Radiotherapy drives lung metastasis in PDAC via a RhoGTPase signaling shift towards MRCK-dependency

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with an extremely poor prognosis, partly owing to its highly metastatic nature. Although radiotherapy is an effective and potentially curative treatment modality, research over the last few decades has indicated that it may induce a more metastatic phenotype in surviving cancer cells. We demonstrate for the first time that clinically relevant doses of image-guided targeted radiotherapy induce metastasis in a genetically engineered KPC mouse model of PDAC. Furthermore, this induction is largely driven by an organotropic switch in lung metastasis. Using an *in vitro* RNAi screening approach, we identified a key role for myotonic dystrophy-related Cdc42-binding kinase (MRCK) in driving this response. MRCK activity was spatially upregulated at the plasma membrane in response to radiotherapy in PDAC cell lines as well as at the invasive margins of PDAC tumors. This upregulation of activity was maintained in metastases, suggesting an important role for MRCK in not only triggering local invasion in response to radiotherapy but also promoting distant metastases. Importantly, inhibition of MRCK with a small-molecule inhibitor (BDP9066) specifically opposed radiation-driven MRCK upregulation and pro-metastatic response.

59 **Introduction**

60 Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive and metastatic malignancy with a
61 dismal five-year survival rate of less than 10% (1). Despite the low incidence rates, it is projected to
62 become the second leading cause of cancer-related death within the next five years with survival
63 outcomes having not significantly improved since the 1970s, highlighting the urgent need for novel
64 therapeutic strategies (2). PDAC is largely refractory to current treatments, and surgical resection is
65 the only potentially curative option. Chemotherapy offers only modest survival benefits, leading to a
66 dismal median survival of less than six months (3). The role of radiotherapy (RT) in PDAC treatment
67 remains controversial owing to inconsistent clinical trial outcomes regarding its efficacy. While some
68 evidence suggests improved overall survival in the neoadjuvant setting, as demonstrated by the
69 PREOPANC phase III trial, in which a gemcitabine-based chemoradiotherapy regimen resulted in
70 superior survival outcomes compared to upfront surgery and gemcitabine alone in resectable and
71 borderline-resectable cases, other studies have reported conflicting findings (4,5). The LAP07 trial, for
72 instance, demonstrated that although chemoradiation delayed local tumor progression in locally
73 advanced PDAC, it did not confer an overall survival advantage (6). As a result, the current
74 recommendation is for RT to be utilized in patients who are poor surgical candidates or have locally
75 advanced disease (7).

76 The limited efficacy of RT in PDAC can be partially attributed to its intrinsic radioresistance, which
77 arises from a dense, fibrotic, and immunosuppressive tumor microenvironment with acquired
78 resistance mechanisms that also contribute to treatment failure (8,9). Another factor that may impact
79 the lack of efficacy is RT-induced invasion and metastasis, a phenomenon that has been documented
80 preclinically in multiple malignancies and reviewed in various articles (10-12). However, despite
81 growing evidence supporting this phenotype in other malignancies, studies investigating RT-induced
82 metastasis in PDAC have largely been confined to *in vitro* analyses, with no *in vivo* preclinical studies
83 investigating its impact on the metastatic burden in clinically relevant models (13,14).

84 RhoGTPases are a family of 26 signaling proteins that drive actin cytoskeletal dynamics to promote
85 cell migration and invasion and consequently play a critical role in tumor metastasis (15,16).
86 Downstream signaling from RhoGTPases is mediated by a panel of effector kinases, including LIM
87 domain kinase (LIMK), Rho-associated protein kinase (ROCK), and myotonic dystrophy kinase-related
88 Cdc42-binding kinase (MRCK), to create a complex and dynamic network (17-19). This complexity
89 leads to a high degree of plasticity that allows cells to adapt to changing conditions within the
90 microenvironment (ME). For example, the RhoA-ROCK signaling axis is used by cells to adopt
91 amoeboid migration to allow navigation through small gaps in the stroma. However, when this form

92 of migration is insufficient to allow cells to pass through dense, complex stroma, cells can switch to a
93 Rac1/CDC42-MRCK driven mesenchymal phenotype that allows extracellular matrix (ECM)
94 degradation and pseudopod formation (20,21).

95 Interestingly, the RhoA-ROCK axis has been demonstrated to drive invasion in preclinical studies of
96 PDAC under physiological conditions, although how the network responds to radiotherapy to support
97 metastasis remains to be elucidated (19). In glioblastoma, a tumor in which cells adopt a more
98 mesenchymal mode of invasion, MRCK was demonstrated to drive local dissemination of disease
99 under standard of care radiotherapy in preclinical models (22,23). However, the role of other
100 RhoGTPase members and downstream effectors in supporting invasion and metastasis is yet to be
101 comprehensively assessed under physiological or irradiated conditions.

102 In this study, we demonstrated a pro-migratory phenotype induced by RT in multiple PDAC cell lines.
103 Importantly, we demonstrated for the first time that this increased motility correlates with a
104 significant increase in metastatic burden in a clinically relevant PDAC model, with a tropism switch to
105 the lung. We showed that this effect is mediated by altered RhoGTPase signaling, with RT promoting
106 a dependency on MRCKα signaling to drive the pro-invasive phenotype. Specifically, we showed that
107 RT increases MRCK activity both *in vitro* and *in vivo*, with spatially localized activation at the invasive
108 edge of irradiated KPC tumors and metastases. Furthermore, both RNAi and pharmacological
109 inhibition of MRCK significantly inhibited RT-induced motility, highlighting the potential of anti-
110 invasive strategies for improving RT efficacy. Importantly, given the established role of MRCK in RT-
111 induced invasion of other malignancies, our findings suggest that MRCK signaling may represent a
112 more central mechanism underlying RT-induced invasion across different cancer types.

113
114 **Materials and Methods**
115
116 **Cell culture**
117 cell lines were maintained in incubators at 37°C with 5% CO_2 , tested regularly for mycoplasma, and
118 authenticated using STR profiling. The PANC-1 (male) and MiaPaCa-1 (female) cell lines were cultured
119 in DMEM (ThermoFisher Scientific #21969035) supplemented with 10% FBS, 1%
120 penicillin/streptomycin (ThermoFisher Scientific 15140-122) and 2mM L-glutamine (ThermoFisher
121 Scientific #25030-024). AsPC-1 cells (female) were cultured in RPMI 1640 medium (ThermoFisher
122 Scientific #11875093) supplemented with 10% FBS, 1% penicillin/streptomycin (ThermoFisher
123 Scientific #15140- 122) and 2mM L-glutamine (ThermoFisher Scientific #25030-024). NBC12.2f
124 (female), NBC12.2g (female), and NBC13.3a (male) murine cell lines were generated in-house from

125 C57Bl/6 KPC mice and cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin and
126 2mM L-glutamine. The primary patient lines, TKCC04 and TKCC05, were kindly gifted by Professor
127 David Chang (University of Glasgow), were characterized as the squamous subtype, and were cultured
128 in RPMI 1640 medium (ThermoFisher #11875093) supplemented with 10% FBS, and 20ng/mL
129 Epidermal Growth Factor (Life Technologies #PHG0311L) (24).
130
131 **Subconfluent migration assay**
132 Cells (1×10^4 cells/mL) were seeded in an appropriate culture dish for the experimental assay (35 mm
133 dishes or 12-well plates) and left to adhere for 24hours. Drug treatments and irradiation were carried
134 out 4 h prior to imaging unless otherwise stated. Timelapse microscopy images were acquired in phase
135 contrast every 20 mins for 16 hours at 10 X magnification (10x/0.30 Plan Fluor Ph1, Nikon). Cell speed
136 was calculated using single-cell tracking performed in ImageJ. All *in vitro* experiments were conducted
137 in biological triplicates, and statistical analysis details are within the corresponding figure legends.
138
139 **Automated cell tracking**
140 Automated tracking was conducted using scripts developed by Dr. Ryan Corbyn (Beatson Advanced
141 Imaging Resource Facility RRID:SCR_023875, CRUK-SI). Time-lapse imaging data were acquired using
142 an IncuCyte S3 system. IncuCyte data was preprocessed to collate the individual time points from the
143 experiment into a single time-lapse image stack followed by drift correction to stabilize the image
144 stacks (Fiji macro “correct 3D drift.”). Bespoke Python scripts were written by Dr. Ryan Corbyn and run
145 in a Jupyter integrated development environment (IDE) for cell segmentation and cell tracking. "We
146 retrained the livecell model from cellpose 2.0 on a subset of the imaging data for automated
147 cell segmentation(25,26). Automated cell tracking was subsequently performed using BTracks,
148 which employs a Bayesian single-cell tracking algorithm and generates deep lineage tree analyses as
149 described by Ulicna et al. ((27)). Tracks of cells present in >90% of the total number of frames were
150 used for further analysis. Further analysis of track lengths and cell speeds was performed for all
151 tracked cells, with the resulting data exported as. csv files.
152
153 **High-throughput immunofluorescence**
154 Cells were seeded at a density of 3×10^3 cells per well in 96-well optically clear plates (Greiner
155 #655892) and allowed to adhere overnight prior to irradiation and/or drug treatments, as specified in
156 the experimental conditions. Treated cells were incubated for an additional 24 h unless otherwise
157 stated. After treatment, the cells were washed with PBS before fixation with 4% formaldehyde at room
158 temperature for 15 min. Cells were permeabilized using 0.1% Triton X-100 in PBS for 10 min, washed

