EMBO reports

A genetic system for biasing the sex ratio in mice

Ido Yosef, Liat Edry-Botzer, Rea Globus, Inbar Shlomovitz, Ariel Munitz, Motti Gerlic, and Udi Qimron

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Transaction Report:

Please note that the manuscript was previously reviewed at another journal and the reports were taken into account in the decision making process at EMBO Reports. Since the original reviews are not subject to EMBO Press' transparent review process policy, the reports and author response cannot be published.

1st Editorial Decision 11th Apr 2019

Thank you for the submission of your revised manuscript, the referee reports and your point-by-point response from your previous submission (to a journal outside EMBO press). I read your manuscript, went through the other files, and discussed your manuscript with my colleagues. We feel that the submitted revised version adequately addresses the concerns of the referees. We have also contacted an expert advisor, who examined your manuscript, the referee reports and the point-by-point response, and indicated that the revised paper is technically sound.

Moreover, your paper has been seen by advisors for bioethics and animal welfare. Considering their feedback, we would like to invite you to revise your manuscript, addressing the following points:

- Please provide a more critical evaluation of the experiments and a thorough discussion. The preliminary nature of these data, in particular for breeding applications (e.g. chicken), needs to be more clearly noted.

- Please discuss application caveats in more detail. E.g., the resulting female animals are genetically modified. Thus, farmers would need to be very careful that they do not spread to nature. Does it mean that such female farm animals need to be inside all their life? And, if there is a risk that the unwanted sex may still be born, is there still a need to check the sex of all new-borns? And, do we need extra welfare checks of female animals born? The focus of this paper is rather commercial, but the welfare of the animals is not very much discussed.

- Please discuss explicitly the problem that some male animals are born. Our advisor states: 'It is a serious problem if part of the unwanted sex is born with serious welfare problems, like in this study, even if these were few animals and there seems to be a possibility to sort this problem. But, we do not know how well this method would be applicable to birds or cows, for example. Having animals born without legs or other severe malformations even at low numbers is not acceptable.'

- Please provide detailed information on animal welfare. In the methods section the information on the mice is very poor. Please refer to the ARRIVE guidelines (see link below). E.g. information on

housing and care is missing. Animal welfare and the welfare assessment should be discussed. Further, detailed information when the embryos are dying needs to be provided:

* At what time point the majority of the individuals of unwanted sex die during the pregnancy? Is it at early stages or later?
* What type of welfare assessment was done on the female pups born?
* How long did the pups live, and have there been any health problems later?

Please follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://embor.embopress.org/authorguide#livingorganisms

Moreover, I have these editorial requests:

- Please add up to five key words and a short running title (not more than 40 characters) to the title page.

- Please make sure that in the final manuscript all figures are correctly called out.

- We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- Please format the references according to our journal style. See: http://embor.embopress.org/authorguide#referencesformat

- Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://embor.embopress.org/authorguide#referencesformat

- Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See: http://embor.embopress.org/authorguide#statisticalanalysis

Finally, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

1st Revision - authors' response 14th May 2019

Please see our response in **Bolded blue** font.

Thank you for the submission of your revised manuscript, the referee reports and your point-by-point response from your previous submission (to a journal outside EMBO press). I read your manuscript, went through the other files, and discussed your manuscript with my colleagues. We feel that the submitted revised version adequately addresses the concerns of the referees. We have also contacted an expert advisor, who examined your manuscript, the referee reports and the point-by-point response, and indicated that the revised paper is technically sound.

We are pleased that both the editors and an expert advisor find that the revised version of the paper adequately addresses previous concerns and that the paper is technically sound.

Moreover, your paper has been seen by advisors for bioethics and animal welfare. Considering their feedback, we would like to invite you to revise your manuscript, addressing the following points:

- Please provide a more critical evaluation of the experiments and a thorough discussion.

In response to this comment and the following comments, we have added the following paragraph listing the requirement from a GMO system, with emphasis on how the current system complies or not with these requirements.

