FIGURE LEGENDS

Figure 1. Outline of the chemical exposure experiments. The scheme of culture conditions for mouse ESCs or EBs is illustrated as Exp. 1–4. ESCs were cultured under stem cell conditions (+LIF) with one of the 25 chemicals that have been detected in the serum of pregnant mothers or fetal cord blood (Table 1), at serum level or tenfold higher than serum level concentrations. After 48-hr exposure of cells to the individual chemicals, the heterochromatin status in the nuclei was analyzed to assess whether the chemicals have potential as epimutagens (Exp. 1). Next, to test whether chemical exposure causes alteration in DNA methylation at unique gene loci (Exp. 2) and whether chemical-induced heterochromatin changes are reversible or not (Exp. 3), we cultured ESCs with five chemicals that were determined as epimutagens in Exp. 1. Finally, cells were exposed to chemicals at serum level during EB formation to investigate whether the chemicals affect cellular differentiation (Exp. 4)

Figure 2. Establishment of an epimutagen screening method and identification of epimutagen candidates by heterochromatin staining in mouse ESCs.

(*A*) Visualization of heterochromatin structure in nuclei by DAPI staining, immunofluorescence, and DNA-FISH. Signal intensities of DAPI, HP1a, or major satellite on the dotted lines (a–b or c–d) were measured and plotted. The strong DAPI signals (filled triangles) were confirmed to merge with the two heterochromatin markers, HP1a, and major satellite signals.

(*B*) Confirmation of altered heterochromatin by DAPI staining of mouse ESCs with a known epimutagen, TSA. After 48-hr exposure of ESCs to TSA (0, 0.2, or 1.0 nM), the cells were stained with DAPI, and the number of DAPI dense signals per interphase nucleus was determined with ImageJ software. The number of signals is shown as a box plot. Statistical comparisons of signal numbers were performed using the Wilcoxon test. *, $p < 0.01$.

(*C*) Effect of solvent on number of DAPI signals. The numbers of DAPI signals were counted and plotted as in (*B*). −, without solvent

(*D*) Number of DAPI signals in nuclei of ESCs exposed to chemicals. ESCs were cultured with chemicals that were categorized into seven groups (A–G) for 48 hr at serum level or tenfold higher

level than serum concentrations. The number of DAPI signals in the nuclei was determined with ImageJ software. Statistical comparisons of signal numbers were performed by the Wilcoxon test. *, $p < 0.01$. ND, Not determined; cells died of chemical exposure.

(*E*) Confirmation of heterochromatin changes in nuclei of mouse ESCs, exposed to DEP, Hg, cotinine, Se, or S-421 by major satellite staining. Heterochromatin in interphase nuclei was visualized by DNA-FISH with a specific probe for a heterochromatin marker, major satellite (red), and by DAPI staining (blue). Scale bar = 10 μm.

(*F*) Size of heterochromatin signals in nuclei of chemical-exposed ESCs. In the graph, the longitudinal and horizontal axes represent the average size and number of major satellite signals in nuclei by DNA-FISH analysis, respectively.

Figure 3. Effect of chemicals on DNA methylation in ESCs.

(*A*) Analysis of DNA methylation status in chemical-exposed ESCs by COBRA assay. ESCs were cultured with chemicals (DEP, Hg, cotinine, Se, and S-421) at the serum level concentrations or their solvents for 96 hr. Inset indicates the workflow of COBRA. DNA methylation degree (%) was calculated by the ratio of digested (methylated) and undigested (unmethylated) fragments. In the graph, each plot shows the methylation level of the individual gene locus that was examined. The longitudinal and horizontal axes represent the methylation level of chemical- and solvent-exposed samples, respectively. Plots that are located near the diagonal line mean similar methylation levels between chemical-exposed and solvent-treated cells. In the case of plots that are away from the diagonal line, methylation levels are different between the two samples. Open and closed plots located inside or outside the dotted line show that the difference in the methylation level between chemical- and solvent-exposed cells was within or over 5%, respectively.

