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Supplemental Information

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

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Supplemental Information

Supplemental Figures

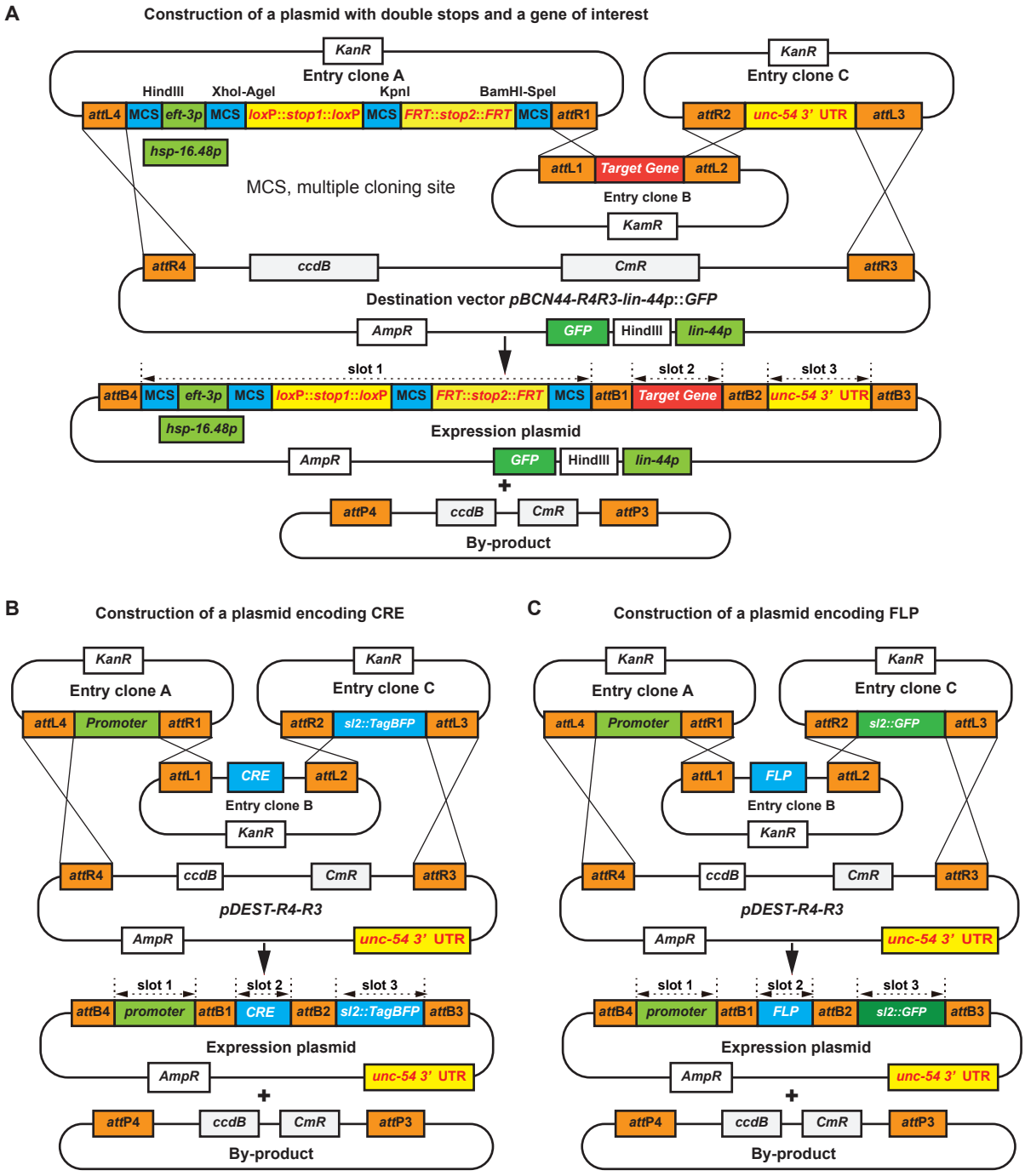

Figure S1. Schematic diagram of the construction of expression plasmids used in the tripartite spatiotemporal gene control system, Related to Figure 1

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.

