

# “Recombomice”: The past, present, and future of recombination–detection in mice

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## Abstract

Homology directed repair (HDR) provides an efficient strategy for repairing and tolerating many types of DNA lesions, such as strand breaks, base damage, and crosslinks. Recombinational repair and lesion avoidance pathways that involve homology searching are integral to normal DNA replication. Indeed, it is estimated that at least ten HDR events take place each time a mammalian cell divides. HDR is associated with the transfer and exchange of DNA sequences. Usually, homologous sequences are aligned perfectly and flanking sequences are not exchanged. However, those sequence misalignments and exchanges that do occur can lead to rearrangements that contribute to cancer (e.g. deletions, inversions, translocations or loss of heterozygosity (LOH)). In order to reveal genetic and environmental factors that modulate HDR in mammals, several approaches have been used to detect recombination events *in vivo*. Here, we briefly review three methods for detecting homologous recombination in mice, namely: sister chromatid exchange (SCE), LOH, and recombination at tandem repeats. We conclude with a more detailed description of the recently developed “Fluorescent Yellow Direct Repeat” (FYDR) mouse model, which exploits enhanced yellow fluorescent protein (EYFP) for detecting mitotic homologous recombination *in vivo*. Applications of the FYDR mice are described, as well as the broader potential for using fluorescent proteins to detect recombination in various tissues/cell types *in vivo*.

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## 1. Recombination and human health

In recent years, homologous recombination has emerged as a topic of intense interest to anyone studying factors that affect genome stability. Homology directed repair (HDR) is integral to DNA replication and is critical for repairing and tolerating DNA lesions. If coding information is missing or corrupted, cells can extract functional sequence information that is available on the sister chromatid (during S and G2 phases of the cell cycle) or on the homologous chromosome (for recent reviews of HDR, see [1–5]). Although HDR is generally highly accurate, and thus beneficial to cells, transfer of genetic information carries with it a certain amount of risk. A significant portion of the human genome is comprised of repeat sequences, and inappropriate recombination between such sequences can lead to insertions, deletions,

inversions or translocations [6]. In addition, recombination between homologous chromosomes can cause loss of heterozygosity (LOH). LOH is particularly problematic in cells that carry one wild type and one mutant copy of a tumor suppressor allele, since loss of the wild type allele (i.e. LOH) can promote cancer (e.g. retinoblastoma) [7,8]. Although LOH can be caused by several different molecular mechanisms (including deletions, point mutations, non-disjunction or HDR), HDR has been estimated to be the underlying cause of LOH in mammalian cells 25–80% of the time [9–12].

People who are born predisposed to spontaneously high levels of recombination are prone to cancer (e.g. Bloom’s Syndrome, Ataxia Telangiectasia, and Werner’s Syndrome) [13–20]. Furthermore, dozens of chemicals that are potent inducers of homologous recombination are also carcinogenic (e.g. [21–24]). Thus, whether caused by an inherited predisposition or by exposure, recombination events are an important class of mutations that contribute to cancer. Here, we will briefly describe various strategies for measuring recombination events in mice, and we will conclude by

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describing a recently developed transgenic mouse line that exploits fluorescence to detect recombinant cells in vivo.

## 2. Recombination detection in mice

One method for assessing homologous recombination in mice in vivo is to measure reciprocal exchanges that occur between sister chromatids (SCEs). Although we do not yet fully understand the underlying molecular basis for SCE formation, it is nevertheless clear that SCEs are homologous recombination events [25]. To measure SCEs in vivo, mice are injected with 5-Bromo-2'-deoxyuridine (BrdU), a base analog that is incorporated into the DNA of dividing cells. After two cell cycles, sister chromatids can be distinguished by differential staining and SCEs can be visualized by microscopic examination of metaphase spreads [26]. Using this approach, spontaneous and DNA damage-induced SCEs have been quantified in bone marrow, lymphocytes, spermatogonia, salivary cells, and regenerating liver [26–29]. Although there are hundreds of reports that include measurements of SCEs in cultured cells, there are relatively few reports of SCE measurements in vivo, presumably because this assay is technically difficult, time consuming, and has only been used to assess crossovers in rapidly dividing cells (cells must divide in the presence of BrdU in order to be evaluated) (reviewed in [26]). Therefore, almost nothing is known about the frequency of recombination in cells that have low turnover rates in vivo, such as somatic stem cells. Furthermore, SCEs only reveal recombination events that have occurred within the previous one or two cell divisions, so it is not possible to learn about the accumulation of recombinant cells in vivo, which is essential for studies of long-term low-dose genotoxic exposures or aging.

