Enhanced Golic+: Gene targeting with 100% recovery in *Drosophila* male germ cells

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ABSTRACT

The efficiency of gene targeting can vary drastically. Even utilizing CRISPR/Cas9 does not ensure rapid, successful gene targeting. In *Drosophila*, we previously established Golic+ to augment gene-targeting productivity simply through fly pushing. This transgene-based system elicits gene targeting in germ cells. It further implements lethality selection to enrich for progeny with accurate gene targeting. However, limitations still remain. Here we deliver an improved Golic+ technique that we name Enhanced Golic+ (E-Golic+). E-Golic+ incorporates genetic modifications to eliminate false positives while simultaneously boosting efficiency. Strikingly, male germ cells are exceptionally susceptible to gene targeting using E-Golic+. With male founders, we easily achieve 100% recovery of correct gene targeting. Given the superior efficiency/specificity and relatively effortless scalability, E-Golic+ promises to triumph in any challenging and otherwise unattainable gene targeting projects in *Drosophila*.

KEY WORDS: *Drosophila*, Gene targeting, Homologous recombination, CRISPR, Male germline, Lethality-based selection

INTRODUCTION

41 The prokaryotic immune system, CRISPR/Cas9, has been successfully adopted for
42 genome editing in diverse species (Komor et al. 2017). An engineered, widely used
43 CRISPR/Cas9 system consists of two components: a single-molecule guide RNA
44 (gRNA) and the Cas9 DNA endonuclease (Jinek et al. 2012; Hwang et al. 2013). The
45 gRNA binds with Cas9 and directs Cas9 to produce double-strand DNA breaks in
46 specific DNA sequences determined by base pairing between the gRNA and a 20bp
47 DNA target. The only additional requirement in the DNA is the presence of
48 protospacer adjacent motif (PAM, canonically NGG) immediately following the 20bp
49 target sequence. One can therefore utilize CRISPR/Cas9 to target almost any
50 genomic region with extremely high selectivity. The resultant DNA breaks are often
51 repaired by non-homologous end joining (Lieber 2010), leading to deletions or (less
52 frequently) insertions until the target sequence is lost. Notably, the likely indel
53 profiles can be largely predicted based on local DNA sequences (Allen et al. 2018).
54 The simplicity, robustness and predictability of Cas9-induced indels have made
55 CRISPR as the most favored strategy for targeted gene disruption. Further, one can
56 try to edit the genome around the Cas9 cut site via homology directed repair (HDR)
57 of DNA breaks (San Filippo et al. 2008). With HDR, one can replace endogenous
58 sequences with some designer sequences by supplying an exogenous template
59 carrying the desired DNA sequence flanked by homology arms. Such tailored
60 genome modifications are versatile but can be difficult if not impossible to achieve
61 even with the CRISPR technology (Horlbeck et al. 2016; Isaac et al. 2016).
62

63 Gene targeting is context-dependent and offers little flexibility in the design. Some
64 gene-targeting experiments are intrinsically more challenging than others. For
65 instance, certain manipulations strive for deletion of a sizable defined DNA fragment
66 or insertion of a long DNA sequence at a specific nucleotide position. This can be
67 extremely challenging if suitable gRNA sites are not available. Moreover, it can be
68 difficult to obtain and insert long homology arms into an already lengthy donor
69 DNA. In addition, the engineered gene products (made through correct gene
70 targeting) may unexpectedly compromise organism viability even in heterozygous
71 conditions. To recover rare gene-targeting events in those challenging cases
72 requires (1) generation of numerous offspring, each with independent trials, and (2)
73 enrichment of offspring with correct gene targeting (especially those with decreased
74 viability) by selection against 'unperturbed' progeny.
75
76 Golic+ is a transgene-based gene targeting system designed to achieve the above
77 two objectives (Chen et al. 2015). First, it employs a *bam* promoter to confine gene
78 targeting to germ cells rather than germline stem cells (Chen and McKearin 2003;
79 Lehmann 2012). This theoretically guarantees independent gene targeting events in
80 individual offspring. Second, it carries two conditional toxic genes: one to eliminate
81 offspring that did not incorporate the donor DNA and the other to select against the
82 incorporation of donor DNA in off-target sites. These lethality-based selections
83 should therefore allow only offspring with correct gene targeting to survive into
84 adults. We envisioned that a low probability gene-targeting event would occur
85 eventually, and that assuming no recovery of false positives in Golic+, patience and

simple fly pushing would be all that is needed to ensure success. The induction of gene targeting in germ cells further eliminates the need for single-founder crosses, a practice to avoid recovery of clonally identical lines. The amount of fly pushing can therefore be greatly reduced. Thus, for complex editing of genes in their native environment, Golic+ is particularly affordable compared to embryo injections.

Nonetheless, since its debut in 2015, the original Golic+ failed to succeed at all gene-targeting experiments. We suspended several trials due to the inability to recover correct gene targeting events after proving many candidates as false positives. In this study, we deliver an enhanced Golic+ (E-Golic+) with (1) much more stringent lethality selections plus (2) superior gene targeting efficiency. Strikingly, the E-Golic+ acts much more potently in male than female germ cells. From male founders, we easily achieve a 100% success-rate with previously failed gene-targeting experiments. Only in the most challenging case did we detect any false positives. In this case, offspring with off-target integration were outnumbered two-fold by offspring with correct gene targeting. Therefore, for extremely intractable or large-scale gene targeting experiments, one can perform group crosses to drastically reduce the labor required for making numerous independent trials with minimal false-positive contamination. In conclusion, E-Golic+ guarantees successful gene targeting in *Drosophila*.

