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Production of knockout rats using ENU mutagenesis and a yeast-based screening assay

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The rat is a widely used model in biomedical research and is often the preferred rodent model in many areas of physiological and pathobiological research. Although many genetic tools are available for the rat, methods to produce gene-disrupted knockout rats are greatly needed. In this study, we developed protocols for creating *N*-ethyl-*N*-nitrosourea (ENU)-induced germline mutations in several rat strains. F_1 preweanling pups from mutagenized Sprague Dawley (SD) male rats were then screened for functional mutations in *Brca1* and *Brca2* using a yeast gap-repair, *ADE2*-reporter truncation assay. We produced knockout rats for each of these two breast cancer suppressor genes.

The rat is an important murine model for studies in physiology, pathobiology, toxicology, neurobiology and a variety of other disciplines[1]. The rat is of value in these fields because it is larger than the mouse and because a plethora of organ-specific physiologic and disease models have been developed for it over the last century. The importance of the rat as a biological model has led to an intense effort to also establish it as a strong genetic model. A key genetic technology available for the mouse but not for the rat is the production of animals in which specified genes have been disrupted (knockout animals)[1]. This is due in part to the inability to produce functional rat embryonic stem cells. In addition, rats have not been generated to date by nuclear transfer (National Institutes of Health Meeting on Rat Model Priorities, May, 1999, http://www.nhlbi.nih.gov/resources/docs/ratmtgpg.htm). Here we report a method to produce knockout rats using an alternative approach.

The first step of our method consists of mutagenizing male rats with ENU. In mice, ENU is currently the mutagen of choice for the production of heritable altered phenotypes[2,3]. ENU was the most efficient mutagen tested[4] and was estimated to cause one functional mutation per 1,000 alleles tested (0.5–1.5 mutations per locus per progeny)[2,3]. It is important to stress the word 'functional' because the total number of mutations is much higher. Beier *et al.* calculated that theoretically there would be 10 actual sequence changes per 1,000 alleles, but that only 1 in 10 of these would result in a functional change leading to a phenotypic variant[5]. A main goal in this study was to develop a method that not only identifies F_1 rats with mutations in selected genes, but also prequalifies mutations that are likely to alter function, thus reducing wasted effort in downstream characterization of mutations that do not alter gene function. Thus, the second step of our approach involves yeast-based screening assays that select for various classes of functional mutations. These assays use gap-repair cloning to integrate either genomic DNA (gDNA) or cDNA of a selected gene between the yeast promoter ADH1 and the reporter gene *ADE2* to form a chimeric protein. If the DNA from a specific allele contains functional mutations that interfere with translation, then an active ADE2 chimeric protein is not produced, resulting in small, red yeast colonies instead of the large, white colonies found when screening wild-type DNA. We have combined ENU mutagenesis and yeast-based screening assays to generate two knockout rats for the breast cancer suppressor genes *Brca1* and *Brca2*.

RESULTS

Development of ENU mutagenesis protocols for the rat

Genome-wide mutagenesis protocols using ENU were established for three rat strains: inbred Wistar-Furth (WF), inbred Fischer 344 (F344) and outbred SD. Sexually mature 9-week-old male rats were given either a single intraperitoneal injection of ENU or a split dose with injections spaced a week apart. Fertility was determined at various times after ENU treatment (**Table 1**). The strains differed in their sensitivity to ENU-induced permanent sterility in a dose-dependent manner, with the WF strain being the most sensitive and the SD strain able to tolerate the highest doses. In all strains tested, ENU-treated male rats rarely recovered fertility after a period of complete sterility, unlike many strains of ENU-treated mice[6]. Average litter size was reduced in both the SD and F344 strains around weeks 7–9 after ENU treatment, the same time period in which we observed reduced fertility in the ENU-treated males. All fertile mutagenized male rats provided viable litters up to 1 year after ENU treatment; however, their lifespan was shortened, with many developing skin and kidney tumors and lymphomas at approximately 1 year of age. None of the doses listed in **Table 1** were acutely toxic to the rat strains tested.

Mutagenized male rats were used to generate F_1 offspring, and phenotypically variant mutant pups were visually identified before weaning at 3–4 weeks of age. Abnormalities of the eyes, tail and growth were

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Table 1 Effects of ENU treatment on male rat fertility and determination of heritable, phenotypic mutations of F_1 rats derived from ENU-treated male rats

Rat strain	ENU dose (mg/kg)	% male rats fertile[a]	No. phenotypic mutants observed[b]	Heritable	Non-heritable	Sterile	Unknown[c]
SD	75	100%	nd				
SD	100	80%	5/1068	0	1	0	4
SD	120	33%	2/347	0	1	0	1
SD	150	0%	nd				
SD	200	0%	nd				
SD	2 × 50	100%	4/524	1	2	0	1
SD	2 × 60	100%	74/4758	13	8	4	49
SD	2 × 75	20%	1/112	0	0	0	1
SD	2 × 100	0%	nd				
SD	0	100%	3/849	0	0	2	1
F344	75	100%	nd				
F344	100	67%	16/587	1	1	5	9
F344	120	0%	nd				
F344	2 × 50	60%	15/297	1	1	1	12
F344	2 × 60	40%	5/145	0	0	0	5
F344	2 × 75	0%	nd				
F344	2 × 100	0%	nd				
F344	0	100%	2/372	0	0	0	2
WF	25	30%	3/366	1	1	0	1
WF	35	33%	1/36	0	1	0	0
WF	50	25%	2/25	0	0	0	2
WF	75	0%	nd				
WF	100	0%	nd				
WF	2 × 15	17%	nd				
WF	2 × 25	17%	2/28	0	0	1	1
WF	2 × 50	0%	nd				
WF	2 × 75	0%	nd				
WF	0	100%	0/51	n/a	n/a	n/a	n/a

