

Supplementary Information

Supplementary Fig. S1. (A) The DNA sequence of the targeting vector used to generate H3t-3× FLAG-knock-in mice. Green, coding region of H3t; light blue, linker sequence; orange, 3×FLAG DNA sequence. Mutations were introduced into the target vector to prevent the knock-in sequence from undergoing cleavage by the designed gRNA. The red lower-case bases indicate the location of mutation insertion, and the bracketed bases indicate the location of mutation deletion. **(B)** The knock-in allele was sequenced using primers described in the diagram. The arrows indicate the positions of primers, and the numbers indicate the positions on the sequence of the targeting vector noted in (A). Genomic DNA extracted from the tail of homozygous H3t-3×FLAG knock-in mice was subjected to PCR using KOD FX Neo DNA polymerase (Toyobo) and primers (a) and (b). Sequence analyses were performed using the FASMAC DNA Sequencing Service (Kanagawa, Japan). The sequence was divided into five regions using primers (a)–(e), and the target sequence was confirmed by alignment with ApE (A Plasmid Editor v2.0.52a) (blue bars). Original electrophoretic image Supplementary Fig. S2D.

Supplementary Fig. S2. Full-length / uncropped electrophoresis gel images. (A) Dotted white rectangle is used in Fig. 1B upper panel. WT, wild-type; Het, heterozygous knock-in; Homo, homozygous knock-in; M, molecular weight marker. **(B)** Dotted white rectangle is used in Fig. 1B lower panel. WT, wild-type; Het, heterozygous knock-in; M, molecular weight marker. **(C)** Dotted white rectangle is used in Fig. 1C. WT, wild-type; Het, heterozygous knock-in; M, molecular weight marker. **(D)** Dotted white rectangle is used in Supplementary Fig. S1B. WT, wild-type; Het, heterozygous knock-in; Homo, homozygous knock-in; M, molecular weight marker.

Supplementary Fig. S3. Full-length / uncropped electrophoresis gel images. Dotted black rectangles are used in Fig. 1D. WT, wild-type; KI, heterozygous knock-in.

Supplementary Fig. S4. H3t-FLAG is incorporated into the nucleosome in the testis of knock-in mice. (A) Extraction of mononucleosomes from the testes of wild-type and heterozygous knock-in mice. MNase-digested nucleosomes were fractionated by a

continuous sucrose gradient centrifugation. Nucleosomal DNAs were extracted and analyzed by agarose gel electrophoresis. Black squares indicate mononucleosome-containing fractions. **(B)** Immunoblot analysis of FLAG-tagged H3t proteins in mononucleosomes. Histones in the purified mononucleosome fractions prepared from the testes of wild-type and heterozygous knock-in mice were separated by SDS-PAGE and probed with anti-FLAG antibody (right). An anti-H3 antibody was used as a control (left).

Supplementary Fig. S5. Differences in testis size derived from the background differences in mice.

(A) The appearance of testes after five backcrosses of heterozygous H3t-3×FLAG knock-in mice to C57BL/6N background and then back to BDF1. Scale, 1 mm. WT, wild-type; KI Het, heterozygous knock-in; KO Homo, homozygous knockout. **(B)** Comparison of testicular weights of wild-type and heterozygous knock-in mice generated on several different backgrounds. Heterozygous FLAG-tagged H3t knock-in male mice backcrossed onto a C57BL/6N background exhibit significantly smaller testes. However, backcrossing these mice onto a BDF1 background partially restores the testicular weight. Statistical significance was determined using the Steel-Dwass test. Black dots represent individual results. WT, wild-type; Het, heterozygous knock-in. **, $P < 0.01$, ***, $P < 0.001$. **(C)** The number of pups generated upon mating wild-type or heterozygous FLAG-tagged H3t knock-in male mice. Heterozygous FLAG-tagged H3t knock-in male mice backcrossed onto a C57BL/6N background are completely sterile. Statistical significance was determined using the Steel-Dwass test. Black dots represent individual results. WT, wild-type; Het, heterozygous knock-in. ***, $P < 0.001$. **(D)** Haematoxylin- and eosin-stained images of testis sections from wild-type and heterozygous knock-in mice on C57BL/6N background. Elongating spermatids were observed in the seminiferous tubules of wild type but not in the knock-in mice, indicating that the knock-in mice are azoospermia (asterisks).

