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SHORT COMMUNICATION

Interaction between Radioadaptive Response and Radiation-Induced Bystander Effect in *Caenorhabditis elegans*: A Unique Role of the DNA Damage Checkpoint

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Although radioadaptive responses (RAR) and radiation-induced bystander effects (RIBE) are two important biological effects of low-dose radiation, there are currently only limited data that directly address their interaction, particularly in the context of whole organisms. In previous studies, we separately demonstrated RAR and RIBE using an *in vivo* system of *C. elegans*. In the current study, we further investigated their interaction in *C. elegans*, with the ratio of protruding vulva as the biological end point for RAR. Fourteen-hour-old worms were first locally targeted with a proton microbeam, and were then challenged with a high dose of whole-body gamma radiation. Microbeam irradiation of the posterior pharynx bulbs and rectal valves of *C. elegans* could significantly suppress the induction of protruding vulva by subsequent gamma irradiation, suggesting a contribution of RIBE to RAR in the context of the whole organism. Moreover, *C. elegans* has a unique DNA damage response in which the upstream DNA damage checkpoint is not active in most of somatic cells, including vulval cells. However, its impairment in *atm-1* and *hus-1* mutants blocked the RIBE-initiated RAR of vulva. Similarly, mutations in the *atm-1* and *hus-1* genes inhibited the RAR of vulva initiated by microbeam irradiation of the vulva itself. These results further confirm that the DNA damage checkpoint participates in the induction of RAR of vulva in *C. elegans* in a cell nonautonomous manner.

INTRODUCTION

Radioadaptive responses (RAR) and radiation-induced bystander effects (RIBE), two conflicting biological phenomena, are important in determining the biological effects of low-dose radiation, and may also affect the dose-response relationship (*1*). These phenomena have been extensively investigated in multiple cell and organism systems (*2, 3*). At the same time, there exist two types of interactions between RAR and RIBE. In one interaction, RAR is initiated in bystander cells by directly irradiated cells through the bystander effect, rendering the bystander cells more resistant to subsequent challenging irradiation (*4*); in the other interaction, these bystander effects are attenuated when the bystander cells are pretreated with low-dose radiation (*5*). However, these interactions are mainly demonstrated in cell culture systems *in vitro*, and little is known about their manifestations in the context of whole organisms.

The model organism *C. elegans* has been extensively used to investigate the adaptive responses induced by various environmental stresses (*6*). The *C. elegans* vulva, located on the middle ventral surface of the body, is an ideal tissue model *in vivo* for radiation-induced reproductive cell death (RCD). This radiation-induced cell death in the process of divisions, or RCD, results in abnormal phenotypes of the vulva, including protruding vulva and vulvaless phenotypes (*7*). Unlike cell and mouse models, *C. elegans* is highly resistant to ionizing radiation, which leads to a requirement of higher radiation doses (100–400 Gy) for induction of the vulva abnormality (*7*). For the same reason, RAR for RCD in the *in vivo* vulva model is also initiated by a higher priming dose of gamma rays (5 Gy) that suppresses the induction of protruding vulva by subsequent gamma irradiation (75 and 100 Gy) (*8*). Notably, the DNA damage checkpoint was found to participate in the induction of RAR

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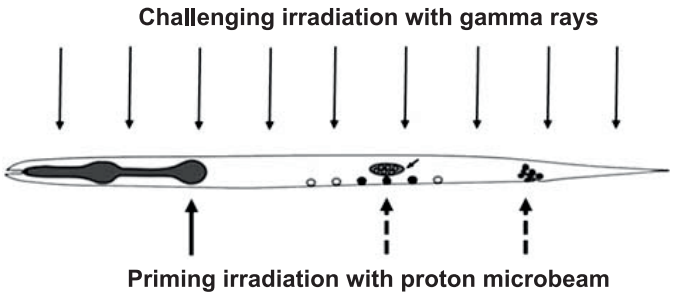

FIG. 1. Schematic representation of the combined irradiation of *C. elegans* with proton microbeam and gamma rays. From left to right: The microbeam-targeted sites are the posterior pharynx bulb, vulva and rectal valve. The circles in the middle of the body represent the vulval precursor cells P5.p, P6.p and P7.p, and the gonad is indicated by the arrow.

of vulva, despite the absence of an active DNA damage checkpoint in the vulval cells (*8*). We therefore propose that the vulval RAR might be induced in a cell nonautonomous manner, which further raises the question of whether vulval RAR can be initiated by irradiation of other body tissues through RIBE.

