





Transgenic *Drosophila* lines for LexA-dependent gene and growth regulation

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Abstract

Conditional expression of short hairpin RNAs with binary genetic systems is an indispensable tool for studying gene function. Addressing mechanisms underlying cell–cell communication in vivo benefits from simultaneous use of 2 independent gene expression systems. To complement the abundance of existing Gal4/UAS-based resources in *Drosophila*, we and others have developed LexA/LexAop-based genetic tools. Here, we describe experimental and pedagogical advances that promote the efficient conversion of *Drosophila* Gal4 lines to LexA lines, and the generation of LexAop-short hairpin RNA lines to suppress gene function. We developed a CRISPR/Cas9-based knock-in system to replace Gal4 coding sequences with LexA, and a LexAop-based short hairpin RNA expression vector to achieve short hairpin RNA-mediated gene silencing. We demonstrate the use of these approaches to achieve targeted genetic loss-of-function in multiple tissues. We also detail our development of secondary school curricula that enable students to create transgenic flies, thereby magnifying the production of well-characterized LexA/LexAop lines for the scientific community. The genetic tools and teaching methods presented here provide LexA/LexAop resources that complement existing resources to study intercellular communication coordinating metazoan physiology and development.

Keywords: *Drosophila*; LexA; LexAop; shRNA; CRISPR/Cas9

Introduction

Binary gene expression systems are a cornerstone in *Drosophila* investigations of gene regulation and function. The most widely-used binary expression system in *Drosophila* relies on 2 separate components: a yeast Gal4-based transcriptional transactivator whose expression is under control of cell-type-specific enhancers, and Gal4-responsive upstream activating sequence (UAS) that regulates the expression of target genes (Brand and Perrimon 1993; Hayashi et al. 2002; Gohl et al. 2011). To investigate the impact of targeted gene suppression, investigators have used Gal4/UAS-mediated gene knockdown to express short hairpin RNAs (shRNAs) in specific cell types (Ni et al. 2011). Studies of many biological questions require simultaneous manipulation of 2 or more independent cell populations or genes (reviewed in Kim et al. 2021). These studies often benefit from multiple binary systems combined in a single fly to study genetic perturbations in

multiple tissues. Such a multiplex approach requires systems that function independently of the UAS/Gal4 system, such as the LexA/LexAop binary system, comprised of a bacterial transactivator (LexA) expressed from cell-type-specific *Drosophila* enhancers and a *sulA*-derived LexA operator-promoter (LexAop) that drives the expression of adjacent target genes (Pfeiffer et al. 2010).

To address this need, we and others have made systematic efforts to expand the number of well-characterized, publicly available LexA fly lines, either by linking defined enhancers to sequences encoding LexA (Pfeiffer et al. 2010) or by mobilizing a LexA-containing transposable element to insert at genomic sites near enhancers, resulting in “enhancer trap” LexA lines with unique expression patterns (Kockel et al. 2016, 2019). Although these resources are slowly growing, there are many well-characterized Gal4 enhancer lines that lack cognate LexA lines.

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Here, we adopted a CRISPR/Cas9-mediated gene-editing approach (Lin and Potter 2016) to develop a genetic conversion system to replace Gal4 with LexA in unique Gal4-based enhancer trap lines.

Compared to existing LexA-expressing lines, the number of fly lines harboring a LexAop-regulated transgene is even smaller, and unlike the Gal4/UAS system, there is no public fly strain resource for LexAop-based shRNA expression. Thus, when a shRNA is proven to be highly specific and robust in generating phenotypes by the Gal4/UAS system, there should be a simplified cloning step to generate a counterpart in LexA/LexAop system. Here, we developed a streamlined workflow to move functionally validated shRNA sequences from UAS-based transgenic lines to LexAop-based transgenic lines. To expand the development of these genetic tools and methods, we created secondary school and undergraduate courses in fly transgenesis. From student researchers and instructors comprising an international scholastic network, we generated novel LexA and LexAop-based fly lines permitting tissue-specific expression of shRNAs, and functionally characterized a subset of these in wing development.

Materials and methods

Drosophila strains

Except for transgenic lines that were generated in this study, all other *Drosophila* lines were obtained from the Bloomington *Drosophila* Stock Center.

Construction of White-AttB-LexAop vector

To replace 10×UAS in the pWALIU20 vector with 13×LexAop2 (*sulA*-derived LexA DNA-binding motifs), a 675 bp product was amplified from pJFRC19-13×LexAop2-IVS-CD4-myr::GFP (Pfeiffer et al. 2010) using primers LexAop_F1 (5'-CACCCATGCATAGGG CCGCAAGCTTGCATG-3') and hsp70p_R (5'-CCTTTTATGATC TATTCAGAGTCTCTTCTGTATTCAATAATTACTTCTTGGC-3'). The polymerase chain reaction (PCR) product was inserted to the PstI and BglII sites on the pWALIU20 vector (Perkins et al. 2015) by Gibson-assembly (NEB HiFi DNA Assembly Kit) to generate White-AttB-LexAop (pWALEXA20 for shRNA expression), a phiC31 integrase-mediated transformation vector with mini white as a selection marker.

Cloning of shRNA sequences from genomic DNAs of VALIU20 based TRiP-3 lines

pWALEXA20 vector (0.5 µg) was digested with XbaI and NdeI and run on 0.8% agarose gel in 1× Tris-acetate-EDTA (TAE). The resulting 9,947-bp fragment was purified in 10 µl water using the Zymoclean Gel DNA Recovery Kit.

To extract genomic DNA from shRNA-based Transgenic RNAi Project (TRiP-3) transgenic lines, 1 male fly was ground in 50 µl of Squishing Buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 25 mM NaCl, and 0.2 mg/ml Proteinase K). The sample was incubated at 37°C for 30 min, heat-inactivated at 95°C for 3 min, and then centrifuged at >18,000g for 5 min; 1 µl of the clear supernatant containing the extracted genomic DNA was added to 19 µl of PCR master mix containing 7 µl of water, 10 µl of Q5 Hot Start High-Fidelity 2× Master Mix (NEB M0494S), 1 µl of 10 µM shRNA_GA_F primer (5'-GAGAAGCTTGAATAGATCTGTCTAGAAAACATCCCA TAAAACATCCCATATTC-3'), and 1 µl of 10 µM shRNA_GA_R1 primer (5'-CTCTAGTCTAGGTGCATATGTCCACTCTAGTA-3'). Furthermore, 1 µl of Squishing Buffer was added to 19 µl of PCR master mix as a control reaction. After a 30-s denaturing period at 98°C, 40 cycles of PCR amplification were performed with a 10-s denaturing period at 98°C, a 20-s annealing period at 62°C, and

a 30-s extension period at 72°C; 5 µl of the PCR reaction was then run on 3% agarose gel in 1× TAE to confirm the 202 bp PCR product. A total of 49 VALIU20-based TRiP-3 lines from the Bloomington *Drosophila* Stock Center were processed as above, and we successfully amplified the shRNA region in all but 3 lines (Bloomington Stock ID 43283, 57215, and 57519), resulting in a 93.8% cloning success rate. Obtaining replacement lines for the 3 lines that went uncaptured did not solve the problem.

