

# Cytoplasmic Irradiation Induces Mitochondrial-Dependent 53BP1 Protein Relocalization in Irradiated and Bystander Cells

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## Abstract

**The accepted paradigm for radiation effects is that direct DNA damage via energy deposition is required to trigger the downstream biological consequences. The radiation-induced bystander effect is the ability of directly irradiated cells to interact with their nonirradiated neighbors, which can then show responses similar to those of the targeted cells. p53 binding protein 1 (53BP1) forms foci at DNA double-strand break sites and is an important sensor of DNA damage. This study used an ionizing radiation microbeam approach that allowed us to irradiate specifically the nucleus or cytoplasm of a cell and quantify response in irradiated and bystander cells by studying ionizing radiation-induced foci (IRIF) formation of 53BP1 protein. Our results show that targeting only the cytoplasm of a cell is capable of eliciting 53BP1 foci in both hit and bystander cells, independently of the dose or the number of cells targeted. Therefore, direct DNA damage is not required to trigger 53BP1 IRIF. The use of common reactive oxygen species and reactive nitrogen species (RNS) inhibitors prevent the formation of 53BP1 foci in hit and bystander cells. Treatment with filipin to disrupt membrane-dependent signaling does not prevent the cytoplasmic irradiation-induced 53BP1 foci in the irradiated cells, but it does prevent signaling to bystander cells. Active mitochondrial function is required for these responses because pseudo- $\rho^0$  cells, which lack mitochondrial DNA, could not produce a bystander signal, although they could respond to a signal from normal  $\rho^+$  cells. [Cancer Res 2007;67(12):5872–9]**

## Introduction

The standard paradigm for radiation effects has been based on direct energy deposition in nuclear DNA driving biological response (1). Previous studies using radioisotope incorporation have shown that the DNA within the nucleus is a key target as <sup>131</sup>I-concanavalin A bound to cell membranes was very inefficient at cell killing, in contrast to <sup>131</sup>I-UdR incorporated into the nucleus (2). These authors also found that dose delivered to the nucleus, rather than cytoplasm or membranes, determined the level of cell death. Recently, it has been shown that irradiation of the cytoplasm alone can induce an effect. Wu et al. (3) found increased levels of mutations in A<sub>L</sub> cells after cytoplasmic irradiation using an  $\alpha$ -particle microbeam. The types of mutations were similar to those that occurred spontaneously in unirradiated cells and were

formed as a consequence of increased reactive oxygen species (ROS). In addition, a considerable number of studies have now reported evidence for nontargeted responses to radiation exposure, where biological response is not in direct proportion to energy deposition in the DNA. One of these responses is the radiation-induced bystander effect, which is defined as the response of cells that were not directly irradiated but were in contact with irradiated cells. A large variety of responses can be triggered in bystander cells, including the induction of sister chromatid exchanges (SCE) (4), chromosomal aberrations (5), changes in protein expression (6), genomic instability (7, 8), and mutations (9). Recently, it has been shown that the irradiation of the cell cytoplasm leads to the production of a bystander response, the extent of which is similar to that triggered by irradiation of the nucleus (10). This proves that direct DNA damage is not required to trigger a bystander response. Understanding the role of nondirect DNA damage-dependent responses to irradiation has highlighted new potential targets for cancer therapy.

Experimental approaches for studying the bystander effect fall into two main categories: (a) use of conventional irradiation approaches with high or low LET (linear energy transfer); transferring media from irradiated cells to unirradiated bystander cells, or irradiating with a low fluence of  $\alpha$  particles where only a few percent of cells are randomly irradiated; (b) targeted irradiation with sophisticated microbeams that allow irradiation of a precise number of cells in specific subcellular locations. In general, studies on sparsely and confluent populations of cells report that bystander responses can be mediated either through soluble factors released from the targeted cells and/or by direct cellular interaction through gap junctions (11–14). However, little is known regarding early events in DNA damage and signaling in bystander or cytoplasmically irradiated cells.

Among the wide variety of DNA lesions induced by IR, double-strand breaks (DSB) are generally accepted to be the most significant lesions because they lead both to cell death and stable genetic alterations if left unrepaired. H2AX phosphorylation on Ser<sup>139</sup> (called  $\gamma$ -H2AX) has been shown to be an early step in the response of cells to DNA damage (15). Use of an antibody to  $\gamma$ -H2AX revealed that discrete nuclear foci appeared 1 min after exposure of cells to ionizing radiation, and that the number of these foci corresponds to the number of induced DNA DSBs (16, 17). The use of antibodies against  $\gamma$ -H2AX has become a common approach to detect the presence of DSBs (18). This method has been used to detect DNA damage in bystander cells after whole cell (19, 20) or nuclear-targeted irradiation (21). Thus far,  $\gamma$ -H2AX ionizing radiation-induced foci (IRIF) formation has been shown to be induced in bystander cells as quickly as 2 min after IR (22) and remain for up to 48 h (23).

p53 binding protein 1 (53BP1) is a member of the BRCT (BRCA1 C-Terminal) repeat family, wherein many members include the

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DNA damage response proteins NBS1 and BRCA1. 53BP1 is required for the phosphorylation of numerous ATM (ataxia-telangiectasia mutated) substrates during the DSB response (24, 25). Like  $\gamma$ -H2AX, 53BP1 has been shown to relocalize into foci shortly after irradiation, with the number of foci closely paralleling the number of DNA DSBs (26). Retention, but not the initial recruitment, of 53BP1 proteins is dependent on the phosphorylation of H2AX (27).

