THE ART AND DESIGN OF GENETIC SCREENS: MOUSE

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Abstract | Humans are mammals, not bacteria or plants, yeast or nematodes, insects or fish. Mice are also mammals, but unlike gorilla and goat, fox and ferret, giraffe and jackal, they are suited perfectly to the laboratory environment and genetic experimentation. In this review, we will summarize the tools, tricks and techniques for executing forward genetic screens in the mouse and argue that this approach is now accessible to most biologists, rather than being the sole domain of large national facilities and specialized genetics laboratories.

MOUSE FANCY
A collection of enthusiasts interested in mice with unusual traits.

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Not surprisingly, given its experimental advantages (BOX 1), the mouse has been by our side as we have walked down the road of biological self-discovery. Mouse mutants have been a source of fascination for millennia[1]. A century ago, the mouse was there with Cuénot to provide the first confirmation that Mendel's newly rediscovered laws applied to animals as well as plants[2]. Since then, the mouse has become the pre-eminent model for human physiology and disease. A few years ago, the sequence of the mouse genome was completed, providing a detailed, if furry, reflection of our own evolutionary history, and leading to the realization that all but a few hundred of our approximately 25,000 genes have direct mouse counterparts[3]. The challenge now is to resolve the relationship between our genome sequence and physical form and to harness this knowledge to find treatments for disease. Genetics offers an unsurpassed method for unravelling complex biological problems, and over the next 100 years, as we chip away at understanding our own biology and ameliorating disease, the mouse will be there beside us.

Mouse genetic screens allow the dissection of many biological processes — from disease resistance, onset and progression, to general phenomena such as development, self-renewal and ageing. Independent of the biological particulars, the ultimate aim of all genetic screens is to link a change in phenotype with a defined change in genotype, and all genetic screens therefore have common elements. Our goal in this review is to capture the state-of-the-art of genetic screens in mice, providing readers with a practical guide to the three steps of the screening process: mutagenesis, screen design and execution, and to its dénouement: the identification of the causative genetic change.

Mutagenesis

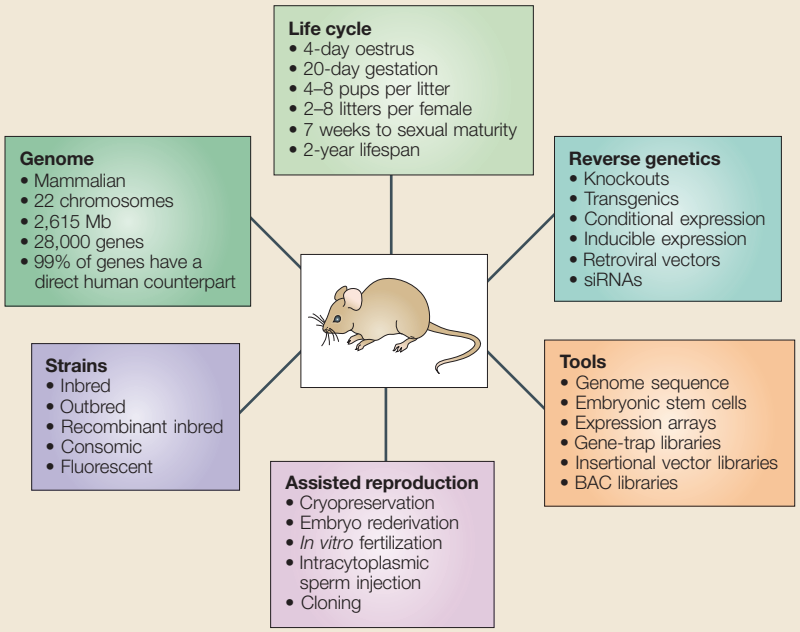
Historically, mouse genetics has relied on spontaneous mutation for introducing genetic variety into populations being screened. A steady stream of mouse mutants was identified in wild populations, both among the collections of the MOUSE FANCY and in the colonies maintained at the Jackson Laboratory. The low rate of spontaneous mutation in mouse germ cells, however, precluded genetic screens from being feasible for any but the largest mouse production facilities and the most patient of investigators. For the mouse to become a widely used genetic model, a method for accelerating the rate of mutagenesis was essential.

In a series of landmark papers, Bill Russell and colleagues showed, in the mouse, that the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) has three advantages over previously used mutagens[4–6]. First, it is very efficient, inducing a loss-of-function mutation at a given locus in approximately 1 sperm in 1,000 (REFS 4,7) and a point mutation every 1 to 2 Mb throughout the genome[8–11]. This rate is ~100-fold higher than the spontaneous mutation rate and 3-fold higher than X-irradiation. This means that an investigator who is interested in a process that is controlled by 10 genes is likely to be able to identify mutants by screening 1,000 mice, a task that is readily achievable by many laboratories (BOX 2). The extent to which this rate can be increased before

Box 1 | The mouse as an experimental system

Despite having diverged some 75 million years ago, mice and humans share a remarkable degree of genomic, anatomical and physiological similarity. As mammals, mice facilitate the study of processes and pathologies that cannot be modelled in yeast, worms or flies. The figure illustrates some of the features and technologies that together make the mouse an exceptionally tractable system for determining mammalian gene function and modelling human disease.

Apart from its small size, the mouse has, compared with other mammals, a short generation time and is relatively fecund. This makes it feasible to carry out genetic studies that necessarily involve significant numbers of animals and considerable breeding efforts. Another advantage is that a large number of well-characterized inbred strains exist, allowing investigators in different laboratories to carry out controlled experiments on the same genetic background and facilitating genetic mapping. Assisted reproduction technologies mean that strains can be cryogenically preserved, transferred and reconstituted at any time. In addition, an unsurpassed array of technologies and tools exist in the mouse, which allow a host of physical and genetic manipulations to be carried out.



Box 2 | The growing accessibility of mouse genetics

For the investigator using mouse genetics for the first time there are two alternatives: obtaining a mutation that has already been generated by a large consortium, or embarking on a genetic screen of their own. One of the criteria by which large mutagenesis centres will be judged is the extent to which the scientific community accesses their library of mutant mice. As a result, a great deal of effort has been spent by these groups in developing broad, hierarchical and standardized phenotyping assays that allow a range of biological processes to be investigated (for example, SHIRPA[95] and EMPReSS[96]) and the bioinformatic tools required to handle the large amounts of data generated. By contrast, for a small laboratory embarking on a genetic screen, it is neither cost-effective nor desirable to isolate mutations that affect a wide range of biological processes, and screens are therefore likely to be highly focused.

