

A powerful method combining homologous recombination and site-specific recombination for targeted mutagenesis in *Drosophila*

Guanjun Gao, Conor McMahon*, Jie Chen, and Yikang S. Rong[†]

Laboratory of Biochemistry and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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Gene targeting provides a powerful tool for dissecting gene function. However, repeated targeting of a single locus remains a practice mostly limited to unicellular organisms that afford simple targeting methodologies. We developed an efficient method to repeatedly target a single locus in *Drosophila*. In this method, which we term “site-specific integrase mediated repeated targeting” (SIRT), an *attP* attachment site for the phage ϕ C31 integrase is first targeted to the vicinity of the gene of interest by homologous recombination. All subsequent modifications of that gene are introduced by ϕ C31-mediated integration of plasmids carrying an *attB* attachment site and the desired mutation. This highly efficient integration results in a tandem duplication of the target locus, which is then reduced into a single copy carrying the mutation, likely by the efficient “single strand annealing” mechanism, induced with a DNA double-strand break (DSB). We used SIRT to generate a series of six mutations in the *Drosophila nbs* gene, ranging from single amino acid replacements and small in-frame deletions to complete deletion of the gene. Because all of the components of SIRT are functional in many different organisms, it is readily adaptable to other multicellular organisms.

gene targeting | position effect | site-specific integration | rare-cutting endonuclease | single strand annealing

A comprehensive understanding of gene function may require, in addition to null mutations, other mutations that disrupt specific domains or residues. Ideally, these mutations should be generated at the endogenous location of the gene to ensure their proper expression. This type of mutational analysis has been limited mostly to simpler organisms, in which targeted mutagenesis is efficient and relatively easy. However, the importance of such studies in higher eukaryotes is unequivocal. In organisms for which efficient transgenic techniques exist, such as *Drosophila*, an alternative is to introduce transgenes carrying different mutations into a null background. The drawback of this approach has been that each transgene is inserted at different positions and is subject to different degrees of chromosomal position effect. In addition, many location specific effects on transcription, such as transvection, are difficult to reproduce by using transgenes. Moreover, the effect of long-range regulatory elements may not be included in the transgene constructs. Over the years, the *Drosophila* research community has developed several methods to combat position effect. The most effective ones use site-specific integration to compare the effect of different transgenes placed at a single preselected genomic location (1–3). To this end, the bacterial phage ϕ C31 integrase was recently introduced into *Drosophila* (4). The integrase catalyzes unidirectional recombination between its *attP* and *attB* DNA targets that is irreversible under normal conditions (5). ϕ C31-mediated integration experiments have been carried out by direct embryo injection. This makes it the simplest and most efficient integration method currently for use in *Drosophila*. More recently, insulator elements were used in combination with the ϕ C31 system to further reduce the influence of position effects (6).

Although ectopic expression from transgenes can be useful in certain cases such as overproducing dominant negative mutants or knocking down tissue specific expression by RNAi, the endogenous locus is undoubtedly the best position in which to compare the effect of different mutations of a particular gene. Therefore, *Drosophila* researchers are in need of an efficient method that would allow repeated modifications of a gene at its endogenous locus. Traditional schemes in higher eukaryotes are inherently labor intensive, requiring several months for a single targeting experiment in *Drosophila* (7). In this study, we describe the SIRT (site-specific integrase-mediated repeated targeting) method, which combines the ϕ C31-mediated site-specific integration and the endogenous homologous recombination machinery. Here we demonstrate the power of the SIRT method by generating a series of six precise mutations in the *nbs* gene, which encodes the *Drosophila* homolog for the human NBS1 protein that is defective in Nijmegen breakage syndrome patients (8). Mutations range from single amino acid changes to a complete deletion of the gene. The SIRT method is also adaptable for other organisms. To facilitate the placement of *att* sites and mutations in DNA vectors, we devised a two-step bacterial recombineering method.

