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## Suppression of homologous recombination sensitizes human tumor cells to IGF-1R inhibition

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### Abstract

Inhibition of type 1 IGF receptor (IGF-1R) sensitizes to DNA-damaging cancer treatments, and delays repair of DNA double strand breaks (DSBs) by non-homologous end-joining and homologous recombination (HR). In a recent screen for mediators of resistance to IGF-1R inhibitor AZ12253801, we identified RAD51, required for the strand invasion step of HR. These findings prompted us to test the hypothesis that IGF-1R-inhibited cells accumulate DSBs formed at endogenous DNA lesions, and depend on residual HR for their repair. Indeed, initial experiments showed time-dependent accumulation of  $\gamma$ H2AX foci in IGF-1R -inhibited or -depleted prostate cancer cells. We then tested effects of suppressing HR, and found that RAD51 depletion enhanced AZ12253801 sensitivity in PTEN wild-type prostate cancer cells but not in cells lacking functional PTEN. Similar sensitization was induced in prostate cancer cells by depletion of BRCA2, required for RAD51 loading onto DNA, and in BRCA2<sup>-/-</sup> colorectal cancer cells, compared with isogenic BRCA2<sup>+/-</sup> cells. We also assessed chemical HR inhibitors, finding that RAD51 inhibitor BO2 blocked RAD51 focus formation and sensitized to AZ12253801. Finally, we tested CDK1 inhibitor RO-3306, which impairs HR by inhibiting CDK1-mediated BRCA1 phosphorylation. RO-3306 suppressed RAD51 focus formation consistent with HR attenuation, and sensitized prostate cancer cells to IGF-1R inhibition, with 2.4-fold reduction in AZ12253801 GI<sub>50</sub> and 13-fold reduction in GI<sub>80</sub>. These data suggest that responses to IGF-1R inhibition are enhanced by genetic and chemical approaches to suppress HR, defining a population of cancers (PTEN wild-type, BRCA mutant) that may be intrinsically sensitive to IGF-1R inhibitory drugs.

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## Introduction

Type 1 insulin-like growth factor receptor (IGF-1R) signals via multiple effectors including phosphatidylinositol 3 kinase (PI3K)-AKT to promote cell survival, and IGF-1R overexpression is associated with clinical radioresistance<sup>1–3</sup>. IGF-1R targeting enhances chemo- and radio-sensitivity, attributed to apoptosis induction<sup>4–7</sup>. Data from our group and others indicate that IGF-1R targeting influences the DNA damage response (DDR), with evidence for delayed repair of DNA double strand breaks (DSBs) by both non-homologous end-joining (NHEJ) and homologous recombination (HR)<sup>8–11</sup>.

Despite activity in preclinical models and early phase clinical trials, IGF-1R inhibitors have shown limited benefit in Phase 2/3 trials of unselected patients, and there are no biomarkers to predict response<sup>3</sup>. We recently screened for regulators of response to IGF-1R inhibition; of the hits, the only authentic repair protein was RAD51, the recombinase that catalyzes the strand invasion step of HR<sup>12</sup>. Here, we aimed to validate RAD51 as a screen hit, and understand how IGF-1R inhibition sensitizes to RAD51 depletion.

## Materials and Methods

Prostate cancer cell lines DU145 and PC3 were from Cancer Research UK Laboratories (Clare Hall, Hertfordshire, UK), and 22Rv1 and LNCaP from Professor Sir Walter Bodmer (Dept. of Oncology, University of Oxford, UK). Cell line identity was validated by STR genotyping. DLD-1 colorectal cancer cells expressing (BRCA2<sup>+/-</sup>) or lacking (BRCA2<sup>-/-</sup>) BRCA2 were from Dr. Scott Kern (Laboratory of Cellular and Molecular Biology, NIH, Baltimore, MA, USA). We used IGF-1R inhibitor AZ12253801 (AstraZeneca, Alderley Park, UK), described in<sup>11</sup>, human R3 IGF-1 (Sigma-Aldrich, USA), CDK1 inhibitor RO-3306 and RAD51 inhibitors RL-1 and BO2 (Calbiochem, Merck Millipore, Watford, UK). Cells underwent Cesium-137 irradiation in an IBL 637 irradiator (CIS Bio International, Bagnols/Seze, France). Gene silencing and western blotting were performed as<sup>11</sup> using siRNAs and antibodies listed in Supplementary information. Cell viability was quantified by CellTiter-Glo (CTG) Luminescent assay (Promega, USA), and clonogenic survival, cell cycle distribution and immunofluorescent detection of  $\gamma$ H2AX and RAD51 foci as in<sup>11</sup> and Supplementary information.

