Supporting Information

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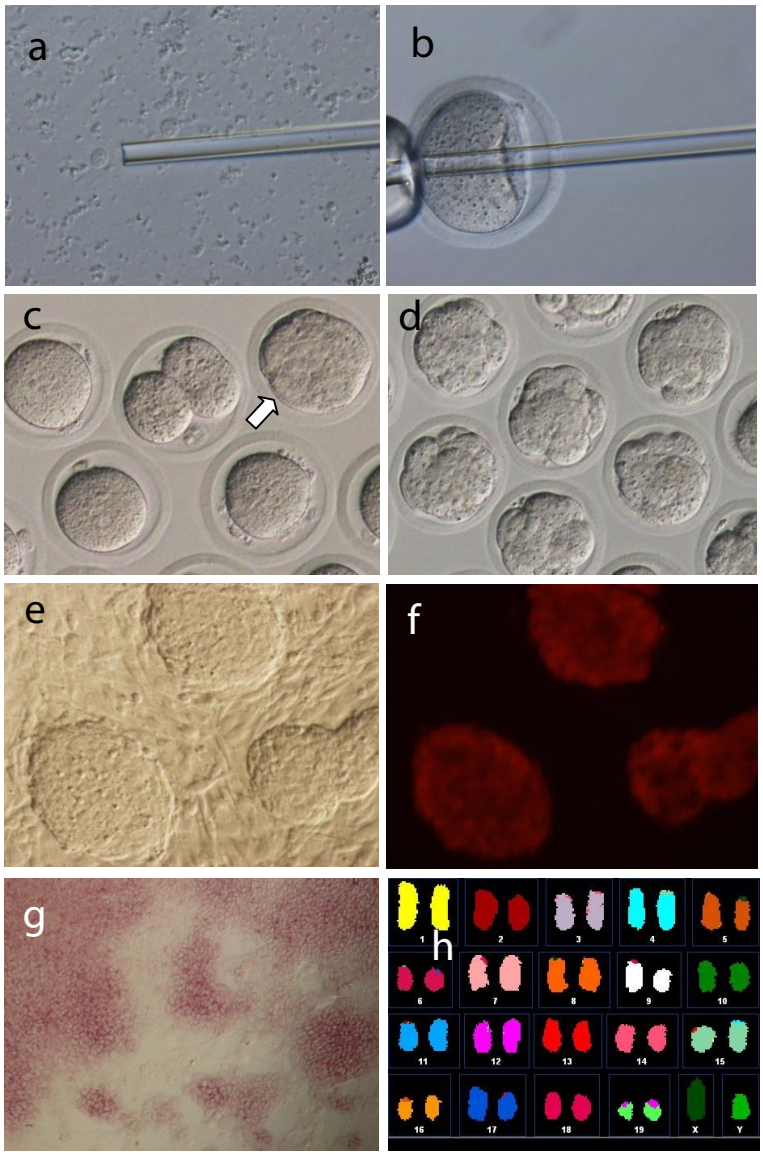

Fig. S1. Nuclear transfer, embryo development and characterization of ntES cells. (*a* and *b*) A denuded nucleus was collected using a large diameter pipette in NIM medium (see *Methods*) and injected into an enucleated oocyte without any additional injection medium. (*c*) Cloned embryos derived from thymus cell nuclei. Only one embryo (arrow) developed to the morula stage at 72 h after nuclear transfer. (*d*) Cloned embryo derived from a brain nucleus. About half of the reconstructed oocytes developed to the morula/blastocyst stage. (*e*–*f*) The ntES cells showed pluripotency. (e) Phase contrast micrograph of ntES cells. (*f*) The same cells showing positive immunostaining for expression of the pluripotency marker Oct3/4 under UV light. (*g*) Alkaline phosphatase staining. (*h*) Spectral karyotyping with fluorescence *in situ* hybridization painting of a C3H-derived ntES cell showing a normal karyotype.

