Evaluation of Mosaicism and Off Target Mutations in CRISPR-Mediated Genome Edited Bovine Embryos

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ABSTRACT (200 words)

The CRISPR/Cas9 genome editing tool has the potential to improve the livestock breeding industry by allowing for the introduction of desirable traits. Although an efficient and targeted tool, the CRISPR/Cas9 system can have some drawbacks, including off-target mutations and mosaicism, particularly when used in developing embryos. Here, we introduced genome editing reagents into single-cell bovine embryos to compare the effect of Cas9 mRNA and protein on the mutation efficiency, level of mosaicism, and evaluate potential off-target mutations utilizing next generation sequencing. We designed guide-RNAs targeting three loci (POLLED, H11, and ZFX) in the bovine genome and saw a significantly higher rate of mutation in embryos injected with Cas9 protein (84.2%) vs. Cas9 mRNA (68.5%). In addition, the level of mosaicism was higher in embryos injected with Cas9 mRNA (100%) compared to those injected with Cas9 protein (94.2%),

with little to no unintended off-target mutations detected. This study demonstrates that the use of Cas9 protein, rather than Cas9 mRNA, results in a higher editing efficiency in bovine embryos while lowering the level of mosaicism. However, further optimization must be carried out for the CRISPR/Cas9 system to become feasible for single-step embryo editing in a commercial system.

INTRODUCTION

CRISPR-mediated genome editing in livestock zygotes offers an attractive approach to introduce useful genetic variation into the next generation of cattle breeding programs. However, genetic mosaicism is particularly problematic for CRISPR-mediated genome editing in developing zygotes[1,2]. Genetic mosaicism complicates phenotypic analysis of F0 animals and may complicate screening multiple founders and breeding mosaic founders to produce an F1 generation. While this is routine in plant and mouse research, such approaches are time-consuming and essentially cost-prohibitive in large food animal species with long generation intervals like cattle.

A limited number of genome editing studies have been reported in bovine zygotes[3], and indicate the frequent production of mosaic embryos. The frequency of mosaicism varies depending upon the type of site-directed nuclease used, the timing of editing relative to embryonic development, the form and efficiency of the targeting regents, the intrinsic properties of the target locus, and the method of delivery[1].

Correspondingly, there are a number of experimental variables that need to be optimized to improve the efficiency of obtaining non-mosaic, homozygous genome edited founder cattle. In this study, we focused on the type of CRISPR/Cas9 system delivered (i.e. mRNA or protein) and report the impact on mutation efficiency, levels of mosaicism, and off-target mutations based on next generation sequencing when using CRISPR-mediated genome editing of bovine zygotes.

RESULTS

Guide construction and testing

To determine the optimal parameters for CRISPR/Cas9-mediated genome editing in bovine zygotes, efficiency following microinjection was investigated for three gRNA per locus on three different chromosomes. Three gRNAs were designed targeting the POLLED locus on chromosome 1, a safe harbor locus (H11) on chromosome 17 and a locus (ZFX) on the X-chromosome downstream of the Zinc Finger, X-linked gene (Supplementary Information, Table S1). Three gRNAs per locus were independently injected alongside Cas9 protein in groups of 30 zygotes, 18 hours post insemination (hpi). Groups of 50 non-injected embryos were cultured as controls. The highest mutation rates were 76.9% for gRNA2 targeting the POLLED locus, 83.3% for gRNA1 targeting the H11 locus, and 77.8% for gRNA3 targeting the ZFX locus (Supplementary Information, Table S2; χ^2 test, $P < 0.05$). Overall, there was a decrease in the number of embryos that reached the blastocyst stage as the rate of mutation for a given gRNA increased. For each locus, the gRNA with the highest mutation rate was associated with the lowest developmental rate (Supplementary Information, Table S2). gRNAs with the highest mutation rate were selected for further analysis.

Guides targeting the POLLED locus, the H11 locus and the ZFX locus were then injected in groups of 30 *in vitro* fertilized embryos 18hpi alongside either Cas9 mRNA or protein (Table 1). While there was no significant difference in development to the blastocyst stage when comparing embryos injected with Cas9 mRNA (16.2%) or Cas9 protein (16.4%), there was a significant decrease in the proportion of zygotes reaching the blastocyst stage for both groups compared to non-injected controls (30.7%; Fig. 1a; χ^2 test, $P < 0.05$). Mutation rates were

significantly higher for Cas9 protein (84.2%) compared to Cas9 mRNA (68.5%) (Fig. 1b; χ^2 test, $P < 0.05$) for all three loci located on different chromosomes (Fig. 1c).

Evaluation of mosaicism and off-target insertions and deletions

To evaluate the level of mosaicism, 69 blastocysts (19 gRNA2 targeting the POLLED locus (10 Cas9 mRNA, 9 Cas9 protein), 26 gRNA1 targeting the H11 locus (11 Cas9 mRNA, 15 Cas9 protein), and 24 targeting the ZFX locus (13 Cas9 mRNA, 11 Cas9 protein)) were collected, barcoded by PCR amplification and sequenced on a PacBio sequencer (Supplementary Information, Table S3). Consensus sequences were called from raw reads using circular consensus sequencing (ccs) with a minimum of 3 passes, a minimum predicted accuracy of 99% and a maximum length of 700bp (Supplementary Information, Table S4). Unsorted ccs reads were aligned to each of the target sequences to analyze the types of insertions/deletions (indels) surrounding the predicted cut site with 26,460 reads aligned to the POLLED target site; 78,305 reads aligned to the H11 target site; and 66,780 reads aligned to ZFX target site (Supplementary Information, Table S5). About half of the aligned sequences for the POLLED locus were wild type sequences (47.8%), while almost three quarters of the H11 and ZFX reads were wild type sequences (75.7% and 71.3%, respectively). The primary indels for reads aligned to the POLLED locus were 7bp deletion (1672 reads), 11bp deletion (1751), 4bp deletion (6356 reads) and 1bp insertion (2250 reads); aligned to the H11 locus were 11bp deletion (3246 reads), 6bp deletion (3813 reads), 3bp deletion (4091 reads), and 1bp deletion (7853 reads); and aligned to the ZFX locus were 14bp deletion (4222 reads), 9bp deletion (2998 reads), 3bp deletion (3198 reads), 1bp deletion (2194 reads) and 1bp insertion (6532 reads) (Supplementary Information, Table S5).

Ccs reads were then sorted by barcode and analyzed by individual embryos (Fig. 2). Seven samples were discarded from further analysis due to a lack of reads following the quality filtering

step (Supplemental Table S3). A total of 10 samples contained only wild type sequence (7 Cas9 mRNA and 3 Cas9 protein), resulting in an overall mutation rate of ~84% (Table 2). Of the 62 samples injected 18hpi, four contained only mutated alleles, without evidence for any wild type sequence. All four samples were from embryos injected with Cas9 protein (Supplementary Information, Table S6). Three of these samples contained only one allele and were presumably non-mosaic homozygous, although our analyses could not rule out an unmappable mutation (e.g. large insertion) at the second allele. Each of the mutated embryos containing more than a single allele had at least three individual alleles or a disproportion of reads for each allele, for example 75% wildtype and 25% mutant (Supplementary Information, Figure S1), suggesting these embryos were mosaic rather than heterozygous. This translates to 94.2% mosaicism when injecting Cas9 protein compared to 100% mosaicism when injecting Cas9 mRNA.

There was a decreased average number of alleles (3.0 ± 0.4) when targeting the POLLED locus using Cas9 protein (Fig 1d; Table 2). There was no significant difference in the number of alleles for the other loci when injecting Cas9 mRNA or protein. However, there was a significant increase in the number of alleles when comparing polled samples of embryos injected 18hpi with guides alongside Cas9 mRNA (5.23± 0.268), as compared to protein (4.23 ± 0.268) (ANOVA, $P < 0.05$). In addition, there was a significant increase in the percentage of wild type alleles present when injecting Cas9 mRNA compared to Cas9 protein for each of the three loci (42.5% vs. 9.1%, 70.9% vs. 33.7% and 79.7% vs. 43.5%, respectively; $P < 0.05$).

A total of 24 potential off-target sites were predicted across 11 bovine chromosomes (1, 4, 7, 8, 10, 12, 14, 18, 21, 27 and X) (Supplementary Information, Table S7) for the three loci. The 24 predicted off-target sites were PCR amplified, barcoded and sequenced using an Illumina MiSeq sequencer for each of the 69 samples (Supplementary Information, Table S3). HTStream

processed reads were aligned to the 24 predicted sites with 10,399,614 reads mapped with coverage ranging from 1X to 112X per sample per site (Supplementary Information, Table S7). Genetic variation was found throughout the samples in each of the 24 predicted off-target sites with almost no indels present at the predicted off-target cut site with the exception of two targets. A 12bp deletion 26bp downstream from a predicted off-target cut site for the H11 gRNA targeting chr1: 7454978 was detected in 69,434 reads (6.8%) (Supplemental Information, Table S7). Additionally, 2,397 reads (0.51%) contained a 3bp deletion 11bp downstream from the predicted off-target cut site of the ZFX gRNA target chr21: 28506796 (Supplemental Information, Table S7).

DISCUSSION

The ability to efficiently generate non-mosaic, homozygous founder animals is important for the production of genome edited livestock. The use of the CRISPR/Cas9 system has been reported across many livestock species[3], but few reports have characterized its use in bovine embryos. In this study, using the CRISPR/Cas9 system, we identified gRNAs that resulted in high rates of mutation at target locations in two autosomes and the X chromosome in bovine embryos with an overall high efficiency (81-90%). Significant differences were observed in gRNA efficiency within a locus, but not between loci. Microinjection of CRISPR/Cas9 editing reagents in zygotes reduced development to the blastocyst stage compared to non-injected controls. However, no difference was observed in the number of embryos that reached the blastocyst stage when comparing embryos injected with Cas9 mRNA or protein (16.2% vs. 16.4%). This finding was important because we observed a significantly higher rate of mutation in blastocysts when injecting Cas9 protein compared to Cas9 mRNA (84.2% and 68.5%, respectively). This difference is likely due to the immediate availability of the gRNA/Cas9 ribonucleoprotein (RNP) complex to

induce mutation in the embryo. When Cas9 mRNA is injected, there is a delay in genome editing as Cas9 mRNA must be translated into protein before it can combine with the gRNA to induce a DSB[4].

