RESEARCH ARTICLE

A Conditional Knockout Toolkit for Caenorhabditis elegans Based on the Cre/loxP Recombination

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Abstract

Conditional knockout (cKO) based on site-specific recombination (SSR) technology is a powerful approach for estimating gene functions in a spatially and temporally specific manner in many model animals. In Caenorhabditis elegans (C. elegans), spatial- and temporal-specific gene functions have been largely determined by mosaic analyses, rescue experiments and feeding RNAi methods. To develop a systematic and stable cKO system in C. elegans, we generated Cre recombinase expression vectors that are driven by various tissue-specific or heat-shock promoters. Validation using Cre-mediated fluorescence protein inactivation or activation systems demonstrated successful Cre-dependent loxP excision. We established a collection of multi-copy Cre transgenic strains for each evaluated vector. To evaluate our Cre/loxP-based cKO system, we generated sid-1 deletion mutants harboring floxed sid-1 single-copy integration (SCI) using ultraviolet trimethylpsoralen (UV/TMP) methods. sid-1 mutants that were rescued by the floxed sid-1 SCI were then crossed with the Pdpy-7::Cre strain for cKO in the hypodermis. The sid-1 cKO animals were resistant to bli-3 RNAi, which causes the Bli-phenotype in the hypodermis, but they were sensitive to unc-22 RNAi, which leads to twitching of the body wall muscle. Our system, which is based on the combination of a transgenic Cre collection, pre-existing deletion mutants, and UV/TMP SCI methods, provided a systematic approach for cKO in C. elegans.
Introduction

The spatial and temporal control of gene inactivation is an essential approach for assessing gene functions in multicellular organisms. Conditional knockout (cKO) strategies based on site-specific recombination (SSR) technologies have been intensively used thus far. A cKO resource that covers 9,000 targeted alleles has been produced in mouse embryonic stem cells [1], and it has promoted systematic studies of mouse genes. In Caenorhabditis elegans (C. elegans), various genetic approaches such as mosaic analyses using extrachromosomal arrays [2], transgenic rescue driven by cell-type specific or heat-shock promoters, and feeding RNAi methods [3], have been largely used to investigate spatial- and temporal-specific gene functions. Recently, conditional genome editing methods using the TALENs or CRISPR-cas9 systems have been developed [4–6]. The somatic TALEN or CRISPR-Cas9 provides a quick approach to generate cKOs. These somatic genome editing methods are, however, heterogeneous and not heritable.

C. elegans Deletion Mutant Consortium and the worm community established a collection of deletion or null mutation in approximately 7,000 genes [7]. The large mutant collection could be a powerful toolkit for systematic cKO when combined with single-copy integration (SCI) of floxed transgenes. Frøkjaer-Jensen et al. have developed an elegant SCI technique based on targeting integrations using Mos1 transposons [8]. We previously developed an alternative single/low-copy integration, ultraviolet trimethylpsoralen (UV/TMP) method [9, 10] that was based on the random integration of transgenes into the chromosomes from multi-copy extrachromosomal arrays, which should be familiar to most C. elegans researchers. Although our method exhibited lower frequency of SCI when compared to MosSCI, we adopted vps-45/ben-1 as a positive/negative selection marker that provides sharp and efficient selections [9].

Site-specific recombination systems, such as the Cre/loxP [11] and FLP/FRT [12] systems, have been used to control gene expression/inactivation [13]. The Cre/loxP system that has an optimum temperature of 37°C and greater [14] has been widely used for genetic manipulation in mammals. The optimum temperature for FLP recombinase is 30°C [14], which is closer to growth temperature of invertebrates. C. elegans has a growth temperature of around 20°C, and FLP/FRT-mediated gene expression systems have been developed [15–17]. Meanwhile, several studies have demonstrated that Cre-mediated loxP excision successfully occurs [9, 18, 19].