Results

Method Overview. The first step of SIRT is the placement of an *attP* site close to the locus of interest, which is accomplished by the current ends-in targeting scheme (7, 9). A P element is constructed, which carries the *white*⁺ (*w*⁺) marker gene and a genomic fragment as targeting homology (Fig. 1A). These two fragments are flanked by *FRT* targets for the FLP recombinase. An *attP* and a cut site for the I-SceI endonuclease are placed within the target homologous fragment. Flies carrying this donor element are crossed with flies expressing FLP and I-SceI under the control of the heat shock promoter. Their progeny are heat shocked early in their development. The combined action of FLP and I-SceI generates an extrachromosomal linear DNA. Recombination between this donor and its chromosomal target generates a tandem duplication of the target locus.

Flies with the duplication, which have red eyes (*w*⁺), are then crossed to flies expressing the I-CreI rare-cutting endonuclease under the control of the heat shock promoter, and their progeny are heat shocked. The original donor P element carries a strategically placed cut site for I-CreI, so that a future double-

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*Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

[†]To whom correspondence should be addressed. E-mail: rongy@mail.nih.gov.

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Construct	Mutation	phiC31 integration	Orientation in pTV2	Reduction (-FLP)	Reduction (+FLP)
	none	NA	Figure 1B	NA	NA
	Δ FHA	5.9% (5/76)	Figure 1B	0% (0/42)	34.6% (9/26)
	SQ to AQ	6.6% (7/119)	Figure 1B	4.4% (2/45)	36.4% (8/22)
	Δ BRCT1	8.1% (7/74)	Reverse	16.0% (4/25)	11.5% (3/26)
	Δ FHA-BRCT1	16.9% (21/124)	Reverse	2.0% (1/54)	25.0% (8/32)
	Δ BRCT2	11.2% (10/89)	Reverse	8.7% (2/23)	15.8% (3/19)
	Δ nbs	6.5% (12/185)	Figure 1B	8.7% (4/46)	23.3% (7/30)
	none	14.5% (9/62)	Figure 1B	ND	ND

Fig. 2. *nbs* constructs for SIRT. The genomic regions used to construct the allelic series are shown in the diagram. These regions were cloned into the pTV2 targeting vector giving rise to the different M constructs. Coding regions are shown as hatched boxes, with the direction of transcription marked with arrows. Only the first intron of *nbs* is shown. M0 was used for *attP* targeting. In M2, serine residues at amino acid (aa) 266 (aa266) and aa268 were mutated to alanine. In M1, M3–5, protein regions deleted were marked with the starting and the ending residues. M40 carried a 40-bp *attB* site (*attB40*). The orientation of the *nbs* inserts are either identical to what is depicted in Fig. 1B or reversed. Frequencies for *vasa-phiC31*-mediated integration by microinjection are calculated as the percentage of fertile crosses with at least one pigmented progeny. The reduction frequencies were calculated as the number of independent reduction lines that retained the desired mutation divided by the total number of independent white-eyed lines established. –FLP, reduction frequencies without an FLP-mediated vector excision step; +FLP, with the FLP step; NA, not applicable; ND, not done.

soma, leading to mosaic eyes. Mosaic males were individually mated to white females to recover germ-line *white* loss events. With the exception of the Δ FHA mutation (M1), we were able to recover precisely reduced events that had the engineered mutations, although at frequencies that were generally lower than we expected (Fig. 2, the “–FLP” column). The reduced efficiency of *white* loss was evident in the soma, so that we observed a smaller degree of eye mosaicism than that found with reduction of similar *nbs* duplications during the gene targeting step (Fig. 1A). We used data obtained from M3 reduction to document this reduced efficiency in the male germ line. Previously, we observed that essentially all mosaic males gave rise to white-eyed progeny after germ-line induction of I-CreI, with an average of 36% of progeny showing white eyes and harboring precisely reduced events (9). For M3 reduction, only 77% (23/30) of the mosaic males gave rise to white-eyed progeny, and most male parents gave rise to less than 5% of the progeny with white eyes. In addition, we recovered many events in which part of the *white* gene was lost due to deletions possibly generated during nonhomologous end joining (NHEJ) repair of the I-CreI-induced DSB. In these “imprecise” events, both *nbs* copies were still present, as evidenced by Southern blot and PCR analyses (Fig. 3). These NHEJ events were recovered in previous experiments, but very rarely. However, for M3 reduction, 8/25 independent white-eyed events were NHEJ events. The other 17 events were precise, and 4 of them retained the Δ BRCT1 mutation.

