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Mouse mutants from chemically mutagenized embryonic stem cells

Robert J. Munroe¹, Rebecca A. Bergstrom¹, Qing Yin Zheng¹, Brian Libby¹, Richard Smith^{1,2}, Simon W.M. John^{1,2}, Kerry J. Schimenti¹, Victoria L. Browning¹, and John C. Schimenti¹

¹ The Jackson Laboratory, Bar Harbor, Maine, USA

² The Howard Hughes Medical Institute, Bar Harbor, Maine, USA

Abstract

The drive to characterize functions of human genes on a global scale has stimulated interest in large-scale generation of mouse mutants. Conventional germ-cell mutagenesis with *N*-ethyl-*N*-nitrosourea (ENU) is compromised by an inability to monitor mutation efficiency, strain¹ and interlocus² variation in mutation induction, and extensive husbandry requirements. To overcome these obstacles and develop new methods for generating mouse mutants, we devised protocols to generate germline chimeric mice from embryonic stem (ES) cells heavily mutagenized with ethylmethanesulphonate (EMS). Germline chimaeras were derived from cultures that underwent a mutation rate of up to 1 in 1,200 at the *Hprt* locus (encoding hypoxanthine guanine phosphoribosyl transferase). The spectrum of mutations induced by EMS and the frameshift mutagen ICR191 was consistent with that observed in other mammalian cells. Chimaeras derived from ES cells treated with EMS transmitted mutations affecting several processes, including limb development, hair growth, hearing and gametogenesis. This technology affords several advantages over traditional mutagenesis, including the ability to conduct shortened breeding schemes and to screen for mutant phenotypes directly in ES cells or their differentiated derivatives.

EMS induces predominantly point mutations in mammalian cells, causing null mutations at frequencies exceeding 1 in 1,000 cells^{3–5}. We treated ES-cell cultures of genotypes 129/Sv and (129×C57BL/6J)F1 with increasing concentrations of EMS, then selected surviving cells in 6-thioguanine (6TG) to measure the mutation rate at the X-linked *Hprt* locus (Table 1; all three ES lines are XY). We observed mutation frequencies of up to 1 in 1,200, similar to those in Chinese hamster fibroblasts⁴.

To investigate the mutational spectrum, we sequenced *Hprt* coding regions (amplified by RT-PCR) from clones resistant to 6TG. This revealed 17 classes of mutations (Table 2): 11 G→A transitions, 2 C→T transitions and 4 putative splicing mutations. This spectrum is typical of mammalian cells⁴. We also induced mutations with ICR191, which induces primarily +1 frameshifts in stretches of guanines⁶. Indeed, all 6TG-resistant clones we sequenced contained an additional guanine in a stretch of five or six guanines. Together with the characterization of ENU-induced mutations in ES cells in the accompanying paper¹¹, we conclude that most mutagens will induce lesions in ES cells, consistent with other mammalian cell types.

To determine if ES cells treated with EMS retain the ability to colonize the germ line, we injected surviving cells into blastocysts to create chimaeras. Germline chimaeras (24) were

derived from both 129 and F1 hybrid ES cells exposed to a range of EMS treatments (Table 1).

We intercrossed G1 siblings from each of the four germline chimaeras derived from v6.4 cells (*Hprt* mutation rate=1/2,000) to generate offspring with recessive mutations (Fig. 1, left). Such matings yielded few or no progeny (maximum of 3; Table 3). This low fecundity was not attributable to infertility of either parent, because the average size of their backcross litters was 5.3 (Table 3). This suggests that G1 mice bore a high load of recessive lethal mutations, and that most or all of the G1 mice from each chimaera were derived from a single, mutagenized ES-cell lineage. There was no evidence of a high load of recessive lethal mutations in G1 siblings sired by chimaera CT129-C, which was derived from a culture bearing more than 30% fewer (1/14,600) *Hprt* mutations (Tables 1 and 3). It is possible, however, that the germ line of this chimaera was derived from multiple independent ES cells. These data suggest that ES cells are tolerant to higher levels of mutagenesis than are practically required, and that two-generation recessive screens should ideally use ES-cell clones mutagenized at an intermediate level.

