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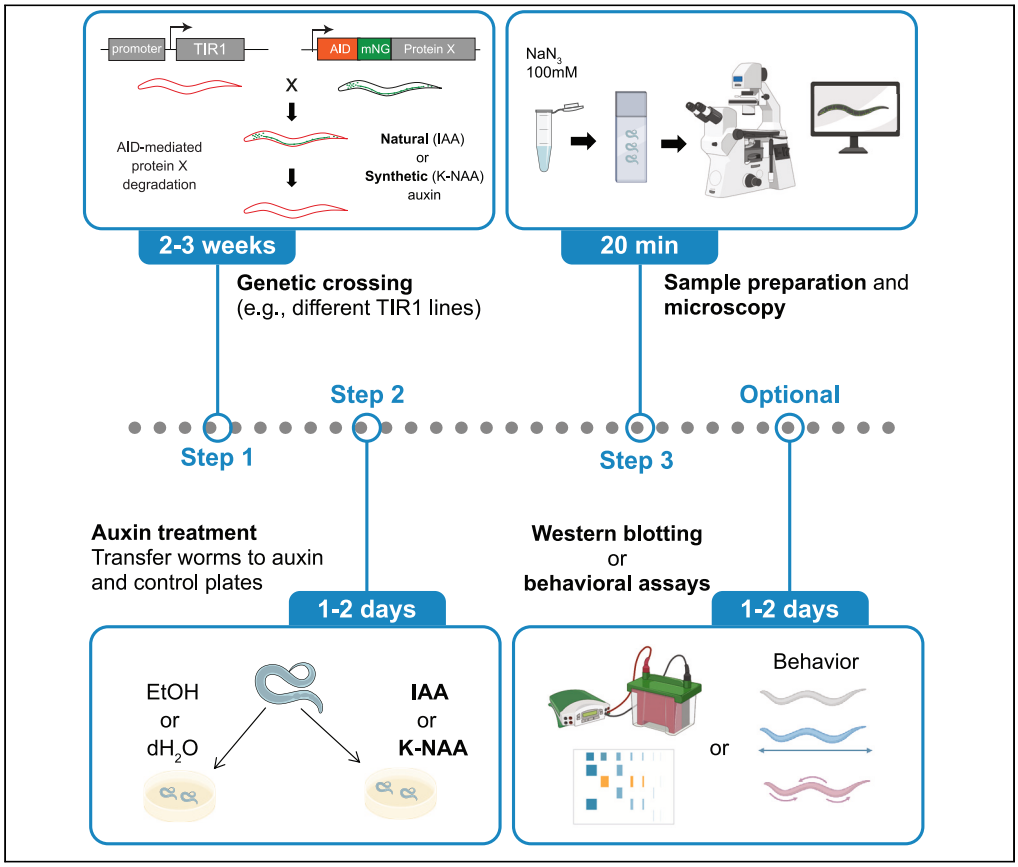
Protocol for auxin-inducible protein degradation in *C. elegans* using different auxins and TIR1-expressing strains



The auxin-inducible degron (AID) system is a powerful tool to deplete proteins *in vivo*. Here, we present a protocol for AID-mediated depletion of two proteins (CFI-1/AT-rich interaction domain 3 [ARID3] and Y47D3A.21/density-regulated re-initiation and release factor [DENR]) in *C. elegans* tissues using different auxins and transport inhibitor response 1 (TIR1)-expressing strains. We describe steps for genetic crossing, sample preparation, fluorescent microscopy, and treatment with either natural (indole-3-acetic acid [IAA]) or synthetic (1-naphthaleneacetic acid, potassium salt [K-NAA]) auxins. We then detail procedures for comparing the degree of CFI-1 depletion in *C. elegans* neurons upon panneuronal or pansomatic TIR1 expression.

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

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Highlights

Procedures for *C. elegans* genetic crossing, sample preparation, and auxin treatment

Steps for protein depletion using different auxins and TIR1 strains

Steps to evaluate AID-driven protein depletion using fluorescent microscopy

Adaptable for microscopy, western blotting, and behavioral assays

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Protocol for auxin-inducible protein degradation in *C. elegans* using different auxins and TIR1-expressing strains

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SUMMARY

The auxin-inducible degron (AID) system is a powerful tool to deplete proteins *in vivo*. Here, we present a protocol for AID-mediated depletion of two proteins (CFI-1/AT-rich interaction domain 3 [ARID3] and Y47D3A.21/density-regulated re-initiation and release factor [DENR]) in *C. elegans* tissues using different auxins and transport inhibitor response 1 (TIR1)-expressing strains. We describe steps for genetic crossing, sample preparation, fluorescent microscopy, and treatment with either natural (indole-3-acetic acid [IAA]) or synthetic (1-naphthaleneacetic acid, potassium salt [K-NAA]) auxins. We then detail procedures for comparing the degree of CFI-1 depletion in *C. elegans* neurons upon panneuronal or pansomatic TIR1 expression.

For complete details on the use and execution of this protocol, please refer to Li et al.[1,2]

BEFORE YOU BEGIN

The protocol below describes the steps necessary for achieving auxin-inducible protein depletion in *C. elegans* tissues. As proof-of-principle, we focus on two conserved proteins with different subcellular localization: (a) the transcription factor CFI-1 (human ARID3) is expressed in the nucleus of *C. elegans* neurons and head muscle cells,[1–3] and (b) the ubiquitously expressed translation initiation factor Y47D3A.21 (human DENR) that localizes in the cytoplasm. The AID system is tripartite and requires (a) the plant hormone auxin or a synthetic analog, (b) the protein of interest must be tagged with the auxin inducible degron, and (c) the *Arabidopsis thaliana* F-box protein, transport inhibitor response 1 (TIR1), must be expressed in the cell type or tissue of interest (Figure 1A).[4] Upon exposure to auxin, TIR1 acts as a substrate to ubiquitinate the AID-tagged protein, enabling its proteasomal degradation. The AID sequence can be introduced into the endogenous locus of the gene of interest by CRISPR/Cas9 gene editing, as detailed in a recent protocol.[5] With this method, we successfully tagged CFI-1 with a green fluorescent protein (mNeonGreen) fused to AID and Y47D3A.21 (DENR) with GFP::AID (Figure 2). Next, we obtained two transgenic strains that express TIR1 either in all *C. elegans* somatic cells or exclusively in neurons (see key resources table) (Figure 1B). Through genetic crossing and/or CRISPR/Cas9 gene editing, we generated homozygous animals for the AID allele (e.g., *cfi-1*(*kas16*[*mNG*::*AID*::*cfi-1*]) I) and the TIR1 transgenes. The steps we detail here for CFI-1/ARID3 and Y47D3A.21/DENR can be applied to any *C. elegans* protein.

