirmed by RT-qPCR and western blotting (Figure 6h, 6i,
411 and Supplementary Figure S8d). Interestingly, the inhibitory effect of decitabine on cell proliferation
412 was decreased by PRKCZ knockdown (Figure 6j and Supplementary Figure S8e). The same result was
413 not observed in SW1353 cells because siRNA could not sufficiently reduce the expression of PRKCZ
414 (Supplementary Figure S9i–k). Furthermore, a siRNA against ATM produced the same results as a
415 siRNA against PRKCZ (Supplementary Figure S11). These results indicated that growth inhibition by

administration of decitabine of NDCS-1 might be mediated predominantly through expression of PRKCZ and subsequent activation of the ATM/CHK2 pathway.

Discussion

This study demonstrates that increased DNA methylation and reduced expression of PRKCZ prevent apoptosis via inactivation of the ATM/CHK2 pathway in DDCS. Our results also show that decitabine induces apoptosis in DDCS by increasing PRKCZ expression levels.

The relationships between certain genes, including RUNX3, p16, FHIT, and E-cadherin,³⁹⁻⁴¹ with methylation in high-grade chondrosarcomas are known. In our study, we adopted a bioinformatic strategy to identify PRKCZ as a target gene by analysing multiple datasets; this approach could be used to discover candidate genes that have not been previously examined and would strengthen the reliability of the results.

We then investigated the biological significance of PRKCZ in the oncogenesis of DDCS. PRKCZ plays an oncosuppressive role in other cancers.^{25,30,32} Consistent with these studies, forced expression of PRKCZ also produced an oncosuppressive effect in DDCS by inducing apoptosis; however, our study showed that PRKCZ activated ATM/CHK2, which was not activated by PRKCZ in other studies that reported PRKCZ-mediated activation of ADAR2,²⁵ c-Myc,³⁰ p38 MAPK,⁴² STAT3, Akt, and Erk.^{43,44} Interestingly, PRKCZ induced apoptosis exclusively in DDCS tumours, but not in the CCS tumours with low-grade histology in our study, implying that the presence of ATM/CHK2 activation by PRKCZ followed by the induction of apoptosis depends on cellular context. Although the mechanism of this cellular context dependence remains unclear, our results strongly suggest that reduced expression of PRKCZ helps DDCS escape from apoptosis.

We then used decitabine to express PRKCZ in DDCS tumours, keeping in mind future clinical applications, because it is used as a treatment for myelodysplastic syndromes. Decitabine produced oncosuppressive effects following PRKCZ expression in only DDCS, which may explain the

controversies over whether DNMTi suppresses oncogenicity or not in chondrosarcoma; that is, DNMTi cannot induce PRKCZ expression, and PRKCZ does not induce apoptosis even if PRKCZ is expressed in CCS tumours. Hence, in malignant cartilaginous tumours, the use of decitabine as an effective treatment may be limited to the treatment of patients with DDCS tumours.

There were several limitations in this study. First, although we showed that PRKCZ could bind ATM directly, it is not clear whether PRKCZ phosphorylates ATM. *In vitro* kinase assays may solve this problem, and we are currently working to overcome the technical difficulties of that experiment. Second, although the promoter region of *PRKCZ* was hypermethylated, there are no IDH mutations in NDCS-1, and the reason for hypermethylation is unclear. A multi-omics study revealed several DDCS cases that exhibited hypermethylation without IDH mutation.¹⁵ Thus, unknown mechanisms of hypermethylation might exist in DDCS. In addition, the mechanism by which decitabine inhibits oncogenicity in DDCS was not clearly elucidated, as decitabine is a non-specific DNMTi that causes global demethylation of DNA. To solve this problem, we are considering carrying out targeted demethylation of the *PRKCZ* promoter region using the CRISPR-dCas9 system.⁴⁵ Finally, because of the rarity of the disease, we examined only a small number of clinical DDCS cases. Therefore, in order to validate the clinical significance of PRKCZ in the biology of DDCS, it will be necessary to examine more clinical samples.

Acknowledgments

We appreciate the technical assistance from The Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences.

Authors' contributions

YM and YN supervised the study; YM, MN, KY, AK, TH, MK, and RO provided ideas for the research design; ES performed the *in silico*, *in vitro*, and *in vivo* experiments and analysed the data; YS and YO

466 collected clinical samples of chondrosarcoma; ES wrote the original draft; YM, ME, NS, TF, KI, and
467 AN reviewed and revised the manuscript; YM, MN, and ME acquired funding All authors approved
468 the final version of the manuscript.

469

470 **Ethics approval and consent to participate**

471 All patients provided written informed consent before undergoing the study procedures. The
472 Institutional Review Board at Kyushu University approved the use of human specimens for this study
473 (approval number: 27–420). This study was conducted in accordance with the Declaration of Helsinki.

474

475 **Consent for publication**

476 All patients provided written informed consent for publication.

477

478 **Data availability**

479 Data used in the study are available from the corresponding author upon reasonable request.

480

481 **Conflict of interest**

482 No potential conflicts of interest were disclosed.

483

484 **Funding information**

485 This work was supported in part by AMED (#JP20ck0106523 (M.N.)), Grant-in-Aid for Young
486 Scientists (#JP18K16627), Grant-in-Aid for Scientific Research (#JP21K09325), and Grant-in-Aid for
487 Research Activity Start-up (#JP21K20838) from the Japan Society for the Promotion of Science, Grant
488 of the Clinical Research Promotion Foundation (2021).

