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Abbreviations: CHO, Chinese hamster ovary; DSB, double-strand break; GJIC, gap-junction mediated intercellular communication; HPRT, hypoxanthine-guanine phosphoribosyltransferase; HRR, homologous recombinational repair; IR, ionizing radiation; NHEJ, non-homologous end joining; MMC, mitomycin C; SCE, sister chromatid exchange

ABSTRACT

We reported previously that the homologous recombinational repair (HRR)-deficient Chinese hamster mutant cell line *irs3* (deficient in the Rad51 paralog Rad51C) showed only a 50% spontaneous frequency of sister chromatid exchange (SCE) as compared to parental wild-type V79 cells. Furthermore, when irradiated with very low doses of alpha particles, SCEs were not induced in *irs3* cells, as compared to a prominent bystander effect observed in V79 cells (Nagasawa *et al*., *Radiat. Res*. **164**, 141-147, 2005). In the present study, we examined additional Chinese hamster cell lines deficient in the Rad51 paralogs Rad51C, Rad51D, Xrcc2, and Xrcc3 as well as another essential HRR protein, Brca2. Spontaneous SCE frequencies in non-irradiated wild-type cell lines CHO, AA8 and V79 were 0.33 SCE/chromosome, whereas two Rad51C-deficient cell lines showed only 0.16 SCE/chromosome. Spontaneous SCE frequencies in cell lines defective in Rad51D, Xrcc2, Xrcc3, and Brca2 ranged from 0.23–0.33 SCE/chromosome, 0–30% lower than wild-type cells. SCEs were induced significantly 20–50% above spontaneous levels in wild-type cells exposed to a mean dose of 1.3 mGy of alpha particles (<1% of nuclei traversed by an alpha particle). However, induction of SCEs above spontaneous levels was minimal or absent after α-particle irradiation in all of the HRR-deficient cell lines. These data suggest that Brca2 and the Rad51 paralogs contribute to DNA damage repair processes induced in bystander cells (presumably oxidative damage repair in S-phase cells) following irradiation with very low doses of alpha particles.

1. Introduction

Homologous recombinational repair (HRR) has been shown to play an important role in the repair of DNA double-strand breaks (DSBs) generated by ionizing radiation (IR) exposure in the S and G2-phases of the mammalian cell cycle, as well as collapsed and broken replication forks during S-phase [1-3]. An increasing number of proteins are being identified as mediators of HRR including the Rad51 recombinase, BRCA1 and BRCA2/FANCD1-PALB2/FANCN, and the Rad51 paralogs Rad51B, Rad51C, Rad51D, Xrcc2, and Xrcc3, all of which have crucial non-redundant roles in this pathway [1,4,5]. The Rad51 protein family likely plays an important role in preventing carcinogenesis, especially in view of the links between Rad51 and the BRCA1/2 proteins (mutations in which predispose to breast, ovarian, and other hereditary cancers) [6-8]. A number of Rad51 paralog and Brca2 mutants and complemented derivatives have been generated in Chinese hamster cells primarily by random chemical mutagenesis [9-13] although an isogenic pair of Rad51D-deficient (*rad51D*) and gene-complemented cell lines was recently generated by gene-targeting in CHO AA8 cells [14].

The formation of sister chromatid exchanges (SCEs) is presumed to result from homologous recombinational events occurring at sites of stalled or broken replication forks during S-phase in order to re-establish an intact replication fork and resume DNA replication [15-17]. Curiously, unlike the other Rad51 paralog mutants that have been characterized, spontaneous SCE frequencies in both *rad51d* CHO cells and mouse primary embryonic fibroblasts (MEFs) were the same as wild-type controls [14,18], suggesting that Rad51D may not directly participate in recombination events in S-phase when SCEs are presumably generated. Alternatively, since SCEs arise only when Holliday junction intermediates are resolved in one of two possible orientations, a compensatory shift in the Holliday junction resolution bias may

account for the observed spontaneous SCE phenotype in *rad51d* cells [14,17]. Mutant cell lines deficient in the Rad51 paralogs and Brca2 have also been generated in chicken B-lymphocyte DT40 cells, all of which exhibited reduced levels of spontaneous and mitomycin C (MMC)-induced SCEs [19-22]. In all the hamster and chicken Rad51 paralog mutants, the formation of Rad51 nuclear foci is severely impaired following treatment with DNA damaging agents (typically IR and MMC). However, it is noteworthy that many of these studies employed lethal doses of genotoxic agents to induce DNA DSBs and interstrand crosslinks, thereby possibly preventing the majority of cells from reaching mitosis for subsequent collection and cytogenetic analysis.

