Initiation of epigenetic reprogramming of the X chromosome in somatic nuclei transplanted to mouse oocyte

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Supplementary information

Materials and Methods

Animal and MII oocytes

[C57BL/6 x CBA] F1 mice were used to obtain MII oocytes were superovulated females, for nuclear transfer and IVF as previously described (Bao et al, 2000).

Pre-implantation embryos prepared by SCNT and IVF

A female cell line was established from tail tip tissue of 4-5 week old F1 female mice, which were used as nuclear donor cells. SCNT procedure was as described previously (Ogura et al, 2000). The enucleated MII oocytes that fused successfully with donor cells were activated by treatment with 10mM strontium (Sigma) in Ca^{2+} -free CZB medium containing 5μg/ml cytochalasin B (Sigma) for 6 hours. Reconstructed embryos were cultured in CZB medium, fixed at different stages and analysed for RNA FISH and RT-PCR. Same stage embryos were used as controls following IVF and culture *in vitro*.

Immunofluorescence and RNA FISH

Embryos were fixed in 4 % paraformaldehyde for 15 min, and permeabilised with AB-buffer (1% Triton-X100, 0.2% SDS, 10 mg/ml BSA in PBS) for 30min. They were then incubated in primary antibodies diluted in the AB-buffer overnight at 4°C. The following antibodies were used: H3-3meK27 (anti-rabbit, T. Jenuwein), Eed (anti-

mouse, a kind gift from A. Otte), Ezh2 (anti-rabbit, Upstate), H3-2meK9 (anti-rabbit, Upstate), G9a (anti-goat, Abcam) and H3-meR26 (anti-rabbit, Abcam). Following incubation with primary antibodies, the embryos were washed three times for 10 min each, and incubated in secondary antibodies conjugated with Alexa Fluor 488 (anti-rabbit, Vector Laboratories) and Alexa Fluor 568 (anti-mouse, Vector Laboratories) for 1 hour in AB-buffer at RT. Finally the embryos were incubated in 0.1 mg/ml RNase A (Roche) in PBS at 37°C for 30 minutes and mounted on slides in Vectashield (Vector Laboratories) containing TOTO3 (Molecular Probes) to stain DNA.

For the detection and co-localisation of *Xist* RNA with H3-3meK27, we applied the method of immunofluorescence combined with RNA-FISH as previously described (Okamoto et al, 2004).

RT-PCR/RFLP analysis

Total RNA from individual early stage embryos and adult tail fibroblast cells (C57BL/6, *Mus Spretus* and [B6xSpr] F1) was isolated using Cells-to-cDNA^{TM} II kit (Ambion) or TRIZOL (GIBCO BRL) with some modifications. 20pg of spike RNA (chloramphenicol acetyltransferase mRNA, INVITROGEN) was added to each embryonic sample before isolation of total RNA for evaluating the efficiency of RNA extraction and RT-PCR. cDNA synthesis by Superscript II reverse transcriptase (INVITROGEN) using a cocktail of oligo d(T) and random hexamer primers (50 ng and 5 ng each per sample, respectively) was carried out, according to the manufacture's protocol. Allele specific RT-PCR/RFLP analyses for *Xist* gene were carried out as described previously (Kay et al, 1993).

References

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Supplementary Figure legends

Figure S1. Characteristics of X chromosome in XX female fibroblast cells. (A) *Xist* RNA coating (green) and (B) a single domain of H3-3meK27 (green) were detected in XX female tail fibroblast cells (100% and 40% of cells, respectively), while neither (C) Ezh2 (green) nor (D) G9a (red) was detectable. DNA was stained with either TOTO3 (blue) or PI (red).

Figure S2. Detection of H3-R26 methylation in 1-2 cell SCNT embryos. SCNT-embryos from female tail fibroblast cells were immunostained at 0 hour (A), 30 min (B), 2 hours (C), 8 hours (D) and 24 hours (E). (A) H3-meR26 was detectable in somatic cell nucleus but (B) global histone demethylation was observed within 30 min after SCNT. (B to E) Methylation of H3-R26 then recovered at 2 hours. (F) H3-meR26 was not detectable in control embryos at the similar stages.

