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Distinctive transforming genes in x-ray-transformed mammalian cells

(oncogenes/radiation carcinogenesis/gene transfer)

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ABSTRACT **DNAs from hamster embryo cells and mouse C3H/10T1/2 cells transformed *in vitro* by x-irradiation into malignant cells transmit the radiation transformation phenotype by producing transformed colonies (transfectants) in two mouse recipient lines, the NIH 3T3 and C3H/101/2 cells, and in a rat cell line, the Rat-2 cells. DNAs from unirradiated cells or irradiated and visibly untransformed cells do not produce transformed colonies. The transfectants grow in agar and form tumors in nude mice. Treatment of the DNAs with restriction endonucleases prior to transfection indicates that the same transforming gene (oncogene) is present in each of the transformed mouse cells and is the same in each of the transformed hamster cells. Southern blot analysis of 3T3 or Rat-2 transfectants carrying oncogenes from radiation-transformed C3H/10T1/2 or hamster cells indicates that the oncogenes responsible for the transformation of 3T3 cells are not the Ki-*ras*, Ha-*ras*, or N-*ras* genes, nor are they *neu*, *trk*, *raf*, *abl*, or *fms*, although quick blot analysis using 11 oncogene probes detected increased transcripts of c-*abl* and c-*fms* in the 3T3 transformants containing oncogenic sequences from the x-ray-transformed C3H/10T1/2 cells. The work demonstrates that DNAs from mammalian cells transformed into malignancy by direct exposure *in vitro* to radiation contain genetic sequences with detectable transforming activity in three recipient cell lines. The results provide evidence that DNA is the target of radiation carcinogenesis induced at a cellular level *in vitro*. The experiments indicate that malignant radiogenic transformation *in vitro* of hamster embryo and mouse C3H/10T1/2 cells involves the activation of unique non-*ras* transforming genes, which heretofore have not been described.**

The molecular mechanisms in radiation carcinogenesis are poorly understood. Cell cultures provide powerful models for investigating the process of radiation-induced malignant transformation under conditions free from host-mediated effects (1–4). In this situation, irradiated single cells give rise to transformed and ultimately tumorigenic populations (1–5).

Our earlier studies on diploid hamster (1–4) and human cells (5) implicated DNA as a target in radiogenic transformation. However, direct proof was beyond reach until methods of DNA-mediated gene transfer (transfection) became available (6–21). The development of these methods using NIH 3T3 or rat cells as DNA recipients in the focus assay (9) led to the identification of oncogenic DNA sequences in cells of a variety of human and rodent tumors (refs. 10–14 and 16–20; reviewed in refs. 11 and 16) and in rodent cells transformed in culture by chemical carcinogens (9, 19, 21). With few exceptions, the transforming genes were shown to be activated forms of the *ras* gene family, including cellular counterparts (c-*ras*) of viral oncogenes in the Kirsten (22) or Harvey (23) murine sarcoma viruses, designated Ki-*ras* and Ha-*ras*, respectively, as well as the N-*ras* gene (17, 18).

The present work was undertaken to establish whether mammalian cells transformed *in vitro* by x-irradiation into malignant cells contain detectable transforming genes in their DNA and, if so, to identify the oncogenic sequences that arose in the cells after their direct interaction with the ionizing radiation.

MATERIALS AND METHODS

Cell Cultures and Transformation. The induction of malignant transformation by 3 Gy (300 rad) of x-rays in freshly explanted cultures of cloned hamster cells (1–4) and in mouse C3H/10T1/2 clone 8 (24) at passage 7 served as a starting point in these experiments. The protocols for transformation of these two systems have been detailed elsewhere (1–4, 24, 25). Morphologically transformed hamster clones (1–4) and type III transformed foci of the C3H/10T1/2 cells (4, 24, 25) were isolated and propagated into transformed cell lines as detailed (4, 25). Unirradiated cells and irradiated nontransformed cells grown under the same experimental conditions served as controls. All cells were tested for their ability to grow in 0.3% agar and to form tumors in nude mice (4, 5, 25). Cell lines derived of five independently transformed hamster clones and five independently transformed foci of C3H/10T1/2 cells with growth potential *in vivo* were used for transfection experiments. In addition, cells cultured from a tumor induced by a hamster x-ray-transformed line (4) and a tumor induced by a C3H/10T1/2 x-ray-transformed line (4) served as representatives of *in vitro*-transformed cells that had undergone further replications *in vivo*.