## Results and Discussion

Data from our group and others indicate that IGF-1R targeting sensitizes tumor cells to ionizing radiation and cytotoxic drugs, and delays DSB repair by NHEJ and HR<sup>4, 7, 10, 11</sup>. DSBs also arise from endogenous damage, typically following collapse of stalled replication forks, and depend on HR for repair due to their one-ended structure<sup>13</sup>. Given these findings and our identification of RAD51 as a candidate mediator of resistance to IGF-1R inhibition in a screen conducted in the absence of exogenous DNA damage<sup>12</sup>, we speculated that IGF-1R inhibition leads to accumulation of DSBs that form at endogenous DNA lesions. To investigate this hypothesis, we used AZ12253801, an IGF-1R inhibitor that shows ~10 fold selectivity over the insulin receptor<sup>11</sup>. In DU145 cells, AZ12253801 inhibited phosphorylation of IGF-1R and its downstream effectors (Figure 1a), confirming previous

results<sup>11</sup>. Using  $\gamma$ H2AX as a DSB marker, we assessed whether IGF-1R inhibition influences accumulation of endogenous damage. Compared with controls, AZ12253801-treated cells showed progressive accumulation of  $\gamma$ H2AX foci over a 3-day time-course (Figure 1b,c). To assess the specificity of this effect, we repeated the experiment using siRNA to deplete IGF-1R. There was no difference 1-2 days after siRNA transfection, but by 3 days IGF-1R depleted cells contained more  $\gamma$ H2AX foci than controls (figure 1d,e). The relative delay compared with effects of AZ12253801 may be because IGF-1R depletion was achieved only after 2-3 days (Figure 1f), consistent with the relatively long half-life (~16-20hr) of IGF-1R protein<sup>14</sup>.

While  $\gamma$ H2AX foci can form at single-stranded DNA, their detection correlates closely with numbers of DSBs<sup>15</sup>. Therefore, these results suggest that IGF-1R inhibition or depletion enhances accumulation of DSBs induced by endogenous DNA damage. These data are consistent with the initial report from Reiss and colleagues, and later work from our group, showing that IGF signaling influences HR, and consequently HR is impaired in cells in which IGF-1R is depleted or inhibited<sup>8, 10, 11</sup>. This effect may contribute to reduced cell survival in IGF-1R depleted cells (Figure 2a). In light of the identification of RAD51 in a screen for proteins whose depletion sensitizes to IGF-1R inhibition<sup>12</sup>, we speculated that IGF-1R inhibited cells are capable of residual HR, albeit with reduced efficiency, and are susceptible to manoeuvres that further comprise repair. Therefore, we tested effects of depleting RAD51 using two RAD51 siRNAs from the screen. Both mediated effective gene silencing, and RAD51-depleted DU145 cells showed reduced cell survival (Figure 2a), consistent with the known toxicity of RAD51 loss in mammalian cells<sup>16</sup>. As in other siRNA screens<sup>17</sup>, our screen included a cut-off for hits that were toxic in the absence of IGF-1R inhibition. RAD51 had not been excluded on this basis, suggesting that *RAD51* silencing had not crossed a threshold that impairs fitness, and that IGF-1R inhibited cells are more sensitive to RAD51 depletion than controls<sup>12</sup>. Indeed, both RAD51 siRNAs sensitized DU145 cells to AZ12253801 (Figure 2b). Depletion of IGF-1R itself did not affect AZ12253801 sensitivity (Figure 2b), consistent with findings that IGF-1R expression has little predictive value for response to IGF-1R inhibition<sup>3, 12</sup>. RAD51 depletion also inhibited cell viability, and induced ~2-fold reduction in AZ12253801 GI<sub>50</sub> (Figure 2c,d), comparable to the siRNA screen results<sup>12</sup>. We then tested three further prostate cancer cell lines, confirming RAD51 depletion by western blotting (Supporting Information Figure S1a). The 22Rv1 cells were sensitized to IGF-1R inhibition by both RAD51 siRNAs, with ~2-fold reduction in AZ12253801 GI<sub>50</sub>, as in DU145. There was no change in AZ12253801 sensitivity in RAD51-depleted LNCaP cells, and a minor effect in PC3, unlikely to be biologically significant (Figures 2e, S1b-d). PC3 and LNCaP lack functional PTEN, while DU145 and 22Rv1 express wild-type (WT) PTEN (Figure 2e), as do 40% of prostate cancers<sup>18</sup>. Given that PTEN encodes a phosphatase that inactivates PI3K<sup>18</sup>, we tested whether RAD51 depletion affects IGF signalling, as we found for other screen hits<sup>12</sup>, but did not detect altered activation of IGF-1R or its effectors in RAD51-depleted DU145 cells (Supplementary Figure S2e). It is possible that a link with PTEN status is provided by the reported ability of AKT and PTEN to influence DSB repair, although the nature of the effect apparently varies with cellular context<sup>17, 19</sup>.

