

## Contextual Synthetic Lethality of Cancer Cell Kill Based on the Tumor Microenvironment

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### Abstract

Acute and chronic hypoxia exists within the three-dimensional microenvironment of solid tumors and drives therapy resistance, genetic instability, and metastasis. Replicating cells exposed to either severe acute hypoxia (16 hours with 0.02% O<sub>2</sub>) followed by reoxygenation or moderate chronic hypoxia (72 hours with 0.2% O<sub>2</sub>) treatments have decreased homologous recombination (HR) protein expression and function. As HR defects are synthetically lethal with poly(ADP-ribose) polymerase 1 (PARP1) inhibition, we evaluated the sensitivity of repair-defective hypoxic cells to PARP inhibition. Although PARP inhibition itself did not affect HR expression or function, we observed increased clonogenic killing in HR-deficient hypoxic cells following chemical inhibition of PARP1. This effect was partially reversible by RAD51 overexpression. PARP1<sup>-/-</sup> murine embryonic fibroblasts (MEF) showed a proliferative disadvantage under hypoxic gassing when compared with PARP1<sup>+/+</sup> MEFs. PARP-inhibited hypoxic cells accumulated  $\gamma$ H2AX and 53BP1 foci as a consequence of altered DNA replication firing during S phase-specific cell killing. In support of this proposed mode of action, PARP inhibitor-treated xenografts displayed increased  $\gamma$ H2AX and cleaved caspase-3 expression in RAD51-deficient hypoxic subregions *in vivo*, which was associated with decreased *ex vivo* clonogenic survival following experimental radiotherapy. This is the first report of selective cell killing of HR-defective hypoxic cells *in vivo* as a consequence of microenvironment-mediated "contextual synthetic lethality." As all solid tumors contain aggressive hypoxic cells, this may broaden the clinical utility of PARP and DNA repair inhibition, either alone or in combination with radiotherapy and chemotherapy, even in tumor cells lacking synthetically lethal, genetic mutations. *Cancer Res*; 70(20); 8045-54.

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### Introduction

Solid tumors contain subregions of dynamic O<sub>2</sub> gradients, which can be described as acute and chronic hypoxia. Severe acute hypoxia (and subsequent reoxygenation) results from temporary changes in blood perfusion. Chronic hypoxia

arises in regions distant from the blood vasculature as a function of decreased oxygen diffusion and leads to gradients of moderate to severe hypoxia.

Intratumoral hypoxia is an adverse clinical prognostic factor associated with decreased disease-free survival for many solid cancers (1-5). Hypoxia can increase resistance to both radiotherapy and chemotherapy (6). Additionally, hypoxia can increase metastatic spread and decrease DNA repair (7-9).

Severe hypoxia can lead to an S-phase arrest in the absence of DNA damage, whereas subsequent reoxygenation increases reactive oxygen species (ROS) production and DNA damage to trigger a CHK2-dependent G<sub>2</sub> arrest (10-14). Alternatively, tumor cells can adapt to moderate chronic hypoxia with minimal changes in proliferation, cell cycle distribution, or clonogenic survival, suggesting a bypass of cell cycle checkpoints (15, 16). In both types of hypoxia, the homologous recombination (HR) pathway involved in the repair of DNA double-strand breaks (DSB) is compromised (15-17). Recent studies have shown that HR defects (e.g., *BRC1/2* deficient) are synthetically lethal with inhibition of the DNA single-strand break (SSB) repair protein, poly(ADP-ribose) polymerase 1 (PARP1; refs. 18-20). In response to DNA breaks, PARP1 catalyzes the addition of poly(ADP-ribose) (PAR) polymers from NAD<sup>+</sup> onto nuclear acceptor proteins, including histones,

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p53, and PARP1 itself, to attract repair proteins and change chromatin conformation (21). Inhibition of PARP1 results in the accumulation of SSBs, which are converted to DSBs when encountered by a replication fork. These collapsed replication forks require HR for repair and continued DNA replication (22). As *BRCA1/2*-deficient cells are HR deficient and incapable of repairing DNA replication-associated DSBs, treatment of these tumors with PARP inhibitors leads to synthetic lethality (18).

At present, the use of PARP inhibitors in the clinic is limited to trials in patients with genetic defects in *BRCA1/2* (23). Given the existence of HR-defective hypoxic cells, we hypothesized that these cells might also be sensitized to PARP inhibition. To investigate this hypothesis, we studied both acutely and chronically hypoxic tumor cell cultures and hypoxic subregions of tumor xenografts and show that repair-defective hypoxic cells are sensitized to PARP inhibition (a consequence of "contextual synthetic lethality"). As all solid tumors contain hypoxic cells, our observations may increase the clinical utility of PARP (or other) inhibitors in combination with radiotherapy or chemotherapy to target treatment-resistant hypoxic tumor cells.

## Materials and Methods

### Cell culture, hypoxia treatments, and PARP inhibitors

The origin and culture of HCT116, 22RV1, DU145, MCF-7, PC3, and H1299 cell lines have been reported previously (13, 15, 16). Immortalized murine embryonic fibroblasts (MEF) wild-type or deficient for PARP1 or hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) were derived from day 13.5 embryos; derivation, culture, and characteristics were as previously described (24, 25).

Logarithmically growing cells were exposed to 0.2% O<sub>2</sub> with 5% CO<sub>2</sub> and balanced N<sub>2</sub> using an Invivo2 400 Hypoxic Workstation (Ruskin). To achieve lower oxygen levels, cells were plated on glass dishes and incubated in a Bactron II anaerobic chamber (Shell Labs) at <0.02% O<sub>2</sub>.

ABT-888 was obtained from Abbott Laboratories through the NIH Cancer Therapy Evaluation Program (CTEP) and reconstituted in water. 4-Amino-1,8-naphthalimide (ANI) was purchased from Acros Organics and reconstituted in DMSO. ABT-888 and ANI were used at concentrations of 2.5 and 10  $\mu$ mol/L, respectively, unless otherwise stated. Enzymatic PARP activity was assessed using the Universal Chemiluminescent PARP assay kit (Trevigen) as previously described (26).