Once PCR amplification was confirmed, 1 µl of the PCR reaction was added to 9 µl of Gibson-assembly reaction containing 3.5 µl of water, 5 µl of NEBuilder HiFi DNA Assembly Master Mix, and 0.5 µl of the linearized and purified pWALEXA20 vector; 1 µl of water was added to 9 µl of Gibson-assembly reaction as a control reaction. After incubating the Gibson-assembly reaction at 50°C for 1 h, 25 µl of competent cells (NEB E5520S) were transformed with 1 µl of the reaction, and all cells were plated on LB agar plate with ampicillin using L-shaped bacterial cell spreader.

Bacterial colonies were picked and grown in 10 ml LB broth with ampicillin for 16 h at 37°C. Amplified plasmids were purified by Zippy Plasmid Miniprep (Zymo Research) and sequenced from the *hsp70* basal promoter using the primer hsp70p_F (5'-CTGCCAAGAAGTAATTATTGAATACAAGA-3') to confirm successful cloning of shRNA fragments. All cloned shRNA sequences in the pWALEXA20 vector were identical to the reported sequences except 2 sequences (originated from Bloomington Stock # 33613 for *white* and 41883 for *Sur-8*) in which there were single base pair mismatches within the complementary region (Supplementary Table S1). We found that identical mismatches were also present in the genomic DNA of the originated stocks. We do not know when these changes were introduced to these stocks, but we do not expect these changes would affect their ability to knock down their target genes since the *white* shRNA sequence with a single mismatch was shown to be functionally active (Fig. 1c).

Generation of transgenic LexAop2-shRNA lines

All sequence-verified plasmids were injected to either $y^1 w^*$ P{nos-phiC31}; P{CaryP}attP40 (#79604) or $y^1 w^*$ P{nos-phiC31}; P{CaryP}attP2 for integration into the second or third chromosome, respectively. To verify the sequences of the newly generated transgenic lines, 232 bp PCR products were amplified from genomic DNA using hsp70p_F and shRNA_GA_R1 primers, purified using DNA Clean and Concentrator-5 (Zymo Research), then sequenced using shRNA_GA_R1 primer.

Functional test of white gene knockdowns in adult eyes

To knockdown *white* gene expression in adult eyes, P{y[+t7.7] w[+mC]=GMR16H11-Gal4}attP2 (BL#47473) or P{y[+t7.7] w[+mC]=GMR16H11-lexA}attP40 (BL#61516) was intercrossed to either y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00004}attP2/TM3, Sb[1] (BL#33613) or y[1] v[1]; P{y[+t7.7] w[+mC]=LexAop2.HMS00004}attP40 (this study); 5-day-old F1 female eyes were imaged by QCapture software (Quantitative Imaging Cooperation).

Generation of MS1096-LexA.G4H line

The second chromosome balancer, CyO, carrying a PBac{y⁺ = attP-9A} was generated by mobilizing PBac{y⁺ = attP-9A} VK00006 on the X chromosome to the chromosomal location 2R (42A13) and the molecular location at 2R: 6,219,947 on the CyO. Using the primers LexA_HACK_F1 (5'-AGAACCCCGGGCCCTAGGATGCCACCCAAGA AGAAGCG-3') and LexA_HACK_R1 (5'-TGAATAATTTCTATTTGGC TTTAGTCGACGGTATCGATAAG-3'), 2,926-bp fragment was