Mosaicism, the presence of more than two alleles in an individual, is a common problem in livestock genome editing[5], with a high rate of embryos resulting in multiple alleles (Table 3). Studies utilizing transcription activator-like effector nucleases (TALENs) have demonstrated lower mosaicism rates than we observed here; however, the proportion of edited embryos tends to be lower as well[6,7]. A study employing a zinc finger nuclease (ZFN) in bovine embryos demonstrated both high embryo editing efficiency and mosaicism rates as compared to those found in TALEN edited embryos[8]. However, the prevalence of mosaicism was reduced when injecting embryos at 8hpi compared to 18hpi, before S-phase had occurred[8]. While we were able to induce mutations in embryos at a high rate, we also observed a high level of mosaicism when injecting 18hpi. Many studies of editing in livestock zygotes similarly report high levels of mosaicism when utilizing CRISPR/Cas9 (Table 3). Many of these studies characterized mosaicism by sequencing the PCR amplicon of the genomic regions flanking the gRNA target sequence and then decomposing the resulting chromatogram data with the TIDE bioinformatics package[9]. Although this approach is cost-effective and rapid, next generation sequencing of the PCR products allows for a more accurate characterization of the different alleles that are present in a mosaic individual, and their relative abundance[10].

In bovine embryos, DNA replication occurs approximately 12-14 hours after fertilization[11]. When injecting at 18hpi, as is often done when using traditional *in-vitro* fertilization (IVF) protocols, most zygotes would be expected to have completed DNA replication[12] and there would likely be more than two copies of each chromosome, thus more opportunities for multiple genomic

edits to occur, resulting in mosaicism. Additionally, following cytoplasmic injection, the gRNA/Cas9 ribonucleoprotein (RNP) complex needs time to enter the nucleus, find its target and cleave the DNA. Furthermore, if injecting Cas9 mRNA, translation to Cas9 protein must also occur, further delaying the editing process, thus resulting in a higher rate of mosaicism. It has been suggested that injection of the CRISPR/Cas9 RNP prior to the S-phase of DNA replication could reduce mosaicism[1].

One recent study with bovine embryos reported low rates (~30%) of mosaicism when introducing Cas9 RNA or protein into early stage zygotes (0 or 10hpi) prior to the S-phase of DNA replication[12]. In that study, allele identification was first made by Sanger sequencing of an amplicon of the targeted region, and then by clonal sequencing of 10 colonies derived from the PCR product per embryo. PCR and cloning-based approaches can identify that a range of alleles exist but cannot accurately quantitate the abundance of each allelic species. The authors went on to employ next generation sequencing on 20 embryos per group to characterize the alleles in non-mosaic embryos. The authors considered embryos that contained biallelic mutations resulting in a gene knockout to be non-mosaic, regardless of the proportion of alleles.

In the current study, we employed next generation sequencing to quantitate the abundance of each allele. The fact that we observed multiple alleles occurring in only a small percentage of reads (< 25%) in many samples analyzed in this study (Figure 2) suggests that editing continued in some subset of cells after the first cleavage division. Further, we considered an embryo containing more than one population of genetically distinct cells to be mosaic irrespective of whether the edit resulted in a missense or nonsense mutation. It is important to determine if founder animals are mosaic because mosaicism complicates the interpretation of the effect of a given genome alteration[5], and subsequent breeding of mosaic founder animals to achieve non-mosaic

184 animals can take years[13]. Additionally, mosaics do not fit easily into the proposed regulatory
185 framework for genome edited food animals[14].

186 Along with the level of mosaicism, one of the concerns raised with the generation of
187 genome edited animals is the potential for off-target mutation events. Typically, online prediction
188 tools are used to calculate the likelihood of off-target sites[15-17]. The top predicted sites can then be
189 PCR amplified and the presence of a mutation determined by either next generation sequencing,
190 TA cloning followed by Sanger sequencing, or mismatch cleavage assays followed by Sanger
191 sequencing[18]. In this study, we used the targeted approach using online predictive tools to identify
192 off-target sites rather than a genome-wide approach. Off-target cleavage can occur in the genome
193 with three to five base pair mismatches in the PAM-distal sequence[15,19-21]. Cas9 specificity is
194 determined by the seed region, or the 8 to 11-nt PAM-proximal sequence, making it the most vital
195 part of the gRNA sequence[19,22]. In our gRNA design, we excluded all gRNAs with less than three
196 mismatches across the off-target sequence. We determined this threshold based on previous studies
197 showing reduced Cas9 activity in regions with at least three mismatches[23].

198 In the 69 samples analyzed, there were two potential off-target mutations detected. One of
199 these (H11) was in a region that had known annotated wild type 12bp deletions (rs876383581 and
200 rs521367917) around the potential cut-site. Additionally, 0.51% of total reads contained a 3bp
201 deletion 11bp downstream from the predicted off-target cut site for the ZFX gRNA target chr21:
202 28506796 (Supplemental Information, Table S7). This predicted site does not have any annotated
203 variation. It is important to note that although this off-target location had three mismatches to the
204 gRNA sequence, all three of the mismatches were located outside the seed region (8-11bp
205 upstream of the PAM sequence). This guide was designed using off-target prediction software and
206 the Btau 4.6.1 bovine reference genome[24], which was the only *Bos taurus* reference genome

available with the online tool at the time. When the off-target prediction software was re-run for the off-target analysis, the most recent reference genome available was UMD 3.1.1[24]. Using the new reference genome, this locus on chromosome 21 was identified as having the requisite three mismatches, but there were no mismatches in the seed region, as specified by our guide design criteria. More recently, an improved reference bovine genome ARS-UCD1.2 was published[25]. Using the online tool with the updated reference genome resulted in the same predicted off-target sites as UMD 3.1.1.

One of the stated concerns with off-target mutation events is that if they occur in functional regions, such as coding sequences or regulatory regions, they could potentially be detrimental to the health or development of the resulting animal. Neither of these two off-target deletions were in a region of annotated function. As there were approximately 20 individual blastocysts included in these analyses, these deletions may also have been the result of naturally occurring polymorphic variation. A detailed sequence analysis of 2,703 individuals from different breeds of cattle revealed a high level of genetic diversity including 84 million single-nucleotide polymorphisms (SNPs) and 2.5 million small insertion deletions[26]. Data like these are essential to put naturally occurring variation, like that seen at the H11 locus, in context. Various studies in humans[27,28], monkeys[29], and rodents[30,31] suggest that the off-target frequency of Cas9-mediated mutagenesis does not differ from the de novo mutation rate.

Overall, we demonstrated efficient CRISPR/Cas9 genome editing across three different loci on three different chromosomes. We found that injecting zygotes with Cas9 protein results in a significantly higher mutation rate compared to Cas9 mRNA (82.2% vs 65.4%). In addition, zygotes injected with Cas9 protein displayed a significantly lower number of alleles compared to those injected with Cas9 mRNA (4.2 vs 5.2). Although off-target events did not appear to be an

issue, the rate of mosaicism was still high, and further optimization needs to be done before this technique is feasible in a livestock production setting.

MATERIALS AND METHODS

Guide Construction

Guides sequences were designed using the online tools sgRNA Scorer 2.0[32,33] and Cas-OFFinder[34] and targeting the POLLED locus on chromosome 1, a safe harbor locus (H11) on chromosome 17 and in the 3' UTR of the Zinc-finger X-linked (*ZFX*) gene (ZFX) on the X-chromosome. Guides were selected with no less than three mismatches in the guide sequence for off-target sites using the UMD3.1.1 bovine reference genome[24], and at least one mismatch in the seed region (8-11bp upstream of the PAM sequence). Oligonucleotides were ordered from Eurofins USA (Louisville, KY) for the top four guides for construction of the gRNA and were used for *in vitro* transcription using the AmpliScribe T7-Flash Transcription kit (Lucigen, Palo Alto, CA) and purified using the MEGAclear Transcription Clean-Up kit (Thermo Fisher, Chicago, IL) as described by Vilarino et al[10]. Cleavage efficiency was tested using an *in vitro* cleavage assay by combining 60ng of PCR amplified product, 100ng of gRNA, 150ng of Cas9 protein (PNA Bio, Inc., Newbury Park, CA), 1μL of 10X BSA, 1μL of NEB Buffer 3.1 and water bringing the total volume to 10μL in a 0.2μL tube and incubating at 37°C for 1 hour. The incubated product was then run on a 2% agarose gel with 5μL of Sybr Gold at 100V for 1 hour and visualized using a ChemiDoc-ItTS2 Imager (UVP, LLC, Upland, CA).

Embryo Production

Bovine ovaries were collected from a local processing plant and transported to the laboratory at 35-37°C in sterile saline. Cumulus-oocyte complexes (COCs) were aspirated from follicles and groups of 50 COCs were transferred to 4-well dishes containing 400µL of maturation media[35]. COCs were incubated for 21-24hr at 38.5°C in a humidified 5% CO_2 incubator. Approximately 25 oocytes per drop were fertilized in 60µL drops of SOF-IVF[35] with $1x10^6$ sperm per mL and incubated for 18hr at 38.5°C in a humidified 5% CO_2 incubator. Presumptive zygotes were denuded by light vortex in SOF-HEPES medium[35] for 5 min. 25 zygotes per drop were incubated in 50µL drops of KSOM culture media (Zenith Biotech, Glendale, CA, USA) at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7-8 days.