"A commercial GMO must meet several statutory requirements for safety and effectiveness under the FDA act. First, the GMO products must be safe to consume. Second, the introduced DNA must be safe to the modified organism itself. Third, the modified organism should be superior, at least in one trait, over a non-GMO. Lastly, the potential environmental impact of the GMO should be non-significant. Thus, before the proposed genetic system could be used for producing, e.g., cattle, a food safety comparison between the meat and milk from non-GM and GM cows must first be conducted. The test should compare key hormones, such as estradiol, testosterone, insulin-like growth factor-1, and other hormones in the samples. It should also assess the key nutritional constituents such as protein, carbohydrate, and fat levels. No significant difference between the samples should be found in both tests. We expect that animals produced by the proposed genetic system will pass these tests, as the transgenes that are used are not known to have any direct impact on the levels of these factors. The transgenes in the proposed system are harmful for the GM male animal, as few males are born with genetic defects and deformations (Fig. 2). Although only a minor percentage of males are born with defects, such a system cannot be approved in its present form. A more robust elimination system should be applied, which kills all males *in utero*. One way to achieve such elimination is by targeting the essential genes with more than a single gRNA for each target. As shown in Figure 2, all gene targets were disrupted to some extent in the males, however, in some cases, this was insufficient to kill them *in utero*. Using, multiple gRNAs against each gene, should ensure that at least one gene would be deactivated at the early embryo stage, thus resulting in lethality, and consequently in a transgene that does not affect the safety of the male GMO. It is noteworthy that most of the male embryos probably dye early during the pregnancy. A thorough study characterizing 5,000 knockout mice lines showed that only 4% of 410 lethal embryonic phenotypes occurred between E12.5 to E18.5 whereas the rest are lethal at earlier stages [20]. We speculate that in our experiments too, lethality of most of the male embryos occurred early (i.e., prior to E12.5) and were absorbed during the pregnancy. This speculation is supported by the fact that only one deformed male pup was born (Fig. 2) and also with the choice of target genes: *Atp5b*, *Cdc20*, and *Casp8*, all shown to be essential for mouse early development [20-22]. The safety of the transgene to the female GMOs, which are the desired animals in the system, is arguably sound. Each of the transgenes (Cas9 or gRNAs) alone does not cause any DNA damage by itself. The female embryo does not produce at any stage the gRNAs, as they are encoded on the Y chromosome. We have further validated by DNA sequencing of a control female that the target genes are intact (Fig. EV3). In addition, female offspring of the Cas9 and Y-line cross showed no sign of illness over a monitoring

period of four months. Furthermore, these females were examined at 6 weeks of age for fertility by crossing them with Cas9 males and monitoring the pregnancy and sex ratio of their pups. All examined females were fertile, providing normal litter size and normal sex ratio, as expected (average litter size was 6.75 totaling 14 males and 12 females and 1 infanticide). Thus, it is probable that the safety and welfare of the GMO females is not affected compared to their non-GMO counterparts. Regarding efficacy, the system produces significantly higher proportion of desired females compared to the non-GMO. This ratio will further be increased when a more robust elimination system is used, as indicated above. A system producing solely females without males will completely overcome the requirement for manual separation of the sexes. Lastly, environmental safety should be maintained by multiple redundant containment conditions to prevent their escape into the wild and consequent propagation there. For example, the animals should be fenced at all time and the husbandries should be locked to prevent theft and unauthorized and untraceable distribution of the animals. Small location devices can also be individually applied for large animals such as cows, in which the device's cost is minor compared to the animal's cost. It is important to note that a GM cow inadvertently released to the wild or escaping the husbandry, will unlikely cause a significant long-term damage, and will most likely be recaptured. This is in contrast to smaller fast-reproducing animals such as fish in which case the containment methods should be multi-layered including biological barriers (e.g., infertility) in addition to physical barriers of escape. Thus, it may be assumed that the environmental impact of GM cows would be considered insignificant. Overall, the proposed system at its current form would only partially meet the statutory requirements for safety and effectiveness. Nevertheless, we believe that upon further improvements listed above, it may satisfy these requirements."

The preliminary nature of these data, in particular for breeding applications (e.g. chicken), needs to be more clearly noted.

