

Agouti C57BL/6N embryonic stem cells for mouse genetic resources

Stephen J Pettitt¹, Qi Liang¹, Xin Y Rairdan², Jennifer L Moran^{3,4}, Haydn M Prosser¹, David R Beier³, Kent C Lloyd^{2,4}, Allan Bradley¹ & William C Skarnes¹

We report the characterization of a highly germline competent C57BL/6N mouse embryonic stem cell line, JM8. To simplify breeding schemes, the dominant agouti coat color gene was restored in JM8 cells by targeted repair of the C57BL/6 nonagouti mutation. These cells provide a robust foundation for large-scale mouse knockout programs that aim to provide a public resource of targeted mutations in the C57BL/6 genetic background.

C57BL/6 is one of the best characterized inbred strains of mice and is the reference strain for the mouse genome sequence¹. However, genetic manipulation of the mouse genome is carried out predominantly in embryonic stem cells derived from the 129 strain of mice. A reliance on 129 embryonic stem cells is not ideal, particularly for genetic studies of immunology, neurobiology and physiology. Recently, large-scale knockout programs have been established to mutate all protein-coding genes in mouse using a combination of gene-trapping and gene-targeting strategies². For these resources, C57BL/6 embryonic stem cells had been chosen for the production of mutant alleles as this genetic background is better suited for the large-scale phenotyping efforts that will follow.

Several laboratories have reported the establishment of C57BL/6 embryonic stem cell lines from the C57BL/6J and C57BL/6N substrains of mice^{3–10}, but the use of these cell lines for high-throughput genetic engineering in mice has met with limited success owing to low germline transmission rates¹⁰. The value of embryonic stem cell resources critically depends on achieving high germline transmission rates among individual clones. In contrast to C57BL/6 cells, embryonic stem cells derived from the 129 strain of mice have proven to be robust. For example, 80% of clones from the BayGenomics and Sanger Institute gene trap resources were able to colonize the germline of mice after blastocyst injection (W.C.S. and K.C.L., unpublished data)⁸.

As a foundation for the international mouse knockout program², we sought to establish feeder-dependent and feeder-free C57BL/6 embryonic stem cells with reliable and robust germline colonization. We established several male cell lines from C57BL/6J and C57BL/6N blastocysts on fibroblast feeder cells using standard methods¹¹. C57BL/6N embryonic stem cells showed better growth and morphology compared to C57BL/6J embryonic stem cells (data not shown). Furthermore, attempts to culture C57BL/6J embryonic stem cells on gelatin in the presence of leukemia inhibitory factor (LIF) were unsuccessful, whereas C57BL/6N embryonic stem cells readily adapted to feeder-free conditions.

An embryonic stem cell line derived from C57BL/6N blastocysts, JM8, possessed a normal male (XY) karyotype (data not shown) and exhibited a normal undifferentiated morphology when cultured on feeder cells and on gelatin-treated plates (Fig. 1a). Injections of early-passage JM8 cells into albino C57BL/6-*Tyr^{c-Brd}* blastocysts produced chimeras with high coat color chimerism, a sex distortion in favor of males and a high proportion of chimeras with 100% contribution to both somatic tissues and the germline (Supplementary Data 1). To confirm the genetic purity of the JM8 cell line, we genotyped 19 single-nucleotide polymorphism (SNP) markers that distinguish the N and J substrains of C57BL/6 mice (Fig. 1b and Supplementary Table 1; J.L.M. and D.R.B., unpublished). This analysis confirmed their C57BL/6N origin. Although the J and N substrains are very closely related (only 102 of 139,561 genotyped SNPs are discordant) several phenotypic differences have been noted^{12,13}.

Genetic variability in a population of embryonic stem cells is the critical determinant of their performance and this can only be assessed by the analysis of large numbers of subclones. We therefore isolated a set of clonal sublines either on feeders or on gelatin and tested these for their germline transmission potential (Supplementary Data 1). In these experiments, most clones exhibited very high rates of chimera formation, producing large numbers of chimeras in which a high percentage or even all of the cells were derived from the injected embryonic stem cells. Notably, 80% of the chimeras were male, indicating that sex conversion of female host blastocysts had occurred in most cases. Upon breeding, we observed an exceptionally high rate of clonal germline transmission, calculated as the fraction of total clones injected that produce at least one male chimera with embryonic stem cell contribution to the germline. From injections of approximately 40 blastocysts per subline, 76% (16 of 21) of the sublines gave rise to at least one germline male chimera. Moreover, culturing cells under feeder-free conditions does not compromise the pluripotency of JM8 embryonic stem cells.