Synchronized cell populations were generated by the G<sub>2</sub> mitotic shake-off technique and confirmed with flow cytometry. Clonogenic assays were performed to determine cell viability as previously described (15).

### Western blotting, small interfering RNA treatments, and microscopy

Western blot analysis was performed as previously described (13, 15). Primary antibodies included ACTIN (Sigma-Aldrich, Inc.), PAR and PARP1 (Trevigen), HIF-1 $\alpha$  (BD Transduction Laboratories), and RAD51 (Santa Cruz). RAD51 small interfering RNA (siRNA) were obtained from

Invitrogen and used at a concentration of 0.25 nmol/L for 24 hours with Lipofectamine 2000 (Invitrogen). Immunofluorescent microscopy was carried out as previously described (13, 26). The primary antibodies included RAD51 (Santa Cruz),  $\gamma$ H2AX (Epitomics), PAR (Trevigen), and 53BP1 (Novus Biologicals).

### Direct repeat-green fluorescent protein HR assay

The direct repeat-green fluorescent protein (DR-GFP) assay was used to evaluate HR as previously described (15). Briefly, H1299 cells containing the DR-GFP construct were transfected with a vector encoding for the I-SceI endonuclease to generate a DSB. Flow cytometry was used to detect GFP-positive cells that have undergone HR.

### Human xenograft assays

A 200- $\mu$ L solution containing  $2 \times 10^6$  HCT116, 22RV1, or RKO cells were injected s.c. into the hind flank of CD1 nude mice (Charles River). Tumors were grown to a volume of 250 mm<sup>3</sup>, and tumor-bearing mice were given an i.p. injection with 30 mg/kg EF5 (Varian) 3 hours before sacrifice. Tumors were excised and fixed in 10% formalin, paraffin embedded, and sectioned to 4- $\mu$ m thickness. For ABT-888 treatments, RKO xenografts were treated twice daily with 50 mg/kg ABT-888 or vehicle for 5 days. Tumors were excised 2 hours after the final ABT-888 treatment and prepared for immunohistochemical staining for  $\gamma$ H2AX (Epitomics), RAD51 (Santa Cruz), and cleaved caspase-3 (CC3; Cell Signaling), as previously described (17).

### Normal gut epithelium toxicity assay

Normal tissue toxicity was determined by measuring intestinal clonogenic survival *in vivo*. Tumor-bearing mice were treated with 5 days ABT-888 or vehicle as described above. Where indicated, whole-body irradiation with 5 Gy (Gamma-cell 40 Extractor) was given 24 hours after the final ABT-888 dose. Three days after radiation, the small intestines were removed, washed, and fixed in formalin. Gut cross sections were stained with Ki-67 (brown) and hematoxylin (blue, nuclear counterstain). Analysis was based on five cross sections per mouse for three mice per treatment group.

### DNA fiber assay

DNA fiber spreads were obtained, as previously described (27), with the following modifications. Aerobic samples were sequentially labeled with 25  $\mu$ mol/L CldU and 250  $\mu$ mol/L IdU (30-minute pulses). For hypoxic samples, CldU-containing medium was added to cells immediately before hypoxic treatment and incubated for 5 hours, after which the medium was replaced with hypoxic equilibrated IdU-containing medium for 1 hour. Reoxygenation samples were treated with CldU, as in the hypoxic samples, and incubated for 6 hours before reoxygenation, at which point the medium was replaced with aerobic IdU-containing medium and further incubated for 1 hour. Fiber spreads were imaged using a Bio-Rad Radiance confocal microscope and analyzed using ImageJ software (NIH). At least 100 replication tracks were measured, and 200 replication structures were counted per experiment.

## Statistics

Results are presented as the mean  $\pm$  SEM with significance calculated by Student's *t* test with a standard software package (GraphPad Prism). Significance was assigned for a *P* value of  $<0.05$ .

## Results

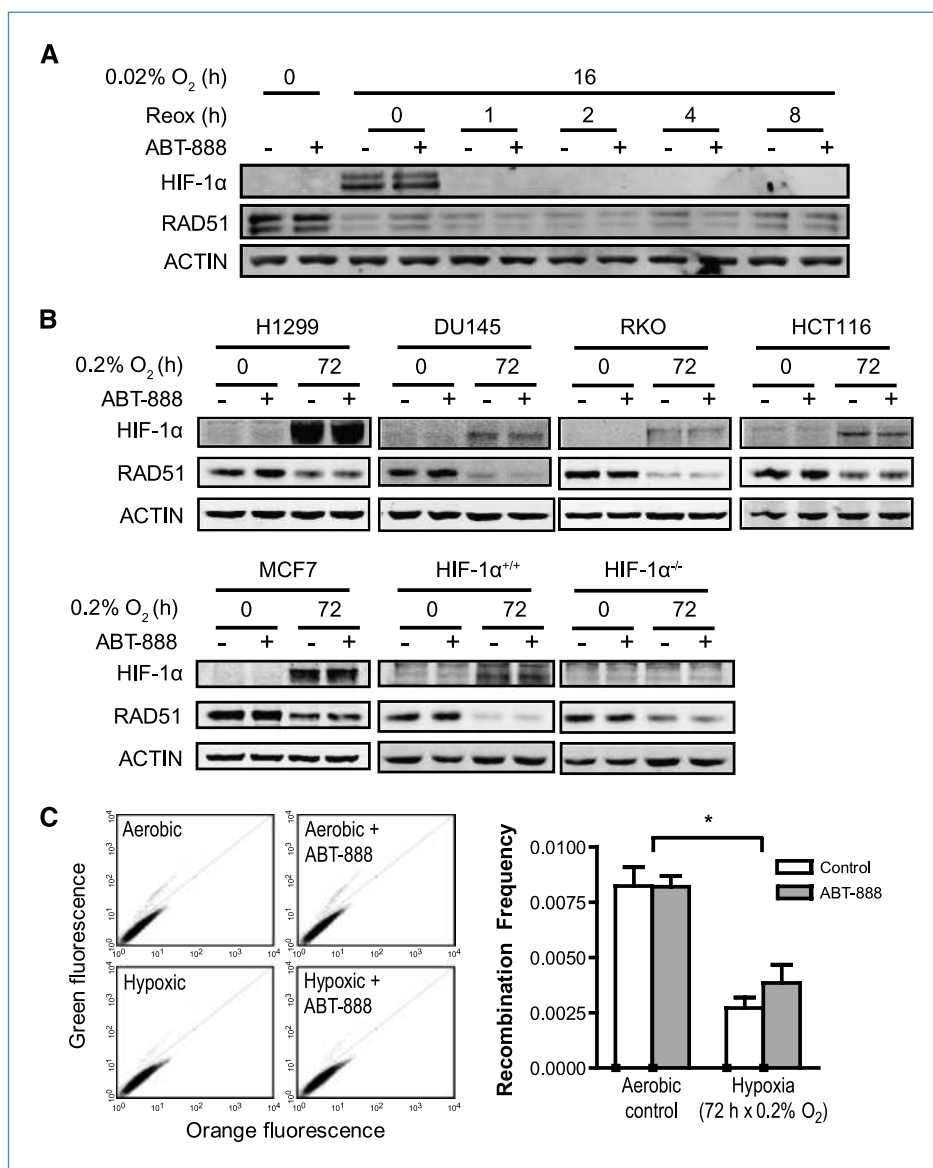
### Hypoxia decreases HR independent of PARP

