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Combining recombineering and ends-out homologous recombination to systematically characterize Drosophila gene families

Rab GTPases as a case study

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Evaluating how an individual gene contributes to a particular biological process benefits greatly from a comprehensive understanding of all members of its gene family. Such knowledge is ideally obtained using multicellular model organisms, which provide rapid and decisive platforms for determining gene function. We recently established a novel transgenesis platform in Drosophila to systematically knock out all members of the Rab small GTPase family of membrane regulators. This platform combines BAC transgenesis/recombineering with ends-out homologous recombinations and Gateway™ technologies and provides a new rapid and scalable method that eases the manipulation of endogenous loci. This method not only allows for the generation of molecularly defined lesions, but also the precise replacement or tagging of genes in their endogenous loci. Using this method, we found that up to half of all Rab GTPases exhibit enriched expression at synapses in the nervous system. Here we provide critical details about the underlying recombineering and transgenesis method, new cassettes for tagging endogenous loci and information on important parameters that will allow Drosophila researchers to target members of other gene families.

Keywords: Drosophila, homologous recombination, recombineering, Rab GTPase, gene targeting

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Homologous recombination techniques in Drosophila have hitherto been limited by difficult vector construction and inefficient in vivo recombination. To alleviate some of these difficulties, we engineered a new recombineering-based targeting vector. Recombineering has been widely used in mouse genetics to clone and manipulate large fragments of DNA.[1] Various selection-based systems allow one to easily insert or delete specific sequences at single base-pair resolution anywhere within a construct using recombination competent bacteria. Recombineering was recently adapted for Drosophila, through the use of the P[acman] vector and φC31-mediated transgenesis.[2,3] Historically it has been difficult to transform Drosophila with large pieces of DNA (> 30 kb). This has limited the length of homology arms in targeting vectors used for homologous recombination. However, the new P[acman] vector allows transformation with DNA fragments of up to 100 kb at predefined target locations within the Drosophila genome. We re-engineered the Drosophila recombineering plasmids by generating a P[acman] vector with FRT and I-Sce I sites necessary for ends-out homologous recombination[4] (P[acman]-KO 1.0[5]; **Fig. 1**). This new vector allowed us to create targeting vectors with homology arms that are several-fold larger than the PCR-generated arms previously used. Furthermore the vector allowed us to target endogenous loci for knock-in/out rather than being limited to predefined φC31 landing sites. We also incorporated a Gateway™ cloning cassette into this vector, allowing us to rapidly and efficiently introduce new genomic DNA into the system. This feature also makes the vector amenable to high-throughput (96 well) manipulations. In this article we discuss additional

cassettes and important considerations in the application of these methods in comparison with previously established ends-out homologous recombination techniques.

New Replacement and Tagging Cassettes for Recombineering

For our analysis of Rab GTPases, we exclusively used a Gal4 targeting cassette (**Figs. 1**, **2A**). Here we provide four additional cassettes: (1) a simple 3xP3 RFP/ Kan cassette for making molecularly defined deletions and (2) Hemagglutinin (HA), (3) FLAG and (4) Green Fluorescent Protein (GFP) cassettes for tagging gene products (**Fig. 2A**). These cassettes, along with the Gal4 cassette, all have the 3xP3 RFP/Kan sequences flanked by loxP sites, which allows for positive selection in both bacteria and in flies and has proved to be invaluable for a number of different reasons (see below). Moreover, this cassette can be removed in vivo using available Cre transgenic lines (Bloomington Stock Center: BL#1501[6]). The activity of Cre leaves behind a 34 bp scar that should be taken into account if one attempts to tag gene products at their N-termini. The GFP, HA and FLAG tagging cassettes share