159 with TBS containing 0.1% Tween-20 (TBS-T), and blocked in 1% BSA in TBS-T for 30 min at room
160 temperature. For immunofluorescence staining, cells were incubated with the primary antibody
161 diluted in blocking solution overnight at 4°C. The details of the primary antibodies are listed in Table
162 S1. After primary antibody incubation, cells were washed three times with PBS and then incubated
163 with secondary antibody along with 1:1000 DAPI (Thermo Scientific #D1306), Texas Red-X Phalloidin
164 (Thermo Scientific #T7471) and 1:10,000 HCS CellMask Deep Red Stain (Thermo Scientific #H32721)
165 for 1 h at room temperature. After incubation, the cells were washed and stored in PBS. High-content
166 imaging was performed using the PerkinElmer Opera High-Content Screening System, and image
167 analysis was conducted using the Columbus™ image analysis software.
168
169 **Cell viability assays**
170 Cells were seeded at a density of 1×10^4 cells/mL in 96-well plates (Corning) and allowed to adhere
171 overnight. Cells were irradiated at either 0 or 5 Gy using the Xstrahl RS225 and treated with BDP9066
172 for 24 h. Cell viability was assessed using the CellTiter-Glo® assay (Promega #G9241) and Promega
173 Glomax Multi detection system.
174
175 **siRNA transfections**
176 Cells used for transfection were maintained in medium free of penicillin/streptomycin for at least one
177 week prior to the start of experiments. PANC-1 and Mia PaCa-2 cells were transfected with siRNA
178 targeting CDC42BPA (MRCKα) or control scrambled siRNA (Dharmacon #L003290-00; MRCKα
179 AAGAAUAUCUGCUGUGUUU) using DharmaFECT 2 transfection reagent (Dharmacon #T-2005-01) and
180 incubated for 48 h prior to imaging or protein extraction.
181
182 **Proteome analysis**
183 Fifteen-centimetre dishes were pre-coated with Matrigel prior to cell seeding to increase cell
184 adherence. Matrigel was diluted at a ratio of 1:40 in cold DMEM medium without additional
185 supplements. A total of 5 mL Matrigel dilution was added to each dish and incubated at 37°C and 5%
186 CO_2 for 1 hour. Excess medium was aspirated prior to cell seeding.
187 PANC-1 cells were seeded at a density of 2×10^5 cells/mL in Matrigel-coated dishes, with 15 mL of cell
188 suspension per dish. Three biological replicates were plated. Cells were left to adhere overnight at
189 37°C and 5% CO_2, resulting in ~80% confluency. Six hours before analysis, the media were aspirated,
190 and plates were washed six times with PBS to ensure all cell debris was removed. Media was then
191 replaced with 10mL FBS-free culture medium. Immediately after media replacement, cells were

192 irradiated with either 0 Gy or 5 Gy and incubated for 6 hours. Following this incubation, cell lysates
193 were collected for proteome and secretome analysis, respectively.
194 Ultra-High Performance Liquid Chromatography/Tandem Mass Spectrometry (UHPLC-MS/MS)
195 analysis and data analysis conducted by the CRUKSI proteomics facility. See supplemental data for
196 details.
197
198 **RNAseq**
199 NBC12.2g, NC12.2f and NBC13.3a were irradiated +/- 5 Gy. RNA was extracted 24 hours post
200 irradiation using Rneasy kit (Qiagen) kit. Truseq (Illumina) mRNA-seq libraries were prepared from
201 total RNA and these were then sequenced using the Illumina HiSeq 4000 using 50M read pairs,
202 2x75bp read length. DEseq2 was utilised for differential gene analysis. Volcano plot created in
203 GraphPad Prism v10.60. See supplementary methods for full analysis details.
204
205 **RNAi screen**
206 PANC1 cells were seeded at a density of 1×10^5 cells/mL in two 12-well plates and allowed to adhere
207 overnight. Cells were transfected with 25 nM custom siRNA ON-TARGETplus SMARTpool library
208 (Horizon) using DharmaFECT 2 transfection reagent and incubated overnight. The details of the siRNA
209 are shown in Table S2. After incubation, transfected cells were washed twice with PBS, detached from
210 the wells using TrypLE (250 µL per well), and resuspended in an additional 750 µL of antibiotic-free
211 medium. Cells were plated at a density of 1×10^4 cells/mL in 24-well plates for the subconfluent
212 migration assay. In parallel, selected transfected cells were replated into additional 6-well plates for
213 protein extraction to verify successful knockdown. All replated cells were allowed to adhere overnight
214 at 37°C and 5% CO_2 and subject to 0 Gy or 5 Gy after 16hrs and 4 h prior to imaging.
215
216 **Western blot**
217 Cells were lysed with 1% SDS and 50 mmol/L Tris (pH 6.8) supplemented with protease and
218 phosphatase inhibitors (Thermo Scientific #1861280) followed by SDS-PAGE using 4–12% Novex Bis-
219 Tris gels or 8% BOLT Bis-Tris Plus gels (Thermo Fisher Scientific), and transfer onto nitrocellulose
220 membranes and western blotting with enhanced chemiluminescence (ECL) detection. Primary
221 antibodies were diluted in 5% bovine serum albumin (BSA). The details of the primary antibodies used
222 are shown in Table S1.
223
224 ***In vivo* experiments**

225 All animal work was conducted under the guidance of the UK Home Office regulations and carried out
226 under project license PP8411096. All experiments were approved by the University of Glasgow Animal
227 Welfare and Ethical Review Board. The mice were housed in standard cages with access to food and
228 water *ad libitum*. Environmental enrichment was also provided. KPC mice of both sexes were used in
229 all the experiments. *Pdx1-Cre*; *LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$ (KPC; (28)) mice were bred in-house at
230 the CRUK Scotland Institute, maintained on a mixed background, and genotyped using Transnetyx
231 (Cordoba, TN, USA). The mice were palpated weekly to detect pancreatic tumors, and the presence of
232 a pancreatic tumor was confirmed by 3-dimensional (3D) ultrasound using the Vevo3100 high-
233 frequency ultrasound platform (FUJIFILM VisualSonics) prior to enrolment in the treatment arm. Mice
234 in the treatment group were housed in the same room, and single housing was avoided where
235 possible. In the gemcitabine study, mice were intraperitoneally administered 100 mg/kg gemcitabine
236 twice a week.
237
238 ***In vivo* irradiation of pancreas tumour**
239 The Small Animal Radiotherapy Research Platform (SARRP, Xstrahl) was used to deliver targeted
240 irradiation as previously described (29). Mice were anesthetized using a mixture of medical air and
241 isoflurane for the duration of the radiotherapy session. An iodine contrast-enhanced cone beam
242 computed tomography (CBCT) image was obtained from 1,440 projections using the in-built cone
243 beam CT function of SARRP. To enhance contrast, the mice were administered 0.25 ml Iohexol
244 (Omnipaque™, GE Healthcare) intraperitoneally immediately prior to CT acquisition. Radiotherapy
245 planning was performed using Muriplan software, including tissue segmentation and placement of an
246 isocenter to localize the pancreatic tumor. Arc radiotherapy was delivered at 280 cGy/min (220 kV, 13
247 mA) using three fractions of 4 Gy every other day, three fractions of 6 Gy every other day, or a single
248 fraction of 12 Gy. The collimator aperture size was selected based on tumor shape and size. The mock
249 irradiation controls consisted of mice subjected to 1,440 CBCT acquisitions, which resulted in the
250 delivery of 6.22 cGy (29). Mice were monitored by researchers blinded to the treatment arm and
251 euthanized when they exhibited clinical signs of PDAC (distended abdomen, loss of body conditioning,
252 lethargy, and jaundice).
253
254 **Histology and immunohistochemistry**
255 Tissue samples were fixed in formalin and embedded in paraffin using standard protocols before
256 acquisition of 4 μm sections using a HistoCore MULTICUT microtome (Leica, UK). Tissue sections were
257 placed on poly-L -lysine slides and oven baked at 60 °C for 2 h prior to staining with hematoxylin and

258 eosin (H&E) or immunohistochemistry (IHC) for Zeb1, and pMRCK. The full list of antibodies used is
259 presented in Table S1.
260
261 All liver and lung tissues from control mice and those that received 3 × 4 Gy of radiation were subjected
262 to serial sectioning (five sections at 50 µm intervals). The sections were blinded and double scored for
263 the presence of metastases. For IHC, antigen retrieval and antibody dilution were optimized in-house
264 for each antibody (Table 2.8).
265
266 Automatic quantification of staining was performed using QuPath ((30), v0.05.1). The full slides were
267 scanned and imaged using a Lecia Aperio AT2 slide scanner. Quantification was conducted blinded,
268 with either the full tumor or five areas at the tumor center and tumor border selected at random.
269 These areas were annotated using QuPath annotation tools. The percentage of positive cells was then
270 calculated.
271
272 **Statistical analysis**
273 Statistical analyses are detailed in the figure legends for all experiments. Statistical analyses were
274 performed using the GraphPad Prism software. The number of independent experiments for each in
275 vivo experiment is within the figure legend and refers to experiments repeated on different days with
276 cells at different passages for each cell line reported. Biological repeats refer to independently derived
277 cell lines used as replicates.
278
279 **Data availability**
280 The data generated in this study are publicly available in (https://github.com/Beatson-
281 CompBio/McLay_2025_A/)
282
283 **Results**
284
285 **Radiotherapy induces motility across a panel of PDAC cell lines**
286
287 Initial qualitative morphological analyses indicated that sublethal doses of RT (5 Gy) induced a shift in
288 morphology of PANC1 cells towards a more elongated shape, reminiscent of a mesenchymal transition
289 (Fig. 1A). Indeed, comprehensive and unbiased high-throughput immunofluorescent imaging and
290 automated analysis indicated that radiotherapy significantly increased the cell area in two PDAC cell
291 lines, which may be indicative of a motile phenotype through increased engagement with integrin
292 signaling (Fig. 1B)(31). Previous studies have reported increased PDAC cell motility *in vitro* in response
293 to RT; however, these studies have been limited in terms of cell line number and available technologies,
294 and as such, have yielded conflicting results ((32-34); as summarized in Table S3). Therefore, we used

295 a comprehensive approach across a panel of commercial, mouse-derived, and primary patient cell
296 lines to measure the effect of RT on PDAC cell motility. We used clinically relevant doses of RT (2 and
297 5 Gy) combined with subconfluent migration assays and single cell tracking to eliminate potential
298 confounding effects on proliferation. We identified a significant increase in migration speed across the
299 selection of three commercial, mouse, and primary lines (Fig. 1C). Parallel cell viability assays indicated
300 that RT had a modest impact on the viability of the TKCC04 line only at this short timepoint (24hrs;
301 Fig.1D). Expansion of this panel indicated that, while the effect of RT on migration is not ubiquitous,
302 five out of eight lines showed increased migration speed post RT, with a further two showing a trend
303 towards an increase (Table 1). Of note, the only murine line that did not show a significant increase in
304 speed, NBC12.2g, was more radiosensitive than the other two lines (Fig. S1).