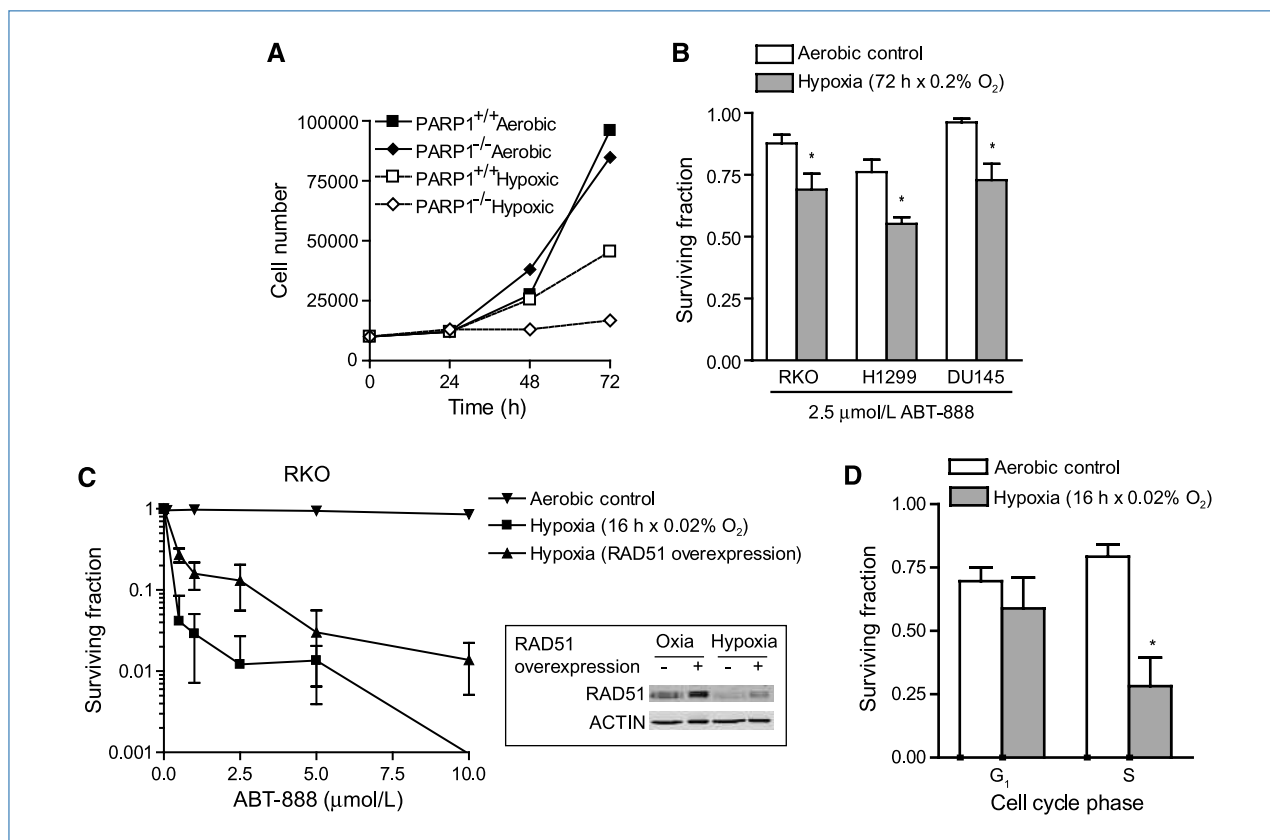
Hypoxia can decrease the expression of a number of HR proteins, including RAD51, RAD51C, XRCC3, RAD52, RAD54, BRCA1, and BRCA2 (15, 17). To explore the relationship between hypoxia, altered HR protein expression, and PARP activity *in vitro*, we assessed the expression of RAD51 in PARP-inhibited cells under aerobic or hypoxic conditions. RAD51 expression was downregulated after treatment with

severe acute hypoxia (16 hours  $\times$  0.02% O<sub>2</sub>), mimicking hypoxic conditions caused by reversible changes in tumor vessel perfusion (Fig. 1A). RAD51 levels remained suppressed for at least 8 hours after reoxygenation. At longer time points, we also observed decreased RAD51 expression across multiple cell lines (H1299, DU145, RKO, MCF7, HCT116, and MEFs), even at less severe oxygen levels (72 hours  $\times$  0.2% O<sub>2</sub>), mimicking hypoxic conditions found distant from the tumor vasculature (Fig. 1B). This RAD51 effect was observed at O<sub>2</sub> concentrations of  $<0.5\%$  (Supplementary Fig. S1A) and is consistent with our previous report of hypoxia-mediated decreases in the translation of HR proteins (15). Additionally, this is the first report using a genetic knockout model to show that this effect is independent of HIF-1 $\alpha$  status (Fig. 1B).