For the uninitiated, there are still two important psychological barriers to embarking on a genetic screen. The first of these is the perception that mouse genetics requires a prohibitive amount of costly animal room space. As an example, consider a simple screen in which mutants are assessed phenotypically before they are weaned, and therefore before they require a separate cage from their parents. If 10 ENU (alkylating agent *N*-ethyl-*N*-nitrosourea)-injected males are housed with 2 females, each of which might generate a litter of 6 pups every 6 weeks, then over a 12-month period, 960 mice would be screened. Clearly, the exact rate at which pups are generated will depend on the strain being used, the dose of ENU injected into males and the condition of the animal rooms; however, this preliminary calculation highlights that space is often not the limiting factor in carrying out a screen.

The second barrier is a misconception that positional cloning of mouse mutations is an almost insurmountable obstacle. Although the mapping of mutations to particular regions of the genome using meiotic recombination occurs in broadly the same manner as it did when genetic linkage was first discovered nearly a century ago[97], the availability of the sequence of the mouse genome has greatly simplified the entire process. Positional cloning is now as routine as building a gene-targeting vector, with the added bonus that the investigator knows what phenotype they are dealing with from the beginning. To appreciate the extent of change, compare the pre-genome strategy described by Silvers[82] and pursued heroically by Takahashi and colleagues to identify the *Clock* gene[58,59], with that used in the post-genome era by Beutler and colleagues to isolate components of the Toll-like receptor signalling pathway[23,24].

lethality and sterility render screening impossible remains to be determined. Second, unlike irradiation, which induces multi-locus deletions, ENU is a point-mutagen and affects single loci. Finally, unlike agents such as chlorambucil, which targets post-meiotic cells, ENU-induced mutations occur in spermatogonial stem cells, allowing males to sire mutant offspring for extended periods. These advantages have made genetic screens of the type that are carried out in lower organisms feasible in the mouse.

Screen design: an overview

FORWARD GENETIC SCREENS can be classified according to the proportion of the genome being screened, ranging from a single gene to the entire genome (FIG. 1). Although ENU treatment induces mutations throughout the genome, various approaches can increase the likelihood of recovering mutations in a particular genomic region. The choice of screening strategy depends on the goal of the study. Region-specific screens are often used to generate an ALLELIC SERIES or when investigating the functional content of a particular genomic interval, whereas genome-wide screens are favoured when the goal is to understand the genetic regulation of a specific biological process. These two types of screen are discussed here in subsequent sections. Irrespective of the exact biological focus of a screen, utmost care must be given to the process of screen design, as this will ultimately determine the success or failure of the endeavour. Several important issues that influence the ease, cost and ultimate success of the screen require consideration.

Biological process. Is the process of interest amenable to investigation in the mouse? With the exception of higher-order neurological functions, such as speech,

FORWARD GENETIC SCREEN
A genetic screen in which mutants are isolated on the basis of their phenotype. The mutation responsible is identified by positional cloning or by a candidate-gene approach.

ALLELIC SERIES
A series of alleles that are present at the same locus, which produce a gradient of phenotypes.

SHIRPA
A hierarchical protocol for the behavioural and pathological phenotyping of potential mouse models of neurological dysfunction.

EMPReSS
Individual phenotyping protocols that provide a platform for the systematic and standardized primary screening of mouse mutants.

EPIGENETIC
Any heritable influence (in the progeny of cells or of individuals) on chromosome or gene function that is not accompanied by a change in DNA sequence.

mice represent a treasure trove for investigators who are pursuing the molecular basis of human biology. Screens for mutations that affect development[12,13], neurological function and behaviour[14–18], reproduction[19,20], metabolism[21,22], immunology and haematopoiesis[23–27], chromosomal instability[28] and EPIGENETICS[29,30] have all been executed in the mouse, and many other screens are underway[31]. In any screen, one must consider whether the assay that is used can measure the desired parameter or, if a surrogate marker is chosen, whether the marker is truly indicative of the process. When modelling human diseases in the mouse, the age-old question of how truly relevant the model is must be considered. Assuming answers of a positive nature can be given to these questions, the purely practical issue of phenotyping arises.

Simplicity. How simple, sensitive and specific is the assay to be used? With increasing complexity comes increasing difficulty when dealing with large numbers of animals, not only during the screening process, but particularly during mapping and mutation identification. In the mouse, a range of genomic tools that can simplify the analysis of otherwise complex biological processes is available. An example is the generation of lines that carry transgenic reporters, which were recently used to recover mutations that affect cortical development and epigenetic inheritance[29,32]. Ultimately, however, it is for the investigator to decide how much effort they are prepared to put into a phenotypic screen — where the rewards are greatest, more significant exertions will be justified.

Life and death. Can the screen be carried out without compromising viability or fertility? If not, care must be taken to ensure that mutations of interest can be recovered, either by sparing closely related mice that are likely to carry the mutation, or by preserving the gametes of the mice to be screened. As sperm freezing is significantly easier than oocyte freezing, this limits screening to male mice. Screens for developmental defects represent an extreme case, as not only are the mice to be screened sacrificed, but so are their mothers, eliminating one class of relatives from which the mutation can be recovered.

Normal variation and robustness. How much does the phenotype of interest vary in the starting strain when it is housed and treated in an identical manner to the mutants that will ultimately be screened? Understanding the extent of normal variation will dictate the cut-off used for examining mutants in more detail. The more control animals that are examined, the greater will be the understanding of the variation in the phenotype. Another question relates to the robustness of the mutant phenotype. Is the phenotype stable from week to week and can the same cut-off therefore be used throughout the screen? If possible, a group of control mice should be assayed in parallel with each group of potential mutants; this will alert the investigator to any drift in the phenotype over time.

False positives versus false negatives. The decision of where to draw the line for examining mutants in greater detail depends on whether it is perceived to be preferable to discard a mutant with modest effects or to assess a large number of outliers, the phenotypes of which might not have a heritable basis. This equation will be different for each investigator, each budget and each phenotype.

Age at screening. The age of the mice at the time of screening is an important determinant of the number of animals that can be screened in a given amount of animal-room space. If animals can be screened before they are weaned they can be housed with their parents, never requiring their own cage. By contrast, if animals must be several months of age before screening can commence, there is a concomitant increase in the space required; so, the cost per mouse screened is much higher, as is the cost of mapping any mutations that are found.

Genetic background. To map a mutation, the wild-type and mutant phenotypes must not only be separable on the background strain used for the screen, but also on the mixed background used for mapping. If polymorphisms that are found between the screening strain and the mapping strain alter either the wild-type or mutant phenotype, mapping will be more complicated but might still be feasible. This is an important issue for immunological phenotypes and some complex-disease models. Conversely, although two closely related strains might have a similar phenotype, their genomes might not be sufficiently polymorphic to easily map the mutation.

