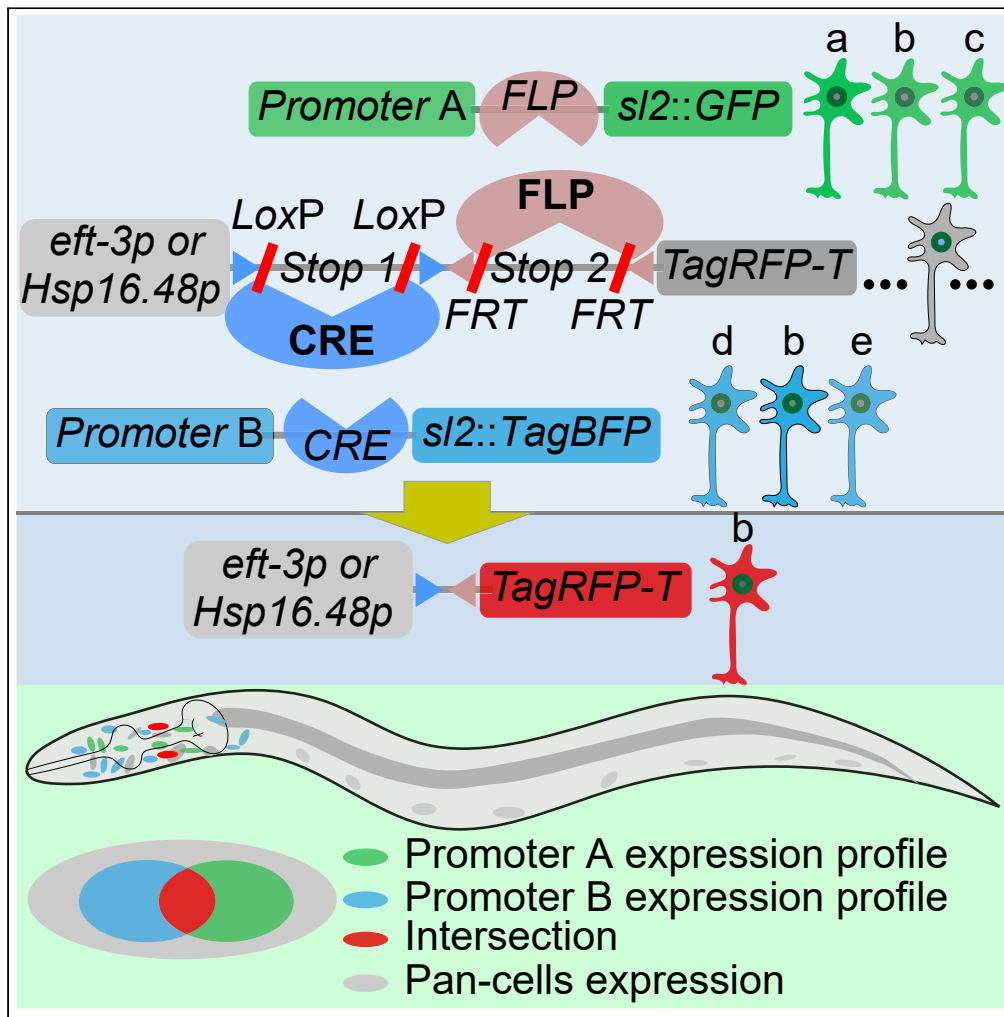


Article

# Dual Recombining-out System for Spatiotemporal Gene Expression in *C. elegans*



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**HIGHLIGHTS**

A tripartite FLP/CRE-out spatiotemporal gene expression system in *C. elegans*

Eight promoter pairs with the targeting of a single-type neuron(s) were identified

The system is easy to use and cost and labor saving



## Article

Dual Recombining-out System  
for Spatiotemporal Gene  
Expression in *C. elegans*

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## SUMMARY

**Specific recording, labeling, and spatiotemporal manipulating neurons are essential for neuroscience research. In this study, we developed a tripartite spatiotemporal gene induction system in *C. elegans*, which is based on the knockout of two transcriptional terminators (stops in short) by two different recombinases FLP and CRE. The recombinase sites (*loxP* and *FRT*) flanked stops after a ubiquitous promoter terminate transcription of target genes. FLP and CRE, induced by two promoters of overlapping expression, remove the stops (subsequent FLP/CRE-out). The system provides an "AND" gate strategy for specific gene expression in single types of cell(s). Combined with an inducible promoter or element, the system can control the spatiotemporal expression of genes in defined cell types, especially in cells or tissues lacking a specific promoter. This tripartite FLP/CRE-out gene expression system is a simple, labor- and cost-saving toolbox for cell type-specific and inducible gene expression in *C. elegans*.**

## INTRODUCTION

Genetic methods are widely used to study functions and neurosignal integration of neural circuits in the nervous system (Dymecki and Kim, 2007; Luo et al., 2008, 2018). Spatiotemporally specific (i.e., cell type-specific and inducible) expression of genes is required to identify neuron type, measure the activity of genetically defined neurons, analyze function(s) of the tested gene(s) in neurons, construct neuronal circuit diagrams, and dissect the process of signal integration in neuronal circuits. The genes expressed include the tested genes of living organisms and those encoding indicators, markers, and proteins used in optogenetic and chemogenetic manipulation of neurons. Owing to the conservation of genes in the animal genera, experimentally tractable animal models are generally used to study the relationships among genes, proteins, and neural circuits that underlie behaviors. *Caenorhabditis elegans* (*C. elegans*) is a prevalent animal model for studying conserved molecular and neural bases of the nervous system. The limited specific promoters of single/paired neuron(s) hinder the pace and veracity of neuroscience studies, not only in *C. elegans* but also in other species (Dymecki and Kim, 2007; Luo et al., 2008, 2018).

For spatial and/or temporal gene expression in *C. elegans*, several strategies have been developed. Bipartite interaction systems of spatial gene expression provide cell type-specific gene expression, which employ two promoters of intersectional expression patterns to drive two interactional parts of a molecule (Chelur and Chalfie, 2007; Zhang et al., 2004). A recently developed strategy of native and tissue-specific fluorescence (NATF) combined with genome editing and split-GFP allows cell-specific fluorescence labeling of native proteins in *C. elegans* (He et al., 2019). Another method used cell-specific rescue of heat shock factor-1 (*hsf-1*) mutants to regulate spatial and temporal transgene expression in *C. elegans* (Bacaj and Sham, 2007).

The bipartite FLP-out or CRE-out systems induce temporal or spatiotemporal gene expression in *C. elegans*. It is based on the knockout of a transcriptional terminator (stop) by recombinase FLP (flipase) or CRE (cyclization recombination) derived from the yeast *Saccharomyces cerevisiae* and bacteriophage P1, respectively (Buchholz et al., 1998; Dymecki, 1996; Golic and Lindquist, 1989; Hoess et al., 1984; Raymond and Soriano, 2007; Sauer and Henderson, 1988). FLP-out and CRE-out are the processes of recombinase-catalyzed intramolecular excision of spacer DNA that lies between tandemly oriented *FRT* (FLP

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<https://doi.org/10.1016/j.isci.2020.101567>



recognition target) and *LoxP* (locus of crossing over in P1) sites (Davis et al., 2008; Guo et al., 2015; Hubbard, 2014; Lopez-Cruz et al., 2019; Macosko et al., 2009; Schmitt et al., 2012; Voutev and Hubbard, 2008; Wang et al., 2017). However, these systems need an inducible promoter combined with a cell type-specific promoter for spatiotemporal gene expression. Moreover, bipartite systems of CRE-out of *mCherry* were designed for lineage tracing with varied fluorescence in *C. elegans* (Ruijtenberg and van den Heuvel, 2015). Other bipartite expression systems were also developed to achieve spatiotemporal transgene control in *C. elegans*, such as GAL4-UAS (cGAL) system (Wang et al., 2017), split intein based split cGAL system (Wang et al., 2018), Q system together with split Q system (Wei et al., 2012), and recently the tetracycline-dependent ribozyme switch system (Wurmthaler et al., 2019). An interesting strategy combining heat shock promoter *hsp-16.2* with infrared laser illumination (Churgin et al., 2013, 2014; Singhal and Shaham, 2017) was also implemented for spatiotemporal gene induction. Nevertheless, a feasible, reliable, labor- and cost-saving toolbox for cell type-specific and inducible expression of genes of interest in *C. elegans* is still needed.

Here, a tripartite spatiotemporal gene expression system was developed, which works on dual knockout of two distinct recombinase sites flanked stops by two different recombinases FLP and CRE (and subsequent FLP/CRE-out). Vectors used in the system were constructed by the flexible Three-Fragment Multisite Gateway method. This tripartite FLP/CRE-out gene expression system not only offers highly refined spatial and temporal control of gene expression, but is also feasible and labor and cost saving.