Transfection Assay. Transfection experiments with high molecular weight DNA extracted from x-ray-transformed hamster and C3H/10T1/2 cells were carried out using the calcium phosphate precipitation method (8, 26–30). In each transfection, 40 μg of donor cell DNA, isolated and purified as reported (28), was cotransfected with a selectable marker (15, 29–31), the pSV2gpt (29) (1 μg per 100-ml plate) onto 5 × 10^5 NIH 3T3 cells, C3H/10T1/2 cells, or Rat-2 cells (18). The transfected cells handled and subcultured as reported (27) were maintained continuously in the presence of selective medium containing mycophenolic acid (15, 29, 30).

In some experiments, before transfection each of the DNAs was cleaved with one of a series of five restriction endonucleases using reported methods (32).

Three weeks after transfection, transformed colonies growing in 5% serum in mycophenolic acid-containing medium were picked from among the mycophenolic-resistant cells and expanded into large cell populations. The DNA was extracted from some of the cells; other cells from the same population were tested for their ability to grow in agar and produce tumors in nude mice.

Analysis of Clonal Transformed Cells. DNA isolated from representative transfectants or from parental normal and

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transformed cells were subjected to Southern blot analysis (33) following reported methods (18) and were probed with Ki-*ras*, Ha-*ras*, and N-*ras* probes or *neu* (31), *trk* (34), *raf* (35), *abl* (36), or *fms* (37). Oncogene expression was analyzed by the quick blot method (38) by molecular hybridization of DNA probes to mRNA immobilized on nitrocellulose filters directly from NaI-solubilized cells.

Probes. The probes used in the Southern blot analysis were kindly provided as follows: The *ras* genes, which have been described earlier (18), were provided by A. Pellicer; the *neu* gene, a 0.42-kilobase (kb) *Bam*HI fragment, by R. A. Weinberg (31); the *trk* gene (34), a 1.2-kb *Eco*RI *Sma* I fragment, by M. Barbacid; the *raf* gene, a 0.7-kb *Sal* I fragment, by U. R. Rapp (35); the *abl* gene (36), a 1.2-kb *Bgl* II fragment, by E. P. Reddy; and the *fms* gene (37), a 1.4-kb *Pst* I, by C. J. Sherr.

RESULTS AND DISCUSSION

Transformed Colonies Induced by DNA from *in Vitro* X-Ray-Transformed Cells. High molecular weight DNAs extracted from five independently x-ray-transformed hamster lines, a tumor produced by hamster-transformed cells, three mouse C3H/10T1/2 transformed lines, and a tumor produced by a C3H/10T1/2-transformed line, induced the appearance of transformed colonies in mouse NIH 3T3, C3H/10T1/2, or Rat-2 recipient cells (Fig. 1; Tables 1 and 2). The procedure using a selective marker indicated to us that the transformants were a direct result of transfection rather than spontaneous transformations arising in the recipient cultures.

While the competence for transfection by gpt was similar in the cells of the two mouse lines 3T3 and the C3H/10T1/2, as observed by others (30), the efficiency of transformants produced by transfection with DNAs from the radiation-transformed cells was somewhat lower in the C3H/10T1/2 recipients than in the NIH 3T3 cells (Table 1). DNA from unirradiated or irradiated untransformed cells did not produce transformants in the recipient lines (Table 1). A representative transformed colony induced in C3H/10T1/2 cells by DNA of hamster x-ray-transformed cells is shown in Fig. 1.

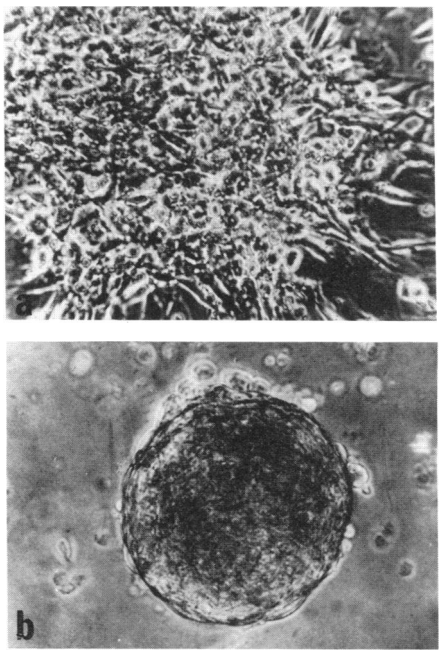

FIG. 1. (*a*) A transformed colony of recipient C3H/10T1/2 mouse cells cotransfected with DNA from *in vitro* x-ray-transformed hamster embryo cells and PSV2gpt. (*b*) C3H/10T1/2 transformants from *a* growing on agar.