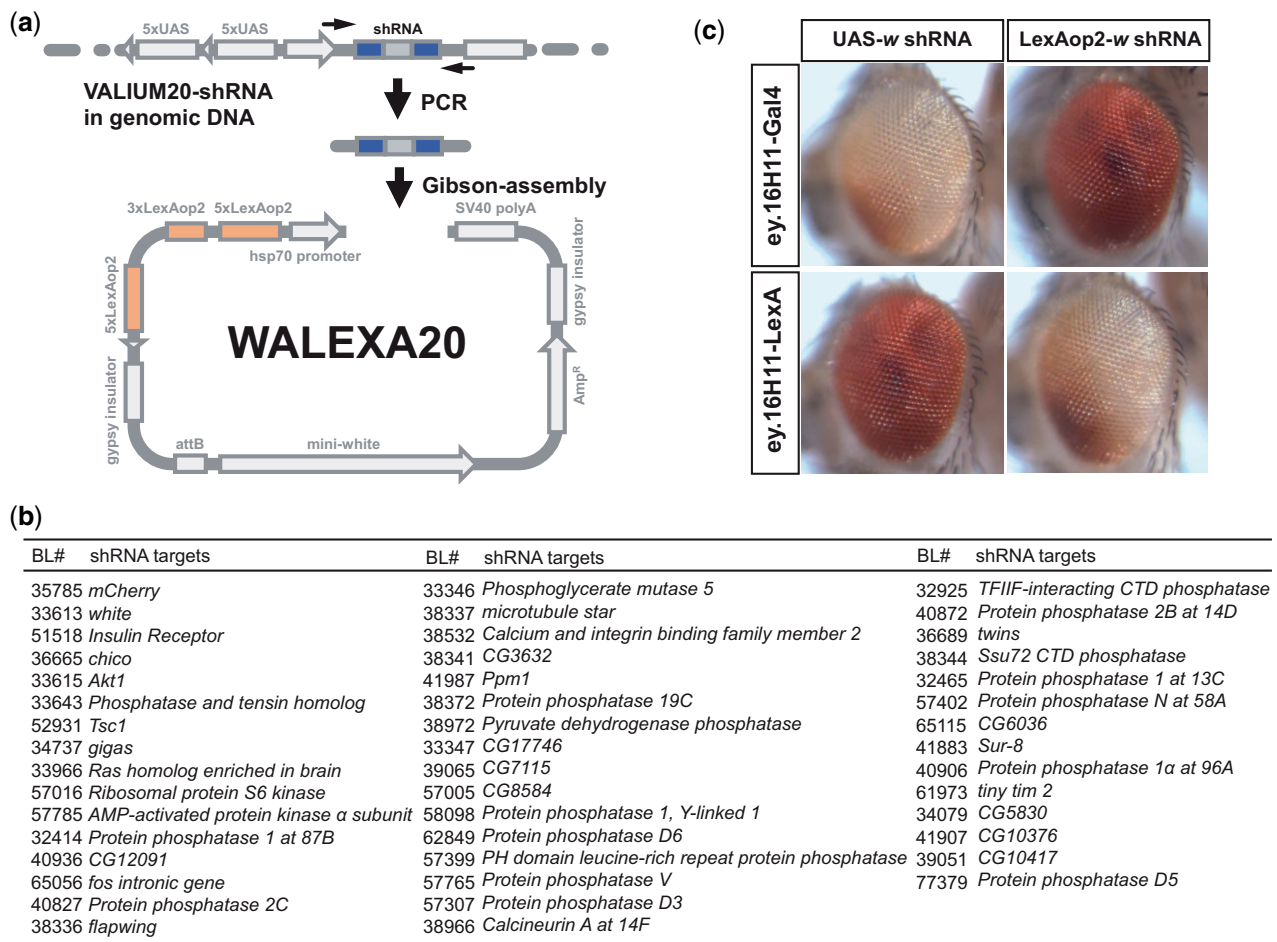


Fig. 1. Graphical summary of cloning gene-specific shRNAs to a LexAop2-based shRNA expression vector and functional verification of LexAop-based shRNA system in adult eyes. a) Gene-specific shRNA sequence (blue) was amplified from genomic DNA of VALIUM20-based transgenic flies using universal primer pairs (black arrows). PCR products were directly used in Gibson-Assembly reaction to clone shRNA sequences (blue) to pWALEXA20 vectors harboring LexAop2 enhancers (orange). b) List of target genes for VALIUM20-based UAS-shRNA transgenic lines and their identifiers in Bloomington Drosophila Stock Center (BL#). shRNA sequences of the target genes are listed in [Supplementary Table 1](#). c) Functional comparison of UAS- and LexAop-based shRNA transgenic lines targeting the *white* gene in adult eyes. Adult eyes are oriented anterior to the left. Eyeless enhancers (16H11) are more effective in knocking down *white* gene expression in the dorsal posterior (white eye area in the top-left and bottom-right panels) than ventral anterior areas of the eye in Gal4/UAS and LexA/LexAop combinations. Wild-type eye colors are maintained in Gal4/LexAop and LexA/Gal4 combinations, indicating these 2 systems are functionally independent.

amplified from the template pBPnlsLexA::GADflUw vector (Addgene #26232) and the amplified product was assembled to 9,083-bp fragment of pHACK-Gal4>QF2 (Addgene #104873) that was digested with AvrII and Sall. The resulting construct called pHACK-Gal4 >nlsLexA::GADfl was inserted to the PBac{y⁺ = attP-9A} site located in the CyO chromosome. The resulting donor transgenic line, called “LexA.G4H” for brevity, was combined to the vas-Cas9 transgene in the presence of L¹ and TM2 to distinguish the chromosomes by visible markers in a y¹ w¹¹¹⁸ genetic background. w¹¹¹⁸ P{w⁺ = GawB}Bx^{MS1096} (Bloomington stock #8860) females were crossed to y¹ w¹¹¹⁸; L¹/CyO, PBac{y⁺ RFP⁺ = LexA.G4H}; TM2/P{y⁺ = vas-Cas9}VK00027 males. F1 male progeny were crossed to y¹ w¹¹¹⁸ females individually, and about 50 Non-CyO F2 virgin females were screened for w⁺ and RFP⁺ eye markers per cross (Fig. 2b). Four independent MS1096-LexA.G4H lines were established from 80 individual F1 crosses and the lines were functionally tested by overexpressing LexAop2-Akt shRNA in wings. Female wings were mounted as described previously (Park et al. 2014) and imaged in AxioImager microscope (Zeiss). Wing length was measured from the wing hinge to the distal end of L3 vein (the red line in Fig. 3b) using an AxioVison microscope (Zeiss) and ImageJ software (NIH).

Reporter expression and Immunofluorescence on larval wing discs

To visualize the expression patterns of MS1096-Gal4 and MS1096-LexA.G4H in the third instar wing discs, males of a dual reporter line, UAS-Stinger LexAop-tdTomato.nls (Bloomington stock #66680) were crossed to females of either MS1096-Gal4 or MS1096-LexA.G4H. Wing discs from F1 progeny were fixed in 4% paraformaldehyde, 1× PBS, and 0.1% Triton X-100 for 1h, then mounted in Antifade mounting medium with DAPI (Vectashield H-1200). Both GFP and RFP channel images were acquired and overlaid in AxioVision to visually check for any leaky interactions between Gal4/UAS and LexA/LexAop expression systems.

To assess cell proliferation and apoptosis in larval wing discs expressing Pp1-87B or mts shRNA, females of MS1096-LexA.G4H were crossed to males harboring LexAop2-shRNA transgenes. Wing discs from F1 progeny were fixed as described above, then permeabilized in PBS with 1% Triton X-100 for 1h. Wing discs were incubated at 4°C for 16h in PBS with 0.1% Triton X-100 containing either antiphospho-Histone H3 (Ser10) antibody (Cell Signaling Technology #9701, 1:1,000) for a proliferation marker or anticlaved caspase-3 (Asp175) antibody (Cell Signaling

