Molecular Therapy
Letter to the Editor

Targeted Deletion of an Entire Chromosome Using CRISPR/Cas9

The recent emergence of gene editing technologies, in particular CRISPR/Cas, has enabled rapid generation of disease models and provides a novel approach for the treatment of monogenic disorders through correction of disease-causing mutations.[1,2] In contrast, the therapeutic potential of CRIPSR/Cas technology for aneuploidies, such as Down syndrome (Trisomy 21), remains unexplored. Indeed, disorders that are caused by supernumerary chromosomes represent a significant challenge, because genetic correction requires targeted ablation of an entire chromosome, which, to our knowledge, has not been demonstrated using genome editing technology.[1]

To assess the potential of CRISPR/Cas technology to effect chromosomal loss, we investigated the hypothesis that simultaneous generation of multiple DNA double-strand breaks (DSBs) at targeted chromosomal locations can induce directed chromosomal deletion.[3] We selected the 90 Mb acrocentric mouse Y chromosome for deletion because loss of this chromosome does not overtly impact cell/mouse viability and it is only present in one copy in male cells, thus facilitating screening.[4]

Our first strategy used CRISPR/Cas to fragment the centromere, which is indispensable for chromosome segregation during mitosis.[5] We screened the 90 kb Y centromere for guide RNA (gRNA) sequences in repetitive elements that would enable targeted cleavage at multiple sites. We identified two gRNA candidates that target the centromere 140 or 41 times (centro 140X and centro 41X, respectively; Figure 1A). For comparison, we also tested a gRNA pair targeting two unique sequences immediately flanking the centromere (centro 2X; Figure 1A). Cas9 and single-guide RNA (sgRNA) were expressed in R1 XY mouse embryonic stem cells (ESCs) using plasmid PX459 V.2, followed by transient puromycin selection, to ensure only transfectants were harvested.[6] Quantification of Y chromosome dosage was performed by genomic qPCR amplification of *Uba1y* and *Erdr1*, genes located at the end of the Y chromosome short and long arm, respectively (Figure 1A). Strikingly, *Uba1y* and *Erdr1* qPCR signal was reduced by 80%–85% for both centro 140X and centro 41X compared with the sgRNA-expressing negative control (Neo-gRNA; Figure 1B). Further, a reduction of ~40% was achieved using the centro 2X gRNA (Figure 1B). To confirm that the reduction of qPCR signal was caused by Y chromosome loss, we performed fluorescence in situ hybridization (FISH) using Y chromosome paint on centro 41X-treated samples. Consistent with the qPCR data, the Y chromosome was not detected in 90% of centro 41X cells compared to 13% of control cells (Figure 1C). We also noted that 6% of control cells had two Y chromosomes, and this was reduced to less than 1% in centro 41X-treated cells. These findings confirm that CRISPR/Cas-mediated centromere cleavage leads to Y chromosome loss at high efficiency.

Next, we tested an alternative strategy for chromosome deletion in which the long arm is targeted for fragmentation by cleavage at multiple sites. As this approach does not target the centromere, it has potential for application in both dividing and non-dividing cells. We again identified gRNAs that targeted repetitive sequences in the Y chromosome (Figure 1A). However, the selected gRNAs sequences were specific to the long arm to ensure the centromere was left intact. Expression of sgRNAs that targeted the long arm 298X, 116X, 45X, 8X, and 2X resulted in *Uba1y* qPCR signal loss of 69%, 40%, 26%, 27%, and 3%, respectively, and *Erdr1* qPCR signal loss of 82%, 68%, 68%, 52%, and 27%, respectively (Figure 1B). These data indicate that targeted fragmentation of a chromosomal arm can induce chromosome deletion and the frequency of deletion is proportional to the number of cuts. Notably, apart from long arm 298X *Edr1*, all long gRNAs resulted in significantly higher *Uba1y* and *Edr1* signals than the centro 41X and 140X gRNAs (Table S3). Given that *Uba1y* qPCR signal was significantly higher than *Erdr1* for all long gRNAs (Table S3), we speculate that fragmentation of the long arm occasionally results in chromosome truncation or translocation, with retention of the Y short arm sequence containing *Uba1y*. FISH Y painting analysis in 298X-treated samples revealed 95% of cells contained no Y chromosome signal, confirming that the long arm fragmentation strategy was indeed effective (Figure 1C).