Mutagenesis screens in the mouse

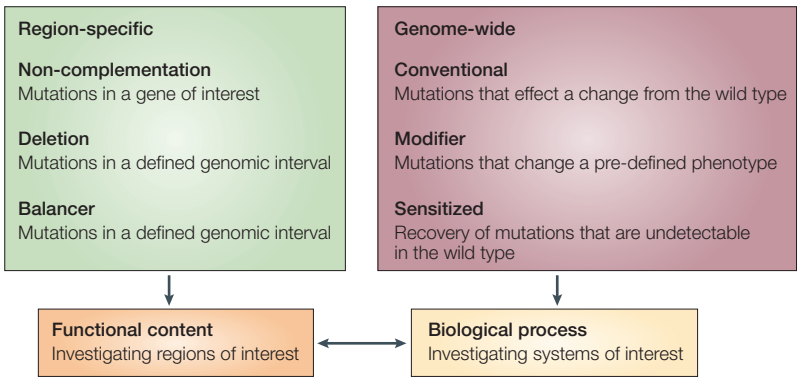


Figure 1 | **Classification of genetic screens.** Current strategies can be grouped according to their genomic focus: region-specific or genome-wide. Within these broad categories, there are several approaches to the identification of new mutations. In general, region-specific screens are designed to examine the functional content of a genomic segment, whereas genome-wide strategies are better suited to the dissection of a specific biological process. There is a large overlap between the two approaches, and in some cases, a region-specific screen might be a more appropriate method for recovering mutations that affect a particular process — for example, development — in which a huge number of genes have essential roles. A region-specific approach, although reducing the number of potential mutations that might be identified, can greatly increase the ease with which the screen is conducted and mutations are subsequently maintained. See the main text for details of the individual types of region-specific or genome-wide screen that are indicated in the figure.

COMPOUND HETEROZYGOTE
A diploid genotype in which the two copies of a gene carry different mutations.

Region-specific screens

Non-complementation screens. Complementation is the production of a wild-type phenotype when organisms that carry recessively functioning mutations in different genes are crossed. By contrast, if the mutations are in the same gene, a mutant phenotype results. This is referred to as non-complementation, and the principle is the essence of both allelic non-complementation screens and non-allelic non-complementation screens, as explained below.

Non-complementation screens involve the mutagenesis of wild-type male G_0 mice, which are then outcrossed to females that are homozygous for the mutation of interest (FIG. 2a). G_1 animals inherit a mutant allele at the locus of interest from their mother and a suite of random ENU-induced mutations from their father. If an ENU-induced mutation results in loss of function at the locus of interest, the COMPOUND HETEROZYGOTE will recapitulate the maternal phenotype due to allelic non-complementation. Mutations outside the original locus that produce a phenotype in combination with the original mutation are the result of non-allelic non-complementation. In addition, unrelated dominant mutations might be recovered. Subsequent breeding will establish into which category each mutation falls.

The specific locus test (SLT), pioneered by Bill Russell[33] and used to demonstrate the potency of ENU[5], is the first and best-known example of such a screen, and represents a seminal contribution to mouse genetics. ENU-treated males were crossed to test-strain females that were homozygous for 7 marker genes, and new alleles of all 7 loci were isolated. Initially designed to examine the potential of various mutagens, the SLT yielded extensive allelic series, such as that for the bone morphogenetic protein 5 gene (*Bmp5*) (REF. 34), which have proved useful in protein structure–function studies. A good illustration of the usefulness of small-scale non-complementation screens is the work of Greg Barsh's laboratory in positionally cloning the X-ray induced *kreisler* mutation[35]. A new ENU-induced allele of the *kreisler* gene (also known as v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B, *Mafb*), which was isolated from just 597 G_1 progeny that were screened, provided independent confirmation that a mutation at the candidate locus was indeed responsible for the phenotype.

The simple strategy outlined above becomes more complicated if mice that are homozygous for the allele of interest are infertile or non-viable. In this case, ENU-treated G_0 males can be crossed to females that are heterozygous for the mutation of interest. Any allelic non-complementing mutation that causes a phenotype will be identified in the G_1 generation. However, unless the ENU-induced allele spares viability and fertility, it cannot be propagated. An approach that circumvents this problem of homozygote infertility or non-viability is the establishment of a population of G_1 males that are heterozygous for ENU-induced alleles, which can then be crossed to females that are heterozygous for the mutation of interest. Any phenotypes that are identified in the G_2 generation can then be recovered from the G_1 father (FIG. 2b). A feature of early efforts was the necessity of using marker mutations that are linked to the locus of interest to allow the identification of informative progeny for screening. Although this is still a useful tool where available, the advent of PCR and, more recently, the sequencing of the genome, have made the molecular genotyping of progeny routine and they have allowed much greater flexibility.

Deletion screens. Spontaneous or induced chromosomal deletions are useful tools for recovering recessively functioning mutations in a particular genomic region. In a simple deletion screen, mutagenized G_0 males are crossed to females that are heterozygous for a deletion. G_1 progeny that are heterozygous for the deletion are haploid for the interval that is defined by the deletion, allowing loss-of-function ENU-induced mutations in the region to be manifested in a dominant

