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Supplemental information

Deficiency of primate-specific *SSX1* induced asthenoteratozoospermia in infertile men and cynomolgus monkey and tree shrew models

Chunyu Liu, Wei Si, Chaofeng Tu, Shixiong Tian, Xiaojin He, Shengnan Wang, Xiaoyu Yang, Chencheng Yao, Cong Li, Zine-Eddine Kherraf, Maosen Ye, Zixue Zhou, Yuhua Ma, Yang Gao, Yu Li, Qiwei Liu, Shuyan Tang, Jiaxiong Wang, Hexige Saiyin, Liangyu Zhao, Liqun Yang, Lanlan Meng, Bingbing Chen, Dongdong Tang, Yiling Zhou, Huan Wu, Mingrong Lv, Chen Tan, Ge Lin, Qingpeng Kong, Hong Shi, Zhixi Su, Zheng Li, Yong-Gang Yao, Li Jin, Ping Zheng, Pierre F. Ray, Yue-Qiu Tan, Yunxia Cao, and Feng Zhang

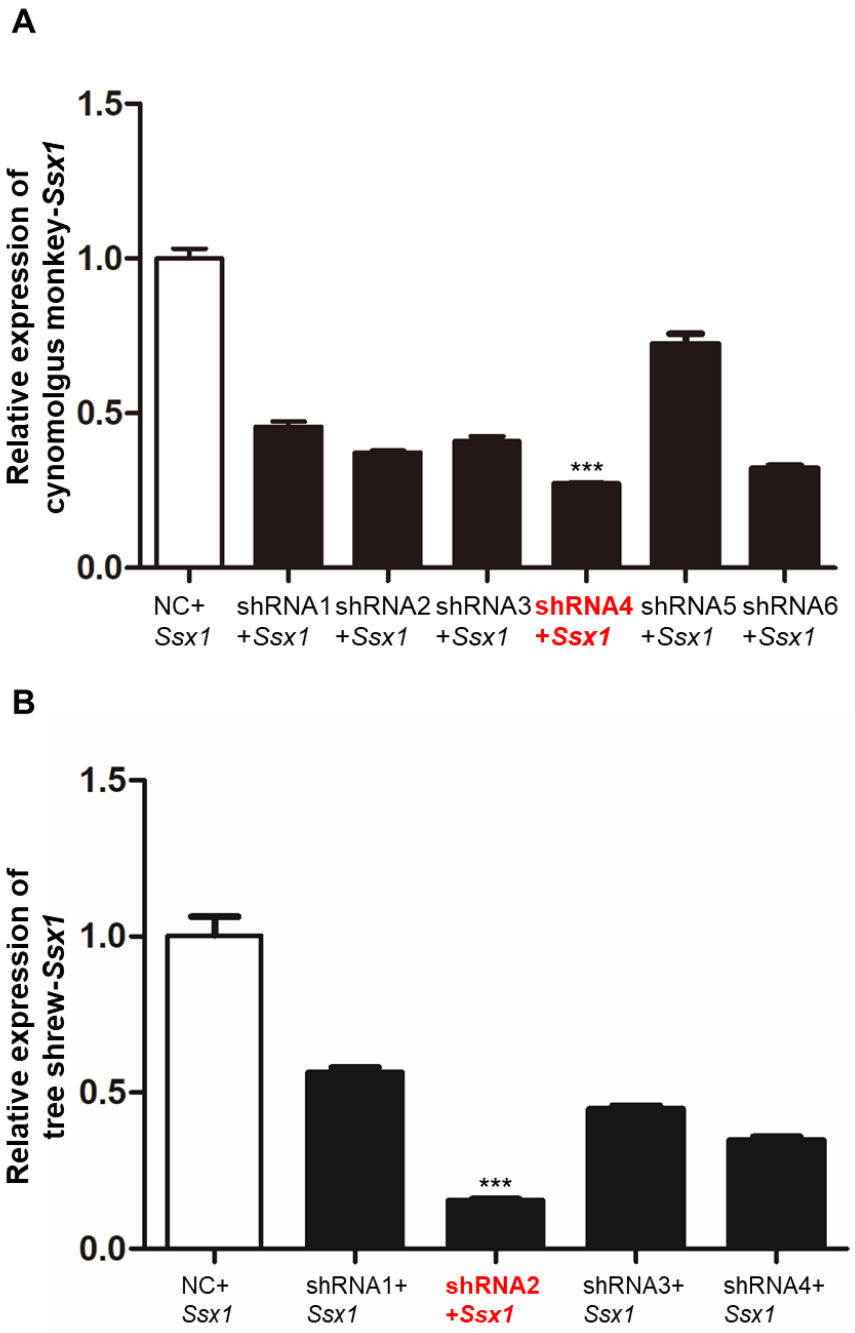

Figure S1. RT-qPCR analysis of knockdown efficacy for *Ssx1* by different shRNAs of cynomolgus monkey (A) and tree shrew (B). Data represent the means ± standard error of measurement (SEM) of three independent experiments. Two-tailed Student's paired or unpaired *t* tests were used as appropriate (***$P < 0.001$). NC, negative control.