Notably, the degree of Y chromosome depletion induced by 8X and 45X are similar. This is significant, because targeted deletion of potentially any chromosome could be achieved relatively easily by transfection of a single vector expressing eight unique gRNAs.[7] We were also impressed with the activity of the long arm 2X gRNA. Although this gRNA induced negligible loss of short arm signal (3%), it appears to truncate the Y long arm relatively efficiently based on an *Erdr1* qPCR signal loss of 27%.

Having successfully deleted an entire chromosome in vitro, we next tested our centromere deletion strategy in vivo in mouse zygotes with the expectation that successful Y chromosome deletion in male zygotes would result in an XO female phenotype.[4] We selected gRNA centro 41X due to its high efficiency in vitro and low off-target prediction (Table S2).[8]

After zygote injection of centro 41X gRNA and Cas9 mRNA, we collected 27 E15.5 embryos, of which 11 were phenotypically male and 16 were female based on gonadal assessment (Figure S1). We then screened the female embryos for X chromosome dosage and identified five embryos with only one X chromosome (Figures 1D and 1E). No evidence of XO karyotype was detected in control females injected with autosomal targeted gRNAs (Figure S2).

To directly assess Y chromosome loss in the five single X females, we performed Y chromosome genomic qPCR. Y short and long arm signals were undetectable in two of these embryos, indicating an XO karyotype. The remaining three embryos contained approximately 50% Y short arm signal and no long arm signal, suggesting that these mice were mosaic, with half of the cells containing