The above paragraph that we added demonstrates the overall pros and cons of the genetic system. We have further added explicitly that the commercialization of the genetic system requires the passing of many obstacles, and that the proof of principle study is a first step in the direction: "Sexing the semen is nevertheless superior to the current proposed genetic system, as the litter size is not reduced, and the offspring is not GMO. There are therefore further obstacles for optimizing the current methodology for biasing the sex and for making it commercially sound. Nevertheless, the proposed proof of principle is a first step in this direction"

- Please discuss application caveats in more detail. E.g., the resulting female animals are genetically modified. Thus, farmers would need to be very careful that they do not spread to nature. Does it mean that such female farm animals need to be inside all their life?

This is now discussed in the added paragraph above. Specifically: "Lastly, environmental safety should be maintained by multiple redundant containment conditions to prevent their escape into the wild and consequent propagation there. For example, the animals should be fenced at all time and the husbandries should be locked to prevent theft and unauthorized and untraceable distribution of the animals. Small location devices can also be individually applied for large animals such as cows, in which the device's cost is minor compared to the animal's cost. It is important to note that a GM cow inadvertently released to the wild or escaping the husbandry, will unlikely cause a significant long-term damage, and will most likely be recaptured. This is in contrast to smaller fast-reproducing animals such as fish in which case the containment methods should be multi-layered including biological barriers (e.g., infertility) in addition to physical barriers of escape. Thus, it may be assumed that the environmental impact of GM cows would be considered insignificant."

And, if there is a risk that the unwanted sex may still be born, is there still a need to check the sex of all new-borns?

This issue is now addressed. We reiterate that only a system with no male production should be approved, and consequently there should be no need for sexing the animals: "The transgenes in the proposed system are harmful for the GM male animal, as few males are born with genetic defects and deformations (Fig. 2). Although only a minor percentage of males are born with defects, such a system cannot be approved in its present form. A more robust elimination system should be applied, which kills all males *in utero*. One way to achieve such elimination is by targeting the essential genes with more than a single gRNA for each target. As shown in Figure 2, all gene targets were disrupted to some extent in the males, however, in some cases, this was insufficient to kill them *in utero*. Using, multiple gRNAs against each gene, should ensure that at least one gene would be deactivated at the early embryo stage, thus resulting in lethality, and consequently in a transgene that does not affect the safety of the male GMO. It is noteworthy that most of the male embryos probably dye early during the pregnancy. A thorough study characterizing 5,000 knockout mice lines showed that only 4% of 410 lethal embryonic phenotypes occurred between E12.5 to E18.5 whereas the rest are lethal at earlier stages [20]. We speculate that in our experiments too, lethality of most of the male embryos occurred early (i.e., prior to E12.5) and were absorbed during the pregnancy. This speculation is supported by the fact that only one deformed male pup was born (Fig. 2) and also with the choice of target genes: *Atp5b*, *Cdc20*, and *Casp8*, all shown to be essential for mouse early development [20-22]."

And, do we need extra welfare checks of female animals born? The focus of this paper is rather commercial, but the welfare of the animals is not very much discussed.

Likewise, this issue is now addressed: "The safety of the transgene to the female GMOs, which are the desired animals in the system, is arguably sound. Each of the transgenes (Cas9 or gRNAs) alone does not cause any DNA damage by itself. The female embryo does not produce at any stage the gRNAs, as they are encoded on the Y chromosome. We have further validated by DNA sequencing of a control female that the target genes are intact (Fig. EV3). In addition, female offspring of the Cas9 and Y-line cross showed no sign of illness over a monitoring period of four months. Furthermore, these females were examined at 6 weeks of age for fertility by crossing them with Cas9 males and monitoring the pregnancy and sex ratio of their pups. All examined females were fertile, providing normal litter size and normal sex ratio, as expected (average litter size was 6.75 totaling 14 males and 12 females and 1 infanticide). Thus, it is probable that the safety and welfare of the GMO females is not affected compared to their non-GMO counterparts."