Supplementary Fig. S6. Full-length / uncropped electrophoresis gel images. Dotted red rectangles are used in Fig. 1E.

Supplementary Fig. S7. Full-length / uncropped CBB stained gel image. Dotted red rectangle is used in Fig. 4A.

Supplementary Table S1. A list of the type and number of peptides detected by mass spectrometry analysis. List of types and number of posttranslational modifications of each amino acid detected by mass spectrometry. *For peptides containing K18 and K23, we were able to distinguish whether the peptides were derived from H3t or canonical H3 by the presence or absence of V24.

		H3t KI					
		H3t-FLAG		Control		WT	
position	modification	number of peptide	abundance (%)	number of peptide	abundance (%)	number of peptide	abundance (%)
K9	un	1	14.3	1	11.1	0	0.0
	ac	2	28.6	1	11.1	2	18.2
	me	1	14.3	2	22.2	2	18.2
	me2	1	14.3	2	22.2	2	18.2
	me3	2	28.6	3	33.3	5	45.5
	ub	0	0.0	0	0.0	0	0.0
K14	un	0	0.0	0	0.0	0	0.0
	ac	11	100.0	15	100.0	15	100.0
	me	0	0.0	0	0.0	0	0.0
	me2	0	0.0	0	0.0	0	0.0
	me3	0	0.0	0	0.0	0	0.0
	ub	0	0.0	0	0.0	0	0.0
K18*	un	5	62.5	4	50.0	3	60.00
	ac	2	25.0	2	25.0	1	20.00
	me	1	12.5	2	25.0	1	20.00
	me2	0	0.0	0	0.0	0	0.00
	me3	0	0.0	0	0.0	0	0.00
	ub	0	0.0	0	0.0	0	0.00
K23*	un	1	9.1	1	7.7	1	9.1
	ac	10	90.9	12	92.3	9	81.8
	me	0	0.0	0	0.0	0	0.0
	me2	0	0.0	0	0.0	1	9.1
	me3	0	0.0	0	0.0	0	0.0
	ub	0	0.0	0	0.0	0	0.0
K27	un	2	6.9	3	9.1	3	7.1
	ac	3	10.3	8	24.2	10	23.8
	me	11	37.9	10	30.3	14	33.3
	me2	11	37.9	6	18.2	12	28.6
	me3	2	6.9	6	18.2	3	7.1
	ub	0	0.0	0	0.0	0	0.0
K36	un	20	51.3	24	58.5	41	68.3
	ac	2	5.1	1	2.4	0	0.0
	me	13	33.3	12	29.3	15	25.0
	me2	4	10.3	3	7.3	4	6.7
	me3	0	0.0	1	2.4	0	0.0
	ub	0	0.0	0	0.0	0	0.0
K37	un	9	56.3	7	41.2	9	33.3
	ac	1	6.3	0	0.0	1	3.7
	me	6	37.5	9	52.9	11	40.7
	me2	0	0.0	1	5.9	5	18.5
	me3	0	0.0	0	0.0	1	3.7
	ub	0	0.0	0	0.0	0	0.0
K56	un	4	57.1	3	50.0	5	50.0
	ac	3	42.9	3	50.0	4	40.0
	me	0	0.0	0	0.0	0	0.0
	me2	0	0.0	0	0.0	0	0.0
	me3	0	0.0	0	0.0	0	0.0
	ub	0	0.0	0	0.0	1	10.0
K79	un	28	45.2	21	36.8	20	22.2
	ac	3	4.8	2	3.5	3	3.3
	me	26	41.9	28	49.1	54	60.0
	me2	5	8.1	6	10.5	13	14.4
	me3	0	0.0	0	0.0	0	0.0
	ub	0	0.0	0	0.0	0	0.0
K122	un	19	86.4	17	89.5	24	92.3
	ac	3	13.6	2	10.5	2	7.7
	me	0	0.0	0	0.0	0	0.0
	me2	0	0.0	0	0.0	0	0.0
	me3	0	0.0	0	0.0	0	0.0
	ub	0	0.0	0	0.0	0	0.0