Guide Testing

Mutation rate for each guide was determined by laser-assisted cytoplasmic injection[36] of *in vitro* fertilized embryos with 6pL of a solution containing 67ng/µL of *in vitro* transcribed gRNA alongside 133ng/µL of Cas9 mRNA or 167ng/µL of Cas9 protein (PNA Bio, Inc., Newbury Park, CA) incubated at room temperature for 30 minutes prior to injection. Injected embryos were incubated for 7-8 days. Embryos that reached blastocyst stage were lysed in 10µL of Epicenter DNA extraction buffer (Lucigen, Palo Alto, CA) using a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, California) at 65°C for 6 minutes, 98°C for 2 minutes and held at 4°C. The target region was amplified by two rounds of the polymerase chain reaction (PCR) using primers developed using Primer3 (Supplementary Information, Table S1)[37,38]. The first round of PCR was performed on a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, California) with 10µL GoTAQ Green Master Mix (Promega Biosciences LLC, San Luis Obispo, CA), 0.4µL of each primer at 10mM and 9.2µL of DNA in lysis buffer for 5 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at anneal temp (Supplementary Information, Table S1), and 30 sec at

72°C, followed by 5 min at 72°C. The second round of PCR was run with 10μL GoTAQ Green Master Mix (Promega Biosciences LLC, San Luis Obispo, CA), 4.2μL of water, 0.4μL of each primer at 10mM and 5μL of first round PCR for 3 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at anneal temp (Supplementary Information, Table S1), and 30 sec at 72°C, followed by 5 min at 72°C. Products were visualized on a 1% agarose gel using a ChemiDoc-ItTS2 Imager (UVP, LLC, Upland, CA), purified using the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA) and Sanger sequenced (GeneWiz, South Plainfield, NJ).

Allelic Variation and Off-Target Analysis

Embryos that reached the blastocyst stage were lysed and underwent whole-genome amplification using the Repli-G Mini kit (Qiagen, Inc., Valencia, CA). To determine presumptive off-target sites, guide sequences were mapped against the bosTau8 bovine reference genome using the online tool Cas-OFFinder[34]. A total of 24 off-target sites were predicted using the online tool: eight off-target sites for the POLLED gRNA, eleven off-target sites for the H11 gRNA and five off-target sites for the ZFX gRNA (Supplementary Information, Table S7). Whole-genome amplified samples were used for PCR amplification of cut-sites and presumptive off-target sites using a dual round PCR approach described above to barcode each sample. Primers were designed to amplify each region using Primer3[37,38] with a 15bp adapter sequence attached to the forward (AGATCTCTCGAGGTT) and reverse (GTAGTCGAATTCGTT) (Supplementary Information, S1). The second round of PCR amplified off the adapters adding an independent barcode for each sample to identify reads for pooled sequencing (Supplementary Information, Table S1).

PCR samples targeting the gRNA cut site underwent SMRTbell library preparation and were sequenced on a PacBio Sequel II sequencer by GENEWIZ, LLC (South Plainfield, NJ, USA). Consensus sequences were called, reads sorted by barcode and BAM converted to individual

FASTQ files using SMRT Link v8.0.0.80529 (https://www.pacb.com/support/software-downloads/). Reads were aligned to each target site using BWA v0.7.16a[39]. SAM files were converted to BAM files, sorted and indexed using SAMtools v1.9[40]. Number and types of alleles were determined for each sample using CrispRVariants v1.12.0[41].

Off-target PCR samples underwent library preparation using the Illumina TruSeq library kit and were sequenced (300bp paired-end) on an Illumina MiSeq Next Generation Sequencer by the DNA Technologies and Expression Analysis Cores at the UC Davis Genome Center. Paired-end reads were processed and overlapped to form high quality single-end reads using HTStream Overlapper v1.1.0 (https://github.com/ibest/HTStream). Processed reads were aligned to each target site using BWA v0.7.16a[39]. SAM files were converted to BAM files, sorted and indexed using SAMtools v1.9[40]. Insertions and deletions were called using CrispRVariants v1.12.0[41].

Statistical Analysis

Comparison between development for guide analysis and mutation rates were evaluated using a linear model and statistical significance was determined using a Chi-square test. To analyze the level of mosaicism, an ANOVA test was used to determine significance between number of alleles per sample and percent wild type when injecting alongside Cas9 mRNA or protein. Samples with only wild type alleles were removed from analysis. Differences were considered significant when $P < 0.05$.

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Author's contribution

SLH and JRO performed the experiments with additional input from PJR, ALV and JDM. SLH, JRO, JCL and AEY participated in sample processing and data analysis. SLH, JRO, ALV and JDM wrote the manuscript with suggestions from all the co-authors. All authors read and approved the final version.

Competing Interests

The authors declare no competing interests.

Data Availability

Raw sequence reads from PacBio Sequel II and Illumina MiSeq sequencing are available in the NCBI Sequence Read Archive as BioProject PRJNA623431 and SRA accession number SRR11850065. Individual results for the blastocyst development and mutation rate from each replicate (~ 30 embryos) of control and microinjected embryos are available in Supplementary Table S8.

References

1 Mehravar, M., Shirazi, A., Nazari, M. & Banan, M. Mosaicism in CRISPR/Cas9-mediated genome editing. *Dev Biol* **445**, 156-162, doi:10.1016/j.ydbio.2018.10.008 (2019).

2 Vilarino, M. *et al.* Mosaicism diminishes the value of pre-implantation embryo biopsies for detecting CRISPR/Cas9 induced mutations in sheep. *Transgenic Res* **27**, 525-537, doi:10.1007/s11248-018-0094-x (2018).

3 Mclean, Z., Oback, B. & Laible, G. Embryo-mediated genome editing for accelerated genetic improvement of livestock. *Frontiers of Agricultural Science and Engineering*, doi:doi: 10.15302/J-FASE-2019305 (2020).

4 Glass, Z., Lee, M., Li, Y. & Xu, Q. Engineering the Delivery System for CRISPR-Based Genome Editing. *Trends Biotechnol* **36**, 173-185, doi:10.1016/j.tibtech.2017.11.006 (2018).

5 Teboul, L., Murray, S. A. & Nolan, P. M. Phenotyping first-generation genome editing mutants: a new standard? *Mamm Genome* **28**, 377-382, doi:10.1007/s00335-017-9711-x (2017).

6 Carlson, D. F. *et al.* Efficient TALEN-mediated gene knockout in livestock. *Proc Natl Acad Sci U S A* **109**, 17382-17387, doi:10.1073/pnas.1211446109 (2012).

7 Proudfoot, C. *et al.* Genome edited sheep and cattle. *Transgenic Res* **24**, 147-153, doi:10.1007/s11248-014-9832-x (2015).

8 Wei, J. *et al.* Efficient introgression of allelic variants by embryo-mediated editing of the bovine genome. *Sci Rep* **5**, 11735, doi:10.1038/srep11735 (2015).

9 Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic acids research* **42**, e168-e168 (2014).

10 Vilarino, M. *et al.* CRISPR/Cas9 microinjection in oocytes disables pancreas development in sheep. *Sci Rep* **7**, 17472, doi:10.1038/s41598-017-17805-0 (2017).

11 Comizzoli, P., Marquant-Le Guienne, B., Heyman, Y. & Renard, J. P. Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes. *Biol Reprod* **62**, 1677-1684, doi:10.1095/biolreprod62.6.1677 (2000).

12 Lamas-Toranzo, I. *et al.* Strategies to reduce genetic mosaicism following CRISPR-mediated genome edition in bovine embryos. *Scientific Reports* **9**, 14900, doi:10.1038/s41598-019-51366-8 (2019).

13 Bishop, T. F. & Van Eenennaam, A. L. Genome editing approaches to augment livestock breeding programs. *J Exp Biol* **223**, doi:10.1242/jeb.207159 (2020).

14 Van Eenennaam, A. L., Wells, K. D. & Murray, J. D. Proposed U.S. regulation of gene-edited food animals is not fit for purpose. *NPJ Sci Food* **3**, 3, doi:10.1038/s41538-019-0035-y (2019).

15 Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**, 827-832, doi:10.1038/nbt.2647 (2013).

16 Doench, J. G. *et al.* Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* **34**, 184-191, doi:10.1038/nbt.3437 (2016).

17 Haeussler, M. *et al.* Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol* **17**, 148, doi:10.1186/s13059-016-1012-2 (2016).

18 Jamal, M. *et al.* Keeping CRISPR/Cas on-Target. *Current Issues in Molecular Biology* **20**, 1-12, doi:10.21775/cimb.020.001 (2016).

19 Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823, doi:10.1126/science.1231143 (2013).

20 Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823-826, doi:10.1126/science.1232033 (2013).

21 Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* **31**, 839-843, doi:10.1038/nbt.2673 (2013).

22 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).

23 Anderson, E. M. *et al.* Systematic analysis of CRISPR-Cas9 mismatch tolerance reveals low levels of off-target activity. *J Biotechnol* **211**, 56-65, doi:10.1016/j.jbiotec.2015.06.427 (2015).

24 Elsik, C. G. *et al.* The genome sequence of taurine cattle: a window to ruminant biology and evolution. *Science* **324**, 522-528, doi:10.1126/science.1169588 (2009).

25 Rosen, B. D. *et al.* De novo assembly of the cattle reference genome with single-molecule sequencing. *Gigascience* **9**, doi:10.1093/gigascience/giaa021 (2020).

26 Hayes, B. J. & Daetwyler, H. D. 1000 Bull Genomes Project to Map Simple and Complex Genetic Traits in Cattle: Applications and Outcomes. *Annu Rev Anim Biosci* **7**, 89-102, doi:10.1146/annurev-animal-020518-115024 (2019).

27 Veres, A. *et al.* Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell Stem Cell* **15**, 27-30, doi:10.1016/j.stem.2014.04.020 (2014).

28 Yang, L. *et al.* Targeted and genome-wide sequencing reveal single nucleotide variations impacting specificity of Cas9 in human stem cells. *Nat Commun* **5**, 5507, doi:10.1038/ncomms6507 (2014).

29 Luo, X. *et al.* Trio deep-sequencing does not reveal unexpected off-target and on-target mutations in Cas9-edited rhesus monkeys. *Nat Commun* **10**, 5525, doi:10.1038/s41467-019-13481-y (2019).

30 Iyer, V. *et al.* Off-target mutations are rare in Cas9-modified mice. *Nat Methods* **12**, 479, doi:10.1038/nmeth.3408 (2015).

31 Willi, R., Smith, H. E., Wang, C., Liu, C. & Hennighausen, L. Mutation frequency is not increased in CRISPR–Cas9-edited mice. *Nature Methods* **15**, 756-758 (2018).

32 Aach, J., Mali, P. & Church, G. M. CasFinder: Flexible algorithm for identifying specific Cas9 targets in genomes. *bioRxiv*, doi:10.1101/005074 (2014).