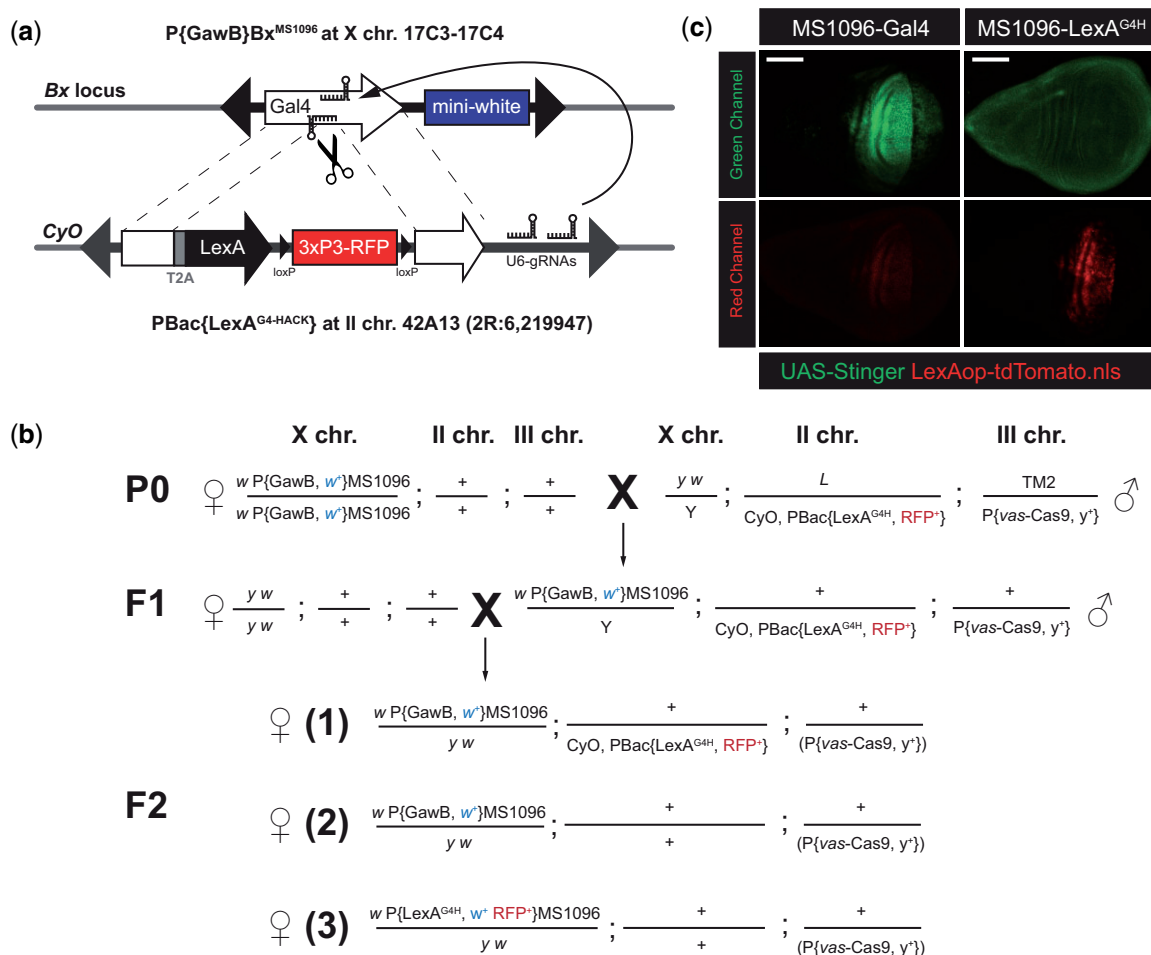


Fig. 2. Schematic outlines of trans-chromosomal Gal4 to LexA.G4HACK conversion system and genetic crosses for identifying the conversion events. a) The LexA.G4HACK donor is located at 42A13 on the second balancer chromosome, *CyO*. In the germline, Cas9 (scissors) and 2 guide RNAs (U6-gRNAs) make double-strand breaks in the middle of *Gal4*-encoding sequence (the white arrow at *Bx* locus on the upper X chromosome), allowing it to be repaired by the donor sequence carrying *Gal4* homology sequences (the white bar and arrow on the lower *CyO* chromosome) that are in-frame with T2A-*LexA* encoding sequence. Successful repair events are identified by co-segregation of both mini white transgene eye color (blue box) and red fluorescent protein eye markers (red box, 3xP3-RFP). b) Homozygous females for the target P{GawB} element harboring *Gal4*-encoding sequence and mini white (w^+) were mated to males carrying both a donor LexA.G4H transgene marked by an eye fluorescence marker 3xP3-RFP (PBac{LexA.G4H}) and a germline-specific Cas9 (P{vas-Cas9}) marked by a body-color marker y^+ . The tri-transgenic F1 male progeny were mated to $y^1 w^{1118}$ females individually. Of the different F2 possibilities (1–3), Gal4 to LexA.G4H conversion events were identified in F2 females without *CyO* balancer, but with mini white eye color and RFP eye fluorescence marker (3). c) Comparison of the original MS1096-Gal4 and converted MS1096-LexA.G4H expression by nuclear GFP (UAS-Stinger) and nuclear tdTomato (LexAop-tdTomato.nls) reporters in larval wing discs. The red channel for Gal4 and the green channel for LexA.G4H are over-exposed to show a little or no cross-activation of the reporter expression. The reporter expressions are mostly restricted in the dorsal half of the wing disc pouch area in both Gal4 and LexA.G4H driver lines. The scale bars indicate 100 μ m.

Technology #9661, 1:1,000) for an apoptosis marker. After washing 3 times in PBS with 0.1% Triton X-100 for 10 min, the discs were incubated at 22°C for 1 h in PBS with 0.1% Triton X-100 containing anti rabbit Alexa Fluor 488 (Invitrogen #A11034). After washing 3 times again, discs were mounted and imaged as described above.

Secondary school class and university partnerships

To facilitate the generation of new LexA and LexAop strains, we formed scholastic collaborations between Stanford University investigators and 2 secondary schools in the United States (Lawrenceville School and Phillips Exeter Academy), and with the University of Oxford Sir William Dunn School of Pathology. Briefly, course work at Lawrenceville for the “Hutchins Scholars Program” summer term was developed by Stanford, Oxford, Lawrenceville,

and Exeter instructors, with the goal of generating WALEXA20-based constructs harboring validated shRNA’s amplified from relevant TRiP-3 stocks (see above). This course included instruction in DNA amplification and cloning, fly transgenesis, multigeneration intercrosses to generate genetically “balanced” stocks, larval and adult dissection, tissue mounting, wing morphometry, and tissue immunohistology. Alongside the protocols taught, students were introduced to fundamental concepts in cell and developmental biology utilized, such as the central dogma and gene expression systems. To develop a separate course (Bio670) at Exeter with the goal of generating new LexA-expressing fly strains, instructors and staff at Stanford worked with Exeter instructors to develop homology assisted CRISPR knock-in (HACK)-based CRISPR/Cas9 replacement of *Gal4* with LexA sequences (to be published elsewhere). The instruction manuals for students are posted online (<https://www.stan-x.org/publications>, last accessed on January 24, 2022).

