Table S1. CRISPR/Cas-Mediated Quintuple Gene Targeting in V6.5 mES cells,

Related to Table 1

Mutant genes	***Tet1, 2, 3***	***Tet1, 2, 3 + Sry***	***Tet1, 2, 3 + Sry + Uty***
No. Mutant alleles	6 and more	7	8
No. Mutant clones / Total clones	54/96	37/96	10/96

Plasmids encoding Cas9 and five PCR products expressing sgRNAs targeting *Tet1*, *Tet2*, *Tet3*, *Sry*, and *Uty* were co-transfected into mES cells. The number of clones containing mutations in all six *Tet* alleles is listed in the *Tet1, 2, 3* column; the number of clones containing mutations in all six *Tet* alleles and *Sry* allele is listed in the *Tet1, 2, 3 + Sry* column; the number of clones containing mutations in all six *Tet* alleles and both *Sry* and *Uty* allele is listed in the *Tet1, 2, 3 + Sry +Uty* column.

The increased efficiency of generating *Tet1, 2, 3* triple targeted mES clones in this quintuple targeting experiment, compared to the triple targeting experiment (Table 1), is likely due to the use of short PCR products instead of plasmids that express sgRNAs. The much smaller size of pooled PCR products may lead to more efficient delivery into transfected cells.

Table S2. Potential Off Targets of *Tet1* and *Tet2* sgRNAs, Related to Table 2

MatchName	**Coordinate (mm9)**	**Strand**	**SEEDPAM**	**Gene**
Tet1				
Tet1_1_TGG_3	chr10:62296293-62296308	-	ggctgctGTCAGGGAGCTCATGG	Tet1
Tet1_1_AGG_1	chr16:8891779-8891794	+	ctgtttgGTCAGGGAGCTCAAGG	33kb 3' of 1810013L24Rik
Tet1_1_AGG_2	chr18:75130318-75130333	-	gggccaaGTCAGGGAGCTCAAGG	9.4kb 5' of Lipg
Tet1_1_GGG_4	chr2:36287584-36287599	+	gtttagtGTCAGGGAGCTCAGGG	9.8kb 3' of Olfr339
Tet2				
Tet2_1_AGG_2	chr3:133148617-133148632	-	gaaagtgCCAACAGATATCCAGG	Tet2
Tet2_1_AGG_1	chr2:120696599-120696614	+	gcaaagaCCAACAGATATCCAGG	Intron of Ubr1
Tet2_1_CGG_3	chr10:95206326-95206341	+	aggaaacCCAACAGATATCCCGG	2.4kb 5' of AK169506
Tet2_1_TGG_4	chr19:39098539-39098554	+	ccacctcCCAACAGATATCCTGG	Intron of Cyp2c55
Tet2_1_TGG_5	chr15:59188892-59188907	+	gagataaCCAACAGATATCCTGG	Intron of E430025E21Rik

Table S3. Oligonucleotides Used in This Study, Related to Experimental Procedures

Oligonucleotides used for cloning sgRNA expression vector

Gene target	**Direction**	**Sequence (5' to 3')**
Tet1	F	CACCGGCTGCTGTCAGGGAGCTCA
	R	AAACTGAGCTCCCTGACAGCAGCC
Tet2	F	CACCGAAAGTGCCAACAGATATCC
	R	AAACGGATATCTGTTGGCACTTTC
Tet3	F	CACCGAAGGAGGGGAAGAGTTCTCG
	R	AAACCGAGAACTCTTCCCCTCCTTC
Sry	F	CACCGCATTTATGGTGTGGTCCCG
	R	AAACCGGGACCACACCATAAATGC
Uty	F	CACCGTTTCTTTTCCTCATTACCTA
	R	AAACTAGGTAATGAGGAAAAGAAAC

Oligonucleotides used for Suveryor assay and RFLP analysis

Gene target	**Direction**	**Sequence (5' to 3')**
Tet1	F	TTGTTCTCTCCTCTGACTGC
	R	TGATTGATCAAATAGGCCTGC
Tet2	F	CAGATGCTTAGGCCAATCAAG
	R	AGAAGCAACACACATGAAGATG
Tet3	F	CCACCTCTGAGCGCAGAGTG
	R	GATGAACACAGTTCCTGACAG
Sry	F	GTCTGTCTTTGTCTGTCTGTC
	R	GGGTATTTCTCTCTGTGTAGG
Uty	F	GAGTTCTTCTTGCGTTCACC
	R	AATGAGCACTTTCAGAGTAGG

Oligonucleotides used for making template for in vitro transcription

Template	**Direction**	**Sequence (5' to 3')**
Cas9	F	TAATACGACTCACTATAGGGAGAATGGACTATAAGGACCACGAC
	R	GCGAGCTCTAGGAATTCTTAC
Tet1 sgRNA	F	TTAATACGACTCACTATAGGCTGCTGTCAGGGAGCTC
	R	AAAAGCACCGACTCGGTGCC
Tet2 sgRNA	F	TTAATACGACTCACTATAGGAAAGTGCCAACAGATATCC
	R	AAAAGCACCGACTCGGTGCC
Tet3 sgRNA	F	TTAATACGACTCACTATAGGAAGGAGGGGAAGAGTTCTCG
	R	AAAAGCACCGACTCGGTGCC

Oligonucleotides used for HDR-mediated repair through embryo injection

Gene target	**Sequence (5’ to 3’)**
Tet1	aaagaaaaaggcccatattatacacaccttggggcaggaccaagtgtggctgctgtcaggGAatTCatggagactaggtgaggaactctgcttcccgctaacccattcttcccggtgacctggctc
Tet2	tcactctgtgactataaggctctgactctcaagtcacagaaacacgtgaaagtgccaacaGAatTCcaggctgcagaatcggagaaccacgcccgagctgcagagcctcaagcaaccaaaagcaca