We conclude from these results that the DNAs of hamster embryo and mouse C3H/10T1/2 cells, transformed in culture by x-irradiation, were altered relative to the DNAs of the untransformed counterparts. These alterations took place following a direct interaction of the physical carcinogen with the cells and resulted in the generation of potent transforming genes, which are not detectable in the normal cellular DNA.

We tested the ability of the transformants to grow in agar and form tumors in nude mice by procedures described elsewhere (4, 5). Cells from the transfected colonies isolated from 3T3, C3H/10T1/2, or Rat-2 recipients formed colonies in 0.3% agar and produced rapidly growing solid tumors. Control recipient NIH 3T3, C3H/10T1/2, or Rat-2 cells or cells transfected with DNA from normal or irradiated but untransformed cells did not proliferate in agar or induce tumors in the animals.

Upon isolation of the transformed foci and retransfection of the cells into the same recipient line, we found that the efficiency of focus formation was not significantly altered in secondary and tertiary rounds of cotransfection with pSV2gpt in the three recipient lines.

Effects of Restriction Enzyme Cleavages on Transfection with DNA. The cleavage of hamster and mouse DNA by site-specific restriction endonucleases provides a useful method for comparing the structures of transforming genes being transferred from the x-ray-transformed cells. Before transfection, each of the DNAs was cleaved with one of a series of five restriction enzymes. Each of these enzymes recognizes a different nucleotide sequence at its site of cleavage (32). The results of these experiments are summarized in Table 2. The data indicate an identical pattern of sensitivity and resistance to the restriction enzymes among the DNAs of the lines of the same species.

The enzymes *Eco*RI, *Kpn* I, and *Hin*dIII inactivated the transforming activity of each of the mouse C3H/10T1/2 DNAs. In contrast, *Bam*HI and *Xho* I had no effect. These results strongly suggest that the same transforming principle is present in each of these cells. Similar results were obtained with the x-ray-transformed hamster embryo cells. In this case, each DNA was resistant to *Xho* I and sensitive to the other endonucleases. Identity in the sensitivity or resistance of transforming DNA to restriction endonucleases has been used as criteria to establish oncogene identity (11). Unfortunately, different sensitivity patterns cannot be used to compare oncogenes of different species. For instance, Ha-*ras1* oncogenes of human, rat, and mouse origin exhibit distinct patterns of sensitivity in spite of the high degree of conservation of this locus during evolution.

Cleavage of DNAs from normal, unirradiated, or irradiated but untransformed hamster and mouse cells by the five restriction enzymes did not create transfectable transforming genes (Table 2). These results indicate that radiation has reproducibly induced the activation of specific transforming genes in both mouse and hamster cells.

Analysis of DNAs from *in Vitro* X-Ray-Transformed Cells. Studies on mouse C3H/10T1/2 transformed *in vitro* by chemical carcinogens (19) and on thymomas induced in mice by exposure to γ irradiation (18) have reported the activation of the c-Ki-*ras* gene in the neoplastic cells.

To determine whether *ras* genes are activated during *in vitro* radiogenic transformation of the hamster embryo and mouse C3H/10T1/2 cells, DNAs from 3T3 or Rat-2 cells cotransfected with pSV2gpt and hamster or mouse DNA and following two cycles of transfection were analyzed by Southern blotting (33) and hybridized with Ki-*ras*- Ha-*ras*-, or N-*ras*-specific probes (18, 39). DNAs from original normal and transformed parental hamster and mouse x-ray transformants as well as DNAs from tumors produced in nude

Table 1. Transforming activity of hamster embryo and mouse C3H/10T1/2 tumor and transformants of DNA

