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Replication stress is an actionable genetic vulnerability in desmoplastic small round cell tumors

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Abstract (233 / 250 words)

Desmoplastic small round cell tumor (DSRCT) is an aggressive sarcoma subtype of high unmet need that is driven by the EWS-WT1 chimeric transcription factor. To identify novel therapeutic approaches to the targeting of DSRCT, we conducted a high-throughput drug sensitivity screening assessing chemosensitivity profiles for 79 small-molecule inhibitors on the JN-DSRCT-1 cell line. We found DSRCT cells to be sensitive to PARP and ATR inhibitors (PARPi, ATRi), as a monotherapy and in combination. These effects were recapitulated using multiple clinical PARPi and ATRi in three biologically distinct, clinically-relevant models of DSRCT, including cell lines, a patient-derived xenograft (PDX)-derived organoid, and a cell line-derived xenograft mouse model. Mechanistically, exposure to a combination of PARPi and ATRi caused increased DNA damage, G2/M checkpoint activation, micronuclei, replication stress, and R-loop formation. EWS-WT1 silencing abrogated these phenotypes and was epistatic with exogenous expression of the R-loop resolution enzyme RNase H1 in reversing the sensitivity to PARPi and ATRi monotherapies, suggesting that EWS-WT1-dependent increase in R-loop formation could be a mechanistic cause of drug sensitivity in DSRCT cells. Combination of PARPi and ATRi further induced an EWS-WT1-dependent cell-autonomous activation of the cyclic GMP-AMP synthase/stimulator of interferon genes (cGAS/STING) innate immune pathway and cell surface expression of programmed-death ligand-1 (PD-L1). Taken together, our findings point towards a role for EWS-WT1 in generating R-loop-dependent replication stress and provide a rationale for the clinical assessment of PARPi and ATRi in DSRCT.

Statement of significance: 29 / 32 Words

We show that EWS-WT1, the unique oncogenic driver of DSRCT, confers sensitivity to PARP and ATR inhibitors, thus providing a rationale for assessing these drugs in patients with DSRCT.

1 Introduction

2 Desmoplastic small round cell tumor (DSRCT) is a rare and aggressive subtype
3 of sarcoma, affecting predominantly young males (1). DSRCT classically
4 presents as a large abdominal mass and is most often diagnosed at advanced
5 or metastatic stages with multiple peritoneal metastatic nodules, and sometimes
6 distant metastases. Clinical prognosis for advanced DSRCT remains poor with
7 a 5-year survival rate below 15% (2). No major therapeutic advance has
8 occurred for DSRCT over the past 20 years, and currently, DSRCT patients
9 undergo a standard Ewing sarcoma regimen, consisting of highly aggressive
10 poly-chemotherapy and extensive surgical debulking (1). Therefore, the
11 development of novel therapeutic strategies is urgently needed.

12

13 DSRCT is molecularly characterized by the t(11;22)(q13;q12) chromosomal
14 translocation, which fuses the transactivation domain of *EWSR1* to the DNA
15 binding domain of *WT1*, encoding an aberrant chimeric transcription factor (2,3).
16 The presence of *EWSR1::WT1* rearrangement is pathognomonic of the disease
17 and provides the diagnosis of DSRCT over other small round cell sarcomas (2).
18 Recent genomic sequencing identified rare additional secondary mutations,
19 notably in genes encoding proteins involved in chromatin remodeling and DNA
20 repair such as *ARID1A*, *KMT2C*, and *MSH3* (4–7). EWS-WT1 conditional
21 expression in mesenchymal stem cells – the putative cell-of-origin of DSRCT –
22 is necessary and sufficient to generate a DSRCT phenotype (8), and EWS-WT1
23 is considered as the unique driver in this simple-genomics sarcoma (9). As such,
24 this chimeric aberrant transcription factor represents the most evident

25 therapeutic target in DSRCT. However, the direct targeting of transcription
26 factors is extremely challenging (10), and one of the most promising strategies,
27 which consists of degrading the target transcription factor, is just entering the
28 clinic and has not yet been evaluated in transcription factors-driven sarcomas.
29 Therefore, targeting downstream consequences of EWS-WT1 presence, such
30 as transcription factor-induced oncogenic programs or replication stress, is an
31 attractive strategy.

32

33 In this study, we aimed to identify novel actionable targeted dependencies in
34 DSRCT, using functional genomics and small-molecule inhibitor screening. We
35 found that two distinct DSRCT cell lines, one newly-established patient-derived
36 xenograft (PDX)-derived organoid (PDX-O) model and one cell line-derived
37 xenograft mouse model were selectively sensitive to poly-ADP-ribose
38 polymerases inhibitors (PARPi) and ataxia-telangectasia and Rad3-related
39 inhibitors (ATRi). Mechanistically, we found that the presence of EWS-WT1
40 increased DNA replication stress and R-loop formation, thereby causing
41 enhanced reliance upon the ATR/CHK1 pathway. Exposure to PARPi and ATRi
42 further activated the cyclic GMP-AMP synthase/stimulator of interferon genes
43 (cGAS/STING) pathway and caused PD-L1 upregulation in DSRCT cell lines,
44 suggesting potential for these drugs as DNA repair-targeted therapies and
45 immunomodulators in DSRCT.

46

47

48

49

50 **Materials and Methods**

51

52 **Cell lines**

53 The DSRCT JN-DSRCT-1 (JN1) cell line was purchased from American Type
54 Culture Collection (ATCC). The DSRCT R cell line was created in-house,
55 derived from a patient-derived xenograft (PDX) shared by Dr. Armelle Logié-
56 Dishington (Champions Oncology, France). Briefly, the PDX was finely minced
57 into tiny pieces that were subsequently washed in fetal bovine serum (FBS) and
58 centrifuged. The pellet was resuspended in DMEM/F12 supplemented with 20%
59 FBS, 1X MEM non-essential amino acids (#11140050, Gibco) and 1X Pen-
60 Strep (#15070063, Gibco), and incubated in a 10 cm² petri dish at 37°C and 5%
61 CO₂. The culture medium was changed every other day with recovery of
62 suspended cells by centrifugation at 1200rpm. After 6-8 weeks, a partially
63 homogeneous cell layer was obtained; from this primary culture, cells were
64 washed with 1X phosphate buffered saline (PBS), dissociated in trypsin-EDTA
65 solution (#25200056, Gibco), and seeded into a new culture flask for
66 subsequent cell culture. JN1 and R cells were cultured in DMEM/F12,
67 supplemented with 10% or 20% of FBS, respectively. A673 and SaOS-2 cells
68 were cultured in DMEM, supplemented with 10% FBS. All cells were grown at
69 37°C and 5% CO₂. Mycoplasma testing was performed bimonthly using the
70 MycoAlert Mycoplasma Detection Kit (Lonza). All cell lines were short-tandem-
71 repeat typed using StemElite ID (Promega) to confirm identity.

72 The JN1 and R cell lines were originally derived from human tumors that were
73 histopathologically diagnosed as DSRCT (11): the JN1 cell line was established

74 from a 7-year old male metastatic DSRCT patient's pleural effusion and harbors
75 the following pathognomonic *EWSR1::WT1* fusion 3'-
76 [CCCATGGATGAAGGACCAGATCTTGATCTAG]-[GTGAGAAACCATAACCACT
77 GTGACTTCAAGG]-5' (**Supp Fig. S1**); the R cell line was established from a
78 20-year old male metastatic DSRCT patient's lymph node and harbors the
79 following pathognomonic *EWSR1::WT1* fusion 3'-
80 [GGAGAGCGAGGTGGCTTCAATAAGCCTGGTG]-[GTGAGAAACCATAACCACT
81 TGTGACTTCAAGG]-5' (**Supp Fig. S2**). The Ewing sarcoma A673 cell line was
82 gifted by Dr. Olivier Delattre (Institut Curie, France) and the osteosarcoma
83 SaOS-2 cell line was gifted by Dr. Olivia Fromigue (Gustave Roussy, France).

84

85 **Generation of RNase H1-overexpressing JN1 cells**

86 To generate stable RNase H1-expressing JN1 (JN1-RNaseH1 cell line), the
87 ppyCAG-RNaseH1-V5 plasmid (Addgene, #111906) was transfected in JN1
88 cells with Lipofectamine 2000 (Thermo Fisher) according to manufacturer's
89 instructions. Stable pools of transfectants were generated by selection with
90 hygromycin B and the resultant selected populations were submitted to clonal
91 isolation using the limiting dilution method. Clones were recovered and profiled
92 for RNase H1 expression by western blot.

93

94 **Drugs and chemicals**

95 The PARP inhibitors olaparib (AZD2281), talazoparib (BMN-673) and veliparib
96 (ABT-888), the ATR inhibitors gartisertib (M4344), ceralasertib (AZD6738) and
97 berzosertib (M6620), the CHK1 inhibitors prexasertib (LY2606368) and SRA-

98 737, as well as cisplatin, topotecan, and SN-38, were purchased from Selleck
99 Chemicals. The ATR inhibitor tuvusertib (M1774) was provided by Merck
100 (Darmstadt, Germany). Inhibitor stock solutions were prepared in
101 dimethylsulfoxide (DMSO) and stored in aliquots at -80°C. Mitomycin C (MMC),
102 thymidine, iodo-deoxyuridine (IdU), and 5-chloro-2'-deoxyuridine (CldU) were
103 purchased from Sigma-Aldrich. PicoGreen® was purchased from ThermoFisher.

104

105 **Small-molecule inhibitor and drug screen**

106 The small-molecule inhibitor and drug screen was performed as described
107 previously (12). Briefly, small molecules were purchased as solid from suppliers
108 listed in **Supp Table S1** and stored in DMSO. Prior to the 384 well-plate screen,
109 solid small molecules were resuspended in DMSO as 10 mM stocks, prior to
110 further dilution in DMSO to create 384 well-plates containing a titration (0.5, 1, 5,
111 10, 50, 100, 500, 1000 nM). A Hamilton Microlab Star liquid handling platform
112 was used for this and all subsequent liquid handling steps except for cell
113 seeding.

114 JN1 cells growing in log phase were seeded in 384 well-plates at 250 cells per
115 well in 50 µL of culture medium using a Thermo Fisher Multi-Drop Combi. This
116 plating density was optimized to ensure that the cells were in growth phase by
117 the end of the five-day treatment. 24 h after seeding, the medium was removed
118 and replaced with medium containing the small molecule inhibitor library, as
119 detailed above. Cells were then continuously cultured in the presence of small
120 molecule inhibitors for a period of five days, at which point cell viability was
121 estimated by adding 20 µL of CellTiter Glo® (Promega), diluted 1:4 in PBS to

122 the medium. After 10 min incubation at room temperature, the CellTiter Glo®-
123 generated luminescence was captured using a Victor X-Light plate reader.
124 Luminescence values from each well were normalized to the median of signals
125 from wells exposed to DMSO only (in absence of small molecule inhibitor) to
126 generate surviving fractions (SF). In total, the cell line was screened three times,
127 generating triplicate SF data sets. Surviving fractions were then used to plot
128 dose-response survival curves, generated using 3-parameter logistic regression
129 analysis via the drc R-package²⁰. Using drc, Area under the curve (AUC)
130 values were calculated from dose-response survival curves. AUC values were
131 expressed as the proportion of the maximum area, representing no response to
132 a drug. They were further scaled to lie between 0 and 1. AUC values that were
133 greater than 1 were capped to 1. Unscaled AUC values for each drug were also
134 standardized, generating robust Z-scores based upon the median AUC effect in
135 a panel of 92 cancer cell lines (**Supp Tables S2-S4**) and the median absolute
136 deviation of these effects. Z-scores were then plotted as a waterfall plot.