A key step in HR is the loading of RAD51 nucleofilaments onto DNA, a process that requires BRCA2. BRCA2 was present in the DNA repair set used in our siRNA screen, but was not identified as a hit<sup>12</sup>. We used two models to investigate whether BRCA2 influences sensitivity to IGF-1R inhibition. Aiming to deplete DU145 cells of BRCA2, we tested screen siRNAs BRCA2\_6 and \_7, and found that both were ineffective, while a third (siBRCA2) did mediate effective target depletion (Figure 2f), as previously reported<sup>21</sup>. In viability assays, the response of DU145 cells to AZ12253801 was enhanced by siBRCA2 but not by the ineffective screen siRNAs (Figure 3f). This suggests that depletion of BRCA2, like RAD51, is capable of sensitizing to IGF-1R inhibition, and also that BRCA2 was a false negative in the screen<sup>12</sup>. As a second approach, we used isogenic DLD1 colorectal cancer cells that express WT PTEN and are BRCA2 WT (BRCA2<sup>+/-</sup>) or null (BRCA2<sup>-/-</sup>)<sup>22</sup>. The cell lines had similar expression of IGF-1R and downstream effectors, undetectable AKT phosphorylation consistent with WT PTEN status, and detectable BRCA2 only in BRCA2<sup>+/-</sup> cells (Figure 2g, left). The effect of BRCA2 loss was tested by measuring the ability to generate irradiation-induced repair foci. Both cell lines formed  $\gamma$ H2AX foci, indicative of damage induction, and BRCA2<sup>+/-</sup> cells contained RAD51 foci that co-localized with  $\gamma$ H2AX, while RAD51 foci were clearly reduced in BRCA2<sup>-/-</sup> cells, indicating defective HR (Figure 2g, right). Consistent with effects of BRCA2 knockdown in DU145 cells, BRCA2<sup>-/-</sup> DLD1 cells were more sensitive than isogenic BRCA2<sup>+/-</sup> cells to AZ12253801 (Figure 2h), raising the possibility that patients with BRCA2 mutant cancers may be sensitive to IGF-1R inhibition.

We next explored chemical approaches to inhibit HR. First, we used small molecule drugs BO2 that inhibits the DNA strand exchange activity of human RAD51, and RL-1, which binds covalently to RAD51 protein and is thought to destabilize the formation of RAD51 nucleofilaments<sup>23, 24</sup>. Viability assays were used to determine GI<sub>50</sub> values for use in subsequent experiments (Supplementary Figure S2a-d). In DU145 cells, radiation-induced RAD51 foci were inhibited by BO2 but not by RL-1 (Figure 3a). BO2 sensitized to IGF-1R inhibition, with 2.2 fold reduction in AZ12253801 GI<sub>50</sub> (Figure 3b), similar to effects of RAD51 depletion (Figure 2d), while RL-1 did not affect the response to AZ12253801 (Figure 3b). These data suggest that the sensitization effect tracks with the ability to suppress RAD51 foci. BO2 also suppressed RAD51 focus formation in PC3, LNCaP and 22Rv1 cells (Supplementary Figure S2e), and induced AZ12253801 sensitization in 22Rv1 and PC3 cells, and no change in LNCaP (Figure S3a-c).