To ‘dilute’ the high load of lethal mutations in the v6.4 cells, we conducted a three-generation screen for recessive mutations (Fig. 1, right, and Table 4). We assessed G3 animals for visible dysmorphology, ocular lesions, hearing and male fertility, and recovered the following mutants: Polydactyly ems (*Pde*), an autosomal dominant mutation that arose in CJ7 cells (Fig. 2a); syndactyly ems (*sne*), an autosomal recessive mutation affecting hindpaw digits (Fig. 2b); twisted legs (*twl*), an apparently recessive mutation causing a bowed tibia and a malformed, triangular shaped fibula (Fig. 2d); and peachfuzz (*pfz*), in which homozygotes lose nearly all hair by the time of weaning (Fig. 2e).

We screened 310 G3 males for fertility and identified 22 that failed to produce offspring. Of these, 11 had sperm or testis abnormalities. One male produced sperm in which all the heads were separated from the tails. Another had no epididymal sperm, apparently caused by defective flagellar formation. The remaining nine animals represent three mutations, called *meil* (meiosis defective 1), spermatogonial depletion (*sgdp*) and round sperm heads (*rsph*).

Mutant *meil* males are devoid of epididymal sperm and have small testes. Testis sections revealed a complete absence of post-meiotic cells (Fig. 2f). The most advanced seminiferous tubule section contained spermatocytes apparently arrested at the zygotene/pachytene stage of meiosis. Homozygous females are also sterile, having ovaries depleted or devoid of oocytes. Affected *rsph* males have sperm with rounded, rather than hooked, heads as visualized by light microscopy (Fig. 2c). Examination by scanning electron microscopy revealed that this is apparently due to a retention of cytoplasm around the sperm heads (Fig. 2c). Many sperm also have curled flagella (data not shown). The *sgdp* mutation causes some seminiferous tubule sections to become devoid of germ cells (Fig. 2c), reminiscent of Sertoli-cell-only syndrome in humans (MIM 305700). Other tubule regions remain relatively normal, although spermatid elongation appeared to be aberrant.

We screened 423 G3 mice from 28 families for deafness, identifying 12 animals with poor Preyer reflex. Of these, four had elevated auditory brainstem response (ABR) thresholds (Table 3): two were homozygous for a single recessive mutation originating from chimaera v6.4-C1 (Table 4), exhibiting high-frequency hearing loss; one carried an autosomal recessive mutation characterized by a subtle, unbalanced gait and elevated ABR threshold at low frequency (8 kHz); and the remaining mouse died before mating.

We failed to observe any retina or optic nerve abnormalities in 367 G3 mice. The lack of phenotypes such as retinal degeneration may reflect the young age (2–5 months) of the analysed animals. Several mice with other visible aberrations were born as single cases, and it is not yet

known if their phenotypes are heritable. These include the following phenotypes (Table 4): curled tails, absent or small eyes, progressive ataxia/paralysis and unusually shaped heads or trunks. Many did not breed, and others are now being analysed.

Our finding that highly mutagenized ES cells retain germline competence provides unique options for the creation of mouse mutants. A wide range of mammalian-cell mutagens are available to achieve a desired mutational spectrum; this may be used to circumvent the mutational bias of ENU that confounds attempts to achieve saturation mutagenesis², and to simplify detection of unselected mutations in genes of interest⁷ using frameshift mutagens such as ICR191. Most significant is the possibility of screening treated ES cells for mutations affecting basic cellular processes (such as DNA-damage repair) or following differentiation. Selection of recessive mutations could be achieved in deletion-bearing cells^{8,9}. The ability to generate, screen and preserve clones at the cellular level affords enormous logistical benefit.

Methods

Treatment of cells

We maintained cultures of ES cells on primary embryonic fibroblasts under standard conditions. When they reached ~50% confluence, we applied EMS (methanesulphonic acid ethyl ester, 1.17 g/ml; Sigma) to the media for 16–20 h. In a typical experiment, we applied a range of EMS concentrations to a parallel series of ES cell cultures, each containing $\sim 2 \times 10^6$ cells. After exposure, the cells were washed in PBS, trypsinized and counted. The bulk of cells were passaged onto fresh feeder plates and frozen for later use (to generate chimaeras). We seeded 1,000 of the remaining cells onto gelatin-coated 60 mm plates and counted the number of resulting colonies to determine the percentage survival of treated cells, following adjustments for plating efficiency (determined by plating untreated cells in parallel) and death during the treatment period (by comparing cell numbers following trypsinization of exposed and control cultures).