(*B*) Methylation status at the *Rnd2*, *Aebp2*, and *Prickle2* T-DMRs by COBRA assay. DNA methylation degree (%) is shown as the average ± SE. *Rnd2* became more methylated upon Hg exposure, and *Aebp2* and *Prickle2* exhibited reduced methylation degrees after Se exposure. The positions of the *Hpy* CH4 IV site are illustrated as triangles. Black boxes represent exons, and the first

exon is only represented in the *Aebp2* and *Prickle2* genes. Statistical comparisons of DNA methylation were performed using the Student's *t* test. *, $p < 0.01$.

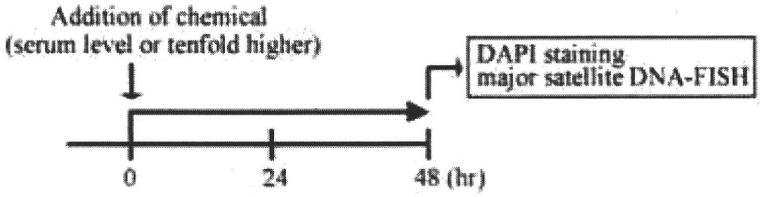
Figure 4. Reversibility of heterochromatin alteration and developmental effect of the five chemicals.

(*A*) Effect of chemicals on heterochromatin states after removal of chemicals. ESCs were cultured with either of the chemicals (DEP, Hg, cotinine, Se, and S-421) at the serum level-concentrations or solvent only for 48 hr, and then continued to culture without chemicals or solvent (control) for another 48 hr. The results are shown as a box plot. DNA-FISH analysis with a specific DNA probe for major satellite repeats in ESCs after removal of DEP. Localizations of major satellite repeats (red), DAPI-stained DNA (blue), and the merged images are shown. Scale bar = 10 μm. Statistical comparisons of DAPI signal number were performed using the Wilcoxon test. *, $p < 0.01$.

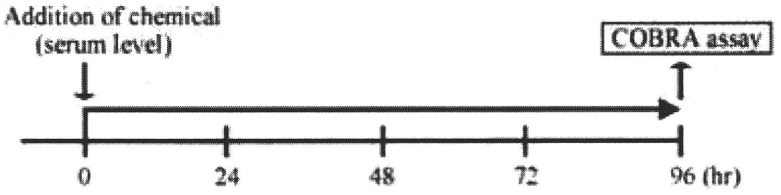
(*B*) Impaired EB formation by chemical exposure at the serum level concentrations. ESCs induced differentiation into EBs in the presence of one of the five chemicals (DEP, Hg, cotinine, Se, and S-421). Size of EBs exposed to chemicals at the serum level concentrations was measured as their areas by ImageJ. Average and SE of areas in EBs were calculated. Relative areas in chemical-exposed EBs were normalized to average areas of solvent-exposed control EBs. Experiments were performed at least twice independently for each chemical. −, solvent only; +, exposure to chemicals at the serum level concentrations. Scale bar = 250 μm. Statistical comparisons of area of EBs were performed using the Student's *t* test. *, $p < 0.01$.

(*C*) Summary of heterochromatin analysis based on the DAPI staining (Exp. 1, 3 in Fig. 1) and EB formation (Exp. 4 in Fig. 1). ↑, increase; ↓, decrease; →, no change of DAPI signal number or size of EBs compared with solvent-treated controls; ND, not determined.

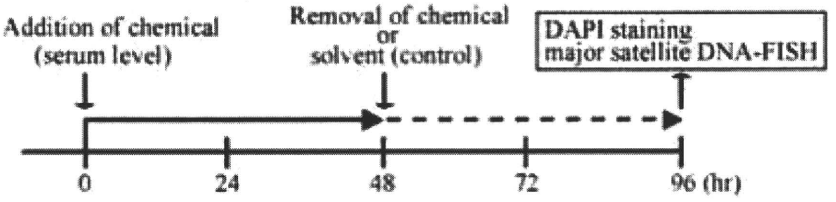
Exp.1 Epimutagen screening
(25 chemicals detected in human samples)



Exp.2 DNA methylation analysis
(DEP, Hg, cotinine, Se or S-421 exposed cells)