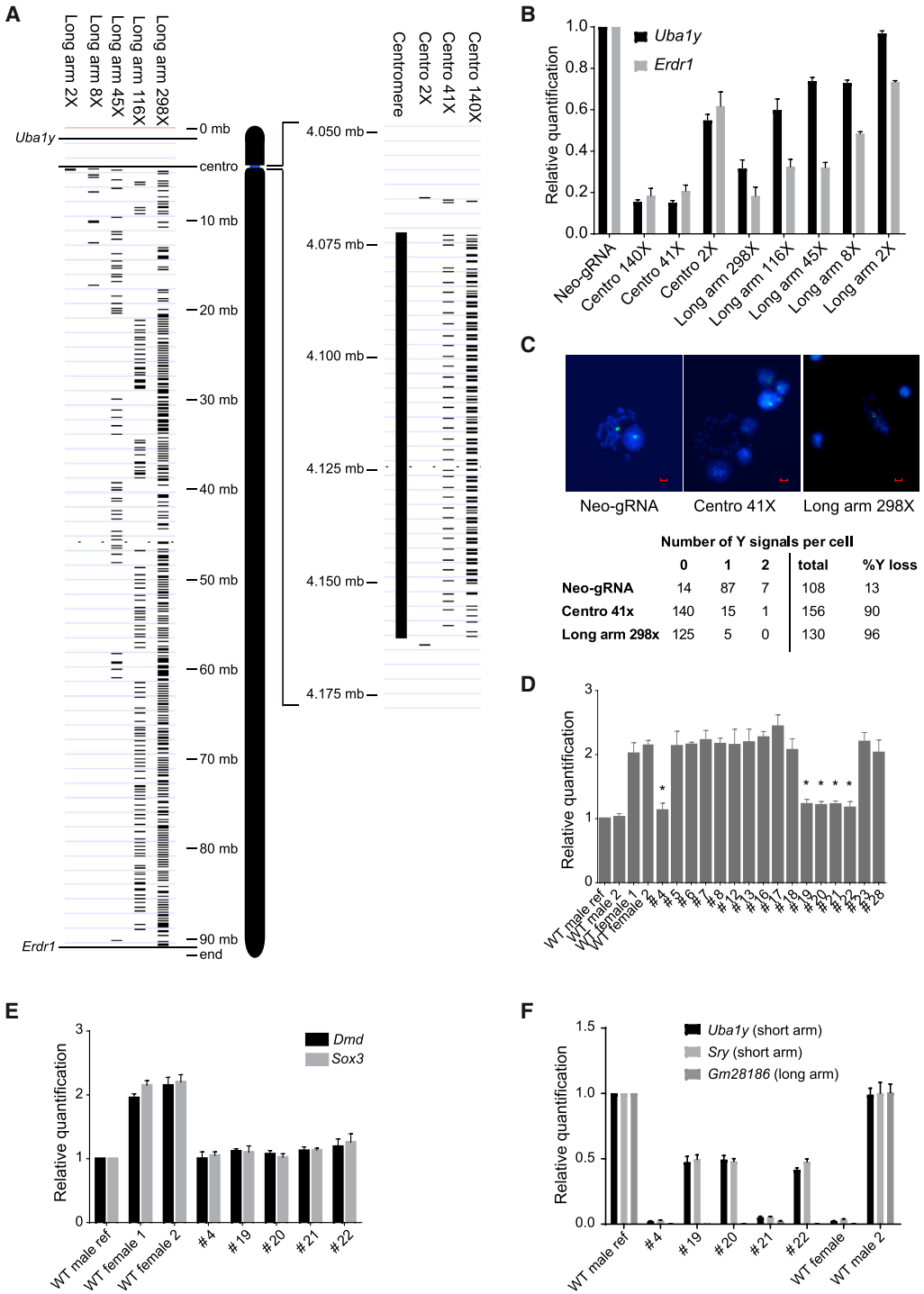

Figure 1. Deletion of Y Chromosome Using CRISPR/Cas9 in Mouse ESCs and In Vivo Mouse Zygote Injection

(A) Schematic showing the position of gRNA target sites in the long arm and centromere of the Y chromosome. (B) qPCR of genomic DNA to quantify Y chromosome dosage. *Sox1* qPCR was used as the internal reference control. Data are presented as mean ± SEM from n ≥ 3 biological replicates. Statistical analysis using two-way ANOVA is presented in Table S3. (C) FISH analysis detection of Y chromosome loss. Y chromosome and DAPI staining was indicated by green and blue signals, respectively. Scale bar, 5 μm. (D) *Xist* genomic qPCR of phenotypically female mice generated through zygote injection of centro 41X gRNA. Asterisks indicate female candidates with single X. (E) *Dmd* and *Sox3* genomic qPCR confirming single X chromosome in female XO candidates. (F) Genomic qPCR quantifying dosage of Y short and long arms. *Sox1* qPCR was used as the internal reference control. Results are presented as mean ± SD from n ≥ 3 replicates.

translocated/truncated Y short arm and the other half containing no detectable Y (Figure 1F).

Given mosaic outcomes are common following CRISPR/Cas zygote injection,[9] we extended our screening to look for phenotypic males that were mosaic for Y chromosome loss. We identified 3 of 11 males with 10%–20% reduction of Y dosage (Figures S3A and S3B). Testis development in these embryos is unsurprising given this level of XY cells.[10] In summary, from 27 embryos, we identified 11 XX females, 8 XY males, 2 XO females, 3 mosaic XO females, and 3 mosaic XO males (Table S1). These results provide proof of concept for efficient chromosome deletion in vivo.

This study shows that targeted chromosome deletion is achievable and relatively efficient both in vitro and in vivo using CRISPR/Cas genome editing. This approach should be applicable for other chromosomes and could be utilized in a variety of cellular contexts and species. Accordingly, we envisage that this strategy will be applied to modeling of aneuploidy syndromes and therapeutic intervention by targeting parental-specific polymorphisms.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.05.021.

AUTHOR CONTRIBUTIONS

F.A., J.H., and P.T. conceived the study. F.A. performed all of the experiments apart from the FISH analysis, which was performed by N.W. and F.G. F.A., J.H., and P.T. drafted the manuscript, which was reviewed and edited by all authors.

CONFLICTS OF INTEREST

The authors declare that they have no competing financial interests.

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Supplemental Information

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Figure S1 Gonadal phenotypes of E15.5 mouse embryos from CRISPR/Cas9 zygote injection**.** Phenotypically male (blue box) and female (pink box) gonads are shown with their sex chromosome dosage.