Mutation rate at *Hprt*

Following exposure to EMS, cells were grown for 10 d and passaged as needed to allow for dissipation of remaining *Hprt* activity. We then plated them at concentrations of $0.5\text{--}1 \times 10^6$ cells/150 mm gelatin-coated plate (no feeders) in media containing 6TG. Selection was removed after 3 d, and the number of colonies was counted 2 weeks after seeding. Untreated control cells yielded no 6TG-resistant colonies in $v6.4$ cells, and a maximum of one per plate with 129 cells. This background was subtracted in the reported frequency of EMS-induced mutations. The concentration of plated cells was critical; higher concentrations of cells plated caused the death of certain fractions of true *Hprt*-deficient cells, as determined in pilot experiments involving plating a constant number of *Hprt*-deficient ES cells with increasing numbers of wild-type cells.

Sequencing of *Hprt* mutations

We extracted total RNA from 6TG-resistant ES cell clones grown in individual wells of a 24-well culture dish, without feeders, using an RNeasy kit (Qiagen). Reverse transcription with Superscript (Gibco-BRL) was primed with random hexanucleotides. cDNA was amplified using the primers 5'–CGTCGTGATTAGCGAT GATG–3' and 5'–TCTACCAGAGGGTAGGCTGG–3', resulting in 852-bp amplicons. The PCR reaction was processed in a Qiaquick PCR cleanup columns (Qiagen), and sequenced from both ends using an ABI Model 377 automated sequencer.

Generation of chimaeric mice

We injected ES cells into C57BL/6J blasto-cysts by standard methods.

Fertility testing of males

We mated G3 males at ~2 months of age to a single (C57BL/6J×A/J) F1 female. The male was deemed infertile if no litters occurred over the following 2 months, and was sacrificed for examination of epididymal sperm. If the sperm appeared abnormal or were absent, then the testicles were fixed in Bouin's, sectioned, and stained with haematoxylin and eosin.

Hearing

We first screened G3 mice with a 'click box', which emanates a high-frequency (18.6 Hz), pure-tone sound burst that is the optimum of mouse hearing sensitivity. This elicits a Preyer reflex (pinna twitch) in mice with normal hearing. An ABR test was performed on those that failed to display this reflex¹⁰. Mice with ABR thresholds above 55 (for click stimulus), 40 (for 8 kHz), 35 (for 16 kHz) or 60 (for 32 kHz) dB SPL were considered to have defective hearing¹⁰.

Clinical eye examinations

A slitlamp was used for evaluation of the anterior segment and an indirect ophthalmoscope for assessment of the retina¹¹. We found a variety of ocular abnormalities (including unilateral and bilateral mid-dilated pupils; oval, eccentric pupils; diffuse corneal opacity with a central clear area, and one mouse with persistent tunica vasculosa lentis and cataract), and we are investigating two to determine if the defects are genetic. The remainder were not pursued, as they had defects characteristic of C57BL/6J mice, the incidence and severity of which varies between colonies and due to environmental factors¹².

Skeletal preparations

We carried out Alizarin red and Alcian blue staining of bone and cartilage as described¹², except the skeletons were stored in a solution of 2:2:1 95% ethanol:glycerol:benzyl alcohol.

Testes histology

We fixed testes in Bouin's for ~24 h, washed in 70% ethanol and embedded in paraffin. We stained sections (5 µM) with haematoxylin and eosin.