In this study, we established a collection of Cre-expression vectors and transgenic strains that are driven by tissue-specific and heat-shock promoters. We also generated tissue-specific knockout mutants in a combination of Cre-transgenic lines, deletion mutants, and UV/TMP methods, and demonstrated the cKO of the targeted gene.
Materials and Methods

Strains

C. elegans strains were cultured using standard techniques [20]. The wild-type strain Bristol N2 was obtained from the Caenorhabditis Genetics Center. The sid-1 deletion mutant strain was obtained from the UV/TMP mutagenized library, which has been previously described [21], and this strain was identified by PCR amplification with primers spanning the deletion region of sid-1(tm2700)V. The mutants were backcrossed five times with N2, and the ben-1(tm234);sid-1(tm2700)V;vps-45(tm246)X mutants were generated by crossing sid-1(tm2700) into ben-1(tm234);vps-45(tm246) [9]. The primers that were used for PCR genotyping were as follows: tm2700_1stround, 5'-CAGTGGCTTCACCTGTCTTA-3', 5'-CGTACATTTCGCCGGCACAGT-3'; tm2700_2ndround, 5'-CGACGTTAAACACATCTCAC-3', 5'-CGCCGGGCACAGTTATCAGAT-3'; tm234_1stround, 5'-ACGTGGGAATGGAACCATGT-3', 5'-TCTCCATTTCCTCTCC-3'; tm234_2ndround, 5'-CTCGGACATTGTAACGGAA-3', 5'-CCCTCCATTGGAAAGAGTCC-3'; and tm246, 5'-CGCAATTGGATACTACCTGT-3', 5'-TCTCCTGCTCTACTTCTGCT-3'.

Constructs and multi-copy transgenic strains

pFX_HBG_Lw_dpy-30p_NLS_GFP, a floxed pan-NLS::GFP plasmid, was constructed as previously described [10]. Briefly, approximately 1.2 kbp of the upstream genomic region of the dpy-30 gene was subcloned into pPD96.04, and the Pdpy-30::NLS::GFP::LacZ sequence was amplified from the plasmid and then subcloned into pFX_HBG_Lw [9]. pFX_HBG_Lw_sid-1 was constructed by subcloning the sid-1 genomic fragment into the BamHI/NotI sites of pFX_HBG_Lw. The positive selection marker (Pef3-::vps-45) and negative selection marker plasmid (pGEMT_ben-1(+)) were constructed as described in our previous study [9]. The positive/negative selection marker plasmids can be provided upon request. To generate tmEx3452 transgenic animals, 60 ng/μl each of Pef3-::vps-45, pGEMT_ben-1(+) and pFX_HBG_Lw_sid-1 and 20 ng/μl of the injection marker Pmyo-2::venus were co-injected into ben-1(tm234);sid-1(tm2700);vps-45(tm246). Cre recombinase cDNA that was amplified from AxCANCre (TaKaRa) or NLS::Cre cDNA that was amplified from pPGK-Cre-bpA (Addgene) were cloned into the NotI/BglII sites of the pFX vector [22]. The genomic fragments for the tissue-specific and heat-shock promoters that are listed in Table S1 were amplified from the N2 genome and cloned 5' into the Cre or NLS::Cre in the pFX_Cre or pFX-NLS::Cre. The Cre multi-copy integration strains were generated as previously described [23], and the injection markers are listed in Table S1. Each strain was outcrossed twice with N2.

Single/low-copy integration by UV/TMP methods

The UV/TMP methods were conducted as previously described [10] with some modifications. Mixtures of young adults and L4 larvae of ben-1(tm234); sid-
1(tm2700); vps-45(tm246); tmEx3452(floxed sid-1) animals were treated at 0.5 μg/ml TMP in M9 buffer and then irradiated with 365 nm UV at 200 J/m² at room temperature. UV/TMP-treated animals were plated onto nematode growth media (NGM) agar dishes that were seeded with *Escherichia coli* OP50 and allowed to lay eggs at 20°C. After 24 h, the P0 adults and newly hatched F1 larvae, which were possibly derived from the eggs that were fertilized before the UV/TMP treatment, were washed off using M9 buffer. The eggs remaining on the plates were incubated at 20°C for another 24 h, until the F1 animals hatched. The hatching rate was approximately 80%. The F1 animals were cultured at 20°C for 2 days; after which the majority of ts-rescued F1 animals developed into L4 larvae (positive selection). The animals were harvested, washed three times with M9 buffer and plated onto OP50-seeded modified NGM plates (containing 8-fold Bacto Peptone and 10 μg/ml of benzimidazole (Wako)) (positive/negative selection). The worms were then cultured at 20°C on benzimidazole-containing NGM plates for 1–2 weeks. The transformed animals were cloned and further selected by PCR, which amplified intact floxed *sid-1* transgene.