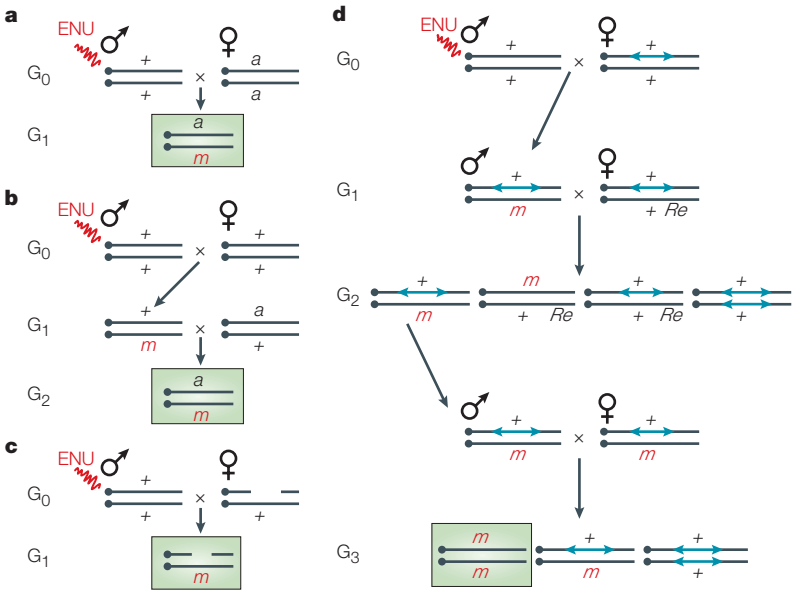


Figure 2 | **Region-specific genetic screens.** The class of animals to be examined for novel phenotypes in each screen is shown in a shaded box. *a* represents a pre-existing allele at the locus of interest, *m* represents a new ENU (alkylating agent *N*-ethyl-*N*-nitrosourea)-induced mutation and + indicates the wild-type allele. **a** | Allelic non-complementation. Each G_1 animal is potentially a compound heterozygote for both mutations. **b** | An allelic non-complementation strategy that can be used in the case of homozygote non-viability. A pool of G_1 animals must first be established by crossing wild-type G_0 females to ENU-treated males. These founder G_1 mice can then be screened by crossing them to animals that are heterozygous for the mutation of interest. **c** | A simple deletion screen for non-lethal mutations. By crossing ENU-treated males with females that are heterozygous for the deletion, recessively functioning mutations can be isolated in the G_1 generation. Screening for lethal or otherwise detrimental mutations requires a strategy that is based on that shown in part **b**. **d** | A balancer screen. The example shown illustrates the chromosome 11 balancer screen[20]. The balancer is represented by the chromosome that carries the two-headed blue arrow. Mice carrying the balancer can be distinguished from littermates by their yellow ears and tail, a trait conferred by the *K14–agouti* transgene, which is carried on the balancer (not shown here). Crossing G_1 mice to animals that are heterozygous for the balancer and *rex* (*Re*; a dominant mutation conferring a curly coat) results in four genotypic classes. Mice that are homozygous for the balancer die *in utero* owing to the disruption of wingless-related MMTV integration site 3 (*Wnt3*) on the balancer chromosome. Animals that have a curly coat carry *rex* in *trans* to either the balancer or the ENU-treated chromosome 11, and are discarded. Animals that are heterozygous for the balancer and ENU-treated chromosome 11 are brother–sister mated in order to make any mutations homozygous. Homozygotes can be distinguished from heterozygotes by coat colour. Animals that are homozygous for the balancer are not recovered.

fashion (FIG. 2c), an important advantage over the three-generation screens that are usually required to make recessive mutations homozygous.

The issue of mutant viability must be considered in this type of screen. If developmental or reproductive mutations are desired, then, like the non-complementation strategy, a pool of G_1 male mice must first be established before crossing them to females that are heterozygous for the deletion. Eugene Rinchik, a pioneer in this area and one of the only investigators so far to execute a deletion mutagenesis screen successfully, has taken this approach. He conducted separate two-generation screens to isolate mutations around the albino (also known as tyrosinase, *Tyr*) and pink-eyed dilution (*p*) loci on chromosome 7 (REFS 36–38).

There are two practical limitations on the feasibility of deletion screens. First, although only a minority of genes in the mouse are HAPLOINSUFFICIENT, deletions necessarily render multiple genes HEMIZYGOUS and often have deleterious effects, ranging from subfertility to non-viability. For each deletion, physical size and genomic location will dictate the number of genes and the collective functions that are eliminated. Only empirical testing can determine whether mice carrying a specific deletion are sufficiently viable to be used in a screen. Second, deletions currently cover only a limited fraction of the genome. Using embryonic stem (ES) cells, several groups have attempted to address this deficit by irradiation and screening for loss of heterozygosity at predetermined loci[39,40], targeting of negative selection cassettes to specific loci (by homologous recombination followed by irradiation)[41,42], and CRE–*LOXP* SYSTEMS that are based on recombination between a targeted and a randomly integrated *loxP* site[43,44]. Despite varying degrees of success, these efforts have significantly increased the deletion resources that are available to the community, and should see a concomitant rise in the popularity of deletion screens.

Balancer screens. First used in *Drosophila melanogaster*, the purpose of a balancer chromosome is to suppress meiotic recombination between itself and its homologue. This is achieved by an inversion or series of inversions, which span part or all of the balancer chromosome. In genetic screens, the use of a balancer allows the germline transmission of a mutagenized chromosome without it undergoing meiotic recombination. It is important to make the distinction between the balancer and the balanced: the balancer is merely a tool, a genetic sleight of hand that prevents recombination of the real (mutagenized) target, the balanced chromosome. Balanced mutagenized chromosomal regions can then be followed through the multiple-generation breeding schemes that are necessary to yield homozygotes, allowing the recovery of recessive phenotypes caused by mutations in the interval that is delineated by the inversion endpoints (FIG. 2d).

In addition to carrying inversions, balancer chromosomes have two other defining properties: a marker that allows animals carrying them to be distinguished from littermates, and a mutation that either prevents recovery of animals that are homozygous for the balancer, or allows heterozygotes to be differentiated from them. This means that genotyping can be done by visual inspection, which makes the selection of pairs for subsequent breeding simple. In addition, at the G_3 generation, it allows mutations to be easily assigned either to the balanced interval or elsewhere in the genome. The balancer breeding scheme enriches for mutations in the balancer interval, but does not preclude the isolation of mutations that map elsewhere in the genome. If a recessive phenotype is seen in animals that are heterozygous for the balancer (that is, heterozygous for the original mutagenized chromosome), the mutation must, by definition, lie outside the balancer interval.

The first mouse balancer screen, which was conducted in Monica Justice's laboratory[20], used a specifically engineered chromosome 11 that was constructed by Alan Bradley's group, Inv(11)8Brd$^{Trp53-Wnt3}$. The balancer contained a 34-Mb inversion between the transformation related protein 53 (*Trp53*; also known as the tumour supressor p53) and wingless-related MMTV integration site 3 (*Wnt3*) loci, and a transgene conferring yellow pigmented ears and tails on otherwise black mice[45]. The screen yielded 231 new mutations that cause developmental, cardiovascular, craniofacial, neurological, fertility-related and haematopoietic phenotypes. Eighty-eight of these mapped to chromosome 11, representing an approximately 300-fold enrichment for mutations within the balancer interval over that which would have otherwise been expected.

This screen showed that the balancer approach is feasible in mice, and also confirmed the supreme effectiveness of balancers in isolating lethal mutations. From conception to adulthood, the presence of lethal mutations in a pedigree is indicated by the simple absence of animals that are homozygous for the original ENU-treated chromosome. Because these are distinguished from littermates on the basis of coat colour, the system represents a fail-safe approach to identifying lethal mutations. Furthermore, mutations are automatically mapped to a chromosomal subregion on isolation. Alan Bradley has since engineered further balancers[46,47], two for chromosome 11 and two for chromosome 4. Others, inspired by the approach, have begun modifying existing radiation-induced inversions to produce functional balancer chromosomes[48,49].