Previously we reported that spontaneous SCE frequencies for the V79-derived *rad51c* cell line *irs3* were 0.16 SCE per chromosome, approximately 50% lower than wild-type V79 cells [23]. Following irradiation with very low doses of α-particles (<2.6 mGy, <2% of cells directly hit), SCEs were not significantly increased above background levels in *irs3* cells whereas SCE frequencies increased significantly 30–40% above background in both wild-type V79 and non-homologous end joining (NHEJ)-deficient V3 (deficient in DNA-PKcs) CHO cells. In addition, mainly chromatid-type aberrations were induced in the bystander cells (*i.e.*, cells neighboring directly-irradiated cells) when cultures of V79, V-3, and *xrs*5 (deficient in Ku70/80) cells were irradiated in G0/G1-phase, which suggested increased levels of single-stranded DNA damage in the bystander cells [23-25]. Similarly, the spectrum of *hprt* mutations induced in bystander cells following low dose α-particle irradiation were nearly all point mutations, in contrast to directly irradiated cells for which total and partial gene deletions predominated [26,27].

Assuming that SCEs generated in bystander cells likewise result from HRR events, it seems unlikely this process involves directly induced ("frank") DNA DSBs as previously suggested [28,29]. The chromosome aberration and mutation spectra observed in bystander cells support our hypothesis that single-stranded oxidative lesions are the source of HRR-derived SCEs in S-phase cells. Further support for this hypothesis comes from a recent report that demonstrated γ-H2AX foci, a marker for DNA DSBs, were generated only in S-phase bystander cells (presumably at collapsed replication forks) in an ATR-dependent manner [30]. In an attempt to better delineate the nature of DNA damage generated in bystander cells following low dose α-particle irradiation, we compare SCE induction among two wild-type Chinese hamster cell lines, mutant cell lines deficient in core members of the HRR pathway including Brca2 and the Rad51 paralogs Xrcc2, Xrcc3, Rad51C, and Rad51D, and their complemented derivatives.

2. Materials and Methods

Cell lines and culture conditions

The wild-type, HRR-deficient mutant and complemented derivative Chinese hamster cell lines used in this study are listed in Table 1. Cell lines were cultured at 37°C in a humidified 5% CO_2 atmosphere with Eagle's minimal essential medium (MEM, GIBCO/Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma, St. Louis, MO). To achieve cell synchrony, cells were seeded in growth medium at a density of 10^5 cells on specially designed stainless steel dishes with a 1.5-μm-thick Mylar base (3.8 cm in diameter, 11.3 cm^2 surface area) coated with fibronectin to facilitate cell attachment. After incubation, the culture medium was replaced with isoleucine-deficient MEM containing 5% 3X dialyzed FBS and antibiotics to synchronize cells

in G0/G1 phase. Isoleucine-deficient medium was changed twice at 24-hour intervals. Irradiation experiments were initiated 30 hours after the second medium change when the cultures reached ~30–50% confluence.

Alpha-particle irradiation

For α-particle irradiations, the Mylar-based culture dishes were placed in the exposure well of a custom-built bench-top α-particle irradiation system described previously [36]. This system consists of approximately 265 MBq of $^{238}PuO_2$ electroplated on a 10-cm-diameter stainless steel disk enclosed in a Plexiglas box filled with helium at ambient air pressure. The cells were irradiated from below by α-particles traversing a rotating collimator and the sealed Mylar window of the exposure well. The doses delivered to the cells were controlled by a timer and a high-speed photographic shutter system, allowing precise irradiation times to achieve doses as low as 0.1 mGy with high accuracy. Revised dosimetric measurements of this system yield a calculated dose rate of 83 mGy/min and a fluence of 0.47 α-particle tracks/nucleus•min^{-1} (average LET of 113 keV/μm; [23]) for CHO and V79 Chinese hamster cells (~80–100 μm^2 nuclear surface area; [37]). Alpha-particle spectroscopy and CR-39 track-etch dosimetry measurements provide a value of 3.86 MeV for the mean α-particle energy at the cell surface with a fluence of 0.0045 α-particles/μm^2•min^{-1} [23]. The dose rate and LET were obtained by Monte Carlo transport code and energy transport (SRIM-2003.26) calculations.