[a]ENU-treated male rats (n = 3–12) were paired with fertile female rats every 2 weeks from weeks 7–26 after ENU administration. Vaginal plugs were observed for all infertile breeding pairs. Fertility was based upon ability to produce a viable litter when bred with females of the same strain. [b]All F_1 pups from litters conceived at least 10 weeks after ENU treatment were visually examined for gross abnormalities in physical development or behavior at least twice before weaning at approximately 21 d of age. Details of the mutants are given in **Supplementary Table 1** online. nd, not determined; n/a, not applicable. [c]Includes all phenotypic mutant F_1 rats that were not evaluated or that died before producing a litter.

those most commonly observed in the F_1 pups (see **Supplementary Table 1** online). Using a split dose protocol of 2 × 60 mg ENU/kg body weight in SD male rats, a screen of visually apparent phenotypes revealed a rate of phenotypically detectable mutants of 1 in 64 F_1 rats (**Table 1** and **Supplementary Table 1** online). A subset of the phenotypic mutant F_1 rats was tested for inheritance. Approximately one-half of those that produced viable litters showed heritability of the trait (**Tables 1** and **2** and **Fig. 1**).

Development of a yeast-based assay for mutation screening

We chose to use the outbred SD rat for the mutation-screening studies owing to its tolerance of ENU treatment, to the variety of ENU-induced, heritable phenotypic mutants identified and to its large litter sizes. We used a split dose of ENU (2 × 60 mg/kg) to mutagenize male SD rats. These rats were then bred to wild-type female SD rats to produce F_1 pups that were screened for mutant alleles of *Brca1* and *Brca2*.

Two related truncation assays[7,8] were developed to screen the *Brca1* and *Brca2* genes of these F_1 pups for functional mutations that could interfere with protein translation (**Fig. 2**). The first assay uses gDNA as a starting macromolecule, whereas the second assay begins with total RNA that is reverse-transcribed to cDNA. In both assays, PCR is used to amplify fragments of the gDNA exon or the cDNA targeted for knockout (**Fig. 2**). The gap-repair vectors are customized for each targeted fragment by cloning in small 5′ and 3′ sequences from the fragment of interest. For *Brca1*, three vectors were generated, and the third vector (used for the cDNA assay) is shown in **Fig. 2**. For *Brca2*, three vectors were also generated and the second is shown in **Fig. 2**. The 5′ and 3′ end sequences from each fragment were cloned in tandem and separated by a unique *Sma*I restriction enzyme site, which allows the plasmid to be linearized. The linearized vector is then transformed together with unpurified PCR product of the gene-specific fragment into competent yeast (*S. cerevisiae*, yIG397 strain) cells. Following transformation, the gene-specific fragment is cloned *in vivo* into the gap-repair vector by homologous recombination. Once incorporated into the vector, the gene fragment is then located behind the yeast promoter *ADH1* and in front of the reporter gene *ADE2*, with which it jointly codes for a functional chimeric protein. This yeast strain lacks ADE2 function that can be restored by this chimeric protein. Yeast cells that produce chimeric ADE2 protein grow efficiently and form large white colonies when plated on selective medium. In the absence

Table 2 ENU-induced heritable phenotypes

Line	Strain	F_1 founder sex	Initial ENU dose (mg/kg)	Observed phenotype	Confirmed in multiple litters
9	F344	Female	100	No left eye	Yes
18	SD	Female	2 × 60	Crooked tail and slit eyes	Yes
19	SD	Male	2 × 50	Growth on tail[a]	Yes
28	SD	Female	2 × 60	Red ring eyes[a]	Yes
29	SD	Female	2 × 60	Oblong face	Yes
32	SD	Female	2 × 60	Slit eyes[a]	Yes
38	SD	Male	2 × 60	Curved tail	Yes
42	SD	Female	2 × 60	Bald spots	Yes
44	F344	Female	2 × 50	Hooklike tail[a]	Yes
54	SD	Female	2 × 60	Scaly skin	Yes
56	WF	Female	25	Head tilt	Yes
60	SD	Female	2 × 60	Scaly skin	Yes
61	SD	Male	2 × 60	Swollen feet	Yes
63	SD	Male	2 × 60	Additional digits on hind feet[a]	Yes
64	SD	Male	2 × 60	Additional digits on hind feet	Yes
68	SD	Male	2 × 60	Kinked tail	No[b]
71	SD	Male	2 × 60	Curly hair and whiskers[a]	No[b]

[a]Observed altered phenotypes are shown in **Figure 1**. [b]Only one litter has been produced to date; however, breeding of founder rat is ongoing.

of functional chimeric protein the yeast cells grow poorly and form small red colonies. Thus, for *Brca1* and *Brca2*, if the DNA donor F_1 pup is wild type for the incorporated gene fragment, the assay yields large white colonies. If, however, the donor rat DNA contains a functional mutation in one allele of *Brca1* or *Brca2* in the assayed fragment, the translation of a functional hybrid ADE2 protein is prevented and small red colonies are produced. In this assay, a functional mutation for *Brca1* and *Brca2* in a rat will be heterozygous; therefore, approximately half the colonies will be red and half white after accounting for a background rate of red colonies.