489

490 Supplementary information is available at the British Journal of Cancer's website

References

1. CDM, F., JA, B., PCW, H. & F, M. *WHO Classification of Tumours of Soft Tissue & Bone*, (WORLD HEALTH ORGANIZATION, 2013).
2. Chow, W.A. Chondrosarcoma: biology, genetics, and epigenetics. *F1000Res* 7(2018).
3. Bovee, J.V.M.G., Cleton-Jansen, A.-M., Rosenberg, C., Taminiau, A.H.M., Cornelisse, C.J. & Hogendoorn, P.C.W. Molecular genetic characterization of both components of a dedifferentiated chondrosarcoma, with implications for its histogenesis. *The Journal of Pathology* **189**, 454-462 (1999).
4. Evans, H.L., Ayala, A.G. & Romsdahl, M.M. Prognostic Factors in Chondrosarcoma of Bone. *Cancer* **40**, 818-831 (1977).
5. Giuffrida, A.Y., Burgueno, J.E., Koniaris, L.G., Gutierrez, J.C., Duncan, R. & Scully, S.P. Chondrosarcoma in the United States (1973 to 2003): an analysis of 2890 cases from the SEER database. *J Bone Joint Surg Am* **91**, 1063-72 (2009).
6. Yokota, K., Sakamoto, A., Matsumoto, Y., Matsuda, S., Harimaya, K., Oda, Y. *et al.* Clinical outcome for patients with dedifferentiated chondrosarcoma: a report of 9 cases at a single institute. *J Orthop Surg Res* **7**, 38 (2012).
7. Kawaguchi, S., Sun, T., Lin, P.P., Deavers, M., Harun, N. & Lewis, V.O. Does ifosfamide therapy improve survival of patients with dedifferentiated chondrosarcoma? *Clin Orthop Relat Res* **472**, 983-9 (2014).
8. Dienstmann, R., Rodon, J., Serra, V. & Tabernero, J. Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. *Mol Cancer Ther* **13**, 1021-31 (2014).
9. Duffaud, F., Italiano, A., Bompas, E., Rios, M., Penel, N., Mir, O. *et al.* Efficacy and safety of regorafenib in patients with metastatic or locally advanced chondrosarcoma: Results of a non-comparative, randomised, double-blind, placebo controlled, multicentre phase II study. *Eur J*

- 517 *Cancer* **150**, 108-118 (2021).
- 518 10. Italiano, A., Le Cesne, A., Bellera, C., Piperno-Neumann, S., Duffaud, F., Penel, N. *et al.* GDC-
519 0449 in patients with advanced chondrosarcomas: a French Sarcoma Group/US and French
520 National Cancer Institute Single-Arm Phase II Collaborative Study. *Ann Oncol* **24**, 2922-6
521 (2013).
- 522 11. Campbell, V.T., Nadesan, P., Ali, S.A., Wang, C.Y., Whetstone, H., Poon, R. *et al.* Hedgehog
523 pathway inhibition in chondrosarcoma using the smoothened inhibitor IPI-926 directly inhibits
524 sarcoma cell growth. *Mol Cancer Ther* **13**, 1259-69 (2014).
- 525 12. L, M.G., Boulay, K., Topisirovic, I., Huot, M.E. & Mallette, F.A. Oncogenic Activities of
526 IDH1/2 Mutations: From Epigenetics to Cellular Signaling. *Trends Cell Biol* **27**, 738-752
527 (2017).
- 528 13. Lu, C., Venneti, S., Akalin, A., Fang, F., Ward, P.S., Dematteo, R.G. *et al.* Induction of sarcomas
529 by mutant IDH2. *Genes Dev* **27**, 1986-98 (2013).
- 530 14. Klutstein, M., Nejman, D., Greenfield, R. & Cedar, H. DNA Methylation in Cancer and Aging.
531 *Cancer Res* **76**, 3446-50 (2016).
- 532 15. Nicolle, R., Ayadi, M., Gomez-Brouchet, A., Armenoult, L., Banneau, G., Elarouci, N. *et al.*
533 Integrated molecular characterization of chondrosarcoma reveals critical determinants of
534 disease progression. *Nat Commun* **10**, 4622 (2019).
- 535 16. Nakagawa, M., Nakatani, F., Matsunaga, H., Seki, T., Endo, M., Ogawara, Y. *et al.* Selective
536 inhibition of mutant IDH1 by DS-1001b ameliorates aberrant histone modifications and
537 impairs tumor activity in chondrosarcoma. *Oncogene* **38**, 6835-6849 (2019).
- 538 17. Sujiker, J., Oosting, J., Koornneef, A., Struys, E.A., Salomons, G.S., Schaap, F.G. *et al.*
539 Inhibition of mutant IDH1 decreases D-2-HG levels without affecting tumorigenic properties
540 of chondrosarcoma cell lines. *Oncotarget* **6**, 12505-19 (2015).
- 541 18. Li, L., Paz, A.C., Wilky, B.A., Johnson, B., Galoian, K., Rosenberg, A. *et al.* Treatment with a