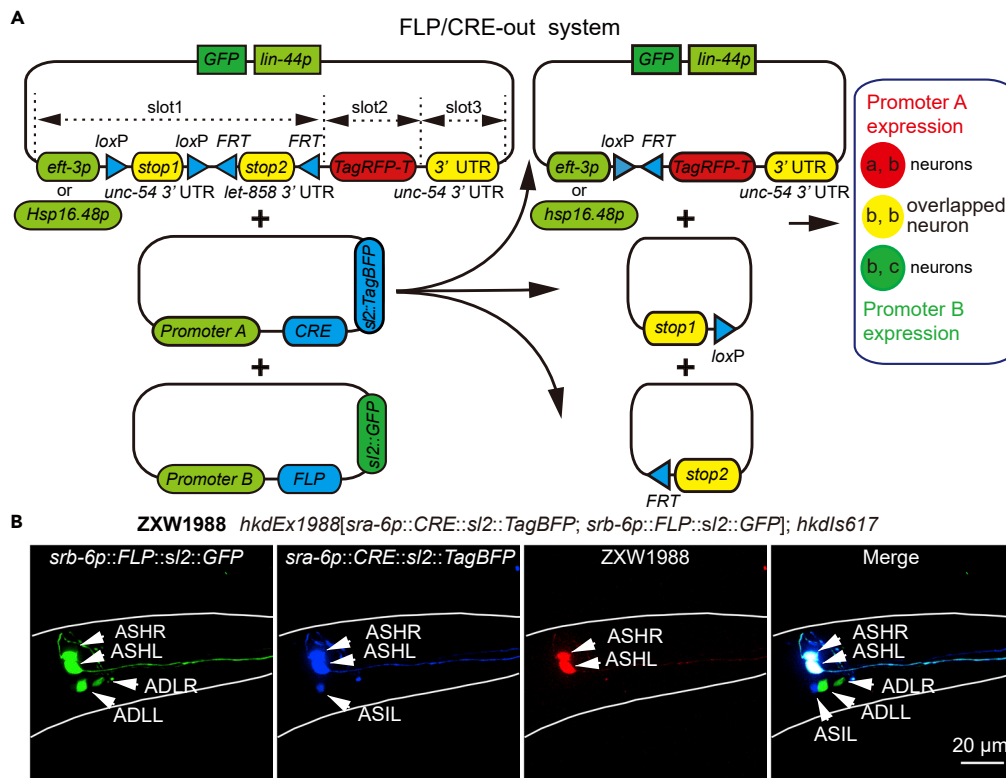
## RESULTS

### Construction and Work Principle of the Tripartite FLP/CRE-out Gene Expression System

Recombining-out transcriptional terminator with recombinase enables spatial, temporal, or spatiotemporal gene expressions (Davis et al., 2008; Guo et al., 2015; Hubbard, 2014; Lopez-Cruz et al., 2019; Macosko et al., 2009; Schmitt et al., 2012; Voutev and Hubbard, 2008; Wang et al., 2017). However, these bipartite systems have challenges providing spatial and temporal gene expression in the neuron(s) or tissue lacking a specific promoter. Therefore, to induce spatiotemporal gene expression in *C. elegans*, we developed a tripartite gene induction system (Figure 1A). The system is composed of a core vector for the expression of a tested gene and two recombinase-encoding vectors. The core vector contains *eft-3* (strong pan-expression) or *hsp-16.48* promoter (heat shock inducible), two different tandem recombinase-sites-flanked transcriptional terminators (stops), and a gene of interest. For an easy change of promoters and genes, we used the Three-Fragment Multisite Gateway method (Magnani et al., 2006) to construct these three vectors (Figures S1A–S1C), using the Invitrogen system from Thermo Fisher Scientific (Thermo Fisher Scientific, MA, USA). To construct the core vector, we used the following sequences: *eft-3* or *hsp-16.48* promoter with *loxP*-flanked stop1 and *FRT*-flanked stop2 as slot1 (*promoter::d-stops*, in short); the gene of interest as slot2; and *unc-54 3' UTR* as slot3. The *loxP* and *FRT* are recombination sites of CRE and FLP. The transcriptional terminators stop1 and stop2, which block the transcription of the target gene, consist of *unc-54 3' UTR* and *let-858 3' UTR*. Recombining-out two stops by recombinase CRE and FLP driven by two distinct promoters with overlapping expression in a type of cell(s), enabled gene transcription in the defined cell(s). CRE and FLP used in the FLP/CRE-out system derived from a nucleolus-transport-enhanced Cre (*nCre*) of yeast *S. cerevisiae* (Hoess et al., 1984; Sauer and Henderson, 1988) and mouse codon-optimized FLP (FLPo) (Raymond and Soriano, 2007). We made further modifications to improve their expression and referred to as CRE and FLP. This system provides an "AND" gate strategy for intersectoral targeting of a single cell type, i.e., controls gene expression in a type of cell(s) that lacks a specific promoter (Figure 1A).

### The FLP/CRE-out Gene Expression System Is Efficient to Label and Identify a Single Type of Neurons

Spatiotemporally manipulating and recording of neuronal activity is vital for studies of neuronal circuits and circuitual neurosignal integration. The FLP/CRE-out system should be a convenient tool for the studies. Neuron-type-specific promoters or promoter pairs are essential for the FLP/CRE-out system. In *C. elegans*, many neurons, especially sensory neurons, have been identified to possess specific promoters or promoter pairs. However, numerous neurons, especially interneurons, still lack known specific promoters or promoter pairs. Identifying promoter pairs for these neurons will facilitate research in these cells. For easy identification of promoter pairs, we constructed a chromosomally integrated *C. elegans* transgene ZXW617 strain using a standard UV/trimethylpsoralen (UV/TMP) integration method (Yandell et al., 1994). Its genotype is *hkdIs617[eft-3p::d-stops::TagRFP-T::unc-54 3' UTR::lin-44p::GFP]*. Co-injection of



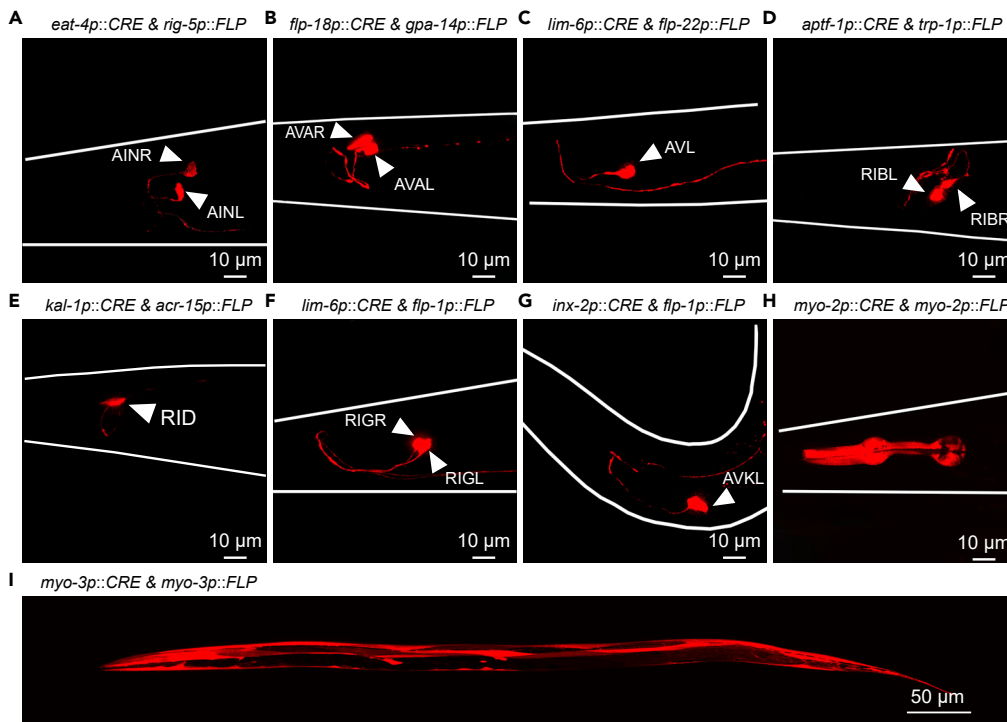
**Figure 1. A Tripartite Spatiotemporal Gene Expression Control System based on Dual Knocking Out Two Transcriptional Terminators**

(A) Design and working principle of the tripartite FLP/CRE-out-mediated gene expression control system. The system consists of three vectors: a core vector and two plasmids encoding CRE and FLP recombinases. The core plasmid consists of sequences of a strong pan-expression *eft-3* or a promoter of the *hsp-16* family, two transcription terminators flanked by *loxP* or *FRT* recombination sites, a target gene and *lin-44p::GFP*.

(B) Confocal fluorescence images of a ZXW1988 transgenic worm. Different fluorescent channels show expression profile of green, *srb-6p* in ASHs and ADLs; blue, *sra-6p* in ASHs and ASIs; and red channels, TagRFP-T in ASHs. The merged image (right) indicated the co-localization of the three fluorescence colors. Scale bar, 20  $\mu$ m.