Sister chromatid exchange analysis

To measure SCEs following α-particle irradiation, the culture medium was replaced with MEM/10% FBS medium containing 10 μM 5-bromo-2′-deoxyuridine (BrdU, Sigma). The

Mylar-based culture dishes were then immediately returned to the incubator to allow the synchronized cells to progress into the second post-irradiation mitosis (approximately 20–24 hours) at which time 0.2-μg/ml colcemid (GIBCO/Invitrogen) was added to each dish for three successive 6-hour mitotic collection intervals. Metaphase chromosome spreads were collected and prepared by the air-dry method. Differential staining of the chromosomes was obtained by the fluorescence-photolysis-Giemsa (FPG) technique [38], and SCEs in the second post-irradiation mitosis were scored as described previously [23,39]. All cell lines examined had a modal chromosome number of 21 (for CHO AA8 and derivative cell lines) or 23 (for V79 and derivative cell lines).

3. Results

Spontaneous SCE frequencies measured in isoleucine-deprived G0/G1-synchronized cultures of wild-type CHO, AA8 and V79 Chinese hamster cells were 0.33 SCE per chromosome (Table 1). Spontaneous SCE frequencies measured in the *rad51c* mutant cell lines *irs3* and CL-V4B were 0.16 SCE per chromosome, approximately 50% lower than those observed in wild-type V79 cells (Table 1). CL-V4B cells corrected with human *RAD51C* cDNA showed a 50% increase in spontaneous SCE levels compared with CL-V4B cells (Table 1) but did not fully restore spontaneous SCE frequencies to parental V79 levels, consistent with previous studies showing partial correction for other endpoints [13,40]. Spontaneous SCE frequencies in *xrcc2* *irs1*, *xrcc3* irs1SF, and *brca2* V-C8 cells ranged from 0.23–0.30 SCE per chromosome (Table 1), 10–30% below those observed in the corresponding wild-type cells and similar to values reported in the literature [11,12,41]. Complementation of the V-C8 cell line with human chromosome 13 (*BRCA2* is located on 13q13.1) failed to restore spontaneous SCE frequencies to

parental V79 levels, as has been shown for this and other endpoints [12]. As we have shown previously [14], spontaneous SCE frequencies in both the *rad51D* 51D1 and complemented 51D1.3 cells (corrected with the Chinese hamster *RAD51D* gene) were identical to those observed in parental CHO AA8 cells.

Dose response curves for SCE induction by low dose α-particle irradiation ($\leq$2.8 mGy) for the cell lines examined in this study are shown in Figure 1. SCE frequencies increased sharply in wild-type CHO, AA8 and V79 cells reaching a maximum induction at a dose of ~0.5–1.0 mGy (*i.e.*, <1% of the cells are directly irradiated; Figure 1A). Although spontaneous SCE frequencies in these cell lines were identical, the maximum induced SCE frequencies after 2.8 mGy α-particle irradiation ranged from 0.06 to 0.16 SCE/chromosome over spontaneous levels. On the other hand, the *rad51c* mutants *irs3* and CL-V4B show no (or minimal) SCE induction after low dose α-particle irradiation (Figure 1B). As observed for the spontaneous frequencies, SCEs were induced in corrected CL-V4B cells to approximately one-half the levels observed in parental V79 cells, indicating partial complementation (Figure 1B). The *xrcc2 irs1* and *xrcc3* irs1SF cell lines also showed no SCE induction following α-particle irradiation (Figure 1C). The *rad51d* 51D1 cells showed only minimal SCE induction following α-particle irradiation, whereas induction in corrected 51D1.3 cells was similar to that observed in parental AA8 cells (Figure 1D). Similar to the Rad51 paralog mutants, *brca2* V-C8 cells also showed no SCE induction following low-dose α-particle irradiation (Figure 1E). SCEs were induced in corrected V-C8 cells containing human chromosome 13, however at frequencies approximately 25% that observed in parental V79 cells (Figure 1E).

Histograms of the distributions of SCE/chromosome for both spontaneous (0 mGy; light bars) and 1.3 mGy α-particle irradiated cells (total SCE frequencies, spontaneous plus IR-

induced; dark bars) are shown in Figure 2 for the wild-type (panels A–C) and the HRR-deficient and complemented derivative (panels D–L) cell lines examined in this study. Poisson distributions of the mean SCE frequency per chromosome for each cell line (light line, spontaneous; dark line; 1.3 mGy α-particle irradiated) are shown to the same scale as insets in Figure 2. The wild-type and (partially) complemented derivative cells all show an appreciable shift in the SCE distributions following 1.3 mGy, while the HRR-deficient mutant cells show no or only a nominal increase in the distributions after irradiation.