Another approach for studying homologous recombination in vivo is to measure the frequency of recombination events that lead to LOH. Using traditional gene targeting approaches, several laboratories have created mice that are heterozygous at the *Aprt* or *Tk* loci (*Aprt*<sup>+/-</sup> and *Tk*<sup>+/-</sup> mice, respectively). In these mice, cells that have undergone LOH (to become *Aprt*<sup>-/-</sup> or *Tk*<sup>-/-</sup>) become resistant to the toxic effects of certain base analogs [30–33]. Thus, using a colony forming assay, the frequency of LOH can be determined for any cell type that can be cultured ex vivo. The *Aprt*<sup>+/-</sup> mice have been integral for studies of environmental and genetic factors that modulate LOH in T-lymphocytes and skin fibroblasts in vivo, and for showing that animals accumulate *Aprt*<sup>-/-</sup> cells with increasing age ([12,32,34–36] and reviewed in [37,38]). As LOH can be caused by any of several different mechanisms, detailed genetic analyses of clonally expanded cells are necessary to differentiate recombination events from other causes of LOH (such as point mutations) [12].

A more direct approach for specifically detecting homologous recombination is to create mutant expression cassettes that, when recombined, give rise to transgene expression (e.g. [21,22,39]) (an example of a direct repeat recombination substrate is shown in Fig. 1A). For example, using direct repeat substrates in cultured cells, it has been shown that homologous recombination plays a critical role in the repair of double strand breaks in mammalian cells [40]. Furthermore, direct repeats have been used to demonstrate that many tumor suppressor genes, including *BRCA1*, *BRCA2*, *p53*, *ATM*, *WRN*, *NBS1*, and *MSH2*, modulate mitotic homologous recombination in cultured mammalian cells [13,14,41–47]. Despite the proven efficacy of direct repeat recombination substrates in vitro direct repeats have rarely been exploited to measure HDR in vivo.

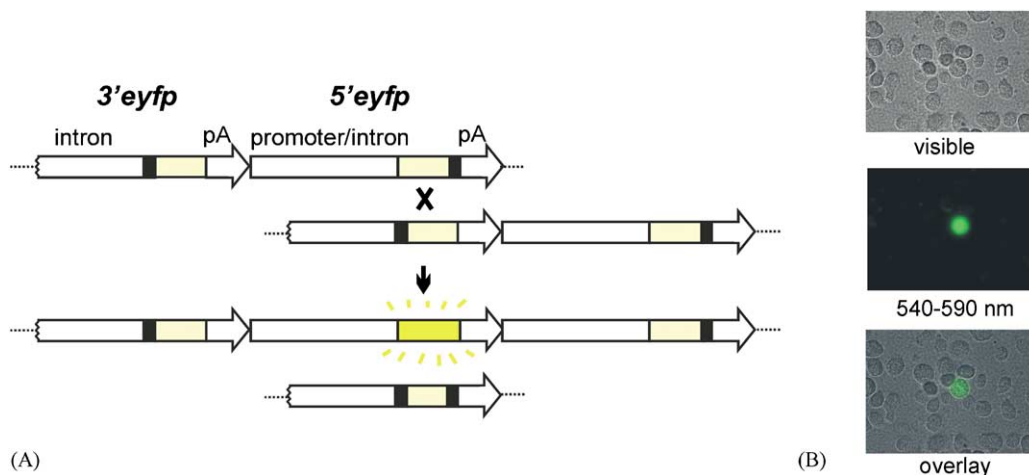


Fig. 1. Recombination at the Fluorescent Yellow Direct Repeat (FYDR) substrate is detected by fluorescence. (A) Homologous recombination restores full length enhanced yellow fluorescent protein (EYFP) coding sequence at the FYDR recombination substrate (unequal sister chromatid exchange is shown). The 5'-*eyfp* and 3'-*eyfp* expression cassettes, which each carry truncated coding sequences, are represented by two large arrows with emphasis on the coding sequences (yellow) and deleted regions (black). Expression is driven by the chicken  $\beta$ -actin promoter and cytomegalovirus enhancer [73]. (B) Images of trypsinized fibroblasts from the FYDR founder mouse.