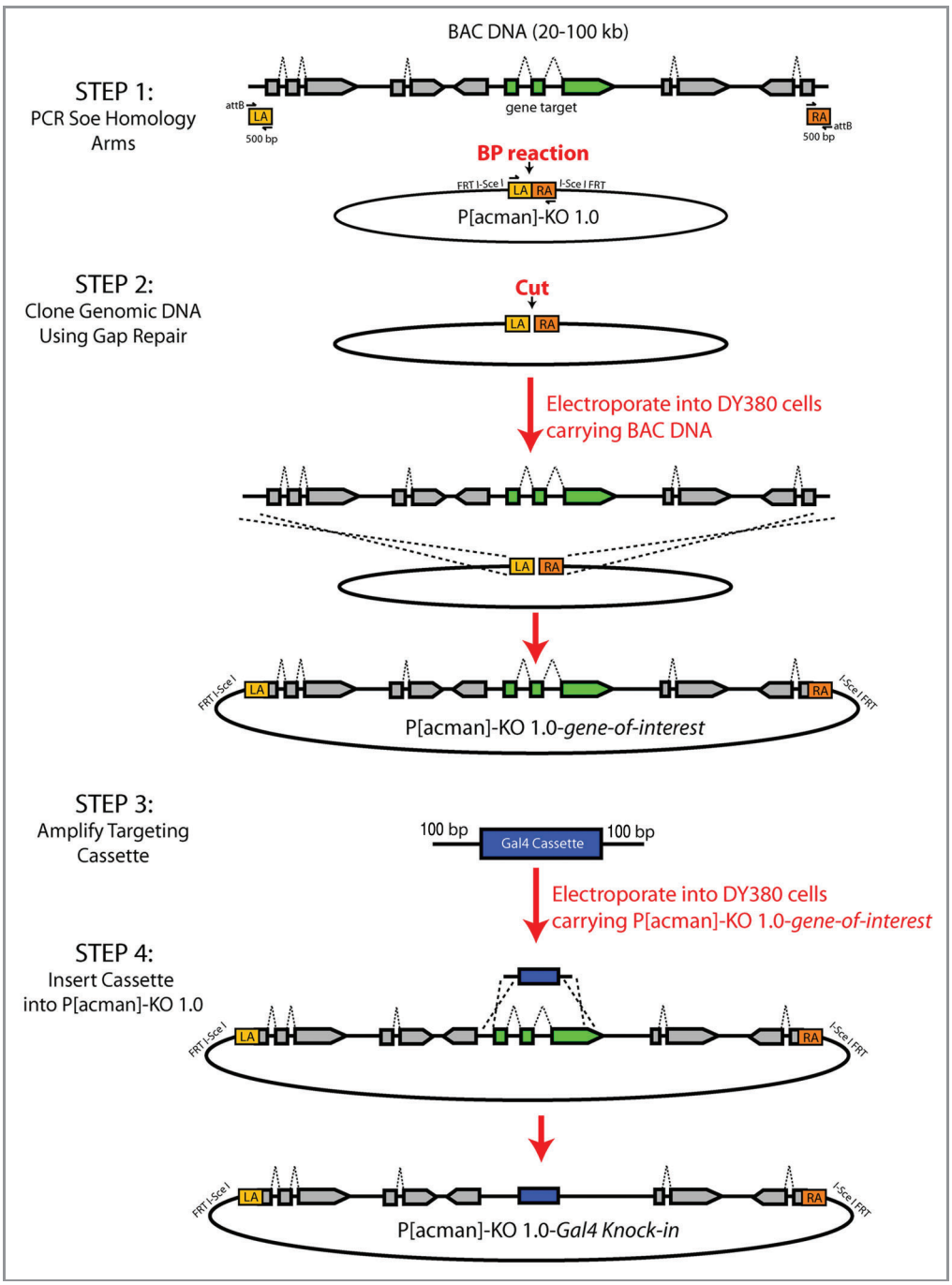

Figure 1. Outline of method for generating Drosophila targeting vectors using recombineering and P[acman]-KO 1.0.

common 5' and 3' sequences, allowing one set of locus specific primers to be used across the entire cassette collection (**Fig. 2B**).