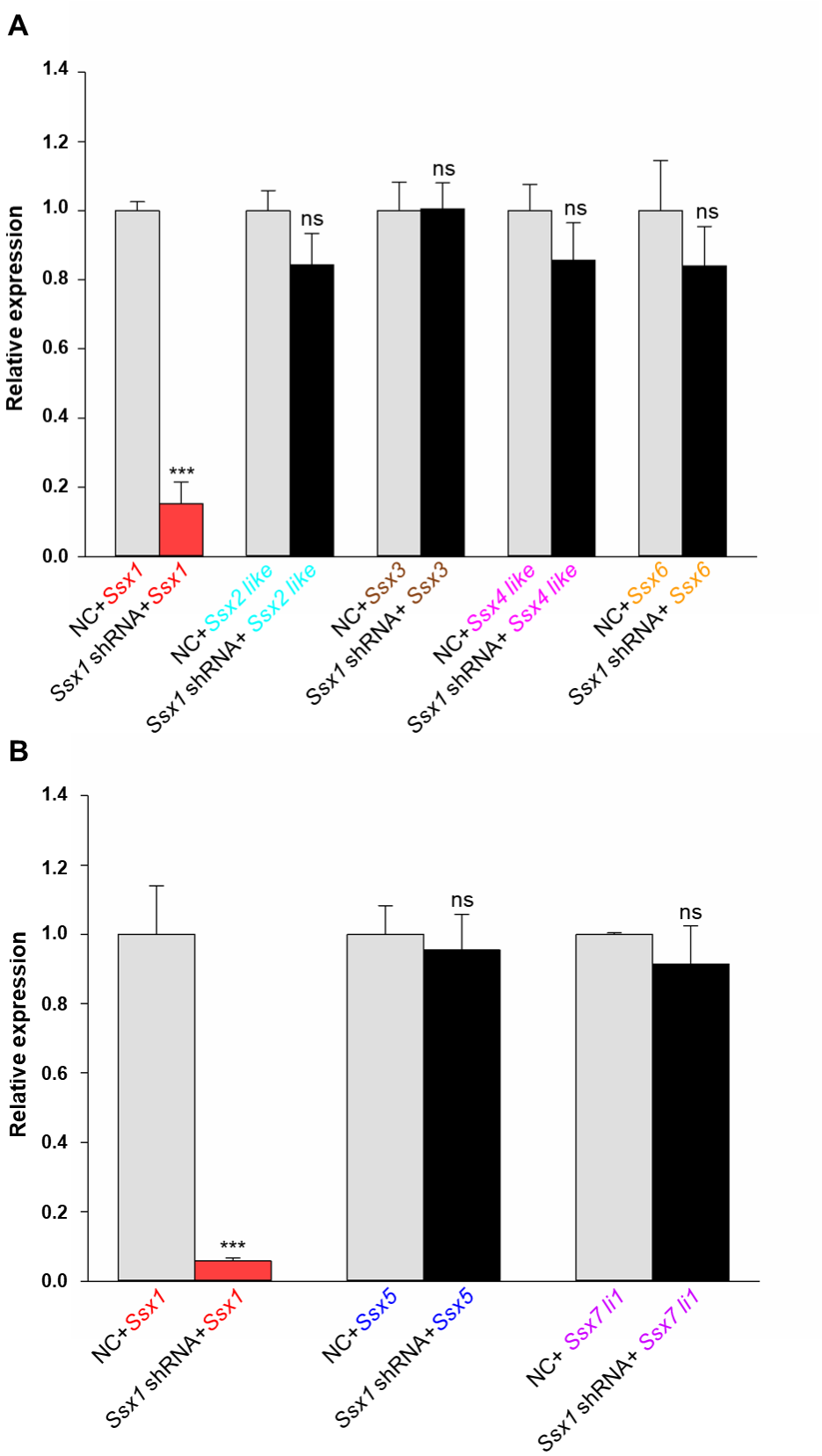

Figure S2. RT-qPCR analysis indicated the significantly reduced expressions of *Ssx1* in cynomolgus monkey (A) and tree shrew (B) models. Data represent the means ± standard error of measurement (SEM) of three independent experiments. Two-tailed Student's paired or unpaired *t* tests were used as appropriate (*** $P < 0.001$).

A

WT

Donor site predictions for NG_012528.2:5031-17083 :

Start	End	Score	Exon	Intron
9	23	0.94	aaaattg	gtgagaat
77	91	1.00	aaacaag	gtatgcct
121	135	0.51	aaaggag	gtatcggt
139	153	0.98	tgttccc	gtaagtga
151	165	0.71	tgaagag	gttggtaa
493	507	0.97	taactcc	gtaagtga
718	732	0.97	accccag	gtcagccc

***SSX1* splice-site mutation c.*4+1G>A**

Donor site predictions for NG_012528.2:5031-17083 :

Start	End	Score	Exon	Intron
9	23	0.94	aaaattg	gtgagaat
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139	153	0.98	tgttccc	gtaagtga
151	165	0.71	tgaagag	gttggtaa
718	732	0.97	accccag	gtcagccc

B

variant	gene	Δ type	Δ score ⓘ	pre-mRNA position ⓘ
X-48125827-G-A UCSC, gnomAD	SSX1 (ENSG00000126752.8_1 / ENST00000376919.4_1 / NM_005635.4, NM_001278691.2) biotype: protein coding canonical transcript OMIM, GTEx, gnomAD, ClinGen, Ensembl, Decipher, GeneCards	Acceptor Loss	0.00	
		Donor Loss	1.00	-1 bp
		Acceptor Gain	0.00	
		Donor Gain	0.01	3 bp

C

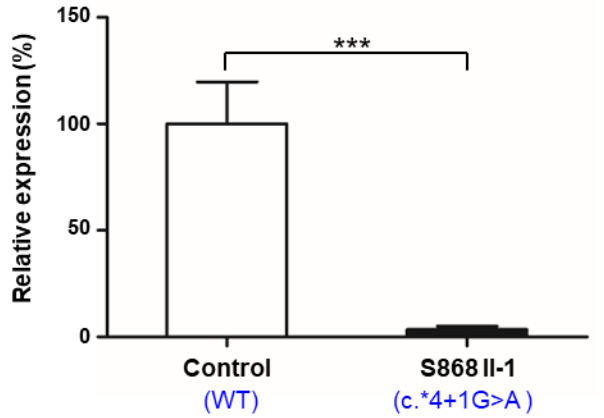

Figure S3. The effect of the mutated site (c.*4+1G>A) on the splicing of *SSX1* mRNA.

(A) A prediction for *SSX1* c.*4+1G>A using the online tool of Splice Site Prediction by Neural Network (NNSPLICE 0.9) at http://www.fruitfly.org/seq_tools/splice.html. The red box represents the missing donor site caused by the splicing variant c.*4+1G>A in *SSX1*.

(B) A prediction for *SSX1* c.*4+1G>A using the SpliceAI tool (a 32-layer deep neural network to predict splicing from a pre-mRNA sequence; https://spliceailookup.broadinstitute.org/). The delta score higher than 0.8 indicated the prediction of a donor loss with high precision.

