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Supplementary Information for

Formation of clustered DNA damage *in vivo* upon irradiation with ionizing radiation: visualization and analysis with atomic force microscopy

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Supplementary Information Text

Supplementary text I. Preliminary study on the concentration of biotin-labeled DNA

The conditions to concentrate biotin-labeled DNA with streptavidin magnetic beads were investigated using a model DNA, as outlined in SI Appendix, Fig. S1 A.

Preparation of the model DNA: biotin-labeled DNA fragments (117, 527, and 1045 bp) were synthesized by PCR using a pGL4.50 plasmid (Promega) as a template, forward primers (Fw117, Fw257, and Fw1045), and reverse primers (Rv and RvBio) (see below). The reverse primer RvBio contained a thymidine in which a biotin residue was attached to the C5 position of the pyrimidine ring via a spacer (Biotin-dT, Glen Research; SI Appendix, Fig. S1 B). DNA fragments (117, 527, and 1045 bp) without a biotin label were also synthesized using the reverse primer Rv.

PCR primers used to prepare the model DNA:

FW117 5′-GGCACCAAAATCAACGGGAC–3′

FW527 5′-GTTCCGCGTTACATAACTTACGGTAAATG–3′

FW1045 5′-AGAAGGGAATGAGTGCGACACG–3′

Rv 5′-GGTTCACTAAACGAGCTCTGC–3′

RvBio 5′-GGTTCACXAAACGAGCTCTGC–3′ X=Biotin-dT

The concentration of DNA with magnetic beads: The 5’ end of the DNA prepared above was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and was suspended in a phosphate-buffered saline (PBS). The conditions to concentrate the biotin-labeled DNA by pulldown with streptavidin magnetic beads were investigated using a Dynabeads kilobaseBINDER kit (Invitrogen), following the manufacturer’s instructions. Briefly, Dynabeads M-280 streptavidin (5 µL from the vial) was prewashed and resuspended in 20 µL of binding solution (the composition is proprietary). The DNA (0.5 pmol) in 20 µL of PBS was added to resuspended beads, and the sample was incubated at 25 °C for 3 h on a roller to keep the beads in the suspension. The supernatant was removed, and the beads were washed with 20 µL of washing solution (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 2.0 M NaCl) and 20 µL of MilliQ water. The beads were resuspended in 20 µL of 10 mM EDTA (pH 8.2). The 95% formamide suggested in the protocol of the kit was omitted in the 10 mM EDTA solution to avoid the denaturation of duplex DNA. The sample was briefly heated at 90 °C for 2 min, and the supernatant was recovered. A part of the supernatant was measured for radioactivity to evaluate the recovery of DNA. The recovered biotin-tagged DNA fragments (117, 527, and 1045 bp) were 26–41% (average, 33%) of the input DNA, while the recovered control DNA fragments (without biotin tag) were 0.8–3.3% (average, 2.0%) of the input DNA (SI Appendix, Fig. S1 C). Thus, biotin-tagged DNA was recovered about 16-fold more efficiently than that those without the tag.

Analysis of DNA released in the supernatant: A part of the supernatant was also analyzed by native polyacrylamide gel electrophoresis (PAGE). PAGE analysis revealed that biotin-labeled DNA was released from the magnetic beads as a complex with streptavidin after a brief heating at 90 °C for 2 min. Thus, the link between the immobilized streptavidin and magnetic beads was cleaved, and the DNA/streptavidin complexes were maintained in the heat treatment. The typical PAGE data for the 527-bp DNA/streptavidin complexes released in the supernatant are shown in SI Appendix, Fig. S1 D (lane 3), together with a separately prepared marker (lane 5).

