Super–Mendelian inheritance mediated by CRISPR–Cas9 in the female mouse germline

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A gene drive biases the transmission of one of the two copies of a gene such that it is inherited more frequently than by random segregation. Highly efficient gene drive systems have recently been developed in insects, which leverage the sequence-targeted DNA cleavage activity of CRISPR–Cas9 and endogenous homology-directed repair mechanisms to convert heterozygous genotypes to homozygosity. If implemented in laboratory rodents, similar systems would enable the rapid assembly of complex genetic configurations. To our knowledge, however, such a system has not yet been demonstrated in mammals. Here we use an active genetic element that encodes a guide RNA (gRNA), which is embedded in the mouse tyrosinase (\(\text{Tyr}\)) gene, to evaluate whether targeted gene conversion can occur when CRISPR–Cas9 is active in the early embryo or in the developing germline. Although Cas9 efficiently induces double-stranded DNA breaks in the early embryo and male germline, these breaks are not corrected by homology-directed repair. By contrast, Cas9 expression limited to the female germline induces double-stranded breaks that are corrected by homology-directed repair, which copies the active genetic element from the donor to the receiver chromosome and increases its rate of inheritance in the next generation. These results demonstrate the feasibility of CRISPR–Cas9-mediated systems that bias inheritance of desired alleles in mice and that have the potential to transform the use of rodent models in basic and biomedical research.

A cross between mice that are heterozygous for each of three unlinked genes must produce 146 offspring for a 90% probability that one will be a triple homozygous mutant. The likelihood decreases further if any of the three mutations are genetically linked but on opposite homologous chromosomes, because recombination events that combine alleles onto the same chromosome would be very infrequent. The cost, time and requirement for a large number of mice to obtain a few individuals of the desired genotype are therefore prohibitive for certain complex models of multigenic evolutionary traits or human diseases, such as arthritis and cancer.

Recently, CRISPR–Cas9-mediated gene drive systems were developed in Drosophila and anopheline mosquitoes that increase the frequency of inheritance of desired alleles. These used genetic elements, which we refer to broadly as active genetic elements, that can carry transgenes or orthologous sequences from other species. Notably, an active genetic element includes a guide RNA (gRNA) and is inserted into the genome at the precise location that is targeted for cleavage by the encoded gRNA. In a heterozygous animal that also expresses the Cas9 nuclease, the gRNA targets cleavage of the wild-type homologous chromosome. Genomic sequences that flank the active genetic element then correct the double-stranded break (DSB) by homology-directed repair (HDR), which copies the active genetic element from the donor to the receiver chromosome and converts the heterozygous genotype to homozygosity. The frequency of transmitting the active genetic element to the next generation is therefore greater than expected by the random segregation of homozygous alleles and is referred to as ‘super-Mendelian’. In addition to the potential to overcome the obstacles of assembling complex genotypes in laboratory rodents, variations of a CRISPR–Cas9-mediated system have been proposed that might help to suppress invasive rodent populations and/or reduce the prevalence of rodent-borne disease.

Despite the high efficiency observed in insects, the approximately 790 million years of divergence since their last common ancestor with mammals presents two potential obstacles to the implementation of active genetics in mice; the frequency of DSB formation using a genetically encoded Cas9 and gRNA and/or the frequency of HDR may prevent efficient gene conversion. The alternative DSB repair pathway, non-homologous end joining (NHEJ), frequently generates small insertions and deletions (indels) that make CRISPR–Cas9 an effective means of mutating specific sites in the genome. Although HDR of CRISPR–Cas9–induced DSBs does occur in vitro and in vivo in mammalian cells and embryos, usually from a plasmid or single-stranded DNA template, NHEJ is the predominant mechanism of DSB repair in somatic cells.

