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1 **CRISPR/Cas9 gene editing to generate *Drosophila* LexA lines in secondary school classes**

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20 Running Title: HACKy-mediated GAL4 to LexA conversion

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22 **ABSTRACT**

23 Genome editing *in vivo* with CRISPR/Cas9 generates powerful tools to study gene regulation
24 and function. We developed CRISPR-based methods that permitted secondary school student
25 scientists to convert *Drosophila* GAL4 lines to LexA lines. Our novel curricula implement a new
26 donor strain optimizing Homology-assisted CRISPR knock-in (HACK) that simplifies screening
27 using light microscopy. Successful curricula adoption by a consortium of schools led to the
28 generation and characterization of 16 novel LexA lines. This includes extensive comparative
29 tissue expression analysis between the parental Gal4 and derived LexA lines. From this
30 collaboration, we established a workflow to systematically generate LexA lines from frequently-
31 used GAL4 lines. Modular courses developed from this effort can be tailored to specific
32 secondary school scheduling needs, and serve as a template for science educators to innovate
33 courses and instructional goals. Our unique collaborations highlight that resources and expertise
34 harnessed by university-based research laboratories can transform experiential science
35 instruction in secondary schools while addressing research needs for the community of science.

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36 **INTRODUCTION**

37 *Drosophila melanogaster* is a powerful organism to investigate gene function in diverse
38 biological settings, including embryonic development and metabolism. To study genes in specific
39 *Drosophila* organs, compartments, or cell populations, investigators have developed binary gene
40 expression systems (Brand and Perrimon 1993; Lai and Lee 2006; Potter et al 2010; Kim et al
41 2021). These systems combine (1) cell-specific *cis*-regulatory elements that drive the expression
42 of a transgene encoding an exogenous transcriptional activator (e.g., GAL4), and (2) a responder
43 transgene whose expression is directed by the transcriptional activator. However, novel
44 challenges in studying more complex biological contexts like inter-cellular or inter-organ
45 communication necessitate parallel genetic manipulations of two, or more, independent cell
46 populations. Multiple independent binary expression systems can be combined in a single fly to
47 study genetic perturbations of multiple tissues simultaneously. This approach has led to powerful
48 epistasis experiments between different tissues (Shim et al. 2013), simultaneous clonal lineage
49 analysis of multiple cell populations (Lai and Lee 2006; Bosch et al. 2015), visualization of
50 specific physical cell-cell contacts (Gordon and Scott 2009; Bosch et al. 2015, Macpherson et al.
51 2015), and measures of hormonal responses in target cells (Tsao et al 2022).

52 Simultaneous use of orthogonal binary expression systems requires generation of
53 independent cell-specific transgenic transcriptional activators. For the LexA/LexAop binary
54 expression system, diverse tissue-specific LexA activator lines have been systematically
55 generated by cloning and linking putative enhancers to *LexA* (Pfeiffer et al 2010) or by inserting
56 LexA-encoding transposons near endogenous enhancers ("enhancer trapping"; Kockel et al
57 2016; Kockel et al 2019; Kim et al 2023). This work enabled detailed studies of tissue-specific
58 *LexA* expression. To expand the collection of activator lines, and to exploit the thousands of
59 extant GAL4 lines (FlyBase) as potential targets, Lin and Potter (2016) developed Homology-
60 assisted CRISPR knock-in (HACK) to replace GAL4 with an orthogonal transcriptional activator.
61 Similar CRISPR/Cas9-based approaches have been successfully applied to generate LexA lines
62 from existing GAL4 lines with well-characterized tissue expression patterns (Chang et al 2022;
63 Karuparti et al 2023). However, no systematic approach to convert frequently used GAL4 lines to
64 LexA has been described.

65 We postulated that university-based research laboratories had unrealized potential to develop
66 and strongly influence traditional science instruction covering biology and genetics in secondary
67 schools, and simultaneously address unmet needs - like new LexA driver lines - of the scientific
68 community. In 2011, Stanford University investigators developed courses (hereafter, the Stan-X

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69 curricula) to introduce students to discovery-based experimental investigations based on fruit fly
70 genetics and biology (Kockel et al 2016, 2019; Chang et al 2022; Wendler et al 2022; Kim et al
71 2023). Currently, there are 15 Stan-X partner schools that run semester-or year-long courses,
72 based on 'enhancer traping' to generate novel LexA-expressing lines. Recently, multiple
73 partnering schools requested diversification of Stan-X course content, including the development
74 of 'modular' courses that could run for 12 weeks or less, but still provide opportunities for
75 discovery through experimental science. In response, we developed an *in vivo* CRISPR-based
76 curriculum with fruit flies for a consortium of schools described here. To enable brightfield
77 microscopy-based genetic screens to score LexA flies "converted" from a GAL4 line, we
78 developed a new yellow^{+} HACK donor strain and tested it with our secondary school consortium.
79 Here, we report the successful generation and characterization of novel LexA lines by student
80 scientists. This effort has established a productive paradigm of university researchers and
81 secondary schools collaborating to generate new LexA lines based on the extensive enhancer
82 characterization and use of its parental GAL4 line.

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83 **MATERIALS AND METHODS**

84 ***Drosophila* strains**

85 Except for LexA.G4HACK donor lines, all other *Drosophila* lines in **Table 1**, **Figures 3,** and **S2**
86 were obtained from the Bloomington Drosophila Stock Center (BDSC).

87 **Generation of version 1 and version 2 LexA.G4HACK donor strains**

88 The construction of pHACK-GAL4>nlsLexA::GADfl (v1) and its insertion into PBac{y+-attP-
89 9A}42A13 on the CyO balancer chromosome were described previously (Chang et al 2022). The
90 CyO balancer chromosome with the v1 donor transgene was combined with the
91 PBac{y[+mDint2] GFP[E.3xP3]=vas-Cas9}VK00027 transgene on the third chromosome (BDSC
92 51324) to make a fully functional v1 donor strain as previously reported (Chang et al 2022)

93 4965 bp PCR fragment was amplified from pCaryP (Groth et al 2004) using the primers y[+]_F2
94 (5'-ATTAGTCTCTAATTGAATGACGTCGCATACTTACATTTTTTCCGCTTTTTCCG-3') and
95 y[+]_R (5'-GCTATACGAAGTTATGACGTCGTCGACTATTAAATGATTATCGCCCGATTACC-3')
96 and inserted to AatII site on pHACK-GAL4>nlsLexA::GADfl (v1) using the HiFi DNA Assembly
97 Cloning Kit (New England BioLabs, E5520S). The resulting construct carrying both 3xP3-RFP
98 and *yellow* transgene markers, pHACKy-GAL4>nlsLexA::GADfl (v2), was inserted into the
99 PBac{y+=attP-9A}42A13 site on the CyO chromosome (the same site as the v1 donor construct).
100 The CyO balancer chromosome with the v2 donor transgene was combined with the
101 M{GFP[E.3xP3]=vas-Cas9.RFP-}ZH-2A transgene on X chromosome (BDSC 55821) to make a
102 fully functional v2 donor strain.

103 **Intercross strategy for CRISPR/Cas9-based conversion of GAL4 to LexA.G4HACK**

104 For the F0 intercross, each vial contained four males of the GAL4 line and four virgin females of
105 the LexA donor line (either v1 or v2). The F0 intercross was transferred to new vials every three
106 days for two weeks. When F1 progeny emerged, each male progeny carrying w[+] and CyO was
107 mated to two virgin females of y[1] w[1118] (BDSC 6598). At least 20 mating pairs were set up to
108 identify independent conversion events from different males. These F1 mating pairs were
109 transferred to new vials once after 5 days of mating to extend the number of F2 male progeny to
110 screen for, but we found this may not be necessary if 40 or more mating pairs were initially set
111 up. For the v1 HACK donor line, F2 male progeny with w[+] and non-CyO markers were selected
112 and screened for RFP expression in ocelli under a fluorescence stereo microscope. For the v2
113 HACK donor line, we screened for males carrying w[+], y[+], and non-CyO markers under a light

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114 stereo microscope, then confirmed their RFP expression in ocelli under a fluorescence stereo
115 microscope. All F2 male progeny with w[+] and non-CyO markers were counted to calculate the
116 overall conversion rates in **Table 1**. To assess the HACK-mediated gene conversion efficiency in
117 independent male germlines, we measured frequencies of gene conversion events from each
118 mating pair and plotted them in **Figure S1**. GAL4 stocks usually carry a wild-type Y
119 chromosome, but we have noted that some GAL4 stocks harbor undocumented Dp(1;Y)y^+
120 chromosomes, and could interfere with body color-based screening in the F2 generation. Two
121 independently converted males per each GAL4 line were saved for further analysis.

122 **Removal of loxP cassette from HACK-converted LexA.G4 lines**

123 A single converted F2 male was mated to two virgin females carrying P{Crey} on the X
124 chromosome (BDSC 766). A single F3 male carrying the w[+] marker was mated to two virgin
125 females of y[1] w[1118] (BDSC 6598). A single founder F4 male with w[+], but without the y[+]
126 cuticle color marker or RFP expression in the ocelli was mated to a balancer line (e.g. BDSC
127 59967) to isolate the chromosome carrying LexA.G4H with only w[+] marker. Even without a heat
128 shock, all F4 males that we have seen were without RFP and y[+] markers, indicating high
129 expression of Cre in F3 male germlines harboring the P{Crey} transgene.