The  $p^{un}$  (pink-eyed unstable) mice were the first mice to be used for studies of homologous recombination at a direct repeat in vivo. Mice that carry the  $p^{un}$  allele were first identified by the occasional appearance of dark spots of fur on an otherwise light gray background [48]. More recent studies have shown that the  $p^{un}$  mice carry a spontaneous duplication of  $\sim 70$  kb at the  $p$  locus, a gene that is essential for normal melanin production [49–51]. Homologous recombination events that delete one copy of the 70 kb repeat can restore expression of the  $p$  gene. If this event occurs early in development, clonal expansion of recombinant cells can result in the appearance of dark spots on either the fur or the retinal epithelium [49,52–54]. The Schiestl laboratory has been instrumental in using the  $p^{un}$  mice to reveal that numerous environmental chemicals and cancer therapeutics induce homologous recombination during development in vivo (reviewed in [53]). Studies using the  $p^{un}$  mice have also revealed that  $Atm^{-/-}$ ,  $Gadd45^{-/-}$ , and  $Wrn^{-/-}$  mice suffer increased susceptibility to spontaneous mitotic homologous recombination during embryogenesis [13,18,55]. However, since recombinant cells must arise during embryonic development in order to give rise to visible changes in pigmentation, the  $p^{un}$  mice have not been used to study recombination events that occur in mature animals.

The  $p^{un}$  mice demonstrate the feasibility and utility of studying recombination at a direct repeat, and as such, the  $p^{un}$  mice set the stage for the development of transgenic mice that carry engineered repeats. In the 1990s, several transgenic mouse lines were engineered to carry  $lacZ$  sequences that, when rearranged, give rise to a detectable phenotype. The first mice were created to detect V(D)J recombination at immunoglobulin recombination signal sequences that flank an inverted  $lacZ$  substrate, and studies have shown that DNA damage induces inversions in these mice [56–60]. While these mice demonstrate the utility of  $lacZ$  as a marker for genetic rearrangements in hematopoietic cells, immunoglobulin rearrangements are not homologous recombination events, since they are initiated by a site-specific recombinase.

In order to specifically detect homologous recombination, mice have been created that carry two different mutant  $lacZ$  alleles that can recombine to restore full length  $lacZ$  coding sequence. Recombinant cells that express  $\beta$ -galactosidase are labeled by histochemical staining and detected by either microscopy or by flow cytometry. Interestingly, one of the first such mouse models was not created to study homologous recombination, but rather was created in order to trace myotome and neuronal cell lineages during development [61,62]. Since homologous recombination at this direct repeat substrate is very rare in somatic cells ( $\sim 5$  per million cell divisions [61]), only those recombinant cells that have undergone clonal expansion have been observed in these mice (visualized as blue patches). Given the technical difficulty of detecting rare recombinant cells that have not undergone clonal expansion during development, these mice have only been used to trace cell lineages, and they have not been used for studies of mitotic homologous recombination.

The first engineered  $lacZ$  mice to be used for studies of homologous recombination in vivo were designed to exploit the fact that germ cells have a naturally high frequency of recombination. The Schimenti and Jasin laboratories independently created “Recombomice” that carry  $lacZ$  recombination substrates expressed from spermatid-specific promoters [63–66]. Homologous recombination is elevated during meiosis. Consequently, the frequency of recombinant germ cells is quite high (between  $\sim 1/10,000$  and  $\sim 1/100$ ), making it possible to detect fluorescently labeled recombinant cells by flow cytometry [63,65]. The Schimenti Recbomice have been used to show that two cancer chemotherapeutics, chlorambucil and cisplatin, induce recombination in the male germ line [64,67]. In addition, one of the transgenic lines created in the Jasin laboratory carries a  $lacZ$  substrate arranged as a palindrome, and this mouse line has been instrumental in monitoring the instability of palindromes in vivo [66,68].

