LETTERS

Glioma stem cells promote radioresistance by preferential activation of the DNA damage response

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Ionizing radiation represents the most effective therapy for glioblastoma (World Health Organization grade IV glioma), one of the most lethal human malignancies[1], but radiotherapy remains only palliative[2] because of radioresistance. The mechanisms underlying tumour radioresistance have remained elusive. Here we show that cancer stem cells contribute to glioma radioresistance through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. The fraction of tumour cells expressing CD133 (Prominin-1), a marker for both neural stem cells and brain cancer stem cells[3–6], is enriched after radiation in gliomas. In both cell culture and the brains of immunocompromised mice, CD133-expressing glioma cells survive ionizing radiation in increased proportions relative to most tumour cells, which lack CD133. CD133-expressing tumour cells isolated from both human glioma xenografts and primary patient glioblastoma specimens preferentially activate the DNA damage checkpoint in response to radiation, and repair radiation-induced DNA damage more effectively than CD133-negative tumour cells. In addition, the radioresistance of CD133-positive glioma stem cells can be reversed with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases. Our results suggest that CD133-positive tumour cells represent the cellular population that confers glioma radioresistance and could be the source of tumour recurrence after radiation. Targeting DNA damage checkpoint response in cancer stem cells may overcome this radioresistance and provide a therapeutic model for malignant brain cancers.

Glioblastomas are the most lethal primary brain tumour with a median survival of less than 12 months because of resistance to radiation and other treatments[1]. Glioblastomas present as diffuse tumours with invasion into normal brain, but frequently recur or progress after radiation as focal masses[2], suggesting that only a fraction of tumour cells is responsible for regrowth. Identification of a crucial cellular subpopulation of brain tumour cells with potent tumorigenic activity[3–5,7] supports the cancer stem cell hypothesis in solid tumours. As glioma subpopulations expressing Prominin-1 ($CD133^+$) are enriched for cancer stem cells and show greater tumorigenic potential than do $CD133^-$ cells[3–5], we have examined the role of glioma cancer stem cells in the development of radioresistance.

We found that ionizing radiation (IR) treatment of short-term cultures from human glioma xenografts enriched the $CD133^+$ subpopulation fourfold relative to untreated cultures (Fig. 1a, b). Likewise, glioma xenografts irradiated *in vivo* were enriched 3–5-fold for $CD133^+$ cells relative to untreated xenografts (Fig. 1c). Similar results were seen in freshly isolated glioblastoma tumour specimens: the basal fraction of $CD133^+$ cells was 2–3% and increased to 6–10% after IR treatment (Fig. 1d). Irradiation did not induce CD133 expression in $CD133^-$ tumour cells (Supplementary Fig. S1), confirming that increased $CD133^+$ fractions after IR were caused by enrichment of original $CD133^+$ subpopulations. In addition, two radioresistant human glioma cell subpopulations derived from short-term cultures of glioma xenografts subjected to three serial cycles of IR also contained greater percentages of $CD133^+$ cells than parental populations (Supplementary Fig. S2). Thus, tumours surviving IR are enriched in $CD133^+$ cancer cells.

To define the biological significance of $CD133^+$ enrichment after IR, we implanted a constant number of tumour cells with increasing percentages of $CD133^+$ cells into the frontal lobes of immunocompromised mice. Increased $CD133^+$ cell fractions dose-dependently decreased tumour latency and enhanced tumour growth and vascularity (Fig. 1e, f, and data not shown). Consistent with these results, viable tumour cells from irradiated xenografts were enriched for $CD133^+$ cells and formed secondary tumours with decreased latencies relative to untreated xenografts (Supplementary Fig. S3). Thus, enrichment of $CD133^+$ cells is crucial in glioma recurrence after radiotherapy.