A similar phenomenon is observed *in vivo* as RAD51 expression is inversely correlated with hypoxia (EF5 staining)

**Figure 1.** Hypoxia decreases HR independent of PARP activity. A, RAD51 protein expression is decreased in RKO cells following 16 h  $\times$  0.02% O<sub>2</sub> and remains suppressed for at least after 8 h of reoxygenation with or without ABT-888. B, RAD51 protein expression is decreased in H1299, DU145, RKO, HCT116, and MCF7 cancer cells as well as HIF-1 $\alpha$  isogenic MEFs following 72 h  $\times$  0.2% O<sub>2</sub> with or without ABT-888. C, functional HR (as assessed by the DR-GFP assay) is decreased following 72 h  $\times$  0.2% O<sub>2</sub> but is unaffected by ABT-888 in H1299 cells. Representative dot plots. Columns, mean of three experiments; bars, SEM; \*, *P*  $< 0.05$ .





**Figure 2.** PARP suppression kills HR-defective hypoxic cancer cells in S phase. A, PARP1-deficient MEFs proliferate slower under hypoxic (0.2% O<sub>2</sub>) conditions than their isogenic counterparts. B, pharmacologic inhibition of PARP1-sensitized RKO, H1299, and DU145 cells when treated with 72 h × 0.2% O<sub>2</sub>. C, pharmacologic inhibition of PARP1-sensitized RKO cells when treated with 16 h × 0.02% O<sub>2</sub>. Partial rescue is achieved by overexpression of RAD51. D, hypoxic S-phase RKO cells generated by mitotic shake-off were more sensitive than G<sub>1</sub> phase cells to PARP inhibition. Points and columns, mean of three to five experiments; bars, SEM; \*, *P* < 0.05.

in multiple xenograft models (Supplementary Fig. S1B–D). These conditions were also sufficient to decrease functional HR as assessed by the DR-GFP HR reporter assay (Fig. 1C). However, in contrast to a recent report (28), PARP inhibition itself did not alter RAD51 expression or HR function under either aerobic or hypoxic conditions (Fig. 1A–C; Supplementary Fig. S2). We conclude that hypoxia leads to defective HR function and that this is independent of PARP activity.

#### PARP suppression kills HR-defective hypoxic cancer cells in S phase

As cells with genetic defects in HR proteins such as BRCA1/2 are exquisitely sensitive to PARP inhibition due to genetic synthetic lethality (18, 19), we assessed whether HR-defective hypoxic cells are also sensitive to PARP inhibition to illustrate the concept of “contextual” synthetic lethality due to the tumor microenvironment. We observed that PARP1<sup>-/-</sup> MEFs had a profound proliferation defect under hypoxic conditions compared with matched PARP1<sup>+/+</sup> MEFs (Fig. 2A), indicating an inability of PARP-deficient cells to adapt to hypoxic conditions. As an important translational end point, we tested PARP inhibitors as po-

tential sensitizers of HR-deficient hypoxic cells. Proliferating cells gassed under conditions of moderate chronic hypoxia, which led to suppressed HR, had decreased clonogenic survival when treated with PARP inhibitors across multiple tumor cell types (Fig. 2B; Supplementary Fig. S3A). Similarly, siRNA knockdown of RAD51 expression to levels observed under hypoxic conditions also resulted in increased sensitivity to PARP inhibition (Supplementary Fig. S3B). A more profound sensitization was observed when cells were treated with PARP inhibitors under severe acute hypoxia followed by reoxygenation (Fig. 2C). The increased clonogenic cell kill may be due to synergy between PARP inhibition and oxidative damage caused by ROS generated on reoxygenation from severe hypoxia or anoxia (11). To understand the role of RAD51 in this phenotype, we overexpressed RAD51 in hypoxic cells and observed partial rescue of cellular lethality (Fig. 2C). Complete rescue is probably not achieved due to suppression of multiple members of the HR pathway by hypoxia, in addition to RAD51 (15).

Given the role of PARP1 in preventing the collapse of replication forks into replication-associated DSBs, we hypothesized that PARP inhibition is toxic to hypoxic cells