Since its initial establishment in *C. elegans* in 2015,[4] the AID system has continued to evolve.[5–8] A battery of cell type-specific TIR1-expressing strains is now available, enabling auxin inducible protein depletion in specific *C. elegans* tissues (e.g., germline, intestine, nervous system, muscle).[9]

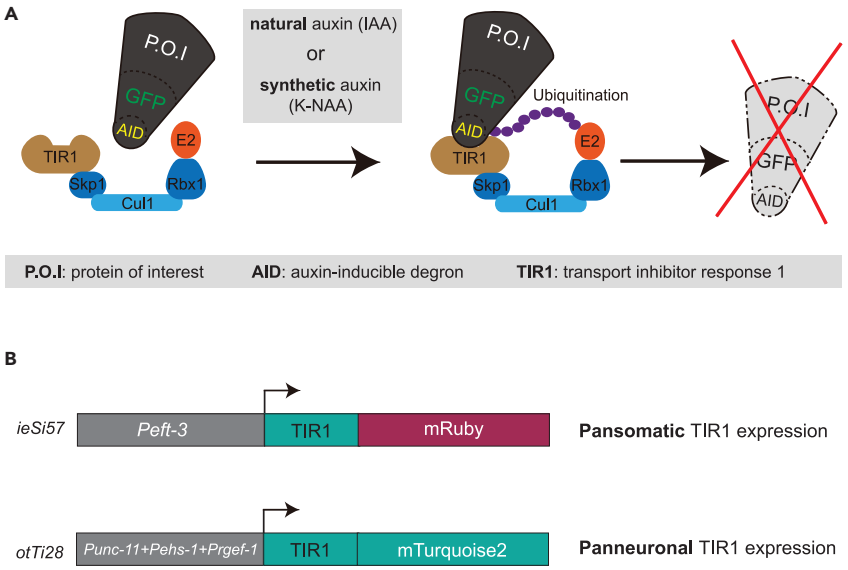

Figure 1. AID-mediated protein depletion using different auxins and TIR1-expressing lines

(A) Schematic of auxin-inducible degradation system. Natural (IAA) or synthetic (K-NAA) auxin treatment was employed in this protocol.

(B) Schematic of the two TIR1 lines used in this protocol.

Because natural auxin (indole-3-acetic acid [IAA]) has several disadvantages (light-sensitivity, no penetrance of eggshell, requires ethanol as a solvent), synthetic auxin analogs, such as 1-naphthaleneacetic acid, potassium salt (K-NAA) and 5-phenyl-indole-3-acetic acid (5-Ph-IAA), have been developed.[5,8,10–12] However, a side-by-side comparison of depleting the same protein using different TIR1 lines, as well as natural and synthetic auxins, is lacking in *C. elegans*.

In this protocol, we first outline all necessary steps for achieving AID-mediated protein depletion in *C. elegans*, ranging from strain generation to auxin administration. Further, we compare the efficacy of natural auxin (indole-3-acetic acid (IAA) and a water-soluble synthetic auxin analog (K-NAA) in depleting a nuclear (CFI-1) and a cytoplasmic (Y47D3A.21[DENR]) protein (Figure 1A). Last, we directly compare the degree of AID-mediated CFI-1 depletion in *C. elegans* tissues when TIR1 is provided either in neurons or all somatic cells (Figure 1B).

Genetic crossing of *C. elegans* strains

Timing: 2–3 weeks

This section describes the necessary steps to generate *C. elegans* strains simultaneously carrying the AID allele and the TIR1 transgene.

1. Genetic crossing entails the following steps:
   a. Generate male animals for the AID allele (e.g., *cfi-1*(*kas16*[*mNG*::*AID*::*cfi-1*]) I) by crossing into N2 control hermaphrodites or via heat-shock exposure.
   b. Cross fifteen males that carry the AID allele with 5 hermaphrodite animals homozygous for the TIR1-expressing transgene.
   c. At the next generation, pick 5 hermaphrodites that are heterozygous for both the AID allele and TIR1 transgene.
   d. Allow worms to generate self-progeny and continue to pick and single 5 hermaphrodites until both the AID allele and the TIR1 transgene become homozygous.

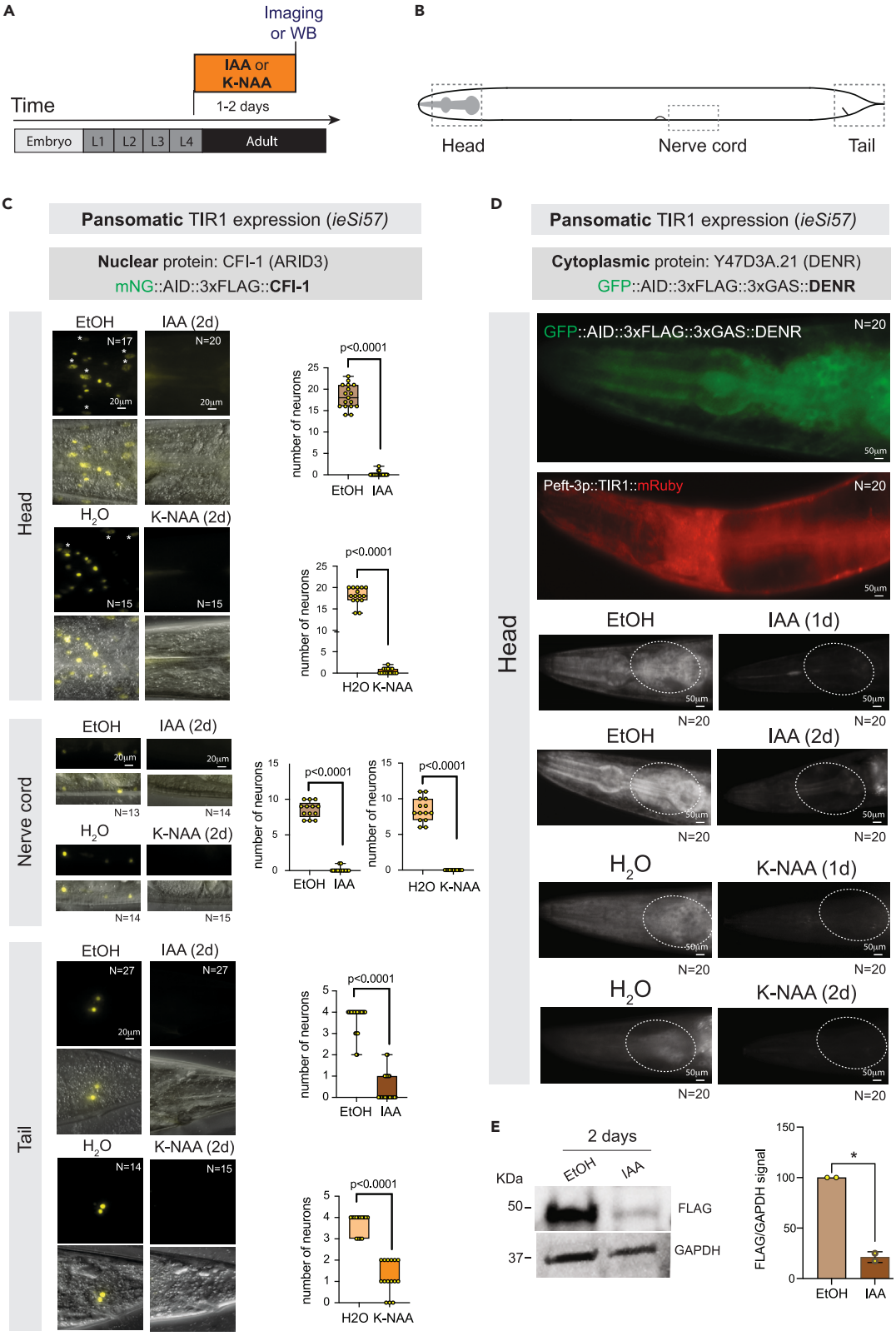
A
Imaging or WB
IAA or K-NAA
Time
1-2 days
Embryo
L1
L2
L3
L4
Adult
B
Head
Nerve cord
Tail
C
Pansomatic TIR1 expression (ieSi57)
Nuclear protein: CFI-1 (ARID3)
mNG::AID::3xFLAG::CFI-1
Head
Nerve cord
Tail
EtOH
IAA (2d)
H2O
K-NAA (2d)
N=17
N=20
N=15
N=13
N=14
N=27
20µm
p<0.0001
number of neurons
EtOH IAA
H2O K-NAA
D
Pansomatic TIR1 expression (ieSi57)
Cytoplasmic protein: Y47D3A.21 (DENR)
GFP::AID::3xFLAG::3xGAS::DENR
Peft-3p::TIR1::mRuby
Head
EtOH
IAA (1d)
IAA (2d)
H2O
K-NAA (1d)
K-NAA (2d)
N=20
50µm
E
2 days
KDa
50-
37-
FLAG
GAPDH
FLAG/GAPDH signal
150
100
50
0
*