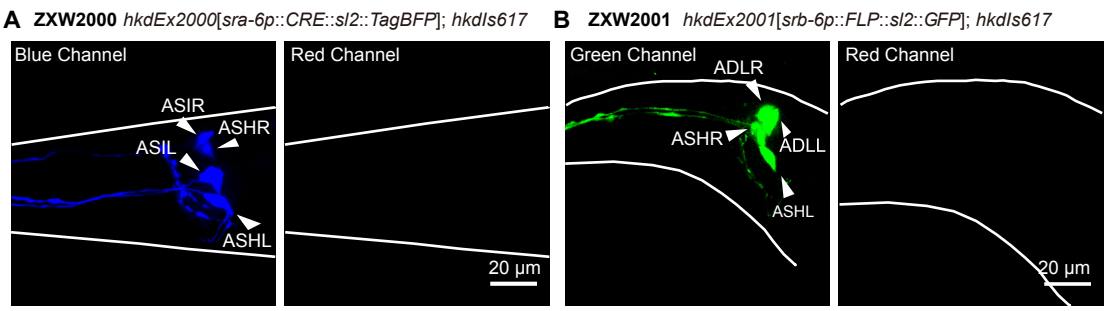

Figure S2. Single recombinase FLP or CRE could not induce TagRFP-T expression in FLP/CRE-out system, Related to Figure 1

(A and B) Confocal fluorescence images of *hkdEx2000*[*sra-6p*::*CRE*::*sl2*::*TagBFP*]; *hkdIs617* and *hkdEx2001*[*srb-6p*::*FLP*::*sl2*::*GFP*]; *hkdIs617* 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.