(C) Expression analysis of *SSX1* in the spermatozoa from men harboring the hemizygous splicing variant c.*4+1G>A in *SSX1*. The primers used for RT-qPCR assay were designed between exons 4 and 6 of *SSX1* (Transcript ID: ENST00000376919.3). Data represent the means ± standard error of measurement (SEM) of three independent experiments. Two-tailed Student's paired or unpaired *t* test was used as appropriate (***$P < 0.001$).

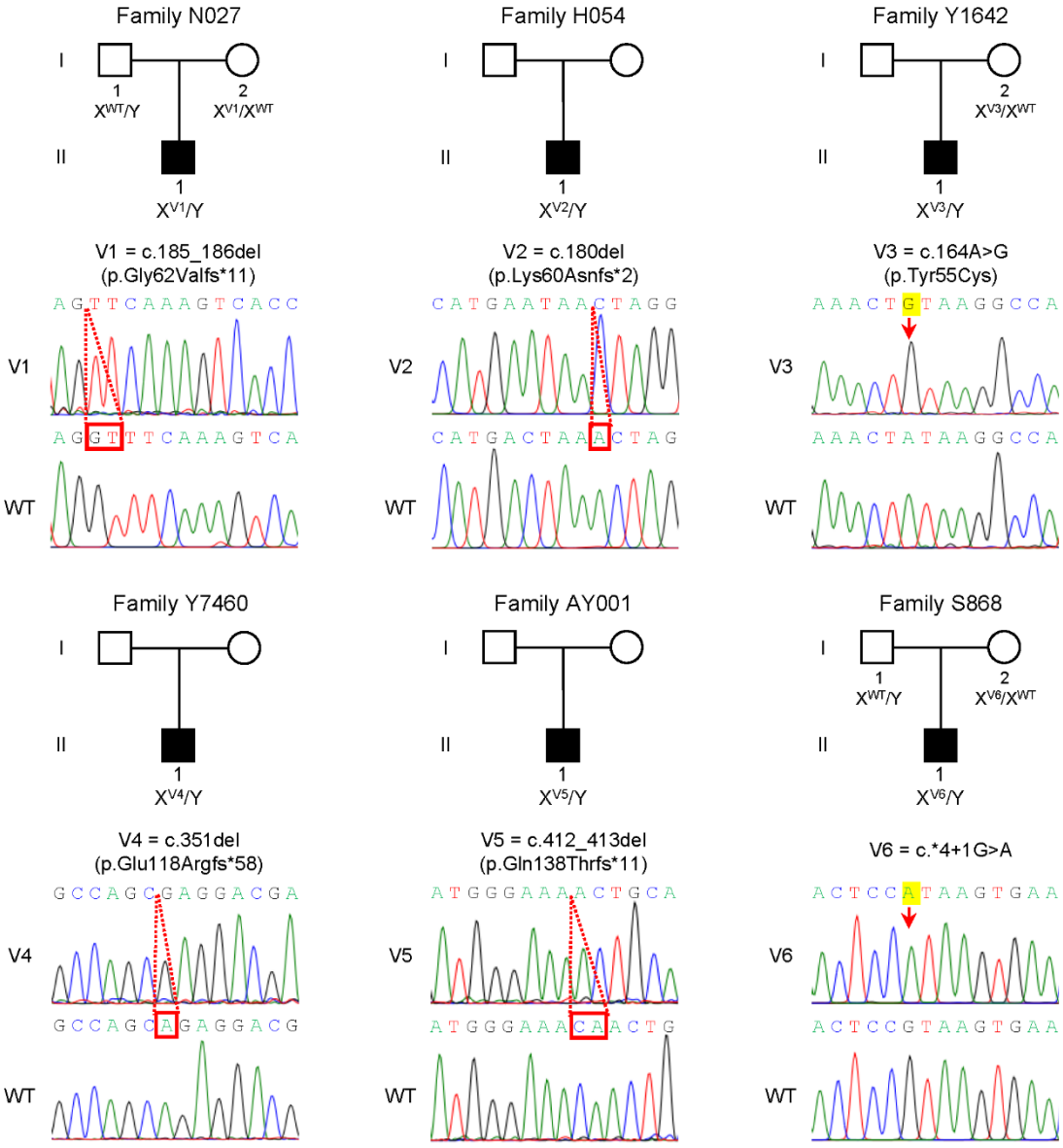

Figure S4. Sanger sequencing confirmed hemizygous *SSX1* variants (V1–V6) in infertile men N027 II-1, H054 II-1, Y1642 II-1, Y7460 II-1, AY001 II-1 and S868 II-1.

The positions of the variants are indicated by red arrows or boxes. Abbreviations: V1, variant 1; V2, variant 2; V3, variant 3; V4, variant 4; V5, variant 5; V6, variant 6; WT, wild type.