Figure 2. AID-mediated depletion of nuclear and cytoplasmic proteins using two different auxins

(A) Schematic of auxin treatment.

(B) Schematic of *C. elegans* body. Boxed regions indicate images shown in panels C and D.

(C) Representative images showing mNG::AID::3xFLAG::CFI-1 expression in head neurons and head muscle cells (asterisks) upon IAA or K-NAA treatment for two days. Controls: Two day-long exposure to either 100% ethanol or dH_2O. Strain genotype: *cfi-1*(*kas16*[*mNG*::*AID*::*cfi-1*]) I; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3′ UTR, cb-unc-119(+)] II. Images from the *C. elegans* head, ventral nerve cord, and tail are shown. Quantification of the number of neurons expressing mNG is provided on the right. Student's t test, N.S: Not significant. N: number of animals. All animals analyzed are lateral views, with the exception of the tail images where 24 of the 27 animals are lateral. Scale bar: 20 μm. Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum values. Yellow circles depict individual values.

(D) Representative images showing *GFP-AID-3Xflag-3xGAS:: Y47D3A.21 and Peft-3::TIR1::mRuby expression in the C. elegans head region (top panels).* Expression of *GFP::AID::3XFLAG::3xGAS::Y47D3A.21* in the cytoplasm of most cells and tissues is observed. Representative images are shown upon IAA or K-NAA treatment for two days. Controls: Two day-long exposure to either 100% ethanol or dH_2O. Fifteen of the 20 analyzed animals are lateral views. Scale bar: 50 μm.

(E) Western blotting with antibodies against FLAG and GAPDH on homozygous GFP::AID::3XFLAG::3xGAS::Y47D3A.21 animals with pansomatic TIR expression upon IAA treatment for two days. Quantification is provided on the right. The experiment was repeated twice. Student's t test, $*p < 0.05$.

⚠ CRITICAL: It is important to use only well-fed (not starved animals) for genetic crosses. Set up crosses by picking animals at larval stage 4 (L4).

Note: As described above, we introduced the TIR1 transgene via genetic crossing to *cfi-1*(*kas16*[*mNG*::*AID*::*cfi-1*]) animals. However, for the cytoplasmic protein (DENR), we employed CRISPR/Cas9 gene editing - on homozygous animals for the pansomatic TIR1 line *(ieSi57)* - to generate the *Y47D3A.21(syb6129[GFP-AID-3Xflag-3xGAS::Y47D3A.21])II* allele following established procedures.[5]

Preparation of solutions

Timing: 1 h

This section describes how to prepare stock solutions of 400 mM IAA or 400 mM K-NAA.

2. Prepare fresh nematode growth medium (NGM). The NGM composition is:
   a. NaCl, Agar, Peptone, 5 mg/mL Cholesterol (prepared in 100% ethanol), 1 M $MgSO_4$, 1 M KPO_4 buffer pH 6.0.
3. Prepare fresh solutions containing natural auxin (indole-3-acetic acid (IAA):
   a. Store the natural auxin indole-3-acetic acid (IAA) at −20°C up to a year.
   b. Prepare a 400 mM IAA stock solution in 100% ethanol and store at 4°C for up to one month.
4. Prepare fresh solutions containing a water-soluble synthetic auxin analog (K-NAA):
   a. Store the water-soluble synthetic auxin analog (K-NAA) at −20°C up to a year or the temperature recommended by the manufacturer.
   b. Prepare a 400 mM K-NAA stock solution in deionized water (dH_2O) and store at 4°C for up to one month.

⚠ CRITICAL: The auxin (IAA) solution should be protected from light exposure and shielded with aluminum foil. However, a recent protocol indicates that the photostability of synthetic auxin (K-NAA) prevents light-induced compound degradation during storage.[11]

Note: Check reagents details in the key resources table.

Preparation of NGM plates for auxin experiments

Timing: 3–4 days

This section describes how to prepare NGM plates containing 4 mM IAA or K-NAA, as well as respective control NGM plates.

5. Prepare NGM plates with auxin (NGM-auxin) and auxin-solvent (NGM-control) (1 day) (Figure S1).
   a. Cool down autoclaved NGM to 50°C and split into two bottles of equal volume (500 mL) for auxin (IAA or K-NAA) and solvent (ethanol or dH_2O).
   b. Add 5 mL of 400 mM auxin (IAA or K-NAA) to 500 mL of NGM to the final concentration of 4 mM.
   c. As a control for natural auxin (IAA), add 5 mL of 100% ethanol into 500 mL of NGM.
   d. As a control for synthetic auxin (K-NAA), add 5 mL of dH_2O into 500 mL of NGM.
   e. Pour 10 mL of prepared NGM-auxin and NGM-control to 60 mm petri dish and let it dry until next day.
6. Seed all plates with OP50 bacteria (2–3 Days).
   a. Add 200 μL of concentrated live OP50 on NGM plates and let it dry for 2–3 days.