130 **PCR genotyping and sequencing of converted LexA.G4 lines**

131 Genomic DNAs from the original GAL4, HACK donor, and converted LexA male flies were
132 extracted as previously reported (Chang et al 2022). 1 µl of the extracted genomic DNA was
133 added to 19 µl of PCR master mix containing 7 µl of water, 10 µl of Q5 Hot Start High-Fidelity 2x
134 Master Mix (NEB M0494S), 1 µl of 10 µM Primer 1 (5'-
135 ATGAAGCTACTGTCTTCTATCGAACAAGC-3') for a GAL4 sequence, and 1 µl of 10 µM Primer
136 2 (5'- GGCATACCCGTTTGGGATATATGATCC-3') for a HACK donor sequence. After a 30-
137 second denaturing period at 98°C, 35 cycles of PCR amplification were performed as a 10-
138 second denaturing period at 98°C, a 30-second annealing period at 60°C, and a 1-minute
139 extension period at 72°C. The PCR reactions from GAL4, donor, and converted flies were
140 resolved in TAE-agarose gel electrophoresis. 1367 bp-long PCR product was amplified only from
141 converted flies, isolated using Zymoclean Gel DNA Recovery Kit (Zymo Research D4008), and
142 sequenced from both ends using Primer 1 and Primer 2.

143 **Imaging LexAop-GFP reporter gene expression**

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144 P{10XUAS-IVS-mCD8::GFP}attP2 (BDSC 32185) and P{13XLexAop2-mCD8::GFP}attP2 (BDSC
145 32203) were used to compare the expression patterns of the original GAL4 and converted
146 LexA.G4HACK line pairs. Four virgin females carrying GFP reporters were mated to a single
147 male of either GAL4, LexA.G4H (RFP^+), or LexA.G4H ($RFP^–$) lines. The mating pairs were
148 transferred to new vials every two days until imaging of expression patterns had been completed.
149 For imaging larval tissues, inverted third instar larvae at the wandering stage were fixed at 4%
150 paraformaldehyde in PBS for >16 hours at 4°C and washed three times in PBS containing 0.1%
151 Triton X-100. Larval brains and imaginal discs were dissected from the washed carcass,
152 transferred onto a glass slide, immersed in 6 µl of the mounting media with DAPI (Vectashield H-
153 1200) for 1 minute, and mounted under an 18 x 18 cover glass. Images of GFP, RFP, and DAPI
154 channels were captured on a compound fluorescence microscope and edited using ImageJ
155 software (NIH). For live imaging of early pupal hemocytes, third instar larvae at the wondering
156 stage were starved on a 2% agar plate for 4 hours, and circulating hemocytes in pupating larvae
157 were imaged under a fluorescence stereo microscope for 30 seconds (Movie S1 and
158 https://youtu.be/Bk__EaKTiVE).

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160 **RESULTS**

161 **A simplified genetic strategy for identifying successful gene conversion *in vivo***

162 A red fluorescent eye marker, 3xP3-RFP, was used in the original HACK study to detect
163 successful editing of GAL4 (Lin and Potter 2016, Chang et al 2023; hereafter the version 1
164 donor, or "v1"). However, genetic screening requiring fluorescence microscopy could prevent
165 adoption of HACK by schools with limited budgets. To permit screening for successful HACK
166 gene conversion with light microscopy, we produced a new transgenic donor strain harboring a 5
167 kb transgene carrying the *yellow* gene enhancer and intronless coding sequence, inserted next
168 to 3xP3-RFP transgene (**Figure 1A**: see Methods). Briefly, we generated a plasmid construct
169 called pHACKy-GAL4>nlsLexA::GADfl (version 2 donor or "v2" hereafter) and inserted this in the
170 attP42A13 genomic site on the CyO balancer, the same position as pHACK in v1 donors (**Figure**
171 **1A**: Methods). Unexpectedly, adults harboring the v2 donor had enhanced RFP expression in
172 eyes and ocelli compared to the v1 donor at the same molecular location (**Figure 1B**), indicating
173 that the 5 kb *yellow* transgene may have improved the expression of the neighboring 3xP3-RFP
174 transgene in this genomic location.

175 To determine whether the additional 5 kb payload in the v2 donor and the use of a different
176 *Cas9* transgene would affect the overall HACK efficiency, we measured GAL4>LexA.G4H
177 conversion in six GAL4 lines (red font in **Table 1**) using v1 and v2 HACK donors. For the v1
178 donor experiment, the *PBac{vas-Cas9}VK00027* transgene located on the third chromosome
179 (BDSC 51324: Port et al 2015) was used (Chang et al 2022). With the v2 donor, we switched to
180 the X-linked *M{vas-Cas9.RFP-}ZH-2A* transgene in a *yellow* background (BDSC 55821, Port et
181 al 2015) to facilitate screening of *yellow* transgene integration events (**Methods**). Overall HACK
182 efficiencies of v2 were slightly lower (1.4%, n = 10,054) than those of the v1 donor (2.3%, n =
183 3,861). However, the relative HACK efficiencies among the different target locations appeared
184 similar between v1 and v2 except for the 32F1 location, indicating that the v2 HACK donor is
185 comparable to v1 in GAL4 target-gene conversion efficiency.

186 To assess the frequency of gene conversion (GAL4 to LexA) in the germ cell lineage of
187 individual male flies, we measured the frequencies of conversion events stemming from
188 individual male matings. This was contrasted with the measurement of the overall conversion
189 rate (**Table 1**), which reflects data pooled from a standard-sized F1 intercross (n = 40); this
190 quantification scheme differs slightly from a prior study (Lin and Potter, 2016), which combined
191 data from 4 males to determine conversion rates. Conversion frequency from an individual F1

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192 male was scored (red number on each bar in **Figure S1**). In the lines with higher overall
193 conversion rates (OK371-GAL4 and Hml-GAL4 in **Figure S1**), we observed that only a few male
194 germ lines (12/94 and 3/59) produced a large number (3 or more) of conversion events, while
195 conversions were more frequent from independent males (40/94 and 17/59). Conversely, lines
196 with lower overall conversion rates produced conversions less often from independent males
197 (2/23 for 459.2-GAL4 and 2/37 for dimm-GAL4), but did not necessarily produce a smaller batch
198 of conversion events (all 22 events found in 1/16 mating for Ilp215-1-GAL4 at attP40). Our
199 finding that conversion events occur “infrequently in many crosses” rather than “frequently in a
200 few crosses” suggests that parallel screening of a relatively large number (e.g., n = 40) of
201 germlines would be the more efficient approach rather than a serial screening of a smaller
202 number (e.g., n = 20) of germlines (**Methods**).

203 HACK-mediated gene conversions on second chromosome-linked GAL4 lines (*cis*-
204 chromosomal HACK) were all successful (n = 7/7), with efficiencies averaging between 0.1 and
205 5.3% (**Table 1**). For third chromosome-linked GAL4 lines (*trans*-chromosomal HACK) 6/7
206 conversions were successful, but the average conversion efficiency was lower (0 to 0.8%: **Table**
207 **1**). Prior studies of *cis*-chromosomal HACK found that HACK donors more proximal to *cis*-targets
208 converted at higher efficiency than distal donors (Lin and Potter, 2016). However, with v1 or v2
209 HACK donors on the CyO balancer second chromosome, we did not observe this proximity effect
210 on two homologous chromosomes. For example, using distally-located (42A13) donors on the
211 CyO balancer, two GAL4 targets closely located at 22A8 and 22E1 show respective HACK
212 efficiencies of 0.4-0.5% versus 2.5-2.8%. Thus, gene conversion efficiencies for GAL4 insertions
213 did not reflect the mere chromosomal distance between the donor and target in *cis*-chromosomal
214 HACK, even when compensating for inversions and translocations on the CyO balancer
215 chromosome. This further supports the strategy of using a single HACK donor embedded in a
216 balancer chromosome, even for *trans*-chromosomal conversions, rather than providing specific
217 HACK donors closely located to a particular GAL4 position for each *cis*-chromosomal HACK. In
218 sum, the v2 HACK donor on the CyO balancer showed comparable performance to v1 and can
219 be used for both *cis*- and *trans*-chromosomal HACKing of GAL4 lines to LexA.G4H.

220

221 **Visible phenotypes permit the detection of successful HACKing**

222 Based on our observation of brighter RFP expression in v2 donor flies compared to v1 donors
223 (**Figure 1B**) we postulated that this difference might persist after CRISPR-based

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GAL4>LexA.G4H conversion. We compared RFP expression after conversion at four different genomic locations (22A8, 22E1, 68C13, and 94D3). In each, the integrated v2 donor showed bright RFP expression in ocelli (arrows, **Figure 2A**). To assess RFP expression after CRISPR/Cas9-mediated targeting with v2 donors at diverse genomic target locations, we compared heads of ten converted GAL4>LexA.G4H flies (**Figure 2B**). After the successful conversion of all ten lines, we observed that RFP expression in compound eyes was variable at different loci, as previously reported (Horn et al 2000), but RFP expression in ocelli cells was observed in all integration sites. Thus ocelli-based screening provides a reliable method for identifying v2 donor-generated conversion events with a fluorescence stereomicroscope.

In addition to RFP expression, conversion with the v2 donor also led to progeny with visibly darker pigmented abdominal segments, consistent with expression of the *yellow* transgene (y^+) in a *yellow* mutant (y^1) genetic background (**Figure 2A**). Thus, the yellow transgene embedded in the v2 donor sequence simplified screening for HACKy-mediated gene conversion events with bright-field microscopy (**Figure 3**). In summary, two markers in the v2 donor - *yellow* and RFP - facilitated screening of HACKy-mediated gene conversion events using light or fluorescence microscopy.