The RIBE in *C. elegans* have been demonstrated using microbeam irradiation with protons (*9*) and $^{12}C^{5+}$ carbon ions (*10*). In addition, we have previously reported that proton microbeam irradiation of the posterior pharynx bulbs and rectal valves in *C. elegans* could increase the level of germ cell apoptosis in gonads, indicating radiation-induced bystander signaling from somatic cells to germ cells (*11*). Given that the *C. elegans* vulva is located in a small region at the central part of the body, far from the pharynx bulb in the head and rectal valve in the tail, microbeam irradiation might be a suitable approach for examining the interaction between RAR and RIBE in *C. elegans*.

In this study, the proton microbeam was used to locally irradiate the posterior pharynx bulb, rectal valve and vulva in young worms. The worms were then completely exposed to high doses of gamma radiation. The ratio of protruding vulvas was examined in the adult *C. elegans*. Microbeam irradiation of the posterior pharynx bulbs and rectal valves both initiated the RAR of the vulva. Notably, although the DNA damage checkpoint is absent in vulval cells, its deficiency can inhibit the RAR of vulva, which is initiated by microbeam irradiation of the pharynx bulb, rectal valve and vulva itself.

MATERIALS AND METHODS

Worm Strains and Synchronization

The *C. elegans* strain N2 variety Bristol was used for general experiments. In addition, the mutant strains VC381: *atm-1*(*gk186*)I and WS2277: *hus-1*(*op241*)I were employed for investigating the role of the DNA damage checkpoint in RIBE-initiated RAR of vulva. To detect the DNA damage checkpoint in body tissues, we used worm strains transgenic for *hus-1::gfp*, WS1433: *hus-1*(op241)I; *unc-119*(ed3)III; opIs34 and *cep-1::gfp*, TG11:*cep-1* (*lg12501*)*I*; *unc-119* (*ed4*)III; *gtEx2*.

All strains were provided by the Caenorhabditis Genetics Center (St. Paul, MN). The worms were cultured and manipulated according to the standard procedures as described by Brenner (*12*).

Synchronized young worms were obtained according to a previously described method (*7*). Briefly, gravid hermaphrodites were washed off plates and were digested with NaOH and bleach solution. The resulting embryos were washed with 1× PBS, plated on unseeded agarose-containing Petri dishes and placed at 20°C for 14 h. The larvae were transferred to *E. coli* OP50-seeded plates and upon placement of food, they were considered 1 h old.

Irradiations

Figure 1 shows a schematic representation of the combined irradiation of *C. elegans* with proton microbeam and gamma rays.

Local Irradiation of Worms with Proton Microbeam

The 14-h-old worms were locally irradiated using the proton microbeam facility in our laboratory at the Chinese Academy of Sciences [Key Laboratory of Ion Beam Bioengineering (CAS-LIBB)] as previously described (*11*). Briefly, worms were selected and placed on 2% agarose gels and then anesthetized by exposure to fumes from 60 μl volatile liquid (mixture of ethanol and ethyl ether in equal volumes) for 3 min. After anesthesia, the worms were placed on a 3.5 μm thick Mylar® film bottom of a radiation dish, then covered with a thin piece of agarose gel. Before microbeam irradiation, the worm images were captured by an integrating CCD camera (CoolSNAP HQ2; Roper Scientific Inc., Tucson, AZ), and the posterior pharynx bulb, rectal valve and vulva were marked manually using an X-Y sample platform controller, respectively. Upon irradiation, the treated worms and their controls were removed from the Mylar film with M9 buffer and then transferred to NGM plates for further analysis.

The average energy of incident protons was 3.2 MeV with a linear energy transfer (LET) of 11 keV/μm at the front surface of the worms, and the average beam diameter on the samples measured 8.7 μm using CR-39 solid detectors (Fukuvi Chemical Industry Co., Fukui Japan) for 10,000 protons.

