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Methyltransferases mediate cell memory of a genotoxic insult

Rebecca E. Rugo[1], **James T. Mutamba**[1], **K. Naga Mohan**[2], **Tiffany Yee**[1], **J. Richard Chaillet**[2], **Joel S. Greenberger**[3], and **Bevin P. Engelward**[1]

[1]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

[2]Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA

[3]Department of Radiation Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA

Abstract

Characterization of the direct effects of DNA damaging agents shows how DNA lesions lead to specific mutations. Yet, serum from Hiroshima survivors, Chernobyl liquidators, and radiotherapy patients can induce a clastogenic effect on naive cells, showing indirect induction of genomic instability that persists years after exposure. Such indirect effects are not restricted to ionizing radiation, as chemical genotoxins also induce heritable and transmissible genomic instability phenotypes. While such indirect induction of genomic instability is well described, the underlying mechanism has remained enigmatic. Here, we show that mouse embryonic stem (ES) cells exposed to γ-radiation remember the insult for weeks. Specifically, conditioned media from progeny of exposed cells can induce DNA damage and homologous recombination in naive cells. Notably, cells exposed to conditioned media also elicit a genome destabilizing effect on their neighbours, thus demonstrating transmission of genomic instability. Moreover, we show that the underlying basis for the memory of an insult is completely dependent on two of the major DNA cytosine methyltransferases (MTases), Dnmt1 and Dnmt3a. Targeted disruption of these genes in exposed cells completely eliminates transmission of genomic instability. Furthermore, transient inactivation of *Dnmt1*, using a tet-suppressible allele, clears the memory of the insult, thus protecting neighbouring cells from indirect induction of genomic instability. We have thus demonstrated that a single exposure can lead to long-term, genome destabilizing effects that spread from cell to cell and we provide a specific molecular mechanism for these persistent bystander effects. Collectively, our results impact current understanding of risks from toxin exposures and suggest modes of intervention for suppressing genomic instability in people exposed to carcinogenic genotoxins.

Introduction

It is well established that DNA damaging agents, such as ionizing radiation and chemical genotoxins, can directly induce mutations that in turn promote cancer and ageing (Friedberg, 2006; Hoeijmakers, 2009). Less well understood, but increasingly appreciated, are the indirect effects of such exposures on genomic stability. For example, cells can suffer a persistent, increased frequency of mutations, many cell generations after the original exposure (Kadhim, 1992; Little *et al*., 1990). Additionally, naïve cells cultured in the

Correspondence and requests for materials should be addressed to B.P.E. (bevin@mit.edu).

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presence of the descendents of exposed cells similarly display an increased frequency of genetic changes (Huo, 2001; Nagasawa, 1992; Zhou *et al*., 2000). These indirect effects of exposure to DNA damaging agents are conventionally described as persistent or bystander effects (Bender, 1962; Morgan, 2003).

A variety of phenotypes have been observed to persist, long after an initial genotoxic exposure. A classic example is delayed reproductive cell death, and reduced plating efficiency, which can persist for more than fifty generations after exposure (Chang and Little, 1992). In addition, de novo genetic changes occur many cell divisions after exposure (Kadhim, 1992; Pampfer, 1989; Seymour and Mothersill, 2004). As with persistent effects, many different phenotypes have been associated with the bystander effect. Naïve bystander cells cultured in the presence of either cells that have been previously exposed to a genotoxic agent, or to media from exposed cultures, are prone to genomic instability, toxicity and malignant transformation (Huo, 2001; Lewis, 2001; Little, 2003; Nagar, 2003; Nagasawa, 1992; Zhou *et al*., 2000).

