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1 **Precise genome engineering in *Drosophila* using prime editing**
2
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47 **Abstract:**
48
49 Precise genome editing is a valuable tool to study gene function in model
50 organisms. Prime editing, a precise editing system developed in mammalian
51 cells, does not require double strand breaks or donor DNA and has low off-target
52 effects. Here, we applied prime editing for the model organism *Drosophila*
53 *melanogaster* and developed conditions for optimal editing. By expressing prime
54 editing components in cultured cells or somatic cells of transgenic flies, we
55 precisely installed premature stop codons in three classical visible marker genes,
56 *ebony*, *white*, and *forked*. Furthermore, by restricting editing to germ cells, we
57 demonstrate efficient germ line transmission of a precise edit in *ebony* to ~50%
58 of progeny. Our results suggest that prime editing is a useful system in
59 *Drosophila* to study gene function, such as engineering precise point mutations,
60 deletions, or epitope tags.
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93 **Keywords:** genome editing, genome engineering, precise editing, *Drosophila*,
94 prime editing, pegRNA, CRISPR
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139 **Introduction:**
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141 Genome editing is a versatile tool to study gene function in model organisms. For
142 example, targeted gene deletions or point mutations can be used to disrupt gene
143 function, create gain of function alleles, or model human disease mutations
144 (PICKAR-OLIVER AND GERSBACH 2019). Furthermore, insertions can be used for
145 gene tagging to detect or manipulate endogenous proteins (VANDEMOORTELE *et*
146 *al.* 2019). *Drosophila melanogaster* is an excellent model to study gene function
147 because of its easy genetic manipulation, rich genomic resources, and
148 conservation of cellular, developmental, and physiological processes with
149 humans (HALES *et al.* 2015; UGUR *et al.* 2016). Importantly, genome editing tools
150 involving clustered regularly interspaced short palindromic repeats (CRISPR)
151 have been successfully applied in *Drosophila* to study gene function (VENKEN *et*
152 *al.* 2016; KORONA *et al.* 2017; BIER *et al.* 2018).
153
154 Prime editing is a recently developed CRISPR-based tool to engineer precise
155 edits in the genome (ANZALONE *et al.* 2019). Unlike precise editing using Cas9
156 and homology-directed repair (HDR), prime editing does not induce double
157 strand breaks and does not require DNA template containing the edit. In addition,
158 this method appears to have low off-target effects. Prime editing consists of two
159 components, 1) a single guide RNA (sgRNA) with a 3' extension encoding the
160 edit, referred to as a prime editing guide RNA (pegRNA), and 2) a nickase
161 mutant of Cas9 (nCas9^{H840A}) fused with an engineered Moloney murine leukemia
162 virus (M-MLV) reverse transcriptase (RT) enzyme, referred to as prime editor 2
163 (PE2). The pegRNA/PE2 complex induces a nick at the target site and reverse
164 transcribes the edit from the pegRNA into the genome via the RT domain. Like
165 Cas9/HDR, many types of precise edits are possible with prime editing, such as
166 single base changes, deletions, or insertions.
167
168 While prime editing was originally developed in human cells (ANZALONE *et al.*
169 2019), it has been quickly adopted in other organisms including mice (ANZALONE
170 *et al.* 2019; LIU *et al.* 2020; SURUN *et al.* 2020) and plants (BUTT *et al.* 2020; CHEN
171 2020; HUA *et al.* 2020; LI *et al.* 2020; LIN *et al.* 2020; TANG *et al.* 2020; VEILLET *et*
172 *al.* 2020; WANG *et al.* 2020; XU *et al.* 2020). Prime editing has been used to help
173 correct disease mutations (ANZALONE *et al.* 2019; ROUSSEAU *et al.* 2020),
174 introduce herbicide resistant alleles (BUTT *et al.* 2020; CHEN 2020; HUA *et al.*
175 2020; XU *et al.* 2020), alter plant morphology (BUTT *et al.* 2020), and model
176 human disease mutations in organoids (LIU *et al.* 2020; SCHENE *et al.* 2020).
177 Adapting and testing prime editing in additional organisms, particularly model
178 systems, has great potential to improve the study of gene function. Here, we
179 develop reagents and optimized conditions to conduct prime editing in
180 *Drosophila*.
181
182 **Results:**
183
184 **Prime editing in cultured S2R+ cells**

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185
186 To initially test prime editing in *Drosophila*, we expressed prime editing
187 components in cultured S2R+ cells by transfection. We used the S2R+ PT5 line
188 (NEUMULLER *et al.* 2012) that constitutively expresses mCherry fluorescent
189 protein and which has been previously used for CRISPR/Cas9 genome editing
190 (VISWANATHA *et al.* 2018). To express PE2 in S2R+ cells, we constructed two
191 plasmids for constitutive expression. *pAct-PE2* expresses PE2 under the
192 *Drosophila Actin5c* promoter (Supplemental Figure 1A), and *pUAS-PE2* (Figure
193 1A) expresses PE2 when used in combination with *pAct-Gal4* (abbreviated as
194 *pAct>PE2*). This should result in high levels of PE2 expression due to signal
195 amplification of the Gal4/UAS system (BRAND AND PERRIMON 1993). In addition, to
196 express pegRNAs in cells, we constructed an empty expression vector (*pCFD3-*
197 *NS*) that lacks the sgRNA scaffold sequence (NS=No scaffold) (Figure 1B), which
198 is a modified version of the sgRNA expression plasmid *pCFD3* (PORT *et al.*
199 2014).
200
201 First, we designed a pegRNA to insert a 23bp barcode (BC) sequence into the
202 *ebony* gene (Supplemental File 1). This strategy was chosen to enable sensitive
203 detection of insertion events by PCR. Four days after transfection of PE2 and
204 pegRNA plasmids into PT5 cells, genomic DNA was collected and insertion-
205 specific primers were used to amplify the putative insertion (Supplemental Figure
206 1B). Gel images of PCR products confirmed the presence of the *ebony*^{23bpBC}
207 insertion using either *pAct-PE2* or *pAct>PE2* (Supplemental Figure 1C). To
208 determine the insertion rate, we performed amplicon sequencing of the target
209 region from transfected cells. Transfections using *pAct>PE2* resulted in an
210 insertion efficiency of 0.42%, whereas transfections using *pAct-PE2* were
211 substantially lower (0.006%) (Supplemental Figure 1D). Although our editing
212 efficiencies were lower than reported in mammalian cells with an equivalent sized
213 insertion (ANZALONE *et al.* 2019), these initial results demonstrated that prime
214 editing was possible in *Drosophila* S2R+ cells.
215
216 Next, we designed a pegRNA to introduce a premature stop codon in *ebony*
217 (*ebony*^{G111X}) (Figure 1C). In addition, we designed an sgRNA that nicks the non-
218 edited DNA strand, since this approach, known as the Prime Editor 3 (PE3)
219 system, can bias mismatch repair and boost editing efficiencies in mammalian
220 cells (MODRICH 2006; CHAKRABORTY AND ALANI 2016; ANZALONE *et al.* 2019). To
221 simultaneously co-express a pegRNA and sgRNA, we constructed a dual
222 expression vector called *pCFD5-NS* (Figure 1D). This vector uses tRNA
223 processing to produce both pegRNA and sgRNA, and is a modified version of the
224 multiplex sgRNA expression plasmid *pCFD5* (PORT AND BULLOCK 2016).
225
226 After transfecting PT5 cells with *pCFD5-PE3-ebony*^{G111X}, *pAct>PE2*, and *pAct-*
227 *GFP*, we isolated GFP+ cells using FACS and performed amplicon sequencing
228 from their genomic DNA (Figure 1E). Under these conditions, precise editing
229 efficiency of *ebony* was 6.0%. Furthermore, by comparing alternate conditions,
230 we found that editing efficiency was ~2.5x lower without FACS enrichment and

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231 ~12x lower using a stable PE2 cell line (*Act-PE2*) (Figure 1E). Like in mammalian
232 cells (ANZALONE *et al.* 2019), the PE3 system caused a low percentage of
233 insertions and deletions (indels) (0.86%) (Figure 1F). Finally, we compared
234 editing efficiency using only a pegRNA (*pCFD3-PE-ebony*^{G111X}). Unexpectedly,
235 editing efficiency was slightly higher (8.4%) without a nicking sgRNA (Figure 1F).
236 As expected, excluding the sgRNA reduced the frequency of indels to
237 background levels.
238
239 To test prime editing at other genomic sites, we designed pegRNAs to introduce
240 premature stop codons into *white* and *forked* (*white*^{A134X} and *forked*^{D111X}), along
241 with sgRNAs to nick on the non-edited strand (Supplemental Figure 1E). Editing
242 efficiencies using both pegRNA and nicking sgRNA were roughly similar to
243 *ebony*, producing 2.5% and 6.7% precise editing of *white* and *forked*,
244 respectively. In addition, results with pegRNA only showed 4.0 and 0.8% precise
245 editing of *white* and *forked*, respectively. Therefore, unlike *ebony* and *white*,
246 *forked* editing efficiency was substantially improved by including a nicking
247 sgRNA. In conclusion, using optimized prime editing conditions, we demonstrate
248 precise editing efficiencies in S2R+ cells of ~4-8%.
249

250 **Prime editing in vivo**

251
252 To test prime editing in vivo, we performed crosses between PE2 and pegRNA
253 expressing transgenic flies. This strategy has been used with Cas9 (BIER *et al.*
254 2018), and Cas12a (PORT *et al.* 2020a) to edit somatic and germ cells, and it is
255 generally associated with higher editing efficiencies than embryo injection. To
256 express PE2 in vivo, we generated *UAS-PE2* transgenic flies, which express PE2
257 when crossed with a Gal4 driver line (Figure 2A). In addition, we generated
258 transgenic flies expressing pegRNAs to introduce premature stop codons into
259 *ebony*, *white*, and *forked*. These genes/edits were chosen to enable easy
260 identification of mutant flies with body phenotypes. In addition, transgenic
261 pegRNA flies were created using the same plasmids validated in S2R+ cells
262 (*pCFD3-PE-gene*^{edit} and *pCFD5-PE3-gene*^{edit}).
263
264 Many groups have reported toxicity in *Drosophila* from expression of Cas9
265 (HUYNH *et al.* 2018; POE *et al.* 2019; PORT *et al.* 2020b) and Cas13 (BUCHMAN *et*
266 *al.* 2020). To test for toxicity from PE2 expression, we crossed *UAS-PE2* to two
267 ubiquitous Gal4 drivers (*Act-Gal4* and *tub-Gal4*) and analyzed the resulting
268 progeny (abbreviated as *Act>PE2* and *tub>PE2*). *Act>PE2* and *tub>PE2* larvae,
269 pupae, and adults were morphologically normal (not shown). Furthermore, the
270 observed number of *Act>PE2* and *tub>PE2* adult progeny was similar to negative
271 control crosses when raised at 25˚C or 29˚C, and when using two different *UAS-*
272 *PE2* transgenes (Figure 2B). Finally, *Act>PE2* and *tub>PE2* flies were fertile and
273 could be propagated as a stock. Therefore, ubiquitous expression of PE2 does
274 not result in obvious toxicity in flies.
275

