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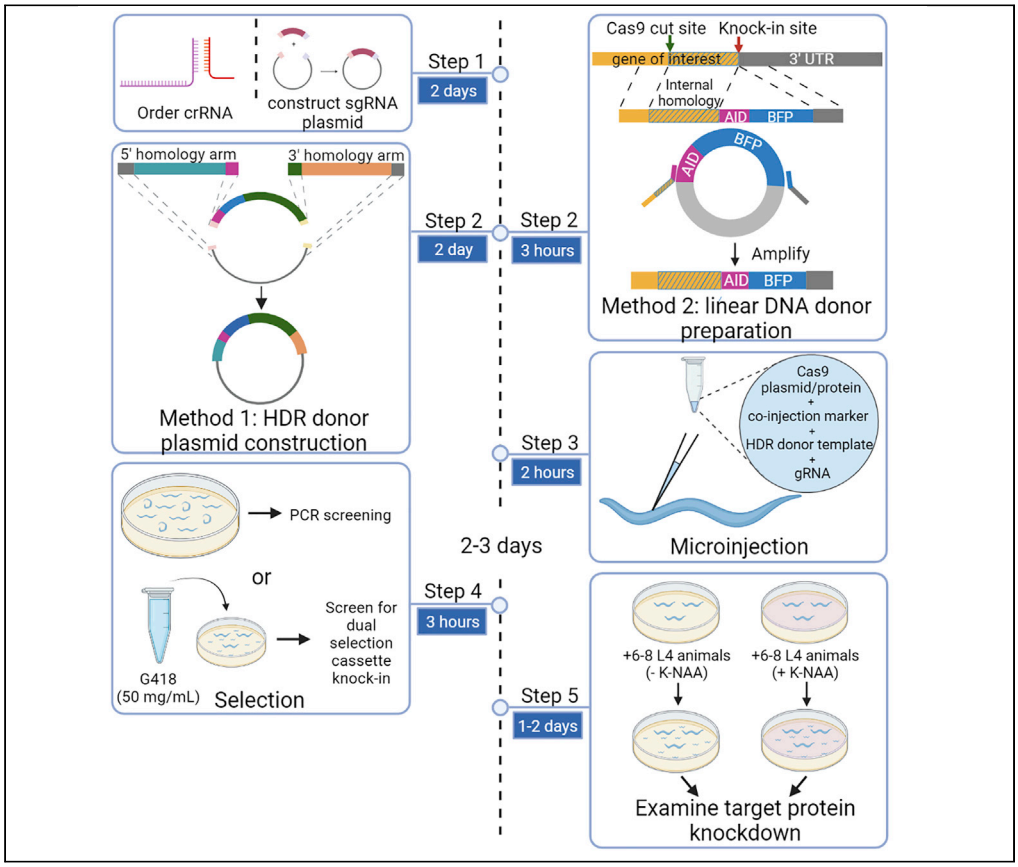
Targeting endogenous proteins for spatial and temporal knockdown using auxin-inducible degron in *Caenorhabditis elegans*



The auxin-inducible degron (AID) provides reversible, spatiotemporal control for the knockdown of target proteins. Here, we present a protocol for AID-mediated protein knockdown in *Caenorhabditis elegans*. We describe steps for generating the knock-in mutants using two CRISPR-Cas9 genome editing techniques and preparing the auxin-containing nematode growth media (NGM) plates. We also detail AID-mediated spatiotemporal protein knockdown.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Mizuki Kurashina, Kota Mizumoto

mizumoto@zoology.ubc.ca

Highlights

Protocol for auxin inducible degron (AID) tagging in *C. elegans*

Steps for generating knock-in mutants with two CRISPR-Cas9 approaches

Spatiotemporal degradation of AID-tagged proteins

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Targeting endogenous proteins for spatial and temporal knockdown using auxin-inducible degron in *Caenorhabditis elegans*

Mizuki Kurashina[1,2,3] and Kota Mizumoto[1,2,3,4,5,6,*]

[1]Department of Zoology, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

[2]Graduate Program in Cell & Developmental Biology, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

[3]The Life Sciences Institute, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

[4]Djavad Mowafaghian Centre for Brain Health, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

[5]Technical contact

[6]Lead contact

*Correspondence: mizumoto@zoology.ubc.ca
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SUMMARY

The auxin-inducible degron (AID) provides reversible, spatiotemporal control for the knockdown of target proteins. Here, we present a protocol for AID-mediated protein knockdown in *Caenorhabditis elegans*. We describe steps for generating the knock-in mutants using two CRISPR-Cas9 genome editing techniques and preparing the auxin-containing nematode growth media (NGM) plates. We also detail AID-mediated spatiotemporal protein knockdown.

For complete details on the use and execution of this protocol, please refer to Kurashina et al. (2021).[1]

BEFORE YOU BEGIN

The auxin-inducible degron (AID) system enables rapid degradation of endogenous proteins by tagging them with a 44-amino acid degron sequence from *Arabidopsis thaliana* IAA17[2] and has been shown to induce protein degradation effectively in many animal models including *C. elegans*.[3–5] The degradation of AID-tagged proteins requires an *Arabidopsis thaliana* F-box protein, TIR1. TIR1 is the substrate recognition subunit of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, which targets AID-tagged proteins for ubiquitin-mediated degradation only in the presence of the plant growth hormone, auxin (indole-3-acetic acid, or IAA).[6] By expressing TIR1 from a cell- or tissue-specific promoter, we can specify the cell types where an AID-tagged protein undergoes degradation (Figure 1). By treating animals with auxin at specific time points, we can control the timing of protein degradation.

Before generating the AID knock-in strains, it is important to know which region of the protein of your interest AID can be fused to, so that the AID-tagged protein is fully functional. For example, adding AID at the C-terminus of a protein which contains a PDZ-binding domain at its C-terminus may disrupt its protein-protein interaction. Also, if the gene has isoforms with multiple translational initiation and/or termination sites, N-terminal or C-terminal AID tagging to one of the isoforms may leave other isoforms to be untagged with AID. In such cases, inserting the AID tag in a region shared among the isoforms may be required for knocking-down all isoforms. Selecting the appropriate promoter to express TIR1 is also critical for precisely controlling the auxin-inducible protein degradation. Tissue-specificity and expression timing/strength of the promoter will affect the degradation of AID-tagged proteins.

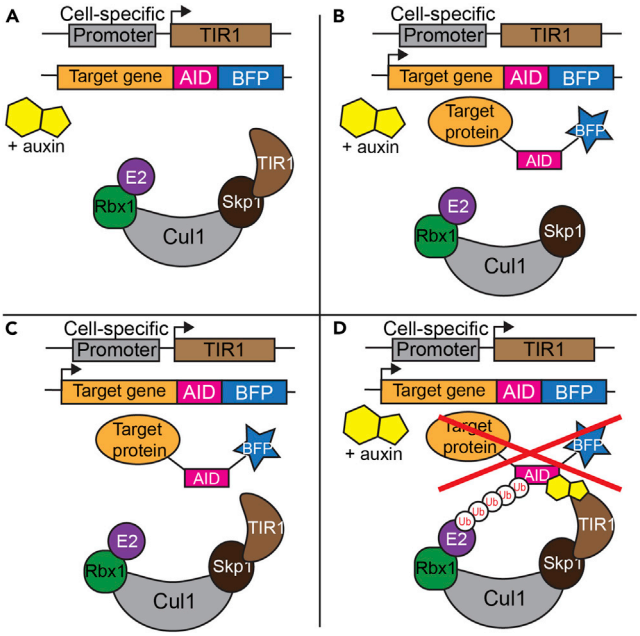

Figure 1. Schematics of auxin-inducible degradation

(A) A cell that expresses *TIR1* but not AID-tagged gene. As the AID-tagged protein is not expressed, auxin-dependent degradation does not occur.