⚠ CRITICAL: Before adding auxin, the temperature of NGM should be 50°C. Otherwise, it may cause a rapid degradation of auxin. In addition, auxin (IAA or K-NAA) concentration in the NGM plate may vary from 500 μM to 4 mM; 4 mM is the highest concentration used in this protocol. However, we recommend optimization of auxin concentrations for each experiment.

Note: NGM plates containing auxin (IAA or K-NAA) can be stored at 4°C up to a month. In our experiments, we shielded both NGM-auxin plates from light (Figure S1). However, a recent protocol indicates that the photostability of K-NAA prevents light-induced compound degradation during storage.[11]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
OP50 Escherichia coli (live)	Caenorhabditis Genetics Center (CGC)	RRID:WB-STRAIN:WBStrain00041969
Chemicals, peptides, and recombinant proteins		
Agar, bacteriological	Lab Scientific bioKEMIX	A466
NaCl	Sigma	71376-1KG
Peptone	Thermo Fisher Scientific	BP1420-2
$CaCl_2$	Acros	CAS 10043-52-4
$MgSO_4$	Sigma-Aldrich	M7506-500G
Potassium phosphate, monobasic	Acros	CAS 7778-77-0
Cholesterol	Sigma-Aldrich	C8667-5G
Indole-3-acetic acid, 98+% (auxin)	Alfa Aesar	CAS 87-51-4
1-naphthaleneacetic acid, potassium salt (K-NAA)	PhytoTech Labs	N610
Tryptone	Sigma-Aldrich	T-7293-250G
Yeast extract	Sigma	Y1625-250G
Sodium azide (NaN_3)	Acros	CAS 26628228
Agarose (protein electrophoresis grade), Fisher BioReagents	Fisher Scientific	BP164-500
Ethanol 200 Proof	Decon Labs	2701
Experimental models: Organisms/strains		
C. elegans: *cfi-1*(*kas16*[*mNG::AID::cfi-1*]) I	This study	KRA345
C. elegans: *otTi28 [unc-11prom8+ehs-1prom7+rgef-1prom2::TIR1::mTurquoise2::unc-54 3′UTR] X*	Hobert Lab (Columbia University)	OH14930
C. elegans: *unc-119(ed3); ieSi57 [Peft-3::TIR1::mRuby::unc-54 3′ UTR, cb-unc-119(+)] II*	Caenorhabditis Genetics Center (CGC)	CA1200
cfi-1(*kas16*[*mNG::AID::cfi-1*]) I; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3′ UTR, cb-unc-119(+)] II	This study	KRA830

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
cfi-1(kas16[mNG::AID::cfi-1]) I; otTi28 [unc-11prom8+ ehs-1prom7+rgef-1prom2::TIR1::mTurquoise2::unc-54 3′UTR] X	This study	KRA842
C. elegans: *Y47D3A.21(syb6129[GFP-AID-3Xflag-3x GAS:: Y47D3A.21])III; ieSi57II*	This study	PHX6129
Software and algorithms		
Version 2.3.69.1000, Blue edition	Carl Zeiss Microscopy	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
ImageJ/Fiji software	Fiji – open source	https://fiji.sc/
GraphPad Prism	GraphPad – subscription based	https://www.graphpad.com/
Other		
Dissection microscope	Carl Zeiss	Stemi 305
Glass microscopic slides (plain)	Fisher Scientific	12-544-1
Falcon Bacteriological Petri dishes with lid (60 mm)	Fisher Scientific	08-757-100B
Zeiss, Axio Imager Z2	Carl Zeiss Microscopy	https://www.zeiss.com/microscopy/en/products/light-microscopes/widefield-microscopes/axio-imager-2-for-life-science-research.html
Aluminum foil	N/A	N/A
Microscope cover glass (22 × 30 mm)-No. 1	Fisher Scientific	https://www.fishersci.com/shop/products/rectangular-cover-glasses-23/12541018
Carl Zeiss Immersol Immersion oil	Fisher Scientific	https://www.fishersci.se/shop/products/carl-zeiss-immersol-immersion-oil-6/10539438
Glass Pasteur pipets ($5^{3/4}$) disposable Pasteur pipets	Fisherbrand	Cat No. 13-678-20B

MATERIALS AND EQUIPMENT

Nematode growth medium (NGM)		
Reagent	Final concentration	Amount
Agar	N/A	17
NaCl	N/A	3
Peptone	N/A	2.5
Milli-Q Water	N/A	972 mL
Autoclave at 121°C for 50 min and supplement with the following:		
1 M $CaCl_2$	1 mM	1 mL
1 M $MgSO_4$	1 mM	1 mL
5 mg/mL Cholesterol (prepared in 100% ethanol)	N/A	1 mL
1 M Potassium phosphate, monobasic (KPO_4) pH 6.0	25 mM	25 mL
Total	**N/A**	**1000 mL**
Storage conditions for 5 mg/mL Cholesterol: stored at 4°C; Storage conditions for remaining reagents: stored at room temperature (20°C–25°C)		

400 mM auxin (IAA or K-NAA) stock solution	
Reagent	Amount
Indole-3-acetic acid (IAA), Alfa Aesar, A10556	0.70
1-naphthaleneacetic acid, potassium salt (K-NAA), PhytoTech Labs, N610	0.89
100% ethanol for IAA or dH_2O for K-NAA	10 mL
Total	**10 mL**
Storage conditions for 400 mM Auxin (IAA or K-NAA) stock solution: at 4°C and shielded from light for up to one month. Fresh preparation each time is recommended for better results. Storage conditions for 100% ethanol: stored at room temperature (20°C–25°C)	

STEP-BY-STEP METHOD DETAILS

Auxin treatments

Timing: 1–2 days

This section describes how to transfer *C. elegans* animals onto NGM-auxin and NGM-control plates.

This step requires transferring the worms onto the previously prepared NGM-auxin and NGM-control plates with ethanol (EtOH) and 4 mM natural auxin (IAA), or onto NGM plates with dH_2O and 4 mM synthetic auxin (K-NAA).

1. Transfer worms to treatment plates.
   a. Pick worms with the platinum wire picker at L4 (or younger). N = 25 or higher.
   b. Transfer them to bacteria seeded NGM-auxin (4 mM) and NGM-control plates.
2. Keep the worms for 1–2 days at 20°C. See problem 1, problem 2, problem 3, and problem 4.