Finally, we exploited the knowledge that cyclin-dependent kinase (CDK) activity is required for serine phosphorylation of BRCA1 and 2 prior to RAD51 nucleofilament formation<sup>20</sup>. CDK1 inhibitor RO-3306 was reported to impair BRCA1 localization to DSBs<sup>25</sup>, and consistent with this, suppressed RAD51 focus formation in DU145 cells when applied at the GI<sub>50</sub> (1 $\mu$ M; Figures 3a, S2c-e). Analysis of cell cycle distribution showed that cells co-treated with 100nM AZ12253801 and 1 $\mu$ M RO-3306 manifested a marked increase in pre-G1 DNA, indicating apoptosis induction (Figure 3c). In viability assays, RO-3306 induced a significant shift to the left of the AZ12253801 dose-response curve, with 2.4-fold reduction in GI<sub>50</sub>, comparable to RAD51 depletion or inhibition. At sub-50nM AZ12253801 concentrations there was more striking sensitization, with 13-fold reduction in GI<sub>80</sub> (Figure 3d). Paralleling effects of RAD51 knockdown, RO-3306 did not affect AZ12253801

sensitivity of PC3 or LNCaP cells (Supplementary Figure S3 d, e), but did sensitize 22Rv1 cells (Supplementary Figure S3f); as in DU145, CDK1 inhibition was more effective than RAD51 depletion at sensitizing to low AZ12253801 concentrations, suggesting that CDK1 may influence response to IGF-1R inhibition via processes in addition to HR modulation.

These results indicate that the response to IGF-1R inhibition is enhanced in PTEN WT cells by manipulations that suppress BRCA2 and RAD51 function. To explore the clinical relevance of these data, it will be important to test these findings in primary human tumor cells and *in vivo* models. In terms of mechanistic insights, we recently reported that IGF-1R inhibited cells show delayed repair of radiation-induced damage, with inhibition of repair by both NHEJ and HR in reporter assays<sup>11</sup>. IGF-1R inhibition was phenotypically silent in cells with an NHEJ defect induced by inhibition or loss of DNA-PK11, implying that with respect to its role in DSB repair, IGF-1R acts primarily through NHEJ. This is consistent with the results of the current study, which support the concept that IGF-1R inhibited cells depend on residual HR for repair of endogenous damage, and are sensitized to its inhibition. These findings suggest that HR-deficient tumors may be intrinsically sensitive to IGF-1R inhibitory drugs.