Whole-Body Irradiation of Worms with Gamma Rays

After microbeam irradiation, the worms were grown on OP50-seeded plates ($\Phi = 6$ cm) for 2 h, and then whole-body gamma irradiated at a dose rate of 3.37 Gy/min using a BIOBEAM Cesium-137 (Cs^{137}) irradiator (cat. no. GM 2000; Gamma-Service Medical, Leipzig, Germany). After gamma irradiation, the worms were immediately transferred to fresh OP50-seeded plates and grown at 20°C to adulthood without disturbance and with adequate food (*8*).

Phenotypic Characterization of Worm Vulva

On approximately day 3 after gamma irradiation, the adult worms were anesthetized with 40 m*M* of NaN_3, and placed onto glass slides with some M9 solution. The protruding vulva was examined using a 20× or 40× optic microscope (*8*). The final data represent the average of at least four independent experiments, and more than 100 worms were used for each experiment.

Statistical Analysis

All data were evaluated in terms of mean ± standard deviation (SD). The Student's *t* test was used to determine the statistical significance between treated and control groups or between treated groups. A *P* value of 0.05 or less between groups was considered statistically significant.

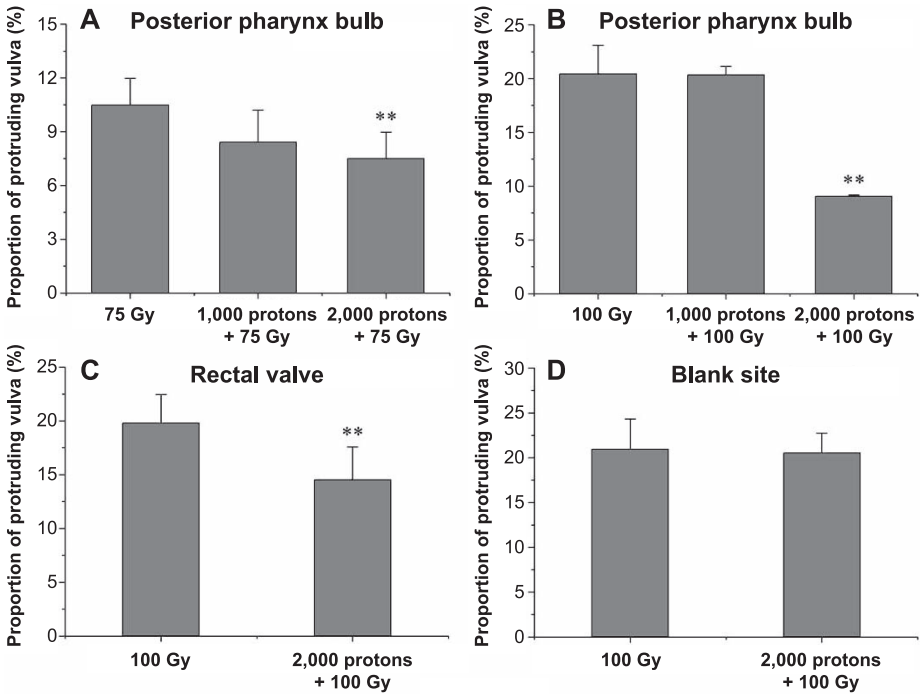

FIG. 2. Induction of the RAR of vulva by combined irradiation with proton microbeam and gamma rays. Panels A and B: The ratio of protruding vulva induced by the dose combinations of 1,000 or 2,000 protons with 75 Gy (panel A) or with 100 Gy (panel B) gamma radiation, with the posterior pharynx bulb as the microbeam target. Panel C: The ratio of protruding vulva induced by the dose combination of 2,000 protons with 100 Gy gamma radiation, with the rectal valve as the microbeam target. Panel D: The ratio of protruding vulva induced by the dose combination of 2,000 protons with 100 Gy, with the blank site close to the posterior pharynx bulb as the microbeam target. Results are means ± SD ($n \geq 4$, *t* test, $^{**}P < 0.01$).

