



# A multimodal treatment of carbon ions irradiation, miRNA-34 and mTOR inhibitor specifically control high-grade chondrosarcoma cancer stem cells

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1 **A multimodal treatment of carbon ions irradiation, miRNA-34 and mTOR inhibitor specifically**  
2 **control high-grade chondrosarcoma cancer stem cells**

3  
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34 **Declarations of interest**

35 None

36  
37 **Abbreviations**

38 ALDH: aldehyde dehydrogenase; CFE: colony forming efficiency; CS: chondrosarcoma; CSC:  
39 cancer stem cell; LET: linear energy transfer; mTOR: mammalian target of rapamycin; NTCP:  
40 normal tissue complication probability; OER: oxygen enhancement ratio; PARP: poly ADP ribose  
41 polymerase; RBE: relative biological effectiveness; TCP: tumor control probability

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42 **Abstract**

43 *Background and purpose*

44 High-grade chondrosarcomas are chemo- and radio-resistant cartilage-forming tumors of bone  
45 that often relapse and metastase. Thus, new therapeutic strategies are urgently needed.

46 *Material and methods*

47 Chondrosarcoma cells (CH-2879) were exposed to carbon-ion irradiation, combined with miR-34  
48 mimic and/or rapamycin administration. The effects of treatment on cancer stem cells, stemness-  
49 associated phenotype, radioresistance and tumor-initiating properties were evaluated.

50 *Results*

51 We show that high-grade chondrosarcoma cells contain a population of radioresistant cancer stem  
52 cells that can be targeted by a combination of carbon-ion therapy, miR-34 mimic administration and/or  
53 rapamycin treatment that triggers FOXO3 and miR-34 over-expression. mTOR inhibition by  
54 rapamycin triggered FOXO3 and miR-34, leading to KLF4 repression.

55 *Conclusion*

56 Our results show that particle therapy combined with molecular treatments effectively controls  
57 cancer stem cells and may overcome treatment resistance of high-grade chondrosarcoma.

58 **Keywords**

59 cancer stem cell, chondrosarcoma, particle therapy, mTOR inhibitor, miR-34

60

## 61 **1. Introduction**

62 Despite striking improvements in the diagnosis and care of human cancer, treatment resistance  
63 remains to this day an issue in some hard-to-treat cancers. Chondrosarcomas (CSs) constitute the  
64 second most common primary bone tumor in adults [1]. Because these cartilaginous tumors exhibit  
65 resistance to chemotherapy and conventional radiation therapy, complete surgical resection still  
66 remains the primary treatment, with a 10-year survival rate comprised between 30% and 80%  
67 depending on the grade. A significant number of patients experience relapse, metastasis or present  
68 unresectable disease with poor clinical outcome and high lethality (grade III). For those reasons, the  
69 clinical management of CS is considered to be particularly challenging, and new therapeutic  
70 approaches are urgently needed. Some subtypes, such as mesenchymal CS, may be more responsive  
71 to chemotherapy, while surgery of dedifferentiated CS may be more successful when combined with  
72 chemotherapy [2]. Radiation therapy has been used in skull-base and spinal CS [3,4]. Recently  
73 published molecular therapy targets for CS have included IDH mutations, Hedgehog, Src and PI3K-  
74 Akt-mTOR pathways, histone deacetylase inhibitors, angiogenesis or immunotherapy with immune  
75 checkpoint inhibition [5]. Some of those targets yielded promising results in preclinical studies, but  
76 early phase clinical results were less conclusive.

77 Cancer stem cells (CSCs) are defined as the subset of dedifferentiated cells within a tumor that  
78 possess the ability to self-renew and reconstitute tumor heterogeneity[6]. CSCs are more resistant than  
79 their non-CSC counterparts and were suggested to be at least partially responsible for treatment  
80 resistance, relapse and metastasis[7]. Cancer treatments that do not effectively target CSCs might  
81 ultimately fail, thus it is of paramount importance to develop new treatment strategies that include  
82 CSCs. Transformed mesenchymal stem and progenitor cells with multipotent differentiation potential  
83 are likely to be cells of origin in CS [8]. CSCs have been characterized in osteosarcomas [9], but are  
84 not well defined in CS.

85 New high linear energy transfer (LET) radiation therapy modalities (such as heavy-ion particle  
86 beams) have emerged, which provide a number of physical and biological advantages over  
87 conventional X-ray therapy (including an improved relative biological effectiveness RBE and a lower  
88 oxygen enhancement ratio OER) and might finally contribute to overcoming treatment resistance [10].  
89 High LET radiation treatment, in combination with other therapies (for example, the chemotherapeutic  
90 agent cisplatin or the PARP inhibitor talazoparib), has shown favourable results in bypassing tumor  
91 and CSC radioresistance [11–15]. Although we have recently shown that low- and high-LET low-dose  
92 exposures of CS cells can trigger bystander responses in non-irradiated neighbouring normal  
93 chondrocytes [16], the high RBE of carbon ions might allow lower normal tissue complication  
94 probability (NTCP) than protons, for the same local tumor control (TCP) [17], indicating that carbon  
95 ion therapy might be an appropriate CS treatment modality. In this study, we investigated the ability  
96 of high LET radiation combined with targeted treatments to target CS cells and CSCs.

## 97 **2. Material and methods**

98 **Cell culture, treatment and sorting of cancer stem cells.** CH-2879 chondrosarcoma cells [18]  
99 were authenticated by Short Tandem Repeat (STR) profiling. Cells were grown in RPMI1640 medium  
100 (Nacalai, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Cosmo Bio, Tokyo, Japan)  
101 and antibiotic-antimycotic solution (Penicillin, Streptomycin, Amphotericin B. Gibco ThermoFisher,  
102 Carlsbad, CA, USA). Cultures were grown in 5% CO<sub>2</sub> at 95% humidity. Cells were treated for 48h  
103 with rapamycin at a final concentration of 1 nM. Cells were transfected with miRCURY LNA miR-  
104 34a Mimic (Qiagen, Hilden, Germany) using Lipofectamine RNAiMax reagent (Invitrogen),  
105 according to the manufacturer's instructions, at a final concentration of 5 nM. Cells not transfected  
106 with the mimic were treated with lipofectamine alone. ALDH activity in the cells was measured by  
107 flow cytometry using the ALDEFLUOR kit (Stemcell technologies, Vancouver, BC, Canada) as  
108 previously [19]. Cells with low and high levels of ALDH enzymatic activity (respectively ALDH<sup>-</sup> and  
109 ALDH<sup>+</sup> cells) were sorted using a FACS Aria cell sorter (BD Biosciences, Franklin Lakes, NJ, USA).

110 As a negative control, cells were treated with diethylaminobenzaldehyde (DEAB), a specific ALDH  
111 inhibitor.

112 **Radiation exposure.** X-ray irradiations were conducted using an M-150WE X-ray generator  
113 (Softex, Tokyo, Japan) at 140 kVp, 8 mA, 80V. Irradiation dose-rate was 1.3 Gy/min. Particle therapy  
114 experiments were performed at the Heavy Ion Medical Accelerator in Chiba (HIMAC). Cells were  
115 irradiated with a 290 MeV/n carbon-ion beam at the center of a 6 cm Spread-Out Bragg Peak (SOBP)  
116 as previously [11].

117 **Sphere formation assay.** Cells were seeded in triplicate in ultra-low attachment plates (Corning,  
118 Corning, NY, USA) with serum-free culture medium at defined densities and grown for 10 days.  
119 Spheres larger than 60  $\mu\text{m}$  in size were counted.

120 **Colony Forming Efficiency (CFE) assay.** After irradiation, cells were seeded at defined densities  
121 and incubated for 10–14 days then stained. Colonies with more than 50 cells were scored and surviving  
122 fractions were determined after correcting for the plating efficiency as previously described [20].  
123 Survival curve data were fitted to the linear (carbon-ion) or linear-quadratic model (X-rays) and are  
124 presented as the mean of at least three independent experiments.

125 **Invasion scratch assay.** Cells were seeded in triplicate in 24-well plates. 16 hours before assays,  
126 culture medium was replaced with serum-free medium, then a wound was introduced into the confluent  
127 monolayer with a pipette tip. Percentage of wound closure 24 hours later was measured with ImageJ  
128 software.

129 **Oxidative stress quantification.** Intracellular levels of reactive oxygen species (ROS) were  
130 measured using 5-(and-6)-chloromethyl-29,79-dichloro- dihydrofluorescein diacetate, acetyl ester  
131 (CM-H2DCFDA, Molecular Probes, Eugene, OR, USA). Cells were plated in 6-well plates, then 24  
132 hours later 10 mM CM-H2DCFDA was added and cells were incubated for 40 minutes. Fluorescence

133 intensities were measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale,  
134 CA, USA) (excitation at 493 nm, emission at 520 nm). Unstained cells were used as negative control.  
135 Results are presented as the mean of three independent experiments.