4. Discussion

We have shown that synchronized G0/G1-phase cultures of HRR-deficient Chinese hamster cell lines have spontaneous SCE frequencies that are 0–30% lower than corresponding wild-type controls, except for the *rad51c* cell lines *irs3* and CL-V4B, which have spontaneous frequencies ~50% lower than parental V79 cells (Table 1). Rad51 paralog and BRCA2 mutants generated in the chicken DT40 cell system were reported to have lower spontaneous SCE frequencies (~30-70% reduction) compared to wild-type cells [15,19-22], depending on the particular HRR protein. Our results also show differences in spontaneous SCE frequencies among the various HRR-deficient Chinese hamster cell lines, providing further support of the notion that the Rad51 paralogs and Brca2 play distinct roles in homologous recombination. Previous biochemical work has shown that the Rad51 paralogs exist in at least two sub-complexes, Rad51B/C/D/Xrcc2 and Rad51C/Xrcc3, with multiple roles in HRR from Rad51 nucleoprotein filament formation to Holliday junction resolution [5,19,42-44]. Recent evidence suggests BRCA2 may also play multiple roles in this pathway, including its primary function of loading Rad51 onto 3′ single-stranded DNA [42,45]. Since its identification as the Fanconi

anemia D1 (FANCD1) protein [46], BRCA2 has been shown to interact directly with FANCD2 and FANCG, members of the Fanconi anemia pathway, and XRCC3 [47-50].

We have also shown that synchronized G0/G1-phase cultures of wild-type CHO, AA8 and V79 cell lines have identical spontaneous SCE frequencies (Table 1). The significant increases in SCE frequency observed in the wild-type hamster cell lines following low-dose α-particle irradiation indicate a prominent bystander response in these cultures, since less than 1% of the cells would have been directly traversed by an α-particle for a dose of 1.3 mGy. However, the dose response for SCE induction differed between these two cell lines, with the AA8 cells showing an ~20% increase, V79 cells showing an increase of ~30%, and CHO cells showing an ~50% increase. This difference is not surprising since the cell lines are distantly related. More importantly, SCE induction following irradiation with low doses of α-particles was abolished or greatly reduced in all the Rad51 paralog and Brca2-deficient mutant lines, demonstrating a key role for HRR in the repair of DNA damage generated in bystander cells (most likely at collapsed replication forks in S-phase).

It had been reported that SCE induction in hamster and chicken DT40 Rad51 paralog mutants following treatment with the interstrand cross-linking agent mitomycin C (MMC) was also reduced compared to wild-type cells, but not abolished [12,15,19,20,40,51]. MMC has been reported to induce both DNA interstrand and DNA-protein crosslinks as well as DNA base damage mediated by the production of reactive oxygen species (ROS) following bioreductive activation [20,51-53]. However, using approximately equitoxic MMC concentrations (~1% survival level), nearly 10-fold reduced SCE frequencies were observed in *irs3* cells relative to parental V79 cells [40], as compared to only a 2-fold decrease observed between these cell lines following low dose α-particle irradiation in this study. The difference in the extent of this

reduction between MMC and low dose α-particle exposures is likely related to the types and levels of DNA damage induced by these agents and specific repair systems employed to resolve this damage. It also must be noted that all cells are exposed in MMC-treated cultures whereas in the case of low dose α-particle irradiations, only a small percentage of cells are directly irradiated ($\leq$2%) and a portion of non-irradiated cells may not be exposed to bystander molecules as a result of the limited diffusion potential of these signaling factors through gap-junction mediated intercellular communication (GJIC) or the cell culture medium [25,29,54].