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276 Next, we crossed *Act>PE2* or *tub>PE2* to transgenic pegRNA lines and analyzed
277 progeny for evidence of editing in somatic cells (Figure 2C). Crosses involving
278 expression of a single pegRNA (*pCFD3-PE-gene*^{edit}) resulted in progeny that
279 were wild-type in appearance (Figure 2D, Supplemental Figure 2A). In contrast,
280 somatic editing using the PE3 system (*pCFD5-PE3-gene*^{edit}) resulted in progeny
281 with mutant phenotypes similar to classical alleles (Figure 2D, Supplemental
282 Figure 2A). In all cases, mutant phenotypes appeared slightly more severe at
283 29˚C compared to 25˚C (not shown). To determine the type and frequency of
284 DNA changes at target sites, we performed amplicon sequencing from single
285 adult fly genomic DNA. For *ebony*, *forked*, and *white,* precise editing efficiency
286 using *Act>PE2* was highest with the PE3 system, resulting in 35.2%, 11.6%, and
287 21.9% reads, respectively, with the intended edit (Figure 2E). Comparable results
288 were obtained using *tub>PE2* (Figure 2E). In addition, editing of *ebony* using
289 *Act>PE2* was higher at 29˚C than 25˚C, but slightly lower in females compared to
290 males (Figure 2E). The PE3 system led to a significant percentage of indels at
291 the target site, with an exceptionally high percentage for *forked* (67.9%). Since
292 both the precise edit and frameshift indels would cause loss of gene function, our
293 sequencing results explain the strong mutant phenotypes when using the PE3
294 system in somatic cells.
295
296 Adapting prime editing to the germ line could enable the creation and
297 propagation of edited fly stocks. To accomplish this, we generated transgenic
298 flies with PE2 under the control of the germ cell-specific *nanos* (*nos*) promoter,
299 either as a single transgene (*nos-PE2*) (Supplemental Figure 2B), or by
300 combination of *nos-Gal4* with *UAS-PE2* (*nos>PE2*) (Figure 2A). We crossed *nos-*
301 *PE2* or *nos>PE2* to *pCFD5-PE3-ebony*^{G111X} to generate G1 progeny with editing
302 components expressed in germ cells (Figure 2F). Next, pools of 10 G1 progeny
303 were crossed with *ebony*^{1} and the percentage of mutant *ebony* G2 progeny
304 (*ebony*^{mut}/*ebony*^{1}) was calculated. Using this assay, we compared *nos-PE2* (two
305 separate insertions) vs. *nos>PE2*, three temperature conditions (25˚C, 29˚C, and
306 29˚C with 37˚C heat shocks (hs)), and male vs. female germ line editing. We
307 observed the highest transmission rate (42.2%) of *ebony* mutations from the G1
308 male germ line using *nos>PE2* and raising G1 animals at 29˚C+hs (Figure 2G).
309 Furthermore, single fly G1 crosses using *nos>PE2* and 29˚C+hs produced similar
310 results to pooled G1 crosses (Figure 2G) and 9/9 (100%) G1 flies were founders
311 for mutation of *ebony*.
312
313 Next, we sequenced the *ebony* target site from 72 mutant *ebony* G2 progeny
314 (Figure 2G). For each single fly G1 cross, the number of mutant *ebony* G2 flies
315 with a correct edit ranged from 75% to 100% and was similar for male and
316 female G1 crosses (Figure 2H). Combining sequencing results from all single fly
317 G1 crosses and both G1 sexes, 63/72 (88.0%) of mutant *ebony* G2 flies had the
318 desired edit, 7/72 (9.7%) had a frameshift indel, and 2/72 (2.8%) had wild-type
319 sequence (Figure 2I). Taking the average frequency of mutant *ebony* G2 flies
320 from single fly G1 crosses (56.2% from G1 males, 26.0% from G1 females) and
321 multiplying by the frequency of *ebony* mutant flies with the *G111X* edit (88.0%),

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322 we estimate that male and female founders on average transmit the desired edit
323 to 49.5% and 22.9% of progeny, respectively. Finally, homozygous *ebony*^{G111X}
324 flies exhibited dark body pigment (Figure 2J) and could be propagated as a
325 viable stock (not shown). These results demonstrate that prime editing is
326 effective for engineering precise genomic edits in the *Drosophila* germ line.
327
328 **Discussion:**
329
330 Currently, precise genome editing in *Drosophila* is performed by CRISPR/Cas9
331 and homology directed repair (HDR) (BIER *et al.* 2018). HDR enables a wide
332 variety of edits, yet is a relatively low-efficiency process, and a number of
333 unintended side-effects have been documented, such as off-target mutations
334 (CARROLL 2013), imprecise integration of the donor DNA (SKRYABIN *et al.* 2020),
335 or genome rearrangement (LEDFORD 2020). In addition, HDR is not as useful for
336 tissue-specific editing because HDR events only occur in dividing cells.
337 Furthermore, molecular cloning of donor constructs can be technically
338 challenging and time-consuming.
339
340 Prime editing has the potential to address some of these limitations. PE2 uses a
341 nickase mutant of Cas9 (H840A) that induces single strand breaks, which are
342 known to decrease undesired genome changes and increase HDR:indel ratios
343 (MAIZELS AND DAVIS 2018; ANZALONE *et al.* 2019). In addition, prime editing does
344 not require cell division and functions in post-mitotic cultured cells (ANZALONE *et*
345 *al.* 2019). pegRNAs contain both targeting sequence and edit template and are
346 simple to generate, thus facilitating multiple editing experiments in parallel.
347 Furthermore, transgenic pegRNAs enable temporal and spatial control of precise
348 editing, similar to transgenic sgRNAs used for CRISPR/Cas9 knockout (KONDO
349 AND UEDA 2013; PORT *et al.* 2014; MELTZER *et al.* 2019; POE *et al.* 2019; PORT *et*
350 *al.* 2020a). Generating transgenic pegRNA fly lines takes ~1 month, and thus
351 delays germ line editing experiments compared to direct injection of genome-
352 editing components into embryos. Injecting pegRNA plasmids or synthesized
353 pegRNAs into PE2-expressing embryos, similarly to what is commonly done for
354 Cas9-based HDR, could speed up the recovery of edited strains, but this
355 approach remains to be tested for prime editing. One important caveat is that
356 prime editing is currently limited to small (<100bp) edits that are identified by
357 molecular assays (e.g. PCR).
358
359 Precise editing efficiencies in S2R+ cells were ~4x lower than in mammalian
360 cells, and nicking sgRNAs (PE3 system) did not always increase efficiency. It is
361 not clear if this is due to biological differences (e.g. DNA repair pathways) or
362 technical differences (e.g. transfection method, promoter use, temperature)
363 between these two culture systems. Further optimization of prime editing will
364 likely improve its efficiency in cultured *Drosophila* cells. Regardless, our results
365 suggest that prime editing can be used as a tool to generate edited S2R+ cells
366 lines. Furthermore, pegRNAs could be stably integrated in S2R+ cells and used

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367 for pooled screening, as has been done with Cas9/sgRNAs (VISWANATHA *et al.*
368 2018).
369
370 Ubiquitous PE2 and pegRNA expression in whole animals led to editing
371 efficiencies of 10-40% for *ebony*, *white*, and *forked*. Although nicking sgRNAs led
372 to higher editing frequencies, they also caused frequent indels (26-68%), which
373 presumably contributed to the robust loss of function phenotypes we observed.
374 Conversely, single pegRNAs did not cause obvious mutant phenotypes despite
375 evidence of precise editing (4-26%). Therefore, unlike existing transgenic
376 crossing techniques for somatic knockout (PORT *et al.* 2014; PORT AND BULLOCK
377 2016; MELTZER *et al.* 2019; POE *et al.* 2019; PORT *et al.* 2020a; PORT *et al.*
378 2020b), we were unable to install a precise edit in the majority of cells in the fly
379 using ubiquitous expression of prime editing components. Nevertheless, some
380 applications may be compatible with our reported somatic editing efficiencies,
381 such as screening edits that drive tumorigenesis or affect cell competition.
382
383 By restricting expression of PE2 to germ cells, we demonstrated efficient
384 transmission of a precise edit (*ebony*^{G111X}) from transgenic founder flies to
385 progeny. 100% of founder flies transmitted the *ebony*^{G111X} edit, with 49.5% of
386 progeny from male founders inheriting the allele. This transmission rate is
387 comparable to, if not higher than, using HDR and embryo injection to install
388 similarly sized edits (GRATZ *et al.* 2014; PORT *et al.* 2014; GE *et al.* 2016; LEVI *et*
389 *al.* 2020) and facilitates molecular screening of a small number of progeny.
390 Similar to S2R+ and somatic cells, transmission rate was increased using
391 Gal4/UAS-based PE2 expression and higher temperature, respectively. Further
392 manipulating this temperature sensitivity will be useful to optimize germ cell
393 editing. It will also be important to determine the generality of this method by test
394 editing of additional genes, especially essential genes.
395
396 Currently, designing an effective pegRNA for precise editing is less
397 straightforward than for sgRNAs. We deliberately selected pegRNA spacer
398 sequences based on previously validated sgRNAs (see methods), but this might
399 have led to better than average editing efficiency. The recent introduction of
400 online tools have made pegRNA design easier, with options to optimize GC
401 content and RNA stability (CHOW *et al.* 2020; HSU *et al.* 2020). When possible,
402 we recommend testing editing efficiency in S2R+ cells before proceeding in vivo.
403 While amplicon sequencing produces high quality quantitative data, there are
404 faster and cheaper molecular assays such as the Dinucleotide signaTurE
405 CapTure (DTECT) (BILLON *et al.* 2020) or Tracking of Indels by Decomposition
406 (TIDE) (SENTMANAT *et al.* 2018).
407
408 In summary, we have developed genetic tools to express prime editing
409 components in *Drosophila*, and optimized conditions for efficient editing in
410 cultured cells and in vivo. By designing/cloning a pegRNA and optional sgRNA,
411 *Drosophila* researchers can generate a wide variety of precise genome
412 modifications such as point mutations, epitope tag insertions, or deletions.