Two sublines in particular, JM8.F6 (feeder-dependent) and JM8.N4 (feeder-free) produced favorable results, and we tested

¹Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, UK. ²Center for Comparative Medicine, School of Veterinary Medicine, University of California, Davis, California, USA. ³Brigham and Women's Hospital, Genetics Division, Harvard Medical School, Boston, Massachusetts, USA. ⁴Present addresses: Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA (J.L.M.) and Stanley Center for Psychiatric Research, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA (K.C.L.). Correspondence should be addressed to W.C.S. (skarnes@sanger.ac.uk).

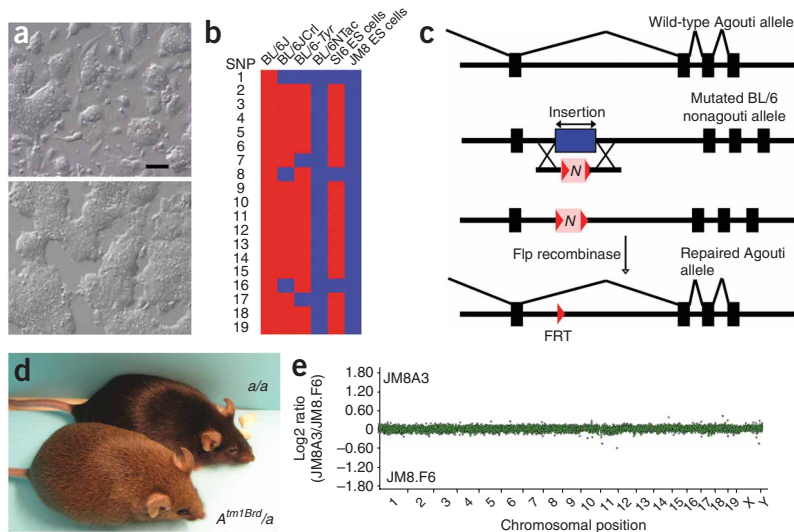


Figure 1 | Properties and origin of Agouti JM8 C57BL/6N embryonic stem cells. **(a)** Phase-contrast images of JM8 parental embryonic stem cells cultured on fibroblast feeder cells¹⁶ (top) and gelatin-coated plates (bottom). Scale bar, 100 μ m. **(b)** Genotype of embryonic stem cells and mice using 19 SNPs that differentiate between the N and J substrains of C57BL/6. JM8 embryonic stem (ES) cells were homozygous for all N alleles (blue). **(c)** Schematic of targeting strategy to restore Agouti function in JM8 cells. Exons, black boxes; retrotransposon, blue box, neo-TK selection cassette, *N*. FRT, Flp recombinase target site. **(d)** F₁ offspring from a male chimera, generated by injection of JM8A3 cells into a C57BL/6-*Tyr^{c-Brd}* blastocyst, crossed to a C57BL/6-*Tyr^{c-Brd}* female. Both mice shown are embryonic stem cell-derived and the mouse carrying the corrected allele (*A^{tm1Brd}/a*, bottom) has an agouti coat. **(e)** CGH plot showing no copy number variants in JM8A3 cells compared to JM8.F6 cells. Relative copy number is plotted as the log ratio of hybridization signals of probes from JM8A3 DNA compared to JM8.F6.

them for their ability to support high-throughput gene targeting. We expanded targeted JM8 embryonic stem cell clones generated for the large-scale knockout program (W.C.S. *et al.*, unpublished data) and microinjected them as above. From injections of 320 targeted clones, we determined the clonal germline transmission rate to be at least 65% (Table 1 and Supplementary Data 2). This should be regarded as a minimum estimate of germline potential since the re-injection of clones is expected, in some cases, to recover failures. Targeted clones derived from the feeder-free JM8.N4 cell line appear to be particularly proficient, producing a 70% clonal rate of germline transmission.