33 Chari, R., Yeo, N. C., Chavez, A. & Church, G. M. sgRNA Scorer 2.0: A Species-Independent Model To Predict CRISPR/Cas9 Activity. *ACS Synth Biol* **6**, 902-904, doi:10.1021/acssynbio.6b00343 (2017).

34 Bae, S., Park, J. & Kim, J. S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* **30**, 1473-1475, doi:10.1093/bioinformatics/btu048 (2014).

35 Bakhtari, A. & Ross, P. J. DPPA3 prevents cytosine hydroxymethylation of the maternal pronucleus and is required for normal development in bovine embryos. *Epigenetics* **9**, 1271-1279, doi:10.4161/epi.32087 (2014).

36 Bogliotti, Y. S., Vilarino, M. & Ross, P. J. Laser-assisted Cytoplasmic Microinjection in Livestock Zygotes. *J Vis Exp*, doi:10.3791/54465 (2016).

37 Koressaar, T. & Remm, M. Enhancements and modifications of primer design program Primer3. *Bioinformatics* **23**, 1289-1291, doi:10.1093/bioinformatics/btm091 (2007).

38 Untergasser, A. *et al.* Primer3--new capabilities and interfaces. *Nucleic Acids Res* **40**, e115, doi:10.1093/nar/gks596 (2012).

39 Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv* **1303.3997v** (2013).

40 Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079, doi:10.1093/bioinformatics/btp352 (2009).

41 Lindsay, H. *et al.* CrispRVariants charts the mutation spectrum of genome engineering experiments. *Nat Biotechnol* **34**, 701-702, doi:10.1038/nbt.3628 (2016).

42 Petersen, B. *et al.* Efficient production of biallelic GGTA1 knockout pigs by cytoplasmic microinjection of CRISPR/Cas9 into zygotes. *Xenotransplantation* **23**, 338-346, doi:10.1111/xen.12258 (2016).

43 Zhang, X. *et al.* Disruption of the sheep BMPR-IB gene by CRISPR/Cas9 in in vitro-produced embryos. *Theriogenology* **91**, 163-172 e162, doi:10.1016/j.theriogenology.2016.10.025 (2017).

44 Crispo, M. *et al.* Efficient Generation of Myostatin Knock-Out Sheep Using CRISPR/Cas9 Technology and Microinjection into Zygotes. *PLoS One* **10**, e0136690, doi:10.1371/journal.pone.0136690 (2015).

45 Tao, L. *et al.* Efficient biallelic mutation in porcine parthenotes using a CRISPR-Cas9 system. *Biochemical and Biophysical Research Communications* **476**, 225-229 (2016).

46 Wang, Y. *et al.* Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. *Sci Rep* **5**, 8256, doi:10.1038/srep08256 (2015).

47 Daigneault, B. W., Rajput, S., Smith, G. W. & Ross, P. J. Embryonic POU5F1 is Required for Expanded Bovine Blastocyst Formation. *Sci Rep* **8**, 7753, doi:10.1038/s41598-018-25964-x (2018).

48 Namula, Z. *et al.* Genome mutation after the introduction of the gene editing by electroporation of Cas9 protein (GEEP) system into bovine putative zygotes. *In Vitro Cell Dev Biol Anim* **55**, 598-603, doi:10.1007/s11626-019-00385-w (2019).

49 Tanihara, F. *et al.* Effects of concentration of CRISPR/Cas9 components on genetic mosaicism in cytoplasmic microinjected porcine embryos. *Journal of Reproduction and Development* **65**, 209-214 (2019).

50 Tanihara, F. *et al.* Generation of a TP53-modified porcine cancer model by CRISPR/Cas9-mediated gene modification in porcine zygotes via electroporation. *PLoS One* **13**, e0206360, doi:10.1371/journal.pone.0206360 (2018).

Legends

Table 1. Number of zygotes reaching the blastocyst developmental stage following microinjection of either Cas9 mRNA or protein and gRNAs targeting three loci (POLLED, H11, and ZFX) on different chromosomes. I*n vitro* fertilized bovine embryos were injected 18 hours post insemination, and the percentage of blastocysts with Cas9-induced mutations was determined by sequence analysis. Letters that differ in the same column are significantly different ($P < 0.05$).

Table 2. Editing efficiencies, mosaicism, average number of alleles and percent wild type reads as determined by PacBio sequencing of 63 blastocysts following microinjection of Cas9 mRNA or protein alongside gRNAs targeting three loci (POLLED, H11, and ZFX) on different chromosomes. I*n vitro* fertilized bovine embryos were injected 18 hours post insemination. Letters that differ in the same column are significantly different ($P < 0.05$). SEM = standard error of the mean.

Table 3. Published results of genome editing targeting the NHEJ pathway in livestock zygotes, and rates of mosaicism (where available). Modified from Mclean et al.[3]. [a]Transcription activator-like effector (TALE), zinc finger (ZF). [b]Nuclease delivered as plasmid, mRNA, or ribonucleoprotein (RNP) complex. [c]Cytoplasmic injection (CI) or electroporation (E). [d]*In vitro* fertilized (IVF) or parthenogenetic (PG) embryos. [e] normalized on the total number of edited embryos or not determined (ND).

494 **Figure 1.** Percentage of uninjected control and microinjected zygotes reaching the blastocyst
495 developmental stage following microinjection of either Cas9 mRNA or protein into *in vitro*
496 fertilized bovine embryos 18 hours post insemination, and percentage analyzed blastocysts with
497 Cas9-induced mutations. (a) Blastocyst developmental percentage of CRISPR injected zygotes
498 for all three loci compared to control non-injected zygotes. (b) Percentage of blastocysts with
499 Cas9-induced mutations when injecting either Cas9 mRNA or protein alongside gRNAs
500 targeting all three loci. (c) Percentage of blastocysts with Cas9 mRNA or protein-induced
501 mutation by and gRNAs targeting three loci (POLLED, H11, and ZFX) in the bovine genome.
502 Error bars = standard error of the mean. (d) Average number of alleles per blastocyst when
503 injecting Cas9 mRNA or protein targeting three loci (POLLED, H11, and ZFX) in the bovine
504 genome. *P < 0.05 **P < 0.005 ***P < 0.0005.
505
506 **Figure 2.** Bar graph depicting the percentage of alleles determined by PacBio sequencing in each
507 of the 62 blastocysts microinjected 18 hours post insemination with either Cas9 mRNA or protein
508 and gRNAs targeting the POLLED, H11 and ZFX loci. For ZFX locus: dotted bars are female;
509 solid bars are male.

Tables and Figures

Table 1. Number of zygotes reaching the blastocyst developmental stage following microinjection of either Cas9 mRNA or protein and gRNAs targeting three loci (POLLED, H11, and ZFX) on different chromosomes. I*n vitro* fertilized bovine embryos were injected 18 hours post insemination, and the percentage of blastocysts with Cas9-induced mutations was determined by sequence analysis. Letters that differ in the same column are significantly different ($P < 0.05$).

Cas9	gRNA	Injected Groups	Total Embryos	Total Blasts (%)	Total Analyzed	Total Mutation (%)
mRNA	control	-	492	131 (27)[a]	-	-
	POLLED	4	109	21 (19)[b]	22	16 (73)[a]
	H11	7	191	28 (15)[b]	27	19 (70)[a]
	ZFX	14	372	60 (16)[b]	62	41 (67)[a]
protein	control	-	749	250 (33)[a]	-	-
	POLLED	12	316	53 (17)[b]	42	36 (86)[b]
	H11	6	162	27 (17)[b]	39	35 (90)[b]
	ZFX	22	562	91 (16)[b]	90	73 (81)[b]

Table 2. Editing efficiencies, mosaicism, average number of alleles and percent wild type reads as determined by PacBio sequencing of 63 blastocysts following microinjection of Cas9 mRNA or protein alongside gRNAs targeting three loci (POLLED, H11, and ZFX) on different chromosomes. I*n vitro* fertilized bovine embryos were injected 18 hours post insemination. Letters that differ in the same column are significantly different ($P < 0.05$). SEM = standard error of the mean.

Locus	n	Cas9	% non-edited	% edited non-mosaic	% mosaic embryos	Alleles	SEM	% Wild Type	SEM
POLLED	10	mRNA	0.0	0.0	100.0	5.4[a]	±0.365	42.5[a]	±7.52
	7	protein	0.0	14.3	85.7	3.0[b]	±0.398	9.1[b]	±8.11
H11	11	mRNA	36.4	0.0	100.0	5.1[a]	±0.396	70.9[a]	±7.01
	13	protein	15.4	7.7	92.3	4.8[a]	±0.353	33.7[b]	±6.69
ZFX	12	mRNA	25.0	0	100.0	5.1[a]	±0.375	79.7[a]	±6.94
	9	protein	11.1	11.1	88.9	4.5[a]	±0.386	43.5[b]	±7.47

Table 3. Published results of genome editing targeting the NHEJ pathway in livestock zygotes, and rates of mosaicism (where available). Modified from Mclean et al.[3]. [a]Transcription activator-like effector (TALE), zinc finger (ZF). [b]Nuclease delivered as plasmid, mRNA, or ribonucleoprotein (RNP) complex. [c]Cytoplasmic injection (CI) or electroporation (E). [d]*In vitro* fertilized (IVF) or parthenogenetic (PG) embryos. [e] normalized on the total number of edited embryos or not determined (ND).