Establishment of a *Brca2* knockout rat line

We looked for disruption of the *Brca2* gene with a gDNA assay, focusing on exon 11 (the largest exon, representing roughly half of the cDNA) (**Fig. 2**). This large exon was divided into three regions of ~1,700 base pairs (bp) each, and the second and third fragments were used for screening. Primer sequences for each fragment are given in **Supplementary Table 2** online. We screened gDNA from 788 preweanling F_1 rat pups before finding a mutated *Brca2* allele using the second-fragment vector (**Fig. 2**). The knockout rat was the only one identified with this *Brca2* mutation out of 296 F_1 offspring screened from this specific mutagenized father, indicating that this mutation was not a preexisting germline mutation in this SD father. Similarly, the female parent produced over 40 offspring, including 10 littermates of the knockout rat, none of which carried the *Brca2* mutation. The *Brca2* knockout rat was detected in our gDNA assay by a yeast plate that had approximately 45% red colonies and 55% white colonies (**Fig. 3a**, right dish). The average background of red colonies was very low (**Fig. 3a**, left dish) for this gDNA assay (0.5% ± 0.6%, $n = 10$). Next, individual red and white yeast colonies were sequenced. A nonsense transversion mutation was detected at nucleotide T4254 of the *Brca2* cDNA that converted TAT (tyrosine) to TAA (stop codon) at Tyr1359 (**Fig. 3a**, lower panel, upper and center sequences). A/T→T/A transversion mutations are the most common mutation type (44%) found in mice bearing ENU-induced, phenotypically detectable germline mutations[2,3]. Genomic DNA from the founder rat 3983 was sequenced and found to contain the identical mutation as detected in the yeast red colonies (**Fig. 3a**, lower panel, lower sequence).

In conjunction with the gDNA assay, we used the cDNA yeast assay with the same *Brca2* fragment 2 vector to screen N_2 pups resulting from the breeding of the *Brca2* knockout founder male rat 3983 to SD females. Both methods identified the same 9 out of 14 pups from the first litter of rats carrying this *Brca2* mutation, and these results were confirmed by the direct sequencing of gDNA from each N_2 pup. This verified the utility of the yeast assay starting from either gDNA or RNA. This cDNA assay had a background of 15.3% ± 2.0% ($n = 20$) for wild-type pups and 48.5% ± 2.1% ($n = 36$) red colonies for knockout pups. Sequencing *Brca2* fragment 2 DNA of 60 red colonies from the cDNA assay of the knockout pups confirmed this background frequency, in that 17% (10/60) of the sequenced clone fragments lacked the specific stop codon mutation. Interestingly, the

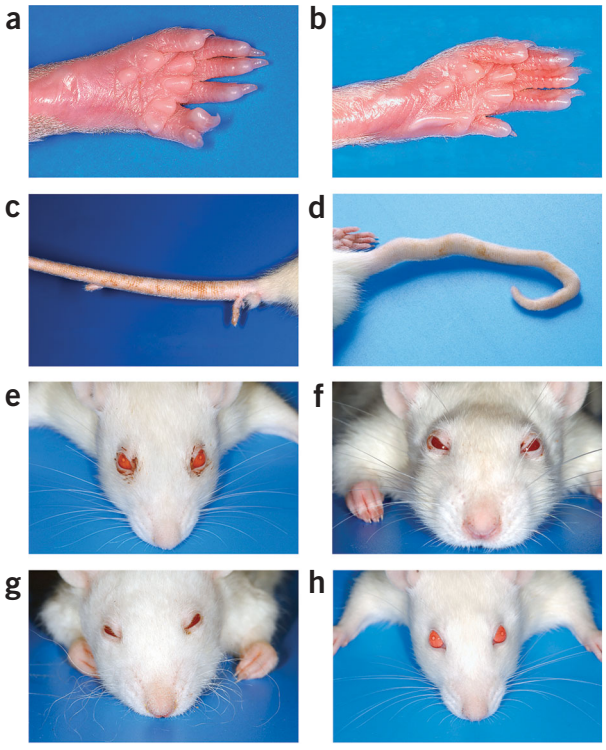

Figure 1 Heritable phenotypic mutant rats. Male rats were given ENU and then bred to produce F_1 pups that were observed for visible altered phenotypes. Details of these derivations are listed in **Table 2**. The phenotypic mutants and control rats shown include: (**a**) line 63 rat with multiple digits on hind foot; (**b**) control rat hind foot; (**c**) line 19 rat with growths on tail; (**d**) line 44 rat with hook-like tail; (**e**) line 28 rat with red ring eyes; (**f**) line 32 rat with slit eyes; (**g**) line 71 rat with curly hair and whiskers, no eye abnormality; (**h**) control rat.

