

Enhanced Golic+: Highly effective CRISPR gene targeting and transgene HACKing in *Drosophila*

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ABSTRACT

Gene targeting is an incredibly valuable technique. Sometimes however, it can also be extremely challenging for various intrinsic reasons (e.g. low target accessibility or nature/extent of gene modification). To bypass these barriers, we designed a transgene-based system in *Drosophila* that increases the number of independent gene targeting events while at the same time enriching for correctly targeted progeny. Unfortunately, with particularly challenging gene targeting experiments, our original design yielded numerous false positives. Here we deliver a much-improved technique named Enhanced Golic+ (E-Golic+). E-Golic+ incorporates genetic modifications to tighten lethality-based selection while simultaneously boosting efficiency. With E-Golic+, we easily achieve previously unattainable gene targeting. Additionally, we built an E-Golic+ based, high-efficiency genetic pipeline for transgene swapping. We demonstrate its utility by transforming GAL4 enhancer-trap lines into tissue-specific Cas9-expressing lines. Given the superior efficiency, specificity and scalability, E-Golic+ promises to expedite development of additional sophisticated genetic/genomic tools in *Drosophila*.

KEY WORDS: *Drosophila*, Gene editing, Transgene swapping, Cas9, Male germline, Lethality-based selection

INTRODUCTION

The prokaryotic immune system, CRISPR/Cas9, has been successfully adopted for genome editing in diverse species (Komor et al., 2017). An engineered, widely used CRISPR/Cas9 system consists of two components: a single-molecule guide RNA (gRNA) and the Cas9 DNA endonuclease (Hwang et al., 2013; Jinek et al., 2012). The gRNA/Cas9 complex can cut specific DNA sequences determined by base pairing between the gRNA and a 20 bp DNA target next to a protospacer adjacent motif (PAM, canonically NGG). The resulting DNA breaks are subject to homology directed repair (HDR) (San Filippo et al., 2008). With HDR, one can replace endogenous sequences with designer sequences by supplying an exogenous template carrying the desired DNA sequence flanked by homology arms. Such tailored genome modifications are versatile but can be difficult if not impossible to achieve even with the CRISPR technology.

Gene targeting (GT) is context-dependent and offers little flexibility in the design. For example, certain manipulations strive for deletion of a sizable defined DNA fragment or insertion of a long DNA sequence at a specific nucleotide position. This can be extremely challenging if suitable gRNA sites (PAM requirement) are not available in the proximity. New Cas9 variants, such as xCas9 (Hu et al., 2018), can relax this requirement with less restrictive PAMs, but they often show reduced on-target editing efficiency in practice (Ni et al., 2020). In addition, it can be technically demanding to flank the already lengthy donor DNA with sufficiently long homology arms for efficient ends-out homologous

recombination (Beumer et al., 2013). Furthermore, some CRISPR GT experiments are intrinsically more challenging than others. For instance, gRNA target sites can become inaccessible to Cas9 binding due to nucleosome occupancy (Horlbeck et al., 2016; Isaac et al., 2016). Also, unsuspected gRNA secondary structure can impede CRISPR editing efficiency (Jensen et al., 2017; Thyme et al., 2016). Moreover, the engineered gene products (made through correct GT) may unexpectedly compromise organism viability even in heterozygous conditions. While no practical solutions exist for these intrinsic difficulties (heterochromatin, gRNA folding, etc.), we intend to overcome such impediments by mass production of GT trials and selection of rare GT events. We believe, to recover rare GT events in challenging cases requires (1) generation of numerous offspring, each with independent trials, and (2) enrichment of offspring with correct GT (especially those with decreased viability) by selection against unedited and incorrectly targeted progeny.

Transgenic CRISPR consistently shows higher targeting efficiency than embryonic injection (Bier et al., 2018; Kondo and Ueda, 2013), and further permits tissue-specific mutagenesis (Meltzer et al., 2019; Port et al., 2014) and gene drive technologies (Champer et al., 2019; Gantz and Bier, 2015). For example, Lin and Potter reported a higher rate of targeted insertion with a transgenic CRISPR setup (18.2%) than direct embryo injection (5.8%) (Lin and Potter, 2016). Moreover, a transgenic system can be optimized for best possible performance by refining individual transgenic components. This manuscript exemplifies the optimization paradigm; here we perfect a GT technique we name Enhanced-Golic+ (E-Golic+). Finally, transgenic systems can be fashioned for

large-scale genetic modifications with identical target and desired insertion, for example transgene modification using HACK (Lin and Potter, 2016).

Previously, we designed Golic+ as a transgenic CRISPR pipeline to recover rare GT events (Chen et al., 2015). First, Golic+ employs a *bam* promoter to elicit GT in germ cells rather than germline stem cells (Fig. 1A) (Chen and McKearin, 2003; Lehmann, 2012). This should guarantee independent GT events in individual offspring. Second, Golic+ carries two conditional toxic genes: one to eliminate offspring that did not incorporate the donor DNA and the other to select against the incorporation of donor DNA in off-target sites (Fig 1C,D). These lethality-based selections should in theory allow only offspring with correct GT to survive into adults. The assumption being that a low probability gene-targeting event will eventually occur, and that, patience and simple fly pushing would be all that is needed to ensure success. The induction of GT in germ cells further eliminates the need for single-founder crosses, a practice used with embryo injection experiments to avoid recovery of clonally identical lines. The amount of fly pushing is therefore greatly reduced. Thus, for complex editing of genes in their native environment, Golic+ is particularly affordable compared to embryo injections.

Despite some successes (Baumann et al., 2017; Koles et al., 2016) since its debut in 2015, the original Golic+ failed to succeed in the most difficult GT experiments. We suspended several trials due to our inability to recover correct GT events after determining numerous candidates to be false positives. In this study, we deliver E-Golic+ with much more stringent lethality selections plus superior GT efficiency. Strikingly, E-Golic+ acts

much more potently in male than female germ cells. Through male founders, we achieved previously unattainable gene targeting with ease. E-Golic+ is also suitable for HACKing preexisting transgenes (transgene editing based on (Lin and Potter, 2016)). Using a common pre-integrated DNA donor, we readily transformed various GAL4 enhancer-trap lines into tissue-specific Cas9 lines by fly pushing alone. In conclusion, E-Golic+ is a highly effective GT method in *Drosophila*.

RESULTS

Enhanced Golic+ reduces false positives while boosting efficiency

One major innovation of Golic+ was GT in the germ cell with *bamP*, rather than the germline stem cell. This design, referred to as the *bamP* pipeline (Fig. 1A) enabled us to set up large group crosses without fear of clonally related GT events. However, despite lethality-based selections (Fig. 1D), most GT 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+, Cas9, FLP, and I-SceI are co-expressed in female germ cells under a minimal *bam* promoter (*bamP(198)-Cas9-P2A-FLP-E2A-I-SceI*) (Fig. 1B). Directed by a gRNA, Cas9 makes a DNA double strand break in the target gene. FLP should mediate formation of a circular donor DNA from a pre-integrated FRT cassette, and I-SceI should subsequently linearizes the donor. Golic+ also employs three LexA-dependent transgenes for lethality-based progeny selection,

including a repressible and an irrepressible toxic gene as well as a repressor gene (Fig. 1C,D). The repressible toxic gene exists in two parts separated by an FRT cassette; after activation of FLP, the gene is reconstituted. The FRT cassette contains (in order) a 5' homology arm, the repressor gene, a 3' homology arm, and the irrepressible toxic gene. After excision of the FRT cassette, the organism's survival should be contingent upon re-integration of the repressor gene and subsequent repression of the repressible toxic gene. Only the DNA flanked by homology arms (the DNA fragment of interest being inserted and the repressor gene) should be integrated with HDR-mediated GT. Since the irrepressible toxic gene is outside the homology arms, it should not be integrated with correct HDR. As Golic+ should enrich for correct GT events, we wondered why there were cases with many survivors lacking correct GT (false positives).