- Small Molecule Mutant IDH1 Inhibitor Suppresses Tumorigenic Activity and Decreases Production of the Oncometabolite 2-Hydroxyglutarate in Human Chondrosarcoma Cells. *PLoS One* **10**, e0133813 (2015).
19. Bui, C., Ouzzine, M., Talhaoui, I., Sharp, S., Prydz, K., Coughtrie, M.W. *et al.* Epigenetics: methylation-associated repression of heparan sulfate 3-O-sulfotransferase gene expression contributes to the invasive phenotype of H-EMC-SS chondrosarcoma cells. *FASEB J* **24**, 436-50 (2010).
20. Sheng, W., Zhang, Z.C., Shi, D.Y., Wang, B.C., Wu, Q., Shao, Z.W. *et al.* Epigenetic silencing of SFRP5 promotes the metastasis and invasion of chondrosarcoma by expression inhibition and Wnt signaling pathway activation. *Chem Biol Interact* **296**, 1-8 (2018).
21. Hamm, C.A., Xie, H., Costa, F.F., Vanin, E.F., Seftor, E.A., Sredni, S.T. *et al.* Global demethylation of rat chondrosarcoma cells after treatment with 5-aza-2'-deoxycytidine results in increased tumorigenicity. *PLoS One* **4**, e8340 (2009).
22. Venneker, S., Kruisselbrink, A.B., Baranski, Z., Palubeckaite, I., Briare-de Bruijn, I.H., Oosting, J. *et al.* Beyond the Influence of IDH Mutations: Exploring Epigenetic Vulnerabilities in Chondrosarcoma. *Cancers (Basel)* **12**(2020).
23. Pollack, S.M., Li, Y., Blaisdell, M.J., Farrar, E.A., Chou, J., Hoch, B.L. *et al.* NYESO-1/LAGE-1s and PRAME are targets for antigen specific T cells in chondrosarcoma following treatment with 5-Aza-2-deoxycytidine. *PLoS One* **7**, e32165 (2012).
24. Reina-Campos, M., Diaz-Meco, M.T. & Moscat, J. The Dual Roles of the Atypical Protein Kinase Cs in Cancer. *Cancer Cell* **36**, 218-235 (2019).
25. Shelton, P.M., Duran, A., Nakanishi, Y., Reina-Campos, M., Kasashima, H., Llado, V. *et al.* The Secretion of miR-200s by a PKCzeta/ADAR2 Signaling Axis Promotes Liver Metastasis in Colorectal Cancer. *Cell Rep* **23**, 1178-1191 (2018).
26. Deevi, R.K., Javadi, A., McClements, J., Vohhodina, J., Savage, K., Loughrey, M.B. *et al.*

- 567 Protein kinase C zeta suppresses low- or high-grade colorectal cancer (CRC) phenotypes by
568 interphase centrosome anchoring. *J Pathol* **244**, 445-459 (2018).
- 569 27. Seto, K.K. & Andrulis, I.L. Atypical protein kinase C zeta: potential player in cell survival and
570 cell migration of ovarian cancer. *PLoS One* **10**, e0123528 (2015).
- 571 28. Llado, V., Nakanishi, Y., Duran, A., Reina-Campos, M., Shelton, P.M., Linares, J.F. *et al.*
572 Repression of Intestinal Stem Cell Function and Tumorigenesis through Direct
573 Phosphorylation of beta-Catenin and Yap by PKCzeta. *Cell Rep* **10**, 740-754 (2015).
- 574 29. Ma, L., Tao, Y., Duran, A., Llado, V., Galvez, A., Barger, J.F. *et al.* Control of nutrient stress-
575 induced metabolic reprogramming by PKCzeta in tumorigenesis. *Cell* **152**, 599-611 (2013).
- 576 30. Kim, J.Y., Valencia, T., Abu-Baker, S., Linares, J., Lee, S.J., Yajima, T. *et al.* c-Myc
577 phosphorylation by PKCzeta represses prostate tumorigenesis. *Proc Natl Acad Sci U S A* **110**,
578 6418-23 (2013).
- 579 31. Valkov, A., Sorbye, S.W., Kilvaer, T.K., Donnem, T., Smeland, E., Bremnes, R.M. *et al.* The
580 prognostic impact of TGF-beta1, fascin, NF-kappaB and PKC-zeta expression in soft tissue
581 sarcomas. *PLoS One* **6**, e17507 (2011).
- 582 32. Galvez, A.S., Duran, A., Linares, J.F., Pathrose, P., Castilla, E.A., Abu-Baker, S. *et al.* Protein
583 kinase Czeta represses the interleukin-6 promoter and impairs tumorigenesis in vivo. *Mol Cell*
584 *Biol* **29**, 104-15 (2009).
- 585 33. Kudo, N., Ogoose, A., Hotta, T., Kawashima, H., Gu, W., Umezumi, H. *et al.* Establishment of
586 novel human dedifferentiated chondrosarcoma cell line with osteoblastic differentiation.
587 *Virchows Arch* **451**, 691-9 (2007).
- 588 34. Iida, K., Fukushi, J., Matsumoto, Y., Oda, Y., Takahashi, Y., Fujiwara, T. *et al.* miR-125b
589 develops chemoresistance in Ewing sarcoma/primitive neuroectodermal tumor. *Cancer Cell*
590 *Int* **13**, 21 (2013).
- 591 35. Hallor, K.H., Staaf, J., Bovee, J.V., Hogendoorn, P.C., Cleton-Jansen, A.M., Knuutila, S. *et al.*

592 Genomic profiling of chondrosarcoma: chromosomal patterns in central and peripheral tumors.
593 *Clin Cancer Res* **15**, 2685-94 (2009).

594 36. W, R. & HE, S. Recommendation for uniform definition of an immunoreactive score (IRS) for
595 immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologie*
596 **8**, 138-140 (1987).

597 37. Kantidze, O.L., Velichko, A.K., Luzhin, A.V., Petrova, N.V. & Razin, S.V. Synthetically Lethal
598 Interactions of ATM, ATR, and DNA-PKcs. *Trends Cancer* **4**, 755-768 (2018).

599 38. Loehberg, C.R., Thompson, T., Kastan, M.B., Maclean, K.H., Edwards, D.G., Kittrell, F.S. *et al.*
600 *Ataxia telangiectasia-mutated and p53 are potential mediators of chloroquine-induced*
601 *resistance to mammary carcinogenesis. Cancer Res* **67**, 12026-33 (2007).

602 39. Jin, Z., Han, Y.X. & Han, X.R. Loss of RUNX3 expression may contribute to poor prognosis
603 in patients with chondrosarcoma. *J Mol Histol* **44**, 645-52 (2013).

604 40. Asp, J., Sangiorgi, L., Inerot, S.E., Lindahl, A., Molendini, L., Benassi, M.S. *et al.* Changes of
605 the p16 gene but not the p53 gene in human chondrosarcoma tissues. *International Journal of*
606 *Cancer* **85**, 782-786 (2000).