RESULTS

Enhanced Golic+ reduces false positives while boosting efficiency

With Golic+, we can readily expand fly crosses to increase independent gene-targeting trials. However, despite lethality-based selections, most gene targeting trials yielded a significant number of false positives; and some Golic+ crosses produced very few survivors in total. We therefore re-examined the Golic+ design for potential shortcomings. In Golic+, a minimal *bam* promoter, bamP(198) co-expresses Cas9, FLP, and I-SceI in female germ cells. Cas9 directed by gRNA makes specific DNA cuts in the target gene. FLP mediates formation of the circular donor DNA from a pre-integrated FRT cassette, and I-SceI subsequently linearizes the donor. Golic+ further employs three LexA-dependent transgenes, including one repressible and one non-repressible toxic gene as well as a repressor gene, for lethality-based progeny selection. The repressible toxic gene exists in two parts separated by an FRT cassette that contains 5' homology arm, the repressor gene, 3' homology arm, and the non-repressible toxic gene in sequence. Excision of the FRT cassette would automatically reconstitute the repressible toxic gene at the original integration site of the donor DNA. We can then render organism survival contingent upon re-integration of the repressor gene. However, the liberated donor DNA carries the repressor gene as well as a non-repressible toxic gene. Given only the repressor gene flanked by homology arms, HDR-mediated gene targeting would naturally segregate the repressor gene from the non-repressible toxic gene and

131 selectively place the repressor gene back to the genome. Thus, Golic+ permits
132 enrichment of correct gene targeting.
133
134 Given the dependence of all key enzymes on the *bam* promoter, we first wondered if
135 the strength of bamP(198) is a key limiting factor in the performance of Golic+. We
136 addressed this issue by trying bamP(898), a longer and presumably stronger *bam*
137 promoter (Chen and McKearin 2003). Notably, co-induction of Cas9, FLP, and I-SceI
138 by bamP(898) yielded many more survivors including false positives at even higher
139 ratios (Supplemental Table). The predominance of false positives overshadowed the
140 evidently more potent bamP(898). To improve the efficiency of Golic+ we need to
141 further identify and eliminate the source(s) of false positives.
142
143 We detected two categories of false positives. The first group consisted of escapers,
144 those without donor DNA incorporation. Errors in the donor DNA liberation step
145 resulted in defective reconstitution of the repressible toxicity gene (Fig. 1A).
146 Without a functional repressible toxic module, organism viability was no longer
147 coupled to genome incorporation of the donor DNA. To eliminate these escapers, we
148 need to ensure presence of an intact, repressible toxic gene ideally at the same
149 homologous site as the pre-integrated donor DNA. We met this requirement by
150 making and placing the 3xP3-RFP-marked *3XLexAop2-riTS-Rac^{V12}* transgene at the
151 same *attP* sites used for holding donor DNAs (Fig. 1B). This guarantees that all
152 3xP3-RFP-marked survivors carry an intact repressible toxic gene. Organism
153

