ENGINEERING CHROMOSOMAL REARRANGEMENTS IN MICE

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The combination of gene-targeting techniques in mouse embryonic stem cells and the Cre/*loxP* site-specific recombination system has resulted in the emergence of chromosomal-engineering technology in mice. This advance has opened up new opportunities for modelling human diseases that are associated with chromosomal rearrangements. It has also led to the generation of visibly marked deletions and balancer chromosomes in mice, which provide essential reagents for maximizing the efficiency of large-scale mutagenesis efforts and which will accelerate the functional annotation of mammalian genomes, including the human genome.

MOUSE GENOMIC TECHNOLOGIES

The mouse has become an important model for studying genetics and disease because it shares physiological, anatomical and genomic similarities with humans. In both organisms, alterations of chromosomal structure can occur spontaneously or after exposure to specific DNA-damaging agents, causing, in many cases, significant biological consequences. In humans, chromosomal abnormalities are a principal cause of fetal loss and developmental disorders[1,2], and chromosomal translocations are involved in the genesis of many types of human tumour[3]. Chromosomal rearrangements in mice can be used to model these diseases and enable the fine genetic dissection of their causes.

Chromosomal deletions[4,5], duplications[6], inversions[7] and translocations[8] can be induced in mice by using radiation or chemical mutagens, such as chlorambucil[5]. Some useful rearrangements have been induced using these approaches, one of which has served as a mouse model of trisomy 21 (REFS 6,9). Deletions that overlap a handful of mouse chromosomal loci, such as the albino and pink-eyed dilution loci on chromosome 7, have been used for fine genetic mapping and genetic screens[10,11]. However, the usefulness of radiation or chemical mutagens for inducing rearrangements is limited by the fact that the end points of the induced rearrangements cannot be predetermined.

Strategies have recently been developed to introduce defined chromosomal rearrangements into the mouse genome by engineering them in embryonic stem (ES) cells using the Cre/*loxP* site-specific recombination system[12] (BOX 1). Using these strategies, mouse models that accurately recapitulate human chromosomal rearrangements have been developed[13–18]. These engineered mouse models, together with the availability of the human genome sequence, will significantly enhance our ability to uncover the specific molecular mechanisms of the defects caused by human chromosomal rearrangements.

Chromosomal engineering technology has also led to the generation of novel genetic reagents for the functional analysis of the mouse genome. Deletion chromosomes that are visibly marked by, for example, coat-colour markers, can be engineered to provide SEGMENTAL HAPLOIDY in the diploid mouse genome. Recessive mutations that are induced in these deletion intervals from mutagenesis experiments can be detected by crossing mutant mice to mice that are hemizygous for different regions of the genome[12,19]. Mouse BALANCER CHROMOSOMES have also been developed using Cre/*loxP* technology by tagging chromosomal inversions with recessive lethal mutations and coat-colour markers[20]. As inversions suppress recombination, these balancer chromosomes can be used to

SEGMENTAL HAPLOIDY
When a diploid organism is haploid for a certain chromosomal region after its deletion or loss.

BALANCER CHROMOSOME
A chromosome with one or more inverted segments that suppress recombination. They are used as genetic tools because they allow lethal mutations to be maintained without selection.

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Box 1 | Cre/*loxP* site-specific recombination

5' – ATAACTTCGTATAGCATACATTATACGAAGTTAT – 3'
3' – TATTGAAGCATATCGTATGTAATATGCTTCAATA – 5'

The reaction catalysed by the P1 bacteriophage Cre recombinase leads to site-specific recombination between two *loxP* sites[51,52]. The *loxP* sequence consists of two 13-bp inverted repeats and an 8-bp asymmetrical core spacer region, which determines the orientation of the site (as shown). The recombination reaction is initiated by Cre binding specifically to the inverted repeat sequences at *loxP* sites, which leads to the formation of a synapse that consists of four Cre subunits and two *loxP* sites in the same orientation. Cre catalyses exchange between the pair of sites in the core spacer region by concerted cleavage and rejoining reactions. A *cis* recombination event between two *loxP* sites in the same orientation will lead to the excision of the *loxP*-flanked DNA sequence as a circular molecule. If *loxP* sites are orientated in opposite directions, the *loxP*-flanking sequence will be inverted. Recombination between two *loxP* sites *in trans* will lead to the reciprocal exchange of the regions that flank the *loxP* sites. Cre can also induce these recombination events when the *loxP* sites are located several megabases apart on the same chromosome, or on two homologous or non-homologous chromosomes[12,24,38,39].

prevent CROSSING OVER in an inverted region — a property that can be used to facilitate large-scale mutagenesis screens[21] (as discussed in more detail below). In this review, we discuss the experimental strategies associated with these recent advances and the contributions that mice with engineered chromosomes are making to functional genomics, and to the study of human genetics and diseases such as cancer.

Chromosomal engineering

The strategy of chromosome engineering is based on the techniques of gene targeting in ES cells and the Cre/*loxP* system (FIG. 1). Using gene targeting, two *loxP* sites are inserted sequentially into two loci in the ES-cell genome. The transient expression of the gene that encodes Cre recombinase in double-targeted ES cells induces recombination between the two targeted *loxP* sites to generate the rearranged chromosome. Using various methods, such as drug selection, Southern blot analysis and fluorescent *in situ* hybridization (FISH), the ES-cell clones that carry the desired chromosomal rearrangement are identified and characterized. Chimaeras are generated by injecting these ES cells into mouse BLASTOCYSTS, from which the progeny that carry engineered chromosome are derived (FIG. 1).

Deletions, duplications and inversions. Defined chromosomal deletions, duplications and inversions are important rearrangements not only because they constitute prevalent classes of genomic anomaly in humans, but also because they provide powerful reagents for mouse functional genomics. Generating these types of genomic alteration begins with defining the two end points of the rearrangement. For regions greater than 1 Mb, end points might be selected from more than 6,000 simple sequence length polymorphism (SSLP) markers that have been mapped in the mouse genome (see link to STS Physical Map of the Mouse at the Whitehead Institute). The primers that are designed to amplify these markers can be used to isolate genomic clones for constructing the end-point targeting vectors[22]. Genes might also be used as end points using high-resolution mapping information that is available for the mouse genome[23] (see link to Genetic and physical maps of the mouse genome at the Whitehead Institute). Both SSLP markers and genes have successfully been used as the end points for engineering numerous chromosomal rearrangements[12,24,25].

In the first step of Cre/*loxP*-mediated chromosomal engineering, a *loxP* site, a POSITIVE SELECTION cassette, one of two complementary but non-functional fragments of a hypoxanthine phosphoribosyl transferase (*Hprt*) gene[12] are introduced into the first end point, in the ES-cell genome, by gene targeting (FIG. 2). To accomplish this, gene-targeting vectors, such as those shown in FIG. 2 are required. These targeting vectors can either be generated in the conventional way, by sequentially inserting various genetic components into a plasmid construct[12,26], or they can be isolated directly from genomic libraries of pre-made targeting vectors[19]. The targeting vectors from these libraries contain all the genetic elements that are required for chromosome engineering, as illustrated in FIG. 2, they require a minimal amount of manipulation before use and they are available from A.B.