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413 Furthermore, the ability to use prime editing in the fly germ line makes it useful to
414 create custom fly strains for gene function analysis. Since CRISPR-based tools
415 are continually engineered for optimal efficiency or new functions, it is likely that
416 future variant prime editor systems will improve this method in *Drosophila*.
417 Finally, the tools and optimized conditions we developed for prime editing in
418 *Drosophila* may be useful in other model organisms.
419

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436
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438 Investigation, J.A.B. and G.B.; Writing – Original Draft, J.A.B.; Writing – Review &
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441

442 Methods:
443

444 pegRNA and sgRNA design
445
446 pegRNA spacer sequences were selected based on previously validated sgRNA
447 target sites for *ebony* (PORT *et al.* 2015), *white* (KONDO AND UEDA 2013), and
448 *forked* (PORT AND BULLOCK 2016). 13bp was used for the pegRNA prime binding
449 site (PBS). For the reverse transcribed (RT) region, we used either a 34bp
450 (*ebony*^{23bpBC}) or 18bp (*ebony*^{G111X}, *white*^{A134X}, *forked*^{D111X}) region. In all of our
451 pegRNA designs, the pegRNA PAM is disrupted by the edit. Nicking sgRNAs
452 were designed to nick the DNA strand opposite to the pegRNA-nicked strand
453 within 40-90 bp of the pegRNA nick (*ebony*^{G111X}: +57, *white*^{A134X}: +70,
454 *forked*^{D111X}: +57). See Supplemental File 1 for pegRNA and sgRNA sequences.
455 See Supplemental File 3 for additional pegRNA and sgRNA design parameters.
456

457 Plasmid cloning
458

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459 Plasmid DNAs were constructed and propagated using standard protocols as
460 follows. PCR fragments were amplified using Phusion polymerase (New England
461 Biolabs M0530). Plasmids were digested with restriction enzymes at 37˚C for 2-
462 16hrs. Linearized plasmid and PCR fragments were gel purified using QIAquick
463 columns (28115, Qiagen). Inserts and backbones were assembled using Gibson
464 assembly (New England Biolabs E2611) or T4 ligation (New England Biolabs
465 M0202). Gateway-compatible expression and entry vectors were recombined
466 using LR Clonase II (ThermoFisher Scientific 11791020). Chemically competent
467 TOP10 *E.coli.* (Invitrogen, C404010) were transformed with plasmids containing
468 either Ampicillin or Kanamycin resistance genes and were selected on LB-Agar
469 plates with 100µg/ml Ampicillin or 50µg/ml Kanamycin. *ccdB* resistant chemically
470 competent *E.coli* (Invitrogen, A10460) were transformed with plasmids containing
471 a Gateway cassette (*ccdB*, Chlor.R.) and were selected on LB-Agar plates with
472 100µg/ml Ampicillin and colonies grown with 100µg/ml Ampicillin and 20µg/ml
473 Chloramphenicol. Plasmid DNA was isolated from bacterial cultures using
474 QIAprep Spin Miniprep Kit (Qiagen 27104) and Sanger sequenced at the
475 DF/HCC DNA Resource Core or GeneWiz. Oligo and dsDNA sequences are
476 listed in Supplemental File 2.
477
478 *pCFD3-NS* (Addgene #149545)*: pCFD3* (Addgene #49410) (PORT *et al.* 2014)
479 was digested with BbsI (Fermentas ER1011) and XbaI (New England Biolabs
480 R0145), which removes the sgRNA scaffold and *Drosophila U6* downstream
481 region, and the backbone was purified using a QIAquick column (28115,
482 Qiagen). A gBlock (IDT) containing two BbsI sites and the *U6* downstream region
483 was inserted into digested *pCFD3* backbone by Gibson assembly.
484
485 *pCFD5-NS* (Addgene #149546): *pCFD5* (Addgene #73914) (PORT AND BULLOCK
486 2016) was digested with BbsI (Fermentas ER1011) and XbaI (New England
487 Biolabs R0145), which removes the sgRNA scaffold, *O.s.* Gly tRNA, sgRNA
488 scaffold, and *U6* downstream region. The backbone was purified using a
489 QIAquick column (28115, Qiagen). A gBlock (IDT) containing two BbsI sites and
490 the *U6* downstream region was inserted into the digested *pCFD5* backbone by
491 Gibson assembly. The *D.m.* Gly tRNA sequence remains 5' to the first BbsI site.
492
493 *pEntr_PE2* (Addgene #149548): *PE2* coding sequence was PCR amplified from
494 *pCMV-PE2* (Addgene # 132775). *pEntr* backbone was PCR amplified from
495 *pEntr_D-TOPO* (Invitrogen K240020). *PE2* coding sequence was cloned into
496 *pEntr* backbone by Gibson assembly.
497
498 *pNos-PE2-attB* (Addgene #149549): *PE2* coding sequence was PCR amplified
499 from *pCMV-PE2* (Addgene # 132775) and gel purified. *pNos-Cas9-attB* (REN *et*
500 *al.* 2013) was digested with XbaI/AvrII (New England Biolabs R0145, R0174) to
501 remove Cas9 sequences and the backbone fragment was gel purified. *PE2*
502 coding sequence was inserted into digested *pNos-attB* by Gibson assembly.
503

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504 *pAct-GW-HygroR* (Addgene #149610): *Act5c* promoter was amplified from *pAWF*
505 (Murphy lab, unpublished, https://emb.carnegiescience.edu/drosophila-gateway-
506 vector-collection) and gel purified. Backbone was PCR amplified from *pMK33-*
507 *GW*, using primers that exclude the *Metallothionein* promoter, and gel purified.
508 The *Act5c* fragment was inserted into the *pMK33-GW* backbone by Gibson
509 assembly.
510
511 *pUAS-PE2-attB* (Addgene #149550) and *pAct-PE2-HygroR* (Addgene #149552)
512 were generated by Gateway reactions between *pEntr_PE2* and *pWalium10-roe*
513 (PERKINS *et al.* 2015) or *pAct-GW-HygroR*, respectively.
514
515 To clone the *pCFD3*-PE-*ebony*^{23bpBC} expression plasmid, oligos encoding the
516 spacer, scaffold, and extension were inserted into *pCFD3-NS* by ligation. Briefly,
517 *pCFD3-NS* was digested with BbsI and purified on a QIAquick column. Top and
518 bottom oligo pairs encoding either the spacer, scaffold, or extension sequence
519 (Supplemental File 2) were designed such that they had overlapping sticky ends
520 with each other and digested *pCFD3-NS*. Oligo pairs were separately annealed
521 and all were ligated into digested *pCFD3-NS* using T4 ligase (NEB, M0202). See
522 Supplemental File 3 for detailed cloning protocols.
523
524 To clone *pCFD3-PE-ebony*^{G111X}, *pCFD3-PE-white*^{A134X}, and *pCFD3-PE-*
525 *forked*^{D111X}, gBlock (IDT) dsDNA fragments encoding the entire pegRNA were
526 inserted into *pCFD3-NS* by Gibson assembly. Briefly, *pCFD3-NS* was digested
527 with BbsI and purified on a QIAquick column. gBlock fragments were designed
528 such that the pegRNA sequence was flanked by sequence homologous to
529 digested *pCFD3-NS* (Supplemental File 2). For each gene target, a gBlock was
530 inserted into digested *pCFD3-NS* by Gibson assembly. See Supplemental File 3
531 for detailed cloning protocols.
532
533 To clone *pCFD5-PE3-ebony*^{G111X}, *pCFD5-PE3-white*^{A134X}, and *pCFD5-PE3-*
534 *forked*^{D111X}, two overlapping gBlock (IDT) dsDNA fragments encoding the
535 pegRNA and nicking sgRNA were inserted into *pCFD5-NS* by Gibson assembly.
536 Briefly, *pCFD5-NS* was digested with BbsI and purified on a QIAquick column.
537 gBlock #1 encoded the sgRNA sequence flanked by sequence homologous to
538 *pCFD5-NS* and a partial sequence encoding the *O.s.* Gly tRNA, and gBlock #2
539 encoded the pegRNA flanked by the *O.s.* Gly tRNA and sequence homologous
540 to *pCFD5-NS* (Supplemental File 2). For each gene target, gBlocks #1&2 were
541 inserted together into digested *pCFD5-NS* by Gibson assembly. See
542 Supplemental File 3 for detailed cloning protocols.
543
544 **Cell culture**
545
546 *Drosophila* S2R+ cells were cultured at 25˚C using Schneider's media (21720-
547 024, ThermoFisher) with 10% FBS (A3912, Sigma) and 50 U/ml penicillin-
548 streptomycin (15070-063, ThermoFisher). S2R+ cells were transfected using
549 Effectene (301427, Qiagen) following the manufacturer's instructions.

