cGAL, a temperature-robust GAL4–UAS system for *Caenorhabditis elegans*

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The GAL4–UAS system is a powerful tool for manipulating gene expression, but its application in *Caenorhabditis elegans* has not been described. Here we systematically optimize the system’s three main components to develop a temperature-optimized GAL4–UAS system (cGAL) that robustly controls gene expression in *C. elegans* from 15 to 25 °C. We demonstrate this system’s utility in transcriptional reporter analysis, site-of-action experiments and exogenous transgene expression; and we provide a basic driver and effector toolkit.

Bipartite gene expression systems offer fine spatial and temporal control of gene expression, and they are crucial for understanding gene function. The GAL4–UAS system, which has proven highly useful in *Drosophila* studies¹, uses the *Saccharomyces cerevisiae* Gal4p protein, which contains a DNA-binding domain (DBD) and a transcriptional activation domain (AD). Gal4p homodimers bind a 17-nucleotide upstream activating sequence (UAS), resulting in transcription of a downstream gene². Cell-specific promoters placed upstream of GAL4 constitute a ‘driver’ construct, while UAS sites placed upstream of the gene of interest constitute an ‘effector’ construct. Incorporation of these constructs into separate transgenics creates standardized driver and effector lines, which can be crossed together, generating progeny with desired expression patterns (Fig. 1a).

The bipartite nature of GAL4–UAS offers three major advantages over direct promoter–gene fusions. Large numbers of strains can easily be generated by crossing driver and effector lines. Incorporating novel components into pre-existing driver and effector libraries requires minimal effort. Verified driver and effector strains become community reagents that foster experimentation and reproducibility. However, the GAL4–UAS system has not been successfully adapted for use in *C. elegans*. We optimized three components of the GAL4–UAS system to make it functional for *C. elegans* across 15–25 °C, and we demonstrated its robustness for tissue-specific gene expression, site-specific genetic rescue and channelrhodopsin experiments.

Previous unpublished attempts using *S. cerevisiae* Gal4p DBD (residues 1–147, henceforth termed GAL4SR) with human herpes virus activation domain (VP16)³ as the driver components exhibited poor performance in *C. elegans*. Since stronger activation domains improve performance of the GAL4 driver⁴, we tested VP64 (ref. 5), which has four tandem copies of VP16. We fused GAL4SR to VP16 and VP64 and placed them both under the pharyngeal muscle promoter (Pmyo-2), designating them Pmyo-2::GAL4SR::VP16 and Pmyo-2::GAL4SR::VP64, respectively. Comparing their performance in a 15xUAS::gfp effector transgene strain (syIs300), the Pmyo-2::GAL4SR::VP64 driver yielded seven-fold more GFP fluorescence in pharyngeal muscles at room temperature (Fig. 1b,c) than did Pmyo-2::GAL4SR::VP16. The fluorescence observed requires both driver and effector transgenes (Supplementary Fig. 1). We therefore adopted VP64 as our activation domain.

We optimized UAS copy number by comparing constructs with 5x, 10x, 15x and 20x UAS copies upstream of gfp at equal concentrations in a strain with a Pmyo-2::GAL4SR::VP64 driver (syIs301). We found successively increasing GFP fluorescence until 15x UAS copies (~2.3-fold versus 5x UAS, P < 0.0001; ~1.3-fold versus 10x UAS, P < 0.01, one-way ANOVA, Tukey’s post-test), beyond which it appeared to saturate (~1.1-fold versus 20x UAS, P = 0.5, one-way ANOVA, Tukey’s post-test; Fig. 1d,e). Thus, 15x UAS was adopted for effector constructs.

To determine the robustness of our GAL4–UAS system under standard *C. elegans* growth temperatures (15–25 °C), we assayed our Pmyo-2::GAL4SR::VP64 driver and 15xUAS::gfp effector combination, and we found its transcriptional efficacy depended heavily on temperature; it performed well only at 25 °C (Fig. 2a,b); ~67% decrease in fluorescence at 20 °C, ~80% decrease in fluorescence at 15 °C, P < 0.0001, two-way ANOVA with Tukey’s correction), consistent with findings in *Drosophila*⁶.