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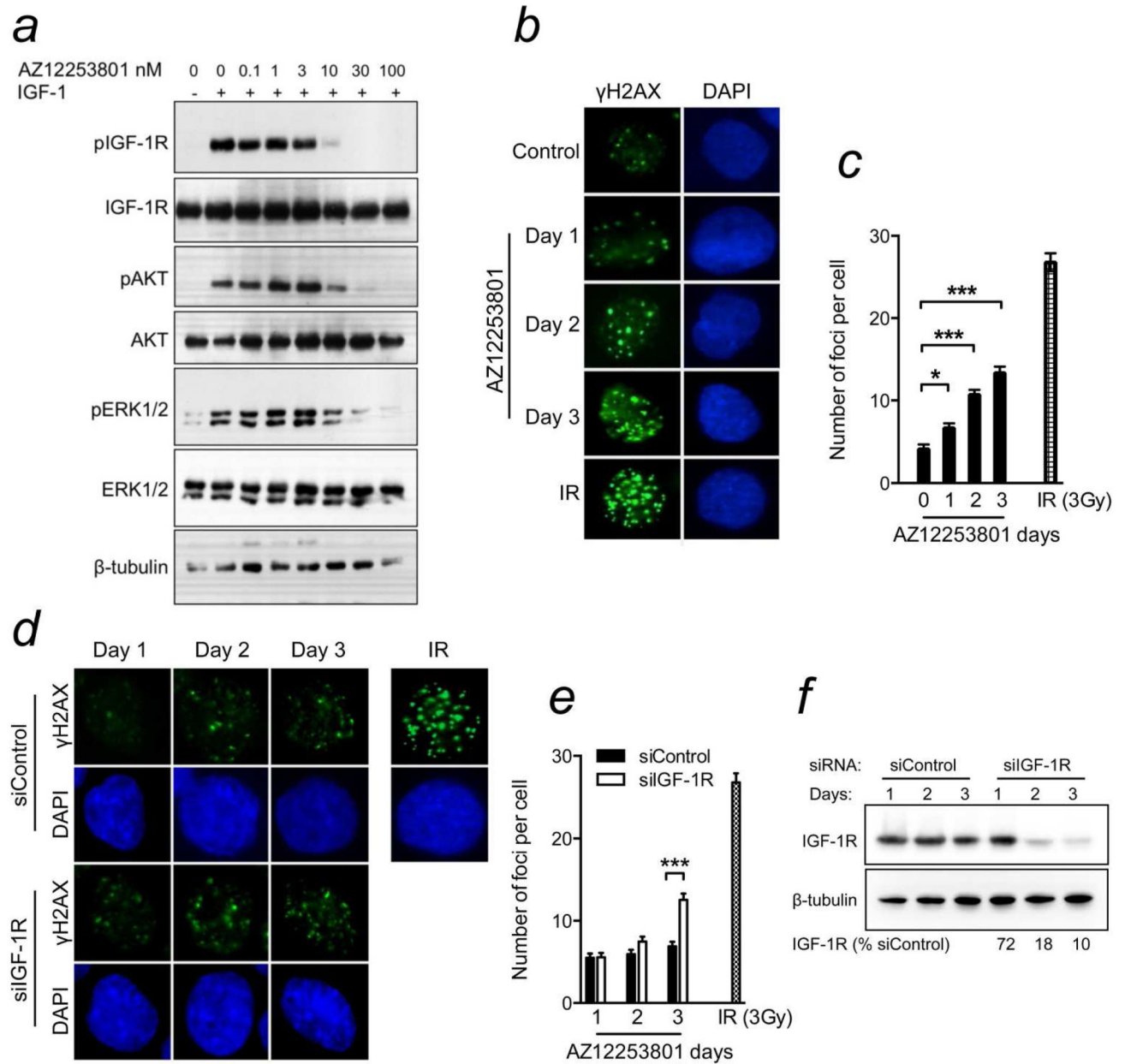
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**Brief description of novelty and impact**

In a screen for biomarkers that predict response to IGF-1R inhibition, we identified RAD51, required for homologous recombination (HR) repair of DNA damage. Here, we show that sensitivity to IGF-1R inhibition is enhanced by multiple approaches to suppress HR, including RAD51 depletion, depletion/loss of BRCA2, or inhibition of RAD51 or CDK1. These data suggest that IGF-1R-inhibited cells depend on HR to repair endogenous damage, and that HR-deficient tumors may be sensitive to IGF-1R inhibitory drugs.