⚠ CRITICAL: It is important to shield the NGM-IAA plates from light because natural auxin (IAA) is light sensitive. Further, worms grown on auxin plates may present a slight delay in growth. This is particularly important if you need to image worms at a specific developmental stage. Because dH_2O (not ethanol) is used to dissolve K-NAA, the worms on K-NAA NGM plates appear healthier compared to worms on IAA (dissolved in 100% ethanol) NGM plates. The solvent of IAA (ethanol) may confound *C. elegans* lifespan and behavioral assays.

Sample preparation for fluorescence microscopy

Timing: 15 min

This section describes how to prepare imaging slides.

This step involves the preparation of anesthetized worms for imaging and mounting them on a 4% agarose pad on glass slides. These pads prevent worms from being crushed between the coverslip and glass slide, and help avoid desiccation.

3. Prepare 4% agarose solution.
   a. Add 4 g agarose to 100 mL dH_2O in a 250-mL Erlenmeyer flask.
   b. Heat up, very carefully, in a microwave until all agarose dissolves.
   c. Place the melted agarose on a heat block, at approximately 95°C, while preparing the slides, to prevent solidification.

Optional: Alternatively, dissolve the agarose on a stirring hotplate, with a stir bar.

Note: The 4% agarose solution can be reused. Microwave upon solidification.

Pause point: Turn on microscope (e.g., Zeiss Axio Imager Z2), computer, laser source and associated equipment before starting to prepare the samples.

4. Prepare the imaging slides (Figure S2).
   a. Prepare two support slides by placing two layers of laboratory tape on each slide. Place these support slides on each side of a blank side.
   b. Place one drop (~600 µL) of warm 4% agarose onto the blank slide using a laboratory pipette with a cut blue tip.

c. Quickly place a second blank slide on the top of the agarose drop, at a 90° angle to the three slides, and gently press the edges of this slide such that the agarose spreads uniformly. Let the agarose dry for a minute.
d. Separate the glass slides without perturbing the agarose pad. Use a razor blade to trim excessive parts and adjust the agarose pad.

Optional: Use a glass Pasteur pipette to drop the warm agarose on the blank slide.

Optional: Mount the control and treatment samples on the same slide by dividing the agar pad into several sections. The agar pad can be divided into 9 smaller squares if done carefully, allowing faster imaging since the slides do not have to be changed out between each condition.

Note: The support slides ensure that the pad thickness (~0.2 mm) will remain the same across all samples.

Note: Prepare fresh agarose pads for each imaging session.

5. Mount the worms.
   a. Place a 10 µL droplet of 100 mM sodium azide (NaN_3) on the agarose pad. See problem 5.
   b. Pick 10–20 worms to be imaged under the dissection scope and put them in the NaN_3 droplet. See problem 6.
   c. Use the platinum wire picker to move the worms around within the droplet so that they do not overlap with each other.
   d. Gently cover the droplet with a coverslip. The worms are ready for imaging. Most of the worms naturally orient themselves in a lateral position See problem 7.

Note: This droplet volume of anesthetizing agent is sufficient for 10–20 worms but respectively increase or decrease the volume if more or less worms are analyzed. See problem 8.

Microscopy and image acquisition

Timing: 15 min

This section describes how to conduct fluorescent microscopy imaging in order to evaluate AID-driven protein depletion.

This step includes the microscope set-up and image acquisition.

Images can be obtained with an automated fluorescence microscope (e.g., Zeiss Axio Imager Z2) and a camera (e.g., Zeiss Axiocam 503 mono) using the ZEN software (Version 2.3.69.1000, Blue edition).

6. Turn on the microscope and associated equipment.
   a. Turn the power on for the laser source.
   b. Turn on the computer.
   c. Open software (e.g., ZEN) for image acquisition.
7. Once calibration of the microscope system is complete, begin imaging samples.
8. Turn the bright-field light source on and direct light to the eyepiece of the microscope.
9. At the lowest magnification, gradually adjust the position of the stage to bring the sample to the center of view, search the worms and then focus using coarse or fine focus adjustment. See problem 9.
10. Switch to the desired magnification and re-focus the sample. We used a 40x objective for data shown in Figures 2C and 3C, and a 20x objective for data in Figure 2D.

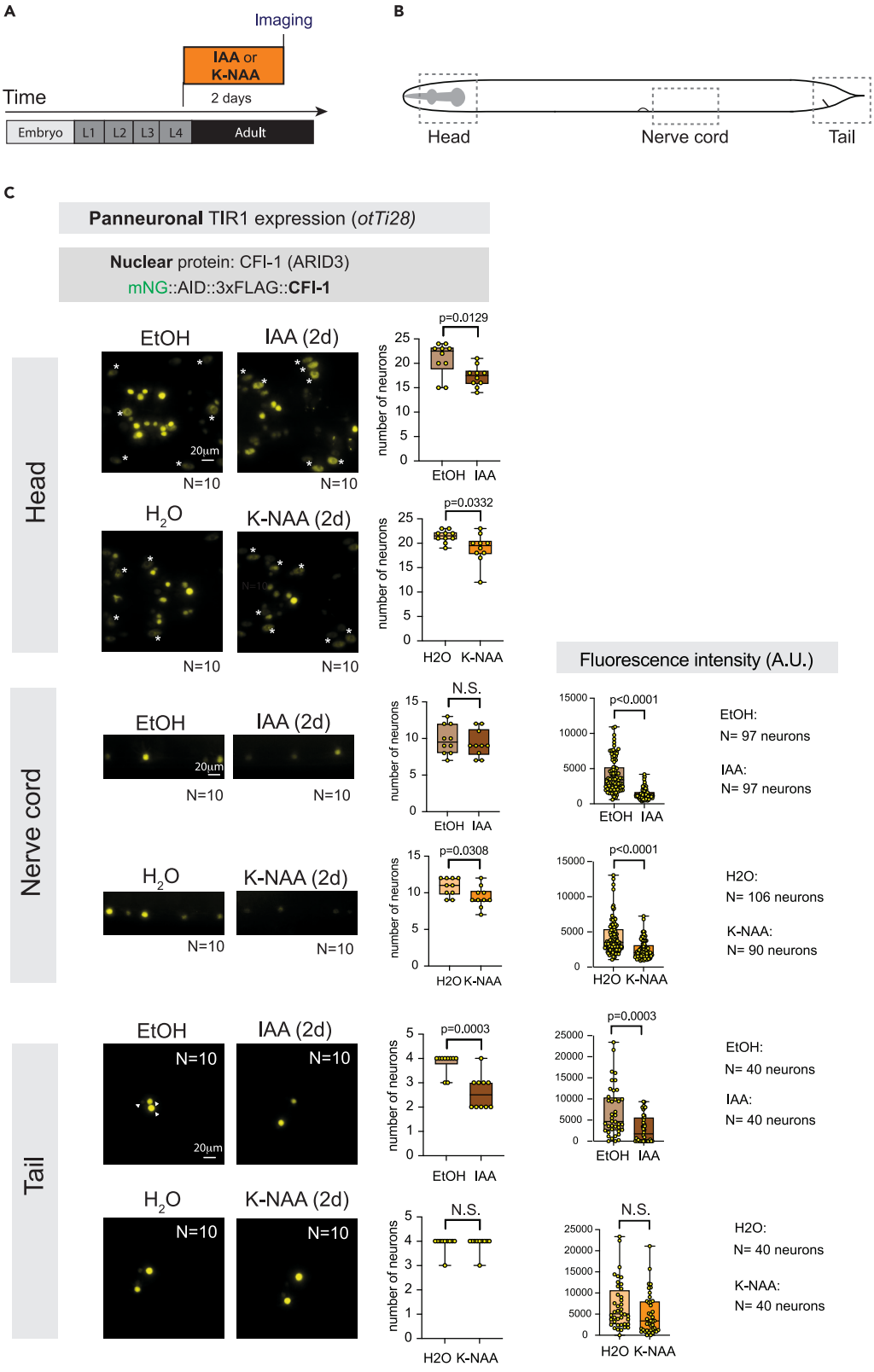
A
Imaging
IAA or
K-NAA
2 days
Time
Embryo
L1
L2
L3
L4
Adult
B
Head
Nerve cord
Tail
C
Panneuronal TIR1 expression (otTi28)
Nuclear protein: CFI-1 (ARID3)
mNG::AID::3xFLAG::CFI-1
Head
EtOH
IAA (2d)
H2O
K-NAA (2d)
20µm
N=10
number of neurons
p=0.0129
p=0.0332
EtOH IAA
H2O K-NAA
Nerve cord
N.S.
p<0.0001
p=0.0308
Tail
p=0.0003
Fluorescence intensity (A.U.)
EtOH:
N= 97 neurons
IAA:
N= 97 neurons
H2O:
N= 106 neurons
K-NAA:
N= 90 neurons
EtOH:
N= 40 neurons
IAA:
N= 40 neurons
H2O:
N= 40 neurons
K-NAA:
N= 40 neurons