FLP and CRE plasmids with a promoter pair of a single neuron-type intersectional expression into the transgenic worms should enable single neuron-specific fluorescent labeling. As expected, the *hkdIs617* [*sra-6p::CRE::sl2::TagBFP*; *srb-6p::FLP::sl2::GFP*] transgene displayed ASHs-specific TagRFP-T labeling (Figure 1B). The expression patterns of *sra-6p* and *srb-6p* were ASHs/ASIs and ASHs/ADLs with an intersection of ASH neurons. To identify whether each of the two stops is sufficient to prevent the expression of the downstream reporter gene, the strains of *hkdEx2000*[*sra-6p::CRE::sl2::TagBFP*]; *hkdIs617* and *hkdEx2001* [*srb-6p::FLP::sl2::GFP*]; *hkdIs617* were generated, respectively. As shown in Figures S2A and S2B, no red fluorescent protein was detected; however, blue and green channels showed the normal expression of CRE and FLP, respectively. This result illustrates that each of the two stops was able to prevent the expression of the downstream reporter gene.

Next, we used the integrated strain ZXW617 to identify various promoter pairs for intersectional targeting of single-neuron type. We successfully identified eight promoter pairs with a single neuron-type intersection. Shown in Figures 2A–2G and Table S1, the following promoter pairs enabled TagRFP-T expression in single types of neuron(s): *eat-4p* & *rig-5p* in AINs (paired L/R), *flp-18p* & *gpa-14p* in AVAs (paired L/R), *lim-6p* & *flp-22p* in AVL (single), *aptf-1p* & *trp-1p* in RIBs (paired L/R), *kal-1p* & *acr-15p* in RID (single), *lim-6p* & *flp-1p* in RIGs (paired L/R), and *inx-2p* & *flp-1p* in AVKs (paired L/R). An FLP-out approach, which used single-copy transgenic strains that stably express an optimized version of FLP in specific tissues or by heat induction, reached 100% recombination efficiencies in several cell types such as muscles, intestine, and serotonin-producing neurons (Munoz-Jimenez et al., 2017). Compared with this system, the FLP/CRE-out system offers efficiencies of 100% in muscle cells and 53%–86% in neurons. The differential efficiencies in



**Figure 2. Identifying Promoter Pairs of Spatial Targeting Using Integrated ZXW617 Worms**

(A–G) Confocal fluorescence images of TagRFP-T in AIN (A, paired L/R), AVA (B, paired L/R), AVL (C, single), RIB (D, paired L/R), RID (E, single), RIG (F, paired L/R), and AVK (G, paired L/R) neurons, respectively. Neuron-specific TagRFP-T expression was controlled by the FLP/CRE-out system with the indicated vectors of CRE and FLP. "Paired L/R" and "single" indicate the type of neuron(s). Scale bar, 10 µm.

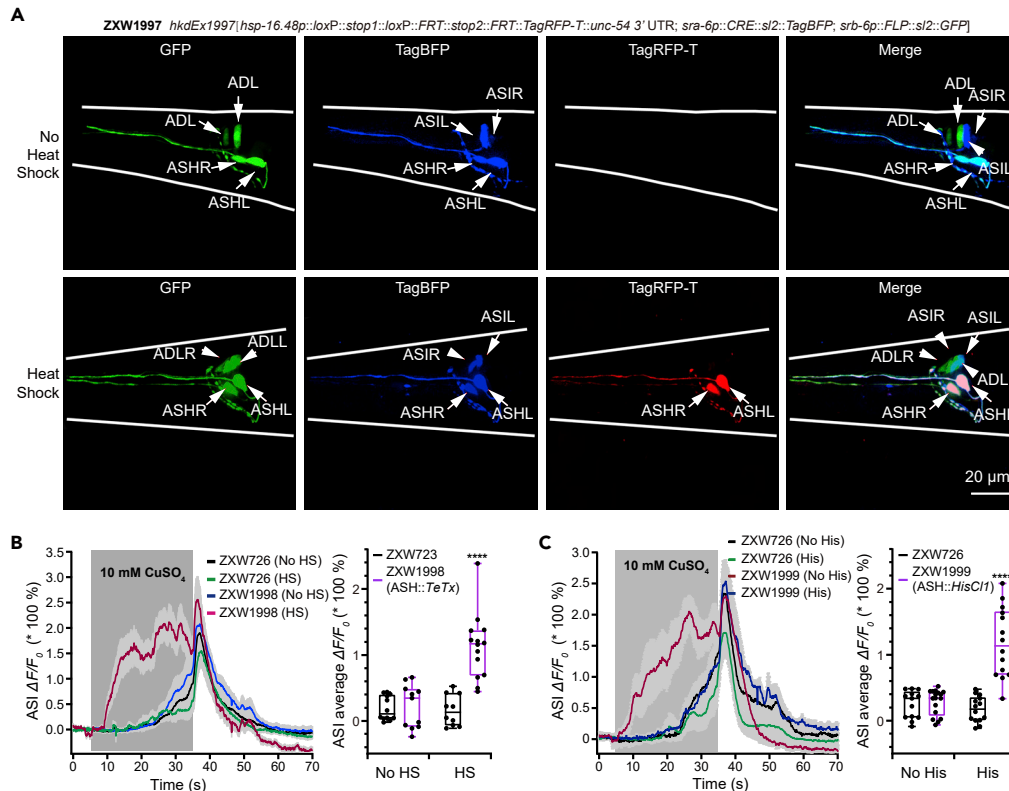
(H and I) Confocal fluorescence images of TagRFP-T in pharyngeal muscle cells (H) and body wall musculature (I). Scale bars, 10 µm (H) and 50 µm (I).

neurons may be caused by the varied expression of the recombinases. The ZXW617 strain may be an array of TagRFP-T. In the array, both local excision and excision between different integrated plasmids should be possible. Thus, the array could be reduced to just a single plasmid over time, since every recombination event leaves behind a *LoxP* or *FRT* site that can recombine again. After some observations, we observed no obvious variability of TagRFP-T fluorescence in different worms, no gradual dimming of the fluorescence over time, and even brighter fluorescence in older animals.

Since the core plasmid used a strong pan-expression *eft-3* promoter to drive target gene expression, the FLP/CRE-out system should be able to control specific gene expression in cells/tissues other than neurons. We thus set out to induce TagRFP-T expression in pharyngeal muscle and body wall musculature. As shown in Figures 2H and 2I, two types of muscle cells showed specific TagRFP-T labeling. In summary, a tripartite cell-specific gene expression system based on recombining-out two different stops was developed. We also have identified eight promoter pairs with intersectional targeting of a single-type neuron(s).

### The Highly Refined Spatial and Temporal Control System of Gene Expression Based on FLP/CRE-out

The FLP/CRE-out system is reliable for inducing cell type-specific gene expression. Replacement of *eft-3* promoter in the core vector with an inducible element or promoter should empower the trinary system to control both spatial and temporal gene expression. Heat shock promoters were widely used to induce temporal gene expression in *C. elegans* (Bacaj and Shaham, 2007; Churgin et al., 2013, 2014; Davis et al., 2008; Hubbard, 2014; Jones et al., 1989; Monsalve et al., 2019; Munoz-Jimenez et al., 2017; Shim et al., 2003; Singhal and Shaham, 2017; Stringham et al., 1992; Voutev and Hubbard, 2008; Wang et al., 2018). So, we replaced *eft-3p* with *hsp16.48p* in the "A" entry clone to construct an *hsp16.48p::d-stops::TagRFP-T* vector (Figure S3B). To examine the inducibility and neuron-specificity of TagRFP-T expression, we tested



**Figure 3. Inducible and Cell Type-Specific Expression of Target Genes Controlled by FLP/CRE-Out System and Uses in Genetic and Chemogenetic Manipulation of Single-Type Neurons**

(A) Confocal fluorescence images of GFP, TagBFP, and TagRFP-T before and after the treatment of heat shock; 12 h after the 2-h heat shock treatment, a single pair of ASH neurons were specifically labeled by red fluorescence. The expression patterns of *sra-6p* and *srb-6p* were ASHs/ASIs and ASHs/ADLs, respectively. Scale bar, 20  $\mu\text{m}$ .

(B) Changes in  $\text{Ca}^{2+}$  transients (in curves in the left panel, and box-and-whisker in the right panel) in response to  $\text{CuSO}_4$  solution (10 mM) in ASI neurons in worms of the indicated genotypes. No HS, without heat shock treatment; HS, with heat shock treatment. The genotypes of ZKW726 and ZKW1998 are *hkdEx726[gpa-4p::R-GECO1; lin-44p::GFP]* and *hkdEx1998[hsp16.48p::loxP::stop1::loxP::FRT::stop2::FRT::TeTx; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP; gpa-4p::R-GECO1]*, respectively.