**Quantitative PCR**

The genomic DNA was isolated from adult animals using the DNeasy Tissue and Blood kit (QIAGEN). A quantitative PCR (qPCR) analysis was performed in a 7500 Real-time Thermal cycler (Applied Biosystems) using the Power SYBR master mix (Applied Biosystems) with the following parameters: 95°C for 10 min and 40 cycles of 95°C for 5 s, 58°C for 10 s and 72°C for 34 s. The primers for *ama-1*, *vps-45*, *ben-1*, *sid-1* were designed within an exon for each gene using the Primer3 software. The utilized primers were as follows: qPCR#ama-1_forward, AGATGGACCTCACCGACAAC; qPCR#ama-1_reverse, 5'-CTGCAGATTACA-CGGAAGCA-3'; qPCR#vps-45_forward, 5'-TGCGTGAGGTTCAAGAAGTG-3'; qPCR#vps-45_reverse, 5'-AACAGCTGGAGCCTTTTTCA-3'; qPCR#ben-1_forward, 5'-TTGCTCGCTTTGATTACCTTG-3', qPCR#ben-1_reverse, 5'-TGTTTCCCCTCTAGGACC-3', qPCR#sid-1_forward, 5'-ACTGACGGAAA-CTGCTCAATC-3'; and qPCR#sid-1_reverse, 5'-AAAGCCTACCGCTATC-CTG-3'.

The data were normalized to the *ama-1* gene, and the copy number of each transgene was calculated by comparing it to the amount of wild-type N2 endogenous genes.

**Bacterial feeding RNAi**

Feeding RNAi was conducted as previously described [3] using RNAi clones from the Ahringer Library. L3 animals were transferred to RNAi plates and cultured at 20°C. RNAi phenotypes were examined 48 h later. For bli-3 RNAi, the Bli phenotype was examined and for *unc-22* RNAi, the twitch of body wall muscle was analyzed.
Microscopy
Differential interference contrast and fluorescence images were obtained using a BX51 microscope that was equipped with a DP30BW CCD camera (Olympus Optical Co., Ltd).

Results and Discussion
Evaluation of Cre expression vectors using floxed pan-NLS::GFP SCI
Although several studies have demonstrated Cre/loxP-mediated gene excision in *C. elegans*, a systematic characterization has not been reported. We previously generated strains bearing the SCI of floxed *Pdpy-30::nuclear localization signal::green fluorescent protein (NLS::GFP)* transgenes (referred to as “floxed pan-NLS::GFP SCI”) [10]. To evaluate the tissue-specific Cre/loxP-mediated gene excision, a Cre expression vector that was driven by the ges-1 promoter was constructed, and injected into the floxed pan-NLS::GFP SCI with *Pges-1::DsRed*. The Ex animals that were obtained were cultured at 25°C until the L4 or young adult stage and then analyzed. We observed that NLS::GFP had disappeared in the intestinal cells that were expressing DsRed but remained in the intestinal cells not expressing the DsRed marker (Fig. 1A, referred to as “GFP-OFF” in Table 1). The nucleic GFP signals were not affected in other tissues such as the body wall muscle (Fig. 1A). Similarly, when *Pscm::Cre* was expressed, the GFP signals were selectively diminished in the nuclei of the seam cells that expressed marker fluorescence proteins (Fig. 1B, Table 1). DNA excision was examined by PCR, in which a 200-bp band was produced only when the floxed region was excised. Products of the 200 bp were observed selectively in *Pges-1::Cre* and *Pscm::Cre* transgenic animals (Fig. 1C, D), and the results indicated that tissue-specific Cre/loxP-mediated gene inactivation occurred. Because Cre recombinase does not seem to contain NLS, artificial NLS-added Cre recombinase was used in the previous report [24]. We constructed *Pges-1::NLS::Cre* and determined its activity, and the results showed that *Pges-1::NLS::Cre* exhibited almost the same activity as *Pges-1::Cre* (data not shown). We further assessed the Cre/loxP-mediated gene inactivation at lower temperatures such as 20°C and 15°C. In many cases, Cre/loxP-mediated GFP elimination occurred at the same level that was found at 25°C. In contrast, *Pmyo-3::Cre* caused GFP elimination at 15°C but not at 25°C (data not shown), although the optimum temperature of Cre recombinase was 37°C and greater [14]. Because the growth rate of *C. elegans* is slower at lower temperatures, such conditions might offer enough time to react to Cre/loxP recombination and GFP turnover.