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550
551 S2R+ cells stably expressing PE2 (PT5-PE2) were generated by transfecting
552 *pAct-PE2-HygroR* into the PT5 line (NEUMULLER *et al.* 2012), which expresses a
553 mCherry-Clic protein trap. PT5 cells were transfected in a 6-well dish at a
554 concentration of 1.8x10^6 cells/ml (2ml total volume). 24 hours after transfection,
555 200 µg/ml Hygromycin B (Calbiochem 400051-1MU) was added to the media. 5
556 days after transfection, cells were resuspended and transferred to a T75 flask
557 with fresh media containing 200 µg/ml Hygromycin B. 1 week later, cells were
558 resuspended, centrifuged at 100g for 10min, and resuspended in 3ml fresh
559 media containing 200 µg/ml Hygromycin B. Resuspended cells were transferred
560 serially into each well of a 6-well plate as a dilution series. Visible colonies were
561 resuspended and expanded after ~3 weeks.
562
563 Plasmids were transfected into PT5 or PT5-PE2 cells. Briefly, PT5 or PT5-PE2
564 cells were seeded at 600,000 cells/well of a 24-well plate and transfected with a
565 total of 200 ng plasmid DNA. PT5 cells were transfected with *pAct-Gal4*
566 (unpublished, Dr. Y. Hiromi, National Institute of Genetics, Mishima, Japan),
567 *pUAS-PE2*, pegRNA plasmid, and *pAct-GFP* (aka pLib6.6, unpublished) at a
568 3:3:3:1 ratio. PT5-PE2 cells were transfected with pegRNA plasmid and *pAct-*
569 *GFP* at a 3:1 ratio. To increase the chances that GFP+ cells contained prime
570 editing plasmids, we transfected less *pAct-GFP* plasmid relative to the other co-
571 transfected plasmids.
572
573 4 days after transfection, GFP+ cells were isolated by fluorescence-activated cell
574 sorting (FACS). Cells were first resuspended in culture media and pipetted into a
575 cell straining FACS tube (352235 Corning) to break up cell clump. 50,000 cells
576 with GFP fluorescence in the 60-80th percentile of fluorescence intensity were
577 sorted on an Aria 561 instrument into a single well of a 96-well plate and
578 incubated at 25˚C for 24hr.
579
580 5 days after transfection, genomic DNA was isolated from sorted and non-sorted
581 cells using the QuickExtract reagent (Lucigen QE09050). In addition, genomic
582 DNA was isolated from non-transfected PT5 cells as a negative control. Briefly,
583 culture media was removed and replaced with the same volume of QuickExtract
584 reagent. The solution was resuspended by pipetting, transferred to a PCR strip
585 tube, incubated at 65˚C for 15min, and then 98˚C for 2min.
586
587 **Fly culture and crosses**
588
589 Flies were maintained on standard fly food at 25˚C, or at 29˚C when noted. Fly
590 stocks were obtained from individual labs or the Bloomington Drosophila Stock
591 Center (BDSC) (indicated with BL#). Stocks used in this study are as follows: *yw*
592 (Perrimon Lab), *yw; Sp hs-hid/CyO* (derived from BL7757), *yw;; TM3,Sb/TM6, Tb*
593 (Perrimon Lab), *ywf* (BL1493), *yv nos-phiC31int; attP40* (BL25709), *yv nos-*
594 *phiC31int;; attP2* (BL25710), *yw*; *tub-Gal4* (BL5138), *yw*; *Act-Gal4* (BL4414), *yw*;
595 *nos-Gal4* (BL4442), *UAS-emptyVK37* (Bellen lab).

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596
597 Transgenic flies generated in this study (submitted to the BDSC):
598
599 *yw; UAS-PE2,w+ attP40* (BL#XXXXX)
600 *yw;; UAS-PE2,w+ attP2* (BL#XXXXX)
601 *yv; pCFD3-PE-ebony^{G111X},v+ attP40* (BL#XXXXX)
602 *yv; pCFD3-PE-white^{A134X},v+ attP40* (BL#XXXXX)
603 *yv; pCFD3-PE-forked^{D111X},v+ attP40* (BL#XXXXX)
604 *yv; pCFD5-PE3-ebony^{G111X},v+ attP40* (BL#XXXXX)
605 *yv; pCFD5-PE3-white^{A134X},v+ attP40* (BL#XXXXX)
606 *yv; pCFD5-PE3-forked^{D111X},v+ attP40* (BL#XXXXX)
607 *yscv; nos-PE2,v+ attP40*
608 *yv;; nos-PE2,v+ attP2*
609
610 Fly stocks with multiple transgenes (submitted to the BDSC):
611
612 *w*; *Act-Gal4*/CyO; *UAS-PE2,w+ attP2* (BL#XXXXX)
613 *w; UAS-PE2,w+ attP40; Tub-Gal4/TM6b* (BL#XXXXX)
614 *w; nos-Gal4; UAS-PE2,w+ attP2* (BL#XXXXX)
615
616 Transgenic flies were generated by phiC31 integration of attB-containing
617 plasmids into either attP40 or attP2 landing sites. Briefly, plasmid DNA was
618 purified twice on QIAquick columns and eluted in injection buffer (100 μM
619 NaPO4, 5 mM KCl), at a concentration of 200 ng/μl. Plasmid DNA was injected
620 into ~50 fertilized embryos (*yv nos-phiC31int; attP40* or *yv nos-phiC31int;; attP2*)
621 and resulting progeny were outcrossed to screen for transgenic founder progeny.
622 *nos-PE2* and pegRNA insertions were isolated by screening for *vermillion*+ eye
623 color. *UAS-PE2* insertions were isolated by screening for *white*+ eye color.
624
625 For PE2 toxicity experiments*, Act-Gal4/CyO* or *tub-Gal4/TM3-Sb* was crossed
626 with either *UAS-empty* (ChrII), *UAS-PE2* (ChrII), or *UAS-PE2* (ChrIII) and
627 progeny were raised at either 25˚C or 29˚C starting at egg deposition. The
628 frequency of PE2 expressing progeny was determined by counting the number of
629 adult non-balancer progeny and dividing by the total number of flies (# non-
630 balancer/# non-balancer + # balancer).
631
632 For somatic editing experiments, *Act>PE2* or *tub>PE2* flies were crossed with
633 pegRNA flies and adult PE2/pegRNA progeny analyzed for mutant phenotypes.
634
635 For germ line editing experiments, *nos-PE2* or *nos>PE2* flies were crossed with
636 *pCFD5-PE3-e^{G111X}* flies and G1 progeny were crossed with *TM3,e^1/TM6b,e^1*. To
637 screen different germ cell PE2 genotypes and temperature conditions, G1
638 crosses were performed as pools of 10 PE2/pegRNA males or females. G1
639 crosses were performed as single PE2/pegRNA male or female crosses for
640 optimal conditions (*nos>PE2*, 29˚C + h.s.). The phenotypes of G2 progeny were
641 scored as either wild-type or *ebony* (dark cuticle pigment) on a fly dissecting

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642 scope. To heat shock G1 larvae, we incubated larvae at 37˚C for 1hr in five
643 separate treatments after egg deposition: 24hr, 48hr, 72hr, 96hr, and 120hr.
644
645 Focal stack images of adult flies were obtained using a Zeiss Axio Zoom V16
646 fluorescence microscope and merged using Helicon Focus 7. Images were then
647 processed using Adobe Photoshop CS6.
648
649 Fly genomic DNA was isolated by grinding a single fly in 50µl squishing buffer
650 (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl) with 200µg/ml Proteinase K
651 (3115879001, Roche), incubating at 37˚C for 30 min, and 95˚C for 2 minutes. For
652 somatic editing experiments, genomic DNA was collected from adult male flies
653 unless otherwise noted. For germ line editing experiments, genomic DNA was
654 collected from both male and female G2 adult flies.
655
656 **Amplicon sequencing**
657
658 Genomic edit sites were amplified by PCR to yield amplicons for NGS. Briefly,
659 1µl of S2R+ or fly genomic DNA was used in a PCR reaction using Q5 High-
660 Fidelity DNA Polymerase (NEB M0491L). Primer pairs (Supplemental File 2)
661 were designed to yield amplicons ~200-280 bp in size with the intended editing
662 site located within 100 bp of either the forward or reverse primer. PCR fragments
663 were purified using QIAquick columns (28115, Qiagen) and submitted to the
664 MGH CCIB DNA Core (CRISPR Sequencing), or Genewiz (Amplicon-EZ).
665
666 NGS reads were analyzed using CRISPResso2 (version 2.0.38) (CLEMENT *et al.*
667 2019). To calculate the percent of reads with the precise edit, we used the
668 following parameters: "--prime_editing_pegRNA_spacer_seq", "--
669 prime_editing_pegRNA_extension_seq", "--
670 prime_editing_pegRNA_scaffold_sequence", "--ignore_substitutions", and "--
671 discard_indel_reads". The precise editing frequency was calculated from
672 "CRISPResso_quantification_of_editing_frequency.txt", for the "Prime-edited"
673 amplicon, as the # unmodified/reads aligned all amplicons. To determine the
674 percent of reads with indels, we ran CRISPResso2 with standard settings and
675 the --ignore_substitutions parameter. The indel frequency was calculated from
676 "CRISPResso_quantification_of_editing_frequency.txt", as the # modified/#
677 reads_aligned.
678
679 For S2R+ and fly experiments involving the edits *ebony*^{G111X}, *white*^{A134X},
680 *forked*^{D111X}, we specified a quantification window ("-qwc") that encompasses the
681 region between the pegRNA and nicking sgRNA (spanning the -6 position
682 relative to the pegRNA PAM to the -6 position relative to the sgRNA PAM)
683 (*ebony*: 96-158; *forked*: 97-159; *white*: 112-187).
684
685 Fastq files containing amplicon reads will be deposited at the NCBI SRA.
686
687 **References:**

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811
812 **Figure legends:**
813
814 **Figure 1. Prime editing in cultured S2R+ cells. A.** Diagram of PE2 expression
815 plasmid *pUAS-PE2*. *UAS*, *Upstream activating sequence*; NLS, Nuclear
816 localization sequence; SV40, 3' UTR; *w+*, *white+* rescue transgene; *attB*, phiC31
817 recombination site. **B.** Diagram of *pCFD3-NS* pegRNA expression plasmid. *BbsI*
818 sites indicate cloning site for pegRNA encoding sequence. *dU6:3*, *U6* promoter;
819 *U6* 3', *U6* downstream region; *v+*, *vermillion+* rescue transgene. **C.** *ebony*
820 genomic region showing target site and installed edit (*ebony*^{G111X}). **D.** Dual
821 sgRNA and pegRNA expression plasmid *pCFD5-NS*. tRNA, *D.m.* and *O.s.* Gly
822 tRNA sequence. **E.** Schematic of S2R+ prime editing experiment. **F.**