Given the dependence of all key enzymes on the *bam* promoter, we first wondered if the strength of bamP(198) is a key factor limiting the performance of Golic+. We addressed this issue by trying bamP(898), a longer and presumably stronger *bam* promoter (Chen and McKearin, 2003). Notably, co-induction of Cas9, FLP, and I-SceI by bamP(898) yielded many more survivors. Unfortunately, the increased survivors included false positives at even higher ratios (Supplementary Table 1). We decided to use the evidently more potent bamP(898) in our future design. However, to improve the effectiveness of the technique we needed to identify and eliminate the source(s) of false positives.

We discovered two categories of false positives from Golic+ (Fig. 1E) (Chen et al., 2015). The first type of false positives results from non-specific insertions of the donor

DNA. In the Golic+ design, HDR at the correct target site should segregate the repressor and the irrepressible toxic gene, as they are separated by one of the paired homology arms. If this works correctly, organisms with non-specific insertions should retain the irrepressible toxic gene and fail to survive upon selection with LexA expression (Fig. 1D). However, the non-specific insertions we recovered had somehow selectively lost the irrepressible toxic gene (Fig. 1E, top). While we do not know how this occurred, we know that linear DNA can promote non-specific insertion and circular DNA is still competent as a template for HDR (Beumer et al., 2008). Thus, we should be able to better preserve the integrity of the flippase liberated donor DNA by keeping it in a circular form. To this end, we made *bamP(898)-Cas9-T2A-FLP* that drives only Cas9 and FLP, thus excluding I-SceI (Fig. 1F, top).

The second type of false positive consisted of escapers, those without donor DNA incorporation (Fig. 1E, bottom). Errors in the donor DNA liberation step (e.g. imprecise flip-out or premature I-SceI cutting) resulted in defective reconstitution of the repressible toxicity gene. We eliminated any issue of premature I-SceI cutting by eliminating I-SceI and making the donor circular (Fig. 1F, top), however imprecise flip-out may still distort the reconstitution of the repressible toxic gene. Without a functional repressible toxic module, organism viability is no longer coupled to genomic incorporation of the donor DNA. To eliminate these escapers, we need to ensure the presence of an intact, repressible toxic gene, ideally at the same homologous site as the pre-integrated donor DNA. We met this requirement by placing an added failsafe, the 3xP3-RFP-marked *3XLexAop2-riTS-Rac^{VI2}* transgene at the same *attP* site used to hold the donor DNA (Fig.

1F, bottom). This guarantees that, even if FLP recombination did not reconstitute the repressible toxic gene, we have a failsafe. All 3xP3-RFP-marked survivors carry an intact repressible toxic gene. Organism survival therefore depends on relocation of the repressor-marked donor DNA onto a different (hopefully the desired) chromosome.

We combined all of these solutions 1) the stronger bam promoter 2) the circular donor and 3) the added failsafe to our previous design to create Enhanced Golic+ (E-Golic+, summarized in Fig. 1B). Please see Supplementary Table 2 for transgenes required to implement E-Golic+ and Figure 2A for representative targeting schemes. We performed a direct comparison of Golic+ with E-Golic+ to see if we could eliminate false positives and increase efficiency. Using E-Golic+, we effectively eliminated virtually all false positives observed in three previously failed Golic+ experiments (Fig. 3A and Supplementary Table 3). Further, we were able to recover multiple correct gene-targeting events in one of the three challenging genes we tested. These results substantiate the success in eliminating false positives with the newly introduced transgenes and the use of circular donor templates. However, two of the three repeated trials were still unproductive. These intractable genes would thus demand either larger scale experiments or increased GT efficiency.

Males make superior founders

One laborious step of performing E-Golic+ is the collection of copious virgin females to serve as founders. Conversely, using males as founders would significantly reduce the

load of fly pushing when many founders are needed to obtain rare GT 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; Lehmann, 2012). Bam protein is expressed transiently in male 4-, 8-, and 16-cell cysts (Insko et al., 2009). Hence, use of *bamP898* in E-Golic+ should also effectively confine GT to male germ cells. We therefore repeated all three GT experiments with E-Golic+ in male founders.

Surprisingly, not only did male founders decrease the amount of labor needed to set up an E-Golic+ GT experiment, but male founders also increased GT efficiency. Using male founders, we readily recovered numerous correctly targeted offspring from each of the three GT trials (Fig. 3B and Supplementary Table 3). None of these trials were successful with Golic+, and only one was successful with E-Golic+ using female founders. To make *vnd-T2A-KD* (see donor design in Fig. 2C), we utilized two gRNA choices and 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. To engineer *Nkx6-T2A-DBD*, we obtained 37 offspring with *Nkx6-T2A-DBD* from a total of 175 male founders, after failing to recover any 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. Expressing GAL4 continuously in all GABAergic neurons could be harmful. In fact, an earlier study has reported challenges in maintaining a similar fly stock generated through recombinase-mediated cassette exchange (Diao et al., 2015). After gaining sufficient confidence in the performance of E-Golic+, we chose only one of the two established Golic+ *{Gad1-T2A-GAL4, gRNA}* donors to target *Gad1*. Given the known challenges,

we screened through progeny from 300 male founders and recovered six offspring with *Gad1-T2A-GAL4*. In addition to six correct gene-targeting lines carrying *Gad1-T2A-GAL4*, we found three false positives with non-specific insertions. With the success of this most difficult case we have tried, we believe future E-Golic+ users should feel comfortable using only one gRNA in their GT designs. Please refer to Figure 2 for details on how to carry out E-Golic+ gene targeting experiments.

Of the three genes we used E-Golic+ to modify, *vnd-T2A-KD* and *Nkx6-T2A-DBD* have since been exploited to study neurodevelopment. We utilized *vnd-T2A-KD* for lineage studies in the *Drosophila* central brain by genetic immortalization (Lee et al., 2019) to label a subset of the 18 Vnd lineages (unpublished data). Lacin and others validated the activity of *Nkx6-T2A-DBD* in the developing ventral ganglion (Lacin et al., 2019). Here we show one of the lines produced by E-Golic+, which inserts T2A-GAL4 downstream of *Gad1*. *Gad1* encodes the glutamic acid decarboxylase enzyme needed to produce the GABA neurotransmitter. We examine the GAL4 expression pattern, highlighting GABAergic neurons (anti-GABA immunostaining) in adult brains (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 pronounced 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).

Taken together, our data demonstrate that E-Golic+ in the male germline is a particularly powerful tool to achieve successful GT. We succeeded in targeting genes that before had seemed futile using other methods. The lethality-based selections against false positives are highly stringent, giving us confidence that each experiment will yield correctly targeted genes. Moreover, with male founders, the GT efficiency is greatly enhanced while the fly pushing labor is reduced. E-Golic+ requires the production of a new transgene *{donor, gRNA}* for each target to insert a DNA fragment of interest at a given gRNA directed locus (see Fig. 2B). With this consideration, we reflected on ways to expand E-Golic+ into an easily exploitable tool for the fly community. As there are tens of thousands of GAL4 transgenic fly lines readily available, we decided to demonstrate that E-Golic+ can transform fly lines containing GAL4 into other tissue specific transgenic lines.

E-Golic+ achieves high-efficiency transgene HACKing

HACK (homology assisted CRISPR knock-in) has pioneered CRISPR-assisted transgene hacking. However, even utilizing a collection of donor transgenes to reduce positional effects, HACK showed variable performance in the conversion from GAL4 to QF2 (Lin and Potter, 2016). Given that HACK acts through GT, we envision that E-Golic+ can deliver a simplified and extremely effective ‘HACK’ system. We also envision the need for diverse Cas9 drivers to facilitate tissue-specific genome editing. Combining these two visions, we established a high-efficiency pipeline to produce diverse Cas9 drivers through HACKing existing GAL4 transgenes.