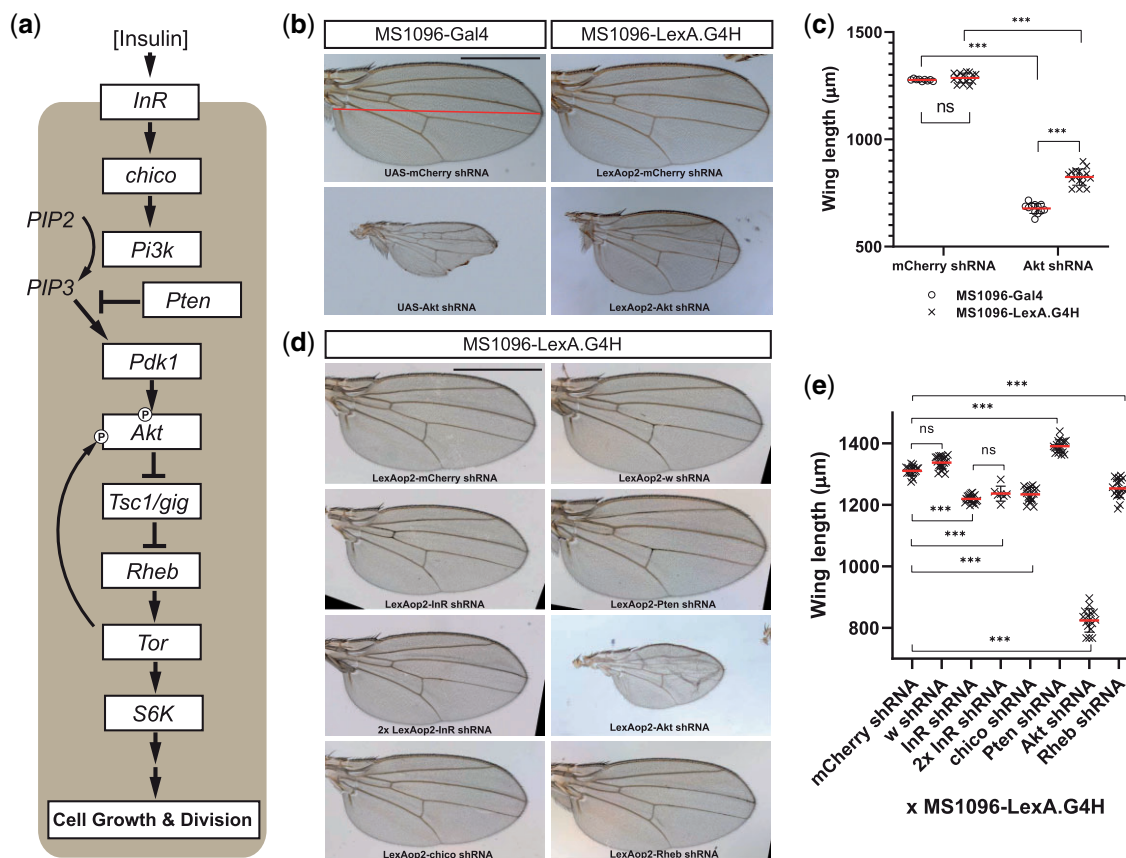


Fig. 3. Functional validation of LexA/LexAop shRNA system in adult wings for insulin signaling regulators. a) The insulin signaling cascade and its components regulating cell size and proliferation during animal development. The arrows indicate positive regulations between signaling components, and “T” symbols between components indicate negative regulations. The 2 “P” symbols on the Akt protein indicate different phosphorylation sites by Pdk1 and Tor. b) Comparison of adult wings expressing control shRNA (*mCherry* shRNA) or *Akt* shRNA by Gal4/UAS or LexA/LexAop systems. Compared to *mCherry* shRNA expression, *Akt* shRNA expression resulted in smaller wing sizes in both systems. The black scale bar indicates 500 μm. Wing length was measured from the wing hinge to the distal end of L3 vein (red bar). c) Quantification of wing length in animals expressing *mCherry* or *Akt* shRNAs by either Gal4/UAS or LexA/LexAop systems. *** indicates the statistical significance of $P < 0.001$ in Student’s t-test and ns indicates statistically not significant. The error bars are SDs. The red bars are the average length of $n \geq 9$ wings. d) Adult wings expressing shRNAs targeting insulin signaling component genes by LexA/LexAop system. The black scale bar indicates 500 μm. e) Quantification of wing length expressing shRNAs by LexA/LexAop system. Compared to *mCherry* shRNA controls, all shRNAs targeting insulin signaling component genes either increased or decreased the wing length significantly while the shRNA targeting *white* gene did not. Increasing the shRNA copy number did not make the wing smaller than a single copy of shRNA for the Insulin receptor gene (ns). *** indicates the statistical significance at $P < 0.001$ in Student’s t-test, and ns indicates statistically not significant. The error bars are SDs. The red bars are the average length of $n \geq 9$ wings.

Results

Generation of LexAop-based transgenic flies for shRNA expression

To develop LexAop-dependent suppression of selected genes, we constructed a plasmid vector pWALEXA20 (Fig. 1a) permitting expression of shRNAs from *sula*-derived LexA DNA-binding motifs (LexAop2; Pfeiffer et al. 2010). The vector design and use of universal PCR primers enables insertion of validated shRNA sequences from the TRiP-3 collection (Ni et al. 2011) adjacent to multimerized LexAop elements. This streamlined cloning process has been successfully adopted in the US secondary schools, and has facilitated the construction of multiple TRiP-3 shRNA-based lines (“see Materials and Methods”; Fig. 1b).