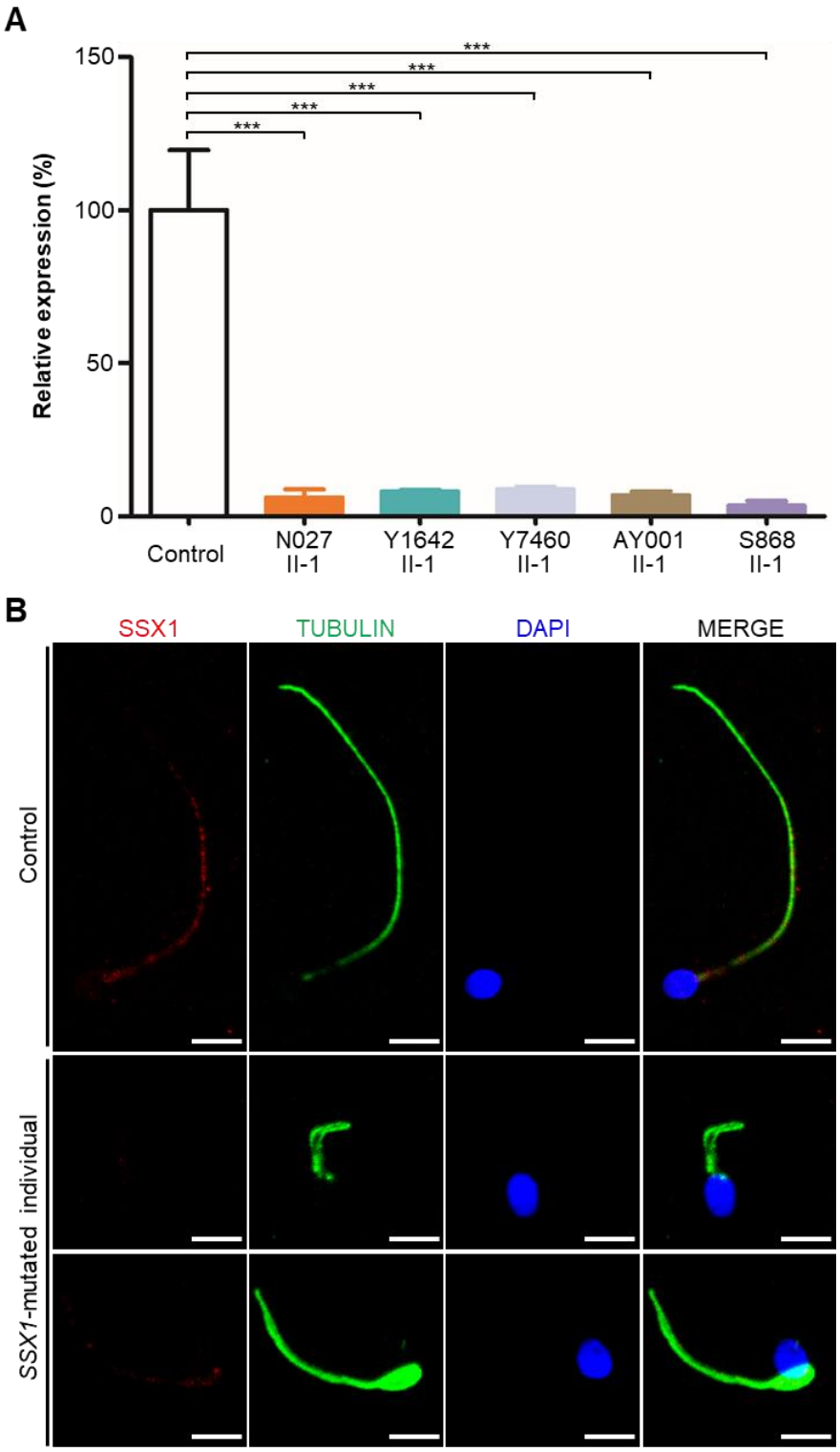

Figure S5. Analysis of *SSX1* mRNA and protein levels in the spermatozoa from a male control and men harboring hemizygous *SSX1* variants.

(A) RT–qPCR analysis indicated that the abundance of *SSX1* mRNA was dramatically reduced in the spermatozoa from men harboring hemizygous *SSX1* variants when compared to that in the spermatozoa from a control male. The data are presented as the mean ± standard error of three independent experiments. Two-tailed Student's paired or unpaired *t* tests were used as appropriate (***$P < 0.001$).

(B) IF staining of SSX1 in the spermatozoa from a male control individual and men harboring hemizygous *SSX1* variants. SSX1 staining (red) is concentrated at the midpiece and principal piece of sperm flagella from the control individual but absent in the sperm flagella from men harboring hemizygous *SSX1* variants. Sperm flagella were stained with anti-α-tubulin (green) antibodies, and DNA was counterstained with DAPI as a nuclear marker. Scale bars: 5 μm. The data of subject N027 II-1 were used as an example.

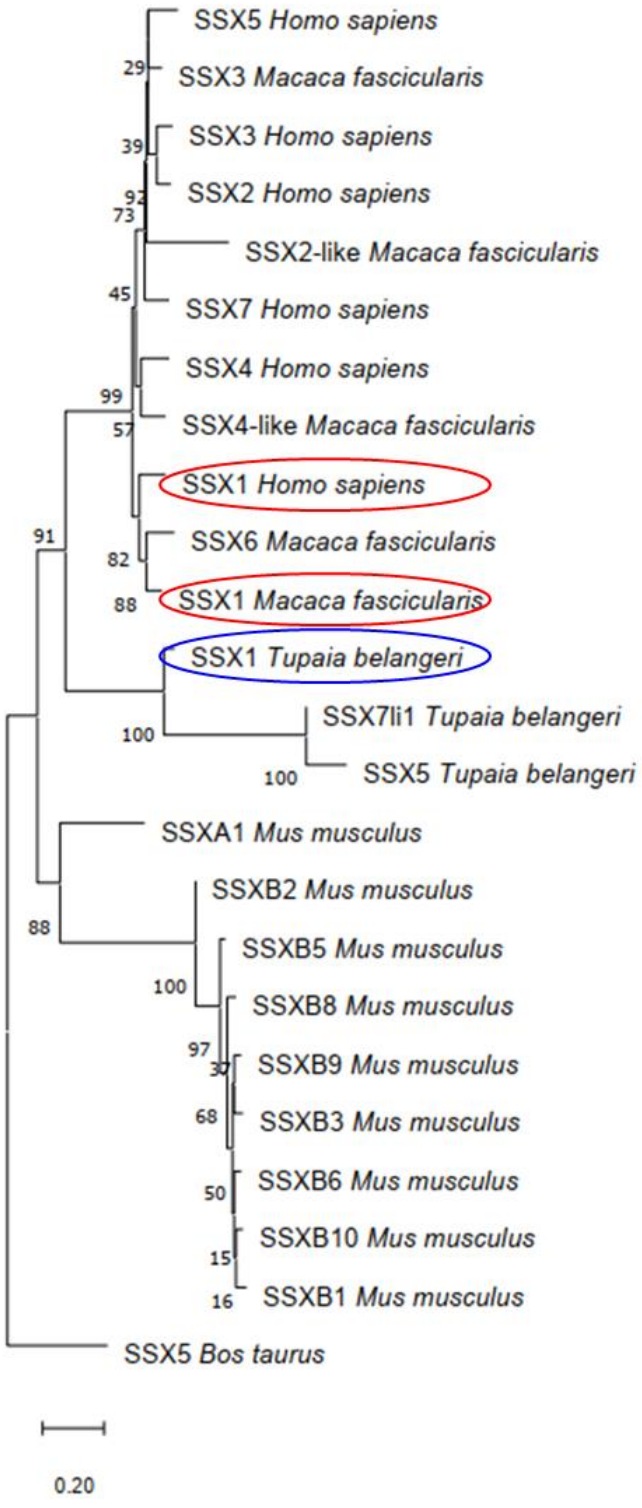

Figure S6. Phylogenetic tree of SSX family members from mouse (*Mus musculus*), tree shrew (*Tupaia belangeri*), cynomolgus monkey (*Macaca fascicularis*) and human (*Homo sapiens*) with the Neighbor-Joining algorithm using *Bos taurus* SSX5 as an outgroup. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