Integration events mediated by phiC31 result in the entire plasmid being integrated, creating a tandem duplication of *nbs*. The structure of this duplication is slightly different from the one resulting from the integration of the *attP*-containing donor molecule during gene targeting (compare Fig. 1A and Fig. 1B). We hypothesized that the “extra” DNA (*FRTs*, P ends and vector backbone) had an adverse effect on the reduction efficiency, due either to the extra length that it added between the *nbs* copies or to a particular feature of the “extra” sequences. To directly test the effect of the “extra” sequences on reduction efficiency, we excised these sequences by FLP-mediated recombination between *FRTs*. This excision improved reduction efficiency in the soma, as evidenced by eyes with much greater degrees of

mosaicism, and in the germ line, as (i) all males gave rise to white-eyed progeny ($n > 100$), and (ii) each male generally had more white-eyed progeny. In addition, most if not all white-eyed flies were the result of precise reduction events. For M3 reduction after FLP-mediated vector excision, all 26 events were precise reduction, of which 3 retained Δ BRCT1. We repeated the reduction experiments for all mutations after having used FLP to excise the vector sequences, and recovered generally much improved frequencies for obtaining the desired mutations (Fig. 2, the “+FLP” column). We also attempted to induce reduction using the P transposase, but did not obtain higher reduction efficiency over the “–FLP” control (see *SI Results*).

All *nbs* point mutations support viability. However, all except the “SQ to AQ” mutation (in M2) impair fertility.

Deleting the *nbs* Locus. Deleting the entire coding region ensures a null allele of a gene. We designed a scheme to generate a gene deletion using SIRT, which is depicted in Fig. 4. We placed an *attB* site in the first intron of *nbs*, less than 50 bp away from the start codon (M7 in Fig. 2). The region to the left of *attB* corresponds to fragment A in Fig. 4A; the *nbs* coding region corresponds to fragment B; and the region to the right of *attP* in M0 corresponds to fragment C. We recovered integration events at a rate (7%) similar to that of other M constructs. We recovered four precise deletion events out of 46 reduction events without implementing an FLP-mediated vector excision step, and 7/30 with such a step. In addition, we also recovered events with a duplicated *nbs* locus (Fig. 4B). Therefore, SIRT can be used to make gene deletions and duplications. Animals homozygous for the *nbs* deletion die as pupae, similar to those with the previously identified *nbs*¹ mutation (10).

Discussion

Systematic mutational analysis is a powerful tool for dissecting gene function. In this study, we developed the SIRT method to make such analysis more efficient in the multicellular organism *Drosophila melanogaster*. A summary flow chart for carrying out SIRT mutagenesis is shown in Fig. S2.