136 **Real-time PCR gene expression profiling.** RNA was extracted using TRIzol reagent and  
137 PureLink RNA Mini Kit (ThermoFisher). cDNA was synthesized using the PrimeScript RT kit (Takara  
138 Bio, Kusatsu, Japan) for mRNAs or the Mir-X First-Strand Synthesis Kit (Takara Bio) for microRNAs.  
139 Then quantitative real-time polymerase chain reaction (qRT-PCR) was run in triplicate in 384-well  
140 plates, using SYBR Premix Ex Taq II (for mRNAs, Takara Bio) or TB Green Advantage qPCR Premix  
141 (for microRNAs, Takara Bio), on a ViiA 7 real-time PCR system (ThermoFisher). Relative mRNA  
142 levels were calculated using the  $\Delta\Delta C_t$  method and normalized to GAPDH (for mRNAs) or U6 (for  
143 microRNAs).

144 **Western blotting.** CH-8279 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer  
145 (Santa Cruz Biotechnology, Dallas, TX, USA). Protein concentrations were determined using the  
146 Protein Assay CBB solution (Nacalai) using bovine serum albumin (BSA) as a standard. Protein  
147 expression levels were measured using a Wes Simple Western instrument (ProteinSimple, San Jose,  
148 CA, USA), with KLF4 (Cell Signaling Technologies, Danvers, MA, USA, #4038S) and GAPDH (Cell  
149 Signaling #2118L) antibodies, according to the manufacturer's instructions [21]. Raw  
150 electropherograms were used to generate blot-like images.

151 **Reporter assays.** CH-2879 cells were transfected using Lipofectamine 3000 reagent with pGL3-  
152 FOXO3-Luciferase reporter, pRL-TK control reporter and/or pCMV-FOXO3 expression vector.  
153 Firefly and Renilla luciferase activities were measured using the Dual Luciferase Assay System  
154 (Promega, Madison, WI, USA). Normalized luciferase activities were obtained as previously.

155 **Mouse experiments.** 4-week old BALB/c nu/nu male mice (Japan SLC, Hamamatsu, Japan) were  
156 distributed 3 animals to a cage and maintained on a 12-hr light/12-hr dark cycle in a temperature-  
157 controlled (22°C) barrier facility with free access to water and a normal diet (CLEA Japan, Tokyo,  
158 Japan). Mice were allowed to acclimatize for 5 days before the experiment. Variable numbers of  
159 ALDH- and ALDH+ chondrosarcoma cells mixed 1:1 with Matrigel Growth Factor Reduced (Corning,  
160 Corning, NY, USA) were injected subcutaneously into mouse flank on both sides under isoflurane  
161 anesthesia. 4 to 9 mice were used per experimental group (for a total of 31 mice). Five days later,  
162 miRCURY LNA miR-34 Mimic (Qiagen) was injected on one side with MaxSuppressor in vivo  
163 RNALancer II delivery system (Bioo Scientific, Austin, TX, SA), according to manufacturer  
164 instructions; 25 µL of mimic/phospholipid-oil emulsion diluted in PBS were injected (1 nmol total  
165 miRNA mimic). On the other side, phospholipid-oil emulsion without mimic was injected. Tumor  
166 volumes were measured using calipers [22]. After experiments, mice were euthanized by carbon  
167 dioxide inhalation. Mouse experiment protocols were approved by the Animal Care and Use  
168 Committee at Okinawa Institute of Science and Technology Graduate University.

169 **Statistical analysis.** Clonogenic survival curve data were fitted to the linear-quadratic model (for  
170 X-ray irradiations) or linear model (for carbon-ion irradiation), using the CS-Cal software  
171 ([www.oncoexpress.de](http://www.oncoexpress.de)), as previously [23]. Statistical significance of the difference between dose-  
172 response curves was performed using one-sided 2-class t-test, Welsh and Sattlewaith approximation  
173 for each dose, with SigmaPlot software ([systatsoftware.com/products/sigmaplot/](http://systatsoftware.com/products/sigmaplot/)) (\*  $P < 0.05$ ). Other  
174 significant differences were assessed using Student's t-test (\*  $P < 0.05$ ). Errors bars represent standard  
175 deviation.

### 176 **3. Results**

177 Subpopulations of cancer stem cells (CSCs) have been identified in sarcomas [24]. Among a panel  
178 of four chondrosarcoma cell lines (CH-2879, OUMS27, L835, SW-1353) (Table A1), CH-2879 cells



179 and, to a lesser extent, OUMS27 cells, could grow as spheres, indicating that they may contain CSCs  
180 [25,26]. The CH-2879 cell line was established from a recurrent Grade III chondrosarcoma of the chest  
181 wall [18], with tumorigenic and invasive abilities. Because CSCs have been associated with  
182 tumorigenicity and metastasis, CH-2879 may be a good model to study treatment responses of grade  
183 III chondrosarcomas.

184 We and others have previously shown that aldehyde dehydrogenase (ALDH) activity is a useful  
185 marker for CSC-like populations in various cancer models [19]. Here, CH-2879 cells contained an  
186 ALDH<sup>+</sup> subpopulation (around 1%) that exhibited increased sphere formation and invasion abilities  
187 associated with a low level of reactive oxygen species (ROS) (Figure 1abcd). Exposure of CH-2879  
188 cells to carbon-ion beam resulted in significant cell death and the induction of stress response pathways  
189 (Figure A1). We then compared clonogenic survival of ALDH<sup>-</sup> and ALDH<sup>+</sup> cells after exposure to X-  
190 rays and carbon-ion beam (Figure 1e). As expected, particle therapy had a higher relative biological  
191 efficiency (RBE) and ALDH<sup>+</sup> cells were markedly more radioresistant to X-rays and carbon-ion beam  
192 (Table 1). However, relative biological efficiencies (RBEs) for ALDH<sup>-</sup> and ALDH<sup>+</sup> cells were similar,  
193 suggesting that particle therapy alone was not sufficient to target CSCs in CS.

194 miR-34 is a tumor-suppressive micro-RNA associated with the regulation of stem-like cells in  
195 prostate cancer, pancreatic cancer or glioblastoma [28–30]. Administration of a synthetic miR-34  
196 mimic decreased sphere formation and invasion capabilities of CH-2879 ALDH<sup>+</sup> cells (Figure 1bc),  
197 and increased ROS levels (Figure 1d), indicating that the maintenance of chondrosarcoma stem-like  
198 phenotype may rely also on miR-34 repression. Indeed, miR-34 expression levels were lower in  
199 ALDH<sup>+</sup> cells than in ALDH<sup>-</sup> cells (Figure 2a)

200 The effect of miR-34 mimic did not rely on the selective elimination of CSCs via apoptosis (Figure  
201 A2), but rather on the disturbance of the dynamic equilibrium between CSCs and non-CSCs.  
202 Expression of miR-34 target genes [31] *NOTCH1*, *C-MYC*, *LMTK3* and *KLF4* were repressed in

203 response to miR-34 mimic treatment (Figure 2b), resulting in lower proportion of ALDH<sup>+</sup> cells (Figure  
204 1a). KLF4 protein expression was detected in ALDH<sup>+</sup> cells, but not in ALDH<sup>-</sup> cells or ALDH<sup>+</sup> cells  
205 treated with miR-34 mimic (Figures 2c, A3). As observed in breast cancer [19], KLF4 seems to play  
206 a role in CSC maintenance in CS, since treatment with *KLF4* siRNA partially recapitulated the effects  
207 of miR-34 mimic (Figure 2d).

208 CH-2978 cells were able to generate tumor xenografts in nude mice (Figure 2e). Tumors were  
209 observed when as few as 10,000 ALDH<sup>+</sup> cells were injected (with 100% of the mice developing  
210 tumors), whereas ALDH<sup>-</sup> cells had very low tumor initiation potential. Administration of miR-34  
211 mimic together with tumor cells resulted in a significant decrease in tumor formation, with less than  
212 half of mice developing tumors after injection of 100,000 ALDH<sup>+</sup> cells (Table 2). However, when  
213 miR-34 mimic was delivered one month after xenograft, it couldn't shrink tumors and could only slow  
214 down tumor growth (Figure 2f). This suggested that miR-34 treatment alone may not be sufficient in  
215 established tumors (or that mimic delivery efficiency needs to be improved) and that combination  
216 therapies might be necessary.