Proteinase K treatment of the DNA/streptavidin complexes: To remove streptavidin from the DNA/streptavidin complexes released from the magnetic beads, the supernatant was treated with 500 ng/μL proteinase K after heating at 90°C for 2 min. This converted the complexes to free DNA. The typical PAGE data for the free 527-bp DNA produced by the proteinase K treatment are shown in SI Appendix, Fig. S1 D (lane 4), together with a 527-bp DNA marker (lane 1). A comparison of the gel mobilities of native and denatured DNA samples (lanes 1 and 2) with that of the recovered DNA showed that the 527-bp DNA obtained from the proteinase K treatment maintained a duplex structure (lane 4).

Supplementary text II. Recovery of damage-containing genomic DNA

TK6 cells were treated with ionizing radiation or Fenton's reagents, and genomic DNA was isolated from the cells. DNA base lesions were labeled with ARP, and DNA was digested with RsaI. DNA fragments with ARP labels were recovered using streptavidin magnetic beads, as described in SI Appendix, Fig. S1. DNA was released from the beads (released as a streptavidin/DNA complex) by a brief heating at 90 °C for 2 min; then, the DNA concentration in the supernatant was measured by fluorometric analysis using a Qubit fluorometer (Thermo Fisher Scientific). The DNA recovery percentage was calculated as the ratio of the amount of DNA recovered by the magnetic beads relative to that of input DNA.

The percentage of recovered DNA from cells treated with X-rays, Fe-ion beams, and Fenton's reagents ranged from 2.67 ± 0.68% to 4.37 ± 1.12% (approximately 3–4%), which was greater than that from untreated control cells (2.37 ± 0.67%; approximately 2%). However, no apparent systematic dose-dependent changes in the percentage of recovered DNA were observed in cells treated with X-rays, Fe-ion beams, and Fenton's reagents.

It was noted that DNA (ca. 2% of input DNA) was recovered from untreated control cells (0 Gy and 0 mM). This can be attributed to (i) endogenous base lesions in the genomic DNA. These lesions were produced by the depurination and reactions of DNA bases with damaging agents, such as reactive oxygen species and methylating agents. This can also be attributed to a (ii) non-specific capture of DNA fragments with no lesions by streptavidin magnetic beads. Although the efficiencies were low, the non-specific capture of intact DNA by streptavidin magnetic beads was observed in experiments with a model DNA (SI Appendix, Fig. S1 C), where 0.8–3.3% of intact DNA (without biotin labels) was recovered.

Supplementary text III. Estimation of the yields of DNA damage

Estimation of DNA length: In the AFM analysis of genomic DNA damage, the fields of AFM observation were randomly selected, and all DNA fragments with and without damage in the field were observed for individual data points. The total number of observed DNA fragments was N for individual data points. According to the agarose gel electrophoretic analysis (SI Appendix, Fig. S2), the average size of DNA fragments used for AFM analysis was 1100 bp. The percentage (p) of genomic DNA fragments recovered by pulldown with streptavidin magnetic beads was determined for each sample (Supplementary text II). By considering the total number of observed DNA fragments (N), the percentage of recovered genomic DNA fragments (p), and the average size of DNA fragments (1100 bp), the total length (L in bp) of DNA before pulldown was estimated using Equation (1).

$$L = N \times (100/p) \times 1100 \quad (1)$$

Estimation of the number of DNA damage: The number of observed DNA fragments containing a given type of DNA damage was n_1 (isolated base damage), n_2 (simple BDCs), n_3 (complex BDCs), and n_4 (complex DSBs). In total, 300 damage-containing fragments were analyzed to determine