Tissue expression patterns of originating GAL4 and converted LexA lines

To test if LexA expression in converted lines was identical to that in the originating GAL4 line, we performed intercrosses to assess and compare LexA-dependent and GAL4-dependent reporter gene expression. A single male from each original GAL4 line was mated to virgin females carrying 10xUAS-mCD8::GFP, and a single converted LexA.G4H male from each screen was mated to virgin females carrying 13xLexAop2-mCD8::GFP (**Figure 3**). To minimize the positional effects of reporter transgene expression, we used GFP reporter transgenes located at the same genomic location on the third chromosome, attP2 (Pfeiffer et al 2010).

The expression patterns of GFP in the converted LexA lines matched that of the original GAL4 lines (**Figure 4A and B**), an assessment that was less ambiguous after Cre-mediated excision of the donor *loxP*-flanked 3xP3-RFP and *yellow* transgene cassettes (see RFP$^+$ cells in **Figure 4A and B**; **Figure S2**). While this *loxP*-flanked transgene cassette did not appear to alter the expression of LexA lines, we removed this cassette in all converted LexA lines.

In the third instar larval brains of converted LexA.G4H lines, GFP reporter expression patterns appeared indistinguishable from reporter expression in the original GAL4 lines (**Figure 4**). However, the intensity of GFP signal of some converted LexA lines (**Figure 4E and H**) appeared

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256 slightly reduced, compared to reporter GFP signal in the original GAL4 lines. In the third instar
257 wing discs, the converted LexA lines that drive reporter expression in the dorsal compartment of
258 the wing disc (**Figure 5A**), the entire wing disc (**Figure 5B**), or the dorso-ventral boundary of the
259 wing disc (**Figure 5C**) all showed identical patterns to the original GAL4 lines. In whole animal
260 live imaging, mCD8::GFP signals on circulating hemocytes that migrate from anterior to posterior
261 in early pupa (Movie S1; **Methods**) also appeared identical between GAL4 and LexA lines
262 (**Figure 5D**). Compared to the original GAL4 lines, converted lines expressing LexA in the adult
263 abdomen and head fat body also showed similar reporter GFP expression patterns (**Figure 5E**).
264 Taken together, our analysis confirmed that the transactivation functions of converted LexA.G4H
265 lines are indistinguishable from the original GAL4 fly lines.

266

267 **Innovating secondary school curricula for systematic generation of LexA enhancer lines**

268 To develop science classes that used CRISPR/Cas9 mediated gene conversion to generate
269 novel LexA-expressing fly lines, we partnered with secondary schools that had previously
270 collaborated with us to develop relevant fruit fly-based science instruction curricula (**Methods**:
271 https://www.stan-x.org). As conversion targets, we selected six GAL4 lines whose expression
272 patterns were previously well-characterized**.** We sequentially developed two courses for teaching
273 fly genetics covering CRISPR/Cas9-mediated gene conversion, larval tissue dissection, and
274 fluorescence imaging techniques (**Figure 6**). The first course (2021) used the v1 donor, while the
275 following class (2022) used donor v2.

276 In the first course using the v1 donor, six students focused on experimental design, execution,
277 and interpretation, and successfully converted assigned GAL4 lines (red font in **Table 1**) over a
278 10.5 week schedule (Phillips Exeter Academy, NH; **Figure 6A**). Students performed intercrosses
279 and screened for a "HACKed GAL4", then stabilized the chromosome carrying each converted
280 LexA driver over a balancer chromosome. Subsequently, the class imaged and compared
281 LexA.G4H-dependent GFP reporter expression with the original GAL4-dependent expression in
282 larval tissues. The class performed genomic PCR and sequencing to genotype converted lines
283 (confirming substitution of LexA.G4H for GAL4: **Methods**), but prioritized functional verification of
284 the converted LexA line by GFP reporter expression. Other class time was devoted to
285 understanding prior usage of the original GAL4 lines. Suggestions from students and instructors
286 for improving the course workflow included: (1) enhancing RFP expression in future studies to
287 ease screening and identification of converted LexA lines, and (2) considering additional visible

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288 phenotypes to identify converted flies, since access to fluorescence stereomicroscope during this
289 course was a significant 'bottleneck'.

290 To address these suggestions, we developed the v2 donor and tested its use in a second
291 course (The Lawrenceville School, NJ; **Figure 6B**). To perform two generations of mating within
292 a 4.5 week summer schedule, an instructor and a teaching assistant intercrossed F0 mating
293 pairs two weeks before the course commencement: this allowed students on the first day of class
294 to identify the correct F1 male progeny, and to set up intercrosses with virgin female flies
295 (**Methods**). Despite the demanding schedule, 5/6 students identified at least one LexA.G4H
296 convertant. Removal of the loxP-RFP cassette was performed separately by a university
297 research partner (**Figure S2**). This subsequent work (1) established balanced, 'genetically stable'
298 LexA lines in a uniform genetic background (y^1 w^{1118}), (2) verified LexA.G4H-dependent tissue
299 expression of a GFP reporter, and (3) distributed new lines to a *Drosophila* stock center, which
300 makes stocks available for an international community of scientists. In summary, these
301 interscholastic curricula and collaborations established CRISPR/Cas9-based strategies to
302 generate well-characterized, novel LexA fruit fly lines ready for use by the science community.
303 Additionally, both classes provided 'proof of concept' for the feasibility of applying an *in vivo*
304 CRISPR/Cas9 genome editing curriculum in a secondary school setting.

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306 **DISCUSSION**

307 To expand the collection of LexA drivers, we and others have generated novel LexA lines
308 using enhancer trap screens (Kockel et al 2016, Kockel et al 2019, Kim et al 2023) or by cloning
309 enhancers to direct LexA expression (Pfeiffer et al 2010; Wendler et al 2022). While these
310 approaches are sound, the novel lines generated by random transposon insertion or putative
311 genomic enhancer fragments require extensive characterization, including insertion site mapping
312 or expression specificity. As an alternative, complementary approach, CRISPR/Cas9 'HACK'
313 strategies to generate LexA lines that recapitulate tissue expression patterns of existing GAL4
314 lines were recently developed. We have modified these approaches (Lin and Potter 2016; Chang
315 et al 2022) to generate new LexA lines, substantially simplifying the screening of HACK events
316 using visible body color phenotypes (HACKy). GAL4>LexA.G4H gene conversion can be
317 subsequently confirmed by detecting eye/ocelli expression of a second RFP marker. In multiple
318 cases, we observed identical tissue expression patterns of reporter genes induced by the original
319 GAL4 and the cognate converted LexA.G4H line, demonstrating the high fidelity of HACKy-
320 mediated conversion. To address demands for experiential science instruction, we worked with
321 secondary school partners to develop curricula that systematically generated new LexA lines
322 with well-characterized gene expression patterns. GAL4 lines were prioritized based on the
323 characterization of the desired expression, and frequency of cited usage
324 (http://flybase.org/GAL4/freq_used_drivers/). Our work with student scientists demonstrates how
325 university-based research could be leveraged to achieve educational outreach that also
326 generates useful tools for the community of science.

327 Using the second chromosome-based v2 donor, gene conversion efficiencies of second
328 chromosome-linked GAL4 lines were higher on average than those observed with third
329 chromosome-linked GAL4 lines. This indicates that *cis*-chromosomal HACKy remains more
330 efficient than *trans*-chromosomal HACKy. Thus, additional lines to achieve *cis*-chromosomal
331 HACKy of third chromosome-linked GAL4 lines could be useful.

332 Prior studies showed that most non-converted F2 males contain small deletions at target
333 GAL4 sequences, indicating the prevalence of non-homologous end joining repair during HACK
334 (Lin and Potter 2016). Thus, we speculate that after CRISPR/Cas9 DNA targeting, biasing
335 homology-directed repair over non-homologous end joining (NHEJ) at double strand breaks
336 could improve conversion efficiency. One possibility to achieve this would be to construct donor
337 strains with impaired NHEJ (Beumer et al 2013).

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338 Recent exciting advances in biology, like CRISPR gene editing, provide opportunities for
339 secondary school instructors to refresh and invigorate curricula targeting nascent student
340 scientists. To leverage this progress, we developed an experimental curriculum that: (1)
341 incorporated several vibrant areas of bioscience, including genetics, molecular biology, bio-
342 informatics, developmental biology, and evolutionary biology, (2) centered around a powerful
343 modern gene editing technology (CRISPR/Cas9 and homology-directed repair) widely-known to
344 the general population that captured the interest of students and their instructors, (3) was based
345 in fruit flies, a cost-effective, safe experimental system with rapid generation times suited for
346 secondary school laboratory classes, that can (4) foster links between school-based data and
347 discoveries with a global community of professional researchers. These courses benefitted from
348 accompanying web-based instruction (see below) and could be readily adapted to suit shorter or
349 longer instructional timeframes. For example, after generating, then improving donor fly
350 characteristics (**Fig 1**), and streamlining curricula (**Fig 6**), we have updated our course at two
351 Stan-X partner schools. These modifications are perhaps better-matched to shorter instructional
352 timeframes like summer terms, or the inclusion of fruit fly experiments as a part of an existing
353 advanced biology class. Although we focused on frequently-used GAL4 lines in this study,
354 university-based research laboratories could also nominate their own GAL4 lines for students to
355 convert, thus fostering direct communication, and a feeling of 'ownership' and purpose in student
356 collaborators.