Inspired by the method HACK, we built a GAL4-to-Cas9 donor by flanking a much larger T2A-Cas9 fragment (~6 kb, compared to 2.7 kb for T2A-QF2 in HACK) with the same 5' and 3' GAL4 homology arms as in HACK. We integrated the universal donor at the *attP40* and *VK00027* sites. We demonstrate its utility by converting three enhancer trap GAL4 lines, *repo-GAL4*, *gcm-GAL4* (both glia-GAL4s) (Awasaki et al., 2008) and *NP21-GAL4* (*fruitless*) (Kimura et al., 2005) into “enhancer trap” Cas9s. We recovered many candidates from each of the GAL4-to-Cas9 HACKing experiments (Fig. 5A). The candidates were molecularly confirmed by a common pair of primers (Fig. 5B) and their Cas9 activities were accessed using a Cas9-dependent CaSSA-GFP reporter (Garcia-Marques et al., 2019). CaSSA-GFP reports Cas9 activity in a given cell (or its precursor), as fluorescence is dependent upon CRISPR/Cas9 cutting and repair by single-strand annealing (Fig 5C). Both *repo-Cas9* and *gcm-Cas9* induced pan-glial CaSSA labeling (Awasaki et al., 2008), and *NP21-Cas9* generated a broad neuronal labeling that exhibits features characteristic of *fruitless*-expressing neurons (Jai et al., 2010).

In conclusion, the high-efficiency GT of E-Golic+ can be easily extended to modify existing transgenes. Here, we created the {*donor*, *gRNA*} transgene needed to transform existing GAL4 lines into tissue-specific Cas9-expressing genes. Furthermore, we generated and verified three such lines. Tissue specific Cas9 lines may accelerate CRISPR applications in *Drosophila*. This application eliminates the need for re-making donor transgenes and thoroughly demonstrates the power of E-Golic+ in genome editing through genetic pipelines.

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 the superiority of E-Golic+. There are two major enhancements. First, E-Golic+ offers a highly stringent lethality selection to expedite the recovery of correct GT. Second, E-Golic+ achieves an exceptionally high efficiency of GT in the male germline. E-Golic+ is probably the most complex fly GT system to date. However, the additional investment in time to produce the transgenic donor can be particularly rewarding when low efficiency is expected to target a known difficult genomic locus and/or to insert a sizeable DNA fragment. In return for the added labor/cost needed to produce the unique *{donor, gRNA}* transgenic flies, E-Golic+ promises relatively effortless delivery of any complex gene editing. E-Golic+ has thus greatly enhanced our ability to engineer the *Drosophila* genome for advanced research questions.

Besides guaranteed endogenous gene targeting, E-Golic+ can readily transform existing transgenes (such as the incredibly large GAL4 collections) into novel transgenes. In such applications, a universal *{donor, gRNA}* should be sufficient to target all transgenes with common homology arms, a much more economical way than repetitive embryo injections. Furthermore, in contrast to HACK, where donor transgenes needed to be

distributed throughout the fly genome to transform various GAL4 transgenes, the E-Golic+ *{donor, gRNA}* transgene can work efficiently at only two genome locations. This is the case even though the donor we integrated for T2A-Cas9 was even larger than the T2A-QF2 donor used with HACK. Furthermore, we recovered only correct GT alleles from 30-60% of E-Golic+ founder males in the creation of three distinct Cas9 drivers. These results substantiate the superior performance of E-Golic+ in fly genome editing, including modification of endogenous genes as well as pre-existing transgenes.

Additional technologies can be incorporated to further improve E-Golic+. Currently, a selectable or screenable marker is indispensable for sophisticated knock-ins with expected lower targeting efficiency. In E-Golic+, we employ the loxP/Cre system for subsequent removal of the rCD2i repressor/marker (see Figure 2C and Materials and Methods). Successful removal of rCD2i still leaves a residual loxP site in the target locus. If a residual “scar” poses a concern for a particular GT design, the current pTL2 backbone design is not ideal. E-Golic+ could be adapted as a scarless design, similar to TTAA/PiggyBac transposase (<http://flycrispr.molbio.wisc.edu/scarless>) or microhomology-assisted scarless genome editing (Kim et al., 2018). Although cloning the *{donor, gRNA}* vector is straightforward (Fig. 2B), generating transgenic lines takes 1-2 months from injection to establishing *{donor, gRNA}* stocks. To increase throughput, one can consider a barcoding strategy (Bischof et al., 2013) to inject pools of donor plasmids to generate multiple transgenes by a single injection.

Previous studies have shown higher levels of GT in the female germline (Rong and Golic, 2000), but more efficient targeted mutagenesis in the male germline (Bibikova et al., 2002). Gene targeting depends on homologous recombination, while gene disruption occurs through non-homologous repair. Such mechanistic distinctions had promoted the idea that the lack of meiotic homologous recombination in *Drosophila* male germline may underlie the previously published gender differences in GT versus gene disruption. However, our data suggest that male germ cells are much more susceptible than female germ cells to Cas9-mediated genome editing via HDR. This gender difference could not be simply explained by a production of more progeny (and thus more independent trials) per male than female founder, because a much higher CRISPR mutagenesis rate in male founders was also observed with direct screening of all progeny (Chen et al., 2019). In addition, a recent paper reported that CRISPR-induced DSBs can be repaired through recombination across homologous chromosomes in germline stem cells (Brunner et al., 2019). Further, it has been shown that homolog pairing coincides with germline differentiation in females during the pre-meiotic mitotic divisions (Joyce et al., 2013). Given these phenomena, we speculate that the homologous chromosomes in male germ cells might not be intimately paired for recombination and thus individually more susceptible to repairs by donor DNA. Regardless of the biological mechanism, it is clear that male germ cells are the top choice for *Drosophila* germline genome editing using E-Golic+.

In our efforts to eliminate false positives, we confirmed that one could effectively prevent off-target integration of the liberated donor DNA by keeping it in the intact circular form.

(Supplementary Table 3). Once linearized, the donor DNA becomes prone to non-specific insertion. Notably, the rate of non-specific insertion for linearized donor DNA varies depending on the donor. Seemingly, there is an inverse correlation between the non-specific insertion rate and the success rate of GT. By contrast, it appears that the off-target integration of circular donor DNA remains persistently suppressed regardless of GT efficiency. These phenomena implicate differential fates for linear versus circular extra-chromosomal DNA, further elucidation of which may help improve future GT techniques.

In sum, E-Golic+ in male germ cells has succeeded in previously failed GT experiments. Impressively, nearly every recovered candidate carried the desired genome modifications at the correct sites. With straightforward cloning, two rounds of crosses, and easy screening, E-Golic+ can routinely deliver the desired gene editing results without delay. Moreover, to achieve really intractable GT, one can readily continue the attempts by simple fly pushing. Given its unparalleled efficiency, specificity, and scalability, we are confident that E-Golic+ will enable further sophisticated genome editing in *Drosophila* and beyond.

MATERIALS AND METHODS

Fly strains

The following fly strains were used in this study: (1) *bamP(198)-Cas9:2A:FLP:2A:I-SceI* in *su(Hw)attP8* and *attP2* (Chen et al., 2015); (2) *bamP(898)-Cas9:2A:FLP:2A:I-SceI* in *su(Hw)attP8* and *attP2* (this study); (3) *GMR3-LexA::GADd* in *attP40* and *VK00027* (Chen et al., 2015); (4) *nSyb-LexA::p65* in *attP16* and *VK00027*; (5) *bamP(898)-Cas9:2A:FLP* in *su(Hw)attP8* and *attP2* (this study); (6) *3X-riTS-RacI^{V12}(3xP3-RFP)* in *attP40* and *VK00027* (this study); (7) *repo-GAL4* (BDSC #7415) , (8) *gcm-GAL4* (Paladi and Tepass, 2004), (9) *NP21-GAL4* (BDSC #30027), and (10) *Actin5Cp4.6-5'GFP-#3-3'GFP/CyO; MKRS/TM6B* and *Sp/CyO; J28-dU6-3-gRNA(target#3)/TM6B* (Garcia-Marques et al., 2019). We plan to deposit fly strains required for implementing E-Golic+ to Bloomington *Drosophila* Stock Center.

Molecular biology

{Donor, gRNA} construction:

We selected gRNA sites within the proximity of the stop codons (vnd gRNA#1 being the farthest one, 327 bp) using DRSC Find CRISPRs (<https://www.flyrnai.org/crispr>). gRNA spacers were placed between the dU6 promoter and gRNA scaffold of pTL2 by annealing two complementary oligos to create TCG and AAG overhangs for ligation with SapI-digested pTL2. 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;
gRNA-GtoC: GATGGATCGACCATAAAGCA.