Next, we examined whether Cre/loxP-mediated gene inactivation can be temporally controlled. A Cre expression vector driven by the *hsp-16.2* promoter was constructed and injected into the floxed pan-NLS::GFP SCI. The Ex animals
were heat-shocked at 33°C for 1 h and cultured at 15°C until the adult stage. As a result, the NLS::GFP signals were diminished depending on the heat-shock (Fig. 1E, F). Taken together, our data demonstrate that Cre/loxP-mediated gene inactivation is spatially and temporally controlled in C. elegans.
Establishment and validation of a collection of Cre expression vectors and transgenic strains

For systematic experiments, we constructed a collection of Cre expression vectors that were driven by the promoters that are listed in Table 1. The vectors were validated by PCR and GFP elimination in the floxed pan-NLS::GFP SCI. Unexpectedly, GFP elimination was not observed in many cases, although Cre/loxP-mediated DNA excision was detected by PCR in all of the tested vectors (Table 1). We also noticed that NLS::GFP was abolished in some tissues, such as the intestine and hypodermis, but not in the other tissues, such as the neurons and pharynx (data not shown). We speculated that nucleic GFP might persist in these tissues even if the DNA is excised out. To visualize Cre/loxP-excision more clearly, we constructed the tester vector Pdpy-30, mCherry, venus::H2B, which expresses Venus::H2B when floxed mCherry was excised (Fig. 2A). Cre expression vectors were co-injected with the tester vector and corresponding enhanced cyan fluorescent protein (ECFP) markers, and then examined to determine whether they cause Venus::H2B expression. As a result, Venus::H2B expression was detected in the nuclei of the pharyngeal cells which express ECFP in Ex[Pdpy-30<mCherry<venus::H2B;Pmyo-2::Cre;Pmyo-2::ECFP] transgenic animals (Fig. 2B, referred as “Venus-ON” in Table 1). Similarly, Venus::H2B was observed

Table 1. Cre recombinase expression vectors driven by tissue specific and heat-shock promoters and transgenic strains.

| Promoter | Expression  | Excision (PCR) | Excision (GFP-OFF) | Excision (Venus-ON) | Cre multi-copy integration strains |
|----------|-------------|----------------|--------------------|---------------------|-----------------------------------|
| ges-1    | intestine   | Yes            | Yes                | Yes                 | tls1000-1002                      |
| myo-2    | pharynx     | Yes            | No                 | Yes                 | tls1076-1078                      |
| myo-3    | BWM         | Yes            | Yes/No             | Yes                 | tls1058,107,1097                  |
| scm      | seam cell   | Yes            | Yes                | NA                  | tls969-972                        |
| dpy-7    | hypodermis  | Yes            | Yes                | NA                  | tls1027-1029                      |
| unc-122  | coelomocyte | Yes            | No                 | NA                  | tls0960-0963                      |
| hsp-16.2 | heat shock  | Yes            | Yes                | NA                  | tls1054,1055,1057                 |
| rgef-1   | pan-neuron  | Yes            | No                 | Yes                 | tls777-778                        |
| snb-1    | pan-neuron  | NA             | NA                 | Yes                 | -                                 |
| unc-4    | DA, VA      | NA             | No                 | Yes                 | tls1068-1070                      |
| unc-25   | DD          | NA             | No                 | Yes                 | tls1072,1073                      |
| eat-4    | glutamatergic neurons | NA | No | Yes | tls1062,1064,1065 |
| che-2    | sensory neurons | NA | NA | Yes | tls1092,1094-1096 |
| ttx-3    | AY          | NA             | NA                 | Yes                 | tls1091                          |
| mec-4    | touch receptor neurons | NA | NA | Yes | tls1074,1075 |
| fig-1    | glia        | Yes            | No                 | Yes                 | tls1024-1026                      |
| lag-2    | DTC         | Yes            | Yes                | NA                  | -                                 |

NA = not analyzed.