Donor DNA	Efficiency of transformation					
	NIH 3T3		C3H/10T1/2		Rat-2	
	Tr col/ μg of DNA	Tr col/ no. of plates	Tr col/ μg of DNA	Tr col/ no. of plates	Tr col/ μg of DNA	Tr col/ no. of plates
HE (secondary cultures)	<0.001	0/80	<0.001	0/80	<0.001	0/30
HE irradiated, untransformed	<0.001	0/70	<0.001	0/85	<0.001	0/30
HE x-ray-transformed line H_1	0.23	147/16	0.20	144/18	0.012	14/30
HE x-ray-transformed line H_2	0.25	160/16	0.17	126/18	0.015	18/30
HE x-ray-transformed line H_3	0.18	127/18	0.14	112/20	ND	ND
HE tumor induced by line H_1	0.15	158/26	0.09	116/30	0.008	10/32
NIH 3T3 normal	<0.001	0/70	ND	ND	ND	ND
C3H/10T1/2 normal	<0.001	0/80	<0.001	0/80	<0.001	0/32
C3H/10T1/2 irradiated untransformed	<0.001	0/90	<0.001	0/90	<0.001	0/28
C3H/10T1/2 x-ray-transformed line C_1	0.15	120/20	0.10	116/29	0.009	11/30
C3H/10T1/2 x-ray-transformed line C_2	0.18	122/17	0.13	130/25	0.011	13/29
C3H/10T1/2 x-ray-transformed line C_3	0.20	120/15	0.15	120/17	ND	ND
C3H/10T1/2 tumor induced by line C_1	0.12	125/26	0.09	101/28	0.007	8/29

In each transfection, 40 μg of DNA was cotransfected with 1 μg of pSV2gpt and the cells were grown in selection medium containing mycophenolic acid (15, 31, 34). Transformed foci were scored 21 days later. Tr col, transformed colonies; ND, not determined; HE, hamster embryo.

mice by the *in vitro* x-ray-transformed hamster or C3H/10T1/2 cells were also analyzed.

Newly acquired *ras* genes in transformed foci can be identified either by comparing band intensities with appropriate controls on genomic Southern blot or by the presence of additional restriction fragments containing the extra genes.

On analyzing the DNA of the NIH 3T3 and Rat-2 cells containing x-ray-transformed hamster or mouse DNA with probes of Ki-*ras*, Ha-*ras*, or N-*ras*, no extra bands were observed and the intensities of the patterns of hybridization were found to be identical to those in the 3T3 or Rat-2 controls (see Fig. 2 for the Ki-*ras* gene; results on N-*ras* and Ha-*ras* genes are similar but not shown). Our data are consistent with the fact that the oncogenic sequences activated in the *in vitro* x-ray-transformed hamster and mouse lines are not the Ki-*ras*, Ha-*ras*, or N-*ras* genes. Analysis of DNAs from the tumors induced by the hamster or mouse x-ray-transformed lines (data not shown) gave similar results, indicating that *ras* gene activation did not occur even after the expansion of the transformed cell population *in vivo* under host-mediated responses.

Analysis of the transformants with three additional molecular probes from oncogenes known to be active in the focus forming assay, *neu* (31), *trk* (34), and *raf* (35), were also negative (data not shown), indicating that the *in vitro* radiation-induced transformants contain transforming genes that have heretofore not been described.

Detection of donor DNA sequences in cells transformed by gene transfer techniques is often considered as sufficient evidence for the existence of dominant transforming genes. Donor sequences are identified by the presence of repetitive sequences specific for the donor species. This experimental approach was first used to demonstrate human repetitive (*Alu*) sequences (40) in NIH 3T3 cells transformed by human tumor DNAs. Subsequently, these *Alu* repetitive sequences have served as markers for the molecular cloning of several human oncogenes.

Unfortunately, this experimental approach could not be used in our present studies, where DNA of x-ray-transformed mouse C3H/10T1/2 cells or hamster embryo cells were transfected onto mouse or rat cells. There are no repetitive DNA probes capable of identifying hamster or mouse DNA over a mouse or rat background (40). Therefore, demonstration of transforming genes in radiation transformed mouse and hamster cells must rely, at the present, on biological studies.