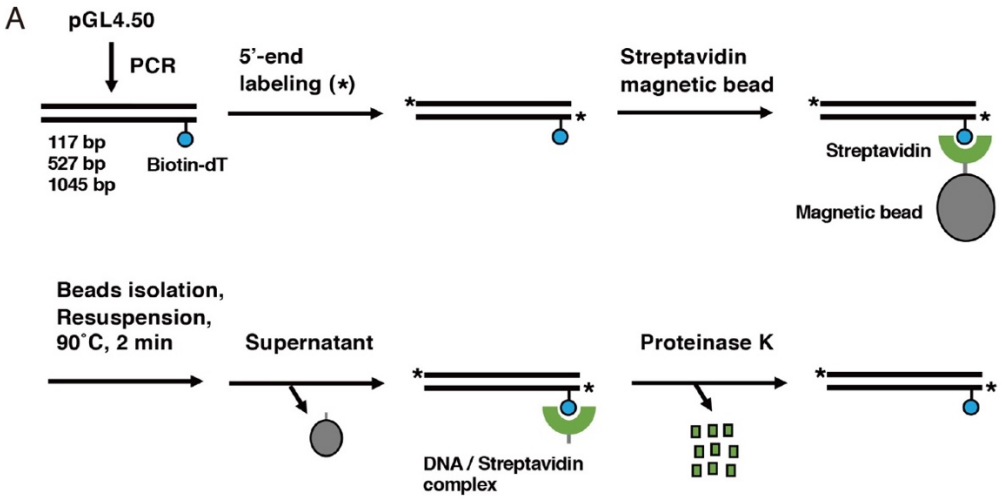
the damage spectra for individual data points. According to the experiment using the model DNA (SI Appendix, Fig. 1C), the average percentage of recovered biotin-labeled DNA was 26.5% for 1045-bp DNA, whose size approximately corresponded to that (1100 bp) of DNA fragments used for the AFM analysis. Thus, the actual number (a_i) of DNA fragments containing a given type of DNA damage before pulldown was estimated using Equation (2).

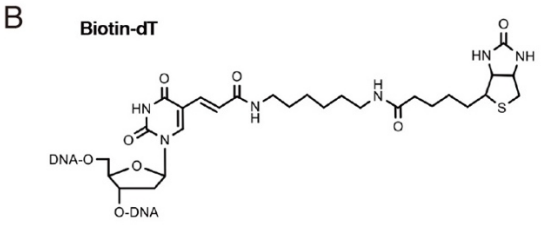
$$a_i = n_i \times (100/26.5) \quad i = 1,2,3,4 \quad (2)$$

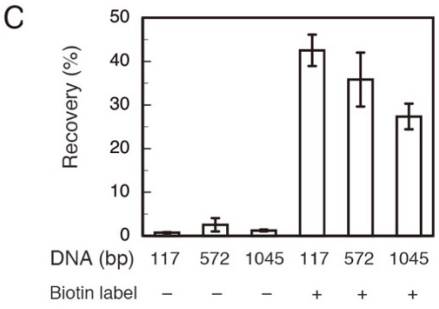
Calculation of the yield of DNA damage: L represents the estimated total length of DNA [Equation (1)] before pulldown, while a_i represents the estimated total number of a given type of DNA damage [Equation (2)] before pulldown. From a_i and L, the yield of a given type of DNA damage (y_i, sites/10^6 bp) can be calculated using Equation (3).

$$y_i = a_i \times 10^6/L = n_i \times (100/26.5) \times 10^6/[N \times (100/p) \times 1100] \quad i = 1,2,3,4 \quad (3)$$

Figs. 4B, 5B, 5E, 6B, and 6C show the calculated values of y_i.


A
pGL4.50
PCR
117 bp
527 bp
1045 bp
Biotin-dT
5'-end
labeling (*)
Streptavidin
magnetic bead
Streptavidin
Magnetic bead
Beads isolation,
Resuspension,
90˚C, 2 min
Supernatant
DNA / Streptavidin
complex
Proteinase K


B
Biotin-dT
DNA-O
O-DNA


C
Recovery (%)
50
40
30
20
10
0
DNA (bp) 117 572 1045 117 572 1045
Biotin label – – – + + +