To evaluate LexA-regulated shRNA function, we first used a transgenic fly line harboring an *eyeless* enhancer (GMR16H11) driving LexA (Pfeiffer et al. 2010) to direct expression of *white* shRNA in eyes, then assessed the loss of eye pigment as a readout for *white* gene knockdown efficiency. In GMR16H11-LexA, LexAop-*white* shRNA flies, we observed a darker eye color in the ventral eye field, and lighter in the dorsal field (Fig. 1c), indicating

localized suppression of *white*. This was comparable to the pattern of eye color in GMR16H11-Gal4, UAS-*white* shRNA flies (Fig. 1c). Thus, tissue-specific suppression of *white* was comparable in the LexA/LexAop and Gal4/UAS systems, though the degree of eye color reduction was more severe in Gal4/UAS progeny. By contrast, we observed no detectable change in eye color of control GMR16H11-Gal4, LexAop-*white* shRNA flies, or in GMR16H11-LexA, UAS-*white* shRNA flies (Fig. 1c), providing additional evidence that LexA/LexAop and Gal4/UAS systems function independently without any cross-activation or leaky expression of shRNA.

Gal4 to LexA conversion in an enhancer trap line by homology assisted CRISPR/Cas9 knock-in

Adult wing size and morphology have been extensively used to identify genes regulating cell growth and patterning in *Drosophila* development. Assessments of wing phenotypes for their size, shape, and vein patterns are highly reproducible and are measurements readily adoptable by early-stage scientists, including secondary school researchers (see “Materials and Methods”). There are well characterized wing-specific enhancer trap Gal4 lines

such as P{GawB}bbg^{C96} and P{GawB}Bx^{MS1096} ("MS1096-Gal4" hereafter; Capdevila and Guerrero 1994; Neumann and Cohen 1996) in which expression of the Gal4 sequence in P{GawB} is regulated by wing-specific enhancers, which remain unidentified. To generate comparable LexA lines with these unique enhancer trap Gal4 lines, we developed approaches to achieve HACK (Lin and Potter 2016: Methods) to replace the existing Gal4-encoding DNA sequence with a LexA.G4H. To assess this approach, we targeted the MS1096-Gal4 enhancer trap line (Fig. 2, a and b). The trans-chromosomal conversion of MS1096-Gal4 target on the X chromosome to LexA.G4H using a HACK-based donor on the second chromosome was efficient (4 independent conversion events in 80 individual crosses; Fig. 2). To address the specificity of LexA expression in the converted lines, we assessed expression of a dual fluorescence reporter in the wing discs of MS1096-LexA.G4H, LexAop-tdTomato, UAS-Stinger larva. The expression of LexAop-based reporter by MS1096-LexA.G4H (Fig. 2c, red) revealed a comparable pattern to the original MS1096-Gal4. Moreover, we did not observe cross-activation of the UAS-based reporter, confirming prior reports that LexA/LexAop and Gal4/UAS systems function independently (Lai and Lee 2006; Yagi et al. 2010). We also noted little to no activation of LexA reporter by the original Gal4 driver (the red channel of MS1096-Gal4 in Fig. 2c). In summary, we generated fly lines permitting CRISPR/Cas9-mediated conversion of Gal4- to LexA-expressing lines, demonstrated the feasibility and efficiency of this genetic conversion, and confirmed the maintenance of the tissue-specific LexA expression in the LexA-converted progeny.

Functional tests of LexAop-based shRNA transgenic lines in developing wings

Insulin signaling regulates the growth of developing tissues by modulating both cell number and size (Rulifson et al. 2002; reviewed in Kim et al. 2021). Measurement of adult wing size has been used to quantify disruption of insulin signaling during *Drosophila* development (Park et al. 2014). To assess if the new wing-specific LexA line is suitable for shRNA-mediated suppression of genes encoding insulin signaling pathway regulators, we generated fly lines harboring LexAop-shRNA transgenes targeting *Insulin Receptor (InR)*, *chico* (an orthologue of mammalian *Insulin Receptor Substrates 1/2*), *Phosphatase and tensin homolog (Pten)*, *Akt*, and *Ras homolog enriched in brain (Rheb)* (Fig. 3a).

To compare LexAop-dependent shRNA-based gene suppression to the original UAS-driven shRNA lines, we intercrossed MS1096-LexA.G4H lines to LexAop-shRNA lines: progeny from intercrosses of the original MS1096-Gal4 and corresponding UAS-shRNA lines served as controls. In adult flies, we observed that shRNA targeting of a crucial insulin signaling gene, *Akt*, resulted in significantly smaller adult wings compared to the control shRNA targeting *mCherry* in both Gal4/UAS and LexA/LexAop systems, although wings after Gal4/UAS-based knockdown of *Akt* were significantly smaller compared to wings deploying LexA/LexAop knockdown (Fig. 3, b and c). In contrast, wings expressing the control *mCherry* shRNA by the 2 systems did not differ in size (Fig. 3, b and c), indicating the measurement of wing size is highly reproducible and LexA expression itself does not interfere with wing development. In addition, adult flies with wing-specific suppression of *Akt* enclosed without any discernable developmental defect, other than wing size changes. These findings are consistent with restricted expression of MS1096-LexA.G4H and MS1096-Gal4 to wing discs.

To assess the regulatory function of other genes in the insulin signaling pathway, we next assessed the impact of LexAop-

shRNA lines targeting *InR*, *chico*, *Pten*, or *Rheb* on wing size. In adult flies, we observed that shRNAs targeting of *InR*, *chico*, and *Rheb* resulted in significantly smaller adult wings (Fig. 3, d and e), consistent with the known requirement of these genes for cell growth while shRNA targeting *white* whose function is not involved in insulin signaling did not change the size. In contrast, shRNA targeting of *Pten* increased wing size (Fig. 3, d and e), confirming the prior observation that loss of *Pten* function leads to increased eye and wing size (Goberdhan et al. 1999). These results confirm the efficiency and reproducibility of the LexA/LexAop-based gene silencing strategy. To test if the degree of the observed loss-of-function phenotype can be further exerted by increasing the copy number of the LexAop-shRNA transgene, we scored the wing size of adult fly carrying 1 and 2 copies of LexAop-shRNA targeting *InR*. This effectively tested whether the LexA-driven expression levels of the shRNA, or, alternatively, the downstream cellular components of the RNA interference pathway are the limiting factor for the phenotypic strength in the wing development context. A single copy of the transgene encoding shRNA that targeted *InR* resulted in smaller wings; doubling the copy number of the same transgene did not reduce wing size further (Fig. 3, d and e). These results suggest that LexA-dependent shRNA expression from 1 copy of the LexAop-*InR* shRNA transgene may be sufficient to saturate the capacity of the RNA interference pathway. In summary, we generated a novel fly line expressing LexA in the larval wing disc to assess the impact of LexAop-based shRNA gene suppression on wing development and growth. Generation and functional validation of LexAop-shRNA lines targeting insulin signaling motivated the development of curricula at partnering secondary schools to generate additional resources, and characterize growth regulators of wing disc development, as detailed below.