Methodological Considerations for Recombineering and Transformation

Details on recombineering methods have been presented elsewhere.[1-3] Briefly, our protocol follows four basic steps for vector building (**Fig. 1**): (1) PCR amplify and PCR-SOE 500 bp left arm (LA) and right arm (RA) homology arms that flank both ends of the genomic region of interest together and clone them into the FRT/I-Sce I modified P[acman]-KO 1.0 vector. (2) Use gap repair within recombination competent bacteria (DY380) to clone the entire region of interest into P[acman]-KO 1.0. (3) Amplify the targeting cassette using oligos containing 100 bp of homology to either side of the gene being targeted. (4) Transform DY380 cells carrying the P[acman]-KO-*region of interest*-plasmid with the PCR amplified targeting cassette. We simplified the first recombineering step by incorporating gateway cloning sequence into our modified P[acman]-KO vector. When designing primers, 25 bp of attB sequence is included on the 5' end of the forward LA primer and the reverse RA primer. Including these attB sequences on either end of the PCR-SOE product allows one to clone this DNA into P[acman]-KO using a BP reaction (Invitrogen).

Incomplete digestion of the P[acman]-KO- *PCR-SOE product* plasmid used for gap repair in the first recombineering reaction (**Fig. 1**; Step 2) will result in a large number of false positives. Therefore we use high-fidelity enzymes and allow the digestion of the P[acman]-KO- *PCR-SOE product* to continue for at least three hours. While the number is variable from construct to construct, we typically obtain < 50 AMP/TET resistant DY380 colonies after the first recombineering reaction. A large number of AMP/TET resistant colonies after the gap repair reaction usually indicates the presence of the original unrecombineered P[acman]-KO *PCR-SOE product* plasmid.

When creating a targeting cassette specific for a particular gene, it is imperative to amplify from a completely linearized and purified cassette template. Attempting to PCR directly off the plasmid carrying a particular cassette often results in contamination of the DY380 cells with this plasmid. Kanamycin resistance conveyed by a contaminating plasmid results in a high number of false positive DY380 colonies after the second recombineering reaction (**Fig. 1**; Step 4). Because of this observation, we often run DNA from colonies obtained after Step 4 on an agarose gel to check for the presence of a low molecular weight high copy plasmid before transforming the final vector into EPI300 cells.