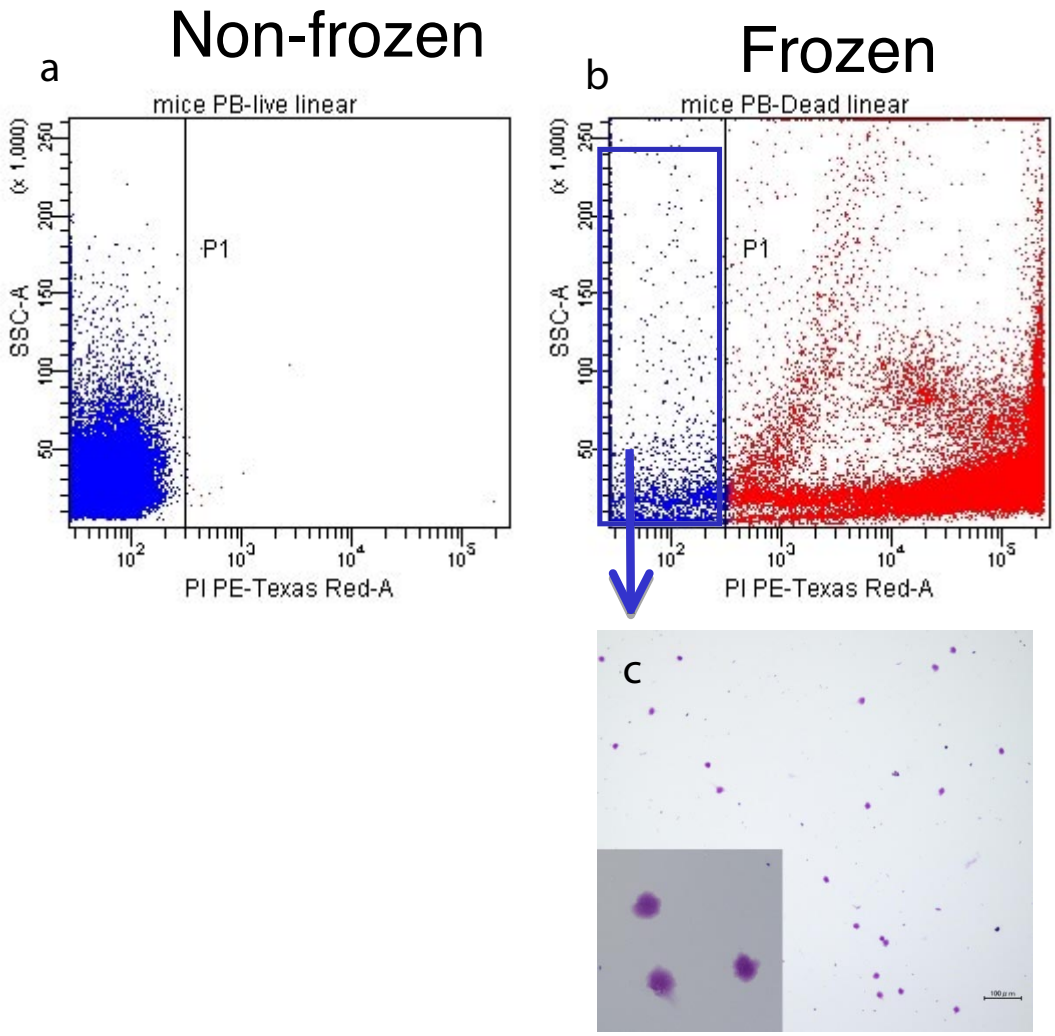

Fig. S2. Fluorescence activated cell sorting (FACS) analysis of frozen blood cells. (*a*) FACS analysis of fresh cells. (*b*) FACS analysis of frozen cells. (*c*) Picture of cells fractionated as live. The blood cells were frozen at −20 °C for two days. After thawing, live or dead cells were determined by side scatter and the dead cell marker, propidium iodide (PI) labeled phycoerythrin–Texas red-A (PI PE–Texas RED-A) using FACSaria (BD Biosciences). In the fresh sample, 100% of the cells were alive, whereas after freeze–thawing, 96.5% of cells were dead and all of the remaining cells had no nucleus. Thus, we could not find any live cells from frozen body samples.