Results from our work and others argue that low-dose α-particle irradiation induces primarily oxidative DNA damage in bystander cells, as evidenced by the spectrum of gene mutations (primarily point mutations) and chromosomal aberrations (primarily chromatid-type) [23-27,54]. Oxidative damage is repaired primarily via the DNA base excision repair (BER) and single-strand break repair pathways (mediated by PARP, XRCC1, DNA glycosylases, DNA polymerase β, DNA ligases, and other proteins) throughout the cell cycle (reviewed in [55-58]). However, any unrepaired base damage (including single-strand nicks and gaps generated as repair intermediates) encountered by replicative DNA polymerases in S-phase may stall or collapse replication forks at the site of damage, forming a so-called one-sided DSB [5]. Recovery of the replication fork is mediated by a complex interplay of the Fanconi anemia and HRR proteins, the resolution of which may result in crossover events between parental strands, *i.e.*, SCEs [17,50,59]. In conclusion, our findings further support the hypothesis that oxidative damage constitutes the major type of DNA damage induced by the IR-induced bystander effect, and that an intact HRR pathway is required for efficient processing of this damage in replicating bystander cells.

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Table 1. Average spontaneous and 1.3 mGy α-particle-irradiated SCE frequencies measured in wild-type, HRR-deficient and complemented derivative Chinese hamster cell lines (mean SCE/chromosome ± SEM for 3–6 independent experiments).

Cell line	Defective gene	Origin	Mean SCE/chromosome ± SEM	
			Spontaneous (0 mGy)	1.3 mGy α-particles
CHO	—	Wild-type [31]	0.33 ± 0.01	0.49 ± 0.03
AA8	—	Wild-type [32]	0.33 ± 0.01	0.39 ± 0.01
V79 (V79-4, V79B)	—	Wild-type [33-35]	0.33 ± 0.01	0.44 ± 0.04
irs1	*Xrcc2*	V79-4 [10]	0.30 ± 0.01	0.32 ± 0.02
irs1SF	*Xrcc3*	AA8 [11]	0.27 ± 0.01	0.28 ± 0.01
irs3	*Rad51C*	V79-4 [10]	0.16 ± 0.01	0.17 ± 0.01
CL-V4B	*Rad51C*	V79B [9]	0.16 ± 0.01	0.16 ± 0.02
Complemented CL-V4B	—	CL-V4B + *HsRAD51C* cDNA [13]	0.25 ± 0.01	0.29 ± 0.07
51D1	*Rad51D*	AA8 [14]	0.33 ± 0.02	0.35 ± 0.01
51D1.3	—	51D1 + *CgRAD51D* gene [14]	0.34 ± 0.01	0.40 ± 0.06
V-C8	*Brca2*	V79B [9]	0.23 ± 0.02	0.22 ± 0.01
Complemented V-C8	—	V-C8 + human chromosome 13 [12]	0.23 ± 0.01	0.25 ± 0.01

FIGURE LEGENDS

Figure 1. Dose-response curves for SCE induction by low dose α-particle irradiation in synchronized G0/G1-phase cultures of wild-type, HRR-deficient and complemented derivative Chinese hamster cell lines. (**A**) CHO (▲), V79 (■), and AA8 (●). The mean number of tracks per nucleus plotted on the upper abscissa. Each data point represents the mean of 5–6 individual experiments. (**B**) *irs3* (●), CL-V4B (▲), corrected CL-V4B + *HsRAD51C* cDNA (■). (**C**) *irs1* (●) and irs1SF (▲). (**D**) 51D1 (●) and complemented 51D1.3 (+ *CgRAD51D* gene, ▲). (**E**) V-C8 (●) and corrected V-C8 + human chromosome 13 (▲). Each data point for panels B–E represents the mean of 3−5 individual experiments.

Figure 2. Histograms of spontaneous (grey bars) and 1.3 mGy α-particle-irradiated (black bars) SCE/chromosome in synchronized G0/G1-phase cultures of wild-type, HRR-deficient and complemented derivative Chinese hamster cell lines. Inset shows Poisson distributions for spontaneous (grey line) and 1.3 mGy-irradiated SCE/chromosome (black line) of mean μ to the same scale. Each panel represents pooled results of 125–250 cells per experiment from 3–6 independent experiments. (**A**) CHO, (**B**) AA8, (**C**) V79, (**D**) *irs3*, (**E**) CL-V4B, (**F**) CL-V4B + *HsRAD51C* cDNA, (**G**) *irs1*, (**H**) irs1SF, (**I**) 51D1, (**J**) 51D1.3, (**K**) V-C8, (**L**) V-C8 + human chromosome 13.