5' and 3' homology arms of approximately 1.5 kb in length and right before and after the *vnd*, *Nkx6*, and *Gad1* stop codons were PCR amplified from genomic DNA. To create the common "GAL4 to Cas9" conversion donor, the HACK GAL4 homology arms (1.2 and 1.4 kb) were used (Lin and Potter, 2016). Homology arms were cloned into pTL2 (Fig. 2B) using 5'MCS and 3'MCS (Primer sequences can be found in Supplementary Table 4). pTL2 will be deposited to Addgene. Within the homology arms, the sequences corresponding to the gRNA target sites were mutated (QuikChange XL Site-Directed Mutagenesis Kit, Agilent) to avoid CRISPR cutting of the donor. Nucleic acid sequences were modified without changing amino acid sequences (silent mutations) or 3'UTR nucleotides conserved between *Drosophila* species.

T2A-effectors were introduced by amplifying effector coding sequence (CDS) with long 5' primer containing T2A sequences. 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). Cas9 CDS was cloned from bamP(898)-Cas9 (Chen et al., 2019), and a synthesized GAL4-T2A-Cas9 fragment (GenScript) was ordered to link the CDSs of GAL4 and Cas9.

bam898-CF generation:

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, the CDS of Cas9:2A:FLP:2A:I-SceI was replaced by a PCR amplificon of only the Cas9:2A:FLP portion to generate bamP(898)-Cas9:2A:FLP (bam898-CF).

GT candidate confirmation:

GT candidates were confirmed by genomic PCR with one primer located upstream or downstream of the homology arms, and the other one positioned within the CDS of the effectors. Primer information is summarized in Supplementary Table 4. Correct GT events place the effector CDS perfectly, which lead to successful PCR amplification and products of predicted sizes.

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, gRNA} were all integrated in *attP40* to target *vnd* on the X chromosome, *Nkx6* and *Gad1* on the third chromosome. For GAL4 to Cas9 conversion, {GAL4toCas9, gRNA-GtoC} was integrated in both *attP40* and *VK00027*. To recover transgenic flies, we raised {donor, gRNA}-injected larvae (Rainbow Transgenic Flies) at room temperature, crossed the eclosed adults with *GMR-LexA::GADD*, and searched for rough or bar eye progeny as successful transformants.

Transgenic donors were mated with flies carrying either *bam198-CFI* to create Golic+ founders or *bam898-CF* and *3X-riTS-Rac1^{VI2}(3xP3-RFP)* to create E-Golic+ founders. For GAL4 to Cas9 conversion, *repo-GAL4*, *gcm-GAL4*, and *NP21-GAL4* (all *white⁺*) were first crossed to corresponding *bam898-CF* plus *3X-riTS-Rac1^{VI2}(3xP3-RFP)* stocks, and then the progeny were mated with *{GAL4toCas9, gRNA-GtoC}* donors to create male founders (*white⁺* and 3xP3-RFP⁺). For lethality selection, we crossed young founders (age 3-7 days) to *nSyb-LexA* of similar ages, collected and screened their progeny continuously until the females stopped producing eggs. Vials were examined daily for eclosed survivors. For E-Golic+, survivors were checked for RFP eye fluorescence. ONLY 3xP3-RFP⁺ GT candidates were maintained for propagation, and genomic PCR confirmation.

The loxP-flanked *lexAop-rCD2i* repressor can be removed by crossing successful knock-ins to a transgenic Cre (*y^lw^{67c23}P{Crey}1b; sna^{ScO}/CyO*, BDSC #766). However, out of the three GT cases, we only managed to remove the *lexAop-rCD2i* cassette for *vnd-T2A-KD* with this strategy.

Immunostaining and fluorescence microscopy

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 Hybridoma Bank or DSHB). Secondary antibodies include: Alexa-488-conjugated goat anti chicken (1:500; ThermoFisher Scientific, A-11039),

Cy3-conjugated goat anti Rabbit (1:200; Jackson ImmunoResearch, #111-165-144), and Cy5-conjugated goat anti mouse (1: 200; Jackson ImmunoResearch, #115-605-146).

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).

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

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Competing interests

The authors declare no competing interests.

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Author contributions

H.-M.C. and T.L. conceived the project. H.-M.C. performed the experiments. X.Y. and C.-C.C. generated E-Golic+ constructs. Q.R. analyzed the Gad1-T2A-GAL4 expression patterns. L.-Y.L. assisted in E-Golic+ screening. H.-M.C., R.L.M., and T.L. wrote the manuscript. T.L. supervised the project.

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Figures

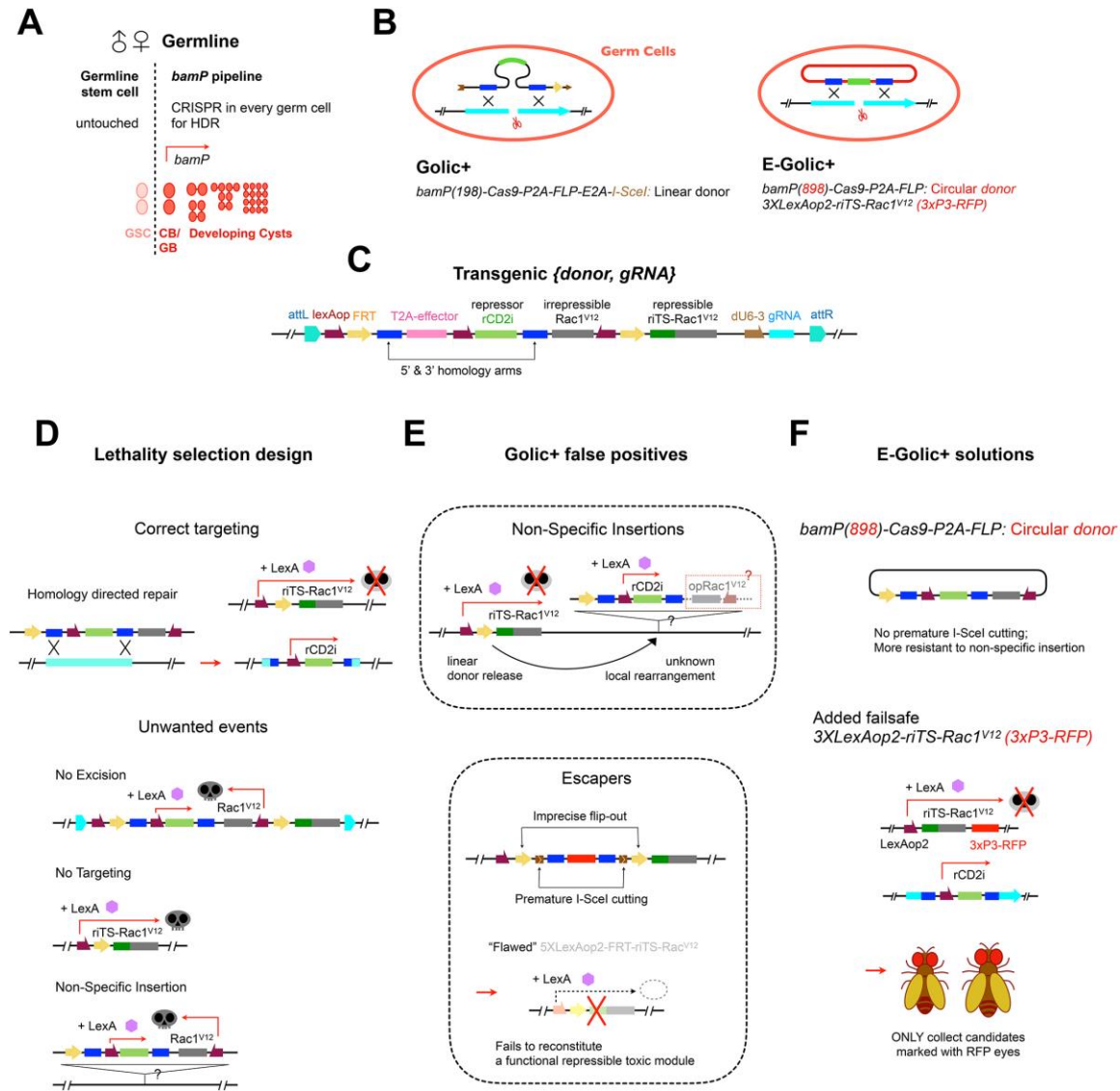


Figure 1. Principles and improvements of E-Golic+. (A) Golic+ and E-Golic+ are transgenic CRISPR pipelines for knock-in gene targeting. The *bamP* expression profile promotes release of donor DNA and successful HDR in cystoblast (CB), gonialblast (GB), and developing cysts. (B) Overview comparing Golic+ and E-Golic+. E-Golic+