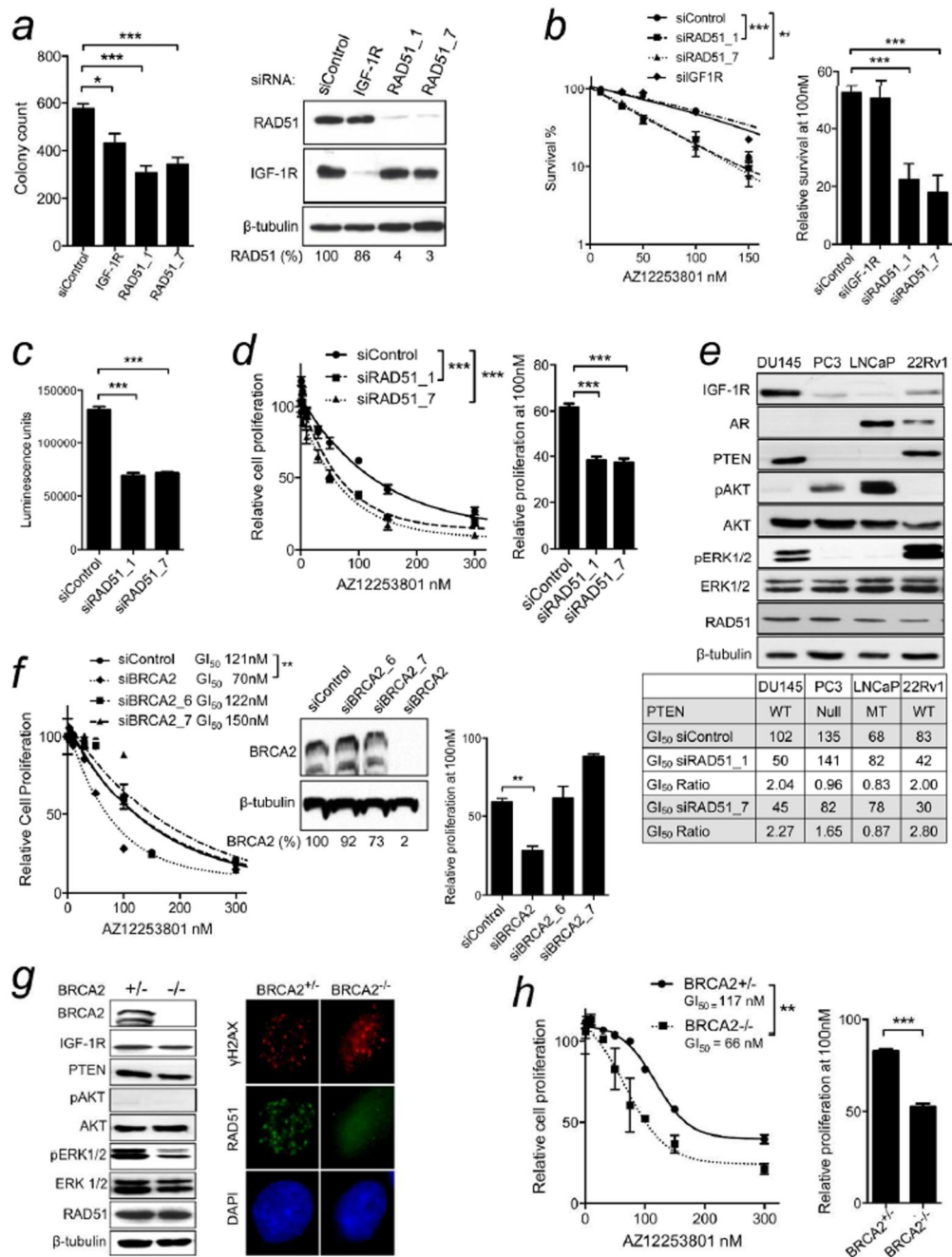




**Figure 1. IGF-1R influences repair of endogenous DNA damage.**

**a)** Serum-starved DU145 cells were treated with AZ12253801 for 1hr and in the final 15min with 50nM IGF-1. **b)** DU145 cells were treated with solvent (control) or 100nM AZ12253801, fixed 0-3 days later and stained for  $\gamma$ H2AX (green) and DAPI (blue). Irradiated cells (3Gy, 6hr) served as positive controls for  $\gamma$ H2AX foci. **c)** Graph: mean  $\pm$  SEM foci per cell from 2 independent experiments. Foci increased with time in IGF-1R inhibited cells (\* $p$ <0.05, \*\*\* $p$ <0.001). **d)** DU145 were transfected with siControl or siIGF1R, and fixed and stained 1-3 days later as b), with irradiated cells as positive controls. **e)** Cells transfected as d) were analysed as c). Graph: mean  $\pm$  SEM foci per cell. Three days post-siRNA transfection, IGF-1R depleted cells contained more  $\gamma$ H2AX foci than controls