Exp 3 Reversibility of heterochromatin changes
(epimutagen candidate DEP, Hg, cotinine, Se, S-421)



Exp.4 EB formation
(DEP, Hg, cotinine, Se or S-421 exposed cells)

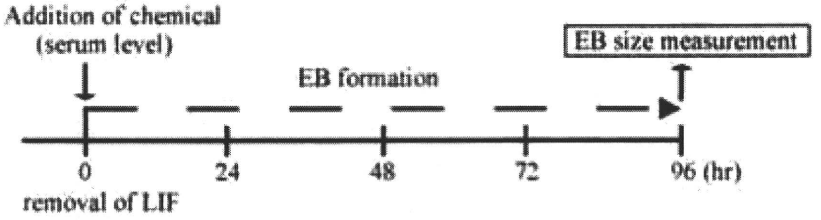


Fig. 1

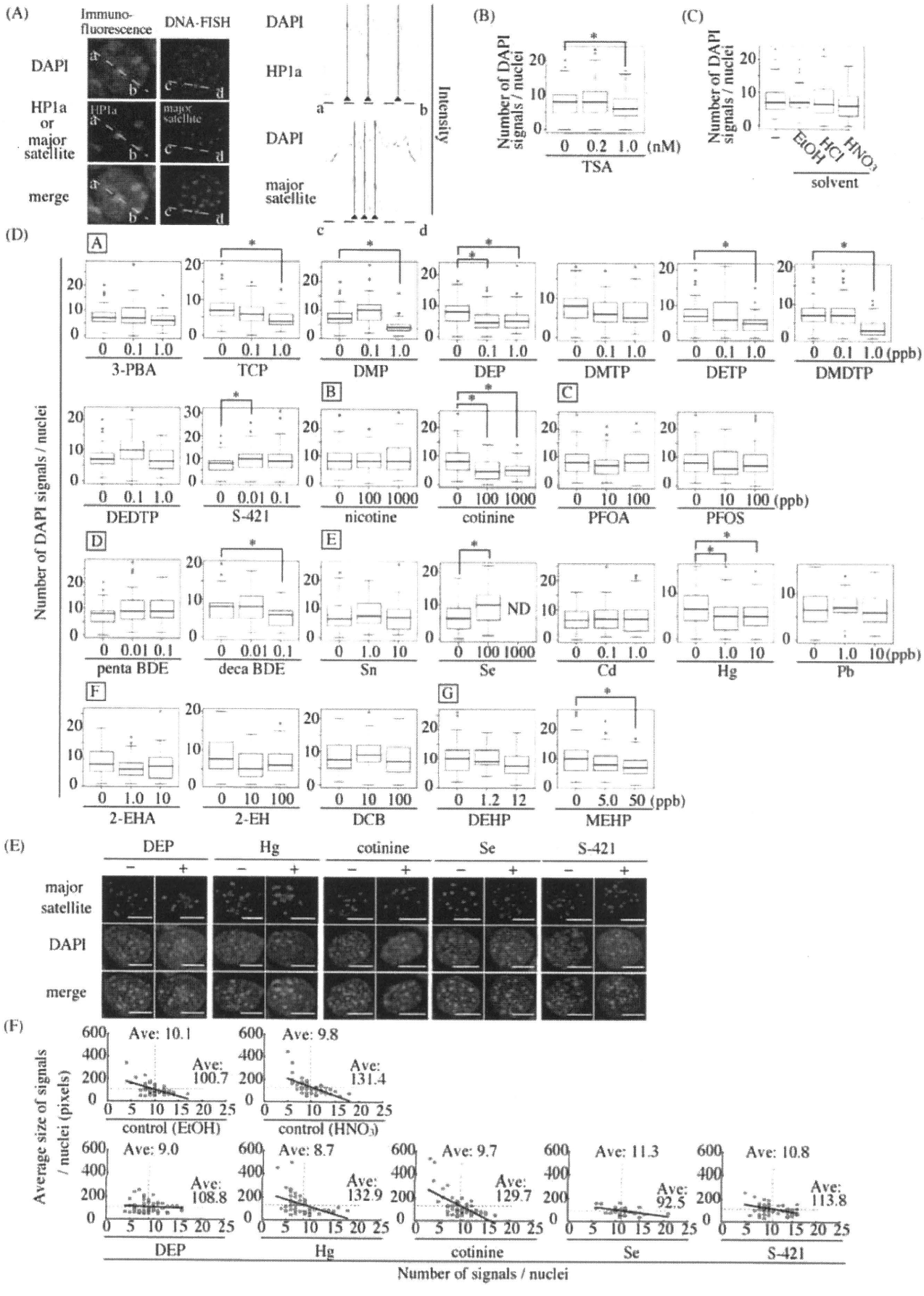

(A)
Immuno-fluorescence
DNA-FISH
DAPI
HP1a or major satellite
merge
major satellite
Intensity
(B)
Number of DAPI signals / nuclei
TSA
(nM)
(C)
EtOH
HCl
HNO3
solvent
(D)
Number of DAPI signals / nuclei
3-PBA
TCP
DMP
DEP
DMTP
DETP
DMDTP
DEDTP
S-421
nicotine
cotinine
PFOA
PFOS
penta BDE
deca BDE
Sn
ND
Se
Cd
Hg
Pb
2-EHA
2-EH
DCB
DEHP
MEHP
(ppb)
(E)
DEP
Hg
cotinine
Se
S-421
major satellite
DAPI
merge
(F)
Average size of signals / nuclei (pixels)
Ave: 10.1
Ave: 100.7
control (EtOH)
Ave: 9.8
Ave: 131.4
control (HNO3)
Ave: 9.0
Ave: 108.8
DEP
Ave: 8.7
Ave: 132.9
Hg
Ave: 9.7
Ave: 129.7
cotinine
Ave: 11.3
Ave: 92.5
Se
Ave: 10.8
Ave: 113.8
S-421
Number of signals / nuclei