357 To train instructors with little to no experience with *Drosophila* or CRISPR, we developed a
358 week-long, intensive teacher training academy, called *Discover Now*. This approach of 'teaching
359 the teachers' has fostered the autonomy of Stan-X instructors and their schools (Kim et al 2023;
360 Chang et al 2022; Wendler et al 2022). Currently, partnering teachers from four additional
361 schools are training to adopt HACKy-based experiments and instruction (S.P., N.L., unpubl.
362 results). To provide practical guides for prospective research scientists and instructors interested
363 in adopting this curriculum in their laboratory classes, the course manual is posted on the Stan-X
364 website (https://www.stan-x.org/publications) and is periodically updated. In summary, we
365 developed experiment-based courses to provide genuine science experiences to secondary
366 school students while generating useful tools for the community of science. This experiential
367 instruction has introduced the wonder, anxiety, and joy of scientific discovery to secondary
368 school students, and informed their choices to pursue additional science training.

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369 **FIGURE AND TABLE LEGENDS**

370 **Figure 1. Designs of LexA.G4HACK donors for CRISPR/Cas9-mediated GAL4 gene**
371 **conversion and chromosomal locations of GAL4 targets.**

372 (A) Genetic designs of two LexA.G4HACK donors for HACK-mediated gene conversion. A DNA
373 double-strand break generated by vas-Cas9 and gRNAs targeting the GAL4 sequence in
374 germline chromosomes can be repaired by homology-assisted CRISPR/Cas9 knock-in of a
375 donor transgene located in a balancer chromosome. The version 2 donor carries a loxP-flanked
376 dual transgene cassette. Both versions of the donor are inserted in the same attP site on the
377 CyO balancer to enable an unbiased comparison of the donor efficiency differences potentially
378 generated by different repair template sizes.

379 (B) The version 2 donor transgene at the genomic location of 42A13 on the CyO balancer
380 showed an improved 3xP3-RFP expression compared to the version 1 donor at the same
381 location. *yellow*$^{+}$ phenotypes in both flies shown are from PBac{y+-attP-9A}42A13 on the CyO
382 balancer.

383 (C) Chromosomal locations of selected GAL4 targets for HACK-mediated gene conversion and
384 the donor location

385

386 **Table 1. Genotypes of the original GAL4 and converted LexA lines and their conversion**
387 **rate using v1 and v2 donors.**

388 Source IDs and genotypes of GAL4 lines selected for the gene conversions and their conversion
389 rates by donor version; red color indicates lines with data for both donor versions. Following the
390 convention of FlyBase genotype nomenclatures, converted lines from P{GawB}-based enhancer
391 trap GAL4 insertions were named as P{ET-lexA::GAD.GB} and converted lines from cloned
392 enhancer-driven GAL4 transgenes were named by replacing GAL4 in the original genotypes with
393 LexA.G4H. n.d. = not determined.

394

395 **Figure S1. Frequencies of conversion events from independent male germlines**

396 Phenotypic counting of F2 male progeny was plotted for each mating pair (Independent F1 Cross
397 ID on X-axis). Black bars represent counts of F2 males with mini-white and non-curly phenotypes
398 while red bars indicate F2 males with mini-white, non-curly, and RFP^{+}, with frequencies written in

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399 red. For the second chromosome-linked GAL4 lines, each mating pair of a single F1 male and
400 two y^1 w^{1118} virgin females produced about 30 F2 males with desired phenotypes in a vial; the
401 progeny size can be increased to 60 if F1 mating pairs are flipped once to a new vial after six
402 days of initial mating. For X or third chromosome-linked GAL4 lines, these numbers decrease by
403 a factor of one-half due to the independent segregation of donor and target chromosomes.

404

405 **Figure 2. Improved RFP expression of integrated version 2 donor at various genomic**
406 **locations**

407 (A) Phenotypic comparison of F2 males with successful donor integrations at different targets.
408 RFP expression in ocelli (white arrows) was more consistently observed in version 2 integration
409 sites than in corresponding version 1 integration sites. The version 2 integration events can also
410 be identified by yellow transgene expression in tail segments in the y^1 w^{1118} mutant genetic
411 background (the bottom row).

412 (B) RFP expression of integrated version 2 donor at different genomic locations. Adult heads of
413 converted males were arranged based on target locations. RFP expression in ocelli was
414 consistently high in all locations, but the expression in compound eyes was highly variable in
415 different locations. Note that the expression of mini-white and 3xP3-RFP was inversely
416 correlated in compound eyes (see text).

417

418 **Figure 3. Mating scheme for converting second chromosome-linked GAL4 lines to**
419 **LexA.G4H and imaging reporter expression.**

420 The parental mating (F0) was set up with a male carrying the GAL4 transgene (“Target”) and
421 virgin females carrying vasa-Cas9 on the X chromosome and a HACK donor on the CyO
422 balancer (“Donor”). In parallel, a male carrying the same “Target” GAL4 transgene was also
423 mated with virgin females carrying the UAS-GFP transgene (BDSC 32185) for documentation of
424 the GFP expression pattern of the “Target” GAL4. These mating pairs were transferred to new
425 vials every two days 6 times. Larval, pupal, and adult progeny from UAS-GFP mating were
426 imaged for GFP expression patterns based on prior characterizations of the “Target” GAL4 line.
427 Up to 80 individual mating pairs were set up for an F1 male progeny carrying all three transgenes
428 and two virgin females of y^1 w^{1118} (BDSC 6598). In the F2 generation, non-curly male flies
429 carrying mini-white transgene were scored for RFP expression in ocelli and/or yellow transgene

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430 expression in tail segments. If identified, a single F2 male carrying the mini-white and RFP
431 transgenes was first mated with virgin females carrying LexAop-GFP transgene (BDSC 32203)
432 for 3 days. The same male was mated again with different virgin females carrying balancer
433 chromosomes (e.g., BDSC 59967) to isolate the chromosome with the modified transgene
434 ("Converted (RFP^{+})").

435

436 **Figure S2. Mating scheme for removing loxP-flanked transgene cassette and establishing**
437 **stable LexA.G4H lines.**

438 A single F2 male carrying the converted LexA.G4H transgene was mated to Cre-expressing
439 virgin females (BDSC 766). A single F3 male with two transgenes was mated with virgin females
440 of y^{1} w^{1118} (BDSC 6598). All F4 males carrying the mini-white transgene were without RFP and
441 yellow transgene expression, but a single F4 male with the mini-white transgene was selected to
442 mate with virgin females carrying balancer chromosomes (e.g. BDSC 59967) to isolate the
443 chromosome with LexA.G4H transgene without RFP and yellow transgenes. In the F5
444 generation, the chromosome carrying LexA.G4H was balanced to establish a "stable stock" in
445 the y^{1} w^{1118} genetic background.

446

447 **Figure 4. Comparison of larval brain reporter expression for original GAL4 and converted**
448 **LexA.G4H lines.**

449 (A) GFP reporter expression in ventral nerve cords of larval brains driven by ppk-GAL4 (left),
450 ppk-LexA.G4H with RFP transgene (middle), and ppk-LexA.G4H with RFP cassette removed
451 (right). The scale bar is 100 μm.

452 (B) GFP reporter expression in neuroendocrine cells of larval brains driven by dimm-GAL4 (left),
453 dimm-LexA.G4H with RFP transgene (middle), and dimm-LexA.G4H with RFP cassette removed
454 (right).

455 (C-H) GFP reporter expression in larval brains driven by GAL4 (left) and LexA.G4H with RFP
456 cassette removed (right) showing expression in vGlut neurons by OK371 enhancer (C), corpora
457 cardiaca cells by Feb36 enhancer (D), brain hemispheres by GH146 enhancer (E), pan-neuronal
458 cells by C155 enhancer (F), ventral nerve cords by D42 enhancer (G), and pan-glial cells by a
459 cloned repo enhancer (H).

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460

461 **Figure 5. Comparison of reporter expression for original GAL4 and converted LexA.G4H**
462 **lines in larval wing discs, pupal hemocytes, and adult fat bodies**

463 (A-C) GFP reporter expression in larval wing discs driven by GAL4 (left) and LexA.G4H with RFP
464 cassette removed (right) marking cells in the dorsal pouch by MS1096 enhancer (A), broad
465 anterior-posterior boundaries by 459.2 enhancer (B), and dorsal-ventral boundaries by C96
466 enhancer (C). The scale bar in (A) is 100 μm.

467 (D) GFP reporter expression in early pupae driven by GAL4 (left) and LexA.G4H with RFP
468 cassette removed (right) showing expression in circulating hemocytes by a cloned Hml
469 enhancer. The image is a still frame from 30-second-long live imaging (Movie S1),

470 (E) GFP reporter expression in the fat body of adult males driven by r4-GAL4 (left side of each
471 image) and r4-LexA.G4H with RFP cassette removed (right side of each image).