To confirm that purified $CD133^+$ subpopulations are enriched for cancer stem cells, we characterized $CD133^+$ and $CD133^-$ cells derived from glioma xenografts and primary human glioblastoma samples. Murine host contamination of $CD133^-$ cellular subpopulations from xenografts was ruled out with the pan-human antibody 3B4, which binds to a glycoprotein only on human cells[8] (Supplementary Fig. S4). The neoplastic origin of cells derived from the human biopsy specimens was confirmed through fluorescent *in situ* hybridization (FISH) analysis of genetic markers altered in the original patient tumour specimen (Supplementary Fig. S5 and data not shown). $CD133^+$ tumour cells showed characteristics consistent with cancer stem cells[3–7,9–11]: namely, neurosphere formation (Fig. 2a); expression of neural and/or cancer stem cell markers, including CD133, Sox2, Musashi and Nestin (Fig. 2b); and multilineage differentiation with markers for astrocytes (GFAP, S100β), neurons (Map-2, TUJ1) or oligodendrocytes (O4, GalC) (Supplementary Figs S6 and S7a). $CD133^+$ cells derived from xenografts or biopsy specimens formed neurospheres (76–89%; Supplementary Table 1), whereas $CD133^-$ cells rarely formed neurospheres. $CD133^+$ tumour cells were highly tumorigenic in brains of immunocompromised mice with characteristics of glioblastomas (Fig. 2c–e and Supplementary Table 1) in concordance with previous reports[3–5]. $CD133^-$ cells did not form detectable tumours even when implanted at 2×10^6 cells per mouse, except for occasional tumours from a single xenograft source (D456MG paediatric xenografts; Fig. 2c and Supplementary Table 1). Of note, we used a shorter incubation period in our mouse studies than some other researchers[3,7]. Longer incubation periods may permit greater regrowth of poorly

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tumorigenic cells but increases the probability of secondary genetic changes. Thus, $CD133^+$ subpopulations derived from patient glioma specimens and xenografts are enriched for characteristics of cancer stem cells, including tumorigenesis *in vivo*.

To identify further the cell subpopulations that contribute to glioma radioresistance, we studied the radiosensitivity of $CD133^+$ and $CD133^-$ tumour cell subpopulations. Colony formation assays confirmed that $CD133^+$ cells isolated from xenografts and a biopsy specimen were more resistant to IR treatment than were corresponding $CD133^-$ cells (Fig. 3a and Supplementary Fig. S8a). The differential radioresistance between the two subpopulations was consistent regardless of the presence of growth factors (Supplementary Figs S9 and S10). The preferential survival of $CD133^+$ cells after irradiation was due to lower rates of apoptosis, as indicated by decreased activation of caspase-3 in $CD133^+$ cells from irradiated cultures or xenografts (Fig. 3b and Supplementary Fig. S8b). In addition, annexin V staining showed that IR-induced apoptosis in $CD133^+$ cells was 4–5-fold lower than that in matched $CD133^-$ cells isolated from primary glioblastomas (Supplementary Fig. S11).

To verify the preferential survival of $CD133^+$ cells after IR, matched $CD133^+$ and $CD133^-$ cells from a xenograft or patient biopsy specimen were differentially labelled with fluorescent dyes[12,13] and mixed in defined ratios. In the absence of IR, the relative percentage of cells derived from $CD133^+$ cells increased only modestly over time. By contrast, the percentage of $CD133^+$-derived cells after irradiation increased more than fourfold (Fig. 3c, d, and Supplementary Fig. S12), confirming that $CD133^+$ tumour cells have greater radioresistance and repopulation potential than do $CD133^-$ cells *in vitro*.

We examined the capacity of $CD133^+$ glioma cells to form tumours after irradiation in several assays. In an *in vivo* limiting dilution tumour formation assay, irradiated $CD133^+$ cells from a