### 3. Fluorescence detection of mitotic recombination in mice

A major obstacle to studies of recombination in mice has been the difficulty of detecting rare recombinant cells in somatic tissues (which are expected to occur at a frequency of about  $\sim 2$  per 100,000) [69–72]. The discovery that enhanced green fluorescence protein (EGFP) can be expressed in nearly every tissue of a mouse [73] raised the possibility that EGFP could serve as a potential marker for detecting rare recombinant cells. Using flow cytometry, it is possible to detect as few as one fluorescent cell per million [74]. In contrast, the lower limits of detection using fluorescently labeled  $LacZ$  positive cells is about  $2/10,000$ , due to non-specific staining that creates a high background of fluorescent cells [65,68]. Therefore, EGFP increases the detection limits by two orders of magnitude when compared to  $LacZ$ , thus making it feasible to use flow cytometry to detect rare recombinant cells in somatic tissues. We therefore set out to develop Recbomice that exploit fluorescent proteins as markers for recombinant cells.

In order to create a mouse model in which recombinant cells are fluorescent, we constructed a direct repeat recombination substrate comprised of two truncated enhanced yellow fluorescent protein ( $eyfp$ ) expression cassettes, each carrying a different deletion within the  $EYFP$  coding sequence. (Note that the yellow and cyan variants of EGFP can readily be differentiated. Therefore, we selected EYFP to facilitate ECFP co-expression in future experiments.) Recombination between the truncated  $eyfp$  cassettes can restore full length  $EYFP$  coding sequence, resulting in a fluorescent phenotype. The  $eyfp$  direct repeat substrate was microinjected into mouse pronuclei, and candidate mice were identified by PCR. To determine if any of the transgenic mice had integrated the  $eyfp$  substrate at a locus that is expressed, cultures of primary ear fibroblasts were assayed by flow cytometry

and fluorescence microscopy. Much to our delight, we found recombinant fluorescent cells among fibroblasts from one of the candidate mice ( $\sim 1/100,000$ ) (Fig. 1B). This founder was crossed with wild-type C57BL/6 mice to establish the Fluorescent Yellow Direct Repeat (FYDR) mouse line [74].

Characterization of the arrangement of the recombination substrate within the FYDR genome revealed that two copies of the *eyfp* recombination substrate had integrated in tandem (when DNA is microinjected, it is often the case that multiple copies are integrated end-on-end). However, there had also been significant degradation during integration. The resulting recombination substrate is composed of a promoter-less *eyfp* cassette that carries the 3' end of the *EYFP* coding sequence, followed by a complete copy of an expression cassette for the 5' end of the *EYFP* coding sequence (Fig. 1A, top) [74]. With this arrangement, mitotic homologous recombination can reconstitute full length *EYFP* by sister chromatid exchange (Fig. 1A), gene conversion, and break-induced replication (note that neither the single-strand annealing sub-pathway nor non-homologous end joining can reconstitute full length *EYFP*).

The FYDR mice have been used to measure spontaneous and DNA damage-induced homologous recombination in primary somatic cells. Primary fibroblasts derived from either mouse embryos or from ear tissue of mature animals were cultured and analyzed by flow cytometry. Using the method of  $p(0)$  [75,76], the spontaneous rate of recombination in embryonic fibroblasts was found to be about an order of magnitude higher than that of cells from adult animals. These results are consistent with the possibility that embryos are more reliant on HDR [77], possibly due to differences in HDR regulation. In addition to revealing differences in the levels of spontaneous recombination at different stages of development, primary cells from FYDR animals can also be used to measure recombination at different ages and to monitor DNA damage-induced recombination (for example, exposure to mitomycin C, a cancer chemotherapeutic, induces up to a 10-fold increase in the frequency of recombinant cells [74]). The FYDR mice thus provide a rapid assay for measuring both spontaneous and damage-induced mitotic homologous recombination in primary somatic cells.