ES-cell clones with a *loxP* site targeted to a first end point can be identified by positive selection and by Southern blot analysis[27]. The subsequent procedures used in our laboratory for generating deletions, duplications and inversions are outlined in FIGS 3 and 4. Only ES-cell lines with an inactivated *Hprt* gene, such as the AB2.2 line[28], can be used in these procedures (see link to the Cell Line Request Form for more information on accessing these ES-cell lines). This is because the Cre/*loxP*-mediated recombination event generates a functional *HPRT* MINIGENE, which is used to select ES-cell clones that contain the desired rearrangement (see below). After isolating the clones targeted at a first end point, a second *loxP* site and the complementary *Hprt* fragment are targeted to a second end point. About six to eight double-targeted clones need to be identified by using the positive selectable markers in the second targeting vector and by Southern blot analysis. We expect half of these clones to be targeted on the same chromosome (*in cis*) as the original targeted insertion, whereas the other half will be targeted to the homologous chromosome (*in trans*).

The type of chromosome rearrangement derived from double-targeted cells will be determined by the *loxP* configuration (see supplementary Table 1 online for the possible outcomes of Cre-mediated recombination), which depends on the orientation of the *loxP* site in a targeting vector. To induce *loxP* recombination, a *cre*-expression vector, such as pOG231 (REF. 29), is electroporated into double-targeted clones. Recombination between the *loxP* sites unites the 5′*Hprt* and 3′*Hprt* cassettes and reconstitutes a functional *Hprt* gene. Culturing these ES cells in a medium that contains hypoxanthine, aminopterin and thymidine (HAT) selects for clones that carry the functional *Hprt* gene, and therefore the rearranged chromosomes.

CROSSING OVER
The exchange of genetic material between two homologous chromosomes.

BLASTOCYST
A preimplantation embryo that contains a fluid-filled cavity called a blastocoel.

POSITIVE SELECTION
When a specific chemical is added to a culture medium, the cells that express a positive selectable marker gene, such as the neomycin or puromycin resistance genes, survive and are selected for.

HPRT MINIGENE
(Hypoxanthine phosphoribosyl transferase gene). This is divided into two complementary, but non-functional, fragments: 5′*Hprt* contains exons 1–2 and 3′*Hprt* contains the remaining exons, 3–9. Each *Hprt* fragment is linked to a *loxP* site, and Cre-mediated recombination unites the 5′ and 3′ cassettes, and restores Hprt activity, which is required for purine biosynthesis and allows desired recombination events to be selected for in HAT (hypoxanthine, aminopterin and thymidine) medium.

ACENTRIC
A chromosome or chromatid without a centromere.

DICENTRIC
A chromatid or a chromosome that has two centromeres.

K14–AGOUTI
A transgene in which the agouti gene is under the control of the keratin 14 promoter. Its expression produces a yellowish coat colour in mice.

If the relative orientations of the two end-point loci (with respect to the centromere) are known, a specific *loxP* configuration can be designed. If the orientations of the two loci are unknown, as is the case for many chromosome-engineering projects, targeting vectors with different orientations of *loxP* sites will need to be tested (FIGS 3 and 4). The complexity of the recombination products that are generated when the orientations and orders of the selection cassettes are unknown is considerable (see FIGS 3 and 4, and supplementary Table 1 online).

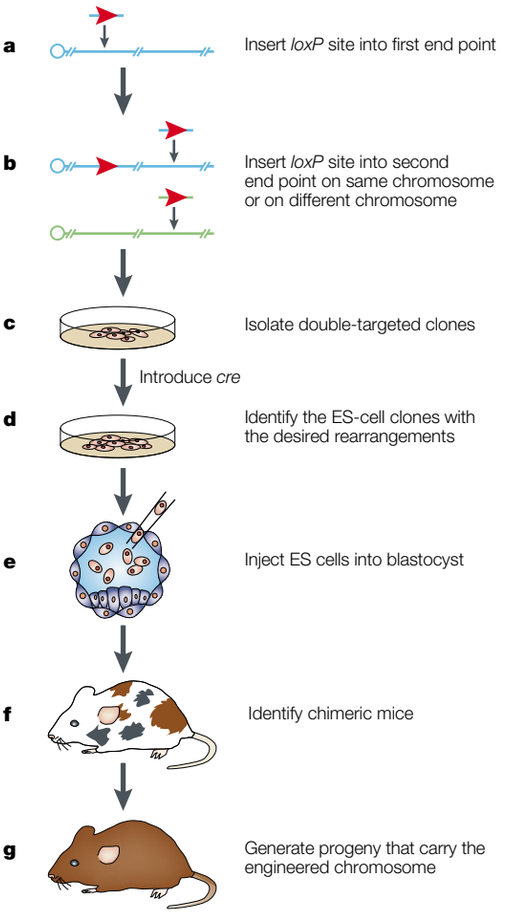

Figure 1 | **A general strategy for chromosomal engineering in mice. a** | A *loxP* site is inserted into the first end point in the embryonic stem (ES)-cell genome using a targeting vector that carries a positive selectable marker gene. **b** | A second *loxP* site, linked to a different positive selectable marker gene, is targeted to the second end point, either on the same chromosome or on a different chromosome by gene targeting or by random insertion. **c** | The expression of *cre* in double-targeted ES cells catalyses recombination between *loxP* sites at the rearrangement end points. **d** | ES-cell clones that carry the desired chromosomal rearrangements are identified and molecularly characterized. **e** | The selected ES cells are injected into mouse blastocysts and the embryos are transferred into the uteri of pseudopregnant foster mothers. **f** | Chimaeras that are generated from blastocyst injection are mated with wild-type mice to establish germline transmission of the modified genome. **g** | The progeny derived from the chimaeras are characterized, and a mutant mouse line that carries an engineered chromosome is established.

To obtain clones that carry a chromosomal deletion and/or a chromosomal duplication, the two targeted *loxP* sites should be orientated in the same direction. If the *loxP* sites are orientated in opposite directions, Cre-mediated recombination between *loxP* sites *in cis* and *in trans* will generate an inversion and inviable recombination products (ACENTRIC and DICENTRIC chromosomes), respectively (FIG. 4 and supplementary Table 1 online). Therefore, if after *cre* expression, HAT-resistant colonies are not recovered from some of the double-targeted clones, this usually indicates that the two targeted *loxP* sites are located in opposite orientations. Given such an observation, deletions and duplications can be generated by inverting the *loxP* selection cassette and re-targeting the second end-point vector.