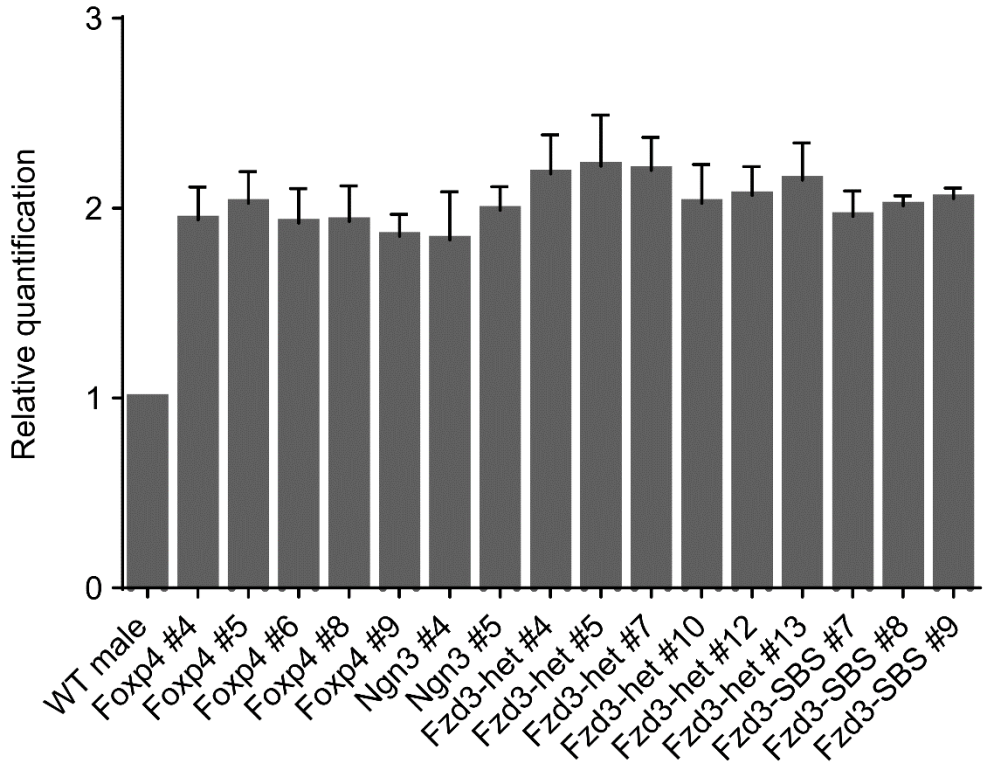

Figure S2 Assessment of X chromosome copy number by *Sox3* qPCR in female mice injected with autosomal gRNAs**.** Sixteen phenotypically female founder mice generated from injection of autosomal gRNAs targeting *Ngn3, Foxp4* and *Fzd3* genes had two copies of the X chromosome. These data indicate that spontaneous loss of the X chromosome does not occur in zygotes injected with CRISPR/Cas9 reagents. Data were presented as mean ± SD from $n \geq 3$ replicates.