137

138 **2D cell-based assays**

139 Cells were plated in 96-well plates at 7000 cells per well for JN1 cells and
140 10000 cells per well for R cells and continuously exposed to the drugs for a
141 period of 7 days in culture. In the case of siRNA transfection, cells were
142 transfected in 6-well plates 48 h prior to drug exposure and trypsinized and
143 reseeded at the density specified above in 96-well plates 24 h prior to drug
144 exposure. Cell viability was estimated by the addition of 50µL of CellTiter-Glo®
145 Luminescent Cell Viability Assay (Promega), diluted in 1:4 in PBS. After 10 min

146 incubation at room temperature, the CellTiter Glo®-generated luminescence
147 was captured using a Victor X-Light plate reader. Luminescence values from
148 each well were normalized to the median signal of wells exposed to DMSO
149 (vehicle) to generate surviving fractions (SF). Surviving fractions were then
150 used to plot dose-response survival curves using GraphPad Prism.

151 For synergy analyses, cells were seeded in 96-well plates and continuously
152 exposed to increasing concentrations of talazoparib (1:4 serial dilution, range:
153 0-500nM) and/or M4344 (1:3 serial dilution, range: 0-1000nM) for 7 days in
154 culture. Cell viability was assessed as described above. The median response
155 of replicates was normalized per median marginal value (i.e. response in
156 absence of treatment). Synergy analysis was performed using R package
157 synergyfinder. Dose-response curves for single drugs were fitted to a four-
158 parameter log-logistic model. Synergy scores were calculated using the Bliss
159 independence model.

160

161 **3D spheroids assay**

162 To form spheroids, 500 JN1 cells in 200 μ L of media were plated into each well
163 of 96-well ultra-low attachment plates (#7007, Corning). Once spheroids
164 reached an area of \sim 200,000 μ m², they were subjected to treatment with
165 increasing concentrations of M6620 or SRA-737, in presence or absence of
166 SN-38 (at 0.25 or 0.5 nM) for 5 days, with drug-containing media replenishment
167 after 3 days. At day 5, the media was removed and replaced with fresh media,
168 and spheroids size was monitored for up to 19 days after treatment start, using
169 a Celigo™ imaging cytometer (Revvity).

170

171 Development of patient-derived xenografts (PDXs)

172 The establishment of PDXs was conducted as previously described (13). All
173 animal procedures and studies were performed in accordance with the
174 approved guidelines for animal experimentation by the ethics committee at
175 University Paris Sud (CEEA 26, project 2014_055_2790) following EU
176 regulation. Animals were housed under pathogen-free conditions with food and
177 water ad libitum. At 1–12 h following the patient biopsy, fresh tumors fragments
178 were implanted under the renal capsule of 6–8-wk-old male NOD scid gamma
179 (NSG) mice obtained from Charles River Laboratories.

180

181 Derivation of DSRCT 3D organoid cultures from a PDX tumor biopsy

182 A PDX model was first established from the primary peritoneal tumor of an 11-
183 year-old male patient with DSRCT. From this PDX, a tumor biopsy was taken
184 and divided into various pieces for downstream processing, including the
185 derivation of DSRCT 3D primary organoid cultures, referred to as GR_13 PDX-
186 derived organoid (PDX-O). For cell dissociation, a sample of the biopsy (~100
187 mm³) was preserved in tissue storage solution (#130-100-008, Miltenyi Biotech)
188 at 4°C, and processed in less than 1 h. The sample was minced into small
189 pieces that were subsequently digested in 5 mL of HBSS Hank's buffer with
190 calcium and magnesium (#24020091, Gibco), containing 7.4 mg/mL
191 collagenase type-II (#17101-015, Gibco) for 1 hour at 37°C. The digestion was
192 stopped by adding 20 mL of Advanced DMEM/F-12 (#12634028, Gibco)
193 supplemented with 1X Pen-Strep (#15070063, Gibco) and 10% FBS

194 (#SV30160, Hyclone). The homogenate was passed through a 100- μ m cell
195 strainer (#542000, Greiner Bio-one) to remove debris and cell clumps, and the
196 cell suspension was then centrifuged for 10 min at 450g. After aspiration of the
197 supernatant, the cell pellet was re-suspended in 1 mL of the above-mentioned
198 blocking medium.

199 To obtain DSRCT human tumor cells and separate them from mouse cells, we
200 used a cell depletion kit (#130-104-694, Miltenyi Biotech). Briefly, the cell
201 suspension was centrifuged for 10 min at 450g and re-suspended in 80 μ L of
202 PBS containing 0.5% w/v BSA. Mouse cells were magnetically labeled by
203 incubating the cell suspension with 20 μ L of mouse depletion cocktail for 15 min
204 in the refrigerator. Human tumor cells were obtained from the flow-through, after
205 passing the labelled cell suspension using magnetic separation and LS columns
206 (#130-122-729, Miltenyi Biotec).

207

208 **PDX-O culture**

209 The cells were counted and plated in 96-well U-bottom ULA wells (#7007,
210 Corning; or #650970, Greiner Bio-one) to ensure the formation of organoids in
211 each well (5,000 viable cells in 100 μ L of complete organoid medium per each
212 well). The medium was refreshed every week by aspirating and adding 50 μ L of
213 complete organoid medium in each well, and the organoids were passaged
214 every 3-4 weeks. The basal organoid medium formulation consisted of
215 Advanced DMEM/F12 (#12634028) supplemented with 10 mM HEPES
216 (#15630049), 1% GlutaMAX (#35050038), 1X B27 supplement (#17504044),
217 1% penicillin/streptomycin (#15140122), 1X N-2 (#17502048; all from Thermo

218 Fisher Scientific); 5% FBS (#F7524, Sigma Aldrich); 50 µg/mL Primocin (#Ant-
219 pm-05, Invivogen) and 10 µg/mL Fungin (#Ant-fn-1, Invivogen). To obtain the
220 complete organoid medium, the basal medium was supplemented with 1 mM N-
221 acetylcysteine (#A72250, Sigma), 10 mM Nicotinamide (#N0636, Sigma), 10
222 ng/mL recombinant human RSPO-3 (#120-44, Preprotech), 10 ng/mL
223 recombinant human Wnt3a (#HZ-1296, Proteintech), 10 ng/mL LIF (#HZ-1292,
224 Proteintech), 25 ng/mL (#HZ-1325, Proteintech), 10 pg/mL IL6 (#HZ-1019,
225 Proteintech), 50 ng/mL recombinant human FGF-basic (#100-18B, Preprotech)
226 and 100 ng/mL recombinant human IGF (#100-11, Preprotech). 10 µM ROCK
227 Inhibitor Y-27632 (#S1049, Selleckem) was added at the initial culture. The
228 cells were plated using 8-channel VIAFLO electronic pipettes (#4624 and #4626,
229 Integra). Finally, the plates were centrifuged for 5 min at 450g.

230

231 **PDX-O drug combination survival assay**

232 After 3 weeks of culture, 240 GR_13 organoids were manually collected from
233 the 96-well plates, transferred into an Eppendorf tube and centrifuged for 5 min
234 at 450g. The pelleted organoids were washed three times with 1X PBS and
235 dissociated with TrypLE Express enzyme (#12604-013, Thermo Fisher
236 Scientific). Next, the cells were filtered using a 70-µm cell strainer (#542070,
237 Greiner Bio-one) and re-suspended in complete organoid medium. For the drug
238 combination survival assay, 4,000 cells were seeded in 40 µL of complete
239 organoid medium per well in U-bottom 96-well plates (#4515, Corning). The
240 formation of organoids was monitored for 3 days through brightfield acquisition
241 every 24h using an Incucyte® SX1 (Sartorius), prior to adding the drugs. Serial

242 five-fold dilutions of talazoparib or M4344 were prepared to yield final
243 concentrations ranging from 50 μ M to 16 nM (talazoparib) or 10 μ M to 64 nM
244 (M4344) in complete organoid medium. A 7x7 dose-response matrix was
245 constructed, and each drug was also used alone to generate reference curves
246 for each individual compound. DMSO at a concentration of 0.3% was included
247 as a negative control (mock) for normalization purposes. Topotecan at a
248 concentration of 1 μ M was included as a positive control and to evaluate the
249 quality of the assay. All treatments were prepared at 10X concentration, and 4.4
250 μ L of each mixture were added to the initial 40 μ L of organoid culture in the
251 wells. Three technical replicates were used in each experiment. All plates were
252 imaged through brightfield acquisition every 24h for 7 days using an Incucyte®
253 SX1 (Sartorius) to monitor the PDX-Os response to treatments. To visualize the
254 live/dead nucleated cells in PDX-Os, the dual-fluorescence Cyto3D Live-Dead
255 assay (#BM01, Tebubio) was applied at 1% v/v in each well, following
256 manufacturer's recommendations. Dual-fluorescence viability signal and
257 brightfield images were acquired with Incucyte SX1 (Sartorius), and correlative
258 measures of cell viability were subsequently obtained by use of CellTiter Glo®
259 3D (#G9682, Promega) on the same wells after 7 days, following
260 manufacturer's instructions.

261

262 **Immunofluorescence and image analysis**

263 For the detection of γ H2AX, RAD51 foci and micronuclei, cells were seeded in
264 black 96-well plates (Greiner Bio-One #655090) at a density of 12,000 cells per
265 well and exposed to the indicated drugs for 72 h. Cells were then fixed in 4%

266 paraformaldehyde for 20 min at RT, washed twice with PBS and permeabilized
267 with 0.5% Triton X-100 in PBS for 10 min. Cells were then blocked in IFF (2%
268 BSA, 2% FBS in PBS) for 1 h at RT and incubated with primary antibodies
269 (RAD51, Abcam ab133534; γ H2AX, Millipore 05-636; dilution 1:1000 in IFF) at
270 4°C overnight. Cells were then washed twice with PBS and incubated with
271 Alexa Fluor 488-conjugated rabbit (ThermoFisher A-11008, dilution 1:1000) or
272 Alexa Fluor 647-conjugated mouse secondary antibodies (Thermofisher A-
273 21235, dilution 1:1000) and 1 μ g/mL DAPI. For micronuclei assessment, cells
274 were incubated with PicoGreen® (1:400 with IFF). Cells were then washed
275 twice with PBS, and 100 μ l PBS was added to each well prior to imaging. Plates
276 were imaged using ImageXpress Micro Confocal High-Content Imaging System
277 (Molecular Devices). Nine independent and randomly selected sites were
278 scanned per well. Quantification of the number of γ H2AX foci, RAD51 foci and
279 micronuclei was performed under identical microscopy settings between
280 samples, using the MetaExpress image analysis system (MolDev).

281

282 **DNA fiber combing**

283 JN1 cells were grown in 100mm dishes and synchronized using a double-
284 thymidine block. Synchronized cells were transfected with EWS-WT1 or CCND1
285 siRNAs as described above. After 8 h, cells were continuously exposed to either
286 DMSO control, talazoparib, M4344 or a combination of both for 6 h. For
287 replication fork labeling, cells received pre-warmed medium containing 100 μ M
288 CldU and were incubated at 37°C, 5% CO₂ for 30 min. Cells were then rinsed 3
289 times with pre-chilled PBS and incubated with 100 μ M IdU for 30 min. Cells were

290 collected in cold PBS, counted and adjusted to 50,000 cells per 50 μ L PBS on
291 ice. Plugs were generated by adding 50 μ L of pre-warmed 1% low-melting point
292 agarose to the cells. The resulting 100 μ L mix was gently homogenized and
293 quickly transferred into a casting mold and incubated for 1 h at 4°C to solidify.
294 Subsequent steps were performed as previously described (14). For the
295 analysis, initiation, termination and cluster patterns of replicative forks were
296 considered to measure fork velocity.

297

298 **Statistical analyses**

299 Apart from the mouse xenograft experiment, no statistical methods were used
300 to predetermine sample size and experiments were not randomized. The
301 investigators were not blinded during xenograft experiments. Unless otherwise
302 stated, all graphs show mean values with error bars (standard deviation, SD);
303 95% confidence intervals were used and significance was considered when * P
304 < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant.

305

306 **Data availability**

307 The raw data generated in this study are available upon request from the
308 corresponding authors.

309

310 Additional methods details are available in Supplementary Information. All
311 uncropped images of the blots included in this study are also available in
312 Supplementary Information.