Nuclease[a]	Reagent[b]	Animal	Delivery Method[c]	Delivery time (post IVF)/h[d]	Target locus	Edited embryos %	Mosaic embryos %[e]	Edited offspring	Mosaic offspring	Reference
TALE	mRNA	Bovine	CI	19	*ACAN* or *GDF8*	52	20	-	-	[6]
TALE	mRNA	Bovine	CI	24	*GDF8*	31-67	ND	3/4	1/3	[7]
TALE	mRNA	Ovine	CI	24	*GDF8*	ND	ND	1/9	0/1	[7]
ZF	Plasmid	Bovine	CI	8	*LGB*	71	100	-	-	[8]
ZF	Plasmid	Bovine	CI	18	*LGB*	83	100	-	-	[8]
ZF	mRNA	Bovine	CI	8	*LGB*	70	75	-	-	[8]
Cas9	Plasmid	Porcine	CI	17	*GGTA1*	ND	ND	11/12	4/11	[42]
Cas9	mRNA	Ovine	CI	0	*PDX1*	67	38	2/4	2/2	[10]
Cas9	mRNA	Ovine	CI	6	*PDX1*	60	67	-	-	[10]
Cas9	mRNA	Ovine	CI	14	*BMPR-IB*	38	86	-	-	[43]
Cas9	mRNA	Ovine	CI	22	*MSTN*	50	80	10/22	4/10	[44]
Cas9	mRNA	Porcine	CI	3	*Tet1*	94	30	-	-	[45]
Cas9	mRNA	Porcine	CI	8	*Tet1*	100	33	-	-	[45]
Cas9	mRNA	Porcine	CI	18	*Tet1*	83	100	-	-	[45]
Cas9	mRNA	Porcine	CI	17	*Npc1l1*	88	ND	11/11	9/11	[46]
Cas9	RNP	Bovine	CI	10 (IVF), 1 (PG)	*POU5F1*	86	34	-	-	[47]
Cas9	RNP	Bovine	E	10	*GDF8*	27-67	75-100	-	-	[48]
Cas9	RNP	Bovine	E	15	*GDF8*	19-67	92-100	-	-	[48]
Cas9	RNP	Porcine	CI	0	*GalT*	21	100	-	-	[49]
Cas9	RNP	Porcine	CI	0 + 6	*GalT*	23	100	-	-	[49]
Cas9	RNP	Porcine	CI	6	*GalT*	65	82	-	-	[49]
Cas9	RNP	Porcine	E	12	*TP53*	88	52	6/9	5/6	[50]

Cas9	mRNA	Bovine	CI	0	*PAEP* or *CSN2*	88	30	-	-	[12]
Cas9	RNP	Bovine	CI	0		87	30	-	-	[12]
Cas9	RNP	Bovine	CI	10		83	35	-	-	[12]
Cas9	mRNA	Bovine	CI	20		84	100	-	-	[12]
Cas9	RNP	Bovine	CI	20		83	100	-	-	[12]
Cas9	mRNA	Bovine	CI	18	POLLED	73	100	-	-	This study
Cas9	RNP	Bovine	CI	18	POLLED	86	86	-	-	This study
Cas9	mRNA	Bovine	CI	18	H11	70	100	-	-	This study
Cas9	RNP	Bovine	CI	18	H11	90	91	-	-	This study
Cas9	mRNA	Bovine	CI	18	ZFX	67	100	-	-	This study
Cas9	RNP	Bovine	CI	18	ZFX	81	88	-	-	This study

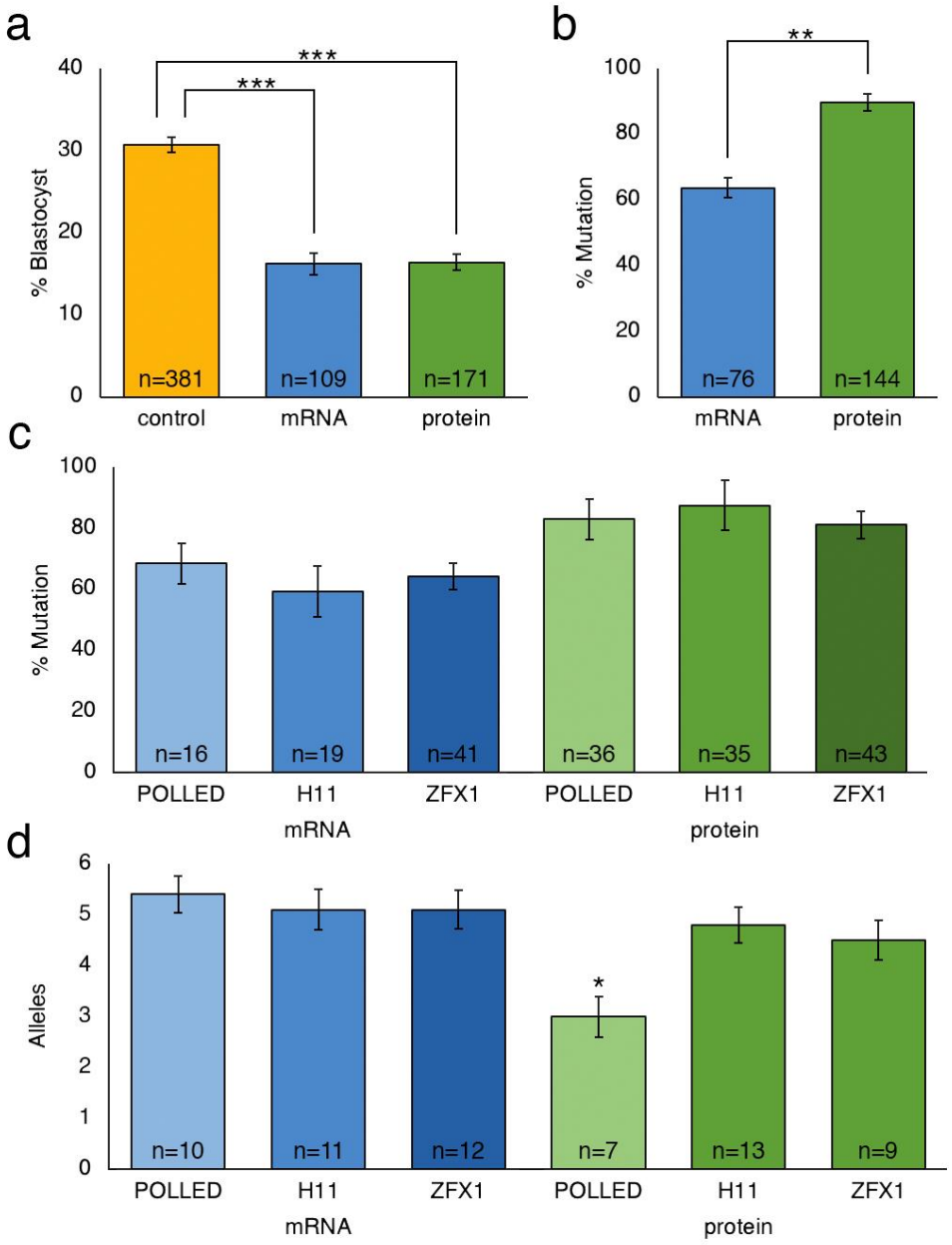

Figure 1. Percentage of uninjected control and microinjected zygotes reaching the blastocyst developmental stage following microinjection of either Cas9 mRNA or protein into *in vitro* fertilized bovine embryos 18 hours post insemination, and percentage analyzed blastocysts with Cas9-induced mutations. (a) Blastocyst developmental percentage of CRISPR injected zygotes for all three loci compared to control non-injected zygotes. (b) Percentage of blastocysts with Cas9-induced mutations when injecting either Cas9 mRNA or protein alongside gRNAs targeting all three loci. (c) Percentage of blastocysts with Cas9 mRNA or protein-induced mutation by and gRNAs targeting three loci (POLLED, H11, and ZFX) in the bovine genome. Error bars = standard error of the mean. (d) Average number of alleles per blastocyst when injecting Cas9 mRNA or protein targeting three loci (POLLED, H11, and ZFX) in the bovine genome. *P < 0.05 **P < 0.005 ***P < 0.0005.

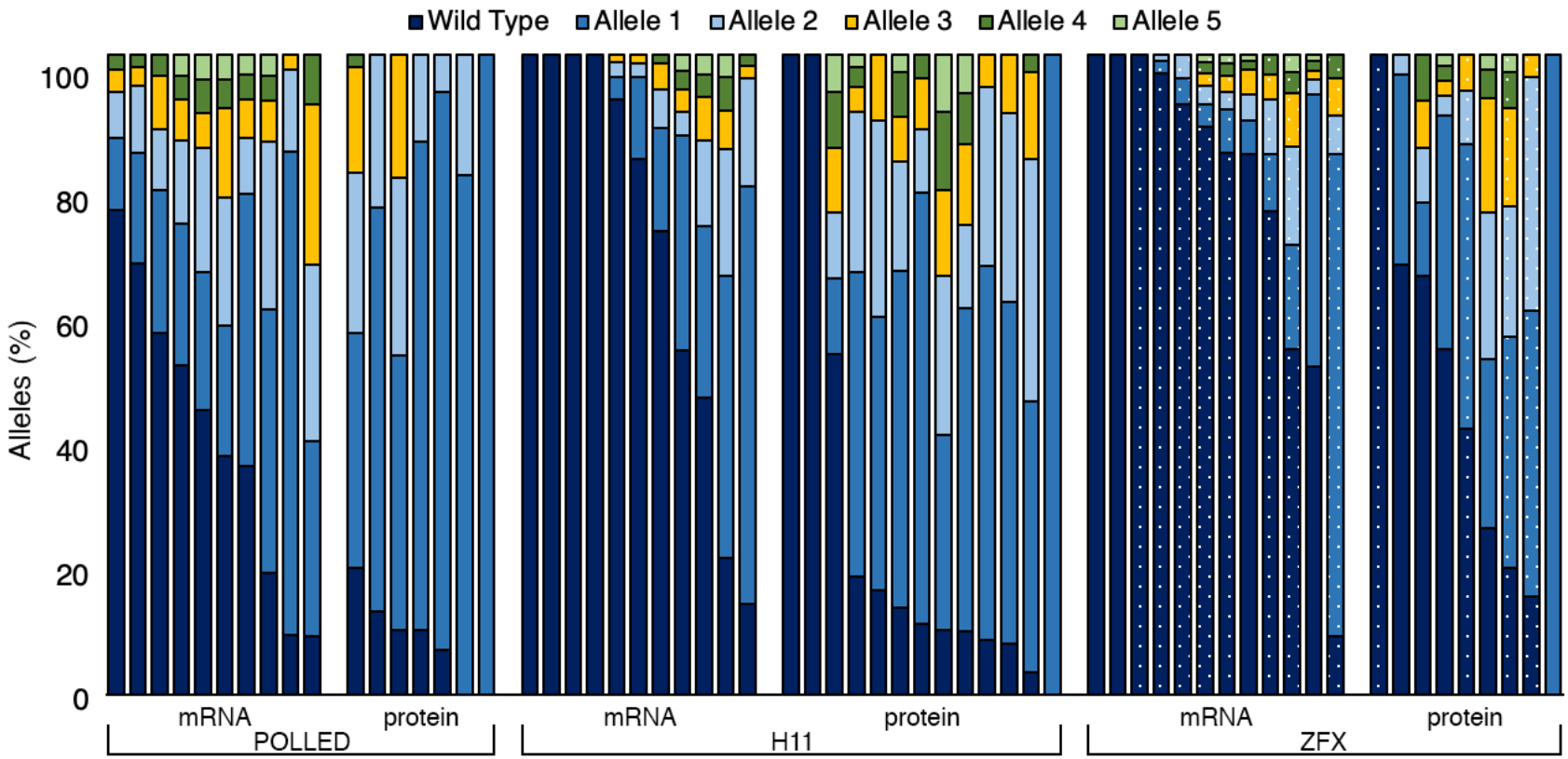

Figure 2. Bar graph depicting the percentage of alleles determined by PacBio sequencing in each of the 62 blastocysts microinjected 18 hours post insemination with either Cas9 mRNA or protein and gRNAs targeting the POLLED, H11 and ZFX loci. For ZFX locus: dotted bars are female; solid bars are male.