RESULTS

The Systemic Induction of RAR of Vulva by Microbeam Irradiation of Worms

In our previously published studies, we showed that the RAR of vulva in *C. elegans* could be induced by whole-body exposure of 14-h-old worms to 5 Gy of gamma rays, and then 75 or 100 Gy of gamma rays with a time interval of 2 h (*8*); bystander effects in *C. elegans* were significantly induced by microbeam irradiation of the posterior pharynx bulbs with 1,000 and 2,000 protons (*11*). In the current study, the posterior pharynx bulbs of 14-h-old worms were first targeted by a microbeam of 1,000 and 2,000 protons, respectively. After 2 h, the worms were whole-body gamma irradiated with 75 and 100 Gy, respectively. Single microbeam irradiation did not cause a phenotype of protruding vulva, as shown in Supplementary Table S1 (http://dx.doi.org/10.1667/RR14548.1.S1). Compared to a single 75 Gy gamma-radiation exposure, the ratio of protruding vulva was significantly decreased by the dose combination of 2,000 protons with 75 Gy ($P < 0.01$), but not by 1,000 protons with 75 Gy ($P = 0.06$), as shown in Fig. 2A. The dose combination of 2,000 protons with 100 Gy also resulted in significantly repressed induction of protruding vulva ($P < 0.01$); however, this was not the case for the combination of 1,000 protons with 100 Gy ($P > 0.05$) compared to that of a single 100 Gy gamma-radiation expsoure, as shown in Fig. 2B. Hereafter, the dose combination of 2,000 protons with 100 Gy was employed in the following experiments, unless otherwise specified. In our previously published study, we showed that microbeam irradiation of the rectal valves in the tails of *C. elegans* could also cause RIBE, although to a weaker extent relative to the posterior pharynx bulbs (*11*). Therefore, we performed another experiment of the RAR of vulva with the dose combination of 2,000 protons with 100 Gy gamma radiation, in which the worm rectal valves were locally irradiated with 2,000 protons. As shown in Fig. 2C, the RAR of vulva was significantly induced by this dose combination ($P < 0.01$). Nevertheless, the vulval RAR may be directly initiated by the scattering dose of the microbeam radiation on the vulva. To exclude this possibility, a mock experiment of the RAR of vulva was performed, in which the proton microbeam was targeted to blank sites close to the posterior pharynx bulbs of the worms. The ratio of protruding vulva was not changed by the mock dose combination of 2,000 protons with 100 Gy compared to that of worms treated with 100 Gy of gamma rays alone ($P > 0.05$), as shown in Fig. 2D. These results suggest that the RAR of vulva could be systemically induced through RIBE in *C. elegans*.