Genome-wide screens

The history of genetics attests to the fact that, for biological processes of apparently imponderable complexity, novel mutations can provide tremendous insights into the critical pathways, opening up entire fields for study. For those interested in specific biological processes, genome-wide screens represent the most powerful approach for the isolation of new mutations. The argument for the use of genome-wide screens is as simple as it is compelling: if interested in genes that control a given process, why restrict your search to anything less than the entire genome?

HAPLOINSUFFICIENT
A gene is haploinsufficient when loss of one functional copy in a diploid organism results in a phenotype.

HEMIZYGOUS
A diploid genotype that has only one copy of a particular gene, as in X-chromosome genes in a male, or when the homologous chromosome carries a deletion.

CRE–*LOXP* SYSTEM
A site-specific recombination system. Two short DNA sequences (*loxP* sites) are engineered to flank the target DNA. Activation of the Cre recombinase enzyme catalyses recombination between the *loxP* sites, which leads to the excision of the intervening sequence.

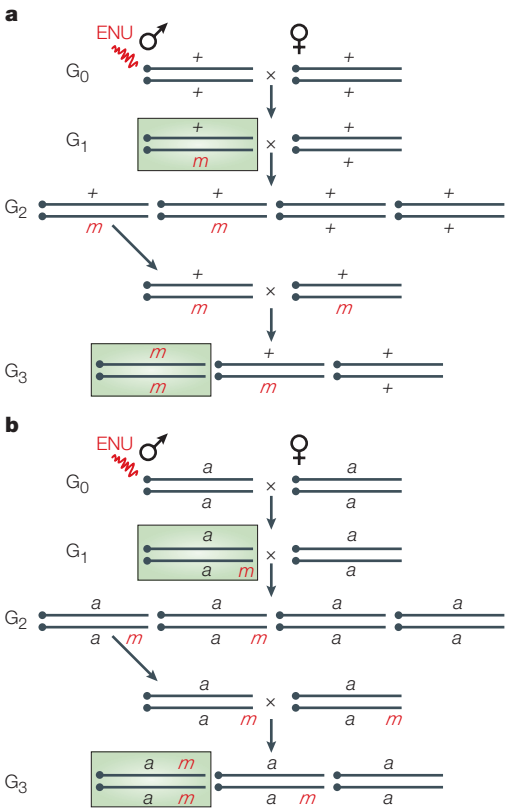


Figure 3 | **Genome-wide genetic screens.** The class of animals to be examined for novel phenotypes in each screen is shown in a shaded box. In both panels, *m* represents a new ENU (alkylating agent *N*-ethyl-*N*-nitrosourea)-induced mutation and *+* indicates the wild-type allele. **a** | Conventional strategy for isolating dominantly or recessively functioning mutations. ENU treatment of wild-type males followed by crossing to wild-type females will facilitate the isolation of dominantly functioning mutations in individual G_1 animals. To make recessively functioning mutations homozygous (and therefore identifiable), pedigrees must be established from individual G_1 mice. This is most efficiently achieved by randomly intercrossing G_1 mice, and then brother–sister mating G_2 mice. A quarter of the G_3 offspring will be homozygous for a mutation that is present in both G_2 parents. **b** | The modifier strategy. *a* represents a pre-existing recessive allele that causes a phenotype of interest. In this simple strategy, mice that are homozygous for the mutation of interest are viable and fertile. Instead of screening for deviations from the normal wild-type phenotype, the screen is designed to isolate mutants in which the pre-existing phenotype of interest is enhanced or suppressed, that is, more or less severe. In this way, genes that function in the same pathway as *a* can be identified. The approach is similar to that in **a**, with dominantly functioning mutations being isolated in the G_1 generation, and recessively functioning mutations being isolated in the G_3 generation. For simplicity, the ENU-induced mutation shown in this example is linked to the recessive allele *a*.

Conventional genome-wide screens. General strategies for executing genome-wide screens to examine dominant and recessive phenotypes are well established (FIG. 3a). In both types of screen, male G_0 mice are injected with ENU and crossed to untreated wild-type females to yield G_1 progeny that can be screened for dominant phenotypes. From this point there are two strategies that can be used to generate G_3 animals to be screened for recessive traits. Which of the two alternatives to use depends on whether it is considered to be more important to screen as many mutations as possible or to screen the highest possible proportion of the mutations that are carried by an individual founding G_1 mouse[50]. To achieve the former aim, G_1 mice are inter-crossed, introducing twice as many mutations into the pedigree. The resulting G_2 mice are then intercrossed to yield G_3 progeny. To achieve the latter aim, traditionally, G_1 males have been mated with non-mutant females and are then crossed to several of the resulting G_2 daughters to produce G_3 animals. As more G_2 daughters are used and more G_3 animals are produced, the number of mutations sampled in the G_3 population approaches the number of mutations that are carried by the G_1 male. From an efficiency viewpoint, there is little to choose between the two screens; however, the generation of G_3 mice by intercrossing G_2 mice does have the important advantage of reducing the reproductive pressure that is placed on the G_1 male as it ages.

The success of pioneering mouse mutagenesis programmes[51,52] has seen the emergence of an increasing number of large-scale centres for ENU mutagenesis[53] that are aimed at filling the 'phenotype gap'[54] and providing access to mutant mice for groups that might not themselves be able to carry out screens. Results from two of these large-scale genome-wide screens conducted at the GSF in Munich, Germany, and the Medical Research Council centre in Harwell, UK[15,26], captured the attention of many biologists. Both groups used simple one-generation screens of wild-type mice to identify mutations with dominant effects. The GSF ran an integrated battery of tests for changes in visible, clinical–chemical, biochemical, immunological, haematological and behavioural parameters. From 14,000 G_1 progeny, they isolated 182 confirmed mutants. The Harwell screen focused on visible, clinical–chemical and neurobehavioural phenotypes, and confirmed 196 heritable traits from 35,000 G_1 mice, of which 85 mutations have been mapped and 20 identified[55].

Many other groups have carried out screens that are focused on their own particular area of biological interest. Elegant and influential early screens led to the identification of the multiple intestinal neoplasia (*Min*; also known as adenomatosis polyposis coli, *Apc*) gene by Bill Dove and colleagues[56,57] and the circadian locomotor output cycles kaput (*Clock*) gene by the Takahashi group[16,58,59]. Despite the inherent difficulties in analysing development *in utero*, successful screens for embryonic lethal mutations have also been carried out[12,13,60–62]. Two of the most exciting and successful ongoing screens are aimed at dissecting the molecular regulation of the immune system. Bruce Beutler's group is focused on understanding the regulation of the innate immune system, and has had outstanding success in identifying components of Toll-like receptor signal transduction pathways, providing invaluable insights into the system[23,24,63]. These studies exemplify

the power of CONDITIONAL SCREENS, where mice are challenged (in Beutler's case with specific pathogens such as mouse cytomegalovirus) to reveal phenotypes that cannot be observed in the steady state.