Copy-number changes are common in cultured embryonic stem cells and, if transmitted, are likely to contribute to phenotypic variation¹⁴. We expanded early-passage JM8.F6 and JM8.N4 cells and examined them by comparative genome hybridization (CGH) using a tiling path bacterial artificial chromosome (BAC) array (0.2 megabase (Mb) resolution; Supplementary Fig. 1). Both sublines

exhibited a normal XY karyotype. For JM8.F6, CGH analysis revealed no copy-number variants in comparison with genomic DNA from C57BL/6NTac mice. The JM8.N4 subline harbored a copy-number gain of a 1.7 Mb region (positions 79225351–80900647) of chromosome 10. However, the hybridization signal suggested that this variant was present only in a subset of the cells.

Blastocysts of various strains can be used in combination with C57BL/6 embryonic stem cells. We routinely used albino C57BL/6-*Tyr^{c-Brd}* blastocysts as hosts; these mice have been maintained as a closed colony for 20 years and selected to be highly fecund for blastocyst production. The injection of C57BL/6N embryonic stem cells into C57BL/6-*Tyr^{c-Brd}* blastocysts was a particularly favorable strain combination for germline transmission. However, in test crosses to detect embryonic stem cell contribution to the germline we crossed the chimeras to albino C57BL/6-*Tyr^{c-Brd}* mice, producing hybrid F₁ mice from the C57BL/6-*Tyr^{c-Brd}* and C57BL/6N substrains. To obtain pure inbred C57BL/6N G₁ mice, a separate cross with C57BL/6N mice is required after identifying chimeras with germline colonization.

Although this can be avoided with strains like C57BL/6J *A^{W-J}*, C57BL/6J-*Tyr^{c-2J}* or BALB/c, they are specialist strains with limited availability and poor fecundity; thus their use should be avoided.

Currently, most transgenic facilities inject strain 129-derived embryonic stem cells into C57BL/6 host blastocysts. To capitalize on the widespread experience with and availability of the C57BL/6 host blastocyst, we repaired the Agouti locus in JM8.F6 embryonic stem cells by gene targeting. Restoring Agouti function to C57BL/6N embryonic stem cells allows visualization of embryonic stem cell-derived mice by coat color (Supplementary Note) and permits the recovery of pure inbred mice from test crosses with C57BL/6N mice.

The nonagouti mutation in C57BL/6 strains is due to an 11.8 kilobase pair retrotransposon in the first intron of the Agouti gene, which abolishes transcription of Agouti mRNA¹⁵. We designed a targeting strategy to delete the retrotransposon from the locus and restore Agouti gene function (Fig. 1c and Supplementary Fig. 2).

Table 1 | Germline transmission of JM8 targeted clones

Cell line	Microinjections			Male chimeras		Test crosses		
	Number of clones injected	Number of clones at birth	Percentage of clones injected	Number of clones at weaning	Percentage of clones injected	Number of clones set up	Number of clones with GLT ^a	Percentage of clones injected with GLT ^b
JM8	61	61	100%	52	85%	52	38	62%
JM8.F6	108	104	96%	87	81%	85	67	62%
JM8.N4	151	151	100%	133	88%	126	104	69%
JM8A3	11	11	100%	10	91%	10	9	82%
Total	331	327	99%	282	85%	273	218	67%

^aGLT, germline transmission. ^bFraction of total clones injected that produce at least one male chimera with germline transmission.