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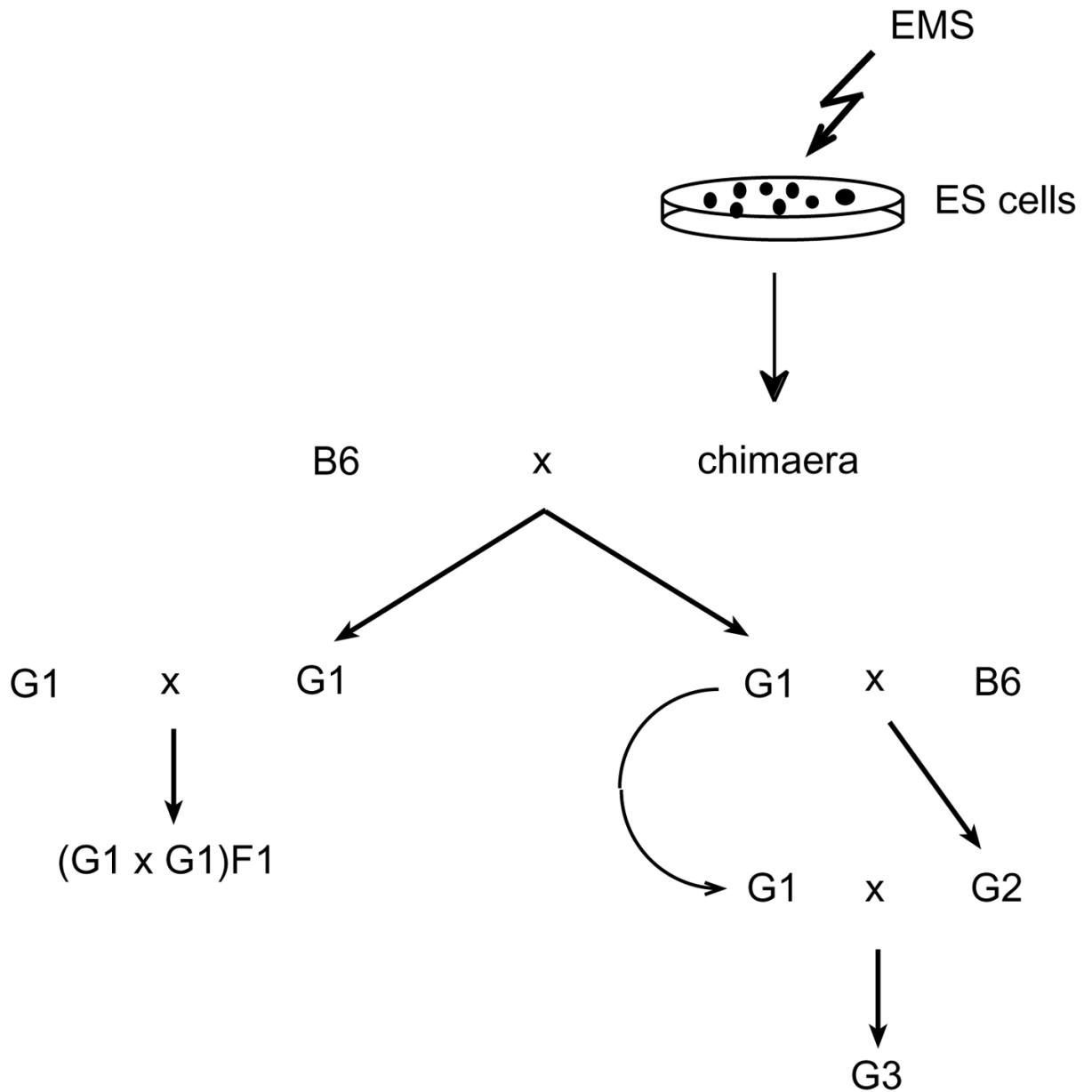


Fig. 1. Breeding strategy for recovery of recessive mutations. Chimaeras generated from EMS-treated ES cells were mated with C57BL/6J (B6) partners. Agouti G1 progeny derived from gametes of ES cell origin were mated with either opposite sex G1 siblings (left) or B6 (right). We backcrossed G2 progeny with the opposite sex G1 parent to yield the G3 generation. Because each of the founding chimaeras could transmit two different alleles at each locus (assuming a germ line derived from a single founder ES cell), several G1 progeny from each chimaera were used to initiate these screens. Furthermore, to optimize the likelihood that most mutagenized alleles would be captured in this screen, we sought to generate 6–8 G2 progeny from each G1 male (up to four G2s from a female G1) for backcrossing.