In another example of the power of genome-wide screens, Chris Goodnow and his team are executing a screen to identify regulators of the acquired immune system, and have also isolated crucial regulators of T- and B-cell function[25,64–66]. Goodnow's screen has beautifully illustrated the ability of forward genetics to shed new light on known pathways, with several mutations revealing new functions for proteins previously thought to have been fully characterized through gene-targeting studies[64]. ENU has the capacity to generate a range of alleles at any locus, and subtle HYPOMORPHIC variations can provide functional insights that binary all-or-nothing gene knockouts often obscure.

Modifier (suppressor/enhancer) screens. Unlike the screens described above, modifier screens do not start with wild-type organisms. Instead, they begin with individuals that have a pre-existing phenotype and aim to identify mutations that enhance or suppress its severity (FIG. 3b). Analyses of coat colour in mice have provided many examples of genetic modifiers[67], among the most spectacular being the modification of the yellow coat colour and obesity observed in a gain-of-function allele of agouti (A_y) by a mutation in the attractin gene (*Atrn*) known as mahogany (*mg*)[68]. In addition, in a series of elegant studies that used the ENU-induced *Min* mutation, Bill Dove's group and others showed that the severity of the neoplastic phenotype produced by this mutation was dependent on the genetic background, indicating that polymorphisms present in different strains influenced the phenotype. Three modifiers of *Min* — *Mom1*, *Mom2 and Foxl1* — have been isolated[69–72].

Despite the success of modifier screens in lower organisms[73–76] and the abundant evidence for mouse genetic modifiers, there is only one published example of this type of screen in the mouse: a screen for mutations that suppress THROMBOCYTOPENIA, which is caused by targeted deletion of the thrombopoietin receptor myeloproliferative leukaemia virus oncogene (*c-Mpl*)[77]. In this study, ENU-treated M*pl*$^{-/-}$ mice were mated with ISOGENIC females and the number of platelets in the peripheral blood of approximately 1,500 G_1 progeny was measured. Two independent mutations in the myeloblastosis oncogene, *c-Myb*, were isolated, which resulted in dominant amelioration of thrombocytopenia[27]. Modifier screens in mice might prove to be especially valuable in investigating the causes of human disease because of the proliferation of excellent mouse models of disease over the past 20 years.

Sensitized screens. A sensitized screen uses a modified genetic background to allow the recovery of mutations that would be undetectable using wild-type organisms. In many biological systems, generation of an observable phenotype requires a reduction of more than 50% in the activity of key components; that is, the genes involved are haplosufficient. It follows that even mutations that result in complete loss of function will not yield phenotypes in a dominant screen, and a more costly and time-consuming recessive screen must be carried out to detect them. An alternative, which forms the basis of sensitized screens, is to reduce the level of activity of the pathway before beginning the screen, so that small changes in the function of pathway components that are caused by mutagenesis will yield a detectable dominant phenotype. This strategy has been extremely successful in dissecting signalling pathways in lower organisms[75,76] and the papers that describe the definition of the *sevenless* (*sev*) signalling pathway in flies are excellent examples of the genre[78–80]. A similar strategy might be used where there is redundancy rather than haplosufficiency — that is, where multiple homologous genes are capable of filling the same biochemical role or where two non-homologous biochemical pathways contribute to the same biological outcome[75]. Reducing the activity of one of the homologues or one of the biological pathways before embarking on the screen for other components can increase sensitivity.

There is an overlap between sensitized and suppressor screens, which is illustrated by the screen for suppressors of thrombocytopenia described above. This screen was also sensitized, as mutations in *Myb* that increase platelet production were detected when they were heterozygous in the G_1 population in *Mpl*$^{-/-}$ mice[27], but only when they were homozygous in the G_3 population on a wild-type background[81].

Identification of the causative genetic change

Heritability, inheritance and penetrance. Understanding whether a phenotype is heritable and, if so, whether it is dominant or recessive, is crucial to the design of subsequent mapping studies. Mice that have aberrant or outlying phenotypes can arise from a screen for several reasons. If the screen involves measurement of a normally distributed QUANTITATIVE TRAIT in large numbers of animals, a small and predictable fraction of the animals will have phenotypes that are in the tail of the distribution, irrespective of the mutations they harbour. Other animals will have outlying phenotypes because of differences in the environment they have experienced, whether *in utero* or *post partum*. A third group will have a distinct phenotype because of the effect of an inherited spontaneous or ENU-induced mutation. Setting up test crosses and examining the phenotype of the resultant progeny can distinguish between these possibilities. If the phenotype is heritable, information can also be gleaned about the mode of inheritance and the PENETRANCE of the phenotype.

Mapping. Mutations are identified by a combination of mapping and sequencing (FIG. 4). Mapping aims to identify polymorphic markers of known chromosomal location that are linked to the phenotype caused by the mutation of interest. The more frequently a particular marker is associated with the phenotype, the closer, genetically, the two loci must be. Mapping now relies on highly polymorphic molecular markers —

CONDITIONAL SCREEN
A phenotypic screen in which animals, or cells taken from them, are challenged in order to test responses that are not ordinarily detectable in the steady state, for example, by exposure to a chemotherapeutic agent. Both hypo- and hyperresponsive mutants can be recovered.

HYPOMORPHIC
A partial loss-of-function allele, sometimes known as a 'weak' or 'leaky' allele.

THROMBOCYTOPENIA
Refers to a reduction in the number of circulating blood platelets.

ISOGENIC
Genetically identical; for example, two mice of the same inbred strain.

QUANTITATIVE TRAIT
A biological trait that shows continuous variation (such as weight) rather than falling into distinct categories (such as diseased or healthy).

PENETRANCE
The proportion of affected individuals among the carriers of a particular genotype. If all individuals with a disease genotype show the disease phenotype, then the disease is said to be 'completely penetrant'.

SIMPLE SEQUENCE LENGTH POLYMORPHISMS (SSLPs) and SNPs. These markers make it feasible to map ENU-induced mutations that are generated in one inbred strain by using conventional intercrosses and backcrosses with a second inbred strain[82]. Some investigators have achieved increased efficiency by combining the crosses used to generate mice for screening with crosses used to map the mutations[12,13]. This efficiency might be offset by the confounding effect of a mixed genetic background on the reproducibility of the trait being measured in the screen and the complications of dealing with outbred mice in later studies. This is particularly relevant in studies of the haematopoietic and immune systems, in which transplantation studies are often of central importance and differences in genetic background can lead to graft rejection or GRAFT-VERSUS-HOST DISEASE.