Figure S3. Co-localization of H3-3meK27 and Eed from 4-cell to morula stage SCNT embryos, compared with embryos obtained from IVF. IF analysis for H3-3meK27 (green) and Eed (red), and merged images with TOTO3 (Blue). (A) Co-localization of H3-3meK27 and Eed was seen at 4-cell stage in SCNT embryo. (B) This state continued to the morula and blastocyst stage (not shown). (C) In IVF embryos, there was no signal for H3-3meK27 and Eed at 4-8-cell embryo. (D) A single domain of Eed co-localised with H3-3meK27 was detectable in some blastomeres at 16-cell to morula stage.

Figure S4. Detection of H3-2meK9 (green) and G9a (red) in 1-2 cell SCNT embryos. White arrows indicate signals for H3-2meK9 on X chromosome. (A) Co-localisation of H3-2meK9 and G9a on X chromosome was not detectable in somatic nucleus. The co-localization of H3-2meK9 and G9a were detected at (B) 2 h and (C) 8 h after SCNT. (D) Both H3-2meK9 and G9a were not detectable at the 2-cell stage in SCNT-embryos.

Figure S5. Detection of H3-3meK27 (green) and G9a HMTase (red). White arrows indicate signals for H3-3meK27 on X chromosome. (A) G9a was not detectable on somatic nucleus. (B and C) H3-3meK27 and G9a co-localisation was detected at 2hrs and 8hrs after nuclear transfer. (D) However, G9a then decreased in 2-cell stage SCNT-embryos.

Figure S6. Detection of H3-3meK27 (green) and Eed (red) in 2-cell SCNT-embryos derived from different donor cells. (A) XX ES cells or (B) XY male tail were used for SCNT as donors. No co-localization signals for of H3-3meK27 and Eed were detected.

Fig. S1 S. Bao, *et al*.