472

473 **Figure 6. Genetics laboratory class schedules deployed for 8 weeks and 4 weeks in**
474 **secondary schools**

475 (A) A 90-minute-long class was held twice a week for 8 weeks. In week 1, students were
476 introduced to *Drosophila* genetics including understanding genotypes, identifying associated
477 markers, and setting up mating with follow-up maintenance. In week 2, students learned the
478 anatomy of the third instar larva, micro-dissection, and imaging of slide-mounted tissues. In
479 weeks 3 and 4, students generated a series of F1 intercrosses, while participating in discussions
480 of prior characterizations of assigned GAL4 lines. In weeks 5 and 6, students started screening
481 for conversion events in F2 progeny. In week 7, if a converted male was found, they set up a
482 mating with LexAop-GFP reporter and documented GFP expression in the resulting progeny with
483 RFP. In week 8, instructors and teaching assistants shipped the "converted (RFP^+)" lines to
484 research laboratories.

485 (B) A 6-hour-long daily class was held five days a week for 4 weeks. To start week 1 with F1
486 mating, instructors and teaching assistants initiated F0 mating 3 weeks before the class started
487 while collecting and maintaining virgin females of y^1 w^{1118}. Since the daily class schedule
488 permitted students to master micro-dissection and imaging techniques more thoroughly, all
489 students successfully documented GFP expression by assigned GAL4 lines by the end of week

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490 2. Students had hour-long daily remote meetings with a research scientist to troubleshoot and
491 discuss primary research articles.

492

493 **Movie S1. Live imaging of early pupa GFP expression in circulating hemocytes driven by**
494 **either Hml-GAL4 (left) or Hml-LexA.G4H (right).** https://youtu.be/Bk__EaKTiVE

495

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496 **DATA AVAILABILITY STATEMENT**

497 Strains and plasmids are available upon request. The course teaching materials and syllabuses
498 are posted on the Stan-X website (https://www.stan-x.org/publications) and periodically updated.
499 The authors affirm that all data necessary for confirming the conclusions of the article are
500 present within the article, figures, and tables.

501

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520

521 **COMPETING INTERESTS**

522 The authors declare no competing interests.

523

524 **AUTHOR CONTRIBUTIONS**

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525 AER, EF, TC, and NL were course instructors. AR and WP were teaching assistants. EG, JH,
526 CS, MT, JW, AY, ESK, NKAA, PC, ACKL, MEL, JL, and KP were students. EW and PHC were
527 undergraduate research assistants who generated final images. LK, SP, and SKK designed and
528 managed the project. PHC, LK, SP, and SKK analyzed the data and wrote the manuscript. All
529 authors read and approved the final manuscript.

530

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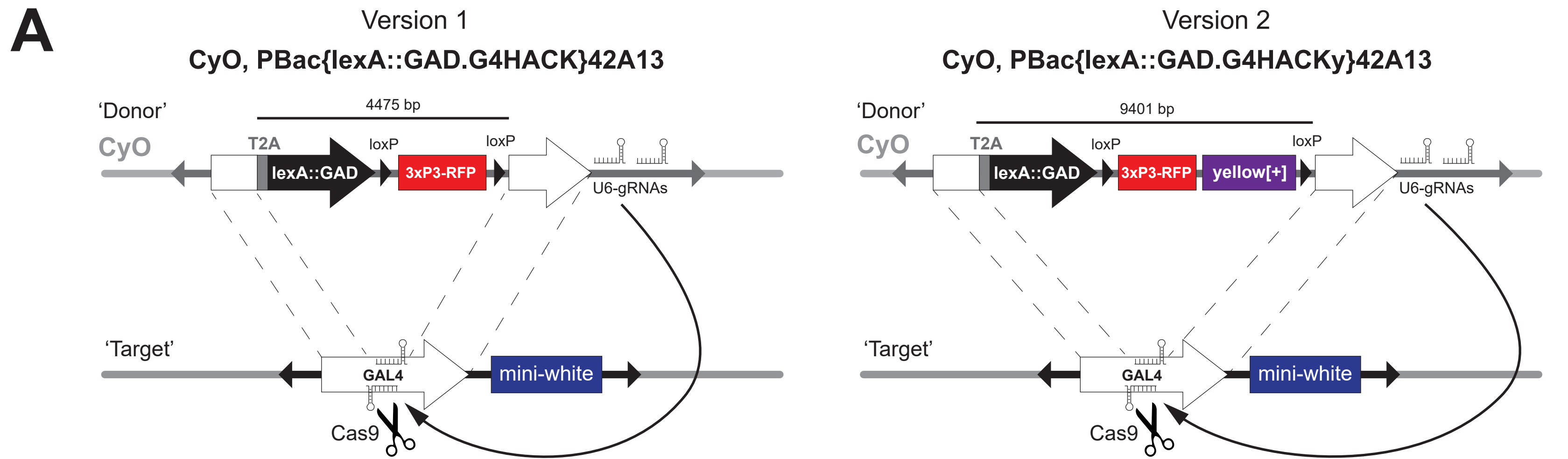

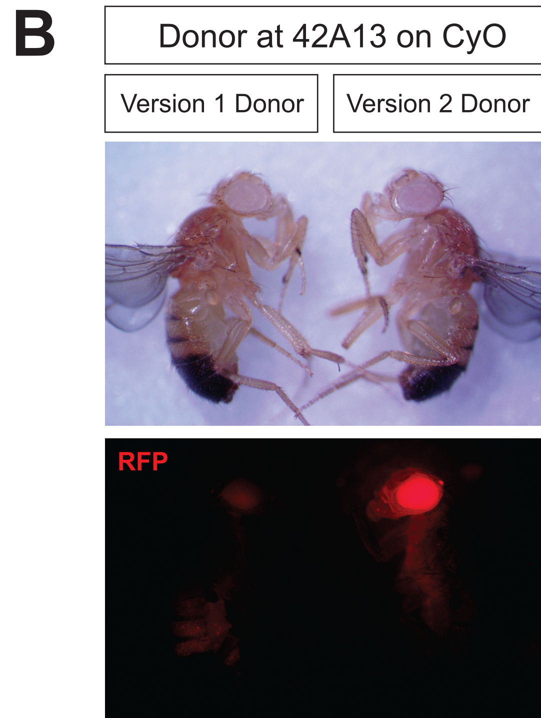

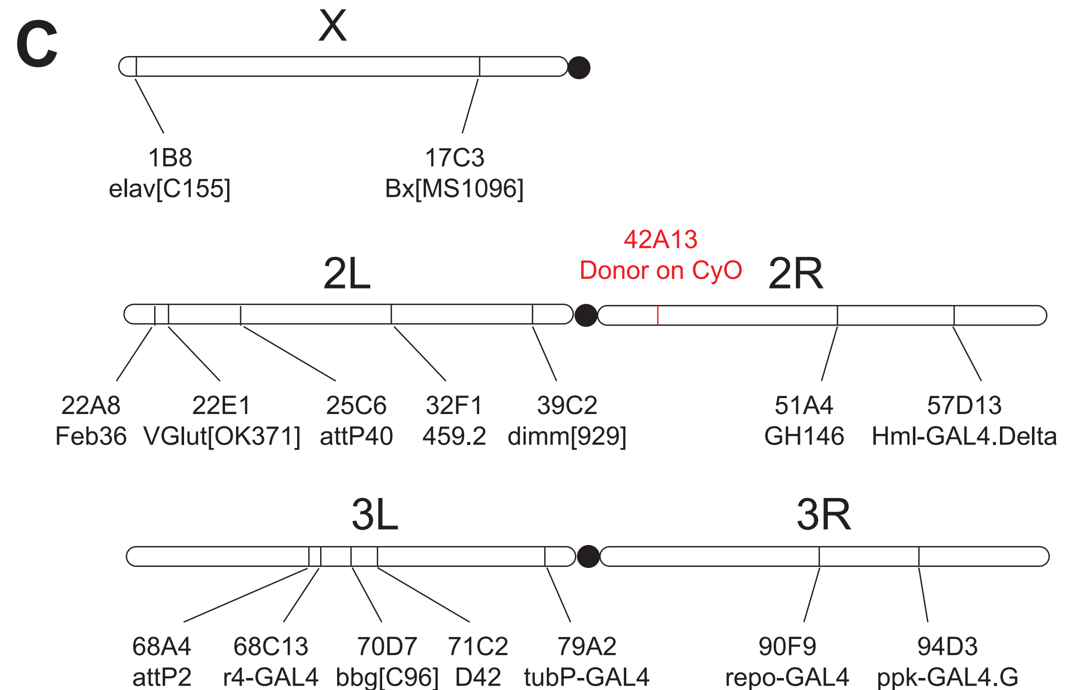

Figure 1. Designs of LexA.G4HACK donors for CRISPR/Cas9-mediated Gal4 gene conversion and chromosomal locations of Gal4 targets.