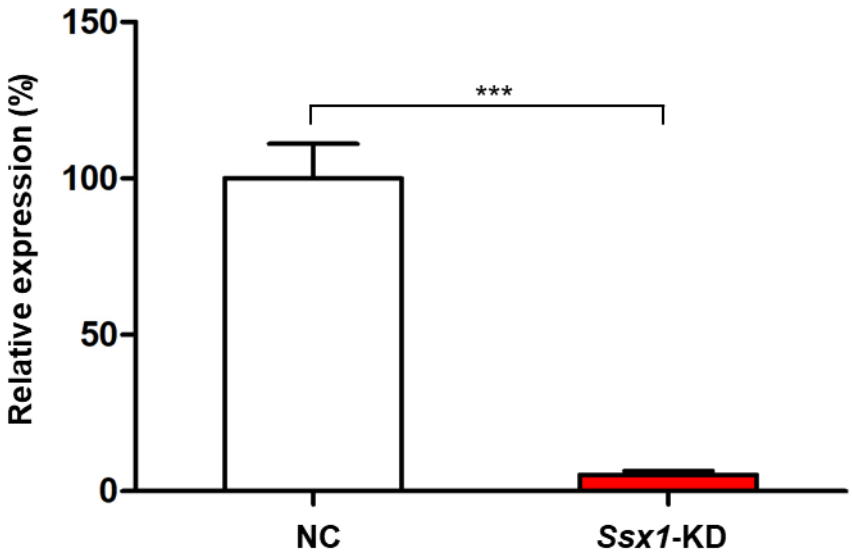

Figure S7. Expression analysis of *Ssx1* mRNA in the testes of negative control (NC) and *Ssx1*-KD male cynomolgus monkeys.

RT-qPCR analysis indicated that the abundance of *Ssx1* mRNA was dramatically reduced in the testes of *Ssx1*-KD male cynomolgus monkeys when compared to that of NC group. Data represent the means ± SEM of three independent experiments. Two-tailed Student's paired or unpaired *t* test was used as appropriate (***$P < 0.001$).

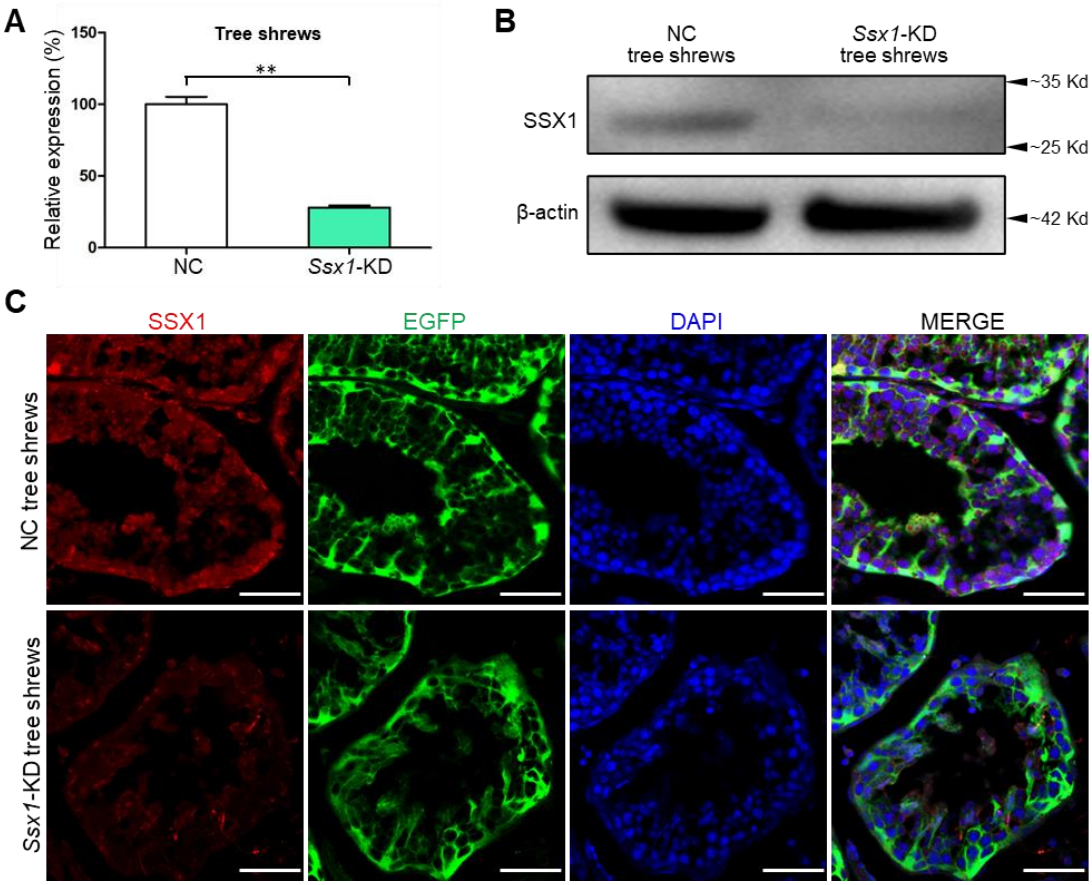

Figure S8. Detection of the efficacy of *Ssx1* expression KD in the testes of adult male tree shrews.
(A) RT–qPCR analysis revealed a significant reduction of *Ssx1* mRNA abundance in the testes of *Ssx1*-KD male tree shrews when compared to that in the testes of negative control (NC) male tree shrews. The data are presented as the mean ± standard error of three independent experiments. Two-tailed Student's paired or unpaired *t* tests were used as appropriate (**$P < 0.01$).
(B) Immunoblotting assays indicated that SSX1 was dramatically reduced in the testes from *Ssx1*-KD male tree shrews. β-actin was used as a loading control.
(C) IF staining of SSX1 in the testes from NC male tree shrews and *Ssx1*-KD male tree shrews. SSX1 immunostaining (red) was mainly concentrated in the spermatogonia or spermatocytes from NC male tree shrews but was dramatically decreased in testicular sections from *Ssx1*-KD male tree shrews. DNA was counterstained with DAPI as a nuclear marker. Scale bars: 50 μm.