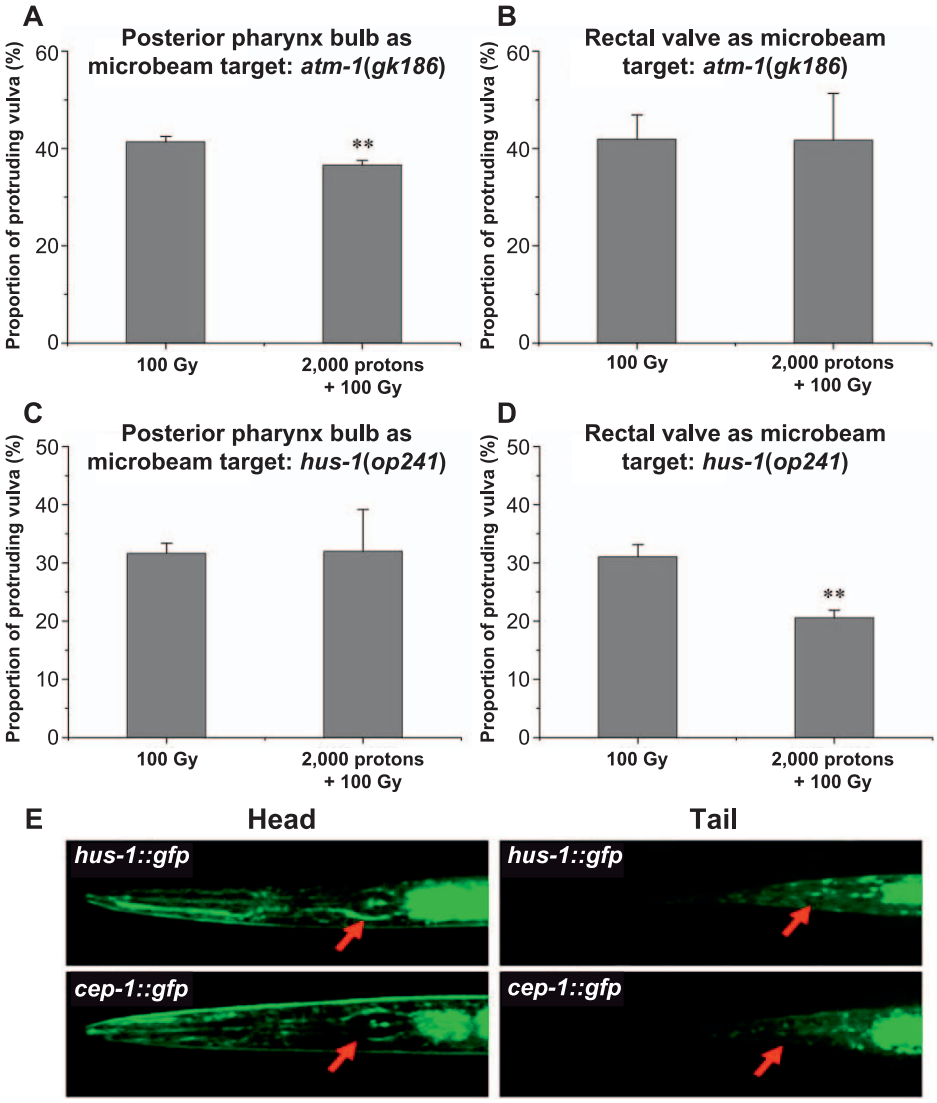

FIG. 3. Induction of the RAR of vulva by combined irradiation with proton microbeam and gamma rays in worms deficient in the DNA damage checkpoint. Panels A and B: The ratio of protruding vulva in *atm-1* worms induced by the dose combination of 2,000 protons with 100 Gy, with the posterior pharynx bulb (panel A) and rectal valve (panel B) as microbeam targets. Panels C and D: The ratio of protruding vulva in *hus-1* worms induced by the dose combination of 2,000 protons with 100 Gy, with the posterior pharynx bulb (panel C) and rectal valve (panel D) as microbeam targets. Panel E: GFP fluorescence in the worms transgenic for *hus-1::gfp* and *cep-1::gfp*. Results are means ± SD ($n \geq 4$, *t* test, $^{**}P < 0.01$).

Involvement of DNA Damage Checkpoint in Systemic Induction of RAR of Vulva

Although the DNA damage checkpoint has been shown to participate in the induction of the RAR of vulva by whole-body gamma irradiation of worms (*8*), its role in the systemic induction of the RAR of vulva remains to be determined. For this purpose, worm mutants of *atm-1*(*gk186*) and *hus-1*(*op241*) that are deficient in the DNA damage checkpoint were used. Microbeam irradiation of the posterior pharynx bulbs of *atm-1* worms with the dose combination of 2,000 protons with 100 Gy led only to a slight (1.13-fold) decrease in the ratio of protruding vulva compared to that by single 100 Gy gamma irradiation ($P < 0.01$; Fig. 3A), while also exhibiting significantly inhibited induction of RAR relative to the 2.26-fold decrease in N2 worms (Fig. 2B). For microbeam irradiation of the rectal valves, the vulval RAR was completely blocked by the mutation of the *atm-1* gene ($P > 0.05$), as shown in Fig. 3B. However, in *hus-1* worms, the vulval RAR was significantly blocked in the case of microbeam irradiation of the posterior pharynx bulbs ($P > 0.05$), but not of the rectal valves ($P < 0.01$; Fig. 3C and D). These results suggest that the DNA damage checkpoint might also participate in the systemic induction of RAR of vulva. Notably, in worms transgenic for *hus-1::gfp* and *cep-1::gfp*, low, but detectable GFP fluorescence was observed in partial regions of the posterior pharynx bulbs and rectal valves (Fig. 3E), indicating the presence of the active DNA