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in the ventral nerve cord neurons of Punc-4::Cre transgenic animals (Fig. 2C, Table 1). Venus::H2B was expressed by all of the tested Cre expression vectors (Table 1). In certain experiments, Venus::H2B was ectopically observed; however, it mostly exhibited specific expression patterns. For example, Venus::H2B was expressed by Peat-4::Cre not only in the head and pharyngeal neurons (Fig. S1A), which are consistent with reports that eat-4 is expressed in glutamatergic neurons, but also in the hypodermal cells (Fig. S1B). Similarly, Venus::H2B was occasionally found in the intestine in Psnb-1::Cre animals (Fig. S1C, D). Unknown or embryonic/developmental expression driven by promoters may cause the ectopic Cre/loxP recombination. Another possibility is that rearrangement in the extrachromosomal arrays may lead to the unexpected reaction.

For stable Cre/loxP experiments, we generated a series of Cre multi-copy integration strains (Table 1, Table S1). Multiple strains for each promoter eliminated conflicts in the Cre-insertion sites and allowed genes to be analyzed.
All of the listed strains and plasmids are available upon request. For Snb-1p::Cre and lag-2::Cre, only the plasmids are available.

**Floxed sid-1 SCI using UV/TMP methods**

To validate our system, we performed cKO on the sid-1 gene. We selected sid-1 because the systemic RNAi-defective phenotype of the mutants is clear and can be assessed throughout the entire body except for in the neurons. First, we generated floxed sid-1 SCI using UV/TMP (Fig. 3A). Approximately 10,000 P0 animals from the parent Ex strain (tm234;tm2700;tm246;tmEx3452[floxed sid-1]) were treated with UV/TMP, and approximately 200,000 F1 animals were collected and cultured under the selection conditions. As a result, 47 stable transformants were obtained. The isolated transformants were further selected by PCR, which resulted in 6 integrated strains with the floxed sid-1 transgene. The integration frequency (integrants/P0 animals) was 0.06%. A qPCR analysis of the sid-1 sequence showed that the five strains, tmIs1004, tmIs1005, tmIs1006, tmIs1007, and tmIs1008 had a single-copy integration of the floxed sid-1 transgene (Fig. 3B). In contrast, tmIs1009 contained a low-copy (3 copies) of the transgenes. A qPCR analysis of the vps-45 revealed a single copy insertion of the vps-45 mini gene in tmIs1004, tmIs1007 and tmIs1008 and several-copy insertion in tmIs1005, tmIs1006 and tmIs1009 (Fig. 3B). When examining the tmEx3452 parental strain, it was revealed that the parental Ex strain contained 32, 139 and 52 copies of transgenes for sid-1, vps-45 and ben-1, respectively. The ben-1 gene was nearly undetectable in the integrant strains, which was expected (Fig. 3B). In this experiment, we successfully obtained multiple single-copy integrations of the floxed sid-1, the size of which is approximately 10 kbp.