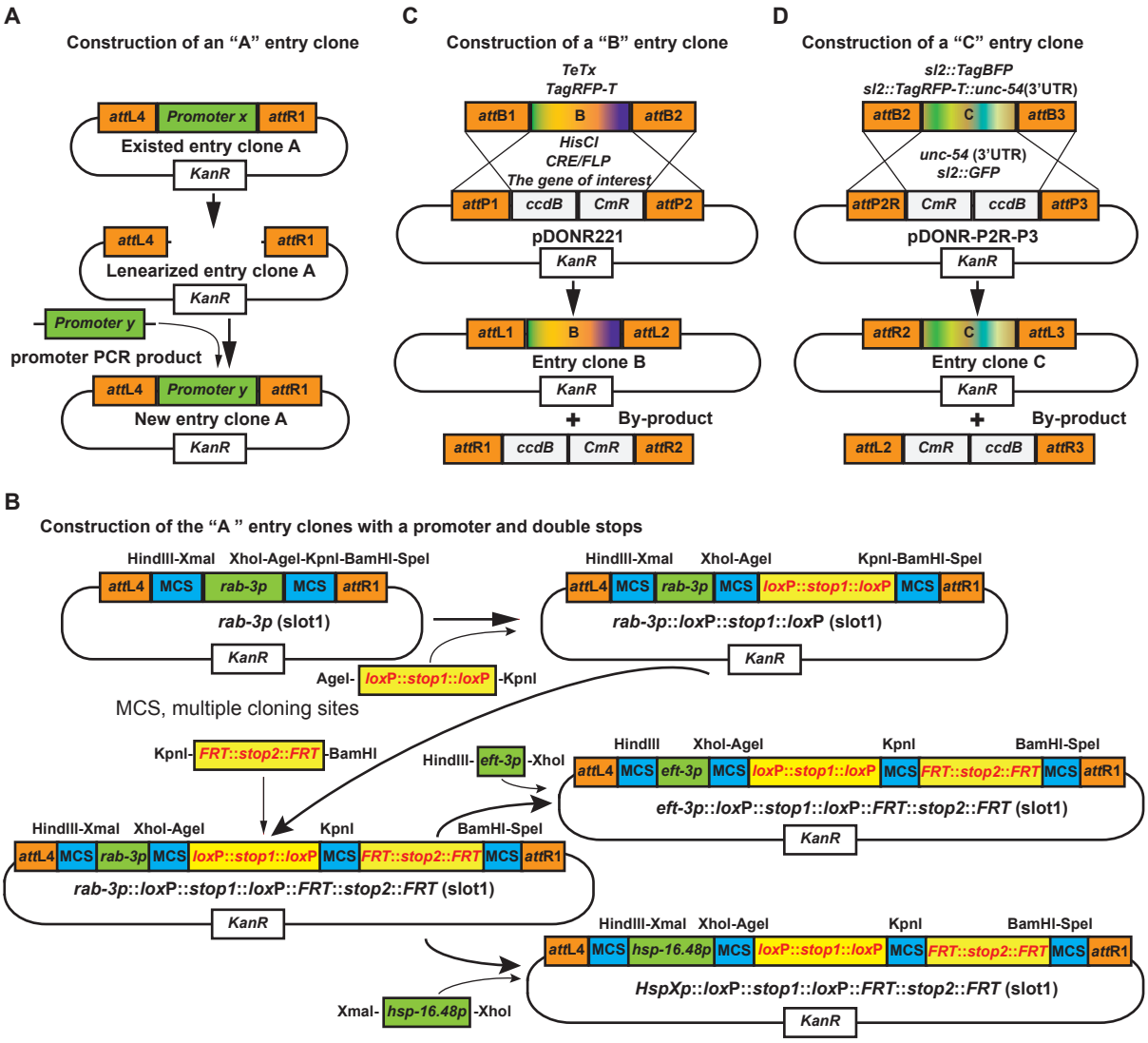

Figure S3. Schematic construction of entry clones used in the FLP/CRE-out tripartite spatiotemporal gene expression system, Related to Figure 1

(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.

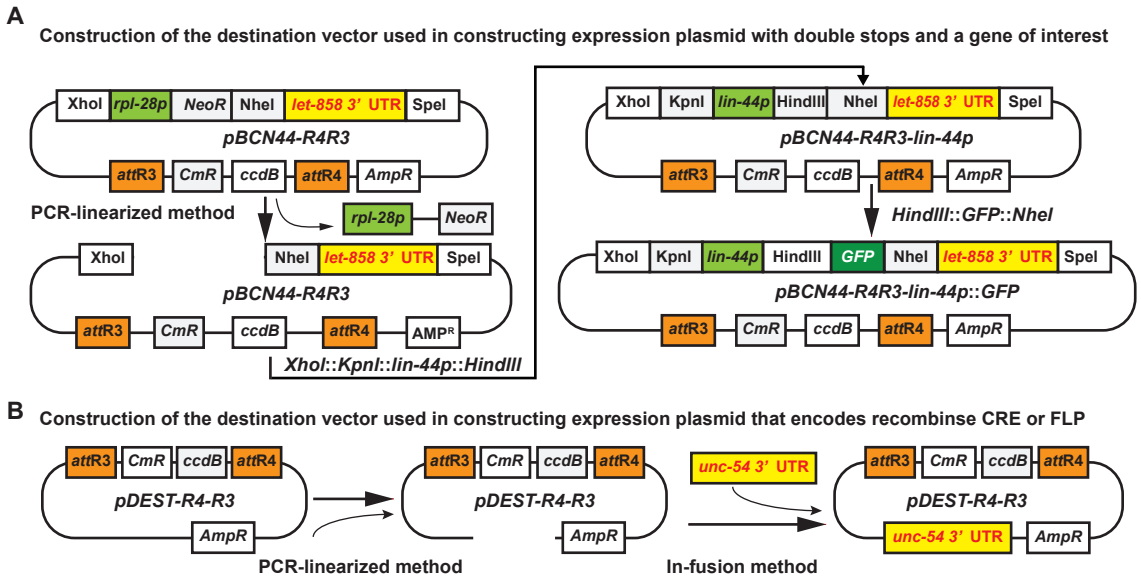

Figure S4 Schematic construction of destination vectors used in the FLP/CRE-out-mediated tripartite spatiotemporal gene induction system, Related to Figure 1