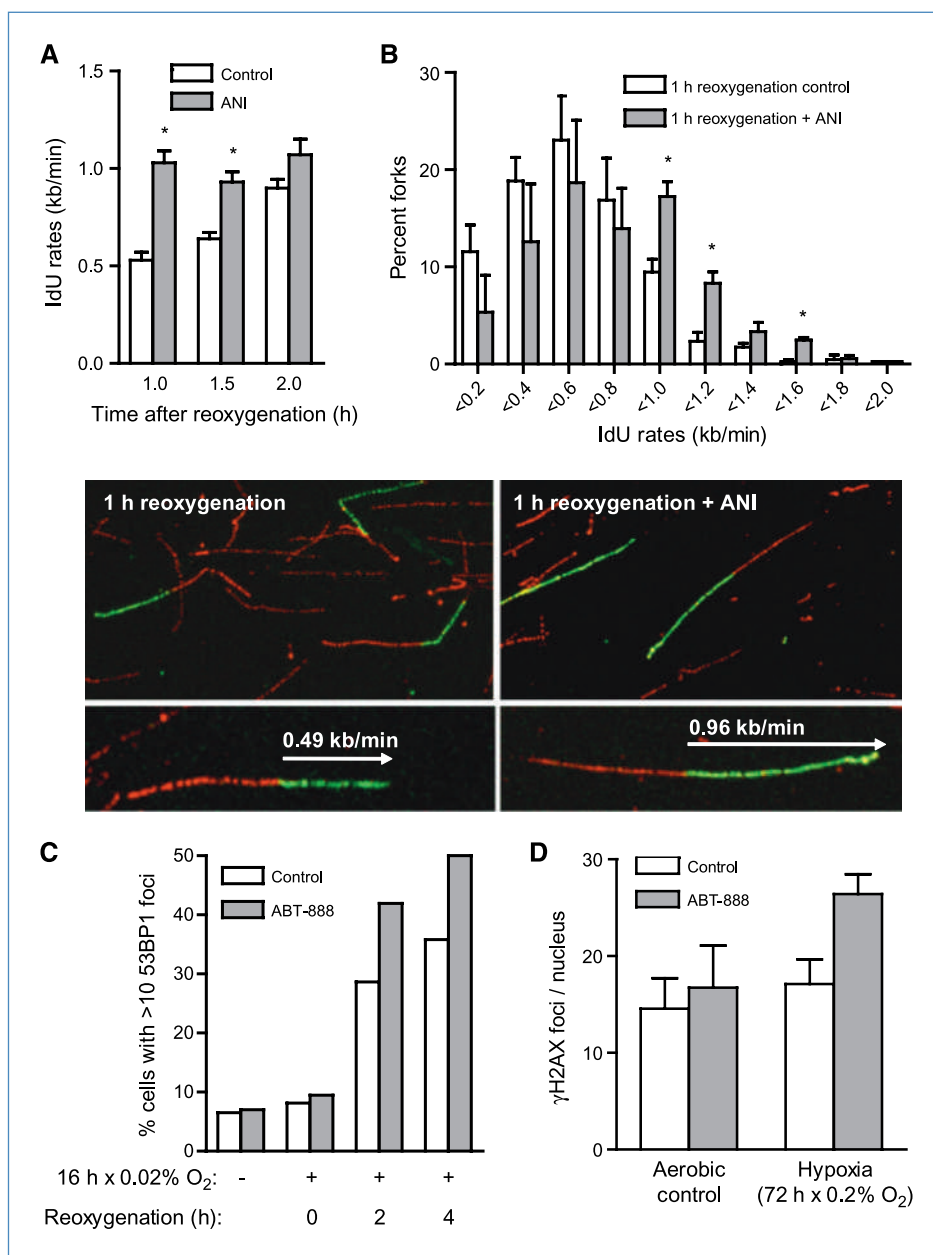
in a cell-cycle specific manner. Using synchronized cell populations, we observed that hypoxic cells in S phase, but not G<sub>1</sub> phase, were preferentially sensitized to PARP inhibition when compared with aerobic cells (Fig. 2D).

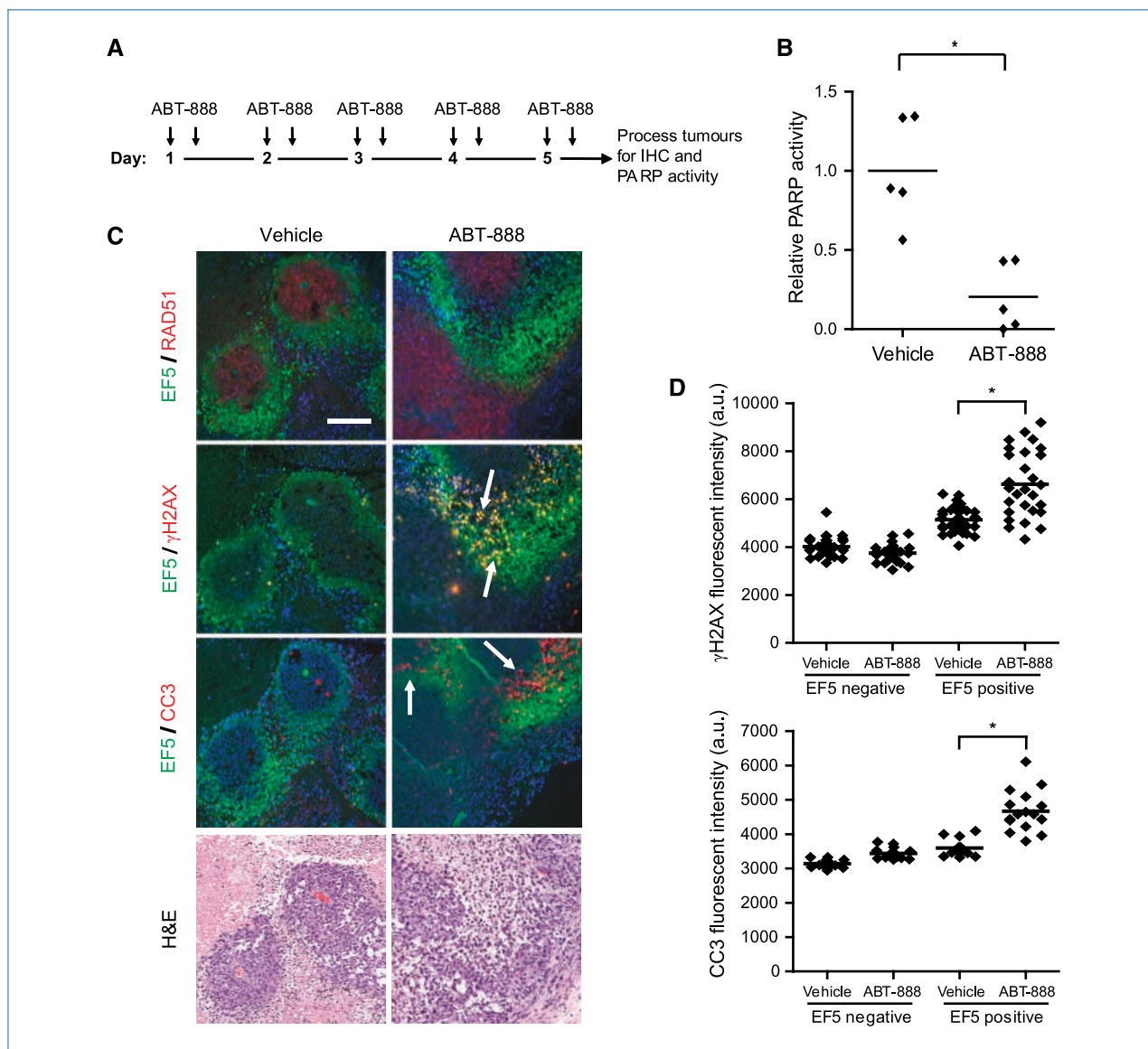
### PARP inhibition of hypoxic cells induces DNA damage in proliferating cells during reoxygenation or chronic adaptation to hypoxia