217 The PI3K-Akt-mTOR pathway has recently emerged as a promising target for intervention in  
218 chondrosarcoma [32–35]. Compelling evidence also indicates an important role of mTOR pathway in  
219 CSC maintenance. Rapamycin, an mTORC1 inhibitor, inhibited the proliferation of CH-2879 cells  
220 (Figure A4). Although rapamycin had a slight radio-sensitizing effect (Figure A5), it decreased the  
221 proportion of ALDH<sup>+</sup> CSCs (Figure 1a). Interestingly, rapamycin administration led to slightly  
222 increased levels of miR-34 (Figure 3a) and lower miR-34 target gene levels (*NOTCH1*, *C-MYC*,  
223 *LMTK3*, *KLF4* and *Rictor*), with a significant repression of *KLF4* expression (Figure 3b).

224 Forkhead box O (FOXO) transcription factors are crucial regulators of cell signaling, and  
225 coordinate Akt and mTOR activities [36]. Over-expression of FOXO3 enhanced FOXO3 promoter  
226 activity (Figure 3c) and was associated with ALDH<sup>+</sup> cells losing sphere forming abilities (Figure 3d).

227 Finally, FOXO3 over-expression resulted in higher miR-34 expression levels (Figure 3e) and lower  
228 levels of miR-34 target genes, including KLF4 transcript (Figure 3f) and protein (Figure 2c). The effect  
229 of Rapamycin on CSCs was counteracted when using an siRNA for FOXO3 (Figures 1 and 3d).  
230 Altogether, these results suggest that rapamycin effects in chondrosarcoma rely on FOXO3 activity.

231 Combined action of rapamycin and miR-34 mimic led to sustained inhibition of sphere-forming  
232 abilities of CH-2879 cells, compared to individual treatments (Figure 4a). Using combined treatment,  
233 it was possible to effectively control CSC subpopulations after exposure to carbon-ion doses as low as  
234 1 Gy, as the resulting cell populations contained at least 10 times less CSCs than non-treated population  
235 (Figure 4b). Such control was not observed when cells were exposed with a roughly equivalent X-ray  
236 dose of 2 Gy, based on an RBE of  $\sim 1.9$  (Table 1). By altering CSC-like phenotype, rapamycin and  
237 miR-34 mimic treatments (alone or combined) prompted the radio-sensitization of ALDH<sup>+</sup> cells.  
238 Although the overall effect of those treatments on the global radio-sensitivity is limited (Figure A5),  
239 the near-complete elimination of CSC-like phenotype after combination treatments may effectively  
240 address CSC-associated treatment resistance.

#### 241 **4. Discussion**

242 Surgical resection constitutes the cornerstone of treatment for chondrosarcoma (CS), as  
243 chemotherapy is most often ineffective. Histologic grade is considered to be the most important  
244 indicator of prognosis, and the outcome for grade III CS with surgical resection alone is usually  
245 relatively poor [37]. CH-2879, a cell line isolated from recurrent grade III CS, was selected as a suitable  
246 model for the development of new therapeutic strategies in hard-to-treat CSs.

247 Cancer stem cells (CSCs) have long been presented as an important culprit for treatment resistance  
248 [38]. Indeed, different tumors harbor various CSC contents, and the proportion of CSCs may be  
249 correlated with radio-resistance [39]. Stem-like properties of CSCs confer them a survival advantage  
250 during cancer therapy. Those include higher reactive-oxygen species (ROS) scavenging abilities

251 (resulting in lower radiation-induced ROS) and improved DNA damage repair activation [40]. It is  
252 therefore of utmost importance to properly identify and target the stem-like population when  
253 establishing new treatment regimen. In CS, a subpopulation of CD133+ cells have been identified that  
254 display stem-like characteristics and were capable of inducing and sustain tumor growth *in vivo* [24].  
255 Significant evidence indicates that enhanced aldehyde dehydrogenase (ALDH) activity is a hallmark  
256 of CSCs and is directly involved in CSC-associated resistance [41]. ALDH+ breast cancer cells exhibit  
257 increased DNA repair abilities and higher survival in response to radiation exposure, associated with  
258 the stimulation of Nanog, BM1, Notch1 and Akt [42]. For these reasons, identification of ALDH+  
259 cells is generally considered to be a reliable marker for stem-like subpopulations [43]. Moreover, the  
260 identification of ALDH as a key player in resistance to radiation therapy and tumor recurrence suggest  
261 that ALDH may be considered as a potential therapeutic target [44]. Here, sorted ALDH+ CH-2879  
262 cells exhibited a number of CSC distinctive features, such as lower ROS levels, increased self-  
263 renewing abilities (as indicated by sphere formation assay), enhanced invasiveness, radioresistance  
264 and *in vivo* tumorigenicity. This suggested that in addition to CD133, ALDH expression should also  
265 be an appropriate marker for the identification of stem-like radioresistant subpopulations in CS.

266 The relative biological efficiency (RBE) of the spread-out Bragg peak (SOBP) carbon-ion beam  
267 at the Heavy Ion Medical Accelerator in Chiba (HIMAC), relative to conventional X-rays, was within  
268 the previously observed range (1.5-2.5) in other experimental models [11–13]. It was lower than the  
269 RBE of the monoenergetic carbon-ion beam at the Grand Accélérateur National d'Ions Lourds  
270 (GANIL) [16]. Although ALDH+ cells were more radioresistant than ALDH- cells, their respective  
271 RBEs (whether at D10 or at D37) were not significantly different. While carbon-ion beam alone may  
272 be more efficient against CSCs in some models [45,46], these results demonstrated that the treatment  
273 of CS should not rely solely on particle therapy and therefore combination treatments may be needed.

274 The relationship between CSCs and non-stem cancer cells (NSCCs) has been a matter of  
275 enormous attention. CSCs and NSCCs coexist in a highly dynamic, bidirectional equilibrium state,  
276 whose maintenance is under the control of a not fully understood molecular crosstalk between CSCs,  
277 NSCCs and the tumor microenvironment [47]. microRNAs closely regulate pluripotency and  
278 differentiation mechanisms, and a number of CSC-associated microRNA regulations have been  
279 described [48]. miR-34 is a well-known tumor-suppressor transcriptionally activated by p53, which  
280 has been associated with cancer stem cell homeostasis in several experimental models [29,30,49]. miR-  
281 34 expression is downregulated in chondrosarcoma cell lines, compared to primary non-tumorous  
282 articular chondrocytes [50]. Here, we show that in CH-2879 CS cell line, administration of a miR-34  
283 mimic was capable of decreasing stem-like radioresistant subpopulations.

284 Hundreds of direct miR-34 targets have been identified, with an over-representation of mRNAs  
285 involved in cell cycle control, DNA damage response and apoptosis [51]. Notch homolog 1 (NOTCH1),  
286 C-MYC, Lemur Tyrosine Kinase 3 (LMTK3) and Krüppel-like factor 4 (KLF4) have all been  
287 identified as having a role in maintenance of self-renewal, chemoresistance, invasion and/or stem-like  
288 properties in cancer [52–55]. KLF4, one of the so-called Yamanaka pluripotency factors, was  
289 described either as a tumor-suppressor or as an oncogene, depending on the cancer type [56]. In  
290 osteosarcoma, KLF4 enhances proliferation and metastasis via alpha-crystallin B chain (CRYAB) [57].  
291 In breast cancer, expression of KLF4 is determinant for the maintenance of CSCs [19,58] and KLF4  
292 seems to play a similar role in CS. Because KLF4 siRNA only partially recapitulated the effect of miR-  
293 34 on CSC-like phenotype, we can hypothesize that while KLF4 is a probably a major effector of miR-  
294 34 in CSCs, other pathways regulated by miR-34 are expected to be involved. As a matter of fact, miR-  
295 34 is able to suppress stem-like characteristics in breast cancer by downregulating Notch pathway [59].  
296 Furthermore, ALDH mRNA levels are reduced in tumor tissues of miR-34-treated mice [60]. Because  
297 ALDHs are involved in ROS scavenging [41,61], miR-34 effects might rely on ROS accumulation,  
298 leading to increased radiosensitivity. Although miR-34 expression levels may not be directly correlated

299 with survival in TCGA data of sarcoma patients, low expression of several miR-34 target genes (C-  
300 MYC, Cyclin-dependent kinase 4 – CDK4, Cyclin-dependent kinase 6 – CDK6, E2F Transcription  
301 Factor 3 – E2F3) is associated with better sarcoma survival (Table A3, Figure A6). miR-34 therapy  
302 may be effective mainly as a combination with other treatment modalities.

303 Mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that is regulated in an extensive list  
304 of functions, including proliferation, survival, cytoskeleton organization or metabolism. mTOR is the  
305 catalytic subunit of two functionally distinct protein complexes: mTOR complex 1 (mTORC1) and  
306 mTORC2. The aberrant activation of mTOR activity is observed in multiple cancer types, resulting  
307 from phosphoinositide 3-kinase (PI3K) amplification/mutation, phosphatase and tensin homolog  
308 (PTEN) loss of function, or from the overexpression of Akt, Ribosomal protein S6 kinase beta-1  
309 (S6K1), eukaryotic translation initiation factor 4E-binding protein 1 (eIF4EBP1) or eIF4E. For this  
310 reason, mTOR pathway inhibition is regarded as an important target for the development of new cancer  
311 therapies. Phosphorylation of S6K1 was detected in 69% of conventional CS and 44% of  
312 dedifferentiated CS [32], suggesting that mTOR inhibition may be a good strategy for CS therapy.