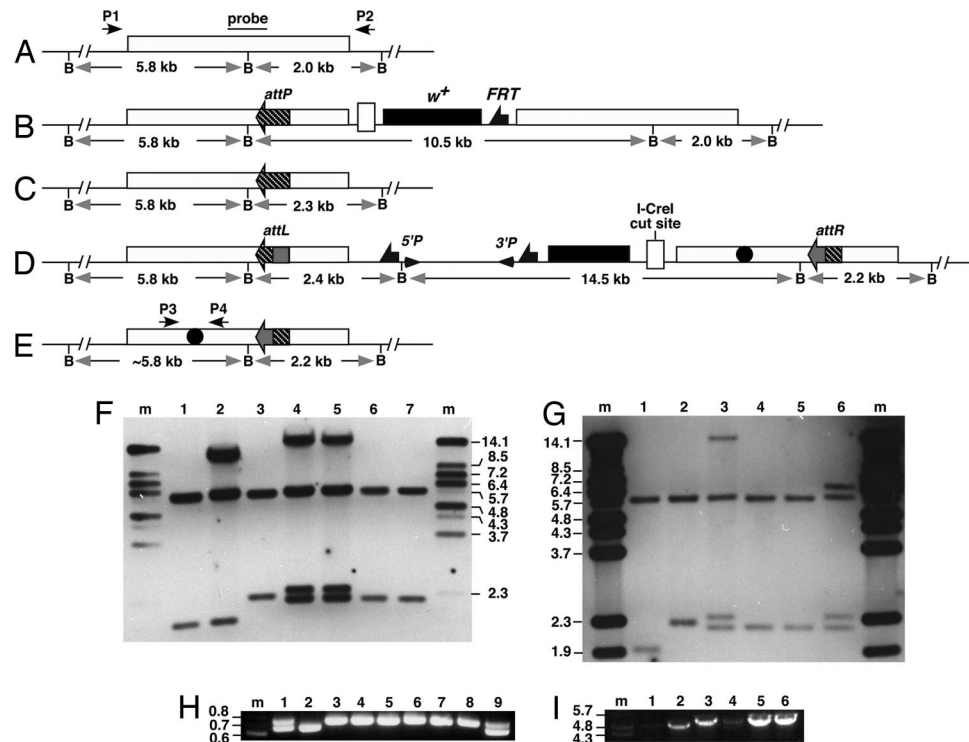


Fig. 3. Molecular analyses of products from various SIRT steps. (A–E) Genomic structures for DNA recovered at various steps of SIRT. The distance between the BamHI (B) sites is labeled. The *nbs* region used as targeting homology is shown as unfilled rectangular boxes. (A) The starting unmodified *nbs* region. Probe for Southern blotting used in F and G and primer pair (P1 and P2) for long-range PCR in I are indicated. (B) The *nbs* duplication after ends-in targeting for *attP* placement. (C) The *nbs* locus in line *nbs*⁴³⁴, a reduction product from B that carries *attP*. (D) The *nbs* duplication after phiC31-mediated integration. The 5' and 3' ends of P element are shown as arrowheads. An *nbs* mutation is shown as a filled circle. (E) The final SIRT product carrying the mutation and *attR*. Primer pair (P3 and P4) used in H are shown. (F) Southern blot analysis of M5. Lane m, markers in kb; lane 1, wild-type as in A; lane 2, *nbs* duplication as in B; lane 3, *nbs*⁴³⁴ as in C; lanes 4 and 5, two independent M5 integration lines as in D; lanes 6 and 7, two independent M5 reduction lines as in E. (G) Southern blot analysis of M3. Lane m, markers; lane 1, wild-type; lane 2, *nbs*⁴³⁴; lane 3, an M3 integration line as in D; lanes 4 and 5, two independent M3 reduction lines (the original 5.8-kb band is slightly smaller due to the deletion); lane 6, a white-eyed line resulted from NHEJ repair. The 14.5-kb mini-*white* band harbors a deletion about 8 kb in size (compare lanes 3 and 6). (H) PCR characterization of potential M3 reduction events without the FLP-mediated excision step. The top band was from the wild-type *nbs* copy, the lower one from the M3 mutant copy. Sample 1 is an M3 integration line as in D. Sample 9 retains both *nbs* copies. All of the other samples have a precisely reduced *nbs* locus. (I) Long PCR amplification of reduction events using P1 and P2. Lanes 1 and 2, wild-type control; lane 3, *nbs*⁴³⁴ (the product is slightly bigger than wild type due to the presence of V5 and *attP*); lanes 4–5, two independent final reduction events with the Δ *BRCT1* mutation (M3); lane 6, a final reduction event with the Δ *BRCT2* mutation (M5).

The Advantages of SIRT over Traditional Gene Targeting. SIRT greatly extends the utilities for the current gene targeting method in *Drosophila*. Under the current scheme (7), an allelic series for a gene has to be generated, with each modification carried out as independent targeting experiments, each of which could take several months to complete. For loci that are less susceptible to targeted mutagenesis, such an allelic series would be a daunting task. In SIRT, the initial rate-limiting step is placing an *att* attachment site close to the gene of interest by using standard targeting methods. All subsequent modifications are introduced by means of two highly efficient processes: phiC31-mediated site-specific integration and I-CreI-induced recombination between direct repeats, each of which are many orders of magnitude more efficient than integration mediated by the endogenous recombination machinery. We recovered integrants at frequencies in line with phiC31-mediated integration at other genomic sites (4, 11, 12), although at the lower end. This could be due to the particular *attP* position, or simply our inexperience in embryo injection (G.G. and C.M. had not performed embryo injection before this study).