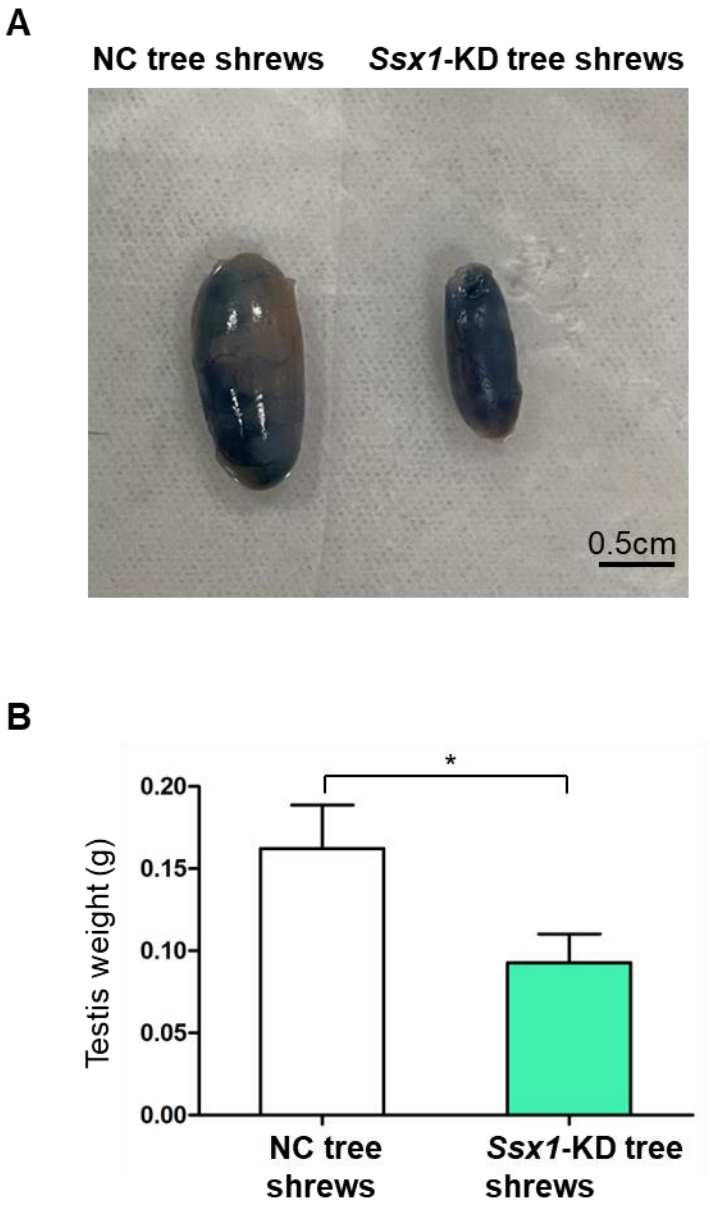

Figure S9. Testis sizes and weights in negative control (NC) and *Ssx1*-KD male tree shrews.
(A) Testis sizes in tree shrew models.
(B) Reduced testis weights were observed in *Ssx1*-KD male tree shrews when compared to those in NC group. For each group, at least five tree shrews were counted and the data represent the means ± SEM; *$P < 0.05$.