Figure 3. AID-mediated depletion of CFI-1 in the nervous system using natural (IAA) and synthetic (K-NAA) auxin

(A) Schematic of auxin treatment.

(B) Schematic of *C. elegans* body. Boxed regions indicate images shown in panel C.

(C) Representative images showing mNG::AID::3xFLAG::CFI-1 expression in neurons and head muscle cells (asterisks) upon IAA or K-NAA treatment for two days. Controls: Two day-long exposure to either 100% ethanol or dH2O. Strain genotype: *cfi-1*(*kas16*[*mNG::AID::cfi-1*]) I; otTi28 [unc-11prom8+ehs-1prom7+rgef-1prom2::TIR1::mTurquoise2::unc-54 3′UTR] X. Images from the *C. elegans* head, ventral nerve cord, and tail are shown. Quantification of the number of neurons expressing mNG and fluorescence intensity are provided on the right. Student's t test, N.S: Not significant. All images are lateral and the animal's head faces left. Fluorescence intensity was calculated using the "sum of stacks" function. Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum values. Yellow circles depict individual values. For images in C, N = number of animals. For the quantifications in nerve cord and tail, N = number of neurons analyzed in total per condition. Scale bar: 20 μm.

11. Switch to the desired laser filter. For example, to visualize mNG::AID::3XFLAG::CFI-1 signals, we used excitation of 506 nm and emission of 517 nm (Figures 2C and 3C). To visualize GFP::AID::3XFLAG::3XGAS ::DENR signals, we used excitation of 488 nm and emission of 509 nm (Figure 2D).
    a. Adjust the exposure time to your fluorescent reporter and avoid saturation.
    b. Avoid photobleaching by minimizing the time that a worm is exposed to light.
12. Acquire Z-stack images with 0.5–1 μm intervals between stacks.
13. Save all images and carefully remove the slide.
14. Once all samples have been imaged turn off all the equipment.

Note: Allow approximately 20 min for the laser to power up and equilibrate before image acquisition.

Note: The optimal thickness is ~0.25 μm (recommended by Zen software), however that will increase the file size and acquisition time, as well as file size.

Note: The imaging parameters might need to be adjusted depending on the specific platform used. Some microscopes require the user to specify the exact wavelength if using a white light laser, and those using advanced detectors might also have to specify the emission range for detection.

Note: Generally, at least 20 worms should be analyzed for each condition.

⚠ CRITICAL: Recalibrate the microscopy system every time the system is restarted.

⚠ CRITICAL: Place the imaging slide that contains the sample on the microscope stage with the glass coverslip facing the objective lens.

⚠ CRITICAL: The same imaging parameters (e.g., exposure time, temperature) should be used across all samples throughout the experiment. Any change may significantly alter signal strength and complicate results interpretation. All imaging for this study was conducted at 20°C–25°C.

Quantification analysis of fluorescent microscopy signals

Images can be obtained using ZEN software (Zeiss). This section includes guidance on quantification of fluorescent microscopy signals using FIJI/ImageJ (Figure S3), an open-source software.[13,14]

15. Open the image files to be quantified in ImageJ. Go to tab "File → Open" to select files individually, or select all files (in Windows, use "Control" plus mouse click; in Mac OS, use "Command" plus mouse click), drag the files, and drop into the ImageJ window.
16. Choose the parameters to be analyzed under the tab "Analyze → Set Measurements…". Check the desired parameters and click OK to close the window.

a. Typical parameters to be measured include "Area", "Mean gray value", "Min and Max gray value", 'Standard deviation" and "Integrated density".

17. Use a background subtraction method to enhance the differences between similar images and to remove undesired signaling from the background. Go to tab "Process → Subtract background…". Check Sliding paraboloid, keep the rolling ball value at 50 pixels and click OK to close the window.
18. If a Z-stack image was acquired, go to tab "Image → Stacks → Z project…". Select Sum Slices (SUM) as projection type and click OK to close the window. A new image window will show up with your SUM projection.
19. In the ImageJ window, click on the freehand selection tool. On the SUM projection image, carefully outline the region to be quantified.
    a. If it helps, the image can be zoomed in to get a closer look when outlining the region of interest. Go to "Image → Zoom → In [+]", or put the mouse cursor in the region where you want to zoom in and use the keyboard shortcut (in Windows, use "Control" and +; in Mac OS, use "Command" and +).
20. To measure the parameters selected previously in step 2, go to "Analyze → Measure" or use the keyboard shortcut (in Windows, use "Control plus M", in Mac OS, use "Command plus M". A new "Results" window will open displaying the measure values.
21. Close the image window and open a new image (select another image in case it's already open).
22. Repeat steps 17–20 for each open image.
23. In the results window, select all the measurements (click on the Results window, then "Edit" → Select all". Copy all measurements to the clipboard ("Edit" → Copy").
24. Paste the results into a new Microsoft Excel file. Proceed with quantification and statistical analysis as desired.