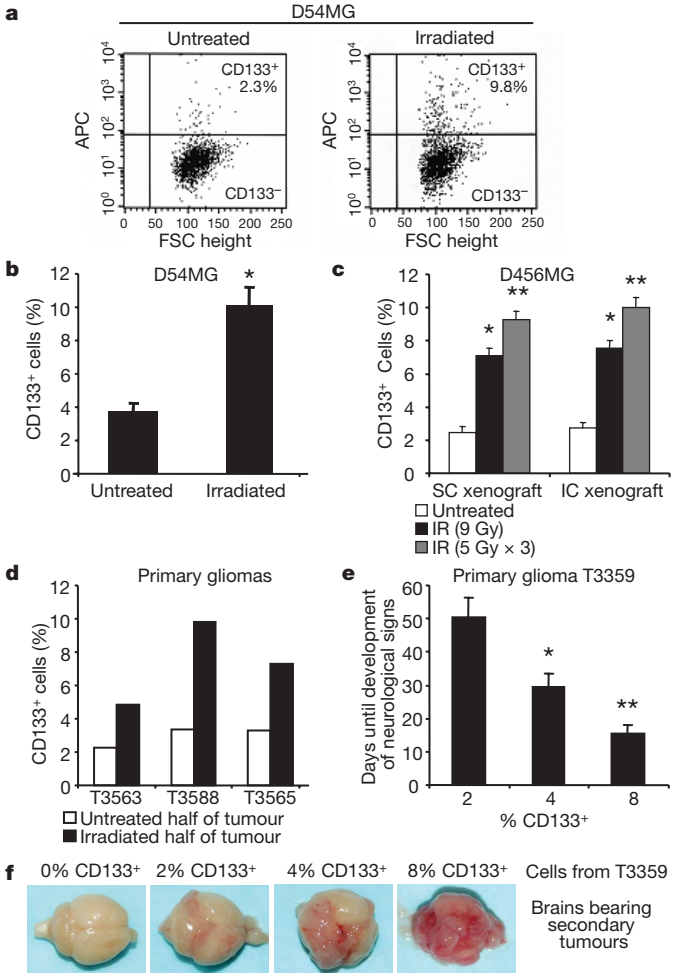


Figure 1 | Enrichment of $CD133^+$ tumour subpopulations after irradiation *in vitro* and *in vivo*, and enhancement of intracranial tumour formation by increased $CD133^+$ fraction. **a**, Glioma cultures from D54MG xenografts were untreated or irradiated (5 Gy). $CD133^+$ fractions were assayed by FACS after 48 h. **b**, Mean ± s.d. results from **a** ($n = 3$; $^*P < 0.001$). **c**, Subcutaneous (SC) and intracranial (IC) D456MG xenografts were irradiated (3×5 Gy or 1×9 Gy) or untreated. $CD133^+$ fractions were quantified after 48 h (mean ± s.d., $n = 3$; $^*P < 0.002$; $^{**}P < 0.001$). **d**, Individual patient tumour specimens were halved and either irradiated with 2 Gy or untreated. The $CD133^+$ fraction in each sample was quantified by FACS. **e**, **f**, $CD133^+$ and $CD133^-$ cells from glioblastoma specimen T3359 were mixed in different ratios and xenotransplanted into mouse brains (100,000 total cells per mouse). **e**, Survival until development of neurological signs (mean ± s.d., $n = 5$; $^*P < 0.002$; $^{**}P < 0.001$). **f**, Representative images of brains bearing secondary tumours.