(C) Changes in the  $\text{Ca}^{2+}$  signals (in curves in the left panel, and box-and-whisker in the right panel) in ASI neurons in response to  $\text{CuSO}_4$  solution (10 mM) in worms of the indicated genotypes. Histamine of 10 mM was used to activate the HisCl1 channel. The genotype of ZKW1999 is *hkdEx1999[eft-3p::loxP::stop1::loxP::FRT::stop2::FRT::HisCl1; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP; gpa-4p::R-GECO1]*. No His, without histamine treatment; His, with histamine treatment. Data of  $\text{Ca}^{2+}$  signals were expressed as the means  $\pm$  SEM as indicated by solid traces  $\pm$  gray shading and box plots with each dot representing those in each individual tested worm. The significance of statistical difference was analyzed by two-way ANOVA analysis with post hoc test of Tukey's multiple comparison correction and indicated as \*\*\*\* $p < 0.0001$ .

gene induction in ASH neurons. We generated a transgenic strain ZKW1997 of genotype *hkdEx1997[hsp-16.48p::loxP::stop1::loxP::FRT::stop2::FRT::TagRFP-T::unc-54 3' UTR; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP]*. As expected, no obvious red fluorescence in ASH neurons was detected in heat shock-untreated worms. In contrast, bright red fluorescence in ASHs was observed in heat shock (2 h heat shock at 34°C and 12 h post-treatment culture under standard 20°C) treated worms (Figure 3A).

Spatial and temporal manipulation of neurons is essential for neuroscience studies. Interruption of neurotransmitter release in a tested neuron(s) with the light chain of tetanus toxin (TeTx) and optogenetic and chemogenetic manipulation is commonly used (Boyden et al., 2005; Guo et al., 2015; Kim et al., 2017; Luo et al., 2008, 2018; Macosko et al., 2009; Pokala et al., 2014; Wang et al., 2016; Yizhar et al., 2011). One primary goal of our study is to develop a toolbox for spatiotemporal manipulation of neurons. TeTx, a specific synaptobrevin protease that blocks vesicle fusion with the plasma membrane and thus

neurotransmitter release (Schiavo et al., 1992), has been successfully used to inhibit chemical synaptic transmission of tested neurons in *C. elegans* (Guo et al., 2015; Liu et al., 2019; Macosko et al., 2009; Wang et al., 2016). Continual inhibition of neurotransmission by TeTx, beginning from embryonic periods, may interfere with the development of the nervous system. Therefore, the inducible expression of the toxin might be an appropriate solution.

In *C. elegans* nociception and avoidance of copper ion, ASIs and ASHs reciprocally inhibit. The inhibition of ASHs augments the activity in ASIs (Guo et al., 2015). Here we used this neuronal interaction to probe further spatiotemporal gene induction of the FLP/CRE-out system. We blocked ASH neurotransmission with inducible expression of TeTx, chemogenetically inhibited ASHs, and examined changes in ASI  $Ca^{2+}$  transients evoked by  $Cu^{2+}$ . We generated ZXW1998 transgenic strain of genotype *hkdEx1998[hsp-16.48p::d-stops::TeTx; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP; gpa-4p::R-GECO1]*. Expectedly, heat-shock treatment (34°C for 2 h) of the transgenic worms caused a significant increase in ASI  $Ca^{2+}$  signals evoked by 10 mM  $CuSO_4$ . In contrast, the heat shock-untreated ZXW1998 and heat shock-treated *hkdEx726 [gpa-4p::R-GECO1; lin-44p::GFP]* worms (Figure 3B) did not display an obvious change in  $Cu^{2+}$ -elicited  $Ca^{2+}$  signals in ASIs. This result indicated a successful temporal induction of TeTx in ASHs.

The *Drosophila* histamine-gated chloride channel subunit 1 (HisCl1) and administration of exogenous histamine are used to inhibit neurons acutely in *C. elegans* (Guo et al., 2015; Pokala et al., 2014). Next, we chemogenetically inhibited ASHs and examined ASI  $Ca^{2+}$ -responses to the  $Cu^{2+}$  treatment. We generated ZXW1999 transgenic strain of genotype *hkdEx1999[eft-3p::d-stops::HisCl1; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP; gpa-4p::R-GECO1]*. As shown in Figure 3C, the administration of 10 mM exogenous histamine for 10 min activates HisCl1 channel and thus acutely inactivates ASHs, resulting in a significant increase in  $Cu^{2+}$ -evoked  $Ca^{2+}$  signals in ASIs. In comparison, the HisCl1 expression (in histamine-untreated ZXW1999) or the treatment of histamine (in ZXW726) alone did not significantly change the  $Cu^{2+}$ -evoked  $Ca^{2+}$  transients in ASIs. In summary, the above experiments corroborate the feasibility and efficiency of the tripartite FLP/CRE-out system of spatiotemporal gene expression.

## DISCUSSION

In the present study, we develop a tripartite FLP/CRE-out gene induction method/system for spatiotemporal control of transgene expression in single/single type of neuron(s) and other cells or tissues in *C. elegans*. In addition, the system provides an "AND" gate strategy for the spatial expression of a gene. The advantages of the FLP/CRE-out method are as follows. (1) Ability to spatiotemporally control gene expression in the neuron(s) or other cells that lack a specific promoter. Especially this system is useful for optogenetic and chemogenetic manipulation of single types of neuron(s). (2) Simple and cost saving. Changes of promoter pairs, which direct CRE and FLP expression, are needed only for expressing a target gene in varied types of neurons and other cells. Meanwhile, the change of a target gene in the core vector is needed to control the spatiotemporal expression of different genes in defined cell types. Replacing promoters and genes in the FLP/CRE-out system is easy by the use of the MultiSite gateway method. All plasmids and worms used in this study are available to the scientific community. The integrated worm strain ZXW617 of genotype *hkds617[eft-3p::d-stops::TagRFP-T::unc-54 3' UTR::lin-44p::GFP]* is a useful tool to identify new promoter pairs for spatial control of gene expression in a single type of neuron(s). Using this worm strain, we identified eight promoter pairs for controlling spatial gene expression in the neuron(s) lacking a known specific promoter or promoter pair. Our work lays an important foundation for studies in these neurons. Importantly, ~6,000 promoters in the *C. elegans* promoterome (Dupuy et al., 2004) are available for constructing recombinase vectors by the three fragment gateway method. Moreover, single-copy transgenic strains that stably express an optimized version of FLP in specific tissues or by heat induction (Munoz-Jimenez et al., 2017) are useful for the FLP/CRE-out system. (3) The strong pan-expression *eft-3p* and *hsp-16.48p* promoters in the core gene expression plasmid of the FLP/CRE-out system lay a foundation for the use of the system in cells and tissues other than neurons in *C. elegans*. (4) In some bipartite systems, the effector transgene is placed downstream of a tissue-specific promoter; therefore, it can only be expressed as long as the promoter is active. Compared with these bipartite induction systems, the use of *eft-3* promoter in the FLP/CRE-out system offers the possibility of prolonged and continuous expression of the effector transgene.

Multiple methods have been developed for controlling spatial or spatiotemporal gene expression in *C. elegans*. Bipartite systems, such as FLP-out and CRE-out systems, are not able to induce spatial and temporal gene expression in a single type of neuron(s) and other cells lacking a specific promoter (Davis et al.,

2008; Guo et al., 2015; Hubbard, 2014; Lopez-Cruz et al., 2019; Macosko et al., 2009; Schmitt et al., 2012; Voutev and Hubbard, 2008; Wang et al., 2017), HSF-1 system (Bacaj and Shaham, 2007), GAL4-UAS system (Wang et al., 2017), QF-GR system (Monsalve et al., 2019). Moreover, the tripartite Q system and split Q system (Wei et al., 2012) empower to control spatiotemporal gene expression. However, this system lacked tight regulation. QS and QF have to be expressed constitutively, which require a minimum of 6 h for quinic acid to alleviate QS repression and allow transgene induction, whereas complete restoration of transgene expression takes 24 h (Monsalve et al., 2019; Nance and Frokjaer-Jensen, 2019). Tripartite split cGAL confers spatiotemporal control of transgene expression but is likely to introduce a temporal delay, compared with a direct heat shock promoter::gene fusion (Wang et al., 2018). The dynamic range of the tetracycline-dependent ribozyme switch system (with maximal gene 3.75-fold  $\pm$  0.85-fold induction) is weaker compared with the classical use of heat shock promoters (Wurmthaler et al., 2019). To temporally and spatially control gene expression and protein activity/localization are essential in modern biology and even modern medicine. Given the advantages of the FLP/CRE-out-mediated gene control system, this system expands the genetic toolbox for *C. elegans*.

### Limitations of the Study

All of the transgenic strains except ZXW617 were not integrated. The ZXW617 strain was generated by a standard ultraviolet/trimethylpsoralen (UV/TMP) integration method, not by single-copy insertion. The strain may be an array of TagRFP-T.

### Resource Availability

#### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Zheng-Xing Wu ([ibbwuzx@mail.hust.edu.cn](mailto:ibbwuzx@mail.hust.edu.cn)).

#### Materials Availability

All data generated or analyzed in this study are included in this published article (and its [Supplemental Information](#) files). The strains and plasmids will be deposited at CGC (<https://cgc.umn.edu/>) and Addgene (<https://www.addgene.org/>), respectively.

#### Data and Code Availability

The published article includes all data generated or analyzed during this study.

## METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101567>.

## ACKNOWLEDGMENTS

We thank Caenorhabditis Genetic Center (CGC) and National BioResource Project (NBRP) for the worm strains used in this study, Dr. B.F. Liu for the support in the fabrication of microfluidic devices, Dr. C. Bargmann for the plasmids containing of HisCl1 cDNA, and Dr. Peter Simms for revising the manuscript. This work was supported by grants from the National Science Foundation of China (31471034) and Fundamental Research Funds for the Central Universities (2016YXZD062).

## AUTHOR CONTRIBUTIONS

Z.-X.W. supervised the project. M.-H.G., T.-H.W., and W.W. performed the majority of the plasmid construction and imaging experiments. Z.-X.W., M.-H.G., and U.A.-S. wrote the manuscript with help from all of the other authors.

## DECLARATION OF INTERESTS

The authors declare no competing interests.



Received: July 7, 2020  
Revised: August 12, 2020  
Accepted: September 11, 2020  
Published: October 23, 2020

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## Supplemental Information

### Dual Recombining-out System

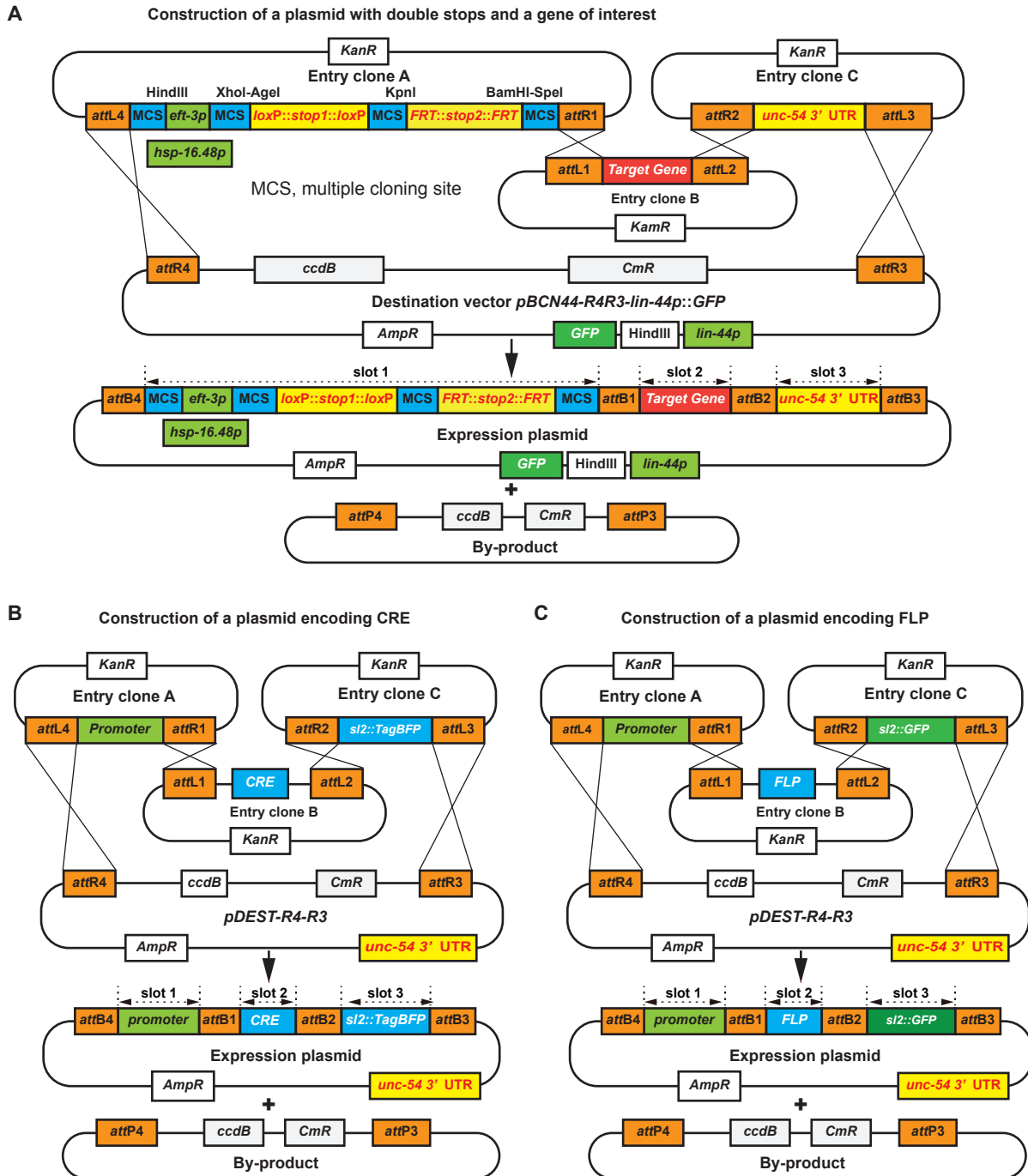
### for Spatiotemporal Gene

### Expression in *C. elegans*

Ming-Hai Ge, Wei Wang, Tai-Hong Wu, Xin Wen, Umar Al-Sheikh, Li-Li Chen, Sheng-Wu Yin, Jing-Jing Wu, Jia-Hao Huang, Qing-Qin He, Hui Liu, Rong Li, Ping-Zhou Wang, and Zheng-Xing Wu

# Supplemental Information

## Supplemental Figures

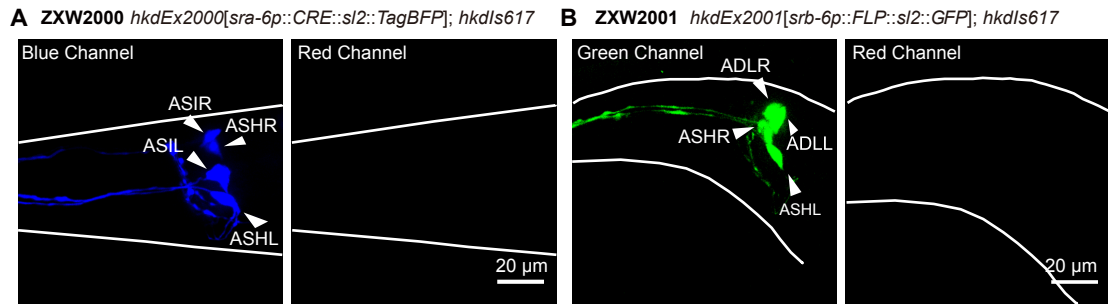


**Figure S1. Schematic diagram of the construction of expression plasmids used in the tripartite spatiotemporal gene control system, Related to Figure 1**

The system works on the base of recombining-out two transcriptional terminators by recombinases FLP and CRE, and subsequent FLP/CRE-out.

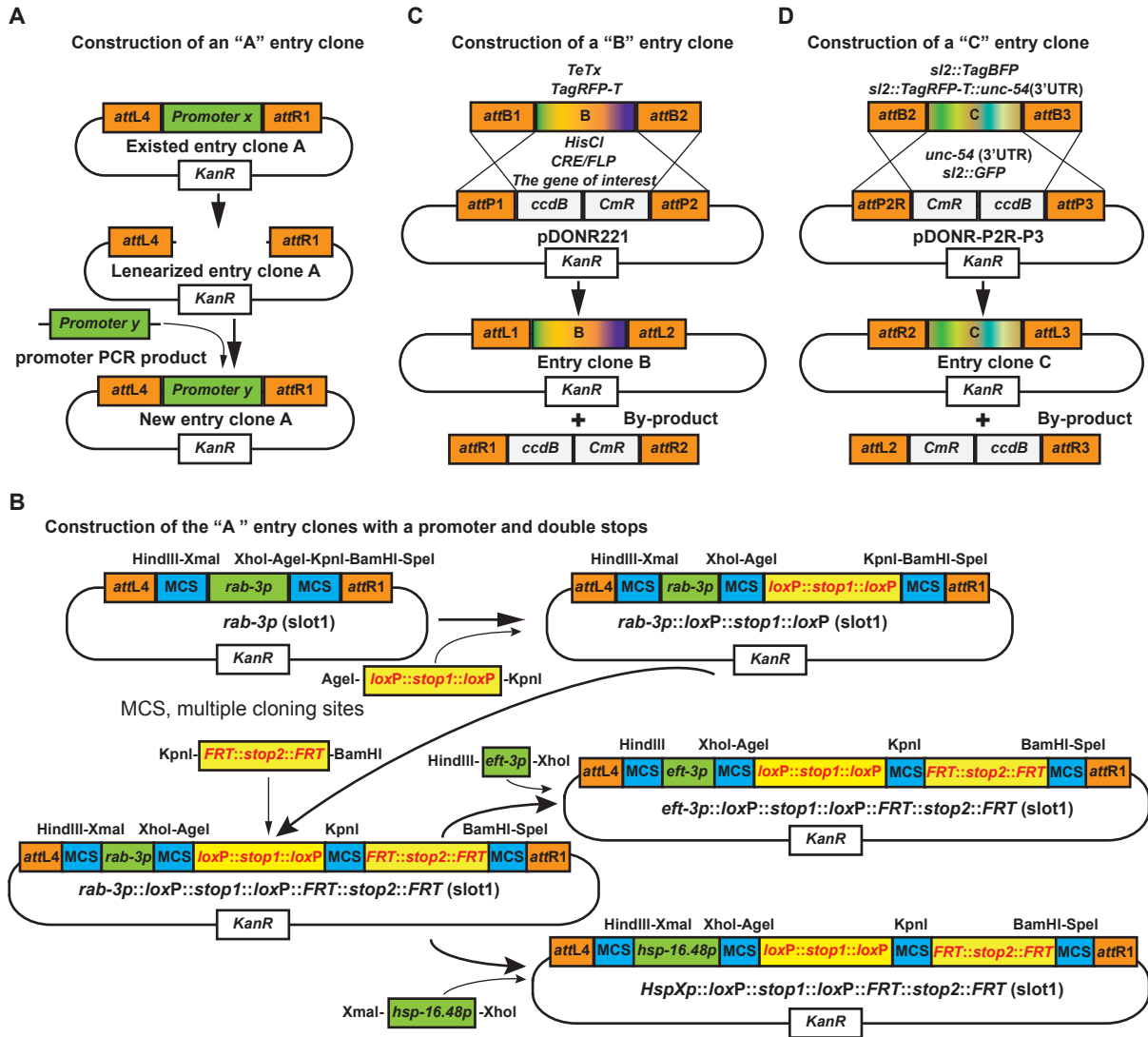
(A) Construction of an expression plasmid used in the FLP/CRE-out gene induction system by L-R recombination. The *lin-44p::GFP* in the expression plasmid acts as a gene marker for transgene encoding a tail-expression fluorescence indicator. The stop1 and stop2 contain sequences of *unc-54* 3' UTR and *let-858* 3' UTR. MCS, multiple cloning site.