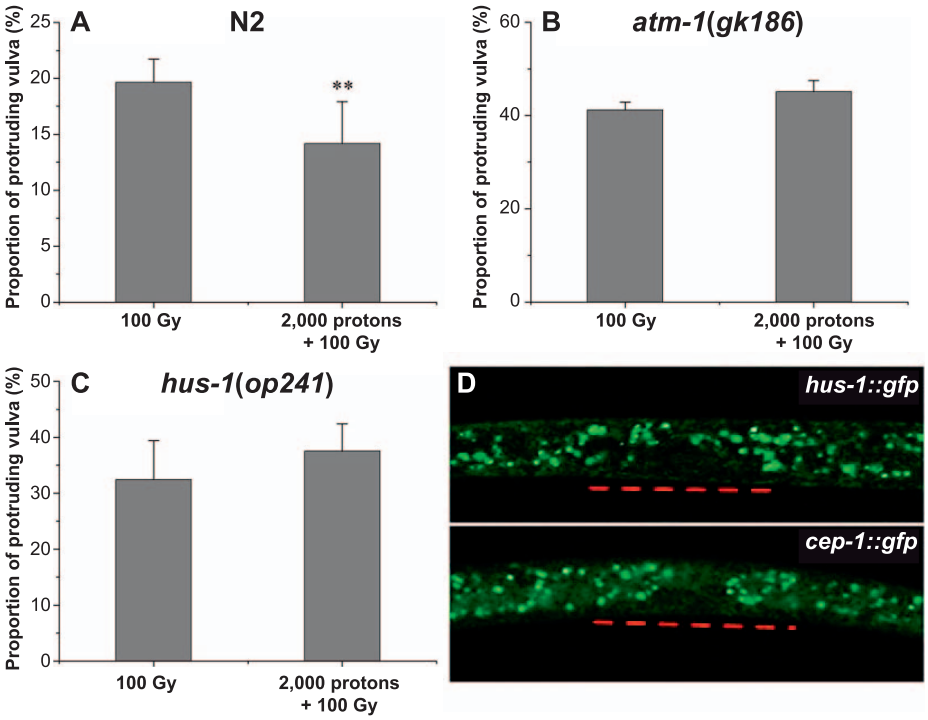

FIG. 4. The RAR of vulva initiated by microbeam irradiation of the vulva itself. Panels A–C: The ratio of protruding vulva induced by the dose combination of 2,000 protons with 100 Gy, with the vulva itself as the microbeam target, in N2 (panel A), mutant *atm-1* (panel B) and mutant *hus-1* (panel C) worms. Panel D: GFP fluorescence in the vulval region of the worms transgenic for *hus-1::gfp* and *cep-1::gfp*. The dashed red line indicates the position of the P5.p, P6.p and P7.p cells. Results are means ± SD ($n \geq 4$, *t* test, ** $P < 0.01$).

damage checkpoint in these body tissues, at least in the 14-h-old worms.

Induction of the RAR of Vulva by Microbeam Irradiation of the Vulva Itself

After confirming the systemic induction of RAR of vulva through RIBE, we then examined whether the vulval RAR can be initiated by microbeam irradiation of the vulva itself. For this purpose, the vulval precursor cell P6.p in the middle vulval region was irradiated with 2,000 microbeam protons, possibly with irradiation of the partial P5.p and P7.p cells flanking P6.p. As shown in Fig. 4A, the vulval RAR was likewise induced by the dose combination of 2,000 protons with 100 Gy ($P < 0.01$). However, there was no detectable GFP fluorescence in this vulval region of the worms that are transgenic for *hus-1::gfp* and *cep-1::gfp*, as shown in Fig. 4D, which therefore raised the question of whether the DNA damage checkpoint is also involved in the vulval RAR initiated by microbeam irradiation of the vulva itself. To address this, the mutant worms of *atm-1* and *hus-1* were irradiated with the combination of 2,000 protons with 100 Gy, with the vulva itself as the target of microbeam irradiation. Surprisingly, the induction of vulval RAR was completely blocked in both mutant worms (in both cases, $P > 0.05$), as shown in Fig. 4B and D. These results further suggest that the DNA damage checkpoint might participate in the induction of RAR of vulva in a cell nonautonomous manner.