An understanding of the mechanisms involved in persistent and transmissible responses to genotoxins is clearly important to human health, given the ubiquitous presence of DNA damaging agents endogenously, in our environment, and in the clinic. Indeed, since the initial discovery of genotoxicity-associated persistent and bystander phenotypes, the underlying causes, physiological impact, and mechanistic aetiology of these responses have been intensively studied (Morgan and Sowa, 2005; Mothersill and Seymour, 2005; Mothersill, 2006). Traditionally, persistent and bystander phenotypes have been studied in response to high doses of ionizing radiation (Mothersill, 2001). However more recently, these phenotypes have also been generated by non-ionizing radiation e.g. ultra violet (UV) radiation (Limoli, 1998; Mothersill, 1998), reactive oxygen and nitrogen species (Azzam, 2002; Dickey, 2009), cytokines (Dickey, 2009) and other genotoxic, chemical exposures (Rugo, 2005). Thus, because endogenously generated chemical species (e.g. cytokines and reactive oxygen and nitrogen species) and exogenous agents to which cells are physiologically exposed (e.g. UV and low dose IR radiation), are capable of initiating persistent and bystander phenotypes alike, it is reasonable to posit that these responses represent normal, physiologically relevant, cellular responses to stressors. Consistent with this viewpoint, are observations of persistent and bystander phenotypes not only at the cellular, but at the tissue (Goldberg, 2002; Koturbash, 2006; Mothersill, 2002; Pant, 1977; Watson *et al*., 2000a) and even organism level of organisation (Mothersill *et al*., 2007). Further, these responses appear to be evolutionarily conserved across different kingdoms and species (Yang et al., 2008).

Intense interest in the underlying mechanism of the bystander effect has prompted studies that have revealed many of the agents capable of inducing persistent and bystander phenotypes, as discussed above. Much less is known, however, about the mechanism by which cells retain and consequently transmit ‘memory’ of an insult, becoming genomically unstable for a long time after exposure. Earlier work in our laboratory showed that genomic instability was transmissible from one cell to the next (i.e., a bystander can induce genomic instability in a naïve cell over multiple generations) (Rugo, 2005). This transmission of genomic instability, while implying heritability, clearly is not consistent with genetic inheritance. Thus our findings, and related observations by others (e.g., (Kovalchuk, 2008; Lorimore, 2003), suggest that persistent and bystander effects might be propagated by an hitherto unknown epigenetic mechanism.

Epigenetic mechanisms of heredity include DNA methylation, histone modification and the functions of certain non-coding RNAs (Goldberg, 2007). Importantly, DNA methylation has been implicated in heritable, persistent changes in phenotype. For example, the persistent

and heritable change in coat colour, and conferred obesity-resistance in the progeny of female mice that were fed genistein during gestation, were found to be DNA methylation-dependent (Dolinoy, 2006). Here, we show that DNA methyltransferases (DNMTs), the enzymes responsible for the epigenetic methylation of mammalian DNA, mediate the propagation of an instability phenotype on exposure to a genotoxin. Specifically, we find that DNA methyltransferases 1 and 3a mediate murine embryonic stem (ES) cell memory of an exposure to ionizing radiation.

Results and discussion

Ionizing radiation is of great societal importance, both in the context of the environment and the clinic. To learn if γ-radiation leads to persistent transmissible instability in ES cells, DNA damage was assessed in bystanders. Naive cells, designated primary bystanders (Fig. 1a), that shared media with cells descended from irradiated cultures showed increased DNA damage (Fig. 1b), while naïve cells that shared media with sham-irradiated cells showed no increase in DNA damage by comet assay. To test for transmission of this DNA damage, a second group of naive WT cells, designated secondary bystanders, were co-cultured with the primary bystanders (Fig. 1a). These secondary bystanders had increased DNA damage when exposed to media from primary bystanders to irradiated cells (Fig. 1c), thus demonstrating transmission of radiation-induced genomic instability from exposed cells, to naive cells (primary bystanders), to naive cells (secondary bystanders).