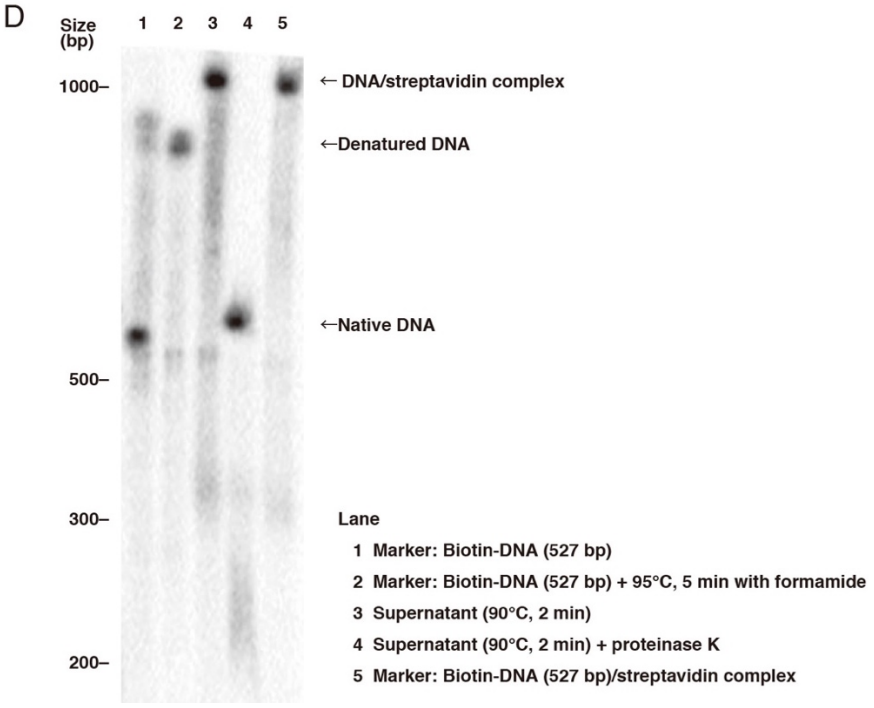

D
Size
(bp)
1 2 3 4 5
1000–
500–
300–
200–
← DNA/streptavidin complex
←Denatured DNA
←Native DNA
Lane
1 Marker: Biotin-DNA (527 bp)
2 Marker: Biotin-DNA (527 bp) + 95°C, 5 min with formamide
3 Supernatant (90°C, 2 min)
4 Supernatant (90°C, 2 min) + proteinase K
5 Marker: Biotin-DNA (527 bp)/streptavidin complex

Fig. S1. Concentration of biotin-labeled model DNA with streptavidin magnetic beads.

(A) Scheme for the concentration of biotin-labeled DNA fragments (117, 527, and 1045 bp) with streptavidin magnetic beads. DNA fragments containing biotin-dT in place of ARP were used to investigate the conditions to concentrate DNA.
(B) Structure of biotin-dT.
(C) Recovery of control (no biotin label) and biotin-labeled DNA fragments (117, 527, and 1045 bp) with streptavidin magnetic beads. The radioactivity of the supernatant after heating at 90 °C for 2 min (see panel A) was measured to quantify the DNA recovered by streptavidin magnetic beads. The percentage of recovered DNA relative to the input DNA was calculated based on the radioactivity, and it was plotted against the sample types.
(D) PAGE analysis of 527-bp DNA samples recovered by streptavidin magnetic beads. Samples with ^{32}P-end labels were separated by 10% native PAGE. Lane 1: marker, biotin-labeled DNA (527 bp); lane 2: marker, biotin-labeled DNA (527 bp) was heat-denatured in formamide at 95 °C for 5 min; lane 3: biotin-labeled DNA (527 bp) was concentrated with magnetic beads. The suspension of the beads was heated at 90°C for 2 min, and the supernatant of the sample was analyzed; lane 4: the supernatant obtained after the heat treatment (lane 3) was treated with proteinase K and was analyzed; lane 5: marker: biotin-labeled DNA (527 bp)/streptavidin complexes were separately prepared by mixing biotin-labeled DNA (527 bp) and free streptavidin.