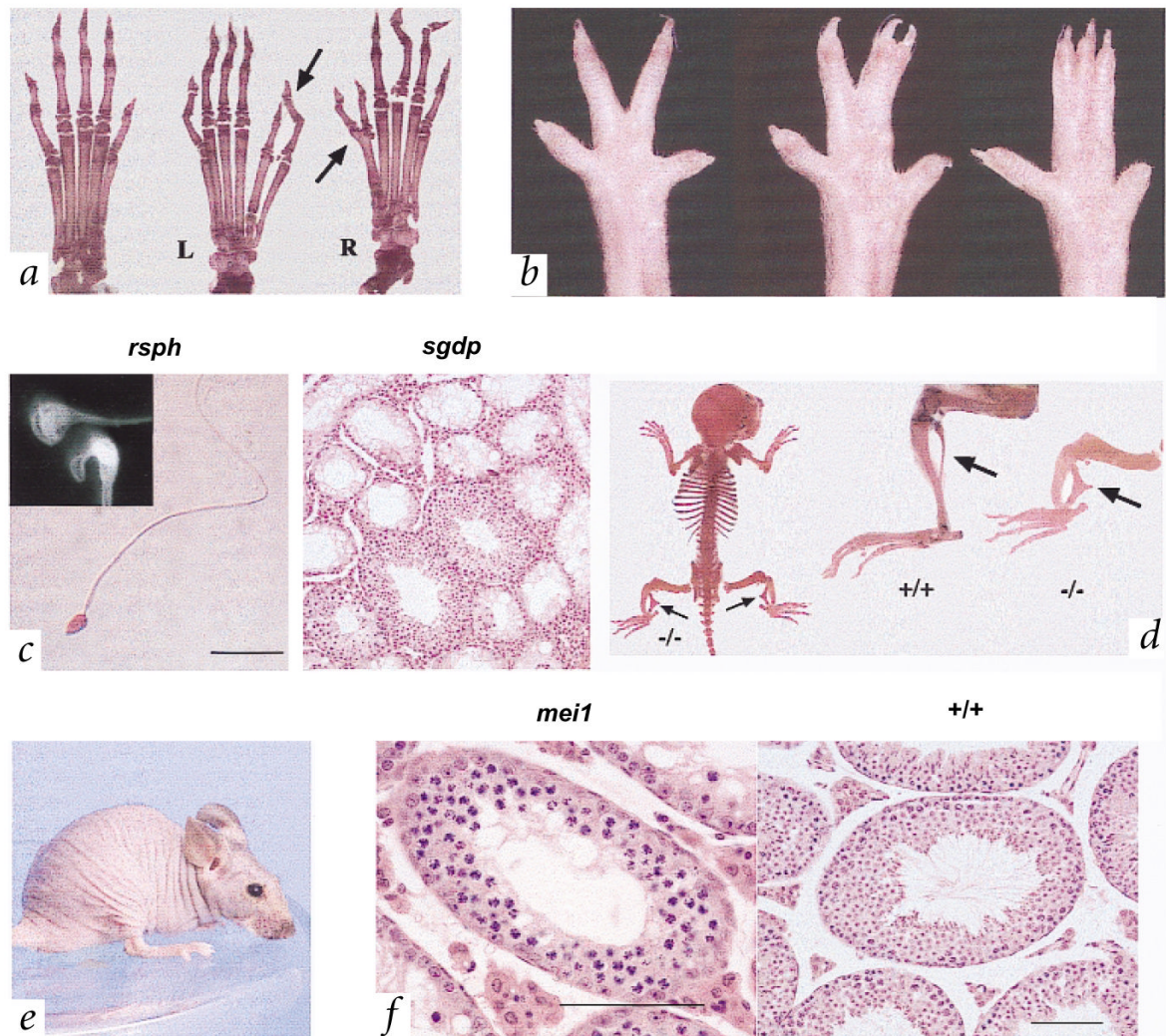


Fig. 2. Phenotypes of mutants. **a**, Bone preparations of hindfeet from normal (+/+, left) and *Pde* (+/-) mutant mice. The length of the extra toe can vary, having either two phalanges, as is seen in the normal first digit, or three phalanges, as in digits two through five. In some cases, the extra toe arises as a bifurcation of a digit 1 metacarpal or phalange. Arrows highlight the variable manifestation of the poly-dactyly phenotype in hindfeet of one individual. L, left; R, right. **b**, Examples of *sne* phenotypic expression in hindfeet of homozygous mutants. **c**, Light microscopic and electron microscopic (EM; inset) images of sperm from the *rsph* mutant and associated *sgdp* phenotype. Normal sperm heads in mice are hook-shaped; an apparently normal shaped head is enveloped in the residual cytoplasmic sac in the EM photograph. Right, an example of an *sgdp* testis section in which relatively normal (albeit with apparently abnormal flagellar development in spermatids) seminiferous tubule segments are juxtaposed with agametic tubule segments. **d**, Skeletal preparations of the *twl* mouse. Arrows indicate the malformed fibulae in the mutant (-/-) compared with a normal animal (+/+). Also note the shortened and thickened tibiae in the affected mice. **e**, A *pfz* homozygote. **f**, Cross-section through the testes of a *mei1* mutant and a normal animal (+/+). Note the accumulation of spermatocytes with condensed chromosomes in the most mature mutant tubules, and how others are either vacuolated or lined only by a thin spermatogonial layer. Presumed apoptotic cells appear as large, dark cells. Scale bars in *c,f*, 100 μ M.

Table 1

Chimaeras from EMS-treated cells and *Hprt* mutation rate

Cells	Genotype	[EMS]	% dead	<i>Hprt</i> -	Chimaeras	Germ line
v6.4 *	129/SvJae×C57BL/6J	600	95%	1/2,000	6	4
CT129 c *	129/Sv + ^c , +P	237.5	38%	1/14,600	9	1
CT129 d *	129/Sv + ^c , +P	475	92%	ND	6	1
CJ7	129/SvEv	200	41%	ND	4	2