(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	*sra-6p* & *srb-6p*	*sra-6p*::*CRE*::*sl2*::*TagBFP*	*srb-6p*::*FLP*::*sl2*::*GFP*	80 % (24/30)
AINs	L/R	*eat-4p* & *rig-5p*	*eat-4p*::*CRE*::*sl2*::*TagBFP*	*rig-5p*::*FLP*::*sl2*::*GFP*	53 % (16/30)
AVAs	L/R	*flp-18p* & *gpa-14p*	*flp-18p*::*CRE*::*sl2*::*TagBFP*	*gpa-14p*::*FLP*::*sl2*::*GFP*	70 % (22/31)
AVKs	L/R	*inx-2p* & *flp-1p*	*inx-2p*::*CRE*::*sl2*::*TagBFP*	*flp-1p*::*FLP*::*sl2*::*GFP*	86 % (33/38)
AVL	Single	*lim-6p* & *flp-22p*	*lim-6p*::*CRE*::*sl2*::*TagBFP*	*flp-22p*::*FLP*::*sl2*::*GFP*	60 % (25/42)
RIBs	L/R	*aptf-1p* & *trp-1p*	*aptf-1p*::*CRE*::*sl2*::*TagBFP*	*trp-1p*::*FLP*::*sl2*::*GFP*	76 % (23/30)
RID	single	*kal-1p* & *acr-15p*	*kal-1p*::*CRE*::*sl2*::*TagBFP*	*acr-15p*::*FLP*::*sl2*::*GFP*	60 % (21/35)
RIGs	L/R	*lim-6p* & *flp-1p*	*lim-6p*::*CRE*::*sl2*::*TagBFP*	*flp-1p*::*FLP*::*sl2*::*GFP*	83 % (31/37)
pharyngeal muscle	multiple	*myo-2p & myo-2p*	*myo-2p*::*CRE*::*sl2*::*TagBFP*	*myo-2p*::*FLP*::*sl2*::*GFP*	100 % (36/36)
body wall muscle	multiple	*myo-3p& myo-3p*	*myo-3p*::*CRE*::*sl2*::*TagBFP*	*myo-3p*::*FLP*::*sl2*::*GFP*	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	*Wild-type*
ZXW617	*hkdIs617*[*eft-3p*::*lox*P::*stop1*::*lox*P::*FRT*::*stop2*::*FRT*::*TagRFP-T*::*unc-54 3'* UTR]
ZXW723	*hkdEx723*[*sra-6p*::*R-GECO1*; *lin-44p*::*GFP*]
ZXW726	*hkdEx726*[*gpa-4p*::*R-GECO1*; *lin-44p*::*GFP*]
ZXW1988	*hkdEx1988*[*sra-6p*::*CRE*::*sl2*::*TagBFP*; *srb-6p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1989	*hkdEx1989*[*eat-4p*::*CRE*::*sl2*::*TagBFP*; *rig-5p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1990	*hkdEx1990*[*flp-18p*::*CRE*::*sl2*::*TagBFP*; *gpa-14p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1991	*hkdEx1991*[*lim-6p*::*CRE*::*sl2*::*TagBFP*; *flp-22p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1992	*hkdEx1992*[*aptf-1p*::*CRE*::*sl2*::*TagBFP*; *trp-1p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1993	*hkdEx1993*[*kal-1p*::*CRE*::*sl2*::*TagBFP*; *acr-15p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1994	*hkdEx1994*[*lim-6p*::*CRE*::*sl2*::*TagBFP*; *flp-1p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1995	*hkdEx1995*[*myo-2p*::*CRE*::*sl2*::*TagBFP*; *myo-2p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1996	*hkdEx1996*[*myo-3p*::*CRE*::*sl2*::*TagBFP*; *myo-3p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*
ZXW1997	*hkdEx1997*[*hsp-16.48p*::*lox*P::*stop1*::*lox*P::*FRT*::*stop2*::*FRT*::*TagRFP-T*::*unc-54 3'* UTR; *sra-6p*::*CRE*::*sl2*::*TagBFP*; *srb-6p*::*FLP*::*sl2*::*GFP*]
ZXW1998	*hkdEx1998*[*hsp-16.48p*::*lox*P::*stop1*::*lox*P::*FRT*::*stop2*::*FRT*::*TeTx*::*unc-54 3'* UTR; *sra-6p*::*CRE*::*sl2*::*TagBFP*; *srb-6p*::*FLP*::*sl2*::*GFP*; *gpa-4p*::*R-Geco1.0*]
ZXW1999	*hkdEx1999*[*eft-3p*::*lox*P::*stop1*::*lox*P::*FRT*::*stop2*::*FRT*::*HisCl1*::*unc-54 3'* UTR; *sra-6p*::*CRE*::*sl2*::*TagBFP*; *srb-6p*::*FLP*::*sl2*::*GFP*; *gpa-4p*::*R-Geco1.0*]
ZXW2000	*hkdEx2000*[*sra-6p*::*CRE*::*sl2*::*TagBFP*]; *hkdIs617*
ZXW2001	*hkdEx2001*[*srb-6p*::*FLP*::*sl2*::*GFP*]; *hkdIs617*