607 41. Ropke, M., Boltze, C., Neumann, H.W., Roessner, A. & Schneider-Stock, R. Genetic and
608 epigenetic alterations in tumor progression in a dedifferentiated chondrosarcoma. *Pathol Res*
609 *Pract* **199**, 437-44 (2003).

610 42. Mazumder, S., De, R., Debsharma, S., Bindu, S., Maity, P., Sarkar, S. *et al.* Indomethacin
611 impairs mitochondrial dynamics by activating the PKCzeta-p38-DRP1 pathway and inducing
612 apoptosis in gastric cancer and normal mucosal cells. *J Biol Chem* **294**, 8238-8258 (2019).

613 43. Butler, A.M., Scotti Buzhardt, M.L., Li, S., Smith, K.E., Fields, A.P. & Murray, N.R. Protein
614 kinase C zeta regulates human pancreatic cancer cell transformed growth and invasion through
615 a STAT3-dependent mechanism. *PLoS One* **8**, e72061 (2013).

616 44. Zang, G., Mu, Y., Gao, L., Bergh, A. & Landstrom, M. PKCzeta facilitates lymphatic metastatic

617 spread of prostate cancer cells in a mice xenograft model. *Oncogene* **38**, 4215-4231 (2019).
618 45. Morita, S., Noguchi, H., Horii, T., Nakabayashi, K., Kimura, M., Okamura, K. *et al.* Targeted
619 DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain
620 fusions. *Nat Biotechnol* **34**, 1060-1065 (2016).

621

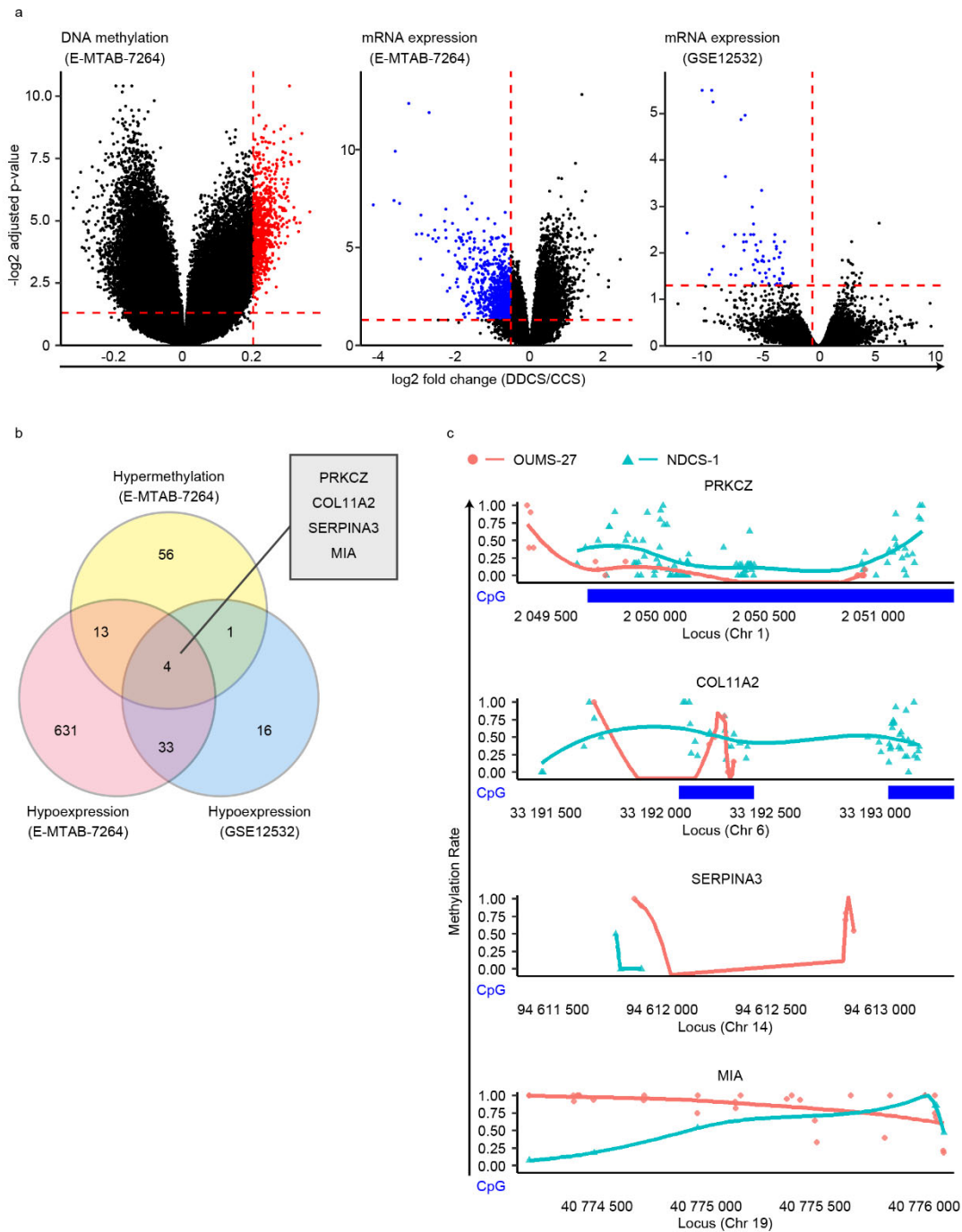
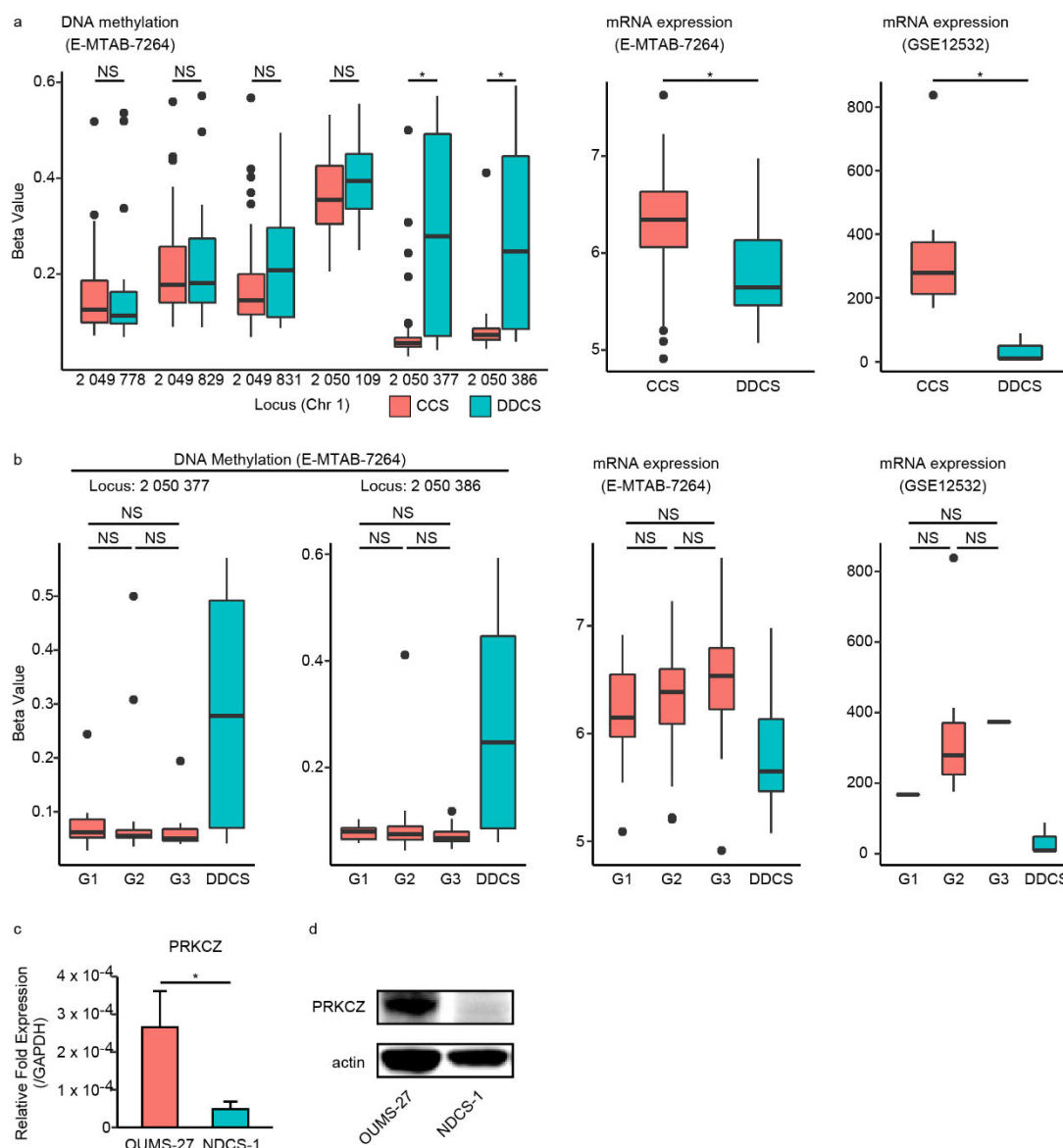