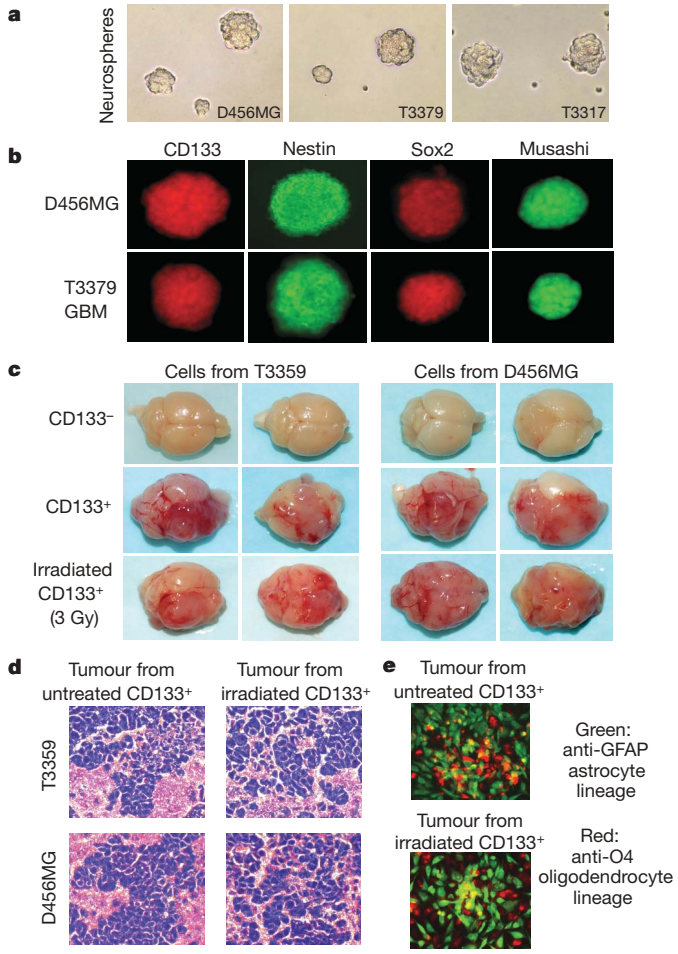


Figure 2 | Characterization of $CD133^+$ and $CD133^-$ cells from human glioma xenografts and primary glioblastoma specimens. **a**, Representative images of neurospheres from $CD133^+$ cells from D456MG xenografts and glioblastoma specimens (T3379, T3317). **b**, Neurospheres from **a** expressed neural stem cell markers (CD133, Nestin, Sox2 and Musashi), as assessed by immunofluorescence. **c**, Untreated or irradiated (3 Gy) $CD133^+$ cells (10^4) from glioblastoma specimen T3359 and from D456MG xenografts were transplanted into brains of immunocompromised mice ($n = 5$). Mice were killed on development of neurological signs or after 8 weeks. $CD133^+$ cells formed haemorrhagic masses regardless of irradiation. T3359 $CD133^-$ cells did not generate tumours. D456MG $CD133^-$ cells (2×10^6) formed small tumours in two out of five brains. **d**, Representative photomicrographs of tumours from **c** (stained with haematoxylin and eosin). **e**, Immunofluorescent staining of frozen sections of the tumours generated by untreated and irradiated $CD133^+$ cells in **c**. Cells were stained for the GFAP astrocyte marker (green) and the O4 oligodendrocyte marker (red).

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xenograft or patient specimen treated with a clinically relevant IR dose (2 Gy) formed tumours with similar potency to untreated $CD133^+$ cancer cells, although treatment of $CD133^+$ cells with 5 Gy of IR reduced tumorigenecity (Supplementary Table 2). 2-Gy-irradiated $CD133^+$ tumour cells derived from human glioblastoma biopsy specimens or xenografts formed tumours with similar latencies to non-irradiated $CD133^+$ tumour cells (Fig. 2c–e and Supplementary Fig. S13a). In addition, irradiated $CD133^+$ cells retained multilineage differentiation potential (Supplementary Fig. S7b) and formed tumours with heterogeneous tumour cell populations expressing markers of different lineages *in vivo* (Fig. 2d, e). Viable $CD133^+$ cells from *in vivo* irradiated xenografts or patient specimens transiently grown in mice formed secondary tumours with similar latencies to $CD133^+$ tumour cells from matched non-irradiated control tumours (Supplementary Fig. S13b). By contrast, the same dose of irradiation abolished the tumorigenic capacity of D456MG $CD133^-$ tumour cells that showed weak basal tumour formation (Supplementary Fig. S13c). Thus, $CD133^+$ glioma cell subpopulations are enriched with cancer stem cells resistant to radiation *in vitro* and *in vivo* in comparison to matched $CD133^-$ glioma cells.

Although IR damages tumour cells through several mechanisms, IR kills cancer cells primarily through DNA damage. Thus, DNA damage checkpoint responses play essential roles in cellular radiosensitivity[14–20]. To determine the role of DNA damage checkpoint responses in glioma cancer stem cell radioresistance, we compared early DNA damage checkpoint responses in $CD133^+$ and $CD133^-$ glioma tumour cell subpopulations. In both $CD133^+$ and $CD133^-$ cells derived from human glioma xenografts, DNA damage induced by IR or a radiomimetic, neocarzinostatin, potently initiated activating phosphorylation of the ataxia-telangiectasia-mutated (ATM), Rad17, Chk1 and Chk2 checkpoint proteins (Fig. 4a and Supplementary Fig. S14a). However, activating phosphorylation of these checkpoint proteins was significantly higher in $CD133^+$ cells than in $CD133^-$ cells (Fig. 4a and Supplementary Fig. S14a), indicating that $CD133^+$ cells show greater checkpoint activation in response to DNA damage. Similarly, $CD133^+$ cells isolated from several human glioblastoma specimens showed a marked increase in activation of several checkpoint proteins in response to DNA damage relative to $CD133^-$ cells (Fig. 4b, c, and Supplementary Fig. S14b). Rad17, a