Cre-mediated recombination can occur in a cell at the G1 phase of the cell cycle or after DNA replication has occurred (S/G2). After chromosome replication, four *loxP* sites will be present in the double-targeted ES-cell genome, and Cre-mediated recombination can occur between sister or non-sister chromatids depending on whether the *loxP* sites are inserted *in cis* or *in trans*. These post-replication events might lead to several recombination outcomes (as shown in FIGS 3 and 4, and supplementary Table 1 online), some of which will not survive selection in HAT medium.

We have found that, when the *cre* expression vector — pOG231 — is used, the efficiency of Cre-mediated *cis* recombination is ~10% and does not alter appreciably if the distance between two targeted *loxP* sites is changed from a few kilobases to up to 10 Mb (REF. 25) (Y.Y. and A.B., unpublished data). When the *loxP* sites are on homologous chromosomes (*trans*), recombination is approximately two to three orders of magnitude less efficient[25] than when they are *in cis*. So, when *loxP* sites are believed (or known) to be in the same orientation, it is possible to identify double-targeted clones in which the *loxP* sites are inserted *in cis* or *in trans* by analysing recombination efficiencies. Selection analysis of the HAT-resistant clones with G418 and puromycin can also be used to classify the clones that carry various types of chromosomal rearrangement. These rearrangements can then be further analysed by Southern blot analysis and by FISH using mouse bacterial artificial chromosome (BAC) clones as probes.

The same strategy can be used to generate inversions (FIG. 4), although in this case the two *loxP* sites remain at the end points of the rearrangement after an inversion has been generated. In principle, the inverted region could revert back to its non-inverted state; however, although this might occur in a small percentage of cells, these cells will not survive in the HAT selection medium.

Variations of the aforementioned strategy have been reported by other groups[24,30–32]. Besides pOG231, several other *cre*-expression vectors, such as pBS185

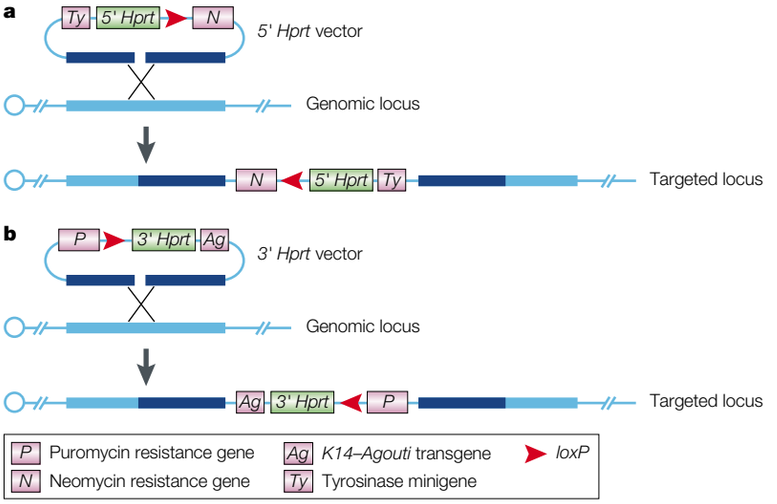

Figure 2 | **Gene targeting in embryonic stem cells.** Insertional targeting vectors, as shown, can be used to insert *loxP* sites, positive selectable markers, the *Hprt* gene fragments and coat-colour markers (such as *Ty* and *Ag*) to predetermined loci in the embryonic-stem-cell genome. **a** | Expression of the neomycin resistance and **b** | puromycin resistance genes allows different targeting events to be selected. The complementary, but non-functional, 5′*Hprt* and 3′*Hprt* fragments are derived from a *Hprt* minigene[12]. The dark blue and light blue bars represent regions of homology between the vector and the genomic locus. The vector is linearized in the region of homology (gap) to stimulate targeted insertion into the locus. X represents recombination between the vector and the genome. *Ag*, the *K14–AGOUTI* transgene; *Hprt*, hypoxanthine phosphoribosyl transferase; *Ty*, the TYROSINASE minigene.

TYROSINASE
Tyrosinase is required for melanin biosynthesis, and the expression of its gene leads to pigment production, and is therefore used as a coat-colour marker.

HSVTK
The herpes simplex virus thymidine kinase (HSVtk) is essential for thymidine nucleotide biosynthesis through a salvage pathway and is often used as a negative selectable marker in gene targeting.

NEGATIVE SELECTABLE MARKER
A negative selectable marker gene, such as *HSVtk*, allows cells that express it to be killed when a specific chemical is added to a culture medium, whereas cells that no longer express the marker gene survive.

HAPLOINSUFFICIENCY
A phenotype that arises in diploid organisms owing to the loss of one functional copy of a gene.

HYBRID ES-CELL LINE
An embryonic stem (ES) cell line isolated from F_1 hybrid embryos, such as from crosses between the strains C57BL/6-Tyr^{c1Brd} × 129S7 or 129S1 × CAST/Ei. These lines facilitate simple sequence length polymorphism analysis.

(REF. 24), pBS500 (REF. 30) and pIC-*CRE*[32], have been used in chromosomal-engineering experiments in ES cells. Other strategies also select for the desired recombination products in different ways. In some cases, a herpes simplex virus thymidine kinase (HSVTK) gene, which acts as a NEGATIVE SELECTABLE MARKER, is inserted between the rearrangement end points. Cre/*loxP*-mediated recombination events that result in a deletion and the loss of this marker can be selected by culturing ES cells in medium that contains 1,2′deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodouracil[24,30–32], which kills cells that express HSVtk. However, negative selection cannot be used to isolate duplications or inversions. Positive-selection strategies facilitate the isolation of cells with reciprocal recombination products (such cells would not survive negative selection), which allows ES cells with balanced genetic changes to be recovered. These rearrangements can then be assessed independently of each other after their segregation in the germ line of mice. This is particularly useful because duplications can rescue mice that inherit a corresponding HAPLOINSUFFICIENT deletion.

One factor that limits the generation of deletions in ES cells is the size of the rearranged interval. Available evidence indicates that large deletions, such as those deletions larger than 22 cM, might lead to ES-cell lethality or to a severe growth disadvantage of the cells in culture[25]. Although Cre/*loxP* recombination will occur readily over these large distances, clones will often emerge from these experiments that have undergone a compensatory genetic change, such as a chromosomal duplication[25].

Nested chromosomal deletions. These are a series of overlapping deletions that surround a predetermined genomic locus. These deletions vary in size and have different end points (nested end points), but many of them will overlap. If the genomic locations of the end points are known, nested deletions can be extremely useful for mapping novel recessive mutations. By crossing mice that carry a hemizygous deletion with a mouse line that carries a novel recessive mutation, progeny that harbour both the deletion and the novel mutation *in trans* can be generated. If a recessive mutant phenotype is observed in the progeny of such a cross, it indicates that the chromosome that carries the deletion cannot complement the novel mutation; the novel mutation is therefore located in the deletion interval. Using this approach, novel mutations can be rapidly mapped to a specific deletion interval by crossing mutant mice with mice that carry nested deletions.