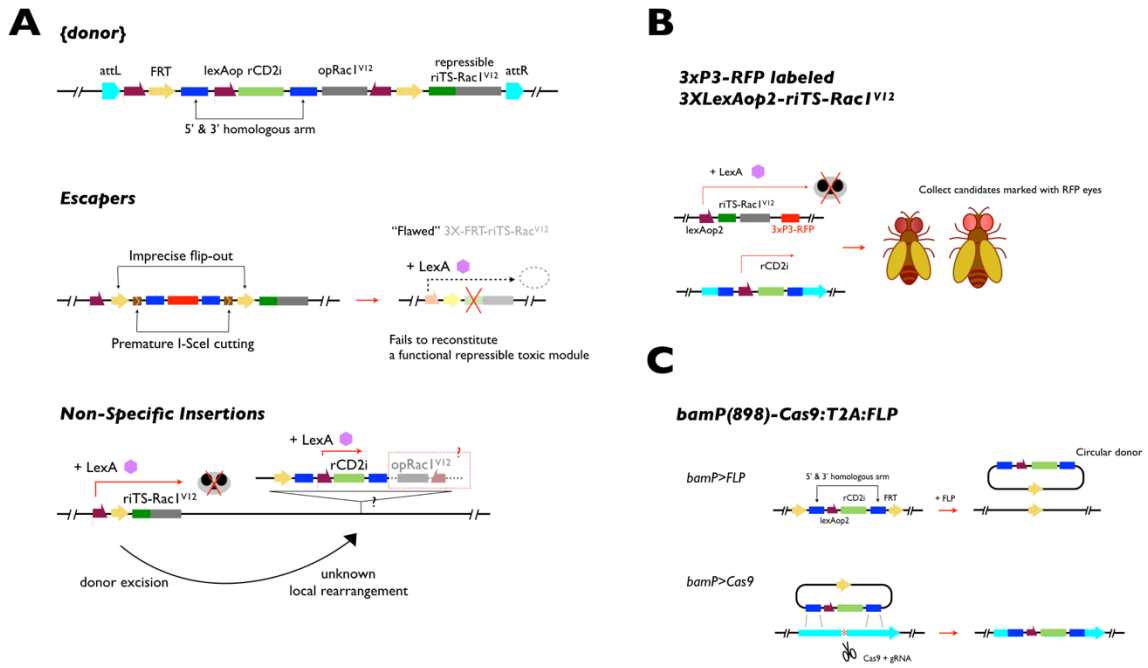

Figure 1. Improvements of E-Golic+ to remove two types of false positives, escapers and non-specific insertions. (A) In Golic+, {donor} was designed with two built-in toxicity modules and integrated in pre-characterized *attP* sites for efficient donor DNA release. We detected two false positive scenarios that produced progeny evading lethality selection. For escapers, they originated from failures in the reconstitution of a toxic module at the {donor} residual site, due to either imprecise flip-out or destructive premature I-SceI cutting. Therefore, they eclosed without ever being challenged by the lethality selection. For non-specific insertions, they retained the rCD2i suppressor and primarily relocated into the same chromosome. Yet they lost the ancillary non-repressible toxic module over this process, and survived the lethality selection without going through HDR. (B) We created 3xP3-RFP labeled *3XLexAop2-riTS-Rac1^{V12}* transgenic lines, and purposefully only collected surviving candidates marked with red fluorescent eyes. Hence, we effectively screened for candidates carrying the rCD2i suppressor, and avoid escapers completely. (C) Using *bamP(898)-Cas9:T2A:FLP*, we induced HDR in germ cells with CRISPR and circular donor DNA, hence directly relieved ourselves from the occurrence of non-specific insertions originating from linear donor DNA.

174 survival would therefore depend on relocation of the repressor-marked donor DNA
175 onto a different (hopefully the desired) chromosome.
176
177 The second group of false positives resulted from non-specific insertions of the
178 donor DNA. Per Golic+ design, HDR at the correct target site should segregate the
179 repressor and the non-repressible toxic gene, as they are separated by one of the
180 paired homology arms. By contrast, organisms with non-specific insertions should
181 retain the non-repressible toxic gene and fail to survive upon selection with some
182 broad LexA driver. However, the non-specific insertions we recovered had
183 somehow selectively lost the non-repressible toxic gene (Fig. 1A). While we do not
184 know how this occurred, we may be able to better preserve the integrity of the
185 liberated donor DNA by keeping it in a circular form. Further, linear DNA can
186 promote non-specific insertion and circular DNA is competent as a template for HDR
187 (Beumer et al. 2008). To this end, we made *bamP(898)-Cas9:T2A:FLP* that drives
188 only Cas9 and FLP, thus excluding I-SceI (Fig. 1C). We refer to Golic+ with
189 *bamP(898)-Cas9:T2A:FLP* plus 3xP3-RFP-marked *3XLexAop2-riTS-Rac*^{V12} as
190 Enhanced Golic+. Please see Table 1 for transgenes required for implementing
191 enhanced Golic+ and Figure 2 for representative targeting schemes.
192
193 We performed a direct comparison of Golic+ with E-Golic+ to see if we could
194 eliminate false positives and increase efficiency. Using enhanced Golic+, we
195 effectively eliminated all false positives observed in three previously failed Golic+
196 experiments (Fig. 3). We were further able to recover multiple correct gene-

197 **Table 1. List of transgenic lines required for implementing Enhanced Golic+**
198

Full Name	Abbreviation	Integration Site(s)	Notes
donor DNA plus gRNA in pTL2	{donor, gRNA}	attP40, VK00027	Needs to be created for each gene-targeting experiment.
GMR3-LexA::GADd	GMR3-LexA	attP40, VK00027	Cross with {donor, gRNA} injected adults to create rough eyes for {donor} transformant screening.
bamP(898)-Cas9-P2A-FLP	bam898-CF	su(Hw)attP8, attP2	Expresses Cas9 and FLP under bamP control to introduce DSB at the target locus and release donor DNA in every germ cell.
3XLexAop2-rCD2miRNATS#6-Rac1^{V12} (3xP3-RFP)	3X-riTS-Rac1^{V12}	attP40, VK00027	Together with {donor, gRNA}*, provides a homozygous suppressible "Toxic" background.
Residual {donor, gRNA}	{donor, gRNA}*		After donor release, it will reconstitute as a suppressible toxic module, 5X-FRT-riTS-Rac1^{V12}.
nSyb-LexA::p65	nSyb-LexA	attP16, VK00027	Induce larval/pupal lethality selection

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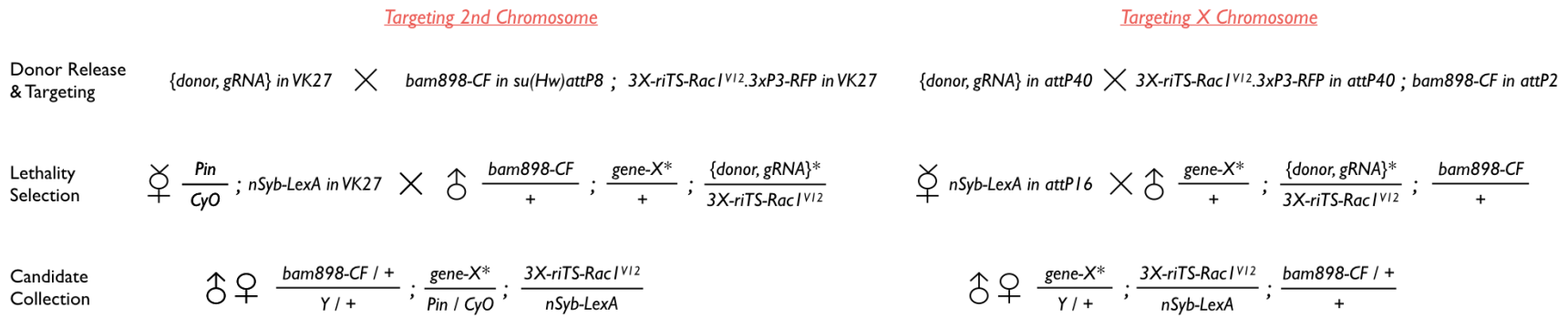