Figure 1. Bioinformatic analysis identified epigenetic silencing of *PRKCZ* in DDCS. **a.** Volcano plots illustrating differences in DNA methylation (**left**) or mRNA expression (**middle and right**). Red dashed lines indicate thresholds: 0.05, 0.2, and -0.5 for adjusted p-value, $\Delta\beta$ value of methylation in DDCS vs. CCS, and log fold change of mRNA expression in DDCS vs. CCS, respectively. Hypermethylated genes are indicated by red dots. Hypoexpressed genes are indicated by blue dots. **b.**

627 Venn diagram illustrating the number of genes detected by analysis of differential DNA methylation
628 or mRNA expression. **c.** Bisulfite sequencing to detect differences in DNA methylation levels in the
629 promoters of the candidate genes in NDCS-1 and OUMS-27. Methylation rates of each C in CpGs are
630 indicated by dots. Curved lines indicate smooth local regression of methylation rates in each cell line.
631 CpG island are indicated by blue bands. The locus was defined by GRCh38 Reference Sequence.
632



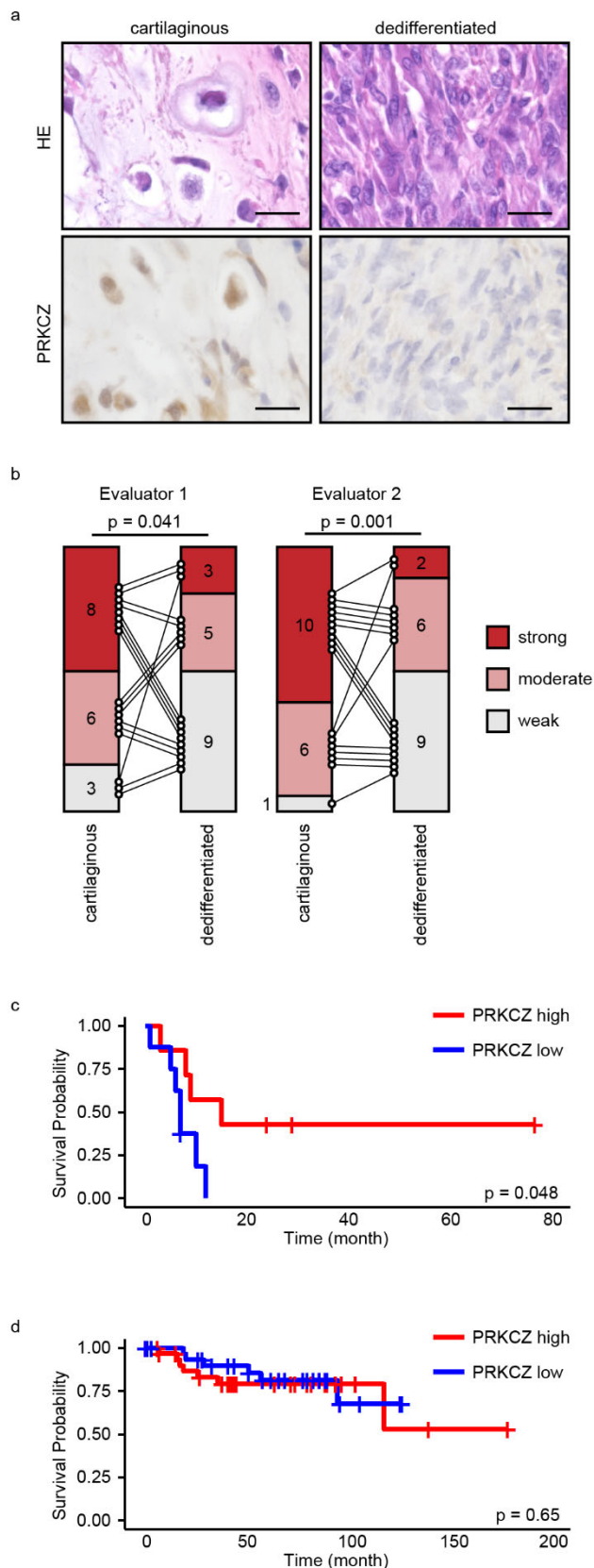
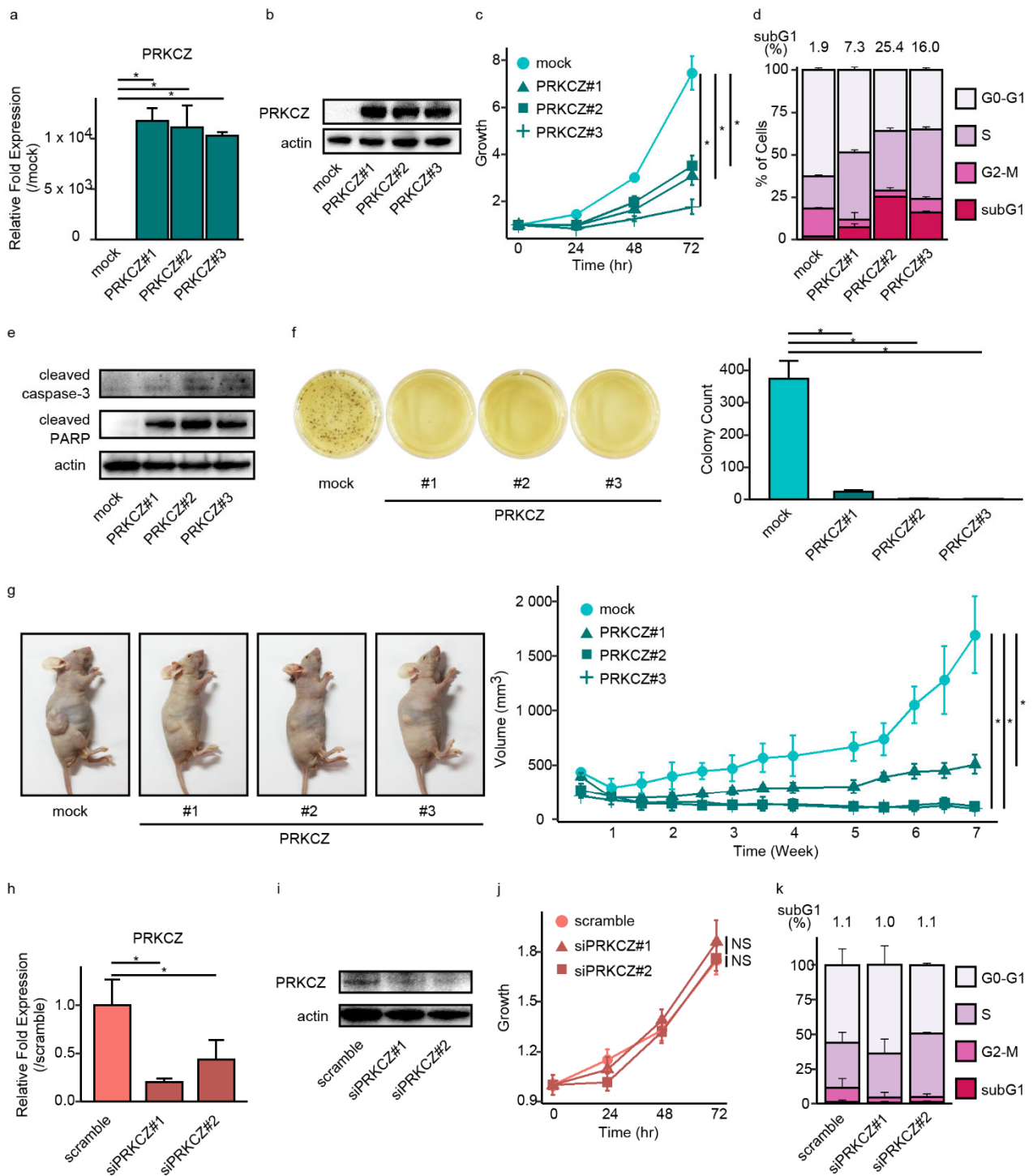


Figure 3. PRKCZ is suppressed in dedifferentiated component of DDCCS relative to the cartilaginous component. **a.** Representative images of HE staining and PRKCZ IHC staining in DDCCS. Normal cartilage is for comparison. Scale bars, 20 μ m. **b.** Evaluation of staining in each component of DDCCS using immunoreactive score. Wilcoxon signed-rank test.