(B) A cell that expresses AID-tagged gene but not *TIR1*. AID-tagged protein does not undergo auxin-dependent degradation as TIR1 protein is not present in this cell.

(C and D) A cell that express *TIR1* and AID-tagged gene. In the absence of auxin, AID-tagged proteins undergo minimal basal degradation (C). This allows for temporal control of auxin-inducible degradation. (D) Upon auxin treatment, AID-tagged proteins will undergo auxin-dependent degradation.

Basic methodologies for *C. elegans*, including worm lysis and microinjection, are available in WormMethods (http://www.wormbook.org/toc_wormmethods.html).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
OP50 *Escherichia coli*	https://cgc.umn.edu/strain/OP50	
DH5-alpha *Escherichia coli* competent cells	Thermo Scientific	EC0111
Chemicals, peptides, and recombinant proteins		
Geneticin (G418)	Sigma-Aldrich	A1720
α-Napthaleneacetic acid (K-NAA)	PhytoTech Lab	N610
Alt-R® S.p. Cas9 Nuclease V3	IDT	1081058
HEPES	Sigma-Aldrich	H3375
KCl	Sigma-Aldrich	P9541
Phusion High-Fidelity DNA Polymerase	NEB	M0530
M9 buffer		
Gibson Assembly Kit (in-house)		
*Bsa*I-HF	NEB	R3733
*Sac*II	NEB	R0157
*Not*I-HF	NEB	R3189
*Srf*I	NEB	R0629

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
T4 DNA ligase	NEB	M0202
rCutSmart Buffer	NEB	B6004S
Critical commercial assays		
GeneJET Gel Extraction Kit	Thermo Fisher	K0691
Nucleospin Plasmid Purification Kit	Macherey-Nagel	741588
Experimental models: Organisms/strains		
C. elegans N2 strain	Caenorhabditis Genetics Center	
Oligonucleotides		
Alt-R® CRISPR-Cas9 tracrRNA, 5 nmol	IDT	1072532
CRISPR-Cas9 crRNA, 2 nmol	IDT	N/A
M13(-41) F primer		
M13 R primer		
Recombinant DNA		
pRB1017 (empty vector for sgRNA)	Arribere et al.[7]	Addgene #59936
pTK73 (empty vector for sgRNA(F+E))	Obinata et al.[8]	
pTK73_oxTi365	Kurashina et al.[1]	Addgene #194052
pTK73_ttTi5605	This study	Addgene #194058
Peft-3::cas9-SV40_NLS::tbb-2 3′UTR	Friedland et al.[9]	Addgene #46168
AID::BFP_loxP_myo2_neoR	Kurashina et al.[1]	Addgene #194054
AID::mTagBFP2_loxP_myo2_neoR	This study	Addgene #194055
oxTi365_loxP_myo2_neoR	Kurashina et al.[1]	Addgene #194057
ttTi5605_loxP_myo2_neoR	This study	Addgene #194056
pDD104 (P*eft-3::Cre*)	Dickinson et al.[10]	Addgene #47551
P*unc-4c::TIR1::unc-54 3′utr*	Kurashina et al.[1]	Addgene #194053
pCFJ90 (P*myo-2::mCherry*)	Frokjaer-Jensen et al.[11]	Addgene #19327
pRF4 (*rol-6(su1006dn)*))	Mello et al.[12]	
Software and algorithms		
C. elegans CRISPR gRNA selection tool*		
Note: This tool is now incorporated into the Wormbase J browser. Select 'CRISPR_Cas9 sgRNA predictions' from the available tracks on the right.		http://genome.sfu.ca/crispr/about.html
Wormbase		https://wormbase.org/tools/genome/jbrowse-simple
IDT gRNA Design Tool		https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system
ApE: A plasmid Editor	Davis and Jorgensen[13]	https://jorgensen.biology.utah.edu/wayned/ape/
Other		
Dissection microscope	Carl Zeiss	Stemi 305
Fluorescence dissection microscope	Carl Zeiss	SteREO Discovery V8
Laser scanning confocal microscope	Carl Zeiss	LSM800
Glass capillaries	World Precision Instruments	1B100F-6
Needle puller	Sutter Instrument	Model P-97
Microinjector	Carl Zeiss	Axiovery 200M
Thermocycler	Bio-Rad	Tetrad 2

MATERIALS AND EQUIPMENT

2× Cas9 buffer	
Reagent	Final concentration
HEPES (pH 7.5)	20 mM
KCl	150 mM

Worm Lysis buffer	
Reagent	Final concentration
Tris-HCl (pH 8.3)	10 mM
KCl	50 mM
$MgCl_2$	2.5 mM
Tween-20	0.45%
NP-40	0.45%
gelatin	0.01%
Proteinase K	60 μg/mL

Thermal cycling steps (Phusion polymerase)			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25 cycles
Annealing	53°C	20 s	
Extension	72°C	30 s/kb	
Final extension	72°C	1 min	1

Gibson Assembly reaction (in-house)		
Steps	Temperature	Time
Incubation	50°C	60 min

Worm Lysis reaction (in-house)		
Steps	Temperature	Time
Incubation	60°C	60 min
Proteinase K inactivation	95°C	15 min

STEP-BY-STEP METHOD DETAILS

Generating AID::BFP or AID::mTagBFP2 knock-in mutants using CRISPR/Cas9 genome editing technology

Timing: 1–5 days

In our original paper,[1] we used a plasmid-based CRISPR/Cas9 system combined with a plasmid containing a dual selection marker cassette as a homology-dependent repair (HDR) donor template (Method 1). According to the recent technological advancements which greatly enhances genome editing efficiency in *C. elegans*,[14–16] we have updated our CRISPR/Cas9 genome editing method to Method 2, which uses a ribonucleoprotein (RNP) complex containing Cas9 protein and gRNA, and PCR-amplified linear DNA as an HDR donor template. In this section, we describe both methods with some benefits and potential limitations of each method (Tables 1 and 2).

Method 1. A plasmid-based AID::BFP/AID::mTagBFP2 knock-in

Timing: 5 days

In this method, we deliver Cas9, single guide RNA (sgRNA), and HDR donor template into the *C. elegans* germ line as plasmids (Figure 2). The plasmid-based Cas9 and sgRNA delivery method is cost-efficient and works reasonably well for many genetic loci in *C. elegans*. However, direct

Table 1. Advantages and disadvantages of dual selection marker plasmid-based knock-in and linear DNA-based knock-in methods

	Dual selection marker plasmid-based knock-in (method 1)	Linear DNA-based knock-in (method 2)
Advantages	- No need for PCR-based screening - Selection marker can be used as a dominant marker	- Easy to prepare - Perfect insertion without additional sequences such as loxP - Higher knock-in efficiency
Disadvantages	- Require multiple steps in HDR donor plasmid construction - Selection cassette may affect gene expression pattern -Leaves a loxP site even after the excision of dual selection cassette	- Requires PCR-based screening - gRNA sequence must be close to the knock-in site - Repair template is prone to PCR-errors

injection of *in vitro* assembled Cas9-gRNA RNP complex increases the editing efficiency compared with the plasmid delivery method,[14] which we describe in Method 2.

As an HDR donor plasmid, we used the dual-marker selection cassette plasmid[17] containing 5′ and 3′ homology arms for HDR, AID::BFP or AID::mTagBFP2, and two selection markers (neomycin resistance (neoR) gene and P*myo-2*::GFP) flanked by two loxP sites. Animals with successful knock-in of the AID::BFP and a selection cassette can be screened for neomycin resistance and pharyngeal GFP expression.[17,18] The dual selection marker allows for the high-throughput screening of the potential knock-in animals without the need for PCR-based screening. The dual selection marker cassette can be excised by injecting a plasmid containing the *Cre* recombinase gene into successful knock-in animals. This dual selection marker system can be combined with direct injection of the Cas9-gRNA RNP complex as described in Au et al.[18]

1. sgRNA plasmid construction (A detailed protocol is described in Arribere et al.)[7]
   a. Find gRNA sequence near the knock-in site using the web-based CRISPR guide RNA selection tools.

   Note: Many design tools provide several parameters: for example, GC content and folding energy are provided in the *C. elegans* CRISPR gRNA selection tool (http://genome.sfu.ca/crispr/about.html). According to Dr. Stephane Flibotte, who developed the database, all gRNAs on this database should be usable (personal communication). From our experience, the length of sequence between the gRNA target site and the knock-in site (closer is better) is critical for efficient knock-in.

   b. Order oligo DNAs containing the 19 nt gRNA sequence (remove one base at the most 5′ end from 20 nt gRNA sequence on design tool databases) with the following overhangs:
   Forward: 5′-TCTTG-NNNNNNNNNNNNNNNNNNN- 3′.
   Reverse: 5′ -AAAC-NNNNNNNNNNNNNNNNNNN-C- 3′ (N denotes the reverse complement of the gRNA sequence).

Table 2. Advantages and disadvantages of plasmid-based gRNA-Cas9 delivery and direct injection of gRNA-Cas9 RNP complex

	Plasmid-based sgRNA-Cas9 delivery (method 1)	Direct injection of gRNA-Cas9 RNP complex (method 2)
Advantages	- Inexpensive - Less sensitive to multiple freeze-thaw cycles	- Easy to prepare - Higher genome editing efficiency
Disadvantages	- Lower genome editing efficiency - Requires cloning of sgRNA plasmid	- Expensive to purchase crRNA, tracrRNA and Cas9 protein - May be susceptible to multiple freeze thaw cycles

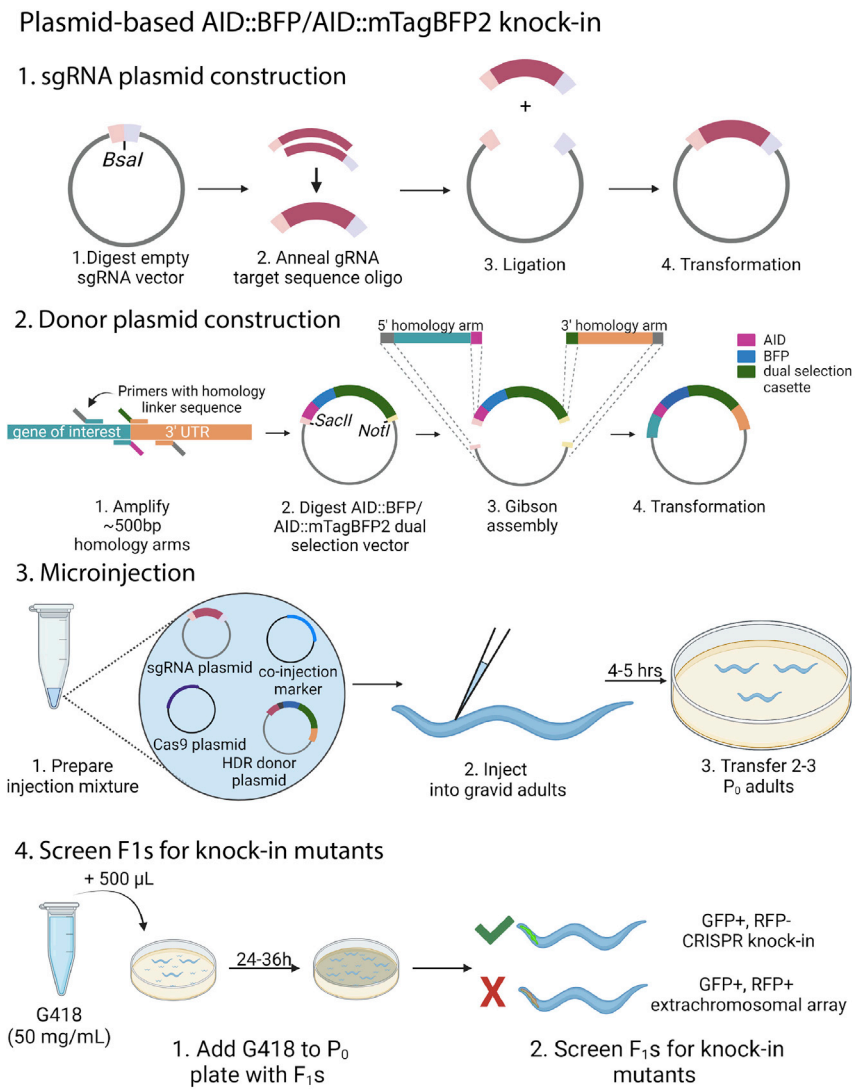

Figure 2. Workflow of Method 1: plasmid-based AID::BFP/AID::mTagBFP2 knock-in

c. Digest the pRB1017[7] or pTK73[8] sgRNA plasmid with *Bsa*I (NEB).
d. Anneal the forward and reverse primers and ligate them into the digested sgRNA plasmids using T4 DNA ligase (NEB).
e. Add ligation mixture to the DH5-alpha Escherichia coli competent cells for transformation.
f. Purify plasmids using plasmid purification kits such as NucleoSpin Plasmid, Mini kit for plasmid DNA.
g. Use Sanger sequencing to confirm the plasmid sequence using the M13 Forward primer.

2. HDR donor plasmid construction.
a. Using high-fidelity DNA polymerases and N2 genomic DNA as a template, amplify approximately 500 bp of 5′ and 3′ homology arms flanking the knock-in site using specific primers with the following linker sequences for Gibson assembly.
5′ homology arm forward: 5′- AAAACGACGGCCAGTGAATTCCCGCGG-(20–25 nt gene-specific primer sequence)- 3′.
5′ homology arm reverse: 5′- GGGCTCCGGCTCCGGCTCCGGCTCC-(20–25 nt gene-specific primer sequence)- 3′.

3′ homology arm forward: 5′- ACGAAGTTATAGTTGCAGGACCACT -(20–25 nt gene-specific primer sequence)- 3′.
3′ homology arm reverse: 5′- CCATGATTACGCCAAGCTTGCGGCCGC -(20–25 nt gene-specific primer sequence)- 3′.

b. Purify the PCR product using a gel purification kit.

⚠ CRITICAL: Make sure to remove the stop codon of the target gene in the 5′ reverse primer such that the coding sequence of AID::BFP/AID::mTagBFP2 is in frame with the target gene.

c. Digest the AID::BFP/AID::mTagBFP2 plasmid with *Sac*II and *Not*I according to the manufacturer's protocol.
d. Using the Gibson assembly method,[19] clone the gel-purified 5′ and 3′ homology arm fragments into the *Sac*II and *Not*I sites of the digested plasmid, respectively.
e. Add the Gibson assembly mixture to the DH5-alpha Escherichia coli competent cells for transformation.
f. Purify the plasmid using plasmid purification kits such as NucleoSpin Plasmid Mini kit for plasmid DNA.
g. Using Sanger sequencing, confirm the sequence of the homology arms using the M13(-41) Forward (5′-GGTTTTCCCAGTCACGAC-3′) and the M13 reverse (5′-GGAAACAGCTATGAC CATG-3′) primers.