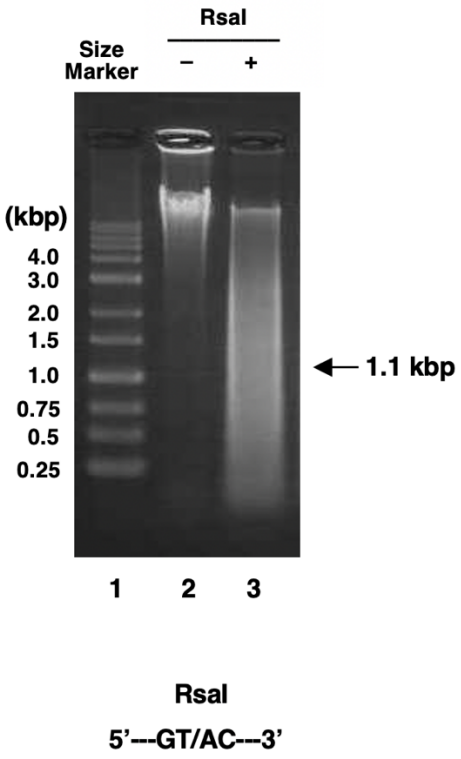


Fig. S2. RsaI digestion of ARP-labeled genomic DNA.

Analysis of ARP-labeled genomic DNA with agarose gel electrophoresis. Genomic DNA was isolated from TK6 cells. DNA damage sites were labeled with ARP, and the resulting DNA was digested with RsaI (Fig. 1). Lane 1, DNA size markers; lane 2, genomic DNA before RsaI digestion; lane 3, genomic DNA after RsaI digestion. DNA was separated with 1% agarose gel electrophoresis.