BDSC	GAL4 trangene or insertion	Location	Version 1 Conversion % (# males RFP[+] w[+] / w[+])	Version 2 Conversion % (# males RFP[+] w[+] / w[+])	loxP cassette removed LexA lines
25750	P{w[+mW.hs]=GawB}elav[C155]	Chr X, 1B8	1.2% (4/~300)	n.d.	P{w[+mW.hs]=ET-lexA::GAD.GB}elav[C155-LG]
8860	P{w[+mW.hs]=GawB}Bx[MS1096]	Chr X, 17C3	1.0% (6/~600)	n.d.	P{w[+mW.hs]=ET-lexA::GAD.GB}Bx[MS1096-LG]
29968	P{w[+mW.hs]=GawB}Feb36	Chr 2, 22A8	0.5% (2/459)	0.4% (12/2967)	P{w[+mW.hs]=ET-lexA::GAD.GB}Feb36
26160	P{w[+mW.hs]=GawB}VGlut[OK371]	Chr 2, 22E1	2.8% (12/425)	2.5% (82/3238)	P{w[+mW.hs]=ET-lexA::GAD.GB}VGlut[OK371-LG]
	P{y[+t7.7] w[+mC]=Dilp215-1-GAL4}attP40	Chr 2, 25C6	n.d.	3.0% (22/736)	P{y[+t7.7] w[+mC]=Dilp215-1-LexA.G4H}attP40
5818	P{w[+mW.hs]=GawB}459.2	Chr 2, 32F1	3.5% (23/651)	0.4% (3/829)	P{w[+mW.hs]=ET-lexA::GAD.GB}459.2
25373	P{w[+mW.hs]=GawB}dimm[929] crc[929]	Chr 2, 39C2	n.d.	0.1% (2/1781)	P{w[+mW.hs]=ET-lexA::GAD.GB}dimm[929-LG] crc[929-LG]
30026	P{w[+mW.hs]=GawB}GH146	Chr 2, 51A4	0.7% (5/683)	0.9% (10/1114)	P{w[+mW.hs]=ET-lexA::GAD.GB}GH146
30139	P{w[+mC]=Hml-GAL4.Delta}2	Chr 2, 57D13	5.3% (42/799)	2.4% (34/1417)	P{w[+mC]=Hml-LexA.G4H.Delta}2
47473	P{y[+t7.7] w[+mC]=GMR16H11-GAL4}attP2	Chr 3, 68A4	n.d.	0.0% (0/543)	
33832	P{w[+mC]=r4-GAL4}3	Chr 3, 68C13	n.d.	0.8% (4/516)	P{w[+mC]=r4-LexA.G4H}3
43343	P{w[+mW.hs]=GawB}bbg[C96]	Chr 3, 70D7-70E1	n.d.	0.5% (4/784)	P{w[+mW.hs]=ET-lexA::GAD.GB}bbg[C96-LG]
8816	P{w[+mW.hs]=GawB}D42	Chr 3, 71C2	n.d.	0.5% (2/381)	P{w[+mW.hs]=ET-lexA::GAD.GB}D42
5138	P{w[+mC]=tubP-GAL4}LL7	Chr 3, 79A2	n.d.	0.1% (1/688)	P{w[+mC]=tubP-LexA.G4H}LL7
7415	P{w[+m*]=GAL4}repo	Chr 3, 90F9	n.d.	0.4% (2/534)	P{w[+m*]=ET-lexA::GAD}repo[LG]
32079	P{w[+mC]=ppk-GAL4.G}3	Chr 3, 94D3	0.4% (3/844)	0.2% (1/489)	P{w[+mC]=ppk-LexA.G4H.G}3

Table 1. Genotypes of the original GAL4 and converted LexA lines and their conversion rate using v1 and v2 donors.

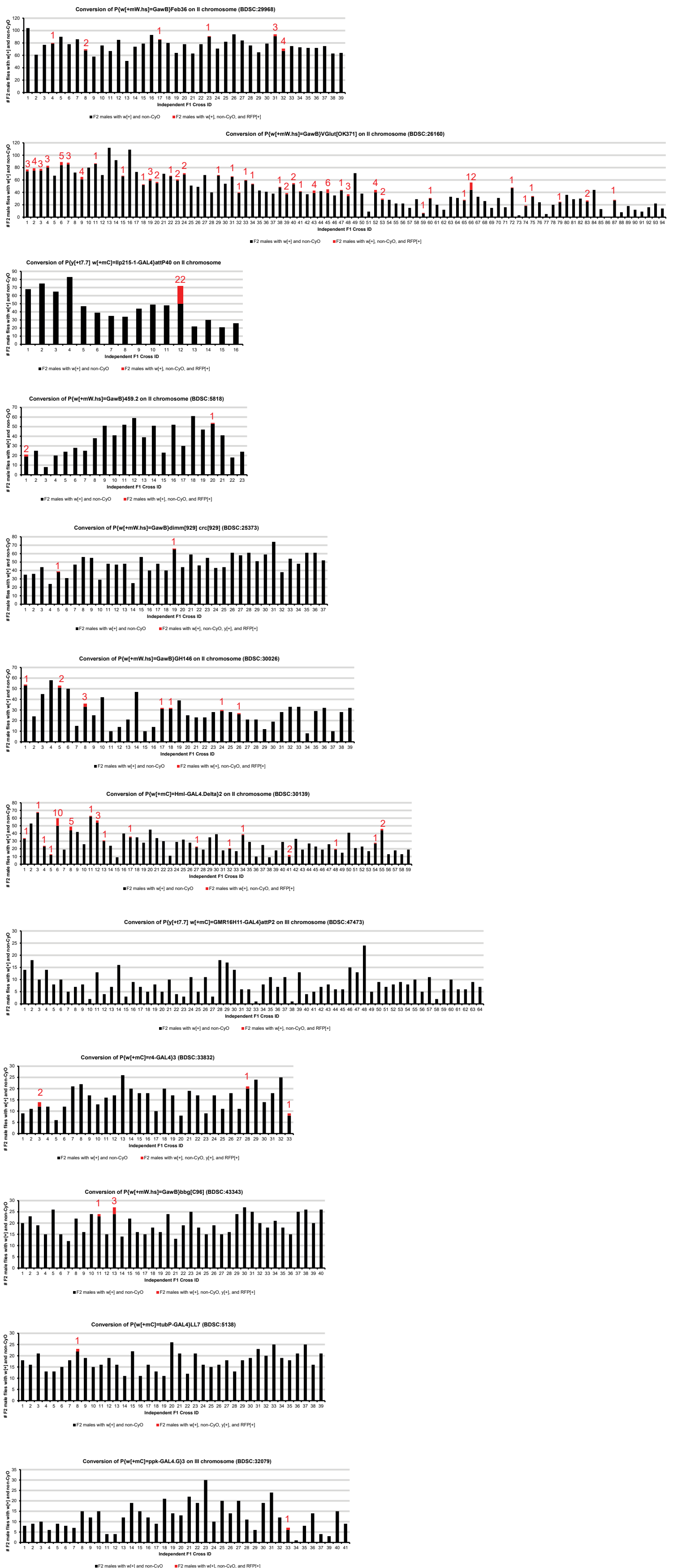

Figure S1. Frequencies of conversion events from independent male germlines

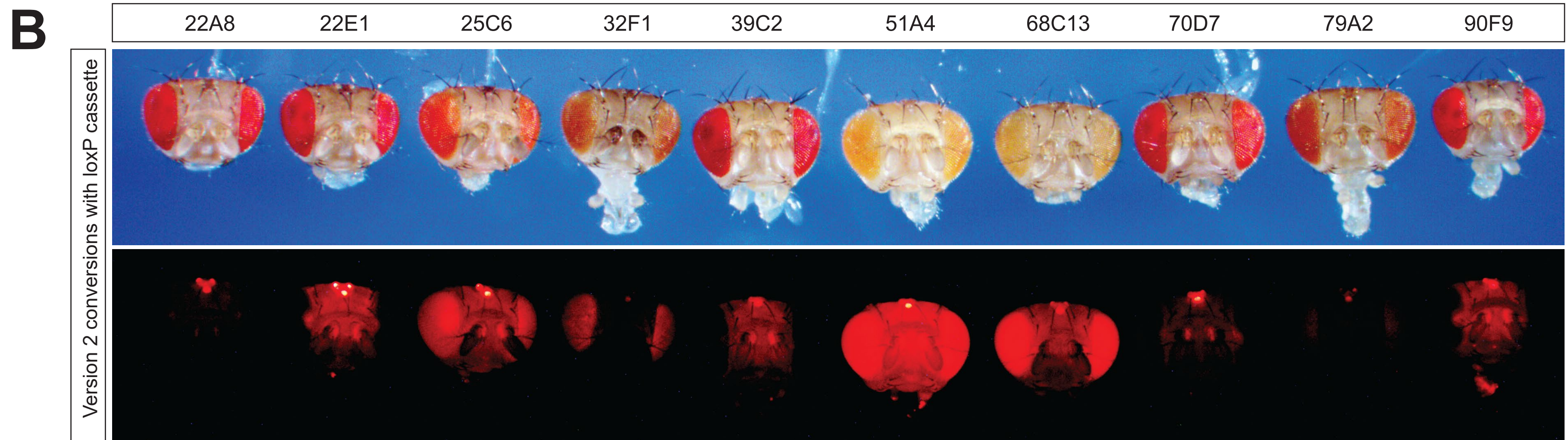

Figure 2. Improved RFP expression of integrated version 2 donor at various genomic locations