To efficiently engineer these types of reagent, we have developed an approach for constructing deletion complexes that does not require that targeting vectors be made for the nested end points[33]. Deletion complexes can be anchored to a predetermined location in the genome by targeting the 5′*Hprt–loxP* cassette as described previously. The 3′*Hprt–loxP* cassette is then inserted randomly into the ES-cell genome by retrovirus-mediated integration (FIG. 5), which generates a library of ES clones with the same targeted end point and a collection of random end points. Only a subset of random insertions will occur on the same chromosome as the original targeting event. However, Cre/*loxP* recombination efficiency is several orders of magnitude more efficient when *loxP* sites are inserted on the same chromosome. So, after the expression of *cre*, most HAT-resistant clones will be derived from retroviral insertions that have occurred *in cis* to the targeted insertion. Cre/*loxP* recombination efficiencies will also decrease if the sites are separated by more than 10 Mb. So, most HAT-resistant clones will have rearrangements that are less than 10 Mb. Clones that are generated by using this strategy carry a random distribution of deletion sizes that range from a few kilobases to several megabases[33]. Clones that contain chromosomal deletions lose the *neo* and *puro* cassettes, and so can be distinguished from other types of rearrangement by sib-selection in G418 and puromycin.

This nested deletion strategy has also been repeated using electroporation to insert the *loxP* cassette randomly into the ES-cell genome[34]. Compared with retrovirus-mediated integration, insertion by electroporation might increase the risk of genomic rearrangements occuring at the insertion site and tandem repeats of a vector might be introduced into the insertion site, although these should be reduced to a single locus by the activity of Cre on a head-to-tail concatenate.

Deletions that are generated by the random insertion of the second end point are usually characterized by Southern blot analysis and by FISH and, if a HYBRID ES-CELL LINE is used, by SSLP analysis. The end points can be defined by cloning the genomic DNA that flanks the deletion end points and by mapping these junction fragments onto a physical map of the region.

Nested deletions have also been generated by irradiation[35–37]. Deletions that are induced by irradiation can be localized and made selectable by targeting a vector that carries a negative selection cassette (such as *HSVtk*) to a predetermined locus. Before irradiation, the cells can be cultured under positive selection pressure to retain the targeted locus. After irradiation, clones that carry the desired deletion can be identified by loss of the negative selection marker. Using this approach, deletions can be produced efficiently; however, they require extensive additional characterization to define each deletion interval.

Chromosomal translocations. One of the main reasons for engineering defined chromosomal translocations is to develop mouse models for human translocations, which underlie certain forms of cancer by causing the abnormal expression of cellular oncogenes or by creating novel fusion genes[3].

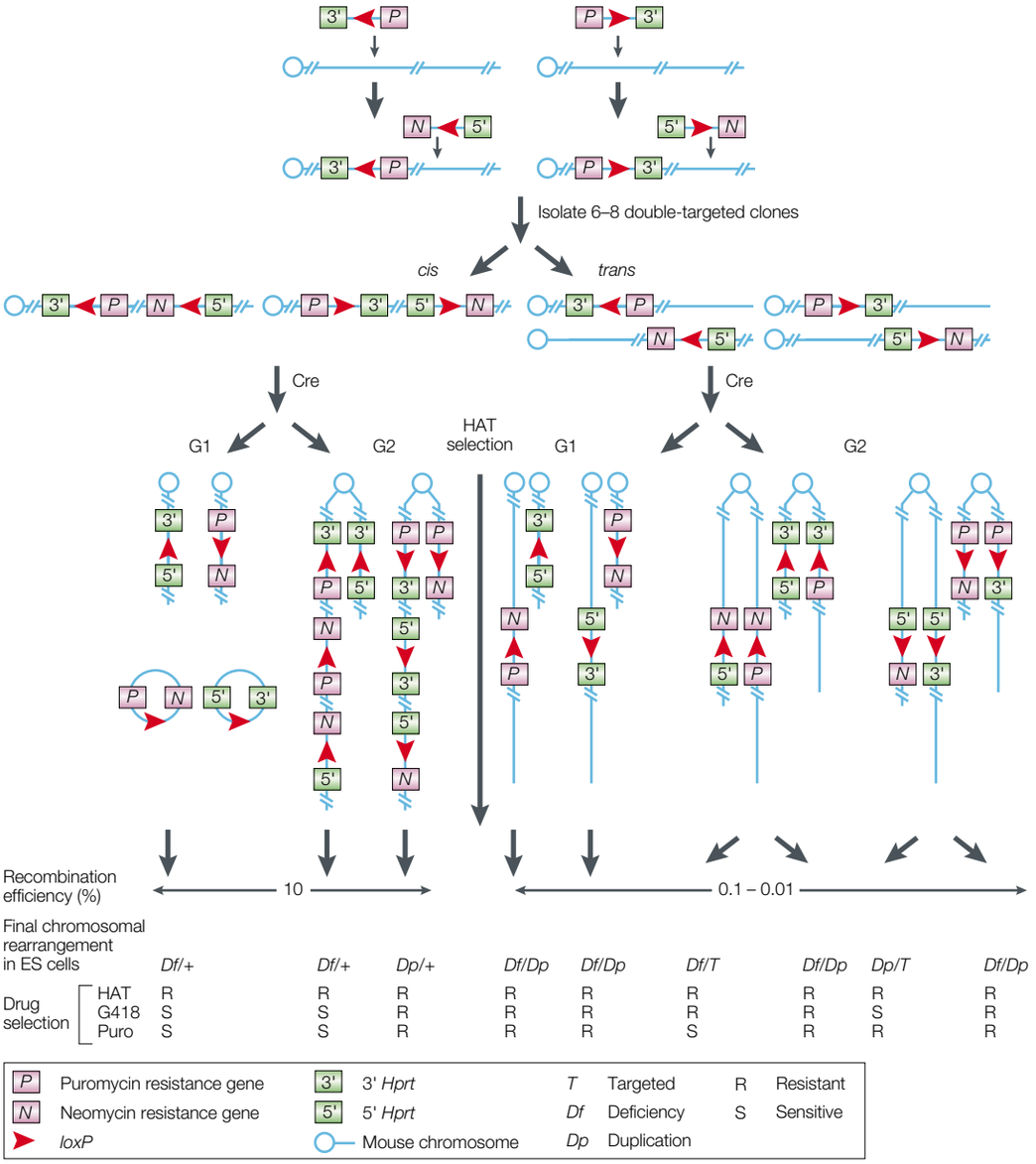

Figure 3 | **Engineering a deletion and/or a duplication in embryonic stem cells.** An experimental procedure for engineering chromosomal deletions and duplications. The cassettes can be targeted in two orientations, only orientations that result in deletions or duplications are illustrated. G1 and G2 indicate the different phases of the cell cycle in which recombination occurs. In G2, four *loxP* sites are located on duplicated chromatids, and recombination events will result in various products. Drug selection will help with identifying the desired rearrangements. For clarity, only the drug-resistance characteristics of the HAT-resistant clones are shown. After *trans* recombination in G2, the chromosome that carries the *Hprt* resistance marker will either segregate with the reciprocal product (carrying the duplication) to give a *Df/Dp* cell, or it will segregate with a non-recombined chromatid that carries the targeting vector (*T*). These cells (*Df/T* or *Dp/T*) are therefore resistant to either G418 or puromycin (Puro) but not both. HAT, hypoxanthine, aminopterin and thymidine; *Hprt*, hypoxanthine phosphoribosyl transferase.