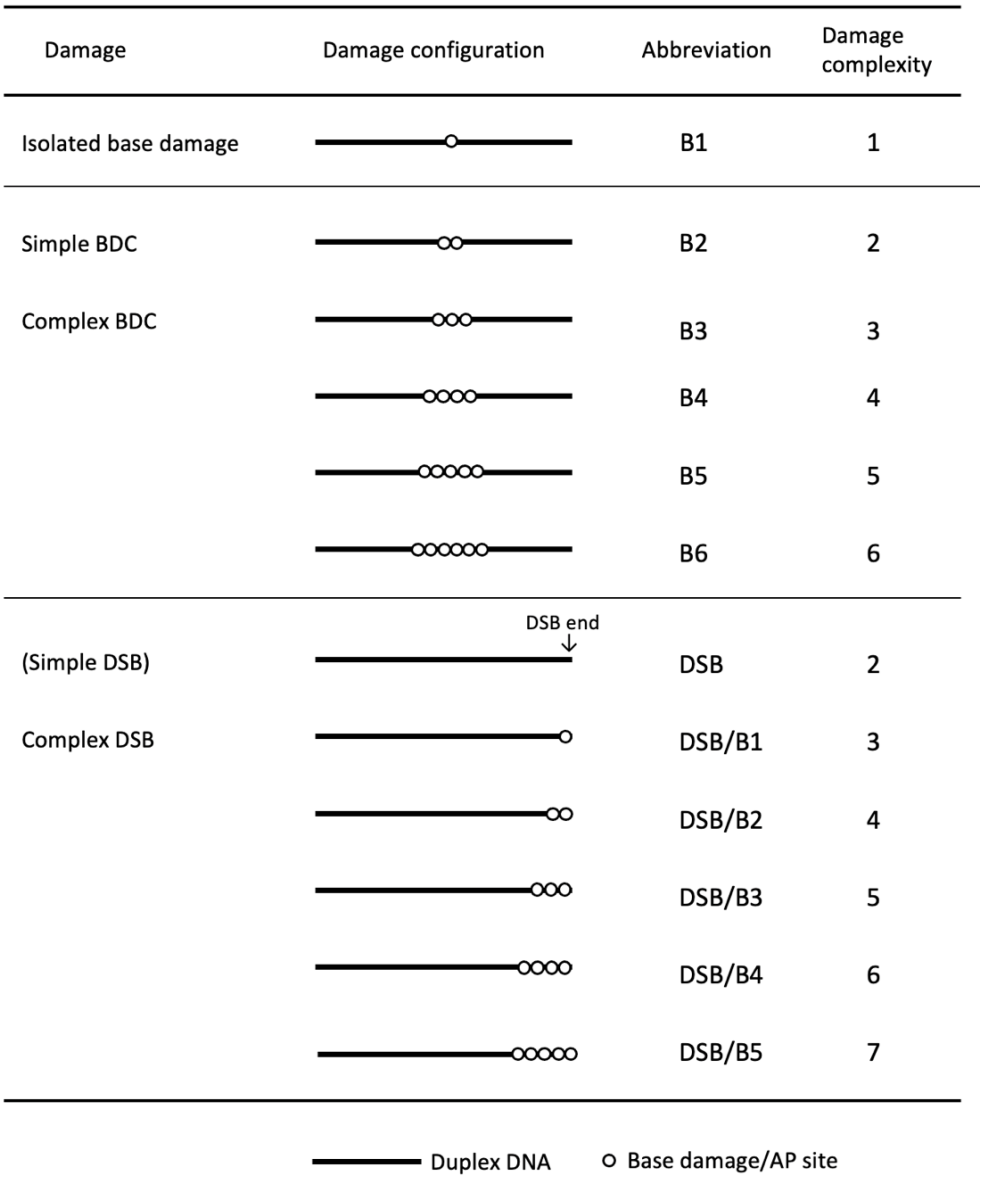


Fig. S3. Schematic presentation of isolated and clustered DNA damages observed in this study.

Damage, damage configuration, abbreviation, and damage complexity are shown. The solid line and circle denote duplex DNA and base damage/AP sites, respectively. Simple DSBs were not observed with AFM, since they were exclusively produced by the RsaI digestion of the genomic DNA. The damage complexity indicates the number of lesions per DNA damaged site