- Please discuss explicitly the problem that some male animals are born. Our advisor states: 'It is a serious problem if part of the unwanted sex is born with serious welfare problems, like in this study, even if these were few animals and there seems to be a possibility to sort this problem. But, we do not know how well this method would be applicable to birds or cows, for example. Having animals born without legs or other severe malformations even at low numbers is not acceptable.'

We certainly agree, and now explicitly state this in the text: "The transgenes in the proposed system are harmful for the GM male animal, as few males are born with genetic defects and deformations (Fig. 2). Although only a minor percentage of males are born with defects, such a system cannot be approved in its present form. A more robust elimination system should be applied, which kills all males *in utero*. One way to achieve such elimination is by targeting the essential genes with more than a single gRNA for each target. As shown in Figure 2, all gene targets were disrupted to some extent in the males, however, in some cases, this was insufficient to kill them *in utero*. Using, multiple gRNAs against each gene, should ensure that at least one gene would be deactivated at the early embryo stage, thus resulting in lethality, and consequently in a transgene that does not affect the safety of the male GMO. It is noteworthy that most of the male embryos probably dye early during the pregnancy. A thorough study characterizing 5,000 knockout mice lines showed that only 4% of 410 lethal embryonic phenotypes occurred between E12.5 to E18.5 whereas the rest are lethal at earlier

stages [20]. We speculate that in our experiments too, lethality of most of the male embryos occurred early (i.e., prior to E12.5) and were absorbed during the pregnancy. This speculation is supported by the fact that only one deformed male pup was born (Fig. 2) and also with the choice of target genes: *Atp5b*, *Cdc20*, and *Casp8*, all shown to be essential for mouse early development [20-22].”

- Please provide detailed information on animal welfare. In the methods section the information on the mice is very poor. Please refer to the ARRIVE guidelines (see link below). E.g. information on housing and care is missing.

We have followed the guidelines and added the required information:

“Ethical statement: All animal experiments conformed with the guidelines of the Tel Aviv University’s Animal Ethics Committee, which follow the state law of prevention of animal cruelty (1994), the guidelines for preventions of animal cruelty published by the council for animal experimentation (2001), and The NRC Guide for the Care and Use of Laboratory Animals. The Animal Facilities in Tel Aviv University also work under a permit by the NIH [F16-00009 (A5010-01)].

Housing and husbandry: All mice were housed and bred under specific pathogen-free conditions maintained in the accredited animal facility in the Sackler Faculty of Medicine at Tel Aviv University. Mice were housed with an inverse 12-hour day-night cycle with lights on at 7:00AM in a temperature (22±1°C) and humidity (55±5%) controlled room. All mice were allowed free access to water and food including sunflower seeds. All cages contained wood shavings, bedding and a cardboard tube for environmental enrichment.

Experimental animals: For all breeding experiments males and females (>20 g), 8-52 weeks of age, were used (n=29). All obtained mice were acclimatized for at least 96 h. Vendor health reports indicated that the mice were free of known viral, bacterial and parasitic pathogens.

Two independent mice of the Y-line (generated from two positive ES cloned: 2E6 and 2H8) were constructed by Cyagen Biosciences (California, USA). These C57BL/6N mice encode the following guide RNAs on their Y chromosome: 5’-CACTGCCACCGGGCGAATCG-3’; 5’-CAGACCTGAATCTTGTAGAT-3’; 5’-TGCAGAGATGAGCCTCAAAA-3’ targeting the genes *Atp5b*, *Cdc20*, and *Casp8*, respectively. These guides were cloned into a vector targeting the reverse orientation of the 2nd exon of the Y chromosome *Uty* gene, which is not part of the pseudoautosomal Y region. Fig. EV1 provides a schematic summary and the Appendix provides detailed description of the Y-line construction.

Mice of the Cas9-line were purchased from Jackson laboratories (Stock No: 026179; Rosa26-Cas9 knockin on B6J) [19]. These mice encode a cassette in the Rosa26 locus on chromosome 6 constitutively expressing the SpCas9 endonuclease from a CAG promoter.

Study design: Ten Cas9-line and five B6J females were crossed with Y-line males. Six F1 females from the Cas9 and Y lines cross were further crossed with Cas9 males to confirm their normal breeding and offspring sex ratio.