Mouse chromosomal translocations with predetermined breakpoints have been created using Cre/*loxP* recombination[15,16,38,39]. Translocations are generated when *loxP* sites are targeted to non-homologous chromosomes. To obtain the desired chromosomal translocation, these targeted *loxP* sites need to be orientated in the same direction relative to their respective centromeres. If two targeted *loxP* sites are in opposite orientations, recombination will result in acentric and dicentric chromosomes (FIG. 6a). The efficiency of Cre/*loxP* recombination between non-homologous chromosomes is several orders of magnitude lower than that of the recombination between *loxP* sites on the same chromosome. The frequency of Cre/*loxP*-mediated recombination between non-homologous chromosomes is also lower than that obtained when *loxP* sites are inserted within a few megabases of each other on homologous chromosomes.

To generate a fusion protein from a chromosomal translocation, the targeting vectors need to be specially designed so that after translocation, two genes originally located on two non-homologous chromosomes can be linked through their introns, with the *loxP* site embedded in the junction region of the breakpoint. After RNA splicing, an in-frame fusion mRNA and protein are generated as a result (FIG. 6b). To prevent the generation of acentric and dicentric chromosomes, only pairs of genes with the same transcriptional orientations relative to their centromeres can be engineered to generate fusion proteins.

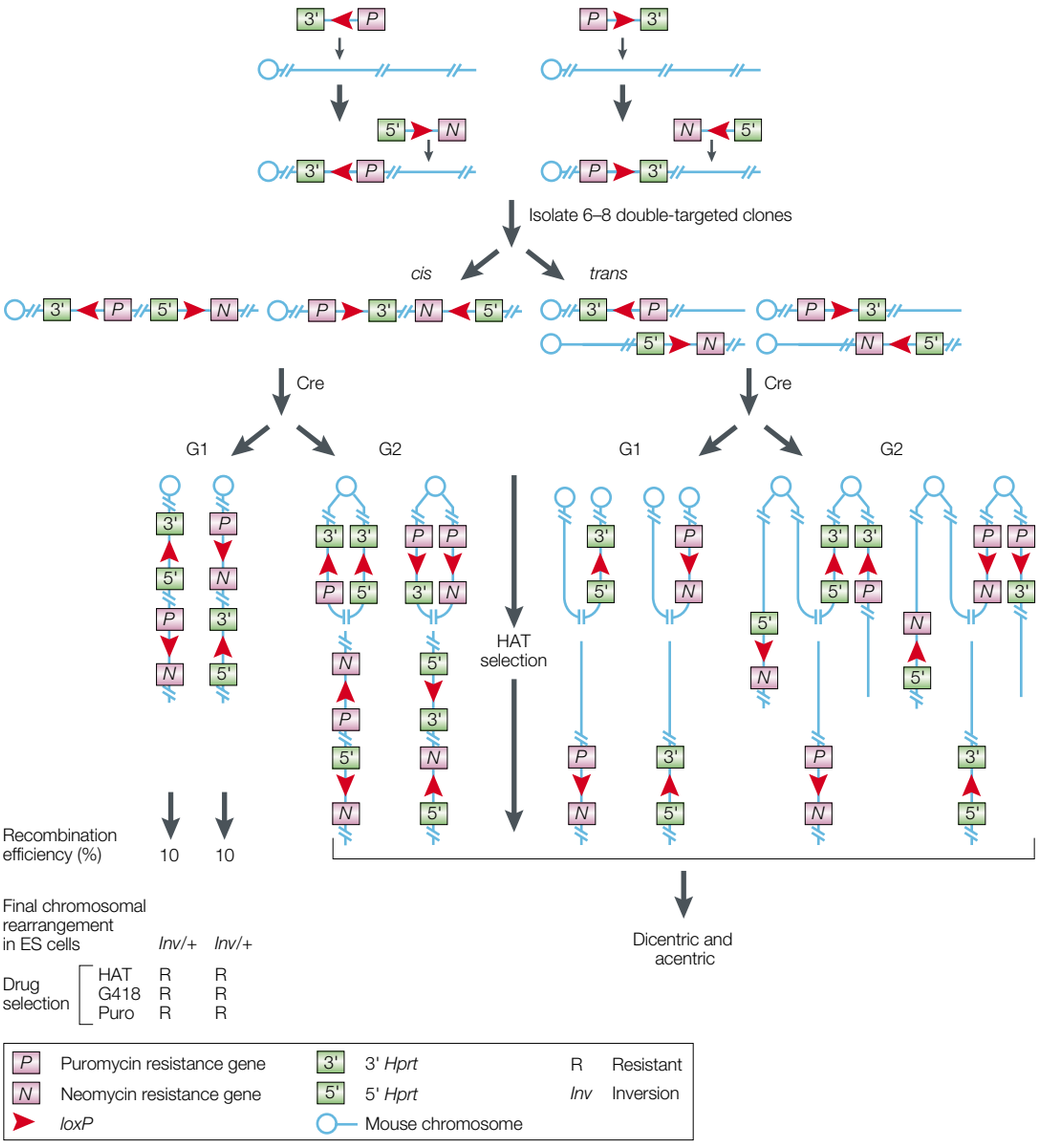

Figure 4 | **Engineering an inversion in embryonic stem cells.** An experimental procedure for engineering chromosomal inversions. G1 and G2 indicate the different phases of the cell cycle in which recombination occurs. Only orientations of the cassettes that result in inversions are illustrated. Cre-mediated recombination at G1 or G2 will result in various products. HAT, hypoxanthine, aminopterin and thymidine; *Hprt*, hypoxanthine phosphoribosyl transferase; Puro, puromycin.