313 Surprisingly, inhibition of mTORC1 by rapamycin lowered the proportion of CSCs. The role of  
314 S6K1 and eIF4eBP1 in mTORC1-mediated regulation of translation is well known. Moreover,  
315 forkhead box O (FOXO) transcription factors are crucial regulators of cellular homeostasis and are  
316 known tumor suppressors in human cancers [62]. The complex interplay between FOXO, mTOR and  
317 Akt has been described [36]. FOXOs decrease ROS levels and inhibit mTORC1 via Sestrin3 [63]. On  
318 the other hand, it was also reported that the mTOR pathway is capable of regulating FOXO3 activity  
319 by downregulating glucocorticoid-inducible kinase 1 (SGK1), which is responsible for FOXO3  
320 phosphorylation. The inactivation of mTORC1 induced by p18 depletion led to FOXO3  
321 hypophosphorylation at Ser314 [64]. Here, we show that inhibition of mTORC1 led to increased  
322 FOXO3 promoter activity and that it directly led to the reversal of CSC-like phenotype. FOXO3 is a

323 transcriptional regulator of miR-34 [65,66] and its activation led to the inhibition of miR-34 targets  
324 like KLF4.

325 Therefore, inactivation of mTORC1 by rapamycin has direct effects on miR-34-associated  
326 pathways. Rapamycin treatment together with miR-34 mimic administration had a sustain inhibitory  
327 effect on CSC-like phenotype. However, the fact that the combination of miR-34 mimic and rapamycin  
328 administration is more potent than rapamycin alone suggests that alternative molecular mechanisms  
329 are also likely to be involved. In non-treated cells, only higher irradiation doses led to significant  
330 effects (such as the induction of cell death pathways). However, high dose exposures can lead to a  
331 relative CSC enrichment [67,68]. By delivering a combination treatment, it was then possible to further  
332 decrease irradiation doses while efficiently suppressing CSC-like attributes.

333 Altogether, these results suggest that mTOR inhibition by rapamycin supplemented with miR-34  
334 mimic treatment may be able to overcome CSC-associated radioresistance in chondrosarcoma during  
335 carbon-ion therapy. Combination treatments might also improve the effectiveness of carbon-ion  
336 therapies at lower doses, decrease risks of relapse and metastasis, and better preserve surrounding  
337 normal tissues against non-targeted effects.

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532 **Figure legends**

533 **Figure 1.** CH-2879 chondrosarcoma cells contain a radioresistant cancer stem cell subpopulation  
534 suppressed by miR-34. **a** Sorting of ALDH<sup>+</sup> cancer stem cells. DEAB-treated cells served as negative  
535 control. The proportion of ALDH<sup>+</sup> cells was measured after carbon-ion irradiation and/or treatment  
536 with miR-34 mimic (34m), rapamycin (rap) or rapamycin + FOXO3 siRNA (rap+Fsi). **b,c,d** Invasion  
537 scratch assay (**b**), sphere-formation assay (**c**) and reactive oxygen species (ROS) level measurements  
538 (**d**) were performed in ALDH<sup>-</sup> and ALDH<sup>+</sup> cells after treatment with miR-34 mimic (34m). **e** Dose-  
539 response curves for clonogenic survival of CH-2879 chondrosarcoma cells. ALDH<sup>-</sup> and ALDH<sup>+</sup> cells  
540 were exposed to X-rays or carbon-ion beam (the differences of clonogenic survival between ALDH<sup>-</sup>  
541 and ALDH<sup>+</sup> cells were significant for every dose after X-ray irradiation and after 2-5 Gy carbon-ion).  
542 Results are expressed as the mean  $\pm$  SD of three or more independent experiments.

543 **Figure 2.** miR34 exerts its effects via KLF4 and protects against tumor formation. **a,b** Expression  
544 of miR-34 (**a**) and miR-34 target genes (**b**) in ALDH<sup>+</sup> cells, relative to ALDH<sup>-</sup> cells. **c** KLF4 protein  
545 expression levels in ALDH<sup>-</sup> and ALDH<sup>+</sup> cells after treatment with miR-34 mimic (34m) or  
546 transfection with FOXO3 expression vector (fox). **d** Sphere formation assay in ALDH<sup>+</sup> cells after  
547 treatment with KLF4 siRNA. Results are expressed as the mean  $\pm$  SD of three or more independent  
548 experiments. **e,f** Growth of chondrosarcoma subcutaneous xenografts (**e**) was measured by external  
549 caliper in nude mice, after injection of ALDH<sup>-</sup> or ALDH<sup>+</sup> cells and/or miR-34 administration (**f**).  
550 Results are expressed as the mean  $\pm$  SD for five or more animals.

551 **Figure 3.** mTOR inhibition by Rapamycin targets chondrosarcoma stem cells via FOXO3 and  
552 miR-34. **a,b** Expression of miR-34 (**a**) and miR-34 target genes (**b**) after rapamycin treatment, relative  
553 to non-treated cells. **c** FOXO3 promoter activity after transfection with FOXO expression vector or  
554 rapamycin treatment. **d** Sphere formation assay in ALDH<sup>+</sup> cells after treatment with FOXO expression  
555 vector (FOXO3), rapamycin (rap) or rapamycin + FOXO3 siRNA (Fsi). **e,f** Expression of miR-34 (**e**)

556 and miR-34 target genes (**f**) in cells transfected with FOXO expression vector, relative to cells treated  
557 with control vector. Results are expressed as the mean  $\pm$  SD of three or more independent experiments.

558 **Figure 4. a** Sphere formation assay after treatment with miR-34 mimic and/or rapamycin, when  
559 cells were plated at various times following treatment. **b** Proportion of remaining ALDH<sup>+</sup> CSCs after  
560 exposure to 1 Gy carbon-ion, equivalent dose 2 Gy X-rays in cells treated with rapamycin alone or in  
561 combination with miR-34 mimic, relative to the proportion of ALDH<sup>+</sup> cells in non-irradiated,  
562 untreated cells. Results are expressed as the mean  $\pm$  SD of three or more independent experiments.

### 563 **Annex A. Supplementary data**

564 Supplementary data available.

565 **Table A1.** Chondrosarcoma cell lines.

566 **Table A2.** Primers for quantitative real-time PCR.

567 **Table A3.** TCGA survival Cox regression results for miR-34 and for 18 miR-34 target genes in  
568 sarcoma patients.

569 **Figure A1.** Transcriptional response of CH-2879 cells to carbon-ion irradiation (a) Number of  
570 deregulated genes 24h after exposure to various irradiation doses (b) Multi-dimensional scaling (MDS)  
571 plot showing sample relations. (c) Top Regulator Effect Networks in Ingenuity Pathway Analysis  
572 (IPA). p53-associated networks are highlighted in pink.

573 **Figure A2.** Apoptosis of CH-2879 cells after miR-34 mimic administration.

574 **Figure A3.** Electropherograms for KLF4 and GAPDH expression.

575 **Figure A4.** CH-2879 cell proliferation after rapamycin treatment.

576 **Figure A5.** Dose-response curves for clonogenic survival of CH-2879 cells after rapamycin  
577 treatment and carbon-ion irradiation.

578 **Figure A6.** Kaplan-Meier survival curves of sarcoma patients with high (top third) or low (bottom  
579 third) expression.