In this work, we used fluorescent detection of foci formation using 53BP1 as a marker of DNA damage. Use of a microbeam allowed us to selectively irradiate the cytoplasm of one cell or every cell in a population to generate biological responses in hit and bystander cells. By mixing unstained cells with cells stained with the lipophilic dye DiI, we were able to distinguish between targeted and neighboring bystander cells, within the same population, and follow 53BP1 IRIF formation in both populations.

Our results show that following microbeam irradiation, the number of cells having  $\geq 4$  53BP1 foci per cell (fpc) increased in the bystander population, and that this increase is independent of whether the targeted cells were irradiated through the nucleus or cytoplasm only. Three hours after irradiation, bystander cells showed the same percentage of cells with increased numbers of 53BP1 foci as cells that had been directly irradiated through the cytoplasm. Attenuation of bystander and direct responses with ROS and NO inhibitors also showed that these species play a role in mediating the bystander signal and in mediating the intracellular signal from the targeted cytoplasm to the nucleus. However, a key difference is that the bystander response was mediated through membrane-dependent signaling, whereas the response in cells receiving direct cytoplasmic irradiation was not. Additional studies using pseudo- $\rho^0$  cells, which lack mitochondrial DNA (mtDNA), revealed that intact mitochondrial function is needed for the cells to induce a nuclear response to direct cytoplasmic targeting.

## Materials and Methods

**Cell culture and reagents.** HeLa cells were grown in EMEM supplemented with 10% FCS, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin. Cytoplasmic staining of cells was done using either 0.1  $\mu$ g/mL Nile red added to cells 6 min before irradiation, or 5  $\mu$ mol/L DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate diluted in DMSO, Abs: 549 nm, Em: 565 nm; Molecular Probes), with cells being stained for 45 min before being plated onto the microbeam dish. At these concentrations, the dyes do not cause any measurable cell perturbation and give a clear cytoplasmic signal. Hoechst 33342 (0.2  $\mu$ mol/L, Molecular Probes) was used for nuclear staining. In some experiments, cells were treated with 1% DMSO or 50  $\mu$ mol/L aminoguanidine (Sigma) 30 min before irradiation or 0.5  $\mu$ g/mL Filipin-III (Sigma) added 15 min before irradiation.

**Creation of mtDNA-depleted cell.** Normal HeLa cells were depleted of mtDNA as described previously (28) with minor modifications (29). Briefly, cells were cultured for a minimum of 7 days in the presence of 250 ng/mL ethidium bromide (EB) under 5% CO<sub>2</sub> in DMEM supplemented with 4.5 mg/mL glucose, 50  $\mu$ g/mL uridine, and 2 mmol/L pyruvate to compensate for the respiratory metabolism deficit. Cells treated in this manner are referred to as pseudo- $\rho^0$ .

**mtDNA quantification.** Total DNA was isolated using DNA Tissue Kit (Qiagen). The status of EB-treated cells as mtDNA depleted was verified using PCR to examine the presence of the cytochrome *b* gene, which is encoded by mtDNA. Previously described primers for the cytochrome *b* gene were used (30). Total DNA (10 ng) was amplified for each sample. An initial denaturation of 5 min at 95°C was followed by 35 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The final extension step was at 72°C for 7 min. DNA amplifications were visualized after electrophoresis on

EB-stained 1.5% agarose gels. Images were captured, and bands were quantified using a Kodak 4000 MM Image-Station and Kodak Molecular Imaging Software.