305

306 **Radiotherapy increases the metastatic burden of pancreatic cancer in a clinically relevant genetic**
307 **mouse model**

308

309 While the activation of cancer cell migration is the initiating step in metastasis, a number of key
310 processes are required within the context of an entire organism, including local extracellular matrix
311 invasion, dissemination via the vasculature/lymph system, and seeding of distant sites. In light of this,
312 we assessed whether the increased motile phenotype translates to an increased metastatic burden *in*
313 *vivo* in a genetically engineered KPC mouse model of PDAC (*LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx1-Cre*)
314 in combination with image-guided targeted RT using a Small Animal Irradiation Platform (SARRP) (Fig.
315 S2A). This model simulates the entire metastatic cascade following targeted irradiation of the primary
316 tumor, allowing us to assess the response to RT in a clinically relevant model and to gain insight into
317 the potential clinical impact of RT on metastasis (Fig. 2A). To ensure a robust analysis, we cut five H&E
318 sections of liver and lungs from control and irradiated (3 × 4 Gy) PDAC mice at 50µm intervals, which
319 were blinded and double scored for the presence of metastases (Fig. 2B). The results indicated that
320 RT induced a significant increase in not only local invasion (Fig. 2C) but also metastatic burden (Fig.
321 2D). Importantly, 3 × 4 Gy did not prolong the survival of KPC mice, as shown in Fig. S2B and previously
322 published data (29), ruling out the possibility that the radiation-associated increase in metastasis may
323 be due to the additional time for tumor cells to seed metastases. In agreement, there was no
324 association between the lifespan of the animal and the presence of metastases on post-mortem
325 examination (Chi Square, P=0.564).
326 Radiotherapy is given in fractionated doses in the clinic, with a trend towards hypofractionation in
327 recent years. To assess whether fraction size and number impact the effect of RT on metastasis, we
328 extended our analysis to include cohorts of mice that received 3 × 6 Gy (increased fraction dose) and
329 1 × 12 Gy (hypofractionation). However, only a significant increase in survival was observed in the 1 x

330 12 Gy cohort (Fig. S2B), all three RT protocols showed a significant pro-metastatic outcome (Fig. 2E),
331 whereas mice treated with gemcitabine alone showed a reduction in metastatic burden (Fig. S2C).
332 These data suggest that RT promotes metastasis independent of the relative lifespan of the treated
333 animals.
334 PDAC metastasizes primarily to the liver and lungs in both patients and the KPC model. Interestingly,
335 and in support of a radiation-specific metastatic induction mechanism, when we analyzed lung and
336 liver metastases separately, we found that RT induced an organotropic switch towards the lungs
337 across all irradiated cohorts (Fig. 2F).
338
339 **RT augments RhoGTPase signaling towards a dependence on MRCK and RhoU**
340 To unpick the potential mechanisms that drive the rapid motility response that we observed post RT
341 and begin to understand how this might trigger the metastatic cascade, we performed RNA-seq and
342 proteomics on PDAC lines post RT/sham RT. There were relatively few genes (at 24h post RT) or
343 proteins (6h post RT) that displayed significant fold changes greater than 1.5 at the relatively short
344 time points used, and no evidence of an emerging motility signature (Fig. 3A-B). This was the case
345 when the cut-off was reduced to a 1.2-fold change in the proteomic data, with response to cellular
346 stress emerging as the main signature post RT (data not shown). Therefore, we sought to undertake a
347 more targeted functional screen of known drivers of motility that are regulated rapidly and
348 independently of gene expression. RhoGTPases drive cancer cell motility and invasion through direct
349 action on the cytoskeleton and are therefore likely to be required to support the observed changes in
350 motility and metastasis.
351 We developed an unbiased, AI-driven, automated segmentation and single-cell tracking approach to
352 assay the dependence of PDAC cells on RhoGTPase family members and associated kinases pre- and
353 post RT for migration (Fig. 3C). To mitigate the inherent plasticity and redundancy in the RhoGTPase
354 network, we used an siRNA approach to transiently knock down each target in PANC1 cells. Using this
355 approach, we achieved knockdown efficiency of approximately 50% across a range of selected targets
356 (Western blot analysis; Fig. 3C(i); Fig. S3). The results in Fig. 3D indicate that there is an overall shift
357 towards dependency upon RhoGTPase activity for motility following RT, with a significant interaction
358 between the presence or absence of RT and the ablation of RhoU, RhoB, and MRCKα (CDC42BPA)
359 expression. Closer examination of the data revealed that while the RhoB interaction was driven by
360 dependency under control conditions, PANC1 cells showed a shift in dependency towards RhoU and
361 MRCKα following RT, suggesting that they may play a role in driving RT-induced motility (Fig. 4A). We
362 used a second siRNA oligo sequence coupled with manually tracked subconfluent migration assays to
363 validate the dependency of irradiated PANC1 cells on MRCKα for motility (Fig. 4B(i), (ii)).

364

365 **The induction of spatially regulated MRCK signaling by RT is conserved *in vitro***

366 Metastasis is a leading cause of morbidity and mortality in pancreatic cancer and represents an urgent
367 and unmet clinical need. Given the availability of small-molecule inhibitors targeting MRCK, we
368 selected this kinase for further validation (35). Analysis of available patient datasets also indicated
369 that higher MRCKα mRNA expression showed a trend towards association with poorer overall survival
370 ($p = 0.059$; Fig. 5A(i)), unlike MRCKβ, which has no association with survival. MRCK and related kinases
371 are well described to be regulated by phosphorylation and intracellular location, which may explain
372 the weak interaction between outcome and RNA expression in patient datasets. In support of this,
373 preliminary single-cell RNA-seq data from KPC mice indicated a significant but minor increase in
374 MRCKα expression post RT (data not shown).

375 To explore this, we performed a western blot analysis to validate the biomarker of MRCK activity
376 (autophosphorylation of S1003; pMRCK; Fig. 5B). Using the PANC1 cell line, a highly motile cell line in
377 response to radiation (Fig 1C), we found no increase in pMRCK levels in response to 2 or 5 Gy RT (Fig
378 5C), suggesting that spatial regulation of RhoGTPase signaling may be a more useful determinant of
379 downstream signaling and the resultant phenotype. Therefore, we utilized high-throughput
380 immunofluorescence imaging with automated analysis to examine MRCK activity within different
381 cellular compartments. We demonstrated that MRCK activity significantly increased at the cell
382 membrane following irradiation across the two cell lines, indicating the induction of discrete cellular
383 responses to promote migration (Fig. 5C).

384

385 **Pharmacological targeting of MRCK with a small molecule inhibitor opposes radiation driven**
386 **migration**

387 We used a potent and specific small-molecule inhibitor of MRCK, BDP9066, to test the potential
388 therapeutic benefit of targeting MRCK in combination with RT in PDAC (35). The results in Fig. 6A (i) –
389 (iii) indicate that treatment of PANC1 cells with BDP9066 significantly reduced pMRCK at the cell
390 membrane only under irradiated conditions, demonstrating a shift towards MRCK dependency post
391 RT. ROCK inhibitor (Y27632) was used as a negative control. Furthermore, the data in Fig. 6A (iv)
392 revealed a dose-dependent reduction in pMRCK in response to BDP9066 with a EC50 10-fold lower in
393 irradiated cells. These data further indicated that the clinical use of MRCK inhibition may have the
394 greatest benefit when used in combination with RT. This radiation-specific reduction in pMRCK in
395 response to MRCK inhibition was confirmed in an additional cell line (Fig. 6B). Importantly,
396 subconfluent migration assays demonstrated that this reduction in MRCK activity translated into a
397 reduction in radiation-driven migration across a panel of cell lines (Fig. 6C).

398

399 **MRCK activity is upregulated at the invasive tumour margins and metastases post radiotherapy**

400 To determine whether MRCK activity was upregulated post RT *in vivo*, we stained the sections from
401 the KPC GEM model (Fig. 2) for pMRCK. While there was no increase in pMRCK across the tumor bulk
402 (Fig. 7A(i)), pMRCK was significantly upregulated at the tumor margins compared to that at the tumor
403 center (Fig. 7A (ii), (iii), (iv), (v)). Furthermore, this pattern of activation was concomitant with an
404 increase in Zeb1 expression (a marker of epithelial-to-mesenchymal transition; Fig. 7B). These data
405 indicate that RT may induce an MRCK-dependent, pro-invasive signature at the invasive borders of
406 PDAC tumors. Importantly, this increase in pMRCK was maintained in metastases from the
407 radiotherapy-treated cohort, indicating that MRCK signaling may be required to support progression
408 from local invasion to distant metastases (Fig 7C). Finally, using *ex vivo* cultured tumor tissue from KPC
409 GEM mice, we demonstrated that pMRCK levels can be significantly reduced by BDP9066 when used
410 in combination with RT, further highlighting the potential for therapeutic translation (Fig. 7D;
411 Supplemental M&M).

412 Together, our data indicate that RT drives increased metastasis in a clinically relevant model of PDAC,
413 with organotropism towards the lung. This is driven, at least in part, by upregulation of MRCK signaling.
414 Targeting MRCK with a small-molecule inhibitor may present a novel therapeutic avenue for
415 containing spread of disease during radiotherapy.