The temperature dependence of the canonical GAL4–UAS system and the optimal growth temperature of *S. cerevisiae* (30–34 °C)⁷ suggested that activity of Gal4p from a specific yeast species may be adapted to its optimal growth temperature. Thus, a Gal4p from more cryophilic *Saccharomyces* yeast species might prove useful in designing a more robust driver at lower temperatures. We chose the Gal4p DBD from *Saccharomyces kudriavzevii*⁸ (Portuguese reference strain ZP591, residues 1–147, henceforth termed GAL4SK) because *S. kudriavzevii* has an optimal growth temperature (23–24 °C)⁷ close to that of *C. elegans*, and the key residues necessary for UAS binding⁹ are conserved between GAL4SC and GAL4SK (Fig. 2c).

We generated a new Pmyo-2 driver by replacing GAL4SC with GAL4SK, and we compared this driver’s performance with that

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of the GAL4Sc-driver across 15–25 °C using the same integrated 15xUAS:gfp effector strain (syIs300). The Pmyo-2::GAL4Sc::VP64 driver lines exhibited more fluorescence than did the Pmyo-2::GAL4Sc::VP64 driver lines at room temperature; blinded researchers accurately sorted these two drivers using an epifluorescence dissecting microscope. We chose the brightest line from each driver for quantitation. At all temperatures, the Pmyo-2::GAL4Sc::VP64 driver performed more robustly than the Pmyo-2::GAL4Sc::VP64 driver; a performance in some instances comparable to that of strains carrying a direct Pmyo-2:gfp fusion as a benchmark (Fig. 2b and Supplementary Fig. 2). Our GAL4Sc driver had slightly higher fluorescence at 25 °C (Fig. 2a,b) and exhibited only an ~20% decrease in fluorescence at 20 °C (versus ~67% for GAL4Sc) and only an ~40% decrease in fluorescence at 15 °C (versus ~80% for GAL4Sc). We adopted GAL4Sc as our DBD of choice, in conjunction with VP64 and 15x UAS, to comprise our fully optimized GAL4–UAS system. We designated our system cGAL to denote its original implementation in C. elegans and its engineered performance at cooler temperatures.

We tested whether cGAL would perform in other major tissues by generating new driver constructs containing various tissue-specific promoters (Pnpn–40, intestine; Pmyo–3, body wall muscles). When injected into a strain (syIs300) with an integrated 15xUAS: gfp effector, these new drivers produced robust and specific GFP fluorescence in expected tissues (Fig. 3a,b). However, our initial pan-neuronal (Prab-3) and GABAergic (Punc-47) drivers displayed poor, mosaic expression in the nervous system along with intense ectopic GFP fluorescence in the posterior gut (data not shown). We speculated that our vector backbone (a derivative of pPD49.26 containing the unc-54 3’ UTR) might be responsible. We therefore switched to the vector pPD117.01 containing the let-858 3’ UTR and a 5’ decoy sequence (comprising a decoy intron and open reading frame) upstream of the multiple cloning sites (see Online Methods and Supplementary Notes 1 and 2); these modifications reduce ectopic expression and improve expression in a broad range of tissues (A. Fire, personal communication). We generated new Prab-3 and Punc-47 driver constructs in the pPD117.01 backbone and injected them into a strain (syIs390) with a new 15xUAS:gfp::let-858 3’UTR effector (also made using pPD117.01; Supplementary Notes 3 and 4). These strains not only showed decreased ectopic fluorescence in the posterior gut (data not shown), but also displayed dramatically increased fluorescence in the entire nervous system (Fig. 3c) or GABAergic neurons (Fig. 3d). Cholinergic (Punc-17) and glutamatergic (Peat-4) neuronal drivers gave similarly strong and specific results in the syIs390 effector strain (Fig. 3e,f). Thus, cGAL
Figure 3 | Functional studies with the cGAL system. GFP effector expression in a variety of major tissues (a–f), each driven by the indicated cGAL driver. In c–f, both driver and effector constructs (15xUAS::gfp::let-858 3′UTR) are built in the pPD117.01 vector. (g) The signaling pathway in the circuit controlling the final expulsion step in the defecation motor program. AEX-2 is necessary in two GABAergic neurons DVB and AVL (AVL not shown) to mediate expulsion. (h) Quantification of expulsion events per defecation cycle in animals with indicated genotypes. aex-2 rescue experiments with GABAergic driver, Punc-47; muscle driver, Pmyo-3; intestinal driver, Pnlp-40. Bars are mean ± s.e.m. n = 8, 8, 8, 8, 7, 7, left to right. (i) Quantification of paralysis response to pulse train. Each dot represents mean response to three blue light exposures from an individual animal. Bars are mean ± s.e.m. n = 20 and 10, left to right. Mann–Whitney test.

is robust across a variety of tissues, and we recommend using the pPD117.01 vector for driver and effector construction.