utilizes a stronger bam promoter (898) to drive Cas9 and FLP. The donor changed from linear with Golic+ to circular with E-Golic+. A failsafe inducible toxic transgene (3xP3-RFP labeled *3XLexAop2-riTS-Rac1^{V12}*) is added to E-Golic+. (C) Transgenic {donor, gRNA} design is identical for Golic+ and E-Golic+. The transgene contains U6-gRNA (cyan), the desired DNA insert (T2A-effector, pink) and three genes for lethality selection 1) an irrepressible toxic gene (*LexAop2-Rac1^{V12}*, grey) 2) a repressible toxic gene (*LexAop2<Flip-out<-riTS- Rac1^{V12}*; green/grey) and 3) the inducible repressor (*lexAop-rCD2i*; green). After FRT recombination 1) donor DNA is released and 2) the repressible toxic gene is reconstituted. (D) Lethality selection design. Top) If targeting is correct, the irrepressible toxic gene is lost and LexA (pink hexagon) drives expression of the repressor, which represses the repressible toxic gene. The organism survives. Bottom) If targeting is incorrect LexA will drive expression of one of the toxic genes and the organism dies. (E) Failure of lethality selection scheme found with Golic+. Top) Non-specific insertions (primarily onto the same chromosome) retain the rCD2i repressor. The irrepressible toxic module is lost, organisms survive the lethality selection without HDR. Bottom) Escapers originate from failures in reconstitution of the repressible toxic gene due to either imprecise FLP-out or destructive premature I-SceI cutting. Therefore, they are not challenged by lethality selection. F) E-Golic+ solutions. 1) The bamP(898) promoter drives stronger Cas9 and FLP expression promoting more gene targeting events. 2) Keeping the donor circular (removing I-SceI cutting design) eliminates non-specific insertions and the source of some escapers. 3) An added failsafe of a second copy of the repressible toxic gene (*3XLexAop2-riTS- Rac1^{V12}*; green and grey) marked by red eye fluorescence (3xP3-RFP, red) eliminates escapers.

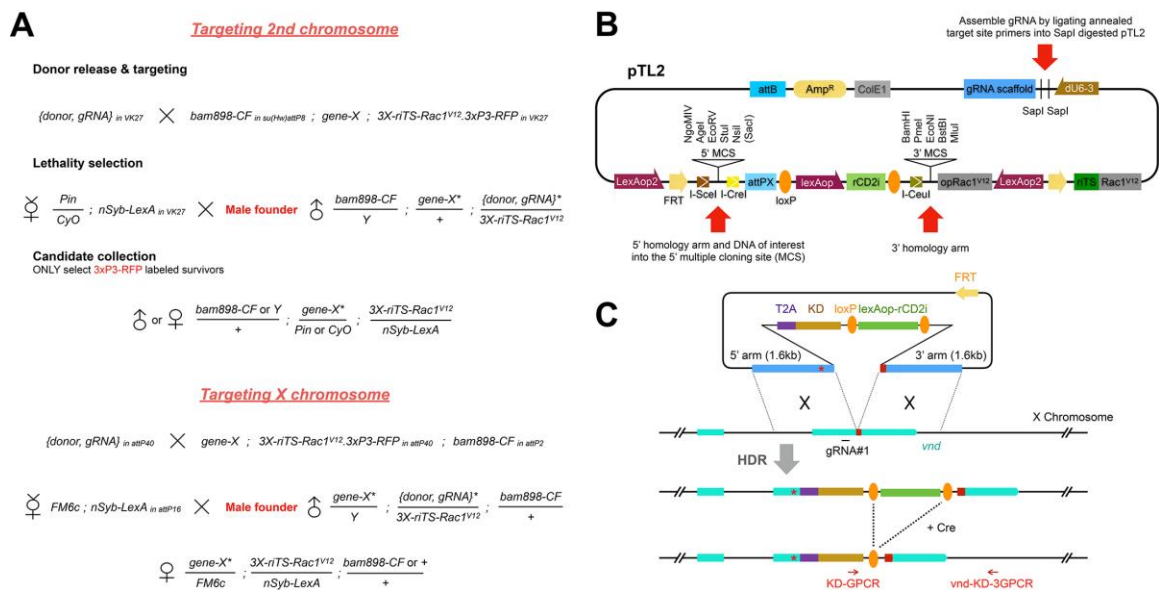


Figure 2. Implementing E-Golic+. (A) Targeting schemes for a second or an X chromosome gene with male founders. E-Golic+ involves two crosses and three steps. Step 1 Donor release & targeting: In the first cross, we create founders that have active CRISPR reactions with circular donor for HDR in their germ cells. *bam898-CF*: *bamP(898)-Cas9-P2A-FLP*. Then, founders are mated with *nSyb-LexA* so that each progeny experiences lethality selection. *gene-X**: targeted gene-X; *{donor, gRNA}**: residual *{donor, gRNA}* after Flip-out, which should result in a reconstituted *LexAop2-FRT-riTS-Rac1^{V12}*. Most, if not all, of the 3xP3-RFP labeled surviving candidates should inherit GT events marked with the rCD2i repressor. (B) Constructing a {donor, gRNA} plasmid with the pTL2 backbone. (C) Donor design for generating *vnd-T2A-KD* knock-in with gRNA#1 is presented here. The same designing principles apply to all E-Golic+ donors. Genomic sequences around 1.6 kb just upstream and downstream of the *vnd* stop codon (red bar) are used as homology arms (TAA is included in the 3' arm). Coding

sequence of T2A-KD is placed in frame so that KD and Vnd can be translated from the same mRNA transcript. gRNA#1 was selected for its proximity to the *vnd* stop codon. Red *: site-directed mutations to avoid CRISPR cutting. After HDR, the *lexAop-rCD2i* cassette can be removed by crossing to a Cre line. After cassette removal, primer *vnd-KD-3GPCR* was chosen farther downstream of the *vnd* 3' arm so that PCR amplicon of *KD-GPCR/vnd-KD-3GPCR* was possible only when T2A-KD is correctly situated at the *vnd* locus.