To assess the feasibility of active genetic systems in mice, we designed a ‘CopyCat’ element that differs from the genetic element used initially in insects in that it cannot self-propagate, because it encodes a gRNA but not the Cas9 protein (Fig. 1a). We designed our strategy to disrupt the tyrosinase gene (\(\text{Tyr}\)), because of the obvious albino phenotype of homozygous loss-of-function mice and to make use of a previously characterized \(\text{Tyr}\) gRNA with high activity. The precise insertion of this element into the gRNA cut site in exon 4 of \(\text{Tyr}\) to obtain the \(\text{Tyr}^{\text{CopyCat}}\) knock-in allele is shown in Extended Data Fig. 1 (see also Supplementary Methods and Supplementary Figs. 1, 2). In brief, the \(\text{Tyr}\) gRNA is transcribed from a constitutive human RNA polymerase III U6 promoter. On the reverse strand of the DNA, to minimize possible transcriptional conflict, \(\text{mCherry}\) is ubiquitously expressed from the human cytomegalovirus (CMV) immediate-early promoter and enhancer. As the 2.8-kb insert disrupts the \(\text{Tyr}\) open reading frame, \(\text{Tyr}^{\text{CopyCat}}\) is a functionally null (albino) allele that is propagated by Mendelian inheritance in the absence of Cas9. Crossing mice that carry the \(\text{Tyr}^{\text{CopyCat}}\) element to transgenic mice that express Cas9 enabled us to test whether it is possible to observe super-Mendelian inheritance of the \(\text{Tyr}^{\text{CopyCat}}\) allele. For this analysis, we assessed eight different genetic strategies that use existing tools to provide spatial and temporal control of Cas9 expression in the early embryo and in the male and female germlines.

We used two ‘constitutive’ \(\text{cas9}\) transgenic lines, \(\text{Rosa26-cas9}\) and \(\text{H11-cas9}\), which express Cas9 in all tissues that have been assessed. Each is driven by a ubiquitous promoter and is placed in the respective \(\text{Rosa26}\) or \(\text{H11}\) ‘safe harbour’ locus. To track the inheritance of the chromosome that is targeted for gene conversion, we bred the \(\text{chinilla}\) allele of tyrosinase (\(\text{Tyr}^{\text{R}}\), here simplified to \(\text{Tyr}^{\text{R}}\)) into each \(\text{cas9}\) transgenic line. \(\text{Tyr}^{\text{R}}\) encodes a hypomorph point mutation in exon 5 that is tightly linked to the gRNA target site in exon 4. \(\text{Tyr}^{\text{R}}\) homozygotes or heterozygotes that also have a null allele have a grey coat colour, and the G to A single-nucleotide polymorphism can be scored with certainty by PCR followed by DNA sequencing (Supplementary Fig. 4).
Female Rosa26-cas9;TyrΔic/Δic and H11-cas9;TyrΔic/Δic mice were each crossed to TyrCopyCat+ males to combine the gRNA and Cas9 protein in the early embryo (Fig. 1b). In absence of a loss-of-function mutation in exon 4 of the receiver chromosome, a TyrCopyCat+ mouse should appear grey (see cas9−;TyrCopyCat+ mice in Fig. 1d). However, we did not observe any grey cas9−;TyrCopyCat+ mice in the F2 offspring of either cross. Instead, all 17 of the Rosa26-cas9;TyrCopyCat+ mice were entirely white. Among H11-cas9;TyrCopyCat+ mice, 21 of the F2 progeny (87.5%) were a mosaic mixture of grey and white fur, and three mice (12.5%) were entirely white (Fig. 1d, e and Extended Data Table 1). The prevalence of mosaicism in the H11-cas9 mice, compared to the all-white mice produced by Rosa26-cas9, suggests that there may be a difference in the level and/or timing of Cas9 expression driven by these two transgenes.

Our next goal was to determine what type of repair events (NHEJ mutations or gene conversions by HDR) were transmitted to the next generation. To assess inheritance in many offspring, we crossed each F2 male Rosa26-cas9;TyrCopyCat+ and H11-cas9;TyrCopyCat+ mouse to multiple albino CD-1 females (Fig. 1c), which carry a loss-of-function point mutation in the Tyr exon 1 (Tyr−, here designated as Tyrnull), to identify those that inherited the Tyrnull-marked receiver chromosome (Supplementary Fig. 4). In the absence of gene conversion, effectively none of these chromosmes would be predicted to also carry the Tyrnull allele, because Tyr exons 4 and 5 are separated by only approximately 9.1 kb.