313 **Results**

314 **Small-molecule inhibitor screening identifies PARP and ATR as targetable** 315 **vulnerabilities in DSRCT**

316 To identify candidate therapeutic targets for DSRCT, we conducted a high-
317 throughput small molecule inhibitor-sensitivity screen in the JN-DSRCT-1 (JN1)
318 cell line, using an in-house curated library of 79 anti-tumor agents and small-
319 molecule inhibitors that are either in clinical use or in late-stage clinical
320 development (12) (**Fig. 1A, B; Supp Table S1**). We calculated normalized Area
321 Under the Curve (AUC) Z-scores from dose-response survival curves of each
322 drug in the JN1 cell line (**Supp Table S3**) and compared them with those of a
323 panel of 92 tumor cell lines previously screened with the same library (**Supp**
324 **Table S4**) (12). This identified several DNA repair inhibitors as being highly
325 toxic to the JN1 cell line, including three clinical PARP inhibitors (PARPi;
326 talazoparib, olaparib and rucaparib, ranked #6, #7, and #9, with Z-scores of -
327 2.018, -1.828 and -1.762, respectively), and one ATM/ATR inhibitor (ATRi;
328 KU60019, ranked #18; Z-score -1.2225). Several conventional cytotoxic agents
329 that are in clinical use for the treatment of DSRCT were also identified, such as
330 etoposide and doxorubicin (ranked #4 and #18, respectively; **Fig. 1B**).

331

332 Because PARPi are already approved in solid tumors and are evaluated in
333 combination with ATRi in multiple clinical trials, including in pediatric populations
334 (15), these small molecule inhibitor classes harbored a high potential for
335 immediate clinical translatability and we selected them for further validation. We
336 conducted validation experiments using several clinical PARPi and ATRi in two

337 DSRCT cell lines: the JN1 cell line and a novel cell line, named “R”, which we
338 created from a patient-derived xenograft (PDX, gift from Champions Oncology).
339 The A673 (Ewing sarcoma) and SaOS-2 (osteosarcoma) cell lines were used
340 comparatively as a sensitive and a resistant control sarcoma model,
341 respectively, based on publicly available PARPi and ATRi sensitivity datasets
342 (GDSC database and Holme et al. (12)). Dose-response survival assays
343 confirmed the sensitivity of JN1 cells to two clinical-grade PARPi (talazoparib
344 and olaparib) and two clinical-grade ATRi (AZD6738 and M4344), with SF50
345 values similar to that of the PARPi-sensitive A673 cell line (**Fig. 1C-F**; JN1 vs.
346 A673: talazoparib, $p = 0.0095$; olaparib, $p < 0.0001$; AZD6738, $p < 0.0001$;
347 M4344, $p < 0.0001$; two-way ANOVA). When comparing the SF50 of PARPi
348 and ATRi found in the JN1 cell line with the corresponding average steady-state
349 or max single-dose plasma concentrations ($C_{ss-mean}$ or C_{sd-max} , respectively)
350 dosed in patients enrolled in pharmacokinetics studies and treated at the
351 recommended phase 2 dose (16–18), we observed that the concentrations we
352 used *in vitro* seemed clinically achievable (talazoparib, $SF50 \approx 10$ nM, $C_{ss-mean}$
353 $= 7$ nM; olaparib, $SF50 \approx 1$ μ M, $C_{ss-mean} = 1.7$ μ M; AZD6738, $SF50 \approx 0.5$ μ M,
354 $C_{sd-max} = 4.5$ μ M; M4344, $SF50 \approx 7$ nM, $C_{sd-max} = 750$ nM) – though no robust
355 conclusion could be drawn at this stage considering the difficulties in comparing
356 *in vitro* data to exposure in patients. We therefore further compared the
357 sensitivity to talazoparib or olaparib of the JN1 cell line with that of other
358 sarcoma cell lines in publicly available datasets (DepMap Broad Institute, **Fig.**
359 **1G, H**; Sarcoma CellMinerCDB (19), **Supp Fig. S3A, B**), and found that JN1
360 was at least as sensitive to the PARPi olaparib and talazoparib as Ewing

361 sarcoma cell lines, consistent with their previously reported sensitivity to
362 PARPi(20).

363

364 R cells also showed sensitivity to ATRi but were resistant to PARPi, with an
365 SF50 similar to that of the PARPi-resistant SaOS-2 cell line (**Fig. 1C-F**; R vs.
366 SaOS-2: talazoparib, $p < 0.0001$; olaparib, ns; AZD6738, $p < 0.0001$; M4344, p
367 < 0.0004 ; two-way ANOVA). This prompted us to explore the known causes of
368 primary resistance to PARPi, such as the loss of PARP1 expression, which
369 abrogates the PARP1 trapping-mediated cytotoxicity of PARPi (21). To test this
370 hypothesis, we first evaluated PARP1 protein expression levels in JN1 and R
371 cells by western blot and found that R cells displayed a significantly lower
372 expression of PARP1 compared to JN1 cells (**Supp Fig. S4A**). To further
373 establish a causative link between PARP1 expression and sensitivity to PARPi
374 in DSRCT cells, we then evaluated the effects of silencing *PARP1* on the
375 sensitivity of JN1 and R cells to PARPi. siRNA-mediated knockdown of *PARP1*
376 conferred resistance to PARPi in JN1 but not R cells (**Supp Fig. S4B, C, E, F**).
377 In addition, we noted that *PARP1* silencing did not affect the sensitivity of JN1
378 cells to veliparib – a PARPi with limited ability to trap PARP1 despite its ability
379 to inhibit PARylation (22,23) (**Supp Fig. S4D, G**). Together, these findings
380 suggest that PARP1 expression is a determinant of PARPi sensitivity in DSRCT
381 cell lines, and that PARP1 trapping contributes to the cytotoxic effect of PARPi
382 in DSRCT.

383

384 To next explore the applicability of our findings to patient's tumors, we analyzed:

385 (i) PARP1 expression by RNA-Seq (29 samples (24)); (ii) PARP1 expression by
386 immunohistochemistry (IHC, 16 samples); and (iii) PARylation levels by IHC
387 (i.e., levels of poly-ADP-ribose, the product of PARP1 activity; 16 samples) in
388 two DSRCT cohorts. This revealed that PARP1 was highly expressed in a large
389 majority of the cases (24 of 29 samples (82,8%) with PARP1 expression >10
390 TPM by RNA-Seq, and 14 of 16 samples (87,5%) with PARP1 H-score \geq 200 by
391 IHC; **Fig. 2A**; **Supp Fig. S5A**) and active (all samples with PARylation H-score
392 \geq 200 by IHC; **Fig. 2B**) as previously reported (20). Since PARPi are mostly
393 toxic by trapping PARP1 onto the DNA, we therefore assumed that our
394 conclusions may be applicable to most DSRCT. We further noted that the
395 patients whose tumors harbored higher PARP1 transcript levels tended to have
396 a longer overall survival, although this did not reach significance (**Supp Fig.**
397 **S5B**).

398

399 **Combination of PARP and ATR inhibitors shows synergistic effects in** 400 **preclinical models that express PARP1**

401 Because several PARPi plus ATRi combinations are currently being
402 investigated in early-phase clinical trials (e.g. NCT04972110, NCT03462342),
403 we evaluated this combination in DSRCT cell lines. Synergy scores calculated
404 according to the Bliss independence method showed a synergistic interaction of
405 the talazoparib and M4344 combination in JN1 (Bliss synergy score = 15.69;
406 **Fig. 2C**, **Supp Fig. S6A**) but not R cells – where a modest additive effect could
407 be observed, consistent with the limited sensitivity of the latter cell line to PARPi
408 monotherapy (Bliss synergy score = 3.96; **Fig. 2D**, **Supp Fig. S6B**). Since

409 CHK1 inhibitors (CHK1i), which control the same cell cycle checkpoint and
410 signaling pathway as ATRi, have also been evaluated in DSRCT in combination
411 with irinotecan (NCT04095221), we further evaluated combination of PARPi
412 with CHK1i. In our original screen, the evaluated CHK1i displayed limited
413 cytotoxic effects in monotherapy in the JN1 cell line (SAR-20106, rank #39, Z-
414 score = -0.697; PF-00477736, rank #55, Z-score = -0.197). We therefore used
415 the clinical-grade CHK1i prexasertib and found additive effects with talazoparib
416 in the JN1 cell line (Bliss synergy score = 6.00) but not R cell line, again
417 consistent with the limited sensitivity of the latter to PARPi monotherapy (**Supp**
418 **Fig. S6C-F**). Since PARPi and irinotecan – which is part of the chemotherapy
419 regimen for patients who suffer from DSRCT – have some partly overlapping
420 mechanism of action through DNA double-strand breaks formation, we
421 assessed the combination of SN-38 (the active metabolite of irinotecan) with
422 two clinical compounds that target the G2/M cell cycle checkpoint: the ATRi
423 M6620 and the CHK1i SRA-737. Using a 3-dimensional (3D) spheroid model
424 derived from the JN1 cell line that represents DSRCT cell physiology better than
425 2D cultures, we found that both inhibitors enhanced the cytotoxic effects of
426 irinotecan – with M6620 showing potentially the most prolonged anti-
427 proliferative potential (**Supp Fig. S7**). The concentrations of SN-38, M6620 and
428 SRA-737 evaluated in these assays were lower than those clinically-achievable
429 in patients based on the C_{sd-max} described for these compounds (SN-38, C_{sd-max}
430 = 33 nM (25); SRA-737, C_{sd-max} = 1.440 μ M (26); M6620, C_{sd-max} = 740 nM (27)),
431 altogether supporting the relevance of our observations made with PARPi.
432

433 To further confirm the sensitivity of DSRCT to PARPi and ATRi, we sought to
434 use a third, independent, biologically distinct and clinically relevant model. Since
435 3D and patient-derived models reportedly better recapitulate the clinical reality
436 than 2D cultures or established cell line models (28–30), we sought to develop
437 a new primary patient-derived organoid model of DSRCT. To do so, we first
438 established a patient-derived xenograft (PDX) model from the primary
439 peritoneal tumor of an 11-year-old male patient with DSRCT, and subsequently
440 created a PDX-derived organoid (PDX-O), referred to as GR_13, in which we
441 assessed the sensitivity to PARPi, ATRi and the combination of both agents
442 (**Fig. 2E, Supp Fig. S8**). These experiments revealed cytotoxic effects of
443 PARPi and ATRi monotherapies against GR_13 PDX-Os and confirmed the
444 synergistic effects of their combination (Bliss independence score = 15.62; **Fig.**
445 **2F, Supp Fig. S9A-C**), albeit at higher concentrations, in line with the known
446 heightened drug resistance of 3D models compared to 2D models (31). In line
447 with PARP1 expression confirmed by western blot in GR_13 PDX-O (**Supp Fig.**
448 **S8C**) and previous findings in the JN1 cell line (**Fig. 2C, Supp Fig. S6A**), this
449 result confirmed our previous observations and the sensitivity of DSRCT to
450 PARPi plus ATRi combinatorial strategy.