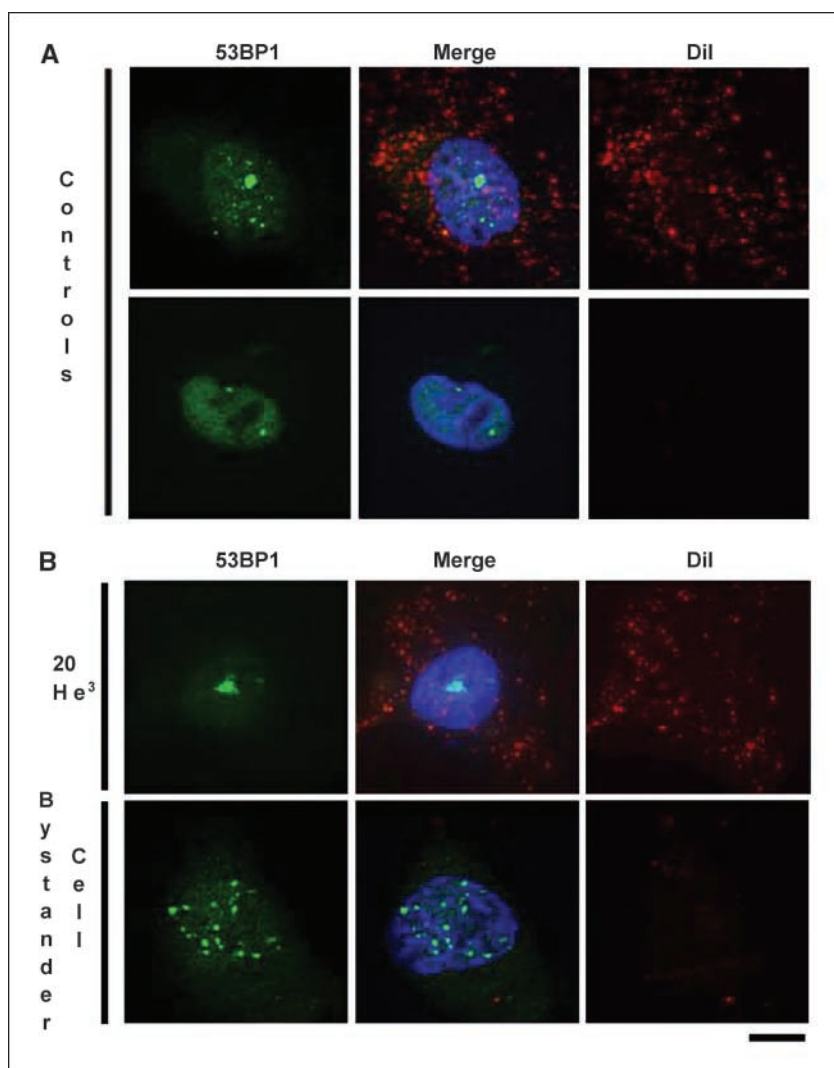
**Microbeam irradiation.** The Gray Cancer Institute charged-particle microbeam facility was used for this study. Details of the experimental setup for nuclear targeting using the microbeam have been described previously (31, 32). Nonconfluent cells were seeded in a 6  $\times$  6-mm area of a specially designed Mylar-based microbeam dish preincubated overnight with cell culture media. For cytoplasmic irradiations, after first scanning for Hoechst-stained nuclear objects, the dish was scanned a second time for DiI- or Nile red-stained objects. To safely exclude the nucleus when targeting, nuclear objects were dilated by 8  $\mu$ m (to take into account the microbeam particle scattering (33) and possible cell movements) and then subtracted from the cytoplasmic image. Then, for each nuclear object, one cytoplasm signal is assigned to irradiate the cytoplasm of each cell at only a single location. The result is a cell file with the number of cytoplasmic objects equal to or less than the number of nuclei found. Practically, in the experiments, an average of 74.2%  $\pm$  7.1 (SE) of cytoplasmic objects were found and irradiated: dishes where <55% of cytoplasmic objects were found were not used. Microbeam irradiation was done at 6–8°C, and cells were irradiated in serum-free media. Serum was replaced immediately after the end of irradiation. In some experiments, normal HeLa and pseudo- $\rho^0$  cells were plated in two distinct areas of the same microbeam dish sharing the same culture medium, and either normal HeLa or pseudo- $\rho^0$  cells were targeted through the nucleus or cytoplasm, with both populations scored for 53BP1 foci formation.

**Immunofluorescence.** Cells were fixed in 3.7% buffered formaldehyde (Sigma) for 15 min at room temperature and permeabilized in PBS supplemented with 0.5% Triton X-100. After overnight incubation in blocking buffer (PBS, 0.1% triton, 5% horse serum, 0.2% milk), cells were incubated with rabbit anti-53BP1 antibody at 1:1500 (Abcam) and then incubated with secondary anti-rabbit Alexa fluor-488 (Molecular Probes). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (0.1  $\mu$ g/mL), and the sample was mounted in MOWIOL, using 1,4-diazabicyclo[2.2.2]octane as an antifading agent. Typically, 150 to 200 cells were counted for each sample, and fluorescent images were captured using a fluorescent microscope (Zeiss Axiovert 200M with apotome, Welwyn Garden City).

**Statistical analysis.** Statistical analysis was done on the data obtained from at least three independent experiments. All of the results are presented as means  $\pm$  SE. Significant levels were assessed using Student's *t* test. A *P* value of 0.05 or less was considered to be significant.

## Results

**Induction of 53BP1 IRIF in bystander cells after nuclear traversal.** Mixed populations of DiI-stained and unstained cells at a 1:1 ratio were used, with only the DiI-stained cells irradiated through the nucleus or cytoplasm with  $\alpha$  particles, whereas the Hoechst-only stained population were classified as bystander cells. In nonirradiated HeLa cells, 53BP1 showed mainly diffuse staining within the cell nucleus, with some cells showing a few foci (Fig. 1A). Moreover, unirradiated DiI-stained cells did not show any differences in the amounts of 53BP1 foci relative to DiI-unstained cells (data not shown), confirming that the dyes and scanning procedures do not cause any perturbations in the cells. After half of the cell population was exposed to 20 <sup>3</sup>He<sup>2+</sup> ions through the nucleus, DNA damage was measured as the increased number of cells showing >4 53BP1 fpc, detected in cells 1 and 3 h following radiation. The threshold of >4 fpc was found to be optimal for determining the fraction of affected cells (data not shown). As expected, 53BP1 relocalization was evident in targeted cells, visible as foci grouped within 2  $\pm$  1  $\mu$ m (microbeam size), each individual focus corresponding to the traversal of a single helium ion. In contrast, in nontargeted neighboring cells, 53BP1 IRIF were located throughout the whole nuclear area (Fig. 1B). It was found on



**Figure 1.** Representative images of HeLa cells stained for 53BP1 immunofluorescence (*green*) and cytoplasmic Dil staining (*red*). After fixation, the red cytoplasmic staining appears punctated instead of the more homogenous staining seen in live cells. *A*, control cells, examples of Dil-stained and unstained cells. *B*, nuclear targeted irradiation of the Dil-stained cells with 20 helium ions 3 h after irradiation, with an example of a bystander Dil-unstained neighboring cell. *Bar*, 10  $\mu$ m.

average that  $12 \pm 6\%$  of the bystander cell population showed an increased number of nuclear 53BP1 foci compared with the nonirradiated controls (Fig. 2*A* and *B*). This bystander effect was evident 1 h following nuclear targeting and remained unchanged after 3 h.