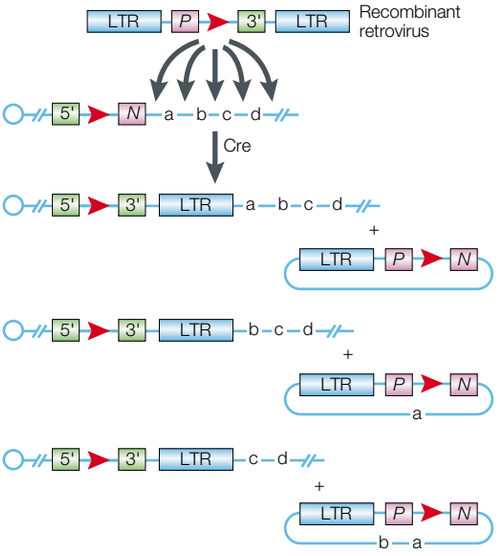

Figure 5 | **Nested chromosomal deletions induced with a retroviral vector.** The first deletion end point is fixed by targeting the 5′*Hprt* cassette and a *loxP* site to a predetermined locus. The 3′*Hprt* cassette and the second *loxP* site are then integrated randomly into the embryonic-stem-cell genome using a recombinant retroviral vector. For clarity, only G1 recombination events from retroviral orientations that result in deletions are illustrated. Cre catalyses recombination between the *loxP* sites (red arrowheads), and HAT medium is then used to select for the clones that carry the recombinant chromosomes. The nested deletions can be identified from a pool of HAT-resistant clones on the basis of their sensitivity to G418 and puromycin. 3′, 3′*Hprt*; 5′, 5′*Hprt*; a–d, genetic markers; HAT, hypoxanthine, aminopterin and thymidine; *Hprt*, hypoxanthine phosphoribosyl transferase; LTR, retroviral long terminal repeat; *N*, the neomycin resistance gene; *P*, the puromycin resistance gene. (Modified with permission from REF. 33.)

To induce a translocation *in vivo*, mice that carry both targeted end points can be crossed with transgenic mice that express *cre* under the control of a regulatory element with the desired tissue and temporal specificity[15,16]. This approach not only has been used to generate better models of human leukaemia-associated translocations, such as the t(8;21)(q22;q22) (REF. 15) and t(9;11)(p22;q23) (REF. 16) translocations, which cause acute leukaemia, but also circumvents the problem of transmitting translocations through the male germ line, as the presence of chromosomal translocations in male germ cells can cause infertility[40]. Recombination events that give rise to chromosomal translocations can reach recombination efficiencies of 10^{-4} to 10^{-6} in tissues that express Cre[15]. Although these recombination rates are low, they can mimic the rare genetic events that are crucial steps in neoplastic transformation (as discussed below).

Applications of chromosomal engineering

About 0.6% of all newborn human infants have cytogenetic imbalances[2], so chromosomal anomalies are a principal cause of human genetic disease. Somatic chromosomal translocations are crucial events in the formation of many types of human tumour, such as leukaemia, lymphoma and sarcoma[3]. Because there are many conserved linkage groups between the human and mouse genomes, the chromosomal rearrangements that are involved in human disease can be modelled in mice. These mouse models can be used to study the molecular events that are associated with these diseases. Mouse models of chromosomal deletions can also be used to analyse rearranged chromosomal regions and can facilitate the identification of the genes that are involved in the clinical features of chromosomal disorders.

The other main driving force behind recent advances in mouse chromosomal manipulation has been the need to generate resources to facilitate genetic screens[28]. Chromosomal rearrangements, such as visibly marked deletions and balancer chromosomes, have been instrumental in the success of genetic screens in *Drosophila*. In mice, mutagenesis efforts to generate and map recessive mutations have been hindered by the lack of marked deletions in most regions of the genome and by the unavailability of balancer chromosomes. The creation of these reagents through chromosomal engineering technology will change future strategies for large-scale, recessive genetic screens in mice and will facilitate the functional analysis of the mouse genome.

Modelling human disease. Among human chromosomal rearrangements, deletions constitute an important class. Deletions are often identified when haploinsufficient gene(s) in the deleted region cause a clinical phenotype. The positive-selection-based Cre/*loxP* strategy for engineering defined chromosomal deletions is uniquely suited for identifying and analysing mammalian haploinsufficient loci. Alternative strategies for generating deletions that use negative selection[24,30–32] and irradiation[35–37] suffer from the disadvantage that they do not generate selectable reciprocal products. However, the positive-selection-based strategy allows duplications to be recovered from *trans* recombination events (see supplementary Table 1 online). Duplications are important experimental tools because they allow haploinsufficient deletions to be maintained, and allow mice that harbour both a deletion and the reciprocal duplication to be recovered because they are genetically balanced.

Using chromosomal-engineering techniques, key genetic elements in several human chromosomal deletion disorders have recently been identified by engineering mouse chromosomal deletions in regions that are homologous with those deleted in certain human deletion disorders, such as DiGeorge syndrome and Prader–Willi syndrome[13,14,17,18]. The power of this approach has been illustrated by modelling the deletion that is involved in DiGeorge syndrome. DiGeorge syndrome is associated with a hemizygous deletion on human chromosome 22, del(22)(q11.2; q11.2). The DiGeorge region had been recalcitrant to molecular dissection in humans[41]. Despite intensive efforts,

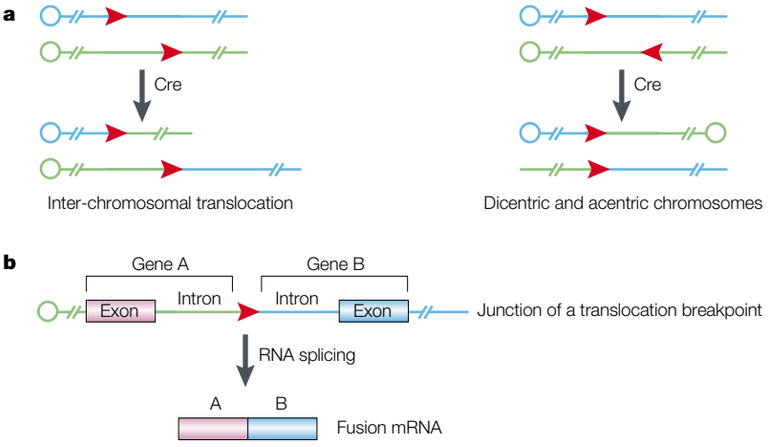

Figure 6 | **Engineering chromosomal translocations.** A strategy for engineering a chromosomal translocation and an associated fusion gene. **a** | Cre-mediated recombination leads to chromosomal translocation or dicentric and acentric chromosomes, depending on the relative orientations of the *loxP* sites (red arrowheads) on two non-homologous chromosomes. **b** | An in-frame fusion mRNA and protein can be generated by engineering an appropriate junction at the translocation breakpoint.

including the analysis of the finished genomic sequence of chromosome 22 (REF. 42), the gene(s) responsible for the clinical features of this disorder have not been identified using human molecular genetic approaches.