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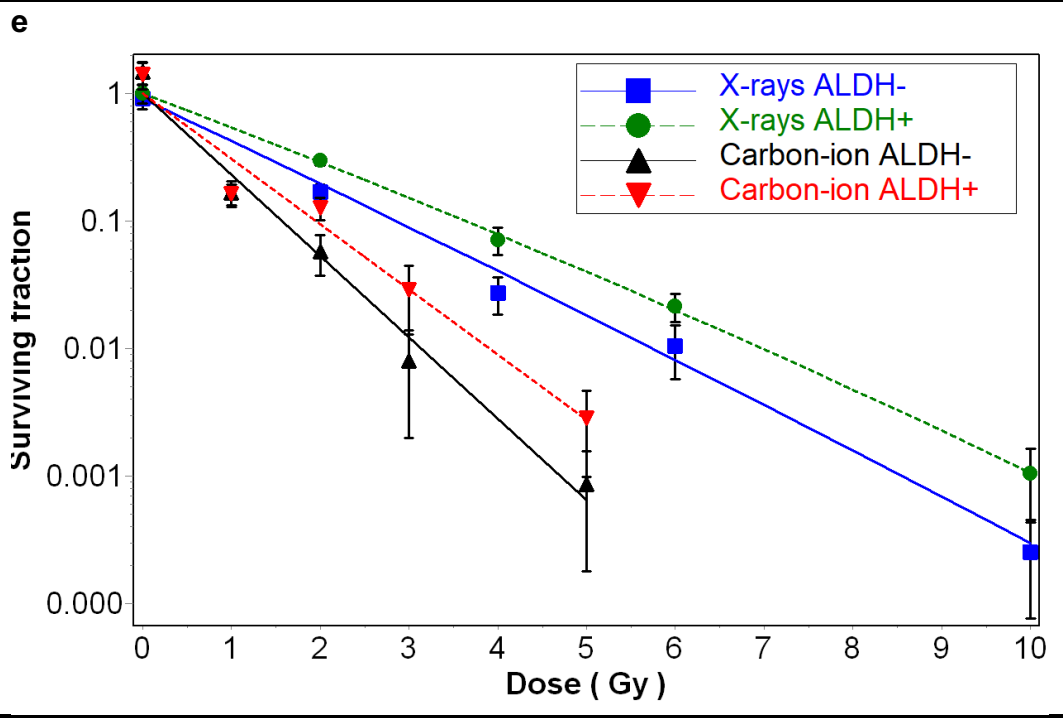
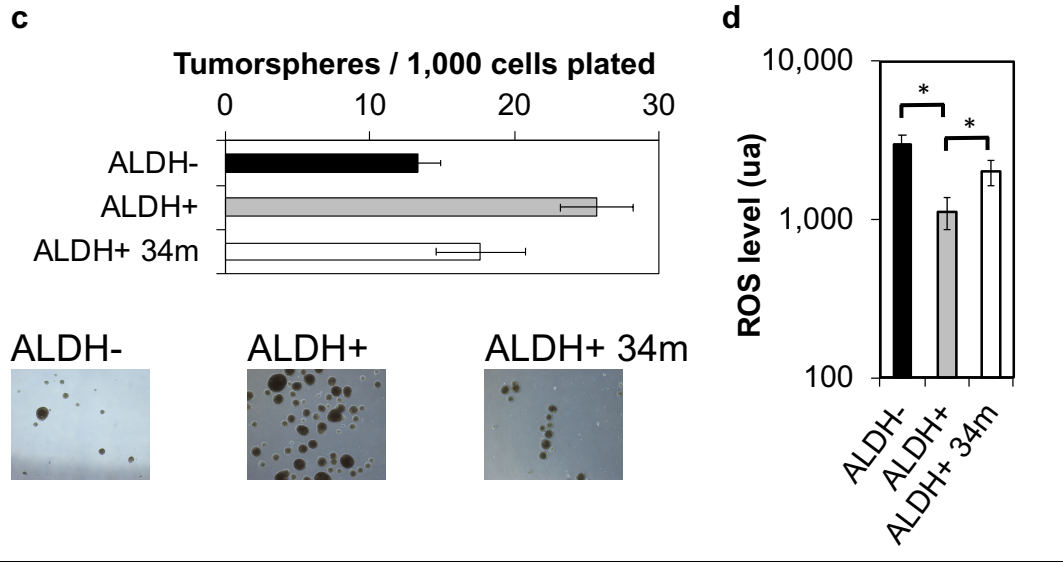
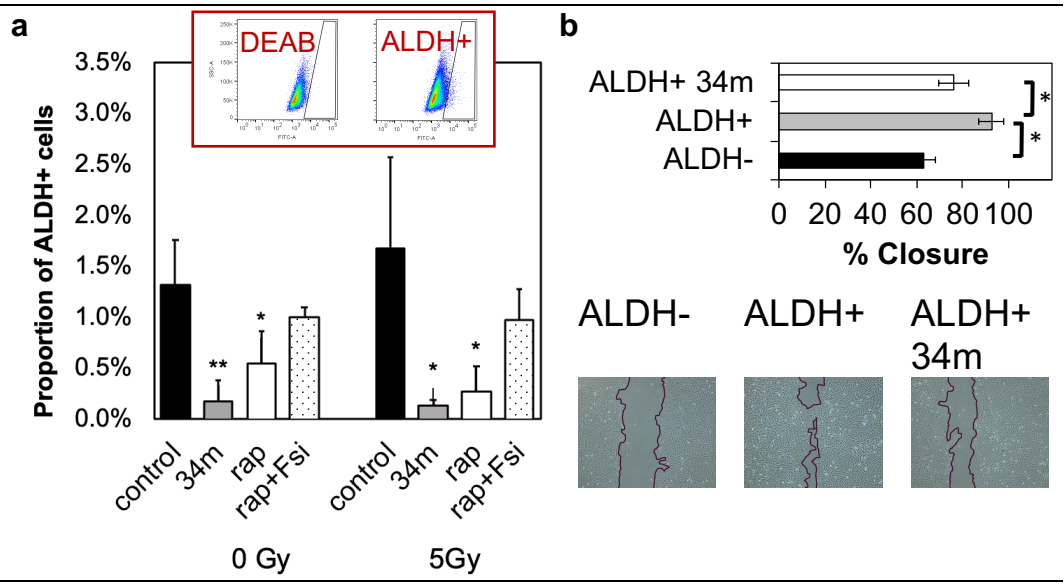
		<b>D10</b>	<b>D37</b>	<b>SF2</b>	<b>RBE<sub>(D10)</sub></b>	<b>RBE<sub>(D37)</sub></b>
<b>X-rays</b>	ALDH-	2.97	1.29	0.21		
	ALDH+	3.64	1.62	0.29		
<b>Carbon-ion</b>	ALDH-	1.57	0.68	0.05	1.89	1.90
	ALDH+	1.96	0.84	0.09	1.86	1.93

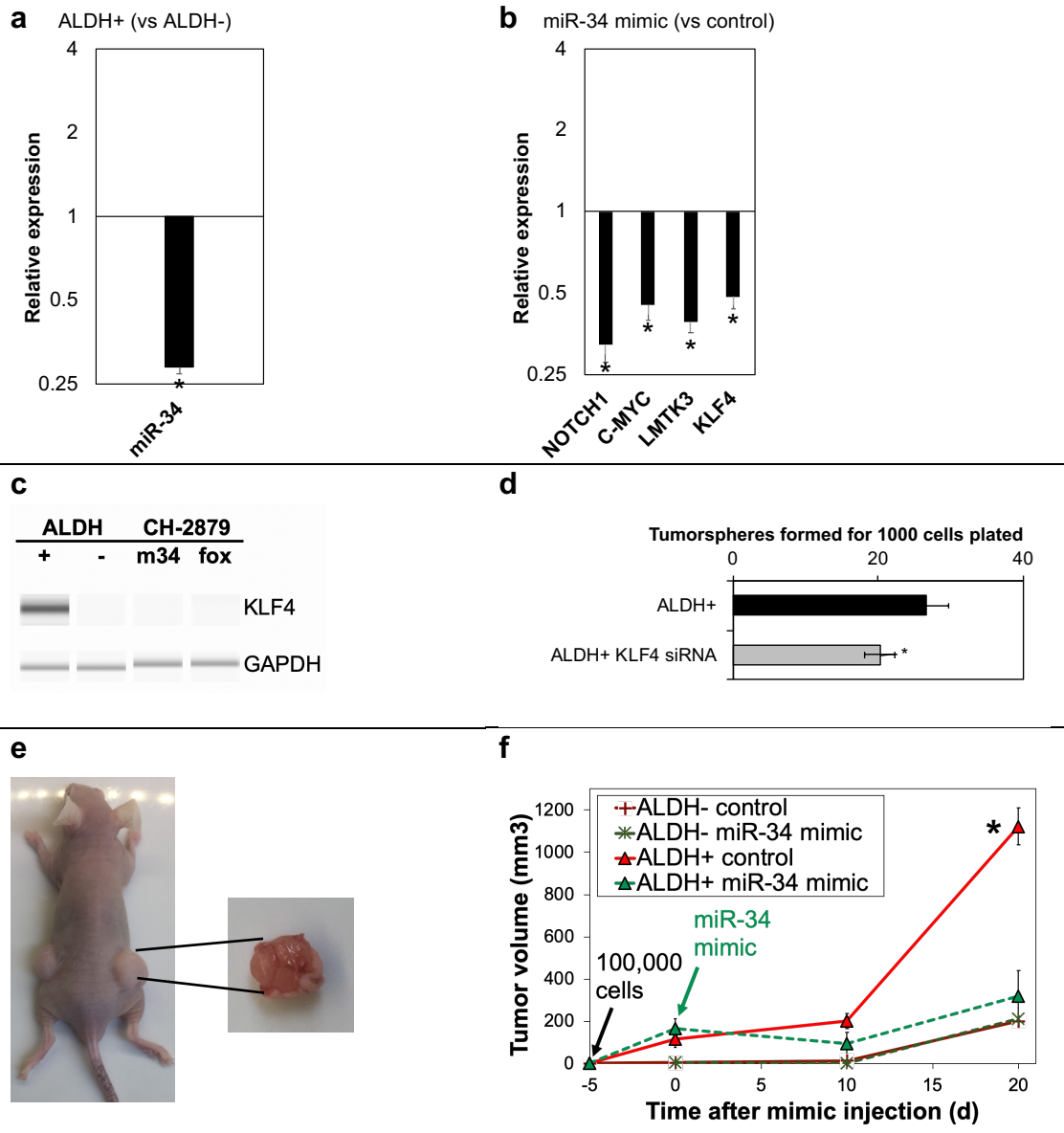
**Table 1:** Clonogenic survival characteristics of CH-2879 cells exposed to X-rays or Carbon-ion beam.