Mapping can be divided into two stages: a low-resolution phase in which the mutation is localized to a large chromosomal interval, and a high-resolution phase in which the size of the interval is reduced to a point at which sequencing can begin. Although it is feasible to use fewer mice and markers, most groups use 20 to 100 animals and 40 to more than 100 markers, distributed throughout the genome, for the first stage[82]. High-resolution mapping then requires the analysis of hundreds to potentially thousands of animals using a panel of molecular markers that are ever more narrowly spaced. If the phenotype is a clear-cut binary trait, in which affected and unaffected mice are easily distinguished, then the location of the mutation should be obvious by visual inspection of the data (FIG. 4). By contrast, if the trait is quantitative and there is overlap in the distributions of affected and unaffected mice, or if penetrance is reduced so that not all mutant mice show the affected phenotype, more sophisticated analytical tools should be used[83–85].

The importance of ensuring that the phenotype and genotype of the animals in which the crucial recombination events have taken place are assigned correctly cannot be overstated. This is especially important if the phenotypes of the affected and unaffected animals overlap, and so cannot be determined with complete confidence. In all cases, a prudent safeguard against error is to breed any animals that carry the results of important recombination events to confirm their inferred phenotype and genotype by assessing the phenotype of their progeny.

Sequencing. During the mapping process, the candidate interval will be narrowed to the point where it is feasible to begin sequencing genes to find the mutation. There are no strict rules to determine when to commence sequencing. The decision is based on several factors including: how small the interval is, and therefore the likelihood of obtaining further recombinants that would narrow the interval; how many polymorphic markers are available in the region to allow further refinement; the gene density within the region; whether there are compelling candidates in the interval; and the cost of sequencing and sequencing facilities that are available compared with the cost of generating and analysing more mice. In practice, refining the interval often proceeds simultaneously with sequencing.

Once a decision to begin sequencing has been made, the question of what to sequence immediately follows (FIG. 4). Given that almost every ENU-induced mutation that has been identified so far has been found in the coding region or intron–exon boundaries of a gene[86], these regions are excellent starting points. The availability of a high-quality and highly annotated sequence of the mouse genome greatly facilitates the identification of exons, which can then be amplified from genomic DNA and sequenced directly. If a mutation is not found in the coding region, then one might need to consider sequencing the introns and 5′ and 3′ untranslated regions of the genes in the interval and, if all else fails, sequencing the presumed intragenic regions. For researchers with access to extensive sequencing facilities, it might be cost-effective to sequence the entire interval in affected and wild-type mice from the outset. This might become the norm if a significant reduction in sequencing costs occurs.

Depending on the resources available, it might be necessary to prioritize the genes in the interval for sequencing. Properties that would influence this might include whether mutations in the gene itself or in genes in the same biochemical pathway are known to yield a similar phenotype to the mutation under investigation, whether the gene is expressed in tissues that are known to be affected by the mutation, whether the level of expression and size of the transcript differ in affected and unaffected mice; and whether there is evidence for the existence of a *bona fide* transcript. Although some of these data might need to be generated *de novo*, an increasing number of web-based resources provide valuable information concerning gene expression, structure and function (see Online links box).

Confirmation. Identification of a mutation in the crucial genomic interval that is defined by mapping does not necessarily mean that it causes the phenotype of interest; independent verification of this link is required. In some cases, indirect evidence might be compelling. For example, there might be parallels between the phenotypes observed when the mouse gene is mutated and those observed when its homologue in lower organisms or humans is mutated, or a similarity between phenotypes that are caused by mutations in other genes that are known to function in the same biochemical pathway. Other evidence includes the expression of the mutated gene in tissues that are affected in the phenotype, and an alteration in the level of expression or activity of the protein that is encoded by the mutant gene. In many cases, direct evidence of the causative nature of the mutation will be required, and two broad strategies are routinely used to obtain this. The first involves analysing further, independent alleles of the gene, which might arise from an ENU-mutagenesis screen[18,27,35], homologous recombination

SIMPLE SEQUENCE LENGTH POLYMORPHISM
Simple dinucleotide and trinucleotide repeats that differ in length and, when amplified using PCR, give rise to the generation of fragments of different sizes.

GRAFT-VERSUS-HOST DISEASE
A destructive attack on host tissues by immune cells that are derived from a bone marrow transplant.

SMALL INTERFERING RNAS
Small antisense RNAs (20–25 nucleotides long), which are generated from specific dsRNAs, that trigger RNA interference. They serve as guides for the cleavage of homologous mRNA in the RNA-induced silencing complex.

INVERSE PCR
A method for cloning DNA that flanks a known sequence. Genomic DNA is digested and ligated into circles, and is then amplified by PCR. The primers used correspond to the known sequence, but point out from this sequence. In a circle that contains the known sequence, the unknown flanking sequence will be amplified.