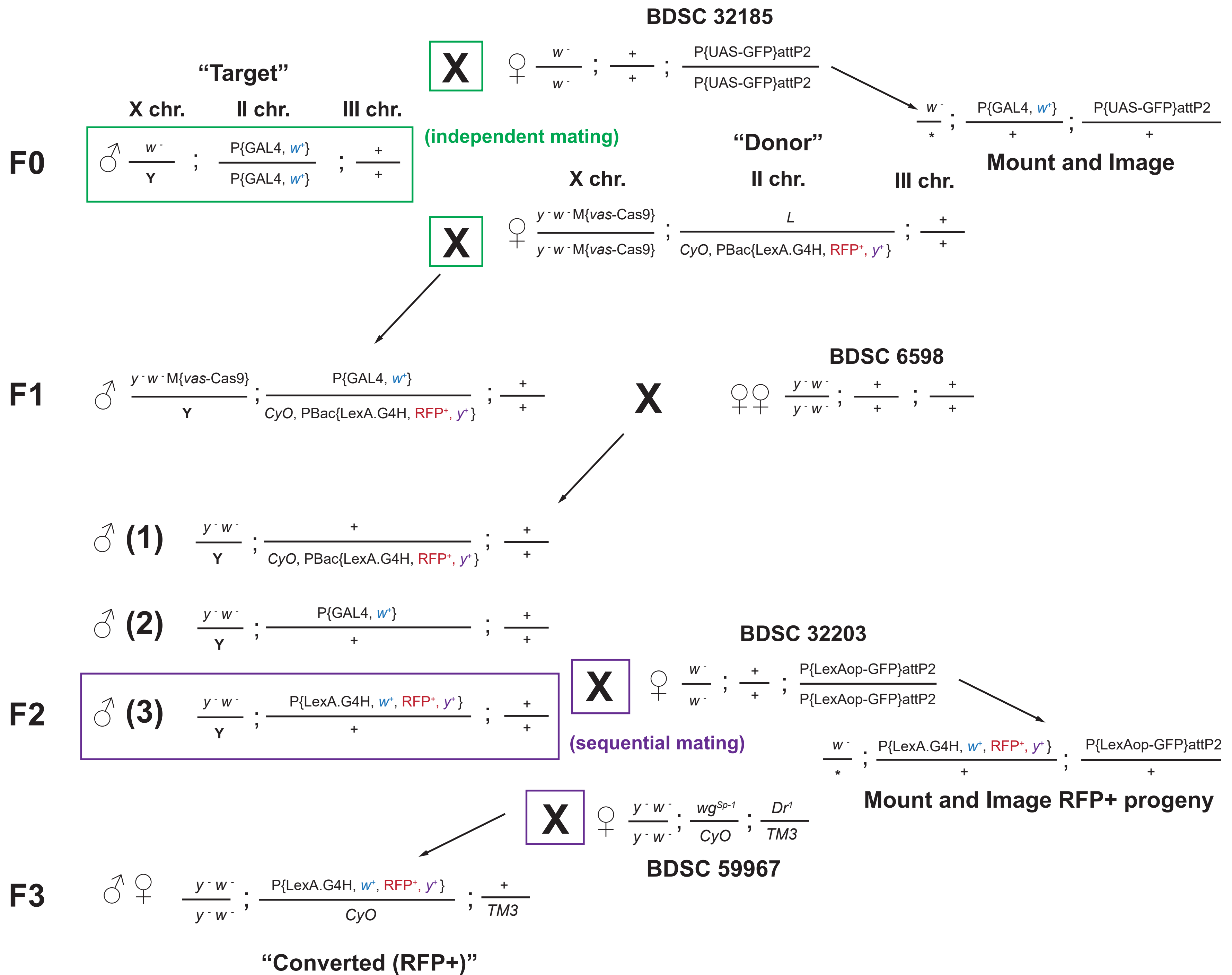

Figure 3. Mating scheme for converting second chromosome linked GAL4 lines to LexA.G4H lines and imaging reporter expression

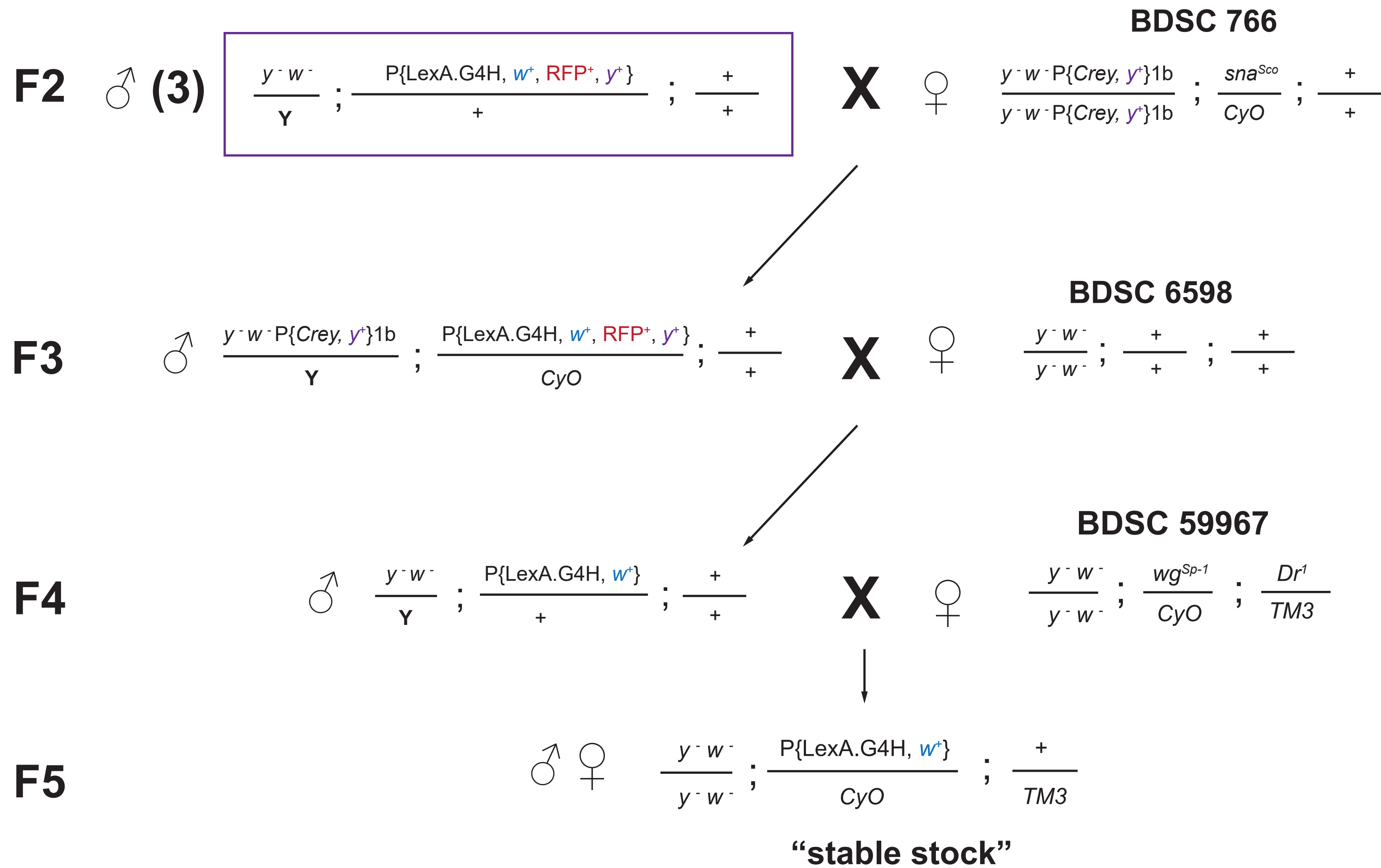

Figure S2. Mating scheme for removing loxP-flanked trangene cassette and establishing stable LexA.G4H lines.