PARP inhibition results in the accumulation of collapsed replication forks requiring HR for their repair (18, 19). We hypothesized that HR-deficient hypoxic cells would have increased difficulty in repairing collapsed replication forks resulting in cell death. The rate of replication restart after

reoxygenation was determined by DNA replication fiber analysis. This confirmed that PARP inhibition increased the rate of replication restart during reoxygenation after severe hypoxia, thus indicating that PARP functions to reduce DNA replication kinetics in the presence of accumulating DNA damage (Fig. 3A and B). Consistent with this finding, PARP inhibition in HR-defective hypoxic cells led to elevated 53BP1 and  $\gamma$ H2AX foci following either acute or chronic hypoxic exposure (Fig. 3C and D). Hypoxia results in replication fork stalling (10, 29), and it has recently been shown that PARP is activated at stalled replication forks (30). To test if the increase in PARP activity in hypoxic cells is related to an increased amount of hypoxia-stalled replication

**Figure 3.** PARP inhibition induces DNA damage in proliferating hypoxic cells. A and B, replication fiber analysis indicates that PARP inhibition using 4-amino-1,8-naphthalimide (ANI) increased the rate of replication restart during reoxygenation after 16 h  $\times$  0.02% O<sub>2</sub> in RKO cells. Representative replication fiber images. C, immunofluorescent staining of 53BP1 foci in RKO cells shows further elevation following reoxygenation after 16 h  $\times$  0.02% O<sub>2</sub> when treated with 2.5  $\mu$ M ABT-888. D, immunofluorescent staining of  $\gamma$ H2AX foci in H1299 cells shows elevated foci following treatment with 72 h  $\times$  0.2% O<sub>2</sub> plus 2.5  $\mu$ M ABT-888. Columns, mean of three experiments; bars, SEM; \*,  $P < 0.05$ .





**Figure 4.** PARP inhibition of hypoxic tumor cells *in vivo* induces DNA damage. A, schematic of ABT-888 treatment. RKO xenografts were treated twice daily for 5 days with 50 mg/kg ABT-888 and then collected for immunohistochemical (IHC) staining and determination of PARP activity. B, relative enzymatic PARP activity of tumor lysates collected 2 h after the final ABT-888 treatment verifies PARP inhibition *in vivo*. C, representative immunohistochemical staining of vehicle- and ABT-888–treated tumors show decreased RAD51 staining in EF5-avid subregions. ABT-888–treated tumors show increased  $\gamma$ H2AX and CC3 staining (white arrows) in EF5-positive subregions compared with vehicle-treated tumors. Scale bar, 100  $\mu$ m. D, quantification of  $\gamma$ H2AX and CC3 fluorescent intensity within aerobic (EF5 negative) and hypoxic (EF5 positive) tumor subregions. Analysis based on four to eight fields per tumor for three to five tumors per treatment group. \*,  $P < 0.05$ .

forks, we colocalized hypoxia-induced PAR foci with induced replication protein A (RPA) foci that form at stalled replication forks. We found that PAR foci colocalize well with RPA foci (Supplementary Fig. S4), suggesting that PARP is indeed activated at hypoxia-stalled replication forks.

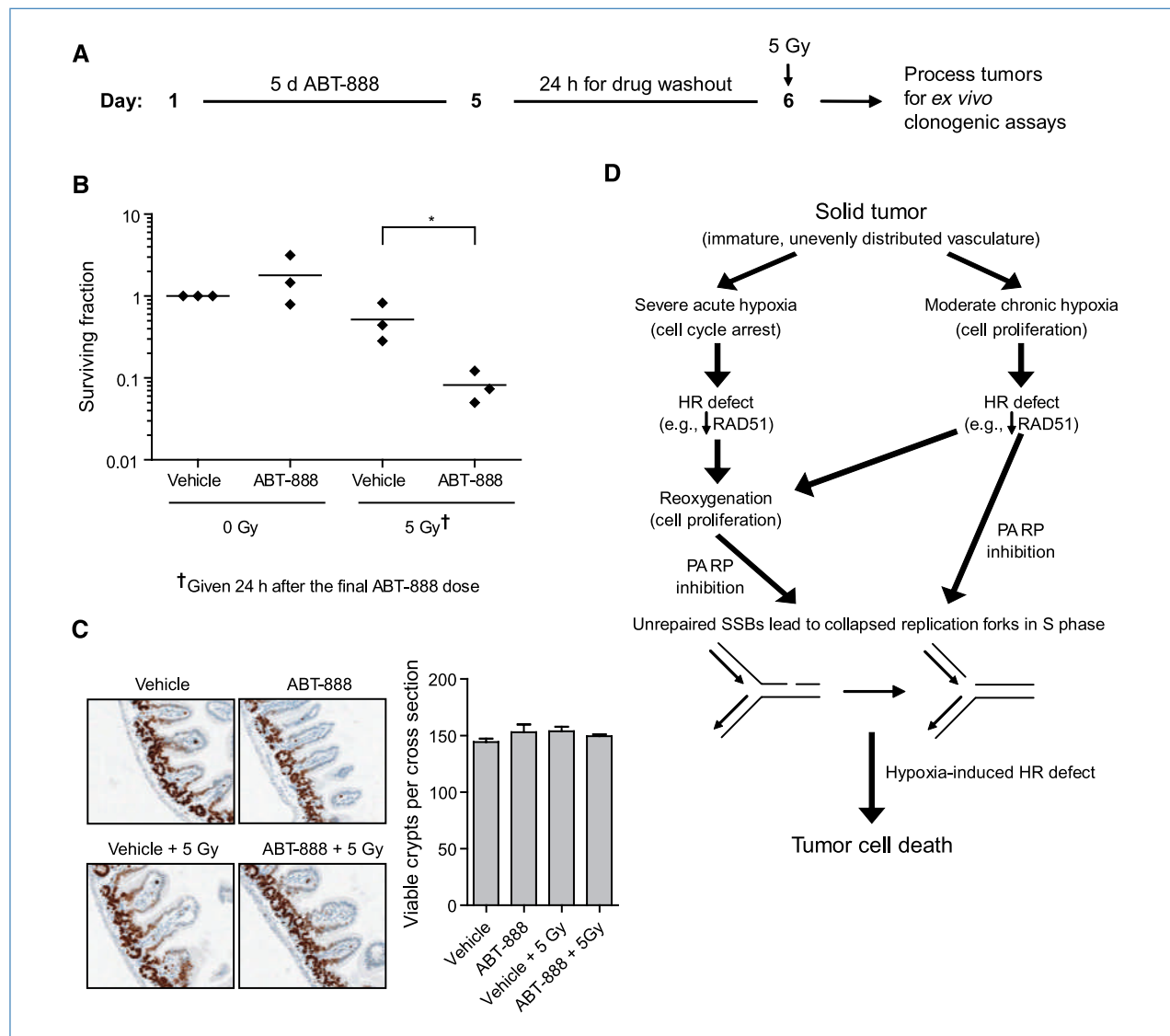
We conclude that PARP inhibition leads to accumulation of DNA breaks in cycling hypoxic cells (during reoxygenation or as a consequence of chronic hypoxic adaptation) similar to that reported for tumor cells that are genetically null for HR (18, 19).