CJ7	129/SvEv	300	44%	1/7,222	8	7
CJ7	129/SvEv	400	64%	1/3,277	14	6
CJ7 *	129/SvEv	400	92%	1/4,524	2	2
CJ7	129/SvEv	500	94%	1/1,200	4	1

The EMS concentration is in $\mu\text{g/ml}$. The two classes of CT129 cells ('c' and 'd') represent different EMS mutagenesis experiments on the same line of cells. ND, not done. Mutation rate at *Hprt* is expressed as a fraction derived from screens of $>5 \times 10^6$ cells, representing $>2 \times 10^4$ founder cells.

* Cell population used in the phenotype screen. The mutation rate is adjusted for the background of spontaneous 6-TG-resistant colonies. These were 1 in 377,777 for CT129, 1 in 187,500 for CJ7 and <1 in 1.1×10^6 for v6.4.

Table 2*Hprt* mutations in EMS- and ICR191-treated ES cells

Chemical	Cells	Change	Position	Mutation
EMS	v6.4	splicing	–	exon 8 skipped
EMS	CJ7	?	?	no PCR product
EMS	CJ7	splicing	–	extra exon betw. exons 5,6
EMS	v6.4	splicing	–	66 bp exon betw. exons 5,6
EMS	CJ7	G→A	47	Gly→Asp
EMS	CJ7	G→A	118	Gly→Arg
EMS	v6.4	G→A	119	Gly→Glu
EMS	v6.4	C→T	202	Leu→Phe
EMS	v6.4	G→A	208	Gly→Arg
EMS	CJ7	G→A	209	Gly→Glu
EMS	v6.4	G→A	209	Gly→Glu
EMS	CJ7	G→A	355	Gly→Arg
EMS	v6.4	G→A	418	Gly→Asn
EMS	CJ7	G→A	419	Gly→Asp
EMS	CJ7	G→A	539	Gly→Glu
EMS	v6.4	C→T	551	Pro→Leu
EMS	CJ7	G→A	601	Asp→Asn
EMS	v6.4	G→A	601	Asp→Asn
EMS	v6.4	G→A	610	His→Tyr
ICR191	v6.4	+1 G	207–212	inc. G run from 6 to 7
ICR191	v6.4	+1 G	333–337	inc. G run from 5 to 6

'Position' refers to the coding region nucleotide number; nt 1 is the 'A' in the initiation codon. The 10 different mutations in EMS-treated v6.4 cells were distributed among 15 sequenced, 6TG-resistant clones, and the 9 different mutations in CJ7 were distributed among 17 clones. We analysed eight 6TG-resistant, ICR191 clones, revealing two classes of mutations. The CJ7 line that failed to yield a PCR product was analysed by Southern blot, revealing no deletions or rearrangements; it is possible that this is a regulatory mutation. The positions of mutations causing splicing defects were not determined.