An interscholastic network for systematic generation of new LexAop flies

To expand the repertoire of extant fly strains permitting LexA-dependent genetics, we next leveraged our multiinstitutional network of secondary schools (see Materials and Methods) to develop curricula that permit students and their instructors to generate additional new lines expressing shRNA. To assess the quality of the data generated in distant classroom settings, we assigned construction of shRNA lines targeting known insulin signaling regulators (*Tsc1*, *gig*, and *S6K*) in addition to the *Drosophila* phosphatases whose function have not been systematically assessed for cell growth and patterning during development. Students and instructors intercrossed these new LexAop-shRNA lines to adults expressing the wing-specific MS1096-LexA.G4H driver, then assessed adult wing length. Flies targeting *Tsc1*, *gig*, and *Pten* (purple bars, Supplementary Figure. S1) had increased average wing length while flies targeting *S6K* and *Rheb* had reduced wing length (green bars, Supplementary Figure. S1), confirming that the students correctly produced shRNA lines targeting insulin signaling regulators with expected results. Additionally, students also identified several phosphatases that altered cell growth or patterning in adult wings (Supplementary Figure. S1), including *Pp1-87B*, *mts*, *CG17746*, *CG8584*, *PpV*, *Pp2B-14D*, *Ssu72*, *Pp1-13C*, and *PpD5* (Supplementary Figure. S1).

We repeated a subset of these intercrosses and confirmed students' results of statistically significant increases or reductions of wing length in adult progeny from these crosses compared to *mCherry* or *white* shRNA controls (Fig. 4a). For example, shRNAs targeting *CG17746*, *CG8584*, *Pp2B-14D*, *Ssu72*, *Pp1-13C*, *PpV*, and *PpD5* resulted in wing size changes without any patterning defect

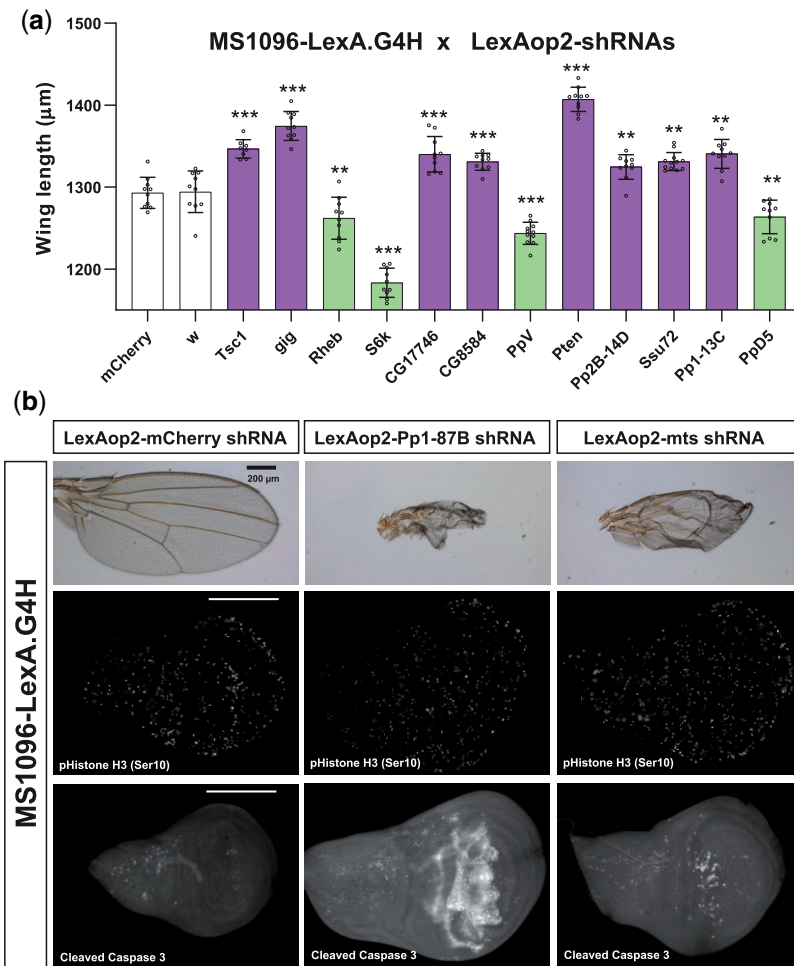


Fig. 4. Identification of growth regulators for *Drosophila* wing development using the LexA/LexAop system. a) Quantification of wing length in flies expressing selected shRNAs that resulted in significantly smaller (green bars) or larger (purple bars) wings compared to the control *mCherry* shRNA expressing wings ($P < 0.01$, Student's *t*-test). The error bars are SDs. The average lengths are based on measurements of 8 individual female wings in each genotype. ** denotes $P < 0.01$, and *** indicates $P < 0.001$. b) Altered adult wing morphology and increased number of apoptotic cells in larval wing discs expressing *Pp1-87B* or *mts* shRNAs compared to the control *mCherry* shRNA. Cleaved caspase-3 staining marks apoptotic cells while phospho-Histone H3 staining marks proliferating cells. *Pp1-87B* and *mts* shRNA expression by MS1096-LexA.G4H resulted in increased apoptotic markers in the dorsal wing pouch where MS1096 enhancer expression is high (Fig. 2c), but the marker expressions are not changed outside of LexA.G4H expression domain in wing discs. The scale bars indicate 200 µm.

assessed by wing hairs and vein locations: further studies should determine if these phosphatases are directly or indirectly involved in the insulin signaling pathway. By contrast, the expression of *Pp1-87B* shRNA or *mts* shRNA led to the development of morphologically abnormal wings (Fig. 4b). *Pp1-87B* and *mts* genes are previously shown to regulate mitotic chromosomal segregation, Hedgehog signaling, and Wingless signaling (Chen et al. 2007; Luo et al. 2007; Zhang et al. 2009). To understand the basis of dysmorphic wing development after shRNA-mediated *Pp1-87B* or *mts* gene knockdown, we immunostained wing discs to assess an earlier stage of wing development in third instar larvae. Expression of phospho-Histone H3, a cell cycle marker of G2/M2 transition, was not detectably altered after *Pp1-87B* or *mts* gene knockdown (Fig. 4b). However, we observed increased expression of Cleaved Caspase-3, a marker of apoptosis, in the wing disc cells expressing *Pp1-87B* shRNA or *mts* shRNA (Fig. 4b). These results suggest that the dysmorphic adult wing phenotypes after *Pp1-87B* or *mts* gene knockdown may originate from a significant increase of cell apoptosis during wing development. The increased expression of Cleaved Caspase-3 in wing discs expressing *Pp1-87B* or *mts* shRNAs was restricted to the central region of the wing disc

where the expression of our MS1096-LexA.G4H is highest, providing additional evidence for the tissue specificity of the new LexA/LexAop-based gene knockdown system. Thus, classroom-based experimental studies revealed several phosphatases that may be required for sustaining cell growth and survival during wing development.