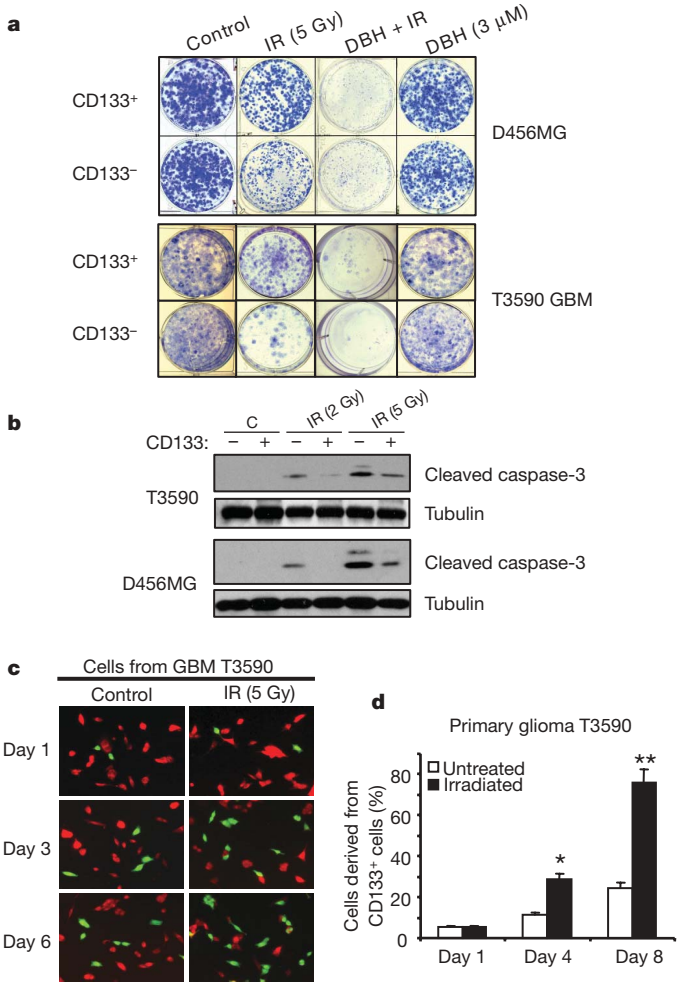


Figure 3 | $CD133^+$ tumour cells show radioresistance and lower sensitivity to radiation-induced apoptosis than $CD133^-$ tumour cells dependent on checkpoint kinase activity. **a**, $CD133^+$ and $CD133^-$ tumour cells were untreated or treated with 5 Gy of IR, a Chk1/Chk2 low molecular weight inhibitor (debromohymenialdisine, DBH, 3 μM), or a combination of both. Representative images of colony formation are shown. **b**, $CD133^+$ and $CD133^-$ cells derived from primary glioblastoma T3590 or D456MG xenografts were untreated or irradiated with 2 Gy or 5 Gy of IR. Whole-cell lysates were collected after 24 h and immunoblotted for cleaved caspase-3, an indicator of cell apoptosis. **c**, $CD133^+$ and $CD133^-$ cells derived from primary glioblastoma T3590 were labelled separately with CFSE (green) and CMTRX (red) fluorescent dyes, mixed in defined ratios (5% $CD133^+$), and visualized by fluorescent microscopy at the indicated time points. **d**, Mean ± s.d. results from **c** ($n = 100$ cells in three trials; $^*P < 0.002$; $^{**}P < 0.001$).