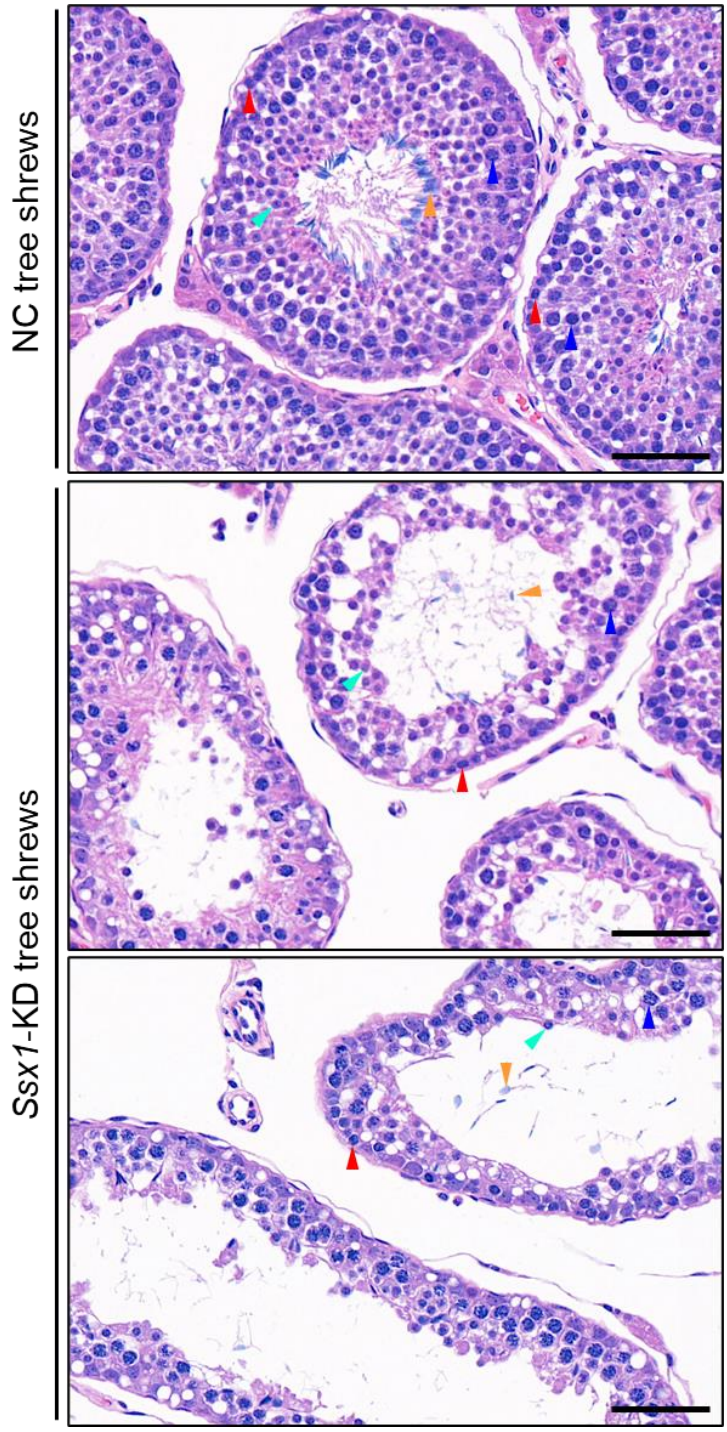

Figure S10. H&E staining of testicular tissue sections obtained from *Ssx1*-KD male tree shrews. All germ cells were regularly arranged in the seminiferous epithelia of negative control (NC) male tree shrews but displayed a loss or disordered arrangement in the testes from *Ssx1*-KD male tree shrews; spermatogonia (red arrowheads), spermatocytes (dark blue arrowheads), round spermatids (light blue arrowheads) and spermatozoa (orange arrowheads). Scale bars: 50 μm.

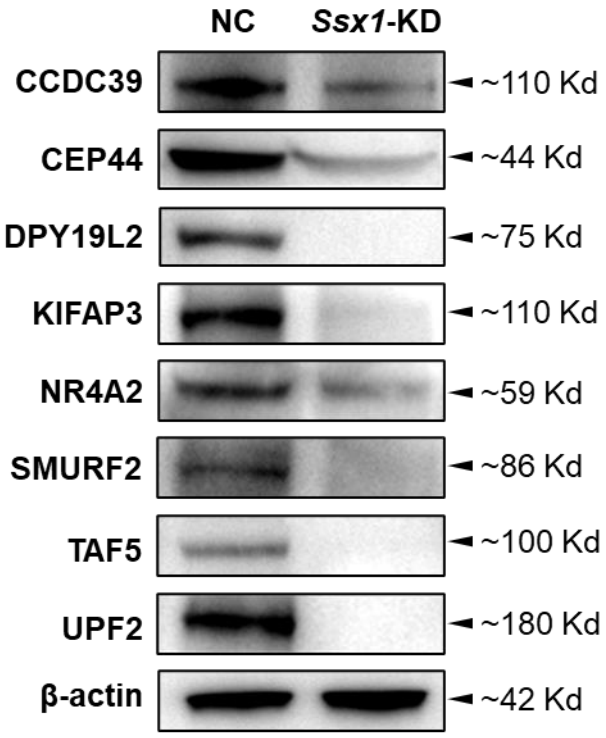

Figure S11. **Western blotting assay revealed significantly reduced levels of multiple selected proteins in the testes from *Ssx1*-KD tree shrews**. β-actin was used as a loading control. NC, negative control.

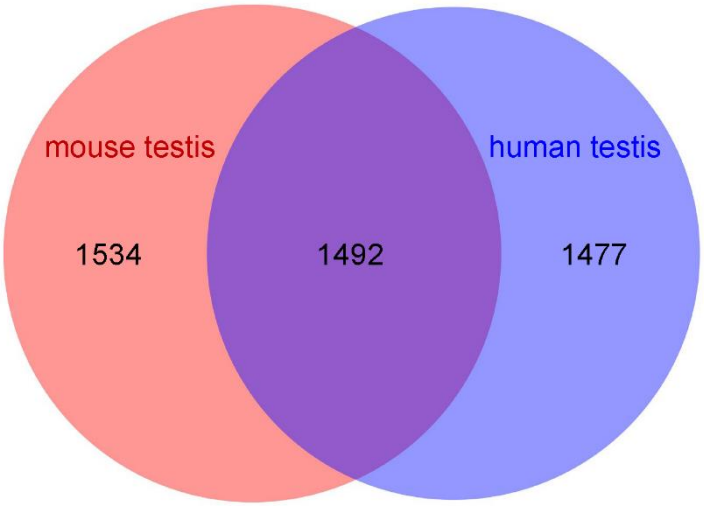

Figure S12. Analysis of the evolutionary divergence of human and mouse testis-enriched genes. The number "1492" represents the number of testis-enriched genes that are evolutionarily conserved between human and mouse. The number "1477" represents the number of human-specific testis-enriched genes, which do not have one-to-one orthologs in mouse or the mouse orthologs of which are not testis-enriched. The number "1534" represents the number of mouse-specific testis-enriched genes, which do not have one-to-one orthologs in human or the human orthologs of which are not testis-enriched.

Primer name	Primer sequence (5'-3')	Tm
V1-F	CAATCCTTCTGCCCAGCCG	56°C
V1-R	AGGTAGCTGAGCTGAAAAGCAAT	
V2-F	AGGCCTTTGATGATATTGCCAC	56°C
V2-R	CCACAGAGACAGCTGGGCTG	
V3-F	TTCTGAGGAGGGGAGGAC	52°C
V3-R	AAAGGAAAATGTGGGGTA	
V4-F	TGTTTTGTCCCCTCCCTA	53°C
V4-R	AGAATCGCTTGAGCACCT	
V5-F	GTCCCCTCCCTATGTTTT	50°C
V5-R	GATTTAATTTAATGCCTCC	
V6-F	AGGTTGGTAATCTAAACGTC	50°C
V6-R	CCACTGCCCAATAACTCA	