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
acr-15p	1.5 kb	From *C. elegans* Promoters Library
aptf-1p	1.8 kb	From *C. elegans* Promoters Library
eat-4p	4.9 kb	F: agttgctccaagctttttattaaatgcaaacggtg R: acttgcacgactagtggtttctgaaaatgatgatga
eft-3p	625 bp	F: ggaagcttgcacctttggtcttttattg R: ggctcgagtgagcaaagtgtttcccaac
flp-1p	2.7 kb	F: gctctagagcccaccagatggccgtgtgta R: tccccccgggggggagataaagtgaagaaaaccaa
flp-18p	3.5 kb	F: agttgctccaagcttaaccaggaaatttaaacg R: acttgcacgactagttgaaacttattggacgcaaac
flp-22p	3.5 kb	F: agttgctccaagctttcttactgatgtttcaaatg R: acttgcacgactagtcatggaacggttcatttttg
FRT::*stop2*::*FRT*	1048 bp	F: ggggtaccgaagttcctatactttctagagaataggaacttcggaataggaacttcgaatcggatgatcgacgccg R: gactagtattccgaagttcctattctctagaaagtataggaacttccgagttttcaccagtttctt
GFP	889 bp	F: cccaagcttatgagtaaaggagaagaact R: gctagctagcctatttgtatagttcatcca
gpa-4p	2.4 kb	F: ctaccatggagctccaaataatgattttattttgac R: acttgcacgactagttgttgaaaagtgttcacaaa
gpa-14p	3.2 kb	F: agttgctccaagcttgccctttttaagtcctcg R: acttgcacgactagttaataaatctcaactataat
hsp16.48p	663 bp	F: tccccccggggaatatccatgttcagtctttaattcttg R: ccgctcgagtcttgaagtttagagaatgaacagtaagc
inx-2p	1.5 kb	F: agttgctccaagcttaaattcaaaacatccatattc R: acttgcacgactagttgctgaaaatgttatgtta
kal-1p	5.3 kb	F: aaaactgcagccaatgggttcgacgacggcaactcac R: tccccccgggggggagtgctgtaagagtaaaaatgtgc
Kozak::*CRE*::*linker*::*ER*	1159 bp	F: ggggacaagtttgtacaaaaaagcaggcttagccgccaccatgggcgcacccaagaagaagaggaaggtgtccaatttactgaccgtacac R: ggggaccactttgtacaagaaagctgggtattacacctcgttctcgtagcagaaggcggcggcatcgccatcttccagcaggc
Kozak::*FLP*::*linker*::*ER*	1372 bp	F: ggggacaagtttgtacaaaaaagcaggcttagccgccaccatggctcctaagaagaagaggaaggtgatgagccagttcgacatcc R: ggggaccactttgtacaagaaagctgggtattacacctcgttctcgtagcagaaggcggcggcgatccgcctgttgatgtagc
lim-6p	1.8 kb	F: agttgctccaagctttagcccttatccaccaacgc R: acttgcacgactagtctggaaaaatggggacggtc
lin-44p	1.3 kb	F: cggggtacctggcaatccaatcttgtgtc R: ctagctagcaagcttcacgctgtgtcacctcgaaaa
linearized pBCN44-R4R3	4.1 kb	F: ctagctagccggccatacaagt R: cggggtaccctcgaggggggcccggca
linearized pDEST-R4-R3	4.6 kb	F: aacgtcgtgactgggaaaac R: gtaaaacgacggccagtga
linearized stop1 (slot1)	2.7 kb	F: actgaccggtggtaccactagtcgtgcaagtttgtacaaa R: ccccccgggaagcttggagcaacttttctatac
*lox*P::*stop1*::*lox*P	825 bp	F: gtcaccggtataacttcgtataatgtatgctatacgaagttatccatctcgcgcccgtg R: ggggtaccataacttcgtatagcatacattatacgaagttattaaacagttatgtttgg
myo-3p	2.3 kb	F: agttgctccctcgagaattattctgtaattaactgc R: cttgcacgactagtggttctagatggatctagtgg
rab-3p	1.3 kb	F: tccccccgggtaaataaaacagctgaca R: gactaccggtctcgagggtcttcttcgtttccgc