Figure 2. Targeting schemes for a second or an X chromosome gene. Like Golic+, E-Golic+ involves two crosses and three steps. In the first cross, we create founders that have active CRISPR reactions with circular donor for HDR in their germ cells. Then, founders are mated with *nSyb-LexA* so that each progeny will experience lethality selection, and most, if not all, of the 3xP3-RFP marked surviving candidates inherit gene targeting events marked with rCD2i suppressor.

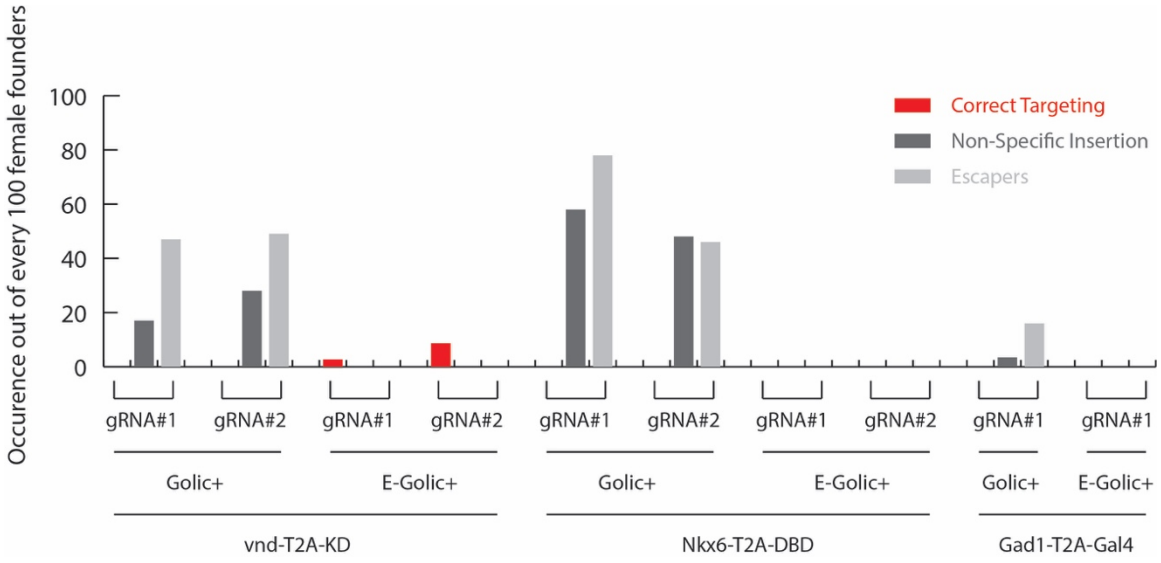

Figure 3. E-Golic+ effectively eliminates the occurrence of false positives. To evaluate the new transgenes introduced in E-Golic+, we performed gene targeting with five different donors using either Golic+ or E-Golic+. Occurrence of three different types of gene targeting candidates (correct targeting, non-specific insertion, and escapers) out of every 100 female founders are numbers interpolated or extrapolated from data in Table 2.

targeting events in one of the three genes we tested. These results substantiate the success in eliminating false positives with the newly introduced transgenes plus use of circular templates instead. However, two of the three repeated trials remained unsuccessful, demanding larger scales of fly pushing or higher gene targeting efficiencies.

Males make superior founders

One laborious step of performing E-Golic+ is the collection of copious virgin females to be founders. Conversely, using males as founders would significantly reduce the load of fly pushing when many founders are needed to obtain rare gene targeting events. Males should be able to be used as founders as *bam* shows comparable restricted expression in both male and female gonads (Fuller and Spradling 2007). Hence, use of *bamP898* in E-Golic+ should also effectively confine gene targeting to male germ cells. We therefore repeated all three gene-targeting experiments with E-Golic+ in male founders.

Using male founders, we readily recovered numerous correct gene-targeting offspring from each of the three gene-targeting trials (Table 2). None of these trials were successful with Golic+, and only one was successful with E-Golic+ using female founders. To make *vnd:T2A:KD*, we recovered 73 offspring with *vnd:T2A:KD* from a total of 200 male founders, as opposed to only 17 from a total of 300 female founders. In the engineering of *Nkx6:T2A:DBD*, we utilized two gRNA choices and

Table 2. Comparison of Golic+ and Enhanced Golic+

	# of Founders	Correct Targeting	Non-Specific Insertion	Escapers
Golic+	Female			
{vnd-T2A-KD, gRNA#1}	100	0	17	47
{vnd-T2A-KD, gRNA#2	100	0	28	49
E-Golic+	Female			
{vnd-T2A-KD, gRNA#1}	150	4	0	0
{vnd-T2A-KD, gRNA#2}	150	13	0	0
E-Golic+	Male			
{vnd-T2A-KD, gRNA#1}	100	49	0	0
{vnd-T2A-KD, gRNA#2}	100	24	0	0
Golic+	Female			
{Nkx6-T2A-DBD, gRNA#1}	50	0	29	39
{Nkx6-T2A-DBD, gRNA#2}	50	0	24	23
E-Golic+	Female			
{Nkx6-T2A-DBD, gRNA#1}	65	0	0	0
{Nkx6-T2A-DBD, gRNA#2}	120	0	0	0
E-Golic+	Male			
{Nkx6-T2A-DBD, gRNA#1}	75	16	0	0
{Nkx6-T2A-DBD, gRNA#2}	100	21	0	0
Golic+	Female			
{Gad1-T2A-Gal4, gRNA#1}	350	0	12	56
E-Golic+	Female			
{Gad1-T2A-Gal4, gRNA#1}	100	0	0	0
E-Golic+	Male			
{Gad1-T2A-Gal4, gRNA#1}	300	6	3	0