451

452 We next assessed the therapeutic potential of an ATRi plus PARPi combination
453 *in vivo*, and evaluated the antitumor effect of PARPi talazoparib, ATRi M1774 or
454 a combination of both agents in mice bearing established xenografts from the
455 JN1 cell line (**Supp Fig. S10A**). Since JN1 tumors do not grow in nude mice, we
456 used NOD scid gamma (NSG) mice that carry the *Prkdc*^{scid} mutation, which

457 confers exquisite sensitivity to DNA damaging agents and chronic exposure to
458 ATRi. This required the use of a minimally toxic schedule of drug administration
459 for a total maximum duration of 33 days. In this experiment, we found that
460 compared to the drug vehicle, both talazoparib and M1774 monotherapies
461 reduced tumor growth of JN1 xenografts (**Supp Fig. S10B, C**; $p < 0.001$, two-
462 way ANOVA). The combination therapy further reduced tumor growth and
463 caused tumor shrinkage (**Supp Fig. S10B-E**; median tumor volume: 151.5 mm^3
464 in vehicle arm, vs. 62.6 mm^3 in combination arm; $p < 0.0001$, two-way ANOVA).
465 Altogether, these results suggested that the combination of PARPi and ATRi
466 could act synergistically in DSRCT that express PARP1, both *in vitro* and *in vivo*.
467

468 **Combination of PARP and ATR inhibitors elicits DNA damage, replication** 469 **stress and genomic instability in DSRCT cells**

470 To understand the molecular mechanisms underlying this vulnerability in
471 DSRCT cells, we first sought to explore the known causes of PARPi and ATRi
472 sensitivity, and assessed DNA damage, homologous recombination (HR)
473 functionality, and replication stress. We found that exposure to PARPi and ATRi
474 led to increased DNA damage, as assessed by immunofluorescence detection
475 of γH2AX foci in JN1 and R cells (**Fig. 3A, B**). This effect was concentration-
476 dependent (**Supp Fig. S11A, B**) and significantly enhanced in the context of
477 PARPi plus ATRi combination using several clinical-grade agents, with γH2AX
478 foci levels being similar to those induced by cisplatin (**Fig. 3A, B**). We further
479 noted that γH2AX foci accumulation was (i) overall more pronounced in JN1
480 cells compared to R cells exposed to the combination therapy, and (ii) limited in

481 R cells exposed to PARPi as a monotherapy, consistent with the low PARP1
482 expression and limited PARPi sensitivity of this cell line. We next assessed HR
483 function by quantifying the levels of RAD51 foci, and found that these were
484 significantly increased in response to PARPi (**Fig. 3C, D, Supp Fig. S11C, D**) –
485 but not ATRi monotherapy, in line with the current literature suggesting that
486 ATR promotes RAD51 accumulation at DSBs (32). This effect was enhanced
487 when both agents were combined, to a higher extent than cisplatin exposure
488 (**Fig. 3C, D**). Altogether, these results suggested that DSRCT cells are HR
489 proficient, and that their sensitivity to PARPi and ATRi does not result from a
490 HR defect.

491

492 By mediating PARP1 trapping onto DNA, PARPi are known to increase reliance
493 upon the ATR/CHK1 pathway due to increased stalled replication forks and a
494 resultant replication stress (33). ATR is a master regulator of the DNA damage
495 response, which coordinates cell cycle transitions with the DNA replication,
496 DNA repair and apoptotic machineries to prevent the deleterious effects of
497 replication stress. ATR activation leads to phosphorylation of CHK1 (p-CHK1)
498 and other ATR effectors, which ultimately slows down origin firing, induces cell
499 cycle arrest in response to DNA damage, and promotes stabilization and restart
500 of stalled replication forks (34,35). We evaluated by western blot the
501 phosphorylation of ATR and CHK1 and found increased p-CHK1 levels upon
502 PARPi and ATRi exposure in JN1 and R cells (**Fig. 3E, F, Supp Fig. S11E**),
503 suggesting an activation of the replication stress checkpoint. To further
504 investigate the presence of ongoing replication stress, we evaluated the levels

505 of RPA2 phosphorylation (p-RPA2) and found increased p-RPA2 levels upon
506 PARPi, ATRi and their combination. This was associated with increased DNA
507 damage and apoptosis (as assessed by γ H2AX and PARP1 cleavage, c-PARP,
508 respectively; **Fig. 3E, F, Supp Fig. S11E**), consistent with our previous
509 observations (**Fig. 3A, B, Supp Fig. S11A, B**).

510

511 To further assess the genomic consequences of PARPi plus ATRi combination
512 in DSRCT, we measured levels of micronuclei – cytoplasmic chromosome
513 fragments that arise during mitosis from lagging chromosomal DNA or
514 chromatin bridges, as a result of unresolved DNA lesions. We found that the
515 combination of PARPi and ATRi significantly increased the number of
516 micronuclei in JN1 cells compared to the DMSO control or either of the
517 corresponding monotherapies (**Fig. 3G, H**). A similar effect was observed in R
518 cells (**Supp Fig. S11F**), although to a lesser extent, in line with their lower level
519 of PARP1 expression. Altogether, these findings indicate that combined
520 exposure of PARPi and ATRi elicits high levels of DNA damage, replication
521 stress and micronuclei in DSRCT cells, in a context of functional HR repair.

522

523 **EWS-WT1 is a determinant of sensitivity to PARP and ATR inhibitors in** 524 **DSRCT**

525 We next sought to explore whether the EWS-WT1 chimeric transcription factor
526 was the cause of PARPi and ATRi sensitivity in DSRCT cells. Indeed, although
527 the *EWSR1::WT1* fusion genes is the known driver of DSRCT, it remained
528 possible that other alterations in DSRCT cells could cause the drug sensitivity

529 effects seen. For example, the t(11;22)(q13;q12) chromosomal translocation,
530 beyond causing *EWS-WT1* fusion, also alters the chromosomal location of
531 genes that flank either *EWSR1* or *WT1*.

532 To do so, we designed siRNAs targeting the specific breakpoints of the EWS-
533 *WT1* fusion in the JN1 and R cell lines, respectively (**Fig. 4A; Supp Fig. S1, S2**)
534 and explored the effect of *EWS-WT1* silencing on the above-described
535 phenotypes. We first assessed cell survival upon PARPi or ATRi exposure and
536 observed that *EWS-WT1* silencing conferred increased resistance to both agent
537 classes (**Fig. 4B-E, Supp Fig. S12A-D**), suggesting the existence of a common
538 *EWS-WT1*-dependent mechanism driving sensitivity to both agents. Of note,
539 silencing of *CCND1* – a direct target of *EWS-WT1* (36) – conferred little
540 increased resistance to PARPi or ATRi compared to *EWS-WT1* silencing in the
541 JN1 cell line (**Supp Fig. S13, Supp Fig. S14A-D**), suggesting that the
542 sensitivity to PARPi and ATRi induced by the fusion was, at least in part,
543 independent from the role of *EWS-WT1* in modulating *CCND1*. Similarly, and in
544 line with this hypothesis, CDK1i-mediated cell cycle blockade failed to
545 phenocopy the effects of siRNA-mediated *EWS-WT1* silencing towards
546 increasing the resistance of DSRCT cells to either PARPi or ATRi (**Supp Fig.**
547 **S14E-F**). This overall suggested that the sensitivity to PARPi and ATRi induced
548 by the fusion was, at least in part, independent from its role in modulating
549 *CCND1* expression and the cell cycle profile (**Supp Fig. S13**). We further found
550 that levels of DNA damage induced by PARPi and ATRi were significantly
551 reduced upon *EWS-WT1* silencing, as assessed by immunofluorescence
552 detection of γ H2AX foci (**Fig. 4F, G**). To confirm the role of *EWS-WT1* in PARPi

553 and ATRi-mediated effects, we next assessed ATR/CHK1 pathway activity by
554 western blot and found that: (i) the PARPi-induced p-CHK1 response was
555 abrogated upon *EWS-WT1* silencing; and (ii) the p-RPA2 and γ H2AX
556 responses elicited by PARPi plus ATRi combination were either reversed or
557 significantly attenuated upon *EWS-WT1* silencing (**Fig. 4H, I**). These findings
558 suggested that *EWS-WT1* is required for the sensitivity of DSRCT cells to
559 PARPi, ATRi and their combination.

560

561 ***EWS-WT1* increases endogenous DNA replication stress and R-loops,**
562 **which drive sensitivity to PARPi and ATRi**

563 Because we observed that the sensitivity to PARPi and ATRi was *EWS-WT1*-
564 dependent and since oncogenic transcription factors have been reported to
565 increase replication stress (37), we next focused on replication forks and their
566 functionality.

567 We first investigated replication fork progression upon silencing of *EWS-WT1*
568 using the DNA fiber combing assay in the JN1 cell line. We found that *EWS*-
569 *WT1* silencing caused a >30% increase in fork velocity (siCNTRL, 0.82 kb/min
570 vs. si*EWS-WT1*, 1.1kb/min; $p < 0.0002$, Mann-Whitney U test; **Fig. 5A**) in the
571 absence of drug exposure. Interestingly, this effect was not observed upon
572 *CCND1* silencing (**Fig. 5A**), suggesting that *EWS-WT1*-induced reduction in
573 replication fork velocity was, at least in part, independent from its effects in
574 driving cell proliferation through the cell cycle (**Supp Fig. S13**). We next
575 assessed replication fork progression upon PARPi and ATRi exposure in the
576 JN1 cell line, and found that their combination decreased fork velocity (siCNTRL

577 DMSO, 0.82 kb/min vs. siCNTRL Tala + M4344, 0.58 kb /min; $p < 0.0001$,
578 Mann-Whitney U test; **Fig. 5B**, **Supp Fig. S15A**), in line with these agents'
579 mechanism of action and increased replication stress. This effect was partially
580 rescued by *EWS-WT1* silencing (siCNTRL Tala + M4344, 0.7 kb/min vs.
581 siEWS-WT1 Tala + M4344, 1.1 kb/min; $p < 0.0001$, Mann-Whitney U test; **Fig.**
582 **5B**). Altogether, these results suggested that *EWS-WT1* expression in JN1 cells
583 increases replication stress, which is further exacerbated by PARPi and ATRi
584 exposure.

585

586 Since aberrant transcription factors not only cause replication stress but also
587 enhance transcription, we next sought to assess R-loops. R-loops are three-
588 stranded nucleic acid structures consisting of an RNA:DNA hybrid and a
589 displaced non-hybridized single-stranded DNA, that form in the genome when
590 an RNA strand invades double-stranded DNA within chromatin. R-loops
591 naturally occur during replication and transcription, where they have important
592 roles in regulating gene expression and chromatin structure. Their aberrant
593 accumulation can also represent a threat to genomic stability, by causing
594 increased replication stress and subsequent DNA damage (38–42).

595 We first assessed R-loop levels in DSRCT cells using RNA:DNA hybrid dot
596 blotting with the S9.6 antibody on genomic DNA extracted from JN1 or R cells.

597 We found that *EWS-WT1* silencing reduced endogenous R-loop levels in both
598 JN1 and R cells (**Fig. 5C**, **D**), while *CCND1* silencing had no such effect (**Supp**
599 **Fig. S15B**). We next compared R-loop levels in cells exposed to PARPi, ATRi
600 or their combination in presence or absence of *EWS-WT1* silencing. This

601 revealed a significant accumulation of RNase H-sensitive R-loops in response
602 to the combination, which was (i) enhanced compared to either of the
603 corresponding monotherapies, and (ii) significantly attenuated in the context of
604 *EWS-WT1* silencing (**Fig. 5C, D**). To further explore the role of R-loops in
605 DSRCT cells, we constructed a JN1 cell line that stably expresses an
606 exogenous cDNA encoding *RNASEH1* – the main ribonuclease responsible for
607 R-loop degradation in humans – herein referred to as JN1-RNaseH1 (**Supp Fig.**
608 **S15C**). In contrast to our previous observations in the JN1 wildtype cell line (**Fig.**
609 **5A**), we noted that *EWS-WT1* silencing had no effect on replication fork velocity
610 in the JN1-RNaseH1 (**Fig. 5E**), suggesting that RNaseH1 overexpression might
611 counteract the replication stress resulting from *EWS-WT1*-driven R-loop burden.
612 Strikingly, dose-response survival assays of JN1 and JN1-RNaseH1 cells
613 exposed to various PARPi or ATRi monotherapies showed that RNase H1
614 overexpression conferred resistance to these inhibitors, supporting a role of R-
615 loops in driving PARPi and ATRi sensitivity in DSRCT cells (**Fig. 5F-I; Supp Fig.**
616 **S16**). Furthermore, we noted that: (i) the magnitude of this effect was similar to
617 that obtained when silencing *EWS-WT1* in JN1 cells, and (ii) silencing *EWS-*
618 *WT1* conferred no further resistance to PARPi or ATRi in JN1-RNaseH1 cells
619 (**Fig. 5F-I; Supp Fig. S17**), supporting an epistasis between *EWS-WT1*
620 silencing and RNase H1 overexpression in driving resistance to PARPi and
621 ATRi. Altogether, these findings show that *EWS-WT1* drives R-loop formation
622 and a resultant increased replication stress in DSRCT cells, which underlies
623 their sensitivity to PARPi and ATRi.