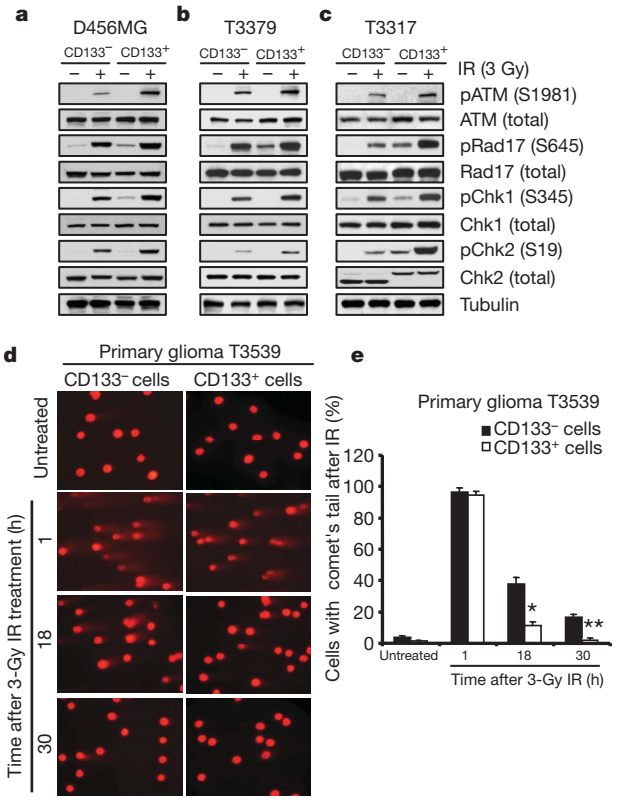


Figure 4 | $CD133^+$ glioma cells preferentially activate the DNA damage checkpoint and repair IR-induced DNA damage more efficiently than $CD133^-$ cells. **a–c**, The activation state of the checkpoint response in matched $CD133^+$ and $CD133^-$ cells from glioma D456MG xenografts (**a**) and glioblastoma specimens (**b**, **c**) was assessed before treatment (−) and 1 h after 3 Gy of IR (+). Whole-cell lysates were immunoblotted for phosphorylated and total amounts of checkpoint proteins (ATM, Rad17, Chk2 and Chk1). **d**, $CD133^+$ and $CD133^-$ cells derived from primary glioblastoma T3539 were irradiated with 3 Gy of IR. The presence of DNA damage at sequential time points after damage was assessed by single-cell gel electrophoresis assay under alkaline conditions (alkaline comet assay). **e**, Quantification of the percentages of cells with comet tails at different time points after IR in $CD133^+$ and $CD133^-$ populations. Data are the means ± s.d. ($n = 100$ cells in three trials; $^*P < 0.001$; $^{**}P < 0.002$).

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crucial regulator of the DNA damage checkpoint[19], also showed significantly greater baseline phosphorylation of a key regulatory residue (Ser 645) in $CD133^+$ cells than in $CD133^-$ cells in most cases, suggesting that the $CD133^+$ subpopulation may be primed to respond to DNA damage. In addition, IR distinctly increased ATM kinase activity in the $CD133^+$ subpopulation derived from primary glioblastoma biopsy specimens in response to IR (Supplementary Fig. S14c). These data show that $CD133^+$ glioma cells can activate checkpoint responses to a greater extent than $CD133^-$ cells, suggesting that the resistance of $CD133^+$ cells to IR is due to preferential checkpoint activation.

The primary downstream effect of checkpoint activation is to induce cell-cycle arrest to repair damaged DNA. We compared the recovery of $CD133^+$ and $CD133^-$ cells derived from both xenografts and biopsy specimens in response to IR-induced DNA damage using the alkaline comet assay[21]. $CD133^+$ and $CD133^-$ cells from glioma xenografts or human patient biopsy specimens were equally susceptible to DNA damage to IR initially, but the percentage of cells with comet tails decreased 4–9 times more rapidly in $CD133^+$ cells than in matched $CD133^-$ cells (Fig. 4d, e, and Supplementary Fig. S15), indicating that $CD133^+$ cells repaired the DNA damage more efficiently than $CD133^-$ cells. This result was further confirmed by assessing the resolution of phosphorylated histone 2AX nuclear foci[22] after IR (Supplementary Fig. S16). The ability to repair DNA damage is essential to cellular survival because maintained DNA breaks induce apoptosis or senescence[14–16]. Thus, the $CD133^+$ tumour cells would be expected preferentially to survive radiation to repopulate the tumour.