**Tissue-specific knockout for the sid-1 gene**

The obtained ben-1(tm234);sid-1(tm2700);vps-45(tm246) mutants harboring the floxed sid-1 SCI were crossed with N2 to generate sid-1; floxed sid-1 SCI. The RNAi of elt-2 (intestine), unc-22 (body wall muscle), bli-3 (hypodermis), and pos-1 (germline) indicated that the RNAi-resistant phenotype of the sid-1 mutant was rescued by the floxed sid-1 SCI (data not shown). sid-1; floxed sid-1 SCI was then crossed into Cre multi-copy transgenic strains. When the sid-1(tm2700);tmIs1005[floxed sid-1 SCI];tmIs1027[Pdpy-7::Cre] animals were examined by bli-3 RNAi, the percentage of animals exhibiting the Bli-phenotype was significantly reduced, demonstrating that sid-1 was at least partially inactivated in the hypodermis (Fig. 3C). In addition, the sid-1(tm2700);tmIs1005[floxed sid-1 SCI];tmIs1027[Pdpy-7::Cre] animals were fully sensitive to unc-22 RNAi (Fig. 3D). These results indicated that the tissue-specific inactivation of sid-1 occurred in our system.
Figure 3. *sid-1* was conditionally knocked out by Cre expression. (A) Schematic overview of floxed *sid-1* SCI by UV/TMP methods. The floxed *sid-1* plasmid was co-injected with positive/negative selection marker plasmids into the recipient strain *ben-1(tm234);sid-1(tm2700);vps-45(tm246)* to establish parental Ex strains. The obtained Ex strain, *ben-1(tm234);sid-1(tm2700);vps-45(tm246);tmEx3452* was treated with a TMP solution followed by UV.
Conclusion

In this study, we provide an approach for *C. elegans* cKO based on the combination of a Cre-transgenic collection, pre-existing deletion mutants, and UV/TMP SCI methods. We established a series of Cre expression vectors and transgenic strains driven by tissue-specific and heat-shock promoters and assessed their activities using Cre/loxP-mediated gene activation and inactivation systems. We also showed that Cre/loxP recombination occurred at 15°C, 20°C, and 25°C, which were within the culture temperature range of *C. elegans*. It is recommended to confirm that the targeted proteins are certainly abolished in Cre/loxP experiments, because we experienced the cases in which the targeted protein persisted despite the excision of the targeted floxed sequence. We further obtained sid-1 deletion strains harboring the single-copy integration of the floxed *sid-1* transgene using UV/TMP methods and showed that the floxed *sid-1* was conditionally inactivated by Cre expression, demonstrating that UV/TMP methods can be used to generate single-copy integration for cKO.

Researchers have now several options, the somatic genome editing using the CRISPR-Cas9 or TALENs, and SSR using the Cre/loxP or FLP/FRT, when generating cKOs. Of these, the somatic CRISPR-Cas9 seems to be the most rapid strategy. This technology enables the analysis of cKO phenotypes within 1 week from the microinjection of Cas9 and sgRNA plasmids [6]. Although the CRISPR-Cas9 system yields higher mutation efficiencies than TALENs in many cases, TALENs may be an alternative strategy to edit a locus that is challenging for the somatic CRISPR-Cas9 method [4, 6]. However, SSR using the Cre/loxP or FLP/FRT should be used in preference when consistent genotypes and phenotypes are desirable, because cKOs using the somatic genome editing methods are heterogeneous and not heritable. The Cre/loxP or FLP/FRT methods are technically both applicable to *C. elegans* cKO, although FLP/FRT-mediated gene inactivation in *C. elegans* has not been reported, to our knowledge. We demonstrated a systematic gene inactivation using the Cre/loxP in this study. A toolkit for the Cre/loxP system will promote efficient and convenient cKO strategies for *C. elegans* researchers.

Supporting Information

Figure S1. Ectopic Venus::H2B expression occasionally observed. (A, B) Venus::H2B expression was detected with ECFP in the head and pharyngeal neurons of the *Ex[Pdpy-30;mCherry<venus::H2B;Peat-4::Cre;Peat-4::ECFP]* animals (A), but occasionally detected in the hypodermal cells (B). (C, D) The
Venus::H2B was detected in pan-neurons of the Ex[Pdpy-30]<mCherry><venus::H2B;Psnb-1::Cre;Psnb-1::ECFP] animals (C) but occasionally expressed in the intestinal cells (D). The arrowheads indicate ectopic expressions.

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Table S1. Detailed information on Cre expression vectors and transgenic strains.
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Author Contributions

Conceived and designed the experiments: EKN RI SM. Performed the experiments: EKN RI YS SY SH. Contributed reagents/materials/analysis tools: EKN RI YS SY SH. Contributed to the writing of the manuscript: EKN RI SM.

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