Experimental procedures: Pregnancy was monitored daily in the above crosses for any sign of stress. Sex was determined by PCR of the Y chromosome on DNA extracted from the animal’s tail. Sex was further confirmed at day 7 and at weaning by observing the genitals. Sanger sequencing of the target regions was carried out following PCR amplification of these regions (see Appendix Tables S1+S2 for oligonucleotides and PCR set-ups).

Breeding pairs were monitored daily and pups were monitored twice a day till weaning and twice a week following weaning. Signs of illness (decreased mobility, ruffled fur, pause in weight gaining, or labored breathing) were monitored by researchers and animal technicians and by consultation with a veterinarian. During the entire duration of the experiments, none of the Y-line males or their pups displayed overt signs of sickness, except the reported deformed male (Fig. 2).

Statistics: Data are presented as the mean ± SD. Comparisons were performed using two-tailed unpaired parametric t-test or two-tailed binomial test, assuming normal distribution or two-tailed t-test of the variance–covariance matrix of the standard errors."

Animal welfare and the welfare assessment should be discussed.

This is now added to the text: "Breeding pairs were monitored daily and pups were monitored twice a day till weaning and twice a week following weaning. Signs of illness (decreased mobility, ruffled fur, pause in weight gaining, or labored breathing) were monitored by researchers and animal technicians and by consultation with a veterinarian. During the entire duration of the experiments, none of the Y-line males or their pups displayed overt signs of sickness, except the reported deformed male (Fig. 2)."

Further, detailed information when the embryos are dying needs to be provided:
* At what time point the majority of the individuals of unwanted sex die during the pregnancy? Is it at early stages or later?

We have now added the day of death due to lack of each of the three targeted genes in the text: "*Atp5b* deficiency in mice results in embryonic lethality prior to organogenesis at embryonic day 9.5 (E9.5) [20]; *Cdc20* deficiency in mice results in metaphase arrest in the two-cell stage embryos and consequently in early embryonic death not later than day E3.5 [21]; *Casp8* deficiency results in necroptosis and consequently in embryonic death at E10.5 [22]"

We have further discussed this issue and provided relevant references supporting our speculation: "It is noteworthy that most of the male embryos probably dye early during the pregnancy. A thorough study characterizing 5,000 knockout mice lines showed that only 4% of 410 lethal embryonic phenotypes occurred between E12.5 to E18.5 whereas the rest are lethal at earlier stages [20]. We speculate that in our experiments too, lethality of most of the male embryos occurred early (i.e., prior to E12.5) and were absorbed during the pregnancy. This speculation is supported by the fact that only one deformed male pup was born (Fig. 2) and also with the choice of target genes: *Atp5b*, *Cdc20*, and *Casp8*, all shown to be essential for mouse early development [20-22]."

* What type of welfare assessment was done on the female pups born?

The monitoring of the welfare of the females is now described in the revised Materials and Methods: "Breeding pairs were monitored daily and pups were monitored twice a day till weaning and twice a week following weaning. Signs of illness (decreased mobility, ruffled fur, pause in weight gaining, or labored breathing) were monitored by researchers and animal technicians and by consultation with a veterinarian. During the entire duration of the experiments, none of the Y-line males or their pups displayed overt signs of sickness, except the reported deformed male (Fig. 2)."

* How long did the pups live, and have there been any health problems later?

We have further elaborated on the health and fertility of the females: "The safety of the transgene to the female GMOs, which are the desired animals in the system, is arguably sound. Each of the transgenes (Cas9 or gRNAs) alone does not cause any DNA damage by itself. The female embryo does not produce at any stage the gRNAs, as they are encoded on the Y chromosome. We have further validated by DNA sequencing of a control female that the target genes are intact (Fig. EV3). In addition, female offspring of the Cas9 and Y-line cross showed no sign of illness over a monitoring period of four months. Furthermore, these females were examined at 6 weeks of age for fertility by crossing them with Cas9 males and monitoring the pregnancy and sex ratio of their pups. All examined females were fertile, providing normal litter size and normal sex ratio, as expected (average litter size was 6.75 totaling 14 males and 12 females and 1 infanticide). Thus, it is probable that the safety and welfare of the GMO females is not affected compared to their non-GMO counterparts."