An alternative strategy exploiting chromosomal engineering was used to generate a 1.2-Mb deletion, Df(16)1, in the region of mouse chromosome 16 that corresponds to the minimal DiGeorge region on human chromosome 22 (REF. 13). The hemizygous deletion mice, Df(16)1/+, develop cardiovascular defects similar to those observed in DiGeorge syndrome patients. Importantly, in mice that harbour both Df(16)1 and the reciprocal duplication, no heart defects are detected, proving that reduced gene dosage in the deleted region is responsible for the mutant cardiovascular phenotype seen in the Df(16)1/+ mice[13]. To locate the gene(s) involved in this phenotype in Df(16)1, smaller overlapping deletions were generated by using known end points or by using randomly generated nested deletions that were induced by a recombinant retrovirus[33]. Mice that carry these sub-deletions were then analysed for heart defects. These studies narrowed down the candidate interval to a region that contained a few genes, one of which — *Tbx1* — went on to be identified as the haploinsufficient gene that causes the principal cardiovascular defects in DiGeorge syndrome[18]. The same conclusion was reached independently by a second group that also used chromosomal engineering techniques[17].

Progress has also been made in efforts to model human leukaemia-associated translocations, such as t(8;21)(q22;q22) (REF. 15) and t(9;11)(p22;q23) (REF. 16). In t(8;21), the breakpoints of the translocation are located in the genes *AML1* (acute myeloid leukaemia 1; also called *RUNX1*, runt-related transcription factor 1) and *ETO* (also called *CBFA2T1*, core-binding factor, alpha subunit 2; translocated to, 1). To model this translocation, the orthologues of these genes, located on mouse chromosomes 4 and 16, were used as the end points for Cre-mediated recombination. In t(9;11)(p22;q23), the translocation generates a fusion gene from the genes *MLL* (myeloid/lymphoid or mixed-lineage leukaemia) and *AF9* (myeloid/lymphoid or mixed-lineage leukaemia; translocated to, 3). To generate such a fusion gene in mice, the mouse *Af9* and *Mll* genes, located on chromosomes 4 and 9, respectively, were used as the rearrangement end points. Mice with double-targeted end points were crossed with transgenic lines that express *cre* in various organs, including the brain, and the desired rearrangements were produced in their progeny. However, *cre* expression has not yet been targeted to the haematopoietic cell lineages and, possibly as a result, leukaemia has not been reported in the mice that carry these translocations[15,16].

These translocations illustrate a further advantage of the Cre/*loxP* chromosomal engineering system. Inducing recombination *in vivo* can generate chromosomal deletions, duplications or translocations[15,16,25,43,44]. This approach can often be essential when the rearrangements cause ES-cell lethality[25] or embryonic death[26], or when modelling human chromosomal rearrangements that occur only in certain somatic cell types[15,16].

Engineered chromosomes for functional analysis. Experimental approaches for the functional characterization of the genome of an organism rely on the generation of mutations. For the mutational analysis of diploid organisms, such as the mouse, genetic tools, such as marked deletions and inversions, are important reagents because they facilitate rapid genetic mapping and maintenance of randomly generated mutations, such as those generated during ethylnitrosourea (ENU) mutagenesis screens. The development of chromosomal engineering techniques has significantly expanded the repertoire of these powerful genetic tools.

In an effort to functionally analyse mouse chromosome 11, 18 deletions have been engineered on this chromosome using Cre/*loxP* technology[26,33] (Y.Y. and A.B., unpublished data; see also link to the Chromosome 11 deletion map). This work has generated mouse lines that carry regions of segmental haploidy, which can be used to screen ENU-mutagenized mice to identify recessive mutations. Smaller nested deletions can then be used for complementation testing, to narrow down the genomic location of a mutation as a prelude to cloning. Thereafter, the mutated gene can be identified by genomic complementation with BACs[45,46] and/or by sequencing the entire mutation-carrying region.

Although many deletions have now been generated on mouse chromosome 11, several of these deletions are haploinsufficient[26] (Y.Y. and A.B., unpublished data). Mice that carry these deletions either die during embryogenesis or show disease phenotypes. This prevents their use in genetic screens, although it does identify regions of the genome that are worthy

ENU (*N*-ethyl-*N*-nitrosourea). A potent mutagen that primarily generates single-base-pair mutations in mouse spermatogonia germ cells.

of further analysis. A deletion can be generated that encompasses a smaller interval as a way to avoid haploinsufficient gene(s), but this reduces the number of genes located in the interval. A mouse line that carries a smaller deletion is therefore not an efficient tool for conducting a genetic screen because the number of recessive mutations that could be detected by using it would be significantly reduced. To overcome this problem, we have generated inversion chromosomes.

Using engineered inversions for mutagenesis screens has several advantages. First, unlike a deletion, a 20- to 30-cM inversion can be generated without causing a detrimental effect to mice. So, by using an inversion, a much larger genomic region can be screened. Second, a heterozygous inversion effectively suppresses crossing over in the inverted genomic segment because a single crossover between loci in the rearranged interval leads to inviable acentric and dicentric chromosomes or aneuploidy. Therefore, inversions can be used to maintain the genomic integrity of a mutagenized region. Third, an inversion can be designed to function as a balancer chromosome by tagging it with a recessive lethal mutation, which prevents animals that carry a homozygous inversion from being viable. Finally, a coat-colour marker can be added to an inversion chromosome so that its inheritance can be followed without requiring the genotypic analysis of progeny.

To facilitate the isolation of ENU-induced recessive mutations on mouse chromosome 11 (see link to Chromosome 11 ENU mutagenesis programme), the first mouse balancer chromosome was constructed on chromosome 11 using Cre/*loxP*-mediated recombination[20] (FIG. 7a). This balancer chromosome is based on a 24-cM inversion between the *Trp53* gene and the *Wnt3* gene. Mice that are homozygous for this inversion die during embryogenesis owing to the disruption of the *Wnt3* gene, which is required for embryonic development[47], at one of the inversion end points. In addition, a coat-colour marker, *K14–Agouti*, has been inserted into the mutated *Wnt3* locus. Such a marked balancer chromosome constitutes an ideal reagent for the isolation of novel ENU-induced recessive mutations in a three-generation breeding scheme[21] (FIG. 7b).