We then applied cGAL to functional studies by examining the rhythmic defecation motor program (DMP), which contains three sequential muscle contractions. The final enteric muscle contraction occurs when the intestine releases neuropeptide NLP-40, which binds its receptor AEX-2 on GABAergic neurons, triggering an expulsion event. We applied cGAL to confirm the site-of-action of aex-2 in GABAergic neurons for expulsion. We generated an effector line (syEx1444) with 15xUAS::aex-2(+):cDNA in the aex-2(sa3) background. This strain was then combined with a Pnlp-40 driver, Punc-47 driver or Pmyo-3 driver to create aex-2(sa3) mutants expressing aex-2(+) cDNA specifically in the intestine, GABAergic neurons or body wall muscles, respectively.

In aex-2(sa3) mutants, expulsion was nearly eliminated. Only the presence of both GABAergic driver and 15xUAS::aex-2(+) cDNA effector rescued the expulsion defect of aex-2(sa3) mutants; neither could rescue the defect alone. Expression of aex-2(+) cDNA in either body wall muscles or intestine did not rescue. These results recapitulate a study using conventional promoter–cDNA fusion transgenes. Therefore, cGAL can be used for tissue-specific rescue experiments.

Next, we tested whether cGAL could be applied to gain-of-function experiments. Channelrhodopsin is a light-sensitive cation channel that depolarizes cells in the presence of all-trans retinal and blue light. Activation of GABAergic neurons in C. elegans causes worms to adopt a flaccid, paralyzed state. We injected the GABAergic driver construct into a strain carrying an integrated 15xUAS::hChR2(H134R)::eyfp::let-858 3′UTR effector to express channelrhodopsin specifically in GABAergic neurons. In the presence of all-trans retinal, animals were subjected to a three-pulse train of 475-nm blue light. Each excitation caused immediate, limp paralysis only in animals possessing both GABAergic driver and effector but not in animals with either component alone. cGAL is thus capable of controlling exogenous transgene expression to interrogate neural circuits in C. elegans.

Finally, we built a basic cGAL driver and effector toolkit (Supplementary Tables 1 and 2). For drivers, we constructed strains and plasmids for major tissues, major neurotransmitter cell types and some individual sensory neurons. For effectors, we have integrated strains for cell labeling (GFP, mKate2, GFP–H2B and mCherry–H2B), cell ablation (ICE), calcium sensing (GCaMP6s), neuronal activation (ChR2), neuronal inhibition (HisCl1) and brief communications
synaptic inhibition (TeTx). All effectors are integrated, and at least one line for each integrated effector was confirmed to be functional (Supplementary Fig. 3 and Supplementary Videos 1–5).

One limitation of cGAL is the absence of Gal80p, a negative regulatory component used to repress Gal4p as a ‘NOT’ gate.14 Gal80p recognizes the C terminus of Gal4p, absent in our drivers. Addition of the minimal C-terminal functional domain that interacts with S. kudriavzevii Gal80p might address this issue.

A similar bipartite expression system, Q,16 has yet to be widely adopted in C. elegans, perhaps because of lack of sufficient drivers and effectors. As with Drosophila,17,18, the cGAL system could be combined with Q or other binary expression systems for control of multiple effectors.

cGAL can already facilitate genetic site-of-action and genome-wide overexpression experiments, but we envision that a growing collection of drivers and effectors will allow analysis of genetic, developmental and neural networks with increased rate and rigor.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

H.W., J.L. and P.W.S. conceived the project. H.W. and J.L. performed the experiments, analyzed the data and wrote the paper. S.G. helped with molecular cloning and strain handling. E.M.S. devised the idea of trying Gal4p from yeast species with lower growth temperatures. C.M.C. and N.P. contributed reagents.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Maintenance of Caenorhabditis elegans strains. Strains were maintained on NGM plates with Escherichia coli OP50 as the food source at room temperature as originally described21, unless noted otherwise. Bristol strain N2 is the wild-type reference strain. The strains used in this study are detailed in the Supplementary Notes 5 and 6, and all the integrated drivers and effectors are listed in Supplementary Table 1.