Tyrnull mice should appear grey because of the partial activity of the hypomorph TyrΔic allele. However, among F2 offspring with this genotype, 100% in the Rosa26-cas9 lineage and 90.4% in the H11-cas9 lineage were completely white, indicating frequent transmission of a CRISPR–Cas9-induced loss-of-function mutation on the receiver chromosome and consistent with the primarily albino coat colour of the F2 parents (Fig. 1c and Extended Data Tables 1, 2). If the induced null alleles resulted from inter-homologue HDR copying the Tyrnull allele from the donor to the Tyrnull-marked receiver chromosome, these white animals should also express the fluorescent mCherry marker. However, in these experiments none of the F2 offspring that inherited the receiver chromosome in either the Rosa26-cas9 or H11-cas9 lineages expressed mCherry. PCR amplification of Tyr exon 4 confirmed that the Tyrnull allele element was not present in white Tyrnull F2 progeny (Extended Data Table 2).

The different propensities to yield a full albino or mosaic coat colour pattern in the F2 generation of Rosa26-cas9 and H11-cas9 lineages were consistent with differences in the number of unique NHEJ mutations that we identified on receiver chromosomes in individuals of each genotype. Sequenced PCR products from Rosa26-cas9;TyrCopyCat+ F2 tails—which are somatic tissues that consist of both ectodermal and mesodermal derivatives—and from individual F2 outcross offspring (representing the germline) typically exhibited only two unique NHEJ mutations, suggesting that many of these Cas9-induced mutations may have been generated in embryos at the 2–4-cell stage (average 2.4 alleles among offspring of five families; Extended Data Fig. 2 and Extended Data Table 3). By contrast, H11-cas9;Tyrnull F2 tails and F3 offspring had significantly more unique NHEJ mutations (average 4.6 alleles in five families; two-tailed Student’s t-test, P = 0.041), consistent with the hypothesis that Cas9 is expressed at a later embryonic stage and/or at lower levels in this lineage.

We considered two explanations for the observation that Tyrnull was not copied to the receiver chromosome in the early embryo. The first possibility is that homologous chromosomes are not aligned for inter-homologue HDR to repair DSBs. Second, the DNA repair machinery in somatic cells typically favours NHEJ over HDR5,9. A possible solution to overcome both potential obstacles is to restrict CRISPR–Cas9 activity to coincide with meiosis in the developing germline. During meiosis, recombination of the maternal and paternal genomes is initiated by the formation of DSBs that are repaired by exchanging regions of homologous chromosomes that are physically paired during meiosis18. Indeed, the molecular mechanisms of NHEJ are actively repressed to favour HDR during meiosis in many species, including mice19.

To test the hypothesis that CRISPR–Cas9 activity will convert a heterozygous active genetic element to homozygosity during meiosis, we designed a crossing scheme to initiate Cas9 expression during germline development in TyrCopyCat+ mice. As no currently available transgenic mice express Cas9 under direct control of a germline-specific
promoter, we crossed mice with a conditional Rosa26- or H11-LSL-cas9 transgene, each with a loxP-Stop-loxP (LSL) site preceding the Cas9 translation start site \(^{15,16}\), to Vasa-cre (also known as Ddx4-cre) or Stra8-cre germline transgenic mice. Vasa-cre is expressed later than the endogenous Vasa transcript in both male and female germ cells\(^{20}\), whereas Stra8-cre is limited to the male germline and is initiated in early-stage spermatogonia\(^{21}\). Although oogonia and spermatogonia are pre-meiotic, and spermatogonia are in fact mitotic, we reasoned that Cre protein must first accumulate before cas9 can be expressed from the LSL-cas9 conditional allele. The possible time delay may require initiation of cre expression before the onset of meiosis so that Cas9-induced DSBs can be resolved by inter-homologue HDR before segregation of homologous chromosomes at the end of meiosis I. We generated each combination of these cre and conditional cas9 lines in case the timing or levels of Cas9 expression were critical variables in these crosses. We also assessed males and females of the Vasa strategies in case there were sex-dependent differences in animals that inherit the same genotype.