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823 Quantification of precise editing and indels from S2R+ transfection experiments
824 by amplicon sequencing. tfx, transfection.
825
826 **Supplemental Figure 1. Related to Figure 1. A.** Diagram of PE2 expression
827 plasmid *pAct-PE2*. **B.** Schematic of experiments to detect and quantify insertion
828 events in transfected S2R+ cells. **C.** DNA gel images of targeted PCR
829 amplification of the insertion site. **D.** Quantification of precise *ebony*^{23bpBC}
830 insertion and indel percentage from S2R+ transfection experiments by amplicon
831 sequencing. **E.** *white* and *forked* genomic region showing target site and installed
832 edits (*white*^{A134X} and *forked*^{D111X}).
833
834 **Figure 2. Prime editing in flies. A.** Schematic of transgenic expression of prime
835 editing components in flies and editing at an endogenous locus. Enhancer-
836 specific Gal4 directs the spatial and developmental timing of PE2 expression. **B.**
837 Quantification of adult fly viability from ubiquitous PE2 expression during
838 developmental stages and raised at either 25˚C or 29˚C. *Act-Gal4/CyO* or *tub-*
839 *Gal4/TM3* were crossed with *UAS-PE2* (Chr. II), *UAS-PE2* (Chr. III), or *UAS-*
840 *empty* (negative control), and the percentage of progeny with or without the
841 balancer was calculated. Number of flies scored from left to right = 748, 687, 655,
842 157, 267, 202, 294, 413, 226, 131, 277, 238. **C.** Schematic of genetic crosses
843 between ubiquitous PE2 and pegRNA transgenic flies. **D.** Images of adult flies
844 with somatic editing using *Act>PE2*. Views of the dorsal side of whole adults
845 (top), scutellum (middle), and eye (bottom). Negative control is *attP40* and
846 classical loss of function allele shown on right. Females shown for editing of
847 *ebony* and *forked*, males shown for *white* editing. e^1 = w^1;; *TM3,e*^{1}/*TM6b,e*^{1}, f^1 =
848 y^1, w^1, f^1. **E.** Quantification of precise somatic editing and indel percentage in
849 adult flies by amplicon sequencing. Error bars show mean with SD. N=3 adult
850 flies. **F.** Schematic of two generation genetic crosses between germ cell PE2 and
851 pegRNA flies. **G.** Quantification of adult cuticle pigmentation (WT vs. *ebony*) in
852 G2 flies for three temperature conditions. Sex of G1 parent(s) is indicated above
853 graph. For the pooled crosses (10 G1 flies each, left of dotted line), the number
854 of G2 flies analyzed was (left to right) 453, 518, 574, 413, 702, 405, 514, 454,
855 514, 405, 376, 493, 557, 492, 510, 562, 471, 481. For single fly G1 crosses (right
856 of dotted line), the number of G2 flies analyzed was (left to right) 209, 109, 76,
857 139, 176, 104, 147, 275, 222. **H.** Quantification of Sanger sequencing analysis of
858 individual G2 flies from single G1 crosses. Eight G2 progeny were analyzed for
859 each of the nine G1 crosses. Sex of G1 parent is indicated above graph. **I.**
860 Sequence of the *ebony* target site in 72 mutant *ebony* G2 flies. **J.** Sequence
861 chromatogram (left) and image (right) of wild-type and *ebony*^{G111X} homozygous
862 adult flies.
863
864 **Supplemental Figure 2. Related to Figure 2. A.** Images of adult flies with
865 somatic editing using *tub>PE2*. Views of the dorsal side of whole adults (top),
866 scutellum (middle), and eye (bottom). Negative control is *attP40* and classical
867 loss of function allele shown on right. Females shown for editing of *ebony* and
868 *forked*, males shown for *white* editing. **B.** Diagram of PE2 expression transgene

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869 *nos-PE2*. *nos, nanos*; NLS, Nuclear localization sequence; 3' UTR, nanos 3'
870 UTR; *v+*, *vermillion+* rescue transgene; *attB*, phiC31 recombination site.
871
872 **Supplemental File 1. pegRNA and sgRNA sequences**
873
874 **Supplemental File 2. Oligo and dsDNA sequences**
875
876 **Supplemental File 3. pegRNA design and cloning protocols**
877
878 **Supplemental File 4. Amplicon sequencing data key**
879
880

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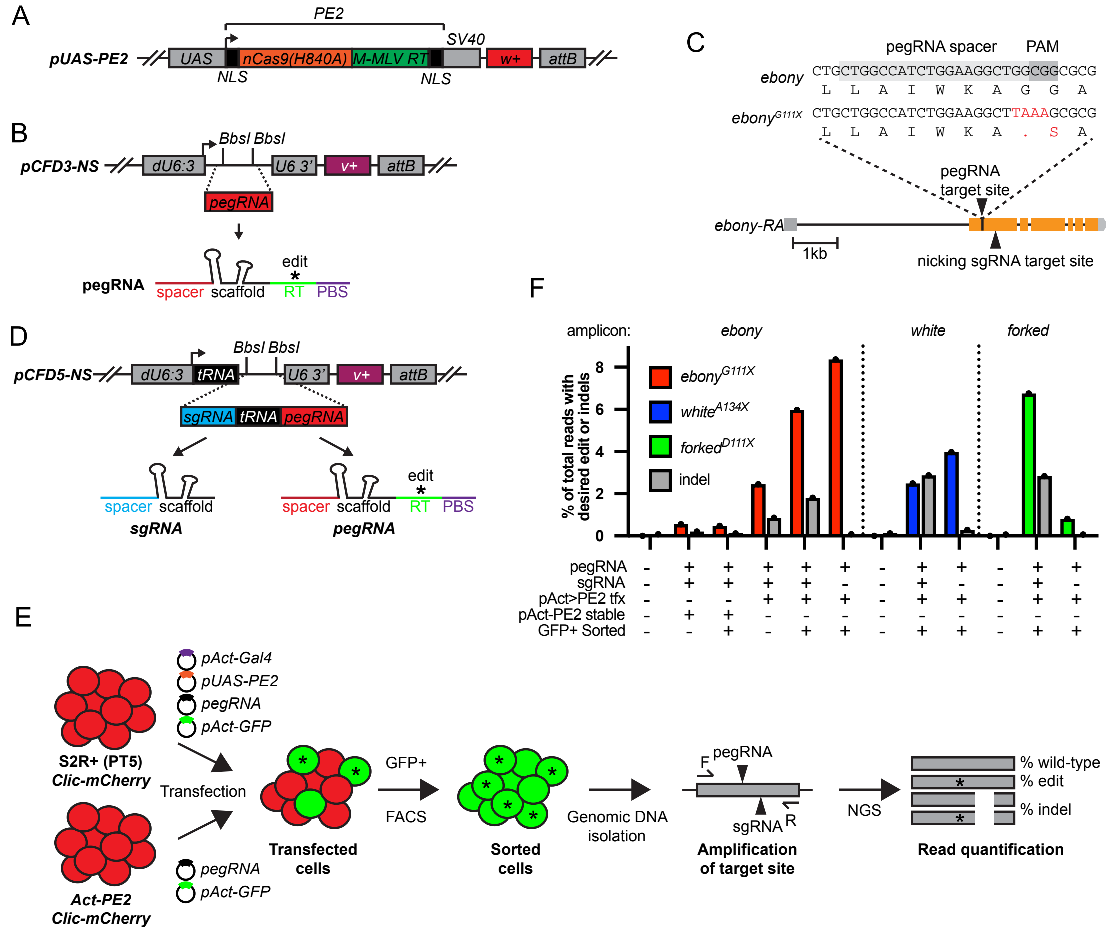

Figure 1

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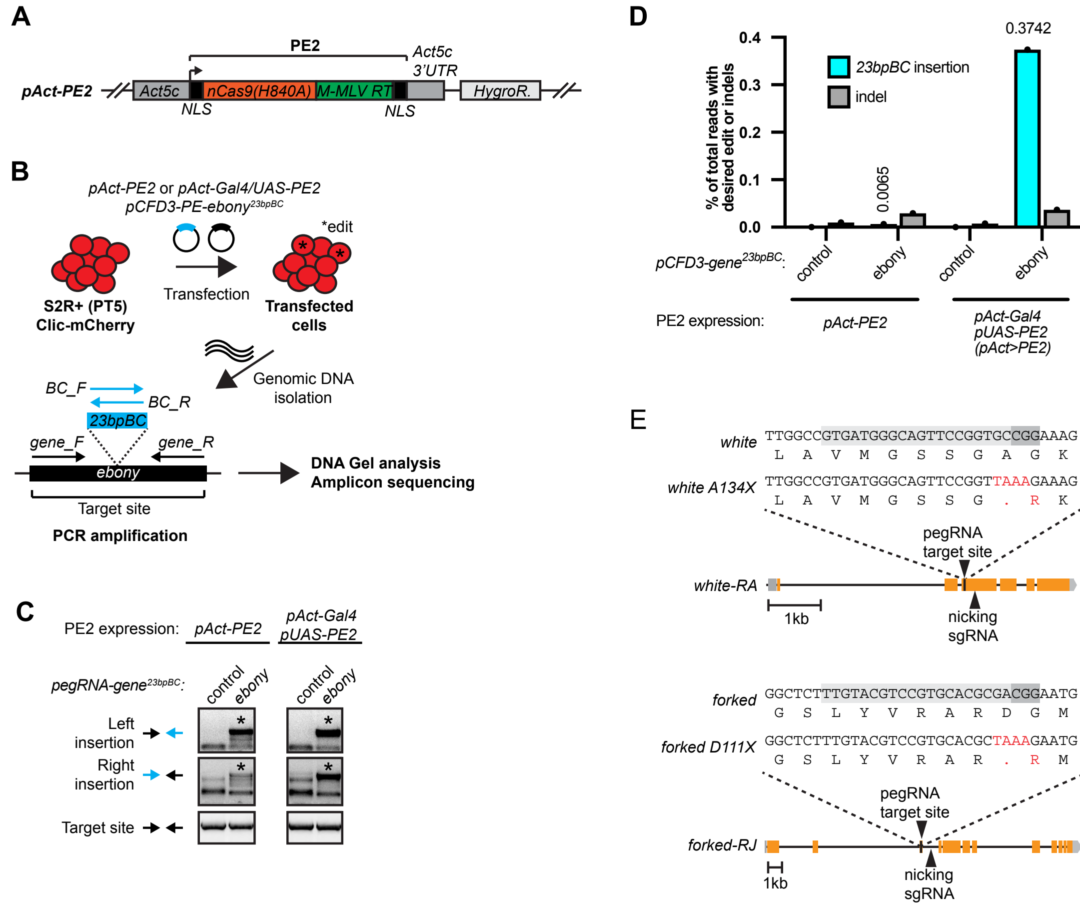