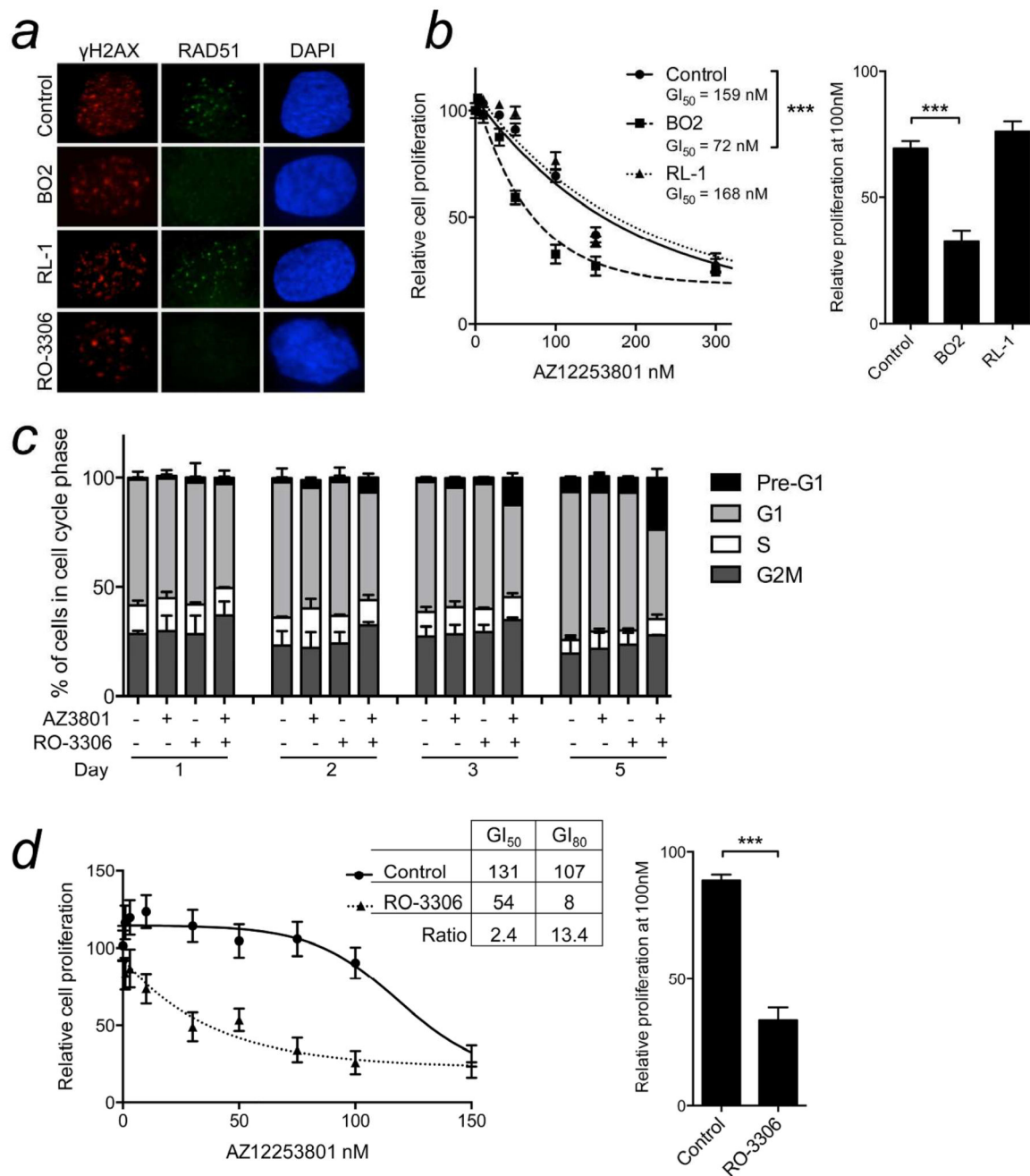
(\*\*\* $p < 0.001$ ). **f**) DU145 cells were siRNA-transfected and lysed on days 1-3 for western blotting. Figures below blot: IGF-1R levels corrected for tubulin loading, expressed as % of siControl-transfectants on the equivalent day.



**Figure 2. PTEN wild-type tumor cells are sensitized to IGF-1R inhibition by depletion or loss of RAD51 or BRCA2.**

**a)** Survival of DU145 cells transfected with siControl, IGF-1R or RAD51 siRNAs. Graph: mean ± SEM colonies showing reduced survival on depletion of IGF-1R (\*p<0.05) or RAD51 (\*\*p<0.001). To right: parallel cultures blotted for IGF-1R and RAD51. Figures below blot: RAD51 levels corrected for loading, expressed as % siControl. **b)** DU145 cells were siRNA-transfected as a) and treated with solvent or AZ12253801. Graph: survival (% untreated) from two independent experiments (6 data points; \*\*\*p<0.001 by two-way