Males heterozygous for Tyr\(^{CopyCat}\) and the Vasa-cre transgene were crossed to females homozygous for both the Tyr\(^{ch}\) allele and one of the two conditional cas9 transgenes (Fig. 2). We avoided the reverse cross using female Vasa-cre mice, because Cre protein maternally deposited in the egg\(^{20}\) might induce recombination of the conditional cas9 allele and induce mutations in the early embryo similar to what we observed in the experiments using constitutive cas9 transgenes. Introducing the Vasa-cre transgene by inheritance from the male instead resulted in most offspring that were entirely grey, owing to the Tyr\(^{CopyCat,ch}\) genotype, and a few mosaic animals (Supplementary Table 4). The presence of mosaicism suggests that this conditional approach to restrict cas9 expression to the germline resulted in some spurious cleavage of the Tyr locus in somatic tissues.

We first tested whether Cas9 in the female germline could promote copying of the Tyr\(^{CopyCat}\) element onto the receiver chromosome by crossing \(F_1\) female mice of each Vasa-cre lineage to CD-1 (Tyr\(^{null}\)) males. In each cross, we identified \(F_2\) offspring that inherited the Tyr\(^{ch}\)-marked chromosome (Fig. 2). As in the crosses to assess the effects of embryonic cas9 expression, we expected that Tyr\(^{ch}\)-marked mice without a loss-of-function mutation in exon 4 of the receiver chromosome would be grey. Mice with a CRISPR–Cas9-induced NHEJ mutation in exon 4 should be white. Mice carrying a CRISPR–Cas9-induced mutation that was repaired by inter-homologue HDR should not only be white, but also show expression of mCherry (red fluorescence) owing to transmission of the mCherry-marked Tyr\(^{CopyCat}\) active genetic element.

Table 1 summarizes the results of these crosses that demonstrate gene conversion upon Cas9 expression in the female germline. In contrast to early embryonic expression of cas9, we observed that the Tyr\(^{CopyCat}\) transgene was copied to the Tyr\(^{ch}\)-marked receiver chromosome in both Vasa-cre;Rosa26-LSL-cas9 and Vasa-cre;H11-LSL-cas9 lineages. However, the observed efficiency differed between genotypes: three out of five females of the Vasa-cre;Rosa26-LSL-cas9 lineage and all five females of the Vasa-cre;H11-LSL-cas9 lineage transmitted a Tyr\(^{ch}\)-marked chromosome that contained a Tyr\(^{CopyCat}\) insertion to at least one offspring. Although there was considerable variation among females with the same genotype, the highest observed frequency of gene conversion (72.2%) within a germline produced 13 out of 18 Tyr\(^{ch}\) offspring with a Tyr\(^{CopyCat}\) insertion in the Vasa-cre;H11-LSL-cas9 lineage (Table 1 and Extended Data Table 4). The probability of obtaining an animal with this genotype by natural meiotic recombination mechanisms is very low (4.7 \times 10^{-5}) owing to ultra-tight linkage between the Tyr\(^{CopyCat}\) and Tyr\(^{ch}\) alleles. Although it seems probable that inter-homologue HDR of Cas9-induced DSBs uses the same DNA-repair machinery that is active during meiotic recombination, these copying events cannot be explained by an increased incidence of chromosomal crossover, because all animals that inherited the donor chromosome lacking the Tyr\(^{ch}\) marker expressed mCherry (Extended Data Table 4).

In contrast to the 41 copying events that we observed out of a total of 132 Tyr\(^{ch}\)-null offspring of female mice, we observed no copying in a total of 113 offspring of males in which conditional cas9 expression was induced by either Vasa-cre or Stra8-cre (Table 1 and Extended Data Table 4).