Fig. 2

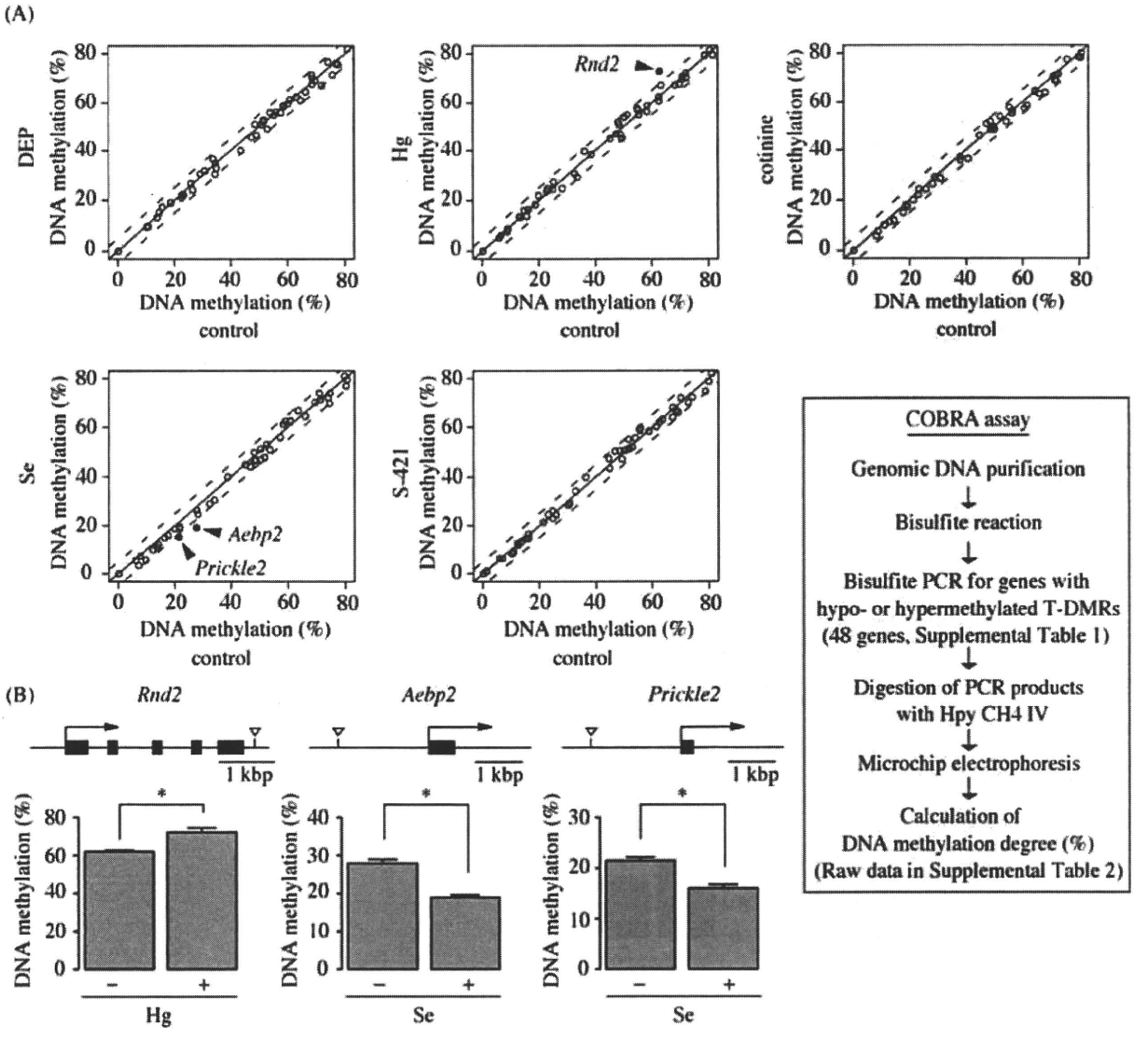