Supplemental Figure 1

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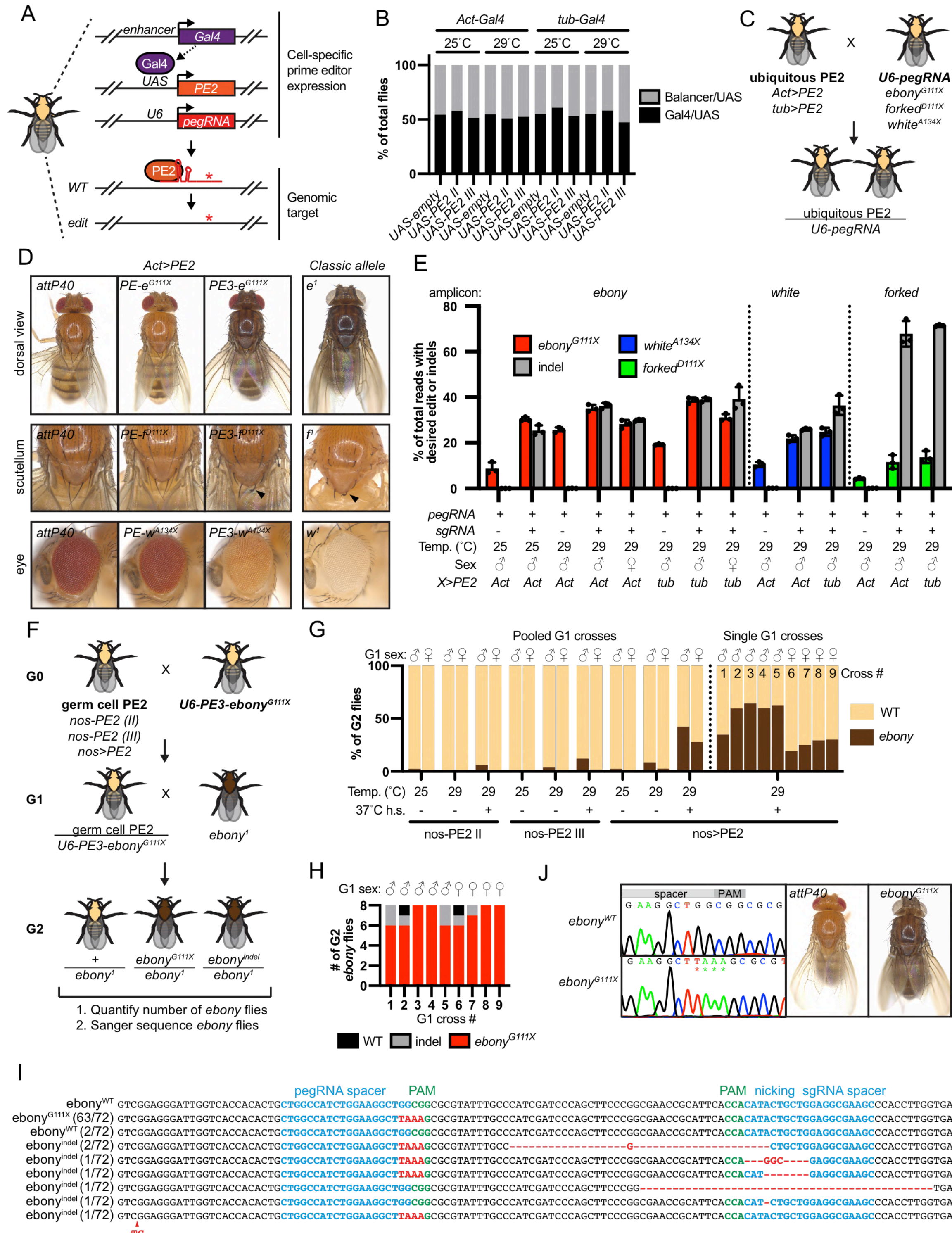

Figure 2

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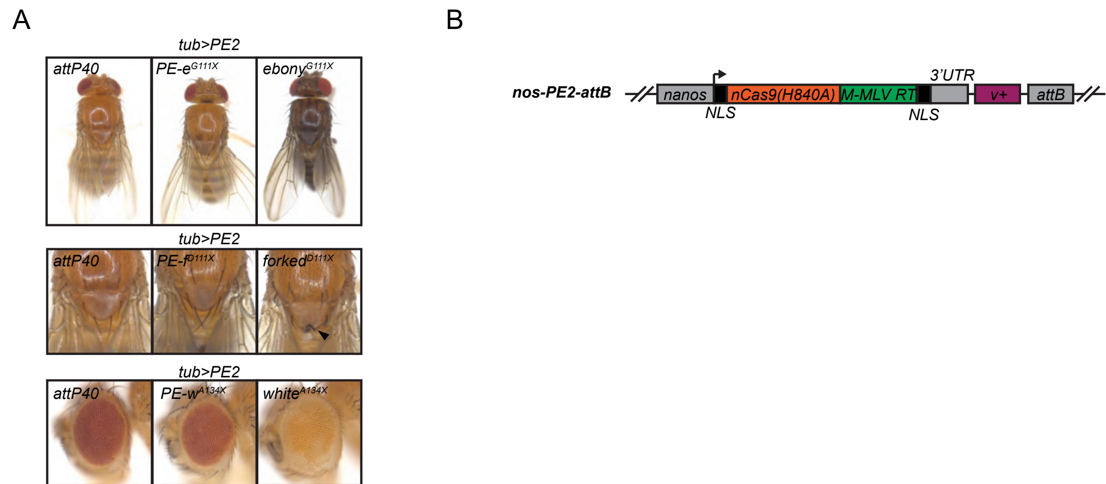

Supplemental Figure 2

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Supplemental File 1 - pegRNA and sgRNA sequences

pegRNA	**pegRNA spacer sequence**	**3' extension**	**PBS length**	**RT template length**	**nicking sgRNA**	**nicking sgRNA spacer sequence**
ebony_pFP545_23bpBC	CTGGCCATCTGGAAGGCTGG	GAAGCTGGGATCGATGGGCAAATACGCGCCGCCAaacaggccgcaagtgttcttaggGCCTTCCAGATGG	13	34		
ebony_pFP545_G111X	CTGGCCATCTGGAAGGCTGG	GGCAAATACGCGCtttaAGCCTTCCAGATGG	13	18	ebony_+57	GCTTCGCCTCCAGCAGTATG
white_ex3-3_A134X	GTGATGGGCAGTTCCGGTGC	AGGGTCGTCTTTCtttaACCGGAACTGCCCA	13	18	white_+70	TTGAGCAGTCGCATCCCGGA
forked_pFP801_D111X	TTGTACGTCCGTGCACGCGA	ATCGGTGCCATTCtttaGCGTGCACGGACGT	13	18	forked_+57	ATCTACTCACCATCCATTTG

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Supplemental File 2 - Oligo and dsDNA sequences		
Name	**Sequence**	**Purpose**
JB1633_pCFD3-NS_gBlock_BbsI-XbaI	agttcgtatatatagacctattttcaatttaacgtcggggtcttcgagaagaccttttttgcctacctggagcctgagagttgttcaataaaataaaaatgtttcgttttttgctttcgccagtatttattatttttcatcaatatgtattcaatttggtatgtatttagtaattgtaatatatagacaatggttttccgttgacgtacatacatctgacgtgtgtttatttagacataatagttatgttttcacatctttttaatgttcgcttaatgcgtatgcattctagacaattgtgctcggcaacagtatatttgt	gBlock to create pCFD3-NS
JB1690_gBlock_pCFD5-NS	agttcgtatatatagacctattttcaatttaacgtcggggctttgagtgtgtgtagacatcaagcatcggtggttcagtggtagaatgctcgcctgccacgcgggcggcccgggttcgattcccggccgatgcagggtcttcgagaagaccttttttgcctacctggagcctgagagttgttcaataaaataaaaatgtttcgttttttgctttcgccagtatttattatttttcatcaatatgtattcaatttggtatgtatttagtaattgtaatatatagacaatggttttccgttgacgtacatacatctgacgtgtgtttatttagacataatagttatgttttcacatctttttaatgttcgcttaatgcgtatgcattctagacaattgtgctcggcaacagtatatttgt	gBlock to create pCFD5-NS
JB265_Gibson_pEntr_1F	AAGGGTGGGCGCGCCGAC	Amplifies pEntr backbone for Gibson assembly
JB266_Gibson_pEntr_1R	GGTGAAGGGGGCGGCCGC	Amplifies pEntr backbone for Gibson assembly
JB1615_PE2_pEntr_F	ccgcggccgccccttcaccatgaaacggacagccgac	Amplifies PE2 coding sequence to assemble with pEntr backbone by Gibson
JB1616_PE2_stop_pEntr_R	gggtcggcgcgcccacccttttagactttcctcttcttcttggg	Amplifies PE2 coding sequence to assemble with pEntr backbone by Gibson
JB1613_PE2-nosbackbone_XbaI-AvrII_F	TCGCCtgAATTgagatctcTCTAGAggtacCGCCACCatgaaacggacagccgac	Amplifies PE2 coding sequence to assemble with pNos backbone by Gibson
JB1614_PE2-nosbackbone_XbaI-AvrII_R	TAAACCTcgagtggatcctcctaggtgctagAttagactttcctcttcttcttggg	Amplifies PE2 coding sequence to assemble with pNos backbone by Gibson
JB157_Gibson_MT-GW_backbone_1F	ACCGAGAGCATCTGGCCA	Amplifies MK33-GW backbone to assemble with Actin5c promoter
JB158_Gibson_MT-GW_backbone_1R	GATCCAGACATGATAAGATACATTGATGAG	Amplifies MK33-GW backbone to assemble with Actin5c promoter
JB159_Gibson_MT-GW_actP_1F	tatcttatcatgtctggatcGCATGCAATTCTATATTCTAAAAACACAAATG	Amplifies Actin5c promoter to insert into MK33-GW by Gibson
JB160_Gibson_MT-GW_actP_1R	attggccagatgctctcggtATCTGGATCCGGGGTCTC	Amplifies Actin5c promoter to insert into MK33-GW by Gibson
ebony_targetsite_F	GAGGGATTGGTCACCACACT	Amplifies indicated target site from genomic DNA, used to amplify in ebony23bpBC experiments
ebony_targetsite_2F	CCGGTTCCTGCAGCCAAACA	Amplifies indicated target site from genomic DNA, used to amplify in ebonyG111X experiments
ebony_targetsite_R	GGGATTTGGCATACAGTTCG	Amplifies indicated target site from genomic DNA
white_targetsite_F	TTCGCAGTCGGCTGATCTGT	Amplifies indicated target site from genomic DNA
white_targetsite_R	CACAGGTTGGCCATTGAGCA	Amplifies indicated target site from genomic DNA
forked_targetsite_F	ACGATGTCACGCCCGTTTAC	Amplifies indicated target site from genomic DNA
forked_targetsite_R	CAACTGCTGCAGTTGGCCAA	Amplifies indicated target site from genomic DNA
JB1647_BC_F	TAAGAACACTTGCGGCCTGT	Binds to inserted 23bp BC for amplification of insertions
JB1647_BC_R	ACAGGCCGCAAGTGTTCTTA	Binds to inserted 23bp BC for amplification of insertions
JB1637_scaffold_top	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG	sgRNA scaffold for annealing and cloning a pegRNA into pCFD3-NS
JB1638_scaffold_bot	GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA	sgRNA scaffold for annealing and cloning a pegRNA into pCFD3-NS
JB1639_ebony_pFP545_spacer_top	gtcgCTGGCCATCTGGAAGGCTGG	ebony_23bpBC pegRNA spacer for annealing and cloning into pCFD3-NS
JB1640_ebony_pFP545_spacer_bot	aaacCCAGCCTTCCAGATGGCCAG	ebony_23bpBC pegRNA spacer for annealing and cloning into pCFD3-NS
JB1641_ebony_pFP545_3'ext_top	gtgcGAAGCTGGGATCGATGGGCAAATACGCGCCGCCAAACAGGCCGCAAGTGTTCTTAGGGCCTTCCAGATGG	ebony_23bpBC pegRNA 3'extension for annealing and cloning into pCFD3-NS
JB1642_ebony_pFP545_3'ext_bot	aaaaCCATCTGGAAGGCCCTAAGAACACTTGCGGCCTGTTTGGCGGCGCGTATTTGCCCATCGATCCCAGCTTC	ebony_23bpBC pegRNA 3'extension for annealing and cloning into pCFD3-NS
JB1721_pCFD5-NS_ebony_pFP545_G111X_PE3_gBlock1	cgggttcgattcccggccgatgcagcttcgcctccagcagtatggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctaacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacc	ebonyG111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1724_pCFD5-NS_ebony_pFP545_G111X_PE3_gBlock2	taacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgcactggccatctggaaggctgggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttttgcctacctggagcctgag	ebonyG111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1722_pCFD5-NS_white_ex3-3_A134X_PE3_gBlock1	cgggttcgattcccggccgatgcattgagcagtcgcatcccggagttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctaacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacc	whiteA134X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1725_pCFD5-NS_white_ex3-3_A134X_PE3_gBlock2	taacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgcagtgatgggcagttccggtgcgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcAGGGTCGTCTTTCtttaACCGGAACTGCCCAtttttttgcctacctggagcctgag	whiteA134X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1723_pCFD5-NS_forked_pFP801_D111X_PE3_gBlock1	cgggttcgattcccggccgatgcaatctactcaccatccatttggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctaacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacc	forkedD111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1726_pCFD5-NS_forked_pFP801_D111X_PE3_gBlock2	taacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgcattgtacgtccgtgcacgcgagttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcATCGGTGCCATTCtttaGCGTGCACGGACGTtttttttgcctacctggagcctgag	forkedD111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS

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Supplemental File 3
pegRNA cloning for Prime Editing in *Drosophila*, June 2020, Version 1.0

Justin Bosch, Perrimon Lab, Harvard Medical School

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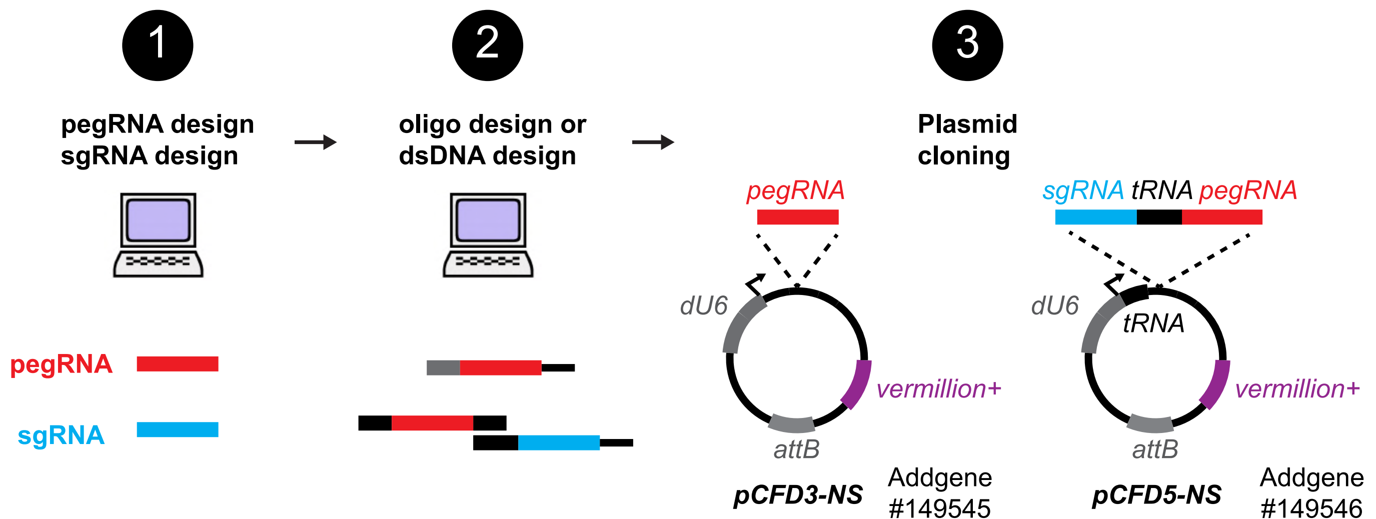
A. Introduction
B. pegRNA and nicking sgRNA design
C. Oligo and dsDNA design
D. Cloning protocol for *pCFD3-NS* using annealed oligos
E. Cloning protocol for *pCFD3-NS* using a dsDNA fragment
F. Cloning protocol for *pCFD5-NS* using two dsDNA fragments

A. Introduction: These protocols are used to assemble plasmids to express pegRNAs under the control of the *Drosophila U6-3* promoter. pegRNAs are designed to make a precise edit in the genome, and optional nicking sgRNAs are designed to enhance prime editing efficiency (PE3 system). To express pegRNAs and sgRNAs, they are encoded in annealed oligos or dsDNA fragments, and then cloned into one of two empty expression plasmids. *pCFD3-NS* is used for expression of a single pegRNA. *pCFD5-NS* is used for expression of a pegRNA/sgRNA pair. *pCFD3-NS* and *pCFD5-NS* do not contain a sgRNA scaffold (NS = No Scaffold), and are slight modifications of the sgRNA-expression plasmids *pCFD3* and *pCFD5* (PORT *et al.* 2014; PORT AND BULLOCK 2016). *pCFD3-NS* and *pCFD5-NS* contain an *attB* site for phiC31 integration and a *vermillion+* marker to select transgenic flies.



Summary of pegRNA-expression plasmids:

Plasmid	Addgene #	Promoter	Used to express	Cloning methods	Fly marker	Bacterial resistance
pCFD3-NS	149545	*dU6:3*	pegRNA	Annealed oligos/T4 Ligase 1 dsDNA fragment/Gibson	*vermillion+*	Ampicillin
pCFD5-NS	149546	*dU6:3*	sgRNA + pegRNA	2 dsDNA fragments/Gibson	*vermillion+*	Ampicillin

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B. pegRNA and nicking sgRNA design

Automatic design (recommended):

PrimeDesign (HSU *et al.* 2020): http://primedesign.pinellolab.org/

pegFinder (CHOW *et al.* 2020): http://pegfinder.sidichenlab.org/

Manual design (optional):

1. Create wild-type (WT) and edited sequence files for annotation
2. WT sequence - select a pegRNA spacer near the desired edit, ensuring the edit is 3' to nick site.
3. Edited sequence - annotate the primer binding site (PBS) by selecting ~13bp 5' to the nick site.
4. Edited sequence - annotate the reverse transcribed (RT) region by selecting ~13-18bp 3' to nick site.
5. Edited sequence – The reverse complement of the PBS-edit-RT sequence is the pegRNA 3' extension.
6. WT sequence - select a sgRNA target on the non-edited strand between +40 and +90 from the pegRNA nick.

Notes:

- Avoid starting pegRNA 3' extension with a "C".
- Edits or silent mutations that affect the PAM or pegRNA spacer sequence increase efficiency.
- Use a shorter RT sequence if region has high G:C content.

Example pegRNA and nicking sgRNA design:

pegRNA spacer
nicking sgRNA spacer
PAM
nick = |
PBS
RT
edit
scaffold

>ebony_WT
CCGGTTCCTGCAGCCAAACAGCGATGGTGACTTCATCGTGGCTGTGTGCATGCAGCCGTCGGAGGGATTGGTCACCACACT
GCTGGCCATCTGGAAGGC|TGGCGGCGCGTATTTGCCCATCGATCCCAGCTTCCCGGCGAACCGCATTCACCACAT|ACTG
CTGGAGGCGAAGCCCACCTTGGTGATTCGCGACGATGACATCGACGCCGGCCGTTTCCAGGGAACTCCCACGTTATCCACC
ACCGAACTGTATGCCAAATCCC

>ebony_GGCG331-334TAAA_G111X
CCGGTTCCTGCAGCCAAACAGCGATGGTGACTTCATCGTGGCTGTGTGCATGCAGCCGTCGGAGGGATTGGTCACCACACT
GCTGGCCATCTGGAAGGCTtaaaGCGCGTATTTGCCCATCGATCCCAGCTTCCCGGCGAACCGCATTCACCACATACTGCT
GGAGGCGAAGCCCACCTTGGTGATTCGCGACGATGACATCGACGCCGGCCGTTTCCAGGGAACTCCCACGTTATCCACCAC
CGAACTGTATGCCAAATCCC

>pegRNA_spacer
CTGGCCATCTGGAAGGCTGG

>pegRNA_extension
GGCAAATACGCGCtttaAGCCTTCCAGATGG

>nicking_sgRNA_spacer
GCTTCGCCTCCAGCAGTATG

>pegRNA
CTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
GCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGG

>nicking_sgRNA
GCTTCGCCTCCAGCAGTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
GCACCGAGTCGGTGC

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C. Oligo and dsDNA design

C1. For cloning into pCFD3-NS by T4 ligation (single pegRNA) (See section D)

Order oligos with overhangs (5' lowercase sequence)

```
>pegRNA_spacer_top
gtcgCTGGCCATCTGGAAGGCTGG

>pegRNA_spacer_bot
aaacCCAGCCTTCCAGATGGCCAG

>Scaffold_top:
gtttTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG

>Scaffold_bot:
gcacCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA

>pegRNA_extension_top
gtgcGGCAAATACGCGCtttaAGCCTTCCAGATGG

>pegRNA_extension_bot
aaaaCCATCTGGAAGGCTtaaaGCGCGTATTTGCC
```