### Table 1 | Observed \(F_2\) outcomes of germline Cas9 strategies

| cre  | cas9      | \(F_2\) parent | No cut or functional repair | NHEJ disruption | HDR conversion | Observed HDR conversion (%) |
|------|-----------|----------------|-----------------------------|-----------------|----------------|-----------------------------|
| Vasa | Rosa26    | M 1            | 25                          | –               | –              | –                          |
| Vasa | Rosa26    | M 1            | 25                          | –               | –              | –                          |
| Vasa | H11       | M 1            | 15                          | –               | –              | –                          |
| Vasa | H11       | M 1            | 15                          | –               | –              | –                          |
| Vasa | H11       | M 1            | 15                          | –               | –              | –                          |
| Vasa | H11       | M 1            | 15                          | –               | –              | –                          |

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It is possible, however, that the number of families and of offspring in each family—which was limited by unexplained low male fertility—was insufficient to detect low-efficiency copying in each of four genetic strategies. If there is indeed a difference between males and females in the efficiency of Tyr<sup>fl/fl</sup> copying, we can consider two potential explanations. First, despite equivalent genotypes in the male and female 

Vasa-cre

lineages, Cre, Cas9 and/or gRNA may not be well-expressed in the male germline. However, the high frequency of white Tyr<sup>fl/fl</sup> offspring suggests that DSB formation is very efficient in males. Second, spermatocytes continually undergo mitosis and produce new primary spermatocytes throughout the life of a male in mammals<sup>23</sup>. By contrast, oogonia directly enlarge without further mitosis to form all of the primary oocytes in the embryo<sup>24</sup>. The difference in the observed efficiency of inter-homologue HDR between females and males at this locus may therefore reflect a requirement for the precise timing of CRISPR–Cas9 activity to coincide with meiosis (Fig. 3). NHEJ indels in males may result from DSB repair that occurs before the alignment of homologous chromosomes in meiosis I. Similarly, the higher observed efficiency of inter-homologue HDR in females of the H11-LSL-cas9 conditional strategy may relate to the lower or delayed <i>cas9</i> expression from the H11 locus, compared to Rosa26, which was evident in the constitutive crosses (Fig. 1d, e and Extended Data Tables 1, 3). Thus, in the Vasa-cre;H11-LSL-cas9 mice, DSB formation may have been fortuitously delayed to fall within a more optimal window during female meiosis.

In summary, we demonstrate that the fundamental mechanism of a CRISPR–Cas9-mediated gene drive is feasible in mice. However, our comparison of eight different genetic strategies indicates that the precise timing of <i>cas9</i> expression may present a greater challenge in rodents than in insects to restrict DSB formation to a window when breaks can be efficiently repaired by the endogenous meiotic recombination machinery. These data are therefore critical to the ongoing discussion about whether CRISPR–Cas9-mediated gene drives could be used to reduce invasive rodent populations, because it appears that both the optimism and concerns are likely to be premature. Further optimization to increase the frequency of gene conversion in both males and females and to reduce the prevalence of drive-resistant alleles (NHEJ indels that alter the gRNA target site) would be necessary to achieve rapid and sustained suppression of wild populations<sup>25–29</sup>

Nevertheless, the copying efficiencies that we observed here would be more than sufficient for a broad range of laboratory applications. For example, the average observed copying rate of 44% using the most efficient genetic strategy in females (Vasa-cre;H11-LSL-cas9) combined ultra-tightly linked tyrosinase mutations such that 22.5% of all offspring inherited a chromosome with both alleles, which would not be possible through Mendelian inheritance. This observed average copying rate would also be expected to increase the inheritance of a single desired allele from 50% to 72%, and the highest rate of gene conversion that we observed (72.2%) would result in an 86% frequency of transmitting a desired allele. If multiple genes could be simultaneously converted to homozygosity, such high transmission frequencies that bypass the onerous constraint of genetic linkage stand to greatly accelerate the production of rodent models for a variety of complex genetic traits.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-0875-2.