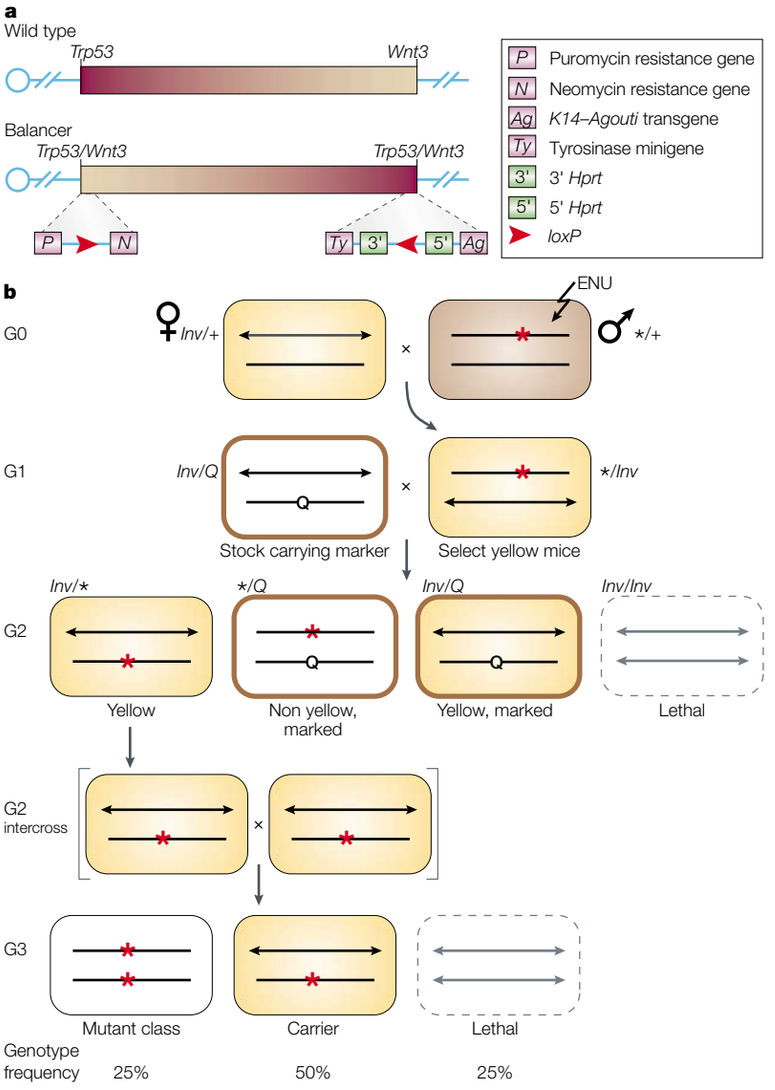

Figure 7 | **A mouse balancer chromosome and its use in ENU mutagenesis screens.** **a** | The mouse balancer chromosome, Inv(11)8, is based on an inversion between *Trp53* and *Wnt3*. Inv(11)8 mice carry an engineered coat-colour marker (agouti) and die when homozygous for the inversion during embryogenesis because of the targeted mutation at the *Wnt3* locus. **b** | A breeding scheme to isolate recessive mutations using a balancer chromosome, such as Inv(11)8. In the first generation (G1), mice hemizygous for Inv(11)8 and an induced mutation are generated by mating ENU-treated males (*/+) to females that carry the balancer chromosome (Inv/+), which is marked by the dominant coat-colour marker, *K14–Agouti*. The G1 mice (*/Inv) are then crossed with mice that carry the balancer chromosome (Inv) and another visible marker (*Q*) on the homologous chromosome that is distinguishable from *K14–Agouti*. The Inv/* mice can be visually identified among the G2 mice, and Inv/*Q* and *Q*/* mice are not used further. Sibling matings between the Inv/* mice generate two classes of G3 mice, Inv/* and */*, which can be distinguished by the presence of *K14–Agouti* on the balancer chromosome. If all G3 animals carry *K14–Agouti*, the induced mutation causes embryonic lethality. *Hprt*, hypoxanthine phosphoribosyl transferase. (Modified with permission from REF. 21.)

Conclusions and perspectives

ES-cell technology has drastically enhanced our ability to engineer various types of mouse genomic alteration, which now include single-gene knockouts, single-base-nucleotide alterations, conditional mutations (see review by Mark Lewandoski on p743 of this issue for more on this technique) and megabase rearrangements. Novel ES-cell-based technologies for genomic manipulation will undoubtedly continue to emerge.

The technologies of chromosome manipulation will become easier to apply as the mouse genome sequencing project progresses (see link to NCBI's mouse genome sequencing page). This is because the availability of a complete mouse genome sequence will facilitate the selection of end points and the construction of targeting vectors for use in engineering-defined chromosomal rearrangements. The mouse genome sequence will also help with identifying the location of random integration sites in nested chromosome deletions.

The applications of these newly developed technologies are still in their infancy. Because they can now be generated in any region of the genome, marked deletions in mice, like their counterparts in *Drosophila*, will become invaluable for mapping genetic loci, such as quantitative trait loci[48]. Marked deletions and balancer chromosomes will also continue to gain importance in large-scale, recessive genetic screens in mice and will have a significant impact on efforts to functionally annotate the mouse genome. Studying DiGeorge syndrome in mice has illustrated the feasibility and benefits of using chromosomal engineering to generate models of human chromosomal rearrangements. Experiments are underway to engineer mouse models for other human congenital chromosomal disorders, such as Smith–Magenis syndrome (K. Walz and J. Lupski, personal communication) and trisomy 21 (REF. 49). The current mouse models of trisomy 21 are trisomic for only a portion of mouse chromosome 16, and mutant mice do not show all the major clinical features of the disorder[9,50]. Because human chromosome 21 orthologues have been located to regions of conserved linkages on mouse chromosomes 10, 16 and 17, a better mouse model could be engineered that would carry segmental trisomies of all these genomic regions. Chromosomal engineering could also be used to generate small overlapping duplications in mouse chromosomal regions conserved with the trisomic human chromosome 21 regions to identify the crucial genomic domain(s) and causative gene(s) that are responsible for the clinical characteristics of the disorder.

Chromosomal-engineering technology has increased our ability to manipulate the mammalian genome, which has special significance in the current genomic era. The unique advantages of using this technology in the functional analysis of mammalian genomes and to develop animal models of human disease have been recognized and will continue to be shown in the coming years.

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Online links

DATABASES
The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
AF9 | *AML1* | *ETO* | *MLL*
MGI: http://www.informatics.jax.org/
albino | *Hprt* | *Mll* | pink-eyed dilution | *Trp53* | *Wnt3*
OMIM: http://www.ncbi.nlm.nih.gov/Omim/
DiGeorge syndrome | Prader–Willi syndrome | Smith–Magenis syndrome | trisomy 21

FURTHER INFORMATION
Cell line Request Form:
http://www.imgen.bcm.tmc.edu/molgen/labs/bradley/cell.htm
Chromosome 11 deletion map:
http://www.mouse-genome.bcm.tmc.edu/ChrEng/deletion.asp
Chromosome 11 ENU mutagenesis programme:
http://www.mouse-genome.bcm.tmc.edu/ENU/ENUHome.asp
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STS Physical Map of the Mouse:
http://carbon.wi.mit.edu:8000/cgi-bin/mouse/index#phys
t(8;21)(q22:q22):
http://www.infobiogen.fr/services/chromcancer/Anomalies/t0821.htm
t(9;11)(p22;q23):
http://www.infobiogen.fr/services/chromcancer/Anomalies/t0911.html
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