#### Cytoplasmic irradiation induces foci in hit and nonhit cells.

Evaluation of cell responses to cytoplasmic irradiation followed the same protocol described above, but with half of the cell population stained with Dil being targeted through the cytoplasm only. Cytoplasmic-targeted cells showed the same 53BP1 IRIF pattern as bystander cells, the foci being located sparsely throughout the nucleus. The presence of the cytoplasmic Dil dye is then the only way to distinguish between hit and nonhit cells within the population.

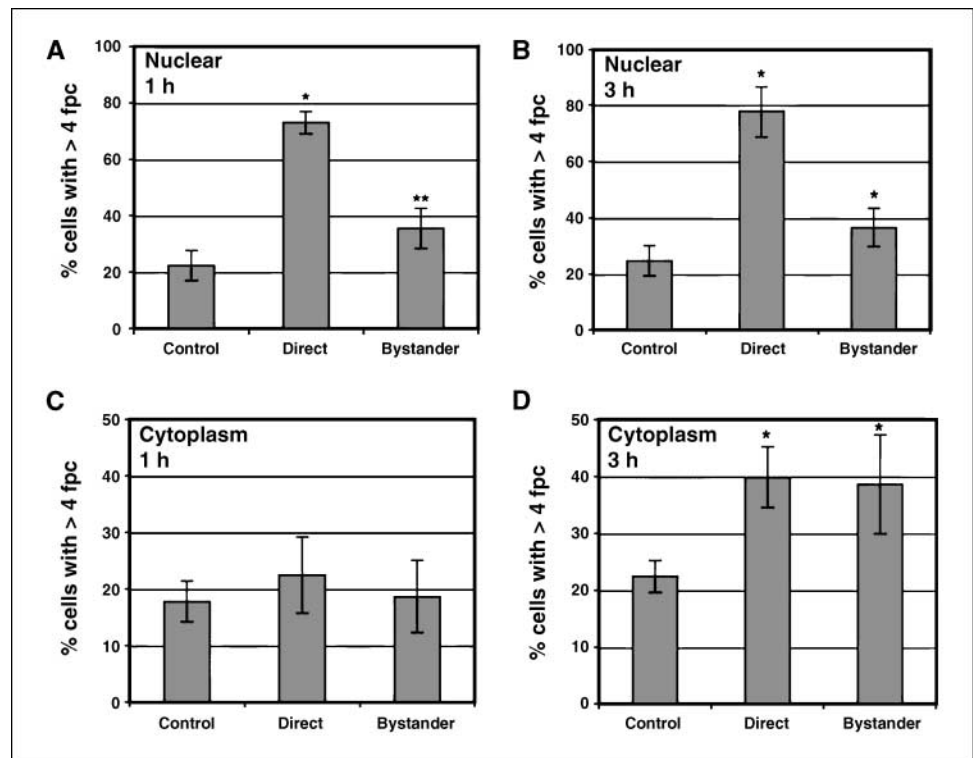
Interestingly, when the cell cytoplasm is irradiated, the biological effect of radiation appears at a later time than after nuclear irradiation. Figure 2*C* shows no effect 1 h after irradiation for both directly hit and bystander cells. Figure 2*D* shows that the formation of nuclear 53BP1 foci significantly increases 3 h after cytoplasmic targeting. The level of response was found to be identical in hit and nonhit cells ( $17 \pm 6\%$  and  $16 \pm 8\%$  increase over control, respectively). These data indicate that targeted cytoplasm can induce damage at the nuclear level. Moreover, when compared with the bystander effect induced by nuclear targeting, the level of

response is not significantly different at 3 h, suggesting that similar mechanisms are involved.

**Response is independent of the dose and number of cells targeted.** The kinetics of 53BP1 focus formation after cytoplasm irradiation (in both directly irradiated and bystander cells) showed a peak at 3 h and decreased after 6 and 18 h, with foci numbers returning close to control values (data not shown); therefore, the 3 h time point was chosen for all further experiments.

We next investigated the influence of dose (particle number) on 53BP1 IRIF. The bystander response following nuclear irradiation was not affected by the number of particles delivered or the number of cells traversed: Fig. 3*A* shows that nuclear traversal through one cell or 50% of the cell population with 1 or 20 helium ions induced the same level of 53BP1 foci increase. In the case of cytoplasmic irradiation, we followed the response of both targeted and bystander cells. Figure 3*B* shows that the response was independent of the number of particles used for irradiation because a single helium ion induced the same level of 53BP1 focus formation in HeLa cells as 20 helium ions, both in directly hit and bystander cells following cytoplasmic traversal. Comparison of the bystander response when only one cell (Fig. 3*B*) or 50% of the cells (Fig. 2*D*) within the population were targeted through the cytoplasm only showed no significant difference, demonstrating

**Figure 2.** Fifty percent of the cell population plated on the microbeam dish was Dil stained and mixed with unstained cells. Every cell in the Dil-stained population was microbeam-targeted either through the nucleus or cytoplasm with 20 helium-3 ions. Cells were fixed 1 and 3 h following irradiation, and the number of cells with 53BP1 foci was scored in both Dil-stained and unstained populations (hit and bystander cells, respectively). *A*, nuclear targeting, 1 h after radiation. *B*, nuclear targeting, 3 h after radiation. *C*, cytoplasmic targeting, 1 h after radiation. *D*, cytoplasmic targeting, 3 h after radiation. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$ , compared with control.



bystander responses were independent of the number of cells targeted. These data are consistent with the work of Shao et al. (10), showing dose independency of micronuclei induction following cytoplasmic traversal of a single cell with one or five helium ions.

**Attenuation of bystander signals with DMSO, NO synthase inhibitor, and filipin after cytoplasmic or nuclear traversal.** The possible role of ROS in mediating the bystander effect following nuclear and cytoplasm irradiation was investigated by using (a) the antioxidant DMSO, which reduces ROS, and (b) the NO synthase inhibitor aminoguanidine.