Analysis of RNAs from *in Vitro* X-Ray-Transformed Cells. We explored the involvement of other genes in radiogenic transformation *in vitro* by investigating the expression of 11 known oncogenes in secondary transformants of 3T3 cells

Table 2. The effect of restriction enzyme cleavages on the transfection of DNAs from normal and *in vitro* x-ray-transformed hamster embryo and C3H/10T1/2 cells

	Number of transformed colonies per μg of DNA					
	No enzyme	*Eco*RI	*Bam*HI	*Kpn* I	*Xho* I	*Hin*dIII
C3H/10T1/2 normal cells	0	0	0	0	0	0
C3H/10T1/2 irradiated untransformed cells	0	0	0	0	0	0
C3H/10T1/2 x-ray-transformed cell line C_1	0.15	0	0.14	0	0.16	0
C3H/10T1/2 x-ray-transformed cell line C_4	0.11	0	0.11	0	0.17	0
C3H/10T1/2 x-ray-transformed cell line C_5	0.09	0	0.09	0	0.13	0
HE secondary, normal cells	0	0	0	0	0	0
HE x-irradiated untransformed cells	0	0	0	0	0	0
HE x-ray-transformed cell line H_1	0.23	0	0	0	0.25	0
HE x-ray-transformed cell line H_4	0.13	0	0	0	0.18	0
HE x-ray-transformed cell line H_5	0.18	0	0	0	0.20	0

In each transfection 40 μg of DNA was applied to 5×10^5 NIH 3T3 cells and the appearance of transformed colonies was scored 21 days later. The numbers shown represent average values from three experiments, the typical standard deviations (not shown) are of the order of 5%. HE, hamster embryo.

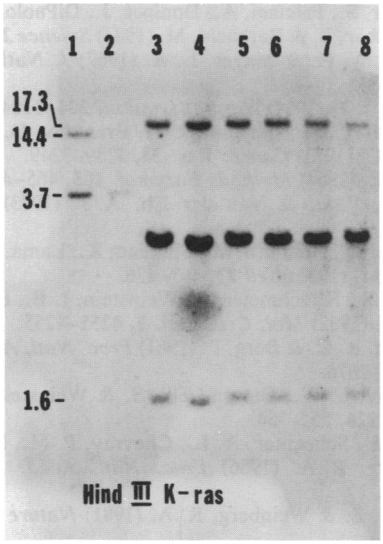

FIG. 2. Analysis of the Ki-*ras* sequences present in DNA of independent NIH 3T3 mouse transformants from *in vitro* radiation-transformed hamster embryo and C3H/10T1/2 mouse cells. Normal and transformant DNAs (20 μg of each) were digested with restriction endonuclease *Hin*dIII, fractionated by electrophoresis through a 0.8% agarose gel, and transferred to nitrocellulose paper. Filters were incubated with 10 cpm of ^{32}P-labeled probe (specific activity, 10^8 cpm/μg). The probe used was an *Sst* II/*Xba* I fragment from the HiHi-3 plasmid containing the cloned Ki-*ras* virus cDNA sequences (18). Lanes: 1, DNA of normal hamster embryo cells; 2, DNA of x-ray-transformed hamster embryo cells; 3, 3T3 transformant obtained with DNA of x-ray-transformed hamster embryo DNA; 4, DNA from 3T3 cells; 5 and 6, 3T3 transformants obtained with DNA from x-ray-transformed C3H/10T1/2 lines 1 and 2, respectively; 7, DNA from x-ray-transformed C3H/10T1/2; 8, DNA from normal C3H/10T1/2 cells.