624

625 **Combination of PARP and ATR inhibitors elicits cell-intrinsic immunity in**
626 **DSRCT cell lines**

627 The cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING)
628 pathway is a component of the innate immune response: by acting as a sensor
629 for cytosolic DNA, cGAS activates a signaling cascade involving STING
630 trafficking, Tank-binding kinase 1 (TBK1) and interferon regulatory factor 3
631 (IRF3) phosphorylation, which culminates in a type I interferon response and
632 the subsequent upregulation of interferon-stimulated genes (ISGs), such as
633 *CCL5* and *CXCL10* (43). More recently, pharmacological manipulation of the
634 cGAS-STING pathway has been proposed as a therapeutic strategy, notably in
635 cancer to render tumors “immunologically hot” as a way to facilitate response to
636 immunotherapies (44).

637 Based on recent reports, including ours, describing that PARPi and ATRi can
638 trigger a cell-autonomous type I interferon response through activation the
639 cGAS-STING pathway following micronuclei formation (41,45–50), we decided
640 to explore the ability of PARPi and ATRi to elicit such response in DSRCT cells.
641 We first observed a concentration-dependent increase in TBK1 and IRF3
642 phosphorylation upon PARPi and ATRi exposure in JN1 cells – an effect that
643 was enhanced in the context of their combination (**Fig. 6A**). We next assessed
644 downstream *CCL5* and *CXCL10* expression by RT-qPCR and found that these
645 chemokines were increased by more than 20- and 5-fold respectively (**Fig. 6B,**
646 **C**) upon combination therapy. This was further accompanied by a
647 concentration-dependent increase in programmed death-ligand 1 (PD-L1) cell-
648 surface expression, as assessed by flow cytometry (**Fig. 6D**). Together with our

649 previous observation that the PARPi plus ATRi combination induces micronuclei
650 formation (**Fig. 3G, H; Supp Fig. S11F**), this data suggests that a cell-
651 autonomous cGAS-STING-mediated type I interferon response is activated in
652 DSRCT cells as a result of PARPi and ATRi exposure. We next investigated the
653 role of EWS-WT1 in such response, and found that *EWS-WT1* silencing
654 attenuated all of the above phenotypes, including TBK1 and IRF3
655 phosphorylation (**Fig. 6E**), CCL5 and CXCL10 upregulation (**Fig. 6F-G**) as well
656 as PD-L1 cell-surface expression (**Fig. 6H**). Altogether, these results indicate
657 that PARPi and ATRi elicit a type I interferon response in DSRCT cells that is
658 dependent upon EWS-WT1 expression.

659 Discussion

660 DSRCT is an extremely aggressive malignancy with very limited therapeutic
661 options. Here, we show that preclinical models of DSRCT are selectively
662 sensitive to clinical PARP and ATR inhibitors (PARPi, ATRi). The use of
663 functional genomics allowed us to propose a model whereby these genetic
664 vulnerabilities are mediated by increased EWS-WT1-dependent replication
665 stress and R-loop formation, which results in cGAS/STING pathway activation
666 and a cell-autonomous type I interferon response (**Fig. 6I**), opening new
667 therapeutic avenues to increase immunogenicity of this genetically simple,
668 immune-cold disease.

669

670 To our knowledge, our work represents the first report of the selective sensitivity
671 of DSRCT cells to ATRi, and of the involvement of EWS-WT1-dependent R-
672 loop burden in this vulnerability. Our work specifically underlines the
673 translational potential of combining PARPi and ATRi in DSRCT, a combination
674 that is currently evaluated in multiple clinical trials, including in children
675 (NCT02813135). Previous literature has suggested a sensitivity of DSRCT to
676 PARPi in combination with the alkylating agent temozolomide, following the
677 observation of a high level of PARP1 and SLFN11 expression in DSRCT (20).
678 Our analysis of 29 and 16 tumor samples by RNA-Seq and IHC confirms these
679 findings, thereby reinforcing the potential of using such DNA damage response
680 inhibitors in the treatment of patients with DSRCT. Still, our observation that
681 some tumors do not express PARP1 – a major mechanism of resistance to
682 PARPi (21,51) – highlights the need for careful molecular selection and

683 verification of adequate PARP1 expression prior to treatment orientation.

684

685 The potential for using CHK1 inhibitors (CHK1i), such as prexasertib, has also
686 been reported in preclinical models of DSRCT (52), and further evaluated in a
687 clinical trial in combination with irinotecan (53) (NCT04095221). In that latter,
688 6/19 (32%) and 9/19 (47%) patients showed partial response and stable
689 disease as best response, respectively. The trial met its primary endpoint,
690 supporting further investigation of this combination. Data regarding the PARPi
691 plus ATRi combination in DSRCT are much scarcer for now: one heavily
692 pretreated patient, who received the PARPi olaparib plus ATRi AZD6738
693 combination as part of the eSMART trial (NCT02813135), presented stable
694 disease for 4 months on study (15). Additional data from this trial are eagerly
695 awaited, to better evaluate the potential of this combination in patients with
696 DSRCT. Since CHK1i and ATRi both act on the G₂/M cell cycle checkpoint, we
697 can anticipate that their mechanism of action is partially overlapping. Based on
698 available clinical data, the PARPi plus ATRi combination may have a better
699 tolerability profile than the CHK1i plus irinotecan combination, notably with
700 regards to fatigue and cytopenias (15,53,54). In the former combination, the oral
701 administration of both drugs also represents an important difference between
702 the two regimens, which may offer the advantage of a higher flexibility in
703 scheduling and dosage adaptations. However, it also represents a limitation for
704 patients who have peritoneal disease – and are therefore at risk of
705 malabsorption, occlusion, etc. – and PARPi have shown disappointing efficacy
706 in pediatric malignancies so far. In this context, we can hope that the use of last

707 generation potent PARP1-selective inhibitors (e.g. AZD5305) will allow to
708 enhance PARPi efficacy while limiting hematological toxicity.

709

710 DSRCT is related to the group of small round cell sarcomas (SRCS), of which
711 Ewing's sarcoma is the prototypic EWS-FLI1-driven disease. PARP1 inhibition
712 has initially been proposed as a therapeutic strategy in Ewing's sarcoma,
713 following the identification of an interaction between PARP1 and the fusion
714 transcripts which potentiated DNA damage (55). EWS-FLI1 was subsequently
715 reported to increase the R-loop burden and disable BRCA1-dependent
716 homologous recombination. Such "BRCAness" phenotype was not observed in
717 our study, where we could detect adequate RAD51 foci formation in DSRCT
718 cells exposed to PARPi. Thus far, PARPi have shown disappointing efficacy in
719 patients with heavily pre-treated Ewing's sarcoma (reviewed in Pearson et al
720 (56)). A few isolated responses have been observed, which deserve further
721 molecular exploration to identify which clinically-relevant biomarkers drive
722 sensitivity in this population. Based on these results, the most recent consensus
723 expert guidelines from the multi-stakeholder Pediatric Strategy Forum on DNA
724 repair (ACCELERATE and European Medicines Agency, with participation of
725 the Food and Drug Administration), recommended to assess CHK1i and ATRi
726 as a high priority, and PARPi only in combination with the latter (56). The
727 synergy observed preclinically upon combination of PARPi and ATRi in the JN1
728 cell line (**Fig. 2C**) and GR_13 PDX-O model (**Fig. 2F**) also supports the latter
729 approach. Beyond SRCS, trabectedin – a cytotoxic drug used in routine
730 sarcoma treatment and which is known to induce R-loops (57) – has been

731 combined with PARPi in various soft-tissue sarcomas in the TOMAS trial
732 (58). Unsurprisingly, these agents could not be used at full dose when
733 combined, but activity (7/50 patients enrolled with PR) supported the evaluation
734 of this combination in an ongoing Phase II trial (NCT03838744). Although this
735 combination is very poorly tolerated as compared to the PARPi plus ATRi
736 combination, authors identified high PARP1 expression as well as an 8-genes
737 signature (including DNA damage response genes such as *SLFN11*, *ATM* and
738 *BLM*) as predictors of better outcome on trabectedin plus PARPi (59). The latter
739 may also be relevant to the PARPi plus ATRi combination.

740

741 We finally found that PARPi and ATRi trigger a cell-autonomous cGAS-STING /
742 type I interferon response and PD-L1 upregulation in DSRCT cells. This
743 immunomodulatory effect of DNA repair inhibitors could be exploited to increase
744 the immunogenicity of DSRCT – which are traditionally devoid of T-cells in the
745 tumor microenvironment – by attracting T-cells within the tumor and favoring
746 sensitivity to anti-PD-1 therapy. Such effect of PARPi and ATRi has been
747 reported in other preclinical models with high replication stress (reviewed in
748 Chabanon et al (60)), as well as in clinical studies evaluating ATRi, notably in
749 non-small cell lung cancer (NSCLC) and melanoma where they can potentiate,
750 or revert resistance to anti-PD-L1, respectively (61–63). For example,
751 translational studies performed in the HUDSON phase II trial showed that the
752 ATRi AZD6738 could both induce inflammatory- and interferon-associated
753 signatures, and decrease exhausted CD8+ T-cells in the blood of patients with
754 NSCLC (62). Still, whether such effects are only observed in traditionally

755 immunogenic diseases, such as NSCLC or melanoma, or also operate in
756 DSRCT, remains to be assessed.

757

758 We should still highlight several limitations to our observations. First, we only
759 had access to a limited number of models. Indeed, DSRCT is an ultra-rare
760 disease (frequency < 1/1M) and cell lines models are challenging to create,
761 probably because the desmoplastic microenvironment of this tumor type also
762 favors cancer cell growth. In this study, we therefore created two previously
763 unpublished models (one PDX-derived cell line and one PDX-derived organoid),
764 which complemented the previously established JN1 cell line. Still, revalidation
765 in additional models would ideally be required. Second, the difference in PARP1
766 expression between our models led to discrepant observations, notably in terms
767 of synergistic or additive cytotoxic effects of the PARPi plus ATRi combination,
768 in which PARP1 expression and trapping plays a crucial role (21,51). Our
769 characterization of PARP1 expression and PARylation in DSRCT patients'
770 tumors shows that PARP1 is expressed and active in the vast majority of cases
771 – in line with previous independent results (20), thereby supporting clinical
772 activity of PARPi in this patient population. Still, other determinants of PARPi
773 and ATRi sensitivity, such as SLFN11 expression and replication stress levels,
774 should also be considered, and the clinical applicability of our findings therefore
775 remains unknown. Finally, we faced technical difficulties in assessing long-term
776 efficacy of the PARPi plus ATRi combination *in vivo*, owing to the systemic
777 toxicity of ATRi in NSG mice caused by their constitutive *Prkdc*^{scid} mutation, and
778 the impossibility to grow DSRCT xenografts in nude mice, which led us to

779 prematurely stop our experiments. If recent clinical trial results show that PARPi
780 and ATRi can be safely combined in adult and pediatric patients, the efficacy /
781 toxicity profile of such combination may need to be compared to that of other
782 regimens that also act on the DNA damage response and replication stress
783 (e.g., CHK1i and irinotecan combinations; NCT04095221 (52,53)), to better
784 define its role in the therapeutic armamentarium. Despite these limitations, we
785 believe our study may have translational utility and clinical impact in DSRCT, a
786 disease where very few therapeutic options and no precision medicine
787 approach are available.

788

789 In conclusion, our findings shed light on EWS-WT1-associated genetic
790 vulnerabilities in DSRCT and provide rationale for evaluating PARPi in
791 combination with ATRi in this deadly disease. Since the replication stress- and
792 R-loop-dependency of this phenotype may also operate in other, more frequent,
793 transcription factor-driven sarcomas – such as Ewing’s sarcoma or synovial
794 sarcoma, we hope that this will favor the development of basket studies
795 enrolling multiple biomarker-selected sarcomas and allow patients to access
796 these therapies despite the rarity of their disease.

Authors' Contribution

Conception and design: AKK, MML, CA, RMC, SPV.

Development of methodology: AKK, MML, CA, MBC, SM, CJL, RMC, SPV.