To examine the improvements made on E-Golic+, both Golic+ and E-Golic+ were applied to create *vnd-T2A-KD*, *Nkx6-T2A-DBD*, and *Gad1-T2A-Gal4* knock-ins. In the last *Gad1-T2A-Gal4* knock-in case, we only construct one {donor, gRNA} for targeting. Female founders were used in both Golic+ and E-Golic+ to reveal the benefits of adopting circular donor plus a 3xP3-RFP marked 3X-riTS-Rac1^{V12} toxicity module to avoid false positives. Additionally, for E-Golic+, male founders showed higher targeting efficiency, which helped us overcome the difficulties of knocking in DBD in *Nkx6* and Gal4 in *Gad1*.

obtained 37 offspring with *Nkx6:T2A:DBD* from a total of 175 male founders, but recovered none from a total of 185 female founders. In the third case, we aimed to insert Gal4 into *Gad1*, which encodes an enzyme characteristic of GABAergic neurons, to make *Gad1:T2A:Gal4*. Expressing GAL4 continuously in all GABAergic neurons could be harmful. In fact, an earlier study has reported challenges in maintaining an analogous fly stock generated through recombinase-mediated cassette exchange (Diao et al. 2015). Given the known challenges, we screened through progeny from 300 male founders and recovered six offspring with *Gad1:T2A:Gal4*. We validated the lines carrying *Gad1:T2A:Gal4* by genomic PCR, and further corroborated their Gal4 expression patterns highlighting GABAergic neurons in adult brains co-stained with anti-GABA antibody (Fig. 4). As expected, we found that *Gad1:T2A:Gal4* labeled several prominent groups of GABAergic neurons reported previously (Okada et al. 2009). We observed prominent labeling of R neurons that innervate the ellipsoid body (Fig. 4A1), neurons dorsal, ventral, and lateral to the antennal lobe neuropil (Fig. 4A2), neurons on the surface of Medulla (Fig. 4A3), and neurons at the interface between medulla and lobula plate in the posterior brain (Fig. 4A4). In addition to six correct gene-targeting lines carrying *Gad1:T2A:Gal4*, we found three false positives with non-specific insertions. It is unclear how a portion of circular DNA that contains the repressor became integrated into the genome in these flies. However, the drastic suppression of non-specific insertion in E-Golic+ is evident, indicating the importance of using extra-chromosomal circular DNAs as templates for HDR.