DISCUSSION

Although RIBE and RAR are two conflicting effects of low-dose radiation, there exist two types of interactions between them (*2, 3*). This study clearly demonstrated one of these interactions in the *in vivo* system of *C. elegans*: bystander cell response to subsequent high-dose irradiation could be attenuated by the hit cells through bystander effects. Single microbeam irradiation of the posterior pharynx bulbs and rectal valves did not cause a visibly abnormal phenotype in the vulva (see Supplementary Table S1; http://dx.doi.org/10.1667/RR14548.1.S1). Therefore, this experimental system of *C. elegans* could not be used to demonstrate the other type of interaction between RAR and RIBE. Moreover, in view of the radiosensitivity of *C. elegans* body tissues in terms of the induction of bystander effects (*11*), only the posterior pharynx bulb and rectal valve were chosen here, to demonstrate the systemic induction of RAR of vulva. In fact, the germline of *C. elegans*, including six germ cells in the 14-h-old worm, is also an important cell group that regulates many aspects of worm development through signal communication with somatic cells, including the vulval cells (*13*). Therefore, it is relevant to examine the RAR of vulva initiated by microbeam irradiation of the germline. However, although the position

of the gonad in the 14-h-old worm is morphologically identifiable under the microscope of the microbeam terminal, targeting this in isolation with the microbeam is very difficult given its adjacency to the vulval region and the large diameter of microbeam used here (>8 μm). Relative to the RAR of vulva that can be effectively induced by a 5 Gy priming gamma-radiation exposure (*8*), the RAR of vulva was initiated here by 2,000 microbeam protons (Fig. 2A and B), which is equal to an absorbing dose of 58.62 Gy at the irradiation site for a 8.7 μm beam diameter (*11*). It has been reported that bystander effects are induced by transmissible factors, which are produced by the cells receiving the actual dose (*14*). Therefore, in this study, the requirement of a higher priming dose for RAR of vulva is possibly because only some body cells that can potentially initiate the RAR of vulva through bystander effects are directly traversed compared to the RAR of vulva initiated by whole-body gamma irradiation. However, it is also possible that the differing radiation quality of gamma rays and protons led to the varied requirement of priming doses for the RAR of vulva.

The DNA damage checkpoint is a vital mechanism for maintaining genomic stability in the cellular response to DNA damage and DNA replication stress through activating downstream DNA repair mechanisms (*15*). However, in somatic cells of *C. elegans*, active DNA repair mechanisms exist (*16*), whereas their upstream DNA damage checkpoint is absent (*17*). In our previous study, the DNA damage checkpoint was proposed to participate in the RAR of vulva in a cell non-autonomous manner (*8*). In the current study, the involvement of the DNA damage checkpoint in the vulval RAR, which was initiated by microbeam irradiation of the posterior pharynx bulbs and rectal valves (Fig. 3), further confirmed this hypothesis. Remarkably, a low activity of the DNA damage checkpoint in the region of the posterior pharynx bulb and rectal valve, but not the vulva, was observed in the 14-h-old worms (Figs. 3E and 4D). It is therefore likely that the DNA damage checkpoint might be involved in the production of bystander signals for initiating the RAR of vulva in these two microbeam-irradiated sites. Moreover, it was shown that ATM-1, but not HUS-1, was involved in the RAR of vulva initiated by microbeam irradiation of the rectal valve in the tail, even though *hus-1* expression was observed in this region (Fig. 3E). Relative to the dictatorial response of the ATM pathway to DNA double-strand breaks (DSBs), the ATR pathway, in which the HUS-1 functions, senses a broad spectrum of DNA damage (*18*). Therefore, it is likely that microbeam irradiation might lead to predominant DSBs in the cells of the rectal valve region, possibly relying on cell cycles. Furthermore, although no active DNA damage checkpoint was observed in the cells of the vulval region (Fig. 4D), mutations in the *atm-1* and *hus-1* genes could also effectively block the RAR of vulva initiated by the microbeam irradiation of the vulva itself. It is therefore likely that vulval cells irradiated with the microbeam might signal other body cells with the active DNA damage checkpoint, which then induce the RAR in the vulva in a feedback manner.

In this study, we initially demonstrated the link between RIBE and RAR in the *in vivo* system of *C. elegans*, and further confirmed that the DNA damage checkpoint is involved in the systemic induction of vulval RAR. However, whether the germ cells also participate in this process remains to be determined, and a more accurate microbeam might be required for addressing this question.

SUPPLEMENTARY INFORMATION

Table S1. The occurrence of protruding vulva induced by single microbeam irradiation of the posterior pharynx bulb and rectal valve.

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