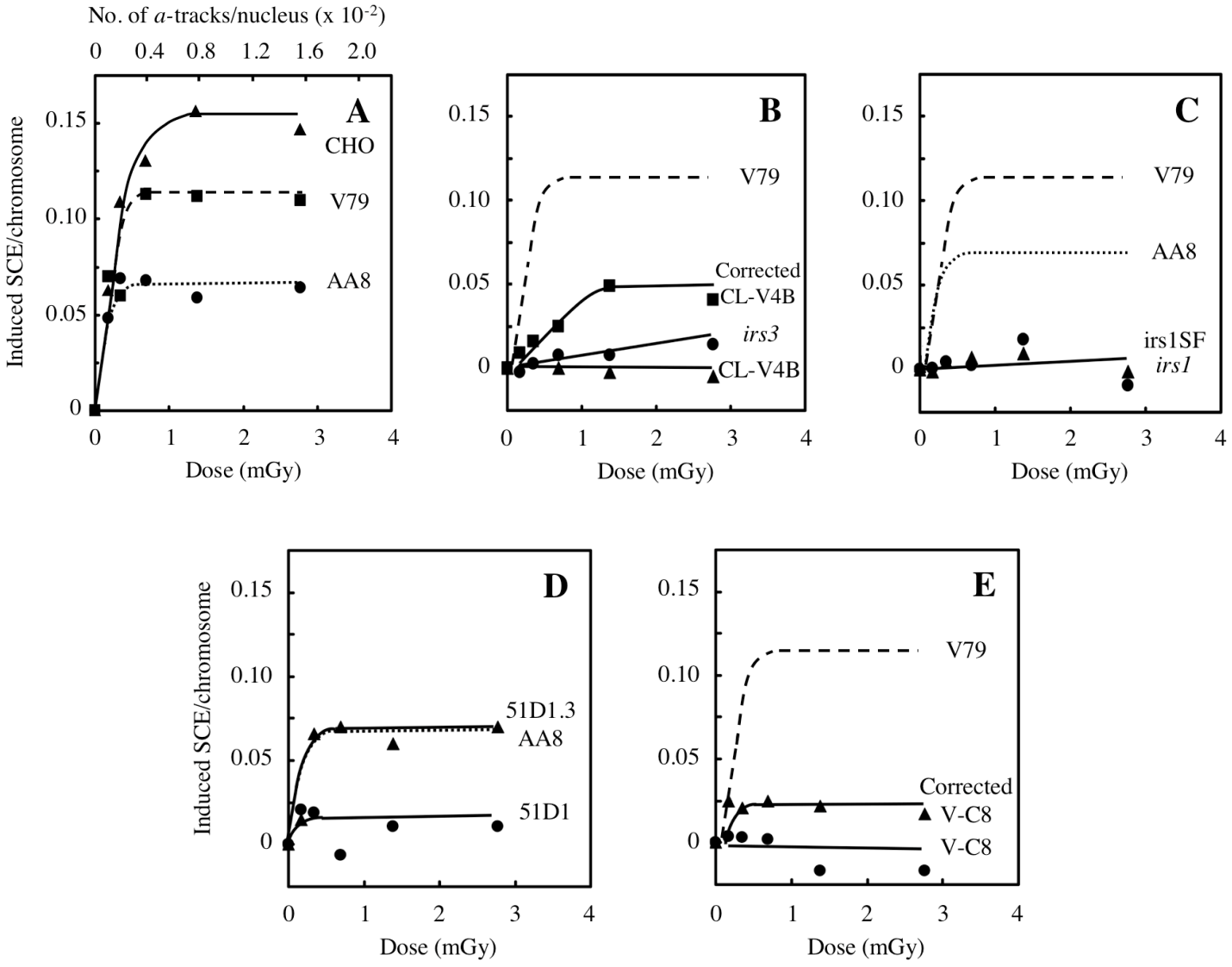
No. of a-tracks/nucleus (x 10-2)
0 0.4 0.8 1.2 1.6 2.0
Induced SCE/chromosome
A
CHO
V79
AA8
B
V79
Corrected
CL-V4B
irs3
CL-V4B
C
V79
AA8
irs1SF
irs1
D
51D1.3
AA8
51D1
E
V79
Corrected
V-C8
V-C8
Dose (mGy)

Figure 1.