Acquisition of data (provided animals, provided facilities, etc.): AKK, MML, CA, NH, MBC, CN, MG, TE, ND, LB, FS, RB, AL, ARM, LCD, MD, ES, OM, GP, AG, RMC.

Analysis and interpretation of data: AKK, MML, CA, NH, MBC, CN, MG, TE, ND, AL, ARM, LCD, MD, ES, AG, RMC.

Writing, review, and/or revision of the manuscript: All authors.

Administrative, technical, or material support: MBC, LCD, JS, SM, AZ, RMC, CJL, SPV.

Study supervision: RMC, SPV.

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Figure Legends

Figure 1. A small-molecule inhibitor and drug screen identifies PARP and ATR inhibitors as candidate therapies for DSRCT. **A.** Schematic illustration of the workflow of small-molecule inhibitor and drug screen performed on the JN-DSRCT-1 (JN1) cell line. **B.** Waterfall plot displaying the difference in AUC between the JN1 cell line (AUC_{JN1}) and the panel of 92 cell lines used for comparison (AUC_{median}) for the 79 evaluated small-molecule inhibitors or drugs. PARP inhibitors are highlighted in red, ATR inhibitors in blue, and conventional cytotoxic agents in green. AUC, Area Under the Curve. **C-F.** Dose-response survival curves of the DSRCT cell lines JN1 and R, and the A673 (Ewing sarcoma) and SaOS-2 (osteosarcoma) cell lines exposed to talazoparib (C), M4344 (D), olaparib (E), or AZD6738 (F) for 7 days. Mean \pm SD; $n = 3$. **G, H.** Violin plots showing the relative sensitivity (\log_2 fold-change of cell viability) of cell lines exposed to the PARP inhibitor talazoparib (G) or olaparib (H) after a single-dose exposure at $2.5\mu\text{M}$ for 5 days in the DepMap database (Prism Repurposing 23Q2), in comparison with that of the JN1 and R cell lines. JN1 and R cell lines sensitivities were extrapolated from the survival assays presented in C and D; surviving fractions were calculated at $2.5\mu\text{M}$ and \log_2 transformed. Ewing Sarcoma cell lines ($n=23$): RDES, A673, SKES1, CADOES1, EWS502, MHHES1, EW8, A673STAG2KO16, A673STAG2KO45, A673STAG2NT14, A673STAG2NT23, CBAGPN, CHLA10, SKNEP1, SKPNDW, TC32; Osteosarcoma cell lines ($n=5$): G292CLONEA141B1, MG63, U2OS,

HOS, SJSA1; Soft-tissue sarcoma cell lines (n=7): S117, TE617T, HT1080, HS729, RD, RKN, RH30, including rhabdomyosarcoma (n=4), leiomyosarcoma (n=1), fibrosarcoma (n=1) and NOS sarcoma cell lines (n=1), respectively. The BRCA1/2-mutant IGROV1 ovarian cancer cell line and BRCA1-mutant MDA-MB-436 breast cancer cell line were used as positive controls for sensitivity to PARP inhibitors.

Figure 2. PARP and ATR inhibitors have synergistic cytotoxic effects in models of DSRCT with high PARP1 expression. A, B. PARP1 expression (A) and PARylation levels (B) as assessed by immunohistochemistry in a cohort of 16 DSRCT samples, compared with those of the JN1 and R cell lines (PARP1 and PAR expression are shown as H-scores). Representative cases (PARP1-high vs. -low tumors; PAR-high vs. -low tumors) are shown to the right, compared with JN1 and R cells. **C, D.** Surface plots of Bliss independence scores calculated for the talazoparib – M4344 combination in JN1 (C) and R (D) cell lines at 7 days. **E.** GR_13-PDX-O model was established from the primary peritoneal tumor of a patient with DSRCT, with confirmation of EWS-WT1 fusion by FISH and WT1-Cter IHC. **F.** Surface plot of Bliss independence score calculated for the talazoparib – M4344 combination in the GR_13 PDX-O at 7 days. The associated dose-response matrix is shown to the right. Mean \pm SD, n=3. Surface plots: the x- and y-axes values indicate drug concentrations, and the z-axis values the associated synergy score; score < -10, antagonistic interaction; score = 0, absence of interaction; score > 10, synergistic interaction.

Figure 3. PARP and ATR inhibitors elicits DNA damage, replication stress and genomic instability in DSRCT cells.

A-D. Quantification of γ H2AX (A, B) or RAD51 foci (C, D) in JN1 (A, C) or R cells (B, D) exposed to DMSO control, PARPi talazoparib, ATRi M4344, or a combination of both for 72 h. Cisplatin was used as a positive control. A minimum of 500 nuclei were analyzed per condition. Violin plots show the absolute number of foci per nucleus. Thick line, median; thin lines, lower and upper quartiles; two-way ANOVA and *post hoc* Dunn's test. **E, F.** Western blot of pCHK1, CHK1, pRPA2, RPA2, γ H2AX, H2AX, and cleaved-PARP1 (cPARP) in JN1 (E) or R (F) cells exposed to DMSO control, PARPi talazoparib or olaparib, ATRi M4344 or AZD6738, or a combination of both for 48 h. **G, H.** Representative immunofluorescence images (G) and quantification of micronuclei-positive cells (H) in JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344, or a combination of both for 72 h. A minimum of 500 cells were analyzed per condition. Mean \pm SD; $n = 3$; one-way ANOVA and *post hoc* Dunn's test. Arrows indicate micronuclei. Scale bar, 20 μ m.

Figure 4. EWS-WT1 is a determinant of DSRCT cells' sensitivity to PARPi and ATRi.

A. Western blot of EWS-WT1 in JN1 and R cells transfected with either siCNTRL or siEWS-WT1. Whole-cell lysates were generated 48h post-transfection. **B-E.** Dose-response survival curves of JN1 or R cells exposed to PARPi talazoparib (B, C) or ATRi M4344 (D, E) for 7 days in presence or absence of siRNA-mediated silencing of EWS-WT1. Mean \pm SD; $n = 3$. **F, G.**

Quantification of γ H2AX in JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344, or a combination of both for 72 h, in presence or absence of siRNA-mediated silencing of EWS-WT1. Cisplatin was used as a positive control. A minimum of 500 nuclei were analyzed per condition. Violin plots show the absolute number of foci per nucleus. Thick line, median; thin lines, lower and upper quartiles; two-way ANOVA and *post hoc* Dunn's test. **H**, **I**. Western blot of pCHK1, CHK1, pRPA2, RPA2, γ H2AX, H2AX, and EWS-WT1 in JN1 (**I**) or R cells (**J**) exposed to DMSO control, PARPi talazoparib, ATRi M4344, or a combination of both for 48 h, in presence or absence of siRNA-mediated silencing of EWS-WT1.

Figure 5. EWS-WT1 drives enhanced DNA replication stress and R-loops, which contribute to DSRCT cells' sensitivity to PARPi and ATRi. A. Assessment of replication fork speed (kb/min) in JN1 cells subjected to siRNA-mediated silencing of EWS-WT1 or CCND1. A minimum of 50 forks were analyzed per condition. Mean \pm SD, each dot represents a single replication fork; $n = 2$, one-way ANOVA and *post hoc* Dunnett's test. **B.** Assessment of replication fork speed (kb/min) in JN1 cells exposed to DMSO control, or a combination of PARPi talazoparib and ATRi M4344 for 6 h, in presence or absence of siRNA-mediated silencing of EWS-WT1. A minimum of 50 forks were analyzed per condition. Mean \pm SD, each dot represents a single replication fork; $n = 2$, two-way ANOVA and *post hoc* Šídák's test. **C, D.** DNA:RNA hybrid dot blot of genomic DNA extracted from JN1 (**C**) or R cells (**D**) exposed to PARPi talazoparib, ATRi M4344 or a combination of both in

presence or absence of siRNA-mediated silencing of EWS-WT1 as in B. S9.6, RNA:DNA hybrids; ssDNA, loading control. **E.** Assessment of replication fork speed (kb/min) in RNaseH1-overexpressing JN1 cells subjected to siRNA-mediated silencing of EWS-WT1. Synchronized cells were collected 14 h post-transfection. A minimum of 50 forks were analyzed per condition. Mean \pm SD, each dot represents a single replication fork; $n = 2$, unpaired t-test. **E.** Dose-response survival curves of JN1 cells exposed to PARPi talazoparib (F) or olaparib (G), and ATRi M4344 (H) or AZD6738 (I) for 7 days in presence or absence of siRNA-mediated silencing of EWS-WT1 and/or RNaseH1 overexpression. Mean \pm SD; $n = 3$. Two-way ANOVA.

Figure 6. The combination of PARPi and ATRi elicits a cGAS/STING-mediated cell-autonomous immune response. **A.** Western blot of pTBK1, TBK, pIRF3, and IRF3 in JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344 or a combination of both for 72 h. **B, C.** RT-qPCR analysis of RNA isolated from JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344 or a combination of both for 72 h. CCL5 (B) and CXCL10 (C) mRNA were analyzed separately relative to RPLP0. Box-and-whisker plots show arbitrary units of gene expression, normalized to the DMSO condition. Boxes indicate median, lower and upper quartiles; whiskers indicate the 5th to 95th percentile range; $n = 4$, two-way ANOVA and post hoc Dunnett's test, relative to the DMSO condition. **D.** Quantification of PD-L1 cell-surface expression by flow cytometry in JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344 or a combination of both for 72 h. Scatter plot shows

the percentage of PD-L1-positive cells within the DAPI-negative population, normalized to the DMSO condition. Mean \pm SD; n=3. Kruskal-Wallis test and post hoc Dunnett's test, relative to the DMSO condition. **E.** Western blot of pTBK1, TBK, pIRF3, and IRF3 in JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344 or a combination of both for 72 h, in presence or absence of siRNA-mediated silencing of EWS-WT1. **F, G.** RT-qPCR analysis of RNA isolated from JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344 or a combination of both for 72 h, in presence or absence of siRNA-mediated silencing of EWS-WT1. CCL5 (F) and CXCL10 (G) mRNA were analyzed separately relative to RPLP0. Box-and-whisker plots show arbitrary units of gene expression, normalized to the siCNTRL DMSO condition. Boxes indicate median and lower and upper quartiles; whiskers indicate the 5th to 95th percentile range; n = 4, two-way ANOVA and post hoc Dunnett's test, relative to the siCNTRL DMSO condition. **H.** Quantification of PD-L1 cell-surface expression by flow cytometry in JN1 cells exposed to DMSO control, PARPi talazoparib, ATRi M4344 or a combination of both for 72 h, in presence or absence of siRNA-mediated silencing of EWS-WT1. Scatter plot shows the percentage of PD-L1-positive cells within the DAPI-negative population, normalized to the siCNTRL DMSO condition. Mean \pm SD; n=3. Kruskal-Wallis test and post hoc Dunnett's test, relative to the siCNTRL DMSO condition. **I.** Model of EWS-WT1-driven DSRCT sensitivity to PARPi and ATRi.

Figure 1.

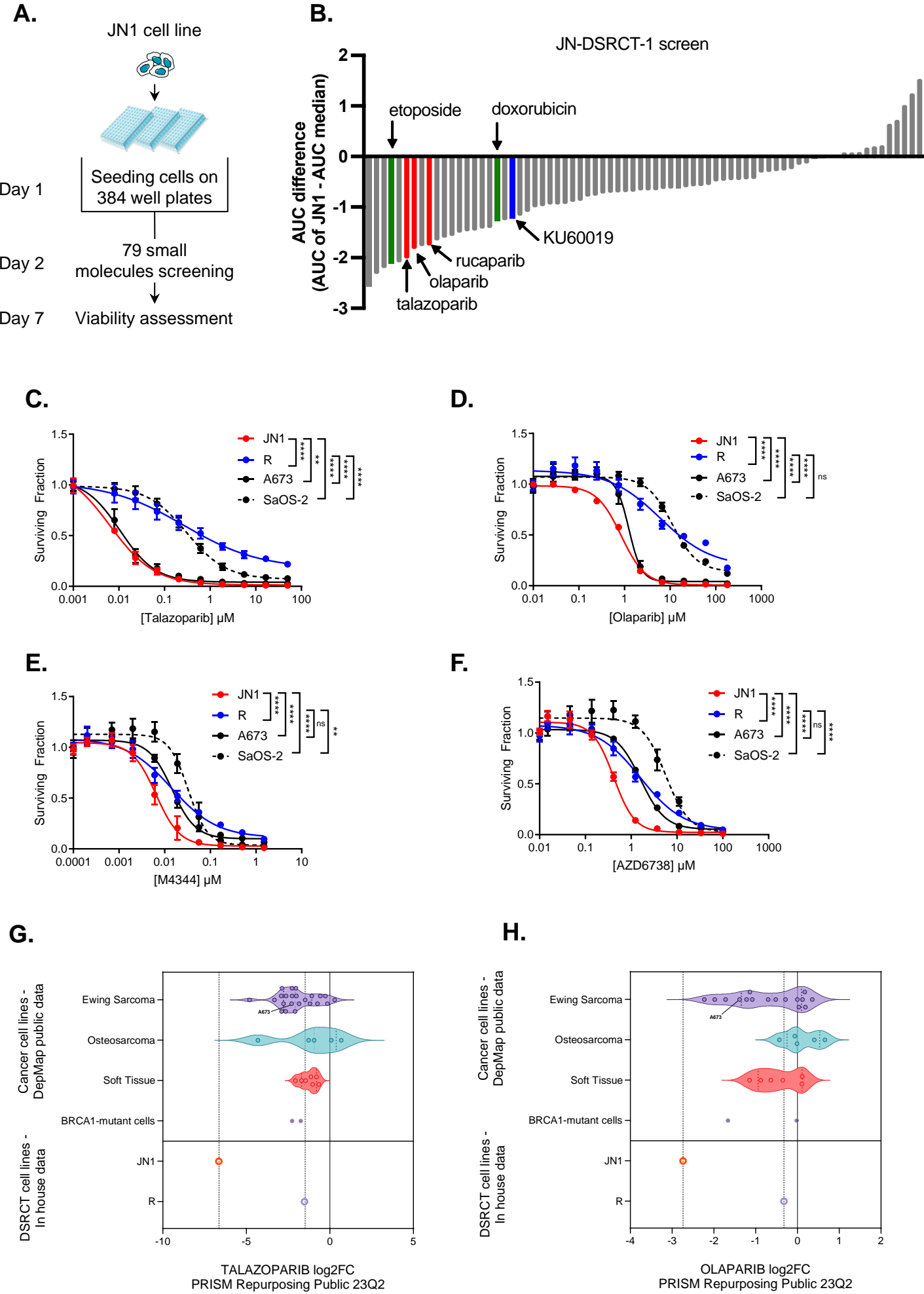
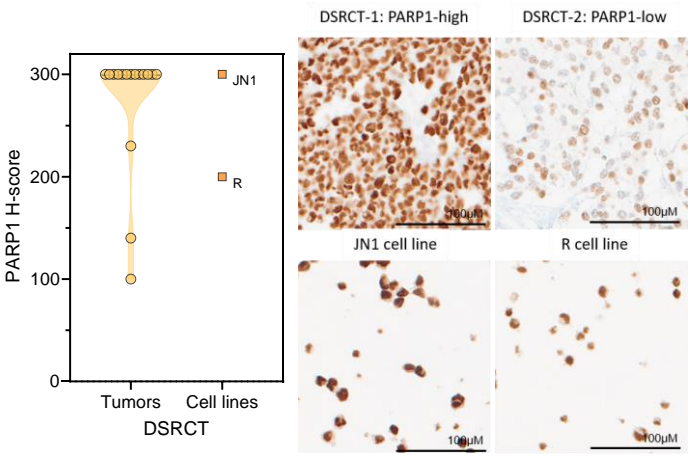
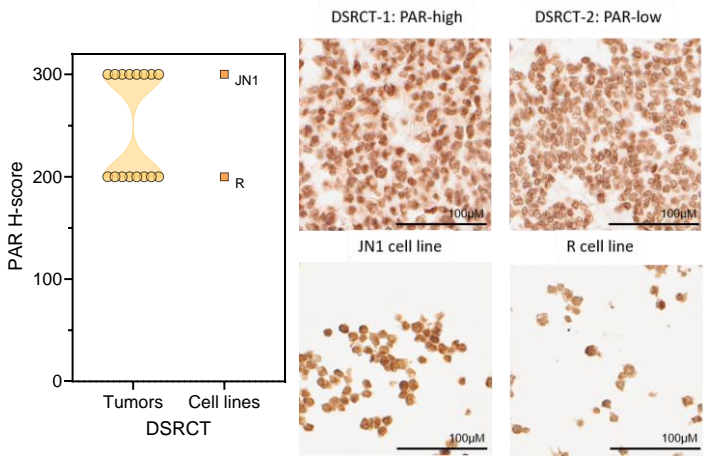


Figure 2.

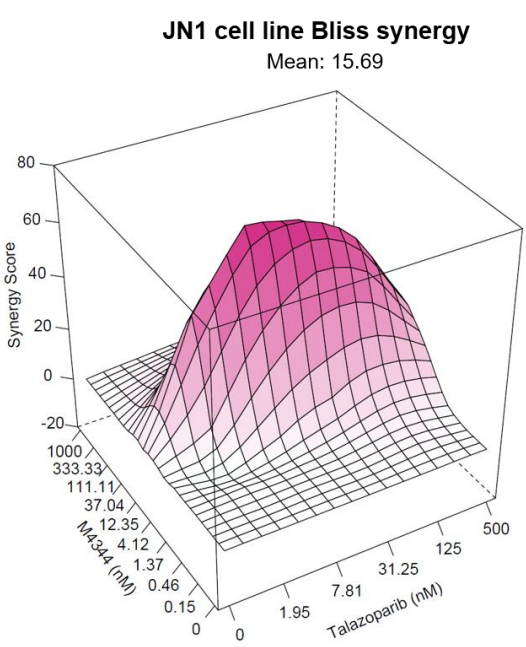
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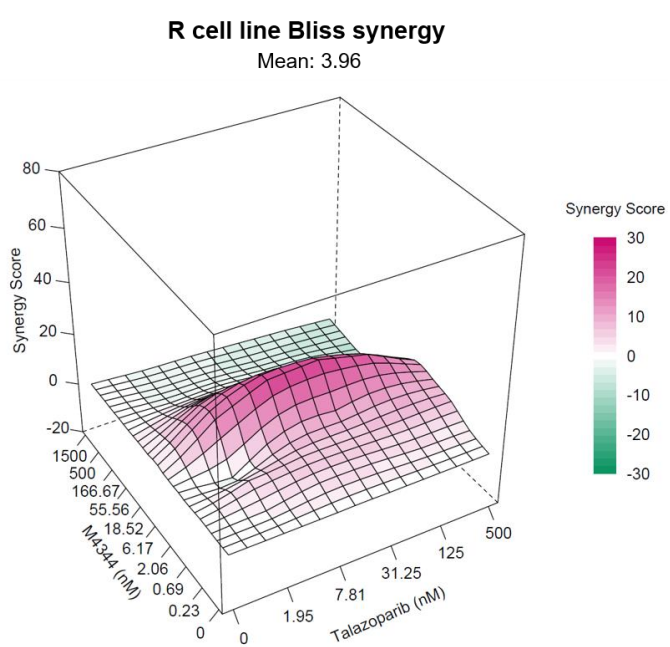
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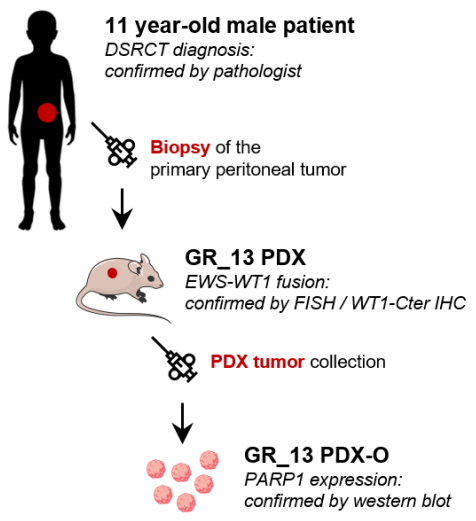
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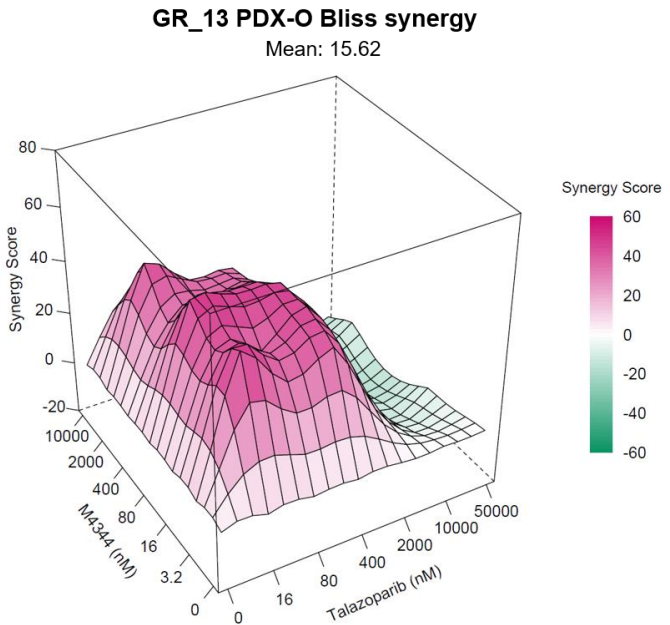