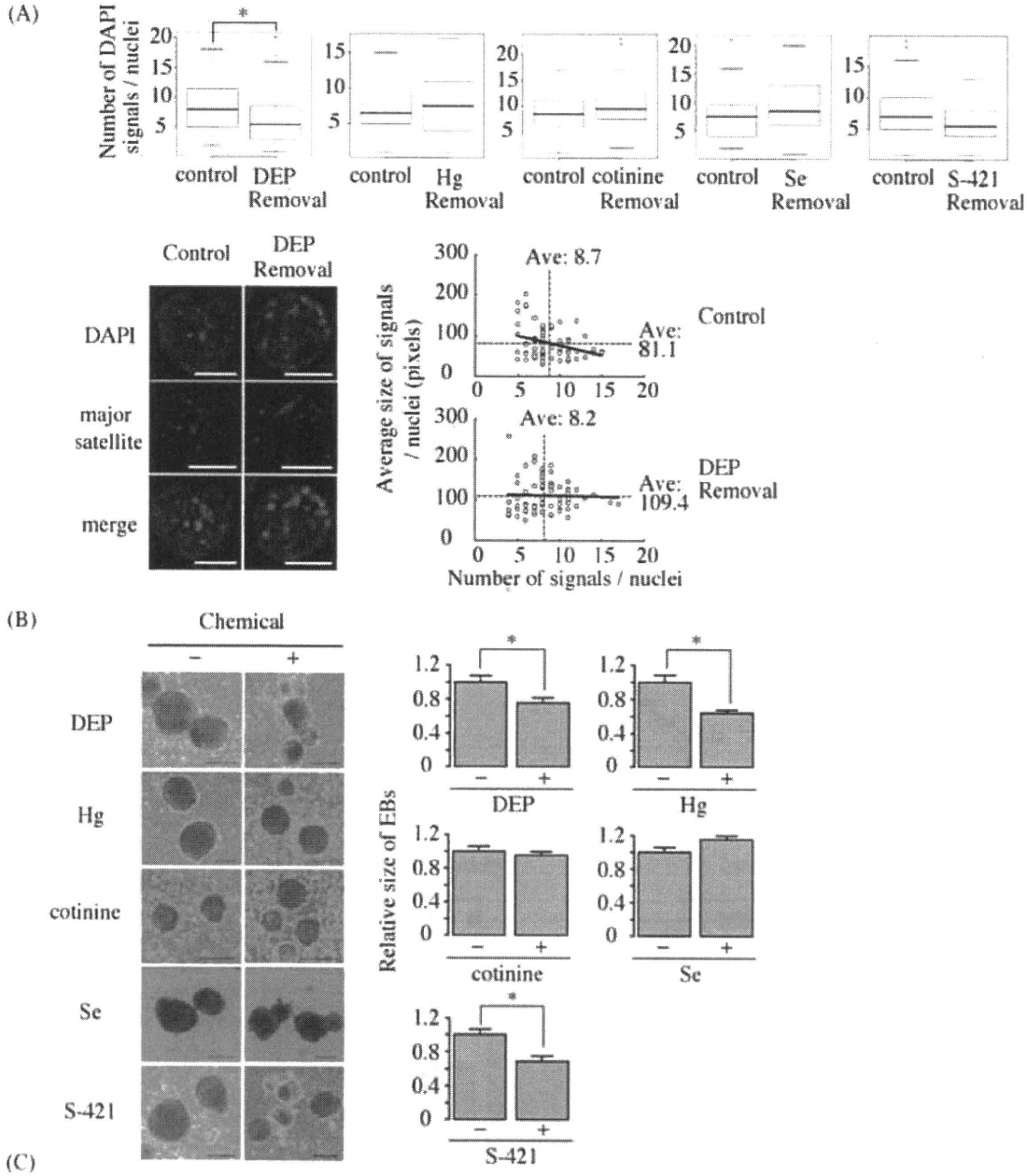