548 **Supplementary Data**

549 Evaluation of Mosaicism and Off Target Mutations in CRISPR-Mediated Genome Edited

550 Bovine Embryos

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560 **Supplementary Table S1.** Sequence of primers used for PCR amplification of the POLLED, H11,
561 or ZFX target regions, predicted off-target regions and gRNA sequences.

	Name	Sequence 5'- 3'	T_m (ºC)
On-Target primers	POLLEDgF	GAAGTGTGGCCGGTAGAAAA	62.8
	POLLEDgR	CGCTCCTTCCAAAACAAAAA	60.4
	H11gF	CCCCAGTGTTGTGCATGTAG	62.4
	H11gR	GTGAATGCCACTGCTGTGTT	60.4
	ZFXgF	AGCAGTGCTTCCAAACTTGAG	60.6
	ZFXgR	GATGAGAGCTTATGTAACTGTTGG	61.2
Off-Target primers	POLLEDoff1F	CAACTTCCCAGCTGTCTGC	59.0
	POLLEDoff1R	CCTTGTATGACGGCAACCTT	59.0
	POLLEDoff2F	TTCACTGCTCAAGGAAATGC	58.4
	POLLEDoff2R	AAGGCTATGAACTTGGGCTTT	58.7
	POLLEDoff3F	TTAAGCTTGGGCGTCTGAGT	59.0
	POLLEDoff3R	CATTTGGCTTTCGGCTACAC	59.0
	POLLEDoff4F	GAGGCAGATTTTGGCTTCAG	60.4
	POLLEDoff4R	GCCTCTGTCCACATGCTCTT	62.4
	POLLEDoff5F	CAGAGTCGGACACGACTGAA	62.4
	POLLEDoff5R	GCTGTGTCCTCCTAGGCTCA	64.5
	POLLEDoff6F	AAGGTTGTGTTGCATGTTGG	59.0
	POLLEDoff6R	AATTCCACTCCTCCAGAATCA	59.0
	POLLEDoff7F	TCTGGCATCACAGCATTTGT	58.4
	POLLEDoff7R	AAGATGCAAGAGACGCAGGT	60.4
	POLLEDoff8F	TTGGCCATGGACCTATGATT	59.0
	POLLEDoff8R	GGAGTGACATGGCACCTCATA	59.0
	H11off1F	GGAACAAAGATCCCACATGC	59.0
	H11off1R	GGCAGTCAAAACCCAAACAC	59.0
	H11off2F	GAATTCTGGGGGCATTGAC	60.2
	H11off2R	GAAGCCTAACCACCTCCACA	62.4
	H11off3F	CTCAGCTGGGTAACATGCAA	60.4
	H11off3R	GAGCAAATTGAGGTGGGTAA	58.4
	H11off4F	AATAAACCCCCAATTTGGCTA	56.7
	H11off4R	GGACTATCCCCTGGAGAAGG	64.5
	H11off5F	AGCCAGAGCTACTTGCTGGT	62.4
	H11off5R	AGGGTTCACTCTTGTTGGTG	60.4
	H11off6F	TGAATGGATAAGCTCCCTGTG	60.6
	H11off6R	GAATGGTCCAGTGGTTGTCC	62.4
	H11off7F	GGCAGAGAGGGAGAGAGACA	64.5
	H11off7R	TTGCCAGACATGAGAAGCAG	60.4
	H11off8F	CATGTAAATTTGGGGGTTGT	57.0
	H11off8R	CCTTCTAATTCTTGTCTGTTTGCTT	57.0
	H11off9F	CCTTGCAGATCAGCTCACAA	60.4
	H11off9R	AATGGCTTCTTCCCTCAGGA	60.4
	H11off10F	GGCTTTTTGCTCTGCTGTTT	58.4
	H11off10R	TCAGAGGACCAGATGATGGA	60.4

	H11off11F	GCACCGGGAGTTAATGTGTAA	60.6
	H11off11R	AAGGGACAAGGTGTGGACTG	62.4
	ZFXoff1F	GCAGCACCCAGAGTATCTCC	64.5
	ZFXoff1R	CCTGAGGTAGGGGGATTGTT	62.4
	ZFXoff2F	CCCCACTCCAGTACTCTTGC	64.5
	ZFXoff2R	TCCCGTGTTTTGTGTGATTT	56.3
	ZFXoff3F	TCATCTGGGCTGTTCTGAAG	60.4
	ZFXoff3R	AAGGTTCCTGCCTGCTTTTT	58.4
	ZFXoff4F	AAGGAAGGGGATTTTCTCCA	58.4
	ZFXoff4R	CACAGGGCTTTCTCCTTGAG	62.4
	ZFXoff5F	CAGCAAACTTTTCAGTGAGCA	58.7
	ZFXoff5R	TCCTCTCCTTTTTGGACATCA	58.7
Barcode Primers	BC1001F	CACATATCAGAGTGCGAGATCTCTCGAGGTT	62.0
	BC1001R	CACATATCAGAGTGCGGTAGTCGAATTCGTT	62.0
	BC1002F	ACACACAGACTGTGAGAGATCTCTCGAGGTT	62.0
	BC1002R	ACACACAGACTGTGAGGTAGTCGAATTCGTT	62.0
	BC1003F	ACACATCTCGTGAGAGAGATCTCTCGAGGTT	62.0
	BC1003R	ACACATCTCGTGAGAGGTAGTCGAATTCGTT	62.0
	BC1004F	CACGCACACACGCGCGAGATCTCTCGAGGTT	62.0
	BC1004R	CACGCACACACGCGCGGTAGTCGAATTCGTT	62.0
	BC1006F	CATATATATCAGCTGTAGATCTCTCGAGGTT	62.0
	BC1006R	CATATATATCAGCTGTGTAGTCGAATTCGTT	62.0
	BC1007F	TCTGTATCTCTATGTGAGATCTCTCGAGGTT	62.0
	BC1007R	TCTGTATCTCTATGTGGTAGTCGAATTCGTT	62.0
	BC1008F	ACAGTCGAGCGCTGCGAGATCTCTCGAGGTT	62.0
	BC1008R	ACAGTCGAGCGCTGCGGTAGTCGAATTCGTT	62.0
	BC1009F	ACACACGCGAGACAGAAGATCTCTCGAGGTT	62.0
	BC1009R	ACACACGCGAGACAGAGTAGTCGAATTCGTT	62.0
	BC1010F	ACGCGCTATCTCAGAGAGATCTCTCGAGGTT	62.0
	BC1010R	ACGCGCTATCTCAGAGGTAGTCGAATTCGTT	62.0
Guide RNA	POLLEDg1	GTCTATCCCAAAAGTGTGGG	-
	POLLEDg2	CCTGTGAAATGAAGAGTACG	-
	POLLEDg3	GATAGTTTTCTTGGTAGGC	-
	H11g1	TAGCCATAAGACTACCTAT	-
	H11g2	CTGGGGCAAAAGTCAACAGT	-
	H11g3	TGACTGGGAGGAGGAAGCCA	-
	ZFXg1	GCTAGTGGGCTAATGCCAGA	-
	ZFXg2	GCCGTCTCTCTATAGCTCAG	-
	ZFXg3	TCTTACAAGGGTGATAGTAC	-

562

563 **Supplementary Table S2.** Mutation rate in embryos for each guide injected 18 hours post
564 insemination alongside Cas9 protein analyzed using PCR and Sanger sequencing. Multiple guides
565 were tested targeting each locus to obtain highest efficiency guide. Letters that differ in the same
566 column are significantly different ($P < 0.05$). Each chromosome independently tested using a two-
567 by-two χ^2 test.

Allele	gRNA	Injected Embryos	Total Blastocysts (%)	Blastocysts Analyzed	Mutation Rate (%)
POLLED	1	47	15 (32[a])	13	0 (0)[a]
	2	75	14 (19[b])	13	10 (77)[b]
	3	90	25 (28[a])	25	2 (8)[a]
H11	1	65	12 (18[b])	12	10 (83)[b]
	2	45	13 (29[a])	13	5 (38)[a]
	3	47	10 (21[b])	10	6 (60)[ab]
ZFX	1	75	22 (29[a])	19	1 (5)[a]
	2	86	22 (26[a])	21	5 (24)[a]
	3	104	18 (17[b])	18	14 (78)[b]

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569

570 **Supplementary Table S3.** List of sequencing barcodes used for PacBio sequencing for embryos
571 injected 18 hours post insemination with gRNAs targeting the POLLED, H11, and ZFX loci
572 alongside Cas9 mRNA or protein and corresponding reads per sample following sorting by
573 barcode. Red highlighted samples were removed from analysis due to insufficient read count.