During homologous recombination mediated targeting, imprecise integration events can be quite frequent for certain loci, generating unanticipated mutations at the target locus (7). Multiple independent events are generally needed to ensure the recovery of a precisely modified gene. On the other hand,

phiC31-mediated integration does not involve new DNA synthesis, thus preserving the integrity of both the donor plasmid and the target locus. For each of the six *nbs* mutations (M1–M5, M7) that we generated, we recovered multiple independent integration events that were structurally identical to each other (Fig. 3 and data not shown). Therefore, even a single integration event may be sufficient for subsequent reduction experiments.

In SIRT, the tandem duplication as the result of a phiC31-mediated integration is reduced by recombination induced by I-CreI cutting. Such recombination likely occurs via the single-strand annealing (SSA) mechanism (9, 13, 14). SSA involves extensive processing of DSBs into 3' overhangs, which is followed by annealing of single-stranded regions from both sides of the DSB. Reduction by SSA can be very efficient in the germ line. For a typical reduction reaction induced by the I-CreI endonuclease, >30% of the progeny would inherit a correctly reduced target locus (9). We observed lower reduction efficiency for the second reduction step in SIRT (Fig. 1B). This was due to the vector sequences present between the target copies as the result of phiC31 inserting the entire plasmid. Presumably, the “extra” sequence required more extensive processing, thus reducing the efficiency of SSA repair. Nevertheless, we were able to recover precise reduction events, although at reduced frequencies. Under unfavorable conditions, we recommend first eliminating the “extra” sequence by FLP-mediated excision.