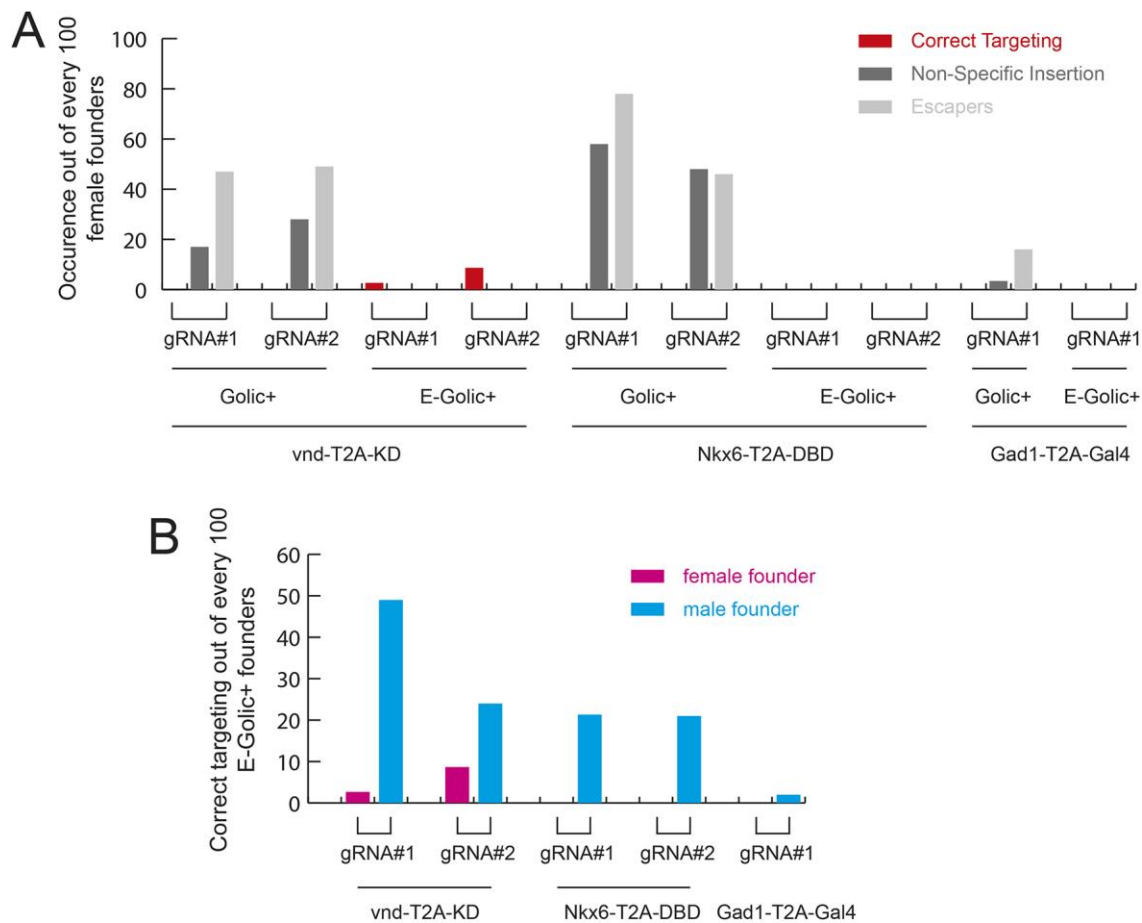


Figure 3. E-Golic+ effectively eliminates the occurrence of false positives and increases targeting efficiency with male founders. (A) E-Golic+ eliminates non-specific insertions and escapers associated with Golic+. Bar graph showing occurrence of three different types of events (correct targeting, non-specific insertion, and escapers) out of every 100 founders. (B) Male founders (blue) increase the number of correct targeting with E-Golic+. See Supplementary Table 3 for raw data.

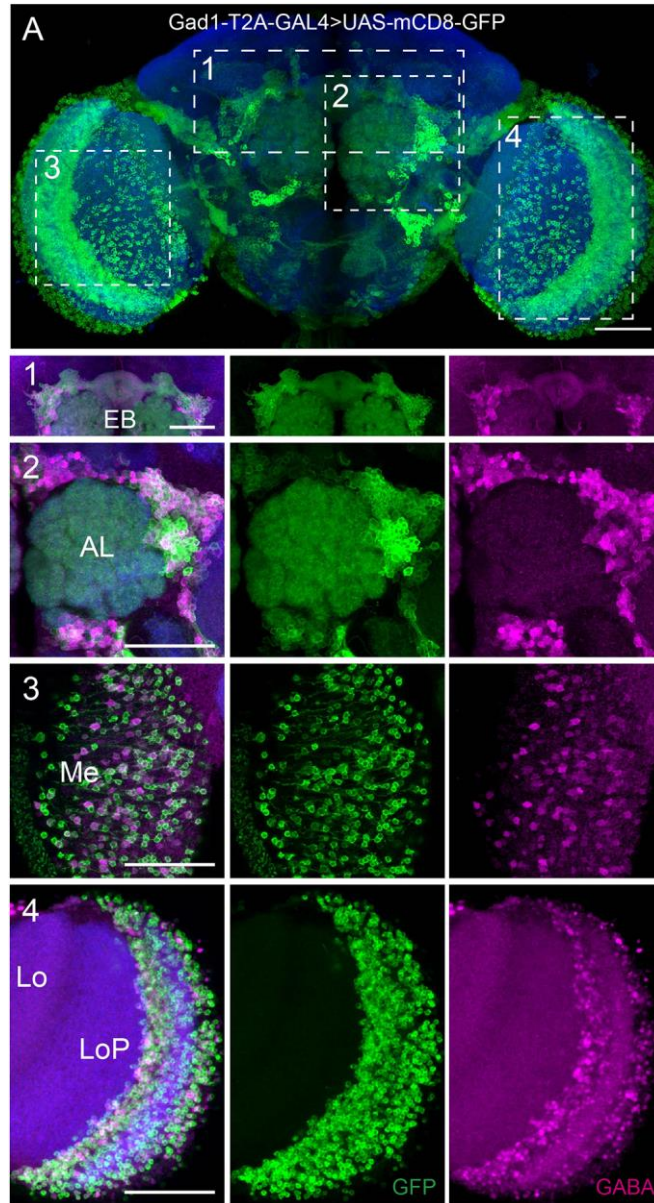


Figure. 4 The expression pattern of *Gad1-T2A-GAL4* in *Drosophila* central nervous system. (A) Composite confocal images of an adult fly brain with *Gad1-T2A-GAL4* driving a neuronal membrane marker (10XUAS-mCD8-GFP, green). The brain was counterstained with nc82 antibody which labels the neuropils (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, 50 μm in all panels.

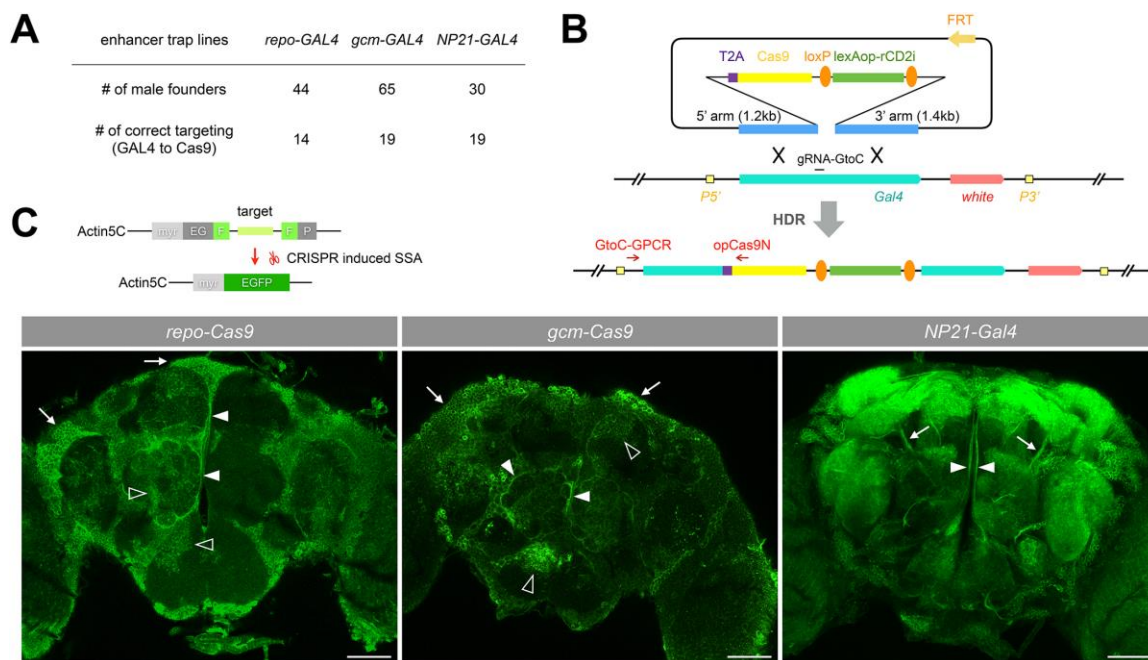


Figure. 5 Convert enhancer trap GAL4s into Cas9s with E-Golic+. (A) Summary of using E-Golic+ to convert three GAL4s into Cas9 versions. (B) Design for the common “GAL4 to Cas9” donor to target transgenic GAL4 lines. PCR amplicon from primer set GtoC-GPCR/opCas9N is evidence of a correct insertion of T2A-Cas9 in the target GAL4 locus. (C) CaSSA reporting from three tissue-specific Cas9s. Processes of cortex glia (arrows), ensheathing (arrowheads), and astrocyte-like (open arrowheads) glia are revealed by repo-Cas9 and gcm-Cas9 (single focal planes) (Awasaki et al., 2008). Under NP21-Cas9, prominent tracts of aDT2 (arrows) and aDT6 (arrowheads) neurons of the fru circuit are labeled (partial z-stack projections) (Jai et al., 2010). Scale bar, 50 μ m in all panels.