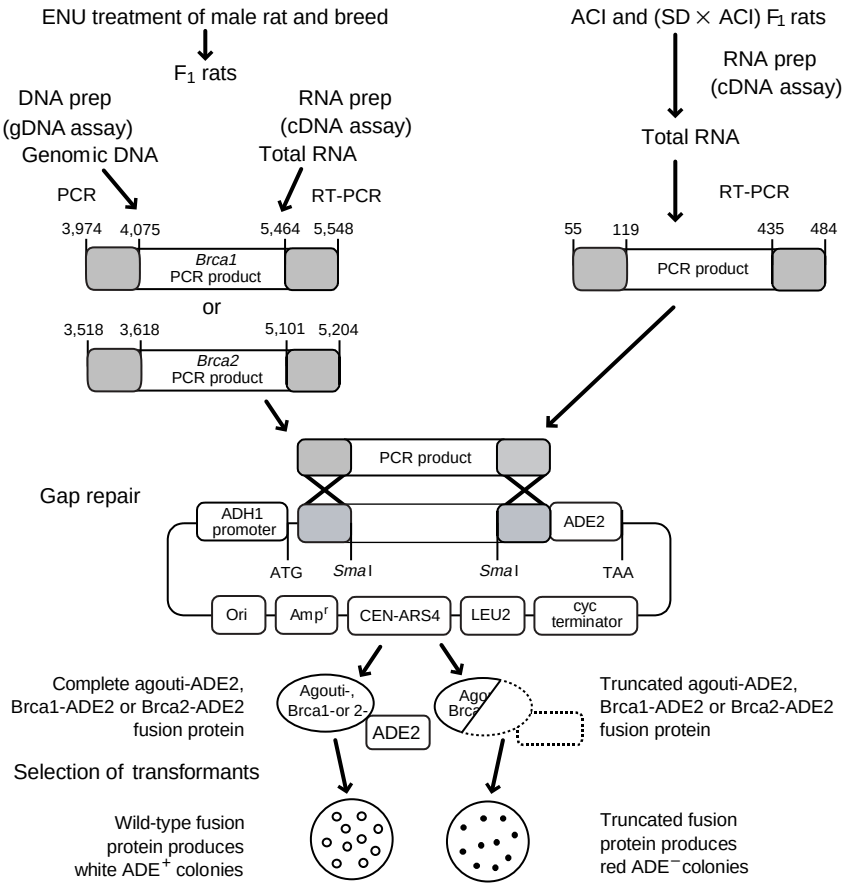

Figure 2 *Brca1*, *Brca2* and *agouti* yeast cDNA/gDNA truncation assays. For *Brca1* and *Brca2* assays, male rats are treated with ENU and bred to produce F_1 pups. DNA and RNA are isolated from tail clips of one-week-old F_1 rats for *Brca1* and *Brca2*. For the *A* (*agouti*) assays, a small piece of ventral skin from ACI or (SD × ACI) F_1 rats is excised and used for RNA isolation. Total RNA is reverse-transcribed and both the resultant cDNA (*Brca1* or *A*) and isolated gDNA (*Brca2*) are amplified using PCR for selected DNA regions. The gap repair vectors are customized for each targeted fragment. The 5′ and 3′ sequences for the *Brca1* vector are derived from nucleotides 3974–4075 and 5464–5548 of the *Brca1* cDNA (GenBank no. AF036760), respectively. The 5′ and 3′ sequences for the *Brca2* vector are derived from nucleotides 3518–3618 and 5101–5204 of the *Brca2* cDNA (GenBank no. U89653, mRNA), respectively. The *Brca1* and *Brca2* vectors shown are those that ultimately led to the identification of the knockouts. A single gap vector was constructed using the 5′ and 3′ sequences derived from nucleotides 55–119 and 435–484 of the *A* mRNA sequence (GenBank no. AB045587), respectively. Following transformation, the gene-specific fragment is cloned *in vivo* into the gap-repair vector by homologous recombination. The wild-type gene fragment codes for a functional fusion protein with the *ADE2* gene of the vector and forms large white colonies when plated. A truncated gene fragment will not form a functional protein and the colonies will be small and red.

reduced in size but otherwise healthy. Histopathological analysis of gonads from the *Brca2* homozygous rats shows severe atrophy that is not observed in the *Brca2* heterozygous and wild-type rat gonads (**Supplementary Fig. 1** online).

Production of a *Brca1* mutant rat line

Customized gap-repair vectors for screening *Brca1* (**Fig. 2**) consisted of two gDNA vectors targeting exon 11 (the largest exon, target fragments 1 and 2) and one cDNA vector targeting *Brca1* from the 3′ end of exon 11 to the end of the open reading frame (ORF) (fragment 3). Primer sequences for the three fragments are given in **Supplementary Table 2** online. After screening 1,965 pups, we identified a *Brca1* mutation in founder rat 5385 using the cDNA assay (**Fig. 2**). This rat was the only one with this mutation identified in 273 offspring from the same mutagenized SD father and in more than 40 offspring, including 14 littermates, from the SD wild-type mother. The background rate of red colony formation in this assay was 12.2% ± 3.3% (n = 1,485) for wild-type DNA compared to 44.3% in the identified mutant. Haploid DNA from red yeast colonies was sequenced, revealing a complete loss of *Brca1* exon 22 (74 bp) (**Fig. 4**). We sequenced introns 21 and 22 in search of a splicing mutation to explain the loss of this exon. A T→C mutation was identified within the splicing branch site of intron 21 (TGGTGAT to TGGCGAT) (**Fig. 4d,e**). A T/A→G/C transition mutation is the second most common type (38%) of ENU-induced mutations[2,3]. The mutation in the branch site of intron 21 caused the splice donor site to skip over exon 22 and find a branch site in intron 22. This led to splicing out of the 74-bp exon 22 and also caused a frameshift downstream from exon 21, exposing a stop codon at the exon 23–24 border (**Supplementary Fig. 2** online). Recently, the female founder 5385 has produced two *Brca1* heterozygous rats out of eight pups, demonstrating germline transmission of this mutation.

background rate for the cDNA assay was over an order of magnitude higher than the gDNA assay, suggesting that most of the background in the cDNA assay comes from DNA replication errors in the reverse transcription reaction.

N_2 pups produced from founder 3983 included 35 heterozygous knockouts out of 64 pups, demonstrating the mendelian inheritance of this knockout gene. *Brca2* heterozygous N_2 male and female rats were bred to produce *Brca2* homozygous knockout pups. The ratio of *Brca2* homozygous knockout rats to *Brca2* heterozygous rats to wild-type rats was approximately 1:2:1. Body weight data were collected for all N_2F_2 pups starting at weaning. The results illustrate a clear phenotype of growth inhibition of male and female *Brca2* homozygous knockout rats (**Fig. 3b**). These rats are sterile and

Nonsense-mediated decay

An anticipated problem using RNA as a starting material for this assay is the potential destruction by cell surveillance mechanisms, such as nonsense-mediated decay (NMD)[9–11], of mRNA transcribed from the mutant allele. We quantified the extent of NMD of the mutated *Brca2* mRNA by comparing the yield of red colonies in the knockout rat samples minus background in the wild-type samples using the cDNA assay (48.5–15.3%) versus the yield of red colonies in the knockouts minus background using the gDNA assay (44.8–0.5%). The same gDNA *Brca2* fragment 2 gap vector was used for both the cDNA and gDNA assays. From these results, NMD is calculated to occur at an approximate rate of [1 – (33/44)] or 25%.