(B and C) Construction of the expression plasmids encoding CRE (B) and FLP (C). The *sl2::TagBFP* or *sl2::GFP* encodes fluorescence indicator for recombinase expression. The expression of CRE and FLP were enhanced by adding sequences of Kozak and linker-ER export to *CRE* and *FLP* in the form of *Kozak::CRE::linker::ER* and *Kozak::FLP::linker::ER*, respectively. Entry clone A, clone B, and clone C contribute the sequences of slot1, slot2, and slot3 in expression plasmids used in the FLP/CRE-out gene induction system.



**Figure S2. Single recombinase FLP or CRE could not induce TagRFP-T expression in FLP/CRE-out system, Related to Figure 1**

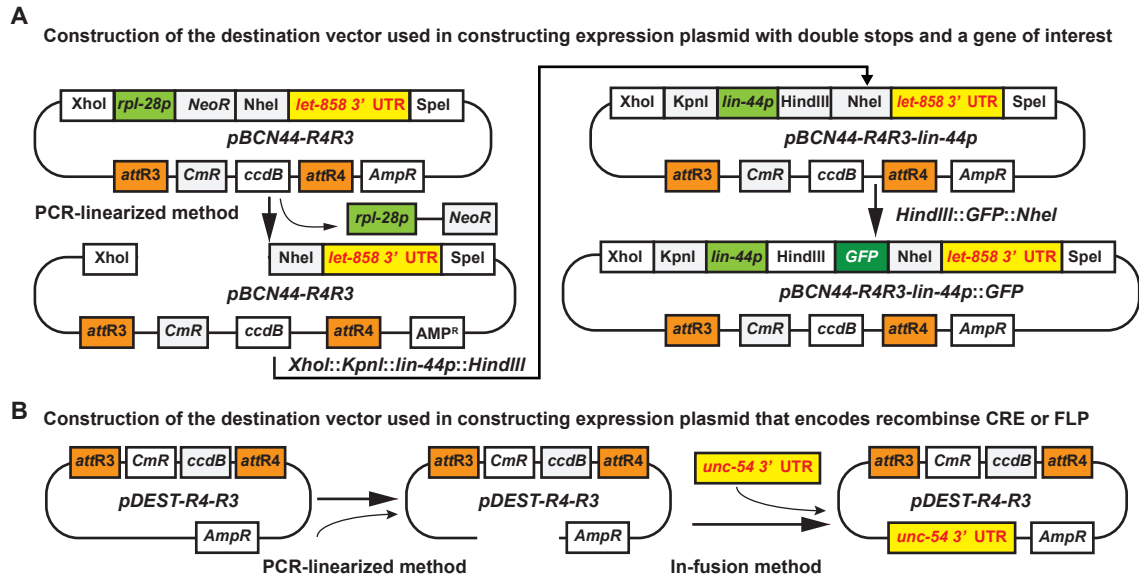
(A and B) Confocal fluorescence images of *hkdEx2000[sra-6p::CRE::sl2::TagBFP]; hkdls617* and *hkdEx2001[srb-6p::FLP::sl2::GFP]; hkdls617* animals. No fluorescence showed in the red channel, hinting no TagRFP-T expression in ASHs, ASIs, or ADLs. The blue channel indicated expression of *sra-6p*-driven CRE in ASHs and ASIs; green, that of *srb-6p*-directed FLP in ASHs and ADLs; and red channels, that of TagRFP-T in neurons. Scale bar, 20 μm.



**Figure S3. Schematic construction of entry clones used in the FLP/CRE-out tripartite spatiotemporal gene expression system, Related to Figure 1**

(A and B) Schematic construction of "A" entry clone used in the FLP/CRE-out gene induction system by the In-Fusion and enzyme digestion methods, respectively.

(C and D) Schematic construction of "B" entry clone (B) and "C" entry clone (C) used in the FLP/CRE-out gene induction system by B-P recombination reactions. "A", "B", and "C" entry clones contribute to the sequences of slot1, slot2, and slot3 in expression plasmids used in the FLP/CRE-out gene induction system, respectively. MCS, multiple cloning site.



**Figure S4 Schematic construction of destination vectors used in the FLP/CRE-out-mediated tripartite spatiotemporal gene induction system, Related to Figure 1**

(A) Destination vector used to construct plasmids containing double recombinase sites-flanked stops and the gene of interest. The stop1 and stop2 contain sequences of the *unc-54* 3' UTR and *let-858* 3' UTR, respectively.

(B) Construction of destination vector used to construct plasmids encoding recombinases CRE and FLP.



## Supplemental Tables

**Table S1. The promoter pairs used for the FLP/CRE-out-mediated gene induction system in this study, Related to Figures 1, 2 and 3**

Neurons or Cells	Neuron type	Combination of promoter pair	Promoter A::Kozak:: CRE::linker::ER	Promoter B::Kozak:: FLP::linker::ER	Efficiency
ASHs	L/R	<i>sra-6p</i> & <i>srb-6p</i>	<i>sra-6p::CRE::sl2::TagBFP</i>	<i>srb-6p::FLP::sl2::GFP</i>	80 % (24/30)
AINs	L/R	<i>eat-4p</i> & <i>rig-5p</i>	<i>eat-4p::CRE::sl2::TagBFP</i>	<i>rig-5p::FLP::sl2::GFP</i>	53 % (16/30)
AVAs	L/R	<i>flp-18p</i> & <i>gpa-14p</i>	<i>flp-18p::CRE::sl2::TagBFP</i>	<i>gpa-14p::FLP::sl2::GFP</i>	70 % (22/31)
AVKs	L/R	<i>inx-2p</i> & <i>flp-1p</i>	<i>inx-2p::CRE::sl2::TagBFP</i>	<i>flp-1p::FLP::sl2::GFP</i>	86 % (33/38)
AVL	Single	<i>lim-6p</i> & <i>flp-22p</i>	<i>lim-6p::CRE::sl2::TagBFP</i>	<i>flp-22p::FLP::sl2::GFP</i>	60 % (25/42)
RIBs	L/R	<i>aptf-1p</i> & <i>trp-1p</i>	<i>aptf-1p::CRE::sl2::TagBFP</i>	<i>trp-1p::FLP::sl2::GFP</i>	76 % (23/30)
RID	single	<i>kal-1p</i> & <i>acr-15p</i>	<i>kal-1p::CRE::sl2::TagBFP</i>	<i>acr-15p::FLP::sl2::GFP</i>	60 % (21/35)
RIGs	L/R	<i>lim-6p</i> & <i>flp-1p</i>	<i>lim-6p::CRE::sl2::TagBFP</i>	<i>flp-1p::FLP::sl2::GFP</i>	83 % (31/37)
pharyngeal muscle	multiple	<i>myo-2p</i> & <i>myo-2p</i>	<i>myo-2p::CRE::sl2::TagBFP</i>	<i>myo-2p::FLP::sl2::GFP</i>	100 % (36/36)
body wall muscle	multiple	<i>myo-3p</i> & <i>myo-3p</i>	<i>myo-3p::CRE::sl2::TagBFP</i>	<i>myo-3p::FLP::sl2::GFP</i>	100 % (35/35)

CRE, Kozak::CRE::linker::ER. FLP, Kozak::FLP::linker::ER. Efficiency = specifically labelled worms (m) / animals tested (n).