Discussion

Intersectional binary expression approaches such as simultaneous use of the LexA/LexAop and Gal4/UAS systems have empowered fly biology, particularly in studies of neuroscience, and intercellular communication coordinating metazoan metabolism (Kim et al. 2021). For example, simultaneous use of 2 independent binary expression systems allowed clonal analysis of multiple cell populations (Lai and Lee 2006; Bosch et al. 2015), studies of tissue epistasis (Yagi et al. 2010), and elucidation of otherwise undetected contacts between cells (Gordon and Scott 2009; Macpherson et al. 2015). To address a growing need for unique fly strains permitting flexible intersectional approaches, we are expanding our efforts to create new genetic tools suitable

for this purpose (Kockel et al. 2016, 2019; Wendler et al. 2020). Here, we describe (1) a genetic tool to convert existing Gal4 lines to LexA.G4H by CRISPR/Cas9-mediated gene editing, (2) a universal cloning method to generate LexAop-based shRNA transgenic lines from functionally validated UAS-based shRNA transgenic lines, and (3) a research-based pedagogy to leverage effort by collaborating students in our secondary school network (Kockel et al. 2016, 2019). Our results demonstrate that *in vivo* gene regulation by LexAop-based gene suppression succeeded in multiple tissue contexts. The resources reported here can be applied to generate additional intersectional binary expression tools in studying genetics, development, physiology, metabolism, and other studies of cells and tissues.

An analysis of HACK-based conversion of Gal4 to QF2.G4H (Lin and Potter, 2016) showed that some target locations had higher conversion rates (conversion “hot spots”) while most donor locations worked equally well for intra-chromosomal or trans-chromosomal conversions. This led us to place our LexA.G4H donor transgene on the second chromosome balancer, CyO, so that the parental donor transgene can be easily identifiable in the F2 progeny while the conversion events at the target location can be detected efficiently. Using this CyO-bound donor transgene on the second chromosome, the trans-chromosomal conversion of MS1096-Gal4 target on the X chromosome to LexA.G4H was successful. Although the conversion rates could vary for other target locations, our donor transgene located on the CyO chromosome may be sufficient for the general purpose of establishing LexA.G4H lines on other chromosomes. In this regard, additional Gal4 lines on the second and third chromosomes have been successfully converted to LexA.G4H through the interscholastic network, and characterization of these new LexA.G4H lines is in progress (Chisholm T, Rankin AE, Kim S, Park S, unpublished results).

To address the challenge of validating the specificity and efficiency of gene suppression using shRNA approaches *in vivo*, we sought to generate a facile cloning step to develop genetic counterparts in the LexA/LexAop system for specific shRNAs that have proven to be robust in the Gal4/UAS system. Here, we designed universal primers that bind immediately outside of the UAS-shRNA region in transgenic flies from the TRiP-3 collection, then streamlined the production of strains harboring functionally validated shRNA sequences expressed from LexAop2 sequences. While characterizing LexAop-based shRNA lines in adult eyes and wings, we consistently observed slightly weaker shRNA activities in LexA/LexAop system compared to the corresponding Gal4/UAS system. Given that both systems share identical transactivation domains, this difference could reflect lower affinity of the LexA DNA-binding domain to multimerized (13 \times) LexAop2 sequences in the pWALEXA20 vector. If so, the shRNA expression system may be further refined by substituting for 8 \times , 16 \times , or 26 \times LexAop2 sequences to decrease or increase the expression level of transgenes (Pfeiffer et al. 2010). Alternatively, the activity of the LexA-encoding sequence used in this study (nlsLexA::GADfl, “see *Materials and Methods*”) may be further optimized (Yagi et al. 2010). In summary, new approaches presented here generated the wing-specific LexA driver line, MS1096-LexA.G4H, and LexAop-based shRNA lines that augment the arsenal of available LexA-dependent expression tools (Pfeiffer et al. 2010; Kockel et al. 2016) and also complement existing Gal4/UAS resources.

Data and biological resources here were generated from partnerships connecting research universities with teachers and students at secondary schools. This illustrates the feasibility of building an interscholastic network to generate unfulfilled

scientific resource needs, while providing a model that contributes to experiential STEM education. Resources and outcomes described here significantly extend, develop, and complement the interscholastic partnerships in experiment-based science pedagogy described in our prior studies, which focused on generating LexA enhancer trap lines (Kockel et al. 2016, 2019). The current study involved collaborations between Stanford and Oxford University investigators with students and teachers at the Lawrenceville School and the Phillips Exeter Academy, 2 US secondary schools. Together, we developed courses focused on shRNA cloning and transgenesis, or CRISPR/Cas9-mediated conversion of Gal4 to LexA strains. These research experiences for students include generating novel fly strains, accompanied by a sense of scientific discovery and ownership (Hatfull et al. 2006), and connection to a broader community of science through the distribution of flies, information, and resources through stock centers and scientific meetings. A curriculum based on classical and molecular fruit fly genetics combined with developmental biology provided a compelling framework of authentic research experiences for student scientists in US grades 9–12 (Redfield, 2012; Kockel et al. 2016, 2019). To the extent that students and their instructors partnered to (1) perform unscripted experiments, (2) develop new curricula, and (3) generate fruit fly genetics resources, the experience was a success, and affirmed the value of practice-based secondary school science education. We speculate such benefits may be amplified from even earlier introduction of experimental science in the US K-12 sequence. In summary, this experience demonstrates how longitudinal studies involving molecular biology, genetics, and developmental biology can build a thriving, interconnected network of teachers, students, and classes that impacts personal growth, and professional development, and in the process generates valuable resources and data for the global scientific community.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplemental material is available at G3 online.

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Conflicts of interest

None declared.

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