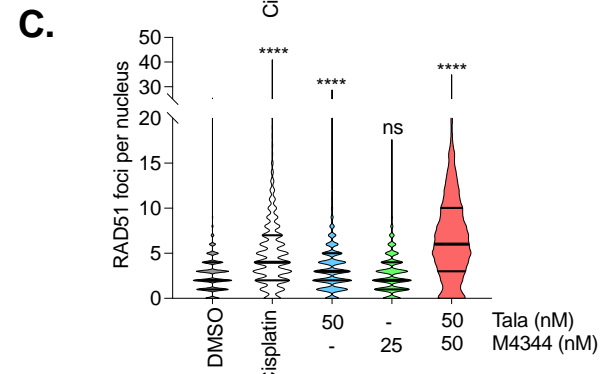
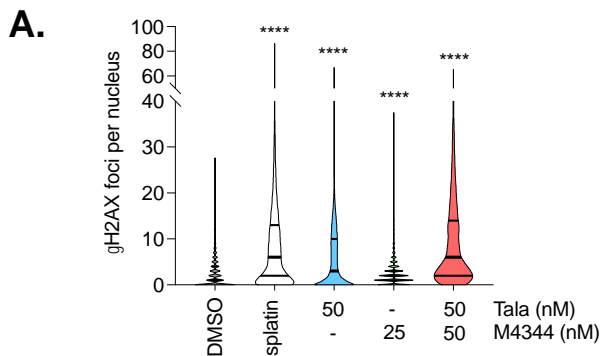
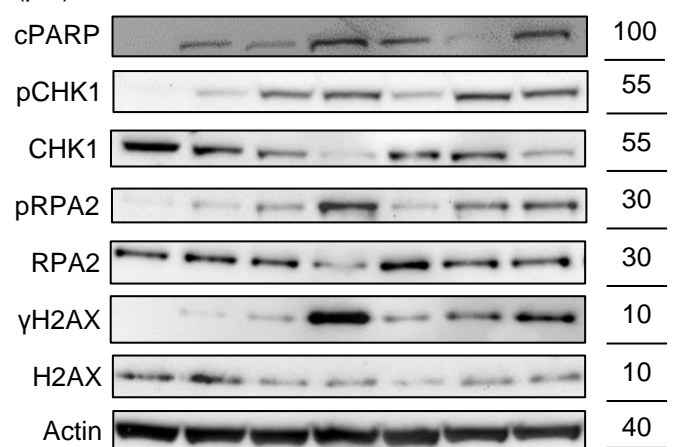
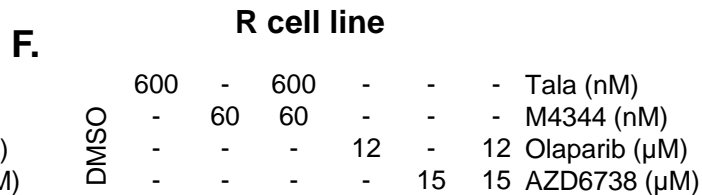
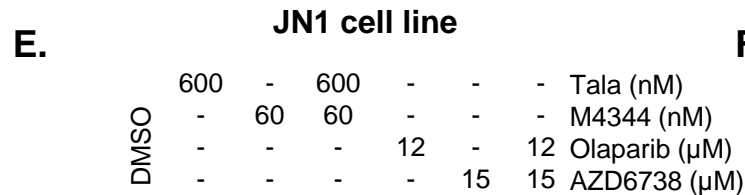
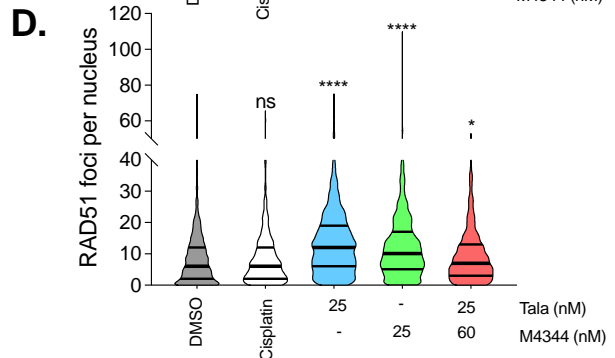
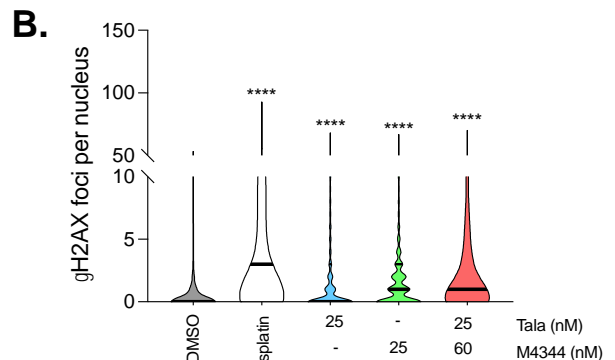
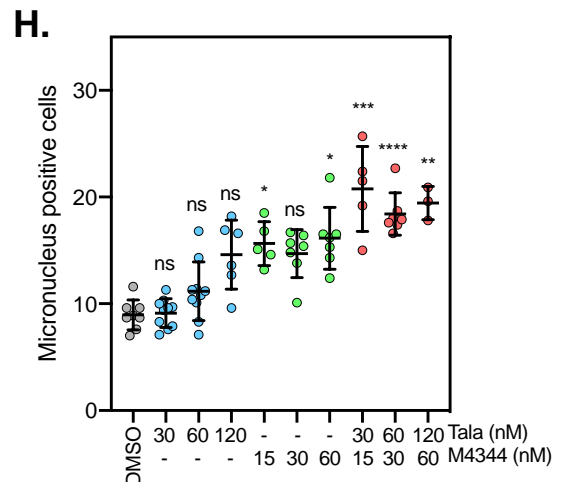
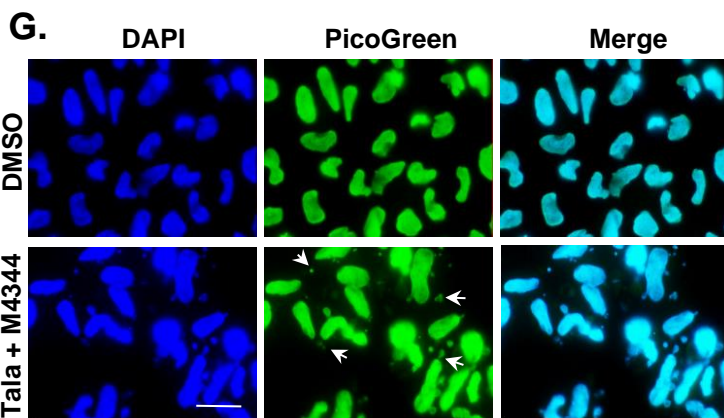
Figure 3.**JN1 cell line****R cell line****JN1 cell line**

Figure 4.

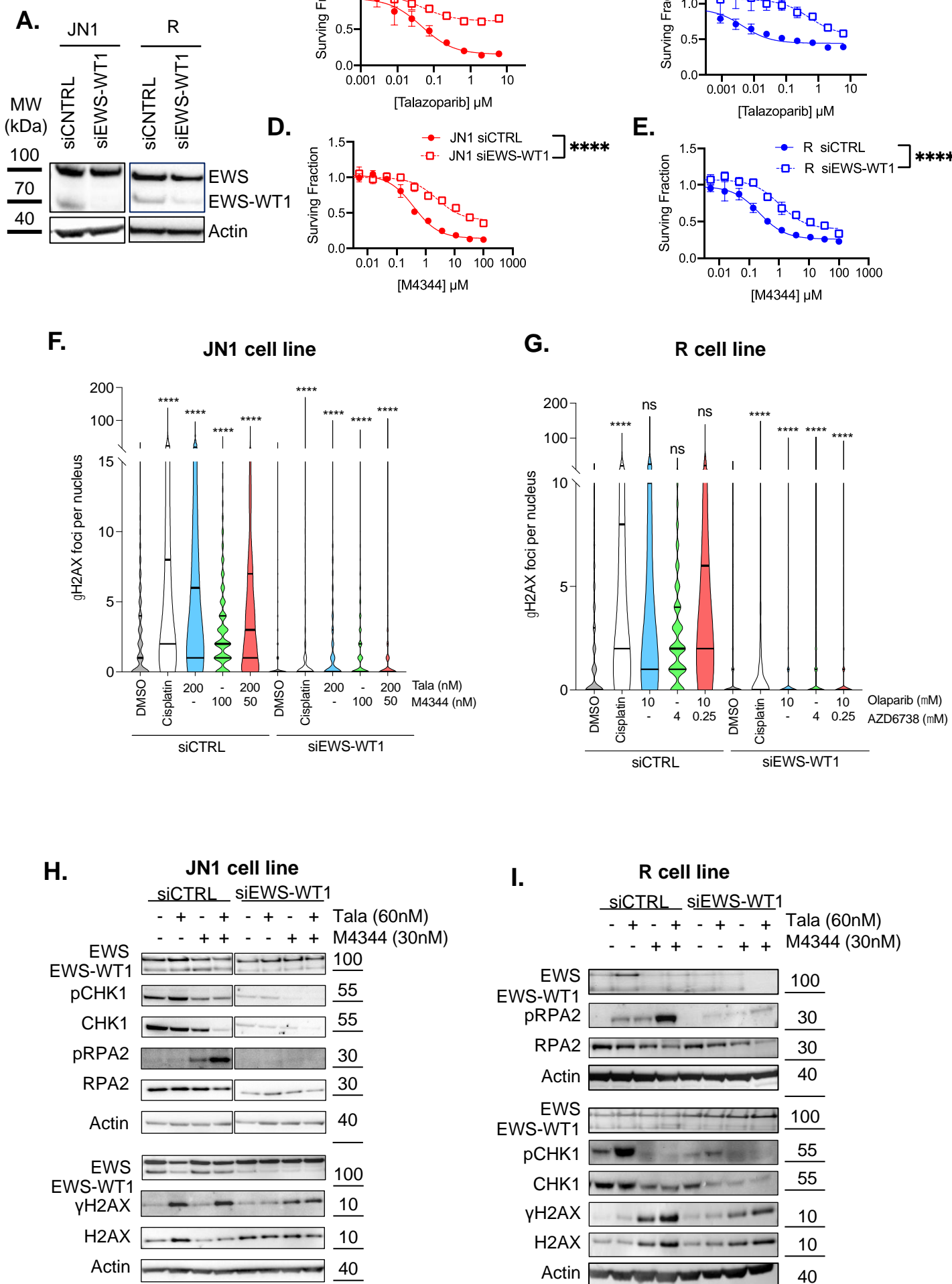
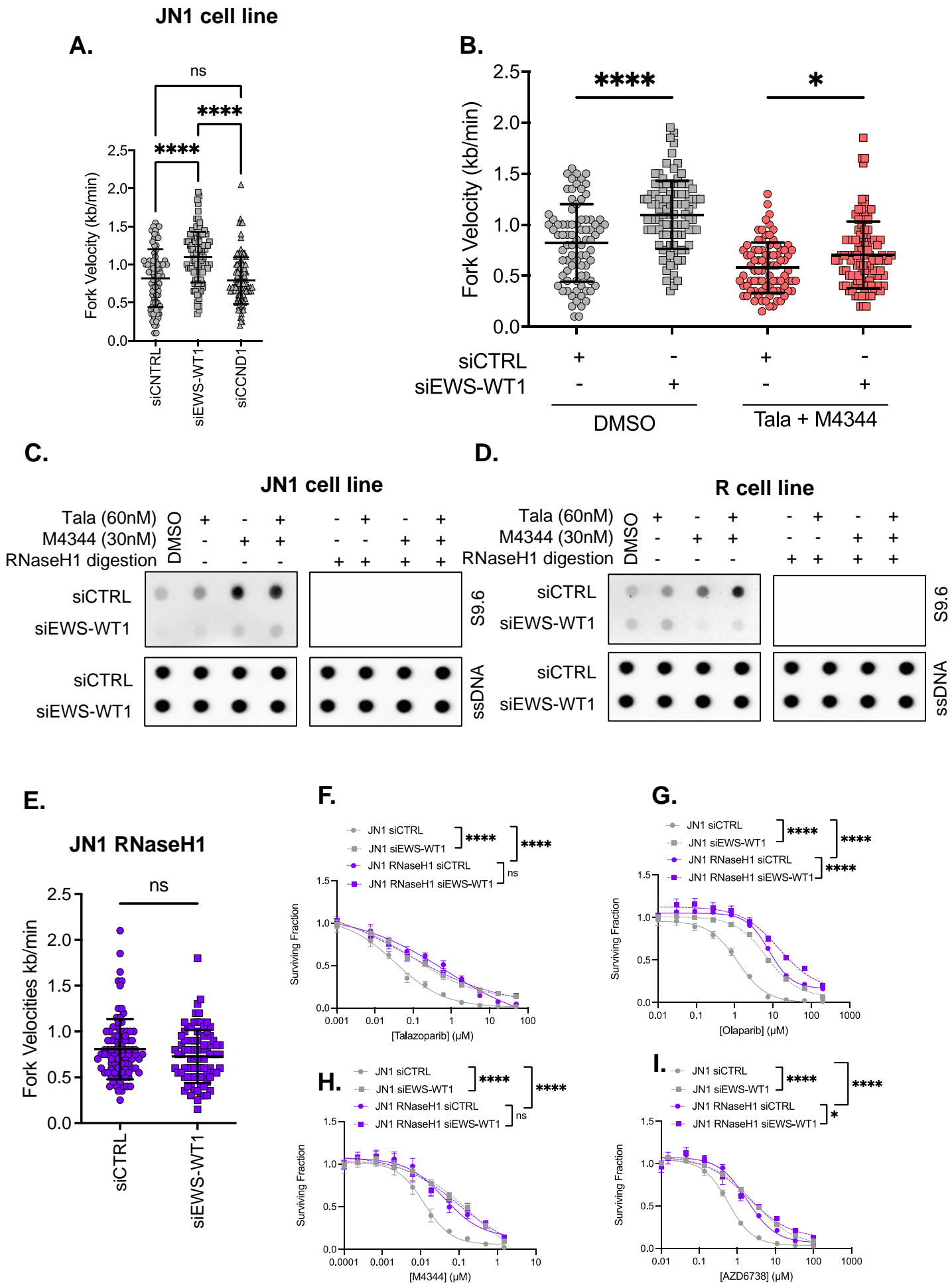


Figure 5.



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