#### PARP inhibition induces killing of hypoxic tumor cells *in vivo*

Single-agent dosing with PARP inhibitors can lead to growth delay in wild-type BRCA1/2 tumor xenograft models (31). We therefore tested whether our observation of synthetic lethality between hypoxia-mediated HR defects and PARP inhibition also occurred *in vivo*. RKO xenografts were treated twice daily with 50 mg/kg ABT-888 or vehicle for 5 days and assayed for DNA damage within hypoxic tumor subregions. A schematic of the treatment protocol is shown

in Fig. 4A. Tumor lysates were collected and used to confirm that inhibition of PARP activity was achieved *in vivo* (Fig. 4B). Immunohistochemical staining confirmed decreased expression of RAD51 in hypoxic (EF5-avid) tumor subregions in both the vehicle and PARP-inhibited tumors (Fig. 4C). Importantly, hypoxic regions of the PARP-inhibited tumors displayed significantly elevated expression of  $\gamma$ H2AX and CC3 selectively across the EF5 gradient (Fig. 4C and D).

To determine if PARP inhibition *in vivo* selectively kills hypoxic tumor cells, we performed *ex vivo* clonogenic assays on ABT-888-pretreated tumors that were exposed to 5-Gy ionizing radiation (IR) 24 hours after the final ABT-888 dose. After drug washout, IR should selectively kill any remaining aerobic cells without bias from PARP inhibitor radiosensitization. A schematic of the treatment protocol is shown in Fig. 5A. Clonogenic survival following tumor irradiation *in vivo* is an established assay to measure changes



**Figure 5.** PARP inhibition of hypoxic tumor cells *in vivo* induces cell death. A, schematic of ABT-888 treatment. RKO xenografts were treated twice daily for 5 days with 50 mg/kg ABT-888. Mice were left for 24 h to allow for drug washout before treatment with 5 Gy of IR followed by *ex vivo* clonogenic assays. This protocol unmasks the hypoxic cell kill without bias from aerobic cell radiosensitization by PARP inhibition. B, *ex vivo* clonogenic assays show decreased survival of ABT-888-treated tumors following exposure to 5 Gy of IR. C, analysis of mouse intestinal crypts shows no toxicity from PARP inhibition or IR. D, model for hypoxia-mediated contextual synthetic lethality with PARP inhibition. Solid tumors have substandard vasculature leading to gradients of moderate to severe hypoxia. Severe acute hypoxia decreases HR capacity and leads to cell cycle arrest, which is reversible on reoxygenation. Moderate chronic hypoxia also decreases HR capacity but still allows for proliferation. PARP inhibition results in unrepaired SSBs, which collapse replication forks in S phase. These collapsed replication forks are lethal to tumor cells with hypoxia-induced HR defects potentially through apoptosis, mitotic catastrophe, autophagy, or terminal senescence. \*,  $P < 0.05$ .

in the hypoxic tumor fraction, as the radiosensitive aerobic tumor cells are preferentially killed over more radioresistant hypoxic cells. The radiation was delivered 24 hours after the final ABT-888 dose, a time when pharmacokinetic and pharmacodynamic studies have shown a return to background levels (32). We observed that ABT-888-pretreated tumors had lower survival than vehicle-treated tumors following irradiation (Fig. 5B). This is consistent with PARP inhibitor-induced killing of hypoxic HR-defective cells before challenge with IR. However, given the effects of PARP inhibition of tumor vasculature (33) and the relatively low hypoxic fraction of the RKO xenografts, it would be difficult to see differences in growth delay that could be directly attributed to sensitization of hypoxic cells to PARP inhibition.

Importantly, this regimen of PARP inhibition, even in combination with the radiation treatment, did not kill normal tissue clonogens as measured by a gut clonogenic assay (Fig. 5C). This shows the increased therapeutic ratio of PARP inhibitor treatment as resistant, hypoxic tumor tissues are targeted without killing normal tissues. We conclude that hypoxic sensitization of tumor cells to PARP inhibition occurs *in vivo*. A model for the proposed mechanism of hypoxic cell death due to contextual synthetic lethality is shown in Fig. 5D.