Figure 4A shows the corresponding foci induced per cell with and without inhibitors. Bystander cells without any inhibitors (labeled NT in Fig. 4A) showed an average of  $1.2 \pm 0.2\%$  (bystander-nuclear) and  $1.3 \pm 0.3\%$  (bystander-cytoplasmic) fpc above unirradiated control cells. Treatment of HeLa cells with 1% DMSO for 30 min before and 3 h after radiation totally prevented 53BP1 focus formation in bystander cells. It has been shown recently that NO production can be involved in the transmission of the bystander signal (14). In our study, treatment with 50  $\mu\text{mol/L}$  aminoguanidine also suppressed the bystander response because the number of induced 53BP1 foci per cell was close to zero. These results show that the bystander signal is mediated through the production of NO and ROS that, in turn, induces an increase in 53BP1 focus formation in bystander cells.

Filipin is an antibiotic that abrogates signaling through glycosphingolipid-enriched membrane microdomains (GEMs), or rafts, in many cell types. As cells are plated sparsely onto the microbeam dishes, soluble signaling factors must be involved in intercellular communication and would be disrupted by the use of filipin. Results showed that disruption of these GEMs by treating cells with 5  $\mu\text{g/mL}$  filipin dramatically reduced the bystander effect (Fig. 4A) after both nuclear and cytoplasmic traversal, indicating that the bystander signal was mediated through membrane GEMs.

#### Attenuation of direct effect with aminoguanidine and DMSO but not with filipin after cytoplasmic traversal.

The increase of 53BP1 foci per cell when every cell in the population is targeted through the cytoplasm can be prevented by the addition of 50  $\mu\text{mol/L}$  aminoguanidine or 1% DMSO when added before radiation (Fig. 4B), suggesting that signaling from the cytoplasm to the nucleus within the irradiated cell is mediated by ROS and reactive nitrogen species (RNS). Addition of DMSO immediately after radiation reduces the number of induced foci, but the difference is not statistically significant.

The number of induced foci per cell was unchanged in the presence or absence of filipin treatment (Fig. 4B), indicating that the direct cytoplasmic effect is mediated through intracellular signaling, and suggests that bystander/external signals do not play a role because no additional effect was seen in the absence of filipin treatment.

The use of these inhibitors on cells directly irradiated through the nucleus did not prevent 53BP1 foci formation (data not shown).

#### Inhibition of mitochondrial function impairs bystander and cytoplasmic signaling.

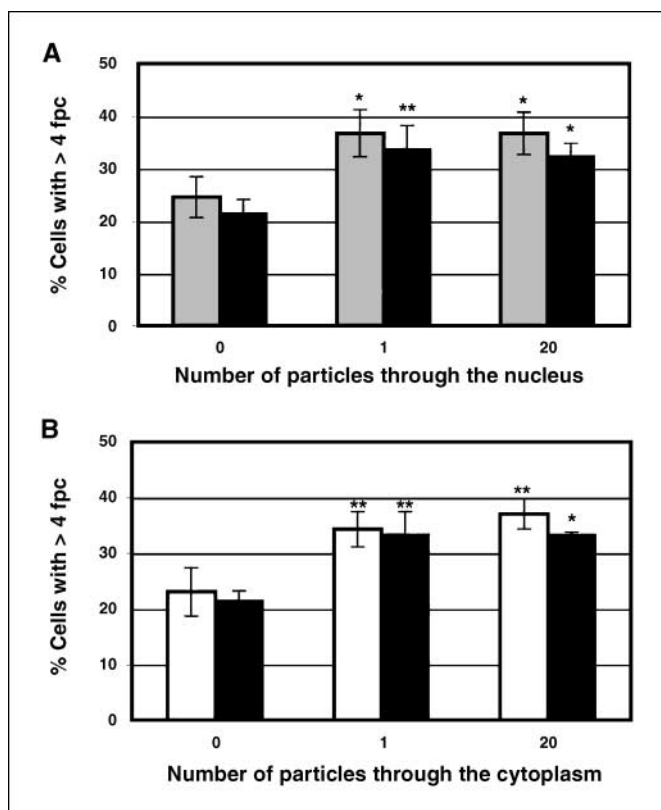
To obtain cells depleted in mtDNA, HeLa cells were exposed to 250 ng/mL EB for 7 to 15 days. PCR analysis of the mtDNA cytochrome *b* gene reveals that EB-treated cells have <5% of mtDNA remaining compared with the normal  $\rho^+$  HeLa cells (Fig. 5A). Thus, as the depletion of mtDNA is not complete, EB-treated cells will be referred to as pseudo- $\rho^0$ . Figure 5B compares bystander (left) and direct (right) effect of pseudo- $\rho^0$  cells and  $\rho^+$  HeLa cells. A total suppression of bystander signal is seen when a few pseudo- $\rho^0$  cells have been targeted through the nucleus or cytoplasm. For direct irradiation, the level of induced foci per cell is significantly lower when pseudo- $\rho^0$  cells are directly targeted through the cytoplasm relative to  $\rho^+$  cells. To know if pseudo- $\rho^0$  cells are still capable of responding to a bystander signal, pseudo- $\rho^0$  and  $\rho^+$  cells were cocultured in two distinct areas of a microbeam

dish. When every  $\rho^+$  cell was targeted and bystander- $\rho^0$  cells were scored for 53BP1 foci (Fig. 5C, left), the results showed that pseudo- $\rho^0$  cells respond to the bystander signal generated from individually targeted  $\rho^+$  HeLa cells. In the reverse experiment, when pseudo- $\rho^0$  cells were targeted (Fig. 5C, right), either through the nucleus or cytoplasm, a bystander effect in neighboring cocultured normal  $\rho^+$  HeLa cells did not occur. This confirms that pseudo- $\rho^0$  cells do not release a bystander signal.