Note: In Figures 2 and 3, we quantified fluorescent signals in neuronal cells. In Figure S4, we quantified fluorescent signal in muscle cells of the *C. elegans* head. In brief, in every animal the same four ventral muscle cells expressing the reporter were quantified for fluorescence intensity with Fiji, as described above. In the fluorescence intensity quantification graphs, each dot represents quantification of fluorescent intensity of individual muscle cells (Figure S4).

EXPECTED OUTCOMES

This protocol provides a reliable method (AID system) to efficiently degrade a protein of interest in *C. elegans*. Because *C. elegans* animals can be placed on NGM-auxin (IAA or K-NAA) plates at different life stages, this system offers temporal control on protein depletion. Moreover, spatial control can be achieved by driving TIR1 expression using tissue-specific promoters (Figure 1B). Because AID is fused to a fluorescent reporter (e.g., *gfp, mNG*), depletion of the AID-tagged protein can be visualized *in vivo* with fluorescent microscopy, leveraging the optical transparency of *C. elegans*. The auxin treatment duration depends on the protein being depleted, but studies have demonstrated rapid depletion even after 30 min of exposure to auxin.[10,11] The degradation rate also depends on the concentration of auxin.

For the nuclear protein CFI-1 (ARID3), we observed efficient mNG::AID::3xFLAG::CFI-1 protein depletion in *C. elegans* neurons and muscle cells when L4 animals are placed on auxin plates for 2 days (Figure 2A). These animals were homozygous for the *ieSi57 [Peft-3::TIR1::mRuby::unc-54 3′ UTR, cb-unc-119(+)] II* line, which expresses TIR1 in all somatic cells (Figure 2C). Importantly, efficient CFI-1 depletion in neurons and muscle cells was observed upon either natural (IAA) or synthetic (K-NAA) auxin treatment (Figure 2C).

For the cytoplasmic protein Y47D3A.21 (DENR), we again observed efficient GFP::AID::3xFLAG:: 3xGAS:: Y47D3A.21 depletion upon either natural (IAA) or synthetic (K-NAA) auxin treatment (Figure 2D). These animals were homozygous for the pansomatic TIR1 line (*ieSi57*). Y47D3A.21 (DENR)

depletion was observed not only by fluorescence microscopy, but also with western blotting (Figure 2E).

In our recent study,[2] we used the AID system for temporal and spatial depletion of CFI-1 in the nervous system. Specifically, we used a pan-neuronal promoter to drive TIR1 expression (*otTi28[unc-11prom8+ehs-1prom7+rgef-1prom2::TIR1::mTurquoise2::unc-54 3′UTR*) in all *C. elegans* neurons (Figure 1B). We performed a genetic cross between this strain and (*kas16*[*mNG::AID::3xFLAG::cfi-1*]), resulting in homozygous *cfi-1*(*kas16*[*mNG::AID::3xFLAG::cfi-1*]); *otTi28[unc-11prom8+ehs-1prom7+rgef-1prom2::TIR1::mTurquoise2::unc-54 3′UTR]* animals. Upon a 2 day-long exposure to natural auxin (IAA), we observed degradation of mNG::AID::3xFLAG::CFI-1 in motor neurons of the ventral nerve cord . Here, we repeated the natural auxin (IAA) treatment to compare it directly with synthetic auxin (K-NAA) treatment. As in our original study,[2] we witnessed significant mNG::AID::3xFLAG::CFI-1 depletion in nerve cord motor neurons – the levels of mNG fluorescence intensity are reduced upon either IAA or K-NAA treatment (Figures 3A–3C). In these same animals, we also assessed mNG::AID::3xFLAG::CFI-1 depletion in head neurons and head muscle cells. Since TIR1 is provided only in neurons, we witnessed a significant reduction in the number of neurons expressing mNG::AID::3xFLAG::CFI-1, but no effect in head muscle cells (Figure S4), highlighting the tissue specificity of the *otTi28* TIR1 line (Figure 3C). Importantly, similar effects were observed in head and nerve cord neurons upon IAA or K-NAA treatment. However, we detected modest mNG::AID::3xFLAG::CFI-1 depletion in neurons located at the tail only after IAA treatment, as K-NAA treatment had no effect (Figure 3C). This is likely due to the very low levels of expression of TIR1 from the *otTi28* line. Consistent with this possibility, we were unable to detect *mTurquoise2* with confocal microscopy (Figure S5). By contrast, pansomatic TIR1 expression resulted in efficient mNG::AID::3xFLAG::CFI-1 depletion in tail neurons upon either IAA or K-NAA treatment (Figure 2C).

More broadly, once efficient depletion of a protein is observed, this protocol is compatible with functional studies for virtually any *C. elegans* protein. Three examples of applicability include.

1. Molecular analysis of TF target genes. For temporally-controlled depletion of mNG::AID::3xFLAG::CFI-1, in our original study,[2] we placed L4 animals on NGM-auxin (4 mM) and control plates for 2 days and examined expression of the cholinergic motor neuron-specific reporter *glr-4 (mScarlet)* with fluorescence microscopy at the adult (Day 2) adult stage. We observed that auxin-mediated CFI-1 depletion was accompanied by an increase in the number of motor neurons expressing *glr-4*. Hence, CFI-1 is required in the adult stage to repress *glr-4* in nerve cord motor neurons.[2]
2. This protocol can be combined with any *C. elegans* **behavioral assays** (e.g., harsh/gentle touch, osmotic avoidance, locomotion with automated worm tracking). Essentially, instead of preparing a microscope slide to evaluate gene expression, behavioral assays can be conducted, like for example the harsh touch used in the original study.[2] We note that K-NAA is dissolved in dH_2O (or M9 buffer), whereas IAA is dissolved in ethanol. Because ethanol could confound certain behavioral or lifespan assays, K-NAA should be preferred for these types of experiments.
3. Western blotting. In addition to microscopy, depletion of the protein of interest can be analyzed by a quantitative method, such as Western blotting (Figure 2E). To do so, place a large number of worms on auxin NGM plates (at least two 60 mm plates). Upon lysis with SDS protein lysis buffer, proceed with determination of protein concentrations. Next, load samples (25–50 mg/mL) on an SDS gel, transfer to immunoblot and probe with protein-specific antibodies. One or two days after auxin (IAA) administration, we witnessed significant reduction of DENR protein levels by Western blotting (Figure 2E).

QUANTIFICATION AND STATISTICAL ANALYSIS

For data quantification, box and whisker plots were adopted to show all points, down to the minimum and up to the maximum value with the horizontal line in box representing the median value. This method also plots each individual value as a point superimposed on the graph. Statistical analyses were performed using unpaired t-test (two-tailed) with Welch's correction and *p*-values were

annotated. Differences with $p < 0.05$ were considered significant. Visualization of data and p-value calculation were performed via GraphPad Prism Version 10.2.1.