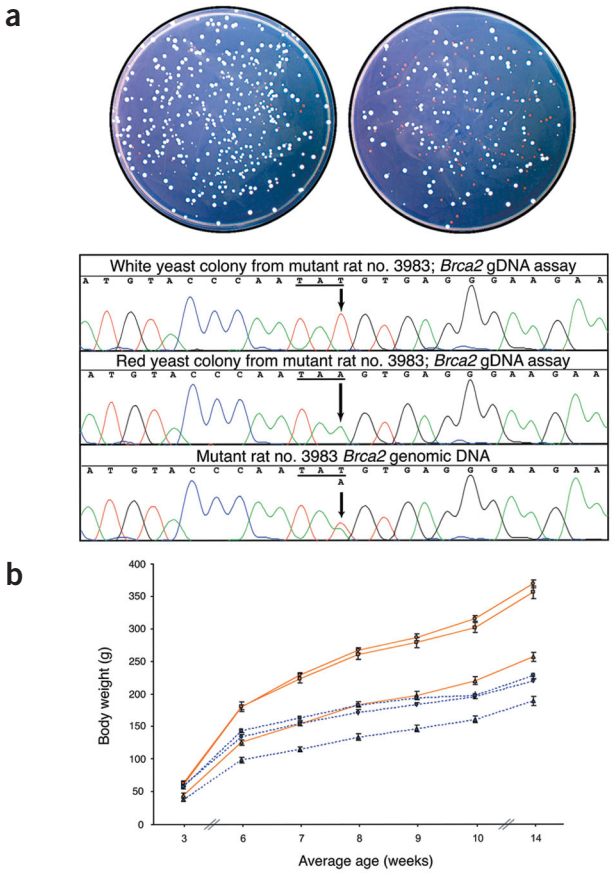

Figure 3 Identification of a *Brca2* knockout rat. (**a**) Screening for a *Brca2* knockout rat. Yeast cells transformed with gap vector and a PCR product enriched for *Brca2* fragment 2 (nucleotides 3518–5204) were plated on selective medium. When gDNA obtained from a rat (SD) with two wild-type alleles was assayed, the resultant plate contained mostly large white colonies (left dish). In contrast, when the DNA is from a rat in which one allele of *Brca2* was functionally mutated, the resultant colonies were an almost equal mixture of red and white colonies (right dish). Red and white colonies from the plate on the right were picked and used to obtain *Brca2* fragment 2 DNA sequence. The sequence from white yeast colonies (lower panel, upper, representative of four colonies tested) is that of wild-type rat *Brca2*, whereas the sequence from red colonies (lower panel, center, representative of eight colonies tested) has a transversion mutation at T4254 (indicated by the arrow) of the cDNA [TAT (tyrosine) → TAA (stop)]. Genomic DNA from the heterozygous knockout rat no. 3983 contains both T and A at nucleotide 4254 as seen in the lower sequence (represents two independent tests). The sequences shown in the lower panel span nucleotides 4242–4266 of the rat *Brca2* cDNA. (**b**) *Brca2* knockout body weight phenotype. Male (solid orange lines) and female (dashed blue lines) *Brca2* homozygous (△), heterozygous (○) knockout rats and wild-type littermates (□) were weighed through their current age of 14 weeks (error bars are ± s.d.).

Because this level of NMD was modest, we challenged our cDNA-based assay using a rat *A* (also known as *agouti*) locus model in which ~85% of the mutant RNA is subject to NMD[11]. Agouti rat strains such as the ACI rat carry two copies of the wild-type locus, whereas nonagouti rats such as SD carry two identical mutant alleles, each with two truncating mutations in the *A* gene. We designed a yeast gap vector for this gene that allowed the entire ORF to be cloned *in vivo* in yeast (**Fig. 2**). We found that our cDNA assay could routinely detect the *A* mutation in (SD × ACI) F_1 pups, which had 12.4% ± 1.8% ($n = 52$) red colonies, whereas the wild-type ACI group had a background of 4.4% ± 1.6% ($n = 40$) red colonies ($P < 0.0001$, unpaired *t*-test). NMD was estimated to remove approximately 80% of the RNA coded from the mutated *A* allele of the F_1 pup, which corresponds well with the above-referenced northern analysis[11]. Note also that the lower background rate of 4.4% red colony formation for the *A* cDNA assay (500 bp) as compared to that of the *Brca1* (12.2%, 1.6 kb) and *Brca2* (15.3%, 1.7 kb) cDNA assays demonstrates that background is proportional to the size of the gene or gene fragment being screened. A second estimate of background was obtained by sequencing for the *A* mutation in individual red colonies from a yeast assay of the (SD × ACI) F_1 pups. Of 61 red colonies evaluated, 5 had random mutations, giving a background of 8%, statistically distinguishable from the F_1 value of 12.4% ± 1.8% red colonies ($P < 0.0001$, one-sample *t*-test).

DISCUSSION

We have established methods to produce knockout rats and have identified knockouts for *Brca1* and *Brca2*. Our technology combines protocols for efficient rat germline mutagenesis by ENU and a yeast-based method to economically (~$18,000 for a 90% chance of success) and rapidly screen preweanling F_1 rat pups from mutagenized fathers for functional mutations in selected genes using yeast truncation assays. The first identified rat gene to be knocked out, *Brca2*^{ochre4254}, was bred to homozygosity and has a phenotype that includes general growth inhibition and gonadal atrophy in both sexes. Interestingly, *Brca2* homozygous knockout mice with similar mutations in exon 11 have shown either embryonic lethality or embryonic survival with premature death[12–14]. We have not yet begun phenotypic evaluation of the *Brca1* knockout rat line.