416

417 **Discussion**

418 Metastasis is a leading cause of morbidity and mortality in PDAC, with approximately 50% of patients
419 having metastasis at diagnosis, increasing to 90% upon autopsy (36). Identifying a therapeutic window
420 for the addition of anti-metastatic therapy has historically been a challenge for the cancer field. The
421 clear induction of metastasis by targeted radiotherapy in a clinically relevant model of PDAC provides
422 strong evidence for the first time that RT-driven metastasis may have an important clinical impact.
423 Importantly, we observed increased metastasis within a short timeframe post-RT (two weeks) in
424 animals that likely already had locally advanced disease at the time of treatment, mirroring the clinical
425 situation. These data indicate a clear window of opportunity for the addition of anti-metastatic
426 therapy concomitant with RT to control the dissemination rates and improve survival outcomes.
427 Indeed, constraining metastatic spread may be predicted to become increasingly urgent as better
428 methods for early, pre-metastatic detection of PDAC arise in the coming years. Although our data
429 provide an important advance in understanding the effect of RT on metastasis, to fully understand the

430 clinical relevance of our observations, the identification and analysis of outcome data from relevant
431 patient cohorts should be performed in the future.

432 Surprisingly, our data indicate that not only does RT drive metastasis, but also a switch in
433 organotropism towards the lungs. Although patients with isolated lung metastases have better
434 survival outcomes, our data clearly indicate a potential therapeutic window whereby the addition of
435 anti-metastatic therapy during RT may abrogate the metastatic spread of the disease and improve
436 survival (37). The observed lung organotropism also provides strong evidence for an RT-specific switch
437 in metastatic drivers. Indeed, our observation of increased pMRCK along with an increase in the
438 mesenchymal marker Zeb1 at the tumor borders indicates a cell signaling switch towards MRCK-driven
439 mesenchymal-like invasion during radiotherapy. Notably, single-cell RNA-seq data generated by our
440 team from irradiated KPC tumors did not identify a clear EMT signature, which may suggest that the
441 MRCK-driven response represents a partial EMT that is unique to the post RT scenario (data not
442 shown). Unpicking this signaling response in the future has the potential to not only further our
443 understanding of RT-driven metastasis but also of what drives lung-specific metastasis under
444 physiological conditions in solid cancers. In addition, the surprising conservation of this response
445 across cancers of starkly different origins (PDAC and glioblastoma) suggests that unpicking the
446 surrounding signaling cascades will further our understanding of radiobiological responses (23).

447 Previous studies have demonstrated that RT drives more fibrotic stroma in solid cancers, including
448 PDAC, which is largely driven by cancer-associated fibroblasts (CAFs) (9,38). This suggests that the
449 switch to mesenchymal-like migration may be a necessary adaptation that allows PDAC cells to locally
450 invade the post-RT landscape, resulting in increased metastatic spread. It is of interest that this switch
451 even occurs *in vitro* in the absence of a stromal compartment, perhaps suggesting that the PDAC cells
452 themselves have a key direct role in the deposition of fibrotic ECM post RT. Indeed, preliminary
453 secretomic and proteomic data from our group indicated that PDAC cells have an altered ECM
454 secretion profile post RT (data not shown).

455 Finally, we demonstrated the potential of utilizing this pro-metastatic switch in PDAC cells as a
456 therapeutic window through the addition of a small molecule inhibitor of MRCK. Although BDP9066
457 is well tolerated *in vivo*, its short plasma half-life limits its translational potential (35). The
458 development of a more stable iteration of the compound, or potentially the targeting of MRCK
459 through alternative approaches, such as PROTAC, is key to moving this approach towards the clinic.

460 In summary, our data highlight the impact of RT on the metastatic landscape of PDAC and identify a
461 novel, exploitable therapeutic approach to improve outcomes in patients with this cancer of current
462 unmet need in the future.

463

464 **Data Availability**

465 The authors confirm that the data supporting the findings of this study are available within the
466 manuscript and its supplementary materials or are available from the authors on request.

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476

477

478 **Disclosure statement**

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482

483 **Author Contributions**

484 **Conception and design:** KMM, MT, JPM, JLB

485 **Development of methodology:** KMM, MT, MO, JPM, JLB, LMC, RC, JM and LMcG.

486 **Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** KM,
487 MT, LD, RC, DG, SL, JM and YS

488 **Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational**
489 **analysis):** KM, MT, LD, RC, DG, YS, SL, and JM

490 **Writing, review, and/or revision of the manuscript:** JLB, KM, JM, MO, and MT.

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Figure. 1 McLay *et al*

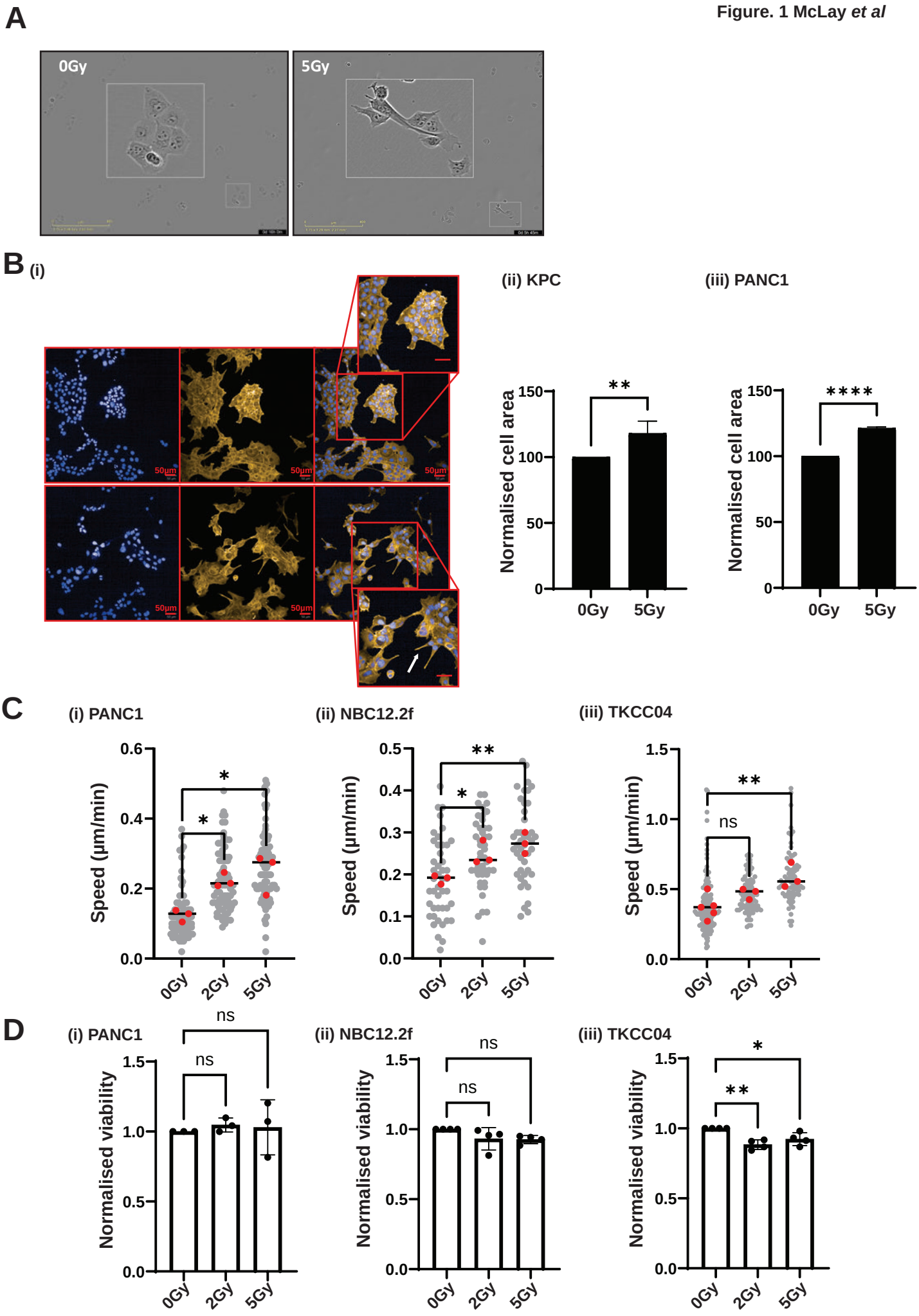


Fig 1.

Radiotherapy induces cell motility across a panel of PDAC cell lines. A) PANC1 cells imaged under phase contrast indicating morphology shift following exposure to 5 Gy RT. **B) (i)** PDAC cells treated with either 0 Gy or 5 Gy RT and imaged via high-throughput imaging. (Blue, DAPI; yellow, actin; last panel, merge) followed by automated analysis of cell area **(ii)** KPC (NBC12.2f) **(iii)** PANC1. Scale bar, 50 µm. Data derived from three independent experiments each containing 4 internal replicates, >1000 cells measured per condition per independent experiment. Error bars = Standard Deviation. Statistical analysis: two-tailed, unpaired *t* test. **, $P < 0.01$; ****, $P < 0.0001$. **C) (i)** PANC1 **(ii)** NBC12.2f (KPC) and **(iii)** TKCC04 cells treated with 0, 2 or 5 Gy RT followed by subconfluent migration assay 24 hrs post RT using time-lapse microscopy and single cell tracking to quantify migration speed. Individual cell speeds plotted in grey, means from independent experiments plotted in red, mean from independent repeats = horizontal bar. Statistical analysis: One-way ANOVA; ns, not significant, *, $P < 0.05$, ** $P < 0.01$. **D)** Viability assays conducted 24hrs post RT on **(i)** PANC1 **(ii)** NBC12.2f and **(iii)** TKCC04. Data from three independent experiments for each cell line. Statistical analysis: One-way ANOVA; ns, not significant, *, $P < 0.05$, ** $P < 0.01$.

Table 1: Migration speed responses to 2 or 5 Gy in a panel of PDAC cell lines.

		2Gy		5Gy	
Cell line	**Origin**	**Δ speed**	**P value**	**Δ speed**	**P value**
PANC1	Commercial	1.822849	0.0027	1.98868	0.0024
Miapaca2	Commercial	1.07396	0.6494	1.019541	0.8727
ASPC1	Commercial	0.879926	0.3841	1.267169	0.0658
NBC12.2f	Murine (KPC)	1.317871	0.0163	1.451858	0.0002
NBC12.2g	Murine (KPC)	1.327689	0.0618	1.044459	0.7157
NBC13.3a	Murine (KPC)	1.692982	<0.0001	2.056329	0.0049
TKC004	Primary patient derived	1.455445	0.0085	1.413566	<0.0001
TKC005	Primary patient derived	1.58574	0.0137	1.558938	0.0099

Table 1.