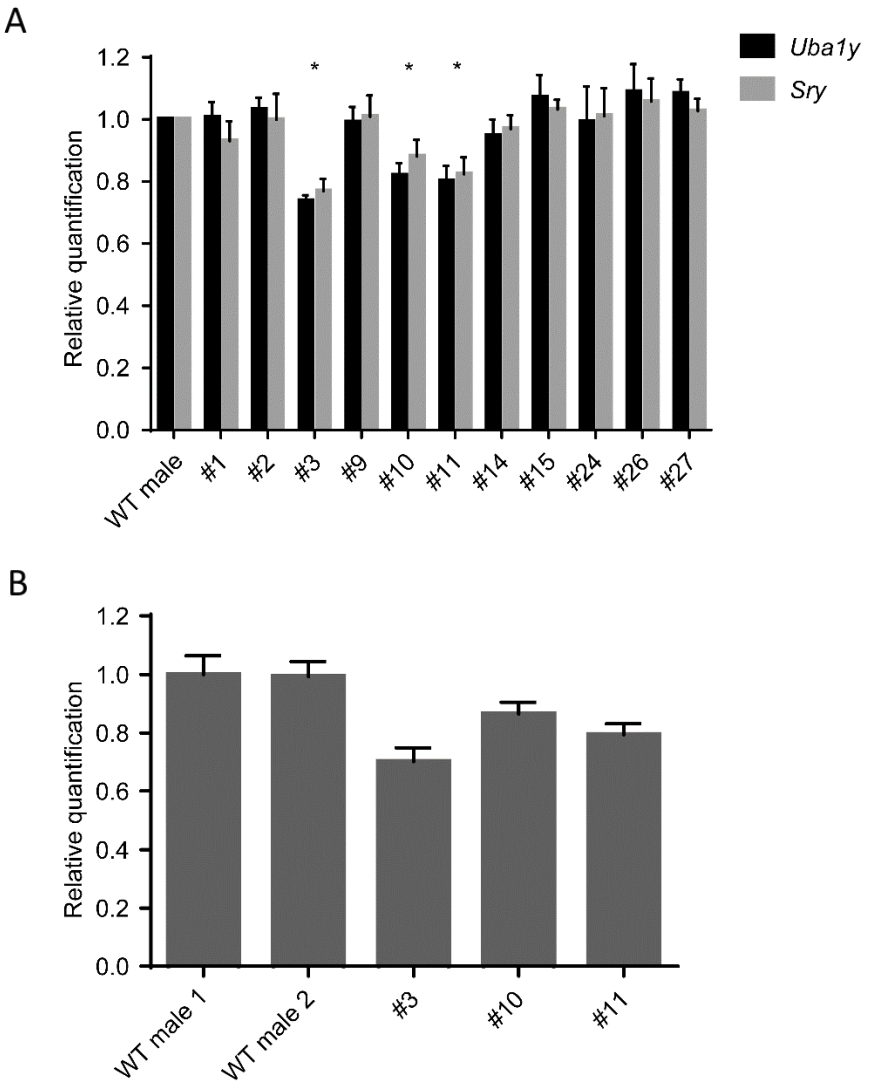

Figure S3 Assessment of Y chromosome dosage in males generated from gRNA centro 41X zygote injection. (**A**) qPCR analysis of *Uba1y* and *Sry* (which are located on Y short arm) revealed males with reduced Y dosage (asterisks) suggesting mosaic XY-XO. (**B**) Confirmation of the mosaicism by qPCR of *Gm28186* (which is located on Y long arm). Data were presented as mean ± SD from n ≥ 3 replicates.

Table S1 | List of gonadal phenotypes and genotypes of all mice generated from gRNA centro 41X injection

Identifier	Gonadal phenotype	Genotype	Additional info
#1	Male	XY	
#2	Male	XY	
#3	Male	XY, XO	Mosaic, with more than 70% XY cells
#4	Female	XO	
#5	Female	XX	
#6	Female	XX	
#7	Female	XX	
#8	Female	XX	
#9	Male	XY	
#10	Male	XY, XO	Mosaic, with more than 70% XY cells
#11	Male	XY, XO	Mosaic, with more than 70% XY cells
#12	Female	XX	
#13	Female	XX	
#14	Male	XY	
#15	Male	XY	
#16	Female	XX	
#17	Female	XX	
#18	Female	XX	
#19	Female	XO, XY_{short_arm}	Mosaic, half XO, half contain truncated Y short arm
#20	Female	XO, XY_{short_arm}	Mosaic, half XO, half contain truncated Y short arm
#21	Female	XO	
#22	Female	XO, XY_{short_arm}	Mosaic, half XO, half contain truncated Y short arm
#23	Female	XX	
#24	Male	XY	
#26	Male	XY	
#27	Male	XY	
#28	Female	XX	

Table S2 | On-targets and potential off-targets all the gRNAs used in this study (provided in excel file)

Table S3 | The two-way ANOVA statistical analysis of *Uba1Y* and *Erdr1* qPCR related to Figure 1B (provided in excel file)

Materials and Methods

gRNA screening and plasmid construction. sgRNAs were identified by manual screening of Y chromosome sequences using the CCTop gRNA design tool http://crispr.cos.uni-heidelberg.de/ provided by Stemmer *et al*. (2015). This tool was also used to predict the off-target potentials containing PAM sequences NGG and NAG (Supplementary information, Table S2). PX459.V2.0 (pSpCas9(BB)-2A-Puro, Addgene #62988) plasmid was used for Cas9 and sgRNA expression. PX459.V2.0 containing sgRNA was prepared as previously described by Ran *et al*. (2013). For dual gRNA centro 2X and long arm 2X, an additional U6-sgRNA cassette was added to the *NotI* site to allow simultaneous expression of two different gRNAs from single plasmid. Plasmid preparations were performed using PureLink® HiPure Plasmid Midiprep Kit (Life Technologies).

Cell culture and transfection. R1 mouse embryonic stem cells were cultured in 15% FCS/DMEM supplemented with 2 mM Glutamax (Gibco), 100 µM non-essential amino acid (Gibco), 100 µM 2-mercaptoethanol (Sigma), 3 µM CHIR99021 (Sigma) 1 µM PD0325901 (Sigma) and LIF (generated in-house). One million of ES cells were nucleofected with 3 µg of plasmid DNA using the Neon™ Transfection System 100 µL Kit (Life technologies) at 1400 V, 10 ms and 3 pulses according to the manufacturer's protocol. 24 hr after transfection, selection was conducted by adding puromycin (2 µg/ml) to the media for the next 48 hr. Surviving cells were cultured for 4-7 days without selection before harvesting.