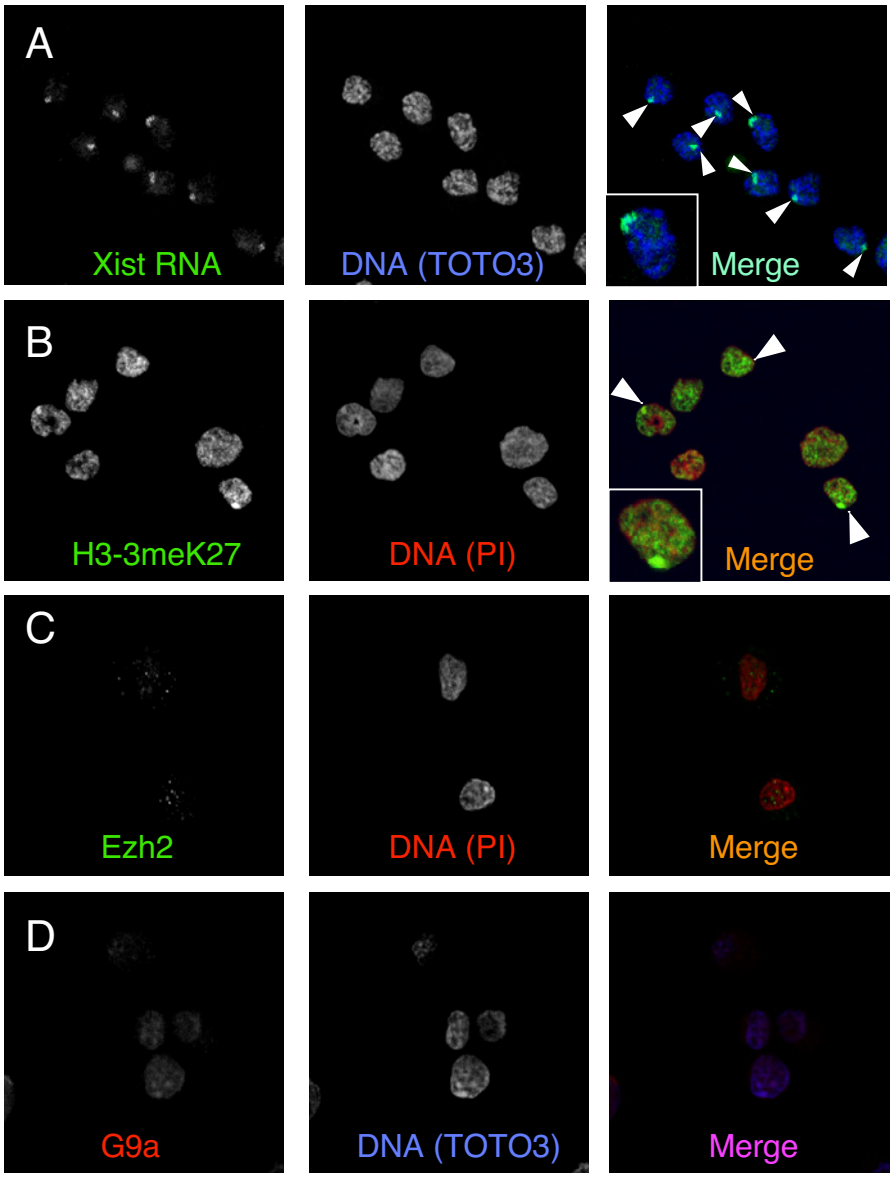


Fig. S2 S. Bao, *et al*.

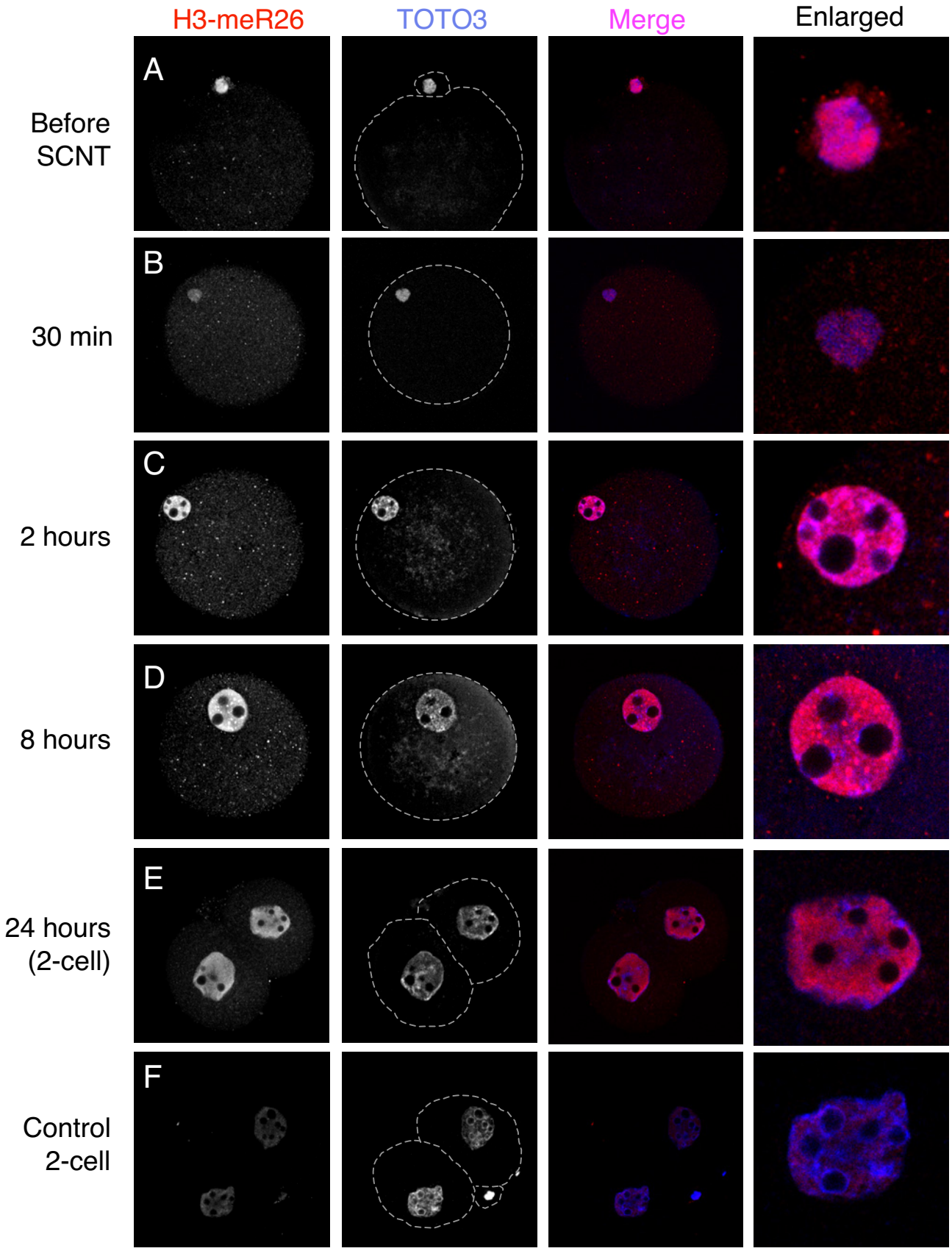
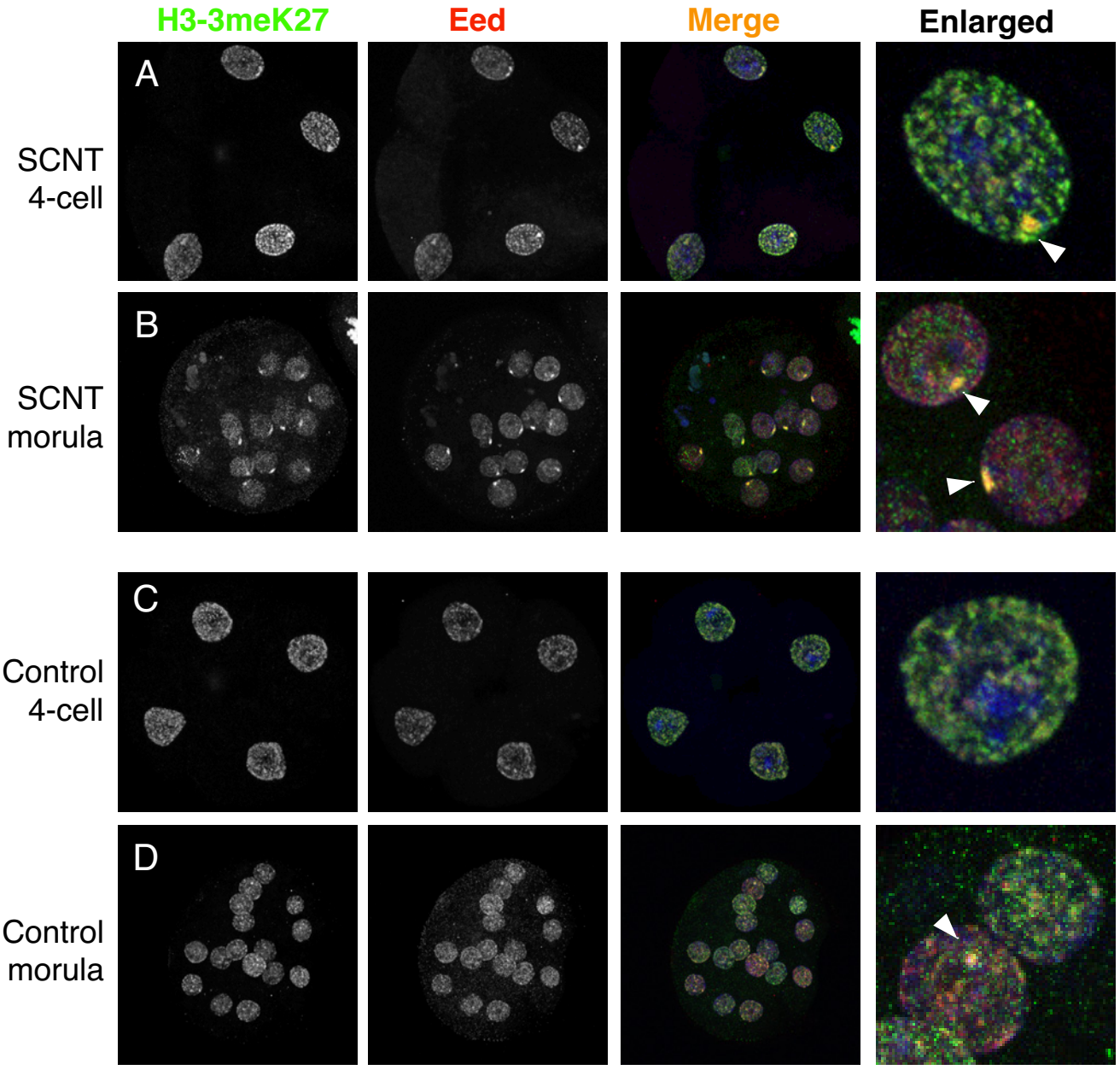


Fig. S3 S. Bao, *et al*.



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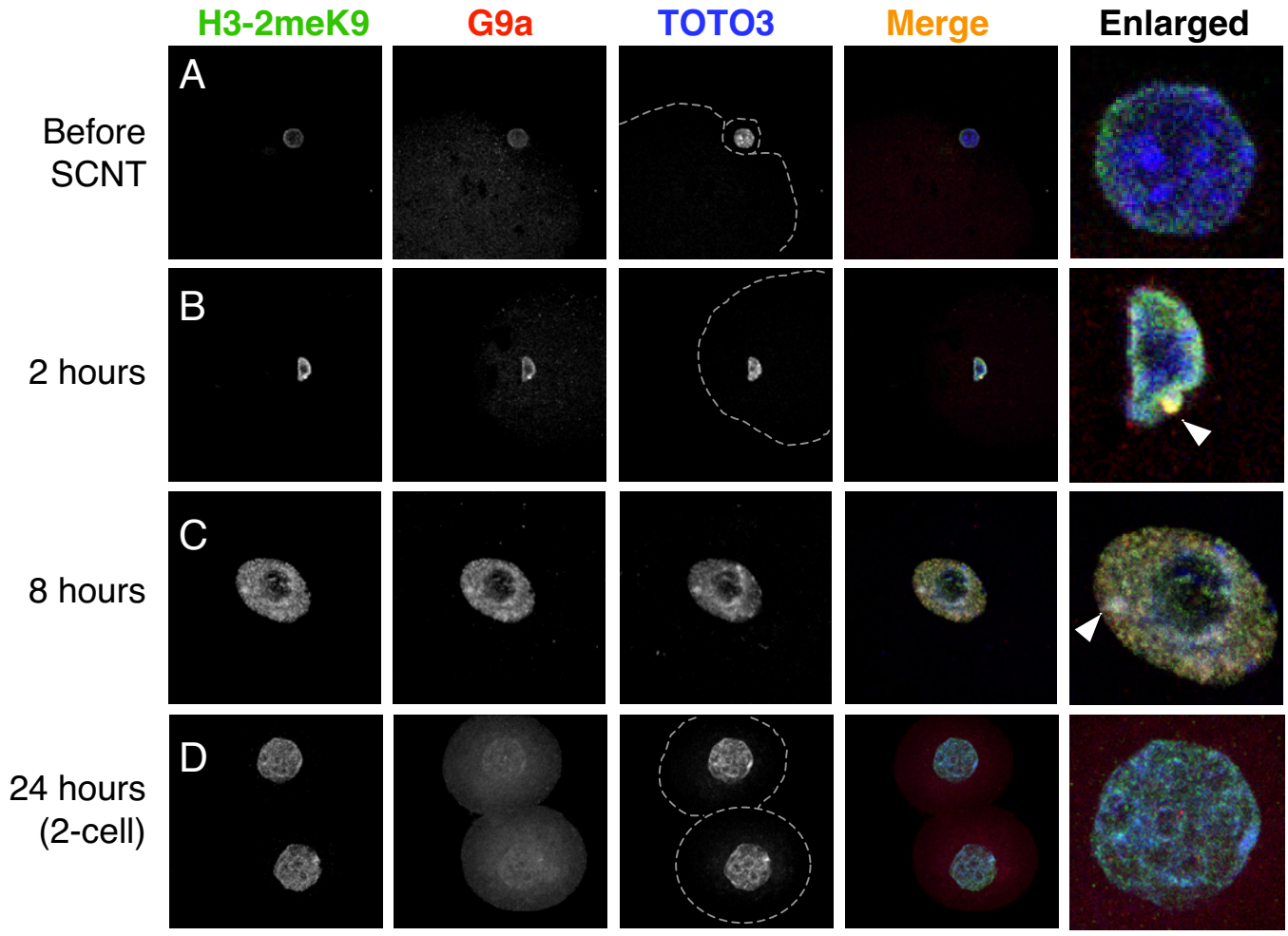

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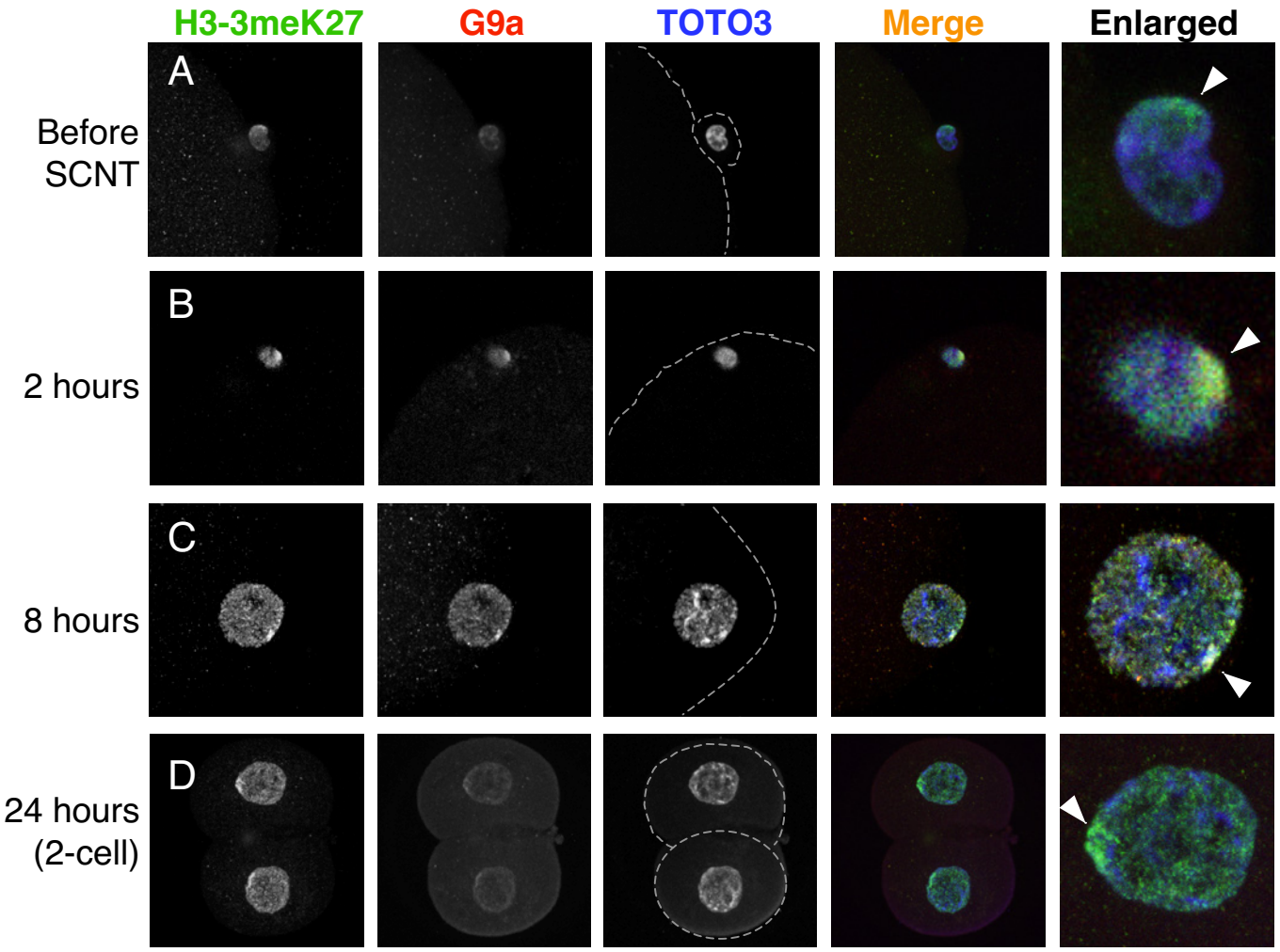

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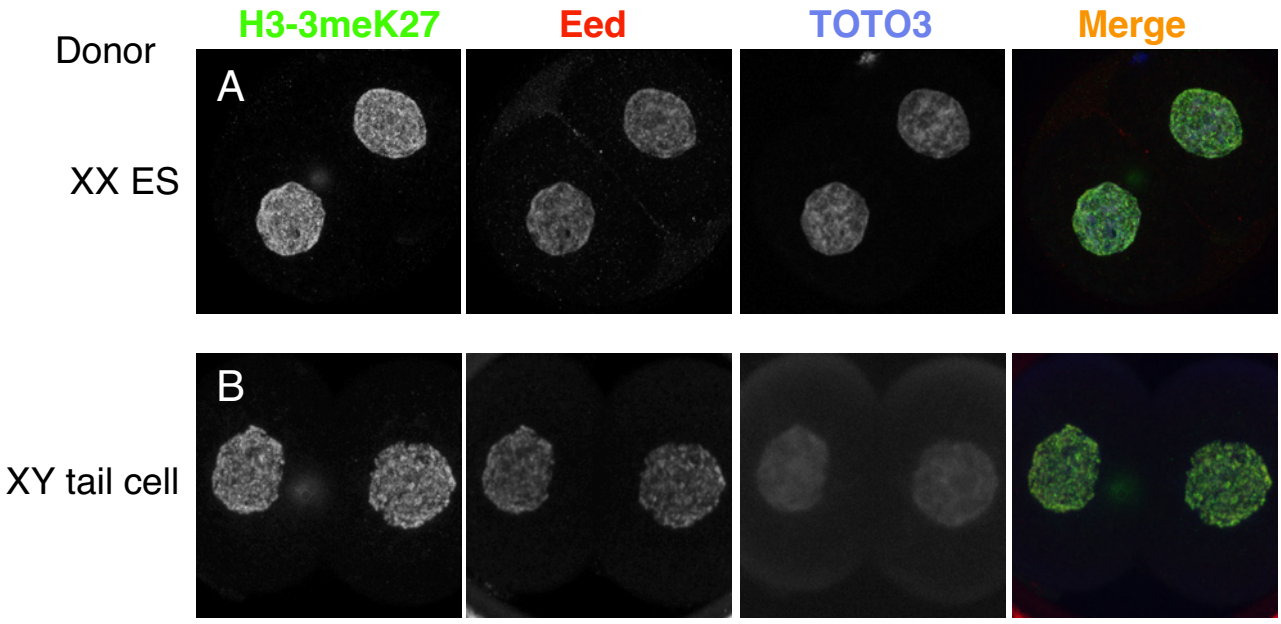


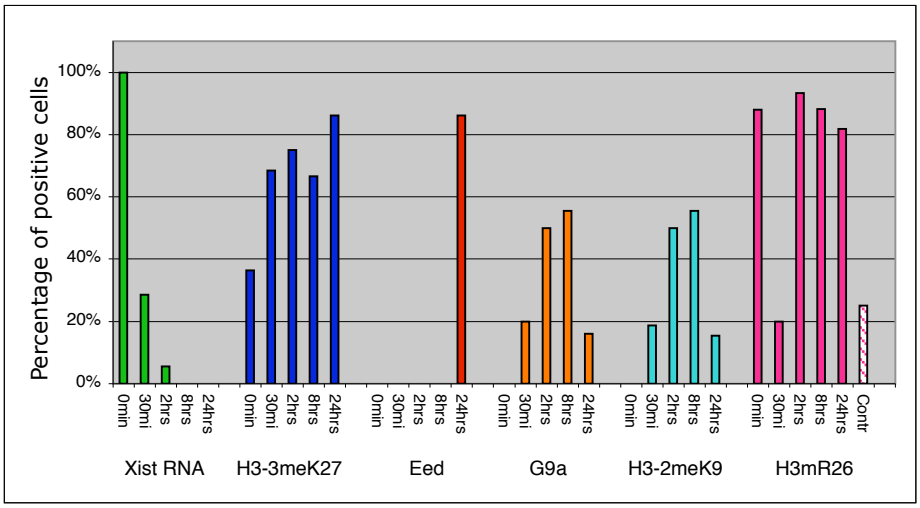
Table S1. Summary of epigenetic modifications on X chromosome following SCNT.

		No. of embryos analysed	No. of cells	accumulation on X		%
				+	-	
Xist	0min	29	29	29	0	100.0%
	30min	14	14	4	10	28.6%
	2hrs	18	18	1	17	5.6%
	8hrs	ND	ND	ND	ND	ND
	24hrs	24	48	0	48	0.0%
H3-3meK27	0min	22	22	8	14	36.4%
	30min	19	19	13	6	68.4%
	2hrs	24	24	18	6	75.0%
	8hrs	27	27	18	9	66.7%
	24hrs	36	72	62	10	86.1%
Eed	0min	25	25	0	25	0.0%
	30min	19	19	0	19	0.0%
	2hrs	22	22	0	22	0.0%
	8hrs	20	20	0	20	0.0%
	24hrs	36	72	62	10	86.1%
G9a	0min	20	20	0	20	0.0%
	30min	15	15	3	12	20.0%
	2hrs	16	16	8	5	50.0%
	8hrs	18	18	10	8	55.6%
	24hrs	25	50	8	42	16.0%
H3-2meK9	0min	14	14	0	14	0.0%
	30min	16	16	3	13	18.8%
	2hrs	12	12	6	6	50.0%
	8hrs	18	18	10	8	55.6%
	24hrs	13	26	4	22	15.4%
H3mR26*	0min	25	25	22	3	88.0%
	30min	20	20	4	16	20.0%
	2hrs	15	15	14	1	93.3%
	8hrs	17	17	15	2	88.2%
	24hrs	11	22	18	4	81.8%
	Control(IVF) 2cell**	12	24	6	18	25.0%

* Detection of H3-mR26 was considered positive for the entire nuclei.