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**Additional information**

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METHODS

Statistics and reproducibility. The five families in each of the constitutive crosses (Extended Data Table 2) are considered five independent experiments with each $F_3$ offspring representing an early embryonic DSB repair event in the $F_2$ parent. Each $F_4$ offspring in Extended Data Table 4 represents a germline DSB repair event, an independent data point and each family is considered an independent trial. No statistical method was used to predetermine sample size. Given breeding limitations, we assessed as many offspring as possible in each family and aimed to assess up to five families for each strategy. To detect gene conversion events, we used genetic linkage rather than a statistical test of inheritance greater than 50% expected by Mendelian segregation. Specifically, the receiver chromosome was marked with a SNP (Tyr<sup>Ch</sup>) located approximately 9.1 kb from the target site for gene conversion. The probability of a naturally occurring recombination event that would unite these ultra-tightly linked loci on the same chromosome is $4.7 \times 10^{-5}$, because the average genetic distance<sup>30</sup> for mouse chromosome 7 is 0.52 cM Mb<sup>-1</sup>.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
All genotyping data for $F_3$ offspring of constitutive crosses and $F_4$ offspring of germline conditional crosses is available at Zenodo with the identifier https://doi.org/10.5281/zenodo.2003087. Annotated sequence data for the Tyr<sup>Ch</sup>-CrdCat transgene is available in GenBank with the accession number MK160997.

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Extended Data Fig. 1 | Knock-in strategy using the TyrCopyCat-targeting vector. The U6-Tyr4a gRNA (tyrosinase exon 4 gRNA a) and CMV-mCherry were inserted into the cut site of the Tyr4a gRNA by HDR after CRISPR–Cas9 DSB formation targeted by the Tyr4a gRNA. See Supplementary Methods and Supplementary Figs. 1, 2 for additional details.
Extended Data Fig. 2 | Rosa26-cas9 and H11-cas9 constitutive lineages have different numbers of unique NHEJ indels. Sanger sequencing of the Tyr a gRNA target exon amplified from tail-tip genomic DNA using TyrHALF2 and TyrHARR2 primers as specified in Supplementary Table 3. Top, a single representative Sanger sequence trace of the bulk PCR product amplified from a Rosa26-cas9;TyrCopyCat-positive F2 mouse (Rosa26 family 1 in Extended Data Table 3) with either major or minor peaks called revealing two distinct alleles. Five Tyr a-positive F3 offspring of this F2 individual each match one of the two alleles (marked 1 (insertion) and 2 (deletion)). Bottom, a single representative sequence trace of the bulk PCR product amplified from an H11-cas9;TyrCopyCat-positive F2 mouse (H11 family 1 in Extended Data Table 3). Alternate alleles cannot be called because of the complexity of overlapping peaks. Five Tyr b-positive F3 offspring each have one of four different alleles (marked 1, 2, 3 and 4). Sequence trace data are representative of all 90 individuals of 5 families of each constitutive strategy described in Extended Data Table 3.
Extended Data Table 1 | Coat colour of $F_2$ individuals that were constitutive cas9-positive and $TyFCopyCat/Ch$

|        | Rosa26>Cas9 | H11>Cas9 |
|--------|-------------|-----------|
| White  | 17          | 3         |
| Mosaic | 0           | 21        |
## Extended Data Table 2 | Analyses of phenotypes and genotypes of all F3 progeny of the constitutive cas9 crosses

|                | F2 parent color | Total F3 Chinchilla- | Total F3 Chinchilla+ | White (NHEJ mutation) | Grey (no cut or functional repair) | mCherry+ (HDR gene conversion) |
|----------------|-----------------|----------------------|----------------------|-----------------------|-----------------------------------|---------------------------------|
| Rosa26 Family 1| white           | 30                   | 20                   | 20                    | 0                                 | 0                               |
| Rosa26 Family 2| white           | 11                   | 15                   | 15                    | 0                                 | 0                               |
| Rosa26 Family 3*| white           | 28*                  | 16                   | 16                    | 0                                 | 0                               |
| Rosa26 Family 4| white           | 22                   | 22                   | 22                    | 0                                 | 0                               |
| Rosa26 Family 5| white           | 16                   | 16                   | 16                    | 0                                 | 0                               |
| H11 Family 1   | prim. white mosaic | 14                  | 15                   | 15                    | 0                                 | 0                               |
| H11 Family 2   | prim. grey mosaic | 23                  | 28                   | 25                    | 3                                 | 0                               |
| H11 Family 3   | prim. white mosaic | 13                  | 9                    | 9                     | 0                                 | 0                               |
| H11 Family 4   | prim. white mosaic | 12                  | 15                   | 15                    | 0                                 | 0                               |
| H11 Family 5   | mosaic           | 23                   | 24                   | 18                    | 6                                 | 0                               |

Genotypes and phenotypes of all F3 progeny in Fig. 1c that are the offspring of a subset of F2 individuals listed in Extended Data Table 1. Prim., primarily; referring to the coat colour that covers the greatest total area when there was obviously not an equal proportion of white and grey.