Molecular biology. Plasmids were constructed by standard molecular cloning techniques with either restriction enzyme cleavage and DNA ligation or Gibson assembly using enzymes from New England BioLabs (Beverly, Massachusetts). The coding region of GAL4, residues 1–147 of Gal4p from Saccharomyces kudriavzevii (the Portuguese reference strain ZP591, a gift from C.T. Hittinger (University of Wisconsin-Madison), was PCR amplified from genomic DNA using 5’ primer ggaGCTAGCatgaagctgttgtcttcaaggg and 3’ primer cggGAATTCcgcgatacactcaacttggc. The synthetic Scal-17mer sequence (CGGAGTACTGTCTCCG)22 was used for the UAS site and was placed upstream of the pes-10 basal promoter in all effector constructs. All constructs were built in either the pSM1 vector (a gift from C. Bargmann, Rockefeller University), a derivative of pPD49.26, which contains the unc-54 3’ UTR, or the vector pPD117.01 (a gift from A. Fire, Stanford University), which contains the let-858 3’ UTR and a 5’ decoy minigene upstream of the MCS for promoter insertion (see Supplementary Notes 1–4 for vector maps and sequences). We generated effector constructs for cell labeling (GFP, mKate2, GFP-H2B and mCherry–H2B), cell ablation (ICE)23, calcium reporter (GCaMP6s)24, neuronal activation (ChR2)25, neuronal inhibition (HisCl1)26 and synaptic transmission blocking (TeTx)27. Other details about plasmids and oligos used in the study are documented in Supplementary Table 2.

Transformation. Transgenic animals were generated using standard microinjection techniques28. Unless noted otherwise, 100 ng/µL total DNA injection samples were prepared, with either 1 kb DNA ladder from New England BioLabs (Beverly, Massachusetts) or the pBluescript II KS+ plasmid, as carrier. Extrachromosomal arrays were integrated into the genome via X-ray irradiation. Most of the integrants were outcrossed at least three times with the wild-type strain N2. Full details about transgenic C. elegans strains in this study are listed in the Supplementary Note 5 and Supplementary Table 1.

Fluorescence imaging. Approximately 25 animals per line were imaged and quantified for the optimization process of the cGAL system using the myo-2 promoter (Pmyo-2). Briefly, L4 or young adult animals grown at corresponding temperatures (15 °C, 20 °C, 25 °C or room temperature) were selected and imaged with Leica DMi6000 inverted microscope equipped with a 40× oil objective and an Andor iXon Ultra 897 EMCCD camera using Metamorph software (Molecular Devices). Images were captured with the same exposure time (20 ms), and the average fluorescence in the pharynx for each animal was analyzed. The representative fluorescent images in Figure 3a–f showing the application of cGAL in different tissues were collected with a Zeiss LSM710 confocal microscope with a 20x objective.

Defecation motor program assay. L4 animals raised at room temperature were picked 1 d before the assay. During the assay, which was performed at 20 °C, each individual worm was picked to a new NGM plate seeded with OP50, and an 18 × 18 mm coverslip was placed over the animal for better optics. After a 2-min acclimation period, each animal was videotaped using a Zeiss Stemi SV11 coupled to a Unibrain Fire-i 501b camera for 5 min, and the number of pBoc and expulsion events were scored. Each pBoc indicated the initiation of each defecation cycle. The ratio of expulsions over pBocs was used to quantify the expulsion phenotype for each animal (n = 7 or 8 for each genotype).

Optogenetics. 1 d before the assay, L4 animals raised at room temperature from each strain were picked individually onto NGM plates seeded with 100 µL OP50 containing 500 µM all-trans retinal (Sigma). During the assay, which was performed at 20 °C, animals were recorded using a Zeiss Stemi SV11 coupled to a Unibrain Fire-i 501b camera. Channelrhodopsin was activated using blue light generated from a Lumen Dynamics X-Cite series 120 lamp and a standard GFP filter set. Blue 475 nm light intensity was measured to be 0.2 mW/mm². After an initial 10-s acclimation period, three light pulses, each 2 s in duration, were delivered to each worm at intervals of 20 s. The researcher doing the assay was blinded to the genotype of the animals.

Histamine chloride inhibition. Histamine plates were made by adding histamine dihydrochloride (Sigma) to the standard NGM recipe for a final concentration of 10 mM. Animals were picked onto plates with a small amount of food. After 30 min, worms were recorded using a Zeiss Stemi SV11 coupled to a Unibrain Fire-i 501b camera.

Data availability. The source data used for the plots in the main and supplementary figures are included in the supplementary materials. Other data are also available from the corresponding author upon request. The coding sequence of Gal4p from Saccharomyces kudriavzevii is in GenBank (GU299177.1). The plasmids for cGAL driver (pHW393, plasmid no. 85583) and effector (pHW394, plasmid no. 85584) are available from Addgene. Source data for Figures 1–3 and Supplementary Figures 1 and 2 are available online.

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