** Control IVF embryos were negative for all modifications at these stages except for H3mR26 at the 2-cell stage.

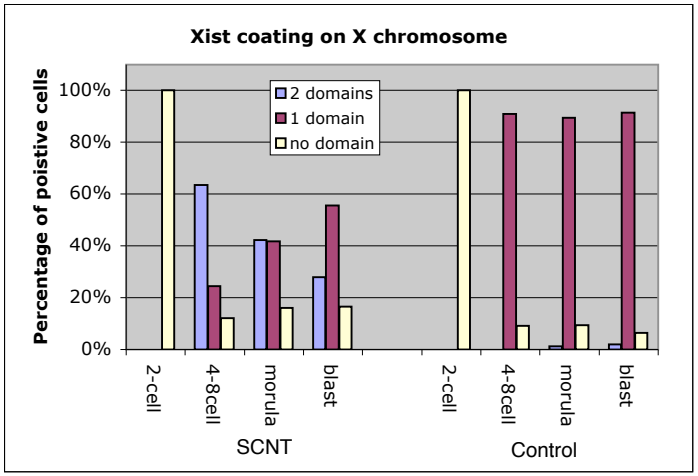
ND; not determined

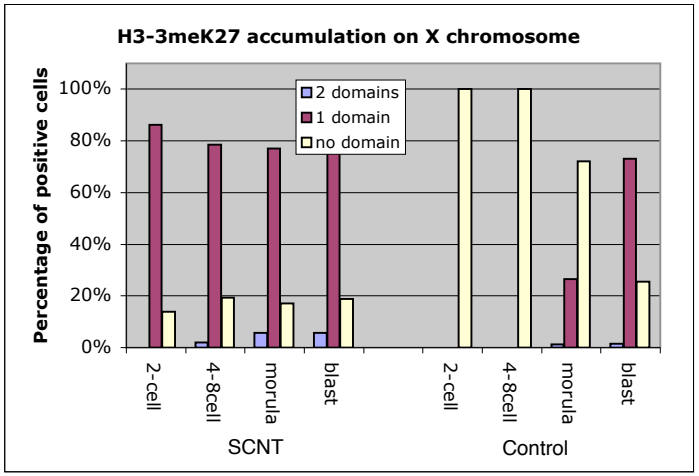


Schematic representation of the data shown in the table.

Table S2. Summary of Xist RNA/H3-3meK27 localisation on X chromosome from 2-cell to blastocyst stage.

			No. of embryos	No. of cells	2 domains	%	1 domain	%	no domain	%
Xist	SCNT	2-cell	24	48	0	0.0%	0	0.0%	48	100.0%
		4-8cell	14	82	52	63.4%	20	24.4%	10	12.2%
		morula	12	156	66	42.3%	65	41.7%	25	16.0%
		blast	10	383	107	27.9%	213	55.6%	63	16.4%
	Control	2-cell	8	16	0	0.0%	0	0.0%	16	100.0%
		4-8cell	6	44	0	0.0%	40	90.9%	4	9.1%
		morula	8	151	2	1.3%	135	89.4%	14	9.3%
		blast	9	626	12	1.9%	573	91.5%	41	6.5%
H3-3meK27	SCNT	2-cell	36	72	0	0.0%	62	86.1%	10	13.9%
		4-8cell	17	98	2	2.0%	77	78.6%	19	19.4%
		morula	10	157	9	5.7%	121	77.1%	27	17.2%
		blast	8	344	20	5.8%	259	75.3%	65	18.9%
	Control	2-cell	12	24	0	0.0%	0	0.0%	24	100.0%
		4-8cell	20	120	0	0.0%	0	0.0%	120	100.0%
		morula	10	169	2	1.2%	45	26.6%	122	72.2%
		blast	9	585	8	1.4%	428	73.2%	149	25.5%





Schematic representation of the data shown in the tables.