651 **Figure 4. PRKCZ overexpression decreases proliferation and induces apoptosis in NDCS-1. a.**
 652 PRKCZ expression in each cell line determined by RT-qPCR. **b.** PRKCZ expression in each cell line
 653 determined by western blotting. C28/I2, human chondrocyte cell line for comparison. In **c-h**, PRKCZ-
 654 overexpressing NDCS-1 vs. mock-transfected NDCS-1. **c.** PRKCZ expression determined by RT-

655 qPCR. **d.** PRKCZ expression determined by western blotting. **e.** Proliferation assay. **f.** Sub G1 fraction
656 rate determined by cell cycle assay. **g.** Levels of apoptosis-related proteins expression determined by
657 western blotting. **h.** Time course of tumor volume of xenograft transplantation. Images were harvested
658 at sacrifice; representative images are shown. In **i–l**, siPRKCZ-transfected OUMS-27 vs. scrambled
659 siRNA-transfected OUMS-27. **i.** PRKCZ expression determined by qRT-PCR. **j.** PRKCZ expression
660 determined by western blotting. **k.** Proliferation assay. **l.** Sub G1 fraction rate determined by cell cycle
661 assay. In **a, c, e, f, h, i, k, and l**, data are means $\pm$ SD; n = 3. Two-tailed t tests. *, p < 0.05. NS, not
662 significant.

663

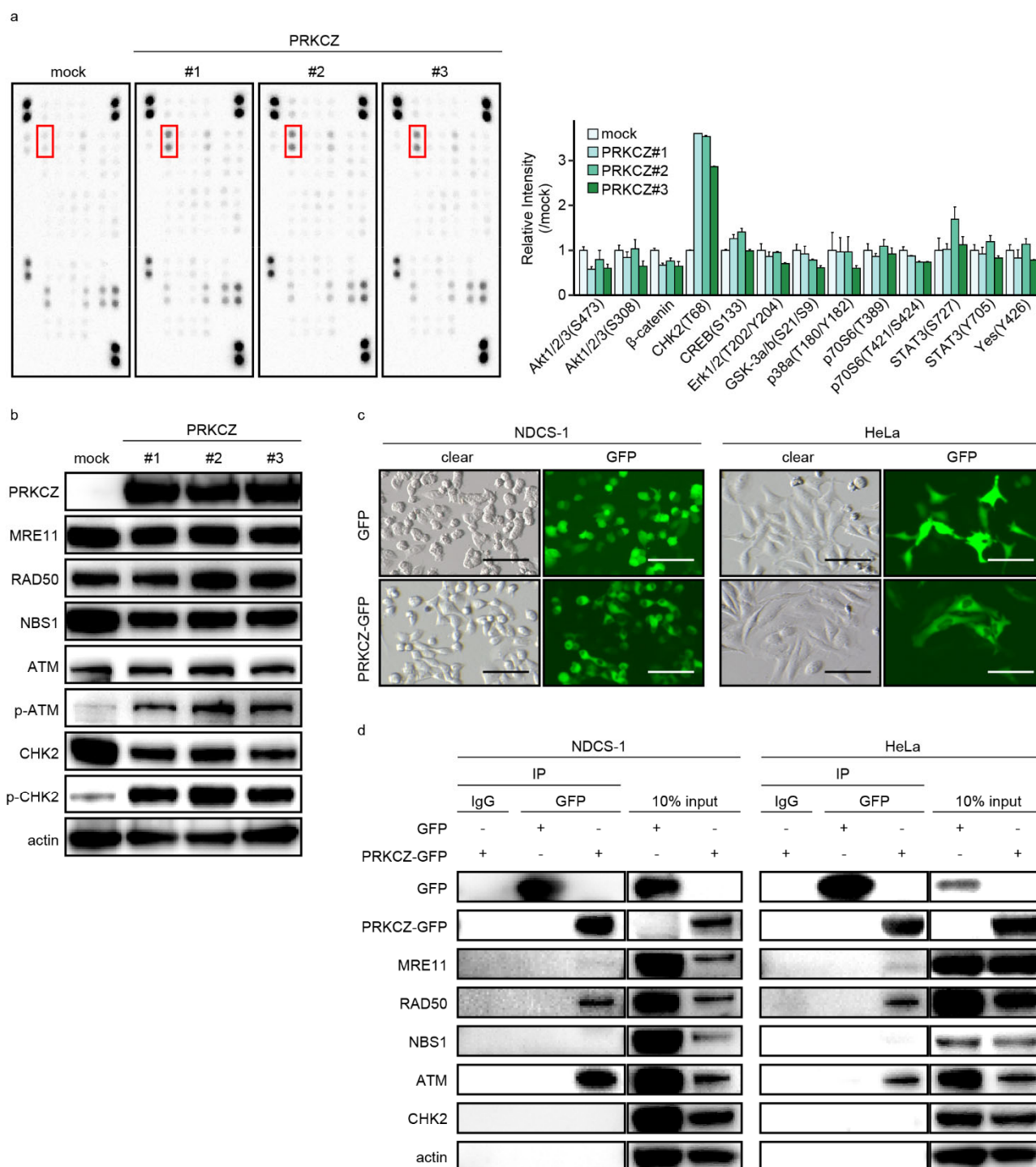
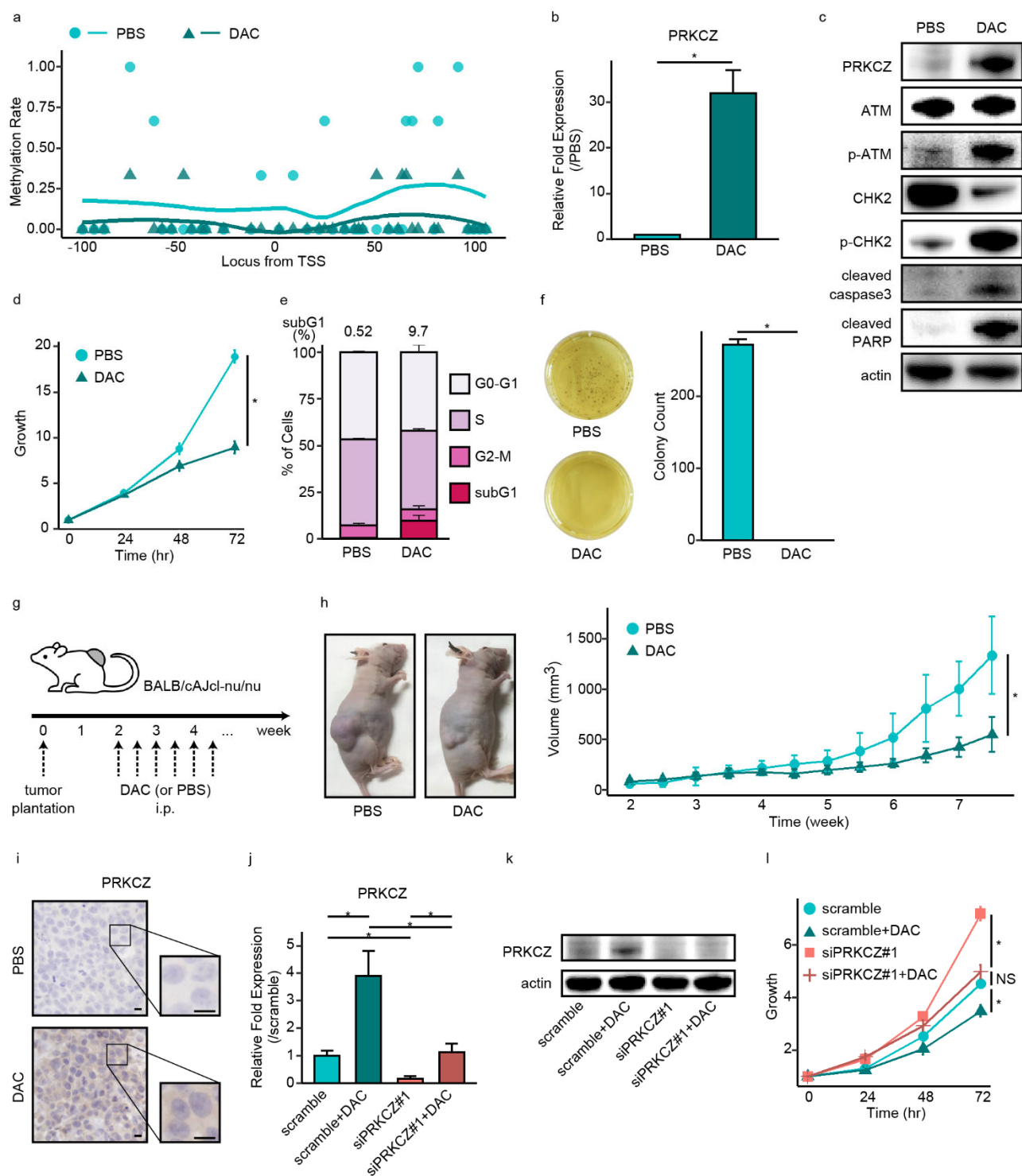