Furthermore, we have also reported of the health of the three surviving males: "Although no sign of illness was observed, one male from the surviving three was found dead after 13.5 weeks. At 4 months of age, the other two males showed no signs of illness."

2nd Editorial Decision 27th May 2019

Thank you for the submission of your revised manuscript to our editorial office. The manuscript has now been seen again by our advisor, and s/he now supports the publication of the study in EMBO reports. Please find his/her comments below.

Thus, I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Advisor:

As far as I can notice, the authors have revised their manuscript very carefully. They have added discussion needed and missing points regarding laboratory animals and their welfare. I think they have done good work.

This is interesting topic and of course there are some (very basic) ethical concerns in this new method. But the discussion part takes care that readers understand that the authors have thought ethical details and animal welfare, which in this case is extremely important.

I think that the paper is now suitable for publication.

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Corresponding Author Name: Udi Qimron, Motti Gerlic
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48269V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The breeding sample size was determined for testing that the Y-line X Cas9-line cross can deliver up to 3-6 litters with no observed health issues in the parents. This breeding stratergy also results in sufficent numbers of pups to ensure adequate power to test our hypotheses regarding litter size and offspring sex ratio. For more details on determining sex ratio see Moore II, DH and Gledhill, BL, Fertility and sterility, 1988.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No Animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Y-line males were randomaly allocated into the breeding cages.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The animal technician who monitors daily the breedings and the occuring births was blind to the experimental hypothesis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	DNA samples were taken at P0 and were labeled by numbers such that PCR was run blindly.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, comparisons were performed using two-tailed unpaired parametric t-test or two-tailed binomial test, assuming normal distribution or two-tailed t-test of the variance–covariance matrix of the standard errors.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Housing and husbandry: All mice were housed and bred under specific pathogen-free conditions maintained in the accredited animal facility in the Sackler Faculty of Medicine at Tel Aviv University. Mice were housed with an inverse 12-hour day-night cycle with lights on at 7:00AM in a temperature (22±1°C) and humidity (55±5%) controlled room. All mice were allowed free access to water and food including sunflower seeds. All cages contained wood shavings, bedding and a cardboard tube for environmental enrichment. Experimental animals: For all breeding experiments males and females (>20 g), 8-52 weeks of age, were used (n=29). All obtained mice were acclimatized for at least 96 h. Vendor health reports indicated that the mice were free of known viral, bacterial and parasitic pathogens. Two independent mice of the Y-line (generated from two positive ES cloned: 2E6 and 2H8) were constructed by Cyagen Biosciences (California, USA). These C57BL/6N mice encode the following guide RNAs on their Y chromosome: 5′-CACTGCCACCGGGCGAATCG-3′; 5′-CAGACCTGAATCTTGTAGAT-3′; 5′-TGCAGAGATGAGCCTCAAAA-3′ targeting the genes Atp5b, Cdc20, and Casp8, respectively. These guides were cloned into a vector targeting the reverse orientation of the 2nd exon of the Y chromosome Uty gene, which is not part of the pseudoautosomal Y region. Fig. EV1 provides a schematic summary and the Appendix provides detailed description of the Y-line construction. Mice of the Cas9-line were purchased from Jackson laboratories (Stock No: 026179; Rosa26-Cas9 knockin on B6J) [19]. These mice encode a cassette in the Rosa26 locus on chromosome 6 constitutively expressing the SpCas9 endonuclease from a CAG promoter.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments conformed with the guidelines of the Tel Aviv University's Animal Ethics Committee, which follow the state law of prevention of animal cruelty (1994), the guidelines for preventions of animal cruelty published by the council for animal experimentation (2001), and The NRC Guide for the Care and Use of Laboratory Animals. The Animal Facilities in Tel Aviv University also work under a permit by the NIH [F16-00009 (A5010-01)].
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirm compliance with ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The data that support the findings of this study are available from the corresponding authors upon request.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA