## Generation of FGFR2 splicing reporter mice

The constructed FGFR2 splicing reporter vector was linearized and injected into the pronucleus of a C57BL/6 oocyte. Mice were genotyped by PCR with primers for EGFP using genomic DNA from the postnatal and late embryonic tails, or yolk sacs from earlier embryos.

## Ethics statement

All experiments involving animals were performed in accordance with the protocols certified by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University (Approval Numbers #0070220, #0080179, and #0090084).

## Cell culture and transfection

Rat AT-3 and DT-3 prostate carcinoma cell lines were kindly provided by Dr. Garcia-Blanco and Dr. Carstein. Cells were maintained in Dulbecco's modified eagle medium (D-MEM) with 10% fetal calf serum (FCS), and vectors were transfected with TransFectin (BioRad). Stealth siRNA was used in knockdown experiments on endogenous Fox2 and was transfected using Lipofectamine RNAiMAX (Invitrogen).

## Microscopy

Fluorescent images of whole embryos of reporter transgenic mice were capture under a fluorescence microscope (MZ16FA, Lecia) with a charge-coupled device (CCD) camera (DP71, Olympus). We also used a confocal microscope (Fluoview FV1000, Olympus) to capture fluorescent images of sectioned transgenic embryos, and the captured images were processed by means of Metamorph (Molecular Devices). Fluorescent images of cultured cells and bright-field images showing in situ hybridization were captured by using a Nikon Eclipse E600 microscope with a CCD camera (DP71, Olympus).

## RT-PCR

Total RNAs and RT-PCR were performed as described previously [34,40]. The identity of all splicing variants was confirmed by sequencing. Amounts of PCR products were measured with a 2100 BioAnalyzer with Agilent DNA1000 kits (Agilent Technology), and the quantitative analyses were performed in more than three independent experiments. Primer sequences used in the RT-PCR assays are listed in Method S2.

## In vitro exon-recognition assay

The PCR products of T7-ex 9 wt (mouse FGFR2 intron 8, 200 nt; exon 9, 145 nt; and intron 9, 105 nt), T7-ex 9 containing UGCAUG and ISE/ISS-3 in intron 8 (intron 8, 237 nt; exon 9, 145



nt; and intron 9, 74 nt), and T7-ex 8 wt (intron 8, 200 nt; exon 8, 149 nt; and intron 9, 100 nt) were used as DNA templates for T7 transcription. A mutated DNA template of 3' ss in intron 8 and 5' ss in intron 9 was prepared from mutated reporter vectors as shown in Figure 3A. The RNA substrates were labeled with $^{32}P$ by in vitro T7 transcription. In vitro splicing reaction and UV cross linking were performed under the conditions described by Sawa [29]. To identify the shifted bands by UV cross linking, pre-heated cDNA oligo (10 μg/mL) for U1 (5'-CGGAGTGCAATG-3') or U2 (5'-CAGATACTACACTTG-3') was added to the RNAs after UV cross linking, and the U1 or U2 cDNA oligo/RNAs mixture were digested with 50 U/mL of RNase H at 30 °C for 10 min. In Figure 6A, highly purified Flag-Fox2 and Flag-ESRP1 were added to RNAs and incubated at room temperature for 5 min before splicing reaction to examine their inhibitory or activating effects on exon recognition of U1 snRNA or U2 snRNA. After these reactions, RNAs were subjected to denaturing PAGE analysis and autoradiography.

## In situ hybridization

In situ hybridization was carried out as previously described [41,42], with modifications. Briefly, embryos were fixed with 4% paraformaldehyde, cryo-protected with 30% sucrose, embedded in optimal cutting temperature (OCT) compound, and cut into sections in 20 μm thickness. Antisense RNA probes labeled with digoxigenin were visualized with Fab fragments from an antibody against digoxigenein conjugated with alkaline phosphatase (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/Nitroblue Tetrazolium (NBT) solutions. Sections were counterstained with Methyl Green. The following cDNA was used as the riboprobe: *Fox1* (131–865 bp from mouse cDNA), *Fox2* (2261–2991 bp from mouse cDNA), *ESRP1* (1168–1708 bp from mouse cDNA), and *ESRP2* (208–807 bp from mouse cDNA).

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**Figure Legends**

Figure 1. Construction of FGFR2 splicing reporter vector and their expression patterns.

(A) Scheme of FGFR2 splicing reporter vector. The genomic fragment of mouse FGFR2 including exon 7 through 10 was amplified and introduced into the reporter vector containing a CAGGS promoter and RFP-EGFP with different reading frames. Modified glutathione-S-transferase gene (indicated as "G") was inserted in front of the exon 7 in-frame. A schematic representation of the mRNA derived from the reporter under the alternative splicing regulation is also shown; the numbers indicate the reading frames. (B) Expression pattern of splicing reporter in vitro. The reporter vector was introduced into two rat prostate cancer cell lines AT-3 and DT-3, which have different cell-type specificities. Scale = 200 μm. (C) Expression pattern of splicing reporter in vivo. Fluorescence images of transgenic reporter mouse embryos at E14.5. Tg(+) is an embryo carrying the reporter vector, and Tg(–) is one of its litter-mate lacking the vector. Arrowheads in Tg(+) indicate EGFP signals with the patterns of whiskers (upper arrowhead) and the edge of a limb (lower arrowhead), both of which are magnified and indicated by white rectangles in the upper left-hand and lower right-hand corners, respectively (scale = 1 mm). (D) Sections from transgenic reporter mouse embryos at E16.5. Each panel shows sections from the indicated tissues, the upper one from Tg(+) and the lower from Tg(–). Portions expressing the EGFP signal are indicated by white arrows (scale = 100 μm).

Figure 2. Unbalanced sequence of 3′ splice sites is essential for mutually exclusive exon selection.

(A) Scheme for 3' splice site mutation on exons 8 and 9. Uppercase letter is intron and lowercase is exon sequence. Red characters indicate mismatches from the conserved consensus sequence in the 3' splice site and poly-pyrimidine moiety, and underline indicate mutated sequence. The yellow arrows with "primer" represent the positions amplified in RT-PCR. (B)

RT-PCR from AT-3 and DT-3 cells transfected the indicated vectors. Splice products were digested with Eco*RV*, which uniquely cuts the PCR product containing exon 9. Each band was identified and indicated with the scheme of splice products. Arrowheads indicate nonspecific PCR products, which was confirmed by sequencing. The asterisk indicates the splice product came from double inclusion of exon 8 and exon 9. The bar graph shows the amount of each splicing product, and is based on calculations from three independent experiments; the mean value for each splice product is show in the respective column with an error bar showing the SD (standard error).

## Figure 3. Promoted selection of exon 8 by disruption of 3′ splice site of exon 9.

(A) Scheme of 3' and 5' splice site mutation on exon 9. Uppercase letter is intron and lowercase is exon sequence, red characters indicates mutated sequence. (B) RT-PCR from AT-3 cells into which the indicated vectors were introduced. Arrowshead indicate aberrant spliced product that used the 5' cryptic splice site inside exon 9. The bar graph, which represents the amount of each splicing product, is based on calculations from three independent experiments; the mean value for each splice product is show in the respective column with an error bar showing the SD (standard error). (C) Results of the in vitro splice site recognition assay. The scheme for exon 9 shows the position of the splice site mutation as "x". "X-link" shows the presence or absence of UV-induced crosslinks in samples after the in vitro splicing reaction. "U1 oligo" and "U2 oligo" represent the digestion of RNA samples by RNaseH with complementary oligos for U1 or U2. The band shown by arrowheads with asterisk may be a probe crosslinked with U1 that binds to the cryptic 5' splice site inside exon 9, because it was detected in the 5' ss mutated probe and digested with U1 oligo.

## Figure 4. Identification of silencing elements for exon 9 recognition.

(A) Scheme of cis-mutation experiment on UGCAUG and ISE/ISS-3 which is located upstream of exon 9. Red characters indicated mutated sequences or deletions. (B) RT-PCR from AT-3 cell into which the indicated vectors were introduced. The bar graph shows the amount of each splicing product, and is based on calculations from three independent experiments; the mean value for each splice product is show in the respective column, with an error bar showing the SD (standard error). (C) RT-PCR from AT-3 and DT-3 cells showing amplified endogenous FGFR2, Fox1, Fox2, ESRP1, and ESRP2. The arrowhead in ESRP1 corresponds to two splice isoforms which was confirmed by sequencing.

## Figure 5. Foxs and ESRPs promote switching from exon 9 to exon 8.

(A)RT-PCR from HeLa cell transfected the wild-type reporter and indicated cDNA expression



vectors with or without Fox2 siRNA. The bar graph shows the amount of each splicing product. (B) Fluorescent microscopy image of HeLa cell transfected the wild-type reporter with indicated cDNA expression vectors (scale bar = 200 μm).

## Figure 6. Foxs and ESRPs promote switching to exon 8 through repressing exon 9 via UGCAUG and ISE/ISS-3.

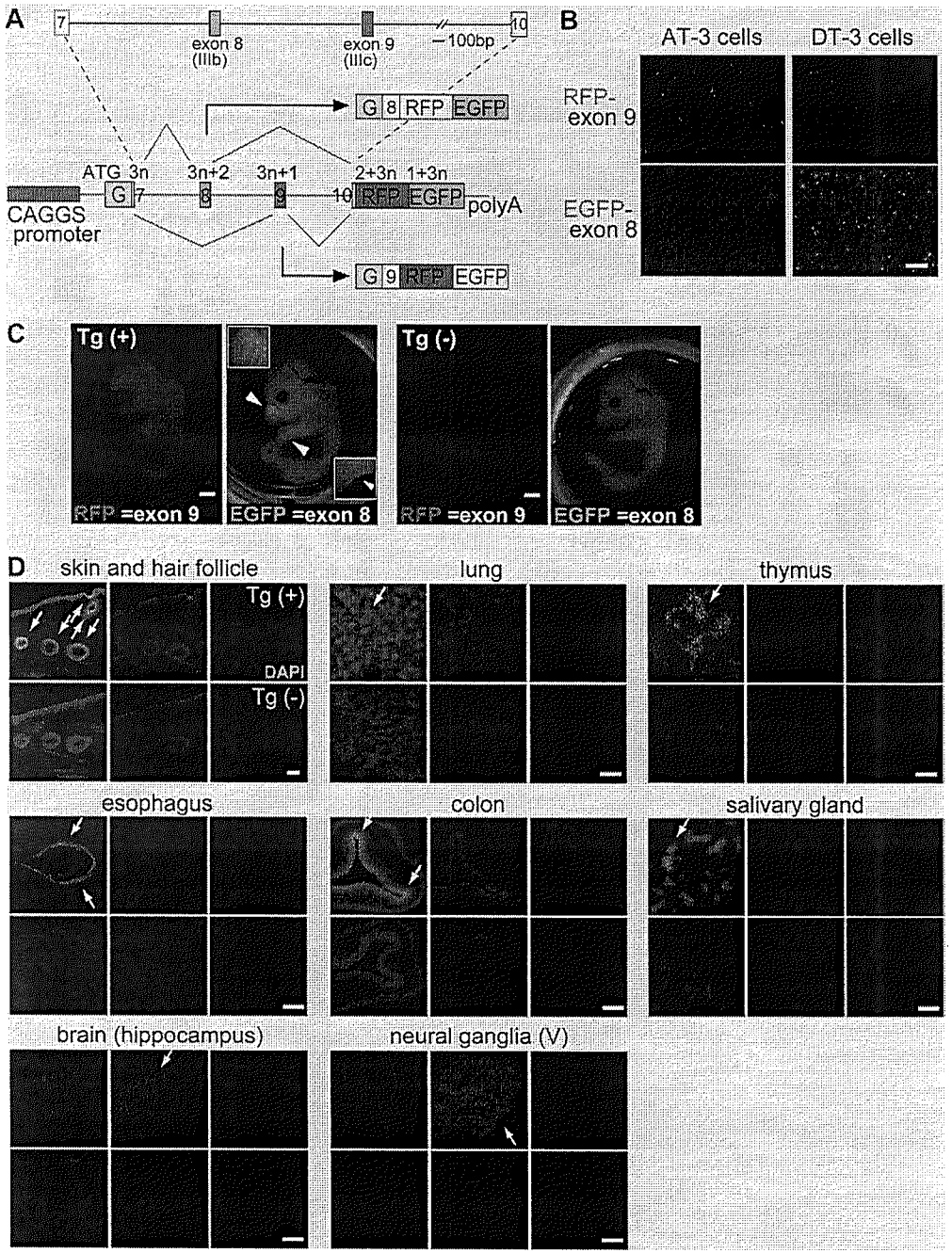
(A) RT-PCR from HeLa cell transfected the indicated wild-type or cis-mutated reporter vectors and Fox2 and/or ESRP1 expression vectors with or without Fox2 siRNA. The bar graph shows the amount of each splicing product, and is based on calculations from three independent experiments; the mean value for each splice product is show in the respective column, with an error bar showing the SD (standard error). (B) Result of an in vitro splice site recognition assay. The scheme for exon 9 RNA probe shows the position of UGCAUG and ISE/ISS-3. The asterisk shows the probe crosslinked with U1, which binds to the cryptic 5' splice site inside exon 9.

## Figure 7. Expression pattern of Foxs and ESRPs in splicing reporter mouse embryos.

Sections from transgenic reporter embryos at E16.5. EGFP signal showed the exon 8 splicing pattern, and in situ hybridization was performed with indicated probes using serial sections. The EGFP signal is indicated by a white arrow. The violet signal, indicating mRNA localization, is shown by arrows, and nuclei were counterstained with Methyl Green (scale bar = 100 μm).

## Figure 8. Model for tissue-specific splicing regulation of FGFR2 gene.

(A) "*The Knight's Fork*" regulation model: In non-epithelial or mesenchymal tissues, exon 9 is chosen as "primary" exon due to its stronger 3' splice site than exon 8 ("default" selection to choose "primary" exon). In epithelial tissues, "key regulators" repress exon 9 utilizing its 3' splice site dependency for exon recognition and cause switch to "secondary" exons ("alternative" selection to choose "secondary" exon). A small number of "key regulators" can control two mutually exclusive exons through modifying ordered splice-site recognitions in a tissue-specific manner, resembling the way that a chess piece can simultaneously attack a rook and check the king. (B) In nematode, mutually exclusive splicing of the worm FGFR homologue gene of *egl-15* is regulated by the cooperation of broadly expressed Fox-1/ASD-1 family and the muscle-specific RNA binding motif protein (RBMs) of SUP-12 (a worm homologue of mRBM24), which act together to repress inclusion of alternative exon 5B to promote muscle-specific expression of exon 5A. In the case of mammalian FGFR2, the Fox family cooperates with the tissue-specific factor ESRPs (RBM35a and b) to repress alternative inclusion of exon 9 and to promote epithelial tissue-specific expression of exon 8.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exon 9 (IIIc)
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ATG 3n
3n+2
3n+1
2+3n 1+3n
CAGGS promoter
RFP EGFP
polyA
G 9 RFP EGFP
B
AT-3 cells
DT-3 cells
RFP-exon 9
EGFP-exon 8
C
Tg (+)
Tg (-)
RFP =exon 9
EGFP =exon 8
D
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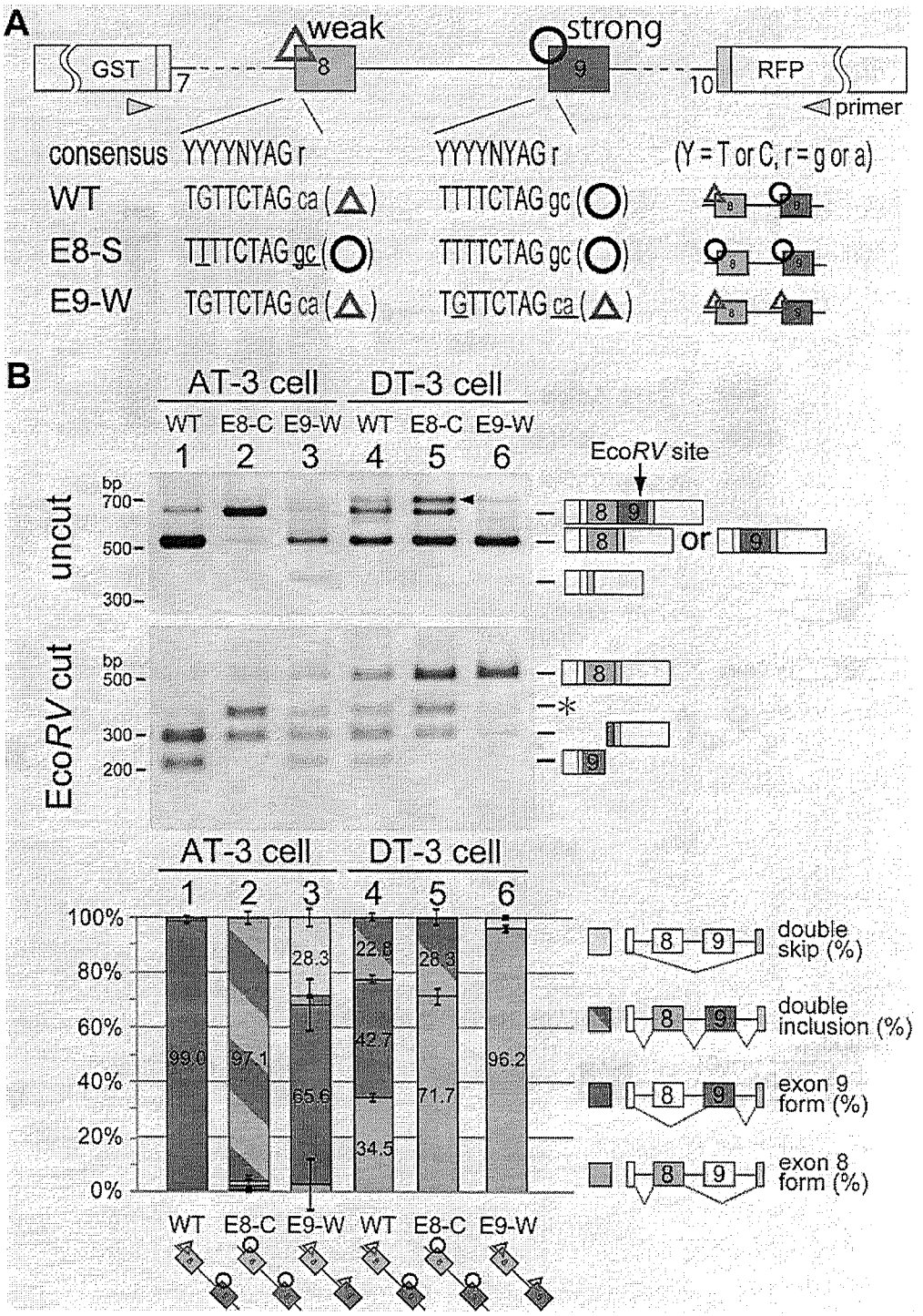
lung
thymus
Tg (+)
DAPI
Tg (-)
esophagus
colon
salivary gland
brain (hippocampus)
neural ganglia (V)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strong
GST
RFP
primer
consensus YYYYNYAG r
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TTTTCTAG gc
E8-S TTTTCTAG gc
TTTTCTAG gc
E9-W TGTTCTAG ca
TGTTCTAG ca
B
AT-3 cell
DT-3 cell
WT E8-C E9-W WT E8-C E9-W
1 2 3 4 5 6
EcoRV site
uncut
bp
700
500
300
or
EcoRV cut
bp
500
300
200
AT-3 cell
DT-3 cell
100%
80%
60%
40%
20%
0%
99.0
97.1
28.3
65.6
22.8
42.7
34.5
28.3
71.7
96.2
WT E8-C E9-W WT E8-C E9-W
double skip (%)
double inclusion (%)
exon 9 form (%)
exon 8 form (%)

A
GST
RFP
primer
UGCAUGCAUG
ISE/ISS-3
1: WT
2: Fox site mutation
UACGUACGUG
3: ISE/ISS-3 deletion
4: both sites mutation
deletion
B
uncut
EcoRV cut
EcoRV site
or
C
endo FGFR2
Fox1
Fox2
ESRP1
ESRP2
AT-3' GT-3'
EcoRV cut
exon 9 (%)
exon 8 (%)


A
3' ss
5' ss
1: WT
2: 3' ss Mut
3: 5' ss Mut
4: 3' & 5' ss Mut
primer
B
uncut
EcoRV cut
EcoRV site
or
(cryptic site)
double skip (%)
exon 9 (%)
exon 8 (%)
cryptic exon 9 (%)
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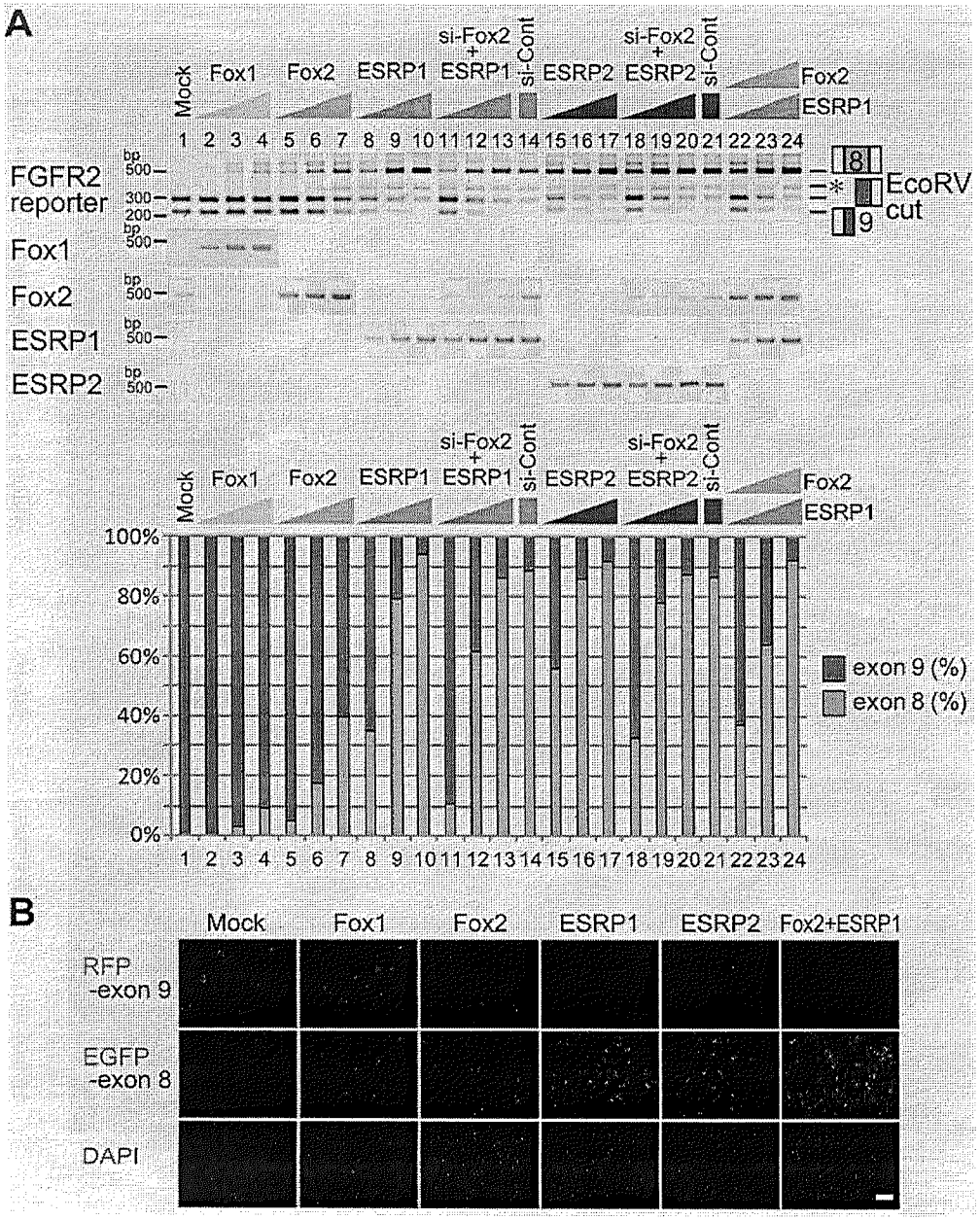
5' ss Mut
X link
Oligo
U1U2
U2+U1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Mock
Fox1
Fox2
ESRP1
si-Fox2 + ESRP1
si-Cont
ESRP2
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si-Cont
Fox2
ESRP1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
FGFR2 reporter
bp 500
300
200
8
9
EcoRV cut
Fox1
Fox2
ESRP1
ESRP2
100%
80%
60%
40%
20%
0%
exon 9 (%)
exon 8 (%)
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ESRP2
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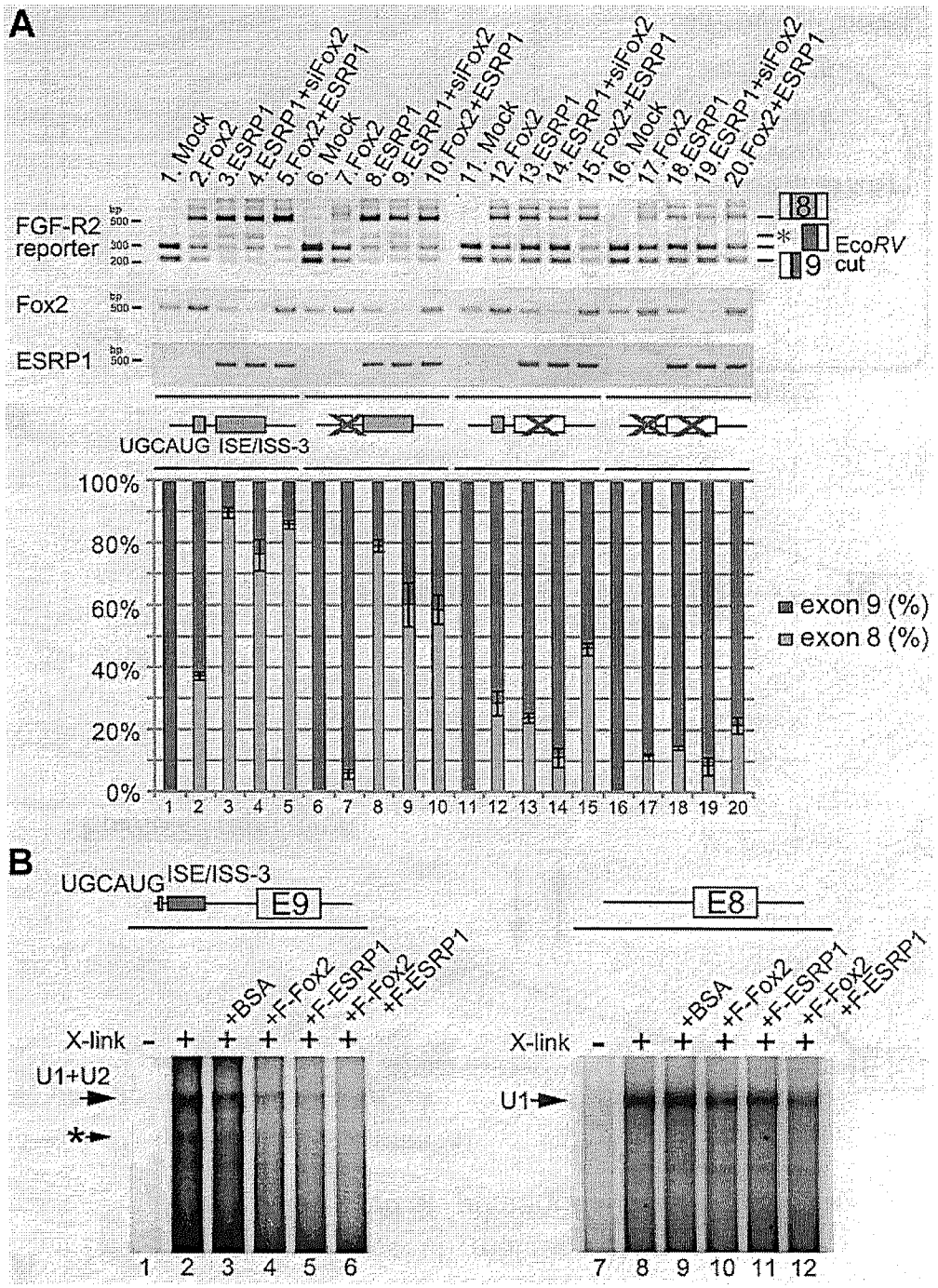
DAPI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3. ESRP1
4. ESRP1+siFox2
5. Fox2+ESRP1
6. Mock
7. Fox2
8. ESRP1
9. ESRP1+siFox2
10. Fox2+ESRP1
11. Mock
12. Fox2
13. ESRP1
14. ESRP1+siFox2
15. Fox2+ESRP1
16. Mock
17. Fox2
18. ESRP1
19. ESRP1+siFox2
20. Fox2+ESRP1
FGF-R2 reporter
bp 500
300
200
8
9
EcoRV cut
Fox2
ESRP1
UGCAUG ISE/ISS-3
100%
80%
60%
40%
20%
0%
exon 9 (%)
exon 8 (%)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
UGCAUG ISE/ISS-3
E9
E8
+BSA
+F-Fox2
+F-ESRP1
+F-Fox2
+F-ESRP1
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U1+U2
*
1 2 3 4 5 6
X-link - + + + + +
U1
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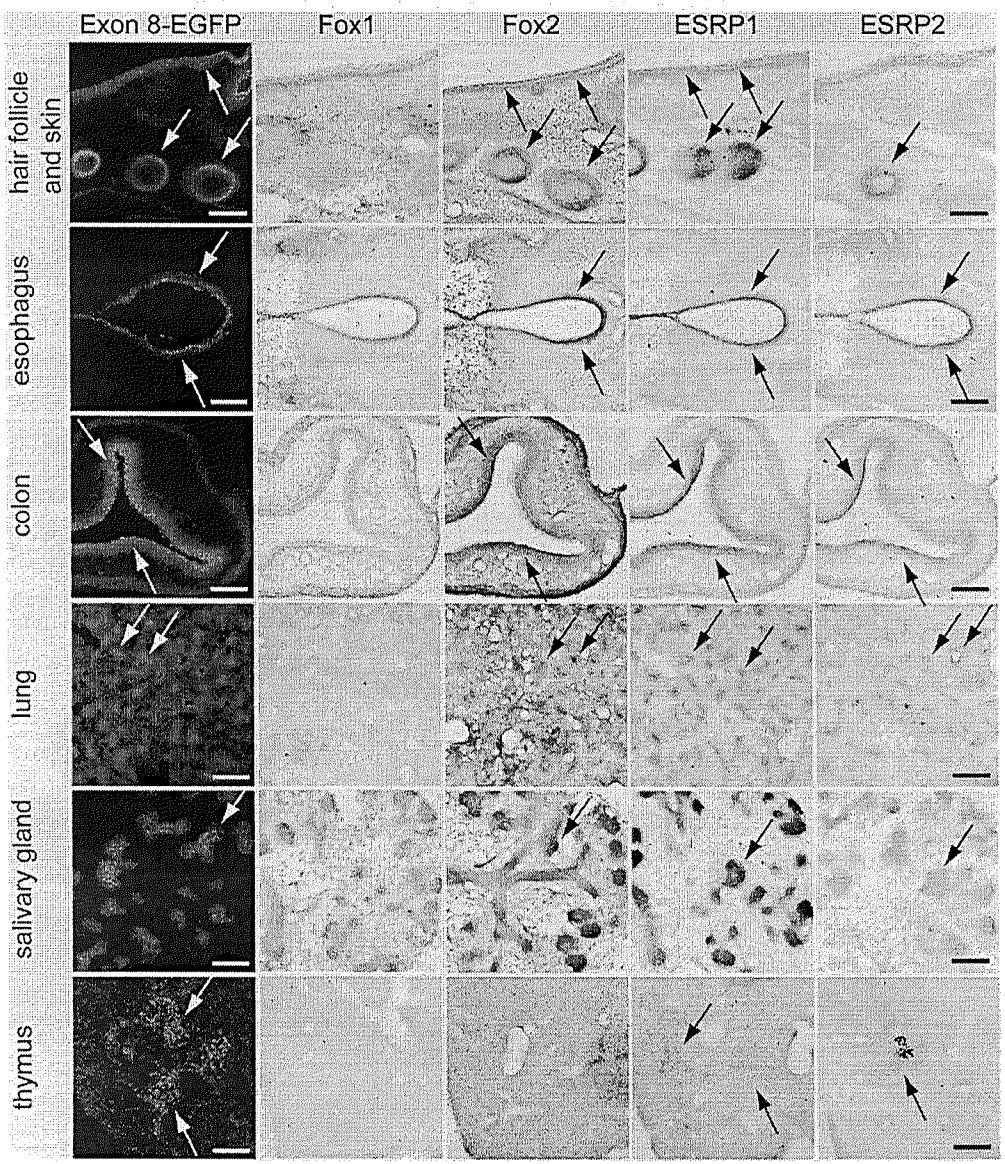
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ESRP2
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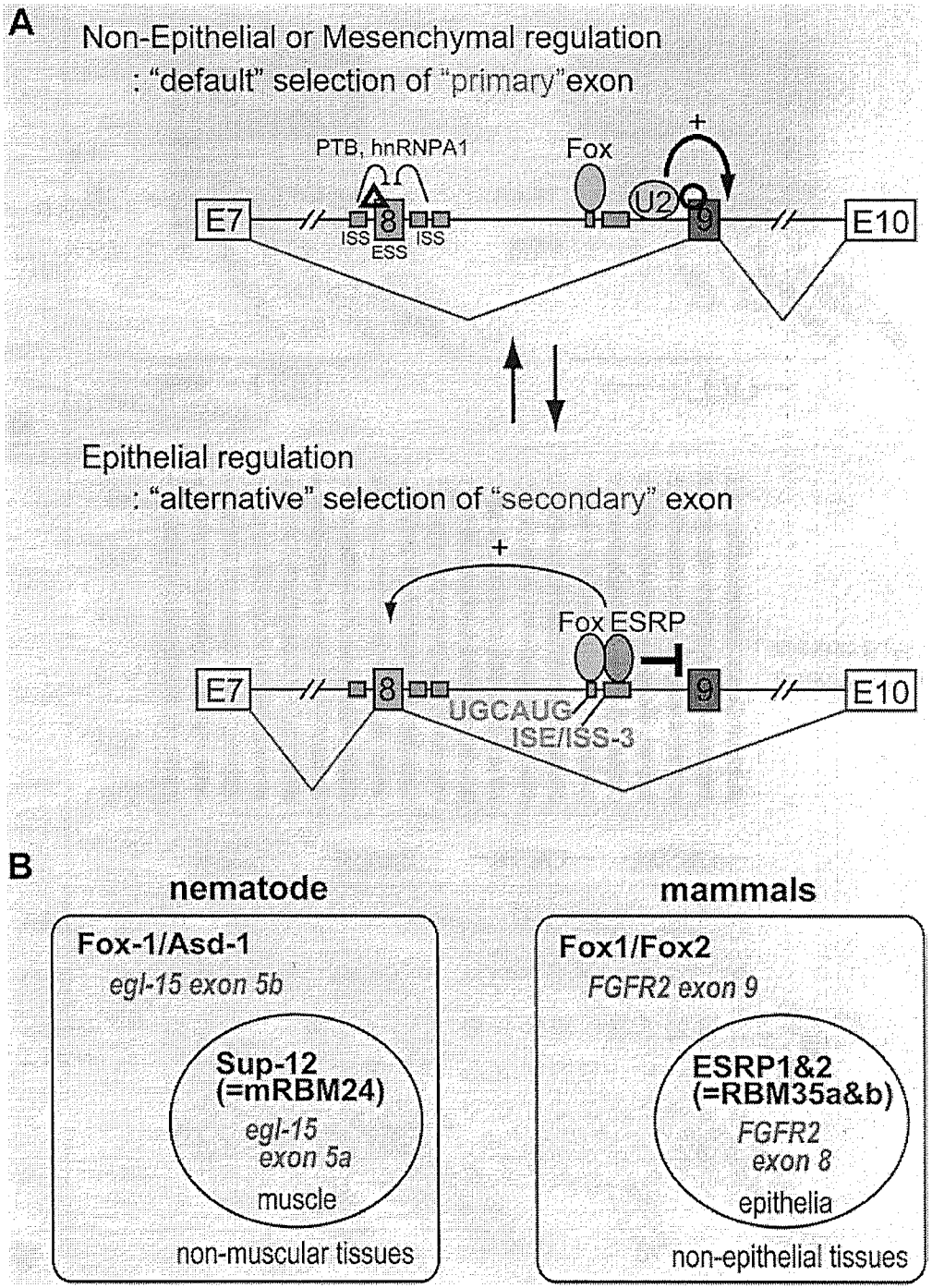
esophagus
colon
lung
salivary gland
thymus


A
Non-Epithelial or Mesenchymal regulation
: "default" selection of "primary"exon
PTB, hnRNPA1
Fox
+
E7
8
9
E10
ISS
ESS
ISS
U2
Epithelial regulation
: "alternative" selection of "secondary" exon
+
Fox ESRP
E7
8
9
E10
UGCAUG
ISE/ISS-3
B
nematode
Fox-1/Asd-1
egl-15 exon 5b
Sup-12
(=mRBM24)
egl-15
exon 5a
muscle
non-muscular tissues
mammals
Fox1/Fox2
FGFR2 exon 9
ESRP1&2
(=RBM35a&b)
FGFR2
exon 8
epithelia
non-epithelial tissues

# Visualization and genetic analysis of alternative splicing regulation *in vivo* using fluorescence reporters in transgenic *Caenorhabditis elegans*.

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Lab HP: http://www.tmd.ac.jp/mri/mri-end/en/



ABSTRACT

Transgenic multi-color fluorescence reporters enable visualization of alternative splicing patterns at a single cell resolution in living organisms and facilitate further genetic analyses to identify *cis*-elements and *trans*-acting factors involved in splicing regulation. Here we describe how to generate fluorescence alternative splicing reporters for the nematode *Caenorhabditis elegans*. We describe strategies for designing the mini-gene reporters and methods for constructing them; DNA fragments ("modules", such as a promoter/3' cassette, a genomic fragment of interest, and a fluorescent protein cassette) that exist in separate vectors are assembled using site-directed recombination. We also describe strategies and methods for mutant screening and SNP mapping utilizing the fluorescence reporters. This is the first detailed description of the design and construction of the fluorescence alternative splicing reporters for *C. elegans* and further genetic analyses. It should take 2~4 months to construct the mini-genes and generate extrachromosomal lines for visualizing spatiotemporal distribution of alternative splicing events *in vivo*. Identification of regulators by integration of the transgenes, mutant screening and mapping the responsible genes will take a further 6~12 months. The fluorescence reporter construction described here can also be applied to vertebrate culture cell system.

## INTRODUCTION

Alternative splicing of pre-mRNAs is an important mechanism for producing proteome diversity in multi-cellular organisms. Recent high-throughput sequencing analysis of human tissue transcriptomes revealed that more than 90% of human genes undergo alternative splicing and that most of these alternative splicing events vary between tissues [1]. Alternative pre-mRNA processing can be classified according to seven basic elements involved in such events: cassette exons which are included in certain mRNA isoforms and skipped in others; mutually exclusive exons; alternative 5' splice sites; alternative 3' splice sites; intron retention; alternative promoters; and alternative poly(A) sites [2,3]. The mechanisms involved in regulating alternative splicing in living cells, in the past, been studied using splicing reporter mini-genes consisting of multiple exons and introns; alternative mRNA isoforms derived from the reporter mini-genes were analyzed by quantifying the amount of reverse transcription (RT)-polymerase chain reaction (PCR) products after isolating total RNAs from transfected cells[4-6]. The laborious nature of these procedures prevented high-throughput analysis of alternative splicing regulation in living cells or organisms.

Analysis of splicing regulation has recently been facilitated by the development of alternative splicing reporters using fluorescent proteins. Initially, mono-chromatic (single-color) fluorescence reporters were designed to monitor correct splicing or skipping of alternative exons, and were successfully used: to isolate mutant cell lines defective in the regulation of alternative splicing [7]; for high-throughput and functional screening for splicing regulatory elements [8]; in the first attempt to visualize tissue-specific splicing regulation in transgenic mice [9]; for visualization of exon skipping in cancer cell lines demonstrating mesenchymal to epithelial transition in grafted rat tissues [10]; and to screen for small chemical compounds affecting alternative splicing regulation [11]. However, expression of the fluorescent protein from the mono-chromatic reporters may also be affected by alterations in other aspects of gene expression such as transcription, mRNA export from the nucleus and translation [12], leading to characterization of false positives in identifying splicing regulators/modifiers.

Multi-chromatic (multi-color) fluorescence alternative splicing reporters have overcome the limitation of the mono-chromatic reporters. For multi-chromatic reporters, expression of each fluorescent protein from the reporter mini-genes indicates a specific splicing event. As all reporters are subject to the same cellular influences, the reporters functions as the internal control for effects unrelated to alternative splicing regulation, and the ratio of the expressed fluorescent proteins in an individual cell represents trends of the splicing events in that cell. Thus, multi-chromatic reporters are suitable for analyzing alternative splicing patterns in a population of cells and in multi-cellular organisms.

Multi-chromatic reporters can be classified into single- and multi-construct types; the former consists of a single mini-gene containing two fluorescent protein cDNAs, while the latter use multiple mini-genes, each of which encodes a single fluorescent protein. A remarkable feature of the single-construct bi-chromatic reporters is that two alternative mRNA isoforms encoding distinct fluorescent proteins are generated from a common pre-mRNA in a mutually exclusive manner, and therefore the single-construct bi-chromatic reporters are sensitive to change in the trend of alternative splicing events. On the other hand, mini-genes for multi-construct reporters generally have simple structures and are easier to construct, but the relative copy numbers of the multiple mini-genes in each cell may affect the ratio of the multiple fluorescent proteins.

Multi-chromatic alternative splicing reporters have been used: for high-throughput analyses of cultured cells with flow cytometry to search for *trans*-acting factors and *cis*-elements [13-15]; as an imaging marker to demonstrate epithelial-mesenchymal and mesenchymal-epithelial transitions of cancer cell lines [16]; and with a plate reader to screen for chemical compounds modifying the splicing regulation [17]. Multi-chromatic reporters were also utilized for the first convincing visualization of tissue-specific skipping of an alternative



exon [18,19] and cell-type specific selection of mutually exclusive exons [20] in mice.

**Development and application of the protocol**

*C. elegans* is intron-rich and may serve as a good model organism for studying regulation mechanisms of alternative splicing *in vivo* [21]. However, only a few studies attempted to analyze alternative splicing events of endogenous genes[22-24] and exogenous reporter mini-genes [25] by RT-PCR. We have recently constructed multi-chromatic alternative splicing reporter mini-genes for *C. elegans*, and successfully visualized cell-type-specific [26] and developmentally regulated [27] alternative splicing events *in vivo*. As *C. elegans* is transparent, it is easy to observe expression patterns of multiple fluorescent proteins in living worms at a single cell resolution. In addition, further identification of *trans*-acting factors, *cis*-elements and partially spliced RNA species is facilitated in *C. elegans* by the availability of genetic tools such as transgenic expression of exogenous proteins, mutant screening and gene mapping, and RNAi-mediated gene knock-down[26-28]. Another advantage of *C. elegans* in studying splicing regulation is that its introns are on average very short [29] and therefore it is easy to construct reporter mini-genes that include all the required elements. Studies on splicing regulation in *C. elegans* have revealed that regulatory mechanisms of alternative splicing is evolutionarily conserved between nematodes and mammals, and further studies in *C. elegans* are therefore likely to help determine cellular codes for alternative splicing in higher organisms [30,31].

We utilized both single- and multi-construct types of multi-chromatic fluorescence alternative splicing reporters in the previous studies [26-28]. From experience, our first choice in designing alternative splicing reporters for *C. elegans* is the multi-construct type (see details explained in Experimental Design). A transgenic worm generated by a standard microinjection method carries hundreds of copies of plasmid DNAs as an extra-chromosomal array [32] and it is generally accepted that injection of a mixture of several mini-genes with the same vector backbone results in proportional incorporation of all the constructs in the extra-chromosomal array [32]. A pair or a set of reporter mini-genes in our multi-construct reporters usually have exactly the same structures and sequences except for fluorescent protein cDNAs and a few, if any, modifications within the genomic fragment of interest; this minimizes the chance that mRNA isoforms derived from the mini-genes will be affected by differences in transcription, stability and/or translation (see below). In this manuscript, we provide protocols for: assembling the multi-construct reporter mini-genes for *C. elegans*; screening for mutant worms, and; single nucleotide polymorphism (SNP) mapping of the responsible genes.

**Features of the technique**

The advantages of the multi-chromatic fluorescence alternative splicing reporters in studying splicing regulation in living organisms over conventional reporter mini-genes and/or direct analysis of endogenous mRNAs include the following:

1. Ease of analysis. In contrast to analysis of splicing patterns by RT-PCR, which includes extraction of RNA, reverse transcription, PCR and electrophoresis, the readout of the multi-chromatic fluorescence reporters can be detected under fluorescence dissection scopes
2. The splicing events can be visualized at a single cell resolution in living organisms. This enables description of splicing patterns in each cell type and leads to discovery of splicing events specific to certain minor cell types or developmental stages. This feature is especially important in analyzing splicing patterns in *C. elegans* because it is practically impossible to dissect single cell types or tissues from worms for RNA preparation. In analysis of average splicing patterns by RT-PCR, a cell-type-specific alternative splicing may appear to be a time-dependent event because the relative population or mass of each cell type varies with the development of tissues, organs or animals [33]. It is possible to distinguish these two patterns with fluorescence alternative splicing

reporters.

3. Transgenic reporter lines are genetically stable and give reproducible results from worm to worm. Transgenic lines can be crossed with existing splicing regulator mutant lines and expression levels of the splicing isoforms can be easily compared in each worm by quantifying the fluorescence in each worm. Transgenic worms can also be used for mutant screening and further genetic analyses.
4. Depending on the promoters used, splicing patterns of the reporter mini-genes can be analyzed in specific cell types and tissues including those where the endogenous gene is not expressed. This feature helps to characterize splicing regulation and to screen for mutants defective in reporter expression.

Points to be considered when interpreting the results of the fluorescence alternative splicing reporters include the following:

1. Expression levels of the mini-genes in each tissue depend on the promoters used and are often higher than the endogenous gene. High expression of the reporter mini-genes may titer *trans*-acting splicing regulators and may in turn affect the ratio of the fluorescent reporter proteins produced. As repertoire and expression levels of splicing regulators vary from cell type to cell type, expression of reporter fluorescent proteins may be differentially affected from cell type to cell type and from reporter to reporter.
2. Stability of the mini-gene-derived mRNAs may affect expression of the fluorescent proteins. mRNAs with premature termination (nonsense) codons (PTCs) are selectively degraded by a quality-control mechanism called nonsense-mediated mRNA decay (NMD). In contrast to exon junction complex (EJC)-dependent NMD in mammals [34,35], long 3' untranslated regions (UTRs) trigger NMD independent of exon-exon boundaries in *C. elegans* [28,36]. It is therefore critical to design fluorescence reporter mini-genes so that mRNA isoforms encoding the fluorescent proteins escape NMD. This is the major reason why single-construct bi-chromatic reporters are not recommended for *C. elegans*.
3. Part of the reporter mini-genes derived from the genomic fragment of interest may affect expression and properties of the reporter fluorescent protein (discussed in the Experimental Design section).

We recommend the following strategy to efficiently determine expression profiles and identify regulators of alternative splicing events:

1. First utilize a ubiquitous promoter for mini-gene construction in order to check expression and splicing patterns of the fluorescence reporter mini-genes and to survey the expression profiles of the fluorescence reporter.
2. Then utilize its own or tissue-specific promoters to validate the expression profiles of the reporter mini-genes and to establish integrant lines for mutant screening.
3. Screen for mutants defective in reporter expression.
4. Check the splicing pattern of the endogenous gene in the mutants.

Limitations of the technique presented here are:

1. It is difficult to construct mini-genes carrying a large genomic fragment. The maximum total size of a plasmid vector is about 20 kb, and therefore the size of the genomic fragment cassettes is limited to a maximum of 15 kb.
2. Some mini-genes do not work probably due to unfavorable features of the genomic fragments of interest as discussed in the Experimental Design section.

**Experimental Design**

**Designing fluorescence reporter mini-genes.** Here we explain the principles of designing fluorescence reporter mini-genes. Figure 1



shows the typical structures of pairs of two-construct bi-chromatic alternative splicing reporter mini-genes constructed and used by us in previous and on-going studies to monitor mutually exclusive exons (Figure 1a) and a cassette exon (Figure 1b). These mini-genes share a common structure: the vector backbone; a promoter; an artificial constitutive intron; and a 3' cassette. The genomic fragment to be analyzed and a cDNA for a fluorescent protein are inserted between the second exon and the 3' cassette. The genomic fragment spans the upstream constitutive exon through the downstream constitutive exon and an in-frame translation initiation codon is artificially introduced at the 5' end to force translation initiation in a specific reading frame; GFP or RFP cDNA should be fused to the genomic fragment in the appropriate frame. In the mini-genes shown in Figure 1a, a termination codon is artificially introduced into one of the two alternative exons in each construct. GFP-fusion protein is produced only from an mRNA isoform with exon 'a' and RFP-fusion protein is produced only from an mRNA isoform with exon 'b'. If both exons are included, neither GFP nor RFP is produced due to the termination codons or a frame-shift. With these mini-genes, expression of GFP and RFP will only exclusively indicate the expression of exon 'a' and exon 'b' isoforms, respectively, if the size of the alternative exons is not a multiple of three bases. In case the size of the alternative exons is a multiple of three bases, GFP and RFP will also be produced from double-skip isoforms. In the mini-genes shown in Figure 1b, the size of the cassette exon is not a multiple of three bases and therefore inclusion of the cassette exon changes the reading frame of the downstream exon. GFP cDNA is connected in frame when the cassette exon is included and RFP cDNA is connected in frame when the cassette exon is excluded. Thus, for these mini-genes, expression of GFP and RFP indicates inclusion and exclusion of the cassette exon, respectively.

It is critical to design mini-genes so that expression of a specific fluorescent protein unambiguously indicates a specific splicing event. For example, if the size of a cassette exon is a multiple of three bases, insertion or deletion of one base in the cassette exon can cause a frame-shift to discriminate between inclusion and exclusion of the exon. Please notice that the reporter mini-genes described here are only a few typical examples of fluorescence alternative splicing reporters. Investigators are encouraged to design reporters according to the alternative splicing events to be visualized. For example, alternative use of multiple 5' splice sites or 3' splice sites could be monitored by utilizing a pair of mini-genes designed to cause a frame-shift by the alternative selection of the splice sites, although we have not constructed these types of reporter yet.

As the genomic fragment of interest is excised from the genome according to the interest in its nucleotide sequence, the amino acid sequences encoded in it is an unnatural internal segment of a protein and may greatly affect folding, stability and/or localization of the fluorescent fusion proteins. It is therefore highly recommended to predict properties of the fusion proteins (such as hydrophobicity profiles, domain structures, localization signals and destabilizing sequences) [37,38] in designing the mini-genes. N-terminal hydrophobic stretches may result in secretion of the fluorescent proteins and therefore should be avoided. Hydrophobic regions within the proteins may cause aggregation or mis-folding of the fluorescent proteins. In such cases, trim the genomic fragment of interest to minimize or compromise the hydrophobic regions. Extra ATG codons in the exonic regions, either in-frame or out-of-frame, may cause aberrant translation initiation resulting in reduced production of the fluorescent proteins (A. Takeuchi, personal communication). N-terminal tags such as glutathionine S-transferase (GST) of *E. coli* may be utilized to stabilize expression of the fusion proteins as in the case of alternative splicing reporters for mammalian cultured cells [20].

**Construction by homologous recombination.** We construct reporter mini-genes by site-specific recombination using the MultiSite Gateway system (Invitrogen). The major advantage of homologous recombination in mini-gene construction is that a variety of Expression vectors, or mini-genes, can be easily and rapidly constructed by assembling modular DNA fragments cloned in Gateway Entry and Destination vectors. For a basic background of the Gateway system, please refer to the provider's website (www.invitrogen.com). We usually

perform a '2-fragment' recombination reaction to construct reporter mini-genes as schematically shown in Figure 2a. The Destination vector provides a promoter, an artificial constitutive intron and a 3' cassette. Entry vectors provide a cassette containing the genomic fragment of interest and a fluorescent protein cassette.

In this protocol, we describe how to clone the genomic fragment into pENTR-L1-R5 vector (steps 1-16). Briefly, we perform a two-step PCR procedure to amplify *att*B-flanked genomic fragments, and perform a site-directed recombination (BP reaction) to clone the fragment (Figure 2b).The first PCR is performed with primers that are template-specific and contain a part of the *att*B sequences at their 5' ends. The first PCR product is then used as a template for the second PCR with *att*B adapter primers that span the entire *att*B sequences. The advantages of the two-step PCR procedure in amplifying *att*B-PCR products are that the template-specific primers are shorter, which lowers a risk of nucleotide error and the cost in the primer synthesis, and that the *att*B adapter primers can be used repeatedly for cloning other DNA fragments in different mini-gene projects. We have a variety of fluorescent protein cassettes in pENTR-L5-L2 vector (see Table 1 and Supplementary Information). The colors of the fluorescent proteins include green, red, yellow and cyan. The manufacturer's instruction of the Gateway system describes the use of one standard reading frame in the *att*B sequences and we usually do so. But we also constructed GFP and RFP cassette vectors in non-canonical frames (Table 1) because there are no termination codons in *att*B sequences in any frame.

We have already constructed a variety of Gateway Destination vectors (Table 2), some of which were used in our previous studies for expression in *C. elegans* [26-28]. In step 18A of this protocol, we describe how we converted a promoter-less expression vector pPD49.26 (originally constructed in A. Fire Lab[39]) into a Destination vector pDEST-PL. Briefly, we performed a two-step inverted *att*B-PCR procedure to amplify *att*B-flanked vector fragments, and performed a site-directed recombination (BP reaction) to clone the PCR product (Figure 2c). This method allows any existing expression vectors containing the ampicillin-resistance gene to be converted into a Destination vectors at the desired position and in the desired reading frame. We have successfully constructed Destination vectors for expression in bacteria[27,28] and in cultured mammalian cells (unpublished observation) by this method. We constructed most of the other Destination vectors listed in Table 2 by ligation-based cloning of a promoter fragment into multiple cloning sites (MCS) of pDEST-PL (Figure 3), as described in Step 18B of the Procedure.

**Generation of transgenic reporter worms and confirmation of splicing patterns.** We generate transgenic worms with extrachromosomal arrays by a standard microinjection method [32,39]. We use *lin-15 (n765ts)* as a host strain and *lin-15(+)* genomic DNA clone[40] as a transformation marker. *lin-15 (n765ts)* mutants are apparently normal at 15°C, but show a fully penetrated multi-vulva (Muv) phenotype at 20°C or higher.

When extrachromosomal lines are established, we strongly recommend analyzing splicing patterns of the mini-gene-derived mRNAs as described in steps 32~43 to validate that splicing of the reporter mini-genes mirrors the endogenous mRNA isoforms, and to confirm that the ratio of alternative mRNA isoforms derived from the mini-genes is consistent with the expression patterns of the fluorescent reporter proteins[26].

We generate integrated lines by ultraviolet (UV)-irradiation essentially as described [41]. We isolate F1 worms as heterozygote candidates. Extrachromosomal lines with a transmission rate of 20~30% are most suitable for screening integrated lines with the protocol described in step 46~53; if the transmission rate is >50%, there will be many false positives in screening for heterozygote candidates in steps 50 and 51; if the transmission rate is 10% or lower, preparation of fluorescent worms for UV-irradiation in step 46 will be laborious.

**Reporter worms for mutant screening.** For effective screening for mutants defective in regulation of the alternative splicing reporter



expression, it is important to design a good screening strategy. The best strategy of mutant screening is to look for worms that gain expression of a fluorescent protein not expressed in the wild type background. Investigators are therefore recommended to postulate a likely mechanism of regulation, i.e. in which tissues is the putative regulator expressed and whether it enhances or silences the splicing event. It is also highly recommended to restrict expression of the reporter to a subset of tissues and to reconstruct the genomic fragment of interest to focus on a specific splicing event. Parallel screening of several reporter strains with different tissue-specific promoters may facilitate the screening process because it is often difficult to predict where the putative regulator is expressed. For example, in the case of the *egg laying defective* (*egl*)*-15* alternative splicing reporter, we mutagenized worms expressing the reporter in either hypodermis or muscles, and successfully isolated various color mutants only from those expressing the reporter in muscles [26,28]. It is not recommended to use a multi-color reporter strain with ubiquitous expression as a parental strain; the effects of mutations in a splicing regulator may be faint or limited to a small number of cells due to redundancy with other regulators and it will be difficult to detect such alterations in a ubiquitously expressing fluorescence reporter worm. It is also not recommended to screen for mutants that lose expression of fluorescent proteins; loss or reduction of expression may also be caused by silencing of transgenes. When utilizing a variety of ubiquitous and tissue-specific promoters, investigators are encouraged to check the consistency of the reporter expression, since different promoters may differentially affect the alternative splicing of the mini-genes [42].

**Mutant screening and SNP mapping.** We usually mutagenize reporter homozygotes. In some cases, however, mutant worms are sterile in the reporter-homozygous background and cannot be established as strains. In such cases, we mutagenize a mixture of reporter heterozygous and homozygous worms or extrachromosomal lines.

We perform SNP-based mapping basically as described [43]. Information about *C. elegans* SNPs and primers for typing are available on a website of the Genome Sequencing Center at Washington University in St. Louis School of Medicine (http://genomeold.wustl.edu/genome/celegans/celegans_snp.cgi) and on WormBase (http://www.wormbase.org/). We first map the gene of interest to a chromosome by analyzing SNPs of pooled lysate from at least 48 single F2 worms. In some cases, we analyzed unverified SNPs in the database. The SNPs we confirmed were deposited to WormBase with information about primers. As the phenotype of the splicing reporter is apparent in the presence of integrated reporter loci, the reporter may also be mapped. Note that presence of a mutation in a certain locus does not indicate that it is the cause of the phenotype - it may be just one of many neutral mutations in the strain. The link between a given gene and the phenotype should be confirmed by reproduction of the color phenotype in an RNAi knockdown experiment or in the preexisting mutant background, or by a rescue experiment.

***Cis*-element search.** To search for *cis*-elements involved in the regulation of alternative splicing, simultaneously modify a pair of mini-genes so that the two have the same mutant *cis*-element, and compare the expression pattern of the mutant pair with that of the wild type pair of mini-genes to observe the effect of the *cis*-element of interest on reporter expression in transgenic worms. The *cis*-elements are often highly conserved among related nematode species [21,25]. We take advantage of sequenced genomes of several related nematode species available in WormBase (http://www.wormbase.org/) to search for candidate *cis*-elements. Although disruption of some single *cis*-elements can significantly alter the splicing pattern of the mini-genes, multiple *cis*-elements are often involved in regulation and simultaneous disruption of multiple elements may be necessary for exerting discernible effects in certain cases.

To correlate *trans*-acting factors to the identified *cis*-elements, it is necessary to test direct binding of the *trans*-acting factors to the *cis*-elements in *in vitro* assays such as the electrophoresis mobility shift assay (EMSA) or UV cross-linking utilizing labeled RNA probes

[44-46]. We use a mutagenized version of the RNA probe as a negative control to confirm sequence specificity of the binding. When using ectopic expression of a *trans*-acting factor to determine its role in alternative splicing *in vivo*, disruption of its corresponding *cis*-element in the mini-genes should eliminate the effect of the ectopic expression [27].



## MATERIALS

### REAGENTS

Agarose-LE, classic type (Nacalai Tesque, cat. no. 01157-95)

Bacto peptone (Becton Dickinson and Company, cat. no. 211677)

BIOTAQ DNA Polymerase (Bioline, cat. no. BIO-21040)

*C. elegans* strains. The wild-type strain N2, *lin-15(n756ts)*, Hawaiian wild-type strain CB4856 and a bacterial strain OP50 are available from *Caenorhabditis* Genetics Center (CGC) (http://www.cbs.umn.edu/CGC/).

$CaCl_2$ (Wako, cat. no. 039-00475)

Carbenicillin disodium salt (Nacalai Tesque, cat. no. 07129-14). Make a 50 mg/ml stock solution in DDW. Sterilize the stock solution by filtering with a 0.22-μm membrane (eg. Millipore, cat. no. SLGS033SS) and store 1-ml aliquots in 1.5-ml tubes at -20°C for months.

Chloramphenicol (Wako, cat. no. 034-10572). Make a 34 mg/ml stock solution in 2-propanol. Store 1-ml aliquots in 1.5-ml tubes at -20°C for months.

Competent cells (see REAGENT SETUP)

Destination vectors for expression in *C. elegans*. Nucleotide sequences of the Destination vectors we constructed for expression in *C. elegans* (Table 2) are available on the *C. elegans* Promoter/Marker Database (http://www.shigen.nig.ac.jp/c.elegans/promoter/index.jsp). These Destination vectors are available from our laboratory upon request to H.K..

N,N-Dimethylformamide (Nacalai Tesque, cat. no. 13016-65)

DNA Ligation Kit Ver.2.1 (TaKaRa, cat. no. 6022)

*Dpn* I (New England Biolabs, cat. no. R0176L)

ECFP, EGFP and EYFP cDNAs (Clontech, cat no. 6900-1, 6081-1 and 6006-1, respectively)

EmeraldAmp PCR Master Mix (TaKaRa, cat no. RR300A)

Ethyl methanesulfonate (EMS) (Sigma-Aldrich, cat. no. M0880) CAUTION EMS is carcinogenic. Wear protective clothing and gloves. Avoid breathing vapors, mist or gas. Prepare EMS solutions in a fume hood and maintain in fume hood while treating worms. All EMS-treated wares should be discarded after inactivation with >2 N NaOH for >24 hrs in the fume hood.

Ex Taq (TaKaRa, cat. no. RR001A)

Fluorescent protein cassettes in Entry vectors. Sequence information of the Entry vectors we constructed (Table 1) is available in Supplementary Sequence Archive. Further information about these vectors is available upon request to H.K.

Gateway BP Clonase II Enzyme Mix (Invitrogen, cat. no. 11789-020)

Gateway LR Clonase II Plus Enzyme Mix (Invitrogen, cat. no. 12538-120)

GoTaq Green Master Mix (Promega, cat. no. M7122)

INA Agar (Ina Food Industry, cat. no. BA-10)

10 x injection buffer (see REAGENT SETUP)

Kanamycin sulfate (Wako, cat. no. 113-00343). Make a 30 mg/ml stock solution in DDW. Sterilize the stock solution by filtering with

a 0.22-μm membrane and store 1-ml aliquots in 1.5-ml tubes at -20°C for months.

$KH_2PO_4$ (Nacalai Tesque, cat. no. 28721-55)

LB medium (Sigma, cat. no. L7275, see REAGENT SETUP)

LB-Agar medium (MP Biomedicals, cat. no. 3002-241)

LB/antibiotics plates (see REAGENT SETUP)

Library Efficiency DB3.1 Competent Cells (Invitrogen, cat. no. 11782-018)

*lin-15(+)* genomic DNA clone, pJMZ (H. Robert Horvitz Laboratory, Massachusetts Institute of Technology, Cambridge, MA, USA)

M9 buffer (see REAGENT SETUP)

2-Mercaptoethanol (Nacalai Tesque, cat. no. 21417-65)

$MgSO_4.7H_2O$ (Nacalai Tesque, cat. no. 21003-75)

mRFP1 cDNA (Roger Tsien Laboratory, University of California, San Diego, CA, USA)

NaCl (Nacalai Tesque, cat. no. 31320-05)

$Na_2HPO_4$ (Nacalai Tesque, cat. no. 31801-05)

NGM plates (see REAGENT SETUP)

Nystatin (Wako, cat. no. 29870)

One Shot *ccdB* Survival T1 Phage-Resistant Cells (Invitrogen, cat. no. C7510-03)

Orange G (Sigma, cat. no. O-3756)

PCR-M (VIOGENE, cat. no. PF1002)

pDONR 221 P1-P5r (A component of MultiSite Gateway Pro 2.0 Kit, Invitrogen, cat. no. 12537-102)

pGEM-T Easy Vector System I (Promega, cat. no. A1360)

Polyethylene glycol (PEG) 6,000 (Wako, cat. no. 169-22945)

tri-Potassium citrate monohydrate (Nacalai Tesque, cat. no. 28524-45)

Primer synthesis (Operon Biotechnologies, see REAGENT SETUP)

PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa, cat no. 6210A)

PrimeSTAR HS DNA Polymerase (TaKaRa, cat. no. R010A)

Quickchange II (Stratagene, cat. no. 200523)

Quickchange II XL (Stratagene, cat. no. 200521)

RNase-Free DNase Set (QIAGEN, cat. no. 79254)

RNeasy Mini - QIAshredder Kit (QIAGEN, cat. no. RNEZ4U)

Sodium azide (Nacalai Tesque, cat. no. 31233-42)

Sucrose (Nacalai Tesque, cat. no. 30404-45)

SuperScript II Reverse Transcriptase (Invitrogen, cat. no. 18064-014)

SYNERGEL (Diversified Biotech, cat. no. SYN-100)

Trehalose dihydrate (Wako, cat. no. 207-14161)

Tris-borate-EDTA buffer (10X) (Nacalai Tesque, cat. no. 35440-44)

Ultrafree-MC Durapore PVDF 0.22 μm (Millipore, cat. no. UFC30GV00)



Venus cDNA (Atsushi Miyawaki Laboratory, RIKEN, Wako, Saitama, Japan)

Wizard Plus SV Minipreps DNA Purification System (Promega, cat. no. A1460)

Wizard SV Gel and PCR Clean-Up System (Promega, cat. no. A9281)

Worm freezing solution (2X) (see REAGENT SETUP)

Worm lysis solution (see REAGENT SETUP)

**EQUIPMENT**

Agarose gel electrophoresis equipment (Mupid-exU, ADVANCE)

Color cooled CCD camera and Software (DP71, DP Manager and DP Controller, Olympus; DFC310 FX and AF6000, Leica)

Compound microscope (DM6000, Leica) equipped with epifluorescence and differential interference contrast (DIC) optics.

Dual-band pass filter set: CFP/DsRed (chroma, cat. no. 52018), YFP/DsRed, (chroma, cat. no. 86025) and GFP/DsRed (chroma, cat. no. 51019).

Electro-Fast Gel Systems (Thermo, cat. no. AB-0934) & Power supply.

Heat block or water bath set to 42°C

High-magnification fluorescence stereo microscopes (MZ16FA and M205FA with Fluo Combi III, Leica)

Incubator set to 20°C or 15°C (MIR-153 and MIR-253, SANYO)

Incubator and incubator shaker set to 37°C

Inverted compound microscope (Axiovert S100, Carl Zeiss) with differential interference contrast (DIC) optics

Laser scanning confocal microscopes (Fluoview 500 and Fluoview 1000, Olympus) with an inverted compound microscope (IX 70, Olympus)

Metamorph (Molecular Devices)

Micro-injector (Transfector 5246, Eppendorf) and glass capillaries (Femtotips/Femtotips II, Eppendorf)

Micro-manipulator (InjectMan, Eppendorf)

Microtube mixer (MT-360, TOMY)

PCR machine (iCycler, BioRad)

Refrigerated centrifuge (MX-301, TOMY)

Spectrophotometer (NanoVue, GE Healthcare)

Ultraviolet crosslinker (CL-1000, UVP)

**REAGENT SETUP**

***C. elegans* genomic DNA.** N2 genomic DNA can be extracted by a standard genomic DNA extraction method utilizing proteinase K and phenol/chloroform[47]. Store at 4°C for frequent use or at -20°C for months. Avoid repeated freeze-thaw cycles.

**Competent cells.** We prepare DH5α competent cells as described[48], but other strains or commercially available competent cells can also be used. Store at -80°C until required.

**10 x Injection buffer.** 200 mM $KPO_4$, 30 mM potassium citrate, 20% (w/v) polyethylene glycol (PEG) 6000, pH 7.5. Store 1-ml aliquots at -20°C for years.

**LB liquid medium.** Dissolve 40 tablets of LB medium in 1 L of water and sterilize 40-ml aliquots in 50-ml polypropylene tubes by

autoclaving. Store at room temperature (22~25°C) for months. Add the appropriate antibiotic(s) (see Table below) before use.

**LB/antibiotics plates.** Add 40 tablets of LB-Agar medium per 1 L of water and autoclave. Allow the medium to cool to 50~60°C before adding the appropriate antibiotic(s) (see Table below). Pour into plates and leave at room temperature until the agar sets. Store at 4°C for upto 2~3 months until needed.

| Antibiotics stock solution | Amount per 1 L medium | Final concentration |
|---|---|---|
| 50 mg/ml Carbenicillin | 1 ml | 50 μg/ml |
| 30 mg/ml Kanamycin | 1 ml | 30 μg/ml |
| 34 mg/ml Chloramphenicol | 0.5 ml | 17 μg/ml |

**M9 buffer.** Dissolve 6 g $Na_2HPO_4$, 3 g $KH_2PO_4$ and 5 g NaCl in 1 L of water. Sterilize by autoclaving. Sterilely add 1 ml of 1 M $MgSO_4$ after cooled to room temperature. Store at room temperature.

**NGM plates.** For 1 L, add 3 g NaCl, 2.5 g peptone and 15 g INA agar to 975 ml distilled water and autoclave. Allow the medium to cool to 60~65°C, and sterilely add 1 ml of 5 mg/ml cholesterol in ethanol, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$ and 25 ml of 1 M potassium phosphate (pH 6.0). Optionally, add 1 ml of 10 mg/ml nystatin in dimethyl formamide as an antifungal reagent; nystatin does not completely prevent fungal contamination. Pour into plates and leave at room temperature until the agar sets. Store at room temperature until use for upto 1 month.

**Worm freezing solution (2X).** 50 mM potassium phosphate (pH 6.0), 100 mM NaCl and 400 mM trehalose. Sterilize by autoclaving. Store at room temperature for months.

**Worm lysis solution.** 25 mM Tris-Cl (pH 8.5), 50 mM KCl, 0.5% (v/v) Tween-20, 1 mM EDTA and 500 μg/ml proteinase K. Store 1-ml aliquots at -20°C.

**Primer design for cloning genomic DNA fragment cassettes into Entry vectors (step 1).** The gene-specific primers (GSPs) for the first PCR have 12 bases of the *att*B site on the 5' end followed by 18 ~ 25 bases of template- or gene-specific sequences (Table 3). Insert Kozak's consensus sequence between a part of the *att*B1 and the gene-specific sequences of GSP-*att*B1F to force translation initiation as shown in Table 3. Exclude termination codons from GSP-*att*B5R. Carefully design the GSPs to maintain the proper reading frame in the *att*B sequences as indicated in Table 3. AT-rich sequence should be avoided in designing the gene-specific sequences because *C. elegans* genome is AT-rich and use of such GSPs may lead to non-specific amplification. Nested PCR works well if it is necessary to use such GSPs. The *att*B adapter primers for the second PCR consist of the following common structure: four guanine (G) residues at the 5' end followed by a 22- or 25-base complete *att*B sequence (Table 3).

**Primer design for converting existing vectors into Destination vectors (step 18A(i)).** The vector-specific primers (VSPs) have 12 bases of the *att*B site on the 5' end followed by one base, which facilitates specific annealing of the *att*B adapter primer in the second PCR, and 18 ~ 25 bases of template-specific sequences (Table 4). Carefully design the VSPs to maintain the proper reading frame in the *att*B sequences as indicated in Table 4 if applicable. The *att*B adapter primers for the second PCR consist of the following common structure: four guanine (G) residues at the 5' end followed by a 25-base complete *att*B sequence and one extra base corresponding to the extra base of the VSPs (Table 4).

**Primer design for amplifying promoter fragments to construct Destination vectors (step 18B(i)).** The gene-specific primers (GSPs) should have three residues and a recognition site for a restriction endonuclease on the 5' end. The restriction site should be



compatible with one of the unique restriction sites in the MCS of pDEST-PL, *Hin*d III, *Sph* I and/or *Xba* I (Figure 3). If the upstream flanking protein-coding gene is in the same direction, we design the forward GSP just downstream of its polyadenylation signal; if the upstream gene is in the opposite direction, we design the forward GSP within its first exon. We usually design the reverse GSP to overlap and disrupt the endogenous translation initiation codon by altering ATG to ATC.

**EQUIPMENT SETUP**

**Microinjection equipment.** We utilize an inverted system microscope with differential interference contrast (DIC) optics equipped with a micro-manipulator and a micro-injector for micro injection.

**Fluorescence stereoscopes.** We utilize a fluorescence stereoscope for maintenance of transgenic lines and screening for mutants. To observe strains expressing two or three fluorescent proteins, we utilize an appropriate dual band-pass filter set, CFP/DsRed, YFP/DsRed or GFP/DsRed, to simultaneously observe two fluorescent proteins.

**Fluorescence microscopy.** We use high-magnification fluorescence stereoscopes MZ16FA and M205FA equipped with Fluo Combi III or a compound microscope to capture images of fluorescence reporter worms with a color cooled charge-coupled device (CCD) camera and an imaging and analysis software.

**Confocal microscopy and image processing.** We utilized confocal microscopes equipped with differential interference contrast (DIC) optics for image scanning, and Metamorph for processing the acquired images.

## PROCEDURE

**Cloning genomic DNA fragment cassettes into Entry vectors. TIMING 2~3 weeks**

**1|** Design and order gene-specific primers (GSPs) for two-step *att*B-PCR (see REAGENT SETUP).

**2|** Prepare the first PCR mixture as tabulated below. We usually use N2 (wild type strain) genomic DNA as a template, but plasmid or cosmid DNAs can also be used. We usually use PrimeSTAR HS DNA Polymerase, but other proofreading polymerases can also be used.

| Reagent | Amount per 25 µl reaction | Final concentration/amount |
|---|---|---|
| Template DNA | 200 ng | 200 ng |
| 5x Polymerase buffer | 5 µl | 1x |
| 2.5 mM each dNTP mixture | 2 µl | 0.2 mM each |
| 2.5 µM GSP-*att*B1F | 2 µl | 0.2 µM |
| 2.5 µM GSP-*att*B5R | 2 µl | 0.2 µM |
| PrimeSTAR HS (2.5 u/µl) | 0.25 µl | 0.025 u/µl |
| DDW | To 25 µl | |

CRITICAL STEP To avoid frequent unintended mutations in the amplified genomic DNA fragment, use proofreading DNA polymerases.

**3|** Run the first PCR in a thermal cycler using the following parameters for PrimeSTAR HS DNA Polymerase:

| Cycle | Denature | Anneal | Extend |
|---|---|---|---|
| 1 | 94°C, 2 min | | |
| 2 - 26 to 31 | 98°C, 10 sec | 55°C, 5 sec | 72°C, 1 min/kb |
| final | | | 72°C, 5 min |

**4|** Verify amplification by running a 5-µl aliquot of the first PCR product on a 1% (w/v) agarose gel.

**5|** Prepare the second PCR mixture as tabulated below. Use the same DNA polymerase as the first PCR in step 2.

| Reagent | Amount per 60 µl reaction | Final concentration/amount |
|---|---|---|
| 5x Polymerase buffer | 10 µl | 1x |
| 2.5 mM each dNTP mixture | 4 µl | 0.2 mM each |
| 2.5 µM *att*B1adapterF | 6 µl | 0.25 µM |
| 2.5 µM *att*B5adapterR | 6 µl | 0.25 µM |
| PrimeSTAR HS (2.5 u/µl) | 0.5 µl | 0.025 u/µl |
| DDW | 23.5 µl | |
| First PCR mixture | 10 µl | |

**6|** Run the second PCR using the following parameters:

| Cycle | Denature | Anneal | Extend |
|---|---|---|---|
| 1 | 94°C, 2 min | | |
| 2 - 6 | 98°C, 10 sec | 45°C, 5 sec | 72°C, 1 min/kb |
| 7 | | | 72°C, 5 min |



CRITICAL STEP The annealing temperature of the second PCR should be 45°C because the annealing sequences are just 12 base pairs.

**7|** Verify amplification in the second PCR by running a 5-µl aliquot of the second PCR product on a 1% (w/v) agarose gel.

**8|** Optionally, add 1 µl *Dpn* I and incubate at 37°C for 1 hour to destroy template DNA.

CRITICAL STEP If the PCR template plasmid or cosmid DNA contains the kanamycin-resistance gene, the PCR mixture should be treated with *Dpn* I before purifying the forward *att*B-PCR products. *Dpn* I recognizes methylated GATC sites in bacteria-derived DNA and the *Dpn* I treatment greatly reduces background colonies in step 12 associated with template contamination.

**9|** Purify the *att*B-PCR product with PCR-M or an equivalent DNA purification column.

PAUSE POINT Store the purified *att*B-PCR product at 4°C or -20°C until use in the next step.

**10|** Prepare the BP reaction mixture in a 1.5-ml microcentrifuge tube as tabulated below and mix well by briefly vortexing or tapping.

| Reagent | Amount per 5 µl reaction | Final concentration/amount |
|---|---|---|
| *att*B-PCR product | 15 ~ 150 ng | 15 ~ 150 ng |
| pDONR 221 P1-P5r (supercoiled) | 75 ng | 75 ng |
| DDW | To 4 µl | |
| BP Clonase II enzyme mix | 1 µl | |

**11|** Incubate the BP reaction mixture at room temperature or at 25°C for 1 hour or overnight. For short *attB*-PCR products of up to 2 kb, 1 hour of reaction is enough; for longer PCR products, longer incubation time will give more colonies in step 12. Although instructed in the manufacturers' manual of BP Clonase II, we usually omit proteinase K digestion because we did not find any difference in the number of colonies in step 12 even if proteinase K digestion is omitted.

PAUSE POINT Store the BP reaction mixture at -20°C until use in the next step.

**12|** Transform *E. coli* strain DH5α or others with 1 ~ 3 µl of the BP reaction mixture using competent cells by a standard heat shock method[48]. Plate transformed cells on LB/kanamycin plates and grow at 37°C overnight to select for kanamycin-resistant clones.

CRITICAL STEP *E. coli* strains with F' episome (e.g. TOP10F') cannot be used for transformation to select Entry clones. These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene in pDONR 221 P1-P5r.

TROUBLESHOOTING

**13|** Set up separate 2-ml LB/kanamycin liquid cultures in 15-ml polypropylene tubes for approximately 6 individual clones. Grow at 37°C in a incubate shaker for 12 ~ 16 hrs by rocking at a maximum speed.

**14|** Purify plasmid DNAs from overnight bacterial cultures using a standard mini-prep plasmid purification kit such as Wizard Plus SV Minipreps DNA Purification System.

**15|** Verify the identity of the purified plasmid DNAs by restriction enzyme digestion. Digest 0.5 ~ 1 µg of the mini-prep plasmid DNA in a 20 µl total volume using 4 ~ 6 U restriction endonuclease(s). Run 10-µl aliquot of the reaction on a 1% (w/v) agarose gel to check for bands of appropriate size.

**16|** Check the sequence of the insert with appropriate sequencing primers.

**Modification of genomic fragment cassettes (Optional). TIMING 2~4 weeks**

**17|** Depending on the design of the mini-genes, introduce a termination codon or a frame-shift into the exon(s) of the genomic fragment cassettes by site-directed mutagenesis; we use Quickchange II or Quickchange II XL according to the manufacturer's instruction.

**CRITICAL STEP** Take care to avoid disruption or creation of *cis*-regulatory elements or splice sites when designing the modified exonic sequences; we introduce termination codons or frame-shifts into less conserved stretches among related nematode species. Check the sequence of the entire cassette after mutagenesis to ensure that only the desired mutations have been introduced.

**CRITICAL STEP** Once the mini-genes have been constructed and validated, the site-directed mutagenesis method described above can be used to modify or disrupt candidate stretches of the genomic fragment cassette to test involvement of putative *cis*-elements. Again, take care to avoid creation of known *cis*-regulatory elements or cryptic splice sites when modifying the sequence of the genomic fragment.

**Constructing Destination vectors (Optional). TIMING 2~4 weeks**

18| Construct Destination vectors by either of the following two methods. Option A describes a useful method to convert existing expression vectors with the ampicillin-resistance gene into Destination vectors by utilizing inverse *att*B-PCR. Option B describes standard restriction digest and ligation-based cloning of a promoter of interest into pDEST-PL.

**(A) Constructing Destination vectors by inverse *att*B-PCR and BP cloning. TIMING 2~3 weeks**

(i) Design and synthesize vector-specific primers (VSPs) for two-step inverse *att*B-PCR (see REAGENT SETUP).

(ii) Prepare the first PCR mixture as tabulated below. We usually use PrimeSTAR HS DNA Polymerase, but other proofreading polymerases can also be used.

| Reagent | Amount per 25 µl reaction | Final concentration/amount |
|---|---|---|
| Template plasmid DNA | 100 ~ 200 ng | 100 ~ 200 ng |
| 5x Polymerase buffer | 5 µl | 1x |
| 2.5 mM each dNTP mixture | 2 µl | 0.2 mM each |
| 2.5 µM VSP-*att*B1R | 2 µl | 0.2 µM |
| 2.5 µM VSP-*att*B2F | 2 µl | 0.2 µM |
| PrimeSTAR HS (2.5 u/µl) | 0.25 µl | 0.025 u/µl |
| DDW | To 25 µl | |

(iii) Run the first PCR in a thermal cycler using the following parameters:

| Cycle | Denature | Anneal | Extend |
|---|---|---|---|
| 1 | 94°C, 2 min | | |
| 2 – 11 to 21 | 98°C, 10 sec | 55°C, 5 sec | 72°C, 1 min/kb |
| final | | | 72°C, 5 min |

(iv) Verify amplification by running a 5-µl aliquot of the first PCR product on a 1% (w/v) agarose gel.

(v) Prepare the second PCR mixture as tabulated below. Use the same DNA polymerase as the first PCR in step 18A(ii).

| Reagent | Amount per 60 µl reaction | Final concentration/amount |
|---|---|---|
| 5x Polymerase buffer | 10 µl | 1x |
| 2.5 mM each dNTP mixture | 4 µl | 0.2 mM each |
| 2.5 µM *att*B1adapterR | 6 µl | 0.25 µM |
| 2.5 µM *att*B2adapterF | 6 µl | 0.25 µM |



| | | |
|---|---|---|
| PrimeSTAR HS (2.5 u/µl) | 0.5 µl | 0.025 u/µl |
| DDW | 23.5 µl | |
| First PCR mixture | 10 µl | |

(vi) Run the second PCR using the parameters shown in step 6.

CRITICAL STEP The annealing temperature of the second PCR should be 45°C because the annealing sequences are just 13 base pairs.

(vii) Verify amplification by running a 5-µl aliquot of the second PCR product on a 1% (w/v) agarose gel.

CRITICAL STEP The amount of the PCR product should increase by at least four-fold in the second PCR.

(viii) Add 1 µl *Dpn* I and incubate at 37°C for 1 hour to destroy template DNA.

(ix) Verify complete digestion by running a 5-µl aliquot of the second PCR product on a 1% (w/v) agarose gel.

CRITICAL STEP A band of the template plasmid detected in steps 18A(iv) and 18A(vii), should disappear by complete digestion. *Dpn* I treatment greatly reduces background colonies in step 18A(xiii) associated with template contamination.

(x) Purify the inverse *att*B-PCR product with PCR-M or equivalent DNA purification column.

PAUSE POINT Store the purified inverse *att*B-PCR product at 4°C or -20°C until use in the next step.

(xi) Prepare the BP reaction mixture as tabulated below in a 1.5-ml microcentrifuge tube and mix well by briefly vortexing or tapping.

| Reagent | Amount per 5 µl reaction | Final concentration/amount |
|---|---|---|
| inverse *att*B-PCR product | 75 ~ 150 ng | 75 ~ 150 ng |
| pDONR 201 (supercoiled) | 75 ng | 75 ng |
| DDW | To 4 µl | |
| BP Clonase II enzyme mix | 1 µl | |

(xii) Incubate the BP reaction mixture at room temperature or at 25°C for 4 hours or overnight. For inverse *attB*-PCR products of up to 4 kb, 4 hours of reaction is enough; for longer PCR products, longer incubation time will give more colonies in step 18A(xiii). Although instructed in the manufacturers' manual, we usually omit proteinase K digestion because we did not find any difference in the number of colonies obtained in step 18A(xiii).

PAUSE POINT Store the BP reaction mixture at -20°C until use in the next step.

(xiii) Transform *E. coli* strain DB3.1 or *ccd*B Survival with 1 ~ 3 µl of the BP reaction mixture as described in the manufacturer's instruction. Plate transformed cells on LB/ampicillin or LB/carbenicillin plates and grow at 37°C overnight to select for ampicillin/carbenicillin-resistant clones.

CRITICAL STEP *E. coli* strains with F' episome must be used for transformation to select Destination clones. These strains contain the *ccd*A gene and will survive in the presence of the *ccd*B gene.

CRITICAL STEP Carbenicillin is a more stable analogue of ampicillin and can be used interchangeably here and elsewhere in this protocol. As colony formation is slow on LB/carbenicillin+chloramphenicol plates, use LB/carbenicillin plates in this step and test chloramphenicol-resistance in step 18A(xiv).

TROUBLESHOOTING

(xiv) Test resistance of 8~16 carbenicillin-resistant colonies from step 18A(xiii) to carbenicillin+chloramphenicol and kanamycin by streaking the bacteria onto LB/kanamycin and LB/carbenicillin+chloramphenicol agar plates (BOX 1) to select for carbenicillin- and chloramphenicol-resistant and kanamycin-sensitive clones.

CRITICAL STEP The Destination vectors should have both ampicillin-resistance and chloramphenicol-resistance genes as schematically shown in Figure 2C and Figure 3. From experience, some carbenicillin-resistant clones from step 18A(xiii) are also resistant to kanamycin and contain aberrant plasmids, which can be excluded in this step.

PAUSE POINT Store the LB/carbenicillin+chloramphenicol master plates at 4°C until use in the next step.

(xv) Set up separate 2-ml LB/carbenicillin+chloramphenicol liquid cultures for approximately 6 individual carbenicillin- and chloramphenicol-resistant clones from step 18A(xiv). Grow at 37°C in an incubator shaker for 16 hrs by rocking at a maximum speed.

(xvi) Mini-prep the plasmid DNAs and check the identity and sequence of the plasmids (See steps 14 ~ 16).

**(B) Constructing Destination vectors by ligating a promoter fragment to pDEST-PL vector. TIMING 2~3 weeks**

(i) Design and synthesize gene-specific primers (GSPs) for amplifying a promoter fragment (See REAGENT SETUP).

(ii) Prepare the PCR mixture as tabulated below. We usually use PrimeSTAR HS DNA Polymerase, but other proofreading polymerases can also be used.

| Reagent | Amount per 50 µl reaction | Final concentration/amount |
|---|---|---|
| N2 genomic DNA | 200 ng | 100 ~ 200 ng |
| 5x Polymerase buffer | 10 µl | 1x |
| 2.5 mM each dNTP mixture | 4 µl | 0.2 mM each |
| 2.5 µM forward GSP | 5 µl | 0.25 µM |
| 2.5 µM reverse GSP | 5 µl | 0.25 µM |
| PrimeSTAR HS (2.5 u/µl) | 0.5 µl | 0.025 u/µl |
| DDW | To 50 µl | |

(iii) Run the PCR in a thermal cycler using the following parameters:

| Cycle | Denature | Anneal | Extend |
|---|---|---|---|
| 1 | 94°C, 2 min | | |
| 2 – 26 to 31 | 98°C, 10 sec | 55°C, 5 sec | 72°C, 1 min/kb |
| final | | | 72°C, 5 min |

(iv) Verify amplification by running a 5-µl aliquot of the PCR product on a 1% (w/v) agarose gel.

(v) Purify the PCR product with PCR-M or equivalent DNA purification column.

(vi) Digest the PCR product and pDEST-PL with appropriate restriction endonucleases.

(vii) Run 1/20 aliquot of pDEST-PL digestion mixture on a 1% (w/v) agarose gel to confirm the digestion.

(viii) Run all the digestion mixture on a 1% (w/v) agarose gel and purify the PCR product and digested pDEST-PL with Wizard SV Gel and PCR Clean-Up System or equivalent gel purification column.

(ix) Run 1-µl aliquot of the PCR product and digested pDEST-PL on a 1% (w/v) agarose gel to confirm the concentration.



PAUSE POINT Store the purified PCR product and pDEST-PL vector at 4°C or -20°C until use in the next step.

(x) Prepare the ligation mixture as tabulated below in a 1.5-ml microcentrifuge tube and mix well by briefly vortexing or tapping. We usually use DNA Ligation Kit Ver.2.1, but other ligases can also be used.

| Reagent | Amount per 10 µl reaction | Final concentration/amount |
|---|---|---|
| Digested pDEST-PL | 50 ng | 50 ng |
| Digested PCR product | 50 ~ 150 ng | 50 ~ 150 ng |
| DDW | To 5 µl | |
| Solution I (DNA Ligation Kit) | 5 µl | 1 x |

(xi) Incubate the ligation mixture at 16°C for 1 hour or more.

(xii) Transform *E. coli* strain DB3.1 or *ccd*B Survival with 1 ~ 3 µl of the ligation mixture by a standard method described in the manufacturer's instruction. Plate transformed cells on LB/carbenicillin plates and grow at 37°C overnight to select for carbenicillin-resistant clones.

CRITICAL STEP *E. coli* strains with F' episome must be used for transformation to select Destination clones. These strains contain the *ccd*A gene and will survive in the presence of the *ccd*B gene.

(xiii) Test resistance of the carbenicillin-resistant colonies to chloramphenicol by streaking the bacteria onto LB/carbenicillin+chloramphenicol plates. Optionally, check insertion of the promoter fragment by performing colony PCR with GoTaq Green Master Mix or EmeraldAmp PCR Master Mix following manufacturers' instruction when the insert is >3 kb and probability of carrying the insert is low.

PAUSE POINT The master plates can be stored at 4°C for up to 1 month until use in the next step.

(xiv) Set up separate 2-ml LB/carbenicillin+chloramphenicol liquid cultures for approximately 6 individual clones resistant to both carbenicillin and chloramphenicol from step 18B(xiii). Grow by rocking vigorously at 37°C for 16 hrs.

(xv) Mini-prep the plasmid DNAs and check the identity of the plasmids (See steps 14 ~ 16).

**Constructing Expression clones. TIMING 1~2 weeks**

**19|** Prepare the LR reaction mixture as tabulated below in a 1.5-ml microcentrifuge tube and mix well by briefly vortexing or tapping.

| Reagent | Amount per 5 µl reaction | Final concentration/amount |
|---|---|---|
| Destination vector | 75 ng | 75 ng |
| Genomic fragment cassette in pENTR-L1-R5 | 15 ~ 100 ng | 15 ~ 100 ng |
| Fluorescent protein cassette in pENTR-L5-L2 | 15 ~ 100 ng | 15 ~ 100 ng |
| DDW | To 4 µl | |
| LR Clonase II Plus enzyme mix | 1 µl | |

**20|** Incubate the LR reaction mixture at 25°C or at room temperature overnight. Although instructed in the manufacturers' manual of LR Clonase II Plus, we usually omit proteinase K digestion because we did not find any difference in the number of colonies in step 21 if proteinase K digestion is omitted.

**21|** Transform *E. coli* strain DH5α or others with 1 ~ 3 µl of the reaction mixture by a standard method [48]. Plate transformed cells on LB/carbenicillin plates and grow at 37°C overnight to select for carbenicillin-resistant clones.

CRITICAL STEP *E. coli* strains with F' episome cannot be used to select Expression clones. These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene in the Destination vectors.

TROUBLESHOOTING

**22|** Test resistance of 6~8 carbenicillin-resistant colonies from step 21 to chloramphenicol, kanamycin and carbenicillin (BOX 1), by re-streaking the bacteria on 3 different LB agar plates, each containing one of the antibiotics, to select for carbenicillin-resistant and chloramphenicol- and kanamycin-sensitive clones.

CRITICAL STEP The Expression clones should have ampicillin-resistance gene and should not have either chloramphenicol- or kanamycin-resistance gene as schematically shown in Figure 2A. From experience, some carbenicillin-resistant clones from step 21 are also resistant to chloramphenicol and/or kanamycin and contain aberrant plasmids, which can be excluded in this step.

PAUSE POINT Store the LB/carbenicillin master plates at 4°C for upto 1 month until use in the next step.

**23|** Set up separate 2-ml LB/carbenicillin liquid cultures for 2~3 individual carbenicillin-resistant clones from step 22. Grow by rocking vigorously at 37°C for 16 hrs.

**24|** Mini-prep the plasmid DNAs and check the identity and *attB1*, *attB2* and *attB5* sequences of the plasmids (See steps 14 ~ 16).

**Generation of transgenic reporter worms by a standard microinjection. TIMING 3~4 weeks**

**25|** Mix a transformation marker DNA, *lin-15 (+)*, and equi-amount of a pair of reporter mini-genes at a total concentration of 100 ng/μl in 1x injection buffer. We prepare 100 ng/μl solution for each plasmid DNA and mix them in a given ratio.

**26|** Filter the injection mixture with a centrifugal filter device. We use Ultrafree-MC Durapore PVDF 0.22 μm.

PAUSE POINT The injection mixture can be stored at 4°C or at -20°C until use in the next step.

**27|** Inject twenty to thirty *lin-15 (n765ts)* adult hermaphrodites grown at 15°C with the injection mixture.

**28|** Recover the injected (P0) worms on an NGM plate at 15°C overnight.

**29|** Transfer the P0 worms to a fresh NGM plate every half day and leave them to lay eggs at 20°C. Culture F1 worms at 20°C.

**30|** Isolate fluorescent or non-multi-vulva (Non-Muv) F1 worms and culture them further at 20°C. As temperature-sensitive (ts) multi-vulva (Muv) phenotype of the *lin-15 (n765ts)* mutant is fully penetrated, rescue of the Muv phenotype or expression of the fluorescent proteins in the F1 generation indicates that the worms carry the injected constructs.

**31|** Establish transgenic lines that express fluorescent proteins or remain Non-Muv at 20°C for at least 3~5 generations.

PAUSE POINT Collect the transgenic worms with M9 buffer and freeze in 1x worm freezing solution at -80°C to be stored for years.

TROUBLESHOOTING

**Checking splicing patterns of mini-gene-derived mRNAs. TIMING 2~3 weeks**

**32|** Harvest well-fed worms of mixed stage with M9 and wash 2~3 times with M9 in a 1.5-ml tube to remove bacteria.

**33|** Freeze 30~100 μl of the worm suspension in liquid nitrogen and grind them to powder with a pestle and a mortar. Occasionally add liquid nitrogen to the mortar to keep the worms frozen if necessary.

**34|** Immediately add 600 μl RLT buffer (from RNeasy Mini kit) supplemented with 1% (v/v) 2-mercaptoethanol to the frozen powder of worms and grind the frozen reagent to powder and continuously mix until thawed.

**35|** Optionally, shear the suspension with QIAshredder Spin Columns. This step reduces viscosity of the lysate.

**36|** Transfer the homogenate to 1.5-ml microtube and rock vigorously on a mixer for 5 min at room temperature.



PAUSE POINT The worm homogenate can be stored at -80°C until use in the next step.

**37|** Extract total RNA from the worms by utilizing RNeasy Mini and DNase following manufacturer's instructions.

**38|** Check the concentration of extracted total RNA with NanoVue or an equivalent spectrophotometer.

PAUSE POINT Total RNA can be stored at -80°C until use in the next step.

**39|** Verify the quality and quantity of total RNA by running 1~2 μg of total RNA on a 1% (w/v) agarose gel after heat denature at 70°C for 5 min.

CRITICAL STEP Make sure that 28S and 18S ribosomal RNAs are extracted without degradation.

**40|** Perform RT. We usually use 1 ~ 2 μg of the total RNA for reverse transcription with PrimeScript II or Superscript II and oligo(dT) as a primer following manufacturers' instructions.

PAUSE POINT The RT product can be stored at -20°C until use in the next step.

**41|** Perform PCR with a reaction mixture tabulated below. We usually use non-proofreading polymerases such as Ex Taq and BIOTAQ.

| Reagent | Amount per 20 μl reaction | Final concentration/amount |
|---|---|---|
| RT reaction mixture | 1 μl | |
| 10 x Ex Taq buffer (Mg plus) | 2 μl | 1x |
| 2.5 mM each dNTP mixture | 1.6 μl | 0.2 mM each |
| 2.5 μM forward primer | 2 μl | 0.25 μM |
| 2.5 μM reverse primer | 2 μl | 0.25 μM |
| Ex Taq (5 u/μl) | 0.2 μl | 0.05 u/μl |
| DDW | 11.2 μl | |

Standard condition is described below. As the transgenic worms carry hundreds of copies of the reporter mini-genes and are expected to express high level of mini-gene-derived mRNAs, 18 to 22 cycles of the PCR are enough in most cases.

| Cycle | Denature | Anneal | Extend |
|---|---|---|---|
| 1 | 94°C, 3 min | | |
| 2 - 19 to 23 | 94°C, 30 sec | 55°C, 45 sec | 72°C, 1 min/kb |
| final | | | 72°C, 5 min |

We use mini-gene-specific forward and reverse primers (see Table below) to specifically amplify the mini-gene derived mRNAs.

| Primer | Sequence | Use |
|---|---|---|
| *att*B1adapterF | see Table 3. | mini-gene-specific forward |
| GSP-*att*B1F | see Table 3. | mini-gene-specific forward |
| EGFP#2 | 5′-TGTGGCCGTTTACGTCG-3′ | EGFP/Venus-specific reverse |
| EGFP#60-*att*B2R | 5′-AGAAAGCTGGGTTTTACTTGTACAGCTCGT-3′ | EGFP/Venus-specific reverse |
| mRFPseqR | 5′-GGAGCCGTACTGGAACTGAG-3′ | mRFP1-specific reverse |
| mRFP#2-*att*B2R | 5′-AGAAAGCTGGGTCTTAGGCGCCGGTGGAGT-3′ | mRFP1-specific reverse |

**42|** Verify amplification and patterns by running a 1/5 ~ 1/2 aliquot of the RT-PCR product on a 1% (w/v) agarose gel.

**43|** To analyze RT-PCR products, directly sequence the purified products, or clone the products in TA-vectors such as pGEM-T Easy and

sequence several clones.

**Observation and imaging of cell-type-specific alternative splicing patterns. TIMING 2 days**

**44|** Paralyze worms on an NGM plate with a drop of 10 mM sodium azide. Observe expression patterns and acquire images of the fluorescent proteins in the living transgenic worms with a high-magnification dissection microscope equipped with an epifluorescence system (see Equipment setup).

**45|** Optionally, observe expression patterns of the fluorescent proteins in single cells with a laser scanning confocal microscope equipped with DIC optics. High magnification view with the laser scanning microscopy will be necessary in order to look into small tissues or to identify cells when analyzing the reporter expression profiles in detail or when the fluorescent reporter proteins are unexpectedly localized to subcellular domains.

**Integration of extrachromosomal arrays by UV irradiation. TIMING 3~4 weeks**

**46|** Culture an extrachromosomal line on an enough number of 90-mm NGM plates for >500 well-fed fluorescent young adult worms. Synchronization is not necessary but may be convenient to prepare that many well-fed fluorescent young adult worms.

**47|** Directly irradiate all the worms on the culture plates with 300 J/m$^2$ (= 300 x 100 μJ/cm$^2$) UV light with the lid open. We use an ultraviolet crosslinker that can preset and monitor the energy of UV to be irradiated.

**48|** Recover worms at 15°C with enough food for 3~6 hours.

**49|** Pool P0 adult hermaphrodites on fresh 90-mm NGM plates. We put ten active fluorescent young adult hermaphrodites per plate and prepare 10~20 plates. Culture the P0 worms at 20°C for 2~3 days.

**50|** Isolate 100~200 fluorescent F1 worms on the plates from step 49 individually on separate 35-mm NGM plates at late larval stages. Culture the F1 worms at 20°C for 2~3 days.

CRITICAL STEP The F1 worms are heterozygote candidates and should express the fluorescent reporter throughout the body in a promoter-dependent pattern.

**51|** Screen for F1 plates with >75% fully fluorescent F2 worms by utilizing a fluorescence stereoscope.

**52|** Isolate three to five fully fluorescent F2 worms individually on separate 35-mm NGM plates from each of the F1 plates selected in step 51. Culture the F2 worms at 20°C for 3~4 days.

**53|** Screen for F2 plates with 100% fully fluorescent progeny by utilizing a fluorescence stereoscope. Lines derived from the same F1 plates should be considered to contain the same integrant alleles.

TROUBLESHOOTING

**EMS mutagenesis and screening. TIMING 3~4 weeks**

**54|** Culture a parental strain on an enough number of 90-mm NGM plates for thousands of well-fed young adult worms. Synchronization is useful for preparing a large number of young adult worms at a time.

**55|** Harvest the well-fed worms with M9 and wash 2~3 times with M9 in a 1.5-ml tube to remove bacteria and young larvae. Resuspend the worms in 1 ml of M9.

**56|** Take 3 ml M9 in a 15~20-ml glass tube. Add 20 μl of ethyl methanesulfonate (EMS) (final 47 mM) and mix by swirling.

CAUTION EMS is carcinogenic. Wear protective clothing and gloves. Avoid breathing vapors, mist or gas. Prepare EMS solutions in a



fume hood and maintain in fume hood while treating worms. All EMS-treated wares should be discarded after inactivation with >2 N NaOH for >24 hrs in the fume hood.

CRITICAL STEP EMS treatment should be in a glass tube. EMS-treated worms stick to plastic tubes and pipettes, and would be seriously damaged or lost.

**57|** Add 1 ml of worm suspension prepared in step 55. Mix by swirling.

**58|** Incubate at room temperature for 4 hours with occasional swirling.

**59|** Wash the worms 3 times with M9.

CAUTION All EMS-containing reagents should be discarded after inactivation with >2 N NaOH for >24 hrs in fume hood.

**60|** Transfer worms to fresh 90-mm NGM plates with a glass pipette.

CRITICAL STEP Use a glass pipette. EMS-treated worms stick to plastic pipettes, and would be seriously damaged or lost.

**61|** Recover worms at 15°C for 6~12 hours.

**62|** Pool either P0 adult hermaphrodites (option A) or F1 embryos (option B) on fresh 90-mm NGM plates and culture at 15°C or 20°C for 4~8 days until a large number F2 worms grow. The more worms are pooled in a plate, the earlier the plate is starved and the F2 worms do not grow to adult. Therefore, the number of worms or embryos per plate and the number of plates to be prepared should be optimized and determined for each parent strain according to the expected phenotype of the mutant worms.

(A) Pooling P0 adult worms.

Pick up and move about 10 gravid P0 adult hermaphrodites per 90-mm NGM plate from the recovery plates in step 61. Prepare 50~100 plates and incubate at 15°C or 20°C until the plates are filled with tens of thousands of F2 worms. Note that the F1 and F2 worms are not synchronized with this method and that the plates will be starved when the most of the F2 worms are at larval stages. This option is recommended when the parent worms are not synchronized in step 54.

(B) Collecting F1 embryos.

Collect gravid adult hermaphrodites from the recovery plates in step 61 with M9 and moved to fresh 90-mm NGM plates. Leave the worms at 20°C to lay eggs for 3~6 hours and remove the P0 adult worms with M9 and move to fresh plates. Repeat this step to prepare required number of plates and incubate at 15°C or 20°C until the F2 worms grow. Optionally, remove F1 adult worms after most of them lay tens of eggs by washing out with M9, if the F2 worms should be synchronized or grown to adult for screening.

**63|** Screen the F2 progeny for worms with altered expression profiles of the fluorescent proteins under a fluorescence stereoscope equipped with an appropriate dual band-pass filter set (See Equipment Setup).

TROUBLESHOOTING

**SNP mapping. TIMING 2~4 months**

**64|** Cross mutant hermaphrodites with males of Hawaiian wild-type strain, CB4856. We usually cross five L4 mutant hermaphrodites with more than ten CB4856 males on a spot of 10-μl OP50 liquid culture on a 60-mm NGM plate at 20°C overnight.

**65|** Isolate the parental mutant hermaphrodites on individual fresh 60-mm NGM plates. Culture at 20°C for 2~3 days.

**66|** Pool F1 L4 hermaphrodites from the plates in step 65 on fresh 90-mm NGM plates. We put ten F1 worms per plate and prepare as many plates as possible. Culture at 15°C or 20°C for 2 days. Remove F1 hermaphrodites for step 67 and culture for another 2~4 days.

CRITICAL STEP Pool only crossed F1 hermaphrodites at L4 stage. Adult F1 hermaphrodites may be crossed with F1 males and this will