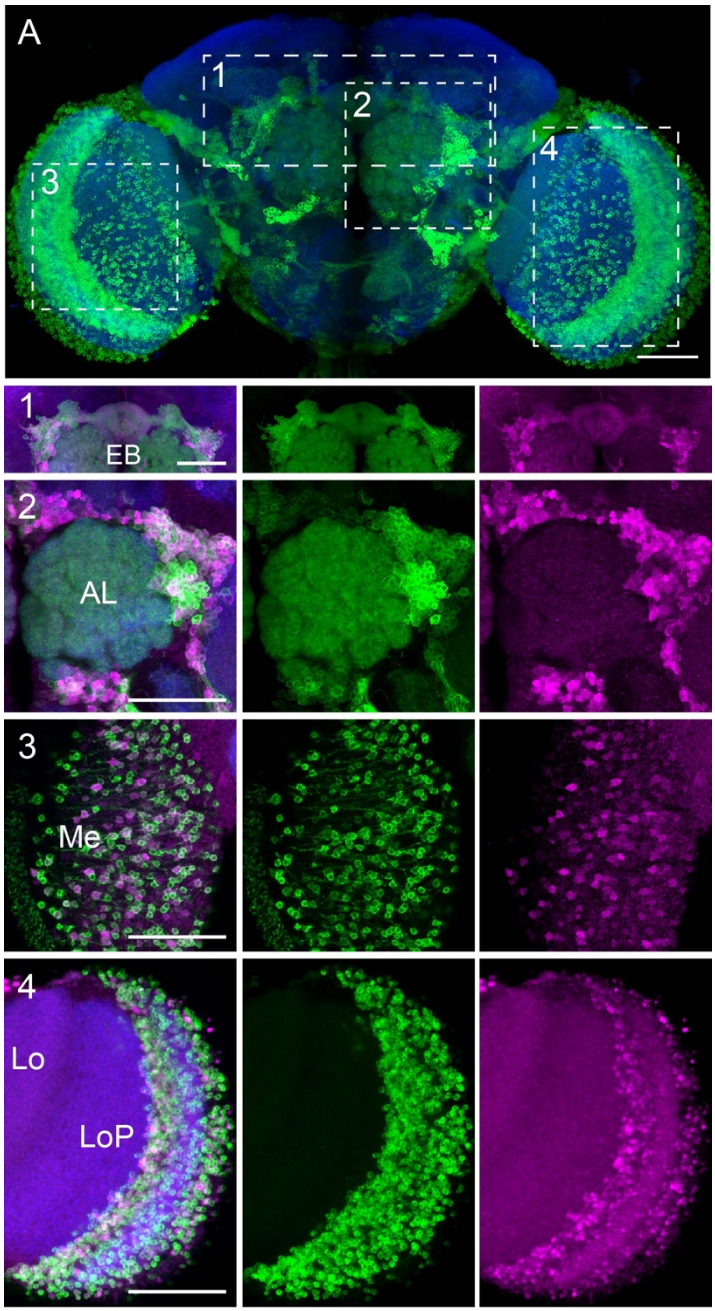

Figure. 4 The expression pattern of *Gad1:T2A:Gal4* in *Drosophila* central nervous system. (A) Composite confocal images of an adult fly brain with *Gad1:T2A:Gal4* driving a neuronal membrane marker (10XUAS-mCD8-GFP, green). The brain was counterstained with an anti-Bruchpilot protein antibody which specifically label presynaptic active zones (blue). Partial projections of the boxed regions in (A) were shown separately below together with anti-GABA staining (Magenta). 1: The Ellipsoid Body (EB) region; 2: The Antennal Lobe (AL) region; 3: The Medulla (Me) surface; 4: The interface between Medulla and Lobula Plate (LoP). Lo: Lobula. Scale bar, 50um in all panels.

In sum, the enhanced Golic+ is particularly powerful in the male germline. The lethality-based selections against false positives remain highly stringent. Moreover, the efficiency of gene targeting is greatly enhanced such that we could readily recuse all previously failed Golic+ experiments with E-Golic+.

DISCUSSION

Homology-dependent gene targeting allows designer genome editing, but suffers from unpredictable success even with modern CRISPR/Cas9 technology. By tackling previously failed gene-targeting trials, we show that the E-Golic+ reaches a 100% success in gene targeting experiments. There are two levels of enhancement. First, E-Golic+ offers absolute lethality selection to expedite the recovery of correct gene targeting. Second, E-Golic+ achieves an exceptionally high efficiency of gene targeting in male germline. E-Golic+ is probably the most sophisticated gene targeting system to date. It amplifies the power of *Drosophila* genetics and exemplifies how to access and modify the genomes of higher organisms.

Past studies have shown higher levels of gene targeting in female germline (Rong and Golic 2000), but more efficient targeted mutagenesis in male germline (Bibikova et al. 2002). Gene targeting depends on homologous recombination, while gene disruption occurs through non-homologous repair. Such mechanistic distinctions have promoted the idea that the lack of meiotic homologous recombination in

305 *Drosophila* male germline may underlie the previously published gender differences
306 in gene targeting versus gene disruption. However, our data suggest that male germ
307 cells are much more susceptible than female germ cells to Cas9-mediated genome
308 editing via HDR. These differences might result from repairs of double-strand DNA
309 breaks in germline stem cells versus germ cells. Interestingly, a recent paper
310 reported that CRISPR-induced DSBs can be repaired through recombination across
311 homologous chromosomes in germline stem cells (Brunner et al. 2019). We further
312 speculate that the homologous chromosomes in male germ cells might not be
313 intimately paired for recombination and thus individually more susceptible to
314 repairs by donor DNA. Nonetheless, we establish male germ cells as the top choice
315 for germline genome editing by E-Golic+ in *Drosophila*.
316
317 In our efforts to eliminate false positives, we confirmed that one could effectively
318 prevent off-target integration of the liberated donor DNA by keeping it in the intact
319 circular form. Once linearized, the donor DNA becomes prone to non-specific
320 insertion. Notably, the rate of non-specific insertion for linearized donor DNA varies
321 depending on the donor. Seemingly, there is an inverse correlation between the
322 non-specific insertion rate and the success rate of gene targeting. By contrast, it
323 appears that the off-target integration of circular donor DNAs remains persistently
324 suppressed regardless of actual gene-targeting efficiency. These phenomena
325 implicate differential fates for linear versus circular extra-chromosomal DNA,
326 further elucidation of which may help improve future gene targeting.
327

In sum, E-Golic+ in male germ cells has triumphed in previously failed gene-targeting experiments with a 100% success rate. Impressively, almost 100% of recovered candidates carried the desired genome modifications at the correct site. Moreover, to achieve really intractable gene targeting, one can readily continue the attempts by simple fly pushing. Given its unparalleled efficiency, specificity, and scalability, enhanced Golic+ in male germ cells promises to enable further sophisticated genome editing in *Drosophila* and beyond.

MATERIAL AND METHODS

Fly strains

Here are the fly strains used in this study: (1) *bamP(198)-Cas9:2A:FLP:2A:I-SceI* in *su(Hw)attP8* and *attP2*; (2) *bamP(898)-Cas9:2A:FLP:2A:I-SceI* in *su(Hw)attP8* and *attP2*; (3) *GMR3-LexA::GADd* in *attP40* and *VK00027*; (4) *nSyb-LexA::p65* in *attP16* and *VK00027*; (5) *bamP(898)-Cas9:2A:FLP* in *su(Hw)attP8* and *attP2*; (6) *3X-riTS-Rac1^{V12}(3xP3-RFP)* in *attP40* and *VK00027.*