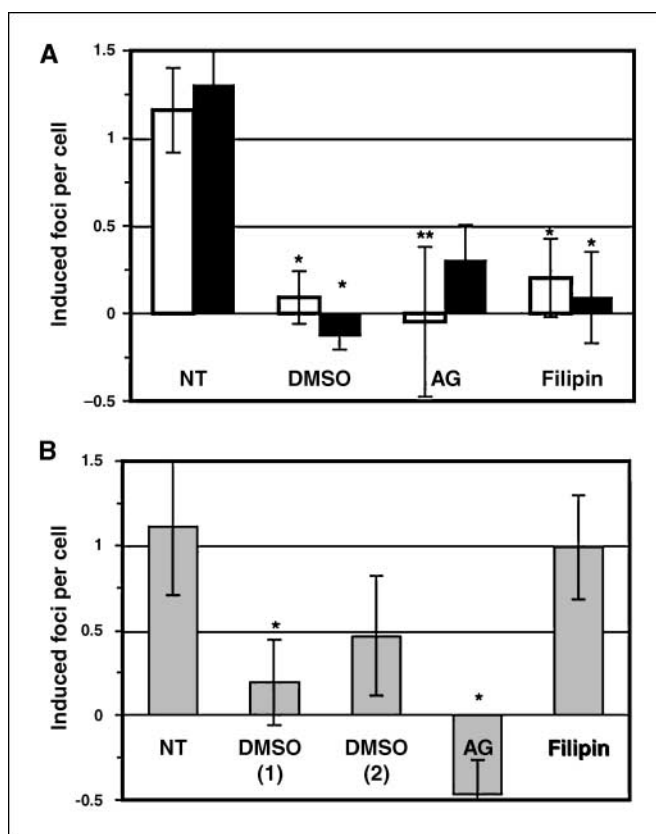
## Discussion

In the past decade, widespread experimental evidence has indicated that production of genetic damage or a biological response from exposure to ionizing radiation is not necessarily just the consequence of direct DNA damage by the radiation. The response whereby nonirradiated cells adjacent to irradiated cells are affected is known as the "bystander effect."

In this work, we have investigated the biological consequences of irradiating the cytoplasm only, in the absence of direct nuclear damage, on both the directly irradiated and neighboring bystander cells. Such experiments are possible by using the Gray Cancer Institute charged-particle microbeam combined with the use of dual fluorochrome labeling (nuclear/cytoplasm), which has allowed us to target and irradiate with high accuracy all or fractions of a cell population through the cytoplasm only.



**Figure 3.** Nuclear (A) and cytoplasmic (B) targeting with 1 or 20 helium ions. Cells were fixed 3 h following irradiation. A, half of the cell population Dil stained (grey) or only 1 cell (solid) was targeted through the nucleus, and bystander cells only were scored for 53BP1 foci. B, direct effect (open): the whole cell population was Nile red-stained and targeted through the cytoplasm, and cells were scored for 53BP1 foci; bystander effect (filled): one cell within the population was targeted through the cytoplasm, and nonhit cells were scored for 53BP1 foci. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$ , compared with control.



**Figure 4.** Modulation of induced 53BP1 foci yields. A, five cells within the population were targeted with one helium ion through the nucleus (white) or the cytoplasm (black). DMSO, aminoguanidine (AG), or filipin were added 30 min before radiation and left for the duration of the incubation time. Cells were fixed 3 h following radiation, and the number of 53BP1 foci was scored in bystander cells. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$ , compared with untreated bystander NT samples. B, direct cytoplasmic irradiation with one helium ion of every cell in the whole population followed by 3 h incubation in the presence of 1% DMSO (1), 5  $\mu\text{g}/\text{mL}$  filipin, or 50  $\mu\text{mol}/\text{L}$  aminoguanidine added 30 min before radiation or with 1% DMSO (2) added immediately after radiation. \*,  $P < 0.01$ , compared with untreated sample.

53BP1 responds to radiation-induced DNA DSBs by quickly relocalizing to discrete nuclear foci, with the number of foci closely corresponding to the number of DSBs (26). We have shown here that 53BP1 protein relocalization appears not only in cells directly hit through the nucleus by helium-3 ions but also in adjacent nonirradiated cells on a sparsely populated dish. Moreover, 53BP1 IRIF do not require direct DNA damage to be induced in cells, as we showed that cytoplasmic irradiation alone is capable of eliciting 53BP1 relocalization to form nuclear foci in both the irradiated and bystander cells.