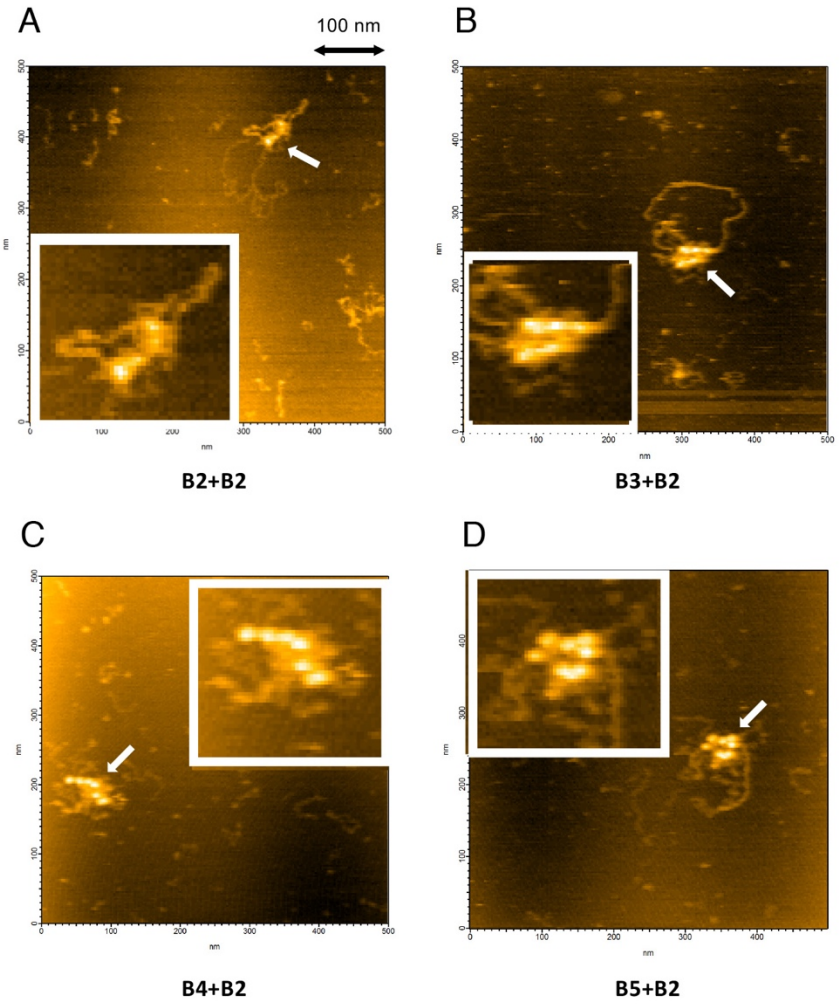


Fig. S4. AFM images of two adjacent BDCs caused by Fe-ion beams.

TK6 cells were irradiated with Fe-ion beams (40 Gy), and genomic DNA damage was analyzed with AFM. Representative images of two adjacent BDCs are shown. The types of two adjacent BDCs are indicated under the AFM image. See SI Appendix, Fig. S3 for the abbreviation of BDC types.

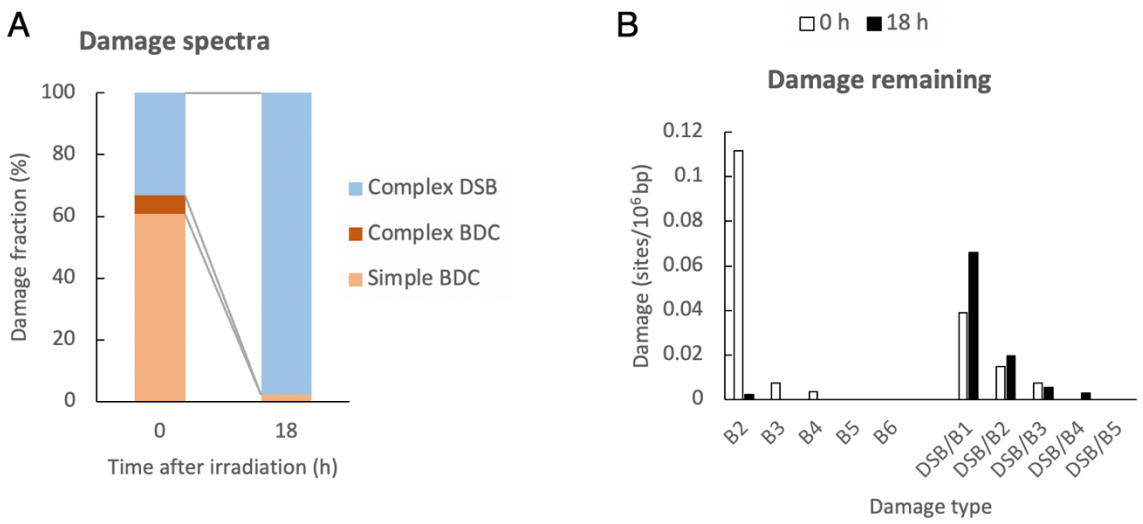


Fig. S5. Repairability of clustered DNA damage induced by Fe-ion beams (5 Gy).

TK6 cells were irradiated with Fe-ion beams (5 Gy) and incubated for 18 h. Clustered DNA damage after 0 and 18 h of irradiation was analyzed by AFM, as shown in Fig. 6. Based on the AFM analysis, 1000 damage-containing DNA fragments were observed, and the clustered DNA damage was analyzed (90 for 0 h and 87 for 18 h). (A) Changes in the spectra of clustered DNA damage after a repair incubation. The percentages of BDCs (simple/complex) and complex DSBs relative to the total clustered damage are shown for 0 and 18 h. (B) Changes in the amount (sites/10^6 bp) of each type of clustered DNA damage after a repair incubation for 18 h. The amount of each type of clustered DNA damage was calculated, as described in Fig. 4B, and is plotted against the type of clustered damage for 0 and 18 h. The values of endogenous damage for untreated cells were subtracted in the calculation of data in panels A and B. In panel B, the differences in the amount of each type of complex DSB for 0 h and 18 h may not be statistically significant due to the limited number of clustered DNA damage sites in the analysis.