To determine the contribution of the preferential activation of DNA damage checkpoint to the increased survival of $CD133^+$ cells after IR administration, we assessed the ability of an inhibitor, debromohymenialdisine (DBH), of checkpoint kinases Chk1 and Chk2 (ref. 23) to prevent $CD133^+$ cell resistance to IR. Pretreatment with DBH minimally impacted the proliferation of both $CD133^+$ and $CD133^-$ cells, but DBH treatment showed synergy with IR to disrupt the radioresistance of $CD133^+$ cells *in vitro* (Fig. 3a). Similar results were obtained in *in vivo* mouse studies (Supplementary Fig. S17). These data confirm that preferential checkpoint response in $CD133^+$ cancer cells is closely associated with the cellular resistance to radiation.

Together, our results show that $CD133^+$ cancer cells contribute to glioma radioresistance and tumour repopulation through preferential checkpoint response and DNA repair, and targeting of checkpoint response in $CD133^+$ cancer cells can overcome glioma radioresistance *in vitro* and *in vivo*, which may provide a therapeutic advantage to reduce brain tumour recurrence. The specific molecular mechanism for the resistance of cancer stem cells to IR can be linked to the function of the DNA damage checkpoint. As the cell cycle of a normal stem cell is tightly controlled by the checkpoint to maintain genomic stability and integrity, the defective checkpoint responses associated with early cancer development[24,25] implicate abnormal checkpoint control as a potential contributor to the transformation of normal cells into cancer stem cells. Future studies may define similar contributions of the cancer stem cell to therapeutic responses in other solid cancers with similar molecular mechanisms. Therapies targeted to the checkpoint kinases in preclinical and clinical development may provide a specific method to disrupt this resistance mechanism to improve overall tumour control with radiation treatment.

METHODS

Additional and more detailed methods are presented in the Supplementary information.

Human glioma xenografts and glioblastoma specimens. D54MG xenografts were derived from A172. D456MG xenografts were derived from a paediatric glioblastoma biopsy directly implanted into immunocompromised mice. Primary glioblastoma samples (designated T3xxx) were obtained from patients undergoing resection in accordance with a protocol approved by the Duke University Medical Center Institutional Review Board.

Isolation of $CD133^+$ and $CD133^-$ tumour cells. Matched subpopulations were separated as described[3,5] with modifications[26].

Alkaline comet assay. DNA damage repair was assessed by single-cell gel electrophoresis assay under alkaline conditions as described[21].

Immunofluorescent staining. Immunofluorescent staining for neural stem cell markers (CD133, Nestin, Sox2 and Musashi) and differentiation markers (GFAP and S100β, astrocytes; O4 and GalC (Galactocerebroside), oligodendrocytes; Map-1 and TUJ1, neurons) was done as described[27].

Radiation treatment. Cells or mice were irradiated at indicated doses with an X-RAD 320 irradiation system (AGFA).

***In vitro* cell mixing and repopulation.** $CD133^+$ derived from glioma xenografts or primary glioblastoma specimens were labelled with CellTracker CFSE green fluorescent dye (Molecular Probes), and the $CD133^-$ cells were labelled with the CellTracker Red CMTPX (Molecular Probes) separately according to the instructions, and then mixed in defined ratios. Triplicate parallel cultures were left untreated or treated with IR (5 Gy). The resulting growth patterns of each tumour cell population at indicated time point was visualized by fluorescent microscopy and analysed by FACS.

Western analysis. Immunoblotting was done as described[18,19].

Intracranial tumour assays. Intracranial transplantation of $CD133^+$ or $CD133^-$ cells into brains of nude mice was done as described[3,26].

Statistical analysis. The level of significance was determined by a two-tailed Student's *t*-test or analysis of variance with $\alpha = 0.05$ (GraphPad software). All quantitative data presented are the mean ± s.d. from at least three samples per data point.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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