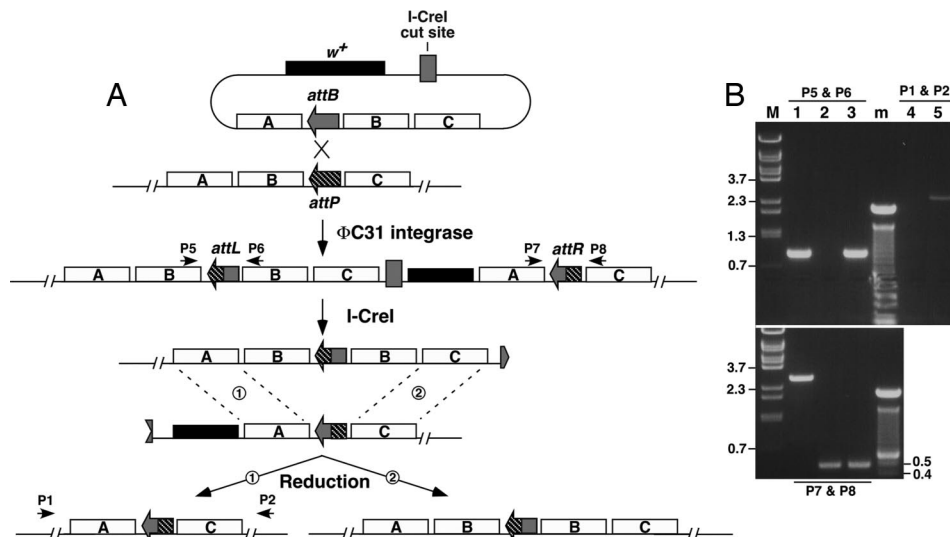


Fig. 4. Using SIRT to precisely delete a gene. (A) Fragment B denotes the gene to be deleted. Fragments A and C are flanking homology used to facilitate recombination. An *attP* placed between fragments B and C is first targeted to the chromosome. On the injected plasmid donor, an *attB* is placed between fragments A and B instead. After ϕ C31-mediated integration, a duplication of fragment B is generated to the left side of the marker gene while a deletion of fragment B is made to the right. During I-CreI-induced reduction, recombination between the two fragment A regions ("region 1") gives rise to a deletion (left), whereas recombination between the fragment C regions ("region 2") results in a duplication (right). The positions for primers P1, P2, P5–8 are labeled. (B) PCR analyses on *nbs* deletion and duplication events. Lane M: markers in kb; lane m, 100-bp ladder. Samples in lanes 1–3 were PCR amplified with P5, P6 (Upper) and P7, P8 (Lower) primer pairs. Samples in lanes 4 and 5 were PCR amplified with P1 and P2. Lane 1 was from a white-eyed fly heterozygous for an *nbs* duplication. The 2.9-kb product in Lower was from the wild-type *nbs* locus on the balancer chromosome. Lane 2 was from an *nbs* deletion heterozygote. Lane 3 was from a homozygous M7 integrant (red-eyed, before reduction). Lane 4 was from a duplication heterozygote, same sample as that in lane 1. No PCR product was expected due to the large size of the fragment. Lane 5 was a deletion heterozygote, same sample as that in lane 2.

SIRT is a multistep process that requires strategic placement of several DNA elements in the plasmid donor, i.e., the cut site for I-SceI, *att* sites, and point mutations. We developed a two-step recombining scheme so that different combinations of these elements could be used to modify a single donor vector, creating a series of very similar constructs for both the initial gene targeting and the subsequent SIRT manipulations (see Fig. S1 and SI Materials and Methods). The relatively large size of *att* sites currently in use (≈ 250 bp) could interfere with normal gene expression of the region. It is encouraging that 39-bp *att* sites can support efficient recombination in human cells (15). Bischof *et al.* (11) showed that a 54-bp *attP* supported recombination with a larger *attB*. Results from using our M40 construct suggest that a 40-bp *attB* can efficiently recombine with a larger *attP*. Therefore, a 40-bp *attB* and a 54-bp *attP* might be sufficient for efficient integration in *Drosophila*.

Additional Utilities of SIRT. Besides small modifications, e.g., point mutations and epitope tags, SIRT is also suitable for making gene deletions. This is most desirable for making a null allele of a gene. We generated a precise deletion of the *nbs* locus using a scheme (Fig. 4), which is similar to a previous study using ends-in targeting to make deletions (16). By changing the distance between the two *att* sites, one could recover deletions with different sizes.

The initial investment of targeting an *att* site renders the entire genomic region, not only the initial gene of interest, susceptible to SIRT-mediated manipulations. For example, one can use the *nbs*-proximal *attP* site to mutate the nearby *CG14174* gene. The size limit for the region that can be modified using SIRT is determined by the clonable size of the genomic fragment under investigation. With the P[acman] system recently developed for *Drosophila* that facilitates cloning and integration of large genomic fragments (17), it is reasonable to speculate that a region 100 kb or more next to a previously targeted *att* site could be susceptible to SIRT-mediated mutagenesis. If one integrates such a large genomic fragment by

ϕ C31-mediated integration, the duplicated target copies would share very extensive sequence homology that is likely to enhance subsequent reduction reactions.

All of the components of SIRT are likely to be universally functional. ϕ C31 integrase and several rare-cutting endonucleases have been introduced into several organisms, from bacteria to plants and mammals (18, 19). SSA repair seems to be operating in every system studied, a process that can be harnessed to reduce target duplications. Therefore, SIRT can be readily adapted for multicellular systems with an existing gene-targeting methodology, such as mouse ES cells. In addition, due to the versatility of the SIRT method, a collection of targeted *att* insertions genome-wide may be a valuable complement to the genome-wide knock-out lines currently under construction.

Materials and Methods

Plasmid construction. Primer sequences are listed in Table S1. Primers used for sequencing are not listed, and their positions and sequences are available upon request.

***nbs* targeting construct.** A 4.8-kb *nbs* genomic fragment was PCR amplified by using primers *nbs2057d* and *nbs6819u*, which added flanking KpnI sites, and cloned by using the TOPO-TA cloning kit from Invitrogen. An I-SceI cut site was cloned into a HindIII site within this fragment by using annealed oligos of I-site-H3plus and I-site-H3minus, with the HindIII site eliminated in the process. A V5 epitope tag was added to the AflIII site at the C terminus of *nbs* by using annealed oligos: *nbs-V5+* and *nbs-V5-*, with the AflIII site eliminated in the process. This KpnI fragment was cloned into the pTV2 targeting vector (9).