Previous studies on cytoplasm-irradiated cells had measured end points following long incubation times after radiation, i.e., 24 to 48 h for micronuclei analysis (10) and 7 to 8 days for the mutagenesis assay (3). Therefore, these studies have not been able to show how quickly after cytoplasmic irradiation the signal can be transmitted to the nucleus within one cell, and from a cell to its neighbors. Interestingly, the kinetics of 53BP1 foci formation depends on the subcellular localization of the radiation. Nuclear-targeted cells show 53BP1 IRIF foci as quickly as 5 min after radiation (data not shown), with a bystander effect arising after 1 h, whereas in cytoplasm-targeted cells, 53BP1 IRIF formation is not significant until 3 h after radiation in both directly hit and the

bystander population (Fig. 2). Thus, cytoplasmic irradiation may require an additional step to manifest a biological response, presumably DNA damage formation, in the nucleus. One possibility is that the generation of free radicals directly in the cell cytoplasm and the interaction of these with the nucleus is not amplified to

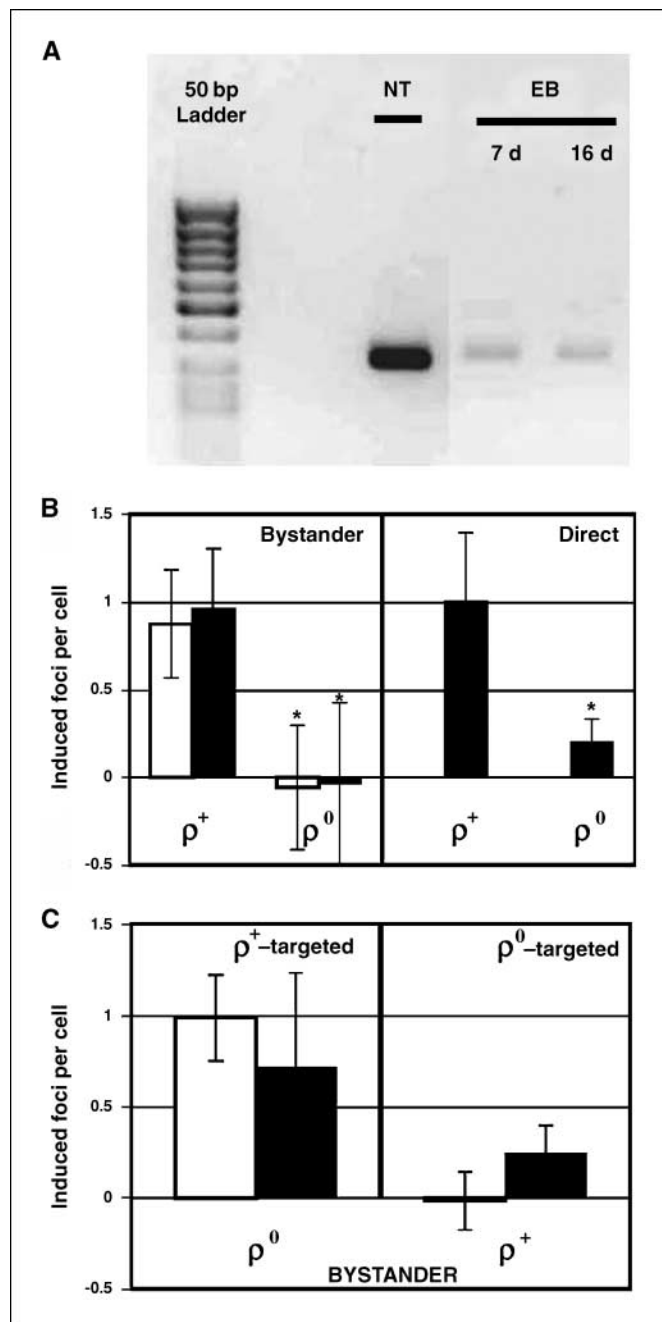
the same extent as a bystander extracellular signaling-driven response. Also, from our studies with filipin (Fig. 4), it is clear that membrane-dependent signaling processes through lipid rafts do not play a role in the direct cytoplasmic response, suggesting that the signaling is predominantly intracellular.

In the case of the bystander population, the percentage of cells affected by the bystander signal is not dependent on the number of helium-3 ions targeted through the cells, and the fraction of cells showing 53BP1 IRIF reached a plateau after only one cell had been irradiated (Fig. 3). Such a response could be achieved if the primary signal released into the medium by the targeted cell is amplified by the recipient bystander cells, leading to the saturation of signaling molecules in the surrounding media. This finding also suggests that the small fraction of cells responding to the bystander signal might be dependent on the cell-cycle phase of the bystander cells at the time receiving the signal. In addition to its function in DSB repair, 53BP1 has been implicated in the ATM-dependent G<sub>2</sub>-M-phase cell cycle checkpoint after radiation, particularly after low doses ( $\leq 3$  Gy; ref. 34). 53BP1 has also been reported to form foci at the sites of stalled replication forks in S-phase cells, together with  $\gamma$ -H2AX and the BLM helicase (35, 36). Taken together, the 53BP1 foci that form in response to cytoplasmic targeting in hit and bystander cells might not be at the sites of direct DSB. In recent studies (23), we have shown that  $\gamma$ -H2AX foci formation in bystander cells is dependent on ATR (ATM and Rad3 related; which plays a central role in controlling responses to replication stress), supporting the hypothesis that these foci are not generated through the same pathway involved in direct radiation. We also reported that the bystander  $\gamma$ -H2AX foci were only detected in S-phase cells. We have also recently found that 53BP1 was able to colocalize with  $\gamma$ -H2AX in bystander cells and not to be induced in ATR-deficient (Seckel) bystander cells.<sup>3</sup> Given that ATR can interact with 53BP1 through the mediation of BLM at sites of stalled replication forks, it is likely that this forms part of the mechanism underlying the data presented here.

There are now substantial data indicating a role for long-lived ROS and RNS in bystander mechanisms (6, 14, 37). The mechanism by which these radicals damage DNA in bystander cells is still not clear, but accumulation of radical damage at stalled replication forks leading to the activation of DSB-dependent markers such as  $\gamma$ -H2AX and 53BP1 is consistent with the data. A recent study showed that NO release from irradiated cells may occur quickly from a constitutive pathway, and that NO radicals may be key signaling molecules (37, 38). In many situations, there is a close interplay between reactive oxygen and nitrogen species in cells after radiation exposure (39). Consistent with those reports, our observations indicate that 53BP1 foci formation is clearly inhibited by the presence, at the time of irradiation, of 1% DMSO, a known scavenger of hydroxyl radicals, and by aminoguanidine, which inhibits nitric oxide synthase (Fig. 4A).