Migration speed responses to 2 or 5 Gy in a panel of PDAC cell lines.

The migration speed was normalized to 0 Gy to determine the fractional change. Statistical analysis: Student's t-test. Green cells: significant increase in speed post RT, blue cells: trend towards increase, white cells: no increase. Data were obtained from three biological replicates for each cell line.

Figure. 2 McLay *et al*

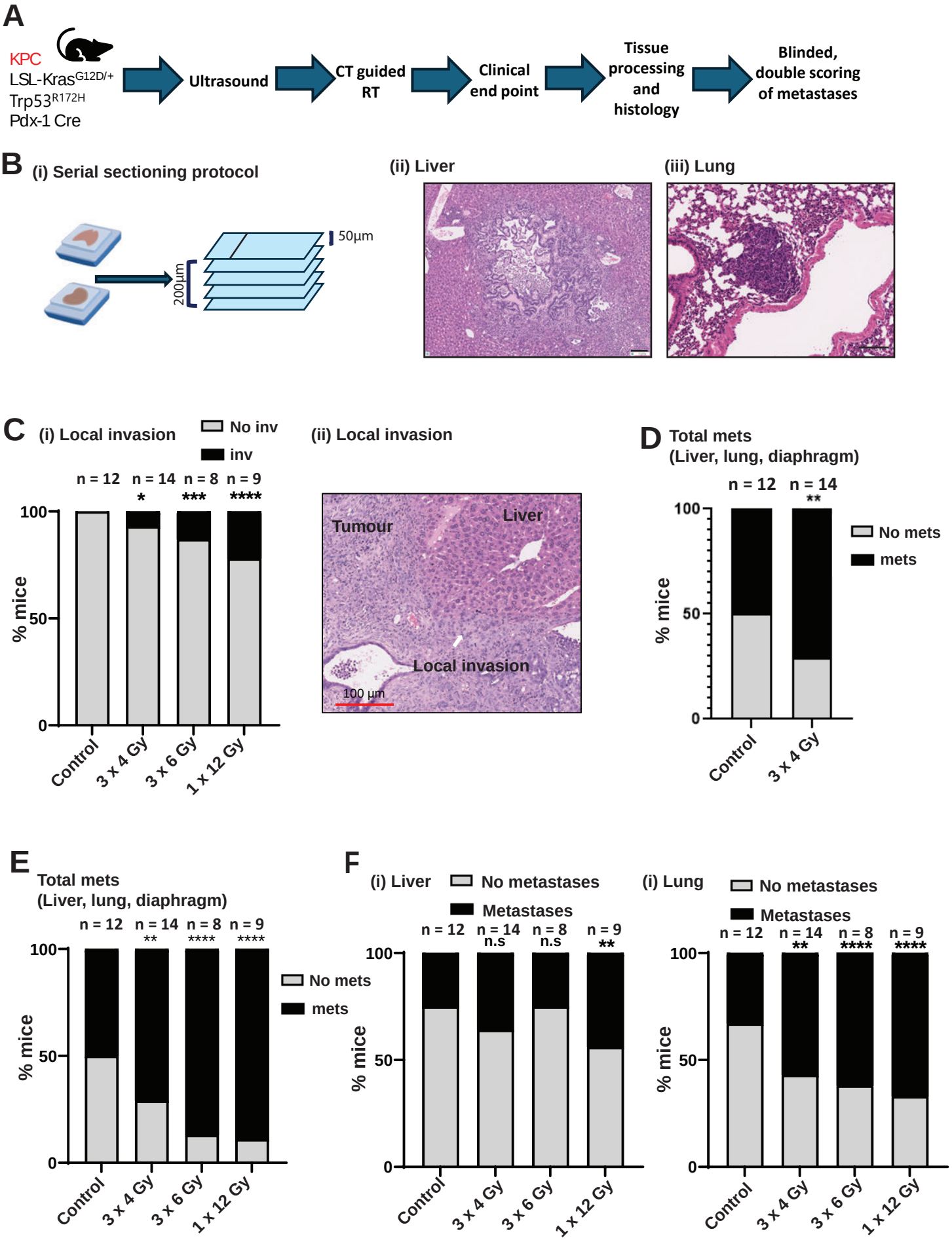
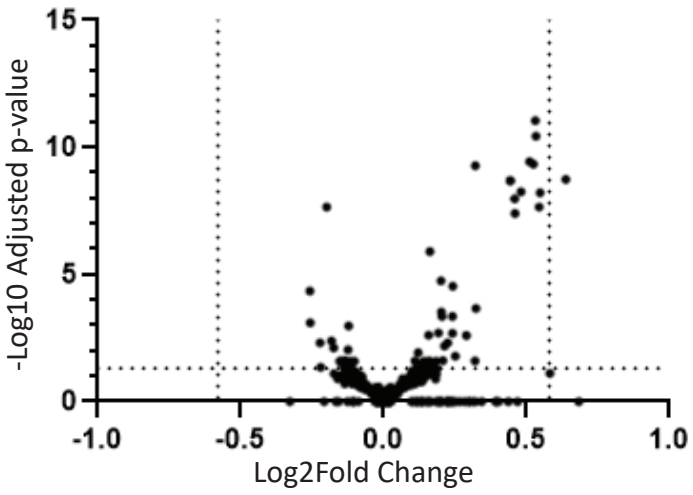


Fig 2.

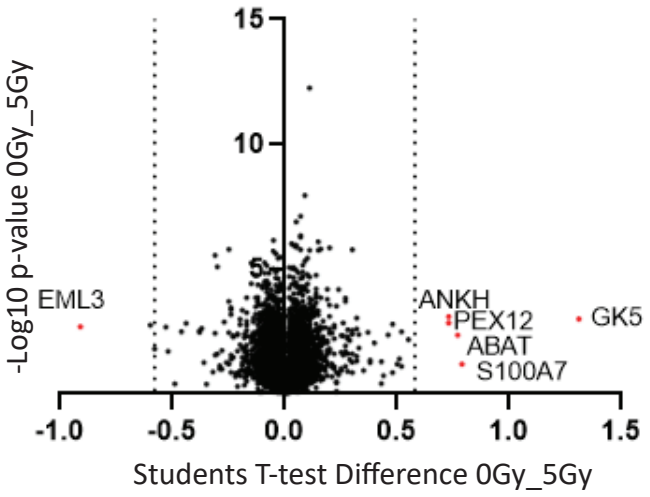
Radiotherapy increases the metastatic burden of pancreatic cancer in a clinically relevant genetic mouse model. A) Tumour bearing KPC mice were subjected to CT-guided targeted RT and sampled at clinical endpoint. Liver and lung sections were blinded and double scored for presence of metastases. **B) (i)** Serial sectioning protocol **(ii)**, **(iii)** example images of liver and lung metastases. **C)** i) H&E sections from KPC mice treated with 0Gy, 3x4Gy, 3x6Gy or 1x12Gy were scored for the presence of local invasion (example in (ii)). **D)** H&E liver, lung, and diaphragm sections from KPC mice treated with 0 or 3x4Gy RT were assessed for presence of metastases. **E)** Metastasis scoring across multiple RT protocols, as indicated. **F)** Breakdown of metastases present in liver **(i)** or lung **(ii)**. Number per cohort indicated on graphs. Statistical analysis: Chi-square test. ns, non-significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ ****, $P < 0.0001$.

Figure 3. McLay *et al*

A



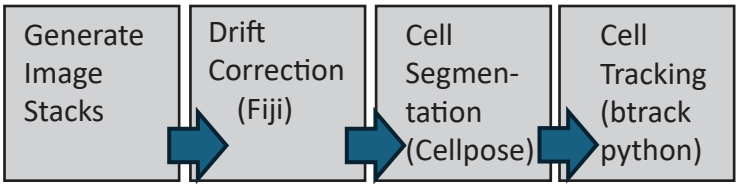
B



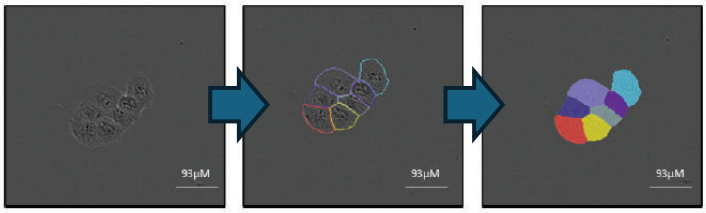
C (i)

	MRCKb	RhoA	ROCK1	GAPDH
NT	1.00	1.00	1.00	1.00
MRCK KD	0.43	0.89	0.76	1.02
RhoA KD	1.17	0.54	0.58	1.01
ROCK1 KD	1.09	0.67	0.56	0.96
GAPDH KD	2.64	1.10	1.73	0.51

(ii)



(iii)



D

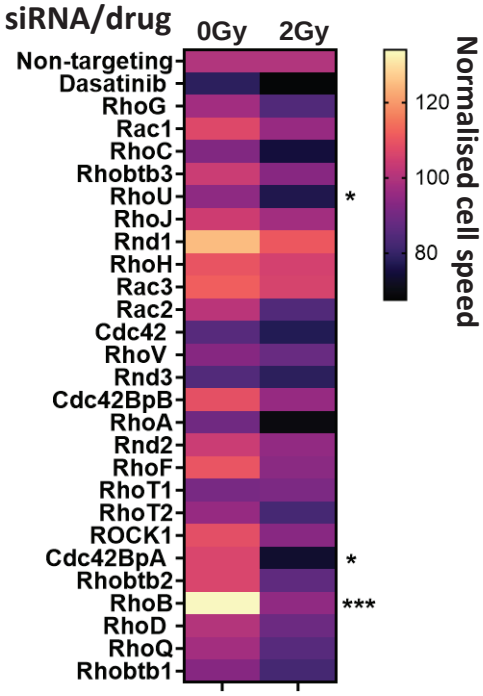


Fig 3.