Table 3

Intercrosses of G1 progeny

Chimaera	Progeny crosses	Days mated	G1×G1 pups	G1×B6 pups/litter
v6.4-C1	C1B×C1A	50	0	6; 7
	C1D×C1C	50	0	0; 5.7
	C1F×C1E	11	0	10; 8.3
		10	2*	
v6.4-C2	C2A×C2B	11	0	7.3; 6.5
		14	0	
	C2C×C2B	20	3	8; 6.5
v6.4-C3	C3A×C3B	48	0	6.5; 3
v6.4-C7	C7B×C7A	27	2*	6; 6
			2	
	C7D×C7C	84	2	3; –
	C7F×C7E	84	3	8.5; 2.5
	C7I×C7H	57	0	0; 7
	C7J×C7K	57	3*	0; 4.2
CT129-C	TC2I×TC2F	68	6	7; 8
	TC2J×TC2G	68	5	–; 7
			8	
	TC2K×TC2H	68	1	–; 7
			11	

The animals listed in 'Progeny crosses' are G1 siblings of the corresponding chimaera, indicated as dam×sire. The length of time these crosses were performed is listed under 'Days mated'. In some cases, the animals were mated twice, as indicated by separate line entries. The number of pups from these intercrosses is listed under 'G1×G1 pups'. The values in 'G1×B6 pups/litter' are the average litter sizes of each G1 parent (dam; sire) when mated with C57BL/6J (a dash indicates that the animal was never outcrossed). The average of these averages is 5.3.

* Pups died before weaning.

Table 4

Mutagenesis screens

chimaera	Expansion of families				Phenotypic screens			
	no. G1	no. G2	pups/mating	no. G3	vis.	fert.	eye	hear.
v6.4-C1	6	38	1.4	93	1 + ser (3)	3/48	2/60	3(2)/69
v6.4-C2	6	29	3.0	161	3	8/78	3/118	0/124
v6.4-C3	1	3	0.0	0	–	–	–	–
v6.4-C7	6	28	1.5	57	2 + Sne (3)	1 + Rsph (2); Sgdp (2)/24	4/44	0/41
CJ7-1	2	9	4.8	53	0	Mei1 (5)/15	0/16	0/16
CJ7-2	1	3	7.7	23	1 + Pde (5)	0/4	0/16	1/18
CT129-C	7	35	6.0	265	1 + Tw1 (3)	0/92	1/89	0/124
CT129-D	3	11	3.1	52	1 + Pfz (5)	1/18	2/24	0/31
Totals	32	156		704	14	16/310	12/367	3/423

The founder chimaeras are listed on the left, with the prefix designation reflecting the ES cells from which they were derived. 'No. G2' lists the total number of sons or daughters from all G1s that were backcrossed to a G1 parent. 'Pups/mating' is the total number of weaned offspring per mating period. A mating period consisted of at least one week in which a G1 and G2 were together in a cage before being separated. If two litters were produced in a mating, it was counted as two mating periods. G3 values indicate the number of pups weaned. All G3 animals were screened for visible mutations ('vis.'): the number of variants not proven to be genetic is indicated and the names of proven mutations are indicated after the '+', with the total number of animals exhibiting that mutation given in parentheses ('ser', a short-eared variant not discussed in the text). For the remaining phenotypic screens, the numerator is the number of variants identified and the denominator is the total number of animals examined. In those cases in which multiple animals in a family have the same heritable mutation, the number of such animals is given in parentheses, in some cases along with the mutant symbol. 'Fert.', infertile males; 'hear.', mice that failed both click box and ABR tests. The totals reflect the number of affected animals (in the numerator; underlined numbers indicate the adjusted number of independent phenotypes) over the total number of animals.