Annealed oligos:

```
>pegRNA_spacer
5'-gtcgCTGGCCATCTGGAAGGCTGG-3'
    3'-GACCGGTAGACCTTCCGACCcaaa-5'

>Scaffold
5'-gtttTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG-3'
    3'-ATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAATAGTTGAACTTTTTCACCGTGGCTCAGCcacg-5'

>pegRNA_extension
5'-gtgcGGCAAATACGCGCtttaAGCCTTCCAGATGG-3'
    3'-CCGTTTATGCGCGaaatTCGGAAGGTCTACCaaaa-5'
```

Cloning:

```
>pCFD3-NS cut w/ BbsI
5'-agacctattttcaatttaac          tttttgcctacctggagcctgag-3'
3'-tctggataaaagttaaattgcagc          aacggatggacctcggactc-5'

>pCFD3-pegRNA_final
agacctattttcaatttaacgtcgCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttg
cctacctggagcctgag
```

C2. dsDNA to clone into pCFD3-NS by Gibson assembly (single pegRNA) (See section E)

Append homology arms (black, lowercase) to pegRNA that overlap with pCFD3-NS cut w/ BbsI.

```
>dsDNA_fragment_pCFD3-NS
agacctattttcaatttaacgtcgCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGt
tttttgcctacctggagcctgag
```

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Cloning:

```
>pCFD3-NS cut w/ BbsI
5'-agacctattttcaatttaac          tttttgcctacctggagcctgag-3'
3'-tctggataaaagttaaattgcagc          aacggatggacctcggactc-5'
```

```
>pCFD3-pegRNA_final
agacctattttcaatttaacgtcgCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttg
cctacctggagcctgag
```

Note: If needed, homology arms can be extended longer (~100bp each). This can help decrease complexity scores using IDT gBlocks.

C3. dsDNAs to clone into pCFD5-NS by Gibson assembly (nicking sgRNA and pegRNA) (See section F)

Append homology arms (black, lowercase) to nicking sgRNA and pegRNA that overlap with pCFD3-NS cut w/ BbsI and encode rice Os-tRNA^{Gly} (lowercase, italic)

```
>dsDNA_fragment1_pCFD5-NS
cgggttcgattcccggccgatgcaGCTTCGCCTCCAGCAGTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCaacaaagcaccagtggtctagtggtagaatag
taccctgccacggtacagacc
```

```
>dsDNA_fragment2_pCFD5-NS
aacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgc
aCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
AAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttgcctacctggagcctgag
```

```
>pCFD5-sgRNA-tRNA-pegRNA_final
cgggttcgattcccggccgatgcaGCTTCGCCTCCAGCAGTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCaacaaagcaccagtggtctagtggtagaatag
taccctgccacggtacagacccgggttcgattcccggctggtgcaCTGGCCATCTGGAAGGCTGGGTTTTAGAGCT
AGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGC
GCtttaAGCCTTCCAGATGGtttttgcctacctggagcctgag
```

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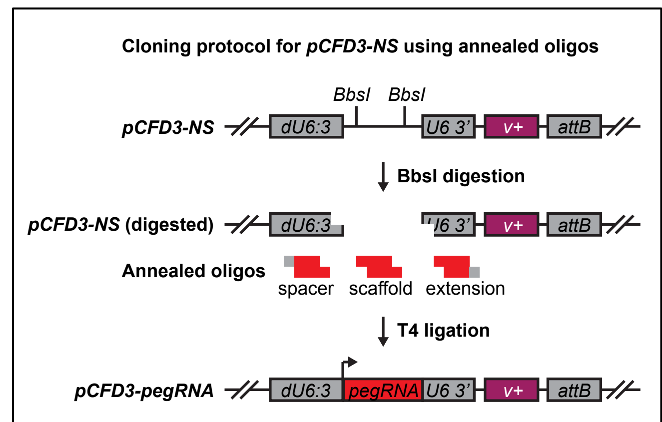
D. Cloning protocol for *pCFD3-NS* (Addgene # 149545) using annealed oligos



D1. Design pegRNA and order oligos (see Sections B&C).

D2. Digest/dephosphorylate *pCFD3-NS*

5μg pCFD3-NS
3μl BpiI (cuts BbsI) (Fermentas, FD1014)
3μl FastAP (Fermentas, EF0651)
6μl 10x FastDigest Buffer
Xμl H20
60ul total

D3. Gel-purify digested *pCFD3-NS* backbone (~6.2kb).

D4. Phosphorylate and anneal each pair of oligos in PCR tubes

1μl Top oligo (100μM)
1μl Bottom oligo (100μM)
1μl 10x T4 Ligation buffer (NEB, B0202S)
6.5μl H20
.5μl T4 PNK (NEB, M0201)
10μl total

37˚C for 30min, 95˚C for 5min, then ramp down to 25˚C at 5˚C/min

D5. Dilute annealed/phosphorylated oligos 1:200 in H20

D6. Ligate annealed oligos into digested *pCFD3-NS*

Xμl digested pCFD3-NS (50ng)
1μl **spacer** diluted annealed oligo
1μl **scaffold** diluted annealed oligo
1μl **3' extension** diluted annealed oligo
1.5μl 10x T4 Ligation Buffer (NEB, B0202S)
Xμl H20
1μl T4 DNA ligase (NEB, M0202)
15μl total

Incubate reaction at room temperature for 30min.

D7. Transform ligation into competent cells and grow colonies on LB-agar Ampicillin plates

D8. (Optional) Colony PCR to identify candidate pegRNA plasmids

pCFD3genoF ACGTTTTATAACTTATGCCCCTAAG
pCFD3genoR GCCGAGCACAATTGTCTAGAATGC

Uncut backbone = 490bp
Correct insert = 638bp (depends on pegRNA length)

D9. Culture colonies with LB + Ampicillin and sequence confirm plasmids

pCFD3seqF ACCTACTCAGCCAAGAGGC

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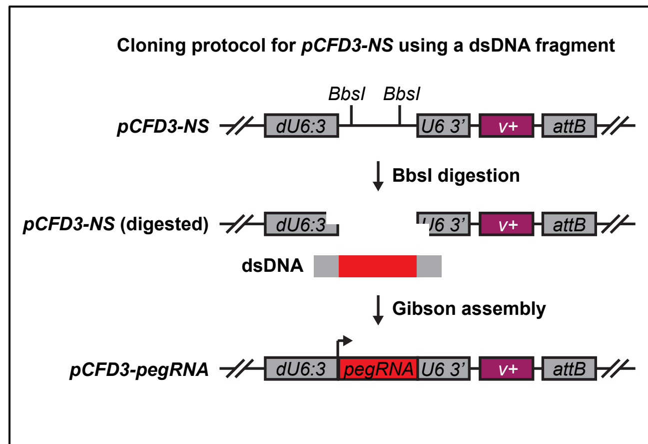
E. Cloning protocol for *pCFD3-NS* (Addgene # 149545) using a dsDNA fragment



E1. Design pegRNA and order dsDNA fragment (see Sections B&C).

E2. Digest/dephosphorylate plasmid

5µg *pCFD3-NS*
3µl BpiI (cuts BbsI) (Fermentas, FD1014)
3µl FastAP (Fermentas, EF0651)
6µl 10x FastDigest Buffer
Xµl H20
60ul total

E3. Gel-purify digested *pCFD3-NS* backbone (~6.2kb).

E4. Gibson assembly

Xµl digested pCFD3-NS (50ng)
Xµl dsDNA fragment (5ng)
2.5µl Gibson master mix (NEB, E2611)
Xµl H20
5µl total

Incubate reaction at 50˚C for 30min.

E5. Transform ligation into competent cells and grow colonies on LB-agar Ampicillin plates

E6. (Optional) Colony PCR to identify candidate pegRNA plasmids

pCFD3genoF ACGTTTTATAACTTATGCCCCTAAG
pCFD3genoR GCCGAGCACAATTGTCTAGAATGC

Uncut backbone = 490bp
Correct insert = 638bp (depends on pegRNA length)

E7. Culture colonies with LB + Ampicillin and sequence confirm plasmids

pCFD3seqF ACCTACTCAGCCAAGAGGC

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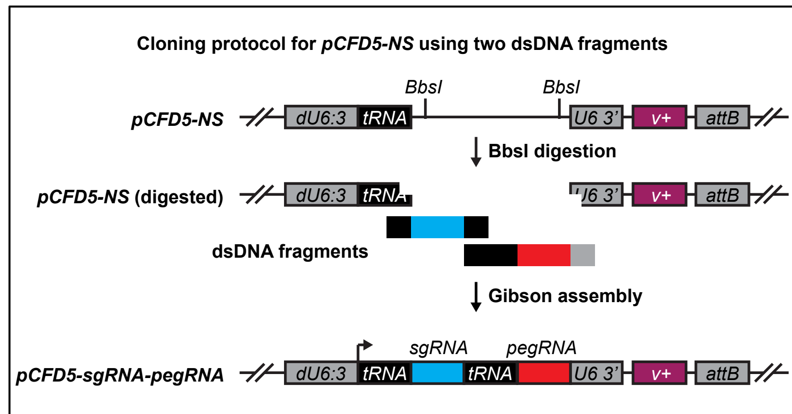
F. Cloning protocol for *pCFD5-NS* (Addgene # 149546) using two dsDNA fragments



F1. Design pegRNA and nicking sgRNA, and order dsDNA fragments (see Sections B&C).

F2. Digest/dephosphorylate plasmid

5µg *pCFD5-NS*
3µl BpiI (cuts BbsI) (Fermentas, FD1014)
3µl FastAP (Fermentas, EF0651)
6µl 10x FastDigest Buffer
Xµl H20
60ul total

F3. Gel-purify digested *pCFD5-NS* backbone (~6.3kb).

F4. Gibson assembly

Xµl digested pCFD5-NS (50ng)
Xul dsDNA fragment 1 (5ng)
Xul dsDNA fragment 2 (5ng)
2.5µl Gibson master mix (NEB, E2611)
Xµl H20
5ul total

Incubate reaction at 50˚C for 30min.

F5. Transform ligation into competent cells and grow colonies on LB-agar Ampicillin plates

F6. (Optional) Colony PCR to identify candidate pegRNA plasmids

pCFD3genoF ACGTTTTATAACTTATGCCCCTAAG
pCFD3genoR GCCGAGCACAATTGTCTAGAATGC

Uncut backbone = 587bp
Correct insert = ~846bp (depends on pegRNA length)

F7. Culture colonies with LB + Ampicillin and sequence confirm plasmids

pCFD3seqF ACCTACTCAGCCAAGAGGC

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