Dependence of RT induced motility on members of the RhoGTPase signaling family.

A-B) Differential gene expression (DGE) was analysed 24 hrs post treatment with 0 or 5Gy RT in three biological replicates (independently derived KPC lines, NBC12.2f, NBC12.2g and NBC13.3a). The proteome of PANC1 cells was analysed using a tandem mass tag approach 6 hrs post treatment with 0 or 5Gy RT**. C (i)** Efficacy of knockdown was confirmed by western blotting for several targets. Expression normalized to actin loading control and shown as ratio of non-targeted control treated cells (NT). Example blot in Fig. S3. **(ii)** Automated cell tracker workflow. Generation of image stacks and application of drift correction via plugin in Fiji followed by cell segmentation using cellpose 2.0 and tracks cells using btrack python package. **(iii)** representative image of cell segmentation from cellpose 2.0. **D)** RhoGTPase siRNA motility screen targeting all members of the RhoGTPase signaling family and select effector kinases in PANC1 cells exposed to either 0 or 5 Gy RT. Presented as a heatmap where each cell speed is normalised to non-targeting control siRNA from the match condition (0 or 5Gy). Statistical analysis: Two-way ANOVA. *, $P < 0.05$, ***, $P < 0.001$. Statistical analysis: two-tailed, unpaired *t* test. ns, non-significant, *, $P < 0.05$, **, $P < 0.01$; ****, $P < 0.0001$.

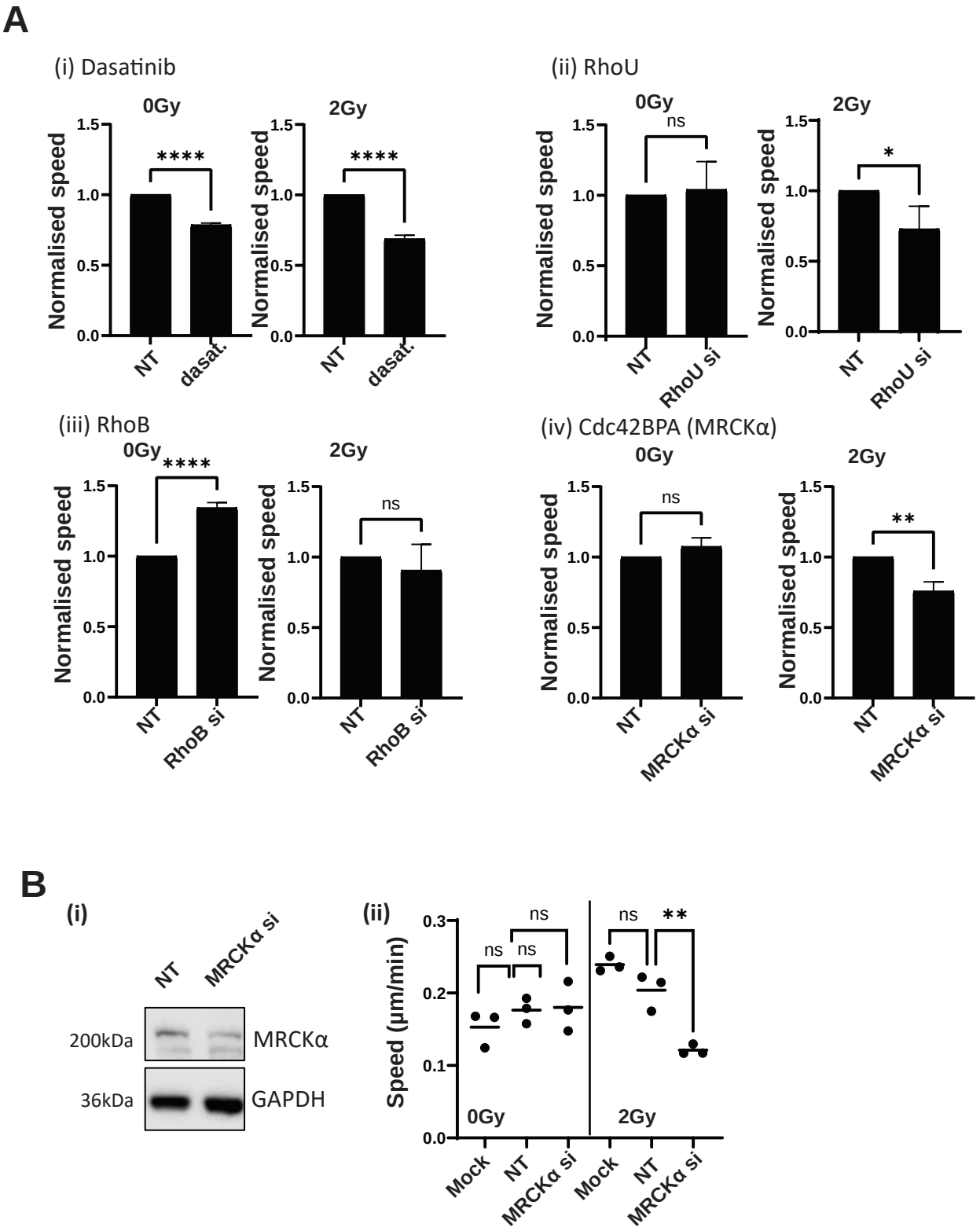

A
(i) Dasatinib
0Gy
2Gy
Normalised speed
1.5
1.0
0.5
0.0
****
NT
dasat.
(ii) RhoU
ns
*
RhoU si
(iii) RhoB
RhoB si
(iv) Cdc42BPA (MRCKα)
**
MRCKα si
B
(i)
NT
MRCKα si
200kDa
MRCKα
36kDa
GAPDH
(ii)
Speed (µm/min)
0.3
0.2
0.1
0.0
ns
**
0Gy
2Gy
Mock
NT
MRCKα si

Fig 4.

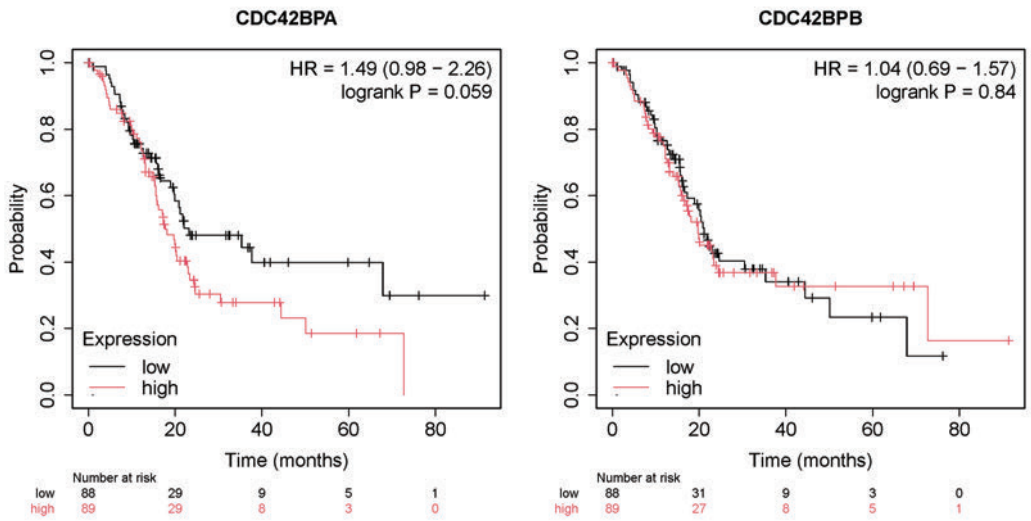
RT-induced motility is dependent on MRCK and RhoU

A) Normalised cell speeds of identified hits from the RhoGTPase siRNA screen. **(i)** Dasatinib (positive control) **(ii)** RhoU **(iii)** RhoB and **(iv)** Cdc42BPA (MRCKα). All data from 3 independent experiments with >50 cells tracked per condition per experiment. **B)** PANC1 cells transfected with an alternate siRNA targeting MRCKα followed by **(i)** Confirmation of MRCKα knockdown via western blotting and **(ii)** subconfluent migration assay. N=3 independent repeats, horizontal bar = mean. Statistical analysis: one-way ANOVA. ns, non-significant, **, $P < 0.01$.

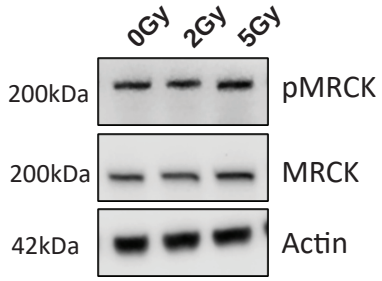
Figure. 5 McLay *et al*

A

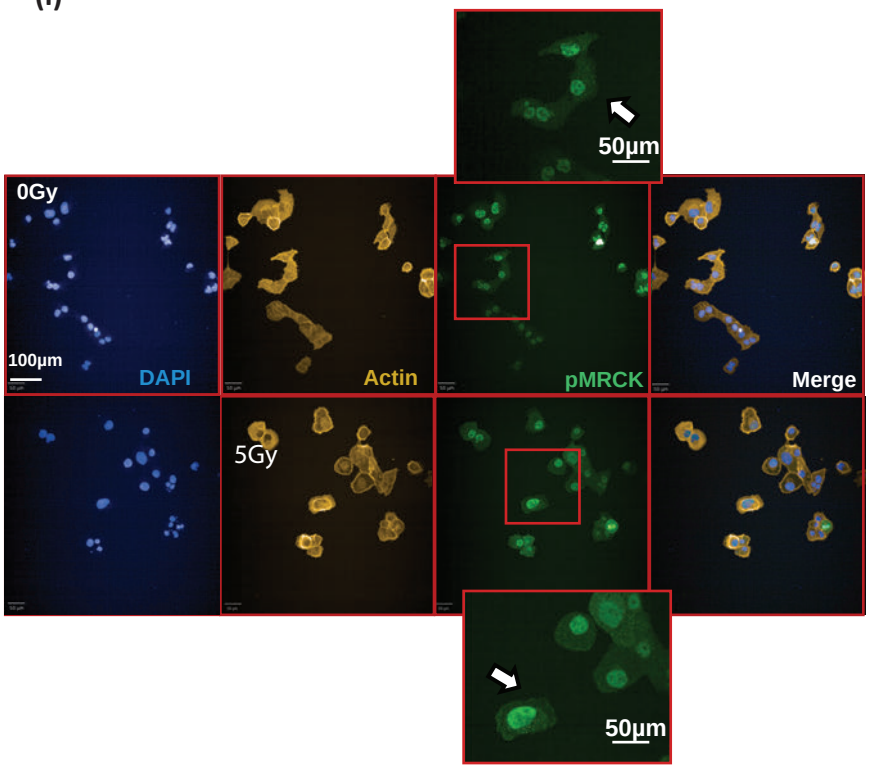
(i) Cdc42BPA (MRCKα) (ii) Cdc42BPB (MRCKβ)