Molecular biology

To create *vnd-T2A-KD*, *Nkx6-T2A-DBD*, and *Gad1-T2A-Gal4* knock-ins, 5' and 3' homology arms of approximately 1.5 kb in length and right before or after the *vnd*, *Nkx6*, and *Gad1* stop codons were amplified from genomic DNA and cloned into pTL2. Homology arms were further mutated to avoid CRISPR cutting on the

donor. The following CRISPR target sites were chosen: vnd_gRNA#1: GCATGGCCGTGCAGTAGACC; vnd_gRNA#2: GTTCCTCACCAGAACTGGAA; Nkx6_gRNA#1: GAAATTAAGTCTTCAGAAGA; Nkx6_gRNA#2: GCCATTTGGTGCGACGATTC; Gad1_gRNA#1: GCTACCAGCCCGACGATCGC. T2A-KD and T2A-DBD were introduced by cloning KD and DBD from pJFRC161-20XUAS-IVS-KD::PEST (Nern et al. 2011) and pBPZpGAL4DBDUw (Pfeiffer et al. 2010). The full *bam* promoter (-898) (Chen and McKearin 2003) was ordered from gBlocks, IDT to create bamP(898)-Cas9:2A:FLP:2A:I-SceI. Afterwards, coding sequence of Cas9:2A:FLP:2A:I-SceI was replaced by a PCR amplification of only the Cas9:2A:FLP portion to generate bamP(898)-Cas9:2A:FLP.

Fly genetics

{vnd-T2A-KD, gRNA#1}, {vnd-T2A-KD, gRNA#2}, {Nkx6-T2A-DBD, gRNA#1}, {Nkx6-T2A-DBD, gRNA#2}, and {Gad1-T2A-Gal4} were all integrated in attP40 to target *vnd* on the X chromosome, *Nkx6* and *Gad1* on the third chromosome. Transgenic *{donor, gRNA}* donors were mated with flies carrying *bam898-CF* and *3X-riTS-Rac1^{V12}(3xP3-RFP)* to create E-Golic+ founders. These founders were then crossed to *nSyb-LexA* flies for lethality selection. Finally, for E-Golic+, only Surviving candidates labeled with 3xP3-RFP were subjected to chromosomal mapping and genomic PCR confirmation.

Immunostaining and fluorescence microscopy

We dissected adult fly brains in ice-cold phosphate-buffered saline (PBS) and immediately transferred them into 4% paraformaldehyde for fixation at room temperature. After 30min fixation and three washes in PBS plus 0.5% Triton-X-100 (PBT), we added blocking solution (PBT with 4% Normal Goat Serum) and blocked the brains for 1 hour. Next, we transferred the brains into blocking solution containing primary antibodies and incubated at 4 °C overnight. After three 30-min wash in PBT, we added secondary antibodies in blocking solution and incubated for two days. Finally, after washing three additional times in PBT, we transferred the brains into PBS and mounted in SlowFade Gold Reagent on charged slides (Fisherbrand, 12-550-15).

Primary antibodies include: Chicken anti-GFP (1:1000; Life Technologies, A10262), Rabbit anti-GABA (1:25; Millipore Sigma, A2052), and mouse anti-nc82 (1:40; Developmental Studies Hydridoma Bank or DSHB). Secondary antibodies include: Alexa-488-conjugated goat antibody to chicken (1:500; ThermoFisher Scientific, A-11039), Cy3-conjugated goat antibody to Rabbit (1:200; Jackson ImmunoResearch, #111-165-144), and Cy5-conjugated goat antibody to mouse (1: 200; Jackson ImmunoResearch, #115-605-146).

We acquired image stacks of whole-mount fly brains using a Zeiss LSM 710 confocal microscope. The images were taken at 1um intervals at 1024x1024 pixel resolution using a 40X C-Apochromat water objective (NA=1.2). The images were further processed with Fiji and Adobe Photoshop.

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397

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404

405

406 ## Competing interests

407 The authors declare no competing interests.

408

409 ## Author contributions

410 H.-M.C. and T.L. conceived the project. H.-M.C. performed the experiments. X.Y. and
411 C.-C.C. generated E-Golic+ constructs. Q.R. analyzed the Gad1-T2A-Gal4 expression
412 patterns. L.-Y.L. assisted in E-Golic+ screening. H.-M.C. and T.L. wrote the
413 manuscript. T.L. supervised the project.

414

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Figure Legends

Figure 1. Improvements of E-Golic+ to remove two types of false positives, escapers and non-specific insertions. (A) In Golic+, {donor} was designed with two built-in toxicity modules and integrated in pre-characterized *attP* sites for efficient donor DNA release. We detected two false positive scenarios that produced progeny evading lethality selection. For escapers, they originated from failures in the reconstitution of a toxic module at the {donor} residual site, due to either imprecise flip-out or destructive premature I-SceI cutting. Therefore, they eclosed without ever being challenged by the lethality selection. For non-specific insertions, they retained the rCD2i suppressor and primarily relocated into the same chromosome. Yet they lost the ancillary non-repressible toxic module over this process, and survived the lethality selection without going through HDR. (B) We created 3xP3-RFP labeled *3XLexAop2-riTS-Rac1*^{V12} transgenic lines, and purposefully only collected surviving candidates marked with red fluorescent eyes. Hence, we effectively screened for candidates carrying the rCD2i suppressor, and avoid escapers completely. (C) Using *bamP(898)-Cas9:T2A:FLP*, we induced HDR in germ cells with CRISPR and circular donor DNA, hence directly relieved ourselves from the occurrence of non-specific insertions originating from linear donor DNA.