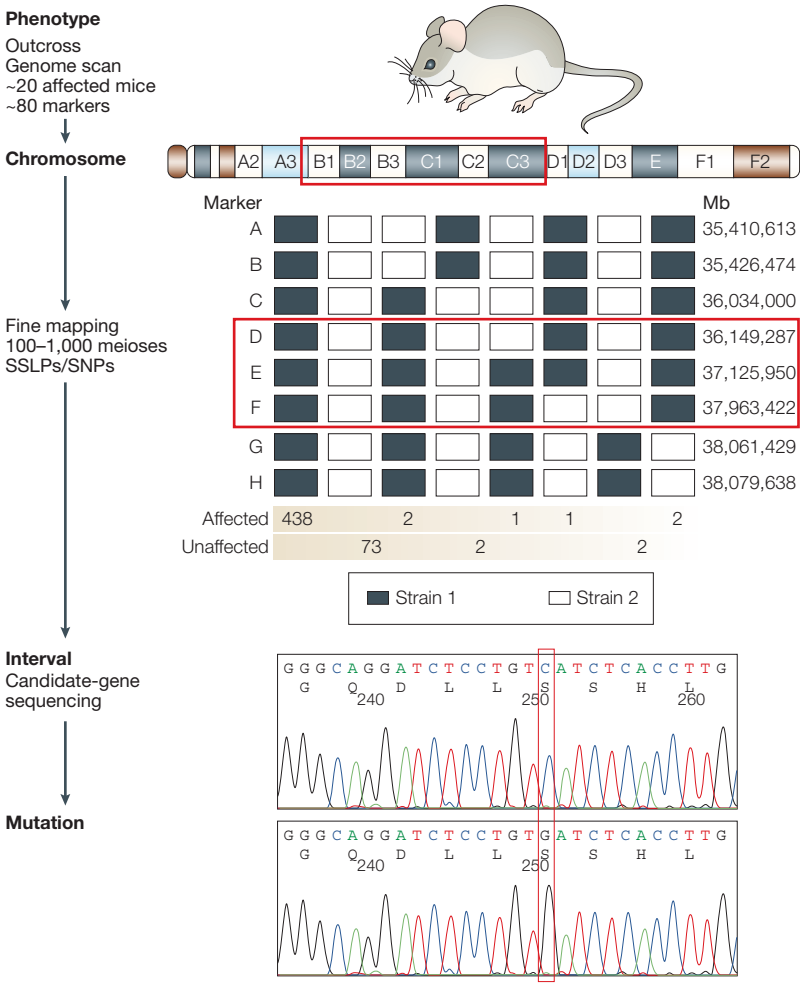


Figure 4 | **Mutation identification.** Once a phenotype of interest is isolated (top panel; here the example of a coat-colour variant is shown), the process of identifying the causative mutation can begin. The first step is a series of outcrosses and/or backcrosses to generate recombinants that will allow a genome-wide scan to be carried out. Genotyping of each informative chromosome (that is, those that have had the potential to undergo meiotic recombination with a homologue that is derived from the mapping strain) is carried out using polymorphic markers (SSLPs (simple sequence length polymorphisms) and SNPs). This allows a chromosomal localization (second panel from top; this example is purely illustrative) to be assigned, and a candidate interval, usually spanning 10–20 Mb to be defined. In the case of region-specific screens, this is unnecessary. Having determined chromosomal linkage, fine mapping can be used to narrow the candidate interval. Depending on centromeric proximity, polymorphism density and gene content, somewhere between hundreds to thousands of informative meioses will be required to narrow the interval to the point at which a manageable number of candidate genes remains — typically somewhere between 1–2 Mb. In the example shown (third panel from top), a recessively functioning mutation that is induced in the C57BL/6 strain has been mapped at high-resolution using animals that are outcrossed to the 129SvEv strain. Here, 521 animals have been genotyped with 8 markers that span the candidate interval of 4.6 Mb, and 10 key recombinants have been identified. The genotypes and phenotypes of the recombinants allow the mutation to be assigned to a refined candidate interval of 1.8 Mb. At this point, DNA sequencing can usually commence, and a careful examination of candidate-gene electropherograms is required to determine the exact location of the ENU-induced mutation (an illustrative example is shown (bottom panel), in which a C to G mutation has occurred).

or insertional mutagenesis (see below), or from the use of SMALL INTERFERING RNAS (siRNAs)[87]. The second strategy centres on ameliorating the phenotype through expression of the wild-type allele using transgenesis[58,88] or retroviral expression in somatic cells[23,24].

Future prospects

There are several developing technologies and approaches that are likely to affect the way that we create mutant mice, the design of genetic screens, and the speed and ease of mutation detection. Although these changes might seem incremental, they will lead to forward genetics becoming a less daunting endeavour, which can be used by most mammalian biology laboratories as they strive to understand their system of interest.

Transposon-mediated mutagenesis. Although it is likely that ENU will continue to be widely used, insertional mutagenesis represents an alluring alternative. Various plasmid and retroviral constructs have been used in mice to interrupt gene function by integration. The low efficiency of insertional mutagenesis so far has seen this technology being used as an alternative to homologous recombination in generating loss-of-function alleles, but not as a serious alternative to ENU in the execution of genetic screens[89]. This might change in the future through the use of mobile genetic elements such as *sleeping beauty*, the movement of which is catalysed by a specific enzyme, a transposase[90]. Transposon-mediated mutagenesis of ES cells[91] and the mouse germline has already been achieved[92]; however, for this approach to rival ENU would require the production of transgenic mice that harbour hundreds of mobile elements. Generation of mice in which transposase expression is limited to the sperm stem cells and their progeny, and is extinguished on fertilization, would also be necessary. This would ensure that all cells of the animal carry the same suite of insertions, and that phenotypes such as cancer formation do not arise because of integration in somatic cells.

The beauty of mobile genetic elements is that, unlike point mutations that are induced by ENU, the site of mutation is tagged with the transposon, increasing the ease of mutation identification. This does not bypass the process of meiotic mapping, as determining which insertion site causes the phenotype would still be required. Instead, the advantage would come after the causative insertion had been localized; its identity could be determined by INVERSE PCR and related strategies[91,93] with greater ease than sequencing can currently identify ENU-induced point mutations.

The rise and rise of suppressor screens. The capacity of disease-causing mutations in humans to be introduced into the mouse genome, with similar consequences, makes the mouse the model organism of choice for many human conditions. Suppressor screens using mouse models of human disease offer particular hope for the identification of targets for therapeutic intervention. Mutations and pharmaceuticals function in the same direction — in most cases, both result in reduced protein function. It follows, therefore, that mutations that ameliorate disease will identify proteins that are potential targets for the design or discovery of pharmaceuticals. As the popularity of suppressor screens increases, it will be interesting to see whether

GENE TRAPPING
A mutation strategy that uses insertion vectors to trap or isolate transcripts from flanking genes. The inserted sequence functions as a tag from which to clone the mutated gene.

ENU-induced mutations can be found that reduce the severity and onset of mouse models of human diseases that currently lack effective treatments.

The $1,000 genome. The publicly available sequence of the mouse genome has revolutionized positional cloning. A similar quantum change will be experienced when an entire genome can be re-sequenced, or when differences between a test and reference genome can be catalogued, for under US$1,000. If all the ENU-induced mutations present in a genome could be rapidly and cheaply determined, then one of the main bottlenecks in equating phenotype with genotype would be relieved. Moreover, the approach of creating a bank of frozen sperm and genomic DNA from a large number of G_1 mice, allowing alleles of specific genes to be identified by sequencing[94], would become highly efficient. All the induced mutations could be catalogued rapidly, rather than the present situation in which genes have to be sequenced on a case-by-case basis. The ultimate outcome of this approach would be a database of mutations with corresponding frozen sperm samples, from which mice that harbour the desired allele could be produced by *in vitro* fertilization. An ENU-induced allele bank would represent a valuable addition to knockout libraries and GENE-TRAP libraries.

A screen in every laboratory, mutants for every graduate student. Mouse genetics is now at the point at which there are no significant technical hurdles or challenges that prevent a rapid progression from mutagenesis to identification of the causative mutation. Carefully conceived screens, in which animals can be assessed before they are weaned, allow large numbers of mutant mice to be examined in a relatively small number of boxes; this level of activity is within reach of most investigators with access to mouse colonies. We look forward to the day when forward genetic screens are embraced as enthusiastically by researchers as knockout technology has been in the past two decades.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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RIKEN (The Institute of Physical and Chemical Research): http://www.gsc.riken.go.jp/Mouse/main.htm
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