Importantly, the FYDR mice also make it possible to detect spontaneous and damage-induced recombinant cells in vivo. For example, to quantify recombinant cutaneous cells, disaggregated skin was directly analyzed by flow cytometry (without growing cells in culture). The frequency of recombinant cells in FYDR skin varies among individual mice (ranging from zero to 188 recombinant cells per million [74]). Variability among mice is expected, since recombination events that occur earlier in development have the opportunity to give rise to higher numbers of fluorescent daughter cells compared to those that arise late in development. Interestingly, preliminary studies comparing the frequency of recombinants in skin from old versus young

mice suggest that the number of recombinant cells is greater in the older mice (Engelward laboratory, unpublished results), which is consistent with recombination taking place throughout an animal's lifetime [38].

The FYDR mice have also been used to measure the effects of ionizing radiation on recombination in vivo. In collaboration with O. Kovalchuk (University of Lethbridge, Canada), animals have been exposed to toxic levels of X-rays. As expected [78–80], acute radiation induces recombination (Engelward and Kovalchuk, unpublished results). Furthermore, the FYDR mice are currently being exposed to X-rays under long-term low-dose conditions. By monitoring the accumulation of recombinant cells over time, the FYDR mice provide a sensitive assay for monitoring long-term low-dose exposures, which more accurately reflect human exposure to carcinogenic agents in our environment.

The FYDR mice are the first mouse model for directly measuring mitotic homologous recombination in the somatic tissues of mature animals (only homologous recombination can reconstitute full length *EYFP* coding sequence and restore fluorescence expression). Using flow cytometry, direct quantification of recombinant cells is simple and rapid. What would take weeks using colony forming assays, now takes just days. These mice can thus be used in numerous applications. For example, by culturing cells from FYDR mice that have been crossed with knock-out animals, simple flow cytometry assays can be used to reveal the effects of specific genes on the spontaneous and damage-induced rates of recombination in primary cells. Such studies can then be extended to reveal the effects of these genes on recombination at different stages of development and at different ages. Importantly, studies of recombination using the FYDR mice are not limited to cell types that can be cultured *ex vivo*. These animals therefore provide an exciting opportunity to test parameters that may affect cellular sensitivity to damage-induced recombination in vivo. For example, FYDR mice can be used to determine if pre-exposure to low levels of DNA damage make mice resistant to subsequent damage-induced recombination events (i.e. an adaptive response).

What are the potential future applications of mice in which recombination can be detected by a fluorescent phenotype. Using newly developed imaging technologies, such as scanning confocal microscopy and scanning two-photon microscopy, it may be possible to identify recombinant cells in situ within intact tissues, which would yield valuable information about the relative susceptibility of different cell types within various tissues. Of particular interest are potential differences between stem cells and transit cells, and between dividing cells and non-dividing cells (does recombination occur in adult neurons?). Ultimately, these exciting applications will reveal the relative susceptibility of various cell types to genotoxic exposures and may help to explain why some cell types are more susceptible to tumorigenesis than others.

#### 4. Conclusions

Given the significance of homology directed repair, both as a fundamental process during mitosis and as a risk factor for cancer, it is clearly important to identify those environmental and genetic factors that influence recombination in mammals. There is widespread concern regarding the possible deleterious effects of synthetic chemicals that are present in our food and in our environment. Furthermore, there is a demand for novel pharmaceuticals that is frustrated by the time it takes to conduct preclinical trials. Consequently, improved systems for genotoxicity testing could be useful for identification of environmental hazards and could also accelerate the process of getting effective new drugs to market.

In this report, we have reviewed several approaches for measuring homologous recombination events in mice *in vivo*. In addition, we have described the recently developed FYDR mouse line, which is the first mouse model that can be used to quantify recombinant cells in the somatic tissues of adult animals. The FYDR mice provide a simple, rapid, and sensitive assay for compounds that impinge on genomic stability *in vivo*. It is hoped that Recombomice such as these will provide a useful tool, both as a sensor of genotoxicity and as an approach for revealing the molecular basis of DNA damage-induced recombination in mammals.

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