(A)
DEP
Hg
cotinine
Se
S-421
DNA methylation (%)
control
Rnd2
Aebp2
Prickle2
COBRA assay
Genomic DNA purification
Bisulfite reaction
Bisulfite PCR for genes with hypo- or hypermethylated T-DMRs (48 genes, Supplemental Table 1)
Digestion of PCR products with Hpy CH4 IV
Microchip electrophoresis
Calculation of DNA methylation degree (%) (Raw data in Supplemental Table 2)
(B)
Rnd2
Aebp2
Prickle2
1 kbp
Hg
Se
Se

Fig. 3



(C)

	chemical	exposure to cells (ppb) (×1, ×10)	DAPI signals (Exp.1) chemical exposure ×1, ×10	DAPI signals (Exp.3) Removal of chemicals	(Exp.4) Size of EBs
A	3-PBA	0.1, 1.0	→ →	ND	ND
	TCP	0.1, 1.0	→ ⇩	ND	ND
	DMP	0.1, 1.0	→ ⇩	ND	ND
	DEP	0.1, 1.0	⇩ ⇩	⇩	⇩
	DMTP	0.1, 1.0	→ →	ND	ND
	DETP	0.1, 1.0	→ ⇩	ND	ND
	DMDTP	0.1, 1.0	→ ⇩	ND	ND
	DEDTP	0.1, 1.0	→ →	ND	ND
	S-421	0.01, 0.1	▲ →	→	⇩
B	nicotine	100, 1000	→ →	ND	ND
	cotinine	100, 1000	⇩ ⇩	→	→
C	PFOA	10, 100	→ →	ND	ND
	PFOS	10, 100	→ →	ND	ND
D	penta BDE	0.01, 0.1	→ →	ND	ND
	deca BDE	0.01, 0.1	→ ⇩	ND	ND
E	Sn	1.0, 10	→ →	ND	ND
	Se	100, 1000	▲ ND	→	→
	Cd	0.1, 1.0	→ →	ND	ND
	Hg	1.0, 10	⇩ ⇩	→	⇩
	Pb	1.0, 10	→ →	ND	ND
F	2-EHA	1.0, 10	→ →	ND	ND
	2-EH	10, 100	→ →	ND	ND
	DCB	10, 100	→ →	ND	ND
G	DEHP	1.2, 12	→ →	ND	ND
	MEHP	5.0, 50	→ ⇩	ND	ND

Fig. 4