Note: Do not use the M13(-21) primer for sequencing as the dual selection marker cassette plasmids possess two M13(-21) sites, which cause mixed sequencing results.

3. Microinjection.
   a. Prepare a 10 μL microinjection mixture with the final concentration of each component as follows:

HDR donor plasmid	50 ng/μL.
sgRNA plasmid	50 ng/μL.
Peft-3::cas9-SV40_NLS::tbb-2 3′UTR Cas9 plasmid	50 ng/μL.
P*myo-2*::mCherry (co-injection marker)	5 ng/μL.

Note: Co-injection marker can be any dominant marker such as pRF4 (*rol-6*) except for P*myo-2::GFP* which is included in the dual selection marker cassette plasmid.

   b. Centrifuge the microinjection mixture at maximum speed (~14000 G) for 10 min.

Note: We recommend centrifuging the original plasmid solutions (~14000 G, 10 min) before preparing the microinjection mixture, to minimize the contamination of resins and small debris, which can clog the needle during microinjection.

   c. Inject the mixture into the gonads of gravid adults (P_0s).[12]

Note: The number of animals you need to inject varies depending on several factors including the microinjection skill of the person who conducts the experiment. We usually inject 20–30 animals (40–50 gonad arms).

   d. Transfer the injected animals to the 60 mm NGM plate.
   e. Wait 4–5 h for the injected P_0 animals to lay the eggs that they already had at the time of microinjection.
   f. Transfer injected P_0 animals to the 60 mm NGM plates with OP50 (P_0 plates: 2–3 P_0s / plate).
   g. Culture injected P_0 animals for 24–36 h at room temperature (22°C).

4. Screening F1s for knock-in mutants.
   a. Prepare a Geneticin (G418) working solution by diluting the stock solution (500 mg/mL in dH_2O) with M9 buffer to a final concentration of 50 mg/mL. The Geneticin stock solution can be stored at −20°C.
   b. Add 500 μL of the G418 working solution to the P_0 plates with many F_1 larvae.
   c. Culture P_0 for 2–3 days at room temperature (22°C).

   ⚠ CRITICAL: Animals that survive the G418 treatment are either 1) proper knock-ins, 2) imperfect knock-ins (such as random insertion of the selection marker), or 3) animals carrying injected donor plasmid DNA as an extrachromosomal array. The following step will exclude animals with extrachromosomal arrays.

   d. Under a fluorescence stereoscope, look for the F_2-F_3 animals that do not express pharyngeal mCherry, but express dim and uniform GFP in the pharynx.

   Note: These are the candidate knock-in animals. Animals that express mCherry and strong GFP carry multiple copies of P*myo-2*::mCherry and P*myo-2*::GFP sequences as an extrachromosomal array.

   Note: You may see some non-GFP animals surviving in the G418-treated P_0 plates. This is because G418 is less effective on L4 larvae and adults.

   e. Single candidate animals to the NGM plates and check for the Mendelian segregation of GFP+ animals in the progeny.

   Note: 100% of the progeny express GFP if the singled candidate animal is homozygous for knock-in, and 75% for heterozygous candidates.

   f. Confirm the perfect knock-in of AID::BFP/AID::mTagBFP2 by sequencing and the BFP/mTagBFP2 expression/localization.

   Note: More than one independent knock-in event may occur in each P_0 plate. However, with this screening method, we cannot distinguish if two knock-in animals obtained from each P_0 plate are derived from a single knock-in from one F_1 animal or two independent knock-ins from two F_1 animals. For this reason, we obtain a maximum of one independent knock-in line per P_0 plate, and the number of P_0 plates sets the maximum independent knock-in lines that could be obtained from each microinjection experiment.

5. Excision of the dual selection marker cassette.
   a. Prepare a 10 μL microinjection mixture with the final concentration of each component as follows:
   P*eft-3*::*Cre* plasmid (pDD104: Addgene #47551) 50 ng/μL.
   pRF4 (*rol-6(su1006dn)*) co-injection marker 50 ng/μL.

   Note: All F_1s from the successfully injected P_0s are the potential candidates for Cre-dependent excision event. As the co-injection marker is for checking the microinjection quality only, any co-injection markers other than P*myo-2*::*GFP* (such as P*myo-2*::*mCherry*) would work.

   b. Centrifuge the injection mixture at the maximum speed (~14000 G) for 10 min.
   c. Inject the mixture into the gonads of homozygous knock-in animals.
   d. Single injected P_0 animals to the new NGM plate, and culture them for 3 days.
   e. Screen F_2 animals for loss of pharyngeal GFP.

f. Single animals without GFP to the new NGM plates.
g. Confirm the excision by sequencing.

Note: While the dual selection marker cassette can be left at the knock-in site and used as a dominant marker for subsequent genetic crosses, we recommend excising the selection cassette, as the insertion of a 5.3kb dual selection marker cassette in between the coding region and the 3′ untranslated region may cause unwanted changes in gene expression and/or mRNA stability in certain cases.

Method 2. RNP and linear donor DNA-based CRISPR/Cas9 mediated genome editing

Timing: 1 day

In the second method of CRISPR/Cas9 mediated knock-in, we use the Cas9 protein and gRNA to assemble an RNP complex *in vitro*, and PCR-amplified linear DNA as an HDR donor template (Figure 3). While the direct injection of the RNP complex greatly increases the efficiency of genome editing in *C. elegans*,[14] this method is more expensive as you need to purchase/synthesize crRNA, tracrRNA and Cas9 protein.

For the linear DNA-based CRISPR/Cas9 method, we use a PCR-amplified linear HDR donor template with short (35–70 nt) homology arms. Compared with the dual selection marker cassette donor plasmid used in Method 1, the preparation of the HDR donor template is significantly simpler. Furthermore, in our experience, we have obtained multiple knock-in mutants from 3–10 injected P_0s, due to the high editing efficiency of the direct injection of RNP complex, and increased HDR efficiency by 'melting' the donor DNA.[16] A detailed protocol for 'melting' the donor DNA is also available in Ghanta et al.[20]

Note: To amplify the linear HDR donor template with homology arms, we need primers with the homology arm sequence and the DNA sequence between the gRNA target site and knock-in site ('internal homology sequence' see Figure 3 - step 2). Therefore, you may be constrained by the maximum length of the oligo DNA sequence you can order. We suggest using a gRNA targeting sequence that is as close to the insertion site as possible (within 30 nt).

6. CRISPR RNA (crRNA) design.
   a. Select a guide RNA target sequence that is close to the knock-in site using guide RNA selection tools.

   ⚠ CRITICAL: When selecting the guide RNA target sequence, make sure to choose a sequence that is close to the site of insertion.

   b. Order/synthesize the crRNA.
7. Ribonucleoprotein (RNP) complex assembly.
   a. Dissolve the crRNA in nuclease-free water to a working concentration of 40 ng/μL.
   b. Dissolve the tracrRNA in nuclease-free water to a working concentration of 40 ng/μL.
   c. Mix 1 μL of 40 ng/μL crRNA and 1 μL of 40 ng/μL tracrRNA and anneal them using the thermal cycler with the following program.
      95°C 30 s.
      90°C 30 s.
      85°C 30 s.
      (5°C decrement).
      35°C 30 s.
      30°C 30 s.

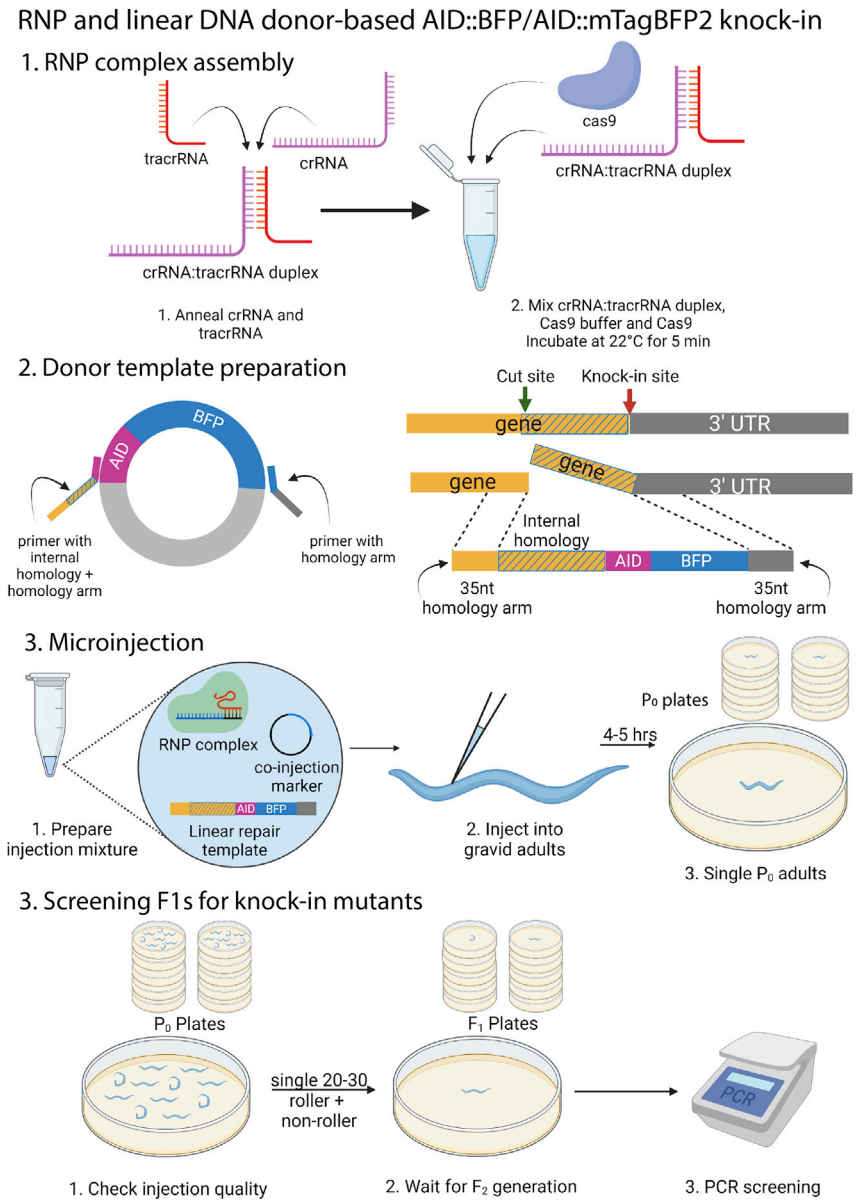

Figure 3. Workflow of Method 2: RNP and linear DNA donor based AID::BFP/AID::mTagBFP2 knock-in

d. Mix 0.5 μL of 61 μM Cas9, 1 μL crRNA:tracrRNA and 2.5 μL of Cas9 Buffer.
e. Incubate the mixture at room temperature (22°C) for 5 min for the RNP complex assembly.

Note: The RNP complex may be kept at −20°C for up to 1 year.

8. HDR donor template preparation.
   a. Using high fidelity DNA polymerases and AID::BFP/AID::mTagBFP2 plasmids as templates, amplify an HDR donor template containing either AID::BFP or AID::mTagBFP2 sequence with a minimum of 35 nt of 3′ and 5′ homology arms. Use the following primer sequences to amplify the HDR template:
   Forward: 5′-(35–70 nt 5′ homology arm)-GGAGCAGGTGCCCCTAAAGATCCAG.

Reverse (BFP)*: 5 '-(35–70 nt 3' homology arm)- TTAATTAAGCTTGTGACCCAGTTTGCT.
Reverse (mTagBFP2)*: 5'-(35–70 nt 3' homology arm)- TTAGTTGAGCTTGTGTCCGAGC TTGG.

Note: underlined sequences correspond to the stop codons of BFP and mTagBFP2, which should be removed if AID::BFP/AID::mTagBFP2 is inserted internally or at the N-terminus of the protein of interest.

⚠ CRITICAL: Make sure to remove the stop codon of the target gene in the BFP reverse or mTagBFP reverse primer such that the coding sequence of AID::BFP/AID::mTagBFP2 is in-frame with that of the target gene.

⚠ CRITICAL: Depending on the location of the gRNA target site, you will need to include the sequence between the Cas9 cut site and the AID knock-in site ('internal homology sequence' See Figure 3 – step 2). For details, see Ghanta et al.[20]

Note: To reduce the risk of mutations caused by polymerase errors, we recommend using fewer PCR cycle numbers (~15 cycles).

b. 'Melt' the gel-purified HDR donor template according to Ghanta et al.[20]

Note: We use the same program as crRNA/tracrRNA duplex formation (step 7c) for 'melting' the donor template.

Note: We recommend centrifuging the HDR donor template (14000 G, 10 min) before microinjection mixture preparation to minimize the contamination of resins and small debris, which can clog the needle during microinjection.

9. Microinjection.
   a. Prepare a 10 μL microinjection mixture with the final concentration of each component as follows:

Component	Volume	Final concentration
'Melted' HDR donor template DNA	5 μL	3.5–5 ng/μL
Cas9-gRNA RNP complex	1 μL	
pRF4 *rol-6(su1006dn)* co-injection marker	x μL	50 ng/μL

Note: According to Ghanta et al.,[20] the microinjection mixture can be stored at 4°C at least up to 4 weeks in our hand.

   b. Centrifuge the microinjection mixture (~14000 G) for 10 min.
   c. Inject the mixture into the gonads of 5 gravid adult animals (P_0s). Make sure to inject into both anterior and posterior gonad arms.
   d. Transfer injected P_0 animals to the NGM plate.
   e. Wait 4–5 h for the P_0 animals to lay eggs that they already had at the time of injection.
   f. Transfer single P0 animals onto new NGM plates.
   g. Culture them for 48–72 h.

10. Screening F_1s for knock-in mutants.
    a. Check each plate for the presence of roller F_1 animals to check the microinjection quality.

b. From each P_0, transfer single 20–30 young roller and non-roller F_1s that are younger than their roller siblings onto NGM plates (F_1 plates).

Note: Ghanta and Mello[16] reported that the likelihood of CRISPR/Cas9 genome editing occurs later in the brood, after the *rol-6(su1006dn)* extrachromosomal array-containing cohort.

c. Culture them for 3–5 days.
d. Lyse ~20 worms from each F_1 plate using the worm lysis buffer.
e. Using the gene-specific forward primer that bind to the sequence upstream of the 5 'homology arm of the HDR donor template, and the BFP or mTagBFP2 reverse primer below, screen F_1 plates for AID::BFP/AID::mTagBFP2 knock-in.
BFP reverse primer: 5 '-GTCCACGGTTCCTTCCATG-3 '.
mTagBFP2 reverse primer: 5 '-TGTGCATGTTCTCCTTGATGAGC-3 '.
f. Confirm the knock-in of AID::BFP/AID::mTagBFP2 via sequencing and examining the BFP/mTagBFP2 expression and localization.

Single copy insertion of *TIR1* transgene using CRISPR/Cas9 genome editing technology

Timing: 2–5 days

Using either of the CRISPR/Cas9 genome editing methods described above, you could insert a single copy of *TIR1* from *Arabidopsis thaliana* with a tissue-specific promoter into one of the universal Mos1-mediated single-copy insertion (MosSCI) sites. TIR1 appears to be toxic to worms when overexpressed from the extrachromosomal array (unpublished observation). Here we describe the protocol to knock-in *(Promoter of interest)::TIR1::unc-54 3′UTR* transgene into the oxTi365 MosSCI site using the plasmid-based CRISPR/Cas9 protocol (Method 1). The oxTi365 MosSCI site is located on chromosome V at position 8,643,066.[8,11] The gRNA target sequence in the sgRNA plasmid (pTK73_oxTi365) is 5′-CGGTCGCGGTACTCCAAATAGG-3′ (the underline shows the PAM sequence). Several *TIR1* single-copy knock-in strains are available from the Caenorhabditis Genetics Center (CGC). There is also another method for generating *TIR1* single-copy knock-in strains.[21]

Note: If the function of the protein of interest is unknown, a single copy *TIR1* transgene with a ubiquitous promoter may be used. For example, you can simply cross your AID-containing strain into the CA1200 strain containing *ieSi57* [P*eft-3::TIR1::mRuby::unc-54 3′UTR + Cbr-unc-119(+)]* II.[3]

11. HDR donor plasmid construction (Figure 4).
    a. Construct *TIR1* transgene using the pSM vector plasmid which is derived from the Fire lab vector plasmid pPD49.26 (Addgene #1686).
        i. Digest the *TIR1* containing pSM plasmid (P*unc-4c::TIR1::unc-54 3′ UTR*) with *Sph*I and *Asc*I restriction enzymes.
        ii. Using high-fidelity DNA polymerases and N2 genomic DNA as a template, amplify your promoter of interest using specific primers with the *Sph*I and *Asc*I sequences (underlined).
        Forward: 5′- GGGGCATGC-(20–25 nt promoter-specific primer sequence)- 3′.
        Reverse: 5′- GGGGGCGCGCC-(20–25 nt promoter-specific primer sequence)- 3′.
        iii. Purify the PCR product using a gel purification kit.
        iv. Digest the PCR product with *Sph*I and *Asc*I for 16 hours.
        v. Gel purify the digested PCR product according to the manufacture's protocol.
        vi. Ligate the purified PCR product with the *TIR1* containing pSM plasmid digested with *Sph*I and *Asc*I from step i using T4 ligase according to the manufacture's protocol.
        vii. Add ligation mixture to the DH5-alpha Escherichia coli competent cells for transformation.

viii. Purify plasmids using plasmid purification kits such as NucleoSpin Plasmid Mini kit for plasmid DNA.

b. Using a high-fidelity PCR polymerase and the *TIR1* plasmid as a template, amplify *(Promoter of interest)::TIR1::unc-54 3′utr* using the following primers:
oxTi365 forward: CGCGGTACTCCAAATGCCC**ATGACCATGATTACGCCAAG**.
oxTi365 reverse: TTATAGGCCGCCTGATGCCC**AAACGCGCGAGACGAAAGGG**.
*Bold letters indicate the primer sequences that bind to the backbone sequences of the pSM vector plasmid located upstream of the promoter (forward) and downstream of the *unc-54* 3′UTR (reverse). Underlined sequences are the adapter sequences for Gibson assembly.

Note: To reduce the risk of mutations caused by polymerase errors, we recommend using fewer PCR cycle numbers (~15 cycles).

c. Digest the oxTi365_loxP_myo2_neoR plasmid with *Srf*I.

Note: The oxTi365_loxP_myo2_neoR plasmid contains 742bp and 946bp of 5′ and 3′ homology arm sequences, respectively. The *Srf*I site is located between the 5′ homology arm and the dual selection marker cassette.

d. Clone the PCR product of *(Promoter of interest)::TIR1::unc-54 3′utr* from step b) into the *Srf*I site of oxTi365_ loxP_myo2_neoR plasmid using Gibson assembly.
e. Check the insert by restriction enzyme digestion and sequencing.

12. Microinjection.

Follow the steps described in step 3.

13. Screening F_1s for knock-in mutants.

Follow the steps described in step 4.

Note: In addition to the *oxTi365* MosSCI site we used in our original work, we also generated sgRNA and HDR donor plasmids for single copy *TIR1* transgene knock-in at another MosCI site on chromosome II (*ttTi5605*).[8,11] The *ttTi5605* MosSCI site is located on chromosome II at position 8,420,204.[8,11]

The gRNA target sequence in the sgRNA plasmid (pTK73_ ttTi5605) is 5′-GATATCAGTCTGTTTCG TAACGG-3′ (the underline indicates the PAM sequence).

Primers to amplify P*unc-4c* (or your promoter of interest)::*TIR1::unc-54 3′utr* to clone the*TIR1* transgene into the ttTi5605_loxP_myo2_neoR HDR donor plasmid is as follows:

ttTi5605 forward: CGATATCAGTCTGTTTCGGCCC**ATGACCATGATTACGCCAAG**.

ttTi5605 reverse: TTATAGGCCGCCTGATGCCC**AAACGCGCGAGACGAAAGGG**.

* Bold letters indicate the primer sequences that bind to the backbone sequences of the pSM vector plasmid located upstream of the promoter (forward), and downstream of the *unc-54* 3′UTR (reverse). Underlined sequences are the adapter sequences for Gibson assembly.

Follow step 11 in this section for cloning the TIR1 transgene into the ttTi5605_loxP_myo2_neoR HDR donor plasmid.

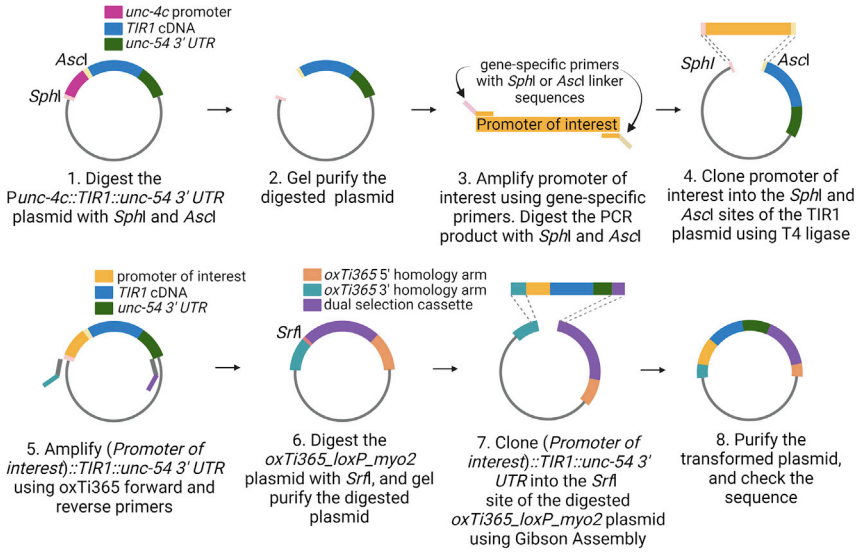

Figure 4. Workflow of HDR donor plasmid construction for *TIR1* single copy insertion

Note: The pTK73_ ttTi5605 and pTK73_oxTi365 plasmids are identical to previously published plasmids, pTK_pOB5 and pTK_pOB4, respectively.[8]

Preparation of synthetic auxin-containing NGM plates

Timing: 20 min

The original work implementing the AID system in *C. elegans* used natural auxin indole-3-acetic acid (IAA), which requires ethanol as a solvent. We found that IAA treatment disrupted the localization pattern of mCherry::RAB-3, a marker to visualize presynaptic vesicles (unpublished). It is also known that IAA causes cytotoxicity.[22,23] For these reasons, we use a water-soluble synthetic auxin analog, 1-naphthaleneacetic acid (NAA), which has been shown to work effectively for the AID system in *C. elegans*.[24]

14. Prepare a stock solution of NAA by dissolving potassium salt of NAA (K-NAA) in distilled water (dH_2O) at a final concentration of 400 mM.

Note: The K-NAA stock solution may be kept at 4°C for up to 3 months.

15. Prepare a 4 mM K-NAA working solution by diluting the stock solution with M9 buffer.

Note: We did not observe growth defects or abnormal localization of presynaptic markers at high concentrations of K-NAA (100 mM) (data not shown). We found that 1 mM (data not shown) and 4 mM K-NAA is sufficient for spatiotemporal degradation of UNC-4::AID::BFP (Figure 5) and UNC-37::AID::BFP.[1]

16. Add 500 µL of 4 mM K-NAA working solution to the 60 mm NGM plates with OP50 bacteria and let the solution dry for 12–16 h at room temperature (22°C).
17. Store the K-NAA NGM plates in a light-blocking container at 4°C for up to four weeks.

Note: Even though NAA is more photostable than IAA,[23] it may still degrade if K-NAA is kept under prolonged light exposure. Storing the K-NAA stock solution, working solution and K-NAA containing NGM plates in the dark at 4°C may help extend their shelf life.

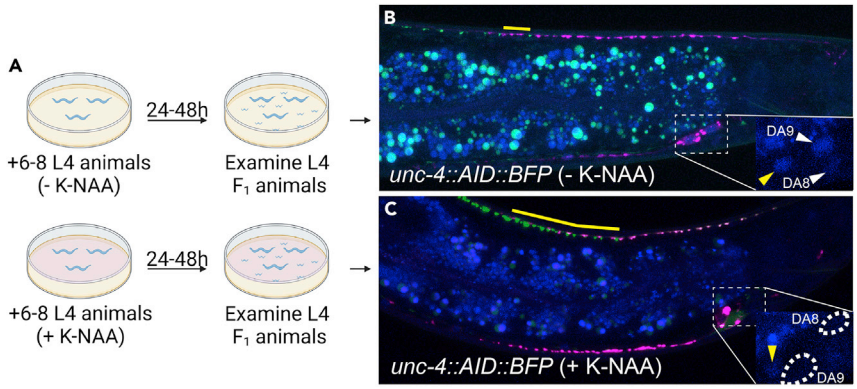

Figure 5. Schematic of auxin-inducible protein degradation

(A) Schematic of negative control (top) and the experimental group (bottom) for auxin-inducible protein degradation. We cultured 6–8 L4 animals on the NGM plates with or without K-NAA and examined the F_1 progeny at the L4 stage for auxin-induced UNC-4::AID::BFP knock-down in DA motor neurons.

(B) Representative image of *unc-4::AID::BFP; mizSi3(Punc-4c::TIR1::unc-54 3′utr)* animals grown on the NGM plates without K-NAA. BFP expression is observed in the DA8 and DA9 (white arrowheads) and VA12 (yellow arrowhead) neurons. DA8 and DA9 presynaptic sites are labeled with GFP::RAB-3 (green) and mCherry::RAB-3 (magenta) on the dorsal side of the worm, respectively. Minimal overlap between the DA8 and DA9 presynaptic domains (yellow line) are observed.

(C) Representative image of *unc-4::AID::BFP; mizSi3(Punc-4c::TIR1::unc-54 3′utr)* animals grown on K-NAA containing NGM plates. Large overlap between DA8 and DA9 synaptic domains is observed (yellow line). Loss of UNC-4::AID::BFP signal in the DA8 and DA9 neurons (dotted white line outlines the cell body) and the presence of UNC-4::AID::BFP signal in the VA12 neuron (yellow arrowhead) indicates DA neuron-specific UNC-4::AID::BFP degradation.

*Opened K-NAA powder bottle can be stored at 4°C for up to one year.

K-NAA treatment for degradation of AID-tagged proteins

Timing: 2–3 days

To test the efficiency of the AID system, conduct continuous knock-down from embryogenesis to adult stage of the gene of interest and examine if this condition reproduces known observable phenotypes (if any) (Figure 5).

18. Confirmation of spatial degradation of AID-tagged protein.
    a. Generate strains with AID::BFP/AID::mTagBFP2 knock-in and the single copy *TIR1* transgene. Check if they exhibit the wild type phenotype of your interest.
    b. Transfer 6–8 L4 P_0 animals onto K-NAA containing NGM plates.
    c. As a control, culture the same stage of P_0 worms on the NGM plate without K-NAA.
    d. Culture them for 24–48 h.
    e. Using a fluorescence microscope, examine the BFP or mTagBFP2 signal in the F_1 animals grown on the NGM plates with and without K-NAA.

Note: Successful protein knock-down will result in a loss of BFP/mTagBFP signal in the cell-types (DA neurons in the case of *unc-4::AID::BFP; mizSi3*) that express both the AID-tagged gene and *TIR1* in the experimental group treated with K-NAA (Figure 5C) .

    f. Examine the progeny for any known observable mutant phenotypes (Ex. dumpy, uncoordinated locomotion, lethality).

⚠ CRITICAL: Once it is confirmed that the continuous degradation of AID-tagged protein causes expected phenotypes, protein degradation can be induced within a specific

developmental time point for examining the temporal requirement of the protein of interest. The degradation of AID-tagged proteins can occur within minutes of treating the animals with auxin.[3] Below we describe the protocol used to knock-down UNC-4::AID::BFP embryonically and post-embryonically to determine the post-embryonic roles of UNC-4 in the spatial organization of synapses.[1]

19. Temporal degradation of the AID-tagged proteins.
    a. Positive and negative control:
        i. For the positive control, we conducted continuous UNC-4::AID::BFP knock-down as described in step 18b.
        ii. For the negative control, we cultured 6–8 L4 P_0 animals on NGM plates without K-NAA as described in step 18c.
    b. Embryonic knock-down:
        i. Place 6–8 L4 P_0 animals onto the K-NAA-containing NGM plates for 16 h.
        ii. Transfer F_1 animals at the early L1 stage to the NGM plates without K-NAA.
        iii. Let the F_1 worms grow on the NGM plate without K-NAA until the L4 stage.
        iv. Examine for their phenotype.
    c. Post-embryonic knock-down:
        i. Place 6–8 P_0 animals at L4 stage onto the NGM plates without K-NAA for 16 h.
        ii. Transfer F_1 animals to the K-NAA containing NGM plates to induce UNC-4::AID::BFP degradation post-embryonically.
            For early post-embryonic knock-down, transfer newly hatched F_1 animals at the early L1 stage to the K-NAA-containing NGM plates.
            For late post-embryonic knock-down, transfer F_1 animals at the L2-L3 stages to the K-NAA containing NGM plates.
        iii. Let the transferred animals grow on the K-NAA-containing NGM plates until the L4 stage and examine their phenotypes.

EXPECTED OUTCOMES

This protocol is used to generate AID::BFP or AID::mTagBFP2 knock-in strains and TIR1 knock-in strains using CRISPR/Cas9 genome editing for auxin-inducible protein degradation. Auxin-inducible degradation allows for the spatial and temporal degradation of proteins of interest.

In our original paper,[1] We used the AID system to spatially and temporally knock down UNC-4. As *unc-4* is specifically required in the A-type motor neurons,[25] we used P*unc-4c::TIR1* knock-in transgene (*mizSi3*) to induce degradation of UNC-4::AID::BFP specifically in the DA-class of cholinergic motor neurons. For continuous UNC-4::AID::BFP knock-down in the DA neurons, we placed 4th larval stage (L4) P_0 animals onto the K-NAA-containing NGM plates and examined their F_1 progeny at the L4 stage. We observed a defect in the presynaptic tiling patterns of DA8 and DA9 neurons[1] (Figure 5C).

LIMITATIONS

Even though the AID system effectively induces the degradation of AID-tagged proteins in *C. elegans*, it may not recapitulate the complete knock-out condition. Therefore, the lack of detectable phenotypes does not necessarily mean that the gene of interest does not play critical roles in specific tissues and/or developmental timings. The AID system requires AID to be fused with the protein of interest. It is possible that the resulting fusion proteins may not function exactly as untagged wild type proteins. It also requires expression of *TIR1* and auxin treatment, which could also affect phenotypes of interest. The observations obtained from the AID-mediated protein knock-down would ideally be confirmed by alternative methods. For example, we used null (*e120*) and temperature-sensitive (*e2322ts)* alleles of *unc-4* to confirm presynaptic patterning defects observed in

the auxin-mediated UNC-4 knockdown.[1] Alternative methods include, but not limited to, tissue specific RNAi[26] and conditional gene knockout using Cre-Lox recombination system.[27]

TROUBLESHOOTING

Problem 1

CRISPR knock-in does not work (related to steps 1-4).

Potential solution

- If Method 1 (plasmid delivery of Cas9 and sgRNA) is used, we highly recommend using direct injection of Cas9-gRNA RNP complex and 'melted' linear DNA as an HDR donor template described in Method 2.
- If Method 2 is used and still does not work, consider using a gRNA closer to the knock-in site. Even the gRNA design tools indicate some issues of these gRNA such as low on-target or high off-target potentials. gRNAs that are closer to the knock-in site generally work better in our hands for getting knock-in animals. The background mutations due to the off-target cleavage can be removed by outcrossing the knock-in animals with wild-type several times. Extending the homology arm length to 60–70 nt can improve the knock-in efficiency.

Problem 2

The dual selection marker cassette cannot be excised (related to step 5).

Potential solution

This can be due to the complex rearrangement within and around the knock-in sites that may affect the sequence and arrangement of the loxP sites. It is reported that complex rearrangements at the Cas9 target site could occur when the dual selection marker cassette plasmid is used as an HDR template.[18] It is important to obtain more than one independent knock-in strain so that you have strains in which the dual selection marker cassette can be excised.

Problem 3

AID::BFP/AID::mTagBFP2 knock-in disrupts the protein's function (related to step 18).

Potential solution

It is possible that the fusion protein of interest and AID::BFP/AID::mTagBFP2 is not functional, and therefore, the knock-in animals exhibit a mutant phenotype on their own. In this case, inserting AID::BFP/AID::mTagBFP2 at different locations of the protein, and/or adding linker sequences between the protein of interest and AID::BFP/AID::mTagBFP2 may be considered.

In some cases, we found that knocking-in AID without BFP or mTagBFP2 makes the AID-tagged protein to be functional. However it is difficult to assess the effectiveness of protein degradation without BFP/mTagBFP2. It is therefore important to confirm that the auxin-mediated protein knock-down reproduces the known mutant phenoytpe before you conduct the spatiotemporal knock-down experiments. Alternatively, inserting AID with a short epitope tag such as 3×FLAG may not disrupt protein function. Western blots can be used to determine the efficacy of the protein knock-down.

Problem 4

Single copy *TIR1* transgene affects phenotypes of interest (related to steps 11-13).

Potential solution

We notice that overexpression of *TIR1* is toxic to worms as many worms with *TIR1* transgene as an extrachromosomal array become sick and sterile within a few generations. It is possible that *TIR1* is overexpressed even if it is integrated into the chromosome as a single copy transgene. In such

cases, avoiding strong promoters to express *TIR1*, or selecting different MosSCI sites such as *ttTi5605* as knock-in destinations may help minimize the effects of *TIR1* overexpression.

Problem 5

Auxin-inducible knock down does not reproduce the known phenotypes of the mutants (related to steps 14-18).

Potential solution

The phenotype of AID-mediated protein knock-down could be affected by the abundance and stabilty of protein of your interest and/or the timing and level of *TIR1* expression.

- Confirm that the K-NAA (or auxin) working solution works for the control AID strains. We use the DLW109 (*wrdSi23 I; unc-104(knu973[unc-104::AID]) II*) strain as a positive control. The DLW109 strain exhibits a severe uncoordinated locomotion phenotype when grown on the K-NAA-containing NGM plate, due to the neuron-specific degradation of UNC-104::AID.[28] If you do not observe the expected phenotype from your positive control, remake the K-NAA stock and working solutions. We note that opened K-NAA powder bottle would expire in 10–12 months.
- Check if the continuous K-NAA treatment would reproduce the known phenotypes of the available mutants when *TIR1* is expressed under the ubiquitous promoter. The transgene *ieSi57 [eft-3p::TIR1::mRuby::unc-54 3′UTR + Cbr-unc-119(+)]* is available from CGC.
- Try a higher concentration of K-NAA solution (10–100 mM). Protein of your interest may be less susceptible to the AID-mediated protein degradation due to its abundance and/or stability. The degradation rate of the AID-tagged proteins depends on the concentration of auxin.[3] We did not notice detectable developmental or behavioral defects when worms are grown on the 100 mM K-NAA containing NGM plate.
- Consider using multiple tissue-specific promoters to express *TIR1*. It is possible that certain tissue-specific promoter may not be expressed in time windows when the protein of interest functions. Using several tissue-specific promoters may mitigate this issue.

Problem 6

Degradation of AID-tagged proteins occurs in the absence of K-NAA (related to steps 18 and 19).

Potential solution

TIR1 has been shown to induce a leaky degradation of AID-tagged proteins in the absence of auxin.[24,29] In such case, we recommend AID version 2 (AID2), which employs a mutant TIR1(F74G) and 5-phenyl-indole-3-acetic acid (5-Ph-IAA), as it has an undetectable level of leaky degradation without 5-Ph-IAA, while achieving faster and more effective degradation of the AID-tagged protein upon 5-Ph-IAA treatment.[30–32] The AID2 system uses the same degron tag as the original AID.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kota Mizumoto mizumoto@zoology.ubc.ca.

Materials availability

Plasmids generated in this study are available from Addgene.

Data and code availability

This study did not generate/analyze datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.M., M.K.; Methodology, K.M., M.K.; Formal analysis, M.K.; Investigation, K.M., M.K.; Writing – original draft, review and editing, K.M., M.K.; Visualization, M.K.; Funding acquisition, K.M.; Resources, K.M.; Supervision, K.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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