<b>Injection</b>	<b>Control</b>	<b>miR-34 mimic</b>
1,000 ALDH-	0/4 (0 %)	0/4 (0 %)
1,000 ALDH+	0/4 (0 %)	0/4 (0 %)
10,000 ALDH-	0/4 (0 %)	0/4 (0 %)
10,000 ALDH+	5/5 (100 %)	1/5 (20 %)
100,000 ALDH-	1/5 (20%)	0/5 (0 %)
100,000 ALDH+	9/9 (100%)	4/9 (45 %)

**Table 2.** Induction of xenograft tumors in nude mice after administration of increasing numbers of CH-2879 cells and miR-34 mimic.

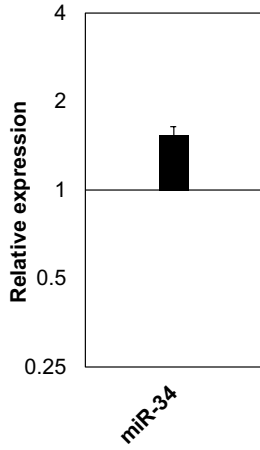




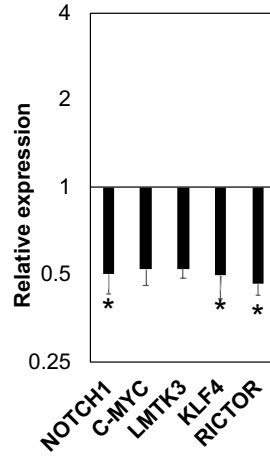


**Figure 2**

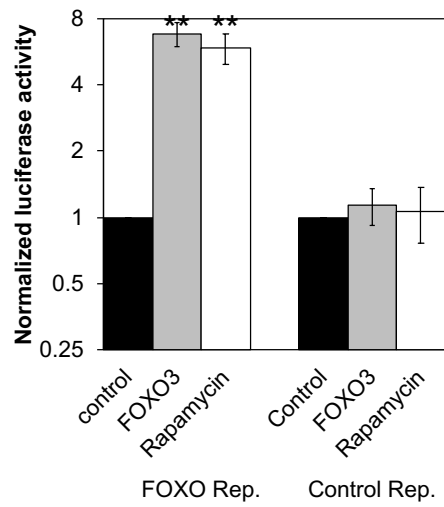
**a** Rapamycin (vs control)



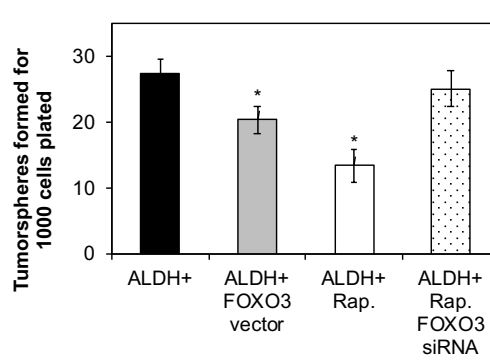
**b** Rapamycin (vs control)



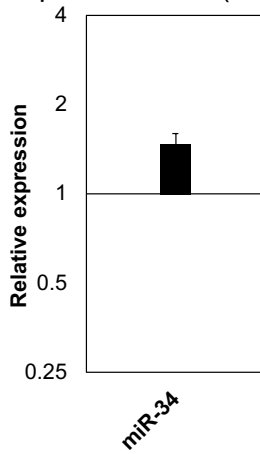
**c**



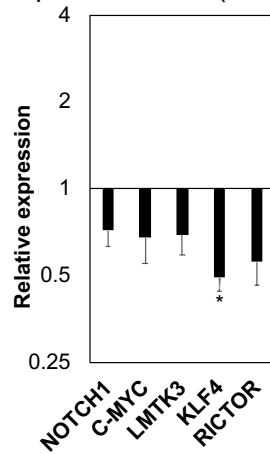
**d**



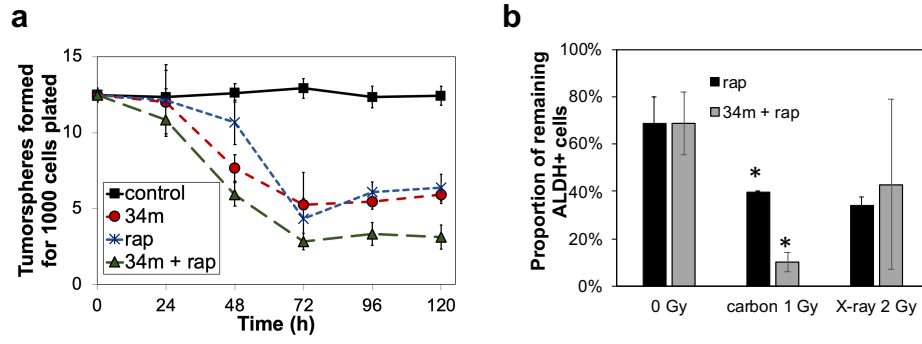
**e** pCMV-Foxo3 (vs control)



**f** pCMV-Foxo3 (vs control)



**Figure 3**



**Figure 4**

## Appendix A. Supplementary Information

### A multimodal treatment of carbon ions irradiation, miRNA-34 and mTOR inhibitor specifically control high-grade chondrosarcoma cancer stem cells

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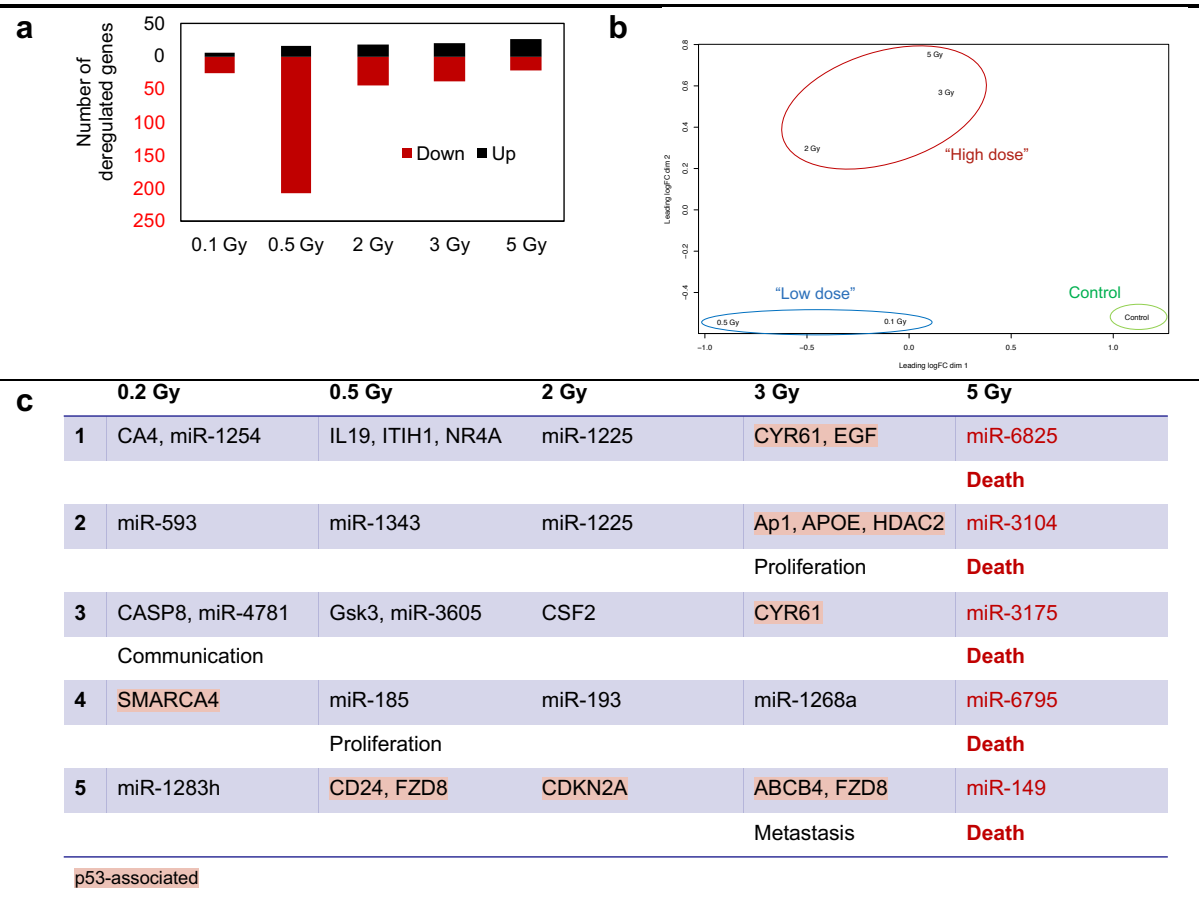
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<b>Name</b>	<b>Diagnosis</b>	<b>Grade</b>	<b>Reference</b>
CH-2879	Chondrosarcoma	III	1
OUMS27	Chondrosarcoma	III	2
L835	Chondrosarcoma	III	3
SW-1353	Chondrosarcoma	II	4