Figure 5. PRKCZ induces apoptosis through activation of the ATM/CHK2 pathway. **a.** Phospho-antibody array showing that PRKCZ overexpression induces CHK2 phosphorylation. Red squares indicate CHK2 spots (**left**). Bar plots indicate semi-quantitative intensity of representative proteins in PRKCZ-overexpressing or mock-transfected NDCS-1 (**right**). Data are means $\pm$ SD; n = 2. **b.**

668 Expression of ATM/CHK2-related proteins in PRKCZ-overexpressing NDCS-1 vs. mock-transfected
669 NDCS-1. Band intensities were quantified and normalized using actin levels. The relative intensities
670 compared to mock are shown under each band. **c.** Microscopic images of GFP-tagged PRKCZ-
671 transfected NDCS-1 and mock-transfected NDCS-1 (**left**) and HeLa (**right**). Scale bars, 100 μ m. **d.**
672 Immunoprecipitation assay to detect interaction between PRKCZ and ATM/CHK2-related proteins in
673 NDCS-1 (**left**) and HeLa (**right**).
674



675 **Figure 6. Decitabine increases PRKCZ expression and induces apoptosis in NDCS-1.** a. DNA
 676 methylation levels on the *PRKCZ* promoter in decitabine-treated vs. PBS-treated NDCS-1, as
 677 determined by bisulfite sequencing. Methylation rates of each C in CpG islands are indicated by dots
 678 (left). Curved lines indicate smooth local regression of methylation rates in each group (left). Average

679 methylation rates (**right**). n = 5. In **b–e**, 10μM of decitabine-treated NDCS-1 vs. PBS-treated NDCS-
680 1. **b**. PRKCZ expression determined by RT-qPCR. **c**. Expression of ATM/CHK2-related proteins and
681 apoptosis-related proteins determined by western blotting. **d**. Proliferation assay. **e**. Sub G1 fraction
682 rate determined by cell cycle assay. **f**. Schedule of tumor implantation and decitabine treatment in the
683 xenograft assay. **g**. Time course of tumor volume of xenografts in decitabine-treated NDCS-1 vs. PBS-
684 treated NDCS-1. Images were obtained at the time of sacrifice; representative images are shown. n =
685 6. In **h–j**, 10μM of decitabine-treated NDCS-1 with or without siPRKCZ. **h**. PRKCZ expression
686 determined by RT-qPCR. **i**. PRKCZ expression determined by western blotting. **j**. Proliferation assay.
687 In **b, d, e, h**, and **j**, n = 3. In **a, b, d, e, g, h**, and **j**, data are means ± SD. Two-tailed t tests. *, p < 0·05.
688 NS, not significant. DAC, decitabine.