Locus	Cas9	Sample	Forward Barcode	Reverse Barcode	Reads per Sample
POLLED	mRNA	1	BC1001F	BC1001R	3359
		2	BC1001F	BC1002R	1049
		3	BC1001F	BC1003R	1446
		4	BC1001F	BC1004R	1075
		5	BC1001F	BC1006R	1118
		6	BC1001F	BC1007R	598
		7	BC1001F	BC1008R	472
		8	BC1001F	BC1009R	2632
		9	BC1001F	BC1010R	2662
		10	BC1002F	BC1001R	2236
	protein	1	BC1002F	BC1003R	24
		2	BC1002F	BC1004R	276
		3	BC1002F	BC1006R	812
		4	BC1002F	BC1007R	654
		5	BC1002F	BC1008R	12
		6	BC1002F	BC1009R	543
		7	BC1002F	BC1010R	1622
		8	BC1003F	BC1001R	1445
		9	BC1003F	BC1002R	417
H11	mRNA	1	BC1003F	BC1004R	3762
		2	BC1003F	BC1006R	3910
		3	BC1003F	BC1007R	1203
		4	BC1003F	BC1008R	3111
		5	BC1003F	BC1009R	3267
		6	BC1003F	BC1010R	2745
		7	BC1004F	BC1001R	7681
		8	BC1004F	BC1002R	1624
		9	BC1004F	BC1003R	1579
		10	BC1004F	BC1004R	1552
		11	BC1004F	BC1006R	1937
	protein	1	BC1004F	BC1008R	37
		2	BC1004F	BC1009R	1693
		3	BC1004F	BC1010R	8
		4	BC1006F	BC1001R	6795
		5	BC1006F	BC1002R	1197
		6	BC1006F	BC1003R	1567
		7	BC1006F	BC1004R	1926
		8	BC1006F	BC1006R	2045

Locus	Cas9	Sample	Forward Barcode	Reverse Barcode	Reads per Sample
H11	protein	9	BC1006F	BC1007R	1108
		10	BC1006F	BC1008R	1472
		11	BC1006F	BC1009R	1213
		12	BC1006F	BC1010R	1937
		13	BC1007F	BC1001R	2163
		14	BC1007F	BC1002R	1838
		15	BC1007F	BC1003R	1500
ZFX	mRNA	1	BC1007F	BC1007R	1630
		2	BC1007F	BC1008R	1603
		3	BC1007F	BC1009R	3973
		4	BC1007F	BC1010R	3531
		5	BC1008F	BC1001R	4960
		6	BC1008F	BC1002R	1720
		7	BC1008F	BC1003R	1521
		8	BC1008F	BC1004R	1530
		9	BC1008F	BC1006R	1039
		10	BC1008F	BC1007R	17
		11	BC1008F	BC1008R	2037
		12	BC1008F	BC1009R	1484
		13	BC1008F	BC1010R	1614
	protein	1	BC1009F	BC1001R	4954
		2	BC1009F	BC1002R	1240
		3	BC1009F	BC1003R	27
		4	BC1009F	BC1004R	1564
		5	BC1009F	BC1006R	1280
		6	BC1009F	BC1007R	1324
		7	BC1009F	BC1008R	2102
		8	BC1009F	BC1009R	2304
		9	BC1009F	BC1010R	74
		10	BC1010F	BC1001R	4014
		11	BC1010F	BC1002R	1812

574

575 **Supplementary Table S4.** Number of PacBio sequencing reads of PCR products from 69
576 blastocysts microinjected with Cas9 editing reagents targeting three loci (POLLED, H11, and
577 ZFX) in the bovine genome, and the percentage of reads that were <700 bp read length, and
578 additionally had a unique blastocyst sample identifying barcode.
579

Filtered By	Locus	Passed	Total	Percent
Read length	Total	171,545	236,518	72.5
Barcode	POLLED	22,416	26,460	84.7
Barcode	H11	58,815	78,305	75.1
Barcode	ZFX	47,236	66,780	70.7

580

Supplementary Table S5. Prevalence of different allele types from PacBio sequencing of targeted PCR products < 700 bp from 69 blastocysts microinjected with Cas9 editing reagents targeting three loci (POLLED, H11, and ZFX) in the bovine genome. Types of mutations = location relative to the cut site (3bp upstream of the PAM sequence): type of deletion; D = deletion, I = insertion. "Other" mutations indicate those with reads too few to report.

Locus	Total Number of Reads	Wild Type Alleles (%)	Type of Mutation				
			# of Reads (%)				
POLLED	26460	12719 (48)	**-16:7D**	**-14:11D**	**-13:4D**	**-10:1I**	**Other**
			1672 (6)	1751 (7)	6356 (24)	2250 (19)	1712 (6)
H11	78305	59302 (76)	**-14:11D**	**-13:6D**	**-12:3D**	**-10:1D**	**-8:1D**
			3246 (4)	3813 (5)	4091 (5)	5061 (6)	2792 (4)
ZFX	66780	47939 (71)	**-10:14D**	**-4:9D**	**-1:3D**	**1:1I**	**2:1I**
			4222 (6)	2998 (4)	3198 (5)	2194 (3)	6532 (10)

588 **Supplementary Table S6.** Number of alleles and percentage of each corresponding allele per sample detected at the cut-site of Cas9
589 mRNA or protein injected embryos. WT = percentage of reads that were wild type sequence. Alleles 1-5 are percent reads with each of
590 the alleles found in the samples. Bold samples contained no wild type sequence. n/a = not applicable; genotypic sex was only determined
591 for samples targeting the X chromosome.

Locus	Cas9	Sample	Sex	# of alleles	% of Reads for Each Allele					
					WT	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
POLLED	mRNA	1	n/a	5	57	22	10	8	3	-
		2	n/a	5	76	11	7	4	2	-
		3	n/a	6	37	44	9	6	4	3
		4	n/a	5	9	31	28	25	8	-
		5	n/a	6	46	22	20	6	6	4
		6	n/a	5	67	17	11	3	2	-
		7	n/a	4	9	75	13	2	-	-
		8	n/a	6	20	43	27	7	4	3
		9	n/a	6	53	23	13	7	4	3
		10	n/a	6	39	21	21	15	5	4
	protein	1	n/a	4	10	43	28	19	-	-
		2	n/a	3	13	63	24	-	-	-
		3	n/a	3	7	87	6	-	-	-
		4	n/a	3	10	76	14	-	-	-
		5	n/a	**2**	**-**	**81**	**19**	**-**	**-**	**-**
		6	n/a	5	20	37	25	16	2	-
		7	n/a	**1**	**-**	**100**	-	-	-	-
H11	mRNA	1	n/a	6	54	34	4	3	3	3
		2	n/a	6	21	44	20	6	5	4
		3	n/a	5	14	65	17	2	2	-
		4	n/a	6	46	27	14	7	3	3
		5	n/a	5	72	16	6	4	2	-
		6	n/a	4	84	13	2	1	-	-
		7	n/a	4	93	4	2	1	-	-
		8	n/a	1	100	-	-	-	-	-

Locus	Cas9	Sample	Sex	# of alleles	% of Reads for Each Allele					
					WT	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
H11	mRNA	9	n/a	1	100	-	-	-	-	-
		10	n/a	1	100	-	-	-	-	-
		11	n/a	1	100	-	-	-	-	-
	protein	1	n/a	1	100	-	-	-	-	-
		2	n/a	6	53	12	10	10	9	6
		3	n/a	6	10	50	13	13	8	6
		4	n/a	6	14	53	17	7	7	3
		5	n/a	1	100	-	-	-	-	-
		6	n/a	6	18	48	25	4	3	2
		7	n/a	4	8	54	29	9	-	-
		8	n/a	4	8	59	28	5	-	-
		9	n/a	**1**	**-**	**100**	-	-	-	-
		10	n/a	5	11	67	10	8	4	-
		11	n/a	6	10	30	25	13	12	9
		12	n/a	5	4	42	38	14	3	-
		13	n/a	4	16	43	31	10	-	-
ZFX	mRNA	1	female	5	9	75	6	6	4	-
		2	male	6	51	43	2	1	1	1
		3	female	5	76	9	9	4	3	-
		4	female	3	97	2	1	-	-	-
		5	male	6	85	5	4	4	1	1
		6	female	6	54	16	15	8	3	3
		7	male	1	100	-	-	-	-	-
		8	female	3	92	4	4	-	-	-
		9	female	6	85	7	3	3	2	2
		10	female	6	89	4	3	2	2	1
		11	female	1	100	-	-	-	-	-
		12	male	1	100	-	-	-	-	-
	protein	1	male	5	65	12	8	7	7	-

Locus	Cas9	Sample	Sex	# of alleles	% of Reads for Each Allele					
					WT	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
ZFX	protein	2	male	6	54	37	3	2	2	2
		3	female	1	100	-	-	-	-	-
		4	female	4	42	44	8	6	-	-
		5	male	6	26	26	23	18	5	2
		6	male	**1**	**-**	**100**	**-**	**-**	**-**	**-**
		7	male	3	67	30	3	-	-	-
		8	female	6	20	36	20	16	6	3
		9	female	4	15	45	36	4	-	-

595 **Supplementary Table S7.** Predicted off-target sites for each of the three guides targeting the POLLED, H11, or ZFX locus. DNA =
596 sequence of off-target site (lower case bases are mismatches). Position is relative to the start of the bosTau8 reference genome. Total
597 reads aligned = number of reads mapped to the off-target sequence from overlapped MiSeq data. Coverage = reads per sample per target.