To determine whether persistent instability results from methyltransferase-dependent epigenetic changes, we exploited the fact that ES cells do not require genome methylation for viability, and are readily cultured, following disruption of the three major DNA MTases: Dnmt1, Dnmt3a and Dnmt3b (Tsumura, 2006). During normal development, the Dnmt3a and Dnmt3b *de novo* MTases catalyze the transfer of a methyl group from S-adenosyl methionine to the 5 position of cytosine at CpG sites (Chen, 1991). Methylation is maintained primarily through the activity of Dnmt1, which efficiently methylates hemimethylated CpG sites (Stein, 1982). Dnmt1 is essential for heritable, epigenetically regulated changes in gene expression that are key to differentiation and development (Li, 1992). To test the possible role of MTases in cellular memory of an insult, we asked if *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells (gift of M. Okano) were able to remember and transmit genomic instability following γ-radiation. Results show that descendents of irradiated *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells were not able to induce DNA damage by comet assay in neighbouring cells, when compared to WT cells (Fig. 2a).

One of the earliest descriptions of a bystander effect in cultured cells revealed that when ~1% of nuclei were irradiated, over 30% of the cells had increased homologous recombination, detected as sister chromatid exchanges (SCEs) (Nagasawa, 1992). Furthermore, ionizing radiation induces a persistent increase in homologous recombination (Huang *et al*., 2007). We therefore asked if SCEs are induced by the progeny of irradiated WT and *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells. Naive cells indeed exhibited a significant increase ($p<0.0001$) in the frequency of SCEs when they shared media with descendents of irradiated cultures (Fig. 2b). However, there was only a very slight, yet significant ($p=0.0168$), increase in SCEs when the irradiated cells were *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells, compared to mock irradiated *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells. Interestingly, SCEs were increased in cells that shared media with unirradiated *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- ES cells, compared to cells that shared media with unirradiated WT ES cells (Fig. 2b). Given that *Dnmt1*-/- cells are genomically unstable (Chen *et al*., 1998; Kim *et al*., 2004), *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- ES cells may be similarly unstable and may thus elicit transmissible responses, analogous to the effects of irradiation. Regardless, progeny of irradiated *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells are less able than WT cells to

induce genomic instability in naive neighbours, showing that one or more MTases are essential for persistent radiation-induced instability.

To discern the roles of individual MTases, we analyzed ES cells carrying targeted disruptions of each MTase (gift of E. Li) (Lei *et al*., 1996; Okano *et al*., 1999). The MTase deficient cells have normal sensitivity to radiation toxicity (data not shown). Interestingly, γ-radiation had no effect on SCEs in primary bystanders to irradiated *Dnmt1*-/- cells, when compared to bystanders to unirradiated *Dnmt1*-/- cells (Fig. 3). The inability of the *Dnmt1*-/- cells to sustain a heritable phenotypic change is consistent with their hypomethylated phenotype (Lei *et al*., 1996). In addition, *Dnmt1*-/- cells induce homologous recombination in neighbouring cells, even without irradiation, which is consistent with their instability phenotype (Chen *et al*., 1998; Kim *et al*., 2004). Although Dnmt1 is a maintenance MTase, it is possible that Dnmt1's ability to perform *de novo* methylation in response to DNA damage (Mortusewicz *et al*., 2005) contributes to the transmissible instability. Similar to *Dnmt1*-/- cells, *Dnmt3a*-/- cells did not transmit genomic instability (Fig. 3), indicating that Dnmt3a-mediated *de novo* methylation is necessary for cells to remember and transmit an instability phenotype. Interestingly, as with *Dnmt1*-/-, *Dnmt3a*-/- cells caused an increase ($P<0.0001$) in SCEs in bystanding WT cells in the absence of radiation, when compared to SCE levels in WT bystanders to unirradiated WT cells. Lastly, unlike Dnmt1 and Dnmt3a, Dnmt3b was not essential for transmissible instability, as a deficiency in this gene still resulted in transmission of an instability phenotype (Fig. 3).