Table S1. Primers used for amplification and verification of *SSX1* variants

Species and locus	Sequence (5'-3')
Cynomolgus monkey-shNC	Top strand: gatccgTTCTCCGAACGTGTCACGTAAttcaagagaTTACGTGAC ACGTTCGGAGAAttttttc
	Bottom strand: aattgaaaaaaTTCTCCGAACGTGTCACGTAAtctcttgaaTTACGTG ACACGTTCGGAGAAcg
Cynomolgus monkey-sh*Ssx1*	Top strand: aattcGCCACAGGAATCAGGTTGAACGTCCTttcaagagaAGGA CGTTCAACCTGATTCCTGTGGttttttg
	Bottom strand: gatccaaaaaaCCACAGGAATCAGGTTGAACGTCCTtctcttgaaAG GACGTTCAACCTGATTCCTGTGGCg
Tree shrew-shNC	Top strand: gatccgTTCTCCGAACGTGTCACGTAAttcaagagaTTACGTGAC ACGTTCGGAGAAttttttc
	Bottom strand: aattgaaaaaaTTCTCCGAACGTGTCACGTAAtctcttgaaTTACGTG ACACGTTCGGAGAAcg
Tree shrew-sh*Ssx1*	Top strand: aattcGCCTTCAACGATATTTCCAAATACTttcaagagaAGTATT TGGAAATATCGTTGAAGGCttttttg
	Bottom strand: gatccaaaaaaGCCTTCAACGATATTTCCAAATACTtctcttgaaAG TATTTGGAAATATCGTTGAAGGCg

Table S2. Sequences of the shRNAs used for knocking down *Ssx1* in male cynomolgus monkeys and tree shrews

Uppercase and lowercase letters indicate the siRNA sequence and shRNA skeleton, respectively, for each shRNA.

Primer name	Primer sequence (5'-3')	Tm
Human-*SSX1*-F	TCACCCTCCCACCTTTCA	60°C
Human-*SSX1*-R	TCGTCCTCTGCTGGCTTC	
Human-*GAPDH*-F	GGAGCGAGATCCCTCCAAAAT	60°C
Human-*GAPDH*-R	GGCTGTTGTCATACTTCTCATGG	
Monkey-*Ssx1*-F	ACCGTAACCACAGGAATC	60°C
Monkey-*Ssx1*-R	ATCTTCTCAGAGGTATTTGC	
Monkey-*actin*-F	ACGTGGACATCCGTAAAG	60°C
Monkey-*actin*-R	GGGCCAGACTCGTCATAC	
Tree shrew-*Ssx1*-F	CCGCAGAAAAGACACTCG	60°C
Tree shrew-*Ssx1*-R	CGGCACGGTAGTTTGGAG	
Tree shrew-*actin*-F	ATTTTGAATGATCAGCCACC	58°C
Tree shrew-*actin*-R	AGGTAAGCCCTGGCTGCCTC	

Table S3. Primers used for RT-qPCR assays

Primer name	Primer sequence (5'-3')	Tm
TS-*Alms1* F	AAAGGCAGTGACTAAGGTT	49°C
TS-*Alms1* R	GACAGAATGAGTAGTATTTTCG	
TS-*Cep44* F	AGAATGACTTGCGCTTTA	48°C
TS-*Cep44* R	ACTACTGAGTTCCTTGTGCT	
TS-*Cep170* F	AAGTGCCAAAAGCATAGA	50°C
TS-*Cep170* R	CATCCACCTCATCATCCC	
TS-*C2cd3* F	TCAAAACGGATGGAAAAG	51°C
TS-*C2cd3* R	TCCTTGGTAGGTGGGTTA	
TS-*Ccdc39* F	CATTGGAAGCCTGGTTAG	50°C
TS-*Ccdc39* R	TACGAAAATCCTGTGCTG	
TS-*Cep164* F	TGACGAACACTATCGGAACT	53°C
TS-*Cep164* R	CGCTTCTAGCCTGCATCT	
TS-*Cfap58* F	ATCAAAAGGCAGAAGTGG	58°C
TS-*Cfap58* R	TATCTGTTTCTGGGTCGC	
TS-*Cmtr2* F	CAAAGGGTACTTCAATAGTTG	49°C
TS-*Cmtr2* R	ATGGAGACAGAAAACGGA	
TS-*Dpy19l2* F	AGCCTCTGAGCCAAGTGC	53°C
TS-*Dpy19l2* R	CGAGGAATGGGTAGGAGA	
TS-*Fndc3a* F	ACCTGGTTTTATTCCTGTC	49°C
TS-*Fndc3a* R	GTGATGATGGTGGACTGC	
TS-*Ica1l* F	GCACGCACGGAATACAGA	55°C
TS-*Ica1l* R	TCCCAGAATCCGAGCAGT	
TS-*Iqce* F	TTTCCACAAACCTCCACC	53°C
TS-*Iqce* R	ACGCCTGAGATTTGACCT	
TS-*Kifap3* F	ACAAGCCTAAAGACCCAC	49°C
TS-*Kifap3* R	TCAACACTCTGTTTCCAGTC	
TS-*Lrrcc1* F	AGAAGACCGAAATCATTAA	48°C
TS-*Lrrcc1* R	TTTTAGCCTGTCTGTGGTA	
TS-*Nr4a2* F	CGCACATGATCGAGCAGA	57°C
TS-*Nr4a2* R	GTTGGGCACAGCGAAGGT	
TS-*Smurf2* F	TCCTCCAGACCTACCAGA	49°C
TS-*Smurf2* R	ATTACGAATCTCCCATCC	
TS-*Taf5* F	GATGGTGGGAAGTTTGGC	54°C
TS-*Taf5* R	GCGTACTCGTTTGGTGGT	
TS-*Ttll10* F	GCCCATTCTTCTACATCG	52°C
TS-*Ttll10* R	CACTTGACCTCGCACCAT	
TS-*Upf2* F	TTTCCTCCAAGTGAAATAA	47°C
TS-*Upf2* R	ATCCAAAAGGTCTGCTAA	
TS-*Usp1* F	AATAAGCAACCCAGCATT	52°C
TS-*Usp1* R	ACATTCCAAGCAGCGAGT	
TS-*Usp47* F	ATTTACCAGCATCTACTCCA	49°C
TS-*Usp47* R	GAACACTATCGTCTACAGCA	
TS-*Wdpcp* F	AACTGGGATACTATGGGC	50°C
TS-*Wdpcp* R	CAGAAGTGGTCTTGTGGG	

Table S4. Primers used for the verification of RNA-seq assays