**Table A1:** Chondrosarcoma cell lines.

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
NOTCH1	CTGAAGAACGGGGCTAACAA	AGTGGTCCAGCAGCACCTT
C-MYC	CCACACATCAGCACAACTACGC	CGGTTGTTGCTGATCTGTCTCA
LMTK3	TCGGCTTCAAGGAATTTGAGA	GGGTGGTCATGTCTGAGTGTGA
KLF4	GCCCCTCGGGCGGCTTCGTGGCCGAGCTC	CGTACTCGCTGCCAGGGGCG
RICTOR	CCGTGTTCGGAGGTTTCATACA	GCCTCTGCTTCTTCATGCATT
GAPDH	GAAGGTGAAGGTCGGAGTCA	TTGATGGCAACAATATCCACTT

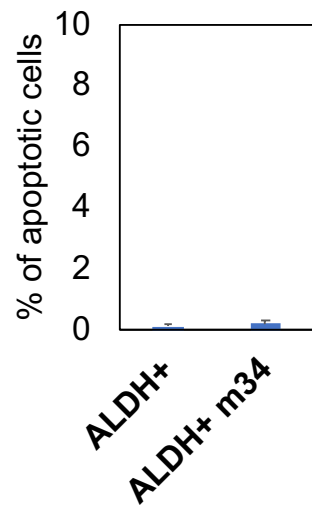
**Table A2:** Primers for quantitative real-time PCR.



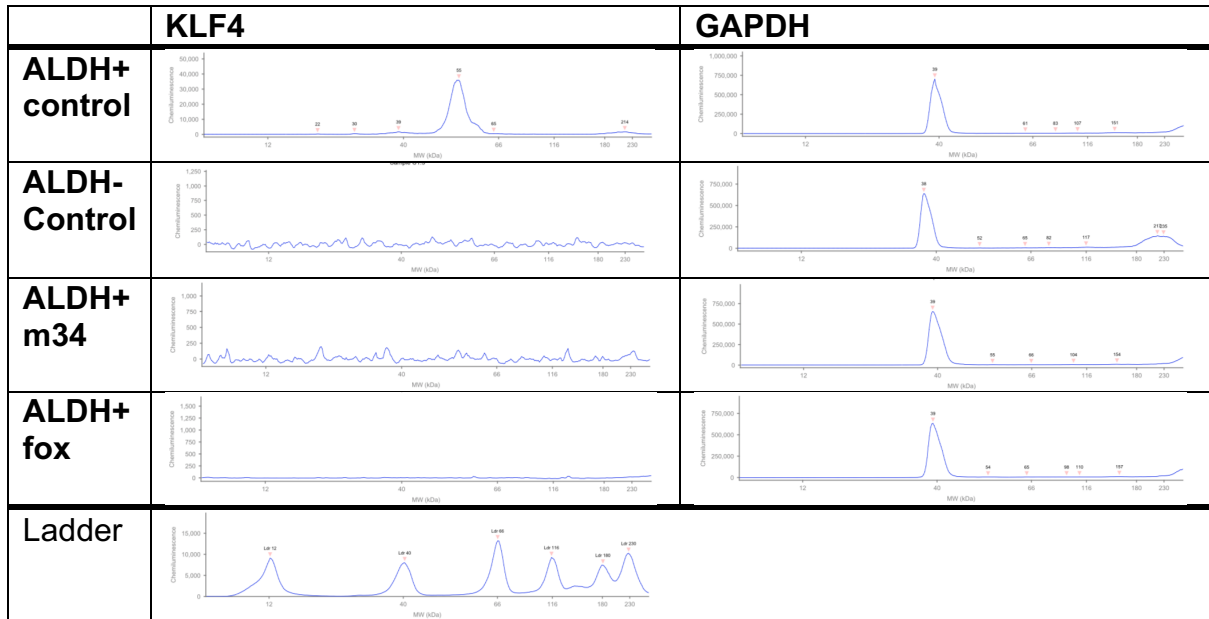
**Figure A1:** Transcriptional response of CH-2879 cells to carbon-ion irradiation (a) Number of deregulated genes 24h after exposure to various irradiation doses (b) Multi-dimensional scaling (MDS) plot showing sample relations. (c) Top Regulator Effect Networks in Ingenuity Pathway Analysis (IPA). p53-associated networks are highlighted in pink.

Using RNA-Seq, we measured gene regulations in CH-2879 cells one day after carbon-ion exposure. A number of genes were up- or down-regulated (Figure A1a), and multi-dimensional scaling (MDS) analysis supported the partition of irradiation doses into two groups (“low doses” and “high doses”). The investigation of individual gene deregulations as well as the determination of regulator effect networks suggested that only high doses (in particular the highest dose of 5 Gy) predominantly activated stress response pathways and cell death-associated mechanisms.

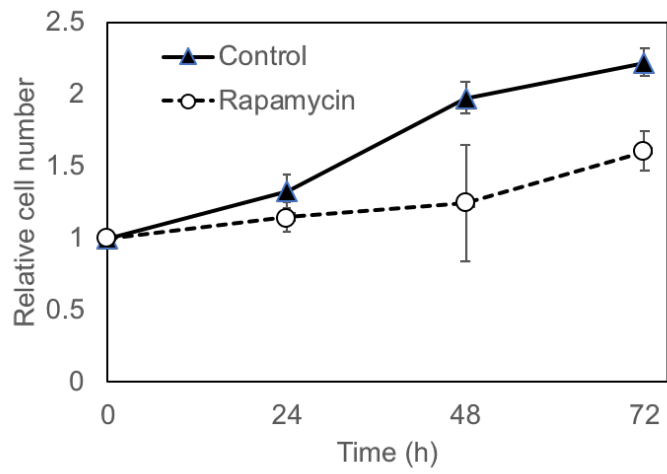




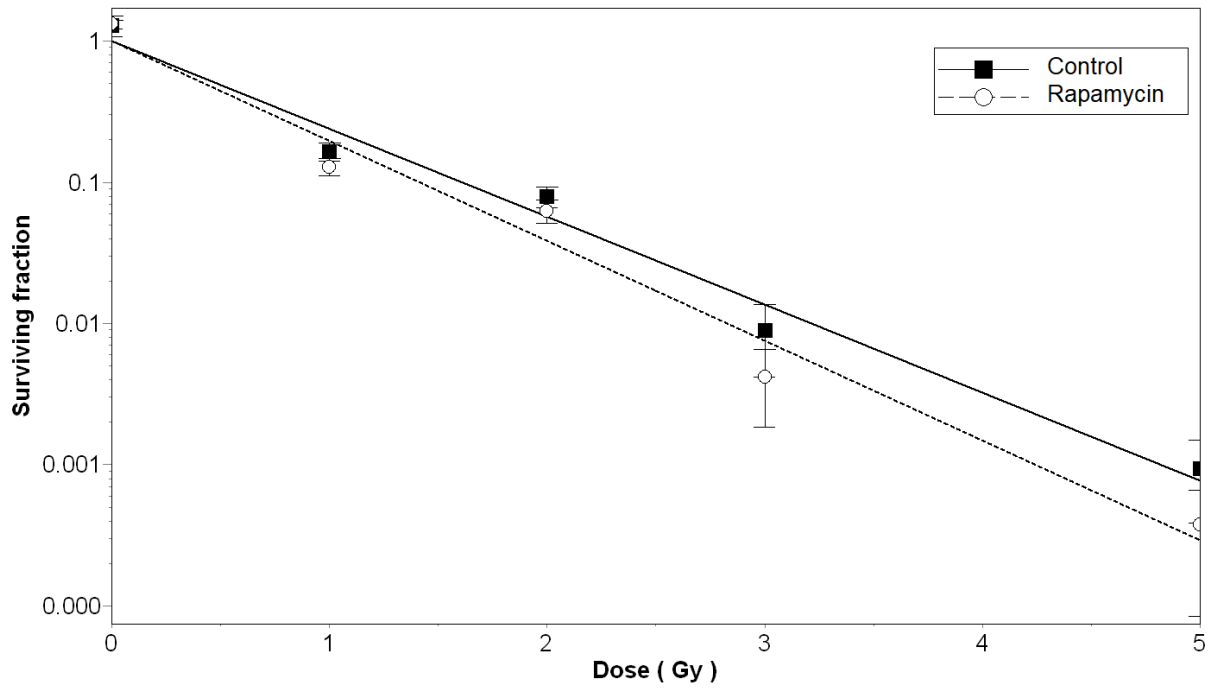
**Figure A2:** Apoptosis of CH-2879 cells after miR-34 mimic administration.