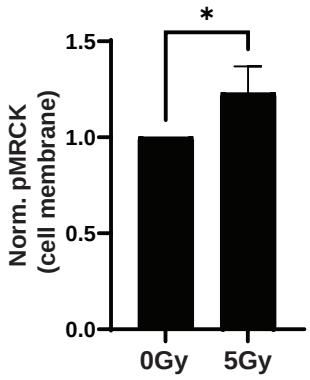
B (i)



C (i)



(ii) PANC1



(iii) KPC

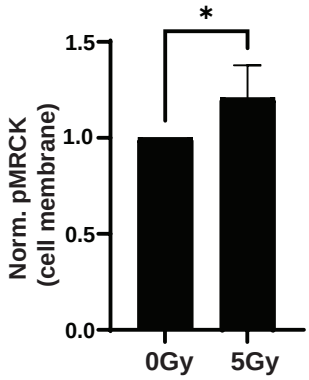
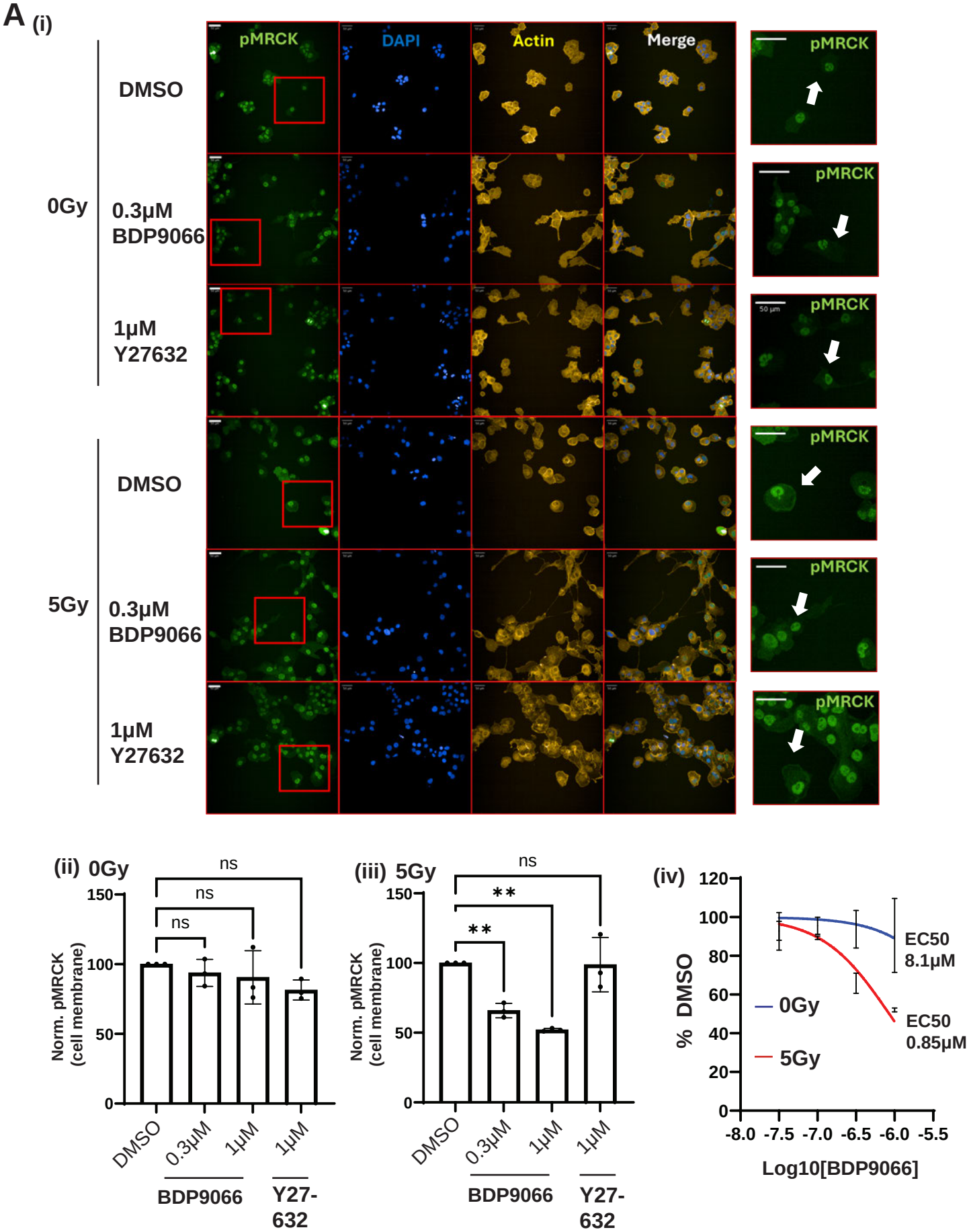


Fig. 5.
MRCK signaling is spatially regulated by RT *in vitro*

A) CDC42BPA (i) and CDC42BPB (ii) mRNA expression and overall survival from available patient datasets plotted using kmplot.com. 177 samples with a median expression cut off. **B)** PANC1 cells treated with 0, 2Gy or 5Gy followed by western blotting of pMRCK total MRCK and actin (loading control). **C) (i)** PANC1 cells treated with either 0 Gy or 5 Gy RT followed by IF for pMRCK and high throughput confocal imaging. Blue, DAPI; yellow, actin; green, pMRCK; last panel, merge. Scale bar, 100 µm. (ii) automated image analysis of membrane associated pMRCK in **(ii)** PANC1 **(iii)** KPC (NBC12.2f) cells exposed to 0 or 2 Gy. All data from 3 independent repeats. Statistical analysis, Students T Test. * $P < 0.05$.

Figure. 6 McLay *et al*



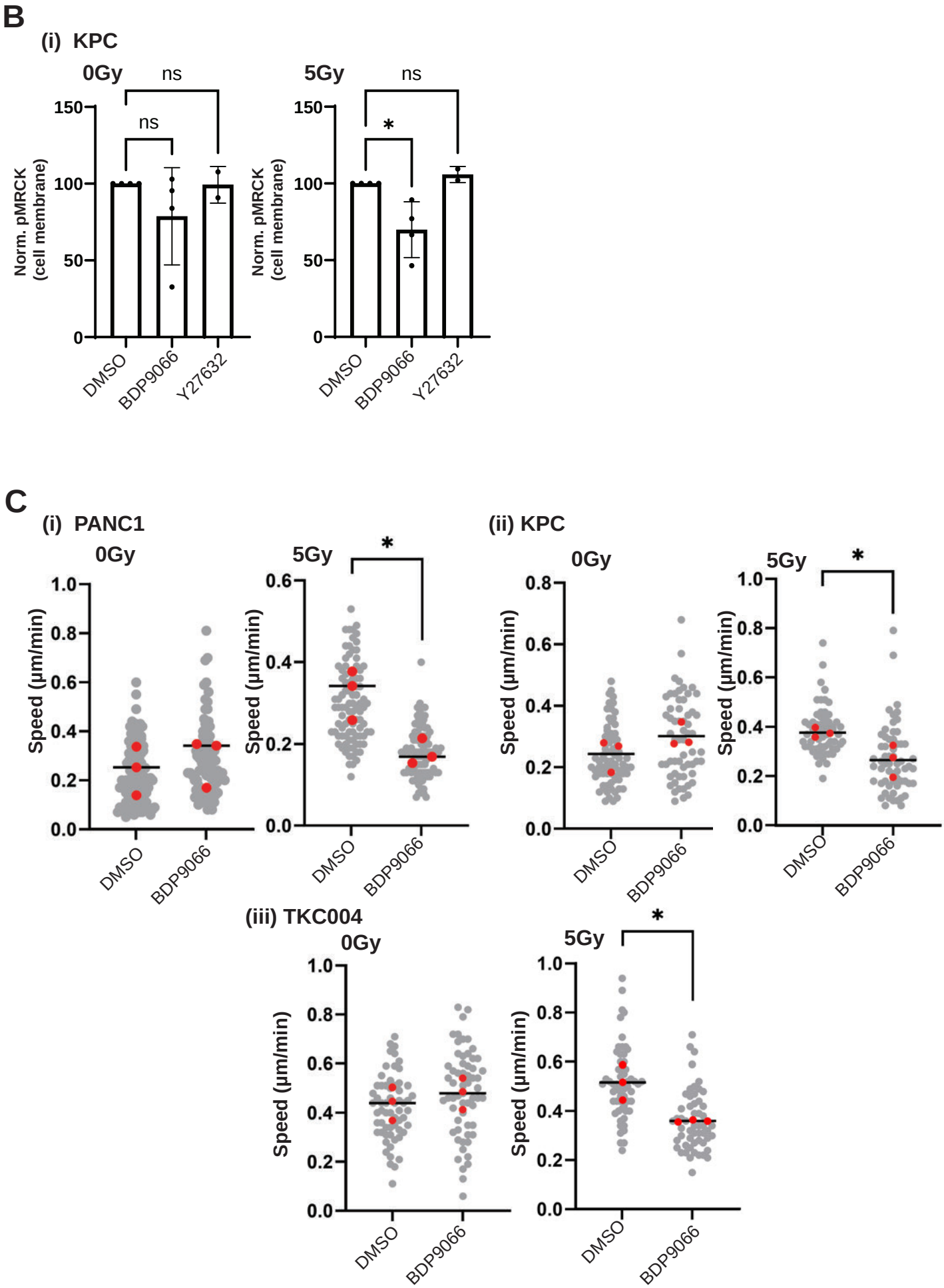

B
(i) KPC
0Gy
ns
ns
5Gy
ns
*
Norm. pMRCK (cell membrane)
150
100
50
0
DMSO
BDP9066
Y27632
C
(i) PANC1
0Gy
5Gy
*
(ii) KPC
0Gy
5Gy
*
(iii) TKC004
0Gy
5Gy
*
Speed (µm/min)
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
BDP9066

Fig 6.