DNA extraction and qPCR. Genomic DNA was extracted from 1-2 million ES cells or tail tissue using High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. qPCRs were performed using Fast SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems 7500 StepOnePlus machine. *Sox1* qPCR was used as internal reference control to normalize qPCR value in all qPCR analyses.

FISH analysis. Cells were cultured in media containing 0.1 µg colcemid (Roche) for 1-2 hours, harvested and incubated in 0.075 M KCl hypotonic solution for 20 minutes. The cells were then fixed using methanol-acetic acid (3:1) solution, dropped onto slides and dried. FISH staining was performed using Mouse IDetect™ Chromosome Y Paint Probe (Empire Genomics) according to the manufacturer's instructions. Y signals were counted from both metaphase and interphase spreads.

Mouse zygote injection. All the experiments involving animal use have been approved by the University of Adelaide Animal Ethics Committee. Cas9 mRNA was produced by *in vitro* transcription of *XhoI*-linearized pCMV/T7-hCas9 (Toolgen) using mMESSAGE mMACHINE® T7 ULTRA Transcription Kit (Ambion). sgRNA centro 41X was generated according to a previously described protocol.[1] In brief, PCR was performed using a T7 containing forward primer 5'-TTAATACGACTCACTATAGAGGAGTTAATATAAAAAACA-3' and a reverse primer 5'- AAAAGCACCGACTCGGTGCC-3' and the PX459.V2 centro 41X plasmid template. The product was purified by QIAquick PCR Purification Kit (Qiagen) and used as a template for in vitro transcription using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB). RNA purification was conducted using RNeasy Mini Kit (Qiagen). Cas9 mRNA (200 ng/µl) and sgRNA centro 41X (100 ng/µl) were injected to the cytoplasm of C57BL/6N zygotes using a Femtojet microinjector. The survival rate of injected zygotes was 89.4% (93/104). 57 zygotes were transferred into 3 pseudo pregnant females (19/recipient). 27 embryos with normal appearance were harvested at E15.5 for gonadal assessment and tissue collection.

List of gRNA sequences used

Name	gRNA sequences 5'-3'	Position in Y
Centro 2X	Left: GGATAAATGTTACATGCAA Right: GATAATAGTTTACTATTCTAA	4.064.613 4.163.810
Centro 41X	GGAGTTAATATAAAAAACA	4.065.169 to 4.159.436
Centro 140X	GAAGAATTACAATGAAAAATA	4.065.349 to 4.161.710
Long arm 2X	Left: GTCCTCTACGTCTATCAGGA Right: GTTTCCAGCCGGGTTTCTTAC	4.312.132 4.412.892
Long arm 8X	GTTCTATGTCAATTTAGGTGG	4.313.453 to 17.275.105
Long arm 45X	GACTGGGTTCTCCTAATCCTT	4.417.594 to 90.167.758
Long arm 116X	GTGGAATTGTGATCTAGATA	5.726.265 to 88.887.822
Long arm 298X	GGCAAAGCACTTCTGCACC	4.596.490 to 90.662.856
Neo	GGCAGCGCGGCTATCGTGGC	None in mouse

Red highlight indicates additional G was added to the gRNAs

List of qPCR primers

qPCR	F (5'-3')	R (5'-3')	Position
Uba1y (Y short arm)	GGCCACAGACTTGGGCCGAC	TGCCTTGTGGTGCCTGTGGC	chrY: 831.667 - 831.891 & 681.963 - 682.187
Erdr1 (Y long arm)	CTGACTGCGTACAGAAATGTCC	GGAAGACACACACACATCTGCA	chrY: 90.822.353 - 90.822.426 & 90.816.416 - 90.816.489
Sry (Y short arm)	CATTTATGGTGTGGTCCCGTG	ATCTTCAATCTCTGTGCCTCCT	chrY: 2.663.492 - 2.663.607
Gm28186 (Y long arm)	CATGCCTCAGACCCCTCAAG	TCTGACCATTGTGACTCAGAC	chrY: 4.372.957 - 4.373.114
Xist	GCCATCCTCCCTACCTCAGAA	CCTGACATTGTTTTCCCCCTAA	chrX
Dmd	ACAGCAAGCAAGCAAGGCTTC	CTCACCAGTGACCCTATGATTGC	chrX
Sox3	CGTTGCCTTGTACCGAAGAT	CGGGACTTCTCGCTTTTGTA	chrX
Sox1	GACTTGCAGGCTATGTACAACATC	CCTCTCAGACGGTGGAGTTATATT	Chr8

Supplemental Reference:

1. Wang, H. et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910-918 (2013).