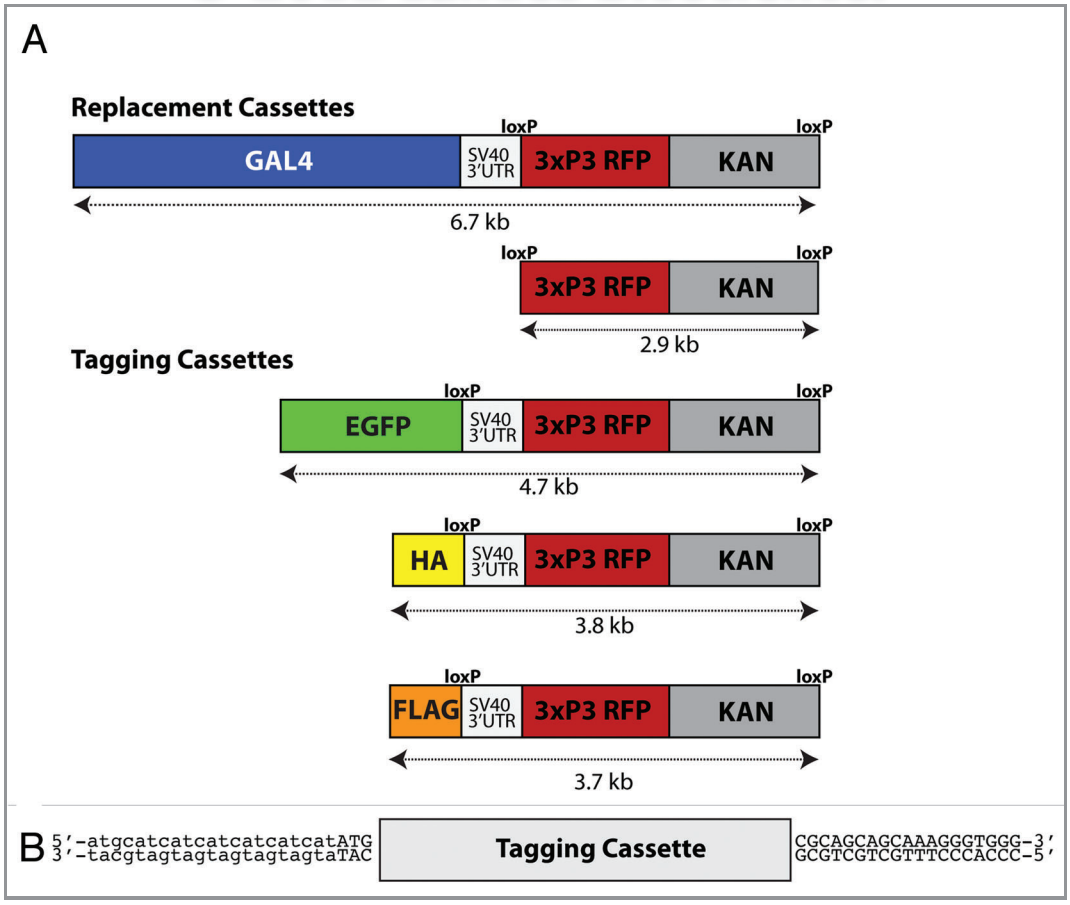

Figure 2. (A) Schematics for targeting and tagging cassettes. (B) Schematic showing the specific sequence flanking each of the specific tagging cassettes.

After sequence verification, correctly recombineered plasmids are transformed into EPI300 cells, amplified using copy control solution (Epicenter) and 'maxi'-prepped for injection as outlined in Chan et al. (2011).[5] Importantly, we found that freshly prepared DNA works best for transformation and therefore always performed the maxi-prep not more than one day before injection. Moreover, the large molecular weight DNA should be stored at 4°C and never frozen. Despite these steps we still find that transformation efficiencies tend to be low for P[acman] vectors over 50 kb (**Table 1**). Because of this low efficiency, we maximize the number of progeny obtained of every G0 fly. Single G0 males were crossed to 3–5 virgin females and single G0 females were crossed to 3 males and all these vials are tossed up to three times.

Gene Targeting of Endogenous Loci

Mobilization of the targeting cassette from the predefined insertion site in vivo is performed using existing transgenic sources of FLPase and I-Sce I (Bloomington stock #s: 6934 and 6935). This mobilization results in the targeting of the knock-in/out cassette to the endogenous locus. P[acman]-KO transformants are identified based on the presence of the *white+* gene contained within the backbone of the P[acman]-KO 1.0 vector. Through the course of our experiments we sometimes isolated transformed flies that expressed the *white+* marker but did not carry the *3xP3-RFP* gene contained within the Gal4 targeting cassette (**Table 1**). Furthermore, we isolated *white+, RFP+* and *white+, RFP-* flies from the same G0 parent. These observations suggested that the DNA used for transformation contained a mixture of P[acman]-KO plasmids: some that contained the *Gal4 3xP3-RFP* cassette and others that did not. We considered the possibility that the P[acman]-KO vector was kept at a low copy number in DY380 cells but not at single copy. Therefore it remained possible that the Gal4 cassette inserted in some fraction of the P[acman] plasmids within a particular cell but not all of them. If DNA isolated after the final recombineering reaction is indeed a mixture, simply diluting the DNA before transforming EPI300 cells should greatly reduce the probability that any resulting bacterial colony would contain both types of plasmids. Subsequent experiments showed that diluting DNA to the point where one obtains 1–50 EPI300 AMP/KAN resistant colonies on a 35 mm Petri dish after electroporation virtually eliminates false positive (*white+, RFP-*) Drosophila transformants. Therefore inclusion of the positive RFP+ marker within the targeting and tagging cassettes provides insurance that Drosophila transformants carry correctly recombineered vectors.