ANOVA). Data were curve-fitted to interpolate AZ12253801 SF<sub>50</sub>: 102nM for controls, and 108, 50 and 45 nM for IGF-1R, RAD51\_1, RAD51\_7 siRNA transfectants respectively. Graph to right: relative survival at 100nM AZ12253801, showing sensitization by RAD51-depletion (\*\*p<0.001). **c**) DU145 cells were reverse-transfected with siControl, siRAD51\_1, or siRAD51\_7 and viability assayed 5 days later. Graph: mean ± SEM luminescence(\*\*p<0.001). **d**) DU145 cells were siRNA-transfected as c), 48hr later treated with AZ12253801, and after 5 days viability assayed. Graph: mean ± SEM viability as % siControl, pooled data from 3 independent assays (\*\*p<0.001), generating AZ12253801 GI<sub>50</sub> values in controls 122nM, RAD51\_1 transfectants 65nM, RAD51\_7 transfectants 55nM. Graph to right: relative viability at 100nM AZ12253801 (\*\*p<0.001). **e**) Cell lysates analyzed for components of IGF axis, androgen receptor (AR), RAD51. Table below: PTEN status, AZ12253801 GI<sub>50</sub> values (3 experiments in each cell line as d), fold sensitization as GI<sub>50</sub> Ratio in siControl/siRAD51 transfectants. **f**) DU145 cells were transfected with siControl or BRCA2 siRNAs (siBRCA2\_6, siBRCA2\_7, siBRCA2), and AZ12253801-treated and viability assayed as d). Graph: mean ± SEM of 2 independent experiments. Only siBRCA2 sensitized to AZ12253801 (\*\* p<0.01). Inset: parallel cultures analysed by western blotting. Figures below blot: BRCA2 levels corrected for loading, expressed as % siControl. Graph to right: relative viability at 100nM AZ12253801 (\*\*p<0.01). **g**) BRCA2<sup>+/-</sup> and BRCA2<sup>-/-</sup> DLD-1 cells analysed by: left, western blotting; right, immunofluorescent staining for γH2AX (red), RAD51 (green), DAPI (blue), 6hr after 5Gy irradiation. **h**) DLD-1 cells were treated with AZ12253801 and viability assayed as d). Graph: mean ± SEM viability, pooled data from 3 experiments. BRCA2<sup>-/-</sup> cells were more sensitive to AZ12253801 than BRCA2<sup>+/-</sup> cells (\*\* p<0.01). Graph to right: relative viability at 100 nM AZ12253801 (\*\*p<0.001).



**Figure 3. Prostate cancer cells are sensitized to IGF-1R inhibition by inhibitors of RAD51 or CDK1.**

**a** DU145 cells were treated with solvent (Control), BO2 (10 $\mu$ M), RL-1 (10 $\mu$ M), or RO-3306 (1 $\mu$ M), after 1hr irradiated (5Gy), and after 6hr stained for  $\gamma$ H2AX (red), RAD51 (green), DAPI (blue). **b** DU145 cells were treated with AZ12253801 with solvent or 10 $\mu$ M BO2 or RL-1, and viability assessed 5 days later. Graph: mean  $\pm$  SEM of 3 independent experiments. Sensitivity to AZ12253801 was enhanced by BO2 (\*\*\*) ( $p < 0.001$  by 2-way ANOVA) but not RL-1. Graph to right: relative viability at 100nM AZ12253801 (\*\*\*)

p<0.001). **c)** DU145 cells were treated for 1-5 days with 100nM AZ12253801 (AZ3801) and/or 1 $\mu$ M RO-3306 and analyzed by flow cytometry. **d)** DU145 cells were treated with AZ12253801 alone or with 1 $\mu$ M RO-3306 and viability assayed after 5 days. Graph: mean  $\pm$  SEM viability from 3 independent experiments. Sensitivity to AZ12253801 was enhanced by RO-3306 (p<0.001 by two-way ANOVA). Legend shows GI<sub>50</sub>, GI<sub>80</sub> and fold sensitization (ratio of GI<sub>50</sub> or GI<sub>80</sub> values). Graph to right: relative viability at 100nM AZ12253801 (\*\*p<0.001).