*A family with offspring that were Tyr<sup>R</sup>-negative and mCherry-negative, suggesting a large deletion in the recipient chromosome that may encompass the Tyr<sup>R</sup> SNP.
### Extended Data Table 3 | Allelic complexity of the constitutive Rosa26- and H11-cas9 families

| Family      | Number of distinct NHEJ alleles in 'n' Tyr<sup>exon2</sup>/mCherry<sup>exo1</sup> offspring |
|-------------|------------------------------------------------------------------------------------------|
| Rosa26 Family 1 | 2 (n=9)                                                                                 |
| Rosa26 Family 2 | 3 (n=10)                                                                                 |
| Rosa26 Family 3* | 2 (n=9)                                                                                 |
| Rosa26 Family 4 | 2 (n=7)                                                                                 |
| Rosa26 Family 5 | 3 (n=13)                                                                                 |
| H11 Family 1   | 4 (n=10)                                                                                 |
| H11 Family 2   | 6 (n=9)                                                                                  |
| H11 Family 3   | 4 (n=6)                                                                                  |
| H11 Family 4   | 2 (n=8)                                                                                  |
| H11 Family 5   | 7 (n=9)                                                                                  |

*A family with offspring that were Tyr<sup>exon2</sup>-negative and mCherry-negative, suggesting a large deletion in the recipient chromosome that may encompass the Tyr<sup>exon1</sup> SNP. This was counted as one of the two unique NHEJ indels.*
Phenotypes and genotypes of all F4 progeny that are the offspring of a subset of F3 individuals listed in Supplementary Table 4.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [ ] Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- [ ] Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

- Data collection: No software was used in this analysis
- Data analysis: We used SnapGene for designing the targeting constructs and for analyzing Sanger sequencing data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. Github). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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All data is available in the main text or the supplementary materials. Primary genotyping data is being curated and will be deposited to Figshare.
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For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  Given the ultra-tight genetic linkage of the Tyr*Ch marker and the Tyr*CopyCat active genetic element, the probability of natural recombination between these alleles is 4.7x10^-5. We include this information in "Statistics and Reproducibility."

Data exclusions  No data were excluded from these results

Replication  Copying of the "CopyCat" element was confirmed by PCR twice from the same sample and again from a separate tissue collected from the same individual. Copying was also evident in multiple animals that were the product of multiple different mating pairs.

Randomization  This study did not include multiple treatment conditions, and therefore random assignment was not necessary. All pups available were analyzed; no random sampling of offspring was necessary.

Blinding  Investigators had no knowledge of phenotype (presence/absence of mCherry, coat color) when performing PCR or Sanger sequencing to assess genotype.

Reporting for specific materials, systems and methods

Materials & experimental systems

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a | n/a |
| ☒ ☒ Unique biological materials | ☒ Chip-seq |
| ☒ Antibodies | ☒ Flow cytometry |
| ☒ Eukaryotic cell lines | ☒ MRI-based neuroimaging |
| ☒ Palaeontology | ☒ |
| ☒ Animals and other organisms | ☒ |
| ☒ Human research participants | ☒ |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials  The "CopyCat" mouse used in this study is a unique transgenic animal. Production of this mouse is detailed in the methods section. Individuals of this mouse line may be available upon request from the corresponding author.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals  Laboratory mice (Mus musculus) were used in this study. The following backgrounds were used: C57BL/6J, FVB, C3H/SWJ. The following specific strains were used: "CopyCat" (transgenic mouse produced for this study), Jackson lab strains #26175, 26179, 26816, 27650, 17490, 6954, 4828. Adult animals were used for breeding, and genotyping was performed from birth to P21.

Wild animals  No wild animals were used in this study.

Field-collected samples  No field collected samples were used in this study.