The observation that genomic instability is induced by unirradiated *Dnmt1*-/- and *Dnmt1*-/-; *Dnmt3a*-/-; *Dnmt3b*-/- cells suggested a possible threshold that prevents further induction of instability after irradiation. We hypothesized that transient loss of Dnmt1 might prevent memory of genotoxic exposure, while protecting bystanders from the instability due to Dnmt1 loss. To test this hypothesis, we exploited mouse ES cells carrying a tetracycline repressible *Dnmt1* allele (Borowczyk *et al*., in press). By three days post doxycycline treatment, Dnmt1 was undetectable, and within three days after removing doxycycline, Dnmt1 expression resumed (Fig. 4a). To suppress Dnmt1 expression before, during and after irradiation, we added doxycycline three days before irradiation and sustained it for seven days. Doxycycline was then removed to restore *Dnmt1* expression (Fig. 4a). Consistent with previous results (Figures 2 and 3), the descendants of irradiated WT cells induced homologous recombination in neighbouring cells. However, under conditions where *Dnmt1* was transiently suppressed, descendents of irradiated cells were not able to induce homologous recombination in their neighbours (Fig. 4b). Importantly, unlike the cells that carried disrupted *Dnmt1* alleles, cells transiently suppressed for Dnmt1 do not induce instability in their neighbours.

The transmissibility of genomic instability through shared media has important implications when considering potential tissue-wide responses *in vivo*. To explore transmissibility, we studied secondary bystanders. Primary bystanders were able to induce homologous recombination in naive cells only if the irradiated target cells had had normal *Dnmt1* expression (Fig. 4b). Thus, transient suppression of *Dnmt1* prevented transmission of instability both to naïve primary bystanders, and to their secondary bystander neighbours.

Characterizing the underlying causes of genomic instability is fundamental in cancer aetiology, prevention of premature ageing, and for understanding the risks of exposures. It is becoming increasingly clear that indirect mechanisms of mutation induction that involve changes in cellular behaviour, in addition to the directly induced DNA lesions, can lead to an increased risk of disease-causing mutations for months or even years after exposure (Lorimore *et al*., 2003; Maxwell *et al*., 2008; Morgan, 2003; Mothersill and Seymour, 2001;

Pant, 1977). Furthermore, at least one study suggests that the extent of bystander-induced DNA damage can be as great as that of the original exposure (Dickey *et al*., 2009).

While the studies described here do not query the exact mechanism by which DNA methylation results in persistent bystander phenotypes, it is possible that changes in gene expression, mediated by DNA methyltransferases (Hermann, 2004), cause cells to secrete factors that impact genomic stability. Specifically, DNA damage is known to alter Dnmt1 and Dnmt3a activity (Maltseva, 2009; Mortusewicz, 2005) and DNA damage can also alter secretion profiles (Rodier *et al*., 2009). Additionally, it is known that cells that secrete TNF-alpha, $NO^{\cdot}$ and TGF-beta can induce DNA damage in nearby cells (Burr, 2010; Dickey, 2009). Thus, as a result of exposure to secreted, genotoxic species, bystander cells could adopt a methylation pattern similar to that of the target cell, and thus both remember and transmit a bystander phenotype. The memory of the genotoxic insult would therefore be stored structurally in DNA in the form of DNA methylation patterns that are created and maintained by DNA methyltrasferases (e.g., Dnmt1 and Dnmt3a). Propagation of the bystander phenotype could then be effected by a change in the secretion profile of the insulted cell. Interestingly, in normal tissues, communication among cells helps to control cell behaviour. Bystander effects may similarly reflect a coordinated response.

The observation that genomic instability can be transmitted from cell to cell, both *in vitro* (Lorimore *et al*., 2003; Mothersill and Seymour, 2004; Nagasawa and Little, 1992) and *in vivo* (Lorimore *et al*., 2005; Watson *et al*., 2000b), opens the possibility that there are tissue wide changes in genomic stability following exposure to a genotoxin, and calls attention to the possibility that persistent and bystander effects are critical risk factors for disease. Here, we have demonstrated that two of the three major MTases, Dnmt1 and Dnmt3a, are essential in order for descendents of irradiated cells to become able to transmit genomic instability to naive cells. Furthermore, we have shown that by temporarily turning off expression of Dnmt1, it is possible to completely eliminate transmission of genomic instability. Interestingly, and indeed consistent with these findings, Dnmt1 and 3a have also recently been shown to play important roles in neurological memory and learning (Feng *et al*., 2010). This finding, albeit apparent specifically in neurons, may represent a general mechanism by which cells store information on, and adapt to, genotoxic and other stimuli.

In conclusion, knowledge of the molecular basis for transmission of genomic instability opens the doors to novel interventions, including the potential administration of Dnmt inhibitors in conjunction with cancer chemotherapy to preserve tissue-wide genomic stability and thus suppress secondary cancers.

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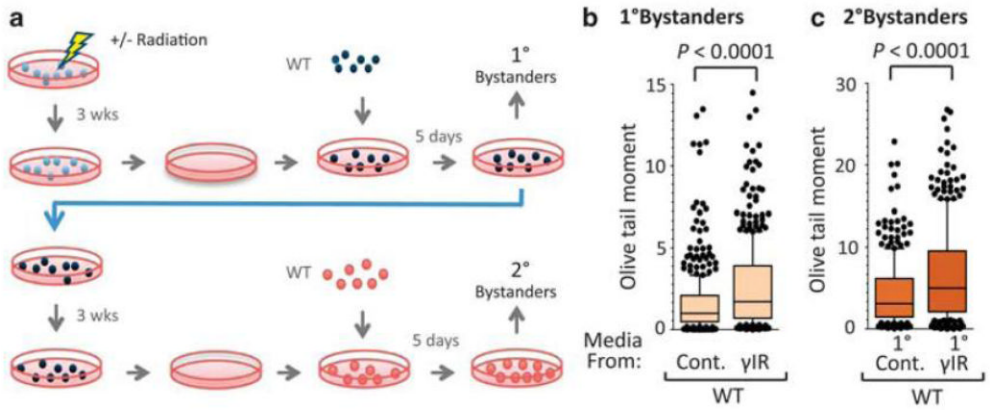

Figure 1. Persistent and transmissible induction of genomic instability

a, Irradiated (or mock irradiated) target ES cells are cultured for three weeks. During the three weeks, cells were passaged three times a week at densities of $0.5\text{-}2\times10^6$ cells per $55mm^2$ dish. After three weeks, 6×10^4 naive WT ES cells subsequently shared media with the progeny of target ES cells for 5 days (to create primary bystanders). Primary bystanders were then cultured for another three weeks. Naive WT ES cells then shared media with the progeny of primary bystanders (to create secondary bystanders). Media was shared via co-culture, employing 1 μm transwell inserts (Corning), or by exposure to conditioned media (filtered [0.25μm]; 1:1, fresh media:conditioned media). ES cells were exposed to ionizing radiation (3 Gy) using a Co-60 source (73 cGy/min). DNA damage was assessed by the alkaline comet assay (Olive, 2006) in primary (**b**), and secondary (**c**), bystanders. For all comet analysis, >100 nucleoids were analyzed per condition using Komet 5.5 (Andor Technology, Ireland) and *P* values were produced by a two-tailed Mann-Whitney. For comet studies, boxes represent the quartiles, whiskers mark the 10^{th} and 90^{th} percentiles, and the median is indicated. For all studies, data was combined from three or more independent experiments.

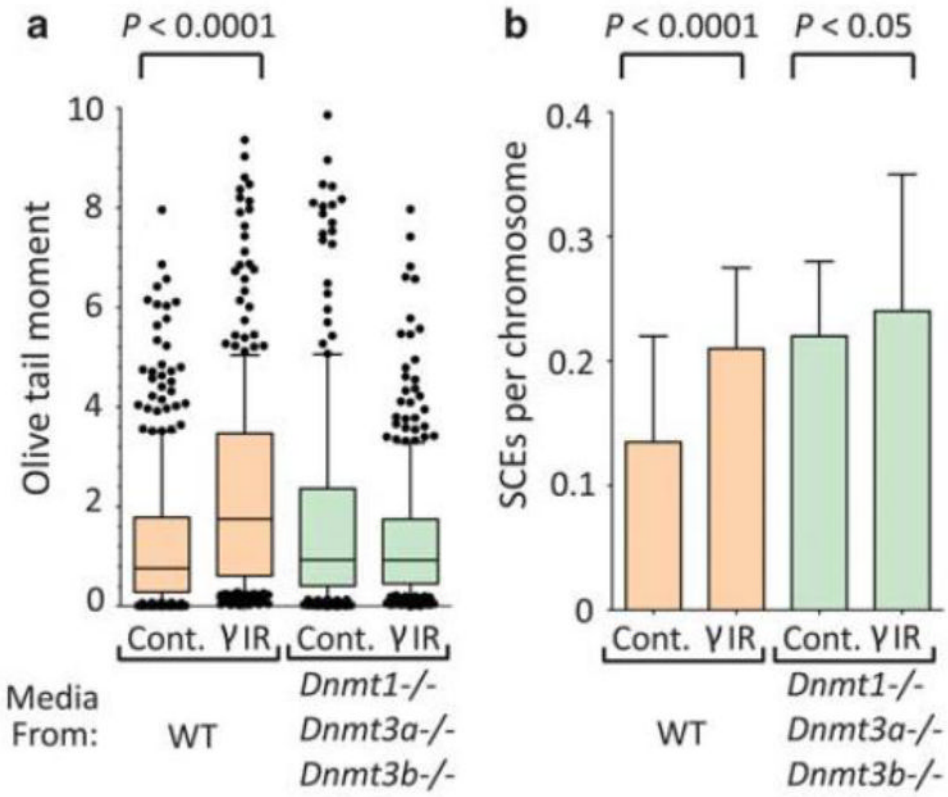

Figure 2. γIR does not lead to the persistent induction of genomic instability in primary bystanders to *Dnmt1-/- Dnmt3a-/- Dnmt3b-/-* cells

DNA damage by comet assay (**a**) and SCEs (**b**) in naive WT ES cells exposed to media from WT and *Dnmt1-/- Dnmt3a-/- Dnmt3b-/-* cells. See Fig. 1 for experimental design. SCEs were counted for ≥80 spreads/condition as previously described (Engelward *et al*., 1996). For SCE studies, median with interquartile range is shown and *P* values were produced by a two-tailed t-test.

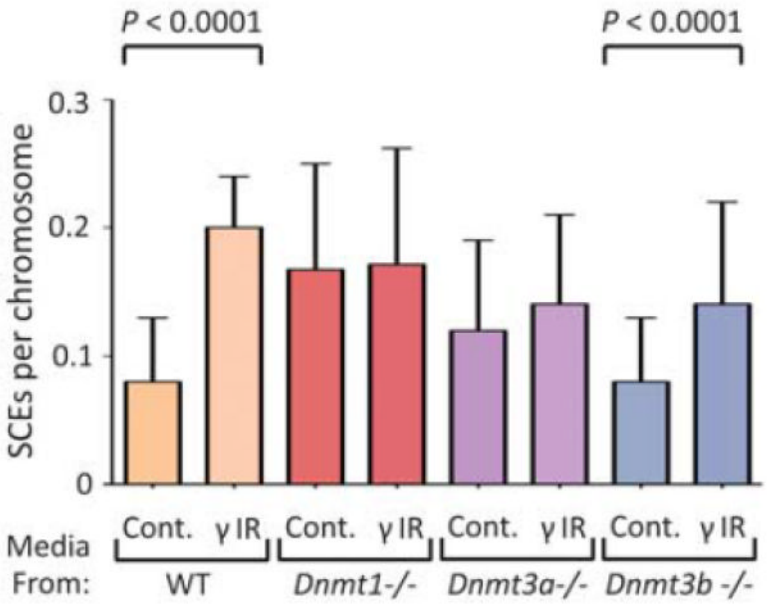

Figure 3. Dnmt1 and Dnmt3a are required for persistent induction of homologous recombination in naïve, primary bystander ES cells

SCEs in naive WT ES cells exposed to media from γIR (and mock irradiated) WT, *Dnmt1-/-*, *Dnmt3a-/-*, and *Dnmt3b-/-* target ES cell populations. See captions from Figs. 1 & 2 for design and analysis.