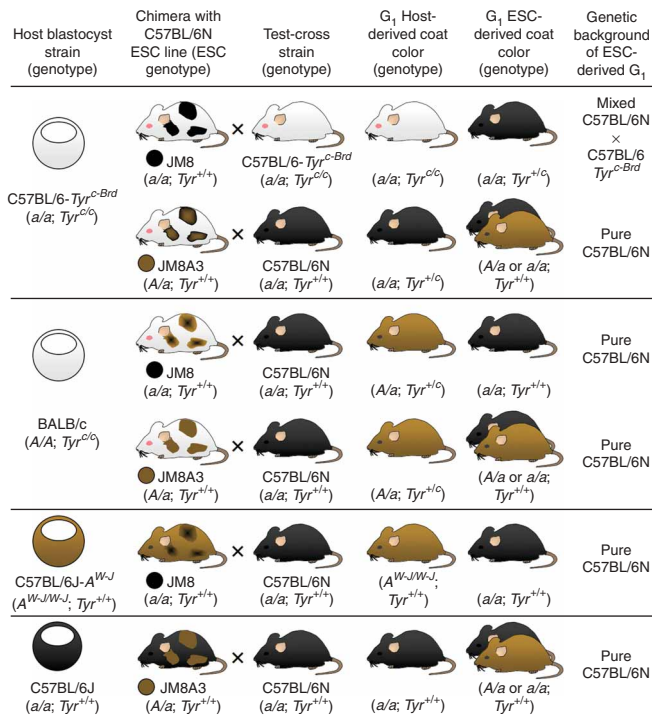


Figure 2 | Coat color of chimeric mice and their offspring from injections of JM8 and JM8A3 cells into blastocysts from several common mouse strains. Possible test crosses to detect germline contribution are indicated, along with the expected coat color(s) and genetic composition of embryonic stem cell-derived (ESC-derived) and host-derived G₁ (first (inbred) generation) offspring. For details, see the **Supplementary Note**.

We replaced the retrotransposon with a selectable marker, which we subsequently removed using Flp recombinase. The resulting Agouti allele contains a net deletion of 607 base pairs of intron sequence relative to the 129 strain Agouti allele and retains a single Flp recombinase target site.

We injected three independent clones into albino C57BL/6-*Tyr^{c-Brd}* blastocysts. All three produced chimeras with high percentage agouti coat color contribution and germline colonization, with one clone, JM8A3, showing particularly high frequencies (**Table 1** and **Supplementary Data 2**). As these cells are heterozygous for the corrected Agouti allele, test crosses with C57BL/6N mice yielded embryonic stem cell-derived offspring with either an agouti or a black coat (**Fig. 1d**). We analyzed gross copy-number variation of JM8A3 DNA compared to the normal JM8.F6 line by array CGH and found no differences at the 0.2 Mb resolution of the assay (**Fig. 1e**). To assess the suitability of JM8A3 cells for high-throughput gene targeting, we performed targeting experiments and measured the clonal transmission rate of targeted clones as above. We obtained a clonal germline transmission rate of 80% from the injection of 11 targeted clones (**Table 1** and **Supplementary Data 2**).

In summary, we derived robust, highly stable, germline-competent embryonic stem cells from the C57BL/6 genetic background that are suitable for high-throughput genetic manipulation. These cells are easily propagated using standard embryonic stem culture conditions, in the presence and absence of feeder cells, obviating the

need for expensive specialty medium⁶. We introduced a dominant agouti coat color marker into the JM8.F6 subline with no adverse effects on genome stability or germline transmission potential. Agouti JM8 cells take advantage of the widespread use of C57BL/6J mice for blastocyst injection by enabling visual assessment of coat color contribution and germline transmission. Moreover, C57BL/6N mice may be used in test crosses to identify chimeras with germline colonization and to produce pure inbred C57BL/6N G₁ mice (**Fig. 2**). This saves one generation of breeding and facilitates the use of this cell line for both small and large-scale mouse genetics programs. JM8, JM8.F6, JM8.N4 and JM8A3 embryonic stem cells are available upon request from the Knockout Mouse Project repository (<http://www.komp.org/>) and the European Conditional Mouse Mutagenesis repository (<http://www.eummcr.org/>).

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank S. Qin, F. Law, L. Delaney, J. Meneses, T. Creek, H. Tharagotnet and A. Beasley for help with embryonic stem cell culture; N. Adams, J. White, R. Ramirez-Solis and the Wellcome Trust Sanger Institute microinjection team for blastocyst injections and mouse breeding; and N. Conte and M. Storer for help with CGH. This work was funded by the Wellcome Trust Sanger Institute (WT077187), grants from the National Institutes of Health (KOMP, U01-HG004080 to W.C.S., K.C.L. and A.B.; U01-42430 to D.R.B.) and a grant from the Sixth Framework Programme of the EU (EUCCOMM, to W.C.S. and A.B.).

AUTHOR CONTRIBUTIONS

S.J.P. repaired the Agouti allele, analyzed the JM8A3 subline by CGH, microinjection and test crosses, and wrote the paper; H.M.P. and A.B. designed agouti targeting strategy and supervised experiments; Q.L. analyzed JM8 subclones by CGH; X.Y.R. and K.C.L. derived the parental JM8 line; J.L.M. and D.R.B. discovered and genotyped SNPs; A.B. designed experiments and assisted in writing the paper; W.C.S. derived JM8.F and JM8.N subclones, designed and supervised targeting and microinjection experiments and wrote the paper.