Supplementary Information

Tables

Table S1. Golic+, comparing *bam198-CFI* and *bam898-CFI* with {*msh-T2A-GAL4 KI*, *gRNA*}

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

Using the same 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. Each founder can produce multiple candidates.

Table S2. 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, gRNA} 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 S3. Comparison of Golic+ and Enhanced Golic+

	# of Founders	Correct Targeting	Non-Specific Insertion	Escapers
Golic+	Female			
{ <i>vnd-T2A-KD</i> , <i>gRNA#1</i> }	100	0	17	47
{ <i>vnd-T2A-KD</i> , <i>gRNA#2</i> }	100	0	28	49
E-Golic+	Female			
{ <i>vnd-T2A-KD</i> , <i>gRNA#1</i> }	150	4	0	0
{ <i>vnd-T2A-KD</i> , <i>gRNA#2</i> }	150	13	0	0
E-Golic+	Male			
{ <i>vnd-T2A-KD</i> , <i>gRNA#1</i> }	100	49	0	0
{ <i>vnd-T2A-KD</i> , <i>gRNA#2</i> }	100	24	0	0
Golic+	Female			
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#1</i> }	50	0	29	39
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#2</i> }	50	0	24	23
E-Golic+	Female			
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#1</i> }	65	0	0	0
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#2</i> }	120	0	0	0
E-Golic+	Male			
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#1</i> }	75	16	0	0
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#2</i> }	100	21	0	0
Golic+	Female			
{ <i>Gad1-T2A-GAL4</i> , <i>gRNA#1</i> }	350	0	12	56
E-Golic+	Female			
{ <i>Gad1-T2A-GAL4</i> , <i>gRNA#1</i> }	100	0	0	0
E-Golic+	Male			
{ <i>Gad1-T2A-GAL4</i> , <i>gRNA#1</i> }	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*} because of enough confidence in E-Golic+. Female founders were used in both Golic+ and E-Golic+ to reveal the benefits of adopting circular donor plus a 3xP3-RFP labeled 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*.

Table S4. Oligos used in this work

Primers to amplify homology arms	
vnd-55-AgeI	ACGT ACCGGT GGCGAACCGGAAGAGACCCGGCTACCATTG
vnd-53-StuI	ACGT AGGCCT GGGCCACCAGGCGGCCCTCTG
vnd-35-BamHI	ACGT GGATCC TAATATTGCTAGGAACTGGCATTACGCGGACTG
vnd-33-MluI	ACGT ACGCGT GACAAGCATGTGGGTCCACTTATCCCAAG
Nkx6-55-AgeI	ACGT ACCGGT TGTCTGTGCACTCTCCCTGC
Nkx6-53-StuI	ACGT AGGCCT ATGACGATAATTATCCTGCTGCTGCT
Nkx6-35-BamHI	ACGT GGATCC TAAAACGAATTTACAACTATGCAATGACATGGAC
Nkx6-33-MluI	ACGT ACGCGT GAGGAGTGAAGCCTCTCTTTTCGTTTAATAG
Gad1-55-AgeI	ACGT ACCGGT GCTCATGGGAGCCCTGCTCCAG
Gad1-53-StuI	ACGT AGGCCT CAAGTCGTCGCCCAGGCGATG
Gad1-35-BamHI	ACGT GGATCC TAAGGGGTTTCAGTCTAGTTTGGCTCGGCTC
Gad1-33-MluI	ACGT ACGCGT TCAATAAAGTGAAGCAGAGAAGCGG
GAL4-55-AgeI	TACG ACCGGT ATGAAGCTACTGTCTTCTATCGAAC
GAL4-53-MfeI	AAGCAGGGA CAATTG GATCTCC
GAL4-35-BamHI	CG GGATCC TCATGGCATCATTGAAACAGCAAG
GAL4-33-PmeI	TACG GTTTAAAC TTA CTCTTTTTTTGGGTTTGGTGGG
Primers for site-directed mutagenesis	
vnd-5arm-m1-F	GCTACGAGGGTGCATCCTGGCCTGCTACATGGCCATGCCACCCATCCG
vnd-5arm-m1-R	CGGATGGGTGGCATGGCCATGTAGCAGGCCAGGATGACCCTCGTAGC
vnd-5arm-m2-F	GCCCCGTCGGGTAGCCGTCCCGGTCCTAGTGAGGAACGGAAAGCCCTGCT TG
vnd-5arm-m2-R	CAAGCAGGGCTTTCCGTTCTCACTAGGACCGGGACGGCTACCCGACGGG GC
Nkx6-3arm-m1-F	TTTTGCCACTTTACAGTCTTCTGAAGACATCATTTTTCCCCTCCAACCTC
Nkx6-3arm-m1-R	GAGTTGGAGGGGAAAAATGATGTCTTCAGAAGACTGTAAAGTGGCAAAA
Nkx6-5arm-m2-F	GTTGCAGGTCTGGTTCCAAAACCGCCGTACCAAATGGCGCAAACGG
Nkx6-5arm-m2-R	CCGTTTGCGCCATTTGGTACGGCGGTTTTGGAACCAGACCTGCAAC
Gad1-5arm-m1-F	ACGCTGATGGTGGGCTACCAGCCTGATGACCGACGGCCCAACTTCTTC C
Gad1-5arm-m1-R	GGAAGAAGTTGGGCCGTCGGTCATCAGGCTGGTAGCCCACCATCAGCG T
Primers to amplify T2AKDPEST, T2AZpGDBD, T2AGAL4	
T2AKDPEST-StuI	ACGT <u>AGGCCT</u> GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGTG GAGGAGAACCCCGGGCCC ATGAGTACTTTTGCAGAAGCAGCGCATCTT ACACCGCATC
T2AKDPEST-SacI	ACGT <u>GAGCTC</u> TTACACGTTGATGCGAGCCGAGGCGCAAG
T2AZpGDBD-StuI	ACGT AGGCCT GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGT GGAGGAGAACCCCGGGCCC ATGCTGGAGATCCGCGCCGCCTTC
T2AZpGDBD-SacI	ACGT <u>GAGCTC</u> TTACGATACCGTCAGTTGCCGTTGAC

T2AGAL4-Stul	ACGT AGGCCT GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGTG GAGGAGAACCCCGGGCCC AAGCTGCTGAGTAGTATTGAACAAG
T2AGAL4-SacI	ACGT GAGCTC TACTCCTTCTTTGGGTTCCGGT
Oligos for gRNA spacers	
Vnd-g1-F	TCGCATGGCCGTGCAGTAGACC
Vnd-g1-R	AACGGTCTACTGCACGGCCATG
Vnd-g2-F	TCGTTCCCTCACCAGAACTGGAA
Vnd-g2-R	AACTTCCAGTTCTGGTGAGGAA
Nkx6-g1-F	TCGAAATTAAGTCTTCAGAAGA
Nkx6-g1-R	AACTCTTCTGAAGACTTAATTT
Nkx6-g2-F	TCGCCATTTGGTGCGACGATTC
Nkx6-g2-R	AACGAATCGTCGCACCAAATGG
Gad1-g1-F	TCGCTACCAGCCCGACGATCGC
Gad1-g1-R	AACGCGATCGTCGGGCTGGTAG
gRNA-GtoC-F	TCGATGGATCGACCATAAAGCA
gRNA-GtoC-R	AACTGCTTTATGGTCGATCCAT
Primers for GT candidate confirmation	
vnd-KD-3GPCR	GCGAGTATTATTTTCGTTGCTGATCG
KD-GPCR	AATTTATTGCGGAATATAGGCGCAA
Nkx6-DBD-5GPCR	AGCAGCCTTAATGATGCCAACTAATG
DBD-GPCR	GCTTGTTCAATACTACTCAGCAGCTTC
GAD1-GAL4-5GPCR	CGTAATTGAATGTCCATTTAACGCCG
GAL4-GPCR	CACACGCTTGTTCAATACTACTCAG
GtoC-GPCR	TGCACGTTTGCTTGTTGAGAG
opCas9N	GTTCTTACGACGGGTGTACCTG

Supplementary Information

Tables

Table S1. Golic+, comparing *bam198-CFI* and *bam898-CFI* with {*msh-T2A-GAL4 KI*, *gRNA*}

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

Using the same 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. Each founder can produce multiple candidates.