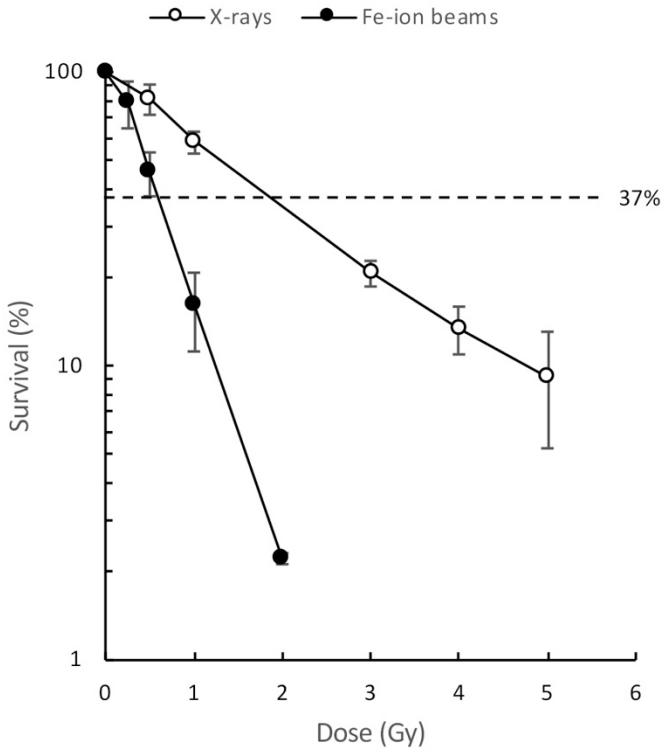


Fig. S6. Survival curves of TK6 cells irradiated with X-rays and Fe-ion beams.

TK6 cells were irradiated with indicated doses of X-rays or Fe-ion beams. The cells were serially diluted and plated in triplicate onto six-well plates containing 5 mL/well of 1.5% (w/v) methylcellulose, Dulbecco's modified Eagle's medium/F-12, and 10% horse serum. The number of colonies was counted at days 10–14. The data shown are from three independent experiments. The error bars represent the standard deviations.

Table S1. Comparison of the yields of DSBs

Radiation	Damage	Yield (DSB/10^6 bp/Gy)	Cells	Method[#]	Refs.
X- or γ -rays	Complex DSB	0.033	TK6	AFM	This study
	Total DSB	0.0119	Human monocytes	PFGE	1
	Total DSB	0.005	Human fibroblasts	PFGE	2
	Total DSB	0.004	V79	PFGE	3
Fe-ion beams	Complex DSB	0.021	TK6	AFM	This study[$]
	Total DSB	0.0109	Human monocytes	PFGE	1[$]
	Total DSB	0.006-0.009	Human fibroblasts	PFGE	2[$]

[#] Method of analysis; AFM, atomic force microscopy; PFGE, pulse-field gel electrophoresis
[$] LET values of Fe-ion beams were 200 keV/μm (This study), 148 keV/μm (1), and 92–440 keV/μm (2).

References

1. Tsao, D., et al. Induction and processing of oxidative clustered DNA lesions in ^{56}Fe-ion-irradiated human monocytes. Radiat Res, 168, 87-97. (2007)
2. Prise, K.M., et al. A review of dsb induction data for varying quality radiations. Int J Radiat Biol, 74, 173-184. (1998)
3. Belli, M., et al. DNA fragmentation induced in human fibroblasts by accelerated ^{56}Fe ions of differing energies. Radiat Res, 165, 713-720. (2006)