**Figure A3:** Electropherograms for KLF4 and GAPDH expression after in ALDH- and ALDH+ cells after treatment with miR-34 mimic (34m) or transfection with FOXO3 expression vector (fox).



**Figure A4:** CH-2879 cell proliferation after rapamycin treatment.

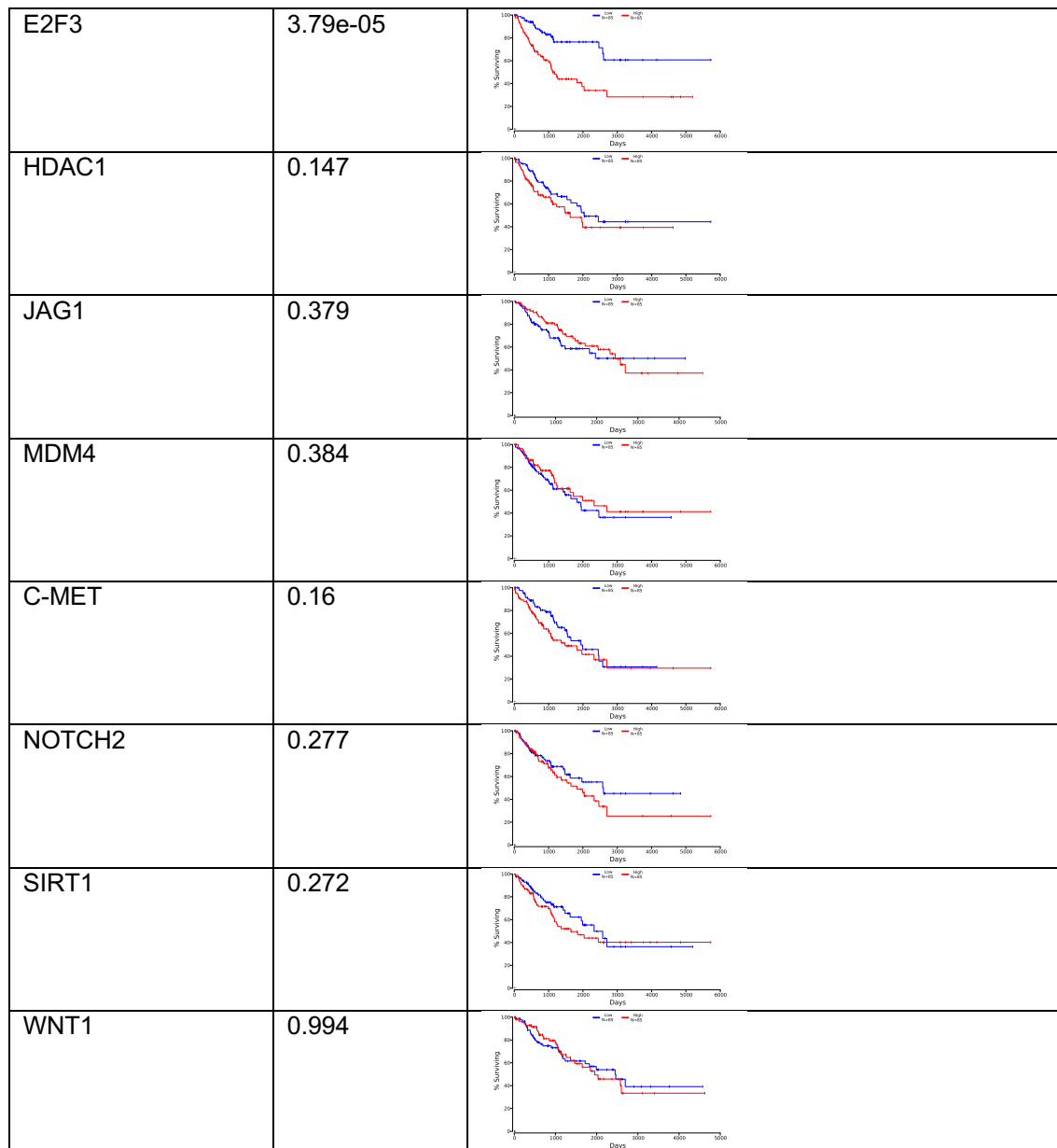


**Figure A5:** Dose-response curves for clonogenic survival of CH-2879 cells after rapamycin treatment and carbon-ion irradiation.

Transcript	Cox Coefficient	P-Value	FDR Corrected	Rank
hsa-MIR-34A-5P	0.069	0.51	0.622	373
NOTCH1	-0.117	0.27	0.565	7716
C-MYC	0.265	0.012	0.113	1703
LMTK3	0.027	0.8	0.915	14126
KLF4	-0.103	0.31	0.605	8240
RICTOR	-0.114	0.29	0.586	8008
BCL2	0.097	0.37	0.654	9054
CCND1	-0.04	0.69	0.863	12876
CCNE2	0.121	0.24	0.532	7204
CDK4	0.213	0.033	0.191	2764
CDK6	0.144	0.18	0.460	6195
E2F3	0.395	$1.80 \times 10^{-4}$	0.0146	197
HDAC1	0.237	0.034	0.193	2825
JAG1	-0.152	0.11	0.352	4947
MDM4	0.004	0.97	0.987	15907
MET	0.101	0.35	0.639	8819
NOTCH2	-0.02	0.86	0.944	14748
SIRT1	0.083	0.43	0.701	9934
WNT1	-0.053	0.62	0.826	12179

**Table A3:** TCGA survival Cox regression results for miR-34 and for 18 miR-34 target genes in sarcoma patients.

Transcript	Logrank p-Value	
hsa-MIR-34A-5P	0.742	
NOTCH1	0.0373	
C-MYC	0.0078	
LMTK3	0.341	
KLF4	0.416	
RICTOR	0.454	
BCL2	0.721	
CCND1	0.627	
CCNE2	0.0558	
CDK4	0.00961	
CDK6	0.0115	



**Figure A6:** Kaplan-Meier survival curves of sarcoma patients with high (top third) or low (bottom third) expression.

## **Supplementary material and methods**

**RNA-Seq.** RNA was extracted using Trizol and a column-based PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). RNA-sequence libraries were prepared with the TruSeq Stranded mRNA NeoPrep kit (Illumina, San Diego, CA, USA). Sequencing was performed on a HiSeq 4000 machine (Illumina) to generate 150 nucleotide paired-end reads at a depth of at least 40 million reads. After quality control with FastQC, reads were mapped to the hg19 reference human genome using tophat2 software (v2.1.1), assembled and quantified using the cufflinks software suite (v2.2.1)<sup>5</sup>. Differentially expressed genes were counted and analyzed with Ingenuity Pathway Analysis (Qiagen, Hilden, Germany)<sup>6</sup>. Mapped reads were also sorted with samtools (v1.3.1), read counts were then quantified with HTSeq-count (v0.9.1) and analyzed with the edgeR package (v3.22.5, using Bioconductor v3.7 on R v3.5.0) for multi-dimensional scaling (MDS)<sup>7</sup>. The sequencing data have been deposited in the Gene Expression Omnibus (GEO) database (Accession Number GSE135371).

**Apoptosis.** Cells were washed with PBS, then stained with Annexin V-FITC and PI at room temperature in the dark using Annexin V-FITC Apoptosis Detection Kit (Nacalai, Kyoto, Japan). The stained cells were analyzed with a FACS Aria flow cytometer (Becton Dickinson, San Jose, CA, USA). Apoptosis was shown as the percentage of apoptotic cells to the total number of counted cells.

**TCGA analysis.** Data from 259 sarcoma patients downloaded from the Cancer Genome Atlas (TCGA) were used to correlate gene expression and survival, using OncoLnc tool (<http://www.oncolnc.org/>). For each gene, Cox regression analysis was performed then sarcoma patients were divided in high (top third) or low (bottom third) expression groups and survival of the two groups was compared using Kaplan-Meier plots and log rank analysis<sup>8</sup>.



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