598

Target Locus	Off-Target Chromosome	DNA	Position	Direction	Total Reads Aligned	Coverage
POLLED	2	CaTGTGAAtTGAAGAGTACc	17859417	+	301211	35X
	10	CCTcTGgAATGAAGAGTACc	23332597	-	94118	11X
	12	CCTGTGAAATGActAGTACa	57833284	+	256177	30X
	14	CCTcTGAAATGAAGAGaACc	83079285	+	85603	10X
	18	CCTGaGAAATGAAGAGgAtG	34058298	-	54877	6X
	18	CtTGTGcAAaGAAGAGTACG	46423386	-	301211	35X
	X	CCTGTGAgATGAtGAtTACG	31206746	+	430923	51X
	X	gCTGTGAAATGAAGAGgAtG	129076601	+	713874	84X
H11	1	TAGCCATAAGcaTACCaAT	3616887	+	21658	2X
	1	TAGCCATAAGtCaACaTAT	7454978	+	1022399	97X
	1	TAGCCAcAAGtCTACaTAT	12203491	-	878840	84X
	1	gAGaCATAAGACTACCcAT	111862587	+	226674	22X
	4	TAGCCATAAGAaTtCCTAa	102992457	-	249056	24X
	7	TAGCaATAAGAgTACCTAa	8578648	-	162124	15X
	7	TAGtCATAAttCTACCTAT	75383424	-	12118	1X
	7	aAGCCATAcaACTACCTAT	75200649	-	265923	25X
	8	TAGCCATcAGACTACCaAg	62416292	-	717812	68X
	10	TAGCCAaAAGACaACaTAT	59699661	+	1104588	105X
	X	TAGCaATAAGAgTAaCTAT	125300735	+	1179081	112X
ZFX	7	TCTTAaAAGGGTGATAaTAt	112332349	+	125193	13X
	12	TCTTACAgaGaTGATAGTAC	22046100	-	474201	47X
	21	aaTTACAgGGGTGATAGTAC	28506796	-	470743	47X
	21	TCTTACAAGaGTcATAGTgC	48414495	-	329628	33X
	27	TCcTAgAAGGGTGATcGTAC	8648733	-	924826	92X

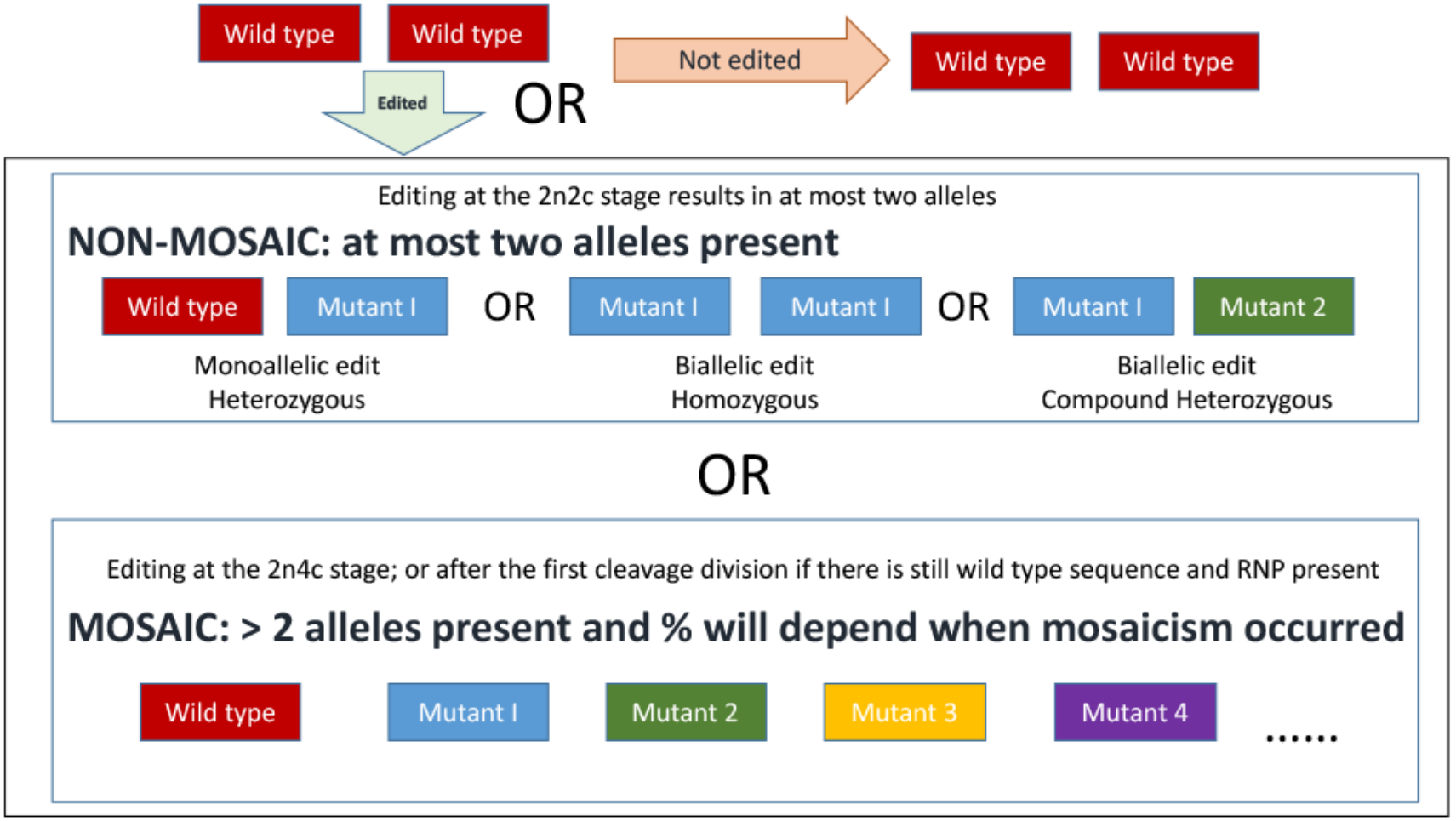
599 **Supplementary Table S8.** Results for development and mutation rate from each replicate of control embryos, and groups injected 18
600 hours post insemination with gRNAs targeting the POLLED, H11, and ZFX loci alongside Cas9 mRNA or protein

Locus	Cas9	# Embryos	# Blastocyst	% Blastocyst	# Blastocysts evaluated	# Blastocysts With mutation	% Mutation
POLLED	mRNA	30	7	23.3	7	4	57.1
POLLED	mRNA	29	5	17.2	5	3	60
POLLED	protein	26	4	15.4	3	3	100
POLLED	protein	27	5	18.5	4	3	75
POLLED	protein	27	3	11.1	2	2	100
POLLED	protein	28	5	17.9	4	3	75
POLLED	protein	26	6	23.1	5	4	80
POLLED	protein	27	4	14.8	3	2	66.7
POLLED	protein	26	4	15.4	3	3	100
POLLED	protein	26	5	19.2	4	3	75
POLLED	protein	27	5	18.5	4	3	75
POLLED	protein	26	4	15.4	3	3	100
H11	mRNA	26	3	11.5	3	2	66.7
H11	mRNA	27	5	18.5	4	3	75
H11	mRNA	26	3	11.5	3	2	66.7
H11	mRNA	27	4	14.8	4	3	75
H11	mRNA	25	3	12	3	2	66.7
H11	protein	26	4	15.4	3	3	100
H11	protein	26	5	19.2	4	3	75
H11	protein	24	3	12.5	3	3	100
H11	protein	26	4	15.4	3	3	100
ZFX	mRNA	26	4	15.4	4	3	75
ZFX	mRNA	26	4	15.4	4	2	50
ZFX	mRNA	27	5	18.5	5	3	60
ZFX	mRNA	26	5	19.2	5	3	60
ZFX	mRNA	26	4	15.4	4	3	75
ZFX	mRNA	27	4	14.8	4	2	50
ZFX	mRNA	26	3	11.5	3	2	66.7
ZFX	mRNA	25	5	20	5	4	80

ZFX	mRNA	26	3	11.5	3	1	33.3
ZFX	mRNA	25	4	16	4	3	75
ZFX	mRNA	26	5	19.2	5	4	80
ZFX	mRNA	26	4	15.4	4	2	50
ZFX	protein	26	4	15.4	4	3	75
ZFX	protein	26	4	15.4	4	3	75
ZFX	protein	27	5	18.5	5	4	80
ZFX	protein	26	5	19.2	5	5	100
ZFX	protein	26	4	15.4	4	3	75
ZFX	protein	24	4	16.7	4	3	75
ZFX	protein	26	3	11.5	3	3	100
ZFX	protein	25	5	20	5	4	80
ZFX	protein	26	3	11.5	3	2	66.7
ZFX	protein	25	4	16	4	3	75
ZFX	protein	26	5	19.2	5	4	80
ZFX	protein	26	4	15.4	4	4	100
ZFX	protein	26	4	15.4	4	3	75
ZFX	protein	25	5	20	5	4	80
ZFX	protein	25	4	16	4	3	75
ZFX	protein	26	4	15.4	4	4	100
ZFX	protein	24	3	12.5	3	2	66.7
ZFX	protein	25	4	16	4	3	75
ZFX	protein	26	4	15.4	4	3	75
ZFX	protein	26	3	11.5	3	2	66.7
control	-	30	9	30	-	-	-
control	-	30	8	26.7	-	-	-
control	-	30	7	23.3	-	-	-
control	-	30	6	20	-	-	-
control	-	30	13	43.3	-	-	-
control	-	30	8	26.7	-	-	-
control	-	30	10	33.3	-	-	-
control	-	29	9	31	-	-	-
control	-	30	8	26.7	-	-	-
control	-	30	13	43.3	-	-	-

control	-	30	8	26.7	-	-	-
control	-	30	9	30	-	-	-
control	-	30	7	23.3	-	-	-
control	-	30	8	26.7	-	-	-
control	-	27	9	33.3	-	-	-
control	-	30	10	33.3	-	-	-
control	-	30	8	26.7	-	-	-
control	-	30	5	16.7	-	-	-
control	-	29	7	24.1	-	-	-
control	-	30	6	20	-	-	-
control	-	30	8	26.7	-	-	-
control	-	29	9	31	-	-	-
control	-	30	8	26.7	-	-	-
control	-	30	14	46.7	-	-	-
control	-	30	8	26.7	-	-	-
control	-	28	13	46.4	-	-	-
control	-	30	8	26.7	-	-	-
control	-	30	7	23.3	-	-	-
control	-	29	8	27.6	-	-	-
control	-	30	9	30	-	-	-
control	-	30	12	40	-	-	-
control	-	30	11	36.7	-	-	-
control	-	30	9	30	-	-	-
control	-	28	9	32.1	-	-	-
control	-	30	10	33.3	-	-	-
control	-	30	13	43.3	-	-	-
control	-	30	7	23.3	-	-	-
control	-	26	8	30.8	-	-	-
control	-	27	9	33.3	-	-	-
control	-	30	12	40	-	-	-
control	-	29	11	37.9	-	-	-
control	-	30	10	33.3	-	-	-

601



Supplementary Figure S1. Schematic representation of possible outcomes from CRISPR-mediated mutation by cytoplasmic injection of an *in vitro* fertilized embryo 18 hours post insemination. 2n = number of homologous chromosomes, i.e. diploid. 2c/4c = number of copies of chromosomes either before DNA replication or after