The key step in a homologous recombination experiment in Drosophila (and the main difference to mouse) is the mobilization of the targeting cassette from a defined site where it is already integrated in the genome. Hence, instead of injection into embryonic stem (ES) cells, the targeting cassette is mobilized by enzymatic excision in germ cells. Consequently, selection screening for a correct targeting event, i.e., insertion of the targeting cassette into the correct endogenous locus, has to occur in flies instead of cells. This process requires three steps: First, the donor DNA (targeting cassette) and transgenes encoding the enzymes needed to mobilize the cassette (*hs-Flp* and *hs-I-Sce I*) are crossed into the same genetic background. Heat shocking the larval progeny causes mobilization of the targeting cassette in the germline. Second, preliminary candidates are screened for mobilization of the targeting cassette away from its original site. This step is greatly facilitated if both the targeting cassette and the site from which it is mobilized are independently marked. Indeed, this feature constitutes a major difference between our technique and other recent efforts,[7,8] as outlined in detail below. The third step comprises mapping new insertions of the targeting cassette to the correct chromosome, followed by molecular and/or genetic verification. It is important to note that most insertions are not the desired clean homologous recombination event. Several modifications have been introduced to optimize this process, which we would like to compare with our recently developed method.

Major improvements to ends-out homologous recombination have recently been introduced by Huang et al. (2008, 2009).[7,8] First, a dominant and heat shock dependent cell lethal construct (hs-hid) present on both the Y and balancer chromosomes kill off all undesired progeny. Second, a dominant cell lethal construct (*UAS-reaper*) is fused behind the 3' homology arm of the targeting cassette. In a correct targeting event, this DNA fragment is deleted; however, in a faulty targeting event its presence leads to the death of the undesired offspring when driven by a neuronal or ubiquitous Gal4 driver. The latter improvement (*UAS-reaper*) has so far not been tested in conjunction with our technique,[5] and a combined method might substantially improve targeting efficiency. A further major difference between our approach and the method by Huang et al.[7,8] is the selection of so-called 'preliminary candidates'. Huang et al.[7,8] use *white+* as a positive selection marker of the targeting cassette but do not mark the site from which it is mobilized, leading to a difficulty in distinguishing failed mobilization

Table 1. Efficiency of *rab-gal4* vector transformation in Drosophila

Construct	$y^+w^+RFP^+$	$y^+w^+RFP^-$	Total Screened
rab5	0	3	~40,000
rab40	0	2	~40,000
rabX1	0	7	~40,000
rab2-ATG	6	2	~40,000
rab18	2	3	~40,000
rab3-ATG	3	0	~20,000
rab5	1	0	~20,000
rab7	1	0	~40,000
rab1-ATG	2	0	~40,000
rab2	2	0	~20,000
rab6	2	0	~40,000
rab11	1	0	~40,000

The DNA corresponding to *rab5, rab40, rabX1, rab2-ATG and rab18 gal4* vectors was not diluted prior to transformation into EPI300 cells, resulting in Drosophila transformants that did not carry the *gal4* targeting cassette. Diluting the DNA of remaining rabs prior to introducing the plasmid into EPI300 cells alleviated this problem so that all the subsequent Drosophila transformants carried the gal4 cassette. 400–500 embryos were injected in each case.

or mobilization and re-insertion based on *white*+ alone. In theory, offspring in which the targeting cassette fails to mobilize should be killed by the UAS-reaper dominant negative selection. Hence, all viable and *white*+ progeny should represent cases where the targeting cassette was mobilized and re-integrated without the *UAS-reaper* sequence. Curiously, only 10% of these potential candidates were on the targeted chromosome.[7] Since random re-insertion is predicted to result in a higher rate of insertion on the correct chromosome, the majority of these false positive 'preliminary candidates' may be from non-excision events. In contrast, our technique employs positive labeling of the targeting cassette with *3xP3-RFP* and *white*+, *yellow*+ as independent markers for the site from which the targeting cassette is mobilized. Selection for the targeting cassette (*RFP*+) and against the site from which it is mobilized (*white*- and *yellow*-) significantly reduces the number of preliminary candidates. For example, Huang et al. report[7] between 116 and 1700 preliminary candidates of which between 1 and 22 could be verified as correct targeting events. In contrast, our method leads to 20–100 preliminary candidates of which 80–90% are on the correct targeted chromosome. The accuracy of these targeting events depends on the homology region and was reported for one locus in Chan et al.[5] as 6 out of 32.

Closing Remarks

The recombineering methods outlined here and in our recent study[5] provide a means to manipulate the Drosophila genome with base-pair precision. Recombineering represents a powerful and scalable method for determining gene function and for exploring how locus dependent and independent parameters contribute to the efficiency of in vivo homologous recombination in Drosophila.

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