Our ENU assay for the rat provides a phenotype-driven, ENU-induced mutation screening for a second murine species. The outbred SD rat tolerated the highest single and split dose of ENU. This and its ability to produce large litters led us to choose it for our genotype-based mutation screening. The inbred F344 strain tolerated higher doses than the very ENU-sensitive WF strain. It will be important in the future to evaluate additional inbred lines for their reproductive tolerance of ENU, as inbred rats provide a more homogenous genome than the more complex outbred rat strains, especially if evaluation of preexisting germline mutations is required. However, with either an inbred or outbred strain, it is important to backcross the knockout founder to either the isologous strain or another of a desired genetic background to eliminate other ENU-induced germline mutations. Switching genetic backgrounds may be more efficient in that it allows the use of speed congenic protocols. Furthermore, to eliminate the possible confounding effects of very closely linked mutations, one can screen for additional alleles of each knockout using this yeast-based technology and evaluate them phenotypically.

Our yeast-based truncation screening assays have advantages and disadvantages that suggest which one should be used to target specific genes. The gDNA assay is most efficient if the selected gene has at least one exon larger than ~400–500 bp. In contrast, the cDNA assay is independent of exon size and can easily incorporate up to ~2,500 bp per vector. However, the background rate of red colony formation is over an order of magnitude lower in the gDNA assay, making it easier to identify mutant rats through red colony formation on the yeast plates. These truncation assays allow screening only for mutations that compromise protein translation, such as nonsense mutations and out-of-frame frameshift deletions or insertions. The *Brca1* knockout rat was identified using a cDNA yeast truncation assay in the 3′ region of the *Brca1* gene that consists of a series of very small exons. None of the

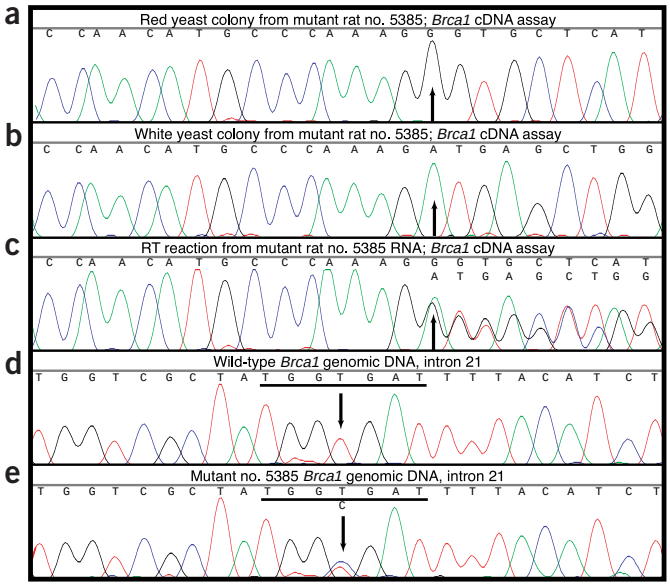

Figure 4 Screening for a *Brca1* knockout rat. Yeast cells were transformed with linearized gap vector and a PCR product enriched for *Brca1* fragment 3 (nucleotides 3974–5548). A plate with 44.3% red colonies (as compared to an average 15.8% red colony background from all other plates, $n = 89$) identified a potential knockout rat, no. 5385. (**a**) Sequence of haploid DNA from a yeast red colony (representative of eight colonies tested) in which exon 22 (74 bp) is deleted. (**b**) The sequence of haploid DNA from a wild-type white colony (representative of two colonies tested). The arrow in panel **a** indicates the first nucleotide (5359) of exon 23, whereas the arrow in panel **b** indicates the first nucleotide (5285) of exon 22. (**c**) This difference is highlighted by sequencing a mixture of cDNA from both rat alleles (+/–) from a reverse transcription reaction of total tail RNA (representative of two independent tests). In panels **a**, **b** and **c**, the sequence before the arrow is the 3′ end of exon 21. (**d**) Results of sequencing gDNA from a wild-type SD rat over a region of intron 21 that contains the splicing branch site (underlined). (**e**) The same sequence from the heterozygous *Brca1* mutant founder rat no. 5385, which includes a T→C mutation (indicated by the arrow) within the splicing branch site. Sequences in **d** and **e** span nucleotides 36–12 upstream of exon 22, with the mutation at nucleotide 24 upstream of exon 22. The mutant sequence is shown in its translated form in **Supplementary Figure 2** online.

exons covered would have been good targets for the gDNA truncation assay because of their small size. In addition, this intronic mutation would not have been found using other screening methods, such as sequencing, heteroduplex analysis or denaturing high-performance liquid chromatography, because these assays are used to screen only the exons from gDNA.

The major drawbacks of the cDNA assay are that the gene-specific RNA may not be produced in an easily collectible tissue and mutant RNA may be lost to a great extent by NMD. In these studies, we demonstrate the ability of a cDNA yeast-based screening assay to detect the *A* mutant allele despite a high level of NMD in this model, and thus show the general ability of a yeast-based screening assay to detect mutants in spite of extensive NMD. NMD can be minimized by pretreating collected cells, such as white blood cells, with a protein synthesis inhibitor before RNA collection. This approach has been successful for the yeast gap-repair p53 assay[15,16] and may be extrapolated to *in vivo* studies by the administration of a protein synthesis inhibitor to rat pups before tissue collection. We have had preliminary success in inhibiting NMD using the protein synthesis inhibitor emetine. The problem of a gene-specific RNA not being produced in tail tissue may be reduced by extending the range of biopsy tissues collected from viable rats (for example, white blood cells, liver and skin). In the future, sperm from F_1 male rats of mutagenized fathers could be cryopreserved[17], and a wide variety of organ-specific RNAs could also be collected and stored, along with DNA from spleens or other tissue from the same male rats. DNAs or RNAs from a large number of rats could thus be screened and the appropriate frozen sperm used to recover mutant rats. Sperm cryopreservation has been established for many mouse strains and crosses[18] and has allowed the recovery of a mutant mouse[19].