Figure 2. Targeting schemes for a second or an X chromosome gene. Like Golic+, E-Golic+ involves two crosses and three steps. In the first cross, we create founders that have active CRISPR reactions with circular donor for HDR in their

germ cells. Then, founders are mated with *nSyb-LexA* so that each progeny will experience lethality selection, and most, if not all, of the 3xP3-RFP marked surviving candidates inherit gene targeting events marked with rCD2i suppressor.

Figure 3. E-Golic+ effectively eliminates the occurrence of false positives. To evaluate the new transgenes introduced in E-Golic+, we performed gene targeting with five different donors using either Golic+ or E-Golic+. Occurrence of three different types of gene targeting candidates (correct targeting, non-specific insertion, and escapers) out of every 100 female founders are numbers interpolated or extrapolated from data in Table 2.

Figure. 4 The expression pattern of *Gad1:T2A:Gal4* in *Drosophila* central nervous system. (A) Composite confocal images of an adult fly brain with *Gad1:T2A:Gal4* driving a neuronal membrane marker (10XUAS-mCD8-GFP, green). The brain was counterstained with an anti-Bruchpilot protein antibody which specifically label presynaptic active zones (blue). Partial projections of the boxed regions in (A) were shown separately below together with anti-GABA staining (Magenta). 1: The Ellipsoid Body (EB) region; 2: The Antennal Lobe (AL) region; 3: The Medulla (Me) surface; 4: The interface between Medulla and Lobula Plate (LoP). Lo: Lobula. Scale bar, 50um in all panels.

Tables

Table 1. List of transgenic lines required for implementing Enhanced Golic+

Full Name	Abbreviation	Integration Site(s)	Notes
donor DNA plus gRNA in pTL2	{donor, gRNA}	attP40, VK00027	Needs to be created for each gene-targeting experiment.
GMR3-LexA::GADd	GMR3-LexA	attP40, VK00027	Cross with {donor, gRNA} injected adults to create rough eyes for {donor} transformant screening.
bamP(898)-Cas9-P2A-FLP	bam898-CF	su(Hw)attP8, attP2	Expresses Cas9 and FLP under bamP control to introduce DSB at the target locus and release donor DNA in every germ cell.
3XLexAop2-rCD2miRNATS#6-Rac1^{V12} (3xP3-RFP)	3X-riTS-Rac1^{V12}	attP40, VK00027	Together with {donor, gRNA}*, provides a homozygous suppressible "Toxic" background.
Residual {donor, gRNA}	{donor, gRNA}*		After donor release, it will reconstitute as a suppressible toxic module, 5X-FRT-riTS-Rac1^{V12}.
nSyb-LexA::p65	nSyb-LexA	attP16, VK00027	Induce larval/pupal lethality selection

Table 2. Comparison of Golic+ and Enhanced Golic+

	# of Founders	Correct Targeting	Non-Specific Insertion	Escapers
Golic+	Female			
{vnd-T2A-KD, gRNA#1}	100	0	17	47
{vnd-T2A-KD, gRNA#2	100	0	28	49
E-Golic+	Female			
{vnd-T2A-KD, gRNA#1}	150	4	0	0
{vnd-T2A-KD, gRNA#2}	150	13	0	0
E-Golic+	Male			
{vnd-T2A-KD, gRNA#1}	100	49	0	0
{vnd-T2A-KD, gRNA#2}	100	24	0	0
Golic+	Female			
{Nkx6-T2A-DBD, gRNA#1}	50	0	29	39
{Nkx6-T2A-DBD, gRNA#2}	50	0	24	23
E-Golic+	Female			
{Nkx6-T2A-DBD, gRNA#1}	65	0	0	0
{Nkx6-T2A-DBD, gRNA#2}	120	0	0	0
E-Golic+	Male			
{Nkx6-T2A-DBD, gRNA#1}	75	16	0	0
{Nkx6-T2A-DBD, gRNA#2}	100	21	0	0
Golic+	Female			
{Gad1-T2A-Gal4, gRNA#1}	350	0	12	56
E-Golic+	Female			
{Gad1-T2A-Gal4, gRNA#1}	100	0	0	0
E-Golic+	Male			
{Gad1-T2A-Gal4, gRNA#1}	300	6	3	0

To examine the improvements made on E-Golic+, both Golic+ and E-Golic+ were applied to create *vnd-T2A-KD*, *Nkx6-T2A-DBD*, and *Gad1-T2A-Gal4* knock-ins. In the last *Gad1-T2A-Gal4* knock-in case, we only construct one {donor, gRNA} for targeting. Female founders were used in both Golic+ and E-Golic+ to reveal the benefits of adopting circular donor plus a 3xP3-RFP marked 3X-riTS-Rac1^{V12} toxicity module to avoid false positives. Additionally, for E-Golic+, male founders showed higher targeting efficiency, which helped us overcome the difficulties of knocking in DBD in *Nkx6* and Gal4 in *Gad1*.

Supplemental Table. Golic+, comparing *bam198-CFI* and *bam898-CFI* with *{msh-T2A-Gal4 KI, gRNA}*

	# of Female Founders	Correct Targeting	Non-Specific Insertion	Escapers
bam198-CFI	95	47	8	15
bam898-CFI	30	37	53	5

Using a common donor *{msh-T2A-Gal4 KI, gRNA}*, we compare the performance of *bam198-CFI* and *bam898-CFI* in terms of the production of correct targeting, non-specific insertion, and escaper candidates.