LIMITATIONS

This protocol demonstrates an effective experimental set up for conditional degradation of AID-tagged proteins in *C. elegans*. Importantly, it also provides side-by-side comparisons of different auxins and TIR1-expressing lines that will benefit future studies of AID-mediated protein depletion in *C. elegans*. However, the AID system may not achieve complete protein elimination, and thereby the lack of a detectable phenotype has to be carefully interpreted. Success of this protocol depends on: (a) the generation of genetically modified *C. elegans* strains where the protein of interest remains functional despite being tagged with the AID degron, (b) TIR1 expression in specific cell types (i.e., neurons, muscles, intestine), and (c) exposure to natural (IAA) or synthetic (K-NAA) auxins. Further, several studies reported hypomorphic effects even in the absence of auxin,[7,15] likely due to: (a) tagging of the endogenous protein may interfere with its normal function, or (b) TIR1 may still be able to drive ubiquitination and subsequent degradation of the AID-tagged protein in the absence of auxin.[7,15] Therefore, alternative approaches are recommended, such as tissue-specific RNAi, and conditional mutagenesis using the Cre/loxP recombination system.[16,17]

TROUBLESHOOTING

Problem 1

Auxin induced protein depletion does not recapitulate expected outcomes or known phenotypes. See Method details – step 2.

Potential solution

Auxin-induced protein depletion can be affected by several factors such as level and timing of TIR1 expression and stability/turnover of the AID-tagged protein.

- Before starting the auxin experiment, confirm the genotype of your strains.
- Include a positive control strain. If you do not observe the expected phenotype in the positive control, consider remaking the IAA or K-NAA working solutions.

Problem 2

Worms growing on auxin plates does not look healthy. See Method details – step 2.

Potential solution

- You can try lower auxin concentrations (0.5–1 mM) and determine the optimal concentration that leads to protein depletion.
- Make sure you pick well-fed worms from NGM plates. Picking worms from starved plates and then transferring to NGM-auxin plate is not recommended.
- Make sure to use NGM-auxin plates that are not older than 3 weeks from the date of preparation and stored in 4°C.
- Before each experiment, let the OP50 on NGM plates dry completely.

Problem 3

Auxin treatment is not effectively depleting the protein of interest. See Method details – step 2.

Potential solution

- You can try higher concentrations of IAA or K-NAA solution (1–4 mM), as in previous publications.[4,5,8–12] For proteins with high stability or high turnover rate, 4 mM is recommended.
- For high concentration of auxin (4 mM), it is recommended to use fresh and highly concentrated OP50 each time, as 4 mM auxin hinders OP50 bacterial growth.

- UV-inactivated OP50 for high IAA concentration could be an alternative approach.
- As an alternative, you can spread/coat 100 μL of 400 mM IAA or K-NAA on NGM plates using a spreader and let it completely dry in the dark. In this case, the 100 mL volume of IAA or K-NAA will be diffused in 10 mL of NGM, resulting in a final concentration of 4 mM IAA or K-NAA.
- To avoid the use of high concentration of IAA/K-NAA, we also recommend the AID2 system, which employs a mutated TIR1 protein (AtTIR1 F79G) and a synthetic auxin (5-Ph-IAA).[7,8] AID2 shows no detectable leaky degradation, requires a 670-times lower auxin concentration, and achieves rapid protein degradation.
- Consider carefully the duration of IAA/K-NAA exposure. You may want to try longer exposure times. However, the original study of AID in *C. elegans* demonstrated effective protein depletion in less than 4 h.[4] Therefore, we recommend assessing protein depletion efficiency at multiple time points.

Problem 4

Endogenous tagging of the protein of interest interferes with the protein's function. See Method details – step 2.

Potential solution

Endogenous tagging of the protein of interest with AID and a fluorescent protein (e.g., GFP, mNG) may interfere with protein function. If that is the case, consider changing the location of the tag (N- versus C-terminus) or replacing the tag with a shorter epitope (e.g., FLAG).

Problem 5

Worms are still mobile in the anesthetizing solution. The cause must be an insufficient concentration of the anesthetizing solution. See Method details – step 5a.

Potential solution

Increase the concentration of sodium azide (NaN_3) and/or leave worms for 5 min in NaN_3 solution before imaging.

Problem 6

High background signal during fluorescent image acquisition. The cause may be an excess of bacteria transferred when picking the worms. See Method details – step 5b.

Potential solution

While transferring the worms from NGM plates onto the agarose pad on microscopy slides, minimize the transfer of bacteria.

Problem 7

Worms burst frequently. This may occur due to several reasons: See Method details – step 5d.

- The worms stayed too long in NaN_3 solution while on the agarose pad of the microscopy slide.
- The volume of the anesthetizing solution is too low, or its concentration is too high.
- The concentration of the agarose solution used for the pad is too high.

Potential solution

The pads help worms stay moist and avoid desiccation, however samples should be imaged within 15–20 min. If worms burst within this timing, the problem must be either the agarose pad or the anesthetizing solution. Lower the concentration of the agarose or the anesthetizing solution. Try increasing the volume of the anesthetizing solution.

Problem 8

Worms scatter too much on the agarose pad. If the agarose pad is too large and the volume of anesthetizing solution is too high. See Method details – step 5.

Potential solution

Consider using a smaller volume of agarose solution to make the pad.

Problem 9

How to distinguish *bona fide* fluorescence signals from autofluorescence during microscopy. See Method details – step 9.

Potential solution

Since autofluorescence of the *C. elegans* intestine is common, it may interfere with detection of fluorescently tagged-proteins fused to AID, such as mNG::AID::3xFLAG::CFI-1. However, autofluorescence in *C. elegance* mostly comes from granules in the intestine, and is more evident in the green channel (488 nm excitation). It also becomes more evident in adult animals. Hence, consider the use of red fluorescent proteins and image younger animals.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paschalis Kratsios (pkratsios@uchicago.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts, Nidhi Sharma (sharman@uchicago.edu) and Filipe Marques (marquesf@uchicago.edu).

Materials availability

The protocol described here generated unique reagents, i.e., new *C. elegans* strains. All *C. elegans* strains used in this protocol are available from the lead contact and/or the Caenorhabditis Genetics Center (CGC) CGC (https://cgc.umn.edu).

Data and code availability

- All data generated for or analyzed in this study are contained in the manuscript and supporting files. Microscopy data will be shared by the lead contact upon request.
- Any additional information required to analyze or reanalyze data in this manuscript is also available upon request.
- No code was generated to analyze the data.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103133.

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, N.S., F.M., and P.K.; investigation, N.S. and F.M.; writing – original draft and writing – review and editing, N.S., F.M., and P.K.; funding acquisition, resources, and supervision, P.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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