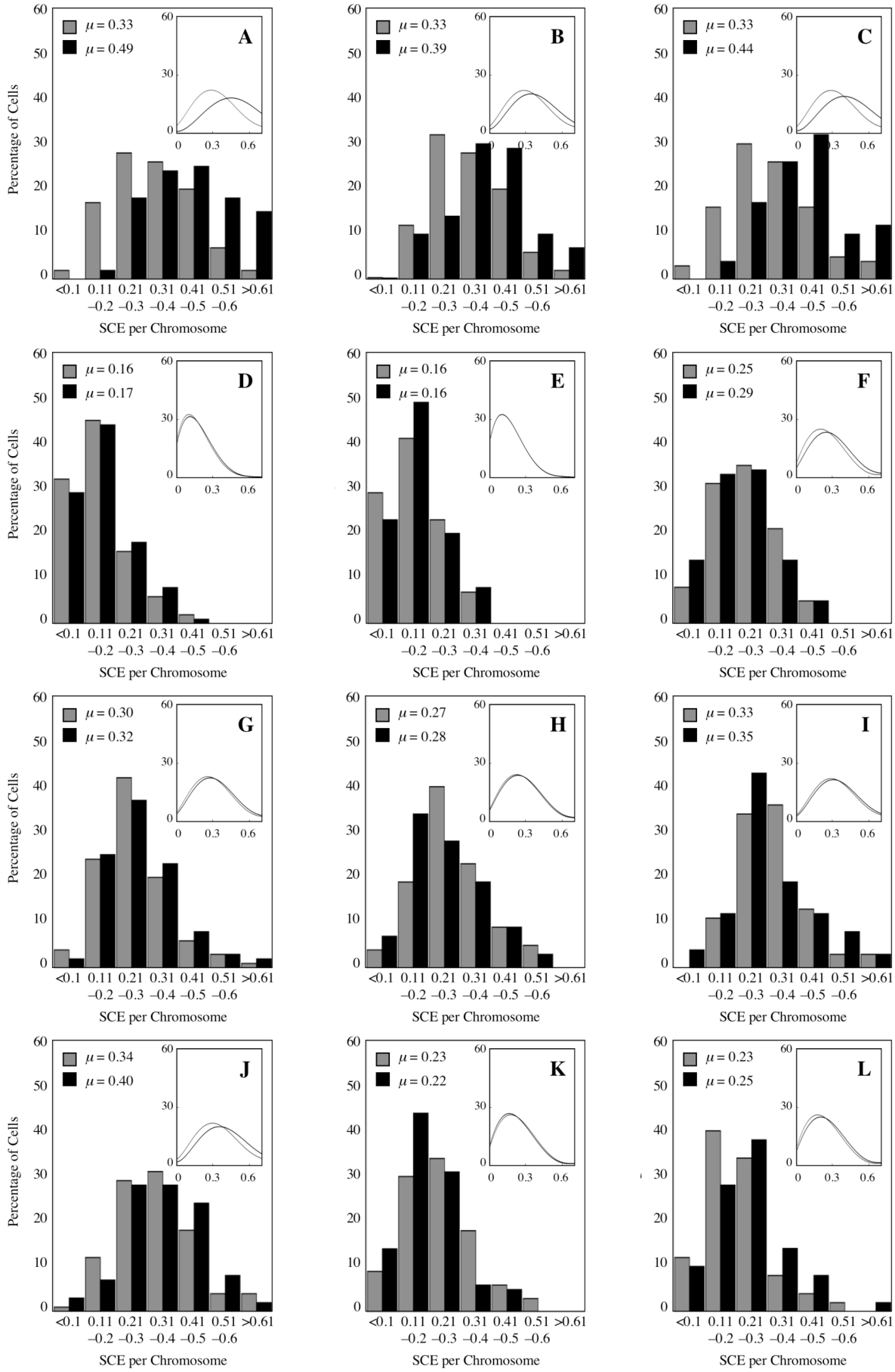
A
μ = 0.33
μ = 0.49
B
μ = 0.33
μ = 0.39
C
μ = 0.33
μ = 0.44
D
μ = 0.16
μ = 0.17
E
μ = 0.16
μ = 0.16
F
μ = 0.25
μ = 0.29
G
μ = 0.30
μ = 0.32
H
μ = 0.27
μ = 0.28
I
μ = 0.33
μ = 0.35
J
μ = 0.34
μ = 0.40
K
μ = 0.23
μ = 0.22
L
μ = 0.23
μ = 0.25
Percentage of Cells
SCE per Chromosome
<0.1 0.11 0.21 0.31 0.41 0.51 >0.61
–0.2 –0.3 –0.4 –0.5 –0.6

Figure 2.