**Table S2. *C. elegans* strains used in this study, Related to Figures 1, 2 and 3**

Strain	Genotype
N2 Bristol	<i>Wild-type</i>
ZXW617	<i>hkdls617[eft-3p::loxP::stop1::loxP::FRT::stop2::FRT::TagRFP-T::unc-54 3' UTR]</i>
ZXW723	<i>hkdEx723[sra-6p::R-GECO1; lin-44p::GFP]</i>
ZXW726	<i>hkdEx726[gpa-4p::R-GECO1; lin-44p::GFP]</i>
ZXW1988	<i>hkdEx1988[sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP]; hkdls617</i>
ZXW1989	<i>hkdEx1989[eat-4p::CRE::sl2::TagBFP; rig-5p::FLP::sl2::GFP]; hkdls617</i>
ZXW1990	<i>hkdEx1990[flp-18p::CRE::sl2::TagBFP; gpa-14p::FLP::sl2::GFP]; hkdls617</i>
ZXW1991	<i>hkdEx1991[lim-6p::CRE::sl2::TagBFP; flp-22p::FLP::sl2::GFP]; hkdls617</i>
ZXW1992	<i>hkdEx1992[aptf-1p::CRE::sl2::TagBFP; trp-1p::FLP::sl2::GFP]; hkdls617</i>
ZXW1993	<i>hkdEx1993[kal-1p::CRE::sl2::TagBFP; acr-15p::FLP::sl2::GFP]; hkdls617</i>
ZXW1994	<i>hkdEx1994[lim-6p::CRE::sl2::TagBFP; flp-1p::FLP::sl2::GFP]; hkdls617</i>
ZXW1995	<i>hkdEx1995[myo-2p::CRE::sl2::TagBFP; myo-2p::FLP::sl2::GFP]; hkdls617</i>
ZXW1996	<i>hkdEx1996[myo-3p::CRE::sl2::TagBFP; myo-3p::FLP::sl2::GFP]; hkdls617</i>
ZXW1997	<i>hkdEx1997[hsp-16.48p::loxP::stop1::loxP::FRT::stop2::FRT::TagRFP-T::unc-54 3' UTR; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP]</i>
ZXW1998	<i>hkdEx1998[hsp-16.48p::loxP::stop1::loxP::FRT::stop2::FRT::TeTx::unc-54 3' UTR; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP; gpa-4p::R-Geco1.0]</i>
ZXW1999	<i>hkdEx1999[eft-3p::loxP::stop1::loxP::FRT::stop2::FRT::HisCl1::unc-54 3' UTR; sra-6p::CRE::sl2::TagBFP; srb-6p::FLP::sl2::GFP; gpa-4p::R-Geco1.0]</i>
ZXW2000	<i>hkdEx2000[sra-6p::CRE::sl2::TagBFP]; hkdls617</i>
ZXW2001	<i>hkdEx2001[srb-6p::FLP::sl2::GFP]; hkdls617</i>

*CRE, Kozak::CRE::linker::ER; FLP, Kozak::FLP::linker::ER.*

**Table S3. List of the primers used in this study, Related to Figures 1, 2 and 3**

Name of DNA fragment	PCR product length	The sequence of primers. F, forward; R, reverse
<i>acr-15p</i>	1.5 kb	From <i>C. elegans</i> Promoters Library
<i>aptf-1p</i>	1.8 kb	From <i>C. elegans</i> Promoters Library
<i>eat-4p</i>	4.9 kb	F: agttgctccaagctttttataaatgcaaacggtg R: acttgacgactagtggtttctgaaaatgatgatga
<i>eft-3p</i>	625 bp	F: ggaagcttgacctttggtctttattg R: ggctcgagtggcaaaaggtttcccaac
<i>flp-1p</i>	2.7 kb	F: gctctagagcccaccagatggccgtgtga R: tcccccgggggagataaagtgaagaaaaccaa
<i>flp-18p</i>	3.5 kb	F: agttgctccaagcttaaccaggaaattaaacg R: acttgacgactagtgaaactattggacgcaaac
<i>flp-22p</i>	3.5 kb	F: agttgctccaagcttctactgatgttcaaatg R: acttgacgactagtcattggaacggttattttg
<i>FRT::stop2::FRT</i>	1048 bp	F: ggggtaccgaagttcctatacttctagagaataggaactcggaataggaactcgaatcgatgatcgacgccc R: gactagtattccgaagttcctattctctagaagataggaactccgagtttccaccagtttct
<i>GFP</i>	889 bp	F: cccaagcttatgagtaaaggagaagaact R: gctagctagcctattgtatagttcatcca
<i>gpa-4p</i>	2.4 kb	F: ctaccatggagctccaaataatgattttttgac R: acttgacgactagttgtgaaaaggttcacaaa
<i>gpa-14p</i>	3.2 kb	F: agttgctccaagctgccccttttaagtcctcg R: acttgacgactagtaataaatcacaactataat
<i>hsp16.48p</i>	663 bp	F: tcccccggggaatatccatgttcagtcttaattcttg R: ccgctcgagtctgaagtttagagaatgaacagtaagc
<i>inx-2p</i>	1.5 kb	F: agttgctccaagcttaaatcaaaacatccatattc R: acttgacgactagttgctgaaaatgttatgta
<i>kal-1p</i>	5.3 kb	F: aaaactgcagccaatgggttcgacgacggcaactcac R: tcccccgggggagtgctgtaagagtaaaaatgtgc
<i>Kozak::CRE::linker::ER</i>	1159 bp	F: ggggacaagttgtacaaaaagcaggcttagccgccaccatgggcccacccaagaagaagaggaaggtgtccaattactgaccgtacac R: ggggaccactttgtacaaagaagctgggtattacacctggtctcgtagcagaaggcggcgccatcgccatctccagcaggc
<i>Kozak::FLP::linker::ER</i>	1372 bp	F: ggggacaagttgtacaaaaagcaggcttagccgccaccatggctcctaagaagaagaggaaggtgatgagccagttcgacatcc R: ggggaccactttgtacaaagaagctgggtattacacctggtctcgtagcagaaggcggcgccatcgccctgttgatgtagc
<i>lim-6p</i>	1.8 kb	F: agttgctccaagcttagcccttaccaccaacgc R: acttgacgactagtcggaaaaatggggacggtc
<i>lin-44p</i>	1.3 kb	F: cggggtacctggcaatccaatctgtgtc R: ctactagcaagcttcacgctgtgtcacctcgaaaa
<i>linearized pBCN44-R4R3</i>	4.1 kb	F: ctactagccggccatacaagt R: cggggtaccctcgagggggccggca
<i>linearized pDEST-R4-R3</i>	4.6 kb	F: aacgtcgtgactgggaaaac R: gtaaaacgacggccagtga
<i>linearized stop1 (slot1)</i>	2.7 kb	F: actgaccggtggtaccactagtcgtgcaagttgtacaaa R: cccccgggaagctggagcaactttctatac
<i>loxP::stop1::loxP</i>	825 bp	F: gtcaccggtataactctgataatgtatgctatacgaagttatccatctcgcccgtg R: ggggtaccataactctgatacattatacgaagttataaacagttatggttg
<i>myo-3p</i>	2.3 kb	F: agttgctccctcgagaattattctgtaataactgc R: ctgacgactagtggtctagatggatctagtg
<i>rab-3p</i>	1.3 kb	F: tcccccgggtaataaaaacagctgaca R: gactaccggtctcgagggtctctctgttccgc

(Continued on next page)

## Continued

Name of DNA fragment	PCR product length	The sequence of primers. F, forward; R, reverse
<i>rig-5p</i>	2 kb	F: agttgctccaagctgcaaaactgcagttgacaag R: acttgcacgactagttttatcctgtttccggt
<i>sra-6p</i>	3.8 kb	F: agttgctccaagcttcctcagtcctacgtgccag R: acttgcacgactagtgcaaaatctgaaataata
<i>sl2::TagBFP</i>	968 bp	F: ggggacaagttgtacaaaaagcaggcttagccatgtgttacctgt R: ggggaccactttgtacaagaaagctgggtattaagctgtgccccag
<i>sl2::TagRFP-T</i>	797 bp	F: ggggacaagttgtacaaaaagcaggcttaatgggtctaaagggcgaa R: ggggaccactttgtacaagaaagctgggtattactgtacagctcgtccatgcc
<i>trp-1p</i>	1.7 kb	From <i>C. elegans</i> Promoters Library
<i>TeTx</i>	1436 bp	F: ggggacaagttgtacaaaaagcaggcttaatgccgatcaccatcaac R: ggggaccactttgtacaagaaagctgggtattaagcggtagcgttga
<i>unc-54</i> 3' UTR	820 bp	F: cactggccgctggtttacgggtaccatggtattgatctgagctccgcatcggccgctg R: gtttcccagtcacgacgttgaacagttatgtttggtat

## Transparent Methods

### Animals

The *C. elegans* worms of various genotypes were cultured on nematode growth medium (NGM) plates at 20 °C using *E. coli* OP50 bacteria as food according to standard methods (Brenner, 1974), unless otherwise indicated. All transgenic worms were generated by microinjection with standard techniques (Mello et al., 1991). Plasmids were injected at concentrations of 50 ng  $\mu\text{l}^{-1}$ . All strains used in this study are listed in Table S2.