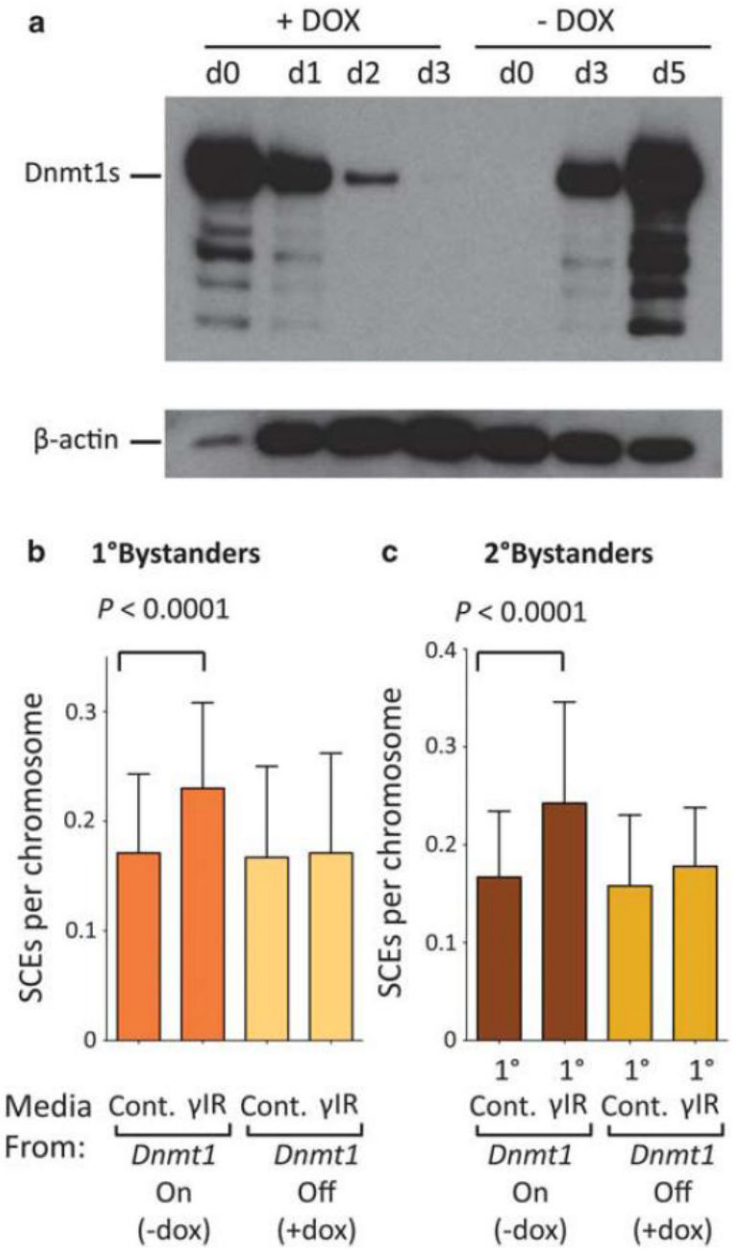

Figure 4. Transient suppression of Dnmt1 protects against radiation-induced genomic instability

a, Doxycycline-dependent repression of Dnmt1 in mouse ES cells assessed by Western blot. **b**, SCEs in primary bystanders to normal and Dnmt1 transiently-deficient cells. **c**, SCEs in naive (secondary bystander) cells exposed to media from primary bystanders to normal and Dnmt1 transiently-deficient cells. Data analysis as per caption for Fig. 2.