Pharmacological targeting of MRCK opposes radiation driven migration

A) (i) PANC1 cells treated with either 0 Gy or 5 Gy +/- DMSO, BDP9066 or Y27632 followed by high-throughput imaging. Blue, DAPI; yellow, actin; green, pMRCK; last panel, merge. Scale bar, 100 µm. White arrows indicate cell membrane. (ii)-(iii) Automated analysis of membrane associated pMRCK from (i). (iv) Dose response curve in the presence/absence of RT (5Gy). All data from 3 independent repeats. Statistical analysis: one-way ANOVA. ns, non-significant, **, $P < 0.01$. **B)** Automated analysis of membrane associated pMRCK in KPC (NBC12.2f) cells. Statistical analysis: one-way ANOVA. ns, non-significant, *, $P < 0.05$. Data from 3 independent experiments, error bars = standard deviation. **C)** Subconfluent migration assay using (i) PANC1, (ii) KPC and (iii) TKC004 cells to measure the response to BDP9066 in the presence/absence of RT (5Gy). 0Gy and 5 Gy plotted separately due to experiment design. All data from 3 independent repeats. Statistical analysis: two-tailed, unpaired *t* test. *, $P < 0.05$.

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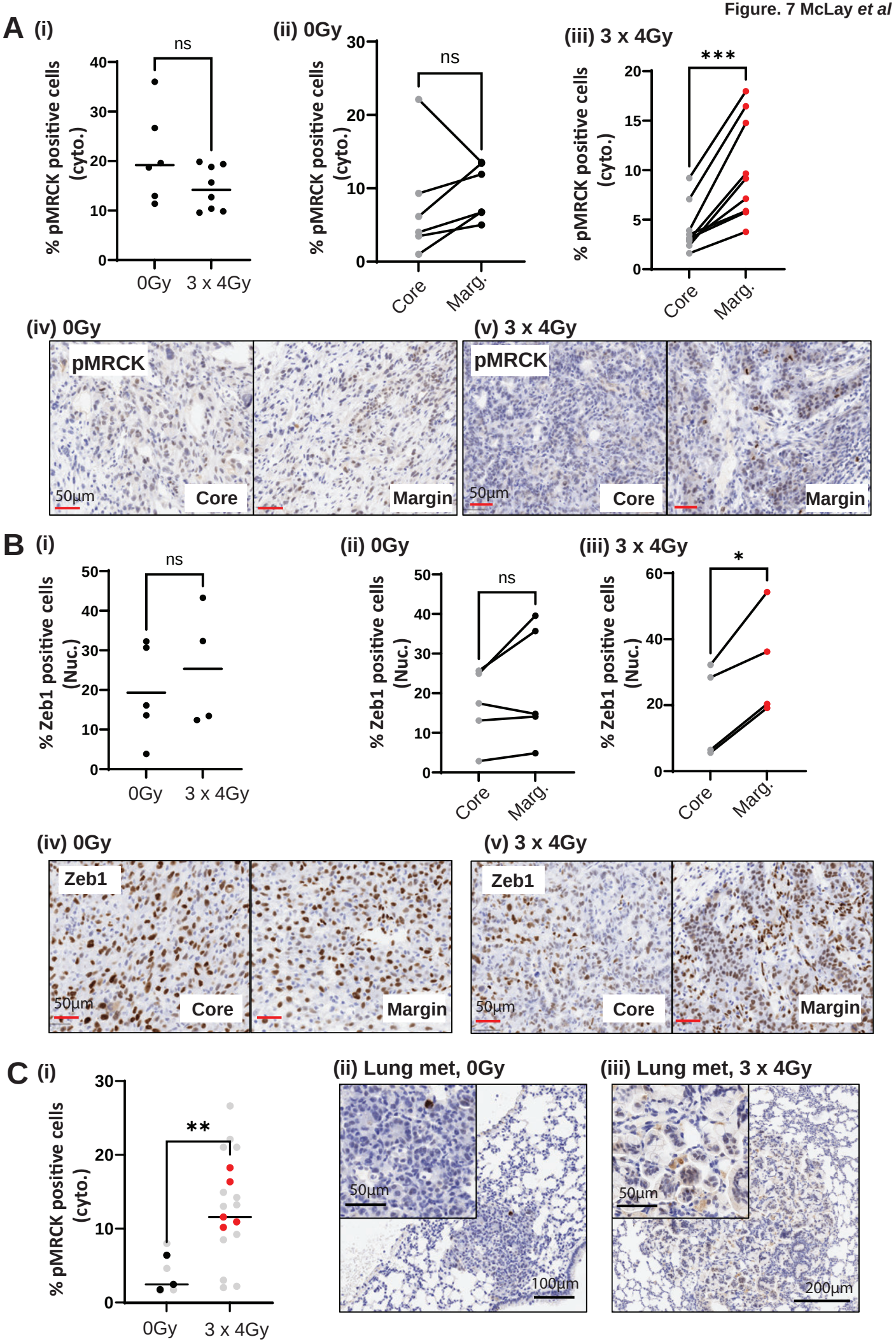
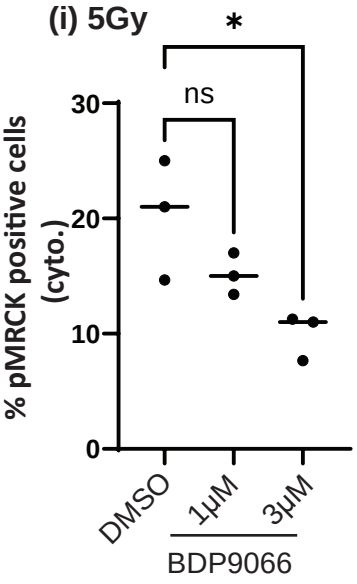


D


(i) 5Gy
*
ns
% pMRCK positive cells (cyto.)
30
20
10
0
DMSO
1μM
3μM
BDP9066

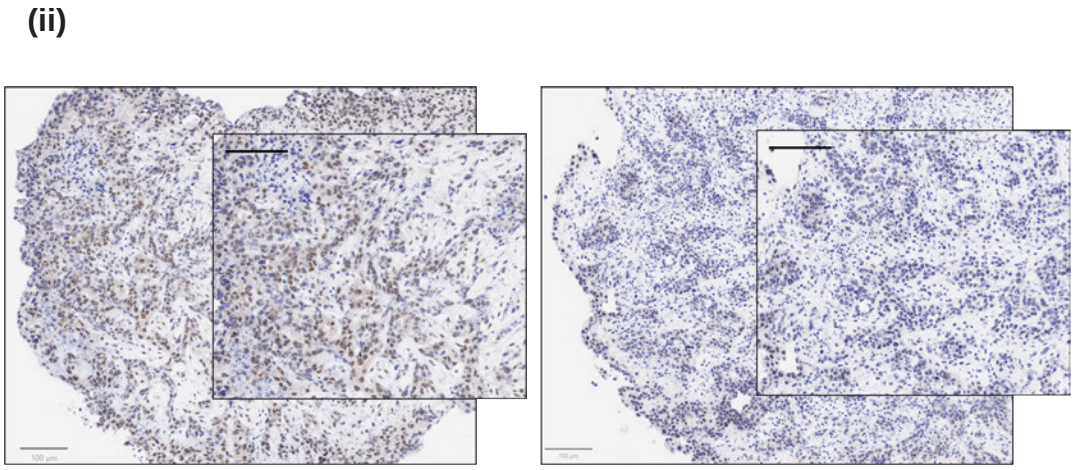

(ii)

Fig 7.

MRCK activity is upregulated at invasive tumour margins post radiotherapy *in vivo*

A) (i) Percentage of cytoplasmic pMRCK positive cells in KPC tumours treated with either 0 Gy or 3 x 4 Gy, n=6 and 8 mice, respectively. (ii, iii) Percentage of cytoplasmic pMRCK positive cells in the core v margin of KPC tumours treated with 0 Gy (ii) or 3 x 4Gy (iii). Means from individual tumours plotted. (iv, v) Example images from (ii)-(iii). Statistical analysis: two-tailed, unpaired *t* test. ns, non-significant, ***, $P < 0.001$. **B)** (i) Percentage of nuclear ZEB1 positive cells in KPC tumours treated with either 0 Gy or 3 x 4 Gy. (ii, iii) Percentage of nuclear Zeb1 positive cells in the core v margin of KPC tumours treated with 0 Gy (ii) or 3 x 4Gy (iii). (iv, v) Example images from (ii)-(iii). Means from individual mice plotted, n=5 0Gy, n=4 3x4Gy. Statistical analysis: two-tailed, unpaired *t* test. ns, non-significant, *, $P < 0.05$. **C)** (i) Percentage of cytoplasmic pMRCK positive cells in liver and lung metastases from KPC mice treated with 0 Gy or 3 x 4 Gy. Individual metastases from mice plotted in grey. Average per mouse in black/red. Statistical analysis: two-tailed, unpaired *t* test. *, $P < 0.05$. (ii, iii) Example images from (i). **D)** (i) Percentage of pMRCK positive cells in sections of 300µm slices from KPC tumours cultured *ex vivo* treated with 0 or 5Gy and DMSO or BDP9066, n=3 mice per group. Statistical analysis: one way ANOVA, ns, non-significant, *, $P < 0.05$. (ii) Example images from (i).