### Plasmids construction

We constructed plasmids by L-R recombination reactions (Fig S1A-C) using the Multisite Gateway system (Invitrogen™, Thermo Fisher Scientific, MA, USA). Three entry clones, entry clones A, B and C, and a destination vector, were used. We utilized BP recombination reactions to construct the B and C entry clones. The destination vectors used in this study were modified from the *pBCN44-R4R3* vector. The primers for PCR products used in the plasmid construction are listed in Table S3.

**Construction of destination vector.** The destination vector employed to construct FLP/CRE-out expression plasmid, *eft-3p* or *hsp-Xp::loxP::stop1::loxP::FRT::stop2::FRT::target gene::unc-54 3'* UTR, was modified from *pBCN44-R4R3* vector (from Addgene, <http://www.addgene.org/vector-database/>). A sequence of *lin-44p::GFP* (serving as a selection marker for transgene) replaced *rpl-28p::NeoR* in the vector by restriction enzyme digestion method. In short, *rpl-28p::NeoR* fragment was removed from the *pBCN44-R4R3* plasmid. Then, two sequences of *XhoI::KpnI::lin-44p::HindIII* and *HindIII::GFP::NheI* were added into the vector by restriction enzyme digestion: *lin-44p* between *XhoI* and *NheI*; *GFP* between *HindIII* and *NheI* (Figure S4A).

The destination vector used to construct *CRE* and *FLP* plasmids was modified from the *pDEST-R4-R3* vector. An *unc-54 3'* UTR was added into the vector between *attR3* and *AmpR*. In short, as shown in Figure S4B, the *pDEST-R4-R3* vector was linearized by the PCR-linearized method. Then, an *unc-54 3'* UTR was added to the location by the In-fusion method (ClonExpress®II One Step Cloning Kit, Vazyme company, Nanjing, Jiangsu Province, China). The *unc-54 3'* UTR sequence was amplified from *C. elegans* DNA genome by PCR.

**Construction of entry clones.** Entry clones B and C used in the MultiSite Gateway system were

constructed by BP reactions. While the most entry "A" clones were constructed by other methods (Figure S3), except some promoter clones in the *C. elegans* Promoters Library (Thermo Fisher Scientific, Waltham, MA, USA) were directly used.

**Entry clone A.** The "A" entry clones contribute to a fragment of slot1 in the expression plasmids. Three types of "A" entry clones were used to construct different expression plasmids. The vector A used in the construction of the recombinase-encoding plasmid contains a promoter sequence. Most parts of "A" entry clones in this study were constructed by the In-Fusion method (Figure S3A), using ClonExpress®II One Step Cloning Kit and primers listed in Table S3. Alternatively, some entry clones in the *C. elegans* Promoters Library were directly utilized. The "A" entry clones used to construct expression plasmids containing sequences of a promoter, two recombinase sites-flanked transcriptional terminators and the gene of interest, in form of *eft-3p::loxP::stop1::loxP::FRT::stop2::FRT* or *hsp-16.48p::loxP::stop1::loxP::FRT::stop2::FRT*. They were modified from *rab-3p* vector by restriction enzyme digestion, as shown in Figure S3B. The transcriptional terminators *stop1* and *stop2* contain sequences of *unc-54* 3' UTR and *let-858* 3' UTR, respectively.

**Entry clone B.** An entry clone B contributing sequences of slot 2 in the expression plasmid contains a fragment of a target gene, i.e., *CRE*, *FLP*, *TeTx*, *TagRFP-T*, or any gene of interest. The "B" entry clones were constructed using pDONR™ 221 donor vector (Invitrogen) by BP reactions (Figure S3C). Modified *CRE* and *FLP* were employed to improve ER-exportation and nuclear translocation of the recombinases. Sequences of *Kozak* and nuclear localization sequence (NLS) were fused to *CRE* and *FLP*, in the form of *Kozak::NLS::recombinase::linker::ER*. The sequences are: *Kozak*, gccgccacc; nuclear localization sequence (NLS) of Simian virus 40, cccaagaagaagaggaaggtg; linker, gccgccgcc; and ER exportation, ttctgctacgagaacgaggtg. *CRE* and *FLP* fragments were amplified by PCR with primers containing *attB1*, *attB2*, *Kozak*, NLS, linker, and ER export: *CRE*; from *pDD104* plasmid (from Addgene); *FLP* from *pPGKFLPpA* plasmid (from Addgene). *TeTx* sequence was amplified from *pWD170* plasmid (by courtesy of Dr. Erik M. Jorgensen) by PCR with the primers containing *attB1* and *attB2* recombination sites. *TagRFP-T* fragment was amplified by PCR with primers containing *attB1* and *attB2* recombination sites.

**Entry clone C.** An entry clone C contributing to sequences of slot 3 in the expression plasmid contains a fragment of *unc-54* 3' UTR, *sl2::GFP* or *sl2::TagBFP*. The corresponding PCR products with *attB2R*

and *attB3* sites were amplified by the PCR method. A pDONR-P2R-P3 donor vector was used to construct "C" entry clones by BP reactions (Figure S3D). The "C" entry clones containing *unc-54* 3' UTR, *sl2::GFP* and *sl2::TagBFP* were used to construct expression plasmids containing a tested gene, *CRE*, and *FLP*, respectively.

### **Confocal fluorescence imaging**

All confocal fluorescence imaging were performed by an Andor Revolution XD laser confocal microscope system (Andor Technology plc., Springvale Business Park, Belfast, UK) based on a spinning-disk confocal scanning head CSU-X1 (Yokogawa Electric Corporation, Musashino-shi, Tokyo, Japan). The confocal system was mounted on an Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan) and controlled by Andor IQ 3.2 software. All fluorescent images were imaged by a  $\times 60$  objective lens (numerical aperture = 1.45, Olympus) and captured by an Andor iXon<sup>EM+</sup> DU-897D EMCCD camera.

### **Calcium imaging**

The calcium responses in neuronal soma were measured by detecting changes in the fluorescence intensity of R-GECO1, a sensitive and rapid kinetic calcium indicator of weak photobleaching (Zhao et al., 2011). A home-made microfluidic device was used for calcium imaging as previously described (Chronis et al., 2007; Guo et al., 2015; Liu et al., 2019; Wang et al., 2016). Briefly, a young adult worm was transferred from food to M13 buffer solution (in mM, Tris-HCl 30, NaCl 100 and KCl 10, PH 7.0) to wash bacteria from the body. Then, the animal was loaded into the worm channel of the microfluidic chip with its nose exposed to the buffer or solution of CuSO<sub>4</sub> dissolved in M13 buffer (final concentration 10 mM) under laminar flow for each Ca<sup>2+</sup> assay. The solutions were delivered via programmable automatic drug-feeding equipment (MPS-2, InBio Life Science Instrument Co. Ltd, Wuhan, Hubei Province, China). R-GECO1 was fluorescently imaged by an Olympus IX-70 inverted microscope (Olympus) with a 40 $\times$  objective lens (numerical aperture (NA) = 1.3, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). R-GECO1 was excited with 525–530 nm light emitted by an Osram Diamond Dragon LTW5AP light-emitting diode (LED) model (Osram, Marcel-Breuer-Strasse 6, Munich, Germany) mounted to a multi-LED light source (MLS102, InBio Life Science Instrument Co. Ltd). Its fluorescence was filtered by a Semrock FF01-593/40-25 emission filter (IDEX Health & Science, LLC, Oak Harbor, WA, USA), An Andor iXon<sup>EM+</sup> DU885K EMCCD camera was used to capture photos and form fluorescence images with 256  $\times$  256 pixels at ten frames per second.

Each animal was exposed under fluorescent excitation light for 1 – 2 min before recording to reduce the light-evoked calcium transients and was used only once. The averaged fluorescence intensity in each frame of the region of interest (ROI) of the soma was captured and analyzed by Image-Pro Plus 6 (Media Cybernetics, Inc., Rockville, MD, USA). The average signal of an adjacent ROI in all frame was used to subtract the background. Average fluorescence intensity within the initial 5 s before stimulation was taken as basal signal  $F_0$ . The percentage changes in fluorescence intensity relative to  $\Delta F/F_0 = (F - F_0)/F_0 \times 100\%$ , were plotted as a function of time for all curves. Mean values of  $\text{Ca}^{2+}$  signals during the administration of  $\text{CuSO}_4$  solution and SEM were plotted in various colors as indicated and in light grey, respectively.

### **Data analyses and display**

Data of the  $\text{Ca}^{2+}$  signals were expressed as means  $\pm$  SEM as indicated by solid traces  $\pm$  grey shading and box-plots with each dot representing data from individual tested worm. The significance of statistic difference was analyzed by two-way ANOV (Analysis of Variance) with post hoc test of Tukey's multiple comparison correction, by the use of software package in Graphpad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). It was indicated as \*\*\*\*  $P < 0.0001$ . The curves of calcium signals were displayed by IGOR Pro 6.10 (WaveMetrics, Inc., Lake Oswego, OR, USA).

### **Supplemental References**

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