Published online at <http://www.nature.com/naturemethods/>
Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

1. Mouse Genome Sequencing Consortium. *Nature* **420**, 520–562 (2002).
2. International Mouse Knockout Consortium. *Cell* **128**, 9–13 (2007).
3. Kontgen, F., Suss, G., Stewart, C., Steinmetz, M. & Bluethmann, H. *Int. Immunol.* **5**, 957–964 (1993).
4. Auerbach, W. *et al. Biotechniques* **29**, 1024–1032 (2000).
5. Schuster-Gossler, K. *et al. Biotechniques* **31**, 1022–1026 (2001).
6. Cheng, J., Dutra, A., Takesono, A., Garrett-Beal, L. & Schwartzberg, P. *Genesis* **39**, 100–104 (2004).
7. Shimizukawa, R. *et al. Genesis* **42**, 47–52 (2005).
8. Hughes, E. *et al. Mamm. Genome* **18**, 549–558 (2007).
9. Keskintepe, L., Norris, K., Pacholczyk, G., Dederscheck, S. & Eroglu, A. *Transgenic Res.* **16**, 751–758 (2007).
10. Hansen, G.M. *et al. Genome Res.* **18**, 1670–1679 (2008).
11. Nagy, A., Gertsenstein, M., Vintersten, K. & Behringer, R. *Manipulating the Mouse Embryo: A Laboratory Manual* 3rd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2003).
12. Stiedl, O. *et al. Behav. Brain Res.* **104**, 1–12 (1999).
13. Khisti, R., Wolstenholme, J., Shelton, K. & Miles, M. *Alcohol* **40**, 119–126 (2006).
14. Liang, O., Conte, N., Skarnes, W. & Bradley, A. *Proc. Natl. Acad. Sci. USA* **105**, 17453–17456 (2008).
15. Bultman, S. *et al. Genes Dev.* **8**, 481–490 (1994).
16. McMahon, A. & Bradley, A. *Cell* **62**, 1073–1085 (1990).

ONLINE METHODS

Mouse strains and embryonic stem cell derivation. C57BL/6J (Jackson Laboratory), C57BL/6JCrI (Charles River Laboratories) and C57BL/6NTac (Taconic) were obtained from commercial breeders. The C57BL/6-*Tyr^{c-Brd}* mice were maintained as a closed colony by A.B.¹⁷. Embryonic stem cell lines were established from C57BL/6J and C57BL/6N blastocysts on feeder cells using standard methods¹¹.

Media and reagents. For embryonic stem cell culture, 500 ml Knockout DMEM (Invitrogen) was supplemented with 5 ml of 100× L-glutamine (Invitrogen), 5 ml of 100× β-mercaptoethanol (0.36 ml β-mercaptoethanol in 500 ml PBS, filter sterilized), 100 U ml⁻¹ LIF (Millipore) and 10% to 15% fetal bovine serum (Invitrogen, lot 40F1150K). Trypsin solution was prepared by adding 20 ml of 2.5% trypsin solution (Invitrogen) and 5 ml of chicken serum (Invitrogen) to 500 ml of filter-sterilized PBS containing 0.1 g EDTA (Sigma) and 0.5 g of D-glucose (Sigma).

Embryonic stem cell culture. JM8 embryonic stem cells were grown either on a feeder layer of SNL7 fibroblasts¹⁶ (neomycin- and/or puromycin-resistant) or on gelatinized tissue culture plates in embryonic stem cell medium containing 15% or 10% serum, respectively. For routine passage of stem cells grown on feeders, confluent cultures were washed twice with prewarmed PBS and trypsinized for 15 min at 37 °C. Ten volumes of prewarmed medium were added, the cells were dispersed by passing gently through a pipette and transferred at a dilution of 1:3 into new plates containing feeders (plated for at least one week before use). Passage of cells on gelatinized plates was carried out in a similar manner, except the cells were trypsinized for 7 min and passed at a dilution of 1:5 into freshly gelatinized plates (treated for 1 min with PBS containing 0.1% gelatin (Sigma)). The medium was replaced the next day, and the cells typically reached confluence 2 d after passaging. For blastocyst injections, embryonic stem cells were grown for 2 d to confluence, trypsinized as described above, pelleted for 3 min at 1,000g, and resuspended in HEPES-buffered embryonic stem cell media (pH 7.6) containing 15% serum.