In summary, the technologies presented here provide the means for producing gene-selected knockout lines for the rat. The generation of unique rat models should extend our knowledge of the genetics underlying human diseases and aid in the development of novel drugs to prevent and treat these diseases.

METHODS

Rat ENU mutagenesis protocol. The University of Wisconsin-Madison Animal Care and Use Committee has approved all experimental animal procedures described in these studies. We administered a single or split dose of ENU by intraperitoneal injection to male rats from Harlan at 9 weeks of age; for a split dose, at 9 and 10 weeks of age. One gram of ENU (Sigma) was dissolved in 10 ml of 95% (vol/vol) ethanol and then diluted with 90 ml of phosphate citrate buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 5.0) before injection. We paired mutagenized males with females of the same strain for consecutive 2- to 3-week periods, beginning 3–5 weeks after the first ENU treatment. We observed female rats for vaginal plugs, gross pregnancy, date of birth and size of litters. For our *Brca1* and *Brca2* mutation screening experiments, we used SD male rats given a split dose of ENU, 2 × 60 mg/kg body weight.

We collected tail clips from the F_1 pups at 1 week of age for macromolecule isolation. We also visually checked all F_1 pups for gross abnormalities in physical development at least twice before weaning at 21–28 d of age. A subset of the F_1 phenotypic mutant rats identified was bred to same-strain rats to determine inheritance of the phenotypic mutation. Several of the rat lines with heritable mutant phenotypes are currently being maintained and backcrossed to eliminate residual ENU-induced genetic changes not associated with the phenotypic mutations.

All breedings to produce ACI and (SD × ACI) F_1 pups were performed at our facility. At 3–7 d of age, pups were killed and ventral skin was collected for the *A* yeast assay.

Vector construction. The gap vector pLSRP53 containing the p53 cDNA[15,20] was digested with *Hin*dIII and *Eag*I to remove the entire p53 coding sequence. A 44-bp linker that contains sequence encoding the first 11 amino acids of rat p53 was inserted at the *Hin*dIII and *Eag*I sites to produce vector pLSK846 with the *Eag*I site converted to a unique *Not*I site. The full length *ADE2* gene was amplified by PCR from yeast strain yIG397 (ref. 15) DNA and integrated into the pLSK846 plasmid at the *Not*I site to generate vector pLSK870. A unique *Not*I site was retained at the 5′ end of the *ADE2* gene. This *Not*I site was used to drop in *Brca1*, *Brca2* or *A* sequence cassettes. Each *Brca1*, *Brca2* or *A* cassette contained two fused ~100 bp fragments, corresponding to the 5′ and 3′ ends of a ~1.6 kb *Brca1* fragment, a ~1.7 kb *Brca2* fragment or the ~500 bp *A* ORF, joined by a unique *Sma*I site. The half-site sequences of the *Brca1*, *Brca2* or *A* cassettes were designed to be in frame with the p53 leader and *ADE2* sequences (**Fig. 2**). Vectors were linearized before yeast transformation by digestion with *Sma*I (20 U/μl) and then purified using a QIAquick PCR purification kit (Qiagen, Inc.).

DNA/RNA extraction. To isolate DNA, small sections of tails were digested overnight at 55 °C in 500 μl of genomic lysis buffer consisting of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA and 1% (wt/vol) SDS. Two hundred μl of Protein Precipitation Solution (Gentra Systems) was added to the lysate solution. DNA in the clear supernatant was precipitated with isopropanol, washed and resuspended in water. Total RNA was isolated from tail or skin sections that were placed in RNAzol B solution (Tel-Test) and homogenized (Polytron PT10-35, Kinematica). The samples were then extracted with

chloroform, precipitated with isopropanol and washed with ethanol. Pellets were resuspended in 30 µl RNA suspension solution (Ambion) for *Brca1* and *Brca2*, and in 60 µl for *A*.

Reverse transcription and PCR. All primers used are listed in **Supplementary Table 2** online. cDNA was synthesized for *Brca1* or *Brca2* from 1–2.5 µg rat tail total RNA at 42 °C for 2 h with 200 U of SuperScript II (Invitrogen). *A* cDNA was synthesized from 1–5 µg of skin total RNA in a 1 h reaction. The 20 µl reaction consisted of 1× reverse transcription buffer (Invitrogen), 0.5× RNA secure reagent (Ambion), 10 mM DTT, 1.25 mM dNTP mix and 0.33 µg *Brca1*-, *Brca2*-, or *A*-specific primers. PCR was performed on 1.0 µl of the cDNA product or ~0.1 µg of gDNA with 1 U of Herculase (Stratagene) in 20 µl reactions containing 1× Herculase Buffer, 0.2 mM dNTP mix and 0.05 µg primers for *Brca1* and *Brca2*. Reaction conditions for *Brca1* and *Brca2* fragments were 95 °C for 2 min, followed by 35 cycles consisting of 1 min at 92 °C, 45 s at 60 °C, and 4 min at 72 °C, followed by 7 min at 72 °C. For the *A* gene PCR, 0.5 U of Failsafe enzyme (Epicentre Technologies) was used with Failsafe buffer J (which contains dNTPs) and 0.1 µg primers. The cycling conditions for *A* were similar to above except that the annealing temperature was 55 °C and the 72 °C extension step was only 1 min. PCR quality and product quantity were estimated by electrophoresis in a 1.2% (wt/vol) agarose gel.