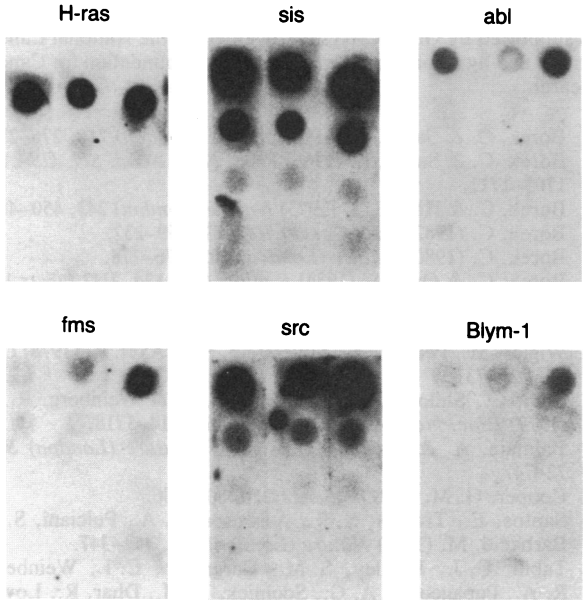

FIG. 3. Oncogene expression in C3H/10T1/2 and NIH 3T3 transfected by DNA from radiation-transformed C3H/10T1/2 cells. Cells were solubilized in NaI, the mRNA was immobilized onto nitrocellulose by standard quick-blot technology, and the blots were hybridized to oncogene probes as described (34). mRNAs from 1 × 10^6 cells and 1:4 serial dilutions (top to bottom) were bound to the filters. For each probe, C3H/10T1/2 mRNA is in the left lane, NIH 3T3 mRNA is in the middle lane, and mRNA of NIH 3T3 transformed by DNA from C3H/10T1/2 radiation-transformed cells is in the right lane.

containing DNA from hamster or C3H/10T1/2 cells (7). Oncogene expression was analyzed using the quick blot method (38) by molecular hybridization of DNA probes to mRNA immobilized on nitrocellulose filters directly from NaI-solubilized cells. Increased mRNA transcripts served as a parameter of altered gene expression. Viral probes of v-*src*, v-*myc*, v-*mos*, v-*myb*, v-*fos*, v-Ha-*ras*, v-Ki-*ras*, N-*ras*, v-*fes*, v-*abl*, and v-*sis* were used (38). Elevated transcripts (5-fold increases) of c-*fms* and c-*abl* genes were found in 3T3 transformants containing DNA from three independently x-ray-transformed C3H/10T1/2 lines (Fig. 3). In view of these results, we attempted to determine whether the transforming genes present in the x-ray-transformed 10T1/2 line were c-*fms* or c-*abl*. Unfortunately, none of several independent 3T3 transformants contains any additional or amplified c-*fms* or c-*abl* sequences as determined by Southern blot analysis, indicating that neither of these two loci was involved in the transformation of these cells (data not shown). No consistent altered expression was observed in 3T3 cells containing x-ray-transformed hamster DNA.

Our present findings show that the *in vitro* malignant transformation of mammalian cells by a single direct exposure to x-irradiation, at a dose relevant to human exposures, results in the activation of oncogenic sequences with detectable transforming activity in three recipient cell lines, the mouse NIH 3T3, C3H/10T1/2 cells, and the Rat-2 cells. The frequency of transfectants in the recipient cells is correlated with the uptake of DNA from the donor x-ray transformants as ensured by cotransfection with the pSV2gpt genetic marker (29).

The transforming sequences activated in three radiation-transformed hamster lines appear to correspond to a single oncogene. A specific oncogene also appears to be present in the three radiation-transformed mouse C3H/10T1/2 lines. Whether these two oncogenes of mouse and hamster cells are identical, related, or completely different must await their molecular characterizations.

The oncogenic sequences activated in the *in vitro* x-ray-transformed lines of the hamster and the mouse C3H/10T1/2 cells are unique. They do not represent activated forms of the *ras* gene family, which have been implicated in neoplastic states including radiation-induced thymomas in mice (18, 39) and chemically *in vitro*-transformed C3H/10T1/2 (9, 19) or guinea pig cells (21). We found no activated *ras* genes even after growing the x-ray-transformed cells into tumors in nude mice. The oncogenic sequences do not represent activated forms of the *neu* (31), *trk* (34), or the *raf* (35) oncogenes, which have been shown to be active in the transfection assay.

The work presented here provides evidence that the *in vitro* radiation-induced phenotype is encoded in the DNA of the radiation-transformed cells. Documentation at the RNA level indicates an enhanced expression of c-*abl* and c-*fms* genes in the transformed mouse cells but not in the hamster. However, at the DNA level no additional or amplified sequences of *abl* (36) or *fms* (37) were observed.

Molecular cloning of the radiation-transforming genes in the cells of both species, defining the sequence of appropriate regions, and establishing where the transforming activity resides will add insight to molecular mechanisms in radiation carcinogenesis and will serve as a powerful tool to dissect steps in the process of radiation-induced malignant transformation.

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