SNP and CGH analysis. Genomic DNA was prepared by standard methods from early-passage embryonic stem cells grown in the absence of feeders or mouse tail samples. SNP analysis was performed with a panel of 768 SNPs using the Illumina Golden Gate platform. Nineteen SNPs are polymorphic between N and J substrains (Supplementary Table 1). CGH analysis was performed as described¹⁴.

Targeting the nonagouti locus. Primers used for initial cloning of the Agouti breakpoint and genotyping the *A^{tm1Brd}* allele were 5'-GCTCCCCGCGGTGCTTCCAGATGTGGAAAGAAGTTC-3' (includes SacII site) and 5'-CGCCGGTACCGATCTGGCACTG CCTTAAAGAGTA-3' (includes KpnI site). The targeting vector endpoints are 154830701 and 154868845 (chromosome 2, NCBI m37 coordinates).

Construction of the targeting vector (ATV-20) was as follows: a fragment of the wild-type agouti locus spanning the insertion site was amplified by PCR (using the primers above) from a 129S7 strain BAC (bMQ-37M9). A positive-negative selection cassette containing Flp recombinase target (FRT)-flanked neomycin phosphotransferase II (*neo*; confers G418/kanamycin drug resistance) and herpes simplex virus thymidine kinase (*TK*; sensitizes to the drug 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil, FIAU) genes was cloned into BglII and EcoRI restriction sites, which closely flank the insertion site. Use of these restriction sites results in a small deletion of a 607 bp BglII-EcoRI fragment in the final allele relative to wild-type agouti. We electroporated the resulting construct into recombination-competent *Escherichia coli*¹⁸ carrying a C57BL/6J BAC (RP23-192A6) that contains the agouti locus (that is, nonagouti allele). This resulted in the replacement of the nonagouti insertion in the BAC by the selection cassette. A fragment of this BAC containing the selection cassette was retrieved by gap repair recombineering to complete construction of the targeting vector. The 5' and 3' homology arms are 9 and 13 kilobase pairs, respectively (Supplementary Fig. 2a). Vector DNA is isogenic with C57BL/6J except immediately around the selection cassette, but targeting is efficient in a C57BL/6N background for the targeting in JM8.F6 cells: 7/50 screened G418-resistant colonies were correctly targeted (14%). Clones were screened by Southern blot for the initial targeting (Supplementary Fig. 2b). A correctly targeted clone was expanded and electroporated with a pFLPe expression plasmid¹⁹ to remove the selection cassette and leave a single FRT site. Clones that had lost the selection cassette were selected in 200 nM FIAU and screened by PCR using the primers above (Supplementary Fig. 2c). The resulting *A^{tm1Brd}* allele (MGI: 3842513) confers an all-over agouti coat, unlike a spontaneously reversion isolated in a C57BL/6J colony, which has a white belly²⁰ (Supplementary Fig. 2d).

Blastocyst injection. Blastocysts (3.5 d post-coitum) were collected from natural matings of C57BL/6-*Tyr^{c-Brd}* albino mice and expanded for 1–2 h in embryonic stem cell medium containing 20% FCS. Up to 25 cells (fewer less expanded blastocysts) were microinjected into the blastocyst cavity and five injected blastocysts transferred to the uteri of pseudopregnant F₁ female mice (five blastocysts per uterine horn). To test for germline transmission, male chimeras were bred to C57BL/6-*Tyr^{c-Brd}* albino female mice. For JM8, JM8.F6 and JM8.N4 the presence of black pups in litters from these crosses indicates germline transmission. In the case of JM8A cells (Agouti heterozygous), a mixture of black and agouti pups are obtained in the case of germline transmission. All animal studies were carried out at the Wellcome Trust Sanger Institute under the UK Home Office licenses 80/2020 and 80/2076.

17. Liu, P., Zhang, H., McLellan, A., Vogel, H. & Bradley, A. *Genetics* **150**, 1155–1168 (1998).

18. Court, D.L. *et al. Gene* **315**, 63–69 (2003).

19. Farley, F., Soriano, P., Steffen, L. & Dymecki, S. *Genesis* **28**, 106–110 (2000).

20. Dickie, M. *J. Hered.* **60**, 20–25 (1969).