Yeast transformation and sequencing. yIG397 (ref. 15) yeast was cultured overnight at 30 °C in YPD medium supplemented with adenine (200 µg/ml) to an OD_{600} of 0.9. The cells were washed and resuspended in a volume of LiOAc/TE solution (0.1 M lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) equivalent to the volume of the cell pellet. For each transformation, 30 µl of yeast suspension was mixed with 10 ng of linearized gap vector, 25 µg of salmon sperm carrier DNA, 150 µl of LiOAc/TE/PEG solution (0.1 M lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40% (wt/vol) PEG) and 2–5 µl unpurified *Brca1*, *Brca2*, or *A* PCR product (total volume ~185 µl). The mixture was incubated for 30 min at 30 °C, then heat-shocked for 15 min at 42 °C. Transformants were then plated on synthetic minimal medium lacking leucine and supplemented with low adenine (5 µg/ml) and incubated for 3 d at 30 °C. An automated colony counter (ProtoCOL, Microbiology International) was used to determine the number of red and white colonies on each plate for the cDNA assays, and the percentage of red colonies per sample was recorded. The background rate of red colonies was determined by averaging the percentage of red colonies from all plates not containing a knockout. gDNA assay yeast plates were generally inspected only visually. The signal-to-noise ratio for the gDNA assay was large (>50:1), whereas that for the cDNA assay was smaller (~3:1). Thus, a criterion for the cDNA assays was set to follow up on samples for which the red colony percentage was at least 2 s.d. above the mean. This conservative criterion was designed to avoid false negatives and on average resulted in two false positives per gene assay for 90 rats screened. Most false positives were eliminated upon repeating the yeast assay using the original RNA sample.

For sequencing, red and white colonies were picked directly into PCR mix, amplified and purified to remove primers and nucleotides. Four microliters of each reaction was then used in a 20 µl cycle-sequencing reaction using BigDye (Applied Biosystems Inc.) chemistry.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Jacob, H.J. & Kwitek, A.E. Rat genetics: attaching physiology and pharmacology to the genome. *Nat. Rev. Genet.* **3**, 33–42 (2002).
2. Justice, M.J., Noveroske, J.K., Weber, J.S., Zheng, B. & Bradley, A. Mouse ENU mutagenesis. *Hum. Mol. Genet.* **8**, 1955–1963 (1999).
3. Noveroske, J.K., Weber, J.S. & Justice, M.J. The mutagenic action of *N*-ethyl-*N*-nitrosourea in the mouse. *Mamm. Genome* **11**, 478–483 (2000).
4. Russell, W.L. *et al.* Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. *Proc. Natl. Acad. Sci. USA* **76**, 5918–5922 (1979).
5. Beier, D.R. Sequence-based analysis of mutagenized mice. *Mamm. Genome* **11**, 594–597 (2000).
6. Justice, M.J. *et al.* Effects of ENU dosage on mouse strains. *Mamm. Genome* **11**, 484–488 (2000).
7. Ishioka, C. *et al.* Screening patients for heterozygous p53 mutations using a functional assay in yeast. *Nat. Genet.* **5**, 124–129 (1993).
8. Kataoka, A. *et al.* Development of a yeast stop codon assay readily and generally applicable to human genes. *Am. J. Pathol.* **159**, 1239–1245 (2001).
9. Culbertson, M.R. RNA surveillance. Unforeseen consequences for gene expression, inherited genetic disorders and cancer. *Trends Genet.* **15**, 74–80 (1999).
10. Frischmeyer, P.A. & Dietz, H.C. Nonsense-mediated mRNA decay in health and disease. *Hum. Mol. Genet.* **8**, 1893–1900 (1999).
11. Kuramoto, T., Nomoto, T., Sugimura, T. & Ushijima, T. Cloning of the rat agouti gene and identification of the rat nonagouti mutation. *Mamm. Genome* **12**, 469–471 (2001).
12. Suzuki, A. *et al. Brca2* is required for embryonic cellular proliferation in the mouse. *Genes Dev.* **11**, 1242–1252 (1997).
13. Connor, F. *et al.* Tumorigenesis and a DNA repair defect in mice with a truncating *Brca2* mutation. *Nat. Genet.* **17**, 423–430 (1997).
14. Freidman, L.S. *et al.* Thymic lymphomas in mice with a truncating mutation in *Brca2*. *Cancer Res.* **58**, 1338–1343 (1998).
15. Flaman, J.-M. *et al.* A simple p53 functional assay for screening cell lines, blood, and tumors. *Proc. Natl. Acad. Sci. USA* **92**, 3963–3967 (1995).
16. Andreutti-Zaugg, C., Scott, R.J. & Iggo, R. Inhibition of nonsense-mediated messenger RNA decay in clinical samples facilitates detection of human MSH2 mutations with an *in vivo* fusion protein assay and conventional techniques. *Cancer Res.* **57**, 3288–3293 (1997).
17. Nakatsukasa, E. *et al.* Generation of live rat offspring by intrauterine insemination with epididymal spermatozoa cryopreserved at –196 °C. *Reproduction* **122**, 463–467 (2001).
18. Nakagata, N. Cryopreservation of mouse spermatozoa. *Mamm. Genome* **11**, 572–576 (2000).
19. Coghill, E.L. *et al.* A gene-driven approach to the identification of ENU mutants in the mouse. *Nat. Genet.* **30**, 255–256 (2002).
20. Yamamoto, K. *et al.* A functional and quantitative mutational analysis of p53 mutations in yeast indicates strand biases and different roles of mutations in DMBA- and BBN-induced tumors in rats. *Int. J. Cancer* **83**, 700–705 (1999).