Table S2. 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, gRNA} 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 S3. Comparison of Golic+ and Enhanced Golic+

	# of Founders	Correct Targeting	Non-Specific Insertion	Escapers
Golic+	Female			
{ <i>vnd-T2A-KD</i> , <i>gRNA#1</i> }	100	0	17	47
{ <i>vnd-T2A-KD</i> , <i>gRNA#2</i> }	100	0	28	49
E-Golic+	Female			
{ <i>vnd-T2A-KD</i> , <i>gRNA#1</i> }	150	4	0	0
{ <i>vnd-T2A-KD</i> , <i>gRNA#2</i> }	150	13	0	0
E-Golic+	Male			
{ <i>vnd-T2A-KD</i> , <i>gRNA#1</i> }	100	49	0	0
{ <i>vnd-T2A-KD</i> , <i>gRNA#2</i> }	100	24	0	0
Golic+	Female			
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#1</i> }	50	0	29	39
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#2</i> }	50	0	24	23
E-Golic+	Female			
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#1</i> }	65	0	0	0
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#2</i> }	120	0	0	0
E-Golic+	Male			
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#1</i> }	75	16	0	0
{ <i>Nkx6-T2A-DBD</i> , <i>gRNA#2</i> }	100	21	0	0
Golic+	Female			
{ <i>Gad1-T2A-GAL4</i> , <i>gRNA#1</i> }	350	0	12	56
E-Golic+	Female			
{ <i>Gad1-T2A-GAL4</i> , <i>gRNA#1</i> }	100	0	0	0
E-Golic+	Male			
{ <i>Gad1-T2A-GAL4</i> , <i>gRNA#1</i> }	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*} because of enough confidence in E-Golic+. Female founders were used in both Golic+ and E-Golic+ to reveal the benefits of adopting circular donor plus a 3xP3-RFP labeled 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*.

Table S4. Oligos used in this work

Primers to amplify homology arms	
vnd-55-AgeI	ACGT ACCGGT GGCGAACCGGAAGAGACCCGGCTACCATTG
vnd-53-StuI	ACGT AGGCCT GGGCCACCAGGCGGCCCTCTG
vnd-35-BamHI	ACGT GGATCC TAATATTGCTAGGAACTGGCATTACGCGGACTG
vnd-33-MluI	ACGT ACGCGT GACAAGCATGTGGGTCCACTTATCCCAAG
Nkx6-55-AgeI	ACGT ACCGGT TGTCTGTGCACTCTCCCTGC
Nkx6-53-StuI	ACGT AGGCCT ATGACGATAATTATCCTGCTGCTGCT
Nkx6-35-BamHI	ACGT GGATCC TAAACGAATTTACAACTATGCAATGACATGGAC
Nkx6-33-MluI	ACGT ACGCGT GAGGAGTGAAGCCTCTCTTTTCGTTTAATAG
Gad1-55-AgeI	ACGT ACCGGT GCTCATGGGAGCCCTGCTCCAG
Gad1-53-StuI	ACGT AGGCCT CAAGTCGTCGCCCAGGCGATG
Gad1-35-BamHI	ACGT GGATCC TAAGGGGTTTCAGTCTAGTTTGGCTCGGCTC
Gad1-33-MluI	ACGT ACGCGT TCAATAAAGTGAAGCAGAGAAGCGG
GAL4-55-AgeI	TACG ACCGGT ATGAAGCTACTGTCTTCTATCGAAC
GAL4-53-MfeI	AAGCAGGGA CAATTG GATCTCC
GAL4-35-BamHI	CG GGATCC TCATGGCATCATTGAAACAGCAAG
GAL4-33-PmeI	TACG GTTTAAAC TTA CTCTTTTTTTGGGTTTGGTGGG
Primers for site-directed mutagenesis	
vnd-5arm-m1-F	GCTACGAGGGTGCATCCTGGCCTGCTACATGGCCATGCCACCCATCCG
vnd-5arm-m1-R	CGGATGGGTGGCATGGCCATGTAGCAGGCCAGGATGACCCTCGTAGC
vnd-5arm-m2-F	GCCCCGTCGGGTAGCCGTCCCGGTCCTAGTGAGGAACGGAAAGCCCTGCT TG
vnd-5arm-m2-R	CAAGCAGGGCTTTCCGTTCTCACTAGGACCGGGACGGCTACCCGACGGG GC
Nkx6-3arm-m1-F	TTTTGCCACTTTACAGTCTTCTGAAGACATCATTTTTCCCCTCCAACTC
Nkx6-3arm-m1-R	GAGTTGGAGGGGAAAAATGATGTCTTCAGAAGACTGTAAAGTGGCAAAA
Nkx6-5arm-m2-F	GTTGCAGGTCTGGTTCCAAAACCGCCGTACCAAATGGCGCAAACGG
Nkx6-5arm-m2-R	CCGTTTGCGCCATTTGGTACGGCGGTTTTGGAACCAGACCTGCAAC
Gad1-5arm-m1-F	ACGCTGATGGTGGGCTACCAGCCTGATGACCGACGGCCCAACTTCTTC C
Gad1-5arm-m1-R	GGAAGAAGTTGGGCCGTCGGTCATCAGGCTGGTAGCCCACCATCAGCG T
Primers to amplify T2AKDPEST, T2AZpGDBD, T2AGAL4	
T2AKDPEST-StuI	ACGT <u>AGGCCT</u> GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGTG GAGGAGAACCCCGGGCCC ATGAGTACTTTTGCAGAAGCAGCGCATCTT ACACCGCATC
T2AKDPEST-SacI	ACGT <u>GAGCTC</u> TTACACGTTGATGCGAGCCGAGGCGCAAG
T2AZpGDBD-StuI	ACGT AGGCCT GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGT GGAGGAGAACCCCGGGCCC ATGCTGGAGATCCGCGCCGCCTTC
T2AZpGDBD-SacI	ACGT <u>GAGCTC</u> TTACGATACCGTCAGTTGCCGTTGAC

T2AGAL4-Stul	ACGT AGGCCT GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGTG GAGGAGAACCCCGGGCCC AAGCTGCTGAGTAGTATTGAACAAG
T2AGAL4-SacI	ACGT GAGCTC TACTCCTTCTTTGGGTTCCGGT
Oligos for gRNA spacers	
Vnd-g1-F	TCGCATGGCCGTGCAGTAGACC
Vnd-g1-R	AACGGTCTACTGCACGGCCATG
Vnd-g2-F	TCGTTCCCTCACCAGAACTGGAA
Vnd-g2-R	AACTTCCAGTTCTGGTGAGGAA
Nkx6-g1-F	TCGAAATTAAGTCTTCAGAAGA
Nkx6-g1-R	AACTCTTCTGAAGACTTAATTT
Nkx6-g2-F	TCGCCATTTGGTGCGACGATTC
Nkx6-g2-R	AACGAATCGTCGCACCAAATGG
Gad1-g1-F	TCGCTACCAGCCCGACGATCGC
Gad1-g1-R	AACGCGATCGTCGGGCTGGTAG
gRNA-GtoC-F	TCGATGGATCGACCATAAAGCA
gRNA-GtoC-R	AACTGCTTTATGGTCGATCCAT
Primers for GT candidate confirmation	
vnd-KD-3GPCR	GCGAGTATTATTTTCGTTGCTGATCG
KD-GPCR	AATTTATTGCGGAATATAGGCGCAA
Nkx6-DBD-5GPCR	AGCAGCCTTAATGATGCCAACTAATG
DBD-GPCR	GCTTGTTCAATACTACTCAGCAGCTTC
GAD1-GAL4-5GPCR	CGTAATTGAATGTCCATTTAACGCCG
GAL4-GPCR	CACACGCTTGTTCAATACTACTCAG
GtoC-GPCR	TGCACGTTTGCTTGTTGAGAG
opCas9N	GTTCTTACGACGGGTGTACCTG