## Discussion

Currently, the use of PARP inhibitors as single agents has been limited to clinical trials for patients with genetic deficiencies in *BRCA1/2* (23). There is active interest in identifying additional genetic, epigenetic, or microenvironmental changes that could lead to a "BRCAness" phenotype with increased sensitivity to PARP inhibitors. To this end, high-throughput screens have identified several potential targets showing sensitivity to PARP inhibition, including the transcription coupled DNA repair proteins DDB1 and XAB2 and the cyclin-dependent kinase 5 (34, 35). Recently, PTEN deficiency leading to suppressed RAD51 has also been shown to sensitize tumor cells to PARP inhibition (36). In this report, we have shown that hypoxia-induced HR defects can also yield a "BRCAness" phenotype. Unlike a recent report from Hegan and colleagues (28), our findings are independent of any direct PARP-mediated effects on RAD51 expression or HR function. Instead, ABT-888 had no effect on RAD51 protein (Fig. 1A and B) or mRNA (Supplementary Fig. S2A) expression nor did it alter the efficiency of DR-GFP measured HR (Fig. 1C) or sensitivity to mitomycin C (MMC) (Supplementary Fig. S2C). Additionally, PARP inhibition alone did not induce a statistically significant increase in  $\gamma$ H2AX expression *in vitro* (Fig. 3D) or *in vivo* (Fig. 4D). Only in conjunction with hypoxia do we observe decreased HR and synthetic lethality that translates to increased clonogenic killing. This may expand the utility of PARP inhibitors when used alone or in combination with radiotherapy or chemotherapy by targeting the hypoxic subpopulation of tumor cells that are otherwise resistant to therapy and possibly responsible for distant metastatic spread (9). Indeed, the use of PARP inhibitors in combined therapy has already shown

promise in preclinical models with improved growth delay in radiation, temolozolomide, cisplatin, carboplatin, or cyclophosphamide-treated tumors (31–33, 37). In future clinical trials, we foresee the need to determine the hypoxic fraction of tumors to select for patients that would most benefit from this strategy.

It has been shown that PARP1 has a role in HIF-1 $\alpha$  stabilization and signaling mediated by nitric oxide and oxidative stress (38, 39). It is conceivable that PARP inhibition could also inhibit HIF-1 $\alpha$  accumulation and signaling leading to a blockade of hypoxic responses and more cell death. However, in our model systems, we do not observe any altered stabilization of HIF-1 $\alpha$  (Fig. 1A and B) or altered HIF-1 transcriptional activity (Supplementary Fig. S2B).

Two groups have reported PARP inhibitor and cisplatin-resistant phenotypes in *BRCA2*-defective cells based on a reverting *BRCA2* mutation (40, 41). In contrast, our contextual synthetic lethality model would not lead to acquired genetic resistance given an alternate pathway of decreased HR based on the decreased transcription and translation of HR genes (15, 42).

HR-compromised hypoxic cells replicating under moderate hypoxia or following reoxygenation showed elevated expression of the DNA damage markers  $\gamma$ H2AX and 53BP1 when treated with PARP inhibitors (Fig. 3C and D). A similar finding was reported for *BRCA2*<sup>-/-</sup> cells exposed to PARP inhibition (18, 19). Severe hypoxia leads to stalled replication, which can be overcome during reoxygenation. However, in the presence of PARP inhibition, our DNA repair foci and replication fiber data support that HR-deficient reoxygenated or chronically hypoxic cells acquire collapsed/damaged replication forks (Fig. 3A and B). Consistent with these data, the toxicity was primarily manifested in S-phase cells (Fig. 2D). Our findings agree with those of Sugimura and colleagues, who showed that PARP1 is required for replication fork slowing on damaged DNA and that fork slowing is HR dependent (43). The epistasis between PARP and HR is explained by PARP being recruited to hypoxia-stalled forks to activate a slow HR repair process (30). This model is also supported by the colocalization of PAR polymers and RPA foci in hypoxia-treated cells (Supplementary Fig. S4).

The development of drugs designed to exploit tumor hypoxia has been focused on prodrugs that are activated by metabolic reduction under hypoxic conditions to form free radical-based cell cytotoxins causing DNA strand breaks, base damage, and DNA-protein cross-links (6). Our work here supports another novel treatment strategy to sensitize traditionally resistant hypoxic cells using PARP inhibitors, which may have a unique therapeutic ratio in killing hypoxic cancer cells over aerobic normal cells. This is specifically true given recent clinical trial data in which PARP inhibition had minimal side effects (23, 44, 45). There may also be a role for combined PARP and HIF-1 $\alpha$ -targeting, as PARP may modify HIF-1 $\alpha$  accumulation through differential oxidative stress under aerobic versus hypoxic conditions and anti-HIF-1 $\alpha$  agents are currently being tested in preclinical and clinical settings (39, 46, 47).



Hypoxic tumor cells may have suppression of other DNA repair pathways beyond HR, including nonhomologous end-joining, mismatch, and base excision repair (48). Further understanding of the contextual synthetic lethality to these and other DNA damage signaling pathways could define new approaches to individualized cancer therapy. Indeed, it has recently been reported that deficiency in the mismatch repair (MMR) proteins MSH2 and MLH1 are synthetically lethal with disruption of the DNA polymerases POLB and POLG, respectively (49). Both of these MMR proteins are downregulated by hypoxia; therefore, inhibition of POLB or POLG may also show contextual synthetic lethality with hypoxia. At the moment, clinically useful inhibitors of POLB or POLG are not yet available, but given the strong inhibition of MSH2 and MLH1 by hypoxia, this is a concept that warrants further study.

Elevated levels of endogenous  $\gamma$ H2AX or 53BP1 foci and decreased RAD51 expression in hypoxic subregions of tumor biopsies may be biomarkers of potential cell kill with PARP inhibitors. The efficacy of this treatment can also be tracked in hypoxic subregions with sequential biopsies during and after treatment similar to the published use of hair follicle  $\gamma$ H2AX foci as a normal tissue biomarker of PARP inhibition (23). Taken together, our data describes a

model of hypoxia-mediated contextual synthetic lethality with PARP inhibition, which supports the development of novel biomarkers for prediction of PARP inhibitor treatment susceptibility and response (Fig. 5D).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Contextual Synthetic Lethality of Cancer Cell Kill Based on the Tumor Microenvironment

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