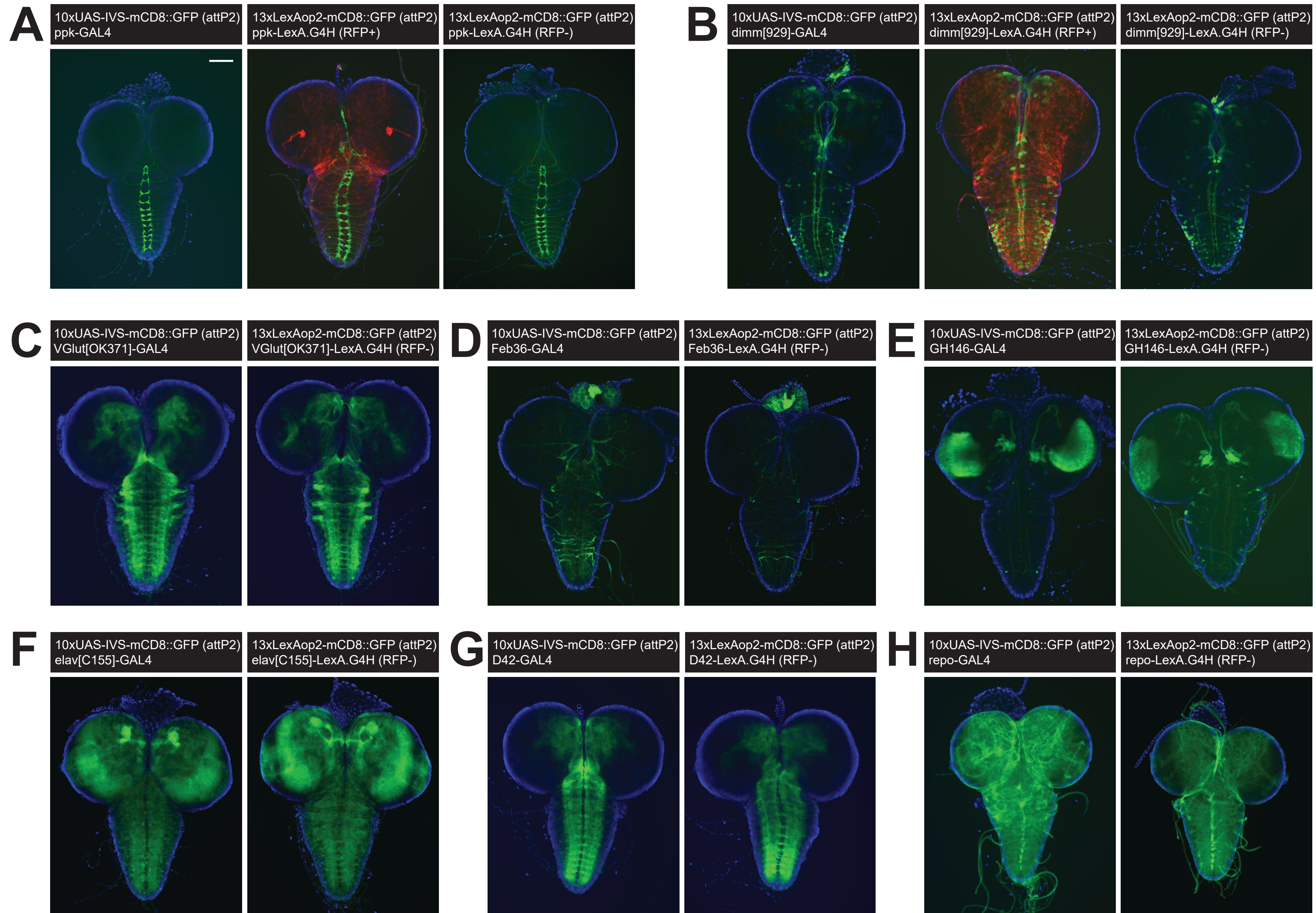

Figure 4. Comparison of larval brain reporter expression for originating GAL4 and converted LexA.G4H lines.

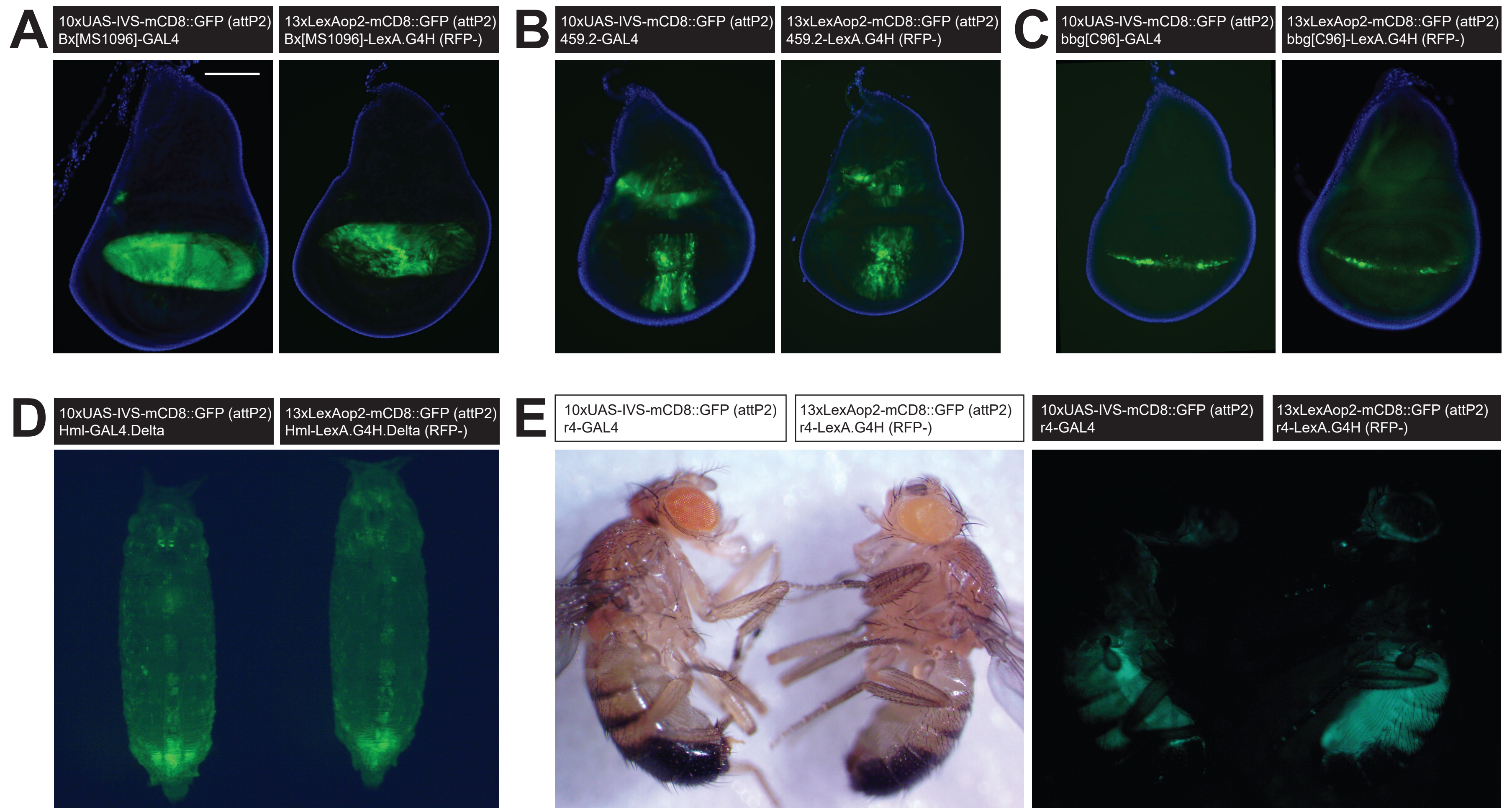

Figure 5. Comparison of wing disc, hemocyte, and adult fat body reporter expression for originating GAL4 and converted LexA.G4H lines.

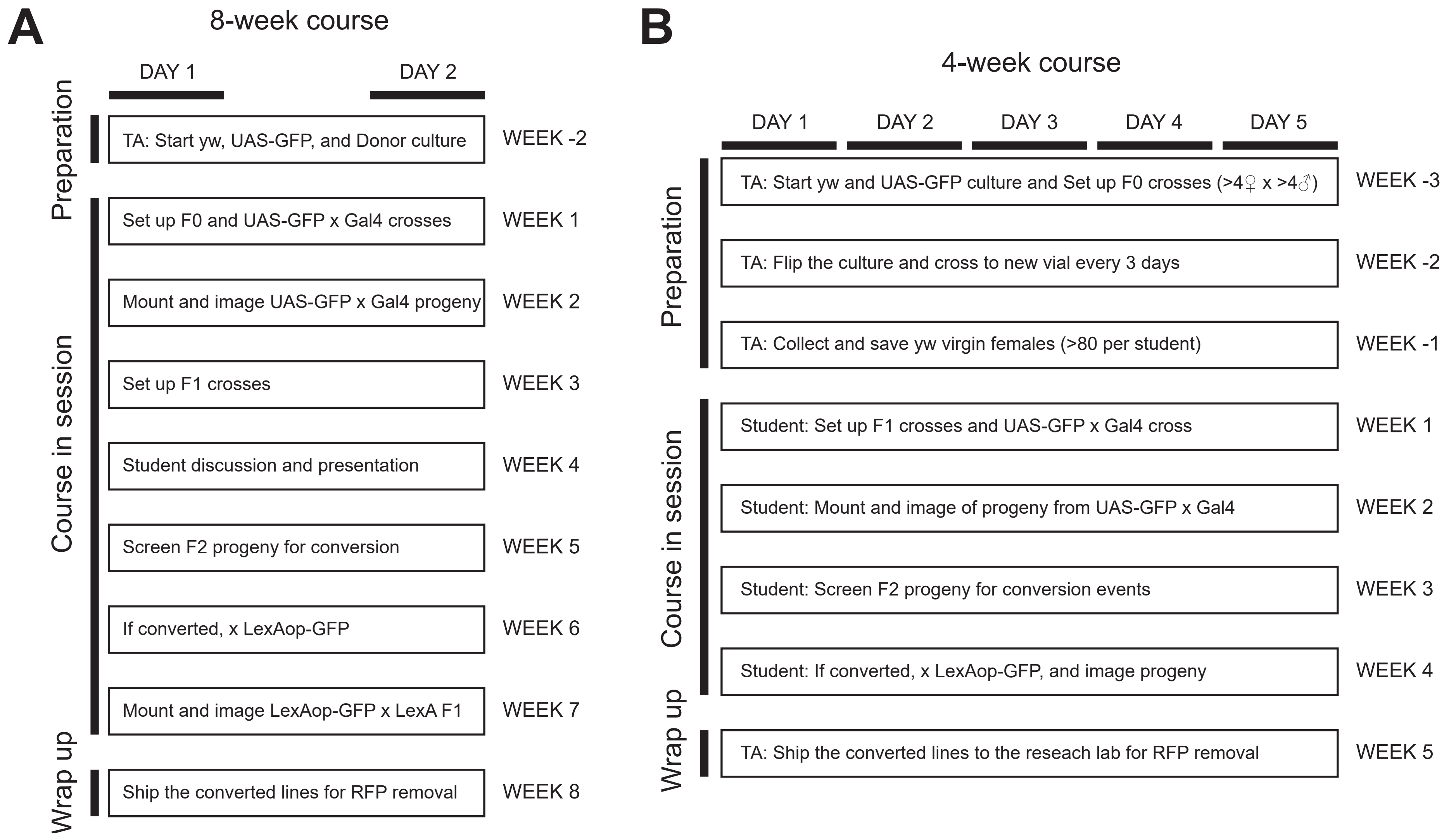

Figure 6. Genetics laboratory class schedules deployed for 8 weeks and 4 weeks in secondary schools