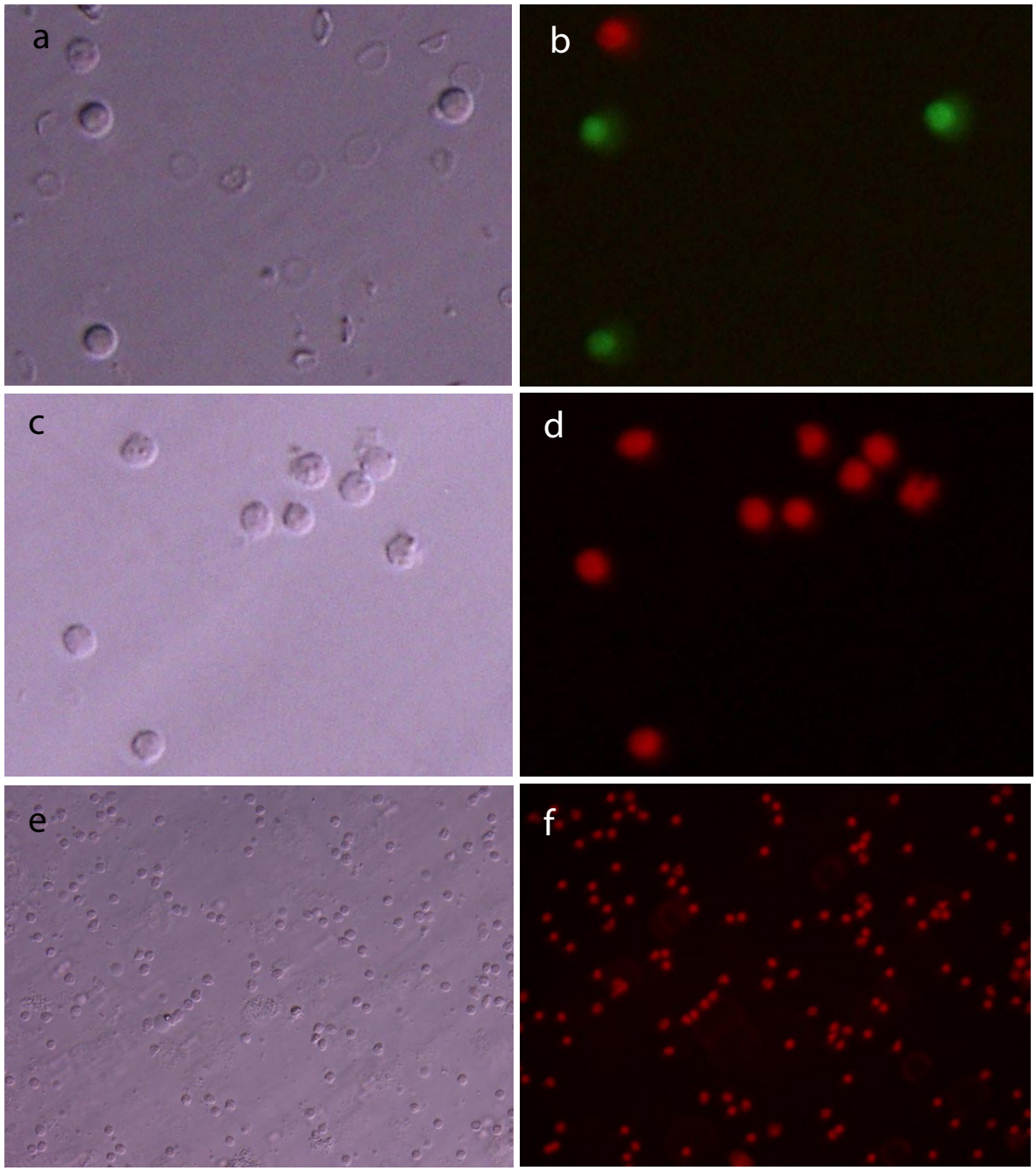

Fig. S3. Vital staining of frozen blood cells. (a and *b*) Live cells collected from a live mouse. (*c–f*) Dead cells collected from seven mice frozen for more than three months. Cells were incubated with erythrocyte lysing buffer (Sigma-Aldrich) and then stained with a Live/Dead Staining Kit (Molecular Probes) according to the manufacturer's protocol. Live cells with an intact cell membrane fluoresce bright green, while cells with damaged cell membranes fluoresce red. We examined over 500 cells from each frozen mouse, but no green cells were observed.

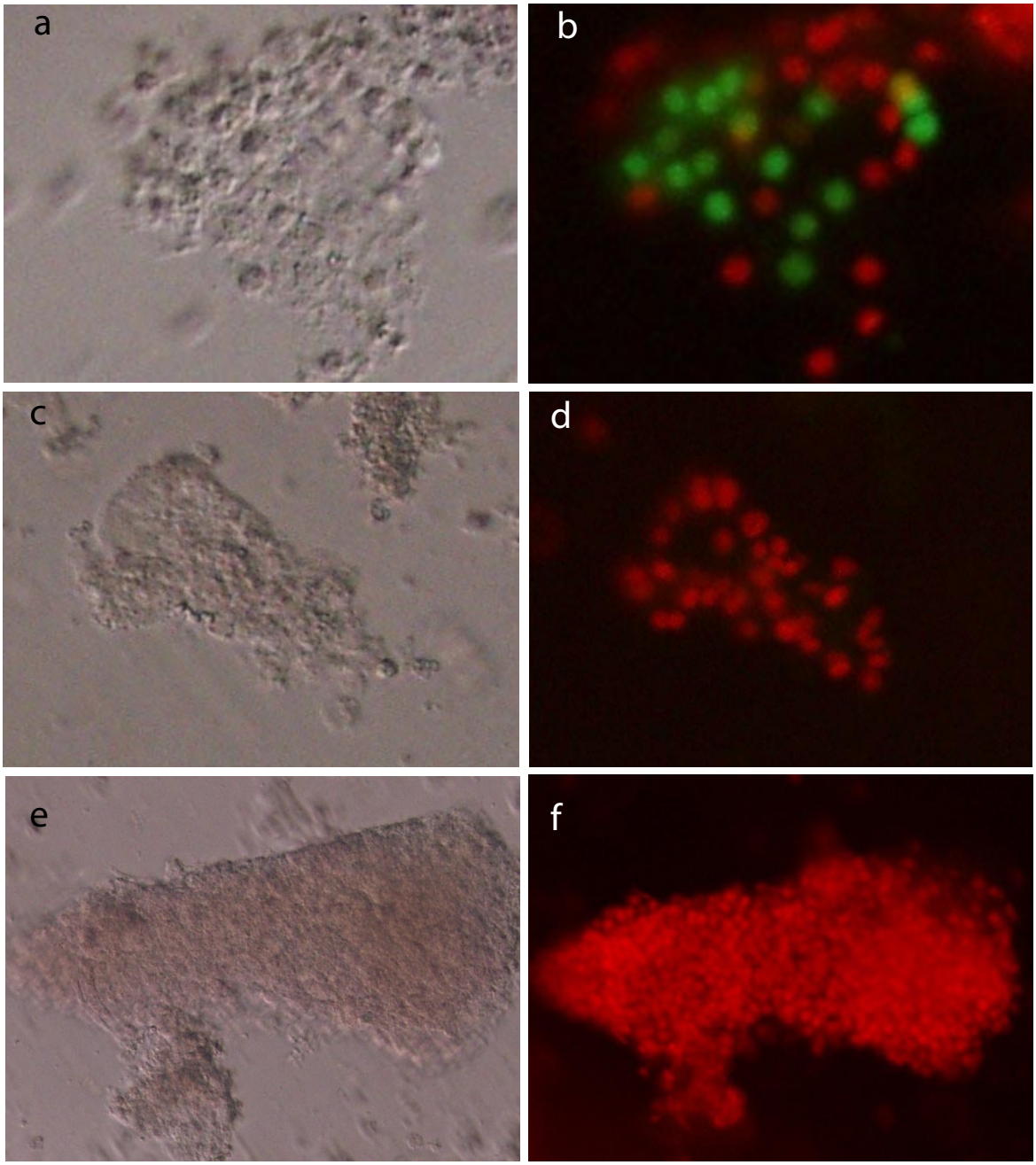

Fig. S4. Vital staining of frozen brain cells. (*a* and *b*) Live cells in a small piece of brain collected from a living mouse. (*c*–*f*) Dead cells collected from six frozen mice. Because the frozen samples were too fragile for separation by enzyme digestion, we used gentle homogenization. We examined more than 100 pieces of brain from each mouse using Live/Dead Staining Kits, but no green cells were observed.

Fig. S5. Diploid and tetraploid chimeras derived from frozen mice. (*a*) Chimeric mice derived from BDF1 ntES cells using diploid ICR (albino) embryos. (*b*) Germline transmission of diploid chimera. (*c*) Chimeric clonal mice from C3H ntES cell line by tetraploid complementation method. All offspring had the agouti coat color.

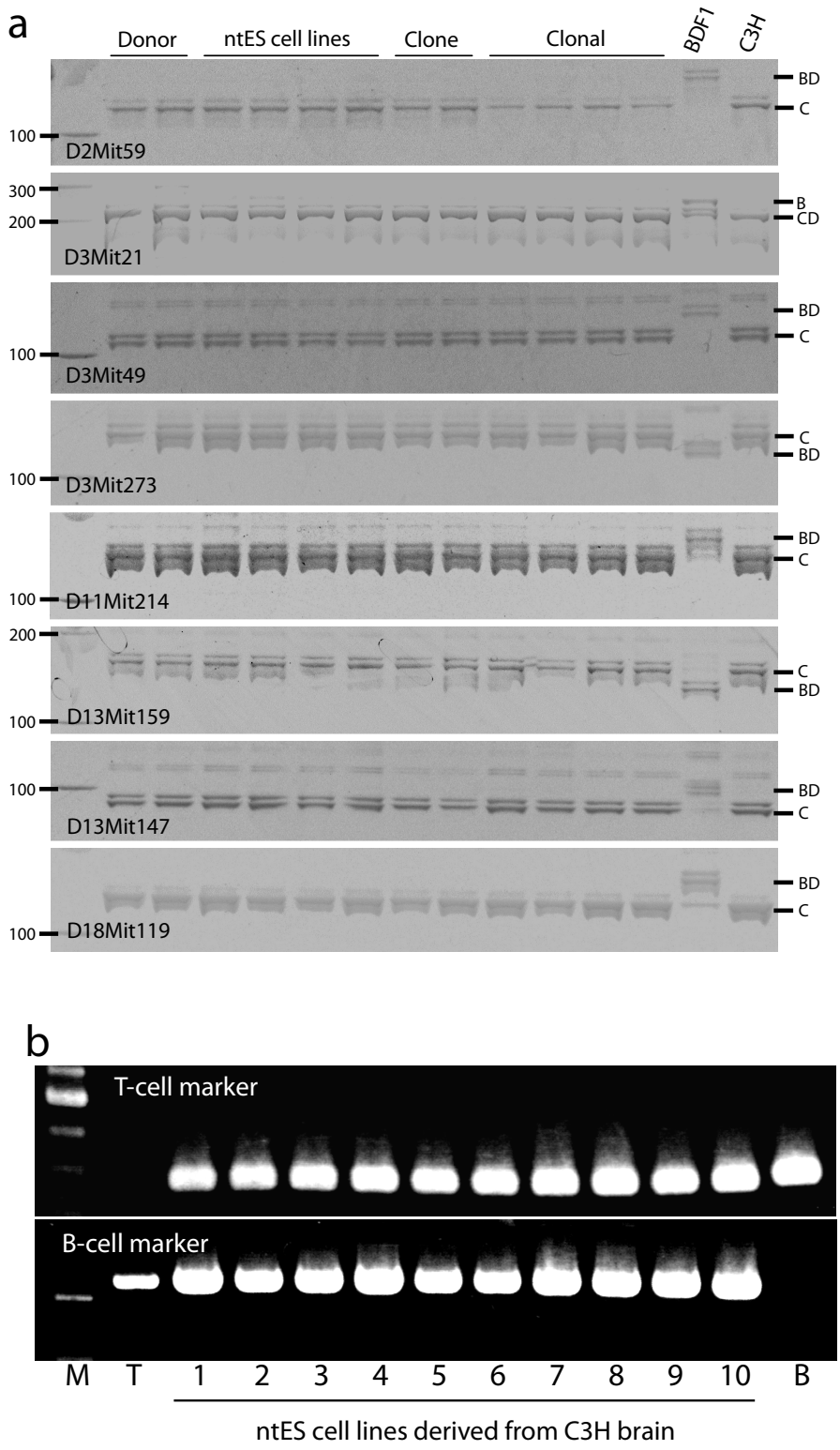

Fig. S6. Genotyping and examination of gene rearrangement. (*a*) Genotyping of cloned and chimeric clonal mice and of ntES cell lines. All samples showed the same pattern as the donor C3H (C) frozen mice but differed from the BDF1 (BD) strain oocyte donors. (*b*) Examination of gene rearrangements. The upper panel shows the deletion of a gene segment of T cells (T) and lower panel showed the Ig rearrangement of B cell (B). None of the ntES cell lines 1–10 derived from C3H strain frozen mice showed any gene rearrangement.

Table S1. Establishment of ntES cell lines from tail blood cells

Duration of freezing	Sex	No. enucleated oocytes	No. activated oocytes	No. embryos developing to morula/ blastocyst, %*	No. established ntES cell lines, %*
Fresh	F	106	48	18 (37.5)	5 (10.4)
	M	106	61	23 (37.7)	5 (8.2)
1 week**	F	225	150	21 (14.0)	7 (4.7)
	M	222	176	37 (21.0)	12 (6.8)
1 month	F	331	255	40 (15.7)	11 (4.3)
3 month	F	237	188	22 (11.7)	8 (4.3)
	M	192	133	11 (8.3)	4 (3.0)

*Based on the numbers of activated oocytes. **This table gives more detail on the tail blood data in Table 1.

Table S2. Diploid chimera and germline transmission