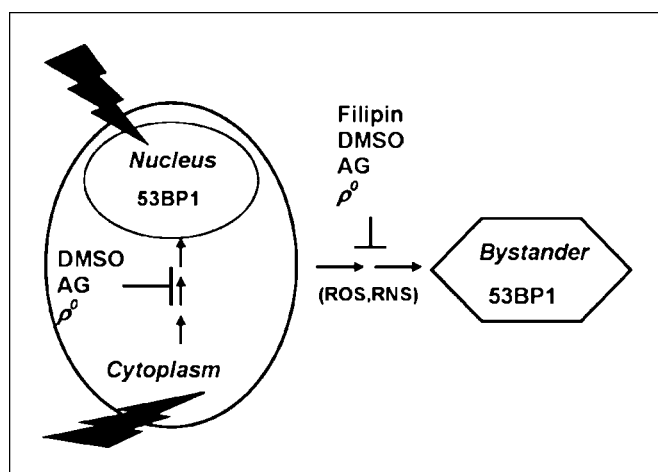
Disruption of rafts (specialized lipidic structures involved in membrane signaling, ref. 40) using the cholesterol-depleting agent filipin has been shown to abrogate a number of cellular signaling processes. Attenuation of 53BP1 IRIF in bystander cells after filipin treatment (Fig. 4A) shows the importance of cell membrane signaling to mediate bystander signals. In contrast, it seems that there is no involvement of membrane signaling in directly irradiated cells. These results are in accordance with data from

<sup>3</sup> S. Burdak-Rothkamm, personal communication.



**Figure 5.** A, PCR product of the cytochrome *b* gene in normal  $\rho^+$  cells and HeLa treated with 250 ng/mL EB for 7 and 16 days. The product is a unique band of 334 bp size. For experiments, cells were used after 10 to 14 days treatment. B, induced foci per cell, cells are fixed 3 h after radiation. Left (bystander effect), five cells within the population of  $\rho^+$  or pseudo- $\rho^0$  are targeted with one helium ion through the nucleus (white) or the cytoplasm (black). Right, direct radiation effect when cytoplasm of every cell are hit by one helium ion. C, induced foci per cell in bystander  $\rho^+$  and pseudo- $\rho^0$  population. Normal  $\rho^+$  cells and pseudo- $\rho^0$  HeLa were cocultured in distinct areas of the microbeam dish. Each cell in one population was targeted either through the nucleus (white) or the cytoplasm only (black), and the second (bystander) population was scored for 53BP1 foci. \*,  $P < 0.01$  compared with  $\rho^+$  sample.





**Figure 6.** Model for 53BP1 activation after nuclear or cytoplasmic irradiation for direct and bystander cells.

Nagasawa et al. (41), showing that the induction of SCEs is abrogated with filipin treatment in the bystander cell population but not in directly irradiated cells. Also, we have previously shown that filipin inhibits micronuclei induction in bystander cells after cytoplasmic irradiation (10).

Mitochondria are the most important source of ROS in mammalian cells. We generated mtDNA-depleted HeLa cells to test the involvement of mitochondria in the signaling pathway after cytoplasmic helium-3 ion traversal. Results obtained from the pseudo- $\rho^0$  cells imply that functional mitochondria are required for downstream cell signaling of cytoplasmic hit cells and for the bystander signal to be generated. The probability of mitochondria being damaged by a direct hit with a single helium-3 ion traversing the cytoplasm is low but cannot be excluded; nevertheless, in that case, the small number of irradiated mitochondria would not be expected to have any effect compared with the overall nonhit mitochondria population. On the other hand, intact mitochondria are not necessary for a bystander response in pseudo- $\rho^0$  cells when

normal  $\rho^+$  cells are targeted. Recent studies have suggested that directly irradiated cells show significant changes in the level of protein import into mitochondria, which are highly dose dependent (42). In bystander cells, mitochondria have been reported to play an important role but only in the triggering of changes involved in apoptosis (43).

Our overall results are summarized in the model presented in Fig. 6, showing that both nuclear and cytoplasmic targeting elicit the formation of nuclear 53BP1 foci in directly irradiated cells and are capable of inducing a bystander response. The response can be inhibited by the presence of 1% DMSO or aminoguanidine at the time of radiation in the case of cytoplasmic hit cells and by DMSO, aminoguanidine, and filipin in all cases for bystander cells. Also, no additional 53BP1 foci appear in bystander pseudo- $\rho^0$  and normal cells when cells lacking mtDNA are hit.

In summary, we have shown for the first time that the direct irradiation of a cell outside of the nucleus elicits a DNA damage response measured using 53BP1 IRIF. This response has many similarities to the bystander phenotype induced in neighboring cells in that it is independent of dose and fraction of cells targeted. However, significant differences exist in the timescale and mechanism of action for cytoplasmic-initiated responses relative to the induction of a bystander response, suggesting that cytoplasmic to nuclear transmission in directly irradiated cells plays a role. Metabolically active mitochondrial function is also required. It is clear that in assessing the overall response of biological systems to radiation exposure, nondirect DNA damage responses need to be considered. A greater understanding of these processes may highlight new pathways and targets for the use of radiation in a therapeutic environment.

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