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Continued

Name of DNA fragment	PCR product length	The sequence of primers. F, forward; R, reverse
rig-5p	2 kb	F: agttgctccaagcttgcaaacttgcagttgacaag R: acttgcacgactagttttattcctgttttccggtt
sra-6p	3.8 kb	F: agttgctccaagcttccttcagtgcctacgtgccag R: acttgcacgactagtggcaaaatctgaaataata
sl2::*TagBFP*	968 bp	F: ggggacaagtttgtacaaaaaagcaggcttagccatgttgttaccttgt R: ggggaccactttgtacaagaaagctgggtattaaagcttgtgccccag
sl2::*TagRFP-T*	797 bp	F: ggggacaagtttgtacaaaaaagcaggcttaatggtgtctaagggcgaa R: ggggaccactttgtacaagaaagctgggtattacttgtacagctcgtccatgcc
trp-1p	1.7 kb	From *C. elegans* Promoters Library
TeTx	1436 bp	F: ggggacaagtttgtacaaaaaagcaggcttaatgccgatcaccatcaac R: ggggaccactttgtacaagaaagctgggtattaagcggtacggttgta
unc-54 3' UTR	820 bp	F: cactggccgtcgttttacggtaccatggtattgatatctgagctccgcatcggccgctg R: gttttcccagtcacgacgttgaaacagttatgtttggtat

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 μl^{-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*::*lox*P::*stop1*::*lox*P::*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 *att*R3 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*::*lox*P::*stop1*::*lox*P::*FRT*::*stop2*::*FRT* or *hsp-16.48p*::*lox*P::*stop1*::*lox*P::*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 *att*B1, *att*B2, 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 *att*B1 and *att*B2 recombination sites. *TagRFP-T* fragment was amplified by PCR with primers containing *att*B1 and *att*B2 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 *att*B2R

and *att*B3 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 ×60 objective lens (numerical aperture = 1.45, Olympus) and captured by an Andor iXon^{EM}+ 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 $CuSO_4$ dissolved in M13 buffer (final concentration 10 mM) under laminar flow for each Ca^{2+} 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× objective lens (numerical aperture (NA) = 1.3, Carl Zeiss MicroImaging GmhH, 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^{EM}+ DU885K EMCCD camera was used to capture photos and form fluorescence images with 256 × 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 Ca^{2+} signals during the administration of $CuSO_4$ solution and SEM were plotted in various colors as indicated and in light grey, respectively.

Data analyses and display

Data of the Ca^{2+} signals were expressed as means ± SEM as indicated by solid traces ± 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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