Organ	Mouse strain	Freezing duration	Sex	Cell line name	No. oocytes injected	No. of pups	No. of high coat color chimeras	Germ line transmission
Tail	BDF1	1 week	F	1–1	16	5	1	Yes
				1–2	17	3	1	
			M	2–1	16	8	3	Yes
				2–2	16	12	7	Yes
		3 months	M	10–1	36	6	2	Yes
				10–2	18	6	4	Yes
				11–1	36	9	7	Yes
				11–2	17	4	3	Yes
				12–1	18	5	0	
			F	13–1	34	11	0	
				13–2	18	5	0	
				14–1	36	19	12	Yes
Brain	C3H	16 years	M	1–1	38	20	7	
				1–2	37	20	8	
				1–3	21	12	3	Yes
				1–4	18	0	0	
				1–5	18	0	0	
				1–6	20	0	0	
				1–7	41	14	2	Yes
				2–1	34	0	0	
				2–2	20	0	0	
				2–3	20	0	0	

Table S3. Production of cloned mice from ntES cell nuclei derived from tail blood cells

Period	Sex	Donor cell type	No. enucleated oocytes	No. activated oocytes	No. embryos developed to 2-cell stage, %*	No. offspring, %**
3 Month	F	ntES	886	704	254 (36.1)	0
	M		186	119	36 (30.3)	3 (8)

*Based on the numbers of activated oocytes.
**Based on the numbers of 2-cell embryos.

Table S4. Production of chimeric clonal mice by aggregation with tetraploid embryos

Duration of freezing	Sex	Donor cell type	No. chimeric embryos	No. offspring, %
Three months	M	ntES	36	3 (8)