***nbs* mutant constructs.** Point mutations of *nbs* were introduced into the KpnI fragment in TOPO vector by using the Phusion site-directed mutagenesis kit from NEB. Primers *nbs5447d* and *nbs5398u* were used for Δ FHA (as in M1); *nbs4726d* and *nbs4725u* for SQ to AQ (in M2); *nbs5148d* and *nbs5147u* for Δ BRCT1 (in M3); *nbs5447d* and *nbs5147u* for Δ FHA-BRCT1 (in M4); and *nbs4630d* and *nbs4629u* for Δ BRCT2 (in M5). The entire *nbs* coding region was sequenced to verify that no other mutation was introduced. These constructs were cloned into pTV2 using the KpnI sites. The *attP* and *attB* sites were introduced by recombining (see SI Materials and Methods).

Stocks and Genetics. Ends-in targeting of *attP* using the M0 vector was performed according to published protocols (9), and I-CreI-induced reduction

crosses were also performed according to published protocols (9). I-Crel-induced reduction occurred in premeiotic germ cells so that multiple events from the same male parent could be identical events that had been amplified by germ cell mitoses. We followed a common practice of retaining only one event per male parent to ensure having independent events for subsequent experiments. Because these males were mated individually to three females, and individual males did not show systematic differences in fertility (we routinely recovered 100–150 progeny per male), the percentages of males with at least one of the desired product (Fig. 2) are likely a reasonable measure of the frequency at which different events of interest occurred. ϕ C31-mediated site-specific integration by microinjection was performed according to published protocols (4, 11). An X-linked *vasa- ϕ C31* line was kindly provided by Drs. J. Bischof and K. Basler (11). The constitutively active *70FLP10* line was used to excise plasmid vector before I-Crel-induced reduction (9). Flies with both an integrated M construct and *70FLP10* were heat shocked during early development at 38°C for 1 h.

Molecular Analyses. Southern blot analyses were performed by using the nonradioactive kit from Amersham Bioscience. DNA was cut with BamHI, AflII, and PstI enzymes individually. A 1-kb *nbs* probe (Fig. 3A) was PCR amplified with primers nbs2970d and nbs3942u. Another probe was amplified with primers nbs4496d and nbs5467u. The presence of the Δ FHA mutation in M1 was verified by PCR with primers nbs5467u and nbs5275d to produce a 200-bp product for wild-type samples and a 180-bp product for mutant samples. The presence of the SQ to AQ mutation in M2 was verified by allelic PCR with primers FR7 and nbs6968u for mutant samples, and FR6 and nbs6968u for

wild-type samples. Each pair gave rise to a 2.2-kb product. The presence of the Δ BRCT1 mutation in M3 was verified by PCR with primers nbs4933d and nbs5663u to produce a 730-bp product for wild-type samples and 650-bp for mutant samples. The presence of the Δ FHA-BRCT1 mutation in M4 was verified by PCR with primers nbs4933d and nbs5663u to produce a 730-bp product for wild-type samples and 356-bp for mutant samples. The presence of the Δ BRCT2 mutation in M5 was verified by PCR with primers nbs4535d and nbs4766u to produce a 230-bp product for wild-type and 200-bp for mutant samples. The primer pair nbs6998d and nbs1991u (P1 and P2, respectively, in Figs. 3A and 4A) lies outside of the *nbs* homology used for gene targeting. They were used to verify the 5-kb fragment from reduced lines. These primers were also used to verify the *nbs* deletion generated using the M7 construct. The deletion resulted in a 2.3-kb fragment. This product was sequenced to verify the deletion junction. Primers nbs2776d and nbs5663u were used to amplify the 500-bp deletion junction in M7 (P7 and P8 in Fig. 4A). Primers 3169u and nbs5275d were used to amplify the 900-bp duplication junction in M7 (P5 and P6 in Fig. 4A).

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